Sensory Neuron–Expressed TRPC4 Is a Target for the Relief of Psoriasiform Itch and Skin Inflammation in Mice

JID Open

Sang Hoon Lee[1], Raquel Tonello[1], Youngin Choi[2], Sung Jun Jung[2,3] and Temugin Berta[1,3]

Psoriasis is an inflammatory skin disease associated with itch, which is a troublesome symptom with a few therapeutic options. TRPC4 is highly expressed in dorsal root ganglia (DRGs). Recently, we have revealed itch signaling in DRG neurons by which TRPC4 mediates itch to serotonergic antidepressants and demonstrated the antipruritic effect of the TRPC4 inhibitor ML204. However, the role of TRPC4 in acute and chronic itch is still largely unknown. Here, we have characterized the expression of TRPC4 in peptidergic DRG neurons and showed that acute itch induced by serotonin and histamine was attenuated in *Trpc4*-knockout mice and ML204-treated mice. We have also shown that silencing *TRPC4* in DRG and its inhibition by intradermal injections were also effective in decreasing psoriatic itch after the repeated application of imiquimod, which is a preclinical model of psoriasis. Of clinical relevance, intradermal injections of ML204 in psoriasiform skin significantly reversed imiquimod-established chronic itch and cutaneous inflammation. Given that TRPC4 is expressed in human DRGs and a specific inhibitor is in clinical trials, our data not only expand our understanding of itch and psoriasis, but also reveal TRPC4 as a potential therapeutic target with considerable translational benefits.

Journal of Investigative Dermatology (2020) **140,** 2221–2229; doi:10.1016/j.jid.2020.03.959

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease affecting 2–3% of the world's population (Hawkes et al., 2017). It is characterized by the presence of scaly skin plaques, increased thickness of the epidermis, infiltration of inflammatory cells in the dermis, and chronic itch (Bowcock and Krueger, 2005; Yosipovitch et al., 2000). Although most preclinical and clinical studies focus on psoriasiform skin inflammation, itch is also a prominent and bothersome symptom in 60–90% of patients with psoriasis (Globe et al., 2009). Yet, the pathogenesis of chronic itch associated with psoriasis is still unclear and is possibly independent of the severity of the inflammation (Roblin et al., 2014).

A number of studies have now identified various neurons and receptors that transduce itch signals. In particular, neurons from dorsal root ganglia (DRGs) that innervate the skin and express numerous pruritogen receptors are emerging as a crucial player for both acute and chronic itch (Bautista et al., 2014). Mechanistically, most pruritogen receptors are coupled with transient receptor potential (TRP) ion channels contributing to various forms of itch (Dong and Dong, 2018). For instance, TRPV1 and TRPA1 are selectively expressed by nociceptive neurons and involved in both acute and chronic itch (Xie et al., 2018). Interestingly, TRPV1 and nociceptive neurons expressing this channel (i.e., TRPV1 expressing DRG neurons) have been implicated in the susceptibility to inflammation in an animal model of psoriasis (Riol-Blanco et al., 2014; Zhou et al., 2018). In contrast, genetic silencing and pharmacological inhibition of TRPA1 resulted in an increased psoriasiform skin inflammation and itch (Kemény et al., 2018), thereby suggesting potential protective actions for TRPA1 and distinct roles for various TRP channels in psoriasis.

TRP cation channels (TRPCs)are divided into four subgroups, TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5, on the basis of sequence similarities. These subgroups are nonselective cationic channels, which are activated by G protein–coupled receptors in various tissues. In sensory ganglia, TRPC3 and TRPC4 can mediate the transmission of different forms of sensory information, including itch (Dou et al., 2017; Lee et al., 2018). Prompted by our recent finding that TRPC4 is present in human and mouse sensory ganglia that control serotonergic itch responses (Lee et al., 2018) and the indication of high serotonin (5-HT) levels in psoriatic skin (Younes and Bakry, 2016), in this study, we investigated the expression of TRPC4 in DRG neurons, its role in acute and chronic itch, as well as identified its genetic silencing and pharmacological inhibition as the potential approaches to relieve psoriasiform itch and skin inflammation in mice.

[1]*Pain Research Center, Department of Anesthesiology, University of Cincinnati Medical Center, Cincinnati, Ohio, USA; and* [2]*Department of Physiology, Medical School, Hanyang University, Seoul, Korea*

[3]*These authors contributed equally to this work.*

Correspondence: Temugin Berta, Pain Research Center, Department of Anesthesiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, ML 0531, Cincinnati, Ohio 45267-0531, USA. E-mail: temugin.berta@uc.edu

Abbreviations: CTRL, control; CGRP, calcitonin gene–related peptide; DRG, dorsal root ganglion; IMQ, imiquimod; siRNA, small interfering RNA; TRP, transient receptor potential; Trpc4 KO, Trpc4 knockout; WT, wild type

Received 16 November 2019; revised 27 March 2020; accepted 30 March 2020; accepted manuscript published online 11 April 2020; corrected proof published online 29 May 2020

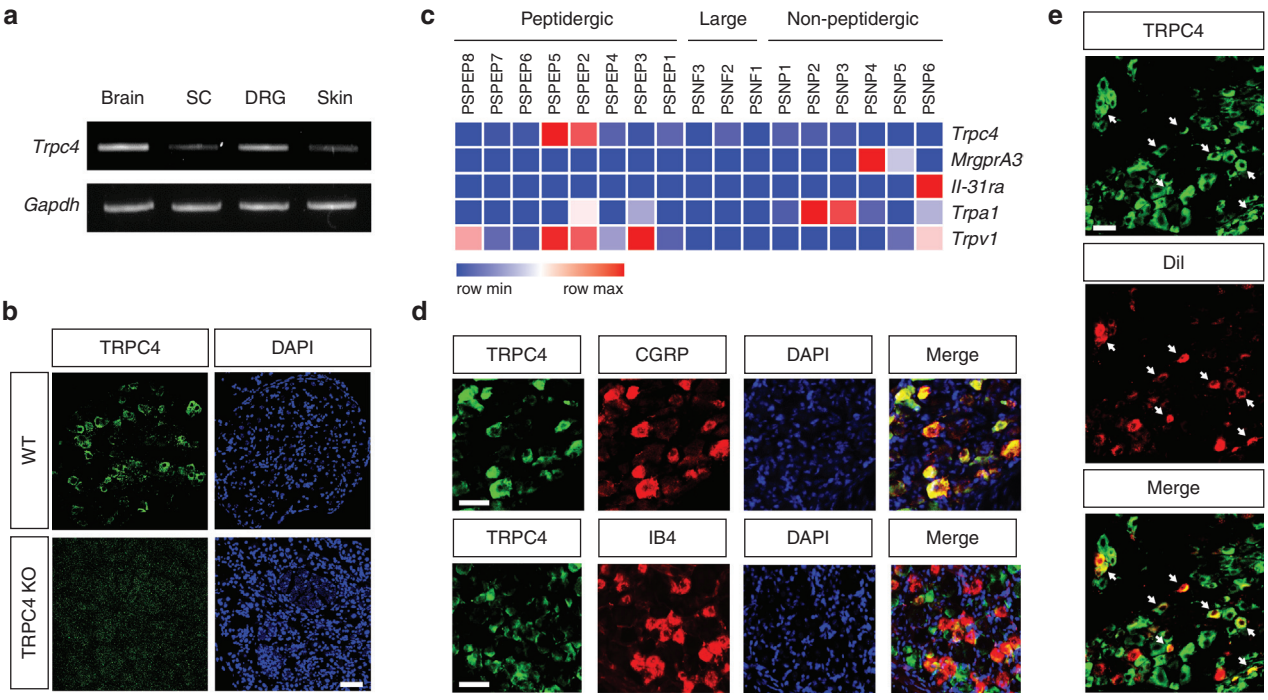


Figure 1. Characterization of TRPC4 expression in peptidergic nociceptive neurons. (**a**) PCR amplification of *Trpc4* transcripts shows expression in the brain, spinal cord, DRG, and skin tissues of mouse. (**b**) Immunostaining of DRG tissues shows the expression of TRPC4 in neurons from WT mice, but not in tissue from *Trpc4*-KO mice. DAPI was used as tissue counterstaining. Bar = 50 μm. (**c**) Heatmap for the expression of selected genes in mouse DRG neuron populations is clustered into the functional subsets that are based on single-cell RNA-seq data. Full datasets and methods are published and available (Zeisel et al., 2018). (**d**) Double immunostaining of TRPC4 with CGRP (peptidergic neurons) or IB4 (non-peptidergic neurons). DAPI was used as tissue counterstaining. Bar = 20 μm. (**e**) Immunostaining of DRG tissues shows the expression of TRPC4 in skin-innervating neurons, which were retrograde traced by the subcutaneous injections of DiI in the nape. Arrows indicate neurons with double staining. Bar = 50 μm. CGRP, calcitonin gene–related peptide; DiI 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate; DRG, dorsal root ganglion; IB4, isolectin B4; RNA-seq, RNA sequencing; *Trpc4* KO, *Trpc4* knockout; WT, wild type.

RESULTS

TRPC4 is expressed in peptidergic DRG neurons

TRPC are mainly expressed in the heart and brain tissues (Clapham et al., 2001). However, we found that transcripts for all TRPC are expressed in the DRG tissue with a high expression for *Trpc3*, moderate expressions for *Trpc1*, *Trpc2*, *Trpc4*, *Trpc7*, and low expressions for *Trpc5* and *Trpc6* (Supplementary Figure S1). In particular, we observed that *Trpc4* transcript is highly expressed in both brain and DRG tissues compared with spinal cord and skin tissues (Figure 1a). We confirmed that TRPC4 protein is indeed expressed in DRG neurons from wild-type (WT) mice, but absent in DRG neurons from *Trpc4*-knockout (*Trpc4* KO) mice, thereby validating the specificity of the TRPC4 antibody (Figure 1b).

