



Acteoside exerts immunomodulatory effects on dendritic cells via aryl hydrocarbon receptor activation and ameliorates Th2-mediated allergic asthma by inducing Foxp3⁺ regulatory T cells

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ABSTRACT

Dendritic cells (DCs) are professional antigen-presenting cells that play a key role in directing T-cell responses and are involved in the pathogenesis of allergic asthma. Acteoside, an active phenylethanoid glycoside, is widely distributed in many medicinal plants. Herein, we explored the immunomodulatory effects of acteoside on bone marrow-derived DCs *in vitro*, and further investigated the immunosuppressive ability of acteoside to manipulate T helper type 2 (Th2)-mediated allergic asthma in mice. Following lipopolysaccharide activation, 50 μ M of acteoside significantly reduced the production of proinflammatory mediators, including interleukin (IL)-12 and tumor necrosis factor (TNF)- α , whereas it enhanced secretion of the anti-inflammatory cytokine, IL-10, by DCs. However, these effects of acteoside on DCs were reversed by pretreatment with CH223191, an aryl hydrocarbon receptor (AhR) antagonist. Additionally, coculture of acteoside-treated DCs with CD4⁺ T cells promoted the generation of forkhead box P3-positive (Foxp3⁺) regulatory T cells (Tregs) via AhR activation. Using a murine asthma model, our results demonstrated that oral administration of 50 mg/kg of acteoside decreased levels of Th2-type cytokines, such as IL-4, IL-5, and IL-13, whereas the level of IL-10 and the frequency of CD4⁺Foxp3⁺ Tregs were augmented. Moreover, acteoside treatment markedly inhibited the elevated serum level of ovalbumin-specific immunoglobulin E, attenuated the development of airway hyperresponsiveness, and reduced inflammatory cell counts in bronchoalveolar lavage fluid. Additionally, histological results revealed that acteoside ameliorated pulmonary inflammation in asthmatic mice. Taken together, these results indicated that acteoside exhibits immunomodulatory effects on DCs and plays an anti-inflammatory role in the treatment of allergic asthma.

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1. Introduction

Asthma is a public health problem worldwide, but particularly in the United States, Britain, Australia, and other developed countries. Allergic asthma is characterized by inflammation of the airways, bronchial hyperresponsiveness, an increased number of allergic-reactive type 2 helper T (Th2) cells, and infiltration by inflammatory cells [1–3]. The pathogenesis of allergic asthma is closely related to an abnormal Th2 immune response. Several mediators, such as interleukin (IL)-4, IL-5, and IL-13, secreted from effector CD4⁺ Th2 cells cause elevation of the immunoglobulin E (IgE) level, eosinophil infiltration, mucus overproduction, and the development of airway hyperresponsiveness (AHR), ultimately leading to an asthma attack. Therefore, drugs that can suppress Th2-type cytokine production in activated CD4⁺ Th cells would prove useful as immunotherapeutic agents for treating allergic asthma.

In addition to Th2 cells, dendritic cells (DCs) also play a pivotal role in the pathogenesis of allergic asthma [4]. DCs are professional antigen-presenting cells (APCs) that have an important role in determining T-cell differentiation after encountering an antigen. Following antigen uptake, DCs mature and present processed antigens to stimulate naïve T cells. Polarization of Th1/Th2 responses and promotion of regulatory T cell (Treg) differentiation by DCs are directed by expressions of various accessory molecules and cytokine secretions [5]. Finally, these DCs may mount a successful immune response against an antigen or induce tolerance. Instrumental cytokines, such as IL-10 and transforming growth factor (TGF)- β , are produced by DCs and are known to promote Treg differentiation. Although various T cells have the potential to mediate immunosuppression, forkhead box P3-positive (Foxp3⁺) Tregs, which express forkhead box P3, a transcription factor, appear to be a dominant cell type. Tregs are the main negative regulatory cells of the immune response, and many studies have demonstrated a tremendous therapeutic potential of Tregs in allergic diseases [6]. Conversely, breakdown of the Treg regulatory mechanism leads to elevated IgE levels and the development of airway inflammation [7,8]. Hence, inhibition of Th2 responses through induction of Tregs is considered a good immunotherapeutic strategy for treating allergic asthma.

Acteoside, a bioactive phenylethanoid glycoside, is widely distributed in many medicinal plants, including *Radix Rehmanniae*, *Plantago lanceolata*, and *Ligustrum purpurascens*, which are used to treat various disorders in traditional Oriental medicine [9–11]. Previous studies showed that acteoside exerts various pharmacological activities [12]. Recent studies revealed that acteoside has neuroprotective activity related to its antioxidative properties and antiapoptotic abilities [13,14]. In mouse peritoneal macrophages, acteoside exerts its anti-inflammatory action through inhibiting lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)- α , prostaglandin E2, and nitric oxide (NO) production [15]. Additionally, Jing *et al.* reported that acteoside exhibited anti-inflammatory effects on LPS-induced acute lung injury in mice via downregulating the nuclear factor (NF)- κ B pathway [16]. On the other hand, one study demonstrated that acteoside inhibited the release of inflammatory cytokines from IgE-sensitized and bovine serum albumin (BSA)-activated rat basophilic leukemia (RBL-2H3) cells [17]. Furthermore, the specific antiasthmatic impacts of acteoside on airway resistance and recruitment of eosinophils into the lungs in ovalbumin (OVA)-sensitized guinea pigs were investigated [18]. Despite those studies, little information is available regarding the immunomodulatory effects of acteoside on DC maturation and function.

DCs constitutively express the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that is involved in regulating immune responses [19]. In addition, acteoside was reported to be a natural AhR ligand and may be a potent immunomodulator. Therefore, in the current study, we were interested in addressing whether acteoside alters the activation and function of DCs through an AhR pathway to further modulate the polarization of CD4⁺ T cells. Thus, bone marrow-derived DCs (BMDCs) were used to clarify whether acteoside exerts an immunomodulatory effect through altering the ability of DCs to regulate Treg

differentiation. Furthermore, the therapeutic effects of acteoside treatment in a mouse model of OVA-induced asthma were investigated. This study of acteoside administration for allergic asthma provides a clear immunomodulatory mechanism and evidence for the use of acteoside as an agent to treat allergic diseases.

2. Materials and methods

2.1. Preparation of acteoside

Acteoside (purity $\geq$ 98% by high-performance liquid chromatography, ChemFaces, Hubei, China) was purified from the herb *Cistanche deserticola*. The structure of acteoside is shown in Fig. 1A. A stock solution (30 mM) was prepared by dissolving acteoside in 99.5% ethanol, and this was diluted by medium before use in cell culture experiments. In the animal model, mice were orally fed acteoside diluted with sterilized ddH₂O.

2.2. Mice

Five-week-old female BALB/c and C57BL/6 mice were obtained from the National Laboratory Animal Center and Laboratory Animal Center of National Taiwan University (Taipei, Taiwan). OT-II mice expressing a transgenic T-cell receptor that is specific for the peptide sequence 323–339 of OVA were kindly provided by Prof. Yungling Lee (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). Mice were maintained in the Animal Center of Taipei Medical University. Animal care and handling protocols were approved by the animal use committee of the College of Medicine, Taipei Medical University (approval no.: LAC-2021-0065).

2.3. Generation of BMDCs

Mouse BMDCs were generated using previously described methods [20]. In brief, bone marrow cells were collected from BALB/c mice and were grown in complete RPMI-1640 medium supplemented with 5% fetal bovine serum, recombinant murine IL-4 (500 U/ml; ThermoFisher Scientific, Rockford, IL, USA), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (500 U/ml; PeproTech, Rocky Hill, NJ, USA). On day 6 of culture, non-adherent immature DCs were harvested and used for further *in vitro* experiments.

2.4. Assay of cell viability

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, DCs (10^5 cells/well) were treated with various concentrations (0.1, 0.5, 1, 10, 50, and 100 μ M) of acteoside and cultured in 96-well plates for 24 h. Untreated DCs were cultured with medium as the control. Cells were then supplemented with 10 μ l of 5 mg/ml CCK8 reagent followed by another 2 h of incubation. The absorbance was determined at 450 nm with a microplate reader. The absorbance of untreated control cells was considered to represent 100% cell viability.

