

# Short-term roxithromycin treatment attenuates airway inflammation via MAPK/NF- $\kappa$ B activation in a mouse model of allergic asthma

Xinxin Ci · Xiao Chu · Xue Xu · Hongyu Li ·  
Xuming Deng

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## Abstract

**Objective** We investigated whether roxithromycin reduces ovalbumin-specific allergic asthma symptoms in mice, and we further investigated the inhibitory mechanism of roxithromycin in ovalbumin-specific allergic asthma.

**Methods** Mice were divided into five groups ( $n = 10$  for each): control group, roxithromycin-treated groups (5, 20 and 40 mg/kg) and ovalbumin-challenged group. We measured the recruitment of inflammatory cells into the bronchoalveolar lavage fluid (BALF) or the lung tissues by Kwik-Diff and hematoxylin and eosin (H&E) staining, goblet cell hyperplasia by alcian blue–periodic acid–Schiff (AB-PAS) staining, airway hyperresponsiveness (AHR) by whole-body plethysmograph chamber, cytokine and immunoglobulin E (IgE) levels by ELISA, and the activation of mitogen-activated protein (MAP) kinases and nuclear factor-kappa B (NF- $\kappa$ B) in the lung tissues by Western blotting.

**Results** Treatment with roxithromycin resulted in fewer inflammatory cells in the BALF and peribronchial areas, and decreased AHR, goblet cell hyperplasia, IgE levels and inflammatory cytokines, as well as MAP kinases and NF- $\kappa$ B activation, which are increased in lung tissues of mice with ovalbumin-induced allergic asthma.

**Conclusions** Our data suggest that oral administration of roxithromycin suppresses ovalbumin-induced airway inflammation and AHR by regulating the inflammatory cytokines via MAP kinases/NF- $\kappa$ B pathway in

inflammatory cells. Based on these results, we suggest that roxithromycin may be used as a therapeutic agent for allergy-induced asthma.

**Keywords** Asthma · Airway inflammation · AHR · NF- $\kappa$ B · MAPKs

## Introduction

Recently, asthma has become one of the most common health problems in the world, especially within industrialized societies. In fact, the prevalence of asthma has been increasing considerably over the last few decades [1–3] for reasons that are not yet completely understood. Allergic asthma is characterized by elevated serum immunoglobulin E (IgE) antibody levels, increased production of cytokines such as interleukin (IL)-4, IL-5 and IL-13 produced by T helper-2 (Th2) cells; and inflammation of the airways with recruitment of inflammatory cells into bronchoalveolar lavage (BAL) fluids [4–6] and lung tissue. Mucus overproduction and airway hyperresponsiveness (AHR) are also hallmarks of asthma [6, 7]. Moreover, there is interplay between each of these features. The Th2 cytokines mentioned above are produced by activated CD4+ T cells, and they play a central role in the pathogenesis of asthma. IL-4 causes B cells to synthesize IgE, which is involved in mast cell degranulation by cross-linking with IgE receptors. IL-5 enables terminal differentiation and proliferation of eosinophil precursors and the eosinophil activation effect [8]. IL-13 also has a pivotal role in triggering asthma symptoms such as AHR, airway inflammation, goblet cell hyperplasia, airway luminal narrowing and subepithelial fibrosis [9].

Current asthma therapies do not inhibit these features in a satisfactory manner. There is therefore a need for

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X. Ci · X. Chu · X. Xu · H. Li · X. Deng (✉)  
Key Laboratory of Zoonosis Research, Institute of Zoonosis,  
College of Animal Science and Veterinary Medicine, Ministry  
of Education, Jilin University, 5333 Xi'an Road, Changchun  
130062, People's Republic of China  
e-mail: xumingdeng@jluph.edu.cn

innovative anti-inflammatory approaches to treating asthma; one potential approach is to utilize macrolides, a family of antibiotics. Macrolides were originally exploited as antibiotics against *Staphylococcus*, *Streptococcus pneumoniae*, *Moraxella*, and *Propionibacterium acnes* [10]. In addition to antimicrobial effects, recent evidence suggests that macrolides also possess distinct anti-inflammatory properties in both infectious and non-infectious airway inflammatory models [11–14]. Roxithromycin, a fourteen-membered macrolide, augments the phagocytotic and bactericidal activities of neutrophils [15]. Clinical studies have shown its efficacy against chronic inflammatory diseases, including diffuse panbronchiolitis [16–18], chronic sinusitis [19], atopic dermatitis and bronchial asthma [20–22]; however, it is unclear if the beneficial effects are related to their anti-microbial or anti-inflammatory properties, because bacteria can often be isolated from the airways in patients with these diseases. To determine if roxithromycin possesses anti-inflammatory properties in a mouse model of allergic inflammation, we tested the ability of roxithromycin to alter ovalbumin-dependent airway inflammation in the mouse.

Despite the prevalence of asthma, its source and mechanism of action remain unclear. Recently, increasing evidence suggests that Rho family kinases might be a potential drug target due to their central role in acute and chronic AHR [23, 24]. Rho family kinases regulate mitogen-activated protein (MAP) kinases [25]. The MAP kinase family is composed of several protein kinases including ERK1 (p44MAPK)/ERK2 (p42MAPK), p38 and JNK. These kinases in turn activate redox-sensitive transcription factors such as nuclear factor-kappa B (NF- $\kappa$ B) [26], a ubiquitous transcription factor complex, which is necessary for directing high-level transcription of many cytokines, adhesion molecules, and other proinflammatory proteins. NF- $\kappa$ B overactivation plays a crucial role in asthma by increasing expression of many inflammatory genes and by perpetuating eosinophilic airway inflammation [27, 28]. This is accomplished by increasing CD4<sup>+</sup> T-cell activation and decreasing CD4<sup>+</sup> T-cell apoptosis. Because the pathophysiological mechanism underlying the attenuating effects of roxithromycin on asthma is not known, we investigated whether roxithromycin reduces ovalbumin-specific allergic asthma symptoms in mice, and we further investigated the inhibitory mechanism of roxithromycin in ovalbumin-specific allergic asthma.

