



Research paper

Effect of mite allergenic components on innate immune response: Synergy of protease (Group 1 & 3) and non-protease (Group 2 & 7) allergens

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ABSTRACT

The major mite allergenic components of protease allergens (group 1,3) and non-protease allergens (group 2,7) derived from *Dermatophagoides pteronyssinus* (Dp) and *D. farinae* (Df) are reported to be capable of sensitizing 80–90% of mite-allergic patients. Although protease and non-protease allergens have been demonstrated to trigger innate and adaptive immune responses through epithelium activation, the simultaneous or sequential effects of both groups of allergens has not been reported. Since all allergens are present in the mite crude extracts, it is important to determine whether these allergens can synergistically trigger the immune responses to cause airway inflammation. A total of 60 house dust mite (HDM)-allergic asthmatic patients were recruited to analyze their serum-specific IgE response to both groups of allergens. Recombinant protease allergen (Der p1 and Der p3) and non-protease allergens (Der p2 and Der p7) were used to activate the human airway epithelium cell (Beas-2B). The cells were analyzed for mRNA expression of IL-6/IL-8 and the culture supernatants were analyzed for neutrophil chemotactic activity (NCA). The results showed 48/60 (80%) HDM-allergic patients were sensitized to all allergenic components of Der p1, Der p2, Der f1, and Der f2. Most of the allergic patients were sensitized to both groups of allergens simultaneously. The associations of Der p1 with Der p2 were 83.3% (50/60) and Der f1 with Der f2 were 80% (48/60). When Beas-2B cells were cultured with Der p2 in conjunction with Der p1 and Der p3, the results showed that there was increased expression of IL-6/IL-8 in comparison with culture with allergen alone. There was only a trivial effect on IL-6/IL-8 expression when Der p2 was co-cultured with Der p7. Similar findings were obtained in the NCA measurement. When Beas-2B was cultured with Der p2 in conjunction with Der p1 and Der p3, there was increased NCA in comparison with culture with allergen alone. There were also trivial effects when Der p2 was co-cultured with Der p7. The allergens (Der p2 and Der p3)-induced IL-6/IL-8 expression and NCA released from Beas-2B could be downregulated by dexamethasone and transcription factor inhibitor SP600125. The allergenic components derived from Dp and Df can sensitize allergic patients simultaneously and activate epithelium through protease allergens (group 1, 3) and non-protease allergen (group 2) synergistically.

1. Introduction

House dust mites (HDM) are major allergens that contribute to the rising incidence of allergic diseases such as allergic bronchial asthma, perennial allergic rhinitis, and atopic dermatitis (D'Amato et al., 2005). Around 50–80% of allergic patients are sensitive to HDM, depending on the geographic location (Heinzerling et al., 2005).

The two predominant species of HDM are *Dermatophagoides pteronyssinus* (Dp) and *Dermatophagoides farinae* (Df), which can be identified in 80% of house dust collected from the carpets in Taiwan. Although at least 23 groups of allergenic components have been identified, these allergens can be further divided by protease and non-protease molecules (Banerjee et al., 2014).

Many protease allergens in HDM have been identified (groups 1, 3,

Abbreviation: Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farinae*; HDM, house dust mite; Der p1, group 1 allergen of *D. pteronyssinus*; Der p2, group 2 allergen of *D. pteronyssinus*; Der p3, group 3 allergen of *D. pteronyssinus*; Der p7, group 7 allergen of *D. pteronyssinus*; NCA, neutrophil chemotactic activity; MD-2, myeloid differentiation-2; PARs, protease activation receptors; LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction; TLR, Toll-like receptor-2; Beas-2B, human airway epithelium cell

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6 and 9). The group 1 allergen exhibits cysteine protease activity and groups 3 and 9 allergens show serine protease activity. More than 50% of HDM-allergic subjects were sensitized by protease allergens. Many non-protease allergens have also been identified (groups 2, 7 and 10). The group 2 allergens have a structural protein that exhibits functional homology with myeloid differentiation-2 (MD-2).

