

# Topical glucocorticoid or pimecrolimus treatment suppresses thymic stromal lymphopoietin-related allergic inflammatory mechanism in an oxazolone-induced atopic dermatitis murine model

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**Abstract** Congenitally or early impaired skin barrier as the first event starting the ‘atopic march’ in atopic dermatitis (AD) patients can increase allergen penetration that results in sensitization, even in the airways, followed by asthma and allergic rhinitis. Thymic stromal lymphopoietin (TSLP) is a cytokine existing in high levels in AD skin and is considered as a novel therapeutic target for atopic disease. We generated oxazolone (Ox)-induced AD-like (Ox-AD) hairless mice and divided them into four groups according to the therapeutic challenges: topical glucocorticoid, pimecrolimus, emollient, and control (acetone-only treated). We assessed the functional studies of skin barrier, epidermal expressions of differentiation markers, IL-1 $\alpha$ , TNF- $\alpha$ , proteinase-activated receptor-2 (PAR-2), TSLP and antimicrobial peptides (AMP), and serum IgE in each group. Topical glucocorticoid or pimecrolimus treatment improved AD-like skin lesions and barrier functions, and restored the epidermal expression of differentiation markers, IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP, in Ox-AD mice. The improvement was relatively better with the glucocorticoid than pimecrolimus. Epidermal AMP expression was restored by topical glucocorticoid, but not pimecrolimus. Our result showed that topical glucocorticoid or pimecrolimus improved the AD-like skin lesions and barrier impairment by suppressing TSLP-related allergic inflammation.

**Keywords** Atopic dermatitis · Glucocorticoid · Pimecrolimus · Protease-activated receptor 2 (PAR-2) · Skin barrier · Thymic stromal lymphopoietin (TSLP)

## Introduction

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease characterized by extreme pruritus, which is considered as the first stage of the atopic march [39]. A recent hypothesis showed that the first event starting the atopic march is an impairment of the epidermal barrier, which increases skin permeation by allergens [3, 37]. Allergen penetration increases sensitization, even in the airways, which contributes to asthma. Thymic stromal lymphopoietin (TSLP) is an epithelial cell-derived IL-7-like cytokine expressed in the skin, gut, lung, and thymus that plays an important role in shaping the allergic inflammatory immune response, as well as in the pathogenesis of AD and asthma [28]. High expression of TSLP was observed in epidermal keratinocytes from acute and chronic AD skin lesions and it triggers dendritic cell-mediated allergic inflammation [38]. Recently, Demehri et al. [8] reported that TSLP overexpression by epidermal keratinocytes is a systemic driver of bronchial hyper-responsiveness in asthma and suggested that early treatment of primary skin barrier defects and selective inhibition of systemic TSLP expression may be key factors in blocking asthma development in AD patients. After allergen exposure, the proteases presenting in the allergen complex activate the protease-activated receptor (PAR)-2, which in turn induces TSLP expression [43]. TSLP converts human epidermal Langerhans cells into antigen-presenting cells that induce pro-allergic T cells [11]. TSLP may also act to recruit IL-4 producing basophils to draining lymph nodes

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that act cooperatively with dendritic cells (DCs) to prime Th2 cytokine responses [43]. Therefore, increased levels of PAR-2 and TSLP are crucial for predicting allergic Th2 inflammation. In addition, the appropriate inhibition of TSLP expression could be a novel therapeutic method for atopic dermatitis.

Topical glucocorticoids have long been widely used to effectively treat acute flares of AD. Recently, topical calcineurin inhibitors (TCIs) such as pimecrolimus and tacrolimus have been widely used for maintenance therapy of AD [12]. However, many AD patients, their parents, and even their physicians do not apply appropriate amounts of topical glucocorticoids, even in acute flares of AD, because of ‘steroid phobia’ [18]. In addition, many physicians do not have confidence in TCIs as a maintenance therapy of AD.

Herein, we investigated the use of topical glucocorticoids and pimecrolimus to suppress allergic inflammatory pathomechanism related to TSLP, a key cytokine in atopic dermatitis, using oxazolone (Ox)-induced AD-like (Ox-AD) mice as an animal model with high epidermal TSLP expression.

## Materials and methods

### Materials

Female hairless mice (hr/hr) aged 6–8 weeks were purchased from Orient Bio Inc. (Seongnam, Korea). 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), acetone and a rabbit anti-mouse antibody against TSLP were purchased from Sigma (St. Louis, MO, USA). Affinity-purified, rabbit anti-mouse antibody against PAR-2 was purchased from Alomone Labs (Jerusalem, Israel). Affinity-purified, rabbit anti-mouse antibodies against loricrin, involucrin, and filaggrin were purchased from Abcam (Cambridge, UK). Affinity-purified, rabbit anti-mouse antibody against CRTH2/DP2 was from Cayman Chemical (Ann Arbor, MI, USA). Serum levels of IgE were measured using a mouse IgE ELISA kit (Koma Biotech, Seoul, Korea). Reverse transcriptase was purchased from Promega (Madison, WI, USA) and a real-time PCR kit from Qiagen (Hilden, Germany). Primers were designed based on retrieved GeneBank sequences using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>, Massachusetts Institute of Technology, Cambridge, MA, USA). Topical pimecrolimus (Elidel® cream) was purchased from Novartis Korea©, while topical methylprednisolone aceponate (Advantan® cream) (mid-potency) was purchased from Invida Korea©, and emollient (Cetaphil® cream) from Galderma©. We used

the emollient to compensate the humectant and occlusive effects of steroid or pimecrolimus cream.

### Experimental protocols and functional studies of the skin barrier state

All animal procedures were approved by the Yonsei University Wonju Campus Institutional Animal Care and Use Committee (IACUC) and performed in accordance with their guidelines. Mice were divided into four groups. An AD-like animal model was established as in previous reports by repeated Ox challenges to hairless mice [24, 29]. Briefly, three groups of animals (each  $n = 6$ ) were sensitized by a single topical treatment with 10  $\mu$ L of 5 % Ox, while the control group was treated with acetone only. After a 1-week sensitization period, Ox-treated groups were treated topically with 60  $\mu$ L of 0.1 % Ox on their backs once a day for 7 days. The control group was continued with acetone-only treatment.

After 7 days of challenge, each group of Ox-AD mice was treated with methylprednisolone aceponate cream (0.2 g; Ox-glucocorticoid group), pimecrolimus cream (0.2 g; Ox-pimecrolimus group), and emollient cream (0.2 g; Ox-emollient group) twice a day for 10 days. During this period, Ox treatments were continued on each group every other day. At 24 h after the final Ox challenge and each treatment, the gross findings, basal TEWL, and SC hydration were evaluated in all mice. Basal TEWL was measured using a Tewameter TM 210 (Courage and Khazaka, Cologne, Germany), and SC hydration was measured using a Corneometer CM 825 (Courage and Khazaka). The environmental conditions of the measurement room ranged between  $22 \pm 2$  °C with a relative humidity between 40 and 45 %. All skin samples were biopsied immediately after this functional study.

### Tissue preparation and immunohistochemical stains

For immunohistochemical staining, we used the methods described in our previous report [19]. Briefly, 5  $\mu$ m paraffin sections were deparaffinized and rehydrated sequentially with 100, 95, 70, and 50 % ethanol for 5 min. Samples were blocked with peroxidases (Thermo Scientific, Fremont, CA, USA) and serum solution (DAKO, Carpinteria, CA, USA). Subsequently, samples were incubated with primary antibodies for the epidermal differentiation marker proteins, AMPs, CRTH2, PAR-2, and TSLP overnight at 4 °C. After three washes, the sections were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Staining was detected with an UltraVision Plus Detection System from Thermo Scientific using 3,3'-diaminobenzidine tetrahydrochloride

as a substrate. Results were examined under a light microscope.