## Materials and methods

### Reagents

IL-4, IL-5, and IL-13 ELISA kits were purchased from BioLegend Inc. (San Diego, CA, USA). Rabbit phospho-

NF- $\kappa$ B/p65, mouse phospho-I $\kappa$ B $\alpha$  and rabbit I $\kappa$ B $\alpha$  were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Rabbit anti-JNK antibody, rabbit anti-ERK antibody, rabbit anti-p38 antibody, mouse anti-phospho-JNK antibody, mouse anti-phospho-ERK antibody, and mouse anti-phospho-p38 antibody were purchased from Santa Cruz (Santa Cruz, CA, USA). Roxithromycin, dimethyl sulfoxide (DMSO) and ovalbumin (OVA, Grade V) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cy3-conjugated sheep anti-rabbit IgG, peroxidase-conjugated Affinipure goat anti-mouse IgG (H + L) and peroxidase-conjugated Affinipure goat anti-rabbit IgG (H + L) were purchased from PTG (Chicago, IL, USA).

### Animals

Female BALB/c mice, weighing approximately 18–20 g, were purchased from Shanghai Jingke Industrial Co., Ltd (Certificate SCXK2003-0003) (Shanghai, China). The mice were housed in microisolator cages and received food and water ad libitum. The laboratory temperature was  $24 \pm 1$  °C, and the relative humidity was 40–80 %. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### Sensitization and challenge with OVA and treatment

These mice were divided into five groups ( $n = 10$  per group) and all mice were sensitized with 20  $\mu$ g OVA adsorbed in 100  $\mu$ g/ml of Imject Alum by i.p. injection on days 0, 7, and 14 (general sensitization). On day 14, mice were anesthetized and 100  $\mu$ g of OVA in 50  $\mu$ l of phosphate-buffered saline (PBS) was administered intranasally in all mice except negative control sensitized with PBS. Mice were again anesthetized before being challenged with 100  $\mu$ g of OVA in 50  $\mu$ l of PBS on each of days 23–26. On days 23–26, roxithromycin at 5, 20, or 40 mg/kg was given by i.p. injection 1 h prior to OVA administration.

### Collection of blood and bronchoalveolar lavage

Twenty-four hours after the last intranasal exposure, mice were anesthetized and bled via the brachial plexus. Collection of blood samples was used to estimate IgE production. Bronchoalveolar lavage was performed twice by intratracheal instillation of 500  $\mu$ l of PBS. The lavage fluid was centrifuged, and the supernatants were used for cytokine measurements. Cell pellets were resuspended in 1 ml of PBS and used for total and differential cell counts. The total cell number in bronchoalveolar lavage fluid (BALF) was counted using a hematology counter (Sysmex SF 3000, Sysmex Co., Kobe, Japan). Eosinophils,

neutrophils, and macrophages in BALF were counted using cytopsin preparations (centrifuged preparations stained with the Kwik-Diff staining set; Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). At least 200 cells in each sample were counted.

#### Lung histology

Histopathological evaluations were performed on the mice. Left lungs were removed by dissection and fixed in 4 % paraformaldehyde. Lung tissues were sectioned, embedded in paraffin, and cut into 3- $\mu$ m sections. Tissue sections were then stained with H&E for general morphology [29] and with AB-PAS for the identification of goblet cells in the epithelium [30]. Quantitative analyses of cell infiltration and mucus production were performed blind as previously described [31].

#### Cytokine levels in BALF

The levels of IL-4, IL-5, and IL-13 levels in BALF were determined by using an ELISA kit (BioLegend). Concentrations were determined in duplicate for each sample.

#### Mouse anti-OVA IgE ELISA

To determine serum levels of OVA-specific IgE, ELISA was carried out using a mouse-specific anti-IgE antibody. Briefly, microplate wells were coated with 1 % OVA in coating buffer (0.05 M sodium carbonate–bicarbonate, pH 9.6) overnight at 4 °C. The wells were then incubated with blocking buffer (1 % bovine serum albumin in PBS, pH 7.2) at room temperature for 1 h and washed. Next, diluted (1/10) serum samples were introduced to the microplate, which was then incubated at room temperature for 2 h, washed, and incubated with biotin anti-mouse IgE, followed by extravidin-peroxidase at room temperature for 30 min and TMB substrate for 15 min. The enzymatic reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and absorbance was read at 450 nm. Units are reported as optical density (OD) at 450 nm.

#### Measurements of airway hyperresponsiveness

Mice were anesthetized, and tracheotomies were performed as described previously [32]. The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine administration. Airway resistance (RI) and lung compliance (C<sub>dyn</sub>) in response to increasing concentrations of methacholine were recorded using a whole-body plethysmograph chamber (Buxco, Sharon, CT, USA) as described [32]. Airway resistance is defined as the pressure driving respiration divided by flow. Lung

compliance refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. Results are expressed as the percentage of the respective basal values in response to baseline.

#### CD4<sup>+</sup> T-cell isolation and Th2 differentiation

To obtain naive CD4<sup>+</sup> T cells, single cell suspensions were prepared from spleens and treated with monoclonal antibodies to CD4 coupled to magnetic beads according to the protocol provided by the manufacturer. For Th2 differentiation, IL-2 (10 ng/ml), IL-4 (5 ng/ml), and anti-IFN- $\gamma$  (4  $\mu$ g/ml) were added.