2.5. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from BMDCs with the TRIzol reagent (ThermoFisher Scientific). First-strand complementary (c) DNA was synthesized from total RNA using a high-capacity cDNA reverse-transcription (RT) kit (ThermoFisher Scientific). The resulting cDNA was further used for the real-time PCR. A qPCR was performed with a SensiFAST™ SYBR® No-ROX kit (Meridian Bioscience, Memphis, TN, USA) according to the manufacturer's instructions. Sequences of utilized primers were as follows: AhR-F (forward): 5'-TTC TTA GGC TCA GCG TCA GCT A-3' and AhR-R (reverse): 5'-GCA AAT CCT GCC AGT CTC TGA T-3'; CYP1A1-F: 5'-GTC CAT CGG AGA GAC CAT TG-3' and CYP1A1-R:

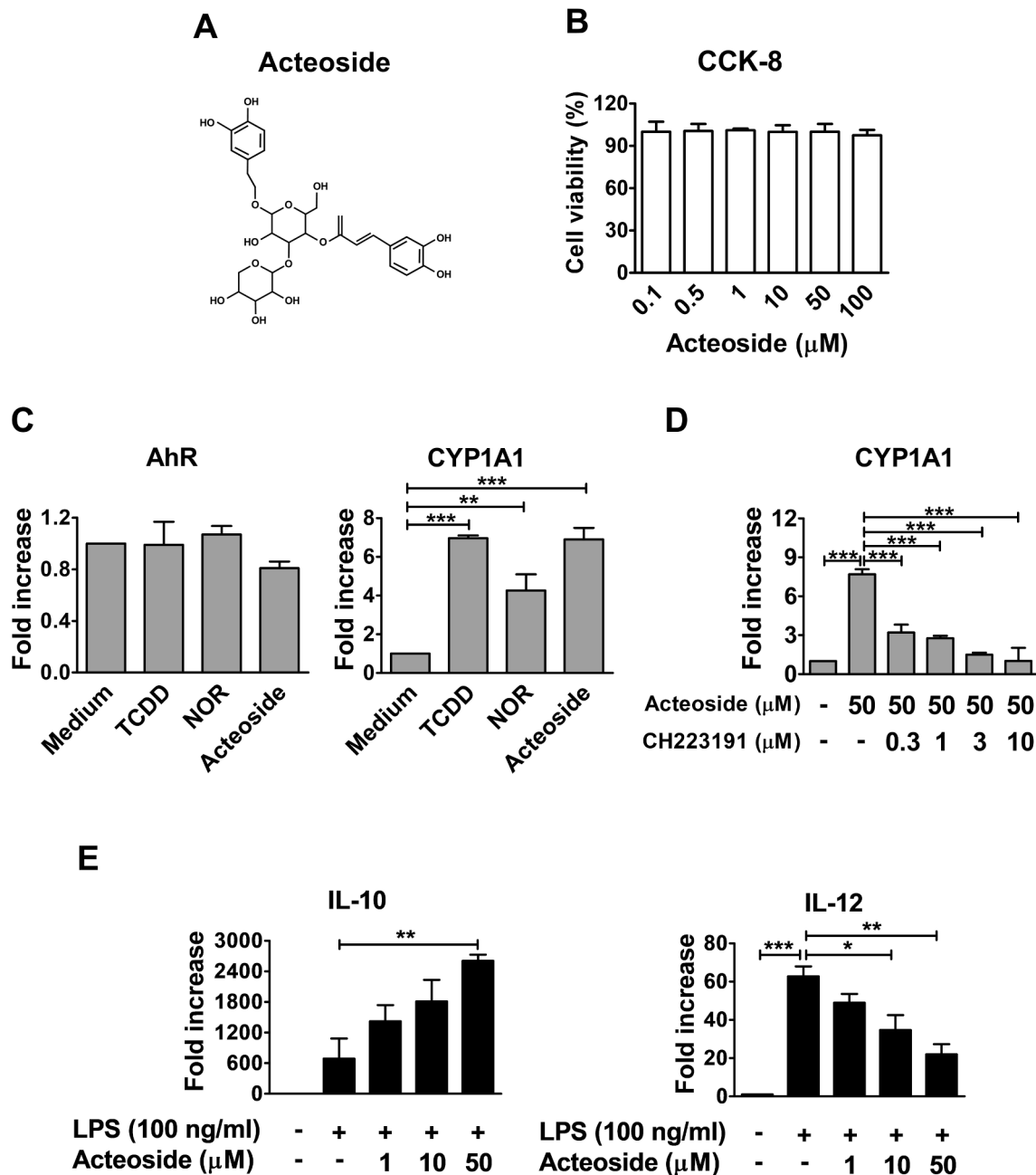


Fig. 1. Acteoside modulates immunoregulatory gene expressions in dendritic cells. (A) Chemical structure of acteoside. (B) Cytotoxicity effect of acteoside on DCs. On day 6 of culture, bone marrow-derived immature DCs were treated with different doses of acteoside for 24 h. Cell viability was detected by an CCK-8 assay. (C) Effects of acteoside on mRNA expression of AhR and CYP1A1 in DCs. DCs were treated with medium alone, TCDD, NOR, or acteoside for 6 h. Then these cells were collected and analyzed by an RT-qPCR method. (D) CH223191 inhibited AhR activation in acteoside-treated DCs. DCs were cultured with acteoside in the presence of various doses (0.3, 1, 3, and 10 μM) of CH223191. After incubation for 6 h, cells were collected, and expression levels of AhR-dependent CYP1A1 genes were evaluated by an RT-qPCR. (E) RT-qPCR analysis of IL-10 and IL-12 gene expressions in DCs. Relative expressions of the AhR, CYP1A1, IL-10, and IL-12 were compared to that of β -actin. Results are expressed as the mean $\pm$ SEM of three independent experiments ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when comparing between the denoted groups.

5'-GGT AGG AGT CAT ATC CAC CTT-3'; IL-10-F: 5'-ATG CAG GAC TTT AAG GGT TAC-3' and IL-10-R: 5'-CCT GAG GGT CTT CAG CTT C-3'; IL-12-F: 5'-ACA TGG TGA AGA CGG CCA G-3' and IL-12-R: 5'-GAA GTC TCT CTA GTA GCC AG-3'; and β -actin-F: CTA GGC ACC AGG GTG TGA TG-3' and β -actin-R: 5'-CTA CGT ACA TGG CTG GGG TG-3'. Data were analyzed using micPCR software (Bio Molecular Systems, Potts Point, Sydney, Australia). β -actin mRNA expression was used as an endogenous control, and resulting data were normalized to β -actin. The fold changes (FCs) were calculated using the $\Delta\Delta C_T$ method. The formulae are: $\Delta C_T = C_T$ (target gene) - C_T (β -actin); $\Delta\Delta C_T = \Delta C_T$ (experiment) - ΔC_T

(control); and $FC = 2^{-\Delta\Delta C_T}$.

2.6. Determination of mediator levels

Supernatants from treated cells were collected, and cytokine levels of IL-10, IL-12, tumor necrosis factor (TNF)- α , IL-6, and IL-1 β in the supernatants were determined with enzyme-linked immunosorbent assay (ELISA) kits. In the animal models, levels of IL-4, IL-5, IL-10, IL-13, and TGF- β in bronchoalveolar lavage fluid (BALF) or OVA-restimulated splenocytes were determined by ELISA kits. IL-10, IL-5, and IL-13 kits

were purchased from R&D Systems (Minneapolis, MN, USA), and IL-12, TNF- α , IL-6, IL-1 β , IL-4, and TGF- β kits were purchased from ThermoFisher Scientific.

2.7. Flow cytometric analysis

A flow cytometric analysis was performed with a FACSCalibur instrument (Becton Dickinson, Mountain View, CA, USA) and the CELL-QUEST PRO program. DCs (10^6 cells/well) were pretreated with or without acteoside for 1 h, followed by 100 ng/ml of LPS (Merck, Darmstadt, Germany) stimulation for another 24 h of incubation. Cells were harvested and stained with fluorochrome-conjugated antibodies for 30 min on ice. The following antibodies were used: anti-CD11c-fluorescein isothiocyanate (FITC), anti-I-A/I-E (major histocompatibility complex (MHC) class II)-phycoerythrin (PE), anti-CD80-PE, anti-CD86-PE, and PD-L2-PE antibodies from BioLegend (San Diego, CA, USA); and anti-CD40-PE, anti-CD54-PE, anti-PD-L1-PE, and anti-ICOSL-PE antibodies from ThermoFisher Scientific. For intracellular Foxp3 staining, T cells were first stained with anti-CD4-PE-cyanine5 (Cy5) and anti-CD25-PE antibodies. Then cells were treated with fixation and permeabilization buffers and incubated with anti-Foxp3-FITC on ice for 30 min. Anti-CD4-PE-Cy5 and anti-CD25-PE antibodies were purchased from BioLegend, and the anti-Foxp3-FITC antibody was purchased from ThermoFisher Scientific.

2.8. Coculture of BMDCs and CD4⁺ T cells

For the T-cell proliferation assay, 10^6 DCs from BALB/c mice were treated with LPS (100 ng/ml), LPS plus acteoside (1 and 50 μ M), or medium only for 24 h. Then DCs (5×10^4 cells/well) were cocultured with T cells at a ratio of 1:4 (DC:T cells) in 96-well plates. After 54 h of culture, cells were pulsed with [³H]-thymidine for another 18 h. Cells were harvested, and [³H]-thymidine incorporation was measured as counts per minute (cpm) with a β -counter. To evaluate Treg-cell polarization, 10^6 allogeneic CD4⁺ T cells from C57BL/6 mice were cocultured with 2.5×10^5 differently treated DCs from BALB/c mice for the mixed lymphocyte reaction (MLR). One group of DCs was treated with LPS plus acteoside (50 μ M) in the presence of 3 μ M CH223191 (Cayman Chemical, Ann Arbor, MI, USA), which is an AhR inhibitor to block AhR activation. For the antigen-specific T-cell response, treated DCs from B6 mice were co-cultured with CD4⁺ T cells from OT-II transgenic mice, and the DC:T-cell ratio was the same as that used in the MLR. After 5 days of culture, numbers of CD25⁺Foxp3⁺ Tregs were analyzed by flow cytometry.

