



Long-term azithromycin ameliorates not only airway inflammation but also remodeling in a murine model of chronic asthma



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ABSTRACT

Objectives: We investigated the effect of long-term treatment with azithromycin on the pathogenesis of chronic asthma with airway remodeling.

Methods: Six-week-old-BALB/c mice were sensitized with ovalbumin (OVA) combined with lipopolysaccharide (LPS) for 1 month, then challenged with OVA for 3 months. Azithromycin at 75 mg/kg was administered via oral gavage five times a week during the challenge period. Inflammatory cells, T helper 2 cytokines in bronchoalveolar lavage fluid (BAL) fluid, and airway hyperresponsiveness (AHR) were measured. Parameters related to airway remodeling were evaluated. The levels of neutrophil elastase, Interleukin (IL)-8, and BRP-39 (human homologue YKL-40) were assessed. The expression of MAPK and NF- κ B signaling were investigated.

Results: Long-term treatment with azithromycin improved AHR and airway inflammation compared with the OVA and the OVA/LPS groups. The concentrations of IL-5 and IL-13 in the OVA/LPS group decreased significantly after azithromycin administration. The levels of neutrophil elastase and IL-8, as surrogate markers of neutrophil activation, were reduced in the azithromycin group compared with the OVA/LPS group. Goblet cell hyperplasia and the smooth muscle thickening of airway remodeling were attenuated after azithromycin treatment. The expression of MAPK/NF-kappaB signal and the level of BRP-39 in the lung decreased remarkably in the OVA/LPS with azithromycin-treated group.

Conclusions: This study suggests that in a murine model of chronic asthma, long-term azithromycin treatment ameliorates not only airway inflammation but also airway remodeling by influencing on neutrophil-related mediators, BRP-39 and MAPK/NF- κ B signal pathways. Macrolide therapy might be an effective adjuvant therapy in a chronic, severe asthma with remodeling airway.

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1. Introduction

Asthma is a chronic airway inflammatory disease that typically has findings of episodic cough, dyspnea, and airway hyperresponsiveness. However, some phenotypes of asthma exist that have only partially reversible or irreversible airway obstruction. They are not well controlled by standard treatment with inhaled

corticosteroids and sometimes have a severe clinical course. Frequently, these conditions can be associated with airway remodeling, including epithelial injury, goblet cell metaplasia, subepithelial fibrosis, smooth muscle hypertrophy/hyperplasia, increased angiogenesis, and alterations to extracellular matrix components [1]. This pathologic change of asthmatic airway is induced by the repeated injury and repair process [2]. It has been thought that airway remodeling occurs in case of chronic asthma in association with persistent inflammation. However, it has been reported that these changes can occur during the first 4–5 years of childhood asthma [3] and that they can be caused by bronchoconstriction without inflammation [4]. Moreover, inhaled corticosteroid treatment to target airway inflammation in asthma cannot prevent airway remodeling [5]. These data suggest that airway remodeling could be a distinct process that occurs parallel to

Abbreviations: OVA, ovalbumin; LPS, lipopolysaccharide; BAL, bronchoalveolar lavage; AHR, airway hyperresponsiveness; IL, Interleukin; MAPK, mitogen-activated protein kinases; NF, nuclear factor; BRP, breast regression protein.

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inflammation in the airway, and that an appropriate therapeutic intervention is required for the better control of asthma. Some drugs including anti-Th2 cytokines therapies, tiotropium, omalizumab, and imatinib, ameliorate remodeling in asthma [2], there have been few reports about the effect of macrolides on it.

In the past, the beneficial effects of macrolide antibiotics in asthma have been thought to be result of their inhibition of the metabolism of exogenous steroid or their antimicrobial activity against commonly atypical pathogens that cause the exacerbation of asthma [6]. However, it is known that macrolides have not only antibacterial properties, but also immunomodulatory and further antiviral properties [7]. Recent animal and human studies [6,8–16] demonstrated that macrolide treatment improved airway inflammation, hyperresponsiveness, further clinical symptoms, or quality of life in asthma. Most of the animal studies [6,14–16] were conducted in an acute asthma model with a short treatment duration of less than 1 month or an in vitro design. Among macrolides, azithromycin has 15-membered ring by the addition of a nitrogen atom into the lactone ring and resultantly has characteristics of good bioavailability, acid stability, and a long half-life [17]. Azithromycin showed effects on chronic obstructive pulmonary disease and asthma [8,9,18–20] in human studies. In particular, Brusselle et al. [9] confirmed the beneficial effect of the drug in patients with severe neutrophilic asthma and in acute exacerbation, suggesting that some specific groups exist whose asthma can be effectively controlled by macrolides. However, previous animal studies focused on the role of macrolides on airway inflammation in asthma, the effects of the drugs, especially azithromycin, on airway remodeling has not been clearly explored.

Regarding the signaling pathways involved in lung disease, Manechotesuwan et al. [21] showed that p38 mitogen-activated protein kinase (MAPK) mediates the phosphorylation of GATA-3, which plays a critical role in allergy by regulating production of cytokines from Th2 lymphocytes. In addition, Morinaga et al. [22] showed that extracellular signal-regulated kinase (ERK) and nuclear factor-kappa B (NF- κ B) pathway were involved in *Chlamydo-philha pneumoniae*-induced MUC5AC production in bronchial epithelial cells and that it is reduced by macrolides, especially clarithromycin and telithromycin. Some previous studies [16,23,24] provided evidence that NF- κ B, a ubiquitous transcription factor complex, could play a central role in asthma through increasing expression of many inflammatory genes, thereby perpetuating eosinophil inflammation. Kano et al. [25] proposed that major three pathways were involved in macrolide immunomodulation as follows: i> receptor tyrosine kinase – MAPK cascade – activator protein-1, ii> toll-like receptors – transforming growth factor-activated protein kinase 1 – NF- κ B, iii> G-protein-coupled receptor-phospholipase C- protein kinase C and calcium/calmodulin signaling. They described that macrolide could inhibit MAPK activation, NF- κ B translocation, and intracellular calcium increase and modulate immune response.

