SHORT COMMUNICATION

Investigating the Role of I Kappa B Kinase ε in the Pathogenesis of Psoriasis

Isabella WEIMAR, Pernille OMMEN, Lars IVERSEN and Claus JOHANSEN*

Department of Dermatology, Aarhus University Hospital, Palle Juul-Jensens Boulevard 99, Aarhus N DK-8200, Denmark. *E-mail: Claus.Johansen@clin.au.dk

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Psoriasis is a common chronic inflammatory skin disease (1). Interleukin (IL)-17A has been identified as a key cytokine mediating the immunopathogenesis of psoriasis (2–4). However, the underlying molecular mechanism by which IL-17A mediates its psoriatic effects is unknown. A distinctive characteristic of psoriasis is the presence of neutrophils in the epidermis. Neutrophils are recruited to the epidermis by neutrophil-recruiting chemokines, such as C-X-C motif chemokine ligand (CXCL)1, CXCL5 and IL-8 produced by activated keratinocytes (5, 6). I kappa B kinase (IKK) ε is a protein encoded by the IKBKE gene. IKKε has been demonstrated to play an important role in the recruitment of neutrophils in IL-17A-induced inflammation (7). By the use of IKKε-deficient mice, it has been demonstrated that IKKε is required for IL-17A-induced lung inflammation and, interestingly, IKKε was found to play an important role in the recruitment of neutrophils (7). To date, the role of IKKε in psoriasis remains unknown; however, the above-mentioned data suggest that IKKε may play a role in the pathogenesis of psoriasis. The aim of the current study was to investigate the role of IKKε in psoriasis.

MATERIALS AND METHODS (see Appendix S11)

RESULTS

In order to examine IKBKE mRNA expression, RNA was isolated from punch biopsies obtained from both lesional and non-lesional skin from 15 psoriatic patients and 6 patients with atopic dermatitis. Furthermore, RNA was isolated from 6 healthy controls. The mRNA expression of IKBKE was significantly increased in lesional psoriatic skin compared with non-lesional psoriatic skin from the same patient; an approximately 2-fold increase was observed. The expression of IKBKE in lesional psoriatic skin was also significantly higher compared with healthy controls (Fig. 1A). To determine whether the observed increase in IKBKE expression was specific to psoriasis or simply due to increased inflammation in the skin we also investigated IKBKE expression in atopic dermatitis. No changes were found in IKBKE expression between lesional and non-lesional atopic dermatitis skin (Fig. 1A). It was also found that the IKKε protein level was increased in lesional psoriatic skin compared with non-lesional psoriatic skin from the same patient (Fig. 1B), supporting a potential role of IKKε in the pathogenesis of psoriasis.

To characterize the role of IKKε in the regulation of specific psoriasis-associated genes, siRNA was used to knockdown IKKε. Transfection of human keratinocytes with IKKε siRNA significantly reduced the mRNA and protein expression of IKBKE/IKKε in tumour necrosis factor alpha (TNFα)- and/or IL-17A-stimulated cells compared with control siRNA transfected cells (Fig. S11). Notably, siRNA-mediated knockdown of IKKε, led to a minor, but significant, reduction in CCL20 mRNA expression after stimulation with IL-17A alone and with IL-17A in combination with TNFα, compared with control siRNA transfected cells (Fig. S21). Likewise, mRNA expression of DEFB4 and CXCL1 was significantly decreased in cells transfected with IKBKE siRNA, after combined stimulation with IL-17A and TNFα, but not when single stimulations were used (Fig. S21).

To further characterize the potential role of IKKε in the pathogenesis of psoriasis the imiquimod-induced psori-
asis-like skin inflammation model was used. First, Ikbke mRNA expression in mice ears treated with imiquimod for 1, 3 and 5 days was investigated. Expression of Ikbke mRNA was increased significantly at both days 3 and 5 in imiquimod-treated mice compared with vehicle-treated mice (Fig. S3\(^1\)). To assess the functional role of IKK\(\varepsilon\) in the imiquimod model, the model was applied on IKK\(\varepsilon\)-deficient mice. IKK\(\varepsilon\) deficiency in the mice was confirmed both by quantitative PCR (qPCR) and Western blotting (Fig. S4\(^1\)). Clinically, imiquimod-treated mice developed erythema and scaling of the skin. However, no differences were observed in the degree of erythema and scaling between IKK\(\varepsilon\)-deficient and wild-type mice treated with imiquimod (Fig. S5\(^1\)). Imiquimod treatment caused weight loss of approximately 15% and increased the ear thickness by approximately 130%; however, no differences in ear thickness were observed between imiquimod-treated wild-type and IKK\(\varepsilon\)-deficient mice (Fig. S6A, B\(^1\)). Haematoxylin and eosin (H&E) staining of skin biopsies revealed no histopathological differences between the 2 mice strains after treatment (Fig. S6C\(^1\)). Immunofluorescence staining revealed presence of neutrophils in skin sections after imiquimod treatment, however, no differences in the number of neutrophils between wild-type and IKK\(\varepsilon\)-deficient mice were observed (Fig. S6D\(^1\)). Finally, the expression of psoriasis-associated genes was examined in the ear biopsies. Although imiquimod treatment increased Nfkbiz, Ccl20 and Cxcl1 mRNA expression compared with vehicle-treated mice, no alterations in Ccl20 and Cxcl1 mRNA expression were observed between imiquimod-treated wild-type and IKK\(\varepsilon\)-deficient mice (Fig. S6E\(^1\)).

