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Defective maintenance of pH of stratum corneum is correlated with preferential emergence and exacerbation of atopic-dermatitis-like dermatitis in flaky-tail mice

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ABSTRACT

Background: Neutralization of stratum corneum (SC) pH, which is induced by a variety of stimuli, such as scratching, use of soap and inflammation, can stimulate activity of serine protease (SPase). Activation of SPase induces production of thymic stromal lymphopoietin (TSLP) through protease-activated receptor-2. Both reduced expression of natural moisturizing factors, which are required for maintenance of SC pH, and the preferential development of atopic dermatitis (AD)-like dermatitis are found in flaky-tail mice (FTM) with a loss-of-function mutation in filaggrin.

Objective: We examined possible correlations between disturbance of responses to an exogenous stimulus of SC neutralization and the preferential emergence of AD-like dermatitis in FTM.

Methods: FTM and wild-type mice (C57BL/6) were subjected to an SC-neutralization stimulus *via* application of 1,1,3,3-tetramethylguanidine (TMG). TMG was applied to young mice at a time when FTM had not yet developed significant dermatitis, and we examined their ability to maintain SC acidity and several parameters associated with AD-like dermatitis.

Results: The recovery of SC pH after the application of TMG was delayed in FTM, presumably because of unchanged expression of Na^+/H^+ antiporter 1, which is involved in maintenance of SC acidity. Cutaneous inflammation with elevated SPase activity and serum levels of TSLP, thymus and activation-regulated chemokine and IgE were induced only in TMG-treated FTM.

Conclusion: Our results suggest that defective maintenance of pH of SC is correlated with emergence and exacerbation of AD-like dermatitis in FTM.

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1. Introduction

Collaboration of genetic background associated with impaired epidermal barrier and environmental factors is required for the emergence of allergic disorders, such as atopic dermatitis (AD) [1]. In fact, substantial pathological relationships between mutations in filaggrin and allergic conditions, including AD, asthma, and food allergy, have been reported [2]. However, precise mechanisms by which the effects of genetic abnormalities, such as mutations in filaggrin, are influenced by environmental factors, with emergence or augmentation of allergic conditions have not been fully elucidated. We examined possible correlations between disturbance of maintenance of pH of stratum corneum (SC) due to genetic abnormality and the preferential emergence of AD-like dermatitis by an exogenous stimulus of SC neutralization in flaky-tail mice (FTM).

The uppermost epidermal layer, the SC, has an acidic surface pH [3]. The normally acidic pH of SC regulates several key protective functions of the skin, including permeability barrier homeostasis, SC integrity/cohesion [4–6], and antimicrobial defenses [7]. In addition, an acidic SC pH inhibits the activity of serine protease (SPase) [8]. Conversely, neutralization of SC adversely impacts these functions and stimulates activation of SPase [3]. Activation of SPase induces degradation of corneodesmosomes, a decrease in SC integrity/cohesion [8], and production of thymic stromal lymphopoietin (TSLP), which is produced by epithelial cells and triggers dendritic cell-mediated Th2-type inflammation, through protease-activated receptor-2 [9]. A variety of environmental stimuli, such as disturbance of the permeability barrier by scratching, use of soap and allergic cutaneous inflammation including AD [3,10–12],

Abbreviations: AD, atopic dermatitis; FTM, flaky-tail mice; NHE1, sodium/proton pump N^+/H^+ antiporter 1; NMFs, natural moisturizing factors; SC, stratum corneum; SPase, serine protease; sPLA2, secretory phospholipase A2; TARC, thymus and activation-regulated chemokine; TEWL, transepidermal water loss; TMG, 1,1,3,3-tetramethylguanidine; TSLP, thymic stromal lymphopoietin; WT, wild-type mice.

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neutralize the pH of SC. On the other hand, recent studies showed that maintenance of acidic SC prevented the emergence of AD-like dermatitis in a murine model [10], and that acute acidification of SC improved the processing of lipids and inhibit the degradation of corneodesmosomes [13]. Thus, changes in SC pH might be important in the pathogenesis of skin disorders associated with permeability barrier dysfunction and inflammation (in particular, Th2-type inflammation), such as AD, and maintenance of the acidity of SC is very important to skin homeostasis.

Several mechanisms are involved in the maintenance of SC acidity. Free fatty acids of pilosebaceous origin [14], microbial metabolites [15], and eccrine gland-derived products [16,17] appear to decrease SC pH. In addition, a sodium/proton pump N^+/H^+ antiporter 1 (NHE1) [11,18], generation of free fatty acids by secretory phospholipase A2-catalyzd (sPLA2-catalyzed) hydrolysis of phospholipid [19,20], and natural moisturizing factors (NMFs), such as urocanic acid generated from histidines of filaggrin by histidase, also participate in this process [20,21]. In particular, both NHE1 and sPLA2 seem to be essential to SC acidity because, when either the sPLA2- or NHE1-mediated pathways to acidification are compromised, the SC pH rises, indicating that other acidifying mechanisms cannot compensate for their disruption [18–20].

While FTM with a loss-of-function mutation in the gene for filaggrin and the *matted* mutation have a greater tendency than wild-type mice (WT) to exhibit AD-like dermatitis with or without exposure to appropriate stimuli, such as haptens or allergens, the mechanistic link between genetic background and environmental factors is unclear [22,23]. Fluhr et al. had investigated the relationship between SC pH and urocanic acid derived from filaggrin, which can work as NMFs, using FTM. In that study, they hypothesized that if NMFs derived from filaggrin were important to regulate SC pH, SC pH of FTM and histidase-deficient mice should increase, however, those mice could maintain SC acidity *via* enhanced expression of NHE1 and sPLA2 despite decrease of NMFs at steady state [20]. Furthermore, recent study showed that up-regulation of NHE1 and sPLA2 acidification pathway can compensate the filaggrin deficiency, maintaining the skin surface acidity in 3D skin construct [24]. Therefore, the pathway of urocanic acid derived from filaggrin seems to be not essential for SC acidification.

Herein, we hypothesized that, if such compensatory mechanisms were fully exploited at the steady state by genetic abnormality, FTM might be sensitive to an exogenous SC-neutralization stimulus, equivalent to an environmental stimulus such as scratching, soap or cutaneous inflammation, with resultant development of allergic inflammation. We show here that the recovery of SC pH after the exogenous SC-neutralization is delayed and the defective maintenance of pH of SC is correlated with preferential emergence and exacerbation of AD-like dermatitis in FTM.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice, which were used as WT (Japan SLC Inc., Hamamatsu, Japan), and female FTM (Jackson Laboratory, Bar Harbor, ME, USA) were used at indicated ages. All animals were housed under conventional conditions and had free access to a commercial diet and water. All experiments with mice were approved by the Ethics of Animal Experimentation Committee of Oita University.

