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Der p 1 Inhibits Spontaneous Neutrophil Apoptosis by Cytokine Secretion of Normal and Allergic Lymphocytes

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Der p 1에 의한 정상인과 알레르기 환자의 림프구에서 사이토카인 분비를 통한 자발적인 호중구 세포고사 억제

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Dermatophagoides pteronissinus (DP) is one of the house dust mites (HDM) associated with allergic diseases. The cysteine protease Der p 1 from DP is a powerful allergen. The pathogenic mechanism of allergy is involved in cytokine secretion of lymphocytes and spontaneous apoptosis of neutrophils. In this study, we investigated whether Der p 1 induces cytokine secretion of lymphocytes and if the release of cytokines is associated with regulation of neutrophil apoptosis. In normal and allergic subjects, Der p 1 increased IL-6, IL-8, MCP-1, and GM-CSF release in a time-dependent course. Supernatants collected from normal and allergic neutrophils after Der p 1 stimulation suppressed the apoptosis of normal and allergic neutrophils, although Der p 1 alone has no effect on neutrophils. Der p 1 suppressed neutrophil apoptosis in coculture of normal neutrophils with normal lymphocytes. Der p 1 more strongly suppressed apoptosis of allergic neutrophils cocultured with allergic lymphocytes than normal neutrophils cocultured with normal lymphocytes. In summary, Der p 1 increases the secretion of cytokines, which has anti-apoptotic effects on neutrophils of normal and allergic subjects. These results will contribute to elucidate the pathogenic mechanism of allergic diseases.

Keywords: Allergy, Cytokine, Der p 1, Lymphocyte, Neutrophil apoptosis

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Introduction

Allergic diseases such as asthma, allergic rhinitis, and allergic conjunctivitis are caused by genetic, environmental, and immunologic factors (Holgate, 2008; Rondón *et al.,* 2012; Kim *et al.*, 2014). House dust mite (HDM) plays as an important allergen in the pathogenic mechanism of allergic diseases by cytokine release and IgE synthesis of lymphocytes (Thomas *et al.*, 2002; Kim *et al.*, 2013; Kang *et al.*, 2014). Der p 1, a cysteine protease, is included in HDM and shows protease activity. Activation of Der p 1 induces cleavage of cell surface proteins, which favors allergen exposure to a body. Der p 1 also increases IgE generation in B lymphocytes, CD25 expression in T lymphocytes (Schulz *et al.*, 1998; Ghaemmaghami *et*

al., 2001; Ghaemmaghami *et al*., 2002).

Lymphocytes include B cells and T cells, and secrete various cytokines that control immune responses. Dysregulation of cytokine production is one of the most important causes in pathogenesis of allergic diseases. HDM induces secretion of IL-6, IL-8/CXCL8, MCP-1/CCL2 and GM-CSF in monocytes and neutrophils, and these cytokines are involved in suppression of neutrophil apoptosis (Lee *et al*., 2008; Yange *et al*., 2012). HDM induces secretion of Th2 cytokines (Thomas *et al*., 2002). Based on the above results, we examined whether Der p 1 activates release of cytokines involved in neutrophil survival in lymphocytes, which alter neutrophil apoptosis.

Materials and Methods

1. Normal and allergic subjects

Allergy patients, including allergic asthma and allergic rhinitis subjects, were recruited from Eulji 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 (≥2+), multiple allergen simultaneous test (MAST) (≥ class 2), or measurement of specific HDM IgE using the Pharmacia Unicap 100 system for common allergens (≥100 IU/ml). The normal subjects had normal lung function with 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 for allergic patients. All participants in this study gave their written informed consent.

2. Isolation of lymphocytes and neutrophils and cell culture

Human lymphocytes and neutrophils were isolated from the heparinized peripheral blood of healthy persons and allergic subjects using Ficoll-Hypaque (Amersham Phamacia biotechnology, Buckinghamshire, U.K.) gradient centrifugation. 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 at 3×10^6/mL and 2×10^6/mL in RPMI 1640 medium with 1% penicillin-streptomycin and 10% FBS (Life Technologies Inc., Gaithersburg, MD). This method routinely yielded greater than 97% neutrophil purity and more than 99% lymphocyte purity as assessed by counting the cells using a cytospin system.

