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Ovatodiolide suppresses allergic airway inflammation and hyperresponsiveness in a murine model of asthma

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ABSTRACT

Asthma, a complex pulmonary allergic disease, major therapy is applied of drugs to control the disease, but quickly recur after the drugs are stopped. In patients with severe asthma may show steroid resistance and would benefit from the development of novel therapeutic drugs.

Ovatodiolide, a unique macrocyclic diterpenoid isolated from *Anisomeles indica*, showed therapeutic potential for the treatment of allergic asthma. As a model of allergic inflammation, we used ovalbumin (OVA)-immunized mice, which displayed T helper cell type 2 (T_H2) cytokine expression in bronchoalveolar lavage fluid (BALF), as well as airway inflammation and hyperresponsiveness (AHR). The results showed that ovatodiolide suppressed T_H2 activation, including cell proliferation and production of the T_H2 related cytokines, interleukin (IL)-4, IL-5, IL-13, IL-33, eosinophil chemotactic protein (eotaxin), and also reduced airway hyperresponsiveness. In this study, ovatodiolide inhibited allergic asthma through downregulation of T_H2 responses in a murine model of asthma.

1. Introduction

Allergic asthma is a chronic airway inflammation disorder characterized clinically by the hyper-secretion of mucus, inflammation of airways, and airway hyperresponsiveness (AHR) (Gandhi et al., 2016), leading to symptoms such as coughing, chest tightness, and wheezing (Lowhagen, 2012). Asthma has become a prevalent public health concern worldwide, according to a World Health Organization (WHO) report, and is a multifactorial disease that is initiated via a cascade of immune responses. It has been suggested that the T helper cell type 2 (T_H2) subset of CD4⁺ cells plays a critical role in allergic asthma. Overproduction of T_H2 cells and associated cytokines, such as interleukin (IL)-4, 5, and 13, plays a crucial role in the pathogenesis of allergic asthma. IL-4 triggers B cell activation and immunoglobulin E (IgE) production, which binds to high-affinity Fcε receptors I (FcεRI) on mast cells. IL-5 activates eosinophil proliferation, maturation, and

recruitment to lung tissue, as well as secretion of inflammatory cation components. IL-13 directly contributes to AHR and mucus hypersecretion (Hammad and Lambrecht, 2015). T_H2 cells are important effector cells that participate in airway inflammation.

Anisomeles indica (L) Kuntze (Labiatae) is a traditional Chinese herbal medicine used as an anti-inflammatory agent in Taiwan. It is used to treat inflammatory disease, gastric dysfunction, and hypertension (Barbosa-Filho et al., 2006; Lin et al., 2011). Ovatodiolide is a bioactive cembrane-type diterpenoid isolated from *Anisomeles indica* (L) (Liao et al., 2012; Rao et al., 2009). Previous studies suggested that ovatodiolide exhibits multiple biological activities, including anti-inflammatory (Rao et al., 2009), anti-cancer (Barbosa-Filho et al., 2006; Hou et al., 2009; Hsieh et al., 2008; Liao et al., 2012), anti-bacterial (Lien et al., 2013; Rao et al., 2012) and anti-HIV activity (Hou et al., 2009). Ovatodiolide has been shown to inhibit bone marrow-derived dendritic cell (BMDC) maturation and T_H2 cell differentiation

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in vitro study (Rao et al., 2013); however, the therapeutic effect of ovatodiolide in vivo is still unclear. Therefore, we investigated the therapeutic effect of ovatodiolide on allergic asthma symptoms in vivo, including airway inflammation, T_H2 cytokine production in bronchoalveolar lavage fluids (BALF), and AHR, in a murine model of allergic asthma.

2. Materials and methods

2.1. Reagents and chemicals

Ovalbumin (OVA) (grade V) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hank's balanced salt solution (HBSS) was obtained from Invitrogen (Carlsbad, CA, USA). Aluminum hydroxide was purchased from ThermoFisher Scientific (AlumImject; Pierce Chemical, Rockford, IL, USA).

2.2. Plant material, extraction and isolation of ovatodiolide

Whole plants of *A. indica* (L) were collected in October 2009 from the Yuli, Hualien County in Eastern Taiwan, and a botanically identified voucher specimen (YMT-09-02) was deposited at the Herbarium of the Institute of Biochemical Sciences and Technology, Chao yang University of Technology, Taiwan. The compound of ovatodiolide was obtained following by the extraction with ethanol and isolation procedures as previously reported (Rao et al., 2012) and its purity (> 95%) was confirmed by HPLC. Ovatodiolide used in this study was endotoxin free. The chemical structure of ovatodiolide is shown in Fig. 1A.

2.3. Animals and experimental protocol

Female BALB/c mice, aged six to eight weeks, were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, Taiwan. The protocols for animal care and handling were approved by the Animal Committee of China Medical University. The animals were divided into two groups, each following a different treatment protocol. In protocol 1 (Fig. 1B), mice were sensitized with a total volume of 200 μ l phosphate buffered saline (PBS) by intraperitoneal injection (i.p.) on days 0, 14, and 28. Mice were then intratracheally instilled (i.t.) with 30 μ l of PBS containing either 1% pure dimethyl sulfoxide (DMSO) (vehicle control), or different concentrations of ovatodiolide (0.25 μ g/mouse, 1.23 μ g/mouse, or 2.46 μ g/mouse) once per day on days 35–39. Ovatodiolide was dissolved in PBS containing 1% pure DMSO. In protocol 2 (Fig. 1C), mice were sensitized by i.p. injection of 50 μ g of OVA bound with 2 mg aluminum hydroxide (AlumImject; Pierce Chemical, Rockford, IL, USA) dissolved in PBS of total 200 μ l volume on day 0, boosted with 25 μ g of OVA combined with 2 mg of aluminum hydroxide on days 14 and 28, and challenged three times with 100 μ g OVA dissolved in PBS of total 30 μ l volume by using intranasal (i.n.) administration on days 40–42. Various doses of ovatodiolide were delivered to anaesthetized animals intratracheally once daily on days 35–39 (five treatments). There were six to eight mice per group.

2.4. Bronchoalveolar lavage and lung histology

Mice were killed by CO_2 . Bronchoalveolar lavage was performed using 1 ml of HBSS instilled by syringe and 1 ml of BALF was gently aspirated. This was repeated three times. All BALF samples were centrifuged. Supernatant from the first BALF sample was collected and

