

Radix Saposhnikovia extract suppresses mouse allergic contact dermatitis by regulating dendritic-cell-activated Th1 cells

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ABSTRACT

Background: Radix Saposhnikovia (RS), called “Fangfeng” in China, is commonly used in Chinese medicinal formulae to treat allergic and inflammatory diseases. However, the underlying mechanisms of RS in ameliorating allergy remain unknown.

Purpose: To study the effects of RS extract on allergic contact dermatitis (ACD) in a mouse model and to investigate the underlying mechanisms *in vivo* and *ex vivo*.

Methods: ACD was induced by sensitizing the mice and treating an ear auricle with 1-chloro-2,4-dinitrobenzene (DNCB). RS extract was administered during the sensitization and/or elicitation phase. Ear swelling was noted and lymphocytic infiltration was investigated with hematoxylin and eosin staining. The cytokines in the sera and the supernatants of lymphocyte cultures were determined with enzyme-linked immunosorbent assays. Lymphocyte proliferation was assessed with a 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The maturation of dendritic cells (DCs) and the differentiation of T cells were examined with flow cytometry. The mRNA expression of *T-bet*, *GATA-3*, and forkhead box p3 (*Foxp3*) was evaluated with real-time PCR.

Results: RS extract (1.3 or 2.6 g/kg) markedly reduced the ear swelling and the intense cellular infiltration of inflammatory cells in the ear tissue. The ratio of interferon γ (IFN- γ)/interleukin 4 (IL-4) was reduced in the sera of the DNCB-sensitized mice and the lymphocyte culture supernatants after treatment with the extract. Further study of the initial stage of ACD revealed that RS extract prevented the differentiation of naïve T cells into Th1 cells, reduced the proportion of CD3⁺CD4⁺ (Th) cells, and suppressed the secretion of IFN- γ and the expression of *T-bet* mRNA in lymphocytes. The RS extract also reduced the proportion of DCs in the sensitized mouse lymphocytes and the expression of CD40⁺CD86⁺ cells in the DCs.

Conclusion: RS extract is effective in treating ACD because it regulates the development of DCs and DC-activated Th1 differentiation.

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Introduction

The prevention and treatment of allergic diseases are a global research focus because these illnesses are recurrent. Allergic contact dermatitis (ACD) is always induced by the delayed-type allergic response, which is part of the skin-sensitization-specific effector-T-cell-mediated immune response (Saary et al. 2005). Dendritic cells (DCs)

play a key role in the initiation of the immune response during the development of ACD. Once haptens or allergens enter the epithelia, DCs react to these antigens and stimulate the acquired immune response. Therefore, DCs play a crucial role in the pathogenesis of allergic diseases (Aiba 2007). Haptens, such as 1-chloro-2,4-dinitrobenzene (DNCB), can induce DC maturation. In this process, DCs undergo significant changes in the expression of the many molecules through which they interact with T cells, including CD86, CD40, CD134/OX40L, and several chemokine receptors (Steinman 2003). Activated DCs start to migrate via the afferent lymphatics to the draining lymph nodes, where they stimulate T-cell differentiation into T-helper 1 (Th1) cells, Th2 cells, Th3 cells, or regulatory T cells (Kadowaki 2007). DCs influence the Th1/Th2 bias, depending not only on their lineage, but also on the microenvironment of the cytokines and/or inflammatory mediators and the maturation status of the DCs. For example, the

Abbreviations: ACD, allergic contact dermatitis; ConA, concanavalin A; Dex, dexamethasone sodium phosphate; DCs, dendritic cells; DNCB, 1-chloro-2,4-dinitrobenzene; DER, drug extract ratio; RS, Radix Saposhnikovia; RSDs, relative standard deviations; RRT, relative retention time; RPA, relative peak area; TCM, traditional Chinese medicine; Th, helper T cells.

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Th1-inducing profile includes the interleukin 12 (IL-12) family members and interferon (IFN), whereas the Th2-inducing profile includes IL-4 and IL-10 (Langenkamp et al. 2000).

Radix Saposhnikovia (RS), the dry root of *Saposhnikovia divaricata* (Turcz.) Schischk (syn. *Ledebouriella divaricata* (Turcz.), Umbelliferae), also called “Fangfeng”, is commonly used in Chinese medicinal formulae for the treatment of some allergic diseases, such as ACD (Lin et al. 2014), urticaria (Lin et al. 2013), and allergic rhinitis (Chan and Chien 2014), in China. RS is a major part of the Chinese drug pair composed of RS and *Schizonepeta tenuifolia* (Benth.) Briq. (syn. *Nepeta tenuifolia* Benth, Lamiaceae), and an ethyl acetate extract of this drug pair lessens ear swelling and capillary permeability, reducing the duration of the itching reaction in mouse allergic dermatitis (Zhe et al. 2013). The chromones of RS are well known as a group of beneficial compounds, including prim-O-glucosylcimifugin, cimifugin, 4'-O- β -D-Glucosyl-5-O-methylvisamminol, and sec-O-Glucosylhamaudol, which are predominantly derived from RS (Dai et al. 2008; Gui et al. 2011). They are used as standards in the quality control of RS and its products (Xiao et al. 2001a; Xiao et al. 2001b). Studies have shown that prim-O-glucosylcimifugin, the most strongly represented chromone in RS, exerts a significant anti-inflammatory effect on xylene-induced mouse ear inflammation (Xue et al. 2000). However, the effects of RS on allergy and the possible underlying mechanisms remain unclear.

In this study, we explored the relationships between the effects of RS extract, the activation of T cells, and the T-cell differentiation mediated by DCs. We addressed this issue by investigating the effects of RS extract on DNCB-induced ACD by observing the pathological changes, and the changes in inflammatory cytokine expression, Th subsets, and the expression of Th-related transcription factors that occur after treatment. We also investigated the surface marker CD11c and the costimulatory molecules of DCs during treatment with RS extract.

Materials and methods

Reagents

4'-O- β -D-Glucosyl-5-O-methylvisamminol, cimifugin, prim-O-glucosylcimifugin, and sec-O-Glucosylhamaudol standard were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. 1-Chloro-2,4-dinitrobenzene (DNCB), concanavalin A (Con A), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Dexamethasone sodium phosphate (Dex) was purchased from Tianjin Kingyork Group Co., Ltd. (Tianjin, China). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Gaithersburg, MD, USA). Penicillin and streptomycin were purchased from Shandong LuKang Pharmaceutical Co., Ltd. (Shandong, China). Fluorescein-isothiocyanate (FITC)-conjugated anti-mouse CD3 and CD11c antibodies, phycoerythrin (PE)-Cy5-conjugated anti-CD4 antibody, PE-conjugated anti-CD40 antibody, allophycocyanin (APC)-conjugated anti-CD86 antibody, mouse regulatory T-cell staining kit, mouse IL-4 enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go! and mouse IFN- γ ELISA Ready-SET-Go! kits were purchased from eBioscience, Inc. (San Diego, CA, USA). TRIzol Reagent was purchased from Life Technologies Corp. (Carlsbad, CA, USA). The RevertAid First Strand cDNA Synthesis Kit was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The QuantiFast SYBR Green PCR Kit was purchased from Qiagen Co., Ltd. (Hilden, Germany).

Animals

BALB/c mice of both sexes were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. All animals were maintained at

Nanjing University of Chinese Medicine under specific-pathogen-free conditions at 22–25 °C and 50–60% humidity, and were used at 6–10 weeks of age. All procedures involving animals were approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine and conducted strictly according to the Guide for the Care and Use of Laboratory Animals (USA, eighth edition).

