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ARTICLE

Knockdown of sodium channel Na_x reduces dermatitis symptoms in rabbit skin

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Abstract

The skin plays a critical role in maintenance of water homeostasis. Dysfunction of the skin barrier causes not only delayed wound healing and hypertrophic scarring, but it also contributes to the development of various skin diseases. Dermatitis is a chronic inflammatory skin disorder that has several different subtypes. Skin of contact dermatitis and atopic dermatitis (AD) show epidermal barrier dysfunction. Na_x is a sodium channel that regulates inflammatory gene expression in response to perturbation of barrier function of the skin. We found that in vivo knockdown of Na_x using RNAi reduced hyperkeratosis and keratinocyte hyperproliferation in rabbit ear dermatitic skin. Increased infiltration of inflammatory cells (mast cells, eosinophils, T cells, and macrophages), a characteristic of dermatitis, was reduced by Na_x knockdown. Upregulation of PAR-2 and thymic stromal lymphopoietin (TSLP), which induce Th2-mediated allergic responses, was inhibited by Na_x knockdown. In addition, expression of COX-2, IL-1β, IL-8, and S100A9, which are downstream genes of Na_x and are involved in dermatitis pathogenesis, were also decreased by Na_x knockdown. Our data show that knockdown of Na_x relieved dermatitis symptoms in vivo and indicate that Na_x is a novel therapeutic target for dermatitis, which currently has limited therapeutic options.

Introduction

Skin plays a critical role in the maintenance of water homeostasis. Disruption of the skin barrier contributes to delayed wound healing, hypertrophic scar formation, and development of various skin diseases including inflammatory dermatoses [1, 2]. Perturbation of the skin barrier results in compensatory changes in the injured skin, including upregulation of inflammatory cytokines. Use of moisturizers and emollients lessens symptoms of many skin disorders and reduces inflammation by improving barrier function [3, 4]. We previously demonstrated that hydration status alters sodium homeostasis and induces sodium flux in keratinocytes through an epithelial sodium channel, ENaC [5]. We also identified a sodium sensor protein expressed in the epidermis, Na_x, which recognizes increases in extracellular sodium concentration caused by disruption of the barrier function of skin [6].

Na_x (SCN7A) is an atypical voltage-gated sodium channel (VGSC) and functions as a sodium concentration-sensing channel, rather than a voltage-dependent channel, in the central nervous system (CNS) [7–9]. Na_x has also been demonstrated to play a critical role in the maintenance of sodium homeostasis in mammals. We demonstrated that Na_x is a key molecule in the epidermis that also acts as a sodium sensor and regulates inflammation [6]. We observed that the expression of many inflammatory genes, such as interleukin-1β (IL-1β), IL-8,

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and cyclooxygenase-2 (COX-2), is upregulated in the epidermis when the barrier function of the skin is disrupted [5, 10]. Microarray analyses of human keratinocytes utilizing upon knockdown of Na_x demonstrated that Na_x regulates many inflammatory genes including IL-1β, IL-8, and COX-2 [5].

Dermatitis is a chronic and relapsing inflammatory skin disease with different subtypes including contact dermatitis and atopic dermatitis (AD). Though the cause of AD is multifactorial, defective skin barrier function is a major contributor to the pathogenesis of AD [11–13]. Impaired epidermal barrier permits allergens and irritants to penetrate the skin, causing pathological changes such as epidermal hyperproliferation, inflammatory cell infiltration into the dermis, and proinflammatory gene overexpression. Interestingly, restoration of epidermal barrier function using emollients improves symptoms of AD, suggesting that loss of skin barrier function is at least partly causal relative to the pathology of AD [11, 14].

Given the complexity of AD, it is important to use animal models that resemble human skin for the elucidation of pathogenesis of AD and for the development of new therapies. We have accrued extensive experience in establishing skin disease models in the rabbit ear, including those simulating wound repair and development of hypertrophic scar [15–17]. The rabbit ear model shares similar characteristics with human skin. For example, the thickness of stratum corneum and the density of hair follicles in the skin of the inner rabbit ear are more similar to that of human skin than that of with rodent skin [18–20]. Furthermore, certain genes that are downstream of Na_x and involved in inflammation in humans, such as IL-8 and S100A12, are expressed in rabbits but not in mice. Recently, we have established AD-like models in rabbit ears using house dust mite (HDM, *Dermatophagoides farina*) extract and oxazolone (OXZ) [21]. However, since we have not characterized elevated serum IgE levels and increased Th2 cytokine expression in the skin of published AD-like models, we have described our models as dermatitis models in this manuscript.

In this study, we addressed whether Na_x inhibition could reverse symptoms in rabbit dermatitis models induced with HDM and OXZ. Knockdown of Na_x by RNAi resulted in alleviation of the gross/histological dermatitis symptoms, decreased infiltration of inflammatory cells into the dermis, and attenuated increase in expression of proinflammatory genes. In addition, expression of PAR-2 and thymic stromal lymphopoietin (TSLP), two important factors involved in the Th2-mediated allergic response and AD progression, was reduced by Na_x knockdown. Our results suggest that Na_x is a novel therapeutic target for dermatitis, a condition that currently lacks effective treatments.

Materials and methods

Alginate–chitosan nanoparticle (ALG/CS-NP)-conjugated RNAi preparation

Dicer-Substrate RNAi (DsiRNA) was synthesized by Integrated DNA Technologies (IDT, Inc, Coralville, Iowa) (Table S1). Na_x-RNAi (SCN7A-1, -2, 3) or sham-RNAi was diluted in a solution of 18 mM $CaCl_2$. RNAi-containing solution was added to a solution of 0.075% sodium alginate (pH 4.9), while stirring continuously, to make a pre-gel. A .05% chitosan solution in 0.05% acetic acid (pH 4.6) was added dropwise into the pre-gel solution with agitation, and the solution was allowed to stir continuously for 45 min at room temperature. Precipitation of nanoparticle-conjugated RNAi was accomplished by centrifugation at 20,000 x *g* at 4 °C for 20 min. The ALG/CS-NP-conjugated RNAi was washed with PBS before resuspension in phosphate buffered saline (PBS), and stored at 4 °C until use.

In vivo inhibition of Na_x in dermatitic rabbit ear skin

Female New Zealand White rabbits that weighed between 3 and 5 kg were purchased from Covance (Princeton, NJ). Treatment of rabbits was carried out following the guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Northwestern University Animal Care and Use Committee. Developing dermatitic symptoms in rabbit ear were observed as described in our previous study [21].

