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4-CMTB Ameliorates Ovalbumin-Induced Allergic Asthma through FFA2 Activation in Mice

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Abstract

Free fatty acid receptor 2 (FFA2, also known as GPR43), a G-protein-coupled receptor, has been known to recognize short-chain fatty acids and regulate inflammatory responses. FFA2 gene deficiency exacerbated disease states in several models of inflammatory conditions including asthma. However, *in vivo* efficacy of FFA2 agonists has not been tested in allergic asthma. Thus, we investigated effect of 4-chloro-α-(1-methylethyl)-*N*-2-thiazoylylbenzeneacetanilide (4-CMTB), a FFA2 agonist, on antigen-induced degranulation in RBL-2H3 cells and ovalbumin-induced allergic asthma in BALB/c mice. Treatment of 4-CMTB inhibited the antigen-induced degranulation concentration-dependently. Administration of 4-CMTB decreased the immune cell numbers in the bronchoalveolar lavage fluid and suppressed the expression of inflammatory Th2 cytokines (IL-4, IL-5, and IL-13) in the lung tissues. Histological studies revealed that 4-CMTB suppressed mucin production and inflammation in the lungs. Thus, results proved that FFA2 functions to suppress allergic asthma, suggesting 4-CMTB activation of FFA2 as a therapeutic tool for allergic asthma.

Key Words: FFA2, Free fatty acid receptor 2, Short-chain fatty acids, Anti-allergic, Anti-asthmatic, Degranulation

INTRODUCTION

Asthma is a chronic inflammatory airways disease, affecting several million people worldwide (Huang *et al*., 2018). Couphing, wheezing, chest tightness, variable airway obstruction, and airway hyper-responsiveness are main symptoms (Wardlaw *et al*., 2002). The airways of asthma patients often show infiltration of eosinophils, mucus overproduction, bronchial mucosal thickening, and bronchial wall remodeling (Pascual and Peters, 2005). Corticosteroids, long-acting β_2-adrenoceptor agonists or leukotriene D_4 antagonists are currently used to control the symptoms (Colucci *et al*., 2007). However, new anti-asthmatic drugs with novel action mechanism in addition to safety and efficacy are needed, considering steroid-induced side effects and steroid-resistant asthma patients (Barnes, 2013).

Metabolites from dietary foods by gut microbiota have drawn attention in receptor pharmacology (Macia *et al*., 2015; Tan *et al*., 2017; Melhem *et al*., 2019). Multiple G protein-coupled receptors (GPCRs) have been reported as sensors for food metabolites such as free fatty acids (Macia *et al*., 2015; Tan *et al*., 2017; Melhem *et al*., 2019). Free fatty acid receptor 1 (FFA1, also known as GPR40) recognizes medium-long chain fatty acids. Free fatty acid receptor 2 and 3 (FFA2 and FFA3, formerly known as GPR43 and GPR41, respectively) recognize short-chain fatty acids such as acetate, propionate, and butyrate (Brown *et al*., 2003; Itoh *et al*., 2003; Le Poul *et al*., 2003; Im, 2004, 2013). Free fatty acid receptor 4 (FFA4, also known as GPR120) recognizes polyunsaturated long-chain fatty acids like DHA and EPA (Hirasawa *et al*., 2005; Talukdar *et al*., 2010). Short-chain fatty acids, dietary metabolites produced by microbiota in the gut, could exhibit anti-inflammatory effects (Trompette *et al*., 2014). Gut microbiota-derived short-chain fatty acids are implicated in modulating respiratory diseases, such as asthma and chronic obstructive pulmonary disease (Young *et al*., 2016; He *et al*., 2017; Qian *et al*., 2019). Actually, levels of short-chain fatty acids were found to be significantly down-regulated in a murine model of asthma (Jin *et al*., 2018). During pregnancy, mothers with higher fecal acetic acid levels compared with other short-chain fatty acids were less likely to have offspring with atopic asthma (Qian *et al*., 2019; Lee-Sarwar *et al*., 2020). FFA2 activation by short-chain fatty acids has been shown to regulate inflammatory responses (Ulven. 2012; Miyamoto *et al*., 2016; Sun *et al*., 2017; Tan *et al*., 2017). Furthermore, FFA2 gene