Plant material

Radix Saposhnikovia, the dry root of *Saposhnikovia divaricata*, was purchased from Nanjing Pharmaceutical Company (Nanjing, China). The plant name has been checked with www.theplantlist.org, and it is called “Fangfeng” in Chinese. The botanical identification was confirmed by Professor Chungen Wang (Nanjing University of Chinese Medicine, China). A voucher specimen (JSLPS 0016) is available at Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica.

Preparation of RS extract

RS (2 kg) was crushed and passed through a 60 mesh sieve. It was then immersed in ethanol:water (80:20, v/v; 100 g per 1.0 l) for 1 h and refluxed for 2 h. This extraction procedure was repeated twice. The herbal residue was then refluxed twice for 2 h each in 1 l of distilled water. The extracts were combined, filtered, and evaporated with a vacuum concentrator system (CH-9230; Buchi Labortechnik, Flawil, Switzerland) at 60 °C. Finally, we obtained 200 ml liquid extract from Radix Saposhnikovia, (DER = 10.2:1). First extraction solvent: ethanol 80% (v/v). The extract with a concentration of 10 g crude drug/ml was stored at –20 °C.

Fingerprint chromatogram generated with high-performance liquid chromatography (HPLC)

The RS extract was dissolved in 50% (v/v) methanol in distilled water to a concentration of 0.5 g crude drug/ml, centrifuged at 10,000 $\times$ g for 5 min, and filtered through a 0.45 μ m nylon membrane filter before HPLC analysis. The standards (4'-O- β -D-Glucosyl-5-O-methylvisamminol, cimifugin, prim-O-glucosylcimifugin, and sec-O-Glucosylhamaudol) were prepared with similar methods, dissolved in 50% methanol to a concentration of 0.5 mg/ml, centrifuged at 10,000 $\times$ g for 5 min, and filtered through a 0.45 μ m nylon membrane filter.

The RS extract was analyzed with HPLC using an Agilent Series 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode-array detector (DAD) connected to the Agilent ChemStation software. An InnovationTM Explorer C18 column (4.6 $\times$ 250 mm, 5 μ m; Chrom-Matrix) was used at a column temperature of 25 °C. The mobile phase consisted of (A) acetonitrile and (B) water, with a gradient elution of 0–3% (v/v) A at 0–5 min, 3–25% A at 5–25 min, 25–50% A at 25–45 min, 50–80% A at 45–70 min, and 80–3% A at 70–90 min. The flow rate was 1.0 ml/min and the wavelength of the DAD detector was 300 nm. The injection volume was 10 μ l. The standard calibration curve was constructed in the concentrations of 250, 125, 62.5, 31.25, 15.625, 7.8125 mg/l for prim-O-glucosylcimifugin, cimifugin, 4'-O- β -D-Glucosyl-5-O-methylvisamminol, and sec-O-Glucosylhamaudol. The chromatographic peaks were confirmed by comparing the retention time with those of a reference standard and quantification was performed by peak integration using the external standard method. All chromatographic operations were performed at room temperature.

Mouse ACD model and the initial stage of ACD

Mouse ACD model: After acclimatization for three days, the abdomens of the BALB/c mice were shaved with a razor over an area of about $2 \times 2 \text{ cm}^2$. The abdominal skin of the mice was treated with 5% DNCB in 80 μl of acetone on days 1 and 2, and the right ear was treated with 50 μl of 3% DNCB solution on day 6. Acetone was used as the vehicle control on the left ears. The mice were killed on day 7 (24 h after elicitation), and a patch (8 mm diameter) was punched from the left and right ears and the ear weight was calculated. Mouse blood was collected and centrifuged at $900 \times g$ for 15 min, and the sera were collected for cytokine analysis. The histopathological changes in the ears were examined with hematoxylin and eosin (H&E) staining. The draining lymph nodes were isolated for lymphocyte culture.

The initial stage of ACD: The BALB/c mice were sensitized with 80 μl of 5% DNCB solution on day 1 and day 2 (40 μl applied to the shaved abdominal skin, and 20 μl to both ears). The mice were killed 12 h after sensitization, and their draining lymph nodes were isolated for lymphocyte culture.

Treatment with RS extract

To investigate the effects of RS on ACD, the mice were randomly assigned to one of seven groups ($n = 8$ per group): normal, model (DNCB-induced ACD mice), Dex (Dex + DNCB-treated mice), and another four groups in which the mice were treated with DNCB + different doses of RS extract (1.3, 2.6, 5.2, or 10.4 g/kg bodyweight). The establishment of the ACD model has been described above. In this model, the RS extract was given intragastrically (0.1 ml/10 g) to the mice on days 1–7 (once daily). Dex (0.65 mg/kg), injected intraperitoneally (0.1 ml/10 g), was used as the positive control. The normal and model mice were given distilled water.

To determine whether the RS extract inhibited the allergic inflammation of ACD when given intragastrically in the sensitization phase of the model, the mice were randomly assigned to one of five groups ($n = 8$ per group): normal, model, Dex, and two RS extract groups (1.3 and 2.6 g/kg bodyweight). The RS extract was given intragastrically to BALB/c mice once daily on days 1–5 during the development of the ACD model. Dex (0.65 mg/kg) was used as the positive control.

To investigate the anti-allergy mechanisms of the RS extract, different RS concentrations (1.3 and 2.6 g/kg) were given intragastrically once daily for four consecutive days (days 0–3) to mice in the initial stage of the model.

Histological observations

The mice were killed and their right ears were removed and immediately fixed in 10% buffered formalin. The tissue was then processed and embedded in paraffin. Tissue sections (5 μm) were prepared and stained with H&E to investigate the histological changes and cutaneous cell infiltration. Micrographs were taken under an optical microscope (Olympus, Tokyo, Japan) at $\times 200$ magnification.

Lymphocyte culture and measurement of cell proliferation

The draining lymph nodes in the neck, armpit, and groin of the ACD mice treated with or without RS extract were removed, ground, and filtered through a 400 mesh sieve under aseptic conditions. Then lymphocytes were washed, collected, and seeded in 96-well plates (6×10^5 cells/well). They were stimulated with Con A (10 $\mu\text{g}/\text{ml}$) for 48 h at 37°C in 5% CO_2 , and the cell culture supernatant were then collected for cytokine quantification. Cell proliferation was measured with an MTT assay. Absorbance was measured at 570 nm with a SynergyTM HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA). All experiments were performed in triplicate.

Measurement of cytokine levels with ELISAs

ELISAs were used to measure the IFN- γ and IL-4 production in the sera and cell culture supernatants, with the Mouse IFN- γ ELISA Ready-SET-Go! and Mouse IL-4 ELISA Ready-SET-Go! kits, respectively (eBioscience), according to the manufacturer's instructions. Absorbance was measured at 450–570 nm and the sample concentrations were determined from a standard curve.