2.9. Allergic asthma animal model and drug treatment

BALB/c mice were assigned to five groups (five mice/group) according to the following treatments: (1) negative control (NC) group injected with phosphate-buffered saline (PBS); (2) asthma group injected with OVA (Merck); (3) Pred group treated with 3 mg/kg body weight (BW)/day prednisolone (Pred) (Merck) as a reference drug for a positive control; (4) Act 25 group treated with 25 mg/kg BW/day acteoside; and (5) Act 50 group treated with 50 mg/kg BW/day acteoside. On day 1, mice were sensitized by an intraperitoneal (i.p.) injection of 100 μ g OVA allergen and 4 mg aluminum hydroxide (alum; ThermoFisher Scientific). Mice were boosted with 50 μ g OVA and the same dosage of alum on days 11 and 22. On days 28 and 29, mice were intranasally (i.n.) challenged with 100 μ g OVA. On days 30–32, mice were exposed to aerosolized 5% OVA in 0.9% NaCl daily for 30 min using a compressed-air nebulizer. On days 21–32, treatment groups were orally treated once daily with acteoside or prednisolone. For oral feeding, acteoside was diluted with sterilized ddH₂O. Control asthmatic mice were administered ddH₂O instead of acteoside. NC mice were neither sensitized with OVA nor administered acteoside treatment although they received an OVA challenge. On day 33, the AHR was measured in all mice, and then they were

sacrificed for subsequent experiments.

2.10. Detection of CD4⁺Foxp3⁺ Tregs and cell proliferation in the spleen

Spleens from each group of mice were collected and ground into single-cell suspensions. The population of CD4⁺Foxp3⁺ Tregs in splenocytes was measured by flow cytometry. In an *ex vivo* OVA-induced T cell proliferation assay, 3×10^5 splenocytes were seeded in 96-well plates and stimulated with OVA (50 μ g/ml). After 72 h of culture, cells were pulsed with [³H]-thymidine, and cpm values were determined with a dry scintillation counter.

2.11. Assay of the OVA-specific IgE antibody

Serum samples were collected from mice on day 33, and the level of OVA-specific IgE in serum was measured with an ELISA kit (Becton Dickinson Biosciences, San Jose, CA, USA) as described earlier [21]. All samples were compared to OVA-specific IgE standard serum. The titer of the OVA-specific IgE antibody was compared to standard serum derived by pooling sera from OVA-sensitized mice. The OD 405 nm reading of standard serum was arbitrarily assigned as 1 ELISA unit (EU). Results are expressed as a ratio of the value of a sample to the standard.

2.12. Determination of the AHR

AHR was assessed by treating mice with inhaled aerosolized methacholine (Merck) stimulation to induce airway constriction. Tracheostomized mice were anesthetized and placed on a ventilator (model 683; Harvard Apparatus, South Natick, MA, USA). After recording the stable baseline pulmonary resistance, increasing doses (1–16 mg/ml) of the methacholine aerosol were administered by nebulization, and pulmonary resistance was recorded for a 3-min period. Values of lung resistance (R_L) were computed (LABVIEW, National Instruments, Dallas, TX, USA) and used to analyze airway resistance. Data are expressed as a ratio of the relative increase in R_L after PBS nebulization.

2.13. Determination of airway inflammation and histological examination

After measuring the AHR in mice, BALF was collected to measure inflammatory mediators and cells as described in a previous study [22]. The collected BALF was analyzed using ELISAs. Total cell numbers in BALF were counted with a hemocytometer. Differential cell counts were distinguished on cytologic preparations. Cell slides were stained with Liu's stain (Chi I Pao, Taipei, Taiwan) after using a Thermo Shandon Cytospin 3 centrifuge (Marshall Scientific, Hampton, NH, USA). In total, 300 cells were counted under microscopy. Inflammatory cells were classified as macrophages, eosinophils, neutrophils, and lymphocytes, based on standard morphological criteria. After the BALF was obtained, the lungs were fixed with 10% formalin-PBS buffer. Lung sections were prepared and stained with a hematoxylin and eosin (H&E) solution to observe infiltrating inflammatory cells, and periodic acid Schiff (PAS) staining was used to evaluate mucus production. Images of stained slices were quantified by IMAGEJ software (National Institutes of Health, Bethesda, MD, USA).

2.14. Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical comparisons were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Acteoside enhances the immunoregulatory gene expression of cytochrome P450 family member 1 (CYP1A1) in DCs

The chemical structure of acteoside is shown in Fig. 1A. We assessed the cytotoxic effect of acteoside against DCs with an CCK-8 assay, and data showed that acteoside exerted no cytotoxicity toward DCs at concentrations of up to 100 μ M (Fig. 1B). Thus, acteoside concentrations of <50 μ M were used for subsequent experiments. Next, expression levels of the *AhR* and *AhR*-dependent *CYP1A1* genes were evaluated in acteoside-treated DCs by an RT-qPCR method. Because 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and norisoboldine (NOR) are *AhR* agonists, we used them in this experiment as positive controls. As shown in Fig. 1C, the *AhR* gene was constitutively expressed in DCs without *AhR* ligand stimulation. Similarly, expression levels of the *AhR* gene in DCs were not affected by *AhR* ligand treatment, such as by TCDD, NOR, or acteoside. In addition, we found that *AhR* activation in DCs caused by TCDD, NOR, and acteoside treatment significantly upregulated expression of its classic target gene, *CYP1A1*. On the other hand, the inhibitory effect of CH223191, an *AhR* antagonist, on *AhR* activation in acteoside-treated DCs was evaluated. As shown in Fig. 1D, CH223191 significantly prevented mRNA expressions of *CYP1A1*-dependent genes in dose-dependent manners. Results demonstrated that CH223191 administration efficiently blocked *AhR* activation in acteoside-treated DCs. Furthermore, we examined whether acteoside regulated expressions of the anti-inflammatory cytokine, IL-10, and proinflammatory cytokine, IL-12, in LPS-activated DCs. Of note, our data showed that acteoside stimulation markedly induced *IL-10* gene expression in LPS-activated DCs in a dose-dependent manner (Fig. 1E). Conversely, acteoside-treated DCs concentration-dependently expressed lower levels of the *IL-12* gene compared to LPS-stimulated DCs. Overall, these results indicated that acteoside treatment of DCs increased expressions of key immunoregulatory genes, and thus acteoside may possess the capacity to suppress inflammation.

3.2. Acteoside regulates cytokine production by DCs via activation of the *AhR* pathway

To identify whether acteoside-induced *AhR* activation alters the profile of DC cytokine production, DCs were treated with different concentrations of acteoside (1, 10, and 50 μ M) following LPS stimulation. In addition, CH223191, an *AhR* antagonist, was used to block *AhR* activation in cells. Protein levels of cytokines in the supernatants from these cultures were assayed by ELISA kits. LPS is well known as an inducer of DC activation and maturation, and we used LPS as a positive control. As expected, LPS-activated DCs displayed increased production of IL-10, IL-12, TNF- α , IL-6, and IL-1 β compared to unstimulated DCs (Fig. 2). In the presence of acteoside, dose-dependent increases in the levels of IL-10 were observed in LPS-activated DCs. In contrast, IL-12 production markedly decreased with acteoside treatment, and similar results were also obtained for the production of the proinflammatory cytokine, TNF- α , by DCs. Nevertheless, CH223191 treatment eliminated all of the above regulatory effects of acteoside on DCs. Taken together, these results demonstrated that acteoside exerts an anti-inflammatory effect on DC cytokine production by a mechanism that is partially dependent on the *AhR*.

