

***Dermatophagoides farinae* Upregulates the Effector Functions of Eosinophils through $\alpha\text{M}\beta_2$ -Integrin and Protease-Activated Receptor-2**

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Keywords

Dermatophagoides farinae · Eosinophils · Integrin · House dust mite · Protease-activated receptor-2

Abstract

Background: Even in subjects who are not sensitized to house dust mite (HDM), allergic symptoms can be aggravated by exposure to dust, suggesting that innate immune responses may be involved in these processes. Since eosinophils express pattern recognition receptors, HDM may directly upregulate eosinophil functions through these receptors. The objective of this study was to examine whether *Dermatophagoides farinae* (*Df*), a representative HDM, or Der f 1, a major allergen of *Df*, modifies the effector functions of eosinophils. **Methods:** Eosinophils isolated from the blood of healthy donors or allergic patients were stimulated with *Df* extract or Der f 1, and their adhesion to recombinant human intercellular adhesion molecule (ICAM)-1 was measured using eosinophil peroxidase assays. Generation of the eosinophil superoxide anion (O_2^-) was examined based on the superoxide dismutase-inhibitable reduction of cytochrome C. Eosinophil-derived neurotoxin (EDN) concentrations in cell media were measured by ELISA as a marker of degranulation. **Results:** *Df* extract or Der f 1 directly induced

eosinophil adhesion to ICAM-1, O_2^- generation, and EDN release. Anti- αM - or anti- β_2 -integrin antibodies or protease-activated receptor (PAR)-2 antagonists suppressed the eosinophil adhesion, O_2^- generation, and EDN release induced by *Df* extract or Der f 1. Eosinophils from allergic patients showed higher adhesion to ICAM-1 than those from healthy donors. **Conclusions:** These findings suggested that *Df* extract and Der f 1 directly activate eosinophil functions through $\alpha\text{M}\beta_2$ -integrin and PAR-2. Eosinophil activation by HDM may play roles in the aggravation of allergic symptoms, not only in HDM-sensitized patients, but also in nonsensitized patients.

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Introduction

Bronchial asthma is characterized by airway inflammation, reversible airway obstruction, and airway hyperresponsiveness [1]. Although a variety of cells are involved in the process of airway inflammation of asthma, eosinophils play important roles in its pathophysiology through the release of a variety of inflammatory media-

Edited by: H.-U. Simon, Bern.

tors including their specific granule proteins, cysteinyl leukotrienes, reactive oxygen species, and cytokines [2, 3].

The house dust mite (HDM) is a significant cause of allergic disease such as bronchial asthma or allergic rhinitis [4]. *Dermatophagoides farinae* (Df)/*Dermatophagoides pteronyssinus* (Dp) is an important HDM, and Der f 1/Der p 1 is a major allergen of Df/Dp [4–6]. HDM plays an important role in the development of allergic sensitization and disease [7–9]. For example, high exposure to HDM increases the possibility of HDM sensitization [7, 8]. Furthermore, in the case of sensitized patients, allergen exposure induces allergic symptoms through degranulation of mast cells or basophils by immunoglobulin (Ig) E cross-linking and subsequent T-cell activation. However, clinically, it has been found that HDM or pollen exposure induces allergic symptoms even in nonsensitized subjects [10, 11]. Although the mechanism of action of this phenomenon has not been fully clarified, one possibility is that innate immune responses may be involved in the development of allergic inflammation in a nonspecific manner.

Eosinophils express pattern recognition receptors such as Toll-like receptors (TLRs) and protease-activated receptors (PARs) [4, 12]. PARs are receptors that are activated by proteolytic cleavage of their extracellular N terminus by a protease. PAR-2 is a PAR that has been associated with inflammatory response to infection and microbial proteases [4]. As HDM has protease activity, several reports have suggested the involvement of PAR-2 in the HDM-induced immune response [4, 13–20]. However, the effect of HDM on eosinophil functions and its mechanism of action have not been fully elucidated.

In this study, we investigated whether Df extract or Der f 1 could directly modify eosinophilic functions. We found that Df extract and Der f 1 induced eosinophil adhesion, superoxide anion (O_2^-) generation and degranulation. These effects were mediated by the $\alpha M\beta_2$ -integrin and partly by PAR-2. Therefore, direct eosinophil activation by HDM may play roles in the induction or aggravation of allergic symptoms.

Material and Methods

Preparation of Eosinophils and Df

Eosinophils were isolated from peripheral blood collected from nonallergic healthy volunteers with a peripheral blood differential eosinophil count of <5%. Healthy volunteers were defined as subjects with no history of allergic disease such as asthma or rhinitis and no symptoms such as coughing or sneezing associated with allergic disease. We measured allergen-specific IgE antibodies (Abs), and no subject had specific IgE Abs to environmental aller-

gens such as HDM (Df/Dp), molds, or Japanese cedar pollen. In selected experiments, we collected eosinophils from allergic subjects who have perennial or seasonal allergic rhinitis without taking any medication. We confirmed that all allergic subjects had serum IgE Abs specific for either HDM (Df/Dp) or Japanese cedar pollen. The numbers of males and females were comparable among the donors. This study was approved by the Ethical Committee of Saitama Medical University, and written informed consent was obtained before collection of each blood sample. Eosinophils were isolated by the combination of Percoll density gradient centrifugation and negative selection using anti-CD16 Ab-coated magnetic beads (Miltenyi Biotec, Auburn, CA, USA), as previously described [21–26]. Over 98% of the cells were eosinophils, as determined by morphological criteria using May-Grünwald-Giemsa staining. Eosinophil viability was >99%, as determined by Trypan blue dye exclusion. Eosinophils were resuspended in Hanks' balanced salt solution (HBSS) supplemented with gelatin to a final concentration of 0.1% (HBSS/gel).

Df was collected and a dried extract prepared as described previously [27]. Concentrations of Der f 1 and Der f 2 were 4.8 $\mu\text{g}/\text{mg}$ extract and 4.0 $\mu\text{g}/\text{mg}$ extract, respectively [27]. We measured lipopolysaccharide (LPS) concentrations of Df extract by using an endotoxin-specific chromogenic Limulus test (Endospecy test, Seikagaku Corporation, Tokyo, Japan) in this study and found that the LPS concentration of Df extract at 1,000 ng/mL was 10.7 ± 0.9 pg/mL ($n = 8$). We confirmed that LPS at 10–1,000 pg/mL did not activate eosinophil functions including adhesion or O_2^- generation (data not shown). Der f 1, purified from mite culture by affinity chromatography, was obtained from Indoor Biotechnologies Inc. (Charlottesville, VA, USA).

Eosinophil Adhesion

The effect of Df extract or Der f 1 on eosinophil adhesion to recombinant human (rh) intercellular adhesion molecule (ICAM)-1-coated plates was assessed based on the residual eosinophil peroxidase activity of adherent eosinophils, as previously described [21–26]. Briefly, eosinophils ($100 \mu\text{L}$ of 1×10^5 cells/mL in HBSS/gel) from nonallergic volunteers or allergic subjects were incubated in the presence or absence of Df extract (10–1,000 ng/mL) or Der f 1 (10–1,000 pg/mL) in rh-ICAM-1-coated plates ($10 \mu\text{g}/\text{mL}$; R&D Systems, Minneapolis, MN, USA) at 37°C for 20 min. Corresponding control wells were coated with HBSS/gel. The plates were washed with HBSS and $100 \mu\text{L}$ of HBSS/gel was then added to the wells. Standards that were comprised of $100 \mu\text{L}$ of serially diluted cell suspensions (1×10^3 , 3×10^3 , 1×10^4 , 3×10^4 , and 1×10^5 cells/mL) were added to the empty wells. The eosinophil peroxidase substrate (1 mM *o*-phenylenediamine, 1 mM H_2O_2 , and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all wells, and the plates were incubated for 30 min at room temperature. The reaction was stopped by adding $20 \mu\text{L}$ of 4 M H_2SO_4 , and absorbance was measured at 490 nm. In some experiments, suspended eosinophils were preincubated with mouse IgG1 (3 $\mu\text{g}/\text{mL}$; clone MOPC-21; Becton Dickinson, Franklin Lakes, NJ, USA), anti- αM integrin monoclonal (m) Ab (3 $\mu\text{g}/\text{mL}$; clone 2LPM19c; Pierce, Rockford, IL, USA), anti- β_2 -integrin mAb (3 $\mu\text{g}/\text{mL}$; clone L130; Becton Dickinson), a cysteine protease inhibitor (100 μM ; E64, Sigma-Aldrich, St. Louis, MO, USA), a serine protease inhibitor (100 μM ; AEBSE, Sigma-Aldrich), or a PAR-2 antagonist (10 μM FSLLRY-NH2 [28–30], R&D Systems, or 10 μM ENMD-1068 [31–33], Enzo Life Sciences, Farmingdale, NY, USA) for 15

min before addition to the wells. Each experiment was performed in quadruplicate using eosinophils from a single donor, and the percentage eosinophil adhesion was determined from mean values that were calculated from log dose response curves. Eosinophil viability after incubation was >98%, as determined by Trypan blue dye exclusion.