For the HDM-induced dermatitis model, the rabbit ear skin barrier was disrupted by application of acetone for 3 h. Treatment with HDM extract solution (Standardized Mite Dermatophagoides Farina, Greer Laboratories, Lenoir, NC) proceeded as described in Fig. 1a. After establishment of the dermatitis model, ALG/CS-NP-conjugated Na_x-RNAi or sham-RNAi was applied to the skin on day 23. The RNAi was mixed with Total Moisture Lotion (Pleasanton, CA) in a ratio of 2 µg DNA per 20 µL lotion. To maintain the inflamed state of the skin, HDM extract was applied initially to the skin for 4 h, and then the RNAi-containing lotion was rubbed gently and symmetrically onto the dermatitis skin areas every other day for three cycles. One ear was treated with Na_x-RNAi while the contralateral ear was treated with control sham-RNAi. The skin on the ventral side of each rabbit ear was harvested at day 29 for histological and molecular analyses (Fig. 1a).

For the OXZ-induced dermatitis model, the stratum corneum of rabbit ear skin was disrupted by tape stripping in order to enhance penetration of OXZ. OXZ (Sigma-Aldrich, St. Louis, MO) was applied as described in Fig. 1e. Once the dermatitic model had been established on day 14,

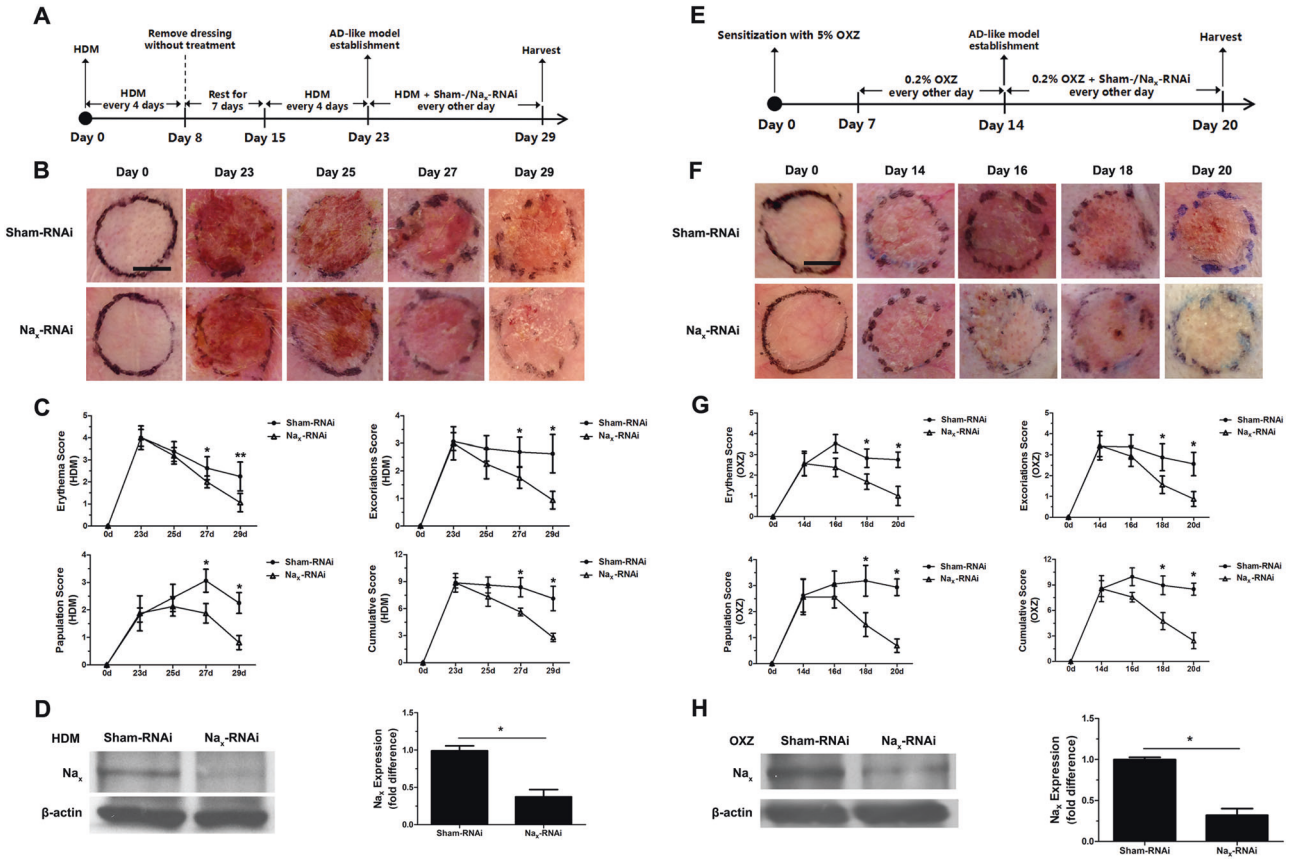

Fig. 1 Knockdown of Na_x expression relieved dermatitic symptoms induced by HDM extract and OXZ in rabbit skin. a, **e** Experimental design for the dermatitis model establishment and Na_x-RNAi treatment regimen. **b**, **f** Gross appearance of dermatitic skin lesions on the rabbit ear after sham/Na_x-RNAi application at different time points. **c**, **g** Three Item Severity (TIS) score. Erythema, excoriations, and papulation of rabbit ear skin lesions were scored during the course of disease progression on a scale from 0 to 4. The cumulative score (erythema plus excoriations plus papulation) was recorded. Scale bar = 0.5 cm. Data are expressed as the mean ± SEM. $n = 6$. $^*p < 0.05$, $^{**}p < 0.01$. Statistical analysis was performed by paired Student's *t*-test (two-tailed) at each time point. **d**, **h** Expression of Na_x in the epidermis of sham-RNAi-treated and Na_x-RNAi-treated skin was analyzed by Western blot analysis and quantified by densitometry using the NIH ImageJ program relative to the loading control β-actin. $n = 4$. $^*p < 0.05$ by paired Student's *t*-test (two-tailed).

the ALG/CS-NP-conjugated Na_x-RNAi or sham-RNAi was mixed with Total Moisture Lotion and applied to the skin. Four hours after application of OXZ, Na_x-RNAi was applied to one ear and sham-RNAi was applied to the contralateral ear. The skin on the ventral side of each rabbit ear was harvested on day 20 (Fig. 1e).

