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Genistein suppresses psoriasis-related inflammation through a STAT3–NF-κB-dependent mechanism in keratinocytes

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ABSTRACT

Psoriasis is a chronic recurrent skin inflammatory disease, and inhibition of inflammation may be an effective means of treating psoriasis. The flavonoid genistein has a clear anti-inflammatory effect. However, the anti-psoriatic effects of genistein and their underlying mechanisms remain unclear. In this study, we investigated the effects of genistein on imiquimod (IMQ)-induced psoriasis-like skin lesions in vivo and explored the mechanisms underlying those effects in vitro. It was found that genistein can significantly improve IMQ-induced pathological scores of cutaneous skin lesions in mice, reduce epidermal thickness, and inhibit the expression of inflammatory factors , including interleukin (IL)-1β, IL-6, tumour necrosis factor-alpha (TNF-α), chemokine ligand 2 (CCL2), IL-17 and IL-23. In vitro studies, genistein inhibited the proliferation of human keratinocyte HaCaT cells and inhibited the expression of inflammatory factors in a dose-dependent manner which induced by TNFα. Further researches showed that genistein could also significantly inhibit phosphorylated STAT3 (pSAT3) expression in IMQ mice dorsal skin and in TNF-α-induced HaCaT cells. The inhibitory effect of genistein on the expression of IL-6, IL-23 and TNF-α was weakened after Stat3 siRNA in HaCaT cells. Genistein could also significantly inhibit TNF-α induced the nuclear translocation of NF-κB, and inhibit the phosphorylation of I-kBα (pI-kBα). After combining with NF-κB blocker BAY 11–7082, the effect of genistein down-regulate the expression of TNF-α and VEGFA was attenuated in HaCaT cells. The results suggest that genistein may be developed for the treatment of psoriasis lesions.

1. Introduction

Psoriasis is a common chronic and recurrent disease with a global prevalence of 1–3% [1–3]. The pathogenesis of psoriasis is not yet clear. Generally, the pathogenesis of psoriasis is related to genetic predisposition, stress, infection, trauma, and drugs [4–6]. As a result of relapse of the disease, with the gradual extension of the course of the disease, the complications of psoriasis are gradually increasing. Common complications include psoriatic arthritis, diabetes, hypertension, dyslipidaemia, non-alcoholic fatty liver disease, and even cancers with high mortality [7]. Current psoriasis treatment drugs, such as vitamin D derivatives, retinoic acid, methotrexate, and glucocorticoids, have side effects such as immunosuppression, severe xerostomia or withdrawal effects, which limit the long-term use of these drugs [8–11]. Clinically, for mild to moderate patients, Vitamin D analogues Calcipotriol (Daivonex) and glucocorticoids are still the first-line drugs for treatment of psoriasis. Therefore, it is very important to find and develop long-term therapeutic drugs for psoriasis.

Several signaling pathways have been found to be abnormally activated in psoriasis. Notably, growing evidence has shown that both STAT3 and NF-κB are overexpressed and activated in skin tissues in psoriasis [12]. One recent study further demonstrated that transgenic

Abbreviations: BAY, BAY 11-7082; CCL2, chemokine ligand 2; Gen, genistein; H&E, haematoxylin and eosin; I-κBα, nuclear factor kappa B inhibitor alpha; IL, interleukin; IMQ, imiquimod; MCP1, monocyte chemoattractant protein1; PASI, psoriasis area severity index; pSTAT3, phosphorylated STAT3; pI-κBα, phosphorylated I-κBα; RT-PCR, real-time polymerase chain reaction; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; TNF-α, tumour necrosis factor-alpha; VEGFA, vascular endothelial growth factor A

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mice with constitutively overexpressed active STAT3 in keratinocytes (K5. STAT3C mice) spontaneously developed a skin phenotype that was highly similar to human psoriasis plaques [13]. In contrast, STA-21, a small molecule inhibitor of STAT3 pathway, could ameliorate skin lesions not only in mice psoriasis but also in human psoriasis [14]. In addition, NF-κB inhibitor BAY 11–7082 has been shown to improve skin lesions in IMQ-induced psoriatic-form mice [15]. Since STAT3-NFκB signaling pathway play a very important role in the pathogenesis of psoriasis, the search for natural products that can inhibit these two signaling pathways has attracted much in recent years.

Genistein is an isoflavone compound extracted from prunus and soybean [16,17]. Previous studies have shown that genistein has anti-inflammatory, antioxidant, anti-light damage, anti-cancer effects, and can be used to treat a variety of inflammatory diseases [18–20]. However, there are a few studies on its therapeutic effect on psoriasis. In this study, we reported the therapeutic effect of genistein on IMQ-induced psoriasis-like lesions in mice, and preliminarily confirmed that its effect is related to the inhibition of STAT3 and NF-kB signaling pathways in keratinocytes.

2. Materials and methods

2.1. Reagents and antibodies

Genistein [PubChem CID: 5280961, 98% purity, verified by high-performance liquid chromatography] was purchased from Nanjing Zelang Medical Technology Company (Nanjing, Jiangsu, China). Daivonex (calcipotriol ointment) was purchased from LEO Laboratories Limited (Dublin, Ireland). The molecular structure of genistein is shown in Supplementary Fig. 1. TNF-α was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Control siRNA (con-siRNA) and STAT3 siRNA II were purchased from Cell Signaling Technology (Danvers, MA, USA). TRIzol reagent and RNase-free water were purchased from Life Technologies (Carlsbad, CA, USA). IL-1β, IL-6, IL-8, IL-23, TNF-α and vascular endothelial growth factor-A (VEGF-A) primers were synthesised by Life Technologies (Carlsbad, CA, USA). BAY 11-7082 (PubChem CID: 5353431) was purchased from Selleck Chemicals (Houston, TX, USA). Human cytokine/chemokine magnetic bead panel and mouse cytokine/chemokine magnetic bead panel kits were purchased from Millipore (Billerica, MA, USA). Minimum essential medium (MEM), trypsin and foetal bovine serum (FBS) were purchased from Gibco (Oklahoma, ME, USA). The following antibodies used for western blotting were purchased from Cell Signaling Technology (Danvers, MA, USA): primary anti-STAT3 (Item: 12640), anti-p-STAT3 (Ser727, Item: 9134), anti-pI-κBα (Item: 2859), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Item: 2118), histone H3 (Item: 4499) and secondary anti-rabbit (Item: 7074) antibodies. Primary anti-I-κBα antibody (Item: ab32518) was purchased from ABCAM (Cambridge, MA, USA).