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deficient mice showed exacerbation of inflammation in multiple animal models including asthma (Maslowski *et al*., 2009). In addition, FFA2 is implied and suggested as a therapeutic target of type 2 diabetes, obesity, and insulin resistance (Tiwari, 2010). However, *in vivo* efficacy of FFA2 agonists has not been investigated in allergic asthma models. Thus, effects of 4-chloro-α-(1-methylethyl)-*N*-2-thiazoylylbenzeneacetanilide (4-CMTB), a specific FFA2 agonist were investigated on antigen-induced degranulation and ovalbumin-induced allergic asthma model (Lee *et al*., 2008; Smith *et al*., 2011).

MATERIALS AND METHODS

Materials

4-CMTB was purchased from Tocris Bioscience (Bristol, UK). Ovalbumin and aluminum hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Rat RBL-2H3 basophilic leukemia cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RBL-2H3 cells were cultured at 37°C in a 5% CO_2-humidified incubator, and maintained in 10% (v/v) heat-inactivated fetal bovine serum containing high-glucose Dulbecco's modified Eagle medium (DMEM) with 2 mM glutamine, 100 U/mL penicillin, 1 mM sodium pyruvate, and 50 μg/mL streptomycin (Huang *et al*., 2018).

Animals

Female five-week-old BALB/c mice were purchased from Daehan Biolink (Seoul, Korea). They were housed in the laboratory animal facility at Kyung Hee University (Seoul, Korea) and provided *ad libitum* water and food. The Kyung Hee University Institutional Animal Care Committee reviewed and approved the protocol with respect to ethical issues and scientific care (Approval Number, KHSASP-20-081).

Assessment of degranulation

By measuring β-hexosaminidase activity in the medium, degranulation of RBL-2H3 cells was assessed. 4-CMTB dissolved in DMSO as a stock solution of 100 mM was diluted in PIPES buffer just before the experiment. We used monoclonal anti-dinitrophenyl mouse immunoglobulin E and human dinitrophenyl albumin to induce degranulation (Huang *et al*., 2018). In order to measure the amount of histamine released from RBL-2H3 cells, 20 μL of 1 M NaOH was added to 100 μL of cell culture supernatant, and then 25 μL of 1% (w/v) o-phthalaldehyde dissolved in methanol was immediately added and mixed. After incubation at room temperature for 4 min, 10 μL of 3 M HCl was added. The amount of histamine was determined by measuring the fluorescence intensity at the excitation wavelength 355 nm and emission wavelength 460 nm using a FLUOstar Omega plate reader (BMG labtech, Ortenberg, Germany).

Induction of asthma in BALB/c mice and administration of 4-CMTB

Six-week-old BALB/c mice (22 g) were randomly divided into four groups (n=5): phosphate-buffered saline (PBS)-injected control group, ovalbumin (OVA)-injected asthma group, OVA-injected and 10 mg/kg 4-CMTB-treated group, and OVA-injected and 20 mg/kg 4-CMTB-treated group. Asthma was induced by intraperitoneal injection of OVA and aluminum hydroxide on D0 and D14. Mice were challenged by exposing to nebulized OVA for D28, D29, and D30 (Aoki *et al*., 2010). 4-CMTB was dissolved in DMSO as a stock solution of 100 mM. The stock was diluted in corn oil (50 μL) just before the experiment. 4-CMTB was treated via intraperitoneal injection 30 min before OVA challenge. We collected bronchoalveolar lavage fluid (BALF) from the lungs on D32, and cell population of BALF cells was analyzed after staining (Heo and Im, 2019).

