Skin pH Is the Master Switch of Kallikrein 5-Mediated Skin Barrier Destruction in a Murine Atopic Dermatitis Model

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Elevated skin surface pH has been reported in patients with atopic dermatitis. In this study, we explored the role of skin pH in the pathogenesis of atopic dermatitis using the NC/Tnd murine atopic dermatitis model. Alkalinization of the skin of asymptomatic NC/Tnd mice housed in specific pathogen-free conditions induced kallikrein 5 and activated protease-activated receptor 2, resulting in thymic stromal lymphopoietin secretion and a cutaneous T-helper 2 allergic response. This was associated with increased transepidermal water loss and development of eczematous lesions in these specific pathogen-free NC/Tnd mice, which normally do not suffer from atopic dermatitis. Injection of recombinant thymic stromal lymphopoietin also induced scratching behavior in the specific pathogen-free NC/Tnd mice. Thymic stromal lymphopoietin production and dermatitis induced by alkalinization of the skin could be blocked by the protease-activated receptor 2 antagonist ENMD-1068. In contrast, weak acidification of eczematous skin in conventionally housed NC/Tnd mice reduced kallikrein 5 activity and ameliorated the dermatitis. Onset of the dermatitis was associated with increased epidermal filaggrin expression and impaired activity of the sodium/hydrogen exchanger 1, a known regulator of skin pH. We conclude that alterations in skin pH directly modulate kallikrein 5 activity leading to skin barrier dysfunction, itch, and dermatitis via the protease-activated receptor 2—thymic stromal lymphopoietin pathway.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease, characterized by xerosis, pruritus, and erythematous lesions accompanied by increased transepidermal water loss (TEWL) and serum IgE concentrations. Skin barrier function is now recognized to be a crucial factor in the pathogenesis of AD (De Benedetto et al., 2012). Impaired skin barrier allows penetration of allergens and irritants, which stimulate local antigen-presenting cells and immune effector cells, leading to T-helper 2 (Th2)-related inflammation (Cork et al., 2009). The association between the impaired skin barrier and Th2 inflammation in AD can be explained by expression of thymic stromal lymphopoietin (TSLP) (Ebner et al., 2007; Nakajima et al., 2012). TSLP is secreted by keratinocytes and directly activates Langerhans cells, which subsequently trigger the differentiation of naïve $CD4^+$ T cells into allergy-promoting $CD4^+$ Th2 cells (Omori and Ziegler, 2007; Rochman et al., 2007). TSLP also induces pruritus by directly stimulating sensory neurons (Wilson et al., 2013). The process by which skin barrier disruption triggers TSLP production in AD has not been fully elucidated.

Filaggrin (FLG) deficiency only partly explains why patients can develop AD (Kawasaki et al., 2012), as many patients with severe AD do not have *Flg* gene mutations. Skin pH is thought to be controlled by a number of factors, including secretory phospholipase A_2, sodium/hydrogen exchanger 1 (NHE1), and urocanic acid produced during FLG degradation and possibly melanin persistence and/or extrusion of cholesterol sulfate (Behne et al., 2002; Elias, 2015; Kezic et al., 2008). Skin pH contributes to skin barrier homeostasis, adhesion of cells in the stratum corneum (SC), and antimicrobial activity (Schmid-Wendtner and Korting, 2006). Patients with AD are known to have elevated skin pH (Eberlein-König et al., 2000). Although deficiency in FLG due

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Abbreviations: AD, atopic dermatitis; FLG, filaggrin; KLK, kallikrein; LBA, lactobionic acid; LEKTI, lympho-epithelial Kazal-type-related inhibitor; Ngf, nerve growth factor; NHE1, sodium/hydrogen exchanger 1; nLBA, NaOH-neutralized 5% LBA; PAR2, protease-activated receptor 2; SC, stratum corneum; SPF, specific pathogen-free; TEWL, transepidermal water loss; Th2, T-helper 2; TMG, 1,1,3,3-tetramethyl guanidine; TSLP, thymic stromal lymphopoietin

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to loss-of-function mutations in the *Flg* gene was previously thought to raise skin surface pH in patients with AD, as surface pH is normal in histidase-deficient mice, the role of this factor has now been called into question (Fluhr et al., 2004; Jungersted et al., 2010; Kezic et al., 2012). The exact mechanism by which elevated skin surface pH occurs in patients with AD without *Flg* gene mutations is currently unclear.

Serine proteases have a critical role in skin barrier function, in the differentiation of keratinocytes, and serine protease activity increases when skin surface pH is more alkali (Ekholm et al., 2000; Hachem et al., 2005b; Mauro et al., 1998). The serine protease kallikrein (KLK) 5 serves as a dominant regulator of the protease cascade in the SC because it is capable of activating KLK7 (Caubet et al., 2004) and KLK14 (Emami and Diamandis, 2008), as well as self-activation (Ekholm and Egelrud, 1998). KLKs are upregulated in inflammatory skin disorders including AD (Komatsu et al., 2007). Lympho-epithelial Kazal-type-related inhibitor (LEKTI) is an endogenous inhibitor of KLK5 (Deraison et al., 2007). Deficiency in LEKTI is associated with upregulation of KLK5, activation of protease-activated receptor 2 (PAR2), and overexpression of TSLP in the epidermis in the mouse model of Netherton syndrome (Briot et al., 2009). Serine proteases have their clinical effects partly by activating PAR2 (Steinhoff et al., 2005) as well as in delayed epidermal permeability barrier recovery (Hachem et al., 2006).

