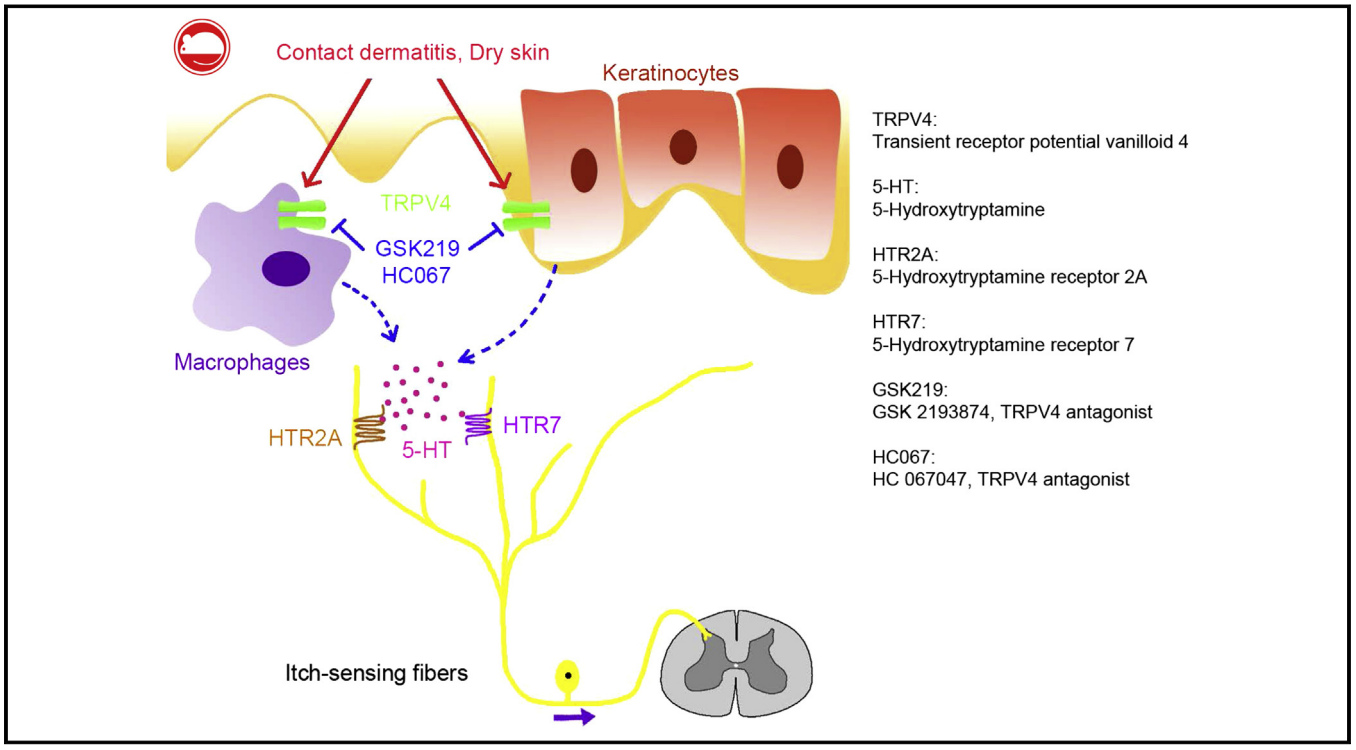
Transient receptor potential vanilloid 4–expressing macrophages and keratinocytes contribute differentially to allergic and nonallergic chronic itch

Jialie Luo, PhD,[a]* Jing Feng, PhD,[a]* Guang Yu, PhD,[a,b] Pu Yang, PhD,[a] Madison R. Mack, BA,[a] Junhui Du, PhD,[c] Weihua Yu, PhD,[d] Aihua Qian, PhD,[e] Yujin Zhang, PhD,[f] Shenbin Liu, PhD,[a] Shijin Yin, PhD,[g] Amy Xu, BS,[a] Jizhong Cheng, PhD,[h] Qingyun Liu, PhD,[i] Roger G. O'Neil, PhD,[j] Yang Xia, PhD,[f] Liang Ma, PhD,[k] Susan M. Carlton, PhD,[c] Brian S. Kim, MD,[a,k] Kenneth Renner, PhD,[l] Qin Liu, PhD,[a] and Hongzhen Hu, PhD[a]

St Louis, Mo; Nanjing, Chongqing, Shanghai, and Wuhan, China; Galveston and Houston, Tex; and Vermillion, SD

GRAPHICAL ABSTRACT



From [a]the Center for the Study of Itch, Department of Anesthesiology, and [k]the Division of Dermatology, Department of Medicine, Washington University School of Medicine, St Louis; [b]the School of Medicine and Life Sciences, Nanjing University of Chinese Medicine; [c]the Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston; [d]the Department of Anatomy, Chongqing Medical University; [e]the Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University, Shanghai; [f]the Department of Biochemistry and Molecular Biology and [j]the Department of Integrative Biology and Pharmacology, the University of Texas Medical School at Houston; [g]the College of Pharmacy, South-Central University for Nationalities, Wuhan; [h]the Department of Medicine, Baylor College of Medicine, Houston; [i]the Brown Foundation Institute of Molecular Medicine and Texas Therapeutics Institute, University of Texas Health Science Center at Houston; and [l]the Center for Brain and Behavior Research, Biology Department, University of South Dakota, Vermillion.

*These authors contributed equally to this work.

Disclosure of potential conflict of interest: R. G. O'Neil's, B. S. Kim's, K. Renner's, and Q. Liu's, institutions received a grant from National Institutes of Health (NIH) for this work and other works. H. Hu's institute received grants from NIH, the Center for the Study of Itch of the Department of Anesthesiology at Washington University School of Medicine and Washington University DDRCC for this work and other works. The rest of the authors declare that they have no relevant conflicts of interest.

Supported in whole or in part by grants from the National Institutes of Health: (R01GM101218A and R01DK103901) and the Center for the Study of Itch of the Department of Anesthesiology of Washington University (to H.H.), grant R01DA019921 (to K.R.), R01EY024704 (to Q.L.), R01AR070116 (to B.S.K.), R01DK098401 (to R.G.O.), and P30DK052574 (to Washington University DDRCC).

Received for publication February 2, 2017; revised May 5, 2017; accepted for publication May 24, 2017.

Corresponding author: Hongzhen Hu, PhD, Or Qin Liu, PhD, Department of Anesthesiology, Center for the Study of Itch, Washington University School of Medicine in St Louis, Campus Box 8054, 660 South Euclid Ave, St Louis, MO 63110-1093. E-mail: hongzhen.hu@wustl.edu. Or: qinliu@wustl.edu.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2017.05.051

Background: Chronic itch is a highly debilitating symptom that underlies many medical disorders with no universally effective treatments. Although unique neuronal signaling cascades in the sensory ganglia and spinal cord have been shown to critically promote the pathogenesis of chronic itch, the role of skin-associated cells remains poorly understood.

Objective: We sought to examine the cutaneous mechanisms underlying transient receptor potential vanilloid 4 (TRPV4)–mediated allergic and nonallergic chronic itch.

Methods: Expression of TRPV4 in chronic itch and healthy control skin preparations was examined by using real-time RT-PCR. *Trpv4*^{eGFP} mice were used to study the expression and function of TRPV4 in the skin by means of immunofluorescence staining, flow cytometry, calcium imaging, and patch-clamp recordings. Genetic and pharmacologic approaches were used to examine the role and underlying mechanisms of TRPV4 in mouse models of dry skin–associated chronic itch and spontaneous scratching associated with squaric acid dibutylester–induced allergic contact dermatitis.

Results: TRPV4 is selectively expressed by dermal macrophages and epidermal keratinocytes in mice. Lineage-specific deletion of TRPV4 in macrophages and keratinocytes reduces allergic and nonallergic chronic itch in mice, respectively. Importantly, TRPV4 expression is significantly increased in skin biopsy specimens from patients with chronic idiopathic pruritus in comparison with skin from healthy control subjects. Moreover, TRPV4-dependent chronic itch requires 5-hydroxytryptamine (5-HT) signaling secondary to activation of distinct 5-HT receptors in both patients with allergic and those with nonallergic chronic itch conditions.

Conclusion: Our study reveals previously unrecognized mechanisms by which TRPV4-expressing epithelial and immune cells in the skin critically and dynamically mediate chronic itch and unravels novel targets for therapeutics in the setting of chronic itch. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Transient receptor potential vanilloid 4, chronic itch, macrophage, keratinocyte*

Abbreviations used

ACD:	Allergic contact dermatitis
AEW:	Acetone/ether mixture followed by distilled water
$[Ca^{2+}]_i$:	Intracellular Ca^{2+}
CIP:	Chronic idiopathic pruritus
C_t:	Cycle threshold
DRG:	Dorsal root ganglion
DTX:	Diphtheria toxin
eGFP:	Enhanced green fluorescent protein
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GFP:	Green fluorescent protein
5-HT:	5-Hydroxytryptamine
Htr2a:	5-HT receptor 2a
Htr7:	5-HT receptor 7
IRB:	Institutional review board
PBS+TX:	PBS with 0.1% Triton X-100
pCPA:	p-Chlorophenylalanine
SADBE:	Squaric acid dibutylester
TPH:	Tryptophan hydroxylase
TRPV4:	Transient receptor potential vanilloid 4
WT:	Wild-type

Chronic itch, a symptom of many primary skin disorders and systemic diseases, is a major medical issue affecting 10% to 20% of the general population and has deleterious effects on both quality of life and productivity.[1,2] Despite decades of research, how chronic itch is generated at the molecular and cellular levels is poorly understood. Recent studies have identified multiple itch-related G protein–coupled receptors and ion channels in the primary sensory neurons, which enable sensory neurons to detect a variety of pruritogens.[3-5] However, upstream pathways, such as the identity of putative receptors that trigger epithelial and immune cells to elicit itch, remain unknown. Lack of this critical information has severely limited the development of effective therapies for most types of chronic itch.

Transient receptor potential vanilloid 4 (TRPV4) is a Ca^{2+}-permeable cation channel in the TRPV family and is abundantly expressed in the skin, renal, and urinary bladder epithelia (www.biogps.org).[6,7] TRPV4 is a polymodal sensory transducer that integrates a variety of thermal, mechanical, and chemical stimuli, including warmth (27°C to 35°C), hypo-osmotic stimulation, and many inflammatory metabolites.[8] As a result, TRPV4 channels are involved in many physiologic and pathologic processes. Although it was recently reported that TRPV4 is involved in acute itch elicited by exogenously applied histamine and 5-hydroxytryptamine (5-HT), its precise mechanism in itch induction remains controversial. Indeed, whether TRPV4 predominantly mediates itch indirectly through skin-associated cells or by directly stimulating dorsal root ganglion (DRG) neurons is an active area of investigation.[9,10] More importantly, the role of TRPV4 in the development of chronic itch remains unexplored.

In the current study we show that these osmosensitive TRPV4 channels are selectively expressed by skin keratinocytes and dermal macrophages and that their activation promotes downstream 5-HT signaling, resulting in itch-specific behavioral responses in mouse models of chronic itch. Importantly, TRPV4-expressing macrophages and keratinocytes are differentially involved in the generation of spontaneous itch behaviors in mouse models of allergic and nonallergic chronic itch, respectively. Furthermore, we identified increased expression of TRPV4 in the skin of patients with chronic idiopathic pruritus (CIP). Collectively, our data demonstrate that TRPV4-expressing cells in the skin are a critical component in the pathogenesis of chronic itch.

