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Pulsatilla saponin inhibits the proliferation of keratinocytes and ameliorates imiquimod-induced psoriasis through the NF-κB and STAT3 signaling pathways

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ABSTRACT

Background and aim: Pulsatilla saponin (Ps) was isolated from Pulsatilla chinensis (Bunge) Regel, a traditional Chinese medicine, that has anti-proliferation, anti-inflammation, anti-tumor and immunomodulation activities. However, the anti-psoriasis activity of Ps and its underlying mechanisms have not been fully elucidated. This study aims to investigate the effect and potential mechanisms of Ps on psoriasis.

Experimental procedure: Ps underwent quality control through HPLC and NMR analysis. Wound healing assay, MTT, clone assay, and EdU staining were used to detect HaCaT cells proliferation. Western blot and immunofluorescence were used to assess the expression of proteins. The th17 cells population was analyzed by flow cytometry. The levels of cytokines in the mice skin tissues were measured by RT-qPCR and ELISA.

Results and conclusion: In vitro, Ps has an inhibition effect on the proliferation of M5-induced HaCaT cells. Ps inhibited proliferation by regulating NF-κB and JAK1/STAT3 pathways. Additionally, Ps decreased TNF-α, IL-1β, and IL-6 mRNA levels in M5-induced HaCaT cells. In vivo, Ps improved the pathological damage of Imiquimod (IMQ)-induced psoriasis BALB/c mice skin and reduced the Ki67 level in mice skin tissue. Further results showed that Ps decreased Th17 cells differentiation and IL-22, IL-17A, IL-6, IFN-γ, TNF-α, and IL-1β secretion. Ps could ameliorate the psoriatic symptoms, decrease M5-induced HaCaT cell proliferation, and decrease the differentiation of Th17 cells in IMQ-induced psoriasis mice. Ps suppressed the release of inflammation cytokines by regulating NF-κB and JAK1/STAT3 pathways. Those results indicate that Ps has promising therapeutic potential for psoriasis treatment.

1. Introduction

Psoriasis, characterized as a chronic, immune-mediated inflammatory disorder, manifests through significant skin thickening, erythema, and scaling.[1] Although the precise pathogenic mechanisms underlying psoriasis remain elusive, emerging evidence suggests a pivotal role for aberrant interactions between keratinocytes and immune cells in its development. During the initial stages of psoriasis, the activated of DCs in the epidermis result in the secretion of Interleukin-23 (IL-23), which is instrumental in the differentiation of naïve T cells into helper T (Th) cells, and producing pro-inflammatory cytokines such as IFN-γ, IL-22, IL-17, and IL-1β, disrupt keratinocyte homeostasis, induce the

Abbreviations: Ps, Pulsatilla saponin; ELISA, Enzyme linked immunosorbent assay; EdU, Ethynyl-2′-deoxyuridine; STAT3, The signal transducer and activator of transcription 3; NF-κB, nucleolar transcription factor κb; IL-22, Interleukin-22; Th17, T help 17; IL-17A, Interleukin-17A; TNF-α, Tumor necrosis factor-α; IFN-γ, Interferon-γ; IL-1β, Interleukin-1β; qRT-PCR, Quantitative real-time reverse transcription PCR; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; IMQ, Imiquimod; DCs, Dendritic cells; FBS, Fetal bovine serum; IL-23, Interleukin-23; DMEM, Dulbecco's modified eagle medium; IL-6, Interleukin-6; IHC, Immunocytochemistry; IF, Immunofluorescence.

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proliferation of keratinocyte and accelerate the characteristic of psoriasis.[2,3] In both psoriatic patients and IMQ-induced murine psoriasis models, an upregulation of inflammatory cytokines has been observed.[4,5] Elevated concentrations of the inflammation cytokines in the keratinocyte lesions of psoriasis, activate various intracellular signaling pathways and stimulate transcription factors, secrete chemokines to facilitate the recruitment of dendritic cells (DCs) and T lymphocytes to the epidermis. These activated immune cells release cytokines (IL-17, IL-22, IL-6 and IFN-γ) in the keratinocyte and eventually leads to inflammatory loop.[6]

The JAK/STAT and NF-κB signaling pathways are fundamental in mediating immune responses and inflammation, playing critical roles in the pathogenesis of psoriasis. Activation of JAKs by cytokines such as IL-22, IL-6, and IFN-γ leads to the selective phosphorylation of STAT proteins, notably STAT3,[7,8] a multifunctional transcription factor closely associated with inflammation, immunodeficiency, and autoimmune diseases,[9] The phosphorylation of STAT3 promotes the hyperproliferation of keratinocytes and significantly affects Th17 differentiation, which is essential in the development of psoriatic lesions.[10,11] Li et al. have reported that inhibiting STAT3 phosphorylation can alleviate the skin damage characteristic of psoriasis.[12] Parallelly, the NF-κB signaling pathway orchestrates a wide array of biological functions including inflammatory responses, immune regulation, and cell proliferation.[13,14] NF-κB signaling pathway is activated in psoriatic skin and drives the production of pro-inflammatory cytokines like IL-17 and TNF-α, exacerbating inflammation and promoting the migration, invasion, and proliferation of keratinocytes in psoriatic lesions.[15,16]

In recent years, Traditional Chinese Medicine (TCM), known for its multi-target characteristics, safety, and efficacy, has emerged as a research hotspot in the development of anti-psoriasis treatments.[17] Pulsatilla chinensis (Bunge) Regel is a traditional Chinese medicine. It mainly treats treating malaria fever, dispelling dampness and heat, cooling blood, and relieving pain functions. In contemporary clinical practice, Pulsatilla chinensis (Bunge) Regel has also been effectively applied in the management of psoriasis.[18] Pulsatilla saponin (Ps), a pentacyclic triterpenoid that was isolated from the dry root of Pulsatilla chinensis (Bunge) Regel and previous studies showed that Ps exhibits anti-inflammation, antitumor, immune-modulation, and antiviral effects,[19–23] quite a few studies involved its effects on psoriasis. Therefore, in this study, we used Ps to explore its effect on psoriasis and the underlying mechanisms both in vitro and in vivo.

2. Methods

2.1. Chemicals and reagents

Methylthiazolyldiphenyl-tetrazolium bromide (MTT)was procured from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), trypsin, and Fetal Bovine Serum (FBS) were procured from Gibco Laboratories (Grand Island, NY, USA). IL-17A and IFN-γ ELISA kits were procured from Fanke (Shanghai, China). All the antibodies utilized in the study were procured from Cell Signaling Technology (Danvers, MA, USA). Real-time quantitative reverse transcription PCR (RT-qPCR) kit was procured from Servicebio (Wuhan, China). Desonide Cream was bought from Chongqing Huapont Pharm. Co., LTD (Chongqing, China). The Bicinchoninic Acid (BCA) kit was bought from Thermo Fisher (Waltham, MA, USA). Imiquimod (IMQ) cream was bought from Sichuan Med-Shine Pharmaceutical Co., LTD (Sichuan, China). Stearic acid was bought from Hunan Huari Pharmaceutical Co., LTD (Human, China). Glycerol monostearate was bought from Jiangxi Alpha High-tech Pharmaceutical Co., LTD (Jiangxi, China). Glycerin and castor oil were bought from Shanghai Aladdin Biochemical Technology Co., LTD (Shanghai, China). Triethanolamine was bought from Chengdu Huayi Pharmaceutical Excipients Manufacturing Co., LTD (Chengdu, China). The ethynyl-2′-deoxyuridine (EdU) kit was procured from Beyotime (Shanghai, China). All human recombinant proteins were bought from PeproTech (Suzhou, China).

2.2. The preparation of Ps

The dry root of Pulsatilla chinensis (Bunge) Regel used in this study was identified by Professor Guoyue Zhong (College of Pharmacy, Jiangxi University of Chinese Medicine). The dry materials of Pulsatilla chinensis (Bunge) Regel (10 kg) were extracted twice with 70 % Ethyl Alcohol (EtOH). D101 macroporous adsorption resin was employed to further isolate the concentrated solution. Deionized water, 30 % EtOH, 70 % EtOH, and 95 % EtOH elute the column in sequence, of which 70 % eluent was further processed by Medium-pressure liquid chromatography (MPLC) to obtain Ps.

