



Protease Activity Enhances Production of Thymic Stromal Lymphopoietin and Basophil Accumulation in Flaky Tail Mice

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Epidermal barrier abnormality due to filaggrin deficiency is an important predisposing factor in the development of atopic dermatitis (AD). In addition, the expression of thymic stromal lymphopoietin (TSLP) in keratinocytes (KCs), induced by barrier disruption, can promote type 2 helper T-cell polarization. Protease activity, including protease-activated receptor-2 (PAR-2), is also known to be involved in epidermal barrier function in AD. However, to our knowledge, the relationship between protease activity and filaggrin deficiency from the perspective of AD has not been elucidated. Flaky tail (*Flg^{ft}*) mice, known to have a mutation in the filaggrin gene, were used to assess the role of protease in KCs in the steady state and the mite-induced AD-like skin inflammation model. In the steady state, the expression and activity levels of endogenous proteases, kallikreins 5, 7, and 14, in the skin and TSLP were higher in *Flg^{ft}* than in control mice. In addition, activation of PAR-2 by its agonist induced the production of TSLP in KCs of *Flg^{ft}* mice, which was abrogated by a newly developed PAR-2 antagonist. Application of the PAR-2 antagonist improved symptoms and basophil accumulation in *Flg^{ft}* mice treated with mite extracts. These results suggest that possibly through the PAR-2 activation in KCs, filaggrin deficiency induces TSLP production and basophil accumulation, which play important roles in the establishment of AD. (*Am J Pathol* 2013, ■: 1–11; <http://dx.doi.org/10.1016/j.ajpath.2012.11.039>)

Q3 A recently discovered link between the incidence of atopic dermatitis (AD) and loss-of-function mutations in the gene encoding filaggrin (*FLG*) has demonstrated that skin barrier dysfunction is a critical driving force in the initiation and exacerbation of AD.¹ In addition to serving as a physical barrier between the environment and the body, keratinocytes (KCs) are thought to play a vital role in both innate and adaptive immune responses.² Stimulated KCs can trigger and modify the activation and differentiation of dendritic cells, B cells, and T cells through the production of cytokines, such as thymic stromal lymphopoietin (TSLP) and IL-33, which induce type 2 helper T-cell (Th2)—polarized immune responses in patients with AD.³

The critical association between the abnormal barrier and Th2 polarization in AD may, in part, be explained by the expression of TSLP.^{4,5} TSLP is mainly produced by

epithelial cells, including KCs, and is highly expressed in the epidermis of patients with AD.^{6,7} In addition, keratinocyte-specific overexpression of TSLP leads to the development of a spontaneous Th2-type skin inflammation in mice⁸ and accumulation of basophils in the skin.⁹ Recently, it has been shown that patients with Netherton syndrome, in which affected individuals experience a significant predisposition for AD, exhibit an elevated level of TSLP in their skin.¹⁰ In patients with Netherton syndrome, the lack of the protease inhibitor, lymphoepithelial kazal-type-related inhibitor,

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results in the up-regulation of epidermal protease kallikreins (KLKs), which, in turn, activates protease-activated receptor 2 (PAR-2) on KCs, causing overexpression of TSLP in the skin. Activated PAR-2 has also induced the expression of TSLP by airway epithelial cells.¹¹

The protease activity in particular allergens, such as papain, has been shown to be required for the induction of Th2 and IgE responses.^{12,13} It has been reported that papain directly activated basophils and recruited them to the lymph nodes during primary immune responses.^{14,15} Despite the fact that basophils represent <0.5% of circulating leukocytes, recent studies have unveiled their potent immunostimulatory functions (ie, their induction of Th2 immunity) by secreting key Th2-inducing cytokines, such as IL-4, and by functioning as professional antigen-presenting cells.^{16,17} Although it has been reported that basophils are often recruited to the affected tissues in allergic disorders, including AD,^{18,19} it is important to clarify the role of the accumulation of basophils to the skin in AD or filaggrin-deficient conditions. Thus far, our understanding of the accumulation of basophils to the skin is limited because basophils cannot be stained in routinely processed histological tissue specimens in human skin and because of the few appropriate available animal models for allergy.¹⁷

We have recently shown that an allergen from the house dust mite [*Dermatophagoides pteronyssinus* (Dp)] is a potent inducer of AD-like skin lesions in Flaky tail (*Flg^{ft}*) mice, which carry a frameshift mutation in the filaggrin gene and a *ma* mutation.²⁰ Because Dp possesses strong protease activity, it is intriguing to investigate the relevance of the activation of protease receptors, especially PAR-2, under filaggrin-deficient conditions. It has been suggested that PAR-2 activation may play a role in the development of AD, based on its production of several inflammatory-related AD cytokines,^{21,22} its direct proinflammatory role in allergic contact dermatitis,²³ and its association with delayed epidermal permeability recovery.²⁴ However, the mechanisms underlying these protease-mediated Th2 skin responses are not fully understood, especially under filaggrin-deficient conditions.

Herein, we found that *Flg^{ft}* mice possessed increased endogenous protease (KLKs 5, 7, and 14) activity, TSLP expression, and basophil accumulation in the skin. In addition, TSLP production by KCs was induced by exposure to a PAR-2 agonist and suppressed by a PAR-2 antagonist. Moreover, stimulation of PAR-2 with Dp induced prominent basophil accumulation to the skin. These findings demonstrate that protease activity in the skin is a critical modulating factor of the development of AD.

Materials and Methods

Mice

C57BL/6J (B6) female mice were purchased from SLC (Shizuoka, Japan). Flaky tail (STOCK *a/a ma ft/ma ft/J*) mice, which carry double-homozygous flaky tail (*ft*) and

matted (*ma*) mutations, were outcrossed onto B6 at Jackson Laboratory (Bar Harbor, ME).²⁵ TSLP receptor (TSLPR)—deficient mice (B6 background) were generated by Dr. Steven Ziegler²⁶ and kindly provided. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine (Kyoto, Japan).

