



Imperatorin exerts antiallergic effects in Th2-mediated allergic asthma via induction of IL-10-producing regulatory T cells by modulating the function of dendritic cells

Chu-Lun Lin^a, George Hsiao^b, Ching-Chiung Wang^c, Yueh-Lun Lee^{d,*}

^a Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan

^b Department of Pharmacology, College of Medicine, Taipei Medical University, Taipei, Taiwan

^c School of Pharmacy, College of Pharmacy, Taipei Medical University, Taipei, Taiwan

^d Department of Microbiology and Immunology, College of Medicine, Taipei Medical University, Taipei, Taiwan

ARTICLE INFO

Article history:

Received 1 February 2016

Received in revised form 22 April 2016

Accepted 29 April 2016

Available online 13 May 2016

Keywords:

Imperatorin

Bone marrow-derived dendritic cells

Interleukin-10

Regulatory T cells

Allergic asthma

ABSTRACT

Imperatorin is a furanocoumarin compound which exists in many medicinal herbs and possesses various biological activities. Herein, we investigated the antiallergic effects of imperatorin in asthmatic mice and explored the immunomodulatory actions of imperatorin on immune cells. We used a murine model of ovalbumin (OVA)-induced asthma to evaluate the therapeutic potential of imperatorin. Additionally, bone marrow-derived dendritic cells (DCs; BMDCs) were used to clarify whether imperatorin exerts an antiallergic effect through altering the ability of DCs to regulate T cells. Oral administration of imperatorin to OVA-sensitized and -challenged mice decreased serum OVA-specific immunoglobulin E (IgE) production, attenuated the airway hyperresponsiveness (AHR), and alleviated airway inflammation in a dose-dependent manner. Notably, secretions of Th2 cytokines and chemokines were reduced, and numbers of interleukin (IL)-10-producing regulatory T cells (Tregs) increased in imperatorin-treated mice. Imperatorin inhibited proinflammatory cytokines and IL-12 production but enhanced IL-10 secretion by lipopolysaccharide (LPS)-stimulated BMDCs. Compared to fully mature DCs, imperatorin-treated DCs expressed high levels of the inducible costimulatory ligand (ICOSL) and Jagged1 molecules, and had the regulatory capacity to promote the generation of IL-10-producing CD4⁺ T cells in vitro. Additionally, imperatorin directly suppressed activated CD4⁺ T-cell proliferation and cytokine production. Imperatorin may possess therapeutic potential against Th2-mediated allergic asthma not only via stimulating DC induction of Tregs but also via direct inhibition of Th2 cell activation. These findings provide new insights into how imperatorin affects the Th2 immune response and the development of imperatorin as a Treg-type immunomodulatory agent to treat allergic asthma.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Asthma is an inflammatory disease of the airways, and many immune cells act together to cause airway hyper-reactivity, mucus overproduction, and airway remodeling. In the asthma population, most children and roughly 50% of adults have allergic asthma, in which the disease causes allergic sensitization and increased serum immunoglobulin E (IgE) levels, and this disease is involved in activation of type 2 helper T (Th2) cells and the production

of Th2 cytokines [1]. Dendritic cells (DCs) are the most potent antigen-presenting cells which affect activation and differentiation of T cells [2]. Antigen-activated DCs display costimulatory molecules and produce different types of cytokines which determine whether T cells differentiate into Th1, Th2, or regulatory T cells (Tregs) [3,4]. In particular, Tregs are implicated in the maintenance of immune homeostasis in the airway, and many studies demonstrated that Tregs dampen immune responses to limit immunopathology [5,6]. There are at least two population of Tregs, Th3 and Tr1, which perform their immunosuppressive function by secreting the anti-inflammatory cytokines, interleukin (IL)-10 or transforming growth factor (TGF)- β [7]. However, in asthmatic patients, there is a breakdown in Treg-regulatory mechanisms which result in the development of airway inflammation

* Corresponding author at: Department of Microbiology and Immunology, College of Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 11031, Taiwan.
E-mail address: yilee@tmu.edu.tw (Y.-L. Lee).

[8]. Therefore, inhibition of Th2 cell responses via the induction of Tregs is considered an effective strategy to treat Th2-mediated allergic asthma.

Imperatorin [9-(3-methylbut-2-enyloxy)-7H-furo[3,2-g]chromen-7-one] (molecular weight 270.28) belongs to a linear furan class of coumarin compounds, and it is the main composition of many traditional Chinese medicines, such as *Angelica dahurica* Linnaeus (L.), *A. sylvestris* L., *A. archangelica* L., and *Peucedanum praeruptorum* L. [9–11]. Imperatorin is known to exert many biological functions. Previous studies showed that imperatorin inhibited human immunodeficiency virus-1 infection in T cell lines and HeLa cells [12]. Imperatorin also possesses anti-inflammatory activities. Some studies reported that imperatorin suppressed proinflammatory cytokine secretion, and reduced nitric oxide (NO) and prostaglandin E2 (PGE2) production in the RAW264.7 cell line [13,14]. Additionally, imperatorin inhibited the growth of H292 and A549 lung cancer cells by enhancing the p53 protein level [15]. More recently, a report provided evidence that imperatorin suppressed degranulation in IgE plus antigen-activated bone marrow-derived mast cells [16]. Thus, we propose that the bioactivities of imperatorin may potentially be able to influence immune cell functions and provide a new alternative option for relieving symptoms of allergic asthma. However, to date, the antiallergic effects of imperatorin against allergic asthma and the modulatory actions on DCs and T cells are still unavailable.

The aims of this study were to identify whether imperatorin treatment can suppress Th2 responses in an ovalbumin (OVA)-induced allergic asthma model and achieve antiallergic activities against the development of airway syndromes. Furthermore, the immunomodulatory effects of imperatorin on T-cell skewing were explored. Our results revealed that imperatorin inhibited Th2-mediated airway inflammation through (i) induction of IL-10-secreting Tregs by modulating DC functions and (ii) direct inhibition of T cell activation. Herein, we provide new evidence for an anti-inflammatory role of imperatorin in inhibiting the immune response and provide a potential use of imperatorin as an antiallergic adjuvant to treat Th2-mediated allergic inflammation.

2. Material and methods

2.1. Preparation of imperatorin

Imperatorin (purity $\geq 98\%$ by high-performance liquid chromatography) was isolated from the dried root of *Saposhnikovia divaricata* (Apiaceae) by previously described purification methods [17]. The structure of imperatorin is shown in Fig. 1A. A stock solution (370 mM) was prepared by dissolving imperatorin in (99.9%) DMSO, and this was then stored at 4 °C until use.

2.2. Animals

Female BALB/c and C57BL/6 mice were purchased from the National Laboratory Animal Center and Laboratory Animal Center of National Taiwan University (Taipei, Taiwan) and maintained in the Animal Center of Taipei Medical University. Animals used were between 5 and 10 weeks of age and were age-matched within each experiment. The animal care and handling protocols were approved by the animal use committee of the College of Medicine, Taipei Medical University (approval no.: LAC-2013-0207).

2.3. Administration of imperatorin to allergen-sensitized mice

Female BALB/c mice at 6 weeks of age were sensitized by an intraperitoneal (i.p.) injection of 50 μ g OVA (Sigma-Aldrich, St. Louis, MO, USA) plus 4 mg alum (Thermo Scientific, Rockford, IL, USA) on day 1. On days 14, 28, 42, and 56, mice were boosted with

OVA (30 μ g) and the same dosage of alum. On days 64 and 65, mice were intranasally (i.n.) challenged with 100 μ g OVA. Then, mice were challenged with 5% OVA in 0.9% NaCl by inhalation for 3 consecutive days (days 66, 67, and 68) for 30 min daily. Finally, the airway hyperresponsiveness (AHR) of mice was measured, and the mice were sacrificed on day 69. To examine the effects of various dosages (0.1, 1, and 5 mg/kg of body weight) of imperatorin, three groups of mice were each day orally fed imperatorin dissolved in soybean oil from days 15–68. OVA-sensitized and –challenged mice were fed soybean oil as an asthmatic positive control (PC). Negative control (NC) mice were neither sensitized with OVA nor administered imperatorin treatment although they received an OVA challenge.

2.4. OVA-specific serum antibody assay

Serum samples were collected from mice on days 0, 21, 49, 63, and 69. OVA-specific IgE, IgG₁, and IgG_{2a} serum antibody titers were measured using enzyme-linked immunosorbent assay (ELISA) kits (Becton Dickinson Biosciences, San Jose, CA, USA). All samples were compared to OVA-specific IgE, IgG₁, and IgG_{2a} standard serum. The concentration of standard serum was arbitrarily assigned as 1 ELISA unit (EU).

2.5. Measurement of AHR

AHR was measured 24 h after the last aerosol exposure. Mice were exposed to increasing doses of aerosolized methacholine (MCh, Sigma-Aldrich), and the resulting AHR was measured by the lung resistance (R_L) in tracheostomized mice that were anesthetized with 100 μ l pentobarbital (10 mg/ml) and 50 μ l zoletil (5 mg/ml). Mice were placed in a ventilator (Model 683; Harvard Apparatus, South Natick, MA, USA) with their breathing controlled via a tracheostomy tube at 150 breaths/min, with a tidal volume of 0.3 ml and a positive end-expiratory pressure of 2 ~ 4 cmH₂O. Increasing concentrations (1–32 mg/ml) of the MCh aerosol were administered by nebulization. The R_L was continuously computed (Labview, National Instruments, Dallas, TX, USA) by fitting the flow, volume, and pressure to an equation of motion.

