

Schisandrin B promotes Foxp3⁺ regulatory T cell expansion by activating heme oxygenase-1 in dendritic cells and exhibits immunomodulatory effects in Th2-mediated allergic asthma

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ABSTRACT

Allergic asthma is induced by T helper 2 (Th2) responses and allergen-specific immunoglobulin E (IgE). In asthma, regulatory T (Treg) cells play a crucial role in controlling immune homeostasis, and induction of Treg cells is a good strategy to treat Th2-mediated allergic asthma. Schisandrin B (Sch B), the main component isolated from *Schisandra chinensis*, reportedly possesses various pharmacological properties, but its immunomodulatory mechanism in allergic asthma remains unclear. In the present study, we explored whether Sch B exerts an antiallergic effect through modifying the function of dendritic cells (DCs) to regulate T-cell polarization and further investigated the immunomodulatory effects of Sch B in allergic asthma. Herein, an *in vitro* study revealed that 20 μ M of Sch B-treated bone-marrow-derived DCs exhibited a semi-mature phenotype that secreted low amounts of proinflammatory cytokines including interleukin (IL)-12, IL-1 β , IL-6, and tumor necrosis factor (TNF)- α , and expressed decreased levels of surface molecules of cluster of differentiation 80 (CD80) and CD86. Compared to fully mature DCs, these Sch B-treated DCs displayed a regulatory ability to promote CD4⁺Foxp3⁺ Treg cell generation via upregulation of heme oxygenase (HO)-1 expression. Of note, in a murine model of ovalbumin (OVA)-induced asthma, levels of Th2-type cytokines such as IL-4, IL-5, and IL-13, and C-C motif chemokine 11 (CCL11) were dampened, whereas numbers of forkhead box P3 (Foxp3)-positive Treg cells were augmented in Sch B-treated mice. Moreover, administration of 5 mg/kg of Sch B alleviated the cardinal features of Th2-mediated allergic asthma, namely, serum OVA-specific IgE production, the development of airway hyperresponsiveness (AHR), and airway inflammation. Collectively, these findings indicate that the effectiveness of Sch B treatment against Th2-mediated allergic asthma was at least partially due to enhancement of DC induction of Treg cells, and Sch B can possibly be developed as an immunomodulatory adjuvant to treat allergic asthma.

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1. Introduction

Allergic asthma is a chronic inflammatory disease that predisposes individuals to reversible episodes of airway obstruction and shortness of breath. In allergic asthma patients, inhaled environmental allergens incite T helper 2 (Th2)-type cytokines and cause the recruitment of inflammatory cells into the lungs. Features of allergic asthma include overexpression of allergen-reactive Th2 cells, elevated allergen-specific immunoglobulin E (IgE), airway hyperresponsiveness (AHR), eosinophilia, and mucus hypersecretion by goblet cells (Finotto, 2019). Importantly, Th2-type cytokines, such as interleukin (IL)-4, IL-5, and IL-13, produced by effector CD4⁺ Th2 cells control key processes of IgE production, activation of mast cells, eosinophil recruitment, the development of AHR, and mucus overproduction (Boonpiyathad et al., 2019; Gans and Gavrilo, 2020).

In addition to Th2 cells, dendritic cells (DCs) also play a crucial role in the development of allergic asthma. DCs, as primary antigen-presenting cells (APCs), are central to immunity and tolerance. Their immunoregulatory role is mainly initiated by ligation of specific receptors on naïve CD4⁺ T cells to further selectively promote Th1-, Th2-, or regulatory T (Treg)-cell responses (Hilligan and Ronchese, 2020). Induction of Treg cells or polarization of Th1/Th2 responses by DCs is directed by their cytokine production and expressions of costimulatory surface molecules (Gill, 2012; Lutz, 2016). Instrumental cytokines, such as IL-12, IL-10, IL-6, and transforming growth factor (TGF)- β , are produced by DCs and are known to participate in T-cell differentiation. On the other hand, immature DCs and semi-mature DCs preferentially induce Treg cells, whereas fully mature DCs drive immunogenic Th1/Th2 populations. Consequently, DCs influence whether the response to an allergen will induce allergic inflammation or tolerance to the allergen.

Treg cells are the main negative regulators by which the body maintains immune tolerance to harmless external antigens and prevents excessive immune responses. Treg cell subsets are generally categorized into two groups: thymus-derived (t)Treg cells and peripherally induced (i)Treg cells (Li et al., 2015b). tTreg cells are characterized by constitutive expression of the transcription factor, forkhead box P3 (Foxp3). iTreg cells are composed of Foxp3⁺ Treg cells and Foxp3-negative but IL-10-producing Tr1 cells. Several studies showed that Treg cells have crucial roles in preventing and regulating Th2-mediated allergic reactions (Khan, 2020; Martin-Orozco et al., 2017). Treg cells perform their suppressive functions through multiple mechanisms, such as secreting suppressive cytokines like TGF- β and IL-10, down-modulating APCs, or competing for growth factors (Khan, 2020; Palomares et al., 2010). Conversely, breakdown of the Treg regulatory mechanism leads to elevated IgE levels and the development of airway inflammation (Bertolini et al., 2021; Lewkowich et al., 2005). Hence, inhibition of Th2 cell responses through induction of Treg cells is considered a good immunotherapeutic strategy to treat Th2-mediated allergic asthma.

Schisandrin (Sch) B, a bioactive dibenzocyclooctadiene lignan, is present in the fruit of *Schisandra chinensis* which is used to treat various disorders in traditional Chinese medicine (TCM) (Sowndhararajan et al., 2018; Szopa et al., 2017). Notably, Sch B has received considerable attention because it has antitumor, anti-inflammatory, and immunomodulatory effects. The antitumor activities of Sch B are associated with antiproliferation and the induction of apoptosis in tumor cells (Li et al., 2015a; Nasser et al., 2020; Xiang et al., 2014). Sch B also possesses considerable anti-inflammatory activities. Xu et al. demonstrated that Sch B reduced the production of IL-1 β , tumor necrosis factor (TNF)- α , and IL-6 in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages (Xu et al., 2018). Additionally, Checker et al. reported that Sch B exhibited anti-inflammatory effects by activating nuclear factor-erythroid 2-related factor 2 (Nrf2) and the heme oxygenase (HO)-1 enzyme in lymphocytes (Checker et al., 2012). On the other hand, one study revealed that Sch B promoted Treg cell expansion in mouse splenic cells stimulated with concanavalin A *in vitro* (Chen et al.,

2016). Furthermore, the direct immunosuppressive impact of Sch B on Th17 cell differentiation was recently investigated (Ma et al., 2021).

Based on those reports, we propose that the bioactivities of Sch B may potentially be able to influence immune cell functions and provide a new alternative option for relieving symptoms of allergic asthma. Thus, bone marrow-derived DCs were used to investigate whether Sch B exerts an antiallergic effect through altering the ability of DCs to regulate Treg differentiation. Meanwhile, we evaluated the potential of the immunomodulatory effects of Sch B treatment to inhibit airway inflammation in a mouse model of ovalbumin (OVA)-induced asthma. We predicted that such studies of Sch B administration for allergic diseases would provide further evidence for the potential use of Sch B in treating allergic diseases.

2. Materials and methods

2.1. Animals

Five-week-old 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). Mice were housed 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-2020-0077).

2.2. Preparation of bone marrow-derived DCs

Bone marrow-derived DCs were prepared according to a previously described method (Lin et al., 2019). Briefly, bone marrow cells were collected from the tibiae and femurs of 5–8-week-old BALB/c mice, and were cultured in RPMI-1640 combined with 5% fetal bovine serum complete medium with recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (500 U/ml; PeproTech, Rocky Hill, NJ, USA) and IL-4 (500 U/ml; ThermoFisher Scientific, Rockford, IL, USA). After 4 days of culture, non-adherent cells were collected, and the old medium was replaced with fresh medium containing GM-CSF and IL-4. On day 6 of culture, non-adherent cells were collected and used for further *in vitro* experiments.