NC/Tnd mice, an inbred strain originating from Japanese fancy mice, spontaneously develop allergic dermatitis from 6 to 8 weeks of age when housed in conventional environments. As previously described, the model has many similarities to AD in humans (Amagai et al., 2013; Matsuda et al., 1997; Tanaka et al., 2012). We previously demonstrated skin barrier dysfunction in conventional NC/Tnd mice with AD, in which the ceramide content of the skin was markedly decreased (Aioi et al., 2001). However, the mechanisms responsible for the induction of skin barrier dysfunction and AD initiation in NC/Tnd mice remain incompletely understood. In this study, we investigated the possible role of skin surface pH on expression of KLK5, PAR2, and TSLP and the spontaneous development of AD in this mouse model of human AD.

RESULTS

Elevated skin surface pH results in the development of AD-like dermatitis in NC/Tnd mice

NC/Tnd mice reared in conventional conditions spontaneously developed AD-like dermatitis after 5 weeks of age, which persists until at least 12 weeks of age. In contrast, no eczema develops in NC/Tnd mice reared in specific pathogen-free (SPF) conditions (Figure 1a). TEWL, an established indicator of skin barrier function, increased in parallel with the development of dermatitis in conventional NC/Tnd mice but not in SPF NC/Tnd mice (Figure 1b). Skin surface pH is reported to be higher in patients with AD than in healthy subjects (Eberlein-König et al., 2000). In view of these data in human AD, we measured skin surface pH in NC/Tnd mice from 5 to 12 weeks of age. The skin surface pH of SPF and conventionally reared NC/Tnd mice were both 6.0 at 5 weeks of age. As the eczema worsened in the conventionally

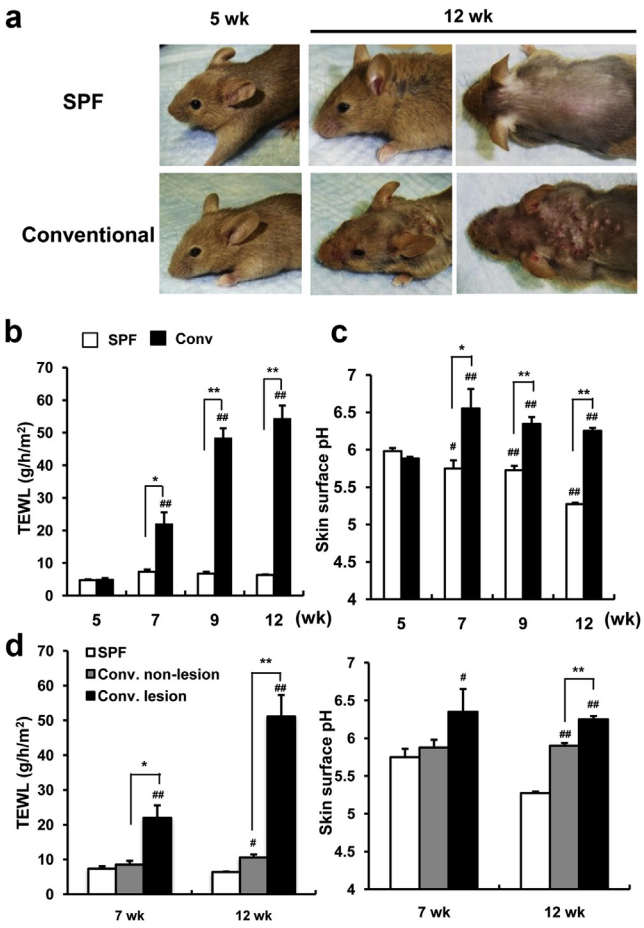

Figure 1. Elevation of skin surface pH accompanied by clinical symptoms and skin barrier dysfunction in the early stage of AD-like dermatitis in NC/Tnd mice. (**a**) Representative photos of SPF and conventional (Conv) NC/Tnd mice at 5 weeks and 12 weeks of age. (**b**) TEWL and (**c**) skin surface pH of SPF and Conv NC/Tnd mice from 5 weeks to 12 weeks of age. Each column represents the mean ± SE of five to six mice in each group. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with age-matched SPF NC/Tnd mice. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with 5-week-old NC/Tnd mice. (**d**) TEWL and skin surface pH of non-lesion or lesion of Conv NC/Tnd mice and those of corresponding sites of SPF mice, and at 7 weeks and 12 weeks of age. Each column represents the mean ± SE of five mice in each group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with age-matched SPF NC/Tnd mice. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with age-matched non-lesion of Conv NC/Tnd mice. AD, atopic dermatitis; SPF, specific pathogen-free; TEWL, transepidermal water loss.

reared NC/Tnd mice, the skin surface pH increased significantly, whereas the pH of the asymptomatic SPF NC/Tnd mice (Figure 1c) became more acidic, as is seen in human infants (Fluhr et al., 2004). At 7 weeks of age, TEWL and skin pH did not differ between SPF NC/Tnd and non-lesion of conventional NC/Tnd mice (Figure 1d). Otherwise, as the eczema worsened in 12-week-old conventional NC/Tnd mice, both TEWL and pH of the non-lesional skin also significantly increased compared with that of SPF NC/Tnd mice (Figure 1c).

Skin FLG expression of conventional NC/Tnd mice

To explore the reason for skin barrier defects observed in conventional NC/Tnd mice, we measured FLG protein expression in the epidermis by Western blotting and immunostaining. At 5 weeks of age, FLG expression did not differ between the two groups (Supplementary Figure S1a online).