2.3. Component identification and content determination of Ps

The Ps sample was completely dissolved with methanol, and then it was separated and purified by preparative liquid chromatography was performed on LC-20ADXR pump with SPD-20A detector and chromatographic column (Cosmosil 5 PE-MS φ10 ID × 150 mm, 5 μm). In the preparation process, the ratio of acetonitrile to water was 23:77 for separation and elution, resulting in three compounds. These compounds are identified and structurally resolved by NMR to determine. The NMR spectra were acquired on a Bruker AVANCE III 600 MHz spectrometer in Pyridine-d5. respectively, based on the 1H and 13C NMR data, which was compared to the literature.

Further determined the contents of the three main pharmacodynamic components identified from the Ps samples. After accurately weighing the Ps sample, methanol was added to dissolve it, and ultrasonic treatment was performed for 30 min using an ultrasonic machine (power 500 W, frequency 40 kHz), the test product solution was obtained by 0.22 μm microporous filter membrane. The content was determined by high performance liquid chromatography (HPLC), waters e2695 with 2998 PDA and Ultimate® Plus C18 (4.6 × 250 mm, 5 μm) were employed to investigate quality control of Pulsatilla saponins at the wavelength of 201 nm. The acetonitrile (A) and water (B) were used as mobile phase to conduct experiments. The isocratic elution was the following: 0–35 min, 26%A-74%B. And the column temperature was 25 °C. According to the chromatographic conditions, the peak area was recorded, and the contents of the above three components were calculated by external standard method.

2.4. Cell culture

HaCaT cells are purchased from American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM (Gibco, Pittsburgh, PA, USA), supplemented with 10 % fetal bovine serum (Gibco, Pittsburgh, PA, USA) and 1 % penicillin (100 IU/mL)–streptomycin (100 μg/mL) (Gibco, Pittsburgh, PA, USA). Cells were maintained at 37 °C in 5 % CO2. Cells were frozen in liquid nitrogen and used for experiments at passages 3 to 10 after thaw.

2.5. Cell viability assay

The cell viability was detected by MTT assay. HaCaT cells were seeded into 96-well plates and cultured over 24 h. Then, Ps at different concentrations were added to the HaCaT cells for 96 h. After which 100 μL 1 mg/mL MTT (Solarbio, China) reagent was added and incubated for another 4 h at 37 °C. After incubation, cells were treated with Dimethyl sulfoxide (DMSO) for 15 min at room temperature. Absorbance was measured at OD = 490 nm by a MicroplateReader (BioTeK, USA).

2.6. Proliferation assay

The HaCaT cells proliferation assay was assessed by MTT. HaCaT cells were pretreated with Ps (5, 2.5, 1.25 μg/mL) for 2 h, then co-

cultured with a mixture of five cytokines (2.5 ng/mL): IL-22 (2.5 ng/mL), IL-1α (2.5 ng/mL), TNF-α (2.5 ng/mL), IL-17 (2.5 ng/mL), and Oncostatin-M (OSM, 2.5 ng/mL) for 72h. This cytokine mixture will be uniformly referred to as M5 hereafter. After which 100 μL 1 mg/mL MTT (Solarbio, China) reagent was added and incubated for another 4 h at 37 °C. After incubation, cells were treated with DMSO for 15 min at room temperature. Absorbance was measured at OD = 490 nm by a Microplate Reader (BioTeK, USA). All experiments were performed in triplicate.

2.7. Cell clone formation assay

HaCaT cells were plated on 6-well plates with a density of 500 per well. Pretreated with Ps (5, 2.5, 1.25 μg/mL), then coculture with M5 (2.5 ng/mL) for 2 weeks. After washing with PBS, then the cells were fixed with 4 % paraformaldehyde and stained with 0.5 % crystal violet solution for 15 min. All experiments were performed in triplicate and the pictures were captured by using a camera.

2.8. Wound healing assay

HaCaT cells were plated on 6-well plates. Using plastic pipettes to scratched the cell monolayer and then pretreated with Ps (5, 2.5, 1.25 μg/mL), then coculture with M5 (2.5 ng/mL), observed by inverted light microscope. All experiments were performed in triplicate. The area of the wound was measured with Image J software (NIH, USA).

2.9. Ethynyl-2′-deoxyuridine (EdU) assay

HaCaT cells were pretreatment with Ps (5, 2.5, 1.25 μg/mL), then cocultured with M5 (2.5 ng/mL) for 48 h. Cells were then treated with 40 μM Edu solution and incubated for 24 h. EdU can be incorporated into newly synthesized DNA in place of thymidine during DNA synthesis. The DNA molecules in newly proliferating cells carry EdU. Then, the ethynyl group on EdU and the fluorescent labeled small molecule Azide Alexa Fluor 594 are catalyzed by Cu(I) to form a stable triazole ring (click reaction).[24] The newly synthesized DNA (newly divided cells) is labeled by the fluorescent probe, and the proliferation of cells is observed under a microscope.

2.10. Western blot analysis

HaCaT cells pretreatment with Ps (5, 2.5, 1.25 μg/mL), and cocultured with M5 (2.5 ng/mL) for 24 h. The RIPA lysis buffer supplemented with 1 % cocktail and 1 % Phenylmethanesulfonyl fluoride (PMSF), was used to lyse the cells and the lysate was separated by centrifugation. HaCaT cells' lyses or mouse skin samples were collected, the concentration was assessed by BCA method. Proteins were loaded on a 6–10 % SDS/PAGE gel and set the program to 80 V/20 min, 120 V/70 min. Then, place the transfer device on ice, and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes at low temperatures. Following the blocking of PVDF membranes with 5 % nonfat milk at room temperature for 2 h, then protein-loaded PVDF membranes were soaked with the first antibodies for a whole night. Finally, the membranes were incubated for 2 h with the second antibody. Final, the PVDF membranes were scanned and analyzed.

2.11. Immunofluorescence (IF) staining

HaCaT cells were pretreated with Ps (5 μg/mL), then cocultured with M5 (2.5 ng/mL). After 24 h, the dish added 4 % paraformaldehyde to fix the cells. The samples were kept for permeabilization with 0.2 % Triton X100 for 30 min, blocked with 5 % BSA for 30 min, incubated with first antibodies at 4 °C overnight and washed with PBS. Next, the samples were stained with fluorescent second antibodies for 1 h at 37 °C.

2.12. Real-time quantitative reverse transcription PCR (RT-qPCR)

HaCaT cells were pretreated with Ps (5 μg/mL) for 2 h, and cocultured with M5 (2.5 ng/mL) for 24 h. Then HaCaT cells or skin samples were collected, TRIzol reagent was used to extract the cellular mRNA and subsequently reverse transcribed into cDNA. The mRNA levels of the cells were quantified using SYBR Green fluorescence. Supplementary Table S4 contains a list of all primers used for RT-qPCR.

2.13. Histopathological analysis and immunohistochemistry

The skin tissues are fixed, embedded in paraffin, and sectioned. Then hematoxylin and eosin were used to stain the sectioned and observed under a microscope. Additionally, immunohistochemistry (IHC) for Ki67. Before incubating with Ki67 primary antibody (Servicebio, Wuhan, China), We blocked tissue sections overnight with 3 % hydrogen peroxide and 5 % BSA at 4 °C followed by overnight incubation with rabbit IgG antibody. Under a microscope, tissue sections stained with 3,3′-diaminobenzidine (DAB) substrate can be observed.

2.14. Flow cytometry

Spleen cells were separated and resuspended at 1 × 106 cells/100 μL in PBS. Then cells were stained with anti-CD4-PE-Cy7 for 30 min. Cells were fixed, permeated, and stained with anti-IL-17A-FITC for 30 min.

2.15. Animal experiments

The male BALB/c mice (6–8 weeks, 20 ± 2 g) were procured from the Guangdong Vital River Laboratory Animal Technology Co., Ltd. (Guangdong, China, No. SCXK (YUE) 2022-0063). All the mice were raised in standard (SPF) conditions. The study was established according to the ethical guidelines and approved by the Ethics Committee on Laboratory Animal Management of Guangxi University of Chinese Medicine (DW20230830-181).