2.3. Evaluation of cell viability and apoptosis

For the *in vitro* study, a Sch B (purity $\geq$ 98%, ChemFace, Wuhan, China) stock solution (50 mM) was prepared by dissolving Sch B in DMSO. This was then stored at -20°C until use. The cytotoxicity of Sch B was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay was performed according to a previously described protocol (Lin et al., 2016). In 96-well plates, DCs (10^5 cells/well) were treated with different concentrations of Sch B (0.01, 0.1, 1, 10, 20, 40, and 100 μM). Untreated DCs were cultured with medium as the control. After incubation for 24 h, cells were pulsed with 10 μl of 5 mg/ml of MTT and incubated for 4 h at 37°C . Then cells were solubilized in DMSO and measured using a microplate reader at 570 nm. In addition, the apoptosis rate of DCs treated with various doses (3, 10, and 20 μM) of Sch B was evaluated using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BioLegend, San Diego, CA, USA) according to instructions from the manufacturer. DCs (10^6 cells/well) were cultured in 24-well plates and treated with medium, LPS (100 ng/ml, Merck, Darmstadt, Germany), and LPS plus Sch B for 24 h. At the end of incubation, cells were harvested and stained with FITC-conjugated Annexin V and PI immediately followed by a flow cytometric assay.

2.4. Surface marker analysis

Expressions of surface molecules on DCs were analyzed by flow

cytometry. DCs (10^6 cells/well) were pretreated with or without Sch B for 1 h, followed by 100 ng/ml of LPS stimulation for a further 24-h incubation. Cells were harvested and stained with FITC-conjugated anti-CD11c or phycoerythrin (PE)-conjugated anti-I-A/I-E (MHC class II), anti-CD80, and anti-CD86 antibodies (Biolegend) at 4 °C for 30 min. Cells were then washed and analyzed using FACSCalibur. Data were analyzed using the CELLQUEST PRO program.

2.5. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells using the TRIzol reagent (ThermoFisher Scientific). First-strand complementary (c)DNA was synthesized from total RNA using a high-capacity cDNA reverse-transcription kit (ThermoFisher Scientific). Dilutions of cDNA were subsequently used for the real-time PCR. A qPCR was performed in a Mic qPCR cycler (Bio Molecular Systems, Potts Point, Sydney, Australia) using a SensiFAST™ SYBR® No-ROX kit (Meridian Bioscience, Memphis, TN, USA) according to the manufacturer's instructions. Briefly, the required amount of cDNA template was mixed with 2 × SYBR Green Master Mix reagent and a final concentration of 100 nM of forward and reverse primers, and then water was added to the required reaction volume. Sequences of utilized primers were as follows: Nrf2-F: 5'-TCA CAC GAG ATG AGC TTA GGG CAA-3', Nrf2-R: 5'-TAC AGT TCT GGG CGG CGA CTT TAT-3', HO-1-F: 5'-AAG CCG AGA ATG CTG AGT TCA-3', HO-1-R: 5'-GCC GTG TAG ATA TGG TAC AAG GA-3', 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). The expression of β -actin mRNA was used as an endogenous control, and the mRNA level was normalized to β -actin expression. The cycle threshold (CT) value indicates the number of PCR cycles that were necessary to detect a fluorescence signal exceeding a fixed threshold. The multiple of change (FC) was calculated using the following formulae: $\Delta C_T = C_T$ (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. Western blot analysis

Proteins were extracted from whole cells using RIPA lysis buffer, and protein concentrations were measured with a BCA protein assay kit (T-Pro Biotechnology, Zhonghe, New Taipei City, Taiwan). Proteins were separated on 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred onto polyvinylidene fluoride membranes, and then blocked via incubation in TBST buffer containing 5% non-fat milk. Membranes were subsequently incubated with primary rabbit anti-mouse antibodies overnight at 4 °C, washed, and then probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (BioLegend) at room temperature for 2 h. The bands were visualized with an enhanced chemiluminescence detection kit (Merck, Darmstadt, Germany), and the intensities of the bands were measured with a luminescent image analyzer (TOPBIO, Linkou, New Taipei City, Taiwan). Anti-Nrf2, anti-HO-1, and anti- β -actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.7. Mixed lymphocyte reactions

Allogeneic CD4⁺ T cells were purified from spleens of C57BL/6 mice. Splenocytes were resuspended as signal cells and stained with anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, CA, USA), and then incubated at 4 °C for 15 min. CD4⁺ T cells were isolated by positive selection and used for the following experiments. For the proliferation assay, 10^6 day-6 DCs from BALB/c mice were treated with medium only, LPS (100 ng/ml), LPS plus Sch B (3, 10, and 20 μ M), and LPS plus Sch B (20 μ M) in the presence of 1 μ M tin protoporphyrin IX (SnPP) (Cayman Chemical, Ann Arbor, MI, USA) for 24 h. Then DCs (0.5×10^5 cells/well) were irradiated with 3000 rad (^{137}Cs source) and cocultured with 2×10^5 T cells/

well in 96-well plates. After 54 h of culture, cells were pulsed with [^3H]-thymidine for another 18 h. Then cells were harvested, and counts per minute (cpm) were measured with a β -counter. To evaluate Treg-cell polarization, 10^6 CD4⁺ T cells were cocultured with 2.5×10^5 differently treated DCs in 24-well plates. After 5 days of culture, numbers of CD25⁺Foxp3⁺ Treg cells were analyzed by flow cytometry.

2.8. Adoptive transfer of DCs

BALB/c mice were assigned to three groups (six mice/group) according to treatment: (1) NC group: mice without treatment; (2) OVA-DC group: mice intravenously injected with OVA-pulsed DCs; and (3) OVA/Sch B-DC group: mice intravenously injected with OVA- and Sch B-pulsed DCs. For the treatment model, 10^6 cells (OVA-treated DCs and OVA/Sch B-treated DCs) were intravenously transferred to mice on days 1–2 and 10–11. On day 16, spleens from each group of mice were collected and ground into single-cell suspensions. The population of CD4⁺Foxp3⁺ Treg cells in splenocytes was measured by flow cytometry.

2.9. Establishment of an asthma model and drug treatment

After 1 week of acclimation, BALB/c mice were randomly divided into five groups (five mice/group) according to treatment: (1) negative control mice injected with phosphate-buffered saline (PBS); (2) OVA-induced asthmatic mice; (3) 1 mg/kg body weight (BW)/day Sch B (SchB1); (4) 5 mg/kg BW/day Sch B (SchB5); and (5) 3 mg/kg BW/day prednisolone as a reference drug for a positive control in mice with OVA-induced asthma. On day 1, mice were sensitized by an intraperitoneal (i.p.) injection of 50 μ g OVA allergen (Merck) plus 4 mg alum (ThermoFisher Scientific). Mice were boosted with 30 μ g OVA and the same dosage of alum on days 14, 28, and 35. On days 42 and 43, mice were intranasally (i.n.) challenged with 100 μ g OVA. Subsequently, mice were exposed to 5% OVA in 0.9% NaCl aerosols for 3 consecutive days (days 44–46) by inhalation. On days 14–46, treatment groups were orally treated once daily with Sch B or prednisolone (Merck). For oral feeding, Sch B was dissolved in DMSO (40 mg/ml) and diluted with soybean oil. Control asthmatic mice were administered sterilized soybean oil instead of Sch B. Negative control (NC) mice were neither sensitized with OVA nor administered Sch B treatment, although they received an OVA challenge. On day 47, AHR was measured in all mice, and then they were sacrificed for subsequent experiments.

2.10. Assay of an OVA-specific serum antibody

Serum samples were collected from mice on day 47 and stored at –20 °C after centrifugation (2000 rpm, 10 min). The level of OVA-specific IgE was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Becton Dickinson Biosciences, San Jose, CA, USA) as described earlier (Hsu et al., 2010). All samples were compared to OVA-specific IgE standard serum. The OVA-specific IgE standard was derived by pooling sera from OVA-sensitized mice. The concentration of standard serum was arbitrarily assigned as 1 ELISA unit (EU). Results are expressed as a ratio of the value to the standard.

2.11. Measurement of AHR

Tracheostomized mice were anesthetized and placed on a ventilator (model 683; Harvard Apparatus, South Natick, MA, USA) at a rate of 150 breaths/min, with a tidal volume of 0.3 ml. After recording stable baseline pulmonary resistance, increasing concentrations (1–32 mg/ml) of the methacholine (Merck) aerosol were administered by an ultrasonic nebulizer, 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 R_L after PBS nebulization.

2.12. Determination of cytokine and chemokine levels

In asthma models, levels of IL-4, IL-5, IL-13, CCL11, and TGF- β in bronchoalveolar lavage fluid (BALF) or OVA-restimulated splenocytes were determined by ELISA kits. Supernatants from treated bone marrow-derived DCs were collected, and cytokine levels of IL-1 β , IL-6, IL-12, and TNF- α in supernatants were determined using ELISA kits. CCL11, IL-5, and IL-13 kits were purchased from R&D Systems (Minneapolis, MN, USA), while IL-1 β , IL-4, IL-6, IL-12, TNF- α , and TGF- β kits were purchased from ThermoFisher Scientific.

