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Gypenosides alleviates HaCaT keratinocyte hyperproliferation and ameliorates imiquimod-induced psoriasis in mice

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Abstract
Background: Psoriasis is an autoimmune skin condition characterized by hyperproliferation of keratinocytes and chronic immune responses. Gypenosides (Gyp) exhibits anti-proliferative and anti-inflammatory effects on different diseases. However, its functioning and mechanism of Gyp on psoriasis remains unknown.
Objective: To explore the effect and mechanism of Gyp on psoriasis.
Material and Methods: The impact and mechanism of Gyp on psoriasis *in vitro* and *in vivo* were probed through cell counting kit-8 (CCK-8), 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay, reverse transcription quantitative polymerase chain reaction, hematoxylin and eosin staining, enzyme-linked immunosorbent serologic assay, immunofluorescence, and Western Blotting assays.
Results: Gyp inhibited cell proliferation and the release of inflammatory cytokinesin interleukin (IL-22)-induced spontaneously transformed human aneuploid immortal keratinocyte cell line (HaCaT). In addition, Gyp demonstrated enhancement in erythema and scaling as well as reductions in the thickness of epidermal layers, release of inflammatory factors, and Ki-67 (a nuclear protein) level in imiquimod (IMQ)-induced mice. Mechanistically, Gyp upregulated nuclear factor erythroid 2-related factor 2 (Nrf-2) expression and diminished the level of p-p65/p65 and p-STAT3/STAT3 in skin tissues from IMQ-induced mice and IL-22-induced HaCaT cells, which were reversed with the application of ML385, an inhibitor of Nrf2. In addition, the administration of ML385 reversed decrease in cell viability and reduced the expressions of IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) in IL-22-induced HaCaT cells caused by Gyp.
Conclusion: In summary, Gyp reduced excessive cell growth and inflammation in psoriasis by suppressing nuclear factor *kappa B* (NF-κB) and signal transducer and activator of transcription 3 (STAT3) through activation of Nrf2.

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Introduction

Psoriasis is a chronic recurring inflammatory disorder affecting approximately 2–3% of global population.[1] Psoriasis is a genetically determined condition characterized by the infiltration of immune cells into the dermis and abnormal growth of keratinocytes in the epidermis. It is also affected by immunological factors and environmental triggers. The common symptoms include burning sensation or discomfort, itching, appearance of lesions with changes in color, and dryness or cracking of the skin.[2,3] The patient's quality of life is greatly affected by these symptoms, which include feeling of despair, worry, and suicidal thoughts.[4,5] Topical corticosteroids and vitamin D analogs are commonly used as the primary treatment for mild psoriasis.[6] In situations of moderate or severe conditions, immunosuppressive medications, such as methotrexate and cyclosporine, together with biological treatments that specifically target proteins, such as etanercept, secukinumab, interleukin (IL)-17A inhibitor, or tumor necrosis factor-α (TNF-α) inhibitors, are utilized.[7] Nevertheless, certain patients are unable to obtain these treatments because of adverse effects, such as lichenification and hypotension as well as exorbitant costs.[2] Hence, it is important that patients suffering from psoriasis undergo treatment by utilizing safer and more efficacious therapeutic approaches.

Owing to their minimal toxicity, wide range of pharmacological effects, and cost-effectiveness, natural compounds have emerged as appealing candidates for pharmaceutical research and development.[8] Gypenosides (Gyp), the principal bioactive ingredient of *Gynostemma pentaphyllum* (Thunb.) Makino, are members of the dammarane-type tetracyclic triterpenoid class, structurally similar to ginsenosides.[9] Pharmacological investigations have discovered several therapeutic properties of Gyp, such as its ability to: prevent atherosclerosis, lower cholesterol levels, modulate the immune system, reduce oxidative stress, protect the liver, and exhibit anti-cancer and anti-depressant properties.[10-16] In addition, Gyp has demonstrated its ability to inhibit the growth of various types of tumors, such as liver cancer,[15], bladder cancer,[17] non-small cell lung cancer[18] as well as esophageal and colon cancers.[19] The inhibitory effect of Gyp on inflammation is reported in ischemic stroke,[20] systemic lupus erythematosus,[21] diabetes mellitus,[22] retinitis pigmentosa,[23] acute lung injury,[24] Graves' ophthalmopathy,[25] etc. Nevertheless, it is still uncertain whether Gyp has the ability to control psoriasis by suppressing the growth and inflammation processes.[26]

In order to investigate the impact and mechanism of Gyp in psoriasis, spontaneously transformed human aneuploid immortal keratinocyte cell line (HaCaT) was stimulated with IL-22 to establish an *in vitro* model, and mice were managed with imiquimod (IMQ) to construct an *in vivo* model of psoriasis. Both *in vitro* and *in vivo* studies were conducted to examine the precise impacts of Gyp on inflammation and proliferation. In addition, the putative mechanism underlying the nuclear factor *kappa B* (NF-κB) and signal transducer and activator of transcription 3 (STAT3) signaling pathways of Gyp was investigated in animal and cell models of psoriasis. We anticipated that the outcomes would offer potential medication candidates for the treatment of psoriasis.

Materials and Methods

Cell culture

The National Collection of Authenticated Cell Cultures in Shanghai (China) provided HaCaT cells (SCSP-5091). The cells were cultured in DMEM (PM150210; Procell, Wuhan, China) and supplemented with 10% fetal bovine serum (FBS; 164210; Procell) and 1% penicillin-streptomycin solution (PB180120; Procell) at 37°C with 5% carbon dioxide (CO_2).

