International Immunopharmacology 32 (2016) 32–38

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Astilbin inhibits Th17 cell differentiation and ameliorates imiquimod-induced psoriasis-like skin lesions in BALB/c mice via Jak3/Stat3 signaling pathway

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ARTICLE INFO

Article history:
Received 10 September 2015
Received in revised form 23 December 2015
Accepted 27 December 2015
Available online 16 January 2016

Keywords:
Psoriasis
Th17
IL-17
Imiquimod
Astilbin
Inflammation

ABSTRACT

The flavonoid astilbin is the major active component extracted from the rhizome of *Smilax glabra*, which has been widely used in China to treat inflammatory and autoimmune diseases, Psoriasis is a common chronic inflammatory disease in which T helper 17 (Th17) cells play an important role, provoking inflammation. We employed an imiquimod (IMQ)-induced psoriasis-like mouse model to investigate the effect of astilbin in inflammation. Mice were administered 25 to 50 mg/kg astilbin. Inflammation of psoriasis-like lesions was assessed by histology, circulating levels of T cells were assessed by flow cytometry and cytokines by bead-based immunoassay. Jak/Stat3 in isolated T cells was assessed by Western blotting and ROR-γt expression was assessed by RT-PCR. Administration of astilbin ameliorated IMQ-induced keratinocyte proliferation, infiltration of CD3+ cells to psoriatic lesions and ameliorated elevations in circulating CD4+ and CD8+ T cells and inflammatory cytokines (IL-17A, TNF-α, IL-6, IFN-γ and IL-2). *In vitro*, astilbin inhibited Th17 cell differentiation and IL-17 secretion of isolated T cells, and inhibited Jak/Stat3 signaling in Th17 cells, while up-regulating Stat3 inhibitor SCOSE3 expression in psoriatic lesions. Thus, astilbin likely alleviates psoriasis-like skin lesions by inhibiting Th17 related inflammation. Astilbin represents as an interesting candidate drug for immunoregulation of psoriasis.

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1. Introduction

Psoriasis is a chronic immune-mediated inflammatory erythematosquamous dermatosis disorder, characterized by uncontrolled keratinocyte proliferation, recruitment of T cells to the skin and release of pro-inflammatory cytokines [1]. Although the molecular mechanisms governing psoriasis pathogenesis are unclear, many studies have indicated that T helper 17 (Th17) cells play an important role in the development of psoriasis lesions [2]. Th17 cells are reported to infiltrate psoriasis lesions, and levels of circulating Th17 cells in the peripheral blood are elevated in psoriatic patients, and positively correlate with the Psoriasis Area and Severity Index (PASI) [3]. Naïve CD4+ differentiate into Th17 cells in the presence of Transforming growth factor (TGF)-β, interleukin (IL)-6, and IL-1β, and their survival and activation are modulated mainly by IL-23. Th17 cells are the main source of IL-17 family cytokines, and IL-17 has been recognized as a key cytokine in establishment and maintenance of the psoriatic phenotype [4]. IL-17A binds receptors expressed on keratinocytes, dendritic cells, dermal fibroblasts and endothelial cells [5]. Three inhibitors targeting IL-17A or its receptor (secukinumab, ixekizumab and brodalumab) have demonstrated encouraging results in phase II clinical trials and have been successfully applied in treatment of psoriasis, psoriatic arthritis and spondyloarthritis [6].

The flavonoid astilbin is a major active component of the rhizome of *Smilax glabra* and other herbs which have been traditionally used to treat psoriasis. Astilbin has been reported to possess multiple clinically relevant bioactivities, including antioxidant [7], anti-inflammatory [8], anti-arthritic [9], and anti-diabetic nephropathy properties [10]. Nevertheless, the molecular mechanisms by which astilbin interacts with inflammatory processes are poorly understood.

Previous studies reported that astilbin down-regulates T cell activity by selectively inducing apoptosis, stimulating negative regulatory cytokine (IL-10) [11] and suppressing activated T cell adhesion and migration [11] in inflammatory disease, such as collagen-induced arthritis [12] and hypersensitivity [13]. In addition, astilbin not only inhibited T lymphocyte function in acute heart allograft rejection, but also inhibited migration and antigen presenting of dendritic cells (DCs) [14], and

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http://dx.doi.org/10.1016/j.intimp.2015.12.035
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Table 1
Primers used in RT-qPCR.

Primers	Sequences (5′ to 3′)	Product (bp)
RORr pf	AACTGGCTTTCCATCATCATCTCTG	157
RORr pr	GGGAAGGCGGCTTGGACCACGAT	
Actin pf	GCCTTCCTTCTTGGGTAT	97
Actin pr	GGCATAGAGGTCTTTACGG	

astilbin was recently reported to reduce activation of both T and B cells in lupus-prone mice [15]. These activities are associated with inhibition of nuclear factor κB (NF-κB) and p38 phosphorylation. However, in a mouse model of dextran sulfate sodium (DSS)-induced acute colitis, administration of astilbin increased the number of DCs detected and increased TGF and IL-10 secretion [16]. Furthermore, DCs pretreated with astilbin enhanced T-regulatory cell (Treg) differentiation.