Eosinophil O₂⁻ Generation

Eosinophil O₂⁻ generation was measured in 96-well ELISA plates as previously described based on the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C [22–24]. We initially added SOD (0.2 mg/mL in HBSS/gel; 20 µL) to SOD control wells and then HBSS/gel to all wells of rh-ICAM-1-coated plates (10 µg/mL) to bring the final volume to 100 µL. The eosinophil density was adjusted to 1.25×10^6 cells/mL of HBSS/gel mixed in a 4:1 ratio with cytochrome C (12 mg/mL of HBSS/gel), and 100 µL of eosinophil suspension was then added to all wells. Immediately after adding *Df* extract (100 or 1,000 ng/mL) or Der f 1 (100 or 1,000 pg/mL) to the eosinophils, the absorbance of the cell suspensions in the wells was measured at 550 nm in an Immuno-Mini (NJ-2300; Japan Intermed Co., Tokyo, Japan), followed by repeated measurements over the next 240 min. In some experiments, suspended eosinophils were preincubated with 3 µg/mL of mouse IgG1, anti-αM-integrin mAb, anti-β₂-integrin mAb, or 10 µM of a PAR-2 antagonist (FSLRY-NH₂ or ENMD-1068) for 15 min before addition to the wells. Each reaction was evaluated in duplicate against the control reaction in wells containing 20 µg/mL of SOD. The results were adjusted for a 1-mL reaction volume, and O₂⁻ generation was calculated at an extinction coefficient of 21.1 mM⁻¹cm⁻¹, as nanomoles of cytochrome C reduced per 1.0×10^6 cells/mL minus the SOD control. The maximum value observed over the incubation time was determined for evaluation of the effects of various factors on eosinophil O₂⁻ generation. Cell viability, as determined by Trypan blue exclusion at the end of each experiment, remained at 95% after a 240-min incubation.

Eosinophil Degranulation

Eosinophils (1×10^6 cells/mL) in 96-well plates were incubated for the 240 min that were required for measurement of O₂⁻ generation and were then immediately centrifuged (1,500 rpm) at 4°C for 10 min. Recovered cell-free supernatants were measured for eosinophil-derived neurotoxin (EDN), as described previously [22–24]. Concentrations of EDN were quantified using ELISA kits (Medical and Biological Laboratory Co. Ltd., Nagoya, Japan).

Statistical Analysis

Values are expressed as means ± SEM. Results were compared using a one-way analysis of variance followed by the Tukey-Kramer test when differences were significant, or a paired *t* test for analysis of the differences between two groups. Values of *p* < 0.05 were considered statistically significant.

Results

Effect of Df Extract or Der f 1 on Eosinophil Adhesion

We initially examined whether *Df* extract directly modifies eosinophil adhesiveness. We found that *Df* ex-

tract (100 or 1,000 ng/mL) significantly enhanced the adhesiveness of eosinophils to plastic plates compared with controls (data not shown), suggesting that *Df* extract modifies the adhesive interactions of eosinophils. To further confirm this hypothesis, we investigated the effect of *Df* extract on eosinophil adhesion to a recombinant adhesion molecule. Eosinophils were stimulated with *Df* extract (10–1,000 ng/mL) in the presence of immobilized rh-ICAM-1, and eosinophil adhesion was then evaluated. Under these conditions, *Df* extract at a concentration of 1,000 ng/mL significantly enhanced adhesion to ICAM-1, compared with the control (spontaneous, $4.5 \pm 0.5\%$; *Df* extract (1,000 ng/mL), $8.5 \pm 0.6\%$; *p* < 0.05; Fig. 1a). Next, we examined whether Der f 1, a major allergen of *Df*, can modify eosinophil adhesion. Der f 1 at a concentration of 100 or 1,000 pg/mL enhanced eosinophil adhesion to ICAM-1 compared with control (spontaneous, $2.3 \pm 0.4\%$; Der f 1 (100 pg/mL), $6.9 \pm 1.1\%$; *p* < 0.05; Der f 1 (1,000 pg/mL), $9.3 \pm 0.7\%$; *p* < 0.01; Fig. 1b). When we compared the adhesion of eosinophils from allergic patients with that from healthy volunteers, adhesion induced by *Df* extract or Der f 1 in allergic patients was higher than that in healthy volunteers (Fig. 1c, d; *p* < 0.05 for both allergens).

Effects of Anti-Integrin mAbs on Eosinophil Adhesion Enhanced by Df Extract or Der f 1

To identify the eosinophil integrin(s) involved in *Df* extract- or Der f 1-induced eosinophil adhesion to ICAM-1, we incubated eosinophils with anti-αM-integrin mAb, anti-β₂-integrin mAb, or an isotype-matched control mouse IgG1 for 15 min and then evaluated eosinophil adhesion. Eosinophil adhesion that was enhanced by *Df* extract (1,000 ng/mL) was inhibited by anti-αM-integrin mAb or anti-β₂-integrin mAb (Fig. 2a). Anti-α₄-integrin mAb did not modify the *Df* extract-enhanced eosinophil adhesion (data not shown). Furthermore, eosinophil adhesion enhanced by Der f 1 (100 pg/mL) was also inhibited by anti-αM-integrin mAb or anti-β₂-integrin mAb (Fig. 2b). Neither anti-αM-integrin mAb nor anti-β₂-integrin mAb modified spontaneous eosinophil adhesion (Fig. 2a, b).

Effect of Cysteine Protease Inhibitor, Serine Protease Inhibitor, or PAR-2 Antagonists on Eosinophil Adhesion Enhanced by Df Extract or Der f 1

We then examined the effect of cysteine protease inhibitor or serine protease inhibitor on *Df* extract- or Der f 1-induced eosinophil adhesion. Eosinophils were incubated with a cysteine protease inhibitor (E64) or serine

