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Tumor Necrosis Factor α-Mediated Induction of Interleukin 17C in Human Keratinocytes Is Controlled by Nuclear Factor κB

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IL-17C is a member of the IL-17 family of cytokines. The expression of IL-17C has been demonstrated to be strongly induced by TNFα in human keratinocytes, and recently the level of IL-17C was found to be increased in the inflammatory skin disease psoriasis. However, little is known about the molecular mechanisms involved in the regulation of IL-17C. Here, we show that pretreatment of cultured human keratinocytes with the inhibitor of κB kinase 2 inhibitor, SC-514, resulted in a significant reduction in both IL-17C mRNA and protein expression, indicating the significance of this pathway in the regulation of IL-17C. NF-κB binding sites were identified upstream from the IL-17C gene, and by electrophoretic mobility shift assay NF-κB was shown to bind to all three identified binding sites. Moreover, NF-κB binding to these sites was inducible by TNFα. Supershift analysis revealed binding of the NF-κB subunits p65 and p50 to all three NF-κB binding sites. To determine the contribution of NF-κB in IL-17C expression, we conducted luciferase gene reporter experiments and demonstrated that a 3204-bp promoter fragment of IL-17C containing three putative NF-κB binding sites was strongly activated by TNFα. Interestingly, mutations of the three NF-κB binding sites revealed that one specific NF-κB binding site was crucial for the TNFα-mediated IL-17C induction because mutation of this specific site completely abolished TNFα-induced IL-17C promoter activation. We conclude that the activation of NF-κB (p65/p50) is crucial for the TNFα-induced stimulation of IL-17C expression in human keratinocytes.

IL-17C is a cytokine first described in 2000 by Li *et al.* (1). It belongs to the IL-17 family of cytokines which consists of six members, IL-17A–F (2, 3). In contrast to IL-17A and IL-17F, the molecular mechanisms involved in the regulation of IL-17C gene expression as well as the biological functions and cellular expression of IL-17C remains poorly characterized. IL-17C has been described to stimulate the transcription of an array of proinflammatory genes, some of which are similar to those induced by IL-17A and IL-17F (1, 4). In addition, studies have shown how ectopic expression of IL-17B and IL-17C by $CD4^+$ T cells exacerbates collagen-induced arthritis (4) and that intranasal administration of adenoviruses expressing IL-17C resulted in bronchoalveolar lavage neutrophilia and inflammatory gene expression in the lung (5), suggesting that IL-17C plays an important role in inflammatory processes. This is supported by a recent study, demonstrating elevated IL-17C mRNA and protein expression in the chronic inflammatory skin disease, psoriasis (6). Furthermore, increased IL-17C mRNA expression in lesional psoriatic skin was significantly reduced as early as 4 days after start of anti-TNFα treatment, *i.e.* before clinical and histological improvement was detectable. Moreover, human keratinocytes were able to produce IL-17C in response to TNFα through a p38 MAPK-dependent mechanism (7). Taken together, these data indicate that IL-17C might play an important role in the pathogenesis of psoriasis and other inflammatory diseases.

Nuclear factor κB (NF-κB) is a transcription factor believed to play a pivotal role in immune and inflammatory responses through the regulation of genes encoding proinflammatory cytokines, chemokines, and growth factors (8–11). Active NF-κB is a dimer formed by members of the Rel family of proteins, consisting of p50, p52, p65(RelA), c-Rel, and RelB (11). In resting cells NF-κB is generally retained in the cytoplasm as an inactive complex bound to its inhibitor, protein inhibitor κB (IκB) (11). Stimulation of cells by a variety of agonists, such as IL-1β and TNFα, results in phosphorylation/activation of a specific IκB kinase (IKK), which phosphorylates the IκBs and thereby tags them for polyubiquitination and subsequent degradation by the 26 S proteasome (12, 13). Degradation of IκB allows NF-κB to translocate to the nucleus where it binds selectively to the consensus sequence G/(T)GGR*NN*YYC/(T)C located in the promoter region of specific genes (N = any base), thereby regulating the transcription of >400 genes involved in inflammation, growth regulation, carcinogenesis, and apoptosis (14, 15). Dysregulations in the NF-κB signaling pathway have been demonstrated to be linked to numerous inflammatory diseases, including psoriasis (8, 16–20). Results from our group have demonstrated an increased NF-κB DNA binding activity to a specific κB binding site in the promoter region of the IL-8 gene and a decreased NF-κB DNA binding activity to a specific κB binding site in the promoter region of the p53 gene in lesional psoriatic skin (20). These data demonstrate that NF-κB regulation is very complex and that there is a high degree of specificity of the genes transactivated by NF-κB.

Because the mechanisms involved in IL-17C regulation are largely unknown, and because IL-17C expression is increased in psoriasis and therefore constitutes a potential target in the treatment of psoriasis, the purpose of this study was to characterize the mechanism by which IL-17C is regulated in human keratinocytes. We show that the NF-κB signaling pathway is involved in the TNFα-mediated induction of IL-17C in human keratinocytes. In addition, we identify a specific NF-κB binding site in the promoter region of IL-17C that is responsible for the production of IL-17C in response to TNFα.

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EXPERIMENTAL PROCEDURES

Quantitative PCR—For reverse transcription we used Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA). Primers and probes were purchased from Applied Biosystems. IL-17C mRNA expression were analyzed using Taqman 20X Assays-On-Demand expression assay mix (assay ID: Hs00171163_m1). The probe was a FAM[2]-labeled MGB probe with a nonfluorescent quencher. As housekeeping gene we used *RPLP0*. *RPLP0* mRNA expression was determined by using Taqman 20X Assays-On-Demand expression assay mix (assay ID: Hs99999902_m1). The probe was a FAM-labeled MGB probe with a nonfluorescent quencher. PCR mastermix was Platinum® qPCR Supermix-UDG (Invitrogen). Each gene was analyzed in triplicate. The real-time PCR machine was a Rotorgene-3000 (Corbett Research, Sydney, Australia). Reactions were run as singleplex. Relative gene expression levels were determined by using the relative standard curve method as outlined in User Bulletin 2 (ABI Prism 7700 sequencing detection system; Applied Biosystems). Briefly, a standard curve for each gene was made of 4-fold serial dilutions of total RNA from punch biopsies from the skin of psoriatic patients. The curve was then used to calculate relative amounts of target mRNA in the samples.

