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Jaceosidin inhibits contact hypersensitivity in mice via down-regulating IFN-γ/STAT1/T-bet signaling in T cells

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ABSTRACT

In the present study, we aimed to investigate the immunosuppressive activity of jaceosidin, a flavone isolated from *Artemisia vestita*, on T lymphocytes both *in vitro* and *in vivo*, and further explore its potential molecular mechanism. Jaceosidin exerted a significant inhibition on the T cell proliferation and activation induced by concanavalin A (Con A) in a concentration-dependent manner and it also inhibited the secretion of the proinflammatory cytokines such as IL-2, TNF-α and IFN-γ of activated T cells. Further study showed that jaceosidin down-regulated STAT1 activation and T-bet expression in activated T cells. Moreover, in order to investigate the immunosuppressive effect of jaceosidin *in vivo*, the picryl chloride (PCl)-induced ear contact dermatitis model was performed on BALB/c mice. Jaceosidin significantly ameliorated PCl-induced ear swelling in a dose-dependent manner, which was due to its inhibition of the STAT1/T-bet signaling pathway. In summary, these findings suggest that jaceosidin exerts its immunosuppressive effect both *in vitro* and *in vivo* through inhibiting T cell proliferation and activation, which is closely associated with its potent down-regulation of the IFN-γ/STAT1/T-bet signaling pathway.

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1. Introduction

Delayed-type hypersensitivity (DTH) is a classic T cell-mediated immune reaction and plays an essential role in the pathogenesis of various inflammatory disorders (Kobayashi et al., 2001). Cytokine-induced signal transducer and activator of transcription factor (STAT) signaling plays a critical role in the activation of T cell proliferation and activation. For example, IFN-γ/STAT1 had been proved essential for $CD4^+$ T cell activation in Con A-induced liver injury model, because such activation was markedly attenuated in IFN-$\gamma^{-/-}$ or $STAT1^{-/-}$ mice (Hong et al., 2002; Siebler et al., 2003a; Tagawa et al., 1998). The transcription factor T-bet has been found to be a key regulator of the IFN-γ gene in Th1 cells, and T-$bet^{-/-}$ mice have impaired Th1 cell development and fail to generate Th1 response *in vivo* (Afkarian et al., 2002; Finotto et al., 2002; Lovett-Racke et al., 2004). Many immune-related diseases, such as multiple sclerosis, rheumatoid arthritis, contact hypersensitivity and transplantation, have been known to involve the DTH mechanism (Kobayashi et al., 2001). These diseases are usually treated by immunosuppressants, which show a strong anti-DTH activity (Allison, 2000). However, these immunosuppressive agents including glucocorticoids, cyclophosphamide, and even cyclosporin A, usually have severe side effects because of their nonselective targeting to the process of immune response and to the cell populations involved (Sitzia and Huggins, 1998; Williams and Coleman, 1995; Brinker et al., 2007). These drugs may alleviate the symptom of the disease, but they also may cause some side effects such as the damage of the immunity system and the increase of the incidence of infection. So the future tendency will be the search for the high effective immunosuppressants with low toxicity.

In our previous studies, we had reported that the extracts of *Artemisia vestita* could alleviate picryl chloride (PCl)-induced contact hypersensitivity through blocking the activation of T lymphocytes (Wang et al., 2005). To our interest, the extract of *A. vestita* exerted immunosuppressive activity without affecting the survival of naïve T lymphocytes. Moreover, the weight index of immune organs such as spleen and thymus from mice was hardly changed after the treatment of this extract. This character is distinct from glucocorticoids such as dexamethasone with inducing thymus atrophy. Under the bio-activity guided isolation, a series of flavones were isolated and identified from *A. vestita* and jaceosidin was one among them (Yin et al., 2008). The anti-inflammatory effect of jaceosidin has been previously reported, it can inhibit the passive cutaneous anaphylaxis reaction induced by IgE *in vivo* and down-regulate the NF-κB signaling pathway *in vitro* (Lee et al., 2007). In addition, previous studies have demonstrated that jaceosidin has anti-tumor (Lee et al., 2005; Lv et al., 2008) and antioxidant activity (Kim et al., 2008). However, the exact mechanism of anti-DTH activity of jaceosidin remains unclear. In this study, we showed that jaceosidin inhibited antigen-specific immune response *in vivo* and *in vitro*, which demonstrated jaceosidin as one of the major

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immunosuppressive components of *A. vestita*, and its anti-DTH activity was proved to be associated with its inhibition of IFN-γ/STAT1/T-bet signaling in T cells.

