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# 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, 182: 841–851; http://dx.doi.org/10.1016/j.ajpath.2012.11.039)*

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 mice[8] 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 Ziegler[26] (Immunology Programme, Benaroya Research Institute, Seattle, WA) 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, Tokyo, Japan), 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 Peptron (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 ($4 \times 10^4$ 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

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