Int J Clin Exp Med 2016;9(2):1639-1647
www.ijcem.com /ISSN:1940-5901/IJCEM0016826

Original Article

Arctigenin attenuates imiquimod-induced psoriasis-like skin lesions via down-regulating keratin17

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Received September 24, 2015; Accepted December 17, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Arctigenin is a phenylpropanoid dibenzylbutyrolactone lignin isolated from Arctium lappa L. It has been demonstrated to have antioxidant, antitumor, anti-inflammatory activities. Keratin17 (K17), an ectopically expressed keratin in psoriatic lesions, plays a critical role in the pathogenesis of psoriasis. In the present study, we optimized the skin-penetrating gel form of arctigenin and applied it on the imiquimod (IMQ)-induced psoriasis-like ear skin in mice. After treatment with arctigenin, IMQ-induced skin incrassation was effectively attenuated accompanying with the reduced weight of spleen. The afflicted skin tissues in the arctigenin-treated group displayed lower K17 expression level and higher AMP-activated protein kinase (AMPK) phosphorylation level compared with those in the model group. In vitro experiments further supported that arctigenin not only delayed keratinocyte proliferation arresting at G2/M cell cycle phase but also induced cell apoptosis. Therefore, arctigenin may be used as a promising drug for psoriasis treatment.

Keywords: Arctigenin, psoriasis, keratin17

Introduction

Psoriasis is a chronic T-cell-mediated inflammatory autoimmune skin disease affecting approximately 2-3% of the worldwide population [1]. The skin changes is characterized with hyperproliferation and aberrant differentiation of keratinocytes (KC), infiltrated with various types of immune cells like Th1, Th17 and $CD4^+$ T cells, and cytokines such as IFN-γ, IL-17A, TNFα and IL-22 [2]. Therefore, the abundant pro-inflammatory cytokines and KC cell proliferations constitute a vicious cycle. Keratin 17 (K17), one of well-known fibrous structural protein family member is a key factor contributing to this inflammation-hyperproliferation feedback loop [3-5]. In psoriasis, the typical keratin expression changes includes reduction of K1 and K10, in contrast with the increase of hyperproliferation-associated keratins K6, K16 and K17 [6, 7]. It was reported that IFN-γ, IL-17A, TNFα and IL-22 could up-regulate K17 expression by activating the signal transducer and activator of transcription (STAT) [8, 9]. Moreover, K17 itself can act as an immunogenic antigen to stimulate immune cell response. Thus, K17 is clearly a major link between psoriasis-associated cytokines and psoriatic phenotypes.

Imiquimod (IMQ) is an innate immune response agent useful for the removal of virus infection in the skin. Application of IMQ on normal mice skin resulted in the influx of various immune cells, as well as hyperplasia of the epidermis. Therefore, IMQ-induced psoriasis-like mice model may provide an ideal tool for the investigation of the pathogenesis and therapeutic reagents of psoriasis [10, 11]. This study was designed to observe the effects of arctigenin gel on an IMQ-induced psoriasis-like skin mouse model.

Arctigenin, a phenylpropanoid dibenzylbutyrolactone lignin, is one of the compounds isolated from Arctium lappa L. It has various known bio-

logical activities, including antioxidant, anti-HIV, and neuroprotection [12, 13]. Arctigenin inhibits the activation of MAP kinases, including p38 kinase, ERK1/2, and c-Jun N-terminal kinase (JNK) through inhibiting MKK and AP-1 activation [14], then depriving TNF-α production [15]. In addition, arctigenin can alleviate immune response in animal models of acute and allergic inflammations via both B-cell and T-cell inhibition [16]. However, it was challenging to answer whether arctigenin can be effectively applied in the treatment of chronic inflammatory disease, such as psoriasis, if so, what's the underlying mechanisms are about.

