

Topical application of *Dermatophagoides farinae* or oxazolone induces symptoms of atopic dermatitis in the rabbit ear

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Abstract Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease characterized by hyperproliferation and abnormal differentiation of the epidermis, and dermal infiltration of inflammatory cells. Appropriate animal models that recapitulate human AD and allow the analysis of disease processes in a reliable manner are essential to the study of AD. In this study, we established two AD models in rabbits by applying an allergen, *Dermatophagoides farinae* (Der f), or a hapten, oxazolone (OXZ). Application of the allergen or hapten induced a rapid onset and a chronically sustained AD-like skin lesion. The clinical symptoms, which include skin erythema, scaling, papula and edema, of AD-like rabbit skin were similar to those in human AD. Histological analysis showed that allergen- or hapten-treated rabbit skin showed increased epidermal thickening and inflammatory cell infiltration. Furthermore, PCNA and keratin 10 (K10) staining revealed excessive proliferation and insufficient differentiation of the epidermis in the rabbit AD-like skin. Western blot analysis showed decreased expression of thymic stromal lymphopoietin (TSLP), an AD cytokine, in the rabbit AD-like skin. Our results suggest that the allergen- or hapten-induced rabbit AD models have

pathological features of human AD-like symptoms and will be useful for evaluating both pathogenic mechanisms and potential therapeutic agents for human AD.

Keywords Atopic dermatitis · *Dermatophagoides farinae* · Oxazolone · Rabbit ear · Thymic stromal lymphopoietin (TSLP) · Inflammatory cell infiltration

Abbreviations

ACD	Acute allergic contact dermatitis
AD	Atopic dermatitis
Der f	<i>Dermatophagoides farinae</i>
H&E	Hematoxylin and eosin
K10	Keratin 10
OXZ	Oxazolone
TIS	Three item severity
TSLP	Thymic stromal lymphopoietin

Introduction

Atopic dermatitis (AD) is a chronic and relapsing eczematous skin disorder that has an increasing prevalence in industrialized countries [3, 20]. Although the precise pathogenesis of AD has not yet been fully understood, immunological abnormality [42], skin barrier dysfunction [17], environmental, pharmacological and psychological factors [1, 34] are believed to contribute to the development and pathogenesis of AD. To further investigate the pathophysiology and explore effective pharmacological therapies for AD, several murine models that display certain features of human AD have been developed. The spontaneous mutants, such as the Nc/Nga mouse, can develop AD-like skin lesions under conventional conditions [2]. The gene manipulated mice, which either over- or

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under-express selective cytokines such as IL-4, IL-18, and thymic stromal lymphopoietin (TSLP), show AD symptoms [7, 8, 16]. However, these models suffer from low efficiency, high expenditure, and unpredictable nature and have not yet been extensively characterized [39]. Currently, the epicutaneous sensitization models induced by antigens, such as ovalbumin, oxazolone (OXZ), dust mite, and trinitrochlorobenzene, have been widely used in mice [26, 28, 29, 37]. These mice presented AD-like dermatosis that mimicked human AD in terms of epidermal hyperproliferation, dermal inflammatory cell infiltration, and pro-inflammatory gene expression. Although these models were considered to be controllable, reproducible, and low-cost, the limited area of mouse skin makes it difficult to be used as a self-control model in pharmacological therapy testing. In addition, the skin structure and the mechanism of wound healing are still significantly different between murine skin and human skin [6]. Therefore, appropriate animal models that mimic human AD and allow the analysis of disease processes, particularly the disease progression and pathological manifestations, in a highly reliable and speedy manner are needed.

Our research group has vast experience in establishing models in the rabbit ear, including hypertrophic scar, bio-film wound, and ischemia models [11, 38, 44]. We have found these rabbit ear models not only share similar characteristics with humans but also possess stability, reproducibility, and handleability. Besides, when compared with rodent skin, the thickness of the stratum corneum and the architecture of collagen fibers in rabbit ear skin were more similar to those of human skin, making rabbit ear a reasonable model for human skin disease research [31, 32]. More importantly, each ear of one rabbit can be treated differentially without mutual interference, which was an ideal self-control model in many experiments.

In this study, we have established two AD models in rabbit ears. We used house dust mite (*Dermatophagoides farina*, Der f) which is a potent aeroallergen in AD pathophysiology; up to 85% of patients with AD have skin prick test reactivity [12]. We also used OXZ that elicits Th2-like hypersensitivity reaction in mice that parallels human AD [26]. Although the exact etiopathogenesis in Der f- or OXZ-induced AD is still unknown, they both can trigger human-like AD in animal models in terms of clinical, histological, and immunological features. We found that repeated challenge of Der f and OXZ causes dermatosis in rabbit ear skin, which is characterized by epidermal thickening, increased TSLP expression (an AD cytokine), and dermal inflammatory cell infiltration. Our novel AD models using the rabbit ear will facilitate studies on both elucidation of AD pathogenesis and evaluation of emerging therapies for AD.

Materials and methods

Allergen- or hapten-induced dermatitis on rabbit ears

New Zealand White female rabbits weighing 3–5 kg were purchased from the Covance (Princeton, NJ). Treatment of the animals was carried out in accordance with the recommendations described in 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 (ACUC). All operations were performed under anesthesia, and all efforts were made to minimize suffering. The hair of the ventral side of each rabbit ear was removed using an electric shaver followed by the depilatory cream application (Nair, Church & Dwight Co., Inc., Ewing, NJ, USA). The stratum corneum of the skin was disrupted by tap stripping in order to enhance the penetration of allergen or haptens [10]. Three circles with diameters of 10 mm were drawn on the ventral side of each ear. One ear was treated with allergen or hapten while the contralateral ear was treated with vehicle alone as controls. An Elizabethan collar (E-collar, Lomir Inc, Malone, NY, USA) was in order to protect treatment sites from grooming.