2.13. Detection of Foxp3⁺ Treg cells in the lungs

Half of a lung from each group of mice was extracted and incubated in 4 ml of 4 mg/ml collagenase A (Merck) with shaking for 90 min at 37 °C. Following digestion, intact tissue was dispersed by repeated pipetting, and the mixture was passed through a 0.07-mm nylon cell strainer. Viable cells were collected by centrifugation and washed twice in Hank's balanced salt solution (HBSS). After washing, viability counts were performed by a trypan blue exclusion method using a hemocytometer. Populations of CD4⁺CD25⁺Foxp3⁺ Treg cells were stained with the following antibodies from ThermoFisher Scientific: PE-conjugated anti-CD25, PE-Cy5-conjugated anti-CD4, and FITC-conjugated anti-Foxp3 antibodies and detected by a flow cytometric assay.

2.14. Detection of OVA-specific cell proliferation in the spleen

Spleens from each group of mice were collected and ground into single-cell suspensions in RPMI-1640 medium. Red blood cells were removed by ACK lysis buffer and washed three times with HBSS. 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 5 days of culture, cells were pulsed with 1 μ Ci/well of [³H]-thymidine, and incubated for 18 h. Then cells were harvested onto glass fiber paper by a semi-automated harvester. cpm values were determined with a dry scintillation counter.

2.15. Inflammatory cell counts in BALF

Mice were sacrificed after measuring the AHR, and the lungs were immediately lavaged three times via a tracheal cannula with 1 ml of HBSS which was retrieved as the BALF. The collected BALF was assayed for chemokines and cytokines. Total cell numbers in BALF were counted with a hemocytometer. Differential cell counts were determined in a Thermo Shandon Cytospin 3 centrifuge (Marshall Scientific, Hampton, NH, USA), followed by Liu's staining (Chi I Pao, Taipei, Taiwan). Two independent, blinded investigators counted the cells using a microscope. Approximately 300 cells were counted in each of five different random locations. Inflammatory cells were classified as macrophages, eosinophils, neutrophils, and lymphocytes, based on standard morphological criteria.

2.16. Lung tissue histopathology

After BALF was obtained, the lungs were immediately removed and one half of a lung was fixed in 10% neutral-buffered formalin. Lung tissues were embedded in paraffin, cut into 4- μ m-thick sections, and stained with hematoxylin and eosin (H&E) solution for inflammatory cells, and periodic acid Schiff (PAS) for goblet cells and mucus production. Stained slices were scanned with a digital camera and analyzed with IMAGEJ software (National Institutes of Health, Bethesda, MD, USA) to quantify the degree of inflammation and mucus production.

2.17. 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. Sch B inhibited LPS-induced maturation and activation of DCs

DCs are the most important APCs involved in interactions with T cells leading to induction of T-cell proliferation and polarization. Therefore, we were interested in identifying whether Sch B could regulate activation of LPS-stimulated bone marrow-derived DCs to further modulate the polarization of CD4⁺ T cells. The chemical structure of Sch B is shown in Fig. 1A. First, DCs were treated with different doses of Sch B (0.01–100 μ M) for 48 h, and then cell viability was determined with an MTT assay (Fig. 1B). Results revealed that Sch B exerted no cytotoxicity on DCs at concentrations of up to 20 μ M, so we used up to 20 μ M Sch B for the following experiments. Next, DCs were treated with different concentrations of Sch B (3, 10, and 20 μ M) following LPS stimulation to induce DC maturation and activation, and the effects of Sch B on cytokine production and surface marker expressions by DCs were examined. Consistent with previous reports, LPS-activated mature DCs exhibited enhanced levels of cytokines (IL-1 β , IL-6, TNF- α , and IL-12) and surface molecules (MHC II, CD80, and CD86) to prime the activation and differentiation of naïve T cells (Lutz, 2016). However, treatment with Sch B significantly reduced the production of IL-12 induced by LPS in a dose-dependent manner (Fig. 1C). Similarly, we found that Sch B treatment significantly suppressed the production of the proinflammatory cytokines, IL-1 β , IL-6, and TNF- α , in LPS-stimulated DCs. Furthermore, we analyzed the effect of Sch B on the phenotype profiles of LPS-stimulated DCs. Compared to LPS-activated DCs, Sch B-treated DCs exhibited marked reductions in expressions of the CD80 and CD86 surface molecules, although no such change in the MHC II level was observed (Fig. 1D). To further identify whether Sch B suppressed DC activation by inducing cell apoptosis, we used an Annexin V/PI double-staining kit to quantify DC apoptosis. As shown in Fig. 1E, although apoptosis had significantly increased after cells were treated with H₂O₂, varying doses (3, 10, and 20 μ M) of Sch B treatment did not induce apoptosis in LPS-activated DCs. This evidence indicated that apoptosis was not associated with the suppressive action of Sch B in activating DCs. Taken together, these data suggested that Sch B treatment reduces the full maturation and activation of DCs and may drive them to become a semi-mature form.

3.2. Sch B-treated DCs expressed upregulated HO-1 and enhanced expansion of Foxp3⁺ Treg cells

Activation of Nrf2 and its dependent gene, HO-1, is known to induce anti-inflammatory responses. In particular, HO-1 was shown to ameliorate OVA-induced asthma by promoting Foxp3⁺ Treg cell differentiation (Xia et al., 2007). Consequently, we were interested in exploring whether Sch B promotes Foxp3⁺ Treg cell expansion by activating Nrf2 and HO-1 in DCs. First, mRNA expression levels of Nrf2 and HO-1 in Sch B-treated DCs were detected by an RT-qPCR. As shown in Fig. 2A, low expressions of these mRNAs were induced in LPS-stimulated DCs. Compared to LPS-activated DCs, we found that Sch B pretreatment showed significant concentration-dependent increases in Nrf2 and HO-1 mRNA expressions in LPS-activated DCs. Likewise, protein levels of Nrf2 and HO-1 were significantly increased in LPS plus Sch B-treated DCs compared to LPS-treated or untreated DCs (Fig. 2B). According to these data, we predicted that Sch B treatment might affect DC maturation by inducing Nrf2 and HO-1 expressions. Since Sch B-treated DCs displayed a semi-mature phenotype and might possess the capacity to induce CD4⁺ T-cell differentiation into Treg cells, we further evaluated the immunomodulatory effect of Sch B-treated DCs on T-cell stimulatory activity and Treg-cell differentiation by an allogeneic mixed lymphocyte