Cell Cultures—Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as described previously (21). Second-passage keratinocytes were grown in K-SFM (Invitrogen). 24 h before stimulation with TNFα (10 ng/ml), the medium was changed to keratinocyte basal medium (same as K-SFM but without growth factors) in which the cells were stimulated. In some experiments the keratinocytes were pretreated with the IKK2 inhibitor SC-514 (50 μM, catalog no. 401479) or the NF-κB inhibitor BMS-345541 (50 μM, catalog no. 401480) (Calbiochem) for 45 min before stimulation. Cells were grown at 37 °C and 5% CO_2 in an incubator.

Western Blotting—Equal protein amounts were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were incubated with anti-phospho-p38 antibody, anti-phospho-MK2 antibody, anti-IκBα antibody (catalog nos. 9211, 3007, and 9242, respectively; Cell Signaling Technology, Danvers, MA) or β-actin (catalog no. A-1978; Sigma-Aldrich). The antibodies were detected with anti-rabbit IgG-HRP (catalog no. 7074; Cell Signaling Technology) or with anti-mouse IgG-HRP (catalog no. p0447; Dako, Glostrup, Denmark) in a standard ECL reaction (Amersham Biosciences) according to the manufacturer's instructions.

ELISA—The IL-17C protein levels in cultured normal human keratinocytes were measured by an IL-17C Duoset ELISA kit (catalog no. DY1234). The ELISA was carried out according to the manufacturer's protocol (R&D Systems). The final result was determined by an ELISA reader (Laboratory systems iEMS Reader MF, Copenhagen, Denmark) at 450 nm. All measurements were performed in doublets.

Electrophoretic Mobility Shift Assay (EMSA)—The promoter region of IL-17C was searched for potential NF-κB binding sites (TFSEARCH version 1.3) (22) resembling the consensus sequence. We identified three potential NF-κB binding sites (bold), and matching oligonucleotides were designed. Furthermore, three corresponding oligonucleotides with a mutated (underlined) NF-κB binding site sequence (bold) were synthesized (DNA Technology A/S, Aarhus, Denmark): hIL-17C(oligo1), 5′-TGGTGC**CGGAATTTCC**CCCAGG-3′ and hIL-17C(MUT1), 5′-TGGTGC**CTTCATGGAG**CCCAGG-3′; hIL-17C(oligo2), 5′-CCCAGC**TGGGCACTTC**CCGAGG-3′ and hIL-17C(MUT2), 5′-CCCAGC**CCTTCAGGAG**CCGAGG-3′; hIL-17C(oligo3), 5′-AACCCA**GGGGAGGCCC**CCAGAG-3′ and hIL-17C(MUT3), 5′-AACCCA**CCCTAGGGAA**CCAGAG-3′.

Gel shift assays were performed as described previously (23). Briefly, oligonucleotides were labeled by T4 polynucleotide kinase (Promega, Madison, WI) and purified on a Nick Spin column (Sephadex G-50; Pharmacia). Nuclear protein (3 μg) preincubated with ^{32}P-labeled oligonucleotides was separated on a 6% Novex® DNA retardation gel (Invitrogen) and visualized by exposure to x-ray film. Supershifts were performed by adding 2 μl of the corresponding commercially available antibodies specific for the individual NF-κB proteins (NF-κB p50, catalog no. sc-7178X; and NF-κB p65, catalog no. sc-7151X; Santa Cruz Biotechnology, Santa Cruz, CA) to the binding reactions. In control experiments a specific competitor (unlabeled hIL-17C(oligo1)) or a nonspecific competitor (unlabeled SP-1 oligo) (E3231, Promega) was added 10 min before addition of labeled hIL-17C(oligo1).

Transfection of Oligonucleotides—Lipofectamine™ 2000 transfection reagent (Invitrogen) was used to transfect cultured normal human keratinocytes. Keratinocytes cultured in 6-well plates were transfected with a pool of hIL-17C(oligo1), hIL-17C(oligo2), and hIL-17C(oligo3) (20 μM, in total) or with a pool of hIL-17C(MUT1), hIL-17C(MUT2), and hIL-17C(MUT3) (20 μM, in total) for 6 h before stimulated with TNFα (10 ng/ml) for 2 h.

IL-17C Reporter Plasmid Construction—To analyze the hIL-17C promoter activity, 3204 bp of the human IL-17C promoter was amplified (GenScript, Piscataway, NJ). The amplification product was subcloned into the promoterless pGL4.10[luc2] vector (Promega) to generate an hIL-17C-2-3204-luc2 reporter plasmid. The functional role of putative binding sites for the transcription factor NF-κB in the IL-17C promoter region was analyzed by introducing mutations in the hIL-17C-2-3204-luc2 plasmid. The three putative NF-κB binding sites at position −135 to −114, −163 to −142, and −2947 to −2926 were mutated at the specific base pairs shown under "Electrophoretic Mobility Shift Assay (EMSA)" (GenScript). The resulting hIL-17C-pGL4.10[luc2] plasmids containing mutated NF-κB binding sites were termed NF-κB-mut1-luc2 (containing one mutated NF-κB binding site at position −135 to −114), NF-κB-mut2-luc2 (containing one mutated NF-κB binding site at position −163 to −142), NF-κB-mut3-luc2 (containing one mutated NF-κB binding site at position −2947 to −2926) and NF-κB-mut1 + 2+3-luc2 (containing three mutated NF-κB binding

[2] The abbreviations used are: FAM, 6-carboxyfluorescein; MGB, minor groove binder; K-SFM, keratinocyte serum-free medium; qPCR, quantitative PCR.

