

Interaction of Der p 2 with Toll-like Receptor 4 and its Effect on Cytokine Secretion

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Der p 2, which is a major allergen of house dust mite, plays an important role in the pathogenesis of allergic disease. There is controversy regarding whether Der p 2 binds to Toll-like receptor 4 (TLR4), and its inflammatory effect has not yet been elucidated. In the current study, we examined the interaction of Der p 2 with TLR4 and the effect of Der p 2 on cytokine release in THP-1 cells and lymphocytes. Among house dust mite extracts, recombinant TLR4 protein interacted with Der p 2. The overall structure of Der p 2 is characteristic of the immunoglobulin superfamily and contains ten β -strands, forming a β -cup fold with two anti-parallel β -sheets, and a short 3_{10} helix. The two sheets can be separated, further allowing the formation of a large internal pocket, which is narrow and suitable for binding large flat molecules such as lipid-like molecules. Der p 2 caused increased secretion of IL-6, IL-8, and MCP-1, which are neutrophil survival factors, in human monocytic THP-1 cells in a time-dependent manner. Der p 2 also induces the release of cytokines in normal and allergic lymphocytes. Supernatant after treatment with Der p 2 inhibited neutrophil apoptosis. In coculture of lymphocytes with neutrophils, Der p 2 inhibited spontaneous apoptosis of allergic neutrophils. In summary, Der p 2 binds to TLR4 and induces an inflammatory response such as cytokine secretion in immune cells. These findings may enable elucidation of allergy pathogenesis by specific allergen of house dust mite.

Key Words: House dust mite, Der p 2, TLR4, Cytokine

INTRODUCTION

Allergic diseases include allergic rhinitis, asthma, and allergic conjunctivitis, and their symptoms are associated with inflammatory response. House dust mite (HDM) is a

main allergen triggering and alleviating allergic diseases (Gaffin and Phipatanakul, 2009; Kemp, 2009; Kim et al., 2014a; Kim et al., 2014b; Lee et al., 2014). *Dermatophagoides pteronyssinus*, an important HDM, includes a variety of allergen proteins (Thomas et al., 2010; Kim et al., 2013). Protease allergens are classified according to cysteine protease, such as Der p 1 and serine protease, including Der p 3, Der p 6, and Der p 9. Non-protease allergen proteins include Der p 2, Der p 7, and Der p 10. Fifty percent of allergic subjects' IgE showed serum IgE binding to Der p 2, indicating that Der p 2 is a main cause of pathogenesis of allergic diseases.

Der p 2 was found as a member of MD2-related lipid-recognition (ML) domain lipid-binding family (Thomas et al., 2010). MD2 protein transports lipopolysaccharide (LPS)

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onto Toll-like receptor 4 (TLR4) and helps LPS to induce an inflammatory signal. Der p 2 increases IL-6 and IL-8 via TLR4 in BEAS-2B cells and also induces IL-1 β , CXCL10, IL-8, and TNF- α in B lymphocytes through activation of TLR4 and MAPK (Tsai et al., 2011; Yin et al., 2015). Although many researchers have considered that Der p 2 functions as a mimic protein of MD2, the exact relationship of Der p 2 and TLR4 remains to be determined.

Neutrophil apoptosis and cytokine secretion are important in deciphering pathogenesis of allergic diseases (Scheel-Toellner et al., 2004; Monteseirín, 2009; Kang et al., 2014). In this study, we examined the secretion of cytokine functioning as neutrophil survival factors due to Der p 2 and anti-apoptotic effect of Der p 2 on neutrophils by cytokine secretion of lymphocytes.

MATERIALS AND METHODS

Normal subjects and asthmatic patients

Allergic patients, including allergic asthma and allergic rhinitis subjects, were recruited from Eulji University Hospital and Konyang University Hospital. Allergic patients had mild to severe symptoms of the disease. Allergic status was based on the presence of positive results of a skin prick test ($\geq 2+$), multiple allergen simultaneous test (MAST) (\geq class 2), or measurement of specific HDM IgE using the Pharmacia Unicap 100 system for common allergens. Additionally, normal subjects were recruited as controls. The normal subjects had normal lung function, no history of asthma or allergic rhinitis, and did not require medication. This study was approved by the Institutional Review Board of Eulji University for normal volunteers and the Institutional Review Board of Eulji University Hospital and Konyang University for allergic patients. All participants in this study gave their written informed consent.

