



BRL-50481 Ameliorates Lung Inflammation in a Murine Model of Ovalbumin-Induced Allergic Asthma with Co-Exposure to Lipopolysaccharide

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Abstract

Asthma is an allergic inflammatory disease of the lungs characterized by eosinophilic inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR). Exposure to environmental endotoxins, such as lipopolysaccharide (LPS), can exacerbate asthma severity. Phosphodiesterase (PDE) inactivates cyclic adenosine 3',5'-monophosphate and cyclic guanosine 3',5'-monophosphate, thereby aggravating inflammation. Accordingly, PDE inhibitors could be used to treat asthma. Herein, we studied the effects of BRL-50481 (BRL), a PDE7 inhibitor, in a murine model of ovalbumin (OVA)-induced allergic asthma with co-exposure to LPS. Mice were sensitized, challenged with OVA, and subsequently exposed to LPS. Mice were administered with BRL prior to the OVA challenge. We observed that BRL treatment could suppress hallmark features of asthma, including mediators of eosinophilic and neutrophilic inflammation, such as expression of antigen-specific immunoglobulin (Ig) E, interleukin (IL)-13, IL-6, and mucus hypersecretion. Mice co-exposed to OVA and LPS exhibited marked AHR, which was improved in BRL-treated mice because of inhibition of mucus overproduction. In conclusion, given that PDE7 inhibitors can regulate allergic inflammatory responses, these agents could be potential candidates for treating asthma.

Key Words: Asthma, BRL-50481, Lipopolysaccharide, Phosphodiesterase

INTRODUCTION

Asthma is a pulmonary allergic disorder that affects more than 300 million individuals worldwide and is characterized by chronic airway inflammation that results in coughing, chest tightness, wheezing, and shortness of breath (Jin *et al.*, 2023). Treatment strategies to combat asthma include inhaled corticosteroids or combination therapy with long-acting β_2 agonists; however, given the adverse effects associated with these agents, there is an urgent need to develop new therapeutic approaches (Miravittles *et al.*, 2021). Additionally, patients with asthma are persistently exposed to air-floating particles such as dust, metals, and endotoxins, resulting in adverse effects, such as exacerbation of inflammation (Ovrevik *et al.*, 2009).

Cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) are well-known second messengers involved in immune responses (Kato *et al.*, 2017). In asthma, increased cAMP levels induce relaxation of airway smooth muscles, inhibit the activation and develop-

ment of T lymphocytes, and inhibit the chemotaxis of eosinophils (Billington *et al.*, 2013; Matera *et al.*, 2021). Furthermore, cGMP regulates vascular smooth muscle relaxation and induces bronchodilation in the small human airways (Lam and Bourke, 2020). cAMP and cGMP are hydrolyzed and inactivated by phosphodiesterases (PDEs), which results in exacerbated inflammation in asthma. Notably, the effects of PDE inhibitors on pulmonary diseases, including acute lung injury, chronic obstructive pulmonary disease, and asthma, have been previously reported (Mokra and Mokry, 2021). Therefore, it can be speculated that PDE inhibitors may be beneficial in asthma therapy (Matera *et al.*, 2014).

PDEs comprise 11 families (PDE1–PDE11) based on their substrates, functions, and tissue distributions. PDE7, an enzyme that hydrolyzes only cAMP, is expressed in the lungs, spleen, and immune cells, such as macrophages and lymphocytes (Huang *et al.*, 2023). PDE7 comprises two subtypes: PDE7A and PDE7B. PDE7A has three splice variants: PDE7A1, PDE7A2, and PDE7A3. PDE7A1 is highly expressed in T lymphocytes, macrophages, eosinophils, and

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airway smooth muscle, whereas PDE7A3 is expressed in the activated CD4⁺ T cells. Considering that PDE7A is broadly expressed in several inflammatory cells, PDE7 inhibitors may be valuable in treating inflammatory diseases (Safavi *et al.*, 2013). However, despite the potential of PDE7 inhibitors in pulmonary diseases, the role of these inhibitors remains poorly explored (Kim *et al.*, 2022). Therefore, we examined the effects of BRL-50481 (BRL), a PDE7 inhibitor, in a murine model of allergic asthma.

In this study, lipopolysaccharide (LPS), the outer membrane of gram-negative bacteria (Wang and Quinn, 2010), was used as an endotoxin to induce lung inflammation. LPS is typically employed to mimic bacterial exposure in experimental models; therefore, LPS is considered the most potent microbial stimulus for inflammation (Rylander *et al.*, 1985). In asthma, LPS exposure has been shown to aggravate the inflammatory response (Braun-Fahrlander *et al.*, 2002). Moreover, we have previously shown that LPS co-exposure can exacerbate mucus hypersecretion and airway hyperresponsiveness (AHR) in an ovalbumin (OVA)-induced murine model of allergic asthma (Lee *et al.*, 2020).

Airway mucus overproduction is involved in airflow obstruction, and many autopsy studies have identified airway mucus hypersecretion as the primary cause of asthma-related deaths (Williams *et al.*, 2006; Evans *et al.*, 2009). AHR is a hallmark clinical symptom of asthma and is defined as an aggravated obstructive response of the airways to stimuli (Meurs *et al.*,

2008). Mediators released from inflammatory cells and large amounts of mucus in the airways are the central causes of AHR in asthma (Shimura *et al.*, 1990). Consequently, in this study, we aimed to demonstrate the potential therapeutic effects of BRL in regulating inflammatory mediators and mucus production in an OVA-induced murine asthma model co-exposed to LPS.

MATERIALS AND METHODS

Mice

Five-week-old male BALB/c mice (Young Bio, Seongnam, Korea) were housed in the animal room at Chung-Ang University with access to pathogen-free food and water under standard laboratory conditions ($24 \pm 2^\circ\text{C}$, $50 \pm 5\%$ humidity and 12 h light-dark cycle). The mice were randomly divided into seven groups ($n=6-8$) as follows: Control, OVA, OVA+BRL, LPS, OVA+LPS, OVA+LPS+BRL, OVA+LPS+DEX group. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chung-Ang University (IACUC-A2022069).

Experimental model

To evaluate the therapeutic effects of BRL (MedChemExpress, 1 Deer Park Dr, NJ, USA), we established a murine model of OVA-induced asthma with co-exposure to LPS (Fig.

