의학박사 학위논문

천식의 점액 과다분비 조절에서 S1PR4 (sphingosine-1-phosphate receptor 4)의 역할

The Role of S1PR4 in the Regulation of Mucus Hypersecretion in Asthma

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이 논문을 의학박사 학위 논문으로 제출함

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Abstract

Goblet cell hyperplasia, a key feature of asthmatic airway remodeling, causes excessive mucus production and plugging, worsening asthma symptoms and lung function, especially in severe acute exacerbations and treatment-resistant asthma phenotypes. As current asthma treatments have limited efficacy in addressing goblet cell hyperplasia and mucus hypersecretion, there is a pressing need for targeted therapies that regulate mucin production.

Sphingosine-1-phosphate (S1P), a sphingolipid metabolite, regulates various cellular processes and S1P levels were elevated in asthma in accordance with the asthma severity. S1P receptor 4 (S1PR4), specifically expressed in immune cells and lungs, is implicated in allergic airway inflammation. Despite reports of decreased S1PR4 expression in asthma and mucin-productive diseases, its precise role in goblet cell hyperplasia and airway remodeling remains elusive. This study aimed to elucidate the impact and mechanisms of the S1PR4 pathway in goblet cell hyperplasia in asthma.

At first, we established an inflammation and bronchoconstriction-driven chronic murine asthma remodeling model to study the role of S1PR4 in regulating this process. Mice exposed to methacholine inhalation during ovalbumin (OVA) challenge after OVA sensitization exhibited exacerbated mucin production and collagen deposition in the airways compared to those in the standard OVA-induced asthma model. And then, to elucidate the role of S1PR4 in airway mucin production, we used heterozygous S1PR4 knockout mice to generate the

model. Depletion of S1PR4 in these mice further aggravated mucin production and collagen deposition in lung, trachea and bronchi tissues. Micro-CT scans further disclosed more pronounced peribronchial patches and greater airway obstruction in the lungs of mice with S1PR4 depletion in this model. S1PR4 depletion significantly enhanced expression of the mucin genes MUC5AC and MUC5B, and a regulator of mucin genes SPDEF in the remodeled airways. Furthermore, S1PR4 depletion was significantly associated with an increase in the expression of the EGF gene and a decrease in the expression of the WNT2 and WNT7a genes.

These findings suggest that the S1PR4 pathway plays a crucial role in regulating goblet cell hyperplasia and mucin production. Upregulating S1PR4 levels in chronic asthmatic remodeling may be a potential therapeutic approach for treating asthma-related goblet cell hyperplasia and mucus hypersecretion.

Keywords: Sphingosine-1-phosphate receptor 4, Asthma, Airway remodeling, Goblet cell hyperplasia, Mucin gene

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1. Introduction

1.1. The pathophysiology of asthma involving airway remodeling

Asthma is a common chronic airway disease characterized by variable respiratory symptoms of wheeze, shortness of breath, chest tightness and cough, accompanied by variable expiratory airflow limitation.[1] The clinical features of asthma are caused by airway hyperresponsiveness (AHR), which involves repetitive smooth muscle contractions, and by chronic airway inflammation. Asthma is a heterogeneous disease that encompasses various phenotypes and endotypes while sharing these clinical and pathophysiologic characteristics.[2-4] It arises from an intricate interaction between genetic predisposition, epigenetic regulation, and environmental factors.[5]

The airway epithelium is the first line barrier against inhaled environmental substances such as allergens, pollutants and microbes.[6] The environmental insult on airway epithelial cells stimulate innate immune responses by secreting chemokines and cytokines, and also cause epithelial cell injury and apoptosis. Disruption of the epithelial barrier induce growth factors such as transforming growth factor (TGF)-β, initiating tissue regeneration to restore homeostasis, however, this repair process is disrupted with persistent damage and prolonged stimulation of epithelium in asthma.[6] This process are known as airway remodeling in asthma. These include the loss of epithelial integrity, thickening of basement membrane, subepithelial fibrosis, goblet cell and submucosal gland hyperplasia, increased smooth muscle mass, and

increased airway vascularity.[7,8] Airway remodeling is present even in mild asthma; however, it is more common and severe in proportion to the severity of asthma.[6,9]

Immune cells, including T cells, mast cells, eosinophils, and macrophages, amplify the inflammatory and airway remodeling responses by releasing cytokines such as interleukin (IL)-4, IL-13, and TGF-β. Signaling from both the damaged epithelium and inflammatory immune cells activates the underlying mesenchymal airway cells, including smooth muscle cells and fibroblasts, leading to pathological airway remodeling in asthma.[10] Studies suggest that mechanical stresses resulting from bronchoconstriction per se may also lead to airway wall remodeling in the absence of inflammatory agents. In patients with asthma, repeated bronchoconstriction experimentally induced using methacholine challenge led to airway remodeling including increased subepithelial collagen-band thickness and mucin production, which was similar to the effects observed in the allergen challenge group.[11]

1.2. Goblet cell hyperplasia in asthma and its clinical impact

One of the key features of airway remodeling in asthma is goblet cell metaplasia and hyperplasia, which refers to the increased number of goblet cells and the transformation of non-mucus-producing cells into goblet cells. This contributes to excessive mucus production, secretion, and mucus-plugging in asthma.[12] In comparison to healthy individuals, patients with mild to moderate asthma exhibit a notable goblet cell hyperplasia.[13] This excessive mucus

production becomes more pronounced in cases of severe asthma or during asthma exacerbations, where it significantly contributes to the obstruction of the airways, further compromising lung function.[14]

Increased expression of MUC5AC and MUC5B, the primary gel-forming mucins secreted by goblet cells and submucosal glands, has been observed in the airways from asthmatic patients compared to healthy controls.[14] Overexpression of MUC5AC increases gel viscoelasticity and tethers mucin to goblet cells leading to mucus plugging in the airways, which are distinct features in asthma, differentiating it from other mucin hypersecretory diseases such as chronic obstructive pulmonary disease (COPD) or bronchiectasis.[14] These features not only contributes to the worsening of asthma symptoms and lung function but also accelerates the process of airway remodeling.[15] Specifically, it has been closely associated with acute severe exacerbations of asthma and a phenotype of severe treatment-resistant asthma.

However, the phenotype of mucus cell hyperplasia and hypersecretion has been undervalued compared to other characteristic features of asthma, such as airway inflammation and AHR, in both clinical and research contexts.[14] Early and more effective inhibition of mucus production during acute asthma exacerbation could reduce hospitalizations, morbidity, and mortality. Furthermore, sustained suppression of mucus overproduction may alleviate the overall burden of asthma and improve the quality of life of patients with the mucin

hypersecretory phenotype.

1.3. Molecular mechanism of goblet cell hyperplasia in asthma

Th2 cytokines, such as IL-4, IL-9, and IL-13, secreted during airway inflammation play a crucial role in the induction of goblet cell hyperplasia in the airways of asthma. In particular, IL-13 is the prime effector cytokine. When IL-13 activates the α chain of the IL-4 receptor, which is a component shared by both the IL-4 and IL-13 receptor complexes, it induces the SAM pointed domain-containing ETS transcription factor (SPDEF) gene via a signal transducer and activator of transcription (STAT)-6 dependent pathway, leading to the upregulation of MUC5AC.[16] On the other hand, compressive mechanical stresses accompanied by bronchoconstriction can lead to goblet cell hyperplasia in airway epithelial cells by activating the epidermal growth factor (EGF) receptor (EGFR), independently of Th2 cytokine.[17] EGFR has the pivotal role in both constitutive and IL-13-induced mucin gene expression. Inhibition of EGFR ligand, TGF-α or EGF, reduced both constitutive and IL-13-induced mucin production, while IL-13 did not affect the release of TGF-α.[18] Based on these findings, a two-signal model has been proposed in which the activation of EGFR by various stimuli, such as allergens, cytokines, oxidative stress, and mechanical injury to airway epithelial cells, inhibits epithelial cell apoptosis and subsequently allows IL-13-dependent goblet cell differentiation via an increase in SPDEF and a reduction in forkhead box protein

A2 (FOXA2) expression.[12,16]

Therefore, mechanical stresses resulting from chronic intermittent bronchoconstriction as well as allergic airway inflammation are responsible for driving goblet cell hyperplasia, one of the key features of asthma.

1.4. Unmet need in managing goblet cell hyperplasia in asthma

Anti-inflammatory treatment with inhaled corticosteroid (ICS) is the standard therapy essential to reduce the risk of serious exacerbations and to control symptoms in asthma.[1,19] It is also the mainstream approach to regulate mucus hyperplasia and to prevent or attenuate airway remodeling. Corticosteroid treatment inhibited IL-13-induced MUC5AC and MUC5B expression and mucus hyperplasia in both experimental studies and bronchial epithelial cells from asthmatic subjects.[20] However, in a longitudinal observation of an asthma patient cohort, a considerable number of patients had persistent mucus plugs on lung computed tomography (CT) scans for years, despite the maintenance of inhaled or systemic corticosteroids, which was associated with a decrease in airflow over time.[21,22] Mechanistically, it has been reported that corticosteroid treatment does not suppress EGFR activation.[23]

Treatment with long-acting β2 agonists (LABA) alone did not significantly suppress the induction of MUC5AC, whereas the combination therapy of ICS with LABA showed additive effects in reducing MUC5AC overexpression compared to ICS alone.[20] However, it is unclear

whether the mechanism is related to an additional anti-inflammatory action or its anti-bronchoconstrictor effect.[24] Long-acting muscarinic antagonists (LAMA), which block acetylcholine signaling, may prevent increased airway smooth muscle contractility induced by cholinergic tone and reduced AHR.[25] Tiotropium, a representative LAMA agent, attenuated IL-13-induced goblet cell metaplasia in human airway epithelial cell, and it was also evident in allergen-challenged animal model.[26,27] However, the role of LAMA in mucus production and hypersecretion in humans is not well studied.

Biologic therapies demonstrated some beneficial effect on airway remodeling in addition to inflammation.[28] Among these, dupilumab, a monoclonal antibody that blocks IL-4 and IL-13 signaling by targeting IL-4 receptor α, reduced goblet cell metaplasia in an allergic asthma mouse model.[29] Tezepelumab, which is an anti-TSLP antibody, have shown to reduce mucus plugging in patients with uncontrolled, moderate-to-severe asthma compared to placebo control group.[30] However, the clinical effectiveness of biologics on mucus gland hyperplasia and mucus plugging has not yet been satisfactory.

Several mucoactive or mucolytic drugs that modify mucus production, secretion, properties, or facilitate mucus clearance have been used. Although these agents showed beneficial effects in experimental or preclinical studies, they were not as effective in the clinical setting as expected.[31]

Taken together, current asthma treatments have shown limited efficacy in significantly

impacting goblet cell hyperplasia and mucus hypersecretion in asthma patients. Therefore, it is necessary to develop new treatments that specifically target mucin production and regulation in asthma.