3.3. No effect of acteoside on expression levels of surface molecules on DCs

We further analyzed whether acteoside treatment affected the phenotype profiles of DCs. As shown in Fig. 3, expressions of CD80, CD86, MHC II, CD40, and CD54 surface molecules during maturation and activation were upregulated in LPS-treated DCs compared to untreated DCs. Compared to LPS-activated DCs, various doses of acteoside treatment showed no differences in CD80, CD86, MHC II, CD40, or CD54 expressions. Moreover, because immunoregulatory molecules such as programmed cell death ligand 1 (PD-L1), PD-L2, and inducible co-stimulatory ligand (ICOSL) expressed by DCs may promote Foxp3⁺ Treg cell differentiation, we also evaluated whether acteoside treatment promoted expressions of immunomodulatory molecules by DCs. Compared to LPS-activated DCs, acteoside had no effect on expressions of PD-L1, PD-L2, or ICOSL by DCs. Collectively, our results indicated

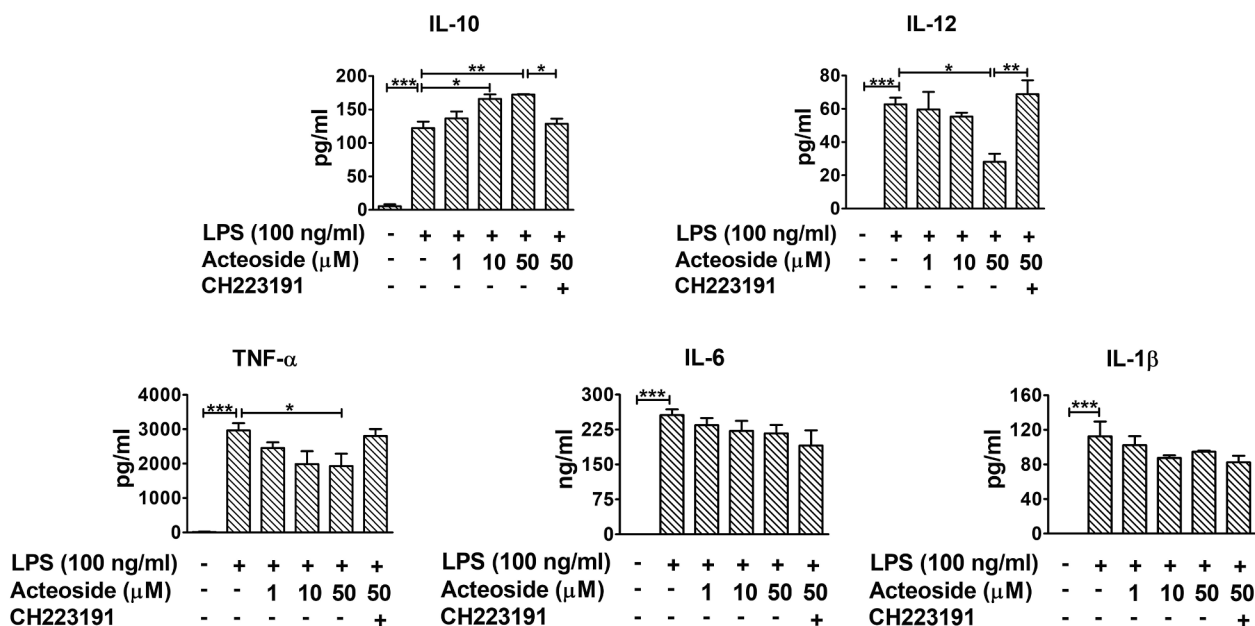


Fig. 2. Activation of the aryl hydrocarbon receptor alters cytokine production of dendritic cells. BMDCs were harvested on day 6 and stimulated for 24 h with medium, LPS (100 ng/ml), or LPS plus acteoside (1, 10, and 50 μ M). CH223191 at 3 μ M was used to block the *AhR* signaling pathway in DCs. Culture supernatants were collected, and IL-10, IL-12, TNF- α , IL-6, and IL-1 β levels were determined by ELISA kits. Results are expressed as the mean $\pm$ SEM of three independent experiments ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when comparing between denoted groups.

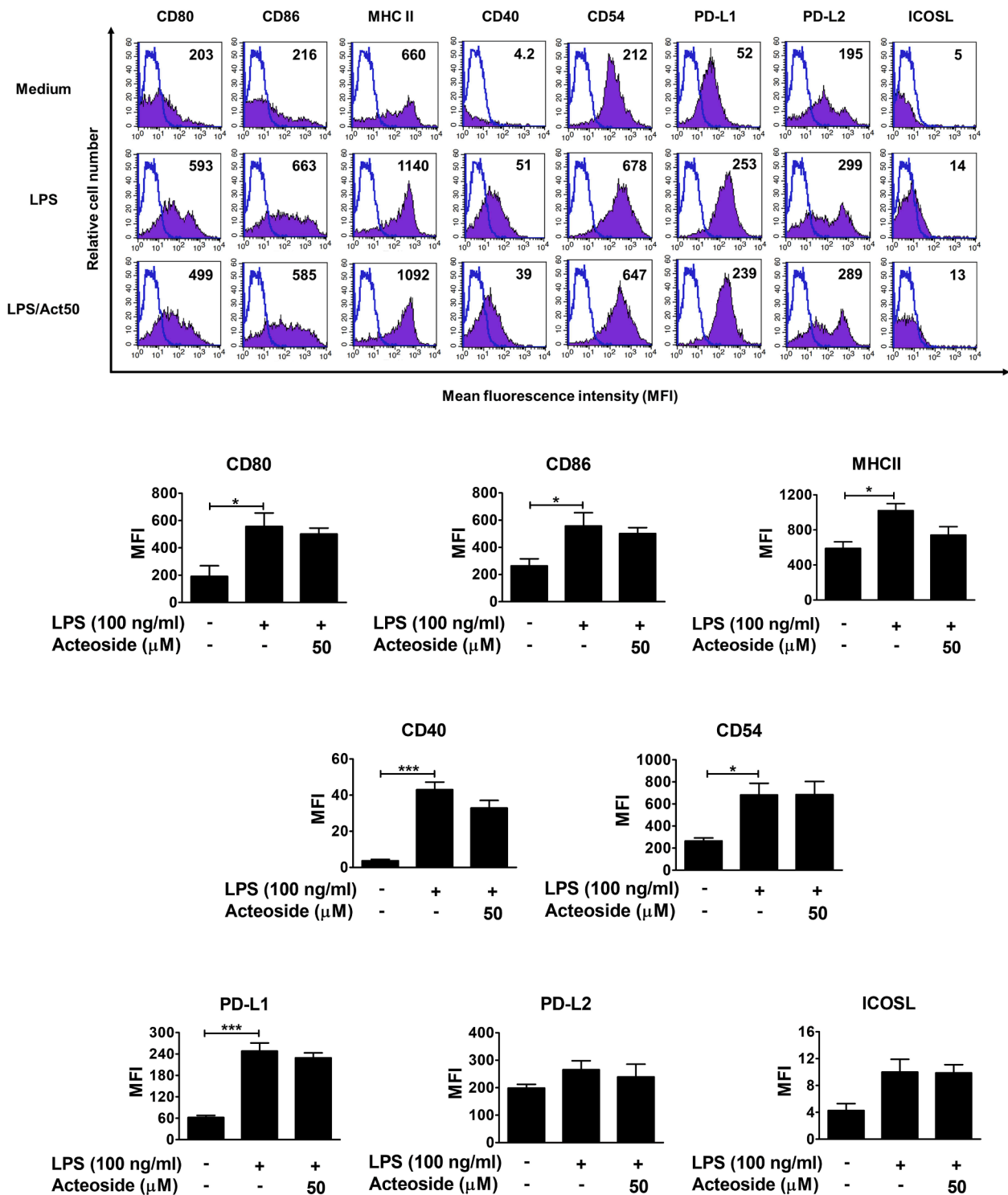


Fig. 3. Effect of acteoside on the phenotype pattern of dendritic cells. DCs were treated with medium, LPS, or acteoside (50 μM) plus LPS. Cells were collected and analyzed by flow cytometry. CD80, CD86, MHC II, CD40, CD54, PD-L1, PD-L2, and ICOSL expressions were characterized. Representative flow cytometric profiles of expressions of these surface molecules on gated CD11c⁺ cells are shown. The mean fluorescence intensity (MFI) was calculated. Results are expressed as the mean ± SEM of three independent experiments ($n = 2$). * $p < 0.05$, *** $p < 0.001$ vs. LPS-treated DCs (LPS group).

that acteoside treatment did not change surface marker expressions of DCs.

