



Immunopharmacology and inflammation

Luteolin attenuates airway inflammation by inducing the transition of CD4⁺CD25⁻ to CD4⁺CD25⁺ regulatory T cells

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ABSTRACT

Regulatory T cells play an important role in autoimmunity and have been shown to exert anti-inflammatory effects in allergic asthma. Mouse model of airway inflammation was used to examine the suppressive activity of luteolin-induced CD4⁺CD25⁺ regulatory T cells (Tregs) *in vivo*. In this study, BALB/c mice were sensitized with ovalbumin antigen (OVA) by aerosol challenge. Then, various biological processes were examined, including airway eosinophilia; mucus hypersecretion; elevation of OVA-specific IgE, expression of Th2 cytokines and chemokine levels; expression of eotaxin 2 and CCR3; and airway hyper responsiveness (AHR). Luteolin significantly inhibited OVA-induced increase in immune cell and eosinophil counts as well as IL-4, IL-5, IL-13, and eotaxin levels in bronchoalveolar lavage fluid (BAL Fluid). Luteolin and cyclosporine A (CsA) which was a positive control also substantially reduced OVA-specific IgE levels, eotaxin 2 levels, and CCR3 expression in BAL Fluid. In contrast, luteolin significantly increased IL-10 and IFN- γ protein levels, as well as IL-10 and TGF- β 1 mRNA expression in the lung. *In vitro* studies showed that the number of luteolin-induced CD4⁺CD25⁺ Treg (iTreg) cells was higher, with elevated levels of TGF- β 1 and *foxp3* mRNA expression in lungs tissue. Transfer of iTreg cells into OVA-sensitized mice reduced AHR, eosinophil recruitment, eotaxin, IgE, and Th2 cytokine expressions, and increased IFN- γ production in BAL Fluid after allergen challenge. Furthermore, adoptive transfer of iTreg cells prevented disease in a CD25-depleted mouse asthma model. Luteolin via induction of *foxp3* and CD4⁺CD25⁺ Treg cells may represent a new strategy in the development of therapies for managing asthma.

1. Introduction

Asthma is a chronic inflammatory disease of the airway that is characterized by airway eosinophilia, goblet cell hyperplasia with mucus hypersecretion, and hyper responsiveness to both inhaled allergens and nonspecific stimuli (Barnes et al., 1998; Busse and Lemanske, 2001). The major effector cells in asthma are eosinophils, mast cells, and Th2 lymphocytes (Gleich, 2000; Wardlaw et al., 1988). CCL11/eotaxin is the first specific chemokine described as an attractant for eosinophils in BAL Fluid (Griffiths-Johnson et al., 1993; Ponath et al., 1996). Airway Th2 cells, mast cells, basophils, and eosinophils appear to be the primary effector cells that orchestrate the clinical

manifestations of disease (Herrick and Bottomly, 2003). Infiltration of the airways by Th2 cells and eosinophils is a predominant feature of the late-phase asthmatic response (Wills-Karp, 1999). Within the airway mucosa, eosinophils, along with Th2 cells and other inflammatory leukocytes releases a wide range of inflammatory mediators that underlie the clinical hallmarks of asthma, such as airway wall remodeling and mucus hypersecretion with airway obstruction and hyper reactivity (AHR) (Holt et al., 1999). Eosinophils have the potential to induce respiratory damage and AHR through the release of highly-charged granular proteins, lipid mediators, and a range of proinflammatory cytokines and chemokines (Lukacs, 2001). It is becoming apparent that eosinophilia can be selectively regulated by integrated signaling events

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involving the chemokines that function through the chemokine receptor 3 (CCR3) and the cytokines produced by Th2 cells (Gerber et al., 1997; Sallusto et al., 1997).

There is now strong evidence for the existence of Th2 subsets that can suppress immune responses (O'Garra and Vieira, 2004). One of the best characterized T cell subsets is the “naturally occurring” CD4⁺CD25⁺ regulatory T cells. The precise mechanisms by which CD4⁺CD25⁺ regulatory cells suppress inflammation are unknown, although there is evidence for the involvement of IL-10 and TGF- β in some settings; administration of antibodies against IL-10 and TGF- β prevent the abrogation of colitis by CD4⁺CD25⁺ regulatory T cells in a mouse model and block the suppressor activity of regulatory T cells isolated from human peripheral blood (Akdis et al., 2004; Asseman et al., 1999; Powrie et al., 1996).

In this study, we screened the natural product library (PB4388.1, Korea Plant Extract Bank) and identified a compound, luteolin, from the root of *Salvia plebeia* R. Br. as a potent immune enhancer. To assess the efficacy of luteolin, mice with OVA-induced asthma were administered luteolin either prophylactically or therapeutically (Jang et al., 2016). Our results demonstrated that luteolin and the positive control cyclosporine (CsA) significantly suppressed chronic AHR of OVA-sensitized control mice (OVA-CTL) and that this action was characterized by increased *foxp3* production and TGF- β expression as well as an increased proportion of CD4⁺CD25⁺ regulatory T cells *in vivo*. We have also demonstrated that the adoptive transfer of iTreg cells inhibited the classical pathology associated with allergic asthma, namely AHR, lung eosinophilia, and Th2 cytokine production.

2. Materials and methods

2.1. Materials

All HPLC-grade reagents, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). A5503 Albumin Grade V and A8222 Aluminum Hydroxide Gel “AL hydrogel,” was from Sigma-Aldrich (St. Louis, MO, USA). Synthetic Cyclosporine A, H&E and periodic acid-Schiff, 4% paraformaldehyde, OVA, aluminum hydroxide, urethane was purchased from Sigma-Aldrich Korea. FBS was from Gibco-BRL (Grand Island, NY, USA). EDTA and collagenase was also from Sigma. MoAbs against CD3e (145-2C11, hamster IgG), CD4 (RM4-5, rat IgG2a), CD8 (53-6.7, rat IgG2a), CD19 (ID3, rat IgG2a), CD25 (3C7, Rat IgG), Gr-1 (RB6-8C5, rat IgG2b), fluorochrome-labeled MoAbs and isotype control IgGs were purchased from BD Biosciences (San Diego, CA, USA), CCR3 (83103, Rat IgG2a), Eotaxin, IgE, IL-4, IL-5, IL-10, IL-13, and IFN- γ ELISA with a MoAbs-based mouse interleukin ELISA kits were purchased from R&D system (Minneapolis, MN, USA). TRIzol reagent was from Invitrogen (ThermoFischer Scientific, Waltham, MA, USA) and DNase I was from Life Technologies (Grand Island, NY, USA). First Strand cDNA Synthesis kit was purchased from Amersham Pharmacia (Piscataway, NJ, USA). Rat IgG was from (Jackson ImmunoResearch, West Grove, PA, USA). FACS buffer with FACS Lysing Solution was from (BD, Franklin Lakes, NJ, USA).

2.2. Identification of luteolin by HPLC analysis

Luteolin was obtained from *Salvia plebeia* R. Br roots as follows: root tissues were ground in a cutting mill, passed through a 50-mesh sieve to obtain a fine powder, and extracted in 10 volumes of distilled water or 50% ethanol (v/w) at 80 °C for 2.5 h in a water bath. The remaining pellets were extracted again with ethanol for 48 h. The resulting ethanol extracts were then extracted with ethyl acetate (EtOAc). The combined EtOAc layers were evaporated to dryness in vacuum, and the residue was separated by chromatography on silica gel to isolate luteolin. The luteolin was further purified by recrystallization from acetonitrile-water. The purity of the luteolin was determined to be > 98% by HPLC analysis (data not shown). The analysis was

performed using a Waters HPLC system equipped with a Waters 600 pump, a Waters 996 PDA detector, an Empower system controller, and an HPLC column (Optima Pak C18 column, 4.6 × 250 mm i.d., 5 mm particle size). The mobile phase consisted of water and acetonitrile (10:90). The column was maintained at 40 °C, and the flow rate was 1.0 ml/min, with PDA detection (200 nm for luteolin and 210 nm for 50% EtOH *Salvia plebeia* R. Br extract analysis). The injection volume was 20 μ l. Sample peaks were assigned according to retention time and the UV spectra of the two standard compounds in the chromatogram.