2.2. Neutralization of SC and physiological assessments

Acute neutralization of SC was achieved with a diluted superbase, 1,1,3,3-tetramethylguanidine (TMG). Topical application of TMG (1:100, v/v) in a mixture of propylene glycol and ethanol (7:3, v/v) on the flank of mice raises the pH of SC without any evidence of toxicity, inflammation or immunological response [5]. In some experiments, we applied TMG which was adjusted with hydrochloric acid into the pH equivalent to that of vehicle (low pH-TMG; pH value is about 5.70). Transepidermal water loss (TEWL), SC hydration and SC surface pH were measured at room temperature (22–26 °C) and 40–55% relative humidity. TEWL was measured with a Tewameter (TM300; Courage & Khazaka, Cologne, Germany). SC hydration was evaluated by analyzing electrical impedance of skin with a Corneometer (CM825; Courage & Khazaka). SC surface pH was evaluated with a skin pH meter (pH 905; Courage & Khazaka). Each value was measured according to each manufacturer's instructions [25,26]. Recovery rate of SC pH was calculated according to the following formula: ([pH value 1 h after TMG treatment] – [pH value at the indicated time])/([pH value 1 h after TMG treatment] – [pH value before TMG treatment]).

2.3. Quantitative assessment of skin morphology

Skin samples from the shaved flank were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4-μm sections were stained with hematoxylin and eosin, toluidine blue and CD3-specific polyclonal antibody (Dako, Tokyo, Japan). The thickness of the epidermis was measured with the scaled ocular lens of a light microscope. The various types of inflammatory cell were counted under high-power magnification.

2.4. Immunohistochemical staining

Skin samples were embedded in Optimal Cutting Temperature compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen in liquid nitrogen. Cryosections of 5-μm thickness (CM3050 cryostat; Leica Microsystems, Wetzlar, Germany) were incubated for 20 min in blocking buffer [PBS plus 5% normal donkey serum (Sigma–Aldrich Co., St. Louis, MO, USA), 2% bovine serum albumin (Sigma–Aldrich Co.) and 0.05% Tween-20]. Then, sections were incubated overnight at 4 °C with primary polyclonal antibodies against NHE1 (Millipore, Temecula, CA, USA), sPLA2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) or TSLP (R&D Systems, Minneapolis, MN, USA). After washing, specimens were incubated for 1 h at room temperature with Alexa Fluor® 488-conjugated goat anti-rabbit or donkey anti-goat second antibodies (Life Technologies, Carlsbad, CA, USA) and counterstained with propidium iodide. Staining was evaluated under a confocal microscope (LSM710 ZEN; Zeiss, Oberkochen, Germany).

2.5. Western blotting

Epidermal sheets were separated from skin samples by treatment with 1000 IU/ml dispase (Godo Shusei, Tokyo, Japan) for approximately 1 h at 37 °C. Each epidermal sheet was homogenized in protein extraction buffer (40 mM Tris–HCl, pH 7.5, 10 mM EDTA containing protease inhibitors and 0.5% Nonidet P-40). The homogenates were centrifuged; the detergent-insoluble pellet was solubilized in protein extraction buffer containing 6 M urea. These samples were separated by 10% SDS–PAGE before transfer to Immobilon®-P Transfer Membrane (Millipore). We used antibodies against NHE1 (see above) and β-actin (Cell Signaling Technology Inc., Danvers, MA, USA) as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc.) as second antibodies, and visualized with ECL™ Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) [27]. Band intensities were determined with Image Quant LAS

4000mini and quantified using Image Quant TL software (GE Healthcare).

2.6. Zymographic assessment of SPase activity

SPase activity was assessed in freshly obtained skin samples by *in situ* zymography [8,27]. Five-micrometer frozen sections of skin samples were incubated with substrate solution (EnzChek®Protease Assay Kit; Life Technologies) for 2 h at 37 °C. After three washes with 0.025% Tween-20, sections were counterstained with propidium iodide and examined under the confocal microscope.

2.7. Quantitative determination of the fluorescence intensities

For quantitative determination of the fluorescence intensities of immunohistochemical staining and *in situ* zymography, confocal images were analyzed in each group, as described previously [28]. Exposure and acquisition setting were fixed and were such that no signal saturation occurred. The fluorescence intensity in the epidermis of each skin biopsy specimen was measured using LSM Software ZEN 2009 (Zeiss) and fluorescence intensity per unit area was calculated.

2.8. Measurements of serum levels of TSLP, TARC and IgE

Serum levels of TSLP, TARC and IgE were determined by ELISA with Quantikine for TSLP (R&D Systems), a TARC immunoassay (R&D Systems) and a mouse IgE quantitation kit (Bethyl Laboratories, Montgomery, TX, USA) according to each manufacturer's instructions. Serum levels of TARC or IgE were measured with 10- or 20-fold diluted serum.

2.9. Real-time PCR

Total RNA was isolated from whole skin samples, which were collected after defatting with scissors, using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to each manufacturer's instructions. Complementary DNA products were amplified in a 20-μl reaction volume containing 10 μl LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH) and 1.0 μM of primers on a LightCycler 480 System (Roche Diagnostics GmbH). The mouse primers used for real-time PCR are as follows: GAPDH forward: 5′-TGAACGGGAAGCTCACTGG-3′ and GAPDH reverse: 5′-TCC ACCACCCTGTTGCTGTA-3′; TSLP forward: 5′-TTTCTAACTGCAACTTCACGTC-3′ and TSLP reverse: 5′-CTCTCA-CAGTCCTCGATTTG-3′. Product specificity was evaluated by melting curve analysis, and relative gene expression levels were calculated using the comparative Ct method with the housekeeping gene GAPDH.

2.10. Statistical analysis

All experiments were analyzed by the two-tailed Student's *t*-test or, when multiple comparisons were made, by ANOVA analysis. All results are presented as means ± SEMs of *n* experiments. A *p*-value of less than 0.05 was considered evidence of statistical significance.

3. Results

3.1. Young FTM (7–8 weeks) did not develop spontaneous dermatitis in our laboratory

Initially, our experiments required mice without significant dermatitis since dermatitis affects SC pH, described above, but FTM develop dermatitis spontaneously with age. The severity of dermatitis and age of onset in FTM differ among laboratories [22,23,29–31]. Such inconsistency might be related to the presence or absence of the *matted* mutation and/or to variations in the genetic background of individual strains and in environmental factors [23,32]. In our laboratory, young FTM (7–8 weeks old) did not have any clinical or histological symptoms of dermatitis, while old FTM (45–46 weeks old) developed dermatitis spontaneously (Fig. 1a). Values of TEWL and SC hydration did not differ between young FTM and WT (Fig. 1b and c). SC pH of FTM was slightly lower than that of WT (Fig. 1d), as reported previously [20], while, Moniaga et al. showed SC pH of young FTM increased in accompany with emergence of dermatitis [23,33]. Serum levels