To initially characterize the DRG neurons that express TRPC4, we reanalyzed a previously published single-cell transcriptional profile database including various populations of DRG neurons (Zeisel et al., 2018). We found that *Trpc4* is highly expressed in peptidergic neurons compared with other DRG neuronal populations (Figure 1c). Surprisingly, *Trpc4* was not present in the canonical pruriceptive neurons expressing the Mas-related G-protein–coupled receptor A3 and IL-31RA (Dong and Dong, 2018), and *Trpc4* expression was confined in *Trpv1*- but not in *Trpa1*-expressing neurons (Figure 1c). Consistent with these findings, we have previously reported that the TRPC4 is coexpressed in a subset of TRPV1-expressing neurons (Lee et al., 2018). We have now found that TRPC4 protein is colocalized with peptidergic and calcitonin gene–related peptide (CGRP)-positive, but not with non-peptidergic and isolectin B4–positive DRG neurons (Figure 1d).

To determine whether TRPC4-expressing neurons innervate the skin, we intradermally injected the neuronal retrograde tracer 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate into the nape and found that about 50% of 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate-labeled somata were also TRPC4-positive neurons (Figure 1e). These results suggest that TRPC4 is primarily expressed in peptidergic DRG neurons innervating the skin, many of which also express TRPV1 and may mediate acute and chronic itch.

TRPC4 mediates serotonergic and histaminergic acute itch

We have reported that TRPC4 mediates itch and calcium responses in DRG neurons elicited by selective serotonin reuptake inhibitors and α-methyl-5-HT, a 5-HT2b receptor agonist (Lee et al., 2018). It is still not known whether other pruritogens are dependent on TRPC4. Common pruritogens were then tested in *Trpc4* KO mice and in mice treated with the specific TRPC4 antagonist ML204 (Alom et al., 2018; Miller et al., 2011).

We found that 5-HT–evoked acute itch (assessed by the number of scratch bouts of the mice hind paw over 30 minutes) was significantly attenuated in *Trpc4* KO and ML204-treated mice (Figure 2a and b). Similarly, histamine-evoked

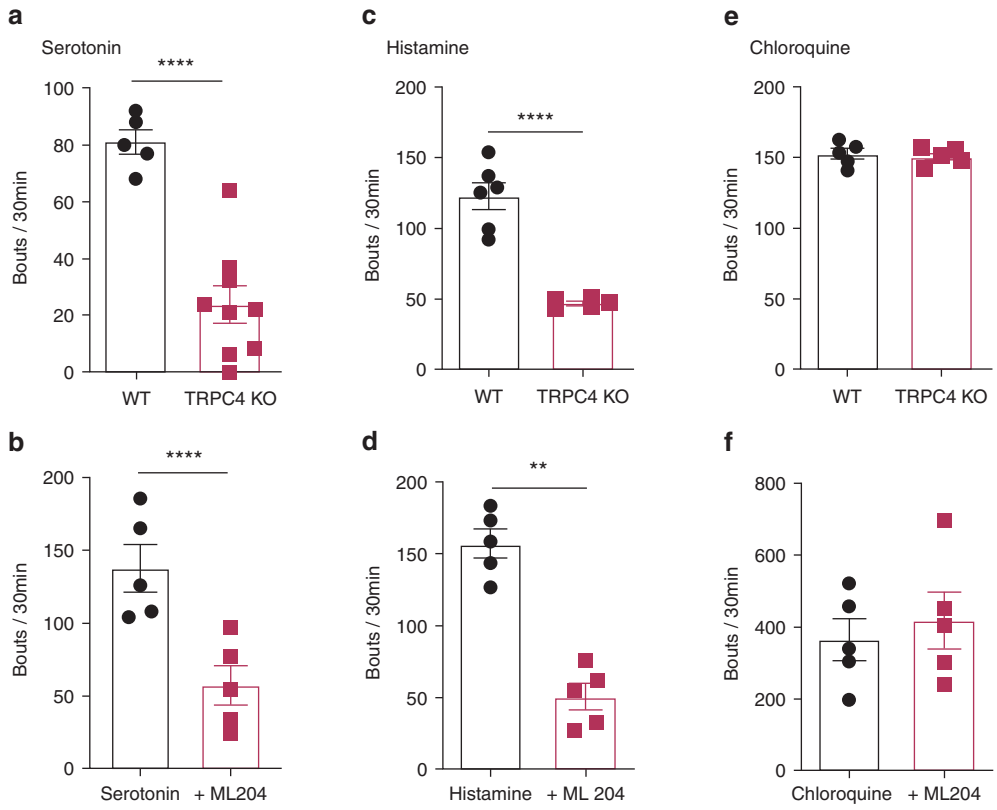


Figure 2. *TRPC4* silencing and inhibition attenuate itch induced by 5-HT and histamine. Nape-directed scratching bouts are elicited in response to the intradermal injection of (**a, b**) 5-HT (10 μg), (**c, d**) histamine (500 μg), and (**e, f**) chloroquine (200 μg) in WT and *Trpc4* KO C57 mice and in CD1 mice with or without TRPC4 inhibitor ML204 (40 μg). Injection volume= 50 μl. Two-tailed, unpaired Student's t-test was used (n = 5 mice per group). Data are depicted as mean ± SEM. $^{**}P < 0.01$, $^{****}P < 0.0001$. 5-HT, serotonin; *Trpc4* KO, *Trpc4* knockout; min, minute; WT, wild type.

itch was also attenuated in *Trpc4* KO and ML204-treated mice (Figure 2c and d). However, chloroquine-evoked acute itch was independent of TRPC4 (Figure 2e and f), and ML204 did not change acute itch induced either by the protease-activated receptor-2 peptide SLIGRL-NH_2 or toll-like receptor 7 agonist imiquimod (IMQ) (see Supplementary Figure S2a and b). This observation is consistent with the lack of colocalization of their respective receptors with TRPC4 (e.g., chloroquine acts at MrgprA3 receptor) and dependence on TRPA1-positive DRG neurons (e.g., IMQ evokes acute itch and/or pain via TRPA1), as shown in the preceding text and previously reported (Han et al., 2013; Park et al., 2014).

To investigate whether the regulation of TRPC4 is functionally expressed in DRG neurons and involved in acute response to pruritogens, calcium imaging responses were recorded in the dissociated DRG neurons of WT and *Trpc4* KO mice stimulated with 5-HT and englerin A, which is an agonist of TRPC4/5 (Akbulut et al., 2015). About 17% (33 of 186) of all DRG neurons from WT mice responded to 5-HT, and almost all these neurons (32 of 33) also responded to englerin A (Figure 3a and c). Additionally, about 35% (65 of 186) of all DRG neurons responded to englerin A, thereby suggesting that a larger cellular population of DRG neurons expressed TRPC4/TRPC5 than 5-HT receptors. However, englerin A (4 of 156) and 5-HT (0 of 156) calcium responses were almost or totally abolished in DRG neurons isolated from *Trpc4* KO mice (Figure 3b and c). Together, these results indicate that TRPC4 regulates histaminergic and serotonergic acute itch and serotonergic functional activity in DRG neurons.

TRPC4 expression in DRG neurons maintains psoriasiform chronic itch

We next asked whether TRPC4 in DRG neurons plays a predominant role in chronic itch and more specifically in psoriasis because this channel (i) is expressed in TRPV1-positive DRG neurons, which contribute to psoriasiform skin inflammation and chronic itch in the mouse model of IMQ-induced psoriasis (Lee et al., 2019 [non-peer reviewed material posted to a preprint server][1]; Riol-Blanco et al., 2014) and (ii) regulates responses to 5-HT, whose altered expression is associated with psoriasis in humans (Younes and Bakry, 2016). To generate an animal model of psoriasis, we exposed the skin of mice to repeated topical applications of IMQ that provoke inflammatory lesions, spontaneous scratches, and alloknesis (i.e., touch-evoked itch) similar to the ones observed in patients with psoriasis (van der Fits et al., 2009; Sakai et al., 2016).

We initially assessed the role of TRPC4 in the development of psoriasiform skin inflammation and spontaneous scratch in WT and *Trpc4* KO mice (Figure 4a). We found that *Trpc4* KO mice not only presented a reduced psoriasiform skin inflammation (Figure 4b), but also completely abrogated the development of spontaneous scratch bouts compared with WT mice (Figure 4c).

[1] Lee SH, Tonello R, Im ST, Jeon H, Park J, Ford Z, et al. Resolvin D3 controls mouse and human nociceptive functions and preclinical progression of psoriasis [e-pub ahead of print]. bioRxivorg 2019; https://doi.org/10.1101/715169 (accessed 28 April 2020).