Cell treatment

Gyp (S5151; 99.33% purity; Selleck, Houston, TX, USA) with varied concentrations of 0, 5, 10, 20 and 40 μM was applied to hatch with HaCaT cells for 24 h to assess the toxic effect of Gyp on HaCaT cells. The concentrations of Gyp used in the incubations were based on a previous study.[21] The Gyp concentration of 0 μM used in the present study indicated that the cells were hatched with phosphate buffer saline (PBS; ST447; Beyotime, Shanghai, China). To establish the *in vitro* model of psoriasis, HaCaT cells were stimulated with 100-ng/mL IL-22 for 24 h as done in previous studies.[27,28] In addition, 10-μM ML385, an inhibitor of Nrf2, was utilized to hatch with HaCaT cells for 24 h to determine the direct role of Gyp in Nrf2 signaling. The concentrations of ML385 (HY-100523; ≥99.96% purity; MedChem Express, Monmouth Junction, NJ, USA) used in the incubations were according to an earlier study.[29] Both Gyp and ML385 were first dissolved in dimethylsulfoxide (DMSO; D8371; Solarbio, Beijing, China) and subsequently diluted with PBS for different assays.

Cell counting kit-8 (CCK-8) assay

After being seeded onto 96-well plates, 5×10^3 HaCaT cells were grown overnight at 37°C with 5% CO_2. Next, 10 μL of CCK-8 reagent (CA1210; Solarbio) was added to each well and incubated at 37°C for 2 h. A Thermo Fisher Scientific (Waltham, MA, USA) microplate reader was utilized to measure absorbance at 450 nm.

5-ethynyl-2'-deoxyuridine (EdU) incorporation assay

BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (C0078S; Beyotime) was used to measure the proliferation of HaCaT cells. Briefly, 6×10^5 HaCaT cells were seeded into six-well plates and grown overnight at 37°C with 5% CO_2. The EdU working solution (20 μM) was then appended to each well of the plates and incubated for 2 h at 37°C. Following the fixation of cells with the immunolabeling fix solution (P0098; Beyotime) and their permeation

with 0.3% Triton X-100 (T8200; Solarbio), the cells were exposed to anti-EdU Click reaction solution for 30 min in the dark. The cell nucleus was stained with Hoechst 33342 (5 μg/mL; P0133; Beyotime). Using a fluorescent microscope (Olympus, Tokyo, Japan), the stained cells were photographed.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent (R0016; Beyotime) was used to extract total RNA from HaCaT cells. Reverse transcription assay was conducted using the complementary DNA (cDNA) synthesis kit (B639252; Sangon Biotech, Shanghai, China). RT-qPCR was done on the Bio-Rad CFX Manager software (Bio-Rad Laboratories Inc., Hercules, CA, USA) with 2× SYBR mix (B110031; Sangon Biotech). The gene expressions were calculated by the $2^{-\Delta\Delta CT}$method with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the control gene. The primer sequence is shown in Table 1.

Animals

BALB/c nude mice, 6–8 weeks old, were purchased from JKbiot (Nanjing, China) and housed in specified pathogen-free (SPF) condition with unlimited access to food and water, a 12-h light–dark cycle, and regulated temperature. The Animal Research Ethics Committee of Southwest Medical University approved all animal experiments. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*.[30]

Animal group and treatment

Six groups, each containing randomly assigned six mice, were prepared. The groups are as follows: control, IMQ, IMQ+Gyp (25 mg/kg), IMQ+Gyp (50 mg/kg), IMQ+Gyp (100 mg/kg) and IMQ+Gyp (100 mg/kg)+ML385. In the IMQ+Gyp (100 mg/kg)+ML385 group, 62.5 mg of IMQ cream was applied topically to the mice's back skin for 7 days in a row. Additionally, the mice were administered 100 mg/kg Gyp intragastrically and 30 mg/kg ML385 intraperitoneally once a day for 4 weeks. The dose of IMQ, Gyp and ML385 was selected based on previous studies.[16,31,32] Mice in the IMQ, IMQ+Gyp (25 mg/kg), IMQ+Gyp (50 mg/kg) and IMQ+Gyp (100 mg/kg) groups were imparted topical doses of 62.5 mg of IMQ cream on their back skin consecutively for 7 days. The animals were administered 25 mg/kg Gyp, 50 mg/kg Gyp, and 100 mg/kg Gyp by oral gavage once daily, as well as the same dose of PBS intraperitoneally, for 4 weeks. Mice in the control group were treated with an identical dose of vaseline on their back skin for seven successive days, and intragastrically administrated with the same dose of PBS once daily for 4 weeks. On days 0, 2, 4, and 7, erythema and scaling were evaluated from 0 to 4 scores using the Psoriasis Area Severity Index (PASI), where 0 score was none, score of 1 and 2 demonstrated moderate, score of 3 meant severe, and score of 4 demonstrated extremely severe condition. Skin samples were taken for the subsequent experiments after the mice were euthanized on day 8.

Hematoxylin and eosin (H&E) staining

Skin tissue samples were treated in 4% paraformaldehyde (P1110; Solarbio) and dried using gradient ethanol. Next, tissues were submerged in paraffin (YA0011; Solarbio) and sliced into 5-μm-thick slices. After staining the slices with H&E (G1120; Solarbio), the same were examined using an Olympus light microscope and the Image-Pro Plus 6.0 image analysis program (Media Cybernetics, USA).