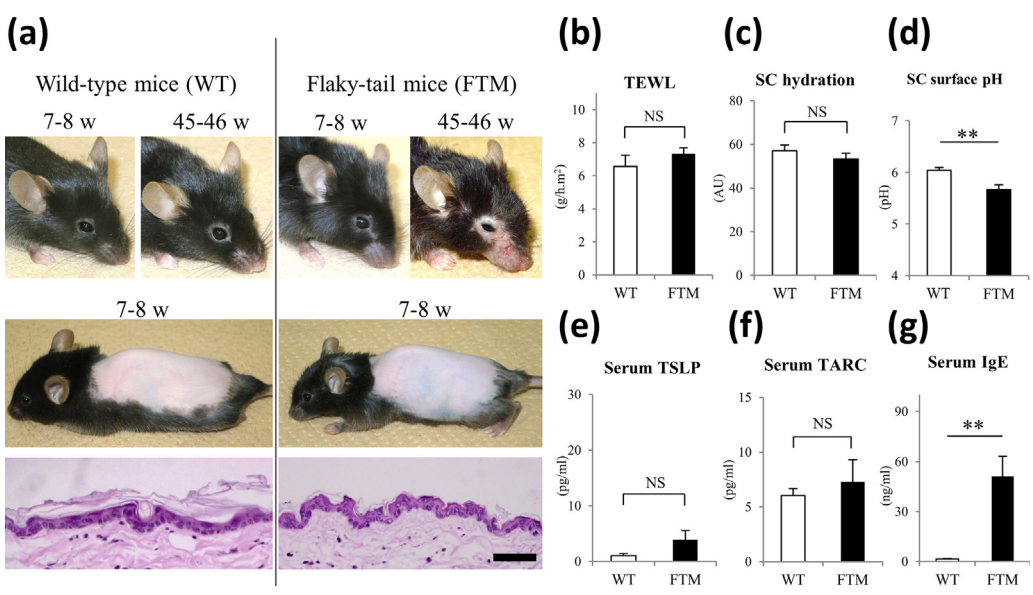

Fig. 1. Characterization of FTM in our laboratory. Clinical and histological findings for WT and FTM at 7–8 and 45–46 weeks of age (a). TEWL (b), SC hydration (c), SC pH (d), and serum levels of TSLP (e), TARC (f) and IgE (g) on WT and FTM at 7–8 weeks old were measured as described in the text. $n = 10$ in (b)–(d) and $n = 6$ in (e)–(g). In our laboratory, young FTM (7–8 weeks old) did not have any clinical or histological symptoms of dermatitis, while old FTM (45–46 weeks old) developed dermatitis spontaneously. Error bars equal to means ± SEMs. $^{**}p < 0.01$. NS, not significant. AU, arbitrary units. Scale bar = 50 μm.

of TSLP and TARC, which are closely related to disease activity in AD [34,35], also did not differ between young FTM and WT, although serum levels of IgE were higher in FTM (Fig. 1e–g). These findings suggested that substantial cutaneous inflammation had not developed in young FTM in our laboratory, therefore, we used only young mice in our experiments.

3.2. Recovery of normal pH after elevation of SC pH was impeded in FTM

We examined the recovery of SC pH after exposure to an SC pH-neutralization stimulus. Initially, we treated mice with a single application of the superbase, TMG and measured the SC pH before and 1, 2, 4, 8, 12 and 24 h after the application. There was no significant difference between the mean value of SC pH of WT (7.12 ± 0.07) and those of FTM (7.26 ± 0.04) 1 h after the application. Interestingly, the SC pH recovery in FTM was delayed significantly. As a result, the SC pH of WT almost recovered at 4 h after the application of TMG, but that of FTM needed 24 h or more time for complete recovery (Fig. 2a). We then subjected mice to daily application of TMG at 24-h intervals and measured SC pH before and 24 h after the each application. Values of SC pH in TMG-treated FTM were higher than those in other experimental groups during the long-term application of TMG (Fig. 2b). Thus, the recovery of SC pH after exposure to the exogenous SC-neutralization stimulus was clearly impeded in FTM, which can induce persistent elevation of SC pH.

3.3. The expression of NHE1 was enhanced by the application of TMG in WT but not in FTM

We attempted to identify the mechanism responsible for the failure of FTM to respond appropriately to the exogenous SC-neutralization stimulus. As reported previously [20], immunohistochemical staining revealed that the steady-state level of NHE1 in the epidermis was higher in FTM than in WT (Fig. 3a, left panels). Interestingly, we detected the apparent enhanced expression of NHE1 after application of TMG in WT but not in FTM (Fig. 3a, right panels). Similar results were obtained by Western blotting (Fig. 3b). On the other hand, immunohistochemical staining revealed no apparent difference in expression of sPLA2 in the same samples as those used to examine expression of NHE1 (Supplementary Fig. S1). These findings raised the possibility that one of the mechanistic factor for maintenance of a low SC pH, namely, the mechanism involving NHE1, is fully exploited in FTM at steady state and, as a result, recovery of SC pH after exposure to an exogenous SC-neutralization stimulus is disturbed in FTM, although precise reasons why NHE1 could not increase in response to further environmental stress remain unclear.

3.4. Two-week daily application of TMG induced substantial skin inflammation with elevated SPase activity and expression of TSLP in epidermis of FTM but not of WT

We subjected mice to 2 weeks of daily application of TMG or vehicle to determine whether FTM would develop AD-like dermatitis more easily than WT in response to an SC-neutralization stimulus. We applied TMG or vehicle every 24 h to flanks of mice (starting at 5–6 weeks of age) for 2 weeks.

We observed elevation of SC pH only in TMG-treated FTM at the end of the treatment period (Fig. 4a). Moreover, epidermal thickness and dermal infiltration by CD3-positive cells and mast cells increased only in TMG-treated FTM (Fig. 4c–e, Supplementary Fig. S2). In addition, SC hydration was reduced only in TMG-treated FTM (Fig. 4b). On the other hand, there was no difference in SC pH, SC hydration and epidermal thickness between vehicle-treated and low pH-TMG-treated FTM, suggesting that the effect of TMG