3.4. Acteoside promotes expansion of *Foxp3*⁺ Tregs by DCs through the AhR pathway

Since acteoside-treated DCs displayed higher expression of IL-10 and

might possess the capacity to induce CD4⁺ T cell differentiation into Tregs, we further explored the immunomodulatory effects of acteoside-treated DCs on T-cell stimulatory activity and Treg-cell differentiation by an allogeneic mixed lymphocyte reaction (MLR). Compared to LPS-stimulated DCs, results revealed that DCs treated with LPS plus acteoside showed a weak activation capacity for allogeneic T-cell proliferation (Fig. 4A). In addition, we found that treatment of DCs with LPS plus

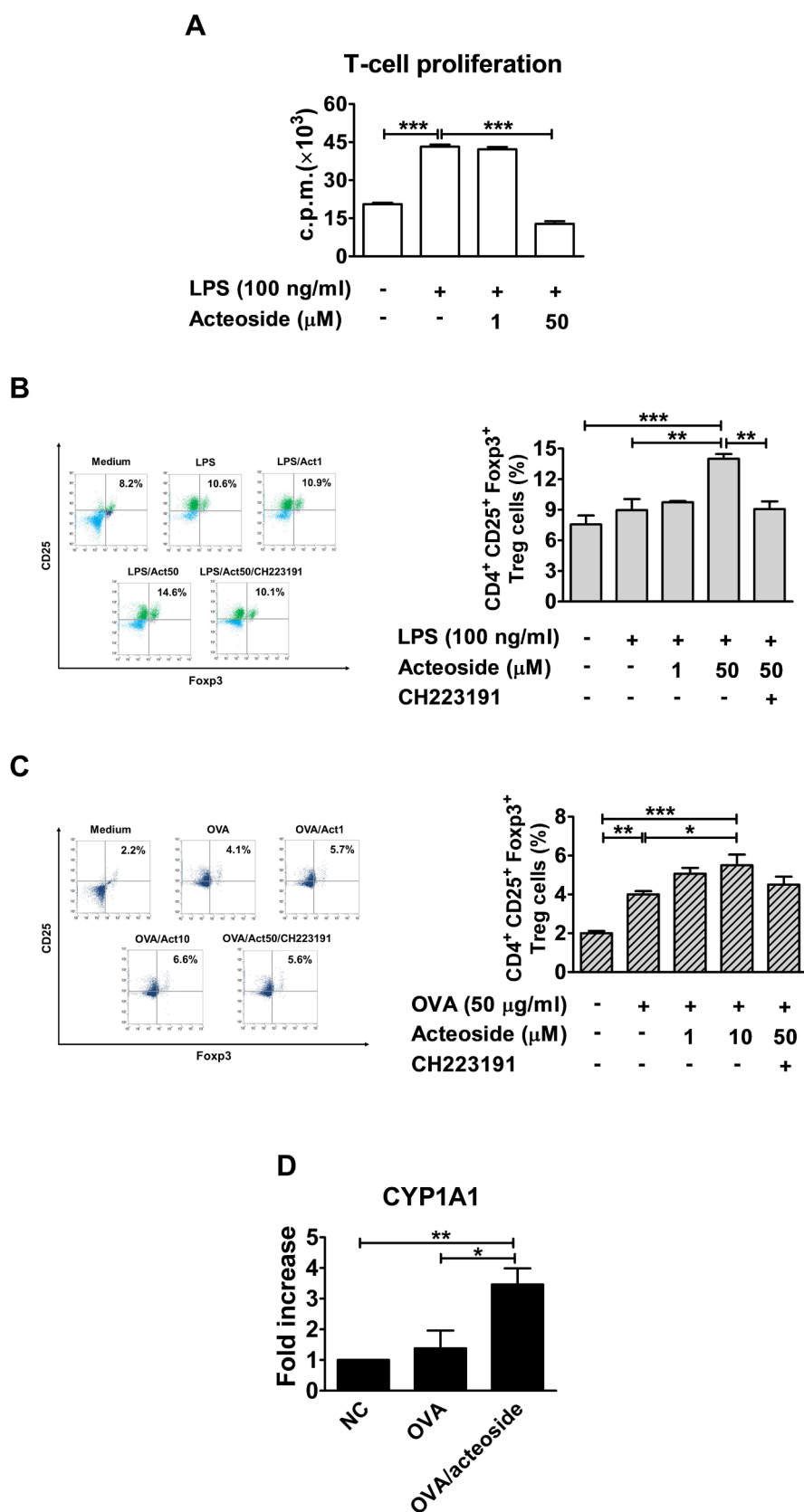


Fig. 4. Acteoside-treated dendritic cells enhance the frequency of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in an AhR-dependent manner. (A) Acteoside-treated DCs displayed low CD4⁺ T-cell stimulatory activity. In a mixed lymphocyte reaction, DCs from BALB/c mice were treated with medium alone, LPS, or LPS plus acteoside (1 and 50 μM) for 24 h. Naive CD4⁺ T cells from C57BL/6 mice were then cocultured with differently treated DCs at a DC:T-cell ratio of 1:4 for 72 h. T-cell proliferation was determined by [³H]-thymidine uptake, and results are expressed as counts per minute (cpm). (B) Acteoside-treated DCs promoted Foxp3⁺ Treg differentiation. In the mixed lymphocyte reaction (MLR), allogeneic CD4⁺ T cells were cocultured with DCs treated with medium alone, LPS, LPS plus acteoside, or LPS plus acteoside in the presence of CH223191 (an AhR antagonist). After 5 days of culture, CD4⁺ T cells were collected and further analyzed by flow cytometry. (C) For the OVA-specific CD4⁺ T-cell response, differently treated DCs from C57BL/6 mice were co-cultured with syngeneic OT-II CD4⁺ T cells. Representative dot plots show expressions of CD25 and Foxp3 by gated CD4⁺ T cells. Results are expressed as the mean $\pm$ SEM of three independent experiments ($n = 2$). (D) Acteoside treatment induced AhR activation in mice. On day 1, mice were sensitized with an injection of 50 μg/mouse ovalbumin (OVA) allergen. One group of OVA-sensitized mice were daily given acteoside (50 mg/kg body weight) by oral administration on days 1 to 4. Naive mice without OVA and acteoside treatment were the negative control group. Results are expressed as the mean $\pm$ SEM of five mice in each group. ** $p < 0.01$ and *** $p < 0.001$ when comparing between denoted groups.

acteoside markedly increased the frequency of Foxp3⁺ Tregs compared to LPS-stimulated DCs (Fig. 4B). Conversely, CH223191 treatment prevented the ability of acteoside-treated DCs to expand Tregs. Consistent with the MLR results, the OT-II CD4⁺ T-cell population driven by OVA and acteoside-pulsed DCs induced a higher number of Tregs than those driven by control DCs (Fig. 4C). On the other hand, AhR inhibitor treatment caused a reduced trend in the population of Foxp3⁺ Tregs. Collectively, these findings suggest that acteoside enhances the expansion of Foxp3⁺ Tregs by acteoside modulation of DCs via AhR activation. Further, we identified whether acteoside treatment induced AhR activation in mice. Thus, the mRNA expression of the *CYP1A1* gene in the lungs was detected by an RT-qPCR. As shown in Fig. 4D, the expression level of *CYP1A1* mRNA in naïve mice was low and slightly induced in OVA-sensitized mice. Of note, in the presence of acteoside treatment, these OVA-sensitized mice expressed a significantly higher level of *CYP1A1* mRNA. These data indicate that acteoside can induce AhR activation *in vivo*.

3.5. Acteoside suppresses activation of Th2 cells in mice with OVA-induced asthma

To evaluate whether acteoside exerts an immunomodulatory effect to alleviate the development of OVA-induced allergic asthma, two groups of OVA-sensitized mice (Act 25 and Act 50) received different doses of acteoside (25 and 50 mg/kg), respectively. Because Th2-type cytokines are responsible for orchestration of the allergic response, levels of IL-4, IL-5, and IL-13 were detected in BALF samples from these mice. As shown in Fig. 5A, the asthma group expressed higher amounts of IL-4, IL-5, and IL-13 in BALF than did negative control mice (NC group). Compared to the asthma group, levels of IL-4, IL-5, and IL-13 were reduced in acteoside-treated mice in dose-dependent manners. As a control agent, prednisolone caused strong suppression of the production of these Th2 cytokines. It is noteworthy that the level of the anti-inflammatory cytokine, IL-10, increased after acteoside administration. Similarly, supernatants from OVA-activated splenocytes also expressed lower levels of Th2-type cytokines and a higher level of IL-10 in acteoside-treated mice compared to the asthma group (Fig. 5B). In addition, we observed that acteoside-treated mice exhibited higher levels of TGF-β, an anti-inflammatory cytokine, in the spleen compared to the asthma group (Fig. 5B). Taken together, these results indicated that acteoside can suppress the Th2 immune response during the development of allergic asthma.

3.6. Acteoside enhances the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in mice with OVA-induced asthma

It was documented that Tregs play important roles in balancing Th1/Th2 cell differentiation and thereby inhibit over-responses of these effector T cells [6]. Therefore, we were interested in identifying whether the suppressive effect of acteoside on the Th2 response was involved in promoting Treg cell generation. Results revealed that percentages of CD4⁺CD25⁺Foxp3⁺ Tregs ranged 4.9%~7.9% in these asthmatic mice (Fig. 6A). Compared to the asthma group, prednisolone-treated mice expressed no difference in the Treg frequency. However, treatment with acteoside significantly promoted CD4⁺CD25⁺Foxp3⁺ Treg cell differentiation in a dose-dependent manner. Furthermore, splenocytes from these differently treated mice were restimulated with OVA, and the suppressive effect of acteoside in OVA-specific T-cell proliferation was determined. We found that acteoside (50 mg/kg) and prednisolone treatment significantly reduced the clonal expansion of OVA-specific CD4⁺ effector T cells (Fig. 6B). Overall, these results implied that acteoside may ameliorate asthma inflammation through inducing CD4⁺Foxp3⁺ Tregs to suppress undesirable effector Th2 responses.

