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Inhibition of phosphodiesterase suppresses allergic lung inflammation by regulating MCP-1 in an OVA-induced asthma murine model with co-exposure to lipopolysaccharide

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Abstract

Objective: Asthma is a chronic inflammatory disease that can lead to severe problems with the respiratory system. This study aimed to evaluate the suppression of allergic airway inflammation of an asthma murine model by 3-isobutyl-1-methylxanthine (IBMX), which is a non-specific cyclic nucleotide phosphodiesterase (PDE) inhibitor.

Methods: Allergic lung inflammation was evoked by ovalbumin (OVA) alone or co-exposure with lipopolysaccharide (LPS) in a murine asthma model.

Results: Compared with the OVA alone-treated control, co-exposure with LPS significantly enhanced allergic inflammatory responses in airway hyperresponsiveness and pathologic changes of the asthmatic mouse lung. Specifically, LPS enhanced allergic asthma through the increase of monocyte chemoattractant protein (MCP)-1 release. However, IBMX significantly suppressed specific airway resistance and tidal volume, the infiltration of eosinophils and mast cells into the lung, the levels of serum immunoglobulin (Ig)E and IgG1, and the release of Th2 cytokines (interleukin (IL)-4, IL-13, and MCP-1). IBMX attenuated the aggravation of inflammation by

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inhibiting MCP-1, and inhibited the infiltration of eosinophils and the production of Th2 cell-associated inflammatory mediators.
Conclusion: These results demonstrate the therapeutic potential of targeting the regulation of PDE activity in an aggravated chronic allergic asthmatic response after bacterial infection.

Keywords
Asthma, IBMX, inflammation, LPS, ovalbumin, phosphodiesterase

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Introduction

Asthma is a common chronic disease worldwide, with over 300 million sufferers.[1] It is a complex long-term inflammatory disease of the respiratory system caused by environmental factors and with genetic susceptibility.[2] Specific environmental exposures including tobacco smoke, infections, air pollutants, and drugs[3,4] can change the lung condition through allergic inflammation, resulting in severe asthma. Impairment caused by asthma can have a considerable impact on a patient's quality of life.[5]

Many mediators associated with inflammation in the bronchus and lungs are involved in the pathophysiology of asthma,[6] such as eosinophils, interleukin (IL)-4, IL-5, IL-13, IL-17A, eotaxin, the Regulated on Activation, Normal T Cell Expressed and Secreted chemokine, and monocyte chemoattractant proteins (MCPs).[7–10] These cytokines stimulate B lymphocytes, resulting in an increase of immunoglobulin E (IgE) [11,12] which sensitizes mast cells to secrete inflammatory mediators.[13] Eosinophils are also activated under this condition, causing severe inflammation. Histamines from mast cells and cytokines from eosinophils cause a hyperactive response that includes smooth muscle contraction and mucus overproduction.[14] This can lead to airway remodeling with chronic epithelial damage and goblet cell metaplasia.

Lipopolysaccharides (LPS), also known as endotoxins, derive from Gram-negative bacteria and provoke broad and non-specific inflammation. The first step of this pathway starts with IL-1 and tumor necrosis factor (TNF)-α, which contribute to endotoxic shock[15] and strong inflammation,[16] and can exacerbate asthma.[17]

3-Isobutyl-1-methylxanthine (IBMX), a methylated xanthine derivative, acts as a non-competitive selective phosphodiesterase (PDE) inhibitor[18] that elevates intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate. IBMX also inhibits TNF-α and leukotriene synthesis, reducing inflammation and innate immunity including T cell activation and proliferation and eosinophil activation.[19–21]

Vinpocetine is a PDE1-selective inhibitor examined in studies of cerebrovascular diseases and anti-inflammatory agents.[22] PDE4-selective inhibitors, including roflumilast and ibudilast, have notable anti-inflammatory effects[23] such as in the infiltration of immune cells in lung injury.[24,25] PDE1, PDE2, PDE3, PDE4, and PDE7 are distributed in the lung,[26] while PDE7 was identified as a target of treatment to alleviate chronic inflammation and airway inflammation.[27,28]

The present study aimed to determine the mechanism of PDE inhibition in

ovalbumin (OVA)-induced allergic lung inflammation, a well-established murine asthma model, using co-exposure to LPS as a potential asthma trigger and IBMX as a non-specific PDE inhibitor.

Materials and methods

Materials

OVA, grade V, from hen egg white (lyophilized powder, ≥98%), and LPS (from *Escherichia coli* O55: B5, purified by gel filtration chromatography) were purchased from Sigma-Aldrich (St Louis, MO, USA). Aluminum hydroxide (Imject® Alum) gel was purchased from Thermo Scientific (Rockford, IL, USA). IBMX was from Tocris Bioscience (Bristol, UK). All chemicals used in this study were of analytical grade.

Animals

Sixty specific pathogen-free 5-week-old male BALB/c mice were purchased from Samtako (Gyeonggi-do, Republic of Korea). They were kept for 7 days under standard laboratory conditions with the purpose of adaptation at $24 \pm 2°C$ with $50 \pm 5\%$ humidity. A light/dark cycle was synchronized to 12:12 hours. Pathogen-free food and water were available *ad libitum*, and sterilized bedding was used for housing. The mice were randomly divided into six groups (n=10 per group): (1) vehicle control, (2) IBMX, (3) OVA, (4) LPS, (5) OVA+LPS, and (6) OVA+LPS+IBMX. The weight of the mice ranged from 18 to 20 g, and there were no significant differences between groups prior to the start of the study. The animal studies were performed in accordance with the Institutional Animal Care and Use Committee of Chung-Ang University and using relevant ethical guidelines and regulations established by the Korean Association for Laboratory Animals (IACUC-2018-00124).