2.6. Analysis of bronchoalveolar lavage fluid (BALF) and lung histology

Mice were sacrificed after measuring the AHR, and the lungs were immediately lavaged via the tracheal cannula with 3 $\times$ 1 ml of Hank's balanced salt solution (HBSS) which was free of ionized calcium and magnesium. The lavage fluid was centrifuged at 400 $\times$ g for 10 min at 4 °C. The supernatants were collected for the chemokine and cytokine assays. Cells were resuspended in 1 ml RPMI-1640 medium and combined with 2% fetal bovine serum (FBS) after washing. Total cell counts were determined by counting at least 200 cells of cytocentrifuged preparations in a hemocytometer, with Liu's staining solution (Chi I Pao, Taipei, Taiwan). Different cell types were classified as macrophages, eosinophils, neutrophils, and lymphocytes, based on standard morphological criteria. The lungs were immediately removed and fixed in 10% neutral-buffered formalin after the lavage, routinely processed, and embedded in paraffin wax. Five-micrometer sections were prepared and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS).

2.7. Determination of cytokine levels

Levels of eotaxin, keratinocyte-derived chemokine (KC), IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ were analyzed using ELISA kits (eotaxin, KC, IL-5, IL-10, and IFN- γ kits from R&D Systems, Minneapolis, MN,

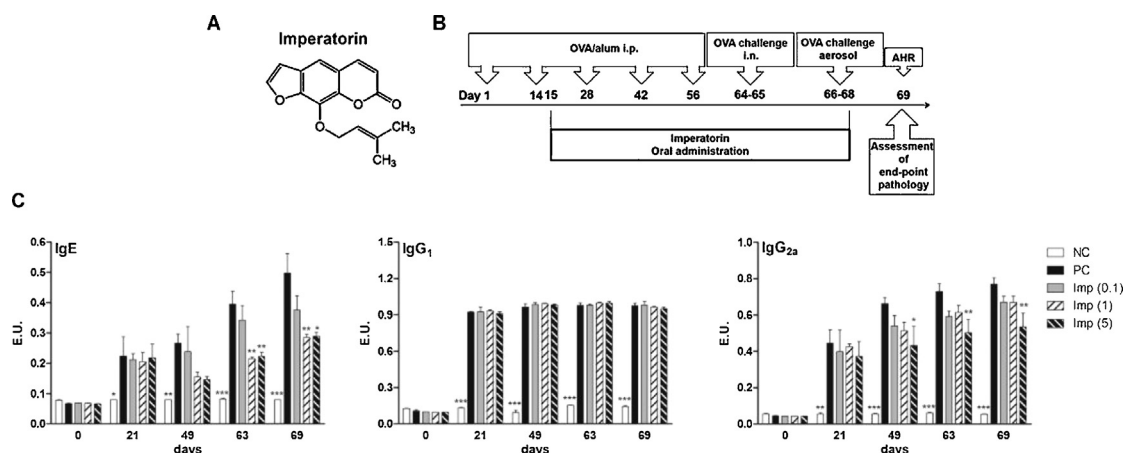


Fig. 1. Expression levels of anti-OVA antibodies in imperatorin-treated allergic asthmatic mice. (A) The structure of imperatorin. The chemical formula is $C_{16}H_{14}O_4$, and molar mass is 278.28 g/mol. (B) Brief protocol of animal sensitization and challenge. On days 1, 14, 28, 42, and 56, all groups of mice were sensitized by an intraperitoneal (i.p.) injection of OVA allergen. Three groups of mice (Imp (0.1), Imp (1), and Imp (5)) were orally fed 0.1, 1 and 5 mg/kg, respectively, of imperatorin on days 15–68. Control mice (PC) were administered soybean oil instead of imperatorin. Mice were then intranasally (i.n.) challenged with OVA on days 64 and 65. Subsequently, mice were exposed to OVA aerosols for 3 consecutive days, and the AHR was measured 1 day after the last challenge. BAL fluid was collected after measuring the AHR. Negative control mice (NC) were neither sensitized with OVA nor administered imperatorin treatment. (C) IgE, IgG₁, and IgG_{2a} anti-OVA antibody expressions of all groups of mice were measured by ELISAs. Results are expressed as the mean $\pm$ SEM of four to six mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

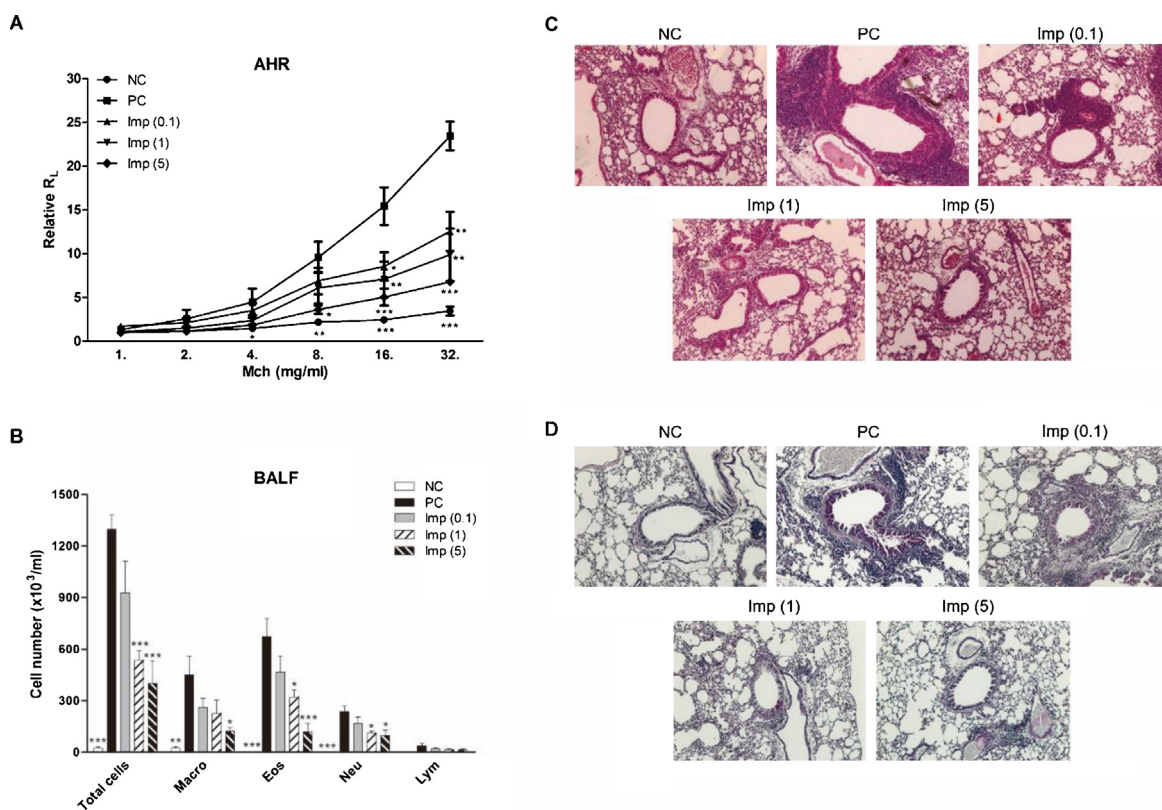


Fig. 2. Effects of imperatorin on the airway function and inflammation. (A) Oral administration of imperatorin suppressed the development of AHR in OVA-induced asthmatic mice. One day after the last OVA challenge, airway resistance was measured in response to increasing concentrations of methacholine (1–32 mg/ml) by invasive body plethysmography. Results are expressed as the pulmonary resistance in the ratio of lung resistance (R_L) after PBS nebulization. (B) Changes in the cellular composition of BAL fluid of mice exposed to an aerosolized allergen. After measuring the pulmonary function parameters, each group of mice was sacrificed, and BAL fluid was collected. Cells from BAL fluid were counted and classified as macrophages (Macro), eosinophils (Eos), neutrophils (Neu), and lymphocytes (Lym). (C) Lung sections were stained with hematoxylin/eosin (H&E) to measure inflammatory cells. (D) Lung sections were stained with periodic acid-Schiff (PAS) staining to measure mucus around the airway. Results are expressed as the mean $\pm$ SEM of four to six mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

USA; IL-1 β , IL-2, IL-4, IL-6, IL-12, IL-13, and TNF- α kits from eBioscience, San Diego, CA, USA).

2.8. Detection of IL-10-producing Tregs

Spleens from each group of mice were harvested and ground into single-cell suspensions. Red blood cells were removed by ACK lysis buffer and washed three times with HBSS. For intracellular

staining, 2×10^6 splenocytes were stimulated with OVA (50 μ g/ml) and cultured for 5 days. Then cells were restimulated with 20 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich) plus 1 μ g/ml ionomycin (Sigma-Aldrich) for 6 h. Intracellular cytokine expressions of IL-4 and IL-10 in cells were measured by flow cytometry. In an OVA-induced T cell proliferation assay, 3×10^5 splenocytes were seeded into each well of 96-well plates and cultured with OVA (50 μ g/ml).

