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Original Article

Oxidative stress induced TSLP production via TRPV4 regulates type 2 inflammation and pruritus in MC903 induced atopic dermatitis mouse model

Keiji Kosaka [a], Akihiko Uchiyama [a,*], Yuta Inoue [a], Mai Ishikawa [a], Takeshi Araki [a], Shintaro Saito [a], Akiko Sekiguchi [a], Yoko Yokoyama [a], Sachiko Ogino [a], Ryoko Torii [a], Yuki Watanuki [a], Koji Shibasaki [b], Sei-ichiro Motegi [a]

[a] Department of Dermatology, Gunma University Graduate School of Medicine, Maebashi, Japan
[b] Laboratory of Neurochemistry, Department of Nutrition Science, University of Nagasaki, Nagasaki, Japan

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ABSTRACT

Background: Transient receptor potential vanilloid 4 (TRPV4) is a calcium ion channel that is widely expressed in various cells, and it regulates multiple physiological and pathological processes. In skin, TRPV4 senses temperature, mechanical and chemical stimuli. Although TRPV4 has been shown to regulate inflammatory in psoriasis, its role in atopic dermatitis (AD) remains unclear.
Objective: We aimed to elucidate the role of TRPV4 is AD pathogenesis and its potential as therapeutic target.
Methods: We used human skin samples from healthy and patients with AD for immunostaining. TRPV4 knock out (KO) mice and MC903-induced AD mouse models were used in vivo. HaCaT cells were used in vitro.
Results: TRPV4 was highly expressed in keratinocytes in lesional skin site of AD. TRPV4 KO mice had less severe dermatitis, barrier dysfunction and pruritus than WT mice in MC903-treated mouse model. TRPV4 KO mice had significantly decreased mRNA expression of type 2 inflammatory cytokines, including TSLP, interleukin (IL)-4, IL-13, and IL-31 via qPCR, and reduced protein levels of TSLP and IL-4 by ELISA. In vitro, oxidative stress promoted expression and activation of TRPV4, following enhanced TSLP expression in HaCaT cells. However, stimulation with IL-4 and IL-13 inhibited TRPV4 activation in HaCaT cells. Finally, treatment with selective TRPV4 antagonist HC-067047 significantly reduced the severity of MC903-induced AD-like dermatitis.
Conclusion: Our findings showed that TRPV4 mediates the expression of keratinocyte-derived TSLP and increases Th2 immunity and pruritus, highlighting TRPV4 as a novel therapeutic strategy for the treatment of AD.

1. Introduction

Atopic dermatitis (AD) is a common chronic and relapsing inflammatory skin disorder, underpinned by a complex interplay of genetic susceptibility, skin barrier impairment, type 2 immune polarization, and persistent pruritus [1]. Keratinocytes play a pivotal role in maintaining cutaneous homeostasis, in part through the production of filaggrin, a critical structural protein involved in epidermal hydration and the maintenance of barrier integrity. In addition, keratinocytes contribute to the formation of tight junctions, which function to minimize transepidermal water loss (TEWL) and

Abbreviations: AD, Atopic dermatitis; Ca^{2+}, Calcium ion; DCs, Dendritic cells; ELISA, Enzyme-linked immunosorbent assay; EtOH, Ethanol; H&E, Hematoxylin and eosin; HRP, Horseradish peroxidase; IL, Interleukin; KO, Knockout; MC903, Calcipotriol; PBS, Phosphate buffered saline; QPCR, Quantitative polymerase chain reaction; SiRNA, Small interfering RNA; TEWL, Transepidermal Water Loss; TSLP, Thymic stromal lymphopoietin

* Correspondence to: Department of Dermatology, Gunma University Graduate School of Medicine, 3-39-22, Showa, Maebashi, Gunma 371-8511, Japan.
E-mail address: akihiko1016@gunma-u.ac.jp (A. Uchiyama).

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provide a physical barrier against the penetration of environmental allergens and microbial pathogens. Various external and internal stimuli, including pro-inflammatory cytokines, allergens, irritants, environmental pollutants, and cigarette smoke, activate keratinocytes, leading to the release of epithelial-derived cytokines known as alarmins, such as interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP). These alarmins activate innate lymphoid cells (ILC2s), thereby initiating type 2 immune responses within the dermis [2].

TSLP, a member of IL-2 cytokine family, is primarily expressed by keratinocytes in the skin. Its receptors are found on a variety of cell types, including T cells, dendritic cells (DCs), mast cells, and keratinocytes. Upon stimulation, TSLP-activated DCs promote T helper 2 (Th2) cell differentiation through the production of chemokines such as CCL17 and CCL22. TSLP can also directly act on $CD4^+$ T cells by inducing transcriptional programs associated with Th2 polarization [3–5]. Th2 cell-derived cytokines, including IL-4, IL-13, and IL-31, downregulate the expression of barrier-related proteins such as filaggrin and claudin-1 in keratinocytes, and promote pruritus, thereby exacerbating epidermal barrier dysfunction [6]. These cytokines also stimulate B cells to produce immunoglobulin E (IgE), which binds to mast cells and triggers the release of histamine and other inflammatory mediators [7]. Furthermore, TSLP, IL-4, IL-13, and IL-31 activate sensory neurons and induce pruritus, leading to scratching behavior that further impairs skin barrier integrity [8]. Consequently, signaling through TSLP, IL-4, IL-13, and IL-31 is considered central to the pathogenesis of AD, and therapeutic strategies targeting these cytokines have led to the development and clinical implementation of novel antibody-based therapies [9]. The transient receptor potential (TRP) channel family consist of six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPP (polycystic), and TRPML (mucolipin). These channels act as pivotal cellular sensors and induce Ca^{2+} influx in response to various physical and chemical stimuli to contribute to chemosensation, cell proliferation, and immune responses, across multiple organ systems [10–13]. In the skin, TRPV4 is expressed by various cell types, including keratinocytes, immune cells, melanocytes, and sensory neurons [14–16]. The activation of TRPV4 is a key factor affecting skin barrier homeostasis, cell proliferation, immune and itch signals [15–19]. TRP channels are also involved in various skin disorders, such as psoriasis, wound healing and AD [11]. Previous studies have shown that ruthenium red, a TRPV1 antagonist, alleviates itch-associated scratching behavior and improves skin barrier dysfunction in a DNFB-induced dermatitis model [20]. In clinical trial, Asivatrep, a topical TRPV1 antagonist, significantly reduced disease severity in patients with AD compared to vehicle [21]. Although TRPV4 is known to play multiple critical roles in skin homeostasis and is implicated in pathological processes such as carcinogenesis, inflammation, fibrosis, and pruritus [22–25], its precise role in the pathogenesis of AD remains unclear.

Hence, the present study aimed to investigate the possible role of the TRPV4 channel in the development of AD using an MC903-induced AD-like dermatitis mouse model using TRPV4 KO mouse. Moreover, we investigated the possible therapeutic potential of selective TRPV4 antagonist in the animal model.

2. Method

The detailed protocols and statistical analysis are described in Supplementary materials and Methods online.