For animal experimentation, Ps cream was prepared. Glycerol monostearate (4 %), stearic acid (12 %), and castor oil (20 %) were mixed and heated to 80 °C and added triethanolamine (1.5 %) slowly. Upon complete dissolution through heating, the mixture was added to the aqueous phase (10 % glycerin, 50 % water and 1 % or 2 % or 4 % Ps) and stirred to achieve homogeneity. Ps cream (1 %, 2 % and 4 %) was applied 62.5 mg to the mice's dorsal area. This application corresponds to dosages of 30, 60, and 120 mg/kg.

A total of six groups of BALB/c mice were randomly divided (n = 6) to conduct the experiment. The dorsal region (25 mm × 25 mm) of the skin was cleanly shaved for all the animals. The control group consisted of untreated normal mice, while the model and treatment groups received topical application of 5 % imiquimod cream (62.5 mg/day) to induce psoriasis. Model groups were applied with blank cream without drugs, while treatment groups of the mice were applied with Desonide Cream (62.5 mg/day) or Ps cream (30, 60,120 mg/kg) once a day for seven days. On day 8, mice were euthanized by cervical dislocation, then mice spleens were harvested and prepared for the determination of Th17 cells. A part of skin tissue samples was collected to determine cytokine levels, western blot, and RT-qPCR. The other parts of the skin tissues were fixed in 4 % formaldehyde for HE staining and IF.

2.16. Data analysis

All experiments were conducted a minimum of three times. Statistical significance was analyzed using GraphPad Prism 9.0 Software. Group comparisons were performed using one-way ANOVA with Dunnett's multiple comparison tests. The P value less than 0.05 ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Component identification and content determination of Ps

According to the NMR data and spectra, the peak attribution and structure analysis of the three compounds were carried out respectively. Comparison with the reported data identified the compound as Pulsatilloside D,[25] Anemoside B4[26] and Hederacoside C.[27] Detailed analytical data and spectrogram are shown in the supplementary file.

Ps underwent quality control through high performance liquid chromatography (HPLC) analysis, indicating that the concentration of pulsatilloside D, anemoside B4, and hederacoside C in Pulsatilla saponin were 4.21 %, 78.64 %, and 12.68 %, respectively. The fingerprint of Ps was shown in Fig. 1 a. The chemical structures of the three components were shown in Fig. 1 b-d.

3.2. Ps inhibits M5-induced HaCaT cell proliferation

HaCaT cells were stimulated by IL-1α, TNF-α, IL-17A, oncostatin M (OSM), and IL-22 (M5) which were used to investigate the anti-proliferation effect and mechanisms of Ps in vitro. The cytotoxicity of Ps in HaCaT cells was assessed by MTT. As shown in Fig. 2 a Ps displayed no significant cytotoxicity. The result indicated that, Ps significantly decreased M5-induced HaCaT cells proliferation (Fig. 2 b). Clone formation assay, EdU assay, and wound healing assay (Fig. 2 c-e) showed the same results. Additionally, as shown in Fig. 2 f-h, the IL-1β, TNF-α, and IL-6 mRNA levels were obviously increased in M5-induced HaCaT cells, and Ps could decrease the mRNA levels of these cytokines. These findings demonstrated that HaCaT cells induced with M5 showed suppression in proliferation and decreased production of inflammatory cytokines when treated with Ps.

3.3. Ps inhibits the activation of NF-κB and JAK1/STAT3 signaling pathways in M5-induced HaCaT cell

NF-κB is a classical signaling pathway in inflammation-mediated disease. NF-κB canonical pathway is activated by cytokines with pro-inflammatory function, like IL-1 and TNF. In the pathogenesis of psoriasis NF-κB has vital function, as it controls the transcription of cytokines and chemokines.[28] Additionally, as a signal transducer, JAK1 is

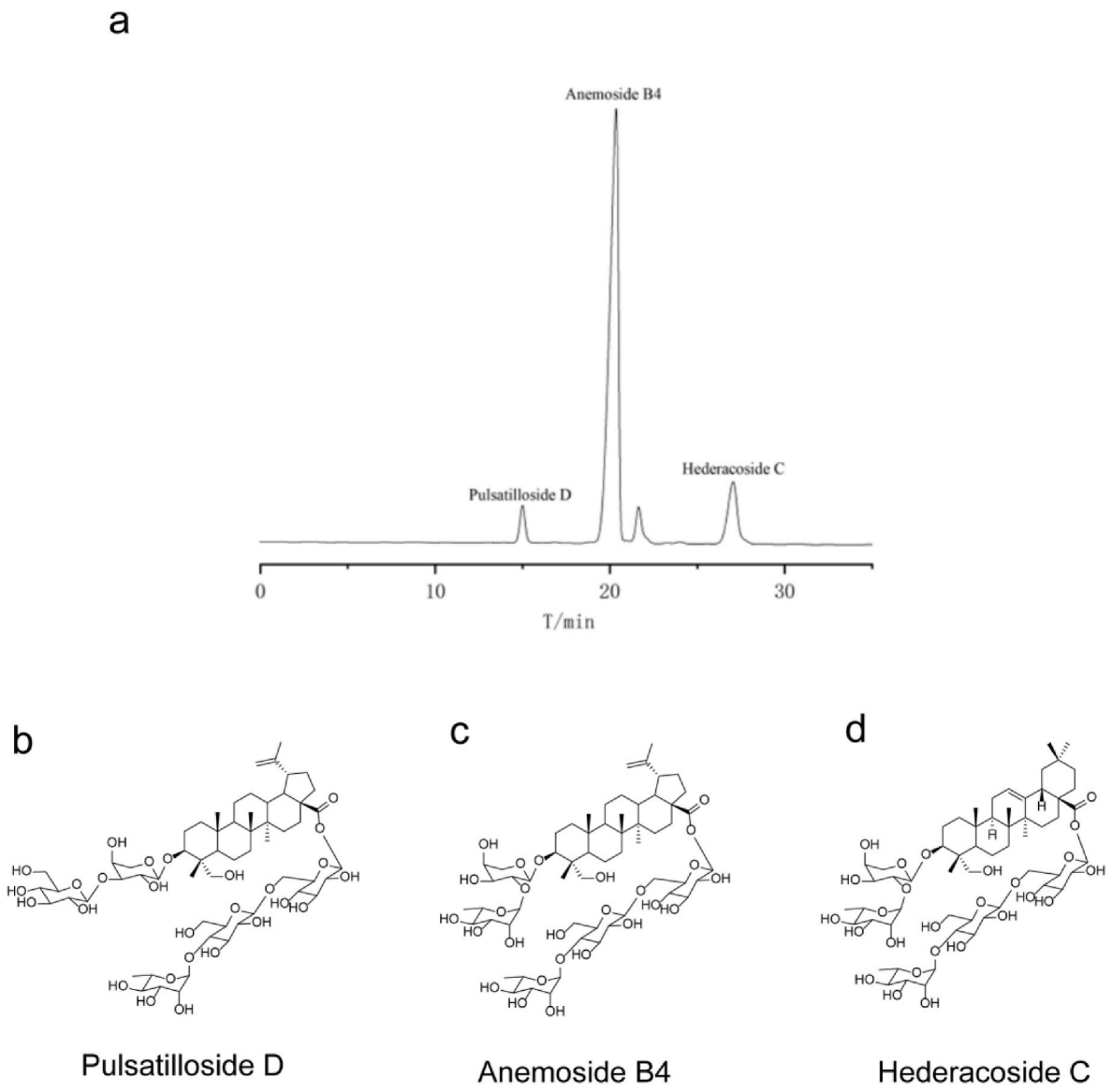

Fig. 1. Component identification and content determination of Ps. (a) The fingerprint of Ps was determined by HPLC. (b) The structure of Pulsatilloside D. (c) The structure of Anemoside B4, (d) The structure of Hederacoside C.

