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TRPV4-expressing Macrophages and Keratinocytes Contribute Differentially to Allergic and Non-allergic Chronic Itch

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Abstract

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 non-allergic chronic itch.

Method—The expression of TRPV4 in chronic itch and healthy control skin preparations was examined by real-time RT-PCR. *Trpv4*^{eGFF} mice were used to study the expression and function of TRPV4 in the skin by immunofluorescence staining, calcium imaging, and patch-clamp recordings. Genetic and pharmacological approaches were employed to examine the role and underlying mechanisms of TRPV4 in mouse models of dry skin associated chronic itch and spontaneous scratching associated with SADBE-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 non-allergic chronic itch in mice, respectively. Importantly, TRPV4 expression is significantly elevated in skin biopsies from patients with chronic idiopathic pruritus (CIP) in comparison to skin from healthy control subjects. Moreover, TRPV4-dependent chronic itch requires 5-HT signaling secondary to activation of distinct 5-HT receptors in both allergic and non-allergic 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.

Keywords

TRPV4; chronic itch; macrophage; keratinocyte

Introduction

Chronic itch, a symptom of many primary skin disorders and systemic diseases, is a major medical issue affecting 10–20% 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 (GPCRs) and ion channels in the primary sensory neurons, which enable sensory neurons to detect a variety of pruritogens. [3, 4, 5] However, the 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.

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TRPV4 is a Ca^{2+}-permeable cation channel in the transient receptor potential vanilloid subfamily (TRPV), and is abundantly expressed in the skin, renal, and urinary bladder epithelia[6, 7] (www.biogps.org). TRPV4 is a polymodal sensory transducer that integrates a variety of thermal, mechanical, and chemical stimuli including warmth (27–35°C), hypo-osmotic stimulation, and many inflammatory metabolites. [8] As a result, TRPV4 channels are involved in many physiological and pathological processes. Although it was recently reported that TRPV4 is involved in acute itch elicited by exogenously applied histamine and 5-HT, its precise mechanism in itch induction remains controversial. Indeed, whether TRPV4 predominantly mediates itch indirectly via 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 non-allergic chronic itch, respectively. Further, we identified elevated 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 mice (Jackson Laboratories), *Trpv4*^{eGFP} (Mutant Mouse Regional Resource Centers, MMRRC), *Trpv4*$^{-/-}$, [11] *Kit*$^{W\text{-}sh/W\text{-}sh}$ (Jackson Laboratories), *Htr7*$^{-/-}$ (Jackson Laboratories), *Htr2a*$^{-/-}$ (a kind gift from Dr Jay Gingrich at the Columbia University) mice were used for the study. The *Cre+ and Cre− Pf4*^{Cre}*;iDTR* (Pf4-Cre^{+} and Pf4-Cre) mice were obtained by crossing the *Rosa26*^{iDTR} mice (Jackson Laboratories) with the *Pf4-Cre* mice (Jackson Laboratories). To generate the *Trpv4*$^{f/f}$ mice, three of the properly targeted ES cell clones were obtained from the KOMP Repository and used for blastocyst injections and one clone led to high contribution chimaeras that produced germline transmitted offspring as assayed by 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} 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 intraperitoneal administration tamoxifen for five consecutive days at 75mg/kg in 0.2 ml 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 the experimental procedures were approved by the Institutional Animal Care and Use Committee of University of Texas Health Science Center at Houston and the

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Institutional Animal Care and Use Committee at Washington University School of Medicine, and were in accordance with the guidelines provided by the National Institute of Health and the International Association for the Study of Pain. All mice were randomly allocated to different experimental groups by the lab managers who are blinded to the experimental design. All mice were included in the analysis unless it is dead.

Human peripheral blood mononuclear cells and skin biopsies

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). De-identified human control skin was obtained from non-cancerous, marginal skin from patients (average age of control subjects was 74.5±4.2 years of age) undergoing routine skin cancer surgery under an IRB-exempt protocol. Human chronic idiopathic pruritus (CIP) skin was obtained directly from patients with a firm diagnosis of CIP (average age of CIP patient was 74.3±2.2 years of age) as approved by the Washington University in St. Louis IRB (Protocol 201412117). The numerical rating scale (NRS) itch score for CIP was examined on a scale of 0 to 10 as a peak value during a week before examination. A thorough work-up was performed to establish the diagnosis of CIP by 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 biopsies were cut and separated using forceps and digested in 0.25 mg/ml Liberase TL (Roche) in DMEM media for 90 minutes at 37°C. Samples were mashed through 70 μm cell strainers and washed with DMEM media supplemented with 5% FBS, 1% L-glutamine (GIBCO), and 1% Pencillin/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 the previous study. In brief, the skin of newborn mouse pups (P0–P2) or human skin biopsy was removed and placed in a Petri dish containing 2.5% dispase II (Life Technologies, NY) and incubated at 4°C overnight. Epidermis was then separated from subcutaneous tissues. Keratinocytes were dissected by gentle scraping and flushing with culture medium. Harvested cells were plated on coverslips coated with collagen IV and cultured in serum-free, fully supplemented keratinocyte medium CnT-07 (CELLnTEC advanced cell systems, Switzerland) (for mouse keratinocytes) or keratinocyte-SFM medium supplemented with BPE and EGF (Invitrogen, Carlsbad, CA, USA) (for human keratinocytes) under a humidified atmosphere of 5% CO_2/95% air at 37°C for 2 days before use.

