



Oral administration of paeoniflorin attenuates allergic contact dermatitis by inhibiting dendritic cell migration and Th1 and Th17 differentiation in a mouse model

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ABSTRACT

Allergic contact dermatitis (ACD) is a hapten-specific CD4⁺ T-cells mediated inflammatory response of the skin. Its pathomechanism involves 2 phases, an induction phase and an elicitation phase. Langerhans cells (LCs) and dendritic cells (DCs) in the skin play key roles in presenting low molecular weight chemicals (haptens) to the lymph nodes. Therefore, inhibition of the migration of LCs or DCs and T-cell proliferation is each expected to control ACD disease. To explore the effectiveness of paeoniflorin (PF) on the migration of LCs and T-cell proliferation *in vivo*, we establish a murine model of ACD, promoted by repeated exposure to an allergen (specifically 1-Chloro-2,4-dinitrobenzene (DNCB)). Administration of PF inhibits DC migration in this DNCB-induced model in the induction phase. As a result, epidermal LC density in the elicitation phase increased in PF-treated mice when compared to PF-untreated mice. At the same time, PF reduced IFN- γ ⁺CD4⁺ and IL-17⁺CD4⁺ T cells proliferation (but not IL-4⁺CD4⁺ T cells proliferation), leading to an attenuated cutaneous inflammatory response. Consistent with this T-cell proliferation profile, secretions of IFN- γ and IL-17 were reduced and IL-10 secretion increased in PF-treated mice, but production of IL-4 and IL-5 remained unchanged in the skin and blood samples. These results suggest that oral administration of PF can treat and prevent ACD effectively through inhibition of DC migration, and thus decrease the capacity of DCs to stimulate Th1 and Th17 cell differentiation and cytokine production.

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1. Introduction

Allergic contact dermatitis (ACD) is a skin disease caused by an allergic reaction to the allergens in contact with the skin, characterized by redness, papules, vesicles, followed by scaling and dryness [1,2]. Approximately, 15–20% of the population worldwide is affected by this disease [3,4], leading to a loss of 4 million work-days per year and almost \$400 million in the United States alone [5–7]. Although

therapeutic application of steroids can often effectively reduce skin inflammation, patients with ACD suffer from chronic disease in some cases.

ACD is a T cell delayed-type hypersensitivity (type IV) response initiated by skin contact with haptens (low-molecular weight chemicals). Haptens penetrate the skin and covalently bind to carrier proteins to become fully immunogenic [8]. Therefore, models of ACD are often employed for studying Ag-specific T cell-mediated immune responses [9,10]. In the sensitization phase, a hapten (*i.e.* an allergen, drug, or metal) is applied to the skin of an animal, usually on the abdomen. Consequentially, the elicitation phase is attained by a subsequent exposure to the same hapten at a remote skin site, such as ear skin [11].

Dendritic cells (DCs) play key roles in the initiation and elicitation of ACD [12]. Sensitization of the skin to allergens occurs when allergens are taken up by antigen-presenting cells in the skin, such as Langerhans cells (LCs) or dermal DCs, and brought to the draining lymph nodes to prime allergen-specific T cells. In lymphoid tissue, hapten-bearing antigen-presenting cells 'educate' naïve T lymphocytes, and in response naïve T lymphocytes become allergen-specific memory or effector T

Abbreviations: DCs, dendritic cells; LCs, Langerhans cells; ACD, allergic contact dermatitis; DNCB, 1-chloro-2,4-dinitrobenzene; PF, paeoniflorin

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cells [4]. When the patient encounters the same hapten again, the hapten diffuses through the skin and is taken up by LCs or dermal DCs, which in turn present the hapten to specific T cells [13]. These specific T cells are then activated and induce cytokine production that further activates the inflammatory response to elicit the typical clinical symptoms of ACD; redness, papules and vesicles.

Experiments for ACD control have focused on prevention of DC migration, inhibition of Th1/Th2/Th17 responses and reduction of local inflammatory cytokine products [12,14]. Attempts to modulate the immune response to ACD have been conducted in a number of clinical trials but have achieved only limited success [15]. Today, topical glucocorticoids still remain an effective remedy for the treatment of ACD. However, prolonged use of high doses of glucocorticoids can cause a variety of side effects.

Paeniflorin (PF), a water-soluble monoterpene glycoside found in the root of *Paonia lactiflora* Pall (the peony), has been suggested to possess a wide range of pharmacological features, including anti-oxidant, anti-inflammatory, and anti-cancer effects [16]. As both Th1 and Th2 immune responses are implicated in the development of ACD skin lesions, PF has been suggested to inhibit inflammatory responses via modulating the balance of inflammatory and anti-inflammatory cytokines in ACD [17]. In our previous study *in vitro*, we also demonstrated that PF inhibited DC maturation and migration, and inhibited Th1 cell proliferation in response to hapten (1-Chloro-2,4-dinitrobenzene (DNCB)) [18].

In this study, we focus on the capacity of PF to influence migration of DCs and T cell proliferation in an *in vivo* DNCB-induced ACD model. The influence of orally administered PF on DC migration, Th1, Th2 and Th17 cell differentiation and cytokine secretions are clearly demonstrated.

2. Materials and methods

2.1. Regents and chemicals

1-Chloro-2,4-dinitrobenzene (DNCB), acetone and olive oil were obtained from Sigma Chemical Co. (USA). RPMI 1640 medium, Hank's balance salt solution, penicillin, streptomycin, L-glutamine, and fetal bovine serum (FBS) were purchased from Gibco (USA).

PF (powder compound, purity determined by HPLC is >98%) was purchased from Silida Technology Ltd. (Tianjin, China) in Fig. 1. PF was dissolved in 50 ml of sterilized PBS to make a 2000 mg/ml stock solution. Working solutions were further diluted in saline.

2.2. Mice and ethics statement

Female BALB/c mice were purchased from the Experimental Center of Yangzhou University (Jiangsu, China). The mice were bred in the animal experimental center of the Institute of Dermatology, Chinese Academy of Medical Sciences (CAMS) in Nanjing, Jiangsu. Animals were housed with six mice per cage at 22 °C with a 12-hours light–

dark cycle. Experiments were conducted with 8 week old mice with body weight 18–20 g. Water and standard diet were available *ad libitum*. The experiments were performed in a pathogen-free environment. The protocols for animal experiments were all approved by the Animal Study Committee of the Institute of Dermatology, CAMS, according to the government guidelines for animal care.