#### Western blot analysis of MAPK and NF- $\kappa$ B

Lung tissues were added to lysis buffer and homogenized. T helper-2 cells ( $1 \times 10^6$ ) were plated into six-well plates and pretreated with 20 and 40  $\mu$ g/ml of roxithromycin [33], 1 h prior to a 30 min treatment with anti-CD3 (5  $\mu$ g/ml) [34]. Nuclear and cytoplasmic fractions of lung were prepared as previously described [34]. Whole cell lysates were prepared in 40 mL of lysis buffer [50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.6 % NP-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM  $\beta$ -glycerophosphate, 1 mM fluoride, 2 mM *p*-nitrophenyl phosphate and 1:25 Complete Mini Protease Inhibitor cocktail (Boehringer, Mannheim, Germany)]. After the lysates were incubated on ice for 30 min, they were centrifuged (12,000g at 4 °C) for 5 min to obtain the cytosolic fraction. Protein concentrations were determined using the Bradford assay (Bio-Rad, Munich, Germany) before storage at  $-70$  °C. Samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were then washed in Tris-Tween buffered saline [TTBS, 20 mM Tris–HCl buffer, pH 7.6, containing 137 mM NaCl and 0.05 % (vol/vol) Tween 20], blocked overnight with 5 % (wt/vol) nonfat dry milk, and probed according to the method described by Towbin et al. [35] with specific antibodies against I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$ , p-NF- $\kappa$ B/P65, p54 JNK, p42 ERK, and p38 antibodies, and monoclonal phospho-specific antibodies to p46–p54 JNK, p42/p44 ERK, and p38 in 5 % (w/v) bovine serum albumin dissolved in TTBS. With the use of a peroxidase-conjugated secondary anti-mouse antibody, bound antibodies were detected by ECL plus (GE Healthcare Buckinghamshire, UK). Band intensities were quantified using Image J gel analysis software. The fold increase in the level of protein expression was calculated by comparing it with that of normal controls. The experiments were repeated three times for each experimental condition.

## Statistical analysis

All values were expressed as mean  $\pm$  standard error of the mean (SEM). Differences between mean values of normally distributed data were assessed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical differences were considered significant at  $p < 0.05$ .

## Results

### Ovalbumin-specific serum IgE levels

We measured OVA-specific IgE levels in the serum by ELISA. The levels in the serum were significantly increased 2.2-fold compared to the control group. The OVA-specific IgE levels were significantly inhibited by roxithromycin administration in a dose-dependent manner (Fig. 1). These results suggest that roxithromycin inhibits OVA-specific IgE in a murine model of asthma.

### Effect of roxithromycin on inflammatory cell recruitment

Inflammatory cell levels (i.e., total cells, eosinophils, neutrophils, and macrophages) in BALF were significantly elevated in OVA-challenged mice versus control mice treated with PBS. Roxithromycin treatment significantly reduced the number of total cells, eosinophils, neutrophils, and macrophages in a dose-dependent manner (Fig. 2).

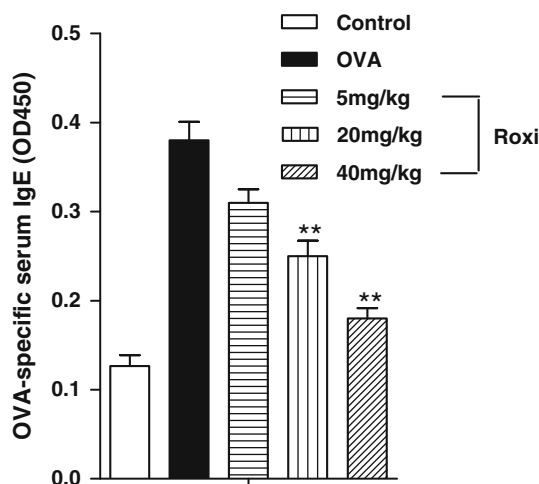


**Fig. 1** Effect of roxithromycin (Roxi) on OVA-specific IgE in serum. OVA-specific IgE levels in the serum were measured by ELISA. Results (mean  $\pm$  SEM) are expressed as optical density values and are representative of at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  versus OVA

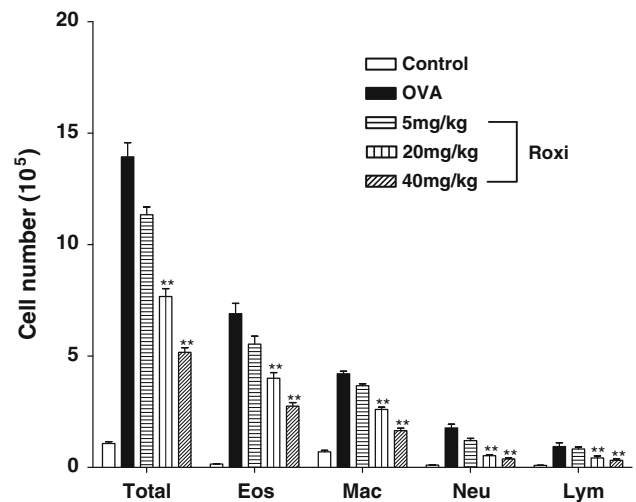


**Fig. 2** Effect of roxithromycin (Roxi) on the recruitment of inflammatory cells in BALF. The lavage fluid was centrifuged, and the cell pellets were resuspended in 1 ml of PBS and applied to a slide by cytospinning to obtain differential cell counts. At least 200 cells were counted per slide. The values represent the mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  versus OVA

Roxithromycin (20 and 40 mg/kg) treatment significantly reduced total cells (from  $12.6 \pm 1.8 \times 10^5$  cells to  $7.8 \pm 0.81 \times 10^5$  and  $5.2 \pm 0.58 \times 10^5$  cells), eosinophils (from  $6.5 \pm 0.45 \times 10^5$  cells to  $4.2 \pm 0.56 \times 10^5$  and  $3.12 \pm 0.21 \times 10^5$  cells), neutrophils (from  $0.9 \pm 0.13 \times 10^5$  cells to  $0.46 \pm 0.031 \times 10^5$  and  $0.22 \pm 0.036 \times 10^5$  cells), and macrophages (from  $5 \pm 0.2 \times 10^5$  cells to  $3.1 \pm 0.25 \times 10^5$  and  $1.82 \pm 0.123 \times 10^5$  cells).

