

ORIGINAL ARTICLE

Anti-inflammatory effects of tilmicosin in a noninfectious mouse model of allergic asthma

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Abstract

Tilmicosin, a semi-synthetic tylosin-derived macrolide antibiotic commonly used by veterinarians, has been shown to possess anti-inflammatory activity. However, possible use in asthma treatment has not yet been studied. In this study, we investigated the anti-inflammatory properties of tilmicosin using a murine asthma model. BALB/c mice were sensitized and challenged by intraperitoneal (i.p.) or nasal administration of ovalbumin. Tilmicosin (10 and 20 mg/kg) treatment resulted in a marked reduction in the presence of several types of immune cells and cytokines in the bronchoalveolar lavage fluids of mice. Levels of ovalbumin-specific Immunoglobulin E (IgE) were significantly decreased following treatment with tilmicosin (10 and 20 mg/kg). Histological studies using H&E (haematoxylin and eosin) and AB-PAS (alcian blue-periodic acid-Schiff) staining demonstrated that tilmicosin substantially inhibited both ovalbumin-induced inflammatory cells in lung tissues and goblet cell hyperplasia in the airway. These findings provided new insight into the immunopharmacological role of tilmicosin in terms of its effects in a murine model of asthma.

Keywords: Allergic airway inflammation, asthma, ovalbumin, tilmicosin, AHR

Introduction

Macrolides, such as erythromycin, roxithromycin, clarithromycin and azithromycin, are a well-established class of antibacterial agents that reversibly bind the 50S ribosomal subunit of susceptible microorganism and inhibit protein synthesis. It has been shown that certain macrolides exhibit anti-inflammatory and immunomodulatory activity *in vitro* and *in vivo* in addition to their antimicrobial properties.^(1–4) The most convincing demonstration of the anti-inflammatory/immunomodulatory effects of macrolide antibiotics has been in the treatment of diffuse panbronchiolitis,⁽⁵⁾ cystic fibrosis^(6,7) and other chronic inflammatory disorders of the lungs, including chronic obstructive pulmonary disease^(8,9) and asthma.^(10–12)

Asthma is a complex chronic airway disorder which is characterized by airway hyperresponsiveness, airway inflammation, lung eosinophilia, mucus hypersecretion by goblets cells and elevated serum IgE. Infiltration of eosinophils, neutrophils, and macrophages into the

bronchial lumen and lung tissues induces inflammation in these tissues.^(13,14) Recruitment of these inflammatory cells from the blood to the site of inflammation is a crucial step in the development and persistence of airway inflammation.^(15,16) This process is accompanied by induction of Th2 cells and up-regulation of IL-4, IL-13 and IL-5 production. Although asthma incidence is increasing rapidly all over the world, there have been no therapeutic strategies available that have been both sufficient and safe for long-term use. Therefore, development of innovative anti-inflammatory approaches for asthma treatment is critical. Macrolides antibiotics represent one potentially useful class of medications. To our knowledge, there are published studies that evaluate the effects of macrolide treatment on allergic airway inflammation in experimental mouse models of allergic asthma, such as clarithromycin and azithromycin.^(17,18)

Tilmicosin, a semi-synthetic macrolide antibiotic derived from tylosin, was proved to be effective in treating

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a broad array of infectious diseases caused by Gram-positive and Gram-negative bacteria, such as pulmonary infections in calves, respiratory infections in sheep and mastitis in ruminant animals.^(19–21) In a recent study, we found that tilmicosin mediated its anti-inflammatory effects via modulation of COX-2 and iNOS gene expression and cytokine production in LPS-activated macrophages and monocytes.⁽²²⁾ To the extent, rather than from anti-bacterial properties, their therapeutic effects might be derived from anti-inflammatory and immunomodulatory properties.⁽⁹⁾ Based on studies investigating the effect of clarithromycin and azithromycin, we found that infectious and non-infectious mouse airway inflammation models support a distinct anti-inflammatory role for macrolides. Thus, we focused on investigating whether tilmicosin had a distinct anti-inflammatory effect in a non-infectious mouse airway inflammatory model.

Materials and methods

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 micro-isolator cages and received food and water ad libitum. The laboratory temperature was $24 \pm 1^\circ\text{C}$ and relative humidity was 40 to 80%.

Animals were housed in a 12-h light/12-h dark cycle (6 AM–6 PM) and provided with rodent diet and water ad libitum. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by US National Institutes of Health.

