

IL-17 promotes proliferation, inflammation and inhibits apoptosis of HaCaT cells via interacting with the TRAF3 interacting protein 2

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Abstract. The present study aimed to investigate the effects of interleukin-17 (IL-17) on the function of keratinocytes and to further investigate its associated mechanism. Human immortalized epidermal cells (HaCaT) were divided into sham control group (Sham), TRAF3 interacting protein 2 (TRAF3IP2)-knockdown with lentivirus group (si-TRAF3IP2), sham control+IL-17 group (Sham+IL-17) and TRAF3IP2-knockdown with lentivirus+IL-17 group (si-TRAF3IP2+IL-17). MTT and flow cytometry assays demonstrated that IL-17 promoted proliferation and inhibited apoptosis of HaCaT cells, while this effect was reversed following knockdown of TRAF3IP2 with lentiviral vectors. In addition, a marked increase in the levels of IL-6, IL-8, IL-23, TNF- α and VEGF was observed in the Sham+IL-17 group compared with that noted in the Sham group ($P < 0.05$). Furthermore, reverse transcription-quantitative polymerase chain reaction and western blotting indicated that the mRNA and protein expression levels of caspase-3 in the si-TRAF3IP2+IL-17 group were significantly increased compared with those of the Sham+IL-17 group ($P < 0.05$). Taken together, the results indicated that IL-17 promoted proliferation and inflammation and inhibited apoptosis of HaCaT cells by interacting with the TRAF3IP2 adaptor protein, while knock-down of the expression of TRAF3IP2 reduced the effects of IL-17 in HaCaT cells.

Introduction

Psoriasis is a chronic inflammatory skin disease with a long course and a tendency to relapse. Various factors, including bacterial infection, and immune-mediated, genetic and environmental factors serve an important role in the development of psoriasis (1). In the general population, the incidence of psoriasis ranges between 0.6 and 4.8% (2). The clinical manifestations of psoriasis are red scaly plaques that can occur on any part of the body (3). Disfiguration, disability and associated comorbidity affect the physical health and mental status of the patients considerably (4). Therefore, the development of novel therapeutic strategies is imperative for the successful treatment of psoriasis.

Interleukin-17 (IL-17) is an inflammatory cytokine with diverse roles in immune protection and immunopathology (5,6). IL-17 promotes the activation of T cells and stimulates epithelial cells, endothelial cells and fibroblasts to produce a variety of cytokines, which leads to inflammation (7). However, during abnormal expression or overexpression of IL-17, chronic inflammation is caused that is associated with several inflammatory disorders and autoimmune diseases (8). IL-17 participates in the occurrence and development of various immune diseases and inflammatory conditions by binding to the IL-17 receptor. This causes signal transduction and the induction of pro-inflammatory factors in order to mediate defense responses (9). Therefore, IL-17 is considered an attractive therapeutic target.

Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are key regulatory proteins in the TNF receptor-associated signaling pathway. They are characterized by a helical coiled structure and a conserved TRAF domain at the C-terminus (10). TRAF3 is one of the most versatile members of the TRAF family. It is an intracellular protein that primarily acts as an adaptor protein with a large variety of binding partners (11). TRAF3 interacting protein 2 (TRAF3IP2), also called CIKS or Act1, is a key intermediate in the normal inflammatory response and is involved in the pathogenesis of various autoimmune and inflammatory diseases (12). Furthermore, TRAF3IP2 is a protein acting downstream of the IL-17 receptor that binds to and stabilizes mRNAs encoding key inflammatory proteins (9).

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Human keratinocytes account for >95% of human epidermal cells. Human immortalized epidermal cells (HaCaT) cells are immortalized cells with human keratinocyte differentiation and proliferation characteristics that have been widely used for studies involving skin biology and differentiation (13). Therefore, the present study aimed to investigate the effects of IL-17 on the proliferation, apoptosis and inflammation of HaCaT cells and to further investigate its mechanism of action. The experiments aimed to identify novel molecular targeted drugs or therapeutic targets that may effectively treat psoriasis.

Materials and methods

Cell culture. HaCaT cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Beijing Solarbio Science & Technology Co., Ltd.) and 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) at 37°C, in the presence of 5% CO₂. Growth phase cells were used for subsequent experiments.