For Der f-induced AD, Der f solution (Standardized Mite *Dermatophagoides Farina*, Greer Laboratories, Lenoir, NC) with 100 µl volume [10,000 allergy unit (AU)/ml] was pipetted onto a small sterile cotton ball and applied to the skin on days 0 and 4. In order to enhance the efficacy of Der f treatment, and to keep the cotton ball in place, a semi-occlusive dressing, Tegaderm™ (3 M Health Care, St. Paul, MN), was applied. On day 8, the Tegaderm and Der f-soaked cotton ball were removed for 7 days. The 7-day rest enhances sensitivity of skin to the allergen [15]. Der f was re-applied twice more, 4 days apart and each application was covered with Tegaderm (Fig. 1a).

For OXZ-induced AD, 5% OXZ (Sigma-Aldrich, St. Louis, MO) dissolved in absolute ethanol was applied topically on the circular areas for sensitization. Seven days later, 0.2% OXZ in ethanol was spread to the circular areas every other day for 14 days or until the sign of AD was observed (Fig. 2a).

Scoring severity of skin inflammation

To score the severity of inflammation and observe the progress of dermatitis, an objective scoring system was employed based on the Three Item Severity (TIS) score [36]. Erythema, edema/papulation, and excoriations were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The

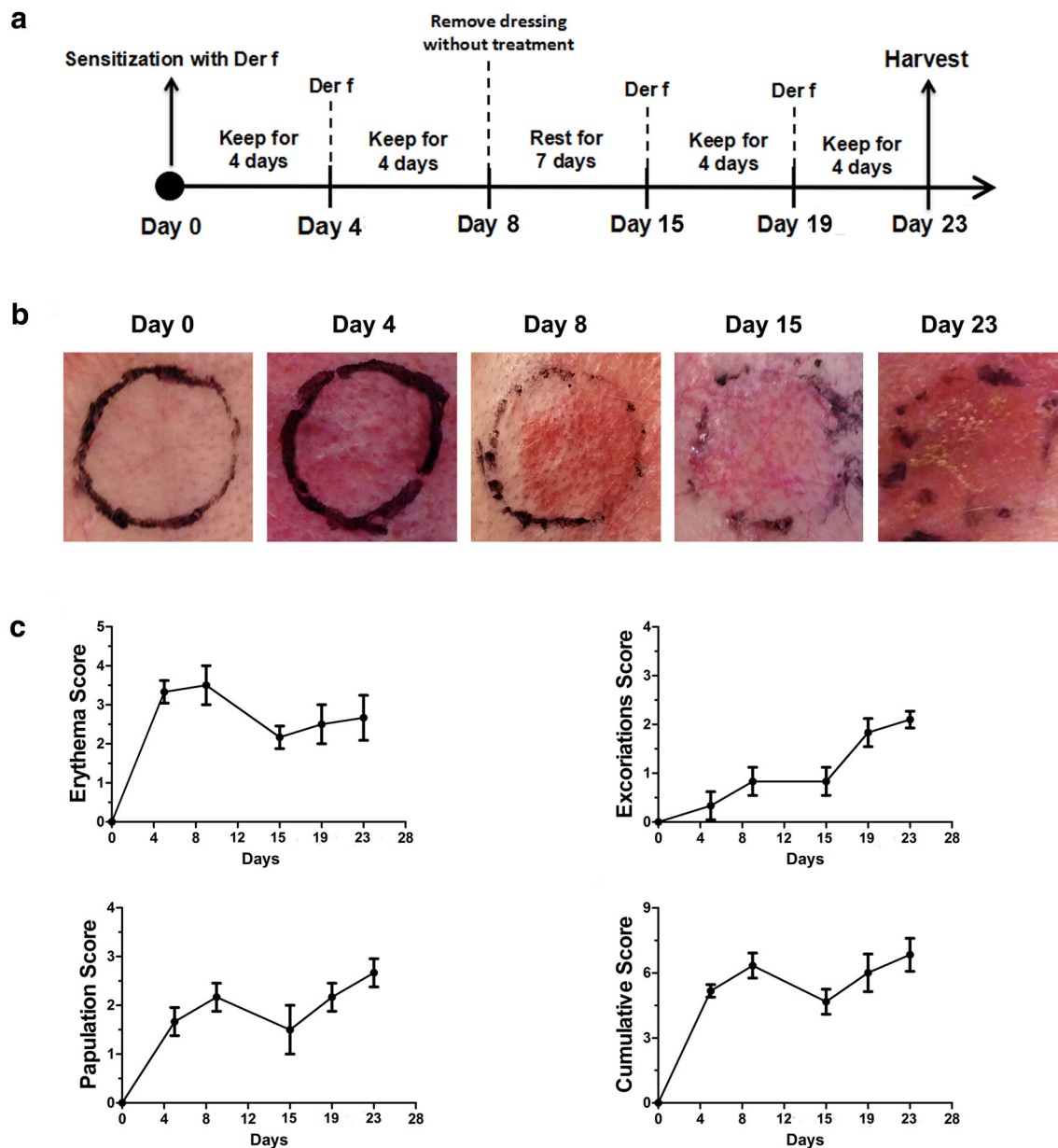


Fig. 1 Der f-induced atopic dermatitis-like symptom on rabbit ears. **a** Methods for the development of an AD model by topical application of Der f on rabbit ear skin. **b** Gross appearance of rabbit ear skin lesion after Der f application at different time points. **c** Erythema,

cumulative score (erythema plus edema/papulation plus excoriations) served as a measure of the severity of inflammation (scale 0–12).