Materials and methods

Reagents

Arctigenin were purchased from Mingxing Pharmaceutical Company (Sichuan, China). Halometasone cream and calcipotriol were obtained from Bright Future Pharmaceutical Laboratories Limited (Hongkong, China). Anti-AMPK antibody (cell signling technology, USA), anti-cytokeratin 17 antibody (Abcam, Cambridge, UK), TNFα (Abingdon, UK), and azone (Shanghai, China) were used. The TPA (Sigma-Aldrich) was dissolved in absolute ethanol to a concentration of 1 mM for the stock. TRIzol reagent (Invitrogen), primescript RT reagent kit, and SYBR premix Ex Taq™ II (Takara) were used to conduct real-time PCR. The imiquimod cream was purchased from 3M pharmaceuticals (Shanghai, China).

Drug preparation

Arctigenin 0.8 g, triethanolamine 0.25 g, carbomer 0.25 g, azone 3 g, ethanol (95%) 15 g, and distilled water q.s. to 100 g. carbomer powder was added to the distilled water while stirring. Azone was added to the ethanol solution containing arctigenin, and the resulting mixture was stirred continuously until the gel formed. Triethanolamine was incorporated into the gel formulation by solubilization in the mix ingredients. This gel formulation of arctigenin was used in the animal experiments.

Animal experiments

Forty male Balb/c mice between 6 and 8 weeks were purchased from the animal experimental center of Fourth Military Medical University and were randomly assigned in cages to receive different treatments. As previously described [17], the psoriasis-like skin mouse model was generated by daily topical application of a dose of 22 mg/cm^2 of commercially available 5% imiquimod cream on the right shaved ear for 8 consecutive days. For therapy, eight mice were received 55 mg/cm^2 of arctigenin gel formulation twice a day for eight days. As a control, the other 16 mice were given vehicle, or halometasone cream combined with calcipotriol. All experiments were approved by the Ethical Committee of Animal Experiments of Fourth Military Medical University. At the end of the experiment period, the animals were sacrificed and their shaved skins were removed and processed for subsequent histological examination.

Scoring severity of skin inflammation

To evaluate the severity of the inflammation of the ear skin, an objective scoring system was developed based on the clinical Psoriasis Area and Severity Index (PASI). Erythema, scaling, and thickening were scored independently on a scale from 0 to 4:0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The level of erythema was scored using a scoring table with red taints. The cumulative score (erythema plus scaling plus thickening) served as a measure of the severity of inflammation (scale 0-12).

Cell culture

Human HaCaT keratinocytes (American Type Culture Collection) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 U/ml of streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2.

Quantitative real-time PCR

Total RNA was extracted from lesion biopsies from ear skin using TRIzol reagent. The 2 μg of total RNA was prepared to first-strand synthesis using random primer and M-MLV reverse transcriptase at 37°C for 2 h. Quantitative real-time PCR amplifications were performed with 1 μl of cDNA, 2×SYBR Green I PCR Master Mix, ddH_2O, 100 nmol/L of primers for K17 (forward, 5'-ACCATGCAGGCCTTGGAGA-3'; reverse, 5'-GTCTTCACATCCAGCAGGA-3'), and β-actin as an

internal control. The reaction condition was as follows: 95°C for 3 min, and then amplified by 45 PCR cycles at 95°C for 5 s, 60°C for 30 s and 55°C for 3 s with a single fluorescence measurement. Reactions were performed on Bio-Rad real-time PCR system. Relative expression levels of target genes were analyzed with normalization to β-actin value using the $2^{-\Delta\Delta Ct}$ comparative cycle threshold method.

Western blot analysis

The expression levels of AMPK and K17 in the mouse ear samples were measured by western-blot. The total proteins were resolved with 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride (PVDF) membrane. Primary antibodies used were anti-K17, anti-AMPK, and anti-phosphorate-AMPK, and horseradish peroxidase-conjugated secondary antibodies were used to develop the membrane.