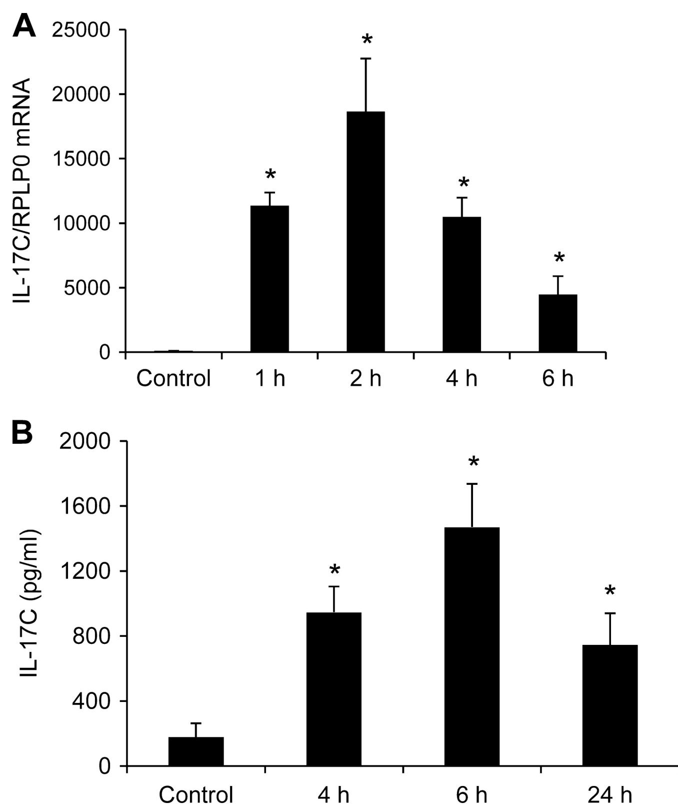

FIGURE 1. **IL-17C expression in cultured normal human keratinocytes is increased in response to TNFα.** Cultured normal human keratinocytes were stimulated with TNFα (10 ng/ml) for the indicated time points. *A*, total RNA was isolated, and the mRNA expression of IL-17C was analyzed by qPCR. *B*, extracellular medium from cultured keratinocytes was isolated, and the IL-17C protein level was analyzed by ELISA. Four separate experiments were conducted, and all results are expressed as mean ± S.D. *, $p < 0.05$ compared with controls. *Error bars*, S.D.

sites). An overview showing the used promoter constructs can be seen in Fig. 6*A*.

Transfection and Determination of Promoter Activity—For IL-17C promoter studies human keratinocytes were cultured in 24-well plates and transfected at 60–70% confluence. Cells were transfected with 0.5 μg of the indicated IL-17C reporter plasmids and 0.025 μg of an internal control *Renilla* luciferase expression plasmid (pRL-TK; Promega) using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's protocol. 24 h after transfection, cells were stimulated with TNFα for 24 h. After stimulation, cells were harvested with 100 μl of passive lysis buffer from Promega, and firefly luciferase activity from the IL-17C-pGL4.10[luc2] reporter vector and *Renilla* luciferase activity were measured by the Dual Luciferase assay system (Promega) on a Fluoroskan Ascent Fl (BIE & Berntsen, Rodovre, Denmark). Promoter activity was reported as the ratio between firefly and *Renilla* luciferase activities in each sample.

Statistical Analysis—In the time studies (Fig. 1 and Fig. 5*A*) statistical analysis was carried out using a one-way repeated measures analysis of variance followed by a Holm-Sidak test. Elsewhere, a Student's *t* test was used. A probability of $p < 0.05$ was regarded as statistically significant.

RESULTS

IL-17C Expression Is Regulated through a NF-κB-dependent Mechanism in Cultured Normal Human Keratinocytes—To analyze the effect of TNFα on IL-17C expression, cultured normal human keratinocytes were stimulated with TNFα for different time points before examining the IL-17C mRNA and protein levels by qPCR and ELISA, respectively. The IL-17C expression was significantly increased in a time-dependent manner with a maximum increase in mRNA and protein levels after 2 and 6 h of stimulation, respectively (Fig. 1, *A* and *B*). Previously, we have demonstrated that TNFα-induced IL-17C expression in human keratinocytes was mediated by a p38 MAPK-dependent mechanism (7). To characterize further the mechanisms by which TNFα regulates the expression of IL-17C, human keratinocytes were preincubated with an IKK2 inhibitor (SC-514) for 45 min before TNFα stimulation. We found that preincubation of the cells with SC-514 significantly inhibited TNFα-induced IL-17C expression at both mRNA ($p = 0.006$) and protein levels ($p = 0.0004$) (Fig. 2, *A* and *B*). Similar results were seen when using another inhibitor of NF-κB (BMS-345541) (data not shown). Because the p38 MAPK signaling pathway has been described to be involved in the regulation of IL-17C (7), we analyzed whether SC-514 or BMS-345541 had a nonspecific inhibitory effect on the p38 MAPK signaling pathway. Preincubation of human keratinocytes with SC-514 or BMS-345541 prior to TNFα stimulation had no effect on the phosphorylation level of p38 MAPK, nor did it have an effect on the phosphorylation level of MK2, a downstream target of p38 MAPK (Fig. 2*C*). As a control for the inhibitory effect of SC-514 and BMS-345541 on NF-κB signaling, we also monitored IκBα degradation. As seen in Fig. 2*C*, both SC-514 and BMS-345541 completely blocked TNFα-induced degradation of IκBα (Fig. 2*C*).

