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RESEARCH PAPER

Shikonin inhibits maturation of bone marrow-derived dendritic cells and suppresses allergic airway inflammation in a murine model of asthma

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BACKGROUND AND PURPOSE
Shikonin exhibits a wide range of anti-inflammatory actions. Here, we assessed its effects on maturation of murine bone marrow-derived dendritic cells (BM-DCs) and on allergic reactions in a murine model of asthma.

EXPERIMENTAL APPROACH
Cultured murine BM-DCs were used to investigate the effects of shikonin on expression of cell surface markers and their stimulation of T-cell proliferation and cytokine production. The therapeutic potential of shikonin was evaluated in a model of allergic airway disease.

KEY RESULTS
Shikonin dose-dependently inhibited expression of major histocompatibility complex class II, CD80, CD86, CCR7 and OX40L on BM-DCs, induced by a mixture of ovalbumin (OVA; 100 $\mu g \cdot mL^{-1}$) and thymic stromal lymphopoietin (TSLP; 20 $ng \cdot mL^{-1}$). Shikonin-treated BM-DCs were poor stimulators of $CD4^+$ T lymphocyte and induced lower levels of interleukin (IL)-4, IL-5, IL-13 and tumour necrosis factor (TNF)-α release by responding T-cells. After intratracheal instillation of shikonin in OVA-immunized mice, OVA challenge induced lower IL-4, IL-5, IL-13, TNF-α and eotaxin release in bronchial alveolar lavage fluid, lower IL-4 and IL-5 production in lung cells and mediastinal lymph node cells and attenuated OVA-induced lung eosinophilia and airway hyperresponsiveness.

CONCLUSION AND IMPLICATIONS
Shikonin effectively suppressed OVA + TSLP-induced BM-DC maturation *in vitro* and inhibited allergic inflammation and airway hyperresponsiveness in a murine model of asthma, showing good potential as a treatment for allergic asthma. Also, our model provides a novel platform for screening drugs for allergic diseases.

Abbreviations
AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; i.n., intranasal; i.t., intratracheal; OVA, ovalbumin; TSLP, thymic stromal lymphopoietin

Introduction

Shikonin and its derivatives are analogues of naphthoquinone pigments, the major components of root extracts of a Chinese medicinal herb (*Lithospermum erythrorhizon*), and are ingredients of several folk medicines (Chen *et al.*, 2002). *L. erythrorhizon* roots have been claimed to be beneficial for burns, anal ulcers, haemorrhoids, infected crusts, bedsores, external wounds and oozing dermatitis (Papageorgiou *et al.*, 1999). Shikonin was first isolated in its acetate form from the roots of *L. erythrorhizon* by Majima and Kuroda (1922). A diversity of pharmacological actions of this compound have been reported, such as inhibition of vascular permeability and acute oedema induced by histamine by topical application of shikonin (Hayashi, 1977), inhibition of cyclooxygenase-2 transcription through down-regulation of extracellular signal-regulated kinase 1/2 and activation protein-1 activities (Subbaramaiah *et al.*, 2001), inhibition of leukotriene B_4 (LTB_4) biosynthesis (Wang *et al.*, 1994), suppression of mast cell degranulation (Wang *et al.*, 1995) and protection of the vasculature by inhibition of the neutrophil respiratory burst (Kawakami *et al.*, 1996), and blocking CCL5 (RANTES) and CCL4 (MIP-1α) binding to human monocytes and chemokine binding to the CC chemokine receptor, CCR1 (Chen *et al.*, 2001; receptor nomenclature follows Alexander *et al.*, 2009). Additionally, shikonin has shown anti-cancer effects, and clinical trials in China showed that shikonin, which inhibited the growth of lung cancer and improved immune function, was effective in the later stages of lung cancer (Guo *et al.*, 1991; Hisa *et al.*, 1998). Shikonin is also involved in wound healing (Ozaki *et al.*, 1994) and inhibition of platelet activation (Chang *et al.*, 1993); it also has antimicrobial effects (Ueba *et al.*, 1993; Yamasaki *et al.*, 1993). Although a broad range of biological and pharmacological activities of shikonin have been reported, the possible effects of shikonin on allergic inflammation have not been determined.

Allergic asthma is characterized clinically by hypersecretion of mucus, chronic inflammation of the airways and airway hyperresponsiveness. Studies in asthmatic humans and animal models of asthma have suggested that $CD4^+$ T helper 2 (T_H2) lymphocytes play a crucial role in allergic asthma (Chung and Barnes, 1999; Wills-karp, 1999). In addition to T_H2 cell effects, dendritic cells (DCs) are the predominant antigen-presenting cells in the lung; inflammatory cytokine-associated eosinophilic airway inflammation, goblet cell hyperplasia, and bronchial hyperreactivity can be completely prevented in the absence of DCs (Lambrecht *et al.*, 1998). Previous studies have shown that DCs play key roles both in T_H2 priming and maintaining allergic airway inflammation (Constant *et al.*, 2002). The ability of DCs to polarize T_H responses is highly dependent on exogenous factors (Banchereau *et al.*, 2000), and a number of molecules have been identified that can convert DCs into T_H2-polarizing antigen-presenting cells, including prostaglandin E_2 (Kalinski *et al.*, 1997) and thymic stromal lymphopoietin (TSLP) (Ito *et al.*, 2005).

In this study, to investigate possible therapeutic effects of shikonin on allergic asthma, we used ovalbumin (OVA) combined with TSLP to convert bone marrow-derived DCs (BM-DCs) into T_H2-polarizing DCs. We analysed DC maturation and DC-stimulated T_H2 differentiation and activation *in vitro*. We also evaluated the *in vivo* effects on T_H2 cytokine expression in bronchoalveolar lavage fluid, airway inflammation, and airway hyperresponsiveness using OVA-immunized BALB/c mice.

Methods

Preparation of BM-DCs

All animal care and experimental procedures were approved by the Animal Committee of China Medical University. The mice were housed in temperature-controlled rooms with a 12 h light/12 h dark cycle and were given food and water *ad libitum*. The preparation of BM-DCs was modified from a previously described method (Inaba *et al.*, 1992; Suen *et al.*, 2001). Briefly, bone marrow cells from the femurs and tibias of female BALB/c mice (3–6 weeks old, 13–17 g from the National Laboratory Animal Center, Taiwan) were first depleted of red cells with lysis buffer. Approximately 10^6 cells were placed in 24-well plates in 1 mL of Rose Park Memorial Institution (RPMI) 1640 medium supplemented with 5% fetal bovine serum, recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) (500 $U \cdot mL^{-1}$), interleukin (IL)-4 (1000 $U \cdot mL^{-1}$) (Pepro Tech, Inc., Rocky Hill, NJ, USA), 4 mM L-glutamine, 25 mM HEPES (pH 7.2), 50 μM 2-mercaptoethanol, 0.25 $\mu g \cdot mL^{-1}$ amphotericin, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. Every other day, fresh medium containing GM-CSF and IL-4 were replaced and non-adherent cells were transferred to a new plate to decrease the contamination by macrophages. On day 6 of culture, non-adherent cells (BM-DCs) were collected and treated with different chemical compounds for 48 h. Then, BM-DCs were analysed by flow cytometry to examine their surface marker expression, and used for other experiments.

Immunofluorescence staining and flow cytometry

BM-DCs ($5–10 \times 10^5$ cells) were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c, anti-I-A/I-E and OX-40L, or phycoerythrin (PE)-conjugated anti-CD80, anti-CD86, anti-CD11c and anti-CCR7 (eBioscience, Inc., San Diego, CA, USA) at 4°C for 30 min.

The cells were washed and suspended in 0.5 mL of phospate buffered saline (PBS) with 0.1% sodium azide, and analysed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA). A total of 2×10^5 cells were counted, and the frequency of each cell surface marker was determined using commercial software. Control cells were suspended in medium only. The flow cytometer was regularly calibrated with CaliBRITE beads (Becton Dickinson).

