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Role of Protease Activated Receptor 2 (PAR2) in *Aspergillus* Protease Allergen Induces Th2 Related Airway Inflammatory Response

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Most allergens have protease activities, suggesting that proteases may be a key link between Th2-type immune reactions in allergic responses. Protease activated receptor (PAR) 2 is activated via the proteolytic cleavage of its N-terminal domain by proteinases. To know the role of PAR2 in *Aspergillus* protease allergen activated Th2 immune responses in airway epithelial cells, we investigated and compared immune cell recruitment and level of chemokines and cytokines between PAR2 knock out (KO) mice and wild type (WT) mice. There were evident immune cell infiltrations into the bronchial alveolar lavage fluid (BALF) of WT mice, but the infiltrations in PAR2 KO mice were significantly lowered than those of WT mice. The IL-25, TSLP, and eotaxin gene expressions were profoundly increased after *Aspergillus* protease, but their expression was significantly lowered in PAR2 KO mice in this study. Compared to PAR2 KO mice, OVA specific IgE concentrations in serum of WT mice were quite increased; moreover, the IgE level of PAR2 KO mice was lower than in WT mice. The IL-25 expression by *Aspergillus* protease stimulation was significantly reduced by p38 specific inhibitor treatment. In this study, we determined that Th2 response was initiated with IL-25 and TSLP mRNA up-regulation in lung epithelial cells via PAR2 after *Aspergillus* protease allergen treatment.

Key words : Protease activated receptor 2 (PAR2), *Aspergillus* protease allergen, allergic airway inflammation, IL-25

Introduction

Asthma and atopic disease are known to be induced by environmental allergens, but the molecular mechanisms through which allergens drive the pathogenesis of these diseases remain unknown. Epithelial cells, as the first cell type to encounter allergens, have been shown to initiate tissue inflammatory responses via the production of a variety of cytokines and chemokines. Moreover, many allergens exhibit intrinsic protease activities [15], and some proteases from infectious agents, parasites, and fungi have been identified as potent allergens [7,23,25]. These different protease allergens have been shown to similarly induce Th2 immune reactions via the activation of several chemokines and cytokines [15,23]. Thus, protease activities may be critical to the initiation of relevant allergic responses.

Most of allergens have protease activities, and suggesting that proteases may be a key link between Th2-type immune responses in allergic response. The protease activities produced by HDM, Aspergillus, or ragweed promoted a Th2 response [5,15]. Moreover, the cysteine proteinase gene from *Leishimania mexicana* has been implicated in the up-regulation of Th2 immunity and the down regulation of Th1 immunity to this pathogen in mice [2]. However, the mechanism by which proteases trigger pro-allergic innate immune responses has not been addressed. We concentrated on some receptors that could be activated by proteases; the protease-activated receptor (PAR)s is one of those. PARs belong to the recently described family of G protein-coupled, seven-trans membrane domain receptors [27]. PARs are activated via the proteolytic cleavage of their N-terminal domain by proteinases, thereby resulting in the generation of a new N-terminal "tethered ligand", which can autoactivate the receptor function [21]. Four members of the PAR family have been cloned thus far. PAR1, PAR3, and PAR4 can be activated by thrombin, and PAR2 can be activated by trypsin, mast cell tryptase, neutrophil protease 3, tissue factor/factor VIIa/factor Xa, membrane-tethered serine proteinase-1, or proteases from *Porphyromonas gingivalis* [12]. There have been studies conducted regarding the relationship between allergic disease and PAR2 [4,17,26]. But there are a few reports about the role of PAR2 in *Aspergillus* protease induced allergic airway inflammation model.

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In this study, to know the role of PAR2 in *Aspergillus* protease allergen activated Th2 immune response on airway epithelial cells, we investigated and compared immune cell recruitment and level of chemokines and cytokines between PAR2 KO (knock out) mice and wild type (WT) mice.

Materials and Methods

Allergen

Aspergillus protease (Asp; Sigma-Aldrich, St Louis, Mo, USA) was reconstituted with sterile PBS to 1 mg/ml and stored at -20°C.

