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TRPA1-Dependent Pruritus in IL-13–Induced Chronic Atopic Dermatitis

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Chronic debilitating pruritus is a cardinal feature of atopic dermatitis (AD). Little is known about the underlying mechanisms. Antihistamines lack efficacy in treating itch in AD, suggesting the existence of histamine-independent itch pathways in AD. Transient receptor potential ankyrin 1 (TRPA1) is essential in the signaling pathways that promote histamine-independent itch. In this study, we tested the hypothesis that TRPA1-dependent neural pathways play a key role in chronic itch in AD using an IL-13–transgenic mouse model of AD. In these mice, IL-13 causes chronic AD characterized by intensive chronic itch associated with markedly enhanced growth of dermal neuropeptide-secreting afferent nerve fibers and enhanced expression of TRPA1 in dermal sensory nerve fibers, their dorsal root ganglia, and mast cells. Inhibition of TRPA1 with a specific antagonist in these mice selectively attenuated itch-evoked scratching. Genetic deletion of mast cells in these mice led to significantly diminished itch-scratching behaviors and reduced TRPA1 expression in dermal neuropeptide containing afferents in the AD skin. Interestingly, IL-13 strongly stimulates TRPA1 expression, which is functional in calcium mobilization in mast cells. In accordance with these observations in the AD mice, TRPA1 expression was highly enhanced in the dermal afferent nerves, mast cells, and the epidermis in the lesional skin biopsies from patients with AD, but not in the skin from healthy subjects. These studies demonstrate a novel neural mechanism underlying chronic itch in AD and highlight the complex interactions among TRPA1$^+$ dermal afferent nerves and TRPA1$^+$ mast cells in a Th2-dominated inflammatory environment. ***The Journal of Immunology*****, 2013, 191: 5371–5382.**

Atopic dermatitis (AD) is characterized by acute flare-ups and chronic eczematous skin lesions associated with refractory chronic itch. The pathophysiology of chronic itch (pruritoceptive) in AD is diverse and involves a complex network of cutaneous and neuronal cells and mediators. Antihistamines are often ineffective in treating chronic itch in AD, pointing to the existence of distinct pruritogens and histamine-independent itch pathways (1, 2). This lack of understanding of the mechanisms underlying itch in AD represents a serious unmet medical need. Little is known about how dermal itch sensory nerves interact with dermal immune cells and keratinocytes in the initiation or aggravation of itch.

Itch is broadly characterized as either histamine dependent or independent, both of which are relayed by subsets of dermal itch-sensitive C-fiber–type nerves. Recent studies on the novel cation channel, the transient receptor potential ankyrin 1 (TRPA1) channel, have shown that TRPA1 functions in cells as a sensor for pain sensation, thermal sensitivity, and neurogenic inflammation. The most recent study shows in a chemical-induced mouse model of itch that TRPA1 is an essential component of the signaling pathways that promote Mas-related G-protein coupled receptor–dependent and histamine-independent itch (3). TRPA1 is activated by a series of by-products of oxidative/nitrative stress, produced under inflammatory conditions or in tissue damage, thus generating neurogenic inflammatory responses (4, 5). In addition, TRPA1 is activated downstream of G protein–coupled receptors, including the proalgesic bradykinin receptor (6, 7). Histamine, serotonin, chloroquine, and BAM8-22 all evoke itch by acting on G protein–coupled receptors (8–10). TRPA1 is the primary transduction channel mediating nonhistamine and endogenous pruritogen-evoked signaling and itch-scratching behaviors (3). Thus, TRPA1 could be a candidate downstream transduction channel onto which multiple histamine-independent itch pathways converge. Little is known about the mechanisms underlying nonhistaminergic itch in chronic inflammatory pruritic skin disease such as AD. Particularly, the role of TRPA1 in the pruritogenesis in AD has not been studied, despite the fact that the majority of chronic itch, such as that seen in AD, is mediated by nonhistaminergic mechanisms (1, 11, 12).

IL-13, a Th2 cytokine, is a critical mediator of human allergic disorders including asthma (13–15) and AD (16–20). We and others have shown that IL-13 plays a critical role in experimental models of asthma, allergic rhinitis (AR), and AD (21–29). IL-13 recently has been implicated in nerve repair in a transected rat spinal cord model (30). However, the role of IL-13 in pruritogenesis of AD has not been defined.

Mast cell–neuronal interactions are important in pruritic conditions (31, 32). Mast cells interact with neuronal cells through pruritogenic mediators such as tryptase, histamine, IL-31, neurotrophins, and neuropeptides, including vasoactive intestinal peptide, calcitonin gene-related peptide (CGRP), substance P (SP) and endothelin-1, adding novel regulatory pathways for the modulation of itch (33–35). Mast cell–derived TNF can promote

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Abbreviations used in this article: AD, atopic dermatitis; AR, allergic rhinitis; BMMC, bone marrow–derived mast cell; CGRP, calcitonin gene–related peptide; DRG, dorsal root ganglia; IHC, immunohistochemistry; MBP, major basic protein; SP, substance P; Tg, transgenic; TRPA1, transient receptor potential ankyrin 1.

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nerve fiber elongation in the skin during contact hypersensitivity in mice (31). Many of the mast cell mediators are involved in the elicitation of itch. Thus, mast cells are important in the cellular network of itch. Questions about how mast cells regulate the expression of TRPA1 and what role mast cell–related TRPA1 plays in itch in AD remain unanswered.

We hypothesized that chronic pruritus in IL-13–induced AD is mediated via a TRPA1-dependent neuronal inflammatory pathway. To test this hypothesis, we first characterized the expression of TRPA1 in the lesional skin from IL-13–induced AD and from patients with AD. We defined the relationships among the expression of TRPA1 in lesional AD skin correlating with itch-evoked scratching behavior, growth of dermal afferent sensory nerves, and the severity of AD in transgenic (Tg) mice in which skin-targeted IL-13 causes chronic pruritic AD, and demonstrated that blockage of TRPA1 with a specific inhibitor significantly attenuated the itch in mice with AD and diminished TRPA1 in dermal afferent nerves and dermal cells in mast cell–deficient AD mice. However, specific blockage of histamine receptor 1 did not. These studies demonstrated that IL-13 induces chronic pruritus via a novel TRPA1-dependent and histamine-independent pathway, and that expression of TRPA1 in AD skin (dermal sensory nerves, mast cells, and epidermis) is critical for the initiation and sustaining of chronic itch in AD.

Materials and Methods

Animals

The generation of K5-tTA-Tight-IL-13 mouse models of AD, the genotyping of these mice, and the activation of the IL-13 transgene were accomplished as we previously described (25, 27). Mast cell–deficient IL-13 Tg mice were generated by crossbreeding c-Kit–deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice (The Jackson Laboratory, Bar Harbor, ME) on C57BL/6 genetic background with K5-tTA mice and TRE-Tight-IL-13 mice. K5-tTA-IL-13 Tg (+) mice carrying null mutation of *c-Kit* (Tg[+]/$Kit^{W\text{-}sh/W\text{-}sh}$) and K5-tTA-IL-13 Tg(+) mice carrying wild type c-Kit (Tg[+]/*c*-$Kit^{+/+}$) were compared in the experiments. Skin mast cells in $Kit^{W\text{-}sh/W\text{-}sh}$ mice usually disappear by the age of 13 wk (36). To ensure that skin mast cells were absent in $Kit^{W\text{-}sh/W\text{-}sh}$ mice, the activation of IL-13 transgene in the skin was initiated at the age of 16 wk by withdrawing doxycycline from drinking water. Mast cells were almost absent (1 mast cell per 10 high power fields) using Toluidine blue staining (Supplemental Fig. 1). In all experiments, Tg(−) littermate controls received the same amount of Dox or no Dox for the same length of time. For the reversibility experiments, after development of AD, the Tg(+) mice were randomly assigned to receive Dox water to turn off the IL-13 transgene in vivo (the transgene On-Off group) or to receive normal drinking water to keep the IL-13 transgene on (the transgene On-On group). Both nonlesional skin samples and lesional AD score–matched, anatomical site–matched skin samples were used in the reversibility experiments (Fig. 5) and disease-kinetic experiments (Fig. 1G–I). Lesional AD skin samples were used for the rest of the experiments. Two different TRPA1-deficient mouse strains were obtained. One strain was from Dr. David Julius (University of California, San Francisco, San Francisco, CA, Source A) and the other was purchased from The Jackson Laboratory. Eight- to 12-wk-old mice were used for the experiments. The procedures involving animals in this study were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University and Yale University.

Histological analysis

Skin tissues from the same anatomical locations from each experimental group of mice were obtained and fixed with Shandon Glyo-Fixx (Thermo Fisher Scientific), embedded in paraffin, and stained with Toluidine blue (Sigma-Aldrich) to identify mast cells.

Clinical scores

After withdrawal of Dox from the drinking water to turn on the IL-13 transgene, Tg(+) mice were examined for skin lesions three times a week, and the clinical scores for disease severity were recorded as described previously with slight modifications (25, 37).

Itch-evoked scratch

As an indicator of pruritus, the scratching behavior of mice was determined as described previously (25, 38). The spontaneous itching behavior of the mice was videotaped 30 min each time, and counted for hind-limb and facial scratching as described previously with slight modification. The number of scratches was quantified by counting facial wiping and lifting of the hind limb directed toward the dermatitis area, regardless of the number of strokes (25, 38).

