

ORIGINAL ARTICLE

Berberine Attenuates Inflammation Associated with Delayed-Type Hypersensitivity via Suppressing Th1 Response and Inhibiting Apoptosis

Zhigang Wang,¹ Zhe Chen,² Tao Chen,³ Tao Yi,⁴ Zhou Zheng,³ Hong Fan,¹ and Zebin Chen^{5,6}

ABSTRACT—Berberine, one of the active alkaloids from *Rhizoma Coptidis*, has been indicated to have anti-inflammatory and immunosuppressive properties. The aim of this study was to determine the role of berberine on ovalbumin (OVA)-induced delayed-type hypersensitivity (DTH) and its potential mechanisms. Berberine treatment significantly reduced footpad swelling, inflammatory cells infiltration, anti-OVA IgG levels, IgE concentration in serum, and the tetramer⁺CD8⁺ cells. In homogenized footpad tissue, the production of Th1-mediated cytokines including IFN- γ , TNF- α , and IL-2 were suppressed following the administration of berberine. Detailed studies revealed that berberine prevented differentiation into Th1 cells in the OVA-primed lymphocytes, resulting from suppressing the expression of T-bet and secretion of IFN- γ but not IL-4. Concanavalin A stimulation assay and MTT assay also indicated inhibiting effect of berberine treatment on IFN- γ production and decreased cytotoxicity in lymphocytes proliferation, respectively. Additionally, berberine obviously decreased the cell apoptosis and enzymatic activity of caspase-3, which was further confirmed by the facts that berberine clearly lowered Bax/Bcl-2 ratio and expression of cleaved caspase-3 protein. On correlation analysis, the percentage of apoptotic cells showed a significant positive relationship with IFN- γ /IL-4 ratio of supernatant from footpad tissue in berberine-treated DTH mice. These results demonstrated that berberine attenuated Th1-mediated inflammation in OVA-induced DTH by curbing Th1 response and inhibiting cell apoptosis, suggesting a therapeutic potential for berberine for the treatment of type IV hypersensitivity.

KEY WORDS: berberine; inflammation; delayed-type hypersensitivity; Th1 response; apoptosis.

¹Department of Pathogen Biology, College of Basic Medicine, Hubei University of Chinese Medicine, Wuhan, 430065, China

²Department of Integrated Chinese Traditional and Western Medicine, Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology, Wuhan, 430030, China

³Department of Anatomy, College of Basic Medicine, Hubei University of Chinese Medicine, Wuhan, 430065, China

⁴College of Basic Medicine, Hubei University of Chinese Medicine, Wuhan, 430065, China

⁵Hubei Provincial Collaborative Innovation Center of Preventive Treatment by Acupuncture and Moxibustion, Hubei University of Chinese Medicine, 1 Huangjiahui West Road, Hongshan District, Wuhan, 430065, Hubei, China

⁶To whom correspondence should be addressed at Hubei Provincial Collaborative Innovation Center of Preventive Treatment by Acupuncture and Moxibustion, Hubei University of Chinese Medicine, 1 Huangjiahui West Road, Hongshan District, Wuhan, 430065, Hubei, China. E-mail: chenzebin-hbctcm@outlook.com

INTRODUCTION

Type IV delayed-type hypersensitivity (DTH), a classic animal model of the T cell-mediated immune response, characterizes predominantly by Th1-driven inflammation and swelling at the site of antigen rechallenge [1–3]. In the sensitization (or afferent) phase, the administration of ovalbumin (OVA) induces the activation and proliferation of T lymphocytes in the regional lymph nodes. In the efferent phase, OVA rechallenge in the footpads or the ear primes T lymphocytes and macrophages recruitment to the location of the challenge, which produces a variety of inflammatory mediators and enhances the inflammatory response in DTH [4]. Because the process of hypersensitivity begins and ends at the location with a relatively short time, the

OVA-induced DTH model is widely used in studies on the immunomodulation and anti-inflammatory effects of a series of new substances and materials [5].

As a traditional Chinese medicine, berberine is a medicinal alkaloid obtained from *Coptis chinensis* (Huanglian) and has been indicated to possess a broad spectrum of pharmacological activities such as anti-inflammatory, anti-oxidant, anti-cancer, anti-allergy, anti-atherogenicity, anti-diabetic activity, hypolipidemic, and cardioprotective [6–8]. Consistent with these characteristics, our previous experiment indicated that berberine could ameliorate collagen-induced arthritis in rats attributed to the inhibition of inflammation and neovasculature development, which emphasized the anti-inflammatory effect of berberine in the chronic inflammatory disease [9]. A recent study showed that berberine had an effective immunosuppressive property, although its immunotoxic effect in DTH, however, its underlying mechanisms were poorly elucidated [10].

In this study, we aim to investigate the role of berberine on OVA-induced DTH and its underlying mechanisms. Our results showed that berberine treatment could attenuate OVA-induced hypersensitivity through suppressing Th1 response and inhibiting cell apoptosis. These studies not only helped understand the mechanism of anti-allergic effect of berberine, but also further supported its anti-inflammatory and anti-apoptosis effects, which may be a potential candidate to treat delayed-type hypersensitivity.

MATERIALS AND METHODS

Materials

Berberine was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in 0.05 % DMSO (Sigma-Aldrich) to produce a stock solution.

Animals

Female C57BL/6 mice, weighing approximately 18–20 g, were obtained from the Laboratory Animal Services Center of the Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The animals were housed in an air-conditioned room (20 ± 1 °C) with a 12-h light/dark cycle and were allowed free access to food and water. All animals were treated in accordance with the international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. All of the protocols were approved by

the Institutional Ethics Committee of Hubei University of Chinese Medicine.