Astilbin appears to target multiple immune processes, but can be summarized by inhibition of T cell induced inflammation. Astilbin may thus pose useful clinically in a variety of inflammatory diseases with less associated toxicities and adverse effects than conventional approaches [17,18].

In this paper, we sought to investigate the effect of isolated astilbin on a mouse model of psoriasis. We found that astilbin significantly inhibited inflammatory responses and keratinocyte over-proliferation in a mouse model of imiquimod (IMQ)-induced psoriasis. *In vitro*, astilbin reduced Th17 cell differentiation and suppressed and IL-17 secretion in a dose dependent manner. Our findings suggest astilbin may improve symptoms of psoriasis by inhibiting Th17 dominated inflammation.

2. Materials and methods

2.1. A mouse model of psoriasis

BALB/c mice (male, 18 to 20 g, 8 week-old) were purchased from Beijing HFK Bioscience (China) (certification NO. SCXK Jing 2014-0004), and housed under specific pathogen-free conditions, with free access to food and water. All animal experiments were performed in accordance with the National Institutes of Health Guidelines on Laboratory Research and approved by the Animal Care Committee of Capital Medical University.

The mice were separated into the following five groups of eight mice. Four groups were administered a daily topical dose of 62.5 mg of a cream preparation containing 5% IMQ (Mingxinlidi Laboratory, China) on hair-free back of mice to establish a model of IMQ-induced psoriasis [19]. The control group (CON) did not receive IMQ. Astilbin (National Institutes for Food and Drug Control, China), was dissolved in DMSO, then diluted in normal saline (NS) to achieve a final DMSO concentration < 0.1% for oral administration. The model group (IMQ) received no astilbin; the methotrexate (MTX) group received 1 mg/kg MTX, a drug used for psoriasis treatment [20]. The astilbin-high group (AH) received 50 mg/kg astilbin, and the astilbin-low group (AL) received 25 mg/kg astilbin. All treatments were operated from the day IMQ was administrated, once a day for 7 days. The severity was monitored and graded using a modified human scoring system Psoriasis Area Severity Index (PASI) [21]. The cumulative score denotes severity of inflammation. After seven days mice were sacrificed by cervical dislocation under sodium pentobarbital anesthesia, and skin lesions and serum samples were collected.