MALDI-TOF/TOF

Recombinant His-tagging TLR4 protein (R&D Systems, Minneapolis, MN, USA) was loaded into a nickel column and extract of *Dermatophagoides pteromyssinus* (DP) (Cosmo Bio, Tokyo, Japan) was added into the column for binding of the extract proteins to TLR4. After incubation, the column

was washed and eluted with washing and elution buffer, respectively. Eluted sample was separated by SDS-polyacrylamide gel electrophoresis and the gel was stained with silver. For MALDI-TOF/TOF MS analysis of the stained bands, samples were applied to the R2, R3 column and eluted with cyano-4-hydroxycinnamic acid (CHCA) (Sigma, St. Louis, MO, USA) dissolved in 70% acetonitrile and 0.1% TFA before MALDI-TOF/TOF MS analysis. Next, mass spectra were acquired on a 4800 Proteomics Analyzer (Applied Biosystems) operated in MS and MS/MS modes. Peptide fragmentation in MS/MS mode was conducted by collision-induced dissociation (CID). For MS analysis, the 800~4,000 m/z mass range was used with 1,000 shots per spectrum. A maximum of 15 precursors with a minimum S/N of 50 were selected for MS/MS analysis. The MS/MS spectra were searched against the NCBI human database (NCBI 20120310) using the MASCOT algorithm (Matrix Science, Boston, MA, USA) for peptide and protein identification.

Structure-Based Sequence alignment of Der p 2 and MD2

The ClustalW2 program was used for sequence alignment and the Pymol software was used to display the superposition of Der p 2 and MD2.

Isolation of lymphocytes and neutrophils

Human lymphocytes and neutrophils were isolated from the heparinized peripheral blood of healthy persons and allergic subjects using Ficoll-Hypaque gradient centrifugation. A CD16 microbeads magnetic cell sorting kit and a monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for neutrophil and lymphocyte isolation, respectively. The cells were washed after hypotonic lysis to remove erythrocytes. Neutrophils and lymphocytes were resuspended in RPMI 1640 medium (Life Technologies Inc, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μ g/ml).

Cell culture

The THP-1 human monocytic cell line was obtained from

the American Type Culture Collection (Manassas, VA). THP-1 cells were grown in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-6, IL-8, and MCP-1 in a cell supernatant after treatment with Der p 2 (INDOOR biotechnologies, Charlottesville, VA, USA) were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) using OptEIA™ Set human IL-6, IL-8, GM-CSF, and MCP-1 (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Detection of apoptosis

An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Diego, CA, USA) was used to detect neutrophil apoptosis. Isolated neutrophils were incubated with an FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Apoptotic neutrophils were analyzed using a FACSCalibur with Cell-Quest software (BD bioscience) and were determined as the percentage of cells showing annexin V+/PI- and annexin V+/PI+.

Statistical analysis

Data were expressed as the means ± S.E.M. Statistical differences were analyzed using a paired *t*-test for two-group comparisons and one-way ANOVA for comparison of more than two groups. All analyses were conducted using the SPSS statistical software package (Version 10.0, Chicago, IL), and a *P* value < 0.05 was considered to indicate statistical significance.

