INTRODUCTION

Allergic diseases including asthma, allergic rhinitis, and allergic conjunctivitis are caused by environmental, genetic, and immunological factors [1,2]. Asthma is an allergic disease characterized by airway obstruction, bronchial inflammation, and allergen-specific IgE. House dust mites were composed of Dermatophagoides pteronissinus and Dermatophagoides farinae, are closely related to asthma pathogenesis [3,4]. Der p 1 and Der p 2 are major allergens of Dermatophagoides pteronissinus. Der p 1 induces cleavage of protease-activated receptor (PAR), which results in allergic inflammation. Der p 1 also facilitates allergen invasion and increases IgE production [5,6]. Der p
2, a group II allergen from of *Dermatophagoides pteronis-sinus*, elicits an inflammatory process including secretion of cytokine such as IL-4, IL-6, and IL-8, and most asthmatic subjects were sensitized to Der p 2 [7,8].

S100A8 and S100A9 belong to the S100 family proteins and are constitutively expressed in monocytes and neutrophils [9–11]. They play as damage-associated molecular pattern (DAMP) via Toll-like receptor 4 (TLR4) and receptor for advanced glycation endproducts (RAGE), and triggers the pathogenesis of asthma, chronic obstructive pulmonary disease, colitis, rheumatoid arthritis, Alzheimer’s disease, and tumor [12,13]. Our reports recently demonstrated that S100A8 and S100A9 induce cytokine secretion, which is involved in regulation of neutrophil apoptosis [14,15].

In this work, we studied the roles of Der p 1 and Der p 2 in production of S100A8 and S100A9 of normal monocytes, as well as constitutive neutrophil apoptosis of normal and allergic subjects due to S100A8 and S100A9 released by Der p 1 and Der p 2.

**MATERIALS AND METHODS**

1. **Reagents**

RPMI 1640 and fetal bovine serum (FBS) were purchased from Life Technologies Inc. (Gaithersburg, MD). Der p 1 and Der p 2 were obtained from INDOOR biotechnologies (Charlottesville, VA, USA). Antibodies against phospho-Lyn and phospho-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against phospho-Akt and ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-S100A8 and S100A9 antibodies were obtained from Abnova (Taipei, Taiwan).

2. **Normal and allergic subjects**

Allergic asthma subjects were recruited from Konyang University Hospital. Asthmatic patients had mild to severe symptoms of the disease. The normal subjects had normal lung function, no history of asthma, and did not require medication. This study was approved by the Institutional Review Board of Eulji University for normal volunteers and by the Institutional Review Board of Konyang University for asthma patients. All participants in this study gave their written informed consent.

3. **Isolation of neutrophils and monocytes, and cell culture**

Human monocytes and neutrophils were isolated from the peripheral blood of normal and asthmatic subjects using Ficoll–Hypaque (Amersham Pharmacia Biotech, Buckinghamshire, UK) gradient centrifugation. CD16 microbeads magnetic cell sorting kit and a monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for neutrophil and monocyte isolation, respectively. The cells were washed after hypotonic lysis to remove erythrocytes. Neutrophils and monocytes were resuspended at 3×10⁶/mL and 2×10⁶/mL in RPMI 1,640 medium with 1% penicillin–streptomycin and 10% FBS (Life Technologies, Gaithersburg, MD, USA). This method routinely yielded greater than 97% neutrophil purity and more than 90% monocyte purity.

4. **Flow cytometry**

After treatment with Der p 1 or Der p 2, human monocytes were harvested and washed twice with PBS. The cells were then fixed with 100 µL of 0.37% paraformaldehyde solution for 15 min at room temperature. Following removal of the fixing solution, the cells were added to 100 µL of 0.2% Triton X–100 in PBS and incubated for 3 min. Next, the cells were washed twice with PBS buffer containing 0.5% BSA, after which non-specific antibody binding was reduced by incubating the cells with normal rabbit IgG. The cells were subsequently separated into new tubes, to which PBS buffer containing anti-S100A8 and anti-S100A9 antibodies was added. Baseline fluorescence was obtained by incubation with normal mouse IgG instead of anti-S100 protein antibodies. After washing three times, the cells were incubated at 4°C for 30 min with FITC-conjugated goat anti-mouse IgG (Molecular Probes: Eugene, OR, USA). Finally, the cells were washed and analyzed on a FACSort cytofluorimeter.
Der p 1 and Der p 2 increase the secretion of S100A8 and S100A9 in normal monocytes. Monocytes isolated from normal were incubated in the absence or presence of Der p 1 or Der p 2 at the concentrations of 10 μg/mL for 24 h, after which the expression of S100A8 and S100A9 was measured by flow cytometry as described in the materials and methods section.
2. S100A8 and S100A9 induced by Der p 1 and Der p 2 suppress spontaneous apoptosis of normal and allergic neutrophils