2. Materials and methods

2.1. Animals

Female BALB/c mice (specific pathogen free), aged 6–8 weeks (18–22 g) were obtained from the Laboratory Animal Center of Shanghai (Shanghai, China). They were maintained with free access to pellet food (Jiangsu Cooperation Medical & Pharmaceutical Company, Nanjing, China) and water in plastic cages at 21 ± 2 °C and kept on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animal's suffering and to reduce the number of animals used.

2.2. Reagents

Mouse $CD3^+$ T cells from lymph nodes were purified by using a mouse T cell Enrichment Column (R&D systems, Minneapolis, MN) with more than 95% purity; picryl chloride (PCl, Nacalai tesque Inc, Kyoto, Japan); 96-well culture plates (Nunclon); ELISA kits for murine IL-2, TNF-α and IFN-γ (R&D systems, Minneapolis, MN); concanavalin A (Con A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St Louis, Mo); cyclosporin A (CsA, Sandoz Ltd, Basel, Switzerland); fetal calf serum (FCS, Maverick, Australia); the phospho-STAT antibody sampler kit was from Cell Signaling (Beverly, Massachusetts); T-bet and anti-α tubulin (TU-02) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); purified anti-mouse CD3 (145-2C11) and purified anti-mouse CD28 (37.51) were from BD PharMingen (San Diego, CA); FITC-anti-mouse CD69 and FITC-anti-mouse CD25 were purchased from Biolegend (San Diego, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.3. Extraction and isolation

The dried aerial part of the *A. vestita* (3 kg) was extracted twice with 75% ethanol by refluence extraction at boiling temperature. The material was extracted with 54 l solvent for 2 h for the first time and then extracted with 36 l solvent for another hour. The extracts were combined and concentrated under reduced pressure to 12 l (named AV-ext) and then AV-ext was suspended in H_2O to 30 l to afford the aqueous solution. The solution was applied on a macroporous adsorption resin HP-20 and eluted following the procedure: water, 30% ethanol, 60% ethanol and 90% ethanol to yield four fractions and evaporate them to dryness under reduced pressure to afford AV1–AV4 (AV1: 30.5 g, AV2: 60.6 g, AV3: 90.6 g, AV4: 33.1 g). Then AV3 was subjected on a silica gel column eluted with CH_2Cl_2–MeOH (100:0 to 30:70) step gradient to give 11 fractions (AV3-1 to AV3-11). AV3-2 was rechromatographed over silica gel (100–200 mesh; Qingdao Oceanic Chemical Plant, China) eluted with CH_2Cl_2–MeOH (99:1) to yield J-02 (jaceosidin, purity >98% by HPLC). The test samples of jaceosidin were 0.2 μm sterile filtered (Millipore, Billerica, MA) and the presence of endotoxin was analyzed using the Limulus assay (kinetic gel-clot assay; ACC Inc). All endotoxin levels were below 0.25 EU/ml in the test samples.

2.4. MTT proliferation assay

Lymph node cell isolated from BALB/c mice were cultured in 96-well plates at a density of 5×10^5 cells/well in RPMI 1640 medium (0.2 ml) and stimulated with 5 μg/ml of concanavalin A (Con A) or anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) in the presence of jaceosidin for 72 h at 37 °C in 5% CO_2/air, then the cell viability was assessed by MTT assay.

2.5. [^{3}H] uptake proliferation assay

Lymph node cell isolated from BALB/c mice were cultured in 96-well plates at a density of 5×10^5 cells/well in RPMI 1640 medium (0.2 ml) and stimulated with Con A (5 μg/ml) in the presence/absence of jaceosidin for 66 h at 37 °C in 5% CO_2/air, then they are incubated with 0.5 μCi/well of [methyl-^{3}H] thymidine (ICN Pharmaceuticals, Costa Mesa, CA) for 6 h before harvesting as previously reported (Wang et al., 2007). The cells are harvested onto filter paper and the uptake was measured as counts per minute (c.p.m.) by a liquid scintillation counter.

2.6. Analysis of CD69 and CD25 cell surface expressions

The expressions of cell surface molecules in T cell cultures were evaluated by flow cytometry. T cells (5×10^5) were stimulated with 5 μg/ml of Con A with the addition of jaceosidin simultaneously. The surface expressions of CD69 and CD25 were assessed after 24 h of culture, respectively. At the end of the culture period, the harvested cells were washed twice with buffer. Cells were stained with specific antibodies for 30 min at 4 °C in the dark. Cells were then washed with buffer to remove the excess stains and analyzed. Samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software.