RESULTS

Der p 2 binds to TLR4

In this study, for the first time, we examined allergen proteins among DP interacting with TLR4. As shown in Fig. 1, six dense protein bands binding to TLR4 were identified, and, among the bands, Der p 2 was characterized using MALDI-TOF-TOF. In order to understand the interaction

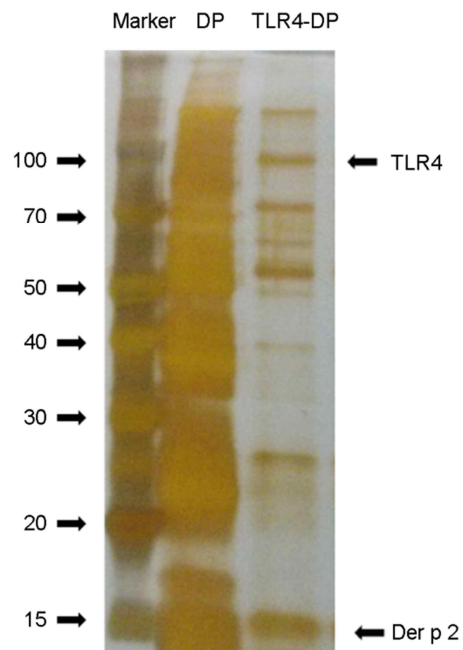


Fig. 1. Der p 2 binds to TLR4. After interacting of DP with TLR4 in a nickel column, eluted sample was separated by SDS-polyacrylamide gel electrophoresis and the gel was stained with silver. The bands were analyzed by MALDI-TOF/TOF MS analysis as described in the Materials and Methods section.

between TLR4 and Der p 2, we compared the structures of Der p 2 and MD2, which showed highly similar structures by superposing them, although their sequence identity showed a very low score. The surface of TLR4, which interacts with MD2, shows an extensive charged network, composed of the A patch and the B patch. Although it is difficult to predict due to missing the negatively charged residues interacting with B patch of TLR4, the interaction between TLR4 and Der p 2 may be forming by some positive residues (Fig. 2).

Der p 2 induces the release of IL-6, IL-8, and MCP-1 in THP-1 cells, and normal and allergic lymphocytes

To investigate the possibility of cytokine secretion by Der p 2, we used the monocytic cell line, THP-1 cells. Der p 2 increased the release of IL-6, IL-8, and MCP-1 in a time-dependent course (Fig. 3). We next examined the effect of Der p 2 on secretion of cytokines of normal and allergic lymphocytes. As shown in Fig. 4A, Der p 2 induced significant secretion of IL-6, IL-8, and MCP-1 in normal

