Enhanced Thermal Sensitivity of TRPV3 in Keratinocytes Underlies Heat-Induced Pruritogen Release and Pruritus in Atopic Dermatitis

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Itch in atopic dermatitis (AD) is aggravated under warm conditions. Transient receptor potential vanilloid (TRPV) 3, a member of the thermosensitive transient receptor potential channels, is activated by innocuous heat and is abundantly expressed in keratinocytes. The potential role of TRPV3 in itch is illustrated in TRPV3 channelopathies of humans and mice. However, the role of TRPV3 in heat-induced itch in AD and the underlying mechanisms are unclear. Here we showed that keratinocytes isolated from patients with AD exhibit enhanced expression and heat sensitivity with hyperactive channel function of TRPV3. Heat stimulus induced enhanced secretion of thymic stromal lymphopoietin, nerve growth factor, and prostaglandin E_2 by keratinocytes from patients with AD through TRPV3 activation. TRPV3 agonists increased thymic stromal lymphopoietin, nerve growth factor, prostaglandin E_2, and IL-33 production in human keratinocytes and induced scratching behavior upon intradermal injection in mice. TRPV3 was upregulated in the skin of MC903-induced AD mouse model. Heat stimulation to MC903-treated mice increased scratching behavior and produced higher levels of thymic stromal lymphopoietin, nerve growth factor, prostaglandin E_2, and IL-33 from the epidermis, which were attenuated by pharmacologic inhibition of TRPV3. Moreover, neutralization of thymic stromal lymphopoietin reduced heat-evoked scratching in MC903-challenged mice. These results suggest that TRPV3 is a potential therapeutic target for heat-induced itch in AD.

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INTRODUCTION

Itch in atopic dermatitis (AD) is characteristically triggered or aggravated at warm temperatures or in the nighttime when the basal body temperature rises (Darsow et al., 2001; Wahlgren, 1991). However, the mechanisms underlying the warmth-provoked itch in AD remain largely unknown.

Certain thermosensitive transient receptor potential (TRP) channels have been implicated in the different types of itch. Many studies have focused on the role of transient receptor potential vanilloid (TRPV) 1 and transient receptor potential ankyrin 1 on sensory nerves in itch (Kittaka and Tominaga, 2017). However, keratinocytes also play an important role in the development of itch by activating the immune cells or by activating itch neurons directly through soluble mediators (Mollanazar et al., 2016). Among the heat-sensitive TRP channels, TRPV3 is most abundantly expressed in keratinocytes and activated by non-noxious warm temperatures, with thresholds of 33−34 °C (Peier et al., 2002; Xu et al., 2002). Gain-of-function mutations of TRPV3 channel in rodents (DS-Nh mice and WBN/Kob-Ht rats) can spontaneously develop hairlessness, pruritus, spontaneous dermatitis characterized by increased infiltration of mast cells and CD4-bearing T cells, and increased serum levels of IL-4 and total IgE, recapitulating human AD (Asakawa et al., 2005; Hikita et al., 2002; Yoshioka et al., 2003). Similar features were observed in $TRPV3^{Gly573Ser}$ transgenic DS mice along with the increased serum levels of IL-13, IL-17, and several chemokine ligands (Yoshioka et al., 2009). Moreover, DS-Nh mice showed enhanced contact hypersensitivity with dendritic cell migration and increased thymic stromal lymphopoietin (TSLP) expression in keratinocytes (Yamamoto-Kasai et al., 2013). Pharmacologic and genetic inhibition of TRPV3 were shown to attenuate 2, 4-dinitrofluorobenzene−induced AD-like dermatitis in BALB/c mice (Qu et al., 2019). These findings indicate a possible role of TRPV3 in AD pathogenesis. Interestingly, $TRPV3^{Gly573Ser}$ on the Th-1 biased C57BL/6J mice (C57BL/6-Nh mice) developed scratching behavior despite the absence

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; 17(R)-RvD1, 17(R)-epimer of reolvin D1; AD, atopic dermatitis; Ca^{2+}, calcium; DPTHF, diphenyltetrahydrofuran; NGF, nerve growth factor; NHEK, normal human epidermal keratinocytes; PGE_2, prostaglandin E_2; siRNA, short interfering RNA; TRP, transient receptor potential; TRPV, transient receptor potential vanilloid; TSLP, thymic stromal lymphopoietin

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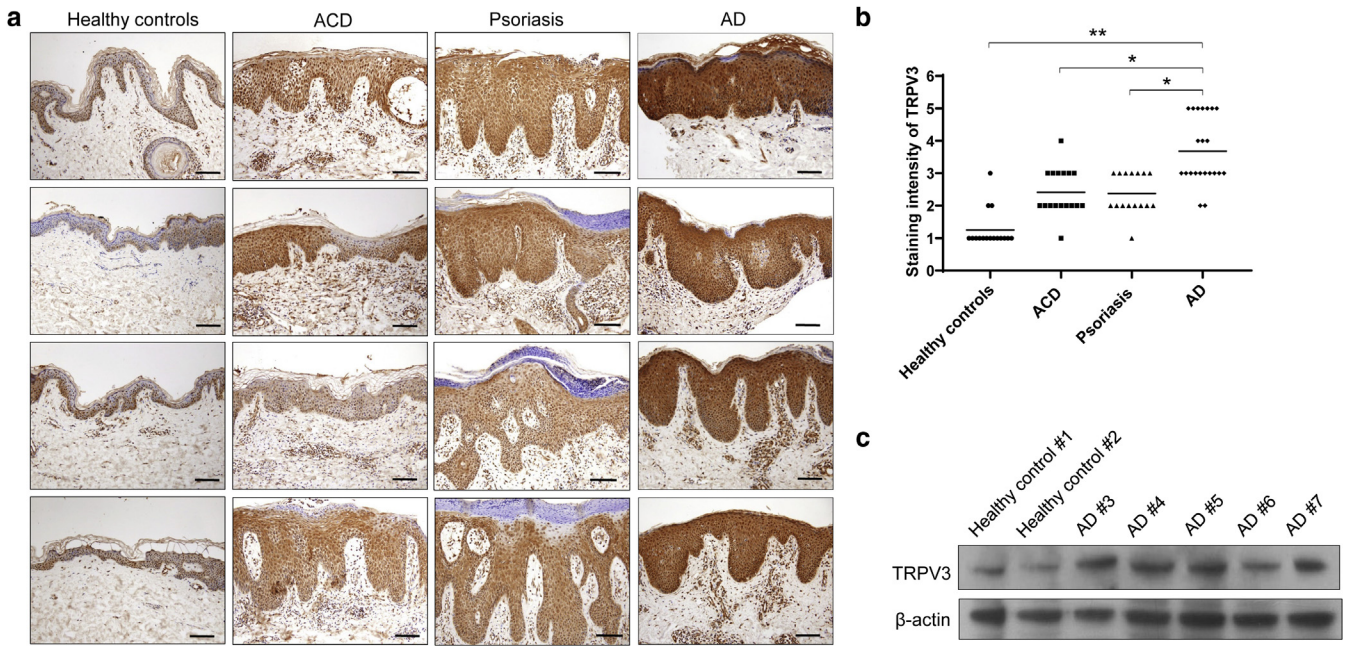

Figure 1. Increased expression of TRPV3 in the epidermal keratinocytes derived from lesional skin of patients with AD. (**a**) Immunohistochemical analysis of TRPV3 expression in lesional skin of patients with AD, ACD, and psoriasis as well as the skin samples from healthy controls (n = 21 in each group). Bar = 100 μm. (**b**) Semiquantitative analysis of TRPV3 immunoreactivity in the epidermis. (**c**) Western blot for TRPV3 expression in keratinocytes derived from AD patients (n = 9) and healthy controls (n = 9). β-Actin was used as a loading control. Numbers 1−7 indicate the patient number. Three independent experiments were performed. Data are mean ± SEM. $*P < 0.05$, $**P < 0.01$. ACD, allergic contact dermatitis; AD, atopic dermatitis; TRPV, transient receptor potential vanilloid.

of dermatitis (Yoshioka et al., 2009), suggesting a role of TRPV3 in itch. In addition, genetic knockout or pharmacologic inhibition of TRPV3 by natural compounds has been shown to reduce dry-skin-evoked chronic itch (Sun et al., 2018; Zhang et al., 2019). A gain-of-function mutation of TRPV3 in human causes Olmsted syndrome characterized by palmoplantar keratoderma and severe pruritus, providing additional insights into the potential role of TRPV3 in itch (He et al., 2015; Lin et al., 2012). However, the mechanism by which TRPV3 activation in keratinocytes induces itch is unclear.

From these findings, we hypothesized that TRPV3 might be involved in the warmth-provoked itch in AD. In this study, we found increased expression and enhanced heat-induced channel activity of TRPV3 in keratinocytes derived from AD lesional skin. We also discovered that heat stimulus triggered the secretion of pruritogens, including TSLP, nerve growth factor (NGF), and prostaglandin E_2 (PGE_2) by keratinocytes from patients with AD through TRPV3 activation. Finally, the role of TRPV3 in warmth-provoked itch and the release of keratinocyte-derived pruritogens was proved in the MC903 mouse model of AD (Li et al., 2006) by pharmacologic intervention and behavioral study.

RESULTS

TRPV3 is highly expressed in the epidermis and keratinocytes from lesional skin of patients with AD

First, we comparatively evaluated TRPV3 expression levels between the lesional skin of patients with AD and skin of patients with other eczematous or chronic inflammatory skin diseases or healthy individuals by immunohistochemistry. As shown in Figure 1a, TRPV3 expression was upregulated in the lesional epidermis of AD, allergic contact dermatitis, or psoriasis than in healthy skin. The semiquantitative analysis revealed that TRPV3 in lesional skin was significantly higher in AD than in allergic contact dermatitis or psoriasis (Figure 1a and b). Consistently, western blot analysis revealed the higher protein levels of TRPV3 in cultured keratinocytes isolated from AD lesional skin than in healthy donors−derived keratinocytes (Figure 1c). Similar to TRPV3, TRPV4 is expressed in keratinocytes and activated by warm temperature (Chung et al., 2004). Immunohistochemistry revealed slightly elevated TRPV4 expression in lesional atopic skin compared with healthy skin but to a lesser extent than TRPV3 (Supplementary Figure S1). The specificity of antibodies for TRP channels was confirmed by using two different primary antibodies, negative control staining without primary antibody or with matched IgG isotype antibody, and by using short interfering RNA (siRNA) transfection technique (Supplementary Figure S2). These findings suggest that TRPV3 in keratinocytes is involved in the pathophysiology of AD.