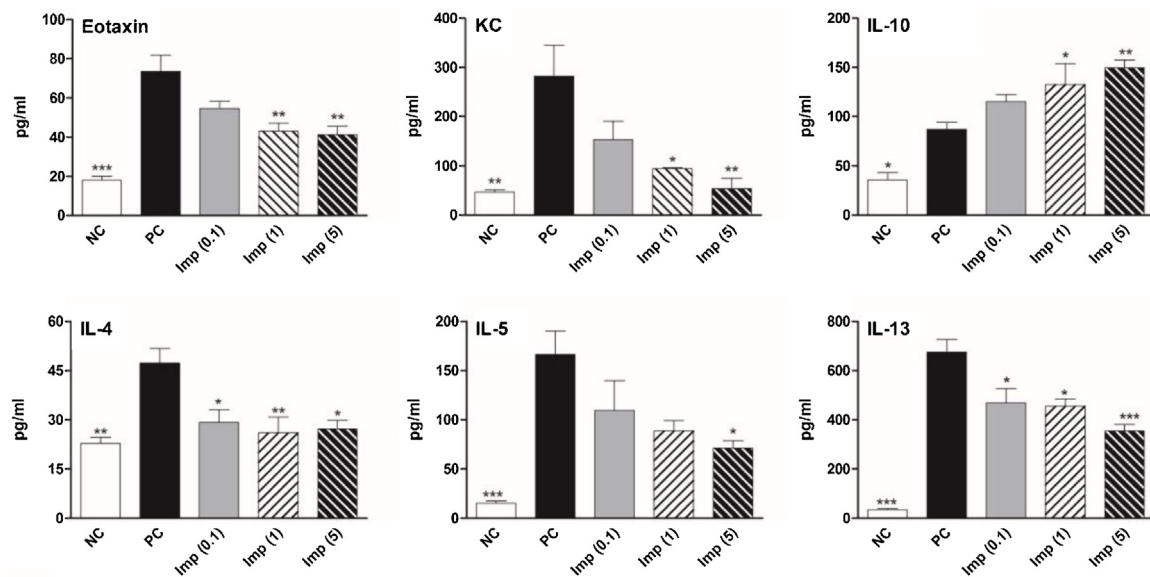


Fig. 3. Effects of imperatorin on levels of inflammatory mediator and cytokine production in BAL fluid. Immediately after measuring pulmonary function parameters, each group of mice was sacrificed, and BAL fluid was collected and analyzed for eotaxin, KC, IL-4, IL-5, IL-10, and IL-13 contents by ELISAs. Results are expressed as the mean $\pm$ SEM of four to six mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

After 72 h of culture, cells were pulsed with [^{3} H]-thymidine for another 18 h. Cells were harvested, and tritiated thymidine incorporation was determined with a dry scintillation counter.

2.9. Generation of bone marrow-derived DCs (BMDCs)

Tibial and femoral bone marrow was isolated from 5–9-week-old BALB/c mice. Bone marrow cells were cultured in RPMI-1640 combined with 5% FBS complete medium with granulocyte-macrophage colony-stimulating factor (GM-CSF) (200 U/ml) on day 0. Fresh medium containing GM-CSF was used to replace the old medium on day 2. Non-adherent cells were removed, and the old medium was replaced with GM-CSF (100 U/ml) and IL-4 (200 U/ml) on day 5. On day 8 of culture, non-adherent cells were collected and washed for further assaying.

2.10. MTT assay

On day 8 of culture, BMDCs (10^5 cells/well) were treated with various concentrations (0.4, 0.7, 3.7, 18.5, 37, 185, and 370 μ M) of imperatorin for 72 h. Untreated DCs were cultured with medium as the control. In another experiment, to assess the cytotoxicity effect of imperatorin on T cells, 10^5 cells/well of CD4 $^+$ T cells were treated with various concentrations (0.4, 0.7, 3.7, 18.5, 37, and 74 μ M) of imperatorin for 72 h. Untreated T cells were cultured with medium alone as the control. Cells were pulsed with 10 μ l of 5 mg/ml of MTT and incubated for another 4 h. Then cells were solubilized in DMSO, and the value was determined by a microplate reader at 570 nm.

2.11. Cytokine measurements

On day 8 of culture, BMDCs (10^6 cells/well) were pretreated in 24-well plates with different doses of imperatorin (0.7, 3.7, and 18.5 μ M), 1 μ M prednisolone (Sigma-Aldrich), or medium only for 20 min, and then treated with or without 100 ng/ml LPS (Sigma-Aldrich) for 24 h. Culture supernatants were harvested, and levels of IL-10, IL-12, and proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) were detected by ELISA kits.

2.12. Surface marker analysis

DCs were pretreated with or without imperatorin, then cultured with 100 ng/ml LPS, and harvested after a 24-h incubation. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c and anti-CD86 or phycoerythrin (PE)-conjugated anti-CD11c, anti-I-A/I-E (MHC class II), anti-CD40, anti-CD54, anti-CD80, anti-CD86, anti-programmed death-ligand (PDL)-1, anti-inducible costimulatory ligand (ICOSL), anti-Jagged1 (eBioscience), anti-Delta-like1, anti-Delta-like4, and anti-Jagged2 (Biolegend, San Diego, CA, USA) at 4°C for 30 min. Cells were washed and resuspended in FACS buffer (1% FBS and 0.1% NaN $_3$ in a 1 $\times$ phosphate-buffered saline (PBS) solution). Staining with isotype control antibodies was performed in all experiments. Flow cytometry was performed using FACSCalibur (Becton Dickinson, Mountain View, CA, USA), and data were analyzed with Cellquest pro software (Becton Dickinson).

2.13. CD4 $^+$ T cell isolation

Splenocytes from BALB/c and C57BL/6 mice were resuspended in MACS buffer (0.5% bovine serum albumin (BSA) and 2 mM EDTA in a 1 $\times$ PBS solution) at 10^7 cells/80 μ L and stained with anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, CA, USA) at 10^7 cells/20 μ L, which was performed for 15 min at 4°C. Cells were washed and resuspended in MACS buffer to positively select CD4 $^+$ T cells.

2.14. *in vitro* T-cell proliferation assay

For mixed lymphocyte reactions, 10^6 day-8 BMDCs from BALB/c mice were treated with LPS (100 ng/ml), imperatorin (18.5 μ M), LPS plus imperatorin, or medium only. After 24 h of culture, DCs were irradiated with 3000 rad (^{137} Cs source). Then allogeneic CD4 $^+$ T cells (2×10^5 cells/well) from C57BL/6 mice were cultured with different proportions of irradiated DCs (0.25×10^4 , 0.5×10^4 , and 10^4 cells/well). Cells were cultured for 72 h and then pulsed with [^{3} H]-thymidine for another 18 h. Cells were harvested, and counts per minute (cpm) were measured with a β -counter. For the cytokine assays, culture supernatants were collected at 24, 48, and 72 h. In

another experiment, to assess the direct effect of imperatorin on T cells, 2×10^5 CD4⁺ T cells from BALB/c mice were stimulated with anti-CD3/anti-CD28 antibodies (1 µg/ml) in 96-well plates and treated with different doses of imperatorin (0.7, 3.7, and 18.5 µM), prednisolone (1 µM), or medium only. After 3 days of culture, cell proliferation was measured by [³H]-thymidine incorporation, and culture supernatants were harvested for cytokine assays.

2.15. Data analysis

Results are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. *p* values of <0.05 were considered statistically significant.

3. Results

3.1. Imperatorin reduced anti-OVA IgE production in serum

We evaluated the therapeutic effects of imperatorin in an OVA-induced asthmatic animal model. The treatment protocol is shown in Fig. 1B. OVA-sensitized mice (Imp (0.1), Imp (1), and Imp (5)) were orally fed different doses (0.1, 1, and 5 mg/kg, respectively) of imperatorin. The PC group was fed soybean oil instead of imperatorin. NC mice were neither sensitized with OVA nor fed imperatorin. Serum samples were collected on the indicated days, and titers of OVA-specific antibodies in serum were assayed by ELISAs. Compared to the PC group, oral administration of a high dose of imperatorin (Imp (5)) to mice significantly decreased OVA-specific IgE and IgG_{2a} production on days 63–69. However, imperatorin had no suppressive effect on OVA-specific IgG₁ expression (Fig. 1C). Previous studies showed that the Th2-type cytokine, IL-4, can induce IgE and IgG₁ production, and the Th1-type cytokine, IFN- γ , can induce IgG_{2a} production in B cells [18,19]. Therefore, these results indicated that imperatorin inhibited both Th1 and Th2 responses in allergic asthmatic mice.

3.2. Imperatorin attenuated allergen-induced airway inflammation

Furthermore, to determine the effects of imperatorin on allergenic-induced asthma, both the AHR and infiltration of inflammatory cells in BALF were investigated. As shown in Fig. 2A, treatment of mice with imperatorin significantly inhibited AHR in a dose-dependent manner, compared to the PC group. On the other hand, PC mice that were sensitized and challenged with OVA exhibited obvious infiltration of macrophages, eosinophils, and neutrophils in BALF (Fig. 2B). In contrast, oral treatment of mice with imperatorin markedly reduced the infiltration of these inflammatory cells. A histological examination of lungs from the PC group showed a large number of infiltrating inflammatory cells and increased mucus secretion from goblet cells (Fig. 2C, D), compared to imperatorin-treated groups. Upon giving imperatorin to mice, imperatorin inhibited inflammatory cell infiltration and mucus production in the lungs.