Because Der p 1 and Der p 2 increase the expression of S100A8 and S100A9 associated with neutrophil survival, we investigated whether S100A8 and S100A9 inhibit constitutive neutrophil apoptosis or not. As shown in Figure 2 and 3, S100A8 and S100A9 were significantly effective on inhibition of normal and allergic neutrophil apoptosis, despite the different degree of inhibition ($p<0.05$). These results indicate that the effect of house dust mite on cytokine secretion of monocytes is involved in inhibition of neutrophil apoptosis.
3. S100A8 and S100A9 have anti-apoptotic effects on neutrophils via Lyn, Akt, and ERK

Because secretory molecules of monocytes, S100A8 and S100A9, after Der p 2 treatment are involved in neutrophil apoptosis, we investigated the exact anti-apoptotic mechanism of S100A8 and S100A9 in neutrophils. As shown in Figure 4, S100A8 and S100A9 induced the phosphorylation of Lyn, Akt, and ERK in a time-dependent manner. These results indicate that Lyn, Akt, and ERK are essential signal molecules in inhibitory effects of S100A8 and S100A9 on neutrophil apoptosis.

DISCUSSION

House dust mites contain a variety of allergen proteins, which function as cysteine and serine proteases, MD-like molecule, α-amylase, and chitinase [17,18]. Der p 1 and Der p 2 are representative allergens and play as important roles in the pathogenesis of asthma [4,19]. We recently demonstrated that S100A8 and S100A9 act as anti-apoptotic factors in neutrophils [4]. In the present study, we investigated that Der p 1 and Der p 2 regulate neutrophil apoptosis by inducing the production of S100A8 and S100A9 in monocytes.

Asthma consists of neutrophilic and eosinophilic subtypes, depending on pathological features. Neutrophilic asthma is characterized by a persistence of airway neutrophilia. Dysregulation of neutrophil apoptosis is one of the most important causes in the pathogenesis of neutrophilic asthma [2,20]. As shown in Figures 1-3, Der p 1 and Der p 2 enhanced the secretion of S100A8 and S100A9 in monocytes comparable to the results of our previous report [20]. S100A8 and S100A9 are effective on suppression of neutrophil apoptosis (Figure 2, 3). The constitutive apoptosis of normal and allergic neutrophils is inhibited by lymphocyte activation due to Der p 1 and Der p 2 [16,19]. The activated lymphocytes secrete the cytokines such as IL-6, IL-8, MCP-1, and GM-CSF, which were essential inhibitory molecules of neutrophil apoptosis. Taken together, alteration of neutrophil apoptosis may be affected by various cytokines such as IL-6, IL-8, S100A8 and S100A9 secreted by monocytes and lymphocytes. The exact mechanism due to Der p 1 and Der p 2 remains to be elucidated. Further study is needed to examine the Der p 1 and Der p 2-mediated signaling related to S100 protein expression and anti-apoptotic signaling.

Since S100A8 and S100A9 have inhibitory effects on neutrophil apoptosis, the fact leads us to examine the exact signal mechanism. As shown in Figure 4, S100A8 and S100A9 inhibit neutrophil apoptosis through Lyn, Akt, and ERK. House dust mite regulates neutrophil apoptosis via TLR4, lyn, PI3K, Akt, ERK, and NF-κB [15,16]. Anti-apoptotic signaling mediated by MCP-1 is involved in the
S100A8 and S100A9 in monocytes. 

Apoptosis of normal and asthmatic subjects by increasing [16], Der p 1 and Der p 2 indirectly regulate neutrophil apoptosis in normal and asthmatic subjects [22]. Based on above results, Lyn, Akt, and ERK are essential proteins in suppression of neutrophil apoptosis. Because Der p 1 and Der p 2 are not directly effective on neutrophil apoptosis, they indirectly promote the activation of Lyn, Akt, and ERK [22]. Leptin delays neutrophil apoptosis via ERK/NF-κB pathway [22]. Based on above results, Lyn, Akt, and ERK are essential proteins in suppression of neutrophil apoptosis. Because Der p 1 and Der p 2 are not directly effective on neutrophil apoptosis, they indirectly promote the activation of Lyn, Akt, and ERK [22].

Conflict of interest: None

Funding: None

Acknowledgements: This paper was supported by Wonkwang Health Science University in 2017.

