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NJK14047 Suppression of the p38 MAPK Ameliorates OVA-Induced Allergic Asthma during Sensitization and Challenge Periods

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Abstract

p38 MAPK has been implicated in the pathogenesis of asthma as well as pro-allergic Th2 cytokines, orosomucoid-like protein isoform 3 (ORMDL3), regulation of sphingolipid biosynthesis, and regulatory T cell-derived IL-35. To elucidate the role of p38 MAPK in the pathogenesis of asthma, we examined the effect of NJK14047, an inhibitor of p38 MAPK, against ovalbumin (OVA)-induced allergic asthma; we administrated NJK14047 before OVA sensitization or challenge in BALB/c mice. As ORMDL3 regulation of sphingolipid biosynthesis has been implicated in childhood asthma, ORMDL3 expression and sphingolipids contents were also analyzed. NJK14047 inhibited antigen-induced degranulation of RBL-2H3 mast cells. NJK14047 administration both before OVA sensitization and challenge strongly inhibited the increase in eosinophil and lymphocyte counts in the bronchoalveolar lavage fluid. In addition, NJK14047 administration inhibited the increase in the levels of Th2 cytokines. Moreover, NJK14047 reduced the inflammatory score and the number of periodic acid-Schiff-stained cells in the lungs. Further, OVA-induced increase in the levels of C16:0 and C24:1 ceramides was not altered by NJK14047. These results suggest that p38 MAPK plays crucial roles in activation of dendritic and mast cells during sensitization and challenge periods, but not in ORMDL3 and sphingolipid biosynthesis.

Key Words: Asthma, Allergy, NJK14047, Immunopharmacology, p38 MAPK

INTRODUCTION

Asthma is a chronic respiratory condition in which airways become narrow and swollen. A total of 339 million people are suffering from bronchial asthma worldwide (Marzhan *et al*., 2022). The signs and symptoms of asthma include shortness of breath, coughing, chest tightness, wheezing, and dyspnea (Wallace-Farquharson *et al*., 2022). Chronic inflammatory responses such as eosinophil infiltration, mucus hyperproduction, bronchial mucosal thickening, and bronchial wall remodeling have been observed in patients with asthma (Bakakos *et al*., 2022). In allergic asthma, initial exposure to sensitizing antigens and subsequent repeated exposure to antigens leads to the development of asthma. Antigen-presenting dendritic cells play a pivotal role in antigen sensitization process (Chiang *et al*., 2022). Activation of mast cells and eosinophils induce T helper 2 (Th2) cells-mediated inflammatory reactions in the airways during antigen exposure (Gilfillan *et al*., 2009). Currently, corticosteroids, long-acting β_2-adrenoceptor agonists, and leukotriene D_4 antagonists are useful in regulating asthma symptoms (Rayees and Din, 2021). However, new therapeutic approaches are needed to overcome the limitations of these therapies such as steroid-induced side effects and steroid-resistant asthma patients (Barnes, 2013).

Genetic variants regulating orosomucoid (yeast)-like protein isoform 3 (ORMDL3) expression were found to be determinants of susceptibility to childhood asthma (Moffatt *et al*., 2007). Later, the ORMDL3 gene in humans was reported to function in sphingolipid metabolism by suppressing serine palmitoyl-coenzyme A transferase, the rate-limiting enzyme for new sphingolipid synthesis (Breslow *et al*., 2010). Higher ORMDL3 expression has been linked to lower sphingolipid synthesis in children with allergic asthma (Ono *et al*., 2020). However, the levels of ORMDL3 and sphingolipids during asthma development have been poorly studied in murine models. Recently, regulatory T cell (Treg)-derived IL-35 has drawn attention (Collison *et al*., 2010; Li *et al*., 2020), because sputum IL-35 level was significantly lower in patients with eosinophilic asthma than that in patients with neutrophilic asthma (Li *et al*., 2020). Therefore, anti-inflammatory cytokines produced by

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regulatory lymphocytes are required to be measured in murine models.

The level of phosphorylated p38 MAPK in epithelial cells was significantly elevated in the airways of patients with severe asthma compared to that in patients with mild asthma and healthy control subjects (Liu *et al*., 2008). Alveolar macrophages from patients with severe asthma showed increased p38 MAPK activation and corticosteroid-insensitivity (Bhavsar *et al*., 2008). The anti-inflammatory cytokine IL-35 induces inhibition of p38 MAPK signaling, which sensitizes monocytes to corticosteroid treatment and reduces asthma injury (Qian *et al*., 2020; Wang *et al*., 2022). Furthermore, recent many studies have suggested the effectiveness of natural products in animal models of asthma due to suppression of p38 MAPK (Bai *et al*., 2022; Park *et al*., 2022; Tirpude *et al*., 2022). Because the p38 MAPK signaling pathway plays a key role in inflammation, and p38α MAPK expression is high in macrophages, monocytes, neutrophils, and $CD4^+$ T cells (Hale *et al*., 1999; Saklatvala, 2004), it is intriguing to investigate whether p38 MAPK plays crucial roles in asthma pathogenesis by regulating ORMDL3 expression, sphingolipid biosynthesis, and anti-inflammatory cytokines. In our previous study, we developed NJK14047, a highly specific inhibitor of p38α/β MAPK (Heo *et al*., 2015). Here, we aimed to investigate whether p38 MAPK is involved in antigen-induced degranulation of RBL-2H3 mast cells and whether p38 MAPK regulates the development of ovalbumin (OVA)-induced allergic asthma, modulates the levels of ORMDL3, sphingolipid biosynthesis, and the levels of anti-inflammatory cytokines in mice, by applying NJK14047, an inhibitor of p38 MAPK, against OVA-induced allergic asthma; we administrated NJK14047 before OVA sensitization or challenge in BALB/c mice.