Delayed-Type Hypersensitivity (DTH)

Mice were immunized s.c. with ovalbumin (OVA, 50 µg in 50 µl saline; Sigma-Aldrich) emulsified with 50 µl complete Freund's adjuvant (CFA; Sigma-Aldrich). After 6 days, mice were challenged by s.c. injection of 200 µg heat-aggregated OVA in 20 µl of PBS in the left hind footpad. Right footpad received PBS as a control. OVA-specific DTH was determined by footpad swelling 24 h after rechallenge with OVA. Footpad swelling was measured as difference in the thickness of OVA-challenged and PBS-injected control footpad for each mouse. Including the control group (control), mice were divided into the delayed-type hypersensitivity group (DTH) and DTH with berberine treatment group (berberine, 10 mg/kg/day, intraperitoneal injection for consecutive 7 days).

Histopathological Observation and Assessment

Mice were sacrificed at the termination of experiment, and the left hind footpad was removed and fixed in 4 % neutral buffered formalin. Following decalcification with 12.5 % EDTA (pH 7.0), the left hind footpad was paraffin-embedded. Tissue sections (5 µm; Cryostat CM3050S, Leica Microsystems, Germany) were stained with hematoxylin and eosin (H&E; Sigma-Aldrich) to investigate histological changes in the epidermal thickness of footpad skin and inflammatory cells infiltration, and pictures were taken using a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan). All sections were randomized and evaluated by two trained observers who were blinded to the treatment groups.

Immunohistochemical Staining

The tissue sections were deparaffinized and rehydrated following a standard procedure. The endogenous peroxidase activity was then quenched with 3 % H₂O₂ in methanol and blocked with normal goat serum. Anti-mouse CD3, CD4, and F4/80 monoclonal antibodies (Santa Cruz, CA, USA) were applied onto each section overnight, respectively. Then, the slides were incubated with poly-HRP reagent. Staining was visualized by incubating with 3,3'-diaminobenzidine followed by hematoxylin counter staining. For the number of positive cells, 100 cells were randomly selected and counted from 5 representative fields of each section. The results are presented blindly by two independent observers.

Preparation of Spleen Lymphocytes

Spleen lymphocytes were prepared by aseptically removing spleens from mice in different group. Homogenized single spleen cells were collected and isolated by density gradient centrifugation (2,000 rpm/min for 30 min; Ficoll-Hypaque density 1.077 g/mL; Sigma-Aldrich), then incubated overnight at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum and antibiotics (100 units/mL streptomycin and 100 units/mL penicillin; Gibco, Grand Island, NY, USA). After 24 h, the nonadherent cells were harvested as spleen lymphocytes for the following experiments.

ConA Stimulation Assay and OVA-Primed Assay

For concanavalin A (ConA) stimulation assay, spleen lymphocytes (2×10^5 per well) of different group were isolated and incubated in the presence of 5 mg/mL ConA (Sigma-Aldrich) for 24 h following experimental progression.

For OVA-primed assay *in vitro*, the lymphocytes (5×10^5 per well) were isolated from DTH mice and cultured in the presence or absence of OVA (100 µg/mL) following the treatment with berberine (0.1–10 µM) for 48 h. The plates were incubated at 37 °C in humidified incubator with 5 % CO₂.

MTT assay

Spleen lymphocytes (2×10^5 per well) of different group were isolated and incubated for 48 h in the presence of 100 µg/mL OVA. Then, MTT working solution (0.5 mg/mL; Sigma-Aldrich) was added, and the plates were incubated for an additional 4 h at 37 °C. After centrifugation, the medium was replaced with dimethyl sulfoxide (Sigma-Aldrich). The absorbance of each well at 570 nm was measured with a plate reader (Thermo Scientific, Waltham, MA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Sera of mice in different groups were collected at the termination of experiment. Anti-OVA IgG titers and IgE levels in serum were determined by enzyme-linked immunosorbent assay (ELISA) analysis (Life Technologies, Grand Island, NY, USA) using a microplate reader (Thermo Scientific).

Footpad was mechanically homogenized, centrifuged, and the supernatant was used for cytokine determination. The expression levels of IFN-γ, TNF-α, IL-2, IL-4, IL-10, and IL-13 were detected by ELISA assay (R&D

systems, Minneapolis, MN, USA) according to the manufacturer's protocol. All experiments were done in triplicate.

RNA Isolation and Real-Time RT-PCR

Total RNA from spleen lymphocytes was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using M-MLV-RTase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR-Green Master PCR Mix (Applied Biosystems, Foster City, CA, USA) on the TP800 qPCR System (Takara, Japan). Real-time PCR of cDNA was performed using the forward (F) and reverse (R) primer sequences: T-bet: F, 5'-TGCC TGCAGTGCTTCTAACA-3'; R, 5'-TGCC CCGCTTCCTCTCCAACCAA-3'; GAPDH: F, 5'-AACT TTGGCATTGTGGAAGG-3'; R, 5'-ACAC ATTGGGGGTAGGAACA-3'. The relative T-bet mRNA levels were normalized to those of GAPDH for mRNA analysis. For relative quantification, $2^{-(C_t-C_c)}$ (C_t and C_c are the mean threshold cycle differences after normalizing to control, which was calculated and used as an indication of the relative expression levels).

Flow Cytometry Analysis

Spleen lymphocytes were prepared as described above. Frequencies of OVA-specific CD8⁺ cells were determined by flow cytometry analysis following staining with APC-conjugated anti-CD8 mAb (Molecular Probes, Eugene, OR, USA) and PE-conjugated SIINFEKL/H-2Kb peptide-MHC tetramers (iTag MHC Tetramer, Beckman Coulter, Fullerton, CA, USA) using a BD FACSCanto II (BD Bioscience, San Diego, CA, USA).