2.7. PCl-induced contact dermatitis in mice

Female BALB/c mice were sensitized by painting 0.1 ml of 1% PCl in ethanol on the shaved skin of their abdomens. Five days later, they were challenged by painting 30 μl of 1% PCl in olive oil on right ear lobe (Fei et al., 2005). Eighteen hours later, ear thickness of right against left was measured with a digimatic micrometer (0.001 mm, Mitutoyo Co., Tokyo, Japan). The control animals were run parallel with other groups except for i.p. the same volume of water.

2.8. Histologic analysis

Formalin-fixed, paraffin-embedded ear tissue was sectioned at 5 mm in thickness, and the sections were stained with hematoxylin and eosin. Following parameters were assessed: 1: the level of leucocyte infiltration and vascular congestion; 2: the erosion and anabrosis of epidermal cells; and 3: the affection on other side of the ears. The 3 parameters were taken into account and each parameter was given a score from 0 to 4, the total score ranged from a minimum of 0 to a maximum of 12. Final data are the average scores of each animal in the same group, and the higher score means more serious inflammation.

2.9. Preparation of lymph node cells, and purification of T cells

This process was performed as previously described (Fei et al., 2005; Sun et al., 2006). T cells were purified with a mouse T cell enrichment column according to the instruction of the kit and more than 95% lymphocytes were CD3-positive T cells as assessed by flow cytometric analysis.

2.10. Cytokine assays

The levels of IL-2, TNF-α and IFN-γ were measured by a mouse ELISA kit (R&D systems, Minneapolis, MN).

2.11. Western blot analysis

Proteins were extracted in lysis buffer (30 mmol/l Tris, pH 7.5, 150 mmol/l sodium chloride, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.12. Statistical analysis

Data are expressed as mean ± S.E.M. of three independent experiments and each experiment includes triplicate sets *in vitro* and of eight or nine animals of per group *in vivo*. Statistically evaluated by Student's *t*-test was performed when only two value sets were compared, and one-way ANOVA followed by Dunnett's test when the data involved three or more groups. Data was considered significant if the *P* value was $P<0.05$.

3. Result

3.1. Effect of jaceosidin on T cell proliferation induced by Con A

Jaceosidin was subjected to HPLC analysis, and the purity was confirmed to be more than 98% (Fig. 1A). The activity of jaceosidin was evaluated on lymphocyte proliferation induced by T cell mitogen Con A *in vitro*. As shown in Fig. 1B, jaceosidin inhibited T lymphocyte proliferation induced by Con A in a dose-dependent manner. A similar result was observed in [^{3}H]-thymidine uptake assay (Fig. 1C), and the IC50 of jaceosidin is 2.22 μM. The positive control cyclosporine A (CsA) also remarkably inhibited T cell proliferation as expected. In addition, jaceosidin dose-dependently inhibited T cell proliferation induced by T cell receptor activator anti-CD3 plus anti-CD28 (Supplemental Fig. 1). It was important to notice that jaceosidin, at the concentrations mentioned before, did not affect the lymphocyte's viability by MTT uptake assay (Fig. 1D) and by Annexin V/PI binding assay (Supplemental Fig. 2). All these results indicated that the immunosuppressive activity of jaceosidin observed here, at the concentrations up to 10 μM, was not caused by its cytotoxicity.

3.2. Effects of jaceosidin on CD69 and CD25 expressions in activated T cells

CD69 and CD25 induction can be triggered by Con A which acts as a T cell mitogen to interact with the T cell receptor/CD3 complex in T cells. It was shown that CD69 and CD25 cell surface expressions were up-regulated in mouse T cells after 24 h incubation with 5 μg/ml Con A, while jaceosidin mediated a potent inhibitory effect on CD69 and CD25 expressions in Con A-treated mouse T cells in a dose-dependent manner (1–10 μM) (Fig. 2).

3.3. Effect of jaceosidin on the production of proinflammatory cytokines in activated T cells

To examine the effect of jaceosidin on the production of proinflammatory cytokines such as IL-2, IFN-γ and TNF-α, ELISA was performed to measure the level of the cytokines in the culture supernatant of activated T cells. Our results showed that the stimulation of mouse T cells with Con A resulted in the considerable production and secretion of IL-2, IFN-γ and TNF-α into the culture medium. Significant reduction of these proinflammatory cytokines was found in activated T cells with the treatment of 1–10 μM jaceosidin (Fig. 3). The inhibitory ratio of jaceosidin (3, 10 μM) on these cytokines is almost equivalent to positive control CsA (1 μM).