2.3. Animal experiments

Male BALB/c mice (6–8-week old) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and were housed under pathogen-free conditions. We selected BALB/c mice for our study instead of any other mouse strain like C57BL/6 because BALB/c strain responds efficiently to respiratory ailments (Gueders et al., 2009). All animals were acclimatized for 1 week before use. The animal protocol was approved by the committee for animal welfare at Daejeon University (DJUAR2016-015). This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the South Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

2.4. Sensitization and challenge

According to a modification of a previously described protocol (Griffiths-Johnson et al., 1993), 50 μ l of 1 mg/ml A5503 Albumin Grade V and 80 μ l of 13 mg/ml A8222 Aluminum Hydroxide Gel “Al-hydrogel,” were dissolved in 870 ml of distilled water. Mice were immunized by intraperitoneal (*i.p.*) injection of alum-precipitated antigen (0.25 ml) containing 12.5 μ g of OVA and 0.26 mg of aluminum hydroxide in PBS on three different days (on the first day of 0, 1, and 2 weeks before inhalational exposure). At 3 and 10 days after the first sensitization by intratracheal injection of 2 mg of OVA on the back of the tongue, mice were exposed to OVA by inhalation of an aerosolized OVA solution using an ultrasonic nebulizer (ME-U12, Omron-Tokyo, Japan) for 30 min per day, 3 days per week for 4 weeks (at a flow rate of 250 L/min, 1% OVA in normal saline for first 3 weeks, and 2% OVA in normal saline for last week). Wild-type mice were injected with 0.9% saline (sensitization), and challenged animals were referred to as naive mice. Inhalation was conducted in a plastic chamber connected to the aerosol output from an Aerogen nebulizer (BUXCO, DSI, MN, USA). 24 h after the last challenge, AHR was measured. The mice were euthanized, and allergic airway inflammation was assessed. In the OVA-sensitized model group, mice that developed clinically detectable asthma were randomly assigned to a treatment group and then injected (*i.p.*) with luteolin (20 mg/kg), and cyclosporine A (CsA; 10 mg/kg) daily for six weeks. Measurements of airway reactivity to inhaled methacholine, BAL Fluid, and collection of immune cells from the lungs were conducted 24 h after the last OVA challenge. The schematic experimental method is shown in Fig. 1.

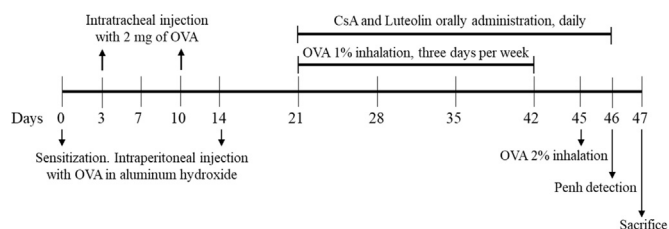


Fig. 1. Schematic figure for experimental method.

2.5. Collection of BAL Fluid and lung cells

BAL Fluid was collected 24 h after the last OVA challenge as follows. Mice were anesthetized by an *i.p.* injection of 10% urethane (100 μ l; Sigma-Aldrich St. Louis, MO, USA). A tracheotomy was performed, and a cannula was inserted into the trachea. Ice-cold DMEM was instilled into the lungs, and BAL Fluid was collected. Total cell counts were obtained with a hemocytometer. For the cytological examination, cells were prepared with a Cytospin (Hanil Science, Gimpo, Korea), fixed, and stained with a modified Diff-Quick stain. Differential cell counts were determined using at least 500 cells on each cytospin slide. Blood was collected by cardiac puncture, allowed to clot, and then centrifuged, and aliquots of serum were stored at -70°C until ELISA. Enzymatic digestion of the lungs was performed as previously described (Hamada et al., 1997; Hamelmann et al., 1997). Briefly, mice were anesthetized, and the lungs were carefully removed. After three washes, the lung was cut into small pieces and then transferred to a 15-ml conical tube containing 20 ml of HBSS with 2% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) and 1 mM EDTA (Sigma, St. Louis, MO, USA) for 30 min at room temperature. After washing, the lung pieces were incubated with 1 mg/ml collagenase (Sigma, St. Louis, MO, USA) with shaking. The lung mixture was then filtered through 70- μ m pore size nylon Cell Strainer (BD Falcon, Bedford, MA, USA) and then centrifuged for 20 min at 450 g. The cell pellet was collected, and the cells were washed twice and stained with various antibodies for FACS analysis.

2.6. Flow cytometric analysis

BAL Fluid cells were incubated with MoAbs against CD3e (145-2C11, hamster IgG), CD4 (RM4-5, rat IgG2a), CD8 (53-6.7, rat IgG2a), CD19 (ID3, rat IgG2a), CD25 (3C7, Rat IgG), and Gr-1 (RB6-8C5, rat IgG2b). All fluorochrome-labeled MoAbs and isotype control IgGs were purchased from BD Biosciences (San Diego, CA, USA), and CCR3 (83103, Rat IgG2a) was purchased from R&D system (Minneapolis, MN, USA). Cells from the lungs and BAL Fluid were incubated with FITC- and PE-labeled MoAbs for 30 min, washed with PBS, fixed with 4% paraformaldehyde for 20 min, washed with PBS, and then stored at 4°C until analysis by two-color flow cytometry on a BD FACSCalibur™ (BD Biosciences, Mountain View, CA, USA).

2.7. Real-time quantitative RT-PCR

Total RNA from lung tissue was isolated with TRIzol reagent (Thermo Scientific, Waltham, MA, USA) and digested with DNase I to remove chromosomal DNA. Then, the DNase I was inactivated by incubation at 75°C for 20 min, and 5 μ g of total RNA was reverse transcribed into cDNA using the First Strand cDNA Synthesis kit (Amersham Pharmacia, Piscataway, NJ, USA). Real-Time quantitative PCR was performed using an Applied Biosystems 7500 Real-Time PCR system according to the manufacturer's instructions. The probe sequences are given in Table 1. Gene expression was analyzed with SYBR Green PCR Master Mix (ABI) and 200 nM primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABI) gene expression was used as an endogenous control. The PCR program was as follows: 2 min at 50°C ,

10 min at 94°C , and 40 cycles of 1 min at 94°C and 1 min at 60°C . The cycle number at which the emission intensity of the sample rose above baseline was referred to as the relative quantity (RQ) and it was proportional to the target concentration. The Real-Time PCR experiment was performed in duplicate and analyzed according to the Applied Biosystems 7500 Fast Real-Time PCR system manual (threshold: 0.05, baseline: 6–15 cycles).

2.8. Measurement of AHR

AHR was measured according to our previously described protocol. Briefly, Penh was estimated using a previously described method with modifications (Kim et al., 2011). A Buxco system (Biosystems XA; DSI Inc., MN, USA) was used to evaluate the extent of airway constriction in mice as described previously. Penh is equal to $\text{Pause} \times \text{PEF}/\text{PIF}$, where $\text{Pause} = (T_e - T_r)/T_r$ (PIF, peak inspiratory flow; PEF, peak expiratory flow; T_e , expiratory time; and T_r , relaxation time). In this experiment, mice were aerosolized with OVA for 30 min per day, 3 days per week for 4 weeks. 24 h after the final inhalation, mice were administered aerosolized normal saline, followed by serial administration of 6.25, 12.5, and 25 mg/ml methacholine (Sigma-Aldrich St. Louis, MO, USA). Airway reactivity was monitored for 30 min.

2.9. BAL Fluid and cytokine measurements

Mice were anesthetized by an *i.p.* injection of urethane (100 μ l), and their lungs were gently lavaged with 1 ml of 0.9% saline via a tracheal cannula. Total and differential BAL Fluid cell counts were determined as previously described (Kim et al., 2011). Samples were centrifuged at 450 g for 10 min, and the supernatants were stored at -80°C . Eotaxin, IgE, IL-4, IL-5, IL-10, IL-13, and IFN- γ production in BAL Fluid and anti-OVA IgE in serum ($n = 5$) was measured by ELISA with a MoAbs antibody-based mouse interleukin ELISA kit (R&D system, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.10. Administration of anti-CD25 and flow cytometric analysis

CD25⁺ cells were depleted by an intraperitoneal injection of CD25-specific, rat IgG1 MoAb. The MoAb (400 μ g/mouse) was dissolved in phosphate buffered saline (PBS) and administered on days 14, 21, 24, and 28 (except where noted) before immunization with 100 μ g of the antigen (OVA). Control animals were administered with rat IgG (Jackson ImmunoResearch, West Grove, PA, USA). CD25⁺ cell depletion was verified by staining blood lymphocytes with antibodies specific for CD4⁺ and CD25⁺ (BD Biosciences, San Diego, CA, USA). Using heparin-coated tubes, blood was collected through a small incision in the lateral tail vein. The blood cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS/0.5% bovine serum albumin) and stained in the dark for 30 min on ice. Subsequently, the cells were washed once with FACS buffer and then treated with FACS Lysing Solution (BD, Franklin Lakes, NJ, USA), which lyses the erythrocytes and fixes the other cells. After washing three more times, the lymphocytes were analyzed using a FACS Caliber and Cell Quest software (BD Biosciences, San Diego, CA, USA).

2.11. Isolation and adoptive transfer of CD4⁺CD25⁺ regulatory T cells

Splenocytes were isolated from naive mice and were enriched for CD4⁺ populations by staining with an anti-CD4 antibody (BD biosciences, San Diego, CA, USA). Then, CD25⁺ cells were isolated from this population by staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD25 MoAb (BD Biosciences, San Diego, CA, USA) and then incubating with magnetic-activated cell sorting anti-FITC beads (Miltenyi Biotec, Auburn, CA). CD4⁺CD25⁺ T cells were selected on a CS column (Miltenyi Biotec, Bergisch Gladbach, Germany), and the flow-through was collected as CD4⁺CD25⁺ T cells. Isolated cells were

Table 1
Sequence of probes for Real-Time PCR.