1.5. Sphingosine-1-phosphate (S1P) and S1P receptor (S1PR) biology

Sphingosine-1-phosphate (S1P) is a bioactive metabolite of sphingolipid that plays a crucial role in various cellular processes, including cell survival, proliferation, migration, angiogenesis, and immune cell trafficking.[32,33] S1P is produced by the phosphorylation of sphingosine either by sphingosine kinases 1 or sphingosine kinases 2, and it is dephosphorylated to sphingosine by S1P phosphohydrolases, or is irreversibly degraded by S1P lyase.[34] S1P acts in the intracellular signaling as a second messenger to regulate cellular biologic processes by interacting with specific intracellular targets, such as histone deacetylases (HDACs), TNF receptor-associated factor 2 (TRAF2), and prohibitin 2 (PHB2).[35]

On the other hand, S1P produced intracellularly can be secreted from cells and it is exported to the extracellular space by several transporters, such as spinster homolog 2 (SPNS2) and ATP-binding cassette (ABC) transporters like ABCC1 and ABCG2. Once outside the cell, extracellular S1P is carried by chaperone proteins, particularly albumin and apoprotein M, which is associated with high-density lipoprotein (HDL).[34] Then, S1P can engage in extracellular signaling via G protein-coupled receptors (GPCRs), specifically the S1P

receptors (S1PRs) present on the surface of the same cell (autocrine signaling) or neighboring cells (paracrine signaling).[36] S1PRs are a family of GPCRs that consist of five subtypes from S1PR1 to S1PR5 at the present time. These receptors are differentially expressed on various cell types, and each S1PR subtype can couple with different G proteins such as G_i, G_q, or $G_{12/13}$, which elicit diverse downstream signaling cascades.[37]

S1P is present in low concentrations in most tissues, including lymphoid tissues, but is highly abundant in the blood and lymph under homeostatic conditions. The S1P gradient between lymphoid tissues and blood/lymph is important for lymphocyte egress from lymphoid organs, with S1PR1 on lymphocytes playing a crucial role.[38] Under inflammatory conditions, S1P levels increase in the tissue which promote the retention of T cells and suppress T cell egress in inflamed peripheral tissues via S1PR1.[39]

S1PR4 is specifically expressed in fetal and adult lymphoid tissues, hematopoietic tissues, and lungs, in contrast to S1PR1, S1PR2, and S1PR3, which are widely expressed across most tissue and cell types.[40] S1PR4 appears to play a minor role in immune cell trafficking, unlike S1PR1, except for its role in the migration of dendritic cells.[34] According to a previous study by Liu et al., S1P regulated protease-activated receptor 1 (PAR1)-mediated human platelet function in a biphasic manner, depending on the concentration of S1P and involving types of S1PRs. S1PR1 mediated the enhancing effect in response to a low concentration of S1P, whereas S1PR4 and S1PR5 mediated the inhibitory effect at high concentrations of S1P.[41]

Another study reported that S1PR4 mediates the immunosuppressive effects of S1P on T cells by inhibiting their proliferation and the secretion of effector cytokines, such as IL-4, IFN-γ, and IL-2, while enhancing the secretion of the suppressive cytokine IL-10.[42]

Given the highly restrictive expression of S1PR4 in lymphoid tissues, peripheral leukocytes, and lung, as well as its role in immune regulation, its involvement in the pathophysiology of asthma, which is a prime example of an inflammatory and immune-related disorder, can be strongly inferred. Moreover, based on existing research, the active role and operating mechanism of S1PR4 may be distinct from those of other S1PRs, such as S1PR1.

1.6. The expression of S1P and S1PR4 in asthma

A robust correlation and reproducibility have been reported between the dysregulation of the sphingolipid synthesis pathway due to a genetic mutation controlling ORMDL sphingolipid biosynthesis regulator 3 (ORMDL3) and childhood-onset asthma.[43-45] Furthermore, the implications of the S1P and S1PR pathway in the pathogenesis of adult asthma and in experimental models have been previously reported in several studies. S1P levels were markedly increased in the bronchoalveolar lavage (BAL) fluids of asthmatic patients compared to healthy controls following segmental allergen challenge.[46] In the metabolomic analysis using serum samples of the adult asthma cohort, S1P was increased in the moderate and severe asthma groups compared to the healthy control and mild asthma

groups, showing an increase with asthma severity independent of steroid use.[47] S1P enhanced mast cell degranulation and cytokine production in response to IgE and antigen challenges in animal model.[48] S1P administration to BALB/c mice induced airway hyperreactivity, increased mucus production, and lung inflammation associated with elevated prostaglandin D_2, IgE, IL-4, and IL-13.[49] Contrary, inhibition of S1P using sphingosine kinase 1 inhibitor significantly reduced OVA-induced airway inflammation and AHR to methacholine.[50]

S1PR4 was predominantly expressed in peripheral blood mononuclear cells (PBMCs) and in the lungs, specifically in the alveoli and macrophages of asthmatic patients.[51] Interestingly, the relative expression of S1PR4 mRNA in the PBMCs of asthma patients was elevated from the baseline following standardized asthma treatment, in contrast to the depressed S1PR1 mRNA expression. The reduced expression of S1PR4 was further confirmed in the lungs and induced sputum of asthma patients compared to the healthy control subjects. This reduction was more remarkable in the neutrophilic asthma patients with less than 3% eosinophils in the induced sputum compared to eosinophilic asthma patients.[51]

The expression of S1PR4 was also reported in mucin-productive diseases other than asthma, such as bronchiectasis and protracted bacterial bronchitis. In this study, the expression of S1PR4 was significantly decreased in bronchiectasis patients compared with control subjects.[52]

Collectively, S1P levels were elevated in asthma in accordance with the asthma severity,

while the expression of S1PR4 was decreased in the lung tissues of asthma patients and in BAL fluids from mucin-productive diseases, suggesting the potential inhibitory or regulatory role of S1PR4.

1.7. The potential role of S1PR4 in goblet cell hyperplasia in asthma

Although the specific role of the S1P and S1PR pathway in asthmatic airway remodeling is not yet well understood, there is some evidence of its involvement in mucous cell hyperplasia in asthma. In an acute allergic asthma model, the inhibition of sphingosine kinase 1, leading to reduced S1P levels, resulted in decreased goblet cell metaplasia in the bronchial epithelium as well as reduced airway eosinophilic inflammation and airway resistance. This effect was achieved through the downregulation of MUC5AC expression via the NOTCH2 and SPDEF signaling pathways.[53] A nonspecific S1PR functional antagonist, FTY 720, showed the same effect on goblet cell hyperplasia in both Th1- and Th2-mediated asthma models.[54] However, the role of specific S1PR in asthmatic airway remodeling, particularly in goblet cell hyperplasia, remains unclear in these studies. Moreover, previous studies on S1PR4 have focused on its role in airway inflammation in acute asthma models, providing limited information regarding mucous cell hyperplasia and airway remodeling.

We hypothesized that down-regulated S1PR4 may contribute to enhanced goblet cell hyperplasia and mucus hypersecretion under chronic airway inflammation and recurrent

bronchial constriction. To study its role in goblet cell hyperplasia as part of asthmatic airway remodeling, it was necessary to establish an appropriate experimental model.

Hence, this study aimed to elucidate the impact and mechanisms of the S1PR4 pathway in mucus hypersecretion and to establish an inflammation and bronchoconstriction-driven chronic asthma remodeling model to study the role of S1PR4 in mucus hypersecretion and goblet cell hyperplasia within the context of chronic airway remodeling in asthma.

2. Materials and methods

2.1. Mice

In experiments to establish an animal model of asthmatic airway remodeling, female C57BL6 mice purchased from Orient Bio (Seongnam, Korea) were utilized. For the experiment to investigate the effects of S1PR4 depletion, both *S1pr4* heterozygous mice and their littermate wild-type (WT) control were used. *S1pr4* heterozygous knockout (*S1pr4*$^{+/-}$) mice were purchased from the Jackson Laboratories (mouse strains, 005799; Bar Harbor, ME, USA). Because homozygous mutation of *S1pr4* results in mid-gestation embryonic lethality, *S1pr4*$^{+/-}$ mice were used.

The depletion of S1PR4 was confirmed by polymerase chain reaction (PCR) genotyping using gene-specific primers on tail tissue samples from their offspring (Table S1). Mating and maintenance of mice were performed in the facility at specific pathogen-free room, and all animal experiments were approved by the Institutional Animal Care and Use Committee of Asan Medical Center.

2.2. Generation of a murine model for inflammation- and bronchoconstriction-driven asthma remodeling

To induce a murine model of asthmatic airway remodeling, on days 0 and 14, ovalbumin (OVA, 100 μg/mouse) and aluminum hydroxide (alum, 2 mg/mouse) were mixed with

phosphate-buffered saline (PBS), and 200 μL of the resulting solution was administered intraperitoneally to induce systemic sensitization. One week after the last sensitization, mice were challenged with OVA (20 μg per mouse) via intranasal administration twice a week for 4 weeks. The dosage of OVA challenge in this model was relatively low at 20 μg per mouse compared to 50 μg of an allergic airway inflammation model in the previous study to reduce the contributions of the inflammatory response in the formation of airway remodeling.[55] In order to induce repeated bronchoconstriction, aerosolized methacholine inhalations were administered three times a week for 4 weeks concurrent with the OVA challenges (Fig 1). Mice were sacrificed 24 hours following the last challenge, on day 49, and the BAL fluids, lung, and airway tissues were used for analysis.

2.3. Preparation and analysis of BAL fluids

To obtain the BAL fluids, the mice were anesthetized with an intraperitoneal injection of avetin (1.2% tribromoethanol). We then washed mouse airway with 2 ml of cold, sterile Dulbecco's phosphate-buffered saline (DPBS). Next, we separated immune cells from BAL fluids by centrifugation at 4 ˚C, 4000 rpm for 10 minutes, and counted total cell with 0.4% trypan blue solution (Gibco) after treatment with RBC lysis buffer (Stemcell, Vancouver, Canada). For differential cell count, we counted 300 inflammatory cells after Diff-Quick staining (Sysmex, Kobe, Japan).

2.4. Cytokine analysis in BAL fluids by ELISA

To assess cytokine concentrations in BAL fluids, we quantify the levels of IL-4, IL-13, and IL-33 in each sample using the mouse Quentikine or mouse ELISA Duoset. All of these kits were purchased from R&D systems and used following the manufacturer's guidelines.

2.5. Micro-Computed Tomography (micro-CT) analysis

Mice were anesthetized with intraperitoneal administration of thiopental sodium and the mouse lung imaging was performed using the micro-CT scanner (SkyScan 1176; SkyScan, Aarselaar, Belgium). The images were acquired at 0.5 aluminum filter, 50 kVp, 200 μA and 300 ms/frame with 360 views. The image field width was up to 68 mm and the voxel size was 35 x 35 x 35 μm. The scan duration was approximately 24 minutes per mouse. The acquired images were reconstructed in each mouse.

2.6. Histopathological analysis

Blood depleted mouse lung tissues perfused with cold, sterile-DPBS were fixed with 4% neutral-buffered formalin solution for 24 h. A paraffin block was prepared with fixed, dehydrated lung, tracheal, and bronchial tissues which were then cut into 5-μm-thick sections. The sectioned tissues were embedded and stained with hematoxylin and eosin (H&E) solution,

Masson’s trichrome (MT) solution, Periodic Acid-Schiff (PAS) solution, and Alcian Blue solution after deparaffinization. All slides were observed under light microscope.

2.7. Reverse transcription quantitative real-time PCR (RT-qPCR) analysis

Total RNA was isolated from lung tissues using Trizol reagent according to the manufacturer’s instructions. Complementary DNA (cDNA) synthesis was performed with random primers using a reverse transcription kit in each sample. The target cDNA levels were quantified by real-time PCR analysis using gene-specific primers for S1PR4, MUC5AC, MUC5B, SPDEF, zona occludens-1 (ZO-1), occludin, claudin-3, 4, 5, 18.1, and 18.2, E-cadherin, N-cadherin (Table S1). The relative expression levels of each gene were normalized to that of β-actin.

2.8. Hydroxyproline assay

The hydroxyproline content in lung tissues was measured using a colorimetric assay kit (BioVision, Catalog #K555-100) according to the manufacturer's instructions. Briefly, lung tissue samples were homogenized in distilled water (1:10, w/v) and hydrolyzed in 12N hydrochloric acid at 120°C for 3 hours. The hydrolysate was then neutralized and oxidized by adding chloramine-T solution and incubated at room temperature for 25 minutes. Next, Ehrlich's reagent was added, and the mixture was incubated at 60°C for 35 minutes. The

absorbance was measured at 550 nm using a microplate reader. The hydroxyproline concentration was calculated based on a standard curve generated with known concentrations of hydroxyproline. The results were expressed as level of hydroxyproline (μg) per weight of lung tissues (mg).