MATERIALS AND METHODS

NJK14047 was synthesized using a previously reported procedure (>97%, HPLC) (Heo *et al*., 2015). OVA and alum were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Rat RBL-2H3 mast 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 Dulbecco's modified Eagle medium (DMEM)-high glucose containing 10% (v/v) heat-inactivated fetal bovine serum along with 2 mM glutamine, 100 U/mL penicillin, 1 mM sodium pyruvate, and 50 μg/mL streptomycin.

Animals

Five-week-old female 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 water and food *ad libitum*. The Institutional Animal Care Committee of the university reviewed and approved the study protocol considering ethical principles and care and use of animals for scientific purposes (Approval Number, KHSASP-21-547).

Assessment of degranulation

Degranulation of RBL-2H3 cells was assessed by measuring β-hexosaminidase activity in the medium. Mouse monoclonal anti-dinitrophenyl immunoglobulin E (DNP-IgE) and human DNP albumin were used to induce degranulation (Lee and Im, 2021b).

Assessment of histamine

RBL-2H3 cells were seeded in a 24-well plate at a density of s 2×10^5 cells/well and sensitized with 0.2 μg/mL DNP-IgE overnight. After washing with phosphate-buffered saline (PBS) three times, the cells were incubated in 400 μL of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer containing NJK14047 for 30 min. After adding 1 μg/mL of DNP-HAS, the cells were incubated for 15 min. The supernatant (100 μL) was mixed with 20 μL 1 M NaOH and 25 μL 1% (w/v) o-phthalaldehyde dissolved in methanol (MeOH) and incubated at room temperature for 4 min. The reaction was terminated by adding 10 μL 3 M HCl. The absorbance was measured at excitation/emission wavelength of 355/460 nm.

Assessment of sphingolipids metabolites in the lung using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The mouse lung was removed, and the lung sample (20-30 mg) was homogenized. An internal standard solution (50 nM C17 ceramide solution) was added to the sample before extraction; total lipids from the mouse lung were extracted using the Folch method. Organic solutions containing lipids were then dried using a vacuum centrifuge and stored at –20°C until LC-MS/MS analysis. The dried matter was reconstituted with MeOH and injected into LC-MS/MS system.

Sphingolipid levels were determined using LC-MS/MS system equipped with 1290 HPLC (Agilent Technologies, Waldbronn, Germany) and QTRAP 5500 (AB Sciex, Toronto, Canada). A reverse-phase column (Pursuit 5 C18, 150×2.1 mm) with mobile phase A (5 mM ammonium formate/MeOH/tetrahydrofuran (500/200/300)) and mobile phase B (5 mM ammonium formate/MeOH/ tetrahydrofuran (100/200/700)) was used. The flow rate through the column was set at 200 μL/min and 35°C. The LC gradient was as follows: 50% of A for 0 min, 50% of A for 5 min, 50-30% of A for 3 min, 30% of A for 7 min, 30-10% of A for 7 min, 10% of A for 3 min, 10-50% of A for 0.1 min, and 50% of A for 4.9 min. Multiple reaction monitoring (MRM) was performed in positive ion mode, and ion chromatogram obtained corresponding to the specific transition for each lipid was used for quantification. The calibration range for each lipid was 0.1-10000 nM ($r^2\geq0.99$). Data analysis was performed using either Analyst 1.7.1 software (AB Sciex, Framingham, MA, USA).

Asthma induction in mice and NJK14047 administration

Following a simple randomization procedure, 6-week-old female BALB/c mice (approximately 22 g) were randomly assigned to four treatment groups (n=5): PBS-injected control group, OVA-injected asthma group, NJK14047 (2.5 mg/kg) treatment before sensitization plus OVA-injected group, and NJK14047 treatment before challenge plus OVA-injected group. Mice were sensitized by intraperitoneal injection of 50 μg OVA and 1 mg aluminum hydroxide on the day 0 (D0) and boosted by the same injection on D14 (sensitization). Mice were challenged by exposure for 30 min to nebulized 1% OVA or PBS alone delivered via an ultrasonic nebulizer (Philips, NJ, USA) on D28, D29, and D30 (challenge). NJK14047 was administered via intraperitoneal injection 30

min before OVA sensitization (D0 and D14) or 30 min before OVA challenge (D28, D29, and D30). Bronchoalveolar lavage fluid (BALF) was collected from the lung on D32, and the cell population of BALF cells was analyzed after staining (Lee and Im, 2021a).

BALF cell counting and analysis

Immune cells in the BALF were adhered to a glass slide using Cellspin® centrifuge (Hanil Electric, Seoul, Korea) and fixed in MeOH for 30 s. Cells were stained with May-Grünwald solution for 8 min, followed by Giemsa solution for 12 min.

Histological examination of the lung

Lung tissue sections were prepared from the lungs of mice of each group. Hematoxylin and Eosin (H&E) and periodic acid-Schiff (PAS) staining were conducted to identify mucus-secreting goblet cells and eosinophil infiltration, respectively. Schiff's regent was used for PAS staining, and hematoxylin and eosin reagent was used for H&E staining.

The degree of lung inflammation was measured by a treatment-blind observer using a subjective scale of 0-3. Mucin-secreting cells in the airways stained with PAS were counted in two lung sections per mouse. Moreover, we measured the length of bronchial basal lamina using the ImageJ software (National Institute of Health, Rockville, MD, USA). Mucous production was expressed as the number of PAS-positive cells per millimeter of bronchiole.

Measurement of the levels of total serum IgE and IL-13

Mouse serum IgE levels were determined using ELISA kits (eBioscience, San Diego, CA, USA). IL-4 and IL-13 levels in the BALF were quantified using ELISA kit. Capture and biotinylated detection antibodies specific for IL-4 and IL-13 were obtained from eBioscience (IL-4: Cat. No. 14-7041-68 and 33-7042-68C, IL-13: Cat no. 14-7043-68 and 33-7135-68B). Avidin-horseradish peroxidase was used, and absorbance was measured at 450 nm.