METHODS

Animals

Adult male and female C57BL/6J (Jackson Laboratories, Bar Harbor, Me), *Trpv4*^{eGFP} (Mutant Mouse Regional Resource Centers), *Trpv4*$^{-/-}$,[11] *Kit*$^{W\text{-}sh/W\text{-}sh}$ (Jackson Laboratories), 5-HT receptor 7 (*Htr7*$^{-/-}$) (Jackson Laboratories), and 5-HT receptor 2a (*Htr2a*)$^{-/-}$ (a kind gift from Dr Jay Gingrich at Columbia University) mice were used for the study. *Cre*$^{+}$ and *Cre*$^{-}$ *Pf4*^{Cre};*iDTR* (Pf4-Cre^{+} and Pf4-Cre^{-}) mice were obtained by crossing *Rosa26*^{iDTR} mice (Jackson Laboratories) with *Pf4-Cre* mice (Jackson Laboratories). To generate *Trpv4*$^{f/f}$ mice, 3 of the properly targeted embryonic stem cell clones were obtained from the Knockout Mouse Project Repository and used for blastocyst injections, and 1 clone led to high-contribution chimeras that produced germline-transmitted offspring, as assayed by using black coat color. This chimera line was then mated to FLPo mice (Jackson Laboratories) to remove the neomycin cassette and generate heterozygous *Trpv4*$^{f/+}$ mice,

which were crossed with *K14^{CreERT}* and *Cx3cr1^{CreERT}* mice to generate both *Cre^{+}and Cre^{-} Cx3cr1^{CreERT};Trpv4$^{f/f}$* (Cx3cr1-Cre^{+} and Cx3cr1-Cre^{-}) and *K14^{CreERT};Trpv4$^{f/f}$* (K14-Cre^{+} and K14-Cre^{-}) mice, respectively. Toamoxifen-inducible *Trpv4* knockdown mice were produced by means of intraperitoneal administration of tamoxifen for 5 consecutive days at 75 mg/kg in 0.2 mL of corn oil.

Experiments were performed 7 days after the last day of tamoxifen administration. Animals were acclimatized to the experimental room in advance of experiments. All behavioral tests were videotaped from a side angle, and behavioral assessments were done by observers blind to the treatments or genotypes of animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee of University of Texas Health Science Center at Houston and the Institutional Animal Care and Use Committee at Washington University School of Medicine and were in accordance with guidelines provided by the National Institutes of Health and the International Association for the Study of Pain. All mice were randomly allocated to different experimental groups by laboratory managers, who were blinded to the experimental design. All mice were included in the analysis unless they died.

Human PBMCs and skin biopsy specimens

Human peripheral blood samples were obtained from patients undergoing routine skin cancer surgery in an outpatient dermatology clinic, as approved by the Washington University in St Louis Institutional Review Board (IRB; Protocol 201507042). Deidentified human control skin was obtained from noncancerous marginal skin from patients (average age of control subjects, 74.5 ± 4.2 years) undergoing routine skin cancer surgery under an IRB-exempt protocol. Skin from patients with CIP was obtained directly from patients with a firm diagnosis of CIP (average age of patients with CIP, 74.3 ± 2.2 years), as approved by the Washington University in St Louis IRB (Protocol 201412117). The numeric rating scale itch score for CIP was examined on a scale of 0 to 10 as a peak value during the week before the examination. A thorough work-up was performed to establish the diagnosis of CIP by means of exclusion of other cutaneous and systemic causes of pruritus, including psychiatric and neurologic conditions, dialysis-dependent renal failure, biliary dysfunction, thyroid abnormalities, HIV/AIDS, hepatitis B, and hepatitis C. None of the patients had a history of malignancy. Informed consent was obtained from each subject before all procedures.

Single-cell suspensions from mouse and human skin tissues

Fresh mouse ear skin preparations and human skin biopsy specimens were cut and separated with forceps and digested in 0.25 mg/mL Liberase TL (Roche, Mannheim, Germany) in Dulbecco modified Eagle medium for 90 minutes at 37°C. Samples were mashed through 70-μm cell strainers and washed with Dulbecco modified Eagle medium supplemented with 5% FBS, 1% L-glutamine (GIBCO, Carlsbad, Calif), and 1% penicillin/streptomycin (GIBCO). Single-cell suspensions were used for subsequent calcium imaging, staining for flow cytometry, and cell sorting for RT-PCR.

Mouse and human epidermal keratinocyte culture

The skin keratinocyte culture was prepared according to a previous study.[12] In brief, the skin of newborn mouse pups (P0–P2) or human skin biopsy specimens were removed and placed in a Petri dish containing 2.5% Dispase II (Life Technologies, Grand Island, NY) and incubated at 4°C overnight. The epidermis was then separated from subcutaneous tissues. Keratinocytes were dissected by means of gentle scraping and flushing with culture medium. Harvested cells were plated on coverslips coated with collagen IV and cultured in the serum-free, fully supplemented keratinocyte medium CnT-07 (for mouse keratinocytes; CELLnTEC Advanced Cell Systems, Bern, Switzerland) or keratinocyte-SFM medium supplemented with bovine pituitary extract and epidermal growth factor (for human keratinocytes; Invitrogen, Carlsbad, Calif) under a humidified atmosphere of 5% CO_2/95% air at 37°C for 2 days before use.

Isolation and short-term culture of mouse DRG neurons

Mouse spinal columns were removed and placed in ice-cold HBSS; neurons were acutely dissociated and maintained, as previously described.[13] In brief, laminectomies were performed, and bilateral DRGs were dissected out. After removal of connective tissues, DRGs were transferred to 1 mL of Ca^{2+}/Mg^{2+}-free HBSS containing 2 μL of saturated $NaHCO_3$, 0.35 mg of L-cysteine, and 20 U of papain (Worthington, Lakewood, NJ) and incubated at 37°C for 10 minutes. The DRG suspension was centrifuged, the supernatant was removed, and 1 mL of Ca^{2+}/Mg^{2+}-free HBSS containing 4 mg of collagenase type II and 1.25 mg of Dispase type II (Worthington) was added and incubated at 37°C for 10 minutes. After digestion, neurons were pelleted; suspended in neurobasal medium containing 2% B-27 supplement, 1% L-glutamine, 100 U • mL^{-1} penicillin plus 100 μg • mL^{-1} streptomycin, and 50 ng • mL^{-1} nerve growth factor; plated on a 12-mm coverslip coated with poly-L-lysine (10 μg • mL^{-1}); and cultured under a humidified atmosphere of 5% CO_2/95% air at 37°C for 18 to 24 hours before use.

Extraction and isolation of mouse platelets

Whole blood was collected in a tube containing 3.8% Na citrate at a ratio of 9:1 after anesthesia was obtained. Anticoagulant whole blood was centrifuged at 100*g* for 10 minutes without brake. Platelet-rich plasma was transferred to a new tube, and 1 μmol/L prostaglandin E_1 was added and incubated for 5 minutes. Another volume of Na citrate was added, and centrifugation was done at 400*g* for 10 minutes. Plasma was discarded, and platelets were resuspended in HBSS containing 4 μmol/L Fura2-AM (Life Technologies). Platelets were plated on coverslips coated with poly-L-lysine and incubated at room temperature for 1 hour. The platelets were used after washing with fresh HBSS for at least 30 minutes by using a calcium imaging assay.

Preparation of skin superfusate

Skin biopsy specimens were dissected from the backs of mice, chopped into small pieces, and incubated in HBSS at 37°C for 30 minutes. The superfusate was collected by means of centrifugation before we carried out the calcium imaging experiment.

Ca^{2+} imaging

Fura-2–based ratiometric measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$) was performed, as described previously.[13] Freshly isolated skin-resident cells, cultured epidermal keratinocytes, cultured DRG neurons, and isolated PBMCs and platelets were loaded with 4 μmol/L Fura-2 AM (Life Technologies) in culture medium at 37°C for 60 minutes. Cells were then washed 3 times and incubated in HBSS at room temperature for 30 minutes before use. Fluorescence values at excitation wavelengths of 340 and 380 nm were recorded on an inverted Nikon Ti-E microscope equipped with 340-, 360-, and 380-nm excitation filter wheels by using NIS-Elements imaging software (Nikon Instruments, Melville, NY). Fura-2 ratios (F340/F380) were used to reflect changes in $[Ca^{2+}]_i$ values on stimulation. Values were obtained from 100 to 250 cells in time-lapse images from each coverslip. The threshold of activation was defined as 3 SDs above the average (approximately 20% greater than the baseline).

Whole-cell patch-clamp recording

Whole-cell patch-clamp recordings were performed with an EPC 10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) or multiclamp 700B amplifier (Molecular Devices, Sunnyvale, Calif) at room temperature (22°C to 24°C) on the stage of an inverted phase-contrast microscope equipped with a filter set for green fluorescent protein (GFP) visualization. Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instrument, Novato, Calif) with a Sutter P-97 pipette puller had resistances of 2 to 4 MΩ when filled with pipette solution containing 140 mmol/L CsCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, and 10 mmol/L HEPES (pH 7.3 and 315 mOsm • L^{-1}). Cells were continuously perfused with standard extracellular solution containing

140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 10 mmol/L HEPES (pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to approximately 340 mOsm • L^{-1} with sucrose). The whole-cell membrane currents were recorded by using voltage ramping from −100 to +100 mV for 500 ms at a holding potential of 0 mV. Data were acquired with PatchMaster (HEKA Elektronik, Lambrecht/Pfalz, Germany) or Clampex 10 (Molecular Devices, Sunnyvale, Calif). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed and plotted with Clampfit 10 (Molecular Devices). Values are presented as means ± SEMs (n represents the number of measurements).

Immunofluorescence

Adult mice, aged 6 to 16 weeks, were asphyxiated with CO_2 and perfused transcardially with 200 mL of PBS (pH 7.3), followed by 200 mL of fixative (4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.3, or Zamboni fixative [2% paraformaldehyde, 15% vol/vol saturated picric acid, and 0.1 mol/L phosphate buffer, pH 7.3]). Tissues were removed and postfixed overnight at 4°C in the same fixative. All tissues were cryoprotected overnight in 30% sucrose in 0.1 mol/L phosphate buffer, pH 7.3, frozen in optimal cutting temperature medium (OCT), sectioned with a cryostat at 20 μm, mounted on a Superfrost Plus slide (Fisher Scientific, Pittsburgh, Pa), and stored at −20°C. Frozen slides were dried at room temperature for 1 hour and washed 3 times in PBS with 0.1% Triton X-100 (PBS+TX), blocked for 30 minutes to 1 hour in PBS+TX containing 10% donkey serum, and incubated overnight at 4°C with primary antibodies diluted in blocking solution (see Table E1 in this article's Online Repository at www.jacionline.org). Sections were then washed 3 times in PBS+TX, incubated for 2 hours at room temperature with secondary antibodies conjugated to Alexa Fluor 488 fluorochrome (Invitrogen) or Cy3 fluorochromes (Jackson ImmunoResearch, West Grove, Pa), and diluted 1:500 in blocking solution. Sections were then washed 3 times in PBS+TX and mounted in anti-fade medium (Vectashield; Vector Laboratories, Burlingame, Calif). All preparations were examined with a Nikon A1 Confocal Laser Microscope System. Images were taken and analyzed in NIS-Elements.

Flow cytometry

Isolated ear skin cells from *Trpv4*^{eGFP} mice were stained with anti-mouse fluorescently conjugated antibodies: CD11b (eBioscience, San Diego, Calif), CD11c (BioLegend, San Diego, Calif), CD3e (eBioscience), B220 (BD PharMingen, San Jose, Calif), and CCR2 (R&D Systems, Minneapolis, Minn). Samples were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (version 10; TreeStar, Ashland, Ore; see Table E1).

Cell sorting and RT-PCR

Isolated GFP^+ and GFP^- skin cells were sorted with a FACSAria II (BD Biosciences). Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RNA was treated with DNase I (Invitrogen), and cDNA was synthesized *in vitro* by using the ThermoScript RT-PCR System kit (Invitrogen). PCR conditions were as follows 95°C for 3 minutes and 40 cycles of 30 seconds at 95°C, 30 seconds at 52°C, and 60 seconds at 72°C. Sequences of the primers used were as follows: GFP forward, 5′-AAGGGCATCGACTTCAAGG-3′; GFP reverse, 5′-TGCTTGT CGGCCATGATATAG-3′; mTRPV4 forward, 5′-CCTGCTGGTCACCTAC ATCA-3′; mTRPV4 reverse, 5′-CTCAGGAACACAGGGAAGGA-3′; murine glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) forward, 5′-GCACAGTCAAG GCCGAGAAT-3′; and mGAPDH reverse, 5′-GCCT TCTCCATGGTGGTGAA-3′.

