

## Interleukin-25 Enhances Allergic Inflammation through p38MAPK and NF- $\kappa$ B Pathways in Mouse Models of Allergic Rhinitis

Zhipeng Li, Hui Wang, and Lisi Liu

*Department of Otorhinolaryngology Head and Neck Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*

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### ABSTRACT

Interleukin (IL)-25, a cytokine of IL-17 family, can activate p38 Mitogen-Activated Protein kinases(MAPK) and Nuclear Factor(NF)- $\kappa$ B pathways to propagate Th2 responses. The allergic rhinitis mouse model was established by stimulating BALB/c mouse with ovalbumin (OVA). Then we detected expression of IL-25 and downstream p38MAPK and NF- $\kappa$ B.

The expression of IL-25, p38MAPK and NF- $\kappa$ B were detected in the OVA-induced allergic rhinitis mouse model. The allergic parameters, such as allergic symptoms, serum OVA-specific immunoglobulin E (IgE) levels and eosinophil infiltration in the nasal mucosa were compared between OVA group and control group.

OVA-induced mice displayed significantly higher allergic responses compared with the saline control group. OVA induced mice demonstrated more allergic symptoms, higher serum OVA-specific IgE levels and eosinophil infiltrations. The increased expression of IL-25, p38MAPK and NF- $\kappa$ B immunoreactivity were detected in epidermal cells in the OVA group. The mRNA measurement of IL-25, p38MAPK and NF- $\kappa$ B showed the same result.

IL-25 enhances the OVA-induced allergic rhinitis by activating p38MAPK and NF- $\kappa$ B pathways.

**Keywords:** Allergic rhinitis; Interleukin-25; Mice; NF-kappa B; p38 Mitogen-Activated Protein kinases

### INTRODUCTION

Allergic rhinitis (AR) (hay fever) is a common clinical condition that affects approximately 30% of

adults and up to 40% of children in industrialized societies.<sup>1</sup>

Still, it is an underappreciated inflammatory disorder of nasal mucosa, which is characterized by pruritus, sneezing, rhinorrhoea, and nasal congestion.<sup>2</sup> AR is mediated by early-phase and late-phase hypersensitivity responses to indoor and outdoor environmental allergens.<sup>3</sup> It is characterized by intense eosinophil and CD4<sup>+</sup> T-cell infiltrates in the nasal

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**Corresponding Author:** Lisi Liu, MD;  
Department of Otorhinolaryngology Head and Neck Surgery,  
Tongji Hospital, Tongji Medical College, Huazhong University of  
Science and Technology, Wuhan 430030, P.R.China.  
Tel: (+86 158) 2718 8199, E-mail: liulisient@139.com

mucosa, mucus hypersecretion, airway remodeling, and airway hyper reactivity.<sup>4</sup> It is well known that antigen-induced AR is mediated by  $T_H2$  cells and their cytokines, IL-4, IL-5, and IL-13.<sup>5</sup>

A current explanation for the increase in allergic disease is offered by the hygiene hypothesis. IL-25 (IL-17E) is a member of the structurally related IL-17 cytokine family. It is produced by activated  $T_H2$  cells and mast cells.<sup>6</sup> Recent studies have revealed that IL-25 potently induces inflammatory cascades that exacerbate allergic inflammation by promoting Th2 cytokine responses and recruiting eosinophils and  $CD4^+$  T-cells.<sup>7-9</sup> Collectively, these findings indicated that IL-25 may play a pivotal role in the pathology of AR. Nuclear factor (NF)- $\kappa$ B and p38 mitogen-activated protein kinase (MAPK) are two transcription factors activated by IL-25.<sup>10</sup> They were found to be involved in the expression of many inflammatory cytokines and adhesion molecules of eosinophils during allergic inflammation.<sup>11, 12</sup> IL-25 activates NF- $\kappa$ B by binding to a newly identified IL-17Rh1.<sup>13</sup> Up-regulation and activation of NF- $\kappa$ B promotes Th2 cell proliferation and cytokine production. The canonical pathway NF- $\kappa$ B complex consists of a p50/p65 heterodimer which is sequestered in the cytosol via its binding to the inhibitory- $\kappa$ B alpha protein (I $\kappa$ B $\alpha$ ).<sup>14, 15</sup> IL-25-induced cytokines and chemokines are mainly regulated by both NF- $\kappa$ B and p38MAPK pathways and also have a wide variety of biological functions.<sup>16</sup>

Despite the numerous contributing factors, the significance of IL-25 in allergic inflammation is obvious and it should be pointed out that IL-25 functions by activating NF- $\kappa$ B and p38 MAPK pathways.<sup>10, 16</sup> Due to this, it is interesting to investigate the possible role of IL-25 and its downstream transcription factors NF- $\kappa$ B and p38 MAPK in AR. We established an AR mouse model to test the expression of IL-25 related factors.