Statistics

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to compare the differences among multiple groups. Differences were considered statistically significant at $p<0.05$.

RESULTS

p38 MAPK inhibitor suppressed degranulation of mast cells

Mast cells play a major role in asthmatic attacks. Multivalent antigen exposure induces mast cell activation through cross-linking of the IgE-FcεRI complex, resulting in degranulation. Degranulation of mast cells releases mediators of allergic responses such as histamine, leukotrienes, cytokines chemokines, and neutral proteases (chymase and tryptase). Degranulation responses were measured in RBL-2H3 rat basophilic leukemia cells; β-hexosaminidase activity in the medium increased following exposure of human serum albumin (antigen) (Fig. 1A). Treatment of NJK14047 inhibited β-hexosaminidase release in a concentration-dependent manner (Fig. 1A). The inhibition of β-hexosaminidase release induced by NJK14047 was significant at a concentration of 0.3 μM (Fig. 1A). This inhibitory effect was further confirmed by measuring the amounts of histamine release from RBL-2H3 cells (Fig. 1B).

p38 MAPK inhibitor did not alter the levels of ORMDL3 and sphingolipids

Genome-wide association studies have identified ORMDL3 as a risk factor for asthma and its function in sphingolipid synthesis (Moffatt *et al*., 2007; Breslow *et al*., 2010). A significant induction of ORMDL3 mRNA expression was observed in the lungs of OVA-treated mice (Fig. 2A), which is consistent with a previous study (Miller *et al*., 2012). However, NJK14047 treatment, both before OVA sensitization and challenge, suppressed the expression of ORMDL3, but the difference was not statistically significant (Fig. 2A). Since *de novo* sphingolipid synthesis can be modulated by ORMDL3, sphingolipid content was measured. Among the tested sphingolipids, C14 ceramide, C16 ceramide, C18 ceramide, C18:1 ceramide, C20 ceramide, C24 ceramide, C24:1 ceramide, 18:0 sphingomyelin, 18:1 sphingomyelin, 16:0 sphingomyelin, 24:0 sphingomyelin, 24:1 sphingomyelin, sphinganine, and sphingosine, the levels of C16 and C24:1 ceramides were significantly increased in the OVA-treated lungs compared to that of the PBS-treated lungs (Fig. 2B). However, NJK14047 administration before sensitization or challenge did not change these contents. The levels of sphingomyelins were not changed by either OVA or NJK14047 treatment (Fig. 2C). Although the levels of sphinganine and sphingosine increased in the OVA group, but the difference was not statistically significant (Fig. 2D).

p38 MAPK inhibitor modulated the production of anti-inflammatory cytokines in the BALF and lungs

Treg-derived IL-35 levels in the sputum were significantly

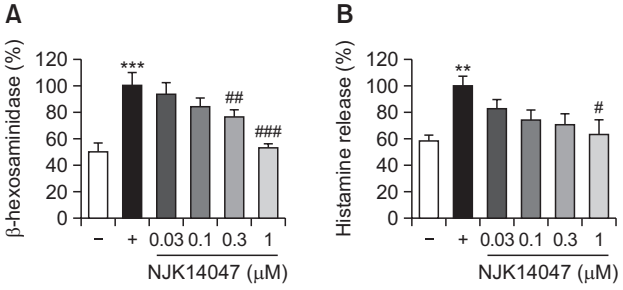

Fig. 1. NJK14047 represses antigen-induced degranulation and histamine release in RBL-2H3 mast cells. (A) After sensitization with anti- dinitrophenyl immunoglobulin E (DNP-IgE) for 18 h, RBL-2H3 cells were challenged with dinitrophenyl-human serum albumin (DNP-HSA). NJK14047 treatment was performed at the indicated concentrations 30 min before antigen challenge. Basal degranulation shows samples without IgE and HSA, and the positive control of antigen-induced degranulation shows samples with IgE and HSA. (B) Released histamine was assessed in the medium after HSA challenges. The results are presented as the mean ± standard error (SE) of three independent experiments. $^{***}p<0.001$, $^{**}p<0.01$ vs. the HSA-untreated group. $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ vs. the HSA-treated group.