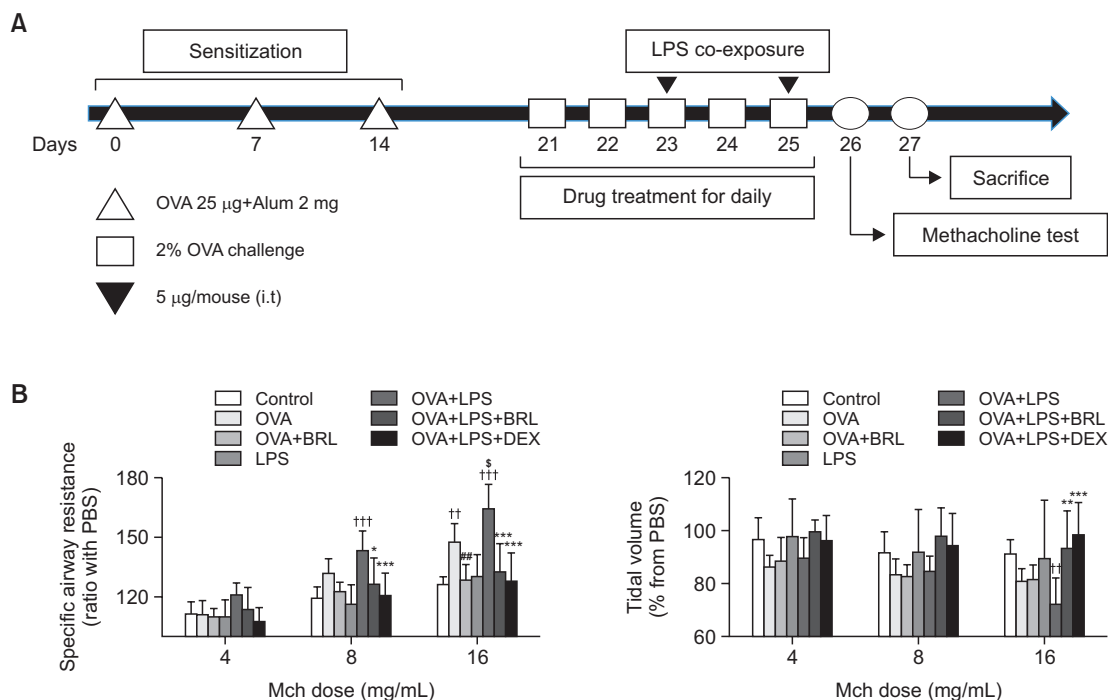


Fig. 1. BRL-50481 (BRL) improved airway hyperresponsiveness. (A) Time schedule to induce a murine model of allergic asthma with co-exposure to ovalbumin (OVA) and lipopolysaccharide (LPS). Mice were sensitized using a mixture of OVA and Alum on days 0, 7, and 14. Mice were intraperitoneally administered BRL (2 mg/kg) and DEX (2 mg/kg) 1 h before the OVA challenge (2 mg/kg). LPS was intratracheally injected 1 h after the OVA challenge on days 23 and 25. (B) A methacholine test was performed 1 day after the last OVA challenge on day 26, and all mice were sacrificed on day 27. Data are expressed as mean $\pm$ standard deviation (SD) ($n=6-8$). Statistical analyses were performed using two-way analysis of variance (ANOVA), followed by Tukey's test. Data were considered statistically significant ($^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared with the Control group, $^{\#}p<0.01$ compared with the OVA group, and $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the OVA+LPS group, and $^{\$}p<0.05$ compared between the OVA and OVA+LPS group).

1A), as described previously with modifications (Kim *et al.*, 2022). The OVA sensitized groups were administered with 25 µg of OVA (cat #A5503, grade V, Sigma-Aldrich, MO, St. Louis, USA) and 2 mg of aluminum hydroxide (Alum; Thermo Fisher Scientific, MA, USA) mixed in 200 µL of normal saline. The mixture was separately injected 100 µL each into intraperitoneal (i.p) and subcutaneous (s.c) routes for sensitization. The sensitization was performed 3 times on days 0, 7, and 14. The Control and LPS group were administered with only the same volume of normal saline. Subsequently, the OVA-sensitized mice were challenged with 2% OVA aerosol for 30 min using a nebulizer (Aerogen®, Galway, Ireland) from days 21 to 25. The Control and LPS group were exposed to 200 µL of normal saline only. BRL and dexamethasone (DEX) (Sigma-Aldrich) were dissolved in 0.1% dimethyl sulfoxide (Sigma-Aldrich). These drugs were administered via i.p. injections 1 h before the OVA challenge. LPS from *E. coli* O127:B8 (cat# 437627, Sigma-Aldrich) was diluted in normal saline (100 µg/mL). For LPS exposure, mice were anesthetized with a mixture of avertin 250 mg/kg and xylazine 5 mg/kg and intratracheally injected 50 µL of LPS stock (5 µg/mouse) 1 h after OVA challenge on days 23 and 25 (Fig. 1A).

Methacholine test

A methacholine test was performed using a Buxco® non-invasive double-chamber plethysmograph (Data Sciences International, MN, USA) to evaluate AHR by measuring the specific airway resistance (sRAW) and tidal volume (TV) 1 day after the last challenge. The sRAW is measurement of airflow that indicates how much effort is required to breathe and TV is the amount of air that moves in and out of the lungs with each breathe. All mice were acclimated for 5 min on a plethysmograph and exposed to methacholine (4, 8, or 16 mg/mL) for 1 min. After methacholine exposure, AHR was automatically measured using FinePointe software (Data Sciences International, MA, USA) for 5 min. All data were expressed as the ratio of the value in phosphate-buffered saline (PBS; 0 mg/mL).

Inflammatory cell counts in bronchoalveolar lavage fluid (BALF)

All mice were sacrificed 1 day after the methacholine test. BALF was collected by lavaging the right lung twice with 700 µL of ice-cold PBS. The collected BALF was centrifuged (1,500×g, 10 min, 4°C) to separate the supernatant and cell pellet. Following the lysis of red blood cells, the cell pellet was suspended in 250 µL of PBS and cytocentrifuged to analyze inflammatory cell infiltration into the lungs. Cytocentrifuged cells were stained using a Kwik-Diff™ Stain kit (Thermo Fisher Scientific) and imaged using a microscope (Leica Microsystems, Wetzlar, Germany) with a Leica DM 480 camera (Leica Microsystem).

Measurement of interleukins in BALF

To measure the level of TH-associated cytokines, IL-1β, 6, 10, and 13 in BALF, an enzyme-linked immunosorbent assay (ELISA) was performed using each Quantikine mouse ELISA kit (R&D Systems, Inc., MN, USA) according to the manufacturer's instructions.

Measurement of OVA-specific immunoglobulin E (IgE) levels in serum

ELISA was performed to measure the serum levels of OVA-

specific IgE. One day after the methacholine test, blood samples were collected from the inferior vena cava with 50 µL of 3.2% sodium citrate as an anticoagulant. Serum OVA-specific IgE levels were measured using an anti-OVA IgE mouse ELISA kit (Cayman Chemical, MA, USA). The serum was diluted 1:100 with the assay diluent according to the manufacturer's instructions.

Histological assays

Briefly, the lungs were fixed in 10% formalin and embedded in paraffin using Tissue-Tek® (Sakura Finetek®, CA, USA). The embedded tissues were cut into 4 µm sections using a Leica microtome 820 (Leica Microsystems), followed by staining with hematoxylin and eosin (H&E), or periodic acid-Schiff (PAS), or Congo red. Subsequently, H&E- and PAS-stained tissues were imaged using a light microscope equipped with a Leica DM 480 camera. The inflammation score was determined based on histological grading criteria (Curtis *et al.*, 1990). Goblet cell hyperplasia and mucus secretion were measured using ImageJ software (NIH Image, MD, USA) (Oliveira *et al.*, 2016). The Congo red-stained tissues were visualized under a microscope using a Toupcam camera (ToupTek, Hangzhou, China). Eosinophils in 20,000 µm² of lung tissue were counted using ImageJ software (Albert *et al.*, 2011).

