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Title Page:

**Inhibition of the Warm-temperature Activated Ca²⁺-permeable TRPV3 Channel
Attenuates Atopic Dermatitis**

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Running Title: TRPV3 inhibition attenuates dermatitis

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Abbreviations: 17(R)-Resolvin D1, 7S, 8R, 17R-Trihydroxy-4Z, 9E, 11E, 13Z, 15E, 19Z-docosahexaenoic acid; 2-APB, 2-Aminoethoxydiphenyl borate; 4% PFA, 4% Polyformaldehyde; AD, Atopic dermatitis; ATP, Adenosine triphosphate; BCA kit, Bicinchoninic acid kit; Car, Carvacrol; DAB, Diaminobenzidine; DNFB, 2, 4-Dinitrofluorobenzene; FPP, Farnesyl pyrophosphate; H&E staining, Hematoxylin-eosin staining; IHC, Immunohistochemistry; IL-6, Interleukin-6; IL-1 α , Interleukin-1 α ; IPP, Isoprene pyrophosphate; NGF, Nerve growth factor; NO, Nitric oxide; Ost, Osthole; PEG2, Prostaglandin E2; RIPA buffer, Radio

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immunoprecipitation assay buffer; TNF- α , Tumor necrosis factor- α ; TRP, Transient receptor potential; TRPA1, Transient receptor potential ankyrin 1; TRPV1, Transient receptor potential vanilloid 1; TRPV3, Transient receptor potential vanilloid 3; WB, Western blot; WT, Wild-type

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by cutaneous lesions and intense pruritus. The warm temperature-activated Ca^{2+} -permeable TRPV3 channel is abundantly expressed in the keratinocytes, and gain-of-function mutations of TRPV3 cause skin lesions and pruritus in rodents and humans, suggesting an involvement of TRPV3 in the pathogenesis of AD. Here we report that pharmacological and genetic inhibition of TRPV3 attenuates skin lesions and dermatitis in mice. In mouse AD-like model induced by topical application of chemical DNFB, we found that TRPV3 proteins together with inflammatory factors $\text{TNF-}\alpha$ and IL-6 were upregulated in the skin detected by Western blot and immunostaining assay. Pharmacological activation of TRPV3 by channel agonist skin sensitizer carvacrol resulted in development of AD in WT mice, but not TRPV3 knockout mice. Furthermore, inhibition of TRPV3 by natural osthole reversed the severity of inflammatory dorsal skin and ear edema in dose-dependent manner, and also decreased the expression of inflammatory factors $\text{TNF-}\alpha$ and IL-6. Taken together, our findings demonstrate the involvement of overactive TRPV3 in the progressive pathology of AD in mice, and topical inhibition of TRPV3 channel function may represent an effective prevention and therapy for AD or inflammatory skin diseases.

Significance Statement

1. Overactive TRPV3 channel is critically involved in the pathogenesis of atopic

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dermatitis.

2. Inhibition of TRPV3 channel function by topical natural osthole may represent an effective therapy for management of atopic dermatitis aimed at preventing or alleviating skin lesions and severe itching.

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Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a common chronic inflammatory dermatologic disease characterized by recurrent eczematous skin lesions and intense itch, severely impairing quality of life (Waldman, Ahluwalia, Udkoff, Borok and Eichenfield, 2018). The pathogenesis of AD, however, is multifactorial with variable environmental and immunologic factors triggering AD in genetically susceptible individuals (Miyagaki and Sugaya, 2015). Because AD is a heterogeneous skin disease and an unmet medical need, identifying and targeting epidermal biosensors aimed at reducing severe itch and inflamed lesions by natural bioactive compounds may lead to an effective management for AD (Waldman et al., 2018; Wang and Wang, 2017).

Previous investigations have demonstrated that gain-of-function mutations of warm temperature-activated Ca^{2+} -permeable TRPV3 channel (Gly573Cys in WBN/Kob-Ht rats and Gly573Ser in DS-Nh mice) can cause skin inflammation, itching and abnormal hair growth in rodents (Asakawa, Yoshioka, Matsutani, Hikita, Suzuki, Oshima, Tsukahara, Arimura, Horikawa, Hirasawa et al., 2006; Yoshioka, Imura, Asakawa, Suzuki, Oshima, Hirasawa, Sakata, Horikawa and Arimura, 2009). These mutations produce AD-like phenotypes such as inflammatory cell infiltration, keratin thickening and high immunoglobulin E (Nilius and Biro, 2013; Toth, Olah, Szollosi and Biro, 2014; Yamamoto-Kasai, Imura, Yasui, Shichijou, Oshima, Hirasawa, Sakata and Yoshioka, 2012). Our and other's identifications of gain-of-function mutations (G573S, G573C, W692G and G573A) in *TRPV3* gene from patients with congenital

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Olmsted syndrome (OS) characterized by palmoplantar and periorificial keratoderma and severe itching further confirms the causative role of thermoTRPV3 channel in cutaneous sensation and inflammatory skin lesions (Duchatelet, Pruvost, de Veer, Fraitag, Nitschke, Bole-Feysot, Bodemer and Hovnanian, 2014; Lin, Chen, Lee, Cao, Zhang, Ma, Chen, Hu, Wang, Wang et al., 2012). It has also been shown that TRPV3 expression is upregulated in the skin lesions of AD patients (Yamamoto-Kasai, Yasui, Shichijo, Sakata and Yoshioka, 2013) and dermal cells isolated from erythrodermic rosacea (Sulk, Seeliger, Aubert, Schwab, Cevikbas, Rivier, Nowak, Voegel, Buddenkotte and Steinhoff, 2012). These investigations suggest that overactive TRPV3 may play an important role in the pathogenesis of AD and inflammatory skin diseases.