Induction of airway inflammatory reaction

Female C57BL/6 mice at the age of 5 weeks were purchased from Samtako Co. (Gyeonggi-do, Korea). $PAR2^{-/-}$ mice (C57BL/6 background) were purchased from The Jackson Laboratory Co. (Bar Harbor, Maine, USA) and were bred in a specific pathogen-free facility at the Institute for Laboratory Animals of Pusan National University. Chicken egg OVA (Sigma) was reconstituted in sterile PBS at 1 mg/ml and stored at −20°C. For intranasal challenge, 10 μl (10 μg) of *Aspergillus* protease was added to 40 μl (40 μg) of OVA immediately before intranasal administration. C57BL/6 mice were induced with airway inflammation by *Aspergillus* protease for one [stage 1] or six total challenges [stage 2], as previously described [3,16,28]. One day after the last challenge, mice were killed for analysis of bronchial alveolar lavage fluid (BALF). BALF cells were collected and further subjected to differential cell counts.

Lung epithelial cell and Mouse embryonic fibroblast cell culture

Mouse lung epithelial (MLE12) cells were obtained from American Type Culture Collection. Cells were treated with 200 ng/ml *Aspergillus* protease. After 2 h of stimulation, cells were collected and lysed, and mRNA was extracted by using TRIzol reagent (Invitrogen). Mouse embryonic fibroblast (MEF) cell were isolated from C57BL/6 WT and PAR2 KO mouse fetus on 10 days after fertilization.

RT-PCR analysis

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

Ova specific immunoglobulin (Ig) analysis

Ova specific Igs (IgG1, IgG2a and IgE) levels were determined via ELISA. 96-well immune-plates (Maxisorp, Nunc, Roskilde, Denmark) were coated at 37°C until dry with 1 μg/ml of anti-mouse Igs (IgG1, IgG2a and IgE respectively) in 0.1M sodium carbonate buffer, at a pH of 9.6. After 3 washes with 0.1% Tween 20 contained PBS (Sigma), the serum samples were diluted (1:15~1:3645 for IgG1, and IgG2a) in sample buffer (PBS supplemented with 0.1% Tween 20 and 2% BSA), then added to the plate and incubated for 2 hr at 37°C. After incubation, the plate was washed 3 times and anti-mouse Ig antibodies (BD Pharmigen™, BD bioscience, USA) were added to the plate and incubated for 1 hr at 37°C. After washing, the plate was incubated for 30 min at room temperature with streptavidin-HRP (BD Pharmigen™, BD bioscience, USA). For color development, tetra-methyl-benzidine (TMB) was utilized as a substrate. The reaction was halted with a half-volume of an H_2SO_4 solution (Merck, Darmstadt, Germany). The absorption was then measured at 450 nm.

Immunoblot analysis

MEF cells were washed with ice cold PBS and lysed in 0.2 ml lysis buffer (20 mM Tris–HCl, pH 8.0, 120 mM NaCl,

Table 1. Primers used for real-time PCR

Primer	Sequence
GAPDH-for*	5′ - TACCCCCAATGTGTCCGTC - 3′
GAPDH-rev†	5′ - AAGAGTGGGAGTTGCTGTTGAAG - 3′
Eotaxin-for	5′ - GCGCTTCTATTCCTGCTGCTCACGG - 3′
Eotaxin-rev	5′ - GTGGCATCCTGGACCCACTTCTTC - 3′
TSLP-for	5′ - GGAGATTTGAAAGGGGCTAAG - 3′
TSLP-rev	5′ - TGGGCAGTGGTCATTGAG - 3′
IL-25-for	5′ - TGGCAATGATCGTGGGAACC - 3′
IL-25-rev	5′ - GAGAGATGGCCCTGCTGTTGA - 3′
PAR2-for	5′ - GACGCTCCGCTCTTCTGCTAT - 3′
PAR2-rev	5′ - GCTCTGCCTCTGGGTTTTGAT - 3′
CCL17-for	5′ - AGAGCTGCTCGAGCCACCAATGTA - 3′
CCL17-rev	5′ - CACCAATCTGATGGCCTTCTTCAC - 3′
CCL21-for	5′ - TTCCCTACAGTATTGTCCGAGGCT - 3′
CCL21-rev	5′ - TCTGTTCAGTTCTCTTGCAGCCCT - 3′
CCL22-for	5′ - GACAGTATCTGCTGCCAGGACT - 3′
CCL22-rev	5′ - CAGATATCTCGGTTCTTGACGG - 3′

