Evaluation of the effects of IL-22 on the proliferation and differentiation of keratinocytes in vitro

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Abstract. Psoriasis is one of the most common chronic inflammatory skin diseases, it is characterized by hyperproliferation of keratinocytes and infiltration of inflammatory cells. Several in vitro studies have reported that interleukin (IL)-22 is involved in excessive proliferation and abnormal differentiation of human keratinocytes. However, the association between IL-22 and CCAAT enhancer binding protein α (C/EBPα) in the pathogenesis of psoriasis remains unclear. Therefore, the present study aimed to investigate the association between IL-22 and C/EBPα, and the effects of IL-22 on the proliferation and differentiation of keratinocytes. Keratinocytes were treated with different concentrations of IL-22 (30, 60 and 90 ng/ml) and subsequently cells were collected at different time intervals. The expression levels of the key molecules of the mitogen-activated protein kinase (MAPK) signaling pathway were determined using western blot analysis. In addition, the effect of IL-22 on the proliferation rate of keratinocytes and reduced the expression levels of cyto
eratin 10 and involucrin. Therefore, these results suggested that IL-22 induces excessive proliferation and abnormal differentiation of human keratinocytes (5-7).

Keratinocytes are an important type of immune cell. The excessive secretion of pro-inflammatory cytokines and chemokines, the aggregation of neutrophils and the formation of new blood vessels often occur in psoriasis (8). Therefore, a vicious circle is formed, resulting in excessive proliferation and abnormal differentiation of keratinocytes (2). In addition, the abnormal activation of signal transduction pathways, associated with immune responses, is considered to play a critical role in the pathogenesis of psoriasis (9). Previous studies have revealed that IL-22 activates the three main mitogen-activated protein kinase (MAPK) pathways, namely p38 kinase, mitogen-activated protein kinase (MEK)/ERK and JNK/stress-activated protein kinase (SAPK) (10,11). Furthermore, it has been reported that IL-22 upregulates the expression of STAT3 and inhibits the differentiation and promotes the proliferation of keratinocytes (12). However, the regulatory effects of IL-22 on the MAPK pathway in keratinocytes have not yet been fully investigated.

CCAAT enhancer binding protein α (C/EBPα), which is a member of the C/EBP transcription factor family, serves a key role in regulating the development, proliferation and differentiation of keratinocytes (13). Additionally, C/EBPα regulates the synthesis and metabolism of phospholipids in several types of cells, including keratinocytes, fat cells and bone marrow cells (14). Previous studies have confirmed that C/EBPα is expressed in the basal lamina of epidermis and its overexpression leads to keratinocyte cycle arrest (15).

Key words: interleukin-22, CCAAT enhancer binding protein α, mitogen-activated protein kinase, keratinocytes, proliferation, differentiation
In the present study, primary cultured keratinocytes were used to evaluate the regulatory effects of IL-22 on the MAPK signaling pathway and to further investigate the role of IL-22 in the pathogenesis of psoriasis.

Materials and methods

Tissues. A total of 12 foreskin specimens were collected from young children (6-18 years old) from September to October 2011 who underwent circumcision in the Qilu Hospital of Shandong University (China). The size of the skin biopsies was 0.6x1.5 cm. The study was approved by the Ethics Committee of the Shandong University, China and all subjects provided written informed consent by their parents or guardians.

Cell culture. Foreskin samples were immediately cultured in Epilife-HKGS medium (M-EPI-500-CA; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with penicillin-streptomycin and stored on ice. Foreskin tissue was removed under aseptic conditions, following which it was rinsed, soaked and shred, then digested with 0.25% pancreatin in PBS at room temperature for ~10 min and unspecific Ab binding was prevented with 5% goat serum (cat. no. ZLI-9021; OriGene Technologies, Inc.). Specific proteins were detected by immunohistochemistry using primary antibody anti-CK10 (1:200; cat. no. ab76318), anti-involucrin (1:2,000; cat. no. ab53112; Abcam). Visualization was performed using a light microscope (magnification, x200; cat. no. CX31; Olympus Corporation).