Quantitative RT-PCR

Total RNA was extracted from human skin tissue by using the RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA was treated with DNase I (Invitrogen), and cDNA was synthesized *in vitro* by using the ThermoScript RT-PCR System kit (Invitrogen). Primer sequences are as follows: hTRPV4 forward, 5′-AGAACTTGGGCATCATCA ACGAG-3′; hTRPV4, reverse 5′-GTTCGAGTTCTTGTTCAGTTCCAC-3′; hTRPV3 forward, 5′- GCTGAAGAAGCGCATCTTTGCA-3′; hTRPV3 reverse, 5′- GTCAGCTTGTGCATGAGGAAG-3′; hGAPDH forward, 5′-GT CGGAGTCAACGGATTT G-3′; and hGAPDH reverse, 5′-TGGGTGGAAT CATATTGGAA-3′. Reactions were carried out in a volume of 20 μL per reaction containing 10 μL of SYBR Green master mix (2×; Applied Biosystems, Foster City, Calif), 0.5 μL of cDNA, 5 μL of 0.4 μmol/L primer mix, and 4.5 μL of water by using the StepOnePlus real-time PCR system (Applied Biosystems). The generated cycle threshold (C_t) value of human TRPV4 was normalized to its respected C_t value of GAPDH (ΔC_t). The value of ΔC_t for the CIP group was further normalized to that for the control group to yield $\Delta\Delta C_t$. Data were expressed as $2^{-\Delta\Delta Ct}$.

Conditional platelet depletion

Platelet depletion was accomplished by using intraperitoneal injections of diphtheria toxin (DTX; 400 ng per mouse) every 48 hours for a total of 3 treatments to *Pf4-Cre*$^+$*;iDTR* mice[14] and confirmed by counting the number of platelets in blood obtained from the facial vein. Only animals with platelet counts reduced to less than 15% of the value before DTX administration were used for further experiments.

Mouse model of chronic dry skin itch

In the dry skin model[15] the rostral backs of mice were treated twice daily with cutaneous application of acetone/ether (1:1) mixture followed by water (AEW). After a 7-day treatment, mice showed robust spontaneous scratching, and the treated skin area exhibited decreased stratum corneum hydration and increased transepidermal water loss, which mimic the symptoms of dry skin in patients. Hind-paw scratching directed toward the treated area was quantified by recording the number of incidences of scratching bouts for 60 minutes daily. After the recording, the videotapes were played back, and all behavioral assessments were done by observers blind to the treatments or genotypes of the animals.

Mouse model of allergic contact dermatitis

The contact sensitizer squaric acid dibutylester (SADBE; Tokyo Chemical, Tokyo, Japan) was used to elicit contact hypersensitivity in the mouse as a model of allergic contact dermatitis (ACD) in human subjects.[16] Mice were sensitized by means of topical application of 20 μL of 0.5% SADBE in acetone to shaved abdominal skin once a day for 3 consecutive days. Five days later, the SADBE-treated group was challenged with a topical application of 20 μL of 0.5% SADBE to the hairy skin on the nape of the neck once a day for 3 consecutive days, whereas acetone alone was used as the vehicle control. Scratching behavior with the hind paw was quantified by recording the number of incidences of scratching bouts for 60 minutes daily.

Statistical analysis

Values are reported as means ± SEMs. The unpaired or paired *t* test was used for comparison between 2 groups or 1-way ANOVA, and repeated measures tests followed by Tukey-Kramer or Bonferroni *post hoc* analysis were used for comparison among multiple groups occurring over time. All tests were carried out as 2-tailed tests. *P* values of less than .05 were considered statistically significant. All experiments were repeated at least twice, unless otherwise noted.

RESULTS

TRPV4 is required for generating spontaneous scratching in mouse models of both allergic and nonallergic chronic itch

TRPV4 has been implicated in both inflammatory pain and acute itch responses elicited by intradermal injections of

histamine or 5-HT.[9,10,17] Although acute itch can serve protective roles against potential threats and dangers in the environment (eg, disease-borne insects), chronic itch is universally pathologic and highly debilitating.[5] To investigate whether TRPV4 is involved in chronic itch, we examined the mRNA expression of TRPV4 in pruritic skin from patients with CIP.[18] Classically, patients with CIP present with chronic pruritus secondary to skin barrier dysfunction in the absence of a clinically defined primary dermatologic disorder.[18] Indeed, TRPV4 mRNA expression was significantly increased in skin biopsy specimens from patients with CIP in comparison with healthy control skin (Fig 1, *A*). On the other hand, expression of TRPV3, another channel implicated in itch, was not significantly increased in the patients with CIP.[19,20] These results suggest that TRPV4 might be involved in the pathogenesis of chronic itch in human subjects.

Next, we examined the role of TRPV4 in a mouse model of dry skin–associated chronic itch induced by AEW treatment,[3,21,22] which produces robust scratching and extensive epidermal hyperplasia in wild-type (WT) mice.[15] We found that the number of spontaneous scratches was markedly reduced in the *Trpv4*$^{-/-}$ mice at each of the measured time points from day 3 to day 7 after AEW treatment (Fig 1, *B*, and data not shown). This was additionally confirmed by pharmacologic inhibition of TRPV4 by HC067,[23] which substantially attenuated spontaneous scratching in the AEW model when applied either systemically or topically (Fig 1, *C*). AEW-elicited scratching critically depends on the application of distilled water,[2] which is reminiscent of a condition called aquagenic pruritus, in which water exposure induces intense itching, especially in elderly patients.[24,25]

Furthermore, exposure to water is known to exacerbate dry skin and itching in patients with conditions such as chronic hand dermatitis.[26] Because distilled water is extremely hypotonic (approximately 17 mOsm),[27] we hypothesized that osmotic stress driven by distilled water promotes the scratching response. Indeed, mice treated with distilled water with increased osmolarity at 0.45% saline (141.5 mOsm) displayed significantly reduced scratching, which was further reduced at a concentration of 0.9% saline (283 mOsm) after the acetone and ether challenge (Fig 1, *D*). Consistent with both *in vitro* and *in vivo* studies showing that TRPV4 is a molecular sensor for extracellular osmolarity in mammals,[11,28,29] these results suggest that TRPV4 is an osmoreceptor in the skin of mice and functionally required to generate dry skin–associated itch, which recapitulates the symptoms of dry skin in human subjects.[15]

Furthermore, we investigated whether TRPV4 is involved in spontaneous scratching associated with ACD by using the contact sensitizer SADBE.[16] WT mice receiving SADBE treatment displayed a robust scratching response when compared with mice treated with vehicle control only (data not shown). The *Trpv4*$^{-/-}$ mice displayed a markedly reduced scratching response after SADBE treatment when compared with WT mice (Fig 1, *E*). Similarly, systemic or topical administration of HC067 significantly reduced spontaneous scratching bouts in WT mice treated with SADBE (Fig 1, *F*). Combined, these results suggest that TRPV4 contributes to both allergic and nonallergic chronic itch in mice.

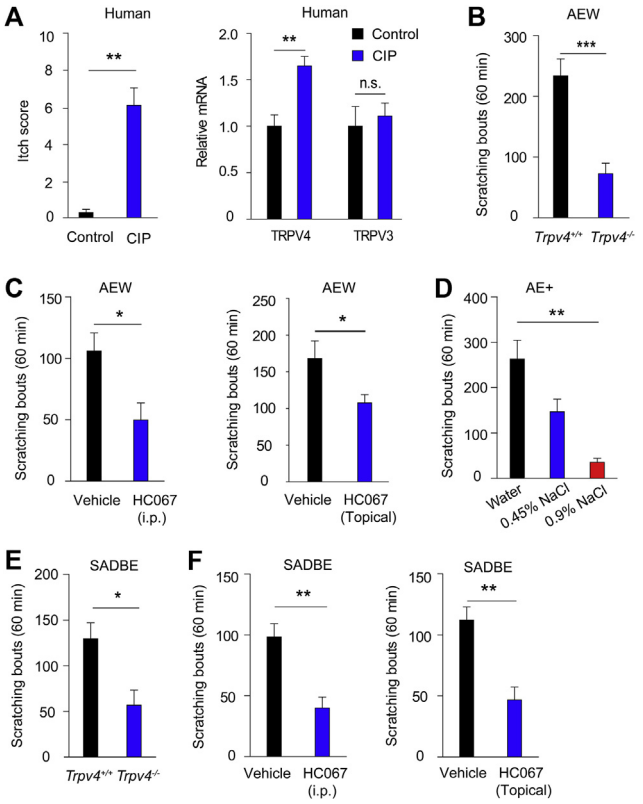


FIG 1. TRPV4 expression is increased in skin biopsy specimens of patients with CIP, and TRPV4 function is required for generating mouse models of both allergic and nonallergic chronic itch. **A,** Bar charts show averaged itch numeric rating scale scores and expression of mRNA transcripts for TRPV4 and TRPV3 in patients with CIP and healthy control subjects. **P* <.05 and ***P* < .01, Student *t* test (n = 8). *n.s.*, Not significant versus control group. **B,** Spontaneous scratches in *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice after 7 days of AEW treatment. ****P* < .001, Student *t* test (n = 7). **C,** Spontaneous scratches in *Trpv4*$^{+/+}$ mice after 7 days of AEW treatment were reduced by HC067 (20 mg/kg, either intraperitoneal *[i.p.]* or topical application) compared with vehicle. **P* <.05, Student *t* test (n = 8-9). **D,** Spontaneous scratches after treatment with the 1:1 mixture of acetone and ether, followed by 0.9% NaCl, 0.45% NaCl, or distilled water, respectively. ***P* < .01, ANOVA (n = 5-7). **E,** Spontaneous scratches in *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice after SADBE treatment. **P* < .05, Student *t* test (n = 6). **F,** SADBE-induced spontaneous scratches in *Trpv4*$^{+/+}$ mice after treatment with HC067 (20 mg/kg, either intraperitoneal *[i.p.]* or topical application) or vehicle. **P* < .05, Student *t* test (n = 5-6).

Both mouse and human keratinocytes and myeloid cells express functional TRPV4 channels

Although TRPV4 is known to be expressed by both neurons and nonneuronal cells, the precise expression pattern of TRPV4 across tissues is controversial.[30,31] Therefore we took a transgenic approach by using BAC transgenic *Trpv4*^{eGFP} mice[32] and determined the expression of TRPV4–enhanced green fluorescent protein (eGFP) in the skin. We found that both K14-expressing keratinocytes[33] in the epidermis and F4/80-expressing macrophages in the dermis were TRPV4-eGFP^{+} (Fig 2, *A*). RT-PCR analysis with total mRNA isolated from sort-purified eGFP^{+} and eGFP^{-} cells from mouse skin single-cell suspensions confirmed a correlation between the expression of TRPV4 and eGFP transcripts, further validating the specificity of the BAC transgenic mice (Fig 2, *B*). Flow cytometry with skin cell suspensions further showed that a subset of TRPV4-eGFP^{+} cells expressed CD11b and CCR2, as well as low levels of CD11c, which is reminiscent of CCR2/CD11b-positive dermal macrophages (Fig 2, *C*, and see Fig E1 in this article's Online Repository at www.jacionline.org).[34] In addition, TRPV4-eGFP^{+} cells were also immunopositive for CD68, CD206, and