### Counting dermal eosinophils in H&E-stained slides

The density of eosinophils was assessed in the area of  $200 \times 200 \mu\text{m}$  on H&E-stained sections and counted in more than ten fields in the dermis of each sample. Then, the relative densities of each treated group were expressed based on the control group.

### Total RNA isolation and cDNA synthesis

To separate the epidermis, the subcutaneous fat was removed from a skin sample using a scalpel, and the skin sample was incubated in 10 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) at 37 °C for 30 min.

Total RNA was isolated from the epidermis samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then resuspended in RNase-free water. RNA concentrations were quantified using a UV/visible spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). Aliquots (1  $\mu\text{g}$ ) of total RNA were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MML-V RTase; Promega, Madison, WI, USA) with 1X RT buffer, 2 mM dNTP (Promega, Madison, WI, USA), 0.2 pM Oligo-dT primer (Bioneer Inc., Daejeon, Korea),

and MML-V RTase (2.5 units/ $\mu\text{L}$ ) in a 20  $\mu\text{L}$  reaction volume, as described in our previous report [19].

### Quantitative real-time reverse transcriptase-polymerase chain reaction

Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to analyze mRNA expression of filaggrin, loricrin, involucrin, IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, TSLP, CRAMP, and mBD-3 using a Rotor-Gene<sup>TM</sup> 3000 (Corbett Life Science, NSW, Australia) and a Quantitect SYBR green PCR kit master mix (Qiagen, Hilden, Germany), 10 pM primer, and 1  $\mu\text{L}$  of template cDNA in a 10  $\mu\text{L}$  reaction volume, as described in our previous report [19]. Primers were designed based on GeneBank sequences using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>, Massachusetts Institute of Technology, Cambridge, MA, USA) (Table 1). Samples were amplified at 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. qRT-PCR data were analyzed as the  $C_t$  values of the target gene and a GAPDH housekeeping gene (Bioneer Inc., Daejeon, Korea).  $\Delta C_t$  values were determined by subtracting the  $C_t$  of the target gene from that of the housekeeping gene.  $\Delta\Delta C_t$  values were determined by subtracting the  $\Delta C_t$  values of Ox group samples from the respective control group. The resulting relative values were determined using the equation  $2^{-\Delta\Delta C_t}$ .

**Table 1** Real-time RT-PCR primer sequences

| Target gene   | Direction      | Sequence (5' → 3')         |
|---------------|----------------|----------------------------|
| GAPDH         | Forward primer | TGCGACTTCAACAGCAACTC       |
|               | Reverse primer | ATGTAGGCCATGAGGTCCAC       |
| mBD3          | Forward primer | GGCTTCAGTCATGAGGATCCATTACC |
|               | Reverse primer | TGCACCGATTCCAGCATCTGCC     |
| CRAMP         | Forward primer | TGAGCCCCAAGGGGACGAGG       |
|               | Reverse primer | GCCGGGTTCAGGGTGACTGC       |
| TSLP          | Forward primer | CGACAGCATGGTTCTTCTCA       |
|               | Reverse primer | CGATTTGCTCGAACTTAGCC       |
| PAR-2         | Forward primer | TCTCTGCACCAATCACAAGC       |
|               | Reverse primer | CTTAGCCTTCTTGCCAGGTG       |
| Filaggrin     | Forward primer | AGACTGGGAGGCAAGCTACA       |
|               | Reverse primer | CCTGCCTCCTTCAGAGTCAC       |
| Involucrin    | Forward primer | CAAGAGCCACAAATGCAAGA       |
|               | Reverse primer | GGAGTTTCTGCTGCTGACC        |
| Loricrin      | Forward primer | CTCATCTTCCCTGGTGCTTC       |
|               | Reverse primer | CAGCTAGAGCCTCCTCCAGA       |
| TNF- $\alpha$ | Forward primer | AGCCCCAGTCTGTATCCTT        |
|               | Reverse primer | CTCCCTTTGCAGAACTCAGG       |
| IL-1 $\alpha$ | Forward primer | CCCGTCCTTAAAGCTGTCTG       |
|               | Reverse primer | AATTGGAATCCAGGGGAAAC       |

## ELISA for serum IgE

The serum levels of IgE were determined as in our previous report [22]. Whole blood samples were centrifuged at 5000 rpm for 5 min at room temperature (RT). The sample and standard solutions were added to plates pre-coated with an antibody specific to IgE and incubated for 1 h at RT. Then, a mouse IgE detection antibody conjugated to HRP was added to each well and incubated for 1 h at RT. Next, a color development solution with TMB substrate was added to each well and allowed to incubate for 20 min. A sulfuric acid solution was added to each well to stop the reaction. The enzyme–substrate reaction was measured using a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT, USA), and the plate was read at  $450 \pm 2$  nm. After triplicate measurements, the concentration of IgE sample was compared with the OD of the standard curve. Student's *t* test was used to analyze the results, with  $p < 0.05$  considered to be statistically significant.

## Results

### Topical glucocorticoid or pimecrolimus treatment improved AD-like skin lesions and epidermal permeability barrier function in Ox-AD mice

We evaluated the effect of topical glucocorticoid or pimecrolimus treatment on AD-like skin lesions and abnormal epidermal permeability barrier induced by repeated Ox treatments. As Ox treatments on hairless mice skin proceeded, their gross appearances showed AD-like lesions of erythema, edema, lichenification, and scale compared with the normal appearance of the acetone-only treated group (control group). Basal TEWL was significantly increased and SC hydration was significantly decreased according to the number of challenges (data not shown). Topical glucocorticoid, pimecrolimus, or emollient were applied to AD mice twice a day (each group  $n = 6$ ) concurrently with Ox challenges every other day for 10 days.

Topical glucocorticoid (Ox-glucocorticoid) or pimecrolimus (Ox-pimecrolimus) treatment improved Ox-AD skin lesions compared with emollient (Ox-emollient) (Fig. 1a). Topical glucocorticoid (Ox-glucocorticoid) or pimecrolimus (Ox-pimecrolimus) decreased basal TEWL (Fig. 1b) and increased SC hydration (Fig. 1c) in AD-like skin lesions compared with emollient (Ox-emollient). In addition, glucocorticoid treatment showed a superior effect on the basal epidermal permeability barrier state compared with pimecrolimus ( $P < 0.05$ ) (Fig. 1b, c).

### Topical glucocorticoid or pimecrolimus restored the epidermal differentiation of AD-like skin

Ox-AD skin (Ox-emollient) showed a marked decrease in epidermal differentiation compared with the control group, which was presented by decreased expression of proteins and mRNAs of epidermal differentiation markers such as filaggrin, loricrin, and involucrin (Fig. 2a, b). Topical glucocorticoid (Ox-glucocorticoid) markedly restored the expression of these epidermal differentiation marker proteins and mRNAs, especially of loricrin and involucrin significantly on real-time RT-PCR ( $P < 0.05$ ) (Fig. 2b). Topical pimecrolimus (Ox-pimecrolimus) also improved the expression of protein and mRNA of the epidermal differentiation markers in AD-like skin compared with emollient (Ox-emollient) (Fig. 2a, b), especially of involucrin on real-time RT-PCR ( $P < 0.05$ ) (Fig. 2b).