Sensitization and challenge with OVA and treatment

These mice were divided into seven groups ($n=10$ each). Control group (Con), mice were sensitized with ovalbumin and nasal administration of phosphate-buffered saline (PBS); ovalbumin-challenged group (OVA), mice were sensitized and nasal administration of ovalbumin (local challenge); dexamethasone group (DXM), ovalbumin-challenged mice were treated with 2 mg/kg of dexamethasone; clarithromycin group (Clar), ovalbumin-challenged mice were treated with 200 mg/kg of clarithromycin; tilmicosin group, ovalbumin-challenged mice were treated with tilmicosin (5, 10 and 20 mg/kg). Hereafter, we used these group abbreviations to clarify the text. Mice were sensitized with 20 μg ovalbumin (Grade V; Sigma-Aldrich, St. Louis, MO) adsorbed in 100 $\mu\text{g}/\text{ml}$ of Imject Alum (Pierce, Rockford, IL) by intraperitoneal injection on days 0, 7 and 14 (general sensitization) in all mice. On day 14, mice were anesthetized and 100 μg of OVA in 50 μl of PBS administered intranasally in all mice excepted negative control sensitized with PBS. Mice were again anesthetized before being challenged with 100 μg of OVA in 50 μl of PBS on each of days 25–27. Control mice were given PBS in place of OVA in challenge stages of the protocol. On days 25–27, tilmicosin (Sigma Chemical Co.) at 5, 10 and 20 mg/kg were given with an oral administration 1 h prior

to ovalbumin administration; 2 mg/kg of dexamethasone (DXM) or 200 mg/kg clarithromycin was given with an intraperitoneal injection as a positive control.

Collection of blood and bronchoalveolar lavage

Twenty-four hours after the last administration, mice were anesthetized and bled via the brachial plexus for collection of blood samples used to estimate the IgE production. Bronchoalveolar lavage (BAL) was performed twice by intratracheal instillation of 500 μl of PBS. The lavage fluid was centrifuged, and the supernatant were kept at -70°C until use for cytokine measurements, the cell pellets were resuspended in a 1 ml of PBS and then applied to a slide by cytopinning for differential cell counts by staining with a modified Giemsa method. At least 200 cells were counted per slide.

Lung histology

Histopathologic evaluation was performed on mice. Left lungs were removed by dissection and fixed in 4% paraformaldehyde. Lung tissues were sectioned, embedded in paraffin and cut at 3 μm . Tissue sections were then stained with haematoxylin-eosin for general morphology, with alcian blue-periodic acid-Schiff (AB-PAS) for the identification of goblet cells in the epithelium.

Cytokine levels in lung tissues

The levels of interleukin (IL)-4, -5 and -13 levels in BAL fluid (BALF) were determined by using an ELISA kit (BioLegend, Inc. Camino Santa Fe, Suite E; San Diego, CA). Concentrations were determined in duplicates of each sample.

Mouse anti-OVA IgE ELISA

To define serum levels of OVA-specific IgE, an ELISA analysis 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 (BSA) in PBS, pH 7.2) at room temperature for 1 h and washed. Then 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 with TMB substrate for 15 min. The enzymatic reaction was stopped with 2 M H_2SO_4 and absorbance was read at 450 nm. Units are reported as optical density at 450 nm.

Determination of airway hyperresponsiveness

Airway hyperresponsiveness to methacholine in conscious, spontaneously breathing animals was measured using a whole-body plethysmograph chamber (Buxco, Sharon, CT) as described.⁽²²⁾ Mice were exposed to aerosolized PBS (for the baseline measurement) or methacholine (6.25 to 50 mg/ml) for 3 min and readings were taken and averaged for 3 min after each nebulization. Airway resistance (RI) is

defined as the pressure driving respiration divided by flow. C_{dyn} 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.

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 (Dunnett's *t*-test) and Student's *t*-test. Statistical differences were considered significant at $P < 0.05$.

Results

Ovalbumin-specific serum IgE levels

Antigen-specific Th2 responses are known to induce antigen-specific IgE antibody production. Therefore, we examined whether tilmicosin influenced ovalbumin-specific IgE production in a murine allergic asthma model using ELISA. OVA-sensitized mice had high levels of serum anti-ovalbumin IgE antibodies compared with control mice (Figure 1). A significant reduction in ovalbumin-specific IgE was observed in mice treated with tilmicosin (10 and 20 mg/kg), clarithromycin and dexamethasone (Figure 1) ($*P < 0.05$, $**P < 0.01$ vs. ovalbumin-challenged mice).