A high proportion of allergic subjects can be sensitized by HDM and most subjects were sensitized by protease allergens and non-protease allergens simultaneously. In addition, both groups of allergens can sensitize human subjects and the activity of each group appears to influence the other. In this study, we sought to explore the mechanisms of the interactions between these two groups of allergens. It has been demonstrated that protease allergen can activate the epithelium through enhanced expression of protease activation receptors (PARs) and similar receptors PAR2 can be activated by Der p3 and Der p9. The relationship between protease activity and chemokine production has also been reported (Kawabata and Kawao, 2005). Our recent study showed that non-protease allergen Der p2 could interact with lipopolysaccharide (LPS) and facilitating airway epithelial inflammation through the upregulation of Toll-like receptor-2 (TLR2) and secretion of IL-6/IL-8 (Yin et al., 2015). Since IL-8 is a potent chemotactic factor for neutrophils in biopsy material from patients of asthma have been reported following allergen exposure (Boulet et al., 1993). Patients that have undergone allergens challenge have shown increased numbers of neutrophils (Park and Jung, 1998; Park et al., 1999). It will be of interest to know whether allergens challenge airway epithelium could contribute to the neutrophils accumulation high IL-8.

In this study we investigated the combination effects of the protease allergen Der p1/Der p3 and non-protease allergens Der p2/Der p7 of HDM on the innate immune response of human epithelium using human epithelium cell lines. Innate immune response genes encoding for IL-6/IL-8 were analyzed by mRNA expression in the cells and neutrophil chemotactic activity (NCA) production were analyzed by measuring the neutrophil chemotactic activity in the culture supernatants.

2. Material and methods

2.1. Patient selection

Sixty Dp-allergic asthma patients were recruited from the allergy clinic of the Division of Allergy, Immunology and Rheumatology of Taichung Veterans General Hospital. Dp-specific IgE was measured using the Pharmacia CAP System (Uppsala, Sweden) and the allergenic components of Der p1/Der p2/Der f1 and Der f2 were measured using the CRD microassay-based sIgE detection assay (ImmunoCap ISAC Thermal Fisher). These subjects had a history of recurrent nasal stuffiness, nasal itching, and its impact on asthma (ARIA) guidelines (Yin et al., 2015).

2.2. Reagents

The transcription signaling factor inhibitors SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD98059 (ERK inhibitor), and BAY11-7082 (I κ B inhibitor) were purchased from Calbiochem (Merck, Germany). LPS, dexamethasone, and calcitriol were obtained from Sigma Aldrich (St. Louis, Mo, USA).

2.3. Bronchial epithelial cell line culture

The human bronchial epithelial cell line Beas-2B, purchased from the American Type Cell Collection (ATCC), was maintained in RPMI-1640 culture medium supplemented with 10% (V/V) heat-inactivated FBS, 2 mM of L-glutamine, 100 U/ml of penicillin, and 100 U/ml of streptomycin. Cells were maintained in 9-cm culture dishes at 37 °C in a humidified atmosphere with 5% CO₂. Before experimental treatments, cells were plated in appropriate culture plates with complete medium

for 24 h to allow attachment and starved in serum-free medium for another 24 h. For the allergen-stimulation studies, Beas-2B cells were treated with protease allergenic component (Der p1 or Der p3) and non-protease allergenic components (Der p2 or Der p7) for 6 h. LPS was used as a positive control and various inhibitors were used to inhibit the Der p3-stimulation including transcription signaling factor inhibitors, dexamethasone, and calcitriol. The cell pellets were collected and extracted for cytokine mRNA determination and the culture supernatant was collected for the neutrophil chemotactic activity (NCA) assay.