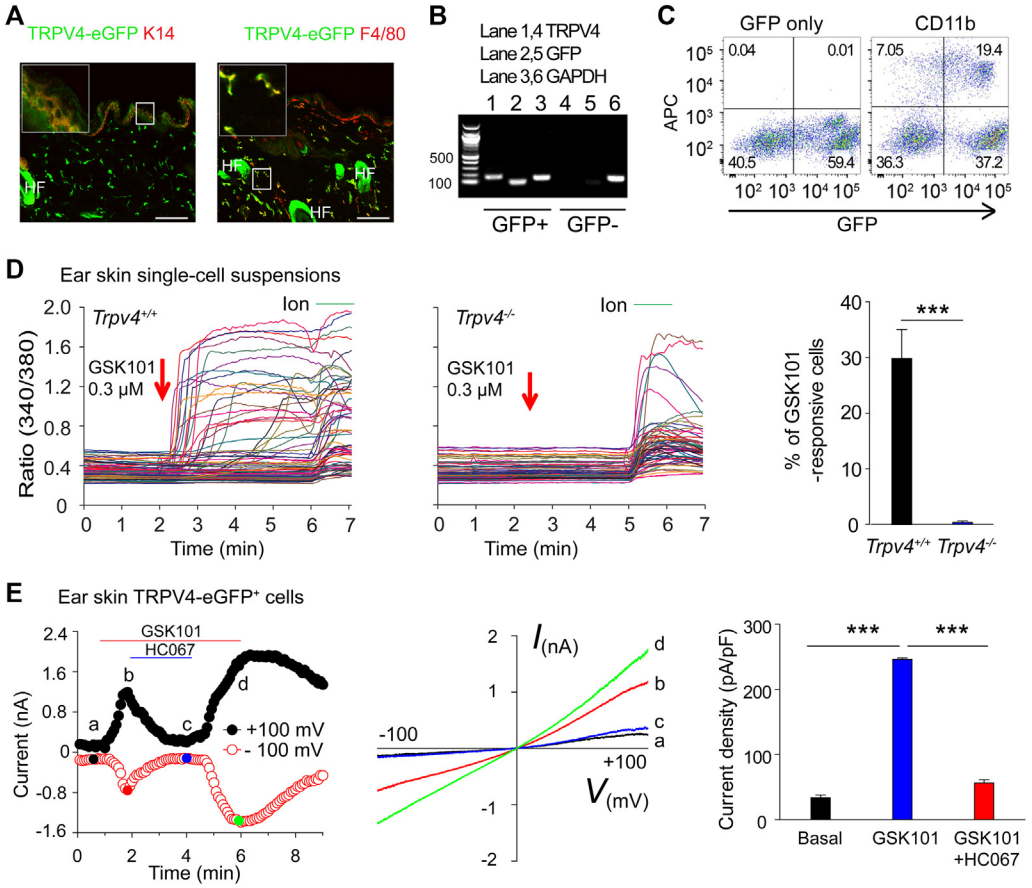


FIG 2. TRPV4 is functionally expressed by mouse skin-resident cells. **A,** Double labeling reveals colocalization of TRPV4-eGFP with K14 *(left panel)* and F4/80 *(right panel)* in skin sections from *Trpv4*^{eGFP} mice. *Bar* = 50 μm. *HF,* Hair follicle. *Inset* shows magnification of *boxed area*. **B,** RT-PCR shows the correlation between TRPV4 and GFP in sorted GFP$^+$ and GFP$^-$ ear skin single-cell suspensions. **C,** Flow cytometry illustrates that TRPV4-eGFP is expressed in CD11b$^+$ skin-resident cells. *APC,* Allophycocyanin. **D,** Representative traces showing GSK101-elicited large $[Ca^{2+}]_i$ responses in freshly dissociated ear skin single-cell suspensions from *Trpv4*$^{+/+}$ *(left)* but not *Trpv4*$^{-/-}$ *(middle)* mice. *Arrows* indicate time points of GSK101 applications. Ionomycin *(Ion)* was used as a positive control. Summarized data from the right show percentages of GSK101-responsive skin cells isolated from the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. ***$P < .001$, Student *t* test (n = 5-8). **E,** Time course *(left)* and representative current-voltage curves *(middle)* elicited by voltage ramps in the absence or presence of 0.3 μmol/L GSK101 in TRPV4-eGFP$^+$ myeloid cells from the ear skin single-cell suspensions. HC067 at 5 μmol/L markedly inhibited GSK101-activated currents. *Bar charts at right* show summarized data. ***$P < .001$, ANOVA (n = 5).

CD163, all of which are cell markers commonly used for tissue macrophages (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org).[35]

We next assessed the function of TRPV4 in freshly isolated skin-resident cells using live cell Ca^{2+} imaging and patch-clamp recordings. Application of GSK101, a potent and selective TRPV4 agonist,[36] induced a robust $[Ca^{2+}]_i$ response in a subset of cells in WT but not *Trpv4*$^{-/-}$ skin single-cell suspensions, which are enriched with resident immune cells (Fig 2, *D*).[37] Moreover, GSK101 also activated large whole-cell membrane currents with a characteristic current-voltage relationship for TRPV4 and $[Ca^{2+}]_i$ responses in freshly isolated TRPV4-eGFP$^+$ cells, which was severely attenuated by either GSK219 or HC067, 2 selective TRPV4 antagonists (Fig 2, *E*, and see Fig E2, *B* and *C*).[23,38] Consistent with previous studies showing the presence of functional TRPV4 in mouse keratinocytes,[33] we found that GSK101 evoked a large $[Ca^{2+}]_i$ response in WT but not TRPV4-deficient mouse primary keratinocytes (see Fig E3 in this article's Online Repository at www.jacionline.org). Similarly, we also demonstrated that GSK101 elicited robust $[Ca^{2+}]_i$ responses in human primary epidermal keratinocytes, freshly isolated human forearm skin single-cell suspensions, and human PBMCs, all of which were abolished by GSK219, suggesting that, as in mice, human skin-resident cells and mononuclear cells also possess functional TRPV4 channels (see Fig E4 in this article's Online Repository at www.jacionline.org). Combined, these results demonstrate that activation of TRPV4 can lead to functional responses in both keratinocytes and dermal myeloid cells in both mice and human subjects.

TRPV4-expressing macrophages and keratinocytes are differentially involved in allergic and nonallergic chronic itch

Consistent with TRPV4-expressing skin-resident cells being critical to the genesis of chronic itch in mice, we showed that

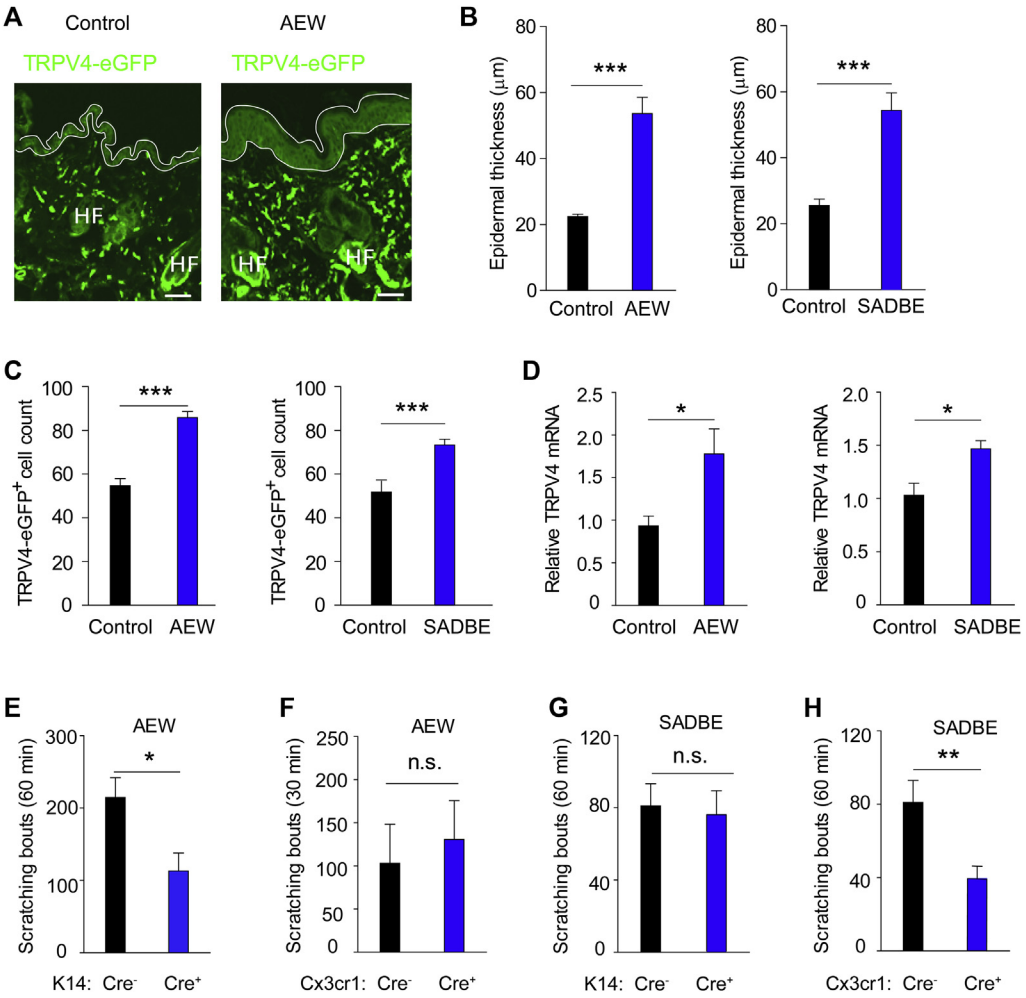


FIG 3. TRPV4 channels expressed by macrophages and keratinocytes contribute differentially to allergic and nonallergic chronic itch. **A,** Representative images showing TRPV4-eGFP$^+$ cells in skin of *Trpv4^{eGFP}* mice treated with vehicle control and AEW. **B,** Epidermal thickness was significantly increased by AEW and SADBE treatments compared with their respective vehicle controls. ***P < .001, Student t test (n = 6-8). **C,** Number of TRPV4-eGFP$^+$ dermal macrophages increased significantly after AEW or SADBE treatment. ***P < .001, Student t test (n = 6-8). **D,** Relative TRPV4 mRNA expression in skin of AEW- or SADBE-treated mice. *P < .05, Student t test (n = 6-8). **E,** Spontaneous scratching in *K14-Cre$^+$* and *K14-Cre$^-$* mice after AEW treatment. *P < .05, Student t test (n = 5). **F,** Spontaneous scratching in *Cx3cr1-Cre$^+$* and *Cx3cr1-Cre$^-$* mice after AEW treatment. Student t test (n = 5). *n.s.*, Not significant. **G,** Spontaneous scratching in *K14-Cre$^+$* and *K14-Cre$^-$* mice after SADBE treatment. Student t test (n = 8-9). *n.s.*, Not significant. **H,** Spontaneous scratching in *Cx3cr1-Cre$^+$* and *Cx3cr1-Cre$^-$* mice after SADBE treatment. **P < .01, Student t test (n = 9).

AEW or SADBE treatment produced epidermal hyperplasia, with a marked increase in the number of TRPV4-eGFP$^+$ keratinocytes, as well as dermal TRPV4-eGFP$^+$ cells, moving toward the epidermal-dermal border (Fig 3, *A-C*). In addition, TRPV4 mRNA transcripts were also increased in the skin of mice treated with either AEW or SADBE (Fig 3, *D*). Collectively, these studies demonstrate that TRPV4 expression is not only functional but also enriched in the skin under chronic itch conditions. To further determine the types of TRPV4-expressing cells contributing to chronic itch, we generated TRPV4 flox mice and crossed them to inducible keratinocyte-specific *K14^{CreERT}* and macrophage-specific *Cx3cr1^{CreERT}* mice. After tamoxifen treatment, the AEW-induced spontaneous scratching response was significantly reduced in the K14-*Cre$^+$* mice compared with their K14-*Cre$^-$* littermates (Fig 3, *E*). However, the AEW-induced spontaneous scratching response was not significantly affected in Cx3cr1-*Cre$^+$* mice (Fig 3, *F*). On the other hand, SADBE-induced spontaneous scratching was significantly attenuated in *Cx3cr1-Cre$^+$* but not in *K14-Cre$^+$* mice compared with their respective *Cre$^-$* littermates (Fig 3, *G* and *H*). These studies suggest that TRPV4 function in macrophages and keratinocytes contributes differentially to the pathogenesis of allergic and nonallergic chronic itch in mice.

