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Acidification of Stratum Corneum Prevents the Progression from Atopic Dermatitis to Respiratory Allergy

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Abbreviations: AD – atopic dermatitis, HDM – house dust mite, Dp – *Dermatofagoides pteronissunus*, SC – stratum corneum, TEWL – transepidermal water loss, LC – Langerhans cell, FLG – filaggrin gene, TSLP – thymic stromal lymphopoietin

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-Abstract-

The presence of congenitally impaired skin barrier followed by atopic dermatitis (AD) is an initial step in the atopic march. The maintenance of acidic pH in the stratum corneum (SC) has been suggested as a therapeutic or preventive strategy for barrier impairment caused by skin inflammation. To determine whether an AD murine model, flaky tail mice, with inherited filaggrin deficiency could develop airway inflammation by repeated topical application followed by nasal inhalation of house dust mite (HDM) antigen (defined as a novel ‘atopic march animal model’), and whether maintenance of an acidic SC environment by continuous application of acidic cream could interrupt the following atopic march. During the course of HDM treatment, acidic cream (pH2.8) or neutral cream (pH7.4) was applied to flaky tail mice twice daily. Repeated applications and inhalations of HDM to flaky tail mice induced AD skin lesions followed by respiratory allergies. Maintenance of SC acidity inhibited the occurrence of respiratory allergic inflammation as well as AD-like skin lesions. Collectively, a novel

atopic march model could be developed by repeated epicutaneous and nasal applications of HDM to flaky tail mice, and that the acidification of SC could prevent the atopic march from AD to respiratory allergy.

-Introduction-

The clinical manifestations of atopic dermatitis (AD), characterized by xerosis, eczematous lesions, pruritus, epidermal barrier dysfunction, immunodysregulation, and IgE-mediated sensitization to exogenous allergens(1), predate the development of asthma and allergic rhinitis in a serial process called the atopic march, suggesting that AD is an entry point for subsequent allergic disease(2, 3). The development of asthma in individuals with AD is secondary to allergen sensitization through a primarily defective epidermal barrier, which enables allergens such as house dust mites(HDM) to penetrate the skin easily and then make contact with Langerhans cells(LCs) in the skin, which in turn promotes Th2 inflammation leading to systemic allergy(4-6). Meanwhile, thymic stromal lymphopoietin(TSLP) secreted by keratinocytes in the disrupted epidermis activate polyclonal T cells. These T cells, then, induce excessive Th2 inflammatory responses, which could aggravate AD and trigger systemic Th2 responses including respiratory allergies(7).

Acidic pH of the stratum corneum(SC) is a key factor in many protective functions including permeability barrier homeostasis, SC integrity and cohesion, and antimicrobial defense, because it is important for the functional activity of enzymes involved in processing SC lipids and modulating the activity of endogenous protease(kallikrein 5, 7, and 14) required for coordinated epidermal differentiation and cornified cell envelope formation(8, 9). In AD, there is an increase of pH in lesional skin resulting in bacterial growth, allergic inflammation and aggravation of AD(10). In a hapten-induced AD model and an immune-dysregulated AD model(NC/Nga mice), simple acidification of SC significantly blocks the progression of barrier impairment followed by downstream immune abnormalities(6, 11, 12). These studies give us the clue and possibility of preventative intervention of atopic march, as

well as a novel AD treatment in that the maintenance of normal or hyperacidic pH could improve or prevent AD in human. However, the oxazolone-induced atopic march model represents an acquired form of AD without inherited or congenital abnormalities, and NC/Nga mice have IgE/Th2 independent pathogenesis that may not represent the immune status implicated in the atopic march(6, 12).

Filaggrin gene(FLG) mutations predispose carriers to increased risk of developing atopic diseases including AD, nickel and food allergies, atopic asthma, and allergic rhinitis, although filaggrin is not found in the bronchial epithelium(13). In general, AD associated with FLG mutations has an earlier onset, is more 'atopic,' and is likely to be associated with asthma as well as more severe and persistent manifestations(14). If FLG mutations occur, the FLG-associated skin barrier is disrupted, resulting in a decrease in lamellar bodies, cornified envelope, corneodesmosome, and tight junction proteins and an elevation of skin surface pH, which lead to increased episodes of percutaneous allergen exposure(5, 15).

Flaky tail mice have the mutations of filaggrin gene and matted gene. The matted phenotype in flaky tail mice results from a mutation in the Tmem79/Matt gene with no expression of the encoded protein(matrin) in the skin. Matrin encoded by Tmem79/Matt gene regulates the assembly of lamellar bodies, a crucial ultrastructure for SC intercellular lipid lamellae(16). Because of these mutations, flaky tail mice spontaneously have dermatitis and atopy caused by a defective skin barrier, which cause systemic sensitization after cutaneous challenge with HDM allergens. In human subjects, a common SNP in MATT gene also has an association with AD(17). They also presents with increases of IgE level, endogenous protease activity, TSLP expression, and basophils in the skin, and has many similarities with the AD phenotype(17, 18). Therefore, we could assume that flaky tail mice with both mutations related with skin barrier would be a better animal model for atopic march started from skin barrier dysfunction.