3.3. Imperatorin suppressed secretion of chemokines and Th2-type cytokines but enhanced IL-10 production

To assess production of mediators in the lungs, BALF was collected and assayed by means of ELISAs. Compared to the PC group, there were significant and dose-dependent decreases in the production of chemokines (eotaxin and KC) and Th2 cytokines (IL-4, IL-5, and IL-13) in imperatorin-treated groups. Notably, imperatorin markedly increased IL-10 production in the lungs (Fig. 3). As expected, similar cytokine patterns were shown in culture

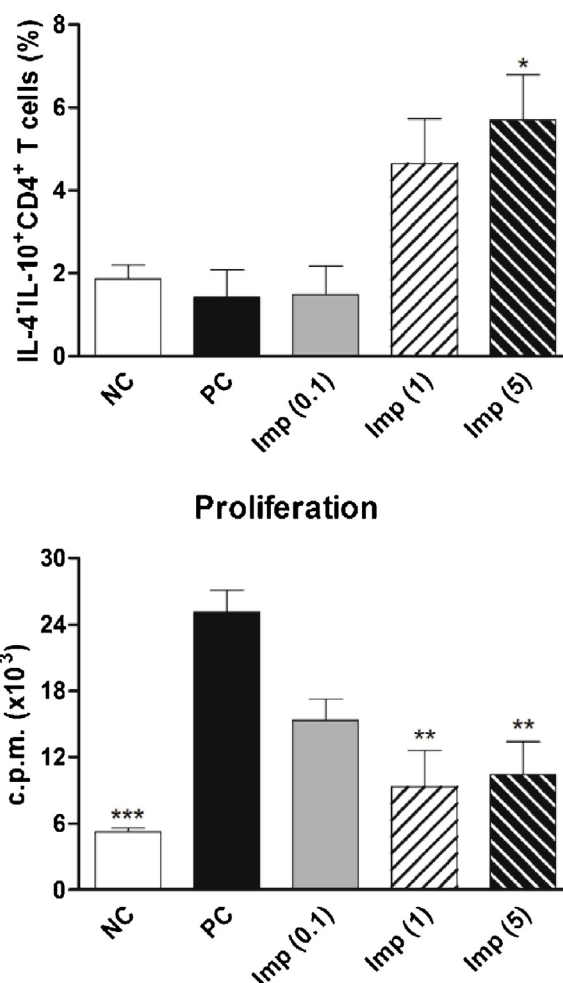


Fig. 4. Imperatorin treatment enhanced the generation of regulatory T cells and reduced OVA-specific T-cell proliferation. (A) The percentage of IL-10-producing Tregs. Splenocytes (10^6 cells/well) from imperatorin-treated and control groups were stimulated with 50 µg/ml OVA in 24-well plates. Cells were cultured for 5 days and restimulated by PMA and ionomycin for 6 h. Restimulated splenocytes were analyzed by flow cytometry. (B) OVA-specific T cell proliferation in allergic asthmatic mice. Splenocytes (3×10^5 cells/well) were cultured in 96-well plates and stimulated with 50 µg/mL OVA for 5 days. Then cells were pulsed with [³H]-thymidine for 16–18 h. Counts per minute (cpm) were measured with a β -counter. Results are expressed as the mean $\pm$ SEM of four to six mice per group. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 vs. the PC group.

supernatants of OVA-restimulated splenocytes in these mice (Fig. S1). These results demonstrated that imperatorin inhibited Th2 responses but enhanced IL-10 production in mice with OVA-induced asthma.

3.4. Imperatorin induced the generation of Tregs in vivo

As the Tr1 Treg cell performs its suppressive ability by producing IL-10, we further identified the possibility of imperatorin suppressing Th2 immune responses by inducing Tr1. Isolated spleen cells were cultured with OVA and restimulated with PMA/ionomycin before being analyzed by flow cytometry. Compared to the PC group, mice administered a high dose of imperatorin (Imp (5)) exhibited significant induction of IL-4⁺IL-10⁺-producing Tregs (Fig. 4A). In the T-cell proliferation assay, imperatorin-treated mice showed a markedly lower proliferative capacity of OVA-specific T cells than did PC mice (Fig. 4B). In conclusion, these data indicated that imperatorin suppressed OVA-specific T cell proliferation by inducing IL-10-producing Tregs in OVA-sensitized mice.

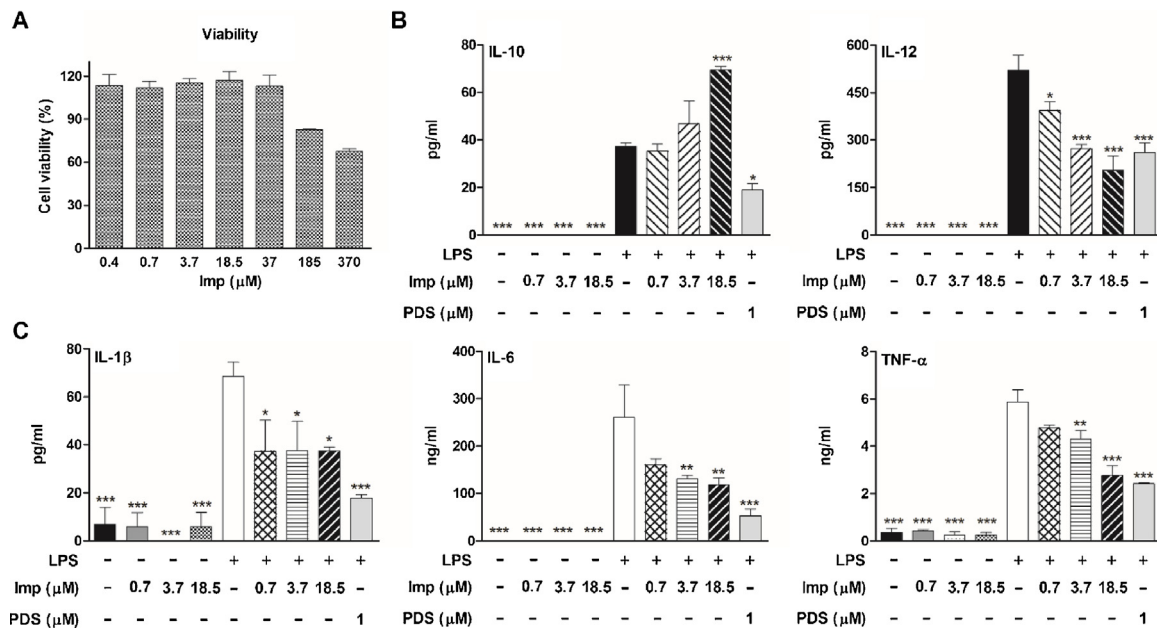


Fig. 5. Imperatorin pretreatment induced IL-10 secretion and reduced proinflammatory cytokines production in LPS-stimulated dendritic cells. (A) Cytotoxicity effect of imperatorin on DCs. On day 8 of culture, DCs were treated with different doses of imperatorin for 24 h. Cell viability was detected by an MTT assay. (B) Expression levels of IL-10, IL-12, and (C) proinflammatory cytokines (IL-1β, IL-6, and TNF-α) from imperatorin-pretreated DCs with or without LPS stimulation. On day 8 of culture, DCs were treated with various concentrations of imperatorin (0.7, 3.7, and 18.5 μM), 1 μM prednisolone (PDS), or medium alone for 20 min and then activated by LPS (100 ng/ml) for another 24 h. Supernatants were collected and analyzed by ELISAs. Results are expressed as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated DCs (LPS group).

3.5. Imperatorin enhanced IL-10 production in LPS-stimulated DCs

DCs play critical roles in determining T-cell differentiation in the face of allergen exposure. Mature DCs secrete IL-10, IL-12, and proinflammatory cytokines (IL-1β, IL-6, and TNF-α), and these cytokines are known to participate in T-cell differentiation [20]. Additionally, variations in expressions of the Notch ligands, PDL-1 and ICOSL, by mature DCs were also shown to be crucial for Treg cell differentiation [21–23]. Thus, we hypothesized that imperatorin might regulate the differentiation route of T cells by affecting the function of DCs. First, we evaluated the possible cytotoxic effects of imperatorin on BMDCs. After 72 h of treatment of DCs with different doses (0.4–370 μM) of imperatorin, cell viability was detected by an MTT assay (Fig. 5A). Because imperatorin showed no cytotoxicity at concentrations of up to 37 μM in DCs, we used up to 18.5 μM imperatorin for the following experiments. Next, we investigated the effect of imperatorin on cytokine production by DCs. DCs were treated with different concentrations of imperatorin (0.7, 3.7, and 18.5 μM) following LPS (100 ng/ml) stimulation. As shown in Fig. 5B, IL-10 production by DCs significantly increased in a dose-dependent manner. In contrast, IL-12 production was markedly decreased by imperatorin treatment (Fig. 5B). Similar data were also obtained when proinflammatory cytokines (IL-1β, IL-6, and TNF-α) were quantified (Fig. 5C). As a control agent, prednisolone caused a strong reduction in the production of IL-10, IL-12, and proinflammatory cytokines by DCs. These data indicated that imperatorin treatment could reduce the inflammatory response via IL-10 production by DCs.

3.6. Imperatorin enhanced ICOSL and Jagged1 surface marker expressions by LPS-stimulated DCs

We further analyzed the phenotype profiles of DCs. In particular, we examined whether imperatorin treatment had any effect on Notch ligand expressions. There are four identified heterodimeric

transmembrane Notch receptors (Notch 1, 2, 3, and 4) and their ligands (Delta-like 1, 3, and 4 and Jagged1 and –2) [24]. Several studies supported delta-like ligands promoting Th1 cell differentiation, whereas Jagged ligands enhance the development of Tregs [21,25]. Compared to LPS-activated DCs, imperatorin (18.5 μM) plus LPS-treated DCs showed no differences in MHC II, CD40, CD54, CD80, CD86, or PDL-1 expressions. However, imperatorin treatment induced an obviously higher level of ICOSL expression by DCs, compared to LPS-treated DCs (Fig. 6A). Furthermore, as shown in Fig. 6B, after treatment with imperatorin, Jagged1 expression by DCs significantly increased. Collectively, imperatorin-treated DCs expressed higher levels of ICOSL and Jagged1 surface molecules, which were correlated with the regulatory ability of DCs on Treg differentiation.