Immunohistochemistry assays

Previously sectioned lung samples on slides were stained using immunohistochemistry (IHC) and evaluated for expression of neutrophils (1:200; ab2557; abcam, MA, USA). The antibody reacted with mouse antigen. Heat-induced antigen retrieval was performed using 10 mmol/L of citrate buffer (pH 6) in a pressure cooker for 20 min. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min at room temperature. Blocking was performed with an universal BSA for 40 min at room temperature. Immunoreactivity for immunohistochemistry was detected using biotinylated IgG secondary antibody followed by incubation with ABC kit (Vector Laboratory, Burlingame, CA, USA) for 40 min, and diaminobenzidine chromogenic substrate for 4-10 min. Slides were counter-stained with hematoxylin in for 30 sec, followed by dehydration and mounting. The IHC-stained tissues were visualized under a microscope using a DPA-M500 camera and ImageView software (DPA, Seoul, Korea). Indux of neutrophils were evaluated using ImageJ software.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the lung tissues using TRIzol reagent (Thermo Fisher Scientific). The extracted RNA was quantified to 1 µg using a NanoDrop ND-1000 (Thermo Fisher Scientific) and reverse transcribed to cDNA using an iScript µcDNA Synthesis Kit (Bio-Rad, CA, USA). Synthesized cDNA was analyzed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with an iQ™ SYBR® Green Supermix (Bio-Rad). All reactions were performed according to the manufacturer's instructions. The housekeeping gene *GAPDH* was used as an internal control. Primer sequences used in this study are listed in Table 1.

Western blot assay

Briefly, proteins from lung tissues were extracted using the Radioimmunoprecipitation assay(RIPA) buffer (Thermo Fisher

Table 1. Primer sets used for qPCR

Gene	Forward sequence (5'–3')	Reverse sequence (5'–3')
<i>IL-13</i>	CAGCAGCTTGAGCACATTTTC	ATAGGCAGCAAACCATGTCC
<i>IL-6</i>	GGACCAAGACCATCCAATTC	GGCATAACGCACTAGGTTTG
<i>IL-1β</i>	GCTGCTTCCAAACCTTTGAC	TTCTCCACAGCCACAATGAG
<i>GAPDH</i>	GGCAAAGTGAGATTGTTGC	AATTTGCCGTGAGTGGAGTC

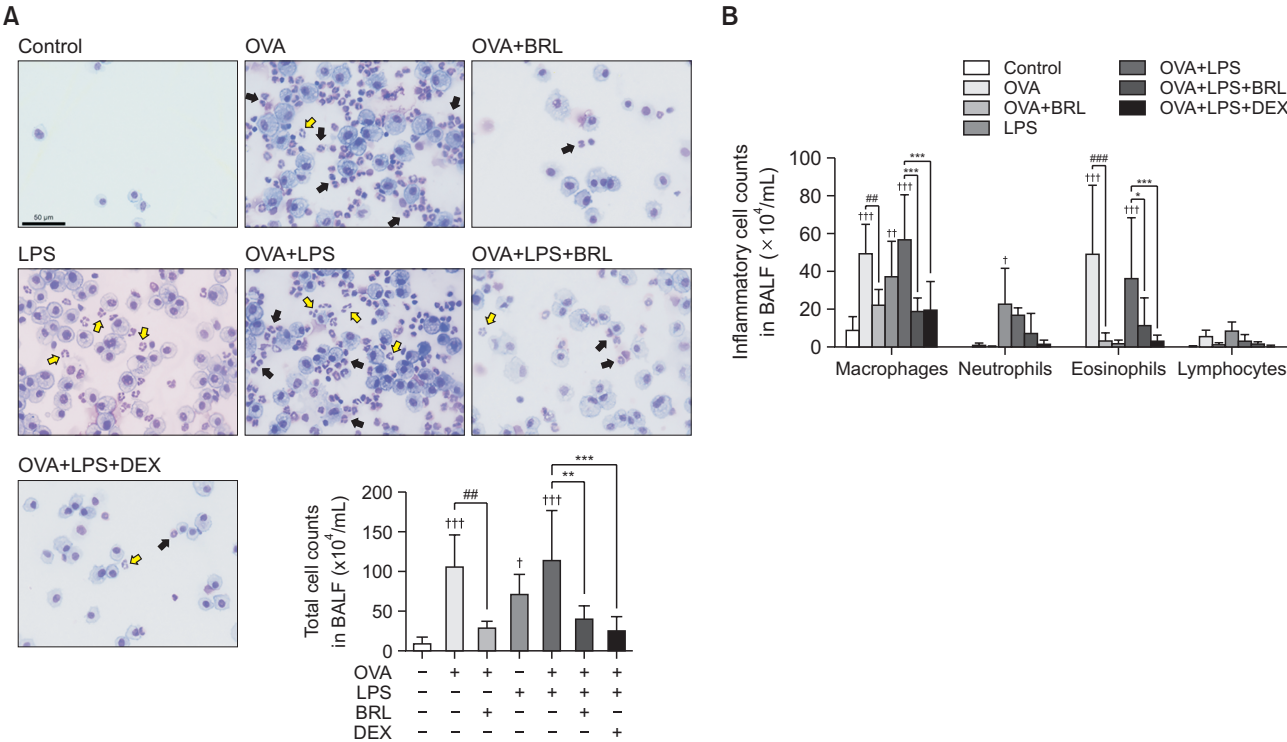


Fig. 2. BRL reduced inflammatory cell infiltration. (A) Inflammatory cells were stained and imaged using a microscope (magnification 400×; scale bar, 50 μm). Black arrows indicate eosinophils and yellow arrows indicate neutrophils. Total cell counts were performed using a hemocytometer. (B) Differential inflammatory cells were counted based on their morphological criteria. Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA, followed by Tukey's test. Data were considered statistically significant (†*p*<0.05, ††*p*<0.01, †††*p*<0.001 compared with the Control group, ##*p*<0.01, ###*p*<0.001 compared with the OVA group, and **p*<0.05, ***p*<0.01, ****p*<0.001 compared with the OVA+LPS group).

Scientific) and quantified using a pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequently, 20 μg of extracted proteins were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride membrane. The protein-transferred membranes were blocked using 5% skim milk in tris-buffered saline-Tween 20 (TBS-T) buffer at room temperature for 1 h, followed by incubation with primary antibodies which diluted at 1:1000 ratio to 5% BSA with 0.5% sodium azide in tris-buffered saline-Tween 20 (TBS-T) against MUC5AC (cat# A17325, ABclonal, MA, USA) and β-actin (Santa Cruz Biotechnology, TX, USA) at 4°C overnight. After incubation with the primary antibody, the membranes were incubated with a horseradish-conjugated secondary antibody which diluted at 1:10000 ratio to 5% skim milk in at room temperature for 1 h. All protein signals were detected using the ECL™ Prime Western Blotting System (Amersham™, Uppsala, Sweden)

and imaged using Fusion Solo X (Vilber, Collégien, France). Band intensity was determined using ImageJ software. β-actin was used as a loading control.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) of 6-8 mice (n=6-8). Statistical analyses were performed using one-way analysis of variance (ANOVA) and two-way ANOVA, followed by Tukey's test, using GraphPad Prism 9.0 software (GraphPad Software, CA, USA). *P* values <0.05 were deemed significant.