Cell transfection and grouping. HaCaT cells in the logarithmic growth phase were adjusted to a density of 5x10⁵ cells/ml with trypsin and incubated in a 6-well plate. When the cells reached 80% confluence, transfection was performed according to manufacturer's protocols of the lip2000 lentiviral transfection kit (Shanghai Jikai Biotechnology Co., Ltd.). The cells were divided into the 5 following groups: i) sham control group (Sham), which included cells transfected with an empty vector (Shanghai Jikai Biotechnology Co., Ltd.); ii) lentiviral-mediated TRAF3IP2-knockdown group (si-TRAF3IP2; 10/250 μl), which included cells transfected with si-TRAF3IP2 (Shanghai Jikai Biotechnology Co., Ltd.); iii) sham control + IL-17 group (Sham + IL-17), which included cells transfected with empty carrier and cultured with 10 ng/ml IL-17A; iv) lentiviral-mediated TRAF3IP2-knockdown+IL-17 group (si-TRAF3IP2+IL-17), which included cells transfected with si-TRAF3IP2 and cultured with 10 ng/ml IL-17A; and v) negative control siRNA group (si-NC), which included cells transfected with scrambled siRNA (Shanghai Jikai Biotechnology Co., Ltd.). All cell groups were cultured for 48 h. The si-NC group was used as the transfection control. The target sequence of si-TRAF3IP2 was 5'-CCCCAAATACAAACAGGACGT-3'. The carrier frame structure of si-TRAF3IP2 was GV248 hU6-MCS-Ubiquitin-EGFP-IRES-puromycin. The si-NC sense strand was 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense strand was 5'-ACGUGACACGUUCGGAGA ATT-3'.

MTT assay. A total of 100 μl of cells were incubated in 96-well plates. The cells were incubated for 24, 36, 48 and 72 h following transfection and IL-17 administration, and 100 μg MTT was added. The cells were incubated at 37°C for 4 h and the supernatant was dissolved with 200 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). A plate reader (Bio-Rad Laboratories, Inc.) was used to assess the optical density of

each well at 495 nm. The relative absorbance of each group was calculated by the following equation:

$$\text{Relative absorbance} = (\text{OD}_{\text{Experiment}} - \text{OD}_{\text{Blank}}) / (\text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}})$$

Flow cytometry. The cells in the different groups were collected and resuspended with pre-chilled PBS (1X) and centrifuged at 11,180 x g for 5 min at 4°C. Following washing twice with precooled (4°C) sterile PBS solution, the cells were fixed overnight with 70% ethanol and incubated at 4°C. Following another wash with PBS, 100 μl Rnase A was added to resuspend the cells. The cells were heated in a waterbath at 37°C for 30 min. Subsequently, 400 μl propidium iodide (PI) was added and the samples were incubated in the dark for 30 min. The cell cycle was detected using a flow cytometer (Gallios; Beckman Coulter, Inc.). The AnnexinV-FITC apoptosis assay kit (BD Biosciences) was used for apoptosis detection. Following washing, 100 μl binding buffer (1X) was added to the cells, and the resulting mixture was incubated with 5 μl Annexin V-FITC in the dark for 15 min. Subsequently, 10 μl PI was added and mixed with the samples. The CellQuest 5.1 software (BD Biosciences) was used to analyze the data.

ELISA. The cell supernatants were collected and the levels of IL-6 (cat. no. HEA079Hu03; v.), IL-8 (cat. no. SEA080Si03; Saihongrui Technology Co., Ltd.), IL-23 (cat. no. SEA384Mu02; Saihongrui Technology Co., Ltd.), TNF-α (cat. no. E-EL-M0049c; Elabscience Biotechnology Inc.) and VEGF (cat. no. GRJ13-009; Guangrui Biotechnology Co., Ltd.) in the cells were detected in strict accordance with the manufacturer's protocols of the ELISA kit. The optical densities at 450 nm were measured using a microplate reader (RT-6100; Lei Du).

Reverse transcription-quantitate polymerase chain reaction (RT-qPCR). The cells in the different groups were collected and centrifuged at 4°C, at 12,000 rpm for 15 min. Total RNA was extracted using the TRIzol reagent (Takara Bio, Inc.). The cDNA was synthesized using the reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The SYBR-Green PCR kit (Qiagen, Inc.) was used as a fluorophore. The RT-qPCR assay was performed in a Mastercycler® nexus X2 (Eppendorf) using 2 μl cDNA as a template under the following conditions: 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min for 40 cycles. The data were processed using the 2^{-ΔΔCq} method (14) and the relative expression levels were calculated using GAPDH mRNA as an internal reference. The sequences of the primers (Shanghai Sheng gong Bioengineering Technology Service Co., Ltd.) used were as follows: TRAF3IP2 forward, 5'-CTGCGTCTG AGTCTGTGGTT-3' and reverse, 5'-TATCCCGTGTCTATG GTTGG-3'; caspase-3 forward, 5'-ATGGAGAACAATAAA ACCT-3' and reverse, 5'-CTAGTGATAAAAGTAGATGTC-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCCCTGTTGCTGTAGCCAAA-3'.