3.4. Effect of jaceosidin on the activation of IFN-γ/STAT1 signaling pathway in T cells activated by anti-CD3 plus anti-CD28 in vitro

To further delineate the molecular mechanisms following T cell receptor activation in T cells, we explored the possible role of jaceosidin on IFN-γ/STAT1 signaling pathway in T cell activation. The result showed that a very low level of phosphorylation of STAT1 could be detected before T cell activation and the level of pSTAT1 significantly increased after the stimulation of anti-CD3 plus anti-CD28. Jaceosidin exerted a dose-dependent inhibition of T cell receptor-mediated phosphorylation of STAT1, but hardly influenced the total STAT1 expression (Fig. 4A). The expression of T-bet was also suppressed by jaceosidin in a dose-dependent manner. In addition, the production of IFN-γ activated by anti-CD3 plus anti-CD28 was significantly inhibited by jaceosidin (Fig. 4B). Thus, jaceosidin appears to be able to interfere with the activation of IFN-γ/STAT1 signaling during T cell activation.

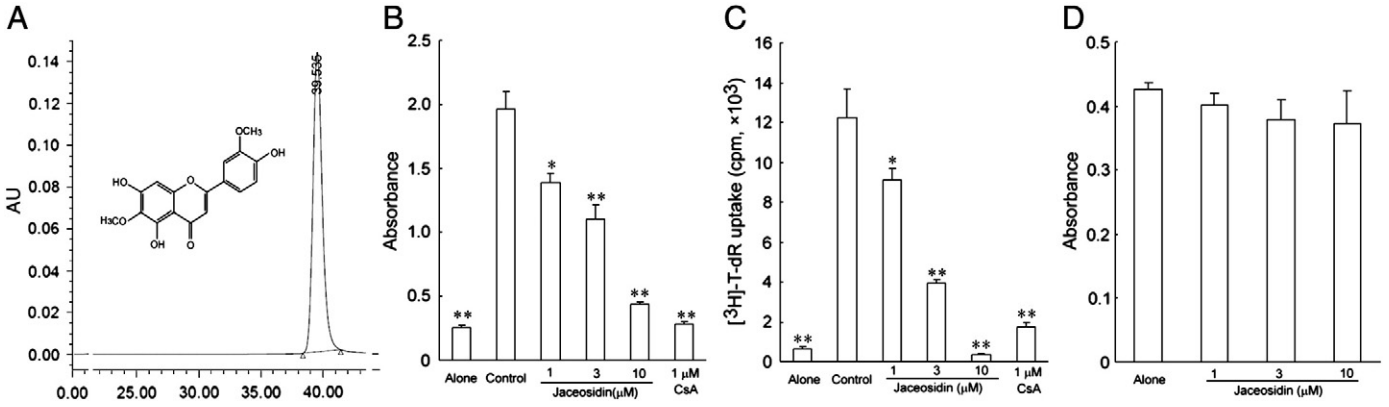

Fig. 1. Effect of jaceosidin on T lymphocyte proliferation induced by Con A. (A) HPLC analysis of jaceosidin and its chemical structure. (B, C) Lymph node cells (5×10^5) were incubated for 72 h at 37 °C and 5% CO_2 in the presence of 5 μg/ml Con A and 1, 3,and 10 μM jaceosidin. Cell proliferation was measured at 540 nm by MTT uptake (B) and [^{3}H]-thymidine uptake assay (C). $^*P<0.05$, $^{**}P<0.01$ versus Control group. (D) Cytotoxicity of jaceosidin on resting lymphocytes from normal mice. Spleen cells (5×10^5) were incubated for 72 h at 37 °C and 5% CO_2 in the presence of 1, 3, and 10 μM jaceosidin. Cell viability was measured at 540 nm by MTT uptake. Data were mean ± S.E.M. of three independent experiments.