As a member of TRPV subfamily and abundantly expressed in the epidermis and keratinocytes, the polymodal TRPV3 is a non-selective cation Ca^{2+} -permeable channel that is activated by warm temperature ($> 33^{\circ}\text{C}$) and a variety of natural skin sensitizers including plant-derived compounds such as carvacrol, camphor, menthol, thymol and also synthesized small molecule 2-APB (Bang, Yoo, Yang, Cho and Hwang, 2010; Gu, Lin, Hu, Zhu and Lee, 2005; Moqrich, Hwang, Earley, Petrus, Murray, Spencer, Andahazy, Story and Patapoutian, 2005; Nilius and Biro, 2013; Peier, Reeve, Andersson, Moqrich, Earley, Hergarden, Story, Colley, Hogenesch, McIntyre et al., 2002; Stotz, Vriens, Martyn, Clardy and Clapham, 2008; Vogt-Eisele, Weber, Sherkheli, Vielhaber, Panten, Gisselmann and Hatt, 2007; Wang and Wang, 2017). Chemical activation of TRPV3 increases the release of many pro-inflammatory

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factors and inflammatory factors such as ATP (Mandadi, Sokabe, Shibasaki, Katanosaka, Mizuno, Moqrich, Patapoutian, Fukumi-Tominaga, Mizumura and Tominaga, 2009), NO (Miyamoto, Petrus, Dubin and Patapoutian, 2011), PGE2 (Huang, Lee, Chung, Park, Yu, Bradshaw, Coulombe, Walker and Caterina, 2008), , NGF (Yoshioka et al., 2009), IL-1 α (Xu, Delling, Jun and Clapham, 2006), TNF- α and IL-6 (Szollosi, Vasas, Angyal, Kistamas, Nanasi, Mihaly, Beke, Herczeg-Lisztes, Szegedi, Kawada et al., 2018). Conversely, TRPV3 can be inhibited by relatively specific natural compounds Osthole and Forsythoside B (Sun, Sun, Qi, Gao, Wang, Wei and Wang, 2018; Zhang, Sun, Qi, Ma, Zhou, Wang and Wang, 2019), and also commonly used nonspecific inhibitors such as ruthenium red (Nilius, Owsianik, Voets and Peters, 2007), 2, 2-diphenyltetrahydrofuran (Chung, Guler and Caterina, 2005), IPP (Bang, Yoo, Yang, Cho and Hwang, 2011), and 17(R)-resolvin D1 (Bang, Yoo, Yang, Cho and Hwang, 2012). It is of interest that pharmacological inhibition of TRPV3 can reduce cell death induced by overactive TRPV3, and relieve pruritus (Sun et al., 2018; Zhang et al., 2019). All these observations suggest an involvement of overactive TRPV3 function in the progressive pathophysiology of AD, and targeting cutaneous TRPV3 may be effective in reduction of AD lesions and intense pruritus.

In this study we attempted to validate cutaneous TRPV3 as an effective therapeutic target for alleviation of AD-like lesions in mice. Our findings show that TRPV3 is upregulated in skin lesions of AD-like mouse model induced by DNFB. Pharmacological activation of TRPV3 by natural skin sensitizer Carvacrol induces skin inflammation in WT mice, but not TRPV3^{-/-} mice. Specific inhibition of TRPV3

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by natural osthole alleviates skin inflammation induced by chemical DNFB or TRPV3 agonist carvacrol. Our results demonstrate that TRPV3 is involved in the progressive pathology of AD, and topical inhibition of TRPV3 may represent an effective prevention and potential therapy for AD or inflammatory skin diseases.

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Materials and Methods

Animals. The C57BL/6 mice (male, 6-8 weeks old, 20 ± 2 g) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice were bred for at least one week before the experiment, so that they could adapt to the new feeding environment. The mice were maintained under stable environment which temperature was kept at $22 \pm 2^\circ\text{C}$. Food and water were obtained arbitrarily, and there was a normal 12-hour circadian rhythm every day. TRPV3^{-/-} mice (female, 8-10 weeks old, 23 ± 3 g) were gifts from Dr. Yong Yang (Peking University First Affiliated Hospital) and Ardem Patapoutian (Scripps Research Institute). TRPV3-knockout mice were genotyped by PCR according as the protocol reported previously (Moqrich et al., 2005; Sun et al., 2018). Briefly, mouse TRPV3 genomic clone was obtained from a 129SVJ BAC library (Genome Systems). The arms of homology were isolated as 4.5 kb and 3.9 kb PCR products using high fidelity Taq polymerase (Invitrogen/GIBCO Life Technologies). The targeting construct was linearized at a unique NotI site and transfected into the CJ7 embryonic stem (ES) cell line derived from 129S1/SV mice. Homologous recombinant clones were identified by Southern blot using probes located at the 5' and 3' end of the construct, and by a neomycin probe. Two of eight targeted clones were injected into C57Bl6/J derived blastocysts. Resulting chimeras were mated to C57Bl6/J females to produce germline-transmission of the allele. Male and female heterozygous mice for the mutated allele were mated to generate the population described in the manuscript previously (Moqrich et al., 2005). All animal tests were authorized by the Institutional

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Animal Care and Use Committee of Qingdao University Health Science Center and were performed in accordance with institutional and national guidelines for the use and care of animals for experiments.

Compounds. Chemical 2, 4-dinitrofluorobenzene (DNFB) in yellow liquid (with MW at 186.10) and natural Carvacrol in pale yellow liquid (with MW: 244.29) were purchased from Sigma-Aldrich Corp (St. Louis, MO, USA). The purity of DNFB is greater than 99%. The purity of Carvacrol is greater than 98%. Osthole in white powder (with MW: 244.29) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). The chemical DNFB was dissolved in solvent (acetone: olive oil= 4:1) (Bhol and Schechter, 2005). Carvacrol was dissolved in saline containing 50% ethanol (Cui, Wang, Wei and Wang, 2018). Stock solution of Osthole (100 mM) was dissolved in DMSO and further diluted in saline for behavior experiments (Sun et al., 2018).

Induction and Treatment of AD in Mice. DNFB was used for induction of dorsal model of atopic dermatitis (AD) in mice as previously described (Bhol and Schechter, 2005; Yuan, Liu, Zhang, Wang and Guo, 2010). Briefly, depilatory cream was used to shave off the hair of mouse dorsal skin with assistance of gas anesthesia device. 15 minutes later, residual depilatory cream on the skin was wiped off with a wet towel before topical application of DNFB for generation of mouse model of AD. Post shaving for 24 hours. 100 μ l of 0.5% DNFB was topically applied to the shaved area of dorsal skin for sensitization. From day 2 to day 4, there was no stimulation until day 5 and 6 when 50 μ l of 0.2% DNFB was topically applied once a day onto the

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same area of the skin. For generation of dorsal AD model, the solvent (acetone and olive oil, 4:1) was used as control.