Western blot analysis. The cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology) and the protein concentration was measured using the bicinchoninic acid kit (Beijing Solarbio Science & Technology Co., Ltd.). Each protein sample (~40 μg/lane) was separated on 10% sodium

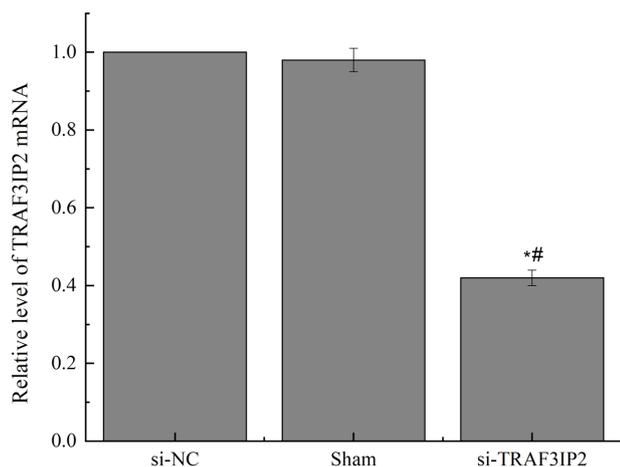


Figure 1. The mRNA expression of TRAF3IP2 in different groups. The values are indicative of the mean \pm standard deviation. * P <0.05 compared with the si-NC group; # P <0.05 compared with the Sham group. si, small interfering RNA; NC, negative control; Sham, Sham control group; TRAF3IP2, TRAF3 interacting protein 2.

dodecyl sulfate polyacrylamide gels by electrophoresis (Mini-protean-3; Bio-Rad Laboratories, Inc.) and transferred onto polyvinylidene difluoride membranes (Merck & Co., Inc.). Subsequently, the membranes were blocked with 5% skimmed milk and incubated overnight at 4°C with primary rabbit anti-human antibodies against caspase-3 (dilution, 1:1,000; cat. no. ab13847; Abcam) and GAPDH (dilution, 1:1,000; cat. no. ab70699; Abcam). The following day, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (dilution, 1:1,000; cat. no. ABIN101988; Antibodies Online) was used at room temperature for 1 h. The membranes were observed and recorded using the enhanced chemiluminescence system (ImageQuant LAS 4000; General Electric Company) for 3-5 min. Protein expression levels were normalized to GAPDH, scanned and quantified using the ImageJ 1.46r software (National Institutes of Health).

Statistical analysis. All experiments were repeated at least three times. Statistical analysis was performed using SPSS 19.0 (IBM Corp.). The mean \pm standard deviation was used to measure the data. Significant differences between two groups were assessed using a Student's *t*-test. Pairwise comparisons were performed using the one-way analysis of variance test, followed by Tukey's post hoc test. P <0.05 was considered to indicate a statistically significant difference.

Results

Expression of TRAF3IP2 mRNA in different groups. The transfection efficiency was detected by RT-qPCR (Fig. 1). The TRAF3IP2 mRNA expression in the si-NC and Sham groups were not significantly different (P >0.05). By contrast, TRAF3IP2-knockdown using a lentivirus significantly decreased the expression of TRAF3IP2 mRNA (P <0.05). This result indicated that the transfections were successful.

Effects of IL-17 on the proliferation of HaCaT cells. Cell proliferation ability was detected by MTT assays (Fig. 2). The

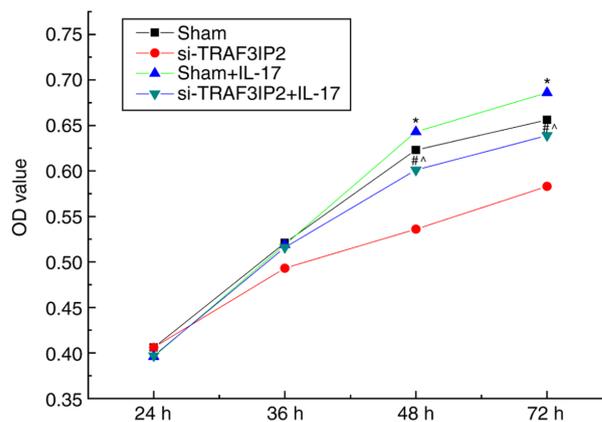


Figure 2. The OD values of different groups. The values are indicative of the mean \pm standard deviation. * P <0.05 compared with the Sham group; # P <0.05 compared with the si-TRAF3IP2 group; #A P <0.05 compared with the Sham+IL-17 group. OD, optical density; Sham, Sham control group; si, small interfering RNA; TRAF3IP2, TRAF3 interacting protein 2; IL-17, interleukin-17.

