

Oxytetracycline Attenuates Allergic Airway Inflammation in Mice via Inhibition of the NF- κ B Pathway

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Abstract Oxytetracycline has been used in the treatment of acute and chronic bronchial inflammation and infectious asthma. However, its potential use for non-infectious asthma has not yet been studied. The objective of this study was to investigate the anti-inflammatory properties of oxytetracycline using a mouse asthma model. Female BALB/c mice, sensitized and challenged with ovalbumin. Naive CD4⁺ T cells from spleen were stimulated for 72 h with anti-CD3 (5 μ g/ml) plus anti-CD28 (2.5 μ g/ml) and differentiated into Th2 cells. IL-4, IL-5, IL-9, IL-13, and ovalbumin (OVA)-specific IgE production were measured by ELISA in BALF and cell supernatants. Histopathological evaluation was used to study the alterations in lung tissue. The mRNA levels of CCL5, CCL11, CCR1, and CCR3 were detected by real-time PCR. In addition, the protein levels of p-Akt, Akt, nuclear factor kappa B (NF- κ B), I κ B α and p-I κ B α in lung tissue and cells were measured by western blot or immunofluorescence analysis. Oxytetracycline treatment caused a marked reduction in IL-4, IL-5, IL-13, immune cells, and the level of ovalbumin-specific IgE. Real-time PCR studies demonstrated that oxytetracycline can significantly reduce CCL5, CCL11 and their specific receptor CCR1 and CCR3. Histological studies demonstrated that oxytetracycline substantially inhibited

ovalbumin-induced inflammatory cell infiltration in lung tissue and goblet cell hyperplasia in airway. Oxytetracycline inhibited the NF- κ B activation via phosphorylation and degradation of I κ B α both in vivo and in vitro. Furthermore, the increased phosphorylated Akt but not Akt protein levels in lung tissues after OVA inhalation were significantly reduced by the oral administration of oxytetracycline. These findings demonstrate an anti-inflammatory effect of oxytetracycline that might be mediated via reduction of inflammatory mediators and activation of transcription factors.

Keywords Allergic airway inflammation · asthma · oxytetracycline · NF- κ B

Introduction

Asthma is one of the most common allergic inflammatory lung diseases worldwide that is characterized by airway inflammation and airway hyperresponsiveness [1]. In most cases, the strength of airway hyper-responsiveness (AHR) correlates with the level of airway inflammation, which is predominated by eosinophil infiltration and production of mucus, Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) and allergen-specific IgE [2].

Th2 cells produce a specific set of cytokines and are important in the control of humoral immunity and allergy [3]. It has been shown that IL-4 plays an important role in B-cell growth and differentiation, as well as secretion of IgE and IL-13 [4]. IL-13 plays a pivotal role in causing asthma symptoms such as airway hyperresponsiveness, airway inflammation, goblet cell hyperplasia, and airway

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luminal narrowing [5]. IL-5 plays a significant role in the process of differentiation, infiltration and activation of pulmonary eosinophils [6]. IL-9 has been reported to contribute to the development of asthma and has been suggested as a potential target for asthma treatment [7]. Signaling pathways controlling Th2 production have been the focus of intense research because they could help to identify novel therapeutic targets for asthma and other allergic diseases.

Inflammation occurs due to the infiltration of eosinophils, neutrophils, and macrophages into the bronchial lumen and lung tissues [8, 9]. Recruitment of these inflammatory cells is regarded as a critical event in the development of airway inflammation. Previous studies have suggested that PI3K contributes to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells [10–12]. Akt, a serine/threonine kinase, is a direct downstream effector of phosphatidylinositol 3-kinase (PI3K) [13]. Akt can be modulated by multiple intracellular signaling pathways and acts as a transducer for many pathways initiated by growth factor receptor-activated PI3K. In addition, CCL5 and CCL11 are both chemokines which binds to a specific receptor CCR1 and CCR3 that participate in eosinophil, neutrophils, macrophages recruitment and Th2 cells infiltration [14].

TCR-mediated Th2 differentiation process and the TCR-induced Th2 cytokine expression process are required for the induction of Th2 response. Some signaling pathways, such as the Lck, LAT, C/EBP β , Ras/ERK MAPK cascade, Ca/calcineurin/NF-AT pathway, and PKC/NF- κ B pathway [15] are play an important role in the two processes. NF- κ B appears to be a critical transcription factor that regulates Th2 cell differentiation and cytokine expression [15]. NF- κ B activity is regulated by an inhibitory protein, I κ B α , which keeps NF- κ B in an inactive state in the cytoplasm. The loss of I κ B α results in the release of free NF- κ B units that translocate from the cytoplasm to the nucleus, where they may trigger the transcription of specific target genes.

Tetracyclines are a broad-spectrum antibiotic that is the drug of choice in infections with *Mycoplasma pneumoniae*, Chlamydiae, Rickettsiae, and some Spirochetes. Recent work has shown that tetracyclines are pluripotent drugs that affect many cellular functions including proliferation, migration, apoptosis, and matrix remodeling [16]. Currently, three groups of tetracyclines are available: tetracycline natural products, tetracycline semisynthetic compounds, and chemically modified tetracyclines [17, 18]. Oxytetracycline is a tetracycline natural product that has been demonstrated to be used in the treatment of acute and chronic bronchial inflammation and

in infectious asthma. Different results from infectious and non-infectious mouse airway inflammatory models indicate a distinct anti-inflammatory role of oxytetracycline. However, no available study has evaluated the effects of oxytetracycline treatment on allergic airway inflammation in an allergic mouse model. Therefore, we used a non-infectious mouse airway inflammatory and bronchial asthma model to examine if there is a distinct anti-inflammatory activity of oxytetracycline and to further investigate the mechanism of the anti-inflammatory effects in this model.

Materials and Methods

Reagents

The mAbs against mouse CD3, CD28, IL-4, IL-5 and IL-13 ELISA kits were purchased from Biolegend (California, USA). Rabbit mAb Phospho-NF- κ B/p65, mouse mAb Phospho-I κ B α , rabbit mAb Phospho-Akt (473), mouse mAb Akt (473) and rabbit mAb I κ B α were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Oxytetracycline, dimethyl sulfoxide, 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

Mice were divided into eight groups ($n=8$ for each). They were sensitized with intraperitoneal ovalbumin plus alum on day 0 and 14, challenged with intranasal ovalbumin on days 23–26, and harvested on day 27. Mice were treated with phosphate-buffered saline (PBS), oxytetracycline (5, 25, 75 mg/kg) or 2 mg/kg dexamethasone on days 23–26, 1 h prior to OVA administration.