For generation of an ear model of AD in mice, the procedure is the same as the above dorsal model without shaving with the solvent (acetone and olive oil, 4:1) used as control. Osthole stock solution (100 mM) was serially diluted in saline for 10 to 1000 times before use and 10% DMSO was used as control for treatment procedure. For experiments of tool compound treatments, first application of Osthole in 100 μ l was applied onto dorsal skin or ear 30 minutes before the last challenge with 0.2% DNFB, and subsequent topical applications of Osthole for 4 days once a day until skin tissue sectioning or measurements of ear thickness or swelling score. Osthole suspension was prepared before use for prevention of chemical delamination.

Ear Thickness Measurement and Ear Swelling Score. Ear thickness was first measured 30 minutes after the last application of 0.2% DNFB before Osthole was used for treatment. The thickness was measured at 24, 48, 72 and 96 hours using Vernier caliper after the first measurement. The degree of mouse ear swelling was observed and scored according to the scale of 0-4 points as previously described (0- no swelling; 1- slight swelling; 2- moderate swelling; 3- sever swelling; 4- extremely severe swelling) (Bhol and Schechter, 2005).

Histology. For histopathological examinations, dorsal skin from mice was fixed in 4% paraformaldehyde (4% PFA), dehydrated in ethanol, embedded in paraffin and cut into 5 and 8 μ m thickness. The tissue sections were stored at 4°C before baked at 60°C in an oven for one hour before H&E staining, which can effectively prevent

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tissue detachment from glass slide. For frozen tissue sections, ear tissues were fixed in 4% PFA, dehydrated for 48 hours in 30% sucrose solution and embedded in OCT embedding agent before cut into 10-15 μ m sections. The sliced sections were stored at -20°C and imaged with a bright-field microscopy (ECLIPSE Ti-S, Nikon) with a CCD-camera (DS-Ri2, Nikon).

Immunohistochemistry (IHC). Paraffin-embedded tissue sections were dewaxed and rehydrated before antigens were repaired in EDTA-buffer (pH 9.0) with high pressure and heat restoration for 2.5 minutes. After antigen retrieval tissue sections were incubated with primary antibodies (Abcam, UK) (TRPV3, 1:100, 4°C ; TNF- α , 1:400, 37°C ; IL-6, 1:500, 4°C) before further incubated with secondary antibodies (ZSGB-Bio, China). Immunoreactions were visualized using DAB reagents (ZSGB-Bio, China). Tissue sections were counterstained by hematoxylin (ZSGB-Bio, China). The negative controls were incubated without primary antibodies.

Western Blot (WB). For determination of expression levels of TRPV3, TNF- α and IL-6, skin or ear tissues were mixed with RIPA buffer (Thermo Scientific, USA) and protease inhibitors (Thermo Scientific, USA) at 100:1 per 10 mg tissues. Tissue lysates were centrifuged at 15000 rpm for 15 minutes. BCA kit (Thermo Scientific, USA) was used to quantify protein amount and followed by SDS-PAGE for WB. The protein transfer condition was 150 mA for 120 minutes, and transferred membranes (Millipore, France) were incubated with primary antibodies (TRPV3 antibody, 1:1000, 8% separating gum; TNF- α antibody, 1:1000, 12% separating gum; IL-6 antibody, 1:2500, 12% separating gum) and secondary antibodies (Abcam, UK) at room

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temperature for 1 hour or at 4°C for 6-8 hours before visualization by ECL system (Thermo Scientific, USA).

Generation of AD-like Model by TRPV3 Agonist Carvacrol. Carvacrol was dissolved in saline solution containing 50% ethanol. Shaving procedure was same as DNFB induced model of AD. Then the mice were induced AD-like symptoms by topical application of Carvacrol. Mouse dorsal skin or ear was swabbed with 3% Carvacrol for consecutive 5 days before measurement of ear thickness and ear swelling scores. For induction of mouse ear edema Carvacrol was topically applied twice onto ear with an interval of half an hour in between.

For observation of Osthole effect, intradermal injection of Osthole (3 mM in 100 μ l) into mouse neck was made 30 minutes before topical application with 3% Carvacrol, and subsequent intradermal injections of Osthole were carried out for 4 days once a day.

Evaluation of skin lesions. Dermatitis scores of AD-like skin lesions in mice were evaluated as previously described (Noguchi, Tominaga, Takahashi, Matsuda, Kamata, Umehara, Ko, Suga, Ogawa and Takamori, 2017). Briefly, the severity of dermatitis was assessed according to four symptoms: (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema and (4) excoriation/erosion. Each symptom was scored from 0 to 3 (none, 0; mild, 1; moderate, 2; severe, 3). The score was defined as the sum of the individual scores, ranging from 0 to 12.

Statistical Analysis. All data are expressed as the means $\pm$ S.D. One-way and two-way ANOVA followed by multiple-comparison test was used to evaluate

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statistical significance using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). A value of $p < 0.05$ was considered to be statistically significant. Spearman's rank correlation coefficient method was used to investigate the correlation between the severity of skin inflammation and ear swelling scores. All Western blot, H&E staining and immunohistochemistry experiments were repeated for three times. Protein samples or tissue sections from different mice were used for each repeat.

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Results

Upregulation of TRPV3 in Mouse Model of AD Induced by Topical DNFB. We started generating a typical model of atopic dermatitis (AD) induced by chemical DNFB (Bhol and Schechter, 2005; Han, Moon, Kim and Jeong, 2018; Kumagai, Kubo, Kawata, Kamekura, Yamashita, Jitsukawa, Nagaya, Sumikawa, Himi, Yamashita et al., 2017; Yuan et al., 2010). As illustrated in Fig. 1A, topical 0.5% DNFB in 100 μ l (acetone: olive oil = 4:1) was applied once to dorsal skin area in mice. After 3 days 50 μ l of 0.2% DNFB was applied to the same area of dorsal skin for 2 days once a day. Phenotypic observations of mice revealed that the pimple-like rash in the dorsal skin was developed (Fig. 1B). Histological examination of dorsal skin tissue sections in H&E staining showed the thickening and dermatitic lesions with infiltration of inflammatory cells (n=3) (Fig. 1C) and increased expression of TRPV3, TNF- α and IL-6 determined by Western blot analysis, as compared with the control (n=3) (Fig. 1D). These results indicate that TRPV3 was involved in the development of mouse atopic dermatitis-like model.