Enzyme-linked immunosorbent serologic assay (ELISA)

The human interleukin-1ß (IL-1ß) ELISA kit (PI305), the human IL-6 ELISA kit (PI330), and the human TNF-α ELISA kit (PT518) (all from Beyotime) were used to measure the amount of IL-1ß, IL-6, and TNF-α in the supernatant of HaCaT cells according to the operating manuals. The absorbance was measured using a 450-nm microplate reader (Thermo Fisher Scientific).

Subsequently, mice skin tissues were washed with pre-cooling PBS and cut into pieces. Then, the skin tissue pieces were mixed with PBS with a weight-to-volume ratio of 1:9 and thoroughly crunched. Next, the homogenates were centrifuged at 5000 g for 10 min at 4°C and the supernatant samples were collected for further examination. The contents in the supernatant of IL-1ß, IL-2, IL-6, IL-10, IL-17, IL-22, IL-23, interferon-γ (IFN-γ) and TNF-α were measured by using Mouse IL-1ß ELISA kit (BMS6002), Mouse IL-2 ELISA kit (BMS601), Mouse IL-6 ELISA kit (KMC0061), Mouse IL-10 ELISA kit (BMS614), Mouse IL-17 ELISA kit (BMS6001), Mouse IL-22 ELISA kit (BMS6022), Mouse IL-23 ELISA kit (BMS6017), Mouse IFN-γ ELISA kit (KMC4021), and Mouse TNF-α ELISA kit (BMS607-3), respectively (Invitrogen, Carlsbad, CA, USA) in keeping with the operating instructions. The absorbance

Table 1 The sequences of primers used in the present study.

Name	Forward (5′-3′)	Reverse (5′-3′)
IL-1ß	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF-□	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

was recorded at 450 nm by a microplate reader (Thermo Fisher Scientific).

Immunofluorescence (IF) staining

Pre-cooling PBS and 4% paraformaldehyde were transcardially infused into the mice. After being separated, skin tissues were submerged in 4% paraformaldehyde. Next, tissues were embedded in optimal cutting temperature compound (OCT; 4583; SAKURA, CA, USA) and divided into 5-μm-thick slices. *Bovine serum albumin* (BSA) blocking buffer (SW3015; Solarbio) and 0.2% Triton X-100 (T8200; Solarbio) were used and the slices were incubated with antibodies against Ki-67, a nuclear protein (1:100; ab15580; Abcam, Cambridge, UK) overnight at 4°C. Next, slices were treated with goat anti-rabbit IgG H&L (Alexa Fluor® 647) (1:1000; ab150079, Abcam) for an hour at room temperature after being cleaned thrice with PBS. Subsequently, the slices were stained with mounting medium and anti-fading 4′,6-diamidino-2-phenylindole (DAPI; S2110; Solarbio), and photographs were taken by fluorescence microscopy (IX71; Olympus).

Western blotting analysis

To extract total proteins, HaCaT cells and skin tissues were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (R0010; Solarbio). The bicinchoninic acid (BCA) protein assay kit (PC0020; Solarbio) was utilized to measure protein concentrations. Protein samples (20 μg) were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and placed on polyvinylidene fluoride (PVDF) membranes (IPVH00010; EMD Millipore, Billerica, MA, USA). The membranes were sealed in 5% BSA blocking buffer for 1 h at room temperature and then treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated at room temperature for 1 h with goat anti-rabbit IgG H&L (HRP) (1:10,000; ab205718; Abcam) as a secondary antibody. The bands were made with a BeyoECL Plus kit (P0018S; Beyotime), and the gray value was determined using the Image-ProPlus software (Media Cybernetics Inc., Rockville, MD, USA). The internal reference was ß-actin. The primary antibodies included antibodies against Nrf2 (1:3000; PA5-27882; Invitrogen), p65, a NF-κB subunit (1:1000; 51-0500; Invitrogen), phosphorylated p65 (p-p65; 1:1000; MA5-15160; Invitrogen), signal transducer and activator of transcription 3 (STAT3; 1:2000; PA5-86208; Invitrogen), phosphorylated-STAT3 (p-STAT3; 1:1000; 44-380G; Invitrogen), and ß-actin (1:2000; PA1-183; Invitrogen).

Statistical analysis

All data were presented as mean ± standard deviation (SD) and analyzed by using the SPSS 20.0 software (IBM, Armonk, New York, USA). Statistical differences were observed with the one-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni test. Differences were considered statistically significant at $P < 0.05$.

Results

Gyp suppressed cell proliferation in IL-22-induced HaCaT cells

To probe the function of Gyp on psoriasis, *in vitro* experiments were first performed on HaCaT cells. Results in Figure 1A show that the HaCaT cell viability was significantly reduced when treated with 40-μM Gyp, compared to 0-μM Gyp. However, there was no statistically significant difference in HaCaT cell viability between the absence of Gyp and the treatment with 5-, 10-, or 20-μM Gyp. Hence, concentrations of 5-, 10-, or 20-μM Gyp were used for the ensuing trials. Following stimulation with IL-22, HaCaT cells were used to establish an *in vitro* model of psoriasis. HaCaT cell viability increased significantly. However, when 10- or 20-μM Gyp was administered, the viability of HaCaT cells decreased noticeably. This reduction was not observed with the administration of 5-μM Gyp (Figure 1B). In addition, the results from Edu experiments demonstrated that the application of either 10- or 20-μM Gyp led to a considerable reduction in the proliferation of HaCaT cells induced by IL-22, as shown in Figures 1C and 1D. Together, these outcomes revealed that Gyp inhibited IL-22-induced HaCaT cell proliferation.