Measurements of Airway Hyperresponsiveness

Mice were anesthetized, and tracheotomy was performed as described [19]. The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine administration. Airway resistance (cmH₂O/ml/s) and lung compliance (ml/cmH₂O) in response to increasing concentrations of methacholine were recorded using the Flexivent system (SCIREQ, Montreal, Quebec, Canada) as described [19]. Results are expressed as the percentage of the respective basal values in response to phosphate-buffered saline.

Collection of Blood and Bronchoalveolar Lavage

Twenty-four hours after the last inhalational exposure, mice were anesthetized and bled via the brachial plexus for collecting of blood samples used to estimate IgE production. Bronchoalveolar lavage was performed twice by intratracheal instillation of 500 µL 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 in a hematology counter (Sysmex SF 3000, Sysmex Co., Kobe, Japan). Eosinophils, neutrophils, and macrophages in BALF were counted on cytopsin preparations (centrifuged preparations stained with the Kwik-Diff staining set; Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). A total of 200 cells in each sample were counted.

Lung Histology

Histopathological evaluations were performed on mice. Left lungs were removed by dissection and fixed in 4% paraformaldehyde. Lung tissues were sectioned, embedded in paraffin, and cut into 3-µm sections. Tissue sections were then stained with H&E for general morphology [20] and with AB-PAS for the identifying of goblet cells in the epithelium [21].

Mouse Anti-OVA IgE ELISA

To define serum levels of OVA-specific IgE, an ELISA analysis was performed 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% BSA in PBS, pH 7.2) at room temperature for 1 h and then washed. Diluted (1/10) serum samples were then added to the microplate, 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 with 3,3',5,5'-tetramethylbenzidine substrate for 30 min. The enzymatic reaction was stopped with 2 M H₂SO₄, and the absorbance was read at 450 nm. Concentrations were determined in duplicates for each sample.

RNA Preparation and Quantitative RT-PCR

Total RNA was isolated from lungs (five mice in each group) using the TRIzol reagent (SIGMA-Aldrich, St. Louis, USA). Real-time PCR was performed on cDNA samples using the SYBR Green system (Bio-Rad; Richmond, CA, USA). Primers used were CCL11 sense 5' AAACCATAAACAACCTCCTC3', antisense 5'CAA TAATCCCAC ATCTCC TT3'; CCL5 sense 5'GGATA G A G G T T T C T T G A T T 3', antisense 5' GCTGATTTCTTGGGTTTG 3'; CCR1 sense 5' CACT CACCGTACCTGTAGCC3', antisense 5' TCTGAT GATCCCTGCATAGC 3'; CCR3 sense 5' TCTGCTGA GATGT CCCAATA 3', antisense 5' TCACCAA CAAAGGCGTAG 3'; β-Actin sense 5' CTGT CCCTGTATGCCTCTG3', antisense 5' ATGTCACGCAC GATTTCC 3'. Parameters of PCR reactions were: 94°C for 3 min for one cycle, then 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 30 cycles. Analysis was performed using the sequence detection software supplied with the instrument. A melting curve analysis was performed to control for the specificity of the amplification products. The gene-specific threshold cycle (Ct) for each sample was corrected by subtracting the Ct for the housekeeping gene β-Actin (Ct). PBS-treated controls were chosen as the reference samples, and the Δ Ct for all experimental samples were subtracted by the Δ Ct for the control samples ($\Delta\Delta$ Ct). The magnitude change of test gene mRNA was expressed as $2^{-\Delta\Delta C_t}$ [22]

CD4+ T Cell Isolation and Th2 Differentiation

To obtain naive CD4+ 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-γ (4 µg/ml) were added.

Cytokine Assays

Naive CD4+ T cells were stimulated for 72 h with anti-CD3 (5 µg/ml) plus anti-CD28 (2.5 µg/ml), and Th2 differentiated cells were restimulated for 24 h with anti-CD3 (5 µg/ml) in the present/absent of oxytetracycline (5, 10, and

20 mg/l), after which supernatants were collected and cytokine concentrations were measured by ELISA using commercially available kits. Control cells were incubated in medium alone for the duration of the experiment. ELISA was also used to determine the levels of Th2 cytokines in BALF.

Immunofluorescence Analysis

Th2 cells (4×10^5) cultured on glass coverslips were plated in 24-well plates and pretreated with 20 mg/l of oxytetracycline, 1 h prior to a 1-h treatment with anti-CD3 (5 μ g/ml). Control cells were incubated in medium alone for the duration of the experiment. Glass coverslips were washed with 0.01 M PBS and fixed in 4% formaldehyde for 30 min at room temperature. Detergent extraction with 3% TritonX-100 was performed for 10 min at room temperature. Cover slips were then saturated with PBS containing 5% BSA for 30 min at room temperature and processed for immunofluorescence analysis with a rabbit anti-phospho-NF- κ B/p65 polyclonal antibody followed by Cy3-conjugated sheep anti-rabbit IgG. Finally, coverslips were mounted on slides and fluorescence signals were analyzed by Fluoview microscopy (OLYMPUS, Japan).

Western Blot Analysis of Akt and NF- κ B

Lung tissues were added to lysis buffer and homogenized. Th2 cells (1×10^6) were plated into 6-well plates and pretreated with 20 mg/l of oxytetracycline, 1 h prior to a 1-h treatment with anti-CD3 (5 μ g/ml). Nuclear and cytoplasmic fractions of lung were prepared as previously described [23]. 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_3VO_4 , 20 mM β -glycerophosphate, 1 mM phenylmethylsulphonyl 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 ($12,000 \times g$ at 4°C) for 5 min to obtain the cytosolic fraction. Protein concentration was 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 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. [24] with specific antibodies against Akt, p-Akt, I κ B α , p-I κ B α , p-NF- κ B/P65 in 5% (wt/vol) BSA 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). To ensure equal loading of proteins among samples, the membranes were probed with peroxidase-conjugated Affinipure goat anti-mouse IgG (H+L), peroxidase-conjugated horse anti-goat IgG (H+L) and peroxidase-conjugated Affinipure goat anti-rabbit IgG (H+L).

Results

Ovalbumin-Specific Serum IgE Levels

OVA-sensitized mice showed higher levels of serum anti-OVA IgE antibodies compared to PBS control mice (Fig. 1). A significant reduction in OVA-specific IgE antibodies was observed in mice treated with oxytetracycline or dexamethasone in a dose-dependent manner (Fig. 1; $*p < 0.05$, $**p < 0.01$).