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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 described.[13] In brief, laminectomies were performed and bilateral DRG were dissected out. After removal of connective tissues, DRG were transferred to a 1 mL Ca^{2+}/Mg^{2+}-free HBSS containing 2 μL saturated $NaIICO_3$, 0.35 mg 1-cysteine and 20 U papain (Worthington, Lakewood, NJ, USA), and incubated at 37°C for 10 min. The suspension of DRG was centrifuged, the supernatant was removed, 1 mL Ca^{2+}/Mg^{2+}-free HBSS containing 4 mg collagenase type II and 1.25 mg dispase type II (Worthington) was added and incubated at 37°C for 10 min. After digestion, neurons were pelleted, suspended in neurobasal medium containing 2% B-27 supplement, 1% 1-glutamine, 100U·mL^{-1} penicillin plus 100μg·mL^{-1} streptomycin, and 50ng·mL^{-1} nerve growth factor, plated on a 12mm 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 18–24 hr before use.

Extraction and isolation of mouse platelets

The whole blood was collected into a tube containing 3.8% Na Citrate at a ratio of 9:1 after the anesthesia of mouse. Centrifuge the anti-coagulant whole blood at 100 g for 10 min without brake. Transfer the platelet rich plasma to a new tube and add 1 μM PGE1 and incubate for 5 min. Add another volume of Na Citrate and centrifuge at 400 g for 10 min. Discard the plasma and resuspend platelets in HBSS containing 4 μM Fura2-AM (Life Technologies). Plate the platelets on coverslips coated with poly-l-lysine and incubate at room temperature for 1 hr. The platelets were be used after washed with fresh HBSS for at least 30 min using calcium imaging assay.

Preparation of skin superfusate

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

Ca^{2+} imaging

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

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Whole-cell patch-clamp recording

Whole-cell patch-clamp recordings were performed using an EPC 10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) or multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) at room temperature (22–24°C) on the stage of an inverted phase-contrast microscope equipped with a filter set for green fluorescence protein visualization. Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instrument Company, Novato, CA, USA) with a Sutter P-97 pipette puller had resistances of 2–4 MΩ when filled with pipette solution containing 140 mM CsCl, 1 mM $MgCl_2$, 0.5 mM EGTA, and 10 mM HEPES with pH 7.3 and 315 mOsm·L^{-1}. Cells were continuously perfused with standard extracellular solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ≈340 mOsm·L^{-1} with sucrose). The whole-cell membrane currents were recorded using voltage ramp from −100 to +100 mV for 500 ms at holding potential of 0 mV. Data were acquired using PatchMaster software (HEKA Elektronik) or Clampex 10 (Molecular Devices, Sunnyvale, CA, USA). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed and plotted using Clampfit 10 (Molecular Devices, Sunnyvale, CA, USA). Values are given as means ± SEM; n represents the number of measurements.

Immunofluorescence

Adult mice, ages 6–16 weeks, were asphyxiated with CO_2 and perfused transcardially with 200 ml of phosphate buffered saline (PBS; pH 7.3) followed by 200 ml of fixative (4% paraformaldehyde in 0.1 M PB, pH 7.3, or Zamboni's fixative [2% paraformaldehyde, 15% (v/v) saturated picric acid, 0.1 M PB, pH 7.3]). The tissues were removed and postfixed overnight at 4°C in the same fixative. All tissues were cryoprotected overnight in 30% sucrose in 0.1 M PB, pH 7.3, frozen in optimal cutting temperature medium (OCT), sectioned with a cryostat at 20 μm, mounted on Superfrost Plus slide, and stored at −20°C. Frozen slides were dried at room temperature for 1 hr and washed three times in PBS with 0.1% Triton X-100 (PBS+TX), blocked for 30 min to 1 hr 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). The sections were then washed three times in PBS+TX and incubated for 2 hr at room temperature with secondary antibodies conjugated to Alexa-488 fluorochrome (Invitrogen) or Cy3 fluorochromes (Jackson ImmunoResearch, West Grove, PA), and diluted 1:500 in blocking solution. Sections were then washed three times in PBS+TX and mounted in anti-fade medium (Vectashield, Vector Laboratories, Burlingame, CA). All preparations were examined with a Nikon Al 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), CD11c (Biolegend), CD3e (eBioscience), B220 (BD Pharmingen), and CCR2 (R&D systems). Samples were acquired on a BD Lsr Fortessa flow cytometer (BD Bioscience) and analyzed using FlowJo software (V10, Tree Star) (see Table E1 in this article's Online Repository at www.jacionline.org).

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Cell sorting and RT-PCR

Isolated GFP^+ and GFP^- skin cells were sorted using a FACSAriaII (BD Bioscience). Total RNA was extracted from using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase I (Invitrogen) and the cDNA was synthesized in vitro using ThermoScript® RT-PCR System kit (Invitrogen). PCR conditions: 95°C for 3 min, 40 cycles of 30 s at 95°C, 30 s at 52°C and 60 s at 72°C. The sequences of the primers used are as follows: GFP forward 5′-AAGGGCATCGACTTCAAGG-3′, reverse 5′-TGCTTGTCGGCCATGATATAG-3′; mTRPV4 forward 5′-CCTGCTGGTCACCTACATCA-3′, reverse 5′-CTCAGGAACACAGGGAAGGA-3′; mGAPDH forward 5′-GCACAGTCAAGGCCGAGAAT-3′ reverse 5′-GCCTTCTCCATGGTGGTGAA-3′

Quantitative RT-PCR

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

Conditional platelet depletion

Platelet depletion was accomplished by intraperitoneal injections of DTX (400 ng per mouse) every 48 hr for a total of 3 treatments to the *Pf4-Cre*$^+$*;iDTR* mice, [14] and confirmed by counting the number of platelets in the 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 back of the mice were treated twice daily with cutaneous application of acetone/ether (1:1) mixture followed by water (AEW). After a 7-d treatment, the mice showed robust spontaneous scratching and the treated skin area exhibited decreased stratum corneum hydration and increased trans-epidermal 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 min 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.