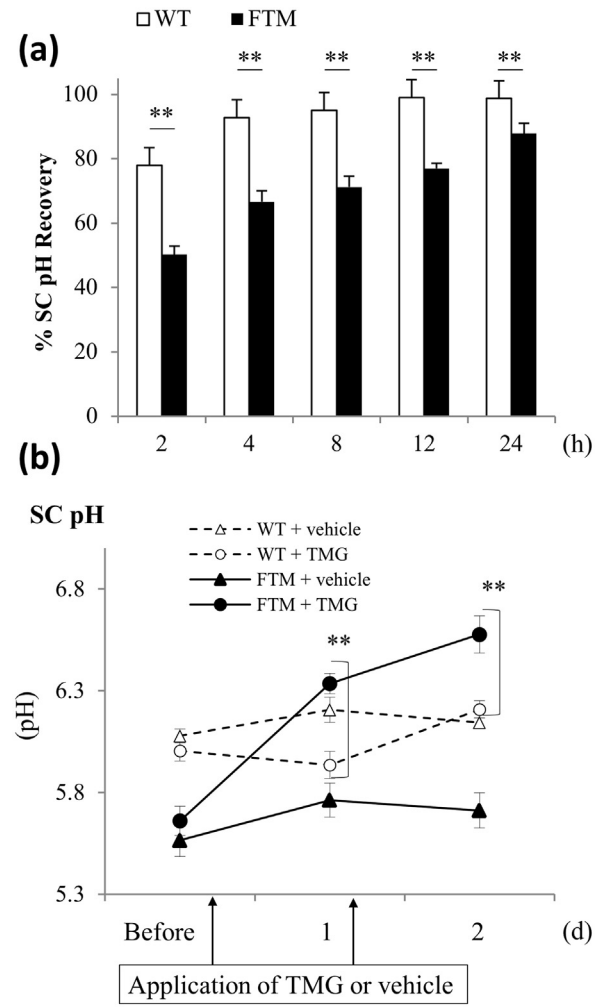

Fig. 2. Measurements of SC pH recovery after the application of TMG. (a) SC pH of WT and FTM was measured before and 1, 2, 4, 8, 12 and 24 h after a single application of TMG. Histogram shows the kinetics of SC pH recovery, and that of FTM was significantly delayed. $n = 10–12$; (b) WT and FTM were subjected to daily application of TMG at 24-h intervals and SC pH was measured before and 24 h after the each application. Values of SC pH in TMG-treated FTM were higher than those in other experimental groups during the long-term application of TMG. $n = 6$. Error bars equal to means ± SEMs. $^{**}p < 0.01$.

treatment on the induction of skin inflammation is exclusively due to SC neutralization by TMG (Supplementary Fig. S3).

Consistent with the rise in SC pH, SPase activity was enhanced only in TMG-treated FTM (Fig. 5a and c). Reflecting the relationship between elevated SPase activity and expression of TSLP [8,9], expression of TSLP in epidermis was enhanced only in TMG-treated FTM (Fig. 5b and d, Supplementary Fig. S4). Taken together, our results suggested that the disturbance of recovery from an SC-neutralization stimulus might be involved in the induction of Th2-type inflammation, namely, AD-like dermatitis.

3.5. Two-week daily application of TMG induced systemic skin inflammation with elevation of serum TSLP, TARC and IgE in FTM

We noted that eyelids of TMG-treated FTM exhibited erythema and edema (Fig. 6a) even though TMG and vehicle had been applied only to flanks in the present study. Such symptoms were very similar to those in our old FTM (Fig. 1a), which spontaneously developed AD-like dermatitis. Furthermore, serum levels of TSLP, TARC and IgE were higher in TMG-treated FTM alone than in both TMG-treated WT and vehicle-treated FTM (Fig. 6b–d). Thus, disturbance of the ability to recover from an SC-neutralization stimulus might be

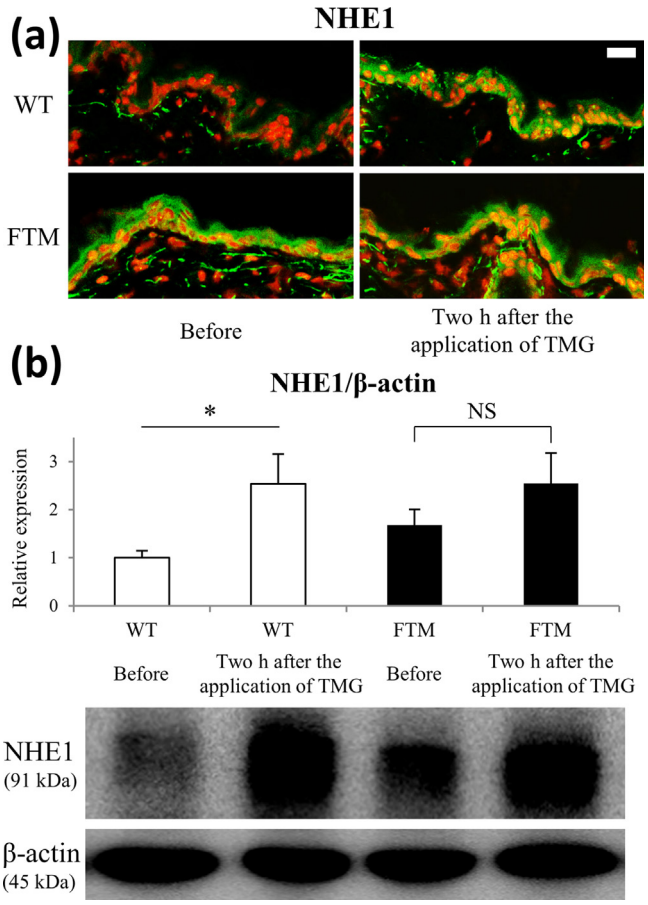

Fig. 3. The expression of NHE1 before and after application of TMG. Skin samples were obtained before (at steady-state) or 2 h after the application of TMG. Immunohistochemical staining and Western blotting were performed as described in the text. (a) Green staining of epidermis represents expression of NHE1 and red counterstaining represents nuclear staining with propidium iodide. The expression of NHE1 in the epidermis was higher in FTM than in WT at steady-state, and was enhanced by the application of TMG in WT but not in FTM. Scale bar = 20 μm. (b) Western blotting analysis revealed similar expression patterns of NHE1 in epidermis as those shown by immunohistochemical staining. Representative bands obtained by Western blotting are exhibited in lower panel. $n = 6–7$. Error bars equal to means ± SEMs. $^{*}p < 0.05$. NS, not significant.

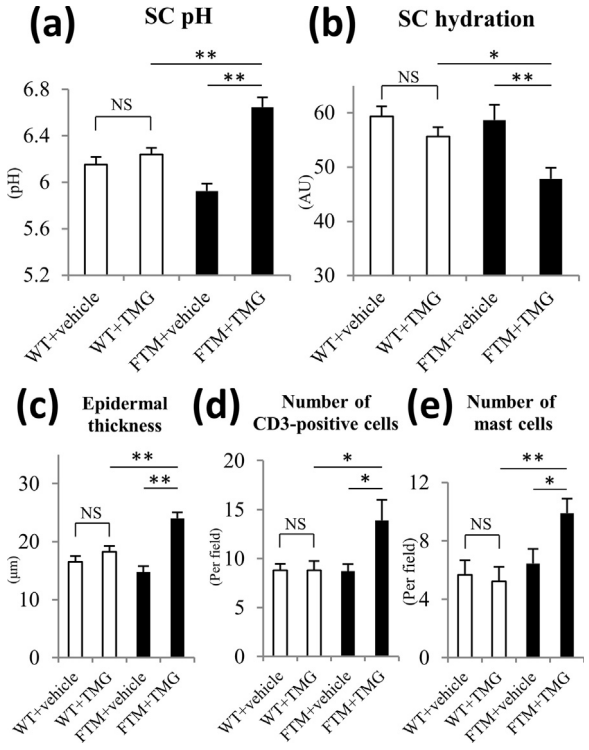

Fig. 4. Substantial cutaneous inflammation, accompanied by elevation of SC pH, in TMG-treated FTM. Measurements of SC pH and SC hydration were obtained and skin biopsies were analyzed in WT and FTM, after 2 weeks of daily application of TMG or vehicle. (a) Elevation of SC pH was observed only in TMG-treated FTM at the end of the treatment period. $n = 7–8$. (b) SC hydration was reduced only in TMG-treated FTM. $n = 10$. (c–e) Hematoxylin and eosin staining and immunohistochemical stainings were performed as described in the text. Epidermal thickness (c) and number of CD3-positive cells (d) and mast cells (e) increased only in TMG-treated FTM. $n = 9–10$. Error bars equal to means ± SEMs. $^{*}p < 0.05$, $^{**}p < 0.01$. NS, not significant. AU, arbitrary units.

involved in the induction of AD-like dermatitis not only in the region challenged by the stimulus but also systemically *via*, at least in part, elevation of serum levels of TSLP and TARC.