2.4. *in vitro* neutrophil chemotactic activity assay

A 24-well modified Boyden chamber (Neuroprobe, Inc., Gaithersburg, MD, USA) with a PVP-free polycarbonate membrane (pore diameter of 8 μ m) was used to assay cell migration for analysis of the neutrophil chemotactic activity. The cell suspension was placed into the upper half of the chamber with 50,000 cells per well, while the medium containing the various concentrations of IL-8 was placed into the lower half of the chamber. Then the chamber was incubated at 37 °C with 5% CO₂ for 90 min. After incubation, non-migrating cells were wiped away, and the filter was fixed and stained according to the manufacturer's instructions. Scion Image software was used to manage and analyze the scanning image of the membrane (Arefieva et al., 2005). After data processing, a ratio of the value of stimulated cell migration to the value of spontaneous cell migration was measured and the results were presented as "increased cell number".

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA extraction was performed using a TRIZOL reagent protocol. The concentration and purity of RNA were determined by measuring the absorbance at a wavelength of 260/280 nm in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE). First-strand cDNA was synthesized from 1 g of total RNA by reverse transcription in a 10-l reaction using a SuperScript III first-strand cDNA synthesis kit according to the manufacturer's instructions. All PCR assays were performed using PCR master mix. Primers used for PCR analyses were designed and synthesized by Clontech Laboratories (Palo Alto, CA) (Table 1). A suitable temperature cycle profile for each PCR reaction was determined using an MJ Mini Personal Thermal Cycler (Bio-Rad, Foster, CA). GAPDH was used as the internal control. The PCR products were loaded onto a 2% agarose gel and separated by electrophoresis. After ethidium bromide staining, DNA bands were visualized and photographed under UV light. The intensities of the DNA bands in the agarose gel were quantified using Gel-Pro Analyzer (Media Cybernetics, Inc., MD).

2.6. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data are presented as mean $\pm$ standard error of the mean (SEM). *p*-values < 0.05 were considered statistically significant. The paired Student's *t*-test was used to determine the statistical differences between groups. Kappa test was

Table 1
Primer sequences and sizes used in RT-PCR.

Species	Gene	F/R	Sequence	Size
Human	IL-6	F	5'-ATG AAC TCC TTC TCC ACA AGC GC-3'	628 bp
		R	5'-GAA GAG CCC TCA GGC TGG ACT G-3'	
Human	IL-8	F	5'-ATG ACT TCC AAG CTG GCC GTG GCT-3'	292 bp
		R	5'-TCT CAG CCC TCT TCA AAA ACT TCT C-3'	
Human	β -Actin	F	5'-GCA AGA GAG GCA TCC TCA CC-3'	240 bp
		R	5'-GCA CAG CCT GGA TAG CAA CG-3'	

F: forward; R: reverse; IL: interleukin.

Table 2

The patient numbers with positive specific IgE to group 1 (Der p1 and Der f1) and group 2 (Der p2 and Der f2) allergens of HDM.

		Der p1		Der p2		Der p1/Der p2	
		+	-	+	-	+	-
Der f1	+	52 ^a	0	50	3	1	0
	-	0	7	1	6	0	6
Der f2	+	48	3	50	1	48	1
	-	4	4	1	8	1	5
Der f1/Der f2	+	48	0	48	0	48	0
	-	0	5	0	5	0	5

A total of patient numbers were N = 60.

^a Data presented as subject numbers.

used for analysis of association between allergy groups 1 and 2.

3. Results

3.1. The relationships of hypersensitivity between two species of HDM (Dp and df) and between two group of allergens, group 1 (Der p1/Der f1) and group 2 (Der p2/Der f2), in allergic patients

A total of 60 patients were recruited for the measurement of specific IgE to group 1 (Der p1 and Der f1) and group 2 (Der p2 and Der f2) allergens. The association of hypersensitivity to Dp and Df was investigated. The results showed 48 patients were IgE positive to Der p1, Der p2, Der f1, and Der f2, 52 patients were IgE positive to Der p1 and Der f1, and 50 patients were IgE positive to Der p2 and Der f2 (Table 2). These results indicated that the allergenic components in the two species of HDM (Dp and Df) showed a strong association which might be due to cross-reactivity in group 1 ($p = 1.56 \times 10^{-14}$) and in group 2 ($p = 10.66 \times 10^{-11}$) (Table 3).