Keratinocytes from patients with AD exhibit enhanced TRPV3 channel activity

TRPV3 is a functionally active calcium (Ca^{2+})-permeable channel in human keratinocytes (Szöllősi et al., 2018); however, the functional activity of TRPV3 in keratinocytes from patients with AD has not been evaluated. The activity of TRPV3 was examined by measuring the change in the intracellular calcium concentration ($[Ca^{2+}]_i$) induced by a combination of two TRPV3 agonists (2-aminoethoxydiphenyl borate and carvacrol) (Cheng et al., 2010), using ratiometric Ca^{2+} imaging. The basal $[Ca^{2+}]_i$ was higher in keratinocytes from patients with AD than in healthy controls−derived

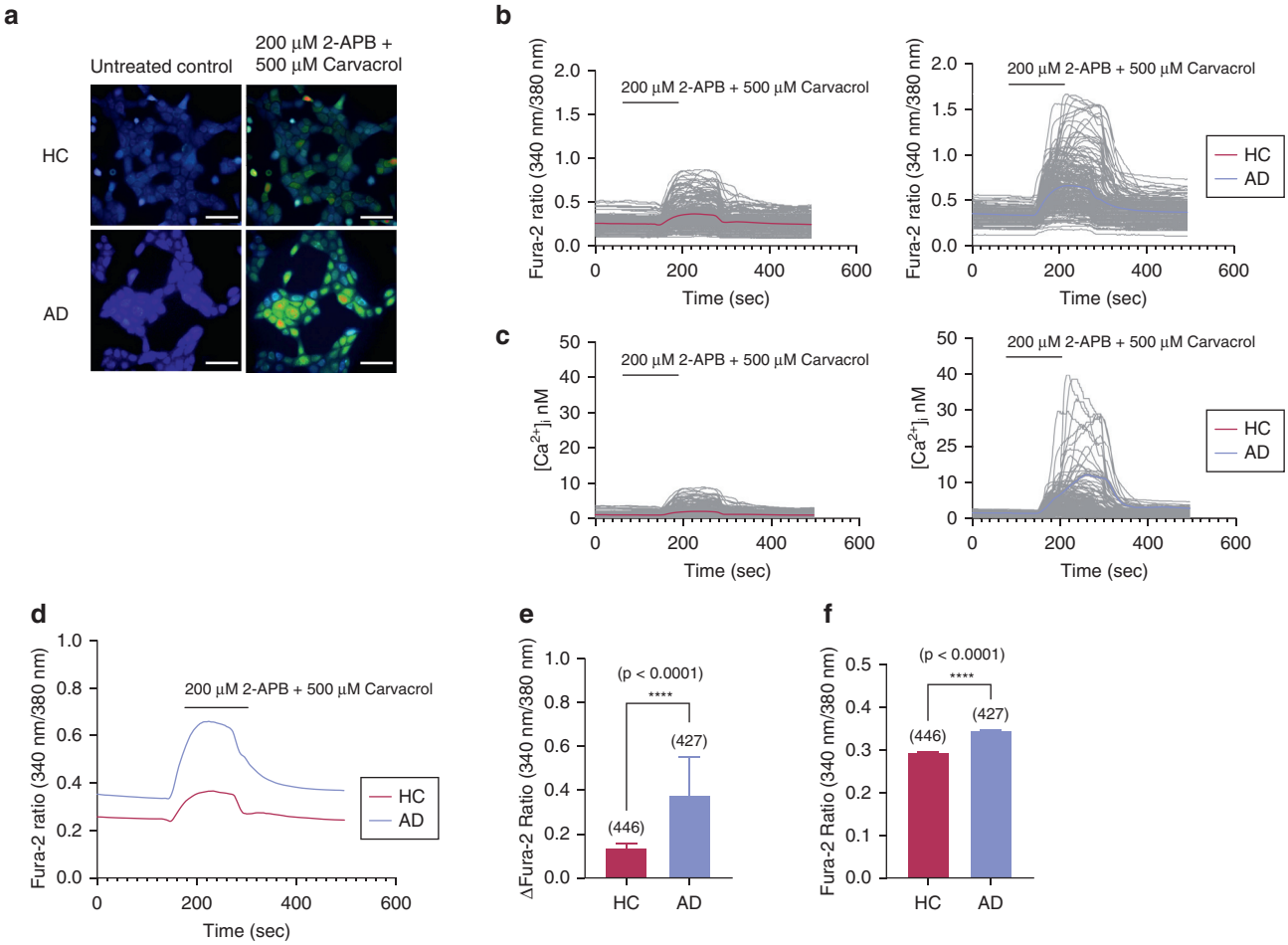

Figure 2. The TRPV3 channel agonists induce greater Ca^{2+} responses in keratinocytes derived from patients with than those in keratinocytes from healthy controls. (**a**) Representative fluorescence images of dynamic Fura-2 signaling changes in keratinocytes from patients with AD and HC. Bar = 200 μm. (**b**) The representative traces showing the effect of agonist-induced $[Ca^{2+}]_i$ increase in keratinocytes from patients with AD and HC. (**c**) Calcium calibration to calculate the changes in $[Ca^{2+}]_i$ in the keratinocytes from patients with AD and HC. (**d, e**) Comparative summary of Ca^{2+} influx induced by chemical agonists in keratinocytes from HC and patients with AD. ΔFura-2 ratio (340/380 nm) denotes the change of $[Ca^{2+}]_i$. (**f**) The basal $[Ca^{2+}]_i$ of keratinocytes from HC and patients with AD. Each data point represents the mean value of the "n" number of cells (in brackets) analyzed from 4–7 replicates from five independent experiments (**e, f**). Error bars represent SEM. $[Ca^{2+}]_i$, intracellular calcium level; AD, atopic dermatitis; APB, aminoethoxydiphenyl borate; Ca^{2+}, calcium; HC, healthy control; TRPV, transient receptor potential vanilloid.

keratinocytes (Figure 2f). More significantly, the amplitude of $[Ca^{2+}]_i$ increase induced by TRPV3 agonist cocktail in keratinocytes from atopic lesional skin was significantly augmented compared with that in keratinocytes from healthy skin (Figure 2a–e), suggesting a higher TRPV3 channel activity in keratinocytes from lesional atopic skin.

Heat-induced TRPV3-mediated Ca^{2+} influx is increased in keratinocytes from patients with AD

We next examined the heat sensitivity of the TRPV3 channel in keratinocytes from AD lesional skin. Given that human TRPV3 is uniquely sensitive at the physiological temperature of 37 °C (Xu et al., 2002), we measured the $[Ca^{2+}]_i$ change induced by 37 °C heat stimulus in keratinocytes from healthy and AD lesional skin. Increasing temperature from room temperature (22–23 °C) to 37 °C elicited an increase in $[Ca^{2+}]_i$ in AD keratinocytes, which gradually returned to the baseline after removal of heat (Figure 3b); however, little response was observed in keratinocytes from healthy skin under the same condition (Figure 3a). The levels of Ca^{2+} influx of individual cells upon heating in AD keratinocytes were significantly greater than those in keratinocytes from healthy controls (Figure 3c and d). To determine the role of TRPV3 in this heat-induced Ca^{2+} response in AD keratinocytes, we employed genetic and pharmacologic approaches. Silencing TRPV3 by siRNA (Figure 3e and f) significantly abolished heat-induced $[Ca^{2+}]_i$ increase in AD keratinocytes, as compared with cells transfected with scrambled siRNA (Figure 3g). To date, there is a lack of selective inhibitors for TRPV3. Diphenyltetrahydrofuran (DPTHF) (Chung et al., 2005), isopentenyl pyrophosphate (Bang et al., 2011), and icilin (Sherkheli et al., 2012) have been used; however, there is no sufficient evidence for the selectivity for TRPV3 for any of these. A recent study (Bang et al., 2012) demonstrated that a 17(R)-epimer of resolvin D1 (17(R)-RvD1) is a TRPV3-specific inhibitor. To use DPTHF and 17(R)-RvD1 for TRPV3 inhibition, we first

