



Total glucosides of paeony inhibit the inflammatory responses of mice with allergic contact dermatitis by restoring the balanced secretion of pro-/anti-inflammatory cytokines



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ARTICLE INFO

Article history:

Received 18 June 2012

Received in revised form 12 October 2014

Accepted 17 December 2014

Available online 31 December 2014

Keywords:

Allergic contact dermatitis

Total glucosides of paeony

Interleukin-2

Interleukin-4

Interleukin-10

Interleukin-17

ABSTRACT

The present study aimed to investigate the regulation exerted by the total glucosides of paeony (TGP) on the production of interleukin-2 (IL-2), IL-4, IL-10 and IL-17 in the serum and lymphocytes of mice with allergic contact dermatitis (ACD). ACD in mice was induced by the repeated application of 2,4-dinitrochlorobenzene (DNCB) to their skins. The mice were orally administered TGP (35, 70, and 140 mg/kg/d) and prednisone (Pre, 5 mg/kg/d) from day 1 to day 7 after immunization. The inflammatory responses were evaluated by ear swelling and histological examination. Thymocyte proliferation was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide assay. The cytokine production in the serum and lymphocytes supernatant was measured by enzyme-linked immunosorbent assay. The results indicated that the topical application of DNCB to the skin provoked obvious inflammatory responses. The oral administration of TGP (70 and 140 mg/kg/d) and Pre (5 mg/kg/d) significantly inhibited skin inflammation, decreased the thymus and spleen indices, and inhibited thymocyte proliferation in mice treated with DNCB. Further study indicated that TGP increased IL-4 and IL-10 production but decreased the production of IL-2 and IL-17 in the serum and lymphocyte supernatant. The correlation analysis suggested significantly positive correlations between IL-2 and IL-17 production and the severity of skin inflammation, whereas negative correlations were obtained for IL-4 and IL-10 production and skin inflammation. In summary, these results suggest that the therapeutic effects of TGP on ACD may result from its regulation of the imbalanced secretion of IL-2/IL-4 and IL-10/IL-17.

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1. Introduction

Allergic contact dermatitis (ACD), an inflammatory skin disease, has been increasing in prevalence in western countries over the past decades [1]. The pathogenesis of ACD is delayed-type hypersensitivity reaction (DTH) mediated by hapten-specific T lymphocytes. Hapten-specific T lymphocytes are activated in an immune organ during the sensitization process (afferent phase). During this stage, interleukin (IL)-2, IL-4 and IL-17 are produced by activatory T lymphocytes stimulated by antigen presentation cells (APC) [2]. A previous study suggested that contact hypersensitivity (CHS) in mice is promoted by the imbalanced secretion of IL-2/IL-4 [3]. Excessive IL-17 and insufficient IL-10 were also recently observed in the serum of ACD patients [4]. It is well known that IL-2 promotes cell-mediated immunity [5]. Moreover, IL-17 not only facilitates the generation of CHS but also increases the

production of inflammatory cytokines in immune disease [6]. In contrast, IL-10 inhibits APC migration and induces immune tolerance to alleviate the inflammatory reaction [7]. In addition, a previous study found that IL-10 can inhibit IL-17 expression [8], and IL-17 has the same effect on the differentiation of Treg cells (the main source of IL-10) [9]. Thus, it has been suggested that the imbalanced secretion of inflammatory/anti-inflammatory cytokines exacerbates the development of ACD.

The total glucosides of paeony (TGP) is the active compound extracted from the roots of *Paeonia lactiflora* Pall, which has been recognized as a valuable herb used in the treatment of rheumatoid arthritis (RA) in ancient China. The main constituents of TGP have been identified as paeoniflorin (Pae), albiflorin, hydroxy-paeoniflorin, paeonin and benzoylpaeoniflorin. A previously published report concluded that TGP is an effective drug with obvious anti-inflammatory and immune-regulatory activities [10]. Compared with glucocorticosteroids, the superiority of TGP for the treatment of inflammatory disease, including improving symptoms with fewer side effects, has been proved in a multi-center clinical trial [11]. Previous studies have found that TGP can reduce IL-2 secretion by T lymphocytes in rats with collagen-

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induced arthritis [12] and restore the balanced secretion of IL-2/IL-4 in the mesenteric lymph nodes of rats with adjuvant arthritis [13]. Moreover, TGP has been reported to inhibit allergic inflammation in the skin of mice [14]. However, further information on the use of TGP for the treatment of ACD has been hardly disclosed. Therefore, the present study aimed to investigate whether there is any connection between the treatment of ACD with TGP and its regulation of the imbalanced secretion of pro-inflammatory (IL-2 and IL-17)/anti-inflammatory cytokines (IL-4 and IL-10).

Prednisone (Pre), which exhibits immunosuppressive and anti-inflammatory activities, is well-known for its wide application for the treatment of allergic and inflammatory disease [15]. In this paper, Pre was used as a suitable positive control drug.

2. Materials and methods

2.1. Animals

The present study was approved by the Ethical Committee on Animal Research at the Institute of Clinical Pharmacology of Anhui Medical University (approved number: 130821). Kunming mice (male, 20 ± 2 g, Grade II, Certificate No. 001) were purchased from the Animal Department of Anhui Medical University (Hefei, Anhui Province, China). All of the mice were housed under standard laboratory conditions at a temperature of $24^\circ\text{C} \pm 1^\circ\text{C}$ and given tap water and commercially available food *ad libitum*. The lighting duration in the breeding room was 12 h (7:00 am to 7:00 pm).

2.2. Plant material

The roots of *P. lactiflora* were collected from plants growing under controlled conditions in Bozhou (latitude $33^\circ47'59.64$ N and longitude $115^\circ50'15.90$ E), China. A voucher specimen was deposited at the

Herbarium of Anhui Medical University (No. 08274). The voucher specimen was identified by Professor Lumin Pan of the Department of Materia Medica, School of Pharmacy, Anhui College of Traditional Chinese Medicine. TGP was extracted [16] from the roots of *P. lactiflora* and purified through the solvent extraction and macroporous resin methods in the Chemistry Laboratory of the Institute of Clinical Pharmacology of Anhui Medical University (Hefei, Anhui Province, China). The dried and powdered roots of *P. lactiflora* were extracted with 70% ethanol under reflux. The concentrated extract was dissolved in water and tandem passed through a macroporous resin column. The column was washed with water until the Molisch reaction stopped and then with 40% ethanol. The concentration of the 40% eluate under reduced pressure yielded the TGP (yellow powder, Fig. 1S, Supporting Information). Pae constituted up to 40.28% of TGP, as determined by HPLC (LC-10AD, Shimadzu Co., Japan) (Fig. 2-1S and Fig. 3S, Supporting Information). The TGP used in this study was free of endotoxin.

2.3. Drug and reagents

TGP was provided by the Institute of Clinical Pharmacology, Anhui Medical University. Pre was purchased from Xian Ju Pharmaceutical Co. Ltd. (Hangzhou, China). Both TGP and Pre were suspended in physiological saline before use. 1-Chloro-2,4-dinitrobenzene (DNCB) was purchased from Shanghai Chemical Agent Co. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-2, IL-4, IL-10 and IL-17 were products of R&D Systems (Minneapolis, MN, USA). Dulbecco's minimum essential medium (DMEM) was purchased from Gibco Co. (CA, USA). Concanavalin A (ConA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All of the other chemicals used in these experiments were of analytical grade and were obtained from commercial sources.