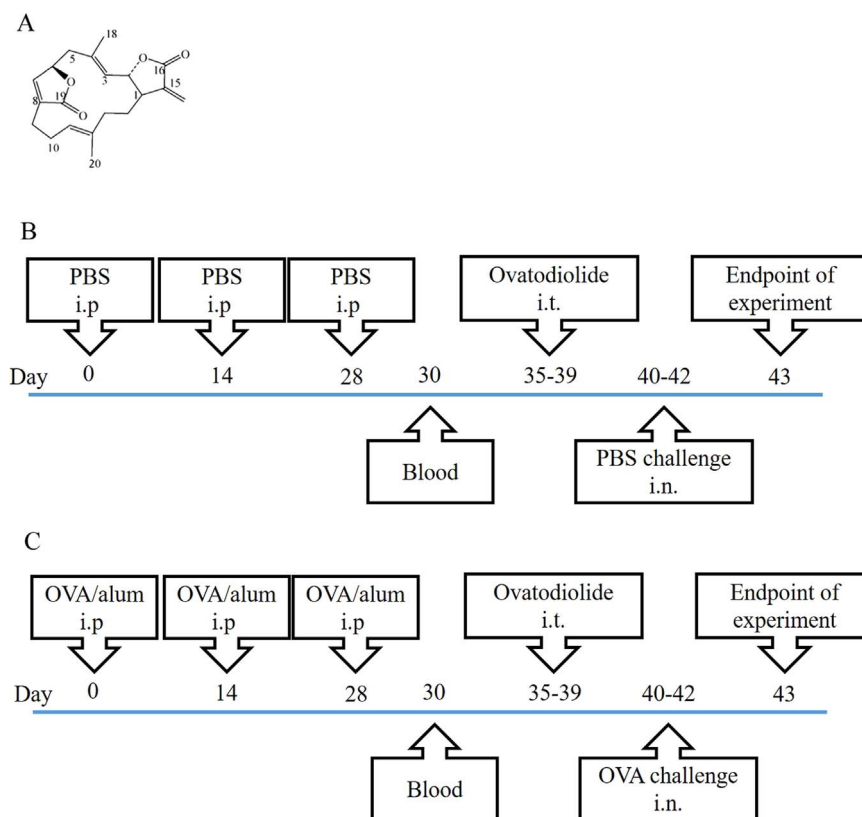


Fig. 1. Ovatodiolide chemical structure and animal study protocol. (A) Chemical structure of ovatodiolide. (B) Brief scheme of animal sensitization and challenge. Mice were separated into four groups: PBS control, 0.25 μ g of ovatodiolide, 1.23 μ g and 2.46 μ g of ovatodiolide; i.n., intranasal; i.t., intratracheal. (C) Animal sensitization and challenge by OVA. Ovatodiolide was dissolved in PBS containing 1% pure DMSO. Mice were intratracheally instilled with 30 μ l of PBS containing 1% pure DMSO (OVA group), 0.25 μ g-mouse⁻¹ group ovatodiolide, 1.23 μ g-mouse⁻¹ group ovatodiolide, or 2.46 μ g-mouse⁻¹ group ovatodiolide once a day from day 35–39. The PBS group represents mice injected intraperitoneally and intranasally with PBS only.

stored at -20°C for further cytokine analysis by enzyme-linked immunosorbent assay (ELISA). BALF cells from all three aspirations were washed three times with HBSS. Total cell numbers were counted with a hemacytometer. Cytospin slides of the BALF cells were prepared using a cytospin and stained with May-Giemsa stain. Differential cell counts were determined by a total count of 300 cells for at least four fields from the slide under a light microscope.

Lungs were fixed with 10% formalin, and sections were prepared for staining with hematoxylin/eosin (H/E) and periodic acid–Schiff (PAS) in order to quantify the number of infiltrating inflammatory cells and mucus production by microscopy. Quantification of airway inflammation, determined by the number of PAS-positive bronchial epithelial cells, was performed according to previous study (Lee et al., 2010). Inflammatory cell infiltration was quantified by counting the number of inflammatory cells in subepithelial and subendothelial area, and expressed as the number of inflammatory cells per subepithelial and subendothelial area (mm^2) in lung sections.

Mucus-producing cells were quantified as the percentage of PAS-positive cells per bronchiole and a mean score was derived from the scores of three sections from each individual mouse.

2.5. Preparation of lymph node cells

Mediastinal lymph nodes were harvested and pooled together from each group after mice kill at day 43. A total of 2×10^5 cells were cultured in 96-well culture plates and stimulated with $100\text{ }\mu\text{g/ml}$ OVA for 72 h. The cell medium was collected for further cytokine analysis.

2.6. Cytokine assay

The cell culture medium and BALF supernatants were collected and stored at -20°C prior to ELISA, according to the manufacturer's instructions (R & D systems, Minneapolis, MN, USA).

2.7. Cell proliferation assay

Mediastinal lymph node cells after re-stimulation of $100\text{ }\mu\text{g/ml}$ OVA for 56 h and were then pulsed with 5-bromo-2-deoxyuridine (BrdU) for measurement of cell proliferation. Briefly, 2 nM of BrdU was added to each well, and the plate was incubated at 37°C for 16 h. The amount of BrdU incorporated into the T-cells was measured using the anti-BrdU monoclonal antibody in the enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Roche Applied Science, Germany).

2.8. Measurement of airway resistance in anesthetized mice

Airway hyperreactivity responses to methacholine (Mch) were measured by a forced oscillation technique (FlexiVent; SCIREQ). Airway resistance (R) increased after challenge with aerosolized MCh (Sigma). Mice were anaesthetized by i.p. injection with 2 mg/kg Rompun (TS, Bayer, Leverkusen, Germany) combined with 4 mg/kg Zoletil (Virbac, Carros, France). An 18 G soft needle was inserted into the trachea to expose the trachea to MCh. Mice were connected to a computer-controlled ventilator and mechanically ventilated with a tidal volume of 0.3 ml at a frequency of 150 breaths/min and a positive end-expiratory pressure of $3\text{--}4\text{ cm H}_2\text{O}$. Mice were then exposed directly through the ventilator to increasing five doses of MCh (0, 1.5, 3.125, 6.25, and 12.5 mg/ml). Data was collected every 20 s for five min and airway resistance was calculated using a software program (Model PNM-PCT100W, LDS PONEMAH Physiology Platform, LDS GOULD).

2.9. Tissue homogenization

Tissue section was weighted immediately when being removed from -80°C . It was placed into 3 ml polypropylene tube and added with 1 ml

ice-cold REzol™ C&T kit (Protech Technology Enterprise Co., Ltd Taipei, Taiwan) or homogenization buffer (25 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM benzamidine, 1 mM phenylmethane sulfonyl fluoride (PMSF), pH 7.4) per 0.1 g lung tissue weight. Tissue section was bursted vigorously in the homogenization at max speed once per 5 s , and the desired homogenate was transferred to 1.5 ml eppendorff and stored at -80°C for further experiment using.

2.10. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was reverse transcribed into cDNA by using the oligo (dT) primer. Quantitative real-time PCR using SYBR Green Master Mix was performed with a model 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). After preincubation at 50°C for 2 min and 95°C for 10 min, the PCR was performed as 40 cycles of 95°C for 10 s and 60°C for 1 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as CT) (ABI system Fast- Real-Time PCR Getting Started Guide). The sequences of the primers are as follows: for IL-4, forward $5'\text{-CTCATGGAGCTGCAGAGACTCTT-3'}$ and reverse $5'\text{-CATTTCATGGTGCAGCTTATCGA-3'}$; for IL-5, forward $5'\text{-TGACC GCCAAAAGAGAAGTG-3'}$ and reverse $5'\text{-GAACCTTTCAG GTAATCCAGGAA-3'}$; for IL-13, forward $5'\text{-CGCAAGGCCCCACTAC-3'}$ and reverse $5'\text{-AAAGTGGGCTACTTCGATTTTGG-3'}$; for GATA-3, forward $5'\text{-CAGAACCGGCCCTTATCA-3'}$ and reverse $5'\text{-ACAGTTCGCGCAGGATGTC-3'}$; for c-maf, forward $5'\text{-AGAGG CGGACCCTGAAAAA-3'}$ and reverse $5'\text{-GTGTCTCTGCACCCTCTT-3'}$; for T-bet, forward $5'\text{-CTGGATGCGCCAGGAAGT-3'}$ and reverse $5'\text{-TGTTGGAAGCCCCCTTGT-3'}$; for IL-33, forward $5'\text{-ACCAGGTG CTACGCTACTATGAG-3'}$ and reverse $5'\text{-CACACCGTCGCTGATTG-3'}$; for β -actin, forward $5'\text{-ACTGCCGCATCTCTTCCT-3'}$ and reverse $5'\text{-ACCGCTCGTTGCCAATAGTG-3'}$.