Reagents

The following primary antibodies were used in this analysis: rabbit anti-KLK7 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-KLK14 (Santa Cruz Biotechnology), rabbit anti-PAR-2 (Santa Cruz Biotechnology), goat-anti-TSLP (R&D Systems, Minneapolis, MN), rat anti-monocyte chemotactic protein (MCP)-8 (TUG8; BioLegend, San Diego, CA), rat anti-FcεRI (MAR-1; eBioscience), rat anti-ckit (2B8; eBioscience), rat anti-CD69 (H1.2F3; eBioscience), and rat anti-CD200R3 (Ba103; Hycult Biotech, Uden, The Netherlands). Alexa Fluor 488 donkey anti-rabbit and anti-goat IgG antibody (Invitrogen, Carlsbad, CA) and biotinylated donkey anti-rat IgG antibody (Vector Laboratories, Burlingame, CA) were purchased. The protease activity was measured using an EnzChek Protease Assay Kit (Molecular Probes, Eugene, OR). Mouse PAR-2 agonist SLIGRL-NH2^{27,28} was purchased from Pepttron (Seoul, South Korea). A newly generated PAR-2 antagonist, NPS-1577, was provided by NeoPharm pharmacy (Daejeon, South Korea). Its antagonistic activity was confirmed by an intracellular calcium influx assay, as described later (Supplemental Figure S1). No antagonistic effect on PAR-1 by NPS-1577 was observed (data not shown).

Intracellular Calcium Mobilization Assay

An intracellular calcium mobilization assay was performed with an FLIPR Calcium 4 Assay Kit (Molecular Devices, Sunnyvale, CA), according to the manufacturer's protocols, with slight modification. Briefly, HCT-15, a PAR-2—overexpressing cell line, was seeded in a black 96-well plate (4×10^4 cells per well) and incubated for 24 hours. Then, loading dye buffer was added and incubated at 37°C for 1 hour. PAR-2 antagonist was added at several doses, 5 minutes before the addition of 25 μmol/L of the PAR-2 agonist, and then fluorescent signal was measured using FlexStation II (Molecular Devices) 7 minutes later.

Amino Acid Analysis, Skin Surface pH, and TEWL

Samples of stratum corneum were collected by tape stripping with adhesive tape (CT-24; Nichiban, Tokyo, Japan), performed five times on a $5 \times 5\text{-cm}^2$ area of mouse dorsal skin. Free amino acids were analyzed on an L-8800 amino acid analyzer (Hitachi, Tokyo, Japan) by discontinuous gradient elution with L-8500 PF-Kit buffer (Hitachi). Amino acids post-labeled with ninhydrin were detected by

measuring the absorbance at 440 and 570 nm. Skin surface pH was measured with a pH meter, D51 (Horiba, Kyoto, Japan). To evaluate inside-to-outside barrier function, transepidermal water loss (TEWL) was measured with a Vapo Scan tewameter (Asahi Biomed, Tokyo, Japan) at 24°C and 46% relative humidity.

Cell Line and Primary Culture of KCs

Mouse KC cell line, PAM 212,²⁹ was seeded in 24-well plates (5×10^4 cells per well) and grown in HuMedia KG2 medium (Kurabo, Osaka, Japan) until 80% confluence, then incubated for an additional 24 hours without hydrocortisone for starvation. After 8 hours of incubation with a PAR-2 agonist or antagonist, cells were collected and used for analysis. For KC primary culture, whole dorsal skin of newborn mice was incubated with 5 mg/mL dispase II (Sanko-Junyaku Co, Tokyo, Japan) at 4°C overnight to separate the epidermis and dermis. Epidermal sheets were then cut into pieces and incubated in a nonenzymatic cell dissociation solution (Sigma-Aldrich, Tokyo, Japan) to obtain a single-cell suspension. Cells were seeded in 96-well plates (8×10^4 cells per well) and grown in HuMedia KG2 medium.

Epicutaneous Sensitization with Ovalbumin

Mice were anesthetized with diethyl ether (Nacalai Tesque, Kyoto, Japan) and then shaved with an electric razor (THRIVE Co Ltd, Osaka, Japan). A single dorsal skin site on each mouse was tape stripped at least five times with adhesive cellophane tape (Nichiban, Tokyo, Japan). A total of 10 μ L of 10 mg/mL ovalbumin (OVA; Hyglos GmbH, Regensburg, Germany) in PBS was applied on the dorsal skin site and covered with transparent film (Suprasorb F; Lohmann & Rauscher, Miami, FL). Each mouse had a total of five 3-day exposures to the OVA, separated by 4-day intervals. Skin sections were obtained after the last OVA application.

Bone Marrow—Derived Basophils and Chemotaxis Assay

Mouse bone marrow cells were cultured with 20% fetal calf serum and 10 ng/mL IL-3 in complete RPMI 1640 medium [consisting of RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum, 5×10^{-5} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 25 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, and 100 μ g/mL streptomycin] for approximately 7 to 10 days to obtain bone marrow—derived basophils (BMBs). Fc ϵ RI⁺c-kit⁺CD200R3⁺ cells were considered as BMBs. For the chemotaxis assay, 1×10^6 BMBs were transferred into the upper chamber of a transwell containing 5- μ m pore filters (BD Biosciences, Franklin Lakes, NJ). Recombinant mMCP-1 (Wako, Osaka, Japan) was added to the lower

chamber and incubated for 2.5 hours at 37°C. The Fc ϵ RI⁺c-kit⁺CD200R3⁺ cells that migrated to the lower chambers were counted by flow cytometry. The migration index was calculated as a percentage of input: number of cells in each lower chamber/total number of cells placed in the corresponding upper chamber.

For the basophil activation assay, BMBs were cultured in a 96-well-plate in complete RPMI 1640 medium plus 3 ng/mL IL-3 for 24 hours. BMBs were stimulated with 10 ng/mL Dp preactivated with 5 mmol/L L-cysteine HCl, pH 7, for 15 minutes at 37°C to regenerate protease activity,²¹ in the presence or absence of 150 μ mol/L PAR-2 antagonist. Cells were collected and analyzed by flow cytometry to observed basophil activation marker CD69.