Cell Proliferation

HaCaT cells were plated in 96-well plates at a density of 2×10^4 cells/well and grown for 24 h. Different concentrations of arctigenin were then added to the cells for final concentrations of 10, 50 and 100 μM, while DMSO (solvent) alone was added for the vehicle control. Cells were then grown at 37°C in 5% CO_2 and 95% air for 24 h, 48 h 72 h and 96 h. In order to determine cell proliferation, CellTiter 96 AQueous One Solution Reagent (20 μl/well) was added, and the plates were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. Absorbance was recorded at 490 nm using a 96-well plate reader. All cultures were assayed in triplicate.

Flow-cytometric analysis

HaCaT cells (1×10^6) were incubated for 30 min at 4°C with the FITC-conjugated Annexin V and propidium iodide (PI) (from Immunotech) to identify apoptotic cells and cell cycle. After washing with PBS, the cells were analyzed by FACS (Beckman Coulter, Brea, CA).

Statistical analysis

Significant differences were analyzed using the unpaired, 2-tailed Student's *t*-test. Graph pad Prism software (Cricket Software, Philadelphia, PA) were used for the above analyses and *P* values less than 0.05 were considered significant.

Results

Arctigenin attenuates IMQ-induced incrassation in mouse ear model

Two to four days after the IMQ application, the ear skin of the treated mice began to display signs of erythema, scales and thickness. As shown in **Figure 1**, these clinical changes were significantly attenuated in the arctigenin treatment group. Compared with the model group (2.1 ± 0.28), the erythema score of the ear was significantly decreased in the arctigenin treatment group (0.6 ± 0.07 mm). There was also a significant difference between the scales score of the model group (2.1 ± 0.15) and the arctigenin treatment group (0.5 ± 0.042). A similar inhibitory effect was also observed with respect to the thickness score (**Figure 1D**) and the cumulative score (**Figure 1E**). Further analysis using H&E staining indicated that the increased skin thickening caused by IMQ treatment was obviously reduced by the arctigenin application (**Figure 1F**). However, the skin thickness in the HA/CO treatment group almost completely alleviated.

Arctigenin down-regulated K17 Expression in IMQ-induced psoriasis-like mouse model

It has been known that arctigenin acts as an anti-inflammatory agent. However, it is still unknown whether it can block the vicious feedback loop between keratinocyte proliferation and immune cells infiltration in psoriasis. When local skin tissue was analyzed, it was found that the phosphorylation of AMPK was reduced in the afflicted area compared to the normal part (shown in **Figure 2A**), whereas K17 expression was remarkably up-regulated (shown in **Figure 2B**, $P<0.05$) in the IMQ-treatment group. Application with arctigenin obviously inhibited K17 positive responses, meanwhile AMPK phosphorylation was rescued. These results indicated that AMPK reactivation may be responsible for K17 inhibition via direct or indirect pathways.

Similiar to the altered proteins, the mRNA level of K17 was down-regulated too (shown in **Figure 2C**), suggesting that K17 reduction induced by arctigenin treatment may occur at transcriptional level. Since previous findings suggested that IMQ could induce splenomegaly through systemic effects. Our results support-