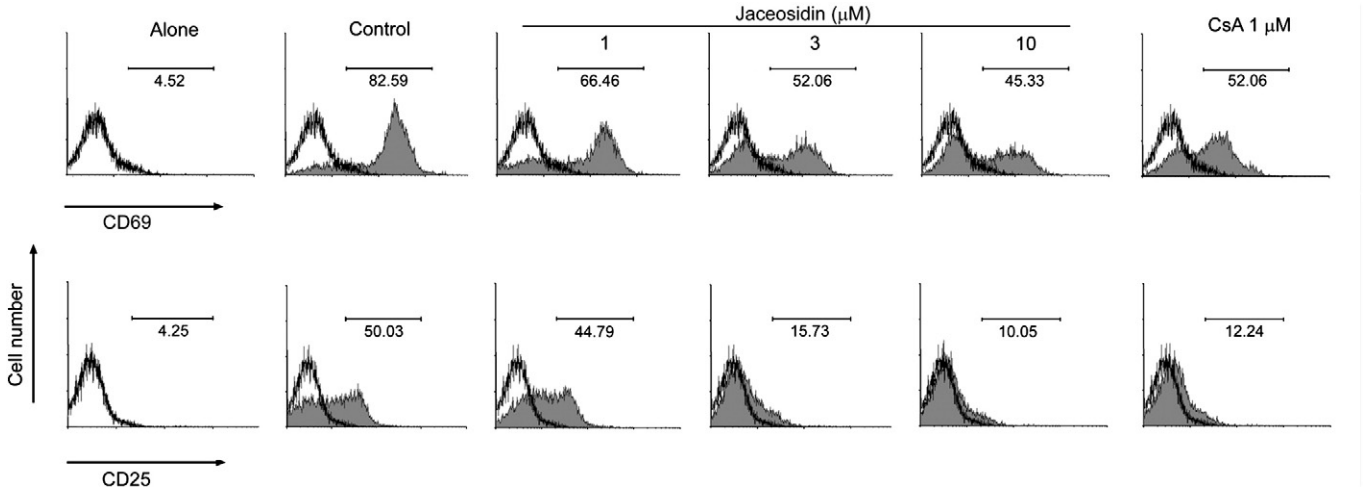

Fig. 2. Effect of jaceosidin on expressions of CD69 and CD25 in T cells. Lymph node cells were prepared from naive mice and T cells were purified using commercial enrichment columns. T cells were stimulated with 5 μg/ml Con A in the presence of 1, 3, and 10 μM jaceosidin for 24 h, then CD69 and CD25 expressions were measured by FACS. Representative histogram of three different experiments was shown.

3.5. Effect of jaceosidin on PCl-induced contact dermatitis in mice

To further assess the immunosuppressive property of jaceosidin *in vivo*, we used the PCl-induced contact dermatitis in BALB/c mice. The *in vivo* dose of jaceosidin (3, 10, and 30 mg/kg) was selected based on our previous report (Sun et al., 2010). Administration for 6 days after the sensitization, jaceosidin significantly inhibited the ear swelling in a dose-dependent manner, and the positive control CsA also showed a strong inhibition (Fig. 5A). Fig. 5B was a representative photo of H&E staining for ear tissues from various groups of mice. The histopathological changes in the ear were mainly observed in the dermis as severe inflammatory infiltration, vascular congestion, and moderate edema in the control group. Compared with the animals in the control group, the mice treated with jaceosidin only showed a mild cellular infiltration and vasodilatation without obvious edema (Fig. 5B,C). Furthermore, the expressions of phosphorylated STAT1 and T-bet were significantly reduced in T cells from draining lymph node of jaceosidin-treated mice (Fig. 5D), indicating that jaceosidin exerts its immunosuppressive activity *in vivo* in the same manner as it works *in vitro*.

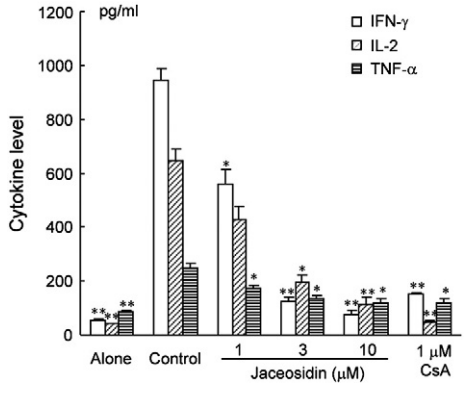

Fig. 3. Effect of jaceosidin on the cytokines production of Con A-activated T cells. Lymph node cells were prepared from naive mice and T cells were purified using commercial enrichment columns. T cells were stimulated with 5 μg/ml Con A in the presence of 1, 3, and 10 μM jaceosidin for 24 h, then the productions of IL-2, IFN-γ and TNF-α were determined by ELISA. Data were mean ± S.E.M. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus control.

3.6. Effect of jaceosidin on the proliferation, cytokine production and STAT1 activation of presensitized T cells induced by TNBS in vitro

The lymph node cells from PCl-sensitized mice were obviously activated and proliferated by TNBS (the liquor form of PCl) *in vitro* (Fig. 6A). Against this, jaceosidin significantly inhibited antigen-specific T cell proliferation (Fig. 6A) and IFN-γ production in a dose-dependent manner (Fig. 6B). STAT1 and T-bet are the key transcription factors of IFN-γ. As a result, jaceosidin dose-dependently inhibited the expressions of phosphorylated STAT1 and T-bet (Fig. 6C).