Compared with the skin of age-matched SPF NC/Tnd mice, the levels of proteolytically cleaved FLG intermediates increased from 9 weeks of age in the epidermis of conventional NC/Tnd mice as dermatitis progressed (Supplementary Figure S1a). Immunostaining for FLG was more extensive within the upper layer within the proliferating layer of the epidermis in conventional NC/Tnd mice (Supplementary Figure S1b). At 12 weeks of age, the protein level of FLG in the skin of conventional NC/Tnd mice was markedly increased, in comparison with that of SPF NC/Tnd mice, where it was almost identical to that of C57BL/6 mice (Supplementary Figure S1c). Because FLG expression is modulated by inflammatory cytokines, including IL-4, IL-13, and IFN-γ (Howell et al., 2009), we measured the mRNA levels of proinflammatory cytokines in the skin of these mice. In 12-week-old conventional NC/Tnd mice, the mRNA levels of not only *Il4* and *Il13* but also *Ifng* were markedly increased compared with those of SPF NC/Tnd mice (Supplementary Figure S1d). Incubation of epidermal tissue with recombinant murine (rm) IFN-γ induced *Flg* mRNA expression, whereas Th2 cytokines (IL-4/IL-13) suppressed *Flg* mRNA levels equally in both C57BL/6 and SPF NC/Tnd mice (Supplementary Figure S1e). To more directly determine whether impairment of the skin barrier was related to dysfunction of FLG processing, we evaluated FLG breakdown products natural moisturizing factor, defined here as pyrrolidone carboxylic acid and trans-urocanic acid in the SC of 12-week-old SPF and conventional NC/Tnd mice. Pyrrolidone carboxylic acid and trans-urocanic acid play a major role in the SC hydration (Elias et al., 2008; Rawlings and Harding, 2004). The concentrations of natural moisturizing factor were significantly higher in the SC of conventional NC/Tnd mice than in those of SPF NC/Tnd mice (Supplementary Figure S1f). To assess the water content of the SC, we measured skin hydration of surrounding areas adjacent to affected sites. As reported in *Flg*-null mice and their corresponding control wild-type mice (Kawasaki et al., 2012), skin conductance in conventional NC/Tnd mice did not differ from that in SPF mice at 12 weeks of age (Supplementary Figure S1g).

Enhanced expression of KLK5, PAR2, and TSLP in conventional NC/Tnd mice with AD

As FLG processing was not impaired in conventional NC/Tnd mice with AD-like dermatitis, we looked for other causes of skin barrier disruption. NHE1 expression was upregulated in 12-week-old SPF NC/Tnd mice without eczema, but not in age-matched conventional NC/Tnd mice with eczema (Supplementary Figure S2a online). We found that serine protease activity was also higher in the epidermis of conventional NC/Tnd mice compared with SPF NC/Tnd mice (Supplementary Figure S2b). KLK5 protein and mRNA expression increased significantly in the epidermis of conventional NC/Tnd mice as their eczema worsened, when compared with age-matched SPF NC/Tnd mice without dermatitis (Figure 2a and Supplementary Figure S2c). Consistent with Western blotting results, KLK5 protein expressed was elevated in the epidermis of conventional NC/Tnd mice with murine AD (Figure 2a). In contrast, the protein level of LEKTI did not differ between SPF and conventional

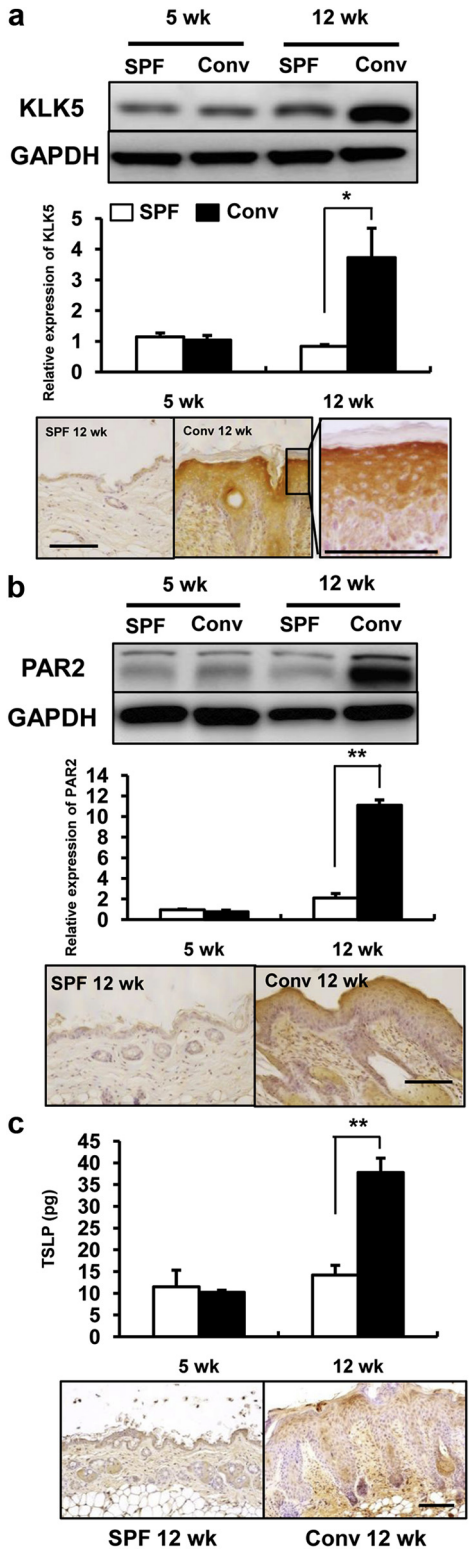

Figure 2. KLK5, PAR2, and TSLP levels in the epidermis of conventional NC/Tnd mice. Expression levels and distribution of (**a**) KLK5, (**b**) PAR2, and (**c**) TSLP in the epidermis of SPF and Conv NC/Tnd mice at 5 weeks and 12 weeks. KLK5 and PAR2 were detected by Western blotting and immunostaining. TSLP concentrations in 100 μg of protein obtained from the isolated epidermis were detected using an ELISA. Data represent three independent experiments with four to five mice in each group. Error bars represent mean ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with age-matched SPF NC/Tnd mice. Bars = 100 μm. KLK5, kallikrein 5; PAR2, protease-activated receptor 2; SPF, specific pathogen-free; TSLP, thymic stromal lymphopoietin.