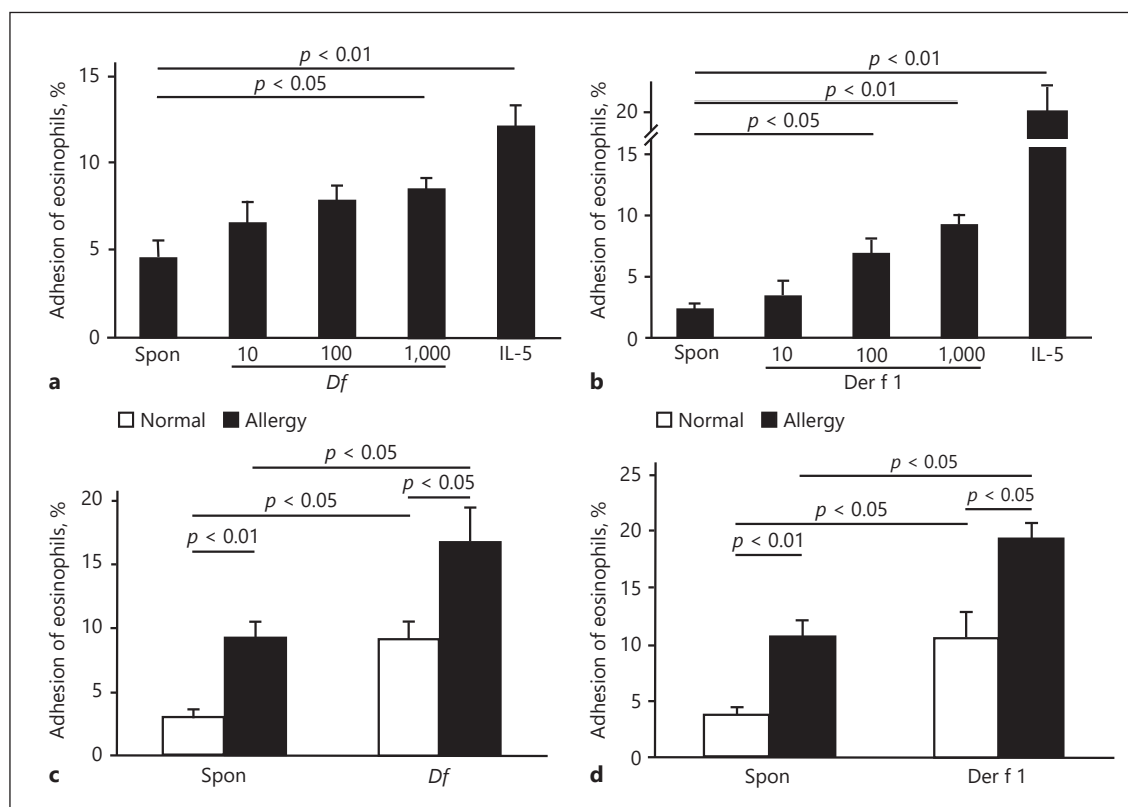


Fig. 1. Effect of *Df* extract and Der f 1 on eosinophil adhesion. **a** *Df* extract significantly augments eosinophil adhesion to rh-ICAM-1-coated plates. Eosinophils ($100 \mu\text{L}$ of 1×10^5 cells/mL in HBSS/gel) obtained from healthy donors were incubated with *Df* extract (10–1,000 ng/mL) or positive control IL-5 (100 pM) in rh-ICAM-1-coated plates. The adhesiveness of the eosinophils was then assessed by assay of residual eosinophil peroxidase activity. Data are shown as means $\pm$ SEM of 4 experiments using cells from different donors. **b** Der f 1 augments eosinophil adhesion to rh-ICAM-

1-coated plates. Eosinophils from healthy donors were incubated with Der f 1 (10–1,000 pg/mL) or IL-5 (100 pM), and then eosinophil adhesion to ICAM-1 was measured ($n = 5$). **c, d** Effect of allergy on *Df* extract- or Der f 1-induced eosinophil adhesion. Eosinophils collected from healthy volunteers or allergic subjects were incubated with *Df* extract (1,000 ng/mL) (**c**) or Der f 1 (100 pg/mL) (**d**), and eosinophil adhesion to ICAM-1 was measured ($n = 7$). Spon, spontaneous adhesion.

protease inhibitor (AEBSF) for 15 min, and then the adhesion was evaluated. The serine protease inhibitor, but not the cysteine protease inhibitor, suppressed *Df* extract-induced eosinophil adhesion (Fig. 3a, c). On the other hand, both cysteine protease inhibitor and serine protease inhibitor suppressed Der f 1-induced eosinophil adhesion (Fig. 3b, d). Combination of cysteine and serine protease inhibitors completely suppressed *Df* extract- or Der f 1-induced eosinophil adhesion (Fig. 3e, f), suggesting that *Df* or Der f 1 induces eosinophil adhesion by protease. These findings suggested that *Df* extract induces eosinophil adhesion mainly through serine protease, whereas Der f 1 activates eosinophils through both cysteine and serine protease.

We next examined whether PAR-2 is involved in *Df* extract- or Der f 1-induced eosinophil adhesion to ICAM-1. PAR-2 antagonists (FSLRY-NH2 or ENMD-1068) inhibited the eosinophil adhesion that was enhanced by *Df* extract (Fig. 3g). Furthermore, PAR-2 antagonists (FSLRY-NH2 or ENMD-1068) also inhibited the eosinophil adhesion that was enhanced by Der f 1 (Fig. 3h). These findings suggested that *Df* extract or Der f 1 could upregulate eosinophil adhesion via PAR-2.

Effect of *Df* Extract or Der f 1 on Eosinophil O_2^- Generation

We next examined whether *Df* extract or Der f 1 modifies O_2^- generation of eosinophils. *Df* extract at a concentration of 1,000 ng/mL significantly activated eosinophil

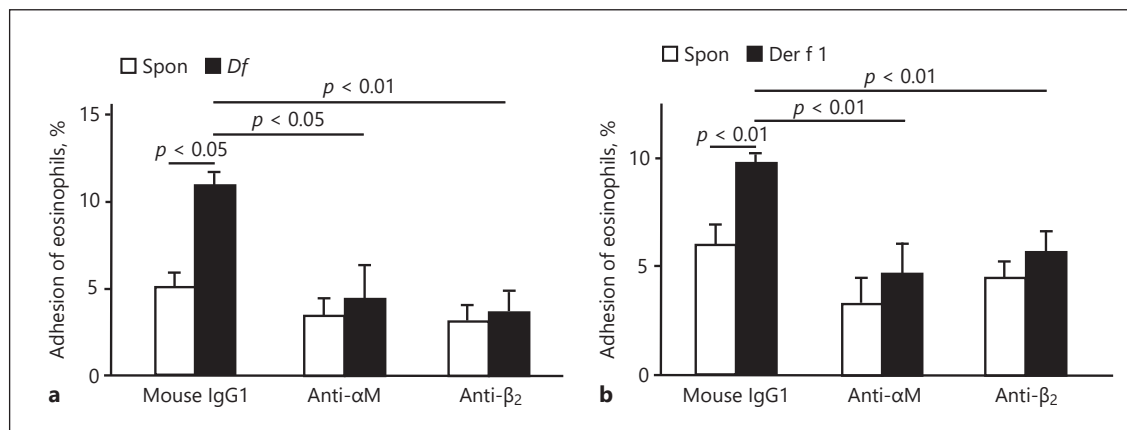


Fig. 2. Effect of anti-integrin mAbs on *Df* extract- or Der f 1-induced eosinophil adhesion. **a, b** *Df* extract or Der f 1-enhanced eosinophil adhesion to ICAM-1 is inhibited by anti-αM- or anti-β₂-integrin mAb. Eosinophils were pre-incubated with anti-αM-integrin mAb, anti-β₂-integrin mAb, or control IgG (3 μg/mL each) for 15 min prior to analysis of adhesion to ICAM-1 in the presence or absence of *Df* extract (1,000 ng/mL) (**a**) (*n* = 4) or Der f 1 (100 pg/mL) (**b**) (*n* = 5). Spon, spontaneous adhesion.

O₂⁻ generation in wells coated with ICAM-1 compared with controls (spontaneous, 0.1 ± 0.0 nmol/10⁶ cells; *Df* extract (1,000 ng/mL), 0.9 ± 0.2 nmol/10⁶ cells; *p* < 0.05; Fig. 4a). Furthermore, Der f 1 at a concentration of 100 or 1,000 pg/mL significantly activated eosinophil O₂⁻ generation compared with controls (spontaneous, 0.4 ± 0.1 nmol/10⁶ cells; Der f 1 (100 pg/mL), 1.7 ± 0.3 nmol/10⁶ cells; *p* < 0.05; Der f 1 (1,000 pg/mL), 2.1 ± 0.3 nmol/10⁶ cells; *p* < 0.01; Fig. 4b). Eosinophil O₂⁻ generation induced by *Df* extract or Der f 1 was blocked by anti-αM-integrin mAb or anti-β₂-integrin mAb (Fig. 4c, d). Furthermore, eosinophil O₂⁻ generation induced by *Df* extract or Der f 1 was partially suppressed by PAR-2 antagonists (Fig. 4e, f). Neither anti-integrin mAb nor PAR-2 antagonists modified spontaneous O₂⁻ generation by eosinophils.

