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# Betulinic acid suppresses Th17 response and ameliorates psoriasis-like murine skin inflammation

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ABSTRACT

Psoriasis is a common inflammatory skin disease. Current treatment for psoriasis relies on conventional immunosuppressive agents. However, long-term treatment with global immunosuppression may cause various side effects. Thus, it is compelling to seek alternative drugs for treating psoriasis with potentially less side effects. Betulinic acid (BA) is a naturally occurring pentacyclic triterpene, an ingredient that originally exists in natural plants and lacks systemic toxicity. BA can regulate immunity with anti-fibrotic, anti-inflammatory and anti-oxidant properties. However, it's unknown whether BA has a therapeutic effect on psoriasis. The objectives of this study were to investigate whether BA attenuates psoriatic skin inflammation and to identify its mechanisms of action. A murine model of imiquimod-induced psoriasis was utilized to evaluate skin lesion while flow cytometry, immunohistochemistry, quantitative RT-PCR and Western blotting analyses were performed for immunoassays. We found that BA treatment alleviated psoriatic symptoms and inflammatory skin lesion. BA lowered the PASI scores, decreased epidermal thickness and reduced T cell infiltration in the skin lesion. Moreover, BA reduced the frequency of IL-17A-expressing $CD4^+$ and γδ T cells in psoriatic mice, but did not alter $CD4^+FoxP3^+$ Treg frequency. BA also reduced IL-17A production but increased anti-inflammatory cytokine IL-10 level in serum of the psoriatic mice. Furthermore, BA inhibited gene expression of pro-inflammatory mediators in skin lesions, including RORγt, IL-17A, IL-6 and TNFα. Importantly, it suppressed NFκB signaling in the skin lesion. Finally, BA inhibited T cell proliferation and IL-17A production by $CD4^+$ T-Cells in vitro. Thus, BA attenuates psoriasis and inhibits Th17 development.

## 1. Introduction

Psoriasis is an immune-mediated, chronic and inflammatory skin disorder that affects approximately 2–3% of the general population worldwide, characterized by thickening and redness of the skin with pronounced keratinocyte hyperproliferation, dermal/epidermal hyperplasia and inflammatory cell infiltration [1–3]. Conventional immunosuppressive agents, including methotrexate (MTX) and cyclosporine, have been widely used to treat psoriasis [4,5]. However, various adverse reactions have limited their clinical application. Furthermore, the pathogenic mechanisms underlying psoriasis have not been fully understood. It is generally accepted that the immunopathology of psoriasis is mainly mediated and propagated by T cells [6,7]. In addition, a variety of proinflammatory cytokines have been implicated in the pathogenesis of psoriasis [8–10]. Thus, the pathogenesis of psoriasis is mainly caused by autoimmune-mediated skin inflammation.

Betulinic acid (BA), 3β-hydroxy-lup-20(29)-en-28-oic acid, is a naturally occurring pentacyclic triterpenoid [11], which was originally extracted from many species of plants [12,13], including the birch tree, birch bark oil (betulae pix) [14–16] and paeoniaceae [17–19]. BA has been reported to possess various biological properties, including anti-inflammatory [20,21], anti-cancer [22,23], anti-fibrotic [24], anti-angiogenic [25] and antioxidant activities [26]. In recent years, BA has been reported to inhibit the production of several pro-inflammatory mediators or cytokines [20,27]. Many studies have demonstrated that BA is also an immunomodulatory agent [28–34]. However, therapeutic effects of BA on psoriasis and its potential mechanisms of action remain unclear. In this study, we found that BA ameliorated imiquimod (IMQ)-

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induced psoriasis-like skin lesion in mice by downregulating Th17 and IL-17+ γδ T cell development. Furthermore, BA inhibited the gene expression of pro-inflammatory mediators in the skin lesion, including RORγt, IL-17A, IL-6 and TNFα. Importantly, it also suppressed NFκB signaling in the skin of the psoriatic mice. Finally, BA inhibited T cell proliferation and IL-17A production by $CD4^+$ T-Cells in vitro. Thus, BA could be a natural anti-psoriatic drug for treating human psoriasis.

## 2. Materials and methods

### 2.1. Animals

BALB/c and C57BL/6 male mice (18–20 g, 6–8 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China). All mice were housed under specific pathogen-free condition, with free access to food and water. This study was carried out in accordance with the National Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Guangdong Provincial Academy of Chinese Medical Sciences.

### 2.2. IMQ-induced murine psoriatic skin disease

Dorsal hair of mice was removed at an approximate size of $2 \times 3\,cm^2$. All mice, including treatment and model groups, were shaved and daubed with 5% imiquimod (IMQ) cream (Ming Xin Li Di Laboratory, China) daily for seven consecutive days with a topical dose of 62.5 mg to establish a murine model of IMQ-induced psoriasis, as described in our previous publication [35].

### 2.3. Treatment of mice with drugs

BALB/c mice were randomly divided into the following groups with six mice per group: control group (without IMQ); model groups (with 62.5 mg of 5% IMQ cream alone); positive control drug group MTX (1.0 mg/kg/day orally); BA groups (Betulinic acid, DASF Inc., Nanjing, China) at either low doses of 25 mg/kg or high doses of 50 mg/kg orally, as used by others [36]. BA is endotoxin free with a purity of over 98%. MTX (Shanghai Sine Pharmaceutical Lab Co., Shanghai, China) was dissolved in the saline. BA was dissolved in 1% (v/v) Tween 20 in the saline. All mice were treated for seven consecutive days.

### 2.4. Evaluation of skin inflammation severity via PASI scoring and H&E staining

The Psoriasis Area and Severity Indexes (PASI), which consist of measurements for skin erythema, scaling and thickness, were used to evaluate the severity of skin inflammation and lesion, as described in our previous study [37]. Briefly, PASI was scored on a scale from 0 to 4 for each category. “0” represents none; “1” represents “slight”; “2” represents “moderate”; “3” represents “marked”; “4” represents “severe”. The cumulative PASI scores were calculated using additive scores based on erythema, scaling and thickening altogether to reflect the severity of the lesion. The mice of each group were scored starting from the day when IMQ was administered for up to eight consecutive days. To visualize cellular infiltration and epidermal thickness, Skin samples were fixed in 4% neutral paraformaldehyde for 24 h and then embedded in paraffin. The samples in paraffin were cut into 3 μm-thick sections and placed on slides that then were subject to H&E staining.

### 2.5. Flow cytometric assays

Draining lymph node (LN) and spleen cells were harvested from control and psoriasis-like mice and stained with these combinations of Abs: anti-CD4-FITC (Clone H129.19)/anti-FoxP3-APC (Clone FJK-16 s, eBioscience), anti-CD4-APC (GK1.5, Biolegend)/anti-IL-17a-PE (Clone ebio17B7, eBioscience), and anti-γδTCR-PE-CF594 (Clone GL3)/anti-IL-17a-PerCP-Cy5.5 (Clone TC11-18H10) (BD Biosciences). Generally, surface staining was performed before the intracellular staining for for FoxP3 and IL-17a. Cells were permeabilized using FoxP3/Fixation/Permeabilization Concentrate and Diluent Kit (eBioscience) before the intracellular staining. Cells were finally analyzed using a flow cytometer (FACSCalibur, BD Biosciences). To purify $CD3^+$ T-cells, they were stained with anti-CD3-APC (Clone 145-2C11), and sorted out using FACSAria III cell sorter (BD Biosciences). The purity of the sorted cells was typically > 96%.