Therefore, we hypothesized that flaky tail mice, as a murine model of congenital AD due to inherited filaggrin deficiency, would develop respiratory inflammation after subsequent inhalation of HDM, similar to the model of atopic march. Then, we secondly hypothesized that this atopic march could be interrupted by maintenance of SC acidity or accelerated by neutralization of the SC.

-Materials and Methods-

Mice and Materials

All animal procedures were approved by the Yonsei University Wonju Campus Institutional Animal Care and Use Committee(IACUC). C57BL/6J(B6) female mice were purchased from OrientBio (Sungnam, Korea). Flaky tail mice (STOCK *a/a ma ft/ma ft/J*), which carry double-homozygous *ft* and *ma* mutations, were outcrossed onto B6 at Kyoto University (Kyoto, Japan)(19). Mice were kept under controlled humidity (40%) and temperature (22±2°C) conditions. Extracted antigen of *Dermatofagoides pteronyssinus*(Dp), a HDM, was purchased from the Department of Environmental Medical Biology, Yonsei University (Seoul, Korea). All laboratory measurements were performed under blinded conditions or by blinded researchers. The acidic cream was composed of citric acid purchased from Sigma-Aldrich (St Louis, MO, USA) and vehicle (Cetaphil®; Lausanne, Switzerland), adjusted using a pH meter. Neutralization of the acidic cream was performed with NaOH.

Development of an atopic march model by topical application and nasal inhalation of *Dermatofagoides pteronyssinus* (Dp) in flaky tail mice and intervention of the atopic march model with an acidic cream

Five-week-old flaky tail mice(n=6, each group) and B6(n=6 each group) mice were used in this study. Once per week, the mice were anesthetized and their dorsal hair was shaved. Dp was mixed with petrolatum to make a 0.5% ointment(pH=3.8). Forty milligrams of 0.5% Dp mixed with petrolatum was applied to the skin of the control group(B6+Dp, n=6) and the Flg^{ft} with Dp groups(n=6, each) twice per week a total of 20 times. During the last 3 days of the

experiment, 100 µg of Dp was given daily by intranasal inhalation(12). While applying Dp on the flaky tail mice, 200 mg of acidic cream (pH 2.8, vehicle cream adjusted with citric acid) was applied topically twice a day on the dorsal skin for 45 days(acidic cream group). For non-treated control groups(B6, n=6), no interventional cream was applied. For the neutralized cream group(pH 7.4, vehicle cream adjusted with sodium hydroxide, n=6), neutralized cream was topically applied twice per day (Suppl. 1). The vehicle was plain Cetaphil® cream with pH 5.5 (Galderma, Lausanne, Switzerland) as previously described(6, 12, 20). After a total of 70 days of Dp application, gross appearance, skin barrier functions such as basal transepidermal water loss (TEWL), SC hydration and skin surface pH, and the degree of allergic inflammation in the skin, blood, and respiratory system were evaluated in all mice. Surface pH was measured with a pH meter (WTW, Weilheim, Germany). Basal TEWL was measured with the Tewameter TM210 (Courage and Khazaka, Cologne, Germany), and SC hydration was assessed as capacitance with the Corneometer CM820 (Courage and Khazaka) 24 h after the final application of Dp, as described previously(21). Twenty-four hours after the last treatment, the mice were sacrificed and skin, blood, and lung specimens were collected to evaluate changes in allergic inflammation as measured by mRNA and protein expression.

Evaluation of macroscopic AD-like skin lesions

Scoring of dermatitis was assessed macroscopically in a blinded fashion using the following scoring procedure described by Saunders *et al.* (22) and adapted from assessments of skin inflammation in the NC/Nga mouse model(6). The total score for each mouse was calculated as the sum of individual scores for each parameter.

Tissue preparation and immunohistochemistry

Immunohistochemical staining was performed to assess epidermal differentiation and inflammation. Briefly, 5-µm-thick paraffin sections were incubated with primary antibodies

against epidermal differentiation marker proteins such as filaggrin, involucrin, and loricrin, protease-activated receptor-2 (PAR-2), and TSLP (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After three cycles of washing, the sections were incubated with the appropriate secondary antibody for 30 min. Staining was detected with the ABC-peroxidase kit (Vector Lab, Burlingame, CA, USA) and counterstaining with hematoxylin was performed. To quantify the density of immunohistochemical staining, three pictures from each section were taken at 100x magnification and labeled. An investigator without any information on the specimen ranked the pictures in order of the intensity of immunostaining (the highest intensity received the first rank). Representative images for each group are provided.