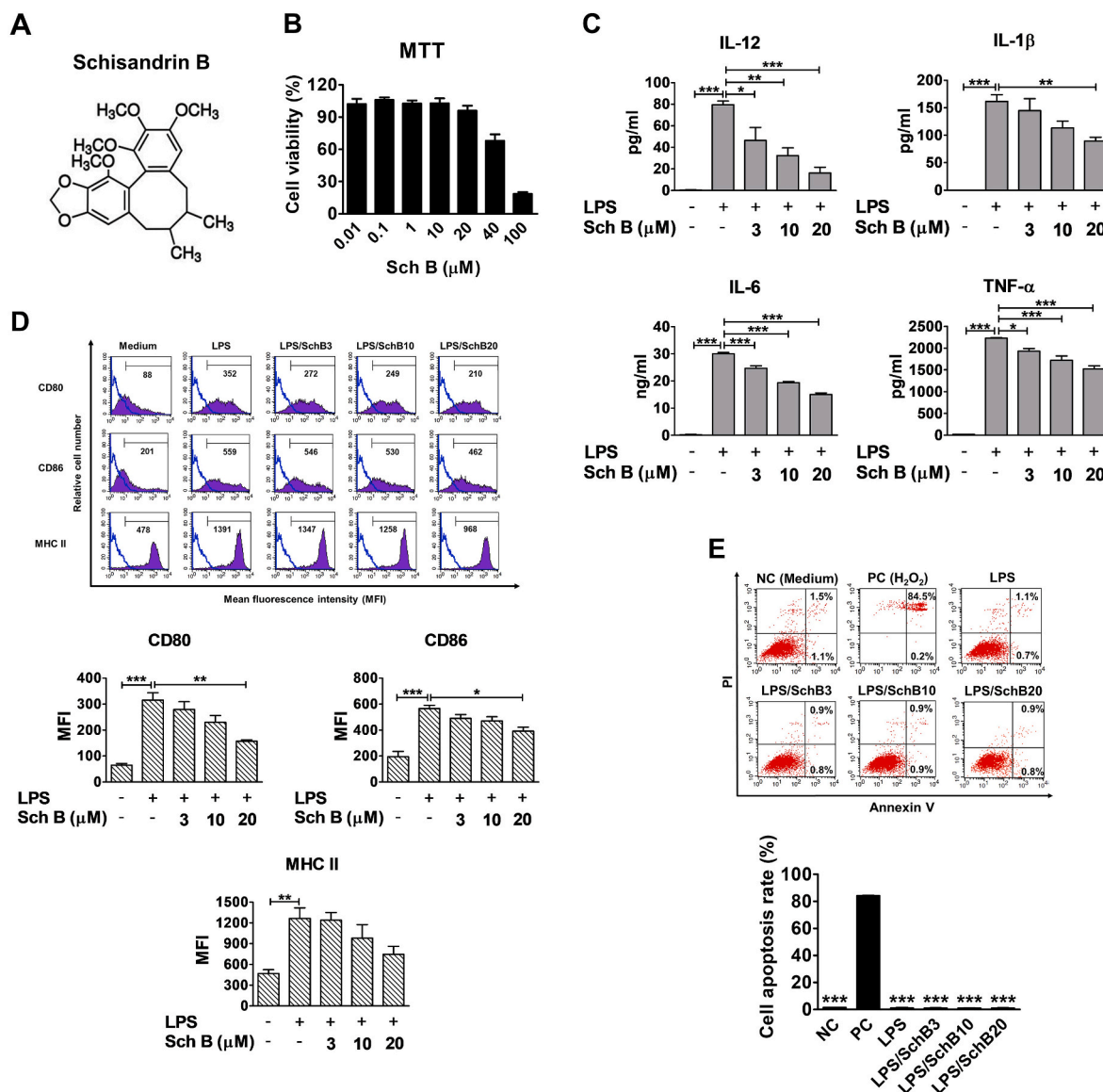


Fig. 1. Cytokine and phenotype patterns of Sch B-treated dendritic cells. (A) The structure of Sch B, with the chemical formula $C_{23}H_{28}O_6$ and a molar mass of 400.46 g/mol. (B) Cytotoxicity effect of Sch B on DCs. On day 6 of culture, bone marrow-derived DCs were treated with different doses of Sch B for 24 h. Cell viability was detected by an MTT assay. (C) Levels of IL-12, IL-1 β , IL-6, and TNF- α from Sch B-pretreated DCs with or without LPS stimulation. Supernatants for the cytokine analysis were determined by ELISA kits. (D) Expression levels of CD80, CD86, and MHC class II by Sch B-treated DCs. DCs were treated with medium, LPS, or Sch B plus LPS. Cells were collected and analyzed by flow cytometry. 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 $\pm$ SEM of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. LPS-treated DCs (LPS group). (E) Flow cytometric analysis of the apoptosis ratio. DCs were treated with H₂O₂, LPS, LPS plus Sch B at various concentrations, and medium only for 24 h. Flow cytometry was used to distinguish different stages of apoptosis by an Annexin V/PI assay. The total apoptotic cell ratio was the sum of cells undergoing early (lower right) and late apoptosis (upper right). Results are expressed as the mean $\pm$ SEM of three independent experiments. *** p < 0.001 vs. H₂O₂-treated DCs.

reaction (MLR). Because HO-1 is involved in inducing Treg cells by DCs, an HO-1 inhibitor (SnPP) was used to identify this possible mechanism. A concentration range of 0.06–10 μ M SnPP was used in many cell-culture systems (Checker et al., 2012; Romero et al., 2019; Xia et al., 2007). Therefore, we examined the suppressive effect of 1 μ M SnPP on the MLR. Results revealed that LPS plus Sch B-treated DCs showed a significantly weak allogeneic T-cell proliferative response compared to LPS-stimulated DCs (Fig. 2C). Instead, SnPP pretreatment reversed the activation capacity of DCs for T-cell proliferation. In addition, we observed that treatment of DCs with LPS plus Sch B significantly promoted the generation of Foxp3⁺ Treg cells compared to LPS-stimulated DCs (Fig. 2D). Conversely, SnPP treatment prevented the ability of Sch B-treated DCs to expand Treg cells. Moreover, LPS plus Sch B-treated

DCs markedly promoted TGF- β production in T cells compared to LPS-stimulated DCs. These findings suggest that Sch B might inhibit the effector Th response through enhancing the expansion of TGF- β -producing Foxp3⁺ Treg cells by Sch B-modulated DCs via HO-1 activation. Furthermore, we designed an experiment to examine whether the adoptive transfer of antigen plus Sch B-treated bone marrow-derived DCs (HO-1-induced DCs) enhanced the generation of Treg cells in mice. As per data shown in Fig. 2E, OVA plus Sch B-treated DCs increased the frequency of CD4⁺Foxp3⁺ Treg cells in mice compared to either untreated or OVA-DC-treated mice. These results revealed that adoptive transfer of HO-1-induced DCs by Sch B treatment efficiently promoted Treg cell generation *in vivo*.