*for; forward
†rev; reverse

1% Triton X-100, 10 mM EDTA, 1 mM EGTA, 0.05% 2-mercaptoethanol, 1X protease inhibitors). Cell debris was removed by centrifugation at 14,000× *g* for 15 min, and the supernatant was boiled in Laemmli sample buffer (Bio-Rad Laboratory, CA, USA) 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). 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, and probed with anti-mouse β-actin (R&D Systems), anti-mouse phospho-ERK, anti-mouse phosphor-JNK, anti-mouse phospho-p38 MAPK, and anti-mouse ikBa (Cell Signaling Technology Inc., MA, USA) specific antibodies at 4°C overnight. After washing, membrane was incubated with secondary rabbit anti-mouse antibody coupled to horseradish peroxidase (Amersham and Pharmacia Biotech) for 1 hr at room temperature. Antibody–antigen complexes were then detected by exposure to X-ray film according to the manufacturer's instructions (Amersham and Pharmacia Biotech).

Flow cytometry analysis

To evaluate the recruitment of neutrophil and eosinophil cells induced by protease allergen, cells isolated from the lung draining lymph node were subjected to CD4, CD11b, and Gr1 staining using the FITC-labeled anti-mouse CD4, APC-labeled CD11b, and Pacific Blue-labeled Gr1 antibodies (eBioscience, USA). The cell surfaces were stained with antibodies (eBioscience, USA) in accordance with the manufacturer's recommendations. Flow cytometry was conducted and analyzed on a FACSCanto™ II (BD) with FACS Diva™ 6.0 software.

Statistics

Data are presented as mean values±SD and are representative of at least 2 independent experiments that used at least 4 mice in each group, unless otherwise indicated. Data were analyzed using the Student's t test (n=2 groups). $p<0.05$ was considered significant.

Results

Protease allergen triggered Th2 response via PAR2 in airway

To know the role of PAR2 in early stage Th2 immune response of airway by *Aspergillus* protease allergen stimulation, the protease administrated once to the airways of wild type (WT) and PAR2 knock out (KO) mice. There were evident immune cell infiltrations, particularly neutrophil, into the WT mice bronchial alveolar lavage (BALF), but, the neutrophil infiltrations into BALF of PAR2 KO mice were significantly reduced (Fig. 1A & B). Also, a $Gr1^{high}CD11b^{high}$ lymphocyte cells (neutrophil) were significantly increased up to approximately 40% by *Aspergillus* protease stimulation, but the $Gr1^{high}CD11b^{high}$ lymphocyte cells recruitment was remarkably reduced in lung draining lymph node of PAR2 KO mice (Fig. 1C). The IL-25, TSLP, and eotaxin gene expressions were profoundly increased after *Aspergillus* protease just 1 time treatment, but their expression was significantly lowered in PAR2 KO mice in this study (Fig. 1D). Also, the levels of the IL-1B of WT mice were quite increased by the *Aspergillus* protease treatment, those of PAR2 KO mice were reduced significantly (Fig. 1D). These result showed IL-1B was also important molecule to initiate inflammation in this study. Interestingly, level of IgE in serum of WT mice was slightly increased by just 1 time *Aspergillus* protease stimulation, but those of PAR2 KO mice was not increased by the protease stimulation (Fig. 1E).