4. Discussion

The present study demonstrated that, in FTM, the recovery from an exogenous SC-pH-neutralization stimulus was impeded, with resultant development of Th2-type inflammation, presumably as a consequence of enhanced SPase activity, with elevation of the level of TSLP in the epidermis, and of serum levels of TSLP and IgE. Our results suggest that defective maintenance of pH of SC might be involved in the preferential emergence and/or development of severer AD-like dermatitis in FTM compared to that observed in normal mice, providing a possible link between failure to maintain an appropriate SC pH and emergence of allergic inflammation. Although precise mechanism of defective maintenance of pH of SC in FTM still remains unclear, one of the possible reasons why the recovery of SC pH after the application of TMG was delayed might be unchanged expression of NHE1 which had already been fully exploited to compensate against internal SC-neutralization stimuli caused by genetic background (*i.e.* loss-of-function mutation in the gene for filaggrin and/or the *matted* mutation) in FTM. In fact, previous studies suggested that the up-regulation of NHE1 was required to maintain SC acidity [11,18,19].

In this study, we used FTM not only with a defect in the synthesis of filaggrin but also with the *matted* mutation [22,23,32]. As noted recently, when FTM are used in studies of AD in the context of filaggrin deficiency, the results need to be interpreted carefully because of the presence of the *matted* mutation [32,36]. In addition, whether the *matted* mutation influences the mechanism of the maintenance of SC pH is unclear. Therefore, the present study does not necessarily account for a mechanistic link between filaggrin mutation and emergence of AD, although it clearly demonstrated a strong association between defective maintenance of SC pH and the emergence and/or exacerbation of AD-like dermatitis. Further studies are required to confirm this issue.

Levels of serum IgE are elevated in most patients with AD, and the level is often correlated with the prognosis and/or severity of AD [34,37]. However, serum levels of IgE were elevated in young FTM in the present study, even though we failed to detect any clinical abnormalities and the skin histology, levels of TEWL, SC hydration and serum levels of TARC were similar to those in WT. The results suggest that some immunological abnormality in organs other than skin is responsible for the elevation of serum IgE in young FTM. Alternatively, minor and undetectable permeability dysfunction and/or cutaneous inflammation might be involved in the elevation of serum levels of IgE in young FTM, contributing in part to the abnormality in pH homeostasis of the SC, resembling cases of "non-lesion" in patients with AD.

A previous study suggested that an elevated level of sPLA2 might be involved in a compensatory mechanism that maintains

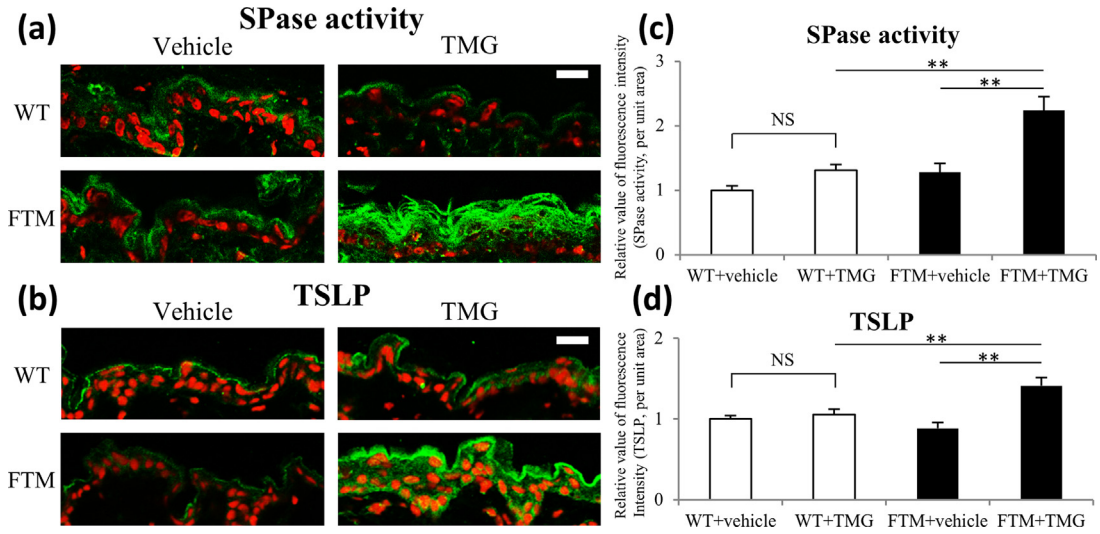

Fig. 5. SPase activity and expression of TSLP in epidermis after 2 weeks of daily application of TMG or vehicle. (a and b) Skin samples were obtained from WT and FTM after 2 weeks of daily application of TMG or vehicle. SPase activity (a) and expression of TSLP (b) in epidermis were examined by *in situ* zymography and immunohistochemical staining, respectively. Green staining represents SPase activity or expression of TSLP and red counterstaining represents nuclear staining with propidium iodide. (c and d) The fluorescence intensity of SPase activity (c) or TSLP (d) in the epidermis was measured as described in the text. $n = 12–13$ (from three mice). SPase activity and expression of TSLP in epidermis were enhanced only in TMG-treated FTM. Scale bar = 20 μm. $^{**}p < 0.01$. NS, not significant.

SC acidity in FTM [20]. However, we found no substantial differences in the steady-state expression of sPLA2 between young WT and FTM or before and after the application of TMG in either type of mice (Supplementary Fig. S1). The reason for this discrepancy is unclear but, in the cited study, authors used older FTM than we did, with slightly thickened epidermis. Thus, the initiation of spontaneous dermatitis might have influenced the expression of sPLA2. In fact, an increase in free fatty acids associated with an elevated activity of sPLA2 in human atopic skin has been reported [38,39]. Although the discrepancy remains to be resolved, the present and previous study suggests that NHE1 pathway might be more rapid and functional than sPLA2 pathway on compensatory mechanism of SC pH normalization [24].