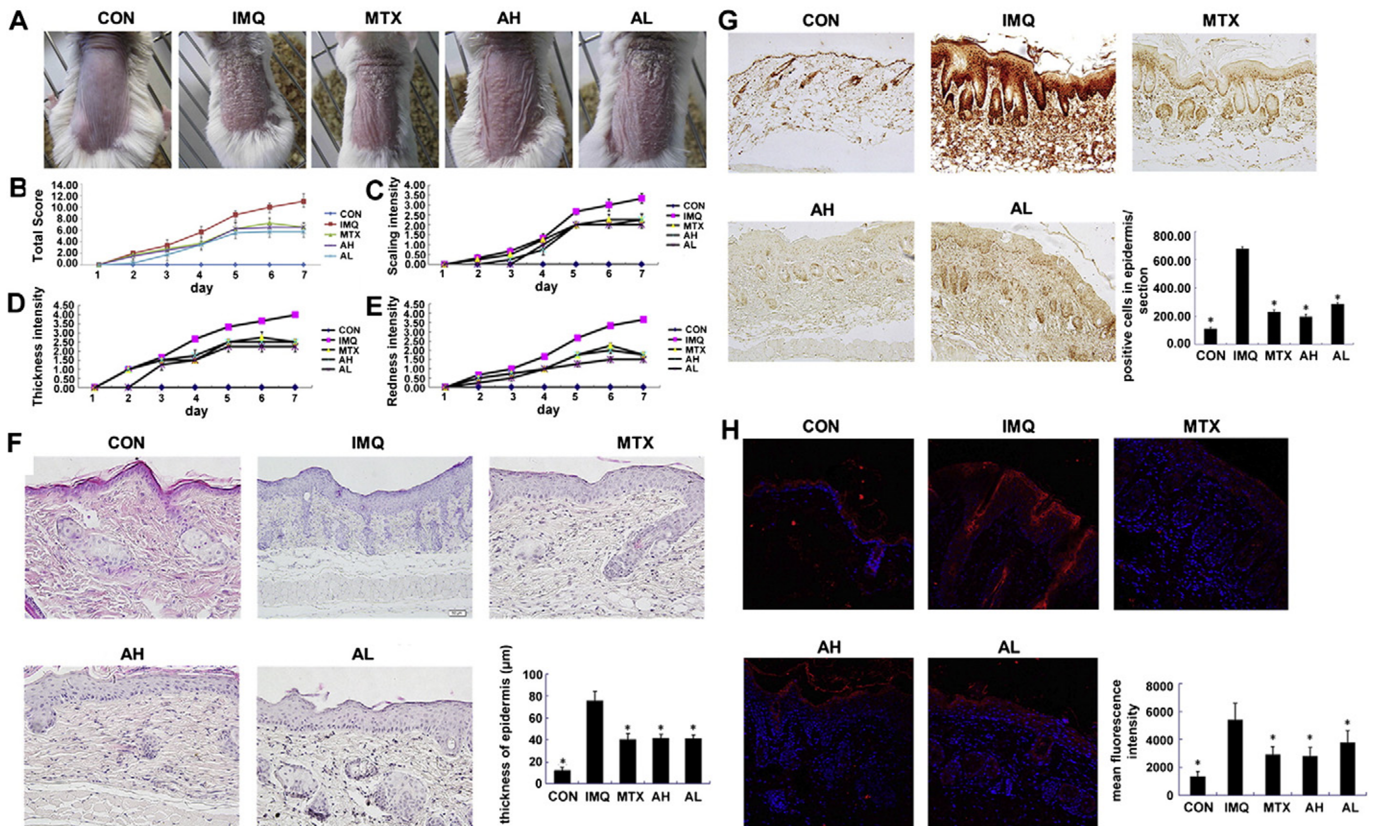


Fig. 1. Astilbin treatment ameliorated IMQ-induced skin lesion. A mouse model of psoriasis was induced in mice by topical application of imiquimod (IMQ). Psoriasis-like skin lesions were observed after 7 days in IMQ-treated animals, but not control animals (CON), and animals also administered 25 or 50 mg/kg astilbin (AL and AH) or 1 mg/kg methotrexate (MTX) exhibited ameliorated symptoms. Bar = 50 μm (A). Means ± SDs of total score (B), scaling (D), skin thickness (C) and redness scores (E) (n = 4 per experiment). Phenotypical presentation and corresponding histological analyses (HE staining) of mouse back skin and epidermal thickness (F). Mouse back skin IHC staining for proliferating cell nuclear antigen (red) (G) and involucrin (red) (H). Blue = DAPI, bar = 50 μm. $^{*}p < 0.05$ vs model mice.

2.2. Isolation of naïve CD4 + T cells and in vitro induction of Th17 differentiation

Total splenic T cells were purified by negative selection with the Mouse naïve CD4 + T Cell Isolation Kit and “The Big Easy” Magnet (EasySep, USA) following the manufacturer's instructions, then further purified to >95%. CD4 + purity by FACS-sorting (BD FACSVerse™, Germany) with anti-CD4 FITC (Miltenyi Biotec, Germany). For gating, viable cells were selected based on their FSC values; then, T-helper cells were isolated as CD4 + cells, which represented >97% total viable cells.

T cells were seeded in 12 well plates containing plate bound anti-CD3 (5 μg/ml, BD Pharmingen, USA) and soluble anti-CD28 (2 μg/ml, BD Pharmingen, USA) antibodies at 2×10^6 cells/well in RPMI 1640 medium (Hyclone, Carlsbad, CA) containing 15% inactivated fetal bovine serum (Hyclone, Carlsbad, CA).

For Th17 cell differentiation, splenic T cells were cultured with 5 ng/ml TGF-β, 20 ng/ml IL-6, 10 ng/ml IL-1β, and 15 ng/ml IL-23