2.2. Preparation of cream

The vehicle cream was composed of 2.62% of glyceryl monostearate, 1.2% of stearic acid, 0.75% of Vaseline, 3.75% of lanolin anhydrous, 4.5% of paraffin liquid, 0.37% of ethyl 4-hydroxybenzoate, 3.75% of glycerine, 0.3% of triethanolamine hydrochloride and 74.96% of pure water. The first six components were prepared in the oil phase by heating to 75 °C, and the seventh to ninth components were prepared in the water phase by heating to 75 °C. The water phase was then gently mixed with the oil phase at 200 rpm to obtain the cream. The cream was then heated to 55 °C and mixed well with genistein by stirring.

2.3. Animals

Male BALB/c mice (7–8 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (Foshan, Guangdong, China) and were grown in a specific pathogen-free clean room under a controlled 12:12-h light/dark cycle. During the entire experiment, mice had free access to chow diet and water. Animal care was in accordance with the guidelines for the care and use of laboratory animals and the principles presented by the Guangdong Academy of Traditional Chinese Medicine. All experimental procedures were conducted in strict accordance with the guidelines of the Animal Experiments Committee.

2.4. IMQ-induced psoriasis-like mouse model

The skin on the back of mice was shaved. After a 3-day acclimatisation, 50 mice were randomly divided into five groups (10 mice/group). In the control group, mice were topically applied only the vehicle cream (no IMQ or genistein). In the model group, mice received a daily topical dose of 62.5 mg commercial IMQ cream (Mingxin Lidi; Sichuan Mingxin Pharma, China) on the shaved back for 7 consecutive days as previously described [21,22]. In the trial groups, mice received IMQ cream along with 0.5% or 2% genistein, respectively. In the positive trial group, mice received IMQ cream and Daivonex. During the entire experiment, the severity of the psoriasis-like skin condition was evaluated daily using the cumulative score. Erythema, scales, and infiltration were scored independently from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The cumulative score was calculated as the sum of three indexes, indicating the severity of inflammation (scale, 0–12) [22,23]. After the treatment, mice were euthanised by diethyl ether inhalation and cervical dislocation, and the shaved skins on their backs were immediately excised. The harvested skin tissues were fixed in formalin and embedded in paraffin for histological analysis. The remaining skin tissues were stored at −80 °C for use in the extraction of RNA and total proteins.

2.5. Cell culture and drug treatment

Human keratinocyte HaCaT cells were obtained from the China Center for Type Culture Collection and cultured in MEM containing 10% heat-inactivated FBS, 100-U/mL penicillin and 100-μg/mL streptomycin (Gibco, Grand Island, NY, USA). The cells were kept in a cell incubator at 37 °C under 5% CO_2 and 95% humidified atmosphere. TNF-α (20 ng/mL) was then used to induce psoriasis-like inflammation in these cells by incubating them with TNF-α for 30 min. The cells were then exposed to genistein (50 or 100 μM) for another 2 h.

2.6. Histopathology and immunohistochemistry

Skin lesions collected from the mouse backs were fixed in 10% formalin and embedded in paraffin. Subsequently, 5–10-μm-thick sections of formalin-fixed paraffin-embedded tissue were obtained. Haematoxylin and eosin (H&E) staining was performed as per the standard protocol. Immunohistochemical staining was performed using primary anti-rabbit CD45 (Servicebio, Wuhan, China) and anti-p-STAT3 antibodies in that order, followed by the corresponding secondary antibodies. Images were taken at random fields using light and fluorescence microscopes (Olympus, Tokyo, Japan). Image-Pro Plus 6.0 was used for quantifying protein abundance.

2.7. Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the skin tissues and HaCaT cells using TRIzol reagent (Takara Biotechnology, Dalian, Liaoning, China). The total RNA concentration in each sample was detected using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). Subsequently, 1 μg of total RNA was reverse-transcribed into cDNA using a Transcriptor Universal cDNA Master kit (Takara Biotechnology, Dalian, Liaoning, China). Target mRNA quantification was performed by high-productivity RT-PCR in ViiATM 7 using a SYBR® Premix Ex Taq™ II Kit (Takara Biotechnology, Dalian, Liaoning, China) as per the manufacturer's protocol. The primers used in this study are listed in

Supplementary Table 1. The $2^{-\Delta\Delta CT}$ method was used to determine the relative expression of target genes after normalisation to GAPDH.

2.8. Western blotting analyses

Total protein and nuclear protein were extracted using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Boston, MA, USA) and a nuclear protein extraction kit (Beyotime Biotechnology, Wuhan, Hebei, China), respectively. The protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific). Subsequently, 40 μg of protein from each sample was separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and then blocked with 5% skim milk in a Tris-buffered saline-0.1% Tween 20 buffer (TBST) for 1 h. The membranes were then incubated with the corresponding primary antibodies (1:1000) at 4 °C overnight. Subsequently, the membranes were washed thrice with TBST (5 min each time) and incubated with a secondary antibody, namely HPR-conjugated goat anti-rabbit IgG (1:2000), at room temperature for 1 h. An enhanced chemiluminescence detection system was used to detect the antibody-bound proteins on the membranes. In certain cases, stripping buffer (Beyotime Institute of Biotechnology) was used to remove the antibodies from the blotted membranes to re-probe the membranes with the loading control.

2.9. Measurement of cytokines

Cell lysis buffer (Cell Signaling Technology, Boston, MA, USA) containing protease inhibitors (Merck, USA) was used to prepare skin homogenates. Briefly, 50 mg of skin tissues from each mouse were cut into pieces in 2 mL microtubes, and then four ceramic beads were added along with 200 μL lysate in the microtubes. Tissue homogenates were prepared using a Precellys 24 multipurpose sample homogenizer (Bertin Technologies, France). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The protein levels of cytokines [IL-1β, IL-6, IL-17, TNF-α, IL-12 p40 and monocyte chemoattractant protein 1 (MCP1)] in dorsal skin tissues and cell culture medium were determined using a mouse cytokine/chemokine magnetic bead panel kit in accordance with the corresponding protocols provided by the manufacturers (Millipore, Billerica, MA, USA). IL-23 concentration in the culture medium was determined using a Human IL-23 Elisa Kit as per the manufacturer's protocol (Cusabio, Wuhan, China).