2.1. Patients and clinical assessments

Human skin samples were obtained from patients who visited Department of Dermatology, Gunma University Hospital. Human skin biopsy samples from patients with AD and extra skin tissue samples obtained during the surgical treatment from non-AD patients. The study was approved by the institutional review board and the local research Ethics Committee of Gunma University (H2022–169). All patients were adults and provided a written, informed consent before participating in the study. This study was conducted according to the principles of the Declaration of Helsinki.

2.2. Mice

All experiments were approved by the Ethical Committee for Animal Experiments of the Gunma University Graduate School of medicine (24–060), and carried out in accordance with the approved guidelines. TRPV4 Knock out (KO) mice were generated as previously [26,27], and kindly provided Dr. K. Shibasaki (Laboratory of Neurochemistry, University of Nagasaki, Nagasaki, Japan).

2.3. Development of MC903 induced AD-like dermatitis mice model

AD-like skin inflammation was induced using commercially available calcipotriol (MC903; Tocris Bioscience, Bristol, UK) described previously [28,29]. A daily dose of 10 μl/cm^2 (0.1 mM) of MC903 in ethanol (EtOH) or the same volume of EtOH as control was applied on the back of each mouse for 6 consecutive days. Transepidermal water loss (TEWL) was measured using the DERMA-LAB TEWL probe (Cortex Technology) on the back skin. TRPV4 antagonist (HC-067047; Sigma-Aldrich St. Louis, MO) with dilution of 300 μg of HC-067047 in 400 μl phosphate-buffered saline (PBS) or the same amount of PBS as a vehicle was subcutaneously.

2.4. Scratch counting in mouse

Scratching behavior was recorded over a 60-minute period. The first 30 min were allotted for the animals to acclimate to the environment, and the number of scratches during the subsequent 30 min was counted. The number of scratching episodes during observation period, along with the duration and number of scratches per episode, was counted by an experienced researcher. One scratch was defined as the scratching motion until the hind legs made contact with the floor (Supplementary media file).

2.5. Histological examination and immunofluorescence staining

Tissues were fixed in formaldehyde, and embedded in paraffin. Four μm sections were stained with hematoxylin and eosin (H&E). Immunofluorescence staining was performed as previously described [30].

2.6. Cell culture

HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and 10 % fetal bovine serum. Mouse primary keratinocytes were obtained as previously described [31]. Cells were incubated in EMEM medium.

2.7. Ca^{2+} imaging

Ratiometric calcium imaging recording was performed in a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH7.4. Fura Red fluorescence was measured by Fura Red -AM (DOJINDO, Kumamoto, Japan) in a standard bath solution. Fura Red -AM was diluted to 5 μM in the solution with 0.1 % pluronic F-127 (Life Technologies Japan, Tokyo, Japan) then loaded into HaCaT cells by incubating for 1 hr at 37°C with 5 % CO_2. After incubation, excess Fura Red -AM dye was washed out with two rinses of standard solution. Fluorescence images of Fura Red -AM (the 525/650 nm

emmition-ratio excited by 488 nm) were captured at 10 sec intervals for 10 min with an upright fluorescence microscope (BX51WI, Olympus, Tokyo, Japan) equipped with a CMOS camera (Neo, Andor, Tokyo, Japan), electronic filter wheel, and filter controller (MAC6000, Ludl, NY, U.S.A.). All images were acquired using a 40x/0.8 NA Plan Apo objective lens (Olympus, Tokyo, Japan) with a 200–300 ms exposure.

2.8. Statistical analysis

P values were calculated using Student's *t*-test (two-sided) or by one-way analysis of variance followed by Tukey-Kramer test. Data analysis was done with GraphPad Prism (version 10.4.1). Error bars represent standard errors of the mean.

3. Results

3.1. The increased expression of TRPV4 in atopic dermatitis in human and mice model

First, we assessed the expression of TRPV4 in human atopic dermatitis. Hematoxylin and Eosin (H&E) staining using human skin biopsy samples showed the characteristic findings of AD such acanthosis and infiltration of inflammatory cells in the dermis compared with healthy normal skin. Immunofluorescence staining revealed the increased expression of TRPV4 in lesional AD patient's skin compared with normal skin (Fig. 1a, Supplementary Fig. S1). Further, we confirmed that the expression of TRPV4 was upregulated in the MC903-treated AD-like skin lesions relative to normal skin in mice (Fig. 1b). The TRPV4 expressing colocalized with AE1/AE3 positive keratinocytes and $CD4^+$ T-cells in patient with AD (Fig. 1c, d). Hence, TRPV4 may have an important role in the pathogenesis of AD.

3.2. The impaired skin dermatitis and itching of MC903MC903-induced AD-like dermatitis in TRPV4 KO mice

We next examined the effect of TRPV4 on the development and severity of AD-like dermatitis induced by topical application of MC903 (Fig. 2a). None of the WT and TRPV4 KO mice in the vehicle-treated control group showed dermatitis. The cumulative dermatitis score was significantly decreased in TRPV4 KO mice from day 5 to day 7 compared with WT mice in MC903 treated group (Fig. 2b, c, and Supplementary Fig. S2). The elevated TEWL (Transepidermal Water Loss) was significantly suppressed in MC903-treated TRPV4 KO mice compared with that in WT mice at day 7 (Fig. 2d). Furthermore, the number of scratching count values was also significantly suppressed in TRPV4 KO mice at day 5 and day 7 compared with that in WT mice in MC903-treated group (Fig. 2e). However, in the MC903-treated group, there were no significant differences between WT and KO mice in the duration or number of scratches per scratching episode (Supplementary Fig. S3). Thus, TRPV4 may regulate MC903-induced AD-like dermatitis, barrier dysfunction and pruritus.

3.3. The decreased TSLP expression and improved skin barrier dysfunction of MC903-induced AD-like dermatitis in TRPV4 KO mice

Histopathological examination using skin samples collected at day 7 revealed that thickened-epidermis was partially suppressed in TRPV4 KO mice compared with WT mice in MC903-treated mice (Fig. 3a). Immunofluorescence staining revealed that the enhanced TSLP expression in MC903-treated WT mice was markedly decreased in TRPV4 KO mice (Fig. 3b). qPCR assay showed that elevated mRNA levels of TSLP expression in MC903-treated WT mice was significantly decreased in TRPV4 KO mice in the lesional skin site (Fig. 3c). Furthermore, immunofluorescence staining revealed that the expression levels of cornified envelope proteins, including filaggrin, loricrin, and involucrin were inhibited by MC903-treatment, and this degression was improved in TRPV4 KO mice (Fig. 3d-f). Thus, TRPV4 might regulate TSLP expression and skin barrier dysfunction of MC903-induced AD-like dermatitis.