Effect of Oxytetracycline on Inflammatory Cell Recruitment

Inflammatory cell levels (i.e., total cells, eosinophils, neutrophils and macrophages) in bronchoalveolar lavage fluid were significantly elevated in OVA-challenged mice versus PBS control mice. Oxytetracycline treatment significantly reduced the number of total cells, eosinophils, neutrophils, and macrophages in a dose-dependent manner (Fig. 2). Oxytetracycline (75 mg/kg) treatment reduced significantly total cells (from $9 \pm 0.79 \times 10^5$ cells to $3.5 \pm 0.8 \times 10^5$ cells), eosinophils (from $5.25 \pm 0.65 \times 10^5$ cells to $2.55 \pm 0.34 \times 10^5$ cells), neutrophils (from $1.25 \pm 0.19 \times 10^5$ cells to $0.1 \pm 0.115 \times 10^5$ cells), and macrophages (from $3.5 \pm 0.58 \times 10^5$ cells to $0.825 \pm 0.17 \times 10^5$ cells; $*p < 0.05$, $**p < 0.01$).

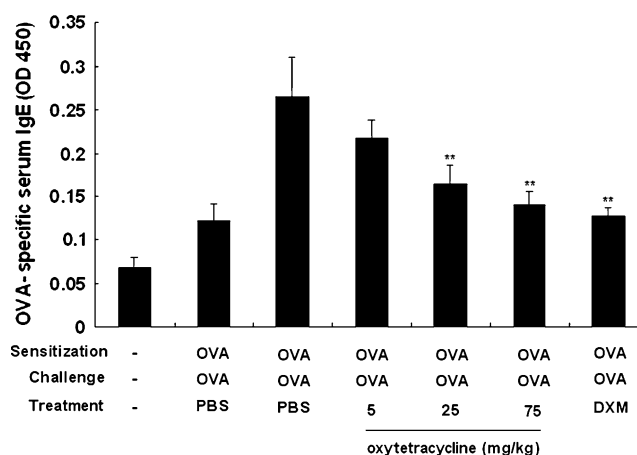


Fig. 1 Effect of oxytetracycline (mg/kg) on OVA-specific IgE in serum. OVA-specific IgE levels in the serum were measured by ELISA. Results (mean $\pm$ SEM) are expressed as optical density values and are representative of at least three independent experiments. $*p < 0.05$, $**p < 0.01$ vs. OVA

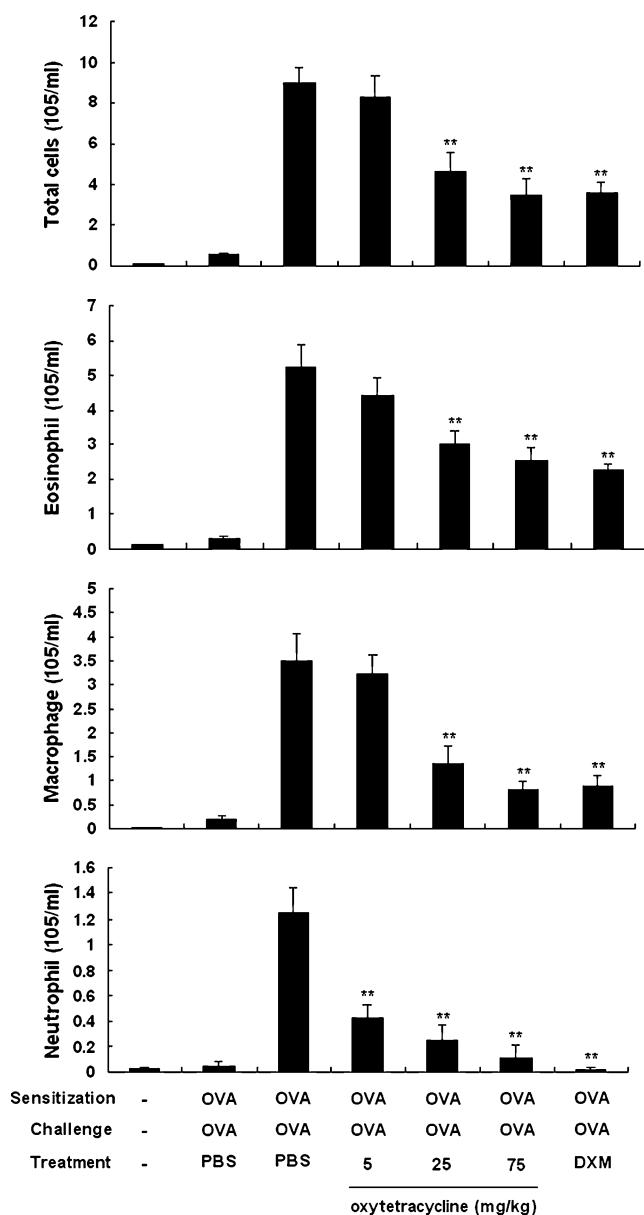


Fig. 2 Effect of oxytetracycline (mg/kg) on the recruitment of inflammatory cells in BALF. The lavage fluid was centrifuged, and the cell pellets were resuspended in 1 ml of PBS and applied to a slide by cytospinning to obtain differential cell counts. At least 200 cells were counted per slide. The values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

Effect of Oxytetracycline 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 notable increase of IL-4 (97.35 ± 11.9 pg/ml), IL-5 (140.882 ± 13.4 pg/ml), and IL-13 (61.658 ± 8.11 pg/ml) concentrations in BALF compared to PBS control mice. Oxytetracycline (25 and 75 mg/kg) treatment significantly decreased the concentrations of IL-4, IL-5, and IL-13 in a dose-dependent manner (* $p<0.05$, ** $p<0.01$).