Cytotoxicity assay

BM-DCs were pretreated with different concentrations of shikonin for 10 min and cultured with or without OVA (100 μg·mL^{-1}) combined with TSLP (20 ng·mL^{-1}) for 48 h. At this time, the number of viable cells was determined using Trypan blue staining (Sugiura *et al.*, 2007). In apoptosis assays, cells were stained with FITC-labelled Annexin V and propidium iodide according to the manufacturer's instructions (Becton Dickinson); cell apoptosis was analysed by flow cytometry.

Quantitative real-time polymerase chain reaction

RNA was converted into complementary DNA and then quantified by quantitative real-time polymerase chain reaction analysis using ABI PRISM 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The signal in this study was generated by the binding of the fluorophore SYBR Green (Applied Biosystems) to double-stranded DNA. The partial cycle giving a statistically significant increase in OX-40L product was determined (threshold cycle; *C*t) and normalized to the *C*t for β-actin. OX-40L and β-actin were amplified using SYBR Green PCR Master Mix (Applied Biosystems). The primers of OX-40L are sense strand 5′-GTCTGCCTGCAACTCTCTTCCT-3′ and antisense strand 5′-CTCCTCTGAGTCTTTG-GATTGGA -3′; the primers of β-actin are sense strand 5′-ACTGCCGCATCCTCTTCCT-3′ and antisense strand 5′-ACCGCTCGTTGCCAATAGTG-3′.

Experimental protocol for asthma model

Female BALB/c mice and DO11.10 TCR transgenic (Tg) mice aged 6–8 weeks were obtained from the Animal Center of the College of Medicine, National Taiwan University. Mice were sensitized by i.p. injection of 50 μg of OVA emulsified with 2 mg of aluminium hydroxide (AlumImuject; Pierce Chemical, Rockford, IL, USA) in a total volume of 200 μL, boosted with 25 μg of OVA emulsified with 2 mg of aluminium hydroxide, and challenged three times with OVA (100 μg in a total volume of 40 μL) by intranasal (i.n.) administrations on consecutive days (see scheme in Figure 4A). Shikonin was delivered to anaesthetized animals (with diethyl ether) intratracheally once daily on days 35–39 (five treatments). There were 8–10 mice per group. Mice were killed with CO_2.

Mixed lymphocyte reaction

Day 6 BM-DCs from BALB/c mice were treated with different concentrations of shikonin for 10 min and cultured with or without OVA (100 μg·mL^{-1}) combined with TSLP (20 ng·mL^{-1}) for 48 h. On day 8, BM-DCs were treated with mitomycin C (50 μg·mL^{-1}) for 30 min and washed three times with Hank's balanced salt solution (HBSS), and then cells were collected for the following proliferation assay. Spleen CD4$^+$ T-cells were isolated from OVA-immunized mice (for the protocol, see Figure 4A) and purified by positive selection using anti-CD4$^+$ microbeads and the MiniMACs system according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA). The purity of T-cells was analysed by flow cytometry (CD4$^+$ T-cells: >95%). Freshly isolated CD4$^+$ T-cells (2×10^5 cells) were co-cultured with BM-DCs at a 1:10 DC/T-cell ratio in the presence or absence of OVA (5 μg·mL^{-1}) medium in round-bottomed 96-well microtiter plates. After 4 days, mixed cell cultures were pulsed with 5-bromo-2-deoxyuridine (BrdU) for measurement of cell proliferation. Briefly, 2 nmol of BrdU was added to each well, and the plate was incubated at 37°C for 8 h. The amount of BrdU incorporated into the T-cells was measured using the anti-BrdU monoclonal antibody in the enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Roche Applied Science, Germany). In siRNA-transfected BM-DC experiment, the numbers of CD4$^+$ T-cells is lower (1×10^5 cells), and cells were co-cultured with 1/10-fold of BM-DCs (1×10^4 cells). In some experiments, freshly isolated DO11.10 spleen CD4$^+$ T-cells were co-cultured with shikonin-treated BM-DCs for 4 days, and cell proliferation and cytokine production were detected as previously described.

Cytokine assays

Cell culture supernatants were collected 48 h after different drug treatments and stored at –20°C before analysis by ELISA according to the manufacturer's

instructions. Standards were prepared from recombinant mouse interferon (IFN)-γ, IL-4, IL-5, tumour necrosis factor (TNF)-α, IL-13, IL-10, and eotaxin (CCL11) (R&D Systems, Minneapolis, MN, USA).

Bronchoalveolar lavage and lung histology

Bronchoalveolar lavage, using 1 mL of HBSS instilled with a syringe, provided bronchoalveolar lavage fluid (BALF); cells were collected by gentle aspiration of the HBSS three times and then centrifuged (Miyabara *et al.*, 1998). Differential cell numbers were counted from cytology preparations. Cell slides were prepared using a cytospin and Liu staining. A total of 300 cells were counted under a light microscope. Supernatants of BALF were assayed by ELISA. Lungs were fixed with 10% neutral phosphate-buffered formalin, and sections were prepared and stained with haematoxylin/eosin (H&E) and periodic acid-Schiff (PAS) to quantify the number of infiltrating inflammatory cells and mucus production by microscopy. Airway inflammation was quantified by the number of inflammatory cells in subepithelial and subendothelial area, and expressed as the number of inflammatory cells per subepithelial and subendothelial area (mm^2) in lung sections. The number of PAS-positive and PAS-negative bronchial epithelial cells was determined in individual airways. Results were expressed as the percentage of PAS-positive cells per bronchiole.

Measurement of airway resistance in anaesthetized mice

Airway resistance was assessed as the increase in pulmonary resistance after challenge with aerosolized methacholine (MCh) in anaesthetized mice using a modification of the techniques described by Glaab *et al.* (2004). Mice were anaesthetized with 70–90 $mg \cdot kg^{-1}$ pentobarbital sodium (Sigma, St. Louis, MO, USA), tracheotomized and mechanically ventilated at a rate of 150 breaths per minute, with a tidal volume of 0.3 mL and a positive end-expiratory pressure of 3–4 cm H_2O with a computer-controlled small animal ventilator (Harvard Rodent Ventilator, model 683, South Natick, MA, USA). PE-50 tubing was inserted into the oesophagus to the level of the thorax and coupled to a pressure transducer (LDS GOULD, Valley View, OH, USA). Flow was measured by electronic differentiation of the volume signal. Pressure, flow and volume changes were recorded. Pulmonary resistance was calculated using a software program (Model PNM-PCT100W, LDS PONEMAH Physiology Platform, LDS GOULD). MCh aerosol was generated with an in-line nebulizer and given directly through the ventilator. Results were expressed as the pulmonary resistance (R_L) of three independent experiments.

OVA-specific and total serum antibody assay

ELISA determined total sera and anti-OVA immunoglobulin (Ig) E, IgG1 and IgG2a antibody titers. Briefly, 96-well microtiter plates were coated with OVA (1 μg per well) or predetermined concentrations of anti-mouse IgE, IgG1 or IgG2a (Pharmingen, San Diego, CA, USA) in $NaHCO_3$ buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed and blocked with 3% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Serum samples were diluted and added to each well overnight at 4°C. Plates were then washed. Either biotin-conjugated anti-mouse IgE, IgG1 or IgG2a (0.5 $mg \cdot mL^{-1}$, Pharmingen) diluted in 3% BSA-PBS buffer (1:500) was added for 45 min at room temperature. Avidin-conjugated horseradish peroxidase (1:5000, Pierce Biotechnology, Rockford, IL, USA) was then added for another 30 min at room temperature. The reaction was developed by peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD, USA) and then stopped with 2N H_2SO_4. Absorbance was determined at 450 nm in a microplate reader. Antibody levels were compared to standard serum, and IgG1, IgE and IgG2a concentrations in standard serum were arbitrarily assigned 1 ELISA unit.

Preparation of lymph node cells

Mediastinal lymph nodes were harvested and pooled from each group at the end of the experiment (day 43). Single-cell suspensions were obtained by mechanical disruption. Cells were stimulated *in vitro* with 10 $\mu g \cdot mL^{-1}$ OVA or 1 $\mu g \cdot mL^{-1}$ CD3 combined with 1 $\mu g \cdot mL^{-1}$ CD28 for 72 h. The cell medium was collected for cytokine analysis.