3.4. The impaired type 2 inflammation of MC903-induced AD-like dermatitis in TRPV4 KO mice

We next investigated the effect of TRPV4 on MC903-induced inflammation in mice. After 7 days of treatment, the numbers of $CD4^+$ T-cells, toluidine blue staining$^+$ mast cells in the dermis of MC903-treated mice were higher than those in vehicle-treated control WT mice, and elevated those inflammatory cells were significantly decreased in TRPV4 KO mice (Fig. 4a, b). The numbers of infiltrating $F4/80^+$ macrophages were significantly increased in MC903- treatment, however there was no difference between TRPV4 WT and KO mice (Fig. 4c). We next assessed the protein levels of TSLP and IL-4 in the lesional skin site from MC903-treated mice using ELISA analysis. Result showed that the expression levels of both TSLP and IL-4 were significantly reduced in TRPV4 KO mice compared to WT mice (Fig. 4d). Consistently, qPCR analysis revealed that the mRNA expression of type 2 inflammatory cytokines, including IL-4, IL-13, IL-31, was elevated in MC903-treated WT mice but significantly suppressed in TRPV4 KO mice. The mRNA expression of IL-4Ra was elevated in MC903-treated groups in both WT and TRPV4 KO mice. In contrast, IL-13Rα expression remained unchanged between WT and TRPV4 KO mice, as well as following MC903 treatment (Fig. 4e). Therefore, TRPV4 might regulate type 2 inflammation of MC903-induced AD-like dermatitis.

3.5. The decreased H2O2 production in MC903-induced AD-like dermatitis in TRPV4 KO mice

We previously reported that oxidative stress plays a key role in the progression of AD-like dermatitis in MC903 mouse model [32]. We found that the increased levels of H_2O_2 in lesional skin of MC903-treated WT mice were significantly suppressed in TRPV4 KO mice (Fig. 4f). In contrast, in vitro experiments showed that MC903 treatment significantly increased ROS production in primary keratinocytes derived from both WT and KO mice, with no significant difference between the two groups (Supplementary Fig. S4).

3.6. Oxidative stress increases the TRPV4 expression and induces TSLP production in keratinocytes

Next, we examined the effects of oxidative stress on TRPV4 expression and TSLP production in human keratinocytes using HaCaT cells. qPCR assay revealed that H_2O_2 stimulation significantly increased the the *trpv4* transcript expressions compared with control one (Fig. 5a). We confirmed that the H_2O_2-induced increase in TRPV4 mRNA expression was abolished by treatment with diphenyleneiodonium (DPI), known as pharmacological ROS inhibitor (Fig. 5b). The knock down of the TRPV4 gene using siRNA transfection resulted in approximately 30 % reduction in TRPV4 mRNA expression compared to the siControl group (Supplementary Fig. S5a)·H_2O_2 stimulation significantly increased mRNA TSLP expression, while siTRPV4 transfection significantly suppressed this upregulation (Fig. 5c). While hypoxia increased TRPV4 mRNA expression, it did not alter TSLP expression, and siTRPV4 had no effect on TSLP levels under these conditions (Fig. 5d). Interestingly, stimulation with IL-4 led to a modest reduction in TRPV4 mRNA expression and did not increase ROS production, nor did it alter TSLP expression levels (Fig. 5e, Supplementary Fig. S5b).

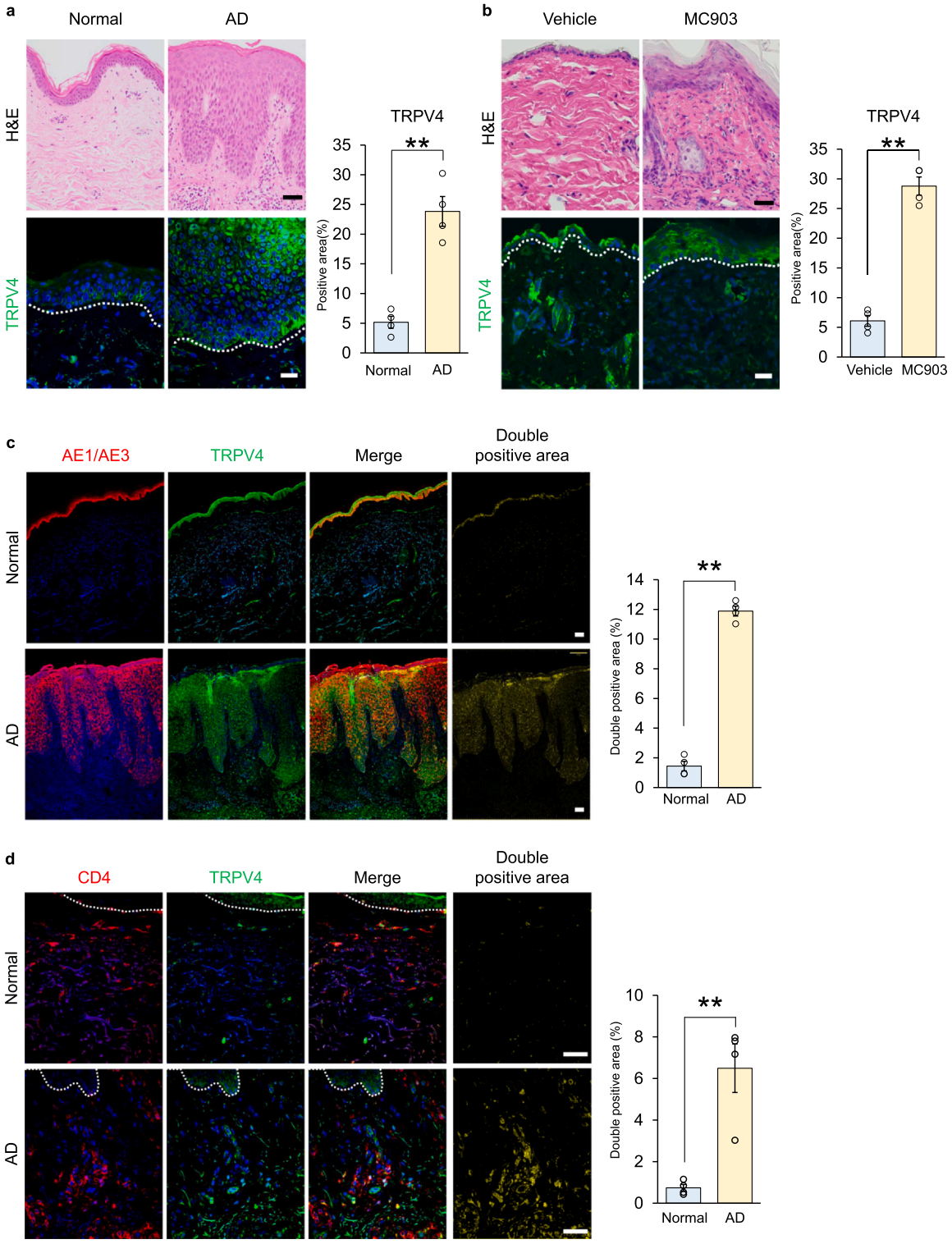

Fig. 1. TRPV4 expression in AD skin site in human and mice. (a-b) Representative H&E (upper panel) and TRPV4 (below panel) stained images of skin sections from biopsy taken from patients with AD (a), and MC903-treated mice at day 6 in vehicle- or MC903-treated WT mice (b). Right panels show the quantification of positive area for TSLP staining with 4 random microscopic fields. n = 4. Epithelium is marked with dotted lines. n = 6. Scale bar = 30 μm. (c-d) Representative images of normal healthy skin and AD patients skin to show the expression of (c) AE1/AE3 (red), (d) CD4 (red), TRPV4 (green), and double positive (yellow) for all. DAPI in blue. Right panels show the quantification of double positive area with 6 random microscopic fields. n = 4. Scale bar = 50 μm. n = 6. * $p < 0.05$, ** $p < 0.01$. Student's T-TEST.