Gyp attenuated inflammation in IL-22-indued HaCaT cells

The levels of inflammatory factors, such as IL-1ß, IL-6 and TNF-α, were analyzed to investigate the role of Gyp in inflammation. HaCaT cells that were stimulated with IL-22 exhibited significantly elevated transcriptional levels of these three inflammatory markers. However, the administration of 10- or 20-μM Gyp significantly neutralized these cells but not 5-μM Gyp (Figure 2A). Additionally, the identical outcomes were verified by ELISA assays (Figure 2B). Collectively, Gyp reduced inflammation in IL-22-indued HaCaT cells.

Gyp ameliorated IMQ-elicited psoriasis skin damage in mice

To construct an *in vivo* model of psoriasis, mice were subsequently managed with IMQ. Erythema and scaling were observed concurrent to IMQ induction; however, application of 50- or 100-mg/kg Gyp alleviated these symptoms, but 25-mg/kg Gyp had no effect (Figure 3A). The IMQ-induced erythema and scaling scores were consistently reduced by 50- or 100-mg/kg Gyp (Figure 3B). Histopathological results showed that IMQ caused thicker epidermal layers, which were lessened by the application of 50- or 100-mg/kg Gyp (Figure 3C). Gyp completely relieved the skin damage caused by IMQ-induced psoriasis in mice.

Gyp inhibited skin tissues inflammation and proliferation in IMQ-induced mice

The levels of inflammatory factors in skin tissues were initially analyzed to investigate the function of Gyp on

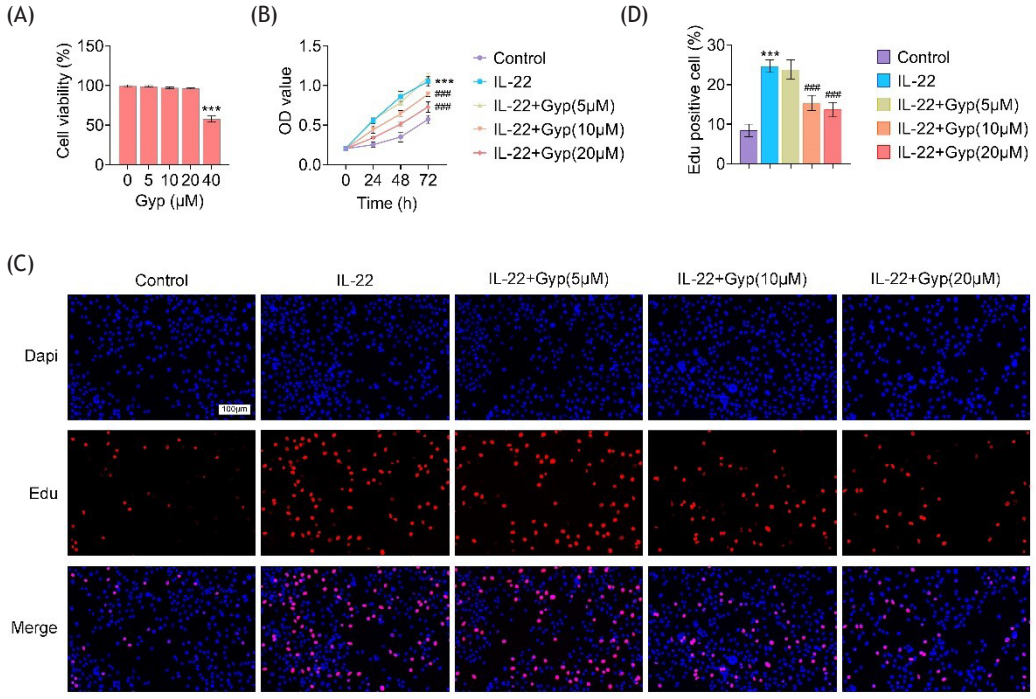

Figure 1 Gyp inhibited IL-22-induced proliferation in HaCaT cells. (A) Cell viability was detected by CCK-8 assays after HaCaT cells were incubated with 0-, 5-, 10-, 20- and 40-μM Gyp for 24 h. $^{***}P < 0.05$ vs. 0-μM Gyp. (B) HaCaT cells were incubated with 0-, 5-, 10- and 20-μM Gyp for 24 h and then stimulated with 100-ng/mL IL-22 for 24 h. The HaCaT cell viability was examined by CCK-8 assays. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. IL-22. (C and D) The HaCaT cell proliferation was determined by Edu assays. Scale bar = 200 μm. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. IL-22.

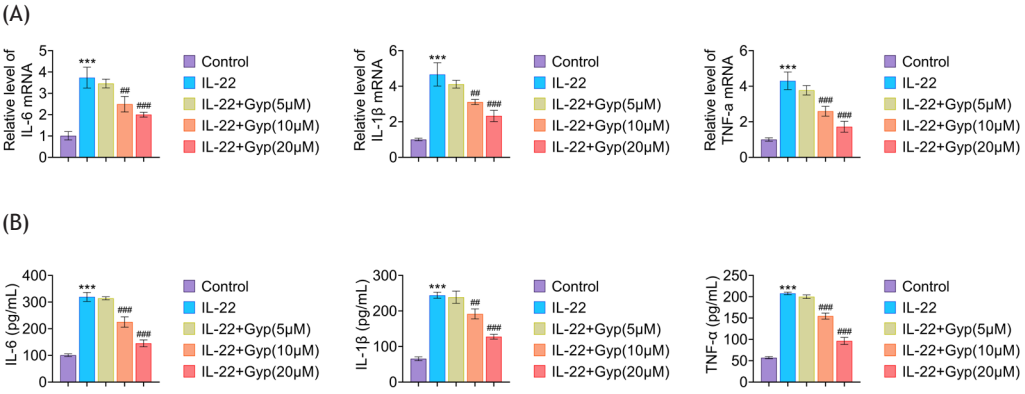

Figure 2 Gyp attenuated inflammation in IL-22-indued HaCaT cells. (A) The relative mRNA expression level of IL-1β, IL-6 and TNF-α was detected by RT-qPCR. Data were expressed after being normalized with GAPDH. (B) The concentration of IL-1β, IL-6 and TNF-α in the cellular supernatant was measured by ELISA. $^{***}P < 0.001$ vs. control; $^{##}P < 0.01$ and $^{###}P < 0.001$ vs. IL-22.