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Mouse model of allergic contact dermatitis (ACD)

The contact sensitizer squaric acid dibutylester (SADBE) (Tokyo Chemical Co.) was used to elicit contact hypersensitivity in the mouse as a model of allergic contact dermatitis in humans.[16] Mice were sensitized by the topical application of 20 μl of 0.5% SADBE in acetone to the shaved abdominal skin once a day for three 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 of the nape of neck once a day for three consecutive days whereas acetone alone was used as the vehicle control. The scratching behavior with the hind paw was quantified by recording the number of incidences of scratching bouts for 60 min daily.

Statistical Analysis

Values are reported as the mean + standard error of the mean (s.e.m.). Unpaired or paired t-test was used for comparison between two groups or one-way ANOVA and repeated measures tests followed by Tukey-Kramer or Bonferroni *post hoc* analysis was used for comparison among multiple groups occurring over time. All tests were carried out as two-tailed tests, $p < 0.05$ was 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 non-allergic 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 may serve protective roles against potential threats and dangers in the environment (e.g., 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 elevated in skin biopsies from patients with CIP in comparison to healthy control skin (Fig 1, A). On the other hand, TRPV3, another channel implicated in itch, was not significantly elevated in the CIP patients. [19, 20] These results suggest that TRPV4 might be involved in the pathogenesis of chronic itch in humans.

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 *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 not shown). This was additionally confirmed by pharmacological 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] Further, exposure to water is

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known to exacerbate dry skin and itching in conditions such as chronic hand dermatitis. [26] Since distilled water is extremely hypotonic (≈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 AE 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 humans. [15]

We further investigated if TRPV4 is involved in spontaneous scratching associated with ACD using the contact sensitizer SADBE. [16] *wt* mice receiving SADBE treatment displayed a robust scratching response when compared to mice treated with vehicle control only (not shown). The *Trpv4*$^{-/-}$ mice displayed a markedly reduced scratching response after SADBE treatment when compared to *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 non-allergic chronic itch in mice.

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

Although TRPV4 is known to be expressed by both neurons as well as non-neuronal cells, the precise expression pattern of TRPV4 across tissues is controversial. [30, 31] We therefore took a transgenic approach by using BAC transgenic *Trpv4*^{eGFP} mice [32] and determined the expression of TRPV4-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 using 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 analysis using skin cell suspensions further showed that a subset of TRPV4-eGFP^{+} cells expressed CD11b and C-C chemokine receptor type 2 (CCR2) as well as low levels of CD11c, which is reminiscent of CCR2/CD11b-positive dermal macrophages [34] (Fig 2, C, and see Fig E1 in this article's Online Repository at www.jacionline.org). In addition, TRPV4-eGFP^{+} cells were also immunopositive for CD68, CD206, and CD163, all of which are cell markers commonly used for tissue macrophages[35] (see Fig E2, A in this article's Online Repository at www.jacionline.org).

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 intracellular Ca^{2+} ($[Ca^{2+}]_i$) 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 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

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HC067, two selective TRPV4 antagonists [23, 38] (Fig 2, E, and see Fig E2, B and C in this article's Online Repository at www.jacionline.org). 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 peripheral blood mononuclear cells, all of which were abolished by GSK219, suggesting that like 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.

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

Consistent with TRPV4-expressing skin-resident cells being critical to the genesis of chronic itch in mice, we showed that 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, the 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, AEW-induced spontaneous scratching response was not significantly affected in the Cx3cr1-Cre^+ mice (Fig 3, F). On the other hand, SADBE-induced spontaneous scratching was significantly attenuated in the *Cx3cr1-Cre*$^+$ mice but not in the *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 non-allergic chronic itch in mice.

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

To identify skin-derived chemical mediator(s) of TRPV4-mediated scratching response, we prepared AEW-treated skin superfusates from both $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice and applied the superfusates directly to cultured wild-type 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

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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] We thus examined if 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 (5-HTP), which is converted to 5-HT without the involvement of TPH. Indeed, administration of 5-HTP 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 the 5-HT receptor 2a (Htr2a), which was shown to mediate 5-HT-elicited scratching in mice, [40] or genetic ablation of the 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*$^{-/-}$ mice 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 pharmacological inhibition or genetic ablation of the function of Htr7, which was also shown to mediate 5-HT-induced itch in mice [41] (Fig 4, J and K), suggesting that Htr7 but not Htr2a signaling likely plays a major role in the 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 concentration leading to activation of TRPV1-positive pruriceptive sensory neurons and generation of histamine-dependent itch. [4] Besides histamine, mast cells also represent a major source of 5-HT. [42] We therefore examined if TRPV4-eGFP was expressed by mast cells using streptin-avidin-rhodamine. [43] However, there was no co-localization of TRPV4-eGFP with streptin-avidin-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* controls (Fig 5, B and C), suggesting that mast cell-derived 5-HT is not a major contributor to TRPV4-dependent chronic itch.

Besides mast cells, platelets are the other major source for 5-HT in the skin. [44] Although it is increasingly understood 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 if platelets are required for

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TRPV4-mediated itch we used a conditional platelet depletion model by crossing the *Pf4*^{Cre} trans genic line with the Cre-dependent *Rosa26*^{DTR} line (*iDTR* followed by DTX injections. [14] The number of platelets in peripheral blood of the *Pf4-Cre*$^{+}$ mice was markedly reduced after DTX injections compared with DTX-treated *Pf4-Cre*$^{-}$ littermates but no other blood cells were affected (Fig 5, D, and 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 rotarod test (see Fig E5 in this article's Online Repository at www.jacionline.org), 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 (ADP) through the P2Y12 receptor and fibrinogen through the platelet glycoprotein IIb/IIIa receptors. [45] We thus investigated if selective inhibition of the P2Y12 or glycoprotein (GP) IIb/IIIa receptors affects TRPV4-mediated chronic itch responses using the selective P2Y12 receptor antagonist clopidogrel or a selective inhibitor of the GP IIb\IIIa receptors, eptifibatide, both of which are antiplatelet drugs approved by Food and Drug Administration (FDA). 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 cutaneous platelets are likely the sources of 5-HT release in response to AEW or SADBE treatment.