Probe	Sequence (5' – 3')
IL-13	CGGGTCTGTGTAGCCCTGGATTCC
IL-10	TGAGGCGCTGTCATCGATTTCTCCC
foxp3	AACTTCTAGGGACCAAGGGGCTCTGG
TNF- α	ATGTCCATTCTGAGTCTGCAAAAGGGA
TGF- β 1	TTCAGCGCTCACTGCTCTGTGTACAG
GAPDH	CATCTGCACCACTGCTTACGC

activated by overnight incubation in 24-well plates coated with 1 $\mu\text{g}/\text{ml}$ anti-CD3 (145-2C11), 1 $\mu\text{g}/\text{ml}$ anti-CD28 (37.51), and luteolin (10 $\mu\text{g}/\text{ml}$), which was added to RPMI medium supplemented with 1 U/ml penicillin, 1 $\mu\text{g}/\text{ml}$ streptomycin, 20 mM L-glutamine, 50 $\mu\text{g}/\text{ml}$ β -mercaptoethanol, and 8% FCS for 48 h after stimulation. After harvesting, dead cells were removed by using Lympholyte-M (Cedarlane, Hornby, Ontario, Canada). The viable CD4⁺CD25⁻ cells were determined to be purified. The cells ($2 \times 10^6/\text{mouse}$) were suspended in PBS, sub lethally irradiated (with 600 rad), and then injected intravenously into mice on the same day as immunization.

2.12. Histological examination

The lungs were infused via the trachea with 1 ml of 10% neutral formalin and immersed in the same fixative for at least 24 h. Tissues were paraffinized, and 6- μm sections were cut and stained with H&E and periodic acid-Schiff to assess cell infiltration and mucus production, respectively, under a light microscope. To determine the severity of inflammatory cell infiltration, peribronchial cell counts extent of mucus production and goblet cell hyperplasia in the airway epithelium was blindly quantified using the 5-point (0–4) grading system described by Tanaka et al. (2001). The scoring system was as follows: 0, no cells; 1, a few cells; 2, a ring of cells 2 cell layers deep; 3, a ring of cells 2–4 cell layers deep; 4, a ring of cells 4 cell layers deep.

2.13. Statistical analysis

Data were analyzed by unpaired Student's *t*-test and analyses of variance (ANOVA) followed by Duncan's multiple range tests using SPSS version 14.0. The difference between the normal and control groups (OVA + vehicle) was evident; however, the significance of the difference between these groups was not shown in the figures and tables to emphasize the statistical differences between the experimental and control groups. The results are presented as mean $\pm$ standard error of the mean, and the significance of differences is denoted as follows: * $P < 0.05$ (compared with WT); and * $P < 0.05$ (compared with OVA-CTL), as determined by Student's *t*-test.

3. Results

3.1. Effects of luteolin on airway inflammation in OVA-sensitized mice

Luteolin (46.3 mg/g) was obtained by extraction of *Salvia plebeia* R. Br roots with 50% EtOH and was quantified by HPLC analysis (Fig. 2A). Airway responsiveness to methacholine was significantly increased in mice after OVA sensitization and OVA challenge. Mice sensitized to OVA via inhalation challenge showed a minimal change in Penh in response to methacholine. As previously reported, luteolin and CsA significantly inhibited airway responsiveness to methacholine (at doses of 6.25, 12.5, and 25 mg/ml) in OVA-CTL mice (Fig. 2B). Luteolin had an inhibitory effect on airway responsiveness 30 min after methacholine administration, and the increase in Penh in response to methacholine (6.25, 12.5, and 25 mg/ml) was inhibited by 57.3%, 51.3%, and 44.7%, respectively. The Penh response to methacholine (25 mg/ml methacholine for 30 min) was significantly reduced in luteolin-treated mice (20 mg/kg, $P < 0.05$) and CsA-treated mice (10 mg/kg, $P < 0.01$; Fig. 2B). OVA challenge dramatically increased OVA-specific IgE production in the BAL Fluid of OVA-sensitized mice (Fig. 2C). However, OVA-specific IgE production in OVA-CTL mice was substantially suppressed by treatment with luteolin ($P < 0.05$) or CsA ($P < 0.01$). These results showed that luteolin treatment suppressed OVA-specific IgE production of B cells in OVA-CTL mice. Fig. 2D, panels a–d shows the histological analysis of airway inflammation with H&E staining and Fig. 2D, panels e–h show Masson's trichrome staining. The lungs of wild-type animals injected with saline showed no inflammation (Fig. 2D, panels a and e, Fig. 2E), whereas the lungs of OVA-CTL mice showed

extensive peribronchial and perivascular inflammatory infiltrates (Fig. 2D, panels b and f, Fig. 2E). In contrast, lung tissue from 10 mg/kg CsA *i.p.*-treated sensitized mice showed minor histologic inflammation (Fig. 2D, panels c and g, Fig. 2E). The majority of the infiltrated inflammatory cells were eosinophils. Histological analysis showed reduced inflammatory infiltrates in the lungs of mice sensitized with 20 mg/kg luteolin *p.o.* (Fig. 2D, panels d and h, Fig. 2E), 10 mg/kg CsA *i.p.* (Fig. 2D, panels c and g, Fig. 2E). Luteolin treatment markedly attenuated eosinophil-rich leukocyte infiltration when compared to that of the OVA-CTL mice (Fig. 2D, panels b and f, Fig. 2E).

3.2. Decreased numbers of immune cells and eosinophilic airway inflammation in OVA plus luteolin-treated mice

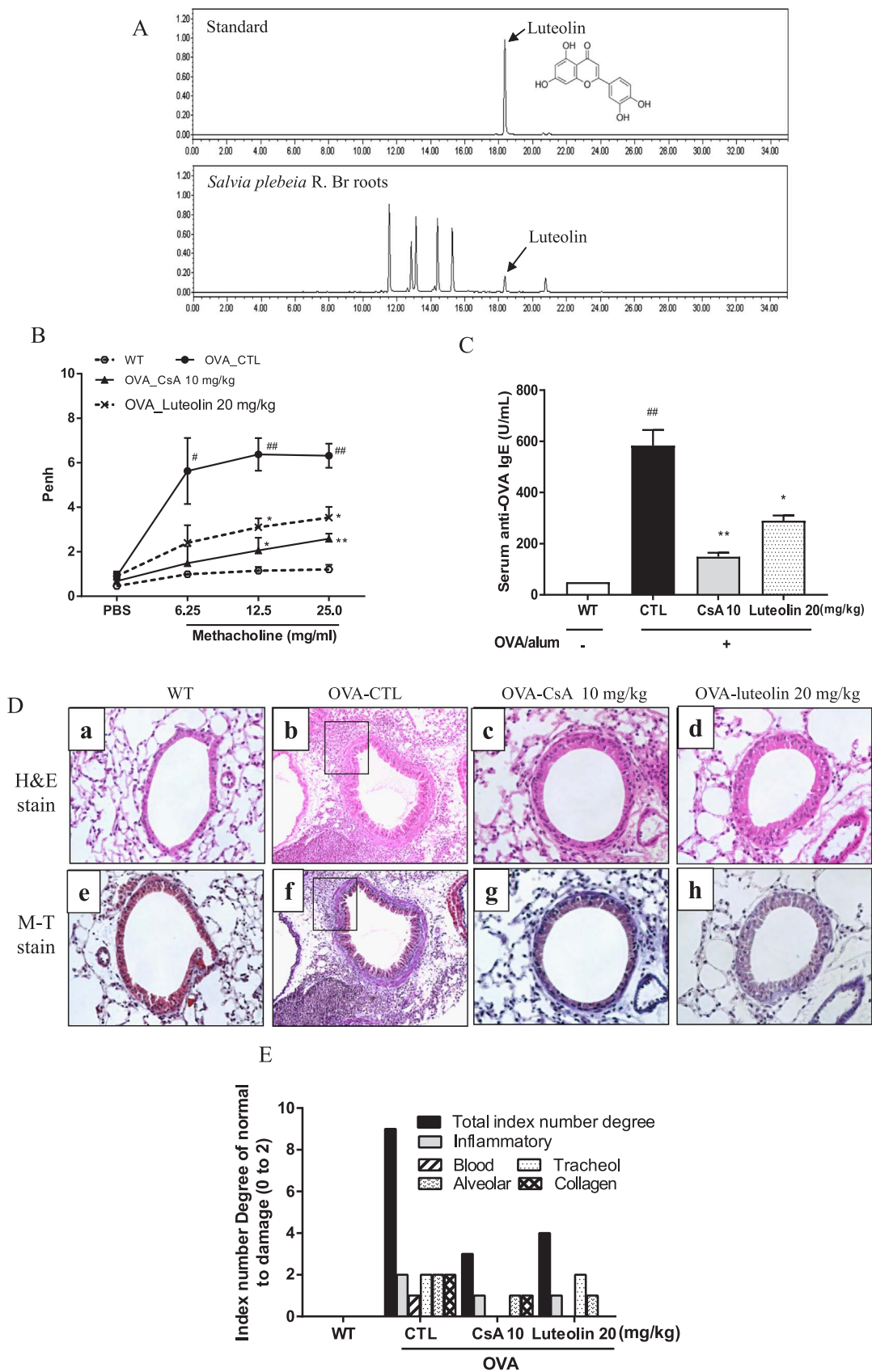
The total numbers of CD19⁺ B cells, CD4⁺ T cells, CD3⁺CCR3⁺, and CD11b⁺Gr-1⁺ cells in the lung were significantly elevated in OVA-CTL mice as compared with the corresponding numbers in naïve mice (Fig. 3A). In contrast, the total numbers of CD19⁺ B cells, CD4⁺ T cells, CD3⁺CCR3⁺, and CD11b⁺Gr-1⁺ cells in the lungs of mice sensitized and treated with luteolin and CsA were significantly lower ($P < 0.05$) than those in OVA-CTL mice. Moreover, luteolin and CsA treatment suppressed the infiltration of CD19⁺ B cells, CD4⁺ T cells, CD3⁺CCR3⁺, and CD11b⁺Gr-1⁺ cells in the lungs of OVA-sensitized mice, however, eosinophils was markedly elevated in the BAL Fluid of OVA-CTL mice as compared to naïve mice (Fig. 3B). In contrast, treatment with luteolin (20 mg/kg, $P < 0.05$) or CsA (10 mg/kg) substantially reduced the number of eosinophils in BAL Fluid compared with that in OVA-CTL mice. The results showed that lungs from luteolin-treated mice had reduced eotaxin 2 (3.3-fold) and CCR3 (2-fold) mRNA levels, as did the lungs from CsA-treated mice had reduced eotaxin 2 (5-fold) and CCR3 (2.5-fold; Fig. 3C). These results, in combination with the effects of luteolin and CsA on inflammatory cell infiltration in the lung, suggest the possibility of bystander suppression in certain aspects of the eosinophil response induced by luteolin. Luteolin (20 mg/kg) treatment substantially reduced the percentage of CD3⁺CCR3⁺ and CD3e⁺CCR3⁺ cells in BAL Fluid compared with the numbers in OVA-CTL mice. However, a higher percentage of CD3e⁺CCR3⁺ cells were detected in the lungs of OVA-CTL mice compared to those in naïve mice (data in naïve mice not shown). Luteolin treatment substantially reduced the percentage of CD3e⁺CCR3⁺ cells in the lung compared with the percentage in OVA-CTL mice (Fig. 3D).