Cell apoptotic were detected using a FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). Spleen lymphocytes were washed and resuspended in buffer containing FITC annexin V and propidium iodide (PI). After incubation, apoptotic cells were analyzed by BD FACSCanto II (BD Bioscience; The early apoptotic cells showed annexin V positive and PI negative, and the late apoptotic or necrotic cells showed both annexin V and PI positive).

Enzymatic Assay for Caspase-3

Caspase-3 activity was detected using a Caspase-3 Activity Assay Kit (Bestbio, Shanghai, China). Cells were harvested and extracted on ice in lysis buffer. The lysates were centrifuged and the supernatants were assessed for protein content. Subsequently, 50 µg supernatant samples

were incubated with 90 μ L reaction buffer and 10 μ L caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide). The OD values were measured at 405 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Western Blot Analysis

Spleens from mice in different groups were obtained and homogenized with lysis buffer (25 mM Tris/HCl (pH 7.4), 150 mM KCl, 5 mM EDTA, 1 % Nonidet P40, 0.5 % sodium deoxycholate, and 0.1 % SDS) containing 1 mM PMSF and a cocktail of protease and phosphatase inhibitors (Cell Signaling Technology Inc., USA). The supernatants were analyzed for protein concentration using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Proteins were separated by electrophoresis after denaturation at 95 °C, and then transferred onto polyvinylidene fluoride (PVDF). After blocked with 5 % skim milk, membranes were incubated with the desired primary rabbit anti-mouse Abs against Bax, Bcl-2, cleaved caspase-3, caspase-3, or GAPDH (Santa Cruz Biotechnology Inc., USA) overnight at 4 °C and then incubated with the appropriate HRP-conjugated secondary antibody. After washing, the membranes were visualized by enhanced chemiluminescence detection (HP Scanjet 7400C; Hewlett-Packard Co., Palo Alto, CA, USA). Optical density for each band was assessed using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA). Sample loading was normalized by quantities of GAPDH detected parallel.

Statistical Analysis

Data are presented as mean $\pm$ S.D. and analyzed by SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Post hoc tests were applied to perform all pairwise comparisons between group means by least significant difference (LSD) *t* test. Pearson's correlation coefficient was used to test the correlations analysis. A statistical difference was considered when *P* value was less than 0.05.

RESULTS

Berberine attenuates disease progression of OVA-induced DTH response

Firstly, we aimed to learn the effect of berberine on OVA-induced DTH response. As shown in Fig. 1a, OVA challenge on day 6 induced a significant amplification in

footpad swelling compared with control. However, berberine treatment led to an incremental decline in footpad thickness (Fig. 1b). In addition, OVA challenge resulted in an obvious increment in the epidermal thickness of footpad skin and filtrations of inflammatory cells into the footpad, but administration of berberine markedly eliminated these changes (Fig. 1c). Consistent with the results, berberine also significantly inhibited anti-OVA IgG antibody and IgE levels in serum (Figs. 1d and 2), which suggested anti-allergic effect of berberine in OVA-induced DTH response.

Berberine Reduces Pro-Inflammatory Cytokines Production Associated with DTH

In DTH reactions, immunologic and inflammatory processes were majorly regulated by T cells and inflammatory mediators. Comparison with DTH group, berberine treatment significantly decreased the percentage of tetramer⁺CD8⁺ cells in CD8⁺ cells (Fig. 3a, b). In line with the result, berberine also evidently restrained the number of CD4⁺ T cells and macrophages recruited to the footpad (Table 1). Furthermore, we observed the effect of berberine on the production of inflammatory cytokines in footpad tissue associated with DTH. As shown in Fig. 3c, the levels of Th1-mediated cytokines, such as IFN- γ , TNF- α , and IL-2, as well as anti-inflammatory cytokine IL-13, were found to be highly augmented in footpad tissue of DTH group. However, berberine treatment lowered the levels of IFN- γ , TNF- α , IL-2, and IL-13. Interestingly, berberine showed little effect on the levels of IL-4 and IL-10 comparison with Th1-typed cytokines (Fig. 3c).

Berberine suppresses Th1-mediated DTH response

Following stimulation with ConA, the levels of IFN- γ in the supernatant of cultured lymphocytes were increased in DTH group. However, the secreted IFN- γ was distinctly decreased in the DTH mice treated with berberine compared with those without berberine treatment (Fig. 4a). Following OVA stimulation, MTT assay indicated there was little cytotoxicity in spleen lymphocytes isolated from DTH mice treated with berberine compared with DTH mice (Fig. 4b). Importantly, OVA-primed lymphocytes produced clearly lower levels of IFN- γ in the presence of berberine but not IL-4 (Fig. 4c, d). Furthermore, the enhancing activity of Tbet, including levels of mRNA and protein, was shown in the DTH group and berberine treatment significantly

