



Paeoniflorin inhibits the maturation and immunostimulatory function of allergen-induced murine dendritic cells



Dongmei Shi^{a,b,1}, Aidong Ma^{c,1}, Haiyan Zheng^{a,1}, Guang Huo^d,
Hongxia Yan^a, Hongjun Fu^a, Ying Qiu^{a,*}, Weida Liu^{b,**}

^a Department of Dermatology, Jining First People's Hospital, Shandong, PR China

^b Department of Mycology, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College, Nanjing, Jiangsu, PR China

^c Department of Radiology, Jining First People's Hospital, Shandong, PR China

^d School of Stomatology, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, PR China

ARTICLE INFO

Article history:

Received 8 November 2013

Received in revised form 31 January 2014

Accepted 2 February 2014

Available online 15 February 2014

Keywords:

Paeoniflorin

Allergic contact dermatitis

Dendritic cells

1-Chloro-2, 4-dinitrobenzene

Regulatory T cells

ABSTRACT

Dendritic cells (DCs) as the front lines of defense play a crucial role in allergic contact dermatitis (ACD). Paeoniflorin (PF) has been clinically proven to be effective in the treatment of inflammatory skin diseases such as ACD. However, the mechanisms underlying the anti-inflammatory effect of PF remain unclear. The aim of this study was to explore the effect of PF on the maturation and immunostimulatory function of DCs in the murine model of ACD *in vitro*. Murine bone marrow-derived DCs were stimulated with the contact sensitizer 1-chloro-2, 4-dinitrobenzene (DNCB) *in vitro*. Surface antigen expression of DCs (MHC II, CD40, CD80, and CD86), as an indicator of maturation DCs and cytokines (IL-12, IFN- γ , IL-10, and TGF- β) after DNCB stimulation in the absence or presence of PF at different doses, was detected. Then, we detected that PF-treated DCs stimulated T cells in response to DNCB. PF inhibited the up-regulation of MHC II, CD80, CD86, and CD40, decreased IL-12p70 secretion, while increased the production of IL-10 and TGF- β , and had no effect on IFN- γ cytokine production by murine bone marrow-derived DCs in response to DNCB. DCs exposed to PF had diminished capacity to stimulate allogeneic T cell proliferation and to activate IFN- γ -producing CD4⁺ T cells and induced CD4⁺CD25⁺Foxp3⁺ T cells and IL-10-producing T cell expansion from naïve CD4⁺ T cells. These results indicate that PF may be effective in preventing and treating ACD *in vitro* and other inflammatory responses possibly through inhibiting maturation of DCs and limiting their capacity to stimulate T cell responses.

© 2014 Elsevier B.V. All rights reserved

1. Introduction

Allergic contact dermatitis (ACD) is one of the most common skin diseases, which accounts for approximately 20% of all work-related health complaints [1]. It is a delayed-type hypersensitivity reaction to a specific allergen [2]. The role of dendritic cells (DCs) in ACD pathogenesis is well documented [3,4]. DCs, as the most common antigen present cells of immune system, play an important role in the initiation of ACD, in which DCs acquire, process, and present antigens to activate naïve T cells that mount a specific antigen immune response. DCs are currently divided into tolerogenic immature and immunogenic mature differentiation stages [5,6]. Although with low levels of co-stimulatory molecule expression, immature DCs (iDCs) have been increasingly recognized in

producing small amounts of proinflammatory cytokines and large amounts of anti-inflammatory cytokines, which result in anergy, apoptosis of effector T cells, or induction and expansion of regulatory T (Treg) cells [7,8]. In contrast, mature DCs, which are able to secrete a potent stimulatory cytokine (e.g., IL-12p70) and express high levels of co-stimulatory molecule expression (CD80, CD86, and CD40), induce strong adaptive immunity. During maturation, DCs lose their capacity to capture soluble antigens but gain T cell stimulatory capacity via increase of antigen processing and up-regulation of MHC, co-stimulatory molecules and cytokines. Therefore, DCs are the central architects of the immune response to induce inflammatory reaction and responsible for tolerogenic immunity upon their activation status. It has been hypothesized that inhibition of DC maturation, in order to induce immune tolerance, may be one mode of certain drugs for ACD treatment.

After T cells are activated by DCs, naïve T cells differentiate into Th1 and Th2 cells, which produce cytokines, such as INF- γ , IL-12, IL-4, IL-10, and TGF- β [9,10]. Cytokines produced by activated T cells lead to further recruitment of T cells, amplifying the inflammation. Meanwhile, DCs induce the proliferation of Treg cells to maintain the balance of the

* Correspondence to: Y. Qiu, Department of Dermatology, Jining First People's Hospital, No. 6 Jiankang Road, Jining 272011, Shandong, PR China. Tel.: +86 537 2253401.

** Correspondence to: W. Liu, Department of Mycology, Institute of Dermatology, No. 12 Jiang Wangmiao Street, Nanjing 210042, Jiangsu, P. R. China. Tel.: +86 25 85470580; fax: +86 25 85414477.

E-mail addresses: qiou_ying@sohu.com (Y. Qiu), liumyc@hotmial.com (W. Liu).

¹ These authors contributed equally to this work.

immune system. The modulation of the inflammatory process is a necessary step, mostly orchestrated by Treg cells, to prevent the development of deleterious inflammatory response [11]. Treg cells play a central role in preventing autoimmunity, facilitating graft tolerance following organ transplantation, and having an impact on the development of inflammation immunity. Therapeutic strategies that focus on inducing Treg cells by iDCs *in vitro* and transferring Treg cells or their expansion *in vivo* have achieved a great success in controlling inflammation in several experimental models [12–14].

Paeoniflorin (PF), a monoterpene glucoside, is one of the principal bioactive components of the Paeonia root. PF has been used for 2000 years as an immune modulator, and an anti-allergic agent [15], and it displays effective anti-inflammatory properties in several animal models [16,17]. PF is also accepted as a safe and efficacious agent in the treatment of ACD. However, to date, the mechanisms of PF in the treatment of ACD are still obscure. Based on the early reports showing that DCs are critical for ACD, we postulated that PF might exhibit effective treatment in ACD through stimulating and modulating the maturation processes of DCs [18,19]. In the present study, we investigated the influence of PF on hapten-stimulated DCs in an *in vitro* model of ACD. We also examined whether PF could repress inflammatory response via differentiation of Th1/Th2, and induce differentiation and proliferation of Treg cells.

2. Materials and methods

2.1. Regents and chemicals

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

PF (Fig. 1) was purchased from Silida Technology Ltd. (Tianjin, China), with a purity of $\geq 98.5\%$ as determined by HPLC. PF was dissolved in 50 ml of sterilized saline as stock solution at 800 $\mu\text{g}/\text{ml}$ concentration. The working solutions at various concentrations were diluted in saline as well.

2.2. Mice

Female BALB/c mice were bred in the animal experimental study center of Zhejiang Chinese Medical University in Hangzhou, Zhejiang, China, and were used at age of 8–12 weeks and 16–18 g in body weight. All experiments were performed under a pathogen-free environment.

The protocols of all animal experiments were approved by the Animal Study Committee of the Zhejiang Chinese Medical University, according to the government guidelines for animal care.

2.3. Cell separation and generation of bone marrow-derived DCs

Bone marrow-derived DCs were generated as described previously [20], with minor modification. Briefly, bone marrow cells were obtained from femurs and tibias of normal BALB/C mice, filtered through nylon

mesh, depleted of RBC with lysis buffer, and washed with PBS. Then, the cells were cultured at 5×10^6 cells/ml in RPMI 1640 containing 10% FBS (Gibco, USA) and 200 U/ml rmGM-CSF (Propetech, USA). At day 2, a 5 ml of fresh medium containing 200 U/ml rmGM-CSF was added to the plates. At days 4 and 6, half of the culture supernatants were collected and centrifuged. The cell pellet was re-suspended in 5 ml of fresh culture medium containing 200 U/ml rmGM-CSF. At day 8, the DCs were harvested for subsequent experiments.

2.4. Cytotoxicity assay

To observe whether PF induces apoptosis or necrosis in DCs, an FITC-conjugated monoclonal antibody (mAb) against activated caspase-3 (BD Biosciences, USA) was used. Briefly, PF-treated (PF, 200 $\mu\text{g}/\text{ml}$) cells were incubated in Cytofix/Cytoperm solution at a concentration of 2×10^6 cells/ml for 20 min on ice. After re-suspension in 100 μl of Perm/Wash buffer (BD Biosciences, USA), the cells were incubated with 20 μl of FITC caspase-3 mAb for 30 min at room temperature in the dark and then analyzed by a flow cytometer. DCs from the same experiments were also stained with trypan blue (Sigma, USA) to determine cell viability.

2.5. Migration of DCs

Transwell experiments were performed using 24-well transwell plates (Millicell, 5.0 μm ; Millipore, Bedford, MA, USA). The suspensions of DCs were placed in the upper well. PF together with complete medium containing GM-CSF (200 U/ml) or with DNCB, or only complete medium were added to the lower chamber. The cultures were performed in 37 °C for 3 h. The cells in the lower wells were collected and counted to calculate the percentage of the cells of migration to the lower well. The above experiments were repeated seven times.

2.6. PF treatment of DCs

At day 8, iDCs were treated with PF at different concentrations (25, 50, 100, and 200 $\mu\text{g}/\text{ml}$) for 18 h at 37 °C. Thereafter, DCs were either stimulated with DNCB (Sigma, USA) for 48 h or left untreated as a control. DNCB was solubilized with non-cytotoxic concentration ($\leq 0.02\%$) of dimethyl sulfoxide (DMSO, Sigma, USA).