Cell counting and analysis in BALF

Using a Cellspin® centrifuge (Hanil Electric, Seoul, Korea), immune cells in BALF were adhered to a glass slide and fixed in methanol for 30 s. Staining with May-Grünwald solution were conducted in the cells on slides for 8 min and subsequently by Giemsa solution for 12 min.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed to analyze the expression levels of Th2 cells. Total RNA was isolated using TRIzol™ reagent (Invitrogen, Waltham, MA, USA), and used for cDNA synthesis. Promega GoTaq® DNA polymerase (Promega Corporation, Madison, WI, USA), primers for each gene, and the synthesized cDNA were reacted to amplify the specific genes. Specific primers for each gene and PCR conditions are described previously (Park and Im, 2019). Forward primer sequence for mouse IL-4 was 5′-CTA GTT GTC ATC CTG CTC TTC TTT-3′ and reverse primer sequence was 5′-CTT TAG GCT TTC CAG GAA GTC TTT-3′. Forward primer sequence for mouse IL-5 was 5′-AGA ATC AAA CTG TCC GTG GG-3′ and reverse primer sequence was 5′-GTC TCT CCT CGC CAC ACT TC-3′. Forward primer sequence for mouse IL-13 was 5′-CAG CAT GGT ATG GAG TGT GG-3′ and reverse primer sequence was 5′-TGG GCT ACT TCG ATT TTG GT-3′. Forward primer sequence for mouse GAPDH was 5′-TTC ACC ACC ATG GAG AAG GC-3′ and reverse primer sequence was 5′-GGC ATG GAC TGT GGT CAT GA-3′. Aliquots (7 μL) were electrophoresed on 1.2% agarose gel and stained with StaySafe™ nucleic acid gel stain (Real Biotech Corporation, Taipei, Taiwan).

Histological examination of the lungs and cell counting in BALF

Tissue sections of lungs from mice of each group were prepared. Hematoxylin and Eosin (H&E) staining and periodic acid-Schiff (PAS) staining were performed to find mucus-secreting goblet cells and eosinophil infiltration, respectively. For PAS staining, Schiff's regent was used and for H&E staining, hematoxylin and eosin regents were used (Heo and Im, 2019; Kim and Im, 2019).

Degree of lung inflammation was evaluated using a subjective scale of 0-3 by a treatment-blind observer, as followings, 0 was assigned when no inflammation was detected, 1 when occasional cuffing and inflammatory cells were observed, 2 when most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and 3 when most bronchi or vessels were surrounded by a thick layer (>five cells thick) of inflammatory cells. Mucin-secreting cells stained with PAS in the airways were counted from two lung sections per mouse. At the same time we also measured the length of the bronchi basal lamina using ImageJ software (National Institute of Health, MD, USA). Mucous production was expressed by the number of PAS-positive cells per mm of bronchiole.

Statistical analysis

Results are expressed as means ± standard errors (SEs). For statistical significance analysis of variance (ANOVA) was used, and followed by Turkey's post hoc test using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). *p* values <0.05 indicated statistical significance.

RESULTS

4-CMTB inhibited antigen-induced degranulation in the RBL-2H3 cells

Mast cells play an important role in the asthma episodes (Theoharides and Kalogeromitros, 2006). Exposure of the antigen makes cross-linking of IgEs on on the mast cell membranes, which results in degranulation. The degranulation of the mast cells releases mediators of allergic responses such as histamine, leukotrienes, and prostaglandins (Naclerio, 1999). RBL-2H3 rat basophilic leukemia cells were used to measure degranulation responses. The β-hexosaminidase activity in the medium was increased by antigen exposure in the RBL-2H3 cells (Fig. 1A). Treatment of 4-CMTB inhibited the release of β-hexosaminidase in a concentration-dependent manner (Fig. 1A). And the inhibition by 4-CMTB was significant at a concentration of 1 μM (Fig. 1A). The inhibitory effect was further confirmed by measuring amounts of histamine released from RBL-2H3 cells (Fig. 1B). Cytotoxicity was not observed up to 1 μM of 4-CMTB in the MTT assay (data not shown).