The consistency of hypersensitivity between house dust mite allergen group 1 (Der p1/Der f1) and group 2 (Der p2/Der f2) was investigated. The IgE-positive sera were analyzed for the consistency of hypersensitivity to Der p1 and Der p2 in Dp, and Der f1 and Der f2 in Df. There were 52/49 patients allergic to Der p1/Der p2 and 52/48 patients allergic to Der f1/Der f2. The consistency of hypersensitivity to group 1 and group 2 was 0.658 ($p = 3.3 \times 10^{-7}$) in Dp and 0.521 ($p = 5.3 \times 10^{-5}$) in Df (Table 4). These results indicate that group 1 and group 2 allergenic components can sensitize patients simultaneously.

3.2. The effects of Der p2 in conjunction with or without Der p1 or Der p3 on the mRNA expression of IL-6/IL-8 from Beas-2B cells

When Beas-2B was cultured with Der p2 alone there were only trivial effects of Der p2 on IL-6/IL-8 expression. However when Beas-2B was cultured with Der p2 in conjunction with Der p1 or Der p3, the Der p1- and Der p3-induced IL-6/IL-8 could be further upregulated by Der p2 (Fig. 1).

Table 3

The agreement of hypersensitivity between group 1 (Der p1 and Der f1) and group 2 (Der p2 and Der f2).

	Der p1	Der p2	Der p1/Der p2
Der f1	1.56×10^{-14}	2.47×10^{-8}	7.25×10^{-14}
Der f2	3.34×10^{-4}	10.66×10^{-11}	1.65×10^{-9}
Der f1/Der f2	3.31×10^{-13}	3.31×10^{-13}	3.31×10^{-13}

* Data presented as p value.

Table 4

The patient numbers with positive/negative specific IgE to Der p1/Der p2 (A) and Der f1/Der f2 (B).

A.		Der p1		B.		Der f1	
		+	-			+	-
Der p2	+	49 ^a	2	Der f2	+	48	3
	-	3	6		-	4	5

^aData presented as subject numbers.

3.3. The effects of Der p2 in conjunction with or without Der p7 or LPS on the mRNA expression of IL-6/IL-8 from Beas-2B cells

In order to example the effect of Der p2 in conjunction with or without Der p7 or LPS, the mRNA expressions of IL-6 and IL-8 from Beas-2B cells were evaluated by RT-PCR. After the Beas-2B was treated with Der p2 alone, the expressions of IL-6 and IL-8 were slightly increased. When Beas-2B was cultured with Der p2 in conjunction with Der p7, the results showed that there were only trivial effects of Der p7 on IL-6/IL-8 expression (Fig. 2). The expressions of IL-6 and IL-8 were more significantly induced by Der p2 in conjunction with LPS.

3.4. The effects of various inhibitory agents on IL-6/IL-8 expression in der p3 and der p2 & der p3-stimulated Beas-2B cells

Beas-2B cells were cultured with Der p3 (25 ng/ml) or Der p2 (5 µg/ml) & Der p3 (25 ng/ml) for 6 h in the presence of various inhibitors, and the cell pellets were then collected for mRNA expression followed by RT-PCR amplification. The results showed the expressions of IL-6 and IL-8 were amount two-fold increased in Der p2 & Der p3 stimulation when comparing with Der p3 alone. When Beas-2B was cultured with Der p2 and Der p3 in the presence of various inhibitors, the expressions of IL-6 or IL-8 were down-regulated. The most effective inhibitors of IL-6/IL-8 expression were transcription-signaling inhibitors SP600125 (JNK inhibitor) and SB203580 (p38 inhibitors) as well as dexamethasone (Fig. 3).