2.7. Flow cytometric measurement of surface antigen expression of DCs

Expression of surface molecules on DCs was quantified by flow cytometry using FITC- or PE-conjugated Ab (anti-CD11c, anti-I-A/I-E, anti-CD40, anti-CD80, and anti-CD86) (eBioscience Inc., San Diego, CA, USA). Dual-color immunofluorescence staining was performed, and the samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (FACSCalibur, Becton Dickinson, USA).

2.8. Assay for cytokine secretion by DCs

For assay of TGF- β , IL-10, IL-12, and IFN- γ , DCs were cultured in six-well plates as above described (2.6). At day 8, the cells were stimulated with PF for 18 h, followed by DNCB. After 48 h, the supernatants were collected, centrifuged at 14,000 rpm to remove cells and debris, and stored at -70 °C. The supernatants were then assayed using a sandwich ELISA (R&D Systems, Minneapolis, MN, USA) (IL-10, IL-12, IFN- γ , and TGF- β), in accordance with the manufacturer's instructions.

2.9. Phagocytic ability

Phagocytic ability was measured as the cellular uptake of FITC/latex beads/rabbit IgG using a phagocytosis assay kit (Cayman Chemical, Ann Arbor, MI, USA). DCs were incubated with FITC/latex beads/rabbit IgG

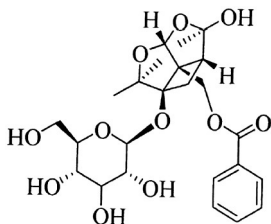


Fig. 1. The chemical structure of paeoniflorin (PF).

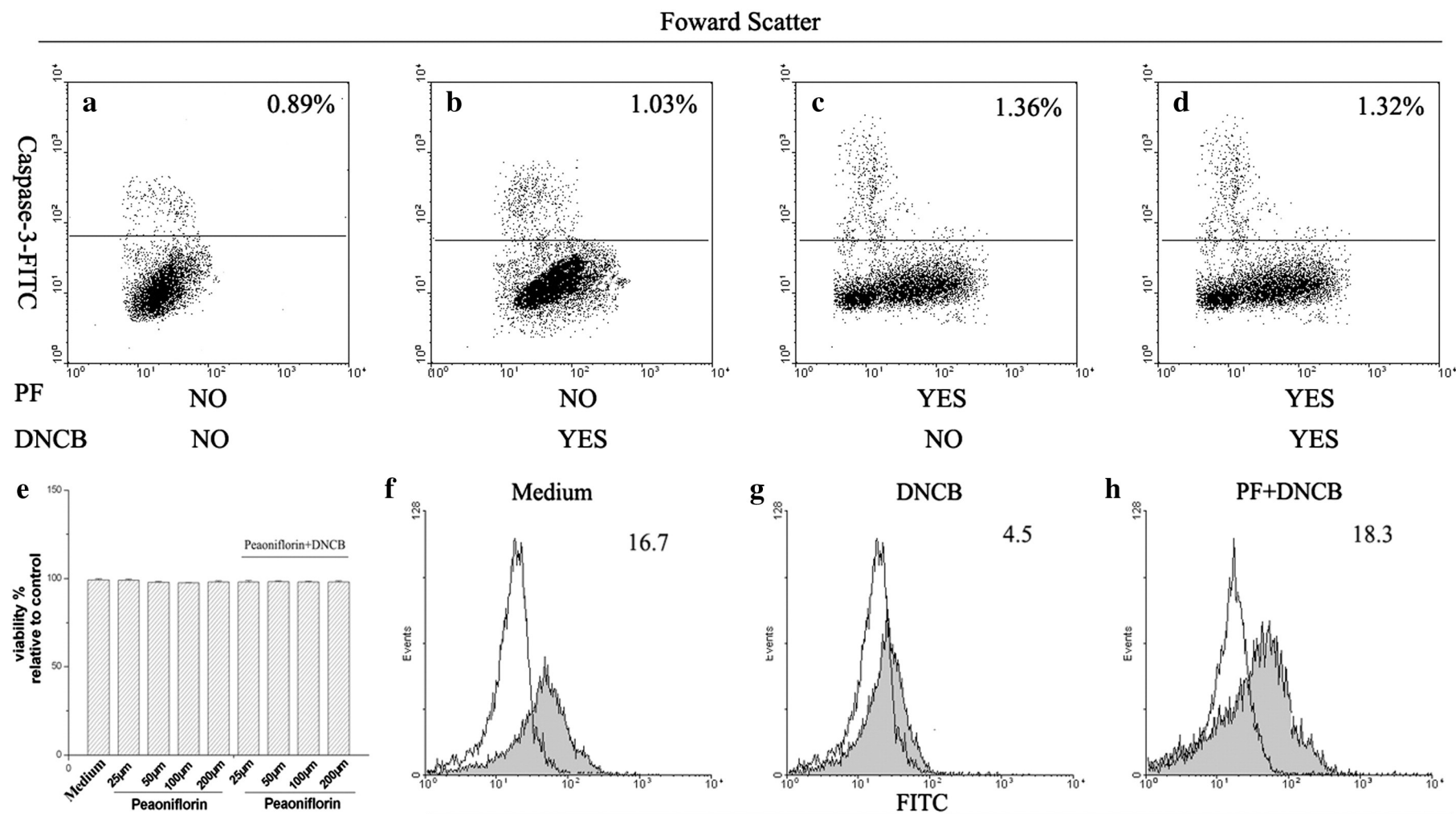


Fig. 2. Peaoniflorin (PF) did not affect the DC viability. DCs were exposed to medium alone or to PF for 66 h (a, c). Some DCs were treated with DNCB for the last 48 h (b, d). The cells were stained with FITC-caspase 3 and analyzed for the caspase-3 activation by flow cytometry. Data from one representative experiment of seven experiments are shown. Cell viability was assessed by trypan blue exclusion assay. Data were expressed as mean $\pm$ SE of seven independent experiments (e). PF did not affect the DC phagocytic capacity (f–h). DCs were incubated with FITC/latex beads/rabbit IgG for 24 h, and then analyzed by flow cytometry. Data from one representative experiment of seven experiments are shown.

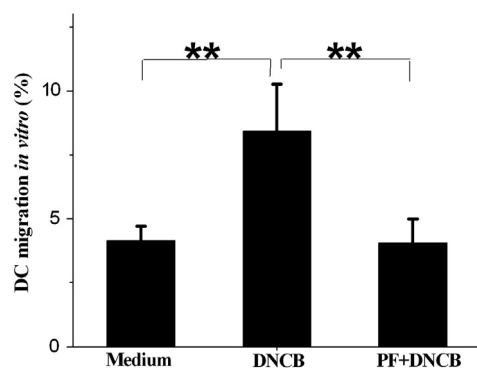


Fig. 3. Paeoniflorin (PF) reduced the migration of DCs. Transwell experiments were performed using 24-well plates (Millicell, 5.0 μ m). The suspensions of DCs were placed in the upper well. PF together with a complete medium alone or DNCB were added to the lower chamber. The cultures were performed in 37 °C for 3 h. The cells in the lower wells were collected and counted to calculate the percentage of the cells of migration to the lower well. Y-axis represents the percentage of DC migration. Data were expressed as mean $\pm$ SE of data from seven independent experiments. $^{**}p < 0.01$.

for 24 h in a CO₂ incubator, and then analyzed by flow cytometry in accordance with the manufacturer's instructions.

2.10. T cell proliferation assays

CD4⁺ T lymphocytes were separated from spleen of BALB/c mice using a CD4⁺ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was assessed by flow cytometry using PE-conjugated anti-CD4 mAbs (eBioscience Inc., San Diego, CA, USA) and was shown to be >95%.

Cell proliferation was assessed by flow cytometry using a dye, carboxyfluorescein diacetate succinimidyl ester (CFSE) (Eugene, OR, USA) as described previously [21]. Briefly, purified T cells were counted and seeded into 96-well round-bottom microtiter plates at 10⁵ cells/well and incubated at room temperature with an equal volume of 2.5 μ M CFSE diluted in PBS. After 5 min, 0.1 ml of 100% FBS was added to quench the reaction. The cells were then washed in PBS, followed by a wash in a complete medium. Irradiated (30 Gy) DCs were added to the T cells at varying ratios in triplicate wells. The final volume of each well was 200 μ l. T cells alone were used as the background control. After 5 days, the cells were collected and then stained with PE-conjugated anti-CD4 mAbs (eBioscience Inc., San Diego, CA, USA). Proliferation was measured as the loss of CFSE fluorescence of CD4⁺ T cells.

2.11. Determination of cytokine production by CD4⁺ T cells

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 (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD4⁺ T cells analyzed by flow cytometry was always >92%. Purified CD4⁺ T cells (1×10^5) were co-cultured with 1×10^4 irradiated DCs (30 Gy). Recombinant murine IL-12 (R&D Systems, USA) at a concentration of 10 ng/ml was added to some T cell cultures stimulated with DCs exposed to PF. After 48 h, cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (50 ng/ml) in the presence of brefeldin A (10 μ g/ml) for 6 h. For intracellular cytokine production 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 0.5 μ g/test of anti-murine IL-4-PE (BD Pharmingen, USA) and anti-murine-IFN- γ FITC (BD Pharmingen, USA) and were analyzed by flow cytometry. IFN- γ and IL-4 production by CD4⁺ T

cells was also assessed using the Cytometric Bead Array kit from BD Pharmingen.