## MATERIALS AND METHODS

### Ethics Statement

All experiments with animals were performed in accordance with the guidelines of the National Institutes of Health (NIH) and Tongji Medical University with all procedures approved by the Institutional Animal Care and Use Committee of Tongji Medical University (Wuhan, China).

### Animals and Experimental Protocol and Sample Preparation

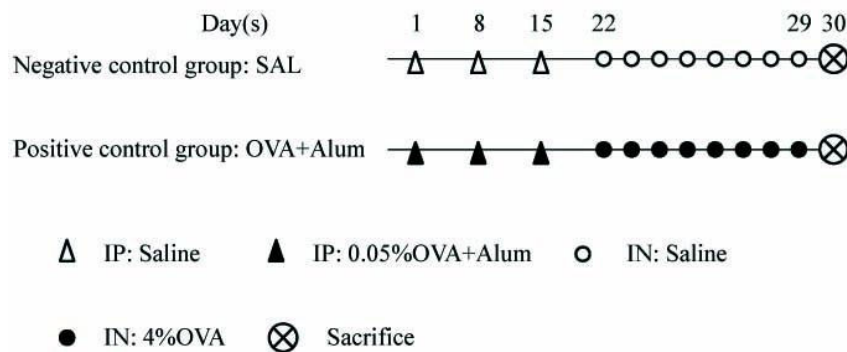
BALB/c mice (SPF grade), including twelve males and twelve females, six weeks old and free of murine-specific pathogens, were obtained from the Experimental Animal Center of Tongji Medical University (Wuhan, China). The mice were housed throughout the experiments in a laminar flow cabinet and were maintained on standard laboratory chow. Mice were divided into two groups consisting of twelve mice each, including 1) negative control group: saline-challenged mice; 2) positive control group: OVA-challenged mice.

The sensitization and antigen challenges of mice for the murine model of AR were performed as previously described. Briefly, mice were administered 0.5 mg/ml OVA (Grade 5, Sigma-Aldrich, St. Louis, MO, USA) and 20 mg/ml aluminum hydroxide (Sigma-Aldrich, St. Louis, MO, USA) in saline at a dosage of 0.2 ml per mouse by intraperitoneal injection. The sensitization was repeated three times on days 1, 8, and 15 followed by daily intranasal instillation of OVA solution (40 mg/ml in saline) into the nostrils (0.02 ml per mouse) on days 22 to 29. The procedure for allergen sensitization is summarized in Figure 1. On days 22-29 after the intranasal administration of OVA or physiological saline, mice were placed individually in observation cages. Twenty-four hours after the final intranasal challenge, mice were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital. The blood samples were obtained via the inferior vena cava and were left for 30 minutes at room temperature and serum was obtained by centrifugation. Samples were stored at -80°C.

The nasal mucosa from seven mice from each group was obtained using a small curette, placed immediately into liquid nitrogen, and stored at -80°C. The mRNA was extracted from these samples.

### Histological Analysis and Immunohistochemistry

The decapitated heads of five mice from each group were fixed in 4% neutral buffered formalin for 24 hours at room temperature. Specimens were decalcified for ten days in 10% EDTA-Na (pH 9.0) and embedded in paraffin. Paraffin-embedded nasal cavities were coronally sectioned (4 $\mu$ m thick) approximately 5 mm from the nasal vestibule. Each section was deparaffinized and stained with



**Experimental protocol.** BALB/c mice were sensitized with 0.05% OVA and 2% aluminum hydroxide (Alum) solution by intraperitoneal injection on days 1, 8, and 15. Mice were challenged with a 4% OVA solution by daily intranasal instillation from day 22 to day 29. SAL: saline; IP: intraperitoneal injection; IN: intranasal instillation.

hematoxylin and eosin (H&E) to evaluate general morphology. The same region of the coronal slice of the septal mucosa in each mouse was observed by light microscopy. The number of infiltrating eosinophils in each animal was obtained by a blind count.