3.5. The effects of protease and non-protease allergens on NCA production from Beas-2B and inhibition by inhibiting agents

When Beas-2B was cultured with Der p2 alone or Der p2 in conjunction with Der p1 or Der p3 or Der p7 to determine the effects on the NCA production from Beas-2B cells, the results showed that there were trivial effects of Der p2 on NCA production. However, when Beas-2B was cultured with Der p2 in conjunction with Der p1 or Der p3, the Der p3-induced NCA could be further upregulated by Der p2. The synergistic effect of Der p2 and Der p1/Der p3 on NCA activity was similar to that of Der p2 and LPS. There was only trivial NCA activity in the supernatant of Der p2 in conjunction with Der p7 (Fig. 4). The NCA activity in the culture supernatant co-stimulated with Der p2 and Der p3 could be down-regulated by SP600125 to a similar extent to that achieved by dexamethasone (Fig. 5).

4. Discussion

In this study we demonstrated that both protease allergenic component (Der p1 and Der p3) and non-protease allergenic component (Der p2) could activate human airway epithelium synergistically to increase the mRNA expression of IL-6/IL-8 and facilitate the secretion of NCA in the culture supernatant. A similar previous finding demonstrated that Der p1 could induce IL-6 and IL-8 production in the cultured human nasal epithelium (Shi et al., 2010). Our previous study showed that Der p2 could induce IL-6/IL-8 expression, especially in the presence of LPS. This further demonstrated that Der p2 could activate epithelium in the presence of Der p1 or Der p3 (Yin et al., 2015). Since

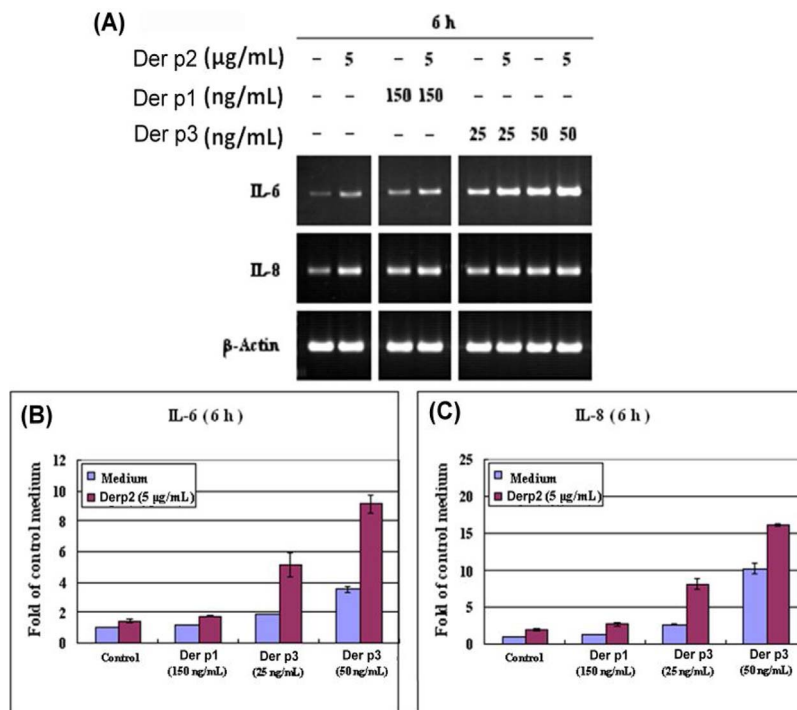


Fig. 1. The effects of Der p2 in conjunction with or without Der p1 or Der p3 on the mRNA expression of IL-6/IL-8 from Beas-2B cells. (A) Beas-2B cells were cultured with Der p2 in conjunction with or without Der p1 or Der p3 for 6 h, and then the mRNA of cell pellets were collected, isolated and analyzed by RT-PCR. (B) The expression of mRNA encoding for IL-6. Quantitative data on band densities were obtained by densitometric analysis and results were presented as the mean $\pm$ SEM of three independent experiments. (C) The expression of mRNA encoding for IL-8, the quantitative data were presented as the mean $\pm$ SEM of three independent experiments.