2.12. Induction of Treg cells by PF-treated DCs

Induction of Treg cells by PF-treated DCs was performed as previously described [22], with minor modifications. Briefly, naïve CD4⁺ T cells were purified from spleen with a negative CD4 T cell isolation kit II (Miltenyi Biotec, Germany). Naïve CD4⁺ T cells (1×10^6) were cultured with PF (200 μ g/ml) and DNCB-treated allogeneic DCs, DNCB alone treated DCs and medium-treated as control in a medium with 10% FBS for 5 days. Then, CD4⁺CD25⁺Foxp3⁺ T cells and IL-10⁺ T cells were analyzed by flow cytometry.

The anti-IL-10 and TGF- β 1 mAbs were titrated prior to experiment. The concentrations of mouse anti-IL-10 and/or anti-TGF- β 1 mAbs resulting in undetectable levels of IL-10 or TGF- β 1 following Ab addition were used in blocking processes.

2.13. Statistical analysis

All the experiments were repeated at least three times. Two-sample Student's *t* test was used to compare the statistical differences in means between two experimental groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. No effect of PF on DC viability and phagocytic capacity

In order to determine the effect of PF on DC viability, DCs were stained with activated caspase-3, a marker of early apoptosis, after exposure to PF. Less than 2% of DCs was stained positively after exposure to 200 μ g/ml PF for 66 h. For toxicity of DNCB, DCs were exposed to PF or medium alone for 18 h, and were then matured with DNCB for 48 h and stained with anti-activated caspase-3. The results showed that low concentrations of DNCB and PF at different doses were not toxic to DCs (Fig. 2a–d). Trypan blue exclusion also showed that DCs were viable after exposure to PF (Fig. 2e).

The phagocytic capacity of PF-treated DCs was assessed using FITC/latex beads/rabbit IgG. As shown in Fig. 2f–h, DCs lost their phagocytic ability during maturation. However, PF-treated DCs retained their phagocytic ability, similar to iDCs.

3.2. PF reduced DC migration

DC migration is essential for the induction of ACD [23]. We test whether PF treatment reduces DC migration *in vitro*. Compared with DNCB-treated DCs, DCs exposed to PF reduced DC migration. There was no significant difference between medium-treated DCs and PF-treated DCs following stimulation by DNCB (Fig. 3).

3.3. PF inhibited the maturation of DCs effectively to respond to DNCB

To ascertain whether PF exposure plays a role in modulating the function of DCs in response to DNCB, cell surface expression of activation markers was analyzed by flow cytometry. We found that cultured DCs used in our experiments exhibited typical immature phenotype: MHC-II^{low}, CD40^{low}, CD80^{low}, and CD86^{low} and underwent maturation after treatment with DNCB, as evidenced by significantly increased expression of MHC-II, CD40, and co-stimulatory molecules such as CD80 and CD86. However, with PF treatment, the surface expression levels of these cell molecules between iDCs exposed to PF and control iDCs were similar, suggesting that iDCs exposed to PF failed to activate DCs under stimulation of DNCB (Fig. 4a–c). Furthermore, this inhibition of the DNCB-induced DC maturation by PF was dose-dependent (Fig. 4b). On the other hand, PF alone without DNCB

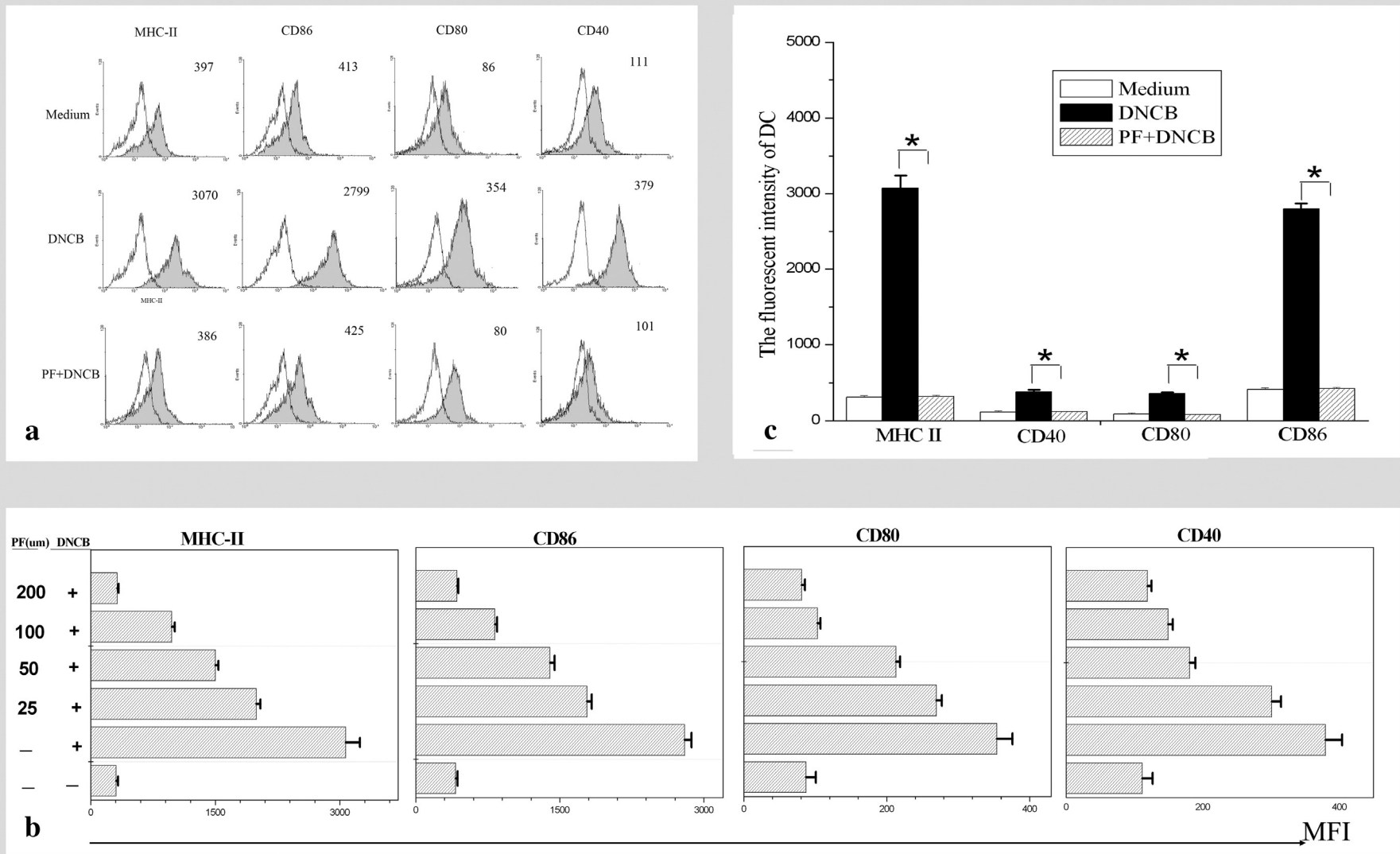


Fig. 4. DCs exposed to paeoniflorin (PF) have a maturation-resistant phenotype. Surface antigen expression by DCs was detected by flow cytometry. (a–c) Immature DCs were exposed to medium alone, to PF for 18 h, and then DNCB was added for 48 h to induce maturation. (a) Expression of maturation and co-stimulatory molecules. Data from one of the seven experiments are shown. The mean fluorescent intensity (MFI) corresponding to the expression of surface antigen is indicated in each histogram. (b) Inhibition of DCs was dose-dependent. Mean $\pm$ SE of data from seven independent experiments is shown. X-axis represents MFI. (c) DCs were exposed to medium alone or to PF (200 μ g/ml) for 66 h. Some DCs were treated with DNCB for the last 48 h. Mean $\pm$ SE of data from seven independent experiments is shown. * $p < 0.05$.