Epidermal Protease Activity

The proteolytic activity was examined by *in situ* zymography and casein gel zymography. For *in situ* zymography, skin biopsy specimens were obtained and frozen sections (5 μ m thick) were rinsed with washing solution (1% Tween 20 in deionized water) and incubated at 37°C for 1 hour with 100 μ L of BODIPY-FL-casein containing digestion buffer (10 μ g/mL; Molecular Probes, CA). The sections were rinsed with Q9 washing solution and observed under a BZ-9000 BIOREVO Q10 (Keyence, Osaka, Japan). For casein gel zymography, mouse epidermis was crushed in 1 mol/L acetic acid solution. After overnight extraction at 4°C, soluble proteins were resuspend in PBS and 5 μ g was loaded onto casein copolymerized acrylamide gels (15% acrylamide and 0.05% α -casein; Sigma-Aldrich) for electrophoresis. Gels were washed with 2.5% Triton X-100 for 1 hour and then incubated for 24 hours at 37°C in reaction buffer containing 59 mmol/L Tris (pH 8.0). Gels were stained with 1% Coomassie Brilliant Blue for 30 minutes. Areas of caseinolytic activity appeared as clear zones against a dark blue background.

Clinical Observations and Histological Characteristics

The clinical severity of skin lesions was scored according to the macroscopic diagnostic criteria.²⁰ Briefly, the total clinical score for skin lesions was designated as the sum of individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), for the symptoms of pruritus, erythema, edema, erosion, and scaling. The presence of pruritus was determined through clinical observation for >2 minutes. For the histological analysis, the dorsal skin was stained with H&E. The histological findings were evaluated as previously reported.³⁰ In brief, samples were scored for the severity and character of the inflammatory response using a subjective grading scale. Responses were graded as follows: 0, no response; 1, minimal response; 2, mild response; 3, moderate response; and 4, marked response. The total histological score was calculated as the sum of the scores concerning inflammation, neutrophils, mononuclear cells, edema, and epithelial hyperplasia. The number of basophils was calculated as the

average from three different fields of each sample (original magnification, $\times 40$).

Quantitative Real-Time PCR and ELISA

mRNA was isolated using TRIzol (Invitrogen, Tokyo, Japan), per the manufacturer's protocol, and subjected to quantitative real-time PCR analysis, using the housekeeping gene *GAPDH* as an internal control.²⁰ All data are presented as fold change against each control (mean of non-stimulated cells or control mice). For the enzyme-linked immunosorbent assay (ELISA), skin samples were chopped and homogenized using a Polytron RT-3000 instrument (Kinematica AG, Bohemia, NY) in cell lysis buffer (Epitomics, Burlingame, CA). The protein concentration was quantified using a Bio-Rad Protein assay (Bio-Rad, Marnes-la-Coquette, France). The concentrations of TSLP protein were measured using a TSLP DuoSet Kit (R&D Systems).

Dermatitis Model

To induce murine AD-like skin lesions, 40 mg of 0.5% Dp (Biostir, Kobe, Japan) in white petrolatum was topically applied to the ears and upper back three times a week for 7 weeks.²⁰ Petrolatum without Dp was used as a control. Alternatively, mice were treated with 0.5% Dp and 1% PAR-2 antagonist in petrolatum. Dp body product (1 g) contained 1.78 mg total protein with 2.47 μ g of Dp protein (Der p1).

Statistical Analysis

Unless otherwise indicated, data were presented as the means $\pm$ SD. *P* values were calculated according to the two-tailed Student's *t*-test. *P* < 0.05 were considered to indicate significant difference between the indicated groups.

Results

Elevation of Skin Surface pH Accompanies with Low Histidine Level in *Flg^{fl}* Mice

In the stratum corneum, filaggrin is proteolytically degraded into a pool of free amino acids, including histidine and glutamine, which are further converted to urocanic acid and pyrrolidine carboxylic acid, respectively. These metabolites are thought to play a key role in maintaining the acidic pH of the stratum corneum.^{31,32} It is already known that the skin surface pH is higher in patients with AD than in healthy controls.^{33,34} In addition, filaggrin concentrations in the stratum corneum are significantly lower in *FLG* mutation carriers than in healthy donors.^{32,35}

Given this information, we evaluated the skin surface pH and the amino acid content of *Flg^{fl}* mice. We found that skin surface pH in *Flg^{fl}* mice was similar to that of control mice during the neonatal period when mice did not exhibit any

clinical dermatitis, but increased with age and with the development of clinical dermatitis (eg, scaling and erythema) (Figure 1A). These data suggest a parallel correlation between skin inflammatory status and the surface pH. Histidine content, meanwhile, decreased in *Flg^{fl}* mice, although glutamine content in *Flg^{fl}* mice was similar to that in control mice (Figure 1B), suggesting that a low histidine level might be involved, at least in part, in the elevation of skin surface pH under filaggrin-deficient conditions.

Enhanced Expression of Endogenous Proteases in *Flg^{fl}* Mice

The elevation of skin surface pH induces the activation of KLK family proteases in the epidermis.^{36,37} KLKs 5, 7, and 14 have been reported to be present in catalytically active forms in the skin of patients with AD.^{38,39} Thus, we measured the expression levels and activity of these KLKs in the skin of adult *Flg^{fl}* mice. The mRNA expression levels of KLKs 5, 7, and 14 were all elevated in *Flg^{fl}* mice compared with controls (Figure 2A). Consistent with this, immunohistochemical (IHC) analysis demonstrated an enhanced expression of KLKs 7 and 14 in the epidermis of *Flg^{fl}* mice. In addition, a proteolytic activity assay, casein gel zymography, revealed that KLK5 activity was significantly increased in *Flg^{fl}* mice (Figure 2A). To evaluate total protease activity in the skin, we performed *in situ* zymography and found enhanced activity in *Flg^{fl}* mice (Figure 2B). These data strongly indicate increased activity of endogenous proteases in *Flg^{fl}* mice in the steady state.