Identification and Characterization of Three NF-κB Binding Sites in the IL-17C Promoter Region—NF-κB initiates gene transcription of target genes containing the classic 10-bp-long κB consensus sequence (Table 1). Therefore, we searched the IL-17C gene sequence on human chromosome 16 and identified three putative NF-κB binding sites (named hIL-17C(oligo1–3)) within a 3.3-kb region upstream of the IL-17C coding sequence (Table 1). The three potential NF-κB binding sites all expressed high homology with the classic κB consensus sequence. By EMSA we demonstrated that NF-κB binds to all three identified NF-κB binding sites. Moreover, we observed a clear time-dependent increase in the NF-κB DNA binding activity in nuclear extracts from cultured keratinocytes stimulated with TNFα compared with vehicle-treated cells (Fig. 3*A*). We also examined the NF-κB DNA binding activity to corresponding oligonucleotides mutated within the κB consensus sequence (Table 1). When nuclear extracts from the keratinocytes were incubated with the mutated oligonucleotides (hIL-17C(MUT1–3)) no NF-κB DNA binding was seen, demonstrating that the specificity of the three identified 10-bp κB sequences are important for NF-κB DNA binding (Fig. 3*B*).

To characterize the NF-κB subunits responsible for the DNA binding, supershift analysis was performed. Incubation of nuclear extract from cultured human keratinocytes with anti-

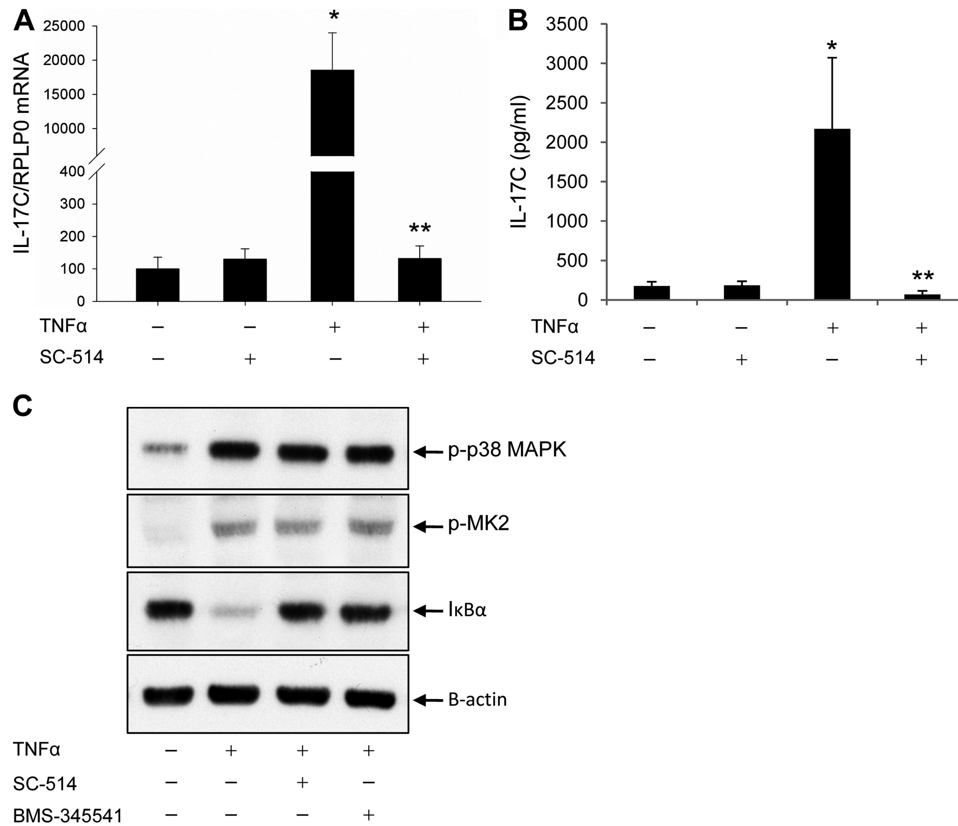

FIGURE 2. **IL-17C is regulated by a NF-κB-dependent mechanism in human keratinocytes.** *A*, cultured normal human keratinocytes were preincubated with an IKK2 inhibitor (SC-514, 50 μM) for 45 min before stimulated with TNFα (10 ng/ml) for 2 h. Total RNA was isolated and analyzed for the expression of IL-17C by qPCR. *B*, cultured human keratinocytes were preincubated with SC-514 as in *A* before stimulated with TNFα for 6 h. The extracellular medium was isolated and the IL-17C protein level examined by ELISA. Four separate experiments were conducted, and all results are expressed as mean ± S.D. (*error bars*). *, $p < 0.05$ compared with nonstimulated cells; **, $p < 0.05$ compared with keratinocytes stimulated with TNFα. *C*, cultured human keratinocytes were preincubated with SC-514 (50 μM) or BMS-345541 (50 μM) for 45 min before stimulated with TNFα for 15 min. Protein extracts were isolated, and Western blotting analysis used to measure the phosphorylated level of p38 MAPK and MK2 as well as the total protein level of IκBα. Equal loading was confirmed by incubation with an anti-β-actin antibody. Data from one representative experiment of three are shown.

TABLE 1

Potential NF-κB binding sites in the promoter region of IL-17C

The 10-bp NF-κB site is shown in bold. Below each original sequence the mutated sequence is shown (mutations are underlined). Consensus sequence is 5′-G/(T)GGR*NN*YYC/(T)C-3′. *N* is any base.

Name of oligonuleotides	Base pair (bp) numbers	Sequence of oligonuleotides
hIL-17C (oligo1)	bp −114 to −135	5′-TGGTGC**CGGAATTTCC**CCCAGG-3′
hIL-17C (MUT1)		5′-TGGTGC**CTTCATGGAG**CCCAGG-3′
hIL-17C (oligo2)	bp −142 to −163	5′-CCCAGC**TGGGCACTTC**CCGAGG-3′
hIL-17C (MUT2)		5′-CCCAGC**CCTTCAGGAG**CCGAGG-3′
hIL-17C (oligo3)	bp −2926 to −2647	5′-AACCCA**GGGGAGGCCC**CCAGAG-3′
hIL-17C (MUT3)		5′-AACCCA**CCCTAGGGAA**CCAGAG-3′

bodies directed against the p50 and/or the p65 NF-κB subunits both revealed a complete supershift of the NF-κB band (Fig. 3*C*), indicating that the p50/p65 heterodimer is the predominant dimer involved in the regulation of TNFα-mediated IL-17C expression.