NC/Tnd mice (Supplementary Figure S2d). Upregulated activity of KLK5 in the skin of patients with Netherton syndrome leads to PAR2 activation as well as overexpression of TSLP (Briot et al., 2009).

We next investigated the expression levels of PAR2 and TSLP in the NC/Tnd mice. In the epidermis of conventional NC/Tnd mice, the levels of cleaved PAR2 in the epidermis were 10-fold higher as the AD-like dermatitis progressed (Figure 2b). Moreover, the protein levels of TSLP increased threefold in the epidermis of conventional NC/Tnd mice with the development of AD-like dermatitis but not in the controls. TSLP expression appeared to be more widespread, occurring in the dermis as well as in the epidermis of conventional NC/Tnd mice (Figure 2c).

Effects of skin acidification on murine AD in conventional NC/Tnd mice

To directly investigate the effects of acidification of the skin on murine AD, we applied 2.5% or 5% lactobionic acid (LBA) twice a day for 2 weeks to the backs of conventional NC/Tnd mice with severe AD-like skin lesions (Hachem et al., 2010; Hatano et al., 2009). The skin surface pH and TEWL in the LBA-treated groups were significantly lower than vehicle and NaOH-neutralized 5% LBA (nLBA)-treated groups (Figure 3a and b). Eczema symptoms were significantly attenuated in mice treated with 2.5% and 5% LBA compared with the vehicle and nLBA-treated groups (Figure 3c). As pruritus is the most troublesome symptoms of AD, we measured itch sensation by analyzing the scratching behavior using a SCLABA-Real system as previously described (Amagai et al., 2013; Ishii et al., 2008). Scratching frequency and duration were significantly reduced in 2.5% and 5% LBA-treated mice compared with vehicle- or nLBA-treated mice (Figure 3d). We found no significant differences in the protein levels and profiles of FLG between the groups (Supplementary Figure S3a online).

The effects of acidification of the skin on the KLK5-PAR2-TSLP cascade in conventional NC/Tnd mice were also assessed. There was no significant difference in LEKTI expression between the groups (Supplementary Figure S3b). The protein levels and distribution of KLK5, PAR2, and TSLP were significantly decreased in the epidermis of 2.5% and 5% LBA-treated mice compared with vehicle- or nLBA-treated mice (Figures 4 and Supplementary Figure S3c). Histology revealed that acidification of the skin reduced both dermal inflammation and epidermal hyperplasia in conventional NC/Tnd mice. The number of both tissue mast cells and eosinophils were decreased in 2.5% and 5% LBA-treated mice (Supplementary Figures S4a and b online). Both 2.5% and 5% LBA treatment also downregulated the mRNA levels of a number of inflammatory cytokines (*Il33*, *Il1b*, *Il4*, *Il5*, *Ifng*, matrix metalloprotease9, and *Il31*) but not *Il25* and *Il13* (Supplementary Figure S4c). Cytokines with growth or chemotactic activities on mast cells, nerve growth factor (*Ngf*) (Sawada et al., 2000), and eosinophil chemotactic cytokine, *Ccl24* were decreased in both 2.5 and 5% LBA treatment groups (Supplementary Figure S4c). On the other hand, the levels of stem cell factor (*Scf*) and *Ccl5* were not altered (Supplementary Figure S4c). Although we also detected the mRNA levels of SCF receptors (*Kit*), no significant change was identified (data not shown).

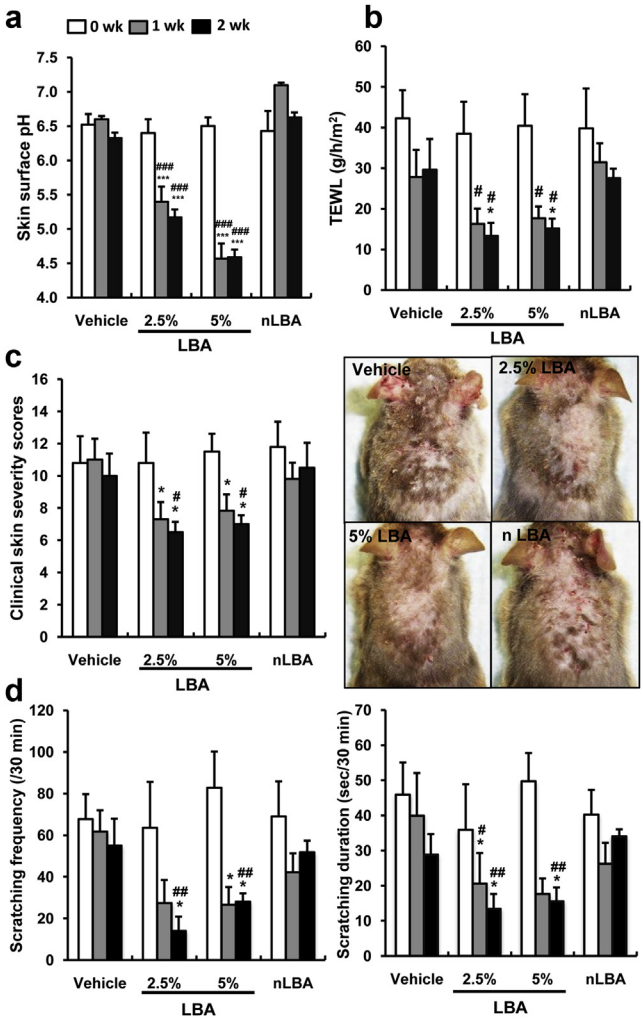

Figure 3. Effects of acidification on the affected skin of NC/Tnd mice. (**a**) Skin pH, (**b**) TEWL, (**c**) clinical score (left) and typical features (right), and (**d**) scratching frequency (left) and duration (right) of mice treated with 2.5% LBA, 5% LBA, or nLBA compared with vehicle-treated control. Each data point represents the mean ± SE of four to five mice in each group. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with the vehicle-treated group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with nLBA-treated mice. LBA, lactobionic acid; nLBA, NaOH-neutralized 5% LBA; TEWL, transepidermal water loss.

