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Shikonin induces apoptosis and suppresses growth in keratinocytes via CEBP-δ upregulation

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A B S T R A C T

Shikonin is an active compound of the oriental medicinal plant, *Leptospermum erythrorhizon,* which has been previously shown to inhibit psoriasis-like inflammation. However, the underlying mechanism is unclear. In the present study, the mechanisms of keratinocyte proliferation and apoptosis in psoriasis in response to shikonin were explored both in vitro and in vivo. Our results showed that shikonin significantly inhibits cell proliferation and induces apoptosis in both HaCaT and LV-STAT3 HaCaT cells by targeting CEBPD, while a decrease in cell survival, proliferation and viability were found through flow-cytometry and MTS assay. Furthermore, gavage with shikonin markedly alleviated psoriasis-like manifestations in IMQ-induced BALB/c mice clinically (PASI Score) and histopathologically. Immunohistochemistry revealed that shikonin potently suppresses the JAK/STAT3 signaling pathway in local skin lesions and increases CEBPD expression. These results imply that shikonin inhibits keratinocyte proliferation and induces apoptosis, which results in psoriasis treatment through the JAK/STAT3 dependent pathway. In addition, the activation of JAK/STAT3 downregulates CEBPD in HaCaT cells and IMQ-induced BALB/c mice. However, shikonin can reverse these effects, suggesting that CEBPD may be a potential therapeutic target for psoriasis.

1. Introduction

Psoriasis is a chronic inflammatory skin ailment that mainly manifests as hyperkeratosis and parakeratosis of keratinocytes, affecting 2–3% individuals worldwide [1,2]. It is also considered to be a systemic inflammatory disease [3,4]. Moreover, transcription factor STAT3 plays a key role in the development and pathogenetic mechanisms of psoriasis and related conditions [5,6].

CCAAT/enhancer-binding protein delta (CEBPD) encodes a 269 amino acid protein of the CEBP family, which includes transcription factors that modulate cell differentiation, metabolism and immune reactions [7,8]. Borrelli et al. [9] through IHC showed that in skin tumors C/EBPd is not expressed in basal cell cancer, while most squamous cell cancers show high C/EBPd protein levels. This indicates that C/EBPd has an important function in the final steps of keratinocyte differentiation.

Shikonin is an active compound of the oriental traditional plant, *Leptospermum erythrorhizon,* which was first used in medicine due to its antitumor effects. Additionally, shikonin and its derivatives display anti-angiogenetic, anti-inflammatory and anti-glycolytic features [10–12]. Liu et al. [13] have indicated that IFN-γ promotes K17 protein overexpression in HaCaT cells via STAT3 activation, with shikonin inhibiting this effect in part by interfering with STAT3. Xu et al. [14] demonstrated that shikonin reduces IL-17-associated VEGF overexpression by blocking JAK2/STAT3.

In a preliminary study, we found that CEBPD expression in psoriasis tissues is lower than that of normal skin tissues. Meanwhile, JAK/STAT3 related genes in the HaCaT cell line were screened after stimulation with IL-17 and shikonin using PCR Array technology. The results show that CEBPD exhibited a decrease after stimulation with IL-17. Interestingly, shikonin can reverse this effect (unpublished results). In the present study, mechanisms of keratinocyte proliferation and apoptosis in psoriasis in response to shikonin were explored. We demonstrated that JAK/STAT3 induction contributes to CEBPD downregulation in psoriasis, by inhibiting or activating STAT3 expression. In addition, shikonin, an effective drug for the treatment of psoriasis, efficiently inhibits JAK/STAT3 activation and increases CEBPD expression, while suppressing proliferation and inducing apoptosis of keratinocytes in psoriasis, both in vitro and in vivo.

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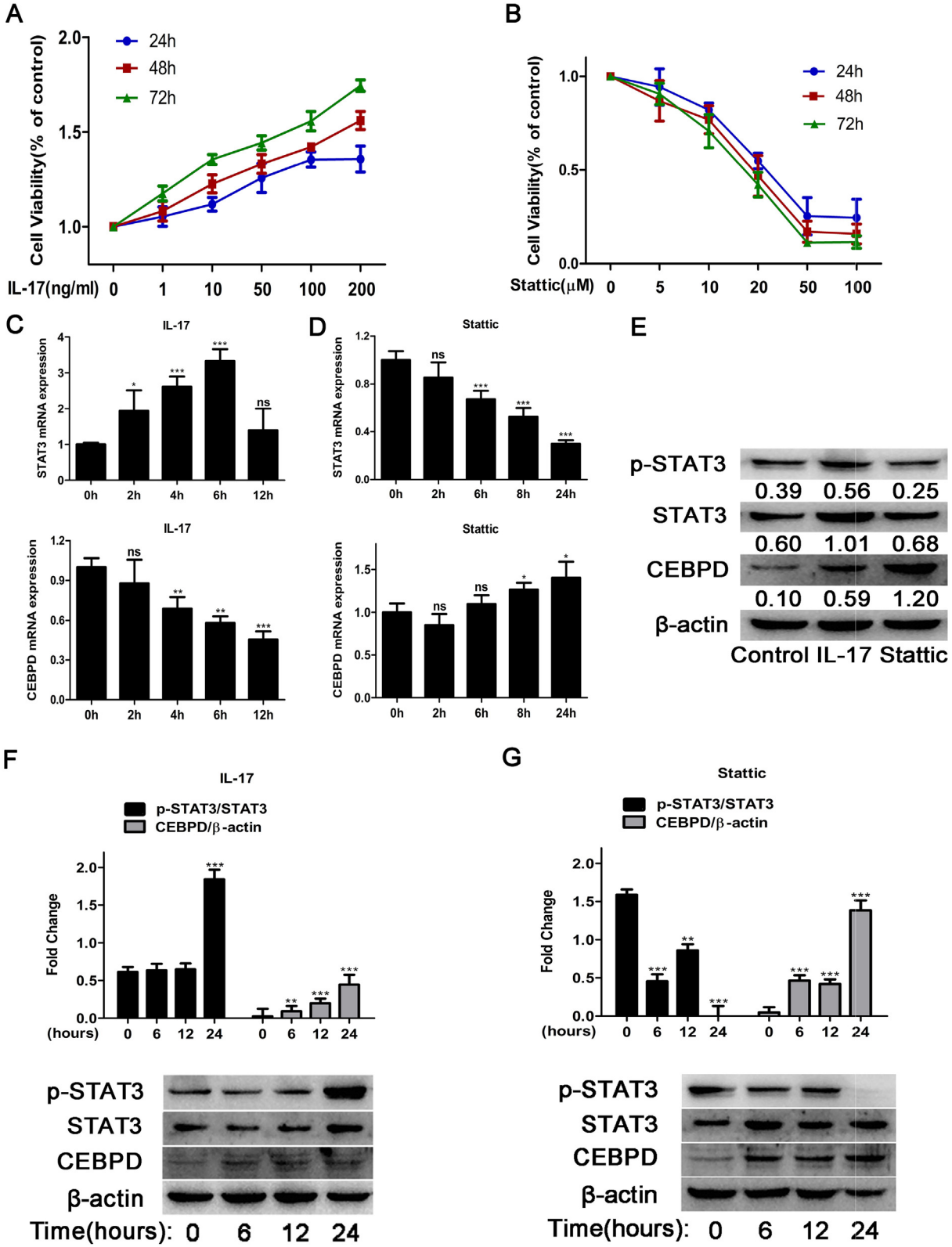