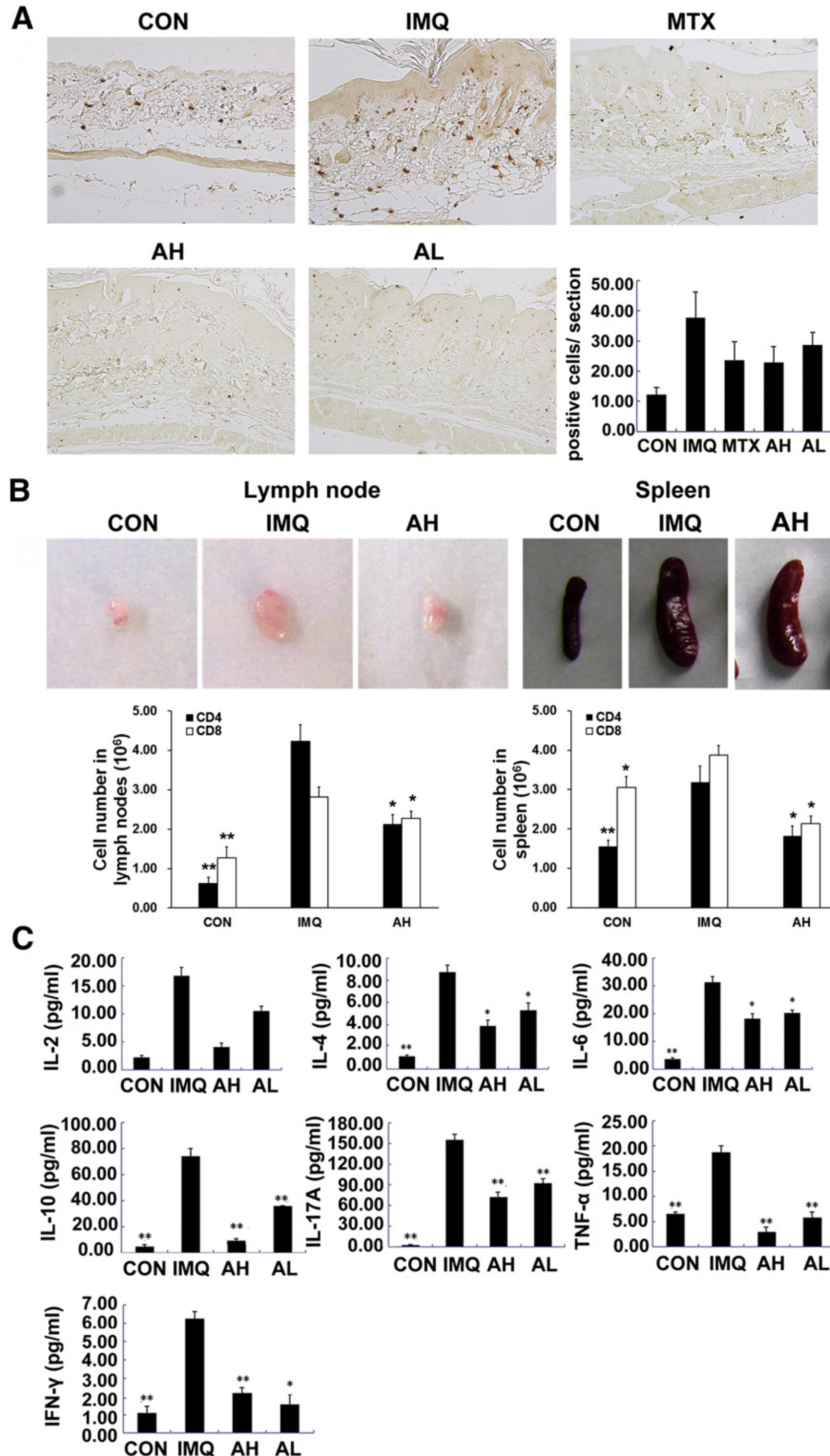


Fig. 2. Astilbin inhibited T cell-mediated inflammatory infiltration. CD3-positive cells in IMQ-induced mice (A). CD4+ and CD8 + T cells in inguinal lymph nodes and spleen (B). Bead-based immunoassay of key psoriasis-related inflammatory cytokines demonstrates significant decreases in IL-17A, IL-6, TNF-α, IFN-γ, IL-2, IL-4 and IL-10 in peripheral blood of IMQ-induced mice treated with astilbin (C). $^*p < 0.05$ and $^{**}p < 0.01$ vs model mice. Scale bar = 50 μm.

(PeproTech, USA), and 5 μg/ml interferon-γ (IFN-γ)-IL-2- and IL-4 directed antibodies (R&D Systems, USA) as indicated [22]. Th17 differentiation was assessed by flow cytometry, as previously described [14].

Astilbin (>98%, molecular weight of 450.40, Chinese institute of food and drug identification) was dissolved in dimethyl sulfoxide (DMSO), and cells were incubated with the indicated concentrations for three days. In control groups, DMSO was used at amounts similar to those of treatment groups.

2.3. Cell viability assays

Cell viability was assessed using the Cell Counting Kit-8 (CCK8) assay (Dojindo Laboratory, Japan) according to the manufacturer's instructions at 3 days after induction of Th17 differentiation. The mean optical density (OD) of the three wells in each group was used to calculate the percentage of cell proliferation.

2.4. Flow cytometry analysis

Cells were stained with anti-CD3-APC, anti-CD8-PE and anti-CD4-FITC antibodies (BD Pharmingen, USA). Prior to IL-17A staining, cells were incubated with Leukocyte Activation Cocktail and BD GolgiPlug for 6 h, then suspended in fixation/permeabilization solution (BD Cytofix/Cytoperm kit-BD Pharmingen, USA), and stained with anti-IL-17A-PE antibody (BD Pharmingen, USA). The concentration of cytokines in peripheral blood was assessed using the Th1/Th2/Th17 CBA Kit (BD Pharmingen, USA) according to the manufacturer's instructions.