Immunostaining for IL-25, P38, P50, and P65 were performed using immunohistochemistry. Sections (4μm thick) were dewaxed, washed in phosphate buffer saline (PBS), and incubated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 15 minutes to block endogenous peroxidases. Sections were then washed with PBS and incubated with BSA for 30 minutes. Blocked sections were incubated with antibodies against IL-25 (polyclonal mouse anti-IL-25 antibody; Millipore, Billerica, MA, USA) or P-P38 (rabbit polyclonal anti-P-P38 antibody, zhongshanjinqiao, China), P50 (rabbit polyclonal anti-P50 antibody, zhongshanjinqiao, China), P65 (rabbit polyclonal anti-65 antibody, zhongshanjinqiao, China) for 24 hours at 4°C. A secondary antibody was applied to each section for 40 minutes at 37°C, and the slides were rinsed in PBS three times after every incubation step. The slides were counterstained with hematoxylin, mounted, and observed under light microscopy.

### Measurement of OVA-Specific IgE in Sera

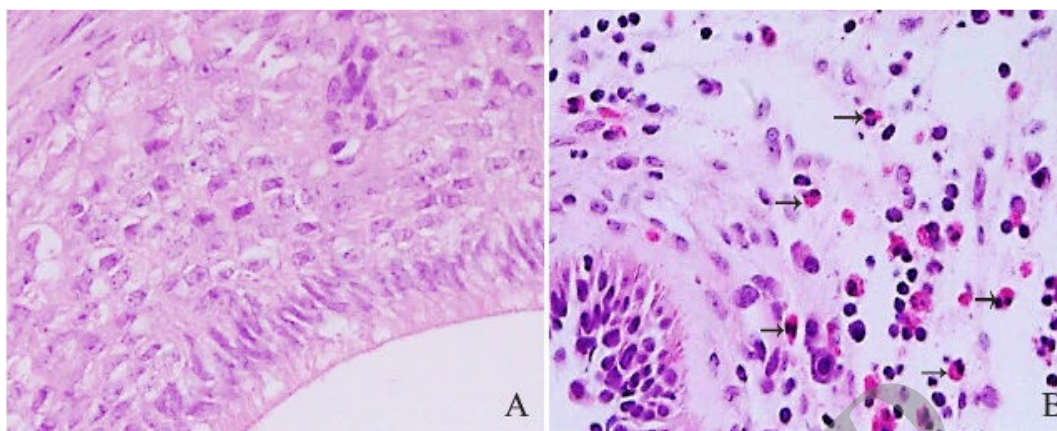
Levels of serum anti-OVA IgE concentrations were measured using anti-OVA-specific IgE ELISA kit. (xitang, shanghai, China),

### Real-time RT-PCR for IL-25, P38, P50, P65 in Nasal Mucosa

Total RNA was extracted by using TRIzol reagent (Molecular Research Center, INC, OHIO, USA) following the standard protocol. The quantity and purity of total RNA were measured by using an ultraviolet spectrophotometer. Subsequently, 1µg total RNA was converted to first-strand cDNA by using revertaid first strand cDNA synthesis kit (Fermentas, Canada). cDNA was amplified by using gene-specific primers and DyNamo Flash SYBR Green qPCR kit (Novogene). Primer sequences were as follows: IL-25, forward: 5'-TCCCACTTTACCACAACCAGAC-3' and reverse: 5'-ACACACACAAGCCAAGGAGACT-3'; P38, forward. 5'-ACCACCCAGTTTCTTCATCA GA-3' and reverse: 5'-CCAGATTACAGCCAAGTTC ACA-3'; P50, forward, 5'-GTGACAGTGGTGTGGA GACATC-3' and reverse: 5'-GGGGCATTTTGTTCAGAGATAG-3'; P65, forward, 5'-GGATGGCTAC TATGAGGCTGAC-3' and reverse 5'-AGGTCTCGC TTCTTCACACACT-3'; GAPDH, forward, 5'-GGTGAAGGTCGGTGTGAACG-3' and reverse: 5'-CTCGCTCCTGGAAGATGGTG -3'. GAPDH was used as an internal control.

## Statistical Analysis

Statistical differences between groups were assessed by the Student's t-test using SPSS17.0. Statistical differences at  $p < 0.05$  were considered significant. Figures were plotted using GraphPad Prism version 5.0 (Graphpad Inc., San Diego, CA, USA).