Penetration analysis of RNAi was performed using fluorescent dye–labeled TYE563 control DsiRNA (IDT, Inc.) that was conjugated to ALG/CS-NP (TYE563-RNAi). TYE563-RNAi was applied to the HDM and OXZ-induced dermatitic skin three times when Na_x-RNAi or sham-RNAi was applied prior to euthanization.

Scoring severity of skin inflammation

The severity of skin inflammation and dermatitis progression were scored using an objective Three Item Severity (TIS) scoring system consisting of gradation of erythema, edema/papulation, and excoriations, (typical clinical symptoms of dermatitis). TIS scores were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The cumulative score (the sum of scores of the three items) served as a measure of the severity of inflammation (scale 0–12).

Histology, epidermal thickness quantification, and monocyte counting

Skin samples harvested from rabbit ears or from human patients were fixed in 10% neutral-buffered formalin overnight and were serially dehydrated, embedded in paraffin, and sectioned into 5 μm sections. Hematoxylin and eosin (H&E) staining was utilized for histological observation and to quantify epidermal thickness and the number of monocytes. Mast cells and eosinophils were stained using the

Eosinophil—Mast Cell staining kit (ScyTek Laboratories Inc., Logan, UT). For mast cell detection, deparaffinized sections were stained with for 30 min with Astra Blue solution and underwent treatment with 0.5% HCl solution to reduce background. For detection of eosinophils, Vital New Red solution was used to stain slides for 30 min. After washing with water, stained sections were serially dehydrated and then mounted in Permount Mounting Medium (Fisher Scientific, Hampton, NH). Images were captured using a Nikon Eclipse 50i light microscope and imported for analysis with NIS Elements BR software (Nikon, Melville, NY).

Immunostaining

For immunohistochemical (IHC) staining, deparaffinized sections underwent antigen retrieval in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) or in a trypsin solution (final 0.05% trypsin in 0.1% calcium chloride). Slides were incubated in a 1% hydrogen peroxide to quench endogenous peroxidase activity, and tissue sections were incubated at 4 °C overnight in a solution containing primary antibody. After washing with PBS thoroughly, sections were incubated in a solution containing biotin-conjugated secondary antibodies (Vector laboratories, Burlingame, CA) and then incubated with avidin–biotin complex (Elite ABC kit; Vector Laboratories, Burlingame, CA). Visualization of signals utilized 3, 3′-diaminobenzidine (DAB) and the nuclei were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO). Primary antibodies to detect rabbit proteins include mouse anti-PCNA (1:1000 dilution, BD Biosciences, San Jose, CA), mouse anti-cytokeratin 10 (1:1000 dilution, DAKO, Santa Clara, CA), mouse anti-TSLP (1:200 dilution, Novus Biologicals, Littleton, CO) and goat anti-S100A9 (1:1000 dilution, Abcam, Cambridge, MA).

For immunofluorescent staining, deparaffinized sections underwent antigen retrieval as described above and were incubated in a solution of primary antibody at 4 °C overnight. After washing, sections were incubated further with fluorophore-labeled secondary antibodies (Invitrogen, Carlsbad, CA). Cell nuclei were stained by incubating in a 1 μg/ml solution of 4′,6-diamidino-2-phenylindole (DAPI). Slides were visualized and images were taken with an EVOS FL Cell Imaging System (ThermoFisher Scientific, Grand Island, NY) and merged using Image-Pro Plus v. 6.0 software (Media Cybernetics, Inc, Rockville, MD). Primary antibodies used for detection of rabbit proteins include mouse anti-CD3 (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX), mouse anti-macrophage (1:500 dilution, Abcam), mouse anti-PCNA (1:100 dilution, BD Biosciences), and mouse anti-TSLP (1:100 dilution, Novus Biologicals).

RT-qPCR and western blot analysis

Epidermis of skin samples was separated from dermis by treatment of the sample with 0.5 M ammonium thiocyanate for 20 min at room temperature.

For RT-qPCR analysis, the tissue was placed in Trizol Reagent (Sigma-Aldrich) and homogenized using Zirconia bead-beating (2.0-mm diameter; Biospec Products Inc., Bartlesville, OK) for 60 s at 5000 rpm using a MagNA Lyser (Roche Molecular Systems, Inc, Indianapolis, IN). Total RNA was isolated following manufacturer's instructions, and genomic DNA was removed using the Turbo DNA-free kit (Life Technologies) according to manufacturer's instructions. Two micrograms of total RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Life Technologies). Quantitative PCR analysis was performed on an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green I dye. Expression of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control, and the $2^{-\triangle\triangle Ct}$ method was used to calculate fold changes in gene expression between analyzed groups. The sequences of primers used for quantitative PCR are listed in Table S2.

For western blot analysis, total protein was extracted from the epidermis was extracted in radioimmunoprecipitation assay (RIPA) buffer and estimated concentrations of protein in the lysates were determined using a BCA kit (Bio-Rad, Hercules, CA). SDS polyacrylamide gels were used to separate equal quantities of total protein, which was then transferred to nitrocellulose membranes and blocked at room temperature for 1 h in nonfat milk. Membranes were incubated overnight at 4 °C in solutions containing primary antibodies including rabbit anti-Na_x (1:1000 dilution, Abcam), mouse anti-TSLP (1:5000 dilution, Novus Biologicals), mouse anti-PAR2 (1:1000 dilution, Santa Cruz Biotechnology) and mouse anti-β-actin (1:5000 dilution, Sigma-Aldrich). Membranes were incubated in solutions containing horseradish peroxidase–conjugated secondary antibodies (Vector Laboratories), and signals were detected using an Enhanced Chemiluminescence detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ). β-actin was used as a loading control for normalization.

Quantification of images and statistical analysis

NIH ImageJ (NIH, Bethesda, MD; https://imagej.nih.gov/ij) was used in order to quantify Western blot and immunofluorescence signals. The signals were translated into total intensities, which were quantified to show differences in protein expression. All data presented in this study are expressed as the mean plus or minus standard error of the

mean (mean ± SEM). Statistical significances of differences between experimental groups were analyzed using two-tailed Student's *t*-tests. Statistical analyses were performed using SPSS 18.0 software (SPSS, Chicago, IL). Differences corresponding to a value of $\alpha < 0.05$ were defined as statistically significant for this report.

