

ORIGINAL ARTICLE

Regulation of Th1/Th2 balance through OX40/OX40L signalling by glycyrrhizic acid in a murine model of asthma

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ABSTRACT

Background and objective: Glycyrrhizic acid (GA) has been reported to have attenuating airway inflammation effects in asthma mouse model. However, the potential molecular mechanisms by which GA exerts anti-inflammatory effects on ovalbumin (OVA)-induced allergic asthma have not been well elaborated.

Methods: The effect of GA on OVA-sensitized and challenged mice was investigated. The effect of GA on anti-OX40 mAb stimulated splenocytes from asthma mice model was also examined.

Results: In OVA-induced asthmatic mice, GA treatment prevented the decrease of T helper1 cytokine (interferon (IFN)- γ) and the increase of T helper2 cytokines (interleukin (IL)-4, IL-5, IL-13) in bronchoalveolar lavage fluid (BALF), reduced serum immunoglobulin (Ig)E and OVA-specific IgE levels, prohibited the protein and mRNA expression of OX40 and OX40 Ligand (OX40L) in lung tissues, and the expression of OX40 in CD4⁺ T cells and OX40L in CD11b⁺ monocytes and CD19⁺ B cells in spleens in a dose-dependent manner compared with the vehicle treatment (all $P < 0.05$). Moreover, OVA significantly increased the activation of p38 mitogen-activated protein kinase (MAPK) in lung tissues, whereas GA and anti-OX40L mAb markedly reduced phosphorylation of p38 MAPK. In addition, GA could inhibit the T cell proliferation and modulate the balance of Th1/Th2 in anti-OX40 mAb stimulated CD4⁺ T cells from asthmatic spleens (all $P < 0.05$).

Conclusions: GA may exert a therapeutic effect on OVA-induced experimental asthma partly by regulating the Th1/Th2 balance through suppressing OX40-OX40L signalling and p38 MAPK activity. GA may be a promising treatment for asthma.

SUMMARY AT A GLANCE

We investigated the effect of glycyrrhizic acid (GA) on an OVA-challenged model of asthma. GA-modulated Th2 and Th1 reduced OX40, OX40L and p-p38 MAPK in OVA-challenged asthma mice model. GA also suppressed OVA-induced and anti-OX40 mAb stimulated splenocyte proliferation and Th2 production. GA has potential as a treatment for asthma.

Key words: asthma, cytokine, glycyrrhizic acid, OX40, OX40 ligand.

Abbreviations: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; DC, dendritic cells; Dex, dexamethasone; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent; FITC, fluorescein-isothiocyanate; GA, glycyrrhizic acid; IFN, interferon; IgE, immunoglobulin E; IgG, immunoglobulin G; IL, interleukin; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear transcription factor κ B; OD, optical density; OVA, ovalbumin; OX40L, OX40 ligand; PAGE, polyacrylamide gel; PBS, phosphate buffer saline; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; TCM, traditional Chinese medicine; Th, T helper.

INTRODUCTION

Bronchial asthma is a common chronic inflammatory disease characterized by airway inflammation, hyperresponsiveness (AHR) and airway remodelling, accompanied by variable airflow obstruction, varying degrees of subepithelial fibrosis and/or mucus hyperproduction.¹ Atopic asthma has long been associated with overexpression of T helper (Th) 2 cytokines including interleukin (IL)-4, IL-5 and IL-13, which are elevated in sputum, bronchoalveolar lavage fluid (BALF) and bronchial biopsies.² Downregulation of Th2 cytokines is one of the major therapeutic strategies in the treatment of asthma.

The activation of T cells requires two coordinated signals: an antigen-specific signal and an

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antigen-independent co-stimulatory signal.³ The co-stimulatory molecule OX40, as a member of the tumour necrosis factor receptor superfamily, plays a key role in a range of memory T cell activities.^{4,5} Although in normal condition, OX40 is not expressed in naïve T cells, it can be induced in the primary immune response 24–48 h after recognition of antigen.^{6,7} In addition, the OX40 ligand (OX40L) may be induced in dendritic cells (DC), macrophage-like cells and activated B cells.^{8–12} OX40–OX40L interactions contribute to augment an ongoing immune response through regulation of Th1 and Th2 cells, involving nuclear transcription factor κ B (NF- κ B), phosphatidylinositol 3-kinase/serine-threonine kinase (PI3K/AKT) and nuclear factor of activated T cells signalling pathways.^{13,14} Pretreatment of anti-OX40L mAb results in a significant inhibition of established asthmatic reaction in a murine model.¹⁵ Therefore, OX40 may be an attractive therapeutic target for suppressing Th2 cell-mediated allergic asthma.

Licorice is a well-known herb plant that has been widely used in food additives, nutraceuticals and traditional Chinese medicine (TCM) for thousands of years. In licorice, glycyrrhizic acid (GA; Fig. 1a) has been identified as the primary bioactive compound.¹⁶ GA has a variety of pharmacologic activities that can attenuate inflammation, oxidant stress, viral infection, tumour growth and angiogenesis.^{17–20} In this study, we investigate GA's inhibition of airway inflammation via OX40/OX40L signalling pathways in a murine model of asthma.

METHODS

Ovalbumin mouse model and experimental protocols

A total of 48 BALB/c mice (female, ages 6–8 weeks) were obtained from the Soochow University Laboratory Animal Centre (China). To induce experimental models of asthma, mice were sensitized intraperitoneally (i.p.) with 20 μ g ovalbumin (OVA; chicken egg albumin, Grade V; Sigma-Aldrich, St. Louis, MO, USA) and 20 mg of aluminium hydroxide gel (Invivo Gen, San Diego, CA, USA) in 0.2-mL saline on days 0, 7 and 14, and challenged with 5% OVA via nebulization for 30 min on days 21–27.²¹

To evaluate the therapeutic effects of GA on the mouse models, intraperitoneal administration of mice with 25, 50, 100 mg/kg GA (Sigma-Aldrich) or 2 mg/kg dexamethasone was performed 30 min before each challenge. Saline-sensitized and challenged mice were used as controls. The mice were sacrificed on day 28, and samples were harvested.