2.10. Proliferation assay

Cell proliferation was assessed using MTT assay (Beyotime Biotechnology, Wuhan, Hebei, China). Briefly, HaCaT cells were treated with different concentrations of genistein (3.12–200 μM) for 24 h and then stimulated with TNF-α (10 ng/mL) for another 12 h. The cell culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). The cells were then treated with different concentrations of genistein (0, 25, 50 and 100 μM) for another 72 h. Subsequently, MTT solution (5 mg/mL) was added to each well, followed by incubation at 37 °C for 4 h. The formazan dyes in the cells were dissolved using 100 μL dimethyl sulphoxide. The optical density (OD) was read at 570 nm (reference, 650 nm) using a Multi-mark microporous plate detector (PerkinElmer, MA, USA). The mean OD of six wells in each group was used to calculate the percentage of cell proliferation. Cell proliferation in the control group (cells treated with neither genistein nor TNF-α) was considered as 100%.

2.11. Transient transfection

HaCaT cells were transfected with STAT3 siRNA using X-tremeGENE siRNA transfection reagent (Roche, Mannheim, Germany) with Opti-MEM I reduced serum medium [Catalog#31985-062] as per the manufacturer's instructions. After 24 h transfection, cells were incubated with 100-μM genistein for 6 h and further treated with TNF-α (20 ng/mL) for another 1 h. The control siRNA was used as a negative control.

2.12. Chemical inhibitors

HaCaT cells were treated with 4 μM BAY 11-7082 (Selleck Chemicals, Houston, USA) for 2 h to inhibit the intracellular NF-κB signaling pathway. The cells were then incubated with 100 μM genistein for 6 h followed by TNF-α (20 ng/mL) for another 1 h.

2.13. Statistical analysis

Data are shown as means ± SEM of at least three independent experiments. Differences between groups were determined by one-way analysis of variance, followed by Bonferroni's test or (two-tailed) Student's *t*-test to compare all pairs of columns. P values < 0.05 were considered statistically significant.

3. Results

3.1. Genistein attenuate IMQ-induced psoriasiform dermatitis

We observed the effect of genistein on *IMQ-induced psoriasiform dermatitis*. Firstly, the cumulative score of skin surfaces preliminarily indicated that psoriasis-like skin lesions developed on day 2 after IMQ administration. More importantly, these lesions became more severe with the passage of experimental time and peaked on day 7. Genistein reduced skin thickness and redness as well as the cumulative score in a concentration-dependent manner (Fig. 1A–D). The body weights of IMQ-treated mice were significantly lower than control group, however, genistein could reverse the body weights in IMQ-treated mice (Fig. 1E). The result indicated that genistein therapy improve the overall health of the mice. H&E staining showed that IMQ induced evident pathological psoriatic lesions, as indicated by the presence of epidermal parakeratosis, thickening of acanthosis cell layer, and downward epidermal extension of in-depth dermis. However, genistein administration diminished these histological changes in a dose-dependent manner (Fig. 1F and G). Immunochemical analyses visually showed that the IMQ-induced aggravation of CD45-positive inflammatory cell filtration in skin lesions was relieved by both genistein and Daivonex (Fig. 1F). Taken together, these results demonstrated that genistein had potent protective effects on IMQ-induced psoriatic mice.

3.2. Genistein suppresses IMQ-triggered skin inflammatory cytokines and chemokines

We examined the effect of genistein on inflammatory cytokines and chemokines in IMQ-induced mouse skin and found that it can significantly inhibit TH1 cytokines, such as IL-1β, IL-6, TNF-α; TH17 cytokines, such as IL-17, IL-23 and chemokine CCL2. RT-PCR analyses indicated that genistein significantly suppressed the mRNA levels of inflammatory factors in a concentration-dependent manner, as indicated by decreased the mRNA abundance of IL-1β, IL-6, TNF-α, CCL2, IL-17 and IL-23 p40 (Table 1). Consistently, IMQ increased the protein abundance of inflammatory factors in mouse dorsal skins, which was reversed by genistein administration (Table 2). Collectively, these data demonstrated that genistein could ameliorate IMQ-triggered skin inflammation.