Experimental protocol

Based on a pilot study and previous research that determined the administration time and dose of OVA and LPS,[29,30] 100 μg OVA, 15 μg LPS, and 1 mg Alum were mixed with 200 μL normal saline. Next, 100 μL of the mixture was injected into the peritoneal cavity of all mice and, simultaneously, 100 μL was injected into the subcutaneous cavity. Sensitization was conducted on days 0, 7, and 14. From days 21 to 31, OVA, LPS, OVA+LPS, and OVA+LPS+IBMX groups were challenged with an aerosol for 45 minutes every other day. The aerosols differed among groups: normal saline for the vehicle control and IBMX groups, 40 mg/mL OVA in normal saline for the OVA group, 20 μg/mL LPS in normal saline for the LPS group, and 20 μg (LPS) + 40 mg (OVA) /mL in normal saline for the OVA+LPS and OVA+LPS+IBMX groups. The aerosol was generated by a nebulizer (Aerogen Pro nebulizer; Aerogen®, Galway, Ireland). IBMX (10 mg/kg) was administered by intraperitoneal injection (i.p.) 1 hour before every antigen challenge. After six challenges, mice were sacrificed following a methacholine challenge on day 32 (Figure 1).

Methacholine test

A methacholine test was carried out on day 32 to discern differences between the groups regarding tidal volume (TV) and specific airway resistance (sRaw). First, 4 mg/mL methacholine was nebulized to conscious mice for 1 minute in a total volume of 0.5 mL. TV and sRaw were then measured for 3 minutes by barometric double-chambered plethysmography (Buxco® FinePointe Non-Invasive Airway Mechanics, DSI™, MN, USA). All

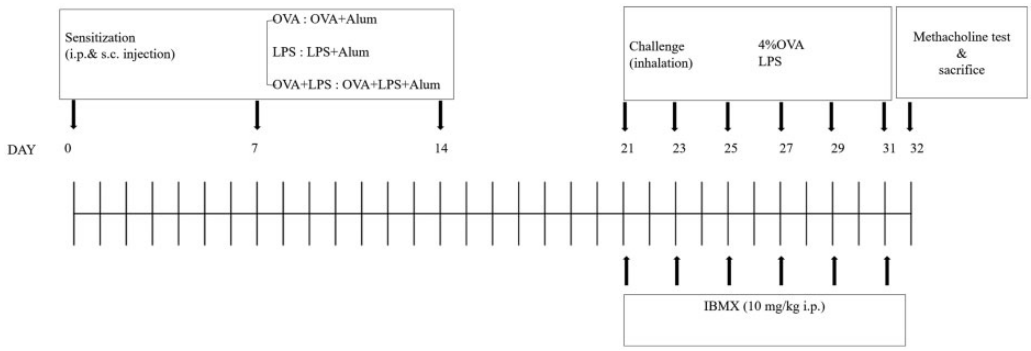

Figure 1. Experimental protocol of the murine asthma model.

measurements were made using non-invasive airway mechanics systems (Buxco® FinePointe Non-Invasive Airway Mechanics, DSI™, MN, USA). sRaw, expressed as mmHg × seconds, was used as the main index of airway hyperresponsiveness. Each value was calculated as the rate of change before and after TV, and was expressed as a percentage.

Bronchoalveolar lavage fluid (BALF) collection and cell count

After the methacholine test, mice were anesthetized with Zoletil 50 (Tiletamine 125 mg / Zolazepam 125 mg) via a 20 mg/kg i.p. injection. After flushing twice with 0.5 mL ice-cold phosphate-buffered saline (PBS), samples were collected into a single volume in a microtube and centrifuged for 10 minutes at 1500 × *g* at 4°C. Supernatants were stored at –78°C for cytokine and chemokine analysis. The BALF pellet was reconstituted for 10 minutes in 100 μL eBioscience™ 1× red blood cell (RBC) lysis buffer (Invitrogen by Thermo Fisher Scientific, Rockford, IL, USA) and centrifuged as before. After removal of the RBC lysis buffer, the pellet was suspended in 100 μL PBS and again centrifuged as before. Each pellet underwent cytocentrifuge preparation in a Shandon Cytospin 2 (Thermo Fisher Scientific) and staining on glass slides using the Kwik-Diff™ Stain Kit (Thermo Fisher Scientific). Inflammatory cells from BALF were counted on a hemocytometer, and a differential cell count was performed on the basis of standard morphological criteria.

Histological analysis

For histological analysis, the left lungs were obtained from mice and fixed with 10% formalin solution. Fixed tissues were embedded with paraffin and cut with a Leica microtome 820 at 4 μm. Hematoxylin–eosin (H&E) staining was performed on sectioned tissues. Inflammation was measured using a previously described scoring system[31] as 0, no inflammation; 1, occasional cuffing with inflammatory cells; 2, most bronchi or vessels surrounded by a thin layer (1–5 cells) of inflammatory cells; and 3, most bronchi or vessels surrounded by a thick layer (>5 cells) of inflammatory cells.

Periodic Acid Schiff (PAS) staining was performed using a PAS stain Kit (Abcam, Cambridge, MA, USA). The mucus score was evaluated using the previously described index[32] as: 0, no mucus; 1, <25% of areas stained by magenta in the epithelium; 2, 25% to 50% of areas stained by magenta in the epithelium; 3, 50% to 75% of areas stained by magenta in the epithelium; and 4, >75% of areas stained by magenta in the

epithelium. The inflammation scores were evaluated by four independent investigators in a blind analysis. The stained area was evaluated using the ImageJ software program. Four tissue sections per mouse were scored, and the inflammation score was expressed as a mean value.