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

The levels of cytokines IL-10, IFN-γ, and IL-17A in the serum of control and psoriasis-like mice were detected using ELISA according to the manufacturer's instructions (Boster, Wuhan, China). The absorbance was read at 450 nm with a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA).

### 2.7. Quantitative real-time reverse transcription PCR (RT-PCR)

Total mRNA was isolated using TRIzol reagents (Invitrogen, USA) and mRNA was then transcribed to cDNA using a PrimeScript™ RT reagent kit (TAKARA Bio Incorporation, Kusatsu, Japan) according to the instructions of the manufacturer. The cDNA was analyzed for the expression of cytokines using a Quantifast SYBR Green PCR kit (TAKARA Bio Incorporation) via an ABI 7500 Fast RealTime PCR System (Thermo Fisher Scientific). The primer sequences were shown in Table 1. The relative mRNA expression levels of cytokines were normalized against β-actin, and analysis was performed through a comparative $2^{\triangle\triangle CT}$ method. All data are shown in the form of relative expression.

### 2.8. Western blot

Total protein samples from the lesion skin or cells were obtained using RIPA lysing buffer followed by centrifugation at 12,000 rpm for 5 min at 4 °C. The concentrations of proteins were measured using a BCA protein assay kit (Pierce, IL, USA). Samples were run on 10% SDS-PAGE gel and then transferred to PVDF membranes. After blocking with 5% BSA for 1 h, the membranes were incubated with primary antibodies anti-P65 or anti-phospho-P65 (1:1000; Cell Signaling Technology) at 4 °C overnight, and subsequently with the appropriate HRP-conjugated secondary antibodies for 1 h. Finally, signals were detected using an ECL method (Promega) and analyzed through Image J Program software (NIH Image, USA).

### 2.9. Immunohistochemistry

The lesion skin was fixed with 4% paraformaldehyde for 48 h and then embedded in paraffin. Tissues were cut into 3.5 μm-thick sections. For immunohistochemistry, slides with sections were incubated with

**Table 1**
Primer sequences of target genes.

| Target gene | Primer sequence (5′ → 3′) |
|---|---|
| RORγt (forward) | AGTGTAATGTGGCCTACTCCT |
| RORγt (reverse) | GCTGCTGTTGCAGTTGTTTCT |
| IL-17 (forward) | GTCCAAACACTGAGGCCAAG |
| IL-17 (reverse) | ACGTGGAACGGTTGAGGTAG |
| TNF-α (forward) | ACGGCATGGATCTCAAAGAC |
| TNF-α (reverse) | GTGGGTGAGGAGCACGTAGT |
| IL-6 (forward) | ACTTCCATCCAGTTGCCTTCTTGG |
| IL-6 (reverse) | TTAAGCCTCCGACTTGTGAAGTGG |
| β-actin (forward) | TGTCCACCTTCCAGCAGATGT |
| β-actin (reverse) | TGTCCACCTTCCAGCAGATGT |

primary antibody anti-CD3 (1:100, Abcam) or anti-FoxP3 (1:100, Cell Signaling Technology) at 4 °C overnight, then secondary antibody HRP-anti-Rabbit IgG (Maxim), followed by diaminobenzidine color development. For quantitative analyses, slides were imaged at a magnification of 200×. The areas of $CD3^+$ cell infiltration were measured using ImagePro plus 6-software and expressed as integrated optical density (IOD).

### 2.10. Analysis of T cell proliferation and cytokine production by $CD4^+$ T cells in vitro

FACS-sorted $CD4^+$ or $CD3^+$ T cells ($4 \times 10^5$/well) derived from the spleen of BALB/c mice were labeled with CFSE dye (eBioscience) and cultured in 96-well plates coated with anti-CD3 Abs in complete RPMI-1640 media (10%FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing anti-CD28 Ab (2.5 μg/ml) in the absence or presence of BA (0.1, 0.3 and 1.0 μM) or MTX (5 μg/ml) in triplicate for four days. For induction of Th17 development, IL-6 (10 ng/ml) and TGFβ1 (5 ng/ml) were also added to the cell culture.

### 2.11. Assays of T cell cytotoxicity in vitro

T cell cytotoxicity was measured using CCK-8 assays. Purified $CD3^+$ T cells were cultured in 96-well plates in complete RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, and stimulated with anti-CD3/anti-CD28 Abs (2.5 μg/ml) and IL-2 (10 ng/ml, Peprotech). BA was added to each well at various concentrations (0.1–30 μM) in quadruplicate. 48 or 96 h later, 20 μl of CCK-8 was added to each well and incubated at 37 °C for 4 h. The absorbance was measured via a microplate spectrophotometer (Thermo Fisher Scientific, USA) at the wavelength of 450 nm.