3.7. H_2O_2 stimulation enhances TRPV4 activation but IL-4or IL-13 reduce the activation in keratinocytes

Next, we examined whether oxidative stress can enhance TRPV4 activation in parallel to the increase of the transcripts. After we applied 1 mM H_2O_2 for 3 min 30 sec, we applied 100 nM GSK (GSK1016790A) for chemical TRPV4 agonist. Compared with control group (Fig. 5f; maximal ratio at 600 sec was 1.105 ± 0.01, n = 400), the H_2O_2 application significantly enhanced the TRPV4 activation (Fig. 5f; maximal ratio at 600 sec was 1.192 ± 0.027, n = 450). These

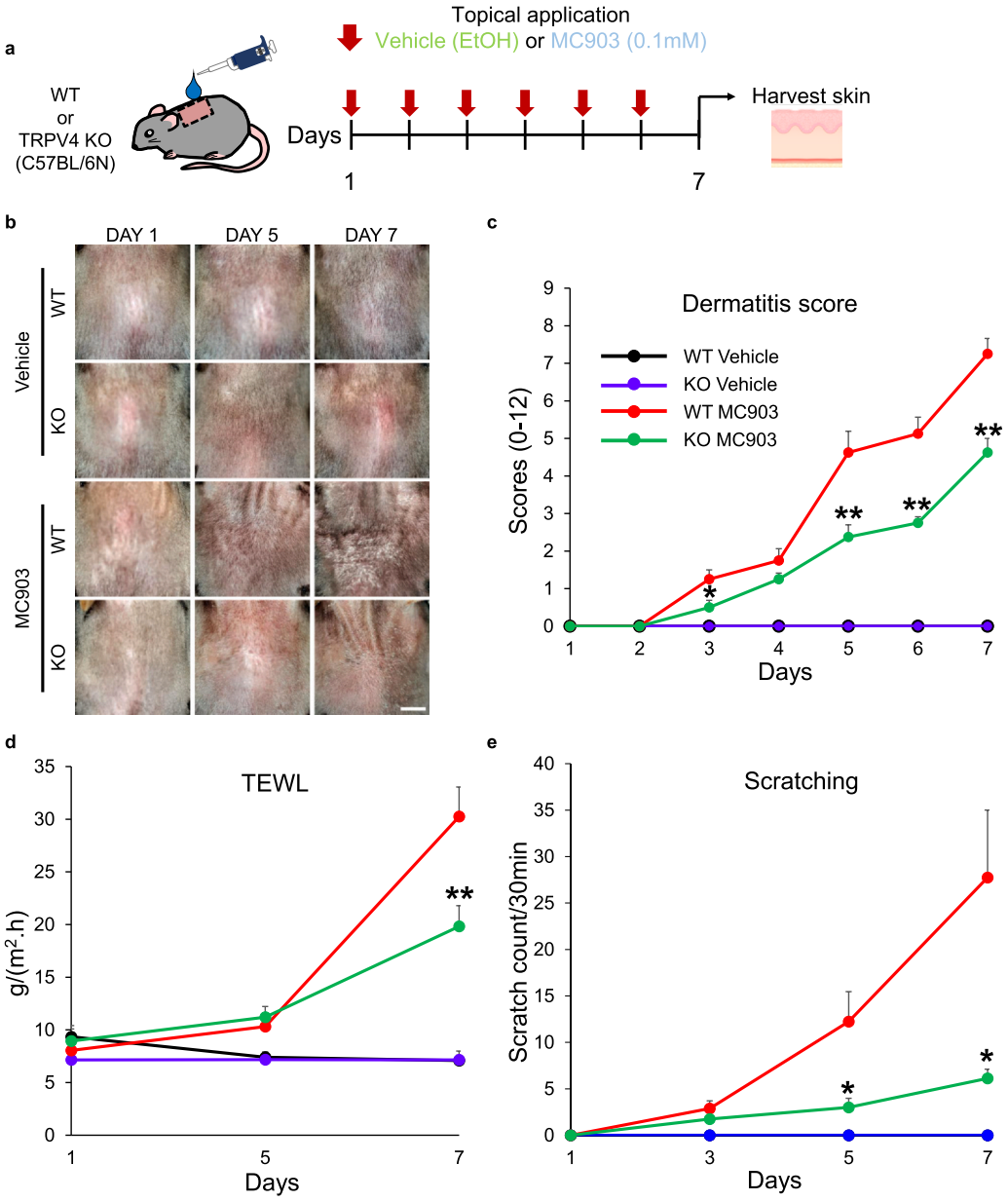

Fig. 2. TRPV4 deficiency attenuated AD-like dermatitis and barrier dysfunction, and itching induced by MC903-treatment. (a) Scheme of the MC903 treatment mouse model. (b) Photographs of skin manifestation at day 1, 5, and 7 in vehicle- or MC903-treated WT and TRPV4 KO mice. Scale bar = 2.5 mm. (c) The cumulative dermatitis score. n = 8. (d) TEWL value at day 1, 5, and 7. n = 8. (e) Scratching count value at day 1, 3, 5, 7. n = 8. * $p < 0.05$, ** $p < 0.01$. One-way ANOVA followed by Turkey post-hoc test.

results indicate that oxidative stress potentiates TRPV4 activation in skin keratinocytes. We also examined whether IL-4 or IL-13 can enhance TRPV4 activation. We pre-incubated the HaCaT cells with human IL-4 (10 ng/mL) or IL-13 (10 ng/mL) for 3 h. After the pre-incubation, we continuously applied IL-4 (10 ng/mL) or IL-13 (10 ng/mL) for the calcium imaging experiments. Compared with control group (Fig. 5g; maximal ratio at 600 sec was 1.606 ± 0.058, n = 400), the IL-4 application significantly reduced the TRPV4 activation (Fig. 5g; maximal ratio at 600 sec was 1.350 ± 0.008, n = 450). Consistent with the IL-4 results, IL-13 also significantly reduced the TRPV4 activation (Fig. 5g; the ratio at 240 sec was 1.227 ± 0.067 (IL-13), n = 450 vs 1.446 ± 0.087 (control)). Taken together with the qPCR results, these results indicate that IL-4 and IL-13 reduces *trpv4* transcription, and leads reduction of the activation in skin keratinocytes.