Effect of *Df* Extract or Der f 1 on EDN Release

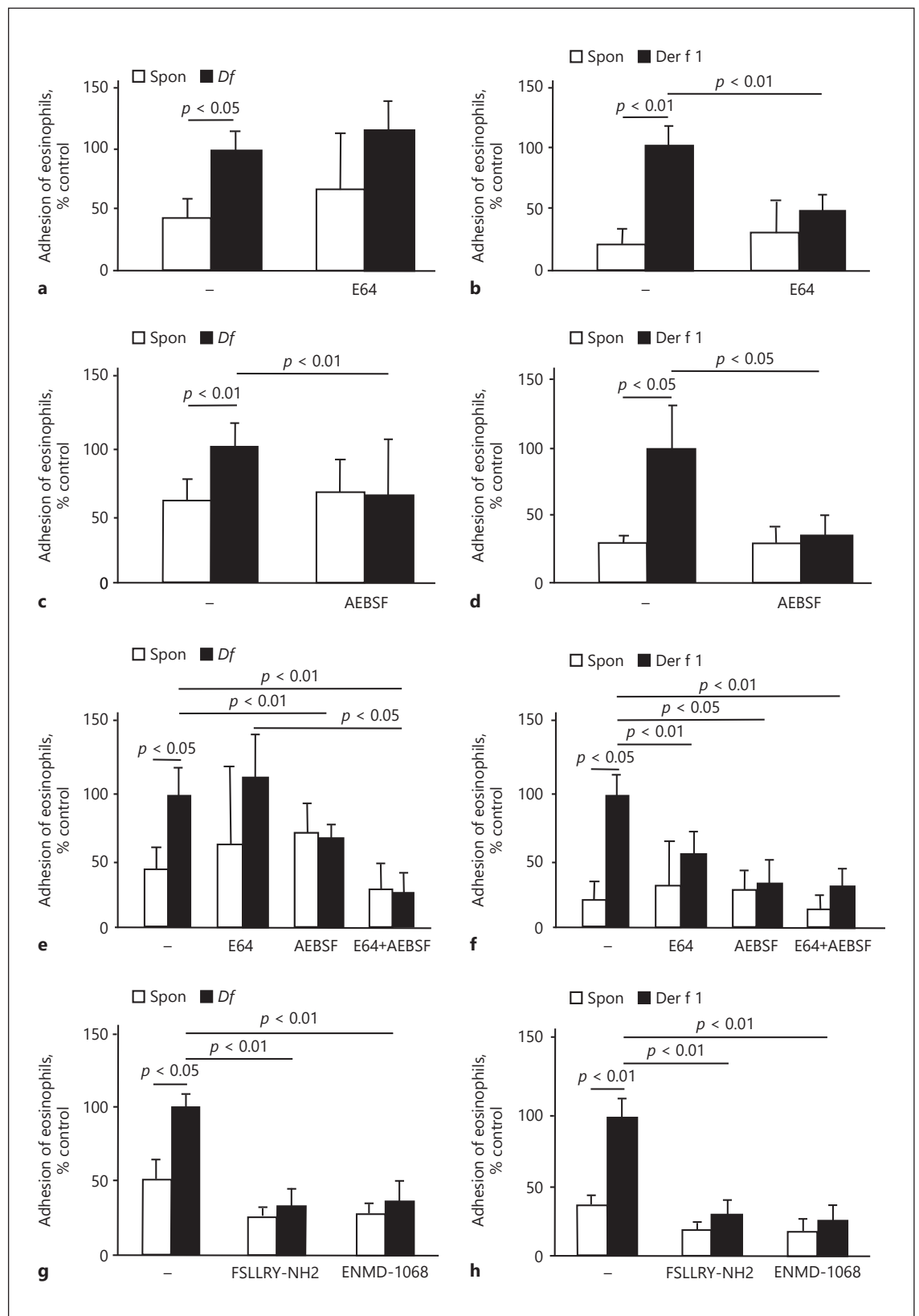
Finally, we examined the effect of *Df* extract or Der f 1 on the degranulation of eosinophils. Compared to control, *Df* extract at a concentration of 100 or 1,000 ng/mL induced significantly higher EDN release (control, 17.2 ± 1.9 ng/mL/10⁶ cells; *Df* extract (100 ng/mL), 51.8 ± 3.5 ng/mL/10⁶ cells; *p* < 0.05; *Df* extract (1,000 ng/mL), 85.9 ± 11.0 ng/mL/10⁶ cells; *p* < 0.01; Fig. 5a). Der f 1 at a concentration of 100 or 1,000 pg/mL also induced higher EDN release compared to control (control, 17.2 ± 1.9 ng/mL/10⁶ cells; Der f 1 (100 pg/mL), 49.8 ± 2.8 ng/mL/10⁶ cells; *p* < 0.05; Der f 1 (1,000 pg/mL), 71.0 ± 3.6 ng/mL/10⁶

cells; *p* < 0.01; Fig. 5b). Anti-αM-integrin or anti-β₂-integrin mAb suppressed the EDN release induced by *Df* extract or Der f 1 (Fig. 5c, d). Furthermore, PAR-2 antagonists partially suppressed EDN release induced by *Df* extract or Der f 1 (Fig. 5e, f).

Discussion

In this study, we found that *Df* extract and Der f 1 induce eosinophil adhesion, O₂⁻ generation and degranulation. Anti-αM-integrin mAb or anti-β₂-integrin mAb suppressed the induction of eosinophil adhesion, O₂⁻ generation, or EDN release, suggesting that the effect of *Df* extract or Der f 1 involves eosinophil αMβ₂-integrin. The effect of Der f 1 on EDN release from eosinophils was consistent with the results of a previous study by Miike and Kita [34]. Furthermore, PAR-2 antagonists partially inhibited eosinophil activation by *Df* extract or Der f 1. These findings suggested that *Df* extract or Der f 1 activated eosinophilic functions by the αMβ₂-integrin and partly via PAR-2. Therefore, direct eosinophil activation by *Df* may play roles in the induction or aggravation of allergic symptoms.

Df extract and Der f 1 significantly induced eosinophil adhesion, even in healthy donors without *Df*-specific IgE (Fig. 1a, b). These findings were consistent with clinical observations that allergens such as HDM or pollen induce allergic symptoms in nonsensitized patients [10, 11].



(For legend see next page.)

However, the mechanism of such an action has not been fully clarified. Local IgE response may be involved in the worsened symptoms in the cases without specific IgE in serum [35, 36]. On the other hand, some reports suggested that HDM may activate eosinophil functions [37, 38]. Nonspecific activation through pattern recognition receptors such as TLRs or PARs may play roles in this process.

PARs are 7-pass transmembrane receptors that are specifically activated by specific proteases and are widely distributed throughout the human body [4]. A serine protease cleaves the N-terminal peptide of each PAR, and the newly exposed N terminus interacts with residues within the remaining receptor, resulting in receptor activation. There are 4 types of PARs (type 1–4) in humans. Of these 4 PARs, PAR-2 in particular has been involved in the development of inflammation and allergy [4, 13–20, 39–46]. For example, PAR-2 activation induces the production of cytokines such as GM-CSF or interleukin (IL)-6 and matrix metalloproteinase-9 from airway epithelial cells or lung fibroblasts [39–42]. Deletion of the PAR-2 gene or PAR-2 antagonist treatment suppressed allergen-induced eosinophilic airway inflammation in mouse models [18–20, 43, 44]. Furthermore, the expression of PAR-2 on

CD14++CD16+ peripheral blood monocytes is increased in severe asthmatics [45]. Recently, Aubier et al. [46] reported that airway smooth muscle enlargement is associated with PAR-2/ligand overexpression in patients with severe asthma, suggesting that PAR-2 may play an important role in the pathogenesis of severe asthma.