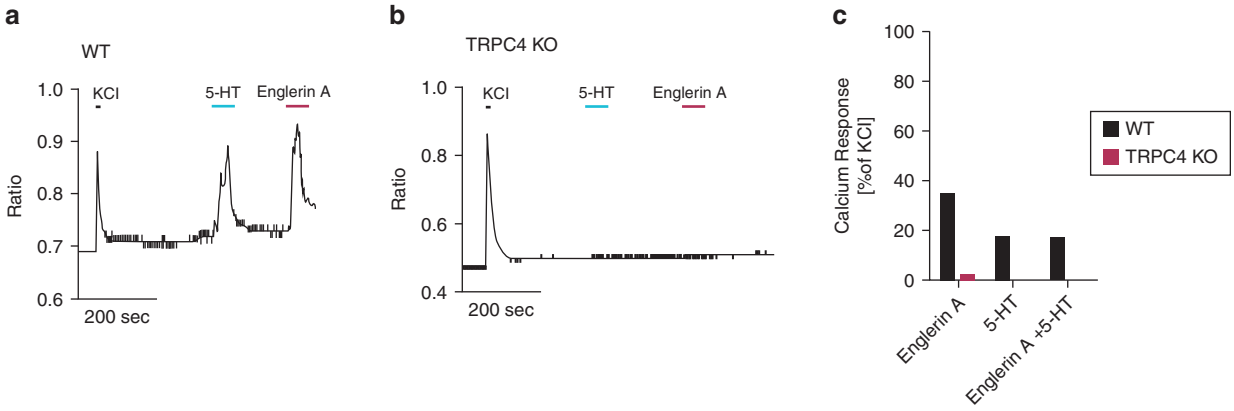
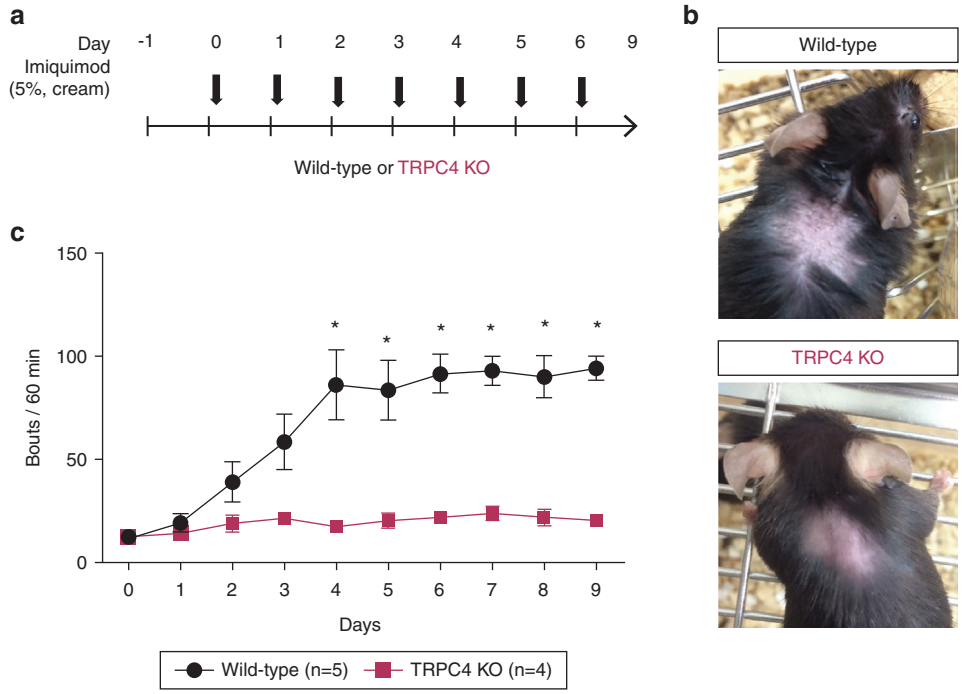


Figure 3. TRPC4 is functionally expressed in 5-HT–sensitive DRG neurons. (**a, b**) Representative Fura-2 ratiometric calcium imaging traces of cultured DRG neurons from WT and *Trpc4* KO mice were identified in response to KCl followed by exposure to 5-HT (100 μM) and englerin A (10 μM), a specific TRPC4/5 agonist. (**c**) Percentage of calcium responders (% of KCl) to englerin A, 5-HT, and to both englerin A and 5-HT in neurons from WT and *Trpc4* KO mice. 5-HT, serotonin; DRG, dorsal root ganglion; KCl, potassium chloride; *Trpc4* KO, *Trpc4* knockout; WT, wild type.

We then tested whether the specific silencing of *TRPC4* in DRG neurons was sufficient to alleviate psoriasiform spontaneous scratch and alloknesis. It should be noted that alloknesis is particularly resilient to current therapies and is measured in mice by counting the number of responses to a light pressure applied using a von Frey filament on the itchy skin (Akiyama et al., 2014; Stinco et al., 2014). To specifically knock down *TRPC4* in DRG, we used a previously well-described small interfering RNA (siRNA) approach (Berta et al., 2012; Lee et al., 2018) and delivered a control (CTRL) siRNA or a *TRPC4* siRNA by intrathecal injections 2 days before the behavioral testing (Figure 5a). As expected, we confirmed the specificity of this approach and found a significant decrease of TRPC4 in DRG but not in spinal cord tissues by *Trpc4* siRNA compared with CTRL siRNA (Figure 5b and Supplementary Figure S3a and b). Most importantly, we found that mice with *Trpc4* siRNA significantly reduced both spontaneous scratch bouts and alloknesis at day 7 (Figure 5c and d).

Similar to the siRNA treatments, spontaneous scratch bouts and alloknesis were also significantly reduced at both days 2 and 7 in mice treated with intradermal injections of ML204 compared with mice treated with a vehicle CTRL (Supplementary Figure S4a–c). Together these results suggest that peripheral TRPC4 in DRG neurons plays a crucial role in mediating both the development and maintenance of spontaneous scratch and alloknesis

Figure 4. *Trpc4* KO mice fail to develop chronic itch and skin inflammation in IMQ-induced mouse model of psoriasis. (**a**) Experimental schematic indicates daily topical applications with 5% IMQ to the nape in WT and *Trpc4* KO mice. (**b**) Representative images of skin in WT and *Trpc4* KO mice 9 days after the first IMQ application. (**c**) Time course for spontaneous scratch bouts in WT compared with *Trpc4* KO mice. Two-way repeated measured ANOVA followed by Sidak's multiple comparisons test was used (n = 4–5 mice per group). Data are depicted as mean ± SEM. *$P < 0.05$. DRG, dorsal root ganglion; IMQ, imiquimod; min, minute; *Trpc4* KO, Trpc4 knockout; WT, wild type.



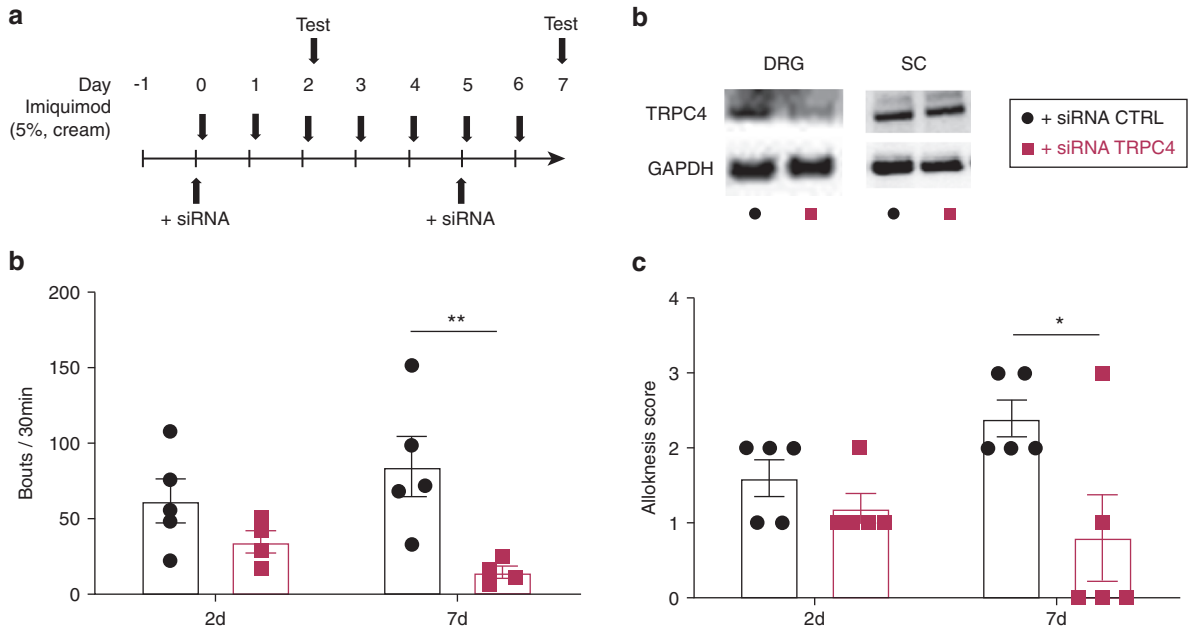


Figure 5. *TRPC4* knockdown in DRG tissue attenuates spontaneous scratch and alloknesis in the IMQ-induced mouse model of psoriasis. (**a**) Experimental schematic indicates daily topical applications with IMQ to the nape, as time for siRNA delivery and behavioral testing. (**b**) Representative western blot images of TRPC4 expression in DRG and SC tissues 7 days after the initial application of IMQ. For raw data and quantification, see Supplementary Figure S3. (**c**) Spontaneous scratch bouts and (**d**) alloknesis score in mice treated with siRNA targeting *Trpc4* (3 μg, 10 μl) compared with mice treated with a CTRL siRNA (3 μg, 10 μl) 2 and 7 days after first IMQ application. Two-way repeated measured ANOVA followed Sidak's multiple comparisons test was used (n = 4–5 mice per group). Data are depicted as mean ± SEM. $*P < 0.05$ and $**P < 0.01$. CTRL, control; d, day; IMQ, imiquimod; min, minute; SC, spinal cord; siRNA, small interfering RNA; *Trpc4* KO, *Trpc4* knockout.

as well as skin inflammation in the mouse model of IMQ-induced psoriasis.

Local inhibition of TRPC4 reverses psoriasiform itch and skin inflammation

To increase the clinical relevance of our study, we next enquired whether targeting TRPC4 locally by a small-molecule inhibitor (i.e., ML204) can also promote the resolution of established psoriasiform itch and skin inflammation (Figure 6a). Mice intradermally treated with ML204 for 3 days showed reduced spontaneous scratch and alloknesis at day 7 after the initial application of IMQ (Figure 6b).

Consistent with previous reports (Lee et al., 2019 [non-peer reviewed material posted to a preprint server][1] Riol-Blanco et al., 2014; Sakai et al., 2016), we found that mice treated with repeated applications of IMQ also developed psoriasiform skin inflammation that worsened over time in redness (i.e., erythema) and excoriation (i.e., scaling). However, mice treated with ML204 exhibited a significant reversal at day 7 in both erythema and scaling compared with mice treated with a CTRL vehicle (Figure 6c). Histological staining also indicated a decrease in psoriasiform skin inflammation as there were reduced thickness of epidermis and number of immune cells in the skin in mice treated with ML204 (Supplementary Figure S5a–c). In particular, we showed a significant decrease in the skin tissue of protein levels for the pro-inflammatory cytokines IL-17F and CCL2 and neuropeptide CGRP (Figure 6d); these pro-inflammatory cytokines and neuropeptide play crucial roles in the local inflammation and pathogenesis of psoriasis (Granstein et al., 2015; Hawkes et al., 2017; Lembo et al., 2014).