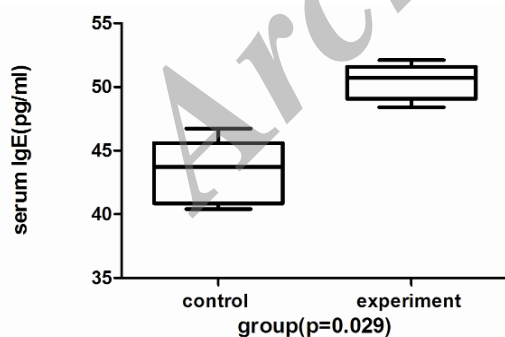


**Figure 2.** HE staining of the model. A The SAL group, columnar epithelium was visible in alignment and there were no obvious glandular hyperplasia or other inflammatory cells in submucosa (400×) B The OVA group, nasal mucosa columnar epithelium were most disappeared, replaced by squamous epithelium, and there were more rod-like or points of leaf eosinophils infiltration in submucosa (400×)

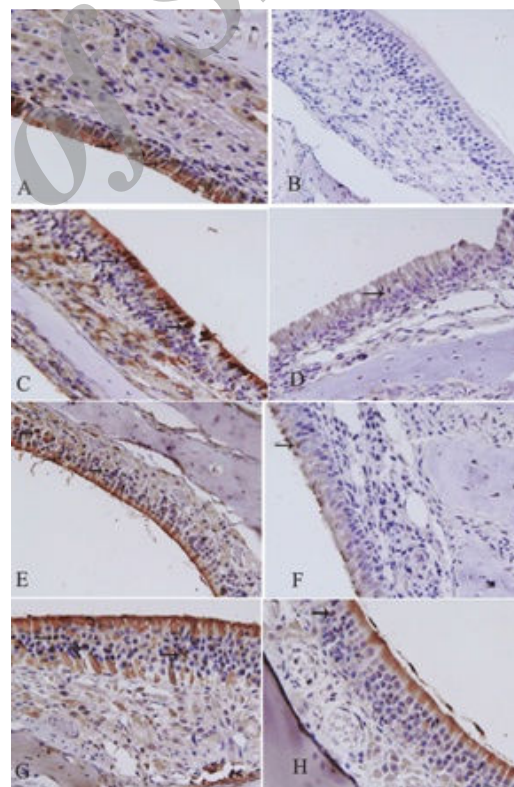
## RESULTS

### The Results of Hematoxylin Eosin (HE) Staining

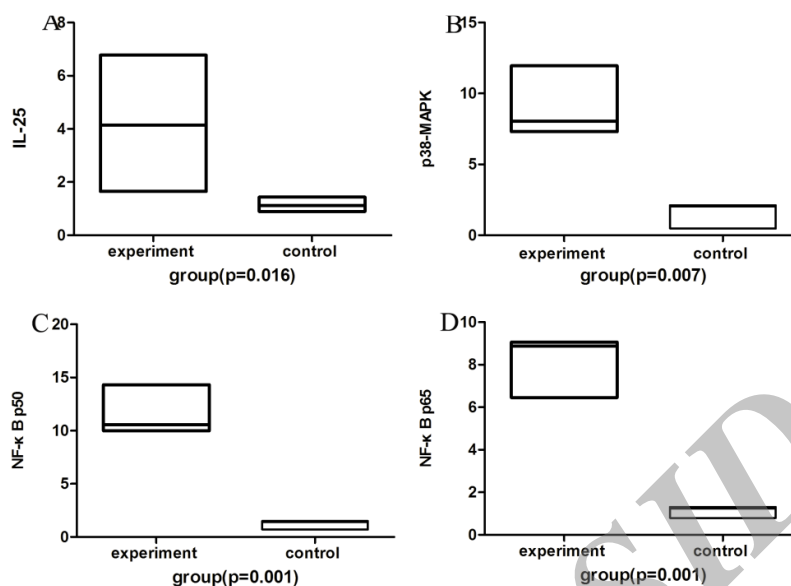
Histological examination showed that the columnar epithelium was visible in alignment and there were no obvious glandular hyperplasia or other inflammatory cells in nasal mucosa of the control group by HE stain while in the experiment group, the nasal mucosa columnar epithelium were mostly disappeared, or replaced by squamous epithelium, and there were more rod-like or points of leaf eosinophil infiltration in submucosa (Figure 2). It was demonstrated to be a successful model of AR.



**Figure 3.** Sampling was performed 24h after the last challenge in saline/OVA-treated mice. Levels of OVA-Specific IgE in sera were analyzed using ELISA (n = 6 mice). There were significant difference between the groups compared by the T-test ( $P_{OVA-sIgE}=0.029$ ).