Fluorescent immunohistochemistry

Mouse skin tissues were fixed with Shandon Glyo-Fixx (Thermo Fisher Scientific), embedded in paraffin, and sectioned at 4-μm thickness. The spinal column was opened and six to eight neck and/or thoracic dorsal root ganglia (DRG) along the whole vertebral column per mouse were dissected. The DRG were collected, cryoprotected in 20% sucrose, frozen in Tissue-Tek O.C.T. (Sakura Finetek), cryosectioned, and mounted on slides. Fluorescent immunohistochemistry (IHC) was performed on deparaffinized mouse skin slides and on acetone-fixed frozen DRG sections. The slides were blocked with 10% donkey serum blocking solution (Sigma-Aldrich) for 1 h. After washing, tissue sections were incubated at 4˚C overnight with goat anti-PGP9.5 (sc-23852; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-GRPR (sc-26836; Santa Cruz Biotechnology), rabbit anti-CGRP (PC205L; Calbiochem), rabbit anti-TRPA1 (NB110-40763; Novus Biologicals), rat anti-CD3 (555273; BD Bioscience), goat anti-Langerin (sc-22620; Santa Cruz Biotechnology), rat anti–c-Kit (14-1172-81; eBioscience, San Diego, CA), rat anti-F4/80 (14-4821-82; eBioscience), rat anti–major basic protein (anti-MBP; a kind gift from Drs. Nancy and James J. Lee, Mayo Clinic, Scottsdale, AZ), and goat anti–mast cell tryptase (MMCP6; sc-32474; Santa Cruz Biotechnology). After rinsing, tissue sections were incubated with secondary Abs, Alexa Fluor 488– or 594–conjugated donkey anti-rat IgG, Alexa Fluor 488– or 594–conjugated donkey anti-goat IgG, and/or Alexa Fluor 488–conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA). Cell nuclei were detected by incubating the tissue sections with DAPI for 10 min. After washing, tissue sections were mounted using PermaFluor (Thermo-Fisher Scientific, Pittsburgh, PA) and examined using a fluorescence microscope.

Quantification of afferent nerves in the skin

The slides were evaluated using micrographs taken with a fluorescent microscope (Olympus BX-5; Olympus America, Center Valley, PA) equipped with a camera (Q-Imaging Retiga Exi; Biovision Technologies, Exton, PA); a micrograph field of view of the entire stained section was taken. Imaging software (iVision; Biovision Technologies) was used to analyze areas of positive staining in each digitized micrograph where simple bilevel thresholding was performed. The total area of the dermal nerve area was outlined by the observer and measured by the software. The software measurement of the positively stained nerve area within the dermal area was calculated as the percentage of the dermal area (positive nerve area divided by dermal area).

TRPA1 blockage and histamine blockage

The role of TRPA1 in AD was assessed by i.p. administration of a selective TRPA1 antagonist, HC-030031 (100 mg/kg; Sigma-Aldrich; 100 mM HC-030031 stock in DMSO and 0.2% Tween 80 in saline), or vehicle control to AD mice (five mice per group). The itch-scratching behavior was video-recorded for 30 min before and for 1 h after the TRPA1 inhibitor injection. Scratching numbers were counted as described earlier. Histamine receptors, H1, H3, and H4, are expressed in the skin, and H1 and H4 appear to be key components in the induction of itch, whereas H3 is an inhibitory receptor of itch induction. The H1 receptor is reported to account for >95% of the histamine receptors in the skin (38). Specific H1 receptor antagonist cetirizine (Pfizer) 15 mg/kg and vehicle (PBS) were administered i.p. to IL-13 Tg(+) mice with AD. The itching behavior was recorded for 30 min before injection. Immediately after administration of cetirizine or vehicle control, scratching behavior was videotaped for 30 min (four mice per group).

Measurement of cytokines in the skin

Protein samples were prepared as described previously (25). In brief, frozen skin tissues were placed in liquid nitrogen, pulverized with a chilled mortar and pestle into powders, and weighted. The skin protein was extracted by adding PBS with 0.25% Triton X-100 and stirring at 4˚C overnight. Debris was removed by centrifugation at 3000 × *g* for 15 min. Supernatants were stored in small aliquots at −80˚C until assayed. All samples were normalized to weight. The expression of IL-13, IL-4, and IFN-γ in the skin

samples were measured using ELISA kits according to the manufacturer's instruction (R&D Systems).

Bone marrow–derived mast cell culture and flow cytometry

Bone marrow–derived mast cells (BMMCs) were obtained by in vitro differentiation of bone marrow cells obtained from femur and tibia of WT mice cultured in RPMI 1640 media (Invitrogen) containing 5 ng/ml IL-3 (Peprotech, Rocky Hill, NJ) supplemented with 10% heat-inactivated FBS (Invitrogen), 100 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 10 μM MEM nonessential amino acids solution, L-glutamine, sodium pyruvate, HEPES buffer (Sigma-Aldrich), and antibiotics. By 4 wk in culture, the purity of BMMCs was >98% as determined by positive Toluidine blue staining and FACS staining of FcεRI and c-Kit (39). Mature BMMCs between 5 and 6 wk in culture, with viability >95% by the trypan blue exclusion assay, were used for experiments. Cells were starved with 0.2% heat-inactivated FBS overnight and then incubated with IL-4 (10 ng/ml), IL-13 (10 ng/ml), IFN-γ (10 ng/ml), or H_2O_2 (10 nM) for 24 h. Stimulated BMMCs were harvested and stained for TRPA1 using rabbit anti-TRPA1 Ab and Alexa Flour 488 donkey anti-rabbit IgG (H+L) Ab, and analyzed by fluorescent IHC and flow cytometry.

Functional imaging of intracellular calcium mobilization

The effect of TPRA1 agonist in intracellular calcium mobilization was studied in murine mast cell line (C57.1). Cultured C57.1 cells were harvested, suspended at 10^7 cells/ml in complete medium, incubated with 3.5 mM Calcium Sensor Dye eFluor 514 (eBioscience, San Diego, CA) for 30 min at 37°C in the dark, and washed and analyzed by flow cytometry as unstimulated. After measuring basal level for 1 min, cells were removed and TRPA1 agonist (100 μM, allyl isothiocyanate; Sigma-Aldrich) was added for 30 min and analyzed by flow cytometry. The maximum intracellular calcium mobilization in cells exposed to treatment was measured, and histogram overlays are displayed as percentage of Max.

Human skin biopsy samples

Deidentified skin biopsy samples from AD patients and healthy subjects were described previously (40). After explanation of the nature of research and obtaining informed consent from patients, 3- to 5-mm punch biopsies were taken from the forearms of healthy individuals (n = 4) and from lesional or nonlesional skin of AD patients (n = 3). Skin samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. The study was approved by the local ethics committee. Cryosectioned human skin samples were analyzed with immunofluorescence staining as described earlier. Acetone-fixed frozen sections were blocked with 10% donkey serum (Sigma-Aldrich) for 1 h and incubated with goat anti-PGP9.5 (sc-23852; Santa Cruz Biotechnology), rabbit anti-TRPA1 (NB110-40763; Novus Biologicals, Littleton, CO), or mouse anti-human mast cell tryptase (Chemicon International-Millipore, Billerica, MA) at 4°C overnight. The slides were rinsed and incubated with Alexa Fluor 594–conjugated donkey anti-goat IgG, Alexa Fluor 488–conjugated donkey anti-rabbit IgG, and/or Alexa Fluor 594–conjugated donkey anti-mouse IgG (Invitrogen). Cell nuclei were stained with DAPI for 10 min. Images were obtained using a fluorescence microscope.

Statistical analysis of the data

Student t test (two-tailed) or, for analysis of groups of data that were not normally distributed, Mann–Whitney U test (two-tailed), was used for statistical evaluation of the results. Differences between samples in comparison with $p < 0.05$ were considered significant. Unless otherwise specified, all data are presented as mean ± SEM.

Supplemental materials

The changes in mast cells in the skin of $Kit^{W\text{-}sh/W\text{-}sh}$ mice, TRPV1 expression in the skin, and the cytokine profiles in the skin of WT and IL-13 Tg(+) mice with or without c-Kit mutation, and TRPA1 expression in the wild type, IL-13 Tg(+), and TRPA1-deficient mice from two different sources are shown in the supplemental figures.

Results

Itch in IL-13–induced chronic AD correlated with the growth of CGRP$^+$, protein gene product (PGP9.5) afferent nerve, the number of cutaneous mast cells, and the severity of AD

We previously demonstrated that Tg expression of IL-13 causes chronic itch and AD in mice (25). To begin to understand the mechanisms in the pathogenesis of itch, we performed fluorescent IHC of PGP9.5 and CGRP with anti-PGP9.5 Ab and anti-CGRP Ab to determine whether IL-13 induces dermal afferent nerve growth. Lesional AD skin samples from the neck of Tg(+) mice shown in Fig. 1A, 1B, and 1D were taken after a 14-wk induction of the IL-13 transgene. As we showed previously (25), remarkable inflammatory infiltrates were present in the epidermis and dermis of IL-13 Tg(+) mice as compared with normal skin of Tg(–) mice (Fig. 1A), and strikingly high amounts of both PGP9.5 and CGRP-immunoreactive nerves were shown in the epidermis ramifying into the dermis with variable staining intensity in IL-13 Tg(+) mice as compared with the scarce presence of these markers in the skin of Tg(–) mice (Fig. 1B, 1C). These responses were associated with significantly increased c-Kit$^+$ mast cells (white arrows) in the Tg(+) AD skin (Fig. 1D, 1E). It is remarkable that itch-evoked scratching behavior in Tg(+) mice with AD correlated with the amount of both dermal CGRP$^+$ (Fig. 1F) and PGP9.5$^+$ sensory C fibers (Fig. 1G), with increased dermal c-Kit$^+$ mast cells (Fig. 1H), and with the severity of clinical AD (Fig. 1I). These findings suggest potential roles for the PGP9.5 and CGRP dermal afferent nerves and mast cells in mediating itch in IL-13–induced AD.