ELISA for serum IgE and TSLP

Serum levels of IgE and TSLP were determined using ELISA kits for mouse IgE (Koma Biotech, Seoul, Korea) and mouse TSLP (R&D Systems, Minneapolis, MN, USA) as previously reported(6, 12).

Bronchoalveolar lavage (BAL)

BAL was performed as previously described(6, 12). The counts of eosinophils, lymphocytes, macrophages, and neutrophils in the BAL fluid were determined by multiplying their percentages in total cells.

Total RNA preparation and cDNA synthesis with blood and lung tissue and quantitative PCR analysis of gene expression

Total RNA preparation and cDNA synthesis were performed as previously described(23, 24). Expression of specific mRNAs were quantified using a Rotor-Gene™ 3000 (Corbett Life Science, Brisbane, Australia), as in our previous reports(23, 24).

Analysis of cytokines in splenocytes

To evaluate the production levels of T-cell-derived cytokines, spleens were obtained from each group, and the isolated splenocytes were resuspended in RPMI 1640 medium containing 10% fetal bovine serum (Gibco®; Life Technologies, Grand Island, NY, USA). Splenocyte suspensions were seeded 3×10^6 cells per well in 24-well flat-bottom microplates and then incubated in the absence or presence of concanavalin A (Con A) (Sigma-Aldrich Co., St. Louis, MO, USA) at 2.5 µg/ml for 48 h. Following incubation, the culture supernatants were collected to determine the production levels of cytokines, including IL-1β, IL-4, IL-5, IL-10, IL-13, and IL-17α using the mouse OptEIATM set (BD Biosciences, San Jose, CA, USA).

Statistical analysis

All data were expressed as mean $\pm$ SEM. ANOVA or Kruskal-Wallis tests were performed when three or more groups were compared or to compare the density of immunohistochemical staining. For individual comparisons, Student's unpaired *t*-test was used in cases of normal distribution, and the Mann-Whitney test was used in cases of non-normal distribution or ranking variables. **p*<0.05 was regarded as statistically significant. Statistical analysis was conducted using SPSS software 10.1 (SPSS, Chicago, IL, USA).

Results

Repeated epicutaneous application of HDM extract provokes remarkable AD-like skin lesions and barrier dysfunction in flaky tail mice, a representative murine model with congenital barrier defects, which are prevented by acidic cream and aggravated by neutral cream.

Repeated epicutaneous application of HDM antigen, Dp antigen, provoked AD-like skin lesions with a high eczema score in flaky tail mice (Fig. 1). However, the control mice (B6 mice) did not exhibit eczema lesions despite the same epicutaneous treatment with Dp antigen. Moreover, application of a neutral cream with Dp extract (Flg^{fl}+Dp+pH 7.4) resulted

in more prominent eczema lesions compared to other treatment groups. In contrast, application of an acidic cream (Flg^{fl}+Dp +pH 2.8) significantly alleviated eczema lesions in Dp-treated flaky tail mice (Fig. 1a). Post-treatment basal TEWL was increased and SC hydration was significantly decreased in the group treated with neutral cream (Flg^{fl}+Dp+pH 7.4 group), but was significantly improved in the group treated with acidic cream (Flg^{fl}+Dp+pH 2.8 group) (Fig. 1b and c). Moreover, there were significant differences in skin surface pH among the cream treated groups, as measured 24 h after the final application of Dp, or 12 h after the final application of cream. Post-treatment SC pH decreased significantly in the group treated with acidic cream (Flg^{fl}+Dp+pH 2.8 group) and increased in the group treated with neutral cream (Flg^{fl}+Dp+pH 7.4 group) (Fig. 1d).

Cutaneous and systemic allergic inflammation in flaky tail mice were aggravated due to HDM and affected by SC acidity.

The number of tissue eosinophils in the skin significantly increased in the Flg^{fl}+Dp+pH 7.4 group and decreased in the Flg^{fl}+Dp+pH 2.8 group compared to the Flg^{fl}+Dp group (Fig. 2a). Immunohistochemical staining and semiquantitative analysis of epidermal differentiation markers such as involucrin and loricrin showed that their expression was significantly decreased in the Flg^{fl}+Dp group compared to the control B6+Dp group (Fig. 2a and Suppl. 2). Application of neutral cream to the Flg^{fl}+Dp group significantly decreased the expression of these markers, whereas acidic cream significantly increased the expression of all except filaggrin. There was minimal expression of filaggrin among Flg^{fl} groups. Semiquantitative analysis of epidermal expression of PAR-2 and TSLP was significantly increased in flaky tail mice compared to the control. Dp treatment further increased their expression. Application of neutral cream to the Flg^{fl}+Dp group significantly increased the expression of PAR-2 and TSLP, whereas acidic cream significantly decreased their expression (Fig. 2b and Suppl. 2).