We finally asked if TRPV4 is functionally expressed by platelets isolated from the peripheral blood of the *Trpv4*^{eGFP} mice. We did not detect TRPV4-eGFP in platelets. Further, 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 the activation of the platelets but rather through the release of unknown proinflammatory mediators following 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 elevated TRPV4 expression in skin biopsies of patients with CIP. Further, we

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demonstrated that 5-HT is a critical downstream mediator of the TRPV4-mediated spontaneous scratching and distinct 5-HT receptors are required for producing TRPV4-mediated allergic and non-allergic chronic itch. Our results support a model in which the TRPV4-positive skin-resident cells are key signaling components in the pathogenesis of chronic itch.

Our data show that TRPV4 expression is up-regulated 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 the 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 of TRPV4 expression in CIP patients, further supporting a general role of TRPV4 in mediating chronic itch in both rodents and humans.

Interestingly, by 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 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. Since TRPV4-expressing epidermal keratinocytes are the first skin cells to access water following barrier disruption, which is an important step in the generation of AEW-induced dry skin model, [15] we speculate that activation of TRPV4-mediated signaling in the epidermal keratinocytes by water is largely responsible for AEW-induced chronic itching. Although the mechanism of SADBE in ACD is not fully understood, activation of T cell-mediated immunity is critically involved in the generation of ACD. [46] Besides 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 contribute to skin inflammation but also chronic itch in 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; 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 the *Pf4-Cre*$^+$ mice severely attenuated spontaneous scratching in mice treated with AEW or SADBE,

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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 via 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 (IBD). [51]

Our results showed that Htr2a, one of many 5-HT-responsive GPCRs, is involved in SADBE-induced spontaneous scratching based on our pharmacological 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 chronic itch might be critical to the development of effective therapies against different forms of chronic itch with distinct etiologies.

In summary, we have shown that the TRPV4-mediated allergic and non-allergic 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. Since chronic itch originates in the skin, identification of TRPV4-dependent itch-specific cellular signaling networks in the skin can guide development of selective pharmacological intervention of these chronic itch pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TRPV4	Transient receptor potential vanilloid 4
GPCRs	G-protein coupled receptors
DRG	dorsal root ganglion
CIP	chronic idiopathic pruritus

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AEW	acetone/ether mixture followed by distilled water
ACD	allergic contact dermatitis
SADBE	squaric acid dibutylester
CCR2	C-C chemokine receptor type 2
TPH	Tryptophan hydroxylase
pCPA	p-chlorophenylalanine
5-HTP	5-hydroxytryptophan
Htr2a	5-HT receptor 2a
DTX	Diphtheria toxin
ADP	Adenosine diphosphate
GP	Glycoprotein
FDA	Food and Drug Administration
IBD	Inflammatory bowel disease
DMEM	Dulbecco modified Eagle medium
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Ct	cycle threshold

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Key Messages

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

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Capsule Summary

TRPV4 in keratinocytes and macrophages contributes to chronic itch through downstream serotonin signaling pathway, suggesting that TRPV4 may be a novel therapeutic target for the treatment of chronic itch.

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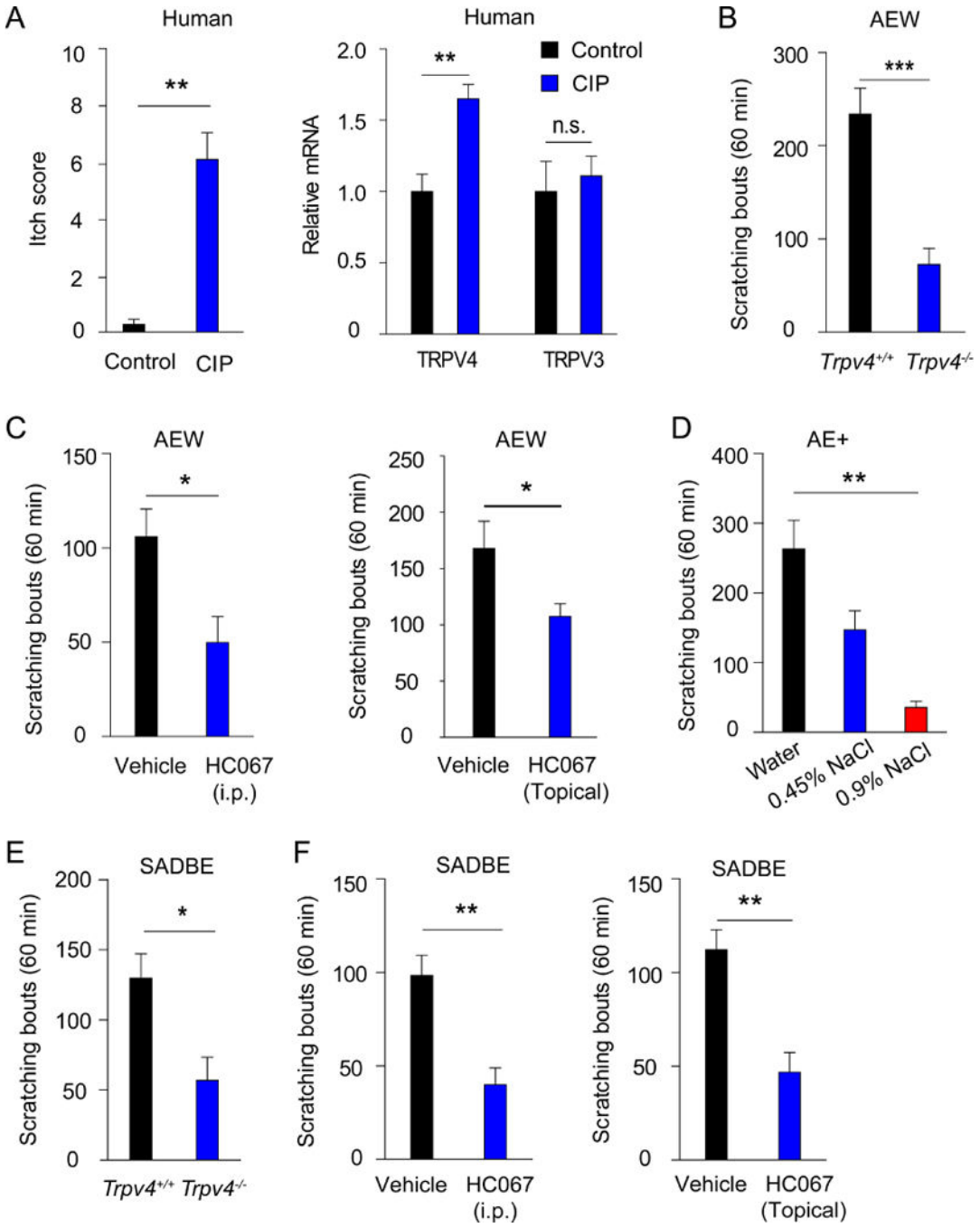