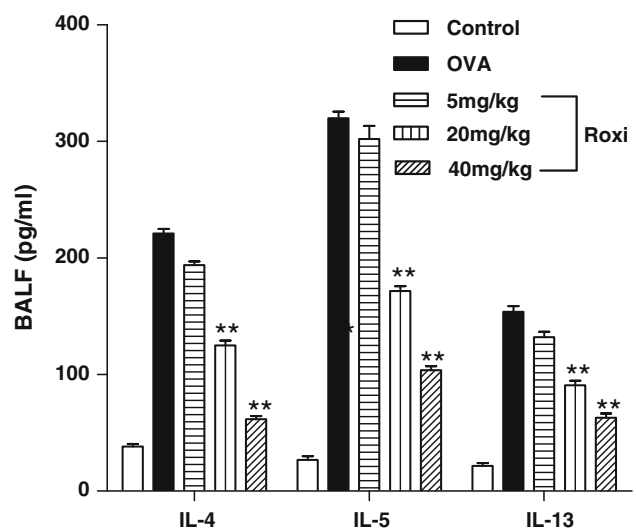


**Fig. 3** Effect of roxithromycin (Roxi) on the secretion of IL-4, IL-5, and IL-13 in BALF. The lavage fluid was centrifuged, and the supernatants were measured by ELISA. The values represent the mean  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$  versus OVA

## Effect of roxithromycin on the release of IL-4, IL-5, and IL-13 in BALF

As shown in Fig. 3, OVA inhalation in sensitized mice caused a marked increase in IL-4 ( $256.908 \pm 22.475$  pg/mL), IL-5 ( $352.55 \pm 33.2$  pg/mL), and IL-13 ( $151.6338 \pm 16.4$  pg/mL) concentrations in BALF compared to PBS control mice. Roxithromycin (20 and 40 mg/kg) treatment significantly decreased the concentrations of IL-4 ( $139.08 \pm 6.17$  and  $94.88 \pm 6.2$  pg/mL), IL-5 ( $215.38 \pm 26.1$  and  $147.442 \pm 20.48$  pg/mL), and IL-13 ( $96 \pm 9.15$  and  $73.96 \pm 6.2$  pg/mL) in a dose-dependent manner.

## Effect of roxithromycin on airway inflammation, goblet cell hyperplasia and mucus production

Histopathological studies were performed to assess the anti-inflammatory effect of roxithromycin on the airway. Inflammatory cell infiltration in the peribronchial and perivascular areas (H&E staining), and mucus overproduction and goblet cell hyperplasia (AB-PAS staining) were observed in OVA-challenged mice (Fig. 4). Roxithromycin treatment (20 and 40 mg/kg) markedly reduced the degree of inflammatory cell infiltration in the peribronchial and perivascular areas, and also reduced mucus