Reversal of DNFB-induced Atopic Dermatitis by Selective Inhibition of TRPV3. To further investigate the role of TRPV 3 in DNFB-induced atopic dermatitis (AD), we used a natural anti-pruritic Osthole that specifically inhibits TRPV3 (Sun et al., 2018), and tested the effect of topical Osthole on dorsal skin inflammation in DNFB-induced AD model in mice. H&E staining revealed that topical applications of Osthole resulted in a dose-dependent reduction of skin inflammation as determined by thickness measurement of skin tissue sections, compared with AD model of DNFB

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group (n=3, *** $p<0.001$) (Fig. 2, A and B). Western blot analysis showed a reversal of increased expressions of TRPV3, pro-inflammatory factors TNF- α and IL-6 from AD model by topical 10 mM Osthole (n=3, *** $p<0.001$) (Fig. 2, C and D), which was further confirmed by immunohistochemical staining of skin tissue sections (n=3) (Fig. 2 E). These results demonstrate that topical inhibition of TRPV3 by Osthole can attenuate DNFB-induced skin inflammation.

Attenuation of DNFB-induced Ear Swelling by Topical TRPV3 Inhibitor Osthole. To confirm the observation that attenuation of DNFB-induced skin inflammation by TRPV3 inhibition, we generated and utilized another mouse model of ear swelling induced by DNFB, and further tested the effects of Osthole on TRPV3 expression and ear inflammation. As shown in Fig. 3A, ear skin sensitization was induced by a topical application of 0.5% DNFB before further challenged by two-time topical applications of 0.2% DNFB. Topical application of DNFB onto right ear resulted in a significant increase of ear swelling, as measured by Vernier caliper (n=13 mice in each group, * $p<0.05$, *** $p<0.001$) (Fig. 3B). Ear tissue sections further confirmed the ear swelling induced by DNFB (n=3) (Fig. 3C), and TRPV3 expression in tissues of swollen ear was also upregulated by Western blot analysis (n=3) (Fig. 3D).

To examine the effect of Osthole on inflammation, we swabbed different concentrations of Osthole (0.1-10 mM) onto the right swollen ear induced by DNFB for consecutive 4 days (Fig. 3A). Vernier caliper reading revealed that the treatment of Osthole caused a significant reduction of ear swelling induced by DNFB on day 4 and

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day 5 in dose-dependent manner ($n=9\sim13$, $***p<0.001$) (Fig. 4A). To further evaluate the relationship between the severity of skin inflammation and ear swelling scores, we calculated the correlation coefficients for ear swelling scores and ear thickness. As expected, the ear swelling scores were positively correlated with the ear thickness ($r=0.7002$, $***p<0.0001$, two-tailed, Gaussian Approximation, $\alpha=0.05$) (Fig. 4B). Further examination of tissue sections from right ears in H&E staining showed reduction of swollen ears as compared from DNFB group or control group ear tissue sections ($n=3$) (Fig. 4C). These results indicate that inhibition of TRPV3 by Osthole can attenuate the development of ear inflammation induced by DNFB.

Suppression of TRPV3 Attenuates Skin Lesions Induced by TRPV3 Agonist Carvacrol. The gain-of-function mutations of TRPV3 cause AD-like lesions in rodents and humans. To further examine the causative role of overactive TRPV3 in dermatitis, we topically applied 3% Carvacrol onto the shaved dorsal skin or left ear for consecutive 5 days (Fig. 5A). In WT mice, topical application of 3% Carvacrol on the lower part of shaved dorsal skin resulted in AD-like lesions compared with the upper part of the skin in the same mouse (Fig. 5B left panel). In contrast, topical application of 3% Carvacrol had no effect on inducing inflammation in dorsal skin area in TRPV3 knockout mice (Fig. 5B right panel). Vernier caliper reading revealed that topical 3% Carvacrol caused the time-dependent development of ear swelling in WT mice, but not in TRPV3 knockout mice ($n=5$, $***p<0.001$) (Fig. 5C). We also calculated the correlation coefficients for ear thickness and swelling scores which were positively correlated with the ear thickness ($r=0.9221$, $***p<0.0001$, two-tailed,

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Gaussian Approximation, $\alpha=0.05$) (Fig. 5D). Similarly, intradermal injection of Osthole (3 mM in 100 μ l) into mouse neck half an hour before challenge by topical Carvacrol (3%) also reduced skin inflammation scores as compared with Carvacrol (3%) treated group (n= 5, $**p<0.01$, $***p<0.001$) (Fig. 5E and F). These results demonstrate that genetic ablation or pharmacological inhibition of TRPV3 prevents development of skin lesions, suggesting TRPV3 as potential therapeutic target for treatment of atopic dermatitis.

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Discussion

The current therapeutic strategy for atopic dermatitis (AD) is primarily aimed at reducing skin lesion severity and recurrence as the pathogenesis of AD is complex (Marsella and De Benedetto, 2017; Waldman et al., 2018). Therefore, our goal of this study was attempted to seek a preventive and therapeutic strategy by validating thermoTRPV3 channel as a target for AD. We took the advantage of both natural skin sensitizer Carvacrol and specific inhibitor Osthole that can modulate TRPV3 channel function (Cui et al., 2018; Sun et al., 2018), and tested the notion that warm temperature-activated Ca^{2+} -permeable TRPV3 channel is critically involved in the pathology of AD. Our findings demonstrate the critical involvement of TRPV3 in development and progression of inflammatory skin lesions. We propose that topical inhibition of TRPV3 channel function by natural Osthole may represent an effective promising strategy for management of AD aimed at preventing or alleviating skin lesions and severe itching (Fig. 6).