2.9. RNA isolation, library preparation, and sequencing

Total RNA was isolated from lung tissues using Trizol reagent (Invitrogen). RNA quality was assessed by TapeStation 4000 System (Agilent Technologies, Amstelveen, The Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA). Libraries were prepared from total RNA using the CORALL RNA-Seq V2 Library Prep Kit (LEXOGEN, Inc., Austria). The isolation of mRNA was performed using the Poly(A) RNA Selection Kit (LEXOGEN, Inc., Austria). The isolated mRNAs were used for the cDNA synthesis and shearing, following manufacture's instruction. The enrichment step was carried out using of PCR. Subsequently, libraries were checked using the TapeStation HS D1000 Screen Tape (Agilent Technologies, Amstelveen, The Netherlands) to evaluate the mean fragment size. Quantification was performed using the library quantification kit using a StepOne Real-Time PCR System (Life Technologies, Inc., USA). High-throughput sequencing was performed as paired-end 100 sequencing using NovaSeq 6000 (Illumina, Inc., USA). Data mining and graphic visualization were performed using ExDEGA (Ebiogen Inc.,

Korea).

2.10. Statistical analysis

All of the data were presented as mean ± standard deviation (SD) using GraphPad Prism software version 8.0.2 (GraphPad, La Jolla, CA). One-way or two-way ANOVA was used to compare variables among multiple groups. Dunn's test of Tukey's test was applied for post-hoc analysis of multiple comparisons. The *p*-values were adjusted and presented for comparisons between groups as follows: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

3. Results

3.1. Establishment of an animal model for inflammation- and bronchoconstriction-driven asthmatic airway remodeling

3.1.1. An asthmatic airway remodeling model induced by OVA sensitization and challenge, with concurrent methacholine inhalation

Previous studies have shown that both airway inflammation and mechanical stress caused by bronchoconstriction can independently reproduce characteristic features of asthmatic airway remodeling, such as subepithelial collagen deposition and goblet cell hyperplasia.[11,16,17] In order to elucidate the impact and mechanisms of the S1PR4 pathway in asthmatic airway remodeling, particularly goblet cell hyperplasia, we developed an animal model that incorporates both airway inflammation and repeated bronchoconstriction. Our experimental model of asthmatic airway remodeling was sensitized with OVA on days 1 and 14. One week later, it was subjected to concurrent exposure to OVA challenges twice a week and methacholine inhalation three times a week over a period of 4 weeks (a 7-week model). (Fig 1A). At the beginning of the experiment, to establish a chronic airway remodeling model, OVA challenges and methacholine inhalation were performed for six weeks following OVA sensitization (a 9-week model). However, in this model, six out of eight animals exposed to concurrent OVA challenges and methacholine inhalation succumbed, leading us to reduce the duration of both OVA challenges and methacholine inhalation to four weeks. In the revised

model, all mice survived until the scheduled time of sacrifice (Fig 1B). In the present study, we named this model the OVA-Mch asthmatic remodeling model.

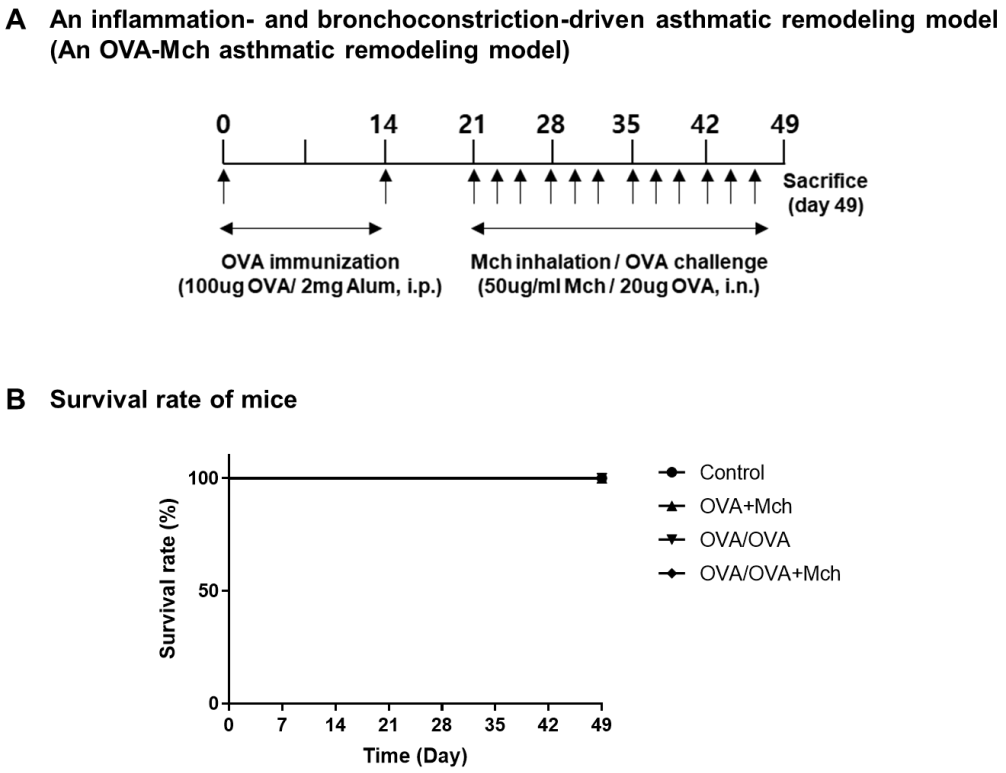


Figure 1. Protocol for an inflammation- and bronchoconstriction-driven asthmatic airway remodeling model.

Wild type (C57BL6) mice were sensitized through intraperitoneal (i.p.) injections of OVA/alum on days 0 and 14, followed by intranasal (i.n.) OVA challenges twice a week and aerosolized methacholine inhalations three times a week for 4 weeks (A). This experiment included three mice in the control group and eight mice in the experimental groups; all mice survived until the time of sacrifice (B).

3.1.2. Phenotypic characteristics of the OVA-Mch asthmatic remodeling model

To assess the effects of chronic methacholine inhalation in the OVA-induced allergic inflammation model, first we evaluated the infiltration of immune cells in BAL fluids. The total number of cells in the BAL fluid was significantly higher in the OVA-Mch asthmatic remodeling group (the OVA/OVA+Mch group) compared to the control group and the group that inhaled methacholine after OVA immunization alone, but there was no statistically significant difference compared to the standard OVA-induction group (Fig 2A). For differential cell counts, the number of eosinophils in the OVA-Mch asthmatic remodeling group was higher than in the control group and the group that inhaled methacholine after OVA sensitization alone, but comparable to the standard OVA group (Fig 2B).

Micro CT scans were performed to evaluate structural changes in the lungs and airways of mice from different experimental groups. Both the standard OVA group and the OVA-Mch induced model showed increased whitish patches on the lungs compared to the control group or the group that methacholine inhalation alone after OVA sensitization. Notably, the OVA-Mch asthma remodeling group exhibited more prominent peribronchial patches and airway narrowing (Fig 3).

Next, the infiltration of cells, collagen deposition, and mucus production were confirmed in mouse lung tissue using H&E, MT staining, and PAS staining (Fig 4). Inflammatory cell infiltrations were increased in the lungs of the OVA-Mch asthma remodeling model, similar

to the degree seen in the standard OVA-induced model. In MT staining, collagen deposition was slightly increased in the OVA-Mch asthmatic remodeling model compared to the standard OVA group. PAS staining of lungs revealed that mucus production was increased in both the standard OVA group and the OVA-Mch asthmatic remodeling group, with a tendency for higher levels following methacholine inhalation. However, methacholine inhalation alone, following OVA sensitization, did not result in increased collagen deposition or mucus production compared to the control group.

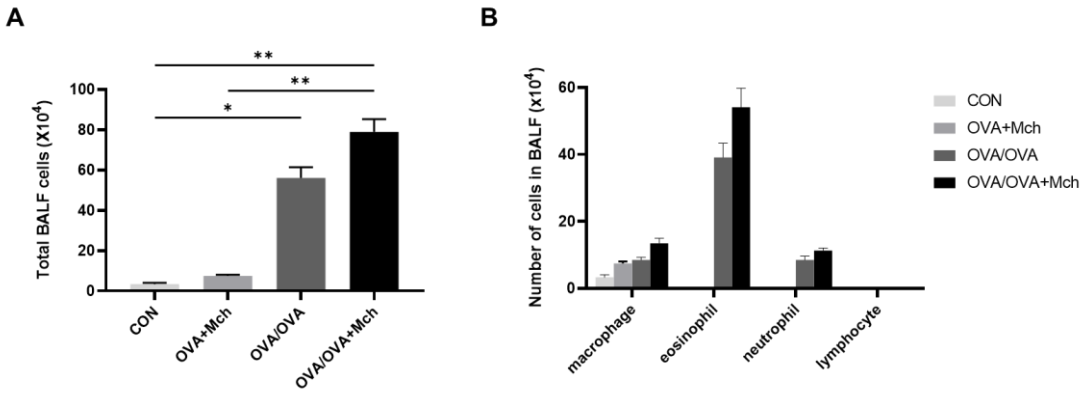


Figure 2. The OVA-Mch asthma remodeling model exhibited airway inflammation comparable to that observed in the standard OVA-induced asthma model.

Total cell counts in BAL fluids from different groups (A), and differential cell counts in BAL fluids (B). One-way ANOVA followed by Dunn's multiple comparison test was used to calculate the adjusted *p*-values for comparisons between groups: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

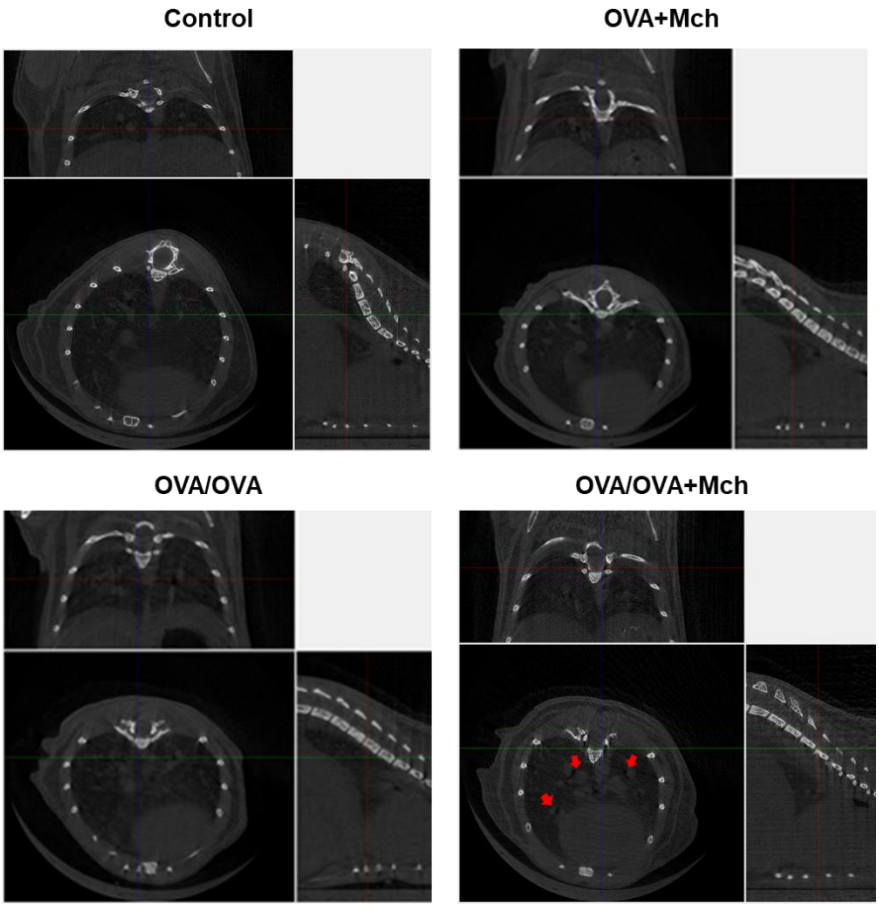


Figure 3. Increased peribronchial patches and airway narrowing in the OVA-Mch asthma remodeling model.