3.3. Genistein inhibits proliferation and inflammatory responses in TNF-α-stimulated keratinocytes

Firstly, the effect of genistein on the proliferation of HaCat cells was

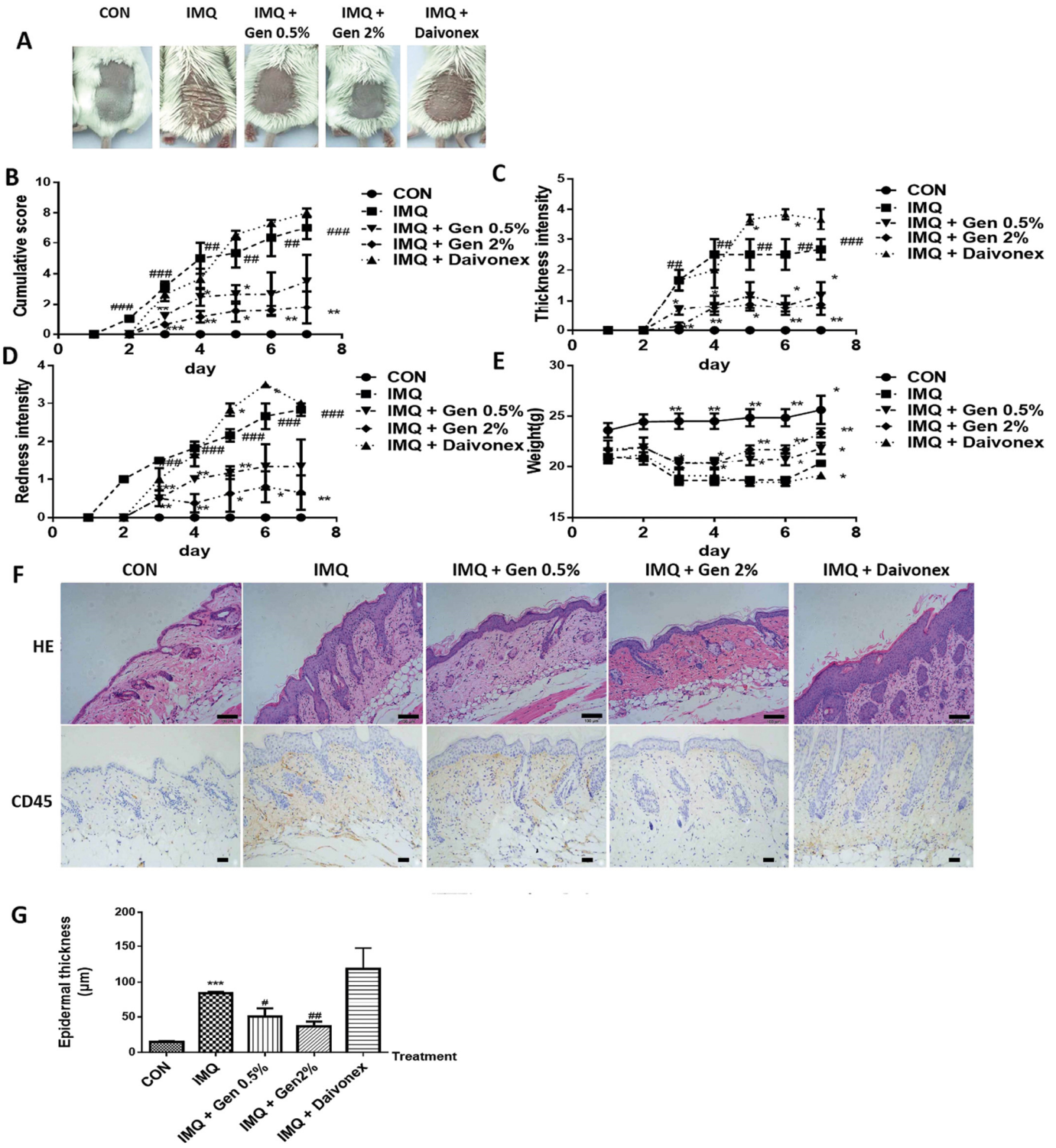

Fig. 1. Genistein attenuates skin lesions in mice with IMQ-induced psoriasis-like skin condition. (A) Photos of mouse dorsal skins were taken after treatment. (B) The cumulative score, (C) skin thickness, (D) redness scores, (E) weight and (F) H&E staining of mouse dorsal skin. Bar: 100 μm. Mouse dorsal skin IHC staining for CD45. Bar: 50 μm. (G) Epidermal thickness of mouse dorsal skin. The values are means ± SEM, with $n = 9$. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus IMQ; $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ versus CON.

detected with or without TNF-α-treating. Cell proliferation detected by MTT assay showed that genistein inhibited the proliferation of HaCaT cells with the IC_{50} of 158.5 μM (Fig. 2A). Genistein significantly inhibited the proliferation of TNF-α-treated HaCaT cells (Fig. 2B). Further, TNF-α-induced mRNA expression of inflammatory cytokines in HaCaT cells, including IL-1β, IL-6, IL-8, IL-23, TNF-α, VEGFA and CCL2, were abolished by genistein (Table 3). Consistently, the contents of IL-1β, IL-6, IL-8, IL-23, TNF-α, VEGFA and MCP1 increased in the culture medium of TNF-α-treated HaCaT cells but decreased in that of genistein-treated cells (Table 4). Overall, these results revealed that genistein suppressed TNF-α-induced proliferation and inflammatory responses in keratinocytes in vitro.

Table 1
Gene expressions in psoriasis-like lesions (mean ± SEM, $n = 3$).

mRNA (RQ)	CON	IMQ	IMQ + Gen 0.5%	IMQ + Gen 2%	IMQ + Daivonex
IL-1β	2.01 ± 0.39	198.80 ± 14.30***	34.90 ± 12.07[a]	35.48 ± 7.23[a]	74.09 ± 12.62[a]
IL-6	12.87 ± 4.45	616.80 ± 63.96***	347.60 ± 116.90[c]	304.10 ± 53.30[b]	363.10 ± 69.12[b]
TNF-α	1.64 ± 0.13	2.31 ± 0.28*	0.78 ± 0.09[a]	0.63 ± 0.07[a]	2.19 ± 0.37
CCL2	0.69 ± 0.08	3.55 ± 0.31***	1.78 ± 0.44[b]	1.66 ± 0.17[a]	2.09 ± 0.20[a]
IL-17	0.66 ± 0.16	1.36 ± 0.15**	0.45 ± 0.14[b]	0.55 ± 0.08[b]	0.42 ± 0.16[b]
IL-23	1.39 ± 0.23	15.63 ± 0.28***	8.57 ± 0.27[b]	4.40 ± 2.29[c]	5.95 ± 0.61[b]

RQ, relative quantitation.
[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$ vs. IMQ.
*** $P < 0.001$.
** $P < 0.01$.
* $P < 0.05$ vs. CON.

Table 2
Cytokines in psoriasis-like lesions (pg/mL, mean ± SEM, $n = 3$).

Cytokine (pg/mL)	CON	IMQ	IMQ + Gen 0.5%	IMQ + Gen 2%	IMQ + Daivonex
IL-1β	27.34 ± 13.87	140.80 ± 42.16*	30.43 ± 9.89[c]	19.24 ± 1.13[b]	68.44 ± 7.24
IL-6	111.10 ± 13.76	872.80 ± 28.79***	263.90 ± 87.74[a]	270.40 ± 108.10[a]	405.70 ± 139.60[b]
TNF-α	4.00 ± 0.95	44.80 ± 10.21**	12.97 ± 4.93[c]	8.50 ± 1.28[b]	28.98 ± 1.054
MCP1	31.21 ± 8.87	191.80 ± 35.95***	85.30 ± 20.49[c]	71.74 ± 14.11[b]	253.10 ± 15.40
IL-17	5.39 ± 1.29	36.92 ± 8.41**	7.77 ± 2.49[c]	6.54 ± 1.165[b]	37.47 ± 2.93
IL-23 p40	26.82 ± 3.22	41.31 ± 1.50*	24.60 ± 4.28[c]	27.23 ± 1.53[a]	25.66 ± 0.86[a]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$ vs. IMQ.
*** $P < 0.001$.
** $P < 0.01$.
* $P < 0.05$ vs. CON.