Figure 1.

TRPV4 expression is elevated in skin biopsies of CIP patients and TRPV4 function is required for generating mouse models of both allergic and non-allergic chronic itch. **A**, The bar charts show the averaged itch numerical rating scale (NRS) scores and mRNA transcripts for TRPV4 and TRPV3 in CIP patients and health controls.. *p<0.05, **p<0.01, Student's *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<0.001, Student's *t* test; n=7.. **C**, Spontaneous scratches in *Trpv4*$^{+/+}$ mice after 7 days of AEW treatment were

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reduced by HC067 (20 mg/kg, i.p or topical application) compared with vehicle. *p<0.05, Student's *t* test; n=8–9. **D**, Spontaneous scratches after the treatment with the 1:1 mixture of acetone and ether followed by 0.9% NaCl, 0.45% NaCl, or distilled water, respectively. **p<0.01, ANOVA; n=5–7. **E**, Spontaneous scratches in *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice after SADBE treatment. *p<0.05, Student's *t* test; n=6. **F**, SADBE-induced spontaneous scratches in *Trpv4*$^{+/+}$ mice after treatment with HC067 (20 mg/kg, i.p or topical application) or vehicle. *p<0.05, Student's *t* test; n=5–6.

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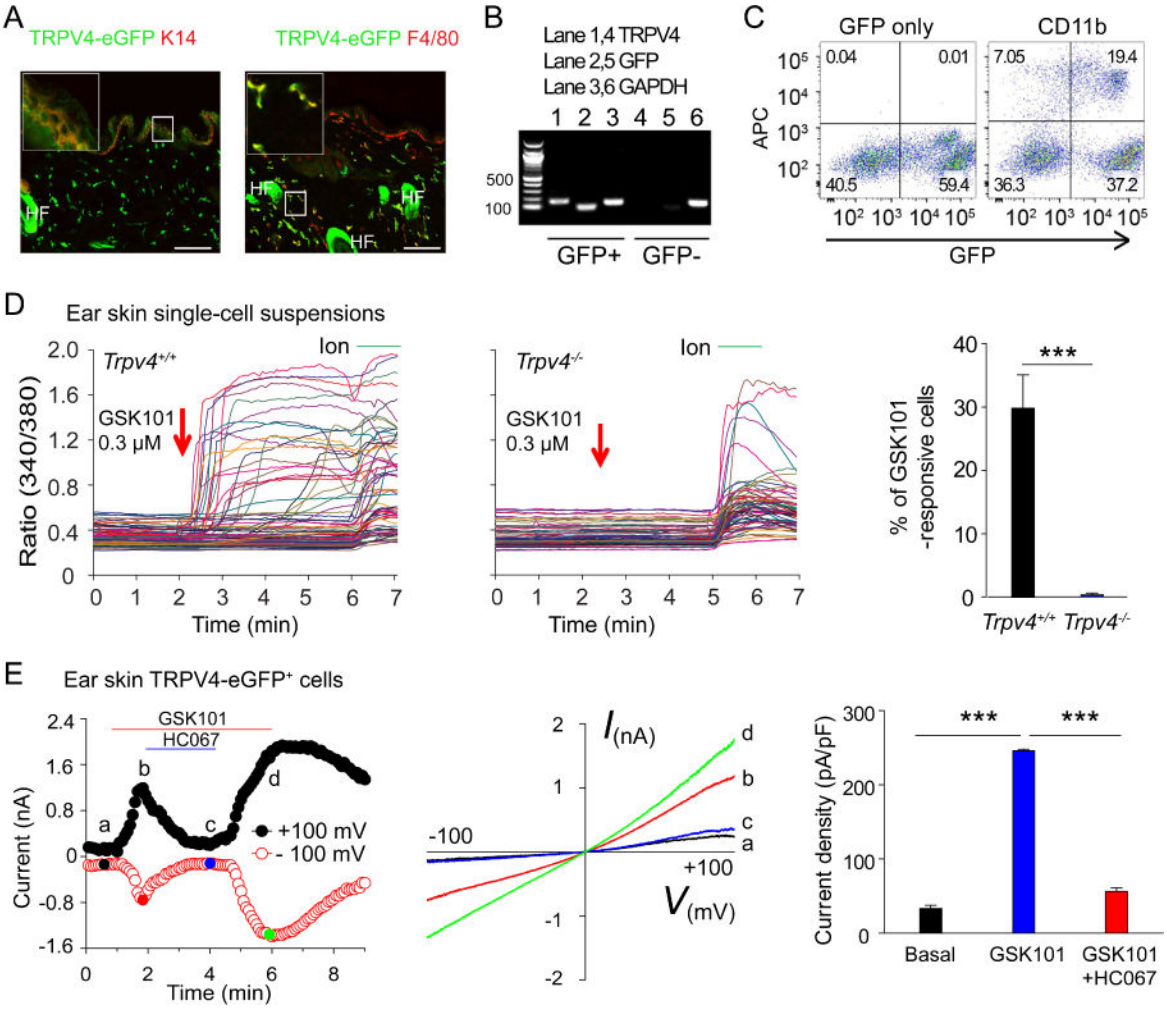

Figure 2.