Histology, epidermal thickness quantification, and monocyte counting

Tissues harvested from rabbit ears were fixed with 10% neutralized formalin overnight followed by dehydration and embedded in paraffin. Serial 5- μ m sections were cut, deparaffinized, and stained for histological analysis.

excoriations, and papulation of rabbit ear skin lesions were scored during the course of disease on a scale from 0 to 4. The cumulative score (erythema plus excoriations plus papulation) was recorded. Scale bar 1 cm. Data are expressed as the mean $\pm$ SEM. $n = 6$

Quantification of epidermal thickness and monocyte counts was performed after staining with hematoxylin and eosin (H&E) by two researchers. Eosinophil and mast cells were stained using the Eosinophil–Mast Cell stain kit (ScyTek Laboratories Inc., Logan, UT). Briefly, for mast cell detection, deparaffinized sections were stained with Astra Blue solution for 30 min and treated with 0.5% HCl solution briefly in order to reduce background staining. For eosinophil detection, sections were stained with Vital New Red solution for 30 min. After washing with water, the stained sections were dehydrated and mounted in synthetic

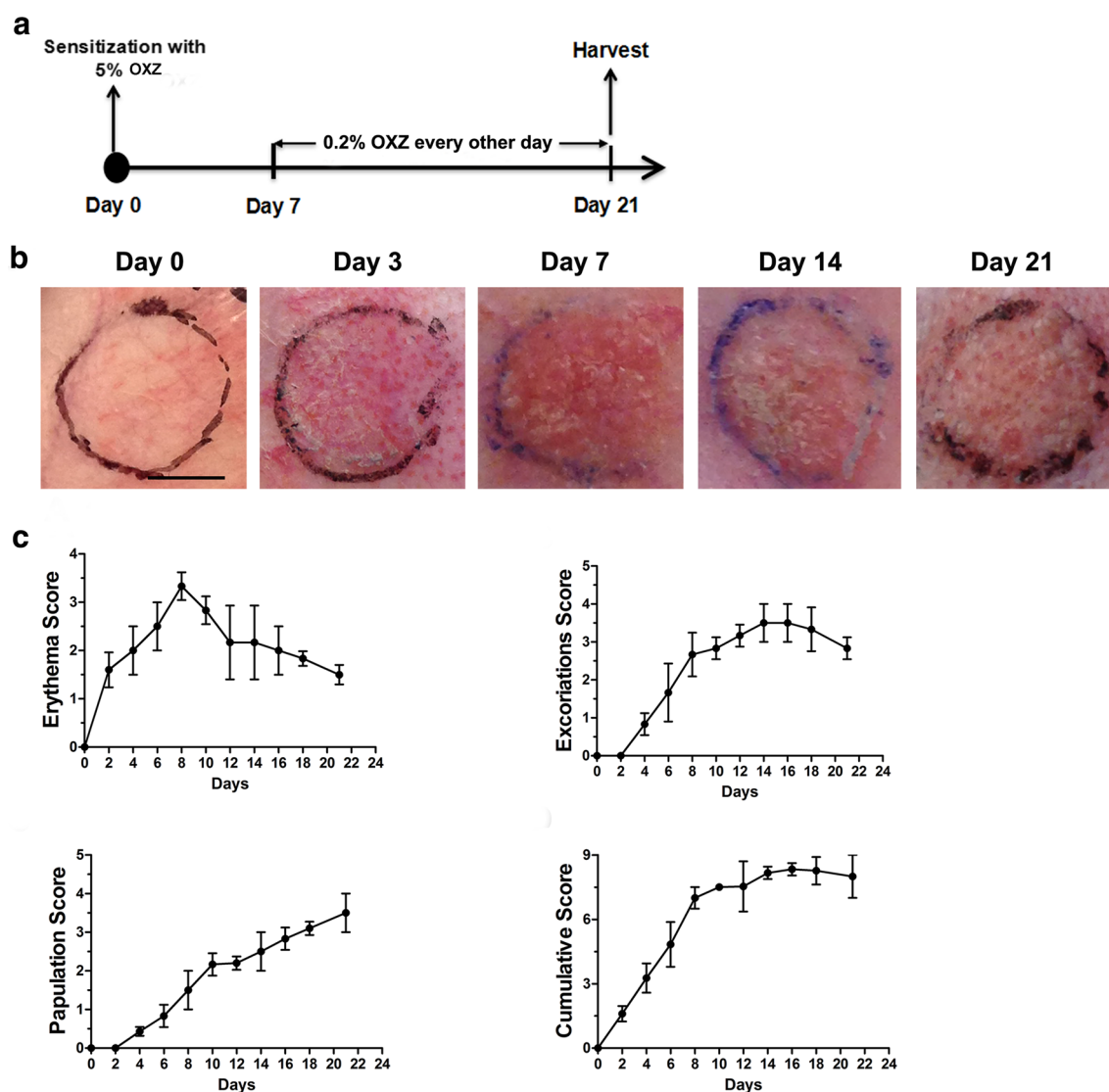


Fig. 2 OXZ induced atopic dermatitis-like symptom on rabbit ears. **a** Methods for the development of an AD model by topical application of OXZ on rabbit ear skin. **b** Gross appearance of rabbit ear skin lesion after OXZ application at different time points. **c** Erythema,

excoriations, papulation, and cumulative score were shown as described in Fig. 1. Scale bar 1 cm. Data are expressed as the mean $\pm$ SEM. $n = 6$

resin (Permunt Mounting Medium, Fisher Scientific, Hampton, NH). Images were captured using a Nikon Eclipse 50i light microscope and NIS Elements BR software (Nikon, Melville, NY) with the NIS Elements program (Nikon Instruments Inc., Melville, NY).

Immunostaining and Western blot

For immunohistochemical staining, the activity of endogenous peroxidase was quenched by incubation with 1% hydrogen peroxide for 15 min followed by treatment of antigen retrieval solution (Dako, Carpinteria, CA). Then, the sections were incubated with mouse anti-PCNA (1:1000 dilution, BD Biosciences, San Jose, CA) and mouse anti-cytokeratin 10 (1:1000 dilution, DAKO)

primary antibodies at 4 °C overnight. After thorough washing, the sections were incubated with biotinylated secondary antibody (Vector laboratories, Burlingame, CA) followed by incubation with avidin–biotin complex (Elite ABC kit; Vector Laboratories) for 1 h. Signal was visualized using 3, 3'-diaminobenzidine (DAB) and nuclei were stained with hematoxylin (Sigma-Aldrich).