2.3. Sensitization and challenge procedures to induce ACD and local lymph node assay

DNCB-induced ACD was achieved as previously described [19,20]. After complete removal of hair in an abdominal area of approximately 4 cm², 100 µl of 1% DNCB solution (dissolved in a 4:1 mixture of acetone: olive oil) was applied to the stripped epidermis for three consecutive days (days 0–2) to sensitize mice. Seven days later (day 9), the elicitation phase was induced by single application of 0.2% DNCB solution (acetone: olive oil; 4:1) to the right ear of each mouse. From day 0 mice were administered PF orally (100 or 200 mg/kg per day) or PBS as control for the subsequent 10 days. At 24 h post-challenge, blood was collected by orbital puncture, and swelling of the ear was measured by a comparison of the ear thickness (cutometer, Mitutoyo, Neuss, Germany). The ear was removed for histopathological and RT-PCR examination, as described below.

To assess the effects of PF on the sensitization phase, a local lymph node assay was performed as previously described, with minor modifications [21,22]. Over a period of 7 days (days 0–7), mice were administered PF or PBS orally. From day 4 mice were sensitized by application of 100 µl of 1% DNCB (acetone: olive oil; 4:1) onto the dorsum of both ears for three consecutive days (days 4–6). On day 7, the mice were

Paeniflorin

[C₂₃H₂₈O₁₁, MW:480.45]

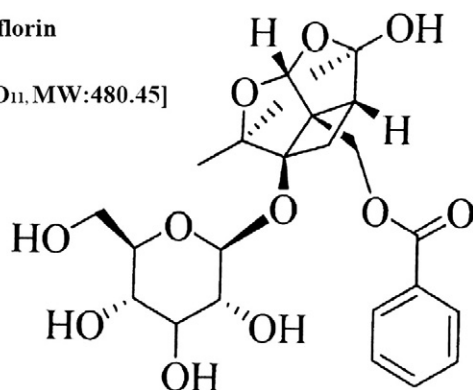


Fig. 1. The chemical structure of paeniflorin.

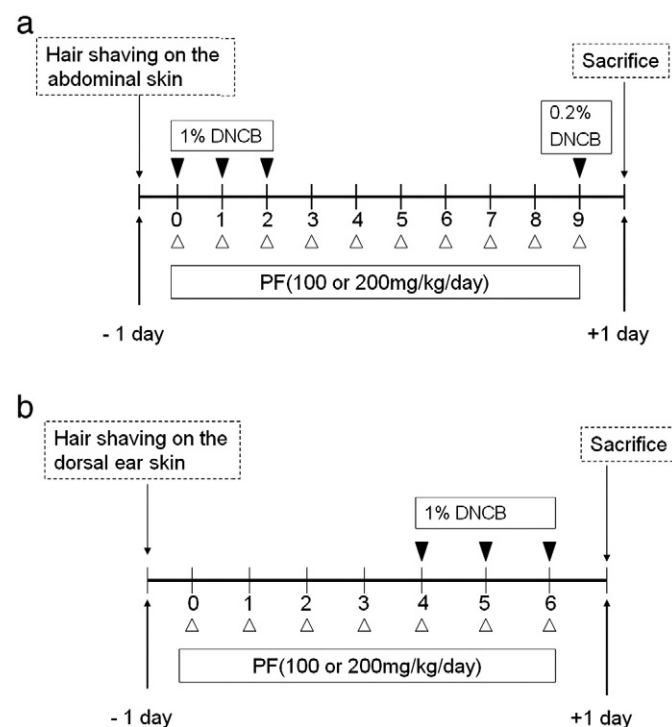


Fig. 2. Design of allergic contact dermatitis model and lymph node assay. (a) Schematic diagram of establishment of a mouse model of allergic contact dermatitis using Th1 contact sensitizers (2,4-dinitrochlorobenzene, DNCB). Mice were administered paeniflorin (PF) (100 or 200 mg/kg/day) or PBS orally from day 0 to 10. 100 µl of 1% DNCB in 4:1 (v/v) acetone/olive oil was administered daily to the stripped abdominal skin to sensitize mice from day 1 to 3. On day 10 the elicitation phase was induced by administration of 0.2% DNCB (acetone: olive oil; 4:1) to the right ear. (b) To assess the effects of PF on the sensitization phase, a local lymph node assay was performed. On days 0–7 mice were administered PF or PBS orally. From day 4, mice were sensitized by application of 100 µl of 1% DNCB (Sigma, USA) (DMSO: olive oil; 4:1) onto the mouse ears for 3 consecutive days (days 4–6). On day 7, mice were sacrificed by cervical dislocation and auricular draining lymph nodes were obtained for the experiment as follows.

sacrificed by cervical dislocation and auricular draining lymph nodes were obtained as schematized in Fig. 2.

2.4. Histopathological studies of the ear skin

Ear skin lesions were dissected and fixed in 10% buffered-neutral formalin for 24 h. The fixed ear tissue slices were embedded in paraffin, sectioned at 5 μ m thickness, de-paraffinized, and dehydrated using standard techniques. Sections were subjected to hematoxylin and eosin (H&E) staining for detection of inflammatory cells. Visual fields were chosen randomly and viewed at a magnification of 100 $\times$ object lens. Histopathological changes were visualized by light microscopy.

2.5. Measurement of skin cytokine mRNA levels by RT-PCR

Semi-quantitative RT-PCR was employed to detect the mRNA levels of INF- γ (Th1), IL-4, IL-5, IL-10 (Th2), and IL-17 (Th17) in ear tissue 24 h after the third challenged with DNCB (Fig. 2b). Total RNA was isolated from the right ear lesion according to the manufacturer's protocol, and stored at -70°C . Then, 0.5 μ g of RNA was used for reverse transcription and amplified by PCR using the access RT-PCR system Mx3005P (Agilent Stratagene). PCR was performed using primers for mouse INF- γ , IL-4, IL-5, IL-10, IL-17 β , and β -actin (Table 1). PCR products were separated on a 2% agarose gel containing ethidium bromide, visualized, and photographed using a gel documentation system (UVP, UK). Band intensity was quantified by densitometry using Image J software (Image Processing and Analysis in Java, NIH, USA). The relative expression levels of target genes were normalized using β -actin as an internal control.