RESULTS

BRL improved airway hyperresponsiveness

To assess the therapeutic effects of BRL in the murine model of allergic asthma, BRL was administered to mice intra-

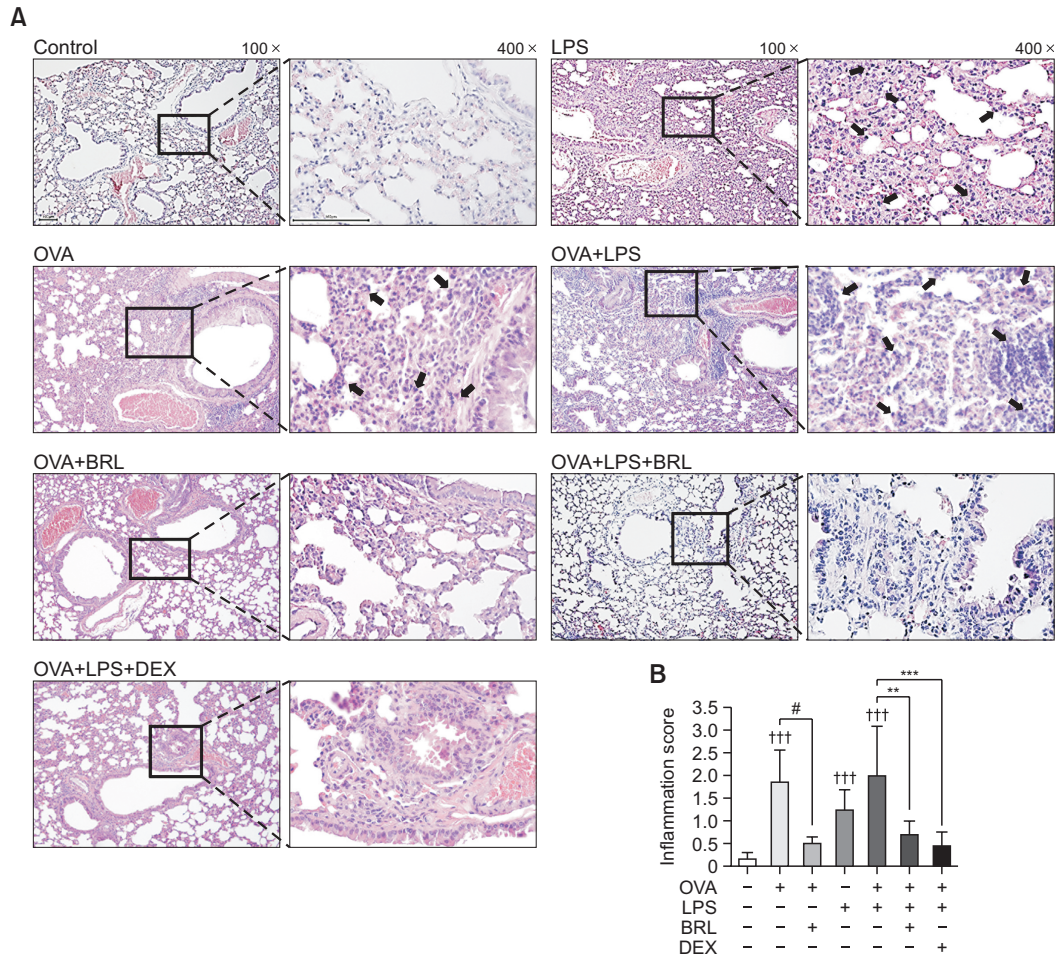


Fig. 3. BRL regulates lung inflammation. (A) Hematoxylin & eosin (H&E)-stained lung tissues were imaged using a microscope and (B) inflammation score was determined based on histologic grading criteria ($n=6-8$; magnification 100 $\times$ and 400 $\times$; scale bar, 100 μ m). Black arrows indicate infiltrated inflammatory cells. Data are expressed as mean $\pm$ SD. Statistical analyses were performed using one-way ANOVA, and Tukey's test was performed post-hoc. Data were considered statistically significant ($^{\dagger\dagger\dagger}p<0.001$ compared with the Control group, $^{\#}p<0.05$ compared with the OVA group, and $^{**}p<0.01$, $^{***}p<0.01$ compared with the OVA+LPS group).

peritoneally 1 h before the OVA challenge (Fig. 1A). A methacholine test was performed to evaluate AHR by measuring the sRAW and TV. OVA and co-exposure to LPS increased the sRAW. In contrast, TV was decreased in the OVA, LPS, and OVA+LPS groups. Our findings revealed that OVA and LPS co-exposure group enhanced asthmatic inflammation than the OVA or LPS single-exposure group, but treatment with BRL significantly decreased airway resistance and increased the tidal volume compared to the OVA and OVA+LPS groups (Fig. 1B).

BRL reduced inflammatory cell infiltration

In the BALF, the total number of cells and differential inflammatory cells, including macrophages, neutrophils, eosinophils, and lymphocytes, were counted and imaged. Exposure to both OVA and LPS increased cell recruitment. OVA exposure induced eosinophil recruitment, whereas LPS exposure induced neutrophil infiltration. The number of infiltrating inflammatory cells was reduced in BRL-treated groups, demonstrating that BRL administration could reduce inflammatory cell infiltration (Fig. 2A, 2B).

BRL regulated lung inflammation

Airway remodeling in a murine allergic asthma model was analyzed using H&E staining of mouse lung tissues. OVA- and LPS-exposed mice had an increased inflammation score and thickened alveoli and bronchioles; whereas, treatment with BRL ameliorated airway remodeling in the lung tissue (Fig. 3A, 3B).

BRL decreased asthma associated cytokines

RT-qPCR was performed to measure the mRNA expression levels of interleukin (IL)-6 and interleukin (IL)-1 β in the lung tissues. OVA and LPS exposure increased mRNA expression levels of IL-6 and IL-1 β , and the IL-6 gene expression was enhanced in the OVA+LPS group compared to the OVA group. Notably, the BRL-treated group exhibited reduced mRNA expression of examined inflammatory cytokines (Fig. 4A). The LPS co-exposure had no significant difference on the levels of IL-6, IL-1 β , and interleukin(IL)-10 compared to the OVA group. But BRL-treated group showed decreased level of IL-6, IL-1 β , and increased level of IL-10 than the OVA+LPS group (Fig. 4B).