TRPV4 is functionally expressed by mouse skin-resident cells. **A**, Double labeling reveals the co-localization of TRPV4-eGFP with K14 (left panel) and F4/80 (right panel) in skin sections from the *Trpv4*^{eGFP} mice. Bar=50 μm. HF: hair follicle. Inset shows the 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 analysis illustrates that TRPV4-eGFP is expressed in CD11b-positive skin-resident cells. **D**, Representative traces showing the 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 the time points of GSK101 applications. Ionomycin (Ion) was used as positive control. Summarized data from in the right showing the percentages of GSK101-responsive skin cells isolated from the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. ***p<0.001, Student's *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 μM GSK101 in the TRPV4-eGFP^{+} myeloid cells from the ear skin single-cell suspensions. HC067 at 5 μM markedly inhibited the GSK101-activated currents. Bar charts in the right illustrate summarized data. ***p<0.001, ANOVA; n=5.

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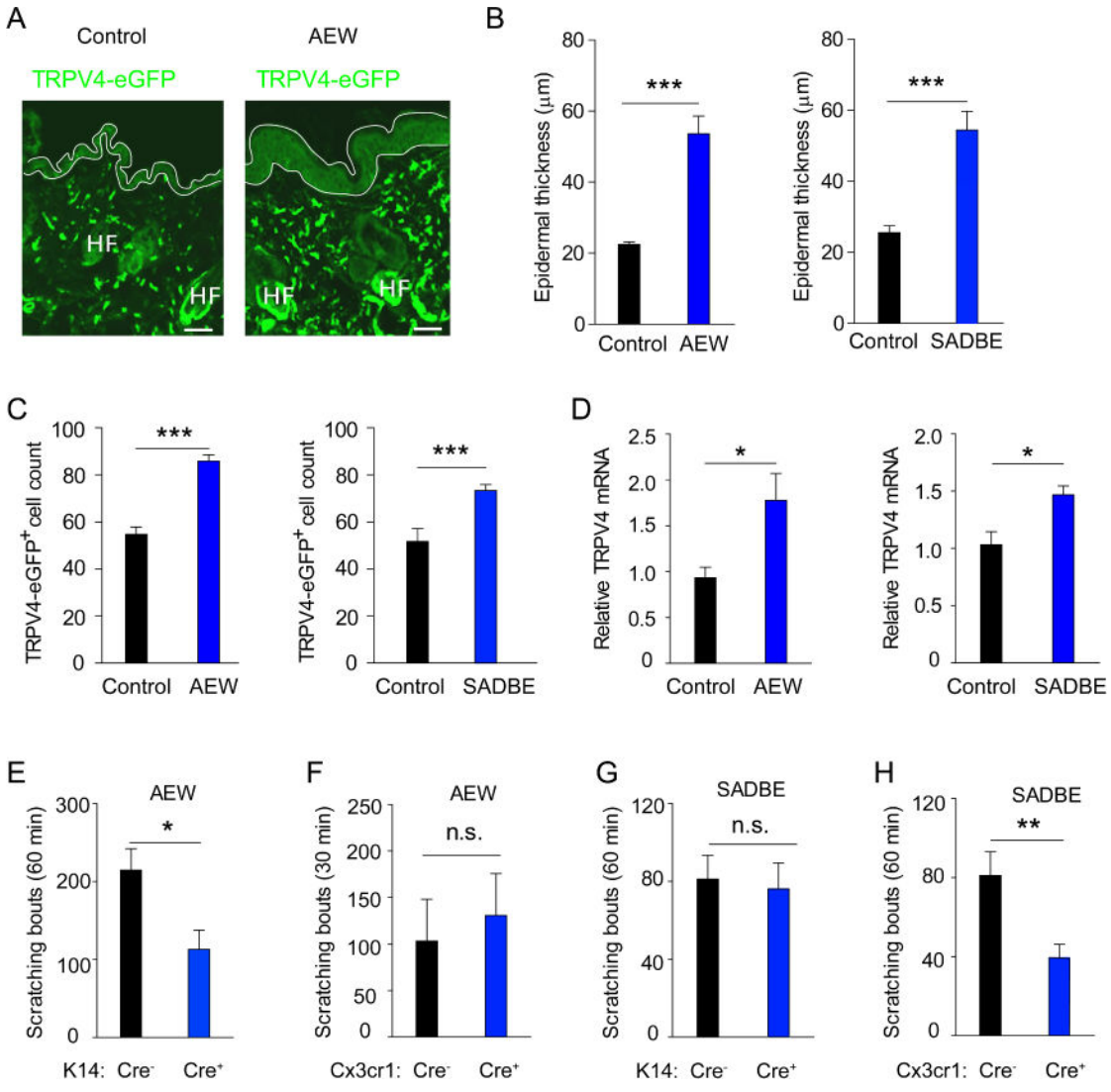

Figure 3.