Conflict of interest: None

REFERENCES

1. Hogate ST. Pathogenesis of asthma. Clin Exp Allergy. 2008;38(6):872-897.
2. Gaffin, JM, Phipatanakul W. The role of indoor allergens in the development of asthma. Curr Opin Allergy Clin Immunol. 2009;9(2):128-135.
3. Kim IS, Lee JS. Suppressive effect of arazyme on neutrophil apoptosis in normal and allergic subjects. Biomed Sci Lett. 2014;20:244-249.
4. Kim IS, Lee NR, Lee JS. Der p 1 inhibits spontaneous neutrophil apoptosis by cytokine secretion of normal and allergic lymphocytes. Korean J Clin Lab Sci. 2015;47:230-236.
5. Ghaemmaghami AM, Robins RA, Gough L, Sewell HF, Shakib F. Human T cell subset commitment determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN-γ. Eur J Immunol. 2001;31(4):1211-1216.
6. Ghaemmaghami AM, Gough L, Sewell HF, Shakib F. The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. Clin Exp Allergy. 2002;32(10):1468-1475.
7. Yu SJ, Liao EC, Shou ML, Chang DT, Tsai JJ. Cell-penetrating peptide derived from human costinophil cationic protein inhibits mite allergen Der p 2 induced inflammasome activation. PLoS One. 2015;10:e0129187.
8. Tsai JI, Shen HD, Chua KY. Purification of group 2 Dermatophagoides pteronyssinus allergen and prevalence of its specific IgE in asthmatics. Int Arch Allergy Immunol. 2000;121(3):205-210.
9. Goyette J, Geczy CL. Inflammation-associated S100 proteins: new mechanisms that regulate function. Amino Acids. 2011;41(4):819-842.
10. Kerkhoff C, Voss A, Scholzen TE, Averill MM, Zänker KS, Bornfeldt KE. Novel Insights into the role of S100A8/A9 in skin biology. Exp Dermatol. 2012;21(11):822-826.
11. Nam AR, Kim DH, Kim MJ, Lee JS, Yang SJ, Kim IS. S100A8 induces secretion of MCP-1, IL-6, and IL-8 via TLR4 in Jurkat T cells. Biomed Sci Lett. 2016;22:60-64.
12. Gebhard C, Németh J, Angel P, Hess J, S100A8 and S100A9 in inflammation and cancer. Biochem Pharmacol. 2006;72(11):1622-1631.
13. Chen B, Miller AL, Bebelatto M, Brewah Y, Rowe DC, Clarke L, et al. S100A9 induced inflammatory responses are mediated by distinct damage associated molecular patterns (DAMP) receptors in vitro and in vivo. PLoS One. 2015;10:e0115828.
14. Kim EH, Lee JS, Lee NR, Baek SY, Kim SJ, Lee SJ, et al. Regulation of constitutive neutrophil apoptosis due to house dust mite allergen in normal and allergic rhinitis subjects. PLoS One. 2014;9:e105814.
15. Lee NR, Baek SY, Gu A, Kim DH, Kim SY, Lee JS, et al. House dust mite allergen suppresses neutrophil apoptosis by cytokine release via PAR2 in normal and allergic lymphocytes. Immunol Res. 2016;64(1):123-132.
16. Kim DH, Choi E, Lee JS, Lee NR, Baek SY, Gu A, et al. House dust mite allergen regulates constitutive apoptosis of normal and atopic neutrophils via Toll-Like Receptor 4. PLoS One. 2015;10:e0129589.
17. Lee JS, Chooi E, Yang EJ, Lee NR, Baek SY, Kim EJ, et al. Induction of the neutrophil migration in normal subjects due to asthma bronchoalveolar lavage fluid (BALF). Biomed Sci Lett. 2014;20:1-6.
18. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. Trends Mol Med. 2010;16(17):321-328.
19. Kim IS, Lee NR, Lee JS. Suppressive effect of Der p 2 on constitutive neutrophil apoptosis by cytokine secretion of normal and allergic lymphocytes. Korean J Clin Lab Sci. 2016;48:102-108.
20. Kim IS, Kim EH, Kim DH, Kim JS, Lee JS. Effect of house dust mite and CCL2 on S100A8 and S100A9 expression in human monocytes. Biomed Sci Lett. 2013;19:344-347.
21. Yang EJ, Choi E, Ko J, Kim DH, Lee JS. Differential effect of
CCL2 on constitutive neutrophil apoptosis between normal and asthmatic subjects. J Cell Physiol. 2012;227(6):2567-2577.

22. Sun Z, Dragon S, Becker A, Gounni AS. Leptin inhibits neutrophil apoptosis in children via ERK/NF-κB-dependent pathways. PLoS One. 2013;8(1):e55249.