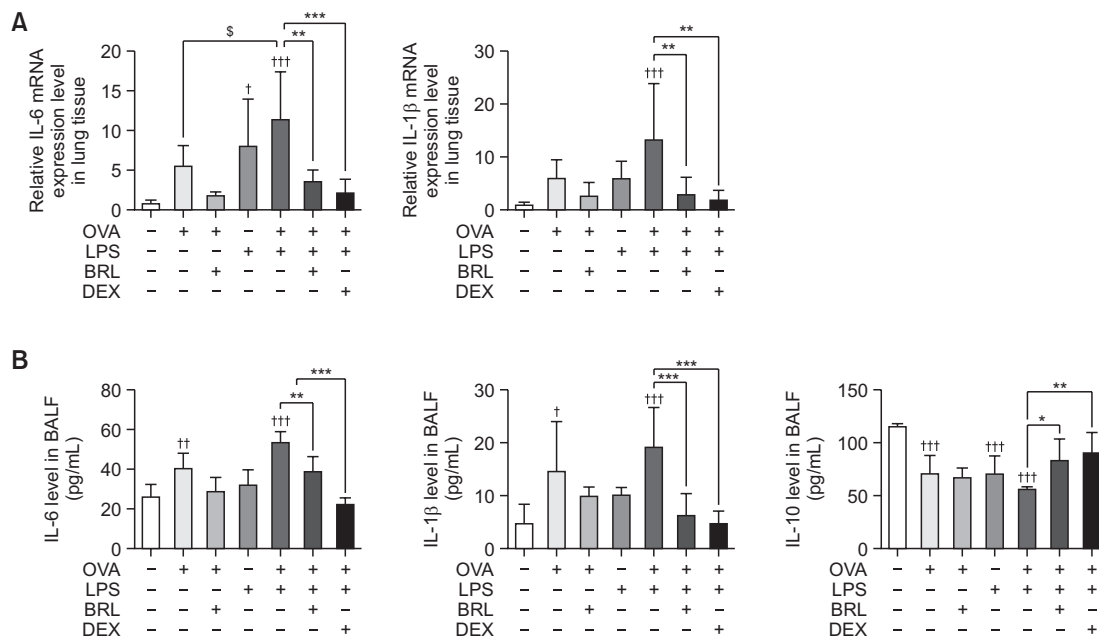


Fig. 4. BRL decreased asthma associated cytokines. (A) Relative mRNA expression level of IL-6 and IL-1 β in the lung were measured by RT-qPCR and (B) IL-6, IL-1 β , and IL-10 levels in bronchoalveolar lavage fluid (BALF). Data are expressed as mean $\pm$ SD (n=6-8). Statistical analyses were performed using one-way ANOVA, followed by Tukey's test ($^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared with the Control group, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the OVA+LPS group, and $^{\$}p<0.05$ compared between the OVA and OVA+LPS group).

BRL decreased eosinophilic allergic biomarkers

To investigate the effect of BRL on eosinophilic inflammation, the infiltrated eosinophils in 20,000 μm^2 of Congo red-stained tissue were counted. Although LPS exposure had no effect, eosinophils had highly infiltrated into the lung tissue of the OVA-exposed group. Treatment with BRL suppressed eosinophil recruitment (Fig. 5A).

Type 2 helper T cell (Th2 cell)-related cytokines, such as IL-13 and antigen-specific IgE, are important markers of eosinophilic allergic inflammation (Ho *et al.*, 2020). The OVA-exposed group showed increased expression of IL-13 mRNA in the lung tissue and elevated IL-13 levels in the BALF. Additionally, OVA exposure increased the serum levels of OVA-specific IgE. LPS exposure did not impact IL-13- or OVA-specific IgE expression. Treatment with BRL decreased the levels of these eosinophilic allergic markers (Fig. 5B).

BRL regulated neutrophilic inflammation

To further investigate the differentiation between the OVA group and the OVA+LPS group and the effect of BRL on neutrophilic inflammation, the percentage of infiltrated neutrophils in IHC-stained tissue were evaluated. The OVA+LPS group showed significantly higher percentage of neutrophils compared to the OVA group. Additionally, treatment of BRL highly reduced the ratio of neutrophils in both the OVA and OVA+LPS group (Fig. 6A, 6B).

BRL suppressed mucus hypersecretion

PAS-stained lung tissues were used to assess goblet cell hyperplasia and mucus hypersecretion (Fig. 7A). OVA exposure increased mucus secretion in lung tissues. Moreover, mucus secretion was significantly increased in the OVA+LPS group. Western blotting was performed to measure the level

of MUC5AC, a mucin protein (Fig. 7A). Protein expression of MUC5AC was increased in the OVA and OVA+LPS groups. Treatment with BRL decreased mucus secretion and MUC5AC protein expression in lung tissues (Fig. 7A, 7B).

DISCUSSION

Allergic asthma is mainly characterized by eosinophilic inflammation; however, bronchial LPS inhalation induces neutrophilic inflammation (Possa *et al.*, 2013; Ray and Kolls, 2017). Upon LPS exposure, proinflammatory cytokines such as IL-6 and IL-1 β are expressed by lung epithelial cells and macrophages (Michel, 2003). Conversely, the production of allergen-specific IgE and Th2 cell-related cytokines, such as IL-13, increases after sensitization during eosinophilic allergic responses (Corren, 2013). IL-13 induces goblet cell hyperplasia and AHR, with the switching of antibody production from IgM to IgE also involved in mucus secretion and constriction of airway smooth muscle, thereby resulting in airflow obstruction (Owen, 2007). In previous study, the exposure of OVA could lead to a rapid increase in airway resistance (Blanchet *et al.*, 2005).

Methacholine is a non-selective muscarinic receptor agonist that acts directly on airway smooth muscle receptors to induce bronchoconstriction. However, methacholine has also been linked to indirect mechanisms of airway response as well, including stimulation of mucous cell secretion. The mechanism of action of derivative methacholine is longer than acetylcholine, thus it is useful in bronchial challenge testing to allow time for assessment of reactivity (Lee *et al.*, 2017). In previous study, the exposure to OVA can increase airway resistance in the methacholine test (Blanchet *et al.*, 2005).