Serum levels of total IgE and TSLP significantly increased in all flaky tail mice with or without Dp treatment compared to control mice. Among flaky tail mice, the mice that were treated with neutral cream showed the most prominent increase in serum IgE and TSLP. In contrast, acidic cream significantly decreased serum IgE and TSLP in flaky tail mice treated with Dp antigen(Fig. 2c and d).

Repeated epicutaneous application followed by nasal inhalation of HDM extract produces asthma-like airway inflammation as well as AD-like skin lesions in flaky tail mice, implying atopic march. This sequence was successfully interrupted by SC acidification, but aggravated by SC neutralization.

Since airway eosinophilia is a hallmark of asthma(6), we performed BAL after 3 consecutive days of nasal inhalation of Dp. The total cell count of the BAL was significantly increased after Dp application. This increase was significantly aggravated by treatment with neutral cream (Flg^{fl}+Dp+pH 7.4 group) and improved by the application of acidic cream(Fig. 3a). Eosinophils, macrophages, and neutrophils were also significantly increased in the Flg^{fl}+Dp group, similar to the results for total cell count(Fig. 3b). Quantitation of mRNA and immunohistochemical staining revealed increased expression of PAR-2 and TSLP in the lung tissue of the Flg^{fl}+Dp group, which was further aggravated by application of neutral cream and improved by acidic cream(Fig. 3c, d, e and Suppl. 3). Moreover, acidic cream significantly decreased IL-1 β , IL-5 and IL-17 α , but increased IL-10 in spleen. However, IL-4 and IL-13 showed no differences after applications of acidic cream(Fig. 4).

-Discussion-

The concept of the atopic march was developed to describe the progression of atopic diseases ranging from AD in infants to asthma and allergic rhinitis in children(3, 25). Abrogation of SC, which initiate allergic sensitization, promotes the penetration of protease-active allergens and the activation of uncontrolled intrinsic proteases, which may activate

PAR-2 and Toll-like receptors on keratinocytes(26, 27). Meanwhile, the dendrites of activated LCs penetrate the weakened tight junctions of the upper stratum granulosum and take up the penetrating allergens(28). Such percutaneous sensitization and chronic repeated allergen challenges via different routes, such as the lungs, nasal cavities, and intestinal tract, are speculated to manifest as the atopic march. In this study, as in our previous studies (6, 12), the epicutaneous treatment and nasal inhalation of HDM in control mice without inherited or acquired barrier defects did not provoke inflammatory lesions in the skin or lung (Figs. 2-4). These results confirmed that the uptake of active allergens and the activation of LCs does not take place when the epidermal barrier is intact and that epidermal TSLP was not increased.

The expression of TSLP is greatly increased in the lesional skin and blood of AD patients, which enables LCs to activate during AD development(5, 29). Since PAR-2 and TSLP expression have been reported in tissues including skin and lung, they also play roles in the occurrence of AD and respiratory allergy(6, 12, 30). TSLP expression is induced when airway epithelial cells are exposed to allergen-derived proteases, and PAR-2 is involved in this process(31). In the present study, repeated application of HDM to the congenitally defective skin barrier of flaky tail mice increases the levels of TSLP, PAR-2, and IgE in the systemic circulation and lung tissue(Fig. 3 and Suppl. 3). These conditions provoke elevations of systemic TSLP and IgE as well as eosinophils, whereas HDM application alone on the intact skin barrier rarely induces asthma. Systemic TSLP levels may affect PAR-2 and TSLP in distant epithelial organs such as the lung, which are readily exposed by HDM allergen. Therefore, we could hypothesize that HDM-induced asthma would occur more easily when the skin barrier is more severely impaired and when AD lesions are more severe.

Epicutaneous application of HDM antigen to flaky tail mice induces skin lesions as AD patients with skin barrier dysfunction(17). Although the relative contributions of *Flg* and *ma* to the compound phenotype of AD have yet to be defined, the matted mouse gene is known as a predisposing gene for AD and a common SNP in MATT is associated with AD in humans(22). In addition, Sasaki et al. (32) showed that the Tmem79(*ma/ma*) mutation is responsible for the spontaneous dermatitis phenotype in matted mice, probably as a result of an impaired LB secretory system and altered skin barrier function. This phenotype is also found in subjects of European descent who have FLG mutations in dose dependent alterations(15). A recent report used flaky tail mice to demonstrate innate immunity-initiating dermatitis and adaptive immunity required for subsequent development of compromised lung function in the atopic march(33). Our results were similar, with increased expression of IL-1 β , Th2 cytokines such as IL-4, IL-5 and IL-13, and IL-17a(Fig. 4). Therefore, flaky tail mice might be a proper candidate animal model of atopic march corresponding with filaggrin mutations in human AD patients.