PAR2 was pivotal molecule for fully induction of Th2 response against protease allergen stimulation in airway

Following 6-time administration of *Aspergillus* protease to the airways, there were evident immune cell infiltrations, particularly eosinophils, into the lung (Fig. 2A). The $Gr1^{low}CD11b^{high}$ lymphocyte cells (activated eosinophils) in lung draining lymph node were significantly increased by 6 times Aspergillus protease stimulation. Activated eosinophil recruitment in PAR2 KO mice was significantly lowered than in WT mice (Fig. 2B). OVA specific IgE concentrations in serum of WT mice were quite increased (from 21 ng/ml to 6.8 μg/ml) by 6 time protease stimulation, also the IgE level of PAR2 KO mice was lowered than WT mice (Fig. 2C). The pattern of OVA specific IgG1 levels of PAR2 KO mice were significantly lower than WT and mice like as OVA specific IgE (Fig. 2D).

Direct induction of CCL17 and CCL21 gene expression by protease allergens

To evaluate the expression level of several chemokine genes related with immune cell recruitment, we checked the

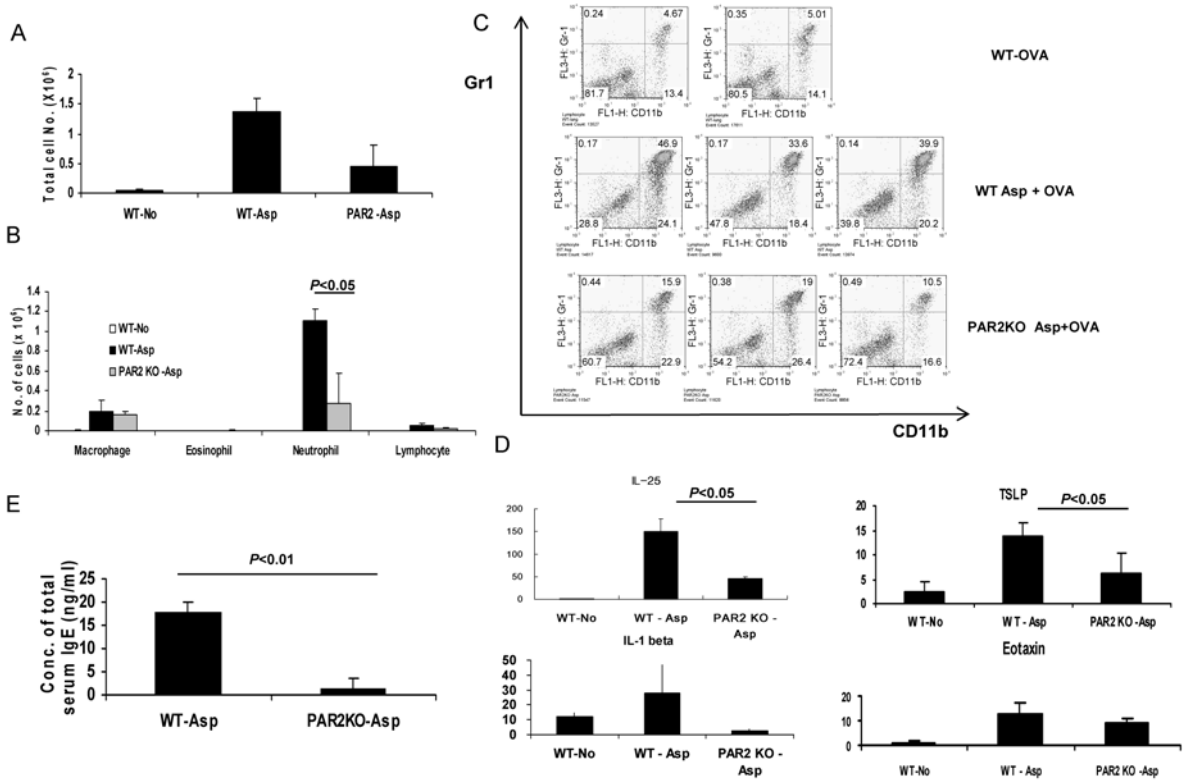

Fig. 1. Initial immune responses by *Aspergillus* protease allergen were related with protease activated receptor (PAR) 2. Cell recruitment of lung by 1 time *Aspergillus* protease I.N. challenge was reduced in PAR2 KO mice (A), especially neutrophil in lung was significantly reduced (B, C). Expression of Th2 related chemokine and cytokine genes (IL-25, TSLP, eotaxin) were reduced in PAR2KO mice (D). Total serum IgE production was reduced in PAR2 KO mice (E).