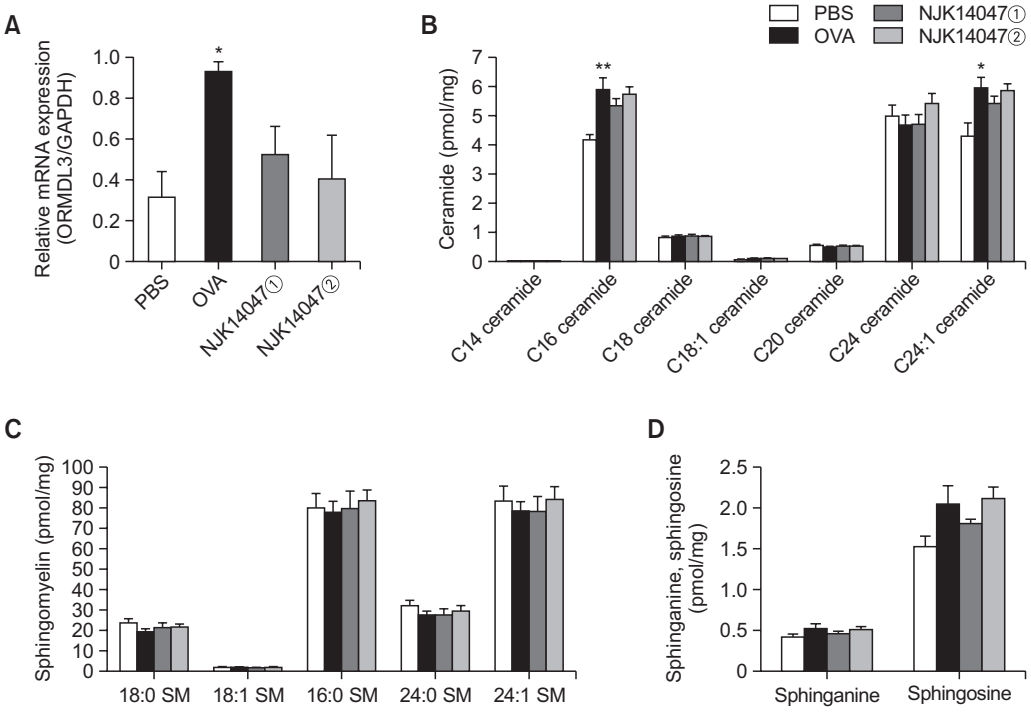

Fig. 2. Changes of sphingolipid content in the lung. (A) qRT-PCR results of ORMDL3. (B) Content of ceramides. (C) Content of sphyngomyelins. (D) Content of sphinganine and sphingosine. ***p*<0.01, **p*<0.05 vs. the PBS-treated group.

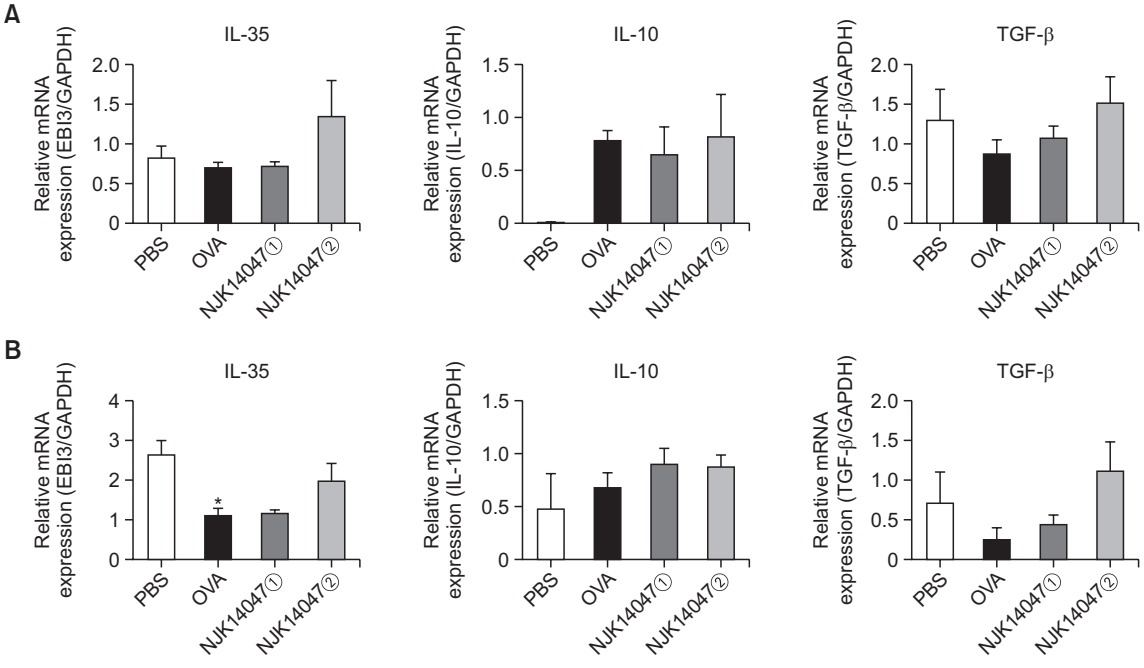

Fig. 3. Changes of the mRNA expression of IL-35, IL-10, and TGF-β in the BALF and the lungs. Analysis of mRNA expression of the IL-35, IL-10, and TGF-β in (A) the BALF and (B) the lung tissues. Relative mRNA levels of cytokines were quantified with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Values are represented as the mean ± standard error (SE) (n=5). **p*<0.05 vs. the PBS-treated group.

lower in patients with eosinophilic asthma than those in patients with neutrophilic asthma (Li *et al*., 2020). In addition, IL-35 inhibits p38 MAPK signaling (Qian *et al*., 2020; Wang *et al*., 2022). Thus, the levels of anti-inflammatory cytokines, including IL-35, IL-10, and TGF-β, were also assessed in the BALF and lung tissues. A tendency of OVA-induced decrease

and NJK14047-induced increase in TGF-β levels in the BALF was observed; however, the difference was not statistically significant (Fig. 3A). In the lung tissues, levels of IL-35 and TGF-β showed a decreasing tendency with OVA treatment and an increasing tendency with NJK14047 treatment before challenge, and the OVA-induced decrease in IL-35 was significant (Fig. 3B). However, NJK14047-induced recovery in IL-35 and TGF-β levels was not significant (Fig. 3B).

p38 MAPK inhibitor suppressed the increase in eosinophil and lymphocyte counts in the BALF

The effects of NJK14047 on antigen sensitization and challenge were investigated in an OVA-induced mouse model of asthma. NJK14047 was administered *via* intraperitoneal injection 30 min before OVA sensitization or challenge. As shown in Fig. 4, NJK14047 administration suppressed the increase in total cell number in the BALF (Fig. 4A). The total cell number in the BALF increased to 231.7% in the OVA-induced asthma group compared to that in the PBS-treated control group (Fig.