Both hypotheses of epidermal barrier dysfunction and immune abnormalities have been debated and suggested to be detrimental for the pathogenesis of AD. Accumulating evidence supports the view that TRPV3 as a cutaneous thermosensor plays an essential role in skin physiology and pathology. TRPV3 is mainly distributed in the skin, and its gain-of-function mutations can cause defective skin barrier formation, AD-like phenotypes in rodents and symptoms of severe skin lesions and itching in humans with congenital Olmsted syndrome (OS) (Asakawa et al., 2006; Lin et al., 2012; Yoshioka et al., 2009). In addition to the described inflammatory skin

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symptoms, Olmsted syndrome patients carrying a gain-of-function mutation of TRPV3 (G573A) also exhibit multiple immune dysfunctions including increased follicular T cells, persistent eosinophilia and elevated immunoglobulin E (Nilius, Biro and Owsianik, 2014). Activation of TRPV3 also triggers a strong proinflammatory response in human epidermal keratinocytes through the NF- κ B pathway (Szollosi et al., 2018), and stimulates production of inflammatory factors and inhibits production of lipids contributing dry skin dermatoses (Szanto, Olah, Szollosi, Toth, Payer, Czako, Por, Kovacs, Zouboulis, Kemeny et al., 2019). In this study we also observed a massive migration of adipocytes to inflammatory skin in response to topical application of DNFB as adipocytes are motile and can actively migrate to skin wounds for repair and prevention from further infections (Franz, Wood and Martin, 2018). All these lines of evidence support the view that thermoTRPV3 is a key player in the progressive pathogenesis of AD.

Chemical DNFB-induced skin and ear swelling models have been extensively used in the study of AD pathogenesis (Han et al., 2018; Heo, Nam, Seo and Lee, 2011; Inagaki, Shiraishi, Igeta, Itoh, Chikumoto, Nagao, Kim and Nagai, 2006; Kumagai et al., 2017; Shin, Son, Kim, Heo, Lee and Kim, 2015). Topical application of DNFB causes significant ear edema with inflammatory cell infiltration in DS-Nh mice that carry the gain-of-function mutation of TRPV3 (G573S) (Yamamoto-Kasai et al., 2013). DNFB-induced phenotypes of AD-like skin lesions also include keratin thickening with biochemical changes of upregulation of inflammatory factors and elevated immunoglobulin E (Yamamoto-Kasai et al., 2013) and such a model was

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used to evaluate anti-inflammatory activities for natural compounds such as aloperine, *uncariae rhynchophylla* and also endogenous substances (alpha-Lipoic acid) (Kim, Jung, Kim, Seo, Jung and Park, 2009; Kim, Kim, Jang, Ahn, Park and Park, 2011; Yuan et al., 2010). However, there are also reports that inhibition of TRPV1 promotes recovery of skin barrier and reduces the expression of inflammatory factors (Lee, Choi, Bae, Choi, Park and Park, 2018; Yun, Seo, Jeong, Bae, Jang, Lee, Kim, Shin, Woo, Lee et al., 2011). In addition, topical camphor is used for treatment of local itch through agonist-induced desensitization of TRPV3 (Misery, 2016; Sherkheli, Benecke, Doerner, Kletke, Vogt-Eisele, Gisselmann and Hatt, 2009), and topical long-term application of extracts from *Tribulus terrestris* plant also reduces oxazolone-induced atopic dermatitis likely through desensitization of TRPV3 activity (Kang, Jung, Nam, Kim, Kang, Kim, Cho, Cho, Park and Bae, 2017).

In summary, we show that pharmacological activation of TRPV3 by natural skin sensitizer Carvacrol induces AD-like phenotypes in mice, but not TRPV3^{-/-} mice. Conversely, inhibition of TRPV3 by natural Osthole alleviates skin lesions and ear edema induced by chemical DNFB that is widely used for generation of AD-like models. Our findings provide a strong evidence that topical inhibition of TRPV3 channels may represent an effective prevention and therapeutic strategy for management of AD or inflammatory skin lesions.

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Author contributions

Participated in research design: YX Qu, XY Sun, KW Wang

Conducted experiments: YX Qu, GX Wang, XY Sun

Performed data analysis: YX Qu, XY Sun

Wrote and revised the manuscript: YX Qu, XY Sun, KW Wang

Conflict of interest

The authors declared no conflicts of interest.

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Figure legends

Fig. 1. Topical applications of chemical DNFB induce dorsal skin inflammation and up-regulation of TRPV3 in mice. (A) Schematic drawing of experimental procedures for generation of mouse AD model of dorsal skin by topical applications of chemical DNFB, and treatment with TRPV3 modulator. **(B)** Phenotypic features of dorsal skin with and without treatment of DNFB at day 7. **(C)** Representative histological H&E staining of paraffin-embedded tissue sections (6 μ m thickness) from mouse dorsal skin with infiltration of inflammatory cells and thickening in DNFB group (n=10 mice in each group). Bar=500 μ m. **(D)** Western blot analysis of expression levels of TRPV3 (~90 kDa), TNF- α (26 kDa) and IL-6 (17 kDa) in lysates of dorsal skin tissues (n=3 repeats).

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Fig. 2. Inhibition of TRPV3 by natural Osthole reverses DNFB-induced atopic dermatitis (AD). **(A)** Representative histological images of H&E staining of paraffin-embedded sections (5~8 μm , n=9~13 mice in each group) from mouse dorsal skin with and without topical skin sensitizer DNFB and treatment of TRPV3 inhibitor Osthole in different concentrations. Bar=500 μm . **(B)** Thickness of skin tissue sections from panel A (n=9~13 mice in each group, *** p <0.001, by one-way ANOVA, followed by Bonferroni's test). Data are presented as the means $\pm$ S.D. **(C, D)** Western blot analysis of expression levels of TRPV3, TNF- α and IL-6 of mouse dorsal skin tissues (n=3 repeats, *** p <0.001, by one-way ANOVA, followed by Bonferroni's test). **(E)** Immunohistochemical staining of TRPV3, TNF- α and IL-6 in paraffin-embedded sections of mouse dorsal skin tissues (5~8 μm , n=3 repeats). Bar=100 μm .