psoriasis *in vivo*. The concentrations of IL-1β, IL-2, IL-6, IL-10, IL-17, IL-22, IL-23, IFN-γ, and TNF-α were significantly increased in the skin tissues of IMQ-elicited mice. However, these concentrations were significantly reduced when 50- or 100-mg/kg Gyp was administered but not when 25-mg/kg Gyp was imparted (Figure 4A). Additionally, the level of Ki-67 in the skin tissues of rodents that was induced by IMQ was significantly reduced by 50- or 100-mg/kg Gyp (Figure 4B). The overall result showed that Gyp suppressed IMQ-induced inflammation and proliferation in mice skin tissues.

Gyp regulated the expression of NF-κB and STAT3 through Nrf2 in psoriasis

To explore the mechanism of Gyp in psoriasis, the expression of Nrf2, whose activation is thought to be a potential strategy in the administration of skin conditions, such as psoriasis, was assayed.[33,34] The Nrf2 expression was significantly downregulated in both skin tissues from IMQ-induced mice and IL-22-indued HaCaT cells, and was prominently restored with the treatment of medium and high concentrations of Gyp (Figures 5A and B). Additionally, it has

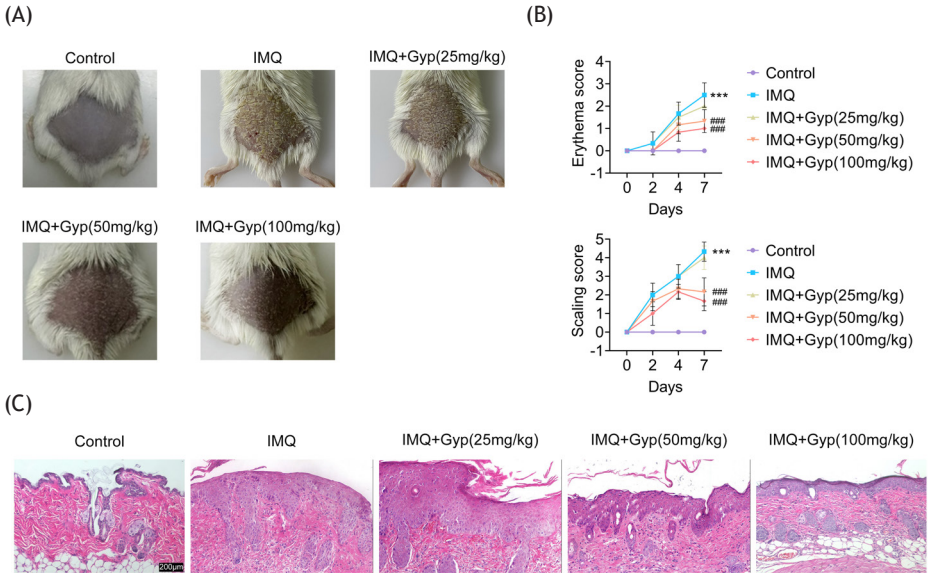

Figure 3 Gyp improved IMQ-induced psoriasis skin damage in mice. 62.5 mg IMQ was topically applied on the mice to induce an *in vivo* model of psoriasis, and received intragastric administration of 100-mg/kg Gyp once a day for 4 weeks. (A) The macroscopic appearance of back skin of mice on day 8. (B) On days 0, 2, 4 and 7, erythema and scaling were evaluated using the PASI. (C) The histopathological assessment by H&E staining. Scale bar = 200 μm. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. IMQ.