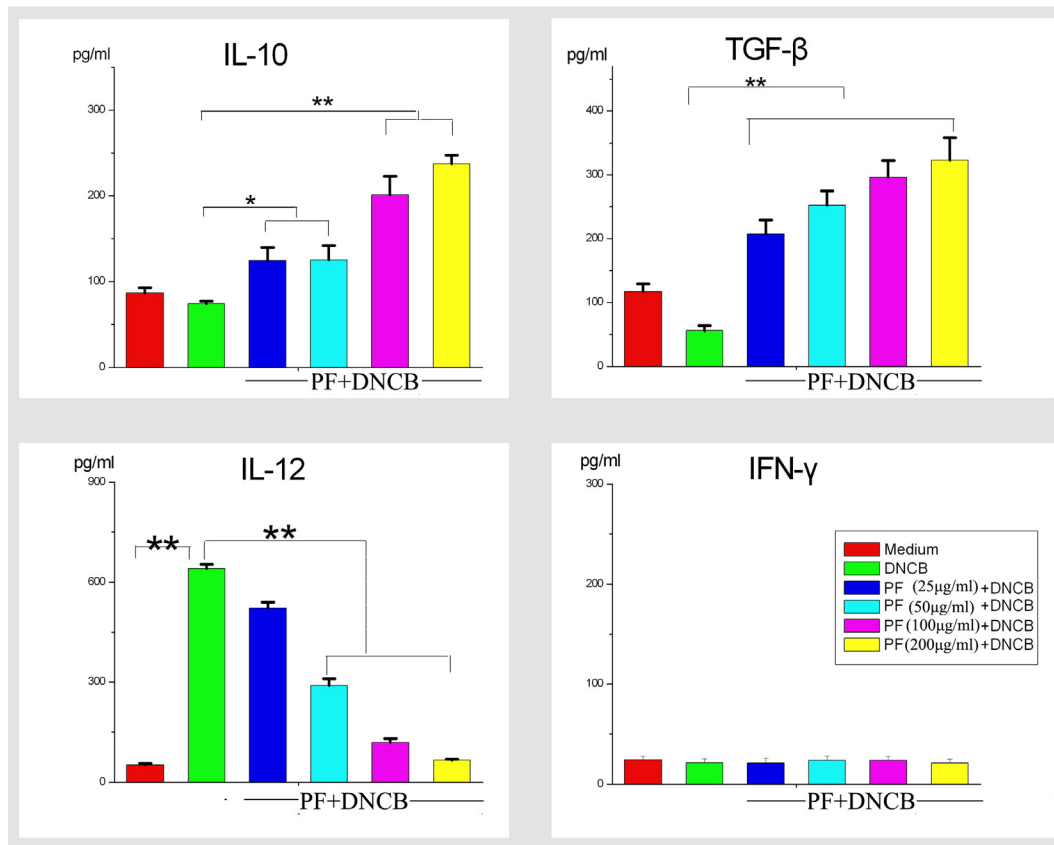


Fig. 5. DCs exposed to paeoniflorin (PF) had diminished capacity to produce IL-12p70, promoted the secretion of IL-10 and TGF- β . Day 5 iDCs were exposed to PF and treated with DNCB for 48 h, and the culture supernatants were collected. The concentrate of cytokines was measured by ELISA kits. Mean $\pm$ SE of data from seven independent experiments is shown. ** $p < 0.01$; * $p < 0.05$.

treatment had no observable effect on DCs, which retained an immature phenotype.

3.4. Cytokine production in PF-treated DCs

Because cytokines produced by DCs in response to DNCB are especially important in determining subsequent T cell responses [24], we next examined the effect of PF on cytokine production by DCs. As shown in Fig. 5, DNCB-treated DCs produced much higher IL-12, but reduced production of IL-10 and TGF- β compared with PF pretreated DCs. In contrast, PF-treated DCs produced higher levels of IL-10 and TGF- β than DCs stimulated by DNCB alone. However, the production of IL-12p70 by PF-treated DCs was much lower than that of those induced by DCs stimulated with DNCB. No significant difference of IFN- γ production was observed between DNCB-treated DCs and PF-treated DCs. The results of immunostaining of the intracellular cytokine are consistent with these observations (data not shown). In summary, PF treatment impaired IL-12 production of DCs and promoted the secretion of IL-10 and TGF- β , but had no effect on IFN- γ level and phagocytic capacity.

3.5. PF-treated DCs had reduced ability to stimulate CD4⁺ T lymphocyte proliferation

We then examined the ability of DCs to induce the proliferation of allogeneic CD4⁺ T cells. Immature DCs were exposed to PF for 18 h, treated with DNCB for 48 h, and used to stimulate allogeneic T cells. As shown in Fig. 6, DCs exposed to PF had an impaired capacity to stimulate the proliferation of allogeneic T lymphocytes at DC-to-T cell

ratios of both 1:50 and 1:200. By contrast, untreated DCs did not affect the allostimulatory capacity of DCs. This indicates that PF-treated DCs are at least partially maturation resistant. However, when the DC-to-T cell ratio was increased to 1:10 or greater, allogeneic T cell proliferation was restored to the level that was achieved by control DCs. The diminished immunostimulatory capacity of DCs exposed to PF may reflect the lower surface expression of MHC-II and co-stimulatory molecules. The presence of a large number of allogeneic DCs may have overcome the effects of reduced expression of MHC-II and co-stimulatory molecules by DCs exposed to PF. Therefore, this hyporesponsiveness of PF-treated DCs was mainly due to down-regulation of co-stimulatory molecules and high production of IL-10 and TGF- β .

3.6. PF-treated DCs showed impaired ability to induce IFN- γ production and exogenous IL-12 restored IFN- γ production by CD4⁺ T cells

Next, we examined whether exposure of DCs to PF affected T cell polarization. IFN- γ production by T cells is a hallmark of Th1 responses, whereas IL-4 production is a hallmark of Th2 responses. DNCB is a well-known contact sensitizer that induces a Th1 response and IFN- γ production. In this experiment, DCs were exposed to PF, treated with DNCB, and then stimulated purified CD4⁺ T cells. As noted above, allogeneic T cell proliferation was not markedly decreased when stimulated at a high DC-to-T cell ratio (1:10). The proportion of CD4⁺ T cells producing IFN- γ or IL-4 was determined by intracellular cytokine staining, and production of IFN- γ and IL-4 by CD4⁺ T cells was determined by ELISA. Compared with control DCs, DCs exposed to PF stimulated lower frequency of IFN- γ -producing CD4⁺ T cells ($p < 0.005$; Fig. 7a). Accordingly, secretion of IFN- γ by CD4⁺ T cells co-cultured with DCs

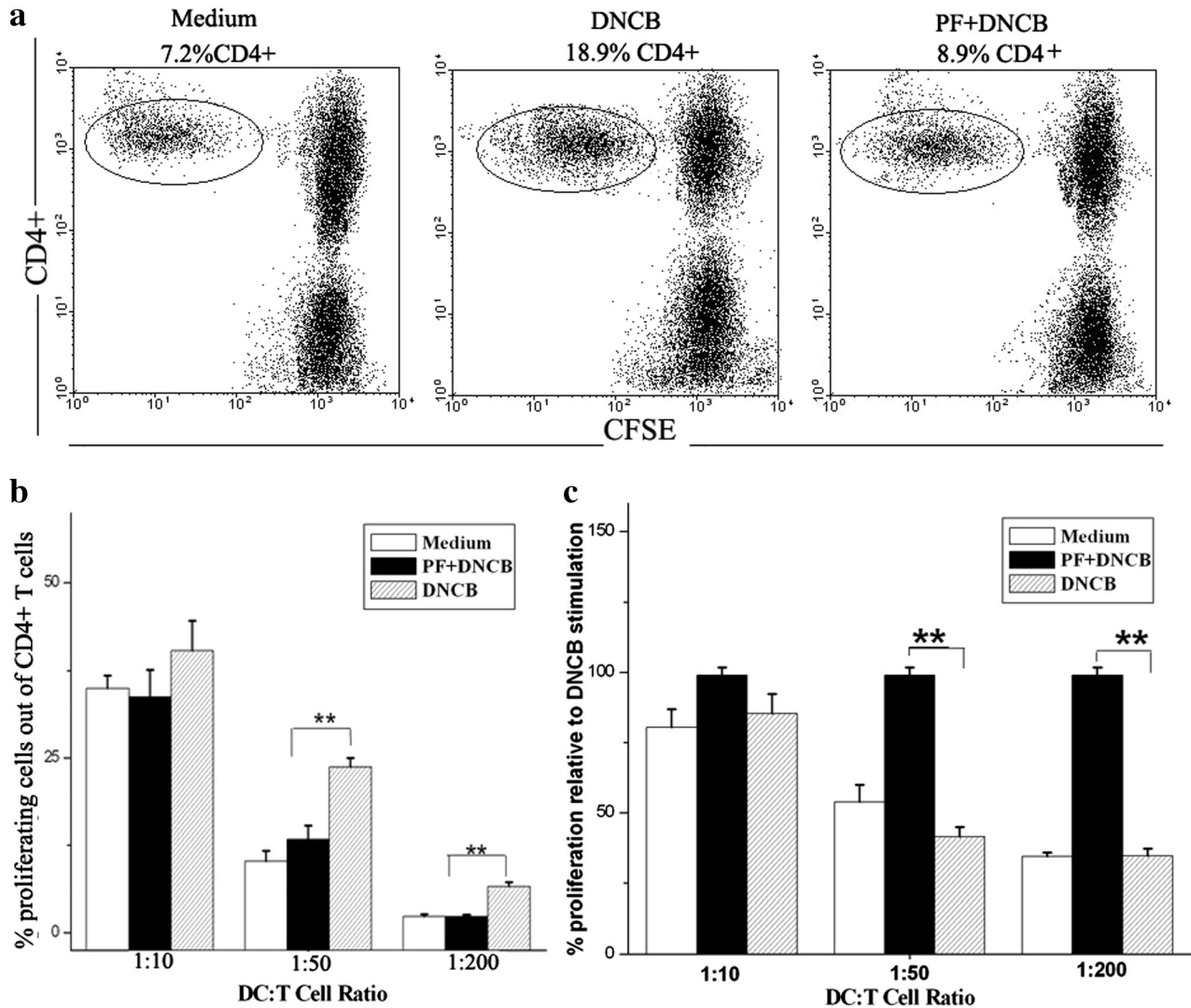


Fig. 6. DCs exposed to paeoniflorin (PF) have reduced capacity to activate T cells. Purified T cells were counted and seeded into 96-well plates at 10^5 cells/well and incubated at room temperature with an equal volume of $2.5 \mu\text{M}$ CFSE. DCs were exposed to medium alone or PF, treated with DNCB for 48 h, and used to stimulate T cells. Five hundred, 2000, or 10,000 irradiated DCs were added to triplicate wells. After 5 days, the cells were collected and stained with PE-conjugated anti-CD4 mAbs for analysis. (a) The numbers in the upper left of each graph represent the percent of CD4⁺ T cells that were proliferating. (b) The results are expressed as the percent of CD4⁺ T cells at different ratios of DC-to-T cells. (c) The results are expressed as a relative value compared with the proliferation of CD4⁺ T stimulated with DNCB. ** $p < 0.01$.

exposed to PF was notably reduced (Fig. 7c). Conversely, we did not observe a significant increase in the average frequency of IL-4-producing CD4⁺ T cells (Fig. 7b) or in IL-4 secretion (Fig. 7d).