2.11. Western blot analysis

Lung tissues were homogenized with lysis buffer (25 mM Tris–HCl, 2 mM EDTA, 1 mM benzamidine, 1 mM PMSF, pH 7.4) containing protease and phosphatase inhibitors. Appropriate volumes of the homogenates, each well containing $50\text{ }\mu\text{g}$ proteins, were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (PVDF). The blots were probed with primary antibodies against trans-acting T-cell-specific transcription factor (also known as GATA-3, SC-268, mouse monoclonal, Santa Cruz, Heidelberg, Germany), phosphorylated signal transducer and activator of transcription 6 (also known as STAT-6, ab13679, mouse monoclonal, Cambridge, UK), STAT-6 (SC-268, mouse monoclonal, Santa Cruz, Heidelberg, Germany), IL-33 (ALX-804-840, mouse monoclonal, Enzo Life Sciences, Farmingdale, NY, USA), and β -actin (#NB300-327, mouse monoclonal, Novus Biologicals, Littleton, CO). Following incubation with horseradish peroxidase-conjugated anti-mouse secondary antibodies, the signals were visualized via enhanced chemiluminescence by using a Millipore ECL kit (Millipore, Bedford, MA, USA) in accordance with the manufacturer's instructions.

2.12. Statistical analysis

All results are given as means $\pm$ standard deviation. ($N \geq 3$). Group comparisons were determined by one-way ANOVA follow by Newman–Keuls post-hoc test. $P < 0.05$ was considered significant. The potential correlation among different groups by Pearson correlation coefficient by using SAS 9.2 software.

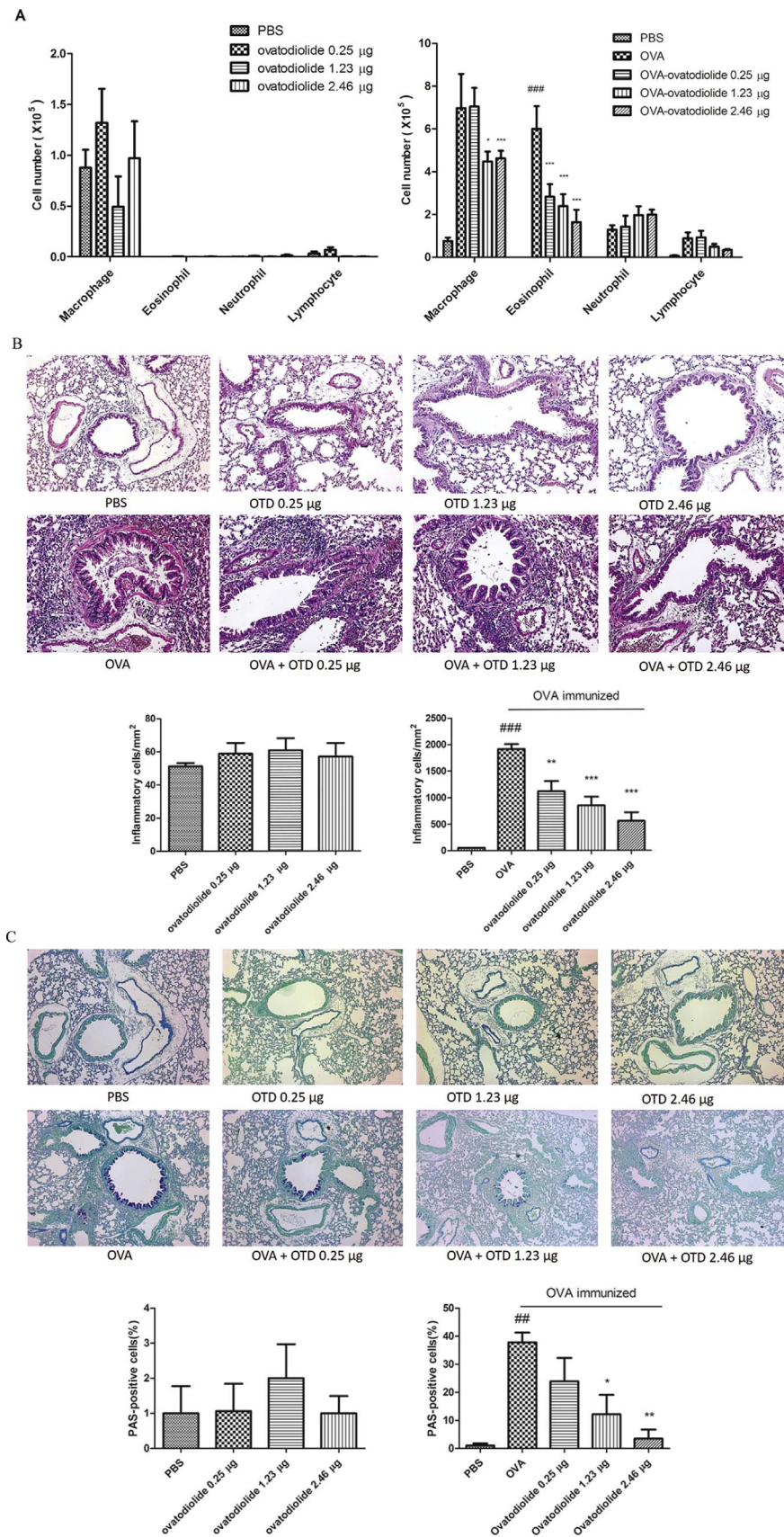


Fig. 2. Airway inflammation was inhibited by ovatodioidide. The cell profile of the BALF and histological analysis of lung tissue in mice after ovatodioidide treatment, shown as the daily dose (μ g) given by intratracheal instillation for five days, are presented. (A) Total cell counts were determined on 3 ml BALF and differential cell counts were assessed by May-Giemsa staining. Data were expressed as the mean $\pm$ standard deviation ($n > 7$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the PBS group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the OVA group without ovatodioidide treatment. (B) Lung sections were stained with hematoxylin/eosin (H & E) for the measurement of inflammatory cells, and the data represented that cellular infiltration into the peri-airway region. (C) Lung sections were also stained with periodic acid–Schiff (PAS) staining for the measurement of mucus production around the airways. Original magnification: 200 $\times$. Quantification of lung inflammation and mucus production as described in Material and Methods. Data were expressed as the mean $\pm$ standard deviation ($n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the PBS group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the OVA group.

3. Results

3.1. Inhibition of allergen-induced airway inflammation by ovatodiolide

First, we examined the effects of ovatodiolide on airway inflammation in a murine model of asthma. The chemical structure of ovatodiolide is shown in Fig. 1A and the animal protocols are presented in Fig. 1B and C. Ovatodiolide was instilled intratracheally at doses of 0.25, 1.23, and 2.46 $\mu\text{g}/\text{mouse}$ once a day for five days from days 35–39. The results show that the BALF cell profile had no obvious inflammatory cell infiltration in PBS-sensitized and challenged mice groups (Fig. 2A, left panel). In OVA-immunized mice, after administration of various doses of ovatodiolide, displayed a dose-dependent and significant decrease in inflammatory cell infiltration, including eosinophils and macrophages (Fig. 2A, right panel). Histology assessment of lung sections from the OVA group revealed a large amount of infiltrated cells (Fig. 2B) and mucus over-production around the airways (Fig. 2C), compared with PBS controls. After treatment with various doses, ovatodiolide remarkably inhibited inflammatory cell infiltration and mucus over-production (Fig. 2B, C).

3.2. Intratracheal instillation of ovatodiolide suppressed AHR in a murine model of asthma

Next, we examined whether ovatodiolide could affect the development of AHR in a murine model of asthma. Airway responsiveness was tested by measurement of pulmonary resistance using an invasive plethysmography. OVA-immunized mice displayed increased lung resistance (RL) after Methacholine inhalation as compared to PBS-sensitized and challenged mice (Fig. 3). After intratracheal delivery of ovatodiolide, lung resistance significantly decreased in the OVA-immunized mice (Fig. 3). We further investigated the potential relationship between the inhibition of the T_H2 -specific cytokines IL-4, IL-5, and IL-13 and airway hyperresponsiveness (AHR). As shown in the Supplementary information (Fig. S3), the protein expression of IL-4, IL-5, and IL-13 in BALF showed strong positive linear correlation with airway hyperresponsiveness.