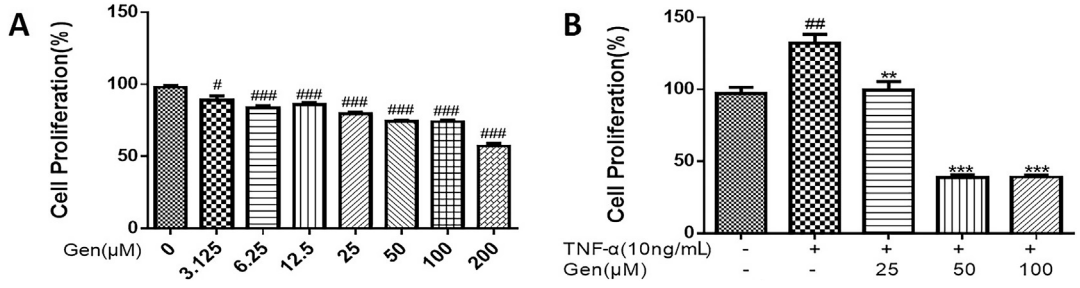

Fig. 2. Genistein inhibits the proliferation and inflammatory responses in TNF-α-treated keratinocytes. (A) HaCaT cells were incubated with the indicated concentrations (0–200 μM) of genistein for 24 h, and cell proliferation was determined by MTT assay ($n = 6$). (B) HaCaT cells were stimulated with 10-ng/mL TNF-α for 12 h, washed twice with PBS, and treated with different concentrations of genistein (0, 25, 50 and 100 μM) for another 72 h; cell proliferation was determined by MTT assay ($n = 6$). The values shown represent the mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus TNF-α; $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ versus blank.

Table 3
Gene expressions in HaCaT cells (mean ± SEM, $n = 3$).

mRNA (RQ)	Blank	TNF-α	TNF-α + Gen(50 μM)	TNF-α + Gen(100 μM)
IL-1β	1.01 ± 0.05	2.80 ± 0.11***	1.28 ± 0.13[a]	1.04 ± 0.03[a]
IL-6	1.11 ± 0.08	5.74 ± 0.32***	1.32 ± 0.04[a]	0.59 ± 0.03[a]
IL-8	0.87 ± 0.06	12.81 ± 2.44**	6.22 ± 0.27[c]	1.95 ± 0.16[b]
IL-23	1.00 ± 0.12	5.79 ± 0.34***	3.11 ± 0.07[a]	1.17 ± 0.06[a]
TNF-α	1.57 ± 0.57	15.18 ± 1.47**	8.15 ± 1.14[c]	4.20 ± 2.13[c]
VEGFA	1.09 ± 0.09	2.84 ± 0.05**	2.29 ± 0.37[b]	1.70 ± 0.10[b]
CCL2	1.13 ± 0.18	43.35 ± 8.67**	2.77 ± 0.34[b]	1.35 ± 0.20[b]

RQ, relative quantitation.
[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$ vs. IMQ.
*** $P < 0.001$.
** $P < 0.01$.

Table 4
Cytokines in cell culture medium (pg/mL, mean ± SEM, n = 3).

Cytokine (pg/mL)	Blank	TNF-α	TNF-α + Gen(100 μM)
IL-1β	1.76 ± 0.35	2.48 ± 0.19	2.22 ± 0.12
IL-6	3.19 ± 0.36	6.55 ± 0.33*	3.21 ± 0.40[b]
IL-8	5.95 ± 0.16	7.05 ± 0.0003*	5.14 ± 0.04[a]
IL-23	3.19 ± 0.36	6.55 ± 0.33*	3.21 ± 0.40[b]
TNF-α	0.86 ± 0.23	7.75 ± 0.65**	2.75 ± 0.10[b]
VEGFA	29.74 ± 3.42	53.33 ± 0.12*	23.92 ± 0.01[a]
MCP1	224.40 ± 3.26	368.60 ± 1.34***	44.48 ± 2.26[a]

[a] $P < 0.001$.
[b] $P < 0.05$ vs. IMQ.
*** $P < 0.001$.
** $P < 0.01$.
* $P < 0.05$ vs. Blank.

3.4. Genistein inhibits STAT3-mediated inflammatory responses

Genistein suppressed STAT3 phosphorylation in the epidermis of mouse dorsal skins in a concentration-dependent manner (Fig. 3A–B). In addition, TNF-α enhanced STAT3 phosphorylation, which was significantly abolished by genistein (Fig. 3C–D). The transfection efficacy of STAT3 siRNA was determined at the protein level. Western blot analyses showed an evident decrease in the protein levels of STAT3 and pSTAT3, suggesting that the transfection was efficient (Fig. 4A). Subsequently, siRNA-mediated STAT3 knockdown greatly reduced the mRNA levels of IL-6, TNF-α and IL-23, but genistein could not further enhance the inhibitory effects of STAT3 siRNA (Fig. 4B–D). In summary, these findings demonstrated that STAT3 inhibition was a prerequisite for genistein to ameliorate psoriasis-related inflammation.

3.5. Genistein suppresses NF-κB signaling in TNF-α-induced HaCaT keratinocytes

Genistein inhibited TNF-α-induced I-κBα phosphorylation in a concentration-dependent manner (Fig. 5A and B). The results showed an evident genistein-induced decrease in the level of NF-κB in HaCaT cells (Fig. 5C and D). In addition, we found that BAY 11-7082, an NF-κB inhibitor, decreased the mRNA expression of TNF-α and VEGFA, but genistein could not further enhance the inhibitory effect of BAY 11-7082 (Fig. 5E and F). In conclusion, these results demonstrated that genistein suppressed psoriasis-related inflammation possibly via NF-κB