Micro-CT scans were performed on the lungs of mice from different experimental groups. Representative images from each group were shown. Red arrows indicate peribronchial patches and bronchial luminal narrowing in the OVA-Mch asthma remodeling model.

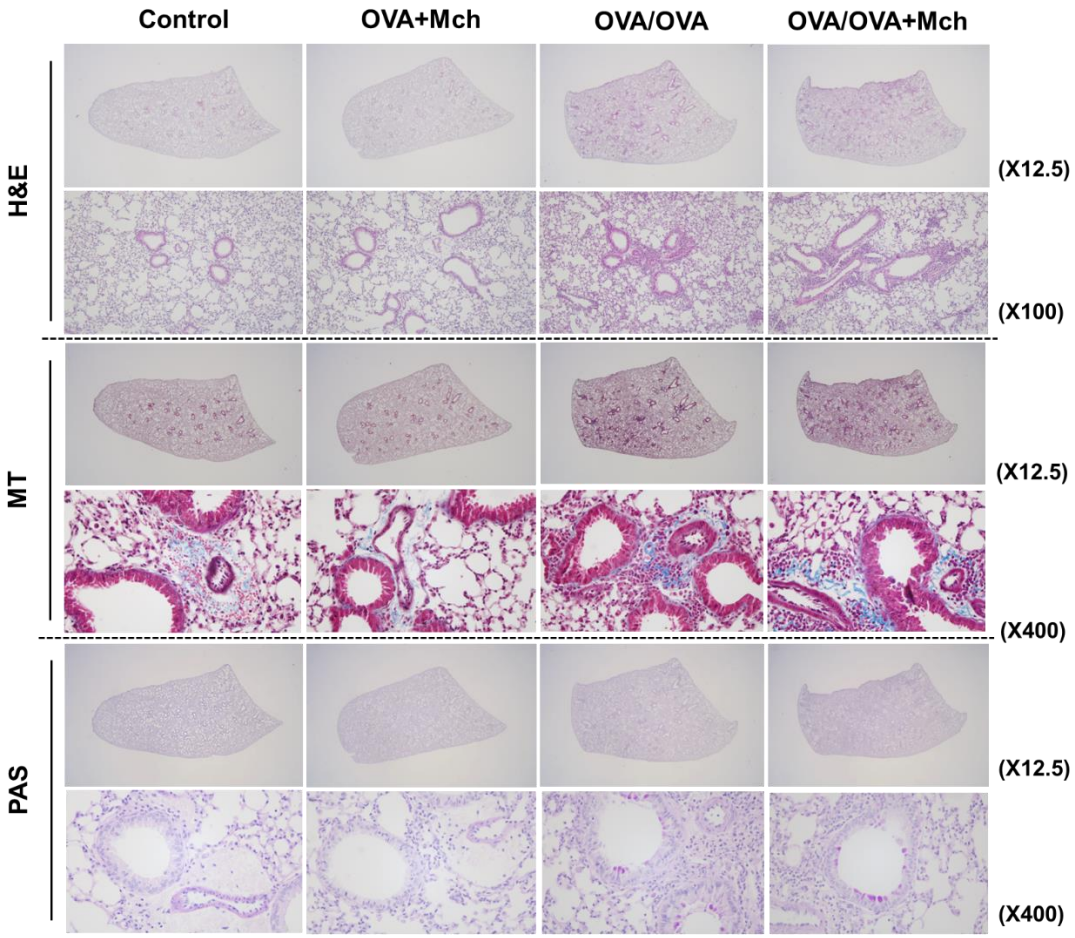


Figure 4. Mucin production and collagen deposition were increased in lung tissue of an OVA-Mch asthma remodeling model.

H&E (x12.5, x100), MT (x12.5, x400), and PAS (x12.5, x400) staining in lungs from different groups were performed. Each set of experiment consisted of three mice for the control group and eight mice for the experimental groups.

3.1.3. The expression of mucins and tight junction-associated genes was altered in the OVA-Mch asthmatic remodeling model

Based on the enhanced peribronchial patches and airway narrowing observed in the micro-CT images, along with increased mucin-positive cells and collagen deposition in the lung pathology of the OVA-Mch asthma remodeling model, the expression of mucin genes and airway epithelial remodeling-associated genes was evaluated by RT-qPCR in the lung lysates.

Relative expression of MUC5AC and MUC5B mRNA was significantly increased in the OVA-Mch asthma remodeling group compared to the control group and the group that inhaled methacholine after OVA sensitization alone. Furthermore, the Mch-OVA asthma remodeling group showed a trend toward higher expression of MUC5AC and MUC5B mRNA than in the standard OVA group (Fig 5). The levels of gene expression related to airway epithelial tight junctions, such as ZO-1, occludin, and claudin-3, 4, 5, 18.1, and 18.2, were compared among groups. Relative ZO-1 mRNA expression was significantly higher in the OVA-Mch asthma remodeling groups compared to the control and group that received methacholine inhalation alone after OVA sensitization (Fig 6A). However, other markers of tight junctions in the airway epithelium did not show statistically significant differences between the groups (Fig 6B-G). The expression of epithelial-mesenchymal transition (EMT)-related genes, including E-cadherin and N-cadherin, also did not differ significantly between groups (Fig 6H and I). In addition, total hydroxyproline levels, a major component of collagen, corrected for lung

weight, did not differ significantly between the groups (Fig 7).

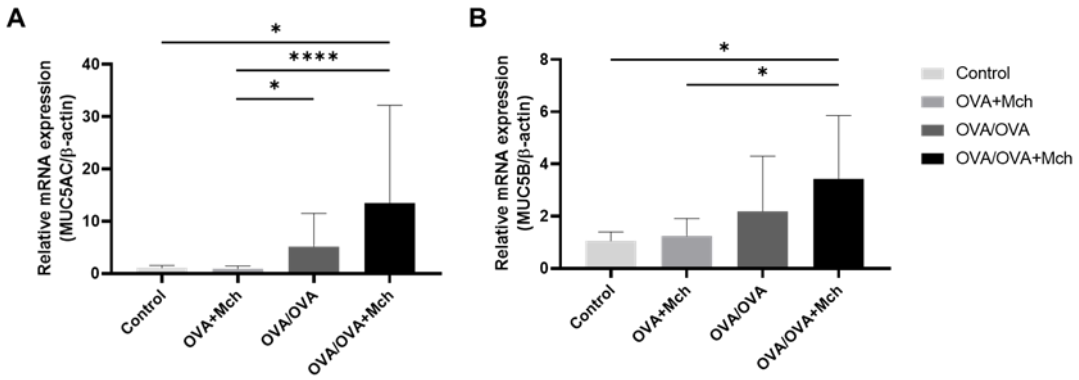


Figure 5. The expression of MUC5AC and MUC5B mRNA was highest in the OVA-Mch asthma remodeling model.

Relative mRNA expression of the MUC5AC gene (A) and the MUC5B gene (B), both compared to the β-actin gene, was measured by RT-qPCR in lung lysates from different groups. This is a combined analysis of the results of two experiments using the same protocol. One-way ANOVA followed by Dunn's multiple comparison test was used to calculate the adjusted *p*-values for comparisons between groups: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

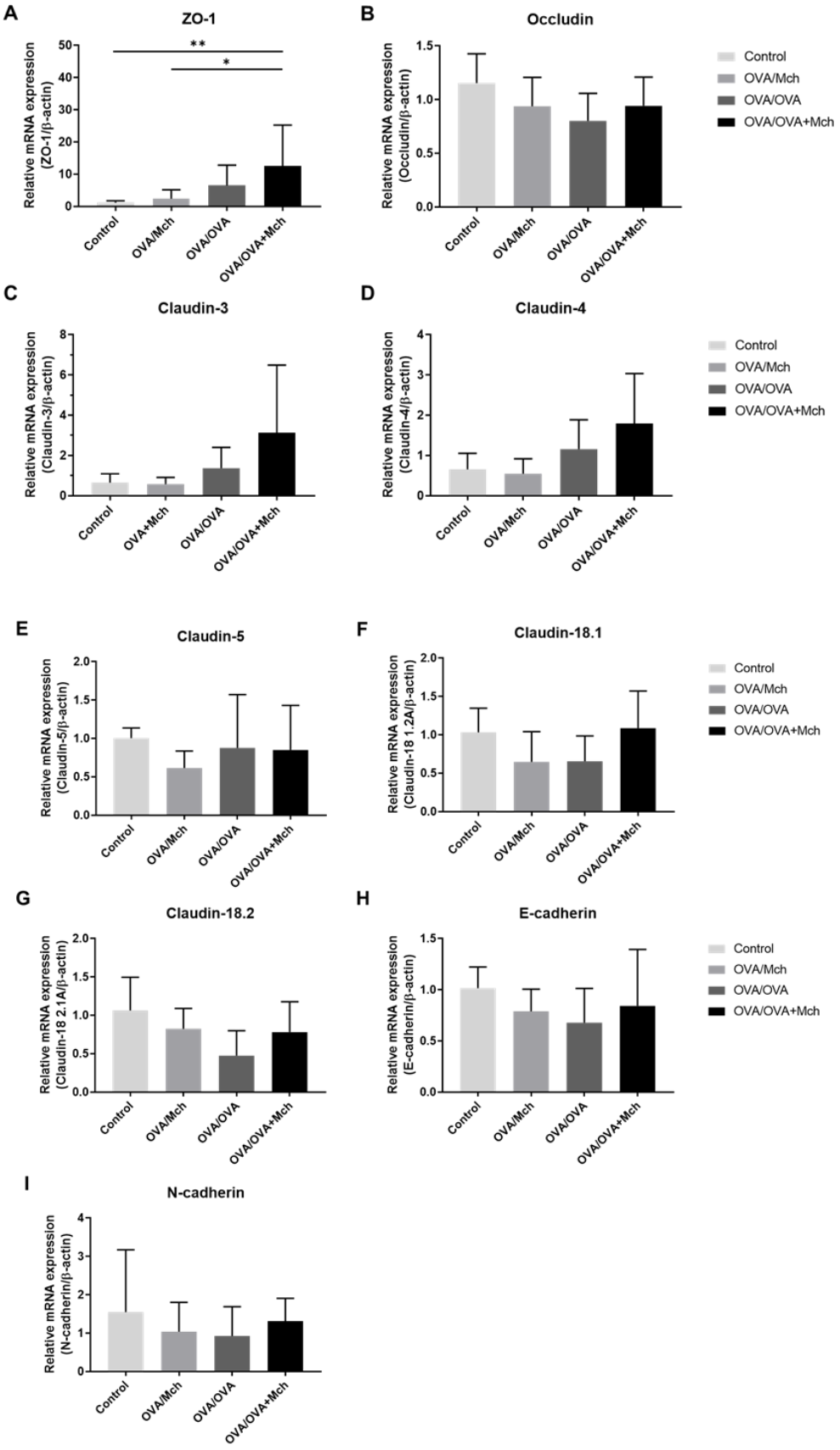


Figure 6. The expression of tight junction-associated genes was altered in the OVA-Mch asthma remodeling model.

mRNA expression levels of genes related to airway epithelial tight junctions (ZO-1, occludin, claudin-3, 4, 5, 18.1, and 18.2) and epithelial-mesenchymal transition (E-cadherin, N-cadherin) were measured relative to β-actin by RT-qPCR in lung lysates from different experimental groups. Each experiment included four mice in the control group and six mice in the other groups. The adjusted *p*-values were reported using one-way ANOVA followed by Dunn's multiple comparison test between groups: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

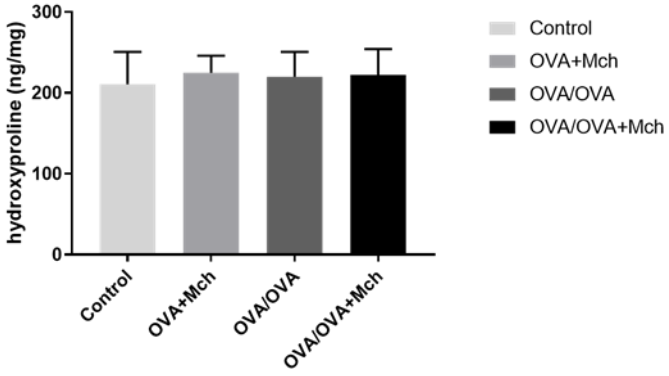


Figure 7. The hydroxyproline level in lung tissues did not differ among the experimental groups.