TNFα-induced NF-κB DNA Binding Activity Is Inhibited by SC-514 in Cultured Normal Human Keratinocytes—Next, we analyzed the impact of the IKK2 inhibitor SC-514 on NF-κB DNA binding to the three oligonucleotides hIL-17C(oligo1–3). Preincubation of cultured human keratinocytes with SC-514 for 45 min prior to TNFα stimulation resulted in a reduction of the NF-κB DNA binding activity to all three oligonucleotides (Fig. 4). Similar results were observed when using the NF-κB inhibitor BMS-345541 (data not shown). Together, these results are consistent with the previously shown reduction in IL-17C mRNA and protein expression after preincubation with SC-514 or BMS-345541 and suggest the IKK2/NF-κB signaling pathway to be part of the regulatory mechanism responsible for the IL-17C gene expression induced by TNFα.

TNFα Induces IL-17C Promoter Activation through NF-κB in Human Keratinocytes—To test whether the observed TNFα-mediated IL-17C induction correlates with IL-17C promoter activation, 3204 bp of the IL-17C promoter was ligated in front of the firefly luciferase gene, and this construct was used to transiently transfect cultured normal human keratinocytes. TNFα treatment of the transfected keratinocytes increased the luciferase activity in a time-dependent manner, indicating activation of the IL-17C promoter. After 24 h of treatment, a ~6-fold increase in the luciferase activity was observed (Fig. 5*A*). To verify the influence of NF-κB on TNFα-mediated

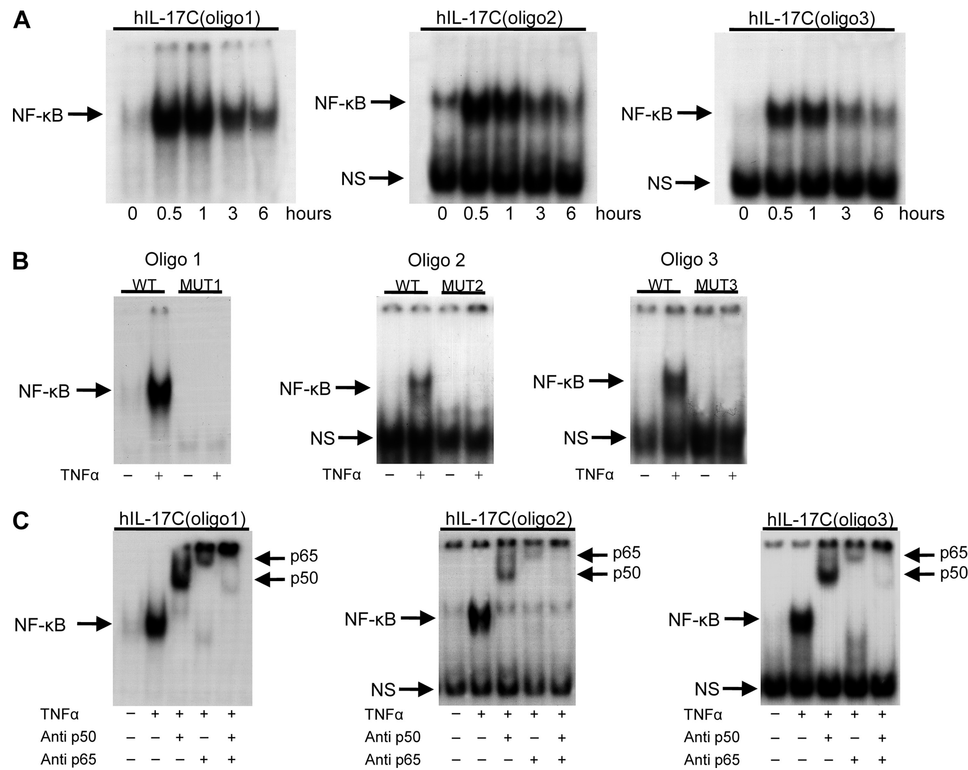

FIGURE 3. **Identification and characterization of three NF-κB DNA binding sites in the promoter region of the IL-17C gene.** *A*, cultured normal human keratinocytes were stimulated with TNFα (10 ng/ml) for the indicated time points before the DNA binding activity to the three putative NF-κB binding sites (oligo1–3) located upstream from the start codon of the IL-17C gene was analyzed by EMSA. *B*, supershift analysis was carried out. Antibodies directed against p50 and p65 were added to the incubation mixture after which the NF-κB DNA binding activity to the three different oligonucleotides was analyzed. *C*, oligo1–3 were mutated as described under "Experimental Procedures," and the NF-κB DNA binding activity was examined by EMSA. Representative gels from four different experiments are shown.

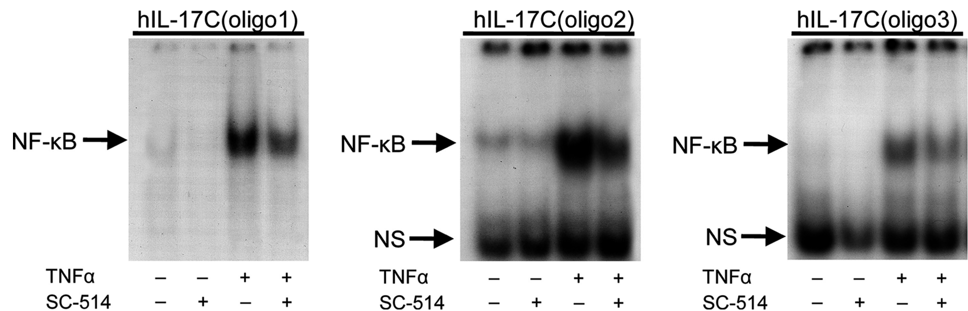

FIGURE 4. **TNFα-induced NF-κB DNA binding activity is inhibited by SC-514.** Cultured normal human keratinocytes were preincubated with SC-514 (50 μM) before stimulated with TNFα (10 ng/ml) for 1 h. The nuclear extract from the cells was isolated, and the NF-κB DNA binding activity to the three putative NF-κB binding sites (oligo1–3) was analyzed by EMSA. Representative gels from four different experiments are shown.