DISCUSSION

Psoriasis is considered a chronic, immune-mediated skin inflammatory disease. As such, most of the current therapies aim to reduce skin inflammation and target pro-inflammatory cytokines (Conrad and Gilliet, 2018). However, patients with psoriasis often describe itch as the most troublesome symptom (Szepietowski and Reich, 2016), which may possibly be independent of skin inflammation (Roblin et al., 2014). This suggests that inflammatory mechanisms and immune cells are not the only players in psoriasis, and therapies are still needed. In this study, we report TRPC4 in sensory neurons as a novel target for relieving psoriasiform skin inflammation and itch.

Recently, we have discovered a previously unknown itch signaling pathway in DRG neurons by which TRPC4 mediates itch to serotonergic antidepressants independently from TRPV1 and TRPA1 (Lee et al., 2018). However, TRPC4 expression in DRG neurons and its function in acute and chronic itch are largely unknown. Here, we found that TRPC4 is expressed in a subset of peptidergic neurons that innervate the skin and express TRPV1, but not TRPA1. In agreement with this restricted expression, the silencing and inhibition of TRPC4 reduced TRPV1-dependent itch to histamine but not TRPA1-dependent itch to chloroquine, SLIGRL-NH_2, and IMQ (Imamachi et al., 2009; Liu et al., 2010; Xie et al., 2018). Similarly to TRPV4 (Akiyama et al., 2016), TRPC4 mediates 5-HT–induced itch and calcium response in DRG neurons, thereby suggesting unique mechanisms and functions for this ion channel.

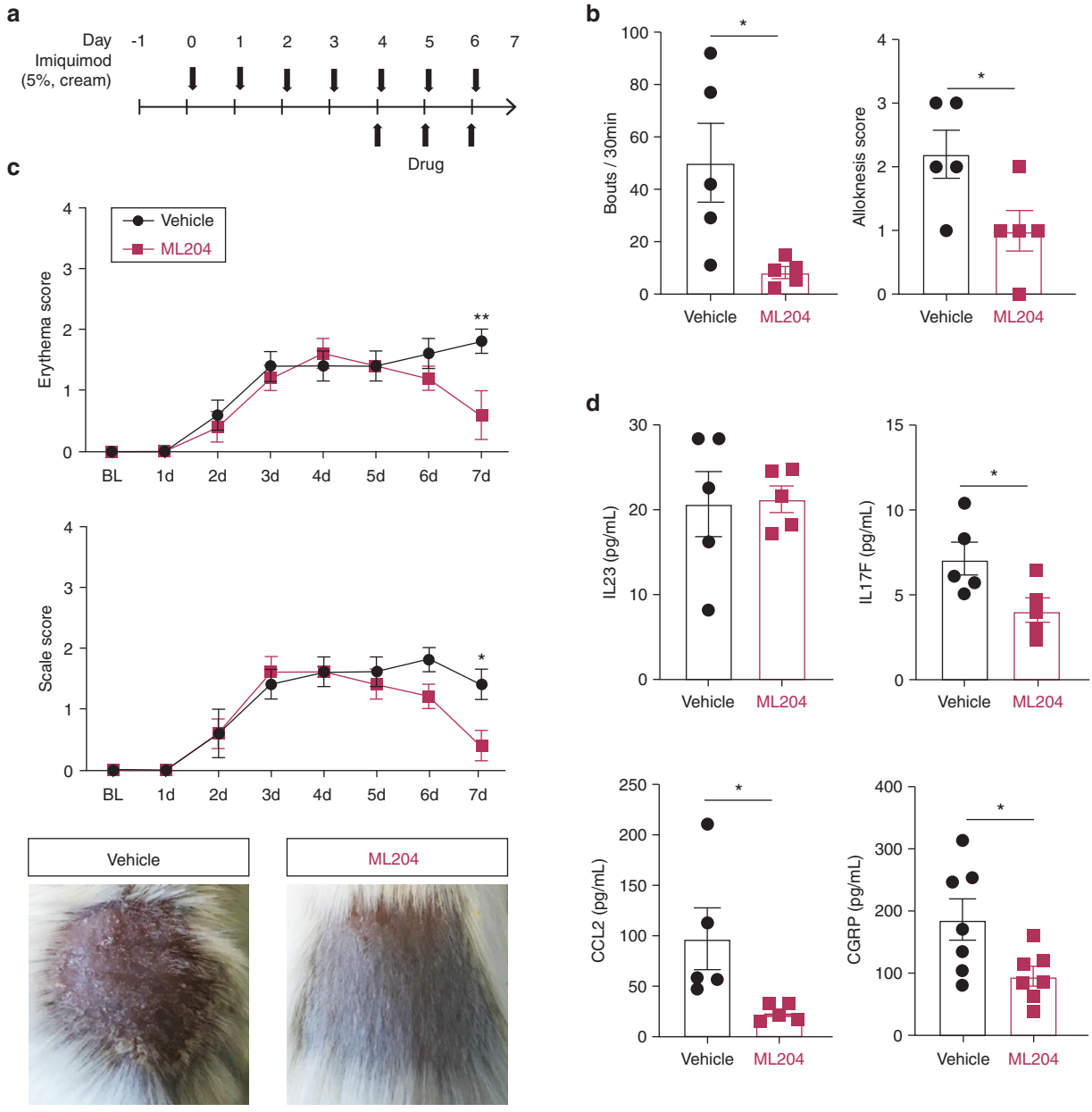


Figure 6. Local inhibition of TRPC4 reverses established chronic itch and skin inflammation in the IMQ-induced mouse model of psoriasis. (**a**) Experimental schematic indicates the daily topical application with IMQ to the nape as well as time for drug delivery. (**b**) Spontaneous scratch bouts and alloknesis score in mice treated with ML204 (40 μg, 100 μl, 3 days) compared with the mice treated with a control vehicle 7 days after initial IMQ application. (**c**) Representative images of skin, and time course erythema and scaling scores with or without ML204 treatment (40 μg, 100 μl, 3 days). (**d**) IL-23, IL-17F, CCL2, and CGRP protein expression levels in the skin of IMQ-treated mice with ML204 or a vehicle control 7 days after the initial application of IMQ. Two-tailed, unpaired Student's *t*-test was used (n = 5−7 mice per group). Data are depicted as mean ± SEM. $*P < 0.05$. CGRP, calcitonin gene−related peptide; IMQ, imiquimod; min, minute.

In our previous study, we found that TRPC4 mediates itch upon the activation of $G_{q/11}$ and PLCβ3 signaling (Lee et al., 2018). In fact, PLCβ3 is critical to histamine-evoked itch (Han et al., 2006), and *Plcβ3* KO mice exhibit reduced responses to 5-HT but not to chloroquine or SLIGRL-NH_2 (Imamachi et al., 2009). These observations agree with our behavioral studies and suggest a major role of PLCβ3 in mediating TRPC4 functions in acute itch. Interestingly, it has been reported that *Plcβ3* KO mice spontaneously develop inflammatory skin diseases because of impairment in mast cell functions (Ando et al., 2014). It should be noted that it has been suggested that *TRPC4* downregulation in keratinocytes can play a role in the pathogenesis of psoriasis (Leuner et al., 2011). However, we did not observe any particular itch and skin inflammation in *Trpc4* KO mice or found changes in TRPC4 protein levels in skin tissue after IMQ treatments (data not shown). In contrast, we demonstrated that *Trpc4* KO mice prevented the development of itch and skin inflammation after IMQ treatments, which may be driven by DRG neurons (Riol-Blanco et al., 2014).

To test the specific role of TRPC4 in DRG neurons, we used previously well-characterized intrathecal delivery of siRNA approach (Berta et al., 2012; Lee et al., 2018). We

found that mice treated with *Trpc4* siRNA scratched significantly less and displayed reduced alloknesis 7 days after the first topical application of IMQ. Previous studies have indicated that both spontaneous itch and alloknesis are resistant to antihistaminic drugs at day 7 (Sakai et al., 2016); however, our data support a neuronal and histaminic-independent role for TRPC4. It has been proposed that 5-HT levels are higher in psoriatic skin (Younes and Bakry, 2016), and we have shown that the inhibition of TRPC4 significantly attenuates itch evoked by the intradermal injection of 5-HT. Mice treated with a single dose of *Trpc4* siRNA still exhibited spontaneous scratching and alloknesis 2 days after the first application of IMQ. This can be due to a diminished effect of a single injection of siRNA or involvement of alternative cellular and molecular mechanisms. An important question to address in future studies is the identification of these mechanisms required for the complete development and/or maintenance of psoriasis.

To increase the potential for clinical translational research of our study, we also examined the effects of local intradermal injections of the small-molecule and specific TRPC4 inhibitor ML204 on established psoriasiform itch and skin inflammation. Local intradermal injections have no or very little systemic effect (Wolf and White, 2012). We delivered ML204 4 days after the first application of IMQ, when mice presented robust psoriasiform itch and skin inflammation (Riol-Blanco et al., 2014; Sakai et al., 2016), and found that these injections significantly attenuated both spontaneous scratch and alloknesis in mice treated with ML204 compare to the ones treated with a CTRL vehicle. Because of the acute effect of ML204 on psoriatic itch (Supplementary Figure S4a–c) and our previous data showing that the peripheral application of ML204 instantly reduced neuronal activity evoked by α-Methyl-5HT in a skin nerve preparation (Lee et al., 2018), we suggest a direct action of ML204 on cutaneous fibers of sensory neurons.