Fig. 1. Cell viability and expression of STAT3 and CEBPD in HaCaT cells under stimulation with IL-17 or Stattic. (A) MTS assay for the cell viability of HaCaT cells treated with 1, 10, 50, 100 or 200 ng/ml of IL-17 for 24, 48 or 72 h. (B) MTS assay for cell viability of HaCaT cells administered 5, 10, 20, 50 or 100 μM of Stattic for 24, 48 or 72 h. (C) mRNA expression levels of STAT3 in HaCaT cells subjected to exposure to 10 ng/ml IL-17 were assessed using real-time qPCR after recovery for 2, 4, 6 or 12 h. GAPDH was employed for normalization, and values are relative to control cells. (D) mRNA expression levels of STAT3 in HaCaT cells administered with 2.5 μM Stattic were assessed using real-time qPCR after recovery for 2, 6, 8 or 24 h. GAPDH was employed for normalization, and values are relative to control cells. (E) Protein levels of p-STAT3, STAT3 and CEBPD in HaCaT cells subjected to exposure to 10 ng/ml IL-17 and 2.5 μM Stattic were assessed using immunoblotting after recovery for 24 h. β-actin serves as an internal control. (F, G) Protein levels of p-STAT3, STAT3 and CEBPD in HaCaT cells subjected to exposure to 10 ng/ml IL-17 or 2.5 μM Stattic assessed using immunoblotting after recovery for 6, 12 or 24 h. β-actin serves as an internal control. *$p < 0.05$ vs. control group; **$p < 0.01$ vs. control group; ***$p < 0.005$ vs. control group.

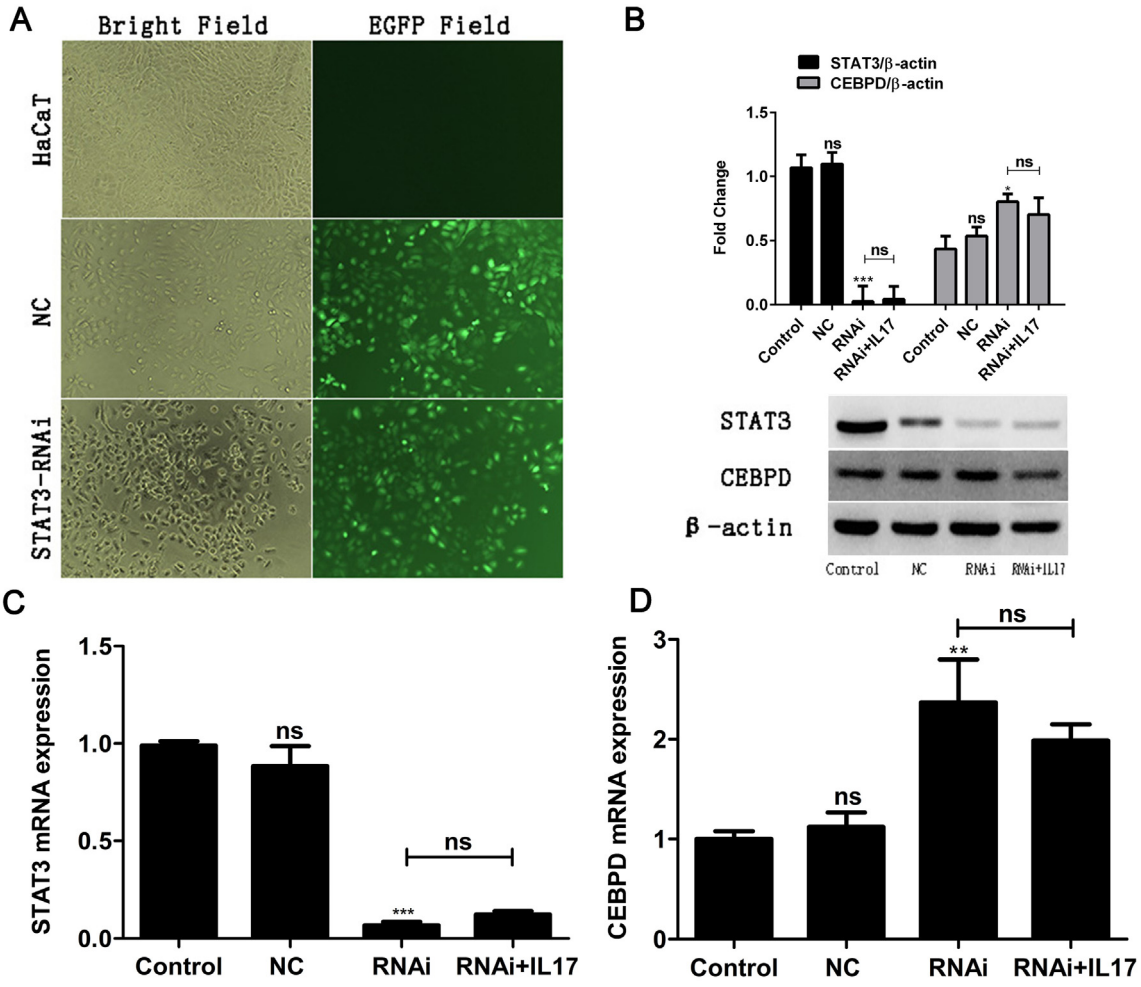

Fig. 2. Detection of STAT3-knockdown HaCaT cells. (A) Bright field and fluorescent micrographs of wild-type (Control), scrambled shRNA transfected (NC) and shSTAT3-RNAi lentivirus transfected (RNAi) HaCaT cells. (B) Western blotting for STAT3 and CEBPD protein levels on Control, NC, RNAi and RNAi + IL17 (10 ng/ml). β-actin was employed for normalization. (C, D) STAT3 and CEBPD mRNA levels assessed using real-time qPCR on Control, NC, RNAi and RNAi + IL17 (10 ng/ml). Values are relative to the control group and normalized with GAPDH. *p < 0.05 vs. control group; **p < 0.01 vs. control group; ***p < 0.005 vs. control group.

2. Materials and methods

2.1. Cell culture

The HaCaT cell line was provided by Kaiji Jiangsu, China, and maintained in RPMI-1640 (HyClone, Logan, UT, USA) medium with 10% fetal bovine serum and 1% penicillin-streptomycin (Biological Industries, Israel), at 37 °C in a humid atmosphere containing 5% CO_2. For JAK/STAT3 activation, recombinant human IL-17 (Peprotech, USA) at various concentrations were incubated with the cells. For JAK/STAT3 inhibition, various concentrations of Stattic (Abcam, Cambridge, MA, USA) were added to the cells. Shikonin [Sigma-Aldrich, USA, purity(HPLC) ≥ 98%] was diluted with DMSO to a stock level of 20 mg/ml and incubated with the cells at various concentrations. Control cells were cultured without an additive.