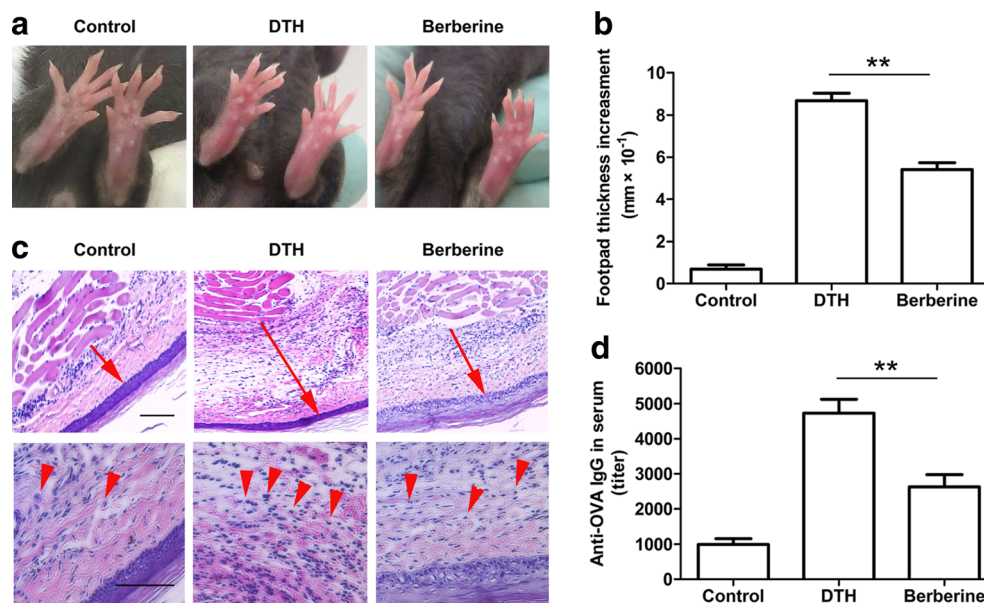


Fig. 1. Berberine inhibits disease progression of OVA-induced DTH reaction. **a** Photographs of footpad swelling and **b** footpad thickness increment were shown in OVA-induced DTH models with or without berberine treatment. **c** Histological changes of the epidermal thickness of footpad skin and inflammatory cells infiltration in OVA-challenged footpads with or without berberine treatment, which were stained using hematoxylin and eosin (H&E). Scale bar = 20 μ m. Red arrow indicated the epidermal thickness of footpad skin and inflammatory cells, respectively. **d** Effect of berberine on serum OVA-specific antibody in OVA-induced DTH models. Data are presented as the mean $\pm$ S.D. from eight separate experiments (** P < 0.01).

reduced the expression of T-bet (Fig. 5). These results suggested that berberine contained an activity via negatively modulating Th1 polarization in OVA-induced DTH, eventually resulting in a decrease in the levels of Th1-typed cytokines.

Inhibiting Apoptosis Participates in the Anti-Inflammatory Effect of Berberine

As shown in Fig. 6a, cell apoptosis was noticeably increased in DTH response. Following treatment with berberine, the percentage of apoptosis cells was obviously

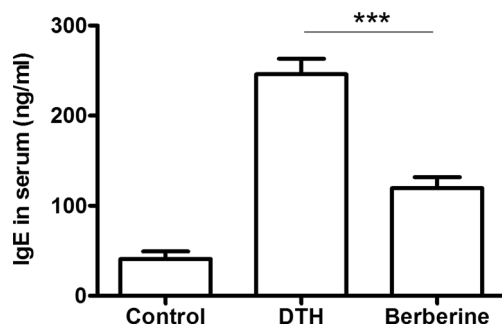


Fig. 2. Berberine shows anti-allergic effect in OVA-induced DTH models. IgE levels in serum from different groups were shown. Data are presented as mean $\pm$ S.D. (n = 8) (** P < 0.001).

decreased (Fig. 6b). Simultaneously, the enzymatic activity of caspase-3, an important marker of apoptosis, was also found to be clearly reduced accompanying with the administration of berberine (Fig. 6c). In line with the results of Annexin V-FITC/PI staining, berberine significantly promoted protein expression of anti-apoptotic Bcl-2 and lowered the level of pro-apoptotic Bax (Fig. 7a, c). In addition, spleen lymphocytes isolated from DTH mice treated with berberine exhibited decreased protein expression of cleaved caspase-3 (Fig. 7b, d), which suggested inhibiting apoptosis was involved in the anti-inflammatory effect of berberine.

Correlation Between Cell Apoptosis and IFN- γ /IL-4 Ratio in Berberine-Treated DTH Mice

Lastly, we explored the correlation between cell apoptosis and activities of Th1 polarization. On correlation analysis, the percentage of apoptotic cells had a significant positive relationship with IFN- γ /IL-4 ratio of supernatant from footpad tissue in berberine-treated DTH mice (r = 0.958, P < 0.01; Fig. 8). The positive correlation firmly demonstrated that berberine may abrade Th1-skewing through the downregulation of IFN- γ production accompanying with inhibiting cell apoptosis.