Total lung hydroxyproline levels, normalized by dividing by the lung weight of the respective group, were compared between groups. This experiment included three mice in the control group and eight mice in each of the experimental groups.

3.2. Investigating the role of S1PR4 in the inflammation- and bronchoconstriction-driven asthma remodeling model

3.2.1. The effect of S1PR4 depletion on modulating airway inflammation in the OVA-Mch asthma remodeling model

To investigate the role of S1PR4 in the inflammation- and bronchoconstriction-driven asthma remodeling model, we subjected WT and *S1pr4*$^{+/-}$ mice to the OVA-Mch asthmatic airway remodeling protocol (Fig 9A). First of all, we confirmed that the relative mRNA expression of S1PR4 in the lung was reduced by about half in *S1pr4*$^{+/-}$ mice compared to WT mice (Fig 8).

Mice had the lowest survival rates in the OVA-Mch asthma remodeling model in both WT and *S1pr4*$^{+/-}$ mice; however, S1PR4 depletion did not further increase mortality (Fig 9B). In BAL fluid analysis, S1PR4 depletion tended to reduce total inflammatory cell counts and eosinophil levels in the standard OVA-induced group. On the other hand, in the OVA-Mch asthma remodeling group, S1PR4 depletion did not affect total inflammatory cell counts or eosinophil counts (Fig 10). The level of IL-13 in BAL fluid was significantly increased in both the standard OVA induction group and the OVA-Mch asthma remodeling group, compared to the control group and group with methacholine alone after OVA sensitization, in both WT and *S1pr4*$^{+/-}$ mice. In addition, the OVA-Mch asthma remodeling group exhibited higher levels of IL-13 than the standard OVA induction group. In particular, S1PR4 depletion exacerbated IL-

13 secretion in BAL fluid in the OVA-Mch asthma remodeling group (Fig 11).

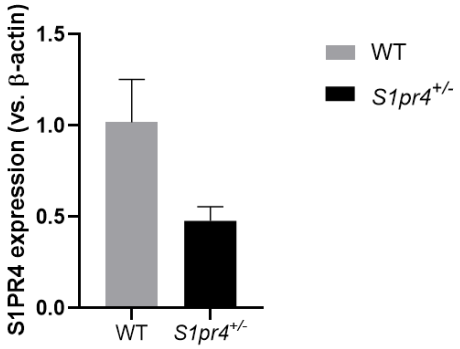


Figure 8. Reduced S1PR4 mRNA expression in lung of *S1pr4* heterozygous mice.

The relative expression levels of S1PR4 mRNA were shown in the lung of WT and *S1pr4*$^{+/-}$ mice using RT-qPCR.

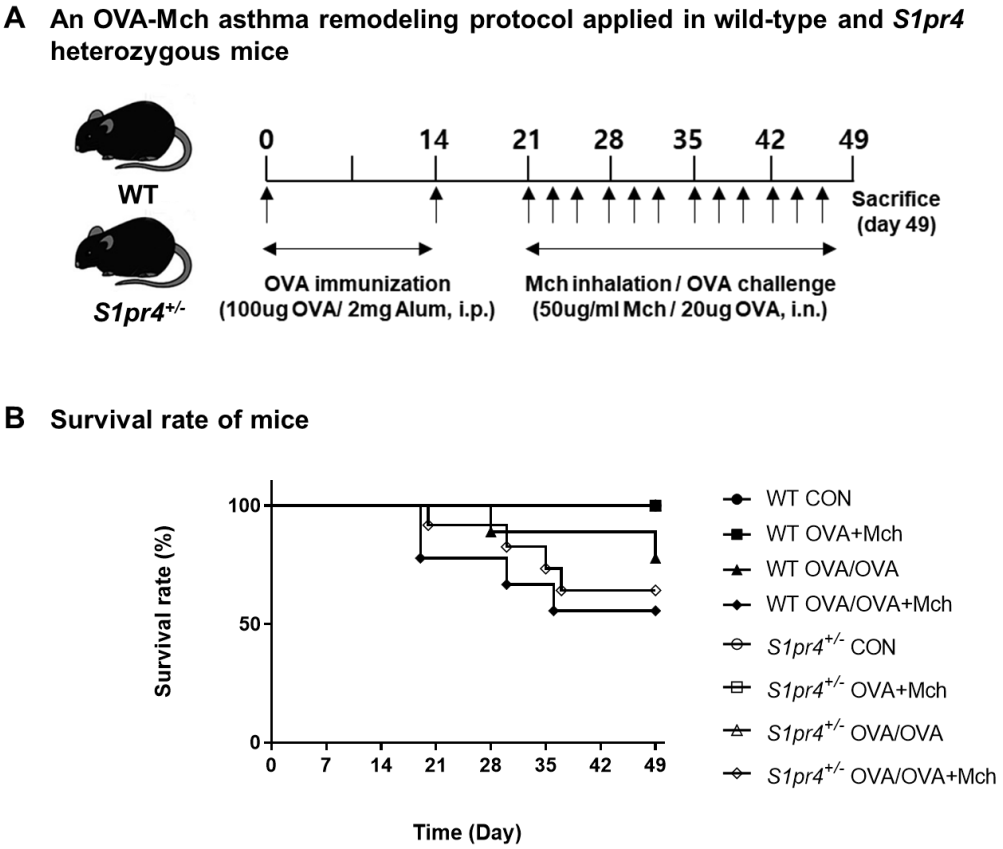


Figure 9. Application of an OVA-Mch asthma remodeling protocol in WT and *S1pr4*$^{+/-}$ mice.

WT and *S1pr4*$^{+/-}$ mice were sensitized through intraperitoneal (i.p.) injections of OVA/alum on days 0 and 14, followed by intranasal (i.n.) OVA challenges twice a week and aerosolized methacholine inhalations three times a week for 4 weeks (A). Each experiment included three mice in the control group and six mice in the OVA+Mch group. For the OVA/OVA group and the OVA/OVA+Mch group, there were nine WT mice and twelve *S1pr4*$^{+/-}$ mice, respectively. Mice had the lowest survival rates in the OVA/OVA+Mch group in both WT and *S1pr4*$^{+/-}$ mice (B).

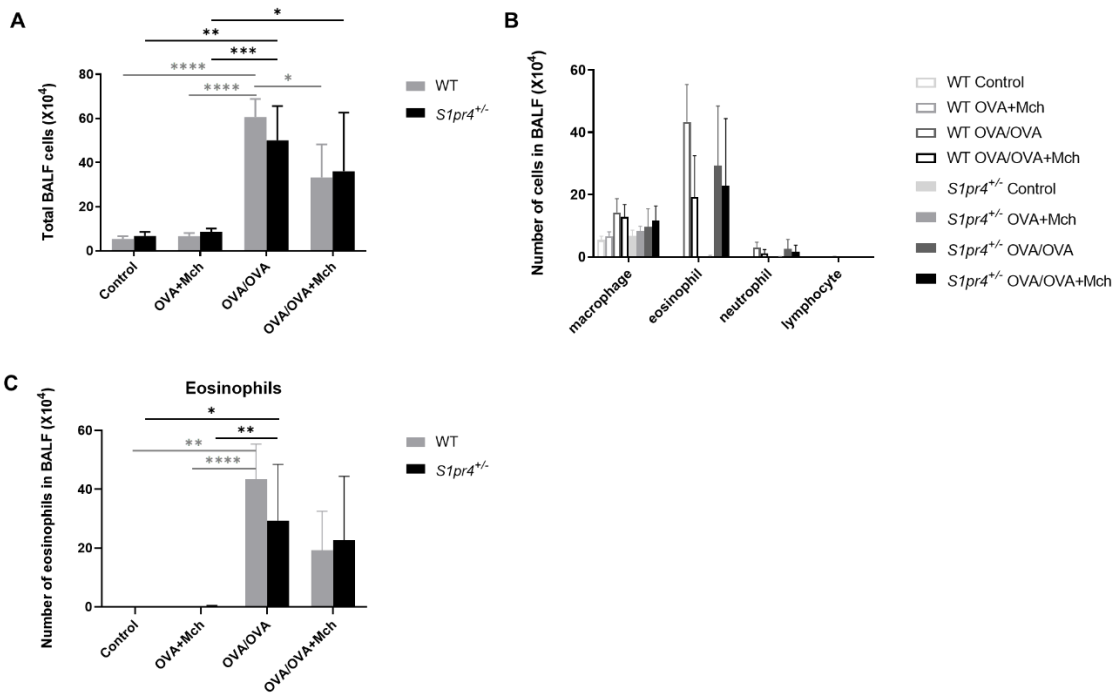


Figure 10. The total cell counts and differential cell counts in BAL fluid did not differ between WT and *S1pr4*$^{+/-}$ mice in each experimental model.

Total cell counts (A), differential cell counts (B), and eosinophil counts (C) in BAL fluid from different experimental groups in WT and *S1pr4*$^{+/-}$ mice. The adjusted *p*-values were reported using two-way ANOVA followed by Tukey's multiple comparison test between groups: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

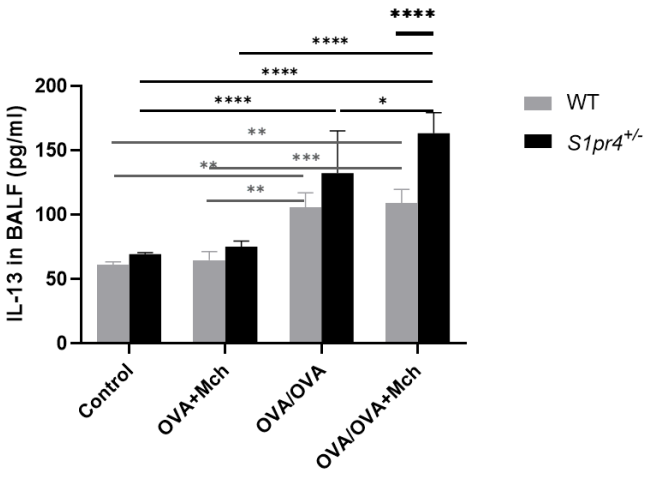


Figure 11. S1PR4 depletion exacerbated IL-13 secretion in the BAL fluid in the OVA-Mch asthma airway remodeling model.

The evaluation of the IL-13 cytokine levels in BAL fluid was performed 24 h after last allergen challenge. This experiment included three mice in the control group and nine mice in the other experiment groups. The adjusted *p*-values were reported using two-way ANOVA followed by Tukey's multiple comparison test between groups: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

3.2.2. S1PR4 depletion exacerbated the impairment of aeration in murine lungs in the OVA-Mch asthma remodeling model

Micro-CT scans were performed on the lungs of WT and *S1pr4*$^{+/-}$ mice from different experimental groups, and 3-dimensional (3D) images were reconstructed to investigate the role of S1PR4 in radiologic changes associated with airway remodeling (Fig 12). The lungs of WT mice without any intervention appear normal with clear airspaces and minimal white patches around bronchi. WT mice that inhaled methacholine only after OVA sensitization showed increased white patches and slightly narrowed bronchial lumen. In the mice of the OVA-Mch asthma remodeling group, the lungs exhibited even more white patches and airway luminal narrowing. In the *S1pr4*$^{+/-}$ mice, the radiologic changes in each experimental group appeared to be more pronounced. Particularly, S1PR4 depletion in the OVA-Mch asthma remodeling group showed increased white patches and severely disrupted airway structures, resulting in airway obstruction with atelectasis, compared to the 3D image of WT mice in the OVA-Mch asthma remodeling group.