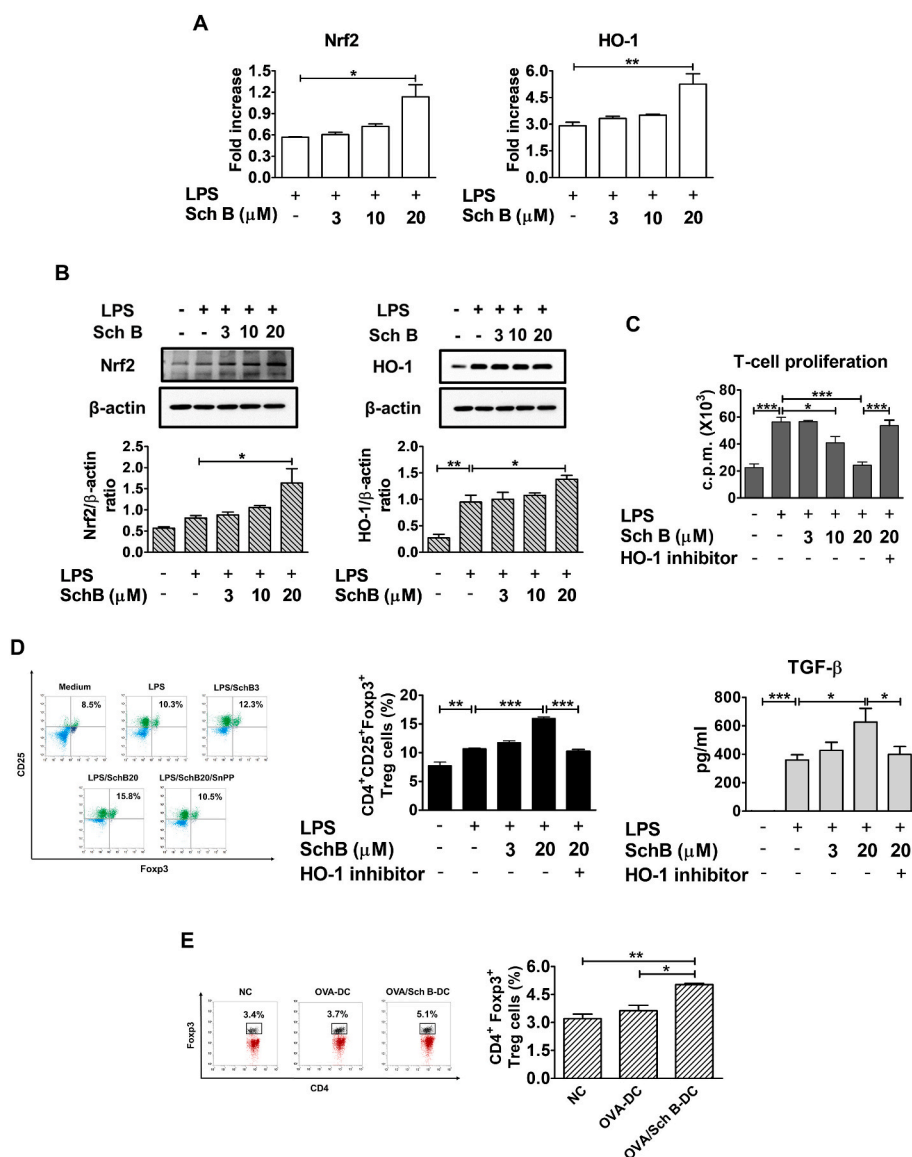


Fig. 2. The expansion of Foxp3⁺ Treg cells by Sch B-treated DCs via activation of HO-1. (A) Effects of Sch B on mRNA expressions of Nrf2 and HO-1 in DCs. DCs were treated with medium alone, LPS, or Sch B plus LPS. Then these cells were collected and analyzed by RT-qPCR method. (B) Effects of Sch B on expressions of the Nrf2 and HO-1 proteins in DCs. DCs were treated with medium alone, LPS, or LPS plus Sch B. Then these cells were collected and analyzed by a Western blot method. (C) Sch B-treated DCs exhibited low CD4⁺ T-cell stimulatory activity. In the mixed lymphocyte reaction, DCs from BALB/c mice were treated with medium alone, LPS, LPS plus Sch B, or LPS plus Sch B in the presence of tin protoporphyrin IX (SnPP; an HO-1 inhibitor) for 24 h. Naive CD4⁺ T cells from C57BL/6 mice were then cocultured with γ-irradiated 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). (D) Sch B-treated DCs promoted TGF-β-producing Foxp3⁺ Treg differentiation *in vitro*. Allogeneic CD4⁺ T cells were cocultured with medium alone, LPS, LPS plus Sch B, or LPS plus Sch B in the presence of SnPP-treated DCs. After 5 days of culture, CD4⁺ T cells were collected and further analyzed by flow cytometry. Meanwhile, levels of TGF-β in supernatants were determined by an ELISA kit. Representative dot plots show expressions of CD25 and Foxp3 by gated CD4⁺ T cells. Results are expressed as the mean ± SEM of three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. (E) Adoptive transfer of OVA- and Sch B-pulsed DCs enhanced the generation of Foxp3⁺ Treg cells in mice. For the treatment model, 10⁶ cells (untreated DCs, OVA-treated DCs, and OVA/Sch B-treated DCs) were intravenously transferred to mice on days 1–2 and 10–11. On day 16, spleens from each group of mice were collected, and the population of CD4⁺Foxp3⁺ Treg cells in splenocytes was measured by flow cytometry. Representative dot plots show expression of Foxp3 by gated CD4⁺ T cells. Results are expressed as the mean ± SEM of five mice per group. **p* < 0.05 vs. the OVA-DC group.

3.3. Sch B treatment reduced anti-OVA IgE production and the severity of AHR in mice with OVA-induced asthma

To explore the *in vivo* impacts of Sch B against asthma, we assessed the therapeutic effects of Sch B in an OVA-induced asthmatic animal model. The experimental protocol is schematically represented in Fig. 3A. Two groups of OVA-sensitized mice (Sch B (1) and Sch B (5)) were fed different doses of Sch B (1 and 5 mg/kg). Serum samples from differently treated mice were collected on day 47, and the level of an OVA-specific IgE antibody in the serum was assayed. IgE is considered to be critical for initiating allergic asthma. Sensitization and challenge with OVA resulted in increased serum levels of OVA-specific IgE in the asthma group compared to negative control animals. As a control agent, prednisolone caused a strong reduction in the production of OVA-specific IgE in serum. Compared to the asthma group, oral administration of a higher dose of Sch B (Sch B (5)) significantly reduced the production of OVA-specific IgE in mice (Fig. 3B). Because previous studies revealed that Th2-type cytokines can promote IgE production (Emson et al., 1998), our results indicated that Sch B diminished the Th2 response in mice with OVA-induced asthma. Furthermore, we investigated whether Sch B administration could attenuate the development of AHR in OVA-induced airway inflammation. AHR was evaluated by

calculation of R_L values 24 h after the final OVA challenge. As shown in Fig. 3C, R_L values of the dose-response curve in the asthma group were markedly induced after methacholine stimulation compared to those of the negative control group. Conversely, Sch B treatment dose-dependently decreased R_L values compared to the asthma group. Similarly, prednisolone treatment decreased the severity of AHR. Overall, these results implied that treatment with Sch B could suppress OVA-induced inflammation-mediated AHR.

3.4. Sch B treatment suppressed the production of Th2-type cytokines and CCL11 in BALF

By studying the production of OVA-induced cytokines and chemokines in BALF and cell culture supernatants from the spleen, we determined whether Sch B really affected the Th2 immune response. Therefore, the release of cytokines (IL-4, IL-5, IL-13, and TGF-β) and the CCL11 chemokine in BALF was observed. As shown in Fig. 4A, levels of Th2-type cytokines (IL-4, IL-5, and IL-13) were found to have significantly increased in the asthma group compared to levels observed in negative control mice, whereas administration of Sch B dose-dependently inhibited production of these cytokines. On the other hand, CCL11, a member of the CC chemokine subfamily, is a

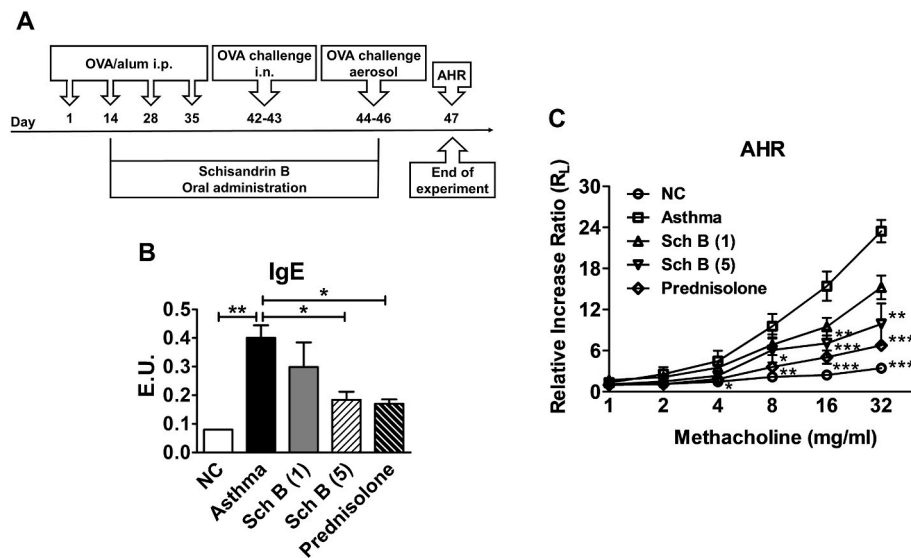


Fig. 3. Sch B treatment reduced OVA-specific IgE antibody production and airway hyper-responsiveness in an OVA-induced mouse asthma model. (A) Brief protocol of animal sensitization and challenge. On days 1, 14, 28, and 35, all groups of mice were sensitized by an intraperitoneal (i.p.) injection of the OVA allergen. Sch B (1 and 5 mg/kg body weight (BW)) or prednisolone (3 mg/kg BW) was daily given orally on days 14–46. Positive control mice (Asthma) were administered soybean oil instead of Sch B. Mice were then intranasally (i.n.) challenged with OVA on days 42 and 43. Subsequently, mice were exposed to OVA aerosols for 3 consecutive days, and AHR was measured 1 day after the last challenge. BALF was collected after measuring the AHR. Negative control (NC) mice were neither sensitized with OVA nor administered Sch B treatment, but were challenged with OVA. (B) Levels of an anti-OVA-specific IgE antibody in all groups of mice were measured by an ELISA. Results are expressed as the mean $\pm$ SEM of five mice in each group. (C) Oral administration of Sch B suppressed the development of AHR in mice with OVA-induced asthma. After the last OVA challenge, the airway resistance of differently treated mice was measured in response to increasing concentrations of methacholine (0–32 mg/ml).

ml) by invasive body plethysmography. Results are expressed as the pulmonary resistance in the ratio of lung resistance (R_L) after PBS nebulization. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. the asthma group.