3.8. TRPV4 antagonist inhibited the development of MC903-induced AD-like dermatitis in mice

Finally, we investigated the therapeutic potential of selective TRPV4 antagonists (HC-067047) in MC903-induced AD-like dermatitis mouse model (Supplementary Fig. S6a). The cumulative dermatitis score of HC-067047-treated mice significantly decreased from days 4–7 compared with that of vehicle-treated mice in MC903 treated groups (Fig. 6a, Supplementary Fig. S6b-e). Similar to TRPV4 KO mice, TEWL and the number of scratching count value were significantly impaired in HC-067047-treated mice compared to vehicle-treated mice in MC903 treated groups (Fig. 6b, c). Histopathological examinations using lesional skin samples revealed that epidermal thickness and the expression of TSLP was impaired in HC-067047-treatemed mice compared with vehicle-treated mice in

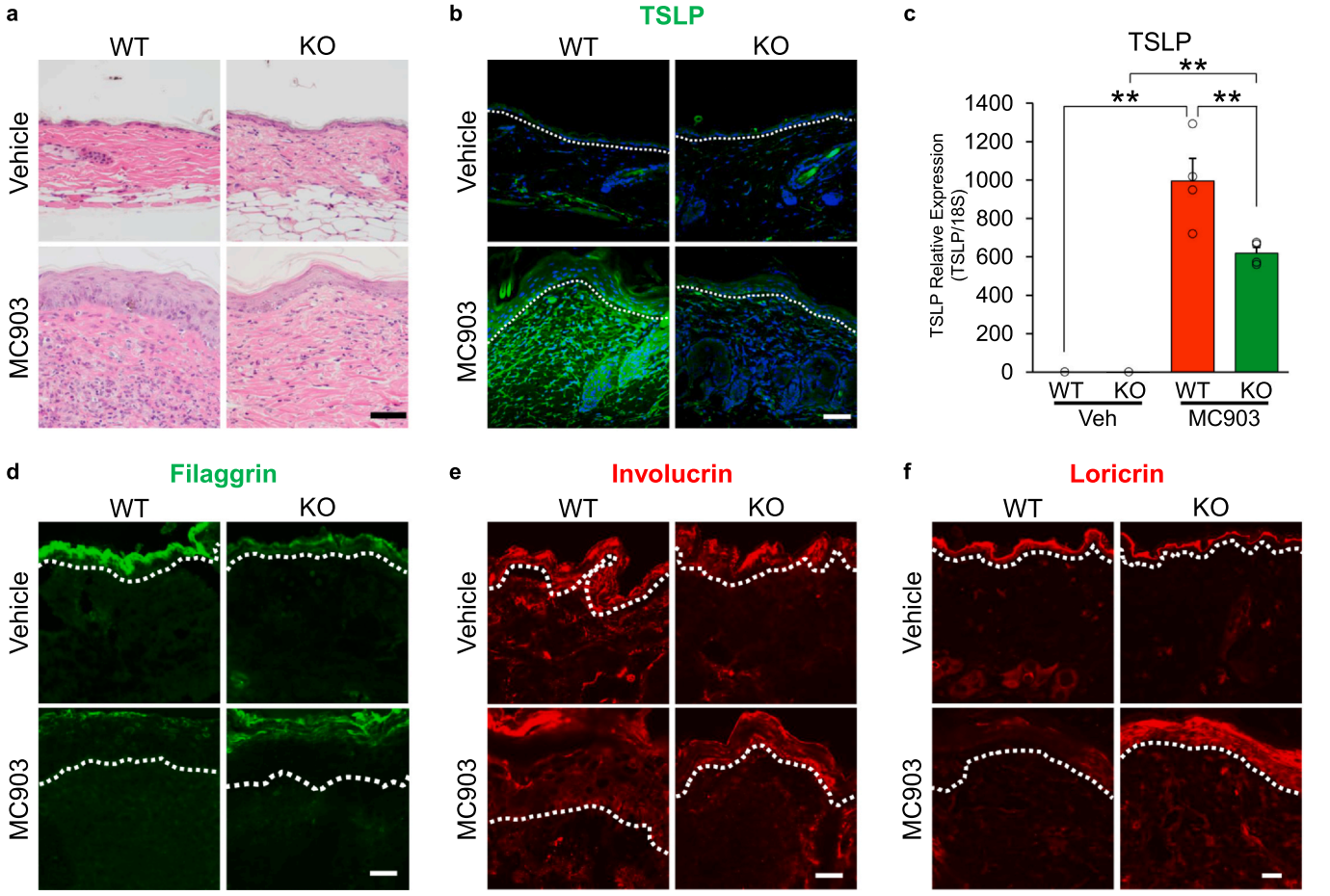

Fig. 3. TRPV4 deficiency improved elevated TSLP expression and skin barrier dysfunction in MC903-treated mice. (a-b) Representative stained images of skin sections taken at day 7 in vehicle- or MC903-treated WT and TRPV4 KO mice for (a) H&E and (b)TSLP. DAPI in blue. n = 6. Scale bar = 40 μm. (c) qPCR quantification of TSLP mRNA levels in lesional skin area at day 7, expressed relative to mRNA levels in vehicle-treated WT mice. All values represent mean ± SEM. n = 4 mice in each group. * $p < 0.05$, ** $p < 0.01$. One-way ANOVA followed by Turkey post-hoc test. (d-f) Representative immunofluorescence staining image of (d) filaggrin, (e) involucrin, and (f) loricrin of skin sections from a biopsy taken at day 7 in each group. n = 4.

MC903 treated groups (Fig. 6d, e). The numbers of infiltrating $CD4^+$ T cells and toluidine $blue^+$ mast cells were significantly decreased in the AD-like skin of HC-067047-treated mice relative to vehicle-treated mice (Fig. 6f). Furthermore, MC903 treatment induced mRNA levels of TSLP and IL-4 expression was significantly suppressed in TRPV4 KO mice (Fig. 6g). Collectively, these findings suggest that TRPV4 contributes to AD pathogenesis by mediating oxidative stress-induced TSLP production in keratinocytes, which in turn promotes type 2 inflammation, mast cell activation, skin barrier impairment, and pruritus (Fig. 6h).

4. Discussion

In this study, we investigated the mechanistic role of TRPV4 in the pathogenesis of AD. In addition, the possible therapeutic effect of TRPV4 antagonist on AD was examined using skin tissue samples collected from an MC903-induced psoriasis-like dermatitis mouse model.

The expression of TRPV4 in the skin of patient with AD was strongly observed in keratinocytes, with some expression also detected in T-cells. Recent studies have revealed that oxidative stress is involved in the pathogenesis of various inflammatory skin diseases, including AD. In addition to ultraviolet radiation and external antigens, physical and psychological factors can also induce oxidative stress in AD [33,34]. Our results showed MC903 induced significantly high accumulation of H_2O_2 in WT but not KO mice. Furthermore, both H_2O_2 and hypoxia stimulation increased TRPV4 mRNA expression in HaCaT cells, and that H_2O_2-induced upregulation of TRPV4 was abolished by DPI treatment. These results suggest that oxidative stress may contribute to the upregulation of TRPV4 in keratinocytes, potentially play a role in the pathogenesis of AD. Another possible factor contributing to the increased expression of TRPV4 could be cell proliferation in the lesional skin of AD. TRPV4 channel may play a role in keratinocyte proliferation and differentiation by facilitating Ca^{2+} influx, which subsequently activates the AKT or MAPK/ERK pathway [22,35–37]. In relation to the mechanisms of cell proliferation, the upregulation of TRPV4 expression may be involved. Regarding differentiation of keraitnocytes, previous study revealed that activation of TRPV4 in keratinocytes regulates tight junction formation and filaggrin expression [15,38]. Immunofluorescence staining looked that filaggrin expression showed slightly decreased trend in stable state in TRPV4 KO mice compared with WT mice. However, there was no difference in TEWL between them. Therefore, the barrier dysfunction in KO mice under stable conditions is considered to be extremely minor, to the extent that it does not manifest a phenotype: however, further investigation is needed to confirm these points.