Lung mononucleocyte preparation

After death, lungs were perfused with 10 mL PBS through the right ventricle. Lungs were then removed and pooled from each group at the end of the experiment (day 43). After removal from animals, lungs were cut into small pieces, and single-cell suspensions were obtained by a stainless steel cell dissociation sieve. Debris was removed by using a cell strainer (100 μm, BD Biosciences, Franklin Lakes, NJ, USA). Then, mononucleocytes were isolated by Ficoll-Plaque Plus according the manufacturer's instructions (GE Healthcare, Sweden). Cells were stimulated *in vitro* with 10 $\mu g \cdot mL^{-1}$ OVA or 1 $\mu g \cdot mL^{-1}$ CD3 combined with 1 $\mu g \cdot mL^{-1}$ CD28 for 72 h. The cell medium was collected for cytokine analysis.

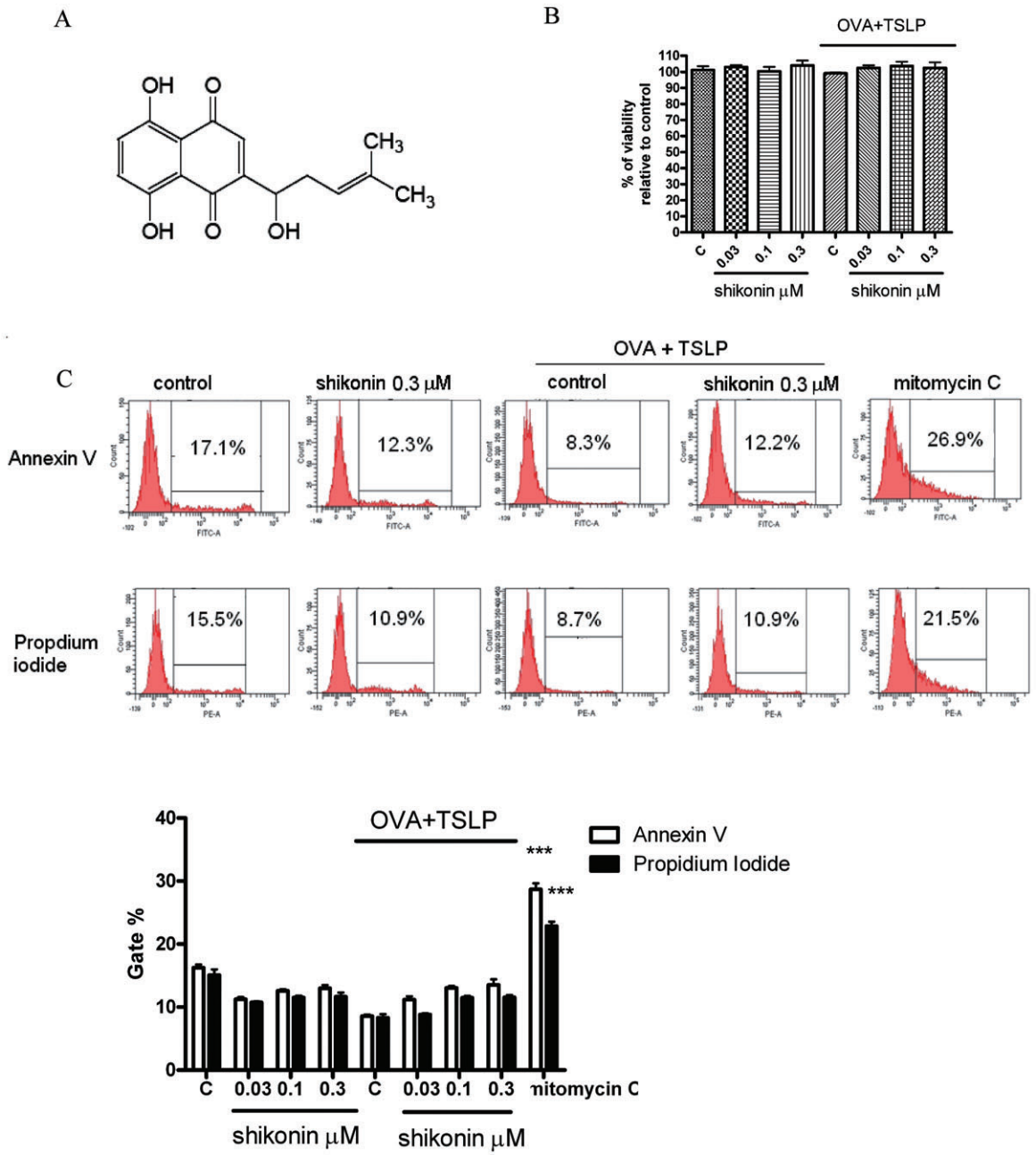


Figure 1

Shikonin inhibited the expression of surface markers on murine bone marrow-derived dendritic cells (BM-DCs). (A) Chemical structure of shikonin. (B) BM-DCs were prepared as described in Methods. BM-DCs were treated with shikonin for 10 min, then cultured with or without 100 μg·mL^{-1} ovalbumin (OVA) combined with 20 ng·mL^{-1} thymic stromal lymphopoietin (TSLP) for 48 h; cell viability was detected by Trypan blue exclusion. (C) Cells were stained with fluorescein isothiocyanate-labelled Annexin V and propidium iodide, and cell apoptosis was analysed by flow cytometry as described in Methods. BM-DCs treated with 25 μg·mL^{-1} mitomycin C for 6 h were used as a positive control. Histograms represent quantification of apoptosis % by gated region, which was analysed by flow cytometry. Data were expressed as mean ± SEM ($n = 3$). ***$P < 0.001$, compared with the control group (no treatment; C). (D) BM-DCs were treated with shikonin for 10 min and then cultured with or without 100 μg·mL^{-1} OVA combined with 20 ng·mL^{-1} TSLP for 48 h. Expression of surface markers was analysed by flow cytometry. The values shown in the flow cytometry profiles are the gated % and the mean fluorescence intensity indexes. Cells were gated on CD11c, and the incidence of CD11c$^+$ cells expressing the antigen of interest is indicated within each histogram. (E) Histograms represent quantification of surface marker expression analysed by flow cytometry. Data were expressed as mean ± SEM ($n = 3$–6). #$P < 0.001$, compared to the control, without OVA + TSLP, group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, compared to the control, with OVA + TSLP, group. MHC, major histocompatibility complex.