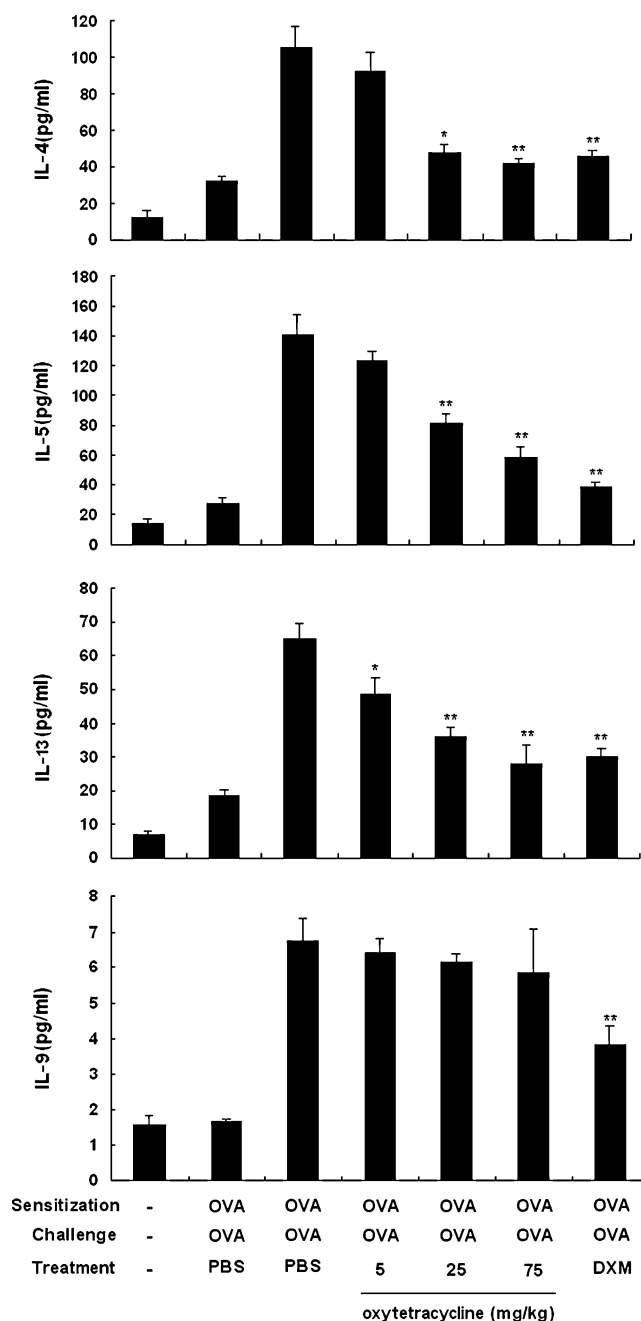


Fig. 3 Effect of oxytetracycline (mg/kg) on the secretion of IL-4, IL-5, IL-9, and IL-13 in BALF. The lavage fluid was centrifuged, and the supernatants were measured by ELISA. The values represent the mean±SEM of three independent experiments. ** $P<0.01$ vs. OVA

Effect of Oxytetracycline on Airway Inflammation, Goblet Cell Hyperplasia, and Mucus Production

In order to assess the anti-inflammatory effect of oxytetracycline on airway, histopathological studies were performed. Inflammatory cell infiltration in the peribronchial and perivascular areas (H&E staining), mucus overproduc-

tion and goblet cell hyperplasia (AB-PAS staining) were observed in OVA-challenged mice (Fig. 4). Oxytetracycline treatment (75 mg/kg) markedly reduced the degree of inflammatory cell infiltration in the peribronchial and perivascular areas, mucus overproduction, and goblet cell hyperplasia.

Effect of Oxytetracycline on Ovalbumin-Induced Airway Hyperresponsiveness

To investigate the effect of oxytetracycline on AHR in response to increasing concentrations of methacholine, we measured both RI and Cdyn in mechanically ventilated mice. RI is defined as the pressure driving respiration divided by flow.

Cdyn 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).

Oxytetracycline treatment (75 mg/kg) dramatically reduced RL and restored Cdyn in OVA-challenged mice in response to methacholine.

Effect of Oxytetracycline on CCL5, CCL 11, CCR1, and CCR3

Whole-lung mRNA expression was determined using quantitative RNA analysis. The levels of chemokines CCL5, CCL11, and their receptors CCR1, CCR3 were greatly enhanced in the OVA group in comparison with the control group. We found that oxytetracycline treatment (25 and 75 mg/kg) significantly reduced the chemokines and their receptors after OVA challenge (Fig. 6).

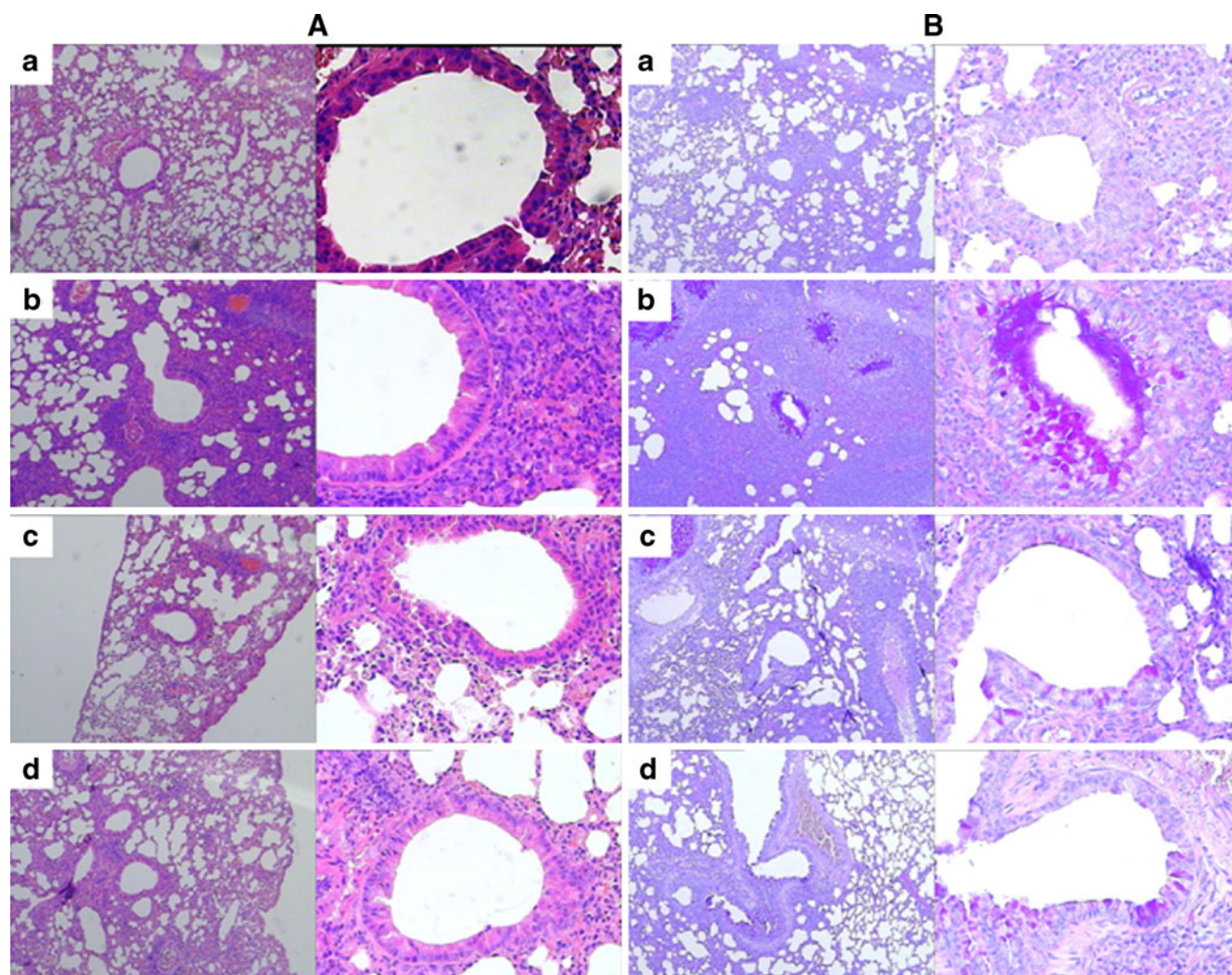


Fig. 4 Effect of oxytetracycline on airway inflammation, airway goblet cell hyperplasia and mucus production. Representative haematoxylin-eosin (**a**) and alcian blue periodic acid Schiff (**b**) stained sections of lung from: *a* PBS-challenged mice, *b* OVA-challenged

mice, *c* OVA-challenged mice treated with oxytetracycline (75 mg/kg), *d* OVA-challenged mice treated with dexamethasone (2 mg/kg). The *left panel* is magnified $\times 100$, the *right panel* is magnified $\times 400$.