Flow-cytometric analysis of cell phenotypes

The draining lymph nodes in the necks, armpits, and groins of the ACD mice treated with or without RS extract were removed, ground, and filtered through a 400 mesh sieve under aseptic conditions. The expression of surface markers on the lymphocytes was then analyzed with flow cytometry. Briefly, 1×10^6 lymphocytes were washed with phosphate-buffered saline (PBS), then incubated with 0.5 μg of FITC-conjugated anti-CD3 antibody and 0.03 μg of PE-Cy5-conjugated anti-CD4 antibody for 15 min in dark. After repeated washes with PBS (1 ml per time), the doubly stained cells were resuspended in PBS and analyzed with a FACSCanto flow cytometer and analyzed with the FACSDive software (BD Biosciences, San Jose, CA, USA). The expression of surface markers and costimulatory molecules on DCs was also determined with flow cytometry. Similar to the method described above, 1×10^6 lymphocytes were incubated with 0.125 μg of FITC-conjugated anti-CD11c antibody, 0.25 μg of PE-conjugated anti-CD40 antibody, and 0.03 μg of APC-conjugated anti-CD86 antibody for 15 min in the dark. All other processes were as described above.

Quantitation of mRNA expression

Total RNA was isolated from the lymph nodes with TRIzol Reagent, according to the manufacturer's protocol, and reverse-transcribed to cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The resulting cDNA was amplified with the QuantiFast SYBR Green PCR Kit. The β -actin gene was used as the housekeeping control against which to normalize the expression of the target genes. The assays were performed in the iCycler iQTM Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The expression of each mRNA was quantified by relating the PCR threshold cycle obtained from the samples to amplicon-specific standard curves. All data are presented as means $\pm$ standard deviations (SD). The primers were synthesized by Invitrogen and the primer sequences were: β -actin, 5'-CCTGTACGCCAACACAGTGC-3' and 5'-ATACTCTGCTTGCTGATCC-3'; Foxp3, 5'-GGAGAAAGCGGATACCAAA-3' and 5'-TCTGTGAGGACTACCGAGCC-3'; T-bet, 5'-GTTGGAGGTGTCTGGGAAGC-3' and 5'-CCGGCCACGGTGAAGGA-3'; and GATA-3, 5'-GGCAGTATCCAGCACAGAA-3' and 5'-TGGTAGAGTCCGAGGCAT-3'.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Data sets with multiple comparisons were evaluated with one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test. Values of $p < 0.05$ were deemed to be significant.

Results

HPLC fingerprint chromatogram of RS extract

We used an HPLC method for the quality control assessment of RS extract. There were 20 common peaks in the chromatograms of the RS extracts recorded at 300 nm (Fig. 1A). Four corresponded to the standards for prim-O-glucosylcimifugin, cimifugin, 4'-O- β -D-Glucosyl-5-O-methylvisaminol, and sec-O-Glucosylhamaudol (Fig. 1B, Table 1). All the main peaks were well separated.

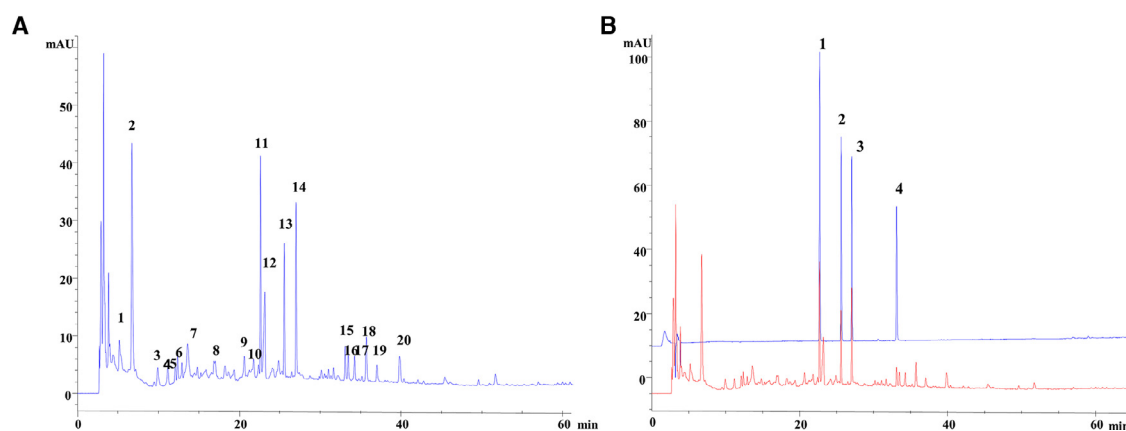


Fig. 1. HPLC fingerprint of RS extract (A) and comparative chromatograms of the fingerprint of RS extract and four standards (1: Prim-O-glucosylcimifugin; 2: Cimifugin; 3: 4'-O- β -D-Glucosyl-5-O-methylvisaminol; 4: Sec-O-Glucosylhamaudol) (B). RS extract was detected with HPLC under optimal conditions to obtain maximum peaks. There were 20 main peaks in the RS extract. Standard solutions of RS extract and the four standards were prepared by dissolving 0.5 g of RS extract/ml or 0.5 mg/ml of the four standards in 50% methanol (HPLC grade). The injection volume was 10 μ l and the samples were detected at 300 nm. All the experiments were performed in triplicate.

Table 1
RRT and RPA of each peak in the fingerprint of the RS extract.

No.	RRT	RPA	Identified compound
1	0.191832	0.583086	
2	0.248593	2.011869	
3	0.366669	0.213155	
4	0.412804	0.173591	
5	0.447238	0.159248	
6	0.457531	0.237883	
7	0.503147	0.888229	
8	0.624111	0.153808	
9	0.763329	0.231454	
10	0.805872	0.207715	
11	0.837863	1.227992	Prim-O-glucosylcimifugin
12	0.85745	1.030663	
13	0.946127	0.800198	Cimifugin
14	1	1	4'-O- β -D-Glucosyl-5-O-methylvisaminol
15	1.224341	0.226014	sec-O-Glucosylhamaudol
16	1.238485	0.182987	
17	1.268106	0.181503	
18	1.321979	0.378833	
19	1.370927	0.148863	
20	1.474082	0.320475	

The injection precision was determined with five replicate injections of the same sample in one day. The relative standard deviations (RSDs) of the relative retention time (RRT) and relative peak area (RPA) were 0.01–0.39% and 0.47–2.05%, respectively, and there were no significant differences. Repeatability was assessed by analyzing five independently prepared samples of RS extract. The RSDs for RRT and RPA were 0.01–0.9% and 1.59–1.91%, respectively, and there were no significant differences. The sample stability was assessed with successive injections of the same sample at 0, 2, 4, 6, and 8 h. During this period, the solution was stored at room temperature. The RSDs for RRT and RPA were 0.02–0.47% and 0.61–2.25%, respectively, and there were no significant changes. The accuracy of analytical method was assessed by the recovery of four major markers. Different concentrations of the four standards (200, 100 and 20 mg/l) were added to RS extract, the recoveries were calculated. The average recoveries ($n = 3$) were 92.72–105.90% with RSD of 0.35–3.65%.

The results for injection precision, repeatability, stability and accuracy indicate that this method is adequate, valid, and applicable to the analysis of RS extract. The RRTs and RPAs of the 20 peaks are shown in Table 1. The content of prim-O-glucosylcimifugin, cimifugin, 4'-O- β -D-glucosyl-5-O-methylvisaminol, and sec-O-glucosylhamaudol in RS was 1.794, 0.825, 1.167 and 0.324 mg/g dry root, respectively. The contents comply with Chinese pharmacopoeia.