IL-17C promoter induction, we pretreated keratinocytes with the IKK2 inhibitor SC-514 for 45 min prior to stimulation with TNFα for 24 h. We found that treatment with SC-514 significantly ($p = 0.003$) reduced the IL-17C promoter activity to a level comparable with vehicle-treated cells (Fig. 5*B*). Similar results were observed when the transfected keratinocytes were pretreated with another NF-κB inhibitor, BMS-345541 (data not shown), indicating that NF-κB is a key player in the TNFα-mediated IL-17C promoter induction.

One Specific NF-κB Binding Site in the IL-17C Promoter Is Responsible for TNFα-mediated IL-17C Induction—To analyze the functional importance of putative binding sites for the transcription factor NF-κB on TNFα-mediated induction of IL-17C, we generated different IL-17C-promoter-luciferase constructs containing mutations of the three NF-κB binding sites (positions −135 to −114, −163 to −142, and −2947 to −2926) (Fig. 6*A*). Transfection of cultured human keratinocytes with the IL-17C-promoter-luciferase construct containing mutations in all three NF-κB binding sites (NF-κB-mut1 + 2+3-luc) significantly ($p = 0.007$) abolished the TNFα-mediated IL-17C promoter activation (Fig. 6*B*). Interestingly, separate mutations of the three NF-κB binding sites revealed that the first proximal NF-κB binding site (positions −135 to −114) is essential for the TNFα-mediated IL-17C promoter activation. When keratinocytes were transfected with the NF-κB-mut1-luc construct, TNFα-induced luciferase activity was significantly reduced to a level comparable with nonstimulated cells (Fig. 6*B*). In contrast, when cells were transfected with IL-17C promoter constructs containing mutations in the second (NF-κB-mut2-luc) or third (NF-κB-mut3-luc) NF-κB

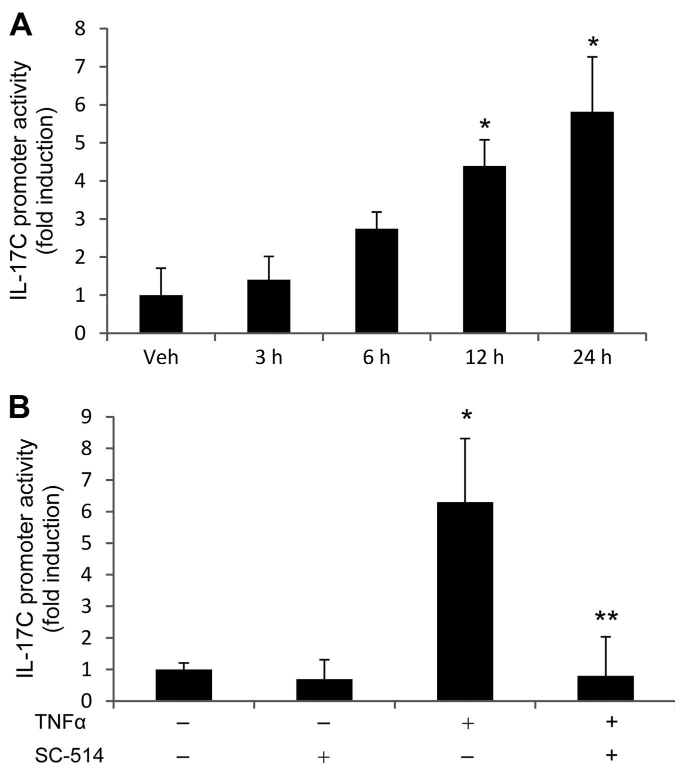

FIGURE 5. **IL-17C promoter activation by TNFα is mediated by a NF-κB-dependent mechanism.** Cultured normal human keratinocytes were transfected with wild-type (IL-17C-2-3204-luc2) IL-17C-promoter-luciferase plasmids together with an internal control (*Renilla* luciferase expression plasmid). After transfection, cells were stimulated with TNFα (10 ng/ml) for the indicated time points (*A*) or preincubated with SC-514 (50 μM) for 1 h (*B*) before being stimulated with TNFα (10 ng/ml) for 24 h. The activity was determined as a ratio between firefly and *Renilla* luciferase activity. Results represent mean ± S.D. (*error bars*) from four different experiments. All measurements were performed in doublets. *, $p < 0.05$ compared with vehicle-treated cells; **, $p < 0.05$ compared with TNFα-stimulated cells.

binding site, no reduction in luciferase activity was observed, demonstrating that these two NF-κB binding sites were not involved in TNFα-induced activation of the IL-17C promoter (Fig. 6*B*).

Transfection of Cultured Human Keratinocytes with Decoy Oligonucleotides Containing the Identified NF-κB DNA Binding Sites Inhibits TNFα-induced IL-17C Expression—To determine the inhibitory effect of NF-κB decoy oligonucleotides on TNFα-induced IL-17C expression, cultured normal human keratinocytes were transfected with an equal mixture of hIL-17C(oligo1), hIL-17C(oligo2), and hIL-17C(oligo3) or an equal mixture of hIL-17C(MUT1), hIL-17C(MUT2), and hIL-17C(MUT3) for 6 h prior to stimulation with TNFα for 2 h. By qPCR we demonstrated, that in keratinocytes transfected with a mixture of decoy oligonucleotides containing the three identified NF-κB binding sites (hIL-17C(oligo1–3)), the TNFα-induced IL-17C mRNA expression was significantly decreased ($p = 0.02$) (Fig. 7). In contrast, when keratinocytes were transfected with a mixture of decoy oligonucleotides mutated in their NF-κB binding site sequence (hIL-17C(MUT1–3)), the IL-17C mRNA expression was unaltered compared with TNFα-stimulated cells (Fig. 7). These experiments demonstrate that it is possible to block the IL-17C promoter activity with NF-κB decoy oligonucleotides.