2.6. Measurement of serum cytokine production

The cytokine content of blood at day 11 (24 h after challenged with DNCB in Fig. 2a) was assessed by ELISA. The blood samples were collected by orbital puncture and serum was stored at -70°C prior to analysis. Serum IFN- γ , IL-4, IL-10, IL-5, and IL-17 contents were measured using ProcartaPlexMul-plex (eBioscience Inc., USA) with Luminex 200, according to the manufacturer's instructions.

2.7. Skin DCs migration assay

The cartilage-free dorsal halves of mouse ear skin at 24 h after the third sensitization with DNCB were split and cultured in 24-well plates as previously described with minor revisions [23,24]. The mouse ear skin was placed onto a table made of sieves with the epidermal side facing upwards. The tables were transferred daily to new wells and fresh media. Cells that migrated from each ear were pooled on days two and three and counted with hemocytometer. The ear halves were weighted, and the number of migrated cells per mg of tissue was

calculated. The cells collected from day 1 were discarded due to a less purity of DC (approximately 30%) [23]. The viability of the cells was evaluated by trypan blue exclusion test.

2.8. Preparation of epidermal sheets for immunohistochemistry

The remaining epidermal sheets were prepared for immunohistochemical examination as previously described [15]. In brief, skin was floated dermal side down on 0.5 M ammonium thiocyanate (Riedel de Haen, Germany) for 10 min at 37°C . The epidermis was separated from the dermis and immediately fixed in cold acetone. DCs were detected by biotinylated monoclonal anti-mouse MHC-II (Heidelberg, Germany). Labeling of the antibodies was visualized using streptavidine-fluorochrome (carbocyanin 3, USA). Analyses were performed using the Kontron KS 400 image analysis system. The density of LCs was analyzed (at $\times 20$ magnifications on a calibrated grid). Sixteen areas were chosen randomly and LCs were counted per ear. Six ears of each group were analyzed for LCs but only three ears were evaluated for the mice treated with PF due to accidental sample loss.

2.9. Measurement of DC migration in lymph nodes

Twenty-four hours after the third sensitization with DNCB (Fig. 2b), the auricular lymph nodes were removed and single-cell suspensions were prepared in phosphate-buffered saline on ice. As DCs in draining lymph nodes migrated from skin are characterized by both CD11c^{high} and CD40^{high} [25], lymph node cells were stained by CD11c and CD40 with corresponding isotype controls as previously described [20]. The percentage of CD11c and CD40 double-positive cells was calculated, and the samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (FACSCalibur, Becton Dickinson, USA).

2.10. Determination of CD4⁺ T cell proliferation and cytokine synthesis in lymph nodes

Auricular lymph nodes were obtained at 24 h after immunization with DNCB. Highly purified CD4⁺ T cells were isolated from lymph node by negative selection using anti-murine CD4⁺ T isolation kit according to the manufacturer's instructions (MiltenyiBiotec, Germany). The purity of CD4⁺ T cells in each sample was >92% when analyzed by flow cytometry. Purified CD4⁺ T cells (1×10^5) were co-cultured with ConA (2 μ g) for 72 h. For intracellular cytokine analysis, cells were harvested, washed, fixed, and permeabilized using the CytoStain kit (BD Pharmingen, USA) according to the manufacturer's instructions. Cells were then stained with a 0.5 μ g/test of anti-murine IL-4-PE (BD Pharmingen, USA), anti-murine-IFN- γ FITC and anti-murine-Th17-PE (BD Pharmingen, USA), respectively and analyzed by flow cytometry.

Table 1
Primers used in quantitative real-time reverse transcription-PCR.

Gene	Sequence 5'–3'	Annealing temperature($^{\circ}\text{C}$)	Gene bank no.
INF- γ	(F)TAT CTG GAG GAA CTG GCA AA (R)ATC AGG TGT GAT TCA ATG ACG	58	BC119065
IL4	(F)CACCTCAAACCACTCTCTGAA (R)CTTGAAGCCCTACAGACGA	59	M25892
IL5	(F)ATGAGACGATGAGGCTTCCT (R)CCCACGACAGTTTGATTCT	58	NM_010558
IL10	(F)AGT GTG TAT TGA GTC TGC TGG (R)TTT CCAAGGAGTTGTTCCG	58	M84340.1
IL17	(F)TGG GTT CTG CGG TAT TTG AC (R)GCT GCT GTG TGA GGT TGA GC	60	NM_010552.3
β -actin	(F)CGC TCT TCC AGC CTT CCT TT (R)CGT TGT TGG CAT ACA GGT CCT	60	M12866

Supernatants were collected at 24 h after DNCB challenge, centrifuged and frozen at -20°C until use. The IFN- γ , IL-4, IL-10, IL-5, and IL-17 contents were quantified by ProcartaPlexMul-plex (eBioscience Inc., USA) immunoassay with Luminex 200 according to the manufacturer's instructions.

2.11. Statistical analysis

All experiments were repeated at least three times. Statistical analyses were performed using SPSS 16.0. Treatment effects were analyzed using one-way analysis of variance followed by Dunnett's test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. PF inhibited ear swelling and T-cell infiltration in the elicitation phase of ACD

A mouse model of DNCB-induced ACD was established as previously described [19,20]. Mice were sensitized to DNCB by topical administration to abdominal skin for three consecutive days (day 1 to 3). Seven days later elicitation was induced by administration of DNCB to the ear skin, and ear swelling was assessed at 24 h later (day 11). As shown in Fig. 3a & c, the ears of mice sensitized to DNCB were significantly swollen in comparison to control animals. However, when mice were administered PF orally from day 0 to 7, ear swelling was reduced in a dose-dependent manner (Fig. 3d & e). In a preliminary experiment, we found that daily administration of 50 mg/kg/day PF could not reduce ear swelling after DNCB induction since ear swelling showed no

difference between PF-treated (50 mg/kg/day) and PF-untreated ACD mice (data not shown). Thus, we administered 100 or 200 mg/kg/day PF in all experiments.