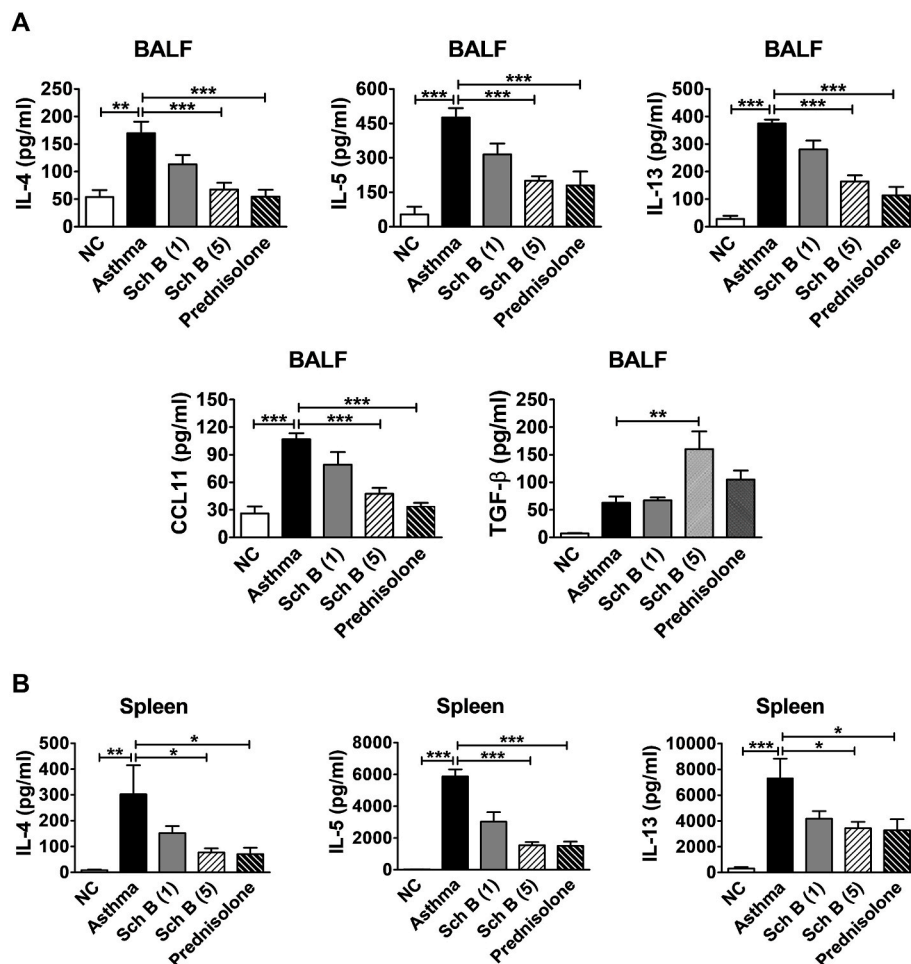


Fig. 4. Sch B treatment relieved Th2 immune responses in mice with OVA-induced asthma. (A) Effects of Sch B on levels of cytokines and a chemokine in BALF. BALF from each group of mice was collected and analyzed for IL-4, IL-5, IL-13, CCL11, and TGF- β contents using ELISA kits. (B) Effects of Sch B on the cytokine profile of OVA-restimulated splenocytes. Splenocytes (10^7 cells/ml) from Sch B-treated and control groups were stimulated with 50 μ g/ml OVA in 24-well plates, and culture supernatants were collected after 3 days. Levels of IL-4, IL-5, and IL-13 in culture supernatants were determined using 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.

chemoattractant and activator of eosinophils, and it is also a main attractant of Th2 cells. The asthma group expressed higher amounts of CCL11 in BALF than did negative control mice. Unlike in the asthma group, treatment with a higher dose of Sch B significantly down-regulated the CCL11 level in BALF. Prednisolone also caused reductions in the abovementioned inflammatory mediators. As expected, similar cytokine patterns were observed in culture supernatants of OVA-restimulated splenocytes in these differently treated mice (Fig. 4B). TGF- β is an inducible anti-inflammatory cytokine which can be produced by Treg cells. Notably, Sch B treatment at 5 mg/kg significantly enhanced the production of TGF- β in BALF. Taken together, these results indicated that Sch B was able to inhibit Th2-mediated responses during the development of OVA-induced allergic asthma.

3.5. Sch B treatment promoted $CD4^+CD25^+Foxp3^+$ T cell generation and reduced OVA-specific T-cell proliferation in mice with OVA-induced asthma

Treg cells possess immunosuppressive abilities which make them key regulators of inflammatory diseases, including asthma. Consequently, we further investigated whether treatment with Sch B could promote the frequency of Treg cells in the lungs of asthmatic mice. Results showed that the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells ranged 0.1%–0.5% in these asthmatic mice. Prednisolone treatment produced no difference in the Treg frequency. Of note, administration of a higher dose of Sch B dramatically increased the population (8%) of $CD4^+CD25^+Foxp3^+$ T cells (Fig. 5A). Furthermore, the suppressive effect of Sch B on OVA-specific effector T-cell proliferation was also evaluated. In the OVA-specific T-cell proliferation assay, splenocytes from these differently treated mice were isolated and further stimulated with OVA *in vitro*. After 5 days of culture, the proliferative situation was measured with a β -counter. Results demonstrated that Sch B-treated mice showed a markedly lower proliferative capacity of OVA-stimulated cells than did asthmatic mice (Fig. 5B). Although prednisolone also reduced the clonal expansion of $CD4^+$ effector T cells, it was reported that the effect of prednisolone in asthma treatment was not mainly mediated by the increased generation of Treg cells, but through depletion of $CD4^+$ effector T cells (Zuska-Prot and Maslanka, 2017). In conclusion, these data indicated that Sch B may relieve allergic syndrome through inducing $CD4^+CD25^+Foxp3^+$ Treg cells to suppress effector Th2 responses.

3.6. Sch B treatment alleviated airway inflammation in mice with OVA-induced asthma

To assess the antiallergic effects of Sch B on allergic asthma, the accumulation of inflammatory cells in BALF was investigated. Sensitization and challenge with OVA in asthmatic mice induced increased numbers of macrophages, eosinophils, neutrophils, and lymphocytes in BALF of mice. In contrast, oral feeding of mice with 5 mg/kg of Sch B

significantly decreased counts of inflammatory cells in BALF, and similar results were also shown in the prednisolone-treated group (Fig. 6A). On the other hand, to clarify the therapeutic effect of Sch B on lung tissues in this animal model, lung sections were stained with H&E solution. In histological examination results of the lungs, marked infiltration of inflammatory cells around the peribronchiolar region was observed in the asthma group compared to negative control mice. However, both Sch B (5 mg/kg) and prednisolone treatment significantly attenuated the accumulation of inflammatory cells (Fig. 6B). In addition, airway mucus occlusion was assessed in lung sections after PAS staining. Results revealed that OVA treatment stimulated the secretion of mucus into the airway lumen in the asthma group, whereas administration of 5 mg/kg Sch B significantly reduced the amount of mucus in the airway lumen (Fig. 6C). Prednisolone treatment also efficiently inhibited peribronchial lung inflammation. To sum up, these results demonstrated that Sch B alleviated the severity of asthma syndrome, including inflammatory cell infiltration and mucus production in the lungs.

4. Discussion

Asthma is a chronic disease of respiratory inflammation with a high incidence, and the prevalence has increased in the past few decades, particularly in Westernized countries. Although anti-inflammatory agents and bronchodilators were developed to treat asthma, many patients have concerns about medication side effects and are frustrated by the lack of preventive and curative therapies. Therefore, increasing numbers of patients are seeking complementary and alternative medicines, such as herbal products and traditional Chinese medicine (TCM). Accumulating publications have revealed that TCMs exert multifarious actions for treating asthma, including modifying the function of DCs, promoting the expansion of Treg cells, and regulating the balance of Th1/Th2 immune responses (Chan and Ng, 2020).

Schisandra chinensis is an important medicinal plant in TCM and other East-Asian medicines. Until now, more than 40 lignans have been isolated from this plant such as Sch, Sch A, Sch B, Sch C, and schisantherin A. Among the different dibenzocyclooctadiene lignans from *S. chinensis*, Sch B is the most important bioactive lignan with low toxicity and low-cost properties. As to the molecular mechanism, the anti-inflammatory activity of Sch B is related to its ability to interact with Toll-like receptor 4 (TLR4) and its adapter proteins that lead to suppression of nuclear factor (NF)- κ B signaling pathways (Zeng et al., 2012). On the other hand, the neuroprotective effect of Sch B was shown to downregulate expressions of IL-1 β , TNF- α , and matrix metalloproteinase (MMP)-2/9 (Lee et al., 2012). Recently, Sch B was reported to reduce IL-17A production by Th17 cells, which are involved in the pathogenesis of autoimmune and allergic diseases (Ma et al., 2021). However, T cells have many kinds of subtypes, and the underlying mechanism of Sch B's regulation of other kinds of T cell subtypes, such as Th2 and Treg cells, remains unclear.