In summary, the present study suggests that defective maintenance of SC pH might be one of the key mechanistic factors in the pathogenesis of allergic inflammation, such as AD. Increased sensitivity to an exogenous SC-neutralization stimulus can induce persistent elevation of SC pH due to several stimuli, such as

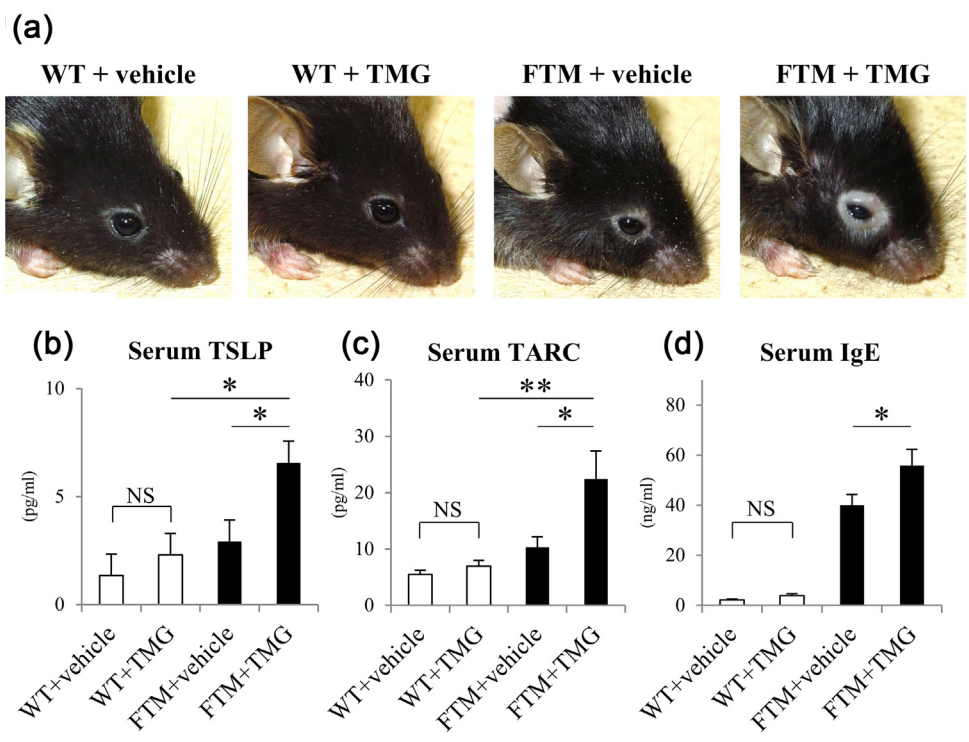

Fig. 6. Clinical findings and serum levels of TSLP, TARC and IgE after a 2 weeks of daily application of TMG or vehicle. (a) After 2 weeks of daily application of TMG or vehicle to WT and FTM, eyelids of TMG-treated FTM alone exhibited erythema and edema even though TMG and vehicle had been applied only to flanks, and this clinical finding was very similar to that in old FTM, which spontaneously developed AD-like dermatitis (see Fig. 1a). (b–d) Serum levels of TSLP (b), TARC (c) and IgE (d) after a 2 weeks of daily application were higher in TMG-treated FTM alone than in both TMG-treated WT and vehicle-treated FTM. $n = 6–8$. Error bars equal to means ± SEMs. $^{*}p < 0.05$, $^{**}p < 0.01$. NS, not significant.

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scratching, use of soap and inflammation, with resultant induction of emergence and/or exacerbation of allergic inflammation, including AD-like dermatitis, *via* elevation of SPase activity in epidermis and serum levels of TSLP, TARC and IgE. Furthermore, skin inflammation increases SC pH and creates a vicious cycle (Supplementary Fig. S5). Up-regulation of NHE1 is induced by permeability barrier abrogation [11]. Therefore, theoretically, any genetic abnormalities related to permeability barrier dysfunction may result in defective maintenance of SC pH, resulting in a prolonged relatively high SC pH following exposure to neutralizing stimuli. Therefore, the present study might provide a possible mechanism for the link between environmental stimuli and genetic background related with compromising permeability barrier function in allergic inflammation. Further investigations to assess whether same mechanisms function in human AD and/or murine AD models which have some genetic backgrounds associated with impaired epidermal barrier other than FTM should therefore be conducted.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2014.01.012.

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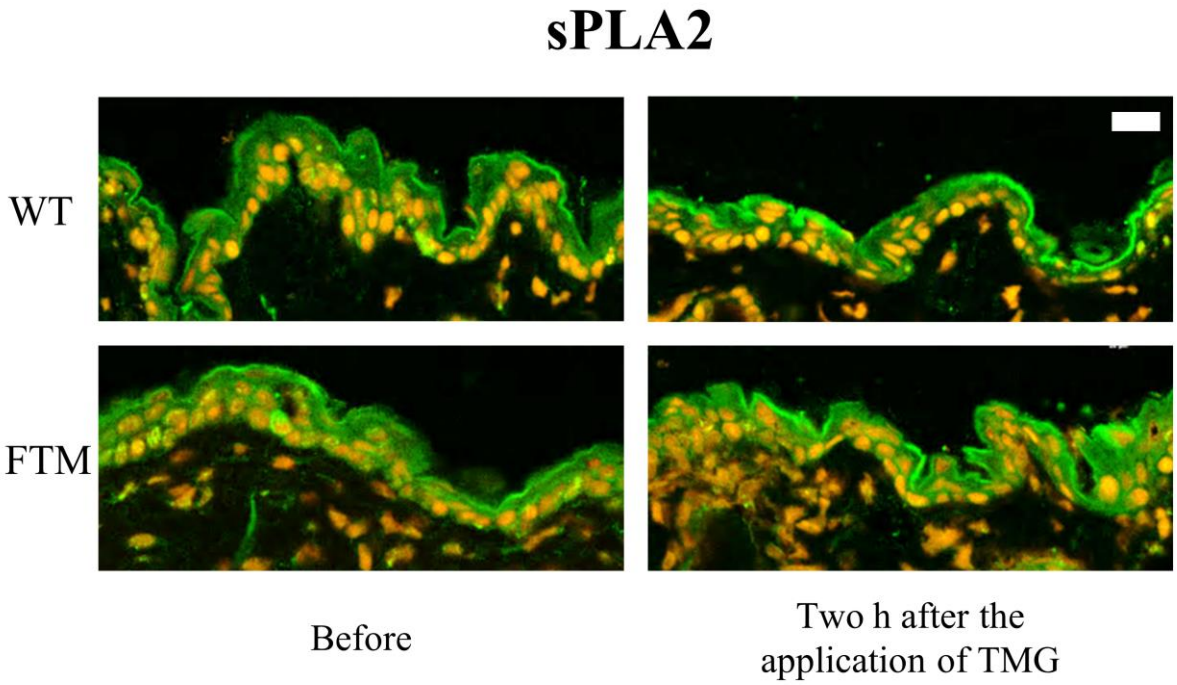
Supplementary data

Defective maintenance of pH of stratum corneum is correlated with preferential emergence and exacerbation of atopic-dermatitis-like dermatitis in flaky-tail mice