3.2. Administration of PF inhibits expression of IFN- γ and IL-17, and enhances expression of IL-10 mRNA in skin lesions

To elucidate the effects of PF on inflammatory cytokines release in ACD, we examined the transcriptional levels of cytokines in skin lesions in the elicitation phase of ACD. Twenty-four hours after DNCB challenge the mRNA levels of IFN- γ , IL-4, IL-5, IL-10, and IL-17 in ear tissues were measured by RT-PCR. We found expressions of IFN- γ and IL-17 increased significantly and IL-10 decreased slightly in the ears of mice sensitized to DNCB in comparison to control animals (Fig. 4). The IL-4 and IL-5 expressions in ear tissues were slightly increased after DNCB induction, but no significant differences were found between the control mice and ACD model. In PF-treated mice, we found the mRNA levels of IFN- γ and IL-17 were significantly decreased in DNCB-induced ACD mice. These results indicated that PF reverses the higher Th1-responses and elevated Th17 response that induced by DNCB antigen. On the other hand, IL-10 expression increased in PF treated mice, but IL-4 and IL-5 expression were not altered with DNCB induction and PF treatment (Fig. 4c & e).

3.3. Administration of PF inhibits secretion of IFN- γ and IL-17 and enhances the level of circulating IL-10

Twenty-four hours after challenge with DNCB, blood samples were collected by orbital puncture to assess the circulating levels of IFN- γ

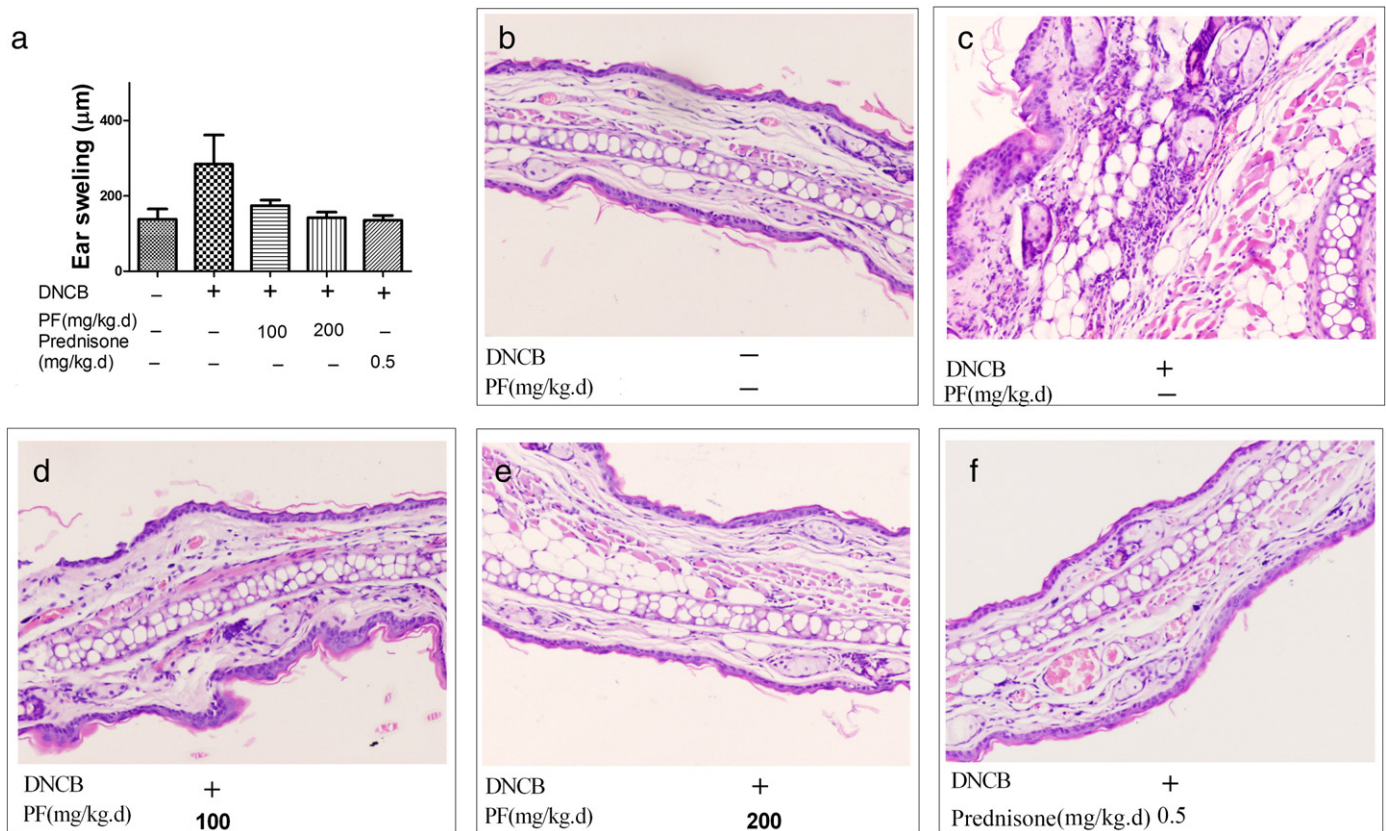


Fig. 3. The influence of paeoniflorin administration on the histopathological features of ear skin lesions in an allergic contact dermatitis mouse model. (a) Ear thickness was measured by micrometer. (b, c, d and e) Ear lesions were removed and fixed with 10% formaldehyde solution. Thin sections were cut and stained with hematoxylin and eosin. (b, c, d and e, original magnification, 100 $\times$). (b) No contact dermatitis was observed normal mice. (c) Contact dermatitis was observed in mice sensitized to and challenged with DNCB to induce a model of allergic contact dermatitis (ACD). (d) ACD model mice administered paeoniflorin (PF) 100 $\mu\text{g}/\text{ml}$ orally following induction by DNCB. (e) ACD model mice administered PF 200 $\mu\text{g}/\text{ml}$ orally following induction by DNCB. Results are presented as mean $\pm$ SD ($n = 6$). ** $p < 0.01$; * $p < 0.05$.