3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazollum bromide (MTT) assay

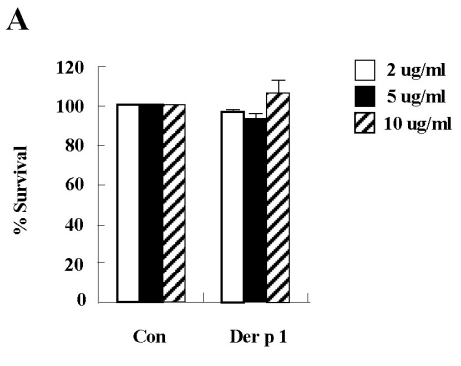
MTT assay was performed to determine cell viability using the cell proliferation kit (Roche, Penzberg, Germany). Lymphocytes in 100 μL of the culture medium were plated into a 96-well culture plate. Der p 1 (INDOOR biotechnologies, Charlottesville, VA, USA) was added to each well. The plate was then incubated for 24 h at 37°C in a CO_2 incubator. 10 μL of MTT solution was added in each well. After incubation of the plate at 37°C for 4 hrs, 100 μL of solubilization solution was added to each well. After 24 hr incubation, the absorbance was measured using an ELISA reader (Bio-Tek Instruments, Winooski, VT) at 550 nm.

4. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-6, IL-8, GM-CSF, and MCP-1 in a cell supernatant 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.

5. Detection of apoptosis

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



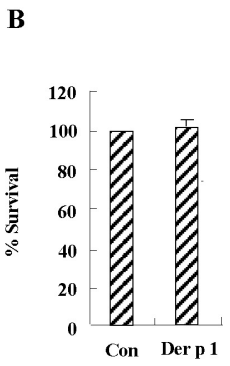
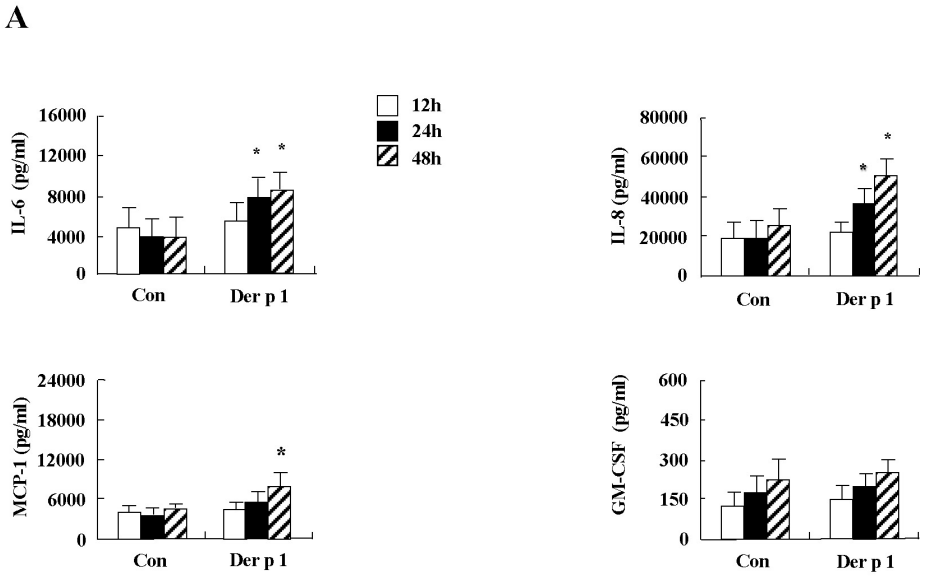


Fig. 1. Derp 1 has no toxicity in lymphocytes. Lymphocytes isolated from peripheral blood of normal (A) and allergic subjects (B) were incubated in the absence or presence of Der p 1 at the indicated concentrations for 24 h., after which the survival rate was measured by MTT assay.