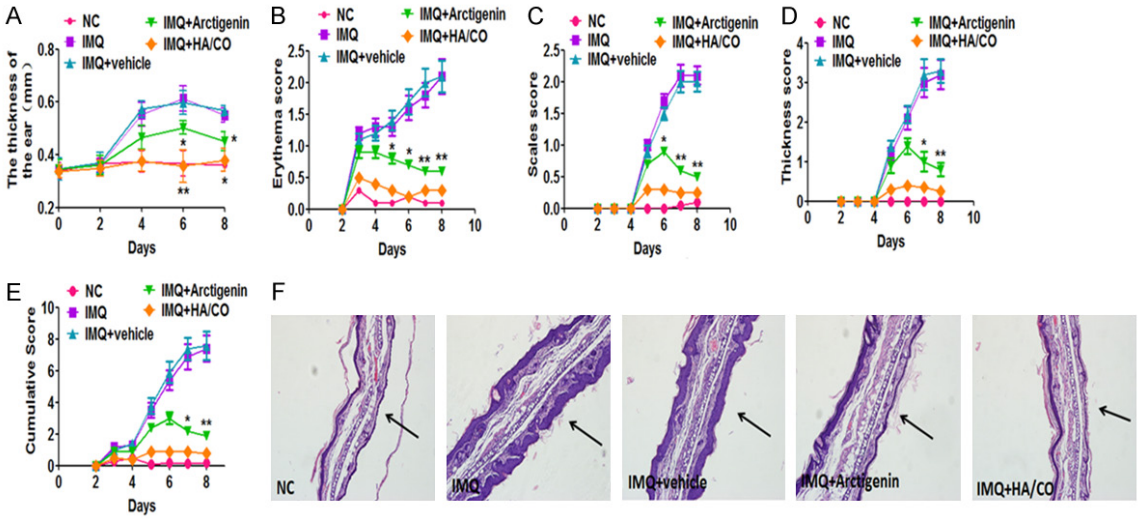

Figure 1. Arctigenin inhibited IMQ-induced psoriasis-like incrassation. (A). The thickness of the right ear skin was measured on the days indicated (data was showed as mean ± SEM). The scores of erythema (B), scales (C), and thickness of the ear skin (D) were evaluated daily on a range from 0 to 4. (E) The cumulative score was the sum of erythema score plus scaling score plus thickness score. Values are the mean ± standard error of the mean of 8 mice per group. (F) H&E staining of the mouse ear skin of different treatment groups (magnification ×200). Arrowheads denote the inside of the ear skin. *P<0.05, **P<0.01 versus IMQ group.

ed that IMQ did increase the spleen cell hyperplasia and weight plus. As shown in **Figure 2D**, after treatment with arctigenin, gain of spleen weight tended to be lessened.

Arctigenin reduced HaCaT keratinocyte proliferation

As above in-vivo experiments suggested, arctigenin may directly affect skin keratinocyte cell proliferation. To verify our hypothesis, in-vitro HaCaT keratinocytes proliferation curve was determined by using the MTT assay. Our data in **Figure 3A** showed that arctigenin significantly inhibited HaCaT keratinocyte proliferation compared with that in the control group after 4-day incubation. Moreover, this inhibition rate increased in a dose-dependent manner.

The inhibition of HaCaT keratinocytes proliferation could result from the induction of apoptosis and/or cell growth arrest. Thus, in order to learn about the cellular processes possibly affected by arctigenin, the effects on cell cycle and apoptosis were investigated by flow cytometry. As shown in **Figure 3B**, the lowest concentration of arctigenin (10 μM) significantly increased the cell apoptosis rate (13.4% ± 1.3%) compared with that in the control group (3.8% ± 0.4%). Then cell cycle analysis results showed that a decreased amount of the cell population in the S phases in the arctigenin group (28.09% ± 3.16%), compared with that in the control group (34.89% ± 4.27%) (shown in **Figure 3C**).

Arctigenin inhibited TNFα, IFNγ-stimulated K17 activation in HaCaT keratinocytes

After the above general cell proliferation test, we set up an inflammation-induced keratinocyte proliferation assay. Stimulation of HaCaT cells with IFNγ (100 U/ml or 300 U/ml) for 24 hours did up-regulate the expression of K17 at both mRNA and protein level (shown in **Figure 4A**). Interestingly, after arctigenin treatment, the level of K17 was significantly suppressed. Similarly, stimulation of HaCaT cells with TNFα (100 ng/ml, 500 ng/ml) for 24 hours also triggered a 2-fold increase in K17 mRNA level compared to that in the control group (shown in **Figure 4B**), while arctigenin addition effectively inhibited TNFα-stimulated K17 activation.

