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Appendix S1

SUPPLEMENTARY METHODS

Cell cultures

Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as described previously (S1). Second-passage keratinocytes were grown in K-SFM (Gibco, Life Technologies, Austin, TX, USA). Twenty-four hours before stimulation with TNFα (10 ng/ml) and/or IL-17A (100 ng/ml), the medium was changed to keratinocyte basal medium (KBM, the same as K-SFM, but without growth factors) in which the cells were stimulated. Cells were grown at 37°C and 5% CO_2 in an incubator.

Biopsies

Four-mm full-thickness punch biopsies were taken from lesional and non-lesional skin from patients with plaque-type psoriasis or atopic dermatitis. Biopsies from lesional psoriatic skin were taken from the centre of a chronic plaque. For each patient, biopsies were taken from only 1 anatomical site and the biopsies from non-lesional skin were taken at a distance of at least 5 cm from a lesional plaque. Biopsies from lesional and non-lesional psoriatic skin were taken as paired samples. The patients had received no topical treatment for a minimum of 2 weeks or no systemic treatment for a minimum of 4 weeks before inclusion, depending on the type of treatment. In addition, 4-mm punch biopsies were collected from normal, healthy controls. The study was conducted in compliance with the Declaration of Helsinki, and signed informed consent was obtained from each patient prior to inclusion in the study.

Mice and treatments

For the experiment shown in Fig. S3[1] mice were purchased and treated as described previously (S2). For the remaining experiments, female *Ikbke*^{tm1Tman} knockout (IKKε-deficient) mice were purchased from The Jackson Laboratory. Female wild-type mice of the same strain were purchased at Charles River Laboratories. All mice were on a C57BL/6 genetic background and used at 4–10 weeks of age. The IKKε-deficient mice were viable and did not display any phenotypic abnormalities. Mice were kept under specific pathogen-free conditions and provided with food and water *ad libitum*. The mice were treated with 45 mg 5% imiquimod (IMQ) cream (Aldara; 3M Pharmaceuticals, St. Paul, MN, USA) or vehicle cream topically on their shaved back along with 8 mg 5% IMQ or vehicle cream on their right ear for 6 days, as described previously (S3). Ear thickness was assessed daily using a Mitutoyo Digimatic Indicator. On day 6 punch biopsies were collected from the right ear for later histological examinations, and the rest of the ear was collected for later qPCR analysis.

H&E staining and immunofluorescence analysis

For H&E staining, 4-µm tissue sections of paraffin-embedded mice ear biopsies were stained with haematoxylin and eosin (H&E) and evaluated by light microscopy.

For immunofluorescence analysis, 4-µm tissue sections of paraffin-embedded mice ear biopsies were deparaffinized, rehydrated and heated in 10 mM sodium citrate buffer (pH 6.0) for antigen unmasking. The samples were blocked for 1 h before incubation with rat anti-Ly6g antibody (cat no. ab25377; Abcam, Cambridge, UK) at 4°C overnight. The samples were then washed and incubated with secondary antibody (#A21210 rabbit anti-rat, Life Technologies) for 1 h at room temperature. Finally, the samples were washed, and nuclear staining was performed by embedding samples in Prolong Gold antifade reagent with DAPI (Life Technologies). Samples were evaluated by epifluorescence microscopy. As negative control, sections were incubated without primary antibody. For isotype control, sections were incubated with rat IgG2b (cat. no. ab18541, Abcam) instead of primary antibody.

Quantitative PCR

For quantitative PCR, Taqman Reverse Transcription reagents, primers and probes were purchased from Life Technologies. Human *DEFB4*, *CCL20* and *CXCL1* mRNA expression was analysed using Taqman 20× Assays-On-Demand expression assay mix (assay ID: Hs00175474_m1, Hs01011368_m1 and Hs00236937_m1, respectively). As reference gene, *RPLP0* (assay ID: Hs99999902_m1) was used. Murine *Nfkbiz*, *Ccl20* and *Cxcl1* mRNA expression was analysed using Taqman 20× Assays-On-Demand expression assay mix (assay ID: Mm00600522_m1, Mm01268754_m1 and Mm04207460_m1, respectively). *Ubc* was used as reference gene (assay ID: Mm02525934_g1). The probe was a FAM-labelled MGB probe with a non-fluorescent quencher. PCR master mix was Platium® qPCR Supermix-UDG (Life Technologies). Each gene was analysed in triplicate. Real-time PCR was performed using the Rotorgene-3000 system (Corbett Research, Sydney, Australia). Reactions were run as singleplex. A standard curve for each gene was made of 4-fold serial dilutions of total RNA. The standard curves were then used to calculate relative amounts of target mRNA.

siRNA transfection

Cultured human keratinocytes were grown to 60–70% confluency. Before transfection, the cells were changed to medium without growth factors (KBM). *IKBKE* siRNA (cat no. (#L-003723-00,

Dharmacon, Lafayette, CO, USA) was pre-incubated with Dharmafect-2 transfection reagent (Dharmacon) in KBM for 20 min. The formed siRNA/transfection reagent complexes were added to the cells to a final concentration of 20 nM. As negative controls, cells were transfected with siControl non-targeting pool siRNA (cat no. D-001810-10-05, Dharmacon). Five hours after transfection, the medium was changed to keratinocyte growth medium (growth factors included). Twenty-four hours before stimulation, the medium was changed to KBM.

Western blotting

Keratome biopsies from psoriatic patients were homogenized in a cell lysis buffer (20 mM Tris-base (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, and 1 mM PMSF) as previously described (S4). The samples were then centrifuged at 10,000 × g for 10 min at 4°C, after which the supernatant constituted the cell lysate. Protein extracts from cultured human keratinocytes were isolated as described previously (S5).

Equal protein amounts were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were incubated with anti-IKKε (cat. no. 2905 (human) or 3416 (mice); Cell Signaling Technology, Danvers, MA, USA). The antibodies were detected with anti-rabbit IgG-HRP (cat. no. 7074; Cell Signaling Technology) in a standard ECL reaction (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Densitometric analysis of the band and background intensities was conducted using Kodak 1D Image analysis software. Results were normalized to β-actin levels.

Statistical analysis

Statistical differences among the experimental groups were analysed by use of 1-way ANOVA or a Student's *t*-test after testing for normality. A probability of $p<0.05$ was regarded as statistically significant.

SUPPLEMENTARY REFERENCES

S1. Johansen C. Generation and culturing of primary human keratinocytes from adult skin. J Vis Exp 2017 Dec 22; 130.
S2. Vinter H, Kragballe K, Steiniche T, Gaestel M, Iversen L, Johansen C. Tumour necrosis factor-alpha plays a significant role in the Aldara-induced skin inflammation in mice. Br J Dermatol 2016; 174: 1011–1021.
S3. Johansen C, Mose M, Ommen P, Bertelsen T, Vinter H, Hailfinger S, et al. IkappaBzeta is a key driver in the development of psoriasis. Proc Natl Acad Sci U S A 2015; 112: E5825–E5833.
S4. Funding AT, Johansen C, Kragballe K, Iversen L. Mitogen- and stress-activated protein kinase 2 and cyclic AMP response element binding protein are activated in lesional psoriatic epidermis. J Invest Dermatol 2007; 127: 2012–2019.
S5. Kjellerup RB, Kragballe K, Iversen L, Johansen C. Pro-inflammatory cytokine release in keratinocytes is mediated through the MAPK signal-integrating kinases. Exp Dermatol 2008; 17: 498–504.