3.3. Cytokine expression in OVA plus luteolin-treated mice

The levels of IL-13, TNF- α , IL-10, and TGF- β 1 mRNA in the lungs of OVA-sensitized mice were upregulated when compared with the corresponding levels in wild type. Treatment with CsA substantially reduced IL-13, TNF- α , IL-10, and TGF- β 1 mRNA levels. Treatment with luteolin substantially reduced IL-13, TNF- α mRNA levels. The most interesting point was that treatment with luteolin substantially upregulated foxp3, IL-10, and TGF- β 1 mRNA levels in the lungs when compared to the levels in OVA-CTL control mice (Fig. 4A). To determine the levels of various cytokines *in vivo*, BAL Fluid samples were collected 24 h after the last OVA aerosol challenge. As shown in Fig. 4B, OVA inhalation in sensitized mice induced substantial Th2 cytokine release in BAL Fluid as compared with the Th2 cytokines levels in naïve mice (wild type). Luteolin and CsA significantly ($P < 0.05$) reduced IL-4, 5, and 13, levels in BAL Fluid as compared with the Th2 cytokines levels in OVA-CTL control mice. In contrast, luteolin did not have any significant effects on IL-10 level, but it significantly upregulated IFN- γ levels.

3.4. Effects of luteolin on regulatory T cells *in vitro*

As shown in Fig. 5A, with or without luteolin pre-incubation, the CD4⁺CD25⁻ starting cell population showed CD25 upregulation at 48 h



(caption on next page)

Fig. 2. Inhibition of airway inflammation by luteolin in an OVA-induced mouse model of asthma (A) Structure of luteolin, purified from the root of *Salvia plebeia* R. Br at 210 nm by HPLC. (B) Inhibitory effect of luteolin on airway hyperreactivity (AHR) in response to methacholine. OVA-CTL mice were administered either 20 mg/kg luteolin, 10 mg/kg cyclosporine A (CsA), or an equal volume of PBS and then were challenged OVA by inhalation. 24 h after the final OVA challenge, AHR was measured using a Buxco system in which mice were exposed to increasing amounts of methacholine (6.25, 12.5, and 25 mg/ml). Values are expressed as mean $\pm$ S.E.M ($n = 8$ mice per group from two separate experiments). (C) Luteolin inhibited OVA specific-IgE production in bronchoalveolar lavage fluid (BAL Fluid) from OVA-CTL mice. BAL Fluid from WT, OVA-CTL, OVA-CsA, and OVA-luteolin sensitized mice was assayed for OVA specific-IgE. (D) Effect of luteolin treatment on lung histopathology in OVA-CTL mice. Lungs were fixed, sectioned, and stained with hematoxylin and eosin (H&E) and Masson's trichrome (M-T) stains. Representative sections from each treatment group are shown. (E) Quantitative analyses of the degree of lung tissue damage in the sections. Data are from individual mice, with the arithmetic mean points shown in histograms. Values are expressed as mean $\pm$ S.E.M ($n = 8$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with WT), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with OVA-CTL) as determined by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests.

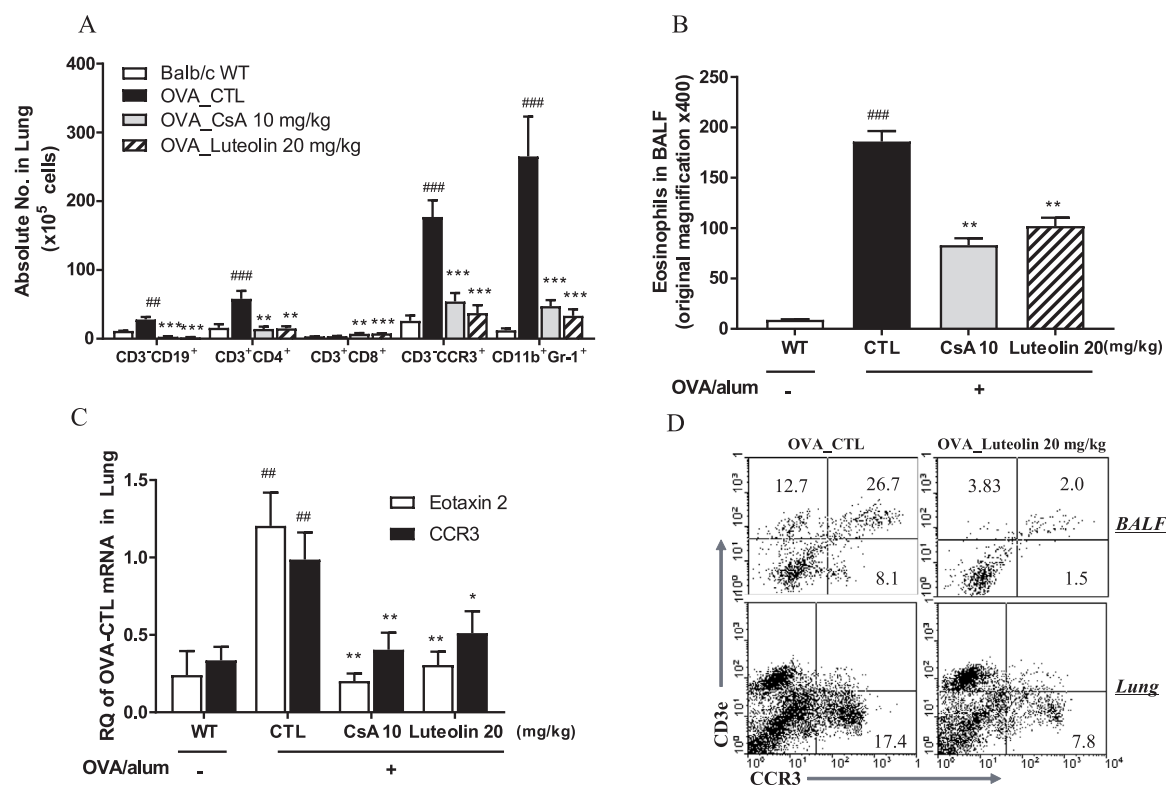


Fig. 3. Protective effects of luteolin against lymphocyte infiltration into the lung during airway inflammation. (A) The absolute numbers of CD3⁺CD19⁺ (B cells), CD3⁺CD4⁺ (helper T cells), CD3⁺CD8⁺ (c/s T cells), CD3⁺CCR3⁺ (eosinophils), and CD11b⁺Gr-1⁺ (granulocytes) lymphocytes are shown. The columns indicate the mean $\pm$ S.E.M from eight mice. (B) Luteolin reduced the numbers of eosinophils in BAL Fluid. Values are expressed as the median ($n = 8$ mice per group from two independent experiments). (C) Expression of eotaxin2 and CCR3 mRNA in OVA-sensitized mice repressed by luteolin. (D) Flow cytometry gated on CD3e⁺, CCR3⁺ cells, with the numbers of the indicated immune cells in the lung and BAL Fluid shown as dot plots. The quadrant markers were set so that > 99% of control Ig-stained cells were included in the lower left quadrant. The numbers in the quadrants indicate the mean percentages of cells that were negative and/or positive for CD3e and/or CCR3. Values are expressed as mean $\pm$ S.E.M ($n = 8$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with WT), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with OVA-CTL) as determined by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests.