2.2. Lentivirus transfection

The HaCaT cell culture was performed in 100 mm Petri dishes in a medium without antibiotics before lentiviral transfection. In order to establish STAT3 knockdown in the HaCaT cells, the cells were infected with a packaged lentivirus containing shSTAT3-RNAi (RNAi) or a control scrambled shRNA (NC). In order to establish STAT3 overexpression in the HaCaT cells, the cells were infected with a packaged lentivirus containing EGFP-STAT3 (LV-STAT3) or a control empty vector (Vector). All lentiviruses were manufactured by GeneChem, Shanghai, China. The HaCaT cells were transfected with Polybrene and Eni. S. (GeneChem). Puromycin (Sigma-Aldrich, USA; 1.0 μg/ml) was employed to screen cells for successful transfection. Lentiviruses were based on the STAT3 sequence (NM_139276) with the sequences being:

STAT3-RNAi: ACAATCTACGAAGAATCAA;
STAT3-P1:GAGGATCCCCGGGTACCGGTCGCCACCATGGCCCAATGGAATCAGCTACAG.
STAT3-P2:TCCTTGTAGTCCATACCCATGGGGGAGGTAGCGCACTCC.

2.3. Inverted fluorescence microscopy

Wild type, STAT3-knockout, STAT3 overexpression and empty vector control HaCaT cells were cultured in 100 mm Petri dishes and were observed under an inverted fluorescence microscope (Leica, Germany).

2.4. Animal model

Male BALB/c mice aged 6–8 weeks were purchased from Liaoning Qianyi Biotechnology Co. Ltd. The animals were randomly divided into

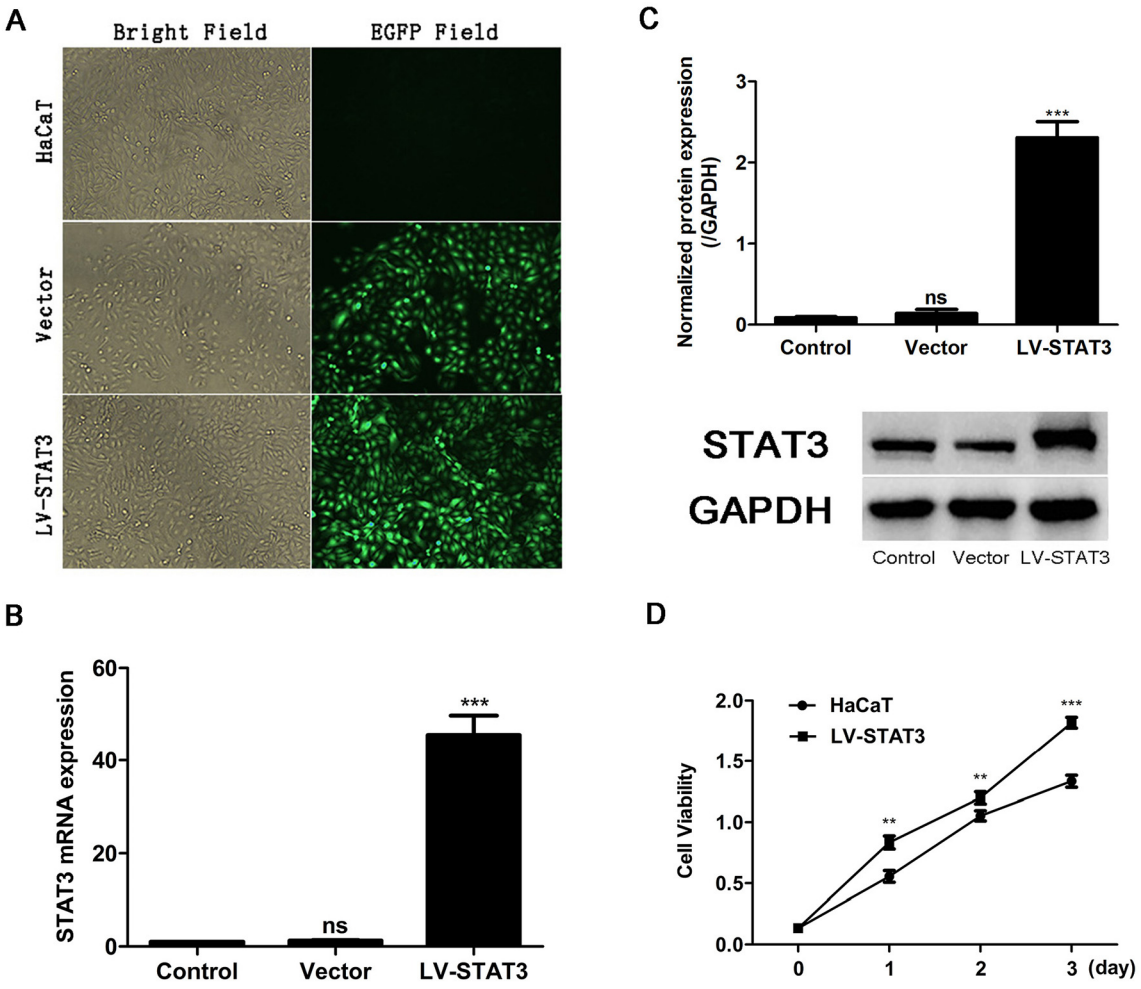

Fig. 3. Verification of STAT3 overexpression in HaCaT cells. (A) Microscopic (bright field and fluorescence) assessment of wild type (Control), empty vector transfected (Vector) and EGFP-STAT3 lentivirus transfected (LV-STAT3) HaCaT cells. (B) mRNA expression of STAT3 in Control, Vector and LV-STAT3 HaCaT cells assessed using real-time qPCR. Values are relative to control cells and normalized with GAPDH. (C) Immunoblotting for total STAT3 protein levels in Control, Vector and LV-STAT3 HaCaT cells. Values are normalized with GAPDH. (D) Cell proliferation was assessed using the MTS assay on HaCaT cells and LV-STAT3 cells treated for 24, 48 or 72 h. **$p < 0.01$ vs. control group; ***$p < 0.005$ vs. control group.

5 groups ($n = 6$): control (CON), model (IMQ), positive drug [Methotrexate at 0.5 mg/kg/d (MTX), Sigma-Aldrich, USA)], low dose shikonin [Shikonin at 5 mg/kg/d (SHI5), Sigma-Aldrich, USA)] and high dose shikonin [Shikonin at 10 mg/kg/d (SHI10), Sigma-Aldrich, USA)] groups. Psoriasis-like inflammatory symptoms were established in mice by topically applying 62.5 mg of 5% imiquimod cream (Zhuhai Lianbang Pharmaceutical co. LTD, China) on a 3.0 cm × 2.0 cm shaved mouse dorsal epidermal area once daily for ten consecutive days, while control animals were treated with Vaseline. For therapy, the drugs were dispensed with edible oil and were administered to mice of the corresponding group through gavage. Meanwhile, the matrix was gavaged into the control and model groups, i.e. edible oil containing 5% DMSO at 1 mg/kg/d. Upon treatment, the animals were euthanized, and skin tissue samples at lesion sites were obtained. All animal experiments were carried out following the National Institutes of Health guidelines for the care and use of Laboratory animals.