Effect of alkalinization of the skin in SPF NC/Tnd mice

To directly assess the effect of increasing skin surface pH on the development of dermatitis, we applied 1,1,3,3-tetramethyl guanidine (TMG) to the skin of C57BL/6 and SPF NC/Tnd mice (Hachem et al., 2005b). The skin surface pH of the TMG-treated group rose from 7.5 (C57BL/6) to 8.5 (SPF NC/Tnd) after treatment (Supplementary Figure S5a online and Figure 5a). The TMG-treated group showed higher TEWL when compared with control groups in both the NC/Tnd and a control C57BL/6 strain (Supplementary Figure S5b and Figure 5b). Notably, SPF NC/Tnd mice treated with TMG showed higher TEWL than TMG-treated C57BL/6 mice. Alkalinization of the skin induced pruritus (Figure 5c), epidermal hyperplasia (Supplementary Figure S6a online), and accumulation of mast cells in the skin of NC/Tnd

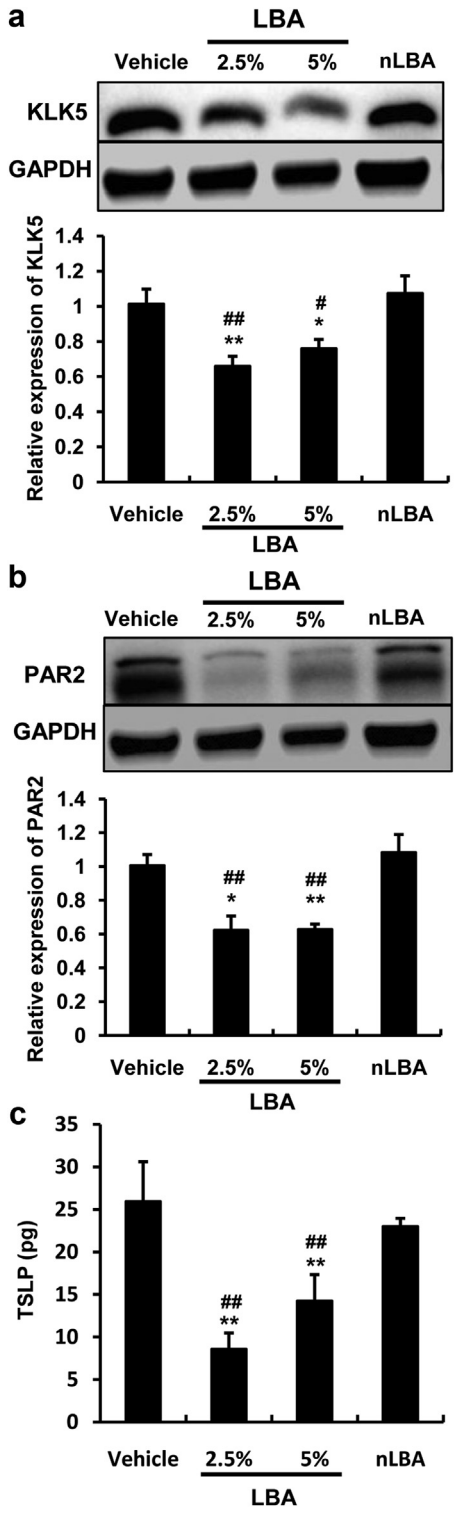

Figure 4. KLK5, PAR2, and TSLP levels after acidification of the affected skin. Expression levels of (**a**) KLK5, (**b**) PAR2, and (**c**) TSLP in the epidermis of vehicle-, 2.5% LBA-, 5% LBA-, and nLBA-treated atopic NC/Tnd mice. The expression of KLK5 and PAR2 was detected by Western blotting, and that of TSLP in 100 μg of protein obtained from the isolates epidermis by an ELISA. Data represent three independent experiments with four to five mice in each group. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with vehicle-treated control. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with nLBA-treated mice. KLK5, kallikrein 5; LBA, lactobionic acid; nLBA, NaOH-neutralized 5% LBA; PAR2, protease-activated receptor 2; TSLP, thymic stromal lymphopoietin.

mice (Supplementary Figure S6b) but not C57BL/6 mice (Supplementary Figures S5c–e). Furthermore, TMG treatment upregulated the mRNA levels of keratinocyte-derived cytokine (*Il33*), (pro-) inflammatory cytokines (*Il1b, Il4, Il5, Il13,* and *Ifng*) and the dendritic cell migration-related matrix metalloprotease9, the itch-related cytokine *Il31*, as well as *Ngf*, but not *Il25, Scf,* and *Ccl5* in the skin of NC/Tnd mice (Supplementary Figures S6c). Changes in *Kit* mRNA expression were not significant (data not shown). We also showed that the protein levels of FLG were significantly increased in the TMG-treated group (Supplementary Figure S6d).