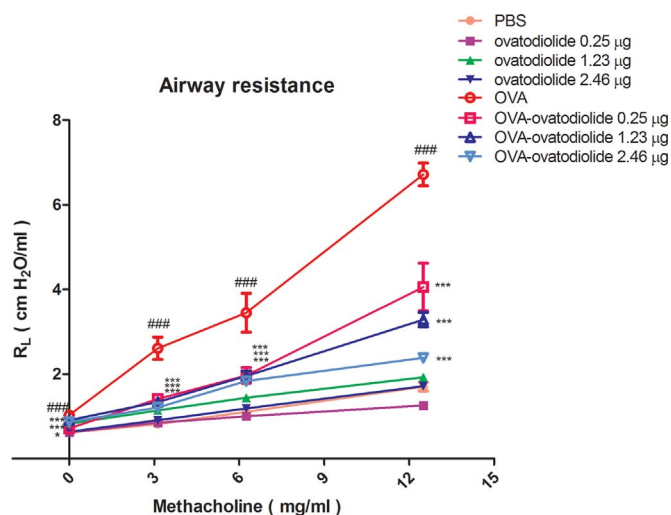


Fig. 3. Suppression of AHR after administration of ovatodiolide in a murine model of asthma. (A) Airway resistance was measured by invasive body plethysmography. Data were expressed as mean $\pm$ standard deviation. of the value of pulmonary resistance (RL) ($n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the PBS group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the OVA group.

3.3. Ovatodiolide reduced OVA-induced IgE production in serum and lung tissue

In our allergic asthma mouse model of allergic asthma, we sensitized mice by intraperitoneal injection to induce systemic lung inflammation and administered ovatodiolide treatment by local intratracheal instillation to inhibit lung inflammation. Our data showed that mice receiving OVA treatment exhibited significantly higher OVA-specific IgE production than the PBS group both in serum and lung tissue homogenate (Fig. 4A and B). After treatment with ovatodiolide, there was no obvious change in serum OVA-specific IgE expression among OVA-immunized groups, but OVA-specific IgE in lung tissue homogenate showed a significant decrease in the 2.46 $\mu\text{g}/\text{mouse}$ ovatodiolide group. As we administered ovatodiolide by intratracheal instillation, we thought that it might only decrease OVA-specific IgE by local, but not systemic, delivery of ovatodiolide.

3.4. Intratracheal instillation of ovatodiolide decreased T_H2 cells activation through GATA-3 gene expression

Next, to investigate the mechanisms of ovatodiolide-induced inhibition of airway inflammation, we measured the cytokines expression profile in the BALF one day after the final mice challenge. The OVA-immunized group displayed a dramatic increase in the level of IL-4, 5, and 13 and eotaxin-1 in the BALF compared with the PBS group (Fig. 5A). After treatment with ovatodiolide, T_H2 -related cytokines, including IL-4, 5, and 13 and eotaxin-1, dose-dependently decreased in the BALF of the OVA-immunized mice. Besides, ovatodiolide did not affect T_H1 -related cytokine- interferon- γ (IFN- γ). We examined the potential correlation between T_H2 cytokine production and mRNA expression and found that the protein expression of IL-4, IL-5, and IL-13 in BALF showed positive linear correlation with their respective mRNA expression; the correlation was strong for IL-5 and IL-13 and moderate for IL-4 (Supplementary information Fig. S2).

Through further investigation of the T cells specific transcription factor mRNA expression in the lung, we found that OVA-immunized mice showed significantly increase T_H2 specific transcription factors GATA-3 (Fig. 6A) and c-Maf (Fig. 6B) mRNA expression but not the T_H1 (Fig. 6C) transcription factor, T-box transcription factor TBX21 (T-bet). Mice intratracheally receive of ovatodiolide resulted in a dose-dependent inhibit GATA-3 and c-maf mRNA expression but not affect T-bet mRNA expression. Previous reports indicated that GATA-3 was a key regulator of the development of T_H2 cell lineage by an increase in the T_H2 cytokine gene expression (Barnes, 2011) and GATA-3 gene expression was regulated by STAT6 activation via the phosphorylation of cytosolic STAT6 (Murphy et al., 1999). As shown in Fig. 6D, ovatodiolide dose-dependently suppressed OVA-induced STAT-6 phosphorylation and GATA-3 protein expression in lung tissue. Therefore, the ovatodiolide regulation might arise from interference in STAT-6 phosphorylation and subsequent decrease in GATA-3 gene expression, which led to the downregulation of the T_H2 response in lung tissue. Further, as shown in Fig. S1, the protein expressions of IL-4, IL-5, and IL-13 in BALF showed positive linear correlation with GATA-3, c-Maf, and T-bet mRNA expression; the correlation was strong for IL-4 and IL-13 and moderate for IL-5. The observed correlation between IL-4, IL-5, and IL-13 protein expression and T-bet mRNA expression was unexpected.

To identify whether ovatodiolide directly affected T_H2 cells activation, we isolated $CD4^+$ T cells from mediastinal lymph nodes to examine. As shown in Fig. 7, $CD4^+$ T cells from mediastinal lymph nodes re-stimulated with OVA for 72 h. The supernatants and cells were analyzed for cytokine production and cell proliferation using ELISA assays and BrdU incorporation. The number of $CD4^+$ T cells in mediastinal lymph nodes increased in OVA-immunized mice as compared to that in PBS control mice. Mice receiving various doses of ovatodiolide intratracheally displayed a significant decrease in $CD4^+$

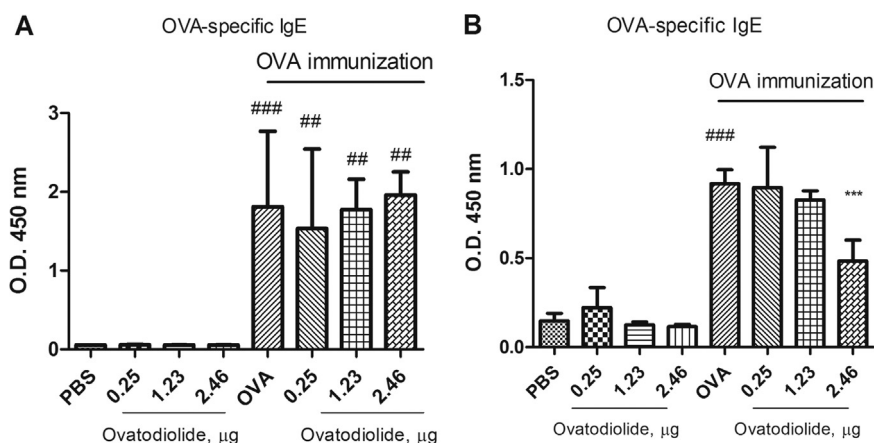


Fig. 4. Suppression of OVA-specific IgE production after administration of ovatodioid in serum and in the lung tissue homogenate of a murine model of asthma. (A) Serum was obtained from blood samples collected on day 43 and (B) lung tissue homogenate and the samples were analyzed by an OVA-specific IgE ELISA assay. Data were expressed as the mean $\pm$ standard deviation (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001, compared with the PBS group; *P < 0.05; **P < 0.01; ***P < 0.001, compared with the OVA group.

T lymphocyte cells proliferation (Fig. 7A). Analysis of type of CD4⁺ T cells activation by cytokine production of CD4⁺ T cells (Fig. 7B), ovatodioid dramatically inhibited T_H2 specific cytokine, IL-4, but did not affect T_H1 cell specific cytokine- IFN- γ .