RS extract attenuated inflammation in mouse ACD and inhibited cytokine production in vivo and ex vivo

Histopathological effects of RS extract on the mouse DNCB-induced ACD model

To observe the effects of RS extract on ear inflammation, a histological analysis was performed and ear swelling measured. The establishment of the ACD model and the dosage regimen for the mice are shown in Fig. 2A. Different doses of RS extract were given intragastrically to the mice in the sensitization phase and elicitation phase of ACD (days 1–7). Dexamethasone (0.65 mg/kg) was used as the positive control. Local edema, angiectasis, and lymphocytic infiltration were observed in the ears of the DNCB-treated mice. The inflammatory symptoms were alleviated in the ears of the RS-extract-treated mice compared with those of the model mice, with less thickening and less lymphocytic infiltration apparent (Fig. 2B). The increased ear swelling in the DNCB-treated mice was significantly inhibited by RS extract treatment (Fig. 2C).

RS extract reduced the elevated IFN- γ levels in sera and lymphocyte supernatants

Th cytokines regulate the immunological and inflammatory processes in ACD. To investigate the effects of RS extract on the production of inflammatory cytokines, the levels of IL-4 and IFN- γ were measured with ELISAs in the sera and lymphocyte culture supernatants, which were collected after treatment with ConA for 48 h. The levels of IFN- γ were greatly increased in the model group. However, the administration of RS extract reduced the levels of IFN- γ , but increased the expression of IL-4 to some extent, thus reducing the IFN- γ /IL-4 ratio (Fig. 3A–F). These results indicate that RS extract negatively modulates Th1 polarization in DNCB-induced ACD, ultimately reducing the Th1/Th2 ratio.

RS extract did not significantly affect the proportions of T and Th cells in lymph nodes in the elicitation phase

According to the results described above, RS extract regulates the imbalance between the Th1 and Th2 cytokines in a model of DNCB-induced ACD, but whether the RS extract influences the proportion of T cells and the subset of Th cells in the lymph nodes was still unknown. Therefore, draining lymph nodes were used to prepare a single-cell suspension, and then incubated with FITC-conjugated anti-CD3 antibody and PE-Cy5-conjugated anti-CD4 antibody. The results were disappointing. We found no significant changes in the proportion of T (CD3 $^{+}$) cells or Th (CD3 $^{+}$ CD4 $^{+}$) cells in the lymph nodes of the mice treated with different doses of RS extract (Fig. 4A–C).

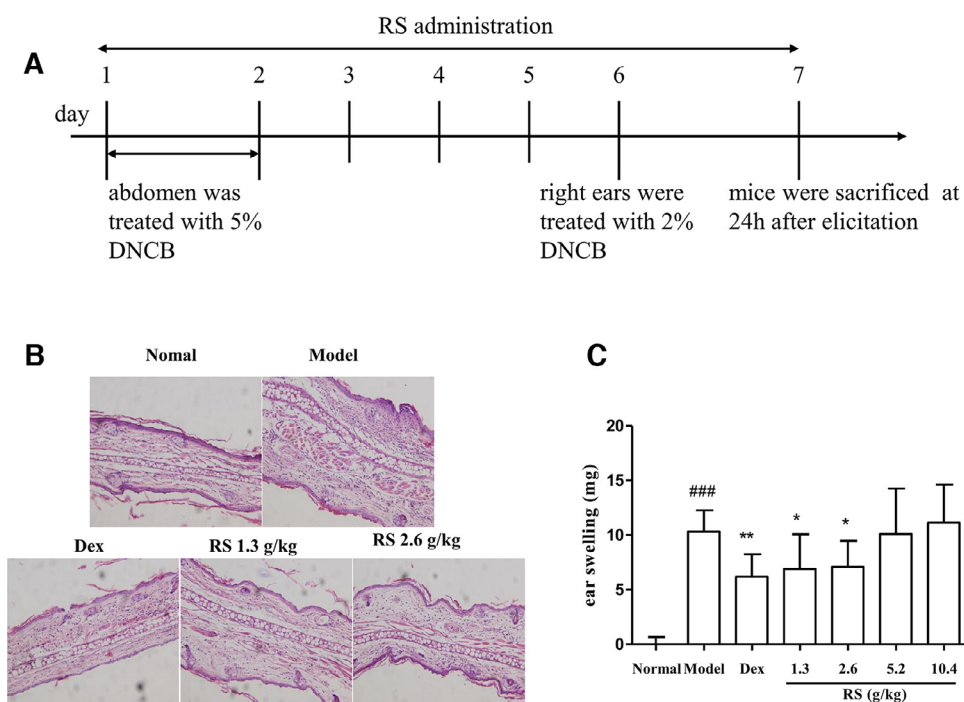


Fig. 2. Ear swelling and histopathological findings with hematoxylin/eosin staining of ear skin sections. (A) Flow chart of the DNCB-induced ACD model and the dosage regimen with RS extract. (B) Effect of RS extract on the histopathological findings in ear skin sections ($n = 6$, magnification: $\times 200$). (C) Effect of RS extract on ear swelling in the ACD model. The differences between the right and left ear weights were calculated (mean $\pm$ SD, $n = 8$, $###p < 0.001$ versus normal; $*p < 0.05$, $**p < 0.01$ versus model). All the experiments were performed in triplicate.

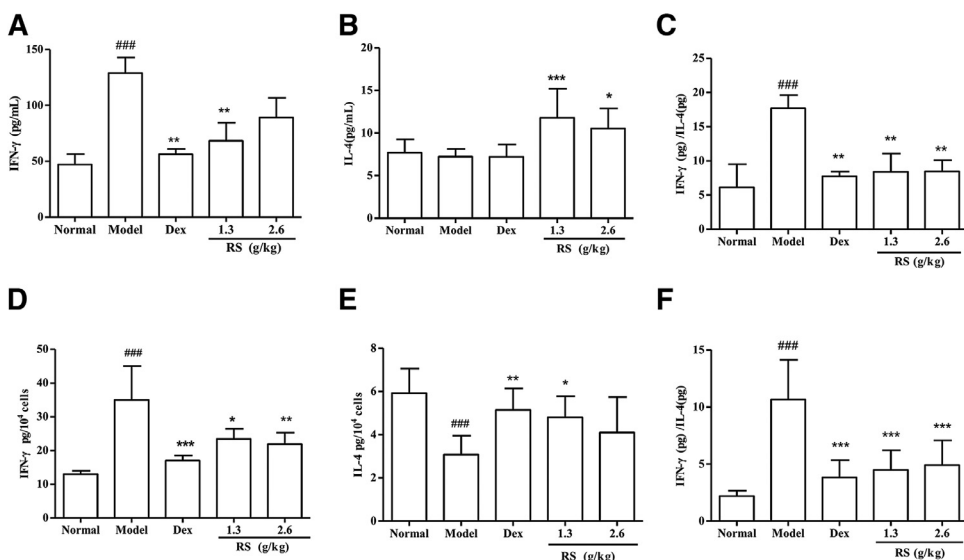


Fig. 3. Effects of RS extract on IFN- γ and IL-4 production *in vivo* and *in vitro*. (A–C) Effects of RS extract on the production of cytokines in the sera of the murine model of ACD. Peripheral blood from BALB/c mice was collected after the intragastric administration of RS extract for seven days; sera were isolated and the IFN- γ (A) and IL-4 (B) in them were analyzed with ELISAs. (D–F) Effects of RS extract on the production of cytokines induced with ConA in lymphocytes from ACD mice. After RS extract was administered for seven days, lymphocytes from the draining lymph nodes of the BALB/c mice were collected and treated with 10 μ g/ml ConA for 48 h. The levels of IFN- γ (D) and IL-4 (E) in the cell culture supernatants and their ratios (F) are shown (mean $\pm$ SD, $n = 8$, $###p < 0.001$ versus normal; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus model).