Human eosinophils also constitutively transcribe PAR-2 mRNA and express the PAR-2 protein [47]. As for the role of PAR-2 in HDM activity, group 3 (Der f 3/Der p 3), group 6 (Der f 6/Der p 6), and group 9 (Der f 9/Der p 9) allergens are components of *Df* or *Dp* and have serine protease activity [4–6]. Fujisawa et al. [17] reported that HDM induced IL-9 production from eosinophils and that this upregulation was suppressed by a serine protease inhibitor, suggesting the involvement of PAR-2. In the present study, *Df* extract-induced eosinophil adhesion was suppressed by the serine protease inhibitor (Fig. 3c). Furthermore, *Df* extract-induced eosinophil activation was suppressed by PAR-2 antagonists (Fig. 3g, 4e, and 5e). As HDM or *Df* has components that have serine protease activity as described above, our results support an important role for serine protease and PAR-2 in the development of HDM or *Df* extract-induced eosinophil activation.

However, as Der f 1 has cysteine protease activity in contrast to the serine protease activity of Der p 3 or Der p 9 [4–6], the role of PAR-2 in the Der f 1-induced immune response is still controversial. Miike and Kita [34] reported that the cysteine protease papain induced eosinophil O_2^- generation; however, this activation was inhibited by the cysteine protease inhibitor E64, and not by an anti-PAR-2 Ab. Therefore, a cysteine protease may activate eosinophils through a PAR-2-independent mechanism. On the other hand, as has been shown for other cells, Asokanathan et al. [13] reported that Der p 1 (which also has cysteine protease activity) induced IL-6 production and receptor internalization in PAR-2-transfected, but not in PAR-1-transfected airway epithelial A549 cells. They also reported that Der p 1 pretreatment abolished trypsin- and PAR-2 agonist-induced Ca^{2+} release, but not thrombin- or PAR-1 agonist-induced Ca^{2+} release, suggesting that Der p 1 activated PAR-2 [13]. Furthermore, Kouzaki et al. [48] reported that the cysteine protease papain induced the production of thymic stromal lymphopoietin from the bronchial epithelial cells BEAS-2B and that this upregulation was partially blocked by transfection of PAR-2 small interfering RNA. In this study, Der f 1-induced eosinophil adhesion was suppressed by both cysteine protease inhibitor and serine protease inhibitor (Fig. 3b, d), suggesting that Der f 1 ac-

Fig. 3. Effect of cysteine protease inhibitor, serine protease inhibitor, or PAR-2 antagonists on *Df* extract- or Der f 1-induced eosinophil adhesion. **a, b** *Df* extract-induced eosinophil adhesion to ICAM-1 is inhibited by serine protease inhibitor, but not by cysteine protease inhibitor. Eosinophils were pre-incubated with or without a cysteine protease inhibitor (E64, 100 μ M) (**a**) ($n = 4$) or a serine protease inhibitor (AEBSF, 100 μ M) (**b**) ($n = 4$) for 15 min prior to analysis of adhesion to ICAM-1 in the presence or absence of *Df* extract (1,000 ng/mL). **c, d** Der f 1-induced eosinophil adhesion to ICAM-1 is inhibited by cysteine protease inhibitor and serine protease inhibitor. Eosinophils were pre-incubated with or without a cysteine protease inhibitor (E64, 100 μ M) (**c**) ($n = 4$) or a serine protease inhibitor (AEBSF, 100 μ M) (**d**) ($n = 4$) for 15 min prior to analysis of adhesion to ICAM-1 in the presence or absence of Der f 1 (100 pg/mL). **e, f** Effect of combination of cysteine and serine protease inhibitors on *Df* extract- or Der f 1-induced eosinophil adhesion. Eosinophils were pre-incubated with a cysteine protease inhibitor (E64, 100 μ M) and/or a serine protease inhibitor (AEBSF, 100 μ M) for 15 min prior to analysis of adhesion to ICAM-1 in the presence or absence of *Df* extract (1,000 ng/mL) (**e**) ($n = 4$) or Der f 1 (100 pg/mL) (**f**) ($n = 4$). **g, h** Effect of PAR-2 antagonists on *Df* extract- or Der f 1-induced eosinophil adhesion. Eosinophils were pre-incubated with or without a PAR-2 antagonist (FSLRY-NH2 or ENMD-1068, 10 μ M each) for 15 min prior to analysis of adhesion to ICAM-1 in the presence or absence of *Df* extract (1,000 ng/mL) (**g**) ($n = 5$) or Der f 1 (100 pg/mL) (**h**) ($n = 5$). The adhesion of eosinophils observed in response to *Df* extract or Der f 1 was used as a control (100%). Spon, spontaneous adhesion; –, without.