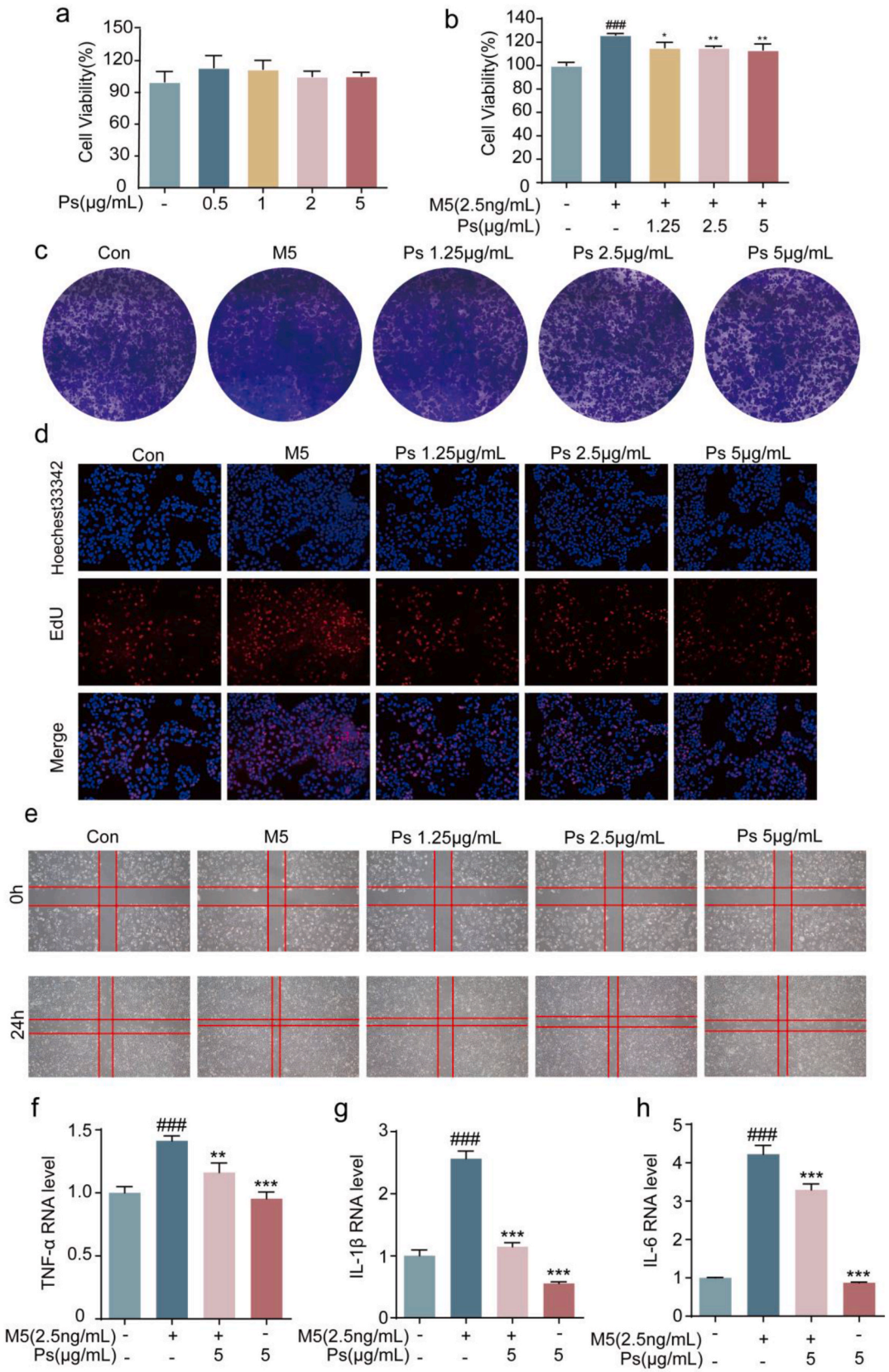

Fig. 2. Ps inhibits M5-induced HaCaT cell proliferation (a) Effects of Ps on cell viability in HaCaT cells. **(b)** Effects of Ps on cell proliferation in HaCaT cells. **(c)** Evaluation of Ps treatment effects on colony formation in M5-induced HaCaT cell proliferation at 2 weeks. **(d)** Representative fluorescence images of EdU assay for HaCaT cell proliferation. **(e)** The inhibitory effect of Ps on M5-induced migration of HaCaT cell was determined by wound healing assay at 0 and 24 h. **(f–h)** The mRNA levels of IL-1β, TNF-α, and IL-6 were detected by RT-qPCR. All experiments were performed in triplicate. $^{###}P < 0.001$ vs. control group, $^{***}P < 0.001$, $^{**}P < 0.01$ vs. model group.

responsible for the upregulation of immune cytokines on psoriatic skin, such as IL-22 and IL-23.[29,30] JAK1 could phosphorylate STAT3 and undergoes nuclear translocation, which then causes the proliferation of cells and immune dysregulation.[31] In this study, Ps inhibited p-p65, *p*-IκBα, *p*-IKKα/β (Fig. 3 a-e), *p*-JAK1 and p-STAT3 (Fig. 4 a-c) proteins expression in M5-stimulated HaCaT cells. Furthermore, immunofluorescence analysis results showed that Ps inhibited the NF-κB (Fig. 3 f-g) and STAT3 (Fig. 4 d-e) translocated into the cell nucleus. Taken together, these results suggested that Ps inhibited NF-κB and JAK1/STAT3 signaling pathways activation in M5-induced HaCaT cells.

3.4. Ps ameliorates IMQ-induced psoriatic skin lesions in mice

Psoriasis induced by IMQ is a common animal model in research.[32] In our research, by applying 5 % IMQ cream, the psoriasis model was established, and positive control was used by administering Desonide cream (62.5 mg/day). On the 7th day, the IMQ-induced mice Psoriasis Area and Severity Index (PASI) score was 7.95 ± 0.90, indicating that