Enhanced expression of TRPA1 in PGP9.5$^+$ and CGRP$^+$ dermal afferents, in DRG and in mast cells in IL-13–induced AD skin

Although a role of TRPA1 has recently been implicated in chemical-induced acute itch, its role in chronic itch in AD has not been demonstrated. To understand the potential role of TRPA1 in neural pathogenesis of itch in AD, we first used fluorescent IHC to ascertain whether TRPA1 was expressed in lesional AD skin of Tg (+) mice as compared with that of Tg(−) mice. The expression of TRPA1 was markedly enhanced in the epidermal and dermal regions (Fig. 2A), and in the CGRP$^+$ and PGP9.5$^+$ afferents of lesional skin of Tg(+) mice; conversely, the expression of TRPA1 was almost absent in the skin of Tg(−) mice (Fig. 2B, 2C). To characterize cutaneous cell types that expressed TRPA1, fluorescent IHC was performed with cellular markers (MBP-eosinophils), (Langerins-Langerhans cells), (F4/80-macrophages), (CD3-T cells), and (c-Kit or mast cell–specific tryptase [MMCP6]-mast cells) for coexpression of TRPA1; the amount of coexpression of TRPA1$^+$/c-Kit$^+$ was significantly higher in AD skin of Tg(+) mice compared with Tg(−) skin (Fig. 2D). However, TRPA1 was minimally expressed or absent in eosinophils, Langerhans cells, macrophages, or T cells (Table I), suggesting that TRPA1 expression may be mast cell specific. The number of dual TRPA1$^+$, c-Kit$^+$ mast cells correlates with the number of itch-evoked scratch behaviors (Fig. 2E). Moreover, the mast cell–specific tryptase MMCP6$^+$ and/or c-Kit$^+$ cells were in close proximity to dermal PGP9.5 and CGRP$^+$ nerve fibers (Fig. 2F and data not shown), suggesting that mast cell–neuronal interactions may be important in itch in chronic AD. Interestingly, the vanilloid, TRPV1, colocalizing with the TRPA1 and important in histamine-induced itch, was not increased in the AD skin of Tg(+) mice (Supplemental Fig. 2).

TRPA1 is expressed in a subpopulation of DRG neurons, the cell bodies of cutaneous sensory neurons, and acts as a sensory receptor for oxidative mediators, hydrogen peroxide, produced endogenously during oxidative stress (5). However, TRPA1 expression in DRG neurons in chronic inflammatory skin diseases such as AD has not been well defined. We assessed the expression of TRPA1 in cervical DRG by IHC. TRPA1 expression was significantly increased in ∼35–40% of DRG from Tg(+) mice with AD as compared with 8.5% in Tg(−) mice (Fig. 2G, 2H). These studies show that IL-13 is a potent stimulator of TRPA1 expression in dermal afferents, mast cells of AD skin, and in the DRG neurons.

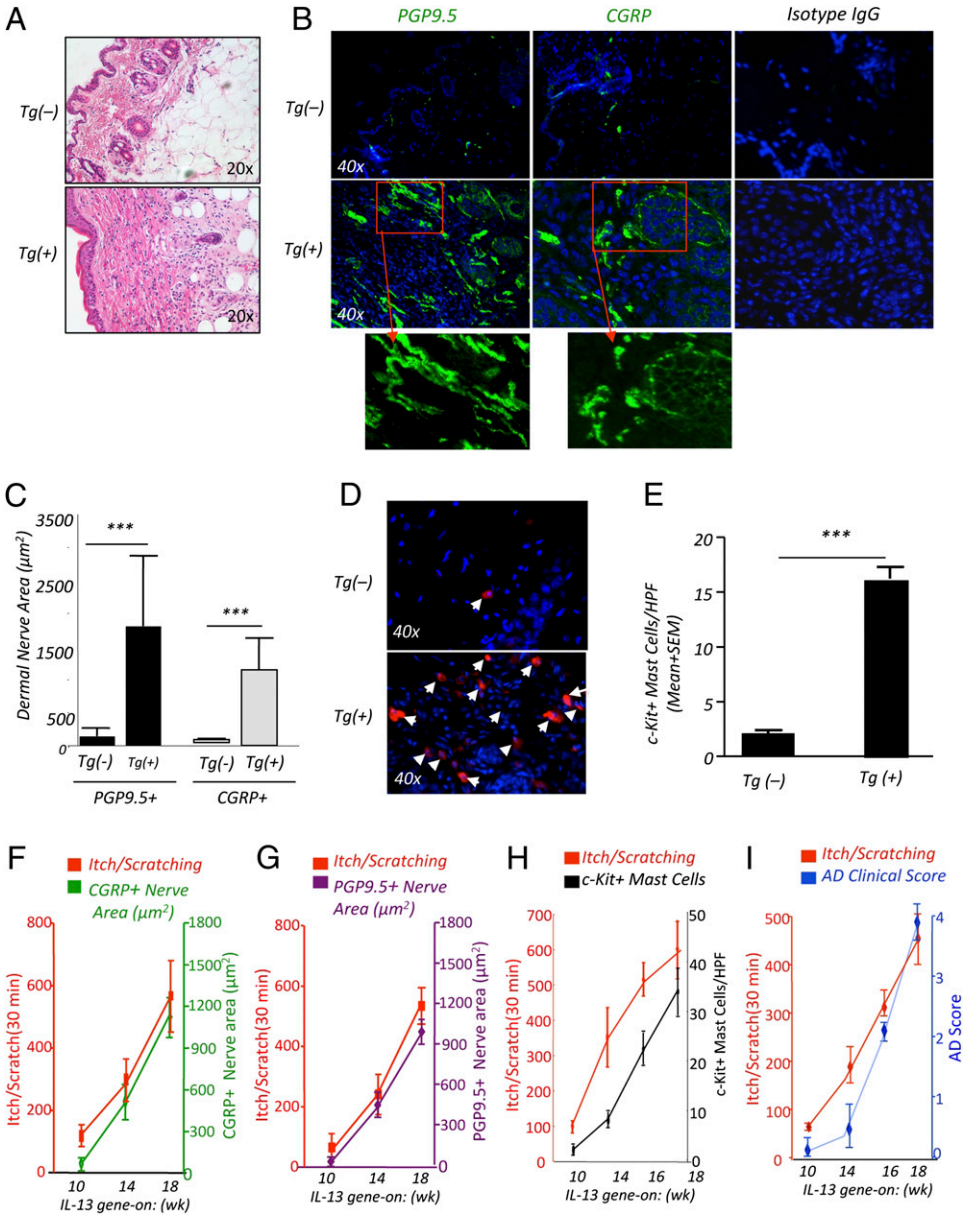

FIGURE 1. Itch-evoked scratching correlates with the amount of cutaneous sensory afferent nerves, the number of dermal mast cells, and the severity of the disease in chronic AD of K5-tTA-IL-13 mice. (**A**) Histology (H&E) of skin samples and (**B**) fluorescent IHC staining with rabbit anti-PGP9.5 IgG (PGP9.5, green), rabbit anti-CGRP IgG (CGRP, green), DAPI (nuclei, blue), or control IgG (rabbit IgG) in Tg(−) or Tg(+) skin from the neck of mice with chronic AD. (**C**) Quantification of changes in dermal PGP9.5$^+$ nerves (*left panel*) and CGRP$^+$ nerves (*right panel*). (**D**) IHC with rabbit anti–c-Kit (c-Kit, white arrows) for mast cells. (**E**) Quantification of c-Kit$^+$ mast cells per HPF in the skin (10 fields per skin sample per mouse; n = 4–5 each group). Correlation of itch-evoked scratching with the dermal nerve growth CGRP$^+$ (**F**), PGP9.5$^+$ (**G**), c-Kit$^+$ mast cells (**H**), and the severity of AD (**I**). (A, B, and D) The lesional AD samples from the neck of Tg(+) mice were used and the data are pooled from three independent experiments, which gave similar results (Tg[−]: n = 4; Tg[+]: n = 5). (C and E) ***$p < 0.001$ versus values from Tg(−) skin. Original magnification ×20 (A), ×40 (B, D).

To assess the specificity of TRPA1 Ab using fluorescent IHC, we collected skin samples from the neck of two different strains of TRPA1-deficient mice (see *Materials and Methods*) and compared them with skin samples of Tg(−) mice. As shown in Supplemental Fig. 4, increased TRPA$^+$ dermal cells were seen in AD skin of Tg(+) animals compared with much fewer TRPA1$^+$ cells in the dermis of wild type mice. In contrast, skin samples from TRPA1 knockout mice of both sources failed to show any TRPA1$^+$ cells.