The ratios of IFN- γ to IL-4-CD4⁺ T cells induced by DCs exposed to DNCB and DNCB plus PF were 2.2 ± 0.6 and 1.2 ± 0.2 , respectively ($p < 0.002$), further highlighting the effect of PF on DCs and reflecting its modest effect on IL-4 production (Fig. 8). These data showed that DCs exposed to PF had a significantly diminished capacity to induce IFN- γ -producing CD4⁺ T cells, even at a high DC-to-T cell ratio, strengthening our hypothesis that PF modulates the immune response by modifying the function of DCs.

Th1 cell development is dependent on the presence of IL-12, as well as on the ability of T cells to respond to IL-12. We hypothesized that the lack of IL-12 production during DC exposure to PF was responsible for the inability of DCs to induce a Th1 response. Because our data showed that PF dramatically inhibited IL-12 secretion by DCs, we examined the effect of restoring IL-12 on the CD4⁺ T cell response. The addition of exogenous IL-12 (10 ng/ml) restored IFN- γ production by T cells stimulated with DCs exposed to PF (Fig. 8). These results suggest that

DCs exposed to PF fail to secrete sufficient IL-12 although stimulated with DNCB. As a result, PF had a diminished ability to stimulate IFN- γ -producing CD4⁺ T cells.

3.7. PF-treated DCs induce IL-10-producing T cells and functional CD4⁺CD25⁺Foxp3⁺ T cells

Because PF-treated DCs produced IL-10 and TGF- β , we examined whether DCs would induce IL-10-producing T cells and CD4⁺CD25⁺Foxp3⁺ T cells [25]. Naïve CD4⁺CD45RA⁺ T cells were co-cultured with DCs for 5 days, and then intracellular CD4⁺CD25⁺Foxp3⁺ T cells were analyzed using flow cytometry. As shown in Fig. 9, PF- and DNCB-treated DCs generated the greater number of CD4⁺CD25⁺Foxp3⁺ T cells and IL-10-producing T cells than did those induced by DNCB-stimulated DCs.

Next, we examined whether this suppression was due to direct cellular contact or soluble factors (IL-10 or TGF- β) or both. In the co-cultured experiments, addition of saturating amounts of anti-IL-10 or anti-TGF- β 1 mAbs reversed the inhibition only moderately. Addition

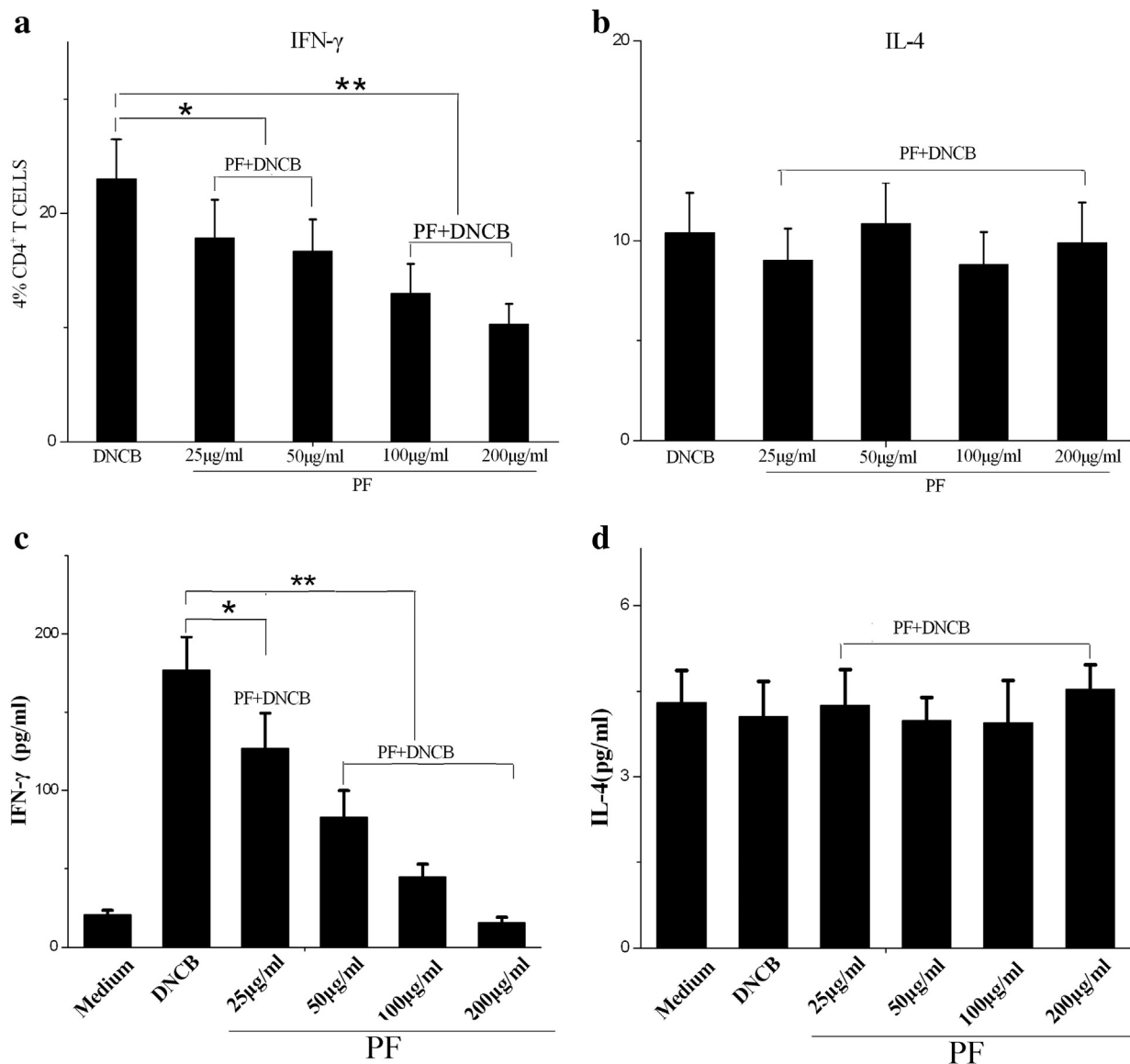


Fig. 7. DCs exposed to various doses of paeoniflorin (PF) (25, 50, 100, and 200 µg/ml) had a reduced capacity to stimulate IFN- γ -producing by CD4⁺ T cells. DCs were exposed to PF and were treated with DNCB for 48 h and to stimulate purified CD4⁺ T cells. DC-to-T cell ratio was 1:20. (a, b) on the day 10, quiescent T cells were stimulated with PMA and ionomycin for 5 h in the presence of brefeldin A. Cells were stained with anti-IL-4-PE and anti-IFN- γ -FITC to detect intracellular cytokine production. Mean $\pm$ SE of data from seven independent experiments is shown. (c, d) supernatants were collected on day 5 of the culture, and IFN- γ and IL-4 contents were analyzed using cytometric bead arrays. * p < 0.05, ** p < 0.01.

of both anti-IL-10 and anti-TGF- β 1 mAbs to the co-cultures reversed the inhibition more markedly, although the inhibition still persisted (Fig. 9). These results indicate that anti-inflammatory cytokines, IL-10, and TGF- β mediate the inhibitory effect of CD4⁺ T cells co-cultured with PF-treated DCs. In addition, the immunosuppressive function of PF-treated DCs was elicited by generating IL-10-producing T cells and functional Foxp3⁺ Treg cells from naïve CD4⁺ T cells.

4. Discussion

ACD, as the most frequent inflammatory skin disease, is also referred to as contact hypersensitivity [26,27]. It has been well established that DCs play an essential role in antigen recognition and T cell priming in the sensitization phase of ACD. PF can be effective in the treatment of ACD. Research has suggested that immunoregulatory drugs could target DCs at multiple levels, including maturation, migration, and differentiation. We suggest that PF, as an immunoregulatory drug, may regulate DC maturation, migration, and differentiation when used in the treatment of ACD.

Migration of DCs from the skin to the lymph node is a critical step in the pathogenesis of ACD. The strategy of targeting the migratory response of DCs represents an emerging option in the treatment of allergic skin diseases like ACD. Some drugs, such as sphingosine-1-phosphate and the immunomodulator FTY720, could attenuate ACD reaction through inhibition of DC migration [28]. In this experiment, we found that DCs could migrate when stimulated by allergen DNCB. When DCs were pretreated with PF *in vitro*, the migration rate was reduced, even though followed by stimulation with DNCB, which indicates that PF inhibiting inflammatory response may be partly due to reduced migration of DCs. Our findings consolidate the strategy that targeting the migratory response of DCs may be a new option in the treatment of allergic skin diseases such as ACD.