3.7. Imperatorin-treated DCs reduced T-cell proliferation and induced IL-10 production by T cells

Because imperatorin treatment promoted DCs to secrete IL-10 and express increased ICOSL and Jagged1 molecules, we predicted that imperatorin-treated DCs might induce CD4⁺ T cell differentiation to Tregs. To clarify the modulatory ability of imperatorin-treated DCs on T cells, LPS- and imperatorin-treated irradiated DCs were cocultured with allogeneic CD4⁺ T cells at various ratios. Imperatorin-treated DCs significantly reduced T-cell proliferation, compared to LPS-stimulated DCs (Fig. 7A). Furthermore, after 72 h of coculture, significantly decreased IFN-γ production and increased IL-10 production were found in these activated T cells (Fig. 7B). These results indicated that imperatorin might induce IL-10-producing Tregs through imperatorin-modulated DCs.

3.8. Imperatorin directly inhibited T-cell activation

We further addressed whether imperatorin directly affected T-cell responses in a DC-independent pathway. The cytotoxic effects

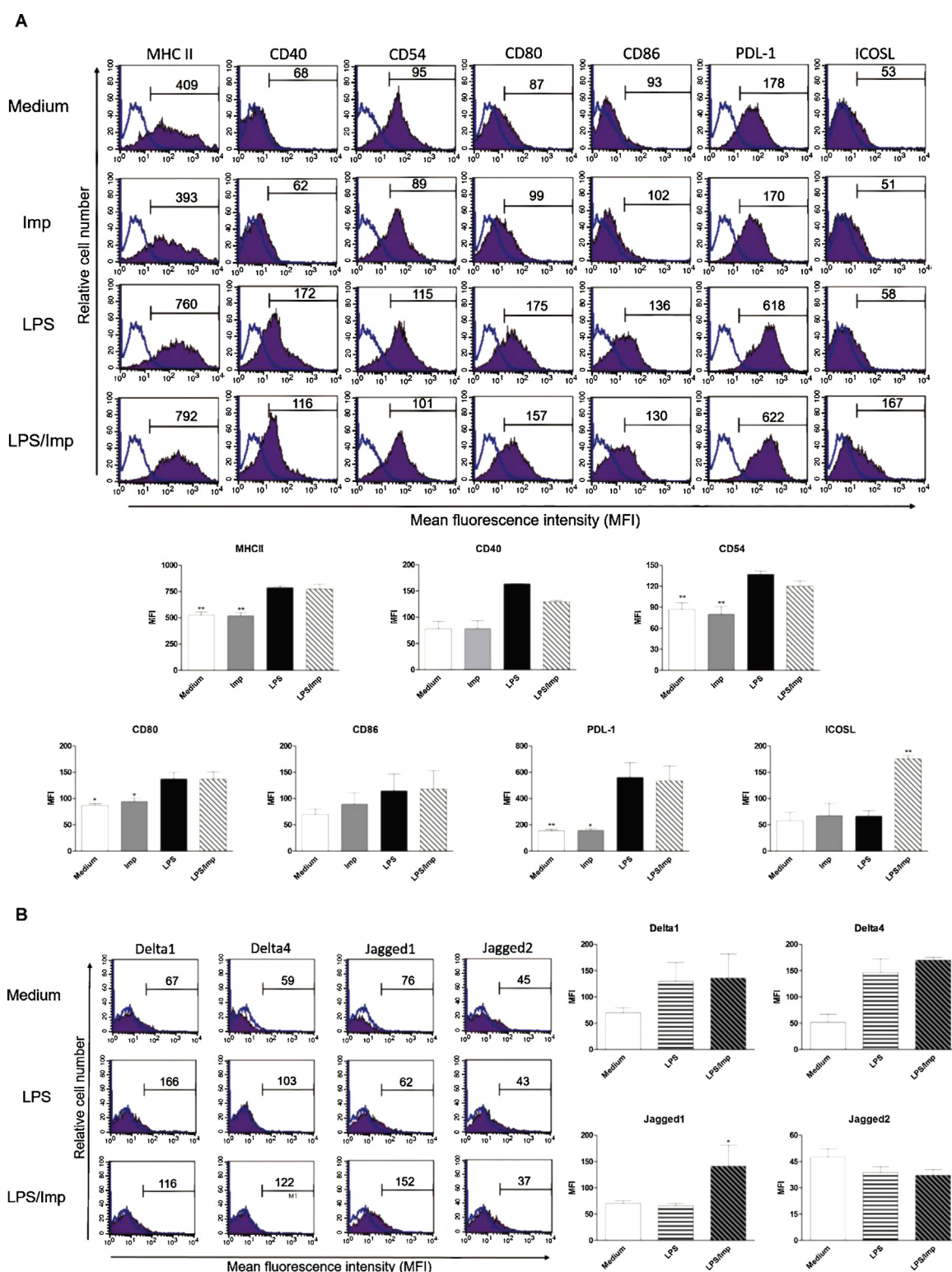


Fig. 6. Imperatorin pretreatment increased the expression of ICOSL and Jagged1 on LPS-stimulated dendritic cells. (A) Expression levels of MHC class II, CD40, CD54, CD80, CD86, PDL-1, and ICOSL on DCs, and (B) Notch ligands Delta1, Delta4, Jagged1, and Jagged2 on DCs. On day 6 of culture, DCs were treated with medium, imperatorin (18.5 μ M), LPS (100 ng/ml), or imperatorin plus LPS for 24 h. After incubation, cells were collected and analyzed by flow cytometry. Representative flow cytometric profiles of expressions of these surface molecules on the gated CD11c⁺ cells are shown. The mean fluorescence intensity (MFI) was calculated, and results are expressed as the mean $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, vs. LPS-treated DCs (LPS group).

of imperatorin on T cells were evaluated by an MTT assay. After treatment with 0.4–74 μ M imperatorin for 72 h, T cells showed no changes in cell viability (Fig. 8A). To analyze the direct effect of imperatorin on T cells, purified CD4⁺ T cells were pretreated with

different doses of imperatorin (0.7, 3.7, and 18.5 μ M) and then activated by anti-CD3/anti-CD28 antibodies. Compared to control T cells (with anti-CD3/anti-CD28 stimulation only), activated T cells cultured in the presence of imperatorin (18.5 μ M) or prednisolone

profoundly lost their proliferation capacity and released lower levels of cytokines (IL-2, IFN- γ , IL-5, IL-10, and IL-13) (Fig. 8B, C). We also confirmed that 18.5 μ M imperatorin did not induce apoptosis in T cells (Fig. S2). These results showed that imperatorin directly suppressed T cell activation; however, it did not drive the differentiation of CD4⁺ T cells into IL-10-producing Tregs.

4. Discussion

Allergic asthma is a chronic inflammatory disease of the airways, together with AHR and airway structural remodeling. There are many cytokines, chemokines, and proinflammatory mediators which can cause airway inflammation. Corticosteroids, such as prednisolone and dexamethasone, are nonspecific anti-inflammatory reagents and are used as the first-line treatment of asthma [26]. Undoubtedly, corticosteroids are the most effective class of drugs to inhibit inflammatory reactions in asthma. However, their side effects are the most frequently reported [27]. Therefore, we need to explore new therapeutic reagents that target pathways involved in asthma pathogenesis, and natural products belonging to traditional Chinese medicine represent a promising resource in this regard.

Imperatorin is the main bioactive compound of the root of *A. dahurica*. *Angelica dahurica* is used in traditional Chinese medicine to treat headaches, bleeding, neuralgia, and menstrual disorders [28]. This natural reagent played a critical role in controlling airway inflammation in an OVA-induced murine asthma model [29]. One report demonstrated that *A. dahurica* suppressed the development of atopic dermatitis-like skin lesions induced by *Dermatophagoides farinae* in NC/Nga mice [30]. Another study further demonstrated that pretreatment of mice with imperatorin significantly reduced IL-1 β , IL-6, and TNF- α production, and induced expression of IL-10 in an LPS-induced acute lung injury animal model [31]. Recently, imperatorin was shown to suppress degranulation of mast cells by inhibiting phospholipase C γ 1 (PLC γ 1) phosphorylation [13]. Overall, those results reveal that imperatorin possesses anti-inflammatory and antiallergic activities; therefore, there is the potential to use imperatorin as an immunomodulatory reagent to treat allergic airway inflammatory diseases.

In the current study, we are the first to identify the therapeutic effect of imperatorin in an asthmatic murine model. A previous study showed that imperatorin had low toxicity in terms of acute neurotoxic effects with a toxic dose of 310–349 mg/kg [12]. In our study, 0.5–5 mg/kg doses of imperatorin were used, and there were no adverse side effects on the outward appearance or organ weights of treated mice (Tables S1, S2). Our results clearly indicated that imperatorin treatment significantly reduced the production of OVA-specific IgE in serum, Th2 cytokine production in BALF and the spleen, and airway inflammation in OVA-sensitized and -challenged mice. Previous studies usually used prednisolone or dexamethasone as control reagents when applying this model. These drugs strongly reduced the number of inflammatory cells in BALF and decreased serum IgE production [32,33]. Other groups also showed that corticosteroids significantly reduced the development of AHR and Th2 cytokine production [34]. Compared to corticosteroid reagents, imperatorin showed a similar therapeutic efficacy, but it had no adverse effects in these treated mice.