Blockade of TRPA1 attenuated itch-evoked scratching in AD

To investigate the function of TRPA1 in itch in AD, we randomly assigned age- and AD clinic score–matched Tg(+) mice to receive either a TRPA1-specific antagonist (HC-030031, 100 mg/kg i.p.) or vehicle control. Behavior of itch-evoked scratching was video-recorded and counted blind to the treatment. The number of itch-evoked scratches was significantly attenuated starting 3 min after administration of HC-030031, and the inhibitory effect lasted for 1 h compared with unaltered itch scratching in mice given vehicle control (Fig. 3A). Conversely, behaviors of itch scratching in Tg(+) mice that were administered (i.p.) a specific histamine receptor 1 antagonist (cetirizine 15 mg/kg) were not significantly altered compared with mice that received control vehicle (PBS) (Fig. 3B). These findings showed that TRPA1 antagonist specifically blocked itch scratching, whereas histamine receptor 1 antagonist failed to do so, indicating that TRPA1 has a role in the pruritogenesis in AD, which is mediated through a histamine-independent pathway and, at least in part, a TRPA1-dependent pathway.

Mast cells are essential for itch and expression of TRPA1 in dermal afferent nerves and DRG

Dermal afferents can both be affected by and contribute to inflammatory responses including those in the skin (41, 42) and the respiratory tract (32). As noted earlier (Fig. 2), mast cells in AD lesions highly expressed TRPA1 and were in close vicinity to TRPA1$^+$ nerve fibers, suggesting a functional role of mast cells in AD. To ascertain the role of mast cells in itch and in regulation of TRPA1 in AD, we generated mast cell–deficient IL-13 Tg mice by crossing IL-13 Tg mice with mast cell–deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (both on C57BL/6 genetic background). As previously reported (36), in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, few cutaneous mast cells were detected in the skin at the age of 12 wk old. To ensure all cutaneous mast cells were absent in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, the IL-13 transgene was not

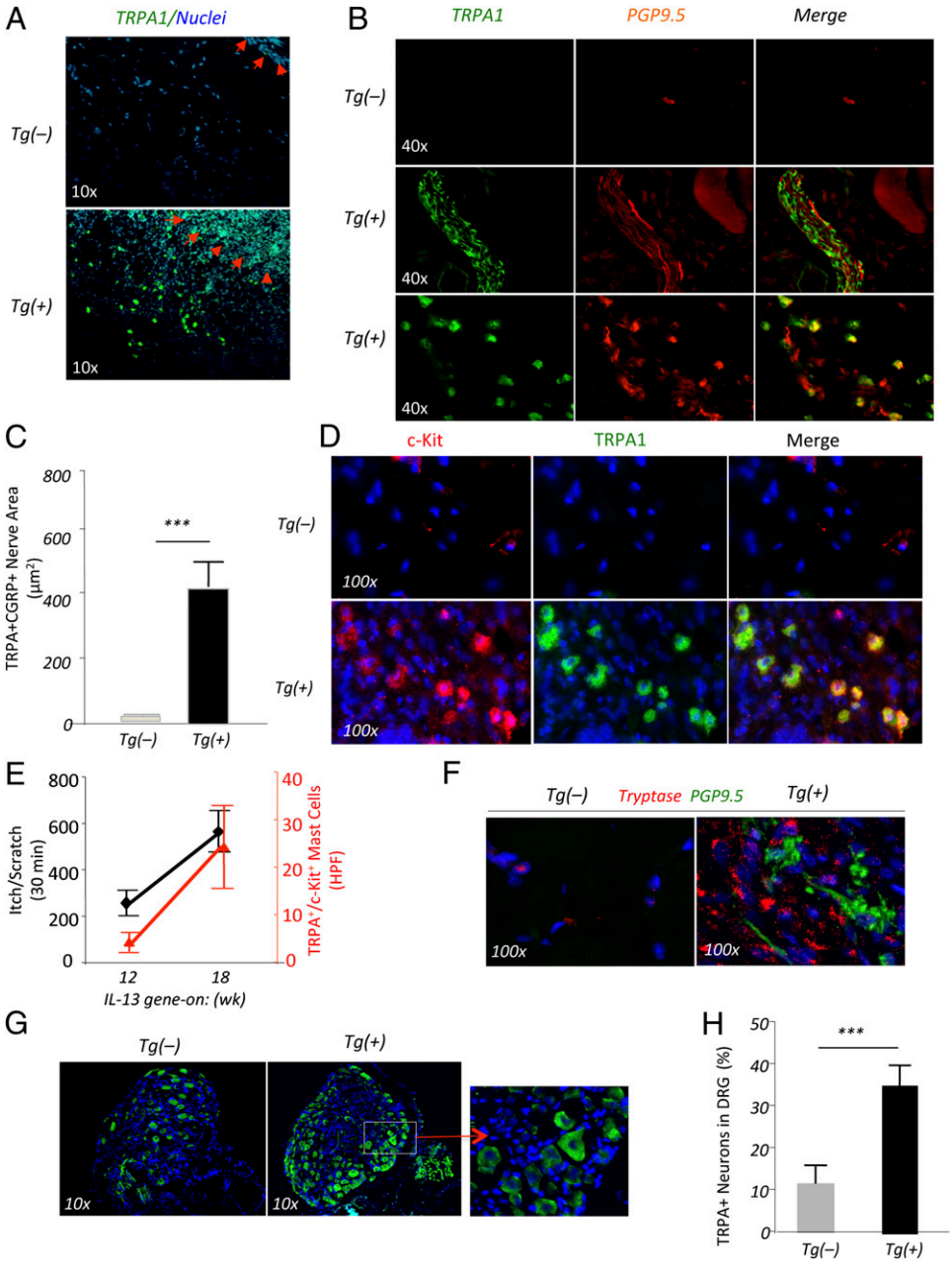

FIGURE 2. Enhanced expression of TRPA1 in the AD skin from K5-tTA-IL-13 mice. (**A**) Expression of TRPA1 in the epidermis and dermis of the lesional AD skin of the neck (arrows to the epidermis) was determined by fluorescent IHC with rabbit monoclonal anti-TRPA1 (TRPA1: green, DAPI for nuclei: blue; original magnification ×10). (**B**) Increased colocalization of TRPA1$^+$ (green)/CGRP$^+$ afferent nerves (red) in the AD skin, particularly in the dermis (original magnification ×40), and (**C**) quantification of TRPA1$^+$/CGRP$^+$ sensory nerves. (**D**) Enhanced coexpression of the dermal TRPA1$^+$ (green) on c-Kit$^+$ (red) mast cells in the AD skin (original magnification × 100), and (**E**) increased TRPA1$^+$/c-Kit$^+$ mast cells correlated with itch-evoked scratching. (**F**) MMCP6$^+$ mast cells (red) by fluorescent IHC with rabbit polyclonal anti-MMCP6 are in close proximity to CGRP$^+$ afferents (green), DAPI (blue for nuclei) in the dermal area of Tg(+) AD skin (original magnification ×100). (**G**) Fluorescent IHC for TRPA1 of cryosections of cervical sensory DRG isolated from Tg(−) and Tg(+) mice showing markedly augmented expression of TRPA1 (green; DAPI for nuclei: blue) in the sensory neurons (original magnification ×10). (G) Photos are representatives of DRG from individual mice. Tg(−) and Tg(+) mice: $n = 5$ for each group. (C and **H**) Data were pooled from three independent experiments with similar results (Tg[−] mice: $n = 5$, Tg[+] mice: $n = 8$). (C and H) ***$p <$ 0.001 versus corresponding values of Tg(−) skin. (A, B, D, F, and G) Results shown are from one of three experiments with identical findings.

activated in IL-13 Tg(+) mice with wild type c-Kit (Tg[+]/c-Kit$^{+/+}$) or mutant *Kit*$^{W\text{-}sh/W\text{-}sh}$ (Tg[+]/*Kit*$^{W\text{-}sh/W\text{-}sh}$) until the mice reached the age of 16 wk when mast cells in c-Kit mutant skin were undetectable (Supplemental Fig. 1). Compared with Tg(+)/c-Kit$^{+/+}$ mice that developed AD, Tg(+)/*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice exhibited a very slow-onset disease, and only 20% of the mice developed AD (Fig. 4A, 4B). Tg(+)/*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice displayed much milder dermatitis associated with significantly less itch scratching (Fig. 4C). Furthermore, TRPA1$^+$ cells in lesional AD skin of Tg(+)/*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were barely seen (Fig. 4D, 4F), and the expression of TRPA1 in the CGRP$^+$ dermal afferent nerves was significantly diminished (Fig. 4E, 4G). Consistently, the expression of TRPA1 in the cervical neurons of the DRG of Tg(+)/*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was markedly reduced compared with Tg(+)/*c-Kit*$^{+/+}$ mice (Fig. 4H, 4I). These findings suggest that mast cells are important for the expression of TRPA1 on the dermal CGRP$^+$ afferents and for the induction of itch in AD. Importantly, the level of IL-13 in the skin samples from Tg(+) mice was not significantly altered in mast cell–deficient IL-13 Tg(+) mice (Supplemental Fig. 3A), indicating that genetic deletion of mast cells in Tg(+) mice did not inhibit the IL-13 transgene expression.