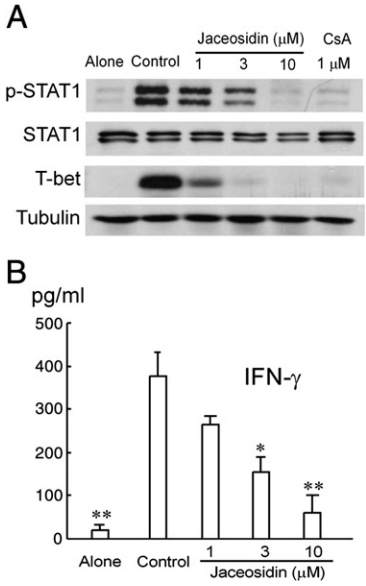

Fig. 4. Effect of jaceosidin on the IFN-γ/STAT1 signaling in T cells activated by anti-CD3 plus anti-CD28 *in vitro*. Lymph node cells were prepared from naive mice and T cells were purified using commercial enrichment columns. (A) T cells were incubated with 1 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 in the presence of jaceosidin for 1 h. Cells were then harvested, lysed and assayed by Western blot. One of three different experiments is shown here. (B) T cells were stimulated with 1 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 in the presence of jaceosidin for 24 h, then the production of IFN-γ was determined by ELISA. Data were mean ± S.E.M. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus control.

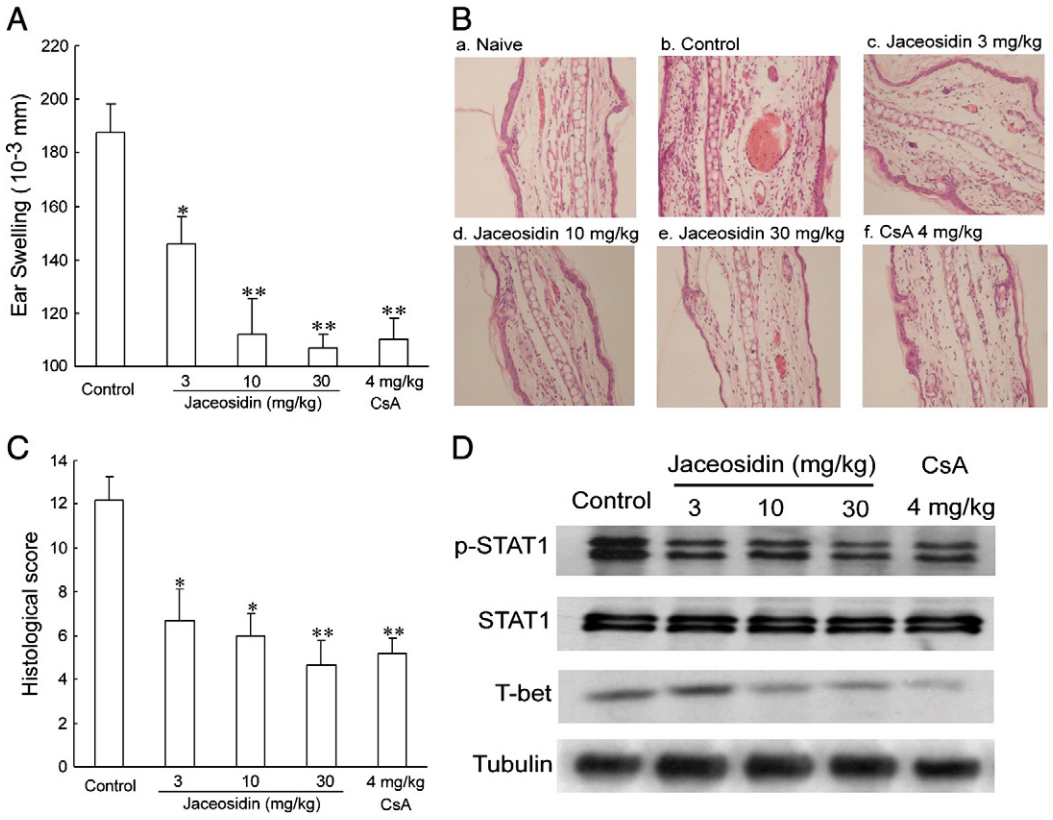

Fig. 5. Effect of jaceosidin and cyclosporine A (CsA) on the PCl-induced contact dermatitis in mice. (A) Mice were sensitized by painting 0.1 ml of 1% PCl in ethanol on the shaved skin of their abdomens, jaceosidin (3, 10, and 30 mg/kg) and CsA (4 mg/kg) were given i.p. for 6 days from the sensitization. On the sixth day, mice were challenged by painting 30 μl of 1% PCl in olive oil on the right ear lobe. Eighteen hours after the challenge, the thickness of right and left ears were measured and the swelling was evaluated by the increase in ear thickness. (B) Hematoxylin and eosin stain (original magnification 200×). (C) Ear histologic scoring. Results are mean ± S.E.M., $n = 9$. $^{*}P<0.05$, $^{**}P<0.01$ versus control group. (D) Effect of jaceosidin on the STAT1 activation and T-bet expression of draining lymph node T cells from mice with contact dermatitis. Draining lymph node cells were isolated at 18 h after PCl challenge, and then T cells were purified and subjected to Western blot. The result shown here is one of three independent experiments.