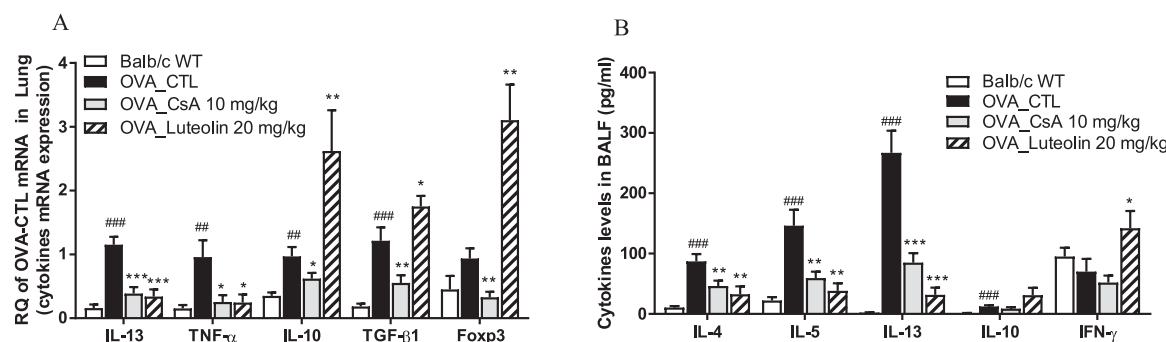


Fig. 4. Changes in cytokine production in BAL Fluid from OVA-induced asthma model mice following luteolin treatment (A) Effects of luteolin on the expression of cytokine mRNA in lung tissues. IL-13, TNF- α , IL-10, TGF- β 1, and *foxp3* expression was determined by quantitative real-time PCR. The changes in gene expression in the lung tissue of CsA- and luteolin-treated mice were compared with their expression in OVA-CTL mice. Expression is presented as relative quantitation (RQ). (B) Luteolin reduced the number of eosinophils in BAL Fluid. IL-4, IL-5, IL-13, IL-10, and IFN- γ levels in BAL Fluid from naïve, OVA-CTL, OVA-CsA, and OVA-luteolin treated mice were measured by ELISA. Values are expressed as mean $\pm$ S.E.M ($n = 8$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with WT), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with OVA-CTL) as determined by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests.

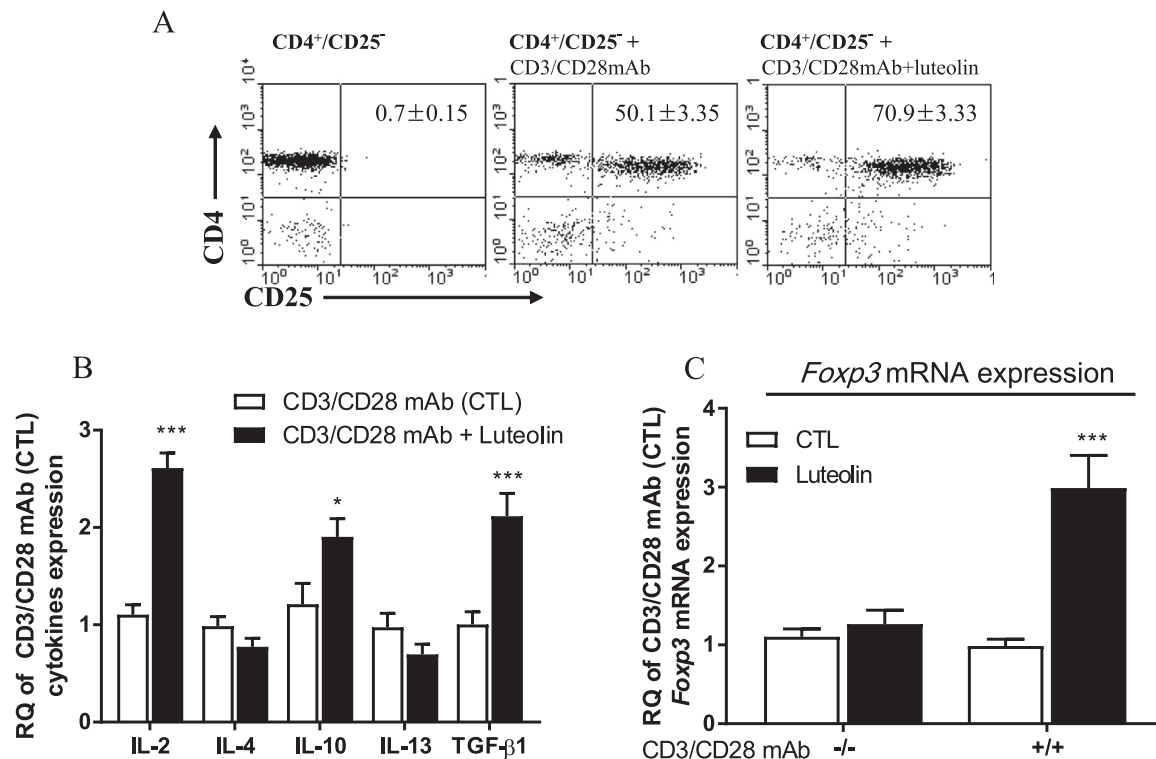


Fig. 5. Luteolin induced phenotype switching of CD4⁺/CD25⁻ cells toward CD4⁺/CD25⁺ cells *in vitro*. (A) Upregulation of CD25 expression in CD3/CD28-activated CD4⁺/CD25⁻ T cells. Isolated CD4⁺/CD25⁻ T cells were stimulated with an anti-CD3/CD28 antibody or an anti-CD3/CD28 antibody and luteolin (10 μg/ml) for 96 h, and then the cell phenotype measured by FACS. (B) Luteolin enhanced CD4⁺/CD25⁺ Treg-related cytokine expression and downregulated cytokine mRNA expression in anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells. IL-2, IL-4, IL-10, IL-13, and TGF-β1 expression was determined by quantitative real-time PCR, which is reported as relative quantitation (RQ). (C) Luteolin synergistically upregulated *foxp3* expression with anti-CD3/CD28 stimulation in CD4⁺/CD25⁻ T cells. *Foxp3* mRNA expression was quantified by Real-time PCR. Values are expressed as mean ± S.E.M (n = 5). *P < 0.05, **P < 0.01, and ***P < 0.001 (compared with CD3/CD28 MoAb [CTL]) as determined by unpaired Student's t-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests.

after stimulation with anti-CD3/CD28 plate-bound Ab. The mean percentages of CD4⁺/CD25⁺ Tregs obtained from three different donors were 0.7 ± 0.15 (CD4⁺/CD25⁻ starting cell), 50.1 ± 3.35 (CD4⁺/CD25⁻ starting cell plus anti-CD3/CD28), and 70.9 ± 3.33 (CD4⁺/CD25⁻ starting cell plus anti-CD3/CD28 plus luteolin). Interestingly, CD25 expression was upregulated in luteolin-treated anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells compared with the expression in untreated anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells. The relative expression of IL-2, IL-10, and TGF-β1 mRNA was upregulated in luteolin-treated anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells compared with the levels in untreated anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells. In contrast, luteolin-treated anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells showed reduced IL-4 and IL-13 mRNA levels compared with untreated anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells (Fig. 5B). We next determined whether luteolin had an impact on the induction of *foxp3* in CD4⁺/CD25⁻ T cells and anti-CD3/CD28-activated CD4⁺/CD25⁻ T cells. As expected, purified, splenocyte-derived, luteolin-treated CD4⁺/CD25⁻ T cells showed higher *foxp3* mRNA levels than CD4⁺/CD25⁻ T cells (control). When CD4⁺/CD25⁻ T cells were co-cultured under the same stimulatory conditions in the presence of anti-CD3/CD28 antibody plus luteolin, *foxp3* mRNA expression was also induced, consistent with the acquisition of Treg suppressive function. Furthermore, 96 h co-culture with luteolin resulted in > 2.989-fold upregulation of *foxp3* (Fig. 5C).

3.5. Isolation and depletion of CD25 MoAb in OVA-sensitized mice

CD25 MoAbs were purified from mouse ascites, and their quality and purity were analyzed by indirect IL-2R ELISA (Fig. 6A). The anti-CD25 MoAb PC61 is capable of depleting CD25 cells *in vivo*. Mice were injected intraperitoneally with 0.5 mg of PC61 (anti-CD25) 2 and 4 days before induction of asthma (OVA). One day after the last injection (day 24), CD25⁺ T cells were markedly depleted from the periphral

blood (Fig. 6B, from 3.94% to 0.38%). The anti-CD25 treatment almost completely depleted cells with high expression of CD25, which are considered Treg cells, in contrast to CD4⁺ T cells with low or intermediate levels of CD25 expression. FACS histograms showed CD25 expression on CD4⁺ cells before (upper dot plot) and 1 week after (lower dot plot) depletion of CD25⁺ cells. The percentages of CD4⁺/CD25⁺ cells in non-depleted (upper dot plot) and anti-CD25 depleted mice (lower dot plot) are shown.