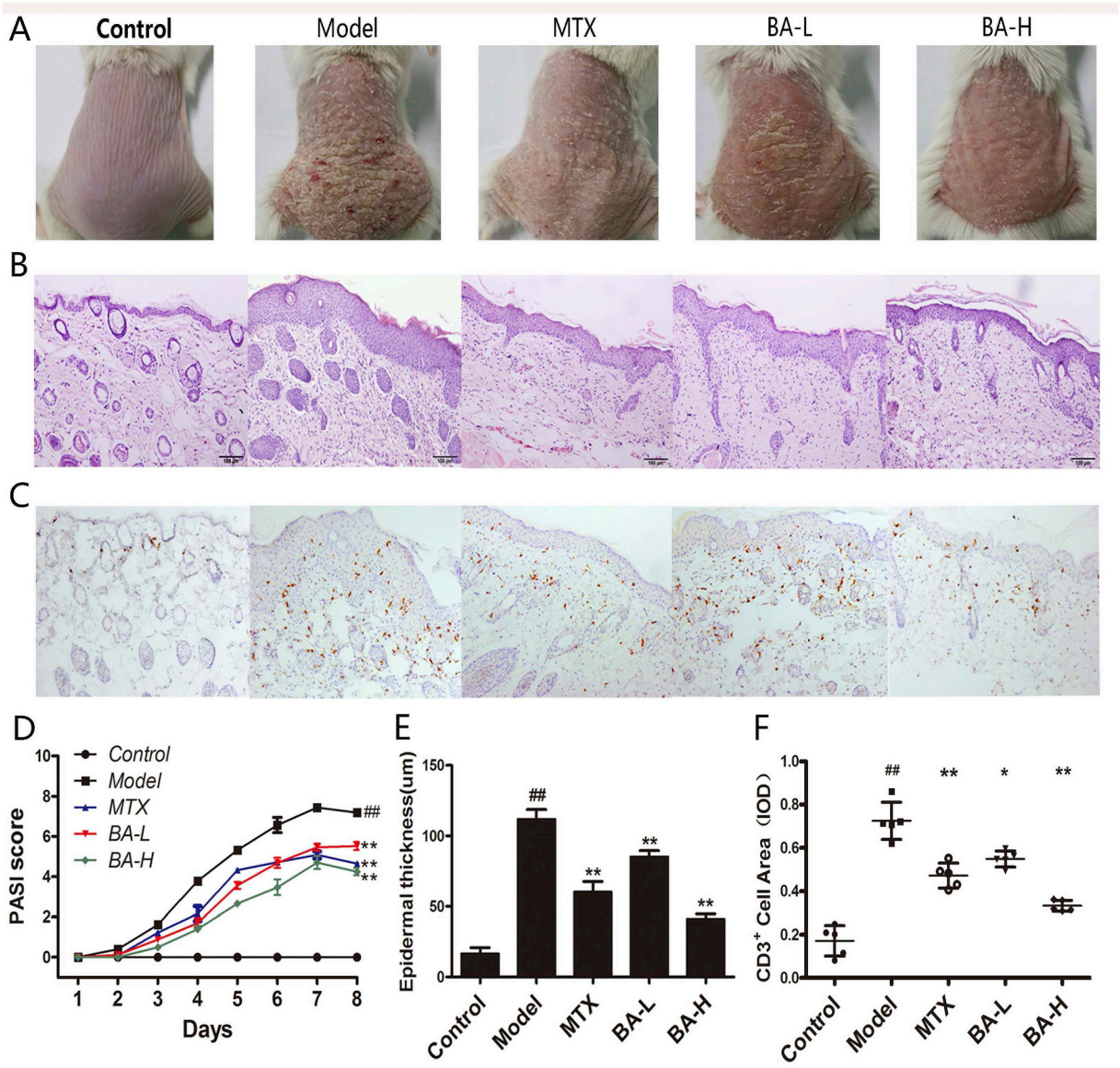


**Fig. 1.** BA ameliorates psoriatic symptoms and skin inflammation in IMQ-induced psoriatic mice.
(**A**) Mice were treated with IMQ plus low-doses of BA (BA-L) or high-doses of BA (BA-H) and back skin photos were taken at 8th day after IMQ painting. (B) H&E staining of the dorsal skin with the original magnification of 200×. (C) Immunohistochemical staining was performed to detect infiltrating $CD3^+$ T cells in dorsal skin at 8th day (magnification of 200×). (D) PASI scores in all groups of mice were evaluated daily and the statistical difference between all groups at 8th day was indicated. (E) Epidermal thickness of the dorsal skin was calculated via Image J software based on H&E staining. (F) The cellular area expressing CD3 was calculated as IOD using Image J based on IHC staining of skin tissue for CD3. Data are expressed as mean ± SD ($n$ = 5–6 mice/group, $^{\#\#}p < 0.01$ vs. Control; $^{**}P < 0.01$ and $^{*}p < 0.05$ vs. Model). One representative of three separate experiments is shown while all results were similar among these three experiments.

### 2.12. Statistical analysis

Comparisons of the means were performed using Student's *t*-test for two groups and one-way analysis of variance (ANOVA) for multiple groups. All data were expressed as the mean ± standard deviation and analyzed through GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). A value of $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. BA ameliorates IMQ-induced psoriatic skin lesion in mice

To determine whether BA would have an effect on psoriasis, we utilized a model of IMQ-induced psoriatic mice that were treated with or without BA. The morphological observations and PASI scores are shown in Fig. 1A and Fig. 1D. Control mice without IMQ had normal skin without any sign of inflammation. However, psoriasis-like lesions, including skin erythema, scaling and thickening, were severe in model group on day 8 following IMQ treatment. Treatments with either low-doses of BA (BA-L: 25 mg/kg) or high-doses of BA (BA-H: 50 mg/kg) attenuated erythema, thickening, scaling and general skin injury, and so did methotrexate (MTX, 1 mg/kg) (Fig. 1A). The PASI scores of BA-treated or MTX-treated mice were also much lower than those of the model group without any drug treatment on day 8 (Fig. 1D). Moreover, H&E staining of skin tissue from the psoriatic mice showed that the thickness of the epidermis in the model group was significantly increased compared with the control group, while BA- or MTX-treated group exhibited a significant decrease in epidermal thickness compared to the model group (Fig. 1B & 1E) on day 8. Immunohistochemistry also revealed an obvious decrease in $CD3^+$ T cell infiltration in the skin of the recipients treated with either BA or MTX compared with that of model group without BA (Fig. 1C & 1F). The carrier of BA itself (the same solution of Tween 20 in saline without BA) did not alter the severity of the psoriatic skin lesion (our primary observation). These findings suggest that BA ameliorates IMQ-induced psoriatic skin lesion and reduces $CD3^+$ T cell infiltration in the skin.

### 3.2. BA does not increase $CD4^+FoxP3^+$ Treg frequency in IMQ-induced psoriatic mice

$CD4^+Foxp3^+$ Tregs are essential for maintaining immunological tolerance and preventing autoimmune diseases, including psoriasis. We therefore asked whether BA would exert its suppressive effects on IMQ-induced psoriasis through inducing $CD4^+Foxp3^+$ Tregs. We measured the frequency of $CD4^+FoxP3^+$ Tregs in the LNs and spleens of IMQ-induced psoriatic mice via flow cytometric analysis on day 8. As shown in Fig. 2A, we found that BA, at either low or high doses, did not significantly alter the percentages of $CD4^+Foxp3^+$ Tregs in the LNs and spleens of the psoriatic mice while MTX increased the Treg percentages in both the LNs and spleens of the mice. Similar findings were also seen on day 4 (data not shown). Importantly, BA also did not alter the frequency of $CD4^+FoxP3^+$ Tregs in the psoriatic skin lesion according to immunohistochemical staining while MTX increased their frequency (Fig. 2B), suggesting that BA treatment does not promote the development of $CD4^+FoxP3^+$ Tregs in the context of psoriasis.