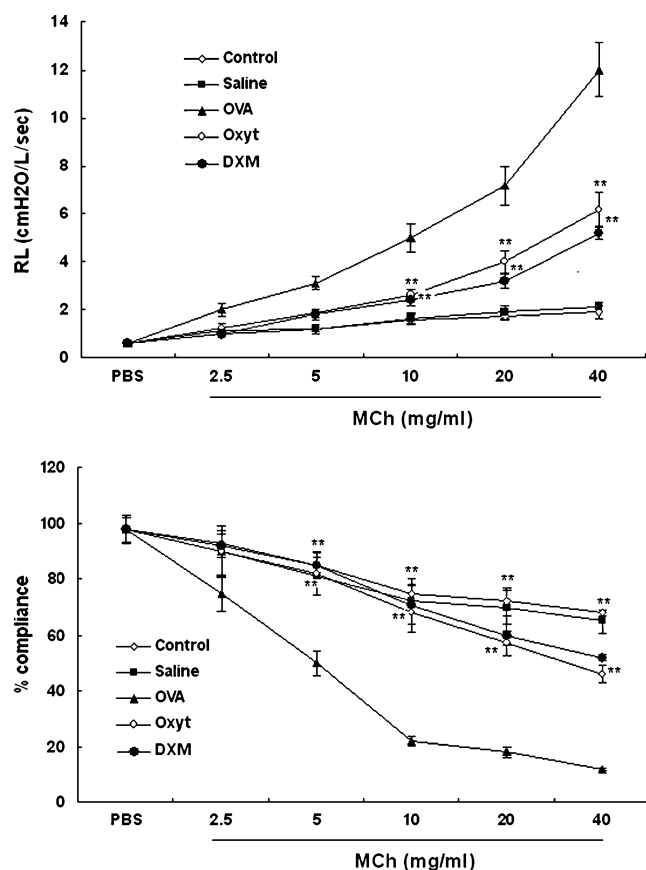


Fig. 5 Effect of oxytetracycline on ovalbumin-induced airway hyperresponsiveness in mice. Airway hyperresponsiveness was assessed by measuring changes in airway resistance (RL) (*upper panel*) and lung compliance (*lower panel*) using the Fexivent system as described in the “Materials and Methods” section. After each methacholine nebulization, the lung resistance (cmH₂O/L/s) and compliance (ml/cmH₂O) were measured and expressed as cmH₂O/L/s and percent of the saline administered group, respectively. The values represent the mean±SEM of three independent experiments. ***P*<0.01 vs. OVA

Effect of Oxytetracycline on Akt Activation In vivo

To evaluate the effect of oxytetracycline on oxytetracycline on the activation in a mouse asthma model, we homogenized the lung tissue to obtain total proteins. The OVA-challenged mice showed marked p-Akt upregulation in their lung tissues compared with control group. Oxytetracycline treatment significantly attenuated these alterations in a dose-dependent manner (Fig. 7, **p*<0.05, ***p*<0.01)

Effect of Oxytetracycline on NF-κB Activation and IκBα Phosphorylation and Degradation In vivo

To evaluate the effect of oxytetracycline on NF-κB activation in a mouse asthma model, we homogenized the lung tissue to obtain total, nuclear and cytoplasmic proteins. In our study, Fig. 8a showed that OVA-challenged mice showed a notably increased nuclear protein level and a decreased cytoplasmic

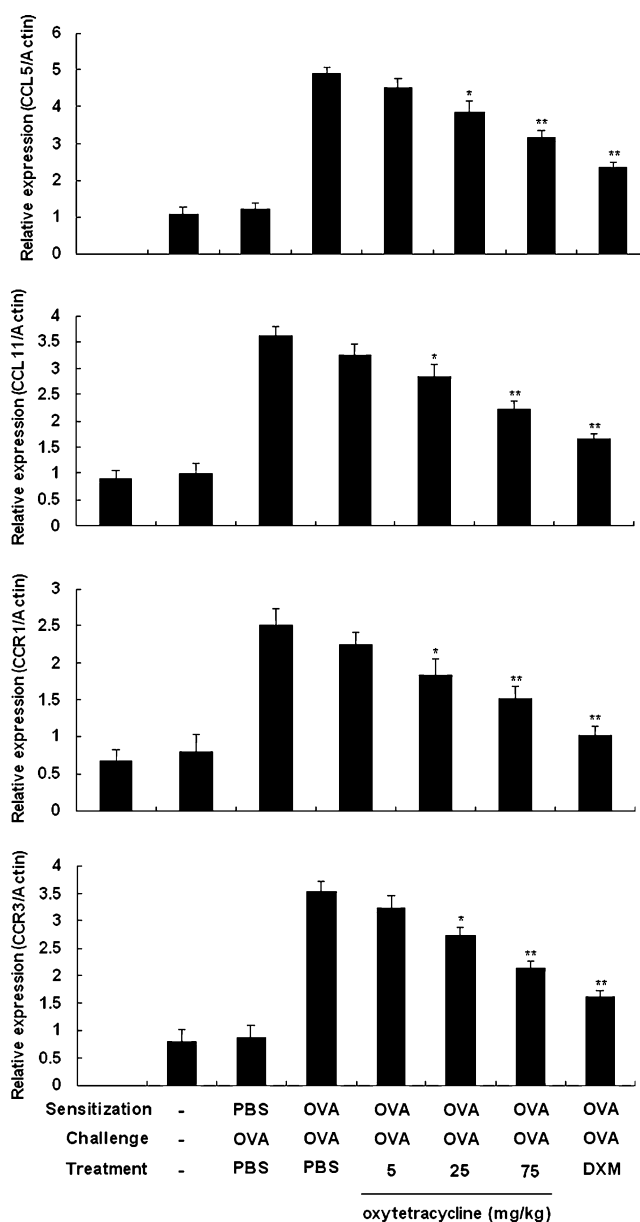


Fig. 6 Effect of oxytetracycline on CCL5, CCL 11, CCR1 and CCR3. The mRNA in whole-lung extracts was measured by real-time PCR. Treatment with oxytetracycline (25 and 75 mg/kg) reduced mRNA expression in the lung. The values represent the mean±SEM of 5 animals in each group. ***P*<0.01 vs. OVA

protein level, compared to PBS-challenged mice. Oxytetracycline treatment significantly attenuated these alterations in a dose-dependent manner (**p*<0.05, ***p*<0.01). To gain further insight into the mechanism of oxytetracycline-mediated regulation of NF-κB in vivo, we examined the effect of oxytetracycline on IκBα phosphorylation and degradation. As shown in Fig. 8b, OVA-induced IκBα degradation was significantly blocked by pretreatment with oxytetracycline, which was related to IκBα phosphorylation.