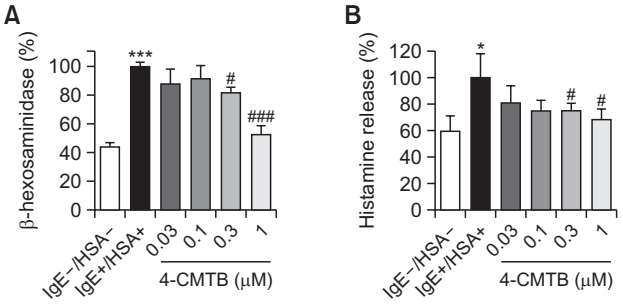

Fig. 1. 4-CMTB inhibited antigen-induced degranulation in RBL-2H3 cells. (A) RBL-2H3 cells were sensitized for 18 h with anti-DNP IgE and then challenged with DNP human serum albumin (HSA). 4-CMTB was treated at the indicated concentrations 30 min before antigen challenge. The samples without IgE and HSA mean basal degranulation from the cells, and samples with IgE and HSA mean positive control of antigen-induced degranulation. The results are presented as means ± SEs of three independent experiments. (B) Amounts of histamine released from RBL-2H3 cells were measured and shown as histograms (n=5). **p*<0.05, ****p*<0.001 vs. the HSA-untreated group. #*p*<0.05, ###*p*<0.001 vs. the HSA-treated group.

4-CMTB inhibited the increases of eosinophils and lymphocytes in the BALF

Next, an OVA-induced mouse model of asthma was applied to verify the inhibitory effect of 4-CMTB. In the animal model, dendritic cells recognize antigen in the sensitization phase and move to lymph nodes. In the lymph nodes, dendritic cells present antigen to naïve T cells. Antigen-exposed T cells differentiate into Th2 cells. Similarly, antigen-recognizing IgEs are produced by B cells. The IgEs are equipped on the surface of mast cells. In the challenge phase, mast cells interact with the antigen and release proinflammatory lipids and cytokines, such as IL-4. Then, eosinophils influx happens to BALF and lungs. Cells of the BALF increased in the OVA-induced asthma group compared to those in the PBS-treated control group (Fig. 2A), and treatment of 4-CMTB (10 mg/kg or 20 mg/kg) inhibited the OVA-induced increase the cell number of BALF (Fig. 2A). The total cell number were assessed in the BALF and the distribution of immune cell populations was calculated. The total cell number of the BALF increased to 363.1% in the OVA-induced asthma group compared to that in the PBS-treated control group (Fig. 2B). 4-CMTB (10 mg/kg or 20 mg/kg) inhibited significantly the OVA-induced increase in the total cell number by 70.6 and 58.8%, respectively (Fig. 2B). The immune cell populations in the BALF were also assessed. The eosinophil number in the BALF was increased by the OVA treatment, and it was significantly reduced by both doses of 4-CMTB by 70.6 and 58.8%, respectively (Fig. 2B). Although the lymphocyte number was lower than the eosinophil number, the OVA induced its increase and 4-CMTB treatment decreased the lymphocyte counts (Fig. 2C). The mac-