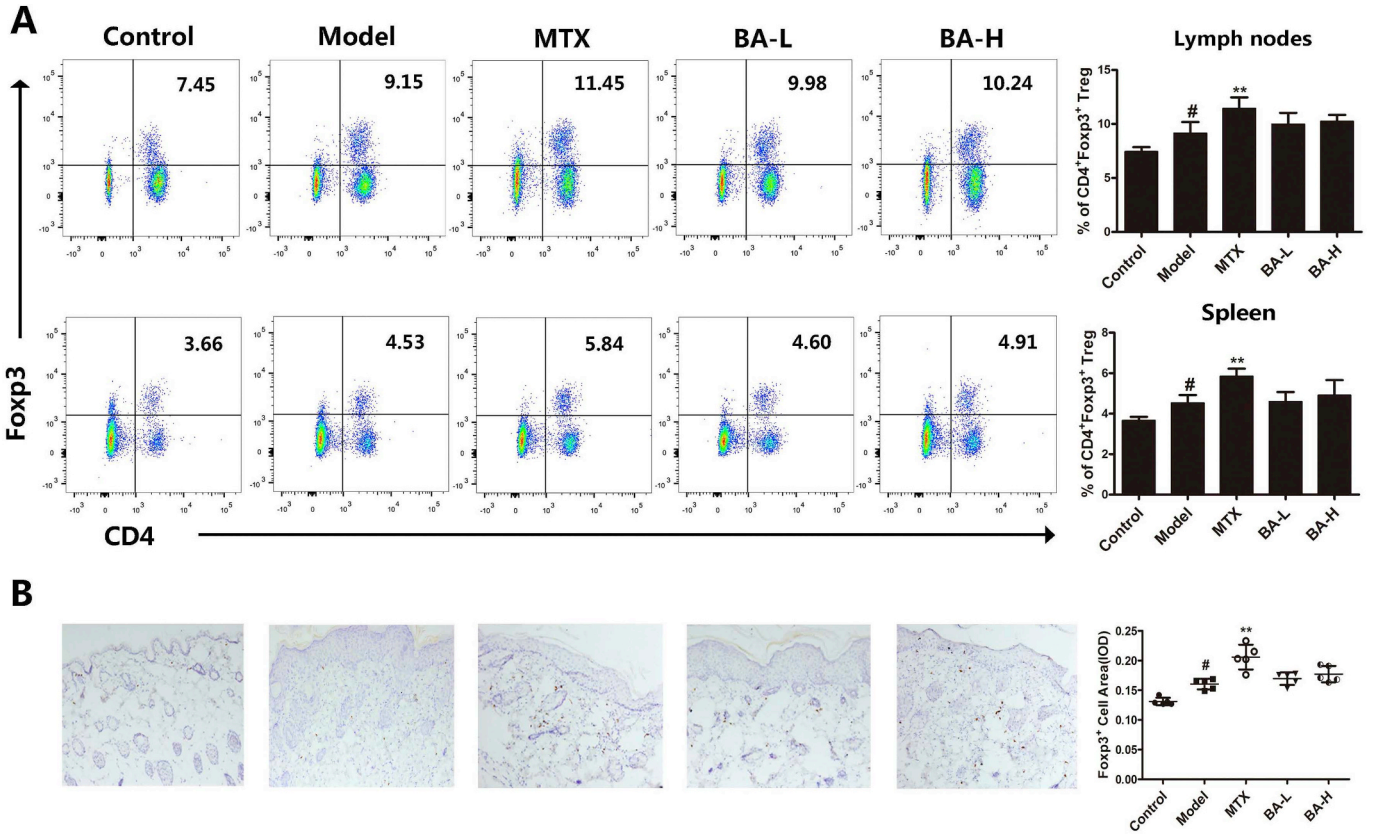


**Fig. 2.** BA does not augment the percentages of $CD4^+FoxP3^+$ Tregs in vivo.
(**A**) Draining lymph node cells and spleen cells were isolated from IMQ-induced psoriasis-like mice on day 8 following daily treatment with BA or MTX (Methotrexate). Cells then were analyzed to determine the percentages of $CD4^+FoxP3^+$ Tregs using a flow cytometer, with column graphs showing the mean frequency of $CD4^+FoxP3^+$ Tregs. (B) Immunohistochemical staining was performed to detect $FoxP3^+$ T cells in dorsal skin at 8th day (magnification of 200×). Data are shown as mean ± SD ($n$ = 4–6 mice/group, $^{\#}p < 0.05$ vs. Control group; $^{**}P < 0.01$ vs. Model group). One representative of three separate experiments is shown while all results were similar among these three experiments.

### 3.3. BA reduces IL-17-expressing cell frequency within both CD4$^+$ and γδT cell populations in the psoriatic mice

Th17 cell development is critical for the pathogenesis of autoimmune diseases. To determine whether BA would hinder Th17 development, we measured the frequency of IL-17-expressing cells within CD4$^+$ and γδT cell populations in the lymph nodes or spleens of IMQ-induced psoriatic mice via flow cytometric analysis. As shown in Fig. 3, we found that either MTX or BA significantly lowered the frequency of IL-17-expressing cells within CD4$^+$ cell population in both draining lymph nodes and spleens of IMQ-induced psoriatic mice while their frequency was dramatically increased in the psoriatic mice compared to normal control mice. Similarly, either MTX or BA significantly lowered the frequency of IL-17-expressing cells within γδT cell population in draining lymph nodes of the psoriatic mice (Fig. 4). However, only high, but not low, doses of BA reduced IL-17-expressing cell frequency within γδT cell population in the spleen of the psoriatic mice.

### 3.4. BA suppresses pro-inflammatory cytokine expression or production in IMQ-induced psoriasis-like mice

Since the Th17/Treg balance plays an import role in the pathogenesis of autoimmune diseases, we specifically measured cytokines IL-17A and IL-10 in the serum of IMQ-induced psoriasis-like mice on day 8. As shown in Figs. 5A, the levels of IL-17A and IFN-γ in the serum from IMQ-induced psoriatic mice were increased compared to the control group of normal mice. Treatment with either BA or MTX reduced the levels of cytokines IL-17 and IFN-γ compared to the model group with IMQ alone. However, BA, but not MTX, increased the level of anti-inflammatory cytokine IL-10 compared to the model group. On the other hand, we also determined the expression of pro-inflammatory mediators in the skin of the psoriatic mice via RT-PCR. As shown in Fig. 5B, we found that either BA or MTX suppressed the gene expression of RORγt, IL-17A, IL-6 and TNFα in the skin lesion. Thus, our results indicate that BA likely ameliorates IMQ-induced murine psoriasis through regulating the balance between pro-inflammatory and anti-inflammatory cytokines.

### 3.5. BA inhibits NFκB signaling in the skin of IMQ-induced psoriatic mice

It has been known that NFκB signaling mediates inflammation and inflammatory diseases. Since we found that BA ameliorated murine psoriatic skin lesion, we then investigated whether BA would alter NFκB activity in IMQ-induced psoriatic mice. As shown in Fig. 6, BA significantly inhibited phosphorylation of p65 (P-p65), the downstream of NFκB signaling, compared to the model group on day 8. Meanwhile, the expression of P-p65 was markedly augmented in the model group (with IMQ) compared to the control group without IMQ. As a positive control, MTX also significantly inhibited P-p65 expression. These results indicate that BA, like MTX, suppresses NFκB signaling.