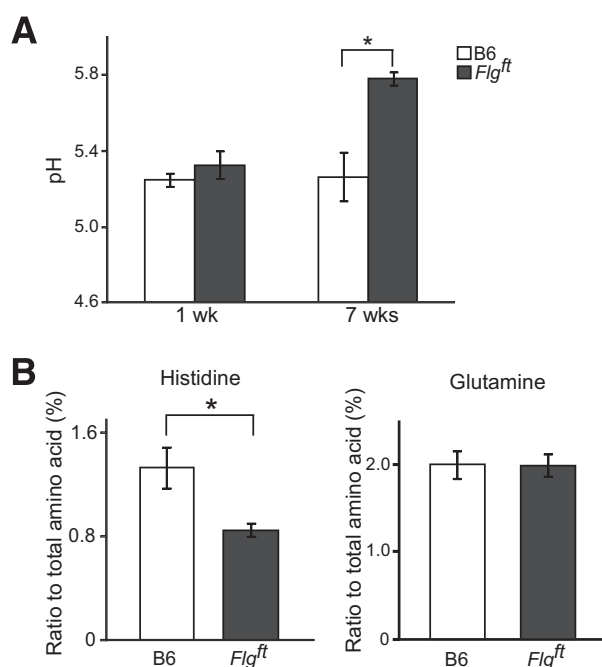


Figure 1 Skin surface pH and amino acid content of *Flg^{fl}* mice in the steady state. **A:** Skin surface pH of neonatal and adult *Flg^{fl}* mice compared with controls (B6 mice) of 1- and 7-week-old mice (*n* = 4 per group). **B:** Histidine (left panel) and glutamine (right panel) ratio (%) to total amino acid of stratum corneum collected by tape stripping (*n* = 3 per group). **P* < 0.05.

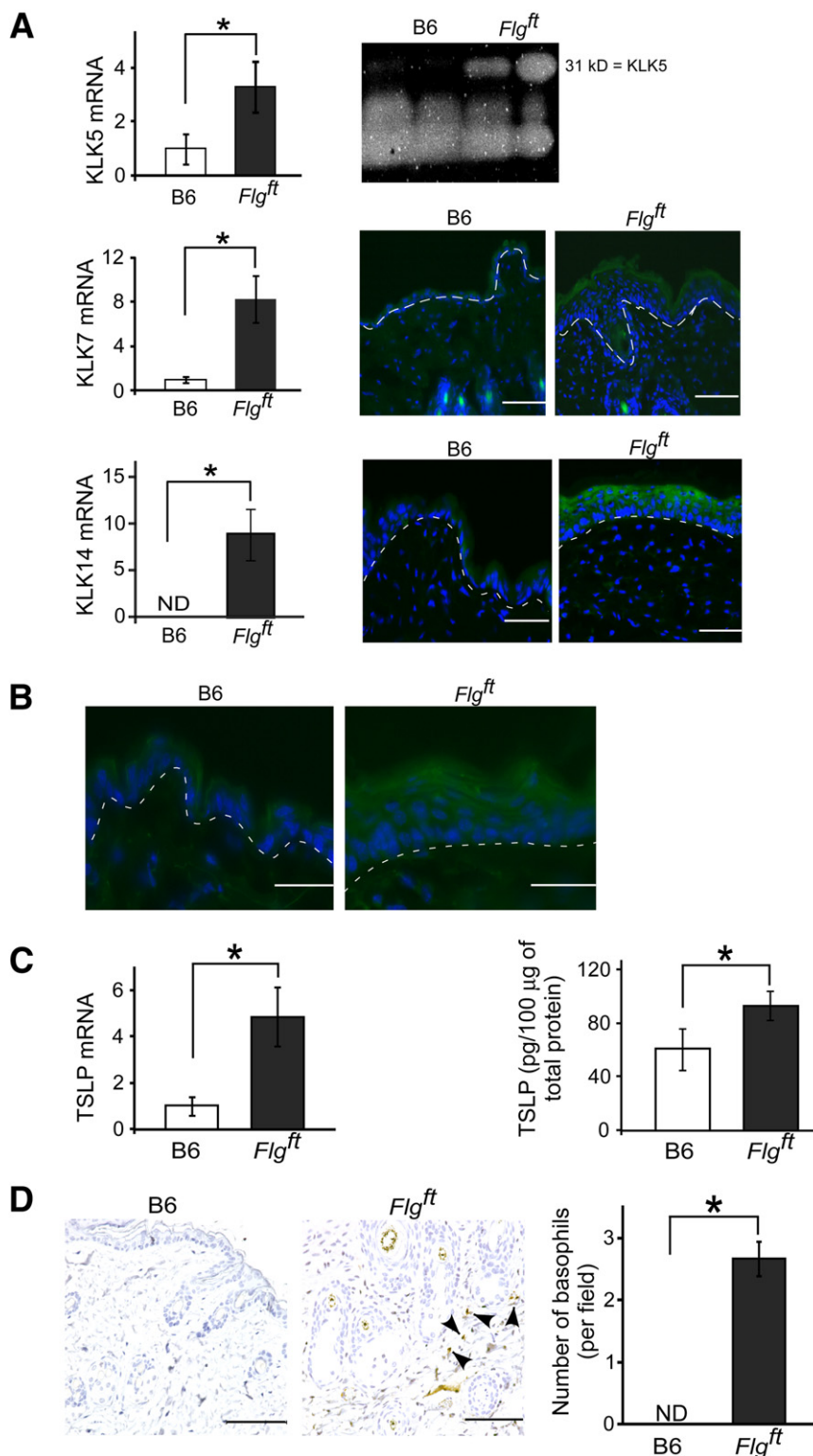


Figure 2 Enhanced KLK and TSLP expression levels and basophil accumulation in *Flg^{fl}* mice. **A:** KLK5 mRNA and enzymatic activity in the mouse skin was evaluated by casein gel zymography (**top panel**). Whole skin of adult mice was collected, and KLK7 (**middle panel**) and KLK14 (**bottom panel**) were measured and detected by mRNA and immunofluorescence (green). **B:** Enhanced epidermal proteolytic activity in *Flg^{fl}* mice, as observed by *in situ* zymography. **C:** Increased TSLP mRNA (**left panel**) and protein (**right panel**) levels from whole skin of *Flg^{fl}* mice ($n \geq 4$). mRNA data are presented as fold increase against control mice (B6). **D:** Basophil accumulation was detected as mMCP-8-positive cells (**arrowheads**) in the dermis part of the *Flg^{fl}* skin, and average numbers were counted. Scale bar = 100 µm (**A** and **B**). * $P < 0.05$.