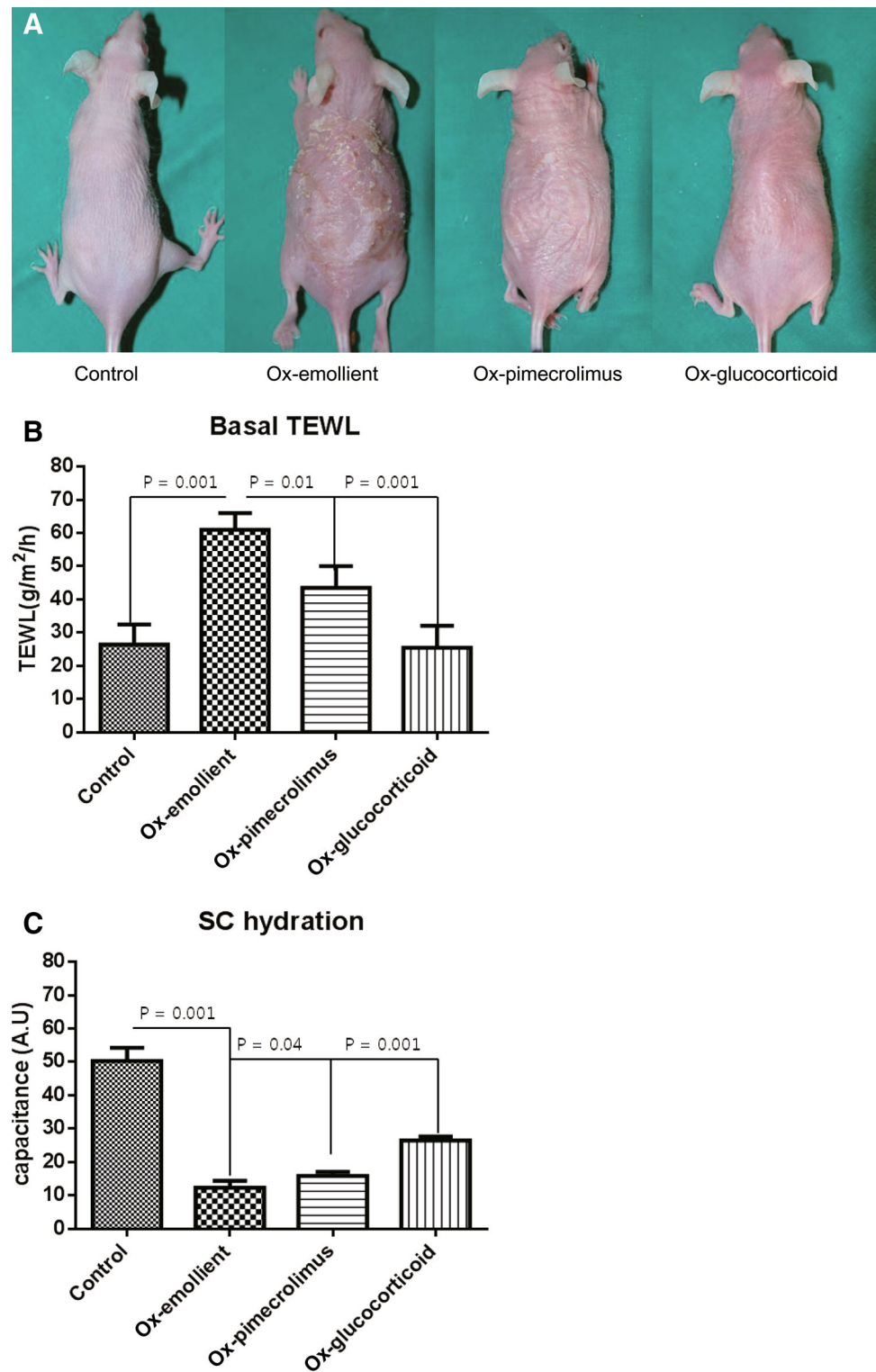
### Topical glucocorticoid or pimecrolimus treatment suppressed the elevated epidermal expressions of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP in AD-like skin

Immunohistochemical stains showed that the proteins of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP were overexpressed in the epidermis of Ox-AD skin compared with control (Fig. 3a). Moreover, qRT-PCR showed that their mRNAs were overexpressed in the epidermis of Ox-AD skin compared with control (Fig. 3b). In AD-like skin treated with topical glucocorticoid (Ox-glucocorticoid) or pimecrolimus (Ox-pimecrolimus), the epidermal expressions of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP were significantly decreased compared with AD-like skin treated with emollient only (Ox-emollient) in immunohistochemical stains and mRNA quantitation ( $P < 0.05$ ) (Fig. 3a, b).

### Topical glucocorticoid, but not pimecrolimus restored the epidermal expression of antimicrobial peptides in AD-like skin

AD-like skin showed a remarkable decrease in the epidermal expression of antimicrobial peptides such as CRAMP and mBD3, as demonstrated by immunohistochemical staining and qRT-PCR results (Fig. 4a, b). Topical glucocorticoid (Ox-glucocorticoid) significantly restored the epidermal expression of mBD3 and CRAMP compared with the emollient (Ox-emollient) (Fig. 4a, b). However, pimecrolimus (Ox-pimecrolimus) did not significantly increase their mRNA expressions in qRT-PCR, although it restored the mBD3 expression in the immunohistochemical stain (Fig. 4a, b).

**Fig. 1** Topical glucocorticoid or pimecrolimus treatments improved AD-like skin lesions and restored the function of the epidermal permeability barrier impaired by repeated Ox treatments. As Ox treatments proceeded, the gross appearances of AD lesions showing remarkable erythema, edema, lichenification, and scales became pronounced compared with the acetone-only treated group (Control) (**a**). Topical glucocorticoid (Ox-glucocorticoid) or pimecrolimus treatment (Ox-pimecrolimus) remarkably improved AD-like skin lesions compared with emollient only (Ox-emollient) (**a**). AD mice receiving topical glucocorticoid (Ox-glucocorticoid) or pimecrolimus (Ox-pimecrolimus) showed lower basal TEWL (**b**) and higher SC hydration (**c**) compared with emollient-treated mice (Ox-emollient). However, topical glucocorticoid showed better improvement in the eczema lesions and permeability barrier functions compared with pimecrolimus