5-HT signaling is involved critically in TRPV4-dependent chronic itch

To identify the skin-derived chemical mediator or mediators of TRPV4-mediated scratching response, we prepared AEW-treated skin superfusates from both *Trpv4$^{+/+}$* and *Trpv4$^{-/-}$* mice and

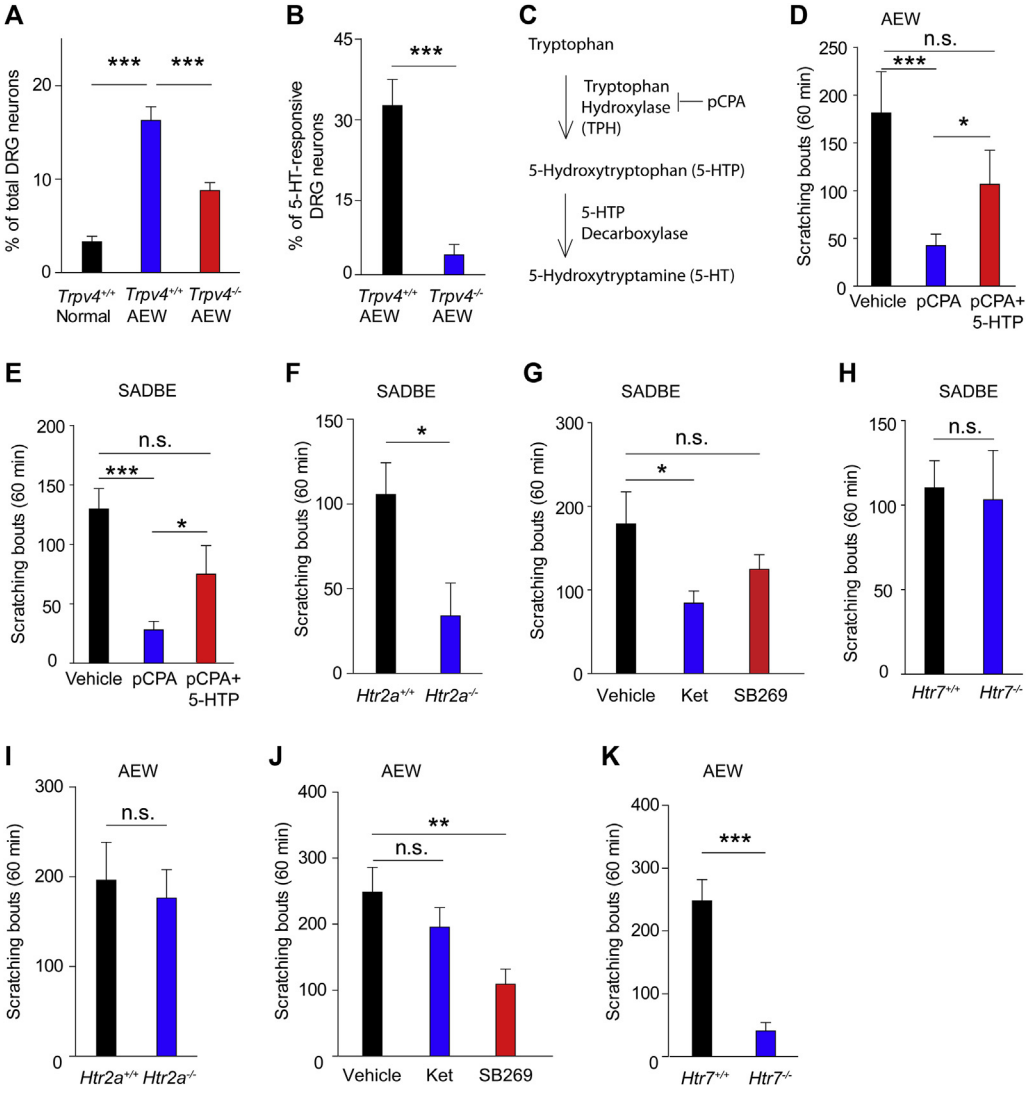


FIG 4. 5-HT signaling is required for TRPV4-dependent chronic itch. **A,** Percentages of DRG neurons responding to skin superfusates from normal *Trpv4*$^{+/+}$, AEW *Trpv4*$^{+/+}$, and AEW *Trpv4*$^{-/-}$ mice. ***P < .001, ANOVA. **B,** Percentages of 5-HT–responsive DRG neurons responding to AEW skin superfusates from *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. ***P < .001, Student t test. **C,** Schematic drawing of the 5-HT synthesis pathway. **D** and **E,** Spontaneous itching in AEW-treated (Fig 4, *D*) and SADBE-treated (Fig 4, *E*) mice with pCPA or pCPA plus 5-hydroxytryptophan *(5-HTP).* *P < .05 and ***P < .001, ANOVA (n = 8-10). *n.s.*, Not significant. **F,** Spontaneous scratching in *Htr2a*$^{+/+}$ and *Htr2a*$^{-/-}$ mice after SADBE treatment. *P < .05, Student t test (n = 5). **G,** Spontaneous scratching in mice treated with vehicle, ketanserin, or Htr7 antagonist SB269970 (SB269) after SADBE treatment. *P < .05, ANOVA (n = 6). *n.s.*, Not significant. **H,** Spontaneous scratching in *Htr7*$^{+/+}$ and *Htr7*$^{-/-}$ mice after SADBE treatment. Student t test (n = 6). *n.s.*, Not significant. **I,** Spontaneous scratching in *Htr2a*$^{+/+}$ and *Htr2a*$^{-/-}$ mice after AEW treatment. Student t test (n = 9). *n.s.*, Not significant. **J,** Spontaneous scratching after AEW treatment in mice treated with vehicle, Htr7 antagonist SB269970 (SB269), or ketanserin. **P < .01, ANOVA (n = 6-7). *n.s.*, Not significant. **K,** Spontaneous scratching after AEW treatment in *Htr7*$^{+/+}$ and *Htr7*$^{-/-}$ mice. ***P < .001, Student t test (n = 10-11).

applied the superfusates directly to cultured WT DRG neurons. As expected, *Trpv4*$^{+/+}$ AEW skin superfusate evoked a robust $[Ca^{2+}]_i$ response in a subset of DRG neurons. By contrast, the *Trpv4*$^{-/-}$ AEW skin superfusate activated significantly fewer sensory neurons, suggesting that TRPV4 mediates the release of neuromediators in the AEW-treated skin (Fig 4, *A*). Interestingly, *Trpv4*$^{+/+}$ AEW skin superfusate activated approximately 30% of all 5-HT–responsive DRG neurons, whereas less than 5% of these neurons were activated by *Trpv4*$^{-/-}$ AEW skin superfusate (Fig 4, *B*). Therefore TRPV4 deficiency leads to significantly fewer 5-HT–sensitive sensory neurons responsive to AEW skin superfusates, suggesting that TRPV4 deficiency might result in significantly reduced 5-HT release in the skin in response to AEW treatment.

Biosynthesis of 5-HT (Fig 4, *C*) is catalyzed by the rate-limiting enzyme tryptophan hydroxylase (TPH), and inhibition of TPH activity by a TPH inhibitor p-chlorophenylalanine (pCPA) has been commonly used to investigate the effects of 5-HT depletion on animal behaviors.[39] Thus we examined whether chemical depletion of 5-HT affected AEW-induced dry

skin–associated chronic itch and SADBE-induced chronic allergic itch. The results revealed that administration of pCPA markedly inhibited spontaneous scratching in mice treated with either AEW or SADBE (Fig 4, *D* and *E*). To validate that deficiency in 5-HT but not other monoamines mediated the pCPA effect, we concomitantly administered pCPA and 5-hydroxytryptophan, which is converted to 5-HT without the involvement of TPH. Indeed, administration of 5-hydroxytryptophan rescued spontaneous scratching in mice treated with pCPA in both AEW- and SADBE-induced chronic itch models (Fig 4, *D* and *E*).

Consistent with the finding that 5-HT is a downstream neuromediator of TRPV4-elicited scratching, administration of ketanserin, a selective antagonist of Htr2a shown to mediate 5-HT-elicited scratching in mice,[40] or genetic ablation of Htr2a function but not inhibition of Htr7 function significantly reduced spontaneous scratching in mice treated with SADBE (Fig 4, *F-H*). To our surprise, the number of spontaneous scratches in the AEW-induced chronic dry skin–associated itch was not substantially changed in the *Htr2a*$^{-/-}$ or WT mice treated with ketanserin (Fig 4, *I* and *J*). In contrast, the number of spontaneous scratches in the AEW model was substantially reduced by either pharmacologic inhibition or genetic ablation of the function of Htr7, which was also shown to mediate 5-HT–induced itch in mice (Fig 4, *J* and *K*),[41] suggesting that Htr7 but not Htr2a signaling likely plays a major role in chronic dry skin–associated itch. Together, these results suggest that both Htr2a and Htr7 are critically involved in TRPV4-dependent chronic itch.

Platelets are required to generate TRPV4-mediated chronic itch

It is well known that mast cell degranulation increases tissue histamine concentrations, leading to activation of TRPV1$^+$ pruriceptive sensory neurons and generation of histamine-dependent itch.[4] In addition to histamine, mast cells also represent a major source of 5-HT.[42] Therefore we examined whether TRPV4-eGFP was expressed by mast cells by using streptavidin rhodamine.[43] However, there was no colocalization of TRPV4-eGFP with streptavidin rhodamine, suggesting that mast cells are not likely to express TRPV4 (Fig 5, *A*). In agreement with this finding, spontaneous scratching responses in mice treated with AEW or SADBE were not significantly altered in the mast cell–deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ "*sash*" mice when compared with WT control mice (Fig 5, *B* and *C*), suggesting that mast cell–derived 5-HT is not a major contributor to TRPV4-dependent chronic itch.

In addition to mast cells, platelets are the other major source for 5-HT in the skin.[44] Although it is understood increasingly that platelets have roles in inflammatory and immune processes in addition to their function in hemostasis and thrombosis, the role of platelets in the pathogenesis of chronic itch has not been studied. To investigate whether platelets are required for TRPV4-mediated itch, we used a conditional platelet depletion model by crossing the *Pf4*^{Cre} transgenic line with the Cre-dependent *Rosa26*^{DTR} line *(iDTR)*, followed by DTX injections.[14] Platelet numbers in peripheral blood of the *Pf4-Cre*$^+$ mice were markedly reduced after DTX injections compared with those in DTX-treated *Pf4-Cre*$^-$ littermates, but no other blood cells were affected (Fig 5, *D*, and data not shown).[14] DTX-treated *Pf4-Cre*$^+$ mice displayed comparable thermal and mechanical sensitivities as DTX-treated *Pf4-Cre*$^-$ littermates in the Hargreaves, hot plate, tail immersion, and von Frey filament tests (see Fig E5 in this article's Online Repository at www.jacionline.org). In addition, *Pf4-Cre*$^+$ mice had no motor function deficit when compared with DTX-treated *Pf4-Cre*$^-$ littermates, as measured by using the rotarod test (see Fig E5), confirming that DTX treatment does not affect acute thermal and mechanical pain sensations. We next tested the responses of *Pf4-Cre*$^+$ mice and their *Pf4-Cre*$^-$ littermates subjected to treatment with AEW or SADBE and showed that DTX-treated *Pf4-Cre*$^+$ mice had substantially attenuated spontaneous itch behaviors induced by AEW or SADBE treatment compared with DTX-treated *Pf4-Cre*$^-$ littermates (Fig 5, *E* and *F*). Together, these results strongly suggest that platelets, but not mast cells, are required for TRPV4-mediated chronic itch responses.