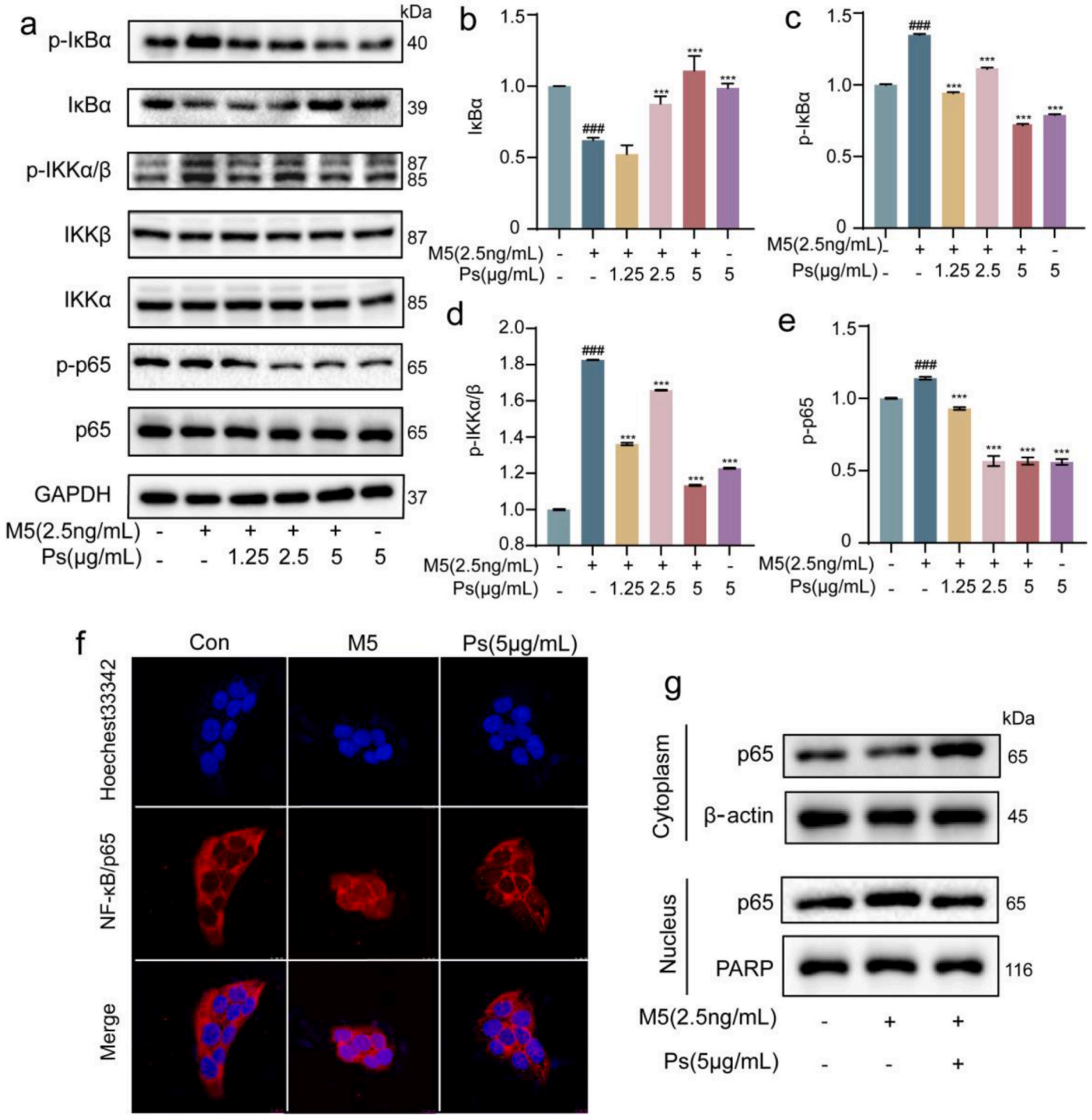

Fig. 3. Ps reduces NF-κB activation in M5-induced HaCaT cell. (a–e) The proteins expression levels of *p*-IκBα, IκBα, *p*-IKKα/β, IKKα, IKKβ, p-p65, and p65 were assessed by western blotting. **(f)** The nuclear translocation of NF-κB/p65 was determined using immunofluorescence analysis. (Scale bar = 10 μm). **(g)** The expression of NF-κB/p65 in cytoplasm and nucleus was assessed by western blotting. All experiments were performed in triplicate. $^{###}P < 0.001$ vs. control group, $^{***}P < 0.001$ vs. model group.

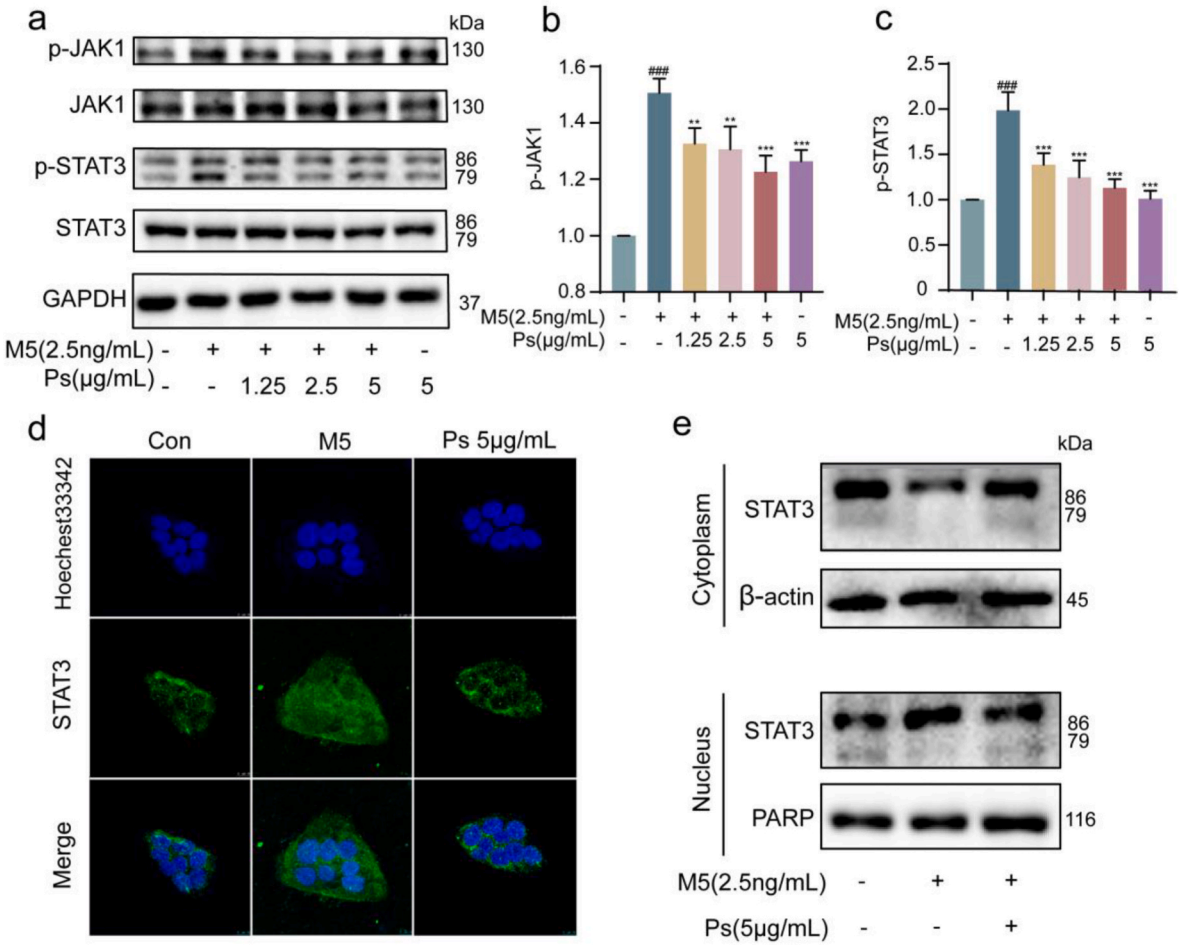

Fig. 4. Ps inhibits JAK1/STAT3 signaling in M5-stimulated HaCaT cell. (a–c) The expression levels of *p*-JAK1, JAK1, p-STAT3, and STAT3 were assessed by western blotting. **(d)** The nuclear translocation of STAT3 was determined using immunofluorescence analysis. (Scale bar = 10 μm). **(e)** The expression level of STAT3 in cytoplasm and nucleus was assessed by western blotting. All experiments were performed in triplicate. $^{###}P < 0.001$ vs. control group, $^{***}P < 0.001$, $^{**}P < 0.01$ vs. model group.

the mice exhibited significantly thickened, erythematous, and scaly skin lesions in comparison to the control group. In contrast, Ps-treated (30, 60 mg/kg) mice's PASI score was 3.9 ± 0.99, and 4.6 ± 1.33 respectively ($P < 0.001$), which had fewer psoriasis skin lesions than IMQ-induced mice model group (Fig. 5 b-c). The efficacy of Ps treatment in ameliorating skin lesions was further validated by HE staining, which revealed that Ps-treated mice had significantly reduced inflammatory cells infiltration and thickness of epidermal compared to the IMQ-induced mice model group (Fig. 5 f). Additionally, immunohistochemistry (IHC) showed that the cell proliferation marker Ki67 expression significantly increased in IMQ-induced mice, Ps (60, 120 mg/kg) remarkably decreased the expression of Ki67, whose effect was comparable to that of the positive control group treated with Desonide. Proliferation induced by IMQ was evidently inhibited by even the lowest dose of Ps (30 mg/kg) (Fig. 5 g). There results indicated that Ps could attenuate IMQ-induced psoriasis in mice.

3.5. Ps decreases the Th17 cells differentiation and inhibited the expression of inflammatory cytokines in IMQ-induced mice

The Th17/IL-17 axis is essential in managing psoriasis.[33] Flow cytometric analysis outcomes showed that the differentiation of Th17 cells was considerably increased in IMQ-induced mice spleen and Ps-treated (30, 60, 120 mg/kg) group decreased the numbers of Th17 cells (Fig. 6 a-b). The ELISA and RT-qPCR results were implemented to further confirm that Ps could obviously suppress the expression level of IL-17 (Figs. 5d–Fig. 6 f). According to these findings, Ps regulates the Th17/IL-17 axis, which may have therapeutic effect on psoriasis. In addition, within the skin tissues of mice induced by IMQ, the expression of IFN-γ protein (Fig. 5 e), the mRNA levels of IL-1β, IL-6, TNF-α, and IL-22 (Fig. 6 c, d, e, g) exhibited notable elevation compared with control mice. However, upon Ps treatment, these levels exhibited significant reduction, highlighting its efficacy in regulating the inflammatory response.