Mammals including human do not synthesize chitin, but do synthesize chitinases [26]. Chitinase-like proteins contain evolutionarily conserved 18-glycosyl-hydrolases similar with chitinases, except the lack of enzymatic activity [27]. In human diseases, it has been known that the proteins has a role on the inflammation and tissue remodeling. In particular, Chupp et al. [28] showed that the expression of human chitinase-like protein YKL-40 increased in the serum and lung in patients with asthma.

In the present study, we used a murine model of chronic asthma to investigate whether azithromycin has an effect not only on airway inflammation but also remodeling. To explore further the underlying mechanisms, we measured the activities of signal pathways such as MAPK and NF- κ B, and the expression of mouse breast regression protein 39 (BRP-39, human homologue YKL-40)

in lung tissue.

2. Materials and methods

2.1. Sensitization and antigen challenge protocol

Female BALB/c mice (Dae-Han Experimental Animal Center, Daejeon, Korea), 6 weeks of age, were used in the experiments. They were sensitized and challenged with ovalbumin (OVA, grade V; Sigma–Aldrich, St. Louis, MO, USA). Lipopolysaccharide (0.1 μ g) (LPS; Sigma–Aldrich) was added during OVA sensitization to induce LPS-enhanced inflammation, as described in the previous studies [29,30]. Mice were divided into five groups: control, azithromycin (Pfizer Pharmaceuticals, Dublin, Ireland), OVA, OVA/LPS, and OVA/LPS with azithromycin treatment ($n = 5$ for the first four groups, and $n = 8$ for the OVA/LPS with azithromycin-treated group). To verify the result more clearly, we used 8 mice in the last treated group. The mice were immunized subcutaneously with mixture of 25 μ g OVA and 1 mg of aluminum hydroxide (Sigma–Aldrich, Milwaukee, WI, USA) in 200 μ l of normal saline once a week for 1 month. 20 ng OVA in 50 μ l of phosphate-buffered saline [PBS] were challenged via intranasal inhalation from day 31 and repeatedly twice a week for 3 months, based on the protocol for inducing a model of chronic asthma described previously [31,32]. LPS (0.1 μ g) was administered intraperitoneally during the sensitization period. Mice were sacrificed 24 h after the final OVA challenge. Airway hyperresponsiveness, bronchoalveolar lavage (BAL) fluid, and lung tissues were analyzed. <http://www.jimmunol.org/cgi/content/full/173/12/7556> – F1#F1All animal experimental protocols were approved by the Animal Subjects Committees of the Catholic University of Korea.

2.2. Administration of azithromycin

Referring to the experiment by Beigelman et al. [15] who used 50 mg/kg of azithromycin, we conducted the preliminary study of a murine model of acute model with three different doses (25, 50, 75 mg/kg). We observed that the best effective dose was 75 mg/kg of azithromycin. Therefore, we used the dose given via oral gavage in a chronic asthma model. From day 38, and 0.5 h before the challenges, 75 mg/kg azithromycin was administered for 5 days a week for 3 months. Control groups and azithromycin groups were treated in the same way with control group receiving PBS. The azithromycin was generously donated by Pfizer.

2.3. Measurement of airway hyper-responsiveness (AHR)

AHR was assessed 24 h after the final OVA inhalation using the flexiVent system (SCIREQ, Montreal, QC, Canada). After the administration of aerosolized methacholine (Sigma) to the mice, we measured the changes in the airway resistance (Rrs; cmH₂O/ml/s) as described elsewhere [33]. In brief, mice were anesthetized, tracheostomized, and then mechanically ventilated (150 breaths/min, tidal volume of 10 ml/kg, and positive end-expiratory pressure of 3 cmH₂O). Mice were challenged with saline aerosol at baseline and then sequentially increasing concentrations of methacholine (6.25, 12.5, 25, and 50 mg/ml). Methacholine aerosol was administered via nebulizer for about 4 min at each concentration, after which Rrs was continuously monitored and recorded. Data were calculated as peak Rrs value for each methacholine concentration.

2.4. Bronchoalveolar lavage (BAL)

Mice underwent anesthesia via intraperitoneal injection of ketamine and xylocaine and were subjected to thoracotomy, after

measurement of airway responsiveness. The trachea was cannulated with a 23-gauge needle on an 800 μ l syringe. For obtaining BAL fluid, the left main bronchus was tied and the right lung was instilled by 1.2 ml of sterile PBS through the trachea. Recovered BAL fluid was centrifuged (10 min at $353 \times g$ at 4°C) and the supernatants were kept at -70°C . The total cells counts in the BAL fluid were measured using a hemocytometer. After the cell number was adjusted to $1 \times 10^6/\text{ml}$, 50 μ l aliquots of BAL fluid were cytospun for 5 min at $43 \times g$ onto microscope slides and stained with Diff-Quik Kit (Sysmax, Kobe, Japan). The percentages of differential cells such as macrophages, eosinophils, lymphocytes, and neutrophils in the BAL fluid were analyzed by numbering 500 leukocytes in randomly distributed fields of the slide under a light microscope.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of T helper 2 (Th2) cytokines, interleukin (IL)-4, IL-5, and IL-13, in the supernatants of BAL fluids were measured by ELISA (R&D Systems, Minneapolis, MN, and BioSource International, Camarillo, CA, USA). The concentrations of IL-8 and neutrophil elastase (NE) were also measured by ELISA in the BAL fluids (Cusabio BIOTECH Ltd., Newark, NJ USA), as were the levels of IL-17 and BRP-39 in lung tissue (R&D Systems). Anti-Ovalbumin IgE and IgG1 (mouse) EIA kit (Item No. 500840 and 500830) were purchased from Cayman Chemical Company (Ann Arbor, MI). The samples were assayed according to the manufacturers' recommendations.