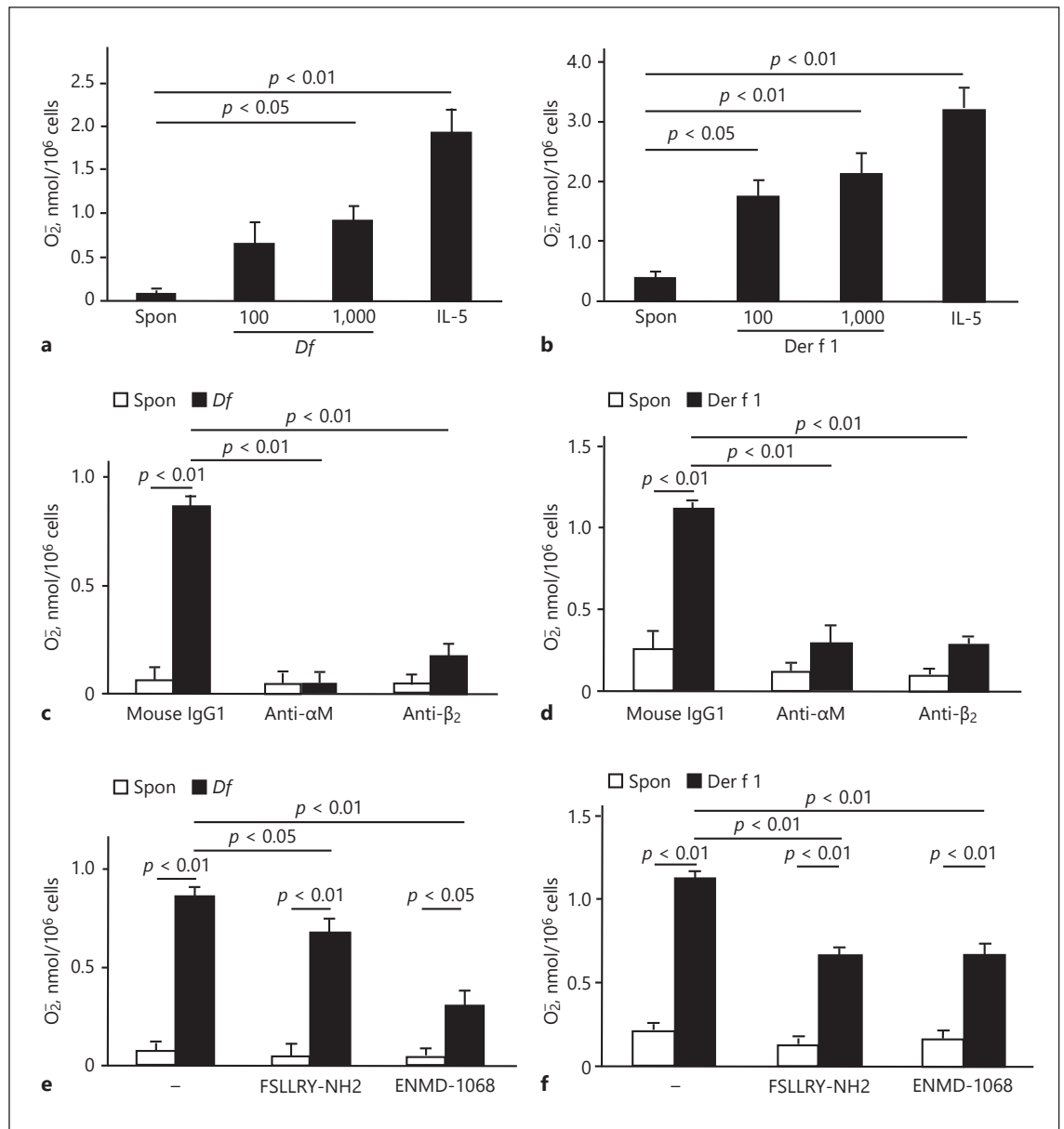


Fig. 4. Effect of *Df* extract and *Der f* 1 on eosinophil O_2^- generation. **a, b** *Df* extract or *Der f* 1 induced eosinophil O_2^- generation in the presence of ICAM-1. Eosinophil cell density was adjusted to 1.25×10^6 cells/mL of HBSS/gel mixed 4:1 with cytochrome C, and the eosinophil suspension was then added to ICAM-1-coated 96-well plates. Immediately after adding *Df* extract (100 ng/mL or 1,000 ng/mL) (**a**) or *Der f* 1 (100 pg/mL or 1,000 pg/mL) (**b**), eosinophil O_2^- generation was measured based on the SOD-inhibitable reduction of cytochrome C, followed by repeated measurements over the next 240 min. Maximum values during the incubation time are shown as means $\pm$ SEM of 5 experiments using cells from different donors. **c, d** *Df* extract- or *Der f* 1-induced eosinophil

O_2^- generation is inhibited by anti- α M- or anti- β ₂-integrin mAb. Eosinophils were pre-incubated with anti- α M-integrin mAb, anti- β ₂-integrin mAb, or control IgG (3 μ g/mL each) for 15 min prior to analysis of O_2^- generation of eosinophils in the presence or absence of *Df* extract (1,000 ng/mL) (**c**) ($n = 5$) or *Der f* 1 (100 pg/mL) (**d**) ($n = 5$). **e, f** Effect of PAR-2 antagonists on *Df* extract- or *Der f* 1-induced eosinophil O_2^- generation. Eosinophils were pre-incubated with or without a PAR-2 antagonist (FSLRY-NH2 or ENMD-1068, 10 μ M each) for 15 min prior to analysis of O_2^- generation of eosinophils in the presence or absence of *Df* extract (1,000 ng/mL) (**e**) ($n = 5$) or *Der f* 1 (100 pg/mL) (**f**) ($n = 5$). Spon, spontaneous O_2^- generation; -, without.

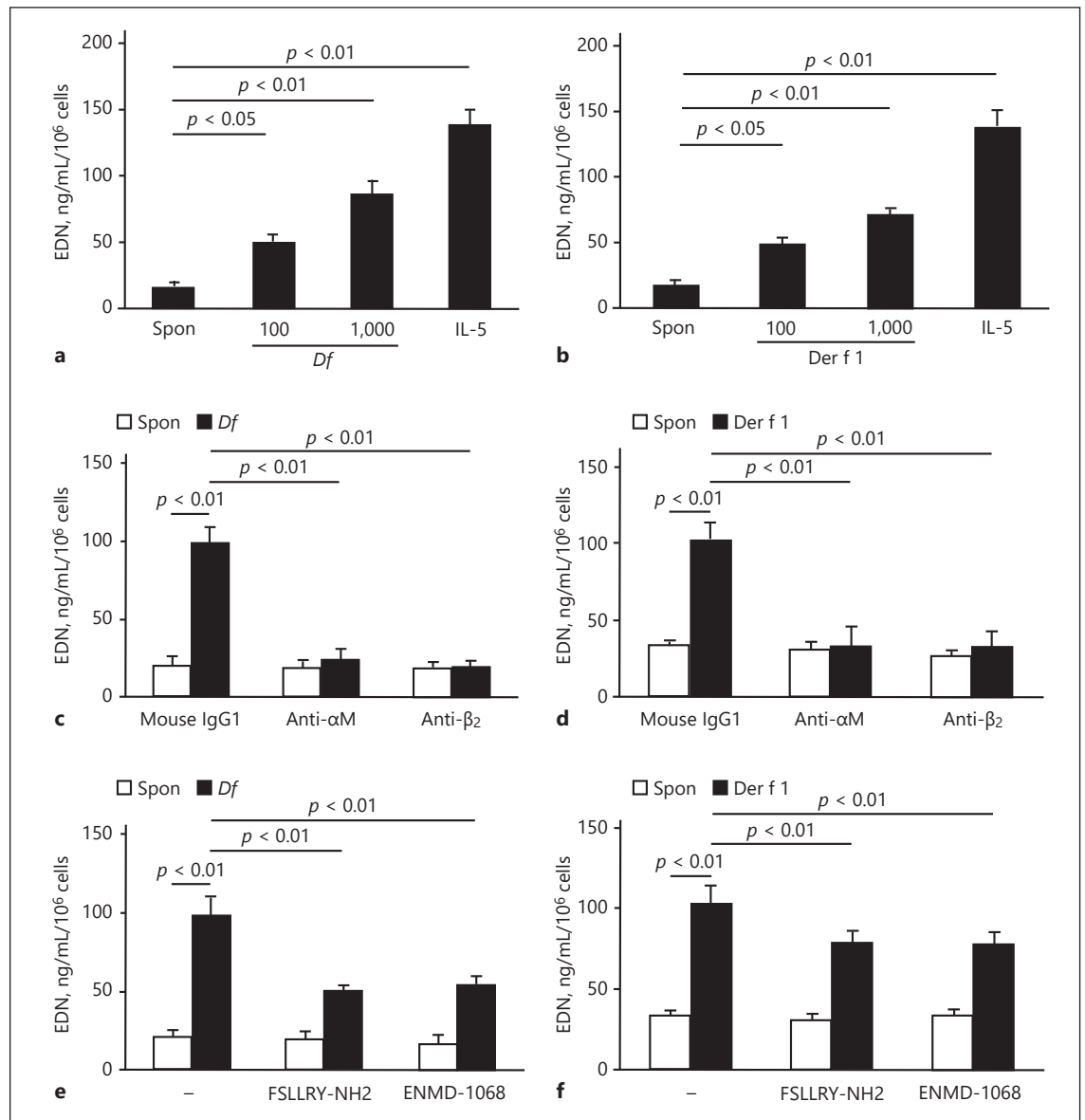


Fig. 5. Effect of *Df* extract and Der f 1 on eosinophil EDN release. **a, b** *Df* extract or Der f 1 induced eosinophil EDN release. Eosinophils (1×10^6 cells/mL) in 96-well plates were incubated with *Df* extract (100 ng/mL or 1,000 ng/mL) (**a**) or Der f 1 (100 pg/mL or 1,000 pg/mL) (**b**) for 240 min. The concentration of EDN in cell-free supernatants was then quantified using ELISA. Data are shown as means $\pm$ SEM of 5 experiments using cells from different donors. **c, d** *Df* extract- or Der f 1-induced eosinophil EDN release was inhibited by anti-αM- or anti-β₂-integrin mAb. Eosinophils were pre-incubated with anti-αM-integrin mAb, anti-β₂-integrin

mAb, or control IgG (3 μg/mL each) for 15 min prior to analysis of EDN release of eosinophils in the presence or absence of *Df* extract (1,000 ng/mL) (**c**) ($n = 5$) or Der f 1 (100 pg/mL) (**d**) ($n = 5$). **e, f** Effect of PAR-2 antagonists on *Df* extract- or Der f 1-induced eosinophil EDN release. Eosinophils were pre-incubated with or without a PAR-2 antagonist (FSLRY-NH2 or ENMD-1068, 10 μM each) for 15 min prior to analysis of EDN release of eosinophils in the presence or absence of *Df* extract (1,000 ng/mL) (**e**) ($n = 5$) or Der f 1 (100 pg/mL) (**f**) ($n = 5$). Spon, spontaneous EDN release; –, without.

tivates eosinophils by both serine and cysteine protease. Furthermore, Der f 1-induced eosinophil activation was partially suppressed by PAR-2 antagonists (Fig. 3h, 4f, and 5f). Therefore, both PAR-2-dependent and indepen-

dent mechanisms may be involved in the Der f 1-induced eosinophil activation. Moreover, additional PARs such as PAR-3, whose mRNA is also transcribed in human eosinophils [47], or a PAR-independent mechanism includ-

ing TLR, may also be involved. We examined the role of TLR4 signaling on *Df* extract- or Der f 1-induced eosinophil adhesion in this study since HDM is contaminated with LPS [49], which is a ligand for TLR4 and activates the functions of dendritic cells and epithelial cells through TLR4 [50, 51]. However, anti-TLR4 mAb did not suppress eosinophil adhesion induced by *Df* extract or Der f 1 (data not shown), which is consistent with the previous reports that eosinophils do not express TLR4 [52, 53].