Many previous studies have attempted to establish an animal model of the atopic march. Although several reports used occlusive patches of ovalbumin(OVA), they required intraperitoneal injections for sensitization, not epicutaneous sensitization via the damaged skin barrier(34, 35). However, in the clinical setting, allergic reactions to OVA disappeared in adulthood, whereas reactions to HDM persist. Recently, we reported that murine models of acquired and inherited AD could progress to asthma-like lesions under more natural and physiologic mechanisms, with favorable results(6, 12). In the present study, we applied HDM antigen, which showed same protein profile after acidic treatment compared to native extracts(36), epicutaneously on flaky tail mice with congenitally disrupted skin barriers, followed by nasal inhalation of HDM, in order to produce asthma-like lung lesions. This technique revealed the physiologic pathways of allergen penetration, systemic circulation, and allergen challenge via different routes, such as the lung and nasal cavities(Suppl. 1)(5).

Our results indicate that no significant changes occur in the lung without inhalation of HDM among flaky tail mice(Fig. 3 and Suppl. 3).

Filaggrin monomers are degraded into natural moisturizing factors to maintain the hydration of the upper SC and to reduce the pH of the skin surfaces(37). If these proteins are decreased or absent due to FLG mutations, the FLG-associated SC barrier is disrupted, which leads to increased episodes of percutaneous allergen exposure(1). Keratinocyte differentiation is also incomplete in AD lesions(38). In our atopic march model, the expressions of involucrin and loricrin were decreased with minimal expression of filaggrin, which may imply further disruption of the skin barrier and provocation of transcutaneous infiltration of allergens such as HDM(Fig. 2b and Suppl 2.). This in turn may induce asthma-like lesions more easily.

The decrease in urocanic acid, a filaggrin degradation product, in AD lesions might contribute to increases of SC pH great enough to activate kallikreins(KLKs) in the SC, which function best under neutral to alkaline pH(39). However, controversy remains over whether filaggrin deficiency increases SC pH(40-42). The increased activity of KLKs such as KLK5 and KLK7(stratum corneum chymotryptic enzyme) under conditions of elevated SC pH contributes to the overdegradation of corneodesmosomes followed by the weakening of SC integrity and cohesion(43). On the other hand, the elevation of SC pH decreases the activity of lipid-processing enzymes, which are crucial for the production of free fatty acids and ceramides in the SC, and thus impairs lipid processing and eventually disrupts the permeability barrier of the epidermis(8, 11). Based on our results, we hypothesize that prolonged skin barrier damage induced by repeated epicutaneous application of HDM in flaky tail mice elevates SC pH. Elevated SC pH could then increase PAR-2 and TSLP in the blood as well as the skin, in turn contributing to the formation of AD-like lesions characterized by elevated IgE and Th2 cytokines.

In this study, the application of HDM antigen through AD-like skin lesions and the respiratory system also induced respiratory allergies, which were represented by airway eosinophilia and elevated PAR-2 and TSLP in the lung tissue. Maintenance of an acidic skin environment could block this cycle early, and hence, the atopic march could be prevented. Interestingly, IL-10, an inhibitory cytokine secreted by regulatory T cells (Treg), showed greater increases in the splenocytes of mice receiving acidic cream applications compared to mice that received neutral cream. Treg cells may suppress Th2 cells effectively(44). Moreover, acidic cream decreased the expression of IL-5, which plays an important role in airway remodeling as well as Th2 inflammatory response(Fig. 4)(45). Based on our cytokine results, we hypothesize that the maintenance of an acidic environment affects immune responses including those of Treg and Th2 cells, and then inhibits systemic allergic reactions and pulmonary remodeling, which also help prevention of the progression to asthma-like lesions in flaky tail mice. Recently, we demonstrated the preventative effect of acid bathing, which favorably restored barrier function and resolved clinical symptoms in cases of severe or refractory AD(46). Similarly, use of emollient with linoleic acid from birth prevents AD by enhancing barrier function(47). Alongside these clinical studies, our results might be applicable in the treatment of young patients with moderate to severe AD who could be at high risk of progressing to asthma.