3.7. Acteoside reduces anti-OVA IgE production and relieves AHR in mice with OVA-induced asthma

Because IgE is critical in the initiation of early asthmatic reactions, serum levels of OVA-specific IgE were measured after the last OVA challenge. The asthma group produced markedly increased levels of OVA-specific IgE compared to unsensitized negative control mice (Fig. 7A). Treatment of asthmatic mice with higher doses of acteoside and prednisolone resulted in significant reductions in OVA-specific IgE production. On the other hand, we investigated whether acteoside administration could attenuate the severity of AHR. As shown in Fig. 7B, R_L values generated as the result of methacholine stimulation increased in the asthma group compared to those of negative control mice. In contrast, treatment with acteoside obviously resulted in a dose-responsive reduction in inflammation-mediated AHR. As expected, prednisolone treatment also significantly decreased the severity of AHR.

3.8. Acteoside attenuates airway inflammation in mice with OVA-induced asthma

Eosinophils are one of the main inflammatory cells involved in airway inflammation. Therefore, the accumulation of inflammatory cells in the BALF of mice was explored. Proportions of macrophages, eosinophils, neutrophils, and lymphocytes significantly increased in BALF samples from asthmatic mice compared to negative control mice (Fig. 8A). When acteoside was administered, numbers of total cells, eosinophils, and lymphocytes dose-dependently decreased, and similar results were also shown in prednisolone-treated mice. Furthermore, to evaluate airway inflammation, lung sections were stained with an H&E solution. As shown in Fig. 8B, the degree of inflammatory cell infiltration was more serious in the asthma group than in the negative control group. However, administration of 50 mg/kg acteoside or prednisolone markedly attenuated the accumulation of inflammatory cells compared to that observed in the asthma group. Meanwhile, lung sections of asthmatic mice stained with PAS showed increased numbers of goblet cells and mucus hypersecretion (Fig. 8C). In contrast, higher doses of acteoside or prednisolone treatment led to marked reductions in abnormal OVA-induced goblet cell hyperplasia and mucus overproduction. Altogether, these results revealed that acteoside relieves airway inflammation, including pulmonary eosinophilia and mucus hypersecretion in the lumen of bronchioles.

4. Discussion

Acteoside is a well-known phenylethanoid glycoside, is available from different sources, and exerts a wide range of pharmacological activities. Recently, it was reported that acteoside from *Callicarpa kwangtungensis* alleviated TNF-α-induced damage in an acute lung injury cell model [23]. In neuroprotective studies, it was proven that acteoside isolated from *Syringa vulgaris* attenuated the inflammatory response following intracerebral hemorrhage by inhibiting NLR family pyrin-containing 3 (NLRP3) [24]. Using an experimental autoimmune encephalomyelitis model, Li et al. demonstrated that acteoside extracted from *Radix Rehmanniae* possessed neuroprotective effects through inhibiting peroxynitrite-mediated mitophagic activation [11]. In addition, acteoside from *Lindernia ruelliioides* was also reported to exhibit anti-hepatitis B virus activity and hepatoprotective effects *in vitro* [25]. In particular, several studies revealed that acteoside possesses direct immunoregulatory effects on immune cells. For example, one study demonstrated that acteoside from *Rehmannia glutinosa* directly suppressed Th22 cell proliferation *in vitro* and alleviated mesangial cell inflammatory injury in patients with IgA nephropathy [26]. Moreover, Song et al. reported that acteoside might promote Th1 immune responses by enhancing interferon (IFN)-γ secretion by mouse CD4⁺ T cells [27]. In addition, acteoside isolated from *Anisomeles indica* exhibited anti-inflammatory activity by inhibiting nitric oxide (NO), IL-12, and

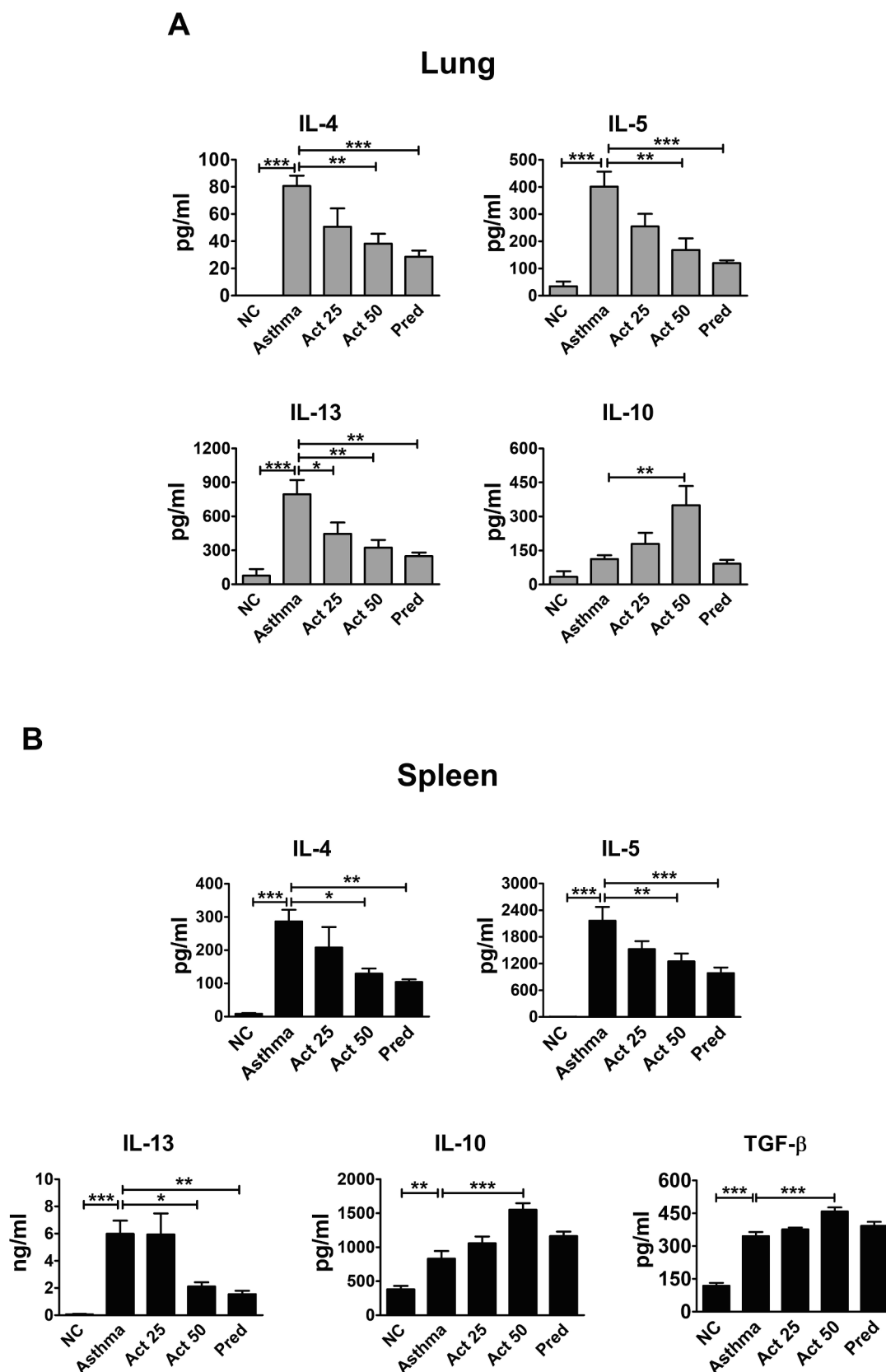


Fig. 5. Acteoside treatment diminishes Th2 responses in a mouse model of ovalbumin-induced asthma. (A) Effect of acteoside on levels of cytokines in BALF. Mice were sensitized and challenged with OVA. Different doses of acteoside (25 and 50 mg/kg) and prednisolone were administrated as described in "Methods". Levels of mediators, including Th2-type cytokines (IL-4, IL-5, and IL-13) and the anti-inflammatory cytokine, IL-10, in the BALF were measured by ELISA kits. (B) Effects of acteoside on the production of cytokines in the spleen. Splenocytes from acteoside-treated and control groups were collected and restimulated by incubation with OVA for 72 h. Supernatants from the cultures were harvested, and levels of cytokine production including TGF- β were determined by ELISA kits. Results are expressed as the mean $\pm$ SEM of five mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the asthma group.