Multiple pathways are involved in platelet activation, including those activated by adenosine diphosphate through the P2Y12 receptor and fibrinogen through the platelet glycoprotein IIb/IIIa receptors.[45] Thus we investigated whether selective inhibition of the P2Y12 or glycoprotein IIb/IIIa receptors affects

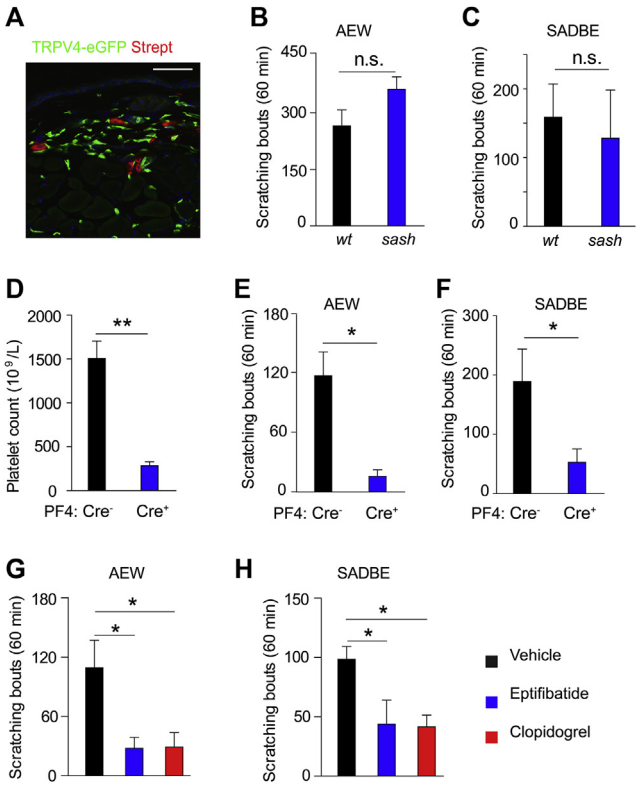


FIG 5. Platelets, but not mast cells, are required for TRPV4-dependent chronic itch. **A,** Immunofluorescent staining shows that TRPV4-eGFP was not colocalized with streptavidin, a mast cell marker. *Bar* = 50 μm. **B** and **C,** Spontaneous scratching induced by AEW (Fig 5, *B*) or SADBE (Fig 5, *C*) treatment was not significantly affected in the *sash* mice. Student *t* test (n = 6-7). *n.s.*, Not significant versus the WT control group. **D,** Platelet count in *Pf4-Cre*$^-$ and *Pf4-Cre*$^+$ mice 6 days after DTX treatment. $^{**}P < .01$, Student *t* test (n = 7-8). **E** and **F,** Spontaneous scratching responses induced by AEW (Fig 5, *E*) or SADBE (Fig 5, *F*) in *Pf4-Cre*$^-$ mice (n = 5 for AEW and SADBE) and *Pf4-Cre*$^+$ mice (n = 6 for AEW and SADBE). $^*P < .05$, Student *t* test. **G** and **H,** Spontaneous scratching responses induced by AEW (Fig 5, *G*) or SADBE (Fig 5, *H*) in the absence or presence of eptifibatide or clopidogrel. $^*P < .05$, ANOVA (n = 6).

TRPV4-mediated chronic itch responses by using the selective P2Y12 receptor antagonist clopidogrel or a selective inhibitor of the glycoprotein IIb/IIIa receptors, eptifibatide, both of which are antiplatelet drugs approved by the US Food and Drug Administration. Indeed, concomitant application of clopidogrel or eptifibatide severely attenuated AEW- and SADBE-induced spontaneous scratching (Fig 5, *G* and *H*). Combined, these results strongly suggest that platelet activation is critical to TRPV4-mediated chronic itch responses and that cutaneous platelets are likely the sources of 5-HT release in response to AEW or SADBE treatment.

Finally, we asked whether TRPV4 is functionally expressed by platelets isolated from the peripheral blood of *Trpv4*^{eGFP} mice. We did not detect TRPV4-eGFP in platelets. Furthermore, live-cell Ca^{2+} imaging also did not detect $[Ca^{2+}]_i$ response elicited by addition of GSK101, although it induced a robust $[Ca^{2+}]_i$ response in TRPV4-eGFP$^+$ blood-derived leukocytes (see Fig E6 in this article's Online Repository at www.jacionline.org). These results suggest that TRPV4 does not directly mediate platelet activation but rather does so through the release of unknown proinflammatory mediators after activation of the TRPV4-expressing skin-resident cells, which induces activation of platelets to release 5-HT and produce chronic itching.

DISCUSSION

Our findings that TRPV4 function is required for producing spontaneous scratching in dry skin–associated chronic itch and SADBE-induced spontaneous scratching[15,16] provide strong evidence for a major role of the TRPV4-expressing skin-resident cells in the pathogenesis of multiple types of chronic itch. We also revealed TRPV4 function in human skin cells and increased TRPV4 expression in skin biopsy specimens of patients with CIP. Furthermore, we demonstrated that 5-HT is a critical downstream mediator of TRPV4-mediated spontaneous scratching and that distinct 5-HT receptors are required for producing TRPV4-mediated allergic and nonallergic chronic itch. Our results support a model in which TRPV4$^+$ skin-resident cells are key signaling components in the pathogenesis of chronic itch.

Our data show that TRPV4 expression is upregulated by treatment of AEW or SADBE on the skin, as reflected by an increase in the numbers of TRPV4-eGFP$^+$ myeloid cells and keratinocytes, as well as increased TRPV4 mRNA transcripts. Moreover, loss of TRPV4 function severely attenuates spontaneous scratching in both AEW- and SADBE-induced chronic itch models, highlighting the importance of TRPV4 in the pathogenesis of chronic itch and suggesting that cutaneous TRPV4 signaling is critically involved in the pathogenesis of chronic itch in mice. Furthermore, our findings that the osmosensitive TRPV4 expressed by skin-resident cells mediates the scratching response elicited by osmotic stress in the AEW-induced chronic itch model might provide the molecular and cellular basis of aquagenic pruritus of the elderly. We also found a significant increase in TRPV4 expression in patients with CIP, further supporting a general role of TRPV4 in mediating chronic itch in both rodents and human subjects.

Interestingly, using conditional knockdown of TRPV4 expression from keratinocytes and dermal macrophages, we found that TRPV4-expressing keratinocytes are critically involved in AEW-induced dry skin–associated chronic itch but not spontaneous scratching in mice with SADBE-induced ACD. Conversely, TRPV4-expressing dermal macrophages are required for generating chronic itch in the SADBE-induced ACD model but not AEW-induced dry skin–associated chronic itch. These results are consistent with our findings that osmotic stress mediates AEW-induced spontaneous scratching. Because TRPV4-expressing epidermal keratinocytes are the first skin cells to access water after barrier disruption, which is an important step in generation of the AEW-induced dry skin model,[15] we speculate that activation of TRPV4-mediated signaling in epidermal keratinocytes by water is largely responsible for AEW-induced chronic itching.

Although the mechanism of SADBE in patients with ACD is not fully understood, activation of T cell–mediated immunity is critically involved in the generation of ACD.[46] In addition to T cells, it was also reported that there is increased migration of dermal innate immune cells, which are important to the sensitization in ACD.[47,48] Indeed, we detected a marked increase in the number of TRPV4-expressing dermal macrophages after treatment of SADBE. Our results suggest that dermal macrophages might not only regulate skin inflammation but also chronic itch in patients with SADBE-induced ACD.

Consistent with previous studies showing that 5-HT– but not histamine-elicited responses are sensitized by AEW treatment,[49] we found that chemical depletion of 5-HT by pCPA severely attenuated spontaneous scratching in mice treated with AEW or SADBE. Although dermal mast cells are rich in 5-HT, our results do not support the involvement of mast cells in TRPV4-mediated scratching responses for the following observations: (1) mast cells do not express TRPV4-eGFP, and (2) consistent with previous studies,[15] we found that the spontaneous scratching responses induced by AEW or SADBE was not altered by mast cell deficiency. Instead, we showed that DTX-induced depletion of platelets in *Pf4-Cre*$^+$ mice severely attenuated spontaneous scratching in mice treated with AEW or SADBE, suggesting that platelet-derived 5-HT is required for TRPV4-mediated chronic itch. In addition, platelets are critical to leukocyte recruitment in chronic skin inflammation through formation of platelet-leukocyte aggregates through P-selectin in peripheral blood and secretion of chemokines at inflamed sites.[50] Furthermore, platelets can also migrate extravascularly and accumulate in inflammatory lesions concomitantly with leukocytes and have been associated with many inflammatory disorders, including asthma, arthritis, and inflammatory bowel disease.[51]

Our results showed that Htr2a, one of many 5-HT–responsive G protein–coupled receptors, is involved in SADBE-induced spontaneous scratching based on our pharmacologic inhibition and genetic ablation studies. However, the Htr7 rather than the Htr2a receptor mediates AEW-induced chronic dry skin–associated itch. Although the exact mechanisms underlying the involvement of multiple subtypes of 5-HT receptors in different mouse models of chronic itch remain to be elucidated, our results show that both AEW- and SADBE-induced chronic itch require 5-HT signaling initiated by activation of TRPV4-expressing epithelial and immune cells in the skin, highlighting heterogeneous modules of chronic itch development through distinct TRPV4/5-HT receptor signaling axes. Recognizing the versatility and selectivity of 5-HT receptor

signaling in patients with chronic itch might be critical to the development of effective therapies against different forms of chronic itch with distinct causes.

In summary, we have shown that the TRPV4-mediated allergic and nonallergic chronic itch involves activation of a series of cellular networks involving TRPV4-expressing keratinocytes, dermal macrophages, platelets, and pruriceptors through paracrine signaling in the skin. Because chronic itch originates in the skin, identification of TRPV4-dependent, itch-specific cellular signaling networks in the skin can guide development of selective pharmacologic intervention of these chronic itch pathways.

We thank Drs M. Suzuki and A. Mizuno for providing the *Trpv4* knockout mice and Dr Zhou-Feng Chen for the breeder pairs of the *Trpv4* knockout mice. We are grateful to Dr Jay Gingrich for providing the *Htr2a* knockout mice. We thank Dr Cristina Strong for providing the primary human epidermal keratinocytes. We also thank Richard Clark, Edgar Walters, and Carmen Dessauer for helpful discussions.

Key messages

- **TRPV4 is expressed predominantly by dermal macrophages in addition to keratinocytes.**
- **TRPV4 in keratinocytes and macrophages contributes to spontaneous scratching associated with AEW-induced dry skin and SADBE-induced allergic dermatitis, respectively.**
- **Platelet-derived serotonin is required for TRPV4-mediated itch sensation.**