2.6. Lung histology

We removed the rib cage and inserted a cannula into the trachea of the mice. The lungs were inflated via the cannula by gentle infusion of the fixative 4% paraformaldehyde, and dissected out by tugging on the trachea. The fixed lungs were immersed into a 50 ml conical tube with enough 4% paraformaldehyde and stayed overnight. After sectioning lung tissues, they were embedded in paraffin and cut to 5–6 μ m thickness. The sectioned left lung were stained with hematoxylin and eosin (H&E) and with periodic acid-Schiff (PAS) for evaluating tissue inflammation and goblet cell hyperplasia/submucosal gland hypertrophy in the epithelium, respectively. Especially, goblet cell hyperplasia was quantified with a method described by Padrid et al. [34], a modified five-point scoring system (grades 0–4). Briefly, the percentage of goblet cells in the epithelium were scored as: grade 0, no goblet cells; grade 1, <25%; grade 2, 25–50%; grade 3, 51–75%; grade 4, >75%. The mean value for goblet cell hyperplasia was calculated for each mouse.

2.7. Assay for hydroxyproline content

Total collagen content in the lung was measured by estimations of hydroxyproline content. An 1.5 ml saline solution was added to 30 mg of the lung tissue collected from the peripheral part of the left lower lobe. The sample was homogenized on ice and the hydroxyproline in the lung homogenate was measured by spectrophotometry.

2.8. Measurement of smooth muscle area

To detect α -smooth muscle actin immunohistochemically, sectioned lungs of 4- μ m thickness embedded in paraffin blocks were deparaffinized with xylene and hydrated in ethanol. The lung samples were incubated overnight at 4°C with either a primary monoclonal antibody against the actin (Immunohistology kit, IMM-2; Sigma–Aldrich), or with a mouse serum instead of the primary antibody as a negative control. The lung sections were

sequentially incubated with a biotinylated secondary antibody, and peroxidase reagent and AEC chromogen (3-amino-9-ethylcarbazole). After the process, immunoreactivity was detected in the lung tissues. To measure the smooth muscle area in the lung, the immunostained area relevant to α -smooth muscle actin in each paraffin-embedded tissue was outlined and quantified using a light microscope with an image analysis system (BX50; Olympus, Tokyo, Japan). The values are presented as the area of immunostaining per micron length of the basement membrane in the bronchioles, which sizes are 150–200 μ m in the internal diameter. In each slide, at least 10 bronchioles were calculated.

2.9. Western blot for MAPK and NK- κ B

After homogenization in PRO-PREP™ protein extraction solution (Intron Biotechnology, Seongnam, Gyeonggi-Do, Korea), total protein was extracted from lung. The lysates were centrifuged for 15 min at 14,000 g at 4°C and the supernatants were isolated. Protein concentrations were quantified by the Bradford assay (Bio-Rad, Munich, Germany) and bovine serum albumin was used as the standard. Equal amounts of protein extracts (50 μ g/ml) were loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane attached to a transfer system (X cell II Module). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% Tween 20 (TBST buffer) for 1 h at room temperature, then exposed overnight to primary antibodies against NK- κ B p65, ERK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), I κ B α (Abcam, Cambridge, MA, USA), JNK, p38 (Cell Signaling technology, Beverly, MA, USA), and monoclonal phospho-specific antibodies for JNK, p38 (Cell Signaling technology) and ERK1 (Santa Cruz Biotechnology) at 4°C in 5% milk in TBST buffer. They were then treated for 1 h at 25° with secondary antibody (horseradish peroxidase [HRP]-linked goat anti-mouse IgG, sc-2005, Santa Cruz Biotechnology, Goat HRP-linked goat anti-rabbit IgG, sc-2004, Santa Cruz Biotechnology). β -actin was used as the loading control. The proteins were analyzed using the Fujifilm LAS-3000 system (Fujifilm Life Science, Stanford, CT, USA).

2.10. Data analysis

All results are expressed as mean $\pm$ standard error of the mean (SEM). A difference between two groups was assessed with a t -test in case of parametric data or Mann–Whitney U test as appropriate. More than two groups were compared with the nonparametric Kruskal–Wallis test and a post-hoc Dunn's multiple comparison test. A P -value < 0.05 was considered significant. All analyses were used by the Statistical Package for the Social Sciences (SPSS) version 12.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Effect of azithromycin on AHR in model of chronic asthma

The mice in the OVA group and the OVA/LPS group showed higher airway resistance than the control group during the methacholine inhalation. After azithromycin administration, the airway resistance showed a trend to a dose-dependent decrease (Fig. 1).

3.2. Effect of azithromycin on airway inflammation in model of chronic asthma

To investigate airway inflammation, we analyzed cell counts in the BAL fluids and lung histology. In the OVA and OVA/LPS groups, the numbers of total cells and eosinophils increased, and the latter group showed a higher percentage of neutrophils in the BAL fluid.

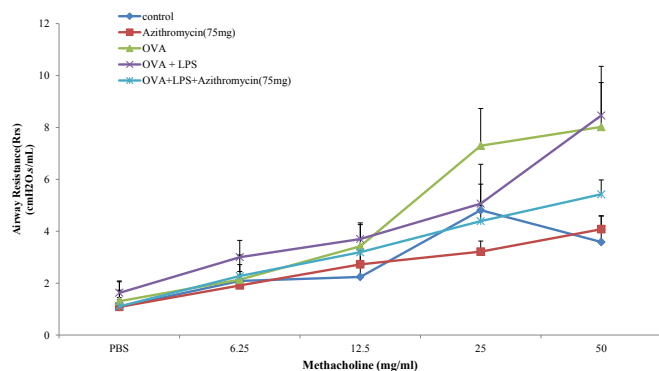


Fig. 1. Effect of azithromycin on airway hyperresponsiveness in a model of chronic asthma. AZI, azithromycin; LPS, lipopolysaccharide; * $p < 0.05$ compared with the OVA/LPS group.