Adhesion of eosinophils from allergic patients by *Df* extract or Der f 1 was higher than that from healthy volunteers (Fig. 1c, d). However, the exact mechanisms of this enhanced upregulation remain unknown. In this study, the titer of *Df*-specific IgE Ab did not affect *Df* extract-induced eosinophil adhesion (data not shown). Furthermore, *Df* extract also induced the adhesion of eosinophils from Japanese cedar pollen-sensitized patients, to a level similar to that from *Df*-sensitized patients (data not shown). Therefore, IgE-mediated mechanisms are unlikely to be involved in the eosinophil activation by *Df* extract. Rather, eosinophils from allergic patients may be already activated in vivo and therefore may be more easily activated by stimulants than those from healthy volunteers.

Miike and Kita [34] reported that Der f 1 induced eosinophil degranulation when cysteine was added into the culture, whereas Der f 1 without cysteine did not induce eosinophil activation. In this context, Kato et al. [15] reported that Der f 1 with cysteine, but not Der f 1 alone, exhibited cysteine protease activity, suggesting that regeneration of the catalytic thiol residue by L-cysteine may play an important role in the cysteine protease activity of Der f 1. Although we have not examined the effect of cysteine addition on Der f 1-induced eosinophil activation, a different origin or extraction method of this antigen and its catalytic thiol residue may contribute to this discrepancy.

A limitation of this study is that there is little information regarding PAR-2 antagonists (FSLRY-NH₂ or ENMD-1068), the cysteine protease inhibitor (E64), or serine protease inhibitor (AEBSF). As for the PAR-2 antagonists, for example, optimal concentrations of PAR-2 antagonists are different among different types of cells [28–33], and there are no data regarding the optimal concentrations for eosinophils. Furthermore, the selectivity of these antagonists has not been fully clarified. Although the concentrations of PAR-2 antagonists that we used in this study were lower than those used in other studies [28–33], the selectivity of these PAR-2 antagonists may affect the results regarding the role of PAR-2 in Der f 1-in-

duced eosinophil activation. Moreover, the only approach that we used to clarify the role of PAR-2 in the *Df* extract- or Der f 1-induced eosinophil activation was to test a PAR-2 antagonist. Other approaches such as transfection of small interfering RNA may be useful to confirm the role of PAR-2.

In conclusion, *Df* extract and Der f 1 activate eosinophilic function through $\alpha M\beta_2$ -integrin and PAR-2. Therefore, direct eosinophil activation by the HDM may play roles in the induction of allergic symptoms, even in nonsensitized patients.

Acknowledgments

The authors thank Ms. Akemi Yokote for her excellent technical assistance.

Statement of Ethics

This study was approved by the Ethical Committee of Saitama Medical University, and written informed consent was obtained before collection of each blood sample.

Disclosure Statement

K.O.-D. is employee of Torii Pharmaceutical Co. Ltd. K.N., T.S., and M.N. received fees for speaking from Torii Pharmaceutical Co. Ltd. The rest of the authors declare no conflict of interest.

Funding Sources

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (15K09228) and from Torii Pharmaceutical Co. Ltd.

Author Contributions

Y.U. carried out the experiments, analyzed the data, and drafted the manuscript. K.N. participated in the direction of the study, analyzed the data, and wrote the manuscript. T.K. and T.N. carried out the eosinophil experiments. T.S. and K.T. participated in the data analyses. K.O.-D. prepared the HDM and participated in the data analyses. M.N. participated in the direction of the study and edited the manuscript. All authors read and approved the final manuscript.