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Fig. 3. Topical applications of DNFB induce mouse ear edema and TRPV3 up-regulation. (A) Schematic drawing of experimental procedures for generation of mouse AD model of ear edema by topical applications of DNFB and treatment with TRPV3 inhibitor Osthole in different concentrations. **(B)** In vivo measurement of ear thickness in mice from panel A with and without topical applications of DNFB (n=13 mice in each group, * $p < 0.05$, *** $p < 0.001$, by two-way repeated measures ANOVA, followed by Bonferroni's test). Data are presented as the means $\pm$ S.D. **(C)** Histological imaging of frozen ear tissue sections (10 μ m) with thickening from AD mice (n=3 repeats). Bar=500 μ m. **(D)** Western blot analysis of TRPV3 protein expression in ear tissues from control and DNFB treated mice (n=3 repeats).

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Fig. 4. Inhibition of TRPV3 by Osthole alleviates DNFB-induced ear swelling. (A)

In vivo measurement of ear thickness in mice treated with and without topical DNFB and treatment of TRPV3 inhibitor Osthole in different concentrations (n=9~13 mice in each group, *** $p < 0.001$, by two-way repeated measures ANOVA, followed by Bonferroni's test). Data are presented as the means $\pm$ S.D. **(B)** The scatter plot illustrating correlation between the ear thickness and ear swelling scores (n=59 mice, Spearman's rank correlation coefficient method, $r = 0.7002$, *** $p < 0.0001$, two-tailed, Gaussian Approximation, $\alpha = 0.05$). **(C)** Representative histological H&E staining of frozen ear tissue sections (10~15 μm , n=3 repeats)) from mice treated with and without topical DNFB and treatment of TRPV3 inhibitor Osthole in different concentrations. Bar=500 μm .

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Fig. 5. Suppression of TRPV3 function attenuates skin lesions and ear edema induced by TRPV3 agonist Carvacrol. (A) Schematic drawing of experimental procedures for generation of mouse AD-like models of dorsal skin lesions and ear edema by topical applications of Carvacrol (3%) for consecutive 5 days. **(B)** Phenotypic features of dorsal skin lesion in WT and TRPV3^{-/-} mice treated with topical Carvacrol for consecutive 5 days. **(C)** In vivo measurement of ear thickness in WT and TRPV3^{-/-} mice with or without Carvacrol (Car) (n=5 mice in each group, *** $p < 0.001$, by two-way repeated measures ANOVA, followed by Bonferroni's test). Data are presented as the means $\pm$ S.D. **(D)** The scatter plot illustrating correlation between the ear thickness and ear swelling scores (n=20 mice, Spearman's coefficient method, $r = 0.9221$, *** $p < 0.0001$, two-tailed, Gaussian Approximation, $\alpha = 0.05$). **(E)** Phenotypic features of dorsal skins before and after topical applications of Carvacrol (3%) for consecutive 5 days (middle panel) with and without intradermal injections of 3% DMSO (left panel) and 3 mM Osthole (right panel). **(F)** Scoring atopic dermatitis from (E) (n= 5 mice in each group, ** $p < 0.01$, *** $p < 0.001$, by two-way ANOVA, followed by Bonferroni's test).

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Fig. 6. A proposed mechanism underlying temperature-sensitive Ca^{2+} -permeable TRPV3 channel in the progressive pathology of atopic dermatitis. For overactive TRPV3-mediated inflammatory signaling, activation of TRPV3 by agonist Carvacrol or gain-of-function mutations (G573S, G573C, W692G and G573A) leads to an elevation of intracellular calcium. In the presence of Ca^{2+} , the phosphorylation of I κ B α (inactivation) and p65 (activation) results in translocation of p65 to the nucleus in the skin keratinocytes, thus activating NF- κ B pathway for inducing the production and release of inflammatory factors aggravates the pathology of AD or skin inflammation. Inhibition of TRPV3 by natural Osthole or silencing reduces the release of inflammatory factors. Topical inhibition of TRPV3 channel function may represent an effective prevention and management for treatment of AD by alleviation of skin lesions, edema and severe itching or other inflammatory skin diseases.