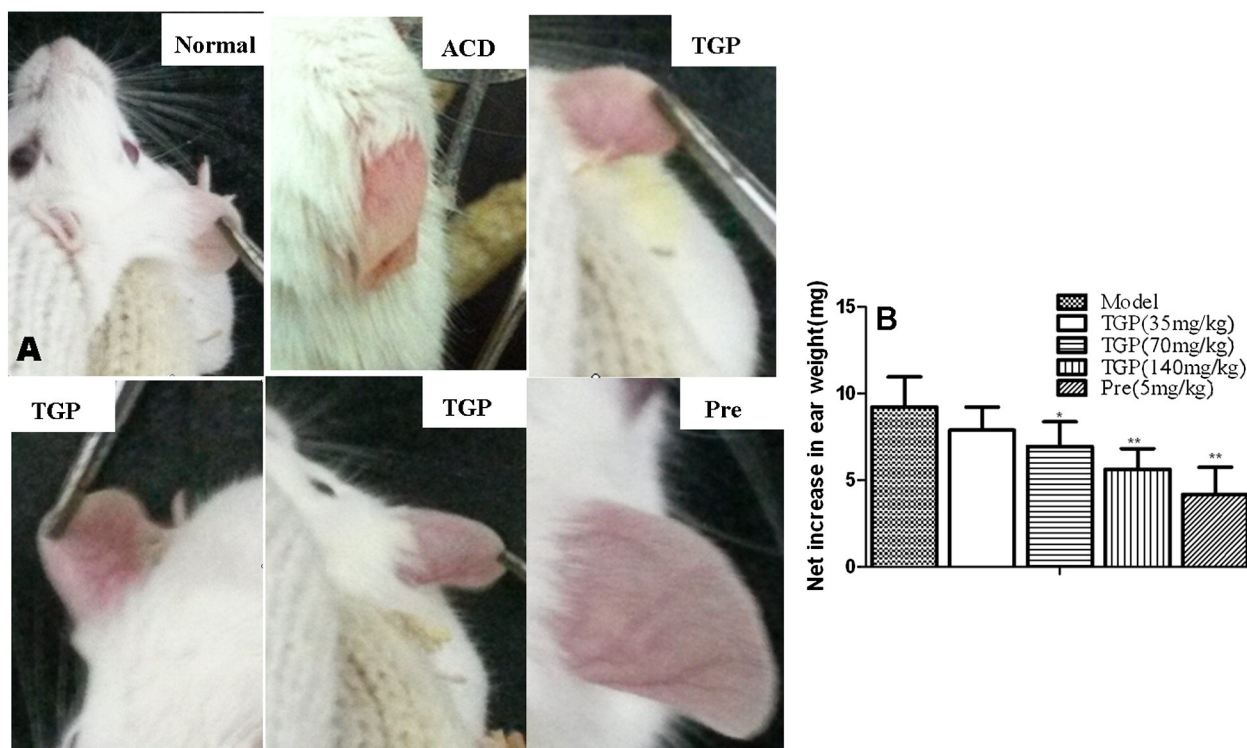


Fig. 1. Effects of TGP on DNCB-induced ear swelling in mice. (A) Appearance of the ear treated with DNCB showed significant swelling at day 7; (B) ear swelling in mice; the data are presented as the means $\pm$ S.D. ($n = 12-14$). * $P < 0.05$ and ** $P < 0.01$ compared with the ACD model group. Treatment with TGP (70 and 140 mg/kg/day) and Pre (5 mg/kg/day) significantly reduced the DNCB-induced ear swelling in mice. TGP: total glucosides of paeony; DNCB: 2,4-dinitrochlorobenzene; ACD: allergic contact dermatitis; Pre: prednisone.

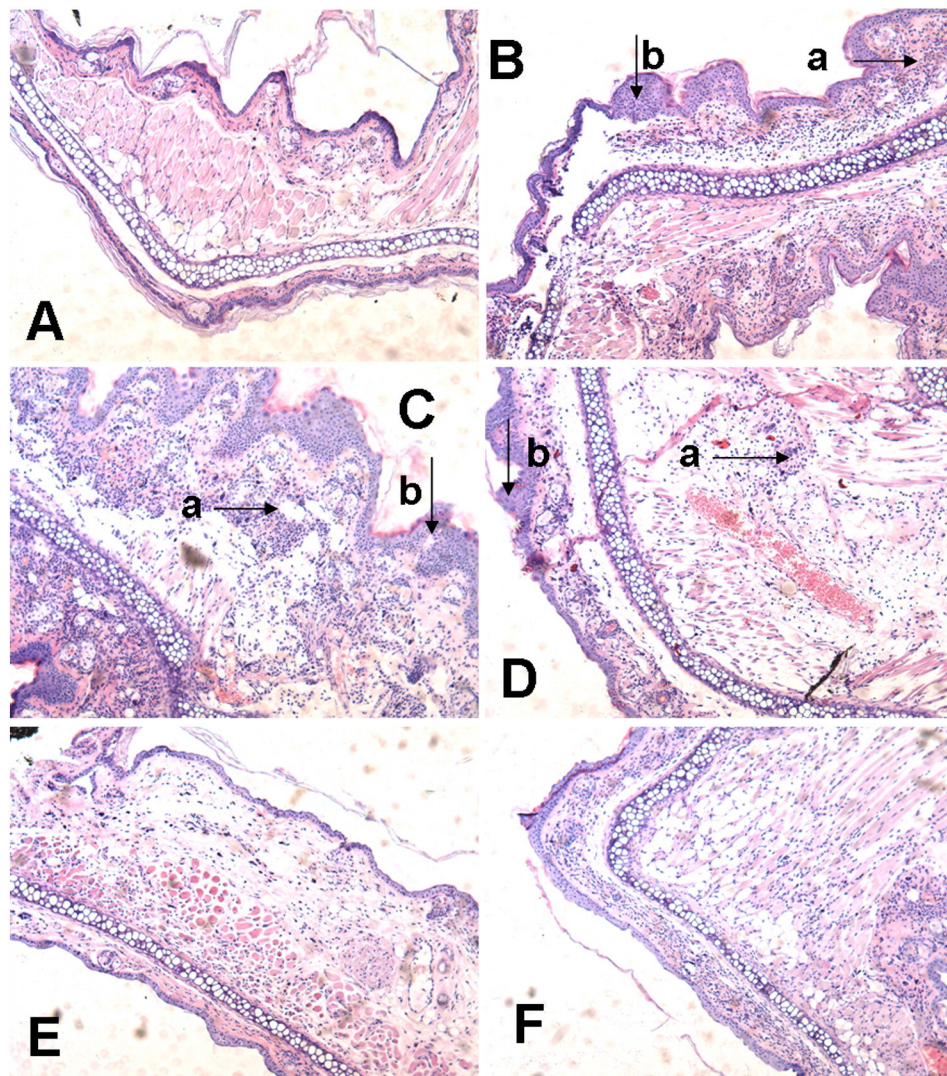


Fig. 2-1. Effects of TGP on skin histopathology in mice treated with DNCB (original magnification 200 $\times$). (A) Normal tissue without inflammation. (B) ACD group: The histopathology displayed obvious monocyte infiltration (a) and epidermal keratinization (b). (C) TGP (35 mg/kg/day): The histopathology showed similar inflammation to that observed in the ACD model. (D) TGP (70 mg/kg/day): The histopathology displayed an obvious decrease in monocyte infiltration. (E) TGP (140 mg/kg/day): The histopathology showed obvious reductions in monocyte infiltration and epidermal keratinization. (F) Pre (5 mg/kg/day): The histopathology showed similar effects to those observed in E.

2.4. Induction and evaluation of ACD

ACD was induced according to a published method [17]. Briefly, DNCB was dissolved in AOO (acetone:olive oil = 4:1). The mice were sensitized by painting their shaved abdomen skin (2 cm $\times$ 2 cm) with 100 μ L of 0.5% DNCB. Five days later, the mice were challenged on both sides of the right ear with 20 μ L of 0.5% DNCB, and rechallenge was performed 24 h later to yield extensive disease. As a control, the left ear was painted with an identical volume of menstruum. Twenty-four hours after the second challenge, small round pieces from the right and left ears were cut using a sharp clamp (diameter 0.8 cm), and the weight of the pieces was determined using an electronic balance. The ear swelling was evaluated by measuring the difference in weight between the right and left ears.

2.5. Treatment [18]

The mice were randomly divided into six groups. The mice treated with DNCB were intragastrically administered TGP (35, 70, and

140 mg/kg/day) and Pre (5 mg/kg/day) from day 1 to day 7 after immunization. The mice in the normal and ACD model groups were given an equal volume of physiological saline.