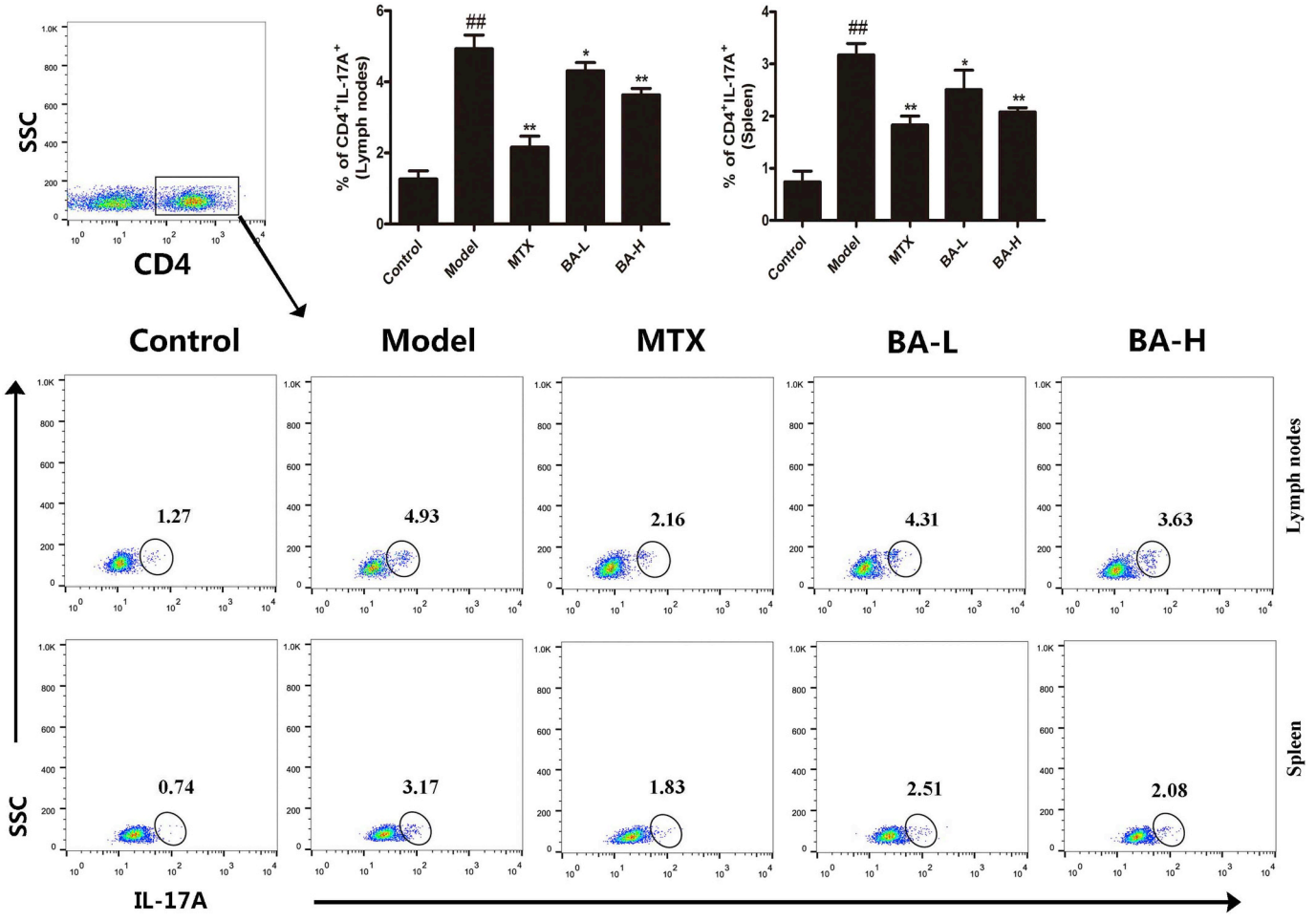


**Fig. 3.** BA treatment downregulates the frequency of Th17 cells in IMQ-induced psoriatic mice.
Draining LN and spleen cells were isolated from IMQ-induced psoriatic mice on day 8 following treatment with BA. Cells were then analyzed via FACS to determine the percentages of IL-17A-expressing cells within CD4$^+$ population. Histograms are gated on CD4$^+$ population. Data of the column graph are shown as mean ± SD ($n$ = 4–6 mice/group, $^{\#\#}p < 0.01$ vs. Control group; $^{**}P < 0.01$ and $^{*}p < 0.05$ vs. Model group). One representative of three separate experiments is shown while all results were similar among these three experiments.

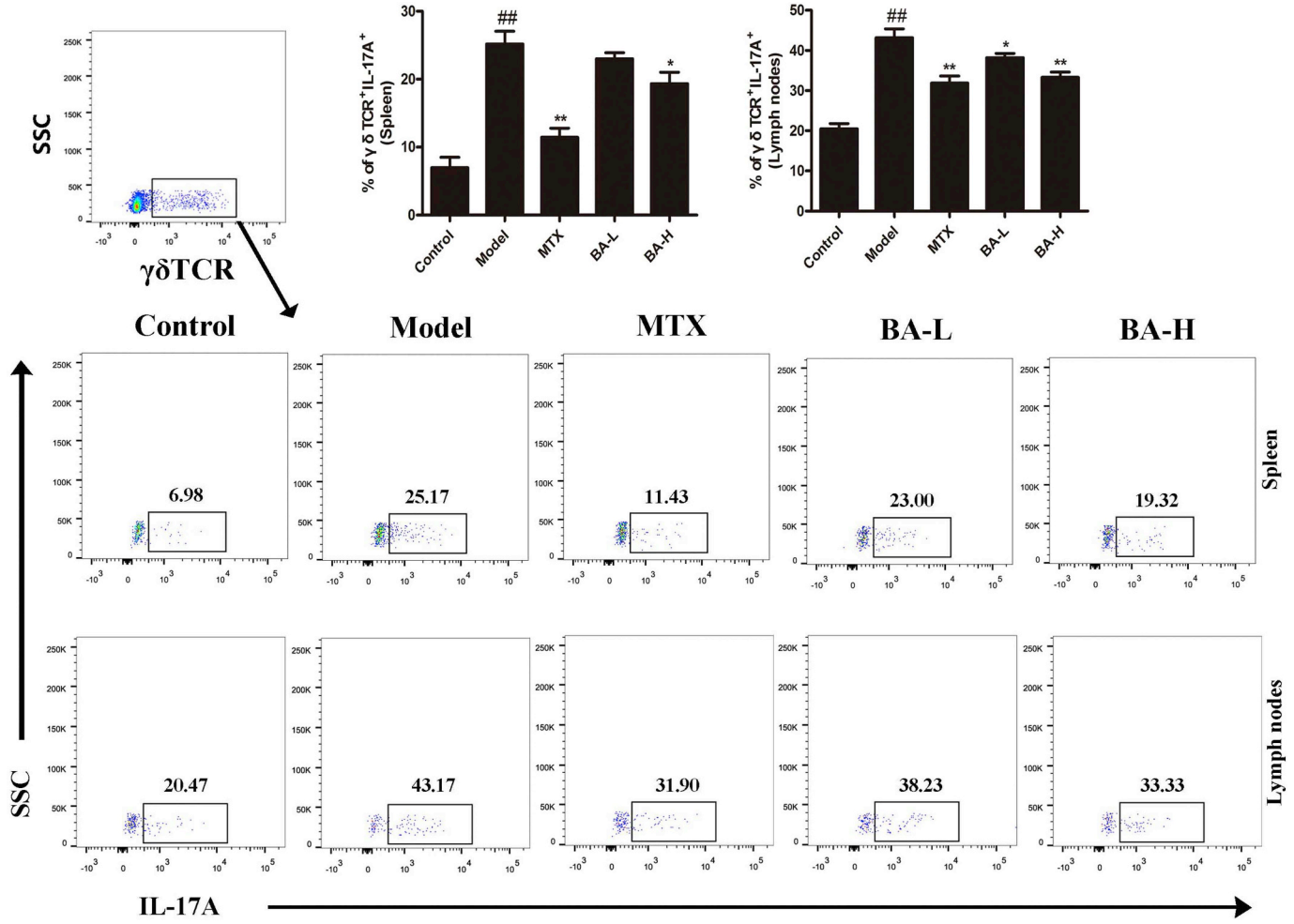


**Fig. 4.** BA also reduces the frequency of IL-17-expressing γδT cells in IMQ-induced psoriatic mice.
Draining LN cells and splenocytes were isolated from IMQ-induced psoriatic mice on day 8 following treatment with BA. Cells were analyzed via FACS to determine the percentages of IL-17A-expressing cells within γδT cell population. Histograms are gated on γδTCR$^+$ population. Data of column graphs are shown as mean ± SD (n = 4–5 mice/group, $^{\#\#}p < 0.01$ vs. Control group; $^{**}P < 0.01$ and $^{*}p < 0.05$ vs. Model group). One representative of three separate experiments is shown while all results were similar among these three experiments.