The degree of eosinophil infiltration was measured by counting the number of eosinophils per area (20,000 μm^2) of lung in Congo red-stained sections, based on red granule staining using a Congo red stain kit (Abcam). Toluidine blue staining was performed for mast cell detection in lung tissues using the NovaUltra toluidine blue stain kit (IHC WORLD, Ellicott City, MD, USA). The number of mast cells in lung tissues was determined by counting violet-stained cells per 20,000 μm^2. Six random fields were counted per section. Stained tissues were observed on a Leica DMR 6000 microscope, and images were taken with a Leica DM 480 camera (Leica, Wetzlar, Germany).

Quantitation of OVA-specific IgE and IgG1 antibodies in serum

Blood was obtained from the inferior vena cava at sacrifice, and 40 μL 3.2% sodium citrate (Sigma-Aldrich) was used for the anticoagulant. Obtained blood samples were centrifuged at $1000 \times g$ at 4°C for 10 minutes. Separated sera were stored at –78°C. A mouse OVA-IgE enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI, USA) and a mouse OVA-IgG1 ELISA kit (Cayman Chemical) were used to determine detect OVA-specific IgE and IgG1 antibodies, respectively, in serum. Absorbance was measured using a FlexStation3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm according to the manufacturer's protocol.

Quantitative analysis of cytokines and chemokines in BALF and serum

Inflammatory cytokines and chemokines were measured by ELISA. IL-4, IL-5, IL-9, IL-13, and MCP-1 were detected using a colorimetric sandwich ELISA kit (Quantikine® ELISA; R&D SYSTEMS, Minneapolis, MN, USA) in BALF. MCP-1 in serum was also detected by ELISA (Quantikine® ELISA; R&D SYSTEMS).

Quantitation of MCP-1 mRNA expression

MCP-1 mRNA expression was measured by quantitative reverse transcription PCR (RT-qPCR). Total RNA extraction was performed from the right lungs of mice using TRIzol® Reagent (Ambion® by Life Technologies, Carlsbad, CA, USA). cDNA was synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). qPCR was performed using the iQ™ SYBR® Green Supermix with primers MCP-1 FW: 5′-GCATCCACGTGTTGG CTCA-3′; RV: 5′-CTCCAGCCTACTCA TTGGGATCA-3′; GAPDH FW: 5′-ACC CAGAAGACTGTGGATGG-3′; and RV: 5′-GGATGCAGGGATGATGTTCT-3′.

Statistical analysis

All values are presented as means ± standard deviation (SD) of the mean (n=10). Quantitative measured values were based on a one-way analysis of variance followed by the Student's t-test. p values were statistically significant at $p<0.05$ and $p<0.01$.

Results

OVA with LPS induced a decrease of TV and an increase of airway resistance which was ameliorated by IBMX

Compared with the control group, OVA, LPS, and OVA+LPS groups each showed a decreased TV, with a significant decrease observed in the OVA group ($p<0.05$).

There was a slightly greater decrease following co-exposure of OVA+LPS than OVA alone, but neither value was statistically significant. Because there was no difference in the TV between IBMX and control groups, IBMX did not cause any allergic reaction. The decrease of TV in the OVA+LPS+IBMX group was almost the same as in the control group. IBMX significantly recovered the TV in an allergic condition triggered by OVA+LPS ($p<0.01$; Figure 2a).

The rate of change before and after sRaw was expressed as a percentage.

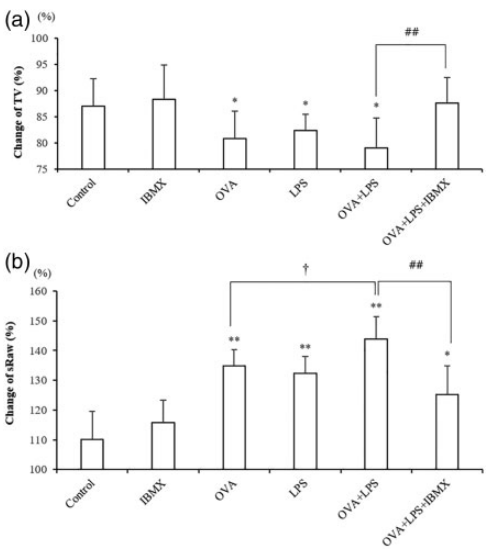

Figure 2. IBMX alleviated the change of TV and airway resistance induced by OVA and LPS. (a) TV was measured as an index of airway responsiveness using a barometric double-chambered plethysmograph (n=10 per group). TV was measured for 3 minutes after methacholine nebulization, then IBMX (10 mg/kg) was intraperitoneally administered before the methacholine test. (b) sRaw was measured as the main index evaluating airway responsiveness by a barometric double-chambered plethysmograph (n=10 per group). IBMX (10 mg/kg, i.p.) was administered 1 hour before the antigen challenge. All values are presented as means ±S.D. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the control. $^{\dagger}p<0.05$ between the OVA and OVA+LPS groups. $^{\#\#}p<0.01$ between the OVA+LPS and OVA+LPS+IBMX groups.

Compared with the control group, OVA, LPS, and OVA+LPS groups displayed remarkable increases in the change of sRaw. OVA and LPS groups each showed a 3-fold higher increase in sRaw compared with the control group (OVA; from 10.1 ± 9.3 to 34.9 ± 5.41, $p<0.01$, LPS; from 10.1 ± 9.3 to 32.2 ± 5.7, $p<0.01$). Co-exposure of OVA and LPS resulted in a higher increase in sRaw compared with OVA alone (from 34.9 ± 5.41 to 44.0 ± 7.5, $p<0.05$). IBMX significantly reduced the increase of sRaw from 44.0 ± 7.5 to 25.1 ± 9.7 in the OVA+LPS+IBMX group ($p<0.01$; Figure 2b).