In our results, the promotion of TSLP production in keratinocytes stimulated by H_2O_2 may be attributed to the enhanced activation of TRPV4 under oxidative stress conditions. However, stimulation with

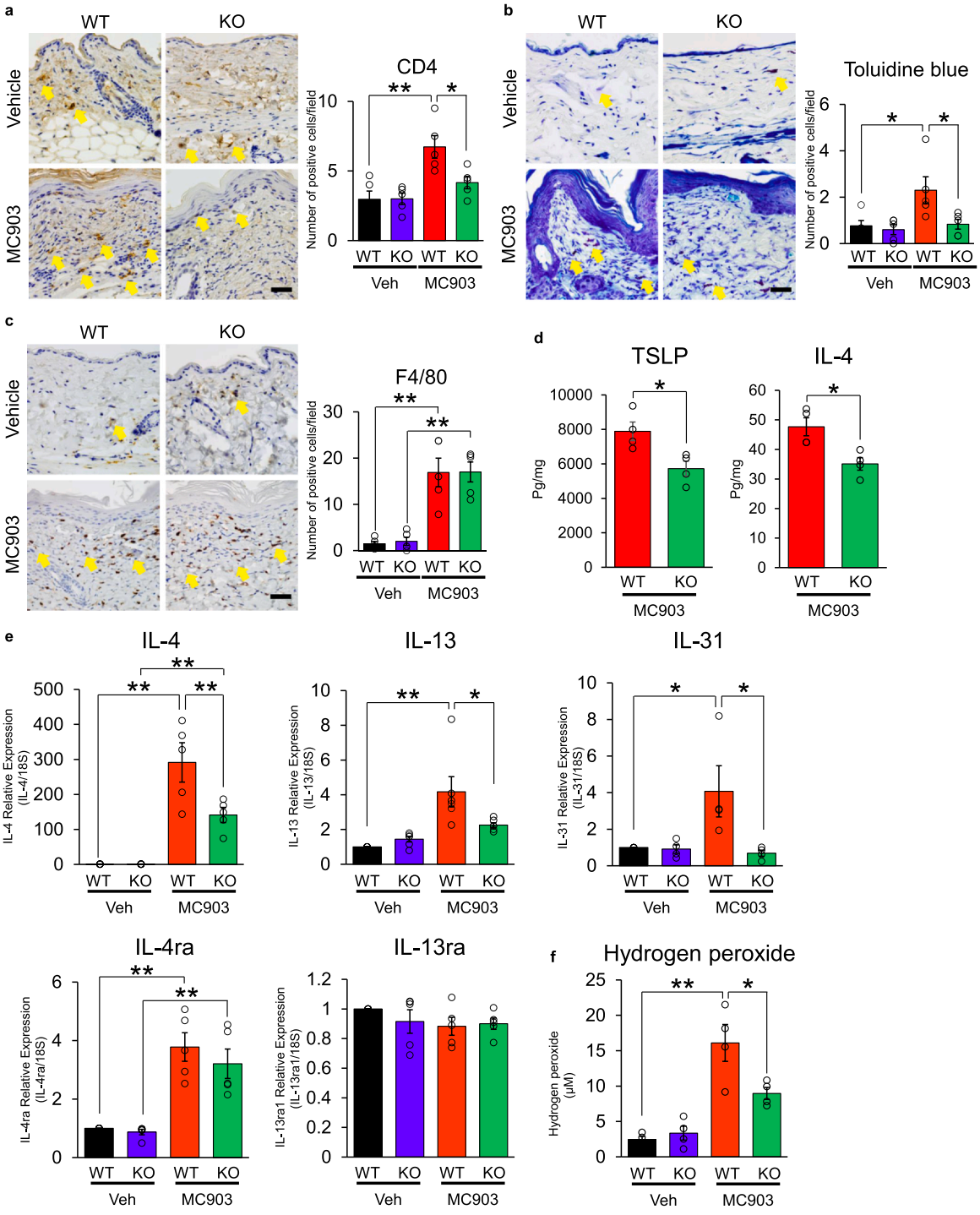

Fig. 4. The impaired type 2 inflammation and H_2O_2 production of MC903-induced AD-like dermatitis in TRPV4 KO mice. (a) Distribution of $CD4^+$ T-cells in the lesional skin site at day 7 in vehicle- or MC903-treated WT and TRPV4 KO mice. (b) Distribution of toluidine blue staining positive mast cells in the lesional skin site at day 7 in each group. (c) Distribution of $F4/80^+$ macrophages in the lesional skin site at day 7 in each group. Arrows indicate positive cells (yellow). (a-c) Right panels show quantification of positive cells per field for each staining in six random microscopic fields. n = 5. (d) ELISA analysis of TSLP and IL-4 in the lesional skin site at day 7 in MC903-treated WT and TRPV4 KO mice. n = 4. (e) qPCR quantification of IL-4, IL-13, IL-31, IL-4Ra, and IL-13Ra mRNA levels in lesional skin area at day 7, expressed relative to mRNA levels in vehicle-treated WT mice. All values represent mean ± SEM. n = 5 for IL-4, IL-4Ra, IL-13Ra, n = 6 for IL-13, and n = 4 for IL-31 in each group. (f) Quantification of H_2O_2 in the lesional skin site at day 7 in vehicle- or MC903-treated WT and TRPV4 KO mice. n = 4. * $p < 0.05$, ** $p < 0.01$. One-way ANOVA followed by Turkey post-hoc test.

IL-4 or IL-13 showed a slight tendency to suppress TRPV4 activation. Previous study revealed that TRPV4 antagonist (HC-067047) stimulation inhibits TRPV4 expression in retina and retinal ganglion cells in rat [39]. Similarly, the mild inhibiting effect of IL-4 on TRPV4 activation could result in the partial downregulation of TRPV4 expression as ROS-independent mechanisms. Regarding the candidate of TRPV4 activator in AD, TRPV4 may be activated by mechanical stimuli associated with scratching. The phospholipase A2 (PLA2)-mediated release of arachidonic acid (AA) and its epoxyeicosatrienoic acids derivative, 5′,6′-epoxyeicosatrienoic acid (5′,6′-EET), has been shown to directly activate TRPV4 [40,41]. Increased levels of AA and its downstream lipid mediators have been observed in AD [42,43]. These results suggest that AA metabolites may contribute to TRPV4 activation in AD skin.