REFERENCES

1. Matterne U, Strassner T, Apfelbacher CJ, Diepgen TL, Weisshaar E. Measuring the prevalence of chronic itch in the general population: development and validation of a questionnaire for use in large-scale studies. Acta Derm Venereol 2009;89:250-6.
2. Yosipovitch G. Dry skin and impairment of barrier function associated with itch—new insights. Int J Cosmet Sci 2004;26:1-7.
3. 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.
4. Han SK, Simon MI. Intracellular signaling and the origins of the sensations of itch and pain. Sci Signal 2011;4:pe38.
5. Luo J, Feng J, Liu S, Walters ET, Hu H. Molecular and cellular mechanisms that initiate pain and itch. Cell Mol Life Sci 2015;72:3201-23.
6. Sokabe T, Tominaga M. The TRPV4 cation channel: a molecule linking skin temperature and barrier function. Commun Integr Biol 2010;3:619-21.
7. Mamenko M, Zaika O, Boukelmoune N, O'Neil RG, Pochynyuk O. Deciphering physiological role of the mechanosensitive TRPV4 channel in the distal nephron. Am J Physiol Renal Physiol 2015;308:F275-86.
8. Garcia-Elias A, Mrkonjic S, Jung C, Pardo-Pastor C, Vicente R, Valverde MA. The TRPV4 channel. Handb Exp Pharmacol 2014;222:293-319.
9. 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.
10. Chen Y, Fang Q, Wang Z, Zhang JY, MacLeod AS, Hall RP, et al. Transient receptor potential vanilloid 4 ion channel functions as a pruriceptor in epidermal keratinocytes to evoke histaminergic itch. J Biol Chem 2016;291:10252-62.
11. Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. J Biol Chem 2003;278:22664-8.
12. Luo J, Stewart R, Berdeaux R, Hu H. Tonic inhibition of TRPV3 by Mg2+ in mouse epidermal keratinocytes. J Invest Dermatol 2012;132:2158-65.
13. Yin S, Luo J, Qian A, Du J, Yang Q, Zhou S, et al. Retinoids activate the irritant receptor TRPV1 and produce sensory hypersensitivity. J Clin Invest 2013;123:3941-51.
14. Wuescher LM, Takashima A, Worth RG. A novel conditional platelet depletion mouse model reveals the importance of platelets in protection against *Staphylococcus aureus* bacteremia. J Thromb Haemost 2015;13:303-13.
15. Miyamoto T, Nojima H, Shinkado T, Nakahashi T, Kuraishi Y. Itch-associated response induced by experimental dry skin in mice. Jpn J Pharmacol 2002;88:285-92.
16. Qu L, Fan N, Ma C, Wang T, Han L, Fu K, et al. Enhanced excitability of MRGPRA3- and MRGPRD-positive nociceptors in a model of inflammatory itch and pain. Brain 2014;137:1039-50.
17. Alessandri-Haber N, Joseph E, Dina OA, Liedtke W, Levine JD. TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. Pain 2005;118:70-9.
18. Xu AZ, Tripathi SV, Kau AL, Schaffer A, Kim BS. Immune dysregulation underlies a subset of patients with chronic idiopathic pruritus. J Am Acad Dermatol 2016;74:1017-20.
19. Steinhoff M, Biro T. A TR(I)P to pruritus research: role of TRPV3 in inflammation and itch. J Invest Dermatol 2009;129:531-5.
20. Lin Z, Chen Q, Lee M, Cao X, Zhang J, Ma D, et al. Exome sequencing reveals mutations in TRPV3 as a cause of Olmsted syndrome. Am J Hum Genet 2012;90:558-64.
21. Wilson SR, The L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. Cell 2013;155:285-95.
22. Akiyama T, Carstens MI, Ikoma A, Cevikbas F, Steinhoff M, Carstens E. Mouse model of touch-evoked itch (alloknesis). J Invest Dermatol 2012;132:1886-91.
23. Everaerts W, Zhen X, Ghosh D, Vriens J, Gevaert T, Gilbert JP, et al. Inhibition of the cation channel TRPV4 improves bladder function in mice and rats with cyclophosphamide-induced cystitis. Proc Natl Acad Sci U S A 2010;107:19084-9.
24. Farage MA, Miller KW, Berardesca E, Maibach HI. Clinical implications of aging skin: cutaneous disorders in the elderly. Am J Clin Dermatol 2009;10:73-86.
25. Berger TG, Shive M, Harper GM. Pruritus in the older patient: a clinical review. JAMA 2013;310:2443-50.
26. Suman M, Reddy BS. Pattern of contact sensitivity in Indian patients with hand eczema. J Dermatol 2003;30:649-54.
27. Vergnolle N, Cenac N, Altier C, Cellars L, Chapman K, Zamponi GW, et al. A role for transient receptor potential vanilloid 4 in tonicity-induced neurogenic inflammation. Br J Pharmacol 2010;159:1161-73.
28. Liedtke W, Friedman JM. Abnormal osmotic regulation in trpv4-/- mice. Proc Natl Acad Sci U S A 2003;100:13698-703.
29. Lechner SG, Markworth S, Poole K, Smith ES, Lapatsina L, Frahm S, et al. The molecular and cellular identity of peripheral osmoreceptors. Neuron 2011;69:332-44.
30. Ryskamp DA, Witkovsky P, Barabas P, Huang W, Koehler C, Akimov NP, et al. The polymodal ion channel transient receptor potential vanilloid 4 modulates calcium flux, spiking rate, and apoptosis of mouse retinal ganglion cells. J Neurosci 2011;31:7089-101.
31. Alexander R, Kerby A, Aubdool AA, Power AR, Grover S, Gentry C, et al. 4alpha-phorbol 12,13-didecanoate activates cultured mouse dorsal root ganglia neurons independently of TRPV4. Br J Pharmacol 2013;168:761-72.
32. Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, et al. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature 2003;425:917-25.
33. Chung MK, Lee H, Mizuno A, Suzuki M, Caterina MJ. TRPV3 and TRPV4 mediate warmth-evoked currents in primary mouse keratinocytes. J Biol Chem 2004;279:21569-75.
34. Tamoutounour S, Guilliams M, Montanana Sanchis F, Liu H, Terhorst D, Malosse C, et al. Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. Immunity 2013;39:925-38.
35. Gros E, Novak N. Cutaneous dendritic cells in allergic inflammation. Clin Exp Allergy 2012;42:1161-75.
36. Thorneloe KS, Sulpizio AC, Lin Z, Figueroa DJ, Clouse AK, McCafferty GP, et al. N-((1S)-1-{[4-((2S)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I. J Pharmacol Exp Ther 2008;326:432-42.
37. Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Kohler A, et al. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. Immunity 2011;34:973-84.
38. Thorneloe KS, Cheung M, Bao W, Alsaid H, Lenhard S, Jian MY, et al. An orally active TRPV4 channel blocker prevents and resolves pulmonary edema induced by heart failure. Sci Transl Med 2012;4:159ra148.

39. Roberts AC. The importance of serotonin for orbitofrontal function. Biol Psychiatry 2011;69:1185-91.
40. Akiyama T, Carstens E. Neural processing of itch. Neuroscience 2013;250:697-714.
41. Morita T, McClain SP, Batia LM, Pellegrino M, Wilson SR, Kienzler MA, et al. HTR7 mediates serotonergic acute and chronic itch. Neuron 2015;87:124-38.
42. Nordlind K, Azmitia EC, Slominski A. The skin as a mirror of the soul: exploring the possible roles of serotonin. Exp Dermatol 2008;17:301-11.
43. Lee MG, Dong X, Liu Q, Patel KN, Choi OH, Vonakis B, et al. Agonists of the MAS-related gene (Mrgs) orphan receptors as novel mediators of mast cell-sensory nerve interactions. J Immunol 2008;180:2251-5.
44. Geba GP, Ptak W, Anderson GM, Paliwal V, Ratzlaff RE, Levin J, et al. Delayed-type hypersensitivity in mast cell-deficient mice: dependence on platelets for expression of contact sensitivity. J Immunol 1996;157:557-65.
45. Lombardi F, De Chaumont C, Shields DC, Moran N. Platelet signalling networks: pathway perturbation demonstrates differential sensitivity of ADP secretion and fibrinogen binding. Platelets 2012;23:17-25.
46. Hill ND, Bunata K, Hebert AA. Treatment of alopecia areata with squaric acid dibutylester. Clin Dermatol 2015;33:300-4.
47. Chun KH, Imai Y, Higashi N, Irimura T. Migration of dermal cells expressing a macrophage C-type lectin during the sensitization phase of delayed-type hypersensitivity. J Leukoc Biol 2000;68:471-8.
48. Sato K, Imai Y, Irimura T. Contribution of dermal macrophage trafficking in the sensitization phase of contact hypersensitivity. J Immunol 1998;161: 6835-44.
49. Akiyama T, Carstens MI, Carstens E. Enhanced scratching evoked by PAR-2 agonist and 5-HT but not histamine in a mouse model of chronic dry skin itch. Pain 2010;151:378-83.
50. Tamagawa-Mineoka R. Important roles of platelets as immune cells in the skin. J Dermatol Sci 2015;77:93-101.
51. McNicol A, Israels SJ. Beyond hemostasis: the role of platelets in inflammation, malignancy and infection. Cardiovasc Hematol Disord Drug Targets 2008;8: 99-117.

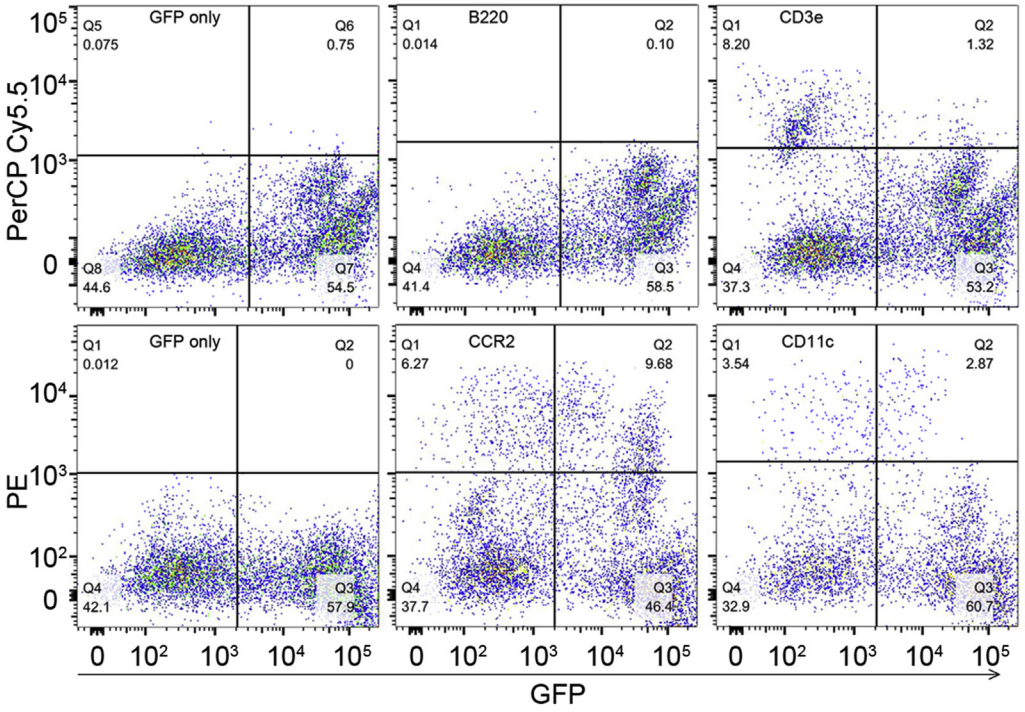


FIG E1. TRPV4-eGFP is expressed by dermal macrophages but not T or B cells. Flow cytometry with ear skin single-cell suspensions from *Trpv4*^{eGFP} mice reveals that TRPV4-eGFP is expressed by a subpopulation of CCR2^{+} or CD11c^{+} macrophages but not by CD3e^{+} T cells or B220^{+} B cells. The experiment was repeated 4 times. *PE*, Phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.

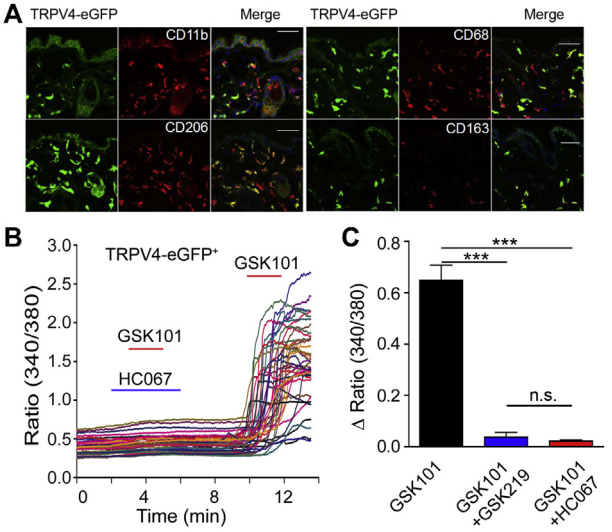


FIG E2. Expression of cellular markers for tissue macrophages in the skin of *Trpv4*^{eGFP} mice. **A,** Double-labeling experiments show that CD11b, CD206, CD68, and CD163 were coexpressed with TRPV4-eGFP^{+} cells in skin. *Bar* = 50 μm. **B** and **C,** Representative traces (Fig E2, *B*) and summarized data (Fig E2, *C*) show that 0.3 μmol/L GSK101 elicited a $[Ca^{2+}]_i$ response in freshly isolated TRPV4-eGFP^{+} ear skin single-cell suspensions, which was inhibited by 0.3 μmol/L GSK219 or 5 μmol/L HC067. ***P < .001, ANOVA (n = 11 coverslips for GSK101 and 5 coverslips for GSK219 and HC067). *n.s.*, Not significant.