Results

Knockdown of Na_x expression relieved clinical symptoms of dermatitis in rabbit dermatitis models

Na_x is a key molecule that regulates inflammation in the epidermis by sensing change in the barrier function of skin characterized by increased trans-epidermal water loss and increased concentration of extracellular sodium [6, 22]. We addressed whether knockdown of Na_x expression alleviates the pathogenesis of dermatitis, of which a major contributor is impaired skin barrier function. Since HDM is a potent aeroallergen that induces AD-like skin lesions in human [23], we used HDM extract to induce dermatitis symptoms in rabbit ear skin. Repeated HDM challenge induced a rapid-onset and chronically-sustained dermatitic skin lesion in the rabbit ear. The symptoms of erythema, scaling, and papules became increasingly apparent, and dermatitis was established at day 23 (Fig. 1a, b). In order to exclude variation among different animals, since rabbits are not syngeneic, each rabbit served as its own control; one ear received sham-RNAi treatment and the other ear received Na_x-RNAi treatment. Both sham-RNAi and Na_x-RNAi were conjugated to alginate-chitosan nanoparticle (ALG/CS-NP). Two days after the first RNAi treatment, erythema, and papules were not relieved by Na_x-RNAi compared with the sham-RNAi control group at day 25, 2 days post first treatment (Fig. 1b). While scaling continually increased in the control group, it was alleviated to some extent by Na_x-RNAi. Na_x-RNAi-treated skin revealed markedly less erythema, scaling, and papules compared with the control at day 27, 4 days post first treatment. While erythema, scaling, and papules were still visible in the control group, these symptoms were drastically reduced by day 29, 6 days post first treatment. The Three Item Severity (TIS) score system was used to evaluate the progression and severity of skin inflammation (Fig. 1c). The TIS score showed that a single Na_x-RNAi application was not sufficient to reduce erythema and papule formation, though it did alleviate scaling. Two or three applications of Na_x-RNAi significantly reduced all measured symptoms of dermatitis including erythema, scaling, and papules. Cumulative score, which represents the progression of dermatitis, demonstrated that HDM-induced dermatitis symptoms persisted through the end of the study in sham-RNAi treated skin, while Na_x-RNAi application relieved the dermatitis symptoms efficiently by day 29, 6 days post first treatment. To ensure the efficiency of Na_x knockdown in vivo, we targeted three different regions of the Na_x gene (Table S1). Western blot analysis demonstrated a significant 63% reduction of Na_x protein in the epidermis that was treated Na_x-RNAi compared with sham-RNAi (Fig. 1d).

Since the etiopathogenesis of dermatitis is multifactorial, we addressed the role of Na_x in another dermatitis animal model that was induced by a hapten, OXZ. OXZ has been used as a sensitizer that elicits allergic contact dermatitis in animals [24]. The typical dermatitis symptoms, such as erythematous papules, skin thickening, and scaling, were observed at day 14 post OXZ treatment (Fig. 1e, f). Sham-RNAi and Na_x-RNAi, which were conjugated to ALG/CS-NP, were used to treat one ear and the contralateral ear, respectively, of each rabbit at day 14. By day 18, the most pronounced erythematous papules and skin scaling were observed in sham-RNAi-treated skin. Erythema and excoriations peaked at day 16, and the erythematous papules remained through day 20 and until the skin was harvested in sham-RNAi treated skin (Fig. 1g). In contrast, in the Na_x-RNAi-treated lesions, the erythematous area was limited and relieved by 2 days post first treatment, while the scaling and papules remained present. The scaling and papules were alleviated at day 18, 4 days post first treatment, by Na_x-RNAi. The typical symptoms of dermatitis, including erythema, scaling, and papules resolved by day 20, 6 days post first treatment in Na_x-RNAi-treated skin (Fig. 1g). Western blot analysis confirmed that the expression of Na_x was reduced significantly by 69% in the Na_x-RNAi-treated epidermis compared with sham-RNAi treated epidermis (Fig. 1h).

We addressed the penetration of RNAi into the epidermis using a fluorescence-labeled control RNAi. Fluorescent signals were found mostly in the epidermis of treated rabbit skin (Fig. S1). This result is in line with the notion that the skin barrier function, which prohibits penetration of foreign materials, is impaired in AD.

Knockdown of Na_x expression decreased hyperkeratosis and inflammatory infiltration in dermatitic skin

Epidermal hyperplasia (hyperkeratosis) is a characteristic feature of AD in humans. Compared with that of sham-RNAi control, hyperkeratosis induced by HDM and OXZ was reduced by 57% and 39%, respectively, in Na_x-RNAi-treated rabbit ear dermatitic skin (Fig. 2a, b). Prominent dermal inflammatory infiltrate, another typical pathological feature of AD, was decreased by 51 and 68% in HDM and OXZ-induced dermatitic rabbit ear skin, respectively, by Na_x-RNAi treatments (Fig. 2a, b). Close examination under