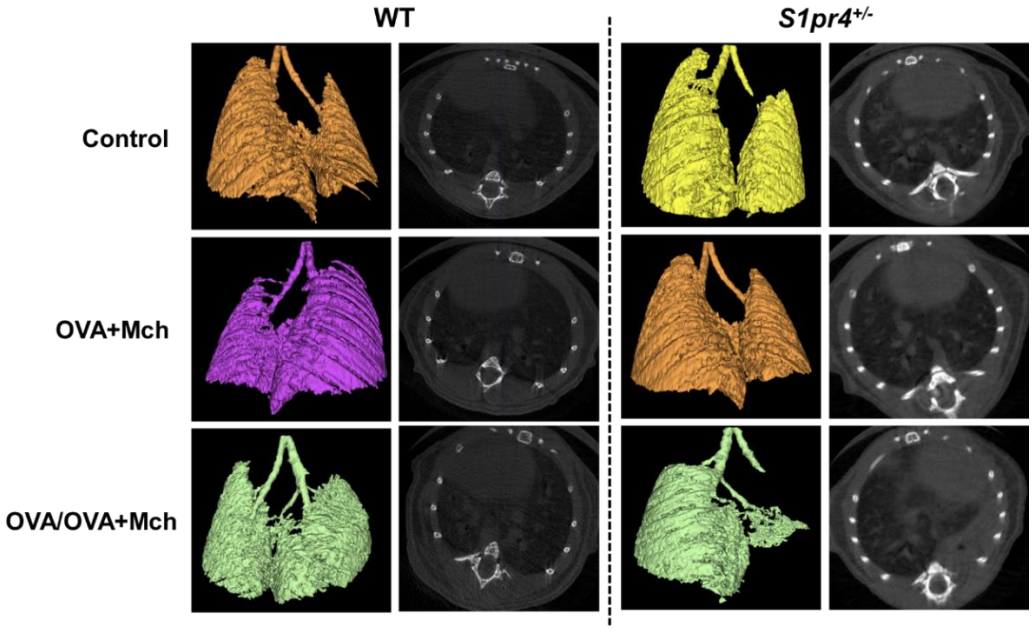


Figure 12. Depletion of S1PR4 exacerbated the impairment of aeration in murine lungs in the OVA-Mch airway remodeling model.

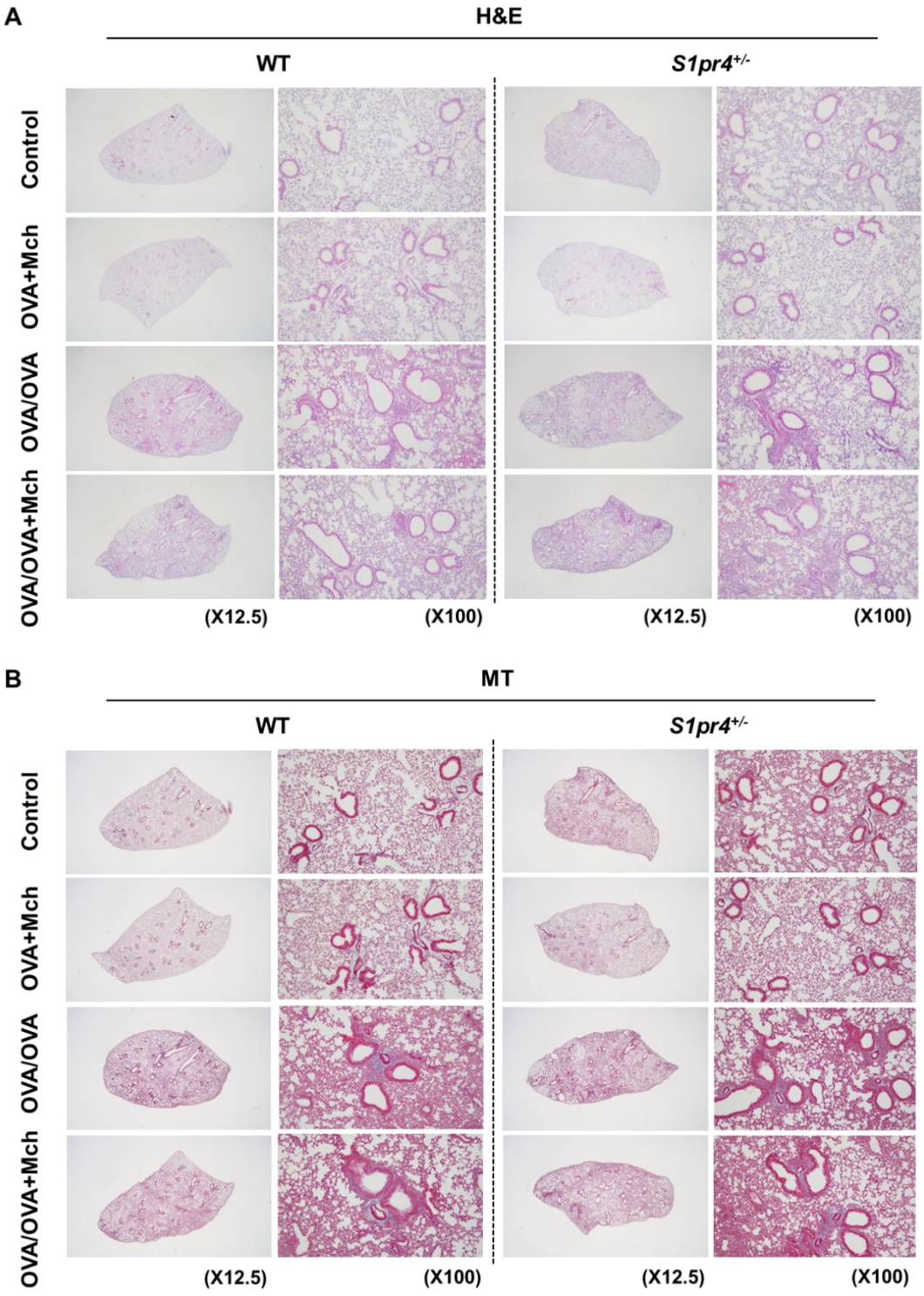
Micro-CT scans were performed on the lungs of WT and *S1pr4*$^{+/-}$ mice from different experimental groups, and 3D images were reconstructed. Representative images from each group were shown.

3.2.3. S1PR4 depletion exacerbated goblet cell hyperplasia and collagen deposition in airway tissue through the induction of OVA-Mch-driven airway remodeling

The effects of S1PR4 depletion in lung tissue were evaluated using H&E, MT, and PAS staining in WT and *S1pr4*$^{+/-}$ mice (Fig 13). The increase in inflammatory cell infiltration into lung tissue in the OVA-Mch asthma remodeling group of *S1pr4*$^{+/-}$ mice was similar to that observed in the corresponding group of WT mice (Fig 13A). Depletion of S1PR4 slightly increased mucus production and collagen deposition in the lung tissue of the OVA-Mch asthma remodeling group compared with the group in WT (Fig 13B and C).

As the animal model of OVA-Mch asthma remodeling mimics the airway remodeling through repetitive methacholine inhalation in OVA-induced allergic inflammation, we further investigated tracheal and bronchial tissues to assess the effects of S1PR4 on the murine airway. In the sagittal cross-sections of murine tracheal and bronchial tissues stained with MT, the collagen, which was stained blue, was more extensively distributed around the subepithelial area due to S1PR4 depletion in the OVA-Mch asthma remodeling model. This deposition was more pronounced in the bronchi than the trachea of mice (Fig 14A and B). PAS staining of murine tracheal and bronchial tissues revealed that mucin accumulation, stained a deep magenta, was more pronounced in the goblet cells and surface epithelium of *S1pr4*$^{+/-}$ mice, particularly in the OVA-Mch asthma remodeling model (Fig 15A and B). Mucus secretion was further assessed using Alcian Blue staining. The mucins, appearing as light blue areas,

were most prevalent in the goblet cells within the epithelium of *S1pr4*$^{+/-}$ murine trachea and bronchi in the OVA-Mch asthmatic airway remodeling group (Fig 16A and B).



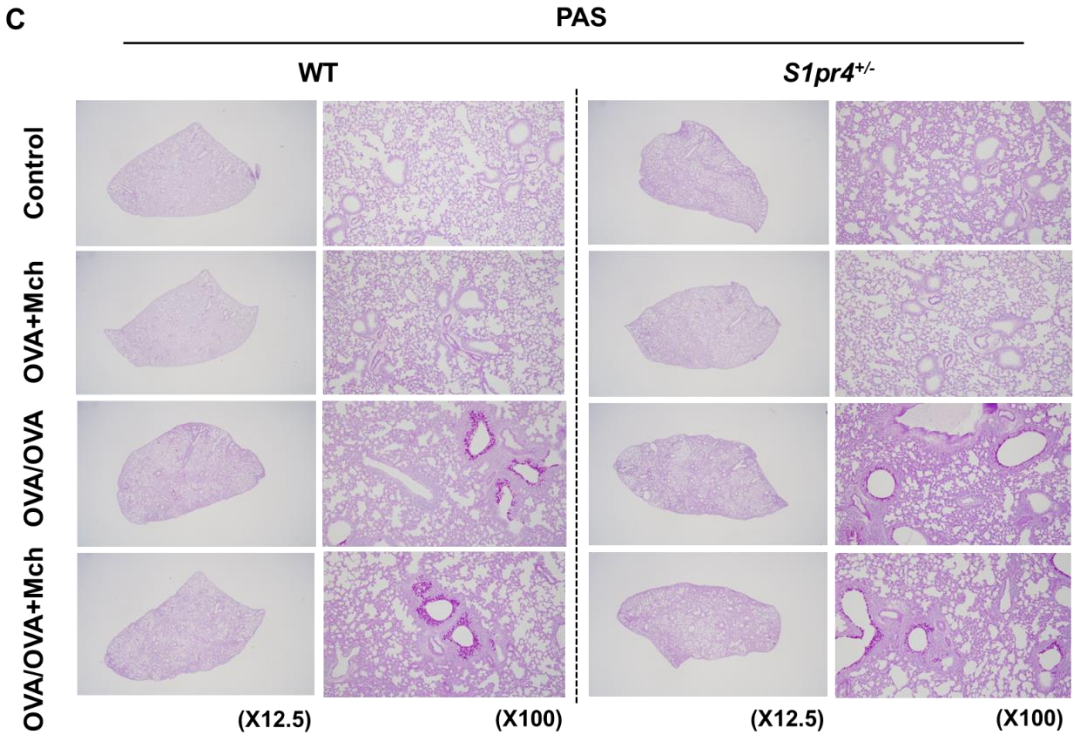
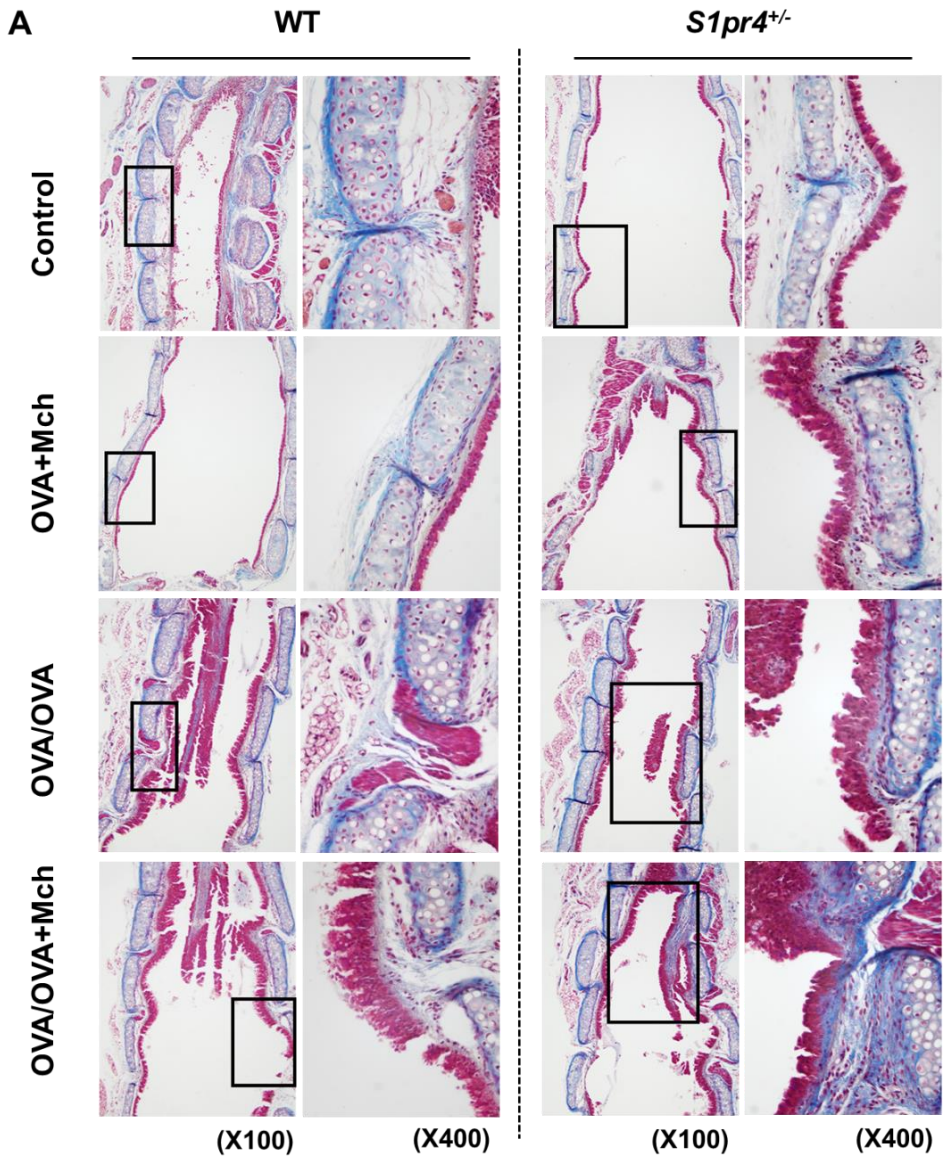