Another key event in ACD is the maturation of DCs which play a pivotal role in inducing response and converting T lymphocytes into functional Th1 cells. DCs activated by DNCB have been shown to stimulate T cell proliferation and induce Th1 cell polarization [29], which is associated with high expression of MHC and co-stimulatory molecules CD40, CD80, CD86, and proinflammatory cytokine IL-12, by DCs. A

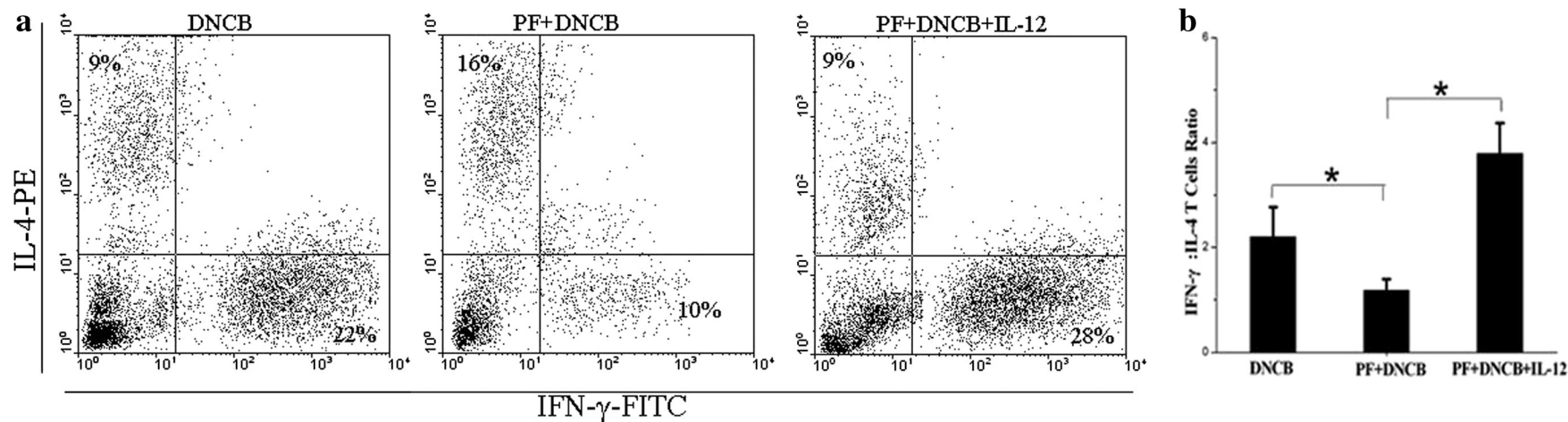
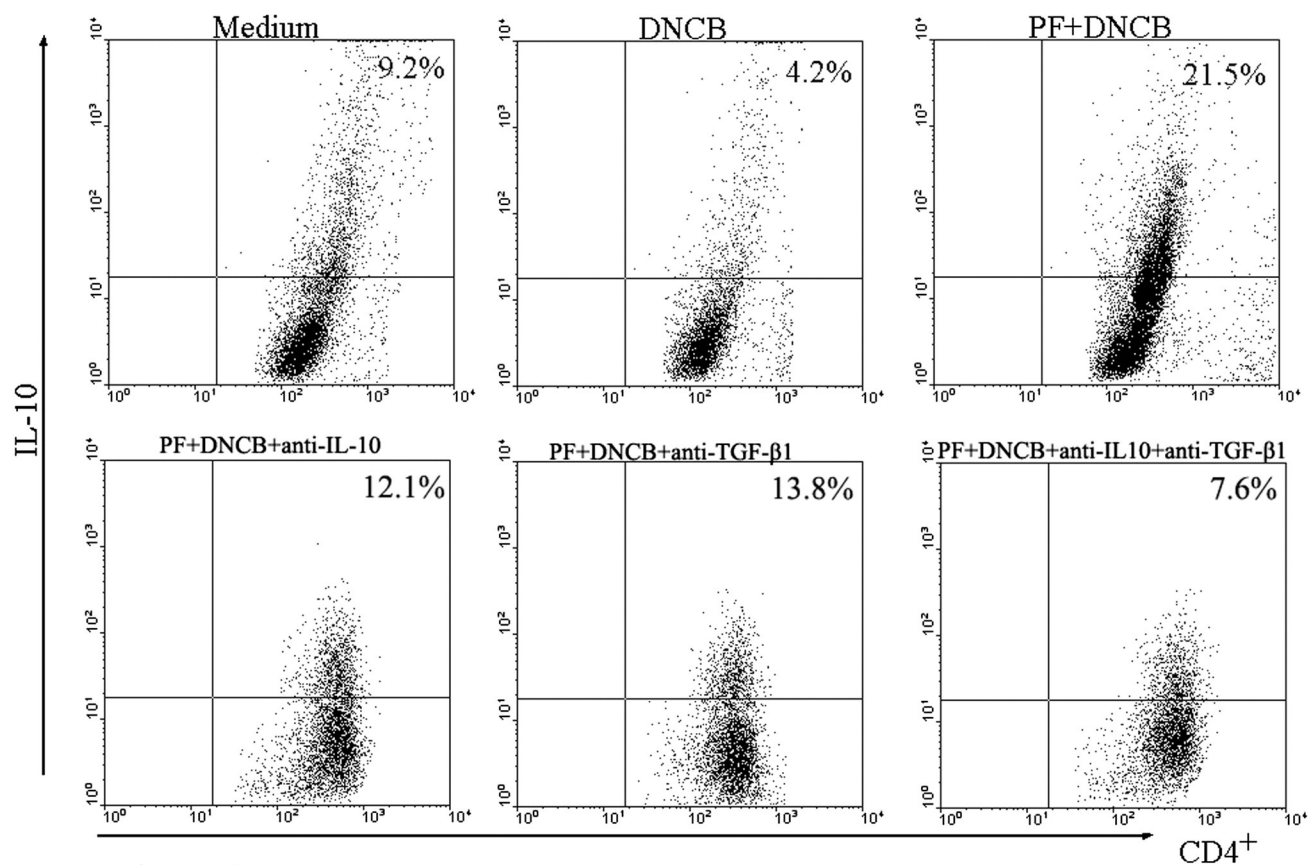
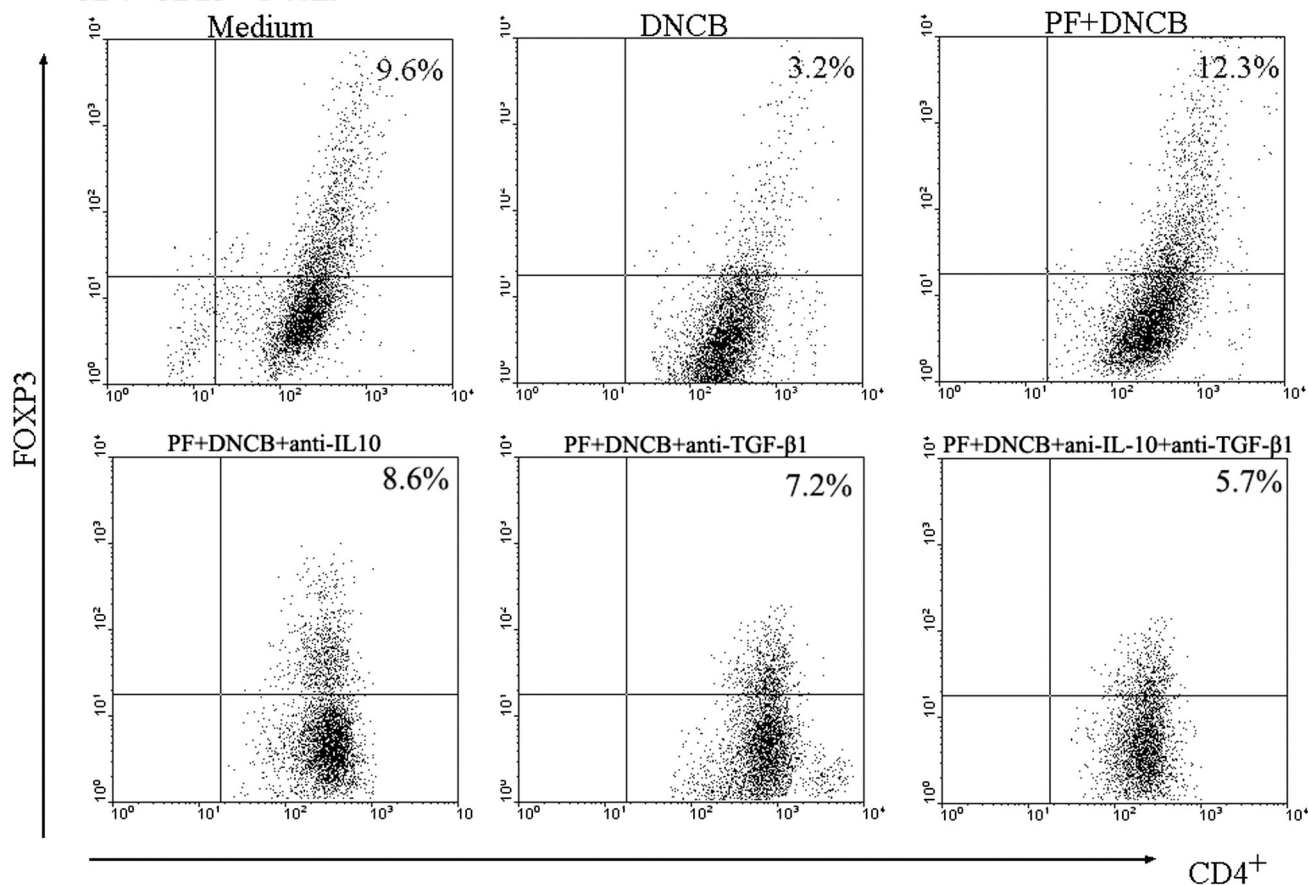


Fig. 8. Exogenous IL-12 restored IFN- γ production by CD4⁺ T cells stimulates with DCs exposed to paeoniflorin (PF). (a) DCs were exposed to PF and were treated with DNCB for 48 h. At day 10, T cells were stimulated with PMA and ionomycin for 5 h in the presence of brefeldin A. Cells were then stained with anti-IL-4-PE and anti-IFN- γ -FITC to detect intracellular cytokine production. One representative experiment of the three experiments with similar results is shown. (b) The ratio of IFN- γ -to-IL-4T cells is depicted. Mean $\pm$ SE of data from seven independent experiments is shown. ** $p < 0.01$.