3.5. Ovatodioid inhibited IL-33 production in lung tissues

IL-33 belongs to alarmin which shows obviously increasing in airway in allergic asthma patients, especially in severe asthma. Study

also reported that IL-33 has steroid-resistance effects and contributes the pathology of severe asthma (Saglan et al., 2013). Therefore, we also detected IL-33 expression in lung tissues in OVA-immunized mice. We found that OVA-immunized mice induced large amount of IL-33 mRNA expression in whole lung homogenate compare with PBS control mice (Fig. 8A). After treatment with 2.46 μ g of ovatodioid, and IL-33 mRNA was dramatically decreased. To further examine IL-33 protein expression in lung tissue of OVA-immunized mice, we analyzed IL-33 protein expression by western blotting. As shown in

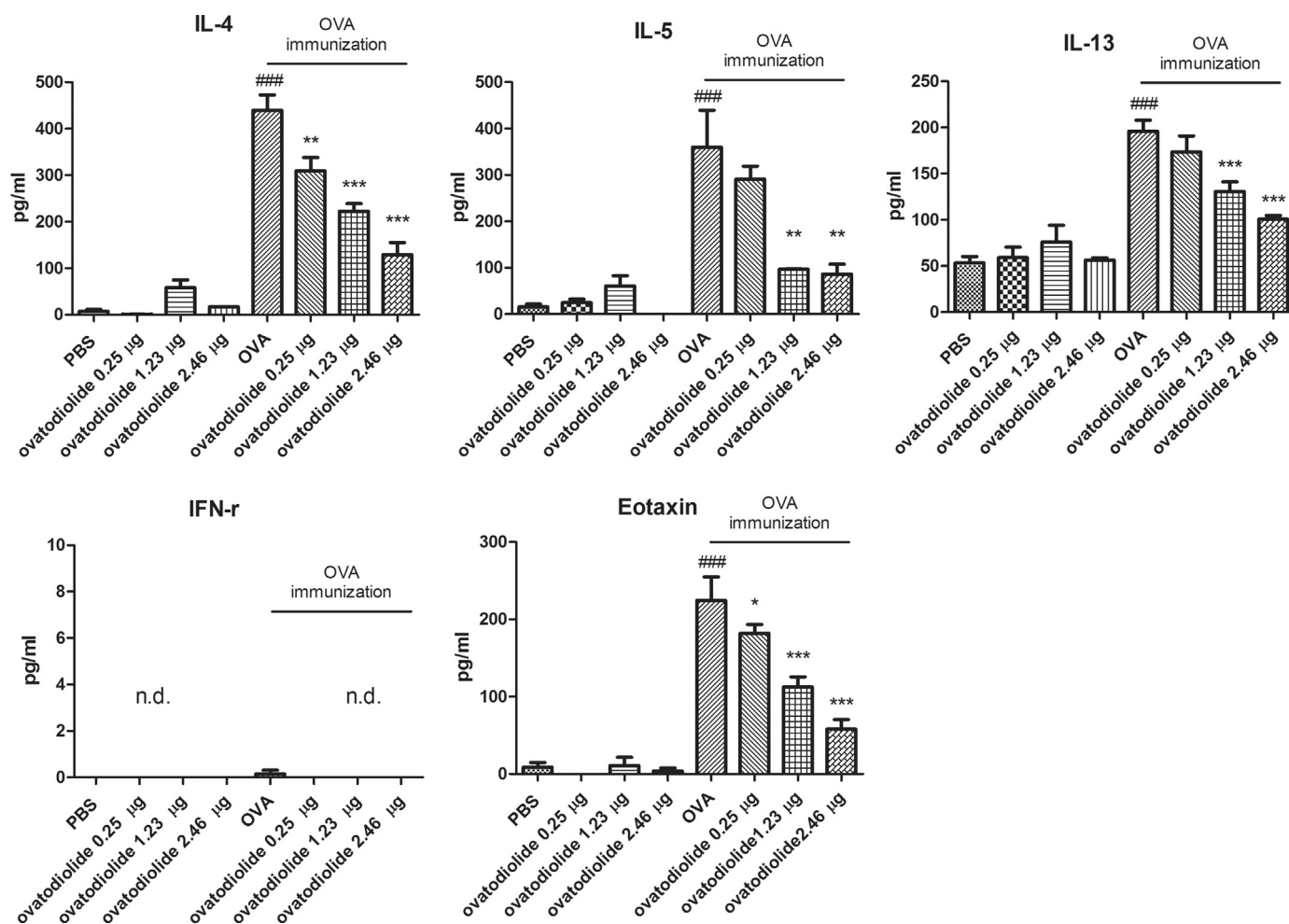


Fig. 5. Suppression of cytokine levels in BALF after administration of ovatodioid in a murine model of asthma. Cytokines in BALF were analyzed by ELISA. Data are expressed as mean $\pm$ standard deviation. (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001, compared with the PBS group; *P < 0.05; **P < 0.01; ***P < 0.001, compared with the OVA group.

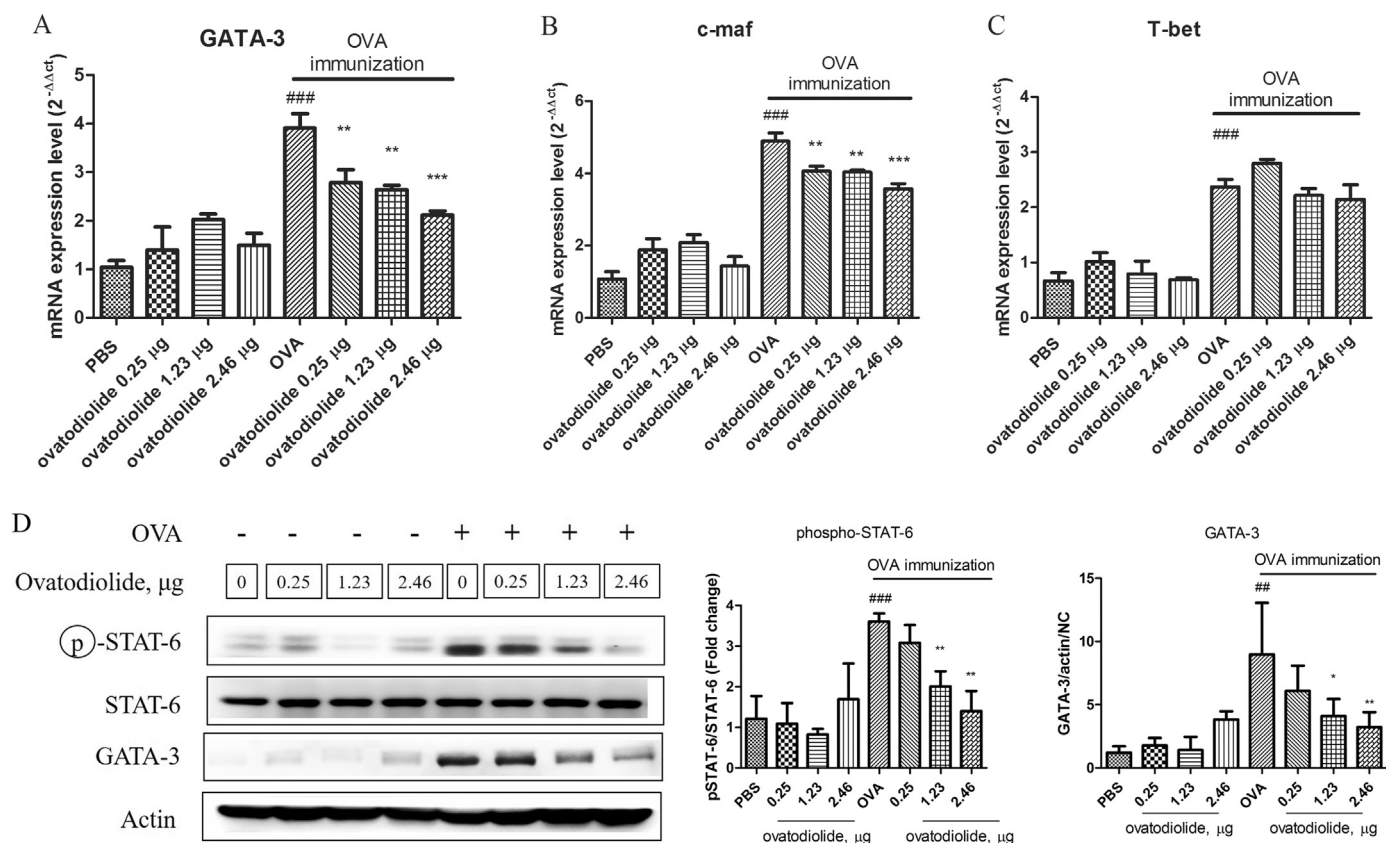


Fig. 6. Ovatodioidide inhibited Th2 cell transcription factor mRNA expression and the expression of signal transduction molecules in a murine model of asthma. The mRNA expression levels of GATA-3 (A), c-Maf (B), and T-bet (C) were measured by real-time PCR. The protein expression levels of phosphorylated STAT-6 (p-STAT-6), STAT-6, and GATA-3 in lung tissues were determined by using western blot and the image data were quantitatively analyzed using Image J quantitative software (D). Data are expressed as mean $\pm$ standard deviation. (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001, compared with the PBS group; *P < 0.05; **P < 0.01; ***P < 0.001, compared with the OVA group.