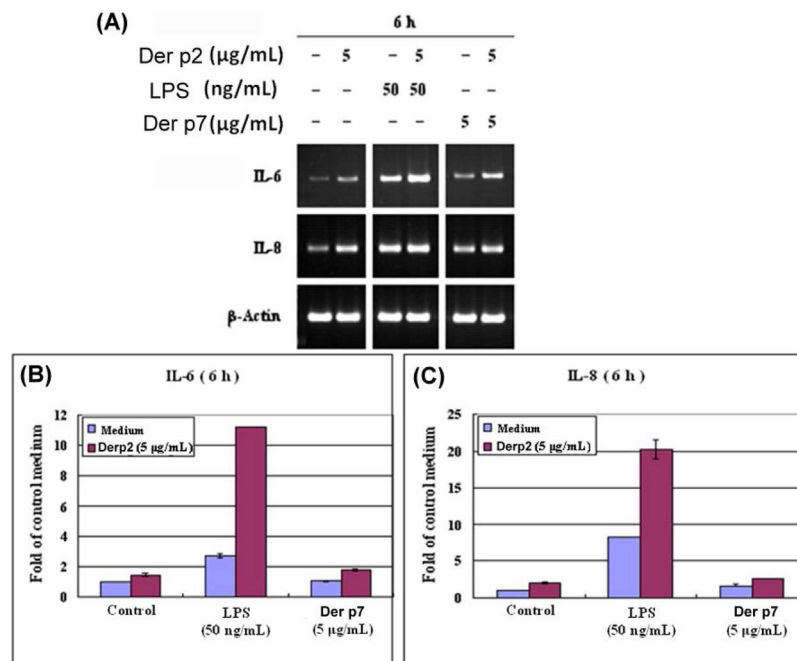


Fig. 2. The effects of Der p2 in conjunction with or without Der p7 or LPS on the mRNA expression of IL-6/IL-8 from Beas-2B cells. (A) Beas-2B cells were cultured with Der p2 in conjunction with Der p7 or LPS for 6 h, and then the mRNA of cell pellets were collected, isolated and analyzed by RT-PCR. (B) The expression of mRNA encoding for IL-6. Quantitative data on band densities were obtained by densitometric analysis and results were presented as the mean $\pm$ SEM of three independent experiments. (C) The expression of mRNA encoding for IL-8, the quantitative data were presented as the mean $\pm$ SEM of three independent experiments.

all allergenic components in HDM are mixed together in the natural environment, and can be inhaled into the airway simultaneously, it is important to determine whether both protease and non-protease allergenic components can sensitize the epithelium together synergistically.

Since there are many allergenic components be classified as protease in HDM including Der p1 (cysteine protease), Der p3 (trypsin-like serine protease), and Der p9 (collagenolytic serine protease) (Thomas et al., 2002). It is conceivable that all protease components are capable of activating protease-activated receptors (PARs). PARs are widely expressed in the airway epithelium and play a variety of pathophysiological roles in inflammation. It was previously reported that protease allergens of HDM, Der p3 and Der p9, are capable of activating PAR-2 and stimulating release of pro-inflammatory cytokines in the human

airway epithelium (Sun et al., 2001; Asokanathan et al., 2002). The mechanism of Der p1-initiated nasal allergic response has been demonstrated through the expression of PAR2, PI3 K, and NF-κB signaling (Shi et al., 2010).

