Appendix S1

SUPPLEMENTARY MATERIALS AND METHODS

Mice. Male and female C57BL/6 mice, K5CreERT2 mice, and *Stat3 flox*/- mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *K5CreERT2* mice were crossed with *Stat3 flox*/- mice to generate *K5CreERT2*;*Stat3 flox*/+ mice. Mice were maintained under specific-pathogen-free conditions in our animal facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Miami.

Keratinocyte STAT3 depletion. *K5CreERT2;Stat3 flox*/+ mice were administered intraperitoneally with tamoxifen (200 µg in 8 µl vehicle [castor oil:sunflower oil = 1:4] /g body weight; Sigma-Aldrich, St. Louis, MO) 2 weeks before experiments.

Ovalbumin-induce murine model of atopic dermatitis. Induction of atopic dermatitis (AD)-like inflammation was described previously (S1). Briefly, mice were sensitized intraperitoneally with ovalbumin (OVA; 100 µg/mice; Sigma Aldrich) in conjunction with aluminum hydroxide (2.9 mg/mice; Sigma-Aldrich) and pertussis toxin (300 ng/mice; List biological laboratories, Campbell, CA) on Day 1. On Day 5, 50 µg of OVA was subcutaneously administered, and fur on the rostral back was shaved. Then, inflammation was elicited from Day 14 to Day 28 as follows. Gauze (1 × 1 cm) soaked with 0.1% OVA (100 µl) in saline was applied to the shaved skin area. The treated skin area was covered with a patch (Tegaderm, 3M Health Care, St. Paul, MN). The next day, the patch was removed, and an identical piece of soaked gauze followed by Tegaderm patch was reapplied to the same skin area.

Behavior test. Mice were habituated twice to a Plexiglas recording arena for 60 min before testing. For acute itch examination, after 30 min of habituation in a Plexiglas, 10-µl of either histamine (50 µg, Sigma-Aldrich), serotonin (10 µg, Alfa Aesar, Heysham, England), or chloroquine (100 µg, Sigma-Aldrich) was injected intradermally into the shaved skin area on the rostral back, and scratching behavior was videotaped from above for 30 min. For OVA-treated mice, spontaneous scratching was recorded for 60 min on Days 14 and 28. The number of scratch bouts was counted by a trained observer. A scratch bout was defined as one or more rapid back-and-forth hind paw motions directed toward and contacting the treated area, ending with licking or biting of the toes or placement of the hind paw on the floor. Hind paw movements directed away from the treated area (e.g., ear-scratching) and grooming movements were not counted (S2).

Antibodies. Anti-STAT3 (D3Z2G) and anti-pSTAT3 (Thy705) antibodies were obtained from Cell signaling technology (Danvers, MA). Anti-TRPV4 antibody (ab39260) was obtained from Abcam (Cambridge, UK).

Epidermal calcium imaging. Earlobes were cut from the euthanized mice and then immersed in 10 mM EDTA in RPMI-1640 complete medium at 37ºC for 1 h. Then the epidermal sheets were collected and incubated with Cal-590 with Pluronic F-127 solved in HEPES medium (AAT Bioquest, Sunnyvale, CA) following the manufacturer's protocol. After incubation, the dye loading medium was replaced with HEPES solution supplemented with 2.5 µM Ca^{2+} and 250 µM glucose. One of the following pruritogens was delivered for 1 min by a perfusion system: histamine (100 µM), serotonin (100 µM), and chloroquine (100 µM). Ionophore, at a concentration of 4.8 µM, was always delivered at the end of each experiment. Images were obtained with a Leica CTR6000 fluorescence microscope at excitation/emission = 540/590 nm and 5 regions of interest (ROIs) of 100 µm diameter per one sample (each ROI includes at least 10 keratinocytes), and a total of 30 ROIs per each phenotype/pruritogen was analyzed using a Leica LAS X software. F/F_0 was determined as the ratio of the fluorescence intensity at the indicated time divided by baseline fluorescence intensity.

For STAT3 inhibition, samples were incubated with 20 µM of STA-21 (Sigma Aldrich) for 1 h before imaging (S3). For inhibition of TRPV4, samples were incubated with 10 µM of GSK205 (Glixx laboratories, Hopkinton, MA) for 15 min before imaging (2).

Immunofluorescence staining. For phospho-STAT3 staining, formalin-fixed paraffin-embedded (FFPE) samples (5 µm-thick sections) were pretreated with target retrieval solution (Dako, Glostrup, Denmark) and incubated with methanol containing 0.3% H2O2 for endogenous peroxidase quenching. For staining of STAT3, 20 µm thick frozen sections were used. For staining of TRPV4, paraformaldehyde (PFA)-fixed epidermal sheets were used. All samples were then treated with a 5% normal donkey serum and 0.2% Triton X-100 in PBS to prevent nonspecific binding of Abs. After blocking, sections were incubated with the indicated antibodies followed by a reaction with Alexa Fluor 488-, 568-, or 594-conjugated secondary antibodies (Life Technologies, Eugene, or Abcam, Cambridge, UK) and mounted with fluoroshield with DAPI (GeneTex, TX, USA). Photomicrographs were captured with a TCS SP8 confocal microscope or CTR6000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

RT-PCR. Epidermal sheets were collected, preserved in RNAlater (Qiagen, Valencia, CA), and stored at –80°C. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and RNeasy Micro Kit (Qiagen, Valencia, CA). Reverse transcription of 0.5 µg total RNA was performed using ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). RT-PCR was performed using Go Taq DNA polymerase (Promega, Madison, WI) on a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA). Forty to fifty cycles of amplification were performed involving sequential denaturation at 95°C for 15 s and annealing/extension at 55°C for 30 s. The primers for PCR were 5'- TCCACTGGCGTCTTCAC-3' and 5'-GGCAGAGATGATGACCCTTTT-3' for mouse GAPDH, and 5'-CCTTGTTCGACTACGGCACTT-3' and 5'-GGATGGGC-CGATTGAAGACTT-3' for mouse TRPV4.

Statistics. All data are expressed as the mean + SEM. For statistical analysis of scratching behavior and the peak fluorescence intensity in calcium imaging, two-tailed unpaired *t*-tests were used. For time course of calcium imaging, two-way repeated measures ANOVA followed by Bonferroni post hoc test was conducted. Results were considered significant at $p < 0.05$.

SUPPLEMENTARY REFERENCES

S1. Akiyama T, Nguyen T, Curtis E, Nishida K, Devireddy J, Delahanty J, et al. A central role for spinal dorsal horn neurons that express neurokinin-1 receptors in chronic itch. Pain 2015; 156: 1240–1246.

S2. Sanders KM, Hashimoto T, Sakai K, Akiyama T. Modulation of itch by localized skin warming and cooling. Acta Derm Venereol 2018; 98: 855–861.

S3. Miyoshi K, Takaishi M, Nakajima K, Ikeda M, Kanda T, Tarutani M, et al. Stat3 as a therapeutic target for the treatment of psoriasis: a clinical feasibility study with STA-21, a Stat3 inhibitor. J Invest Dermatol 2011; 131: 108–117.