For immunofluorescence staining, the sections were permeabilized with Triton X100 (0.3%), blocked with normal goat serum (10%), and incubated with mouse anti-CD3 (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX), mouse anti-macrophage (1:1000 dilution, Abcam, Cambridge, MA), and rabbit anti-loricrin (1:1000 dilution, Abcam) primary antibodies at 4 °C overnight. Then, the sections were further incubated with Alexa Fluor

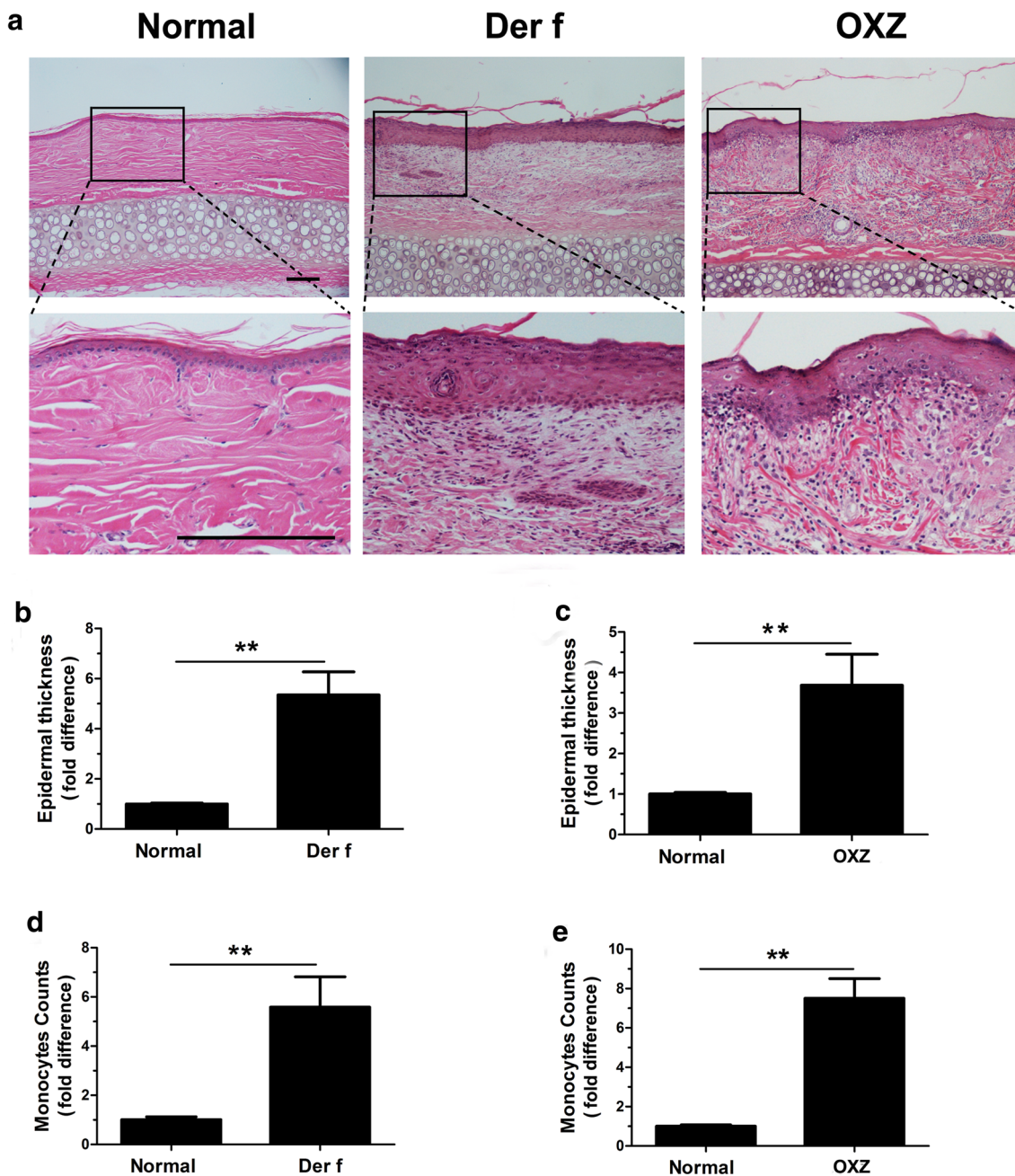


Fig. 3 Histological analysis of the Der f- and OXZ-treated rabbit ear skin lesions. **a** H&E staining. Rabbit ear skin was treated with Der f or OXZ and harvested at days 23 or 21, respectively. Non-treated skin was shown as a control. **b, c** Epidermal thickness was quantified. The

values were compared to control that was set as 1. **d, e** Tissue monocytes count. The number of monocytes in dermis was compared to control that was set as 1. Scale bar 50 μ m. Data are shown as the mean $\pm$ SEM. $n = 6$, ** $p < 0.01$

594-conjugated secondary antibody or Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/ml). Images were obtained with an EVOS FL Cell Imaging System (ThermoFisher Scientific, Grand Island, NY) and were merged using Image-Pro Plus v. 6.0 software (Media Cybernetics, Inc, Rockville, MD, USA).

For Western blot analysis, the epidermis was separated from the dermis by incubating rabbit skin in 0.5 M ammonium thiocyanate at room temperature for 30 min as previously described [45]. Whole cell proteins were extracted using RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate, pH 7.5). Proteins were transferred onto nitrocellulose membranes after separation by SDS-PAGE (polyacrylamide gel