2.6. Histopathology and Immunohistochemical analysis

The histopathological examination was carried out according to published methods [17,19]. Briefly, the punched piece of the right ear (diameter 0.8 cm) and spleen tissue were fixed with 10% formaldehyde and embedded in paraffin, and thin sections were then prepared. After the skin and spleen section were stained with hematoxylin and eosin, a microscopic examination was conducted by a board-certified pathologist unaware of the treatment of the samples. The pathological scores for the ear pieces were assessed using the following scoring system: infiltration of inflammatory cells: Grade 0, no changes; Grade 1, few infiltrations; Grade 2, moderate infiltrations; and Grade 3, extensive infiltrations; cutaneous epidermis: Grade 0, no changes; Grade 1, minor keratinization in epidermal tissue; Grade 2, obvious epidermal keratinization; and Grade 3, severe keratinization in

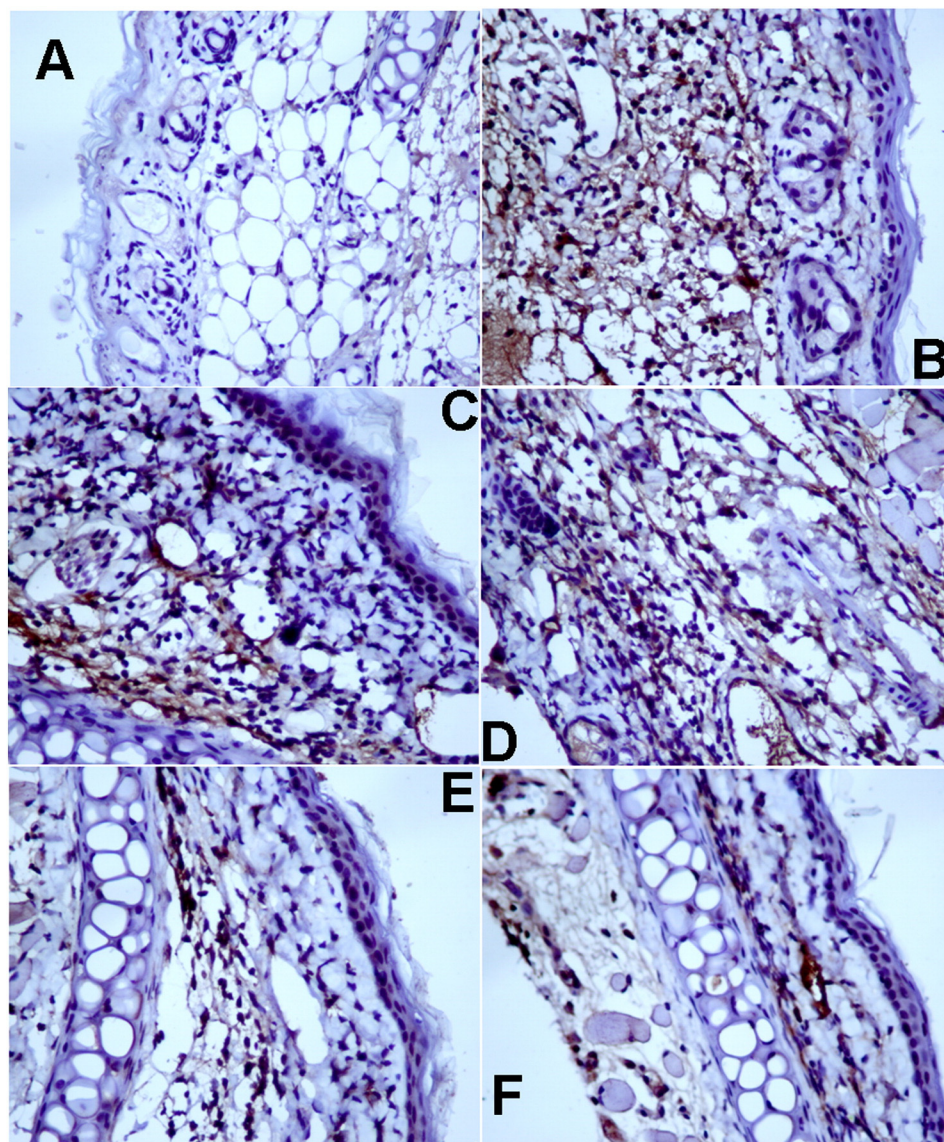


Fig. 2-2. Effects of TGP on monocytes infiltration in ear tissue of mice treated with DNFB (original magnification 400 $\times$). (A) Normal tissue without inflammation; (B) ACD group; (C) TGP (35 mg/kg/day); (D) TGP (70 mg/kg/day); (E) TGP (140 mg/kg/day); (F) Pre (5 mg/kg/day).

epidermal tissue. In addition, five standard evaluations were also applied to the histopathology scores of spleen tissue [19]: cellularity of periarteriolar lymphoid sheaths (PALS), lymphoid follicles, marginal zone, red pulp and the total number of germinal centers (GCs) in each section. The scoring scheme consisted of ordinal categories ranging from 0 (no effect) to 4 (severe effect).

Immunohistochemical analysis [20] was performed with a high temperature antigen unmasking technique. The sections were heated in the unmasking solution (citrate buffer, pH 6.0), washed, and then were incubated with primary monoclonal antibodies (anti-CD14; dilution fold 1/50; ProteinTech Group, Chicago, USA) at room temperature for 60 min. This procedure was followed by incubation with the secondary antibodies (ProteinTech Group, Chicago, USA). These products were developed with the use of diaminobenzidine solution. After the chromogen reactions, the sections were rinsed and then counterstained with hematoxylin. After rinsing, the sections were dehydrated and covered. A blind observer evaluated the serial sections. For quantitative analysis, the cells were counted in three independent microscopic fields ($\times 400$).

2.7. Assay of thymus and spleen indices [21]

On day 7 after immunization, the weight of the mice was measured, and the mice were then killed by cervical dislocation. The thymus and spleen were removed and weighed. The thymus and spleen indices were defined as the thymus and spleen wet weight, respectively, divided by the body weight.

2.8. Thymocyte proliferation assay [21]

After the thymus weight was measured, the thymus was minced aseptically in sterile condition. The thymocytes were collected and suspended in DMEM (Containing 5% (v/v) calf serum) at a concentration of 2×10^7 cells/mL. The cell suspension (100 μ L) and ConA (100 μ L with a final concentration of 5 mg/L) were simultaneously seeded into a 96-well culture plate. Triplicate wells were designed. The cultures were incubated at 37 $^{\circ}$ C in an atmosphere of 5% CO₂ for 48 h. Four hours before the end of the incubation period, 10 μ L of MTT (5 g/L) was added to each well. The absorbance was measured using an ELx808