The major allergen Der p2 has been identified as an MD-2-related lipid recognition protein. MD2 forms a stable complex with TLR4 on the cell surface and both have been implicated in LPS recognition (Trompette et al., 2009). TLR4 stimulation effectively potentiates human B cell activation and production of antibodies in T-dependent immune responses. Our previous reports showed that Der p2 and LPS can synergistically augment TLR-mediated pro-inflammatory signaling (Tsai et al., 2011). In previous reports, Der p2 could activate epithelium to secrete IL-6/IL-8 through the upregulation of TLR2 expression and

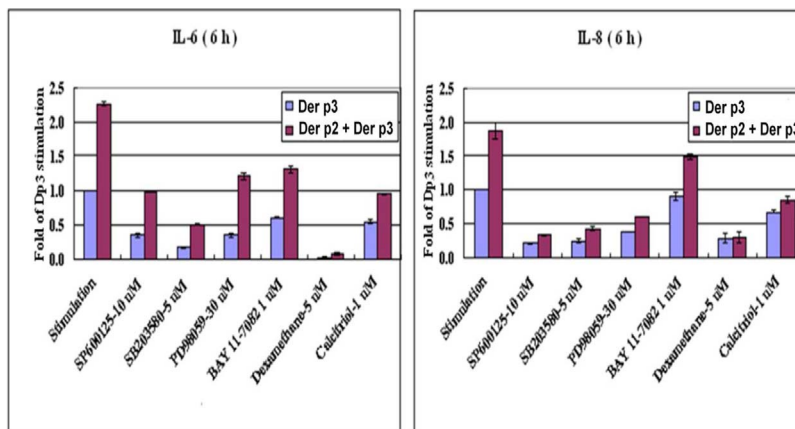


Fig. 3. The effects of various inhibitory agents on IL-6/IL-8 expression in Der p3 and Der p2 + Der p3-stimulated Beas-2B cells. Beas-2B cells were cultured with Der p3 or Der p2 + Der p3 for 6 h in the presence of various inhibitors, and the cell pellets were then collected for mRNA expression followed by RT-PCR amplification. The most effective inhibitors of IL-6/IL-8 expression were dexamethasone and transcription-signaling inhibitors SP600125 (JNK inhibitor) as well as SB203580 (p38 inhibitors). Data presented as the mean $\pm$ SEM of 3 independent experiments.

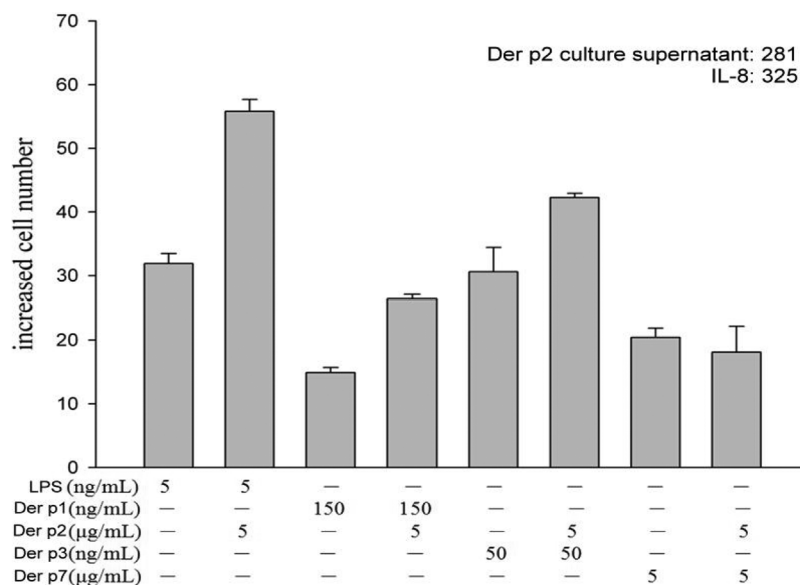


Fig. 4. The effect of Der p2 and combination of Der p2 with Der p1 or Der p3 or Der p7 on the NCA production from Beas-2B cells. Beas-2B cells were cultured with various stimulation conditions for 6 h, and the cultured supernatants were then collected for NCA assay. The neutrophil chemotactic assay was performed to measure the biologic activity of NCA in the supernatant. The increased cell number was expressed in comparison with the medium control. The chemotactic activity of IL-8 was used as the positive control. Data were presented as the mean $\pm$ SEM of 3 independent experiments.