Recruitment of immune cells following co-exposure of OVA and LPS ameliorated by IBMX

In OVA-induced mice, the total number of immune cells from BALF was five times higher than that of the control group, and almost six times higher in the OVA+LPS group. The LPS group showed a slight increase in total immune cells, but a large proportion of these cells were monocytes (data not shown). In the co-exposure group, the administration of IBMX significantly lowered the total number of immune cells ($p<0.01$; Figure 3a). The number of eosinophils in the OVA group was significantly increased compared with those in the control group and OVA+LPS group ($p<0.01$; Figure 3b). IBMX alone did not influence the number of eosinophils, but dramatically reduced eosinophil recruitment under co-exposure conditions. Similar findings were observed after staining immune cells in BALF (Figure 3c).

IBMX improved histological changes induced by OVA with LPS exposure

H&E staining was performed to investigate histological changes of the lung. OVA-induced mice showed thickened bronchial

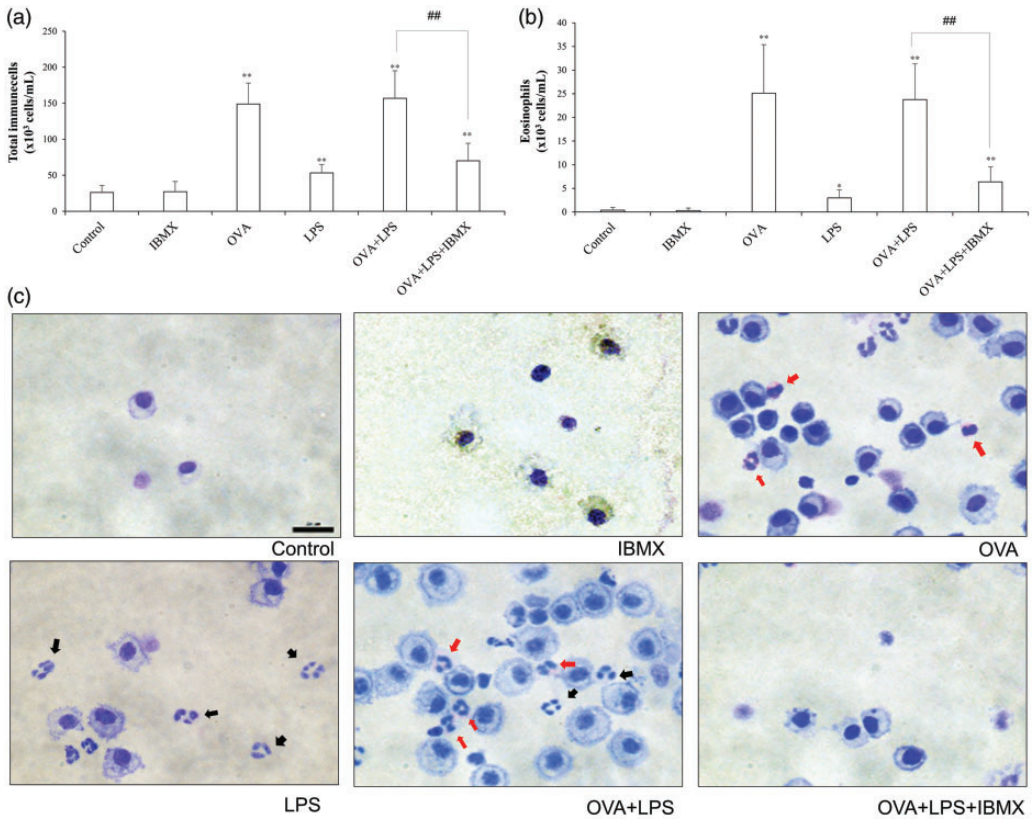

Figure 3. IBMX inhibited the increase of total immune cells and eosinophils in BALF.
The numbers of total immune cells (a) and eosinophils (b) in BALF obtained from immunized mice challenged with OVA and LPS were counted based on differential cell count protocols. Total immune cells stained with the KWIK-DIFF™ stain kit are shown in (c) (magnification ×630, scale bar 20 μm). Red arrow indicates eosinophils and black arrow indicates neutrophils. All values are presented as mean±S.D. $^{*}p<0.05$, $^{**}p<0.01$ compared with the control. $^{\#\#}p<0.01$ between the OVA+LPS and OVA+LPS+IBMX groups.

walls, an altered alveolar structure, and inflammatory cell infiltration in the peribronchial and perivascular areas compared with controls. These changes were aggravated in the co-exposure group. IBMX treatment decreased the thickness of the bronchial walls and the infiltration of inflammatory cells, and significantly ameliorated changes in the alveolar area ($p<0.001$; Figures 4a and 4d). Stained mucus and goblet cell hyperplasia were more often found in the OVA group than the control group, and the co-exposure of OVA and LPS increased the secretion of mucin and hyperplasia of goblet cells. IBMX treatment significantly inhibited mucin secretion and goblet cell hyperplasia ($p<0.001$; Figures 4b and 4e). Congo red staining showed that OVA exposure induced an increase in the number of eosinophils, and this was increased in the lungs following co-exposure to OVA and LPS. IBMX significantly reduced the infiltration of eosinophils ($p<0.01$; Figure 4c and 4f). The infiltration of mast cells into the lungs was also induced by OVA exposure, but not

