Appendix S1

MATERIALS AND METHODS

Patients

All participants provided written informed consent according to the principles of the Declaration of Helsinki. The study was approved by the local ethics committee. Lesional skin biopsies were collected from 24 Caucasian patients with severe extrinsic type of AD (associated with high serum IgE levels, allergen-specific IgE and positive skin-prick test reactions (20)) and from 5 healthy controls. Patients with AD did not have any concomitant skin diseases at the time of examination and had not been treated with any moisturizers for one day, with topical corticosteroids for 3 days and with systemic immunosuppressants for 28 days prior to examination. The characteristics of both AD patient groups are shown in Table I. The severity of AD was determined using OSCORAD and epidermal thickness (ET) and Ki67 expression measurements on biopsies. Two groups were formed according to their *FLG* genotype: patients with severe symptoms without *FLG* mutations (Wt) ($n = 12$, mean OSCORAD: 44.8) and patients with severe symptoms with *FLG* mutation (n=12, all were heterozygotes for one of the 2 alleles [2282del4, R501X], mean OSCORAD: 42.6). Biopsies were taken from all 24 patients with AD, 5–5 samples in both groups were used for immunohistochemistry (IHC) and 12–12 samples were analysed by quantitative PCR (qPCR). Biopsies from 5 healthy controls were investigated in all experiments.

Filaggrin genotyping

Analysis of the *FLG* mutations R501X and 2282del4, which are responsible for 80–99% of all FLG mutations in white European patients with AD (21, 22), was performed for all patients. DNA isolated from peripheral blood mononuclear cells with GenElute Blood Genomic DNA Kit (Sigma, Chemical Co., St Louis, MO, USA) was subjected to polymerase chain reaction (PCR) amplification. Primers for genotyping were: ACG TTC AGG GTC TTC CCT CT and ATG GGA ACC TGA GTG TCC AG for R501X; and CAG TCA GCA GAC AGC TCC AG and AAA GAC CCT GAA CGT CGA GA for 2282del4. PCR amplification conditions were as follows: 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 10 min. All PCR products were purified with QIAquick PCR purification Kit (Qiagen Inc., Hilden, Germany) and bidirectionally sequenced on an ABI Prism 3100 automated sequencer with Big-Dye terminator cycle sequencing reagents (Applied Biosystems, Foster City, CA, USA).

Immunohistochemical staining

For IHC analyses, paraffin-embedded sections from patients and healthy controls (5–5 samples in each groups) were deparaffinized. Heat-induced antigen retrieval was performed and sections were pre-processed with hydrogen peroxide (H_2O_2) for 10 min. Sections were stained with antibodies against human FLG (mouse IgG: Abcam, Cambridge, UK), human Ki67 (mouse IgG: Sigma), human TSLP, human IL-33 (mouse IgG: Abcam, Cambridge, UK), human CCL27 (mouse IgG: Sigma), human CD3 (mouse IgG: UD-Genomed Kft, Hungary) and human CD11c (mouse IgG: Sigma).

Subsequently, HRP-conjugated anti-mouse/rabbit secondary antibodies (Dako, Glostrup, Denmark) were employed. Before and after incubating with antibodies, washing of samples was performed for 5 min, 3 times in each step. Staining was detected with the Vector VIP Kit (VECTOR Laboratories, Burlingame, CA, USA)/DAB (Dako, Glostrup, Denmark). Sections were counterstained with methylene green/haematoxylin. The detection of one protein was carried out on all sections in parallel at the same time to enable us to evaluate comparable protein levels. Positive and negative controls were also used to normalize staining against all proteins.

A routine haematoxylin and eosin staining was also carried out to measure ET as the quotient of epidermal area and epidermal length in each specimen.

Whole-slide imaging

The slides were digitalized using a Pannoramic SCAN digital slide scanner with a Zeiss plan-apochromat objective and Hitachi 3CCD progressive scan colour camera. ET as a well-accepted method for the measurement of the severity of skin inflammation in AD was calculated as the quotient of the field area (FA) of the region of interests (ROI) and the length of epidermis in each ROIs. The protein levels were analysed by 2 independent observers (a dermathopathologist and a biologist) by using Pannoramic Viewer 1.15.2 (3DHistech Ltd., Budapest, Hungary) software's HistoQuant and NuclearQuant applications. The observers did not have information about the FLG genotype of patients with AD. ROIs ($n = 20$/slide) were selected and then the FA (mm^2) and the mask area [MA (mm^2)] were measured by the software. The FA shows the whole area of the ROI, and the MA represents the positive area. The MA/FA values were counted for all ROIs.

RNA isolation and quantitative PCR

All samples (12–12 AD and 5 control) were homogenized in Tri reagent solution (Sigma) with Tissue Lyser (Qiagen) using previously autoclaved metal beads (Qiagen), and total RNA was isolated from skin samples and treated with DNase I (Life Technologies, CA, USA) according to the manufacturer's instructions. The concentration and purity of the RNA were measured by means of NanoDrop spectrophotometer (Thermo Scientific, MA, USA), and its quality was checked using Agilent 2100 bioanalyser (CA, USA). For qPCR, complementary DNA (cDNA) was synthesized using the High Capacity cDNA Archieve Kit and qPCR was carried out in triplicates using pre-designed MGB assays (all from Life Technologies). The following primers were used: PPIA (Hs99999904_m1), TSLP (Hs00263639_m1), IL-33 (Hs00369211_m1) and CCL27 (Hs00171157_m1). All reactions were performed with an ABI Prism 7000 (Applied Biosystems). Relative messenger RNA (mRNA) levels were calculated using either the comparative CT or standard curve methods normalized to the expression of cyclophilin A mRNA. Relative gene expression to healthy controls was calculated as a quotient of the normalized gene expression in Wt AD or *FLG* mutant AD skin and normalized gene expression in healthy skin.

Statistical analysis

To determine the statistical significance between the 3 groups, one-way analysis of variance (ANOVA) test and Newman-Keuls post test were used. Differences between the groups were demonstrated using mean ± 95% confidence interval. p-values < 0.05 were considered statistically significant. Analysis of correlations was performed by Pearson r test. Two-tailed p-values < 0.05 were considered statistically significant.