Thus, we found that the RS extract had a significant effect on ACD mice. This effect may be related to the modulated expression of Th cytokines and the correction of the Th1/Th2 imbalance. However, we failed to observe that RS extract affected the proportions of T cells and Th cells in the lymph nodes. Considering how T cells are activated and the role they played in ACD, the sensitization phase might be the appropriate time point at which to examine the proportion of Th cells and the effect of RS on it.

RS extract inhibited mouse ACD by regulating DC-activated Th1 cells in the initial stage of DNCB-induced ACD

Histopathological effects of RS extract in the sensitization phase in the mouse ACD model

To observe the histopathological effects of RS in the sensitization phase, we modulated the dosage regimen. The mice were only administered RS extract in the sensitization phase of ACD (days 1–5)

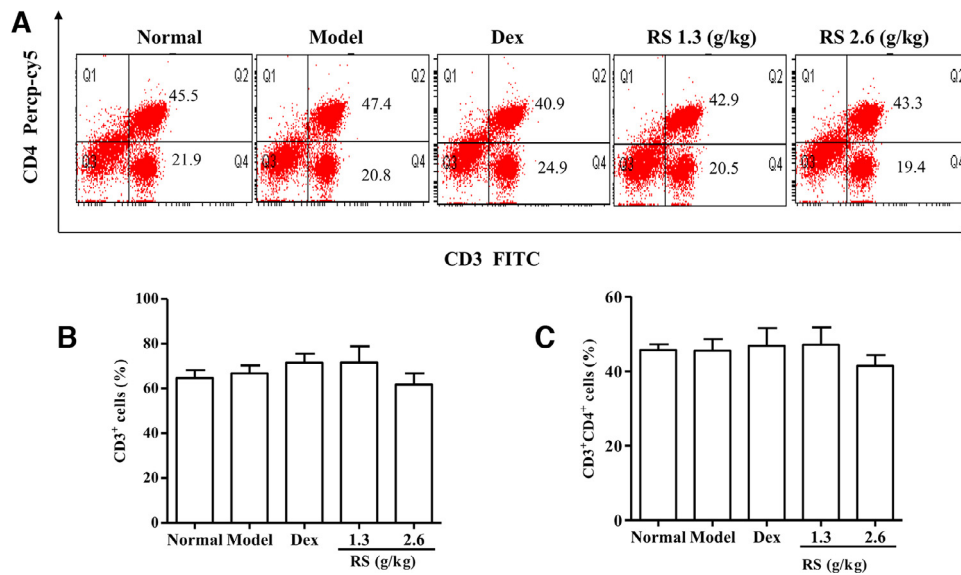


Fig. 4. Effect of RS extract on the proportions of T cells in the lymph nodes of BALB/c mice treated with RS extract for seven days. In the DNCB-induced ACD model, lymphocytes from the draining lymph nodes were separated for flow cytometry or stimulated with ConA (10 μ g/ml) for 48 h. (A) Flow-cytometry results for CD3⁺CD4⁺ cells in lymph nodes. (B and C) The proportions of T cells (CD3⁺) and Th cells (CD3⁺CD4⁺) in the lymphocytes (mean $\pm$ SD, $n = 4$). All the experiments were performed in triplicate.

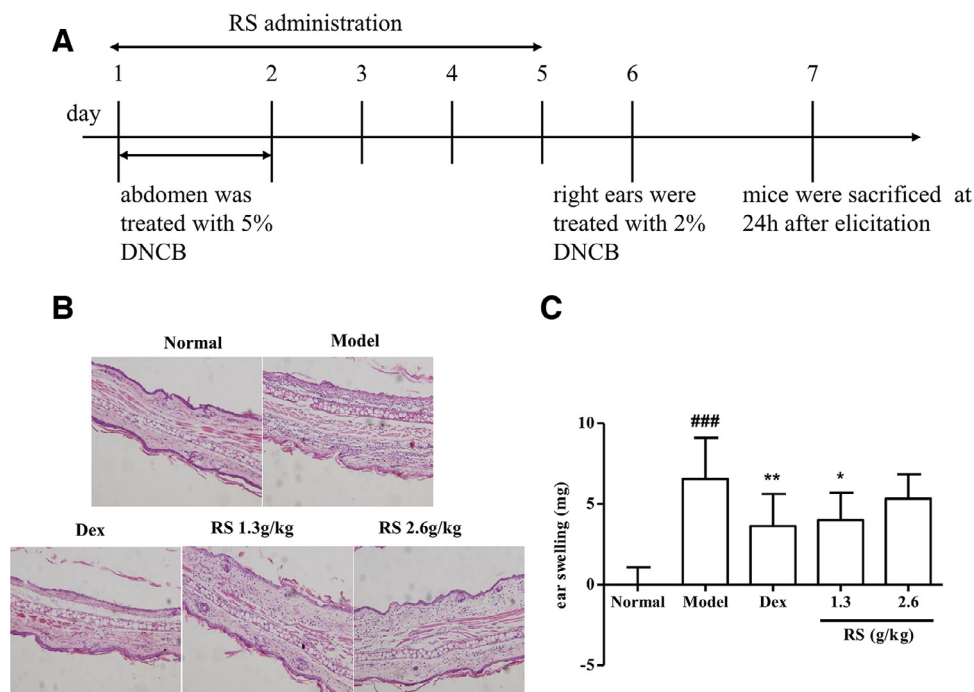


Fig. 5. Ear swelling and histopathological findings after hematoxylin/eosin staining of ear sections. (A) Flow chart of RS extract administered to the murine ACD model. RS extract was only given intragastrically in the sensitization phase of the model. (B) Effect of RS extract on the histopathological findings of ear skin sections ($n = 6$, magnification: $\times 200$). (C) Effect of RS extract on ear swelling in the ACD model. The differences between the weights of the right and left ears were calculated (mean $\pm$ SD, $n = 8$, ### $p < 0.001$ versus normal; * $p < 0.05$, ** $p < 0.01$ versus model). All the experiments were performed in triplicate.

and killed on day 7 (Fig. 5A). Histopathological effects were observed. However, RS extract (1.3 g/kg) alleviated the inflammatory symptoms and the ear swelling in the sensitization phase of ACD (Fig. 5B, C).

RS extract inhibited T-cell proliferation in the initial stage of ACD

To study the effects on T cells and their subsets, we applied DNCB to induce the initial stage of ACD (Fig. 6A), as described in detail in the Materials and methods section. The draining lymph nodes were isolated on day 3 for lymphocyte culture, and the proliferation rate was calculated after stimulation with ConA for 48 h. The RS extract

(1.3 g/kg) markedly inhibited T-cell proliferation in the lymphocytes (Fig. 6E).

RS extract reduced the proportions of T and Th cells in mouse lymph nodes in the initial stage of ACD

The draining lymph nodes from mice in the initial stage of ACD were used to prepare single-cell suspensions, which were analyzed with flow cytometry. The RS extract reduced the elevated proportions of CD3⁺ cells and CD3⁺CD4⁺ cells in the lymph nodes (Fig. 6B–D).