TRPV4 channels expressed by macrophages and keratinocytes contribute differentially to allergic and non-allergic chronic itch. **A**, Representative images showing the TRPV4-eGFP^{+} cells in the skin of *Trpv4^{eGFP}* mice treated with vehicle control and AEW. **B**, The epidermal thickness was significantly increased by AEW and SADBE treatments compared with their respective vehicle controls. ***p<0.001, Student's *t* test; n=6–8. **C**, The number of TRPV4-eGFP^{+} dermal macrophages increased significantly following AEW or SADBE treatment. ***p<0.001, Student's *t*-test; n=6–8. **D**, Relative TRPV4 mRNA in the skin of AEW- or SADBE-treated mice, *p<0.05, Student's f test; n=6–8. **E**, Spontaneous scratching in the *K14-Cre*$^{+}$ and *K14-Cre*$^{-}$ mice after AEW treatment, *p<0.05, Student's f test; n=5. **F**, Spontaneous scratching in the *Cx3cr1-Cre*$^{+}$ and *Cx3cr1-Cre*$^{-}$ mice after AEW treatment. Student's *t* test; n=5. n.s. not significant. **G**, Spontaneous scratching in the *K14-Cre*$^{+}$ and *K14-Cre*$^{-}$ mice after SADBE treatment. Student's *t* test; n=8–9. n.s. not significant. **H**, Spontaneous scratching in the *Cx3cr1-Cre*$^{+}$ and *Cx3cr1-Cre*$^{-}$ mice after SADBE treatment. **p<0.01, Student's *t* test; n=9.

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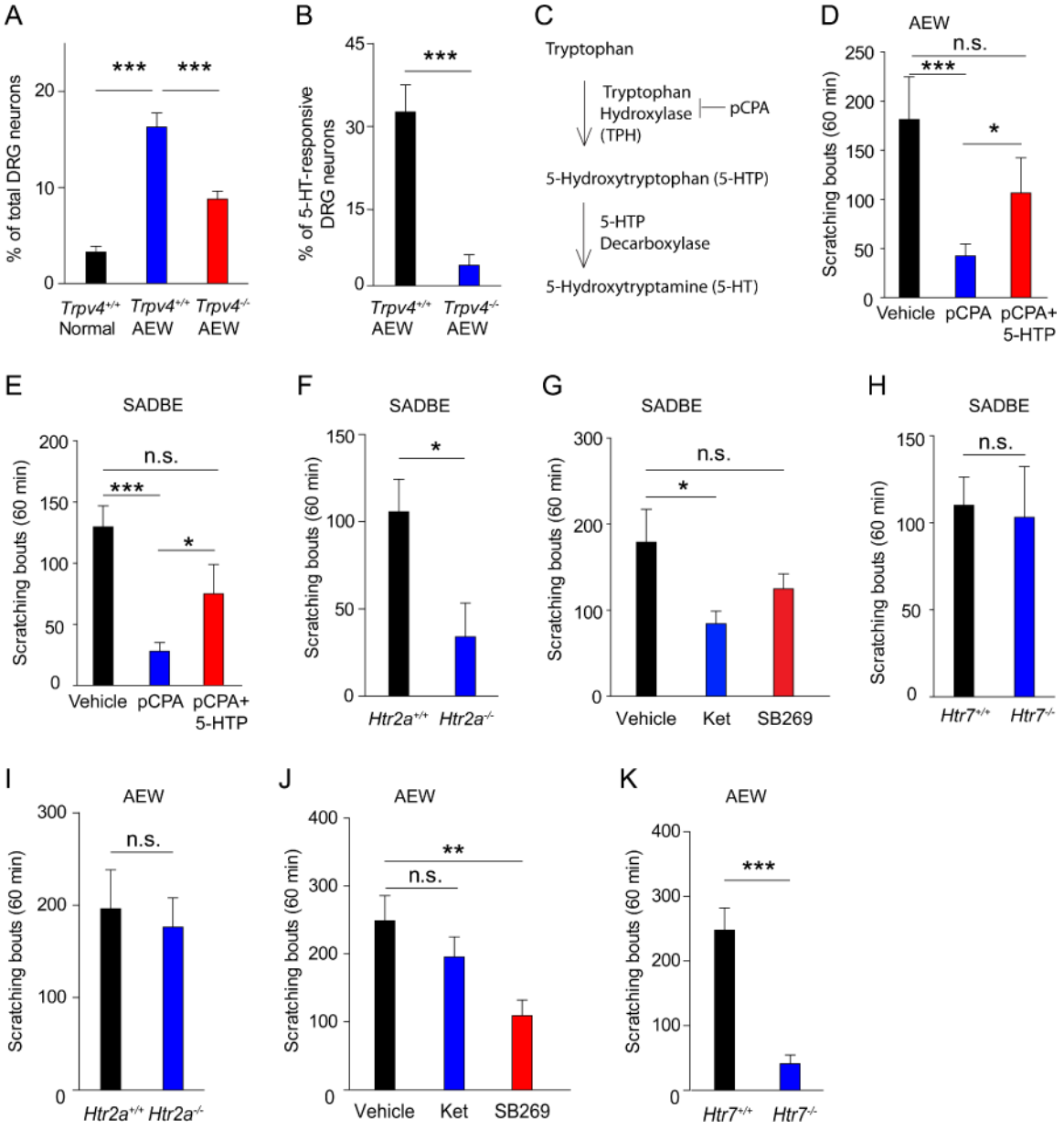

Figure 4.