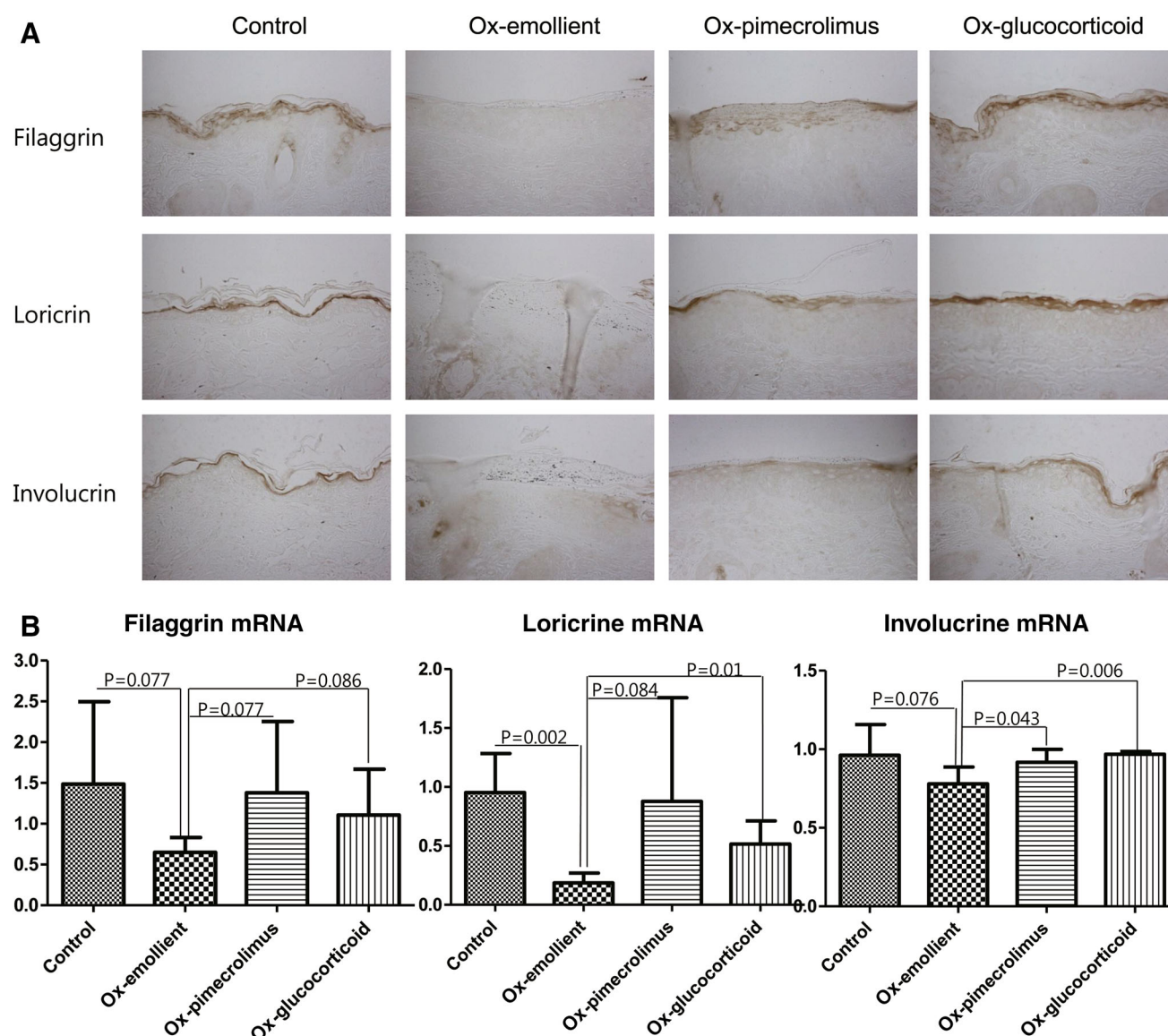


**Fig. 2** Topical glucocorticoid or pimecrolimus restored the epidermal expression of differentiation markers in the Ox-AD skin. Ox-AD skin (Ox-emollient) showed a marked decrease in epidermal differentiation compared with acetone-only treated group (control). Topical glucocorticoid (Ox-glucocorticoid) markedly restored the expression of epidermal differentiation markers such as filaggrin, loricrin, and

involucrin presented with immunohistochemical stain (a) and mRNA quantitation (b). Topical pimecrolimus (Ox-pimecrolimus) also improved their expressions in AD-like skin compared with emollient (Ox-emollient) (a, b) significantly of involucrin on real-time RT-PCR ( $P < 0.05$ ) (b)

### Topical glucocorticoid or pimecrolimus treatment suppressed epidermal hyperplasia and inflammatory cellular infiltration in AD-like skin

AD-like skin showed marked dermal inflammatory cell infiltrations, as well as epidermal hyperplasia. In particular, the numbers of eosinophils in H&E stains and Th2 cells in CRTH2 immunohistochemical stains were increased (Fig. 5a, b). After topical glucocorticoid or pimecrolimus was applied, inflammatory cell infiltration,

including eosinophil and Th2-dominant cells, in the dermis significantly improved and epidermal hyperplasia decreased (Fig. 5a, b).

### Topical glucocorticoid or pimecrolimus treatments on an AD model did not suppress serum IgE level

Serum IgE level was remarkably elevated in Ox-AD mice compared with control mice. Topical glucocorticoid or pimecrolimus treatment on AD mice did not suppress

serum IgE levels. It is likely that the 10 day treatment duration was not sufficient to affect serum IgE levels (Fig. 5c).

## Discussion

Recently, AD has been known as the initial disease of atopic march [39]. TSLP overexpressed in the keratinocytes of AD-affected skin can trigger a mechanism that underlies the progression of the atopic march [13]. In addition to the early protection of skin barrier defects in AD-prone people, a selective inhibition of TSLP might be a key intervention to improve the symptoms of AD or block the progression of atopic march. Therefore, we performed this study to determine whether topical glucocorticoid or pimecrolimus treatment could suppress the pathomechanism related to TSLP overexpression in AD.

For this study, we adopted Ox-AD murine model that we had already used [22]. Similar to human AD, Ox-AD mice showed Th2-dominant inflammatory infiltrates, elevated serum IgE levels, and an impaired permeability barrier. In addition, epidermal and blood TSLP expressions were gradually increased according to the number of Ox challenges after sensitization, which suggested that repeated Ox (hapten) treatment can induce Th2 inflammation via Th1. Previous studies reported that topical glucocorticoid or pimecrolimus application showed potent anti-inflammatory activity on the inflamed skin of Ox-AD mice and restored skin barrier, AMP, and epidermal differentiation [16, 30]. Our study also demonstrated again that topical glucocorticoid or pimecrolimus treatment could improve AD-like skin lesions and restore the function of the epidermal permeability barrier and the epidermal differentiation impaired by repeated Ox treatments. In addition, topical glucocorticoid or pimecrolimus application could significantly decrease the epidermal expression of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP with immunohistochemical staining and their mRNA quantities with qRT-PCR. In the case of hydrocortisone cream, we found that it could not suppress local skin inflammation sufficiently. We also found that the strong-potency steroid cream induced skin atrophy and systemic effect by cutaneous absorption. Therefore, we used a mid-potency steroid topical methylprednisolone cream in this experiment.

TSLP triggers DCs to induce Th2 inflammatory responses [42]. In addition, TSLP could enhance the effector stage of the allergic response [17] and amplify Th2 cytokines secretion in synergy with IL-1 and TNF- $\alpha$  [2]. Previous studies demonstrated that TSLP increases in both of the skin specimens and the serum from acute or chronic AD patients [17, 21]. Overexpression of TSLP in epidermal keratinocytes is a systemic driver of bronchial hyper-

responsiveness [8], and TSLP in airway epithelial cells is correlated with reduced lung function in asthma patients [15]. Moreover, TSLP can be induced by exogenous stimuli such as trauma, defects in barrier differentiation, infection with microbes, and Toll-like receptor (TLR) ligation, as well as by topical application of active vitamin D3 or low-calcemic analog treatment [25]. In particular, the PAR-2-TSLP relationship has been emphasized in some articles [4, 5], and PAR-2 is considered to be a parent signal inducing the overexpression of TSLP in epithelial cells [20]. It is known that skin barrier perturbation stimulates the epidermal production of the cytokines such as IL-1 $\alpha$  and  $\beta$ , TNF- $\alpha$ , and GM-CSF [41], which has a role in the regulation of proinflammatory and homeostatic processes in the skin. Therefore, we believe that repeated Ox treatment induced acute to chronic barrier disruption and then increased epidermal expression of PAR-2 and TSLP, which could drive the Th2 inflammation synergistically in Ox-AD murine model (Fig. 6).

Previous studies about the relationship between glucocorticoids and TSLP reported that inhaled budesonide could inhibit the TSLP expression in the lung tissues of asthmatic rats [27] and that glucocorticoids inhibit TLR3 ligand-induced TSLP in keratinocytes under an atopic cytokine milieu [23]. In addition, TSLP secretion of synovial fibroblasts is negatively regulated by dexamethasone [35]. A recent study by Le et al. [24] showed that glucocorticoids could inhibit the dsRNA-induced release of TSLP in an atopic cytokine milieu at much lower concentrations than calcineurin inhibitors. This in vitro result supports our in vivo experimental result that topical glucocorticoid inhibits the expression of epidermal TSLP. Our result provides further in vivo evidence that topical glucocorticoid application could decrease the epidermal expression of PAR-2 and TSLP and improve other skin barrier-related markers. Topical pimecrolimus can reduce IL-1 expression in mouse skin [19]. We confirmed that topical pimecrolimus can suppress the epidermal expression of PAR-2 and TSLP and proinflammatory cytokines such as IL-1 $\alpha$  and TNF- $\alpha$  in Ox-AD murine model. The relationship between calcineurin inhibitors and TSLP has not been studied, except in one in vitro study [24], in which cyclosporine did not suppress keratinocyte-derived TSLP expression since calcineurin inhibitor is a T cell inhibitor. However, our in vivo study showed that topical calcineurin inhibitor, pimecrolimus, suppressed the epidermal expressions of PAR-2 and TSLP, as both keratinocytes and T cells are involved in AD inflammatory pathogenesis in vivo.