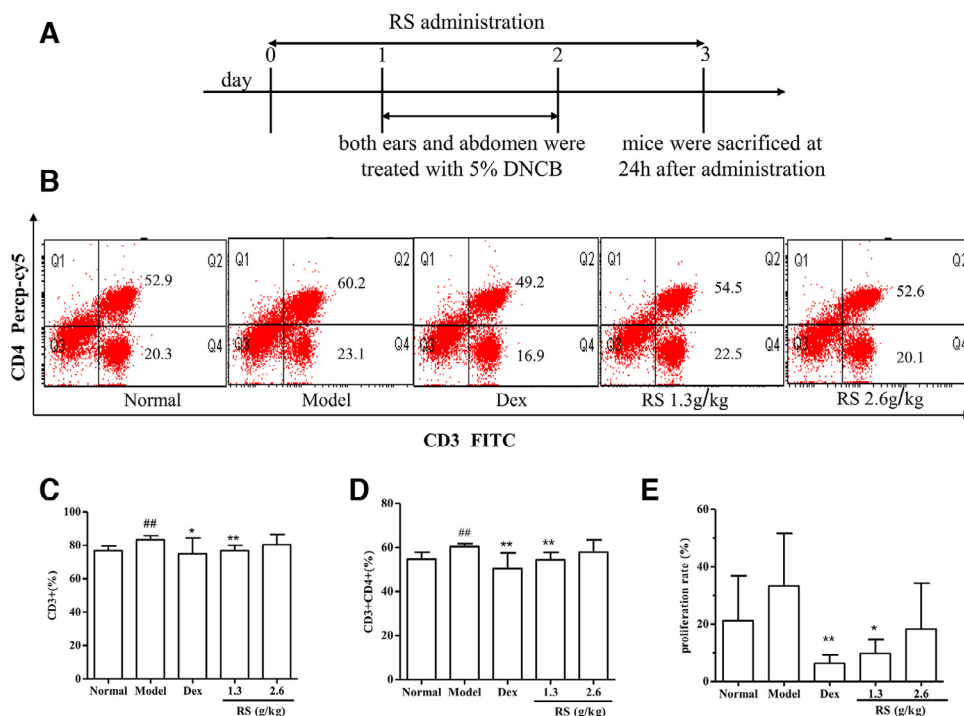


Fig. 6. Effects of RS extract on the proportions and proliferation of T cells in the draining lymph nodes in the initial stage of the ACD model. Lymphocytes from draining lymph nodes were separated for flow cytometry or stimulated with ConA (10 μ g/ml) for an MTT assay. (A) Flow chart of the DNCB-induced model of initial-stage ACD and the administration of RS extract in this murine model. (B) Flow-cytometric results for CD3⁺ and CD3⁺CD4⁺ cells in lymph nodes. (C and D) The proportions of T cells (CD3⁺) and Th cells (CD3⁺CD4⁺) in the lymph nodes ($n = 8$). (E) Proliferation rate of T cells. Proliferation was determined with the MTT assay ($n = 3$) (mean $\pm$ SD, ## $p < 0.01$ versus normal; * $p < 0.05$, ** $p < 0.01$ versus model). All the experiments were performed in triplicate.

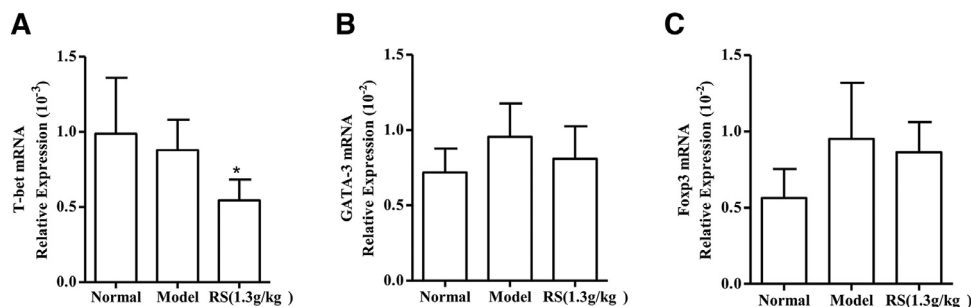


Fig. 7. Effects of RS extract on the expression of Th1/Th2/Treg-specific transcription factors in lymphocytes. RS extract (1.3 g/kg) was given intragastrically to BALB/c mice once daily for four consecutive days, after two rounds of sensitization with DNCB (2%) in the ear skin on day 1 and day 2. The lymph nodes were separated on day 3 and total RNA was isolated. Quantitative real-time PCR was performed. (A) The relative mRNA expression of T-bet. (B) The relative mRNA expression of GATA-3. (C) The relative mRNA expression of Foxp3. The β -actin gene was used as the housekeeping control to normalize the expression of the target genes (mean $\pm$ SD, $n = 4$, * $p < 0.05$ versus model). All the experiments were performed in triplicate.

These results suggest that the RS extract already plays an anti- allergic role in the sensitization phase, and that this role may be related to its effect on the proliferation and differentiation of T cells.

RS extract downregulated a Th1-related transcription factor in the lymph nodes in the initial stage of ACD

We analyzed the mRNA expression of T-bet, GATA-3, and Foxp3, representative Th1, Th2, and Treg transcription factors, respectively, in the draining lymph nodes in the initial stage of ACD, using real-time PCR. The expression of T-bet mRNA was markedly downregulated by RS extract (1.3 g/kg), whereas the expression of GATA-3 and Foxp3 mRNAs was not significantly altered (Fig. 7A–C).

RS extract negatively affected on the number and maturation of DCs in the draining lymph nodes

Because DCs play a key role in the activation and differentiation of T cells, we tested whether the effect of RS on T cells is mediated by its effect on DCs. To do so, the cell surface molecules on DCs

(CD11c, CD40, and CD86) were detected with flow cytometry. RS extract (1.3 g/kg) significantly reduced the amount of CD11c⁺ DCs in the mouse lymph nodes, and inhibited the expression of CD40 ($p < 0.01$) and CD86 ($p < 0.05$) (Fig. 8A–C). Therefore, DCs might be a critical cellular target of RS extract in the initial stage of DNCB-induced ACD.

Discussion

Traditional Chinese medicine (TCM), which has been widely used for many generations in China, has proven efficacy (Koo and Arain 1998; Koo and Desai 2003), wide applications, and few adverse effects, but its homonyms, synonyms, and multiple components make the authentication and quality assessment of TCM essential. Several quality control techniques have been used to achieve this goal, each of which has different advantages (Gao et al. 2011; Zhong et al. 2009). In this study, we used a fingerprinting technique to assess RS, and established an RS fingerprint with HPLC. There were