si-TRAF3IP2 HaCaT cell group exhibited decreased proliferation compared with the Sham control cell group. On the other hand, in si-TRAF3IP2 HaCaT cells treated with IL-17 (si-TRAF3IP2 + IL-17), the cell proliferation was significantly decreased compared with the Sham control cell group (P <0.05). Furthermore, HaCaT cells treated with IL-17 exhibited increased proliferation compared with the Sham group and si-TRAF3IP2 + IL-17 group. In addition, compared with the Sham + IL-17 group, the proliferation ability was significantly decreased in the si-TRAF3IP2 + IL-17 group (P <0.05). These findings suggested that IL-17 promoted the proliferation of HaCaT cells by interacting with the TRAF3IP2 adaptor protein.

Effects of IL-17 on the cell cycle of HaCaT cells. The percentage of cells in the G1 phase of the cell cycle in the Sham+IL-17 group (58.32 \pm 3.21%) was significantly higher than that of the Sham group (Fig. 3; 54.18 \pm 3.19%; P <0.05). However, the percentage of cells in the G1 phase of the si-TRAF3IP2+IL-17 group was decreased to 50.88 \pm 2.36%, which was significantly lower than that in the Sham+IL-17 group (P <0.05). The results further indicated that IL-17 promoted the transition of the cell cycle from the G1 to the S phase and the proliferation of HaCaT cells via interacting with the TRAF3IP2 adaptor protein. Knockdown of TRAF3IP2 expression decreased the efficacy of IL-17 on the proliferation of HaCaT cells.

Effects of IL-17 on the induction of apoptosis of HaCaT cells. Flow cytometric analysis was used to detect the induction of cell apoptosis (Fig. 4). The number of apoptotic cells in the Sham+IL-17 group (4.40 \pm 0.19%) was significantly lower than that in the Sham group (5.02 \pm 0.23%; P <0.05). However, the percentage of apoptotic cells in the si-TRAF3IP2+IL-17 group (6.83 \pm 0.32%) was significantly higher than that in the Sham group (P <0.05), indicating that IL-17 inhibited the apoptosis of HaCaT cells by interacting with the TRAF3IP2 adaptor protein. Knockdown of the expression of TRAF3IP2 decreased the efficacy of IL-17 on the induction of apoptosis of HaCaT cells.

Effects of IL-17 on the levels of inflammatory factors in HaCaT cells. The cell supernatant cytokine levels were