Next, we examined whether skin alkalinization might stimulate the KLK5-PAR2-TSLP cascade. The protein levels of KLK5, PAR2, and TSLP were significantly higher in the epidermis of TMG-treated SPF NC/Tnd mice than in the epidermis of HCl-neutralized TMG (nTMG) and vehicle-treated controls (Figures 5d–f). TMG treatment failed to induce KLK5, PAR2, or TSLP expression in the epidermis of C57BL/6 mice (Supplementary Figure S7a–c online). The levels of LEKTI were unchanged in the epidermis of both mice treated with TMG (Supplementary Figures S7d and S8). We also analyzed skin pH at each time point after single application of TMG to the dorsal skin of C57BL/6 or SPF NC/Tnd mice. After application of TMG to SPF NC/Tnd mice, skin surface pH was significantly higher than C57BL/6 mice and recovery was delayed (Supplementary Figure S9a online). Because secretory phospholipase A_2 and NHE1 are responsible for maintenance of acidic pH of the skin and their activities are known to be regulated by skin pH change (Fluhr et al., 2001; Hachem et al., 2005a), we checked the protein levels of secretory phospholipase A_2 and NHE1 at each time point after TMG application. TMG-induced acute alkalinization enhanced secretory phospholipase A_2 expression in the epidermis of both C57BL/6 and SPF NC/Tnd mice, and there was no difference between the two strains (Supplementary Figure S9b). The protein levels of NHE1 were increased in the epidermis of C57BL/6 mice at 3 and 6 hours after application of TMG (Supplementary Figure S9b). NHE1 was also detected in the epidermis of NC/Tnd mice, but the level did not increase in response to skin alkalinization (Supplementary Figure S9b).

Recently, TSLP has been reported to directly induce itch sensation by stimulating sensory neurons (Wilson et al., 2013). We therefore also examined whether an increase in TSLP after alkalinization of the skin could induce scratching behavior. Intradermal injection of 1 μg of recombinant murine (rm) TSLP in the nape of the neck promoted scratching behavior in SPF NC/Tnd mice (Supplementary Figure S10 online).

Effects of pH on PAR2-TSLP pathway activity

We hypothesized that increased pH would trigger TSLP production via PAR2 activation. Using an epidermal culture system, we investigated whether increased pH could induce TSLP production through the PAR2 pathway. In the epidermis isolated from SPF NC/Tnd mice, we found that the mRNA levels of *Tslp* were significantly enhanced in a pH-dependent manner (Supplementary Figure S11 online). However, pretreatment with 10 mM ENMD-1068, a PAR2 antagonist, markedly suppressed the increase of *Tslp* mRNA expression in the epidermal sheets cultured in medium with neutral to alkaline pH (Supplementary Figure S11).

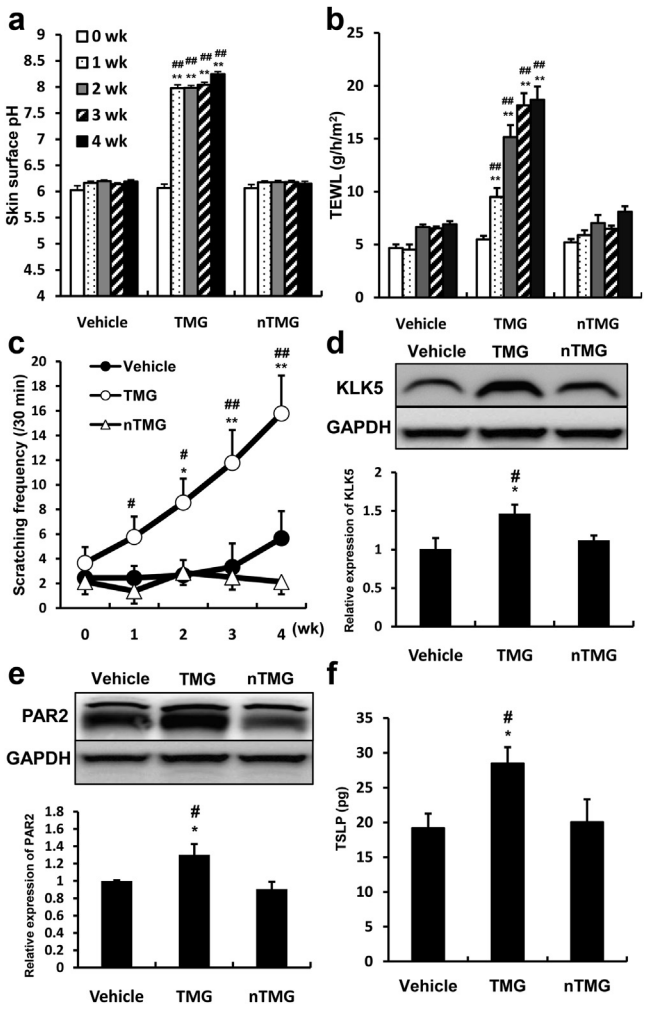

Figure 5. Effects of TMG treatment on the skin of SPF NC/Tnd mice. (**a**) Skin pH, (**b**) TEWL, and (**c**) scratching frequency of vehicle-, TMG-, and nTMG-treated mice. Expression levels of (**d**) KLK5, (**e**) PAR2, and (**f**) TSLP in the epidermis of vehicle-, TMG-, and nTMG-treated mice. The protein expression of KLK5 and PAR2 was detected by Western blotting, and that of TSLP in the epidermis was measured by ELISA. Each data point represents the mean ± SE of five to six mice in each group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the vehicle-treated group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with the nTMG-treated group. KLK5, kallikrein 5; nTMG, HCl-neutralized TMG; PAR2, protease-activated receptor 2; TEWL, transepidermal water loss; TMG, 1,1,3,3-tetramethyl guanidine; TSLP, thymic stromal lymphopoietin.