3.6. Suppression of eosinophilia in CD25-depleted and Treg-adoptive transferred OVA-sensitized mice

The CD25-depleted OVA-sensitized mice were challenged with OVA after administration of the anti-CD25 MoAb (PC61). The absolute numbers of CD19⁺ B cells, CD4⁺ T cells, CD3⁺CCR3⁺ cells, and CD11b⁺Gr-1⁺ cells in the lung were significantly elevated in CD25-depleted OVA-sensitized mice (control) compared to the corresponding numbers in naïve mice, whereas the number of CD8⁺ T cells were limited (Fig. 7A). In contrast, the total absolute numbers of CD19⁺ B cells, CD4⁺ T cells, CD3⁺CCR3⁺ cells, and CD11b⁺Gr-1⁺ cells in the lungs of mice treated with luteolin, CsA, and iTreg adoptive transfer were significantly lower than the numbers in CD25-depleted OVA-CTL mice. The absolute numbers of CD8⁺ T cells in the lungs of mice treated with luteolin and CsA did not change when compared with CD25-depleted OVA-CTL mice whereas significantly increased in iTreg adoptive transfer (Fig. 7A).

The number of eosinophils was markedly elevated in BAL Fluid from CD25-depleted OVA-CTL mice (Fig. 7B, Fig. 7C, (panel b)) compared to the number in naïve mice (Fig. 7B, and Fig. 7C, (panel a)). In contrast, iTreg adoptive transfer (Fig. 7C, (panel e)), CsA (Fig. 7C, (panel c)), luteolin (Fig. 7C, (panel d)) substantially (P < 0.05) reduced the number of eosinophils in BAL Fluid compared to that in CD25-depleted OVA-CTL mice (Fig. 7C, (panel b)).

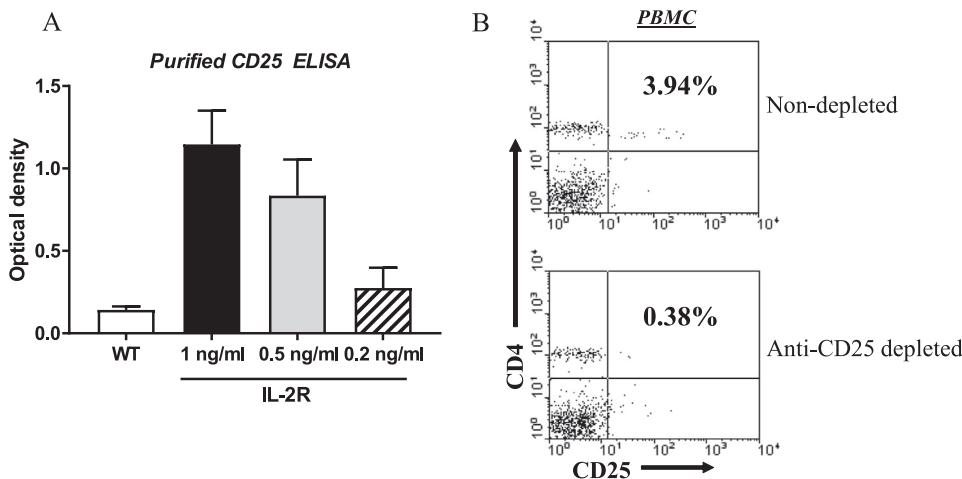


Fig. 6. CD25 MoAb isolation and CD25 depletion in OVA-treated mice. (A) Stepwise increase in IL-2R levels in mice that were administered a purified CD25 MoAb. (B) BALB/c mice were depleted of CD25⁺ cells by treatment with an anti-CD25 MoAb. Mice were injected intraperitoneally with 0.5 mg of PC61 (anti-CD25) or rat IgG for 1 week before OVA/alum *i.p.* administration. Blood cells from non-depleted and CD25 cell-depleted mice were stained with CD4⁺- and CD25-specific MoAb. CD4⁺CD25⁺ T cells constituted 5 ~ 10% of the CD4⁺ cells from naive BALB/c mice.

3.7. Induction of Tregs by iTreg-adoptive transfer in OVA-sensitized mice

foxp3 and TGF- β expression were increased by 1.57-fold and 3.44-fold, respectively, in luteolin-treated CD25-depleted OVA-sensitized mice (Fig. 8A, $P < 0.05$) as compared with the corresponding levels in CD25-depleted OVA-CTL mice (CD25-Depl.-CTL). As shown in Fig. 8B, OVA inhalation in sensitized mice substantially increased eotaxin, IgE, IL-5, and IL-13 levels in BAL Fluid as compared to the corresponding levels in naive mice. Treatment with luteolin or CsA and iTreg adoptive transfer significantly reduced eotaxin, IgE, IL-5, and IL-13 levels in BAL Fluid compared with the corresponding levels in CD25-Depl.-CTL. However, luteolin treatment and iTreg adoptive transfer significantly upregulated IFN- γ levels.

3.8. Suppression of airway inflammation by iTreg-adoptive transfer in OVA-sensitized mice

We next investigated the effect of iTreg adoptive transfer on the development of AHR in BALB/c mice. For this experiment, responsiveness to methacholine was assessed by whole-body plethysmography

and measuring the increase in Penh as an index of airway obstruction. Sensitized animals challenged with OVA (1% as an aerosol) for 20 min daily for 3 consecutive days developed AHR in response to methacholine inhalation. iTreg adoptive transfer dramatically prevented AHR in response to inhaled methacholine (12.5 and 25 mg/ml; Fig. 9A, $P < 0.05$), suggesting that the immune-mediated pathology was modified. Lung tissue was collected 24 h after the last OVA challenge. CD25-Depl.-CTL mice (Fig. 9B, panels b and c) induced marked inflammatory cell infiltration in the blood and in tracheal, alveolar, peribronchiolar, and perivascular connective tissues when compared with the numbers of inflammatory cells in saline-challenged mice (Fig. 9B, (panel a)). The CsA-treated (Fig. 9B, (panel d)), luteolin-treated (Fig. 9B, (panel e)), and iTreg adoptive transfer (Fig. 9B, (panel f)) groups showed marked attenuation of the eosinophil-rich leukocyte infiltration in the blood and alveolar spaces as compared with that in the CD25-Depl.-CTL (CTL; Fig. 9C). In contrast, CD25-depleted OVA-sensitized mice developed marked goblet cell hyperplasia and mucus hypersecretion within the bronchi of the lung (Fig. 9B, (panels b and c)), and also showed mucus plug formation in the bronchial lumen (Fig. 9B, (panel c)). The degree of lung tissue damage was significantly abated by treatment with CsA or

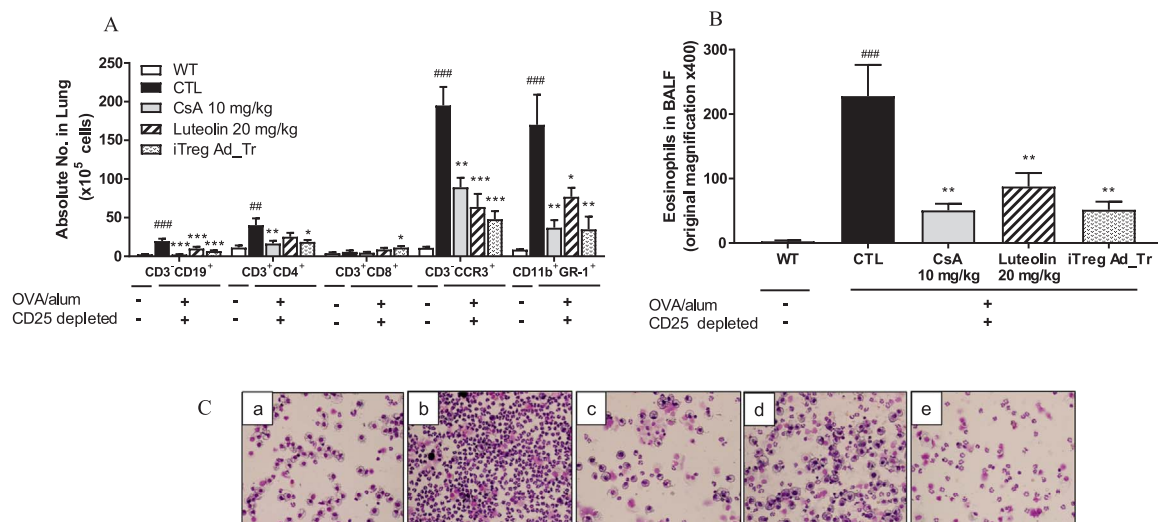


Fig. 7. Adoptive transfer of luteolin-induced CD4⁺CD25⁺ Treg cells (iTreg) in CD25-depleted OVA-mice. (A) iTreg-adoptive transfer attenuated immune cell recruitment in the lungs of OVA-treated mice. The absolute numbers of immune cells in the lungs of mice were CD25-depleted or not in OVA-CTL mice are shown. Values are expressed as mean $\pm$ S.E.M ($n = 5$ mice per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests. (B) Treg-adoptive transfer suppressed eosinophilia in BAL Fluid. (C) Photomicrograph of BAL Fluid cytopins from CD25-depleted OVA-CTL mice. BALB/c normal (a), CD25-depleted OVA-sensitized control mice (CD25-Depl.-CTL) (b), 10 mg/kg cyclosporin A-treated CD25-depleted OVA-sensitized mice (c), 20 mg/kg luteolin-treated CD25-depleted OVA-sensitized mice (d), transfer of luteolin-treated CD4⁺CD25⁺*foxp3*⁺ Treg cells in CD25-depleted OVA-sensitized mice (e) (magnification, 200 $\times$). Values are expressed as mean $\pm$ S.E.M ($n = 5$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with WT), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (compared with CD25-Depl.-CTL) as determined by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests.