In our results, epidermal AMP expression was decreased in AD-like skin, which is similar to AD lesional skin [34]. Epidermal AMP expression is dependent on inflammatory cytokines and the state of the epidermal permeability barrier. Th2 cytokines such as IL-4 and IL-

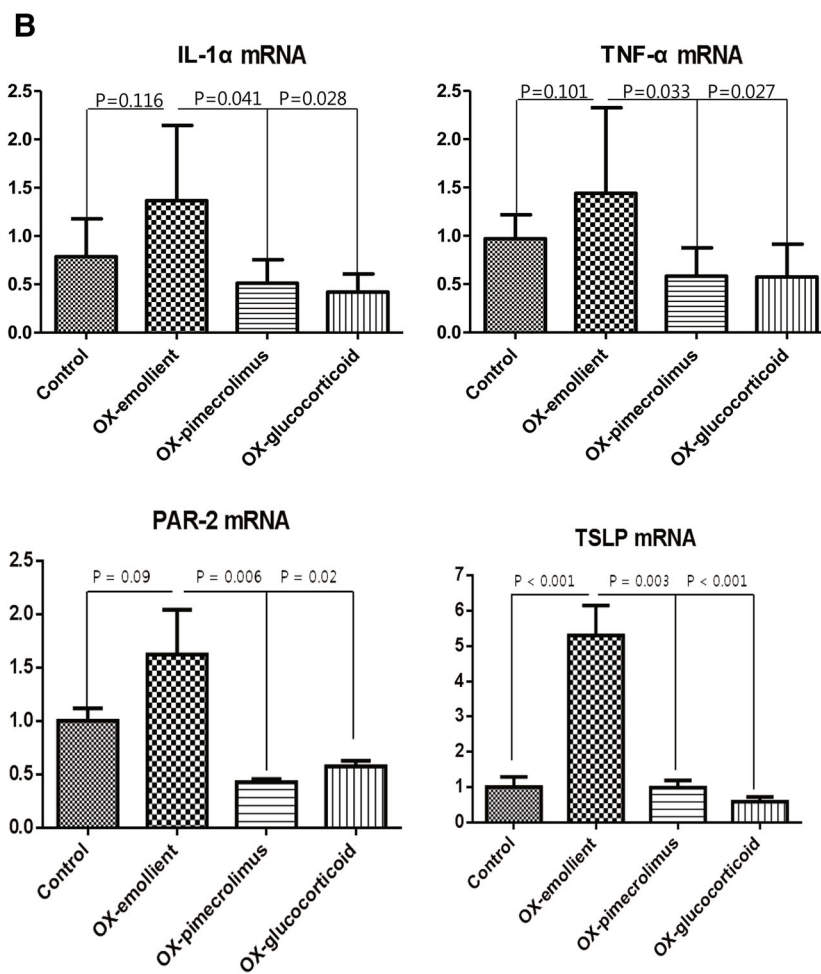
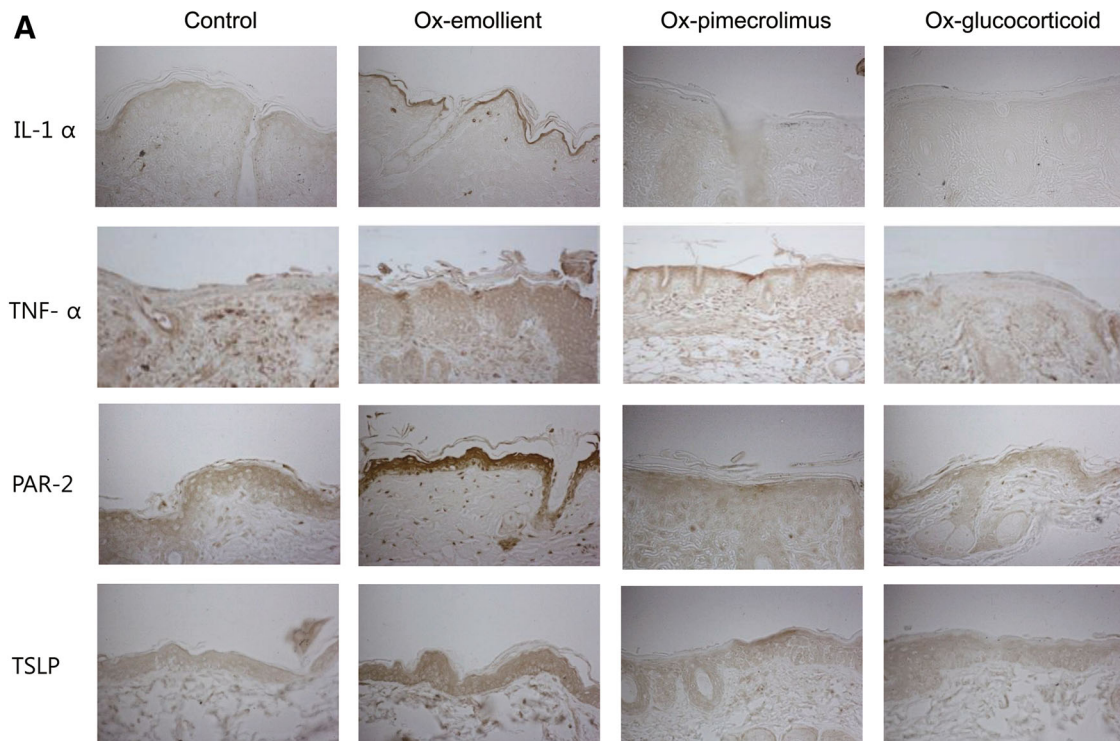


**Fig. 3** Topical glucocorticoid or pimecrolimus treatment suppressed the elevation of epidermal expression of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP in Ox-AD skin. Immunohistochemical stains showed that IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP were overexpressed in Ox-induced AD-like skin compared with control (a). Real-time RT-PCR also demonstrated the overexpression of mRNA of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP in AD-like skin (b). In the topical glucocorticoid (Ox-glucocorticoid) or pimecrolimus (Ox-pimecrolimus) groups, the immunohistochemical stains (a) and mRNA levels (b) of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP were markedly decreased compared with AD-like skin receiving only emollient (Ox-emollient) ( $P < 0.05$ )

13 have an inhibitory effect on AMP expression [36]. Epidermal AMP expression is linked to permeability barrier function, because cathelicidin and beta-defensin are co-packaged along with lipids within epidermal lamellar bodies (LBs) before their secretion and co-localized in SC intercellular lamellae after their secretion [1]. Therefore, decreased epidermal expression of AMP could be due to an impaired epidermal permeability

barrier state and an increase in Th2 cells in Ox-AD mice. Topical glucocorticoid or pimecrolimus treatment can suppress overall T cells, which would account for the reduction of the IL-4, IL-13, and IL-17 cytokines. Thus, our result demonstrated that topical glucocorticoid treatment restored epidermal permeability barrier function and epidermal AMP expression by suppressing inflammatory responses. In our study, topical glucocorticoid treatment significantly restored epidermal AMP expression compared with emollient. However, topical pimecrolimus treatment did not significantly affect epidermal AMP expression in spite of remarkable effects on the permeability barrier, differentiation, and cytokine expression compared with emollient treatment. This result might be due to the sum of their lesser effects compared with glucocorticoid treatment. In our experiment, topical glucocorticoid or pimecrolimus treatment did not reduce serum IgE levels. Similar to our experiments, we have observed that many patients with severe AD have