IL-22 stimulation. Normal human epidermal keratinocytes (NHEKs) at passage two were passaged and harvested using trypsin/EDTA. The culture medium (M-EPI-500-CA; Invitrogen; Thermo Fisher Scientific, Inc.) was prepared to support a single-cell suspension. When cells adhered to the wall and cell fusion reached 60%, IL-22 (cat. no. RC-212-33; Bio Basic, Inc.) was added to 6-well plates at concentrations of 30, 60 and 90 ng/ml, and subsequently total RNA and proteins were extracted from the cells for further analysis.

Cell transfection. Before transfection, 2x10^5 cells were inoculated and cultivated in the incubator at 37°C with 5% CO2 until the cell confluence reached 50%. NHEKs were transfected with 33 nM C/EBPα or control scramble siRNAs (cat. no. siNooooo1-1-10) (both Guangzhou RiboBio Co., Ltd.) with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. The sequence of C/EBPα siRNA was 5’-GCG AAAGAAGAUUGACCCUGG-3’. Subsequent experiments were performed 24 h after cell transfection.

Reverse transcription (RT)PCR analysis. Following stimulation of keratinocytes with IL-22 for 24 h at 37°C, total RNA was isolated using TRIzol (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) and its purity and concentration were measured for further analysis. Subsequently, total RNA was reverse transcribed into cDNA using a BioTeke Super RT kit (cat. no. PR6601; BioTeke Corporation), according to the manufacturer’s instructions. The temperature protocol for reverse transcription was as follows: 30°C for 5 min, 42°C for 60 min, and 95°C for 5 min. The C/EBPα mRNA expression levels were detected using 2% agarose gel electrophoresis. A total of 10 µl/ lane PCR product was loaded with 2 µl 6X loading buffer and separated at a constant voltage of 80 v. Bands were stained with 0.5 µg/ml EB solution (cat. no. E1020; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 10 min. Bands were visualized using a Tanon-2500R gel image analysis system (Tanon Science and Technology Co., Ltd.). The primers used in the present study were synthesized by Sangon Biotech, Co., Ltd., and their sequences are presented in Table I.

Western blot assay. Following stimulation of NHEKs with IL-22 (cat. no. RC-212-33; Bio Basic, Inc.) at 37°C for 30, 60, 90 min and 48 h, cells were harvested, and total protein extracts were isolated using RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). Protein concentration was determined using a BCA protein assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology) and protein expression levels were determined using western blotting. A total of 40 µg/ lane total protein from each sample was loaded and separated via 10% SDS-PAGE, then transferred to a PVDF membrane. The membrane was blocked with blocking-buffer (cat. no. P0023B; Beyotime Institute of Biotechnology) for 1 h at room temperature, and then incubated with the primary antibody overnight at 4°C. The next day, the membrane was washed with TBST (0.1% Tween-20) and subsequently incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The following primary antibodies were used: Anti-C/EBPα (1:1,000; cat. no. ab74404), anti-CK10 (1:1,000; cat. no. ab76318), anti-involucrin (1:2,000; cat. no. ab53112; all from Abcam), anti-phosphorylated (p)-JNK1/2/3 (1:2,000; cat. no. bs-1640R), anti-JNK1/2/3 (1:2,000; cat. no. bs-2592R), anti-p38 (1:2,000; cat. no. bs-0637R), anti-p-p38 (1:2,000; cat. no. bs-5476R), anti-ERK (1:2,000; cat. no. bs-0022R) and anti-p-ERK (1:2,000; cat. no. bs-1522R; all BIOSS) and anti-β-actin (1:1,000; cat. no. 4970s; Cell Signaling Technology, Inc.). The following secondary antibody was used: Goat anti-rabbit IgG H&L (HRP) (1:5,000; cat. no. ab6721; Abcam). Visualization was performed using a BeyoECL Moon kit (cat. no. PB0018FS; Beyotime Institute of Biotechnology). The protein bands were analyzed with ImageJ software.
software (version Java1.8.0_112; imagej.nih.gov/ij/docs/index.html).

Cell viability assay. NHEKs were seeded into 96-well plates at a density of 1x10^4/well and were then incubated at 37˚C for 24 h. Subsequently, IL-22 (RC-142; Bio Basic, Inc.) was added to the wells at a final concentration of 30, 60 and 90 ng/ml. Finally, following stimulation for 12, 24, 48 and 72 h, 10 µl Cell Counting Kit-8 (CCK-8) reagent (Beyotime Institute of Biotechnology) was added to each well and the cells were cultured at 37˚C for an additional 2 h, according to the manufacturer's instructions. Optical density (OD) values were measured at 450 nm.