The OVA/LPS azithromycin-treated group had significantly lower total cells, eosinophils, and neutrophils compared with the OVA/LPS group (Fig. 2A). The hematoxylin and eosin stain of lung tissue showed that the OVA and OVA/LPS groups had comparable increases in inflammatory cells and that azithromycin administration attenuated the infiltration of peribronchial inflammatory cells (Fig. 2B).

3.3. Effects of azithromycin on T helper (Th)2 cytokines and other parameters in model of chronic asthma

With respect to Th2 cytokines, the OVA/LPS group showed a similar pattern to the OVA group with increased levels of IL-4, IL-5, and IL-13 in the BAL fluid (Fig. 3A). The concentration of the above cytokines decreased significantly after the administration of azithromycin. We also investigated the levels of IL-8 and neutrophil elastase (NE), which are parameters to influence on the activity of neutrophils and consequently can be affected by azithromycin. The level of NE decreased markedly and that of IL-8 showed a trend to decrease in the OVA/LPS azithromycin-treated group compared with the OVA/LPS group (Fig. 3B). The level of OVA-specific IgE in

the serum significantly decreased after azithromycin administration in the OVA/LPS group (Fig. 3C).

3.4. Effects of azithromycin on parameters of airway remodeling in model of chronic asthma

We analyzed goblet cell hyperplasia, peribronchial fibrosis, and smooth muscle hypertrophy, important structural changes of airway remodeling in asthma, in lung tissues. Regarding goblet cell hyperplasia, azithromycin administration decreased the area and the score of the PAS stain, compared with the OVA/LPS group (Fig. 4A). To verify peribronchial fibrosis, we quantified it by assaying hydroxyproline in lung tissues. The hydroxyproline content tended to be lower in the OVA/LPS azithromycin-treated group than in the OVA/LPS group (Fig. 4B). For smooth muscle hyperplasia, the area immunostained for peribronchial α -smooth muscle actin in mice of OVA/LPS group was significantly greater than that in control mice. The administration of azithromycin to OVA/LPS mice significantly reduced the area of α -smooth muscle actin immunostaining compared with the corresponding area in the OVA/LPS group (Fig. 4C).

3.5. Effect of azithromycin on signal pathways and BRP-39 in model of chronic asthma

We investigated the effect of azithromycin on the MAPK pathway (JNK, ERK1, and p38). Both OVA and OVA/LPS groups showed greater phosphorylation of the three MAPKs than the control groups. After azithromycin administration, the phosphorylation of the three MAPKs was dramatically decreased (Fig. 5). Regarding NK- κ B activation, the OVA/LPS group showed higher expression of NK- κ B and treatment with azithromycin decreased expression of NK- κ B. As OVA-induced inhibition of I κ B α was attenuated by azithromycin, this alteration in NK- κ B was related to I κ B α activity (Fig. 6). The concentration of BRP-39 in the lung tissue increased in the OVA and the OVA/LPS group compared with controls. The level decreased remarkably after treatment with azithromycin (Fig. 7). The pattern in the BAL fluid was similar (data not shown).

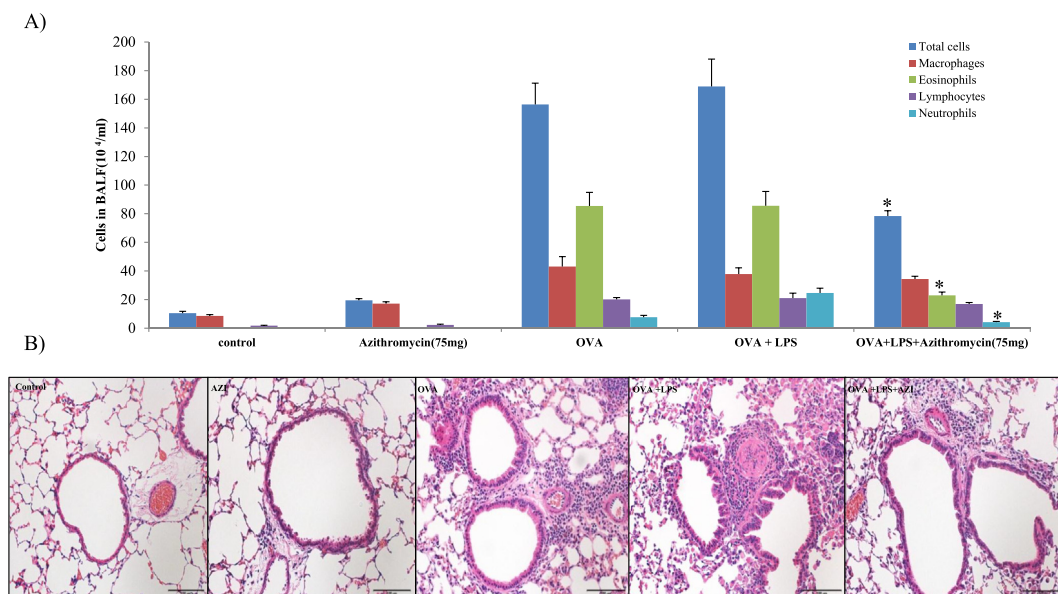


Fig. 2. Effects of azithromycin on pulmonary inflammation in a model of chronic asthma. (A) Inflammatory cell counts in BAL fluids, (B) hematoxylin and eosin-stained section of lungs. Values shown are mean $\pm$ SEM ($n = 5-8$ per group). AZI, azithromycin; LPS, lipopolysaccharide; * $p < 0.05$ compared with the OVA/LPS group.