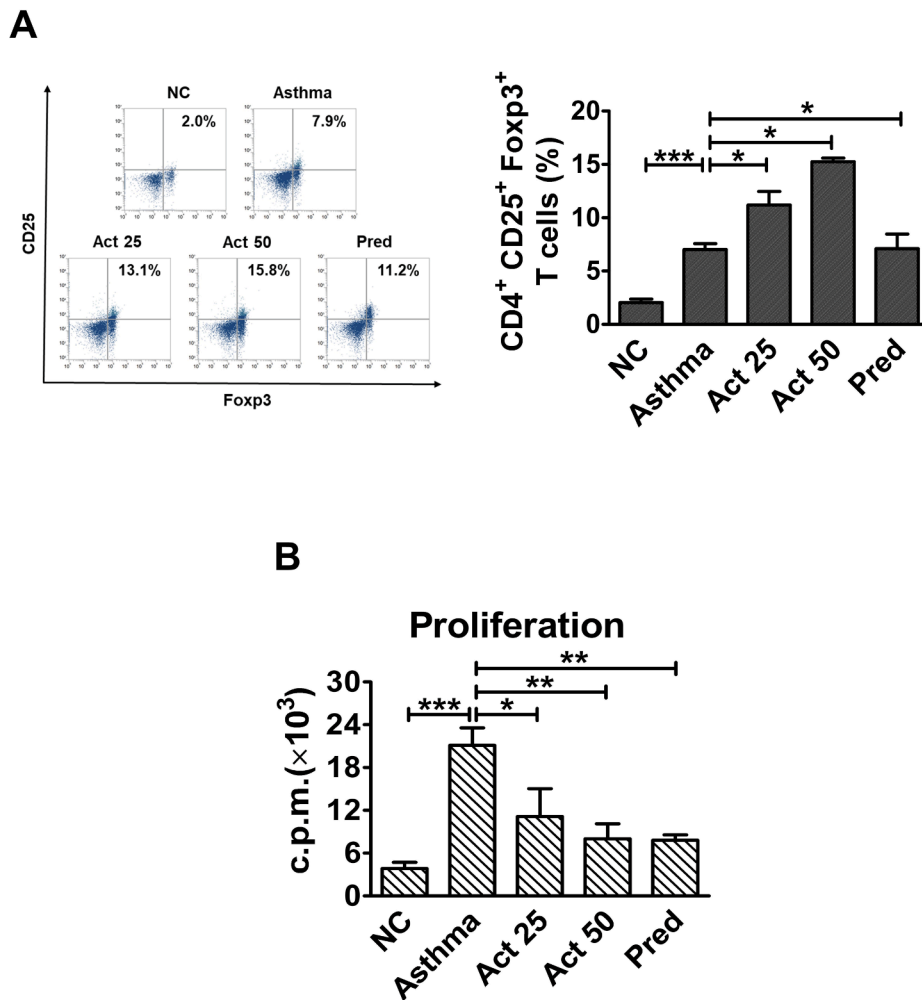


Fig. 6. Acteoside treatment promotes the generation of Foxp3⁺ regulatory T cells and dampens effector T-cell proliferation in mice with OVA-induced asthma. (A) Proportions of Foxp3⁺ Tregs in the spleen increased in acteoside-treated mice. Splenocytes from different experimental groups were collected and analyzed by flow cytometry. Representative dot plots show expressions of CD25 and Foxp3 by gated CD4⁺ T cells. Percentages of CD4⁺CD25⁺Foxp3⁺ Tregs are shown. (B) Effect of acteoside on the proliferative response of OVA-activated splenocytes. Splenocytes from different experimental groups were restimulated with OVA for 5 days. The proliferative response was assessed by a [³H]-thymidine assay. Values of the c.p.m. were determined with a β -counter. Results are expressed as the mean $\pm$ SEM of five mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the asthma group.

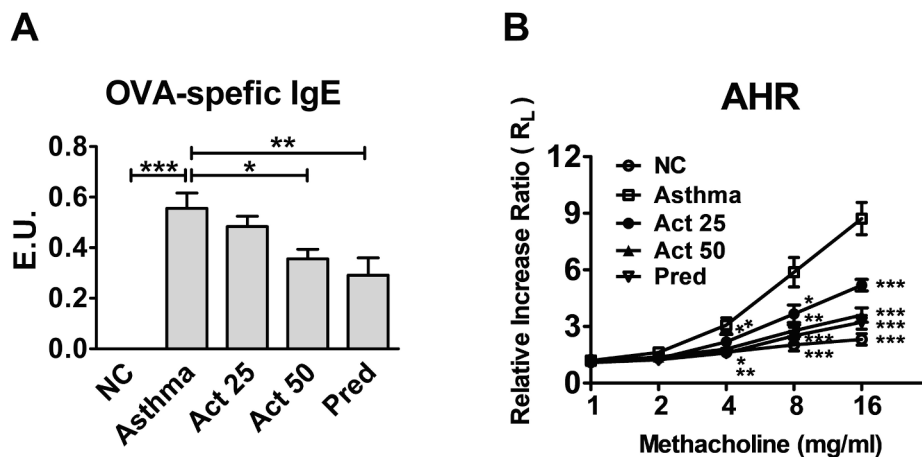


Fig. 7. Acteoside treatment reduces OVA-specific IgE production and relieves airway hyper-responsiveness in mice with OVA-induced asthma. (A) Effect of acteoside on levels of OVA-specific IgE in serum of mice. Blood from different experimental groups was collected after the last airway challenge. Levels of OVA-specific IgE in serum were measured by an ELISA. (B) Effect of acteoside on the development of AHR. After the last OVA challenge, the airway resistance of differently treated mice was measured in response to increasing doses (0–16 mg/ml) of aerosolized methacholine by invasive body plethysmography. Lung resistance (R_L) values were recorded at every stage. Results are expressed as the mean $\pm$ SEM of five mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the asthma group.

TNF- α production from LPS plus IFN- γ -stimulated macrophages [28]. Therefore, our study was designed to investigate whether acteoside from *Cistanche deserticola* exerts an immunomodulatory effect by regulating Treg differentiation and explored the protective effects of acteoside against an animal model of allergic asthma induced by OVA.

It is evident that DCs represent an attractive cell target that can be utilized to dampen aberrant immune responses in the context of allergies, autoimmunity, and chronic inflammation [29,30]. DCs are

professional APCs that capture antigens and then migrate to secondary lymphoid tissues and present the antigens to naïve CD4⁺ T cells. Depending on the cytokine expression pattern and costimulatory molecules by DCs, DCs prime CD4⁺ T cell activation and drive the differentiation into distinct T subsets, such as Th1, Th2, Th17, and Tregs. These DCs influence whether the response to an inhaled antigen entails the induction of allergic inflammation or tolerance to the antigen. Among cytokines produced by DCs, IL-10 is an important anti-