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Figure 1

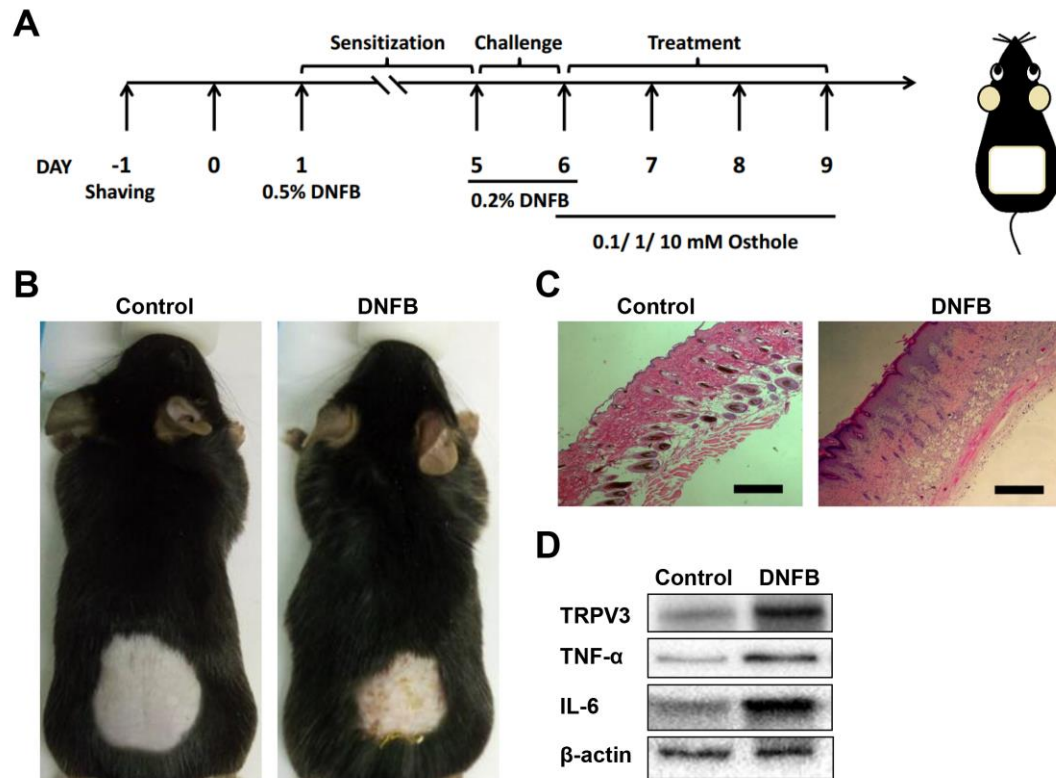


Figure 2

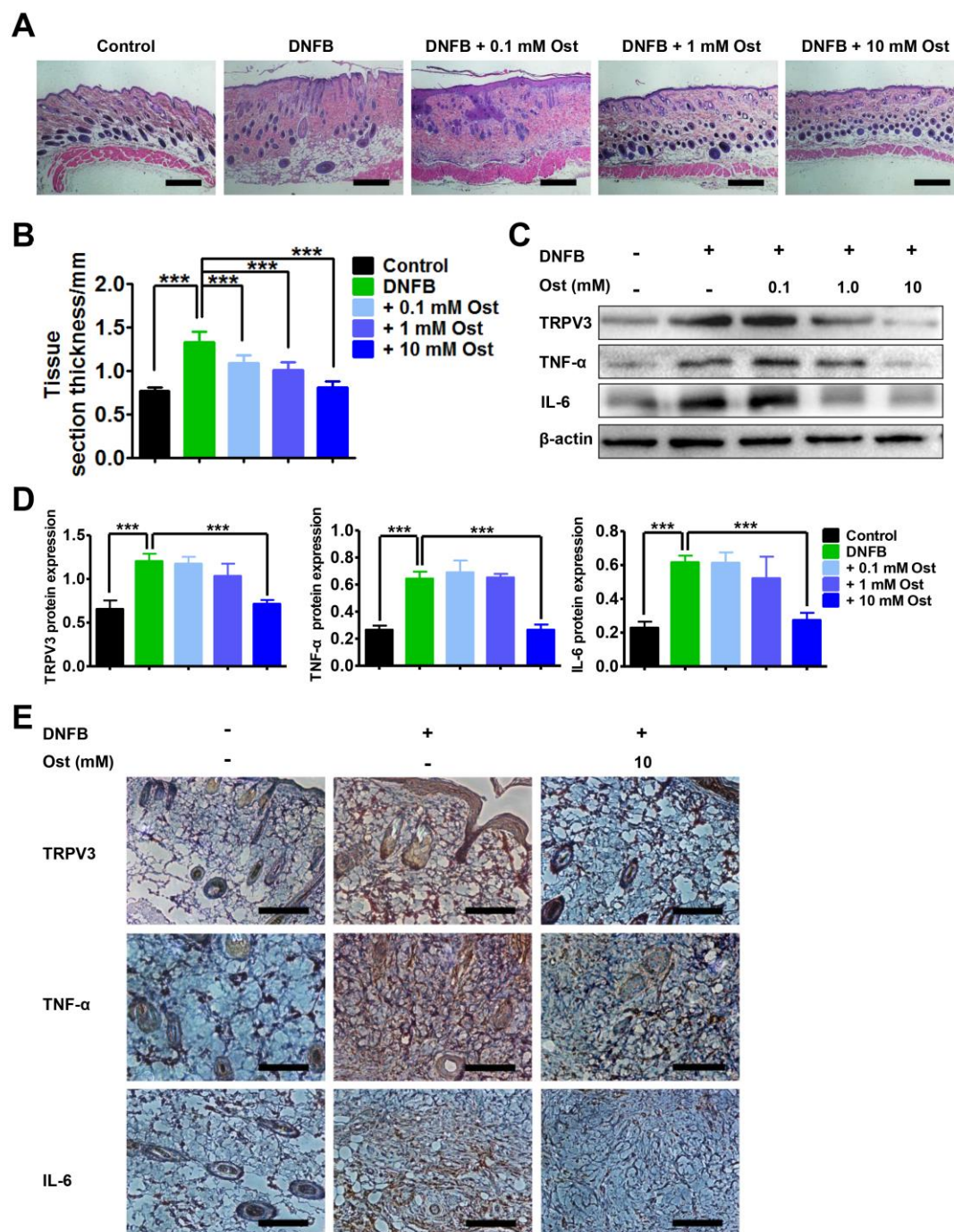


Figure 3