Excessive activation of KLKs in the skin of patients with Netherton syndrome results in PAR2 activation and induces overexpression of TSLP (Briot et al., 2009). We therefore investigated the protein levels of PAR2 and TSLP in the epidermis after treatment of rmKLK5 using epidermal sheet culture. In the epidermis isolated from C57BL/6 mice, PAR2 and TSLP levels were significantly enhanced in a time-dependent manner after treatment with 400 nM rmKLK5 (Figure 6a and b). To examine whether the PAR2 pathway is responsible for TSLP production in keratinocytes, the epidermis was isolated from C57BL/6, SPF NC/Tnd mice, and PAR2-deficient mice, and incubated with rmKLK5. *Tslp* mRNA expression was markedly increased in the epidermal sheets of C57BL/6 and SPF NC/Tnd mice (Figure 6c). However, *Tslp* mRNA expression was not increased in the epidermal sheets of PAR2-deficient mice. In addition, pretreatment of epidermal

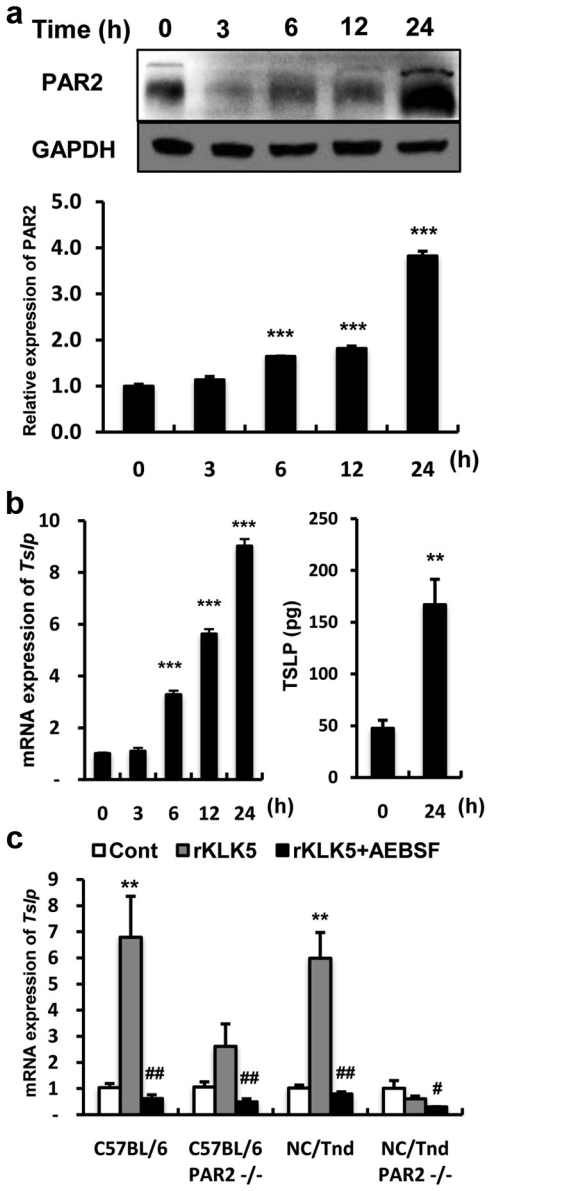

Figure 6. Activation of the PAR2-TSLP pathway by KLK5. (**a**) Time course of PAR2 expression after stimulation with 400 nM rmKLK5 in epidermal sheet cultures obtained from C57BL/6 mice. Each image is representative of three independent experiments. Relative intensities were shown as means ± SE obtained from three independent experiments. $^{***}P < 0.001$ compared with 0 hours. (**b**) TSLP mRNA (left) and protein (right) expression after treatment with rmKLK5 in epidermal sheet cultures from C57BL/6 mice. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with 0 hours. (**c**) TSLP mRNA expression after the indicated treatment in epidermal sheet cultures isolated from C57BL/6, SPF NC/Tnd, and PAR2-deficient mice. Each expression level was shown as means ± SE obtained from three independent experiments with three samples in each group. $^{**}P < 0.01$ compared with the control group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with the rmKLK5-treated group. AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; KLK5, kallikrein 5; PAR2, protease-activated receptor 2; SPF, specific pathogen-free; TSLP, thymic stromal lymphopoietin.

sheets with 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, a serine protease inhibitor, reduced *Tslp* expression in the epidermal sheet of both C57BL/6 and SPF NC/Tnd mice.

DISCUSSION

Elevated skin surface pH has been linked to skin barrier dysfunction and eczema, suggesting the role for a defect in

skin pH homeostasis in the pathogenesis of this condition (Eberlein-König et al., 2000). Maintenance of acidic skin pH reduced the development of dermatitis in a hapten-induced dermatitis model (Hatano et al., 2009). However, the mechanisms by which alteration of skin surface pH in AD leads to skin barrier and immunological dysfunction have until now remained unclear. In this study, we clearly demonstrate that raised skin pH triggers development of dermatitis via the KLK5-PAR2-TSLP pathway in NC/Tnd mice, which can be blocked by the addition of the PAR2 antagonist ENMD-1068. Prolonged elevation of skin surface pH, possibly because of impaired augmentation of NHE1 activity in NC/Tnd mice compared with control mice, is likely to lead to more prolonged KLK5, PAR2, and TSLP activity, contributing to skin barrier disruption and the development of dermatitis (summarized in Supplementary Figure S12 online). Because NC/Tnd mice are not LEKTI deficient, the pathogenesis of AD-like dermatitis in NC/Tnd mice must be explained by other mechanisms. In patients with AD with wild-type *Flg* gene, the expression of Flg was found to be higher than that of healthy controls (Cole et al., 2014). Moreover, skin barrier disruption by sodium lauryl sulfate exposure promoted the expression level of FLG during the repair phase in human skin (Törmä et al., 2008). FLG and natural moisturizing factor have been reported to act on skin permeability barrier rather than skin acidification (Fluhr et al., 2010; Kawasaki et al., 2012). Observations in the current study suggest the possibility that an increase in FLG processing in the epidermis of NC/Tnd mice with AD-like dermatitis as well as SPF NC/Tnd mice with TMG treatment is part of a compensatory response to protect skin barrier function (Cole et al., 2014; Törmä et al., 2008). Although FLG deficiency has been reported to play a central role in the pathogenesis of AD in some patients, we clearly show that the lack of FLG is not the reason for the AD-like dermatitis in NC/Tnd mice. Therefore, NC/Tnd mice represent a model for human AD with normal *Flg* gene expression.