Interestingly, ML204 also significantly reversed the visual signs of skin inflammation such as erythema and scaliness as well as diminished the thickness of the epidermis and infiltration of inflammatory cells. Furthermore, we observed significantly lowered expression of pro-inflammatory cytokines IL-17F and CCL2 in skin tissue after ML204 treatment. Both of these cytokines increase in psoriatic skin (Hawkes et al., 2017; Lembo et al., 2014), and IL-17 inhibitors are currently approved for the treatment of moderate-to-severe plaque psoriasis (Brembilla et al., 2018). How TRPC4 reduces pro-inflammatory cytokine and skin inflammation? We cannot exclude the fact that a decrease in scratching by the inhibition of TRPC4 may alleviate contacts with the inflamed tissues and consequently reduce skin inflammation. However, we found a significant decrease of CGRP in the skin tissue of mice treated with ML204. The effects of ML204 on psoriasiform skin inflammation may result from the regulation of CGRP, which is a well-known mediator of neurogenic inflammation, and its receptors are expressed by multiple immune cells (Blake et al., 2019). Further studies are required to determine the exact role of CGRP in psoriasis, but our preprint work indicates that the knockdown of *CGRP* in sensory neurons significantly prevents the development of psoriasiform skin inflammation (Lee et al., 2019 [non-peer reviewed material posted to a preprint server][1]). It should be noted that increases in CGRP levels have been implicated in the pathogenesis of psoriasis (Assas et al., 2014; Granstein et al., 2015; Saraceno et al., 2006).

What is the relevance of our preclinical findings for the pathogenesis and treatment of human psoriasis? First, we used a mouse model comprising repeated topical applications of IMQ that provokes itch symptoms and skin inflammatory lesions that resemble human psoriasis (van der Fits et al., 2009; Sakai et al., 2016). Although it would be of particular interest to test the inhibition of TRPC4 in other animal models of psoriasis such as the genetically engineered K14-cre $Ikk2^{fl/fl}$ or K5-iκBαSR transgenic mice (Van Hogerlinden et al., 1999; Pasparakis et al., 2002), we found that the inhibition of TRPC4 reduced several pro-inflammatory cytokines, including IL-17F that plays a crucial role in these transgenic models and human psoriasis (Hawkes et al., 2017). Second, it has been reported that TRPV1 expressing neurons regulate immune cells and IL-23/IL-17 pathway (Riol-Blanco et al., 2014), and the inhibition of TRPV1 can reduce the neuronal release of the peptidergic peptide CGRP, reducing skin inflammation (Lee et al., 2019 [non-peer reviewed material posted to a preprint server][1]). Considering the presence of TRPC4 in a subset of TRPV1 expressing neurons and the reduction of CGRP in psoriasiform skin of mice treated with ML204, it is tempting to hypothesize a similar anti-inflammatory action of TRPC4 through CGRP. It should be noted that several CGRP antagonists have now received the approval of US Food and Drug Administration for the treatment of headache and migraine (Tessitore and Russo, 2019), and our studies suggest that it may be worth to repurpose them for the treatment of psoriasis. Third, we have demonstrated that the specific neuronal targeting of TRPC4 in DRGs is sufficient to alleviate psoriatic itch and skin inflammation. Given that we have found TRPC4 is highly expressed in the human DRG tissue (Lee et al., 2018), targeting these neuronal mechanisms may offer additional approaches for the treatment of psoriasis. Finally, we also found that local intradermal injections of TRPC4 inhibitor Ml204, which are an amenable and safe approach in clinical setting, effectively reversed psoriasiform itch and inflammation.

In summary, we have demonstrated that (i) TRPC4 is expressed in a subset of peptidergic neurons involved in serotonergic and histaminergic acute itch, (ii) TRPC4 mediates the pathogenesis of psoriasiform chronic itch, and (iii) local inhibition of TRPC4 can reverse both chronic itch and skin inflammation in an animal model of psoriasis. Because TRPC4/5 has entered clinical trials for the treatment of central nervous system disorders and mammalian TRPC4 structure has been recently resolved (Duan et al., 2018), it is likely that TRPC4 antagonists may soon be useful for the selective attenuation of acute itch and psoriasis.

MATERIALS AND METHODS

Reagents

We purchased 5% IMQ cream from Perrigo (Allegan, MI), whereas 5-HT, histamine, chloroquine, IMQ, and ML204 were purchased from Sigma (St. Louis, MO). CTRL and *Trpc4* siRNA were purchased

from Ambion (Austin, TX) and mixed with in-vivo jetPEI from Polyplus (New York, NY) to increase their uptake by DRGs.

Animals

Adult CD1 mice, c57BL/6 WT and *Trpc4* KO mice (male, 25−35 grams) were used for behavioral and biochemical studies. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Hanyang University, Seoul, Korea, and the University of Cincinnati, Cincinnati, Ohio, USA.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (San Diego, CA), and all data were expressed as mean ± SEM. The sample size of each experimental group was based on previous similar studies (Lee et al., 2018; Sakai et al., 2016), and no outliers were excluded. The two-tailed, unpaired Student's *t*-test was used to compare two groups, whereas two-way repeated measured ANOVA followed by Sidak's multiple comparisons test was used to compare behavioral data, erythema, and scale scores. The criterion for statistical significance was $P < 0.05$.

Details of all experimental procedures are described in Supplementary Materials.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files. Raw data are also available from the corresponding author on reasonable request.

ORCIDs

Sang Hoon Lee: http://orcid.org/0000-0001-6668-9910
Raquel Tonello: http://orcid.org/0000-0002-2175-9804
Youngin Choi: http://orcid.org/0000-0002-6666-3304
Sung Jun Jung: http://orcid.org/0000-0002-1051-6495
Temugin Berta: http://orcid.org/0000-0002-4486-8288

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We acknowledge the National Psoriasis Foundation for funding this research through a Discovery Grant Award to TB. This research was also partially supported by a grant from the National Research Foundation of Korea to SJJ. (2019R1A2B5B01070643). The *Trpc4* KO mice were kindly provided by D.E. Clapham.

AUTHOR CONTRIBUTIONS

Conceptualization: SHL, TB; Formal Analysis: SHL, RT, YC; Funding Acquisition: SJJ, TB; Investigation: SHL, RT, YC; Methodology: SHL, RT, YC; Project Administration: TB; Resources: SJJ, TB; Supervision: SJJ, TB; Visualization: SHL, RT, YC; Writing - Original Draft Preparation: SHL, TB; Writing - Review and Editing: RT, YC, SJJ

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at doi: https://doi.org/10.1016/j.jid.2020.03.959.

REFERENCES

Akbulut Y, Gaunt HJ, Muraki K, Ludlow MJ, Amer MS, Bruns A, et al. (-)-Englerin A is a potent and selective activator of TRPC4 and TRPC5 calcium channels. Angew Chem Int Ed Engl 2015;54:3787−91.

Akiyama T, Ivanov M, Nagamine M, Davoodi A, Carstens MI, Ikoma A, et al. Involvement of TRPV4 in serotonin-evoked scratching. J Invest Dermatol 2016;136:154−60.

Akiyama T, Nagamine M, Carstens MI, Carstens E. Behavioral model of itch, alloknesis, pain and allodynia in the lower hindlimb and correlative responses of lumbar dorsal horn neurons in the mouse. Neuroscience 2014;266:38−46.

Alom F, Miyakawa M, Matsuyama H, Nagano H, Tanahashi Y, Unno T. Possible antagonistic effects of the TRPC4 channel blocker ML204 on M_2 and M_3 muscarinic receptors in mouse ileal and detrusor smooth muscles and atrial myocardium. J Vet Med Sci 2018;80:1407−15.

Ando T, Xiao W, Gao P, Namiranian S, Matsumoto K, Tomimori Y, et al. Critical role for mast cell Stat5 activity in skin inflammation. Cell Rep 2014;6:366−76.

Assas BM, Pennock JI, Miyan JA. Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. Front Neurosci 2014;8:23.

Bautista DM, Wilson SR, Hoon MA. Why we scratch an itch: the molecules, cells and circuits of itch. Nat Neurosci 2014;17:175−82.

Berta T, Liu T, Liu YC, Xu ZZ, Ji RR. Acute morphine activates satellite glial cells and up-regulates IL-1β in dorsal root ganglia in mice via matrix metalloprotease-9. Mol Pain 2012;8:18.

Blake KJ, Jiang XR, Chiu IM. Neuronal regulation of immunity in the skin and lungs. Trends Neurosci 2019;42:537−51.

Bowcock AM, Krueger JG. Getting under the skin: the immunogenetics of psoriasis. Nat Rev Immunol 2005;5:699−711.

Brembilla NC, Senra L, Boehncke WH. The IL-17 family of cytokines in psoriasis: IL-17A and beyond. Front Immunol 2018;9:1682.

Clapham DE, Runnels LW, Strübing C. The TRP ion channel family. Nat Rev Neurosci 2001;2:387−96.

Conrad C, Gilliet M. Psoriasis: from pathogenesis to targeted therapies. Clin Rev Allergy Immunol 2018;54:102−13.

Dong X, Dong X. Peripheral and central mechanisms of itch. Neuron 2018;98:482−94.

Dou R, Liu Z, Yuan X, Xiangfei D, Bai R, Bi Z, et al. PAMs ameliorates the imiquimod-induced psoriasis-like skin disease in mice by inhibition of translocation of NF-κB and production of inflammatory cytokines. PLoS One 2017;12:e0176823.

Duan J, Li J, Zeng B, Chen GL, Peng X, Zhang Y, et al. Structure of the mouse TRPC4 ion channel. Nat Commun 2018;9:3102.

Globe D, Bayliss MS, Harrison DJ. The impact of itch symptoms in psoriasis: Results from physician interviews and patient focus groups. Health Qual Life Outcomes 2009;7:62.

Granstein RD, Wagner JA, Stohl LL, Ding W. Calcitonin gene-related peptide: key regulator of cutaneous immunity. Acta Physiol 2015;213:586−94.

Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, et al. A subpopulation of nociceptors specifically linked to itch. Nat Neurosci 2013;16:174−82.