4. Discussion

Natural products and their derivatives have been used as an invaluable source of therapeutic agents historically (Borchers et al., 1997; Cragg and Newman, 2009). Traditional Chinese medicine has been vastly used for thousands of years in clinic, and many drugs, which derived from them, were proved to possess some special advantages like high activity with low toxicity. *A. vestita* is a representative of the traditional Chinese medicine mentioned earlier. Our previous study revealed that the extract of *A. vestita* exerted the selective immunosuppressive activity, and identified some active components under a series of bio-guided isolation (Yin et al., 2008). Here jaceosidin, one of the active compounds from *A. vestita*, was further investigated both *in vitro* and *in vivo* especially its molecular mechanism.

T lymphocytes were demonstrated to play a central role in T cell-mediated autoimmune disease and chronic inflammatory disorders (Perkins, 1998). So the *in vitro* immunosuppressive activity of jaceosidin was examined on Con A-induced T cell proliferation, and jaceosidin showed a strong inhibition only on the activated T cells (Fig. 1B, Supplemental Fig. 1) but not on naïve T cells (Fig. 1D, Supplemental Fig. 2), indicating the selectivity of the compound to some degree. When T lymphocytes were activated, the expressions of CD25 and CD69 were increased. As shown in Fig. 2, the treatment of jaceosidin significantly reduced the expressions of CD25 and CD69 in a dose-dependent manner. As shown in the result, the expression of CD69 is significantly reduced by jaceosidin, but to a lesser extent than CD25. Since the expression of CD69 is an early activation marker for T cells while CD25 is somewhat later, this result suggests that jaceosidin has its effect relative later during the process of T cell activation. As typical Th1-type cytokines, IL-2, IFN-γ and TNF-α have been implicated in the pathogenesis of several immunological diseases, particularly Th1-mediated diseases (Schulze-Koops and Kalden, 2001). So further study was conducted to examine the action of jaceosidin on Th1 cytokine production. Consistent with the aforementioned result, jaceosidin dose-dependently inhibited the secretion of IL-2, IFN-γ and TNF-α, especially on the production of IFN-γ (Fig. 3). The production of IL-4, which known as a typical Th2 cytokine, was also tested and jaceosidin only showed a slight tendency on the inhibition of IL-4 production (data not shown). IFN-γ is one of the most important endogenous mediators of immunity and inflammation, and plays a key role in Th1 cell response (Hu and Ivashkiv, 2009; Siebler et al., 2003b). The inhibition of IFN-γ secretion indicated the potential of jaceosidin for the treatment of Th1-mediated immune disorders. IFN-γ signals mainly transmit through the Jak-STAT signaling pathway and the major STAT protein activated by IFN-γ is STAT1 (Leonard, 2001; Ortmann et al., 2000; Stark, 2007). Recent studies further revealed that the expression and activation of STAT1 are important in the pathogenesis of colonic inflammation (Schreiber et al., 2002). Results from Fig. 4 demonstrated that the phosphorylation of STAT1 and the expression of its downstream molecule T-bet were significantly decreased with the treatment of jaceosidin.

Although jaceosidin caused inhibition on T cell proliferation and activation *in vitro*, this evidence might not completely reflect their action *in vivo*. Therefore, the PCl-induced murine model of contact dermatitis, which was considered to be a T cell-mediated DTH model, was performed on BALB/c mice. DTH is a $CD4^{+}$ T cell-mediated immune response and is associated with T cell activation and production of Th1-type cytokines (Kobayashi et al., 2001). DTH