Skin alkalinization promotes KLK5, PAR2, and TSLP, leading to impairment of barrier function and Th2-dependent inflammation in SPF NC/Tnd mice but not in C57BL/6 mice. After TMG application, (pro-)inflammatory cytokines and chemokines were upregulated in NC/Tnd mice. Particularly, itch-related cytokines *Il31* and *Ngf* were increased as well as Th2-type cytokines. As having various functions on nerve cells and immune cells, we speculate that *Ngf* may be involved in mast cell recruitment and itch evocation at the onset of AD. NHE1 helps to maintain physiological pH after contact with alkaline substances, including soap and microbes and in FLG-deficient mice (Behne et al., 2002; Fluhr et al., 2010; Hachem et al., 2005a; Sakai et al., 2014). Our study shows the dichotomy between a compensatory increase in NHE1 with skin alkalinization in control mice and no change in NHE1 in the AD-prone NC/Tnd mice. One explanation for this might be the inflammatory response associated with AD. Raised IFN-γ has previously been found to suppress NHE1 in epithelial cells (Son et al., 2009). Impaired NHE1 has previously been shown to result in prolonged skin alkalinization (Fluhr et al., 2004). Impaired pH regulation by NHE1 might exacerbate skin barrier function and affect susceptibility to AD-like dermatitis in NC/Tnd mice. The possible association between impaired pH regulation by NHE1 and susceptibility to AD-like dermatitis has also been reported in flaky tail mice (Sakai et al., 2014). The current observation obtained by using AD model mice endorses the importance of NHE1 in the development of eczema.

KLK5 is enzymatically active at neutral-to-alkaline pH (Ekholm et al., 2000) and induces desquamation by degrading desmosomal cadherins (Caubet et al., 2004; Fortugno et al., 2011). Transgenic mice where KLK5 is overexpressed exhibit not only barrier disruption but also upregulation of TSLP expression and Th2-induced inflammation in the skin (Furio et al., 2014). Increased KLK5 expression in the epidermis of alkalized skin appears to be sufficient to induce skin barrier dysfunction and a proinflammatory response in our NC/Tnd mouse model. Furthermore, TSLP activates TSLP receptors in sensory neurons to induce pruritus (Wilson et al., 2013). KLK5 is an important initiator of PAR2 and TSLP activation after pH elevation in the skin. In support of this, we showed that alkalinization of the skin with TMG increased skin surface pH, TEWL, and scratching behavior, triggering the development of allergic dermatitis in the NC/Tnd mice. Evidence for the possibility of developing a simple therapeutic intervention to treat AD is provided by the fact that acidification of the skin not only leads to significantly decreased KLK5, PAR2, and TSLP but also improvement in skin barrier function and disease severity (scratching behavior and skin damage) in murine atopic NC/Tnd mice. Thus, controlling skin surface pH might be an important treatment strategy for AD.

MATERIALS AND METHODS

Animals

NC/Tnd mice used in this study were housed in either SPF or air-unfiltered conventional conditions. PAR2-deficient mice were generously supplied by Kowa (Aichi, Japan) (Ferrell et al., 2003). Congenic PAR2-deficient NC/Tnd mice were generated by backcrossing SPF NC/Tnd mice onto PAR2-deficient mice for more than 13 generations. PAR2 deficiency in those mice was confirmed by genotyping. The explored area was the dorsal skin, where AD develops in conventional NC/Tnd mice but not in SPF NC/Tnd mice and C57BL/6 mice, unless otherwise indicated. All experiments with animals complied with both the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology (No. 24-94) and the guidelines for the use of laboratory animals provided by Science Council of Japan.

Skin acidification

Male conventional 12-week-old NC/Tnd mice were treated with a topical application of 2.5% or 5% LBA (pH 2.8 or 2.6) dissolved in distilled water (vehicle) to 5−6 cm^2 areas on their flanks twice a day for 2 weeks. Controls were treated similarly with nLBA (pH 7.0) in vehicle or vehicle alone. Skin surface pH and TEWL were evaluated at 3 hours after application on the indicated days. Scratching behavior of mice was quantified using the SCLABA-Real System (Noveltec, Kobe, Japan) every week (Ishii et al., 2008). Clinical skin severity of dermatitis was scored according to the criteria described previously (Matsuda et al., 1997).

Skin alkalinization

Male C57BL/6 mice and SPF NC/Tnd mice, 7−8 weeks of age, were topically administered TMG (6.5/1,000, vol/vol; pH 13.0) dissolved

in propylene glycol:ethanol (7:3 vol/vol; pH 7.0; vehicle) at 12-hour intervals for 4 weeks on 5–6 cm^2 areas on each flank. Controls were treated similarly with nTMG (pH 7.0) in vehicle or with vehicle alone. Skin surface pH and TEWL were evaluated at 3 hours after application on the indicated days. Scratching behavior of mice was quantified using a SCLABA-Real System every week (Ishii et al., 2008).

Other experimental procedures can be found in the Supplementary Materials.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1038/JID.2015.363.

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