Appendix S1

MATERIALS AND METHODS

Water

UPSW was produced using a water softener with a cation-exchange resin (Miura Co., Ltd., Ehime, Japan) that removes both Ca^{+2} and Mg^{+2} from tap water. The UPSW generators were provided by Miura, CO., Ltd. to each patient in the study. The composition of the tap water (supplied by Bureau of Waterworks, Tokyo Metropolitan Government, Japan) and UPSW used in the study are shown in Table SI.

Table SI. *Ion composition of tap water and ultra-pure soft water (UPSW) used in this study*

Measured items	Tap water	UPSW
pH	8.1	8.1
$CaCO_3$, mg/l (*water hardness*)	151.9	0.0
Ca^{2+}, mg/l	45.0	0.0
Mg^{2+}, mg/l	9.6	0.0
Na^{+}, mg/l	14.2	83.3
Cl^{-}, mg/l	18.0	18.0
SO_4^{-2}, mg/l	37.0	39.0

Mice

NC/Tnd mice (15–16 mice in each group) with moderate AD housed in conventional environments were used to evaluate the effect of UPSW on dermatitis severity and scratching score as detailed below. After clipping the hair, the dorsal skin was washed with soap and rinsed with pre-warmed (37–40ºC) tap water or UPSW for 3 min once a day for 3 weeks. NC/Tnd mice (12–16 mice in each group) housed in a specific pathogen-free (SPF) environment were used to examine the pro-inflammatory properties of topical metallic soap. In both sets of experiments, mice were kept in a clear acryl cage and allowed free access to food and water. Temperature and humidity of the conventional animal or SPF rooms were 22 ± 4°C and 40 ± 15%, respectively. The animal rooms were kept on a 12-h light and 12-h dark cycle. All experiments with animals complied with the standards specified in the guidelines of the University Animal Care and Use Committee in Tokyo University of Agriculture and Technology, and were approved by the institutional committee.

Clinical skin severity scores

As previously described (17–19), the clinical skin severity score for AD-like lesions was defined as the sum of the individual scores based on a 4-point grading scale. Grading criteria were applied to each of the following 5 signs and symptoms: itching, oedema, erythema/haemorrhage, excoriation/erosion, and scaling/dryness. Clinical skin severity scores were recorded weekly by researchers who were blinded to the treatment applied to the mice.

Analysis of scratching behaviour

Because itching is one of the primary symptoms of AD, scratching behaviour of individual mice was recorded and analysed using a scratching behaviour quantification system (SCLABA®-Real, Noveltech Inc., Kobe, Japan) according to the manufacturer's instructions. The SCLABA®-Real system measures the frequency and duration of scratching episodes in fixed periods (17–19).

TEWL

TEWL of the dorsal skins of each mouse was measured using a multi probe adapter system (CK electronic GmbH, Germany). Mice were customised to the laboratory for 60 min prior to the measurement where temperature and humidity were set at 23°C and 40% respectively. Triplicate measurements were performed on each mouse and the mean of the values presented.

Quantification of soap deposition on the skin

Forearms of 6 healthy volunteers (2 males and 4 females), aged 25 to 46 years old, with no history of skin disease, were soaked in pre-warmed 0.05 M soap consisted with lauric acid sodium for 5 min and then rinsed in tap water or UPSW at 37°C for the indicated periods. Dose-dependent deposition of lauric acid on the skin was measured after rinsing in water with various concentrations of $CaCO_3$ for 90 s. The stratum corneum was stripped using Sellotape (Scotch®, 3M, Tokyo, Japan) and fatty acids derived from soap on the skin measured by gas chromatography. All experiments with healthy volunteers were also performed in compliance with the standards specified in the guidelines of the University Committee for Human Study, Tokyo University of Agriculture and Technology, and were approved by the institutional committee.

Ultra-pure soft water treatment of adults with mild atopic dermatitis

A pilot study was designed to assess the effects of UPSW on the skin condition of patients with AD. This study was performed by the site management organisation (Cosmex Co., Ltd, Tokyo, Japan). Eight female Japanese patients with mild AD (defined under "The guidelines for the treatment of atopic dermatitis 2004" which was published by the Committee for Guidelines for Management of Atopic Dermatitis of the Japanese Dermatological Association) aged 30–47 (median 37) years were studied. They were all recruited from the dermatology clinic (Tsubasa Dermatology clinic, Tokyo, Japan) and were being treated with moisturisers and mild potency topical corticosteroids only. Dryness, scaling and pruritus of the skin were evaluated by a dermatologist at baseline and then 2 and 4 weeks after daily showers with the UPWS. These 3 parameters were scored according to the score for the eczema area and severity index (EASI) with following criteria: none = 0, slight = 1, mild = 2, moderate = 3. No other changes were made to the patients' care, including topical therapies and soap.