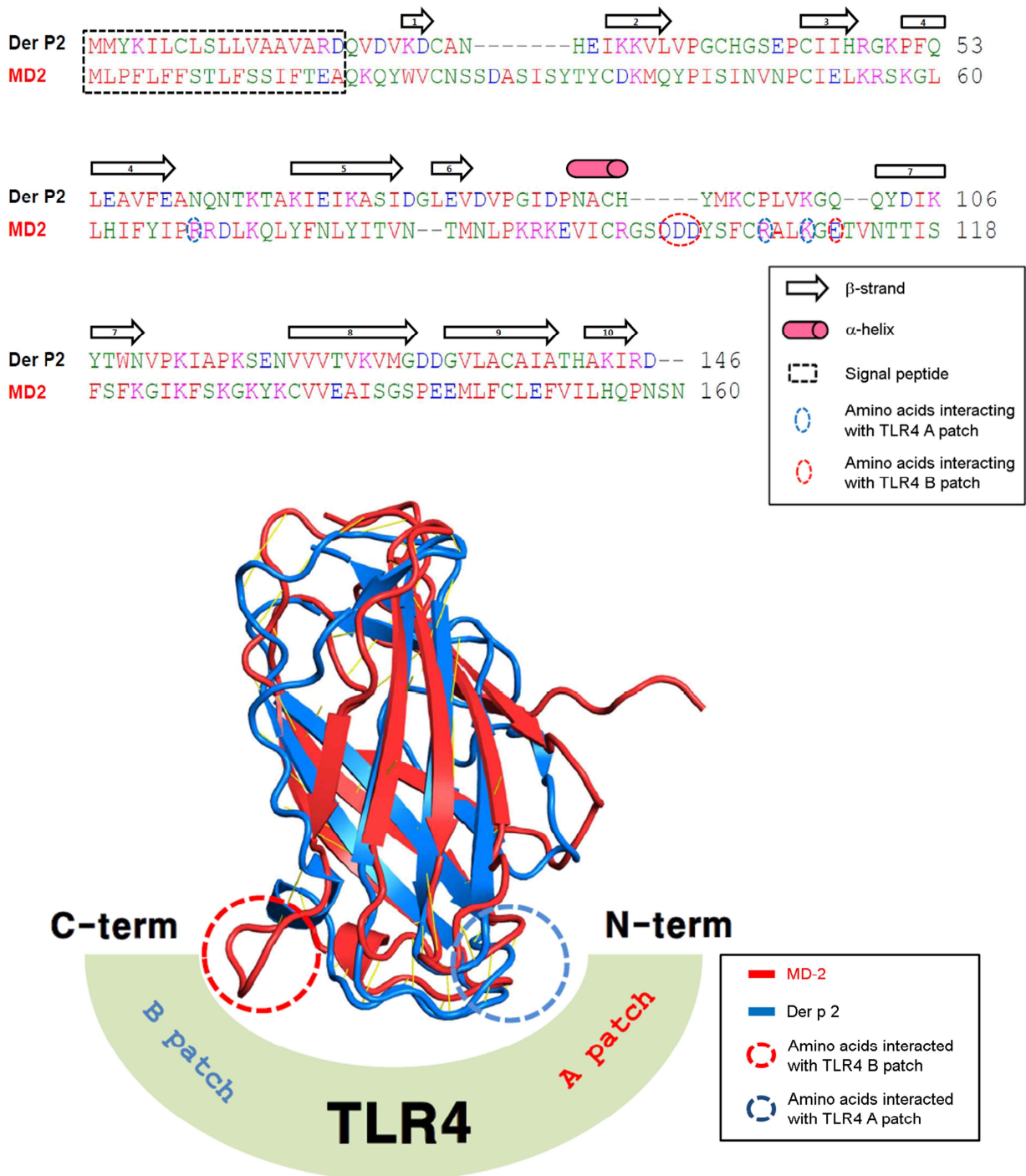


Fig. 2. Structure-based sequence alignment of Der p 2 and MD2. Der p 2 and MD2 are aligned. The secondary structures of Der p 2 are depicted above the sequence alignment as arrows for β -strands, and cylinders, for α -helices. Superimposed ribbon diagram of Der p 2 and MD2 are represented.

lymphocytes ($P < 0.05$). This result leads us to examine the effect of Der p 2 in allergic lymphocytes. Der p 2 increased

the secretion of IL-6, IL-8, and MCP-1 in allergic lymphocytes despite no significant evaluation (Fig. 4B). In com-