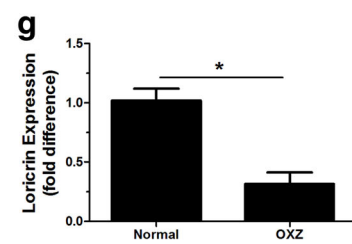
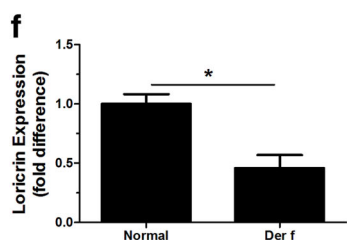
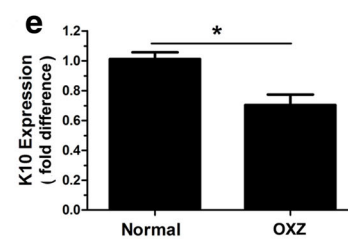
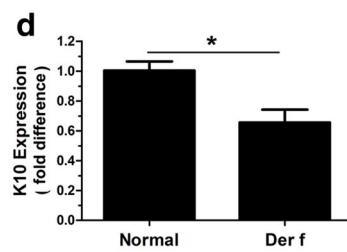
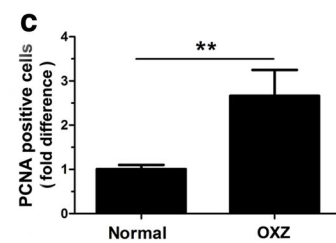
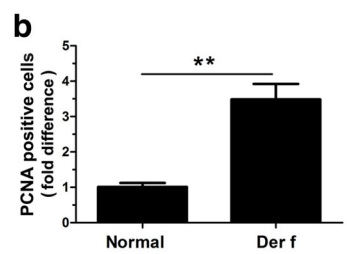
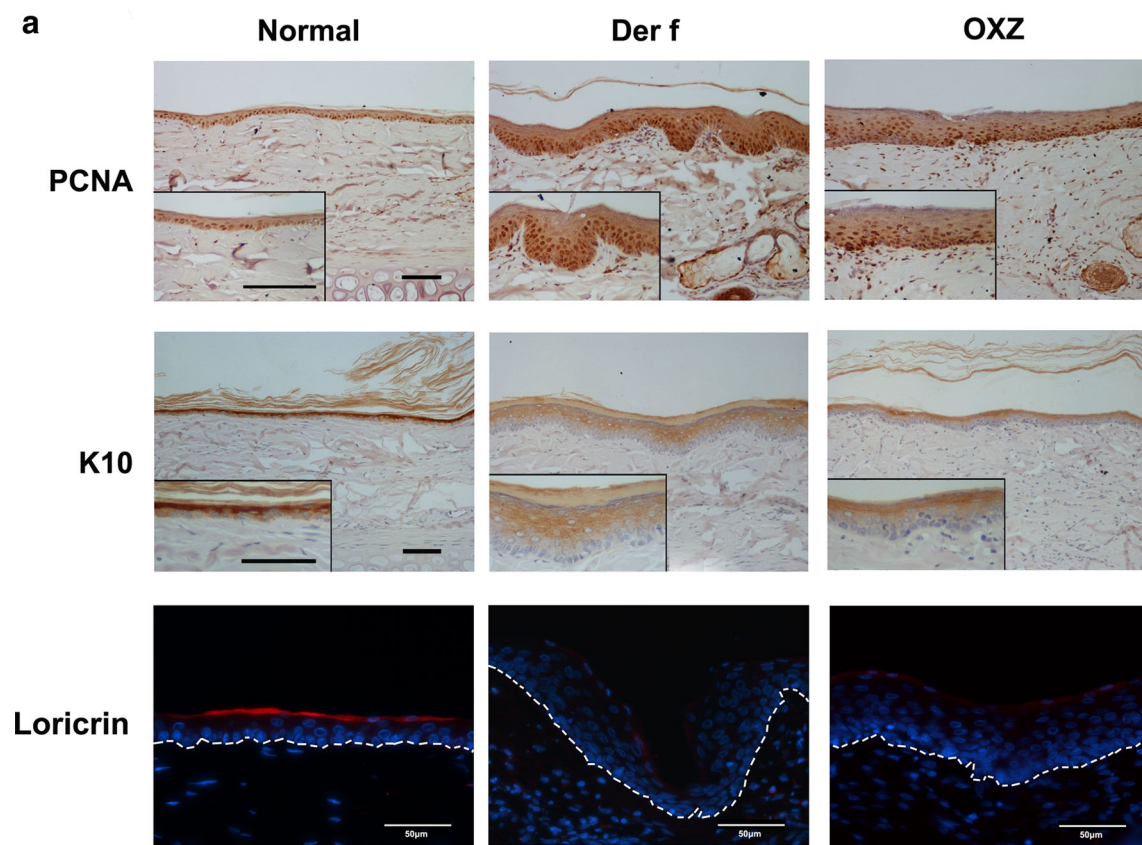


Fig. 4 Proliferation and differentiation of the epidermis in the Der f- and OXZ-treated rabbit skin. **a** Immunostaining of PCNA (a proliferation marker), K10 (an early differentiation marker, cytokeratin 10), and loricrin (a late differentiation marker) was shown. Scale bar 50 μ m. **b, c** Quantification of PCNA-positive cells. The number of positive cells in the allergen (or hapten) treated skin was compared to control that was set as 1. **d, e** Quantification of K10. **f, g** Quantification of loricrin. The expression level of K10 and loricrin was quantified using the NIH ImageJ program and its expression in the allergen (or hapten) treated skin was compared to control that was set as 1. Data are shown as the mean $\pm$ SEM. $n = 6$, $*p < 0.05$, $**p < 0.01$

electrophoresis). Mouse anti-human TSLP (1:1000 dilution, Novus Biologicals, Littleton, CO) and mouse anti-human β -actin (Sigma-Aldrich, 1:5000 dilution) were used as primary antibodies. A horseradish peroxidase (HRP)-conjugated antibody (1:5000 dilution, Vector laboratories, Burlingame, CA) was used as a secondary antibody. The signals were visualized using an Enhanced Chemiluminescence (ECL) detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ).

Statistical analysis

All data in this study are expressed as the mean and standard error of the mean (mean $\pm$ SEM). Significant differences were analyzed using a paired Student's t test. All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The p values lower than 0.05 were considered to be significantly different.