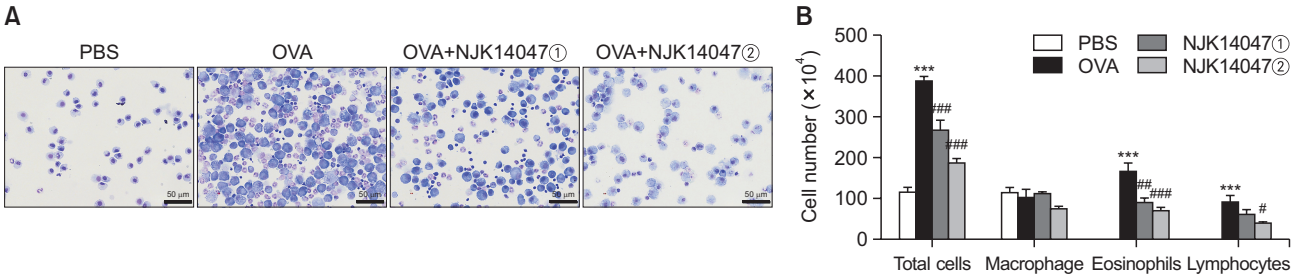

Fig. 4. NJK14047 inhibits ovalbumin-induced immune cell accumulation in BALF. (A) Mice were sensitized with ovalbumin (OVA) twice *via* intraperitoneal injection on day 0 (D0) and D14, and later challenged on D28, D29, and D30 with nebulized OVA. NJK14047 was administrated intraperitoneally at a dose of 10 mg/kg, 30 min before OVA sensitization or challenge. BALF cells were stained with May-Grünwald and counted. (B) Total cell count, macrophage, eosinophil, and lymphocyte counts in the BALF. The results are presented as the mean ± standard error (SE) of cell count values (n=5). ***p<0.001 vs. the phosphate buffered saline-treated group, $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. the OVA-treated group.

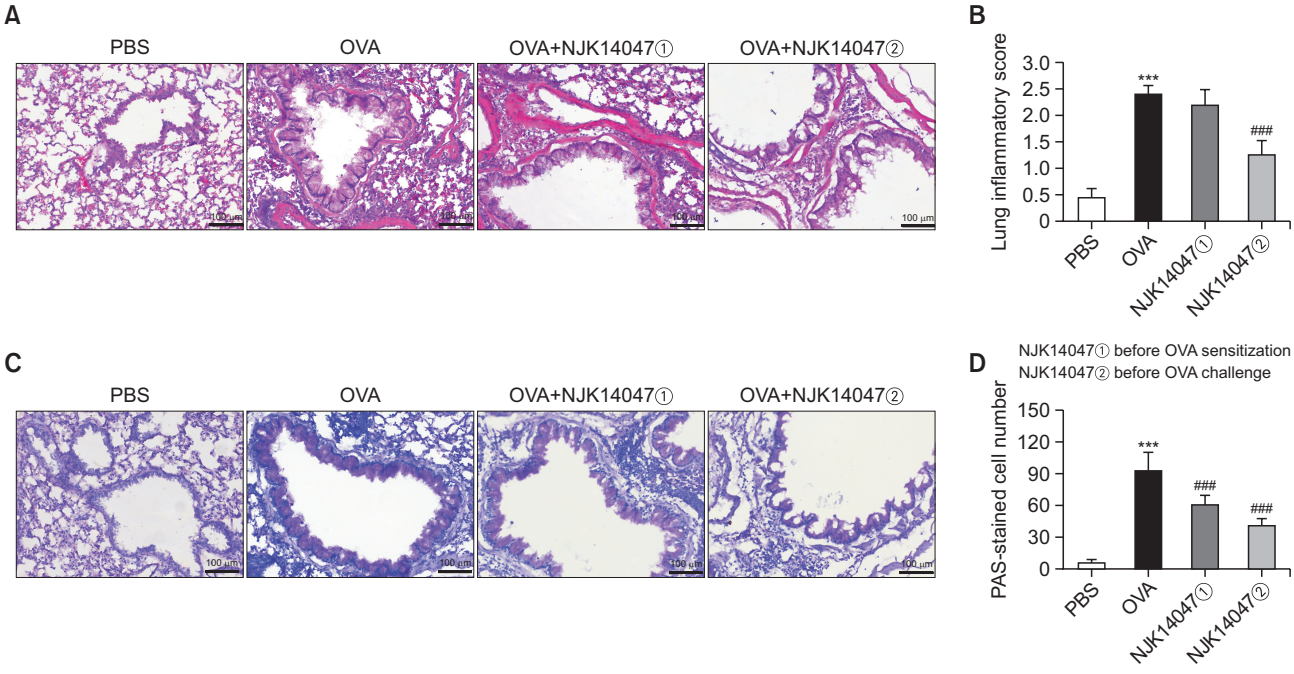

Fig. 5. NJK14047 protects against airway inflammation and mucin production. (A) Panels show H&E-stained sections of the lung tissues from the the phosphate-buffered saline (PBS) group, ovalbumin (OVA) group, and NJK14047-treated OVA groups (before sensitization or challenge). Small dark blue dots around the bronchioles indicate eosinophils, which are scarcely observed in the PBS group, whereas they are densely accumulated around the bronchioles in the OVA group. However, eosinophil accumulation is less obvious in the OVA+NJK14047 groups than in the OVA group. (B) Panels show PAS/hematoxylin-stained sections of the lung tissues from the PBS group, OVA group, and NJK14047-treated OVA groups (before sensitization or challenge). In PAS staining, mucin was stained as purple color. Darker and denser purple color is observed surrounding the bronchiole in the OVA group than that in the PBS group. (C) Lung inflammation was semi-quantitatively evaluated; histological findings were scored as described in the Materials and methods section. (D) Mucous production was measured by counting the number of PAS-positive cells per millimeter of bronchiole (n=5 per group). Values represent the mean ± standard error (SE) (n=5). ***p<0.001 vs. the PBS-treated group, $^{\#\#\#}p$<0.001 vs. the OVA-treated group.