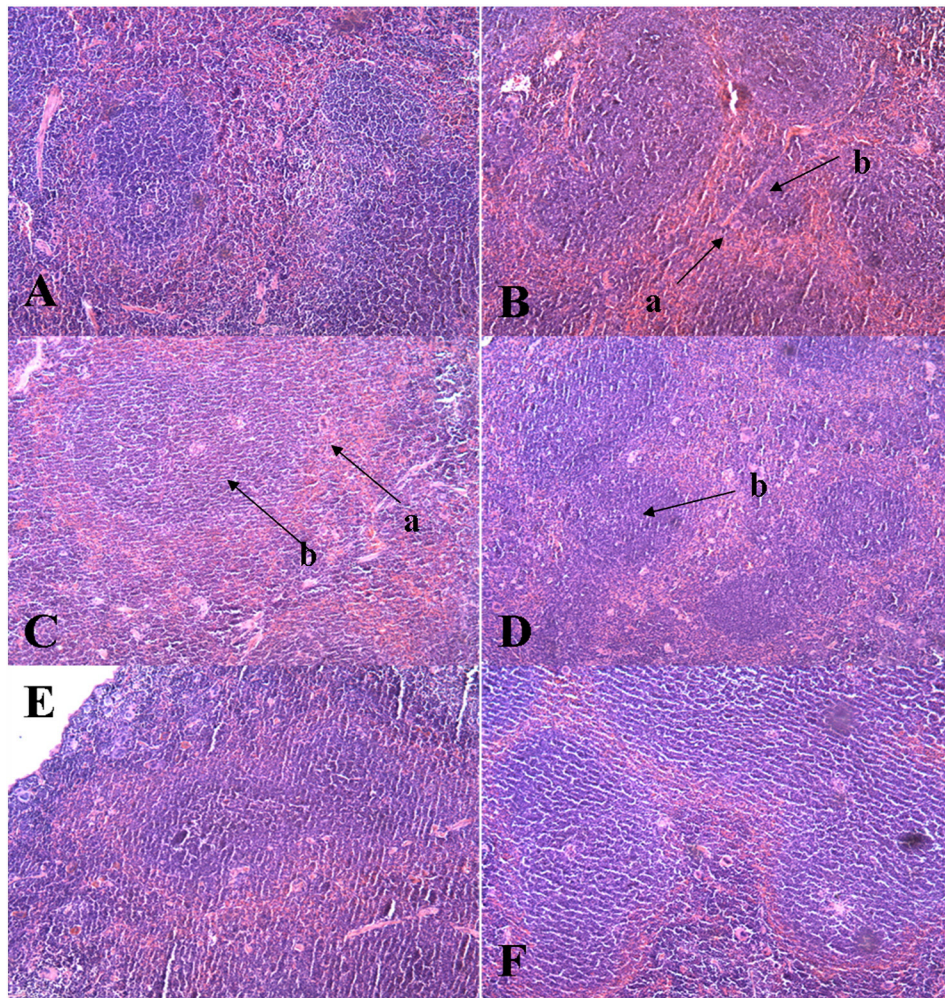


Fig. 2-3. Effects of TGP on spleen histopathology in mice treated with DNCB (original magnification 200 $\times$). (A) Normal spleen tissue. (B) ACD group: The histopathology displayed obvious red pulp hyperplasia (a) and GC appearance (b). (C) TGP (35 mg/kg/day): The histopathology displayed a similar appearance to that observed in the ACD model. (D) TGP (70 mg/kg/day): The histopathology showed an obvious reduction in red pulp hyperplasia. (E) TGP (140 mg/kg/day): The histopathology showed obvious reductions in red pulp hyperplasia and GC appearance. (F) Pre (5 mg/kg/day): The histopathology showed similar effects to those observed in E.

ELISA Microwell Reader (BioTek Co, USA) at 490 nm. The results are described as the average of triplicate measurements of the absorbance.

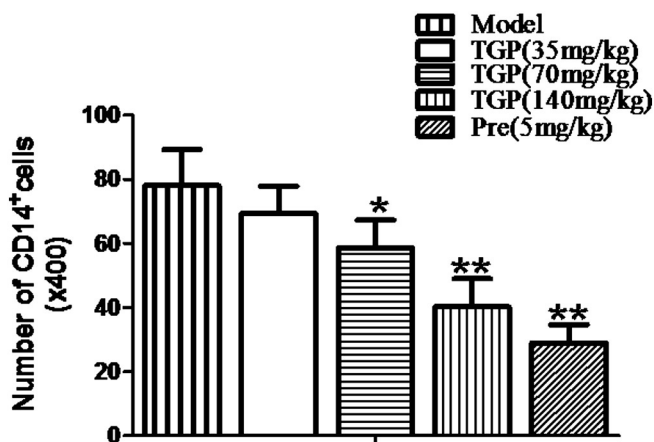


Fig. 2-4. The number of monocytes at 400 magnification. The data are presented as the means $\pm$ S.D. ($n = 6$). * $P < 0.05$ and ** $P < 0.01$ compared with the ACD model group.

2.9. Analysis of cytokine production [21,22]

On day 7 after immunization, blood samples were collected from the mice, stored at room temperature for 30 min, and then centrifuged at 3000 rpm for 15 min to separate the serum. After their weights were measured, the thymus and spleens were minced aseptically under sterile conditions. The thymocytes and splenocytes were then collected and suspended in DMEM (containing 5% (v/v) calf serum) at a concentration of 2×10^7 cells/mL. The cell suspensions (500 μ L) and ConA (500 μ L with a final concentration of 5 mg/L) were simultaneously seeded into a 24-well culture plate. The cultures were incubated at 37 $^{\circ}$ C in an atmosphere of 5% CO₂ for 48 h. At the end of the incubation period, the 24-well culture plate was centrifuged at 2000 rpm (4 $^{\circ}$ C) for 15 min. The supernatant was collected and reserved at -20° C. The concentrations of IL-2, IL-4, IL-10 and IL-17 in the serum and lymphocyte supernatant were measured using ELISA kits according to the manufacturer's instructions.

2.10. Statistical analysis

The data are expressed as the means $\pm$ standard deviation (S.D.). The significance of the differences between the means was evaluated by Student's *t*-test (SPSS Software Products, USA). A difference was considered significant at $P < 0.05$. Spearman's coefficient of correlation

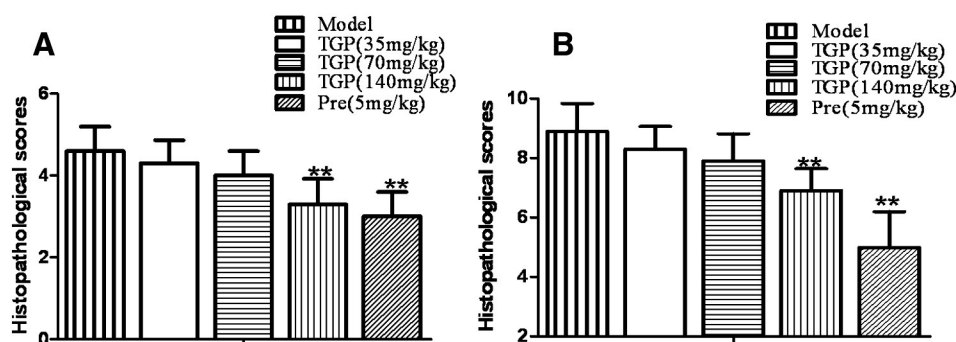


Fig. 2-5. Effects of TGP on histopathological scores of mice treated with DNCB. A: skin histopathology; B: spleen histopathology. The data are presented as the means $\pm$ S.D. ($n = 10$). ** $P < 0.01$ compared with the ACD model group. Treatment with TGP (140 mg/kg/day) and Pre (5 mg/kg/day) significantly reduced the skin and spleen histopathological scores of mice treated with DNCB.

was used to investigate the correlation between the severity of the inflammatory responses and the cytokine levels. Differences were considered significant at $P < 0.05$.

3. Results

3.1. TGP inhibited DNCB-induced ear swelling in mice

The oral administration of TGP (70 and 140 mg/kg/day) significantly inhibited the DNCB-induced ear swelling in mice ($P < 0.05$ and $P < 0.01$, respectively; Fig. 1). In addition, Pre (5 mg/kg/day) showed a similar inhibitory effect.

3.2. TGP ameliorated the skin and spleen histopathology in mice treated with DNCB

Compared with normal mice, the skin histopathology of mice treated with DNCB revealed significant monocyte infiltration (Fig. 2-1B and Fig. 2-2B) and epidermal keratinization (Fig. 2-1B), and the spleen histopathology displayed obvious red pulp hyperplasia (Fig. 2-3B) and GC appearance (Fig. 2-3B). The animals administered TGP (140 mg/kg/day) and Pre (5 mg/kg/day) presented markedly decreased monocyte numbers and epidermal keratinization (Fig. 2-1E–F, Fig. 2-2 E–F and Fig. 2-4), and the degree of spleen injury was also significantly alleviated in the hyperplasia of red pulp and GC appearance (Fig. 2-3 E–F). In addition, compared with the histopathological scores of the model group, the scores for the TGP (140 mg/kg/day) and Pre (5 mg/kg/day) groups were significantly reduced (Fig. 2-5).

3.3. TGP reduced thymocyte proliferation in mice treated with DNCB

As shown in Fig. 3, the thymus and spleen indices of the model group were increased significantly ($P < 0.01$). Further study indicated that

DNCB treatment promoted thymocyte proliferation in mice ($P < 0.01$). However, treatment with TGP (70,140 mg/kg/day) significantly reduced the thymus and spleen indices ($P < 0.05$) and inhibited the abnormal proliferation of thymocytes in mice treated with DNCB ($P < 0.01$) (Fig. 4).

3.4. TGP regulated the IL-2, IL-4, IL-10 and IL-17 levels in serum

As shown in Fig. 5, increases in IL-2 and IL-17 and decreases in IL-4 and IL-10 were observed in the serum after DNCB treatment ($P < 0.05$). However, it was apparent that TGP can reduce the production of IL-2 and IL-17 and increase IL-4 and IL-10 production. In addition, Pre showed a similar regulatory effect on cytokine production (IL-2, IL-10 and IL-17) to that found with TGP.