2.5. Evaluation of IMQ-induced psoriasiform dermatitis

The severity of the dorsal skin lesions of IMQ-induced BALB/c mice were graded using the Psoriasis Area and Severity Index (PASI). A modified PASI score was calculated as a cumulative score (erythema + scaling + thickness) with a range from 0 to 12, for each day of treatment. Scoring was done on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; and 4, severe.

2.6. Histological and immunohistochemical analyses

Skin samples from the mice were excised, washed with PBS and fixed in formalin, embedded in paraffin and sectioned as 5 μm slices. Then, staining with hematoxylin and eosin (H&E) was performed, followed by assessment under a light microscope (Olympus, Tokyo, Japan). Epidermal thickness was determined at 6 randomly selected high-power fields in each sample. For immunohistochemical assessment, the samples were incubated with rabbit anti-mouse Phospho-Stat3 (Tyr705) (1:1000 dilution, CST), anti-STAT3 (1:500 dilution, Abcam) and anti-CEBP Delta (1:200 dilution, Abcam) antibodies, overnight at 4 °C. Then, an EliVision Super Kit (Maixin, China) was employed for detection. Immune complexes were detected using the DAB kit (Maixin, China). A total of 3 randomly selected high-power fields at the center of the slide were assessed under a light microscope (Olympus). Mean optical density (MOD) was obtained by dividing integral optic density (IOD) by the corresponding area, using Image-Pro Plus 6.0.

2.7. MTS assay

The MTS assay (Promega, USA) was employed for measuring the

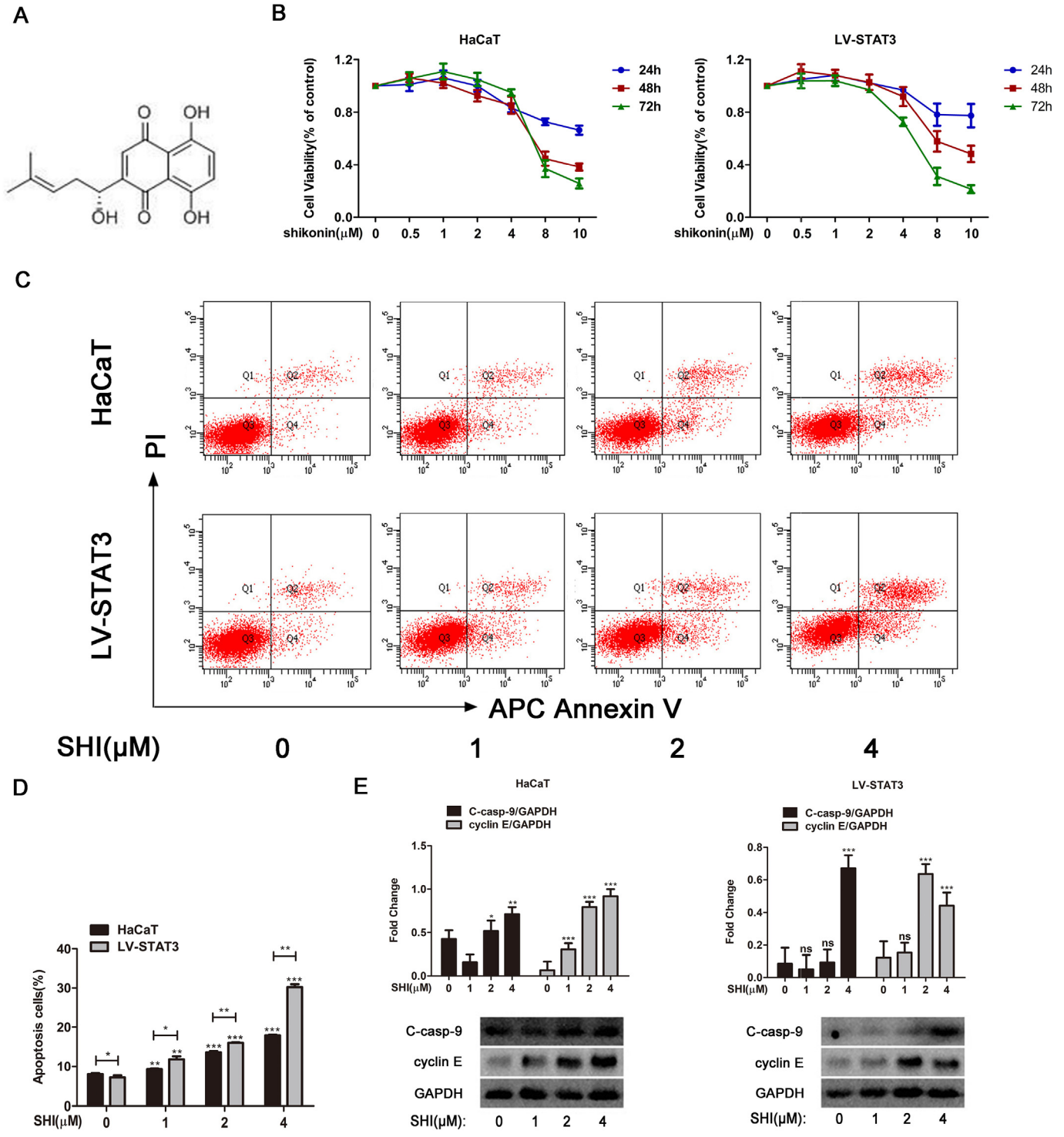

Fig. 4. Cell viability and apoptosis of wild type and LV-STAT3 HaCaT cells assessed using flow-cytometry under treatment with shikonin. (A) Chemical structure of shikonin ($C_{16}H_{16}O_5$). (B) Cell proliferation assessed using MTS assay on wild type and LV-STAT3 HaCaT cells administered with 0.5, 1, 2, 4, 8 or 10 μM shikonin for 24, 48 or 72 h. (C) Apoptosis in wild type and LV-STAT3 HaCaT cells after 12 h treatment with 0, 1, 2 or 4 μM shikonin, were assessed using flow-cytometrically followed by annexin V/PI staining. (D) Quantitation of (C). (E) Western blot assay for the expression levels of cyclin E and cleaved-caspase 9 in wild type and LV-STAT3 HaCaT cells after a 24 h of treatment with 0, 1, 2 or 4 μM shikonin. Values are relative to the control group and normalized with GAPDH. *p < 0.05; **p < 0.01; ***p < 0.005.

viability of HaCaT cells. Following manufacturer recommendations, 3000 cells per well (96-well plates) were cultured at 37 °C in a humid environment containing 5% CO_2. Different concentrations of IL-17, Stattic or Shikonin were added to the cultured cells and incubated with for 24, 48 or 72 h. Then, MTS was added for 3 h of incubation. Cell viability was assessed by detecting optical density at 490 nm using a microplate reader. All experiments were performed in triplicate.