Statistical analysis. All statistical analyses were performed using SPSS 18.0 software (SPSS, Inc.). All data are presented as the mean ± SD from independent experiments performed in triplicate. The differences between 2 groups were analyzed using Student's t-test. In addition, when ≥3 groups were compared, the differences were analyzed using one-way ANOVA, followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of CK10 in cells assessed via immunocytochemistry. Brown staining in the cytoplasm demonstrated that keratinocytes were successfully isolated from foreskin samples (Fig. 1).

Expression levels of JNK, ERK and p38 in NHEKs following stimulation with IL-22. NHEKs were stimulated for 0, 30, 60 and 90 min with 60 ng/ml IL-22. Subsequently, total protein extracts were isolated and western blot analysis was performed to detect the expression levels of JNK, p-JNK, p38, p-p38, ERK and p-ERK. The results revealed that JNK phosphorylation was significantly increased after cell stimulation with IL-22 for 30 min and decreased at 60 min post-stimulation (Fig. 2). Accordingly, the expression levels of p-p38 and p-ERK were significantly increased at 30 min and reached the highest levels at 60 min post-stimulation. However, expression levels decreased following incubation for 90 min in the presence of IL-22.

IL-22 promotes the proliferation of keratinocytes. The effect of IL-22 on the proliferation of keratinocytes was assessed using a CCK-8 assay. Therefore, keratinocytes were stimulated for 12, 24, 48 and 72 h with 30, 60 and 90 ng/ml IL-22. The results demonstrated that IL-22 promoted the proliferation of keratinocytes in a dose- and time-dependent manner (Fig. 3).

IL-22 inhibits the differentiation of keratinocytes. Total protein extracts were isolated from keratinocytes treated with 60 ng/ml IL-22 for 48 h to detect the expression levels of keratinocyte differentiation markers, namely CK10 and involucrin. IL-22 treatment for 48 h significantly reduced the protein expression levels of CK10 and involucrin in keratinocytes compared with the control (P<0.05; Fig. 4).

IL-22 decreases the expression of C/EBPα at the mRNA and protein level. Following stimulation of keratinocytes with 30, 60 and 90 ng/ml IL-22 for 48 h, C/EBPα mRNA and protein expression levels were assessed using RT-qPCR and western blot analysis, respectively. The results showed that mRNA and protein expression levels of C/EBPα were significantly down-regulated in the IL-22 group compared with that in the control group (P<0.05) (Figs. 5 and 6). However, no statistically significant differences were observed among the different concentration groups (P>0.05).

Downregulation of C/EBPα promotes proliferation of keratinocytes. NHEKs, were transfected with C/EBPα siRNAs or control using Lipofectamine 2000 to evaluate the effect of C/EBPα on cell proliferation. The CCK-8 proliferation assay demonstrated that C/EBPα siRNA promoted the proliferation of NHEKs at 24, 48 and 72 h (all P<0.05) compared with that of the control siRNA and empty vector groups (Fig. 7). The results suggested that downregulation of C/EBPα promoted proliferation of keratinocytes.

Downregulation of C/EBPα inhibits the differentiation of keratinocytes. To investigate the effect of C/EBPα on the differentiation of keratinocytes, cells were transfected with C/EBPα siRNAs or control using Lipofectamine 2000.

Table I. Genes and primer sequences.

| Gene       | Primer sequence (5’-3’)                      |
|------------|-----------------------------------------------|
| C/EBPα     | F: ACGTGGAGACGCAGCAGAA                       |
|            | R: GTAGGCAATGGAGCCGGTGA                      |
| β-actin    | F: CACCAACTGGGACGACAT                       |
|            | R: CAGAGGCGTAGAGGGACA                       |

F, forward; R, reverse; C/EBPα, CCAAT enhancer binding protein α.
Western blot analysis revealed that involucrin and CK10 expression levels were significantly decreased in the C/EBPα siRNA group compared with those in the control siRNA and non-transfected groups (Fig. 8). The aforementioned results indicated that C/EBPα downregulation inhibited the differentiation of keratinocytes.

Discussion

Psoriasis vulgaris, one of the most common chronic inflammatory diseases in the clinic, is considered to occur as a consequence of genetic and environmental factors, leading to the induction of keratinocyte proliferation via immune-mediated signaling pathways (1,2).