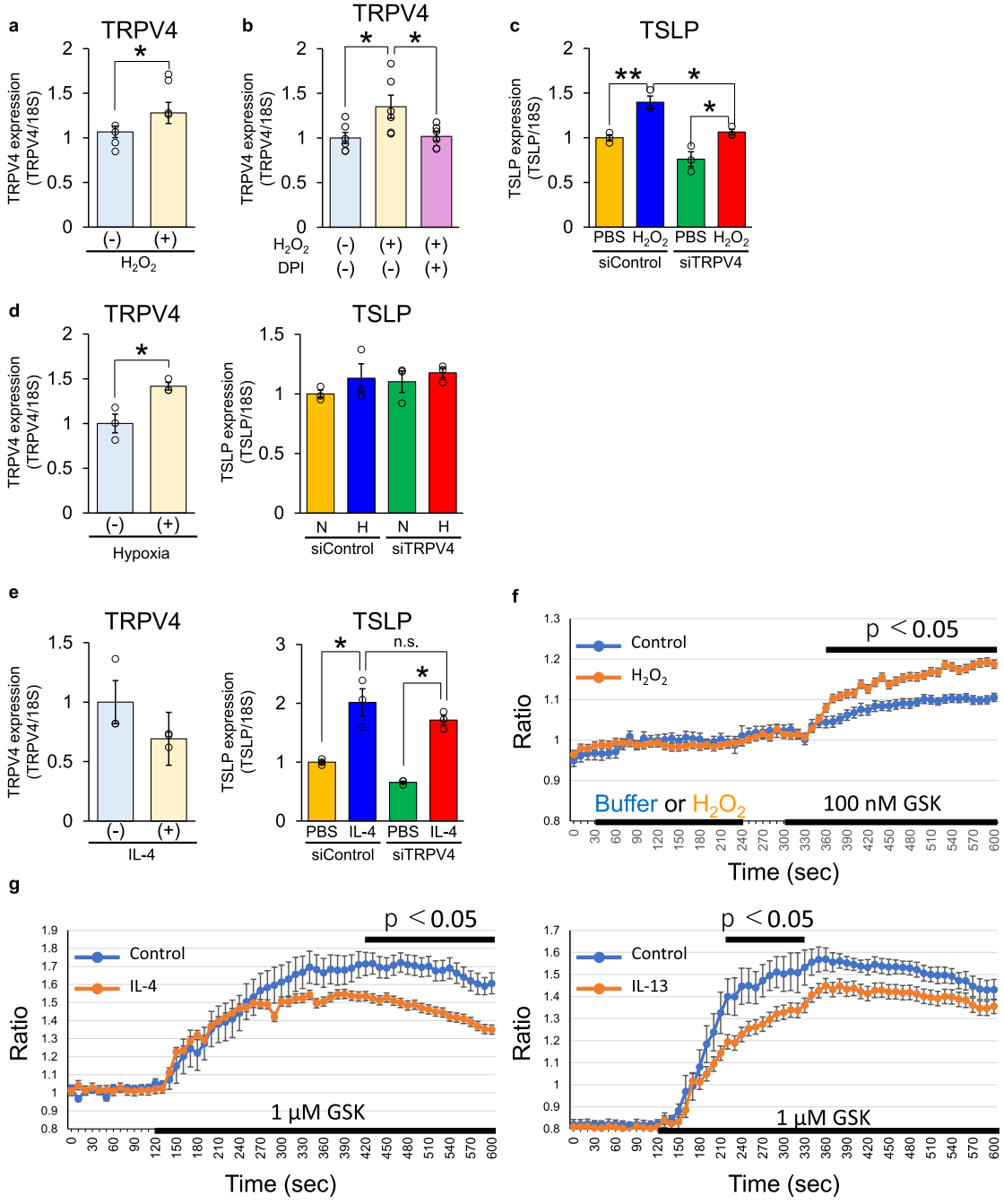

Fig. 5. Oxidative stress activates TRPV4 and induced TSLP production in keratinocyte. (a) PCR quantification of TRPV4 mRNA levels in HaCaT cells with or without H_2O_2 treatment for 1.5 h. n = 4. (b) PCR quantification of TRPV4 mRNA levels in HaCaT cells with or without H_2O_2 treatment and DPI for 1.5 h. n = 4. (c) PCR quantification of TRPV4 mRNA levels in HaCaT cells transfected with siControl or siTRPV4, with or without H_2O_2 treatment for 1.5 h. n = 3. (d) Left panel shows PCR quantification of TRPV4 mRNA levels in HaCaT cells under normoxia or hypoxia for 6 h. n = 3. Right panel shows PCR quantification of TRPV4 mRNA levels in HaCaT cells transfected with siControl or siTRPV4 under normoxia or hypoxia for 1.5 h. n = 3. N: normoxia, H: hypoxia. (e) Left panel shows PCR quantification of TRPV4 mRNA levels in HaCaT cells with or without IL-4 treatment for 6 h. n = 3. Right panel shows PCR quantification of TRPV4 mRNA levels in HaCaT cells transfected with siControl or siTRPV4 with or without IL-4 treatment for 6 h. n = 3. All values represent mean ± SEM. * p < 0.05, ** p < 0.01. One-way ANOVA followed by Turkey post-test. (f) Average traces of [Ca2 +]i changes in HaCaT cells (control; blue circles, n = 400 cells), or H2O2 applied cells (orange circles, n = 450 cells)·H2O2 (1 mM) was applied during recording for 210 sec. The [Ca2 +]i changes were measured by Fura-Red AM. The data were quantified as ratio of 525/650 nm emmition (excited by 488 nm). Upper black line represents significant differences between two groups by Student's T-TEST. (g) Average traces of [Ca2 +]i changes in HaCaT cells (control; blue circles, n = 400 cells), or IL-4 (10 ng/mL, orange circles, n = 450 cells) (left panel), and IL-13 (10 ng/mL, orange circles, n = 450) (right panel). These cytokines were applied during whole recording period.

There have been multiple reports on the involvement of TRPV4 in the regulation of pruritus in inflammatory skin diseases [23,44]. In addition to its expression in keratinocytes, TRPV4 is also expressed in T cells, where it contributes to various functions including T-cell activation, Ca^{2+} influx-mediated effector responses, cytokine production, and the expression of activation markers [45]. TSLP promotes the differentiation of naïve T-cells into Th2 T-cells, which subsequently produce type 2 inflammatory cytokines such as IL-4, IL-13, and IL-31. They are known to stimulate sensory neurons and induce pruritus [46]. Interestingly, a recent study demonstrated that IL-4 and IL-13 are not involved in IL-31-induced itch-associated scratching behavior in mice [47]. Our result showed the down-regulation of mRNA levels of IL-4, IL-13, and IL-31 in the lesional skin of TRPV4 KO mice. Moreover, the results showed that IL-4 and IL-13