**Figure 4.** Immunohistochemical analysis of IL-25, p-p38, NF-κB p50 and p65 expression in OVA-challenged mice and control mice. Histological findings of the nasal mucosa in each group (magnification 400×). A: OVA group, IL-25. B: SAL group, IL-25 C: OVA group, p-p38 D: SAL group, p-p38 E: OVA group, p50 F: SAL group, p50 G: OVA group, p65 H: SAL group, p65



**Figure 5.** Real-time RT-PCR analysis of IL-25, p38, NF-κB p50 and p65 subunits in nasal mucosa. Relative target gene expression was normalized to GAPDH, an internal control.

### The Results of ELISA

AR mouse models had higher serum IgE levels than control models. In the control group, the concentration of OVA-specific IgE was  $43.47 \pm 2.58$  pg/ml whereas in the experiment group, the concentration of OVA-specific IgE was  $50.44 \pm 1.40$  pg/ml. There was significant difference between the groups compared by the t-test (Figure 3).

### Immunohistochemical Analysis of IL-25, p-p38MAPK, p50 and p65

There were both the expression of IL-25, p-p38MAPK and NF-κB p50, p65 subunits in the nasal mucosa of two groups of mice. The positive protein signals were seen as brown granular deposits within nasal mucosal epithelium cells. IL-25 was predominantly in cell membranes but p-p38MAPK and NF-κB p50, p65 subunits were expressed in the nuclear (Figure 4).

### Quantitative RT-PCR Analysis

Significantly higher levels of IL-25 were detected in AR mouse models than controls. P38MAPK was the transcription factor activated by IL-25 and its gene expression was significantly increased in AR mouse models when compared with controls. NF-κB p50, p65 subunits were canonical pathways of IL-25 induced Th2 immune reaction. This experiment showed that

NF-κB p50, p65 subunit levels in AR mouse model were found augmented than controls by quantitative PCR. The results suggested that the expression of IL-25, p38MAPK, NF-κB p50 and p65 subunits mRNA increased significantly in the nasal mucosa of the experiment group (Figure 5). We assessed gene expression of IL-25 in the nasal mucosa samples using quantitative RT-PCR. Interestingly, the expression of p38MAPK was significantly positively correlated with expression of IL-25 in these tissues, suggesting a relationship between expression of the cytokine and local synthesis of the transcription factor ( $p < 0.001$ , Pearson  $r = 0.996$ ). Moreover, IL-25 expression was significantly and positively correlated with expression of the NF-κB p50 and p65 subunits ( $p < 0.05$ , Pearson  $r$  was 0.900 and  $p < 0.001$ , Pearson  $r$  was 0.992, respectively). Taken together, these data suggested that IL-25 is associated with the downstream transcription factor p38MAPK and NF-κB p50 and p65 subunits in nasal tissues.

### DISCUSSION

Based on previous research, the airways of patients with allergic airway inflammation always shows the presence of IL-25, increased eosinophils and Th2 cells, and elevated Th2 cytokines IL-4, IL-5, IL-9.<sup>18-20</sup> But in vitro IL-25 could selectively induce a significant

release of other Th2 cytokine IL-4, IL-5, and IL-10 and inflammatory cytokine IL-6 and chemokines CXCL9, CXCL10, and CCL5.<sup>21</sup> These findings suggest that IL-25 might trigger the allergic inflammation and is involved in the pathogenesis of AR.

These previous studies have demonstrated that IL-25 contributes to allergic immune responses and bronchial asthma. Our studies using a murine model of AR confirmed and extended previous observations that IL-25 plays an important role in regulating nasal allergic immune responses by activating p38MAPK and NF- $\kappa$ B p50 and p65 subunits. First, we showed that IL-25, p38, NF- $\kappa$ B p50 and p65 subunit levels were up-regulated in nasal mucosa during AR in mice. The inflammatory responses to OVA challenge, including nasal symptoms, inflammatory cell infiltration, eosinophil recruitment, and up-regulation of OVA-specific IgE levels in sera, were observed in the OVA-challenged group. Second, the gene expression of the p38, NF- $\kappa$ B p50 and p65 subunits were significantly and positively correlated with IL-25 in the nasal tissues. Therefore, we demonstrated that a relationship between expression of the cytokine and local synthesis of the transcription factor. Taken together, these observations suggested that IL-25 and its downstream transcription factor are intimately involved in the pathogenesis of AR.