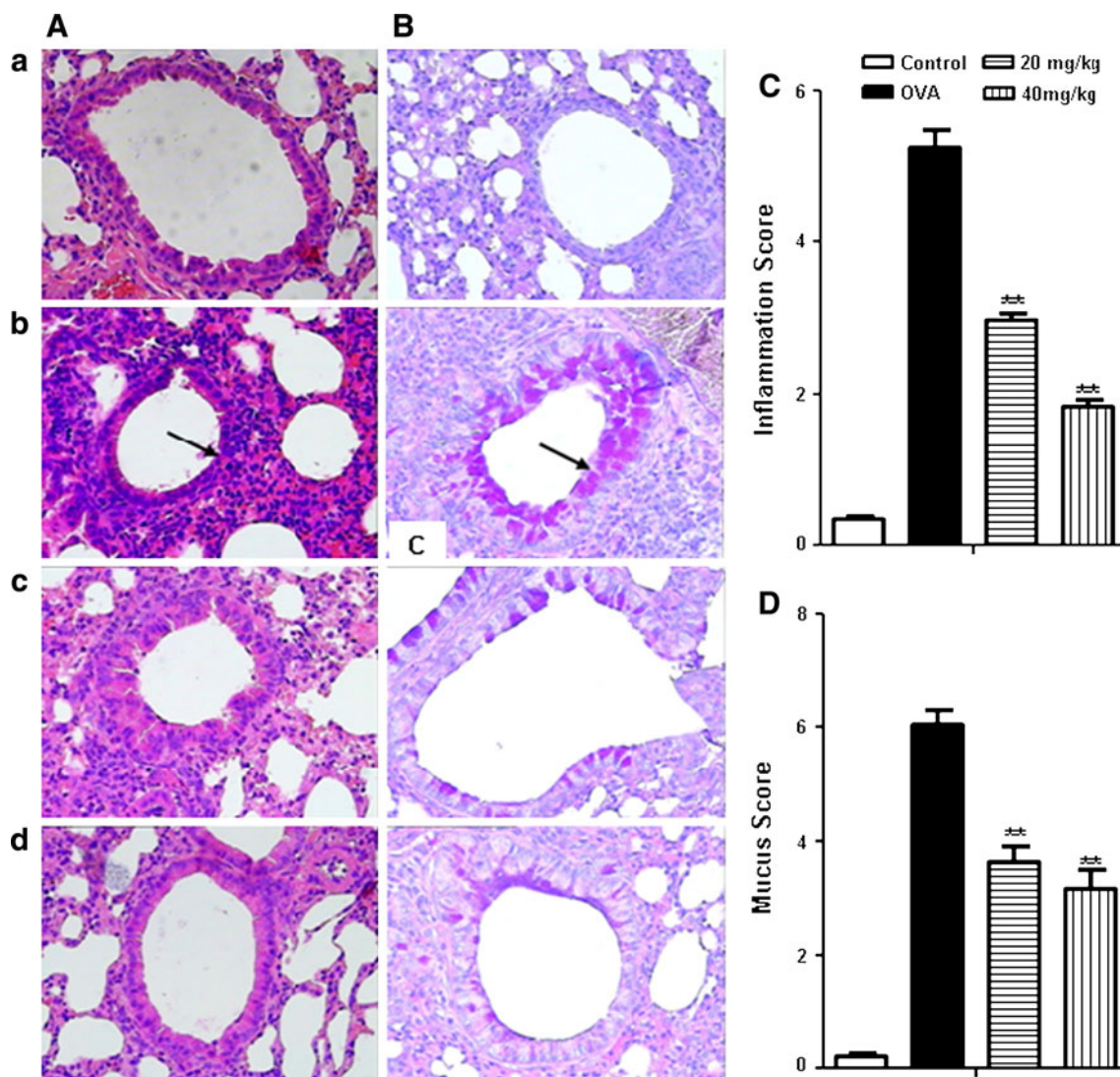


**Fig. 4** Effect of roxithromycin on airway inflammation, airway goblet cell hyperplasia and mucus production. Representative H&E (a) and Alcian blue-periodic acid-Schiff-stained sections (b) of lung from: a PBS-challenged mice; b OVA-challenged mice; c OVA-challenged mice treated with roxithromycin 20 mg/kg; d OVA-challenged mice treated with roxithromycin 40 mg/kg (magnification

$\times 400$ ). Quantitative analyses of inflammatory cell infiltration (c) and mucus production (d) in lung sections were performed as previously described [31]. At least five different fields for each lung section was examined to score the inflammatory cells and goblet cells. Mean scores were obtained from five mice.  $**p < 0.01$  versus OVA

overproduction and goblet cell hyperplasia. Roxithromycin 5 mg/kg did not show any inhibition of mucus overproduction and goblet cell hyperplasia (data not shown).

#### Effect of roxithromycin on ovalbumin-induced airway hyperresponsiveness

To investigate the effect of roxithromycin on AHR in response to increasing concentrations of methacholine, we measured both RI and Cdyn in mechanically ventilated mice. Airway resistance is defined as the pressure driving respiration divided by flow. Lung compliance refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. OVA-challenged mice developed AHR, typically reflected by high RI and low Cdyn (Fig. 5). Roxithromycin treatment (20 and 40 mg/kg) dramatically reduced RI and

restored Cdyn in OVA-challenged mice in response to methacholine.

#### Effect of roxithromycin on NF- $\kappa$ B activation and I $\kappa$ B $\alpha$ phosphorylation and degradation

To evaluate the effect of roxithromycin on NF- $\kappa$ B activation in a mouse asthma model, we homogenized lung tissue to obtain total, nuclear, and cytoplasmic proteins. As shown in Fig. 6a, the lung tissue from OVA-challenged mice showed a notable increase in nuclear protein level and a decrease in cytoplasmic protein level compared to PBS-challenged mice. Roxithromycin treatment significantly attenuated these alterations in a dose-dependent manner. To gain further insight into the mechanism of roxithromycin-mediated regulation of NF- $\kappa$ B in vivo, we examined the effect of roxithromycin on I $\kappa$ B $\alpha$  phosphorylation and degradation. As shown in Fig. 6b, OVA-induced I $\kappa$ B $\alpha$  degradation was significantly blocked by pretreatment with roxithromycin, which was related to I $\kappa$ B $\alpha$  phosphorylation.

#### Effect of roxithromycin on MAPK pathway activation

To evaluate the effect of roxithromycin on MAPK activation in a mouse asthma model, we homogenized the lung tissue of OVA-challenged mice to obtain total proteins. The levels of phosphorylated MAPKs were subsequently measured by Western blot analysis using three different phospho-specific antibodies. The phosphorylation levels of the MAPKs isoforms decreased dramatically in roxithromycin-treated mice compared to OVA-challenged mice; however, there was no change in the phosphorylation levels of the MAPKs isoforms between the different groups (Fig. 7). Total protein levels of MAPKs were used as controls for possible fluctuations in overall protein levels.

#### Effect of roxithromycin on MAPK pathway activation and I $\kappa$ B $\alpha$ phosphorylation and degradation in vitro

After Th2 cells were stimulated with anti-CD3, the levels of phosphorylation of p-p38, p-JNK, p-ERK, and p-I $\kappa$ B $\alpha$  increased, but I $\kappa$ B $\alpha$  decreased compared to the unstimulated group. Treatment with roxithromycin (20 and 40 mg/L) could dramatically downregulate the increase of phosphorylation of p-p38, p-JNK, p-ERK, and p-I $\kappa$ B $\alpha$ , and upregulate the decrease of I $\kappa$ B $\alpha$  (Fig. 8).