a CD4⁺ T cells**b** CD4⁺CD25⁺ T cells

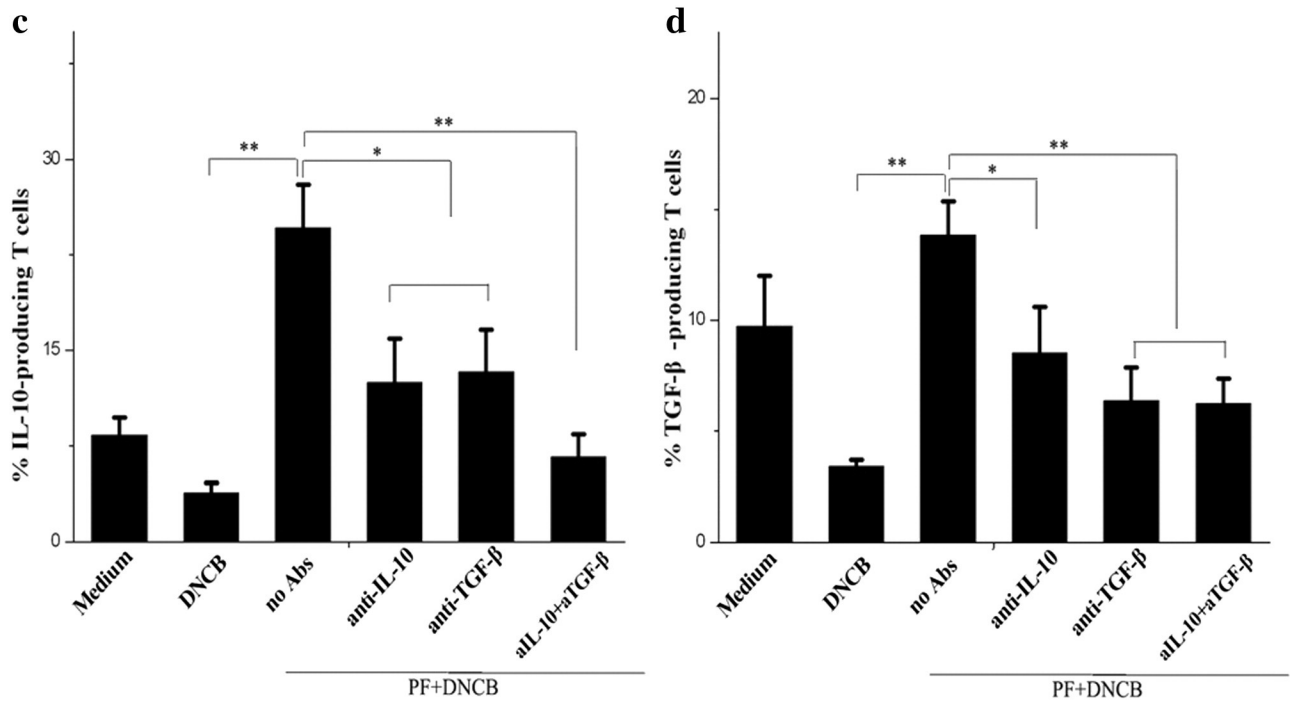


Fig. 9. Paeoniflorin (PF)-treated DCs induce IL-10-producing T cells and CD4⁺CD25⁺Foxp3⁺ regulatory T cells. DCs were exposed to PF and were treated with DNCB for 48 h and co-cultured with naïve CD4⁺CD45RA⁺ T cells. Co-culture experiments were also performed without anti-IL-10 and anti-TGF-β1 mAbs or in the presence of saturating blocking anti-IL-10 and/or TGF-β1 mAbs. Then, the intracellular expression of IL-10 and Foxp3⁺ in CD4⁺CD25⁺ T cells was analyzed by flow cytometry (a, b). Numbers in the corners indicate the percentage of positive cells. One representative experiment of seven is shown (c, d). Data were expressed as mean ± SE of seven independent experiments (e). **p* < 0.05, ***p* < 0.01.

growing body of evidence suggests that both immature and mature DCs play important roles in immune responses. Mature DCs are believed to induce T cell immunity, whereas iDCs induce T cell tolerance, predominantly via Treg cells [30,31]. Maturation of DCs is associated with greatly enhanced expression of MHC, CD40, CD80, CD83, and CD86. Immature DCs are poor T cell stimulators because of their moderate levels of MHC II expression and no or very low levels of co-stimulatory molecules such as CD80, CD86, and CD40 [32] and proinflammatory cytokines such as IL-12, but anti-inflammatory cytokines such as IL-10 and TGF-β in comparison with mature DCs, are highly produced [11].

We found that co-culture of iDCs with PF did not induce the maturation of iDCs even after the subsequent DNCB treatment, as assessed by the expression of MHC II, CD80, CD86, and CD40. This PF inhibition on the DNCB-triggered maturation of DCs was also in a dose-dependent fashion. Meanwhile, we showed for the first time that PF inhibited the DNCB-triggered IL-12 production but enhanced expression of anti-inflammatory cytokines, including IL-10 and TGF-β, indicating that PF inhibits the maturation of DCs triggered by DNCB, and to some extent, promotes their tolerogenicity. Lack of DC maturation was not the result of reduced DC viability since PF was not directly toxic to DCs in our observation. Moreover, PF-treated DCs did not impair the phagocytosis capacity of DCs. Our experiments showed that PF could inhibit the maturation of DCs directly. Indeed, keratinocyte, mast cells and endothelial cells are crucial types of cells for the development of ACD, and produce various chemical mediators required for DC maturation and migration [33]. Meanwhile, PF has immunomodulatory effects on dermal endothelial cells, keratinocytes and mast cells [34–36]. Thus, it is plausible that PF indirectly affects DC function by modulating the responses of these innate immune cells, thus contributing to its anti-ACD properties.

Whether PF directly or indirectly regulate the maturation of DCs, our results suggest that PF may suppress the DC's ability to stimulate responder T cells and induce suppressor T cell proliferation, which further inhibits the initiation of cell-mediated immune response following DNCB challenge. The hypothesis is supported by the

following observations. First, DCs exposed to PF had impaired ability to activate allogeneic T cells and to stimulate IFN-γ production. Second, an increasing ratio of DCs-to-responders restored the proliferation of allogeneic T cells. Third, the presence of a large number of allogeneic DCs overcomes the effects of reduced expression of MHC and co-stimulatory molecules by DCs exposed to PF. Our findings suggest that PF can impair the capacity of DCs to stimulate T cells and selectively modulates DCs' ability to induce IFN-γ-producing T cells.

Different DC subsets may stimulate the proliferation of different populations of Treg cells, such as CD4⁺CD25⁺Foxp3⁺ and IL-10-producing T cells, which have been described as an indispensable cell population in the maintenance of peripheral immunological tolerance [37,38]. Our results showed that the immunosuppressive function of PF-treated DCs was elicited by generating IL-10-producing T cells and functional Foxp3⁺ Treg cells from naïve CD4⁺ T cells.

Previous studies have revealed that multiple cytokines and chemokines are also involved in the regulation of the process in ACD [39]. Cytokines produced by DCs contribute effectively to defining the T cell response. Among these, IL-12 has been widely recognized as a key cytokine in the development of Th1 cell polarization, characterized by IFN-γ production by T cells [40,41]. Our findings show that PF-treated DCs could inhibit the secretion of IL-12 in response to DNCB. DCs exposed to PF had impaired ability to induce IFN-γ-producing T cell induction. Our results demonstrate that addition of exogenous IL-12 could restore the ability of PF-treated DCs to induce IFN-γ-producing T cells, suggesting that diminished capacity to simulate IFN-γ-producing T cells is partly resulted from reduced IL-12p70 secretion.

IL-10 and TGF-β are the most commonly studied immunosuppressive cytokines and induce the proliferation of Treg cells, e.g., IL-10-producing T cells and CD4⁺CD25⁺Foxp3⁺ T cells. Our findings show that PF-treated DCs produce high levels of anti-inflammatory cytokines such as TGF-β and IL-10 in comparison with DNCB-treated DCs alone. To clarify whether PF-treated DCs induce IL-10-producing T cells and CD4⁺CD25⁺Foxp3⁺ T cell proliferation via anti-inflammatory cytokines, we used anti-IL-10 and anti-TGF-β mAbs in a parallel experiment and found that both cytokines

could reverse the inhibition remarkably, although the inhibition still persisted. These results demonstrate that IL-10 and TGF- β mediate the inhibitory effect of CD4⁺ T cells co-cultured with PF-treated DCs.

The current study investigates the effects of PF on the maturation of murine bone marrow-derived DCs in response to DNCB. Recently, Toll-like receptors (TLRs), a new group of receptors belonging to the IL-1 receptor family, have been reported to be involved in the initiation and maturation of the DCs [42]. Several TLRs have been identified in DCs. TLR4 and TLR5 ligands appear to be particularly important for DC activation in total glucosides of paeony-treated collagen-induced arthritis [43]. Since total glucosides of paeony is mainly composed of PF (>90%), we suggest that attenuated TLR4 and TLR5 activation may be involved in decreased maturation and activation of DCs in ACD. However, we did not explore why PF could inhibit the maturation of DCs in the present study. Further investigation of this question will help better understand the molecular mechanisms of immunomodulating action of monoterpenic glucosides from the plant in the treatment of ACD.