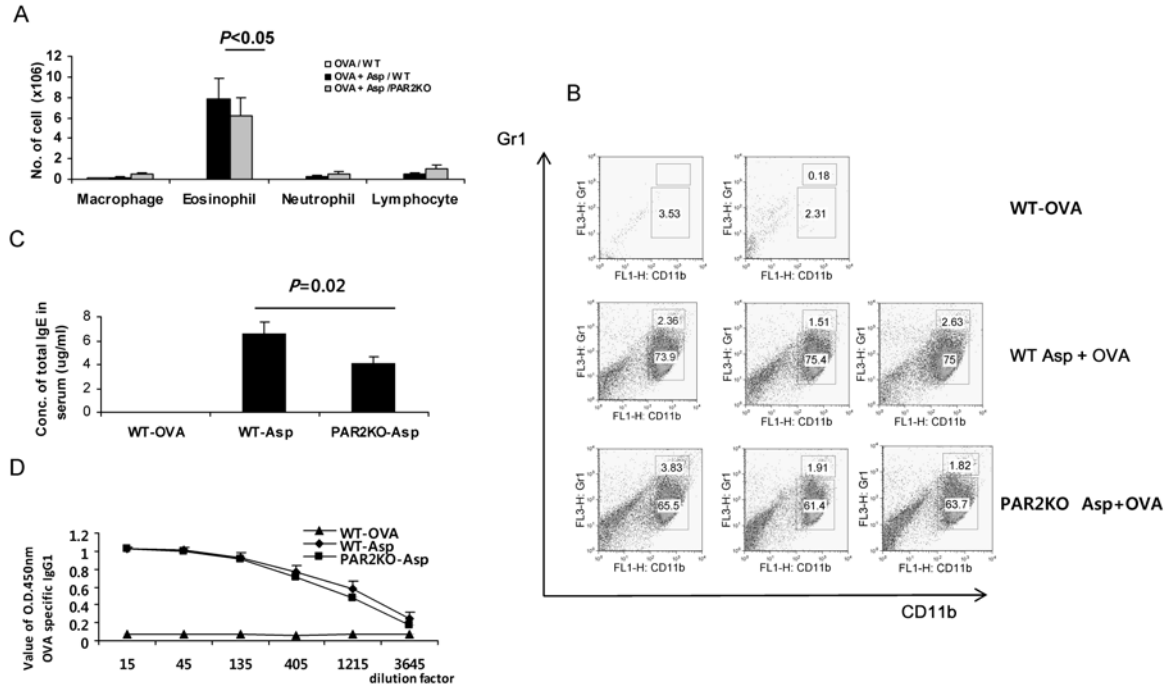

Fig. 2. *Aspergillus* protease induced chronic stage Th2 immune response was regulated by PAR2. Eosinophil recruitment of lung by 6 times *Aspergillus* protease I.N. challenge was reduced in PAR2 KO mice (A, B) and IgE and OVA specific serum IgG1 production were significantly reduced in PAR2 KO mice (C, D).

expression levels of CCL17 (the function is T cell and basophil recruitment), CCL21 (the function is lymphocyte and dendritic cell migration into T zone of lymph node), MDC (the function is T cell and basophil recruitment), and CXCL1 (Neutrophil recruitment). After *Aspergillus* protease stimulation, MLE12 cells expressed CCL17 and CCL21 mRNA at higher levels (Fig. 3A). But MDC and CXCL1 gene expression of MLE12 cells were not increased by *Aspergillus* protease stimulation (Fig. 3A and data not shown). The level of CCL21 gene expression level was slightly increased by *Aspergillus* protease in PAR2 KO mice, while the gene expression level was remarkably increased in WT mice by the protease stimulation (Fig. 3B), but CCL17 gene expression of PAR2 KO mice was not different from WT mice (data not shown).