To identify the mechanisms involved in the functions of GA, we blocked the OX40–OX40L interaction, by an i.p. injection of 300- μ g rat anti-mouse-OX40L mAb (Biolegend, San Diego, CA, USA) or isotype control IgG2b at the time of OVA sensitization and subsequently every 3 days before the first OVA challenge.²² A p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580 (5 mg/kg; Selleck, Houston, TX, USA) in dimethylsulfoxide (DMSO)-

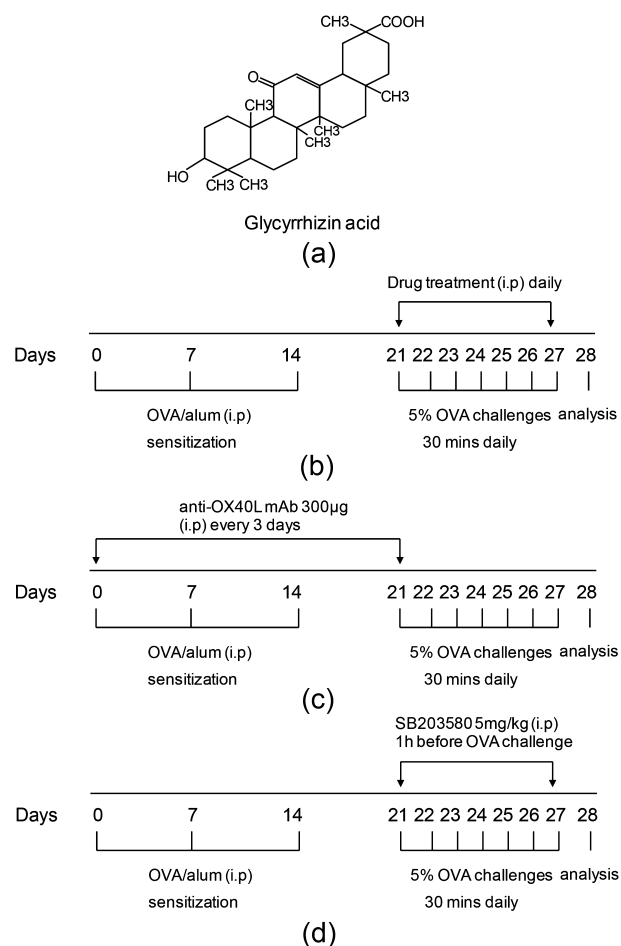


Figure 1 (a) Chemical structure of glycyrrhizic acid (GA). (b) Experimental scheme for ovalbumin (OVA)-induced allergic asthma in bronchoalveolar lavage fluid (BALB)/c mice. Asthma was induced by sensitization and challenge of OVA in BALB/c mice. The mice were treated intraperitoneally (i.p.) with three different concentrations of GA (25, 50 and 100 mg/kg) or Dex (2 mg/kg) 30 min before OVA challenge on days 21–27. (c) To block OX40–OX40L interaction, mice were injected i.p. with 300 μ g of rat anti-mouse-OX40L blocking mAb or isotype control IgG2b at the time of OVA sensitization and subsequently every 3 days before the first OVA challenge. (d) SB203580 (5 mg/kg) dissolved in sterile dimethylsulfoxide (DMSO)-phosphate buffer saline was injected i.p. 1 h before each OVA challenge on days 21–27. The mice were sacrificed on day 28, and samples were harvested.

phosphate buffer saline (PBS) was injected i.p. 1 h before OVA challenge on days 21–27.²³ In this study, isotype control IgG2b or vehicle (0.1% v/v DMSO) had no significant effect on key characteristics of allergic asthma (AHR, inflammation and IgE) and important mediators in an OVA-induced asthmatic murine model (data not shown). The whole protocol was approved by the Ethical Committee of the First People's Hospital of Wujiang (no. 2013-10) (Fig. 1b,c,d).

Measurement of IgE in serum

The mouse sera were isolated and frozen at -80°C before analysis. The concentrations of total IgE and

OVA-specific IgE in serum were determined by using the enzyme-linked immunosorbent (ELISA) kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

BALF collection and measurement of Th cytokines

Mice were anaesthetized, and their tracheas were intubated. The right lung was lavaged three times with 600 μ L of cold PBS. The concentrations of IL-4, IL-5, IL-13 (as Th2 cytokines) and interferon (IFN)- γ (as a Th1 cytokine) in the BALF were analyzed by using the ELISA kits (eBioscience, San Diego, CA, USA).

Lung immunohistochemistry

After dewaxed and hydrated, the lung sections were subjected to high-pressure heat-induced antigen retrieval and incubated overnight at 4°C with anti-mouse OX40 (Biolegend) or anti-mouse OX40L antibody (Biolegend) (1:100). Sections were subsequently washed and incubated with biotinylated rat anti-mouse IgG secondary antibody (Dako, Copenhagen, Denmark) for 30 min, followed by incubation with 3,3'-diaminobenzidine tetrachloride (Dako) for 5–10 min and visualization of haematoxylin staining under light microscopy. Negative-control was used with PBS instead of the primary antibody. The value of average optical density (OD) was calculated by integrated OD versus staining positive area using Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

RT-PCR analysis

Lung tissues were collected 24 h after the last OVA challenge, and total intracellular RNA was extracted using RNeasy Plus (Takara Bio, Shiga, Japan). First strand cDNA synthesis was performed with a PrimeScript RT reagent kit (Takara Bio). Oligonucleotide primers for OX40, OX40L and the internal control Hprt were as follows: OX40, 5'-GTGTACACAGTGC AACC-3' and 5'-GGCCAGGAGGGCAGGG-3'; OX40L, 5'-GTGCTTCATCTATGTCTGCC-3' and 5'-CTCATTCT TGTATGAGCTG-3'; Hprt, 5'-TATGGACAGGACTGAA AGAC-3' and 5'-TAATCCAGCAGGTCAGCAA-3'. The expression of target genes was normalized by the housekeeping gene Hprt.

Flow cytometry analysis

Spleens of mice were harvested, and mononuclear cells were separated by centrifugation with a Ficoll-Conray gradient (Hanyang Biotec, Beijing, China) and double stained with fluorescein-isothiocyanate (FITC)-anti-mouse CD4/PE-anti-mouse OX40, FITC-anti-mouse CD8/PE-anti-mouse OX40, FITC-anti-mouse CD19/PE-anti-mouse OX40L or FITC-anti-mouse CD11b/ PE-anti-mouse OX40L (eBioscience). Samples were analyzed by Beckman Coulter Gallios flow cytometry (Marseille, France) and software FlowJo 7.6 (Tree Star Inc, Ashland, OR, USA).

Isolation of CD4⁺ T cells and T-cell proliferation assay

To obtain CD4⁺ T cells, mononuclear cells were prepared from spleens of asthmatic mice and purified by using the Mouse CD4 Cell Positive Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following to the manufacturer's protocol. Purified CD4⁺ T cells were cultured in triplicate pre-coated with anti-CD3 mAb (1 μ g/mL, Biolegend) in the presence of GA (5, 10 or 100 μ g/mL), anti-OX40 mAb (2.5 μ g/mL, Biolegend), or Rat IgG1 as control at 37°C, 5% CO₂. After 72 h of incubation, cell proliferation was measured by CCK8 kit (Dojindo, Kumamoto, Japan) and IL-4, IL-5, IL-13 and IFN- γ in cultural supernatant were measured by ELISA (eBioscience).

Western blot analysis

Freshly isolated left lung tissues were homogenized in the presence of protease inhibitors and the protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). A 30- μ g sample of protein was electro-blotted onto a polyacrylamide gel (PAGE) membrane following separation on a 10% sodium dodecyl sulphate-PAGE (SDS-PAGE) gel, incubated with primary antibody against p38, p-p38 or β -actin (Cell Signaling, Danvers, MA, USA), followed by incubation with the HRP-conjugated secondary antibody. The signals were detected using an ECL reagent (Bio-Rad) and the quantities of the target bands were normalized by β -actin.