2.8. Real-time qPCR assay

Total RNA was extracted from cultured cells using a miRNeasy Mini Kit (Qiagen, Germany), as recommended by the manufacturer and was

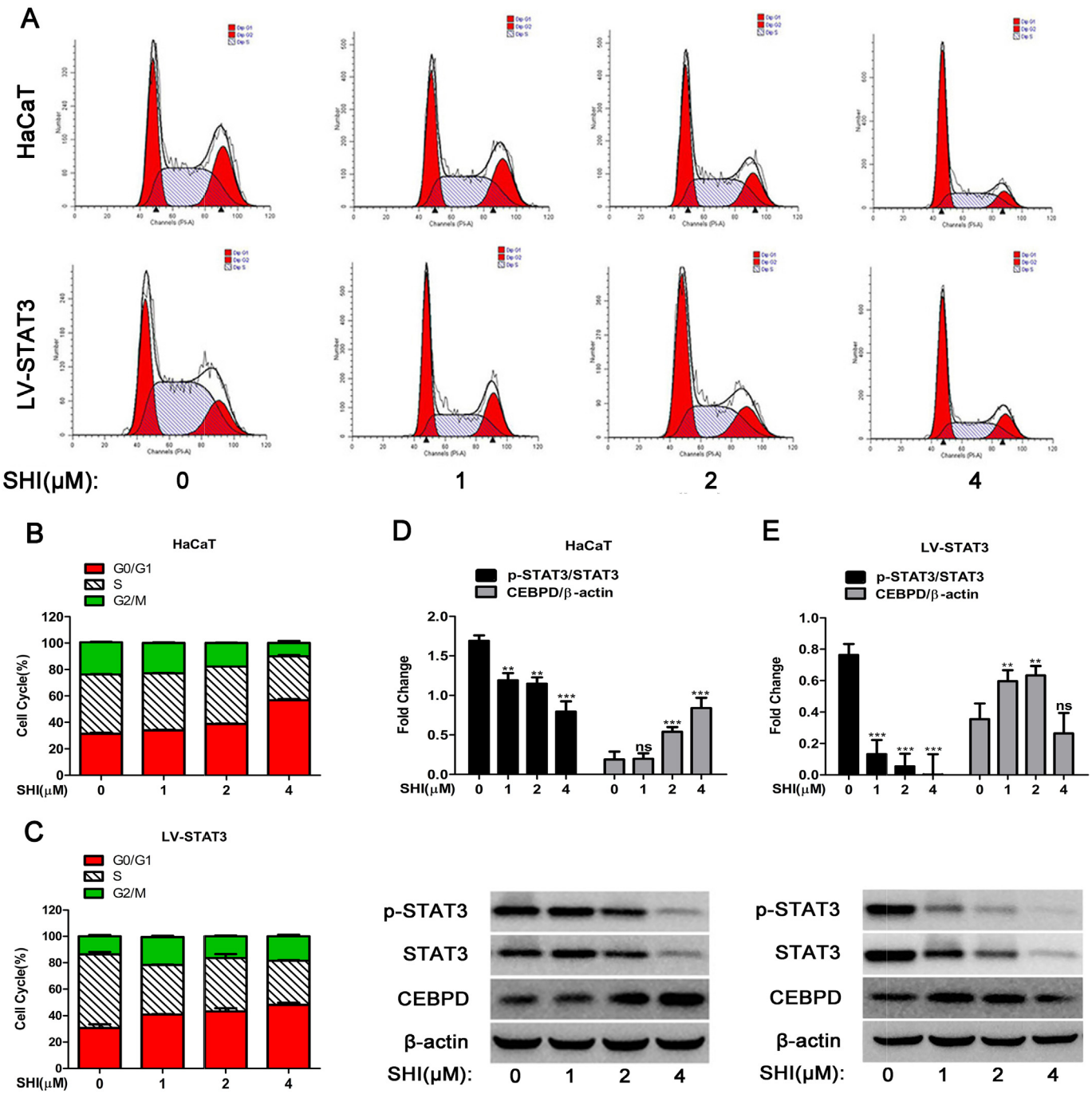

Fig. 5. Cell cycle distribution and protein levels of p-STAT3, STAT3 and CEBPD in wild type and LV-STAT3 HaCaT cells under treatment with shikonin. (A) Cell cycle distribution of wild type and LV-STAT3 HaCaT cells was assessed using flow cytometry after 12 h of treatment with 0, 1, 2 or 4 μM shikonin. (B, C) Percentages of G0/G1, S and G2/M-phase cells in wild type and LV-STAT3 HaCaT cells after 12 h of treatment with 0, 1, 2 or 4 μM shikonin. (D, E) Immunoblotting for assessing p-STAT3, STAT3 and CEBPD protein levels in wild type and LV-STAT3 HaCaT cells after 24 h of treatment with 0, 1, 2 or 4 μM shikonin. β-actin was employed for normalization. *$p < 0.05$ vs. control group; **$p < 0.01$ vs. control group; ***$p < 0.005$ vs. control group.

quantitated using a ND1000 spectrophotometer. First strand cDNA synthesis was carried out with 1 μg of mRNA and the GoScript Reverse Transcription Kit (Promega, USA), based on manufacturer's instructions. Primers for STAT3, CEBPD and GAPDH were designed using data available on PubMed. The primer sequences were as follows:

STAT3-f,5′-CCCTTGGATTGAGAGTCAAGA-3′;
STAT3-r,5′-AAGCGGCTATACTGCTGGTC-3′;
CEBPD-f,5′-GCCATGTACGACGACGAGAGC-3′;
CEBPD-r,5′-CGCCTTGTGATTGCTGTTGAAGAG-3′;
GAPDH-f,5′-TGGAGTCTACTGGCGTCTT-3′;
GAPDH-r,5′-TGTCATATTTCTCGTGGTTCA-3′.

The RT2 SYBR Green qPCR Master mix (Promega, USA) was employed for amplification using a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) on a reaction mixture containing primers (0.4 μl each), 2 × qPCR Master Mix (10 μl), cDNA (2 μl), and nuclease-free water. Amplification was performed as follows: 95 °C (2 min); 40 cycles at 95 °C (15 s) and 60 °C (1 min). Melting curves were generated to confirm specificity. The $2^{-\Delta\Delta Ct}$ method was employed for data