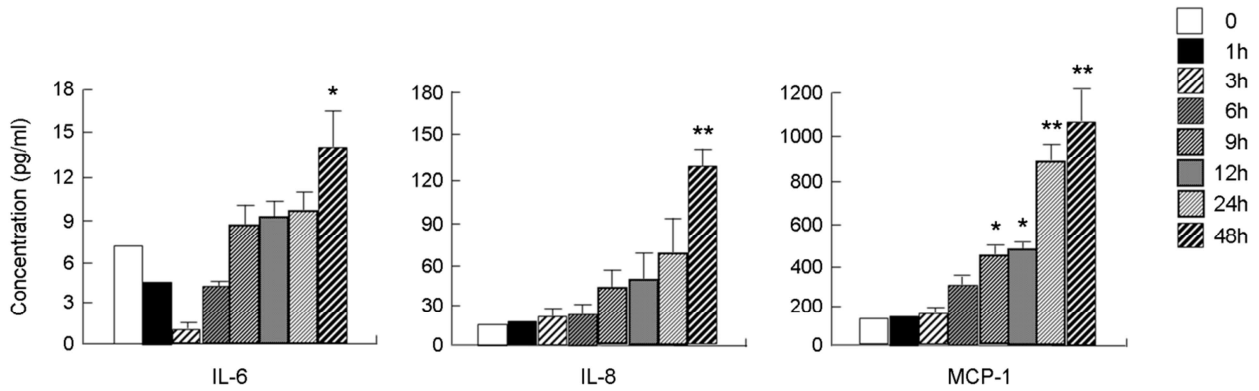


Fig. 3. Der p 2 induces the secretion of IL-6, IL-8, and MCP-1 in a time-dependent manner in THP-1 cells. THP-1 cells were incubated in the presence of 10 µg/ml Der p 2 for the indicated time. The supernatant was collected and analyzed by ELISA. Data are expressed as the means ± S.E.M. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference between the control and Der p 2-treated groups.

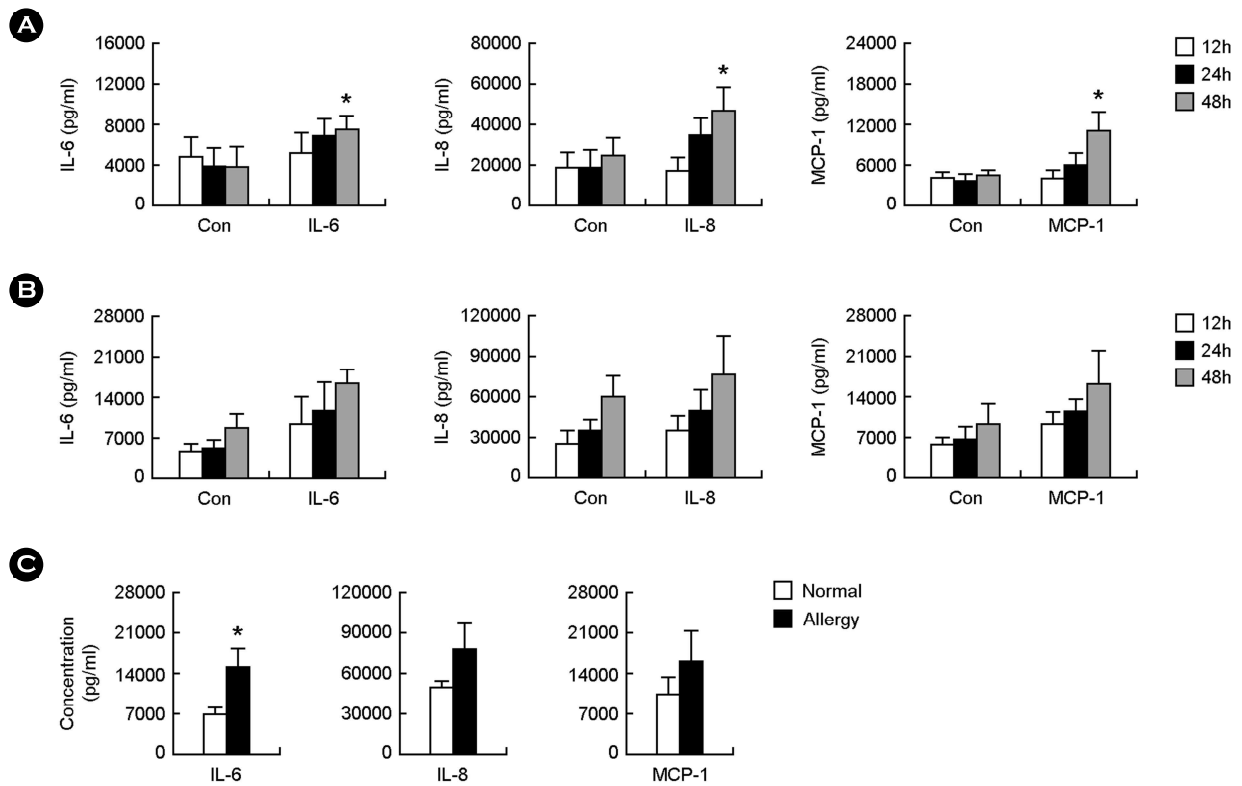


Fig. 4. Der p 2 induces the secretion of IL-6, IL-8 and MCP-1 in a time-dependent manner in normal and allergic lymphocytes. Normal ($7 < n > 13$) (A) and allergic ($5 < n > 9$) (B) lymphocytes were incubated in the presence of 10 µg/ml Der p 2 for the indicated time. The supernatant was collected and analyzed by ELISA. (C) Data from Fig. 4A and B were reconstituted to compare normal with allergic subjects. Data are expressed as the means ± S.E.M. * $P < 0.05$ indicates a significant difference between the control and Der p 2-treated groups.