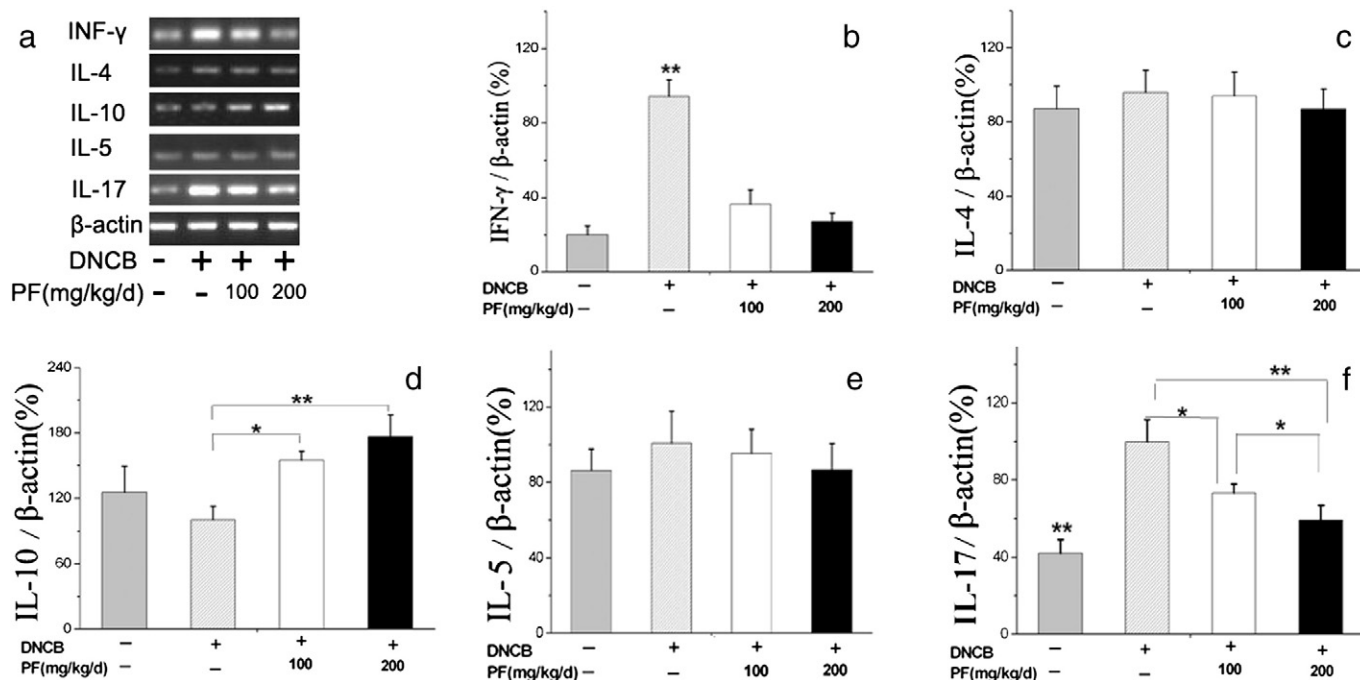


Fig. 4. Influence of paeoniflorin on mRNA expression of Th1, Th2 and Th17 cytokines in a mouse model of allergic contact dermatitis. Mice were sensitized and challenged with DNFB as previously described. (a) Total RNA was extracted from ear tissue to detect levels of INF- γ , IL-4, IL-5, IL-10, IL-17 and β -actin mRNA were determined by semi-quantitative RT-PCR. Results shown are representative of five observations. (b) The intensity of PCR bands was measured using the NIH Image J program. Results are presented as mean $\pm$ SD ($n = 5$). * $p < 0.05$; ** $p < 0.01$.

(Th1), IL-4, IL-5, IL-10 (Th2), and IL-17 (Th17). As shown in the Fig. 5, the levels of INF- γ and IL-17 in the blood are significantly higher in ACD model mice than control mice. However, the IL-4, IL-5 and IL-10 levels in ACD mice are similar to control mice. Consistent with the mRNA expression of cytokines in skin lesions, administration of PF reduced DNFB induced- INF- γ and IL-17, but enhanced IL-10 secretion and had no effects on IL-4 and IL-5 productions.

3.4. PF inhibits skin DC migration

Excised dorsal ear halves were cultured for three days, and cells that emigrated from the specimen on days 2 and 3 were pooled for DC migration study, in which the majority of cells were DC cells. We observed a higher population of DCs in DNFB-sensitized ear specimens than control specimens. However, the level of DC