The results of our present and previous studies indicate that the application of HDM antigen to AD-like lesions and the respiratory organs could induce respiratory allergies more easily in a murine model, regardless of the underlying acquired or congenital etiology. Besides, maintenance of a neutral skin environment could aggravate AD, but acidic skin could interrupt this cycle. Again, we found the importance of maintenance of normal SC acidity to maintain healthy skin barrier, which can prevent the penetration of external allergens through skin barrier. Therefore, maintenance of an acidic environment in the SC could be a novel strategy to prevent progression of the atopic march from barrier disruption to respiratory allergy.

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-Figure legends-

Fig. 1. Repeated epicutaneous application of house dust mite (HDM; Dp) antigen followed by nasal inhalation provokes remarkable AD-like skin lesions and barrier disruption in flaky tail (Flg^{fl}) mice, a representative AD murine model with congenital barrier defects due to inherited filaggrin mutation. Those symptoms were improved by acidic cream application, but were aggravated by neutral cream application. Systemic allergic inflammation induced in flaky tail mice was improved by the acidic cream, but aggravated by the neutral cream. (a) Eczema scores and gross appearance, (b) basal TEWL, (c) SC hydration, and (d) skin surface pH after repeated epicutaneous application of HDM (Dp) extract followed by nasal inhalation were compared among treatment groups. ANOVA showed $p < 0.05$ in all measurements. Results are expressed as mean $\pm$ SEM. * $p < 0.05$, compared to the control (B6 + Dp and Flg^{fl}); + $p < 0.05$, compared to Flg^{fl} + Dp (n=6, each group).

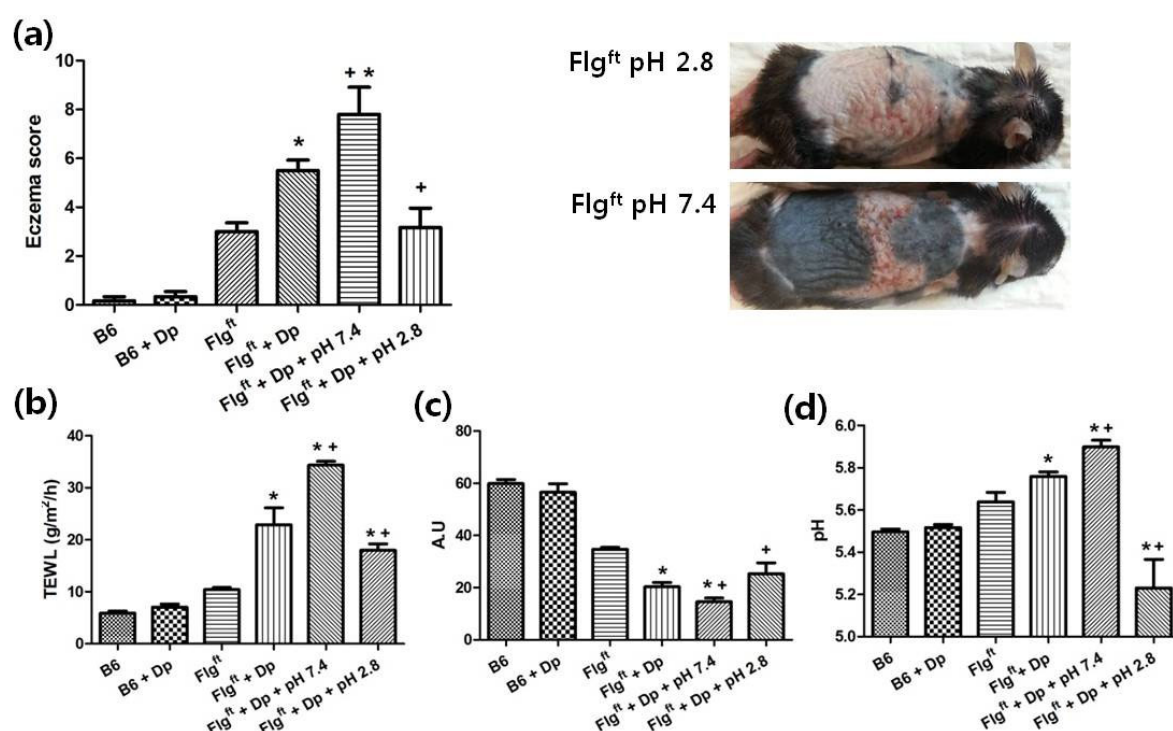


Fig. 2. Cutaneous allergic inflammation and abnormal epidermal differentiation induced in flaky tail mice was aggravated by neutral cream application, but improved by acidic cream application. (a) Tissue eosinophils were counted with H&E staining, (b) the expression of

epidermal differentiation markers such as filaggrin, involucrin, and loricrin, PAR-2, and TSLP were measured by immunohistochemical staining in the skin specimens, (c) serum IgE and (d) TSLP were measured by ELISA in blood samples taken after repeated epicutaneous application and nasal inhalation of Dp. All were compared among treatment groups. ANOVA (a) and Kruskal-Wallis tests (b) showed $p < 0.05$ for all measurements. Results are expressed as mean $\pm$ SEM. * $p < 0.05$, compared to the control (B6 + Dp); + $p < 0.05$, compared to Flg^{fl} + Dp (n=6, each group).