**DISCUSSION**

This study demonstrated an increased mRNA and protein expression of IKK\(\varepsilon\) in lesional psoriatic skin. The increase in IKK\(\varepsilon\) seemed to be specific for psoriasis and not just due to increased inflammation in the skin in general, because IKK\(\varepsilon\) expression was unaltered between lesional and non-lesional skin from patients with atopic dermatitis. Previous data have shown that the function of IKK\(\varepsilon\) in IL-17A-mediated signalling is linked with neutrophilia (7). IKK\(\varepsilon\) was demonstrated to play a role in IL-17A-induced airway inflammation by regulating the expression of neutrophil-mobilizing cytokines and chemokines, including CXCL1 and CXCL2 (7). Here, we also found IKK\(\varepsilon\) to be involved in the expression of CXCL1. However, only an involvement of IKK\(\varepsilon\) on the CXCL1 expression was observed when TNF\(\alpha\) and IL-17A were given in combination, whereas no involvement was found by stimulation with IL-17A alone. The discrepancy between our data and that of Bulek et al. (7) could be because the role of IKK\(\varepsilon\) in IL-17A stimulation is cell-type specific.

To analyse how IKK\(\varepsilon\) was regulated in vitro during psoriasis development, we used the imiquimod-induced psoriasis-like skin inflammation model, a well-described psoriasis mouse model (8). By comparing vehicle- and imiquimod-treated wild-type mice, we observed no increase in Ikbke mRNA over time during imiquimod treatment. Likewise, we demonstrated only a minor, although significant, induction of Ikbke mRNA upon imiquimod treatment at days 3 and 5. This is in contrast to what we observed in biopsies from psoriatic patients, where we demonstrated an almost 2-fold induction of IKBKE mRNA between lesional and non-lesional psoriatic skin. This could suggest that, in this specific psoriasis mouse model, or perhaps in mice in general, IKK\(\varepsilon\) does not play a major role in the induction of psoriasis-like skin inflammation. Although the imiquimod-induced psoriasis mouse model is dependent on IL-17A signalling (8, 9), it is possible that other cytokines might play a more prominent role in mice, and therefore mice might not represent the best model to study IL-17A downstream effects. This theory was also supported by the fact that we observed no difference between Ikbke knockout mice and wild-type mice in their clinical appearance, bodyweight, ear thickness and expression of the inflammatory genes Ccl20 and Cxcl1 after imiquimod treatment. Surprisingly, IKK\(\varepsilon\) deficiency significantly increased the imiquimod-induced Nfkbiz gene expression, suggesting that IKK\(\varepsilon\) might have a protective role in this model.

Although we demonstrated increased expression of Ikbke mRNA in lesional psoriatic skin and that IKK\(\varepsilon\) knockdown in vitro decreased IL-17A/TNF\(\alpha\)-induced proinflammatory gene expression, this proinflammatory role of IKK\(\varepsilon\) in psoriasis was not supported by results from the imiquimod-induced psoriasis mouse model. Thus, further studies are needed in order to fully elucidate the role of IKK\(\varepsilon\) in psoriasis.

*The authors have no conflicts of interest to declare.*

**REFERENCES**

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% CO₂ 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, mice were purchased and treated as described previously (S2). For the remaining experiments, female Ikkβε 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 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 mouse 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 mouse 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-IgG 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, Hs0111368_m1 and Hs00236937_m1, respectively). As reference gene, RPLP0 (assay ID: Hs99999902_m1) was used. Murine Nkbi, Ccl20 and Cxcl1 mRNA expression was analysed using Taqman 20× Assays-On-Demand expression assay mix (assay ID: Mm00465522_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 Platinum® 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 µM PMSF) as previously described (S4). The samples were then centrifuged at 10,000 × g for 10 min at 4°C, and 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 (mouse); 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.
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**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**