Effect of tilmicosin on the inflammatory cell recruitment

We examined the effects of tilmicosin on inflammatory cell infiltration into BAL fluid in ovalbumin-challenged mice. Inflammatory cell numbers (i.e., eosinophils, neutrophils and macrophages) were significantly elevated in the BAL fluid of ovalbumin-challenged mice versus the negative controls (Figure 2). Levels of eosinophil, neutrophil and macrophage infiltration were affected to varying degrees by tilmicosin treatment, clarithromycin and dexamethasone.

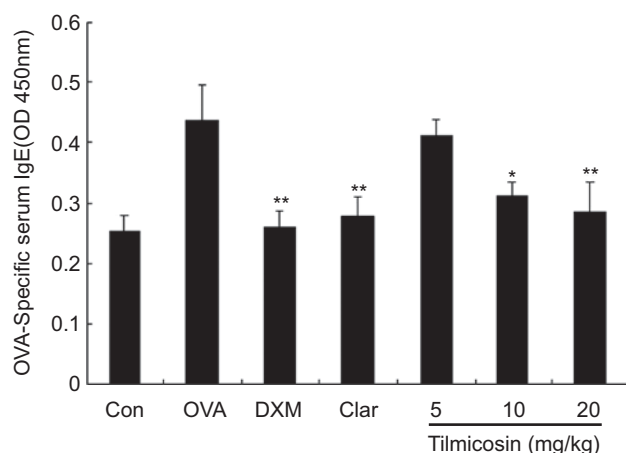


Figure 1. Effect of tilmicosin (different dose) on OVA-specific IgE in serum. OVA-specific IgE levels in the serum were measured by ELISA. Results (mean $\pm$ SEM) are expressed as optical density values and are representative of at least three independent experiments. $*P < 0.05$, $**P < 0.01$ vs. OVA-challenged mice.

Tilmicosin (10 and 20 mg/kg) treatment significantly reduced eosinophil (from $77.4 \pm 6 \times 10^4$ cells to $42.8 \pm 4.4 \times 10^4$ and $25.4 \pm 4 \times 10^4$ cells), neutrophil (from $46 \pm 5.5 \times 10^4$ cells to $27.5 \pm 3.4 \times 10^4$ and $13.68 \pm 2.03 \times 10^4$ cells) and macrophage (from $9.9 \pm 1 \times 10^4$ cells to $6.8 \pm 0.86 \times 10^4$ and $5.7 \pm 1.1 \times 10^4$ cells) infiltration in a dose-dependent manner versus untreated ovalbumin-challenged mice ($*P < 0.05$, $**P < 0.01$ vs. ovalbumin-challenged mice).

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

To determine the effect of tilmicosin on cytokine release in OVA-challenged mice, levels of BALF cytokines