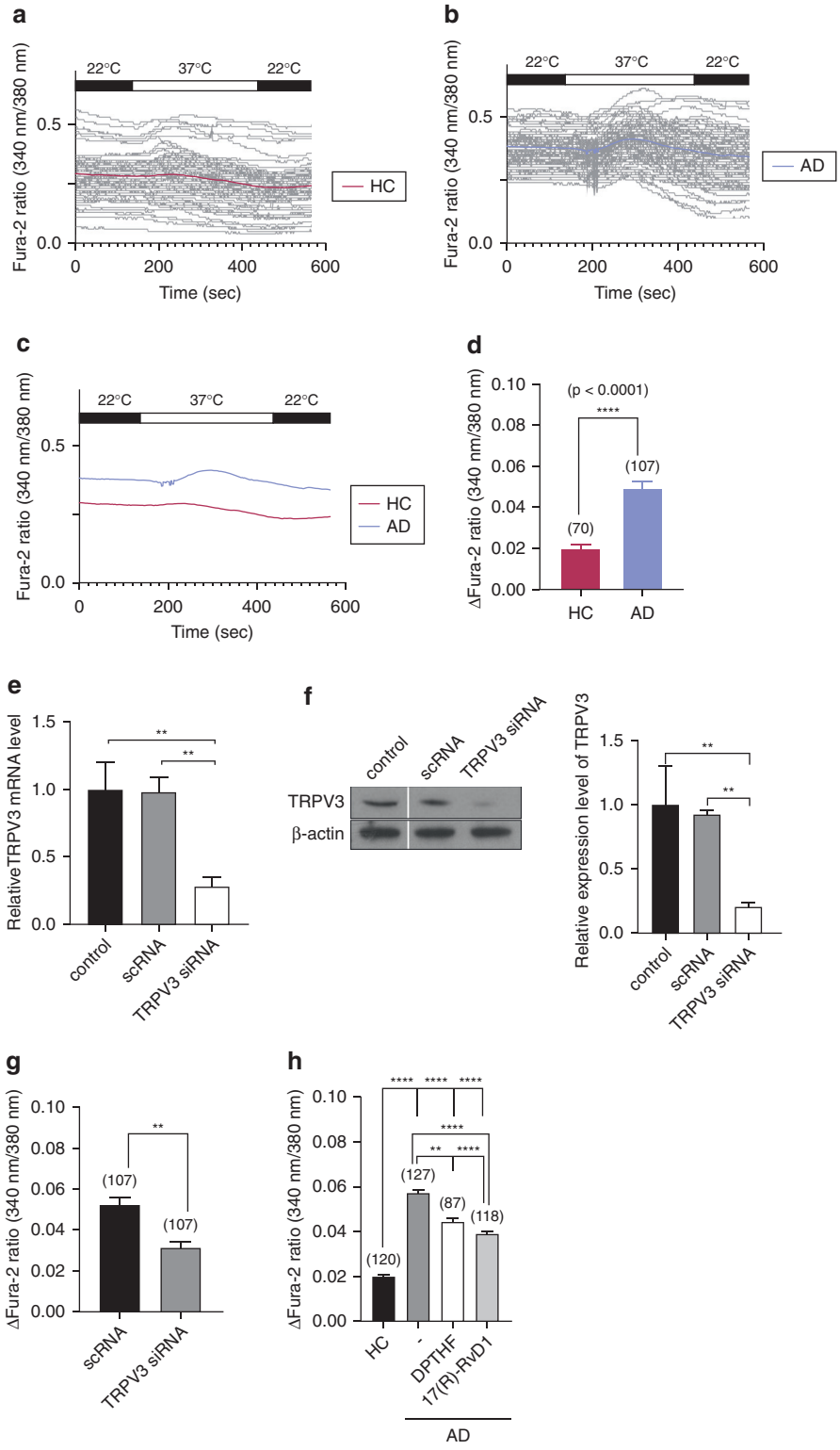

Figure 3. Heat stimulus triggers TRPV3-mediated Ca^{2+} influx in keratinocytes from patients with AD. (**a, b**) The representative traces show the effect of heat-induced Ca^{2+} influx in keratinocytes from the lesional skin of (**a**)HC and (**b**) patients with AD at 37 °C. (**c, d**) Comparative summary of Ca^{2+} influx in the keratinocytes from HC and patients with AD at 37 °C. ΔFura-2 ratio (340/380 nm) denotes the changes in the intracellular Ca^{2+} levels. (**e**–**g**) Keratinocytes from patients with AD were transfected with ScRNA or TRPV3 siRNA and were exposed to increasing temperature from 22 °C to 37 °C. (**e**) qRT-PCR and (**f**) immunoblotting with densitometric analysis confirmed the knockdown of TRPV3. (**g**) Comparative summary of Ca^{2+} influx in scrambled TRPV3- and TRPV3 siRNA-transfected AD keratinocytes at 37 °C. (**h**) Comparative summary of calcium influx in keratinocytes from patients with AD in the absence or presence of 300 μM DPTHF or 300 nM 17(R)-RvD1 upon exposure to increasing temperature from 22 °C to 37 °C. Each data point represents the mean value of the "n" number of cells (in brackets) analyzed from 3–4 replicates from (**g, h**) three to five independent experiments Error bars represent SEM. $*P < 0.05$, $**P < 0.01$. 17(R)-RvD1, 17(R)-epimer of resolvin D1 $[Ca^{2+}]_i$, intracellular calcium level; AD, atopic dermatitis; Ca^{2+}, calcium; DPTHF, diphenyltetrahydrofuran; HC, healthy control; scRNA, scrambled RNA; siRNA, short interfering RNA; TRPV, transient receptor potential vanilloid; qRT-PCR, quantitative real-time PCR.

investigated their selectivity for TRPV3. Fura-2-based Ca^{2+} assays of primary human keratinocytes overexpressing TRPV3 confirmed that DPTHF or 17(R)-RvD1 potently inhibited 2-aminoethoxydiphenyl borate and carvacrol-evoked Ca^{2+} response that was not significantly inhibited by a selective TRPV4 or transient receptor potential ankyrin 1 inhibitor. Moreover, DPTHF or 17(R)-RvD1 had little effects on the Ca^{2+} response evoked by a selective TRPV4 or transient receptor potential ankyrin 1 agonist (Supplementary Figure S3), confirming their selectivity for TRPV3 over other TRP channels at the concentrations used in our study. When DPTHF or 17(R)-RvD1 was applied to AD keratinocytes, a significant reduction in heat-evoked Ca^{2+} responses was observed (Figure 3h), further

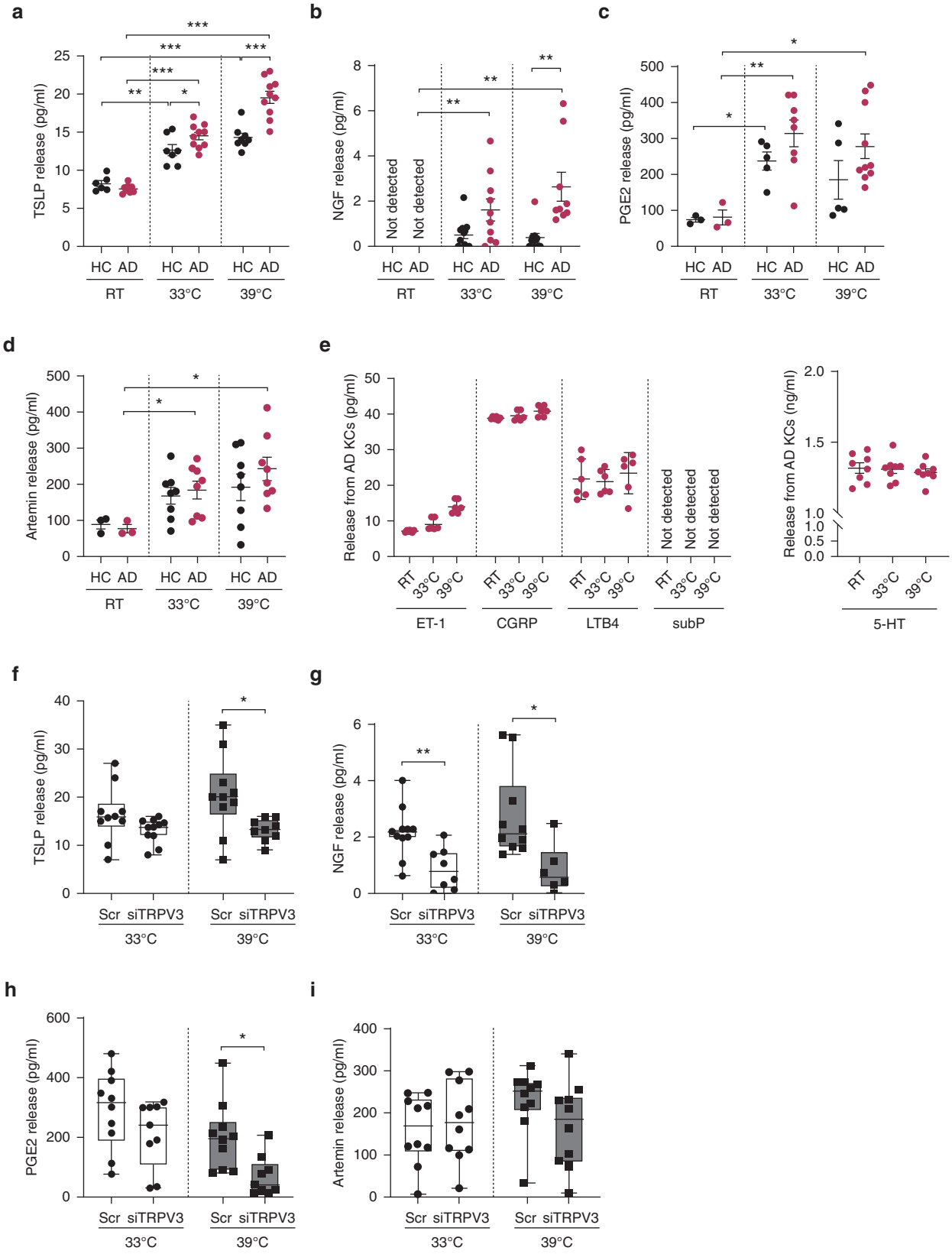

Figure 4. Keratinocytes derived from patients with AD exhibit enhanced release of TSLP, NGF, and PGE2 in response to innocuous heat stimulus by TRPV3 activation. (**a**–**e**) Keratinocytes from patients with AD and healthy controls were incubated at room temperature (22 °C) or two innocuous warm temperatures (33 °C and 39 °C) for 24 hours. (**f**–**i**) Keratinocytes derived from patients with AD treated with Scr or siTRPV3 were incubated at two innocuous warm temperatures (33 °C and 39 °C) for 24 hours. The culture supernatant was subjected to ELISA for measuring the levels of keratinocyte-derived pruritogens. Three independent experiments were performed. Data are mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.0005$. 5-HT, 5-hydroxytryptamine; AD, atopic dermatitis; AD