References

- Bochner BS, Udem BJ, Lichtenstein LM. Immunological aspects of allergic asthma. *Annu Rev Immunol*. 1994;12(1):295–335.
- Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol*. 2000 Apr;105(4):651–63.
- Weller PF. Human eosinophils. *J Allergy Clin Immunol*. 1997 Sep;100(3):283–7.
- Jacquet A. The role of innate immunity activation in house dust mite allergy. *Trends Mol Med*. 2011 Oct;17(10):604–11.
- Holck A, Dale S, Sletten K. Purification and characterization of a major allergen from the house dust mite *Dermatophagoides farinae*. *Allergy*. 1986 Aug;41(6):408–17.
- Le Mao J, Mayer CE, Peltre G, Desvaux FX, David B, Weyer A, et al. Mapping of *Dermatophagoides farinae* mite allergens by two-dimensional immunoblotting. *J Allergy Clin Immunol*. 1998 Oct;102(4 Pt 1):631–6.
- Calderón MA, Linneberg A, Kleine-Tebbe J, De Blay F, Hernandez Fernandez de Rojas D, Virchow JC, et al. Respiratory allergy caused by house dust mites: what do we really know? *J Allergy Clin Immunol*. 2015 Jul;136(1):38–48.
- Platts-Mills TA, Vervloet D, Thomas WR, Aalberse RC, Chapman MD. Indoor allergens and asthma: report of the Third International Workshop. *J Allergy Clin Immunol*. 1997 Dec;100(6 Pt 1):S2–24.
- Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med*. 2003 Oct;349(15):1414–22.
- Andersen I, Lundqvist GR, Proctor DF, Swift DL. Human response to controlled levels of inert dust. *Am Rev Respir Dis*. 1979 Apr;119(4):619–27.
- Andersen I, Mølhave L, Proctor DF. Human response to controlled levels of combinations of sulfur dioxide and inert dust. *Scand J Work Environ Health*. 1981 Mar;7(1):1–7.
- Kvarnhammar AM, Cardell LO. Pattern-recognition receptors in human eosinophils. *Immunology*. 2012 May;136(1):11–20.
- Asokanathan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, et al. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol*. 2002 Oct;169(8):4572–8.
- Adam E, Hansen KK, Astudillo Fernandez O, Coulon L, Bex F, Duhant X, et al. The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. *J Biol Chem*. 2006 Mar 17;281(11):6910–23.
- Kato T, Takai T, Fujimura T, Matsuoka H, Ogawa T, Murayama K, et al. Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes. *Allergy*. 2009 Sep;64(9):1366–74.
- Sun G, Stacey MA, Schmidt M, Mori L, Matoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol*. 2001 Jul;167(2):1014–21.
- Fujisawa T, Katsumata H, Kato Y. House dust mite extract induces interleukin-9 expression in human eosinophils. *Allergol Int*. 2008 Jun;57(2):141–6.
- Davidson CE, Asaduzzaman M, Arizmendi NG, Polley D, Wu Y, Gordon JR, et al. Proteinase-activated receptor-2 activation participates in allergic sensitization to house dust mite allergens in a murine model. *Clin Exp Allergy*. 2013 Nov;43(11):1274–85.
- Post S, Heijink IH, Petersen AH, de Bruin HG, van Oosterhout AJ, Nawijn MC. Protease-activated receptor-2 activation contributes to house dust mite-induced IgE responses in mice. *PLoS One*. 2014 Mar;9(3):e91206.
- de Boer JD, Van't Veer C, Stroo I, van der Meer AJ, de Vos AF, van der Zee JS, et al. Protease-activated receptor-2 deficient mice have reduced house dust mite-evoked allergic lung inflammation. *Innate Immun*. 2014 Aug;20(6):618–25.
- Nagata M, Saito K, Tsuchiya K, Sakamoto Y. Leukotriene D4 upregulates eosinophil adhesion via the cysteinyl leukotriene 1 receptor. *J Allergy Clin Immunol*. 2002 Apr;109(4):676–80.
- Takaku Y, Nakagome K, Kobayashi T, Hagiwara K, Kanazawa M, Nagata M. IFN- γ -inducible protein of 10 kDa upregulates the effector functions of eosinophils through β 2 integrin and CXCR3. *Respir Res*. 2011 Oct;12(1):138.
- Noguchi T, Nakagome K, Kobayashi T, Ueda Y, Soma T, Nakamoto H, et al. Effect of beta2-adrenergic agonists on eosinophil adhesion, superoxide anion generation, and degranulation. *Allergol Int*. 2015 Sep;64 Suppl:S46–53.
- Noguchi T, Nakagome K, Kobayashi T, Uchida Y, Soma T, Nakamoto H, et al. Perioxin upregulates the effector functions of eosinophils. *J Allergy Clin Immunol*. 2016 Nov;138(5):1449–1452.e5.
- Kobayashi T, Takaku Y, Yokote A, Miyazawa H, Soma T, Hagiwara K, et al. Interferon- β augments eosinophil adhesion-inducing activity of endothelial cells. *Eur Respir J*. 2008 Dec;32(6):1540–7.
- Kobayashi T, Kouzaki H, Kita H. Human eosinophils recognize endogenous danger signal crystalline uric acid and produce proinflammatory cytokines mediated by autocrine ATP. *J Immunol*. 2010 Jun;184(11):6350–8.
- Shima K, Koya T, Tsukioka K, Sakagami T, Hasegawa T, Fukano C, et al. Effects of sublingual immunotherapy in a murine asthma model sensitized by intranasal administration of house dust mite extracts. *Allergol Int*. 2017 Jan;66(1):89–96.
- Al-Ani B, Saifeddine M, Wijesuriya SJ, Hollenberg MD. Modified proteinase-activated receptor-1 and -2 derived peptides inhibit proteinase-activated receptor-2 activation by trypsin. *J Pharmacol Exp Ther*. 2002 Feb;300(2):702–8.
- Tripathi T, Abdi M, Alizadeh H. Protease-activated receptor 2 (PAR2) is upregulated by Acanthamoeba plasminogen activator (aPA) and induces proinflammatory cytokine in human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2014 May;55(6):3912–21.
- McLarty JL, Meléndez GC, Brower GL, Janicki JS, Levick SP. Trypsin/Protease-activated receptor 2 interactions induce selective mitogen-activated protein kinase signaling and collagen synthesis by cardiac fibroblasts. *Hypertension*. 2011 Aug;58(2):264–70.
- Kelso EB, Lockhart JC, Hembrough T, Dunning L, Plevin R, Hollenberg MD, et al. Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. *J Pharmacol Exp Ther*. 2006 Mar;316(3):1017–24.
- He RQ, Tang XF, Zhang BL, Li XD, Hong MN, Chen QZ, et al. Protease-activated receptor 1 and 2 contribute to angiotensin II-induced activation of adventitial fibroblasts from rat aorta. *Biochem Biophys Res Commun*. 2016 Apr;473(2):517–23.
- White MJ, Gomer RH. Trypsin, Trypsin, and Thrombin Polarize Macrophages towards a Pro-Fibrotic M2a Phenotype. *PLoS One*. 2015 Sep;10(9):e0138748.
- Miike S, Kita H. Human eosinophils are activated by cysteine proteases and release inflammatory mediators. *J Allergy Clin Immunol*. 2003 Apr;111(4):704–13.
- Rondón C, Campo P, Togias A, Fokkens WJ, Durham SR, Powe DG, et al. Local allergic rhinitis: concept, pathophysiology, and management. *J Allergy Clin Immunol*. 2012 Jun;129(6):1460–7.
- Campo P, Rondón C, Gould HJ, Barrionuevo E, Gevaert P, Blanca M. Local IgE in non-allergic rhinitis. *Clin Exp Allergy*. 2015 May;45(5):872–81.
- Coward WR, Sagara H, Wilson SJ, Holgate ST, Church MK. Allergen activates peripheral blood eosinophil nuclear factor-kappaB to generate granulocyte macrophage-colony stimulating factor, tumour necrosis factor-alpha and interleukin-8. *Clin Exp Allergy*. 2004 Jul;34(7):1071–8.
- Svensson L, Rudin A, Wennerås C. Allergen extracts directly mobilize and activate human eosinophils. *Eur J Immunol*. 2004 Jun;34(6):1744–51.

- 39 Vliagoftis H, Befus AD, Hollenberg MD, Moqbel R. Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated receptor 2. *J Allergy Clin Immunol*. 2001 Apr; 107(4):679–85.
- 40 Asokanathan N, Graham PT, Fink J, Knight DA, Bakker AJ, McWilliam AS, et al. Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. *J Immunol*. 2002 Apr; 168(7):3577–85.
- 41 Vliagoftis H, Schwingshackl A, Milne CD, Duszyk M, Hollenberg MD, Wallace JL, et al. Proteinase-activated receptor-2-mediated matrix metalloproteinase-9 release from airway epithelial cells. *J Allergy Clin Immunol*. 2000 Sep;106(3):537–45.
- 42 Asokanathan N, Lan RS, Graham PT, Bakker AJ, Tokanović A, Stewart GA. Activation of protease-activated receptors (PARs)-1 and -2 promotes alpha-smooth muscle actin expression and release of cytokines from human lung fibroblasts. *Physiol Rep*. 2015 Feb;3(2):3.
- 43 Asaduzzaman M, Nadeem A, Arizmendi N, Davidson C, Nichols HL, Abel M, et al. Functional inhibition of PAR2 alleviates allergen-induced airway hyperresponsiveness and inflammation. *Clin Exp Allergy*. 2015 Dec; 45(12):1844–55.
- 44 Schmidlin F, Amadesi S, Dabbagh K, Lewis DE, Knott P, Bunnett NW, et al. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *J Immunol*. 2002 Nov; 169(9):5315–21.
- 45 Shrestha Palikhe N, Nahirney D, Laratta C, Gandhi VD, Vethanayagam D, Bhutani M, et al. Increased Protease-Activated Receptor-2 (PAR-2) Expression on CD14++CD16+ Peripheral Blood Monocytes of Patients with Severe Asthma. *PLoS One*. 2015 Dec; 10(12):e0144500.
- 46 Aubier M, Thabut G, Hamidi F, Guillou N, Brard J, Dombret MC, et al. Airway smooth muscle enlargement is associated with protease-activated receptor 2/ligand overexpression in patients with difficult-to-control severe asthma. *J Allergy Clin Immunol*. 2016 Sep;138(3):729–739.e11.
- 47 Miike S, McWilliam AS, Kita H. Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. *J Immunol*. 2001 Dec;167(11):6615–22.
- 48 Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol*. 2009 Jul;183(2):1427–34.
- 49 Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature*. 2009 Jan;457(7229):585–8.
- 50 Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med*. 2009 Apr;15(4):410–6.
- 51 Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, et al. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med*. 2010 Sep;207(10):2097–111.
- 52 Sabroe I, Jones EC, Usher LR, Whyte MK, Dower SK. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol*. 2002 May;168(9):4701–10.
- 53 Meerschaert J, Busse WW, Bertics PJ, Mosher DF. CD14(+) cells are necessary for increased survival of eosinophils in response to lipopolysaccharide. *Am J Respir Cell Mol Biol*. 2000 Dec;23(6):780–7.