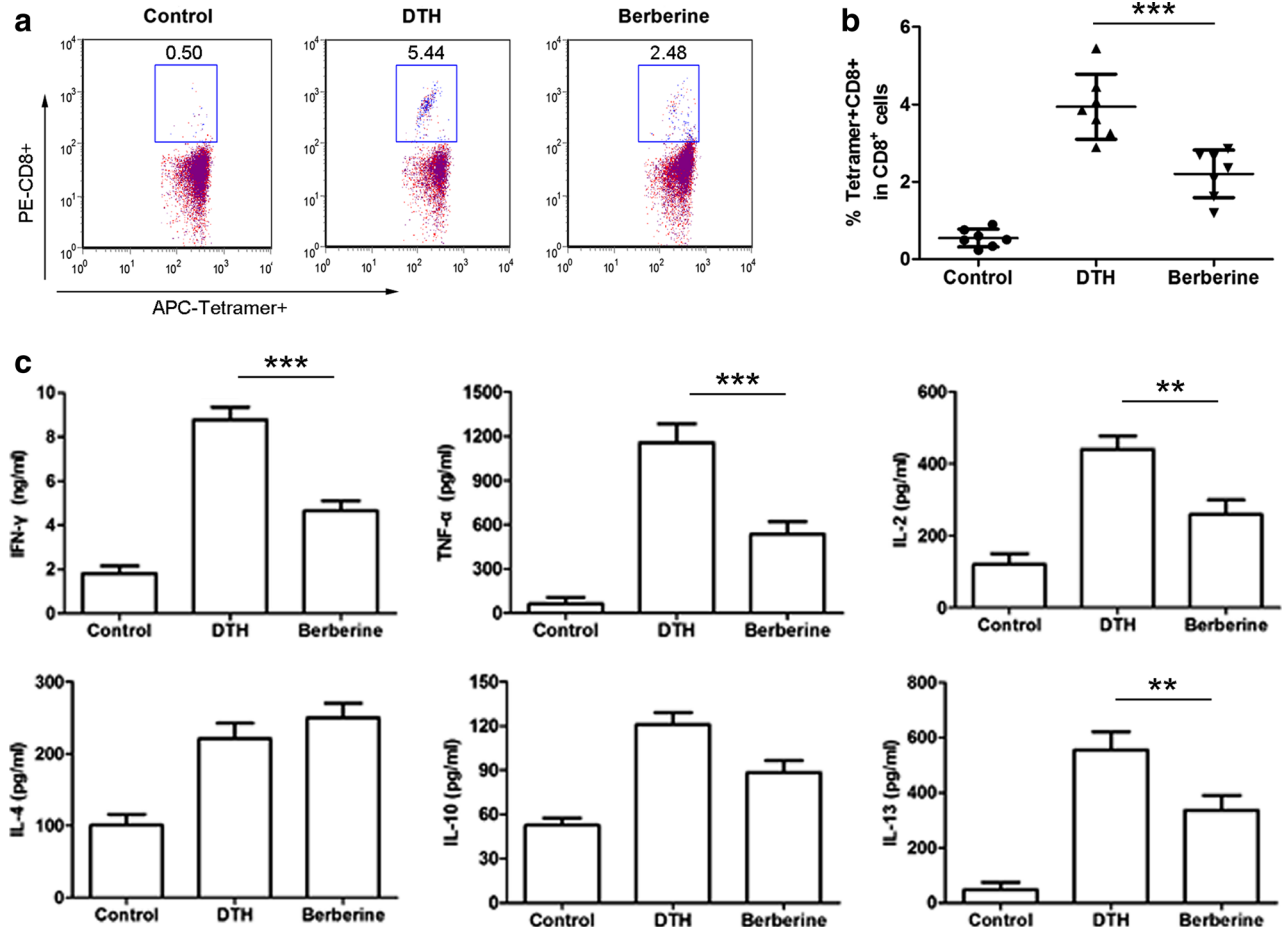


Fig. 3. Berberine attenuates inflammation associated with DTH. **a** Flow cytometric analysis of OVA-specific CD8⁺ cells staining with APC-CD8 and PE-tetramers mAbs was taken and one representative of seven independent experiments was shown. **b** The percentage of tetramer⁺CD8⁺ cells in CD8⁺ cells was statistically analyzed ($n = 7$). **c** Effect of berberine on pro-inflammatory and anti-inflammatory cytokines production of footpad tissue in OVA-induced DTH. All experiments were done in triplicate ($n = 8$). Data are presented as the mean $\pm$ S.D. (** $P < 0.01$ and *** $P < 0.001$).

DISCUSSION

In the present study, we investigated the anti-allergic effect of berberine on OVA-induced DTH response in C57BL/6 mice model. Mechanistic studies indicated that berberine abraded Th1-skewing through negatively modulating Th1 polarization, eventually leading to a decrease in the levels of IFN- γ production. Simultaneously, the correlation analysis revealed that inhibiting cell apoptosis was involved in the anti-inflammatory effect of berberine, which suggested that there might be a cross-talking between existing anti-apoptosis induction and prevention of inflammation response by caspase-3 deactivation and Th1 depolarization.

Anti-allergic effect of berberine had been investigated and reported in recent paper. Berberine and limonin exhibited suppressive effects on IgE production by a human B-

Table 1. Immunohistochemical Staining of CD3⁺, CD4⁺, and F4/80⁺ Cells in the Footpads of Ovalbumin-Sensitized and Challenged Mice

Characteristics	Number of positive cells		
	Control	DTH	Berberine
CD3 ⁺	15 $\pm$ 2	56 $\pm$ 8	26 $\pm$ 6**
CD4 ⁺	8 $\pm$ 2	24 $\pm$ 5	15 $\pm$ 4*
F4/80	4 $\pm$ 2	44 $\pm$ 5	19 $\pm$ 6***

Data are presented as the mean $\pm$ S.D. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