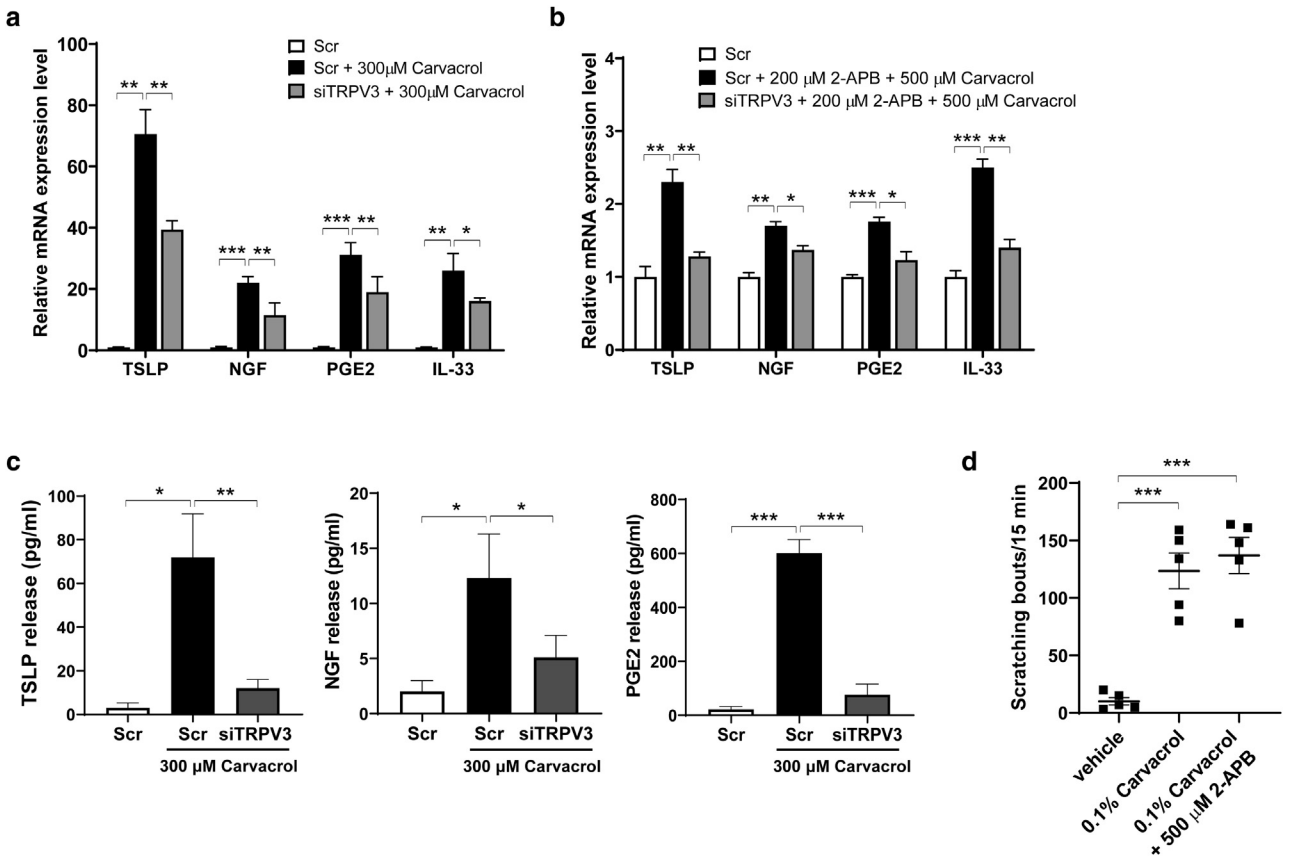

Figure 5. TRPV3 channel activation induces the production of certain pruritogens in normal human keratinocytes. Normal human epidermal keratinocytes transfected with Scr or siTRPV3 were stimulated with the TRPV3 agonist, carvacrol (300 μM), or TRPV3 agonist cocktail (200 μM 2-APB and 500 μM carvacrol) for 6 hours. (**a, b**) qRT-PCR analysis of mRNA levels of *TSLP*, *NGF*, *PGE$_2$*, and *IL-33*. (**c**) The culture supernatant was subjected to ELISA for measuring the levels of pruritogens. Data are representative of three independent experiments. Data are mean ± SEM and normalized to the Scr siRNA-non-treated group. (**d**) Scratching bouts evoked by intradermal injections of either carvacrol (0.1%, 20 μl), TRPV3 agonist cocktail (0.1% carvacrol and 500 μM 2-APB, 20 μl), or vehicle (10% ethanol in PBS, 20 μl) (n = 5 per group) into the cheek of mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0005$. 2-APB; 2-aminoethoxydiphenyl borate; PGE$_2$, prostaglandin E$_2$; Scr, scrambled; siRNA, short interfering RNA; siTRPV, TRPV siRNA; TSLP, thymic stromal lymphopoietin; TRPV, transient receptor potential vanilloid qRT-PCR, quantitative real-time PCR.

supporting the enhanced heat sensitivity of TRPV3 in AD keratinocytes.

Innocuous heat stimulus elicits an enhanced secretion of TSLP, NGF, and PGE$_2$ by keratinocytes from patients with AD through TRPV3 activation

Keratinocytes are a major source of TSLP and IL-33 (Mack and Kim, 2018). In addition, they produce neuropeptides (i.e., substance P, endothelin, and NGF, to name a few), lipid mediators (leukotriene B4 and PGE$_2$), and neurotransmitters (i.e., serotonin [5-hydroxytryptamine]) that underlie the pathology of AD and pruritus (Andoh et al., 2001; Emrick et al., 2018; Gouin et al., 2017; Hägermark and Strandberg, 1977). Calcitonin gene–related peptide (McCoy et al., 2013; Rogoz et al., 2014) and artemin (Hidaka et al., 2017; Murota et al., 2012), which can be produced by keratinocytes, are also implicated in the thermally provoked itch. To investigate the role of keratinocytes in the heat-induced atopic itch, we analyzed the levels of above-mentioned, keratinocytes-derived pruritogens by ELISA in the culture supernatant of keratinocytes from patients with AD and healthy controls after exposure to heat of 33 °C or 39 °C, the temperature range at which TRPV3 is activated. AD keratinocytes secreted significantly higher amounts of TSLP, NGF, PGE$_2$, and artemin upon heating compared with those incubated at room temperature (Figure 4a–d); however, they did not increase the release of endothelin-1, calcitonin gene–related peptide, leukotriene B4, substance P, and 5-hydroxytryptamine (Figure 4e). Exposure of keratinocytes from healthy controls to heat did not alter the release of artemin and NGF, but it increased TSLP and PGE$_2$ secretion (Figure 4a–d). However, heat-induced TSLP secretion by AD keratinocytes was significantly higher than that by keratinocytes from healthy individuals (Figure 4a). siRNA-mediated knockdown of TRPV3 significantly attenuated the heat-induced secretion of TSLP, PGE$_2$, and NGF but not artemin by AD keratinocytes (Figure 4f–i), confirming the role of TRPV3 in the heat-induced secretion of these pruritogens by AD keratinocytes.

KC, keratinocyte from patients with atopic dermatitis; CGRP, calcitonin gene–related peptide; ET-1, endothelin 1; HC, healthy control; LTB4, leukotriene B4; NGF, nerve growth factor; PGE$_2$, prostaglandin E$_2$; RT, room temperature; scr, scrambled; siRNA, short interfering RNA; siTRPV, TRPV siRNA; subP, substance P; TRPV, transient receptor potential vanilloid; TSLP, thymic stromal lymphopoietin.