IL-25 activation is required for inflammatory reactions associated with fungal or helminth infections and allergies.<sup>22</sup> In allergic inflammation, IL-25 is synthesized in response to and is activated by various mediators, including inflammatory cytokines, growth factors, apoptotic cell debris, infectious pathogens, and allergens. Although regulated by different intracellular signaling mechanisms, IL-25 accumulates in both activated Th2 cells and mast cells induced by pro-allergic stimulation, and both the cells are crucial effectors in IgE-associated allergic disorders. Moreover, the mechanism of IL-25 in allergic airway inflammation has recently become evident, such as activate p38MAPK and NF- $\kappa$ B pathways in the airways.<sup>10, 16</sup> These functions are all critical for immune and inflammatory response. In the present study, we showed that the IL-25 levels was significantly elevated in the nasal mucosa of OVA primed BALB/c mice than the control and distributed widely in the mucosa. This suggested that IL-25 plays a pivotal role in regulating nasal allergic immune response. The inflammatory response, including nasal symptoms, inflammatory cell

infiltration, eosinophil recruitment, and up-regulation of OVA-specific IgE levels in sera were observed in the OVA challenged group.

In order to verify this speculation, we tested the expression of p38MAPK and NF- $\kappa$ B p50, p65 subunits in nasal mucosa by immunohistochemistry and RT-PCR. P38, one of the most important target transcription factors preferentially regulated by IL-25, is also an important multifunctional signaling cascade that has been implicated in the regulation of innate immunity and adaptive immunity, particularly in controlling immune cell activation and differentiation. P38MAPK has also been shown to modulate T cell immunity and regulate Th1/Th2 dependent immune response and regulate Th2-specific cytokines such as IL-4, IL-5 and IL-10 and so on. Several studies have shown that increased IL-25 expression causes overproduction of p38MAPK, which results in the recruitment of eosinophils and Th2 cells, the secretion of inflammatory cytokines and mediators. In keeping with these observations, our study demonstrated that changes in p38 mRNA expression correlated with IL-25 mRNA levels, suggested p38MAPK expression in nasal mucosa was regulated by IL-25 in AR. The results have statistical significance. Therefore, one mechanism for the roles of IL-25 in the pathogenesis of AR is via up-regulation of nasal p38MAPK expression.

NF- $\kappa$ B is another transcription factor that functions in the immune response. Recent studies have demonstrated that NF- $\kappa$ B has diverse roles in the proliferation, differentiation, and survival of native T cells and that they are also involved in regulating other functions including the differentiation and proliferation of various cell types of CD4<sup>+</sup> T cells.<sup>25, 26</sup> Previous in vitro studies have shown that IL-25 can stimulate Th cells by activating NF- $\kappa$ B pathway in vitro experiments. Our present mechanistic study showed that elevated NF- $\kappa$ B mRNA levels in the nasal mucosa were probably controlled by IL-25 in vivo in the allergic rhinitis mouse model. Elevated expression of NF- $\kappa$ B indicated the likelihood of a poor outcome and the aggravation of allergic reactions. In summary, according to our findings, such activation of NF- $\kappa$ B can exacerbate AR.

Like previous observations, the gene and protein levels of IL-25 in the nasal mucosa were substantially elevated in our mouse model of AR, suggested that IL-25 was synthesized and secreted. In addition, the increased p38 and NF- $\kappa$ B p50, p65 subunit levels in



nasal mucosa following OVA challenge were also significantly increased. Meanwhile, our present findings show that IL-25 expression was significantly and positively correlated with expression of the p38 and NF- $\kappa$ B p50 and p65 subunits. Taken together, these observations indicated that IL-25 and its transcription factor p38MAPK and NF- $\kappa$ B p50, p65 subunits are intimately involved in AR.

In conclusion, this is the first report about the activation of p38MAPK and NF- $\kappa$ B by IL-25 in the AR model in vivo. Our results suggested that in the OVA-induced mouse model, IL-25 participated in the pathogenesis of AR by the combined activation of p38MAPK and NF- $\kappa$ B pathways, thereby providing new insight for the elucidation of immunopathologic mechanisms of Th2 cell and eosinophil mediated allergic inflammation in nasal mucosa. Further investigation is required for other potential intracellular signaling pathways for the regulation of the release of chemokines and cytokines. In view of the recent advances in the application of IL-25 inhibitors as potential anti-inflammatory agents, our study should provide new insights on the development of therapeutic intervention for AR.

## ACKNOWLEDGEMENTS

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