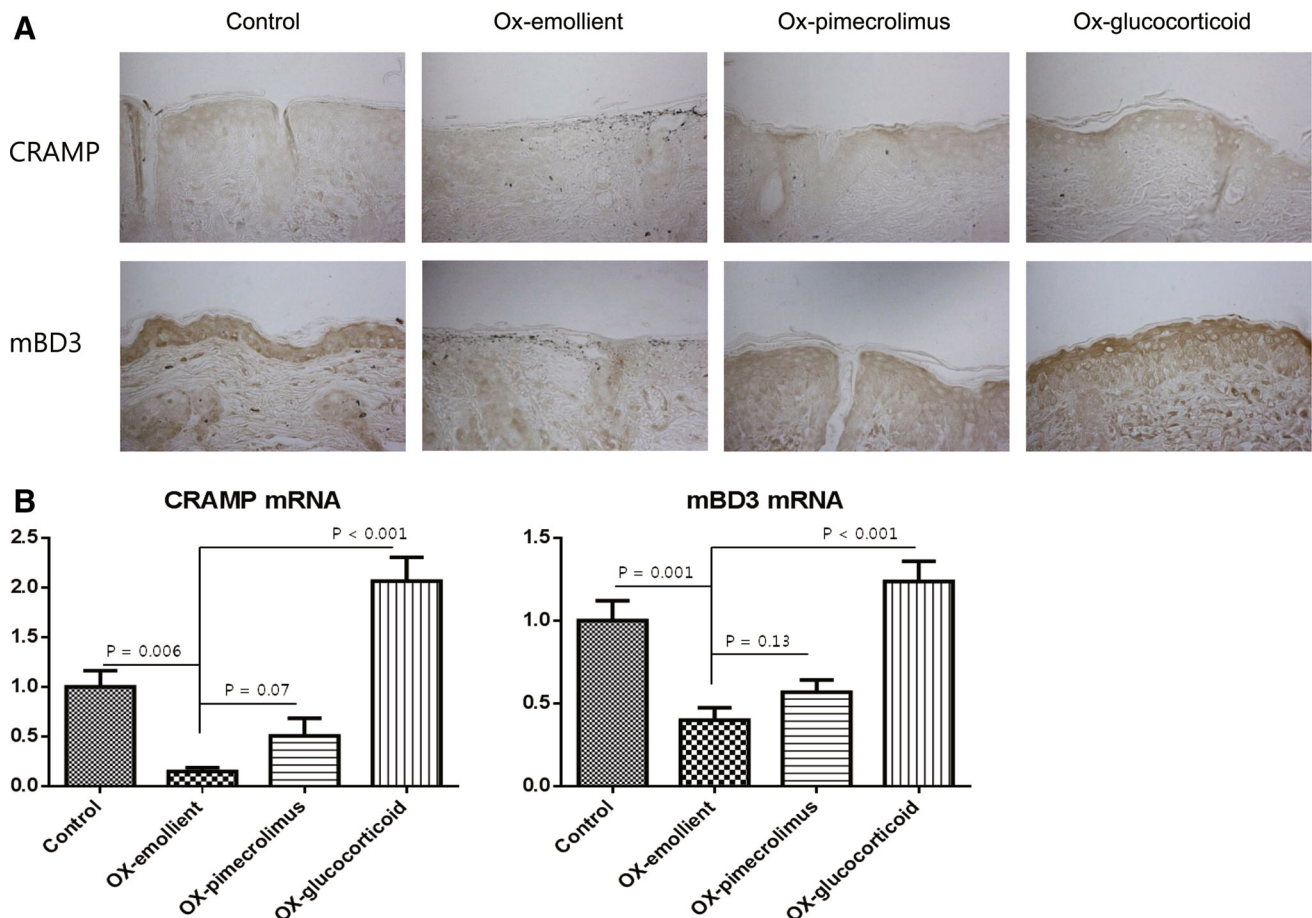


**Fig. 4** Topical glucocorticoid, but not pimecrolimus restored the epidermal expression of antimicrobial peptides in AD-like skin. Ox-AD skin showed a remarkable decrease in epidermal AMPs such as CRAMP and mBD3, as shown by immunohistochemical stains (a) and real-time RT-PCR (b). Topical glucocorticoid (Ox-

glucocorticoid) treatment restored the epidermal expressions of CRAMP and mBD3 compared with emollient (Ox-emollient). However, pimecrolimus (Ox-pimecrolimus) treatment did not increase their expression in real-time RT-PCR, although it restored mBD3 expression in the immunohistochemical stain (a, b)

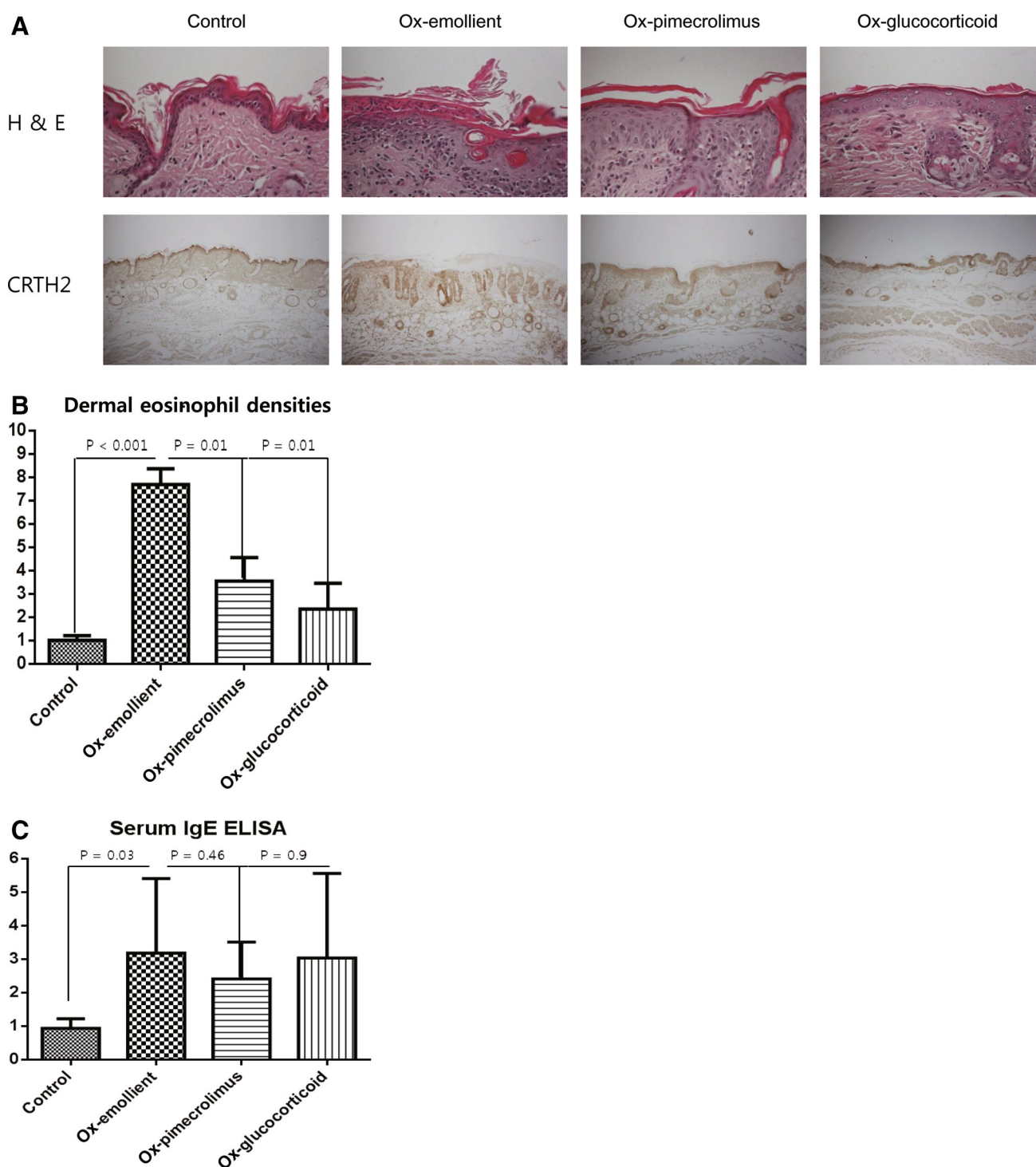


**Fig. 5** Topical glucocorticoid or pimecrolimus treatment suppressed epidermal hyperplasia and inflammatory cellular infiltration in AD-like skin, but not serum IgE level. AD-like skin showed marked dermal inflammatory cell infiltrations, as well as epidermal hyperplasia. Especially, the numbers of eosinophils in H&E stains and Th2 cells in CRTH2 immunohistochemical stains were increased (**a**, **b**).