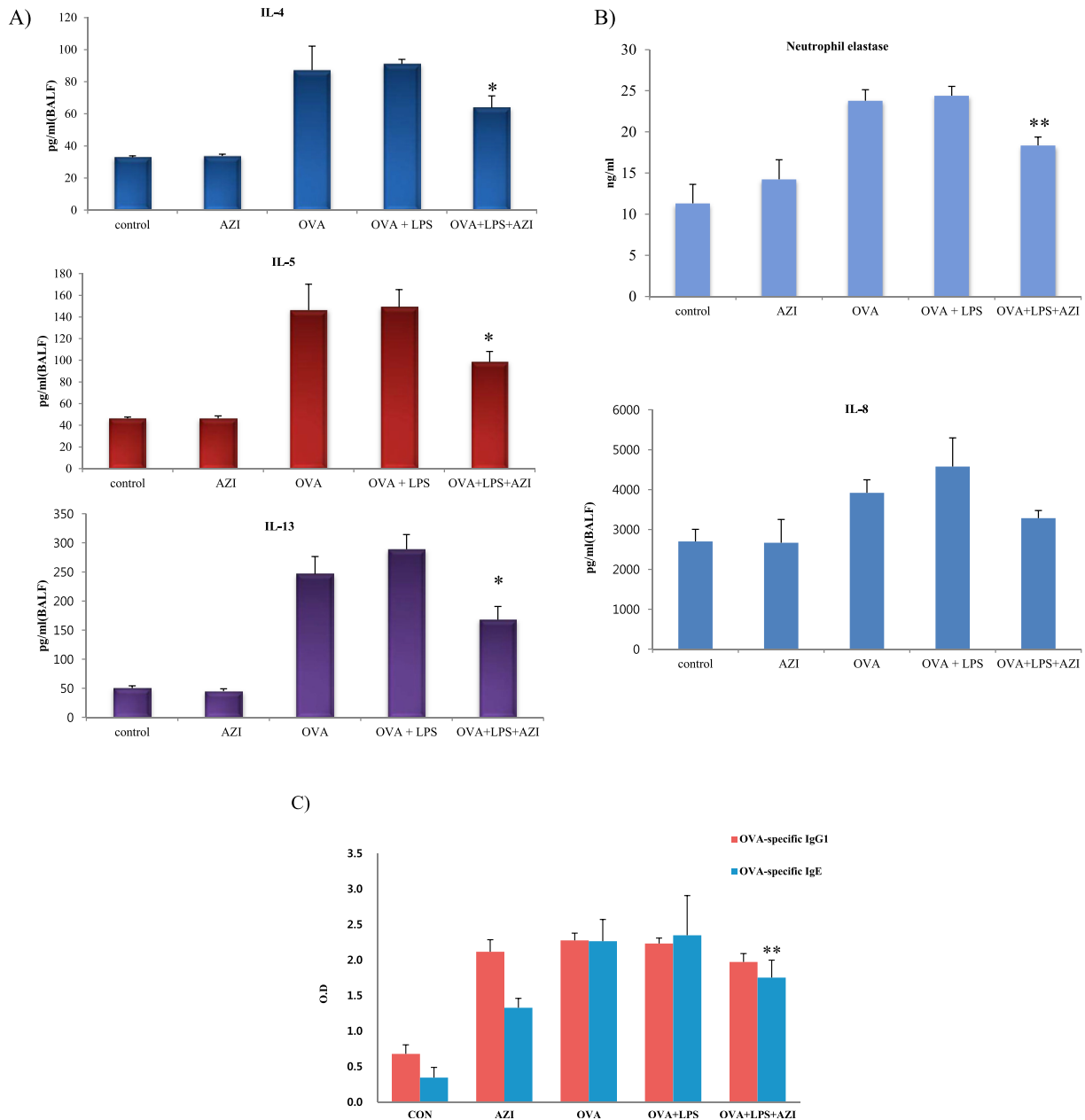


Fig. 3. Effects of azithromycin on the levels of (A) Th2 cytokines (IL-4, IL-5, IL-13) and (B) neutrophil-related parameters (neutrophil elastase, IL-8) in BAL fluid, and (C) OVA-specific IgE in serum in a model of chronic asthma. Values shown are mean $\pm$ SEM ($n = 5-8$ per group). AZI, azithromycin; LPS, lipopolysaccharide; * $p < 0.05$ compared with the OVA/LPS group; ** $p < 0.01$ compared with the OVA/LPS group.

4. Discussion

This study demonstrates that in a murine model of chronic asthma, azithromycin treatment for 3 months has protective effects against both airway inflammation and airway remodeling parameters including smooth muscle hypertrophy. Moreover, these influences may be mediated by effects on neutrophil-related mediators, BRP-39, and the signaling activities of MAPK and NF- κ B pathways.

Whereas the previous animal studies [6,15,16] showed macrolides effect in an acute asthma model, our study demonstrated that the long-term administration for 3 months with azithromycin had also effects on AHR and inflammation with a significant reduction in Th2 cytokines IL-4 and IL-13 in a chronic asthma model. Besides,

neutrophils accumulation and IL-8 production were inhibited by the azithromycin treatment, similar with other chronic inflammatory airway diseases [35].