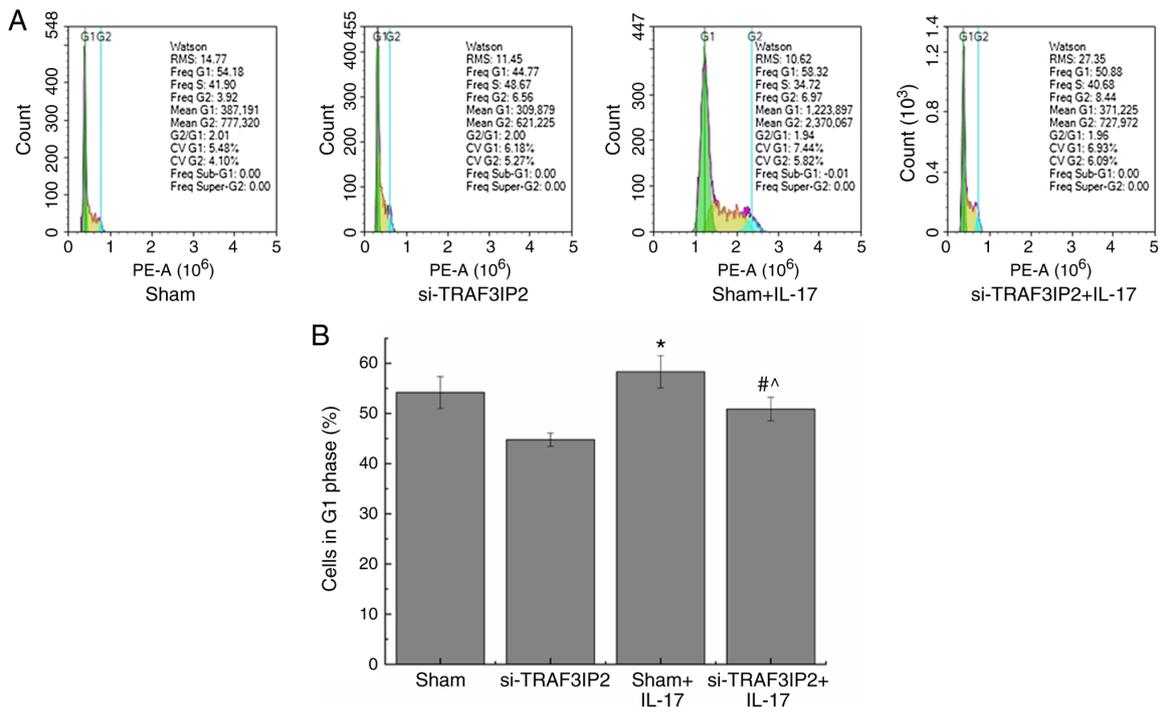


Figure 3. Cell cycle analysis of different cell groups. (A) Cell cycle, (B) Cells in G1 phase (%). The values are indicative of the mean \pm standard deviation. * $P < 0.05$ compared with the Sham group; # $P < 0.05$ compared with the si-TRAF3IP2 group; ^ $P < 0.05$ compared with the Sham+IL-17 group. Sham, Sham control group; si, small interfering RNA; TRAF3IP2, TRAF3 interacting protein 2; IL-17, interleukin-17.

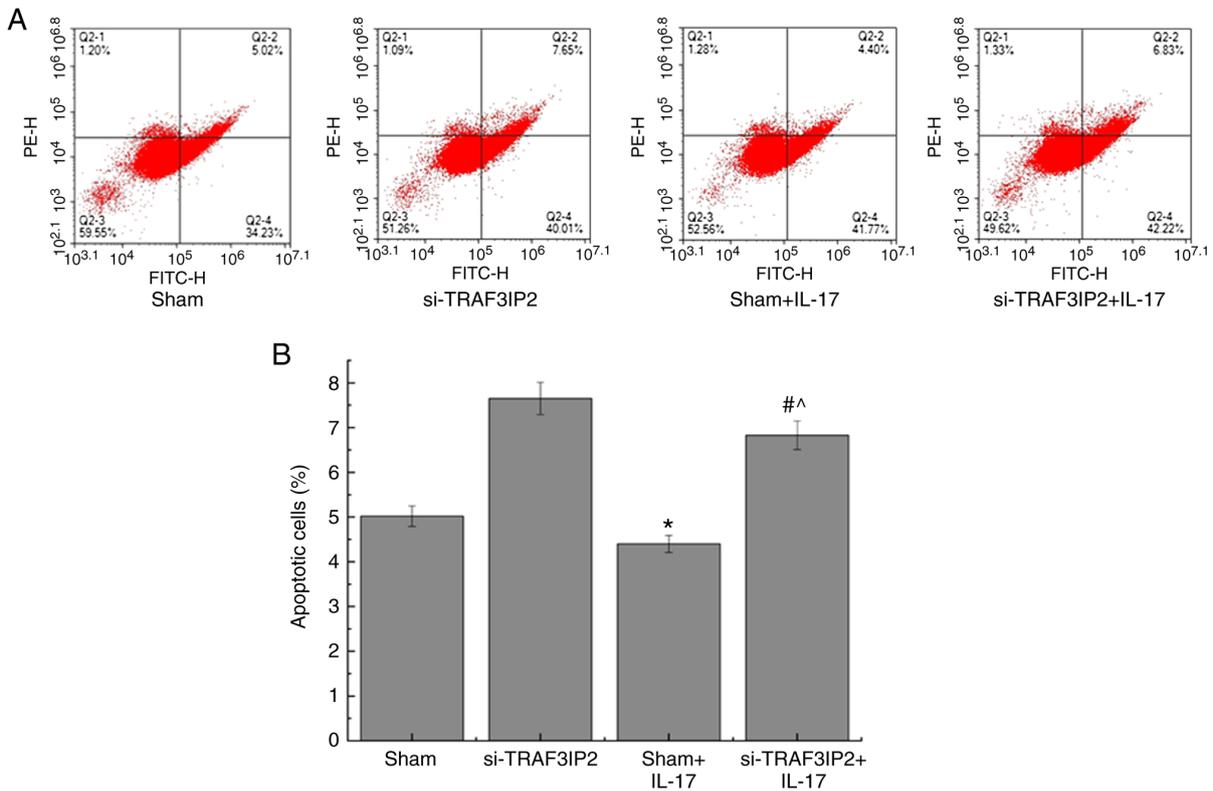


Figure 4. Changes in the percentage of apoptotic cells noted in the different groups. (A) Flow cytometry. (B) Apoptotic cells (%). The values are mean \pm standard deviation. * $P < 0.05$ compared with Sham group; # $P < 0.05$ compared with si-TRAF3IP2 group; ^ $P < 0.05$ compared with Sham+IL-17 group. Sham, Sham control group; si, small interfering RNA; TRAF3IP2, TRAF3 interacting protein 2; IL-17, interleukin-17.