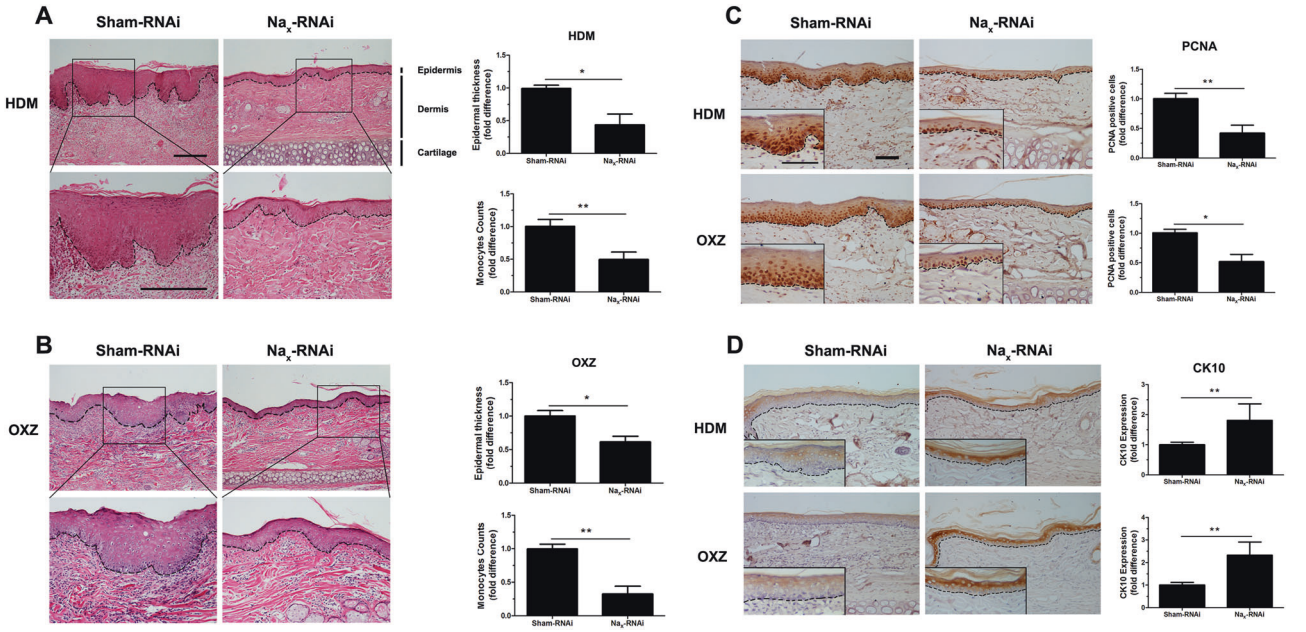

Fig. 2 Epidermal hyperplasia, dermal inflammatory infiltrate, and aberrant differentiation of the epidermis were reduced in dermatitic skin by Na_x knockdown. HDM (**a**, **c**, **d**) or OXZ (**b**, **c**, **d**)-induced rabbit ear dermatitic skins were treated with Na_x-RNAi or sham-RNAi and harvested as described in Fig. 1a and e. **a**, **b** H&E staining. Epidermal thickness and dermal monocytes were quantified. **c** Immunostaining of PCNA, a proliferation marker. **d** Immunostaining of CK10, a differentiation marker. The thickness of the epidermis (**a**, **b**), number of positive cells (**a**, **b**, **c**), and expression level of CK10 (quantified using the NIH ImageJ program), **d** in the Na_x-RNAi treated dermatitic skin was compared with sham-RNAi-treated AD-like skin that was defined as 1. The dermal-epidermal junction is indicated by dashed lines. Scale bar = 50 μm. Data are shown as the mean ± SEM. $n = 6$, $*p < 0.05$, $**p < 0.01$ by paired Student's *t*-test (two-tailed).

high-power magnification demonstrated that the distinct changes in dermal collagen architecture returned to normal in dermatitic rabbit ear skin after Na_x-RNAi treatment.

Knockdown of Na_x expression reduced keratinocyte hyperproliferation and aberrant differentiation of the epidermis in dermatitic skin

Increased epidermal thickening, which is characteristic of AD in humans, is caused by hyperproliferation of keratinocytes. Increased numbers of keratinocytes positive for PCNA, a proliferation marker, were found in the basal cell layer of the epidermis in dermatitic skin induced by HDM and OXZ. The number of PCNA-positive keratinocytes was reduced by 57 and 48% with Na_x-RNAi treatment compared with sham-RNAi treatment, respectively (Fig. 2c). Skin excoriation is an important sign of parakeratosis, which is caused by aberrant epidermal differentiation, another characteristic of AD lesions [25]. Cytokeratin 10 (CK10) is expressed in the suprabasal layer of the epidermis and is involved in the barrier function of skin [26, 27]. Reduced expression of CK10 was found in AD, in which epidermal barrier function is impaired [28]. We also found that the expression of CK10 was reduced and that its expression was distributed throughout the epidermis in the sham-RNAi treated dermatitic skins (Fig. 2d). Na_x-RNAi treatment increased the expression of CK10 in HDM and OXZ-induced dermatitic skins by 1.8- and 2.3-fold, respectively. In addition, limited CK10 expression in the suprabasal layer of the epidermis was recovered in Na_x-RNAi-treated dermatitic skin.

Knockdown of Na_x expression decreased inflammatory cell infiltration in dermatitic skin

Infiltration of mast cells and eosinophils is increased in AD patient skin lesions and is associated with AD severity [29]. We found that infiltration of mast cells was reduced by 35 and 57% in HDM and OXZ-induced dermatitic rabbit skins treated with Na_x-RNAi, respectively, compared with skins treated with sham-RNAi (Fig. 3a). The number of infiltrated eosinophils was also reduced by 45 and 76% in HDM and OXZ-induced dermatitic rabbit skins by Na_x-RNAi treatment, respectively (Fig. 3b). The presence of T cells is a key feature of AD skin lesions, which is not observed in normal skin. Though a small number of cells were detected overall, the infiltration of T cells was reduced by Na_x-RNAi treatment compared with sham-RNAi control in dermatitic skin (Fig. 3c). Furthermore, the infiltration of macrophages, a critical component of the pathogenesis of AD, was reduced in dermatitic skin by Na_x-RNAi treatment (Fig. 3d). The number of macrophages was reduced by 54 and 81% by Na_x-RNAi treatment in HDM and OXZ-induced dermatitic skin, respectively, compared with sham-RNAi control treatment.