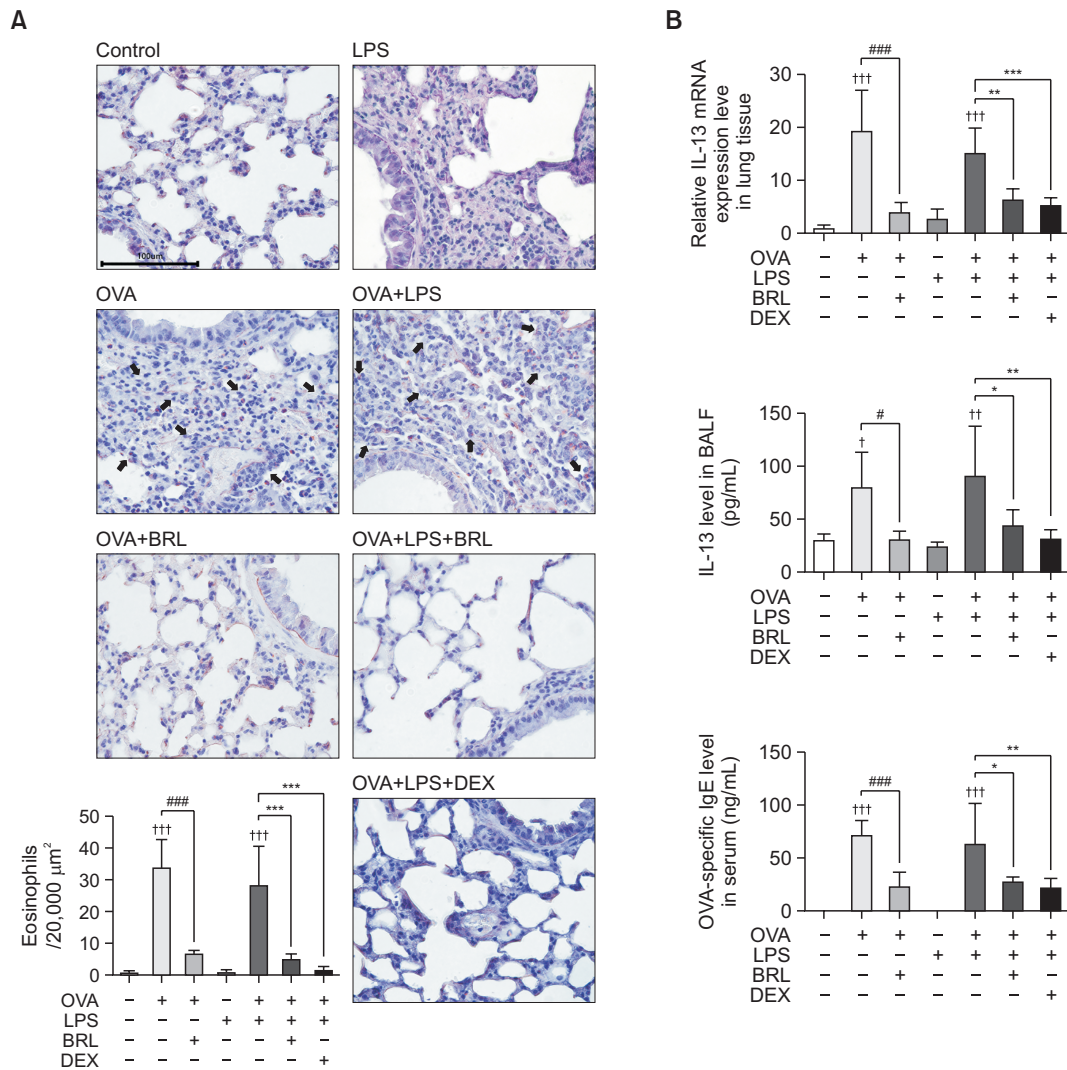


Fig. 5. BRL decreases eosinophilic allergic biomarkers. (A) Infiltrated eosinophils in 20,000 μm^2 of Congo red-stained lung tissues were counted. Treatment with BRL decreased the number of eosinophils in lung tissues ($n=6-8$; magnification 630 $\times$; scale bar, 100 μm). (B) Treatment with BRL reduced interleukin (IL)-13 gene expression in lung tissues and IL-13 levels in bronchoalveolar lavage fluid (BALF) and serum levels of OVA-specific immunoglobulin E (IgE). Data are expressed as mean $\pm$ SD. Statistical analyses were performed using one-way ANOVA, followed by Tukey's test ([†] $p<0.05$, ^{††} $p<0.01$, ^{†††} $p<0.001$ compared with the Control group, [#] $p<0.05$, ^{###} $p<0.001$ compared with the OVA group, and ^{*} $p<0.05$, ^{**} $p<0.01$, ^{***} $p<0.001$ compared with the OVA+LPS group).

As shown in Fig. 1B, the OVA group and OVA+LPS groups showed much higher airway resistance than the Control group, but BRL treatment reduced airway resistance in both the OVA and OVA+LPS groups. In addition, the tidal volume of the OVA and OVA+LPS groups was lower than that of the Control group, but increased in the BRL treated group.

Inflammatory cell infiltration is another feature of allergic asthma (Possa *et al.*, 2013). After sensitization to an allergen, inflammatory cells, such as macrophages and eosinophils, infiltrate the lungs via the action of chemokines (Oliveira and Lukacs, 2001). As shown in Fig. 2A and 2B, OVA or LPS exposure increased the number of inflammatory cells in the BALF. The total number of inflammatory cells was similar between the OVA- and LPS-exposed groups; however, the composition of inflammatory cells differed significantly. OVA exposure increased the number of eosinophils, whereas LPS exposure

induced neutrophil recruitment. Treatment with BRL reduced macrophage, eosinophil, and neutrophil infiltration. Additionally, infiltrated eosinophils in Congo red-stained lung tissues were counted. The number of eosinophils were reduced in the lung tissues of BRL-treated mice (Fig. 2A, 4B).

Airway remodeling, including thickened alveoli and bronchioles induced by cell infiltration and hyperplasia, is a characteristic of lung inflammation (Hough *et al.*, 2020). Our results also revealed the ameliorative effect of BRL on OVA- and LPS-induced airway remodeling (Fig. 3A).

OVA exposure and LPS exposure increased the proinflammatory cytokines such as IL-6 and IL-1 β (Michel, 2003). In this study, LPS co-exposure increased the mRNA expression of IL-1 β and IL-6 gene expression, otherwise the BRL-treated group decreased mRNA expression of IL-1 β and IL-6 gene expression (Fig. 4A). In addition, the level of IL-6 and IL-1 β

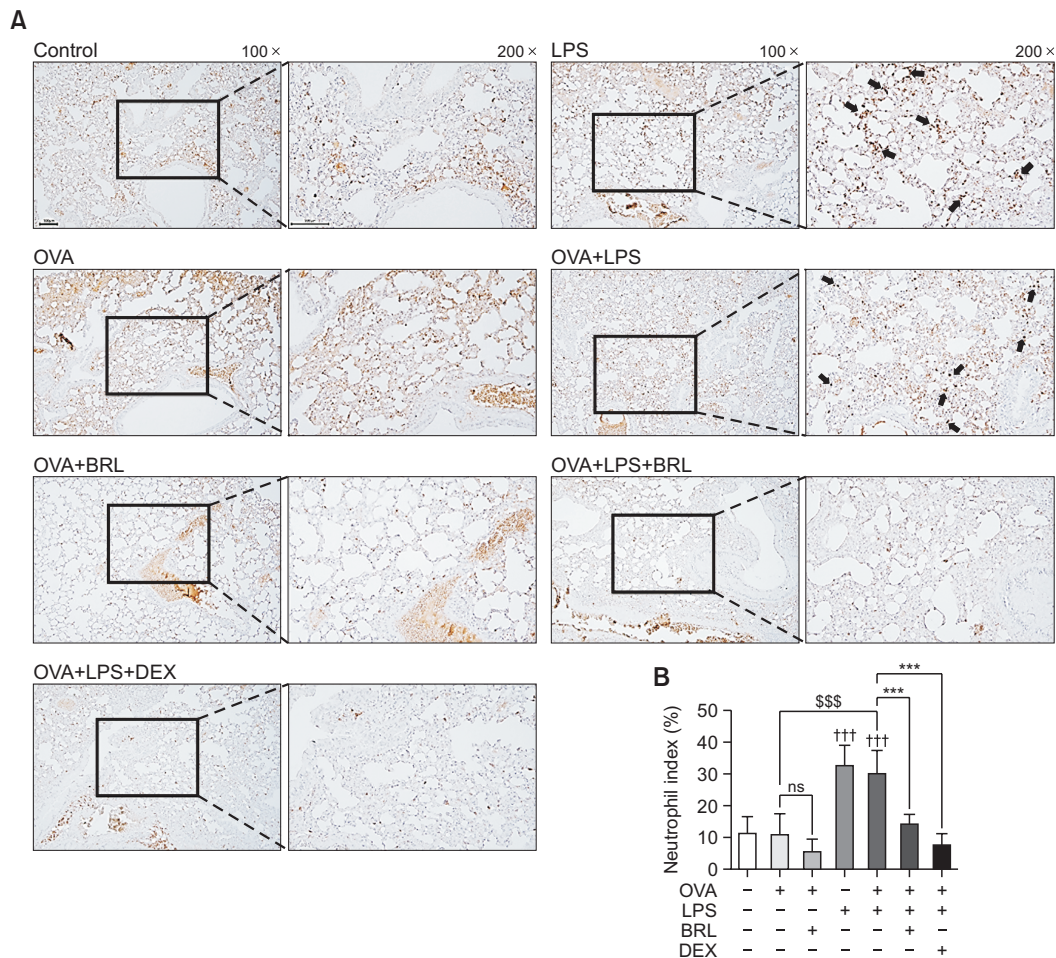


Fig. 6. BRL regulates neutrophilic inflammation. (A) Immunohistochemistry (IHC)-stained lung tissues were imaged using a microscope. (B) Quantitative analysis of infiltrated neutrophils in IHC-stained lung tissues were evaluated. Treatment with BRL reduced the percentage of neutrophils in lung tissues (n=4; magnification 100× and 200×; scale bar, 100 μm). Black arrows indicate infiltrated neutrophils. Data are expressed as mean ± SD. Statistical analyses were performed using one-way ANOVA, and Tukey's test was performed post-hoc. Data were considered statistically significant (†††*p*<0.001 compared with the Control group, ****p*<0.001 compared with the OVA+LPS group, and ^{\$\$\$}*p*<0.001 compared between the OVA and OVA+LPS group).