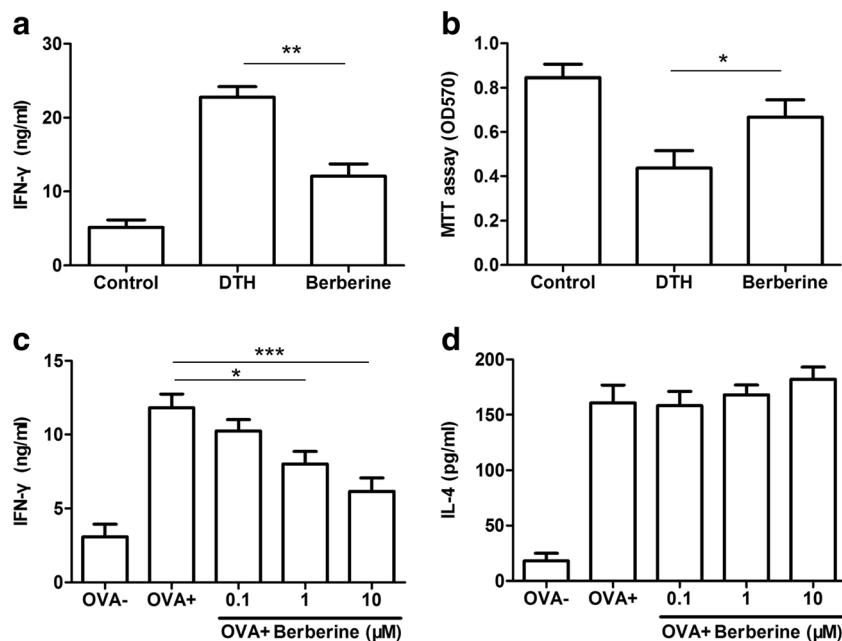


Fig. 4. Berberine reduces cytokine production by Th1 cells. **a** Spleen lymphocytes of different group were cultured for 24 h in the presence of 5 mg/mL ConA, and IFN-γ levels in the supernatant were quantified by ELISA. **b** Spleen lymphocytes of different group were cultured for 48 h in the presence of 100 μg/mL OVA, and cell proliferation was analyzed by MTT assay. **c, d** Spleen lymphocytes were isolated from DTH mice and treated with 0.1–10 μM berberine for 48 h in the presence or absence OVA (100 μg/mL), then **c** secreted IFN-γ and **d** IL-4 levels were measured by ELISA. All experiments were done in triplicate ($n = 8$). Data are presented as the mean $\pm$ S.D. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

cell line and peripheral blood mononuclear cells (PBMCs) from food-allergic pediatric patients [11]. In rat model of OVA-induced allergic asthma, berberine treatment reduced the levels of OVA-specific IgE, inhibited the OVA-induced airway inflammation, and attenuated the OVA-induced infiltration of eosinophils, macrophages, neutrophil, and lymphocytes to airway [12]. Additionally, berberine reduced allergic inflammation, allergic symptom scores, eosinophil counts, and serum specific IgE levels in a house

dust mite allergic rhinitis mouse model [13]. Utilizing sheep red blood cells as antigenic stimulus to induce DTH response in Balb/c mice, a recent study reported that berberine at a high dose (i.e., 10 mg/kg/day for 14 days) imparted an inhibitory effect on the mouse immune system. At a lower dose (i.e., 5 mg/kg/day), berberine only seemed to affect DTH responses and mitogen-induced splenocyte/lymphocyte proliferation [10]. Based on these results, berberine at 10 mg/kg/day was used to explore its potential

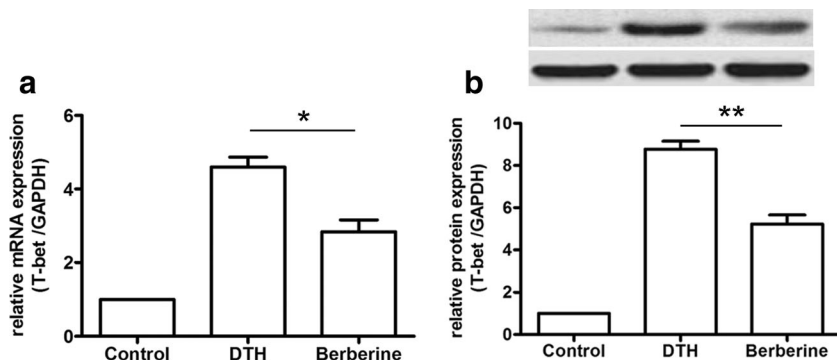


Fig. 5. Effect of berberine on T-bet. Spleen lymphocytes from mice of different group were obtained and total RNA were isolated. **a** Quantitative real-time PCR and **b** the proteins expression of T-bet were performed, and statistical analysis was shown, separately. A representative Western blot was shown ($n = 8$). Data are presented as the mean $\pm$ S.D. (* $P < 0.05$ and ** $P < 0.01$).