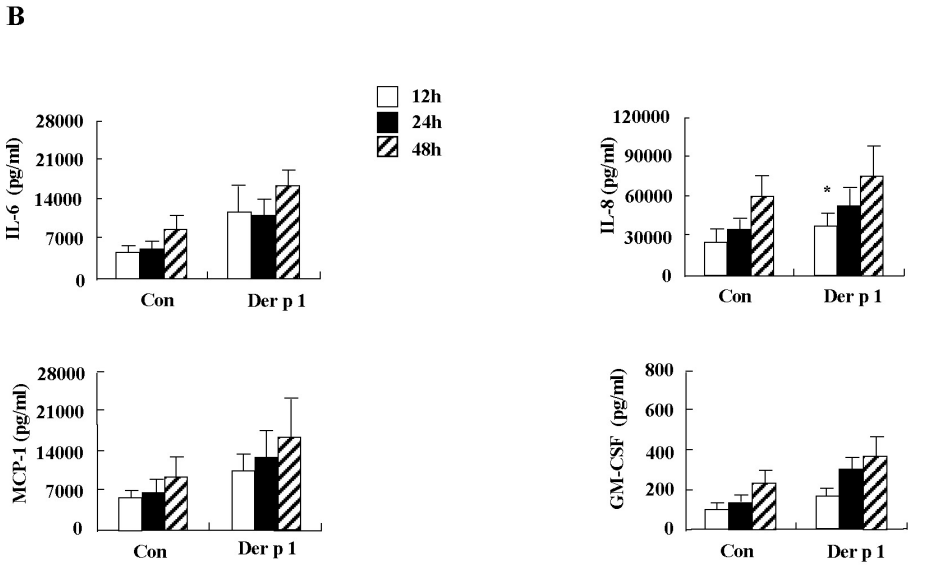


Fig. 2. Der p 1 increases the release of IL-6, IL-8, MCP-1, and GM-CSF in a time-dependent course in normal and allergic lymphocytes. Normal (A) and allergic (B) lymphocytes were incubated with 10 μg/mL Der p 1 for the indicated time. The supernatant was collected and analyzed by ELISA. Data are expressed as the means± S.E.M. *$p<0.05$ indicates a significant difference between the control and Der p 1-treated groups.

6. 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

1. Der p 1 induces the secretion of IL-6, IL-8, MCP-1, and GM-CSF in normal and allergic lymphocytes

Prior to investigating the effect of Der p 1 on cytokine secretion of normal and allergic lymphocytes, we examined the effects of Der p 1on cytotoxicity of lymphocytes. Der p 1 at 2 μg/mL, 5 μg/mL and 10 μg/mL was not effective on the survival of normal and allergic lymphocytes (Fig. 1). Der p 1 significantly increased the secretion of IL-6, IL-8, MCP-1 and GM-CSF in a time-dependent course ($p<0.05$) (Fig. 2). These results indicate that cytokine secretion induced by Der p 1 occurs in allergic lymphocytes as well as in normal lymphocytes.

2. Cytokine secretion induced by Der p 1 delays spontaneous apoptosis of normal and allergic neutrophils

Since cytokines secreted by Der p 1 are associated with neutrophil survival, we examined whether the molecules secreted by Der p 1 delay spontaneous neutrophil apoptosis. We first collected supernatant after Der p 1 stimulation in normal and allergic lymphocytes, then used this supernatant to treat normal and allergic neutrophils. As shown in Fig. 3, Der p 1 alone has no effect on normal neutrophil apoptosis and inhibited allergic neutrophil apoptosis without statistical significance. The Der p 1-treated supernatant of normal and allergic lymphocytes significantly inhibited the spontaneous apoptosis of normal and allergic neutrophils relative to the control supernatant ($p<0.05$). Control supernatants of normal and allergic neutrophils were significantly effective on apoptosis of allergic neutrophils ($p<0.05$). As shown in Fig. 5A, the anti-apoptotic effect of Der p 1-treated supernatants on allergic neutrophils was stronger than on normal neutrophils.