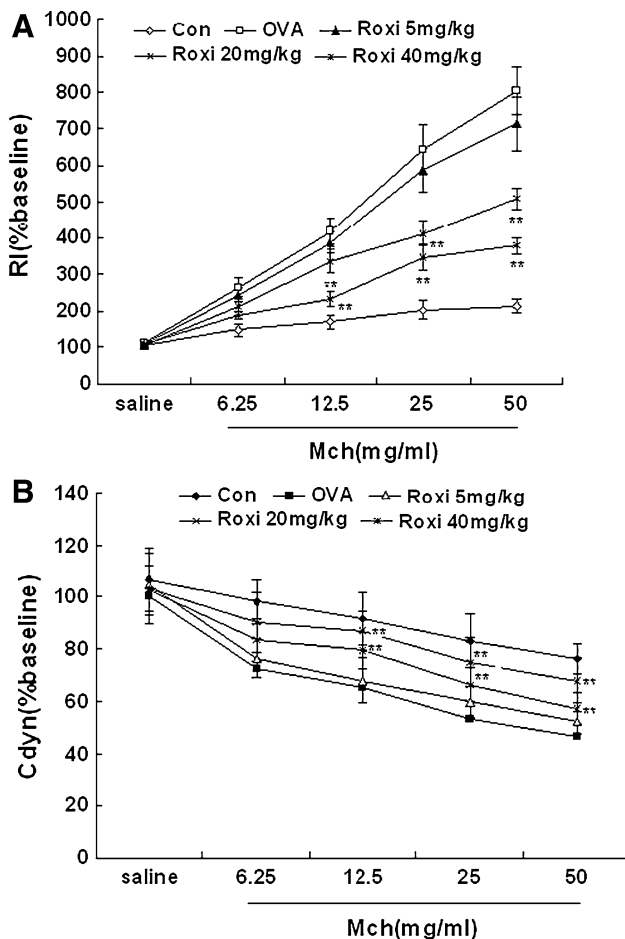


**Fig. 5** Effect of roxithromycin on ovalbumin-induced airway hyperresponsiveness in mice. Airway hyperresponsiveness was assessed by percentage change from the baseline level of **a** lung resistance (RI,  $n = 6$  mice per treatment group) and **b** dynamic compliance (Cdyn,  $n = 6$  mice per treatment group). Mch methacholine, Roxi roxithromycin. \* $p < 0.05$ , \*\* $p < 0.01$  versus OVA

## Discussion

Asthma is a clinical complication of persistent inflammation of the airway and subsequent airway hyperresponsiveness,

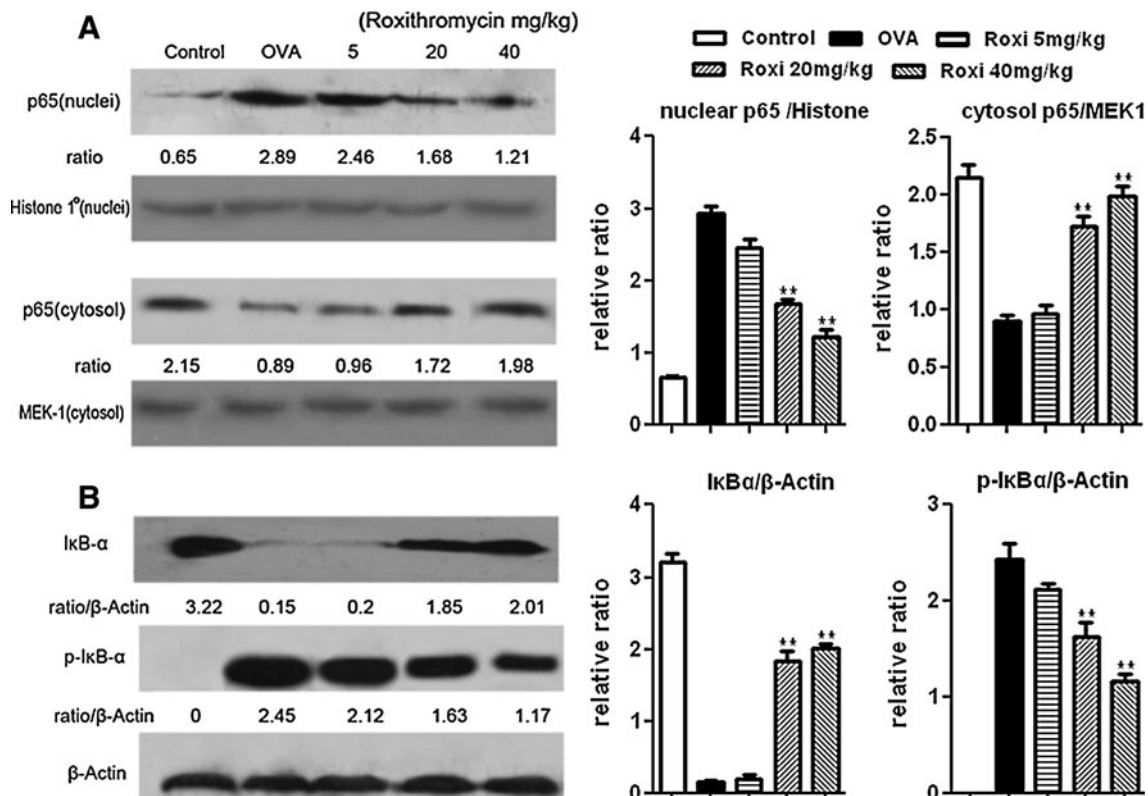


**Fig. 6** Effect of roxithromycin on the activation of NF- $\kappa$ B and I $\kappa$ B $\alpha$  phosphorylation and degradation in vivo. **a** Effect of roxithromycin treatment on nuclear translocation of NF- $\kappa$ B. Nuclear and cytoplasmic proteins from lung were analyzed by Western blotting with specific antibodies. Histone 1 and MEK-1 were used as internal

control. **b** Effect of roxithromycin treatment on I $\kappa$ B $\alpha$  phosphorylation and degradation. Total cellular proteins from lung were analyzed by Western blotting with specific antibodies.  $\beta$ -Actin was used as an internal control. Experiments were repeated three times and similar results were obtained

and it is a leading cause of morbidity and mortality in critically ill patients. Understanding the pathogenesis and mechanism of allergic asthma is important for finding appropriate targets for therapies so that patients can receive symptomatic treatment. There is extensive in-vitro and growing in-vivo evidence that macrolide antibiotics possess anti-inflammatory/immunomodulatory properties that are important for the treatment of various inflammatory disorders of the respiratory tract, including asthma [36]. Roxithromycin is one of the macrolides that has shown efficacy against asthma in clinical settings, but its mechanism was not yet completely demonstrated. Therefore, in our study, we investigated the inhibitory effects and mechanism of roxithromycin in ovalbumin-induced allergic asthma in mice. Our data suggest that roxithromycin attenuates airway inflammation via MAPK/NF- $\kappa$ B activation, thereby indicating its potential use as a therapeutic agent for the treatment of allergic asthma.