Inactivation of IL-13 in vivo led to diminished growth of TRPA1$^+$/PGP9.5$^+$ afferent nerves

We then evaluated whether IL-13–upregulated expression of TRPA1 in AD could be reversed after the IL-13 transgene was turned off in vivo. IL-13 Tg(+) mice with AD were randomly assigned to receive either Dox water to inhibit the IL-13 transgene (IL-13 Tg[+] On-Off group) or normal water to keep the transgene on (IL-13 Tg[+] On-On group) for 4 wk. Expression of TRPA1 and itch-evoked scratching behavior were assessed and quantified. As shown in Fig. 5A and 5B, the mice that continued to receive normal water (the transgene On-On group) developed strikingly intense itch-scratch and enhanced growth of TRPA1$^+$/PGP9.5$^+$ nerve fibers in AD skin. In contrast, the expression of TRPA1$^+$/PGP9.5$^+$ in AD lesions and the number of itch-evoked scratching in the Tg(+) mice given Dox water to turn off the IL-13 transgene

Table I. Enhanced expression of TRPA1 in c-Kit$^+$ mast cells in AD

Skin Cells/ TRPA1$^+$ (HPF)	Tg(−) (mean ± SEM)	Tg(+) (mean ± SEM)	*p*
CD3$^+$/TRPA1$^+$	3.4 ± 0.21/0	14.5 ± 2.1/0	NS
MBP$^+$/TRPA1$^+$	0.5 ± 0.03/0	9.4 ± 1.3/0	NS
Langerin$^+$/TRPA1$^+$	3.7 ± 1.45/0	7.8 ± 0.99/0	NS
F4/80$^+$/TRPA1$^+$	5.4 ± 2.1/0	12 ± 1.1/0	NS
c-Kit$^+$/TRPA1$^+$	10 ± 1.77/1.6 ± 0.4	37 ± 3.9/36 ± 4.1	0.0012

Skin samples from Tg(−) and IL-13 Tg(+) mice were fluorescent IHC stained for TRPA1 and with specific Abs to CD3 (T cells), MBP (eosinophils), F4/80 (activated macrophages), Langerin (Langerhans cells), and c-Kit (mast cells). The data are pooled from two independent experiments with similar findings. Three slides from each animal were stained. Eight to 10 HPFs per slide were blindly counted: Tg(−) mice, $n = 4$, Tg(+), $n = 6$.

(the transgene On-Off group) were markedly diminished (Fig. 5A, 5B). These findings suggest that pruritus in AD and the expression of TRPA1 are mainly dependent on IL-13.

Cytokine regulation of TRPA1 expression in cultured mast cells

To understand how TRPA1 is regulated by inflammatory cytokines important in AD, we obtained BMMCs from wild type C57BL/6 mice by culturing the cells in IL-3–containing medium for 6 wk, at which time >98% of the cells are identified as mature mast cells by FACS for c-Kit/FcεRI (39). The BMMCs were stimulated for 24 h with various cytokines and H_2O_2, reactive oxygen species known to stimulate TRPA1. FACS analysis showed that the expression of TRPA1 was increased when stimulated by H_2O_2, IL-13, or IFN-γ. Among these, IL-13 showed the strongest stimulation. Interestingly, IL-4 downregulated the expression of TRPA1 (Fig. 6A). By fluorescent IHC, consistent with the FACS results, BMMCs stimulated with IL-13 expressed significantly higher TRPA1 (14.7%) compared with H_2O_2 (4.1%) and IFN-γ (7%)–stimulated cells, but IL-4–stimulated cells failed to do so (Fig. 6B, 6C), despite the observation that the IL-4 levels in the AD skin were increased (Supplemental Fig. 3). Taken together, these data demonstrated that IL-13 is a potent stimulator of TRPA1 expression in cultured mast cells, whereas IL-4 did not stimulate TRPA1 expression. Th1 cytokine IFN-γ modestly upregulates the expression of TRPA1 in mast cells.

Mast cells express functional TRPA1 channel

It has been shown that neuronal sensitivity to allyl isothiocyanate (mustard oil) correlates with TRPA1 expression in vivo and TRPA1 can be activated by increases in intracellular calcium (6). Cultured mast cells (murine mast cell line C57.1) were loaded with Calcium Sensor Dye eFluor 514 for 30 min at 37°C in the dark to determine whether TRPA1 has a functional role in mast cells. The cells were then washed and analyzed by flow cytometry as unstimulated (Fig. 6D). The cells were then washed and stimulated with TRPA1 agonist, allyl isothiocyanate (100 μM, blue histogram; Fig. 6E). The cells stimulated with allyl isothiocyanate showed very strong calcium signals indicative of marked intracellular calcium mobilization. These data indicate that TRPA1 plays a functional role in mast cells in response to allyl isothiocyanate stimulation.

Markedly enhanced expression of TRPA1 in dermal afferent nerves and in mast cells in human AD skin

Although several neuropeptides have been implicated in the pathogenesis of human AD, expression of TRPA1 in human AD has not been characterized. We compared skin biopsy samples from chronic AD lesions of the forearms of AD patients and from the forearms of healthy subjects for TRPA1 and PGP9.5 expression by fluorescent IHC. We found that in lesional AD skin, both cutaneous PGP9.5$^+$ and TRPA1$^+$/PGP9.5$^+$ double-positive afferent nerves were highly increased (Fig. 7A–C). In addition, TRPA1 was markedly expressed in the dermal cells and keratinocytes in the lesional skin of AD patients, whereas minimal expression of TRPA1 was noted in the skin of healthy subjects (Fig. 7D). Furthermore, consistent with findings in the Tg(+) AD mice (Fig. 2D), we found that tryptase$^+$ mast cells were markedly increased in the AD skin (Fig. 7E) and that dermal TRPA1$^+$ cells were colocalized with tryptase$^+$ cells, indicating that TRPA1 is expressed in mast cells, and the number of tryptase$^+$/TRPA1$^+$ cells was significantly increased and they were in very close proximity to dermal PGP9.5$^+$ afferents (Fig. 7F, 7G, inset), suggesting a spatial relationship between mast cells and the afferent nerves in human skin lesions in AD. Taken together, these findings in human AD, consistent with those in the AD mouse models, suggest that augmented expression of TRPA1 and increased TRPA1$^+$ mast cells may be important in the generation of itch in AD.

Discussion

Chronic itch, a hallmark of human AD, represents a huge burdensome clinical problem (43). There is no effective treatment for

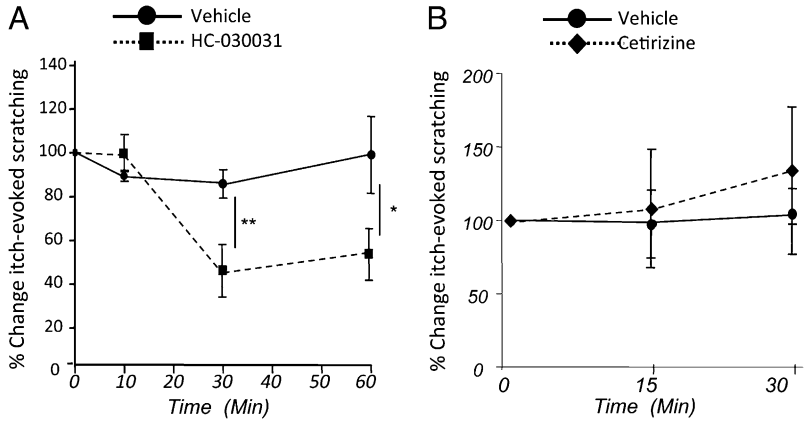

FIGURE 3. Blockade of TRPA1 attenuated itch-evoked scratching. Age- and AD score–matched IL-13 Tg(+) mice were administered with TRPA1-specific antagonist HC-030031 (100 mg/kg i.p., one dose) or vehicle control. Itch-evoked scratching activities were video-recorded, counted, and compared between the two groups. (**A**) HC-030031 significantly inhibited itch-scratching behaviors in Tg(+) mice with AD. (**B**) Histamine receptor blocker (cetirizine 15 mg/kg i.p.) failed to reduce itch-induced scratching. (A and B) Data were pooled from two independent experiments with similar results. *$p < 0.05$, **$p < 0.01$; $n = 5$ for each group.