The water content of the stratum corneum of the patients was measured with Corneometer® CM825 (Courage & Khazaka electronic GmbH, Cologne, Germany), which assesses the dielectric constant of the skin. Dry skin typically has a water content of <40 arbitrary units (a.u.) (20). TEWL was assessed using a VapoMeter® (Delfin Technologies Ltd., Kuopio, Finland). Both measurements were made on the forearms of patients with mild AD at baseline, 2 and then 4 weeks into treatment. All experiments on patients were performed in compliance with the standards specified in the guidelines of the University Committee for Human Study, Tokyo University of Agriculture and Technology, and were approved by the institutional committee. Patients participating in the study provided written informed consent.

Topical application of metallic soap

For the topical application of metallic soap on to the barrier-disrupted dorsal skin of SPF NC/Tnd mice without AD, we generated metallic soap by mixing chemicals as detailed in Table SII. Various fatty acids, lauric acid, stearic acid, and oleic acid, were used as typical constituents of soap. All chemicals used in the study were purchased from Sigma-Aldrich, Tokyo, Japan. The skin barrier of NC/Tnd mice was tape stripped 3 times, followed

Table SII. *Composition of soap and metallic soap used for topical application*

	Concentrations, mg/ml			
Added chemicals	Diluent	Soap	Metallic soap with Ca^{2+}	Metallic soap with Mg^{2+}
NaCl	11.5	–	11.5	12.0
Lauric acid sodium	–	25	–	–
Stearic acid sodium	–	25	–	–
Oleic acid sodium	–	25	–	–
Lauric acid calcium	–	–	25	–
Stearic acid calcium	–	–	25	–
Oleic acid calcium	–	–	25	–
Lauric acid magnesium	–	–	–	25
Stearic acid magnesium	–	–	–	25
Oleic acid magnesium	–	–	–	25

by the application of metallic soap onto the dorsal skin twice a week for 4 weeks. Physiological saline was used as the diluent. Scratching behaviour of mice was recorded and analysed alternate weeks using SCLABA®-Real (Noveltech Inc., Kobe Japan).

After 4 weeks, the mice were sacrificed and blood and tissue samples collected. Lesional skin and axillary lymph nodes were collected and stored in liquid nitrogen for real-time PCR analysis and fixed in 10% buffered-formalin for histological analysis. Plasma samples were isolated by centrifugation and stored at −20°C until analysed.

Plasma total IgE

Measurement of plasma total IgE was performed using a sandwich ELISA as previously described (21, 22).

Haematoxylin and eosin staining

Skin samples stored in 10% buffered-formalin were embedded in paraffin and sectioned. Paraffin sections were treated with xylene and ethanol, rinsed with water, and stained with haematoxylin followed by eosin G.

Reverse transcription and real-time PCR

Total RNA was extracted from the skin or the axlliary lymph nodes of each mouse snap frozen in liquid nitrogen and processed with Isogen (Nippongene, Tokyo, Japan), then reverse-transcribed into cDNA with a PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan) as manufacturer's instruction. Complementary DNA samples were used for real-time PCR. Real-time PCR was performed with SYBR Premix Ex Taq II (Takara Bio) in the presence of 0.2 μM each of the forward and reverse primers for murine IL-4 (5'–TCTCGAATGTACCAGGAGCCATATC–3' and 5'–AGCACCTTGGAAGCCCTACAGA–3'), murine IL-5 (5'–AGCACAGTGGTGAAAGAGACCTT–3' and 5'–TCCAATGCATAGCTGGTGATTT–3'), murine IL-10 (5'–GACCAGCTGGACAACATACTGCTAA–3' and 5'–GATAAGGCTTGGCAACCCAAGTAA–3'), murine transforming growth factor, TGF-β1 (5'–TGTGGAGCAACATGTGGAACTCTA–3' and 5'–TTGGTTCAGCCACTGCCGTA–3'), murine IFN-γ, (5'–CGGCACAGTCATTGAAAGCCTA–3' and 5'–GTTGCTGATGGCCTGATTGTC–3'), murine thymic stromal lymphopoietin (TSLP) (5'–CGAGCAAATCGAGGACTGTGAG–3' and 5'–GCAGTGGTCATTGAGGGCTTC–3'), and murine β-actin (5'–TGACAGGATGCAGAAGGAGA–3' and 5'–GCTGGAAGGTGGACAGTGAG–3') using the following thermal cycling programs: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence intensity was measured in real time during the extension steps using the ABI Prism 7000 Sequence Detector (Applied Biosystems, Tokyo, Japan). Relative expression levels of the target gene were normalised to the endogenous reference (β-actin) and calculated by 2-ΔΔCT.

Statistical analysis

In the pilot study with patients, statistical significance was analysed by Wilcoxon signed-rank test using JMP® 9.0.0 (SAS Institute Inc.). Clinical skin severity scores, scratching analysis, and TEWL values in NC/Tnd mice were compared between the 3 groups by 1 or 2 way-ANOVA and non-parametric Dunnett's multiple comparisons using JMP® 9.0.0 (SAS Institute Inc.). Serum IgE concentrations and expression levels of mRNA were compared by 1 or 2 way-ANOVA and the Tukey-Kramer test as post hoc analysis. Statistical analysis was performed using Ekuseru-Toukei 2008 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). $p < 0.05$ was considered statistically significant. All data are represented as mean ± standard error (SE).