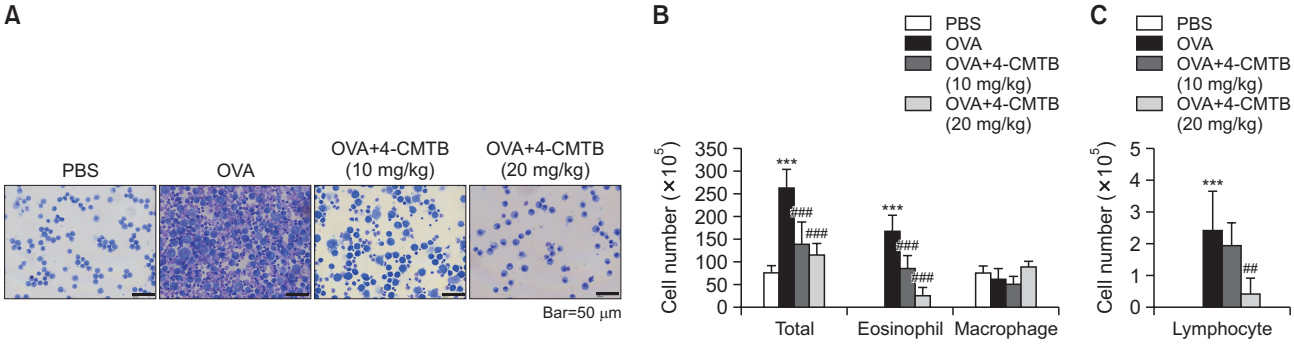

Fig. 2. 4-CMTB inhibited OVA-induced immune cell accumulation in BALF. (A) Mice were sensitized with OVA twice by i.p. injection on D0 and D14, and later challenged on D28, D29, and D30 with nebulized OVA. 4-CMTB was treated intraperitoneally at the dose of 10 mg/kg or 20 mg/kg, 30 min before OVA challenge. Cells in BALF were stained using May-Grünwald stain and counted. (B) Total cell counts, eosinophils, and macrophages in BALF. (C) Lymphocytes counts in the BALF. The results are presented the mean ± SE cell count values (n=5). ****p*<0.001 vs. the PBS-treated group, ##*p*<0.01, ###*p*<0.001 vs. the OVA-treated group.

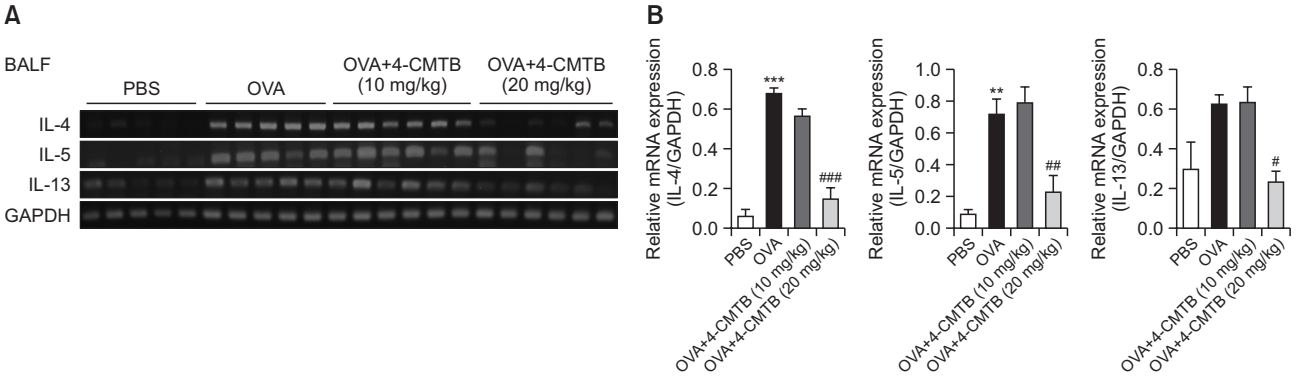

Fig. 3. 4-CMTB inhibited the mRNA expression levels of Th2 cytokine in BALF-associated cells. (A) mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) in the BALF cells. Each lane represents one of five different mice. (B) mRNA levels of cytokines were quantified as ratios to GAPDH mRNA level. Values represent the means ± SEs (n=5). ***p*<0.01, ****p*<0.001 vs. the PBS-treated group, #*p*<0.05, ##*p*<0.01, ###*p*<0.001 vs. the OVA-treated group.