in BALF were decreased in the BRL-treated group compared to the OVA+LPS group. From these results, we can suppose that BRL has a positive effect to ovalbumin-induced allergic asthma with co-exposure to lipopolysaccharide by influencing mainly to IL-6 and IL-1β. Further, we measured the level of IL-10 in BALF to see the difference of IL-10 in each group and correlation between IL-1β, IL-6 and IL-10. As a result, IL-10 made a significant difference between 'OVA+LPS group and OVA+LPS+BRL treated group (Fig. 4B). Additionally, IL-10 is a potent regulator of the innate and adaptive immune responses (Pils *et al.*, 2010) and has a potent of antioxidant and anti-inflammatory features on OVA-induced rats (Cellat *et al.*, 2021). So, we can predict that IL-10 which is influenced by BRL has the inhibitory effect to a murine model of ovalbumin-induced allergic asthma with co-exposure to lipopolysaccharide.

In previous studies, the exposure to OVA resulted in attenuation of airway eosinophilia and hyperresponsiveness (Schramm *et al.*, 2004). Also, LPS is one of the most powerful pro-inflammatory factor and can induce neutrophilic inflammation (Kumari *et al.*, 2015). In this study, OVA and OVA+LPS groups

showed significantly higher eosinophil counts compared to the Control group, and in addition, the eosinophil counts in OVA+BRL group and OVA+LPS+BRL were lower than those in the OVA and OVA+LPS groups (Fig. 5A). Also, LPS co-exposure had no effects on the levels of IL-13 and OVA-specific IgE, rather OVA sensitization increased the levels of IL-13 and OVA-specific IgE. Additionally, the BRL-treated group showed decreased mRNA expression of IL-13 in the lung and decreased level of IL-13 in BALF. The BRL-treated group also showed decreased level of OVA-specific IgE in serum when compared with the OVA and OVA+LPS groups (Fig. 5B).

OVA and LPS caused changes to the structure of the epithelium and inflammatory cell infiltration in the lungs, but the main reason of eosinophil infiltration was OVA exposure (Lee *et al.*, 2020). Without OVA, exposure to LPS can lead to neutrophilic inflammation and increase the production of Th2 cytokines (Radermecker *et al.*, 2019). To identify the difference in neutrophil index of each groups, we performed IHC-staining. The neutrophil index of LPS and OVA+LPS group were higher than the Control group, in addition, the OVA+LPS

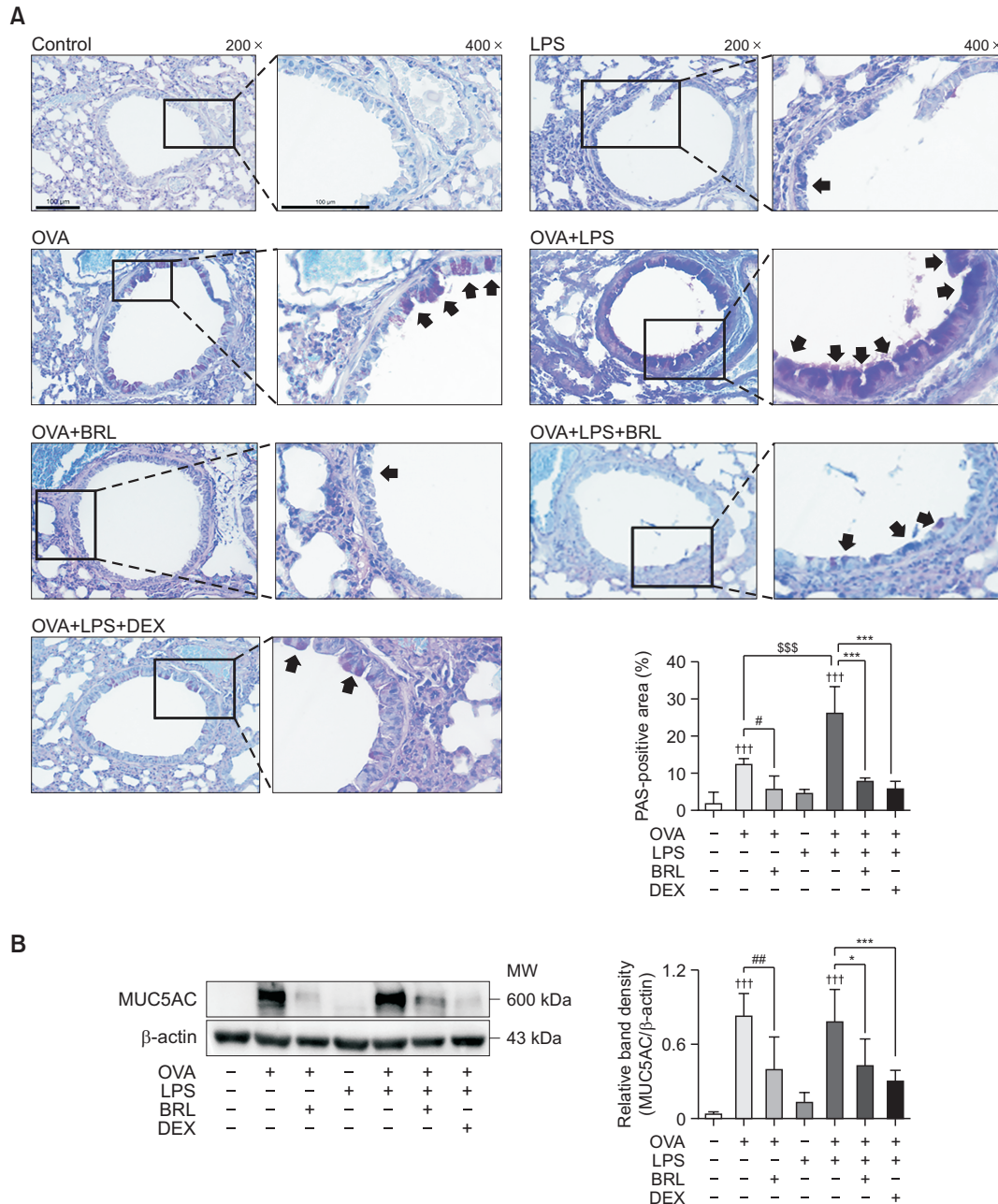


Fig. 7. BRL suppresses mucus hypersecretion. (A) Periodic acid-Schiff (PAS)-stained tissues were imaged using a microscope, and the PAS-positive area was measured using ImageJ software ($n=6-8$; magnification 200 $\times$ and 400 $\times$; scale bar, 100 μ m). The OVA+LPS group exhibited significant mucus production. Treatment with BRL suppressed goblet cell hyperplasia and mucus hypersecretion. (B) MUC5AC, a mucin protein, was assessed using western blotting. Treatment with BRL reduced MUC5AC protein levels in lung tissues. Data are expressed as means $\pm$ SD. Statistical analyses were performed using one-way ANOVA, followed by Tukey's test. $^{+++}p<0.001$ compared with the Control group, $^{\#}p<0.05$, $^{##}p<0.01$ compared with the OVA group, $^{*}p<0.05$, $^{***}p<0.001$ compared with the OVA+LPS group, and $^{sss}p<0.001$ compared between the OVA and OVA+LPS group.

group showed significantly higher neutrophil index than the OVA group. Moreover, the neutrophil index of OVA+LPS+BRL group was much lower than those in the OVA+LPS group (Fig. 6A, 6B). These results suggest that co-exposure to OVA and LPS may induce neutrophilic inflammation and chronic asthma more than exposure to OVA alone, and also that treatment of BRL shows a decrease in neutrophil index, implying that

BRL is a potential treatment for neutrophilic inflammation.