**Fig. S1. Knockdown of I kappa B kinase ε (IKKε) by siRNA.** (A) Cultured human keratinocytes were transfected with IKBKE siRNA (siIKBKE) or control siRNA (siCon) before combined stimulation with tumour necrosis factor alpha (TNFα) (10 ng/ml) and IL-17A (100 ng/ml) for 24 h. Whole-cell protein extracts were isolated from the keratinocytes and the IKKε protein level was measured by Western blotting (n = 3). β-actin was used as a loading control. (B) Densitometric analysis of the band intensities normalized to β-actin. The ratio is presented as mean ± standard deviation (SD). *p < 0.05 compared with siCon, Student’s t-test.
Fig. S2. Effect of I kappa B kinase ε (IKKε) knockdown on the mRNA expression of psoriasis-associated genes. Cultured primary human keratinocytes were transfected with 20 nM IKBKE siRNA (siIKBKE) or control siRNA (siCon) prior to stimulation with interleukin (IL)-17A (100 ng/ml) and/or tumour necrosis factor alpha (TNFα) (10 ng/ml) for 24 h. RNA was extracted from the cells and the mRNA expression of (A) CCL20, (B) DEFB4 and (C) CXCL1 were analysed by quantitative PCR (qPCR) (n=4). RPLP0 mRNA was used for normalization. The relative fold induction is presented as mean ± standard deviation (SD). *p<0.05 compared with siCon, Student’s t-test.
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Fig. S3. *Ikbke* mRNA expression during imiquimod treatment. Wild-type (WT) mice were treated with imiquimod (IMQ) or vehicle (Veh) daily for 1, 3 or 5 days. Biopsies from the ears were collected after the indicated number of days, and RNA extracted. *Ikbke* mRNA expression was analysed by quantitative PCR (qPCR). *Ubc* mRNA was used for normalization. Results are expressed as mean ± standard deviation (SD) from 4 mice. Data were analysed with one-way analysis of variance (ANOVA). *p < 0.05.

Fig. S4. Validation of I kappa B kinase ε (IKKε) deficiency in *Ikbke*tm1Tman knockout mice at protein and mRNA level. *Ikbke*tm1Tman knockout (KO) and wild-type (WT) mice were treated topically with imiquimod (IMQ) or vehicle (Veh) cream daily for 6 days. (A) Skin from the back of the mice was collected and protein extracted. The isolated protein from 15 KO and 7 WT mice was analysed by Western blotting. β-actin was used as a loading control. (B) Biopsies from the ear of the mice were collected and RNA extracted. The mRNA expression of *Ikbke* was analysed by quantitative PCR (qPCR). *Ubc* mRNA expression was used for normalization. The relative fold induction is presented as mean ± standard deviation (SD) of 6 WT and 5 KO mice treated with vehicle and 12 WT and 10 KO mice treated with imiquimod.
Fig. S5. Imiquimod (IMQ)-induced psoriasis-like skin inflammation in IKKε-deficient and wild-type (WT) mice. Representative pictures of WT (n = 6) and Ikbke knockout (KO) mice (n = 5) treated with vehicle daily for 6 days and WT (n = 12) and KO (n = 10) mice treated with IMQ daily for 6 days.
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Fig. S6. Imiquimod (IMQ)-induced psoriasis-like skin inflammation in I kappa B kinase ε (IKKe)-deficient and wild-type mice. (A) Bodyweight and (B) ear thickness of wild-type (WT) and knockout (KO) mice treated with vehicle or IMQ daily for the indicated days. Data points represent mean ± standard deviation (SD) of 6 WT and 5 KO mice treated with vehicle (Veh) and 12 WT and 10 KO mice treated with IMQ. (C) Sections of ears recovered from IMQ-treated WT and Ikbke KO mice were stained with haematoxylin and eosin (H&E). Scale bars: 100 µm. (D) Sections of imiquimod-treated ears from WT and Ikbke KO mice were analysed for the presence of neutrophils by immunofluorescence staining. Green fluorescence is Ly6g-positive cells and blue fluorescence is DAPI nuclei staining. White dotted lines indicate the dermal-epidermal junction. Scale bars: 100 µm. (E) WT and Ikbke KO mice were treated with vehicle or IMQ on the ears daily for 6 days. Biopsies were taken from the ears, RNA isolated and the expression of Ccl20, Cxcl1 and Nfkbiz analysed by quantitative PCR (qPCR). Data points represent mean ± standard deviation (SD) of 6 WT and 5 KO mice treated with vehicle and 12 WT and 10 KO mice treated with IMQ. Data were analysed with one-way analysis of variance (ANOVA). *p<0.05.