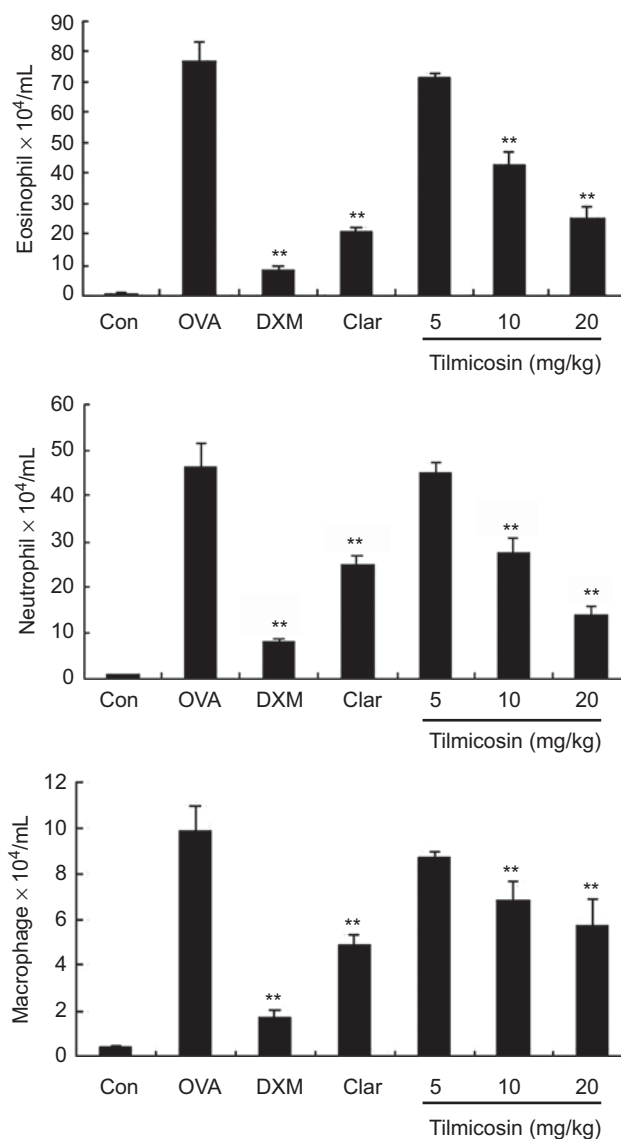


Figure 2. Effect of tilmicosin (different dose) on the recruitment of inflammatory cell in BAL fluid. The lavage fluid was centrifuged, and the cell pellets were resuspended in 1 ml of PBS and applied to a slide by cytospinning to obtain differential cell counts by staining with a modified Giemsa method. At least 200 cells were counted per slide. The values represent the mean $\pm$ SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$ vs. OVA-challenged mice.

were determined by ELISA in each experimental group 24 h after the final challenge. As shown in Figure 3, OVA-challenged mice induced significant elevation of cytokines such as IL-4 (129.6664 ± 9.9 pg/ml), IL-5 (154.45 ± 18.6 pg/ml) and IL-13 (78.9 ± 10.1 pg/ml) in BALF. Treatment with tilmicosin (10 and 20 mg/kg) significantly inhibited production of IL-4, -5 and -13 in a dose-dependent manner (* $P < 0.05$, ** $P < 0.01$ vs. ovalbumin-challenged mice).

Effects of tilmicosin on inflammatory cell infiltration into lung tissue

To assess the anti-inflammatory and/or anti-remodeling effect of tilmicosin, histopathological studies were performed. Inflammatory cell infiltration in peribronchial and perivascular areas was observed in OVA-challenged mice (Figure 4). Treatment with tilmicosin (20 mg/kg) markedly reduced the degree of inflammatory cell infiltration in the peribronchial and perivascular areas.

Effect of tilmicosin on airway goblet cell hyperplasia and mucus production

To evaluate goblet cell hyperplasia, we stained lung sections with AB-PAS stain. Mucus overproduction, goblet cell hyperplasia and epithelial cell disruption were observed in the bronchial airways of OVA-treated mice, but not in the PBS-treated mice (Figure 5). Tilmicosin (20 mg/kg) treatment significantly reduced mucus secretion, goblet cell hyperplasia and epithelial cell disruption.

Effect of oxytetracycline on ovalbumin-induced airway hyperresponsiveness

To investigate the effect of tilmicosin on airways hyperresponsiveness (AHR) in response to increasing concentrations of methacholine, we measured both RI and C_{dyn} in mechanically ventilated mice. OVA-challenged mice developed AHR, typically reflected by high RI and low C_{dyn} (Figure 6). Tilmicosin treatment (10 and 20 mg/kg) dramatically reduced RI and restored C_{dyn} in OVA-challenged mice in response to methacholine (* $P < 0.05$, ** $P < 0.01$ vs. ovalbumin-challenged mice).

Discussion

Allergic asthma is one of the most common diseases encountered in clinical trials and its mortality has increased worldwide over the past 20 years.⁽²³⁾ Thus, it is crucial to figure out its pathogenesis. In the present study, we demonstrated that tilmicosin possesses potent anti-inflammatory effects in an allergic airway inflammation model via the inhibition of AHR and airway inflammation. Tilmicosin (10 and 20 mg/kg) treatment resulted in a marked reduction of inflammation both in BAL fluid and in the lung, as demonstrated by numbers of infiltrating eosinophils, neutrophils