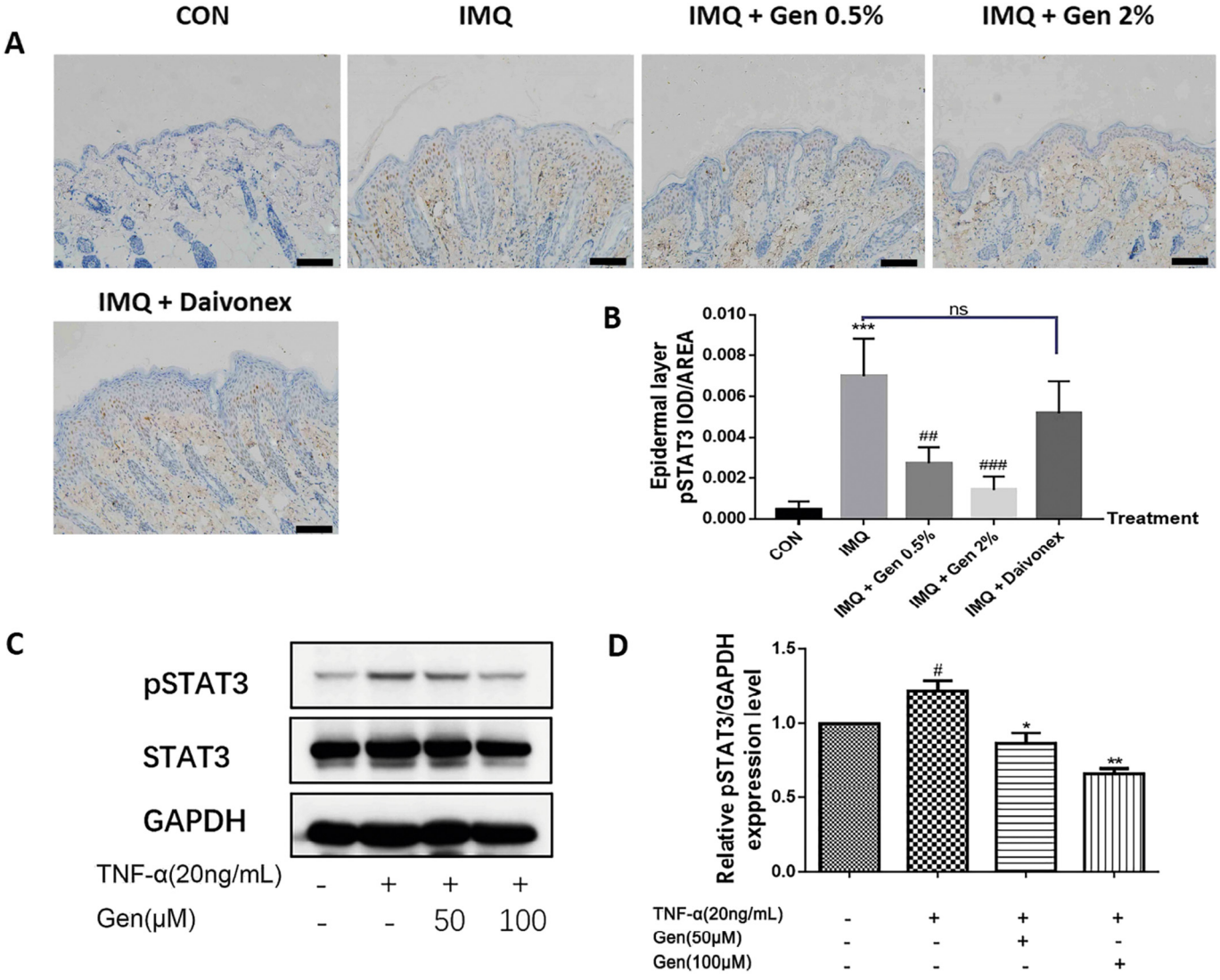

Fig. 3. Genistein inhibits STAT3 signaling in epidermis and HaCaT keratinocytes. (A, B) Immunohistochemical staining and average optical density (AOD) of mouse dorsal skins in the epidermal layer for pSTAT3 using Image-Pro Plus 6.0. Bar: 100 μm. (C, D) HaCaT cells were pre-incubated with genistein (50 and 100 μM) for 5 h and then exposed to TNF-α for 1 h; pSTAT3 and STAT3 levels were assessed by western blotting. Ns = Not significant. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus IMQ or TNF-α; $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ versus CON or blank.