Th2 cells mainly cause eosinophilic asthma by secreting Th2 cytokines, such as IL-4, IL-5, and IL-13. These cytokines can induce asthma syndromes including IgE production by B cells, the development of AHR, eosinophil infiltration in the lungs, and mucus secretion in the airways [35]. It is known that IL-4 induces the switch to IgE production by B cells, and such high levels of IgE can further induce mast cell activation [36]. IL-5 supports eosinophil development in the bone marrow [37]. IL-13 is able to reduce lung

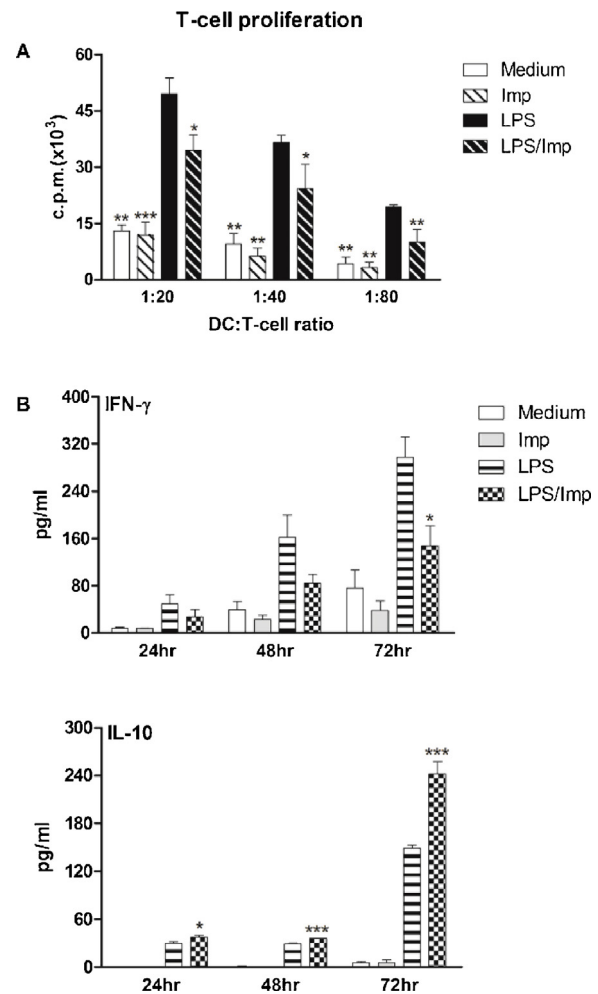


Fig. 7. Imperatorin-treated activated dendritic cells reduced T-cell proliferation but enhanced IL-10 production in an allogeneic mixed lymphocyte reaction. (A) The modulatory effect of imperatorin-treated DCs on T cell proliferation. DCs from BALB/c mice were pretreated with medium, imperatorin (18.5 μ M), LPS (100 ng/ml), or LPS plus imperatorin for 24 h. Naïve CD4⁺ T cells from C57BL/6 mice (2×10^5 cells/well) were then cocultured with γ -irradiated DCs at DC: T-cell ratios of 1:20, 1:40, and 1:80 in 96-well plates for 54 h. Cells were pulsed with [³H]-thymidine for another 18 h. Results are expressed as counts per minute (cpm). (B) The modulatory effect of imperatorin-treated DCs on T cell cytokine production. Naïve T cells were cocultured with DCs in 48-well plates at a DC: T ratio of 1:2.5. Supernatants were collected at 24, 48, and 72 h, and cytokine levels were analyzed by an ELISA. Results are expressed as the mean $\pm$ SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the LPS group.

function and induces AHR by directly acting on airway cells [38]. Thus, we speculated that imperatorin has an antiallergic effect on allergic asthma by reducing these Th2 cytokine secretions. Our results also showed that imperatorin treatment significantly down-regulated IFN- γ production and resulted in low OVA-specific IgG_{2a} production in serum. IFN- γ is produced by Th1 and natural killer cells, and it induces activated B cells to produce IgG_{2a} [39]. Furthermore, we observed that OVA-specific T-cell proliferation was suppressed in imperatorin-treated allergic asthmatic mice. Overall, these findings indicated that imperatorin dampened OVA-induced immune responses, thereby limiting the immunopathology.

We further identified whether the suppressive effect of imperatorin on immune responses is due to the induction of Tregs *in vivo*. Our results showed that IL-10 production was markedly up-regulated in the lungs and spleen of imperatorin-treated mice. In addition, the number of IL-4-IL10⁺ CD4⁺ T cells was particularly increased in these mice. IL-10 is an immunosuppressive cytokine. It can induce differentiation of Tr1 cells and inhibit activation of

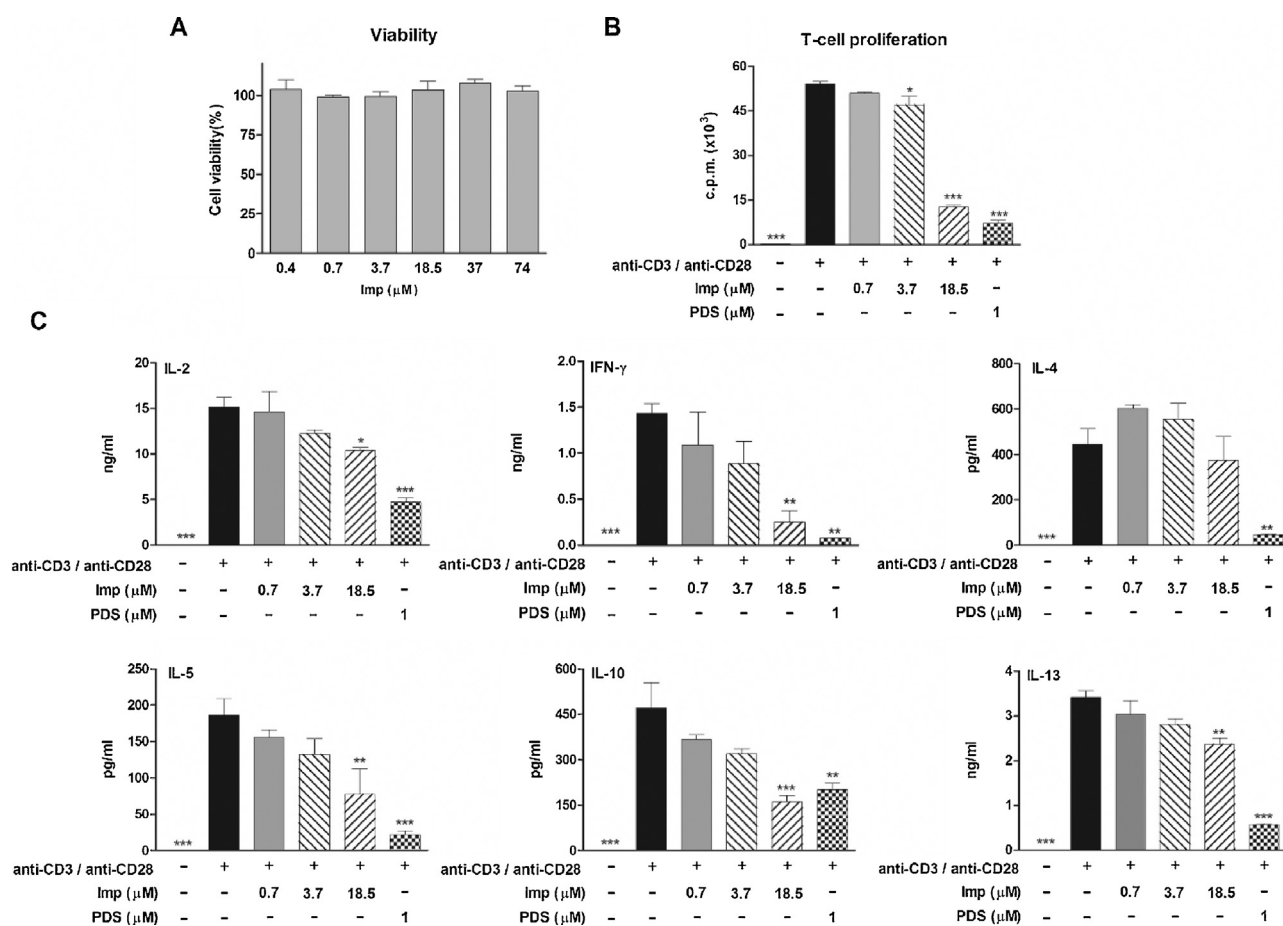


Fig. 8. Imperatorin directly reduced CD4⁺ T cell activation. (A) Cytotoxicity effect of imperatorin on T cells. T cells were treated with different doses of imperatorin for 72 h. Cell viability was detected with an MTT assay. The direct effect of imperatorin on T-cell (B) proliferation and (C) cytokine production. T cells (3×10^5 cells/well) were cultured in 96-well round-bottomed plates and pretreated with imperatorin (0.7, 3.7, and 18.5 μ M) or prednisolone (PDS, 1 μ M) for 20 min, then cultured with anti-CD3/anti-CD28 antibodies for another 72 h. Cells were then pulsed with 1 μ Ci/well of [^{3} H]-thymidine for 18 h. Counts per minute (cpm) were measured with a β -counter. Supernatants were collected at 72 h and analyzed by ELISAs. Results are expressed as the mean $\pm$ SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the anti-CD3/anti-CD28 group.