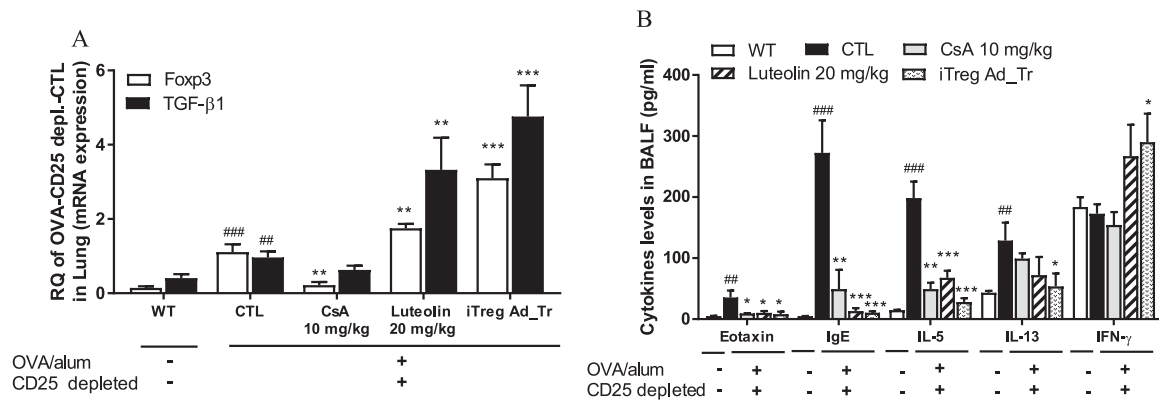


Fig. 8. Induction of Tregs by Treg-adoptive transfer in OVA-treated mice. (A) Gene expression in lung tissues following Treg-adoptive transfer in OVA-treated mice. *Foxp3* and *TGF-β1* expression was determined by real-time PCR, which is reported as relative quantitation (RQ) assays. Data shown are the $CD4^+CD25^+$ cells in the lung tissue that were depleted or not in OVA-sensitized mice. In OVA-CTL mice treated with CsA or luteolin or adoptive transfer of luteolin-treated $CD4^+CD25^+$ T cells, the fold increase in *foxp3* and *TGF-β1* mRNA levels was similar to that in CD25-Depl.-CTL mice. Normalized *foxp3* and *TGF-β1* values were determined as the ratio of *foxp3* and *TGF-β1* expression to GAPDH expression. (B) Inflammatory mediators and IFN-γ levels in BAL Fluid from Treg adoptively transferred CD25-depleted OVA-sensitized mice. Eotaxin, IgE, IL-5, IL-13, and IFN-γ levels were measured in BAL Fluid by ELISA. Values are expressed as the mean $\pm$ S.E.M ($n = 5$). $^{\#}P < 0.05$, $^{##}P < 0.01$, and $^{###}P < 0.001$ (compared with WT), $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (compared with CD25-Depl.-CTL) as determined by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests.

luteolin and iTreg adoptive transfer as compared with the damage in the control CD25-Depl.-CTL (Fig. 9C).

4. Discussion

Salvia plebeia R. Br. (Labiatae) is an annual or biennial herb growing in mountainous regions that is used as a medicinal plant in Asia (Nugroho et al., 2012). In traditional medicine, *S. plebeia* is used for the treatment of hepatitis and inflammation (Lu and Foo, 2002). *S. plebeia*

extract (SPE) has a variety of biological actions, including anti-oxidant, anti-tumor, and anti-inflammatory activities (Jin et al., 2008). Recent studies showed that an ethanol extract of *S. plebeia* ameliorated the inflammatory response to LPS and/or TNF-α stimulation in RAW 264.7 and BEAS-2B cells, and improved the histopathological changes in the lungs in a mouse model of asthma (Jang et al., 2016).

Our present findings demonstrated that induction of $CD4^+CD25^+$ regulatory T cells by luteolin could attenuate OVA-induced pulmonary inflammation, release of Th2 cytokines and chemokines in the airway,

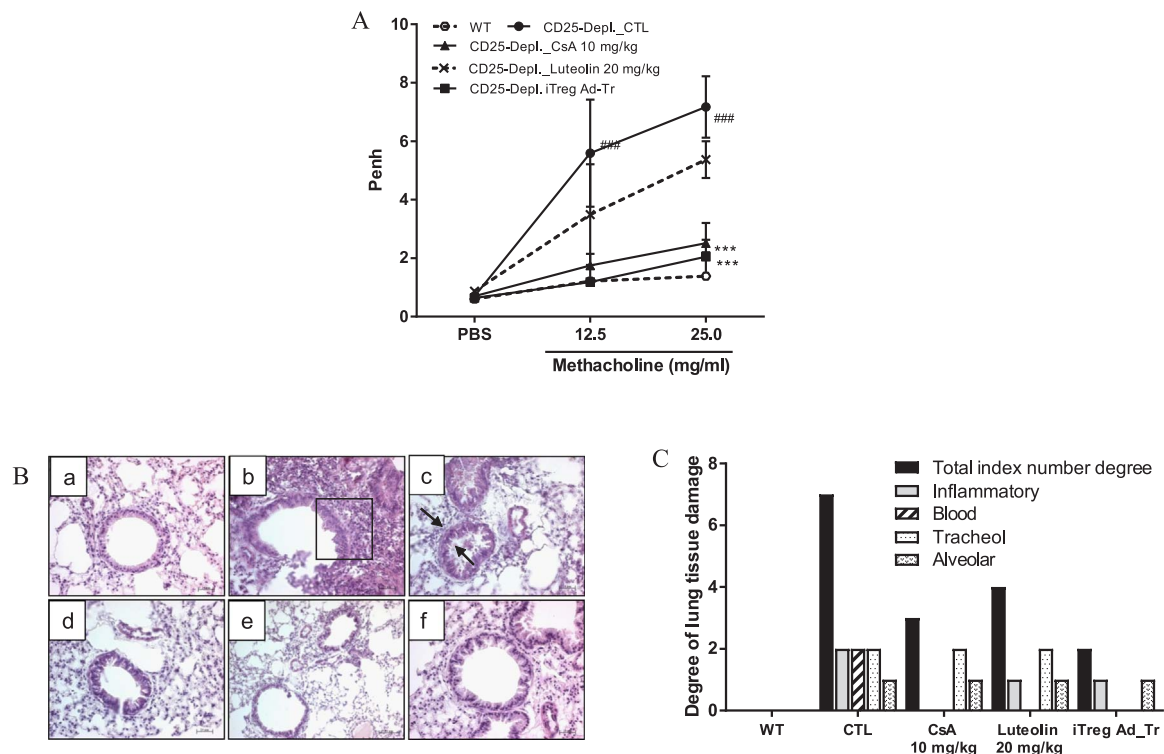


Fig. 9. Suppression of airway inflammation by iTreg-adoptive transfer into OVA-treated mice. (A) Airway responsiveness to methacholine in iTreg adoptively transferred CD25-depleted OVA-sensitized mice. Values are expressed as mean $\pm$ S.E.M ($n = 5$). $^{\#}P < 0.05$, $^{##}P < 0.01$, and $^{###}P < 0.001$ (compared with WT), $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (compared with CD25-Depl.-CTL) as determined by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests. (B) CD25-Depl.-CTL mice (Fig. 9B-(b-c)) induced marked infiltration of inflammatory cells (black square), blood, tracheal, and alveolar into the peribronchiolar and perivascular connective tissues (black arrow) as compared with WT mice (Fig. 9B (a)). The majority of the infiltrated inflammatory cells were eosinophils. CsA (Fig. 9B(d)), luteolin treatment (Fig. 9B(e)), and iTreg adoptive transfer mice (Fig. 9B (f)) markedly attenuated the eosinophil-rich leukocyte infiltration into blood, and alveolar spaces stained with periodic acid-Schiff reagent. (C) Quantitative analyses of the degree of lung tissue damage in the sections were performed based on the method of Tanaka et al. (2001).

airway mucus production, serum IgE levels, and AHR in OVA-sensitized mice. There is now clear evidence that Th2 cells play an essential role in the pathogenesis of allergic airway inflammation (Herrick and Bottomly, 2003; Wilson et al., 2005). Our present data showed that luteolin significantly reduced the levels of IgE, IL-4, IL-5, and IL-13 in BAL Fluid and the absolute numbers of CD19⁺ B cells, CD4⁺ T cells, CD3⁺CCR3⁺ cells, and CD3e⁺Gr-1⁺ cells in the lung tissue. In contrast, the level of IFN- γ , a Th1 cytokine, was increased by luteolin treatment.