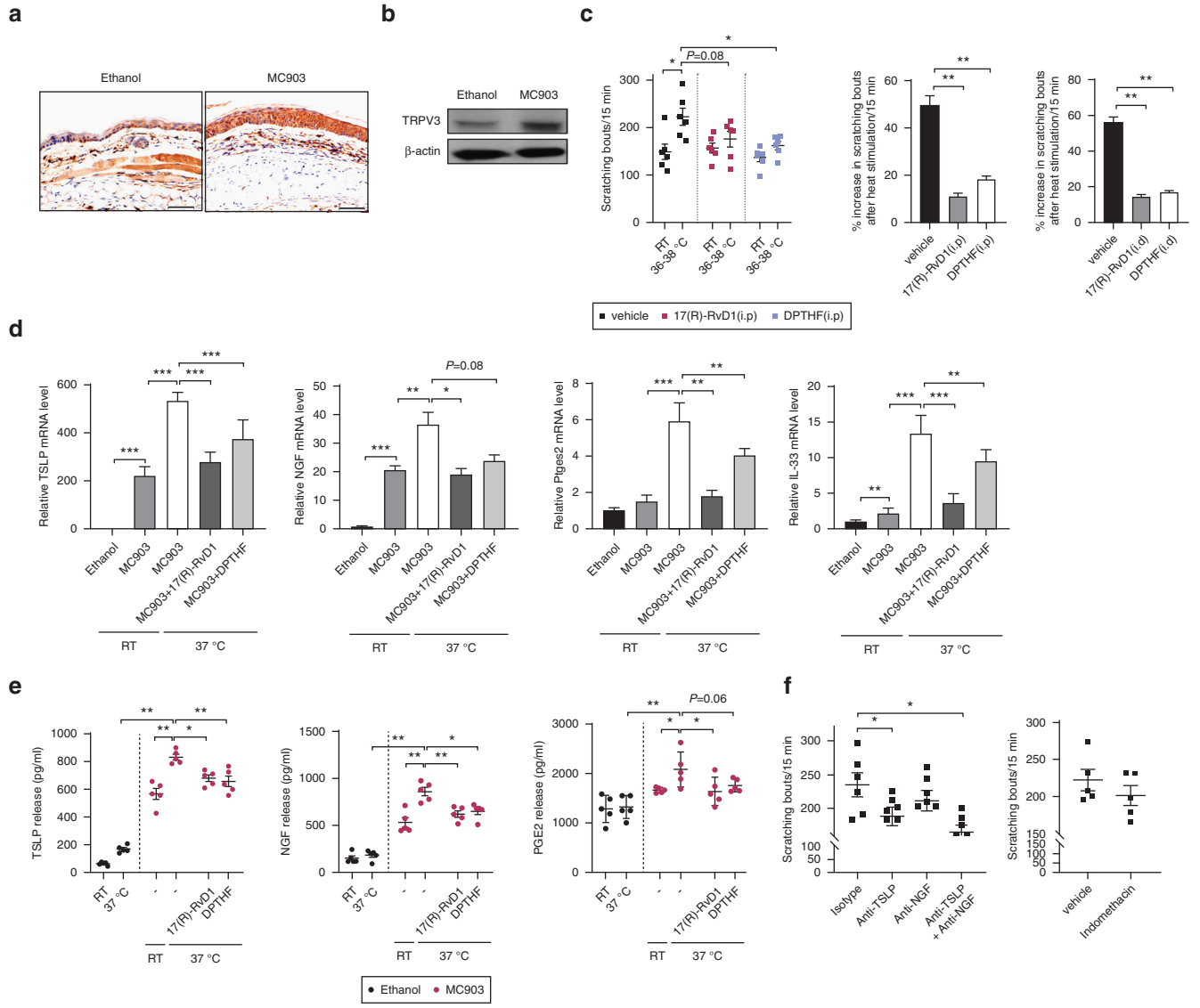

Figure 6. TRPV3 plays a central role in heat-induced itch and pruritogen secretion in the MC903 model of AD. Representative **(a)** immunohistochemical staining and (**b**) western blot analysis for TRPV3 in the epidermis of vehicle (ethanol)- or MC903-treated mice. Bar = 100 μm. (**c**) Scratching behavior in MC903-induced mouse model of AD during heat stimulation after pretreatment with TRPV3 antagonist, 17(R)-RvD1 (1.5 mg/kg) or DPTHF (1.5 mg/kg), or vehicle 30 minutes before heat stimulation on day 7 (either i.p. or i.d.); n = 5−6 mice per group. **(e)** Pruritogens secretion in culture supernatant and (**d**) mRNA expression levels of pruritogens by ex vivo epidermis obtained from vehicle (ethanol)-treated mice and MC903-treated mice pretreated with TRPV3 antagonist, 17(R)-RvD1 (1.5 mg/kg) or DPTHF (1.5 mg/kg), or vehicle, intradermally 15 minutes before heat stimulation were quantified by **(e)** ELISA and (**d**) qRT-PCR after 24 hours of incubation at room temperature (22 °C) or 37 °C. qRT-PCR results are normalized to those of vehicle control mice epidermis incubated at RT; n = 5 mice per group. Three independent experiments were performed. (**f**) To block the endogenous TSLP and/or NGF activity, MC903-treated mice were pretreated with intraperitoneal injection of anti-TSLP, anti-NGF, or a cocktail of anti-TSLP and anti-NGF neutralizing antibodies, or isotype control antibodies at a dose of 200 μg per mouse 30 minutes before heat stimulation. Other groups of MC903-treated mice were injected intraperitoneally 30 minutes before heat stimulation with a cyclooxygenase inhibitor indomethacin (10 mg/kg) or vehicle (acetone). Scratching behavior was recorded during heat stimulation; n = 5−6 mice per group. Data are mean ± SEM. $*P < 0.05$; $**P < 0.01$, $***P < 0.0005$. 17(R)-RvD1, 17(R)-epimer of resolvin D1; AD, atopic dermatitis; DPTHF, diphenyltetrahydrofuran; i.d., intradermal; i.p., intraperitoneal; NGF, nerve growth factor; PGE_2, prostaglandin E_2; RT, room temperature; TRPV, transient receptor potential vanilloid; TSLP, thymic stromal lymphopoietin; qRT-PCR, quantitative real-time PCR.

Activation of TRPV3 channel with agonists induces the production of TSLP, NGF, PGE2, and IL-33 in normal human keratinocytes and scratching behavior in mice

We next addressed whether TRPV3 activation in keratinocytes can trigger the production of keratinocyte-derived pruritogens. Treatment of normal human epidermal keratinocytes (NHEK) with carvacrol or agonist cocktail (2-aminoethoxydiphenyl borate and carvacrol) increased the mRNA levels of *TSLP*, *PGE_2*, *NGF*, and *IL-33*, which were significantly attenuated by TRPV3 knockdown (Figure 5a and b). Consistently, carvacrol treatment of NHEK induced an increased release of TSLP, NGF, and PGE_2, which were markedly attenuated by TRPV3 siRNA transfection (Figure 5c). However, IL-33 was not detected in the culture medium using ELISA (data not shown). These results indicate that TRPV3 plays an important role in the production of certain pruritogens in NHEK. To assess the effect of TRPV3 activation on itch in vivo, we injected carvacrol or TRPV3

agonist cocktail intradermally in the cheek of the mouse. Carvacrol or a combination of carvacrol and 2-aminoethoxydiphenyl borate triggered marked scratching behavior compared with vehicle (Figure 5d), demonstrating that activation of TRPV3 suffices to elicit itch response in mice.

TRPV3 is involved in warmth-provoked itch and pruritogens response in MC903 model of AD

To provide in vivo evidence for a role of TRPV3 in warmth-provoked itch in AD, we used the vitamin D3 analog MC903 to induce AD-like inflammation and itch in mice (Li et al., 2006; Morita et al., 2015; Oetjen et al., 2017). Seven consecutive days of topical application of MC903 to the mouse cheek and nape of the neck developed eczematous lesions and stable scratching behavior. Immunohistochemistry and western blot analysis revealed the upregulation of TRPV3 in the epidermis of MC903-treated mice compared with that of the vehicle (ethanol)-treated mice (Figure 6a and b). To investigate the effect of innocuous heat on MC903-evoked itch, we placed MC903-treated mice in the chamber at a temperature range of 36−38 °C for 15 minutes. Exposure to a warm environment resulted in a significant increase in scratching bouts compared with those at room temperature. The role of TRPV3 in heat-induced itch was evaluated using pharmacologic inhibitors because the genetic knockout of TRPV3 is unlikely to develop the MC903-induced AD phenotype (Qu et al., 2019). Systemic or local administration of 17(R)-RvD1 or DPTHF 30 minutes before heat stimulation significantly attenuated heat-evoked scratching in MC903-treated mice (Figure 6c). To examine the heat-induced production of TRPV3-dependent pruritogens from MC903-treated skin, we incubated the epidermal sheets obtained from MC903 or vehicle-treated mice ex vivo at room temperature or 37 °C. The sheets from MC903-treated mice secreted greater amounts of TSLP, NGF, and PGE_2 at 37 °C than those secreted at room temperature (Figure 6e). The tissue levels of *TSLP*, *NGF*, *PGE_2*, and *IL-33* mRNA in the sheets from MC903-treated mice also increased 3 hours after heat stimulation (Figure 6d). Pretreatment with 17(R)-RvD1 or DPTHF significantly attenuated the heat-induced secretion and the gene expression levels of *TSLP*, *NGF*, *PGE_2*, and *IL-33* in the epidermis of MC903-treated mice (Figure 6d and e). These results suggest an important role of TRPV3 in the heat-induced itch and the secretion of key pruritogens from the skin in the MC903 mouse model of AD.

Furthermore, we investigated whether TRPV3-dependent pruritogens are involved in heat-evoked itch in MC903-treated mice by systemic treatment with neutralizing antibody to TSLP or NGF or the cyclooxygenase inhibitor indomethacin. Pretreatment with TSLP-neutralizing antibody or a combination of neutralizing antibody to TSLP and NGF significantly attenuated the heat-evoked scratching in MC903-treated mice. Administration of NGF-neutralizing antibody or indomethacin reduced heat-induced scratching in MC903-treated mice, but this trend did not reach statistical significance (Figure 6f).

DISCUSSION

Herein, we demonstrate an important role of TRPV3 in keratinocytes in the heat-evoked itch and epithelial cell−derived pruritogens release in AD. Consistent with a previous study (Yamamoto-Kasai et al., 2013), we observed higher levels of TRPV3 in the lesional epidermis, as well as the cultured keratinocytes derived from patients with AD. Lesional atopic skin expressed even higher levels of TRPV3 than the lesional skin of allergic contact dermatitis and psoriasis, indicating the contribution of TRPV3 to the pathogenesis of AD.

Using Ca^{2+} imaging experiments, we found an increase in basal $[Ca^{2+}]_i$ in keratinocytes isolated from AD lesional skin. Given that proinflammatory mediators were shown to elevate basal $[Ca^{2+}]_i$ in several types of cells by modulating TRP channels, store-operated Ca^{2+} entry, or G-protein coupled receptors, we speculate that increased inflammatory mediators might be responsible, but further studies are needed for a clearer understanding of the mechanisms (Hamby et al., 2012; Jia et al., 2013; Mickle et al., 2016; Vriens et al., 2009). More importantly, we demonstrated that keratinocytes from patients with AD exhibit hyperactive channel function and enhanced thermosensitivity of TRPV3. We also demonstrated that TRPV3 activation in vivo by intradermal injection of chemical agonists increased the scratching behavior of mice, indicating an essential role of TRPV3 in itch. Together, enhanced thermosensitivity of TRPV3 in keratinocytes may participate in heat-induced itch in AD.

Keratinocyte-derived pruritogens are important in itch pathways; however, the direct association between heat stimulus and the production of pruritogens from AD lesional skin has not been investigated. We identified that TSLP, NGF, PGE_2, and artemin are highly secreted by keratinocytes from patients with AD in response to heat, suggesting their involvement in the heat-induced itch in AD. Furthermore, we demonstrated that TRPV3 mediates the heat-induced release of TSLP, NGF, and PGE_2 from AD keratinocytes.

Although TRPV3 has been shown to regulate TSLP production in DS-Nh mice and human keratinocytes (Park et al., 2017; Yamamoto-Kasai et al., 2013), the clinical relevance of this has remained unexplored. Here, we demonstrate that heat-induced TSLP secretion by keratinocytes from patients with AD and MC903-induced AD mouse model is primarily mediated by TRPV3 activation and neutralizing TSLP significantly attenuated heat-induced scratching in MC903-treated mice, suggesting that thermally activated TRPV3-induced TSLP secretion plays an essential role in mediating heat-evoked itch in AD.

We also found that heat-evoked release of NGF and PGE_2 from AD keratinocytes was mediated by TRPV3 activation, in line with a previous report using $TRPV3^{Gly573Ser}$ transgenic mice (Yoshioka et al., 2009) and a study using TRPV3-overexpressing keratinocytes (Huang et al., 2008). However, acute blockage of NGF by a single injection of neutralizing antibody did not significantly reduce the heat-induced scratching in MC903-treated mice, suggesting that thermally activated TRPV3-induced NGF may induce cutaneous hyperinnervation rather than act as a direct pruritogen in our

mouse model. Cyclooxygenase inhibitor indomethacin also had no significant effect on heat-induced scratching in MC903-treated mice.

In addition, TRPV3 activation increased IL-33 transcription in NHEK. Similar to TSLP, IL-33 can activate sensory neurons directly through ST2, thereby mediating itch in the poison-ivy allergic contact dermatitis mouse model (Liu et al., 2016), suggesting a potential role of IL-33 in TRPV3-mediated itch. However, we could not detect IL-33 in the culture supernatant of TRPV3 agonist-treated NHEK and heat-stimulated AD keratinocytes using ELISA. This finding may be because IL-33, a nuclear cytokine (Cayrol and Girard, 2018), can be secreted only after cellular damage or necrosis.