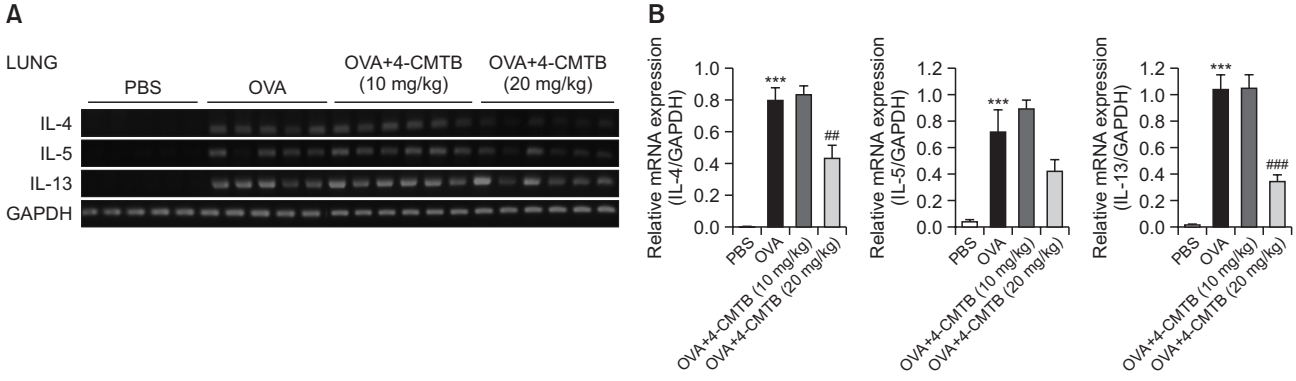

Fig. 4. 4-CMTB inhibited the mRNA expression levels of Th2 cytokine expression in the lung tissues. (A) mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) in the lung tissues. Each lane represents one of five different mice. (B) mRNA levels of cytokines were quantified as ratios to GAPDH mRNA level. Values represent the means ± SEs (n=5). ****p*<0.001 vs. the PBS-treated group, ##*p*<0.01, ###*p*<0.001 vs. the OVA-treated group.

rophage numbers were not significantly changed by OVA or 4-CMTB (Fig. 2B).

4-CMTB suppressed the expression of Th2 cytokines in the BALF and lung tissues

Th2 cytokines such as IL-4, IL-5, and IL-13 play important roles in the development of allergic asthma (Romagnani, 2002). Th2 cytokines induce eosinophils recruitment and activation, hypersecretion of mucus in epithelial cells, metaplasia of goblet cells, and the proliferation of smooth muscle cells (Boucherat *et al*., 2013). The expression levels of Th2 cytokine mRNAs in the cells of BALF and lung tissues were measured by RT-PCR (Fig. 3A). The levels of IL-4, IL-5, and IL-13 mRNAs were increased by 987.5, 713.0 and 917.1%, respectively, in the BALF of the OVA group mice, and these increases were suppressed by 4-CMTB treatment (Fig. 3B). In particular, treatment of 20 mg/kg 4-CMTB suppressed the mRNA expression of IL-4, IL-5, and IL-13 by 46.4, 62.3 and 67.4%, respectively (Fig. 3B). Moreover, as shown in Fig. 4A, the levels of IL-4, IL-5, and IL-13 mRNAs in the lung tissues from the OVA group mice were observed to be increased (Fig. 4A). Quantification of the RT-PCR results showed that OVA treatment increased the levels of IL-4, IL-5, and IL-13 by 922.2, 503.1 and 772.3%, respectively (Fig. 4B). Those increases were also suppressed by 4-CMTB treatment (Fig. 4B).

4-CMTB inhibited the mucin secretion and inflammation in the lungs

Histological examination of the lung samples was also conducted. PAS staining was conducted to show the mucins, mucous glycoproteins, produced by the goblet cells. Secreted or stored mucins are shown as dark violet as shown in Fig. 5A. The mucins are stained in cells surrounding the bronchioles in the OVA group mice. The mucin production, however, was suppressed by treatment of 4-CMTB (Fig. 5A). Further, the semi-quantitative analysis of mucin production was conducted by counting the PAS-positive cells of the bronchioles (Fig. 5B). Stained cells were rare in the PBS-treated group. In the OVA-treated group, however, approximately 100 PAS-positive cells/mm were detected, and 4-CMTB treatment significantly suppressed the number of PAS-positive cells (Fig. 5B).