A previous study has demonstrated that in psoriasis vulgaris the proliferation rate and the number of generated keratinocytes is increased by 2 and 28 times, respectively, compared with normal epidermis (16). However, the exact underlying mechanism of the increased keratinocyte proliferation rate in psoriasis remains unknown. The abnormal differentiation of keratinocytes is one of the most important features of psoriatic lesions, resulting from the abnormal expression of several keratins (17). In addition, it has been reported that transglutaminase 1 kinase, involucrin, peptidase inhibitor 3, ATP binding cassette subfamily C member 8 and CK6/CK16 proteins are upregulated in psoriatic lesions (18). Furthermore, keratin K1/K10, a characteristic protein of the terminal differentiation stage of keratinocytes, is significantly reduced in the psoriatic lesions compared with the normal skin (19). Therefore, in the present study, CK10 and involucrin were selected as differentiation markers to investigate the effect of IL-22 on keratinocyte differentiation. The results revealed that IL-22 significantly decreased the expression of CK10 and involucrin in keratinocytes. In addition, IL-22 significantly promoted keratinocyte proliferation and differentiation in a time- and dose-dependent manner.

Th17 cells, a subpopulation of CD4+ T lymphocytes that differs from Th1, Th2 and regulatory T cells, mainly secrete the cytokines IL-17 and IL-22 (20). Previous studies have confirmed that IL-22 is one of the key pro-inflammatory cytokines in inflammatory diseases (21,22). IL-22 has been found in IL-9-stimulated BW5147 cells (mouse T lymphoma cells) and exerted its biological effects by interacting with its receptor (23). An in vitro study demonstrated that IL-22 promoted the excessive proliferation and abnormal differentiation of human keratinocytes (5). Furthermore, Ma et al (24) established a psoriasis mouse model by transferring CD4+ CD45RBhi T cells from BALB/cBy to C.B-17/Prkdc<sup>scid/scid</sup> mice and revealed that subcutaneous injection of IL-22 antibodies prevented the progression of psoriasis. They also reported that the levels of Th17-related cytokines, namely IL-17A, IL-17F, IL-22 and IL-23p19, were significantly reduced (24). These findings suggest that IL-22 serves an important role in the pathogenesis of psoriasis by promoting the secretion ability of Th17 cells, which in turn acts on keratinocytes. Therefore, IL-22 may be considered the link between the immune system and keratinocytes.

A previous study has confirmed that abnormal signal transduction pathways play a critical role in the pathogenesis
of psoriasis (9). It has also been demonstrated that three main pathways, namely MEK-ERK, JNK/SAP and p38 kinase, are activated by IL-22 (25). Furthermore, IL-22 upregulates the expression of STAT3, inhibits keratinocyte differentiation, promotes keratinocyte proliferation and induces the formation of psoriatic lesions (12). Additionally, the activity of the MAPK signaling pathway is significantly increased in psoriatic lesions (26). Notably, in psoriasis, p-ERK is upregulated in

Figure 4. IL-22 significantly decreases the expression of CK10 and involucrin. Following stimulation for 48 h, the protein expression levels of CK10 and involucrin were determined in the control and IL-22-treated (60 ng/ml) groups using western blotting. β-actin served as an internal control. The intensity of the immunoreactive bands was measured using ImageJ. Experiments were performed independently three times. *P<0.01 vs. control. IL-22, interleukin 22; CK10, cytokeratin 10.

Figure 5. Effect of different concentrations of IL-22 on C/EBPα mRNA expression. The results are presented as the mean ± SD of three independent experiments. **P<0.01 vs. control. IL-22, interleukin 22; C/EBPα, CCAAT enhancer binding protein α.

Figure 6. Effect of different concentrations of IL-22 on C/EBPα protein expression. Following stimulation for 48 h, the protein expression levels of C/EBPα at different concentrations of IL-22 were determined using western blot analysis. β-actin served as an internal control. The intensity of the immunoreactive bands was measured using ImageJ. Experiments were performed independently three times. *P<0.05, **P<0.01 vs. control. IL-22, interleukin 22; C/EBPα, CCAAT enhancer binding protein α.
the base layer and stratum spinosum cell nucleus, while in the normal skin p-ERK is only expressed in the basal layer (27). JNK and p38 kinase are expressed in the granular layer of the normal skin and in both the granular layer and stratum spinosum of the psoriatic lesions (28). The results of the present study revealed that primary cultured keratinocytes treated with 60 ng/ml IL-22 for 30 min upregulated the expression of p-ERK, p-JNK and p-p38, indicating that IL-22 was involved in the activation of the MAPK pathway in keratinocytes.