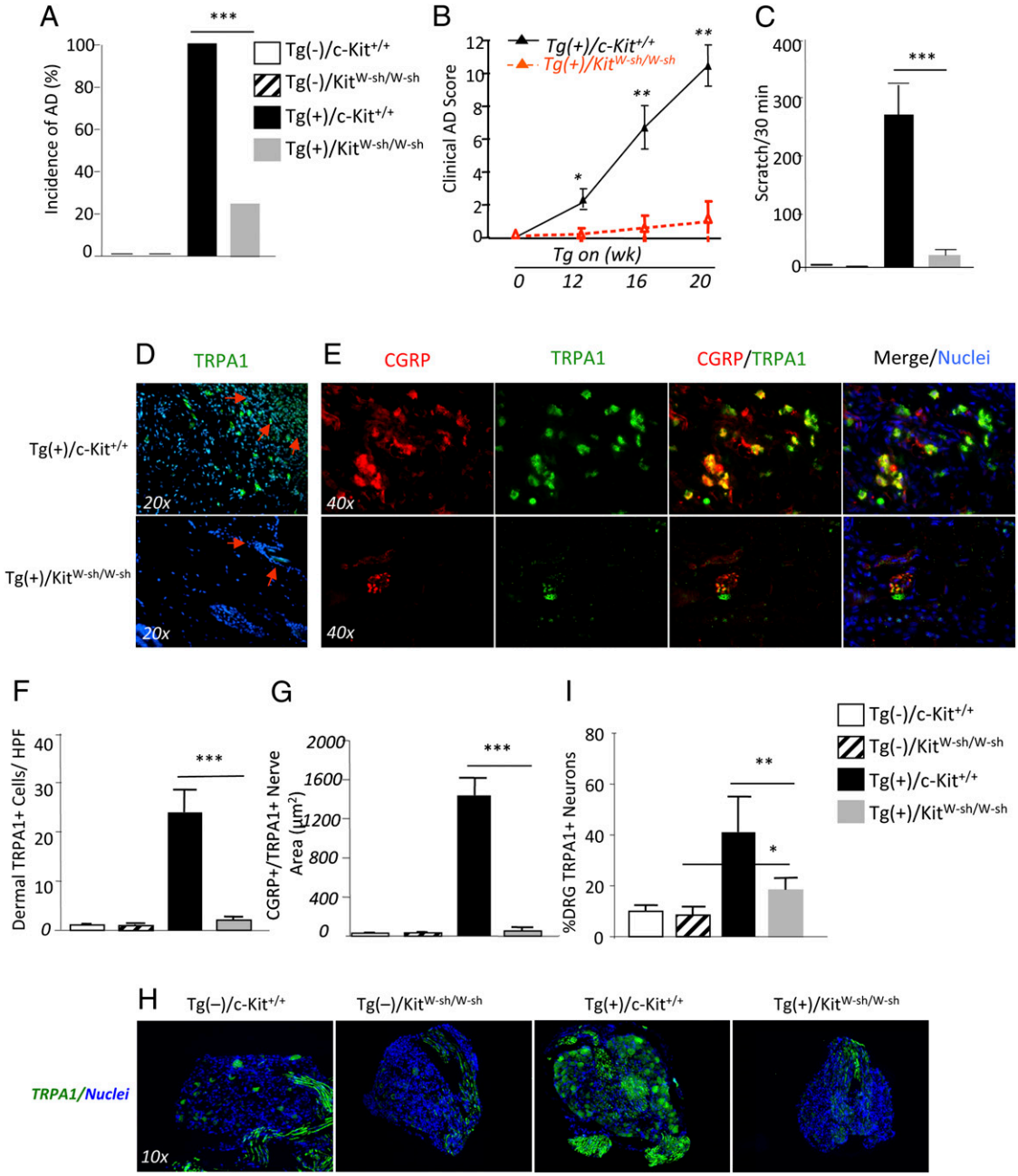

FIGURE 4. Role of mast cells in itch-evoked scratching and TRPA1 expression in the epidermis, dermal cells, and dermal sensory afferents and sensory neurons. (**A** and **B**) Mast cell–deficient IL-13 Tg(+) mice exhibited slow-onset, low-incidence, and less severe AD and (**C**) markedly reduced itch-evoked scratching. Skin samples from the upper portion of the back and sensory neurons from DRG collected from cervical region of IL-13 Tg(+) mice on c-$Kit^{+/+}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ genetic background were fluorescent IHC-stained for TRPA1 (green), neuropeptide-releasing nerves (CGRP, red), and DAPI (blue for nuclei). (**D**) Attenuation and (**F**) quantification of $TRPA1^+$ cells in the epidermis and dermis (original magnification ×20). (**E** and **G**) Diminished dermal coexpression of $TRPA1^+/CGRP^+$ afferent sensory nerves (original magnification ×40). (**H** and **I**) Harvested cervical ganglia (6–8 ganglia/mouse) from Tg(−)/c-$Kit^{+/+}$, Tg(−)/$Kit^{W\text{-}sh/W\text{-}sh}$, Tg(+)/c-$Kit^{+/+}$, and Tg(+)/$Kit^{W\text{-}sh/W\text{-}sh}$ mice were stained for TRPA1 (green), showing reduction of TRPA1 expression in the sensory neurons of DRG from Tg(+)/$Kit^{W\text{-}sh/W\text{-}sh}$ mice as compared with Tg(+)/c-$Kit^{+/+}$ mice (original magnification ×10). Tg(+)/c-$Kit^{+/+}$ mice: $n = 5$, Tg(+)/$Kit^{W\text{-}sh/W\text{-}sh}$ mice: $n = 5$, Tg(−) groups $n = 4$ for each. (A–C) Data are pooled from five independent experiments with similar results. Tg(−) mice: $n = 12$, Tg(+) mice: $n = 18$. (D, E, and H) Each is representative of three independent experiments. Tg(+)/c-$Kit^{+/+}$ mice: $n = 8$, Tg(+)/$Kit^{W\text{-}sh/W\text{-}sh}$ mice: $n = 9$. (F, G, and I) Data are pooled from 3 experiments, $n = 4$–7 for each group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

itch in patients with AD because of a lack of understanding of the mechanisms underlying chronic pruritus. Th2 cytokine IL-13 is a critical mediator in human atopic disorders including asthma and AR. It has a major role in the pathogenesis of both innate and adaptive immunity of AD. This study was designed to enhance our understanding of the mechanisms of pruritogenesis in AD induced by Th2 cytokine IL-13 in a skin-selective Tg model of chronic AD and to begin to characterize the relevance of neuronal abnormality to itch in human AD. Our studies demonstrated that IL-13 is a potent stimulator of pruritogenesis in AD, and that the pruritogenic effects of IL-13 are mediated, in part, by a novel TRPA1-dependent mechanism. They also demonstrated that dermal expression of TRPA1 is critical in mediating itch in IL-13–induced AD, and that IL-13 stimulates expression of TRPA1 in mast cells in vivo and in vitro. Importantly, we found that TRPA1 was highly expressed in the dermal cells, sensory afferents, and keratinocytes

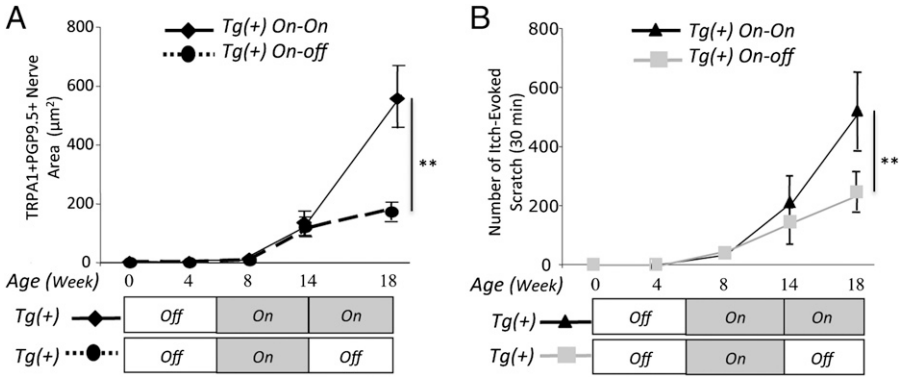

FIGURE 5. Inactivation of the IL-13 transgene in Tg(+) mice with AD over time led to diminished TRPA1 in lesional AD skin associated with diminished itch-induced scratching. After withdrawal of Dox in the drinking water (the transgene was turned on) for 8 wk, IL-13 was induced and Tg(+) mice began to develop AD (clinical score: 2). These mice were then randomly assigned to receive either Dox to inactivate the IL-13 transgene (IL-13 Tg[+] On-Off group) or normal water to keep the transgene on (IL-13 Tg[+] On-On group). Mice were sacrificed, and skin samples were obtained at the age of 18 wk. (**A**) Significantly attenuated TRPA1$^+$/PGP9.5$^+$ afferent nerves in AD skin after the IL-13 transgene was turned off in the Tg(+) On-Off group (dotted line) compared with the On-On group (solid line) in which the disease continued to worsen. (**B**) The number of itch-evoked scratching behaviors was diminished in the Tg(+) On-Off group (gray line) compared with the Tg(+) On-On group (black line; $n = 5$ for each group). $^{**}p = 0.01$.

and was coexpressed in tryptase$^+$ mast cells in lesional skin biopsies from patients with AD. To our knowledge, the findings represent the first evidence showing a novel TRPA1-dependent pathway in IL-13–induced itch in AD.

Antihistamine therapy is often ineffective in treating itch in AD, suggesting that mediators other than histamine such as cytokines and neuropeptides may be involved. Many mediators such as IL-31, SP, and nerve growth factor have been shown to have a pruritogenic role in AD (44, 45). We generated and used an externally inducible skin-specific IL-13 Tg mouse model (K5-tTA-IL-13), because it recapitulates important aspects of human AD, including chronic pruritic dermatitis and systemic Th2 immunity (25–27). In these mice, we found that the number of mast cells and PGP9.5$^+$ and neuropeptide-secreting CGRP$^+$ dermal afferent nerves were significantly increased in the AD skin, and that these correlated with chronic itch-scratching behaviors. We showed strongly increased PGP9.5$^+$ and CGRP$^+$ dermal afferents in the skin of IL-13 Tg(+) mice compared with Tg(−) mice (Fig. 1B), although the intensity of immunofluorescent staining for both dermal PGP9.5 and CGRP nerve fibers was not as intense as seen in other studies where knock-in GFP was used as a tracer or colchicine was used to enhance CGRP accumulation before detection (46–48).