Based on animal studies, the immunological processes involved in airway inflammation associated with asthma are characterized by the proliferation and activation of CD4<sup>+</sup> T cells of the subtype Th2. T helper-2 cells produce cytokines such as IL-4, IL-5, and IL-13, which are

associated with the infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen, causing B cells to synthesize IgE. This leads to AHR, airway inflammation, goblet cell hyperplasia, airway luminal narrowing, and subepithelial fibrosis. In our study, we demonstrated that OVA-induced asthma increased levels of inflammatory cytokines such as IL-4, IL-5, and IL-13, as well as increased levels of IgE and infiltration of inflammatory cells in the BALF and peribronchial areas. In addition, OVA-induced asthma caused AHR and goblet cell hyperplasia. All of these features were significantly decreased by administration of roxithromycin, in a dose-dependent manner.

Previous studies have shown that the production of many inflammatory cytokines is dependent on MAP kinases [37, 38]. The B-cell receptor (BCR) activates primarily extracellular signal-regulated kinases (ERK)-2 when B cells are activated in vitro, resulting in the activation of different transcription factors [39]. A previous study has shown that p38 mitogen-activated protein kinase (MAPK) plays a critical role in phosphorylating GATA-3, which in turn is important for regulating the expression of the cytokines IL-4, IL-5, and IL-13 from Th2 cells and is

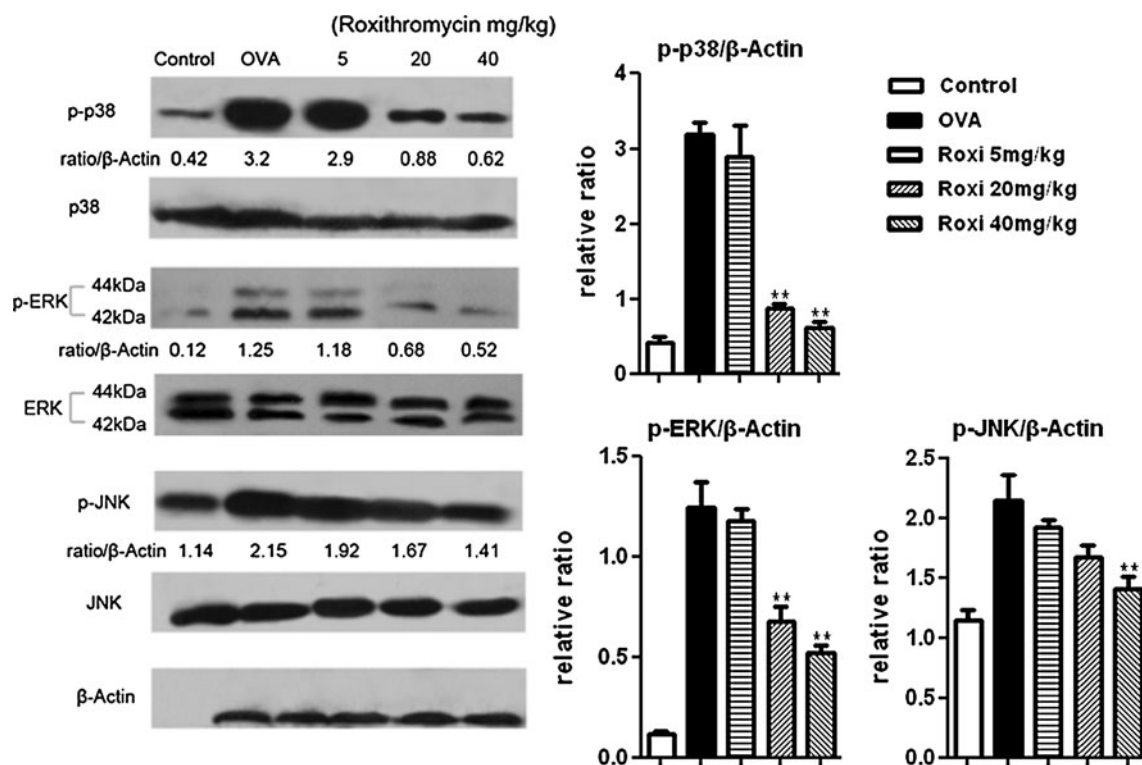


**Fig. 7** Effect of roxithromycin on the activation of MAPK pathway. Total protein samples were analyzed by Western blotting with phosphor-specific antibodies.  $\beta$ -Actin was used as an internal control. Experiments were repeated three times and similar results were obtained