2.5. Immunochemical staining and immunofluorescence staining

Skin samples from the back lesions of mice were collected in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (HE) and anti-Rabbit CD3, proliferating cell nuclear antigen (PCNA), involucrin and IL-17 antibodies (Abcam, USA) diluted 1:500, and staining was assessed using light and fluorescence microscopes (Olympus, Japan).

2.6. Western blotting

Skin samples were lysed and the protein was resolved in 10% SDS-PAGE. The membrane fraction was incubated with mouse-anti-total or phosphorylated Stat3, jak2 and jak3 antibodies, rabbit-anti-SCOSE3 antibody (Cell Signal Technology, Danvers, MA) and rabbit-anti-β-actin antibody (Santa Cruz, CA), then IRDye 700DX- or 800DX-conjugated secondary antibodies (Rockland Inc., Gilbertsville, PA). Immunofluorescence was assessed by Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NB).

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

Total RNA was extracted from skin lesions using TRIzol (Invitrogen, USA) and purified using a NucleoSpin RNA Clean-up Kit (Macherey-Nagel, Germany). Complementary DNA was generated using an AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, USA) and specific primers (Table 1), and the relative expression levels of genes were determined with an ABI 7500 Fast Real-Time PCR System using real-time PCR master mix (Roche, USA). The actin gene was used as a reference to normalize the data.

2.8. Statistical analyses

All data were expressed as mean ± standard deviation (SD) or original data representing one of at least three independent experiments. One-way analysis of variance (ANOVA) followed by Newman–Keul's post hoc test was used to compare multiple groups. Unpaired Student t-test was performed to compare two groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Astilbin ameliorated abnormal proliferation and differentiation of keratinocytes in a mouse model of psoriasis

To investigate whether astilbin has a beneficial effect on psoriasis, we administered astilbin to a mouse model of psoriasis. Psoriasis was induced in mice by topical application of IMQ. After seven days, typical erythema, scaling and thickening were observed in IMQ-induced skin lesions. The weight of mice orally administered 25 or 50 mg/kg astilbin did not differ significantly from control animals, and astilbin-treated animals had smoother skin, more shallow erythema and sparser scales (Fig. 1A–E).

IMQ-treated skin lesions exhibited increased epidermal hyperplasia, elongated 'rete-like' ridges, acanthosis in the epidermis and perivascular infiltration of the inflammatory cells in the upper dermis, a phenotype typical of human psoriatic skin, but astilbin significantly reduced the thickness of the epidermis layer, and attenuated IMQ-induced psoriasis (Fig. 1F).

PCNA is expressed in proliferative cells, especially the basal cell. Expression of PCNA was reduced in the skin lesions of astilbin-treated mice, suggesting that astilbin reduces IMQ-induced proliferation of keratinocytes (Fig. 1G). Expression of involucrin in keratinized epidermis cells was observed throughout the epidermal layer of lesions, but involucrin expression was decreased in astilbin-treated mice and limited to the top layer (Fig. 1H). These results suggest that the administration of astilbin effectively ameliorates IMQ-induced keratinocyte differentiation.