DISCUSSION

The IL-17 family of cytokines is a recently described group of cytokines that have unique structural features distinguishing them from other cytokine families (3, 24). IL-17A and IL-17F are the best characterized members of the IL-17 family and known to play an important role in many inflammatory diseases, including psoriasis and rheumatoid arthritis (25–28). Recently, we have identified IL-17C as the only other member of the IL-17 family with an increased expression in psoriatic skin, suggesting that also IL-17C possesses inflammatory properties (6). However, although IL-17A and IL17F expression and function are well characterized, less attention has been paid to the mechanisms involved in the regulation of IL-17C. In this study, we present both essential and novel findings regarding the regulation of IL-17C expression. We demonstrate a NF-κB-dependent mechanism to be essential for IL-17C expression in human keratinocytes in response to TNFα treatment. Furthermore, one specific NF-κB binding site in the promoter region of IL-17C was identified to be crucial for TNFα-mediated IL-17C induction.

Recently, we demonstrated that stimulation of cultured human keratinocytes with TNFα led to an increased IL-17C expression (7). Because TNFα is known to activate the transcription factor NF-κB (29–31), we asked whether the NF-κB signaling pathway was involved in the TNFα-mediated IL-17C induction. Preincubation with an inhibitor (SC-514) targeting the IKK2 in the NF-κB signaling pathway completely abolished TNFα-mediated IL-17C induction at both the mRNA and protein level in cultured human keratinocytes. Because many chemical inhibitors have been demonstrated not to be entirely specific (32), we used a second IKK2 inhibitor (BMS-345541) to substantiate our data. Pretreatment of the keratinocytes with BMS-345541 before TNFα stimulation strongly reduced IL-17C expression, demonstrating that IL-17C is regulated through the NF-κB signaling pathway in response to TNFα.

We have previously demonstrated that TNFα-induced IL-17C expression in human keratinocytes is mediated by a p38 MAPK-dependent mechanism (7). Blocking the p38 MAPK signaling pathway, however, only resulted in a partial reduction (~60%) of IL-17C expression (7). Interestingly, in this study inhibition of the NF-κB signaling pathway completely reduced the TNFα-induced IL-17C expression. Because p38 MAPK has been described to be involved in the phosphorylation of the p65 subunit of NF-κB through mitogen- and stress-activated protein kinase-1 (MSK1), thereby activating NF-κB (33), it is possible that the reduction observed in IL-17C expression by blocking the p38 MAPK pathway is due to an inhibition of the p38 MAPK/MSK1-mediated phosphorylation of p65.

Upon stimulation, inactive NF-κB complexes kept in the cytoplasm become activated by phosphorylation, leading to nuclear translocation of active NF-κB dimers and eventually transcription of specific target genes (34). In this study we demonstrated binding of the p65/p50 heterodimer to all three NF-κB binding sites of the IL-17C promoter. These findings are in agreement with the fact that the p65/p50 heterodimer generally is believed to be a transcriptional activator because of a powerful transcriptional activation domain on the p65 subunit