Han SK, Mancino V, Simon MI. Phospholipase Cbeta 3 mediates the scratching response activated by the histamine H1 receptor on C-fiber nociceptive neurons. Neuron 2006;52:691−703.

Hawkes JE, Chan TC, Krueger JG. Psoriasis pathogenesis and the development of novel targeted immune therapies. J Allergy Clin Immunol 2017;140:645−53.

Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, et al. TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. Proc Natl Acad Sci U S A 2009;106:11330−5.

Kemény Á, Kodji X, Horváth S, Komlódi R, Szőke É, Sándor Z, et al. TRPA1 acts in a protective manner in imiquimod-induced psoriasiform dermatitis in mice. J Invest Dermatol 2018;138:1774−84.

Lee SH, Cho PS, Tonello R, Lee HK, Jang JH, Park GY, et al. Peripheral serotonin receptor 2B and transient receptor potential channel 4 mediate pruritus to serotonergic antidepressants in mice. J Allergy Clin Immunol 2018;142:1349−52.e16.

Lembo S, Capasso R, Balato A, Cirillo T, Flora F, Zappia V, et al. MCP-1 in psoriatic patients: effect of biological therapy. J Dermatolog Treat 2014;25:83−6.

Leuner K, Kraus M, Woelfle U, Beschmann H, Harteneck C, Boehncke WH, et al. Reduced TRPC channel expression in psoriatic keratinocytes is associated with impaired differentiation and enhanced proliferation. PLoS One 2011;6:e14716.

Liu T, Xu ZZ, Park CK, Berta T, Ji RR. Toll-like Receptor-7 mediates pruritus. Nat Neurosci 2010;13:1460−2.

Miller M, Shi J, Zhu Y, Kustov M, Tian JB, Stevens A, et al. Identification of ML204, a novel potent antagonist that selectively modulates native TRPC4/C5 ion channels. J Biol Chem 2011;286:33436−46.

Park CK, Xu ZZ, Berta T, Han Q, Chen G, Liu XJ, et al. Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1. Neuron 2014;82:47–54.

Pasparakis M, Courtois G, Hafner M, Schmidt-Supprian M, Nenci A, Toksoy A, et al. TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. Nature 2002;417:861–6.

Riol-Blanco L, Ordovas-Montanes J, Perro M, Naval E, Thiriot A, Alvarez D, et al. Nociceptive sensory neurons drive interleukin-23-mediated psoriasiform skin inflammation. Nature 2014;510:157–61.

Roblin D, Wickramasinghe R, Yosipovitch G. Pruritus severity in patients with psoriasis is not correlated with psoriasis disease severity. J Am Acad Dermatol 2014;70:390–1.

Sakai K, Sanders KM, Youssef MR, Yanushefski KM, Jensen L, Yosipovitch G, et al. Mouse model of imiquimod-induced psoriatic itch. Pain 2016;157:2536–43.

Saraceno R, Kleyn CE, Terenghi G, Griffiths CEM. The role of neuropeptides in psoriasis. Br J Dermatol 2006;155:876–82.

Stinco G, Trevisan G, Piccirillo F, Pezzetta S, Errichetti E, di Meo N, et al. Pruritus in chronic plaque psoriasis: a questionnaire-based study of 230 Italian patients. Acta Dermatovenerol Croat 2014;22:122–8.

Szepietowski JC, Reich A. Pruritus in psoriasis: an update. Eur J Pain 2016;20:41–6.

Tessitore A, Russo A. Is targeting CGRP the right pathway to prevent migraine? Lancet 2019;394:984–6.

van der Fits L, Mourits S, Voerman JSA, Kant M, Boon L, Laman JD, et al. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. J Immunol 2009;182:5836–45.

Van Hogerlinden M, Rozell BL, Ährlund-Richter L, Toftgård R. Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. Cancer Res 1999;59:3299–303.

Wolf RF, White GL. Clinical techniques used for nonhuman primates. Nonhuman Primates in Biomedical Research: Biology and Management, 2nd ed. San Diego, CA: Academic Press (Elsevier); 2012. p. 323–36.

Xie Z, Hu H, Xie Z, Hu H. TRP channels as drug targets to relieve itch. Pharmaceuticals (Basel) 2018;11:100.

Yosipovitch G, Goon A, Wee J, Chan YH, Goh CL. The prevalence and clinical characteristics of pruritus among patients with extensive psoriasis. Br J Dermatol 2000;143:969–73.

Younes SF, Bakry OA. Immunohistochemical evaluation of role of serotonin in pathogenesis of psoriasis. J Clin Diagn Res 2016;10:EC05–9.

Zeisel A, Hochgerner H, Lönnerberg P, Johnsson A, Memic F, van der Zwan J, et al. Molecular architecture of the mouse nervous system. Cell 2018;174:999–1014.e22.

Zhou Y, Follansbee T, Wu X, Han D, Yu S, Domocos DT, et al. TRPV1 mediates inflammation and hyperplasia in imiquimod (IMQ)-induced psoriasiform dermatitis (PsD) in mice. J Dermatol Sci 2018;92:264–71.

SUPPLEMENTARY MATERIALS AND METHODS

Reagents

We purchased 5% imiquimod (IMQ) cream from Perrigo (Allegan, MI) and control vehicle cream (Vaseline, 100% pure petroleum jelly) from Unilever (Trumbull, CT). Serotonin (cat. no. H9523), histamine (cat. no. H7125), chloroquine (cat. no. C6628), SLIGRL-NH_2 (cat. no. S9317), IMQ (cat. no. I5159), ML204 (cat. no. SML0400), and compound 48/80 (cat. no. C2313) were purchased from Sigma (St. Louis, MO), whereas mouse *Trpc4*-targeting small interfering RNA (siRNA) (Assay ID s75490) and nontargeting siRNA (cat. no. 4390844) were purchased from Ambion (Austin, TX). In-vivo jetPEI (cat. no. 201-10G) by Polyplus (New York, NY) was mixed with siRNA to increase its uptake by dorsal root ganglion (DRG) tissues.

Animals

All mouse procedures were approved by the Institutional Animal Care and Use Committee of Hanyang University, Seoul, Korea, and the University of Cincinnati, Ohio, USA, and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male CD1 mice (aged 8–10 weeks old) from Charles River Laboratories (Wilmington, MA) were used in our pharmacological and biochemical experiments, whereas male c57BL/6 wild-type mice (8–10 weeks old) from Orient Bio (Seongnam, Korea) were used as controls for experiments with the *Trpc4*-knockout mice (8–10 weeks old). *Trpc4*-knockout animals were previously characterized (Riccio et al., 2014) and obtained from D.E. Clapham.

Acute itch assays

After the fur on the rostral back was shaved, mice were habituated to the recording chambers (15 × 25 × 10 cm) 2 days before testing. On the day of the experiment, mice were placed in the recording chambers (15 × 25 × 10 cm) and allowed to habituate for 30 minutes. Then, mice were removed from the chambers and administered with an intradermal injection of 50 μl in the nape of the following pruritogens: histamine (500 μg), serotonin (10 μg), chloroquine (200 μg), compound 48/80 (100 μg), SLIGRL-NH_2 (100 μg), IMQ (20 μg), all diluted in saline containing 3.5% DMSO. Pruritogens were injected with a vehicle control (saline with 3.5% DMSO) or ML204 (40 μg, diluted in the same solution). Immediately after the injections, mice were placed in the recording chambers and video recorded for 30 minutes and spontaneous scratch response was subsequently quantified by observers blinded to the treatment of the animals. As we have previously described (Lee et al., 2018), a bout was counted when a mouse lifted its hind paw to scratch the shaved region and returned the paw to the floor. Hind paw movements directed away from the injection site and grooming movements were not counted.

Mouse model of psoriasis

We generated psoriasiform skin inflammation and itch in mice by the application of IMQ cream as previously described in a study (Sakai et al., 2016). Briefly, mice received a daily topical application of 62.5 mg of 5% IMQ cream on the shaved back skin (nape area of 2 × 2 cm) for seven consecutive days, whereas control mice were treated similarly but with a control vehicle cream (Vaseline). Doses and time courses of intradermal injections of ML204 or intrathecal injections of siRNA used to investigate the role of TRPC4 in psoriasis are reported in the following method or in the text. Skin inflammation, spontaneous itch, and alloknesis were measured daily or on days 2 and/or 7.

Chronic itch and alloknesis

Mice were habituated to the testing environment daily for at least 2 days before testing. Spontaneous chronic itch was measured for 20–22 hours after each topical application, and mice were videotaped from above for 30 minutes. The number of videotaped scratch bouts was counted by a trained observer blinded to the treatment condition. A scratch bout was defined as one by a rapid back-and-forth hind paw motions directed toward and contacting the treated area, ending with licking or biting of the toes or placement of the hind paw on the floor. Hind paw movements directed away from the treated area (e.g., ear scratching) and grooming movements were not counted. After the 30-minute recording period, alloknesis was assessed as follows: the mouse received five separate innocuous mechanical stimuli delivered by using a von Frey filament (bending force: 0.07*g*, Stoelting, Wood Dale, IL) to five randomly selected sites along the border of the cream application area. The presence or absence of a positive response (a hindlimb scratch bout directed to the site of mechanical stimulation) was noted for each stimulus. The alloknesis score was the total number of positive responses elicited by the five stimuli (0–5).

Skin inflammation score and histopathology

Skin inflammation was evaluated by scoring the erythema and scaling of the skin, a method based on similar and recent studies (Dou et al., 2017; Xu et al., 2017). Briefly, erythema (red taint) and scaling (white plaques) were scored separately on a scale ranging from 0 to 4 according to the degree of severity: 0, absent; 1, mild; 2, moderate; 3, severe; and 4, very severe. At the end of the behavioral experiments, skin inflammation was also assessed by H&E staining. Skin from the nape was fixed in 4% paraformaldehyde, embedded in tissue freezing medium, and cut into 20-μm-thick sections on a microtome. The sections were stained with H&E, and epidermal thickness and number of immune cells were quantified.