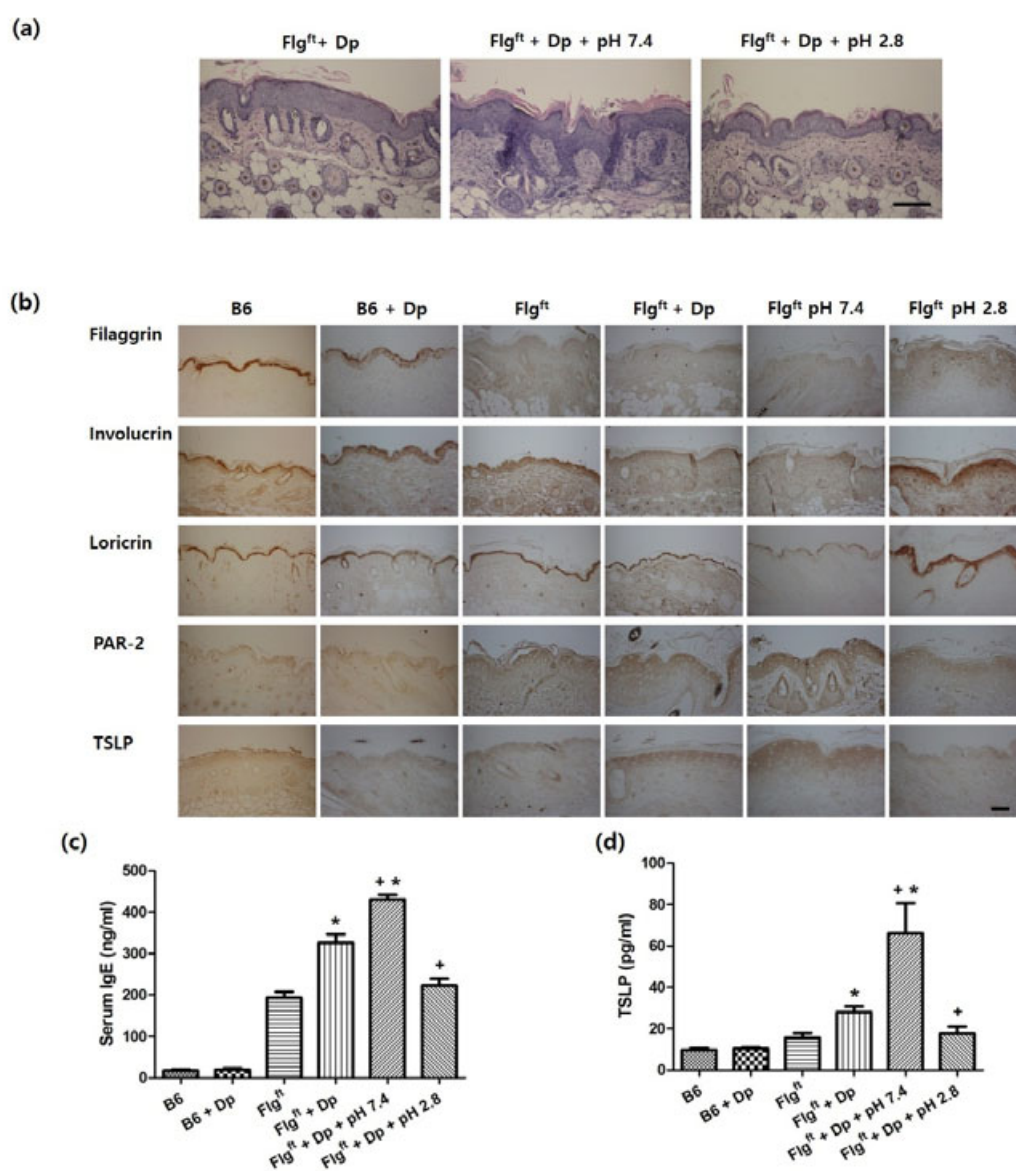


Fig. 3. Repeated epicutaneous application followed by nasal inhalation of house dust mite (HDM; Dp) antigen produces asthma-like airway inflammation as well as AD-like skin lesions in flaky tail mice, implying atopic march. Those symptoms were improved by acidic cream application, but were aggravated by neutral cream application. Bronchoalveolar lavage (BAL), real-time RT-PCR for mRNA expression, and immunohistochemical staining for PAR-2 and TSLP in the lung tissue were performed after epicutaneous application and nasal inhalation of Dp. (a) Total cell count and (b) individual cell count of the BAL, and (c, d) mRNA and (e) tissue expression of PAR-2 and TSLP were measured after treatment. ANOVA (a-d) and Kruskal-Wallis tests (e) showed $p < 0.05$ in all measurements. Results are expressed as mean $\pm$ SEM. * $p < 0.05$, compared to the control (B6 + Dp); + $p < 0.05$, compared to Flg^{fl} + Dp (n=6, each group).