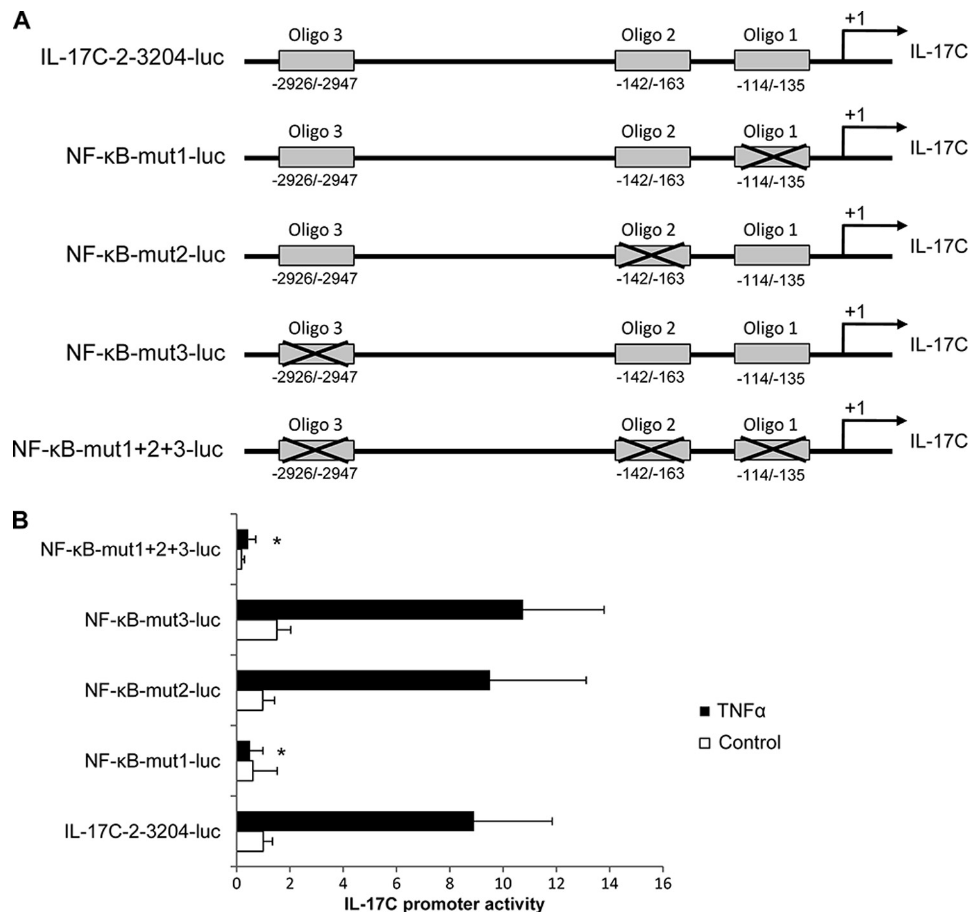

FIGURE 6. **Mutation of a specific NF-κB binding site inhibits TNFα-mediated IL-17C promoter activation.** *A*, IL-17C promoter constructs. Nucleotide positions are marked relative to the IL-17C transcription start. Three NF-κB binding sites in the IL-17C promoter (−3204 to −1 bp) linked to the luciferase gene were mutated in different combinations. *B*, cultured normal human keratinocytes were transfected with wild-type (IL-17C-2–3204-luc2) or mutated IL-17C-promoter-luciferase plasmids together with an internal control (*Renilla* luciferase expression plasmid). After transfection with the indicated plasmids the keratinocytes were stimulated with TNFα (10 ng/ml) for 24 h. The activity was determined as a ratio between firefly and *Renilla* luciferase activity. Results represent mean ± S.D. (*error bars*) from four different experiments. All measurements were performed in doublets. *, $p < 0.05$ compared with IL-17C-2–3204-luc2 reporter plasmid-transfected cells stimulated with TNFα.

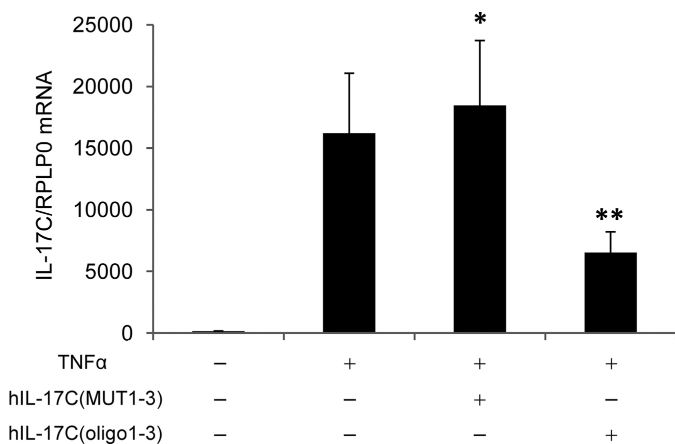

FIGURE 7. **Transfection of keratinocytes with decoy oligonucleotides containing the indentified NF-κB DNA binding sites inhibits TNFα-induced IL-17C expression.** Cultured normal human keratinocytes were transfected with a pool of hIL-17C(oligo1–3) or with a pool of hIL-17C(MUT1–3) for 6 h before being stimulated with TNFα (10 ng/ml) for 2 h. Total RNA from the cells was isolated, and the mRNA expression of IL-17C was analyzed by qPCR and normalized with *RPLP0*. Four separate experiments were conducted, and all results are expressed as mean ± S.D. (*error bars*). *, $p < 0.05$ compared with nonstimulated cells; **, $p < 0.05$ compared with TNFα-stimulated cells.

(33) and the fact that the p65/p50 heterodimer is the most abundant form of NF-κB (35).

Because NF-κB binding activity to an oligonucleotide *in vitro* is not necessarily representative of its functional activity within the cells, we analyzed the functional relevance of these NF-κB binding sites for TNFα-mediated IL-17C induction by a luciferase gene reporter assay. We found that mutation of all three NF-κB sites completely blocked IL-17C promoter activation after stimulation with TNFα, indicating that NF-κB plays a crucial role in the regulation of TNFα-mediated *IL-17C* gene induction. Interestingly, separate mutations of the three NF-κB binding sites revealed that only the first proximal NF-κB binding site (positions −135 to −114) was involved in the TNFα-mediated IL-17C induction in human keratinocytes because mutation of this specific site completely inhibited IL-17C promoter activation upon TNFα treatment. The observation that specific NF-κB binding sites are involved in gene transcription is consistent with a previous study. Wehkamp *et al.* identified three putative NF-κB binding sites in the promoter region of the hBD2 gene and found the first proximal NF-κB binding site to be of major importance for the IL-1β-mediated hBD2 induction followed by the second and the third NF-κB binding sites (36).

The use of specific NF-κB decoy oligonucleotides for blocking NF-κB activity is considered to be a potential new class of antigene therapy (37). In mice, treatment either topically or systemically with NF-κB decoy oligonucleotides followed by exposure to UVB light has been shown to significantly reduce UV-induced cutaneous swelling, epidermal hyperplasia, and secretion of proinflammatory cytokines such as IL-1β, TNFα, and IL-6 (38). In addition, prevention and regression of atopic dermatitis by ointment containing NF-κB decoy oligonucleotides have been demonstrated in chronic atopic dermatitis mice models (39, 40). In this study we showed that transfection of cultured human keratinocytes with a mixture of decoy oligonucleotides containing three identified NF-κB binding sites in the promoter region of the IL-17C gene reduced TNFα-induced IL-17C expression. Thus, it is possible that decoy oligonucleotides blocking NF-κB-induced IL-17C expression may be used as a treatment strategy in inflammatory diseases, showing increased expression of IL-17C.

Not much is known about how the different IL-17 family members are regulated. Here we show that IL-17C is regulated by a NF-κB-dependent mechanism in cultured human keratinocytes, and in a previous study we demonstrated that also p38 MAPK was involved in the regulation of IL-17C (7). Whether the other IL-17 family members are regulated in a similar way is still unknown. However, recently IκBζ, a nuclear IκB family member, was demonstrated to increase IL-17A expression by binding directly to the regulatory region of the IL-17A gene (41). In addition, the transcription factor IFN regulatory factor 4 has been described to directly bind to the IL-17A promoter and thereby regulate the expression of IL-17A (42).

This study strongly indicates that the molecular mechanisms leading to IL-17C production in human keratinocytes are dependent on NF-κB activation and therefore, that IL-17C gene expression is NF-κB-driven. Delineating the molecular mechanisms responsible for the TNFα-mediated induction of IL-17C gene expression is important for a better understanding of the regulation of IL-17C expression in inflammatory diseases and could result in the identification of new potential targets for future therapeutic approaches to inflammatory skin diseases.

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