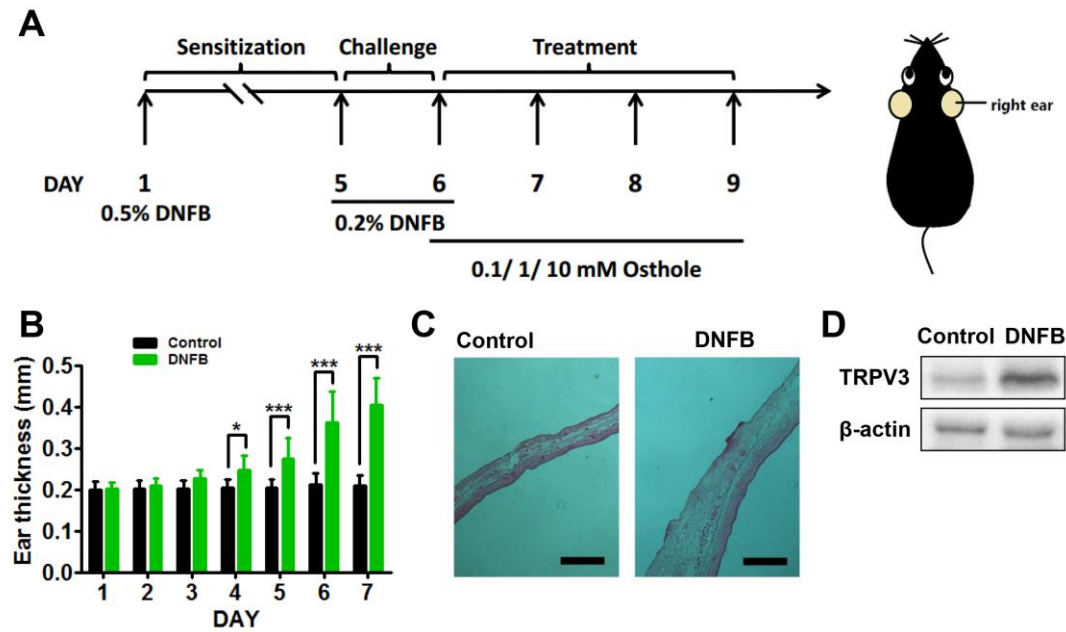


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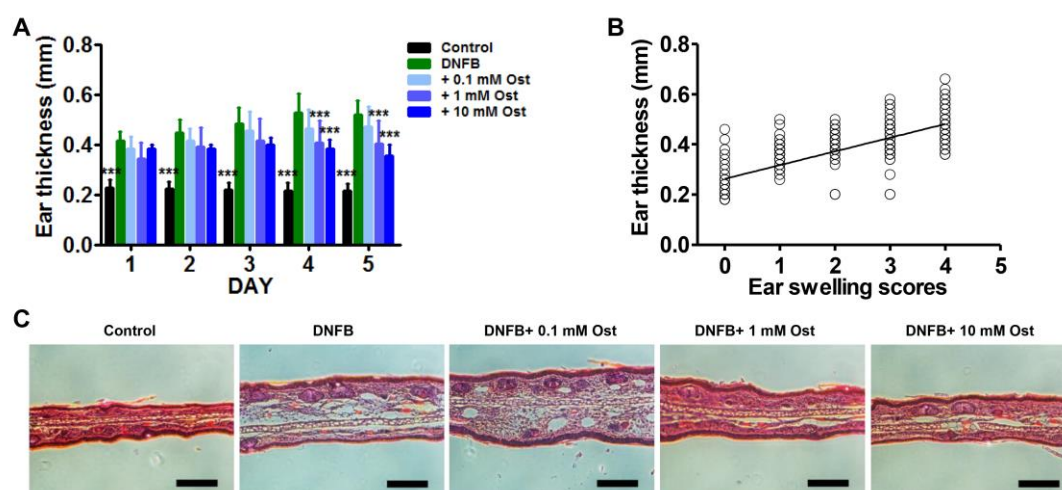


Figure 5

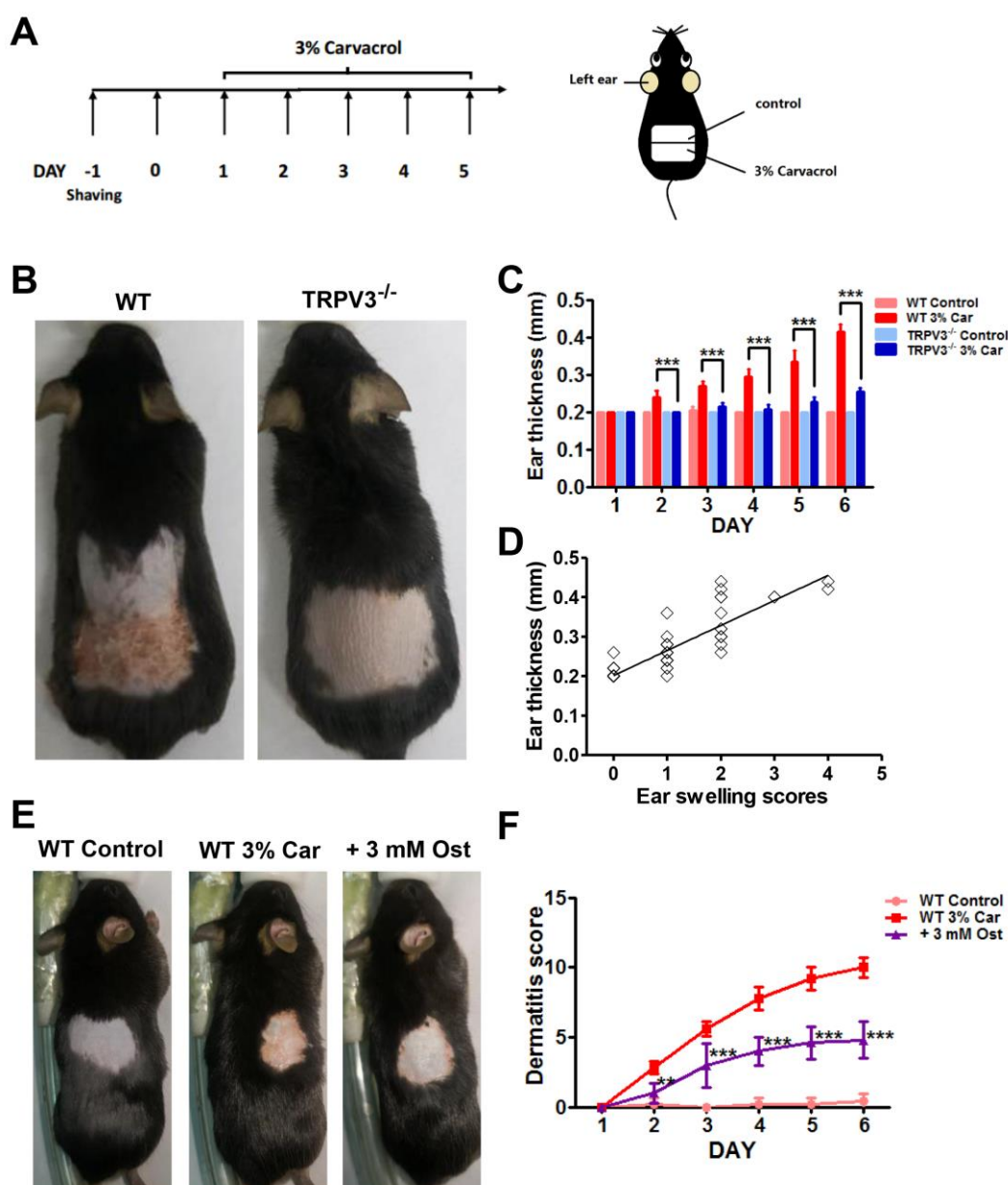


Figure 6