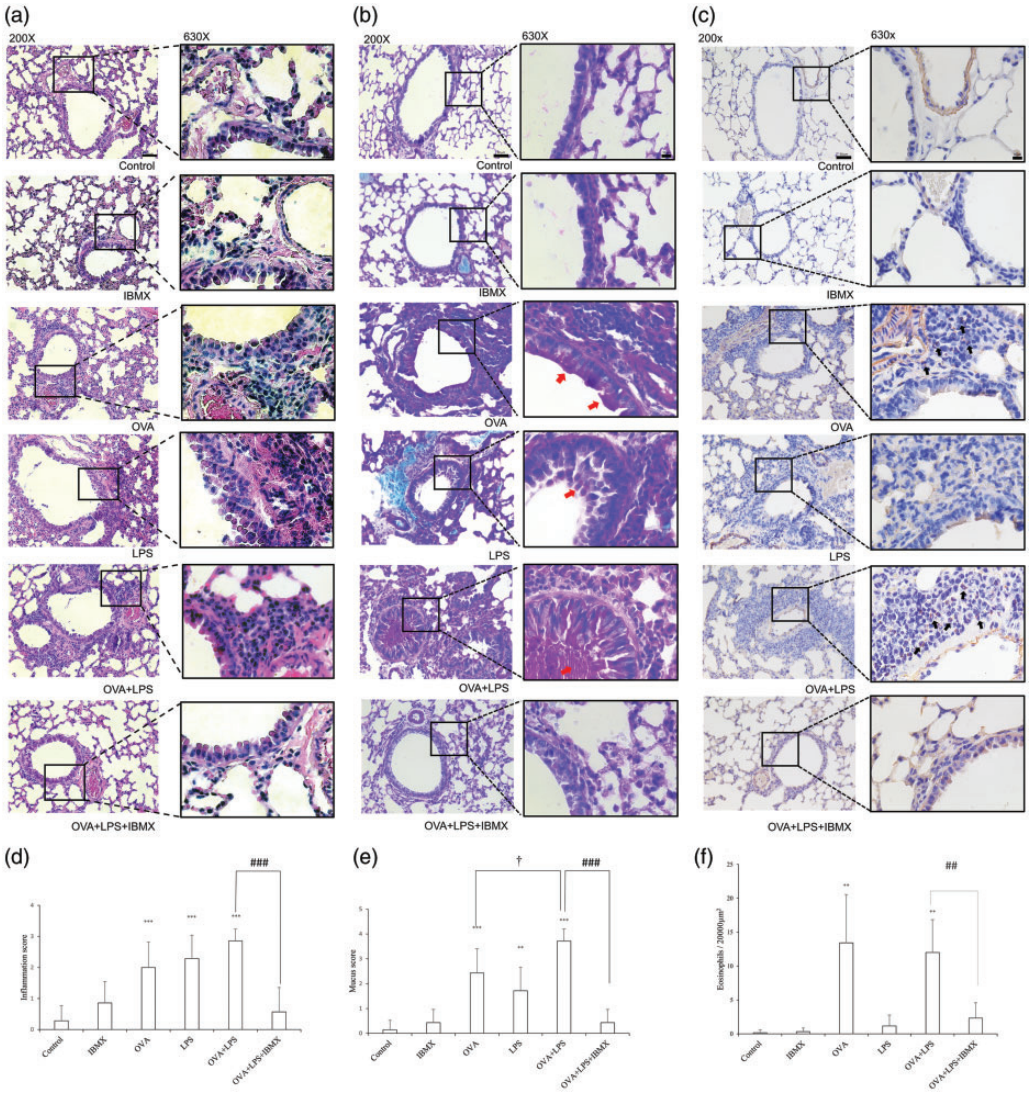

Figure 4. Histological changes were ameliorated by IBMX in lung tissue.
(a) Histological changes of bronchial and perivascular areas were evaluated by H&E staining in lung tissue (left: magnification ×200, scale bar 50 μm; right: magnification ×630, scale bar 10 μm). (b) PAS staining showing mucin (red arrows). Left: magnification ×200, scale bar 50 μm; right: magnification ×630, scale bar 10 μm). (c) Congo red staining measured the infiltration of eosinophils to the lungs. Stained eosinophils are indicated by black arrows (left: magnification ×200, scale bar 50 μm; right: magnification ×630, scale bar 10 μm). (d) Inflammation scores were evaluated in lung sections. (e) Mucus scores were evaluated in lung sections. (f) Eosinophils were counted in six random fields of each stained lung tissue. All values are presented as mean±S.D. $^{**}p<0.01$ and $^{***}p<0.001$ compared with the control. $^{\#\#}p<0.01$ and $^{\#\#\#\#}p<0.001$ between the OVA+LPS and OVA+LPS+IBMX groups. $^{\dagger}p<0.05$ between the OVA and OVA+LPS groups.

by LPS. IBMX significantly inhibited the infiltration of mast cells compared with the OVA+LPS group (Appendix A and B).

Effects of IBMX on the increase of OVA-specific IgE and IgG1 by OVA and LPS in serum

The concentration of IgE in the serum was significantly elevated in OVA-induced asthma mice compared with the control group ($p<0.05$; Figure 5a). There was no significant enhancement in the co-exposure group. However, in co-exposure conditions, the administration of IBMX notably reduced the level of OVA-specific IgE. OVA+LPS groups also showed significantly increased levels of IgG1 in the serum compared with controls, and this was significantly reduced by IBMX ($p<0.01$; Figure 5b).