3.5. TGP regulated the IL-2, IL-4, IL-10 and IL-17 levels in lymphocyte supernatant

As observed in Fig. 6A, compared with thymocytes from normal mice, the mice treated with DNCB exhibited markedly increased production of IL-2 and IL-17 and decreased production of IL-4 and IL-10 ($P < 0.01$). The analysis of cytokine production in the splenocyte supernatant revealed similar results to those discussed above (Fig. 6B, $P < 0.05$). Specifically, TGP obviously reduced the production of IL-2 and IL-17 and increased the production of IL-4 and IL-10 in the supernatants of thymocytes and splenocytes isolated from ACD mice. In addition, similar results were obtained after treatment with Pre. To reveal the relationship between the severity of skin inflammation and cytokine production in the serum and lymphocyte supernatant, we calculated the correlation coefficients between the skin histopathological scores and the cytokine production (Table 1). To our surprise, the scores significantly correlated with the production of cytokines. Specifically, the production of IL-2 and IL-17 displayed positive

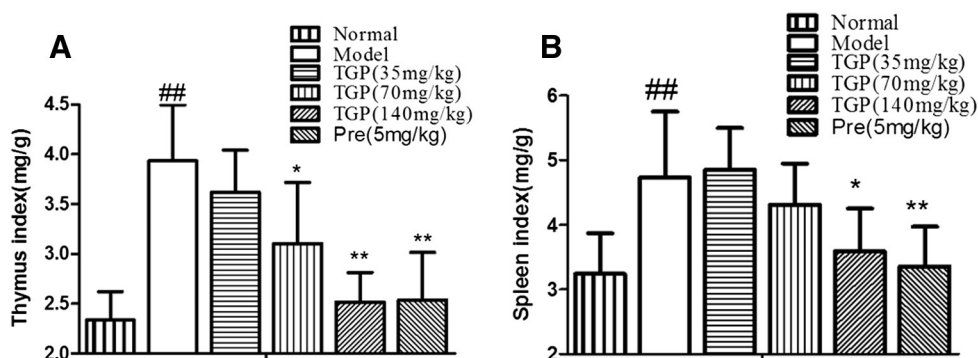


Fig. 3. Effects of TGP on thymus and spleen indices in mice treated with DNCB. The data are presented as the means $\pm$ S.D. ($n = 10-12$). # $P < 0.05$ and ## $P < 0.01$ compared with the normal group; * $P < 0.05$ and ** $P < 0.01$ compared with the ACD model group.

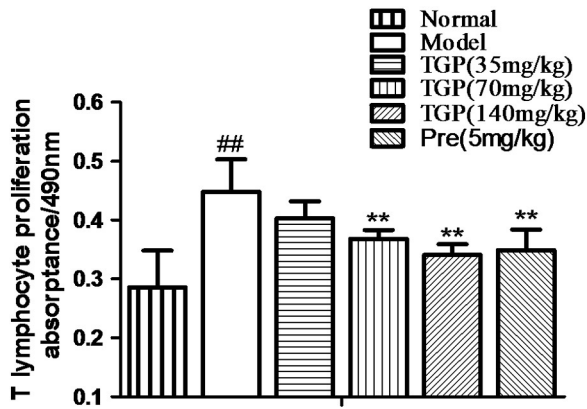


Fig. 4. Effects of TGP on thymocyte proliferation in mice treated with DNCB. The data are presented as the means $\pm$ S.D. ($n = 9-11$). ^{##} $P < 0.01$ compared with the normal group; ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compared with the ACD model group. The thymocytes were incubated for 48 h at 37 °C and 5% CO₂ in the presence of 5 mg/L ConA. The cell proliferation was measured at 490 nm by MTT uptake. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide; Con A: Concanavalin A.

correlations with the scores, whereas the production of IL-4 and IL-10 showed negative correlations (Fig. 4S to Fig. 6S, Supporting Information).

4. Discussion

ACD, which is closely associated with the imbalanced secretion of inflammatory and anti-inflammatory cytokines [23], is a T cell-mediated skin reaction. During the sensitization phase in murine CHS to haptens, IL-2, IL-4 and IL-17 are secreted [2]. It is well known that IL-2 can promote lymphocyte proliferation [24], and highly concentrated IL-2 can induce the activation of the inflammatory cytokine cascade in the epidermal-immune system [25]. In the elicitation phase, IL-4

negatively regulates the CHS response, as demonstrated by a more conspicuous appearance of inflammation in IL-4-deficient mice [26]. Similarly, the CHS response to dinitrofluorobenzene is significantly enhanced in mice lacking STAT6 [27] (the signal transducer and activator of transcription for IL-4). In the present study, a notably negative correlation was obtained between IL-4 production and inflammation scores.

Recent studies have shown that IL-17 can promote the development of ACD [28,29]. Briefly, IL-17 can enhance dendritic cell (DC) maturation [30], promote keratinocyte activation and proliferation, stimulate the expression of adhesion molecules, and up-regulate the secretion of inflammatory cytokines (IL-6) and chemokines (IL-8) [31,32]. Moreover, IL-6 deriving from activatory DCs may promote Th17 cells to secrete IL-17 [33]. Thus, during CHS responses, the neutralization of IL-17 by antibody markedly reduces inflammatory cell infiltration [29], as is markedly observed in mice in which the IL-17 receptors are knocked out [34]. As expected, these results are consistent with the positive correlations between IL-17 production and inflammation scores obtained in the present study (Table 1). Furthermore, IL-10, which is known as an anti-inflammatory cytokine, is secreted by many types of immunocytes [7]. During the elicitation phase of ACD, IL-10 may exert its anti-inflammatory effects by inhibiting APC migration and reducing the production of pro-inflammatory cytokines, such as tumor necrosis factor and IL-1 [7,35]. Therefore, IL-10-deficient mice show enhanced inflammatory responses to hapten, and exogenous rIL-10 significantly reduces the skin inflammation induced by hapten [7,35].

To date, ACD models of mammals have been established successfully by repeatedly applying small molecular haptens to their skin. Among these haptens, DNCB is commonly used to induce sensitization, mainly due to its availability and reproducibility [3]. In the present study, the repeated application of DNCB resulted in obvious skin inflammation (Fig. 1 and Fig. 2-1B). Treatment with TGP (140 mg kg⁻¹) significantly inhibited ear swelling and reduced the monocyte numbers in the ear tissue, similarly to the effects observed after the oral administration of Pre (5 mg.kg⁻¹). Further study showed that TGP can inhibit thymocyte

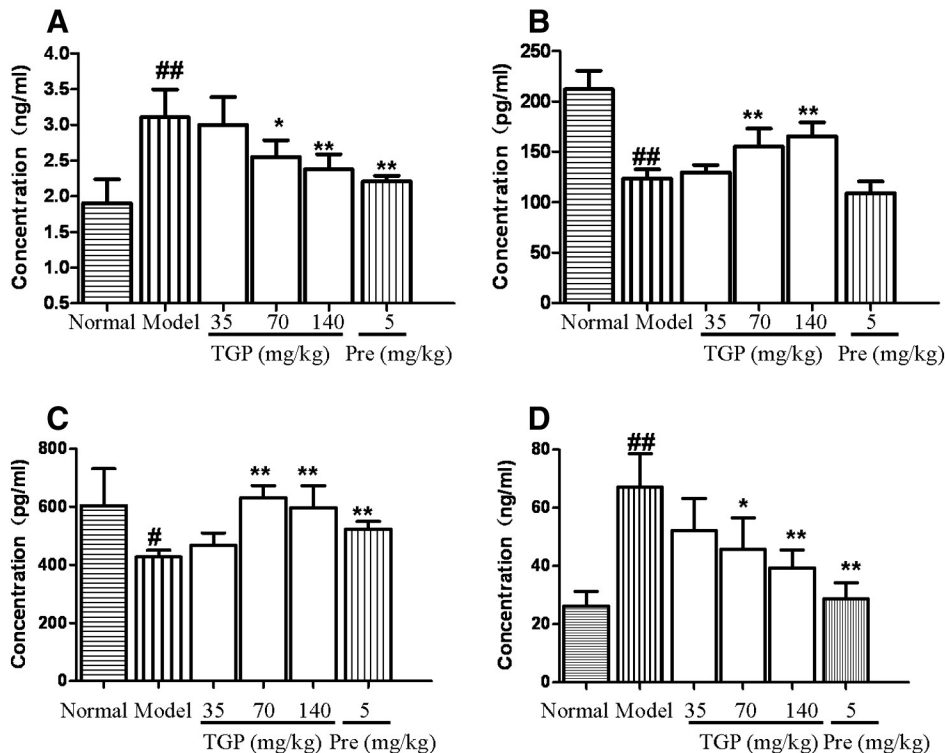


Fig. 5. Effects of TGP on cytokine production in serum of mice treated with DNCB. A: IL-2; B: IL-4; C: IL-10; D: IL-17. The data are presented as the means $\pm$ S.D. ($n = 6-8$). [#] $P < 0.05$ and ^{##} $P < 0.01$ compared with the normal group; ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compared with the ACD model group.