Figure 13. S1PR4 depletion exacerbated mucin production and collagen deposition in lung tissue through the induction of OVA-Mch airway remodeling.

Representative images of H&E (A), MT (B), and PAS (C) (x12.5, x100) staining of lung tissue sections from different experiment groups were compared between WT and *S1pr4*$^{+/-}$ mice.



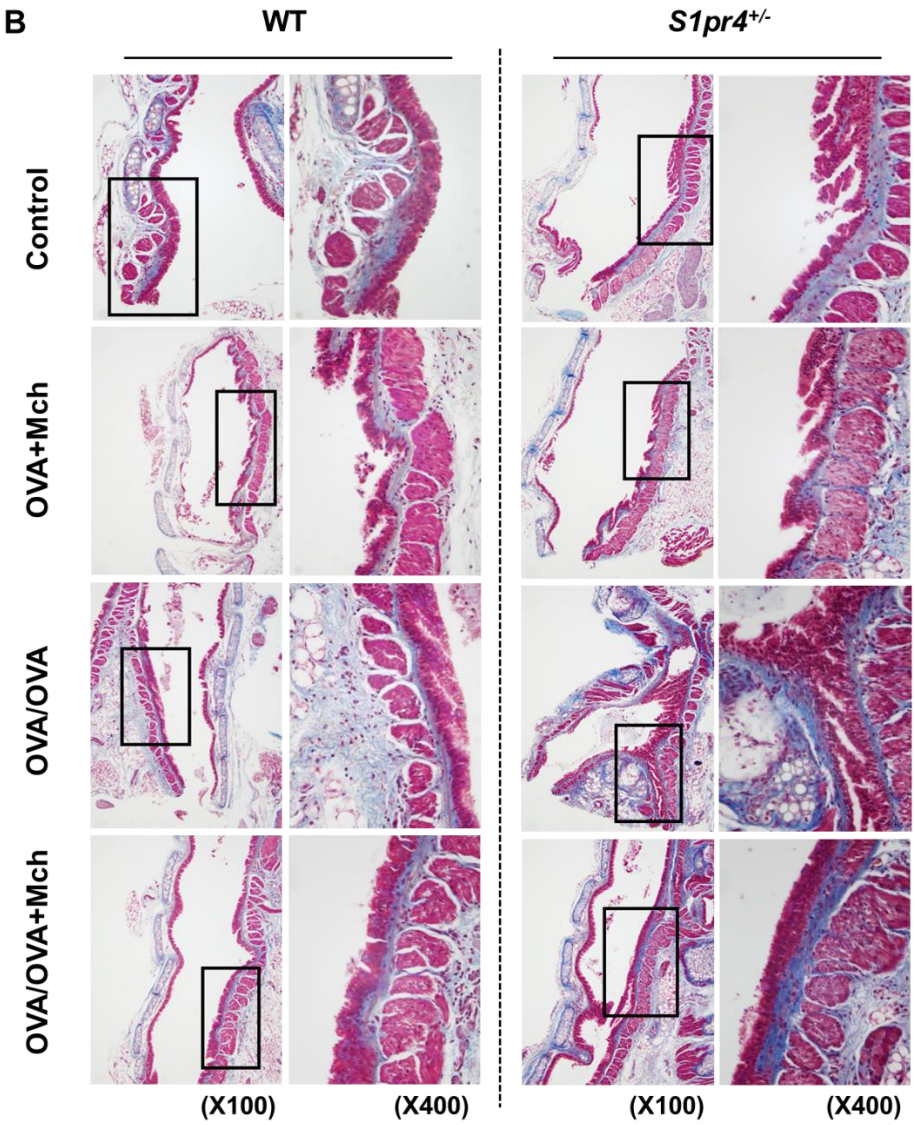
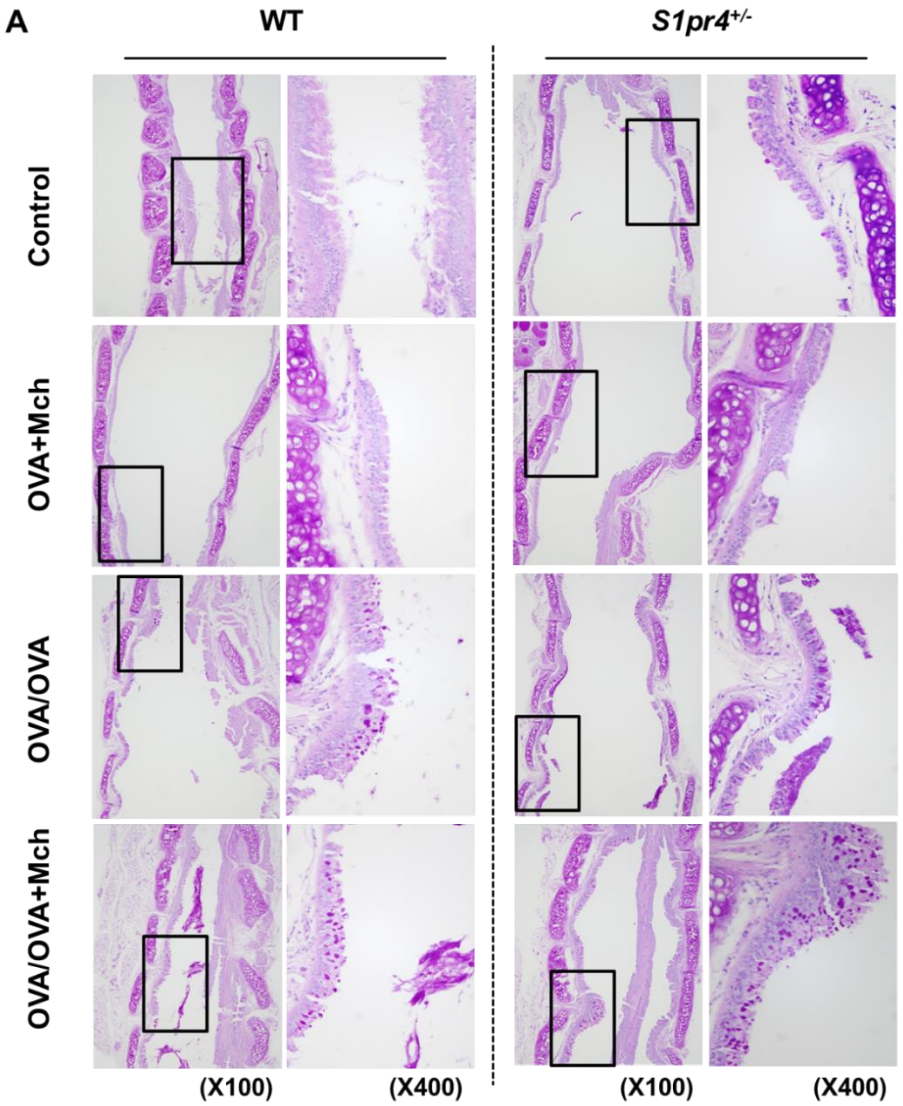


Figure 14. S1PR4 depletion exacerbated collagen deposition in the trachea and bronchi of mice through the induction of OVA-Mch airway remodeling.

Representative images of MT staining of tracheal (A) and bronchial (B) cross-sections from different experiment groups were compared between WT and *S1pr4*$^{+/-}$ mice (x100, x400). Collagen was stained blue.