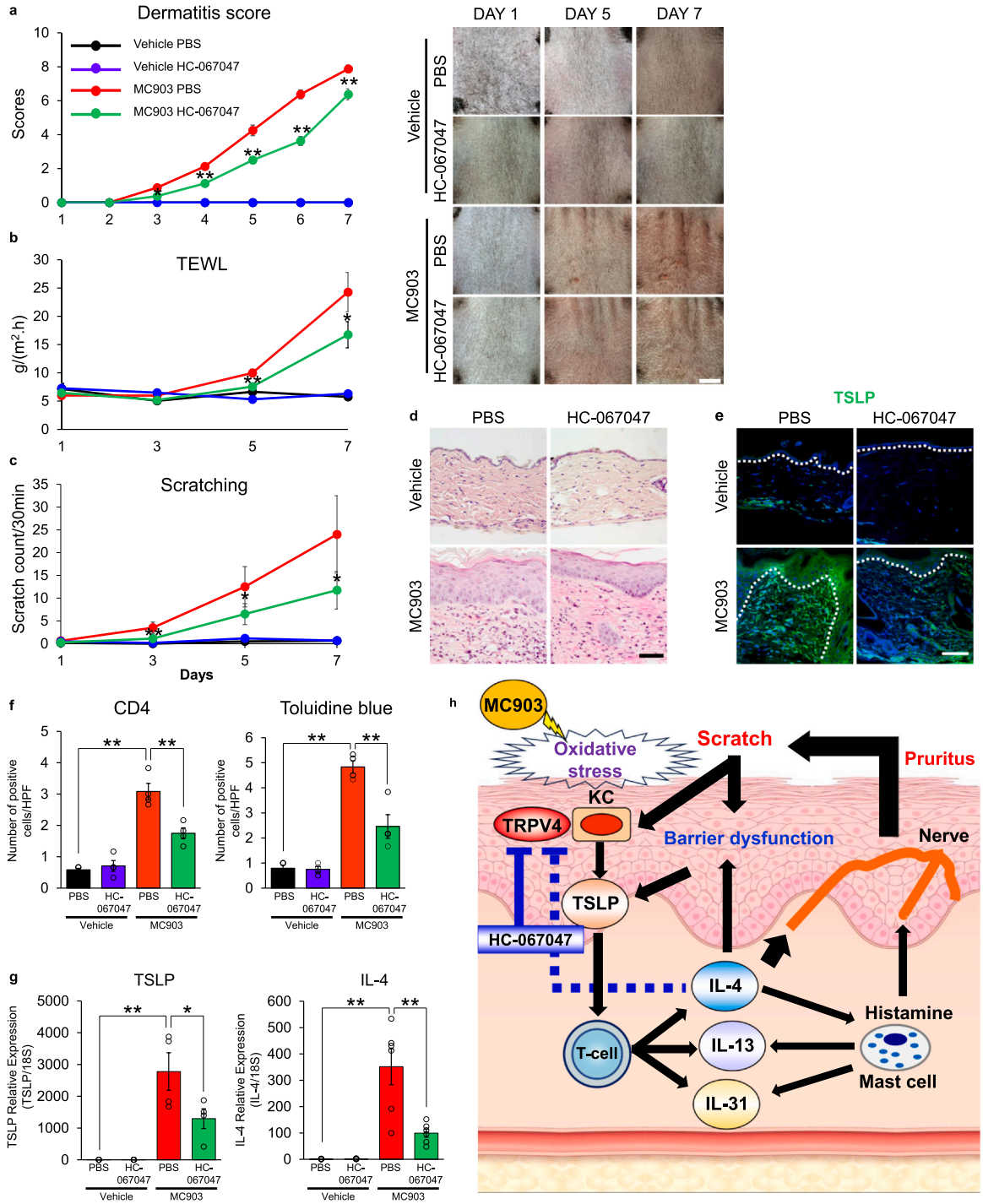

Fig. 6. TRPV4 antagonist (HC-067047) attenuated the development of MC903-induced AD-like dermatitis in mice.(a)The cumulative dermatitis score of skin manifestation at day 1, 5, and 7 in vehicle- or MC903-treated mice that were injected with PBS as control or HC-067047 (left panel), and representative photographs of each conditions (right panel). Scale bar = 2.5 mm. (b) TEWL value, and (c) Scratching count value in vehicle- or MC903-treated mice that were injected with PBS or HC-067047. n = 6 for vehicle-treated group and n = 8 for MC903-treated group. (d-e) Representative stained images of skin sections taken at day 7 in each group for (d) H&E and (e) TSLP. DAPI in blue. n = 6 for each group. Scale bar = 40 μm. (g) Quantification analysis of immunostaining for CD4 + (left panel) and mast cells (right panel) at day 7. Positive cells positive cells per field for each staining in six random microscopic fields in the dermis were counted. n = 4. (g) qPCR quantification of TSLP and IL-4 mRNA levels in lesional skin area at day 7, expressed relative to mRNA levels in PBS and vehicle-treated mice. n = 4 for TSLP and n = 6 for IL-4. All data represent the mean score ± SEM. * $p < 0.05$, ** $p < 0.01$. One-way ANOVA followed by Turkey post-hoc test. (h) Schematic model summarizing the mechanistic roles of TRPV4 regulate AD.

mildly suppressed TRPV4 activation in keratinocytes in vitro. These results suggest that the reduced IL-31 expression in TRPV4 KO mice may contribute to the attenuation of scratching behavior. However, the precise relationship between IL-31, TRPV4 activation, and pruritus remains elucidated and warrants further investigation.

TRPV4 has been reported to play several roles in macrophage function, including the promotion of TNF-α production, and the suppression of IL-1β production and M1 macrophage differentiation through inhibition of NF-κB signaling [48,49]. The absence of TRPV4 in macrophages may contribute to alterations in Th1 immunity in

atopic dermatitis; however, further studies are required to clarify this point.

At last, we demonstrated that treatment with TRPV4 antagonist reduced the severity of dermatitis, barrier dysfunction, pruritus, the expression of infiltrating inflammatory cells in MC903-induced AD-like dermatitis. This finding supported the notion that TRPV4 antagonist can be a potential therapeutic target by regulating various factors such as epidermal proliferative capacity and inflammation in AD.

There are several limitations in this study as described below. First, the therapeutic effects of TRPV4 inhibitors in patients with AD remain unclear, and the factors that regulate TRPV4 expression and activation are in AD still not well understood. In the mouse model, we only used the MC903 model for evaluation, and some experiments were not assessed in a time-course manner. In vitro, the experiment was performed using HaCaT cells, which may not be sufficient for assessing normal keratinocytes. Furthermore, the small sample size resulted in variability between experiments.

In conclusion, TRPV4 in skin may play a role in producing TSLP and following Th2 immunity and itching in the pathogenesis of AD. There is a potential for the therapeutic application of TRPV4 as a novel treatment target for atopic dermatitis.

Author contributions

Keiji Kosaka: Conceptualization; Data curation; Formal analysis; Investigation;Visualization; Writing – original draft. **Akihiko Uchiyama:** Conceptualization; Data curation; Formal analysis; Investigation; Project administration; Software; Supervision; Visualization; Writing – original draft. **Yuta Inoue:** Investigation; Resources. **Mai Ishikawa:** Investigation; Resources. **Takeshi Araki:** Investigation; Resources. **Shintaro Saito:** Investigation; Resources. **Akiko Sekiguchi:** Investigation; Resources. **Yoko Yokoyama:** Investigation; Data curation; Methodology; Resources. **Sachiko Ogino:**Investigation; Resources. **Ryoko Torii:** Investigation; Resources. **Yuki Watanuki:** Investigation; Resources. **Koji Shibasaki:** Data curation; Formal analysis; Investigation;Project administration; Software; Supervision; Visualization; Writing – original draft. **Sei-ichiro Motegi:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Visualization; Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdermsci.2026.01.005.

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