3.2. Astilbin inhibited T cell-mediated inflammatory infiltration in a mouse model of psoriasis

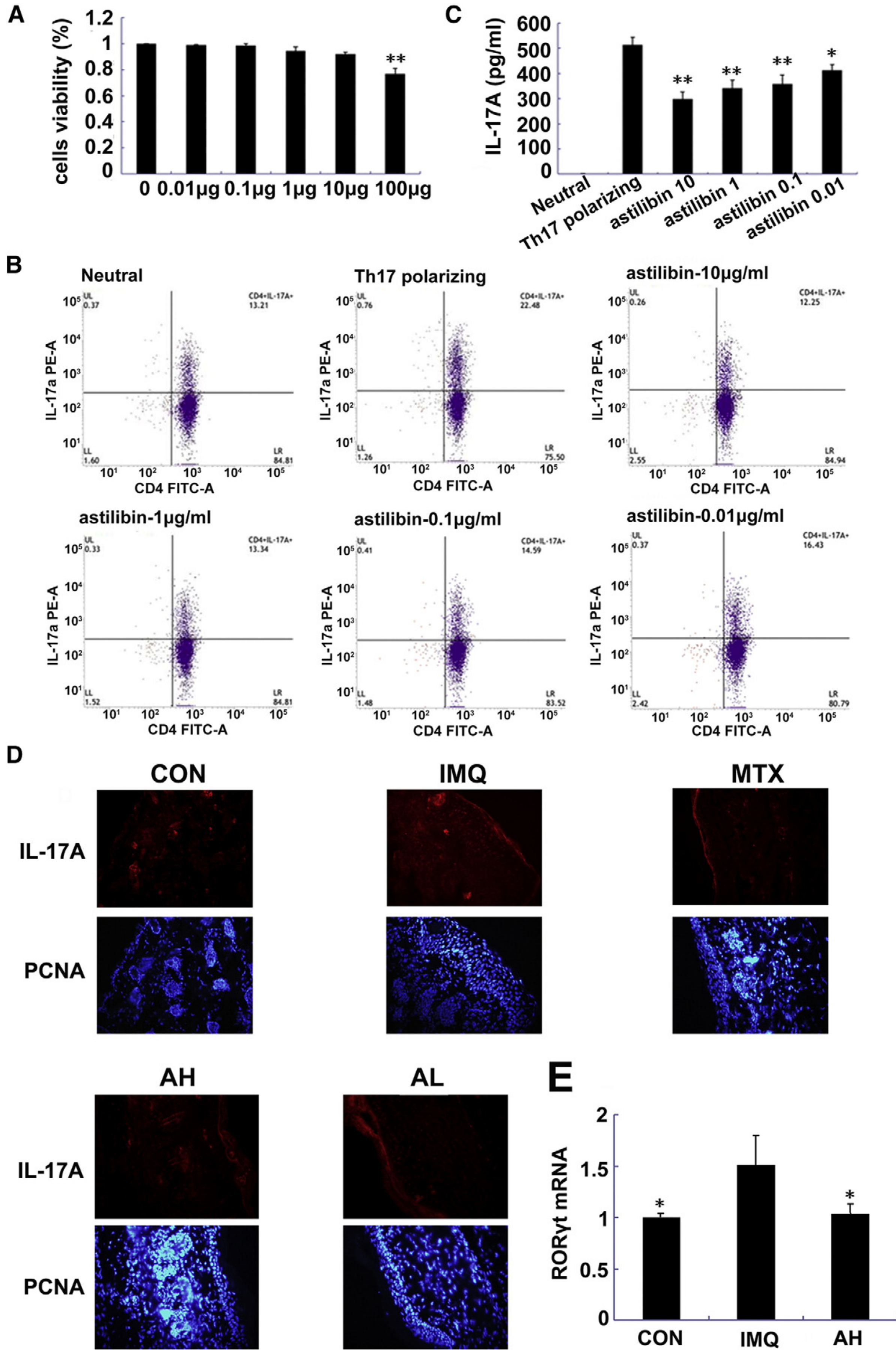
We then investigated the impact of astilbin on inflammatory infiltration. Infiltration of CD3+ cells was observed in IMQ-induced lesions, but reduced in astilbin-treated mice (Fig. 2A). The inguinal lymph nodes and spleen in IMQ-treated mice were enlarged, and absolute numbers of CD4+ and CD8+ T cells were elevated in inguinal lymph nodes and the spleen. The weight of lymph nodes and spleen were reduced in astilbin-treated mice, and the dysregulation of CD4/CD8 cell in both tissues was ameliorated (Fig. 2B).

IMQ up-regulated the levels of psoriasis related cytokines in the peripheral blood of mice, indicating systemic immunity activation. The level of circulating IL-17A was increased by over 25 fold, IL-10 by 15 fold, IL-4 by 9 fold, IL-2 by 8 fold, IL-6 and IFN-γ by 6 fold, and TNF-α by 3 fold. In astilbin-treated mice levels of IL-17A, IL-6, TNF-α, IFN-γ, IL-2 and IL-4 in peripheral blood were significantly decreased ($p < 0.05$). Interestingly, IL-10 levels were also reduced in astilbin-treated mice (Fig. 2C).

3.3. Astilbin inhibited Th17 cell differentiation in vitro and IL-17A secretion in a mouse model of psoriasis

To investigate how astilbin influences Th17 cell function, we first investigated whether astilbin affected the viability of isolated, Th17 cells *in vitro* using the CCK8 assay. While 0.1–10 μg/ml astilbin did not affect Th17 cell viability, at concentrations over 100 μg/ml Th17 viability declined, thus in subsequent experiments we use the dose range of 0.1–10 μg/ml (Fig. 3A).

To investigate the effect of astilbin on Th17 cell differentiation, we measured the fraction of IL-17A+ cells and IL-17A secretion before and after incubation with astilbin by flow cytometry and bead-based immunoassay, respectively. Astilbin reduced Th17 cell differentiation, and IL-17A secretion in a dose-dependent manner (Fig. 3B and C).