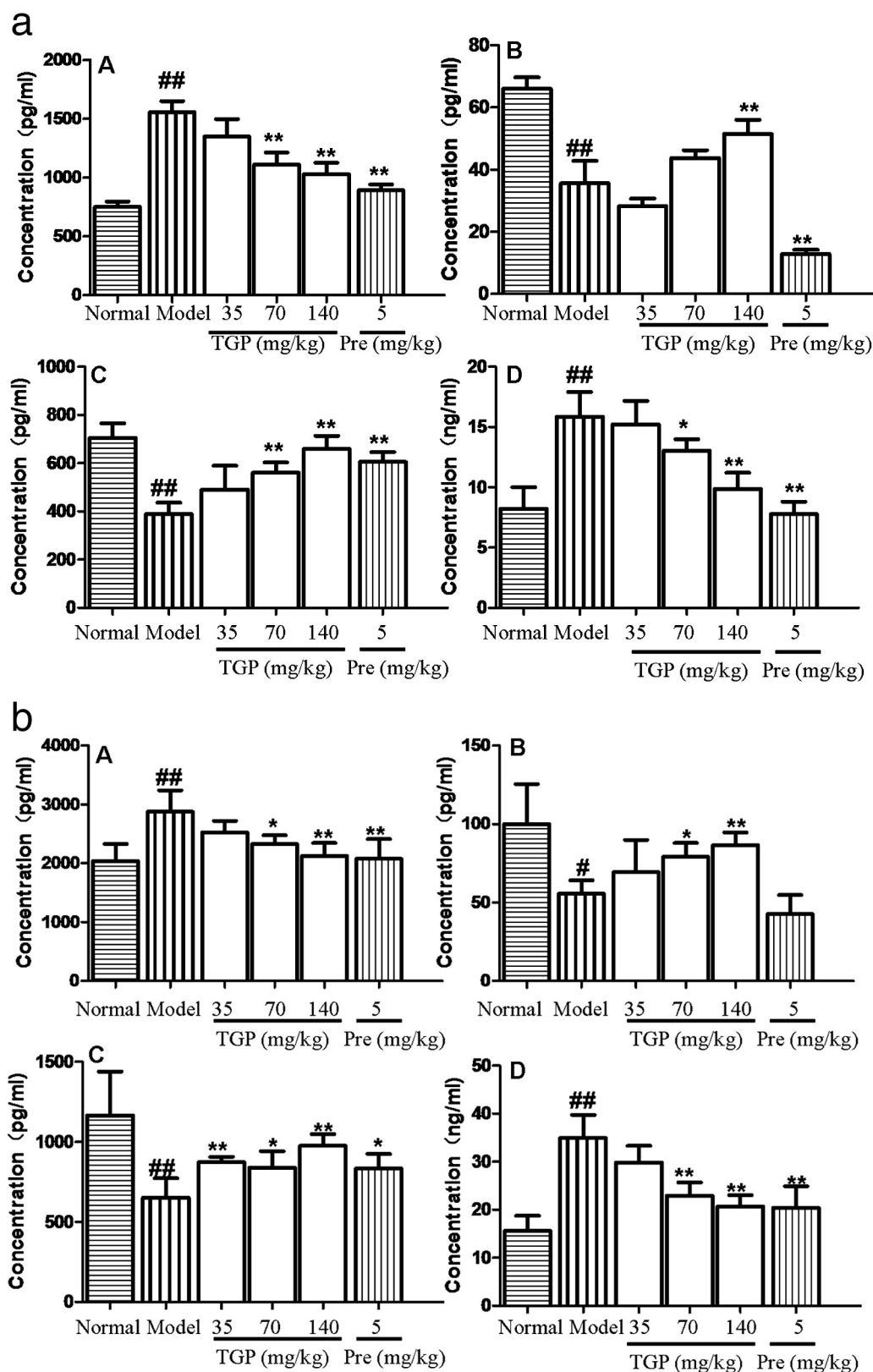


Fig. 6. Effects of TGP on cytokine production in the supernatant of lymphocytes isolated from mice treated with DNCB. (a) Supernatant of thymocytes. (b) Supernatant of splenocytes. A: IL-2; B: IL-4; C: IL-10; D: IL-17. The thymocytes and splenocytes were treated with 5 mg/L Con A for 48 h, and the cytokine production was then measured by ELISA. The data are presented as the means $\pm$ S.D. ($n = 6-8$). # $P < 0.05$ and ## $P < 0.01$ compared with the normal group; * $P < 0.05$ and ** $P < 0.01$ compared with the ACD model group. ELISA: Enzyme-linked immunosorbent assay.

proliferation and restore the balanced secretion of pro-inflammatory cytokines and anti-inflammatory cytokines. In the current study, the anti-proliferative properties of TGP may be partly due to its inhibitory

effects on IL-2 [24]. Moreover, as the main ingredient of TGP, Pae can promote the expression of pro-apoptosis gene (Bax) [36]. Moreover, TGP can restore the balanced secretion of IL-2/IL-4 in ACD mice.

Table 1Correlations between the cytokines levels and the skin histopathological scores ^a.

	Cytokines	Regression equation	Correlation coefficient	P-value
Serum	IL-2	$Y = 2.014X - 1.684$	0.8853	0.046*
	IL-4	$Y = -0.029X + 8.126$	0.9276	0.023*
	IL-10	$Y = -0.009X + 8.597$	0.7652	0.132
	IL-17	$Y = 0.081X + 0.157$	0.9095	0.032*
Thymocyte	IL-2	$Y = 2.959X + 0.228$	0.9102	0.032*
	IL-4	$Y = -0.070X + 6.880$	0.9025	0.036*
	IL-10	$Y = -0.008X + 8.101$	0.9305	0.022*
	IL-17	$Y = 0.314X - 0.305$	0.9578	0.001**
Splenocyte	IL-2	$Y = 3.720X - 4.470$	0.9665	0.007**
	IL-4	$Y = -0.067X - 8.869$	0.9647	0.008**
	IL-10	$Y = -0.006X + 8.348$	0.8451	0.072
	IL-17	$Y = 0.154X + 0.133$	0.9473	0.014*

* Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level.

^a The correlation analysis between the cytokine levels and the skin histopathological scores included the normal group, the model group and the three TGP groups.

However, in comparison with TGP, Pre reduced both IL-2 and IL-4 production, which supports the definitions of TGP and Pre as an immunomodulator and an immunosuppressor, respectively. A recent study suggested that the promotion of IL-10 excretion by TGP partially results in its therapeutic effects on immune disease [37]. The present results show that TGP can increase IL-10 production by lymphocytes. In addition, IL-17 is closely related to many inflammatory diseases, but the effects of TGP on IL-17 in disease remain poorly understood. In this study, we firstly discovered that TGP can prevent the secretion of IL-17 from lymphocytes in ACD mice. More importantly, the results presented in Table 1 strongly suggest that the severity of skin inflammation in mice is promoted by the imbalanced secretion of IL-2/IL-4 and IL-10/IL-17.

In conclusion, these results suggest that the therapeutic effects of TGP on ACD in mice may result from its regulation of the imbalanced secretion of IL-2/IL-4 and IL-10/IL-17.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

The authors would like thank Da-ke Huang for their excellent technical assistance. This work was financially supported by the National Natural Science Foundation of China (Nos. 30572356; 81173075; 81330081 and 81302845), the Anhui Province Nature Science Foundation in University (No. KJ2013A158), and the Grants for Scientific Research of BSKY (No XJ201211) from Anhui Medical University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2014.12.026>.