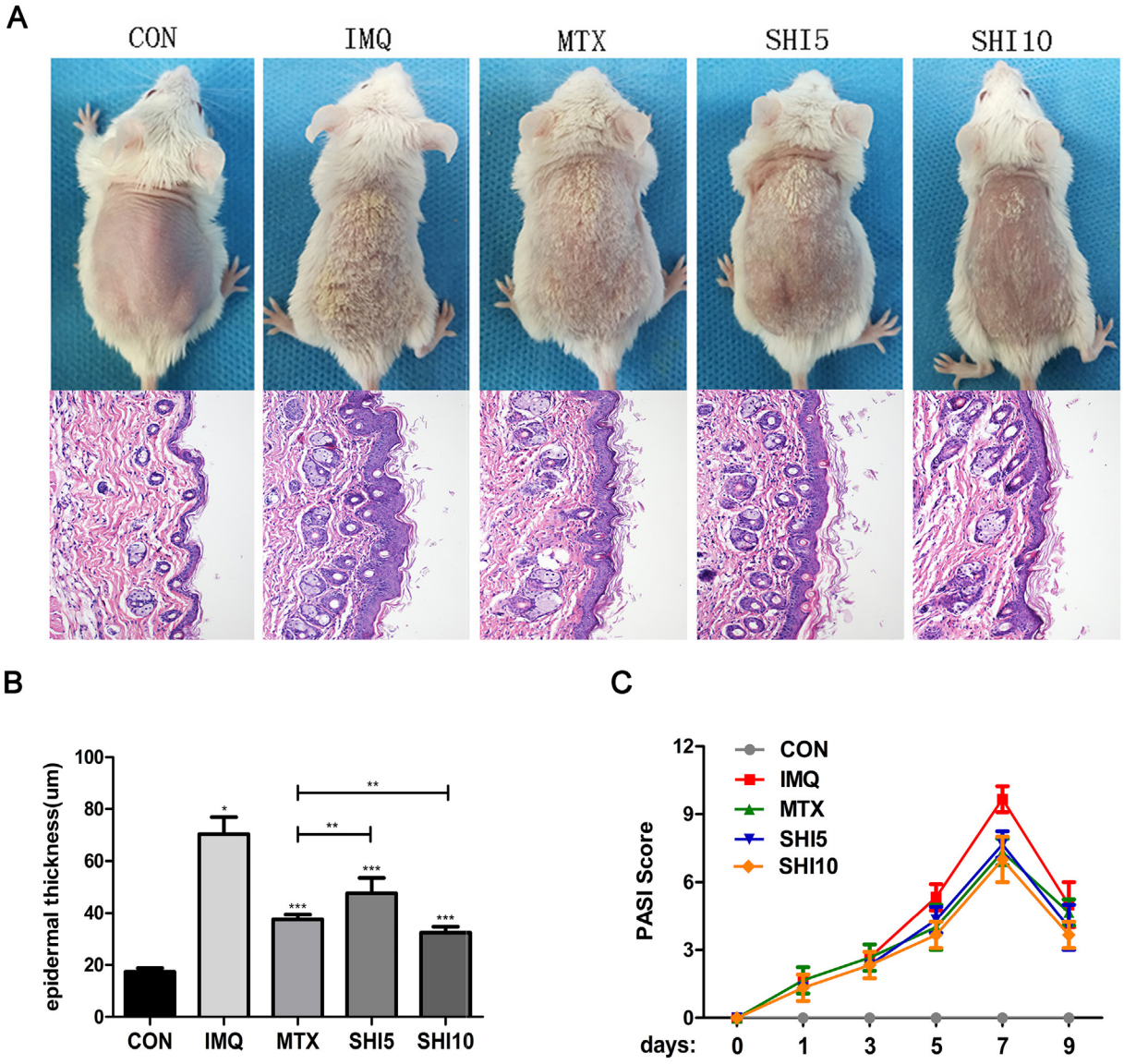

Fig. 6. Effects of shikonin on IMQ-induced psoriasis-like skin inflammation. (A) Images of IMQ-induced psoriasis-like skin lesions acquired using a digital camera. Epidermal thickness was observed through hematoxylin and eosin (H&E) staining. (B) Epidermal thickness of each group was quantitated using Image-Pro Plus 6.0. (C) The severity of the dorsal skin lesion of IMQ-induced Balb/c mice was graded using PASI score. *$p < 0.05$ vs. CON group, **$p < 0.05$ vs. MTX group, ***$p < 0.05$ vs. IMQ group.

analysis, with GAPDH used as the control gene. All experiments were performed in triplicate.

2.9. Immunoblotting

Total protein extraction from cultured cells and mouse skin tissue samples was carried out using a RIPA lysis buffer (Beyotime, China) containing protease/phosphatase inhibitors (Beyotime, China), as recommended by the manufacturer. Protein quantitation was performed using a BCA protein assay kit (Beyotime, China) as directed by the manufacturer. Equal amounts of total protein (20 and 30 μg for cells and tissue samples, respectively) were resolved using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Upon blocking (5% skim milk or BSA in TBST) for 1 h and incubation with respective primary antibodies overnight at 4 °C, the samples were treated with goat anti-rabbit secondary antibodies for 1 h under ambient conditions. Visualization was performed using an ECL kit (Bio-Rad, USA) on a MicroChemi™ Chemiluminescent Imaging System (DNR Bio-Imaging Systems, Israel). Primary antibodies included rabbit anti-phospho-Stat3 (Tyr705) (Cell Signaling Technology, MA, USA), anti-STAT3 (ab68153, Abcam, UK), anti-CEBP Delta (ab198230, Abcam, UK), anti-Beta actin (Abbkin, China) and anti-GAPDH (Abbkin, China) polyclonal antibodies. HRP-conjugated goat anti-rabbit antibody (ZB-2301, ZSGB-BIO, China) was employed as a secondary antibody.

2.10. Flow cytometry

Cells (2×10^5/well) were cultured in six-well plates for 24 h. In order to assess cell cycle distribution, the cells were first incubated in a serum-free medium for 24 h before being transferred into a normal medium containing different doses of shikonin for another 12 h of incubation. Then, the cells were collected and fixed with 75% chilled ethanol overnight at 4 °C, followed by staining with 500 μl of a binding buffer containing 5 μl of RNase A (5 mg/ml; Sigma-Aldrich) for 30 min at 37 °C. Next, 5 μl of PI (5 mg/ml; BD Biosciences, USA) was added for 30 min on ice, away from light. In order to assess apoptosis and senescence, the cells were suspended in 500 μl of the binding buffer and

