Appendix S1

MATERIALS AND METHODS

Animals

Male NC/Nga mice (10–12 weeks old; Oriental Yeast, Tokyo, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine under a 12-h light: 12-h dark cycle at a regulated temperature of 22–24°C with food and tap water provided *ad libitum*. Care and handling of all animals conformed to the National Institutes of Health (NIH) guidelines for animal research, and all animal procedures were approved by the Institutional Animal Care and Use Committee of Juntendo University Graduate School of Medicine.

Reagents

The following reagents were used in this study: Dfb ointment (Biostir Inc., Osaka, Japan), bovine serum albumin (BSA), CsA, nerve growth factor (NGF), poly-D-lysine, laminin, 5-fluro-2-deoxyuridine, uridine, penicillin, and streptomycin (Sigma, St Louis, MO, USA), optimal cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan), normal donkey serum (NDS; Chemicon, Temecula, CA, USA), sevoflurane (Abbott Japan, Osaka, Japan), somnopentyl (Kyoritsu Seiyaku Corporation, Tokyo, Japan), Vectashield® mounting medium (Vector Laboratories Ltd, Peterborough, UK), Dulbecco's modified Eagle's medium (DMEM)/F12 and N-2 supplement (Invitrogen, Carlsbad, CA, USA).

Antibodies

The following antibodies were used in this study: rabbit anti-protein gene product 9.5 (PGP9.5) (1:400 dilution; Enzo Life Science, Inc., Farmingdale, NY, USA), rabbit anti-TSLP (1: 1000 dilution, Millipore), rabbit anti-IL-31 (1: 500; Sigma-Aldrich), guinea pig anti-keratin-14 (1:100 dilution, Progen Biotechnik), guinea pig anti-keratin-10 (1:100 dilution, Progen Biotechnik), rat anti-substance P (SP) (1:100 dilution; Chemicon), and rat anti-CD4 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were purchased from Molecular Probes (Eugene, OR, USA).

Experimental design

AD-like symptoms were induced by repeated application of Dfb twice per week for 3 consecutive weeks (induction phase), followed by repeated application of Dfb twice per week for the following 3 consecutive weeks for maintenance of AD-like symptoms. CsA was injected intraperitoneally 3 times per week for 3 consecutive weeks (treatment phase) from weeks 3 to 6. During the treatment course, dermatitis score and transepidermal water loss (TEWL) were evaluated before each application of Dfb ointment and measured 3 times per week. Scratching behaviour was evaluated after CsA treatment.

Induction of dermatitis

In the first induction, mice were anaesthetized with sevoflurane and the dorsal skin of the nape of the neck was shaved with an electric shaver. The residual hair was depilated by applying hair removal cream, which was then followed by application of ointment containing 100 mg Dfb to the shaved area and both surfaces of each ear. In the second induction, residual growing hair in the shaved area was shaved again with an electric shaver, followed by barrier disruption by treatment with 4% sodium dodecyl sulphate (SDS) solution 2 h before application of Dfb ointment. The above procedure was repeated twice per week for 3 consecutive weeks.

Measurement of TEWL

TEWL was measured by pressing the Tewameter® TM210 (Courage-Khazaka, Cologne, Germany) against the rostral part of the back skin for 30 s. TEWL was measured 3 times per week, starting before CsA treatment and ending after 3 weeks of CsA treatment.

Observation of scratching behaviour

Mice in each group were placed individually in acrylic cages consisting of 4 cells (13×9×35 cm). A camcorder (HDR-SR11; Sony, Tokyo, Japan) was positioned above the cages to record the behaviour of the mice. After an acclimation period of at least 1 h, the behaviour of mice was recorded on video for 2 h with no experimenters present in the observation room. Scratching behaviour was assessed by monitoring and counting the replays of each video. Each incidence of scratching behaviour was defined as the raising to lowering of a leg and changes in number of scratching behaviours after treatment were recorded.

Treatment with CsA

Individual doses of CsA were prepared by dissolving in a minimal amount of 95% ethanol and diluted as a fine suspension by adjusting to the final volume with 30% glycerin in distilled water. In the treatment phase, all of the mice in each group were injected with 1 mg/kg CsA or 5 mg/kg CsA (0.25 ml, intraperitoneal) 3 times weekly for 3 consecutive weeks, immediately followed by application of 100 mg of Dfb ointment twice a week for 3 consecutive weeks. TEWL measurement and dermatitis score were evaluated after each CsA treatment, starting from the 3rd week and ending on the 6th week. Scratching behaviour was observed in the 3rd week (before CsA treatment) and the sixth week (end of CsA treatment).