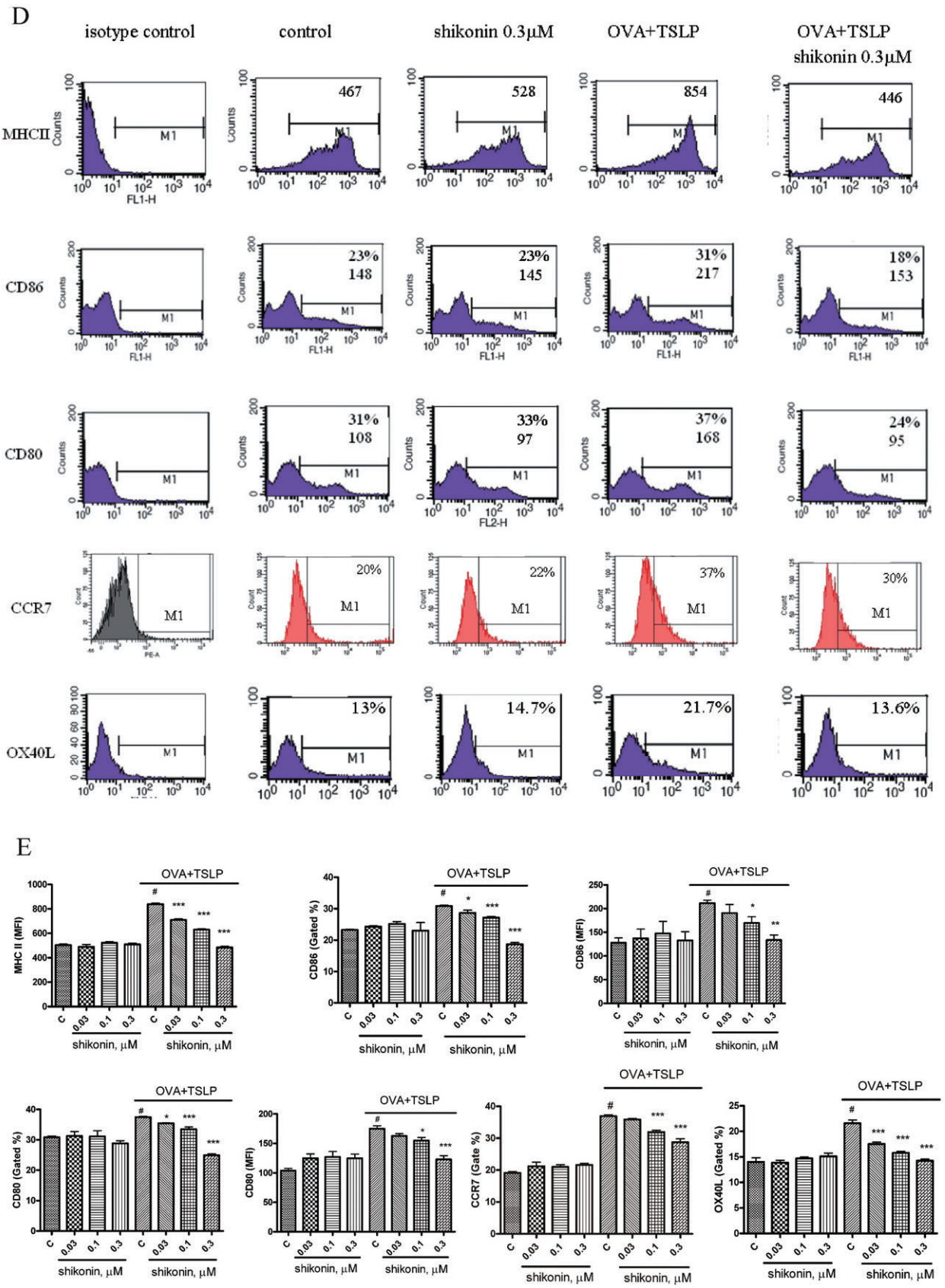


Figure 1

Continued.

Statistical analysis

Results are given as means ± SEM ($n \geq 3$). Group comparisons were performed by one-way ANOVA followed by Newman–Keuls *post hoc* test. $P < 0.05$ was considered significant.

Materials

Shikonin (Figure 1A) was purchased from EMD Chemical, Inc. (Darmstadt, Germany). OVA (grade V) was purchased from Sigma Chemical Co. RPMI 1640 medium, HBSS, penicillin, streptomycin, L-glutamine and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). TSLP was purchased from R&D Systems, Inc. siRNAs (for OX-40L and negative siRNA, designed and chemically modified to not known target genes in human, mouse and rat cells) were purchased from Dharmacon RNAi Technology (Thermo Fisher Scientific, Lafayette, CO, USA).

Results

Shikonin decreases OVA + TSLP-induced BM-DC surface marker expression

First, we evaluated possible cytotoxic effects of shikonin on BM-DCs. After treatments with shikonin at 0.03, 0.1 and 0.3 μM for 48 h, DCs did not show any necrosis as shown by Trypan blue exclusion assay (Figure 1B) or apoptosis by Annexin V and propidium iodide staining assays (Figure 1C). Next, we investigated surface marker expression. DCs treated with different concentrations of shikonin did not show any apparent changes for major histocompatibility complex (MHC) class II, CD80, CD86 or CCR7 expression (Figure 1D and E).

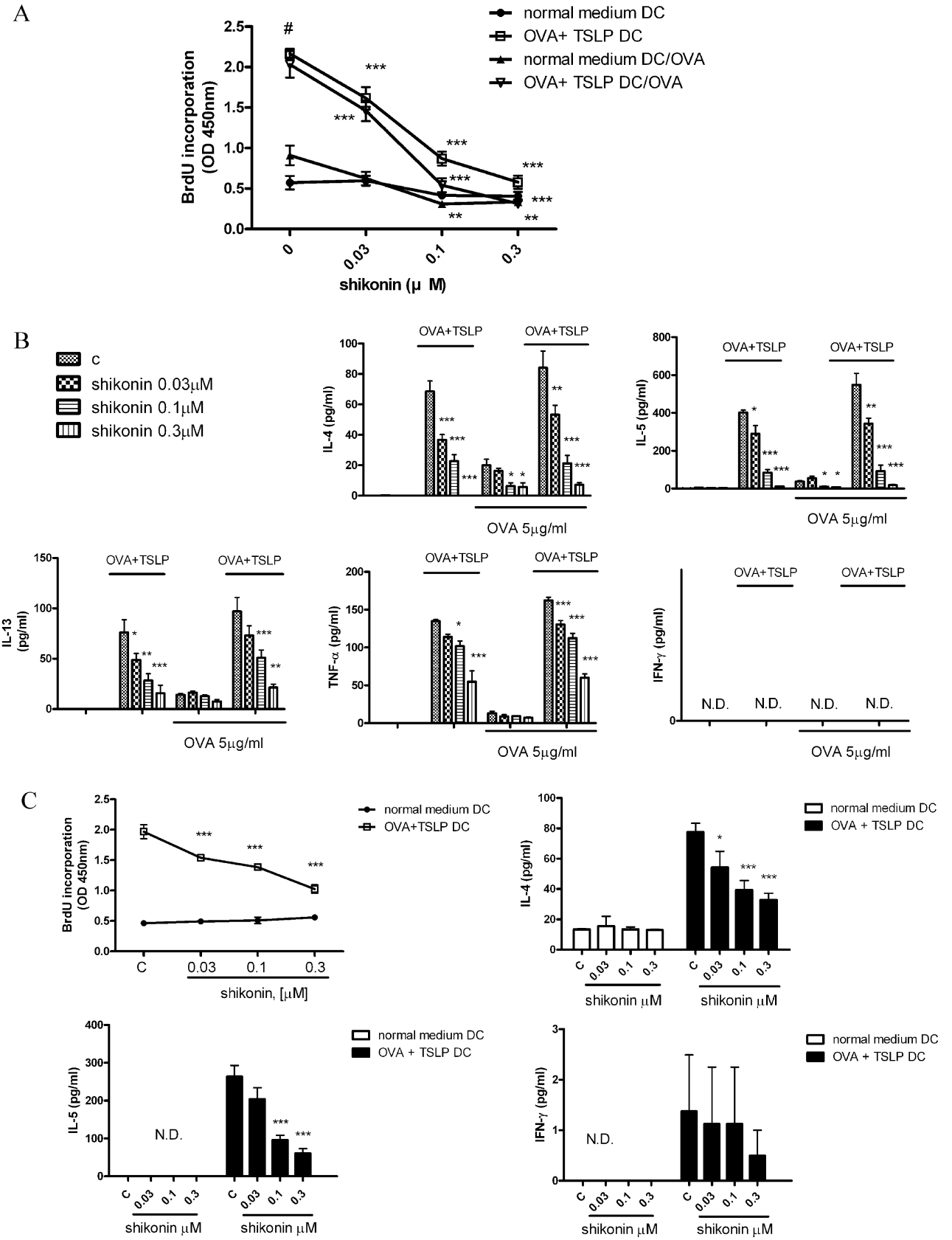
Because OVA is an allergen and TSLP released by epithelial cells can induce DC activation that is involved with T_H2 cell differentiation (Simecka, 1998), we used a mixture of OVA and TSLP (OVA + TSLP) to drive DCs to become T_H2-polarizing antigen-presenting cells. Shikonin (0.03, 0.1 and 0.3 μM) dose-dependently inhibited the OVA + TSLP-induced expression of MHC class II, CD80, CD86 and CCR7 (Figure 1E). Because TSLP-activated DCs can induce an inflammatory T_H2 response via the OX40 ligand (OX40L) (Ito *et al.*, 2005), we also evaluated OX40L expression on BM-DCs. After treatments with shikonin (0.03, 0.1 and 0.3 μM), OX40L expression on BM-DC did not show any apparent differences compared with the control group (Figure 1D and E). After treatment with OVA + TSLP, OX40L expression on BM-DC was significantly increased and shikonin dose-dependently inhibited this OVA + TSLP-induced OX40L expression (Figure 1E).