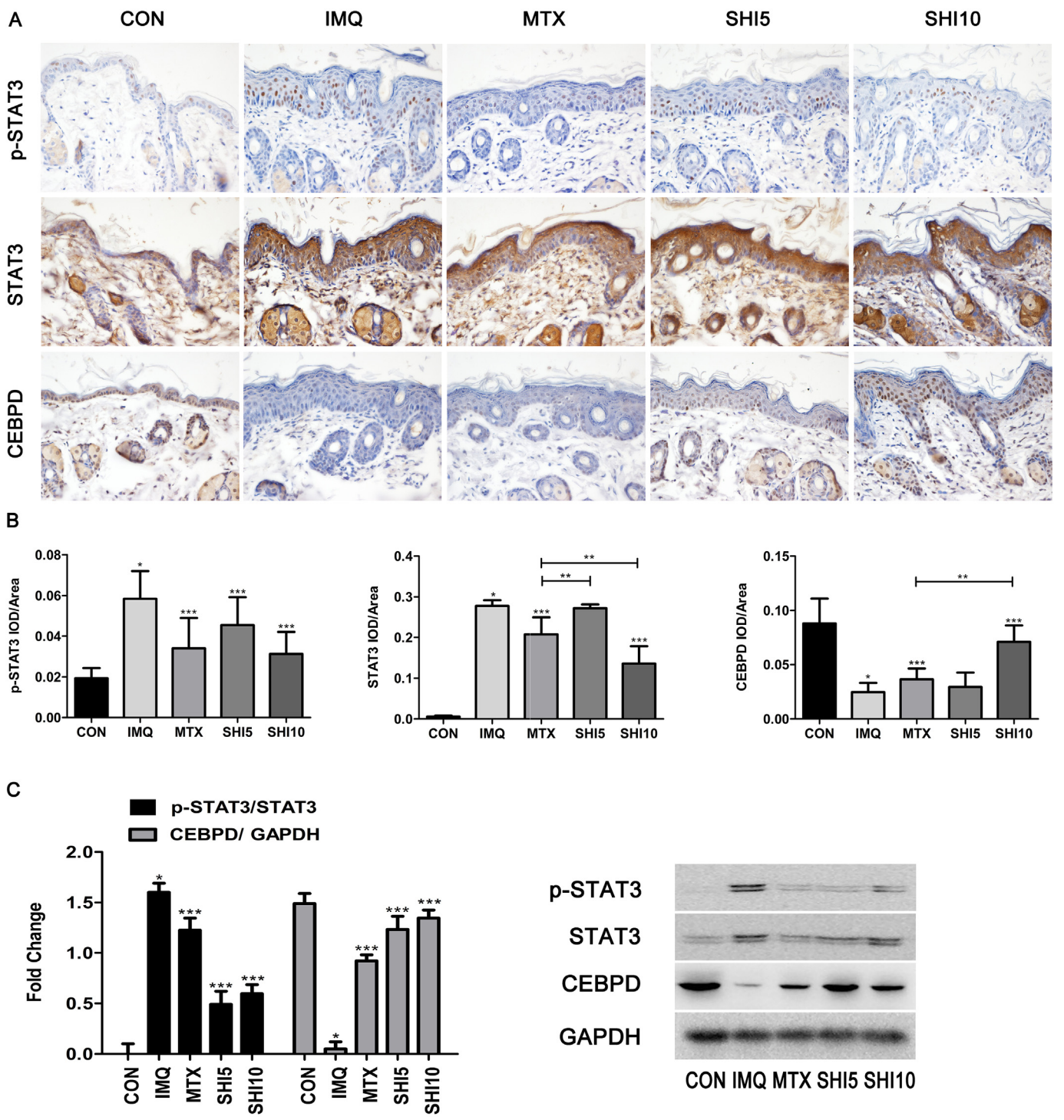

Fig. 7. The expression of p-STAT3, STAT3 and CEBPD in tissues from each group using IHC and immunoblotting. (A) Immunohistochemical staining of p-STAT3, STAT3 and CEBPD in tissues from each group. Analysis was performed using light microscopy. (B) Mean optical density (MOD) was obtained by dividing integral optic density (IOD) by area using Image-Pro Plus 6.0. (C) Immunoblotting for p-STAT3, STAT3 and CEBPD protein level in each group, with GAPDH as an internal reference. *p < 0.05 vs. CON group, **p < 0.05 vs. MTX group, ***p < 0.05 vs. IMQ group.

stained with APC-linked Annexin V (5 μl; BD Biosciences) and PI (5 μl; 5 μg/ml; BD Biosciences) for 30 min, away from light. A BD LSRFortessa (BD Biosciences) was used for analysis, gating 10,000 cells. Cell cycle distribution was assessed using ModFit software (BD Biosciences, USA).

2.11. Statistical analysis

Data are presented as mean ± SEM. SPSS v21.0 (SPSS Inc., Chicago, IL, USA) was employed for all statistical analyses. Group pairs were compared using student's *t*-test. Comparison of more than two groups was done using one-way ANOVA. A p value of < 0.05 was considered as statistically significant.

3. Results

3.1. JAK/STAT3 activation decreases CEBPD expression in the HaCaT cell line

First, we assessed whether JAK/STAT3 activation participates in CEBPD downregulation in psoriasis by successively activating and inhibiting JAK/STAT3 signaling using interleukin-17(IL-17) and Stattic (STAT3 inhibitor), respectively. IL-17 activates the classical JAK/STAT3 signaling pathway, whereas Stattic specifically suppresses STAT3 activation, dimerization and nuclear translocation by interacting with its SH2 domain.

Fig. 1A and B show that IL-17 treated HaCaT cells had significantly elevated viability in comparison with that of control cells, with time- and concentration- dependence, while Stattic treated HaCaT cells presented an opposite trend. Meanwhile, after 24 h of treatment with IL-17(10 ng/ml) and Stattic (2.5 μM), western blotting data in Fig. 1E shows that JAK/STAT3 is significantly activated by IL-17 and is accompanied by a slight increase in CEBPD expression, while in Stattic treated HaCaT cells, CEBPD expression exhibits significant upregulation, reflecting inhibition of JAK/STAT3 signaling.

Furthermore, we measured CEBPD and STAT3 mRNA and protein expression levels on a time gradient. Fig. 1C and D show that STAT3 mRNA is activated by IL-17 (10 ng/ml) in a time-dependent manner, peaking at 6 h, while CEBPD is downregulated. Conversely, STAT3 mRNA is inhibited in a time-dependent manner by Stattic (2.5 μM), while CEBPD is upregulated. Western blotting data shown in Fig. 1F and G are consistent with the results of RT-qPCR. Surprisingly, CEBPD protein expression is slightly upregulated by IL-17.

In order to rule out that IL-17 or Stattic may regulate CEBPD via pathways other than the JAK/STAT3 signaling pathway, STAT3 was silenced with the aid of a packaged lentivirus containing shSTAT3-RNAi (RNAi), and then the transfected cells were treated with IL-17 for 24 h. Transfected HaCaT cells were assessed under an inverted fluorescent microscope (Fig. 2A). We measured CEBPD and STAT3 mRNA and protein levels using real-time qPCR and immunoblotting, respectively. Fig. 2B, C and D suggest that STAT3 is markedly downregulated by shSTAT3-RNAi, while CEBPD shows significant upregulation. In addition, no significant upregulation of these proteins was found upon IL-17 administration. In combination these findings indicate that activation of JAK/STAT3 signaling plays a role in CEBPD downregulation, while JAK/STAT3 inhibition significantly upregulates CEBPD.

3.2. Successful STAT3 overexpression in HaCaT cells with the aid of a packaged lentivirus containing EGFP-STAT3

HaCaT cell transfection was carried out as described above. To assess transfection efficiency, the cells infected with a packaged lentivirus containing EGFP-STAT3 (LV-STAT3) or a control empty vector (Vector) were examined under fluorescence microscopy. Fluorescent cells accounted for > 90% of all cells in all high-power fields (Fig. 3A). Subsequently, real-time qPCR and immunoblotting were employed to quantify the amount of STAT3 in HaCaT cells. As shown in Fig. 3B and C, STAT3 mRNA and protein levels were markedly higher in the LV-STAT3 group. Combined, the above findings suggest successful construction of STAT3 overexpression in HaCaT cells using lentivirus transfection technology. As shown in Fig. 3D, STAT3 hyperactivation markedly promotes cell proliferation.

3.3. Shikonin significantly suppresses proliferation and enhances apoptosis in HaCaT cells via CEBPD regulation

Shikonin is a compound with a molecular weight of 288.30, and its molecular formula is $C_{16}H_{16}O_5$ [5,8-Dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone)]. The chemical structure of shikonin is shown in Fig. 4A. The MTS assay revealed that shikonin significantly decreases cell proliferation in both wild-type and LV-STAT3 HaCaT cells, in comparison with that of the control group, in a time and concentration dependent manner (Fig. 4B). Flow cytometric analysis further confirmed the changes in cell cycle distribution and apoptosis. Indeed, shikonin remarkably increases apoptotic rates of both wild-type and LV-STAT3 HaCaT cells, while LV-STAT3 HaCaT cells were more sensitive to shikonin ($p < 0.05$) (Fig. 4C and D). In agreement, cell cycle analysis (Fig. 5A, B and C) demonstrate that shikonin remarkably arrests the cell cycle at G0/G1. Meanwhile, we assessed cell cycle, apoptosis-related proteins cyclin E and cleaved-caspase 9 using immunoblotting (Fig. 4E). The results corroborate with the results of the flow cytometric analysis. Interestingly, shikonin markedly suppresses the JAK/STAT3 signaling pathway and upregulates CEBPD (Fig. 5D and E). These in vitro findings indicate that shikonin significantly enhances apoptosis and reduces proliferation of HaCaT cells by inhibiting the JAK/STAT3 signaling pathway and upregulating CEBPD expression.