parison of effect of Der p 2 between normal and allergic lymphocytes, Der p 2 induced a greater increase in secretion

of IL-6, IL-8, and MCP-1 in allergic lymphocytes than in normal lymphocytes (Fig. 4C).

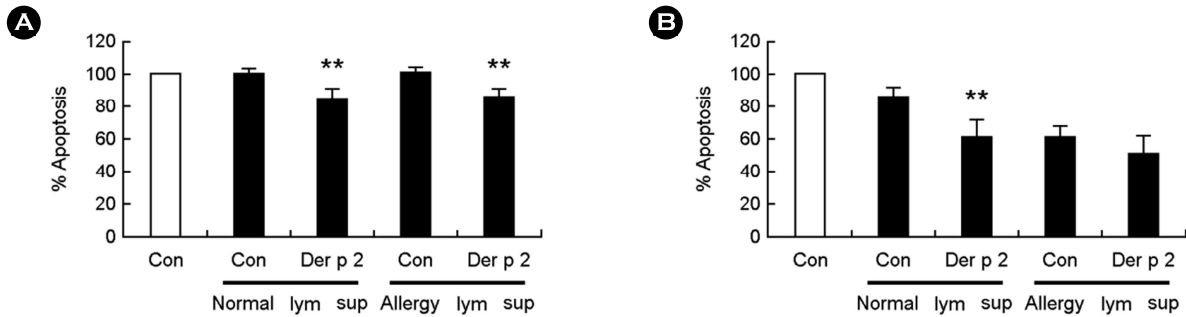


Fig. 5. Cytokine secretion due to Der p 2 inhibits spontaneous apoptosis of normal and allergic neutrophils. Normal and allergic lymphocytes (6<n<13) were incubated with and without 10 µg/ml of Der p 2 for 24 h. The supernatant (sup) was collected and added to normal (A) and allergic (B) neutrophils isolated from the peripheral blood of normal and allergic subjects (3<n<4). Neutrophils apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are presented relative to the control, which was set at 100% of the means ± S.E.M. ***P* < 0.01 indicates a significant difference between the control and supernatant-treated groups.

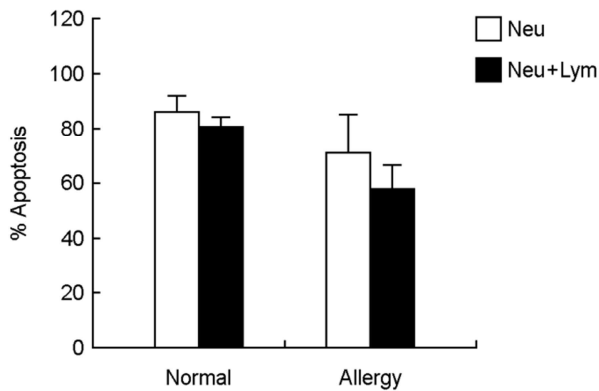


Fig. 6. Der p 2 has anti-apoptotic effects on neutrophils in coculture with lymphocytes. Normal lymphocytes and neutrophils or allergic lymphocytes and neutrophils (1:1 ratio) (n=3) were incubated for 24 h in the presence of 10 µg/mL Der p 2. Neutrophils apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are presented relative to the control, which was set at 100% of the means ± S.E.M.