In the H&E staining, the eosinophils in the lung sections are shown as small, navy-blue dots (Fig. 6A). Although very few

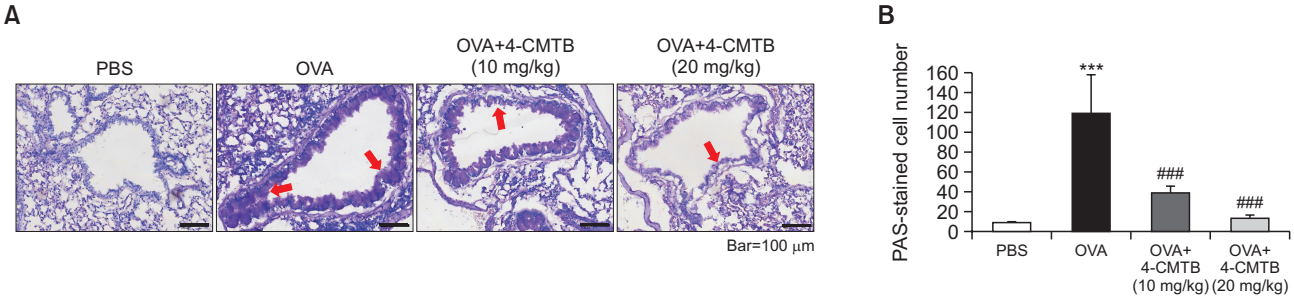

Fig. 5. 4-CMTB protected against mucin production. (A) Panels show PAS/hematoxylin-stained sections of lung tissues from the PBS group, OVA group, and 4-CMTB (10 mg/kg or 20 mg/kg)-treated OVA groups. In PAS staining, mucin is stained as purple color. In OVA group, darker and thicker purple color is observed surrounding the bronchiole compare to PBS group. (B) Mucous production was evaluated by counting the number of PAS-positive cells (red arrows) per mm of bronchiole (n=5 per group). ***p<0.001 vs. the PBS-treated group, $^{###}p$<0.001 vs. the OVA-treated group.

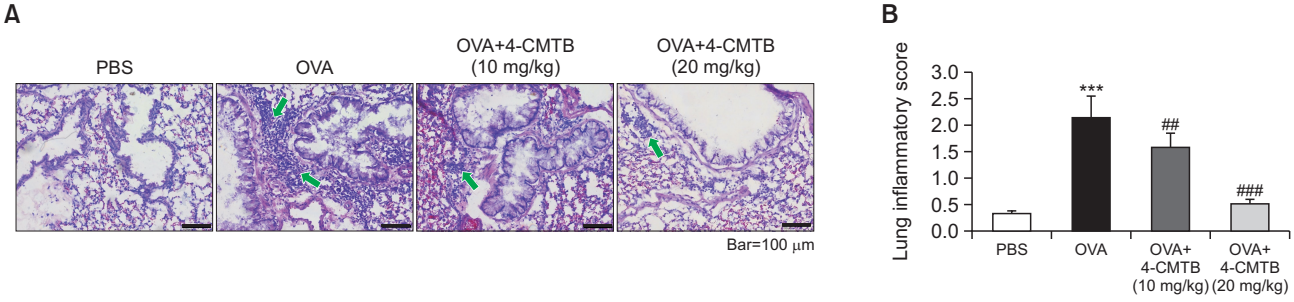

Fig. 6. 4-CMTB protected against airway inflammation. (A) Panels show H&E-stained sections of lung tissues from the PBS group, OVA group, and 4-CMTB (10 mg/kg or 20 mg/kg)-treated OVA groups. Small navy blue dots around the bronchioles are eosinophils. Eosinophils were rarely observed in the PBS group, whereas they accumulated densely around the bronchioles in the OVA group (green arrows). However, eosinophil accumulation was less obvious in the OVA+4-CMTB groups than in the OVA group. (B) Lung inflammation was semi-quantitatively evaluated; histological findings were scored as described in the Materials and methods section. Values represent the means ± SEs (n=5). ***p<0.001 vs. the PBS-treated group, $^{##}p$<0.01, $^{###}p$<0.001 vs. the OVA-treated group.

eosinophils were observed in the PBS control group, many eosinophils surrounded the bronchioles densely in the OVA group (Fig. 6A). Treatment with 4-CMTB (10 mg/kg or 20 mg/kg) reduced the eosinophil numbers (Fig. 6A). By using a subjective scale of 0-3, semi-quantitative evaluation of lung inflammation indicated average inflammation score of 2.3 in the OVA-treated group, and treatment with 4-CMTB reduced the score significantly (Fig. 6B).