3.4. Shikonin alleviates imiquimod-induced mouse skin lesions by upregulating CEBPD

The effects of shikonin were investigated in a IMQ-induced psoriasis-like skin inflammation model. Photographs of the lesions were acquired using a digital camera. Simultaneously, the histological properties of IMQ-induced psoriasis-like skin were assessed using hematoxylin and eosin (H&E) staining. Clearly, IMQ caused psoriasis-like symptoms in the mouse skin, which shows severe keratinocyte hyperplasia (Fig. 6A). In addition, gavage with shikonin markedly reduced symptoms, including scaling and epidermal hyperplasia. Meanwhile, shikonin reduced epidermal thickness compared with that of the model group (IMQ), especially the high dose shikonin group (SHI10) (Fig. 6B). The PASI scores of shikonin-treated mice were significantly lower than that of the model group (Fig. 6C).

Further, psoriasis-like lesions were assessed using IHC and immunoblotting. The results (Fig. 7A, B and C) show that JAK/STAT3 signaling is significantly activated in the IMQ group compared with that of the control samples, whereas CEBPD levels decreased. Furthermore, shikonin restored the expression levels of STAT3 and CEBPD. The above results clearly suggest that shikonin inhibits the proliferation of keratinocytes by upregulating CEBPD in vivo.

4. Discussion

In this study, we generated STAT3 overexpression in HaCaT cells using a packaged lentivirus containing EGFP-STAT3, to mimic abnormal proliferation of keratinocytes in psoriatic lesions. Here, using an IMQ-induced psoriasis-like skin mouse model, we demonstrated the JAK/STAT3/CEBPD axis both in vitro and in mice, and explored the mechanisms behind the effects of shikonin in psoriasis. The above data clearly suggest that JAK/STAT3 activation decreases CEBPD levels both in vitro and in mice, and that these effects are reversed by shikonin. Interestingly, we first showed that JAK/STAT3/CEBPD signaling plays a pathogenic role in psoriasis, documenting the mechanism of action of shikonin on psoriasis in mice. Our findings suggest that CEBPD might constitute a potent therapeutic target for psoriasis.

Psoriasis is a chronic skin inflammatory ailment that is mainly manifested as hyperkeratosis and parakeratosis of keratinocytes [1,2]. As a result of its significant involvement in inflammation and immunity, STAT3 contributes to the pathogenetic mechanisms of psoriasis and related inflammatory diseases. Mounting evidence indicates that STAT3 function is altered in psoriasis, as reflected by elevated levels of activated STAT3 in skin samples from psoriatic patients [15]. Meanwhile, STAT3 participates in T cell subset expansion and homeostasis in inflammation, activating anti-apoptosis and proliferation associated genes and inhibiting regulatory T cell (Treg) conversion downstream of IL-6/IL-23 signaling [16]. Accordingly, as a result of STAT3 overexpression, cell proliferation is markedly enhanced, further resulting in

keratinocyte hyperplasia of IMQ-induced psoriasis-like skin.

Meanwhile, apoptosis decreases in psoriatic lesions [17], leading to the hyperproliferation of keratinocytes. Therefore, we hypothesized that efficient anti-psoriatic agents would suppress hyperproliferation and enhance apoptosis of keratinocytes. As shown above, shikonin markedly reduces HaCaT cell proliferation, with arrest at G0/G1, suggesting that cell cycle arrest might partially explain shikonin-associated HaCaT cell growth suppression.

Shikonin has antitumor, anti-angiogenic [14], anti-inflammatory [18,19] and anti-proliferative features. Tang et al. have demonstrated that a shikonin/gefitinib combination exhibits synergistic anticancer effects in cell cultures as well as in vivo, likely by suppressing PKM2/STAT3/cyclinD1 signaling [20]. In this study, both wild type and LV-STAT3 HaCaT cells that were administered shikonin showed a concentration-dependent increase of apoptosis. Meanwhile, we found that this process involves the JAK/STAT3 pathway and its downstream effector CEBPD (Figs. 4 and 5). Furthermore, these findings were confirmed in vivo: shikonin not only reduced psoriasis-like symptoms, but also inhibited the proliferation of keratinocytes by upregulating CEBPD (Fig. 6).

CEBPD is a critical inflammatory response molecule of innate immunity [21]. CEBPD is a member of the CEBP family of proteins that act by forming homodimers and heterodimers that bind to DNA regulatory sequences, to modulate gene expression [22,23], cell proliferation [24], cell cycle and differentiation [25], apoptosis [26], as well as metastasis and inflammation [27]. However, the CEBPD gene is downregulated in multiple tumors, including HCC [28], leukemia [29] and cervical cancer [30], and CEBPD is considered a survival biomarker for breast cancer [31]. Although the role of CEBPD has been studied for a long time, most reports have focused on tumors, while the function of CEBPD in the psoriatic epidermis that is mainly composed of keratinocytes is poorly understood.

In this study, we focused on CEBPD due to the following reasons. First, shikonin is considered to be an effective therapy for psoriasis that efficiently inhibits JAK/STAT3 activation. We found in a preliminary study that CEBPD is one of the targets of shikonin. Second, STAT3 activation is downregulated by CEBPD in keratinocytes in psoriasis, as reported for the first time in this work. It may be involved in the mechanism that promotes keratinocyte proliferation via STAT3 activation. Third, shikonin suppresses proliferation and induces apoptosis of keratinocytes via CEBPD regulation in psoriasis both in vitro and in vivo.

Surprisingly, there are other studies that have demonstrated that activated STAT3 increases the expression of C/EBPd in cancer cachexia or chronic kidney disease, which is contrary to the results of our study [32,33]. In the afore mentioned study, they added the conditioned medium from cultures of C26 cells to C2C12 myotubes, which indicated that activation of p-STAT3 stimulates muscle protein losses via C/EBPd. Actually, there are various factors in the additive and the C/EBPd gene responds to a variety of extracellular signals [34–37]. Sivko et al. have suggested that C/EBPd is tightly regulated transcriptionally, post-transcriptionally, and post-translationally during G0 growth arrest of human mammary epithelial cells. C/EBPd mRNA and protein both have short half-lives of 40 and 160 min, respectively [38]. Thus, we propose that the discrepancy is due to differences in stimulation time, conditions and cell type.

In summary, this study demonstrates that shikonin upregulates CEBPD, whose deficiency induces HaCaT cell proliferation and psoriasis-like lesions, mostly via JAK/STAT3 signaling. Therefore, CEBPD should be considered a promising therapeutic target for psoriasis.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

Ya-jie Yu performed the experiments and wrote the paper, all authors designed the study, Xing-hua Gao and Long Geng examined the paper.

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