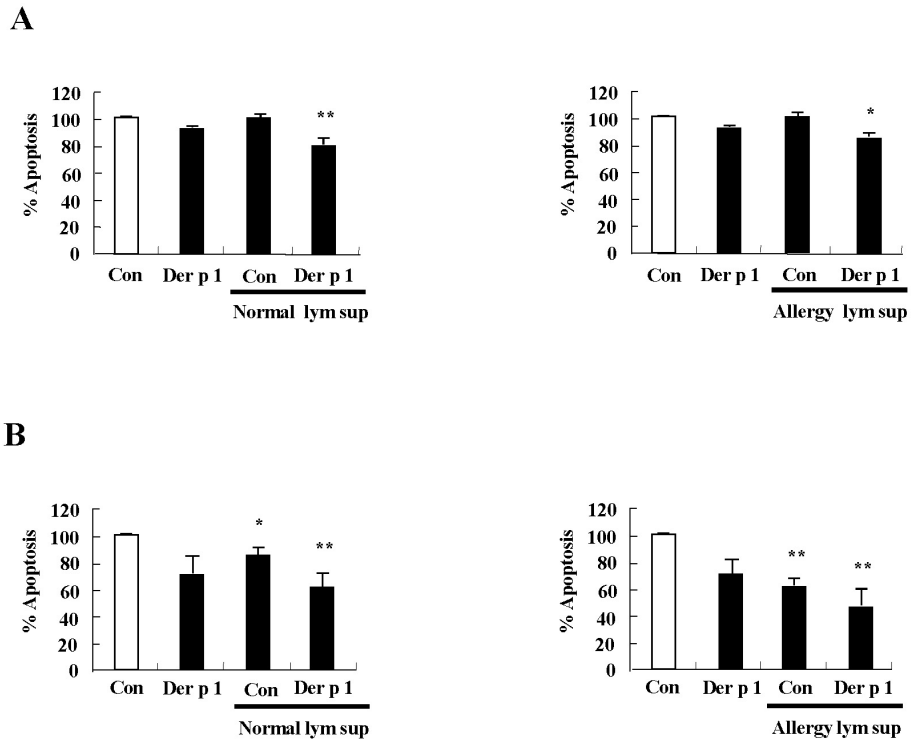


Fig. 3. Cytokine release by Der p 1 inhibits spontaneous apoptosis of normal and allergic neutrophils. Normal and allergic lymphocytes were incubated with and without 10 μg/mL of Der p 1 for 24 h. The supernatant (Sup) was collected and added to fresh neutrophils isolated from the peripheral blood of normal (A) and allergic subjects (B). 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. *$p<0.05$ and **$p<0.01$ indicate a significant difference between the media and supernatant-treated groups.