4B). NJK14047 treatment before antigen sensitization or challenge significantly suppressed the OVA-induced increase in the total cell number by 44.0% and 73.6%, respectively (Fig. 4B). The distribution of immune cell populations was also assessed. OVA-induced increase in eosinophil count was significantly decreased by NJK14047 treatment before sensitization or challenge by 45.3 and 57.9%, respectively (Fig. 4B). Although the lymphocyte count was lower than the eosinophil count, OVA treatment induced an increase, whereas NJK14047 treatment before sensitization significantly decreased the lymphocyte count (Fig. 4B). Macrophage count was not significantly altered after OVA or NJK14047 treatment (Fig. 4B).

p38 MAPK inhibitor suppressed mucin secretion and inflammation in the lungs

Histological studies using H&E and PAS staining of lung samples showed increase in inflammation score and mucin hypersecretion. Eosinophils were observed as small, dark-blue dots in H&E staining of the lung sections (Fig. 5A). There were few eosinophils in the PBS control group, whereas many eosinophils could be easily observed around the bronchioles

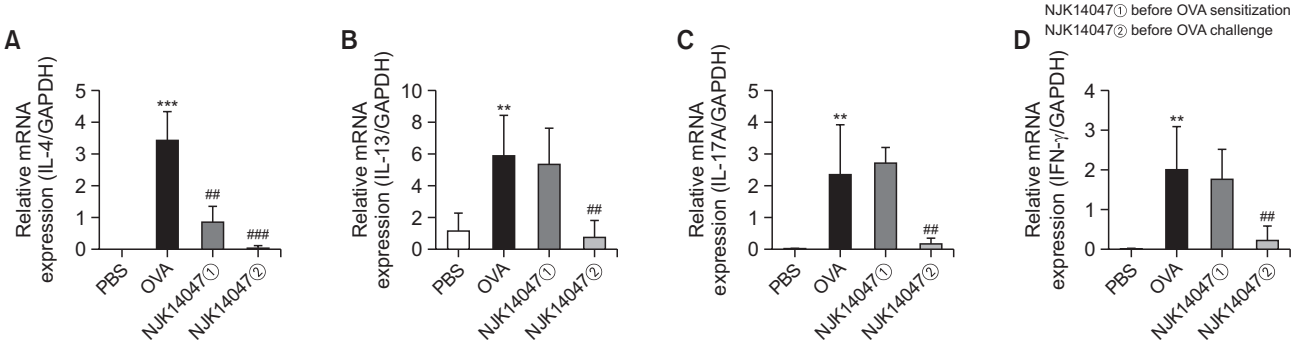

Fig. 6. NJK14047 treatment inhibits the mRNA expression of cytokines in the BALF cells. Analysis of mRNA expression of the Th2 cytokines IL-4 and IL-13 and the Th17 cytokine IL-17A in the BALF cells. (A) IL-4, (B) IL-13, (C) IL-17A, and (D) IFN-γ. Relative mRNA levels of cytokines were quantified with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Values are represented as the mean ± standard error (SE) (n=5). ***p<0.001, **p<0.01 vs. the PBS-treated group, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. the OVA-treated group.

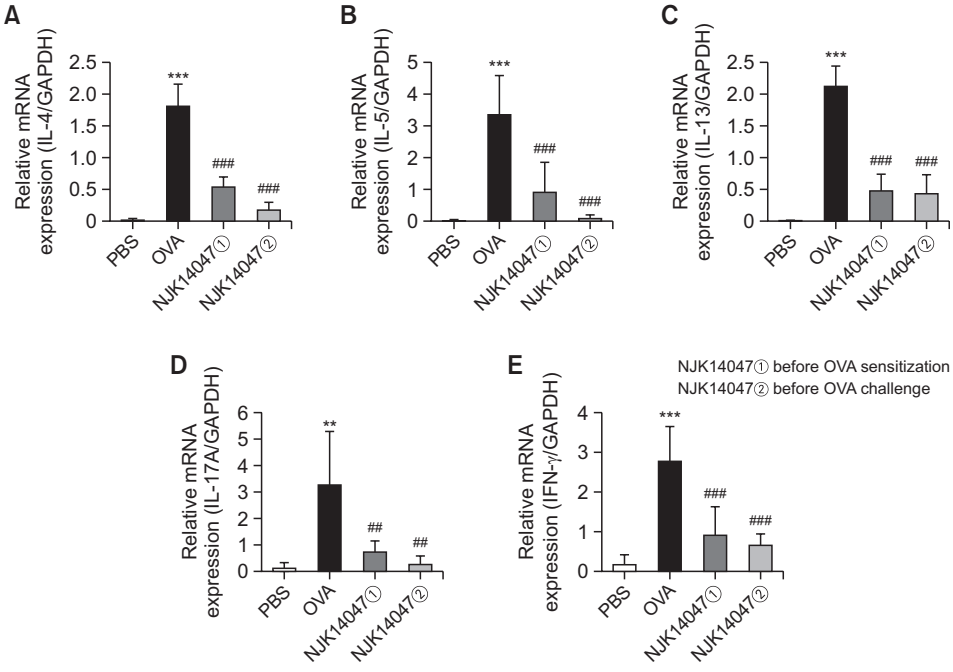

Fig. 7. NJK14047 treatment inhibits the mRNA expression of cytokines in the lungs. Analysis of mRNA expression of the Th2 cytokines IL-4, IL-5, and IL-13 and the Th17 cytokine IL-17A in the lung tissues. (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-17A, and (E) IFN-γ. Relative mRNA levels of cytokines were quantified with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Values are represented as the mean ± standard error (SE) (n=5). ***p<0.001, **p<0.01 vs. the PBS-treated group, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. the OVA-treated group.

in the OVA group (Fig. 5A). NJK14047 treatment before OVA sensitization or challenge inhibited the OVA-induced increase in the eosinophil count (Fig. 5A). Using a subjective scale of 0-3, semi-quantitative evaluation of lung inflammation indicated an average inflammation score of 2.4 in the OVA-treated group, which was significantly reduced after NJK14047 treatment before sensitization or challenge (Fig. 5B).