Statistical analysis

Values are presented as mean $\pm$ standard deviation (SD). Differences between groups were assessed by one-way analysis of variance (ANOVA) test followed by LSD's multiple comparison test. $P < 0.05$ were considered statistically significant.

RESULTS

Effect of GA on serum IgE levels

Total and OVA-specific IgE levels were significantly elevated in OVA-challenged mice, compared with control group. However, the administration of GA, anti-OX40L mAb or SB203580 led to a significant reduction in both parameters (Fig. 2a,b).

Effect of GA on the cytokine levels in vivo and vitro

The Th1/Th2 balance was similar to the Th2-dominant immunity seen in allergic asthma. The OVA-induced group showed a significantly increased levels of IL-4, IL-5 and IL-13 and decreased level of IFN- γ in BALF compared with the saline group. There was a significant dose-dependent decrease of IL-4, IL-5 and IL-13 and increase of IFN- γ in BALF in GA, anti-OX40L mAb or SB203580 treated groups in comparison with OVA-challenged group (Fig. 2c,d,e,f). Similar effects of GA can be seen on cytokine production in splenic CD4⁺ T cells stimulated with anti-CD3

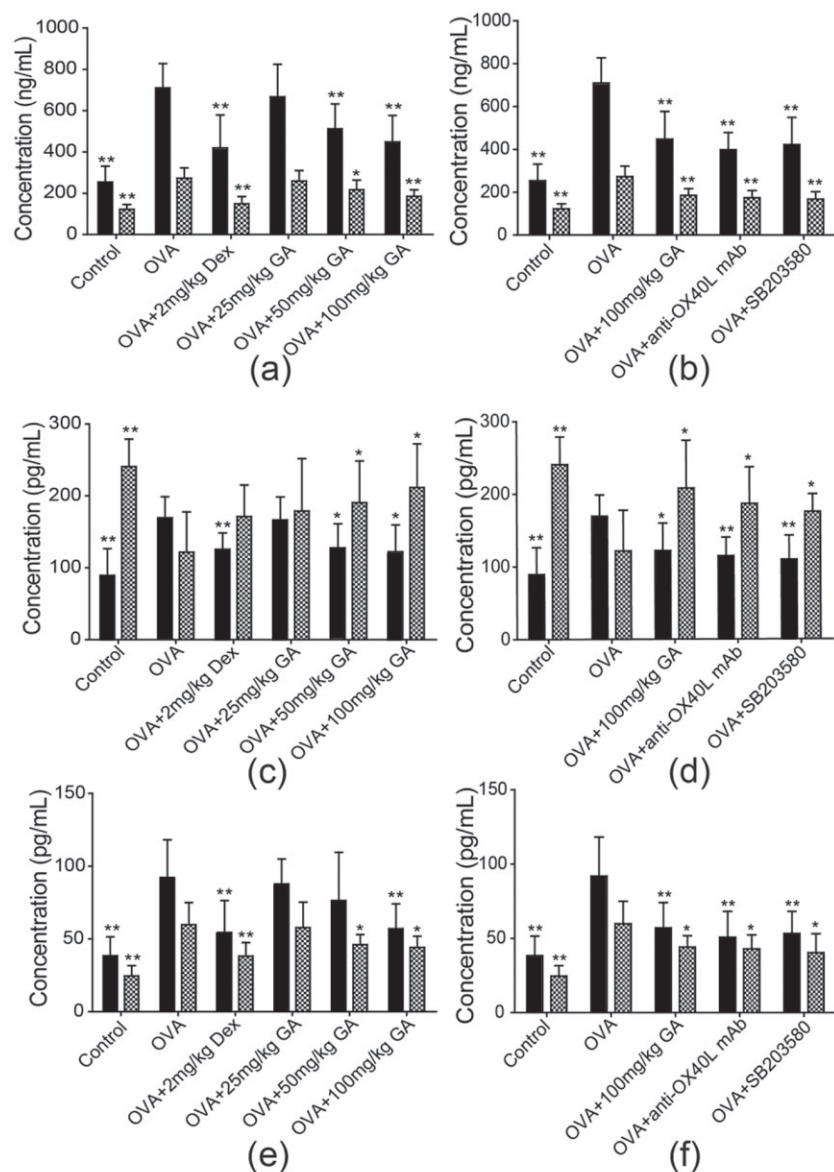


Figure 2 Effect of glycyrrhizic acid (GA) on the secretion of immunoglobulin E (IgE) in serum and cytokines in bronchoalveolar lavage fluids (BALF). Mice were anaesthetized 24 h after the last ovalbumin (OVA) challenge, and the blood samples were collected to estimate the production of (a, b) IgE and OVA-specific IgE. The tracheas were intubated, the left main bronchus was tied and the left lung was removed. The right lung from each group was lavaged three times with 600 μ L of cold phosphate buffer saline through the tracheostomy tube. Of which, the fluid recovery rate was more than 85%. The supernatant of BALF was collected after centrifugation at $4000 \times g$ at 4°C for 5 min, then stored at -80°C until use. Data represented as mean $\pm$ standard deviation (SD) of three independent experiments. Control, saline-sensitized and challenged group receiving vehicle; OVA, OVA-sensitized and challenged group; OVA + 2 mg/kg Dex, OVA-sensitized and challenged group receiving 2 mg/kg Dex; OVA + GA, OVA-sensitized and challenged group receiving 25, 50 or 100 mg/kg GA; OVA + anti-OX40L mAb, OVA-sensitized and challenged group receiving 300 μ g anti-OX40L mAb; OVA + SB203580, OVA-sensitized and challenged group receiving 5 mg/kg SB203580. Each group with 8 mice. * $P < 0.05$, ** $P < 0.01$, compared with OVA mice. Keys: (a) Effect of different concentrations of GA on (■) total IgE, (▨) OVA-specific; (b) Effect of GA, anti-OX40L mAb and SB203580 on (■) total IgE, (▨) OVA-specific IgE; (c) Effect of different concentrations of GA on (■) interleukin (IL)-4, (▨) interferon (IFN)- γ ; (d) Effect of GA, anti-OX40L mAb and SB203580 on (■) interleukin (IL)-4, (▨) interferon (IFN)- γ ; (e) Effect of different concentrations of GA on (■) IL-5, (▨) IL-13; (f) Effect of GA, anti-OX40L mAb and SB203580 on (■) IL-5, (▨) IL-13.

mAb (1 μ g/mL) and anti-OX40 mAb (2.5 μ g/mL) from OVA-induced asthmatic mice (Fig. 3a,b).

Effect of GA on the proliferation of splenic CD4⁺ T cells in vitro

As shown in Figure 3c,d, co-culture with anti-OX40 mAb promoted the proliferation of CD4⁺ T cells from asthmatic spleens compared with the group stimulated by anti-CD3 mAb alone, whereas GA significantly inhibited the augmented T cells proliferation induced with anti-OX40 mAb in a dose-dependent manner.