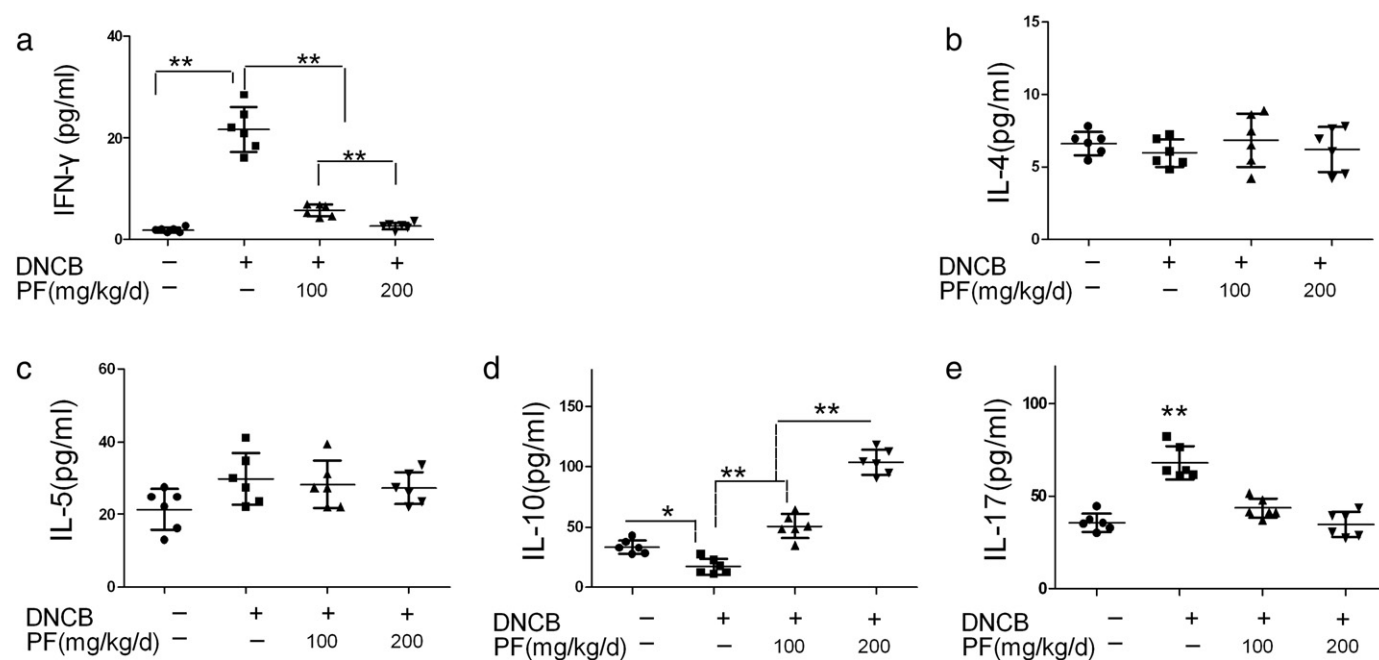


Fig. 5. Influence of paeoniflorin on circulating cytokine levels in a mouse model of allergic contact dermatitis. Mice were sensitized and challenged with DNFB as previously described. 24 h after challenge, blood samples were collected by orbital puncture and expression of INF- γ (Th1); IL-4, IL-5 and IL-10 (Th2); and IL-17 (Th17) was measured using ProcartaPlex Multiplex with Luminex 200 according to the manufacturer's instructions. Results are presented as mean $\pm$ SD ($n = 6$). ** $p < 0.01$; * $p < 0.05$.

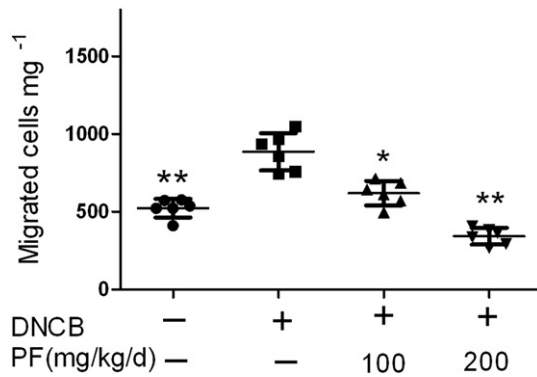


Fig. 6. Migration of skin dendritic cells out of ear explants. Mice were sensitized and challenged with DNCB as previously described. Cartilage-free dorsal ear halves were cultivated for 24 h after the third sensitization with DNCB for 3 additional days. Explants were transferred daily into new wells. Emigrated cells were pooled for each explant and counted with a hemocytometer. The number of migrated cells was calculated per mg of tissue explant. Results are presented as mean $\pm$ SD ($n = 6$). ** $p < 0.01$; * $p < 0.05$.

migration in DNCB-sensitized mice was significantly reduced in mice administered with PF (Fig. 6).

3.5. LC density in skin lesions

Twenty-four hours after sensitization, cartilage-free ear halves were cultivated with DNCB for 3 days in order to assess the influence of DNCB challenge on the quantity of skin DCs [23]. The density of LCs was assessed by MHC staining and we found that the number of MHC-positive cells in the epidermis of murine ear skin was significantly reduced in mice challenged with DNCB when compared to the control mice. However, the number of DC in DNCB-induced ACD mice ear tissues restored to the level of control mice after treated orally with PF (Fig. 7).

3.6. PF decreases DC migration in lymph nodes

In an ACD mouse model, DCs have also been found to carry antigens from the skin to the regional lymph nodes [26] and in which naïve T cells are transformed into Th1, Th2 and/or Th17 cells. We next measured the population of DCs in the lymph nodes. The DC cells migrated from skin are CD11c/CD40 double staining positive [25]. As shown in Fig. 8, the number of CD11c/CD40⁺ positive cells in the local lymph nodes increased after DNCB sensitization. In comparison to

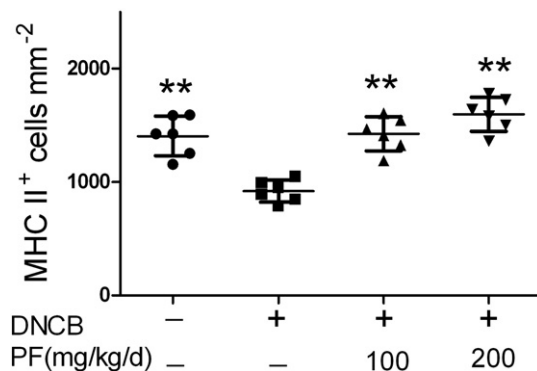


Fig. 7. MHC II⁺ cells in the ear epidermis of mice 24 h after last sensitization with DNCB. Mice were sensitized with DNCB as previously described. From day 7 to 9, ears were treated topically with DNCB (1%). Epidermis was stained for MHC II⁺ cells and density was determined microscopically by means of a calibrated grid, $n = 6$ per group. ** $p < 0.01$.

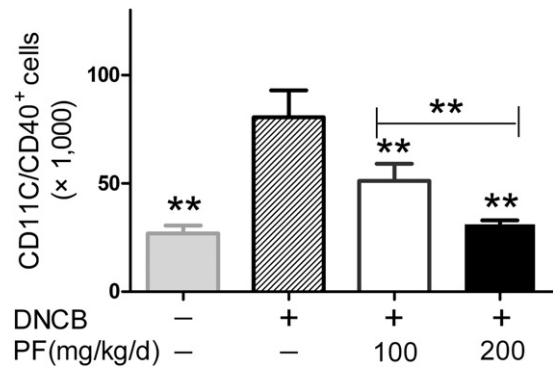


Fig. 8. The number of CD11c/CD40-positive cells in the draining lymph nodes after third sensitization with DNCB. Mice were sensitized with DNCB as previously described. Twenty-four hours after DNCB sensitization, auricular lymph nodes were removed. The single cell suspension was stained for CD11c and CD 40 and staining was assessed by flow cytometry. Results are presented as mean $\pm$ SD ($n = 6$). ** $p < 0.01$.