Using the MC903-induced mouse model of AD, we provide strong evidence that TRPV3 is involved in heat-induced spontaneous scratching and pruritogens release from the epidermis in vivo based on our pharmacologic inhibition studies. However, the lack of potent, selective pharmacologic inhibitors for TRPV3 has been a limitation in understanding the role of this channel in in vivo models where channel function may affect model formation. Thus, we demonstrated that DPTHF and 17(R)-RvD1 used in our study are relatively selective inhibitors of TRPV3 over other itch-related TRP channels and then evaluated the clinically relevant question of whether thermally activated TRPV3 is involved in heat-evoked itch in AD in vivo.

In conclusion, we found the enhanced thermal sensitivity of TRPV3 in keratinocytes from AD lesional skin, which contributes to the heat-induced release of pruritogenic substances such as TSLP, NGF, and PGE_2 from keratinocytes. Our in vivo study demonstrated that TRPV3 and thermally activated TRPV3-induced increase in TSLP are essential for heat-evoked itch in the MC903-induced AD mouse model. Collectively, our results suggest that targeting the TRPV3 channel may be an effective treatment strategy for heat-induced itch in AD.

MATERIALS AND METHODS

Keratinocytes isolation, culture, and heat stimulation

Ten Korean adult patients with AD and healthy volunteers were recruited in this study in accordance with an approved Institutional Review Board (no. IRB 3-2018-0087) protocol of the Yonsei University College of Medicine, Seoul, Korea. All participants provided written informed consent, complying with the principles of the Declaration of Helsinki. The diagnosis of AD was based on Hanifin and Rajka criteria. Skin samples were taken from the lesional skin of patients with AD and normal skin of healthy donors. Primary human keratinocyte culture was performed as previously described by our group (Kim et al., 2019). For temperature stimulation, cells were acclimated in buffer containing 130 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM HEPES, 1.2 mM $NaHCO_3$, and 10 mM glucose, adjusted to pH 7.45 with NaOH at room temperature for 2 hours. Heat was applied by transferring cells from room temperature to a CO_2 incubator with the temperature set at 33 °C, 37 C, or 39 °C for 24 hours. NHEK (#C0015c; Gibco, Rockville, MD) were used in the TRPV3 agonist stimulation experiments.

Knockdown of TRPV3 and TRPV4 expression in keratinocytes

NHEK and keratinocytes from patients with AD were transfected (Lipofectamine RNAiMAX Transfection Reagent, Thermo Fisher Scientific, Waltham, MA) with 25 nM of a non-targeting negative control siRNA (silencer select negative control number 1, #4390843; Thermo Fisher Scientific), siRNA directed against TRPV3 (validated silencer select siRNA, s46346, Thermo Fisher Scientific), or siRNA directed against TRPV4 (validated silencer select siRNA, s34001; Thermo Fisher Scientific), according to the manufacturer's instructions. Cells were incubated for 24 hours at 37 °C, and the efficiency was evaluated by quantitative real-time PCR and western blot.

Overexpression of TRPV3 in primary human keratinocytes

Primary human keratinocytes transiently transfected with pCMV6 expression vector with C-terminal Myc-DDK Tagged TRPV3 (OriGene, Rockville, MD). For genetic transfection, exponentially growing primary human keratinocytes were electroporated with 2.5 μg of TRPV3 cDNA by using Human Keratinocyte Nucleofector Kit, Nucleofector II Device, and Amaxa Nucleofector Solution (Cat. VPD-1002; Lonza, Walkersville, MD). Lonza Nucleofector II electroporation system showed high efficiency in the transfection, applying proprietary electroporation buffers and parameters. Transfected cells were grown for an additional 48 hours before immunoblotting experiments.

MC903 model of AD with chronic itch and drug administration

Female C57BL/6 mice (8–9 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal protocols used in this study were approved by the Yonsei University Institutional Animal Care and Use Committee. To induce AD-like inflammation and chronic itch, we applied MC903 (R&D Systems, Minneapolis, MN) to the mouse cheek and the neck (0.2 mM in 20 μl ethanol [vehicle]) once daily for 7 days (day 0–6). For the ex vivo culture of epidermal sheets, mice were topically treated once daily with MC903 on both ears for 7 days. On day 7, scratching behavior after heat stimulation was evaluated, and tissues were obtained for western blot, immunohistochemistry, and ex vivo culture of epidermal sheets. TSLP and NGF were blocked systemically by intraperitoneal injection of MC903-treated mice with the corresponding neutralizing antibody (mouse monoclonal TSLP-neutralizing antibody [200 μg, MAB555; R&D Systems] and mouse monoclonal NGF-neutralizing antibody [300 μg, N3279; Sigma-Aldrich, St. Louis, MO]) 30 minutes before heat stimulation on day 7. Species- and isotype-matched normal IgG (Sigma-Aldrich) was used as vehicle control. To block PGE_2 synthesis, we administered indomethacin (10 mg/kg; Sigma-Aldrich) or vehicle (acetone) intraperitoneally to MC903-treated mice 30 minutes before heat stimulation. The antagonists and antibody dosages were based on previous studies (Emrick et al., 2018; Han et al., 2012; Takano et al., 2005).

Data availability statement

No datasets were generated or analyzed during the current study.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: SC, SEL; Formal analysis: SHS, SK; Funding acquisition: SEL; Investigation: SHS, SK, SEK, SC, SEL; Project administration: SC, SEL; Supervision: SC, SEL; Writing - Original Draft Preparation: SHS, SK, SC, SEL; Writing - Review and Editing: SC, SEL

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.02.028.

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SUPPLEMENTARY MATERIALS AND METHODS

Reagents

Carvacrol (#282197), 2-aminoethoxydiphenyl borate (#42810), 2,5-dimethyltetrahydrofuran (#D187208) from Sigma-Aldrich (St. Louis, MO), and 17(R)-resolvin D1 (#13060; Cayman Chemical, Ann Arbor, MI) were used for in vitro (vehicle: DMSO) and in vivo (vehicle: 10% ethanol) study. Transient receptor potential vanilloid (TRPV) 4 selective agonist 4α-phorbol-12,13-didecanoate (Watanabe et al., 2002), a transient receptor potential ankyrin 1 selective agonist JT010 (Takaya et al., 2015), a selective TRPV4 inhibitor GSK2193874 (Cheung et al., 2017), and a selective transient receptor potential ankyrin 1 inhibitor, AM-0902 (Schenkel et al., 2016) were obtained from Tocris Bioscience (Bristol, United Kingdom).

Measurement of cytosolic intracellular calcium concentration

Keratinocytes were seeded on the glass coverslips and cultured for 24 hours. Then, they were loaded with Fura-2-acetoxymethyl ester (Thermo Fisher Scientific, Waltham, MA) in darkness for 45 minutes at 37 °C. After dye loading, cells were washed with bath solution and transferred to a perfusion chamber on a fluorescence microscope. Imaging was performed by employing a monochromator Polychrome V (TILL Photonics, Martinsried, Germany) controlled by MetaFluor software (Molecular Devices). Keratinocytes were constantly perfused at 2.5 ml per minute with a normal physiological salt solution containing: NaCl 135 mM, KCl 4.5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, HEPES 10 mM, and D-glucose 10 mM (pH 7.4, 289–295 mOsm). Fura-2 signals were obtained by alternating excitation at 340 nm or 380 nm and detecting emission at 510 nm with a Cascade 512B cooled charge-coupled device camera (Photometrics, Tucson, AZ). The ratiometric fluorescence intensities at 340 nm and 380 nm were acquired every 2 seconds to monitor the changes in calcium concentration in each cell as a function of time. Data analysis was done with background fluorescence subtracted. We conducted the measurements of calcium influx of keratinocytes from healthy skin and atopic dermatitis lesions after sequential thermal stimulation or application of a chemical cocktail of TRPV3 agonists (i.e., 200 μM 2-aminoethoxydiphenyl borate and 500 μM carvacrol) at a fixed temperature. All calcium measurements were performed at room temperature (22 °C) and/or 37 °C using a temperature controller TC-324B (Warner Instruments, Hamden, CT). Calibration of the ratio of Fura-2 fluorescence signals and calculation of the free intracellular calcium concentration were done according to the equation proposed by (Grynkiewicz et al., 1985):

$$[Ca^{2+}]i = (R - Rmin) / (Rmax - R)\, Kd\, \beta$$

R is the ratio of fluorescence excited at 340 nm to that excited at 380 nm, and Kd is the affinity constant of Fura-2 for calcium, which was taken at 135 nM for 22 °C and 224 nM for 37 °C. Rmin was the minimum R-value measured in calcium-free solution, and Rmax was the maximal R-value measured in 10 mM calcium solution with 10 μM ionomycin. β was denoted by the ratio of calcium-free and/or calcium-saturated fluorescence at 380 nm from our experiments. All chemicals for calcium imaging study were purchased from Sigma-Aldrich unless otherwise stated.

Immunohistochemistry and semiquantitative image analysis

Paraffin-embedded skin samples from the lesional skin of patients with atopic dermatitis (n = 21), allergic contact dermatitis (n = 21), and psoriasis (n = 21) were selected in this study based on the clinical and histological reviews. Normal human skin samples (n = 21) were collected from patients undergoing excision for benign neoplasia. All studies using human skin samples were approved by Institutional Review Board (no. IRB 3-2017-0140) of Yonsei University College of Medicine, Seoul, Korea, and all human skin samples were obtained with the written informed consent of the donors. Paraffin-embedded tissue sections (4 μm) were deparaffinized in xylene, rehydrated in an ethanol series, and incubated in a blocking buffer (Novocastra, Newcastle upon Tyne, United Kingdom; 3% hydrogen peroxide) for 20 minutes at room temperature. Using a 1:150 dilution of polyclonal rabbit antibodies for TRPV3 (AP11388PU-N; OriGene, Rockville, MD) or TRPV4 (NB110-55614; Novus Biologicals, Littleton, CO), slides were incubated overnight at 4 °C and washed three times with PBS. Slides were incubated with horseradish peroxidase–conjugated rabbit anti-mouse IgG (#K4063; DAKO, Glostrup, Denmark) at room temperature for 60 minutes. After washing, slides were treated with diaminobenzidine for 5–10 minutes and washed. Slides were stained with hematoxylin and eosin. For specificity control, other different primary antibodies for TRPV3 (ab85022; Abcam, Cambridge, United Kingdom) and TRPV4 (ab191580; Abcam) were used, which showed similar staining patterns. For negative control, the primary antibody was omitted, or slides were incubated overnight with an isotype-matched IgG (R&D Systems, Minneapolis, MN). Then, sections were incubated with corresponding secondary antibodies and detection systems (Supplementary Figure S2). For semiquantitative image analysis, the epidermal intensity of immunostained TRPV3 was rated on a scale of 0–5 (0 = absent, 1 = weak, 2 = low, 3 = moderate, 4 = strong, and 5 = very strong staining).