Results

Der f induces AD-like symptoms in the rabbit ears

Allergens derived from house dust mites have the ability to induce AD-like skin lesion in human and mice [14]. We observed that skin erythema occurred after a single Der f application followed by obvious formation of papules at day 2. The erythema and papules were diminished while the skin scaling was clearly visible, despite the absence of Der f treatment for 7 days. The symptoms of erythema, scaling, and papules became increasingly apparent after subsequent Der f challenges, and the AD was established at day 23 (Fig. 1b).

The TIS score showed that the erythema and papules occurred immediately but became most obvious after two Der f treatments (day 8). The TIS score diminished 3 days after rest on day 15 and then became more pronounced after Der f treatments resumed on days 19 and 23 (Fig. 1c). The skin scaling was not apparent when Der f-treated skin was covered with Tegaderm (day 8), but it progressed continuously and became severe when Der f-treated skin was not covered with Tegaderm (days 8–15) (Fig. 1c). In general, the Der f-induced AD-like skin lesion displayed a rapid onset and a chronically sustained course.

OXZ induces AD-like symptoms in the rabbit ears

OXZ has previously been used as a sensitizer to elicit allergic contact dermatitis model in mouse. We found that the skin became reddish within 12 h when 5% OXZ was applied to rabbit skin. The reddish area exceeded the circular area of OXZ application in the first 24 h. The reddish area was limited and mild erythema was observed at day 2. The erythema became more apparent and peaked at days 7 and 8 when the skin scaling was clearly visible. The most pronounced skin scaling occurred between days 14 and 16, and part of the scaly skin peeled off later. The typical symptoms of AD with evidence of erythematous papules, skin thickening, and scaling were observed at day 21 (Fig. 2b).

We evaluated the progression and severity of OXZ-induced skin inflammation using the TIS score system, which is based on erythema, excoriations, and papulation. As shown in Fig. 2c, the erythema developed quickly after OXZ application with a maximum erythema peak at days 7 and 8. Though erythema was gradually diminished until day 21, it was still obvious when compared with adjacent normal skin. The excoriations appearing at days 3 and 4 peaked at days 14 and 16. The papules emerged at days 3 and 4 and progressed gradually until day 21. The cumulative score, which represents the progress of AD in general, showed that the OXZ-induced AD symptoms progressed quickly during the first week before reaching a plateau, with symptoms persisting through the end of the 21 day study.

Increased epidermal thickness and inflammatory infiltration in the Der f- or OXZ-treated rabbit ear skin

Analysis of H&E-stained sections from the Der f- or OXZ-treated skin harvested at days 23 and 21, respectively, showed increased epidermal thickening and prominent dermal inflammatory infiltrate and edema (Fig. 3a). Analysis under high-power microscopic fields showed hyperplasia of the epidermis in the Der f- or hapten-treated skin. In addition, abundant infiltrates of mononuclear cells and distinct changes in dermal collagen architecture were also observed (Fig. 3a).

To quantify the degree of epidermal hyperplasia, a phenomenon typical for AD, the epidermal thickness was measured. The analysis showed that the epidermal thickness increased by 5.4 ± 0.9 and 3.7 ± 0.8 folds, after Der f and OXZ treatment, respectively, compared to non-treated skin (Fig. 3b, c). The degree of inflammatory infiltration was determined by quantification of monocytes in the dermis. The number of dermal monocytes increased by 5.6 ± 1.2 and 7.5 ± 1.0 folds after Der f and OXZ treatment, respectively (Fig. 3d, 3e).