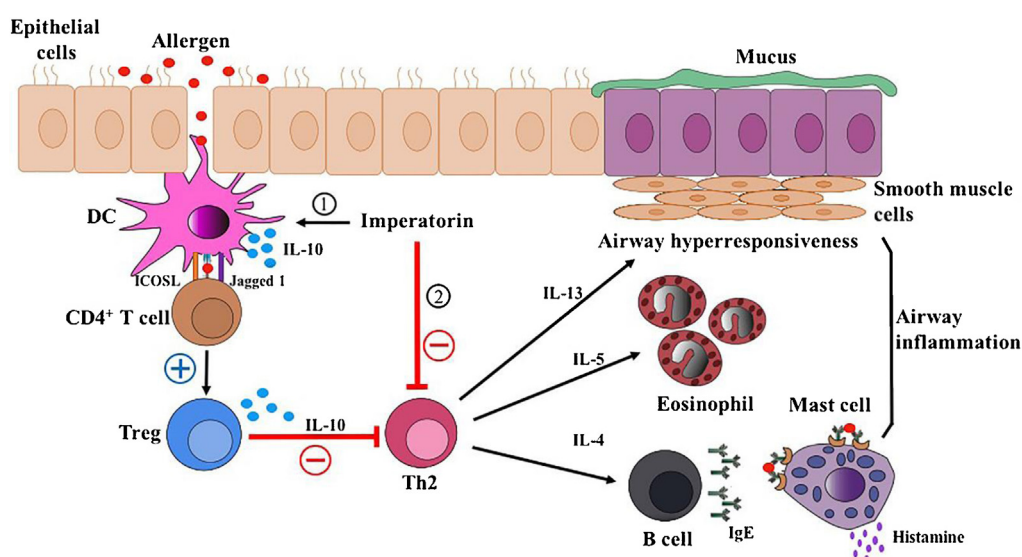


Fig. 9. Imperatorin attenuates Th2-mediated airway inflammation through modulation of DC function and direct inhibition T cell activation. There are two immunosuppressive mechanisms of imperatorin to dampen Th2-mediated airway inflammation. The first pathway is that imperatorin upregulates IL-10, Jagged1, and ICOSL expression by DCs. These modified DCs drive naïve CD4⁺ T cell differentiation into IL-10-producing Tr1 cells. Tr1 cells then suppress Th2 responses by IL-10. The second mechanism is to directly inhibit Th2 cell proliferation and cytokine production.

effector T cells, eosinophils, mast cells, and DCs [40]. Tr1 cells can secrete high levels of IL-10, and the immunosuppressive mechanism of Tr1 cells is IL-10-based [41,42]. A previous study showed that IL-10-secreting Tregs were induced to suppress the allergic immune response with specific immunotherapy of patients allergic to house dust mites [43]. Therefore, our observations suggest that orally feeding mice imperatorin promoted the development of IL-10-producing Tr1 cells to achieve the immunosuppressive function in this Th2-mediated allergic asthma model.

How imperatorin carries out its immunosuppressive role is not fully understood. One of the possibilities is that imperatorin treatment might influence DC function, thereby preventing excessive Th2 responses. DCs are key players effecting immune tolerance by cytokine production or cell-cell interactions with T cells [44]. Thus, we next identified whether imperatorin induced IL-10-producing Tregs by modifying the function of DCs. We found that pretreatment of DCs with imperatorin resulted in reduced production of proinflammatory cytokines (IL-6, TNF- α , and IL-12) but enhanced IL-10 production following LPS activation. In particular, imperatorin-treated DCs expressed high levels of ICOSL and Notch ligand Jagged1 molecules. Furthermore, when these DCs were further cocultured with naïve T cells, they possessed the ability to promote the generation of IL-10-producing T cells. It is well known that fully mature DCs secrete higher levels of proinflammatory cytokines (IL-12, TNF- α , IL-1 β , and IL-6). IL-12 and TNF- α drive Th1 cell differentiation [45], whereas IL-1 β and IL-6 induce Th17 cell differentiation [46]. Our results suggest that imperatorin-treated DCs did not promote Th1 or Th17 cell differentiation. However, it might drive naïve T cells to become IL-10-secreting Tr1 cells by high amounts of IL-10 from DCs. In addition to IL-10, ICOS-ICOSL signaling is critical for Tr1 induction. ICOS is a member of the CD28 family, and its expression is upregulated following T cell activation by an interaction with ICOSL on antigen-presenting cells [47]. DCs upregulate ICOSL upon maturation, which was shown to induce IL-10-producing Tregs [48]. Furthermore, different levels of Delta and Jagged protein expressed by DCs can be induced via different stimuli [49]. Some studies also showed that the Jagged1 ligand enhances the development and function of Tregs. Vigouroux et al. demonstrated that Jagged1-transduced human B cells induced a Tr1 cell population in an *in vitro* study [25]. Cahill et al. identified that Jagged1-expressing mesenchymal stromal cells provided protection against lung inflammation by enhancing Tregs and increasing levels of IL-10 [50]. In summary, based on the above, we predict that imperatorin-pretreated DCs induce IL-10-secreting Treg differentiation through a cytokine effect and/or cell-cell contact on T cells.

A second possibility for the inhibitory effects of imperatorin might be through direct induction of IL-10-producing Tregs. To clarify whether imperatorin can directly drive T cells to differentiate into IL-10-producing Tregs, we analyzed the effect of imperatorin on activated T cells. Our data showed that imperatorin inhibited activated T-cell proliferation and cytokine production *in vitro*. However, there was no effect on Treg induction. Imperatorin inhibited T cell proliferation through inhibiting the nuclear factor of activated T cell (NFAT) binding to the IL-2 promoter and transcriptional activities [51]. Alternatively, previous studies reported that imperatorin inhibited the mitogen-activated protein kinase (MAPK) pathway; therefore, it might inhibit IL-2 production by T cells [52,53]. Overall, we suggest that the therapeutic effect of imperatorin is in part through direct reduction of T cell activation.

In conclusion, imperatorin exerts an immunosuppressive effect in mice with OVA-induced asthma by two regulatory mechanisms (Fig. 9). First, imperatorin can modify DCs to Treg-polarizing antigen-presenting cells. These DCs produce the polarizing cytokine, IL-10, and express higher levels of surface markers (ICOSL and Jagged1) to induce Tr1 differentiation, and then inhibit Th2-

mediated allergic asthma. Second, imperatorin can reduce Th2 responses by directly inhibiting activated Th2 cell proliferation and cytokine production. In the future, imperatorin may have the potential for use as a therapeutic drug for asthma patients.

Acknowledgements

This study was supported by a research grant (MOST 103-2320-B-038-032-MY3) from the Ministry of Science and Technology, Taiwan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2016.04.030>.

References

- [1] B.N. Lambrecht, H. Hammad, The immunology of asthma, *Nat. Immunol.* 16 (2015) 45–56.
- [2] G. Nace, J. Evankovich, R. Eid, A. Tsung, Dendritic cells and damage-associated molecular patterns: endogenous danger signals linking innate and adaptive immunity, *J. Innate Immun.* 4 (2012) 6–15.
- [3] I. Mellman, R.M. Steinman, Dendritic cells: specialized and regulated antigen processing machines, *Cell* 106 (2001) 255–258.
- [4] B.N. Lambrecht, H. Hammad, The role of dendritic and epithelial cells as master regulators of allergic airway inflammation, *Lancet* 376 (2010) 835–843.
- [5] O. Palomares, G. Yaman, A.K. Azkur, T. Akkoc, M. Akdis, C.A. Akdis, Role of Treg in immune regulation of allergic diseases, *Eur. J. Immunol.* 40 (2010) 1232–1240.
- [6] R. Torres, A. Herreras, M. Serra-Pages, A. Marco, J. Plaza, C. Costa-Farre, et al., Locally administered prostaglandin E2 prevents aeroallergen-induced airway sensitization in mice through immunomodulatory mechanisms, *Pharmacol. Res.* 70 (2013) 50–59.
- [7] S. Metenou, T.B. Nutman, Regulatory T cell subsets in filarial infection and their function, *Front. Immunol.* 4 (2013) 305.
- [8] C.M. Lloyd, S. Saglani, T cells in asthma: influences of genetics, environment, and T-cell plasticity, *J. Allergy Clin. Immunol.* 131 (2013) 1267–1274.
- [9] J.Y. He, W. Zhang, L.C. He, Y.X. Cao, Imperatorin induces vasodilatation possibly via inhibiting voltage dependent calcium channel and receptor-mediated Ca²⁺ influx and release, *Eur. J. Pharmacol.* 573 (2007) 170–175.
- [10] S.D. Sarker, L. Nahar, Natural medicine: the genus *Angelica*, *Curr. Med. Chem.* 11 (2004) 1479–1500.
- [11] S. Sigurdsson, H.M. Ogmundsdottir, S. Gudbjarnason, Antiproliferative effect of *Angelica archangelica* fruits, *Z. Naturforsch. C* 59 (2004) 523–527.
- [12] R. Sancho, N. Marquez, M. Gomez-Gonzalo, M.A. Calzado, G. Bettoni, M.T. Coiras, et al., Imperatorin inhibits HIV-1 replication through an Sp1-dependent pathway, *J. Biol. Chem.* 279 (2004) 37349–37359.
- [13] G.J. Huang, J.S. Deng, J.C. Liao, W.C. Hou, S.Y. Wang, P.J. Sung, et al., Inducible nitric oxide synthase and cyclooxygenase-2 participate in anti-inflammatory activity of imperatorin from *Glehnia littoralis*, *J. Agric. Food Chem.* 60 (2012) 1673–1681.
- [14] W. Guo, J. Sun, L. Jiang, L. Duan, M. Huo, N. Chen, et al., Imperatorin attenuates LPS-induced inflammation by suppressing NF- κ B and MAPKs activation in RAW 264.7 macrophages, *Inflammation* 35 (2012) 1764–1772.
- [15] K. Choochuay, P. Chunhacha, V. Pongrakhananon, R. Luechapudiporn, P. Chanvorachote, Imperatorin sensitizes anoikis and inhibits anchorage-independent growth of lung cancer cells, *J. Nat. Med.* 67 (2013) 599–606.
- [16] K.T. Jeong, E. Lee, N.Y. Park, S.G. Kim, H.H. Park, J. Lee, et al., Imperatorin suppresses degranulation and eicosanoid generation in activated bone marrow-derived mast cells, *Biomol. Ther.* 23 (2015) 421–427.
- [17] C.C. Wang, L.G. Chen, L.L. Yang, Inducible nitric oxide synthase inhibitor of the Chinese herb *I. Saposchnikovia divaricata* (Turcz.) Schischk., *Cancer Lett.* 145 (1999) 151–157.
- [18] M. Ochel, H.W. Vohr, C. Pfeiffer, E. Gleichmann, IL-4 is required for the IgE and IgG1 increase and IgG1 autoantibody formation in mice treated with mercuric chloride, *J. Immunol.* 146 (1991) 3006–3011.
- [19] C.M. Snapper, C. Peschel, W.E. Paul, IFN- γ stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide, *J. Immunol.* 140 (1988) 2121–2127.
- [20] A. Schlitzer, F. Ginhoux, Organization of the mouse and human DC network, *Curr. Opin. Immunol.* 26 (2014) 90–99.
- [21] R. Bassil, W. Orent, W. Elyaman, Notch signaling and T-helper cells in EAE/MS, *Clin. Dev. Immunol.* 2013 (2013) 570731.