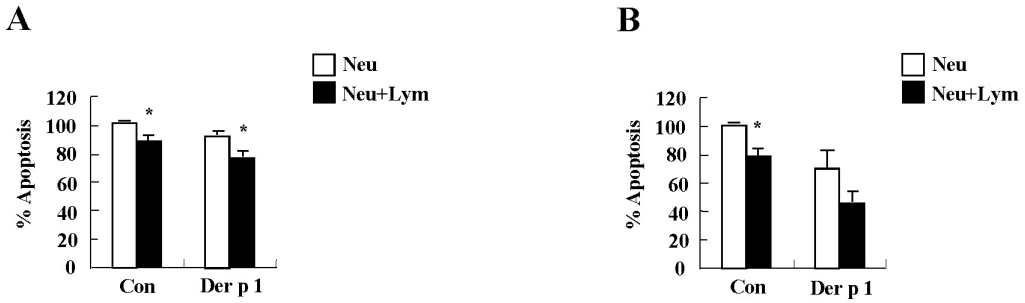


Fig. 4. Der p 1 is effective on neutrophils in coculture with lymphocytes (A) Normal neutrophils or neutrophils and lymphocytes (1:1 ratio) were incubated for 24 h in the absence and presence of Der p 1 (10 μg/mL). (B) Allergic neutrophils or neutrophils and lymphocytes (1:1 ratio) were incubated for 24 h in the absence and presence of Der p 1 (10 μg/mL). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are expressed as the means±S.E.M and are presented relative to the control, which was set at 100%. *$p<0.05$ indicates a significant difference between the neutrophils and neutrophils/lymphocytes groups.

These results indicate that the cytokines secreted by Der p 1 inhibited neutrophil apoptosis.

3. Der p 1 has anti-apoptotic effects on neutrophils in coculture with lymphocytes

Since secretory molecules of lymphocytes after exposure to Der p 1 are related to neutrophil survival, we examined whether neutrophil apoptosis is altered in coculture with lymphocytes. As shown in Fig. 4, neutrophil apoptosis was weakly inhibited by coculture of neutrophil with lymphocytes. Der p 1 inhibited apoptosis of normal and allergic neutrophils in the presence of lymphocytes. As shown in Fig. 5B, the anti-apoptotic effect of Der p 1 on allergic neutrophils in the coculture with lymphocytes was stronger than on normal neutrophils.

Discussion

HDM, one of the essential allergens, induces the pathogenic mechanism of allergic diseases. HDM contains a variety of proteins such as Der p 1, Der p 2, and Der p 3 (Thomas, 2010; Rondón *et al.*, 2012; Lee *et al.*, 2014; Kim and Lee, 2015).We previously demonstrated that allergic diseases are related to regulation of neutrophil apoptosis and HDM, and that HDM inhibits the spontaneous apoptosis of neutrophils via TLR4/Lyn/PI3K/Akt/ERK/NF-κB pathway (Kim *et al.*, 2014; Kim *et al.*, 2015). In this study, we investigated that Der p 1 regulates neutrophil apoptosis by increasing cytokine secretion of lymphocytes.

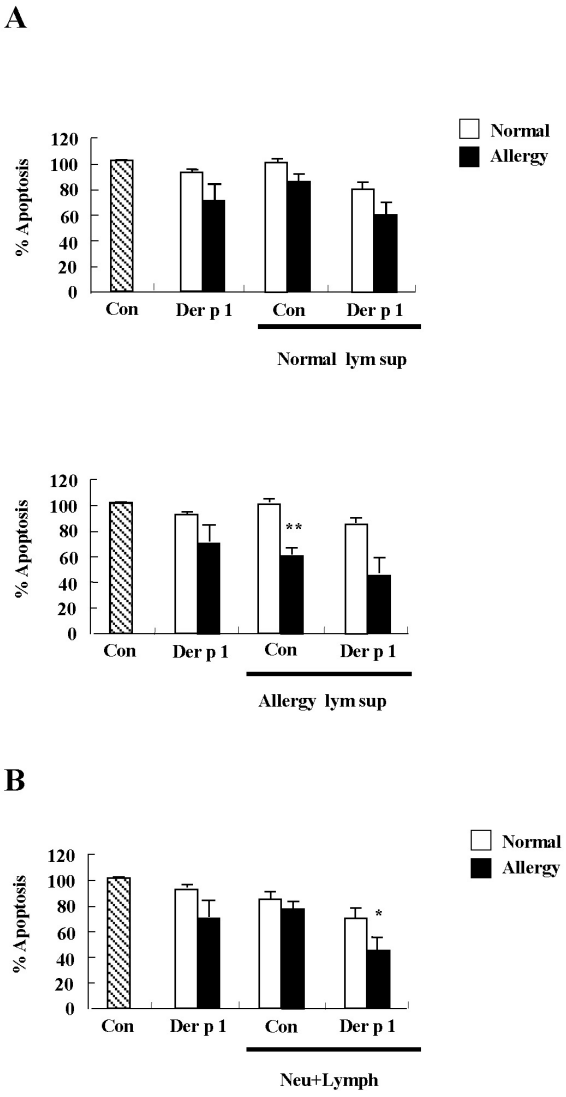


Fig. 5. Comparison between normal and allergic subjects. (A) Data from figure 3 were reconstituted to compare normal with allergic subjects. (B) Data from figure 4were reconstituted to compare normal with allergic subjects..