It has been reported that an unregulated activity of KLKs in the skin of patients with Netherton syndrome results in PAR-2 activation and induces overexpression of TSLP.¹⁰ Therefore, we next investigated the expression of PAR-2 and TSLP in *Flg^{fl}* mice. In the epidermis of *Flg^{fl}* mice, PAR-2 expression was enhanced both in the steady state and after the application

of mite extract (Supplemental Figure S2A). The PAR-2 expression level in KCs from *Flg^{fl}* mice was enhanced by the PAR-2 agonist, which was not seen in KCs of control B6 mice (Supplemental Figure S2B). In addition, *Flg^{fl}* mice exhibited elevated TSLP mRNA and protein levels (Figure 2C) in accord with basophil accumulation in the skin

(Figure 2D). To investigate if TSLP is responsible for basophil accumulation, we performed an OVA-induced epicutaneous sensitization model in TSLP receptor-deficient (*TSLPR*^{-/-}) mice and control B6 mice. On the epicutaneous application of OVA, *TSLPR*^{-/-} mice showed much less basophil accumulation in the skin compared with control mice (Supplemental Figure S3), suggesting an essential role of TSLP in promote basophil accumulation, as previously reported.⁹ Taken together, these data indicate that an elevation of skin surface pH under filaggrin-deficient conditions induced the activation of KLCs, which, in turn, acted on PAR-2 in KCs and induced TSLP to accumulate basophils in the skin.

PAR-2 Activation Induces TSLP in Mouse KCs *in Vitro*

We next aimed to elucidate whether PAR-2 activation directly induced TSLP production using the mouse KC cell line, PAM 212. We cultured PAM212 in the presence of PAR-2 agonist and found that TSLP expression was up-regulated (Figure 3A). Moreover, this up-regulation was completely diminished by the addition of a PAR-2 antagonist. To assess this effect under filaggrin-deficient conditions, we prepared KC primary culture from the neonatal skin of *Flg*^{fl} and B6 mice. Interestingly, TSLP expression levels in *Flg*^{fl} mice were higher in both mRNA and protein levels, even in the absence of the PAR-2 agonist (Figure 3B), compared with those in control mice. By using *Flg*^{fl} KCs, we observed that the induction of TSLP by the PAR-2 agonist was inhibited by the PAR-2 antagonist at both mRNA and protein levels (Figure 3C). In addition, the PAR-2 antagonist itself down-regulated the PAR-2 mRNA level in KCs of B6 mice (Supplemental Figure S4). Taken together, these data suggest that TSLP is induced in KCs by the activation of PAR-2, and that this induction is enhanced under filaggrin-deficient conditions.

Inhibition of Mite-Induced AD-Like Skin Lesions by the PAR-2 Antagonist

We next investigated whether the PAR-2 pathway is involved in the development of the mite-induced dermatitis model. We recently reported that cutaneous applications of mite extract, Dp, induced AD-like skin lesions in *Flg*^{fl} mice, but not in control mice.²⁰ We induced dermatitis in *Flg*^{fl} mice by the application of 0.5% Dp ointment in the presence or absence of the PAR-2 antagonist. Treatment with Dp ointment induced prominent skin inflammation, which was inhibited by the presence of PAR-2 antagonist (Figure 4A). In addition, acanthosis, elongation of rete ridges, and dense lymphocyte and neutrophil infiltrations in the dermis after Dp application were attenuated by treatment with the PAR-2 antagonist (Figure 4B and Table 1). Consistently, histological scores of *Flg*^{fl} skin after Dp exposure were decreased by the addition of the PAR-2 antagonist treatment (Figure 4C). These results suggest that Dp, which has protease activity, activates PAR-2 and induces AD-like skin inflammation.