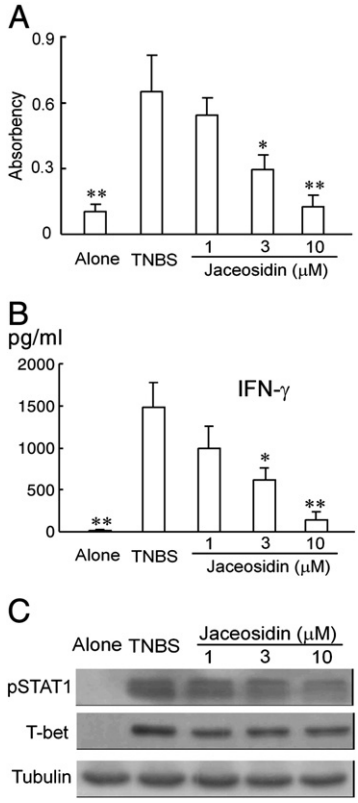

Fig. 6. Effect of jaceosidin on antigen-specific T-cell proliferation, cytokine production and STAT1 activation induced by TNBS *in vitro*. (A) Splenocytes isolated from BALB/c mice 5 days after sensitization were treated with 1 mM TNBS and 25 mg/ml mitomycin C for 30 min at 37 °C. After washing 3 times in medium supplemented with 0.6% glycylglycin, the cells were used as stimulator cells. At the same time, lymph node cells from the previously described sensitized mice, whose adherent cells had been removed, were used as responder cells. The 4×10^5 stimulator cells and 2×10^5 responder cells were co-cultured for 72 h. Then cell proliferation was examined by MTT uptake. Results are mean ± S.E.M., $n=3$. $^*P<0.05$, $^{**}P<0.01$ versus TNBS group. (B) T cells from draining lymph nodes of PCl-immunized mice were incubated with different concentrations of jaceosidin and then exposed to TNBS (1 mM) for 24 h. The supernatants were harvested and assayed for IFN-γ by ELISA kit. Results are mean ± SD, $n=3$. $^*P<0.05$, $^{**}P<0.01$ versus TNBS group. (C) Effect of jaceosidin on the STAT1 activation and T-bet expression of presensitized T cells induced by TNBS *in vitro*. T cells from draining lymph nodes of PCl-immunized mice were incubated with different concentrations of jaceosidin and then exposed to TNBS (1 mM) for 24 h. Cells were then harvested, lysed and assayed by Western blot. One of three different experiments is shown here.

reaction can be divided into induction phase and effector phase, and we found that jaceosidin showed a significant inhibition on the ear swelling when given in the induction phase of the DTH reaction (Fig. 5), while it only showed a slight tendency of inhibition when given in the effector phase (data not shown). These results suggest jaceosidin may mainly inhibit the activation rather than the function of the effector T cells involved in DTH reaction. Moreover, there was no death or any sign of illness observed when the mice were treated with jaceosidin during the whole *in vivo* experimental period. Importantly, jaceosidin did not influence the body weight as well as the spleen weight of the mice *in vivo* (data not shown). On the contrary, positive control CsA shows a tendency to decrease the spleen weight of mice (data not shown), although it also deeply inhibited the DTH reaction (Fig. 5A). This character of jaceosidin is different from that of some immunosuppressants such as CsA, suggesting its selectivity to the activated T lymphocytes. These results reveal that jaceosidin has potential for the treatment of T cell-mediated contact dermatitis for its high activity and low toxicity *in vivo*. Then the molecular mechanism of jaceosidin's action was further elucidated. As shown in Fig. 5D, jaceosidin-treated T cells from the draining lymph node of mice with contact dermatitis exhibited the dampened STAT1 phosphorylation and impaired T-bet expression compared with the control group. In addition, jaceosidin dose-dependently inhibited T cell proliferation induced by specific antigen TNBS (the liquor form of PCl) *in vitro* and prominently decreased the production of proinflammatory cytokine IFN-γ. And consistent with the results observed in cytokine production, jaceosidin significantly inhibited STAT1 phosphorylation and T-bet expression in a dose-dependent manner (Fig. 6). These findings indicate that the inhibition of IFN-γ/STAT1/T-bet signaling pathway by jaceosidin blocks the activation of T lymphocytes and finally contributes to the alleviation of the ear swelling and inflammation. Our present study, which was focused on the activation of STAT1, did not exclude other possible molecular pathways from being influenced by jaceosidin. For instance, jaceosidin also reduced TNF-α secretion (Fig. 2), and it had been reported that jaceosidin inhibited the phosphorylation of IκB as well as blocked IκB degradation in T cell activation (Lee et al., 2007). It is well-known that TNF-α/NF-κB signaling is also involved in T cell activation and NF-κB activation can induce various gene expressions, which in turn, lead to T cell proliferation and cytokine production (Schmitz and Krappmann, 2006). The results of this study suggest that the inhibitory effect of jaceosidin on DTH reaction is at least partly mediated through IFN-γ/STAT1 signaling pathway.

Taken together, we have demonstrated that jaceosidin is one of the major components which are responsible for the immunosuppressive activity of *A. vestita*. The molecular mechanism of jaceosidin is associated with the inhibition on the activation of IFN-γ/STAT1/T-bet signaling pathway in T cells. These findings suggest the potential of jaceosidin as a new effective remedy for the treatment of T cell-mediated immune diseases.

Conflict of interest statement

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2010.10.068.

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