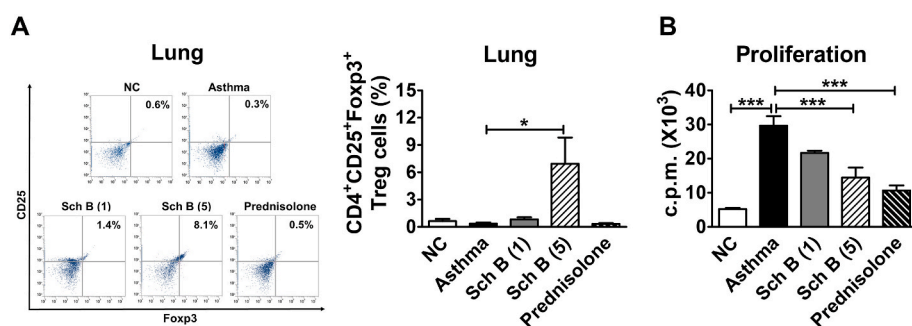


Fig. 5. Sch B treatment enhanced the generation of $Foxp3^+$ Treg cells and reduced effector T-cell proliferation. (A) The frequency of $Foxp3^+$ Treg cells in the lungs. Lung lobes from different experimental groups were enzymatically digested with collagenase A. Single-cell suspensions from the lungs were collected and analyzed by flow cytometry. Representative dot plots show expressions of CD25 and $Foxp3$ by gated $CD4^+$ T cells. Frequencies of Treg cells are shown. (B) The proliferative response of OVA-activated splenocytes was reduced in Sch B-treated mice. Splenocytes from different experimental groups were restimulated with OVA for 5 days. The proliferative response was assessed by [^3H]-thymidine assay. Count per minute (cpm) values were

measured 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.

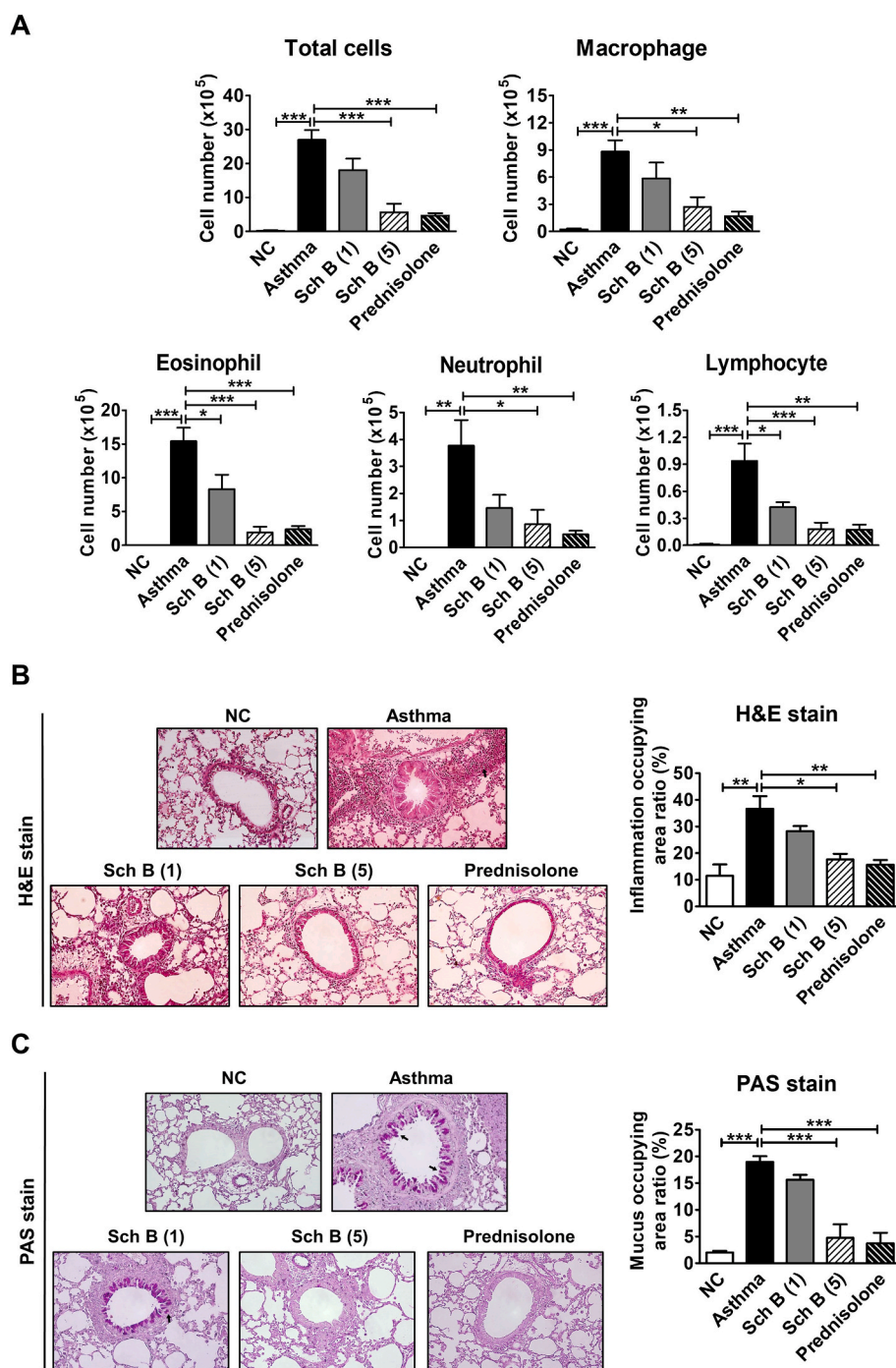


Fig. 6. Sch B treatment attenuated airway inflammation in lungs of mice with OVA-induced asthma. (A) Effects of Sch B on the recruitment of inflammatory cells in BALF. After measuring pulmonary function parameters, each group of mice was sacrificed, and BALF was collected. Cells from BALF were stained with Liu's stain reagent and classified as macrophages, eosinophils, neutrophils, and lymphocytes. (B) Lung sections were stained with hematoxylin and eosin (H&E) to measure the infiltration of inflammatory cells. Black arrows indicate infiltrating inflammatory cells in lung tissues. Inflammatory changes are presented as percentages of the inflamed area by computer-based quantification. (C) Lung sections were stained with PAS to measure mucus around the airway. Black arrows indicate a mucus plug in the airway lumen. 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.

The strength of DC maturation and activation, represented by expressions of MHC class II and costimulatory molecules (CD80 and CD86) and cytokines, is translated into the strength of T-cell stimulation and determines outcomes of T-cell differentiation. For example, DCs stimulated with LPS reached a fully mature form characterized by the release of the IL-12 cytokine that leads to Th1 induction. Alternatively, semi-mature DCs which were generated by Sch B treatment expressed reduced levels of stimulatory cytokines and surface molecules and possessed poor T-cell stimulatory activity, but promoted Foxp3⁺ Treg cells. Similarly, an inhaled corticosteroid also dampened Th immune responses through inhibiting DC maturation (Georas, 2004). Moreover, it was proven that human DCs treated with glucocorticoids become regulatory DCs which promote the expansion of Treg cells *in vitro*

(Hamdi et al., 2007). Thus, we predict that Sch B is critical for the commitment of DCs to differentiate into regulatory DCs for induction of Treg cells, and this mechanism may contribute to the therapeutic effects of Sch B in allergic asthma. In addition, it was reported that naïve CD4⁺ T cells stimulated with anti-CD3/CD28 antibodies in the presence of Sch B exhibited Treg polarization *in vitro* (Chen et al., 2016). Therefore, it is possible that Sch B may upregulate the number of Treg cells in a DC-independent pathway *in vivo*. Recently, Ma et al. reported that Sch B protected mice from colitis by decreasing Th17 induction and IL-17A secretion (Ma et al., 2021). Thus, it is also possible that Sch B could target Th17 cells to exert its antiallergic activity in allergic asthma, but this hypothesis requires further study.

Using *HO-1*-knockout mice, *HO-1* was demonstrated to be an

important target gene in anti-inflammatory and immunomodulatory effects (Poss and Tonegawa, 1997). HO-1 is an intracellular enzyme which degrades heme into carbon monoxide (CO), iron, and biliverdin, and is known for its anti-inflammatory effects in various diseases. Previous studies demonstrated the effects of an HO-1 inhibitor on Treg induction and asthmatic responses *in vivo* (Xia et al., 2006, 2007). Hemin is an inducer of HO-1, whereas SnPP is an inhibitor of HO-1. Using an asthmatic animal model, hemin treatment resulted in induction of Foxp3 expression, but such expansion of Treg cells was specifically inhibited by SnPP. Moreover, the antiasthmatic effects of hemin were also blocked by SnPP. These data suggest that HO-1 may exert its protective effect on asthma by upregulating Foxp3⁺ Treg cells; thus, it is a potential therapeutic target for allergic diseases. Specifically, targeting HO-1 upregulation in DCs suppressed LPS-induced phenotypic maturation and proinflammatory cytokine secretion, resulting in a reduction of allergic T-cell proliferation (Chauveau et al., 2005). Consistent with this finding, we found that Sch B-treated DCs expressed increased levels of HO-1 and displayed a semi-mature phenotype. Additionally, SnPP, an HO-1 inhibitor, abolished the effect of Sch B on DC function of immunomodulating Treg cell differentiation. Therefore, it is likely that Sch B affects Treg cell generation through activation of HO-1 in DCs.