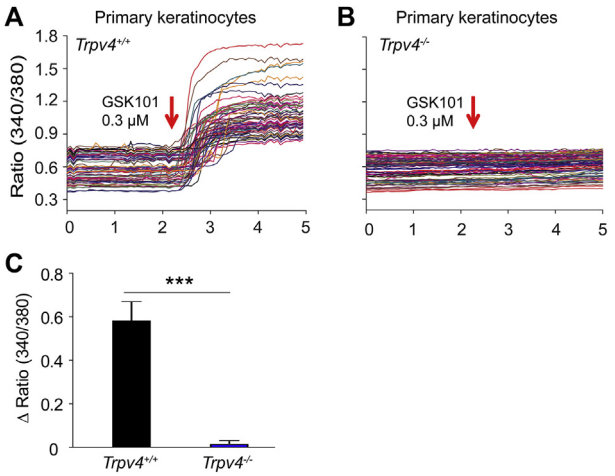


FIG E3. Functional expression of TRPV4 in primary cultured mouse keratinocytes. **A** and **B,** Representative traces showing GSK101-elicited $[Ca^{2+}]_i$ responses in cultured keratinocytes from *Trpv4*$^{+/+}$ (Fig E3, *A*) and *Trpv4*$^{-/-}$ (Fig E3, *B*) mice. **C,** Summarized data showing the averaged response evoked by GSK101 in cultured keratinocytes from *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. ***$P < .001$, Student *t* test (n = 5 coverslips in each group).

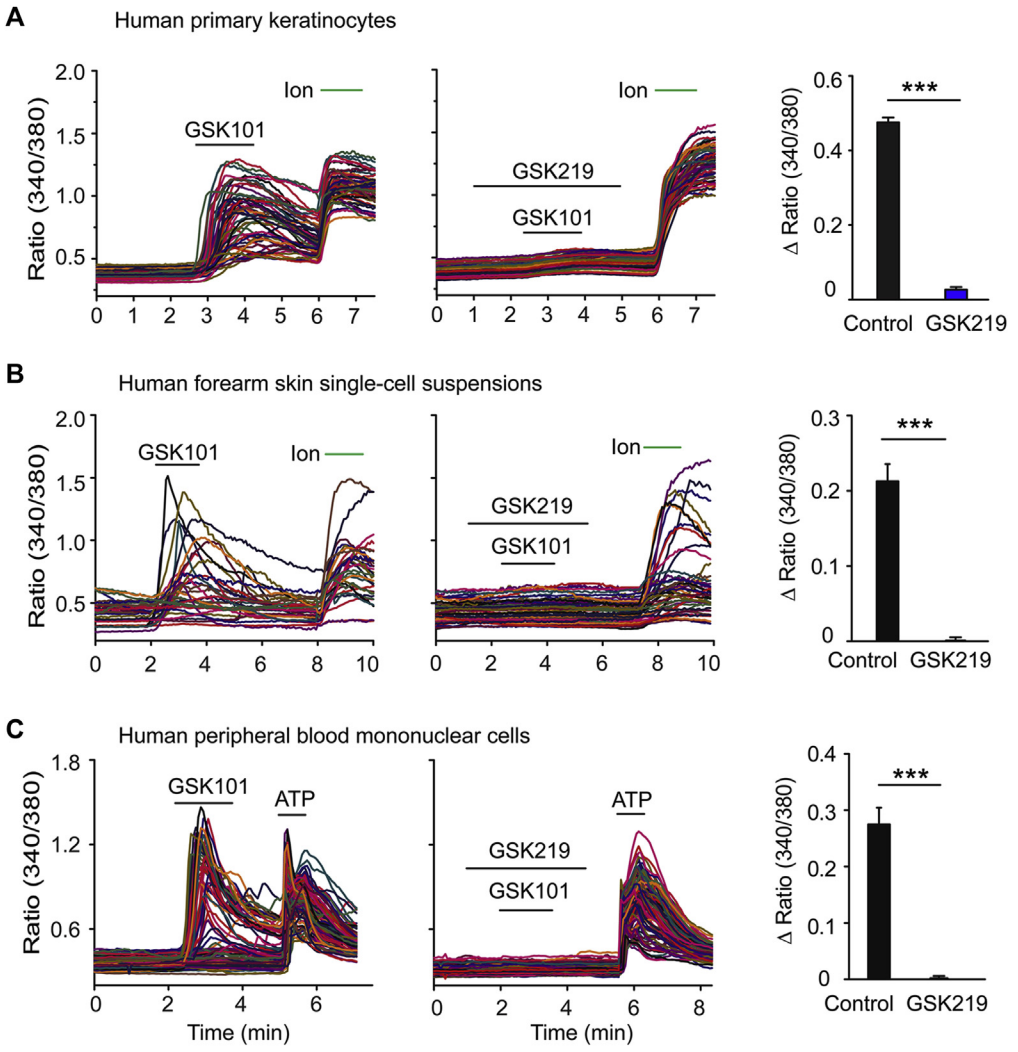


FIG E4. GSK101 elicits $[Ca^{2+}]_i$ responses in human primary keratinocytes, forearm skin cell suspensions, and human PBMCs. Representative traces show the GSK101 (0.3 μmol/L)–elicited $[Ca^{2+}]_i$ response in the absence *(left)* and presence *(middle)* of GSK219 (0.3 μmol/L) in human primary keratinocytes **(A)**, human forearm skin cell suspensions **(B)**, and human PBMCs **(C)**. *Bar graphs on the right* show that GSK219 abolished GSK101 responses in all cell types tested. ***$P < .001$, Student *t* test (n = 5 coverslips in each group). Ionomycin *(Ion)* and ATP were used as positive controls.

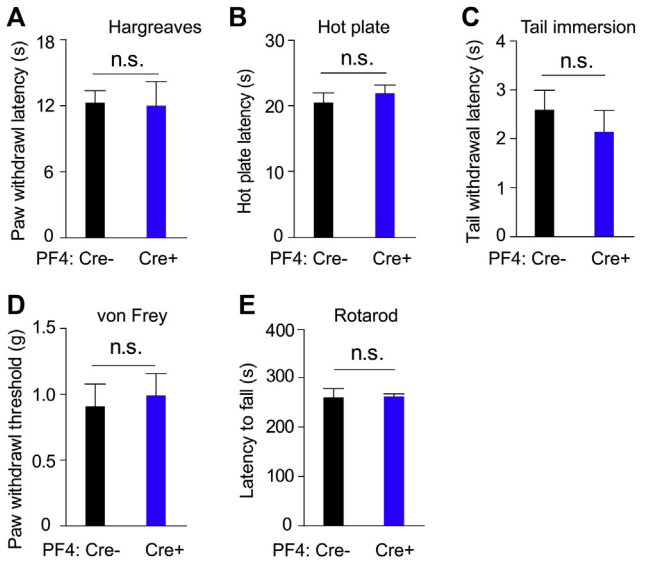


FIG E5. Conditional depletion of platelets does not affect thermal and mechanical sensations and motor function in mice. Paw withdraw latency **(A)**, hot plate latency **(B)**, tail withdraw latency **(C)**, paw withdraw threshold **(D)**, and latency to fall **(E)** in the *Pf4-Cre*$^{-}$ mice (n = 5 mice) were not significantly different from those of *Pf4-Cre*$^{+}$ mice (n = 6 mice). $P > .05$, Student *t* test. *n.s.*, Not significant versus the *Pf4-Cre*$^{-}$ group.

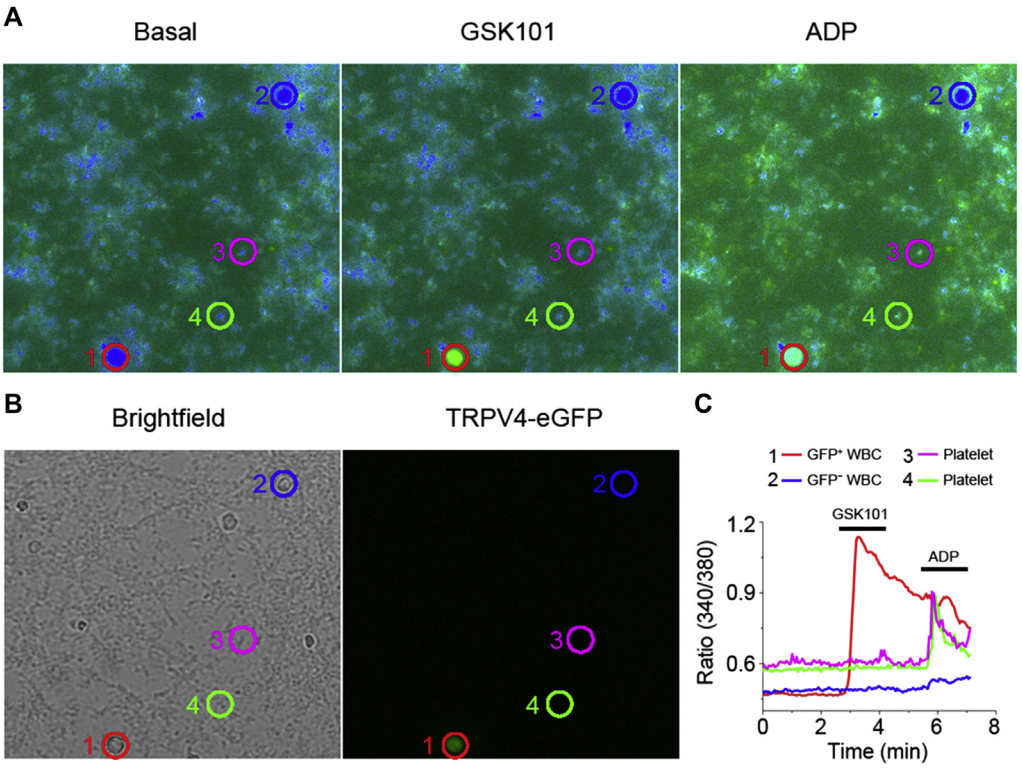


FIG E6. TRPV4 is not functionally expressed by platelets. **A,** Representative images showing the $[Ca^{2+}]_i$ response elicited by 0.3 μmol/L GSK101 and 100 μmol/L adenosine diphosphate *(ADP)*. Cell number 1 represents a GFP^+ white blood cell. Cell number 2 represents a GFP^- white blood cell. Cell numbers 3 and 4 represent platelets. **B,** Bright-field and GFP images show that TRPV4-eGFP is present in a white blood cell (cell number 1) but not in platelets. **C,** Representative traces show that GSK101 elicited a $[Ca^{2+}]_i$ response in GFP^+ white blood cells (cell number 1) but the GFP^- white blood cells (cell number 2) or platelets (cells number 3 and 4). Adenosine diphosphate serves as a positive control. The same experiment was repeated at least 3 times.

TABLE E1. Primary antibodies used for immunofluorescent staining and flow cytometry

Antibody	Host species	Dilution	Source
GFP	Chicken	1:500	Aves Labs, Tigard, Ore; catalog no.: GFP-1020
Purified anti-K14	Rabbit polyclone	1:500	Covance, Madison, Wis; catalog no.: PRB-155P
Purified anti-F4/80	Rat (clone # BM8)	1:500	BioLegend, San Diego, Calif; catalog no.: 123101
Purified anti-CD11b	Rat (clone no. 5C6)	1:500	AbD Serotec, Oxford, United Kingdom; catalog no.: MCA711GT
Purified anti-CD206	Rat (clone no. MR5D3)	1:500	AbD Serotec; catalog no.: MCA2235T
Purified anti-CD68	Rat (clone no. FA-11)	1:200	BioLegend; catalog no.: 137001
Purified anti-CD163	Rabbit polyclone	1:100	Santa Cruz Biotechnology, Dallas, Tex; catalog no.: sc-33560
Rhodamine-conjugated avidin	NA	1:1000	Rockland Antibodies & Assays, Limerick, Pa; catalog no.: A003-00
APC anti-CD11b	Rat (clone no. M1/70)	1:300	eBioscience, San Diego, Calif; catalog no.: 17-0112
PE anti-CD11c	Hamster (clone no. N418)	1:300	BioLegend; catalog no.: 117307
PE anti-CCR2	Rat (clone no. 475301)	1:300	R&D Systems, Minneapolis, Minn; catalog no.: FAB5538P
PerCP Cy5.5 anti-CD3e	Hamster (clone no. 145-2C11)	1:300	eBioscience; catalog no.: 45-0031
PerCP anti-B220	Rat (clone RA3-6B2)	1:300	BD PharMingen, San Jose, Calif; catalog no.: 553933

APC, Allophycocyanin; *NA*, not applicable; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.