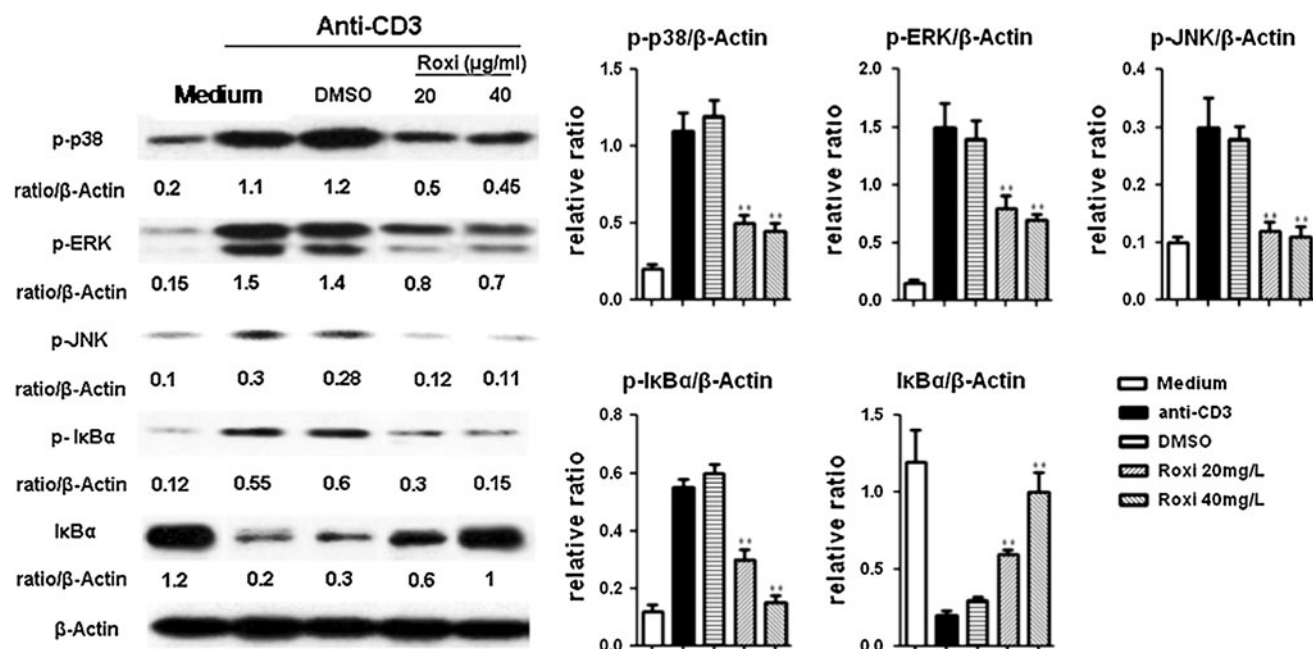


**Fig. 8** Effect of roxithromycin on MAPK pathway activation and IκBα phosphorylation and degradation in vitro. The cells were stimulated with anti-CD3 for 30 min. Total protein samples were analyzed by Western blotting with phosphor-specific antibodies.  $\beta$ -

Actin was used as an internal control. Quantification of protein expression was normalized to  $\beta$ -actin using a densitometer. \* $p < 0.05$ , \*\* $p < 0.01$

therefore a key mediator of allergic diseases [40]. Our data, which describes the increased activation of JNK, ERK, and p38 MAP kinases after ovalbumin challenge, are consistent with the findings in the previous reports [41]. Moreover, our data show that roxithromycin inhibited these signaling pathways and suggest that roxithromycin inhibits MAP kinase pathways in the lung tissues of ovalbumin-challenged mice.

NF- $\kappa$ B is a pleiotropic transcription factor that plays an important role in regulating the expression of multiple genes involved in immune responses. NF- $\kappa$ B plays a central role in asthma, and its activation in airways is necessary to fully induce the recruitment of inflammatory cells and drive a majority of the antigen-induced inflammation that is a significant contributor to adaptive immune responses and structural remodeling of the airway wall [42]. In a previous study, it was found that proteasome-dependent degradation of nuclear p65/RelA is the predominant mechanism of NF- $\kappa$ B signaling termination in the absence of I $\kappa$ B [39]. In our experiment, NF- $\kappa$ B translocated from the cytoplasm to the nucleus after ovalbumin challenge, and roxithromycin inhibited this translocation in a dose-dependent manner. In addition, the phosphorylation and degradation of I $\kappa$ B $\alpha$ , which is required for p65 activation, was regulated by roxithromycin.

The effects of roxithromycin on the signaling pathways mentioned were measured 24 h after the last challenge, when airway and lung inflammation are also markedly inhibited in roxithromycin-treated mice. At that time point, regulation of p38, JNK, MAPK, and NF- $\kappa$ B could be indirectly caused by diminished inflammation or a direct consequence of roxithromycin treatment. To check the indirect or direct effect of roxithromycin treatment on the activation of MAPK and NF- $\kappa$ B, we purified naive CD4<sup>+</sup> T cells and differentiated them to Th2 cells, then pretreated with roxithromycin (20 and 40  $\mu$ g/ml) 1 h prior to a 30-min treatment with anti-CD3 (5  $\mu$ g/ml). In accord with the results *in vivo*, roxithromycin can inhibit the activation of MAPK and NF- $\kappa$ B *in vitro*, which suggests that roxithromycin can directly inhibit the activation of MAPK and NF- $\kappa$ B.

Macrolide antibiotics have potent immunomodulatory activity which can be used as immunomodulatory therapy for chronic lung diseases, including diffuse panbronchiolitis, cystic fibrosis, asthma, and chronic obstructive pulmonary disease [43, 44]. Although macrolide-induced regulation of MAPK and NF- $\kappa$ B may play a key role in regulating production and secretion of proinflammatory cytokines *in vitro*, the molecular mechanisms that underlie the effect of macrolides on asthma are still not clear [45, 46]. We were the first to demonstrate that roxithromycin effectively suppresses AHR and tissue remodeling by regulating inflammatory cytokines and inflammatory cells

via MAP kinases and NF- $\kappa$ B in mice with ovalbumin-induced allergic asthma.

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