Quantitative real-time PCR

Quantitative real-time polymerase chain reaction was performed as previously described by our group (Kim et al., 2019). TaqMan probes for TRPV3, TRPV4, thymic stromal lymphopoietin, artemin, prostaglandin E2, nerve growth factor, substance P, calcitonin gene–related peptide, endothelin-1, and IL-33 (Thermo Fisher Scientific) were used. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (Thermo Fisher Scientific).

Quantification of pruritogens release by ELISA

The protein expression levels of TSLP, artemin, PGE2, NGF, subP, CGRP, ET-1, LTB4, 5-HT, and IL-33 secreted into the supernatants of cultured human primary keratinocytes or ex vivo culture of epidermal sheets from mice were quantified in triplicate using ELISA kits (Supplementary Table S1) following the manufacturer's instructions.

Western blot

Total proteins from mouse epidermal tissue lysate and primary human keratinocytes were isolated using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride. After protein isolation, equal amounts of proteins were loaded onto Nupage Novex Bis-Tris Gels (Thermo Fisher Scientific), and electrophoresis was performed using the X-cell SureLock Mini-Cell (Thermo Fisher Scientific). After that, proteins were transferred onto polyvinylidene fluoride membranes, and the membranes were incubated with a 1:2000 diluted polyclonal rabbit TRPV3 antibody (AP11388PU-N; OriGene), polyclonal rabbit TRPV4 antibody (ab191580; Abcam), or 1:4000 diluted β-actin. After probing the primary antibody, the membranes were washed with tris-buffered saline containing 0.05% Tween 20. Horseradish Peroxidase–conjugated anti-rabbit or anti-mouse secondary antibodies (Thermo Fisher Scientific) were incubated with membranes. Blots were developed using enhanced chemiluminescence reagent (Thermo Fisher Scientific). Quantitation of protein band densities was performed using ImageJ densitometry software (National Institutes of Health, Bethesda, MD).

Preparation of mice epidermal sheets and heat stimulation

On the day of the experiment (day 7), skin samples from the inflamed ear was obtained (1 cm^2) from freshly sacrificed mice 15 minutes after the intradermal injection of TRPV3 antagonists, 17(R)-epimer of resolvin D1 (1.5 mg/kg in 20 μl), diphenyltetrahydrofuran (1.5 mg/kg in 20 μl), or vehicle (10% ethanol, 20 μl). The epidermal sheets were obtained from the ears and further chopped using tweezers and then placed in 6-well plates (Corning Costar Co., Corning, NY) and incubated in keratinocyte growth medium (KGM, Gibco BRL, Rockville, MD) supplemented with bovine pituitary extract, epidermal growth factor, and 1% penicillin and streptomycin at room temperature (22 °C) or 37 °C for 24 hours. The ex vivo culture supernatants and tissue lysates were collected for ELISA analysis and quantitative real-time PCR, respectively.

In vivo heat stimulation and behavioral analysis

An in vivo heat stimulation study was performed in an individual observation glass chamber. A local heater was installed at a certain distance from the chamber to maintain the temperature within the glass chamber constantly at 36–38 °C. On the day of the experiment (day 7), MC903-treated mice were acclimated to the experimental room for 30 min and placed in the warmed chamber for 15 minutes. To investigate the role of TRPV3, we injected TRPV3 antagonists, 17(R)-epimer of resolvin D1 (1.5 mg/kg), diphenyltetrahydrofuran (1.5 mg/kg), or vehicle (10% ethanol) into the inflamed cheek and neck region intradermally (20 μl) or intraperitoneally (200 μl), 30 minutes before heat stimulation. Scratching behavior was recorded with a digital video camera during the heat stimulations. For acute itch assay, C57BL/6, 8-week old female mice (Jackson Laboratory, Bar Harbor, ME) were placed individually in a glass chamber for at least 30 minutes before behavioral analysis. Carvacrol, a combination of carvacrol and 2-aminoethoxydiphenyl borate or vehicle (10% ethanol) was administered intradermally into the cheek of the mouse. Scratching behavior was videotaped for 30 minutes. One bout of scratching by either hind paw was defined as an itch-related scratching behavior.

Data analysis

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA). The difference was considered statistically significant when the P value was less than 0.05. Student's t test or Welch's test was used to compare the data between the two groups. Statistical significance is presented as: not significant $P \geq 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ and error bars indicate the SEM.

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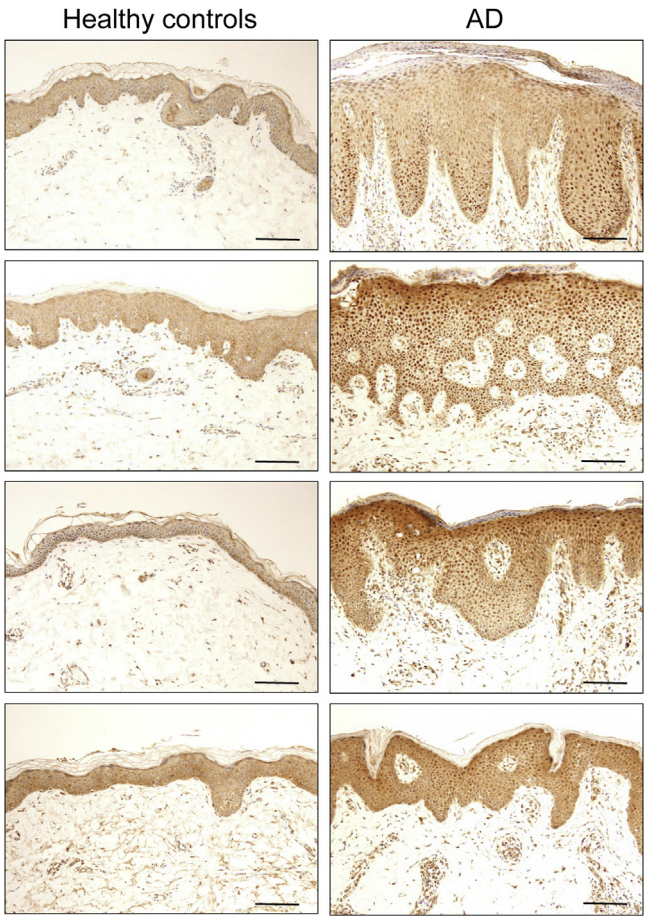
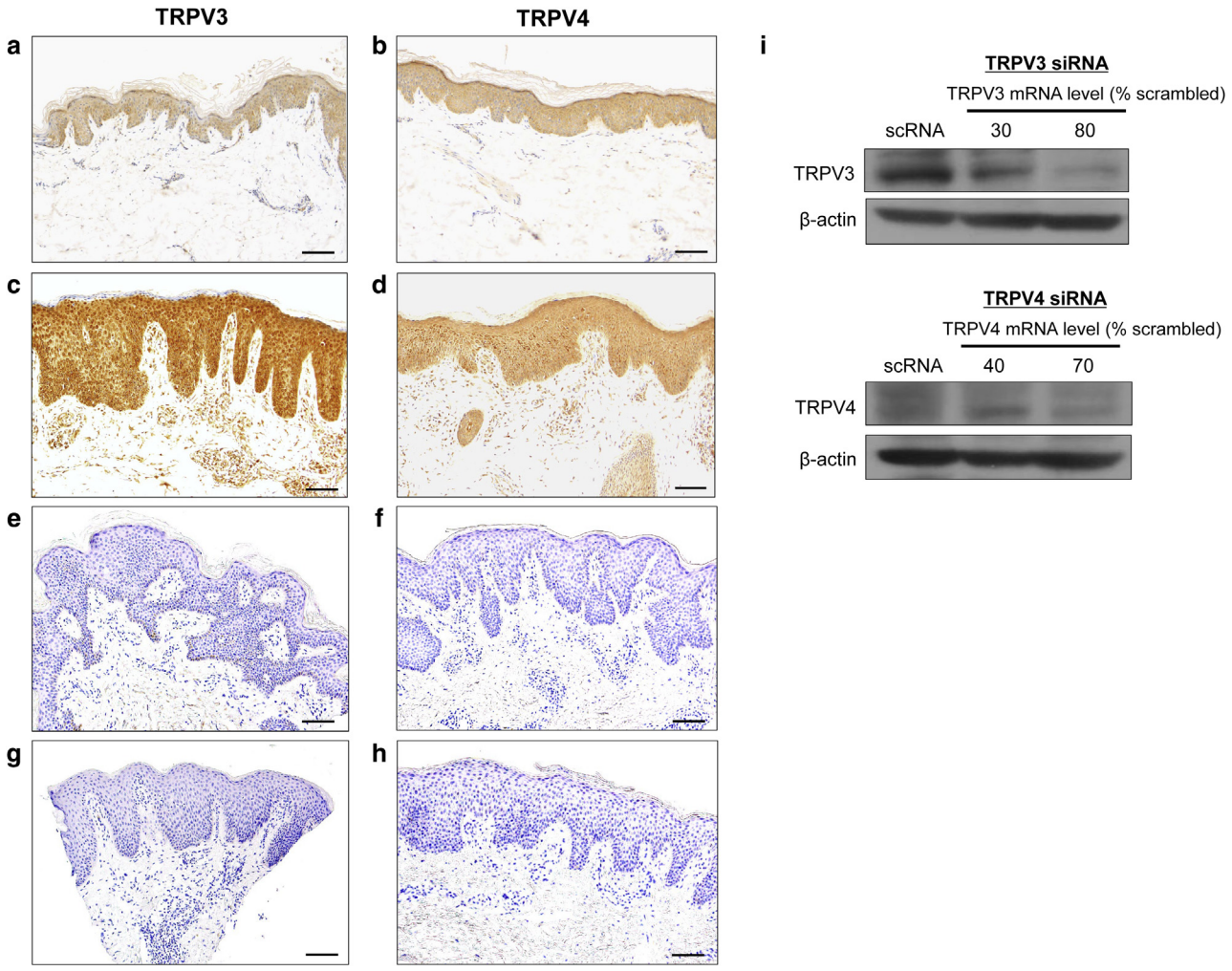
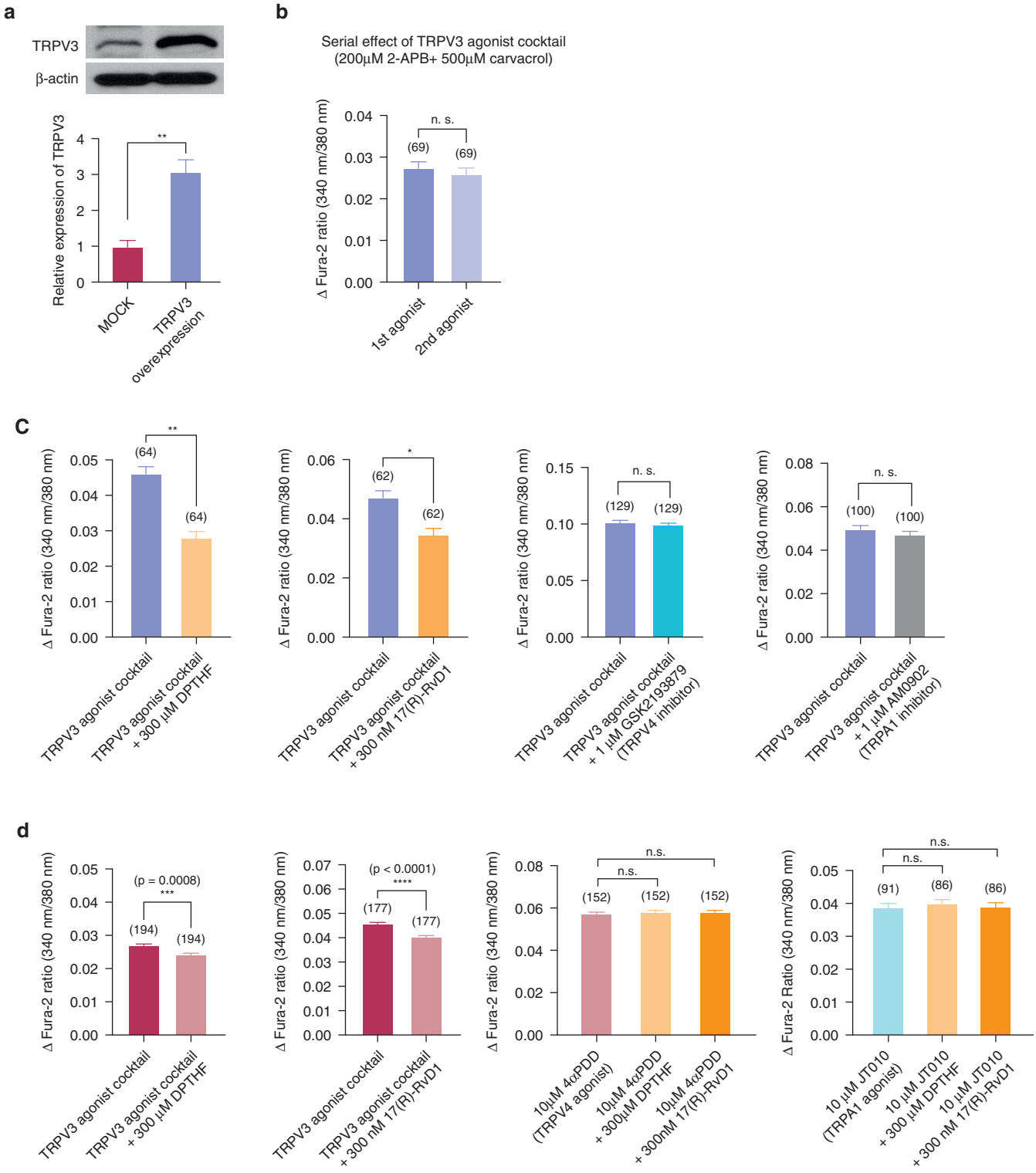