analyzed in order to reflect the inflammatory response. The expression levels of IL-6, IL-8, IL-23, TNF- α and

VEGF were significantly increased in the Sham+IL-17 groups compared with those in the Sham group (Fig. 5;

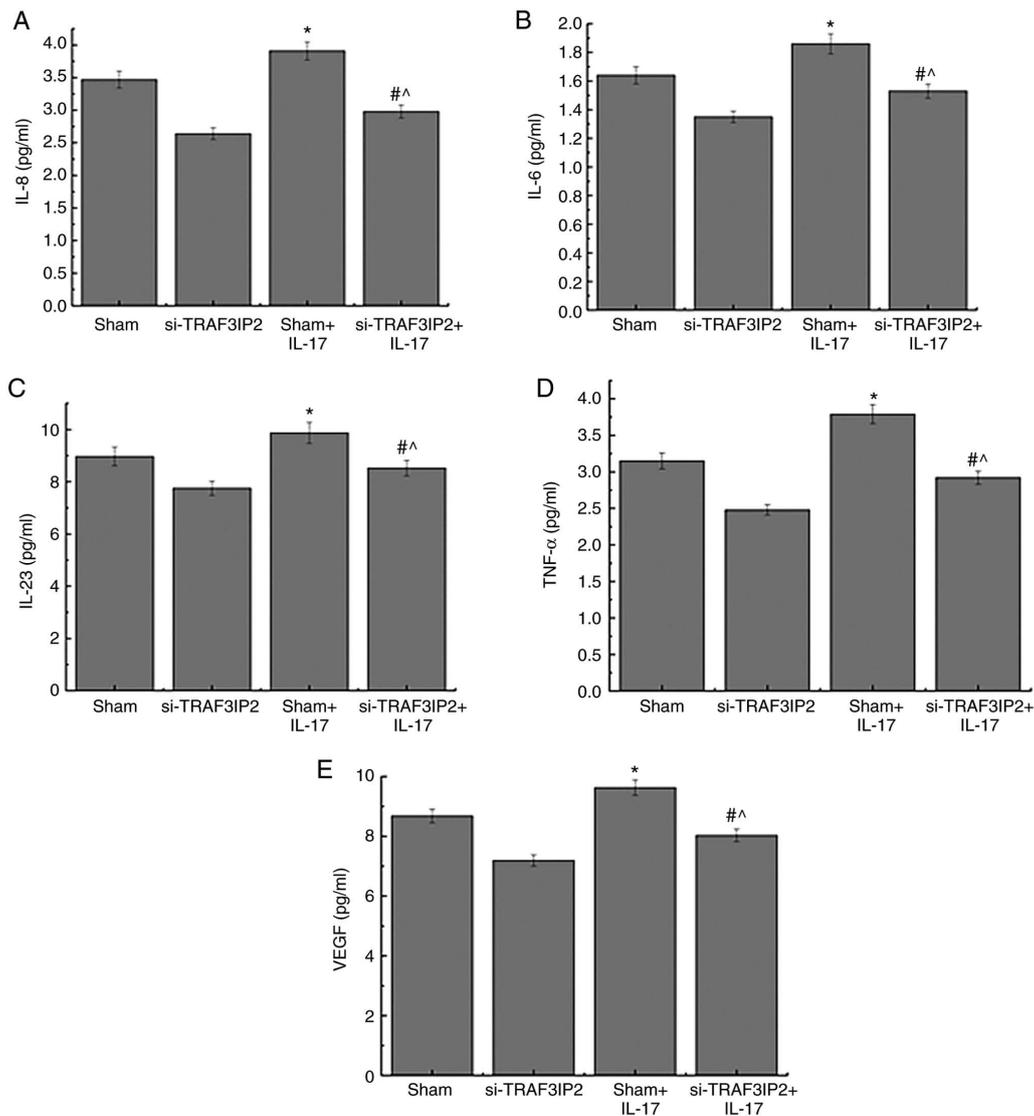


Figure 5. The levels of the inflammatory factors in the different groups. (A) IL-8. (B) IL-6. (C) IL-23. (D) TNF- α . (E) VEGF. The values are indicative of the mean \pm standard deviation. * $P < 0.05$ compared with the Sham group; # $P < 0.05$ compared with the si-TRAF3IP2 group; ^ $P < 0.05$ compared with the Sham+IL-17 group. IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; Sham, Sham control group; si, small interfering RNA; TRAF3IP2, TRAF3 interacting protein 2.