Shikonin-treated BM-DCs are poor stimulators of CD4 T lymphocytes and induce lower levels of cytokine release by responding T-cells

Next, we investigated the ability of shikonin-treated BM-DCs to inhibit the proliferation of OVA-immunized BALB/c spleen $CD4^+$ T-cells. $CD4^+$ T-cells treated with phorbol myristate acetate (5 ng·mL^{-1}) and ionomycin (1 μM) were used as a positive control and cell proliferation monitored by BrdU incorporation (Figure 2A). OVA + TSLP-treated DCs were twofold to 2.5-fold more efficient stimulators of $CD4^+$ T-cells, compared to untreated DCs (Figure 2A) and also induced increased production of IL-4, IL-5, IL-13 and TNF-α (Figure 2B). Shikonin dose-dependently decreased $CD4^+$ T proliferation, and

Figure 2

Shikonin dose-dependently inhibited $CD4^+$ T-cell responses. (A) On day 6 of culture, bone marrow-derived dendritic cells (BM-DCs) were treated with different concentrations of shikonin for 10 min and then cultured with or without ovalbumin (OVA) (100 μg·mL^{-1}) + thymic stromal lymphopoietin (TSLP) (20 ng·mL^{-1}) for 48 h. On day 8, BM-DCs were collected and treated with 50 μg·mL^{-1} mitomycin C for 30 min. To assay proliferation, fresh spleen $CD4^+$ T-cells isolated from OVA-immunized mice (see Figure 4A, for the protocol) were co-cultured with different treatments of BM-DCs at a 1:10 DC/T-cell ratio, in the presence or absence of 10 μg·mL^{-1} OVA for 4 days. Proliferation of $CD4^+$ T-cells was measured by uptake of BrdU with an ELISA kit, as described in Methods. Normal-medium DC groups represent DCs cultured in RPMI medium, with or without shikonin. OVA + TSLP DC groups represent DCs, treated with or without shikonin and then cultured in the RPMI supplement with OVA + TSLP. Normal medium DC/OVA or OVA + TSLP/OVA represent differently treated DCs cultured with T-cells in the presence of 10 μg·mL^{-1} OVA. (B) Cytokine-secreting pattern of OVA-immunized $CD4^+$ T-cells after stimulation with shikonin-treated BM-DCs. Shikonin-treated BM-DCs pulsed with or without OVA + TSLP were collected and treated with 50 μg·mL^{-1} mitomycin C for 30 min. BM-DCs were co-cultured at a 1:10 ratio with OVA-immunized $CD4^+$ T-cells in the presence or absence of 10 μg·mL^{-1} OVA. After 4 days, supernatants were collected, and levels of cytokine production were analysed by enzyme-linked immunosorbent assay (ELISA). Data were expressed as mean ± SEM ($n = 5$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, compared with the OVA + TSLP control group. (C) Shikonin dose-dependently inhibited responses of $CD4^+$ T-cells from spleens of DO11.10 mice. BM-DCs were treated with different concentrations of shikonin for 10 min and then cultured with or without 100 μg·mL^{-1} OVA combined with 20 ng·mL^{-1} TSLP for 48 h. Spleen $CD4^+$ T-cells freshly isolated from D011.10 mice were co-cultured at a 1:10 ratio with different treatments of BM-DCs. $CD4^+$ T-cell proliferation was measured by uptake of BrdU with an ELISA kit, as described in Methods. Supernatants were analysed for cytokine production by ELISA. Data were expressed as mean ± SEM ($n = 5$). #$P < 0.001$, compared to the control, without OVA + TSLP, group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, compared with the OVA + TSLP control group. N.D., not detected.


A
normal medium DC
OVA+ TSLP DC
normal medium DC/OVA
OVA+ TSLP DC/OVA
BrdU incorporation (OD 450nm)
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shikonin 0.03μM
shikonin 0.1μM
shikonin 0.3μM
OVA+TSLP
OVA 5μg/ml
IL-4 (pg/ml)
IL-5 (pg/ml)
IL-13 (pg/ml)
TNF-α (pg/ml)
IFN-γ (pg/ml)
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shikonin μM

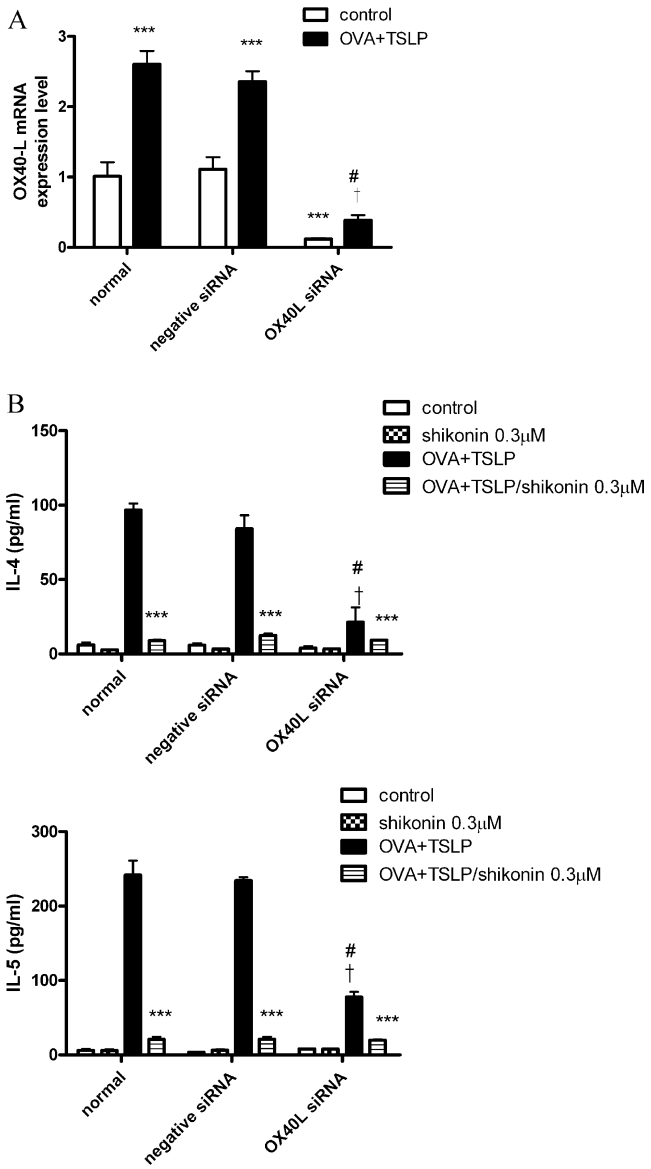


Figure 3

Inhibition of interleukin (IL)-4 and IL-5 production in T-cells by knockdown of OX40L siRNA. (A) Bone marrow-derived dendritic cells (BM-DCs) were transfected with 20 nM OX40L siRNA and negative siRNA by Lipofectamine 2000 for 72 h, and OX40L mRNA expression was detected by real-time PCR as described in Methods. (B) BM-DCs were transfected with 20 nM OX40L siRNA and negative siRNA for 72 h, treated with 0.3 μM shikonin for 10 min, and then cultured with or without ovalbumin (OVA) + thymic stromal lymphopoietin (TSLP) for 48 h. Freshly isolated spleen $CD4^+$ T-cells isolated from OVA-immunized mice (see protocol in Figure 4A) were co-cultured with different treatments of BM-DCs for 4 days. Supernatants were analysed for cytokine production by ELISA, as described in Methods. Data were expressed as mean ± SEM ($n = 3$). ***$P < 0.001$, compared with the OVA + TSLP group, respectively. #$P < 0.001$, compared with normal OVA + TSLP group; †$P < 0.001$, compared with the negative siRNA OVA + TSLP group.