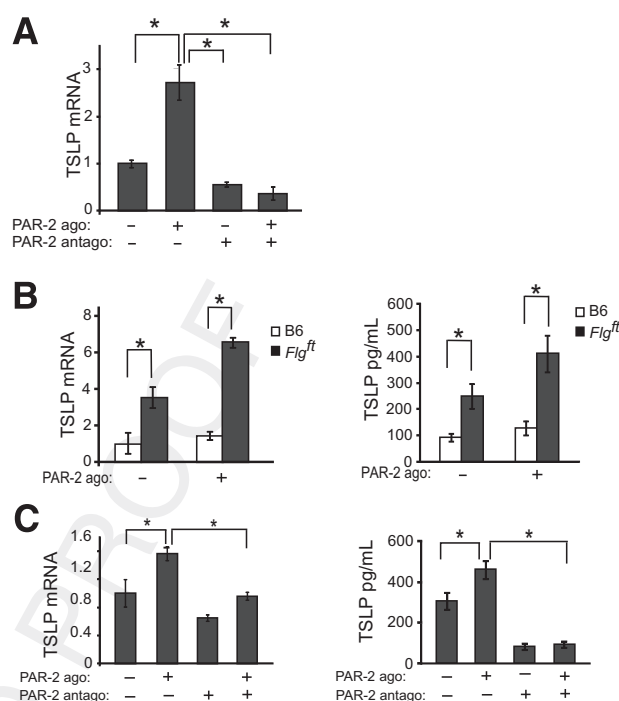


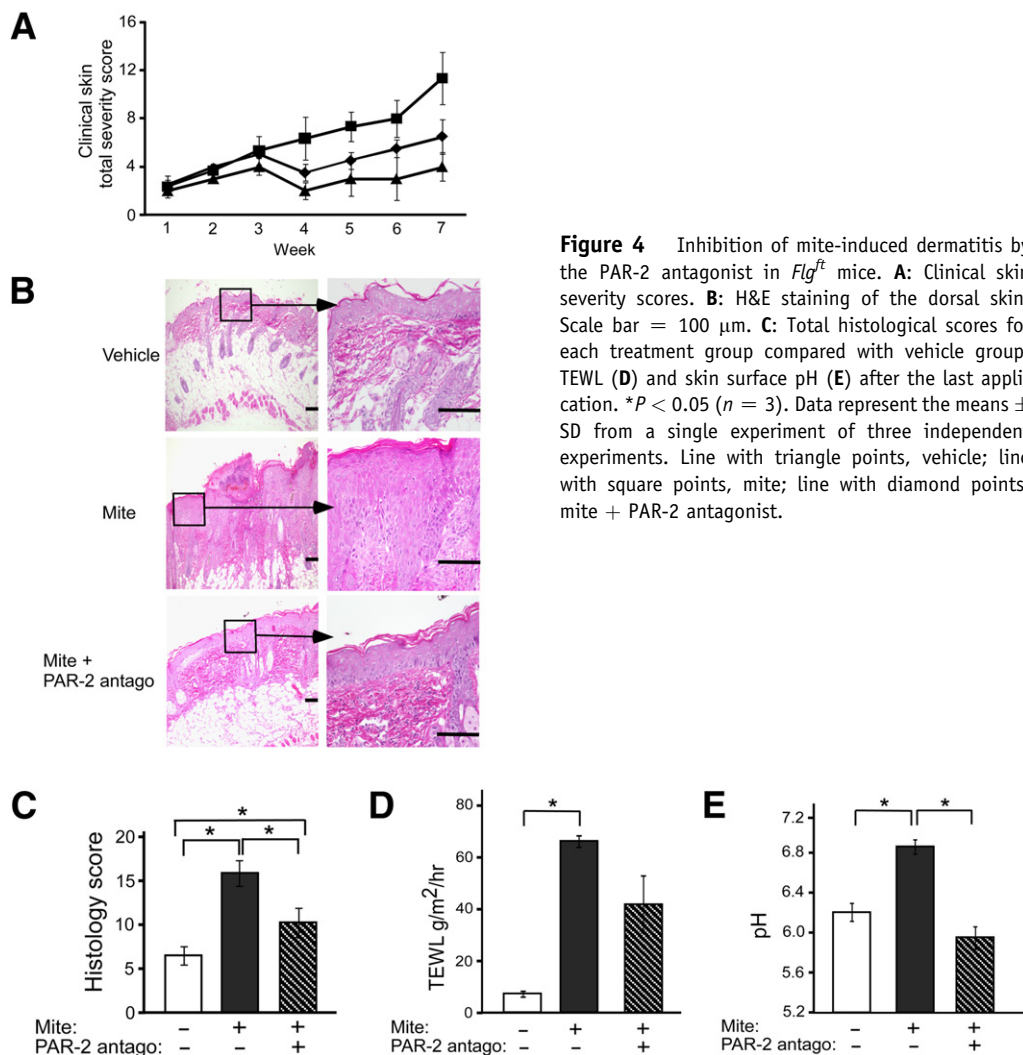
Figure 3 PAR-2 agonist induced TSLP production and was abrogated by PAR-2 antagonist in KCs. **A:** Mouse KC cells, PAM 212, were incubated with the 100 μ mol/L PAR-2 agonist (PAR-2 ago) and 150 μ mol/L PAR-2 antagonist (PAR-2 antago) for 8 hours. **B:** KC primary cultures from neonatal skin of *Flg*^{fl} and control mice were stimulated with 100 μ mol/L PAR-2 agonist (**C**) and from *Flg*^{fl} mice with 150 μ mol/L PAR-2 antagonist (12 and 3 hours of incubation for protein and mRNA levels, respectively). * $P < 0.05$ ($n = 3$). Data represent the means $\pm$ SD from a single experiment of three independent experiments. mRNA data presented as fold increase against non-treated control.

We evaluated barrier function by measuring TEWL and skin surface pH in each group. Dp treatment significantly increased TEWL and skin surface pH, both of which were decreased by PAR-2 antagonist treatment (Figure 4, D and E). Thus, the PAR-2 antagonist treatment improved barrier function, which may result in improvement of the clinical manifestations of mite-induced dermatitis.

We next investigated whether the expression of TSLP was modulated by PAR-2 using the mite-induced AD-like skin inflammation model in *Flg*^{fl} mice. Although TSLP production was marginally increased by the application of mite ointment, TSLP expression was attenuated by the application of the PAR-2 antagonist (Figure 5, A and B). B6 mice, on the other hand, exhibited no expression of TSLP, with or without the application of Dp (Supplemental Figure S5).

Basophil Accumulation in Dp-Induced Skin Inflammation

Because basophils have been reported to be recruited by protease antigens,^{14,40} we investigated whether basophils accumulated in this mite-induced AD model. Intriguingly, we observed an increase in basophil numbers resulting from the application of mite extracts. This accumulation of



basophils was attenuated by treatment with the PAR-2 antagonist (Figure 5, C and D). The accumulation of basophils was accompanied by increased expression in the skin of MCP-1 (alias, chemoattractant of human basophils that is released by KCs) (Figure 5E). We also found that murine BMBs themselves expressed PAR-2 (Figure 5F), suggesting that protease might act on basophils directly. In addition, mMCP-1 recombinant induced murine BMB migration in a dose-dependent manner (Figure 5G). Moreover, we found that stimulation of Dp in BMBs induced the expression level of CD69 (an activation marker of basophils), which was abrogated by the PAR-2 antagonist (Supplemental Figure S6), suggesting a prominent role of basophils in the lesional skin.

Discussion

In our current study, we detected an elevation of skin surface pH in adult *Flg^{fl}* mice, accompanied by increased endogenous protease activity, TSLP expression, and basophil

accumulation. TSLP was highly produced when KCs of *Flg^{fl}* mice were being exposed to a PAR-2 agonist, and was inhibited by a PAR-2 antagonist, suggesting that PAR-2 is at least partly responsible for the induction of TSLP production by KCs. We also found that mite extract, which possesses protease activity and induces skin inflammation in *Flg^{fl}* mice, also attracted basophil accumulation in skin lesions. Both

Table 1 Dermis Phenotypes after DpApplication and on Treatment with the PAR-2 Antagonist

Phenotype	Vehicle	Mite	Mite + PAR-2 antagonist
Inflammation	1.25 $\pm$ 0.50	3.25 $\pm$ 0.50*	1.75 $\pm$ 0.50
Neutrophils	0.50 $\pm$ 0.50	2.00 $\pm$ 0.80*	1.00 $\pm$ 0.00
Mononuclear cells	1.50 $\pm$ 0.50	3.50 $\pm$ 0.50*	2.00 $\pm$ 0.80
Edema	1.50 $\pm$ 0.50	3.50 $\pm$ 0.50*	2.50 $\pm$ 0.50*
Epithelial hyperplasia	1.75 $\pm$ 0.50	3.75 $\pm$ 0.50*	2.75 $\pm$ 0.50*

Data are given as means $\pm$ SD.

* P .

skin inflammation and basophil accumulation can be attenuated by PAR-2 antagonist application. A similar response can be observed in the basophil chemokine, MCP-1, in skin lesions.