Immunohistochemical and histological analyses

Skin from each mouse was removed under anaesthesia with somnopentyl. After removing the skin, samples were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C. After washing with phosphate-buffered saline (PBS, pH 7.4), small pieces of skin were immersed successively in PBS containing 20% sucrose overnight at 4°C. The samples were then embedded in OCT compound and frozen on dry ice. Cryosections 8 μm, and 20 μm thick were cut using a CM1850 cryostat (Leica, Wetzlar, Germany) and mounted on silane-coated glass slides. After blocking in PBS with 5% NDS, 2%BSA, and 0.2% Triton X-100, the cryosections on the glass slides were incubated with primary antibodies overnight at 4°C. The next day, the primary antibodies were washed with PBS containing 0.05% Tween 20 (PBS-T), and incubated with secondary antibodies for 1 h at room temperature. The sections were washed again with PBS-T and mounted in Vectashield® mounting medium. Then, the immunoreactivity was assessed by confocal laser scanning microscopy (DMIRE2; Leica). In histological analyses, cryosections 8μm thick were stained with direct fast scarlet 4BS (DFS), toluidine blue (TB), and haematoxylin-eosin (HE).

Semi-quantitative measurements

Epidermal thickness was measured in 9 random fields (1.0×10^5 μm^2) per mouse. All of these measurements were

performed using BZ-H2A software (Keyence, Osaka, Japan). To perform semi-quantitative measurements of the fluorescence intensity of TSLP in epidermis, 9 random fields (1.6×10^5 μm^2) per mouse were observed with a confocal laser-scanning microscope, with exposure and acquisition settings such that no signal saturation occurred. The sum of the fluorescence intensity of the epidermis and the area of the epidermis in each field was measured using Leica Confocal Software. Fluorescence intensity per unit area was determined by dividing the total fluorescence intensity by the total area. To semi-quantify the number of epidermal nerve fibres, 9 specimens from each mouse were stained with the above-mentioned primary antibodies, and optical sections 0.9 μm thick were scanned through the z-plane of the stained specimens by confocal microscopy. The images were reconstructed in 3 dimensions using Leica Confocal Software (Leica). For measurement of the number of epidermal nerve fibres, we analysed at least 15 confocal images per group per experiment. The number of epidermal nerve fibres in each 1.6×10^5 μm^2 section of the epidermis was quantified. The numbers of inflammatory cells and epidermal nerve fibres were counted manually. All values represent the means ± SD.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA from the DRGs was isolated with an RNeasy Fibrous Tissue Minikit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. Gene expression analyses for itch-related receptors in DRGs were performed by qRT-PCR using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The protocols for qRT-PCR analysis were performed according to the manufacturer's instructions. The primers used in this study were synthesized by Perfect Real-Time support system (TaKaRa, Kyoto, Japan) and are listed in (STable I). The levels of gene expression were calculated relative to expression of ribosomal protein S18 (RPS18). The qRT-PCR analyses in this study were performed at least 3 times.

STable I. *Sequences of primer pairs used for qRT-PCR analysis*

Genes	Sequence (5'–3')
H1R	(forward) ACACATCAGAGGCATGGACAGAC
	(reverse) TCTTCGGAGGCAGAAGAGGTG
H4R	(forward) TTTGTGGTGGACAGAAACCTTAGAC
	(reverse) AACATGCAGATTCCACTTCCAA
Mrgpra3	(forward) AAGCCAGCAACCTACATCCAG
	(reverse) TTCCAGGGATGGTTTCGTTC
IL-31RA	(forward) CCAGAAGCTGCCATGTCGAA
	(reverse) CTCGGTGTCCCAACACTGGATA
Osmr	(forward) GATTCGCATCACAGCCAACAA
	(reverse) CCAGATACGGGCTCCCAAGA
PAR2	(forward) GCTGGGAGGTATCACCCTTCTG
	(reverse) GAGGATGGACGCAGAGAACTCA
TSLPR	(forward) CCAGAAGCTGCCATGTCGAA
	(reverse) CTCGGTGTCCCAACACTGGATA
Trpv1	(forward) CAAGGATGACTTCCGGTGGTG
	(reverse) CCTGGGTCCTCGTTGATGATG
TrpA1	(forward) TGGGCAGCTTATTGCCTTCAC
	(reverse) GGACCTCTGATCCACTTTGCGTA
TGR5	(forward) TGCCCAAAGGTGTCTACGAGTG
	(reverse) GCTGCATTGGCTACTGGTGTG
NK1R	(forward) CAGTCAGCCCTGGGAACCTA
	(reverse) GGTCCACCTCATCTTCGCTCATA

Culture of DRG neurones

Postnatal rat-derived DRG neurones (5×10^3 cells: Cambrex, Walkersville, MD) were placed into each well for neurite growth assay. The DRG neurones were maintained in serum-free medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10 ng/ml NGF, 87.5 ng/ml 5-fluro-2-deoxyuridine, 37.5 ng/ml uridine, 50 U/ml penicillin, and 50 mg/ml streptomycin supplemented with 5% N-2 at 37°C in 5% CO_2.

Assay of neurite outgrowth in cultured DRG neurones

DRG neurones were cultured in basal medium with vehicle (dimethyl sulphoxide), 0.02 μM CsA, and 0.2 μM CsA in the presence of 10 ng/ml NGF. After 3 days, the cells were fixed with 4% PFA in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature, and then examined under phase-contrast microscopy (DMIL; Leica). For semi-quantitative determination of neurite outgrowth, 9 random photographs were taken per well and the length of the longest process of DRG neurones was measured with BZ-H2A software (Keyence). In each experiment, at least 100 cells from triplicate wells per group were analysed. The data from 3 independent experiments were used for statistical analysis.