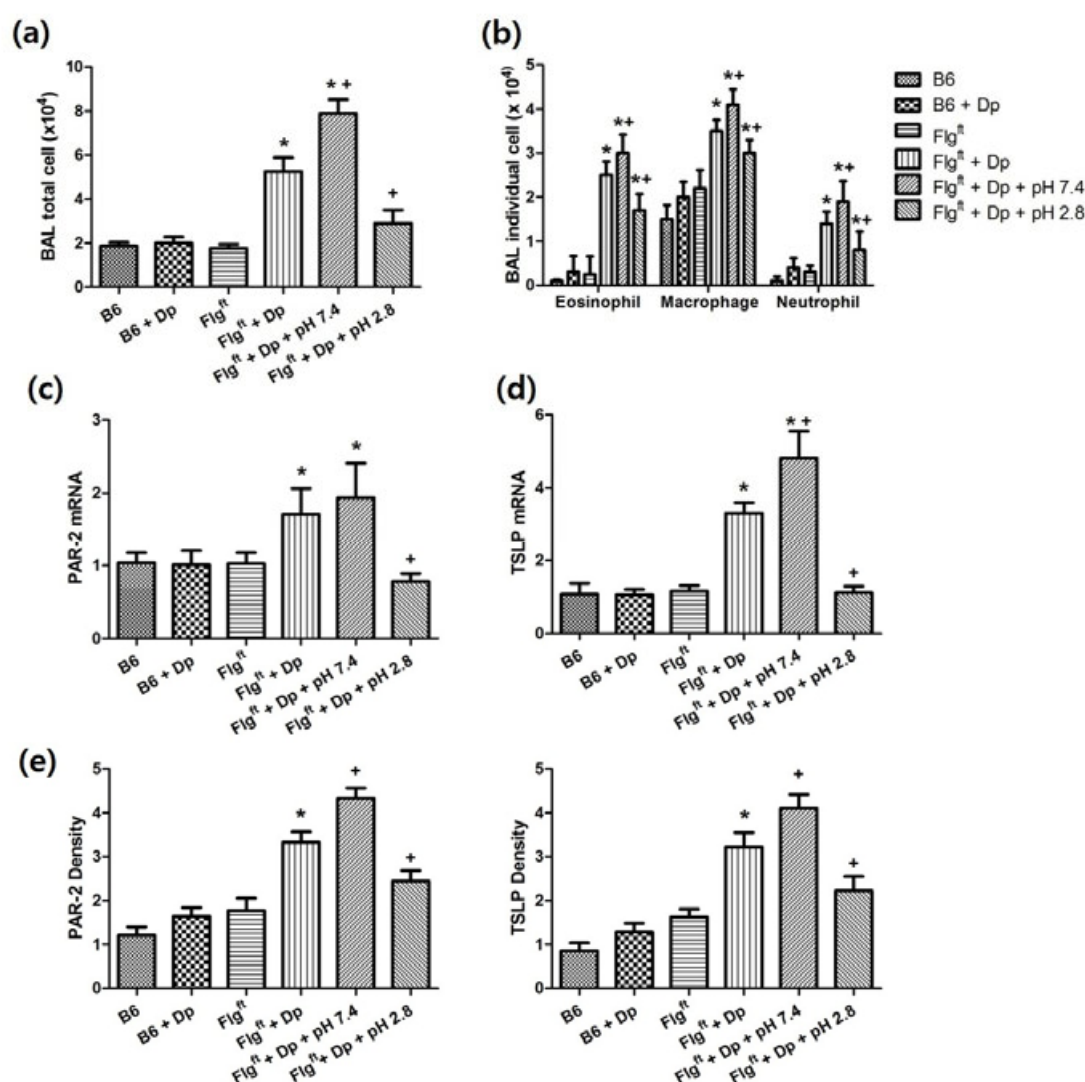


Fig. 4. Splenic cytokines after application of acidic or neutral cream with repeated epicutaneous application of house dust mite (HDM; Dp) antigen followed by nasal inhalation. Results are expressed as mean $\pm$ SEM. * $p < 0.05$, compared to the control (B6 + Dp); + $p < 0.05$, compared to Flg^{fl} + Dp + pH7.4 (n=6, each group).