Recent studies showed that TRPA1 is an essential component of the signaling pathways that promote histamine-independent itch and is also a downstream transduction channel onto which multiple histamine-independent itch pathways converge (3). However, the role of TRPA1 in chronic itch associated with pathological conditions, such as AD, has yet to be determined. In this study, we observed a markedly enhanced expression of TRPA1 in dermal c-Kit$^+$ mast cells and in dermal CGRP$^+$ afferents in the lesional AD skin, which were associated with increased numbers of TRPA1$^+$ sensory neurons in the DRG and enhanced dermal levels of TRPA1. As a point of significance, these changes correlated with itch-induced scratching, highlighting the importance of TRPA1 in IL-13–induced chronic itch. Furthermore, when TRPA1 was blocked by a specific antagonist in mice with AD, itch-induced scratching was significantly attenuated; by contrast, nonsedating H1 histamine receptor blocker failed to inhibit itch in these mice, indicating that the chronic itch in AD induced by IL-13 is, at least in part, through the TRPA1 pathway and not through the histamine-dependent pathway. It is possible that the effective blocking of itch-scratch by TRPA1 antagonist is through blocking itch signal transmission. Nevertheless, this indicates a contributing role for TRPA1 in the generation of itch in AD. It has been shown that TRPA1 is coexpressed in ~50% of TRPV1$^+$ sensory neurons, which are known to transduce histamine-induced itch (49–53). In contrast with enhanced TRPA1 expression in the AD skin, TRPV1 was found minimally expressed in the AD skin, and TRPV1 expression was comparable between WT skin and AD skin (Supplemental Fig. 2), suggesting that TRPV1 may not be important in itch in AD induced by IL-13, despite previous findings that the level of histamine in AD lesions was elevated (25).

In chronic inflammatory skin such as AD, stimulated nerve fibers may activate local mast cells, which, in turn, can regulate local nerve functions (54, 55). In this study, we demonstrated that in both lesional skin from mice with AD and lesional skin from AD patients, not only was the expression of TRPA1 markedly enhanced and the number of TRPA1$^+$ mast cells significantly increased, but also these TRPA1$^+$ mast cells were in close proximity to afferent fibers. Moreover, the number of TRPA1$^+$ mast cells was correlated with itch-evoked scratching in mice with AD. These findings suggest that in the chronic inflammatory lesional skin, such as in AD, their close anatomical association may represent functionally important interactions between dermal TRPA1$^+$ mast cells and TRPA1$^+$ afferent nerves that could biologically be significant in the induction and maintenance of chronic itch in AD. For example, mast cells are found in close proximity to sensory nerves in inflammatory skin lesions from patients with psoriasis (56), AD (57), and contact dermatitis (31). Allergen-induced activation of tissue mast cells is associated with alterations in the phenotype or physiology of nearby primary afferent (sensory) nerves (57–61).

Interestingly, when mast cells were genetically deleted from IL-13 Tg mice, the onset of itch-evoked scratching behavior was markedly delayed and attenuated, and the expression of TRPA1 in the dermal nerves and epidermal and dermal cells in these mice were significantly diminished, indicating that mast cells are essential for the expression of TRPA1 in dermal sensory nerves and epidermal cells in the AD skin, an important but previously unrecognized role in the pathogenesis and pruritogenesis of AD induced by IL-13. In chronic inflammatory skin, stimulated sensory nerves can activate local mast cells, which, in turn, can influence local afferent nerve function. Indeed, other studies showed that neuropeptides released by cutaneous nerves, such as SP, vasoactive intestinal peptide, and somatostatin, can induce mast cells to release inflammatory mediators, such as histamine, TNF, and other inflammatory mediators (62–67). Afferent nerves express specific

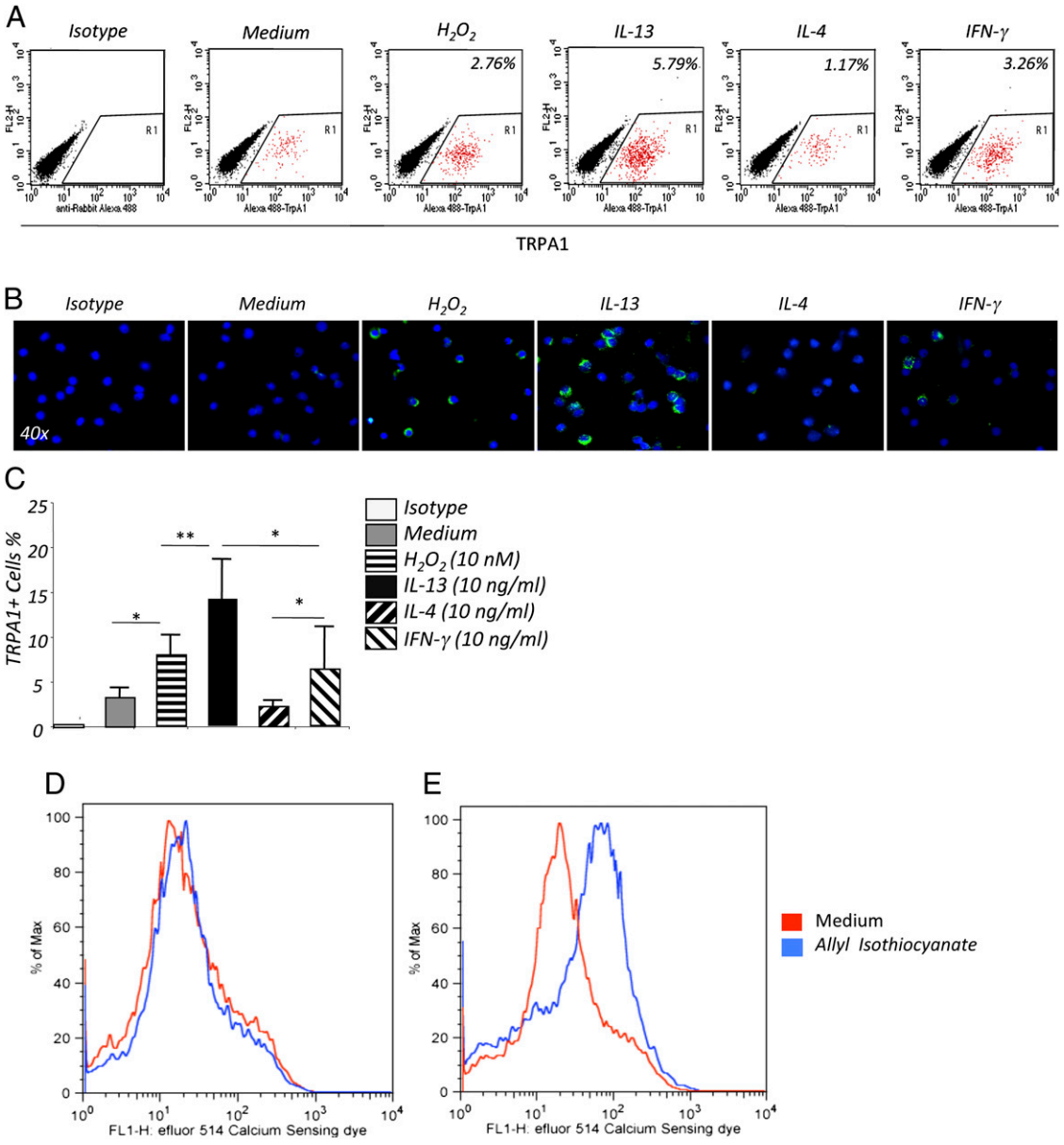

FIGURE 6. Cytokine regulation of TRPA1 expression in mast cells and functional calcium imaging of mast cells stimulated with TRPA1 agonist. BMMCs were obtained by culturing bone marrow cells of wild type C57BL/6 mice in IL-3–containing medium for 4–6 wk, when 98% of the cells were identified as mature mast cells by Toluidine blue staining and by flow cytometry for c-Kit and FcεRI as previously described (39). (**A** and **B**) BMMCs were stimulated for 24 h with H_2O_2 (10 nM), IL-13 (10 ng/ml), IL-4 (10 ng/ml), or IFN-γ (10 ng/ml). (A) Cultured BMMCs were stained for TRPA1 using rabbit anti-TRPA1 Ab and Alexa Flour 488 dye–labeled donkey anti-rabbit IgG (H+L), and analyzed by flow cytometry. (B and **C**) Detection and quantification of $TRPA1^+$ mast cells (green) by fluorescent IHC. Data represent four independent experiments. $*p < 0.05$, $**p < 0.01$ versus medium. Cultured mast cells (C57.1 cell line) were harvested, washed, and loaded with Calcium Sensor Dye eFluor 514 for 30 min at 37˚C, and the cells were washed and analyzed by flow cytometry as unstimulated (**D**) and (**E**) stimulated with media (red) and with 100 μM allyl isothiocyanate (blue) for 30 min. The maximum intracellular calcium mobilization in cells exposed to the agonist was measured, and histogram overlays are displayed as percentage of Max. Shown are representative of three independent experiments.

receptors for neuropeptides (68), PGs (69), histamine (70), proteases (71), neurotrophins (72), and cytokines (73, 74). Levels of IL-13 in the skin samples from Tg(+)/*c-Kit*$^{+/+}$ and Tg(+)/*Kit*$^{W\text{-}s/W\text{-}sh}$ were comparable, indicating that mast cell deficiency in K5-tTA-IL-13 mice did not affect the expression of the IL-13 transgene, and that changes in diminished TRPA1 expression and reduced AD severity in mast cell–deficient Tg(+) mice were not caused by inhibition of the IL-13 transgene (Supplemental Fig. 3A). Our study suggests that mast cell deficiency in IL-13 Tg(+) mice leads to interruption of this positive feedback loop of neuroinflammation-itch, which may be TRPA1 dependent.

TRPA1 was reported to be expressed in different mammalian tissues, including brain, intestine and pancreas (75, 76), human skin keratinocytes (77, 78), and vestibular and auditory sensory epithelia (77, 79). However, functional activity of TRPA1 channels is most consistently characterized in sensory neurons and other cells with sensory functions. Functional TRPA1 has been reported in synoviocytes of joints (80) and endothelial cells (81). Our studies showed that IL-13, besides its immunomodulatory roles in both innate and adaptive immunity and in compromising the dermal barrier immune defense against microbial infections in AD (20, 82–84), has a novel role in the pruritogenesis of AD.