$P < 0.05$). However, significant decreases in the levels of IL-6, IL-8, IL-23, TNF- α and VEGF were observed in the si-TRAF3IP2+IL-17 group compared with those in the Sham+IL-17 groups ($P < 0.05$). These findings indicated that IL-17 promoted the inflammation of HaCaT cells by interacting with the TRAF3IP2 adaptor protein, while knockdown of TRAF3IP2 expression decreased the inflammatory efficacy of IL-17 on HaCaT cells.

Effects of IL-17 on the expression levels of caspase-3 in HaCaT cells. RT-qPCR and western blot analyses were used to investigate the mRNA and protein expression levels of caspase-3 in HaCaT cells, respectively (Fig. 6). The mRNA and protein expression levels of caspase-3 were decreased in the Sham+IL-17 group compared with those in the Sham group. Furthermore, a significant increase in the mRNA and protein expression levels of caspase-3 was noted in the si-TRAF3IP2+IL-17 group compared with those of the Sham+IL-17 group. These results demonstrated that IL-17

decreased the expression levels of caspase-3 by interacting with the TRAF3IP2 adaptor protein in HaCaT cells. These effects were reversed by lentiviral-mediated knockdown of TRAF3IP2.

Discussion

Psoriasis is a programmed pathological interaction among skin cells, and immune-mediated genetic and environmental factors (1). The hallmarks of psoriasis include deep-level epidermal modifications, including hyperproliferation and changes in keratinocyte differentiation, along with significant inflammatory cell infiltration and neovascularization (15). The concept of using anti-IL-17 as a potential treatment agent is not novel. The FDA approved secukinumab in 2015 and ixekizumab in 2016 to treat adults with moderate-to-severe plaque psoriasis (16). Furthermore, TRAF3IP2 is one of the genetic factors implicated in psoriasis that is involved in the IL23/IL-17 axis (17). Therefore, the present study investigated