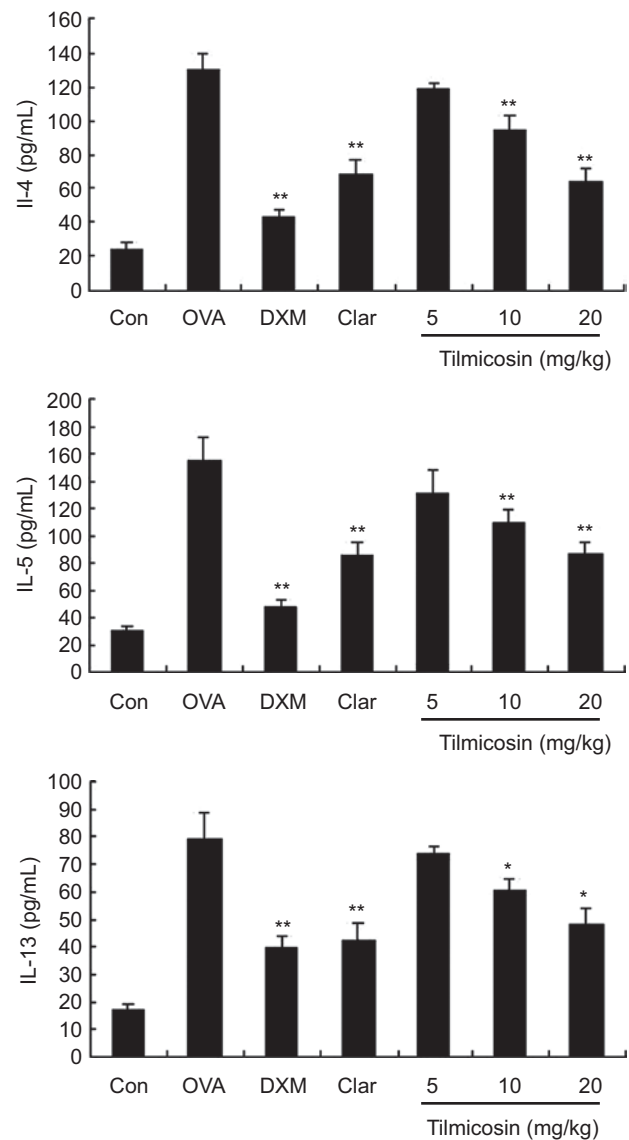


Figure 3. Effect of tilmicosin (different dose) on the secretion of IL-4, IL-5 and IL-13. The lavage fluid was centrifuged, and the supernatants were measured by ELISA. The values represent the mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. OVA-challenged mice.

and macrophages, mucus production and levels of Th2-associated cytokines in BAL and these inhibitory effects are similar to azithromycin and clarithromycin. Previous reports have indicated that azithromycin and clarithromycin have effects on patients with asthma, which are related to their anti-inflammatory properties using the non-infectious mouse airway inflammatory models.^(17,18) This study is initial to demonstrate that tilmicosin, already known as antibiotic, which was widely used in veterinary clinical trials, owns a novel beneficial effect in a non-infectious mouse model of allergic airway inflammation. Further study should be focused on monitoring the effects of tilmicosin in other experimental animal models to justify the antiasthmatic effects of tilmicosin as an important step to put it to clinical use.