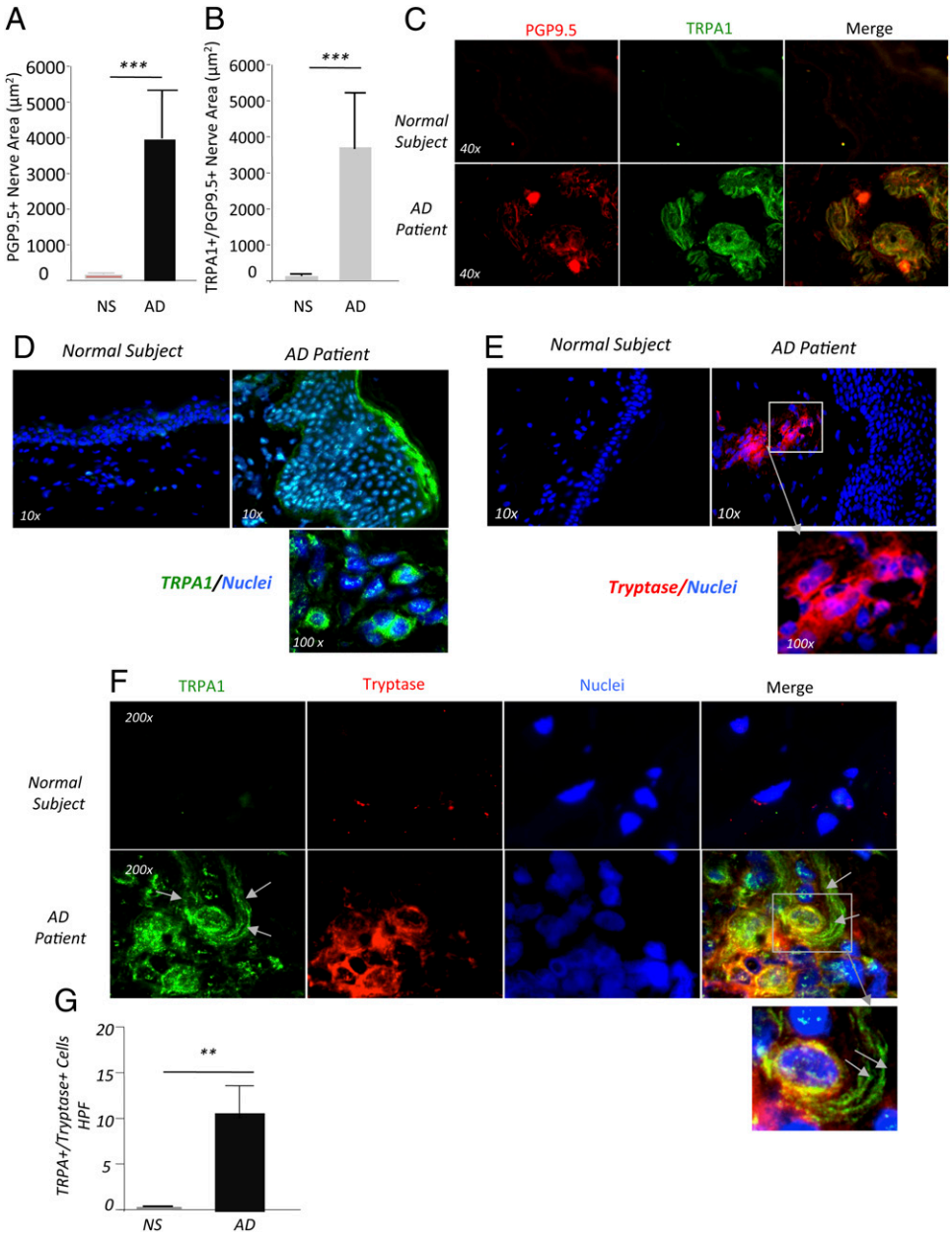

FIGURE 7. Increased dermal sensory nerve growth, TRPA1$^+$ sensory afferent nerves, TRPA1$^+$ epidermal and dermal cells, and TRPA1$^+$/Tryptase$^+$ mast cells in chronic lesional skin biopsies from patients with AD. Fluorescent IHC with rabbit monoclonal anti-TRPA1, anti-PGP9.5, and anti-human Tryptase was performed in skin biopsy samples from normal subjects (NS) and AD patients. The comparison was made between the skin biopsy samples from the forearms of NS and chronic lesional AD biopsy samples from the forearms of AD patients. (**A**) Quantification of dermal PGP9.5$^+$ afferent nerves, and (**B**) TRPA1$^+$ afferent nerves, and (**C**) markedly increased TRPA$^+$ (green)/PGP9.5$^+$ (red) afferent nerves in AD skin. (**D**) Expression of TRPA1 (green), DAPI (blue) for nuclei, in the epidermis (keratinocytes) and dermal cells. (**E**) Increased Tryptase$^+$ (red) mast cells and (**F**) coexpression of TRPA1$^+$ (green) and Tryptase$^+$ (red) mast cells and these TRPA1$^+$/Tryptase$^+$ mast cells in proximity with TRPA1$^+$ afferent nerves (arrows). (**G**) Quantification of TRPA1$^+$/Tryptase$^+$ mast cells. (C–F) Each is a representative of three to four different sections of the skin from each patient. (G) Cells in 10–12 high-power fields per slide were counted (NS: $n = 4$, AD patients: $n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$.

After the IL-13 transgene in vivo was turned off in Tg(+) mice that developed AD, the growth of TRPA1$^+$/PGP9.5$^+$ afferents and the expression of TRPA1$^+$ cells in lesional AD skin were significantly diminished. Our in vitro data on cytokine regulation of TRPA1 expression in cultured mast cells demonstrated that IL-13 is a potent stimulator of TRPA1 expression in mast cells. IFN-γ modestly increased TRPA1 expression in mast cells. Interestingly, IL-4, another Th2 cytokine, failed to upregulate TRPA1 expression. Although Th2 cytokines IL-13 and IL-4, using the same IL-4R type II (heterodimer of IL-4Rα and IL-13Rα1), share many biological activities, there is considerable evidence that IL-13 has some unique functions in vivo not seen with IL-4. IL-13 blockade abolished allergic inflammation independently of IL-4 (21, 22), and IL-13 expels helminth *Nippostrongylus brasiliensis* infection independently of IL-4 (85). Differences between IL-4 and IL-13 responses might be because of differences in their receptors and signaling pathways (86). In a contact dermatitis model, topical application of TRPA1 agonist, allyl isothiocyanate, resulted in suppression of IL-4 production by local lymph nodes (87). The role of IL-4 in TRPA1 in chronic allergic dermatitis needs to be further studied. Importantly, cultured mast cells stimulated with TRPA1 agonist, *Allyl isothiocyanate*, showed markedly increased calcium signals indicating a functional role of TRPA1 in mast cells.

Importantly, in accordance with our findings in mice with AD, we found enhanced expression of TRPA1 in dermal mast cells, dermal afferent nerves, and the epidermis in the lesional skin from patients with AD. The finding of increased mast cells in the AD skin biopsies was consistent with the findings that increased mast cells and degranulation have been shown in the lesional skin of AD patients (88, 89). The finding of heightened TRPA1 expression in the epidermis of lesional human AD skin suggests that TRPA1 in the epidermal cells including keratinocytes could also contribute to chronic itch in AD.

When viewed in combination, these novel observations show that TRPA1 expression in the lesional AD skin (keratinocytes, mast cells, and dermal afferent nerve fibers) is essential for the pruritogenesis in AD, that mast cells positively regulate TRPA1 expression in dermal

sensory nerves in AD skin, and that IL-13 is a potent stimulator of TRPA1 expression in mast cells. It is important to point out, however, that TRPA1 antagonist, HC-030031, administration and the null mutation of c-Kit to delete mast cells, although markedly decreasing IL-13–induced AD-associated itch and TRPA1 expression in AD skin, did not completely abrogate IL-13–induced itch responses. This suggests that mast cell–independent, TRPA1-independent mechanism(s), or other pruritogenic mediators or pathways also contribute to the pathogenesis of IL-13–induced itch in AD. The mechanisms that promote TRPA1 expression in dermal sensory afferents and the epidermis in the AD skin and those that diminish TRPA1 expression in the dermal sensory afferents in the AD skin from mast cell–deficient IL-13 Tg mice remain to be fully defined in the future studies. Importantly, demonstration of enhanced expression of TRPA1 in dermal mast cells, dermal sensory nerves, and in the epidermis in the chronic AD lesions in IL-13 Tg mice was recapitulated in the findings from studies of the biopsy samples from patients with AD. Demonstration of increased TRPA1$^+$ mast cells and cutaneous nerve growth in the lesional AD skin in the present studies highlights their relevance and importance in the cellular network of chronic itch.

In summary, our study revealed a novel neural mechanism underlying chronic itch in AD induced by IL-13. This mechanism is TRPA1 dependent, involves interactions between TRPA1$^+$ dermal mast cells and TRPA1$^+$ dermal afferent nerves in a Th2-dominated inflammatory environment, and is responsible for the pruritogenesis of chronic itch in AD. These studies also indicate a role for TRPA1 in the pruritogenesis of human AD.

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Disclosures

The authors have no financial conflicts of interest.

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