The roles of TSLP in the development of AD have been addressed previously.^{41,42} Other reports have shown that bacteria-derived flagellin via Toll-like receptor 5, Toll-like receptor 2–Toll-like receptor 6 heterodimer in *Staphylococcus aureus* membranes, including diacylated lipopeptide,⁴³ viral double-stranded RNA, and a Th2 cytokine milieu,⁴⁴ tape stripping of the skin,⁴⁵ and KLK5 through the PAR-2 pathway¹⁰ can initiate the production of TSLP. Nevertheless, the role of PAR-2 in the development of AD, specifically regarding KCs, TSLP, and basophils, has not been addressed. Herein, we demonstrated that the enhancement of protease activity under filaggrin-deficient conditions induces production of TSLP and basophil accumulation in the skin and that PAR-2 induces TSLP. Therefore, the PAR-2 pathway is involved in and is at least partly responsible for a Th2-skewed skin condition similar to AD.

We and others recently reported that *Flg^{fl}* mice with *fl* and *ma* mutations spontaneously developed AD-like skin

lesions.^{20,46,47} By using these mice, we investigated the biological function of filaggrin in skin homeostasis. We demonstrated that, although skin surface pH is comparable between *Flg^{fl}* and control mice in the neonatal period, adult *Flg^{fl}* mice showed a higher pH than control mice. This observation is different from the results of a previous report that demonstrated similar skin surface pH regardless of filaggrin deficiency.⁴⁸ A possible explanation for this discrepancy is that the elevation of skin surface pH is derived from the occurrence of skin inflammation, which was not observed in the previous article.⁴⁸ Taken together, our findings show that filaggrin deficiency itself may have a minor effect on the regulation of skin surface pH, although it is likely to cause skin inflammation, which results in the elevation of skin surface pH.

An increase in skin surface pH alone activates the KLK family of serine proteases.^{36,37} At least eight KLKs are expressed in the skin,⁴⁹ among which KLKs 5, 7, 8, and 14 have been reported to be involved in the development of AD.³⁹ Excessive activity of these KLKs has been reported to impair the skin barrier by causing premature breakdown of corneodesmosomes and increased corneocyte desquamation

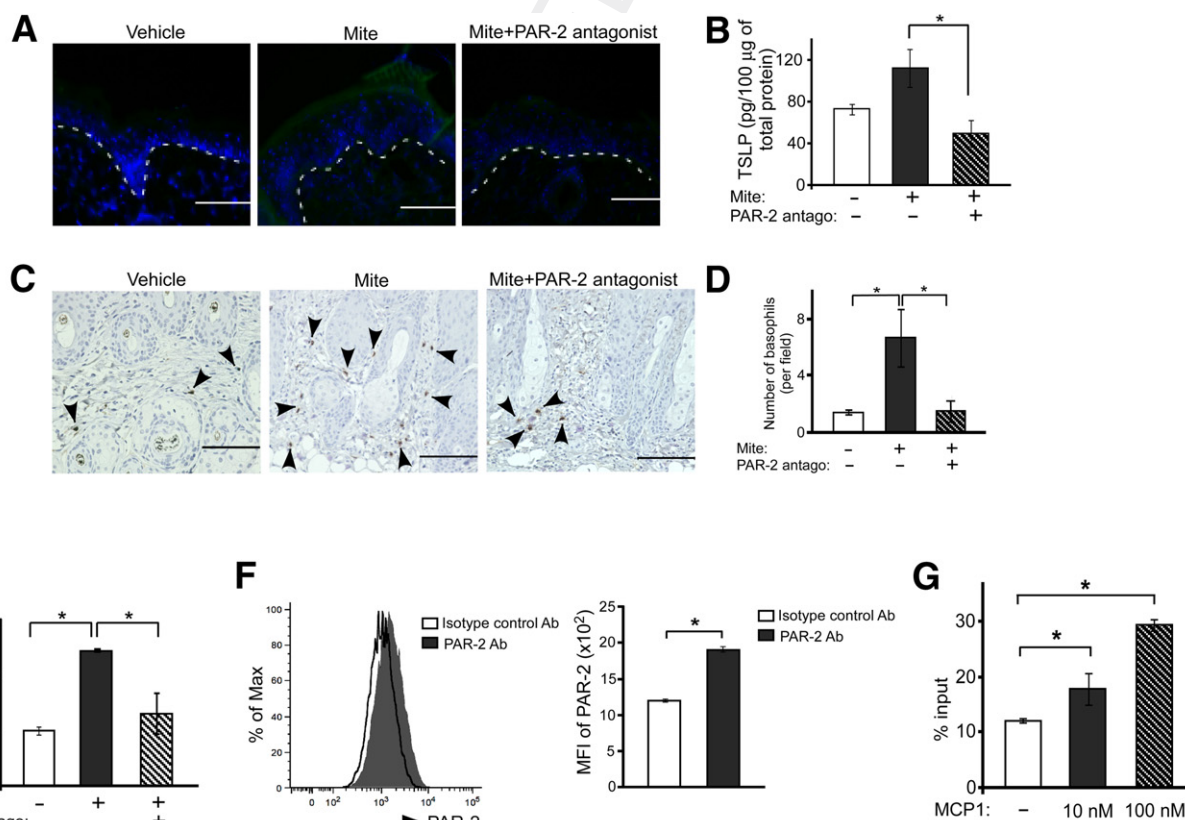


Figure 5 Enhanced TSLP expression and basophil accumulation of mite-induced skin lesions in *Flg^{fl}* mice. **A:** An IHC study of dorsal skin for anti-TSLP antibody (Ab; green). **B:** TSLP protein levels in the skin were measured by ELISA. **C and D:** Basophils were detected as mMCP-8–positive cells (arrowheads) in the dermis of the skin (**C**), and average numbers were counted (**D**). **E:** mMCP-1 mRNA level of the mouse dorsal skin. Presented as fold increase against non-treated control ($n = 3$). Data represent the means $\pm$ SD from a single experiment of three independent experiments. **F:** Murine bone marrow–derived basophils (Fc ϵ RI $^{+}$ c-kit $^{+}$ CD200R3 $^{+}$ –positive cells) expressed PAR-2, as shown by histogram (left panel) and mean fluorescence intensity (MFI) level (right panel), analyzed by flow cytometry. **G:** Basophils migrated toward recombinant mMCP-1 in a dose-dependent manner in a chemotaxis assay ($n \geq 4$). Data represent the means $\pm$ SD from a single experiment of two independent experiments. Scale bar = 100 μ m (**A** and **C**). Max, maximum.