Underlying mechanisms of K17 reductions by argtigenin in HaCaT keratinocytes

To further clarify the mechanism that K17 up-regulation was effectively alleviated by arctigennin, we further tested the phosphorylation of STAT1. As shown in **Figure 5**, our results disclosed that arctigenin significantly inhibited the phosphorylation of STAT1 (P<0.05). Thus presumably, K17 down-regulation maybe mediat-

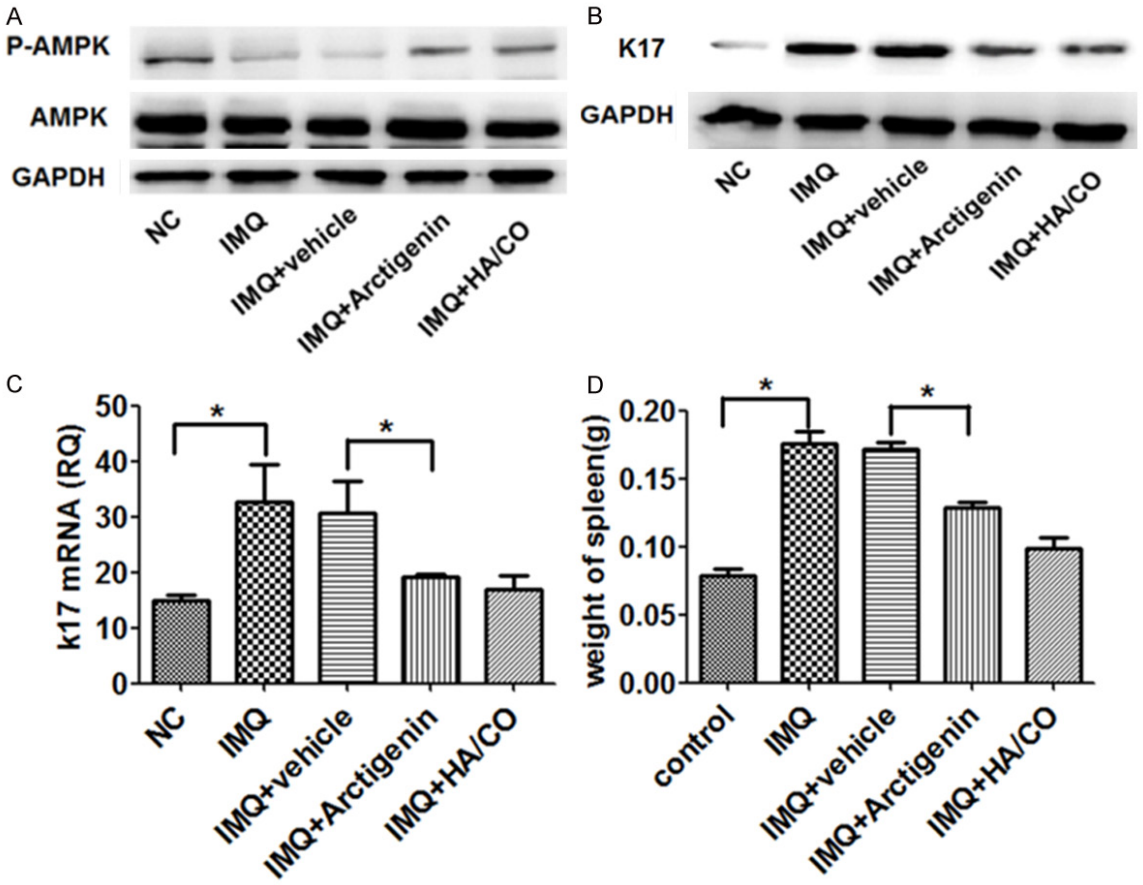

Figure 2. The effects of arctigenin on the expression of AMPK, K17 activation, and spleen weight in the IMQ-induced psoriasis-like mouse model. (A) Phosphor-AMPK expression and K17 expression (B) at the local skin tissues of different treatment groups were detected by western blot. GAPDH served as internal controls. (C) The changes in K17 mRNA between different groups were analyzed by real-time PCR. GAPDH served as internal controls (n=8). (D) The spleen weight at day 8 were measured and data represented as mean ± SEM (n=8). *P<0.05.

ed via the AMPK and STAT1 pathway related signals changes.