GA treatment suppressed OX40/OX40L pathway in asthmatic mice

The OX40 and OX40L mRNA levels of lungs were significantly increased in the OVA-challenged group

compared with those in saline group, whereas GA markedly attenuated OVA-induced OX40 and OX40L mRNA expression in a dose-dependent manner (Fig. 4a,b).

The protein expression of OX40 and OX40L in immunohistochemistry was upregulated in the lungs of the OVA-challenged group as compared with saline group. The increased expression of OX40 occurred predominantly in the cytoplasm of lymphocytes and alveolar epithelial cells, whereas OX40L mainly expressed in the cytoplasm of lymphocytes, alveolar epithelial cells and macrophages. Treatment with GA substantially reduced the upregulation of OX40 and OX40L. Of note, the efficacy was seen significantly at dosage of 100 mg/kg (Fig. 4c,d,e).

As expected, OVA induced a significant increase of OX40 expression in CD4⁺ T cells, and OX40L expression in CD11b⁺ monocytes and CD19⁺ B cells in

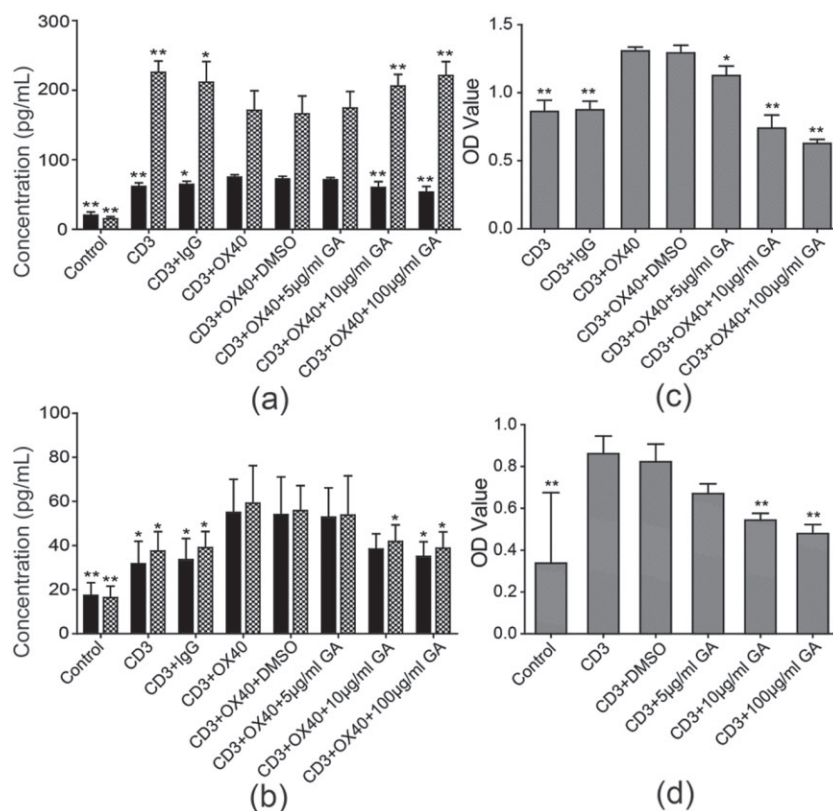


Figure 3 Effect of glycyrrhizic acid (GA) on the secretion of cytokines *in vitro* and proliferation of splenic CD4⁺ T cells. Mononuclear cells were prepared from spleens of ovalbumin (OVA)-sensitized and challenged mice by Ficoll–Hypaque density-gradient centrifugation. Then CD4⁺ T cells were isolated and purified from mononuclear cells by using the Mouse CD4 Cell Positive Isolation Kit following the manufacturer's protocol. Purified spleen CD4⁺ T cells (2×10^5 cells per well) were cultured in triplicate in 96-well flat bottom plates pre-coated with anti-CD3 mAb (1 µg/mL) for 30 min. The levels of (a) interleukin (IL)-4, interferon (IFN)-γ and (b) IL-5, IL-13 in cultural supernatant were detected after incubation with anti-OX40 mAb (2.5 µg/mL) and different concentrations (5, 10 or 100 µg/mL) of GA for 72 h. The proliferation of CD4⁺ T cells was measured by CCK8 after incubation with anti-CD3 mAb and different concentrations of GA for 72 h at the presence of (c) anti-OX40 mAb or (d) not. Control, CD4⁺ T cells; CD3, CD4⁺ T cells incubated with anti-CD3 mAb; CD3 + IgG, CD4⁺ T cells incubated with anti-CD3 mAb and isotype control IgG; CD3 + OX40, CD4⁺ T cells incubated with anti-CD3 mAb and anti-OX40 mAb; CD3 + OX40 + dimethylsulfoxide (DMSO), CD4⁺ T cells incubated with anti-CD3 mAb, anti-OX40 mAb and DMSO; CD3 + OX40 + GA, CD4⁺ T cells incubated with anti-CD3 mAb, anti-OX40 mAb and GA (5, 10, or 100 µg/mL). Data represented as mean ± standard deviation (SD) of three independent experiments. * $P < 0.05$, ** $P < 0.01$, compared with CD3 + OX40 + DMSO (a,b,c) or CD3 + DMSO(d). Keys: (a): (■) interleukin (IL)-4, (▨) interferon (IFN)-γ; (b): (■) IL-5, (▨) IL-13.

spleens of asthmatic mice compared to those treated with saline or Dex. Treatment with GA dramatically decreased the levels of OX40 and OX40L expression in spleens of asthmatic mice, especially those treated with high dosage of GA. No significant difference in OX40 expression was seen in CD8⁺ T cells in any groups (Fig. 5).

Effect of GA on p38 MAPK pathway activation in lungs of asthmatic mice

To further investigate whether the inhibition of asthmatic response by GA was mediated through OX40/OX40L/p38 MAPK, phosphorylation of p38 MAPK was examined in OVA-challenged mice pretreated with GA or anti-OX40L mAb or SB203580. As shown in Figure 6, treatment with GA with a dose of 50, 100 mg/kg or anti-OX40L mAb or SB203580 effectively inhibited OVA-induced phosphorylation of p38 MAPK in allergic mice.

DISCUSSION

Allergic asthma exhibits an elevated level of serum IgE.² Airway eosinophilia, driven by CD4⁺ T cells, plays a central role in the pathogenesis of asthma.²⁴ A better understanding of the nature of T cell activation is essential for development of novel approaches to suspend Th2-dominant inflammation in asthma.²⁵ To our knowledge, it is the first time to demonstrate that GA attenuates experimental asthma by regulating the Th1/Th2 balance via the OX40/OX40L signalling pathway.