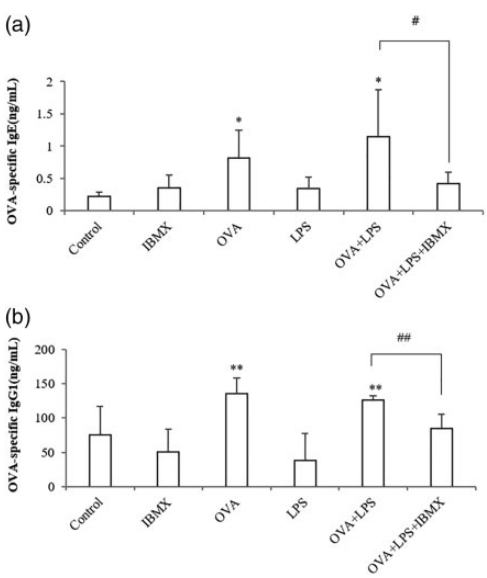

Figure 5. Production of OVA-specific IgE and IgG1 was decreased by IBMX in serum.
Serum OVA-specific IgE (a) and IgG1 (b) were measured using ELISA kits. IBMX (10 mg/kg, i.p.) was administered 1 hour before antigen challenge. All values are presented as mean±S.D. *$p<0.05$ and **$p<0.01$ compared with the control. #$p<0.05$ and ##$p<0.01$ between the OVA+LPS and OVA+LPS+IBMX groups.

IBMX inhibited OVA and LPS-induced release of Th2-mediated cytokines in BALF

OVA-induced mice showed a noticeable rise of Th2-mediated cytokines in BALF, with

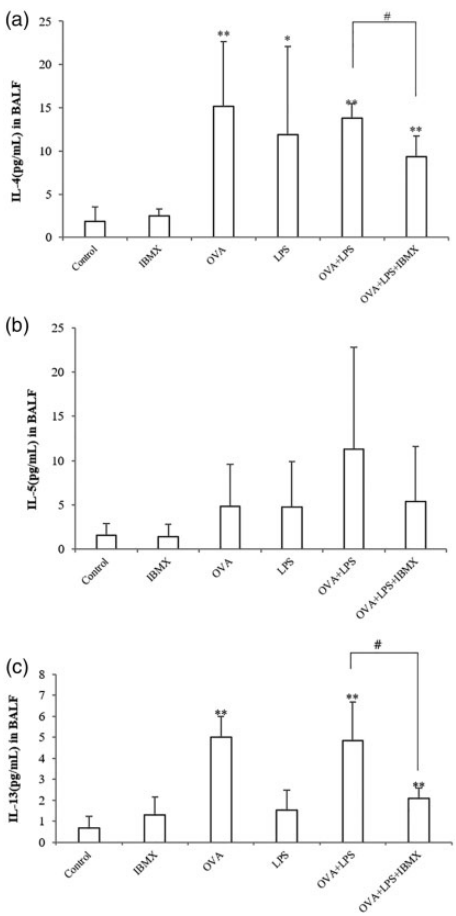

Figure 6. The increase of Th2-mediated cytokines was downregulated by IBMX in BALF.
The levels of IL-4 (a), IL-5 (b), and IL-13 (c) in BALF obtained from immunized mice challenged with OVA and LPS were determined using ELISA kits. IBMX (10 mg/kg, i.p.) was administered 1 hour before antigen challenge. All values are presented as mean±S.D. *$p<0.05$ and **$p<0.05$ compared with the control. #$p<0.05$ between the OVA+LPS and OVA+LPS+IBMX groups.

observed increases in IL-4 and IL-13 in both OVA and OVA+LPS groups ($p<0.05$; Figures 6a, c). The level of IL-5 showed a slight (non-significant) elevation in the co-exposure group (Figure 6b). The administration of IBMX significantly reduced the IL-4 level ($p<0.05$). Simultaneously, the concentration of IL-13 decreased with IBMX administration. IL-5 showed a similar tendency but it was not significant.

IBMX inhibited the OVA and LPS-induced release of chemokine MCP-1 in BALF and serum

MCP-1 levels increased in OVA-induced asthma. The OVA group showed a 3-fold increase in BALF MCP-1 compared with the control group, while the co-exposure of OVA+LPS resulted in a 12-fold increase. The administration of IBMX significantly lowered the MCP-1 concentration ($p<0.05$; Figure 7a). Serum MCP-1 levels also increased in OVA, LPS, and OVA+LPS groups, and IBMX again significantly reduced MCP-1 levels compared with the OVA+LPS group ($p<0.01$; Figure 7b). RT-qPCR confirmed that OVA and LPS induced the expression of MCP-1 in lung tissue. Co-exposure of OVA and LPS also increased the expression of MCP-1, and this was significantly inhibited by IBMX treatment ($p<0.01$; Figure 7c).