Our present findings also showed that luteolin prevented eosinophil infiltration into the airway, as shown by a significant drop in total inflammatory cell counts and eosinophil counts in BAL Fluid (Fig. 3B). Similarly, tissue eosinophilia was also inhibited by luteolin treatment, as revealed by the significant reduction inflammatory cell infiltration. Eosinophil transmigration into the airways is a multistep process that is orchestrated by Th2 cytokines such as IL-4, IL-5, and IL-13, and specific chemokines, such as eotaxin, in combination with CCR3 (Larche et al., 2003; Lukacs, 2001). IL-13 is the most potent inducer of eotaxin expression in airway epithelial cells (Erin et al., 2002). Our results showed that eotaxin and CCR3 mRNA expression was substantially reduced in the lung tissue of luteolin-treated mice, which may be associated with the significant drop in IL-13 levels in BAL Fluid following treatment with luteolin.

In this study, luteolin treatment upregulated IL-10 and TGF- β production *in vivo* as shown in (Fig. 4A). In contrast, the level of IFN- γ in BAL Fluid, a Th1 cytokine, was increased by luteolin treatment (Fig. 4B). It is unclear why IFN- γ was also enhanced in BAL Fluid, but it is possible that an increase in iTreg alone may not be sufficient for downregulation of Th2 cells and Th2 cytokines (Dardalhon et al., 2008). The present data suggest that luteolin induced iTreg was more responsive than IFN- γ under both Th1 and Th2 conditions. Thus, it appeared that luteolin induced iTreg was a more important controller of Th1/Th2 differentiation than T-bet, consistent with reports that treatment with luteolin substantially upregulated IL-10 ($P < 0.01$), TGF- β 1 ($P < 0.05$), and *foxp3* ($P < 0.01$) mRNA levels in the lungs when compared to the levels in OVA-CTL mice (Fig. 4A).

Luteolin treatment significantly increased the numbers of CD4⁺CD25⁺ regulatory T cells among the anti-CD3/anti-CD28-stimulated murine splenic CD4⁺ T cells (< 97%) *in vitro*, and significantly increased IL-10, IL-2, and TGF- β mRNA expression (Fig. 5B), suggesting that luteolin induces the differentiation of CD4⁺CD25⁺ cells into CD4⁺CD25⁺ T cells. An important observation in this study was the elevated proportion of CD4⁺CD25⁺ T cells in the lung tissue of OVA-sensitized mice in response to luteolin treatment. Although CD4⁺CD25⁺ T cells are present at a relatively low frequency in the lung tissue of chronic asthma patients, these cells could control disease progression by inhibiting the functions of other pathogenic activated T cells, granulocytes, and macrophages (Lee et al., 2005). An increase in the proportion of CD4⁺CD25⁺ T cells and a corresponding decrease in CD11b⁺Gr-1⁺ granulocyte infiltration into the lungs of OVA-sensitized mice treated with luteolin suggest the possibility that luteolin may ameliorate asthma, at least in part; by inducing the infiltration of CD4⁺CD25⁺ regulatory T cells into the inflamed tissue. However, CD25 is expressed on both regulatory and activated effector T cells (Shi et al., 2004); therefore, it is insufficient to define the infiltrating CD4⁺CD25⁺ T cells as regulatory T cells. Therefore, we examined the expression levels of *foxp3* as the most specific marker of regulatory T cells (Wei et al., 2004). CD4⁺CD25⁺ regulatory T cells express *foxp3*, a fork head transcription factor that appears to be a master controller of Treg development and function and, thus far, the best Treg marker (Ling et al., 2004). *Foxp3* encodes the transcription factor scurfin, which binds to the promoter region of cytokine genes and attenuates the production of activation-induced cytokines, such as IL-2, thus preventing the proliferation of regulatory T cells upon stimulation (Chen et al., 2003). CD4⁺CD25⁺ T cells exert suppressive activity when transduced with the *foxp3* gene or isolated from *foxp3*-transgenic mice (Hori et al., 2003; Schubert et al., 2001). These findings

support the notion that CD4⁺CD25⁺*foxp3*⁺ cells are a distinct lineage of regulatory T cells (Shi et al., 2004). In this study, anti-CD3/anti-CD28-stimulated murine splenic CD4⁺ T cells (< 97%) showed a significant increase in *Foxp3* mRNA expression after treatment with luteolin *in vitro* (Fig. 5C). This suggests that luteolin treatment may directly drive expansion of the CD4⁺CD25⁺ regulatory T cell population, in the same way that was shown for estrogen in a recent study (Khattri et al., 2003).

Mouse studies have shown that adoptive transfer of a Th2 cell-polarized CD4⁺ T cell population depleted of CD4⁺CD25⁺ cells resulted in increased airway eosinophilia compared with adoptive transfer of unfractionated Th2 cells (Jaffar et al., 2004). In humans, the reduction of allergic symptoms after successful allergen immunotherapy was associated with the appearance of IL-10-producing Treg (Francis et al., 2003; Jutel et al., 2003). In animal studies, a population of CD4⁺ T cells induced by OVA (alum) immunization has been shown to inhibit the development of IgE response (Curotto de Lafaille et al., 2001). Exposure to inhaled allergen before systemic sensitization induces decreased IgE synthesis and airway eosinophilia by promoting the development of CD4⁺ Tregs expressing surface-bound TGF- β (Ostroukhova and Ray, 2005). Interestingly, exposure to heat-killed *Mycobacterium vaccae* gives rise to a population of IL-10- and TGF- β -producing Tregs that limit IgE synthesis, type 2 cytokine production, and AHR after subsequent allergen exposure (Zuany-Amorim et al., 2002). Similarly, systemic allergen sensitization in the presence of killed *Listeria monocytogenes* markedly reduces airway inflammation and AHR by facilitating the development of a unique population of Tregs that produce IL-10 and IFN- γ and express inducible T-cell costimulatory molecule (ICOS), T-bet, and *foxp3* (Stock et al., 2004).

Therefore we used adoptive transfer of luteolin-induced CD4⁺CD25⁺*foxp3*⁺ (iTreg) cells, to demonstrate whether these cells have functional regulatory T cell activities. Transfer of iTreg cells into OVA-sensitized mice reduced eosinophilia (Fig. 7B) as well as the numbers of Th2 cells, Th2 cytokines, eotaxin, and IgE secretion in BAL Fluid (Fig. 8B). *In vitro*, the phenomenon is contact dependent, but can be observed in the absence of either IL-10 or TGF- β (Polanczyk et al., 2004; Thornton and Shevach, 1998). In contrast, we showed that luteolin upregulated TGF- β in the lung after transfer of iTreg cells, and this was observed along with abrogation of AHR and allergic inflammation. Moreover, we highlighted the importance of luteolin treatment as it abolished the suppressive effect of the CD4⁺CD25⁺ regulatory T cells, and AHR, eosinophilia, and Th2 cytokine levels after treatment were comparable to those observed in the absence of regulatory cells.

The data presented in this paper suggest that induction of T regulatory cells by luteolin may represent a novel treatment for allergic asthma. Indeed, it has been previously demonstrated that non-allergic individuals have a higher percentage of iTreg cells, whereas allergic individuals are characterized by a higher percentage of allergen-specific IL-4-producing cells (Akdis et al., 2004). Strategies for inducing expansion of the naturally occurring CD4⁺CD25⁺ population and enhancement of regulatory T cell function by luteolin and other drugs such as corticosteroids, has been demonstrated *in vitro* (Piccirillo et al., 2002). Alternatively, it may be possible to induce T regulatory cell populations with luteolin treatment *in vivo*, as has been described in several different murine systems using mycobacterial exposure by airway delivery, before sensitization and challenge, or *in vitro* by stimulation in the presence of immunosuppressive drugs (Barrat et al., 2002; Nguyen et al., 2007).

5. Conclusion

In conclusion, luteolin was found to be effective for suppression of airway inflammation and upregulation of Th2 in OVA-sensitized mice. Collectively, these observations suggest that the molecules involved in modulating iTreg cell activity are potential targets for the development

of novel therapies for asthma. Here, we provided, for the first time, direct evidence that adoptive transfer of iTreg cells in OVA-sensitized mice abrogates the features of allergic airway disease *in vivo*. Thus, our data suggest that strategies designed to maximize the function of luteolin-induced Tregs *in vivo* could benefit allergic asthmatic patients.

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Conflict of interest

All authors have declared no conflict of interest.

Declarations of interest

none.

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