Glycyrrhizin was extracted from the roots of glycyrrhiza glabra, and for centuries, it has been safely used as an anti-allergic agent in TCM.²⁶ Orally administered glycyrrhizin is usually metabolized to GA by intestinal bacteria that contain β-D-glucuronidase, whereas intravenously administered glycyrrhizin is first metabolized to 3-mono-glucuronide-glycyrrhetic acid by lysosomal β-D-glucuronidase

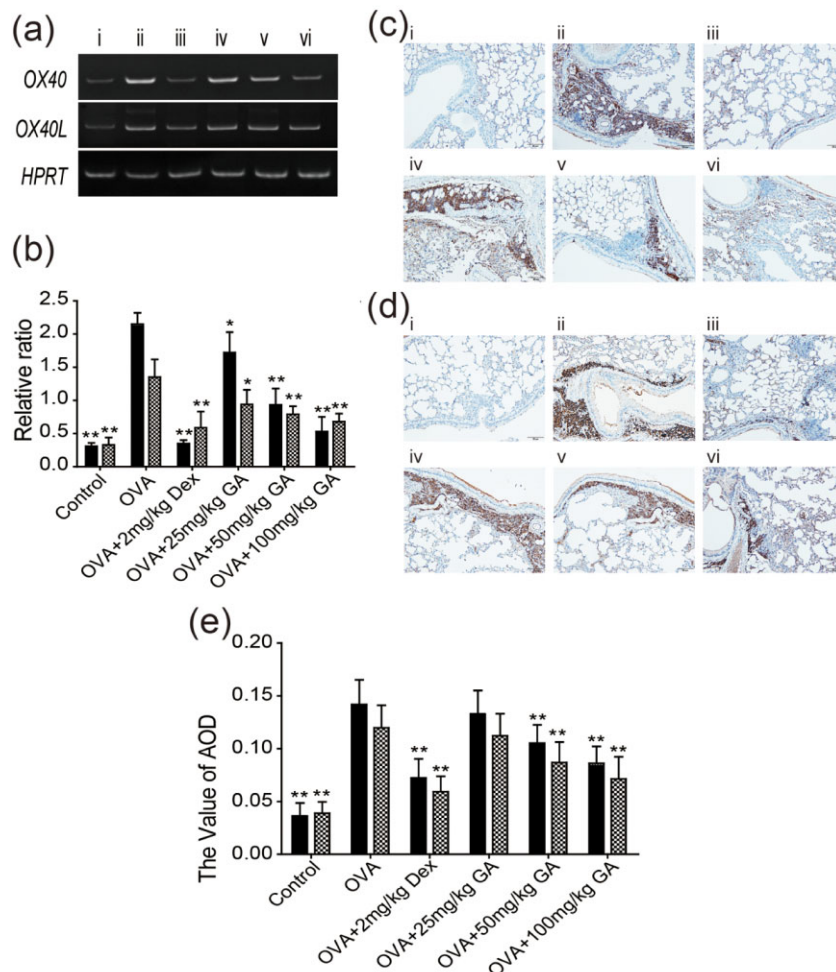


Figure 4 Glycyrrhizic acid (GA) suppressed mRNA and protein expression of OX40 and OX40L in lungs of ovalbumin (OVA)-induced asthma mouse model. The upper lobe of the left lung was collected 24 h after the last OVA challenge and snap frozen in liquid nitrogen. Total intracellular RNA was extracted using 1 mL RNAiso Plus. First strand cDNA synthesis was performed with a PrimeScript RT reagent kit. Hprt was used as an endogenous control. The expression of target genes was normalized by the housekeeping gene Hprt. (a,b) Representative experiments of reverse transcription-polymerase chain reaction (RT-PCR) for OX40 and OX40L expression ($n = 6$ per group). After dewaxed and hydrated, the lung tissue sections were subjected to heat-induced antigen retrieval and then blocked and incubated overnight at 4°C with anti-mouse OX40 or anti-mouse OX40L antibody (1:100). Sections were subsequently washed and incubated with biotinylated rat anti-mouse IgG secondary antibody for 30 min, followed by incubation with 3,3'-diaminobenzidine tetrachloride for 5–10 min and visualization of specific staining under light microscopy. Negative control was used with phosphate buffer saline instead of the primary antibody. Immunohistochemistry of (c) OX40 and (d) OX40L expression in lung tissue was assessed at a 200-fold magnification. (i) Control; (ii) OVA; (iii) OVA + Dex (2 mg/kg); (iv–vi) OVA + GA (25, 50 or 100 mg/kg). (e) Integrated optical density (OD) versus staining positive area was taken for the value of average OD using Image Pro Plus 6.0. * $P < 0.05$, ** $P < 0.01$, compared with OVA mice. Keys: (b): (■) OX40, (▨) OX40L; (e): (■) OX40, (▨) OX40L.

in the liver, and then excreted into the intestine, where bacteria further metabolize it into GA.²⁷ Overall, GA is the bioactive ingredient that exhibits multiple pharmacological actions.

GA-treated DCs can enhance proliferation of allogenic T cells accompanied by production of IFN- γ and IL-10 and reduction of IL-4, suggesting that GA may elevate allostimulatory activity of DCs towards a Th1 immune response.²⁸ Our previous results also indicated that GA inhibited the Th2 responses through modulating the expressions of CD86, MHC-II, CD40, OX40L on CD11c⁺ DCs.²⁹ Tu *et al.* reported that in the liver and spleen of murine fibrosis models, GA upregulated both Th1/Th2 and Treg/Th17 balances to Th1- and Treg-dominant lineages via multi-

ple pathways such as via c-Jun amino terminal kinase, extracellular signal-regulated kinase and PI3K/AKT.³⁰ GA also has protective role on β_2 adrenergic receptor (β_2 AR) agonist-stimulated receptor internalization and cell apoptosis.³¹ The combination of GA and β_2 AR led to a coordinate anti-inflammatory function through the suppression of NF- κ B activation.^{32,33} GA also exhibited a glucocorticoid-like inhibitory effect on inflammation without affecting the serum cortisol level.³⁴ However, before GA is recommended as a treatment for asthma, further basic and clinical studies are still required to clarify its pharmacological effects.

Consistent with previous reports,^{35,36} our study demonstrated that GA could significantly alleviate the