recovered from active disease in spite of unchanged or even increased serum IgE. It may be that IgE is not the key mediator of AD and that IgE is produced after the

Topical glucocorticoid or pimecrolimus significantly suppressed inflammatory cell infiltration, including eosinophil and Th2-dominant cells, in the dermis as well as epidermal hyperplasia (**a**, **b**). Serum IgE level was remarkably elevated in Ox-induced AD mice compared with control. Topical glucocorticoid or pimecrolimus did not suppress serum IgE level (**c**)

production of IL-4 by activated Th2 cells. In addition, our result suggests that topical treatment for 10 days is not sufficient to affect serum IgE levels.

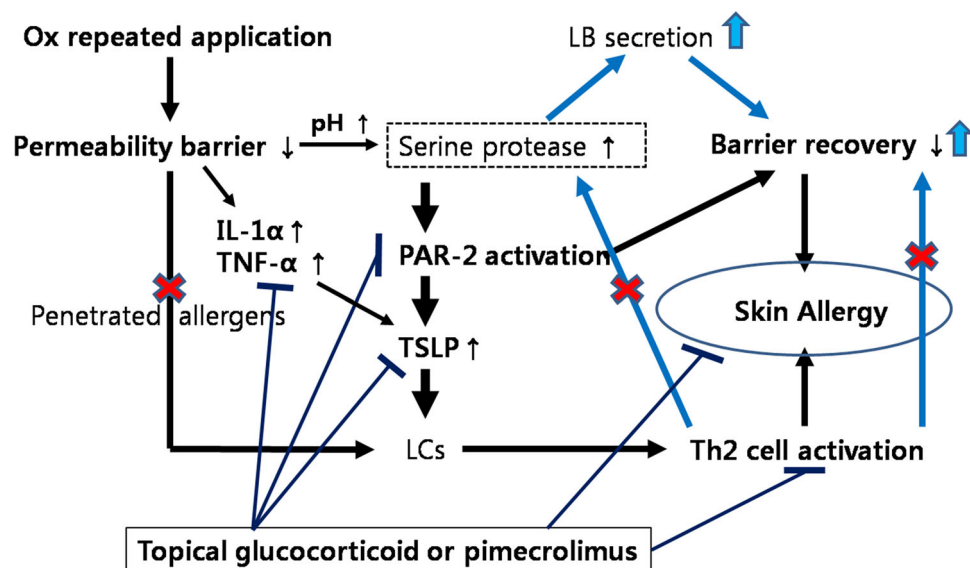


**Fig. 6** Mechanism of action of topical glucocorticoid and pimecrolimus on Ox-AD skin. In this study, repeated Ox treatment disrupted the skin barrier and then elevated skin pH. Proteases such as serine proteases and PAR-2 were activated in chronically disrupted skin, in which a high skin pH was maintained. Activated PAR-2 can provoke TSLP expression, triggering Langerhans cell activation. Th2

inflammation followed and AD-like skin lesions formed. Topical glucocorticoid or pimecrolimus suppresses the epidermal expression of Th2 inflammation-related cytokines such as IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP, which contribute to blocking AD-like skin lesions such as skin allergic reaction

From our results, we observed that repeated Ox treatment disrupts the skin barrier and increases the skin surface pH, which contributes to acute to chronic inflammatory skin lesions. Proteases and PAR-2 are activated in chronically disrupted skin, in which a high skin pH is maintained [7]. Activated PAR-2 can provoke TSLP expression, triggering LC activation. Eventually, Th2 inflammation is induced and AD-like skin lesion is formed. In addition, topical glucocorticoid or pimecrolimus could inhibit cutaneous allergic inflammation by decreasing the epidermal expression of IL-1 $\alpha$ , TNF- $\alpha$ , PAR-2, and TSLP significantly (Fig. 6). Although topical glucocorticoids suppress the activity of PAR2 activators, which are predominantly mediated by the production of PGE2 [6], the action of glucocorticoids on PAR-2 is still debatable.

Ox-AD murine skin pathology closely resembles the immunopathology and the ultrastructural abnormalities observed in Notch-deficient murine models of AD [9, 10, 14, 26, 33, 40]. It has been already known that striking similarities exist between Notch-deficient AD mice and AD patients in immunological aspects (up-regulation of TSLP, IL-4, and IgE), the differentiation-mediated aspects (down-regulation of terminal epidermal differentiation markers such as filaggrin, loricirin, and involucrin), and the barrier aspects (reduced formation of cornified lipid envelope and LB). These similarities contribute to a “Notch-deficiency hypothesis of AD” [32]. Notch-deficient skin induces a lethal systemic B-lymphoproliferative disorder by secreting TSLP, a sentinel for epidermal integrity [9,

10]. Notch1 is a repressor of AP-1, the crucial transcription factor activating the TSLP promoter. Moreover, both AP-1, a target of glucocorticoid treatment, and NFAT, a target of calcineurin inhibitor treatment, form transcription factor complexes (AP-1/NFAT) which synergistically activate the expression of the key Th2-cytokines such as TSLP, IL-4, and GM-CSF in AD pathogenesis. Furthermore, the therapeutic mode of action of glucocorticoids, calcineurin inhibitors, and UV in AD patients has been deduced to converge in the activation of impaired Notch signaling [31]. Because of the above similarities among our Ox-AD murine model, impaired Notch signaling AD model, and AD patients, we may infer Ox treatment in our study could down-regulate epidermal Notch receptors or impair Notch signals. Further study about Notch impairment in Ox-induced AD murine model is needed.

Due to the side effects of glucocorticoid steroids, many patients have ‘steroid phobia’ and thus use them reluctantly in spite of their dramatic efficacy toward AD flare. Our results showed that glucocorticoid application can improve the clinical manifestations of AD through improvement of the disrupted skin barrier, and that early suppression of AD flare by glucocorticoids can treat AD. In addition, our in vivo study showed that topical pimecrolimus treatment can suppress the expression of cytokines, including PAR-2 and TSLP, in AD epidermis. This suggests that therapy with topical calcineurin inhibitors can also assist in treating AD. From our results, we conclude that topical glucocorticoid or pimecrolimus application can treat acute AD

lesion through the inhibition of TSLP-related inflammation in AD patients.

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**Conflict of interest** There is no conflict of interest.