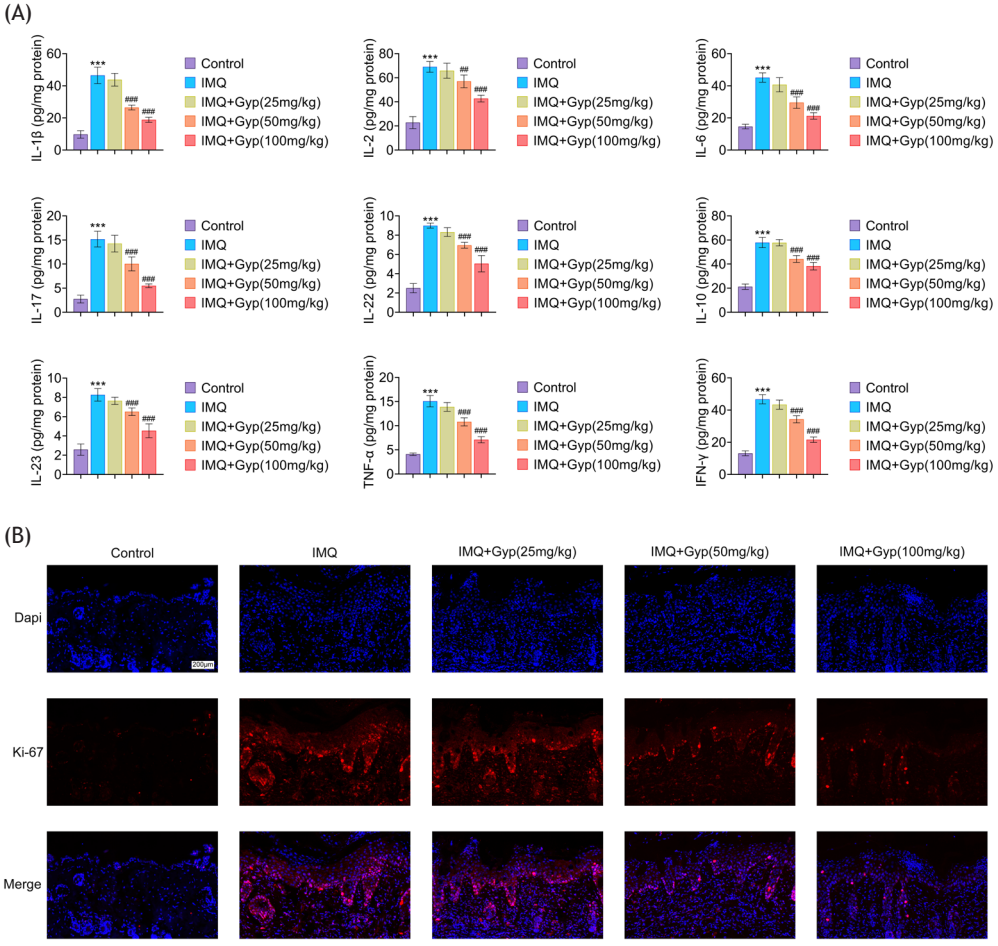

Figure 4 Gyp repressed skin tissues inflammation and proliferation in IMQ-induced mice. (A) The concentrations of IL-1β, IL-2, IL-6, IL-10, IL-17, IL-22, IL-23, IFN-γ and TNF-α in skin tissues of mice were detected by ELISA. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. IMQ. (B) The level of Ki-67 was determined by immunofluorescence. Scale bar = 100 μm.

been demonstrated that NF-κB regulated immune and inflammatory responses,[35] and STAT3 regulated cell proliferation in psoriasis.[36,37] Thus, the expression levels of p65 and STAT3 were determined in skin tissues. The relative levels of p-p65/p65 and p-STAT3/STAT3 were significantly upregulated in both skin tissues from IMQ-induced rodents and IL-22-indued HaCaT cells as illustrated in Figures 5A and 5B. These levels were significantly decreased with the administration of medium and high concentrations of Gyp. Additionally, the administration of ML385, an inhibitor of Nrf2, resulted in a significant recovery of the relative levels of p-p65/p65 and p-STAT3/STAT3 that were reduced by Gyp administration (Figures 5A and 5B). Combined, Gyp activated Nrf2 in psoriasis, which resulted in the downregulation of NF-κB and STAT3.

Gyp attenuated inflammation and over-proliferation via Nrf2 in IL-22-induced HaCaT cells

Furthermore, the administration of Gyp in IL-22-induced HaCaT cells resulted in a significant reduction in cell viability and the presence of IL-1β, IL-6, and TNF-α. However, treatment with ML385 significantly restored these levels (Figures 6A and 6B). Consequently, the findings suggested that Gyp inhibited inflammation and over-proliferation in IL-22-induced HaCaT cells by activating Nrf2.