3.6. Ps suppresses the activation of NF-κB and JAK1/STAT3 signaling pathways in IMQ-induced mice skin

In order to delve deeper into the activation of the NF-κB and JAK1/STAT3 signaling pathways in an IMQ-induced psoriasis mouse model, the levels of proteins associated with these pathways were assessed via western blot analysis in mouse tissues. Notably, the proteins expression of *p*-IκBα, *p*-IKKα/β, p-p65, *p*-JAK1, and p-STAT3 (Fig. 7 a-g) exhibited an increase in the psoriatic mouse skin tissues induced by IMQ. Conversely, Ps treatment resulted in a reduction of these proteins expression in the mouse skin tissues. These findings highlight the inhibitory effect of Ps on the activation of the NF-κB and STAT3 signaling pathways in IMQ-induced psoriasis.

4. Discussion

Given the limited efficacy of existing treatment options and the

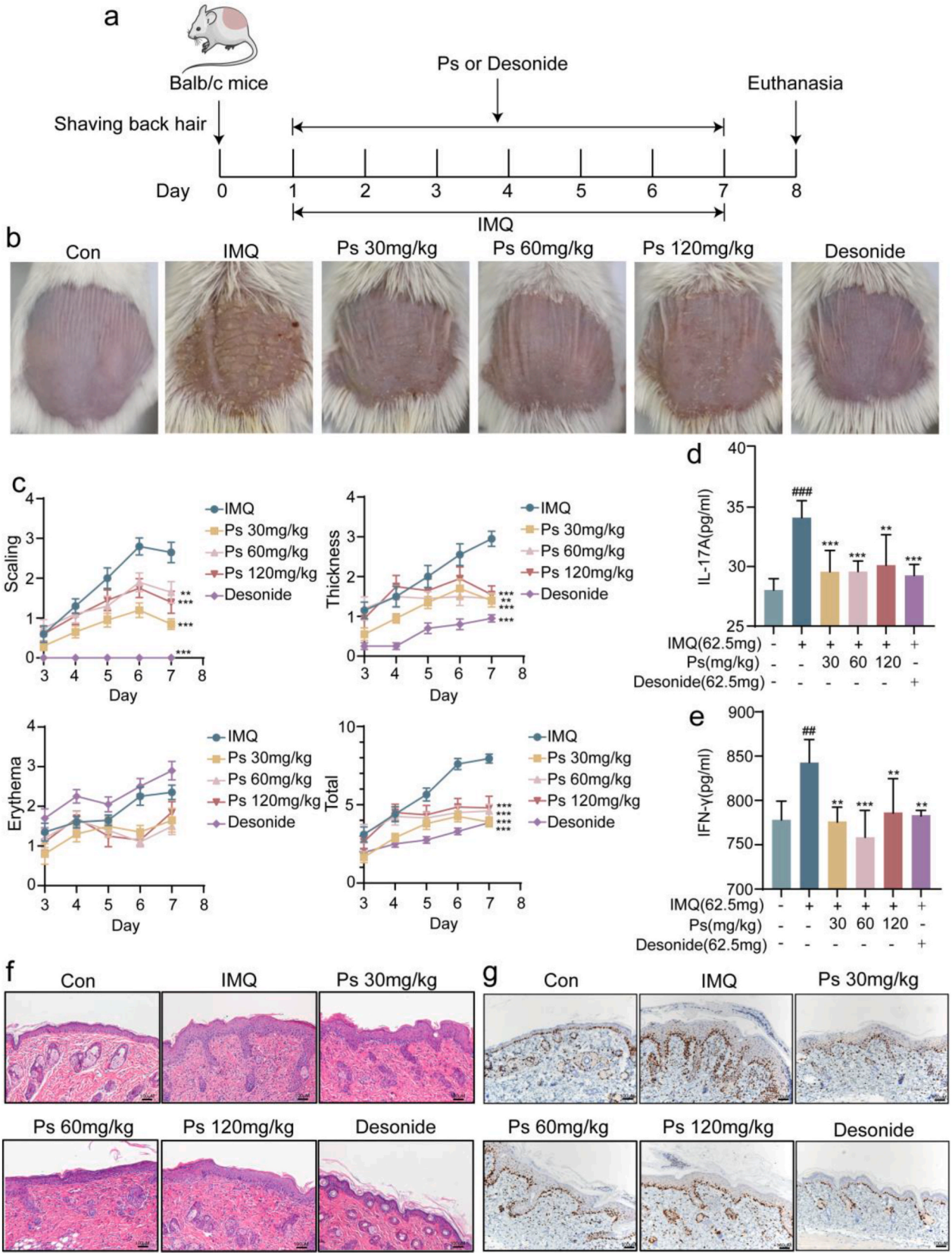

Fig. 5. Ps ameliorates IMQ-induced psoriatic skin lesions in mice. (a) Scheme of animal experiment. (b) Tissue macrograph of Ps effects on skin. (n = 6). **(c)** Statistics of PASI scores in mice skin**. (d–e)** The expression of IL-17A and IFN-γ were detected by ELISA of skin tissues. **(f)** HE staining of skin tissues. (Scale bar = 100 μm). **(g)** Ki67 expression level was analyzed by IHC staining of skin tissues. (Scale bar = 100 μm). $^{###}P < 0.001$, $^{##}P < 0.01$ vs. control group, $^{***}P < 0.001$, $^{**}P < 0.01$ vs. model group.