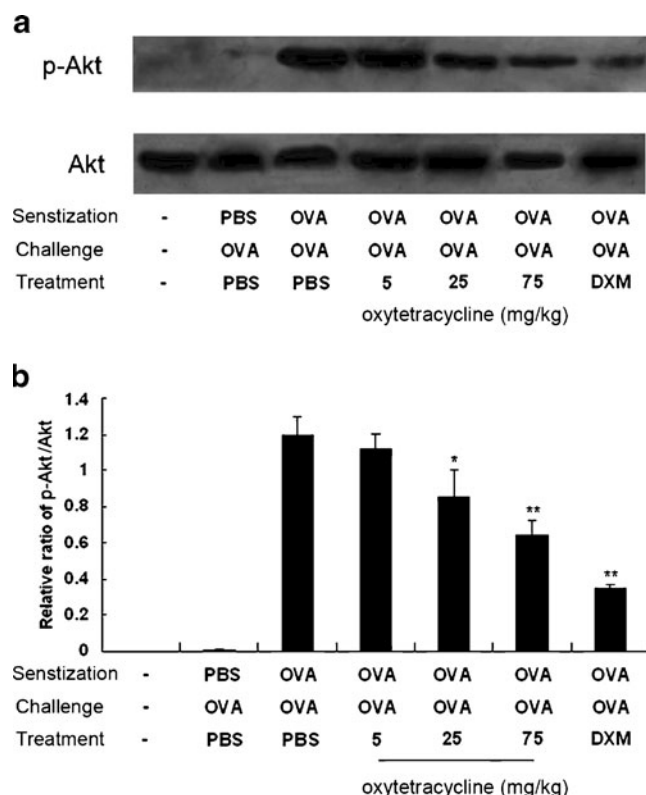


Fig. 7 Effect of oxytetracycline on Akt activation in vivo. Total cellular proteins from lung were analyzed by western blot with specific antibodies. Experiments were repeated three times and similar results were obtained

Effect of Oxytetracycline on the Release of IL-4, IL-5, and IL-13 In vitro

To assess whether oxytetracycline treatment could directly affect lymphocyte function, we examined anti-CD3/CD28-challenged immune responses in Th2 cells. IL-4, IL-5, and IL-13 concentrations in the culture supernatant of Th2 cells were measured by sandwich ELISA (Fig. 9). Treatment with anti-CD3/CD28 alone in Th2 cells resulted in a significant increase of cytokine production compared to that in control cells. However, IL-4, IL-5, and IL-13 levels in the cell supernatant of oxytetracycline-treated cells significantly decreased in a dose-dependent manner (* $p < 0.05$, ** $p < 0.01$).

Effect of Oxytetracycline on NF- κ B Activation and I κ B α Phosphorylation and Degradation In vitro

We evaluated the effect of oxytetracycline NF- κ B activation and found that p65 was distributed in the cytoplasm before anti-CD3/CD28 stimulation. Treatment with anti-CD3/CD28 resulted in the accumulation of p65 in the nucleus 30 min after stimulation with anti-CD3/CD28, the

majority of intracellular p65 translocated from the cytoplasm to the nucleus, as shown in Fig. 10a. In oxytetracycline-treated samples, nuclear translocation of p65 was inhibited (Fig. 10a). To gain further insight into the mechanism of oxytetracycline-mediated regulation of NF- κ B, we examined the effect of oxytetracycline on I κ B α phosphorylation and degradation. As shown in Fig. 8b, anti-CD3/CD28-induced I κ B α degradation was significantly blocked by pretreatment with oxytetracycline. To determine whether this I κ B α degradation was related to I κ B α phosphorylation, we examined the effect of oxytetracycline on anti-CD3/CD28-induced p-I κ B α by western blot and found that oxytetracycline had effect on anti-CD3/CD28-induced I κ B α phosphorylation either.

Discussion

Allergic asthma is currently considered a chronic airway inflammatory disorder associated with the presence of activated CD4⁺ Th2-type lymphocytes and eosinophils. In recent years, allergic disease is a major health issue with high fatality rates. Furthermore, the drug development process is challenging because of the limited understanding of molecular pathogenesis of allergic asthma. The present study was designed to investigate whether negative regulation of NF- κ B by oxytetracycline would affect inflammatory responses related to asthma in vivo and in vitro.

The transcription factor NF- κ B is considered a master regulator of inflammation and immune processes [25, 26] and can regulate multiple proinflammatory genes that may contribute to allergic asthma. NF- κ B activity is controlled by chemical modifications such as phosphorylation and by interactions with other proteins, notably members of the I κ B family (inhibitors of NF- κ B). In our study, we demonstrated that OVA-induced NF- κ B p65 translocation from the cytoplasm to the nucleus was strongly inhibited by oxytetracycline (Fig. 8a) and that phosphorylation and degradation of I κ B α , required for p65 activation, were regulated in oxytetracycline-treated mice in vivo (Fig. 8b). To be more specific, our western blot and immunofluorescence analysis of anti-CD3/CD28-stimulated normal Th2 cells in vitro showed that oxytetracycline inhibited phosphorylation and degradation of I κ B α , which then depressed NF- κ B p65 translocation from the cytoplasm to the nucleus (Fig. 8).

Th2 cytokines play an essential role in the pathogenesis of allergic airway inflammation [27, 28]. Allergen exposure in the airway induces a Th2-dominant response by activating inflammatory cells and increasing concentrations of IL-4, IL-5, and IL-13 [29]. In our study, oxytetracycline treatment significantly decreased high concentrations of IL-4, IL-5, and IL-13 caused by OVA.