Discussion

It was known that psoriasis is a persistent chronic inflammatory skin illness, with features of keratinocyte hyperplasia and immune cell infiltration. Among various known therapy for psoriasis, corticosteroid drugs, as the general immune response blocker, could exert effective treatment response, however relapse and other side effects still perplex this illness therapy. Currently VitA and VitD oinments are also applicable in the treatment, nevertheless, the sustained relapsing natures remained as the main problem irritating the patients and challenging the clinics.

In order to disclose the novel treatment regimen, psoriasis-like skin model was set up. Topical application of IMQ on mice skin leads to the rapid proliferation of plasmacytoid dendritic cells and stimulates keratinocytes to increase cytokine production [18, 19]. These effects in the mouse skin closely resemble human plaque-type psoriasis with respect to ear thickening, erythema, scaling, epidermal alteration (acanthosis, parakeratosis), and so on. Therefore, in many respects, the pathogenesis of IMQ-induced psoriasis-like inflammation was similar with psoriasis. In this study, we employed this model to evaluate the therapeutic effects of arctigenin on psoriasis and explore the underlying mechanism.

Chinese herbs are natural products widely used in clinics, especially in the use of anti-inflammation and autoimmune responses. Arctigenin structurally belongs to be a phenylpropanoid dibenzylbutyrolactone lignin. Both higher hydro-

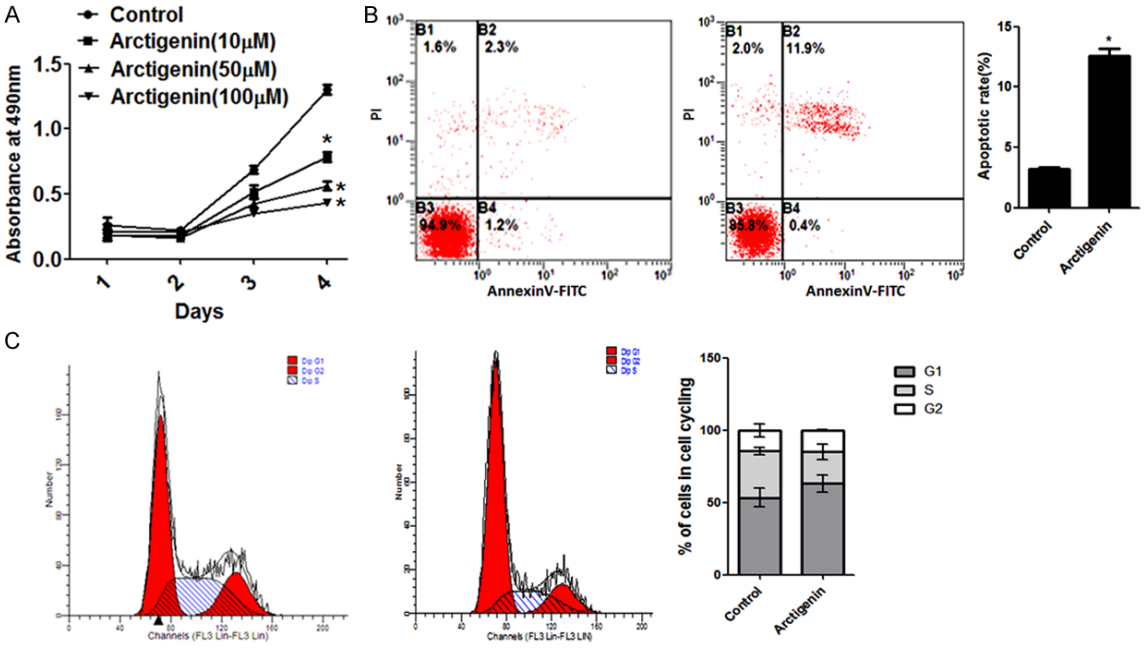

Figure 3. Effects of arctigenin on HaCaT cell proliferation. HaCaT cells were incubated with different concentrations of arctigenin (10, 50, 100 μM) for up to 4 days. A. Cell viability was examined using a MTT-based assay. Data were expressed as the absorbance at 490 nm. Results are the mean ± SD from three independent experiments. *P<0.05 versus vehicle control. B. Apoptotic rates of HaCaT cells were analyzed by FCM. The data presented are representative of three different experiments. C. Cell cycle analysis of HaCaT cell treated with 10 μM arctigenin. The figure shows the DNA content flow cytometric histograms of HaCaT cells and the corresponding percentages of cell cycle distribution after treatment with arctigenin. The pictures are representative of three independent analyses. Results were represented as mean ± SD from three independent experiments. *P<0.05 versus control cells.