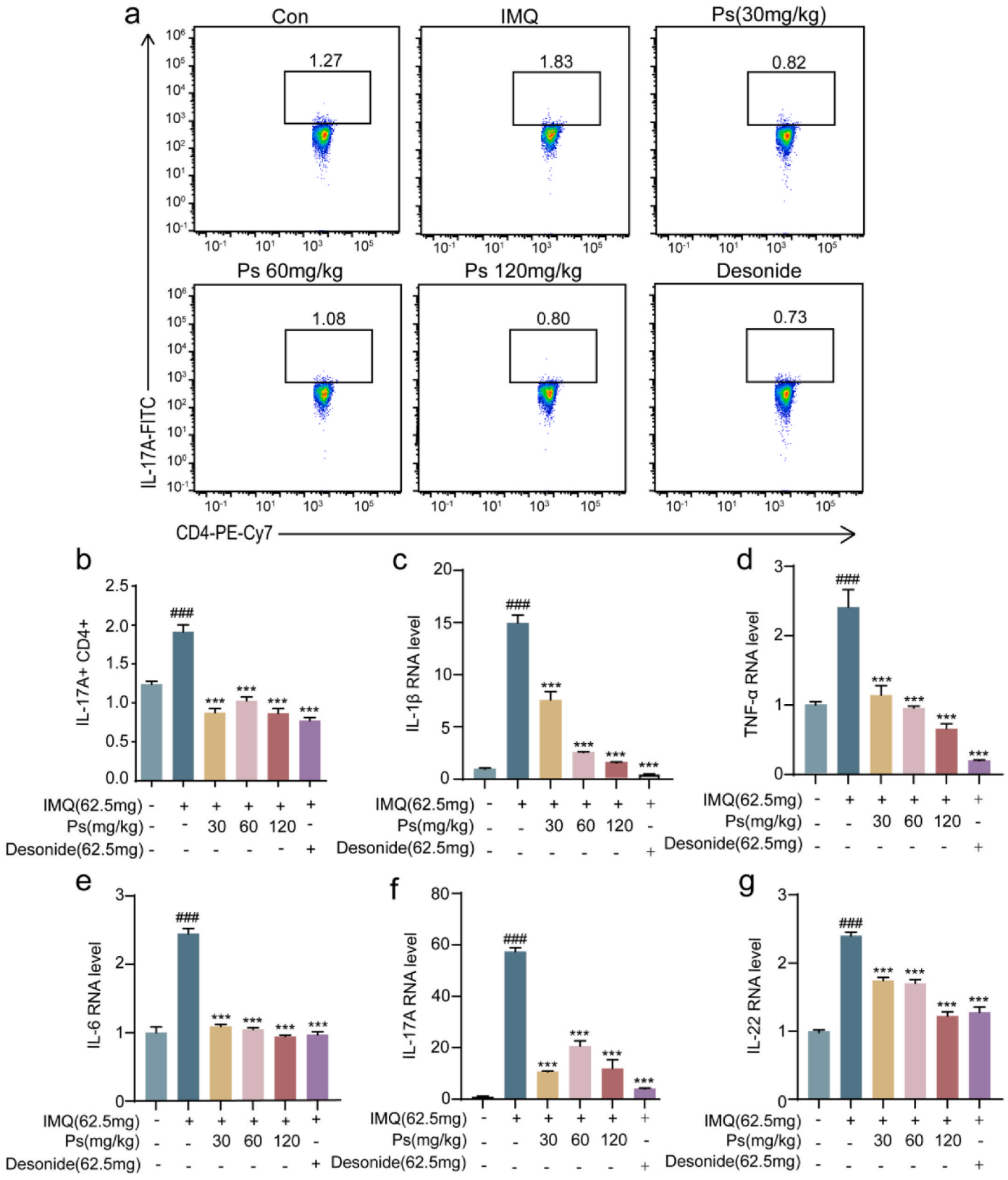

Fig. 6. Ps decreases the Th17 cells differentiation and inhibited the expression of inflammatory cytokines in IMQ-induced mice. (a–b) The population of Th17 cells ($CD4^{+}IL17A^{+}$) in mice spleen was detected by flow cytometry. (c–g) The mRNA levels of IL-1β, TNF-α, IL-6, IL-17A, and IL-22 were detected by RT-qPCR of skin tissues. $^{\#\#\#}P < 0.001$, vs. control group, $^{***}P < 0.001$ vs. model group.

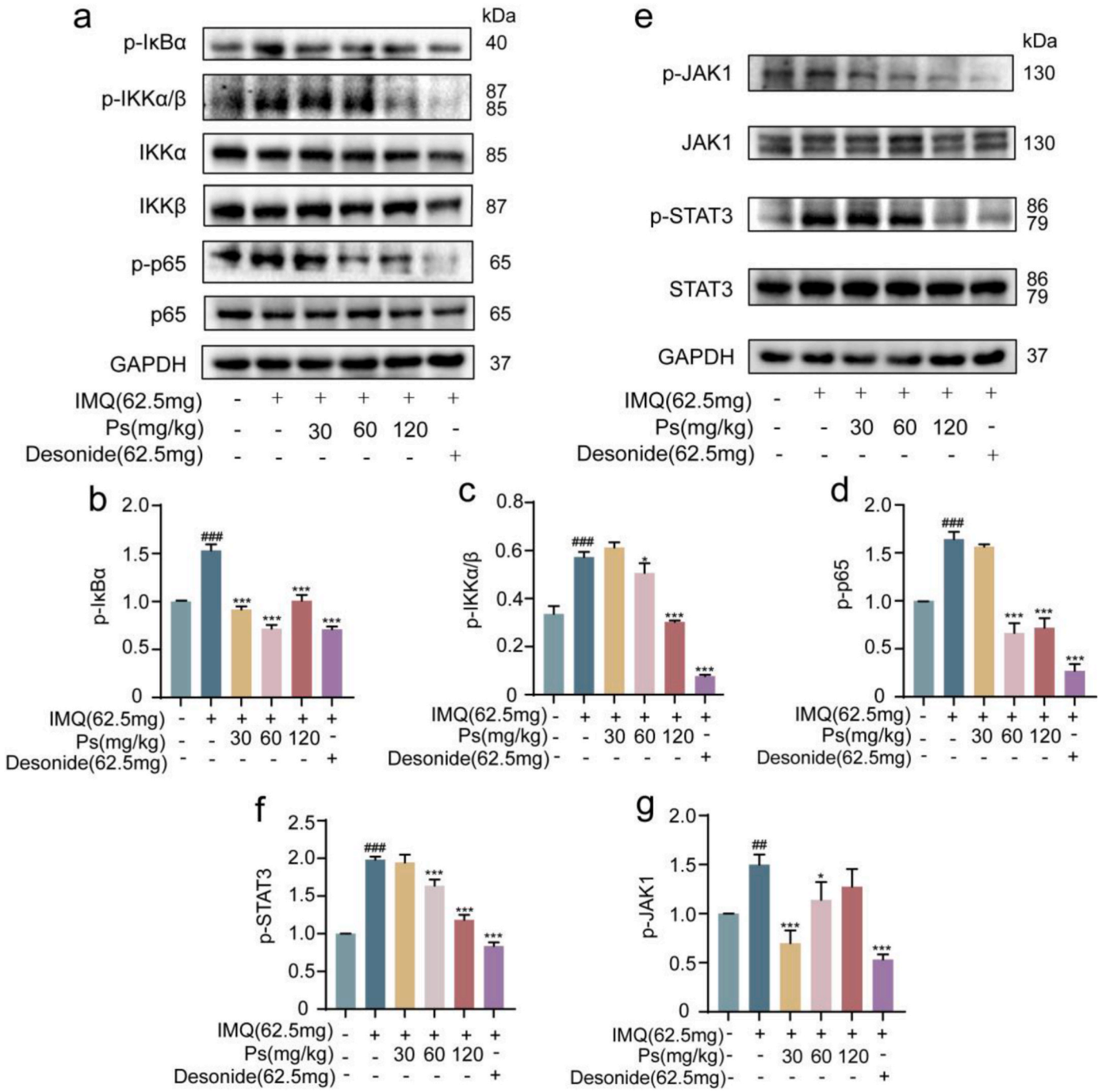

Fig. 7. Ps suppresses NF-κB and JAK1/STAT3 signaling in IMQ-induced mice skin. (a–d) The expression of *p*-IκBα, IκBα, *p*-IKKα/β, IKKα, IKKβ, p-p65, and p65 were detected by western blotting of skin tissues. **(e–g)** The expression of *p*-JAK1, JAK1, p-STAT3, and STAT3 were detected by western blotting of skin tissues. $^{###}P < 0.001$, $^{##}P < 0.01$ vs. control group, $^{***}P < 0.001$, $^{*}P < 0.05$ vs. model group.

current treatment options have serious side effects, psoriasis poses a significant physical and mental burden for patients.[34] Psoriatic lesions are characterized by the accelerated proliferation of keratinocytes, triggering the secretion of cytokines and chemokines. Consequently, this inflammatory response ensues, accompanied by the recruitment of immune cells.[35] The pathophysiological features of psoriasis, including inflammation, thickening, and scaling, can be attributed to the increased presence of immune cells such as Th17, along with elevated levels of inflammatory and immune cytokines like IL-22, TNF-α, IL-6, IL-1β, and IL-17A.[36] But the pathogenesis of psoriasis is not only immune system disorders, there are also other predisposing factors, such as bacterial infection, skin trauma, smoking, etc. The current treatment strategy for psoriasis is not effective and has serious side effects, so further research is needed to find better medication for the treatment of psoriasis. Ps has a variety of pharmacological activities, especially its anti-inflammation and immunomodulatory effects.[19] Therefore, we hypothesized that Ps may have potential in psoriasis treatment.

Keratinocyte proliferation and infiltration of inflammatory cytokines are defining features of psoriasis. In psoriatic skin, the secretion of inflammatory cytokines by immune cells leads to the activation and proliferation of keratinocytes.[37] Therefore, inhibition of keratinocyte proliferation has also been used as a criterion for evaluating drug treatment for psoriasis. The pathogenesis of psoriasis involves immune and inflammatory mediators, such as IL-17A, TNF-α, IL-1α, and IL-22.