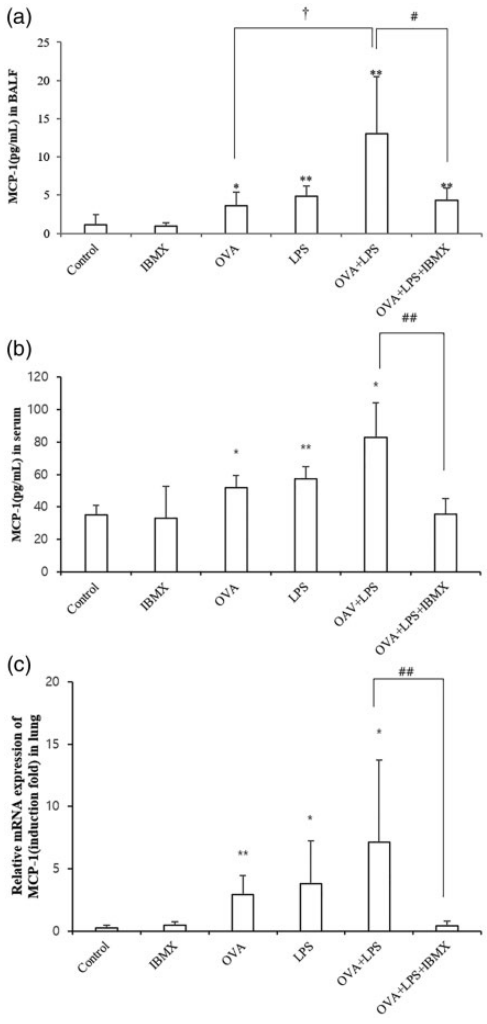

Figure 7. IBMX blocked the MCP-1 expression induced by OVA and LPS.
The levels of MCP-1 release were measured in BALF and serum, and expression levels in the lungs were measured in lung tissues. (a) The MCP-1 level in BALF obtained from immunized mice challenged with OVA and LPS was determined using ELISA kits. (b) The MCP-1 level in serum was also measured by an ELISA kit. (c) MCP-1 mRNA expression in lung tissue was measured using RT-qPCR. IBMX (10 mg/kg, i.p.) was administered 1 hour before antigen challenge. $^{*}p<0.05$, $^{**}p<0.01$ compared with the control. $^{\dagger}p<0.05$ between the OVA and OVA+LPS groups. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ between the OVA+LPS and OVA+LPS+IBMX groups.

Discussion

This study sought to determine the relationship between allergic asthma and infection by Gram-negative bacteria with LPS in their outer membranes. The methacholine test is a simple method of determining airway hyperresponsiveness by inducing contraction in the bronchus and increasing airway resistance with lowered TV.[33] Using this method, we observed significant changes to TV and sRaw in the treatment

groups compared with the control group, resulting from OVA-induced inflammation in the lungs. Co-exposure of LPS and OVA aggravated sRaw but had less of an effect on TV. These findings demonstrate that LPS caused an additional inflammatory condition with OVA in the bronchus and lungs.

A high eosinophil infiltration into the lung, which is a typical feature in asthmatic patients, can cause a severe asthmatic condition.[34] In our study, OVA induced an intense asthmatic condition compared with the control group by increasing both the number of eosinophils and the total number of immune cells. The LPS group also showed a significant increase in these cells, although many were monocytes. LPS did not directly induce an increase of eosinophils compared with the OVA-only group, suggesting that it uses another pathway to trigger additional inflammation.

Histological analysis revealed that OVA and LPS caused changes to the structure of the epithelium and inflammatory cell infiltration in the lungs. Eosinophils were induced to infiltrate into the lungs by OVA exposure, but this was not further increased by LPS. Similarly, the infiltration of mast cells into the lungs was induced by OVA exposure, and not by LPS. Increased mucus secretion is a crucial factor in asthma, and the inhibition of this is important to improve prognosis.[35] PAS staining showed that OVA induced mucus secretion, and that this was increased by LPS and OVA, suggesting that LPS exacerbated lung injury and symptoms in asthma.

Serum IgE and IgG1 levels both increased in the treatment groups compared with the control group. The co-exposure group also showed an increase in IgE levels, but the LPS group did not show a more synergic effect compared with the OVA group. Th2 cells play a key role in asthma because the cytokines and chemokines that they release are associated with airway allergic inflammation.[36] As OVA induced Th2 cell activation, OVA and co-exposure groups showed a remarkable increase of IL-4 and IL-13 in BALF. The level of IL-5 also rose in the co-exposure group, but the values were not significant. LPS only increased IL-4 levels, indicating that it did not directly affect Th2 cells.

MCP-1 is released by many different immune cells including monocytes, macrophages, eosinophils, and mast cells.[37] It plays a critical role in regulating allergic airway inflammation and hyperresponsiveness.[38–40] OVA and LPS elevated the MCP-1 concentration in BALF and serum, respectively, and increased MCP-1 mRNA expression in the lung. Furthermore, OVA and LPS co-exposure led to a striking rise in MCP-1 in BALF compared with the OVA group, while OVA and LPS co-exposure significantly increased the airway resistance compared with OVA-only exposure. LPS increased the number of inflammatory cells, such as neutrophils and monocytes, except eosinophils. These results indicate that MCP-1 induced the increase of inflammatory cells, but not eosinophils, which exacerbated airway hyperresponsiveness and mucus secretion.

IBMX signaling tightly regulated the allergic response pathway induced by OVA and LPS. The inhibition of PDE previously increased cAMP levels in the airway smooth muscle, leading to a reduction in sRaw and TV.[41] In our study, IBMX inhibited the release of IL-4, IL-5, and IL-13 in BALF and also reduced the recruitment of inflammatory cells and eosinophils in BALF. Additionally, it reduced mucus secretion induced by the co-exposure of OVA and LPS. Furthermore, IBMX reduced MCP-1 in BALF, serum, and lung tissue, and controlled the production of OVA-specific IgE and IgG1 in serum. These results suggest that PDE inhibition suppressed the allergic lung inflammation

induced by OVA and LPS through the inhibition of MCP-1.