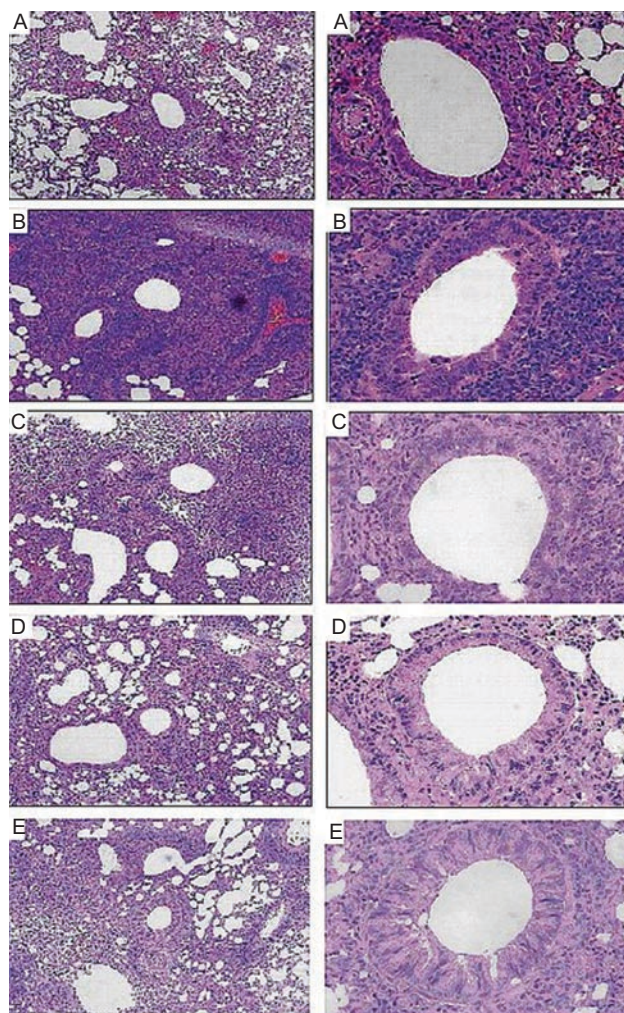


Figure 4. Effect of tilmicosin on lung tissue inflammatory cell infiltration and airway remodeling. Representative haematoxylin-eosin stained sections of lung from: (A) PBS-challenged mice; (B) OVA-challenged mice; (C) OVA-challenged mice treated with tilmicosin (10 mg/kg); (D) OVA-challenged mice treated with tilmicosin (20 mg/kg); (E) OVA-challenged mice treated with dexamethasone (2 mg/kg). The left panel is magnified $\times 100$ and the right panel is magnified $\times 400$.

Our experiments were conducted on specific pathogen-free (SPF) female BALB/c mice referring to previous studies.^(18,24,25) Using SPF mice, we can exclusively attribute the beneficial effects of florfenicol to its anti-inflammatory/immunomodulatory properties (thereby excluding the antimicrobial effects). In this study, tilmicosin was efficacious in reducing various ovalbumin-induced asthmatic responses. It has been shown that IL-4 plays an important role in B cell growth, differentiation and secretion of IgE and IL-13.⁽²⁶⁾ Our data indicate that concentrations of IL-4 and -13 were reduced in lung tissues following treatment with tilmicosin. Also, ovalbumin-specific IgE levels, which increased in mice challenged with ovalbumin, decreased following tilmicosin treatment. Consistent with previously reported data, IL-4 may be responsible for the production of IgE and IL-13. IL-13 plays a pivotal role in causing asthma

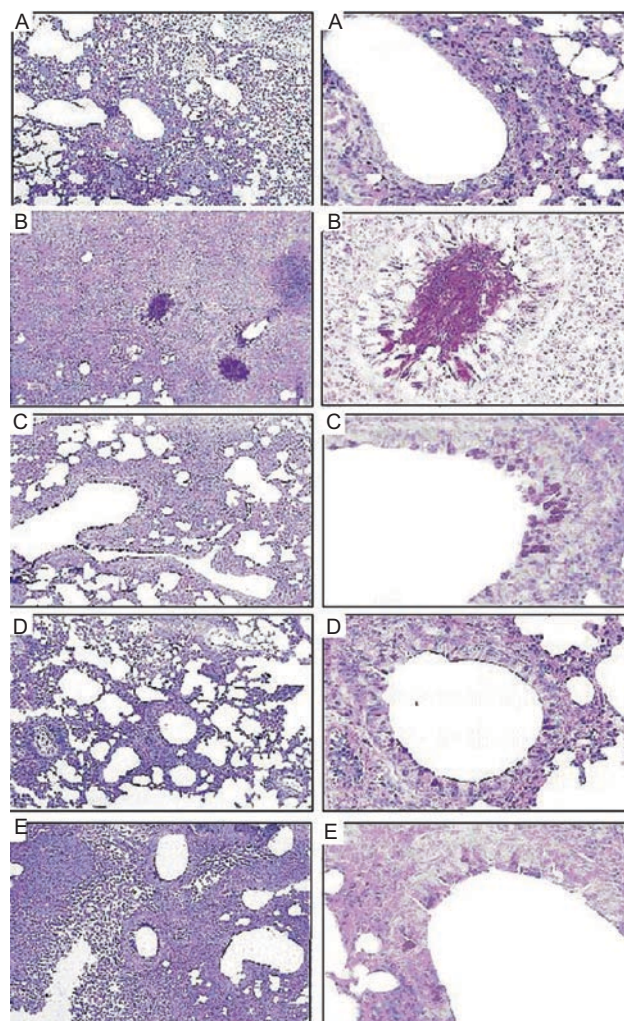


Figure 5. Effect of tilmicosin on airway goblet cell hyperplasia and mucus production. Representative alcian blue-periodic acid-Schiff stained sections of lung from: (A) PBS-challenged mice; (B) OVA-challenged mice; (C) OVA-challenged mice treated with tilmicosin (10 mg/kg); (D) OVA-challenged mice treated with tilmicosin (20 mg/kg); (E) OVA-challenged mice treated with dexamethasone (2 mg/kg). The left panel is magnified $\times 100$ and the right panel is magnified $\times 400$.

symptoms such as airway hyperresponsiveness, airway inflammation, goblet cell hyperplasia and airway luminal narrowing.⁽²⁷⁾ Taken together, these data indicate that the effect of tilmicosin on airway inflammation and goblet cell hyperplasia may be attributed to an inhibition of IL-13.