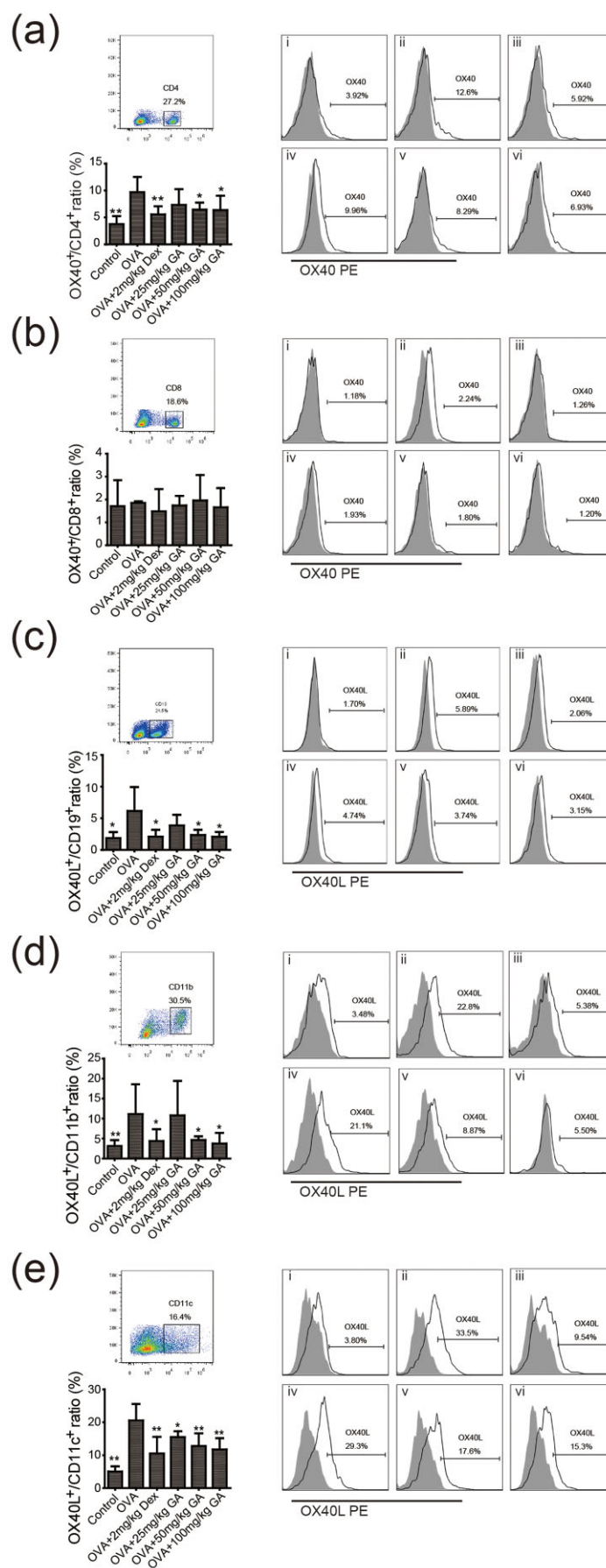


Figure 5 Glycyrrhizic acid (GA) ameliorates the expression of OX40/OX40L in spleens of ovalbumin (OVA)-induced allergic asthma in mice. Spleens of mice were harvested and mononuclear cells were separated and double stained with fluorescein-isothiocyanate (FITC)-anti-mouse CD4/PE-anti-mouse OX40, FITC-anti-mouse CD8/PE-anti-mouse OX40, FITC-anti-mouse CD19/PE-anti-mouse OX40L or FITC-anti-mouse CD11b/PE-anti-mouse OX40L for 20 min at room temperature. After being washed, the stained cells were analyzed by flow cytometry and software FlowJo 7.6. PE-mouse immunoglobulin G (IgG) and FITC-mouse IgG were used as the controls in this experiment. Representative infiltrations of (a) CD4⁺ OX40⁺ cells, (b) CD8⁺ OX40⁺ cells, (c) CD19⁺ OX40L⁺ cells and (d) CD11b⁺ OX40L⁺ cells in murine spleens with or without treatment. (i) Control; (ii) OVA; (iii) OVA + Dex (2 mg/kg); (iv–vi) OVA + GA (25, 50, or 100 mg/kg). Data represented as mean $\pm$ standard deviation (SD) ($n = 8$ per group). * $P < 0.05$, ** $P < 0.01$, compared with OVA mice.

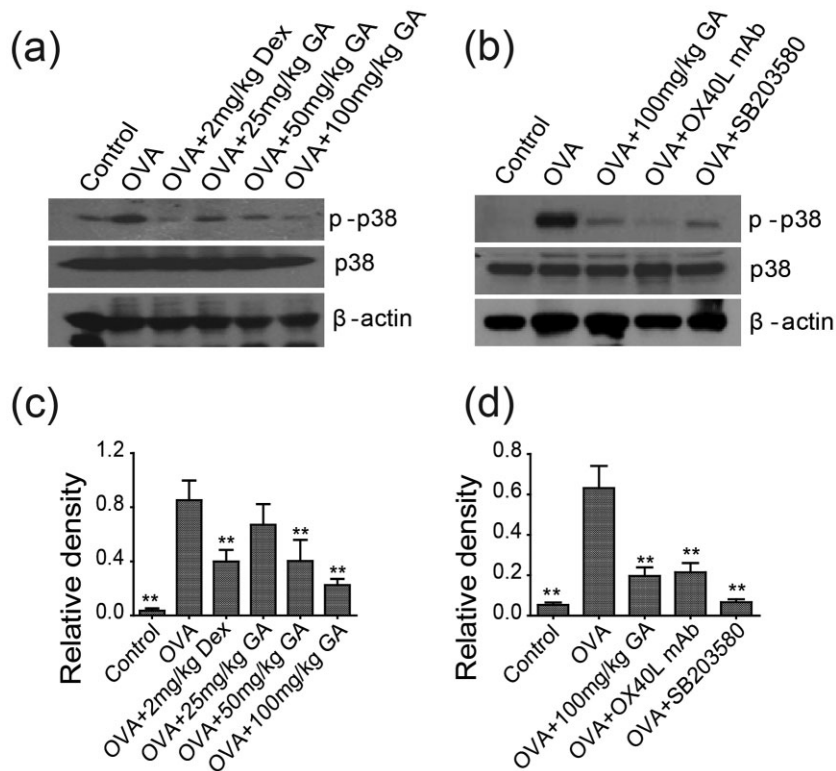


Figure 6 Effect of glycyrrhizic acid (GA) on p38 mitogen-activated protein kinase (MAPK) pathway activation in the lungs of the asthma mouse model. (a, b) Protein expressions of phosphorylated p-p38 and p38 MAPK in lung tissues were evaluated 24 h after the last OVA challenge. (c, d) Density ratio versus β-actin was measured by using a densitometer. Control, saline-sensitized and challenged group; OVA, OVA-sensitized and challenged group; OVA + 2 mg/kg Dex, OVA-sensitized and challenged group receiving 2 mg/kg Dex; OVA + GA, OVA-sensitized and challenged group receiving 25, 50 or 100 mg/kg GA; OVA + anti-OX40L mAb, OVA-sensitized and challenged group receiving 300 μg anti-OX40L mAb; OVA + SB203580, OVA-sensitized and challenged group receiving 5 mg/kg SB203580. Data represented as mean ± standard deviation (SD) ($n = 6$ per group). * $P < 0.05$, ** $P < 0.01$, compared with OVA mice.

severity of asthma, including airway inflammation and hyperresponsiveness (Supplementary Figures S1 and S2). We provide new insights on GA regarding its anti-inflammatory action by targeting costimulatory pathways. Previous scholars have demonstrated an important role of OX40 and OX40L in costimulation of allergen-specific T cells,^{7,9–12} which lead to the development of effector cells and production of high levels of Th2 cytokines, such as IL-4, IL-5 and IL-13. They also demonstrated that OX40- or OX40L-deficient mice exhibited impaired reactivation of memory Th2 immune response and diminished inflammation in lung.^{15,37} In our study, OVA induced a significant increase of OX40 and OX40L in both the lungs and spleens of mouse models. Blockade the interaction between OX40 and OX40L using anti-OX40L mAb attenuated airway inflammation, hyperresponsiveness and modulated the production of Th1 and Th2 cytokines. These findings indicated that the OX40-OX40L signalling pathway was involved in this OVA-induced allergic inflammation. We also demonstrated that GA could significantly downregulate the mRNA and protein expression of OX40 and OX40L and modulate the production of cytokines. To further investigate the relationship between GA and OX40-OX40L, we co-cultured GA with anti-CD3 mAb stimulated splenic CD4⁺ T cells both with and without anti-OX40 mAb. The results indicated that GA, especially at high dose, could inhibit the augmented proliferation and alter the production of cytokines of splenic CD4⁺ T cells induced with anti-OX40 mAb in vitro.