in patients with AD.^{37,50} Herein, we have observed an increased expression of KLKs 5, 7, and 14, and enhanced protease activity in the skin of *Flg^{fl}* mice. The activation of these KLKs then induces the activation of PAR-2⁵¹ and of Th2-mediated allergic inflammation molecules (eg, TSLP).¹¹ Stronger evidence is seen in Netherton syndrome, in which unregulated KLK5 induces TSLP through the PAR-2 pathway.¹⁰ Our data support the hypothesis that TSLP is produced through the KLK–PAR-2 pathway, because TSLP is induced from filaggrin-deficient KCs in a PAR-2–dependent manner. We still do not know its underlying mechanism on how PAR-2 stimulates TSLP expression; however, it was reported that a synergy between NF-κB and STAT6 induces TSLP in response to double-stranded RNA and IL-4⁵² and stimulation of KLK5 in human keratinocytes, with either PAR-2 siRNA or an NF-κB inhibitor, lead to down-regulation of TSLP.¹⁰ Thus, a KLK–PAR-2–NF-κB–TSLP pathway may be an essential mechanism in the development of the Th2-polarized skin inflammation that is observed in patients with AD.

As we previously showed, the topical application of Dp in *Flg^{fl}* mice induces skin lesions that are clinically and histologically similar to those seen in AD.²⁰ In this study, we demonstrated that induction of TSLP in KCs by the PAR-2 agonist was higher than that by Dp. This discrepancy might derive from different protease activity; PAR-2 agonists have only serine protease activity, whereas Dp has cysteine and serine protease activities in combination. Nevertheless, we showed that the application of a PAR-2 antagonist inhibited the development of Dp-induced dermatitis in *Flg^{fl}* mice, and abolished TSLP expression in KCs. These findings suggest that Dp-induced skin inflammation was caused, at least in part, by the activation of PAR-2 in KCs.

Intriguingly, we also found that a significant number of basophils accumulated to the skin lesions induced by mite ointment. Basophils are known to play an important role in the development of immune disorders, such as allergies, by producing Th2-promoting cytokines (eg, IL-4),¹⁷ and their antigen-presenting function is critical to induce antigen-specific Th2 immunity.¹⁶ Some antigens, including papain, exhibit protease activity and induce basophil activation and accumulation; nevertheless, it remains unclear how protease activity induces the accumulation of basophils. Herein, we demonstrated that PAR-2 antagonist treatment attenuated basophil accumulation in a mite ointment–induced dermatitis model, and that the MCP-1 production was abrogated by the PAR-2 antagonist treatment. Although we do not have evidence that PAR-2 directly acts on KCs to induce MCP-1 for basophil accumulation, our findings suggest that the protease activity of a certain antigen induces MCP-1 to attract basophils, which then initiate Th2-skewing skin inflammation, as seen in AD through the PAR-2 pathway.

Flg^{fl} mice express a double mutation of *ft* and *ma*; therefore, we cannot eliminate the possibility that some of the observations could have been influenced by the

concurrent *ma* mutation. Nevertheless, another group succeeded in removing the *ma* early in the course of backcrossing with B6 mice, and showed enhanced immune responses to hapten and protein antigens (OVA) in mice with an *ft* but not an *ma* mutation.⁵³ Furthermore, AD is a complex genetic disease that arises from gene-gene and gene-environment interactions, and we believe that the findings in our mouse model can still be beneficial and reveal important findings for the understanding of AD.

In summary, we have shown that the activation of PAR-2 is a key event in the development of Th2-skewing skin inflammation via TSLP production in KCs and basophil accumulation. Because this pathway is more prominent under filaggrin-deficient conditions, the blockage of PAR-2 activation should be a possible therapeutic target for AD.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2012.11.039>.

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1365 **Supplemental Figure S1** Antagonistic activity of NPS-1577. Antagonistic activity of a PAR-2 antagonist, NS-1577, on the PAR-2 agonist-induced 1384
1366 intracellular calcium changes in HCT-15 cells (PAR-2 overexpressing cell line). RFU, relative fluorescence unit. 1385
1367 1386
1368 **Supplemental Figure S2** Enhanced PAR-2 expression in *Flg^{fl}* mice. **A:** Immunofluorescence of PAR-2 in *Flg^{fl}* mice and control mice, with or without Dp 1387
1369 application. Scale bar = 100 μ m. **B:** PAR-2 mRNA in *Flg^{fl}* and control keratinocyte culture stimulated with 100 μ mol/L PAR-2 agonist at the indicated 1388
1370 incubation period. Data are presented as fold increase against 0 hours. **P* < 0.05 against 0 hours in *Flg^{fl}* mice. 1389
1371 1390
1372 **Supplemental Figure S3** Attenuated basophil accumulation in *TSLPR^{-/-}* mice. *TSLPR^{-/-}* mice showed much less basophil accumulation in the skin 1391
1373 compared with wild-type mice after OVA application. Scale bar = 100 μ m. **Arrowheads**, basophils. **P* < 0.05. ND, not detected. 1392
1374 1393
1375 **Supplemental Figure S4** Down-regulation of PAR-2 by the PAR-2 antagonist. Down-regulation of PAR-2 mRNA in keratinocytes of B6 mice by the PAR-2 1394
1376 antagonist. Data presented as fold increase against non-treated control. **P* < 0.05. 1395
1377 1396
1378 **Supplemental Figure S5** Impaired induction of TSLP in B6 mice. B6 mice showed no expression of TSLP by immunofluorescent staining, with or without 1397
1379 Dp application. 1398
1380 1399
1381 **Supplemental Figure S6** Mite Dp enhanced activation of basophils, which was diminished by PAR-2 antagonist. Murine bone marrow-derived (Fc ϵ RI⁺ 1400
1382 c-kit⁻) were cultured for 24 hours, with or without 10 ng/mL Dp in the presence or absence of PAR-2 antagonist (PAR-2 antago). The basophil activation 1401
1383 marker (CD69) was analyzed by flow cytometry. MFI, mean fluorescence intensity. **P* < 0.05. 1402