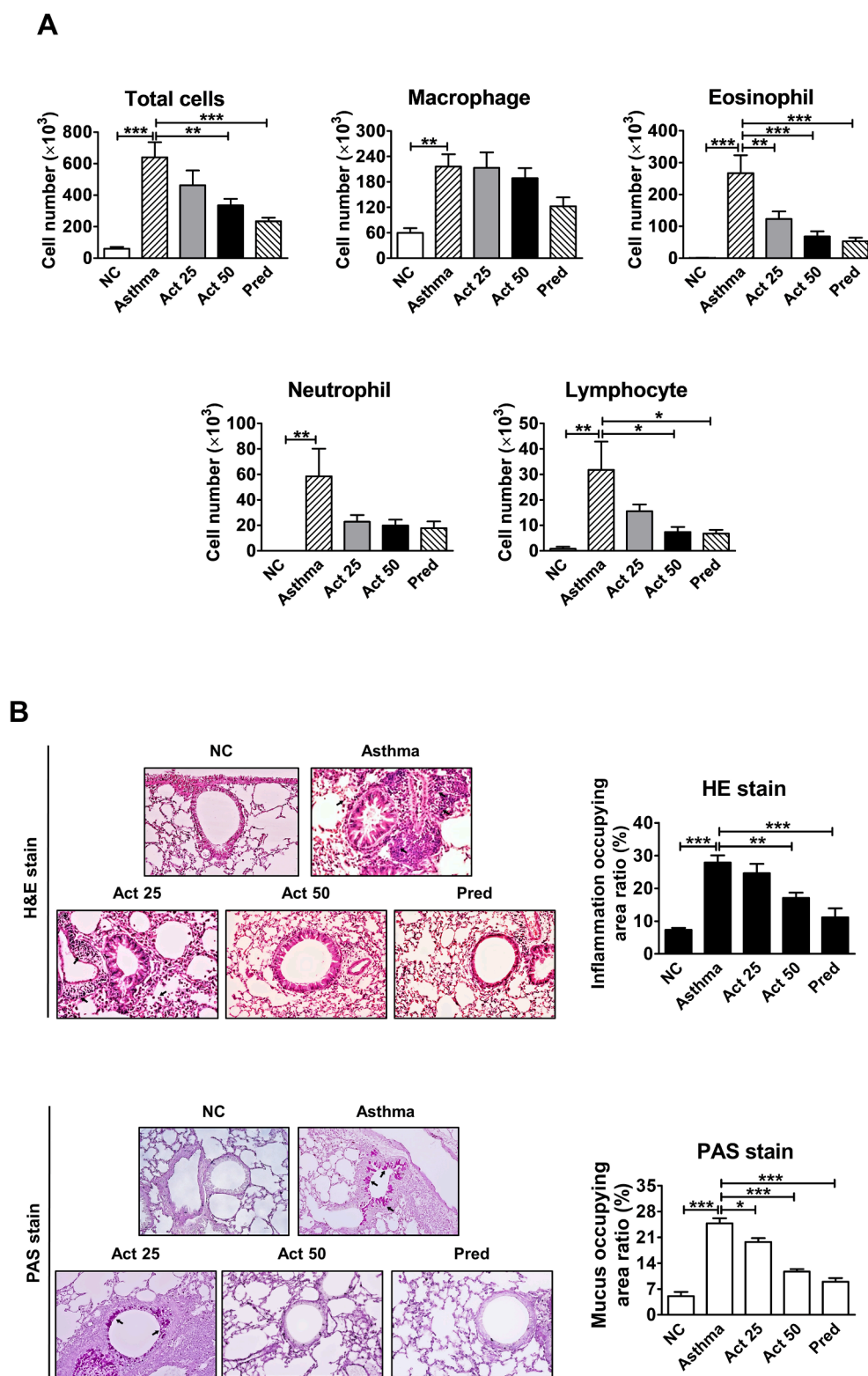


Fig. 8. Acteoside treatment alleviated airway inflammation in mice with OVA-induced asthma. (A) Effect of acteoside on the movement of inflammatory cells into BALF. Cells from the BALF were isolated by centrifugation and stained with Liu's dye. Numbers of differential leukocytes, including macrophages, eosinophils, neutrophils, and lymphocytes, were calculated. (B) The degree of inflammatory cell influx into lung tissues was confirmed by H&E staining (magnification 200 $\times$). Black arrows indicate inflammation. Inflammatory changes are presented as percentages of the inflamed area by computer-based quantification. (C) Mucus plug accumulation in lung tissues was examined by PAS staining (magnification 200 $\times$). Black arrows indicate a mucus plug occupying the central lumen of the bronchioles. The degree of mucus production is presented as percentages of PAS-positive areas. Results are expressed as the mean $\pm$ SEM of five mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the asthma group.

inflammatory cytokine that diminishes excessive T-cell immune responses to prevent chronic inflammation. In the present study, acteoside-treated DCs produced enhanced levels of IL-10 compared to control DCs and possessed the ability to expand the number of CD4⁺CD25⁺Foxp3⁺ Tregs *in vitro*. Evidence from several previous studies showed that certain agents or bacteria can induce IL-10-producing DCs that exhibit an immunoregulatory function. For

example, dexamethasone-treated DCs, which maintained production of a large amount of IL-10 and had lower activity in stimulating T-cell proliferation, are potentially useful for inducing immune tolerance [31]. In addition, Larsen et al. reported that DCs matured in the presence of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) showed enhanced IL-10, and these DCs primed naive T cells to develop into IL-10-producing T cells [32]. Consistent with previous reports, we also

found that acteoside dose-dependently reduced IL-12 production by DCs. IL-12 is a proinflammatory cytokine that induces IFN- γ production, favors the differentiation of Th1 cells, and is recognized as being involved in driving autoimmunity and inflammatory diseases. One study reported that acteoside displayed significant reductions in IL-12 and INF- γ in LPS-activated microglial murine (N9) cells [33]. In addition, TJN-419, a synthetic compound derived from acteoside, dose-dependently inhibited IL-12 production in LPS-stimulated macrophages [34]. Therefore, based on the above-described data, it may be possible to use acteoside-treated DCs as a therapeutic tool to improve treatment of inflammatory diseases in the future.

The AhR is a ligand-activated transcription factor activated by a broad array of natural and synthetic agonists and was shown to be involved in regulating inflammatory responses and inducing tolerance by modulating immune cells [19,35]. In patients with severe asthma, decreased expression of the AhR gene by bronchial epithelial cells was recently reported by Lee et al. [36]. Moreover, a deficiency in the AhR led to a worsening of allergic asthma symptoms in a murine asthma model, and such evidence indicates that the AhR plays an important role in maintaining normal lung function and mediating disease severity. Acteoside, a natural AhR ligand, was proposed to mediate anti-inflammatory properties by modulating DCs. However, the mode of action of acteoside treatment on DCs is still unclear. Therefore, we clarified the potential immunomodulatory effects of the AhR ligand, acteoside, regarding the phenotype and function of mouse DCs. Herein, we found that IL-10 is a critical target of acteoside-treated DCs. In particular, we showed that acteoside upregulated IL-10 production in LPS-matured DCs, which was reversed by pretreatment with CH223191, an AhR antagonist. Additionally, blocking AhR activation in DCs resulted in losing the ability to promote Treg expansion. This indicates that AhR signaling may affect the differentiation of Tregs involved in acteoside-treated DCs. Indeed, one report showed that 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), an AhR ligand, acted on DCs to promote Foxp3⁺ Treg differentiation in a retinoic acid-dependent manner [37]. In another report, BMDCs treated with the dietary AhR ligands, indole-3-carbinol (I3C) and indirubin-3'-oxime (IO), increased the frequency of Foxp3⁺ Tregs when cultured with naïve T cells [38]. Collectively, those studies indicated that AhR signaling in DCs may affect Treg differentiation and favor an anti-inflammatory role for immune responses.

TCDD, a synthetic AhR ligand, released from industrial processes is a chemical contaminant and has long been known to cause immunotoxicity. Although TCDD was reported to possess an antiallergic effect [39], natural AhR ligands including acteoside were suggested to be safer therapeutic agents than synthetic AhR ligands for treating inflammatory and immune diseases. In this study, we proposed that acteoside may be a potentially safe and effective therapeutic treatment for allergic asthma. Therefore, the effects of different doses of acteoside on an OVA-induced asthmatic model were investigated. In the current study, our results showed that administration of 50 mg/kg acteoside inhibited IL-4, IL-5, and IL-13 production in mice with OVA-induced asthma. Allergic asthma is considered a Th2 cell-associated inflammatory disease, and Th2 type cytokines, such as IL-4, IL-5, and IL-13, are key molecules involved in the pathology of allergic asthma. Therefore, these Th2 cytokines have become major targets for asthma therapies. Elevated levels of serum IgE were correlated with the severity of asthma, and reducing IL-4 may decrease IgE production by suppressing the class-switching process in B cells [40]. Further, a decrease in IL-4 production may reduce eosinophil recruitment to the lungs by reducing adhesion of circulating eosinophils to endothelial cells [41]. Apart from IL-4, another pathway which is involved in eosinophilic airway inflammation is orchestrated by IL-5. IL-5 can promote the maturation, survival, and recruitment of eosinophils [42]. Therefore, we speculated that acteoside inhibits the recruitment of eosinophils to the lungs by inhibiting IL-4 and IL-5 secretion. Moreover, it was reported that IL-4 and IL-13 play roles in mucus hypersecretion and the development of AHR [43,44]. We found

that acteoside treatment significantly alleviated OVA-induced AHR in response to inhaled methacholine. Thus, the results indicate that acteoside plays a key role in preventing the development of AHR by inhibiting IL-4- and IL-13-dependent pathways.

In acteoside-treated mice, levels of the anti-inflammatory cytokine, IL-10, in the lungs and spleen significantly increased. In addition, the frequency of CD4⁺CD25⁺Foxp3⁺ Tregs in acteoside-treated mice also increased. There are at least two potential mechanisms that might explain the antiallergic effects of acteoside on allergic asthma. First, acteoside may induce immune cell-derived IL-10 production to directly suppress allergic responses. IL-10 exhibits broad anti-inflammatory effects and can be produced by multiple cell types, including DCs, macrophages, B cells, and Tregs. IL-10 can directly inhibit the proliferation and cytokine production by Th2 cells [45]. In addition, IL-10 reduces eosinophil recruitment to sites of allergic inflammation and IgE production [46]. IL-10 also downregulates IgE-mediated mast cells and basophil activation [47,48]. Therefore, IL-10 is particularly important as a negative regulator of immune responses to allergens. Second, acteoside may facilitate DC-derived IL-10 secretion to induce Foxp3⁺ Tregs which results in suppression of Th2-mediated allergic responses. Indeed, several studies demonstrated that IL-10-producing DCs limit allergic Th2 responses via the induction of Tregs [49,50]. Foxp3⁺ Tregs mainly secrete various soluble suppressive factors, including IL-10 and TGF- β , to suppress heightened immune responses, and further trigger Treg expansion [6]. Therefore, we predict that acteoside exerts immunosuppressive effects through inducing Tregs. However, further study is necessary to clarify whether these two mechanisms play roles in the inhibitory effect of acteoside on allergic responses.

In conclusion, our research showed that acteoside enhanced IL-10 production by DCs via activation of the AhR, and these acteoside-treated DCs promoted Foxp3⁺ Treg differentiation. Furthermore, our data demonstrated that acteoside treatment efficiently ameliorated the symptoms of allergic asthma and increased the frequency of Foxp3⁺ Tregs *in vivo*. These results suggest that acteoside represents a potent immunomodulatory agent for the therapeutic treatment of Th2-associated allergic diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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