Given that IL-13 and OVA-specific IgE participate in mucus hypersecretion, goblet cell hyperplasia and mucus overproduction were measured using PAS-stained lung tissues. Also, recent study shows that there is a correlation between NF- κ B signal and MUC5AC (Hossain *et al.*, 2022). We conducted Western blotting to evaluate MUC5AC levels in lung tissues.

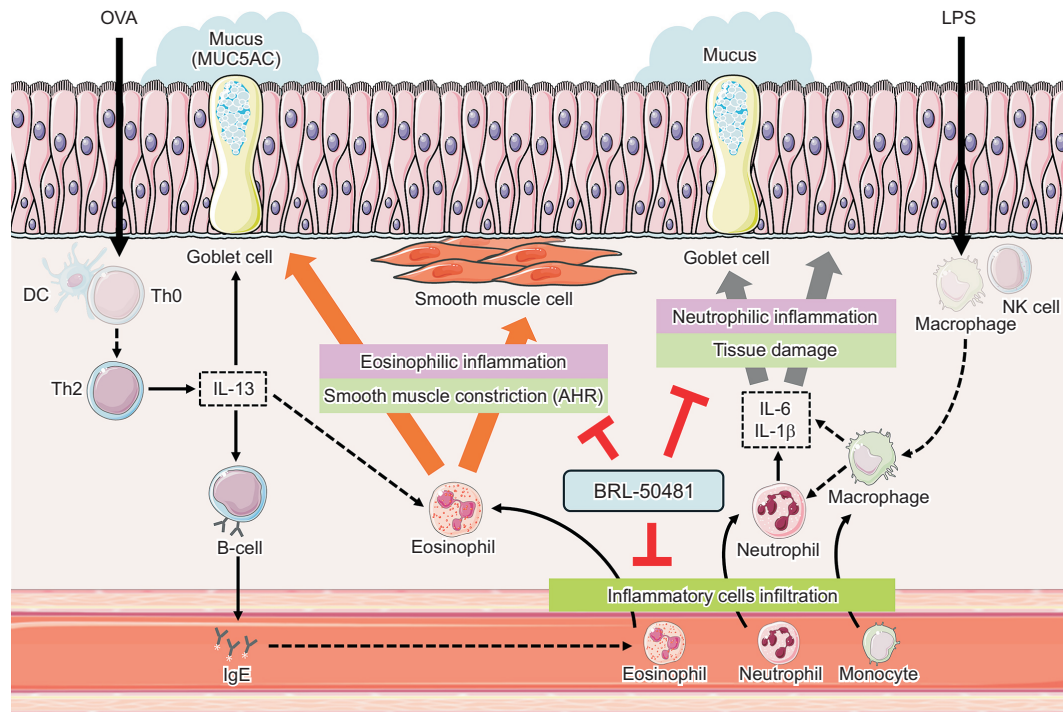


Fig. 8. Illustration of the effect of BRL treatment on a murine model of allergic asthma with co-exposure to OVA and LPS. BRL alleviated both eosinophilic and neutrophilic inflammation. Notably, BRL attenuated mucus hypersecretion, neutrophil infiltration, and the elevated levels of IL-6 and IL-1 β , all of which were synergistically amplified by both types of inflammation. The illustration was created using smart.servier.com. The confirmed signaling pathways in this study were indicated as a solid arrow; the potentially expected signaling pathways were presented to a dashed arrow; the inhibitory effect of BRL in this asthma murine model were presented as a red bar; the main pathogenic pathway of this asthma murine model associated to the effect of BRL were presented to large orange and gray arrows. OVA, ovalbumin; LPS, lipopolysaccharide; DC, dendritic cell; Th0, naïve T cell; Th2, type 2 helper T cell; IgE, immunoglobulin E; IL, interleukin; NK cell, natural killer cell.

IL-13 induces the expression of MUC5AC, resulting in mucus hypersecretion (Seibold, 2018). Goblet cell hyperplasia was observed in the OVA group, and the OVA+LPS group showed significantly increased mucus hypersecretion than the OVA group. Conversely, MUC5AC protein expression did not differ between the OVA and OVA+LPS groups (Fig. 7A, 7B). It is speculated that LPS co-exposure can impact other mucin proteins, resulting in a synergistic increase in mucus secretion in the OVA+LPS group.

Previously, we have reported the anti-inflammatory effects of BRL on OVA-induced asthmatic lung inflammation, exacerbated by co-exposure to Asian sand dust (ASD) in mice (Kim *et al.*, 2022). The effects of BRL in inhibiting eosinophilic inflammation, IL-13 levels, MUC5AC levels and OVA-specific IgE levels have been investigated in both previous and current studies (Kim *et al.*, 2022). The previous study examined the effects of BRL on macrophage recruitment, including macrophage count and MCP-1 levels, which were synergistically increased by ASD co-exposure (Kim *et al.*, 2022). In contrast, the current study evaluated the effects of BRL on the synergistic increase in IL-6 and IL-1 β levels and the infiltration of neutrophils into lung tissue exacerbated by LPS co-exposure. To the best of our knowledge, this is the first study to evaluate the effects of BRL on neutrophilic inflammation induced by infection in allergic asthma. Based on the findings from both studies, eosinophilic inflammation was associated with elevated IL-13 secretion from Th2 cells, which promoted B cell

class-switching and subsequent IgE production, leading to increased eosinophil infiltration. In contrast, neutrophilic inflammation was associated with elevated IL-6 and IL-1 β secretion from macrophages, resulting in increased neutrophil infiltration. Eosinophilic and neutrophilic inflammation synergistically induced mucus hypersecretion in the airway. Notably, BRL treatment alleviated both eosinophilic and neutrophilic inflammation by reducing the levels of IL-13, IL-6, and IL-1 β , along with their associated inflammatory cell infiltration and mucus hypersecretion.

In summary, we established a murine model of allergic asthma model by inducing eosinophilic and neutrophilic inflammation (Fig. 8). Mice co-exposed to OVA and LPS showed increased mucus hypersecretion and neutrophil index resulting in highly elevated AHR compared with that in the OVA-exposed mice. Treatment with BRL in OVA and OVA+LPS model improved the aggravated AHR. The decrease of eosinophil counts and neutrophil index in OVA+BRL group and OVA+LPS+BRL group shows that BRL may treat neutrophilic asthma, not only eosinophilic asthma. In addition, the result of IL-6, IL-1 β , IL-10, IL-13 and MUC5AC levels shows that BRL can be the potent therapeutics of murine model of ovalbumin-induced allergic asthma and chronic asthma model which is induced by co-exposure of OVA and LPS.

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