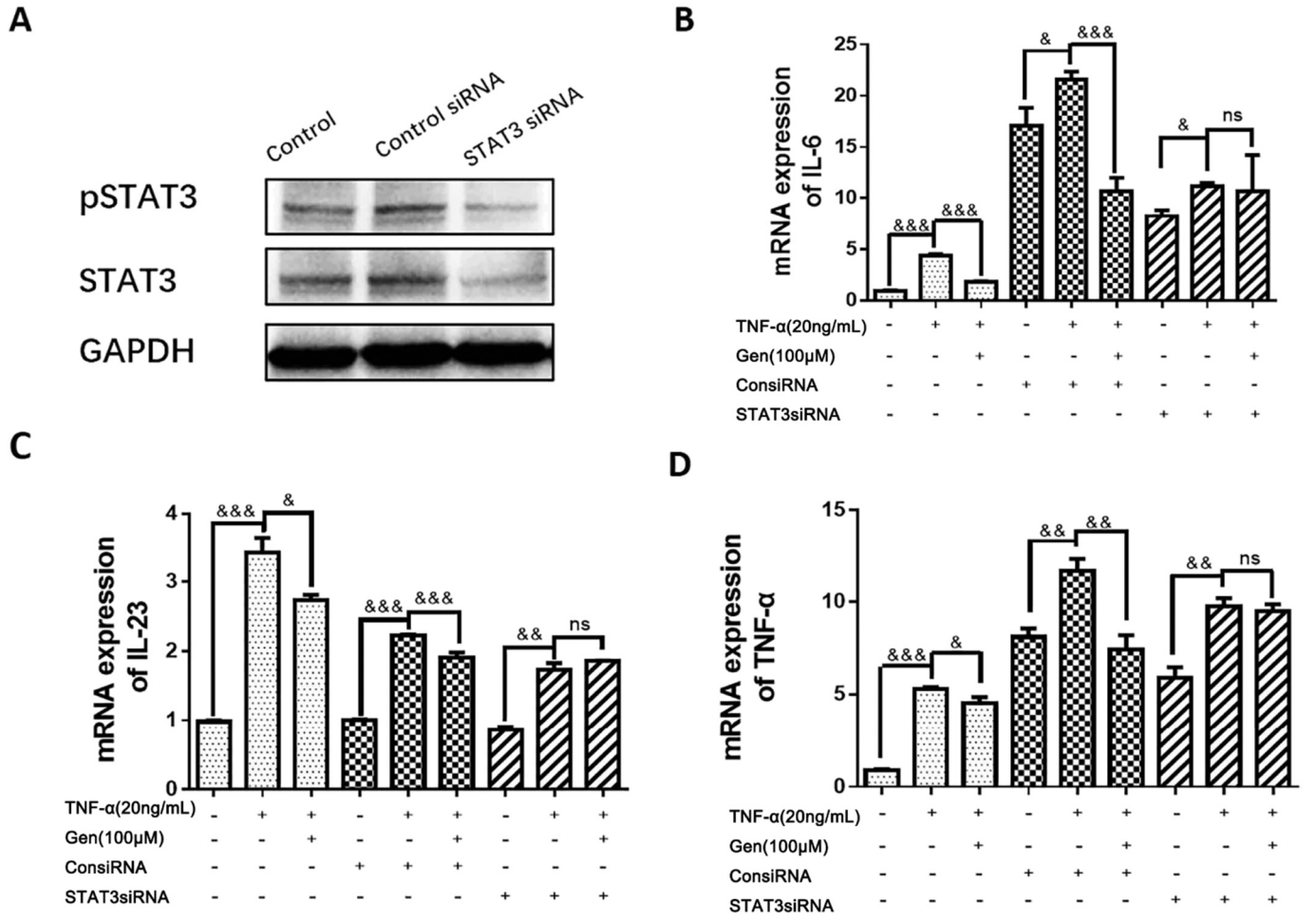

Fig. 4. Genistein inhibits STAT3-mediated inflammatory responses. (A) HaCaT cells were transfected with Con siRNA and STAT3 siRNA, and STAT3 and pSTAT3 expression was verified by western blotting. (B-D) HaCaT cells were exposed to TNF-α for 30 min after transfection, and then incubated with 100 μM genistein for 8 h; mRNA expression of IL-6, IL-23 and TNF-α was determined by RT-PCR. The results are shown as means ± SEM from three independent experiments. Ns = Not significant. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus TNF-α or IMQ; $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus blank; $^{\&\&\&}P < 0.001$, $^{\&\&}P < 0.01$, $^{\&}P < 0.05$.

pathway.

4. Discussion

Psoriasis is a chronic recurrent disease characterized by epidermal hyperplasia, erythema, and scales, affecting approximately 1–3% of the world's population. A growing number of studies have shown that active ingredients from natural herbal medicines can play a role in the prevention and treatment of psoriasis [21,24–26].Genistein is a flavonoid with a variety of pharmacological activities. In the present study, we report the therapeutic effect of genistein on imiquimod-induced psoriasis-like mouse lesions and unravel its possible anti-inflammatory mechanism in HaCaT cells in vitro.

There are many methods to establish psoriasis animal models, commonly include propranolol-induced ear skin hypertrophy model in guinea pigs; imiquimod-induced skin lesion model in mice; and IL-22 subcutaneous injection model. IMQ, an agonist of toll-like receptor (TLR) 7 and TLR8, can cause psoriasis-like lesions by topical applying to the mice bare dorsal skin for 5–7 days [27,28]. The pathological features of the IMQ model are similar to those of human psoriasis, with diffuse epidermal hyperplasia and inflammatory cell infiltration [22]. The types of mice in IMQ model often include Balb/c mice and C57BL/6 mice. Some researchers even prefer to use single male mice for pharmacological studies. For example, Nadeem, A [29] and Di T [24] used single male Balb/c mice to prepare IMQ models. We have also studied the treatment effect of traditional Chinese formula in male mice with IMQ model [25]. Therefore, we also observed the therapeutic effect of genistein on psoriasis-like lesions in male mice.

We found that genistein can significantly improve IMQ-induced pathological scores of cutaneous skin lesions, reduce epidermal thickness, and inhibit CD45 expression in mice skin. Genistein also could down-regulated expression of inflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-17 etc. Our results suggest that genistein may play a role in the treatment of psoriasis skin lesions through anti-inflammatory effects. Interestingly, topical application of Daivonex failed to alleviate the thickness and redness of psoriatic skins in our study. Daivonex, a synthetic vitamin D3 analogue, has been reported to inhibit cell proliferation and stimulate the differentiation of epidermal keratinocytes and lymphocytes in both in vitro systems and psoriatic patients [30]. This is not consistent with our research. We speculate that the unsatisfactory therapeutic effect of Daivonex in our study is due to the short time of IMQ model, which leads to the short treatment time of Daivonex. Clinically, Daivonex hardly worked until 4 consecutive weeks of treatment, and it was found that the area of skin lesions usually became larger during the initial administration [31,32]. Although Daivonex did not improve the skin thickness in IMQ model, Daivonex still showed a strong inhibitory effect on the expression of cytokines and chemokines in inflammatory dermis. This is consistent with the action of genistein.