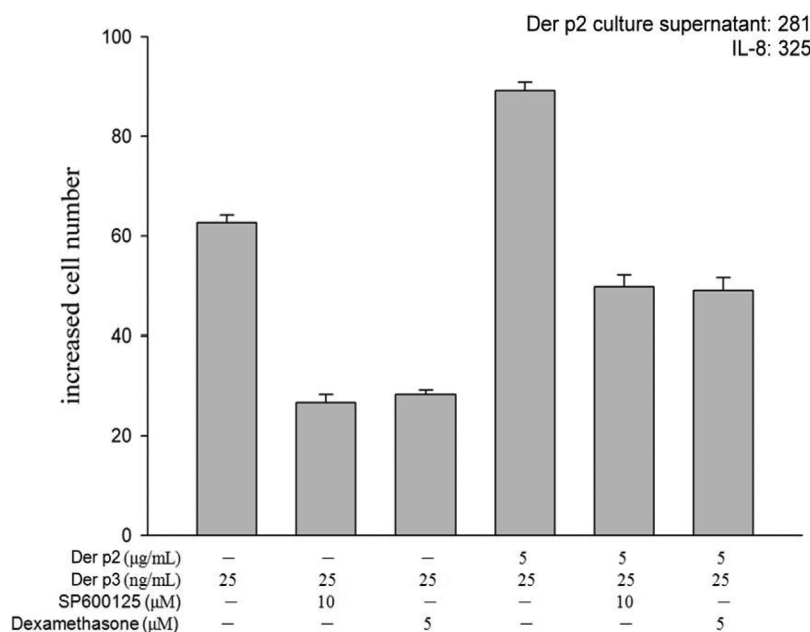


Fig. 5. The effects of SP600125 and Dexamethasone on NCA production from Der p3 or Der p2 + Der p3-stimulated Beas-2B cells. Beas-2B cells were cultured with various stimulation conditions for 6 h, and the cultured supernatants were collected for NCA assay. The neutrophil chemotactic assay was performed to measure the biologic activity of NCA in the supernatant. The increased cell number was expressed in comparison with the medium control. The chemotactic activity of IL-8 was used as the positive control. Data were presented as the mean $\pm$ SEM of 3 independent experiments.

NFkappa B signaling (Yin et al., 2015).

In this study, the epithelium could be synergistically activated by protease Der p1/Der p3 and non-protease Der p2 simultaneously. Whether these two receptors (TLR/PAR) can be activated synergistically remains to be further investigated. Our study showed that the airway epithelium could be activated and produced NCA by protease Der p1/Der p3 and non-protease Der p2 allergens synergistically, indicating that these allergens might play a role in the pathogenesis of neutrophil infiltration in allergic inflammation. Neutrophilic infiltration in the allergic airway has been reported in chronic asthma and has been regarded as the first cell recruited to the site of allergic reaction (Monteseirín, 2009). However, its role has not been fully elucidated. This study provides evidence that protease and non-protease allergens might activate Beas-2B and contribute to the production of neutrophil chemotaxis activity without any other bacteria or virus infection.

In conclusion, the allergenic components of HDM can trigger neutrophil-related allergic inflammation through airway epithelium activation by both protease allergens Der p1/Der p3 in conjunction with the non-protease allergen Der p2. Although a part of allergic components have been used for allergy diagnosis and therapy, our study findings suggest that both protease and non-protease allergens should be used simultaneously in clinical practice in order to obtain better results.

Conflict of interest

There are no financial or other issues that might lead to conflict of interest.

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