A
cells viability (%)
1.2
1
0.8
0.6
0.4
0.2
0
**
0
0.01μg
0.1μg
1μg
10μg
100μg
C
IL-17A (pg/ml)
600
500
400
300
200
100
0
**
**
**
*
Neutral
Th17 polarizing
astilibin 10
astilibin 1
astilibin 0.1
astilibin 0.01
B
Neutral
Th17 polarizing
astilibin-10μg/ml
astilibin-1μg/ml
astilibin-0.1μg/ml
astilibin-0.01μg/ml
IL-17a PE-A
CD4 FITC-A
D
CON
IMQ
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2
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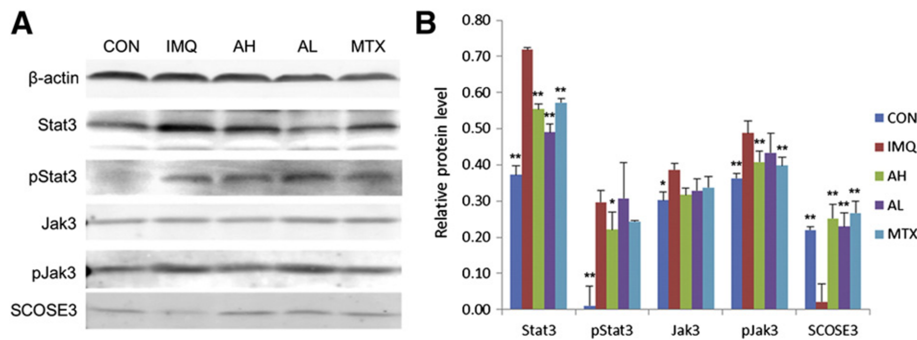

Fig. 4. Astilbin inhibited activation of Stat3 signaling pathway. The levels of Stat3 and phosphorylated Stat3, Jak3 and phosphorylated Jak3 reduced in astilbin treated mice were assessed by Western blotting. The expression of SCOSE3 was increased in the treatment group mice compared with the model mice. A, Immunoblot showing the bands detected for various proteins. B, quantitation of A, after normalization with β-actin. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs IMQ group.

In model mice, IL-17A was mainly observed in the epidermal keratinocytes and dermal layer, but in astilbin-treated mice infiltration of IL-17A + cells into skin lesions was reduced (Fig. 3D) and expression of Th17 specific transcription factor RORγt mRNA was reduced (Fig. 3E). These results suggest that astilbin could directly inhibit the differentiation and function of Th17 cells.

3.4. Stat3 signaling mediated the inhibitory effects of astilbin in a mouse model of psoriasis

Stat3 has emerged as an important regulator of keratinocytes and Th17 cell differentiation [17,18]. To investigate whether astilbin influences Th17 cells by inhibiting Stat3 signaling in psoriasis, in IMQ-induced skin lesions, we observed increased phosphorylation of Stat3 and jak3 by Western blotting. The levels of both the phosphorylated and non-phosphorylated forms of these proteins decreased in high dose astilbin treated mice. Interestingly, expression of SCOSE3 was increased in astilbin-treated mice (Fig. 4). These results suggest that astilbin might inhibit Th17 cells activity by a molecular mechanism involving inhibition or interruption of the Stat3 signaling pathway.

4. Discussion

In this study we investigated the effect of astilbin in a mouse model of psoriasis. We established IMQ-induced psoriasis-like skin lesions in BALB/c mice, and found that astilbin significantly ameliorated keratinocyte proliferation and inflammatory infiltration and the gross, histological, cellular and molecular changes. Astilbin reduced infiltration of CD3 + cells to psoriatic lesions and ameliorated elevations in circulating CD4 and CD8 + T cells and inflammatory cytokines. Astilbin at 50 mg/kg generally conferred slightly higher benefits compared with the low dose of 25 mg/kg. Based on these data, 50 mg/kg astilbin would be more suitable for psoriasis. This dose is similar to that previously used in a DSS induced colitis model [16]. However, the optimal dose should be determined in further studies. *In vitro*, astilbin inhibited Th17 cell differentiation, IL-17 secretion, and Jak/Stat3 signaling in Th17 cells, and up-regulated SCOSE3 expression in psoriatic lesions. Thus, astilbin represents and interesting candidate drug to antagonize local inflammation in psoriasis by intervening in Th17 cell function.

Previous studies reported that astilbin down-regulates T cell activity [15], inducing apoptosis, stimulating negative regulatory cytokine expression, suppressing activated T cell adhesion and migration [11]. In agreement, we found that astilbin reduced the numbers of T cells in skin lesions, and decreased CD4 + and CD8 + T cell numbers in inguinal lymph nodes and spleen specimens. The changes in CD8 + T cells were less obvious, when relative amounts of cells (%) were assessed. Indeed, lymph nodes are composed predominantly of T lymphocytes, while the spleen also contains large numbers of B cells, DCs, and macrophages, so any changes in T cell composition are less easily detected.