Fig. 8B. IL-33 was increased in OVA-immunized mice. When mice were received 2.46 μg ovatodioidide in OVA-immunized mice, IL-33 protein expression has similarly inhibited.

4. Discussion

Ovatodioidide is a cembrane-type diterpenoid compound isolated from *Anisomeles indica*. Compounds isolated from *A. indica* extracts are classified into seven cembrane-type diterpenoids, seven flavonoids, four triterpenoids, five benzenoids, and anisovatodside (Chen et al., 2008; Ho et al., 2009b; Rao et al., 2009). Ovatodioidide belongs to the cembrane class of diterpenoids. Previous study found that among cembrane-type diterpenoids, ovatodioidide exhibited the most anti-inflammatory effects, including antiplatelet aggregation (Chen et al., 2008) and inhibition of NO production by 89% at a concentration of 5 μM, compared to the DMSO control (Lien et al., 2013; Rao et al., 2009). Cembrane-type diterpenoids have anti-inflammatory activity even when isolated from other plant sources, such as *Lobophytum crissum*. A previous study showed that Lobocrassin B inhibited tumor necrosis factor (TNF-α) production in bone marrow-derived dendritic cells (Lowhagen, 2012). In our study, we demonstrated that ovatodioidide suppressed allergic asthma symptoms, including airway inflammation, Th2 cell activation, cytokine release, and AHR in an OVA-induced murine model of allergic asthma. Besides, our results indicated that ovatodioidide alone did not induce mice airway inflammation (Fig. 2A) and had no toxicity effect (data not shown) in mice after five days of daily intratracheal injection of ovatodioidide.

The mechanism of ovatodioidide-inhibited allergic asthma symptoms might be through inhibition of Th2 cell differentiation and activation. In this study, ovatodioidide inhibited the Th2 related cytokines, IL-4, IL-5, IL-13, and eotaxin, in BALF. For confirmation

that ovatodioidide indeed inhibited Th2 cell activation, CD4⁺ T cells were isolated from lung draining lymph nodes and cell proliferation and cytokine production was measured. The data showed that ovatodioidide inhibited allergen-induced Th2 cell activation. In addition, the inhibitory effects of ovatodioidide on allergen-induced Th2 cell activation did not result from increased Th1 cell activation because the level of IFN-γ in BALF and CD4⁺ T cells from lung draining lymph nodes was not affected. Analysis of T-cell-specific transcription factor mRNA expression in lung also showed the coincident result. Ovatodioidide suppressed Th2-cell-specific transcription factor GATA-3 and c-maf gene expression but not affected Th1-cell-specific transcription factor T-bet. In addition, previous studies reported that *A. indica* extracts containing a large amount of ovatodioidide could inhibit *Helicobacter pylori*-induced nuclear factor kappa B (NF-κB) activity in gastric epithelial cells (Lien et al., 2013; Rao et al., 2012). Signals through the T-cell receptors inducing early IL-4 production also probably mediated by activation of NF-κB and STAT-6 (Murphy et al., 1999) which induced GATA-3 gene expression. We found that the ovatodioidide-inhibited GATA-3 protein expression in lung tissue might arise from interference in STAT-6 phosphorylation. However, whether ovatodioidide affects NF-κB activity that increase of GATA-3 mRNA expression still needs further investigation (Ho et al., 2009a). The importance of innate immune responses in asthma pathogenesis has become significantly concern, especially on the innate cytokine IL-33. Recent studies describing the roles of IL-33 regarding the induction and maintenance of Th2 inflammation by activation of s type II innate lymphoid cells (ILC2s) which are a novel population to produce high levels of Th2 cytokines IL-5 and IL-13 (Licona-Limon et al., 2013; Rijt et al., 2016; Sean et al., 2013). Increased IL-33 expression was present in patients with children with severe therapy-resistant asthma despite high-dose steroid therapy. Study indicated that IL-33 mainly expressed

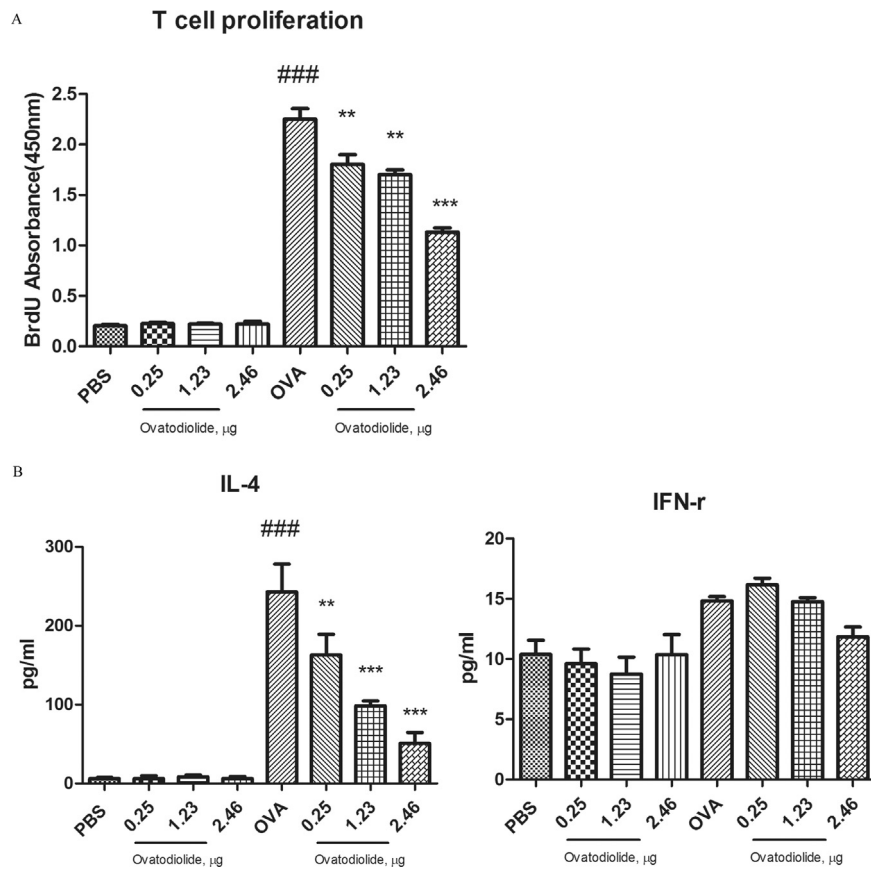


Fig. 7. Ovatodioid inhibited T_H2 cells cell proliferation and cytokines production of mediastinal lymph node cells in a murine model of asthma. Mediastinal lymph node cells were isolated from PBS and OVA-immunized mice. 2×10^5 cells were cultured in 96-well dish and treated with $100 \mu\text{g ml}^{-1}$ ovalbumin for 72 h, and then cell and the medium were collected for analysis of cell proliferation (A) and cytokine production as describe in Materials and Methods (B). Data are expressed as mean $\pm$ standard deviation. (n = 6). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the PBS group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the OVA group.