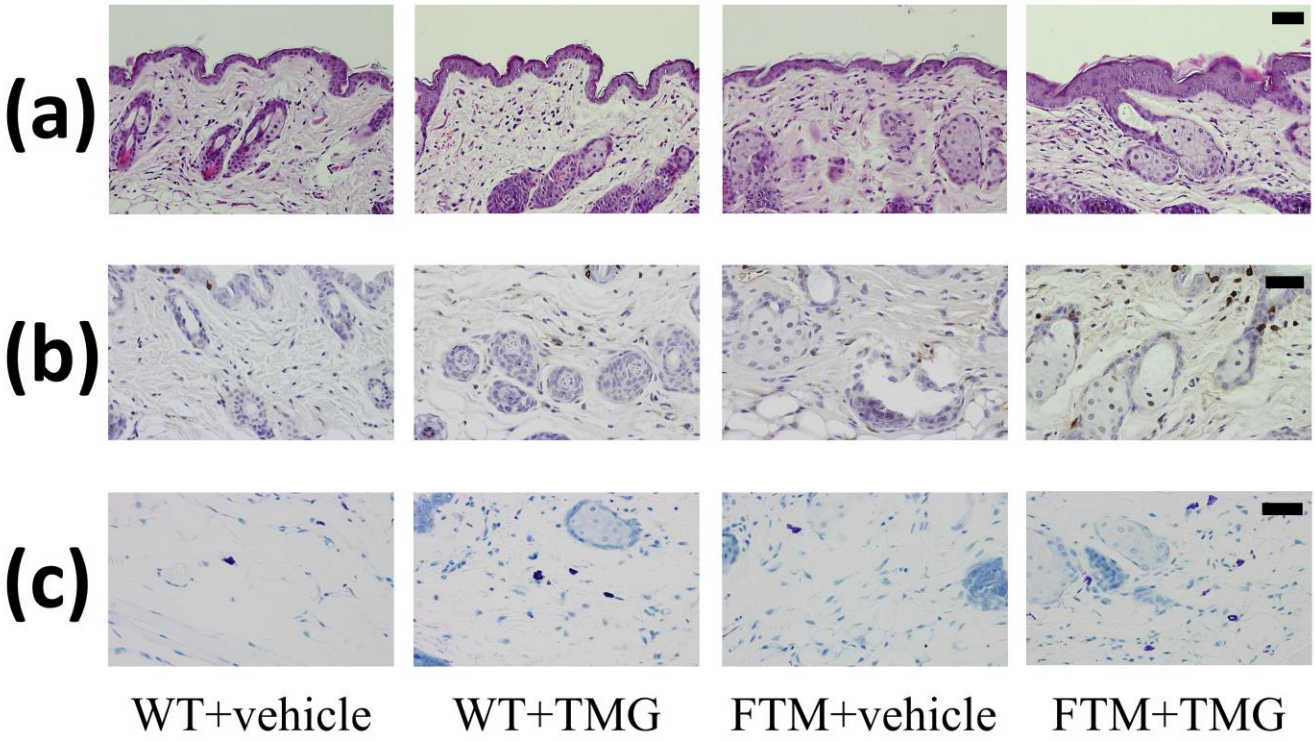
Sakai et al.

Supplementary Figures S1-5



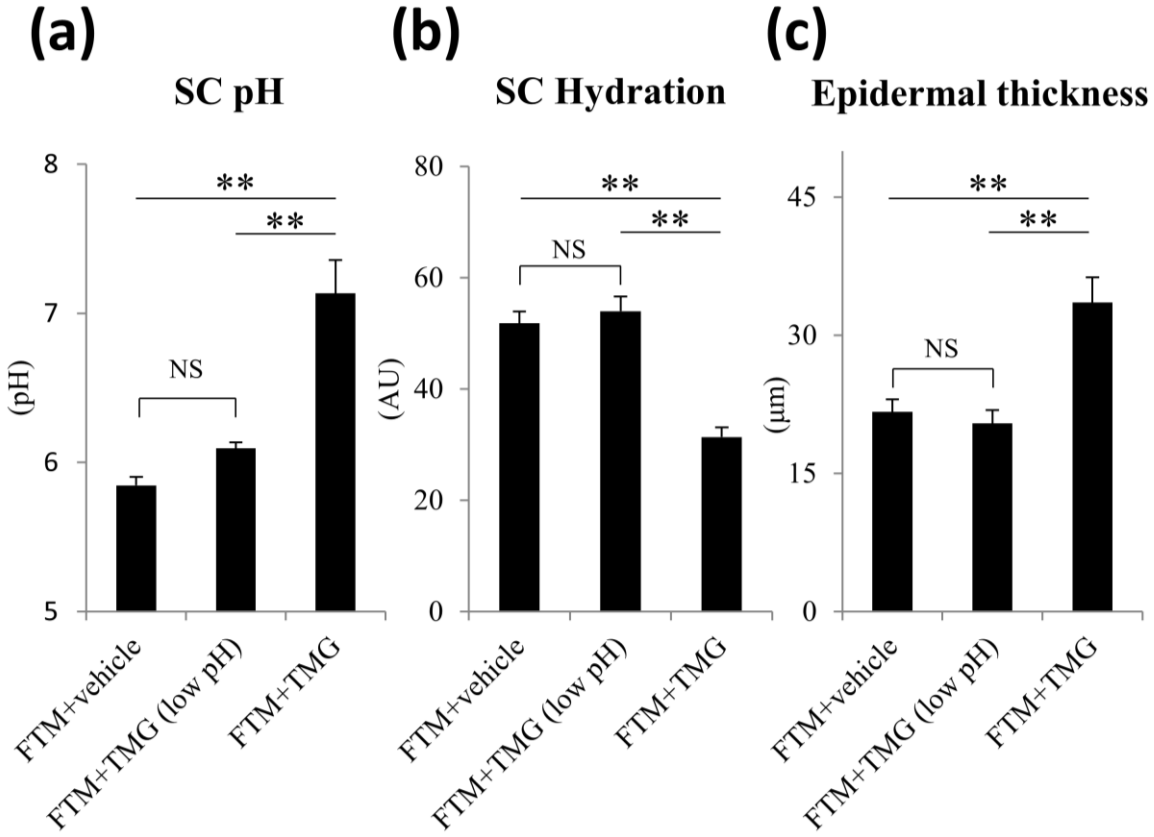
Supplementary Figure S1: The expression of sPLA2 before and after application of TMG

Skin samples were obtained from WT and FTM, before (at steady-state) or 2 h after the application of TMG. Immunohistochemical staining was performed as described in the text. Green staining of epidermis represents expression of sPLA2 and red counterstaining represents nuclear staining with propidium iodide. There was no difference between the both mice at steady state, and before and after the application of TMG. Scale bar = 20 μm.