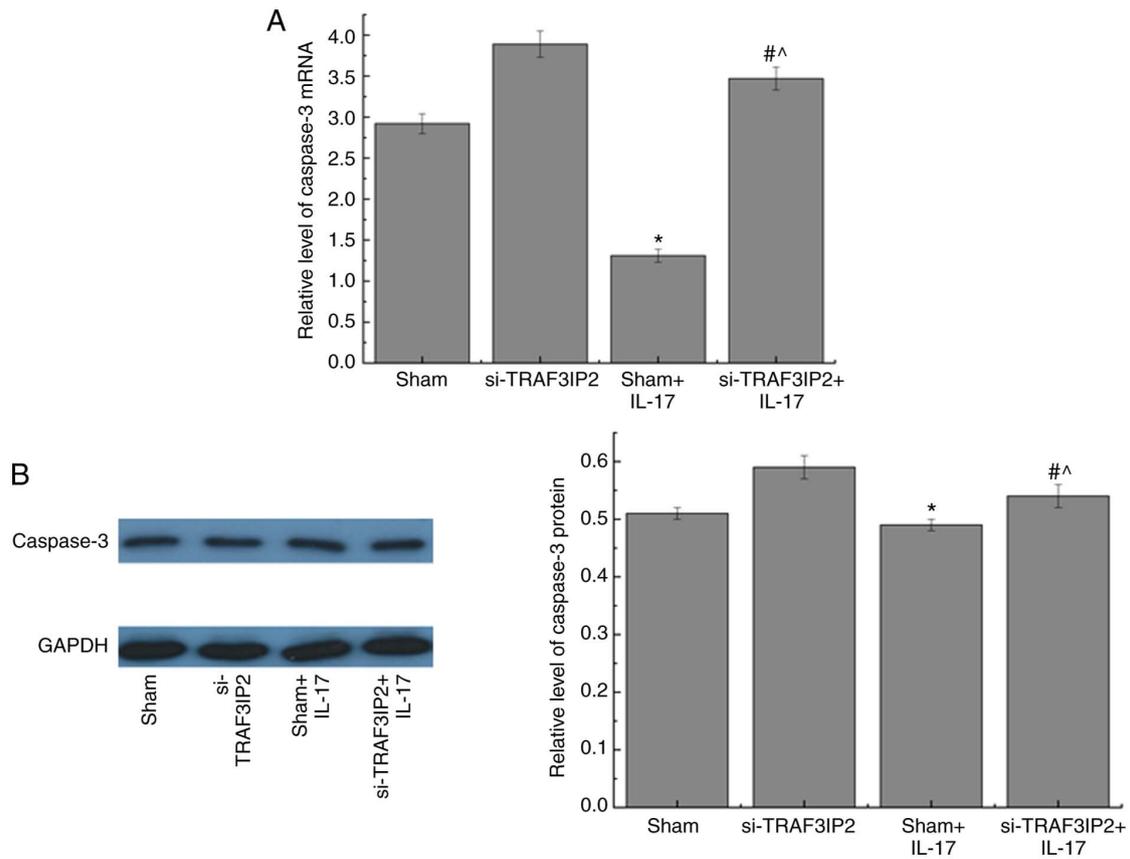


Figure 6. The mRNA and protein expression levels of caspase-3 in the different groups. (A) Reverse transcription-quantitative polymerase chain reaction. (B) Western blotting. The values are indicative of the mean \pm standard deviation. * $P < 0.05$ compared with the Sham group; [#] $P < 0.05$ compared with the si-TRAF3IP2 group; [^] $P < 0.05$ compared with the Sham+IL-17 group. Sham, Sham control group; TRAF3IP2, TRAF3 interacting protein 2; si, small interfering RNA; IL-17, interleukin-17.

the role of IL-17 as a key effector molecule on psoriasis and investigated its association with TRAF3IP2.

Cell proliferation is one of the important physiological functions of viable cells. However, several diseases, including psoriasis, are associated with abnormal cell proliferation, migration and invasion (18,19). The results of the MTT assay indicated that IL-17 promoted the proliferation of HaCaT cells via interacting with the TRAF3IP2 adaptor protein. The complete cell cycle includes the G1, S and G2 phases and the mitotic phase (20). Lentiviral-mediated TRAF3IP2-knockdown can affect the cell cycle and cause abnormal arrest of the cells in the G1 phase, thereby reducing the number of cells entering the S and G2 phases and causing a reduction in the proliferation index. Apoptosis is a basic biological phenomenon of cells that is required to maintain the stability of the intracellular environment. The caspase family of enzymes is an important mediator of programmed cell death (apoptosis) and caspase-3 is directly involved in the transduction of apoptotic signals (21,22). In the present study, the apoptotic rate, and mRNA and protein expression levels of caspase-3 were significantly increased in the IL-17+si-TRAF3IP2 group compared with those in the Sham+IL-17 group. These findings demonstrated that IL-17 promoted proliferation and inhibited apoptosis of HaCaT cells, while knockdown of TRAF3IP2 expression decreased the efficacy of IL-17.

Psoriasis is a chronic recurrent inflammatory skin disease that interacts with multiple factors, including immune-mediated genetic and environmental factors (1). The number of

Th17 cells is increased in the peripheral blood of patients with psoriatic disease and its effector IL-17 serves an important role in the severity of psoriasis (15). IL-17 stimulates the production of VEGF, IL-6, IL-8, granulocyte-macrophage colony stimulating factor, IL-23, TNF- α and other cytokines from macrophages (23,24). VEGF is a highly specific vascular endothelial cell growth factor that promotes increased vascular permeability, extracellular matrix degeneration, and vascular endothelial cell migration, proliferation and angiogenesis (25). IL-6, IL-8, IL-23 and TNF- α are cytokines with multiple biological effects and are considered important inflammatory factors. Various cytokines can cause a chronic inflammatory state in skin cells and alter epidermal hyperproliferation, differentiation, apoptosis and neoangiogenesis that result in the production of skin diseases. In the present study, ELISA indicated that IL-17 promoted the inflammation of HaCaT cells by interacting with the TRAF3IP2 adaptor protein. Taken together, the results indicated that IL-17 served a crucial role in the pathogenesis and development of psoriasis and that knockdown of TRAF3IP2 may be considered a novel therapeutic target for psoriasis.

The present study demonstrated that IL-17 promoted proliferation and inflammation, while inhibiting the apoptosis of HaCaT cells. The possible mechanism of action may be via its interaction with the TRAF3IP2 adaptor protein. In addition, the data provided novel evidence on the role of TRAF3IP2 as a new therapeutic target for psoriasis. IL-17 may regulate proliferation, apoptosis and inflammation of human immortalized

epidermal cells by interacting with the TRAF3IP2 adaptor protein. However, the lack of co-immunoprecipitation experiments to prove the hypothesis that IL-17 interacts with TRAF3IP2 is a limitation of the present study.

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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

JiZ conducted the experiments, data collection and interpretation. YS participated in study design, coordination of the experiments and data collection. JuZ participated in the study design, data collection and data analysis and prepared the manuscript. QL and LC participated in study design, data analysis, data interpretation and wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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