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Supporting Information

**Total glucosides of paeony inhibit the inflammatory responses of mice with
allergic contact dermatitis by restoring the balanced secretion of
pro-/anti-inflammatory cytokines**

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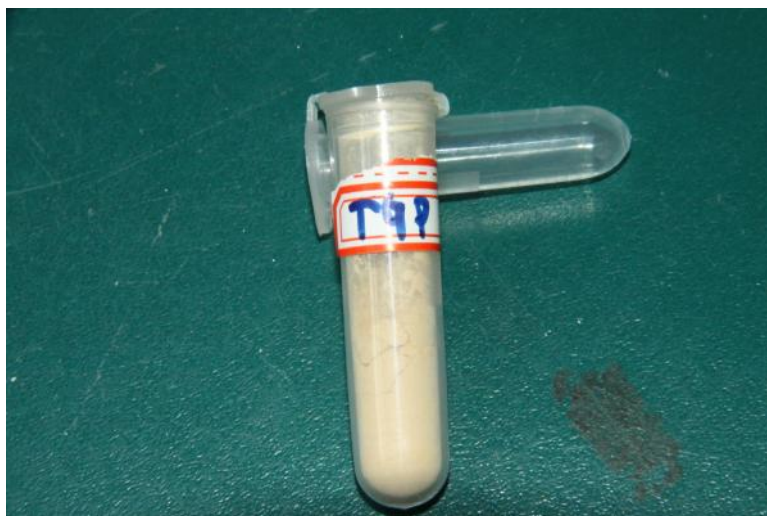


Fig.1S. The appearance of TGP extracted from roots of *Paeonia lactiflora* Pall

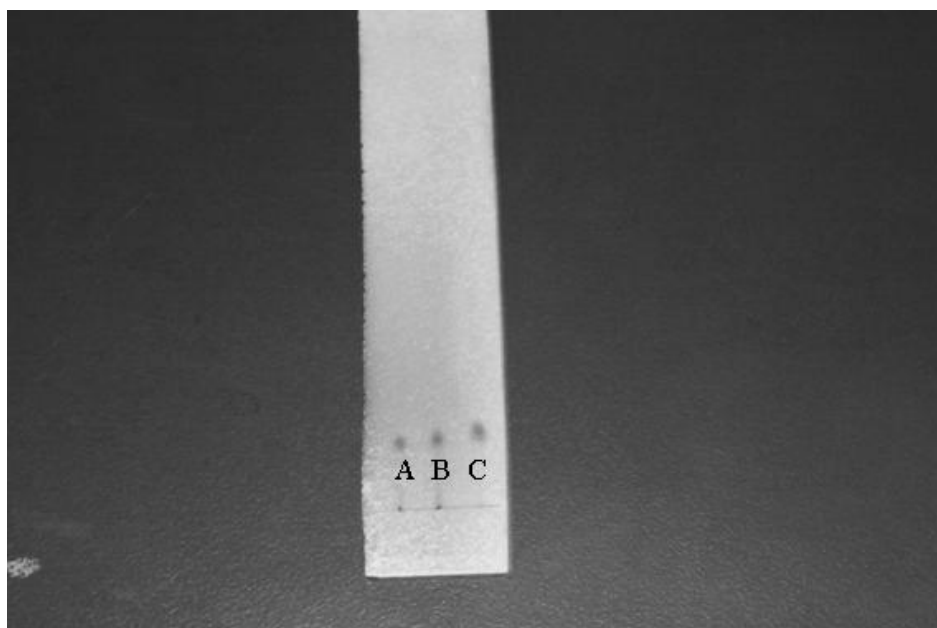


Fig.2S. Thin layer chromatograph of TGP extracted from roots of *Paeonia lactiflora* Pall
A: TGP extracted from roots of *Paeonia lactiflora* Pall; **B:** TGP purchased from market; **C:** standard Pae

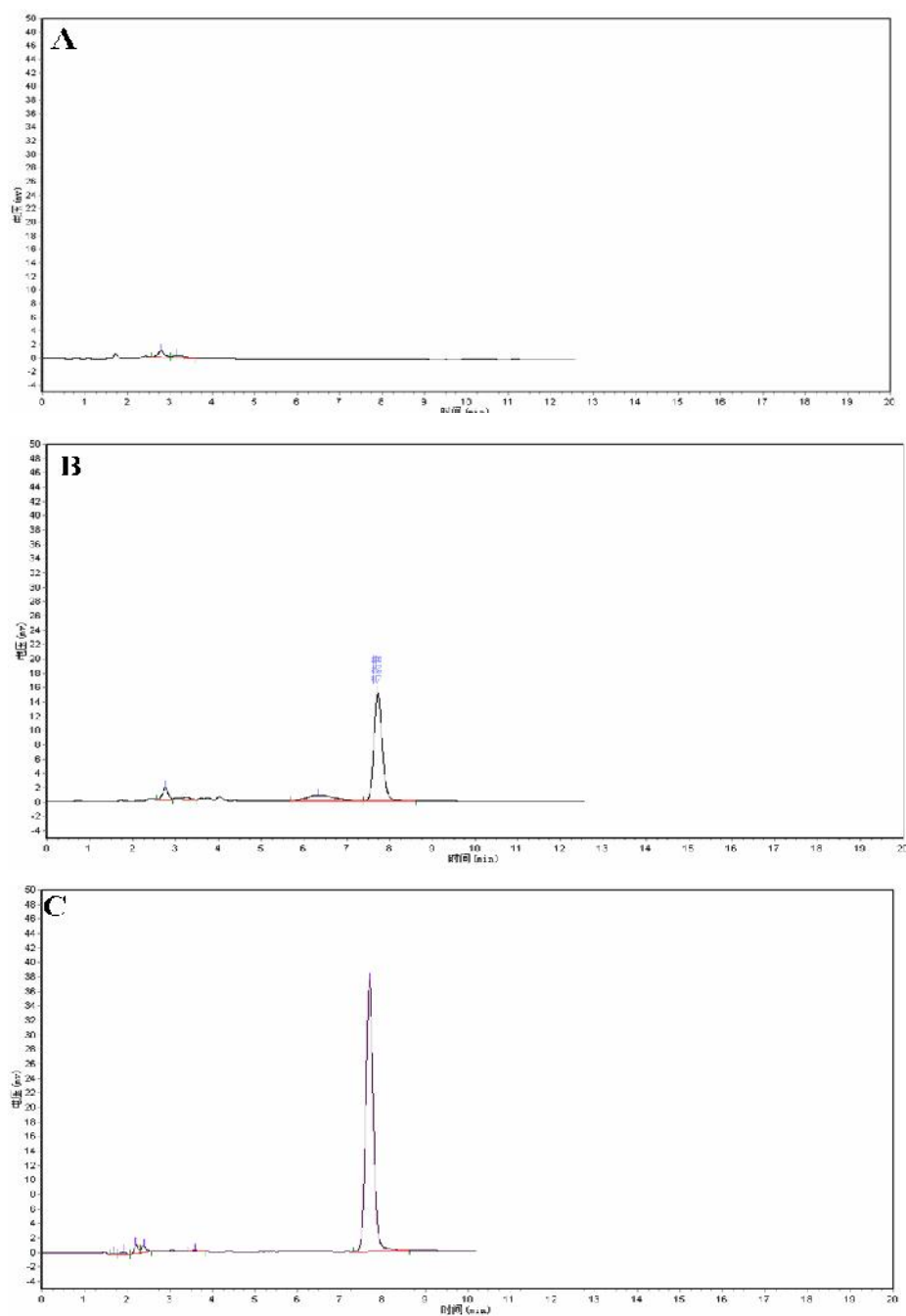


Fig.3S. The HPLC chromatograms of TGP

(mobile phase: methanol/water =40/60; flow rate:0.8 mL·min⁻¹; wavelength:230 nm; column temperature : 30°C; a Hypersil C₁₈ column :4.6mm×250mm, 5μm, Elite analytical instrument Co., Ltd, Dalian, China) The retention time of Pae was 7.7 min approximately. **A:** mobile phase; **B:** TGP extracted from roots of *Paeonia lactiflora* Pall; **C:** standard Pae