Therefore, those cytokines were used as an inducer in vitro. In our study, IL-1α, TNF-α, oncostatin M, IL-22, and IL-17A mix (M5) were used to induce HaCaT cells to develop symptoms of psoriatic epidermis. M5 induces keratinocytes to exhibit hyperproliferation and inflammation characteristics of psoriasis.[38] In this study, we found that M5-induced HaCaT had abnormal proliferation, and the proliferation was inhibited by treatment with Ps (Fig. 2 b-e). And Ps remarkably inhibited the secretion of IL-6, IL-1β, and TNF-α in M5-induced HaCaT cells (Fig. 2 f-h). Thus, Ps may alleviate the symptoms of psoriasis by modulating the secretion of inflammatory cytokines in keratinocytes and decreasing the crosstalk between the keratinocyte and the immune cell.

STAT3 is activated by various immune cytokines, such as IL-22 and IFN-γ leading to translocation into the nucleus, and cell migration, proliferation, and differentiation-related genes are activated.[39,40] Tohyama et al. and Ravipati et al. found that IL-22 and IL-6 could activate the STAT3 in the psoriatic lesions, suggesting that STAT3 inhibition might cause the sympathy of psoriasis.[41,42] Additionally, STAT3 could regulate the differentiation of Th17 cells, and alleviates psoriatic lesions reported in a previous study.[43] Our findings showed that STAT3 signaling pathway activation was significantly upregulated in the lesions skin of IMQ-induced psoriasis mice model, which was remarkably inhibited after treatment with Ps (Fig. 7 e-g). Further research revealed that the population of Th17 cells in the psoriasis mice model was increased, and secreted IL-17 and IL-22, in the skin lesions to promote the process of psoriasis (Fig. 5 d-e, Fig. 6 a-b). The result further illustrated that Ps may reduce the Th17-mediated immune response by inhibiting the STAT3 signaling pathway.

NF-κB is a crucial transcription factor involved in the development of psoriasis, and its activation is heightened in psoriatic lesions.[44,45] Previous research reported that Anemoside B4 exerts an anti-inflammation effect by affecting the NF-κB signaling pathway.[46] We evaluated whether Ps could modulate NF-κB activation to alleviate psoriasis, and results showed that Ps inhibited activation of NF-κB in the M5-induced HaCaT cells (Fig. 3 a-g). TNF-α, IL-17A, and IFN-γ are upstream regulatory factors of NF-κB, and crucial inflammatory factors in the inflammatory response.[47] In our study, Ps significantly inhibited the secretion of IL-17A and IFN-γ in the IMQ-induced mice model (Fig. 5 d-e). We suggest that Ps inhibits cell proliferation by affecting NF-κB pathways and decreasing the IL-17A and IFN-γ expression, therefore relieving the symptoms of skin thickening.

The primary objective of this study is to examine the impact of Ps on an IMQ-induced psoriasis mice model and investigate the in vivo mechanism. IMQ-induced psoriasis mice model can simulate psoriatic-like inflammation and is a commonly used animal model.[48,49] In this study, compared with IMQ-treated, Ps-treated mice have shown less thickened, erythematous, and scaly in skin lesions mice, which was also reflected in the PASI score (Fig. 5 b-c). HE staining showed that IMQ-induced pathological psoriatic lesions in mice, including loss of the granular layer, epidermal hyperplasia, acanthocyte thickening, parakeratosis, and inflammatory cell infiltration. After the Ps were treated, the symptoms were relieved (Fig. 5 f). We use Desonide cream as a positive drug, which is a corticosteroid drug that has an advantage in treating psoriasis because of its anti-proliferative effects, but has side effects such as skin atrophy, contact dermatitis, and other symptoms.[50] Compared to Desonide, Ps is a traditional Chinese medicine extract, which has effectiveness, safety and multiple targets. In this context, Ps can reduce Th17 differentiation and secretion of inflammation and immune cytokines by affecting NF-κB and STAT3 signaling pathways, thus alleviating the IMQ-induced psoriasis in mice (Fig. 8).

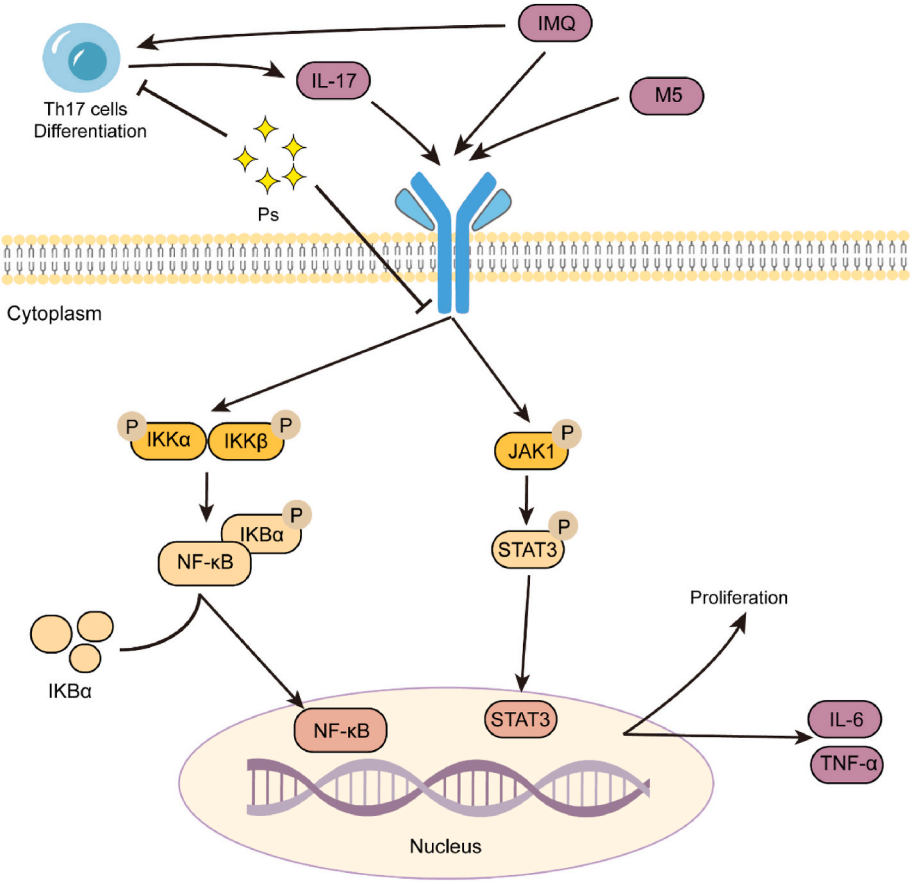

Fig. 8. The schematic of the anti-psoriatic activities of Ps. Ps inhibited the differentiation of Th17 cells and the expression of inflammation cytokines, and inhibited the keratinocyte proliferation by blocking the activation of NF-κB and JAK1/STAT3 signaling pathways.

5. Conclusion

In summary, our research has established that Pulsatilla saponin (Ps) exhibits significant anti-proliferative effects on M5-induced HaCaT cell and mitigates the symptoms of IMQ-induced psoriasis in murine models. Furthermore, Ps significantly reduced Th17 differentiation and decreased the expression of critical pro-inflammatory cytokines, including IL-17A, TNF-α, IL-6, IFN-γ, IL-22, and IL-1β, by inhibiting the NF-κB and JAK1/STAT3 signaling pathways. These results highlight the therapeutic potential of Ps in the treatment of psoriasis, indicating its viability as a foundation for innovative treatment strategies.

Author contributions

Renyikun Yuan, Shilin Yang, and Hongwei Gao provided the experimental ideas. Jilang Li performed in vitro. Siyuan Li, Shan Han, Xiang Gao, and Jia He performed in vivo experimentation. Haixin Qiu, Jilang Li and Yuming He gathered the data and carried out the statistical analysis. Haixin Qiu and Yuming He conducted experiments of the preparation of Ps. Jilang Li and Haixin Qiu wrote the manuscript. Jingjing Li, Jianfang Feng, Renyikun Yuan, and Hongwei Gao revised the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Materials

The following supporting information shown in supplementary data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2024.04.001.

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