Cytokine secretion due to Der p 2 inhibits spontaneous apoptosis of normal and allergic neutrophils

Because cytokine secretion involved in neutrophil survival increases after treatment with Der p 2, we examined whether the cytokines secreted by Der p 2 delay constitutive neutrophil apoptosis. To examine the effects of cytokine release due to Der p 2 on neutrophil apoptosis, supernatant was collected after exposure to Der p 2 in normal and allergic lymphocytes. Der p 2-treated supernatant of normal

and allergic lymphocytes inhibited the constitutive apoptosis of normal and allergic neutrophils compared to the control supernatant (Fig. 5A and B). Control supernatant of normal lymphocytes had no effect on apoptosis of normal neutrophils; however, control supernatant of allergic lymphocytes did have anti-apoptotic effects on allergic neutrophils.

Der p 2 has anti-apoptotic effects on neutrophils in coculture with lymphocytes

Because secretory molecules of Der p 2 treated-lymphocytes affected neutrophil survival, we examined whether Der p 2 can alter neutrophil apoptosis in the presence of lymphocytes. As shown in Fig. 6, Der p 2 did not suppress apoptosis of normal neutrophils; however, it inhibited the apoptosis of allergic neutrophils, despite no statistical significance.

DISCUSSION

The number of patients with allergic diseases increases annually in all countries, and HDM is an important cause of allergic disease. In the current study, we examined whether Der p 2, a representative allergen of HDM, binds to TLR4 and its effect on cytokine secretion. Our results demonstrate that (1) Der p 2 interacts with TLR4 *in vitro* (2) Der p 2 may bind to A patch of TLR4 by some positively charged residues. (3) Der p 2 induces secretion of IL-6, IL-8, and

MCP-1 in normal and allergic lymphocytes. (4) Cytokines secreted due to Der p 2 suppress spontaneous neutrophil apoptosis.

TLR4 is an essential receptor of inflammatory response. DP induces asthmatic features via TLR4 in airway structural cells of an asthmatic mouse model (Hammad et al., 2009).

DP inhibits constitutive apoptosis of normal and allergic neutrophils and it transduces the anti-apoptotic signal via TLR4, Lyn, PI3K, Akt, ERK, and NF- κ B (Kim et al., 2014; Kim et al., 2015). Der p 2 induces cytokine release in BEAS-2B cells and B lymphocytes via TLR4 (Tsai et al., 2011; Yin et al., 2015). There is controversy regarding binding of Der p 2 to TLR4. Der p 2 triggers allergic responses by direct TLR4 binding or by TLR4 binding after binding to LPS (Ryu et al., 2013; Tropmette et al., 2009). However, a recent study demonstrated that Th2-biased action induced by Der p 2 is not associated with functional TLR4 (Stremnitzer et al., 2014). As shown in Fig. 1 and 2, binding of Der p 2 to TLR4 from DP was identified and the interaction site of TLR4 binding to Der p 2 was analyzed by structure modeling. However, the interaction of Der p 2 with TLR4 was not demonstrated in other *in vitro* experiments such as specific plasmon resonance (SPR) and an *in vivo* experiment. To confirm and reinforce the results of this study, we will perform these experiments in the near future.

During inflammatory or allergic response, neutrophils migrate toward the inflamed site and act as immune regulators in the site. Alteration of survival rate of neutrophils is important in allergy pathogenesis (Monteseirín et al., 2009). Der p 2 induced the secretion of IL-6, IL-8, and MCP-1 and it practically showed an anti-apoptotic effect in coculture of lymphocytes with neutrophils (Fig. 4 and 5). We recently reported that regulation of neutrophil apoptosis due to Der p 2 is not altered (Kim et al., 2015). These results indicate that cytokine secretion due to Der p 2 in lymphocytes affects neutrophil survival. Actions of Der p 2 such as cytokine secretion and neutrophil survival differed between normal and allergic subjects (Fig. 4C and 5). These results indicate that normal and allergic states are important criteria in evaluating the effect of Der p 2. In addition, in this study there is a question regarding whether effects of Der p 2 are associated with TLR4. The exact mechanism by

Der p 2 involved in this process was not identified in this study. Further studies are being conducted to more clearly define the mechanisms associated with this reaction.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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