Aspergillus protese allergens induce IL-25 gene expression via the p38 MAP kinase pathways

In order to assess the intracellular mechanism that regulates the IL-25 expression via protease allergen stimulation, we assessed the activation of intracellular MAPK and IkBα. *Aspergillus* protease treatment in MEF cells rapidly induced the phosphorylation of ERK, JNK, and p38 MAPK within 30 min (Fig. 3C). On the other hand, IkBα levels were not different up to 2 hr after *Aspergillus* protease treatment. The IL-25 expression by *Aspergillus* protease stimulation was significantly reduced by p38 specific inhibitor treatment (Fig. 3D). These results showed that *Aspergillus* protease allergens induce IL-25 gene expression via the p38 MAP kinase pathways.

Discussion

In this study, we demonstrated PAR2 had a critical role at initiation stage and fully development stage of Th2 response elicited by *Aspergillus* protease treatment. Most of allergens, including Amb a from rag weed 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, suggesting that proteases may be a key link between Th2 - type immune re-

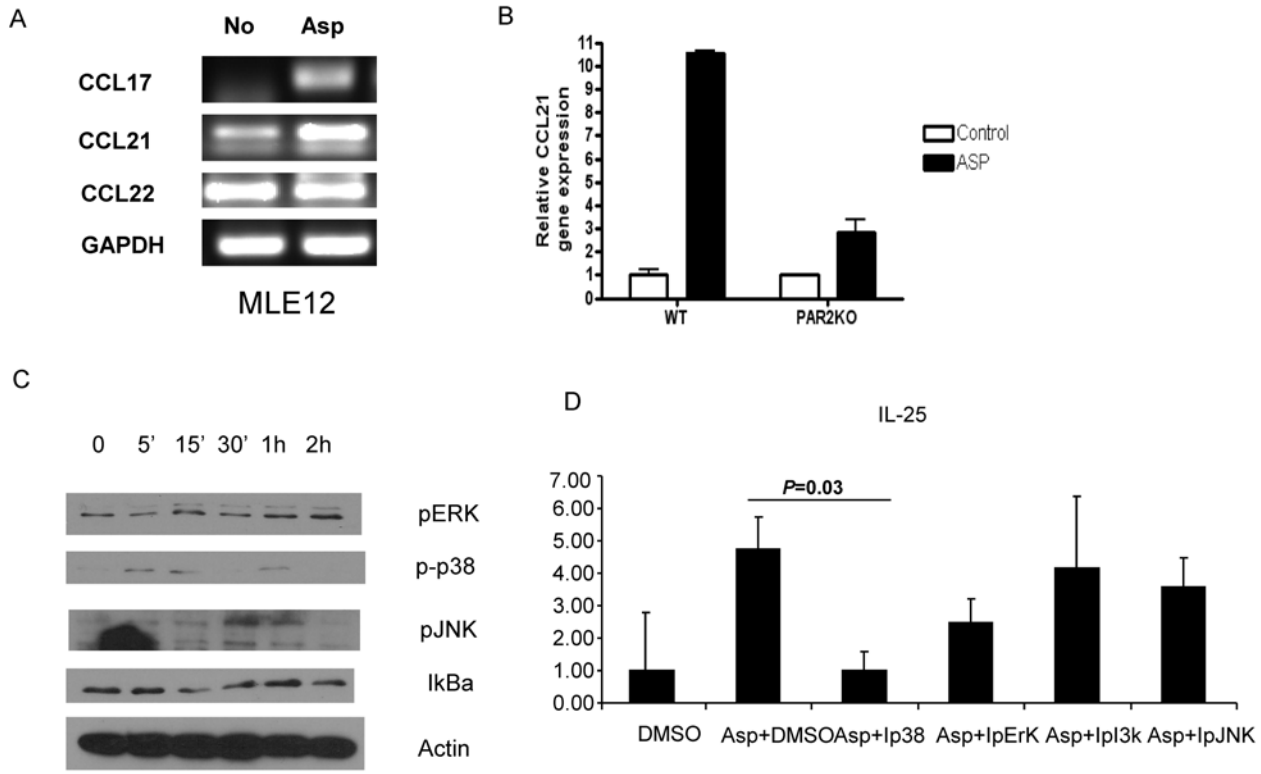

Fig. 3. *Aspergillus* protease activated CCL21 gene expression via PAR2 (A&B), and activated IL-25 gene through Erk, JNK, and p38 MAP kinase pathways (C). IL-25 and expression resulting from *Aspergillus* protease treatment were inhibited by MAP kinase inhibition (D). I-pI3K; inhibitor of pI3kinase (LY294002), I-Erk; inhibitor of Erk MAPK (PD98059), I-p38; Inhibitor of p38 MAPK (SB203580), I-JNK; Inhibitor of JNK MAPK (JNK inhibitor II).

sponses and allergic response [11]. The finding from this study enhance our understanding of the mechanisms of protease allergen elicit Th2 response.

One time of *Aspergillus* protease stimulation could elicit initiation of Th2 response, especially neutrophil recruitment was the most prominently observed in this animal model (Fig. 1A – 1C). Two cytokines- interleukin (IL)-25 and thymic stromal lymphopoietin (TSLP) have been recently identified as initiating cytokines of the Th2 allergic response. In this study, also, we detected the expression levels of IL-25 and TSLP in the lung of WT mice were quite elevated by *Aspergillus* protease stimulation, but these elevations were significantly diminished in PAR2 KO mice (Fig. 1D). IL-25 (also known as IL-17E), a member of the IL-17 family, has been implicated in Th2 cell-mediated immunity [9,13]. Recently, IL-25 has been identified as one of the initiators of the Th2 response [3] and has also been shown to be expressed by mast cells [14]. 