As described previously, p38, one of three major MAPKs, has been considered to play a key role in allergic asthma and also been significantly implicated

in corticosteroid insensitivity.^{38,39} Our study has shown that the augmentation of asthmatic symptoms as well as the phosphorylation of p38 MAPK after OVA challenge is significantly inhibited after the administration of SB203580, a selective p38 MAPK inhibitor. Moreover, anti-OX40L mAb prominently represses the activation of p38 MAPK in OVA-induced lungs, suggesting that OX40-OX40L may act as upstream of the p38 MAPK pathway in allergic airway disease. Likewise, GA dramatically decreases the phosphorylation of p38 MAPK in lung tissues of OVA-challenged mice. Accordingly, these findings support the point that treatment of allergic mice with GA leads to the suppression of OX40/OX40L and the subsequent disruption of p38 MAPK activity, reversing pathophysiologic features of asthma. Further studies are planning to investigate the effects of GA on steroid resistance asthmatic model.

In conclusion, our data suggest that GA exerts a therapeutic effect on OVA-induced experimental asthma partly by regulating the Th1/Th2 balance via OX40/OX40L signalling and p38 MAPK pathway. GA may be a promising therapeutic drug for asthma.

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Supplementary Information

Additional Supplementary Information can be accessed via the online version of this article at the publisher's website:

Figure S1 Effect of GA on OVA-induced airway hyperresponsiveness and airway inflammation in mice. Changes of airway responsiveness in each group after stimulation with different concentrations of methacholine. Airway responsiveness

was measured and expressed as (a1) lung resistance (RL) and (b1) dynamic compliance (Cdyn) after the final challenge. Twenty-seven days after OVA injection and inhalation, mice were exsanguinated and their lungs were lavaged. (c1) Total cells, lymphocytes, neutrophils, eosinophils and macrophages in BALF were analyzed. Each bar represents the mean $\pm$ SD of 8 mice. (d1) Microscopic findings of the lung tissues stained with hematoxylin and eosin (Magnification $\times$ 200). (d1i) Control; (d1ii) OVA; (d1iii) OVA + Dex (2 mg/kg); (d1iv-d1vi) OVA + GA (25, 50, or 100 mg/kg). * P < 0.05, ** P < 0.01, compared with OVA mice.

Figure S2 Effect of GA on OVA-induced airway hyper-responsiveness and airway inflammation in mice. Changes

of airway responsiveness in each group after stimulation with different concentrations of methacholine. Airway responsiveness was measured and expressed as (a2) lung resistance (RL) and (b2) dynamic compliance (Cdyn) after the final challenge. Twenty-seven days after OVA injection and inhalation, mice were exsanguinated and their lungs were lavaged. (c2) Total cells, lymphocytes, neutrophils, eosinophils and macrophages in BALF were analyzed. Each bar represents the mean $\pm$ SD of 8 mice. (d2) Microscopic findings of the lung tissues stained with hematoxylin and eosin (Magnification $\times$ 200). (d2i) Control; (d2ii) OVA; (d2iii) OVA + GA (100 mg/kg); (d2iv) OVA + anti-OX40L mAb (300 μ g); (D2v) OVA + SB203580 (5 mg/kg). * P < 0.05, ** P < 0.01, compared with OVA mice.

SUPPLEMENTARY INFORMATION

Regulation of Th1/Th2 Balance through OX40/OX40L Signaling by Glycyrrhizic Acid in a Murine Model of Asthma

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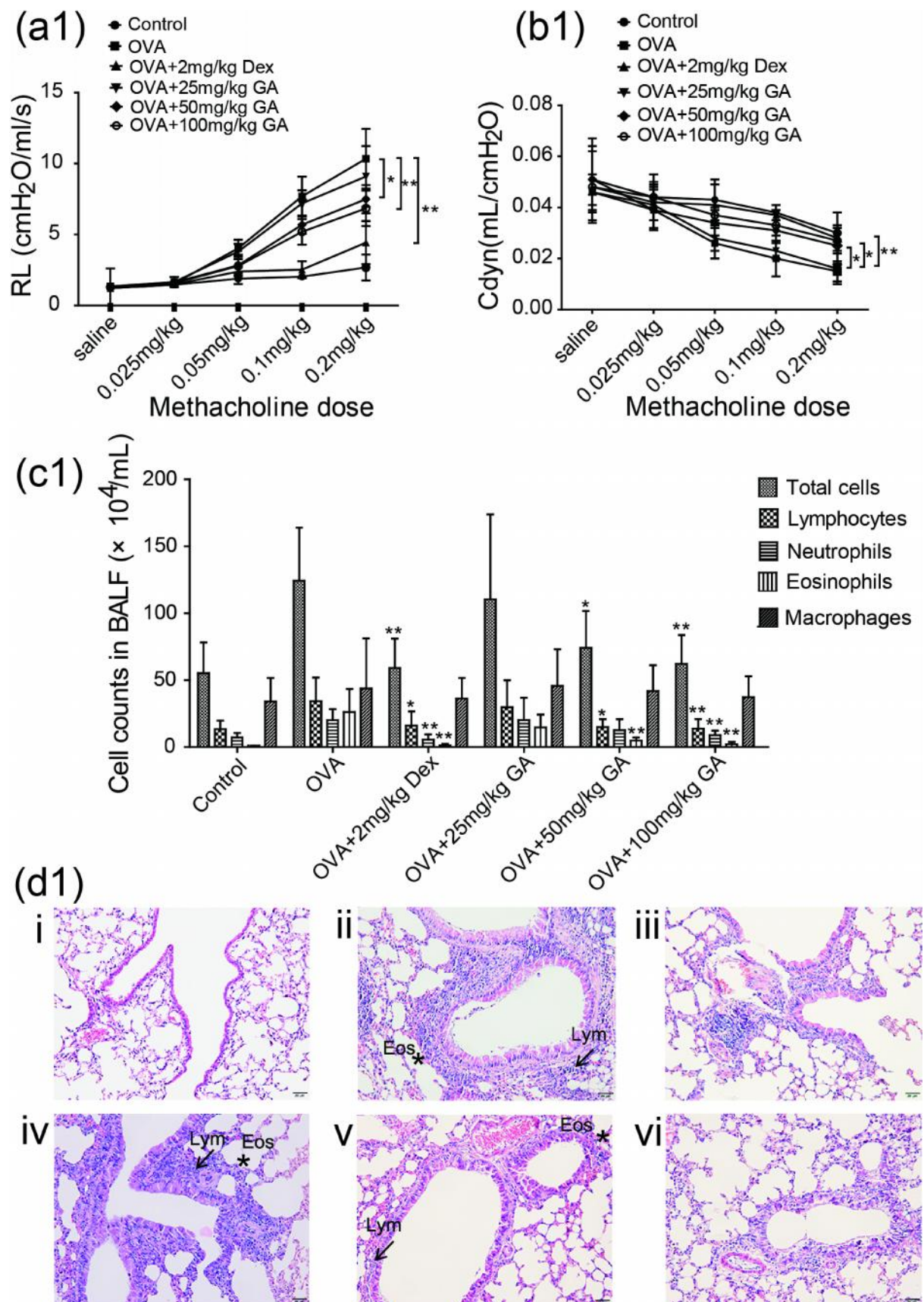
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Figure S1