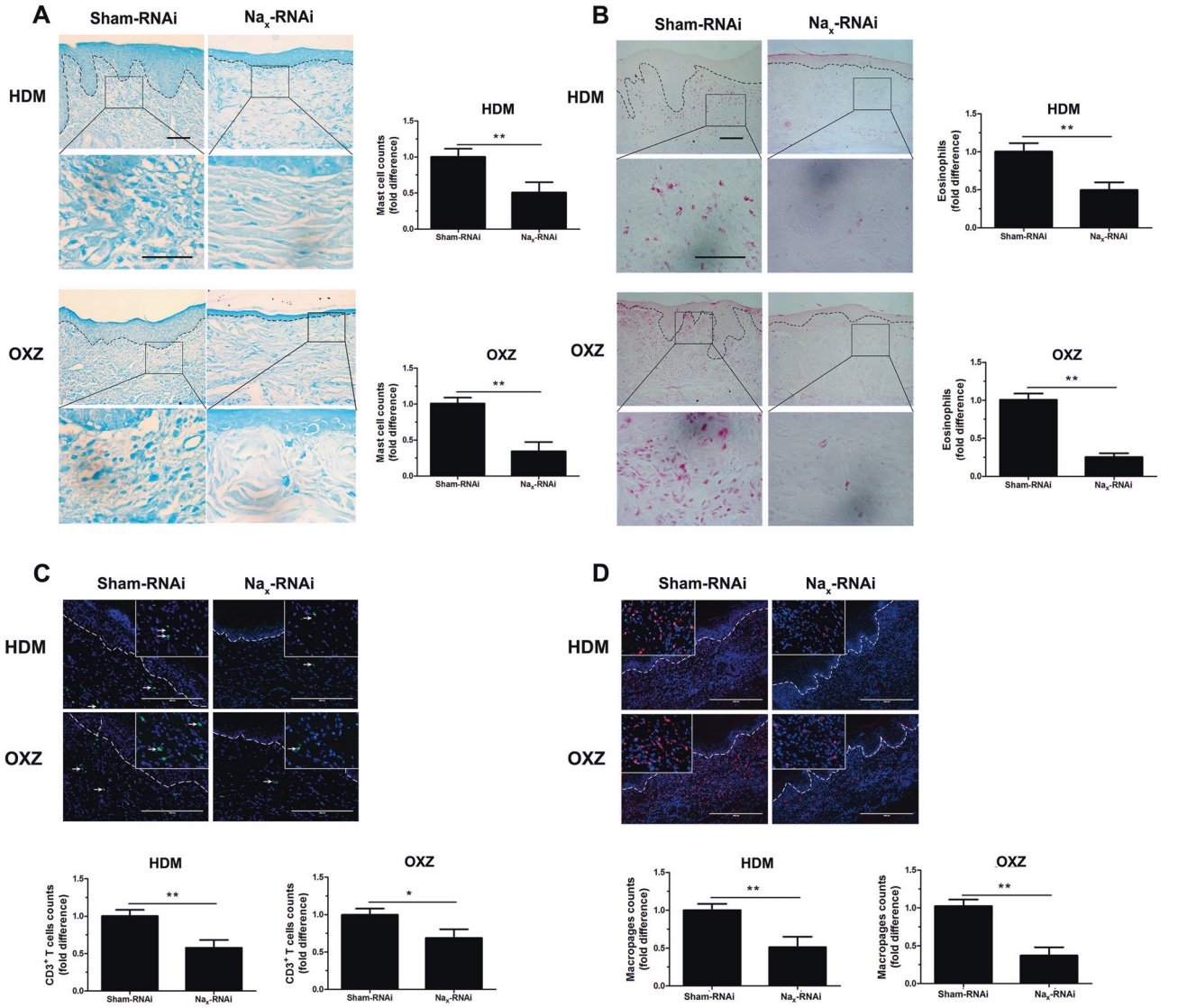

Fig. 3 Infiltration of inflammatory cells was decreased in dermatitic skin by Na_x knockdown. HDM or OXZ-induced rabbit ear dermatitic skins were treated with Na_x-RNAi or sham-RNAi and harvested as described Fig. 1a, e. **a** Mast cell staining with Astra Blue. **b** Eosinophil staining with Vital New Red. **c** $CD3^+$ T-cell immunostaining. **d** Macrophage immunostaining. **a**–**d** Quantification. The number of positive-signal cells in Na_x-RNAi treated dermatitic rabbit skin was counted in high-power fields (20×) and presented number per mm^2. The dermal-epidermal junction is indicated by dashed lines. Scale bar = 50 µm (**a**, **b**) and 200 µm (**c**, **d**). Data are shown as the mean ± SEM. $n = 6$, $*p < 0.05$, $**p < 0.01$ by *p*aired Student's *t*-test (two-tailed).

Knockdown of Na_x expression reduced expression of PAR-2 and TSLP in dermatitic skin

TSLP is a crucial Th2-associated cytokine that contributes to the allergic immune response. TSLP is produced by inflammatory cells as well as epithelial cells and initiates the Th2 immune response in AD [30]. Overexpression of TSLP has been found in the squamous epithelium of AD patients [31, 32]. We found high expression of TSLP dispersed throughout the epidermis in sham-RNAi-treated dermatitic skin (Fig. 4a). In contrast, expression of TSLP was observably reduced in dermatitic skin after Na_x-RNAi treatment. Western blot analysis of epidermal proteins showed that the expression of TSLP was reduced by 81 and 69% in HDM and OXZ-induced dermatitic rabbit skins by Na_x-RNAi treatment, respectively, compared with sham-RNAi control (Fig. 4b). PAR-2 is a protease-activated receptor that promotes Th2-mediated allergic inflammation [33, 34]. PAR-2 is an important sensor of exogenous allergens and is expressed at high levels in skin lesions of AD patients, where it plays a role in AD pathogenesis [35]. PAR-2 activation also leads to secretion of proinflammatory cytokines, such as TSLP, and promotes recruitment of granulocytes and T cells [36]. Western blot analysis demonstrated a reduction of PAR-2 in the epidermis by 84 and 70% in HDM and OXZ-induced dermatitic rabbit skins following