phobicity and liver toxicity limited its clinical applications. Skin usage may provide a best choice to avoid its shortcomings. In order to enhance its skin absorption rate, we prepared several skin-penetrating mixture reagents, after testing and comparing percutaneous absorption rate, the 30% ethanol-saline +3% azone group was selected to exhibit the optimal transdermal delivery efficiency (data not shown).

Previous studies had shown that arctigenin exerted depressive effects on both acute and allergic immune responses, via suppressing both T cell and B cell activations. It was still unknown whether it can be effectively applied in psoriasis skin illness treatment. Applying with the arctigenin regimen, we did observe that it showed obvious therapeutic effects on psoriasis skin illness. Gross and histology observations both demonstrated its efficacies. Besides spleen weight and related immune cell related signaling changes, other important molecular biomarkers of KC derived from tissue samples were determined, the results showed that K17 increases were effectively prohibited, as a contrast, AMPK reactivation was rescued, denoting that K17 down-regulation and AMPK reactivation may contribute to the novel mechanisms related with arctigenin therapeutic effects.

As recent findings indicating, keratinocyte and immune cells constituted a feedback loop, T helper cells, such as, Th1, Th17, and IL-22-producing CD4+ T cells, contribute to the pathogenesis of psoriasis through the release of inflammatory cytokines that promote further recruitment of immune cells, keratinocyte proliferation, and sustained inflammation [20]. As many psoriasis-related cytokines, such as IFN-γ, IL-17A, TNFα and IL-22, were able to up-regulate K17 expression [9], K17 itself harboring the similar antigen epitope with Streptococcus may act as one of auto-antigens to trigger positive reactive T cell clones. Thus K17 become an important mediator in this vicious cycle. Therefore, blocking K17 expression may

