Supplementary data

Supplemental materials and methods

Animals

Male NC/Nga mice (10-week old; Charles River Japan, Yokohama, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine under controlled temperature (22 - 24°C), humidity (50 ± 5%) and light (light on 8:00 - 20:00). Food and tap water were provided ad libitum. All animal procedures were approved by Animal Care and Use Committee of Juntendo University Graduate School of Medicine.

Induction of atopic dermatitis-like symptoms

Dermatitis was induced by application of Dfb ointment (Biostir, Kobe, Japan) twice per week for three weeks. Severity of skin lesion was graded according to criteria described (Matsuda et al., 1997).

Intrathecal treatment with minocycline

After three weeks of Dfb application, mice with a dermatitis score > 5 were selected and divided into three groups: group 1, saline (n=7); group 2, 5 μg minocycline (n=8); group 3, 50 μg minocycline (n=8). Minocycline (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline, and filtrated through a sterile syringe filter. Intrathecal administration was performed as described (Hylden JL et al., 1980). Briefly, 5 µL solution was intrathecally injected into the lumbar spinal area (L5/L6) using a 25 µL Hamilton syringe with 33-gauge needle under sevoflurane anesthesia. Slight flinching of the tail was considered indicative of successful intrathecal administration. The procedure was conducted three times per week for 2 weeks.
Oral treatment with minocycline

After three weeks of Dfb application, mice with a dermatitis score > 5 were selected and divided into three groups: group 1, saline (n=6); group 2, 5 mg kg⁻¹ minocycline (n=6); group 3, 25 mg kg⁻¹ minocycline (n=6). Saline or minocycline (5 or 25 mg kg⁻¹ in 200 μL saline) was administered orally (p.o.) by syringe twice per day for 3 weeks. Minocycline was prepared as described above.

Analysis of scratching behavior

Scratching behavior was recorded and analyzed using a SCLABA®-Real system (Noveltec Inc., Kobe, Japan). In brief, mice were put into an acryl cage and after acclimatization period of at least an hour scratching behaviour was recorded continuously for 12 hours once a week, with no human present.

Clinical skin severity score

Severity of skin lesion was graded according to criteria described (Matsuda et al., 1997). Severity scoring was performed before applying Dfb ointment twice per week in induction and treatment phases. Clinical features of AD-like lesions (erythema/hemorrhage, edema, excoriatio/erosion and scaling/dryness) were graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe), with a maximum score being 12 in total.

Measurement of transdermal water loss (TEWL)

TEWL was measured under sevoflurane anesthesia on the dorsal skin lesion three times per week using a tewameter® TM210 (Courage & Khazaka Electronic, Cologne, Germany) and the median value of three measurements at each time point documented.
**Immunohistochemistry**

Mice were deeply anesthetized with intraperitoneal administration of somnopentyl and perfused through the left ventricle with phosphate saline buffer (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the cervical and upper thoracic spinal cord was harvested, post-fixed in 4% paraformaldehyde solution for 4 hours, and kept in PBS containing 20% sucrose overnight at 4°C. Samples were cut transversely into 20 µm-thick frozen sections on a cryostat, CM3050S® (Leica Biosystems Nussloch, Wetzlar, Germany). Six sections, as one set per animal, were collected for semi-quantitative measurement and mounted onto silane-coated glass slides. After blocking in PBS containing 5% normal donkey serum, 2% bovine serum albumin and 0.2% Triton X-100 (blocking solution), was used as a primary antibody. The sections were incubated with rabbit polyclonal anti-Iba1 (1:500, Wako Pure Chemical, Osaka, Japan) overnight at 4°C. Then, the sections were washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with Alexa Fluor 488-conjugated species-specific secondary antibody (1:300; Invitrogen, Danvers, MA) for one hour at room temperature. After washing with PBS-T, the sections were mounted in Vectashield® mounting medium (Vector Laboratories, Burlingame, CA).

For semi-quantitative measurement, the number of Iba1⁺ microglia was counted in the posterior area of the spinal cord, behind the central canal, in each mouse.

**Statistics**

Data are expressed as mean±standard deviation (SD). The statistical significance of differences among data of control and treated mice was determined by one-way analysis of variance, and Bonferroni’s method or unpaired t test using GraphPad Prism 6 (GraphPad Software, San Diego, CA) with P < 0.05 taken to indicate statistical significance.
Supplemental Figures

Supplemental Figure S1. Distribution of Iba1+ microglia in Dfb-NC/Nga mice spinal cord. (a) The number of scratching bouts in 12 hours was significantly increased after repeated application of Dfb ointment. In Dfb-NC/Nga mice, (b) dermatitis score and (c) TEWL value significantly increased after repeated application of Dfb ointment including preconditioning by sodium dodecyl sulfate (SDS). (d) A representative image of spinal Iba1+ microglia (green) in SDS-NC/Nga mice (control) (e) A representative image of spinal Iba1+ microglia (green) in Dfb-NC/Nga mice. Scale bar, 100 µm. (f) The number of Iba1+ microglia in the posterior area of the spinal cord was significantly increased in Dfb-NC/Nga mice compared with that in SDS-NC/Nga mice. Data (mean ± SD, n = 6 animals) were compared by unpaired t test. **P < 0.01, ****P < 0.0001 vs. control.
Supplemental Figure S2. Effects of oral minocycline on dermatitis, scratching behavior and spinal Iba1⁺ microglia in Dfb-NC/Nga mice. (a) Experiment schedule. After the induction phase, saline or minocycline (5 or 25 mg per kg in 200 μL saline) was administered orally (p.o.) twice per day for 3 weeks. (b) In 25 mg kg⁻¹ minocycline-treated mice, the dermatitis score was significantly decreased after the 3-week treatment. (c, d) No significant difference was found among the three groups in numbers of scratching bouts or Iba1⁺ microglia in the posterior area of the spinal cord. Data (mean ±SD, n = 6 animals) were compared by one-way analysis of variance and Bonferroni’s method.