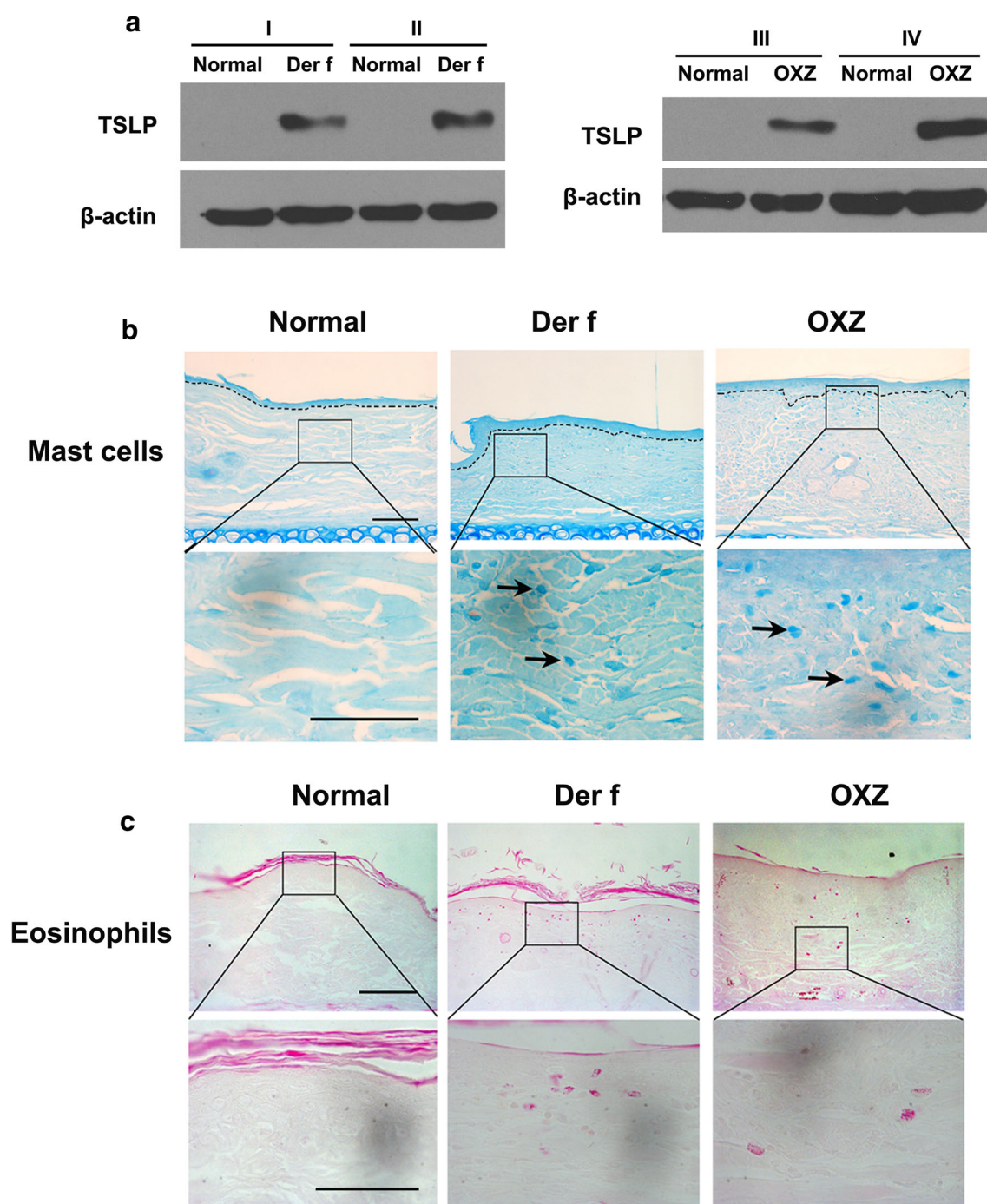


Fig. 5 Increased expression of AD markers in the rabbit skin by Der f or OXZ treatment. **a** Western blot of TSLP was shown. Protein (10 μ g) from the epidermis of rabbit ear skin was loaded in 12% polyacrylamide gel. Two samples (labeled as *I*, *II*, *III*, *IV*) from normal and Der f (or OXZ) treated skin were shown. The expression

of TSLP was detected with its specific antibody. β -Actin was detected as a loading control. **b** Mast cells were stained with *Astra Blue*. Arrows indicate some of positive signals. **c** Eosinophils were stained with *vital new red*. Scale bar 50 μ m (**b**, **c**)

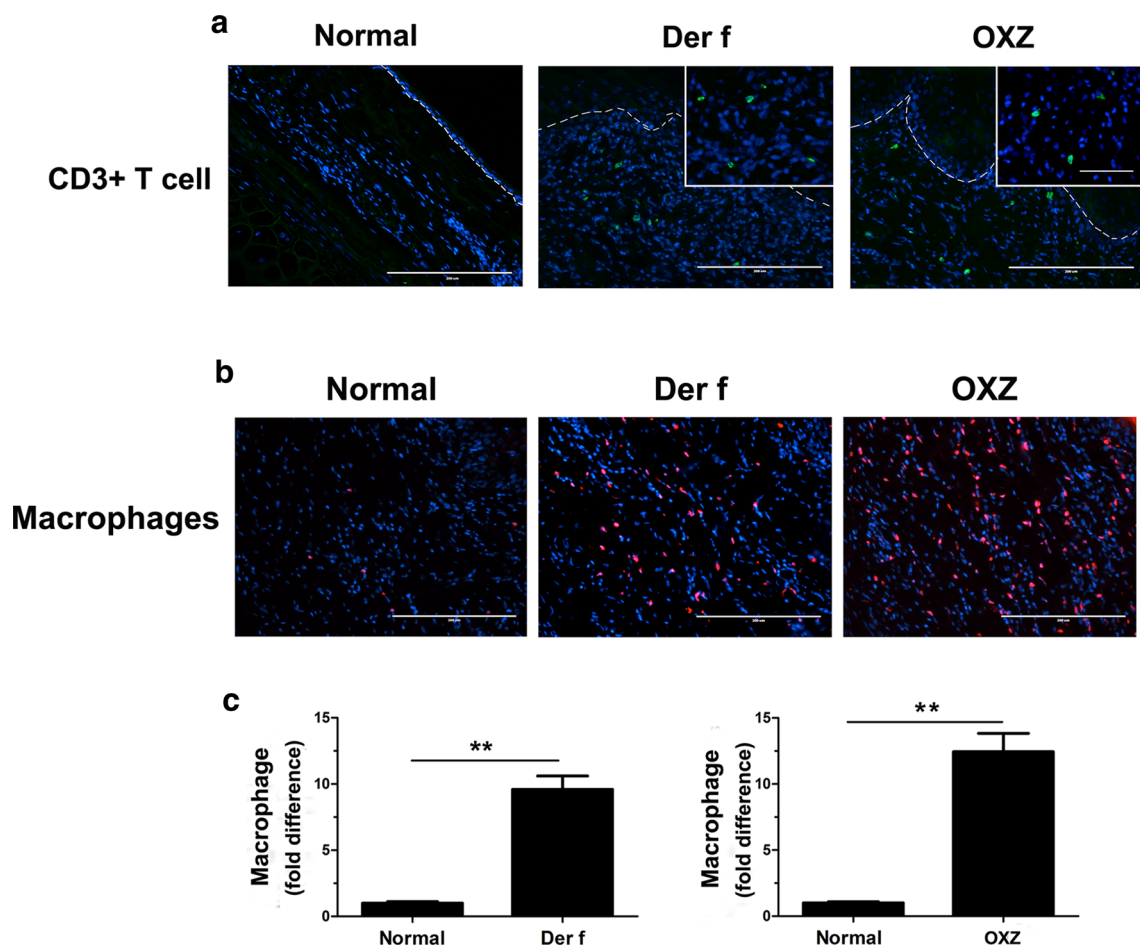


Fig. 6 Infiltration of T cells and macrophages in the Der f- or OXZ-treated rabbit skin lesions. **a, b** The immunostaining of CD3⁺ T cell and macrophages was shown. The epidermis–dermis junction area was indicated by white dashed lines. Scale bar 200 μ m.