Furthermore, we observed that long-term macrolide use significantly improved smooth muscle hypertrophy, a pivotal feature of the structural alterations in the asthmatic airway. Goblet cell hyperplasia and peribronchial fibrosis, two characteristic components of airway remodeling, also tended to be attenuated after azithromycin administration. As these structural changes in asthma have been considered increasingly important in a clinical context due to the persistent increases in airway responsiveness, variable or fixed airflow limitation, and possibly decline in pulmonary function [36], the above findings suggest a beneficial role of azithromycin on remodeling airway of asthma.

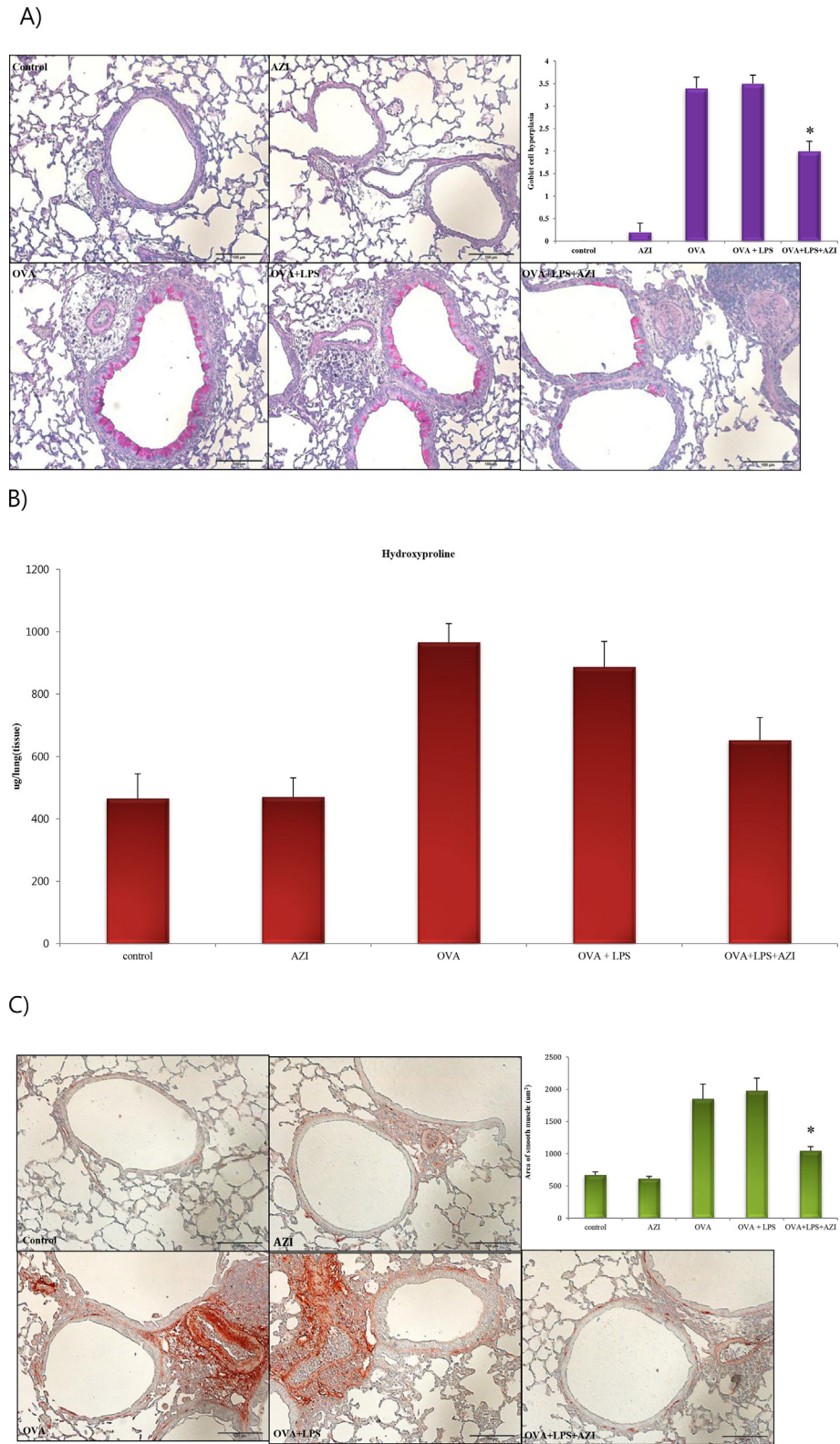


Fig. 4. Effect of azithromycin on parameters of airway remodeling in a model of chronic asthma. (A) Goblet cell hyperplasia. Representative photomicrographs of PAS-stained lung sections (original magnification $\times 200$) and quantification of the PAS-stained area by point scoring as described in Materials and methods. (B) Level of collagen expression of hydroxyproline in lung tissues. (C) Airway smooth muscle area. Representative photomicrographs of lung sections immunostained α -smooth muscle actin (original magnification $\times 200$) and areas of immunostaining per micron length of basement membrane of the bronchioles, as assessed using an image analyzer. Values shown are mean $\pm$ SEM ($n = 5-8$ per group); AZI; azithromycin, LPS; lipopolysaccharide; * $p < 0.05$ compared with the OVA/LPS group.