Supplementary Figure S1. Representative immunostaining for TRPV4 in lesional skin of patients with AD and skin samples from healthy controls. Immunohistochemical analysis of TRPV4 expression in the lesional skin samples from patients with AD and skin samples from healthy controls (n = 21 in each group). Bar = 100 μm. AD, atopic dermatitis; TRPV, transient receptor potential vanilloid.



Supplementary Figure S2. Validation of the specificity of antibodies for transient TRPV3 and TRPV4. The similar staining patterns of TRPV3 and TRPV4 on (**a, b**) healthy skin and (**c, d**) AD lesional skin samples using another different primary antibody against TRPV3 (ab85022; Abcam) and TRPV4 (ab191580; Abcam) as compared with Figure 1 and S1 support the specificity of TRPV3 and TRPV4 immunohistochemical staining in human skin samples. (**e, f**) Negative control staining was performed by omitting the primary antibodies against (**e**) TRPV3 and (**f**) TRPV4 in the lesional AD skin. The images of (**g**) and (**h**) showed the staining of matched IgG isotype antibody for AD lesional skin as negative control. Bar = 100 μm. (i) Validating antibodies against TRPV3 (AP11388PU-N; OriGene) and TRPV4 (NB110-55614; Novus Biologicals) that were employed for the immunohistochemical study (Figure 1 and S1) by siRNA knockdown. Knockdown of TRPV3 and TRPV4 by siRNA was confirmed by mRNA levels measured by qPCR with normalization to *Gapdh* mRNA. TRPV3 and TRPV4 protein levels were determined by western blotting with β-actin as an internal loading control. The combination of siRNA-transfected keratinocytes and antibodies to TRPV3 or TRPV4 reduced the signal compared with cells transfected with scrambled siRNA by western blot according to the rate of decrease in *TRPV3* or *TRPV4* mRNA levels. AD, atopic dermatitis; siRNA, short interfering RNA; TRPV, transient receptor potential vanilloid.



Supplementary Figure S3. Relatively selective inhibition of TRPV3 channel by DPTHF or 17(R)-RvD1 in primary human keratinocytes. (**a**) TRPV3 levels of primary human keratinocytes transfected with human TRPV3 was quantified and confirmed by western blots. (**b**) Comparisons of the amplitude of the Ca^{2+} peak in primary human keratinocytes induced by a serial stimulation with TRPV3 agonist cocktail (200 μM 2-APB and 500 μM carvacrol). The second response to agonist cocktail was not significantly reduced compared with the first response to agonist cocktail. (**c**) Effect of TRPV3 inhibitors (300 μM DPTHF or 300 nM), a selective TRPV4 inhibitor (1 μM GSK2193874), or a selective TRPA1 inhibitor (1 μM AM0902) on the agonist cocktail—evoked human TRPV3 activation was observed in the TRPV3 overexpressed keratinocytes using Fura-2-based Ca^{2+} assays. The second TRPV3 agonist cocktail stimulation was given for 2 minutes and each inhibitor was co-perfused to the bath chamber in the agonist cocktail solution. (**d**) Comparison of intracellular Ca^{2+} changes evoked by TRPV3 agonist cocktail, a selective TRPV4 agonist (10 μM 4αPDD), or a selective TRPA1 agonist (10 μM JT010) in native primary human keratinocytes. The effects of TRPV3 inhibitors either 300 μM DPTHF or 300 nM 17(R)-RvD1 on each TRPV3, TRPV4, and TRPA1 agonist stimulation were analyzed statistically. All values are presented as the mean ± SEM (number in brackets are number cells tested). *P < 0.05, **P < 0.01, ***P < 0.0005, ****P < 0.0001. 2-APB, 2-aminoethoxydiphenyl borate; 4α-phorbol-12,13-didecanoate, 4αPDD; 17(R)-RvD1, 17(R)-epimer of resolvin D1; Ca^{2+}, calcium; DPTHF, diphenyltetrahydrofuran; TRPA, transient receptor potential ankyrin; TRPV, transient receptor potential vanilloid; n.s., not significant.

Supplementary Table S1. List of ELISA Kits

Pruritogen	ELISA for Human Keratinocytes	ELISA for Mouse Keratinocytes
TSLP	DY1398 (R&D Systems, Minneapolis, MN)	MTLP00 (R&D Systems, Minneapolis, MN)
Beta-NGF	DY256 (R&D Systems, Minneapolis, MN)	CYT304 (Chemicon, Temecula, CA)
PGE_2	KGE0048 (R&D Systems, Minneapolis, MN)	514010 (Cayman Chemicals, Ann Arbor, MI)
Artemin	DY2589 (R&D Systems, Minneapolis, MN)	DY1085-05 (R&D Systems, Minneapolis, MN)
Substance P	KGE007 (R&D Systems, Minneapolis, MN)	KGE007 (R&D Systems, Minneapolis, MN)
IL-33	D3300B (R&D Systems, Minneapolis, MN)	M3300 (R&D Systems, Minneapolis, MN)
CGRP	A05481 (Bertin Pharma, France)	A05481 (Bertin Pharma, France)
ET-1	ADI-900-020A (Enzo, Farmingdale, NY)	ADI-900-020A (Enzo, Farmingdale, NY)
LTB4	KGE006B (R&D Systems, Minneapolis, MN)	KGE006B (R&D Systems, Minneapolis, MN)
5-HT	IM1749 (Beckman Coulter, Brea, CA)	IM1749 (Beckman Coulter, Brea, CA)

Abbreviations: CGRP, calcitonin gene–related peptide; ET-1, endothelin-1; HT, hydroxytryptamine; LTB4, leukotriene B4; NGF, nerve growth factor; PGE_2, prostaglandin E_2; TSLP, thymic stromal lymphopoietin.