### 3.6. BA reduces production of IFNγ and IL-17 by CD4$^+$ T cells in vitro

Since BA suppressed Th17 response in vivo, we further examined whether it would also inhibit Th17 development in vitro. FACS-sorted CD4$^+$ T cells derived from naïve mice were cultured in the presence of anti-CD3/anti-CD28 Abs without or with BA for four days. For induction of Th17 development, IL-6 and TGFβ1 were also added to the cell culture. As shown in Fig. 7, at concentrations of 0.3 and 1.0 μM, BA significantly inhibited production of both IL-17A and IFNγ by CD4$^+$ T cells in vitro while at 0.1 μM, BA failed to do so. As a control, MTX also significantly reduced both IL-17A and IFNγ levels.

### 3.7. BA also suppresses T cell proliferation in vitro

Given that BA attenuated the skin lesion in psoriatic mice and that psoriasis is mainly initiated or mediated by T cells, we then asked whether it would also suppress T cell proliferation in vitro. FACS-sorted and CFSE-labeled CD3$^+$ T cells derived from naïve mice were cultured in the presence of anti-CD3/anti-CD28 Abs without or with BA for four days. As shown in Fig. 8A, starting at a concentration of 0.3 μM, BA significantly inhibited T cell proliferation in vitro ($P < 0.05$). At 1.0 μM, BA further suppressed proliferation of T cells ($P < 0.01$). As a control of typical immunosuppressant, MTX also suppressed their proliferation in vitro. To determine potential cytotoxicity of BA, CD3$^+$ T cells were cultured for 48 or 96 h to measure their viability using CCK-8 assays. As shown in Fig. 8B, up to 1.0 μM of BA did not alter cell viability at all. At 3.0 μM, BA slightly reduced cell viability after the culture of 96, but not 48, hours. The cytotoxicity of BA was severe at a concentration of 30 μM at both time points. IC50 was 18.17 and 9.24 for 48 and 96 h respectively. These findings suggest that inhibition of T cell proliferation by BA, at 0.3 and 1.0 μM, is not attributed to its cytotoxicity.

## 4. Discussion

Psoriasis is a common, chronic and inflammatory skin disorder that affects patients physically and psychologically [38]. The precise etiology of psoriasis remains poorly understood although it may result from autoimmunity [39]. Currently, several approaches are available for the treatment of psoriasis, including immunosuppressive agents MTX and cyclosporine. However, due to long-term global immunosuppression, a conventional immunosuppressant may cause severe side effects, including organ toxicities, cancers and infections. On the other hand, biological treatments, such as anti-inflammatory antibodies that inhibit IL-23/IL-17 axis, are much more expensive than conventional drugs. Thus, it is compelling to seek a more efficient, safer and less expensive drug that can be used to treat this refractory inflammatory skin disease.

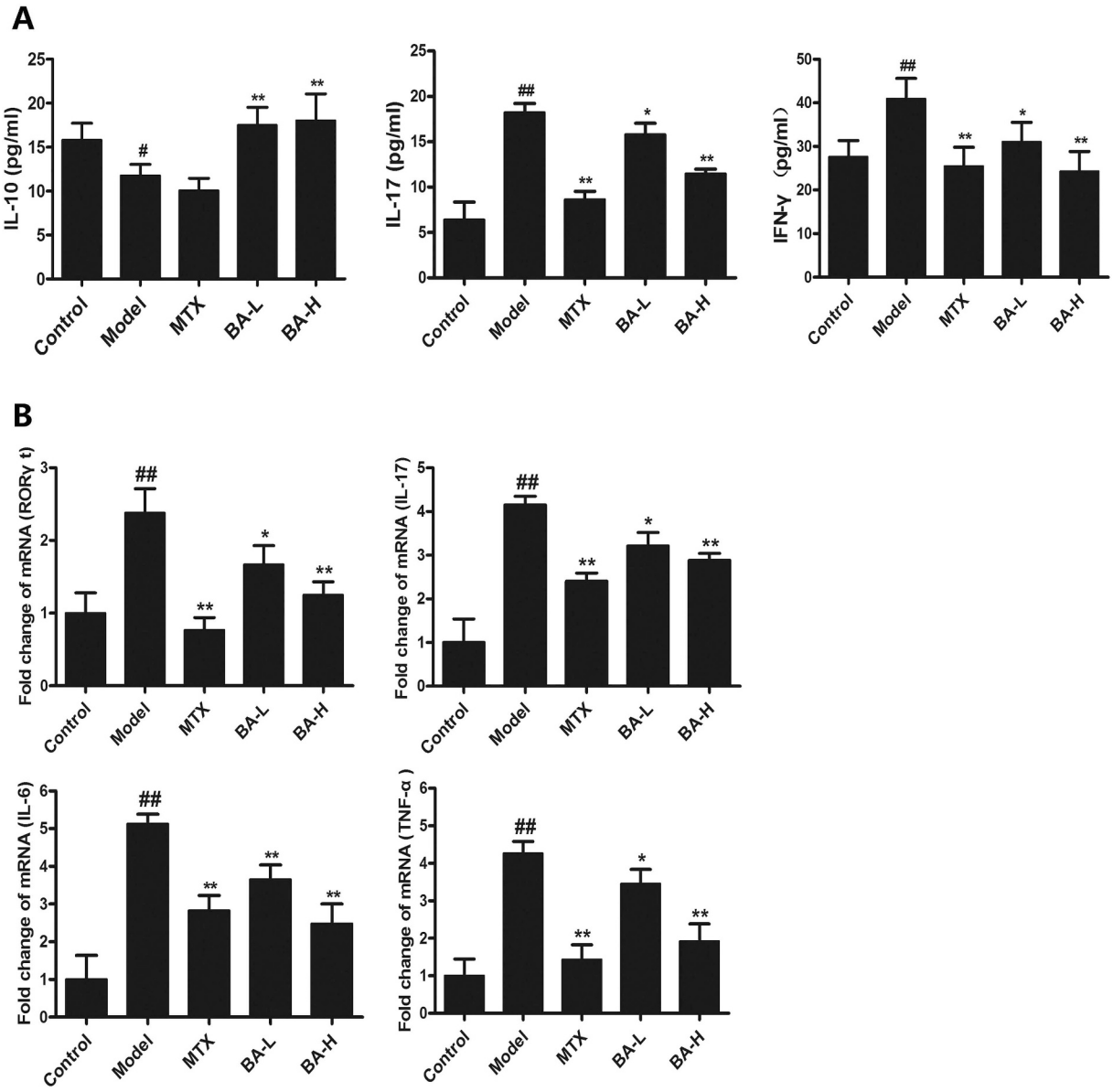


**Fig. 5.** Effects of BA on protein levels of cytokines in serum and mRNA expressions of pro-inflammatory mediators in the skin of IMQ-induced psoriatic mice. (A) The levels of IL-17A, IL-10, and IFN-γ in the serum of IMQ-induced psoriatic mice were determined by ELISA 8 days after the treatment with BA or MTX. (B) Gene expressions of pro-inflammatory mediators in the skin of psoriatic mice were also measured via quantitative RT-PCR on day 8. Data are expressed as mean ± SD derived from four separate experiments (n = 4 mice with 1 mouse per group and per experiment, $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. Control group; $^{**}P < 0.01$ and $^{*}p < 0.05$ vs. Model group).