Increased proliferation and aberrant differentiation of the epidermis in the Der f- or OXZ-treated rabbit ear skin

We hypothesized that hyperproliferation of the epidermis, another typical characteristic of AD, is the underlying mechanism of the increased epidermal thickening that was observed in allergen-treated skin. Analysis of PCNA, a proliferation marker, showed increased numbers of PCNA-positive cells in the basal layer of the allergen- or hapten-treated skin compared to non-treated control skin (Fig. 4a–c). It is known that the aberrant epidermal differentiation is an important pathological characteristic for AD [13]. Keratin 10 (K10), a keratinocyte differentiation maker, is typically expressed in the upper stratum spinosum of the epidermis of normal skin. Interestingly, expression of K10 was detected throughout the epidermis and its expression level was decreased in the Der f- or OXZ-treated skin compared to normal skin (Fig. 4a, d, e). Loricrin is a late

c Quantification of macrophages-positive cells. The number of positive cells in the hapten (or allergen) treated skin was compared to control that was set as 1. Data are shown as the mean $\pm$ SEM. $n = 6$, ** $p < 0.01$

differentiation maker and expressed in the upper stratum granulosum. The expression level of loricrin was decreased in the Der f- or OXZ-treated skin compared to normal skin (Fig. 4a, f, g).

Increased TSLP expression and inflammatory cell infiltration in the Der f- or OXZ-treated rabbit ear skin

TSLP is a key epithelium-derived cytokine in the AD pathogenesis [25]. Overexpression of TSLP has been found in squamous epithelium of AD patients [40]. Here, we found an increased expression of TSLP in the Der f or OXZ-treated skin compared to normal skin (Fig. 5a). It is known that TSLP promotes allergic inflammation in AD by inducing proliferation of mast cells and migration of eosinophils [47]. Increased infiltration of mast cells and eosinophils is one of the crucial features of AD [24, 41]. We detected increased infiltration of mast cells and

eosinophils in the dermis of Der f- or OXZ-treated skin. In contrast, few mast cells or eosinophils were detected in the normal skin (Fig. 5b, c). Previous studies have shown that a cellular infiltrate comprising predominantly T cells is a key feature of AD skin lesions [5]. While T cells were not observed in normal skin sections, their presence was detected in the Der f- or OXZ-treated skin sections (Fig. 6a). In addition, increased infiltration of macrophages, which are critically involved in the pathogenesis of AD, was found in the Der f- and hapten-treated skin compared to control skin (Fig. 6b, c).

Discussion

AD is a multifactorial chronic inflammatory skin disease and a major public health problem worldwide. It is on the rise in many developing countries, affecting over 10% of the population, and has reached a plateau of 20% in western countries [35]. Previous studies have considered the immunologic abnormalities as the main etiopathogenesis for AD. Such abnormalities, including the sensitized dendritic cells [33], the increased IgE level [4], and the Th2 immunophenotype [23] result in skin allergic responses and secondary pathogen colonization (“inside-outside” theory) [22]. On the other hand, many studies have revealed that the skin of AD patients displayed epidermal barrier dysfunction, allowing for the penetration of allergens and microbial pathogens [9], and leading to abnormal immune responses and a relapsing inflammatory skin condition (“outside-inside” theory) [19]. However, the precise pathogenic processes of AD seem much more complicated and our understanding of this disease is far from complete. Therefore, it becomes increasingly important to establish suitable animal models that allow further investigations in the elucidation of pathogenesis of AD and in developing new therapies.

Currently, the most frequently used animal AD model is mouse, which is divided into three categories: spontaneous mutants, genetically modified mice and hapten-treated mice. The spontaneous mutant models only develop AD under conventional, nonspecific pathogen-free conditions, and the incidence of AD lesions drastically varies from facility to facility [27]. The transgenic mouse models are not commercially available and are of limited use for evaluating potential therapeutic approaches for AD [18]. However, the hapten-induced models have proven to be efficient, low-cost and feasible to induce AD in mice. We previously established a mouse AD model by topical application of OXZ [45]. However, we found that the exposed skin lesions on the mice were easily damaged and contaminated. In addition, the limited skin areas made it difficult to subject to many different treatments in the same

animal. Since we have rich experience in establishing wound healing models using rabbit ears [11, 38, 44], we found that the same location in two ears of one rabbit is ideal for self-control experiments. Besides, certain genes that are involved in inflammation in humans, such as S100A12 and IL-8, are expressed in rabbit but not in mice [43].

In this study, we used Der f and OXZ to elicit AD on the rabbit ear skin. AD is a common chronic inflammatory skin disease with typical clinical features, such as pruritus, erythema and scaling and histologic characteristics [30], including epidermis hyperplasia and skin barrier dysfunction. Previous researches have demonstrated that Der f and OXZ are allergens that can induce AD in mice [26, 46]. Here, we showed that initial Der f or OXZ challenge elicits acute allergic contact dermatitis (ACD) in rabbit ears that is characterized by extensive erythema. Further challenges, however, skew the acute ACD to a chronic ACD, with multiple features of human AD, including typical symptoms of scaling and papulation. Histologically, Der f- and OXZ-treated skin lesions showed significant thickening of the epidermis, hyperkeratosis and parakeratosis, accompanied by dermal infiltration of macrophages. Moreover, the expression of TSLP in the epidermis was increased after Der f and OXZ treatment. TSLP is a crucial epithelium-derived cytokine in the AD pathogenesis. TSLP stimulates naïve T-cells to Th2 cells, which further induces the AD pathogenesis by recruiting eosinophils and activating mast cell proliferation. We observed increased number of mast cells and eosinophils in Der f- and OXZ-treated skin lesions, which demonstrated that OXZ or Der f elicits an AD-like skin inflammation. These two AD-like models commendably simulate human AD in terms of clinical symptoms, histological features and immune response [21]. In addition, the scratching behavior, another important symptom in AD, was also observed in our models.

In summary, the AD models established in our present study using rabbit ear skin displayed a rapid onset and a chronically sustained human AD-like course, with the additional advantages of low costs, reproducibility and controllability. Furthermore, the sufficient skin areas and the same location in two ears can be used as a self-control model, which offer the advantage of allowing evaluation of different therapeutic agents in the same animal.

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Compliance with ethical standards

Conflict of interest The authors state no conflict of interest.

Ethical approval Animal experiments were performed in accordance with the recommendations described in 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 (ACUC).

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