In the present study, the effect of azithromycin on airway remodeling could be mediated by its modulation of airway

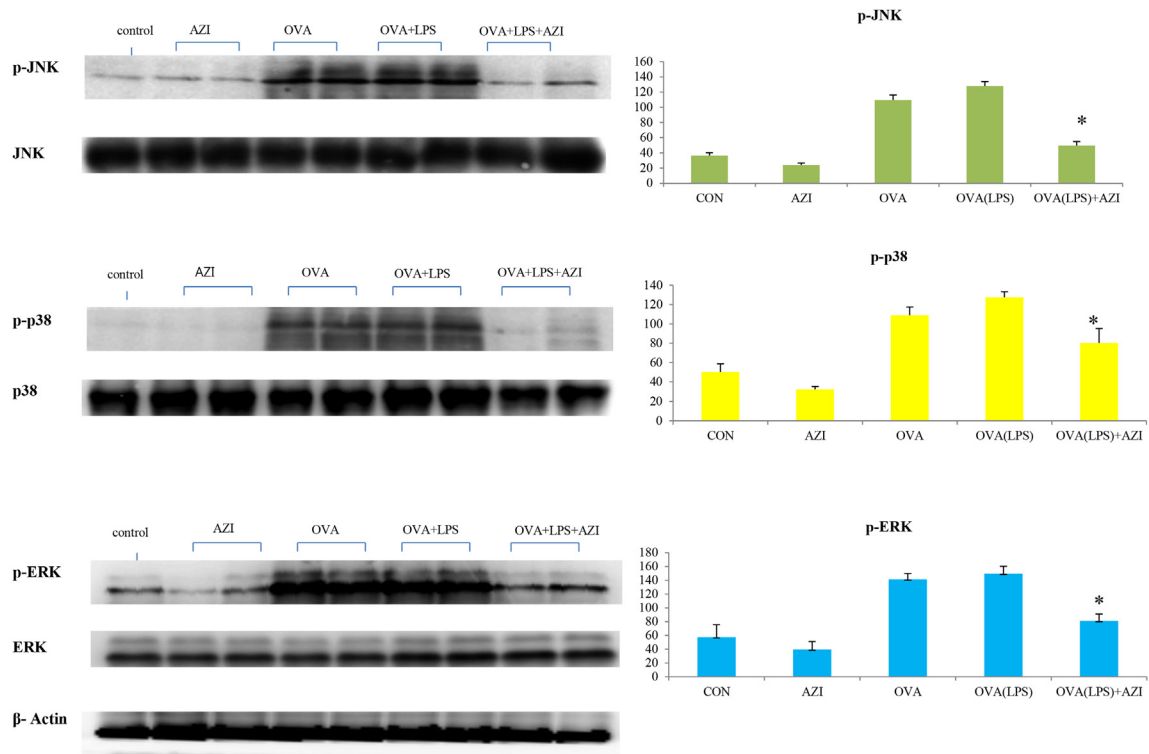


Fig. 5. Effect of azithromycin on the expression of MAPK pathways in a model of chronic asthma. Values shown are mean $\pm$ SEM ($n = 5-8$ per group); AZI; azithromycin, LPS; lipopolysaccharide; * $p < 0.05$ compared with the OVA/LPS group.

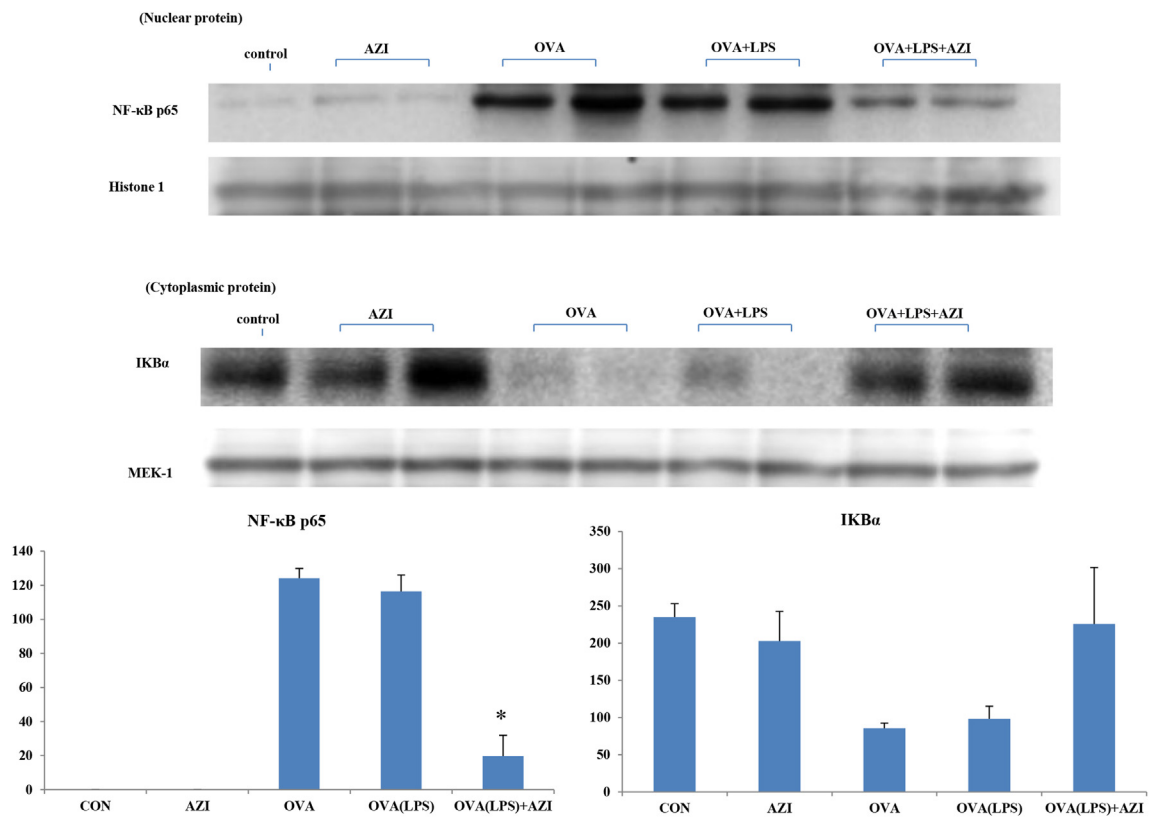


Fig. 6. Effect of azithromycin on the activity of NF-κB signaling in a model of chronic asthma. Values shown are mean $\pm$ SEM ($n = 5-8$ per group); AZI; azithromycin; LPS; lipopolysaccharide; * $p < 0.05$ compared with the OVA/LPS group.