IL-4, IL-5, IL-13 and TNF-α production was stimulated by OVA + TSLP-treated DCs. However, in the OVA + TSLP-treated DC group, OVA (5 μg·mL^{-1}) treatment did not stimulate IL-4, IL-5, IL-13 and TNF-α production. In this experiment, we did not detect any IFN-γ production among different treatment groups. We also compared the levels of cell proliferation and cytokine production in $CD4^+$ T-cells isolated from spleens of DO11.10 mice, which were co-cultured with shikonin-treated BM-DCs (Figure 2C). We found that the trend of $CD4^+$ T-cell proliferation and cytokine production was similar to $CD4^+$ T-cells isolated from OVA-immunized mice, but that the inhibition of OVA + TSLP-induced cell proliferation and IL-4 and IL-5 production after shikonin treatment was less than that observed in $CD4^+$ T-cells, isolated from OVA-immunized mice.

To clarify the contribution of OX40L to the inhibition of cytokine release in $CD4^+$ T-cells by shikonin in BM-DCs, we used RNA interference assays to knockdown OX40L expression. After transfection of OX40L siRNA in BM-DCs for 72 h, OX40L mRNA expression was significantly knocked down in control and OVA + TSLP groups (Figure 3A). BM-DCs were transfected with OX40L siRNA for 72 h and co-cultured with $CD4^+$ T-cells from OVA-immunized mice for 4 days. IL-4 and IL-5 expression was significantly inhibited in all OX40L siRNA-transfected groups, indicating that the IL-4 and IL-5 production induced by OVA + TSLP was mainly dependent on OX40L. Knockdown of OX40L expression in BM-DCs attenuated the stimulatory effects of T_H2 cells. Therefore, in co-cultures of CD4+ T-cells and BM-DCs with knockdown of OX40L, stimulation with OVA + TSLP induced relatively low levels of IL-4 and IL-5 secretion (Figure 3B). This reduced cytokine output was still sensitive to inhibition by shikonin (Figure 3B).

Intratracheal instillation of shikonin decreases bronchial alveolar lavage fluid, lung cell and mediastinal lymph node cell T_H2 cytokine levels in a murine model of asthma

We examined the effects of shikonin in a murine model of asthma. Mice were sensitized to OVA and challenged with i.n. OVA droplet aspiration, as shown in Figure 4A. Mice were treated once a day with shikonin on days 35–39 (five treatments) (Figure 4A). One day after the final challenge, we measured cytokine contents in the BALF.

Mice sensitized and challenged with OVA (OVA groups) showed increases in IL-4, IL-5, IL-13, TNF-α and eotaxin release in BALF compared to control, PBS-sensitized and challenged mice (PBS groups; Figure 4B). After treatment with shikonin, IL-4, IL-5, IL-13, TNF-α and eotaxin levels in BALF were

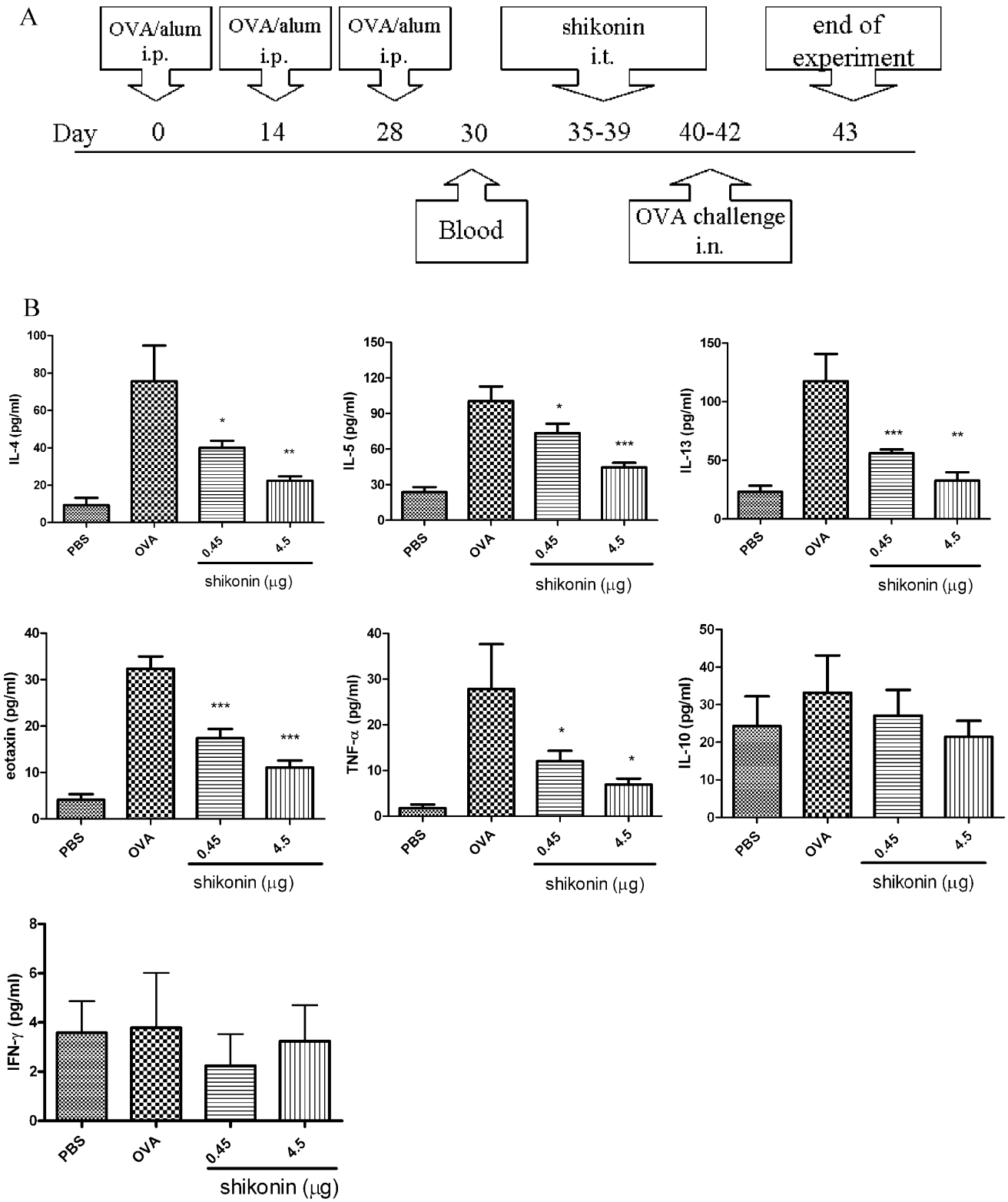


Figure 4

Suppression of cytokine levels in BALF after administration of shikonin in a murine model of asthma. (A) Brief scheme of animal sensitization and challenge. (B) Cytokine levels in BALF were analysed by enzyme-linked immunosorbent assay. Data were expressed as mean ± SEM (n = 8–10). The experimental groups consisted of a PBS control group (PBS sensitized and challenged) and the other groups sensitized and challenged with ovalbumin (OVA) (OVA groups). Shikonin treatment is shown as the daily dose (μg) given i.t. for 5 days. Data were expressed as mean ± SEM (n = 8–10). *P < 0.05; **P < 0.01; ***P < 0.001 different from the OVA group without shikonin.; i.n., intranasal; i.t., intratracheal.

dose-dependently decreased. However, IFN-γ and IL-10 levels in BALF were not affected either by challenge with OVA or by treatment with shikonin.

We also investigated whether shikonin inhibited T lymphocyte activation in lung and mediastinal lymph nodes. As shown in Supporting Information Figure S1 (see Supporting Information), the number of lung and mediastinal lymph node cells were increased in OVA-immunized mice (positive group). After mice were treated with shikonin, the number of lung and mediastinal lymph node cells was significantly decreased, as compared with the positive group (Supporting Information Figure S1A). As shown in Supporting Information Figure S1B and S1C, treatment with a mixture of CD3 and CD28 (CD3 + CD28) induced IL-4, IL-5, and IFN-γ production in lung cells and mediastinal lymph node cells, isolated from OVA-immunized mice. Shikonin inhibited this induced IL-4 and IL-5 production but not that of IFN-γ. Note that in OVA-treated lung cell and mediastinal lymph node cell groups, levels of IL-4 and IL-5 production were lower than those in mice treated with (CD3 + CD28).