PF-untreated ACD mice, the number of such DCs in the lymph nodes reduced significantly in DNCB-induced mice with PF treatment, particularly in high dose of PF. These results suggest that PF interferes with replenishment and migration of antigen-presenting DCs from the skin to the lymph nodes.

3.7. PF inhibits IFN- γ ⁺CD4⁺ T cell and IL-17⁺CD4⁺ T cell proliferation in the lymph node

Given the fact that IFN- γ production by T cells is a hallmark of Th1 responses, IL-4 is a hallmark of Th2 and IL-17 is a hallmark of Th17 responses, we assessed PF affects on T cell polarization by measuring these T cell subgroups in lymph nodes. Lymph node cells from immunized mice with PF treatment or without treatment were cultured with ConA for 72 h before the levels of intracellular Th1 (IFN- γ), Th2 (IL-4) and Th17 (IL-17) were measured. As we described above, DNCB as a contact sensitizer can effectively induce a Th1 responses indicated by a high IFN- γ production. Our previous *in vitro* experiments also demonstrated a dominant CD4⁺ T cell response after DCs are challenged with DNCB.

The proportion of CD4⁺ T cells producing IFN- γ , IL-4 or IL-17 was assessed by intracellular cytokine staining, and production of IFN- γ , IL-4 and IL-17 by CD4⁺ T cells was detected by ProcartaPlex Mul-plex (eBioscience Inc., San Diego, USA) with Luminex 200. In comparison to control group mice, IFN- γ -producing CD4⁺ T cells and IL-17-producing CD4⁺ T cells in the lymph node from the DNCB-induced ACD mice were increased, but decreased after PF treatment ($p < 0.005$; Fig. 9a). Accordingly, CD4⁺ T cell secretion of IFN- γ and IL-17 was notably reduced following co-cultured with DCs that were previously exposed to PF (Fig. 9b). On the other hand, the percentage of IL-4-producing CD4⁺ T cells and IL-4 secretion were not changed a (Fig. 9).

4. Discussion

In this study, we continue our investigation of the effectiveness of PF on ACD and immune pathological mechanisms underlying this treatment. In a DNCB-induced ACD mouse model, our findings are in agreement with an earlier study done by us *in vitro* [18]. First, DNCB induces Th1 and Th17 responses in the *in vivo* animal model. Second, oral administration of PF reduces DC/LC migration from affected epidermis into the lymph nodes. Third, as a result of inhibited LC migration, the immune response in the local lymph nodes is diminished during the sensitization phase, and may lead to a less inflammatory reaction at the later elicitation phase of ACD.

Anti-inflammatory effects of PF on ACD animal models have been observed by us and others [17,18]. In a DNCB-induced ACD mouse model, repeated application of DNCB induced ACD, which was

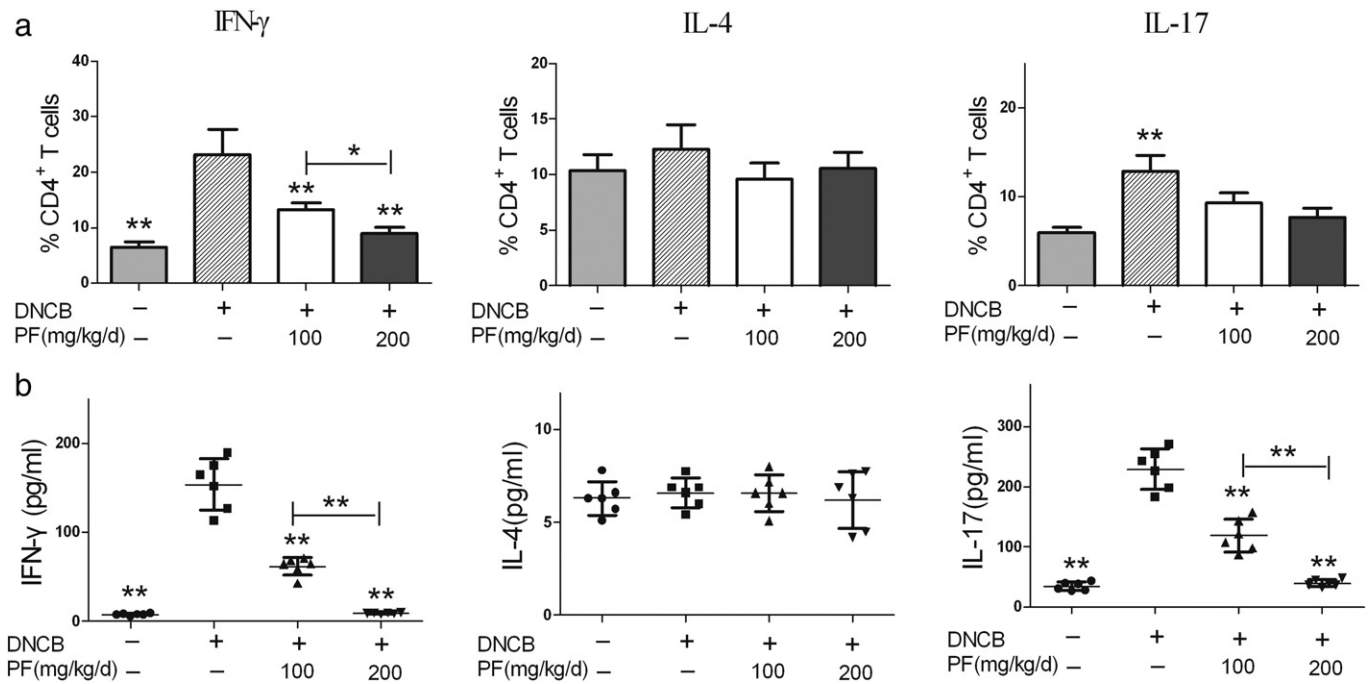


Fig. 9. Detection of differentiation of T cells in lymph node after third sensitization with DNCB. Mice were sensitized with DNCB as previously described. (a) Auricular lymph nodes were obtained 24 h after immunization with DNCB. Highly purified CD4⁺ T cells were isolated from lymph nodes and purified CD4⁺ T cells (1×10^5) were co-cultured in the presence of 2 μ g ConA for 72 h. T cell differentiation was assessed by flow cytometry. (b) The production of IFN- γ , IL-4 and IL-17 by CD4⁺ T cell content was assessed by ProcartaPlexMul-plex with Luminex 200. Results are presented as mean $\pm$ SD (n = 6). **p < 0.01; *p < 0.05.