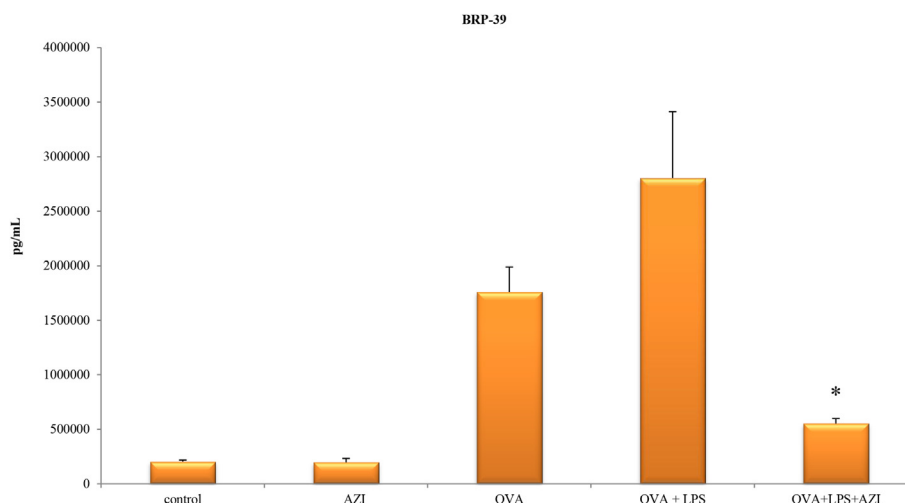


Fig. 7. Effect of azithromycin on the concentration of BRP-39 (human homologue YKL-40) in lung tissue. Values shown are mean $\pm$ SEM ($n = 5-8$ per group); AZI, azithromycin; LPS, lipopolysaccharide; * $p < 0.05$ compared with the OVA/LPS group.

inflammation and hyperresponsiveness, because the chronic inflammation and repetitive mechanical contraction caused by AHR could induce structural alterations in the asthmatic airways [2]. Alternatively, the inhibition of the MAPK/NF- κ B activation by azithromycin treatment could directly induce an anti-remodeling effect. In fact, a study using human airway smooth muscle cells showed that azithromycin had anti-angiogenic effects via the p38 MAPK pathway [37]. Tully et al. [24] showed that the inhibition of NF- κ B in mice with house dust mite-induced asthma attenuated components of airway remodeling including collagen deposition and smooth muscle hypertrophy. The present study also demonstrated that long-term azithromycin treatment attenuated the activation of MAPK/NF- κ B pathways that was induced by OVA challenge. Finally, the decreased expression of chitinase-like protein BRP-39 in the mouse and its human homologue YKL-40 by azithromycin therapy might be one possible explanation of the anti-remodeling effects seen in our model of chronic asthma. Recent evidence [38–40] suggests that the serum levels of YKL-40 in patients with asthma are correlated with the severity of the disease and the thickening of the subepithelial basement membrane, and that this effect is associated with the proliferation and migration of bronchial smooth muscle. In this study, we measured for the first time BRP-39 in lung tissue and BAL fluid in a murine model of chronic asthma treated with azithromycin. We observed that azithromycin administration significantly reduced the levels of BRP-39 in both types of samples. However, the association between the macrolide and BRP-39, and indeed the interplay between this association and the antiremodeling effects of azithromycin in asthma should be verified in a further study.

It was reported that exposure of LPS enhanced inflammation and the level of the dosage could determine Th1 versus Th2 inflammation in asthma [29,30]. The experiments demonstrated that low-dose LPS with OVA during the sensitization period induces in Th2 response associated with the activation of antigen-containing dendritic cells, whereas high levels of LPS with antigen does Th1 response-dominant asthma. Our data using low dose, 0.1 μ g, of LPS, also revealed that Th2 inflammation and remodeling were accentuated in the OVA/LPS group comparable to the OVA group with more neutrophilic inflammation. We confirmed that IL-8 and NE, which are inflammatory mediators related to the activity of neutrophils, the levels of which are elevated in patients with noneosinophilic asthma [41], were

significantly decreased after azithromycin treatment. This is consistent with the results of the previous study about clarithromycin treatment for refractory asthma [10]. Notably, in a study by Tang et al. [42], the expression of IL-8, a neutrophil chemo-attractant, which was decreased significantly by azithromycin, was induced by YKL-40 in bronchial epithelial cells via MAPK and NF- κ B pathways, causing smooth muscle proliferation and migration. This suggests that the effect of YKL-40-induced IL-8 on airway remodeling may be mediated via these signal pathways.

There are some limitations in this study. We chose azithromycin at a concentration of 75 mg/kg, referring to the previous study [15] and our preliminary data. Although the dosage was the highest effective and safe for a long-term use in a murine model of chronic asthma, it seems difficult to apply the same dose in human asthma due to the development of a side effect and a drug resistance. In addition, we administered LPS in the sensitization period following the previous stud, however, there is a possibility that exposure of LPS in the challenge period is more relevant in human asthma with enhanced allergic response than it in the sensitization period. Further research under the setting similar with human asthma could be helpful to verify the effect and the best regimen of the azithromycin.

5. Conclusions

This study provides evidence that long-term azithromycin administration in a murine model of chronic asthma improves not only airway inflammation, but also remodeling. Moreover, these effects of azithromycin may be mediated by effects on neutrophils-related mediators, BRP-39, and the MAPK/NF- κ B signal pathways. This result suggests that macrolide therapy might be an effective adjuvant option in chronic asthma, especially in chronic, severe asthma with remodeling airway.

Conflict of interests

We all authors have declared no conflict of interests.

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