## References

- Aberg KM, Man MQ, Gallo RL, Ganz T, Crumrine D, Brown BE, Choi EH, Kim DK, Schroder JM, Feingold KR, Elias PM (2008) Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. *J Invest Dermatol* 128:917–925
- Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, Paquette N, Ziegler SF, Sarfati M, Delespesse G (2007) Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med* 204:253–258
- Bergmann RL, Wahn U, Bergmann KE (1997) The allergy march: from food to pollen. *Environ Toxicol Pharmacol* 4:79–83
- Briot A, Deraison C, Lacroix M, Bonnart C, Robin A, Besson C, Dubus P, Hovnanian A (2009) Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. *J Exp Med* 206:1135–1147
- Briot A, Lacroix M, Robin A, Steinhoff M, Deraison C, Hovnanian A (2010) Par2 inactivation inhibits early production of TSLP, but not cutaneous inflammation, in Netherton syndrome adult mouse model. *J Invest Dermatol* 130:2736–2742
- Cocks TM, Fong B, Chow JM, Anderson GP, Frauman AG, Goldie RG, Henry PJ, Carr MJ, Hamilton J, Moffatt JD (1999) A protective role for protease activated receptors in the airways. *Nature* 398:156–160
- Cork MJ, Danby SG, Vasilopoulos Y, Hadgraft J, Lane ME, Moustafa M, Guy RH, Macgowan AL, Tazi-Ahnini R, Ward SJ (2009) Epidermal barrier dysfunction in atopic dermatitis. *J Invest Dermatol* 129:1892–1908
- Demehri S, Morimoto M, Holtzman MJ, Kopan R (2009) Skin-derived TSLP triggers progression from epidermal-barrier defects to asthma. *PLoS Biol* 7:e1000067
- Demehri S, Liu Z, Lee J et al (2008) Notch-deficient skin induces a lethal systemic B-lymphoproliferative disorder by secreting TSLP, a sentinel for epidermal integrity. *PLoS Biol* 6:e123
- Dumortier A, Durham AD (2010) Di Piazza M (2010) Atopic dermatitis-like disease and associated lethal myeloproliferative disorder arise from loss of Notch signaling in the murine skin. *PLoS One* 5:e9258
- Ebner S, Nguyen VA, Forstner M, Wang YH, Wolfram D, Liu YJ, Romani N (2007) Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells. *J Allergy Clin Immunol* 119:982–990
- El-Batawy MM, Bosseila MA, Mashaly HM, Hafez VS (2009) Topical calcineurin inhibitors in atopic dermatitis: a systematic review and meta-analysis. *J Dermatol Sci* 54:76–87
- Fehervari Z (2012) TSLP underlies the atopic march. *Nat Immunol* 13:533
- Franzke CW, Cobzaru C, Triantafyllopoulou A et al (2012) Epidermal ADAM17 maintains the skin barrier by regulating EGFR ligand-dependent terminal keratinocyte differentiation. *J Exp Med* 209:1105–1119
- Han H, Xu W, Headley MB, Jessup HK, Lee KS, Omori M, Comeau MR, Marshak-Rothstein A, Ziegler SF (2012) Thymic stromal lymphopoietin (TSLP)-mediated dermal inflammation aggravates experimental asthma. *Mucosal Immunol* 5:342–351
- Hatano Y, Elias PM, Crumrine D, Feingold KR, Katagiri K, Fujiwara S (2011) Efficacy of combined peroxisome proliferator-activated receptor- $\alpha$  ligand and glucocorticoid therapy in a murine model of atopic dermatitis. *J Invest Dermatol* 131:1845–1852
- He R, Oyoshi MK, Garibyan L, Kumar L, Ziegler SF, Geha RS (2008) TSLP acts on infiltrating effector T cells to drive allergic skin inflammation. *Proc Natl Acad Sci USA* 105:11875–11880
- Jenerowicz D, Czarnecka-Operacz M, Silny W (2005) Corticosteroid phobia in patients with atopic dermatitis. *Wiad Lek* 58:607–615
- Jung YJ, Jung M, Kim M, Hong SP, Choi EH (2011) IL-1 $\alpha$  stimulation restores epidermal permeability and antimicrobial barriers compromised by topical tacrolimus. *J Invest Dermatol* 131:698–705
- Kouzaki H, O'Grady SM, Lawrence CB, Kita H (2009) Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol* 183:1427–1434
- Lee EB, Kim KW, Hong JY, Jee HM, Sohn MH, Kim KE (2010) Increased serum thymic stromal lymphopoietin in children with atopic dermatitis. *Pediatr Allergy Immunol* 21:e457–e460
- Lee HJ, Jung M, Kim JH, Yoon NY, Choi EH (2012) The effect of adipose-derived stem cell-cultured media on oxazolone treated atopic dermatitis-like murine model. *Ann Dermatol* 24:181–188
- Le TA, Takai T, Kinoshita H, Suto H, Ikeda S, Okumura K, Ogawa H (2009) Inhibition of double-stranded RNA-induced TSLP in human keratinocytes by glucocorticoids. *Allergy* 64:1231–1232
- Le TA, Takai T, Vu AT, Kinoshita H, Ikeda S, Ogawa H, Okumura K (2010) Glucocorticoids inhibit double-stranded RNA-induced thymic stromal lymphopoietin release from keratinocytes in an atopic cytokine milieu more effectively than tacrolimus. *Int Arch Allergy Immunol* 153:27–34
- Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P (2006) Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. *Proc Natl Acad Sci USA* 103:11736–11741
- Lin HY, Kao CH, Lin KM, Kaartinen V, Yang LT (2011) Notch signaling regulates late-stage epidermal differentiation and maintains postnatal hair cycle homeostasis. *PLoS One* 6:e15842
- Liu H, Pan JH, Lou WL, He JG (2010) Effects of inhaled budesonide on the bronchial-pulmonary pathology and expression of thymic stromal lymphopoietin in lung tissues in asthmatic rats. *Zhongguo Dang Dai Er Ke Za Zhi* 12:816–819
- Liu YJ (2006) Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med* 203:269–273
- Man MQ, Hatano Y, Lee SH, Man M, Chang S, Feingold KR, Leung DY, Holleran W, Uchida Y, Elias PM (2008) Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges. *J Invest Dermatol* 128:79–86
- Meingassner JG, Fahrngruber H, Bavandi A (2003) Pimecrolimus inhibits the elicitation phase but does not suppress the sensitization phase in murine contact hypersensitivity, in contrast to tacrolimus and cyclosporine A. *J Invest Dermatol* 121:77–80
- Melnik BC (2014) Does therapeutic intervention in atopic dermatitis normalize epidermal Notch deficiency? *Exp Dermatol* 23:696–700
- Melnik BC (2014) The potential role of impaired Notch signalling in atopic dermatitis. *Acta Derm Venereol May* [Epub ahead of print]

33. Murthy A, Shao YW, Narala S et al (2012) Notch activation by the metalloproteinase ADAM17 regulates myeloproliferation and atopic barrier immunity by suppressing epithelial cytokine synthesis. *Immunity* 36:105–119
34. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL, Leung DY (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 347:1151–1160
35. Ozawa T, Koyama K, Ando T, Ohnuma Y, Hatsushika K, Ohba T, Sugiyama H, Hamada Y, Ogawa H, Okumura K, Nakao A (2007) Thymic stromal lymphopoietin secretion of synovial fibroblasts is positively and negatively regulated by Toll-like receptors/nuclear factor-kappaB pathway and interferon-gamma/dexamethasone. *Mod Rheumatol* 17:459–463
36. Palatsi R, Hagg P (2011) The immune response against microbial infections in the skin—weak in atopic dermatitis and strong in psoriasis. *Duodecim* 127:127–134
37. Patrizi A, Pileri A, Bellini F, Raone B, Neri I, Ricci G (2011) Atopic dermatitis and the atopic march: what is new? *J Allergy (Cairo)* 2011:279425
38. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, Gorman D, Zurawski S, Abrams J, Menon S, McClanahan T, de Waal-Malefyt RdR, Bazan F, Kastelein RA, Liu YJ (2002) Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 3:673–680
39. Spergel JM, Paller AS (2003) Atopic dermatitis and the atopic march. *J Allergy Clin Immunol* 112:S118–S127
40. Weber S, Niessen MT, Prox J et al (2011) The disintegrin/metalloproteinase Adam10 is essential for epidermal integrity and notch-mediated signaling. *Development* 138:495–505
41. Wood LC, Jackson SM, Elias PM, Grunfeld C, Feingold KR (1992) Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest* 90:482–487
42. Zhang Y, Zhou X, Zhou B (2012) DC-derived TSLP promotes Th2 polarization in LPS-primed allergic airway inflammation. *Eur J Immunol* 42:1735–1743
43. Ziegler SF, Artis D (2010) Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol* 11:289–293