BA can be extracted from many species of plants, including the birch tree [14–16] and Paeonia spp. (Paeoniaceae) [17–19]. BA has been reported to modulate immune responsiveness [28–34]. Importantly, in vivo studies have demonstrated the complete absence of systemic signs of toxicity [40–44]. However, the exact effects of this molecule on specific immune responses, particularly the Treg/Th17 balance and pathogenesis of psoriasis, have not been elucidated. Using a murine model of IMQ-induced psoriasis, we examined the effects of BA on murine psoriasis. We found that BA treatment alleviated psoriatic symptoms and inflammatory skin lesion. BA significantly lowered the PASI scores and reduced skin immunopathology. Moreover, administration of BA suppressed Th17 and γδT development and IL-17 expression but did not induce $CD4^+FoxP3^+$ Tregs in IMQ-induced psoriatic mice. It also inhibited T cell proliferation in vitro. Finally, BA suppressed gene expression of pro-inflammatory cytokines and NFκB signaling in the lesion skin of psoriatic mice, suggesting that BA is an anti-inflammatory NFκB inhibitor.

Autoimmune psoriasis is mainly mediated and propagated by T cells [6,7]. In present study, we also found that BA inhibited T cell proliferation in vitro at concentrations of 0.3 and 1.0 μM, which did not cause any cytotoxicity. At 3.0 μM, however, it started to show moderate cytotoxicity on T cells while its cytotoxicity was severe at a concentration of 30 μM. The IC50 was 18.17 and 9.24 for 48 and 96 h. Previous studies have reported that BA has no systemic toxicity [40–44]. Thus, BA could be potentially developed into a promising natural drug for the treatment of psoriasis without any major side effect.

Psoriasis is generally a T-cell-mediated inflammatory skin disease. Previous studies have shown that Tregs are dysfunctional in psoriasis, with decreased suppressive capacity [45]. $CD4^+CD25^+$ Tregs prevents autoimmune diseases and allograft rejection in many animal models [46–49]. MTX, an immunosuppressive and anti-inflammatory drug, has been widely used to treat psoriasis [4,5]. MTX has been shown to exert its immunosuppressive effects via increasing $CD4^+FoxP3^+$ Tregs

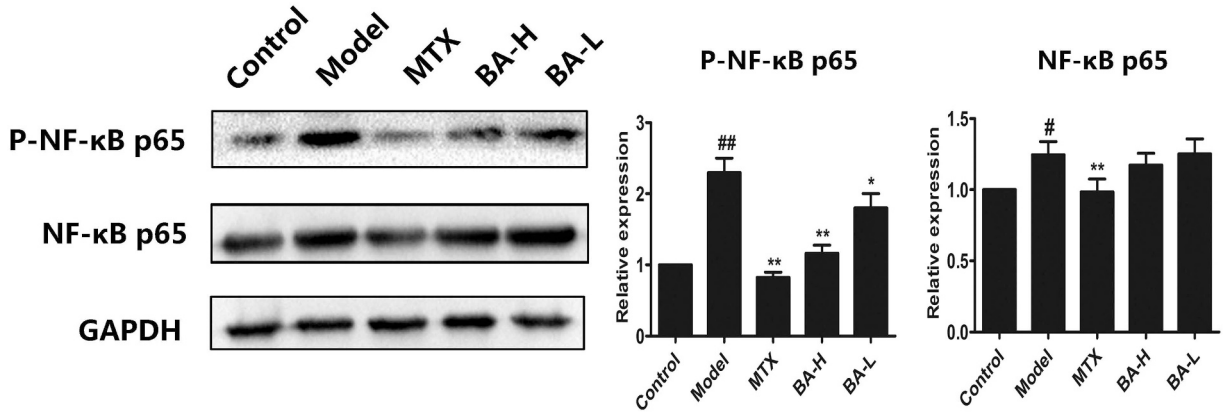


**Fig. 6.** BA inhibits NFκB signaling in IMQ-induced psoriatic skin lesion.
Impacts of BA on protein expression of phosphorylated p65 (P-p65) and total p65 were evaluated by Western blotting analyses of the lesion skin of the psoriatic mice on day 8 following MTX or BA treatment. Data are expressed as mean ± SD derived from four separate experiments (n = 4 mice with 1 mouse per group and per experiment $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. Control group; $^{**}P < 0.01$ and $^{*}p < 0.05$ vs. Model group).

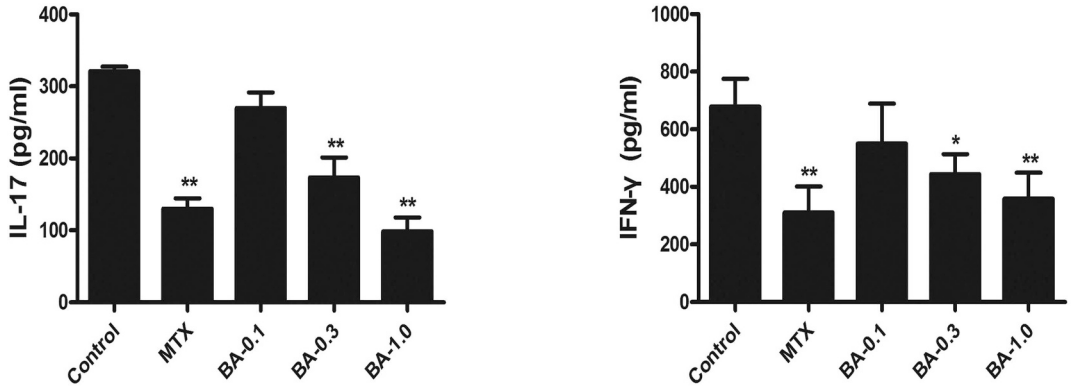


**Fig. 7.** BA suppresses production of IFNγ and IL-17 by $CD4^+$ T cells in vitro.
FACS-sorted $CD4^+$ T cells derived from naïve mice were cultured in the presence of anti-CD3/anti-CD28 Abs without or with BA for four days. For induction of Th17 development, IL-6 and TGFβ1 were also added to the cell culture. IFNγ and IL-17A in the supernatant were measured via ELISA. Data are presented as means ± SD derived from three independent experiments ($n = 3$, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Control).

[5,45,50]. A previous study demonstrated that SH479, a derivative of BA, increased $CD4^+FoxP3^+$ Treg numbers in a mouse model of collagen-induced arthritis [33] although BA itself did not significantly augment their numbers. Our findings showed that BA did not augment $CD4^+FoxP3^+$ cell frequency in the psoriatic mice. Therefore, BA and MTX may exert their therapeutic effects on psoriasis through differential mechanisms. Interestingly, a recent study by Brusotti et al. revealed that BA was a PPARγ antagonist that improved glucose uptake and inhibited adipogenesis [51]. It would be interesting to further determine whether BA improves type 2 diabetes.