Repeated topical application of IMQ, a Toll-like receptor 7/8 ligand and potent immune activator, induces and exacerbates inflammatory skin lesions, and has been widely used to model psoriasis skin inflammation [21]. This model recapitulates hallmarks of human psoriasis, including erythema, hyperkeratosis, scaling, neutrophil micro-abscesses in the epidermis and T cell infiltration in the dermis. Although Th1 T cells are the predominant cell type in psoriasis, Th17 cells are also implicated in the pathogenesis of plaque psoriasis [23]. A greater proportion of plasmacytoid dendritic cells (pDCs), DCs and T cells were detected in the spleen of IMQ-treated mice, and while CD4 + IL-17 + T cells were also up-regulated, the number of CD4 + IFN-γ + T cells did not change significantly. As shown above, astilbin attenuated IL-17A expression both in skin lesions and the peripheral blood. Astilbin directly decreased the percentage of CD4 + IL-17A + cells and inhibited IL-17A expression, suggesting that astilbin not only inhibits Th17 cell differentiation but also inhibits Th17 cell function, further suppressing Th17-associated inflammation. It should be noted that cytotoxic T cells (CD8 +) also produce IL-17 [24]; since CD8 + cell amounts were also altered in spleen and lymph node specimens from astilbin treated mice, further studies should assess the specific impact of astilbin on IL-17 levels in cytotoxic T cells.

The proinflammatory cytokine TNF-α is secreted by Th17 cells and other inflammatory cells, and is considered an important target for psoriasis treatment [25]. In this model, astilbin reduced TNF-α expression, and furthermore, reduced expression of Th1 related cytokine IFN-γ. In addition, astilbin ameliorated IMQ-induced IL-10 expression. We hypothesize that as increased IL-4 expression protects Th1/Th2 balance, IL-10 expression accompanies increased proinflammatory cytokine secretion, thus acting to suppress inflammation and maintain Th17/Treg balance in this mouse model of psoriasis. As astilbin inhibits IL-17, IL-10 expression is also reduced. These observations may, however, be model-specific, as they were not reported by other groups [25].

Fig. 3. Astilbin inhibited Th17 cell differentiation and function. Viability of astilbin-treated CD4 + T cells was assessed by CCK8 assay (A). IL-17A expression of astilibin-treated CD4 + T cells was analyzed with by cytometry (B). The concentration of IL-17A in supernatants measured by ELISA (C). Representative photographs of immunofluorescence of IL-17A-positive cells in IMQ-induced mice (D). RORγt mRNA expression in IMQ-induced skin lesions (E). Flow cytometric and immunofluorescence results are representative of three independent experiments. DMSO control groups were represented by the 0 group (A) or Th17 polarizing group (B and C). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with CD4 + T cells without astilbin treating, polarizing Th17 cells or IMQ-induced mice. Red = IL-17A, Blue = DAPI. Scale bar = 50 μm.

However, our data corroborate previous findings that astilbin exhibits overt inhibitory effects on LPS-mediated gene expression of IL-10, among other inflammation mediators [26].

Numerous studies have shown that Stat3 signaling is up-regulated in both psoriasis and IMQ-induced psoriasis-like lesions [27]. Jak1 and 2 have been implicated in keratinocyte proliferation, activation and apoptosis, while Jak3 is predominantly involved in T cell activation and proliferation [28]. SCOSE3 is a specific negative regulator of Stat3 produced by phosphorylation, and RORγt a downstream effector of the Stat3 pathway involved in Th17 cell activation. We found that astilbin inhibited phosphorylation of both Stat3 and Jak3, and reduced RORγt expression, revealing that astilbin may suppress Th17 through the Jak3/stat3 pathway. In addition, astilbin up-regulated SCOSE3 expression, suggesting that astilbin directly affects multiple processes involved in Stat3 signaling in IMQ-induced psoriasis-like inflammation. Astilbin's use in targeting IL-17A for psoriasis treatment has many advantages: Astilbin has relatively low costs; in addition, not only it regulates IL-17A secretion effectively, astilbin also maintains proper balance of T cell differentiation. Additional research is required to determine the precise molecular pathways altered by astilbin, and assess whether the immune processes underpinning the role of astilbin in IMQ-induced psoriasis are relevant to psoriasis in humans.

In conclusion, we demonstrated that astilbin alleviates IMQ-induced psoriasis-like inflammation by reducing inflammatory cell differentiation and cytokine secretion, in particular directly inhibiting differentiation of Th17 cells and secretion of IL-17A. These findings indicate new avenues for the treatment of inflammatory diseases of the skin, and suggest that astilbin could serve as a candidate drug for inhibition of local inflammation in psoriasis.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 81441098 and No. 81573974), independent-selected topic project of Beijing University of Chinese Medicine for graduate students (2015-JYB-XS015), and Beijing Municipal Science & Technology Commission (No. Z141100002214015).

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