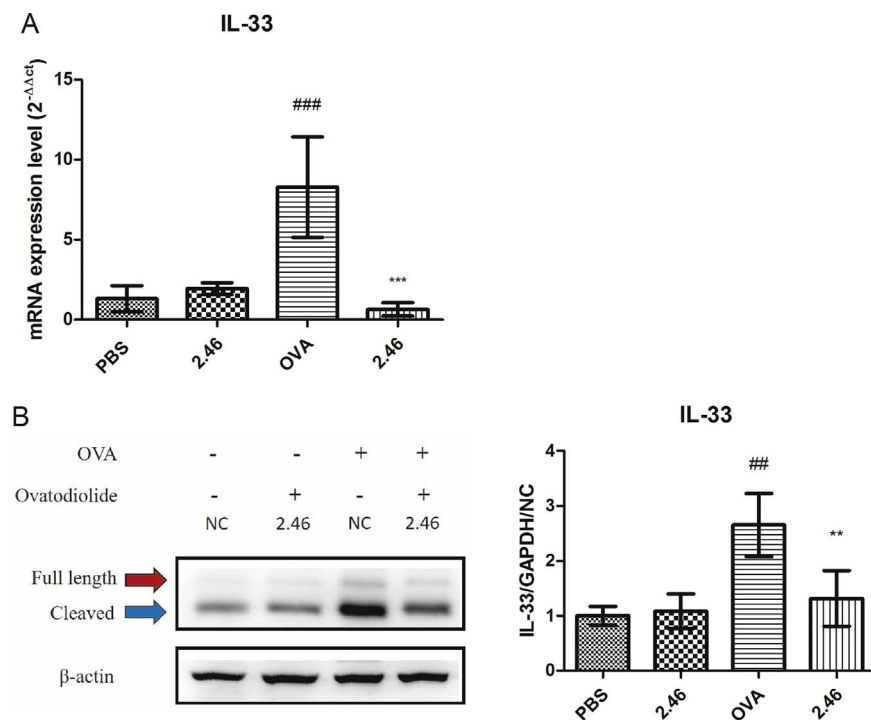


Fig. 8. Ovatodioid inhibited IL-33 production in lung tissues. The mRNA expression levels of IL-33 were detected using real-time PCR (A), protein expression levels of IL-33 in lung tissues by using western blot and quantitative data were analyzed using Image J quantitative software (B). Data are expressed as mean $\pm$ standard deviation. (n = 6). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the PBS group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the OVA group.

in the epithelium and smooth muscle of adults with severe asthma (Prefontaine et al., 2009, 2010). In this study, ovatodiolide strongly attenuated airway IL-33 gene expression in murine model of asthma. It is implicated that ovatodiolide might be a potential drug for severe asthma.

5. Conclusion

The present data demonstrated that ovatodiolide could inhibit airway inflammation, mucus production, and AHR through attenuated of Th2 cells activation in a murine model of asthma. In addition, ovatodiolide also showed that anti-IL-33 effects which might affect ILC2 activation and has therapeutic potential for severe asthma. To sum up, we found that ovatodiolide showed therapeutic potential effects on allergic asthma.

Conflict of interest

The authors announce no conflicts or competing financial interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2017.06.036.

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Supplementary information

Ovatodiolide suppresses allergic airway inflammation and hyperresponsiveness in a murine model of asthma

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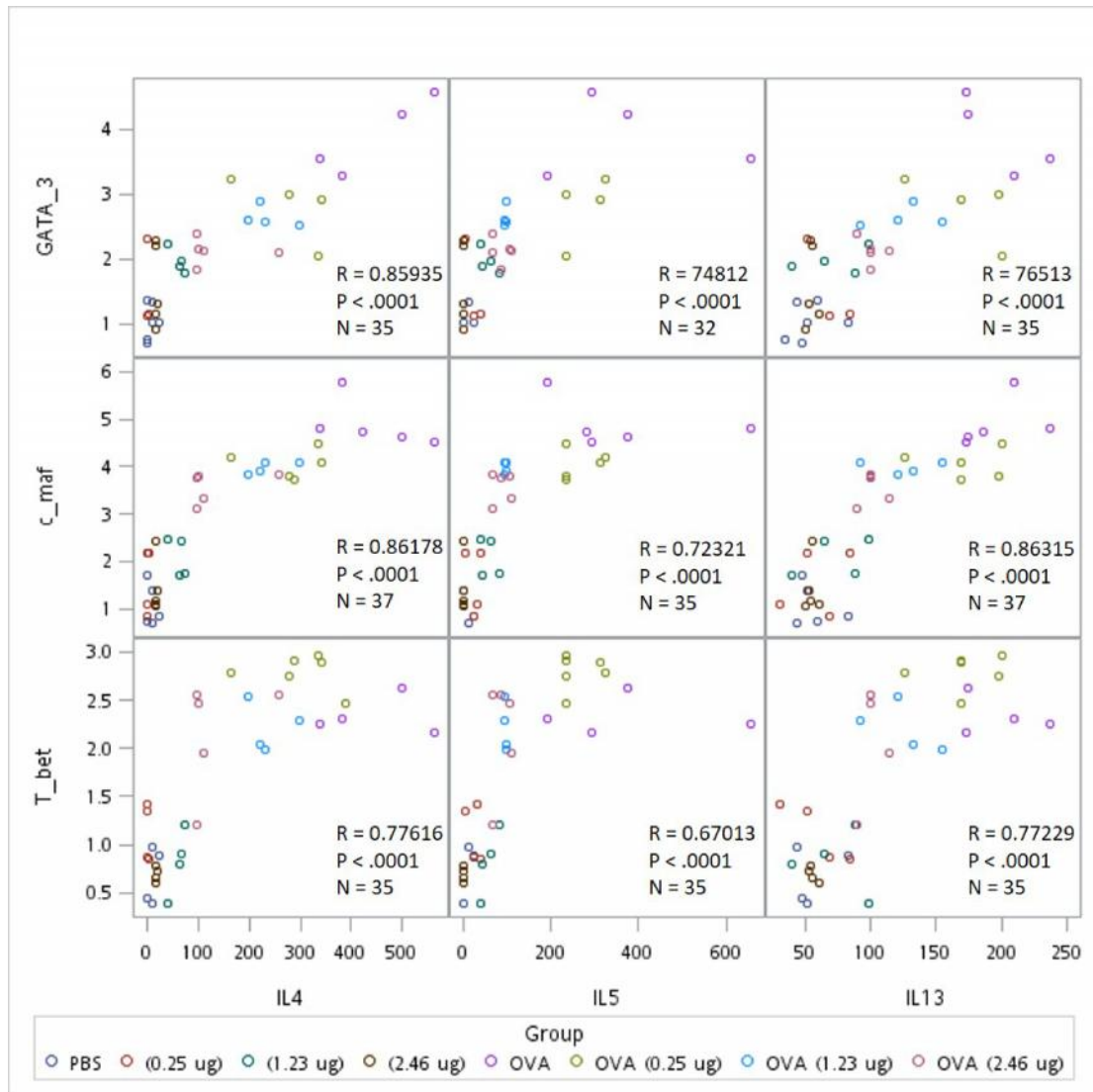


Figure. S1. Pearson's correlation coefficients for the relation between BALF Th2 cytokines release and GATA-3, c-Maf, and T-bet transcription factors mRNA in lung tissue by using SAS 9.2 software. 0.25 μ g, 1.23 μ g, 2.46 μ g indicated ova 0.25, 1.23, and 2.46 μ g/mouse groups, respectively. OVA (0.25 μ g), OVA (1.23 μ g), OVA (2.46 μ g) indicated OVA combined 0.25 μ g, 0.25, 1.23, and 2.46 μ g/mouse groups, respectively.