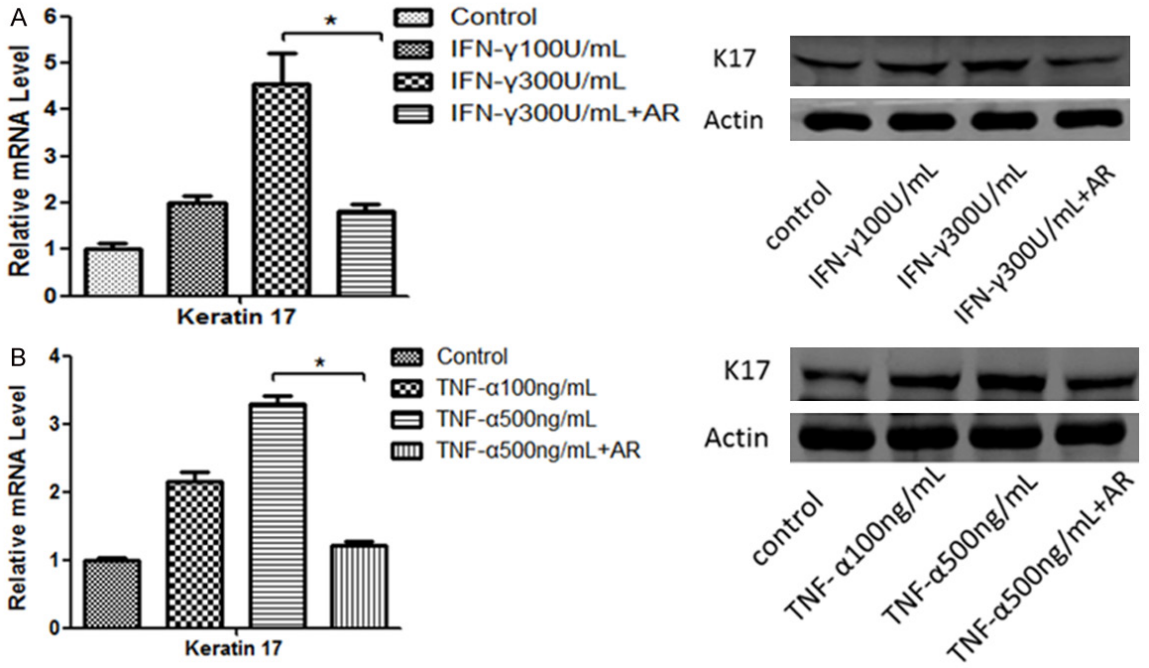

Figure 4. Arctigenin inhibited IFNγ, TNFα-stimulated K17 activation on HaCaT keratinocytes. HaCaT cells were stimulated with IFNγ (A) or TNFα (B) with or without arctigenin (AR) for 24 hours, then the mRNA and protein levels of K17 were analyzed by real-time PCR or western-blot. *P<0.05.

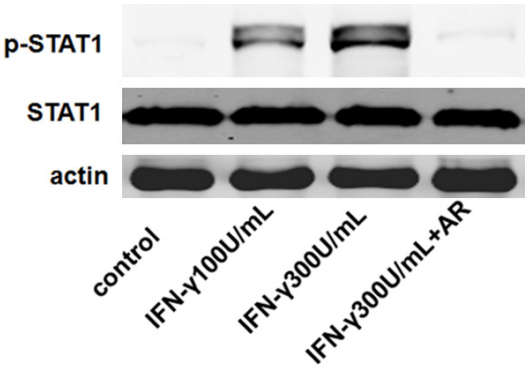

Figure 5. Arctigenin inhibited the activation of STAT1. HaCaT keratinocytes were seeded into 6-well plate at a density of 5×10^5 cells/well and incubated at 37°C in 5% CO_2 for 24 h. Following that, IFN-γ (100 U/ml, 300 U/ml) and arctigennin (10 μM) were added, and incubated at 37°C for another 24 hours. Then proteins were extracted from cells according to the manufacturers' instructions and subjected to western-blot analysis.

become an effective option in psoriasis treatment [21]. Among the topical ointments for psoriasis treatment, both corticosteroids and retinoids were reported to directly repress K17 expression in keratinocytes at the transcriptional level, which is believed to be one of their action mechanisms [22].

In order to define the new machanisms undelying the artigenin treatment, we intended to observe whether it can directly affected keratinocyte cell proliferations and keratin expressions. Firstly, artigenin itself displayed proliferation inhibitory effects, the inhibition was derived by directly arresting the cell cycle at G2/M phase, parallel with shortened S phase, cell death analyses also disclosed that artigenin effectively increased cell apoptosis and necrosis rates. Secondarily, under either TNF-a or IFNγ stimuli in KC cells, K17 up-regulation was obviously prohibited by arctigenin treatment, both at mRNA level and protein levels. As a contrast, AMPK phosphorylation level was shown to be up-regulated, conceivably, AMPK activation could inhibit mTOR and some other pro-inflammation pathways to dampen the K17 expressions. It is well known that STAT1 or JAK1 activation are also both related to the expression of pro-inflammatory cytokines. Thus, the decreased phosphorylation of STAT1 by artigenin treatment may contribute to K17 reductions.

In summary, our results provide substantial evidences disclosing that arctigenin skin formula maybe used for the psoriasis therapy by directly affecting KC and immune cell activation and

interaction, particularly related with K17 and other inflammation signals.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Grant Numbers 31270843, 81030046, 30871423) and the Innovation of Science and Technology project in Shaanxi province (Grant Number 2015KTCL03-13).

Disclosure of conflict of interest

None.

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