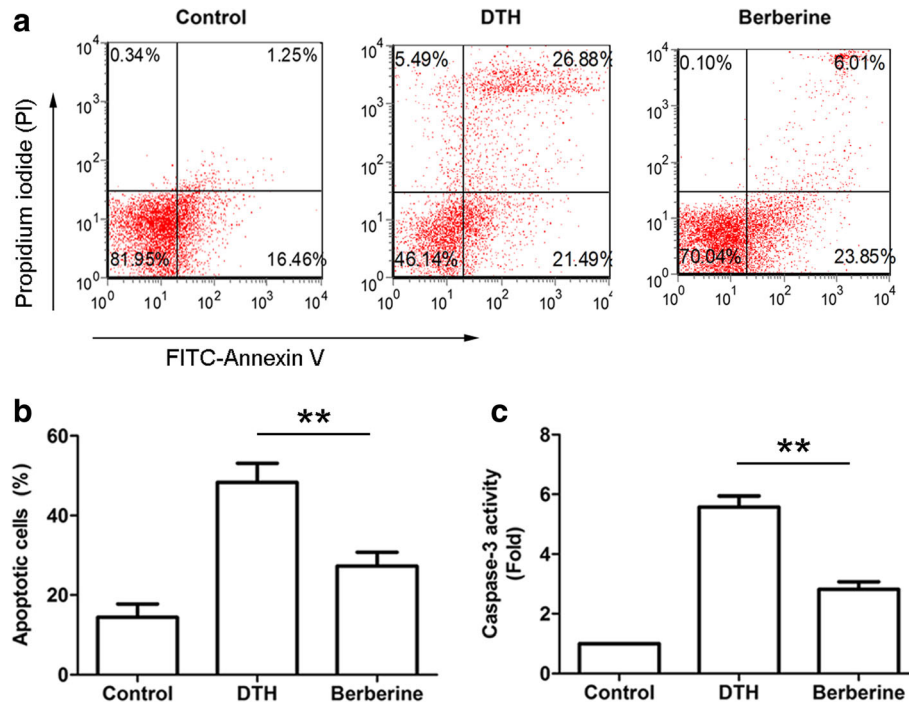


Fig. 6. Inhibiting apoptosis is involved in the anti-inflammatory effect of berberine. Spleen lymphocytes of different group were isolated as described in the “MATERIALS AND METHODS” section. **a** Apoptotic analysis was taken by flow cytometry (annexin V⁺PI⁻ and annexin V⁺PI⁺), and one representative of eight independent experiments was shown. **b** The percentage of apoptotic cells was statistically analyzed ($n = 8$). **c** Enzymatic assay for caspase-3 activity in spleen lymphocytes treated with OVA (100 $\mu\text{g/mL}$) for 48 h were analyzed ($n = 8$). Data are presented as the mean $\pm$ S.D. (** $P < 0.01$).

mechanism in the present study. In line with above results, our data showed that berberine treatment significantly

reduced footpad swelling, inflammatory cells infiltration, anti-OVA IgG levels, IgE concentration in the serum, and

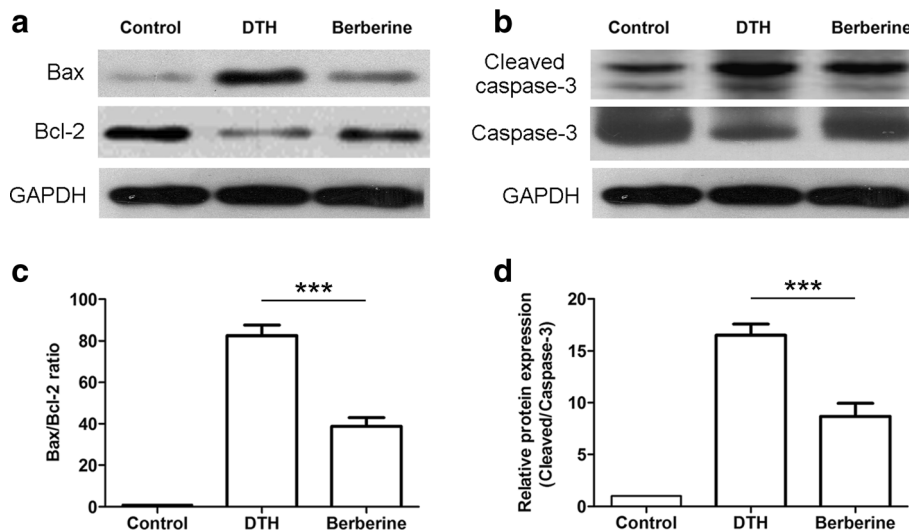


Fig. 7. Berberine regulates the expression of apoptosis-related proteins. **a** The expression of apoptosis-related proteins including Bax and Bcl-2 and **b** cleaved caspase-3 in OVA-induced DTH response was assayed by Western blotting, and one representative of eight independent experiments was shown. **c** The relative protein expression of Bax/Bcl-2 and **d** cleaved caspase-3 were quantified and statistically analyzed ($n = 8$). Data are presented as the mean $\pm$ S.D. (*** $P < 0.001$).

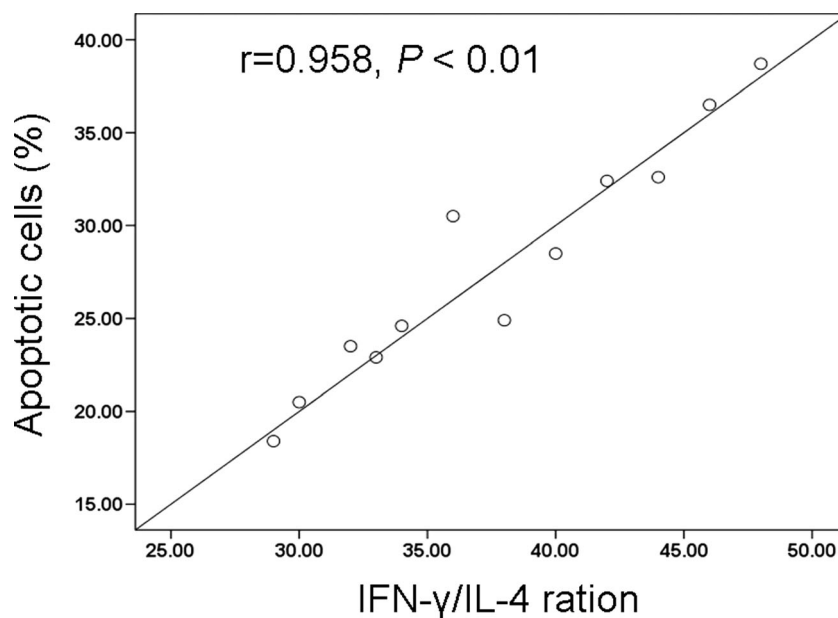


Fig. 8. Relationship between cell apoptosis and IFN- γ /IL-4 ratio. Correlation analysis between the percentage of apoptotic cells and IFN- γ /IL-4 ratio of footpad tissue in berberine-treated DTH mice ($n = 12$).

the tetramer⁺CD8⁺ cells (Figs. 1, 2, and 3a, b; Table 1), which suggested berberine could suppress both cellular and humoral immune functions. Nevertheless, there were some differences in this study comparison with the previous report [10]. Firstly, OVA antigen was used in C57BL/6 mice to induce DTH response, including sensitization and primed phase, replacing sheep red blood cells in Balb/c mice. Secondly, OVA antigen was subcutaneous injection but the sheep red blood cells were intraperitoneal administration. Thirdly, berberine was used for consecutive 7 days instead of 14 days. Lastly, ConA was selected in our study for lymphocyte proliferation test, unlike phytohemagglutinin A (PHA) or lipopolysaccharide (LPS) used in the above paper.