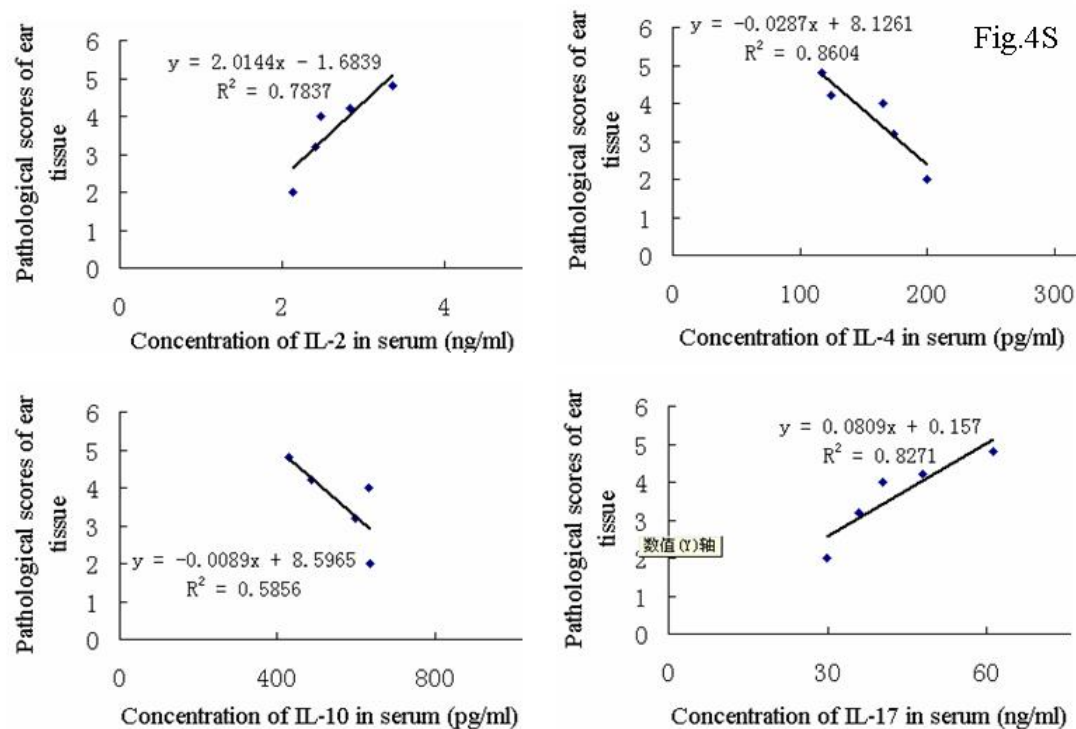


Fig.4S. The scatter plot of the serum cytokines production and skin pathological scores

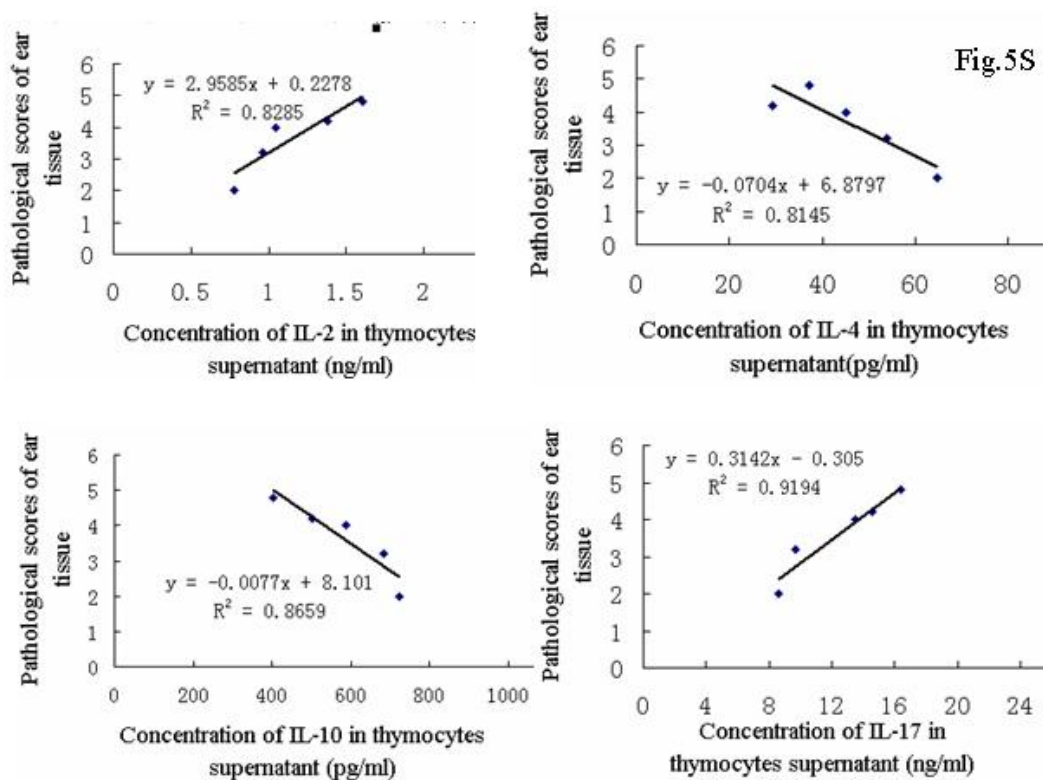


Fig.5S. The scatter plot of the cytokines production in thymocytes supernatant and skin

pathological scores

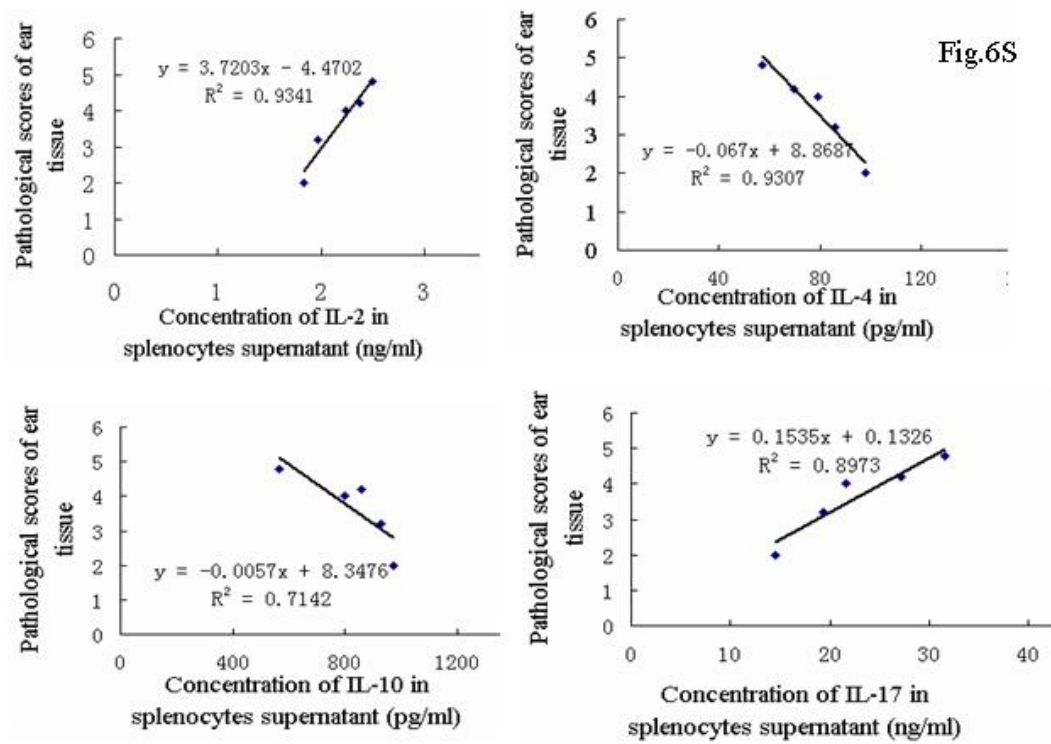


Fig.6S. The scatter plot of the cytokines production in splenocytes supernatant and skin

pathological scores