Eosinophils are the predominant inflammatory cells in asthmatic lung tissues. Eosinophil recruitment into the airways is considered to be one of the most crucial characteristics of asthmatic responses. Additionally, the presence of eosinophils in asthmatic BAL fluid is also associated with the production of IL-5, which plays a significant role in the process of differentiation, infiltration and activation of pulmonary eosinophils.⁽²⁸⁾ Because IL-5 promotes the differentiation and activation of eosinophils, its reduction would logically also decrease eosinophil infiltration, a phenomenon we observed in tilmicosin-treated mice. Apart from this,

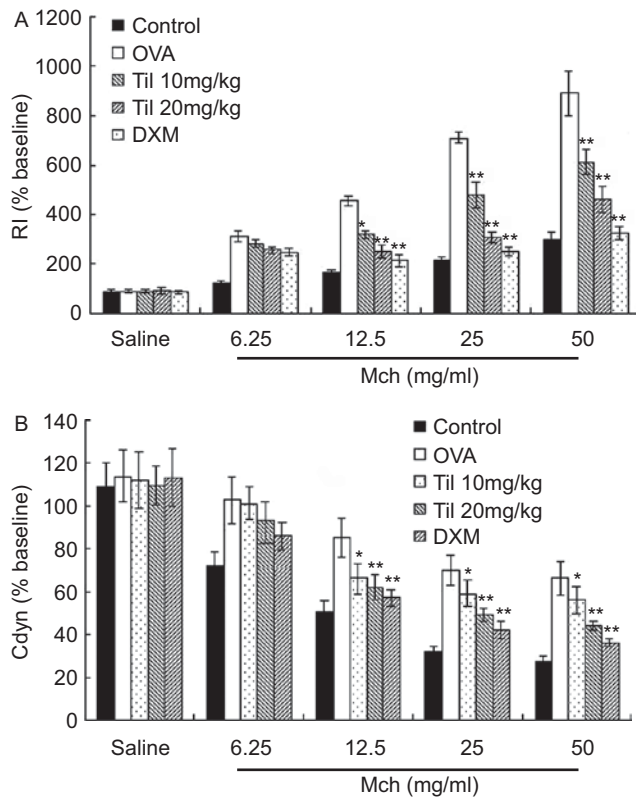


Figure 6. Effect of tilmicosin on ovalbumin-induced airway hyperresponsiveness in mice. Airway hyperresponsiveness was assessed by percentage change from the baseline level of (A) lung resistance (RI, $n=6$ mice per treatment group) and (B) dynamic compliance (C_{dyn} , $n=6$ mice per treatment group). Til, tilmicosin; Mch, methacholine. * $P<0.05$, ** $P<0.01$. vs. OVA.

the neutrophils and macrophages that produce these cytokines are also major players in inflammatory responses. Tilmicosin treatment reduced the numbers of these cells in ovalbumin-challenged mice. Therefore, we hypothesize that tilmicosin is capable of reducing cytokine-mediated recruitment of asthmatic inflammatory cells to the lungs. The cellular mechanisms by which tilmicosin mediates these effects remain to be determined.

In conclusion, tilmicosin effectively inhibited important pathological characteristics of asthma and airway inflammation, decreasing the concentrations of a number of inflammatory mediators, including IL-4, -5 and -13, in BAL fluid. These results indicate that the efficacy of tilmicosin, along with other macrolide antibiotics, in pathogenetic process of asthma could be attributed to their anti-inflammatory or immunomodulatory properties and not only to their anti-microbial activity. Utility of macrolides in asthma may be put in context of the apparently beneficial effects that were observed in complex lung diseases with features of chronic inflammation and infection such as cystic and diffuse panbronchitis. Separating the anti-microbial and anti-inflammatory properties of the macrolides would assist in determining the usefulness of macrolides in treating subjects with asthma.

Declaration of interest

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