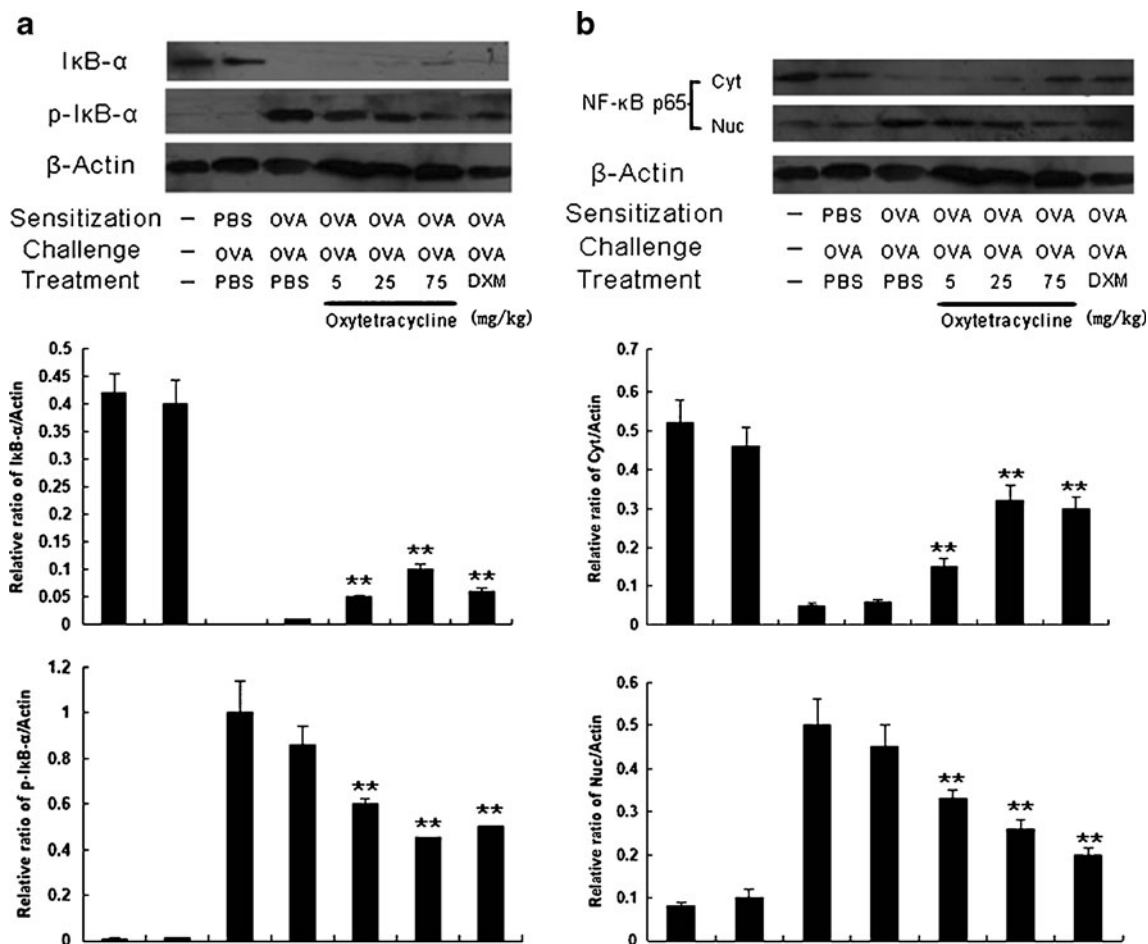


Fig. 8 Effect of oxytetracycline on the activation of NF-κB and IκBα phosphorylation and degradation in vivo. **a** Effect of oxytetracycline treatment on nuclear translocation of NF-κB. Nuclear and cytoplasmic proteins from lung were analyzed by western blot with specific antibodies. **b** Effect of oxytetracycline treatment on IκBα phosphor-

ylation and degradation. Total cellular proteins from lung were analyzed by western blot with specific antibodies. β-Actin was used as an internal control. Experiments were repeated three times and similar results were obtained

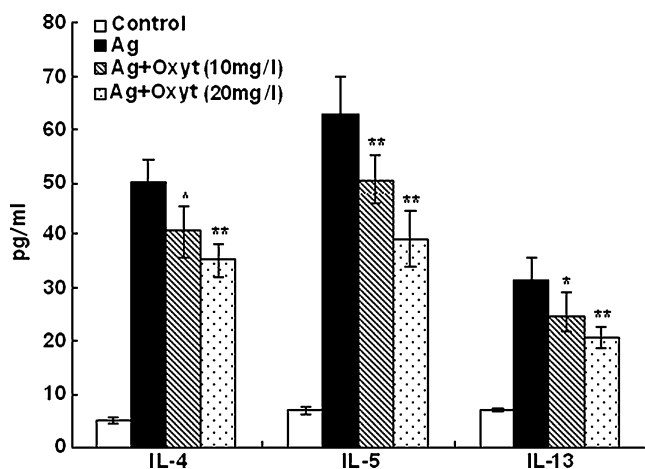


Fig. 9 Effect of oxytetracycline on the release of IL-4, IL-5, and IL-13 in vitro. The supernatant of Th2 cells was measured by sandwich ELISA. The values represent the mean±SEM of three independent experiments. ** $P < 0.01$ vs. Ag control

Furthermore, we investigated the inhibitory effect of oxytetracycline in vitro. Data in murine cells indicated that oxytetracycline significantly decreased high concentrations of IL-4, IL-5, and IL-13 following anti-CD3/CD28 stimulation. These data suggest that the anti-inflammatory effect of oxytetracycline is, at least in part, mediated through a suppressive action on T lymphocytes.

Eosinophils are identified as the major effector cells underlying allergic airway inflammation [30]. It has also been shown that IL-5 enables terminal differentiation and proliferation of eosinophil precursors and exerts an eosinophil activation effect [31]. Chemokines play an important role in allergic inflammatory diseases of the airways through participating in leukocytes recruitment and Th2 cells infiltration. In addition, PI3K contribute to the pathogenesis of asthma by influencing the recruitment of eosinophils and neutrophils. Our present findings showed that oxytetracycline prevented inflammatory cell infiltration in airway as shown by significant decreases in total cell,