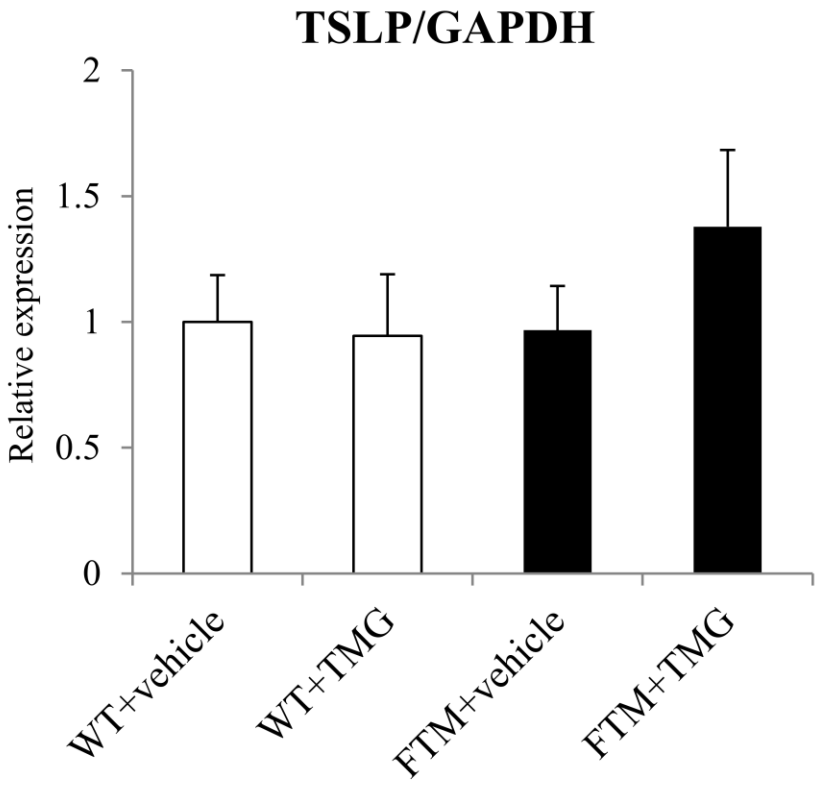
Supplementary Figure S2: Skin morphology after two weeks of daily application of TMG or vehicle

(a-c) Skin samples were obtained from WT and FTM after two weeks of daily application of TMG or vehicle. Hematoxylin and eosin staining and immunohistochemical stainings were performed as described in the text. Epidermis thickened (a), and number of CD3-positive cells (b) and mast cells (c) increased only in TMG-treated FTM. Scale bar = 50 μm.



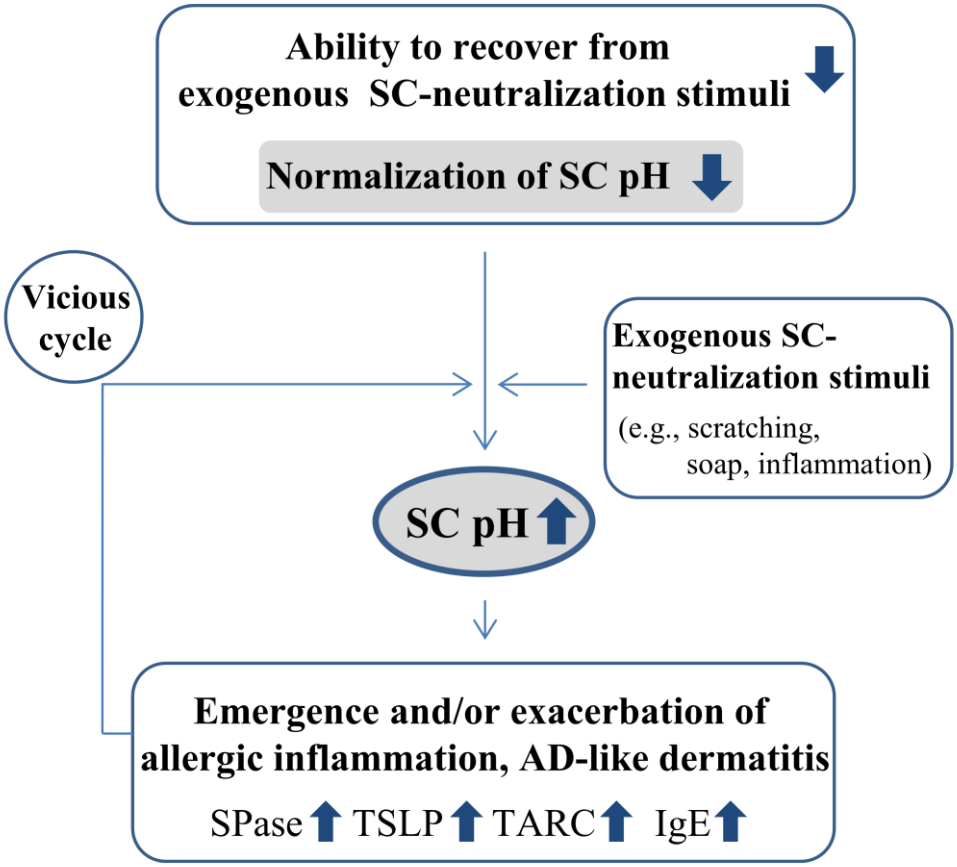
Supplementary Figure S3: Low pH-TMG did not induce substantial skin inflammation

We subjected FTM to two weeks of daily application of vehicle, TMG or low pH-TMG (TMG [low pH]). The pH of low pH-TMG was adjusted into that of vehicle (pH value is about 5.70), as described in the text. (a) Application of low pH-TMG did not elevate SC pH at the end of the treatment period. n=6 to 9; (b) SC hydration was not reduced in low pH-TMG-treated FTM. n=8 to 11; (c) Hematoxylin and eosin staining were performed as described in the text. Epidermal thickness did not increase in low pH-TMG-treated FTM. n=12. These results suggested that neutralization of SC pH is the major factor of skin inflammation in this study. Error bars equal to means±SEMs. ** $p < 0.01$. NS, Not significant. AU, Arbitrary units.



Supplementary Figure S4: Expression of TSLP in whole skin analyzed by real-time PCR after two weeks of daily application of TMG or vehicle

Whole skin samples were obtained from WT and FTM after two weeks of daily application of TMG or vehicle. Relative expression of TSLP in the skin was examined by real-time PCR as described in the text. n=5 to 8. The real-time PCR showed about 35 % increase in TSLP expression compared to that in vehicle-treated WT only in TMG-treated FTM, supporting the results from the immunohistochemical analysis (Fig. 5), although the difference has not reached a statistical significance. Error bars equal to means±SEMs.



Supplementary Figure S5: Proposal for a key role of failure to maintain acidity of SC in the pathogenesis of allergic inflammation and AD-like dermatitis.

In the proposed model, increased sensitivity to an exogenous SC-neutralization stimulus can induce persistent elevation of SC pH by several stimuli such as scratching, use of soap and inflammation, with resultant induction of emergence and/or exacerbation of allergic inflammation including AD-like dermatitis via elevation of SPase activity in epidermis and serum levels of TSLP, TARC, and IgE. Furthermore, skin inflammation increase SC pH, creating a vicious cycle.