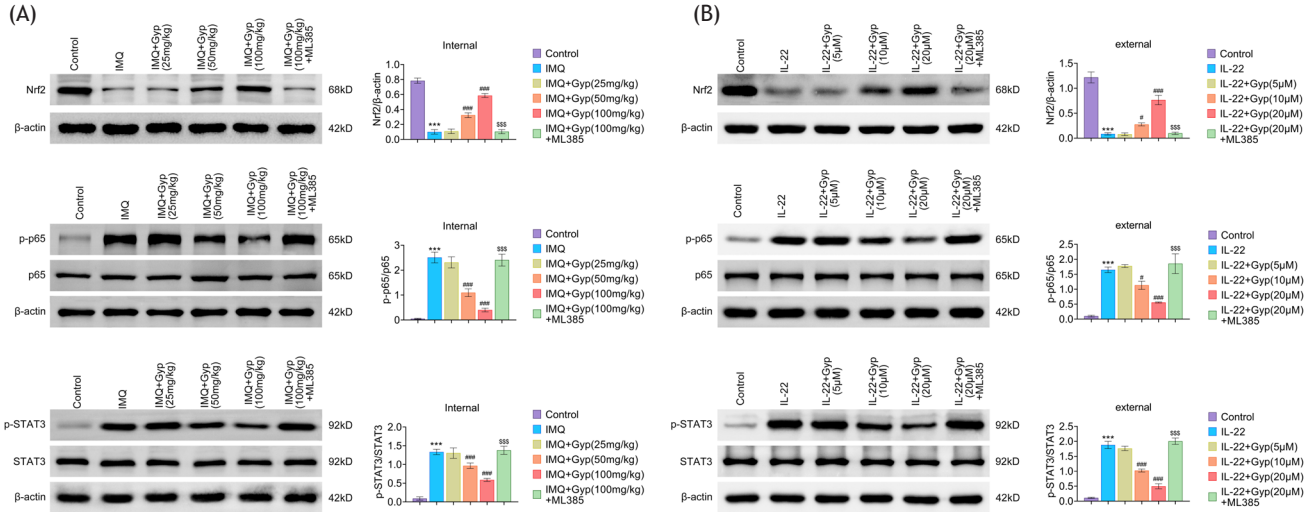

Figure 5 Gyp reduced the expression of NF-κB and STAT3 by activating Nrf2 in psoriasis. Mice were topically applied with 62.5-mg IMQ to induce an *in vivo* model of psoriasis, and received intragastric administration of 100-mg/kg Gyp and intraperitoneal injections of 30-mg/kg ML385 once a day for 4 weeks. (A) The relative protein expression of Nrf2, p-p65, p65, p-STAT3 and STAT3 was examined by Western Blot analysis in skin tissues of mice. Data were expressed after being normalized with ß-actin. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. IMQ; $^{\$\$\$}P < 0.001$ vs. IMQ+Gyp (100 mg/kg). (B) The relative protein expression of Nrf2, p-p65, p65, p-STAT3 and STAT3 was examined by Western Blot analysis in HaCaT cells. Data were expressed after being normalized with ß-actin. $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$ and $^{###}P < 0.001$ vs. IL-22; $^{\$\$\$}P < 0.001$ vs. IL-22+Gyp (20 μM).

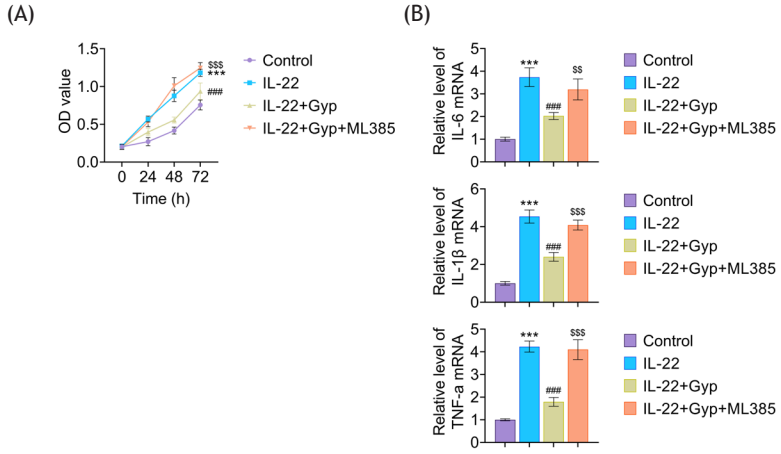

Figure 6 Gyp inhibited inflammation and over-proliferation via Nrf2 in IL-22-induced HaCaT cells. HaCaT cells were stimulated with 100-ng/mL IL-22 for 24 h, incubated with 20-μM Gyp for 24 h and 10-μM ML385 for 24 h. (A) Cell viability was detected by CCK-8 assays. (B) The relative mRNA expression level of IL-1ß, IL-6 and TNF-α was detected by RT-qPCR. Data were expressed after being normalized with GAPDH. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. IL-22; $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ vs. IL-22+Gyp.

Discussion

Psoriasis is a chronic inflammatory skin condition mediated by the immune system and affects a significant proportion of global population.[2] Psoriasis patients are typically treated with corticosteroids or immunosuppressive medications.[38] Nevertheless, significant financial costs and adverse consequences restrict the effectiveness and suitability of various treatment approaches.[2] Thus, development of safer and more effective agents is essential and important for the treatment of psoriasis. Gyp was discovered to decrease the release of inflammatory factors and over-proliferation in both IL-22-induced HaCaT cells and IMQ-induced mice in this study.

Mechanistically, Gyp upregulated the Nrf2 expression and downregulated the level of p-p65/p65 and p-STAT3/STAT3 *in vitro* and *in vivo*, which were reversed with the application of ML385, an inhibitor of Nrf2. Additionally, Gyp decreased cell viability and levels of IL-1ß, IL-6 and TNF-α in IL-22-indued HaCaT cells, which were reversed with the treatment of ML385. Collectively, Gyp attenuated over-proliferation and inflammation via Nrf2-mediated downregulation of NF-κB and STAT3 in psoriatic mice. In the past, inflammatory illnesses, such as psoriasis, were treated with a variety of active compounds from natural herbs, and this practice has continued to this day.[39] Natural products are a highly desirable target for the development of innovative therapeutic treatments against psoriasis because of their enormous abundance and low toxicity.

Ginsenoside Rg1, a ginsenoside, ameliorates psoriasis-like skin lesions by repressing proliferation and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasomes in keratinocytes.[40] Terrestrosin D, a steroidal saponin from *Tribulus terrestris* L. (family: Zygophyllaceae) attenuated inflammation and ameliorated psoriasis-like skin lesions and behavior in a mice model.[41] Saikosaponin A, a triterpenoid saponin from oriental medicinal plant Radix bupleuri, improved IMQ-induced psoriasis in mice.[42] Gyp, a saponin isolated from *Gynostemma pentaphyllum* (Thunb.) Makino, is demonstrated to reduce proliferation of IL-22-induced HaCaT cells. Additionally, Gyp improved erythema and scaling, the thicker epidermal layers as well as Ki-67 level in IMQ-induced mice. Research disclosed that IL-22 encouraged proliferation of keratinocyte, a crucial step in the pathogenesis of psoriasis,[43] thus was widely utilized to establish an *in vitro* model of psoriasis.[27,28]

Mice with psoriasis-like skin dermatitis are commonly studied using the IMQ-induced murine model. IMQ is a strong immunological stimulant and a ligand for TLR7 and TLR8. When IMQ was applied topically, mice developed skin lesions resembling psoriasis that had many of the phenotypic and histological traits of human psoriasis, including parakeratosis, elicitation of acanthosis, and a mixed inflammatory infiltrate mediated by an increase in the IL-23–IL17 axis.[44] Additionally, it was demonstrated that the supplement of IMQ to human non-lesion psoriatic skin produced skin inflammation resembling psoriasis, despite the fact that some distinctions were observed from psoriasis in humans. [45] Consequently, this research established an *in vivo* model of psoriasis in mice through the utilization of IMQ in a manner that was consistent with the results of earlier studies.[31,44] Together, these results suggested that Gyp ameliorated psoriasis *in vitro* as well as *in vivo*.