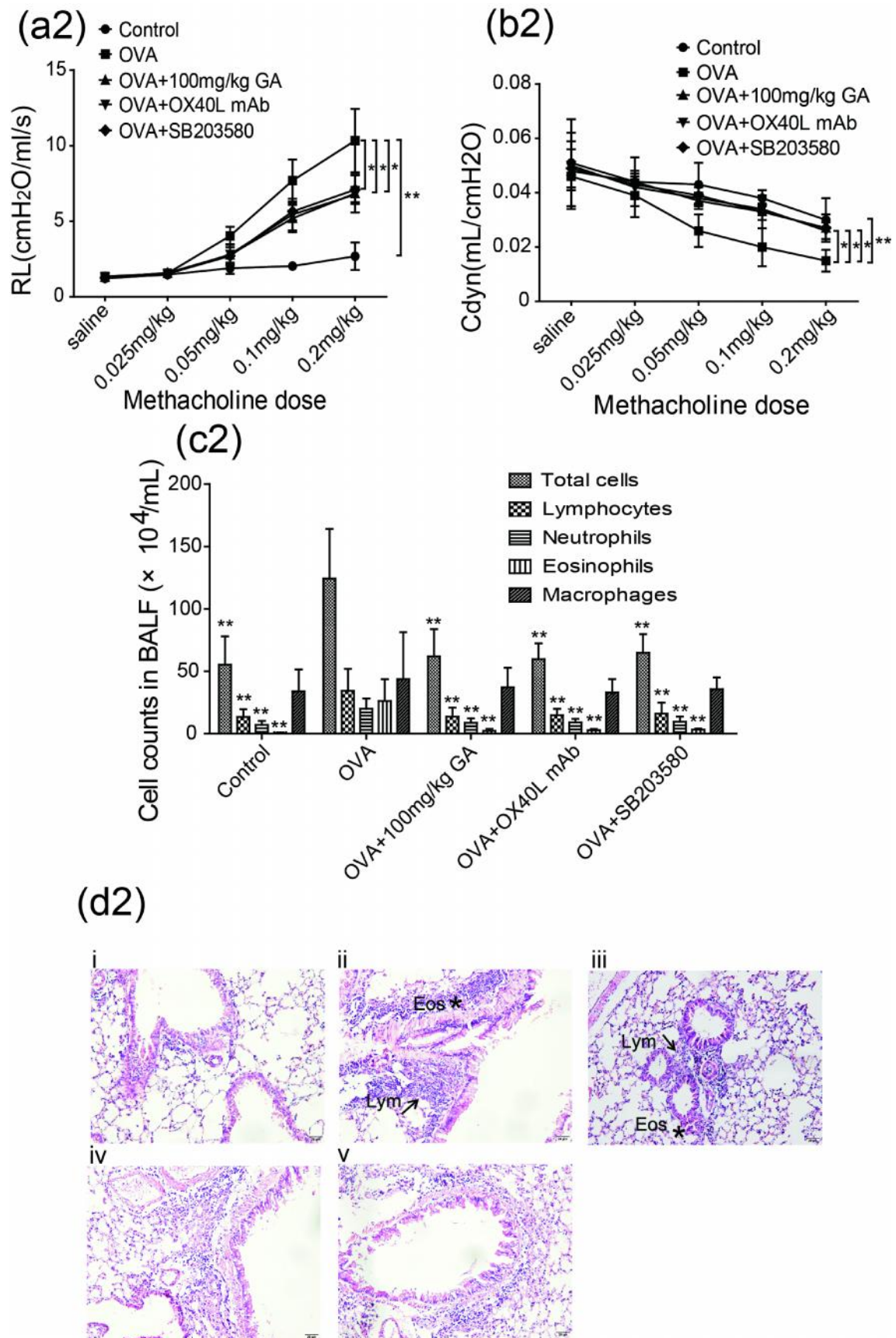


Effect of GA on OVA-induced airway hyperresponsiveness and airway inflammation in mice.

Changes of airway responsiveness in each group after stimulation with different concentrations of methacholine. Airway responsiveness was measured and expressed as (a1) lung resistance (RL) and (b1) dynamic compliance (Cdyn) after the final challenge.

Twenty-seven days after OVA injection and inhalation, mice were exsanguinated and their lungs were lavaged. (c1) Total cells, lymphocytes, neutrophils, eosinophils and macrophages in BALF were analyzed. Each bar represents the mean $\pm$ SD of 8 mice. (d1) Microscopic findings of the lung tissues stained with hematoxylin and eosin (Magnification $\times$ 200). (d1i) Control; (d1ii) OVA; (d1iii) OVA + Dex (2 mg/kg); (d1iv-d1vi) OVA + GA (25, 50, or 100 mg/kg). * $p < 0.05$, ** $p < 0.01$, compared with OVA mice.

Figure S2



Effect of GA on OVA-induced airway hyperresponsiveness and airway inflammation in mice.

Changes of airway responsiveness in each group after stimulation with different concentrations of methacholine. Airway responsiveness was measured and expressed as (a2) lung resistance (RL) and (b2) dynamic compliance (Cdyn) after the final challenge.

Twenty-seven days after OVA injection and inhalation, mice were exsanguinated and their lungs were lavaged. (c2) Total cells, lymphocytes, neutrophils, eosinophils and macrophages

in BALF were analyzed. Each bar represents the mean $\pm$ SD of 8 mice. (d2) Microscopic

findings of the lung tissues stained with hematoxylin and eosin (Magnification $\times$ 200). (d2i)

Control; (d2ii) OVA; (d2iii) OVA + GA (100 mg/kg); (d2iv) OVA + anti-OX40L mAb (300 μ g);

(D2v) OVA + SB203580 (5mg/kg). * $p < 0.05$, ** $p < 0.01$, compared with OVA mice.