Inhibition of allergen-induced airway inflammation by shikonin

To investigate the effects of shikonin on airway inflammation, we analysed the cellular composition in BALF at 48 h after the last three OVA challenges (days 42–44). In PBS-sensitized and challenged mice (PBS group), no obvious inflammatory cells were noted in BALF. However, after sensitization and challenge with OVA (OVA group), the numbers of macrophages and eosinophils in BALF were significantly increased (Figure 5A). After intratracheal instillation of shikonin, challenge of the OVA group induced less eosinophils in BALF, although macrophage numbers were not affected (Figure 5A).

Histological examination of lung sections from the OVA group showed a large number of infiltrating inflammatory cells and increased mucus formation around the airway, compared to that in the PBS group (Figure 5B). In addition, we found that the peribronchial inflammation was more severe than the perivascular inflammation, as shown in the H&E-stained lung sections from the OVA group (Figure 5B). After treatment with shikonin, challenge of the OVA group showed clearly less inflammatory cell infiltration and mucus formation than in OVA mice without shikonin treatment.

Intratracheal delivery of shikonin suppresses the development of airway hyperresponsiveness

We investigated if shikonin could affect the development of airway hyperresponsiveness in a murine model of asthma. One day after the final challenge, airway responsiveness was assessed by pulmonary resistance using invasive body plethysmography. BALB/c mice sensitized and challenged with OVA showed increased lung resistance (R_L) to MCh inhalation compared to PBS-sensitized and challenged mice (Figure 5C; OVA group and PBS group). After treatment with shikonin, R_L levels were significantly inhibited compared with the untreated OVA group.

Shikonin did not affect allergen-specific and total immunoglobulin levels in serum

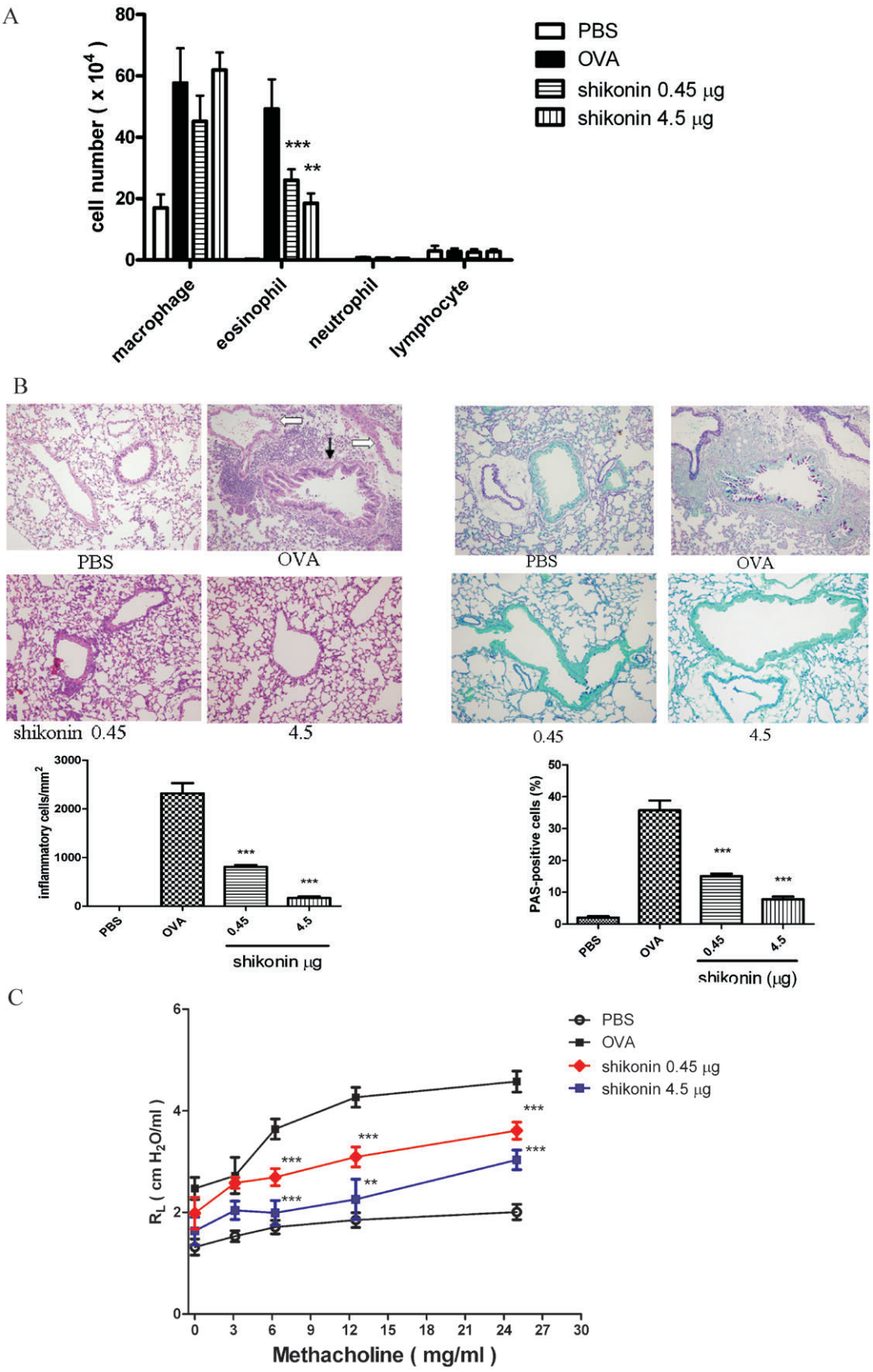
On day 43, mice were bled to examine serum immunoglobulin levels. Compared to the PBS group, OVA-specific and total serum IgE levels in OVA-immunized groups, with or without shikonin-treatment, were significantly increased (Figure 6). However, OVA-specific and total serum IgE levels were not altered by treating the OVA groups with shikonin at either dose (Figure 6). Also, no difference was observed in the OVA-specific and total serum IgG2a and IgG1, T_H1 and T_H2 response-related immunoglobulins.

Discussion and conclusions

In this study, we used OVA + TSLP to activate BM-DCs into T_H2-polarizing antigen-presenting cells. These cells expressed higher levels of surface

Figure 5

Airway inflammation and hyperresponsiveness inhibited by shikonin. Profile of cells in BALF and histological analysis of lung tissue in mice after shikonin treatment, shown as the daily dose (μg) given i.t. for 5 days. (A) Total cell counts were determined on 1 mL BALF and differential cell counts were assessed by Liu staining. Data were expressed as mean ± SEM ($n \geq 10$) a*$P < 0.05$, different from the ovalbumin (OVA) group; b*$P < 0.05$ different from the PBS group. (B) Lung sections were stained with haematoxylin/eosin (H&E) for measurement of inflammatory cells and periodic acid-Schiff (PAS) for measurement of mucus production around the airways. Data revealed a different extent of cellular infiltration of the peri-airway region. Original magnification, ×200. Quantification of infiltration was by counting the number of inflammatory cells per mm^2 of subepithelial and subendothelial area. Mucus-producing cells were quantified as the percentage of PAS-positive cells per bronchiole. Data were expressed as mean ± SEM ($n = 5$). ***$P < 0.001$, compared with the OVA group. In the H&E-stained lung section of the OVA group, the bronchiole is indicated with a black arrow and the vessel is indicated with a white arrow. (C) Airway resistance as measured by invasive body plethysmography. Data were expressed as mean ± SEM of the value of pulmonary resistance (R_L) ($n = 5$). **$P < 0.01$; ***$P < 0.001$, versus the OVA group.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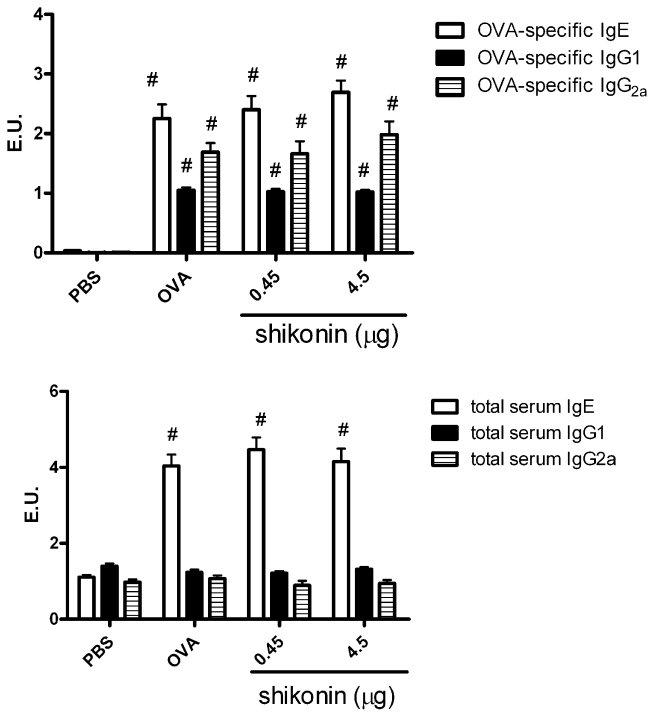