Mucin and mucous glycoproteins produced by goblet cells were visualized by PAS staining of the lung sections. Mucin-secreting cells were observed as dark violet spots surrounding the bronchioles in the OVA group (Fig. 5C). PAS staining was inhibited after NJK14047 treatment before OVA sensitization or challenge (Fig. 5B). Furthermore, semi-quantitative analysis of mucin production was performed by counting the number of PAS-positive cells in the bronchioles (Fig. 5D). Stained cells were scarce in the PBS-treated group. However, approximately 100 PAS-positive cells/mm were detected in the OVA-treated group, which were significantly suppressed after NJK14047 treatment before sensitization or challenge (Fig. 5D).

p38 MAPK inhibitor suppressed the production of Th2 and Th1/Th17 cytokines in the BALF and lungs

Th2 cells contribute to the pathogenesis of allergic asthma by producing Th2 cytokines, such as IL-4, IL-5, and IL-13, leading to eosinophil accumulation in the airway wall, mucus overproduction, and synthesis of IgE. In addition, Th1 and Th17 cytokines are involved in the late-stage pathogenesis of asthma. Therefore, changes in the mRNA levels of the Th2 cytokines IL-4, IL-5, and IL-13, the Th1 cytokine IFN-γ, and the Th17 cytokine IL-17A were measured by qPCR in the BALF cells. As shown in Fig. 5, mRNA levels of the four cytokines were increased in the BALF cells of the OVA group, and NJK14047 treatment before OVA challenge significantly suppressed the OVA-induced increase (Fig. 6). However, NJK14047 treatment before OVA sensitization did not inhibit OVA-induced increase in cytokine levels (Fig. 6), which was consistent with the findings of milder suppression of BALF cell numbers, lung inflammation, and mucin production after NJK14047 treatment before OVA sensitization.

Similarly, changes in mRNA levels of IL-4, IL-5, IL-13, IFN-γ, and IL-17A in the lungs were measured by qPCR. As shown in Fig. 6, mRNA levels of the five cytokines were increased in the BALF cells of the OVA group, and both NJK14047 treatments before OVA challenge and sensitization significantly suppressed the OVA-induced increase (Fig. 7).

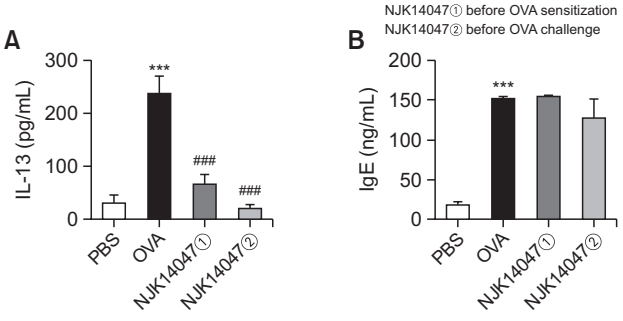

Fig. 8. Effect of NJK14047 on IL-13 levels in BALF and IgE levels in serum. ELISA was used to measure the protein levels of (A) IL-13 in BALF and (B) IgE in serum. Results are presented as the mean ± standard error of the mean (SEM) (n=5). ***p<0.001 vs. the PBS-treated group, ###p<0.001 vs. the OVA-treated group.

p38 MAPK inhibitor suppressed OVA-induced increase in BALF IL-13 levels but not serum IgE

Th2 cytokines, such as IL-13, play major role in the progression of allergic asthma. Th2 cytokines induce eosinophil recruitment and activation, hypersecretion of mucus in epithelial cells, metaplasia of goblet cells, and proliferation of smooth muscle cells. Protein level of the Th2 cytokine IL-13 in the BALF was measured by ELISA; IL-13 level was increased in the OVA-induced group compared to that in the PBS-treated control group, and the increase in IL-13 levels was significantly suppressed by both treatments of NJK14047 (Fig. 8A). Serum IgE level was assessed to confirm immunological effects of OVA and NJK14047. IgE production was increased in the sera of OVA-treated mice (Fig. 8B). However, the OVA-induced increase in serum IgE levels was not suppressed by both NJK14047 treatments, that is before antigen sensitization and challenge.

DISCUSSION

Several novel findings were reported in this study. First, the study, to the best of our knowledge, is the first to reveal the inhibition of *in vitro* antigen-induced degranulation of mast cells by the p38 MAPK inhibitor NJK14047. Second, the p38 MAPK inhibitor NJK14047 inhibited *in vivo* OVA-induced allergic responses in the BALF and lungs. Third, OVA treatment induced ORMDL3 expression and increased C16 and C24:1 ceramide levels in the lungs. Forth, IL-35 levels in the lungs were decreased by OVA treatment but not recovered by NJK14047.

p38 MAPK inhibitors have been used in previous studies using murine allergic asthma models (Escott *et al*., 2000; Underwood *et al*., 2000; Nath *et al*., 2006). The notable difference between NJK14047 and other p38 MAPK inhibitors is related to their potency; NJK14047 showed the highest potency among the tested p38 MAPK inhibitors. The effective doses in previous studies have been reported as 12 mg/kg of SB239063, 10-100 mg/kg of SB203580, 30 and 90 mg/kg of SD282 (Escott *et al*., 2000; Underwood *et al*., 2000; Nath *et al*., 2006), implying that NJK14047 (2.5 mg/kg) has approximately 5-40 folds higher potency than that of the other p38 MAPK inhibitors. In addition, discrepancies in some findings was observed. A common finding was the marked reduction in inflammatory cytokine production (Escott *et al*., 2000; Underwood *et al*., 2000), which was observed in the NJK14047-treated group receiving NJK14047 treatment before OVA challenge and sensitization. Inhibition of eosinophil infiltration in the BALF was observed in a previous study, but not in other studies (Escott *et al*., 2000; Underwood *et al*., 2000; Nath *et al*., 2006); similar results were shown by the NJK14047-treated group before OVA challenge; however, also mild inhibition was observed in the NJK14047-treated group before OVA sensitization. Hence, NJK14047 treatment before OVA challenge showed slightly better outcomes than that by NJK14047 treatment before OVA sensitization.