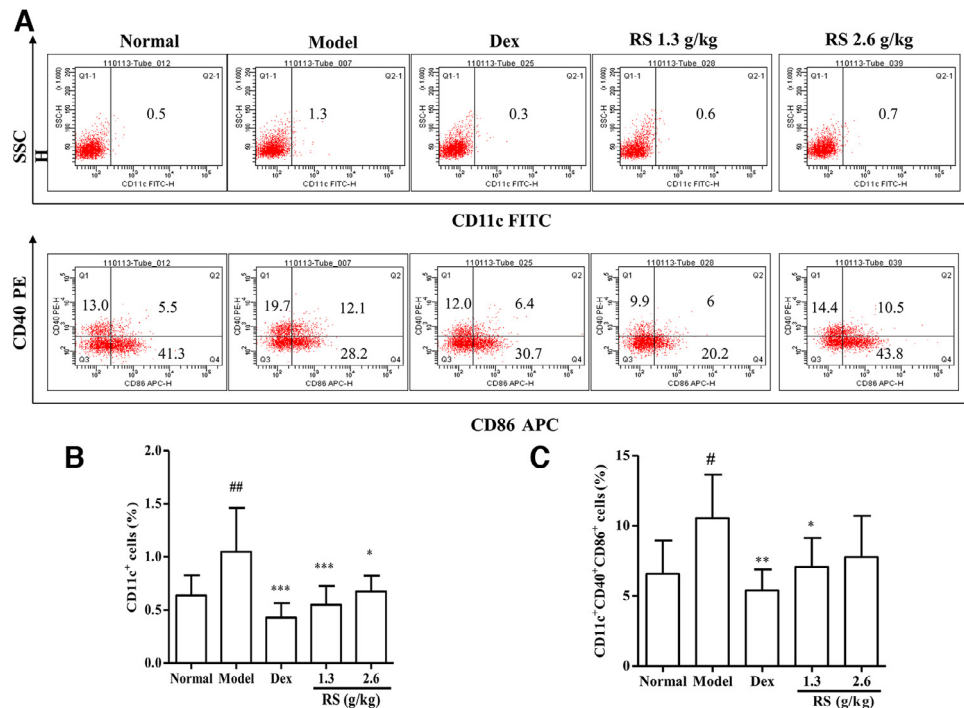


Fig. 8. Effects of RS extract on the numbers and maturation of DCs in the lymph nodes of DNCB-induced allergic mice. (A) Flow-cytometric results for CD11c⁺ and CD40⁺CD86⁺ in the lymph nodes of the DNCB-induced model of initial-stage ACD. (B and C) Proportions of DCs (CD11c⁺) and their costimulatory molecules (CD40⁺CD86⁺) in lymphocytes (mean $\pm$ SD, $n = 8$, ## $p < 0.01$ versus normal; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus model). All the experiments were performed in triplicate.

20 main peaks in this chromatogram, four of which corresponded to the prim-*O*-glucosylcimifugin, cimifugin, 4'-*O*- β -D-Glucosyl-5-*O*-methylvisaminol, and sec-*O*-Glucosylhamaudol standards, so the sample used in this study conformed to the Quality Specification Standards for *Saposhnikovia divaricata* (Turcz.) Schischk (Xiao et al. 2001a, b).

In the clinical context, RS ("Fangfeng" in Chinese) is commonly used as an immunomodulatory agent in Chinese herbal mixtures, such as Yu-Ping-Feng-San, a representative herbal compound for the treatment of allergic diseases. Some reports have shown that Yu-Ping-Feng-San enhances the functions of DCs, modulates the immune functions of mice under low-temperature conditions (Qiao-feng et al. 2014), and adjusts the imbalance in Th1/Th2 in mice with allergic rhinitis (Jun et al. 2006). RS has also been used to cure respiratory-tract infections (Pu Li 2003), allergic rhinitis (Chan and Chien 2014), but the pharmacological efficacy of RS for ACD and its underlying mechanisms have been unclear. Many chemicals can induce ACD (Tanaka et al. 2012), including 1-chloro-2,4-dinitrobenzene (Ju 2009), 1-fluoro-2,4-dinitrobenzene (Yuan et al. 2010), 1-chloro-2,4,6-trinitrobenzene (Lass et al. 2010), and FITC (Larson et al. 2010). Because the symptoms of skin inflammation or itching and the pathological changes in animals treated with DNCB are similar to those of patients with ACD, this experimental model has frequently been used to study the pathogenesis of ACD and potential preventive measures. In this study, we used this experimental model to evaluate the pharmacological efficacy of RS in treating ACD and the underlying mechanisms. Our results showed that RS extract reduces ear swelling and lymphocytic infiltration, and attenuates the pathological changes associated with DNCB-induced ACD. These results indicate that RS exerts a specific therapeutic effect on ACD, even when used alone.

T cells play an important mediating role in the development of ACD. In response to an antigenic/adjuvant stimulus, CD4⁺ T cells differentiate into functionally distinct subsets (Th1 or Th2) of cells that can be identified by monitoring their signature cytokines, IFN- γ and IL-4, respectively, which are the hallmarks of the Th1- and Th2-cell-

mediated responses. In this study, RS extract downregulated IFN- γ levels and upregulated IL-4 levels in the mouse sera and lymphocyte culture supernatants, significantly reducing the IFN- γ /IL-4 ratios, a commonly used index of Th1/Th2 immunity (Piskin et al. 2003). Notably, we did not find that RS extract influenced the proportions of CD3⁺ and CD3⁺CD4⁺ cells in the elicitation phase of ACD, which suggests that the elicitation phase of the disease might not be the appropriate period in which to observe the impact of RS on T-cell subsets.

To effectively investigate the mechanisms by which RS regulates the balance of Th cells, we used a model of the initial stage of ACD. The efficacy of RS extract in treating ACD was confirmed only when it was administered in the sensitization phase of the disease, when it reduced the ear swelling and pathological tissue changes. We observed that the RS extract potently reduced the proportions of CD3⁺ and CD3⁺CD4⁺ cells in the lymph nodes in the initial stage of ACD. Our data also demonstrated that RS extract downregulated the mRNA expression of *T-bet*, a Th1-specific transcription factor that induces the synthesis of IFN- γ and negatively regulates the Th2 pathways (Lighvani et al. 2001; Szabo et al. 2000, 2003). These findings strongly suggest that the *T-bet* and IFN- γ -expression-dependent suppression by RS extract of Th1 differentiation enhances Th2 skewing, modulating the imbalance in the Th1/Th2 ratio.

Finally, we explored the impact of RS extract on DCs. The expression of CD11c, the biomarker of DCs, and its costimulatory molecules (CD40 and CD86) was reduced by RS extract in the initial stage of ACD. Various stimuli promote the maturation of DCs, upregulate several costimulatory molecules, and enhance the release of cytokines by DCs, such as IL-12, IL-6, and IL-23, which drive T-cell polarization. Depending on the cytokine milieu, T cells differentiate into Th1, Th2, or Th17 cells (Dudda et al. 2005). The presence of DCs and their induction of T-cell activation and proliferation are the key events that induce ACD (Straube et al. 2005). Because DCs are important in allergy and T-cell activation and proliferation, we deduced that the effect of RS extract on ACD might be mediated by suppressing T-cell activation and Th-cell differentiation, which is mediated

by DCs, and thus influences the expression of allergic inflammatory cytokines.