Figure 6

Serum levels of ovalbumin (OVA)-specific and total IgE, IgG1 and IgG2a in sensitized (OVA) and non-sensitized (PBS) mice. On day 43, blood samples were obtained, and OVA-specific and total IgE, IgG1, and IgG2a concentrations were measured by enzyme-linked immunosorbent assay (ELISA) ($n = 6$). Shikonin treatment is shown as the daily dose (μg) given i.t. for 5 days. No differences in serum levels of OVA-specific and total IgE, IgG1, and IgG2a were observed among the different groups of shikonin-treated mice. Data were expressed as mean ± SEM. #$P < 0.05$, different from the PBS group. Antibody levels were compared to standard serum, and IgG1, IgE and IgG2a concentrations in standard serum were arbitrarily assigned 1 ELISA unit (1 EU).

markers (MHC II, CD80, CD86, CCR7 and OX40L) and stimulated OVA-immunized $CD4^+$ T-cell proliferation and their release of IL-4, IL-5, IL-13 and TNF-α. This is the first study that has used OVA + TSLP-activated BM-DCs to provide a test system form compounds with therapeutic potential for asthma. Using DCs as a cell model to evaluate potential treatments in immune diseases has been widely applied in cancer (Ding *et al.*, 2009), autoimmune disease (Baldwin *et al.*, 2010) and allergic diseases (Lin *et al.*, 2005). In this study, we found that shikonin showed therapeutic potential for allergic asthma through several mechanisms.

First, shikonin dose-dependently inhibited OVA + TSLP-induced BM-DC maturation by decreasing the cell surface expression of MHC class II molecules, the co-stimulatory molecules CD80, CD86, OX40L and CCR7. Unlike other DC stimuli, such as CD40L and toll-like receptor ligands, TSLP does not stimulate myeloid DCs to produce the polarizing cytokine IL-12 or the pro-inflammatory cytokines TNF-α, IL-1β and IL-6. TSLP triggers myeloid DCs to produce an array of different chemokines, including CCL11, IL-8 (CXCL8), thymus and activation regulated chemokine [TARC (CCL17)] and I-309 (CCL1), which are important for recruiting eosinophils, neutrophils and T_H2 cells, respectively (Ito *et al.*, 2005). OX40L was identified as a key molecule expressed by TSLP-activated DCs using microarray analyses. OX40L is expressed on DC surfaces and acts as a co-stimulatory signal for T_H2 priming and memory induction. In addition, previous studies have demonstrated that DCs activated by TSLP can prime naïve $CD4^+$ T-cells to differentiate into T_H2 cells via OX40L (Ito *et al.*, 2005) and produce chemokines that attract eosinophils, T_H2 cells and neutrophils to induce innate allergic responses and cause additional adaptive allergic responses (Wang and Liu, 2009). In our study, we found that DCs, which had been pulsed with OVA + TSLP, activated $CD4^+$ T-cells to release IL-4 and IL-5 and that this release was mainly dependent on OX40L. In addition, after knockdown of OX40L expression in DCs, IL-4 and IL-5 release in activated $CD4^+$ T-cells was attenuated by shikonin treatment. This indicates that shikonin inhibited T_H2 cytokine release in $CD4^+$ T-cells possibly through other pathways whose elucidation needs further investigation.

In addition, we found that the levels of CD80 and CD86 expression for OVA + TSLP-treated DCs were twofold lower than 100 $ng \cdot mL^{-1}$ lipopolysaccharide-treated DCs, and there was no IL-12 released into the culture medium (data not shown). A previous study also found that allergen-fungal protease-treated DCs expressed lower levels of surface markers (CD80 and CD86) and produced less IL-12 (Lamhamedi-Cherradi *et al.*, 2008). However, the mechanisms by which different extracellular stimuli that activate T_H1-polarizing and T_H2-polarizing DC maturations show different functional properties is still unknown and needs further investigation.

Second, shikonin-treated BM-DCs altered stimulation of T lymphocyte growth and differentiation. Shikonin dose-dependently inhibited OVA + TSLP-induced OVA-immunized $CD4^+$ T-cell proliferation and T_H2 cytokine release. In addition, we did not detect IFN-γ production, which could also account for the absence of IL-12 production in OVA + TSLP-stimulated BM-DCs.

Third, in a murine model of asthma, shikonin showed therapeutic potential *in vivo* by reducing T_H2 cytokine release in BALF and lung and mediastinal lymph node cells, and by inhibiting airway inflammation and airway hyperresponsiveness. We found that shikonin did not inhibit T-cells, stimulated with (CD3 + CD28), from releasing IFN-γ in

lung and mediastinal lymph node cells, which might indicate that shikonin specifically attenuates activation of T_H2 cells induced by (CD3 + CD28). In addition, because serum total and OVA-specific IgE, IgG1 and IgG2a levels did not show any apparent changes after intratracheal instillation of shikonin, we suggest that intratracheal shikonin had a local effect in lung, but not a systemic effect, on allergic responses.

As *in vitro* studies have shown shikonin to inhibit allergic reactions, in terms of inhibition of LTB_4 biosynthesis (Wang *et al*., 1994) and suppression of mast cell degranulation (Wang *et al*., 1995), shikonin might suppress airway inflammation *in vivo* through different mechanisms. In addition, from the data presented here, it is not possible to conclude that there is a direct link between the inhibition of allergic inflammation by shikonin *in vivo* and the *in vitro* effect upon DC maturation. Further investigation is needed. A previous study found that shikonin caused necrosis and apoptosis in HL-60 and K562 cell lines (Han *et al*., 2009). In our study, the highest shikonin dose was 30-fold lower than that used in the study of Han *et al*., and we did not observe any necrotic or apoptosis effects in BM-DCs. Thus, cell apoptosis or necrosis *in vitro* did not contribute to the effects we observed of shikonin in allergic asthma.

In conclusion, we have described a model of BM-DCs stimulated by OVA + TSLP as an experimental system to assess compounds for effects on the development of allergic diseases. We also demonstrated that shikonin effectively suppressed OVA + TSLP-induced BM-DC maturation *in vitro* and inhibited allergic inflammation and airway hyperresponsiveness in a murine model of asthma. Therefore, this study provides a novel platform for screening drugs for allergic diseases and shikonin was shown to have clear potential as a drug for allergic asthma.

Acknowledgements

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Conflict of interest

No conflicts of interest to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Shikonin suppressed IL-4 and IL-5 production in lung cells and mediaterial lymph node cells in a murine model of asthma. (A) Lung cells and mediaterial lymph node cells were isolated from naïve or OVA-immunized mice, and cells were counted by a hemocytometer. Lung cells (B) and mediaterial lymph node cells (C) were treated with 1 μg·mL^{-1} CD3 + 1 μg·mL^{-1} CD28, or 10 μg·mL^{-1} OVA, for 72 h, and medium was collected for

investigation of cytokine production by ELISA. Data were expressed as mean ± SEM (n = 6). #P < 0.05, compared with the respective negative group; *P < 0.05; **P < 0.01; ***P < 0.001, compared with the respective positive group. n.d., not detected.

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