In this study for the first time, we investigated the immunomodulatory effects of Sch B on the Th2/Treg balance in an asthmatic murine model. We chose oral administration of Sch B for 32 days at doses of 1 and 5 mg/kg BW for long-term treatment of mice with OVA-induced asthma. Our data revealed that treatment of asthmatic mice with 5 mg/kg Sch B significantly promoted the generation of Foxp3⁺ Treg cells which suppressed activation of Th2 cells. However, a low dose of Sch B (1 mg/kg) exerted only mild antiallergic effects in asthmatic mice. Thus, we speculated that higher administered doses of Sch B (5–10 mg/kg BW) might be a better choice for allergy therapy. Altogether, our study demonstrated that 5 mg/kg BW Sch B administration could effectively limit the development of immunopathology in asthmatic mice by suppressing OVA-induced Th2-mediated responses.

IL-4, IL-5, and IL-13 are secreted by Th2 cells and were reported to play important roles in allergic airway inflammation. Thus, allergic asthma is recognized as a Th2-mediated inflammatory disease. The immunoglobulin switching process in B cells leading to IgE secretion is typically mediated by IL-4 (Geha et al., 2003). Allergen-specific IgE further promotes progression of inflammation by binding to and activating the high-affinity receptor for IgE (FcεRI) on surfaces of mast cells (Elieh Ali Komi and Bjerrmer, 2019). Apart from IgE synthesis, IL-4 is also responsible for recruiting eosinophils (Gavett et al., 1997). In addition, another pathway which is involved in increased eosinophil infiltration in the lungs is also orchestrated by IL-5 and coordinated by specific chemokines such as CCL11 (Riffo-Vasquez and Spina, 2002). Therefore, decreased expressions of IL-4, IL-5, and CCL11 in Sch B-treated mice were associated with reductions in serum IgE levels and pulmonary eosinophilia, supporting the pivotal role of these cytokines and the chemokine in allergic asthma. Moreover, IL-4 and IL-13 play roles in mucus hypersecretion and AHR development (Dabbagh et al., 1999; Kuperman et al., 2002). AHR to a large number of stimuli is characterized by bronchial constriction that is generally found in asthma patients. We found that Sch B treatment markedly suppressed OVA-induced AHR in response to inhaled methacholine. Consistent with a previous report by Chen et al. (2021), our results indicated that Sch B plays a key role in preventing the development of AHR by inhibiting IL-4- and IL-13-dependent pathways.

In recent years, several studies reported that CD4⁺Foxp3⁺ Treg cells play crucial immunosuppressive roles in maintaining immune tolerance and controlling allergic diseases (Singer et al., 2014; Zhang et al., 2014). Therefore, we observed whether the therapeutic effects of Sch B on allergic asthma might partially be due to induction of Treg cells. Indeed, our results showed that the frequencies of CD4⁺CD25⁺Foxp3⁺ Treg cells in the lungs were significantly higher in mice treated with Sch B than those of asthmatic mice. Various types of Treg cells possess the ability to

mediate targeted immunosuppression, but Foxp3⁺ Treg cells appear to be the dominant cell type. The Foxp3 transcription factor is instrumental in Treg cell development and function. Foxp3⁺ Treg cells can reduce IgE production, inhibit airway eosinophilia, and downregulate the release of cytokines from effector Th2 cells by mechanisms involving TGF-β and IL-10 in allergic diseases (Chiang et al., 2017; Hawrylowicz, 2005; Matsuda et al., 2019; Sakaguchi, 2005). Indeed, our data showed that treatment with 5 mg/kg BW Sch B markedly increased TGF-β production in the lungs. The role of TGF-β in controlling immune responses has been widely studied. TGF-β exhibits a suppressive effect on immune responses by suppressing inflammatory cells and promoting Foxp3⁺ Treg cell activity and generation (Chen et al., 2003; Horwitz et al., 2008). Taken together, our findings suggest that Sch B treatment dampened effector Th2 cell responses to restrict the severity of allergic asthma, at least in part mediated by augmenting the number of Foxp3⁺ Treg cells. Although we did not detect the level of IL-10 in mice, it is possible that the accumulation of neutrophils in the lungs may be inhibited by IL-10 production by Treg cells. IL-10 is an important anti-inflammatory cytokine, and activation of a variety of inflammatory cells is inhibited by IL-10. Many cells have the ability to produce IL-10, including macrophages, B cells, Th1, Th2, and Treg cells. Previous studies demonstrated that administration of IL-10 alleviated allergen-induced airway inflammation in mice (Fu et al., 2006; van Scott et al., 2000). In addition, Nabe et al. proved that IL-10 produced by antigen challenge reduced airway neutrophil infiltration in a murine model of asthma (Nabe et al., 2012). Thus, in the future, it would be interesting to further clarify whether IL-10 plays a regulatory role in Sch B treatment and what types of immune cells are major sources of IL-10.

In conclusion, this study showed that the immunosuppressive effect of Sch B on OVA-induced asthma may in part be mediated by modifying DCs to Foxp3⁺ Treg-polarizing APCs (Fig. 7). These Sch B-treated DCs became semi-mature APCs which expressed low levels of cytokines and surface markers to promote Foxp3⁺ Treg differentiation, which then inhibited Th2-mediated allergic asthma. In the future, Sch B may have the potential for use as an immunomodulatory adjuvant to treat allergic

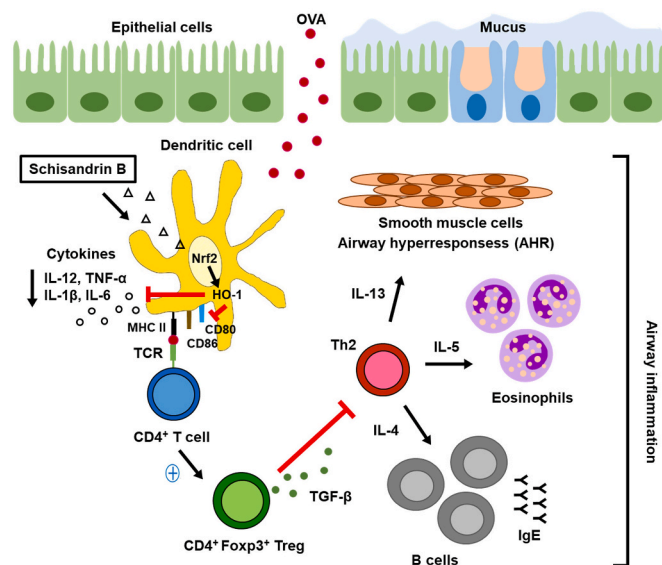


Fig. 7. Sch B treatment exerts immunomodulatory effects on dendritic cells by enhancing HO-1 expression and ameliorates Th2-mediated allergic asthma. The antiallergic effect of Sch B on Th2 responses is partially due to the induction of Foxp3⁺ Treg cells in mice with OVA-induced asthma. Sch B treatment affects DC maturation by increasing HO-1 expression and drives these semi-mature DCs toward antigen stimulation, limiting the magnitude of the immune response by reducing T-cell proliferation and promoting Foxp3⁺ Treg differentiation. Thus, Sch B can potentially be used as an immunomodulatory adjuvant to treat Th2-mediated allergic diseases.

asthma.

Declaration of competing interest

The authors declare that they have no financial conflicts of interest.

CRediT authorship contribution statement

Chen-Yuan Chiang: Conceptualization, Data curation, Writing – original draft. **Jer-Hwa Chang:** Conceptualization, Formal analysis, Writing – original draft. **Hsiao-Chi Chuang:** Investigation, Software, Writing – review & editing. **Chia-Kwung Fan:** Investigation, Methodology, Writing – review & editing. **Tsung-Yun Hou:** Investigation, Methodology, Software, Validation. **Chu-Lun Lin:** Investigation, Methodology, Software, Validation. **Yueh-Lun Lee:** Funding acquisition, Project administration, Supervision, Writing – review & editing.

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