C/EBPα, a member of the C/EBP family, was first isolated from rat liver cell nuclei by McKnight in 1987. C/EBP α is a heat-stable transcription factor that interacts with the CCAAT motif (29). Previous studies have shown that C/EBP α is involved in the regulation of keratinocyte differentiation and its expression is gradually elevated in stratum spinosum, granular layer and stratum corneum keratinocytes in a space- and time-dependent manner (13,15), which indicated that C/EBPα was expressed in basal layer keratinocytes, and is upregulated as keratinocytes exit the basal layer, enter the spinous and granular layers and undergo terminal differentiation. Maytin and Habener (30) found that CK10 was primarily expressed in BALB/MK keratinocytes following stimulation with 0.12 mM Ca²⁺ for 24 h and its expression was decreased after 48 h. However, the expression levels of C/EBPα were increased by 5 times (31). In the terminal differentiation stage, the markers of spinous and granular layer, K1/K10, are induced by C/EBP binding to DNA (13). The expression levels of C/EBPα and CK10 over time (C/EBPα at first, CK10 later) and at different sites (C/EBPα in the basal layer and CK10 in the spinous layer) suggests that these molecules may be involved in the differentiation of keratinocytes. C/EBPα is considered an important negative regulator of cell proliferation and its anti-tumor effects have been reported in numerous types of skin tumors (31). For example, Loomis et al (32) demonstrated that epidermal-specific C/EBP α knockout mice were highly susceptible to skin squamous carcinoma. However, the underlying mechanisms of C/EBPα in regulating cell proliferation remains unclear. It has been considered that C/EBPα interacts with several cell cycle proteins in its dimer form, it can interacts directly with CDK2 and CDK4 enzymes to prevent cyclin binding thus regulating the cell cycle (33). In addition, C/EBPα regulates, stabilizes and activates the cell cycle inhibitor p21 or directly inhibits CDK2-, CDK4- and E2F-induced transcription. These findings indicate that C/EBPα may block the cell cycle process and inhibit cell proliferation (34). In the present study the expression levels of C/EBPα were significantly decreased in keratinocytes following treatment with IL-22, suggesting a potential role in the pathogenesis of psoriasis vulgaris. Additionally, the MAPK signaling pathway may act as a key molecule in the interaction between IL-22 and C/EBPα. Therefore, elevated IL-22 expression levels in psoriasis vulgaris may activate the MAPK
signaling pathway, which in turn may mediate the inactivation of C/EBPα. However, the mechanism of cell signal transduction is complex. A signal transducer may not only participate in the cell signal transduction of one pathway, and functional molecules in one signal transduction pathway may affect and regulate other pathways. The present study primarily investigated the relationship between IL-22 and the MAPK signaling pathway. Moreover, it was found that the effect of IL-22 on the proliferation and differentiation of keratinocytes may be mediated via the regulation of the MAPK signaling pathway. The result of this study cannot exclude whether there are other signaling pathways that play a role in the regulatory mechanism of IL-22. This will be further studied in the future.

In conclusion, the present study demonstrated that IL-22 promoted proliferation and inhibited differentiation of keratinocytes. The C/EBPα expression levels were significantly decreased in IL-22-treated keratinocytes. Furthermore, downregulation of C/EBPα promoted keratinocyte proliferation and decreased the expression of CK10 and involucrin. Therefore, IL-22 may contribute to the abnormal proliferation and differentiation of keratinocytes via downregulating the expression of C/EBPα. Finally, the results suggest that the IL-22/MAPK/C/EBPα axis may be involved in the pathogenesis of psoriasis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

HZ and LZ designed the present study and performed the experiments, and should be regarded as corresponding and first author, respectively. WM analyzed and interpreted patients’ data. JY contributed to the clinical diagnosis and histological examination during the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Qilu Hospital of Shandong University, China and was in accordance with The Declaration of Helsinki (1964) and its later amendments. All patients provided written informed consent for publication of the results.

Patient consent for publication

All patients provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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