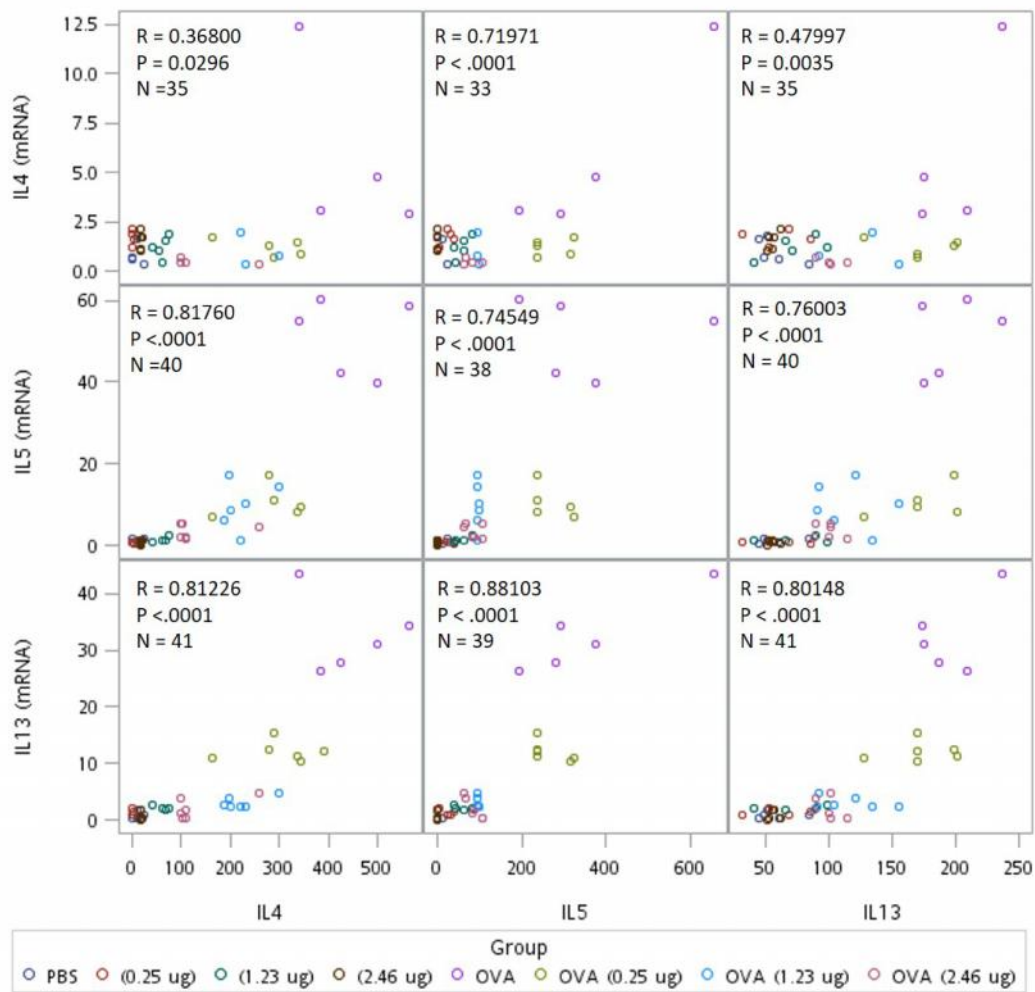


Figure. S2. Pearson's correlation coefficients for the relation between BALF Th2 cytokines release and their mRNA expression in lung tissue by using SAS 9.2 software. 0.25 μ g, 1.23 μ g, 2.46 μ g indicated ovalotriolide 0.25, 1.23, and 2.46 μ g/mouse groups, respectively. OVA (0.25 μ g), OVA (1.23 μ g), OVA (2.46 μ g) indicated OVA combined 0.25 μ g, 0.25, 1.23, and 2.46 μ g/mouse groups, respectively.

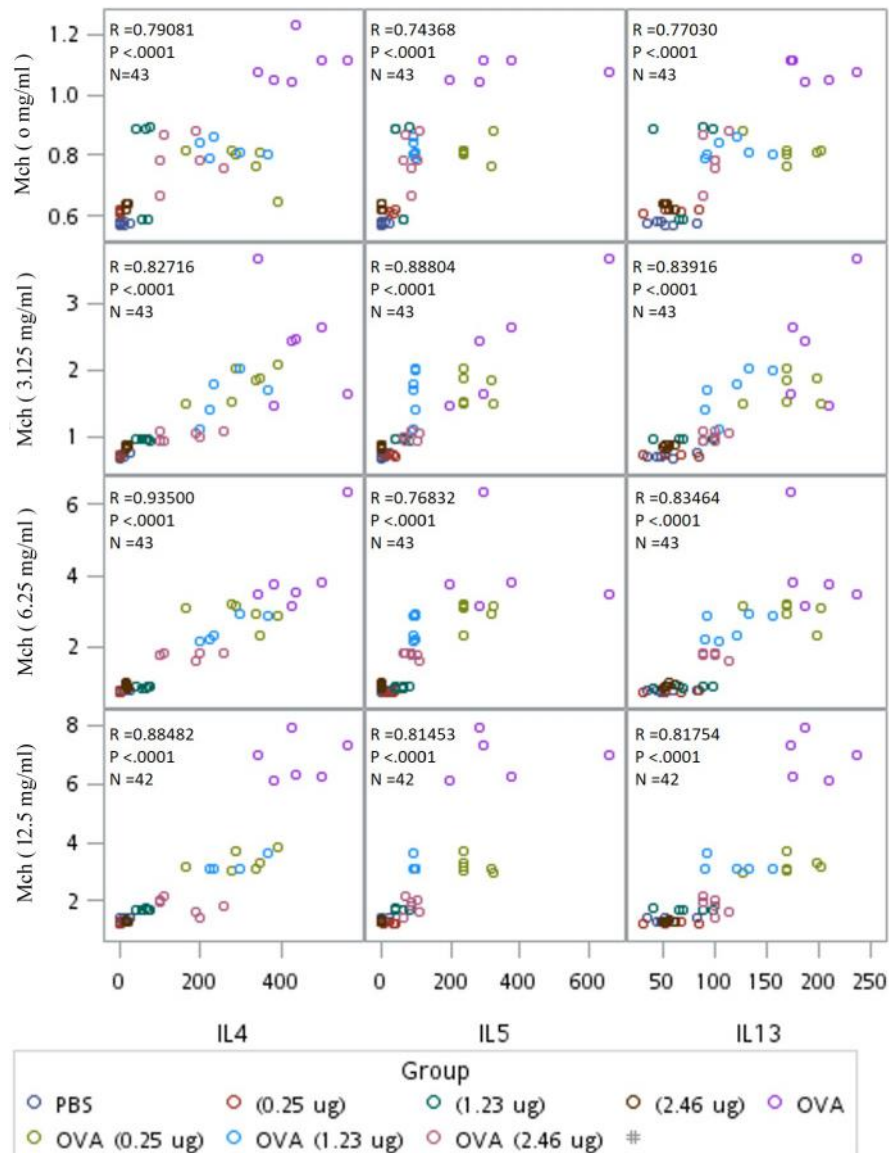


Figure. S3. Pearson's correlation coefficients for the relation between BALF Th2 cytokines release and airway hyperresponsiveness in lung tissue by using SAS 9.2 software. 0.25 μ g, 1.23 μ g, 2.46 μ g indicated ovaodiolide 0.25, 1.23, and 2.46 μ g/mouse groups, respectively. OVA (0.25 μ g), OVA (1.23 μ g), OVA (2.46 μ g) indicated OVA combined 0.25 μ g, 0.25, 1.23, and 2.46 μ g/mouse groups, respectively. Methacholine (MCh)