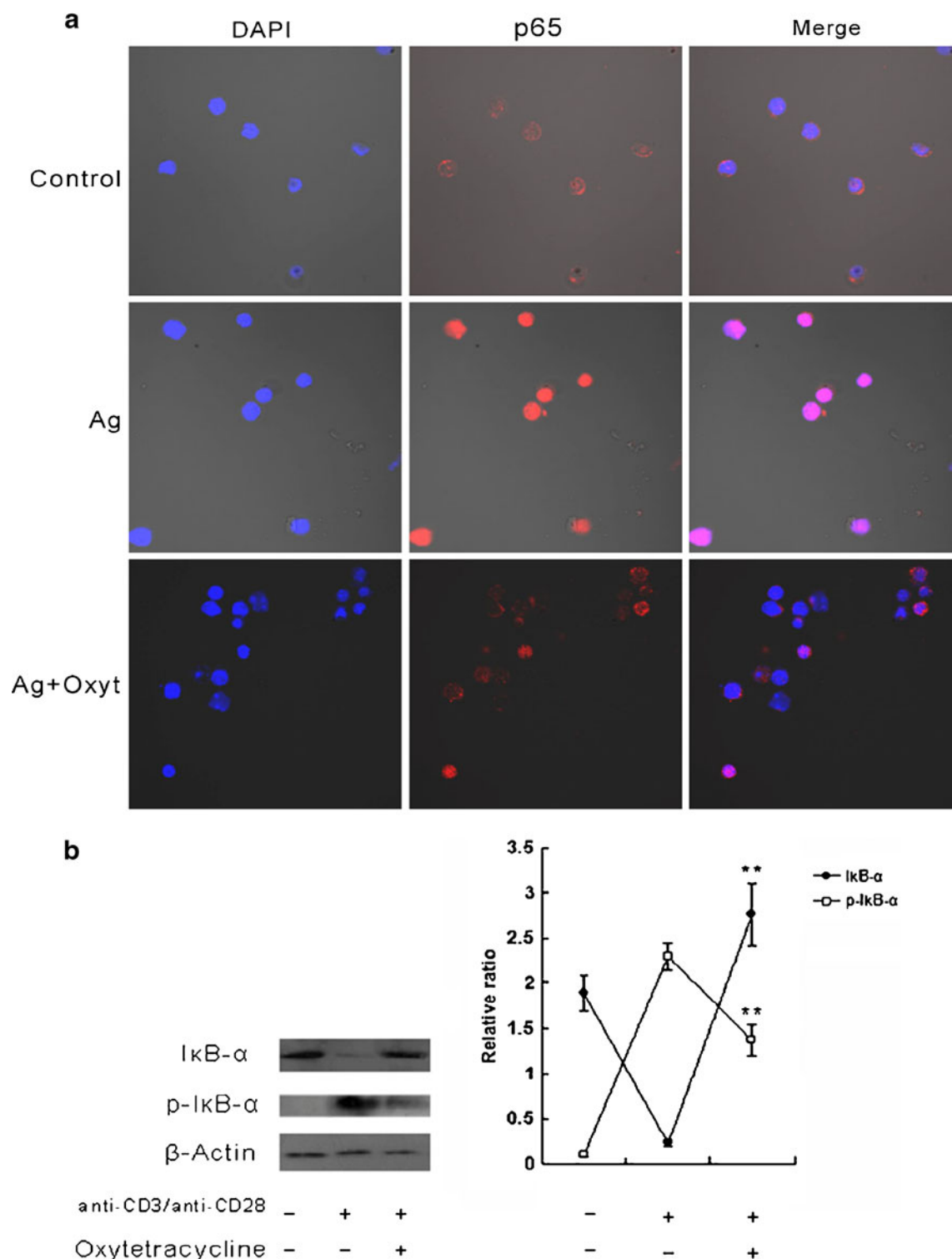


Fig. 10 Effect of oxytetracycline on the activation of NF- κ B and I κ B α phosphorylation and degradation in vitro. **a** Effect of oxytetracycline(mg/kg) treatment on nuclear translocation of NF- κ B. Immunocytochemistry techniques were employed. Th2 cells were cultured with anti-CD3 (5 μ g/ml) for 1 h (1 mg/L), fixed, permeabilized, and incubated with rabbit anti-p65 antibody followed by Cy3-conjugated anti-rabbit Ig (red). The nuclei of the corresponding cells were

visualized by DAPI staining. Magnification for images was $\times 600$. **b** Effect of oxytetracycline treatment on I κ B α phosphorylation and degradation. Th2 cells were cultured with anti-CD3 (5 μ g/ml) for 1 h (1 mg/L), total cellular proteins were analyzed by western blot with specific antibodies. β -Actin was used as an internal control. Experiments were repeated three times and similar results were obtained

eosinophil, neutrophil, and macrophage counts in BALF. Furthermore, oxytetracycline can reduce CCL5, CCL11, CCR1, CCR3, and p-Akt which is a direct downstream effector of PI3K after OVA challenge. The reduction of leukocyte infiltration found in the oxytetracycline-treated group may be due to IL-5, CCL5, CCL11, and PI3K reduction because they influence leukocytes recruitment. CCL5/RANTES and CCL11/eotaxin are both target genes of NF- κ B and upregulated by NF- κ B [32, 33]. Our result suggests that the decreased NF- κ B activation could account for this phenomenon. In addition, PI3K and Akt play an important role in the activation of NF- κ B [34]. The reduction of the p-Akt protein levels treated with oxytetracycline maybe lead to the alleviated NF- κ B activation.

High levels of serum IgE are a sign of Th2 immune responses. Our data showed that serum levels of OVA-specific IgE were substantially decreased by oxytetracycline in OVA-challenged mice. IL-4 and IL-13 cause B cells to synthesize IgE, which is involved in mast cell degranulation by cross-linking with IgE receptors [35, 36]. In addition, NF- κ B plays a crucial role in B-cell proliferation and development [37]. Therefore, the inhibitory effect of oxytetracycline on OVA-specific IgE may be contribute to the inhibitory effect on B-cell activation via inhibition of NF- κ B activation, and on IL-4- and IL-13-mediated class switching to IgE.

It is believed that inflammatory mediators released during allergic inflammation play a pivotal role in causing asthma symptoms such as airway hyperresponsiveness, airway inflammation, goblet cell hyperplasia, airway luminal narrowing, and subepithelial fibrosis [38–41]. In our study, we found that oxytetracycline significantly inhibited OVA-induced AHR in response to increasing concentrations of methacholine, the degree of inflammatory cell infiltration in the peribronchial and perivascular areas and goblet cell hyperplasia. Therefore, we conclude that the effect of oxytetracycline on airway hyperresponsiveness, airway inflammation, and goblet cell hyperplasia may be attributed to the inhibition of Th2 cytokine production, eosinophils, and serum IgE level.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. In our study, we first reported that oxytetracycline effectively reduced Th2 cytokine production, potentially via inhibition of NF- κ B activity in vivo and in vitro. The inhibition of Th2 cytokines in vivo caused the reduction of inflammatory cell recruitment to BALF, CCL5, CCL11, CCR3, CCR5, p-Akt, serum OVA-specific IgE synthesis, mucus hypersecretion, goblet cell hyperplasia, and AHR. These findings suggest a therapeutic potential of oxytetracycline in the treatment of asthma. The more we understand different pharmaceutical effects

and mechanisms of antibiotics, the better we will make full use of antibiotics in clinic.

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