Supplementary material to article by N. Takahashi et al. "Involvement of µ-opioid Receptors and κ-opioid Receptors in Itch-related Scratching Behaviour of Imiquimod-induced Psoriasis-like Dermatitis in Mice"

Appendix S1

MATERIALS AND METHODS

Evaluation of skin condition and dermatitis

Before IMQ application, transepidermal water loss (TEWL) and stratum corneum (SC) hydration were evaluated daily in each mouse using a Tewameter TM300 and a Corneometer CM825 (C Courage and Khazawa, Cologne, Germany), respectively (room temperature; 25.1 ± 0.6°C, relative humidity; 43.4 ± 1.3 %). The severity of psoriasis-like dorsal skin lesions was assessed according to 4 symptoms: erythema/haemorrhage, scarring/dryness, oedema, and excoriation/erosion. Each symptom was graded from 0 to 3 (none, 0; mild, 1; moderate, 2; severe, 3). Dermatitis score was defined as the sum of the individual scores, with total scores ranging from 0 to 12.

Histological analyses

Twenty-four hours after the fifth application of IMQ cream, the mice were anaesthetized with somnopentyl, and skin samples were collected. The 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 in PBS containing 20% sucrose overnight at 4°C. These samples were embedded in O.C.T. compound and frozen on dry ice. Cryosections (20 µm thick for PGP9.5 staining and 6 µm thick for haematoxylin-eosin (HE) staining) were cut using a CM1850 cryostat (Leica Biosystems, Wetzlar, Germany) and mounted onto MAS-GP type A coated glass slides (Matsunami Glass, Osaka, Japan). After blocking in PBS containing 5% normal donkey serum (NDS), 2% BSA, and 0.2% Triton X-100, the cryosections on the glass slides were incubated with rabbit anti-PGP9.5 antibody (1:500 dilution; Enzo Life Sciences, Farmingdale, NY, USA) overnight at 4°C. The sections were washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with AlexaFluor 488-conjugated donkey anti-rabbit IgG (1:300 dilution; Thermo Fisher Scientific, Franklin, MA, USA) overnight at 4°C. The sections were washed again with PBS-T and mounted in Vectashield® mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). In histological analyses, cryosections 6 µm thick were stained with haematoxylin and eosin solution.

Semi-quantification of epidermal nerve fibres

Three skin specimens from each mouse were incubated with anti-PGP9.5 antibody. Using a confocal microscope, optical sections 0.9 µm thick were scanned through the z-plane of the stained specimens (thickness 20 µm), and the images were reconstructed in 3 dimensions using Leica Confocal Software (Leica Microsystems). The numbers of epidermal nerves were determined by analysing at least 6 confocal images from each mouse. The numbers of epidermal nerve fibres in areas measuring 1.6 × 10^3 µm^2 were hand-counted in the images. Number of intraepidermal PGP9.5 fibres (Fig. S3b) indicates the number present within the epidermis. Number of PGP9.5 fibres penetrating into epidermis (Fig. S3c) indicates the number crossing the epidermis basement membrane. All values are reported as mean ± standard deviation (SD).

Semi-quantitative measurements of epidermal thickness

Epidermal thickness was measured in 9 random fields (1.0×10^3 µm^2) per mouse using BZ-H2A software (Keyence, Osaka, Japan). All values represent the mean ± SD.

Quantitative RT-PCR (qRT-PCR) analysis

Dorsal skin samples collected from each mouse under somnopentyl anaesthesia were collected 24 h after the fifth daily application of IMQ cream. For quantitative RT-PCR (qRT-PCR) analysis, total RNA was isolated from skin samples using RNaseasy Fibrous Tissue Mini kits (Qiagen KK, Tokyo, Japan), according to the manufacturer’s instructions. The expression of mRNAs encoding IL-17A, IL-17F and IL-23p19 was assessed by qRT-PCR using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s protocols. PCR primer sequences were: IL-17A, 5'-GAA GGC CCT CAG ACT ACC TCA A-3' (forward) and 5'-TCA TGT GGT CCA GCT TTC-3' (reverse); IL-17F, 5'-TGT GGC ACG TGA ATT CCA GA-3' (forward) and 5'-TAT TGA TGC AGC CCG AGT GTC-3' (reverse); IL-23p19, 5'-ACA TGC ACC AGC CTG AGT GTC-3' (forward) and 5'-GAG CAT ATC TTC GGC CCA CAC-3' (reverse). The levels of gene expression were calculated relative to expression of mRNA encoding ribosomal protein S18 (RPS18).

Evaluation of H1 receptor antagonist in histamine-evoked scratching behaviour

Mice were anaesthetized with sevoﬂurane, and the rostral part of the back was clipped with an electric shaver. After 3 days, mice were orally administered 3 mg/kg bepotastine besilate (Wako Pure Chemical Industries), followed 1 h later by an intradermal injection of 10 µl of 27.1 mM histamine dihydrochloride (Sigma-Aldrich) or vehicle (saline; Otsuka Pharmaceutical Factory, Tokushima, Japan). Scratching behaviour was monitored for 1 h using a SCLABA®-Real system (Noveltec).