During the OVA challenge period, mast cells are very important for inducing an allergic response. According to a previous study, antigen-mediated activation of murine mast cells result-

ed in increased p38 MAPK phosphorylation, and SB203580 inhibition of p38 MAPK reduced IgE-mediated IL-6 production (Boudreau *et al*., 2004). In addition, mast cells induce cytokine production through the p38 MAPK signaling pathway (Kalesnikoff *et al*., 2001). The p38 MAPK inhibitor SB203580 abolished the substance P-induced increase in TNF-α mRNA and protein levels (Azzolina *et al*., 2002). However, the effects of p38 MAPK inhibition on antigen-induced degranulation have not yet been reported. This study is the first time, to the best of our knowledge, to provide evidence that the p38 MAPK inhibitor NJK14047 can inhibit antigen-induced degranulation. In addition, NJK14047 suppression of Th2 cytokine levels during the antigen challenge period might be due to suppression of p38 MAPK in T cells because activation of p38 kinase in Th2 cells contributes to differentiation of Th2 cells and upregulation of Th2 cytokine gene expression (Schafer *et al*., 1999; Chen *et al*., 2000; Skapenko *et al*., 2001; Lu *et al*., 2004). In fact, in human T cells, p38 MAPK activation mediated the phosphorylation and translocation of GATA-3, a key transcription factor for Th2 cell differentiation (Maneechotesuwan *et al*., 2007), and SB203580 p38 MAPK inhibition suppressed Th2 cytokine (IL-4 and IL-13) synthesis in human T cells (Koprak *et al*., 1999; Mori *et al*., 1999). Therefore, suppression of p38 MAPK in both mast cells and Th2 cells may synergistically suppress the allergic response during the antigen challenge period.

During OVA sensitization period, dendritic cells play major roles in antigen recognition, maturation, migration, and presentation of antigens to naïve T cells. Multiple roles of p38 MAPK in these processes have been reported. SB203580 inhibited the LPS-induced upregulation of CD1a, CD40, CD80, CD86, HLA-DR, and the DC maturation marker CD83, implying the critical role of p38 MAPK in maturation of immature dendritic cells (Arrighi *et al*., 2001). p38 MAPK inhibition by SB203580 in dendritic cells leads to the suppression of Treg generation and induction of Th1 cells (Jarnicki *et al*., 2008). Blocking p38 MAPK activation by SB202190 in dendritic cells inhibited the expression of IFN-α/β and IL-12 in innate antiviral response (Mikkelsen *et al*., 2009). Therefore, inhibition of p38 MAPK in dendritic cells may lead to delayed maturation, but suppress Treg response and induce Th1 response. These complex responses in dendritic cells may explain why NJK14047 treatment before OVA sensitization led to mild suppression in the present study.

ORMDL3 genetic variants and their functions in sphingolipid metabolism have drawn great attention in asthma research (Moffatt *et al*., 2007; Breslow *et al*., 2010). Higher ORMDL3 expression was linked to lower sphingolipid synthesis in children with allergic asthma (Ono *et al*., 2020). Several studies using ORMDL3 transgenic mice and serine palmitoyl CoA transferase knockout mice have shown contradictory and complex results, that is, decrease, increase, no change, and no association with sphingolipid levels (Worgall *et al*., 2013; Miller *et al*., 2014; Oyeniran *et al*., 2015; Siow *et al*., 2015; Zhakupova *et al*., 2016; Miller *et al*., 2017). However, a significant upregulation of ORMDL3 mRNA expression was observed in the lungs after sensitization with OVA, house dust mites, and *Alternaria alternata* (Miller *et al*., 2012), which is consistent with the present results. Furthermore, we observed increase in the levels of C16:0 and C24:1 ceramides in the lungs of OVA-treated mice. This observation supports that ORMDL3 regulates systemic levels of ceramides (Debeuf *et al*., 2019). Furthermore, C16:0 and C24:0 ceramides have been identified as sphingolipid metabolic markers in patients with uncontrolled asthma (Kim *et al*., 2020). Although we observed increase in ORMDL3 expression and C16:0 and C24:1 ceramide levels in OVA-treated mice, NJK14047 treatment did not significantly change these levels. Therefore, although an association between ORMDL3 expression and ceramide levels has been observed in a murine model, NJK14047 inhibition of p38 MAPK led to suppression of allergic responses without changes in the ceramide contents.

Further investigation on functional roles of p38 MAPK on ORMDL3 expression, eosinophil recruitment, and Th2 cytokines-mediated airway inflammation is needed (Ha *et al*., 2013), because activation of p38 MAPK regulates the expression of multiple proinflammatory cytokines and chemokines in asthma (Pelaia *et al*., 2021).

Many recent studies have suggested that the efficacy of natural products is due to suppression of p38 MAPK in animal models of asthma (Bai *et al*., 2022; Park *et al*., 2022; Tirpude *et al*., 2022). The present study demonstrated the importance of p38 MAPK in the pathogenesis and suggested the potential of targeting of p38 MAPK for the treatment of allergic asthma, in addition to revealing the role of p38 MAPK in antigen-induced degranulation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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