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<0.001, ANOVA. **B**, Percentages of 5-HT-responsive DRG neurons responding to the AEW skin superfusates from *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. ***p<0.001, Student's *t* test. **C**, Schematic drawing of the 5-HT synthesis pathway. **D** and **E**, Spontaneous itching in AEW- (Fig 4, D) and SADBE- (Fig 4, E) treated mice with pCPA or pCPA+5-HTP. *p<0.05, ***p<0.001, ANOVA; n=8–10. **F**, Spontaneous scratching in the *Htr2a*$^{+/+}$ and *Htr2a*$^{-/-}$ mice after SADBE treatment. *p<0.05, Student's *t* test; n=5. **G**, Spontaneous scratching in mice treated with vehicle, Ketanserin, or Htr7 antagonist SB269970 (SB269) after SADBE treatment. *p<0.05, ANOVA; n=6. **H**, Spontaneous

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scratching in *Htr7*$^{+/+}$ and *Htr7*$^{-/-}$ mice after SADBE treatment. Student's *t* test; n=6. **I**, Spontaneous scratching in *Htr2a*$^{+/+}$ and *Htr2a*$^{-/-}$ mice after AEW treatment. Student's *t* test; n=9. **J**, Spontaneous scratching after AEW treatment in mice treated with vehicle, Htr7 antagonist SB269970 (SB269), or Ketanserin. **p<0.01, ANOVA; n=6–7. **K**, Spontaneous scratching after AEW treatment in the *Htr7*$^{+/+}$ and *Htr7*$^{-/-}$ mice. ***p<0.001, Student's *t* test; n=10–11. n.s. not significant versus vehicle group.

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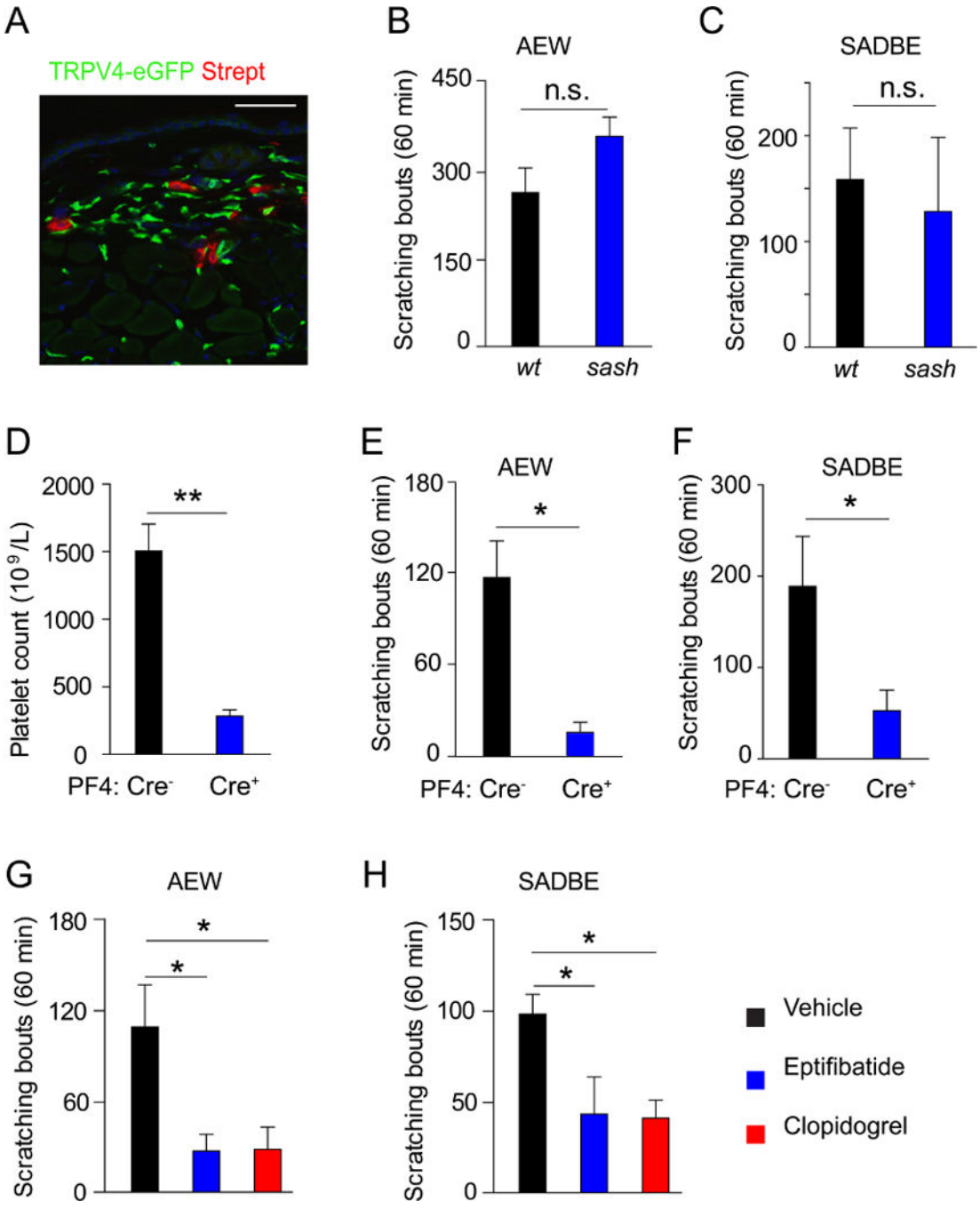

Figure 5.

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's *t* test; n=6 −7. n.s. not significant versus *wt* control group. **D**, Platelet count in *Pf4-Cre* mice and *Pf4-Cre*$^{+}$ mice 6 days after the DTX treatment. **p<0.01, Student's *t* test; n=7–8. **E** and **F**, Spontaneous scratching responses induced by AEW (Fig 5, E) or SADBE (Fig 5, F) in the

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Pf4-Cre^{-} mice (n=5 for AEW and SADBE) mice and the *Pf4-Cre*$^{+}$ mice (n=6 for AEW and SADBE). *p<0.05, Student's *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<0.05, ANOVA; n=6.