Calcium imaging of cultured DRG neurons

We performed Fura-2 acetoxymethyl ester–based (Molecular Probes) calcium imaging experiments as previously described. Briefly, DRG neurons prepared were loaded with Fura-2 acetoxymethyl ester (2 μM) and 0.01% Pluronic F-127 (weight by volume, Life Technologies, Carlsbad, CA) for 40 minutes at 37 °C in DMEM containing 10% fetal bovine serum and 1% penicillin–streptomycin. Extracellular solution contained 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 $CaCl_2$, 1 mM $MgCl_2$, and 10 mM D-(+)-glucose at a pH of 7.3. Acquired images were displayed as the ratio of 340 nm to 380 nm. Cells were illuminated with lamp and were excited by Lambda DG-4 (Shutter Instrument, Novato, CA)

and identified as neurons by eliciting depolarization with high potassium solution (50 mM) at the start of each experiment, followed by exposure to serotonin (100 μM) and englerin A (10 μM). Intracellular calcium concentrations were measured by digital video microfluorometry with the camera (Hamamatsu, Shizouka, Japan, C11440) coupled to the microscope (Olympus, Tokyo, Japan, IX70) and a computer with MetaFluor software (Universal Imaging Crop). All graphs displaying Fura-2 ratios were normalized to the baseline ratio F340/F380 = (Ratio)/(Ratio t = 0), and all drugs were applied via bath perfusion at a flow rate of 3−5 ml/min.

Gene knockdown by siRNA

A total of 3 μg of siRNA targeting *TRPC4* or nontargeting control siRNA were diluted in a 10 μl solution containing 2.62 μl of in-vivo jetPEI and 5% glucose were injected intrathecally on days 0 and 5 after the first application of IMQ to investigate the role of TRPC4 in DRG tissues. A valid spinal puncture and intrathecal delivery of siRNA was confirmed by a reflexive tail flick after the needle entry into the subarachnoid space, as we have previously described (Lee et al., 2018; Tonello et al., 2019).

Western blotting

To validate the knockdown of TRPC4 in DRG tissues (C3−T3), we collected samples of cervical spinal cord and DRG at the end of the behavioral experiments. Samples were homogenized in the radioimmunoprecipitation assay buffer (lysis buffer) (cat. no. 20-188, Sigma, St. Louis, MO) containing MS-SAFE protease and phosphate inhibitors (cat. no. MSSAFE, Sigma) and centrifuged to remove cell debris. Protein concentration was measured using the Qubit protein assay kit (cat. no. Q33211, Fisher Scientific, Hampton, NH), 20 μg of proteins was loaded for each lane and separated by NuPAGE 4−12% Bis-Tris Protein Gel (cat. no. NP0323BOX, Fisher Scientific, Hampton, NH), and proteins were transferred electrophoretically to polyvinylidene fluoride membranes. After blocking with 2% BSA, the membranes were incubated overnight at 4°C with TRPC4 antibody (1:1,000, cat. no. ACC-018, Alomone, Jerusalem, Israel) and GAPDH antibody (1:1,000, cat. no. 3683S, Cell Signaling, Danvers, MA), and then incubated with the appropriate horseradish peroxidase−conjugated secondary antibody (1:2000, Cell Signaling, Danvers, MA). The blots were detected by using a chemiluminescence method (SuperSignal West Dura Extended Duration Substrate, cat. no. 34075, Fisher Scientific) and scanned with the iBright FL1000 Imaging System (Fisher Scientific). Bands were quantified using the National Institutes of Health software Image J, and the relative values of each protein were normalized by the values of the protein levels of GAPDH.

ELISA

Animals were terminally anesthetized with isoflurane and nape skin were rapidly removed 8 days after IMQ or vehicle treatment and homogenized in radioimmunoprecipitation assay buffer (lysis buffer)containing a cocktail of protease inhibitors and phosphatase inhibitors (see western blotting). Protein concentrations were assessed by the Qubit protein assay kit and samples were analyzed using the IL-17F (cat. no. 436107, BioLegend, San Diego, CA), IL-23 (cat. no. M2300, R&D system, Minneapolis, MN), CCL2 (cat. no. MJE00B, R&D system, Minneapolis, MN), and CGRP (cat. no. A05482, Bertin bioreagent, Bretonneux, France). A standard curve was included in the experiment, and the assay was used according to the manufacturer's instructions.

Immunofluorescence

Mice were terminally anesthetized with isoflurane and perfused through the ascending aorta with PBS and then by 4% paraformaldehyde in PBS. DRG tissues (C5−T3) were removed, post-fixed in the same fixative, and placed in 30% sucrose solution for 24 hours at 4 °C. Tissue sections were cut in a cryostat (12 μm), blocked with BlockAid blocking solution (cat. no. B10710, Fisher Scientific), and incubated overnight at 4 °C with the following primary antibodies or reagents: TRPC4 (1:1,000, cat. no. OST00025W, Fisher Scientific), TRPC4 (1:200, cat. no. ACC-018, Alomone), CGRP (1:500, cat. no. ab36001, Abcam, Cambridge, MA), isolectin B4 (1:500, cat. no. I32450, Fisher Scientific), and/or DAPI (300 nmol/L, cat. no. D1306, Fisher Scientific). Sections were then washed and incubated with the appropriate secondary antibodies conjugated to Alexa Fluor 488 or 546 (1:1,000, Fisher Scientific). A BX63 fluorescence microscope (Olympus) was used to analyze 3 to 5 sections, and images were captured with a high-resolution CCD Spot camera (cat. no. DP80, Olympus).

1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate labeling and imaging

To determine the skin of nape innervating neurons, we intradermally injected the retrograde nerve tracer 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (100 μl of 1 mg/ml, cat. no. D282, Life Technologies) into the skin of nape and collected DRG after 3 days. We observed the red signal in neuronal cell bodies in DRG tissues (C5−T3).

Quantitative real-time RT-PCR

Naive mice were deeply anesthetized with isoflurane and perfused transcardially with PBS and DRG tissues (C5−T3) removed immediately. Total RNA was extracted using Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA), the amount and quality of which were assessed by SimpliNano UV-Vis Spectrophotometer (General Electric, Boston, MA) and then converted into cDNA using a high-capacity cDNA reverse transcription kit (cat. no. 4368814, Fisher Scientific). Specific primers for TRPCs as well as GAPDH were obtained from PrimerBank (Wang et al., 2012). Primer sequences are shown in Supplementary Table S1. Quantitative real-time RT-PCR was performed on a QuantStudio 3 Real-Time PCR System (Fisher Scientific) using PowerUp SYBR Green Master Mix (cat. no. A25741, Fisher Scientific). All samples were analyzed at least in duplicate and normalized by GAPDH expression. The relative expression ratio per condition was calculated based on the method described by Pfaffl (2001).

RT-PCR

Naive mice were deeply anesthetized with isoflurane, perfused transcardially with PBS, and brain, spinal cord, and DRG tissues (C5−T3) were removed immediately. Total RNA

was extracted using Direct-zol RNA MiniPrep kit (Zymo Research), the amount and quality of which were assessed by SimpliNano UV-Vis Spectrophotometer (General Electric) and then converted into cDNA using a high-capacity cDNA reverse transcription kit (cat. no. 4368814, Fisher Scientific). Samples were diluted 2:100 and used as a template for RT-PCR experiments. The following primer pairs were used: *Trpc4* (forward, 5′-CGACCATGCAGATATAGAATGGAA-3′; reverse, 5′-TGGTATTGGTGATGTCTTCTCAAG-3′), and *Gapdh* (forward, 5′-TGAAGGTCGGTGTGAACGAATT-3′; reverse, 5′-GCTTTCTCCATGGTGGTGAAGA-3′). The RT-PCR products were displayed on 1.5% agarose gels with SYBR Safe DNA Gel Stain.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (San Diego, CA), and all data were expressed as mean ± S.E.M. Although no statistical power calculation was conducted, the sample size of each experimental group was based on previous similar studies (Lee et al., 2018; Sakai et al., 2016). No outliers were excluded. The two-tailed, unpaired Student's *t*-test was used to compare two groups, whereas two-way repeated measured ANOVA followed by Sidak's multiple comparisons test was used to compare behavioral data, erythema, and scale scores. The criterion for statistical significance was $P < 0.05$ with $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

SUPPLEMENTARY REFERENCES

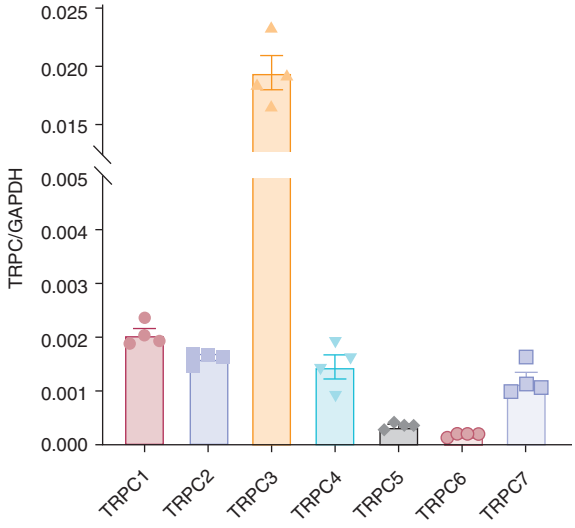
Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.

Riccio A, Li Y, Tsvetkov E, Gapon S, Yao GL, Smith KS, et al. Decreased anxiety-like behavior and G q/11-dependent responses in the amygdala of mice lacking TRPC4 channels. J Neurosci 2014;34:3653–67.