Suppression of autoimmunity or chronic inflammation by $CD4^+CD25^+$ Tregs has been largely mediated by immunosuppressive cytokines, including IL-10 [52]. Some clinical trials revealed modest and transient efficacy of recombinant IL-10 in treating psoriatic patients [53–55]. In this study, we found that BA increased IL-10 production in the serum of psoriatic mice. However, we did not find an increase in $CD4^+CD25^+$ Treg numbers, suggesting that the Tregs may not be responsible for the increase in IL-10 level. On the other hand, a variety of cytokines have been implicated in the pathogenesis of psoriasis, including proinflammatory cytokines IL-17 (Th17) and IFN-γ [9,10,56]. In particular, Th17 cells play an important role in the pathogenesis of autoimmune diseases [57]. We demonstrated that BA suppressed production of IL-17 in the serum and its gene expression in the skin of the psoriatic mice. We also found that it reduced the frequency of $IL\text{-}17^+$ CD4+ or γδT cells and downregulated RORγt mRNA expression. Our results suggest that BA ameliorates psoriasis by mainly suppressing Th17 response. However, our study does not reveal why BA regulates RORγt/Th17 responsiveness. Future studies are needed to further identify in-depth mechanisms underlying its immunoregulatory effects.

Recent studies have shown that IL-17-producing γδ T cells are involved in the pathogenesis of psoriasis and other autoimmune diseases. γδ T cells that were capable of producing IL-17 were increased in the skin of psoriatic patients [58] while IL-17-producing γδ T cells broke tolerance by enhancing inflammation in various autoimmune diseases, including psoriasis and rheumatoid arthritis [59]. Moreover, suppression of inflammation mediated by IL-17-producing γδ T cells attenuated imiquimod-induced psoriasis-like skin lesion in mice [60]. We found that BA reduced the frequency of IL-17-expressing γδ T cells in psoriatic mice and also decreased IL-17 production by activated $CD4^+$ T cells in vitro. Thus, targeting IL-17-expressing γδ T cells may provide a novel strategy for treating psoriasis. On the other hand, IFNγ is a typical Th1 cytokine that is widely involved in various immunopathology, including autoimmune psoriasis [8–10]. We demonstrated that BA suppressed IFNγ production in vivo and in vitro. Therefore, BA may ameliorate murine psoriasis by suppressing both Th1 and Th17 responses.

In conclusion, BA treatment alleviates psoriatic symptoms and skin lesion in IMQ-induced psoriatic mice. It mainly suppresses Th17 response by reducing the frequency of IL-17-expressing $CD4^+$ and γδT cells in the psoriatic mice. BA also inhibits gene expression of pro-inflammatory mediators and cytokines, including RORγt, IL-17A, IL-6 and TNFα, while suppressing NFκB signaling in the lesion skin of the psoriatic mice. Moreover, it inhibits T cell proliferation in vitro at

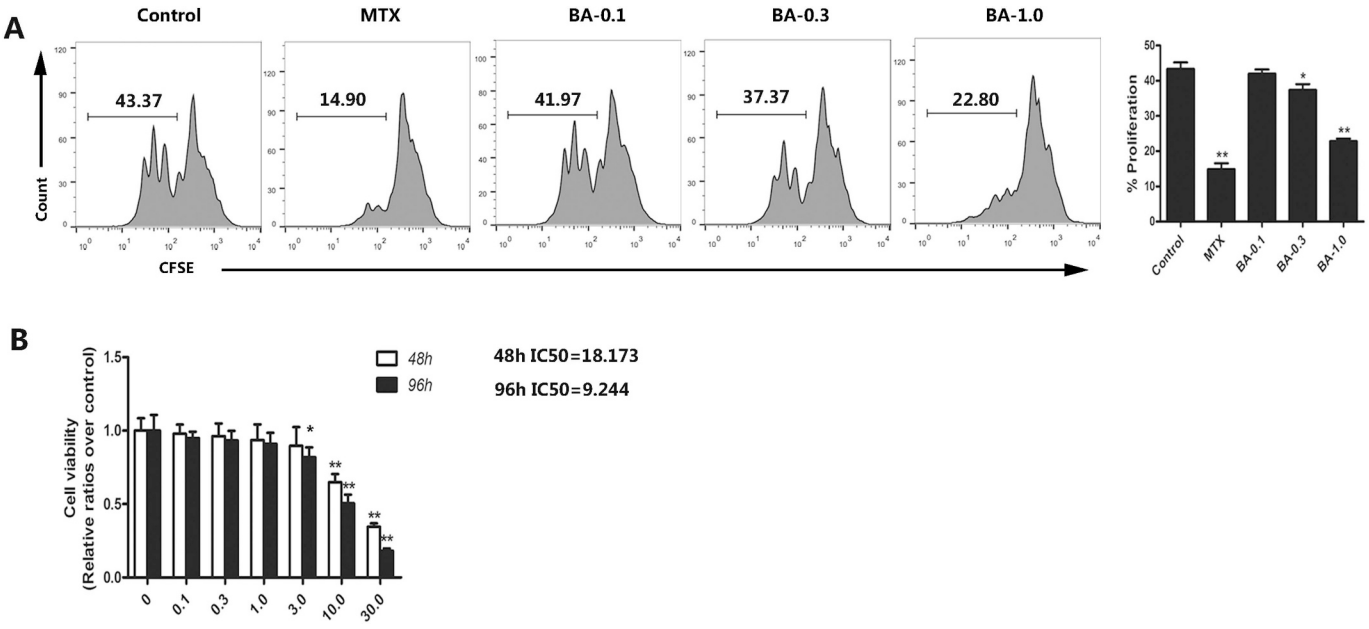


**Fig. 8.** BA inhibits T cell proliferation in vitro.
(**A**) FACS-sorted $CD3^+$ T cells derive from naïve mice were labeled with CFSE dye and cultured in 96-well plates coated with anti-CD3 Abs in complete RPMI-1640 media containing anti-CD28 Ab in the presence of MTX (5 μg/ml) or BA (at a concentration of 0.1, 0.3 or 1.0 μmol/l). After culturing for four days, cell proliferation was analyzed using a flow cytometer. Column graphs show the percentages of proliferated cells. Data are presented as mean ± SD derived from three independent experiments (n = 3, *P < 0.05 and **P < 0.01 vs. Control group). (B) BA cytotoxicity to $CD3^+$ T cells was measured based on cell viability using CCK-8 assays 48 or 96 h after the similar culture plus various concentrations of BA (μM). Data are presented as means ± SD derived from four independent experiments (n = 4, *$P < 0.05$ and ** $P < 0.01$).

concentrations that do not cause cytotoxicity, suggesting that BA is a new NFκB inhibitor. Thus, BA could be used as a potentially new immunosuppressant for the treatment of human psoriasis in the future.

## Author contributions

CL and YC: Formal analysis, investigation, methodology and writing-original draft; ChL: Funding acquisition and supervision; HC, JD, YY and YYX: Formal analysis; HL: Methodology; HH, JW and LH: Resources; and ZD: Conceptualization and writing-review & editing.

## Declaration of Competing Interest

None.

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