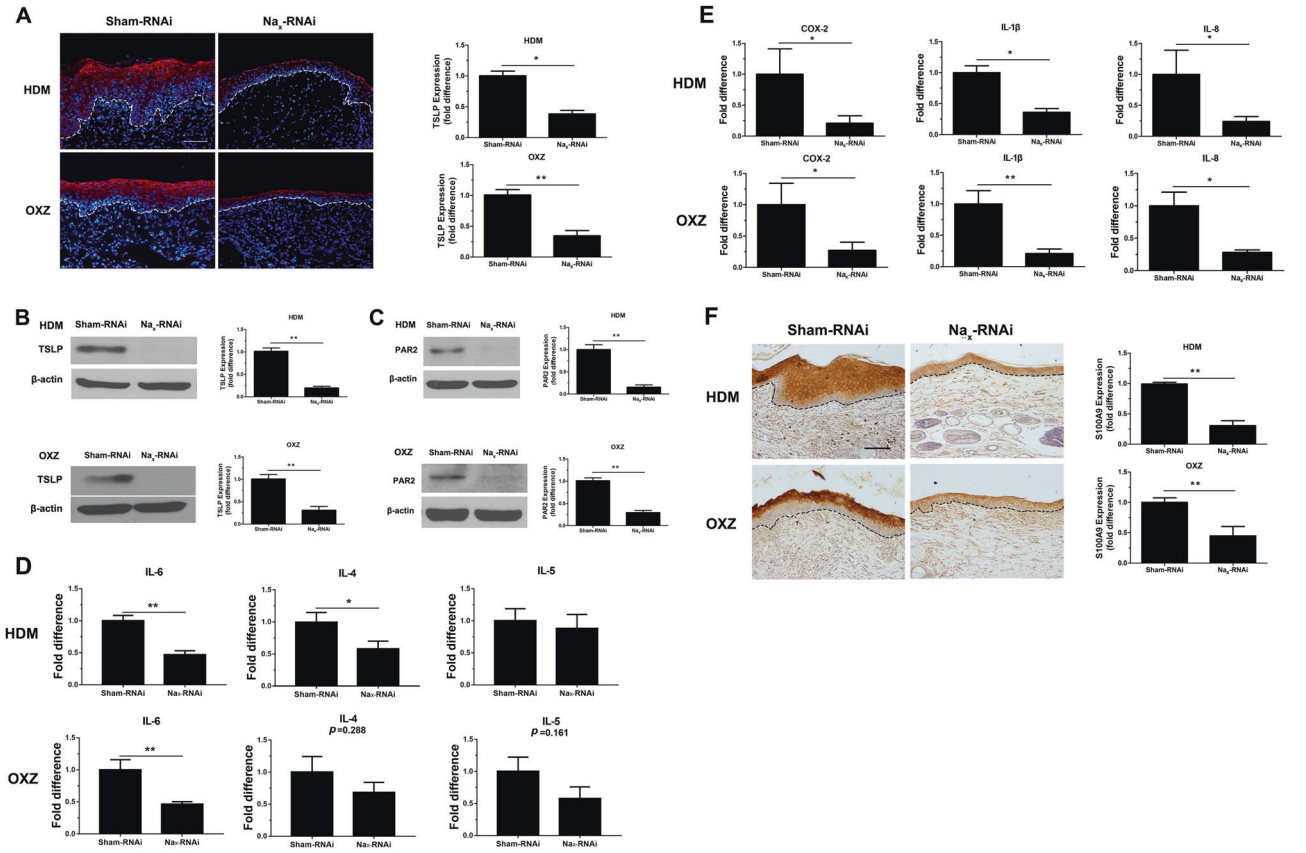

Fig. 4 Upregulation of TSLP, PAR-2, and proinflammatory genes was reduced in dermatitic skin by Na_x knockdown. HDM or OXZ-induced rabbit ear dermatitic skins were treated with Na_x-RNAi or sham-RNAi and harvested as described in Fig. 1a, e. **a** Immunostaining of TSLP. Western blot analysis of TSLP (**b**) and PAR-2 (**c**). Western blot was performed on proteins isolated from the epidermis. The intensity of immunostaining and Western blot was quantified using the NIH ImageJ program relative to loading control β-actin, and expression in Na_x-RNAi treated AD-like skin was compared with sham-RNAi treated AD-like skin that was defined as 1. Scale bar = 50 μm. **a**, **b**, **c** Data are shown as the mean ± SEM. $n = 4$, $*p < 0.05$, $**p < 0.01$ by *p*aired Student's *t*-test (two-tailed). **d** mRNA expression in the dermis. Expression of IL-6, IL-4, and IL-5 in Na_x-RNAi-treated AD-like skin was compared with sham-RNAi-treated skin that was defined as 1. Data are shown as the mean ± SEM. $n = 7$–12, $*p < 0.05$, $**p < 0.01$ by un*p*aired Student's *t*-test (two-tailed). **e** mRNA expression in the epidermis. Expression of COX-2, IL-Iβ, IL-8 in Na_x-RNAi-treated AD-like skin was compared with sham-RNAi-treated skin that was defined as 1. Data are shown as the mean ± SEM. $n = 5$–10, $*p < 0.05$, $**p < 0.01$ by unpaired Student's *t*-test (two-tailed). **f** S100A9 immunostaining and quantification. The expression level of S100A9 was quantified using the NIH ImageJ program and its expression in Na_x-RNAi treated AD-like skin was compared with sham-RNAi-treated AD-like skin that was defined as 1. The dermal-epidermal junction is indicated by black dashed lines. Scale bar = 50 μm. Data are shown as the mean ± SEM. $n = 6$, $*p < 0.05$, $**p < 0.01$ by paired Student's *t*-test (two-tailed).

Na_x-RNAi treatment, respectively, compared with treatment with sham-RNAi control (Fig. 4c). These data suggest that Na_x acts as a key molecule driving the progression and allergic inflammation of dermatitis. We also analyzed expression of Th2 cytokines (IL-4, IL-5, IL-10, IL-13), a Treg cytokine (IL-10), IL-6 (which activates Th2 through Th17), and a proinflammatory cytokine (TNF-α) [37, 38]. RT-qPCR analysis showed that expression of IL-6, IL-4, and IL-5 was reduced in HDM and OXZ-induced dermatitic rabbit skins by Na_x-RNAi treatment compared with sham-RNAi control, though statistical significance was not reached in all samples (Fig. 4d). However, there were no changes in the expression of IL-10, IL-13, and TNF-α in HDM- or OXZ-induced dermatitis-like skin by Na_x-RNAi treatment compared with sham-RNAi treatment (data not shown). Large variations in gene expression, except for IL-6, were found in the samples. This may due to incomplete knockdown of Na_x in the epidermis (Fig. 1d, h), which indirectly regulates T cells in the dermis through secretion of cytokines such as TSLP.