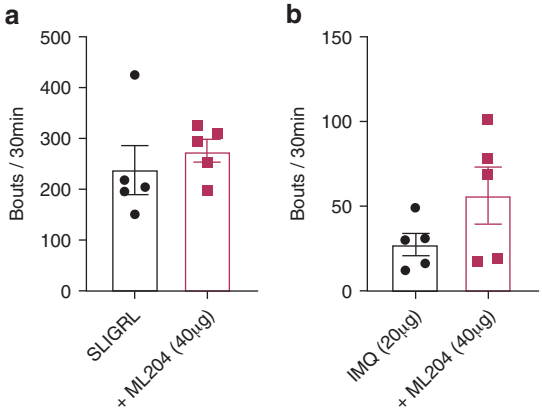
Tonello R, Lee SH, Berta T. Monoclonal antibody targeting the matrix metalloproteinase 9 prevents and reverses paclitaxel-induced peripheral neuropathy in mice. J Pain 2019;20:515–27.

Wang X, Spandidos A, Wang H, Seed B. PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. Nucleic Acids Res 2012;40:D1144–9.

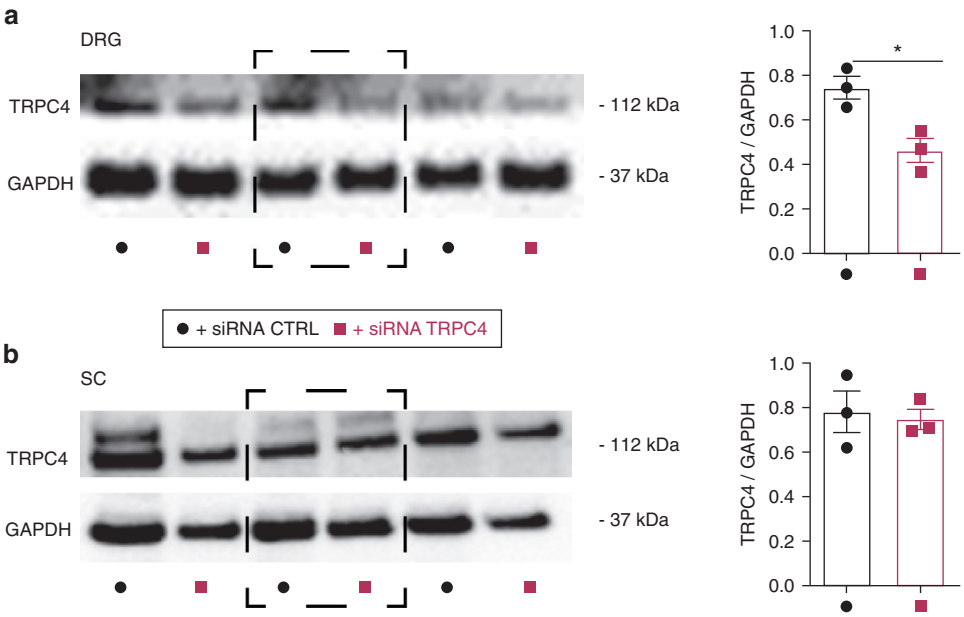
Xu J, Duan X, Hu F, Poorun D, Liu X, Wang X, et al. Resolvin D1 attenuates imiquimod-induced mice psoriasiform dermatitis through MAPKs and NF-κB pathways. J Dermatol Sci 2017;89:127–35.



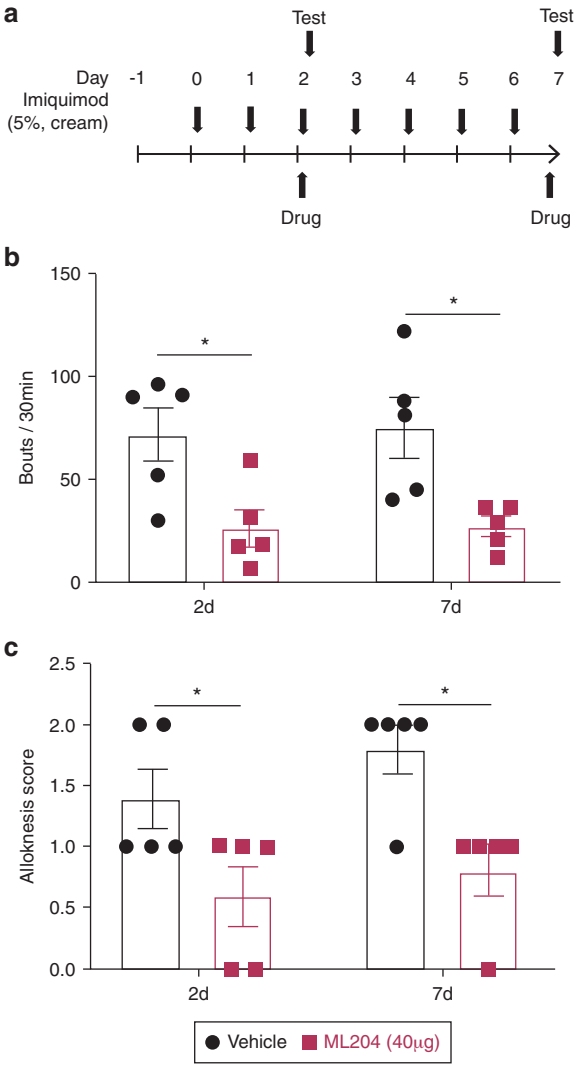
Supplementary Figure S1. Quantitative RT-PCR analysis shows the relative expression of TRPC in mouse DRG tissues. The values are normalized to GAPDH. n = 4 mice. Data are depicted as mean ± SEM. DRG, dorsal root ganglion.



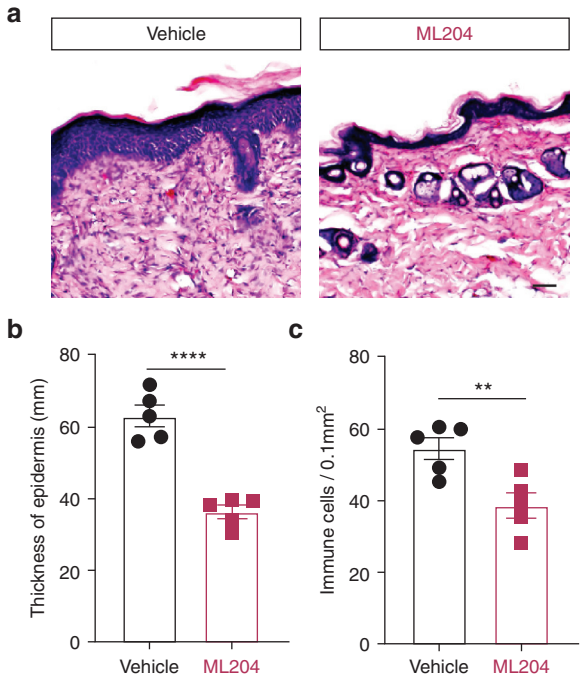
Supplementary Figure S2. Effect of TRPC4 inhibitor in acute itch evoked by SLIGRL-NH_2 and IMQ. Nape-directed scratching bouts elicited in response to the intradermal injection of (**a**) SLIGRL-NH_2 (100 μg), and (**b**) imiquimod (20 μg) with or without TRPC4 inhibitor ML204 (40 μg). Injection volume 50 μl. Two-tailed, unpaired Student's *t*-test was used (n = 5 mice per group). Data are depicted as mean ± SEM. DRG, dorsal root ganglion; IMQ, imiquimod; min, minute.



Supplementary Figure S3. Western blot analyses show the specific knockdown of *TRPC4* in DRG and not in spinal cord tissues after treatment with siRNA targeting *TRPC4*. Western blot images of TRPC4 expression in (**a**) DRG and (**b**) spinal cord 7 days after the initial application of a vehicle control or 5% IMQ. The values are normalized to GAPDH. Two-tailed, unpaired Student's *t*-test was used (n = 3 mice per group). Data are depicted as mean ± SEM. CTRL, control; DRG, dorsal root ganglion; IMQ, imiquimod; siRNA, small interfering RNA.



Supplementary Figure S4. TRPC4 inhibitor attenuates spontaneous scratch and alloknesis in the IMQ-induced mouse model of psoriasis. (**a**) Experimental schematic indicates the daily topical application with IMQ to the nape as time for drug delivery and behavioral testing. (**b**) Spontaneous scratch bouts and (**c**) alloknesis score in mice treated with ML204 (40 μg, 100 μl) compared with mice treated with a control vehicle two and 7 days after first IMQ application. Two-way repeated measured ANOVA followed by Sidak's multiple comparisons test was used (n = 5 mice per group). Data are depicted as mean ± SEM. *$P < 0.05$. d, day; IMQ, imiquimod; min, minute.



Supplementary Figure S5. Histological staining of skin tissue indicates decreases in psoriasiform inflammation. (**a**) Representative H&E histopathology, (**b**) quantification of skin thickness, and (**c**) number of immune cells. Bar = 50 μm. Two-tailed, unpaired Student's *t*-test or two-way repeated measured ANOVA followed by Sidak's multiple comparisons test were used (n = 5 mice per group). Data are depicted as mean ± SEM. **$P < 0.01$, ****$p < 0.0001$. min, minute.

Supplementary Table S1. Quantitative Real-Time RT-PCR Primer Sequences

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Accession Number
Trpc1	TACGGTTGTCAGTCCGCAGA	TCGTTTTGGCCGATGATTAAGTA	NM_011643.4
Trpc2	GCCATGTGGTGTCATTTTCCT	GTTGTCCAGTCTTGTCCTGAG	NM_011644.3
Trpc3	TCGAGAGGCCACACGACTA	CTGGACAGCGACAAGTATGC	NM_019510.2
Trpc4	TGTATCTGGCAACAATCTCCTTG	CATGTCCCATGATTCCCGTGG	NM_016984.3
Trpc5	GTGTATCCAGTTCGGAGGTAGA	CCTCGCTTGATAAGGCAATGA	NM_009428.3
Trpc6	AGCCAGGACTATTTGCTGATGG	AACCTTCTTCCCTTCTCACGA	NM_013838.2
Trpc7	CTTCCTGGACTCGGCTGAGTA	GCGTTCTGCCCCATGTAGT	NM_012035.3
Gapdh	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	NM_001289726.1