As we know, T helper cell differentiation has classically represented distinct T cell phenotypes according to cytokine milieu, Th1, Th2, Th17, and regulatory T cells, leading to terminally differentiated T cell subsets [14]. Th1 cells primarily secrete pro-inflammatory Th1-type cytokines such as IL-2, IFN- γ , and TNF- α ; whereas, Th2 cells principally produce anti-inflammatory Th2-type cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13 [15]. Immunomodulatory effect of berberine on Th17 cells was indicated in the latest research, which showed that berberine exerted significantly beneficial effects on chronic relapsing dextran sulfate sodium-induced colonic injury and inflammation via inhibition of pro-inflammatory Th17 responses

[16]. Additionally, berberine suppressed Th17 responses that were mediated by direct actions on T cells as well as an indirect effect via dendritic cells [17]. In contrast, we demonstrated berberine-suppressed Th1 response in OVA-induced DTH reaction, which was supported by the reduced levels of Th1-mediated cytokines including IFN- γ , TNF- α , and IL-2 in footpad tissue and ConA-stimulated lymphocytes (Figs. 3c and 4a). Consistently with the results, OVA-primed lymphocytes secreted lower levels of IFN- γ in the presence of berberine but not IL-4 in this study (Fig. 4c, d). Moreover, berberine treatment significantly reduced the activity of T-bet in mRNA and protein levels (Fig. 5). These results suggested that berberine contained its anti-inflammatory in OVA-induced DTH attributed to preferentially suppress Th1-driven immune response but little effect on the Th2-based response, eventually resulting in the inhibition of Th1 polarization. If Th17 cells participated in the anti-inflammatory of berberine in OVA-induced DTH response, additional studies remained to be performed to clarify the details.

Up to now, berberine has attracted much attention for its multiple pharmacological properties accompanied by different molecular mechanisms [18]. Apoptosis is a programmed cell death under the control of the activities of individual development, including the death receptor pathway and the mitochondrial pathway [19]. Accumulating evidences showed that cell apoptosis may be contributed

to the biological effects of berberine in different models and disease progression. Berberine could suppress the proliferation and induce the apoptosis of human osteosarcoma cell lines U2OS cells through inhibiting the PI3K/Akt signaling pathway activation, resulting in upregulating the expression of Bax and ADP-ribose polymerase (PARP) and downregulating the expression of Bcl-2 and caspase3 [20]. On the contrary, berberine exhibited neuroprotection via decreasing anoxia-induced apoptosis-modulating proteins caspase 3, caspase 8, caspase 9, PARP, and BCL-2/Bax in rat model of middle cerebral artery occlusion [21]. In addition, berberine protected human umbilical vein endothelial cells from LPS-induced injury by blocking the JNK pathway and subsequently promoting the expression of the anti-apoptotic protein myeloid cell leukemia-1 [22]. Furthermore, renoprotective effect of berberine against gentamicin-induced nephrotoxicity could be partially mediated through its antioxidant, anti-inflammatory, and anti-apoptosis action [23]. Berberine-supplemented diets could attenuate oxidative stress and hepatocytes apoptosis and improve mitochondrial function [24]. Summarizing these findings, we can find that berberine execute pro-apoptosis or anti-apoptosis associated with the difference of cell type and experimental model. In this study, berberine inhibited cell apoptosis, promoted protein expression of anti-apoptotic Bcl-2, lowered levels of pro-apoptotic Bax, and cleaved caspase-3 in OVA-induced DTH response (Figs. 6 and 7), suggesting that inhibiting apoptosis was involved in the anti-inflammatory effect of berberine.

Based on the above-mentioned experiments and our results presented here, we speculated possible relationship between cell apoptosis and Th1 polarization. In berberine-treated DTH mice, the percentage of apoptotic cells had a significant positive relationship with IFN- γ /IL-4 ratio of supernatant from footpad tissue (Fig. 8). Logically, inhibiting cell apoptosis would therefore lead to the development of more T cells, resulting in a larger OVA responding CD4⁺ or CD8⁺ T cells compartment showing increased overall proliferative capacity and cytokine production in response to OVA [25]. However, Th1-type pro-inflammatory IFN- γ production was obviously decreased following berberine treatment although there was an increased tendency in the levels of IL-4 production. It was possible that more naïve T helper cells differentiate into other T cell subtypes such as Th17 cells or Tregs following the negative modulation of Th1 polarization [26]. On the other hand, although the number of T cell phenotypes, excluding Th1 cells, was upregulation, but the function of these T cell subsets was downregulation or anergy [27]. Lastly, there was overlapping reaction

between other molecular mechanisms and the anti-inflammatory effect of berberine. Further studies were needed to elucidate the precious mechanism and which would help to explain clearly the anti-allergic effect of berberine.

In summary, our studies showed that berberine treatment lead to the reduction of cell apoptosis and decrease of Th1 polarization, which negatively target IFN- γ production, thereby suppressing delayed-type hypersensitivity response. These findings indicated that berberine had a beneficial effect on the mediation of the immune system as immune regulator, suggesting its new therapeutic targets to treat Th1-driven autoimmunity, such as type IV hypersensitivity.

ACKNOWLEDGEMENTS

This study was supported by the grants from the Health and Family Planning Commission of Hubei Province (No. 2013Z-Z01) and the Natural Science Foundation of Hubei Province of China (No. 2015CFA096).

COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest. The authors declare no conflicts of interest.

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