HaCaT cells are spontaneously transformed aneuploid immortal

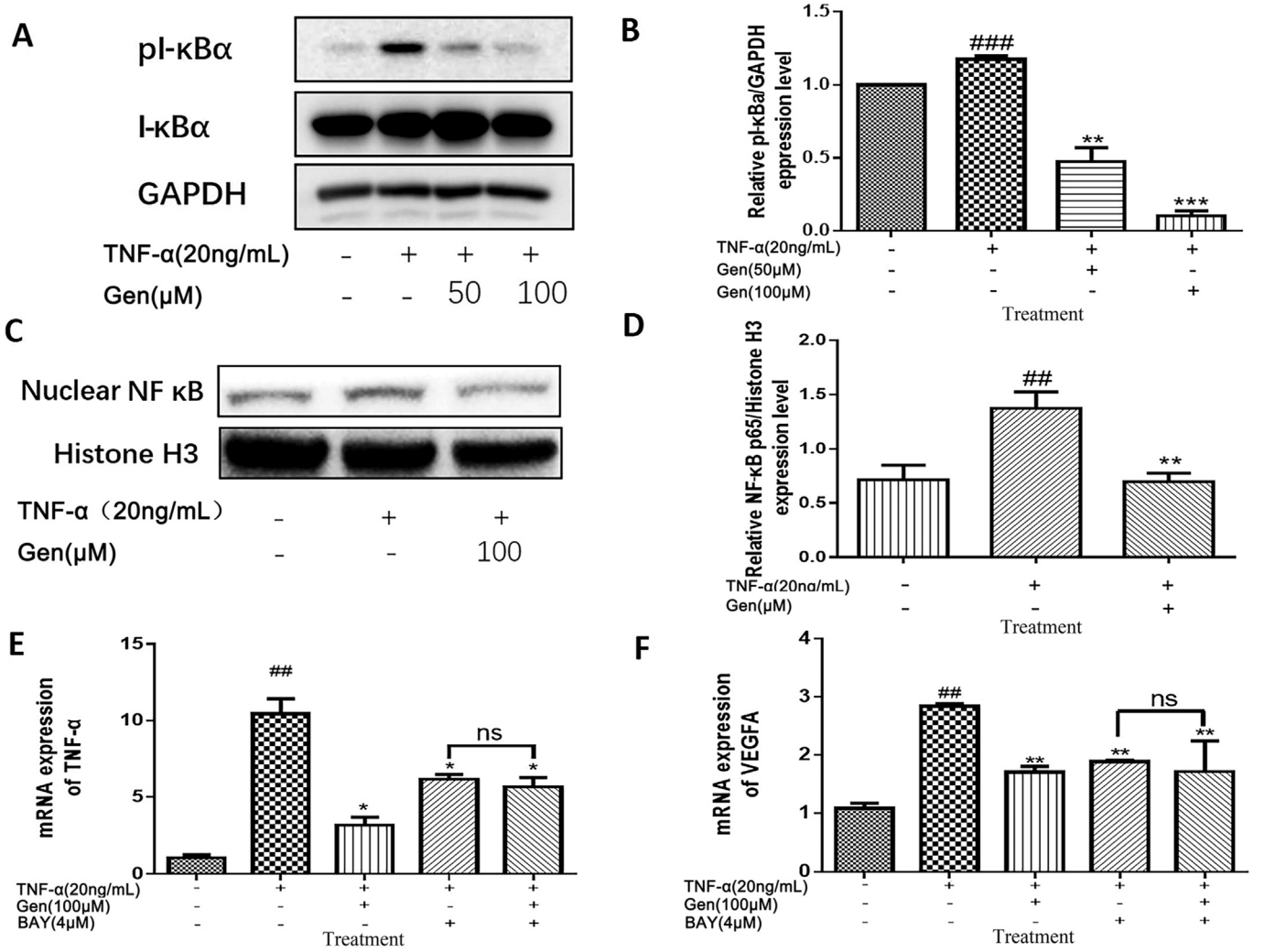

Fig. 5. Genistein suppresses NF-κB signaling in TNF-α-induced HaCaT keratinocytes. HaCaT cells were incubated with 100 μM of genistein for 5 h and then exposed to TNF-α for 1 h. (A, B) The total pI-κBa levels were assessed by western blotting. (C, D) The NF-κB p65 expression in the nucleus. (E, F) HaCaT cells were incubated with 4 μM of BAY 11-7082 (abbreviated as BAY in the figure, sic passim) for 2 h, then exposed to TNF-α for 30 min, and at last, incubated with 100 μM of genistein for 8 h; the mRNA expression of TNF-α and VEGFA was determined by RT-PCR. The results are shown as means ± SEM from three independent experiments. Ns = Not significant. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus TNF-α. $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus blank.

keratinocyte cells isolated from adult skin tissues, which have been widely used in studies on the pathophysiology of keratinocytes in multiple diseases, such as psoriasis and eczema [22,33–35]. TNF-α, IFN-γ and IL-17/22 play very important role in the pathogenesis of psoriasis, so they are often used as in vitro stimulators to induce keratinocyte inflammation or hyperproliferation [22,34].

The increased level of TNF-α can be detected in both serum and skin lesions of psoriasis patients and is positively correlated with the severity of the disease [36,37]. Further, anti-TNF-α neutralising antibody has been successfully applied for treating psoriasis [36,37]. More importantly, TNF-α has been the most commonly used inducer for establishing an in vitro inflammatory model of keratinocytes [22,26]. In current study showed genistein could significantly inhibit the proliferation of HaCaT cells with or without TNF-α induction and abolish 20 ng/mL TNF-α induced inflammatory cytokines and chemokines mRNA upregulated expression, including IL-6, IL-1β, IL-8, IL-23, VEGFA and CCL2. These data indicate that genistein also exhibits a strong anti-inflammatory effect on keratinocytes in vitro.

STAT3 blockade could attenuate inflammation and can thus serve as a strategy for treating psoriasis [14]. In this study, we found that genistein, but not Daivonex, suppressed STAT3 phosphorylation. Next, we found that STAT3 silence could mimic the inhibitory effects of genistein on the expression of inflammatory factors. Consistent with our results, some novel clinical drugs for psoriasis, such as benzothiadiazole and astilbin, have been shown to alleviate psoriatic skin lesions by inhibiting the STAT3 signaling pathway in mouse dorsal skins [24,38]. These data suggest that the anti-inflammatory effects of genistein are associated with inhibition of the STAT3 pathway.

Nuclear factor κB (NF-κB) is a nuclear transcription factor that regulates expression of a large number of genes that are critical for inflammatory responses. NF-κB binds to the I-κB member and is in an inactive state in the cytosol. Once I-κBα is phosphorylated by some other proteins, NF-κB protein separates from I-κBα and translocates into the nucleus to regulate the expression of multiple genes. In current study, we found that genistein inhibited TNFα-induced NF-κB phosphorylation in HaCaT cells. We further used NF-κB inhibitor BAY 11-7082 to clarify the mechanism of action of genistein. BAY 11-7082 had been reported to could improve skin lesions in IMQ-induced psoriasis in mice [15]. Our study also showed that BAY 11-7082 can significantly down-regulate the expression of inflammatory factors induced by TNFα in keratinocytes. When BAY 11-7082 was combined with genistein, the role of genistein to down-regulate the expression of inflammatory factors disappeared. Therefore, it could be reasonable to postulate that NF-κB is a critical regulator to genistein during psoriasis treatment.

In conclusion, our work demonstrates that genistein ameliorated the severity of IMQ-induced psoriasis-like skin lesions in mice and reduced TNF-α-induced cell proliferation and inflammation of keratinocytes. Mechanistically, the therapeutic effects of genestein may be associated with down-regulation of STAT3 and the NF-κB pathway. Our results provide new insight of genistein and may contribute to the development of drugs for psoriasis treatment.

Conflict of interest

The authors state no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.054.

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