- [22] P. Hubert, N. Jacobs, J.H. Caberg, J. Boniver, P. Delvenne, The cross-talk between dendritic and regulatory T cells: good or evil, *J. Leukoc. Biol.* 82 (2007) 781–794.
- [23] A.E. Herman, G.J. Freeman, D. Mathis, C. Benoist, CD4⁺CD25⁺ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion, *J. Exp. Med.* 199 (2004) 1479–1489.
- [24] S. Artavanis-Tsakonas, K. Matsuno, M.E. Fortini, Notch signaling, *Science* 268 (1995) 225–232.
- [25] S. Vigouroux, E. Yvon, H.J. Wagner, E. Biagi, G. Dotti, U. Sili, et al., Induction of antigen-specific regulatory T cells following overexpression of a Notch ligand by human B lymphocytes, *J. Virol.* 77 (2003) 10872–10880.
- [26] R.K. Kumar, C. Herbert, P.S. Thomas, L. Wollin, R. Beume, M. Yang, et al., Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma, *J. Pharmacol. Exp. Ther.* 307 (2003) 349–355.
- [27] L. Bjermer, Evaluating combination therapies for asthma: pros, cons, and comparative benefits, *Ther. Adv. Respir. Dis.* 2 (2008) 149–161.
- [28] N.I. Baek, E.M. Ahn, H.Y. Kim, Y.D. Park, Furanocoumarins from the root of *Angelica dahurica*, *Arch. Pharm. Res.* 23 (2000) 467–470.
- [29] M.Y. Lee, C.S. Seo, J.A. Lee, N.H. Lee, J.H. Kim, H. Ha, et al., Anti-asthmatic effects of *Angelica dahurica* against ovalbumin-induced airway inflammation via upregulation of heme oxygenase-1, *Food Chem. Toxicol.* 49 (2011) 829–837.
- [30] H. Lee, J.K. Lee, H. Ha, M.Y. Lee, C.S. Seo, H.K. Shin, *Angelicae Dahuricae radix* inhibits dust mite extract-induced atopic dermatitis-like skin lesions in NC/Nga mice, *Evid. Based Complement. Altern. Med.* 2012 (2012) 743075.
- [31] J. Sun, G. Chi, L.W. Soromou, N. Chen, M. Guan, Q. Wu, et al., Preventive effect of Imperatorin on acute lung injury induced by lipopolysaccharide in mice, *Int. Immunopharmacol.* 14 (2012) 369–374.
- [32] M. Gurusamy, S. Nasser, H. Lee, B. Jung, D. Lee, G. Khang, et al., Kinin B1 receptor antagonist B1113823 reduces allergen-induced airway inflammation and mucus secretion in mice, *Pharmacol. Res.* 104 (2016) 132–139.
- [33] C.H. Hsu, C.M. Hu, K.H. Lu, S.F. Yang, C.H. Tsai, C.L. Ko, et al., Effect of selective cysteinyl leukotriene receptor antagonists on airway inflammation and matrix metalloproteinase expression in a mouse asthma model, *Pediatr. Neonatol.* 53 (2012) 235–244.
- [34] S.F. Ahmad, B. Khan, S. Bani, K.A. Suri, N.K. Satti, G.N. Qazi, Amelioration of adjuvant-induced arthritis by ursolic acid through altered Th1/Th2 cytokine production, *Pharmacol. Res.* 53 (2006) 233–240.
- [35] G. Pelaia, A. Vatrella, M.T. Busceti, L. Gallelli, C. Calabrese, R. Terracciano, et al., Cellular mechanisms underlying eosinophilic and neutrophilic airway inflammation in asthma, *Mediators Inflamm.* 2015 (2015) 879783.
- [36] M.T. Kasaian, K. Marquette, S. Fish, C. DeClercq, R. Agostinelli, T.A. Cook, et al., An IL-4/IL-13 dual antagonist reduces lung inflammation, airway hyperresponsiveness, and IgE production in mice, *Am. J. Respir. Cell Mol. Biol.* 49 (2013) 37–46.
- [37] A.H. Assa'ad, S.K. Gupta, M.H. Collins, M. Thomson, A.T. Heath, D.A. Smith, et al., An antibody against IL-5 reduces numbers of esophageal intraepithelial eosinophils in children with eosinophilic esophagitis, *Gastroenterology* 141 (2011) 1593–1604.
- [38] M. Crapster-Pregont, J. Yeo, R.L. Sanchez, D.A. Kuperman, Dendritic cells and alveolar macrophages mediate IL-13-induced airway inflammation and chemokine production, *J. Allergy Clin. Immunol.* 129 (2012) 1621–1627.
- [39] R. Haapakoski, P. Karisola, N. Fyhrquist, T. Savinko, S. Lehtimäki, H. Wolff, et al., Toll-like receptor activation during cutaneous allergen sensitization blocks development of asthma through IFN- γ -dependent mechanisms, *J. Invest. Dermatol.* 133 (2013) 964–972.
- [40] M. Saraiva, A. O'Garra, The regulation of IL-10 production by immune cells, *Nat. Rev. Immunol.* 10 (2010) 170–181.
- [41] A. Taylor, J. Verhagen, K. Blaser, M. Akdis, C.A. Akdis, Mechanisms of immune suppression by interleukin-10 and transforming growth factor- β : the role of T regulatory cells, *Immunology* 117 (2006) 433–442.
- [42] C.L. Maynard, C.T. Weaver, Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation, *Immunol. Rev.* 226 (2008) 219–233.
- [43] M. Jutel, M. Akdis, F. Budak, C. Aebischer-Casaulta, M. Wrzyszczyk, K. Blaser, et al., IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy, *Eur. J. Immunol.* 33 (2003) 1205–1214.
- [44] R. Kushwah, J. Hu, Role of dendritic cells in the induction of regulatory T cells, *Cell Biosci.* 1 (2011) 20.
- [45] S.E. Ross, R.O. Williams, L.J. Mason, C. Mauri, L. Marinova-Mutafchieva, A.M. Malfait, et al., Suppression of TNF- α expression, inhibition of Th1 activity, and amelioration of collagen-induced arthritis by rolipram, *J. Immunol.* 159 (1997) 6253–6259.
- [46] D.C. Newcomb, R.S. Peebles Jr., Th17-mediated inflammation in asthma, *Curr. Opin. Immunol.* 25 (2013) 755–760.
- [47] T.R. Simpson, S.A. Quezada, J.P. Allison, Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS), *Curr. Opin. Immunol.* 22 (2010) 326–332.
- [48] T. Ito, M. Yang, Y.H. Wang, R. Lande, J. Gregorio, O.A. Perng, et al., Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand, *J. Exp. Med.* 204 (2007) 105–115.
- [49] P. Cheng, D. Gabrilovich, Notch signaling in differentiation and function of dendritic cells, *Immunol. Res.* 41 (2008) 1–14.
- [50] E.F. Cahill, L.M. Tobin, F. Carty, B.P. Mahon, K. English, Jagged-1 is required for the expansion of CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells and tolerogenic dendritic cells by murine mesenchymal stromal cells, *Stem Cell Res. Ther.* 6 (2015) 19.
- [51] N. Marquez, R. Sancho, M. Ballero, P. Bremner, G. Appendino, B.L. Fiebich, et al., Imperatorin inhibits T-cell proliferation by targeting the transcription factor NFAT, *Planta Med.* 70 (2004) 1016–1021.
- [52] H.O. Pae, G.S. Oh, B.M. Choi, S.C. Chae, Y.M. Kim, K.R. Chung, et al., Carbon monoxide produced by heme oxygenase-1 suppresses T cell proliferation via inhibition of IL-2 production, *J. Immunol.* 172 (2004) 4744–4751.
- [53] Y. Cao, Y. Zhang, N. Wang, L. He, Antioxidant effect of imperatorin from *Angelica dahurica* in hypertension via inhibiting NADPH oxidase activation and MAPK pathway, *J. Am. Soc. Hypertens.* 8 (2014) 527–536.