As shown in Fig. 2 and 3, Der p 1 induced expression of IL-6, IL-8, MCP-1, and GM-CSF in normal and allergic lymphocytes, and the cytokines secreted by Der p 1 inhibited neutrophil apoptosis. In addition, Der p 1 suppressed apoptosis of normal and allergic neutrophils in the coculture with lymphocytes (Fig. 4). Type 2 cytokines such as IL-4, IL-5, and IL-9 is involved in allergy and IL-17 is considered as unexpected key molecule of allergy (Allen *et al.*, 2015; Wynn, 2015). It has been recently reported that IL-6, IL-8, and MCP-1 are important in pathogenesis of allergy (Lee *et al.*, 2008; Yang *et al.*, 2012). IL-6, IL-8, MCP-1, and GM-CSF function as survival factors of neutrophils. In addition, IL-6 is an essential cytokine in the acute phase of inflammation. IL-8 and MCP-1 chemoattract both monocytes and neutrophils. GM-CSF differentiates myeloid progenitor cells into mature neutrophils in bone marrow (Rossi and Zlotnik, 2000; Gabay, 2006; Manz, 2014). Therefore, Der p 1 can affect a variety of immune responses including chemotaxis and hematopoiesis as well as regulation of neutrophil apoptosis.

Because it is important that normal and allergic subjects are different, we evaluated the different aspects between normal and allergic patients. As shown in Fig. 5, the anti-apoptotic effects of Der p 1-treated normal and allergy supernatants on allergic neutrophils were stronger than on normal neutrophils. The anti-apoptotic effect of Der p 1 on allergic neutrophils cocultured with lymphocytes was stronger than on normal neutrophils. These results indicate that Der p 1 differently affect neutrophil regulation in normal and allergic states. Further study is needed to unveil the exact reasons on the difference between normal and allergic subjects.

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요 약

Dermatophagoides pteronissinus (DP)는 알레르기 질환과 연관이 있는 집먼지 진드기 중 하나이다. 집먼지 진드기에 의해 생성되는 Cystein 단백질분해효소(Derp-1)가 강력한 알레르겐으로 작용한다. 알레르기 병인기전은 림프구의 사이토카인 분비와 호중구의 자발적인 세포고사와 연관이 있다. 본 연구에서는 Derp-1이 림프구에서 사이토카인 분비를 유도하는지 여부와 이에 의해 호중구 세포고사 조절에 영향을 주는지를 실험 해보았다. 정상인과 알레르기 환자의 림프구에서, Derp-1에 의해 IL-6, IL-8, MCP-1그리고 GM- CSF의 분비가 증가됨을 보였다. Derp-1이 단독으로 호중구에 영향을 주는 것은 아니지만, Derp-1으로 호중구를 자극한 뒤 모은 상층액이 호중구의 세포고사를 억제시킴을 확인하였다. 정상인의 호중구와 림프구를 co-culture하여 Derp-1을 처리한 결과 호중구의 세포고사가 억제되었고, 이보다 알레르기 환자에서 시행되어진 것이 그 효과가 더 크게 나타났다. 즉, Derp-1은 사이토카인의 분비를 증가시키고, 이로 인해 정상인과 알레르기 환자의 호중구의 세포고사를 억제시킨다. 이를 통해 알레르기 질환의 병인기전을 밝히는데 유용한 자료가 될 수 있을 것으로 사료된다.

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