In summary, RS extract suppressed allergic skin inflammation in a mouse model of DNCB-induced ACD, and exerted its effect by negatively regulating the skewed Th1 response through Th1-cell polarization, which is regulated by DCs and dependent on T-bet expression in the sensitization phase of this model. Our results might explain why RS is always the preferred component in various Chinese antiallergy herbal compounds, and might be an alternative therapeutic option for ACD. The antiallergic mechanisms we have identified in this study may be related to the most important advantage of this TCM, to reduce the recurrence of the allergic disease. However, its molecular mechanisms of action, its active constituents, and whether the active constituents correspond to the fingerprint chromatogram in our results are still unknown. Therefore, more in-depth studies are required to clarify these questions.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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References

- Aiba, S., 2007. Dendritic cells: importance in allergy. *Allergol. Int.: Off. J. Jpn. Soc. Allergol.* 56, 201–208.
- Chan, R.Y., Chien, W.T., 2014. The effects of two Chinese herbal medicinal formulae vs. placebo controls for treatment of allergic rhinitis: a randomised controlled trial. *Trials* 15, 261.
- Dai, J., Chen, X., Cheng, W., Liu, X., Fan, X., Shen, Z., Bi, K., 2008. A sensitive liquid chromatography-mass spectrometry method for simultaneous determination of two active chromones from *Saposhnikovia divaricata* root in rat plasma and urine. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 868, 13–19.
- Dudda, J.C., Lembo, A., Bachtanian, E., Huehn, J., Siewert, C., Hamann, A., Kremmer, E., Forster, R., Martin, S.F., 2005. Dendritic cells govern induction and reprogramming of polarized tissue-selective homing receptor patterns of T cells: important roles for soluble factors and tissue microenvironments. *Eur. J. Immunol.* 35, 1056–1065.
- Gao, H., Wang, Z., Li, Y., Qian, Z., 2011. Overview of the quality standard research of traditional Chinese medicine. *Front. Med.* 5, 195–202.
- Gui, Y., Tsao, R., Li, L., Liu, C.M., Wang, J., Zong, X., 2011. Preparative separation of chromones in plant extract of *Saposhnikovia divaricata* by high-performance counter-current chromatography. *J. Sep. Sci.* 34, 520–526.
- Ju, C., 2009. The role of haptic macrophages in regulation of idiosyncratic drug reactions. *Toxicol. Pathol.* 37, 12–17.
- Jun, G., Chao-Bin, S., Lei, L., Qiang, D., Kuang-Cheng, X., Sheng-Rong, Z., Yu-Fa, W., 2006. Effects of yupingfeng powder on Th1/Th2 balance in murine model of allergic rhinitis. *Chin. Tradit. Pat. Med.* 28, 1163–1166.
- Kadowaki, N., 2007. Dendritic cells: a conductor of T cell differentiation. *Allergol. Int.: Off. J. Jpn. Soc. Allergol.* 56, 193–199.
- Koo, J., Arain, S., 1998. Traditional Chinese medicine for the treatment of dermatologic disorders. *Arch. Dermatol.* 134, 1388–1393.
- Koo, J., Desai, R., 2003. Traditional Chinese medicine in dermatology. *Dermatol. Ther.* 16, 98–105.
- Langenkamp, A., Messi, M., Lanzavecchia, A., Sallusto, F., 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat. Immunol.* 1, 311–316.
- Larson, R.P., Zimmerli, S.C., Comeau, M.R., Itano, A., Omori, M., Iseki, M., Hauser, C., Ziegler, S.F., 2010. Dibutyl phthalate-induced thymic stromal lymphopoietin is required for Th2 contact hypersensitivity responses. *J. Immunol.* 184, 2974–2984.
- Lass, C., Merfort, I., Martin, S.F., 2010. In vitro and in vivo analysis of pro- and anti-inflammatory effects of weak and strong contact allergens. *Exp. Dermatol.* 19, 1007–1013.
- Lighvani, A.A., Frucht, D.M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B.D., Nguyen, B.V., Gadina, M., Sher, A., Paul, W.E., O'Shea, J.J., 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 15137–15142.
- Lin, J.F., Liu, P.H., Huang, T.P., Lien, A.S., Ou, L.S., Yu, C.H., Yang, S.L., Chang, H.H., Yen, H.R., 2014. Characteristics and prescription patterns of traditional Chinese medicine in atopic dermatitis patients: ten-year experiences at a medical center in Taiwan. *Complement. Ther. Med.* 22, 141–147.
- Lin, Y.H., Chen, Y.C., Hu, S., Chen, H.Y., Chen, J.L., Yang, S.H., 2013. Identifying core herbal treatments for urticaria using Taiwan's nationwide prescription database. *J. Ethnopharmacol.* 148, 556–562.
- Piskin, G., Koomeen, C.W., Picavet, D., Bos, J.D., Teunissen, M.B., 2003. Ultraviolet-B irradiation decreases IFN-gamma and increases IL-4 expression in psoriatic lesional skin in situ and in cultured dermal T cells derived from these lesions. *Exp. Dermatol.* 12, 172–180.
- Pu, Li, C.L., Jianhua, Ma, Lanxiang, Wang, 2003. Saposhnikovia Decoction preventing adult spring upper respiratory tract infection in 210 cases. *J. Jilin Univ.* 24, 371–375.
- Qiao-Feng, Z., Yu-Qi, G., Xia, L., Li, W., Hong-hai, Z., Cheng-Fang, Y., 2014. Changes of the function of mouse dendritic cells under the condition of hypothermia and regulation of Yu Ping Feng pulvis. *Shandong Med. J.* 54, 1–3.
- Saary, J., Qureshi, R., Palda, V., DeKoven, J., Pratt, M., Skotnicki-Grant, S., Holness, L., 2005. A systematic review of contact dermatitis treatment and prevention. *J. Am. Acad. Dermatol.* 53, 845.
- Steinman, R.M., 2003. Some interfaces of dendritic cell biology. *APMIS: Acta Pathol. Microbiol. Immunol. Scand.* 111, 675–697.
- Straube, F., Grenet, O., Bruegger, P., Ulrich, P., 2005. Contact allergens and irritants show discrete differences in the activation of human monocyte-derived dendritic cells: consequences for in vitro detection of contact allergens. *Arch. Toxicol.* 79, 37–46.
- Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., Glimcher, L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100, 655–669.
- Szabo, S.J., Sullivan, B.M., Peng, S.L., Glimcher, L.H., 2003. Molecular mechanisms regulating Th1 immune responses. *Annu. Rev. Immunol.* 21, 713–758.
- Tanaka, A., Amagai, Y., Oida, K., Matsuda, H., 2012. Recent findings in mouse models for human atopic dermatitis. *Exp. Anim./Jpn. Assoc. Lab. Anim. Sci.* 61, 77–84.
- Xiao, Y.Q., Li, L., Yang, B., Huang, L.Q., 2001a. Studies on chemical constituents from root of *Saposhnikovia divaricata* (Turcz.) Schischk. *Zhongguo Zhong Yao Za Zhi* 26, 117–119.
- Xiao, Y.Q., Yang, B., Yao, S.T., Li, W., Li, L., Huang, L.Q., Xue, B.Y., 2001b. Studies on quality specification standards for *Saposhnikovia divaricata* (Turcz.) Schischk. *Zhongguo Zhong Yao Za Zhi* 26, 185–187.
- Xue, B.Y., Li, W., Li, L., Xiao, Y.Q., 2000. A pharmacodynamic research on chromone glucosides of fangfeng. *Zhongguo Zhong Yao Za Zhi* 25, 297–299.
- Yuan, X.Y., Liu, W., Zhang, P., Wang, R.Y., Guo, J.Y., 2010. Effects and mechanisms of aloperine on 2,4-dinitrofluorobenzene-induced allergic contact dermatitis in BALB/c mice. *Eur. J. Pharmacol.* 629, 147–152.
- Zhe, W., Liu, Y., Xiping, G., Jing, Y., Min, H., Zhiqiang, W., Xing, P., Xue, W., Nan, Z., 2013. The anti-allergic effects of ethyl acetate extract of Jingfang Powder. *Pharmacol. Clin. Chin. Mater. Med.* 29, 92–95.
- Zhong, X.K., Li, D.C., Jiang, J.G., 2009. Identification and quality control of Chinese medicine based on the fingerprint techniques. *Curr. Med. Chem.* 16, 3064–3075.