DISCUSSION

Previously, several experiments were conducted in inflammatory responses and diseases using FFA2 gene deficient mice. Calcium influx and chemotaxis were induced by acetate in mouse and human neutrophils and eosinophils, however those were not observed in neutrophils from FFA2 gene deficient mice (Maslowski *et al*., 2009). Markedly increased inflammatory responses in FFA2 gene deficient mice were observed in OVA-induced allergic airway inflammation (Maslowski *et al*., 2009). Wild type mice reconstituted with bone marrow of FFA2 gene deficient mice also showed exacerbated inflammatory response, demonstrating that FFA2 in immune cells was largely responsible for increased response (Maslowski *et al*., 2009). Therefore, anti-inflammatory roles of FFA2 has been proved by observing exacerbated responses in FFA2 gene deficient mice. However, whether chemical agonists of FFA2 is effective on inflammatory diseases has not been studied. In the present study, for the first time the *in vitro* efficacy of 4-CMTB has been tested in the RBL-2H3 cells, showing an inhibitory effect on the degranulation. In addition, at the first time the *in vivo* efficacy of 4-CMTB was studied by using ovalbumin-induced asthma model, showing significant suppression of allergic asthma; 1) a significant suppression of the immune cell accumulation in the BALF, 2) a significant inhibition of cytokine expressions in BALF and lung tissue, 3) a significant suppression of lung inflammation, and 4) a significant reduction in mucin production. Through the present study, efficacy of FFA2 chemical agonist on allergic responses was shown, which proves anti-inflammatory roles of FFA2.

Previous studies utilized short-chain fatty acids as natural agonists for FFA2 and FFA3. Feeding a high-fiber diet increased circulating levels of short-chain fatty acids in mice, which protected against allergic inflammation in the lung (Trompette *et al*., 2014). Propionate treatment changed immunological environment in the lungs and influenced the severity of allergic inflammation, that was dependent on FFA3 and independent on FFA2 (Trompette *et al*., 2014). However, this study was conducted in FFA3 or FFA2 gene deficient mice. Therefore, those mice could have compensatory adaptation

to the gene deficiency during development. Also in this study mice were challenged with the house dust mite extract through intranasal administration, which induces a very mild allergic response only on the epithelium of airways, where house dust mite extracts were exposed. On the other hand, systemic OVA sensitization and airway exposure to nebulized OVA in our study induces a very strong allergic response (Trompette *et al*., 2014). Therefore, differences in experimental animal models and sustained gene deficiency or temporal chemical blockage may explain the discrepancy of FFA2 involvement. FFA3-mediated protective effect against allergic airway inflammation was found due to reduced eosinophilia and impaired Th2 cell response (Trompette *et al*., 2014). Compared to studies using short-chain fatty acids, the present study is simple and specific by using 4-CMTB, a FFA2 selective agonist. Although FFA3 involvement was not tested in the present study, efficacy of FFA3 chemical agonist should be studied in the future.

The present results are also supported by FFA2 expression in neutrophils and eosinophils from mouse and human (Maslowski *et al*., 2009; Theiler *et al*., 2019) , and by calcium-increasing actions of acetate and propionate in human eosinophils through FFA2 (Theiler *et al*., 2019). However, actions of butyrate such as eosinophil trafficking and survival, and type 2 lymphoid cell-dependent airway hyper-reactivity were dependent on HDAC inhibition and independent on FFA2 or FFA3 (Thio *et al*., 2018; Theiler *et al*., 2019). Epigenetic modification by butyrate should also be considered as a factor mediating anti-inflammatory responses of short-chain fatty acids as dietary metabolites from gut microbiota.

In summary, it was observed that 4-CMTB has a suppressive effect on the antigen-induced degranulation and anti-allergic effects in the OVA-induced mouse model of asthma. Therefore, the present study provides evidence of the therapeutic efficacy of 4-CMTB in allergic asthma.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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