Knockdown of Na_x expression down-regulates subsequent proinflammatory genes in dermatitic skin

Our previous data revealed that Na_x controls proinflammatory signaling in the epidermis [6]. Expression of IL-1β, IL-8, and COX-2, proinflammatory proteins downstream of Na_x, is upregulated in the epidermis when the barrier function of skin is disrupted [5, 6, 10]. We measured

the mRNA expression of these genes in the epidermis by RT-qPCR. Expression of the genes encoding COX-2, IL-1β, and IL-8 was decreased by 59%, 89%, and 61%, respectively, in HDM-induced dermatitic skin by Na_x-RNAi treatment compared with sham-RNAi control (Fig. 4e). Expression of genes encoding COX-2, IL-1β, and IL-8 was decreased by 66, 79, and 69% in OXZ-induced dermatitic skin after Na_x-RNAi treatment (Fig. 4e). S100A9 is a key epithelium-derived proinflammatory factor that is highly expressed in many skin diseases that have barrier function disruption, including AD [39, 40]. Our previous data indicated that S100A9 is upregulated in human hypertrophic scars and keloids in which the barrier function of the skin is impaired, and that S100A9 is a downstream gene of Na_x and functions as a cytokine secreted from the epidermis [6, 41]. Immunostaining indicated that S100A9 was mainly distributed in the suprabasal layer of the epidermis in dermatitic rabbit skin that was treated with sham-RNAi (Fig. 4f). Expression of S100A9 was reduced by 69 and 55% in HDM and OXZ-induced dermatitic skins by Na_x-RNAi treatment, respectively. These data suggest that Na_x is a key molecule that regulates inflammation in the epidermis of dermatitic skin.

Discussion

Our present study demonstrates the importance of Na_x-related inflammatory pathway in the development and progression of dermatitic skin lesions. Whereas Na_x was detected in the suprabasal layer of normal human skin specimens, it was detected diffusely throughout the epidermis of AD skin specimens (Fig. S2). This suggests that the hyperthickened epidermis seen in AD skin contains more Nax expressing layers. Several key findings support this conclusion. First, Na_x was expressed at a high level in dermatitic skin lesions. Knockdown of Na_x alleviated clinical symptoms of dermatitis, such as erythema, scaling, and papulation. In addition, histologic features characteristic of dermatitic skin lesions, including thickening of the epidermis and inflammation of the dermis, were also lessened after inhibition of Na_x. Second, knockdown of Na_x suppressed the excessive inflammatory response characterized by inflammatory cell infiltration and cytokine expression in dermatitic skin lesions. Third, the upregulation of PAR-2 and TSLP were decreased by Na_x knockdown, demonstrating that Th2-mediated allergic inflammation in dermatitis is regulated at least in part by Na_x.

Na_x is classified by phylogenetic analysis as a member of the family of VGSCs. It is called an atypical VGSC because the important sequences for voltage sensing and inactivation are different from other VGSCs [42, 43]. When cRNA was injected into Xenopus oocytes and a cell line, no functional channel activity was detected upon stimulation with electric potential [44, 45]. Further analysis demonstrated that Na_x functions as a sodium concentration-sensitive channel, rather than a voltage-dependent channel, which responds to increases in extracellular sodium concentration in excitable neuronal cells [7, 8]. One report suggested that Na_x is a sodium leak channel that allows sodium to pass through cell membranes in the nervous system [9]. We recently demonstrated that Na_x is also widely expressed in epithelial cells of many organs and involved in the excessive inflammatory response following sodium perturbation caused by epithelial water loss [6]. Disruption of skin barrier function resulting in increased water loss is an important feature of many skin diseases such as hypertrophic scarring, dermatitis, and ichthyosis. Our previous study identified Na_x as a sodium sensor that regulates proinflammatory gene expression through two major signaling transduction pathways in the skin; Na_x-ENaC and Na_x-GPCR [6]. We demonstrated that ENaC controls the production of prostaglandin E2 (PGE2), a proinflammatory mediator produced by COX-2. Na_x regulates ENaC activity through prostasin, a membrane-bound protease. Though this association is not fully characterized, Na_x also regulates PAR-2, a G protein–coupled receptor (GPCR), in epithelial cells, through modulation of protease activity. Both IL-1β and IL-8 are proinflammatory downstream genes of PAR-2.

Studies have shown that dehydration of the skin surface and exposure to proteolytically active allergens contributes to aberrant protease/PAR-2 signaling in AD [45]. PAR-2 activation leads to abnormal desquamation and antimicrobial peptide degradation, thereby leading to AD progression [46]. In addition, PAR-2 is an important epidermal sensor for detrimental exogenous factors, such as allergens and haptens, and increases expression of proinflammatory cytokines, such as TSLP [47]. TSLP is a key cytokine that induces allergic inflammation in AD by recruiting inflammatory cells, such as mast cells, eosinophils, T cells, and macrophages [48, 49]. Knockdown of Na_x expression reduced the increased expression of PAR-2 and TSLP in dermatitic rabbit skin (Fig. 4). In addition, infiltration of inflammatory cells in the dermis was reduced by Na_x knockdown (Fig. 3). These data suggest that Na_x initiates and perpetuates excessive skin inflammation in response to skin barrier disruption, leading to the development of dermatitis at least in part by activating the PAR-2/TSLP signaling pathway.

We have reported that modulation of Na_x downstream genes, such as those encoding COX-2, IL-Iβ, IL-8, and S100A9, represents a potential therapeutic approach for treatment of hypertrophic scarring [5, 50]. Increased expression of these genes has been reported in AD patients and in AD-like animal models [39, 40, 51, 52]. We anticipate that proinflammatory genes downstream of Na_x in

the epidermis, which are induced by skin barrier disruption, participate in AD pathogenesis. Knockdown of Na_x reduces expression of TSLP as well as proinflammatory genes of downstream of Na_x, which suggests that Na_x is an effective therapeutic target that regulates expression of multiple proinflammatory cytokines.

AD is an increasingly prevalent chronic inflammatory disease characterized by intense skin itching and increased proinflammatory gene expression. Of the various clinical symptoms observed in AD, increased epithelial water loss and dry skin are considered to be important. Many studies have suggested that aberrant epidermal barrier function induces abnormal inflammatory and immune responses that may contribute to the pathophysiology of AD [53]. Emollients have been used to treat AD by restoring the barrier function of skin. Topical calcineurin inhibitors and topical corticosteroids have also been used as non-specific anti-inflammatory therapies to treat AD. However, these agents also have unwanted side effects [54, 55]. Thus, there is a paucity of effective treatments to treat AD at the present time due to the heterogeneity of this condition and the complexity of disease etiology. Our findings have revealed that Na_x mediates the inflammatory pathway in response to the disruption of epidermal barrier function, leading to pathological characteristics of dermatitis in two rabbit models of dermatitic skin. In addition, we showed that knockdown of Na_x by RNAi in this model reversed the progression of dermatitis. Therefore, we anticipate that Na_x is a novel molecular target in dermatitis, which includes AD, which may be considered in the context of clinical treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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