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 [3,28]. Also, 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 not been understood. TSLP has been shown to activate dendritic cells (DCs), which then in turn prime naïve T cells to express Th2 cytokines, resulting in allergic responses [1,19]. In addition, TSLP can act directly on T cells to promote Th2 differentiation [1,18,20]. Therefore, IL-25 and TSLP perform a pivotal role in provoking allergic inflammation, and particularly the Th2 allergic response. The results of previous studies indicated that *Aspergillus* protease could also induce IL-25 gene expression and evoke a profound Th2 allergic response [3,28]. Sokol *et al*. also reported similar results, showing that papain, cystein protease, could induce TSLP gene expression, which is required for basophil-mediated Th2 differentiation [24].

Eotaxin functions systemically and synergistically with IL-5 to stimulate eosinophil release from the bone marrow and locally to selectively recruit eosinophils into inflammatory sites [8]. Additionally, the eotaxin has been reported to be regulated by PAR2 activation in an allergic inflammation mouse model [26], and these results are consistent with our results. In our study, eotaxin gene expression was not increased as the result of *Aspergillus* protease stimulation in PAR2 KO mice, but their expression was increased significantly in WT mice (Fig. 1D). These results showed that the PAR2 was one of the most important molecules at initiation stage of Th2 immune response against *Aspergillus* protease stimulation. We also evaluated the role of PAR2 at fully development stage of Th2 response against 6 times *Aspergillus* protease stimulation. We demonstrated that eosinophil recruitment and OVA specific IgE and IgG1 level of PAR2 KO mice were significantly lowered than those of WT mice (Fig. 2). These result showed that PAR2 was also one of the most important molecules at chronic stage of Th2 immune response against *Aspergillus* protease stimulation. IL-1B have been reported as a potent inflammation inducer in lung diseases. Gangly et al. suggested that IL-1B and IL-18 were one of the most important factors on initiation of lung inflammation evoked by carbon nanoparticle exposure in mice [10].