Psoriasis is characterized by the hyperproliferation of keratinocytes and the infiltration of inflammatory cells into epidermal tissue. There are numerous cytokines, such as IL-1ß, IL-2, IL-6, IL-10, IL-17, IL-22, IL-23, IFN-γ, and TNF-α, all essential for the pathogenesis of psoriasis and are released by activated immune cells.[46] Psoriasis advances by secreted cytokine signaling crosstalk. The intricate interactions between immune cells and keratinocytes through cytokine signaling govern the initiation of psoriasis and the persistence of inflammation in psoriatic skin. Specifically, skin injury, external stimuli and infection induce the influx of IFN-α from populations of plasmacytoid dendritic cells, which, in turn, stimulate the development of myeloid dendritic cells.[47] Maturity of dendritic cells releases TNF-α, IL-1ß, and IL-6, which activate naive T-helper cells (Th1, Th17, or Th22).[48,49]

Th cells release IFN-γ, IL-17a, and IL-22 on migrating to the epidermis, which induces keratinocytes to express different chemokines and antimicrobial proteins.[50,51] This vicious cycle of interaction between immune cells and keratinocytes encourages the infiltration of immune cells, such as T cells, dendritic cells, macrophages, and neutrophils, as well as the hyperproliferation of keratinocytes, exacerbating the development of psoriatic lesions.[52,53] Here, Gyp decreased the levels of IL-1ß, IL-6 and TNF-α in IL-22-induced HaCaT cells. In addition, Gyp reduced the release of inflammatory cytokines containing IL-1ß, IL-2, IL-6, IL-10, IL-17, IL-22, IL-23, IFN-γ and TNF-α in IMQ-induced mice. Likewise, Gyp reduced proliferation of IL-22-induced HaCaT cells and Ki-67 level in IMQ-induced mice. Altogether, these results indicated that Gyp attenuated over-proliferation and inflammation in psoriatic mice.

It was discovered that NF-κB signaling, an essential route to control many cellular functions, such as proliferation and inflammation,[54,55] is active in psoriatic lesions and contributes to the pathophysiology of psoriasis.[35,56] One potential novel psoriasis treatment is to inhibit keratinocyte proliferation and inflammation by blocking NF-κB signaling.([35,57] STAT3 is a therapeutic target for psoriasis because of its role as a critical regulator in the pathophysiology and control of cell proliferation.[36,37] During psoriasis, the transcription of STAT3 stimulates cytokine release in immune cells stimulated by chronic inflammation. The immunological response is heightened by the concurrent development of T lymphocytes and activation of dendritic cells. Within this intricate immune system, IL-6, IL-23, and IFN-γ have a direct bearing on the growth and development of keratinocytes.[58] Here, Gyp downregulated the level of p-p65/p65 and p-STAT3/STAT3 in both skin tissues of IMQ-induced mice and IL-22-indued HaCaT cells, indicating that Gyp suppressed NF-κB and STAT3 signaling in psoriasis.

Moreover, we discovered that Gyp upregulated the expression of Nrf2 both *in vitro* and *in vivo* and decreased the level of p-p65/p65 and p-STAT3/STAT3 in skin tissues of IMQ-induced mice and IL-22-indued HaCaT cells, which were reversed with the application of ML385. These findings showed that Gyp downregulated NF-κB and STAT3 signaling pathways by activating Nrf2 in psoriasis. Ooi et al.[59] reported that ML385 promotes maslinic acid (a natural

pentacyclic triterpene acid)-mediated suppression of NF-κB activity in human umbilical vein endothelial cells. The reduced expression of p65 by the treatment of salvianolic acid A in H_2O_2-stimulated HK-2 cells was partly reprieved with ML385.[60] Zhang et al. [61] found that ML385 attenuates the repressive effect of liensinine on NF-κB signaling in septic liver injury. Moreover, Tong et al.[62] demonstrated that ML385 abrogates the effect of pentagalloyl glucose on STAT3 phosphorylation and STAT3 nuclear translocation in advanced glycated end products-treated mouse mesangial cells. In addition, the administration of ML385 reversed decrease in cell viability and reduction in the levels of IL-1β, IL-6, and TNF-α in IL-22-induced HaCaT cells caused by Gyp. Taken together, Gyp attenuated over-proliferation and inflammation via Nrf2-mediated downregulation of NF-κB and STAT3 in psoriasis.

Conclusion

Gyp inhibited over-proliferation and inflammation through Nrf2-mediated inactivation of NF-κB and STAT3 in psoriasis. However, the direct role of Nrf2-mediated inactivation of NF-κB and STAT3 can be investigated *in vivo*. Furthermore, additional pre-clinical and clinical trials are required in the subsequent assays. In summary, the results offered improved comprehension and prospective options for the pharmaceutical therapy of psoriasis.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Competing interests

The authors stated that there was no conflict of interest to disclose.

Author Contributions

Tao Liu designed the study, completed the experiment and supervised data collection. Yuanmin He analyzed and interpreted the data. Yongmei Liao prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the final manuscript.

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