To our knowledge, this is the first account of effects of PF on the DNCB-induced DCs and the subsequent differentiation of CD4⁺ T cells. This report encourages further evaluation of the *in vivo* function of PF-treated DCs in relation to their potential regulatory influence on delayed-type hypersensitivity reaction caused by a wide range of low molecular weight reactive chemical allergens.

Conflict of interests

The authors have no conflict of interests in connection with this study.

Acknowledgments

We would like to thank Professor Dongmei Li and Dr Chengxuan Qiu for their kind assistance in editing our manuscript. This work was supported in part by a grant from the National Scientific Foundation of China (NM 81071332).

References

- [1] Martin SF, Esser PR, Weber FC, Jakob T, Freudenberg MA, Schmidt M, et al. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 2011;66:1152–63.
- [2] Blauvelt A, Hwang ST, Udey MC. 11. Allergic and immunologic diseases of the skin. *J Allergy Clin Immunol* 2003;111:S560–70.
- [3] Christensen AD, Haase C. Immunological mechanisms of contact hypersensitivity in mice. *APMIS* 2012;120:1–27.
- [4] Kimber I, Basketter DA, Gerberick GF, Dearman RJ. Allergic contact dermatitis. *Int Immunopharmacol* 2002;2:201–11.
- [5] Strioga M, Schijns V, Powell Jr DJ, Pasukoniene V, Dobrovolskiene N, Michalek J. Dendritic cells and their role in tumor immunosurveillance. *Innate Immun* 2013;19:98–111.
- [6] Burastero SE, Paolucci C, Breda D, Ponti J, Munaro B, Sabbioni E. Chromium (VI)-induced immunotoxicity and intracellular accumulation in human primary dendritic cells. *Int J Immunopathol Pharmacol* 2006;19:581–91.
- [7] Mahnke K, Qian Y, Knop J, Enk AH. Induction of CD4⁺/CD25⁺ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 2003;101:4862–9.
- [8] Lutz MB. Therapeutic potential of semi-mature dendritic cells for tolerance induction. *Front Immunol* 2012;3:123.
- [9] Murphy KM, Reiner SL. The lineage decisions of helper T cells. *Nat Rev Immunol* 2003;2:933–44.
- [10] Micallef MJ, Ohtsuki T, Kohno K, Tanabe F, Ushio S, Namba M, et al. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur J Immunol* 1996;26:1647–51.
- [11] Matsumoto T, Hasegawa H, Onishi S, Ishizaki J, Suemori K, Yasukawa M. Protein kinase C inhibitor generates stable human tolerogenic dendritic cells. *J Immunol* 2013;191:2247–57.
- [12] Cai Z, Zhang W, Li M, Yue Y, Yang F, Yu L, et al. TGF-beta1 gene-modified, immature dendritic cells delay the development of inflammatory bowel disease by inducing CD4⁺/Foxp3⁺ regulatory T cells. *Cell Mol Immunol* 2010;7:35–43.
- [13] Zhou F, Ciric B, Zhang GX, Rostami A. Immune tolerance induced by intravenous transfer of immature dendritic cells via up-regulating numbers of suppressive IL-10(+) IFN-gamma(+) -producing CD4⁺ T cells. *Immunol Res* 2013;56:1–8.
- [14] Thome R, Moraes AS, Bombeiro AL, Farias AS, Francelin C, da Costa TA, et al. Chloroquine treatment enhances regulatory T cells and reduces the severity of experimental autoimmune encephalomyelitis. *PLoS One* 2013;8:e65913.
- [15] He DY, Dai SM. Anti-inflammatory and immunomodulatory effects of paeonia lactiflora pall., a traditional Chinese herbal medicine. *Front Pharmacol* 2011;2:10.
- [16] Wu SH, Wu DG, Chen YW. Chemical constituents and bioactivities of plants from the genus *Paeonia*. *Chem Biodivers* 2010;7:90–104.
- [17] Wang C, Yuan J, Wu HX, Chang Y, Wang QT, Wu YJ, et al. Paeoniflorin inhibits inflammatory responses in mice with allergic contact dermatitis by regulating the balance between inflammatory and anti-inflammatory cytokines. *Inflamm Res* 2013;62:1035–44.
- [18] Sun Y, Dong Y, Jiang HJ, Cai TT, Chen L, Zhou X, et al. Dissection of the role of paeoniflorin in the traditional Chinese medicinal formula Si-Ni-San against contact dermatitis in mice. *Life Sci* 2009;84:337–44.
- [19] Kaplan DH, Igyarto BZ, Gaspari AA. Early immune events in the induction of allergic contact dermatitis. *Nat Rev Immunol* 2012;12:114–24.
- [20] Lutz MB, Yuchtman N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999;223:77–92.
- [21] Liu D, Yu J, Chen H, Reichman R, Wu H, Jin X. Statistical determination of threshold for cellular division in the CFSE-labeling assay. *J Immunol Methods* 2006;312:126–36.
- [22] Sato K, Yamashita N, Baba M, Matsuyama T. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood* 2003;101:3581–9.
- [23] Baumer W, Krekeler S, DeVries VC, Niedorf F, Tschernig T, Kietzmann M. Non-steroidal and steroidal anti-inflammatory drugs vary in their modulation of dendritic cell function in the elicitation phase of allergic contact dermatitis. *Exp Dermatol* 2006;15:322–9.
- [24] Cho YC, Lee SH, Yoon G, Kim HS, Na JY, Choi HJ, et al. Licochalcone E reduces chronic allergic contact dermatitis and inhibits IL-12p40 production through down-regulation of NF-kappa B. *Int Immunopharmacol* 2010;10:1119–26.
- [25] Rolland JM, Gardner LM, O'Hehir RE. Functional regulatory T cells and allergen immunotherapy. *Curr Opin Allergy Clin Immunol* 2010;10:559–66.
- [26] Thyssen JP, Linneberg A, Menne T, Johansen JD. The epidemiology of contact allergy in the general population—prevalence and main findings. *Contact Dermatitis* 2007;57:287–99.
- [27] Mowad CM. Patch testing: pitfalls and performance. *Curr Opin Allergy Clin Immunol* 2006;6:340–4.
- [28] Reines I, Kietzmann M, Mischke R, Tschernig T, Luth A, Kleuser B, et al. Topical application of sphingosine-1-phosphate and FTY720 attenuate allergic contact dermatitis reaction through inhibition of dendritic cell migration. *J Invest Dermatol* 2009;129:1954–62.
- [29] Toebak MJ, Moed H, von BMB, Bruynzeel DP, Gibbs S, Scheper RJ, et al. Intrinsic characteristics of contact and respiratory allergens influence production of polarizing cytokines by dendritic cells. *Contact Dermatitis* 2006;55:238–45.
- [30] Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity. *Trends Immunol* 2002;23:445–9.
- [31] Menges M, Rossner S, Voigtlander C, Schindler H, Kukutsch NA, Bogdan C, et al. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J Exp Med* 2002;195:15–21.
- [32] Inaba K, Witmer-Pack M, Inaba M, Hathcock KS, Sakuta H, Azuma M, et al. The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J Exp Med* 1994;180:1849–60.
- [33] Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol* 2013;133(2):303–15.
- [34] Yoshihisa Y, Furuichi M, Ur RM, Ueda C, Makino T, Shimizu T. The traditional Japanese formula keishibukuryogan inhibits the production of inflammatory cytokines by dermal endothelial cells. *Mediators Inflamm* 2010;2010:804298.
- [35] Huang J, Qiu L, Ding L, Wang S, Wang J, Zhu Q, et al. Ginsenoside Rb1 and paeoniflorin inhibit transient receptor potential vanilloid-1-activated IL-8 and PGE(2) production in a human keratinocyte cell line HaCaT. *Int Immunopharmacol* 2010;10(10):1279–83.
- [36] Lee B, Shin YW, Bae EA, Han SJ, Kim JS, Kang SS, et al. Antiallergic effect of the root of *Paeonia lactiflora* and its constituents paeoniflorin and paeonol. *Arch Pharm Res* 2008;31(4):445–50.
- [37] Hirata N, Yanagawa Y, Satoh M, Ogura H, Ebihara T, Noguchi M, et al. Dendritic cell-derived TNF-alpha is responsible for development of IL-10-producing CD4⁺ T cells. *Cell Immunol* 2010;261:37–41.
- [38] Marguti I, Yamamoto GL, da CTB, Rizzo LV, de Moraes LV. Expansion of CD4⁺ CD25⁺ Foxp3⁺ T cells by bone marrow-derived dendritic cells. *Immunology* 2009;127:50–61.
- [39] McFadden JP, Puangpet P, Basketter DA, Dearman RJ, Kimber I. Why does allergic contact dermatitis exist? *Br J Dermatol* 2013;168:692–9.
- [40] Cho YC, You SK, Kim HJ, Cho CW, Lee IS, Kang BY. Xanthohumol inhibits IL-12 production and reduces chronic allergic contact dermatitis. *Int Immunopharmacol* 2010;10:556–61.
- [41] Youssef S, Stuve O, Patarroyo JC, Ruiz PJ, Radosevich JL, Hur EM, et al. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* 2002;420:78–84.
- [42] Watts C, West MA, Zaru R. TLR signalling regulated antigen presentation in dendritic cells. *Curr Opin Immunol* 2010;22:124–30.
- [43] Lin J, Xiao L, Ouyang G, Shen Y, Huo R, Zhou Z, et al. Total glucosides of paeony inhibits Th1/Th17 cells via decreasing dendritic cells activation in rheumatoid arthritis. *Cell Immunol* 2012;280:156–63.