In conclusion, LPS had a synergic effect with OVA to increase the release of MCP-1 in allergic lung inflammation. The inhibition of PDE attenuated the aggravation of inflammation by inhibiting the release of MCP-1, and decreasing the infiltration of eosinophils and the production of Th2 cytokines. These results demonstrate the therapeutic potential of targeting the regulation of PDE activity by inhibiting MCP-1 expression, and provide novel insights into the pathological mechanisms that occur in an aggravated chronic allergic asthmatic response after bacterial infection.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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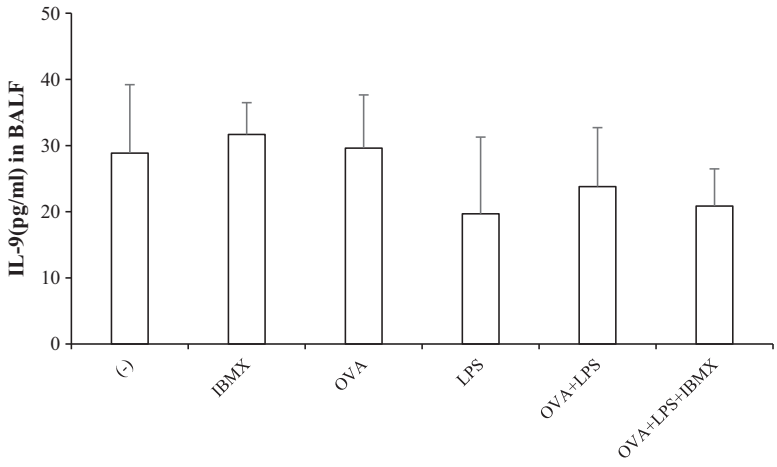
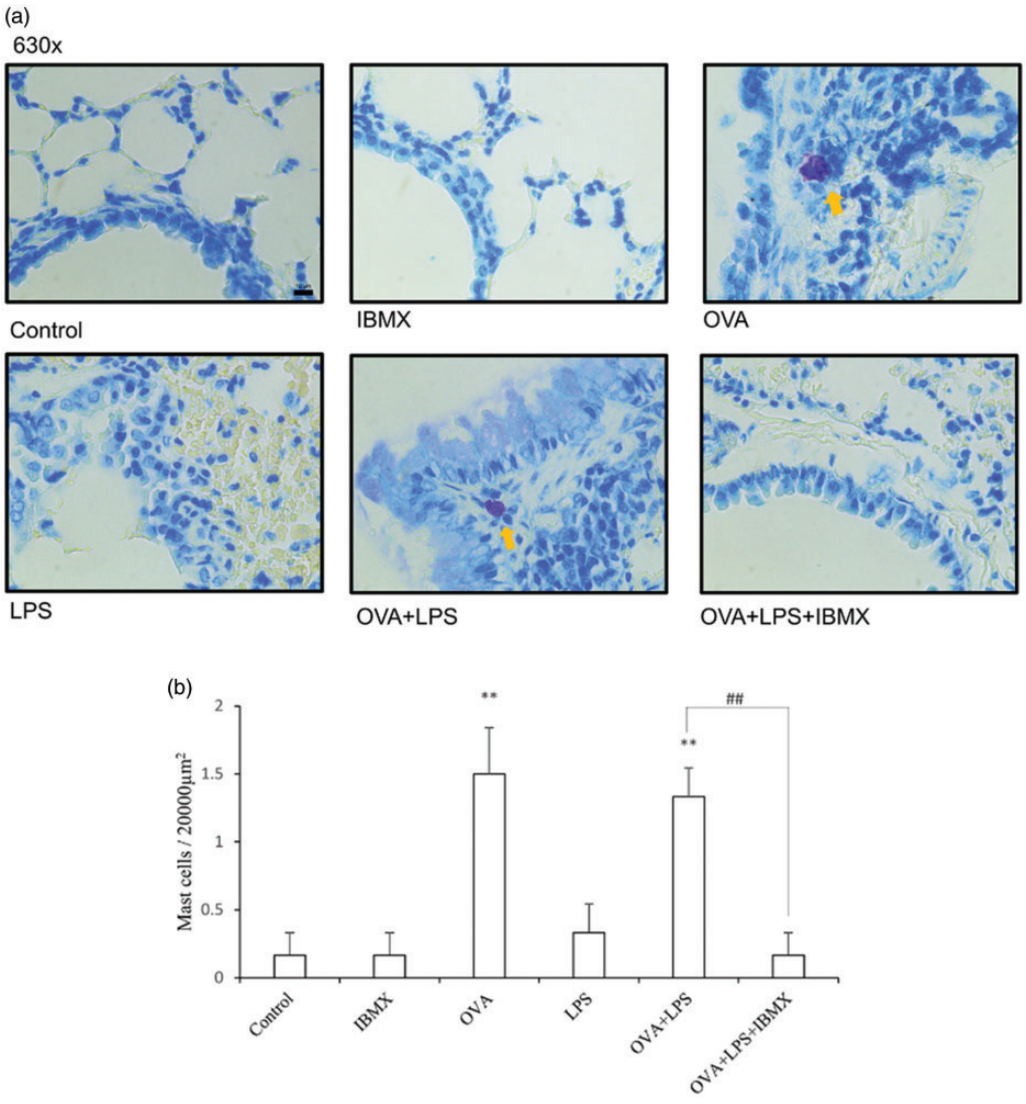
Appendix A. OVA and LPS did not induce IL-9 levels in BALF



The levels of IL-9 in BALF obtained from immunized mice challenged with OVA and LPS were determined using ELISA kits.[42–44] IBMX (10 mg/kg, i.p.) was administered 1 hour before antigen challenge. All values are presented as mean $\pm$ S.D.

Appendix B. The infiltration of mast cells into the lungs was ameliorated by IBMX in lung tissue



(A) Toluidine blue staining was performed to measure the infiltration of mast cells into the lungs. Mast cells stained violet are indicated by yellow arrows (magnification ×630, scale bar 10 μm). (B) Mast cells were counted in six random fields of each stained lung tissue. All values are presented as mean ± S.D. $^{**}p<0.01$ compared with the control. $^{\#\#}p<0.01$ between the OVA+LPS and OVA+LPS+IBMX groups.