characterized by significant skin swelling 24 h after challenge with DNCB. PF is able to ameliorate inflammatory symptoms 24 h after challenge with haptens [17]. In the current study, we find that PF reduces the ear thickness significantly and infiltration of CD3⁺ cells in the elicitation phase of ACD is accordingly decreased.

To better understand the immune response to a contact allergen in the sensitization and elicitation phases of ACD, the DC migration and lymph node assay, T-cell differentiation and cytokines were further studied in both ear induction (sensitization phase, Fig. 2b) and abdominal-ear induction (elicitation phase, Fig. 2a) stages. The migration of dermal DCs/LCs in the ACD immunopathological mechanism has been reported for other antigens as well [19,23]. For example, toluene-2,4-di-isocyanate was traced from the skin to draining lymph nodes in the induction phase through hapten-presenting DCs/LCs and hapten-specific T-cell responses in the elicitation phase [23]. DCs are the major antigen-presenting cells in both the induction and elicitation phases of ACD. In our earlier study, we found that PF not only inhibited maturation of DCs, but also inhibited the migration of DCs induced by DNCB *in vitro* [18]. This *in vivo* study confirms that while DNCB reduced the number of DCs and LCs in the skin lesion, PF treatment was able to restore DC and LC numbers in the skin to levels found in the untreated control group, indicating by a decrease in the population of CD40^{high} and CD11c^{high} cells (i.e., DCs). These results suggest that PF inhibits DC migration which would otherwise lead to impairment of DCs' ability to present antigens and stimulate T cell proliferation, which eventually gives rise to an attenuated inflammatory response. Such PF inhibition of lymphocyte proliferation in local lymph nodes was also observed directly with repeated topical application of test allergens in local lymph nodes [27]. The PF effects on outward contact-sensitizer-induced proliferation of lymph node cells may be useful in the treatment of lymph node cell-mediated allergic responses.

It has long been assumed that hapten-specific CD4⁺ T cells are the critical effectors of ACD [28]. In ACD, LCs (dermal DC) internalize haptens, transport them to regional lymph nodes and present hapten-bound MHC-peptide complex to naïve T cells. Naïve T cells differentiate

into effector T cells such as Th1, Th2 and others. These T cells secrete the inflammatory cytokines, which cause the local inflammatory symptoms in the skin. Therefore, some drugs, such as bisindol and IL-9, which regulate Th1 or Th2, or somewhat balance the Th1/Th2 responses, have been used to treat ACD [29,30]. Meanwhile, Th17 cells, a subset of skin-infiltrating chemically-reactive T lymphocytes, have been suggested to modulate ACD [31,32]. Recent evidence has shown that IL-17⁺ T cells infiltrate at sites of ACD and are found most commonly at the site of severe spongiosis [33]. DNCB and DNFB were commonly used to induce a Th1-dominated inflammation and toluene-2,3-di-isocyanate and FITC for a Th2-promoted inflammation [34,35]. Here, we confirm that DNCB can induce IFN- γ -producing CD4⁺ T cell differentiation in local lymph nodes and enhance IL-7-producing CD4⁺ T cell differentiation as well.

With the local lymph node assay, we demonstrate that PF inhibits Th1 and Th17 cell differentiation in draining lymph nodes after challenge with DNCB [36]. As DNCB enhances the levels of pro-inflammatory cytokines (IFN- γ and IL-17) in circulating blood in our animal model, we find that the secretions of Th1 (IFN- γ) and Th17 (IL-17) cytokines in the lymph node were reduced under PF treatment. However, Th2 cytokines (IL-4 and IL-5) were not affected. These results suggest that PF suppresses DNCB-induced Th1 and Th17 responses and decreases inflammatory cytokines in both sensitization and elicitation phases. IL-10, an anti-inflammatory cytokine, is secreted by various immunocytes [37,38]. IL-10-deficient mice exhibit enhanced inflammatory response in response to haptens, and exogenous rIL-10 alleviates that response [39]. During the course of ACD, IL-10 reduces inflammation by inhibiting antigen presenting cell migration and down-regulating the secretions of pro-inflammatory cytokines [39]. IL-10 is an immunosuppressive cytokine that induces proliferation of Treg cells (e.g., IL-10-producing T cells and CD4⁺CD25⁺Foxp3⁺ T cells). In agreement with our *in vitro* study [18], the secretion of IL-10 in the lymph node and the expression of IL-10 in the skin lesions were increased in PF-treated ACD mice. The increase of IL-10-producing T cells was also noted in the local lymph node assay, suggesting that enhanced IL-10 production may be caused by increase of Th2 cells. Therefore, the anti-inflammatory effects of PF in ACD

contribute by reducing Th1 and Th17 cell differentiation and production of INF- γ (Th1) and IL-17 (Th17) cytokines, and perhaps also simultaneously by the increase of anti-inflammatory cytokines.

This study showed, for the first time, that PF directly inhibits migration of DCs from affected skin lesions and represses the expansion of Th1 and Th17 cells in ACD mice. PF is also able to decrease cytokine productions and reduce pathological inflammatory responses in this animal model. These results seem to recommend PF as a promising new therapeutic agent for ACD with the potential to be therapeutically useful to patients with other acute inflammatory conditions, and we believe this warrants further investigation.

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