The CCL17 and CCL21 gene expression levels were significantly increased by *Aspergillus* protease stimulation (Fig. 3A). Interestingly the CCL21, not CCL17, gene expression level was significantly reduced in PAR2KO mice (Fig. 3B). The CCL21 has potent activity in attracting naïve and mature T cells, B cells, and DCs [6,22]. CCL21 was expressed in high endothelial venules in the peribrochial area and perivascular lymphatics in the lung. The CCL21 deficiency mice did not support T cell sticking, resulting in a marked reduction of T cell homing to peripheral lymph node. Although the CCL21 gene expression level was reduced in PAR2 KO mice, lymphocyte recruitment into lung was increased (Fig. 2 & 3). It might be existence unknown pathway related with lymphocyte recruitment. We have demonstrated that *Aspergillus* protease induces IL-25 gene expression via the phosphorylation of MAPK p38 and ERK1/2 in MEF cells (Fig. 3C), and that their expression was blocked by p38 inhibitors, but not by ERK1/2 inhibitors (Fig. 3D). These results showed that IL-25 expression was regulated by p38 MAPK pathway.

In this report, we have determined that Th2 response was initiated with IL-25 and TSLP mRNA up-regulation in lung epithelial cells via PAR2 after an *Aspergillus* protease allergen treatment. Moreover, we determined that the IL-25 expressions of protease allergen stimulation were mediated via the intracellular p38 MAP kinase pathways.

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초록 : *Aspergillus* 단백분해효소 알러젠에 의해 유도된 Th2 관련 기도염증반응에서 protease activated receptor 2 (PAR2)의 역할

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대부분의 알려진 알러젠들은 단백분해효소의 성격을 가지고 있고 이는 알레르기 반응에서 Th2 면역 반응을 일으키는 데 중요한 역할을 하는 것으로 알려져 있다. 이러한 단백분해효소들과 반응하는 것으로 알려진 protease activated receptor (PAR) 는 4가지 종류가 있으며, 이 중 PAR2의 경우 알레르기 질환과 많은 상관관계를 보여 많은 연구가 되고 있다. 본 연구는 *Aspergillus* protease 알러젠에 의한 초기 및 만성 Th2 면역반응에서 PAR2 의 역할을 규명하기 위해 *Aspergillus* protease 알러젠으로 정상쥐와 PAR2 유전자 결핍쥐 모두 Th2 반응을 유도한 후 면역세포의 침윤 정도 및 Th2 관련 cytokine 및 chemokine 유전자들의 발현 정도를 비교하였다. 그 결과 *Aspergillus* protease 알러젠으로 비강내로 1회 처리했을 경우 중성구의 침윤이 두드러지는데, 이때 PAR2 결핍 마우스는 이러한 면역세포의 침윤이 유의적으로 감소하였다. 또한, 이와 관련된 IL-25, TSLP, Eotaxin 유전자들의 발현 역시 PAR2 결핍 마우스에 현저히 감소하였다. 한편, *Aspergillus* protease 알러젠으로 비강내로 6회 처리했을 경우 중성구 대신 호산구의 침윤이 두드러지지만 PAR2 결핍 마우스에서 그 정도가 유의적으로 낮았다. OVA 특이 IgE와 IgG1 농도 역시 현저하게 PAR2 결핍 마우스에서 낮았고, CCL21의 발현이 PAR2 결핍마우스 MEF cells에서 현저히 감소하였다. Th2 초기 면역반응에서 가장 중요한 IL-25의 발현에 MAKP p38 pathway가 관여한다는 것을 이번 연구에서 알 수 있다. 본 연구를 통해 *Aspergillus* protease 알러젠으로 유도된 알러지성 기관지 염증 반응에서 초기 반응뿐만 아니라 만성반응에서도 PAR2가 중요한 것을 알 수 있다.