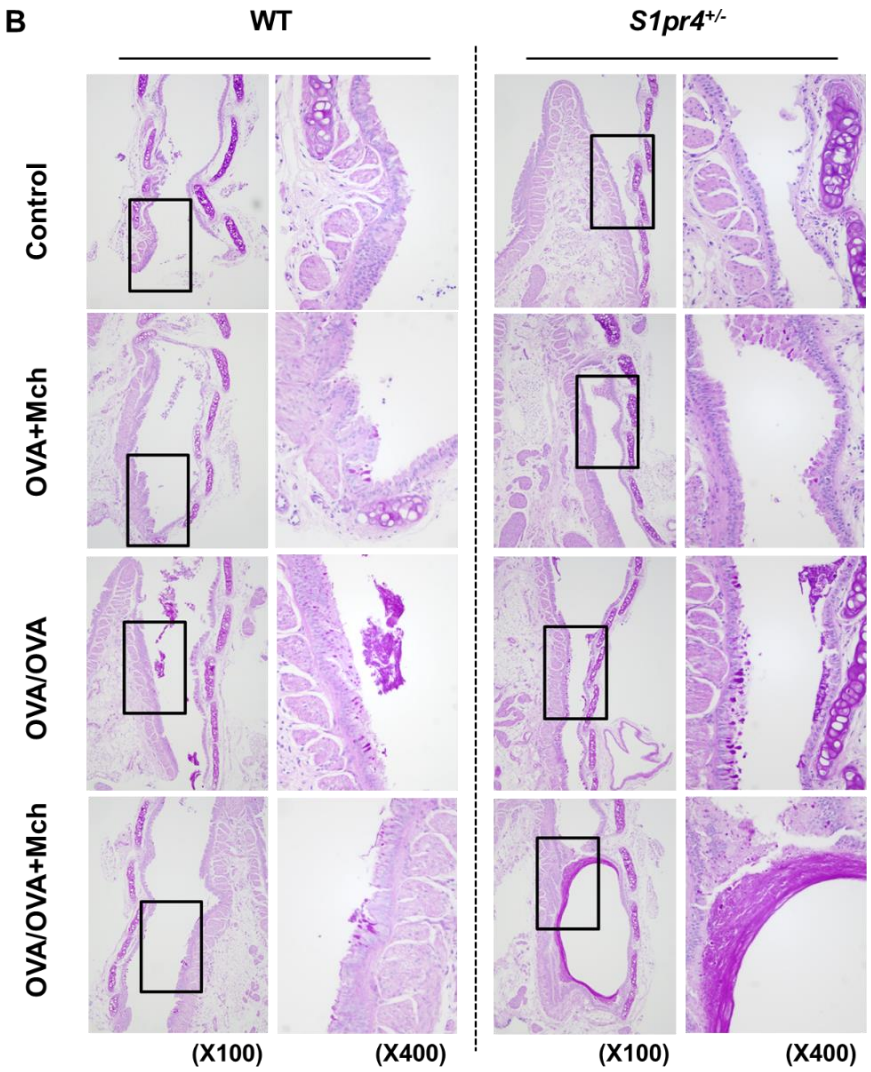
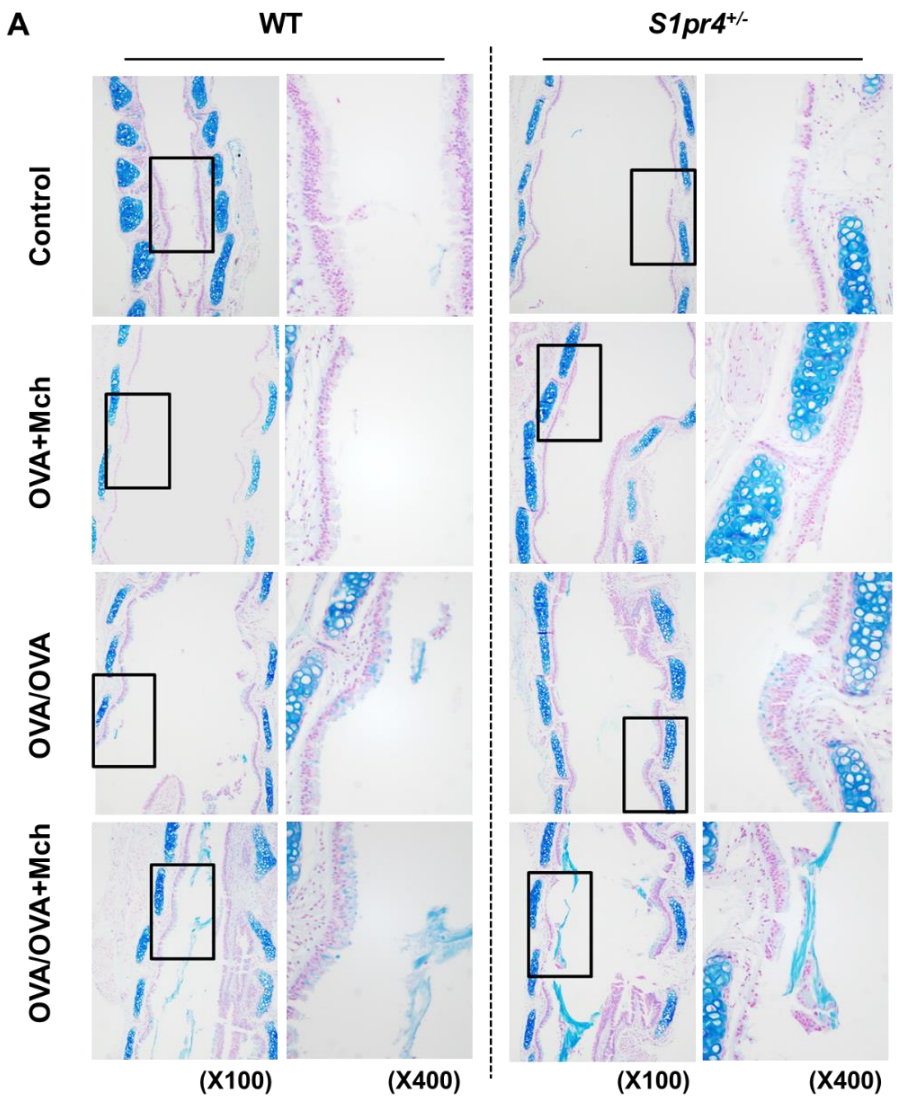


Figure 15. S1PR4 depletion exacerbated mucus production in the trachea and bronchi of mice through the induction of OVA-Mch airway remodeling.

Representative images of PAS staining of tracheal (A) and bronchial (B) cross-sections from different groups were compared between WT and *S1pr4*$^{+/-}$ mice (x100, x400). The mucin was stained a magenta color.



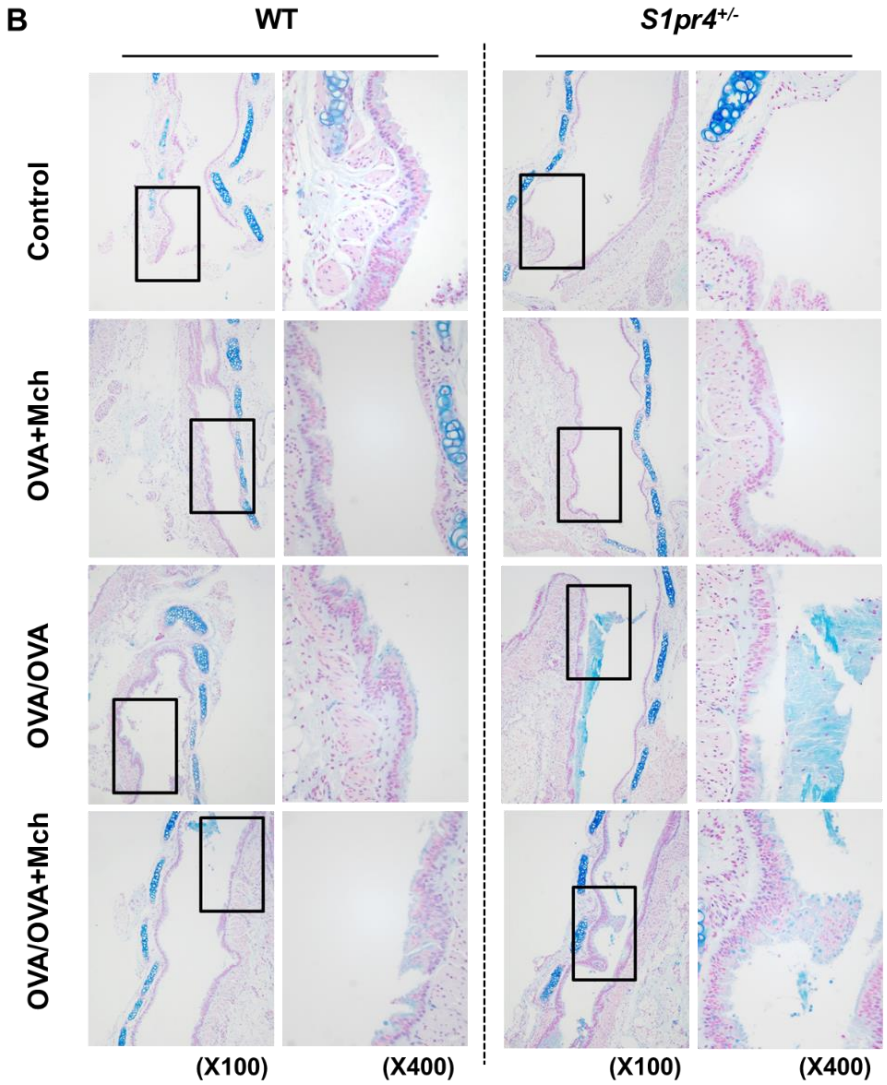


Figure 16. S1PR4 depletion exacerbated mucus production in the trachea and bronchi of mice through the induction of OVA-Mch airway remodeling.

Representative images of Alcian Blue staining of tracheal (A) and bronchial (B) cross-sections from different experiment groups were compared between WT and *S1pr4*$^{+/-}$ mice (x100, x400). The mucin was stained blue.

3.2.4. S1PR4 depletion exacerbated mucin gene expression and altered tight junction genes in the OVA-Mch airway remodeling model

Both WT and *S1pr4*$^{+/-}$ mice exhibited increased expression of the mucin genes MUC5AC and MUC5B, which are the major secretory mucins in the airways of asthma, in the OVA-Mch asthma airway remodeling group compared to the control and methacholine-alone with OVA sensitization groups. S1PR4 depletion significantly exacerbated the expression of MUC5AC and MUC5B genes in the OVA-Mch asthma airway remodeling group (Fig 17A and B). The levels of the SPDEF gene, which regulates the expression of mucin genes, were also much increased in the group with OVA-Mch asthma remodeling induction in both WT and *S1pr4*$^{+/-}$ mice. Furthermore, depletion of S1PR4 significantly aggravated this increase in SPDEF mRNA expression compared to the WT mice in the same model (Fig 17C).

Next, mRNA levels of representative markers for tight junctions such as ZO-1, claudin-18.1, and claudin-18.2, relative to β-actin, were measured by quantitative RT-PCR in lung lysates from different groups of WT and *S1pr4*$^{+/-}$ mice. ZO-1 expression was significantly increased in the OVA-Mch asthma remodeling group compared to the other groups. However, there were no significant differences in ZO-1 expression between the experimental groups in the *S1pr4*$^{+/-}$ mice (Fig 18A). Depleting S1PR4 also altered the mRNA expression levels of claudin-18.1 and claudin-18.2 in the OVA-Mch asthma remodeling group, which was not observed in the other experimental groups (Fig 18B and C).

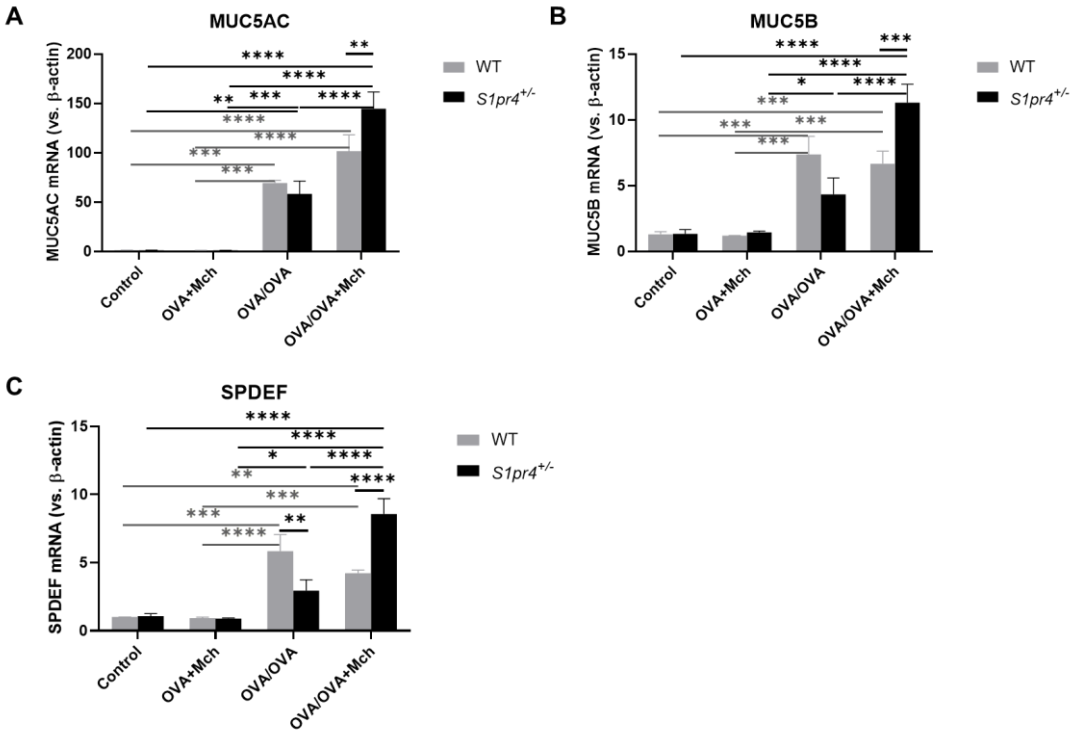


Figure 17. Depletion of S1PR4 exacerbated the increase in gene expression of MUC5AC, MUC5B, and SPDEF in the OVA-Mch asthma remodeling model.

Relative mRNA expression of mucin genes (MUC5AC and MUC5B) (A and B) and the SPDEF gene (C), which regulates the expression of mucin genes, was measured by RT-qPCR in lung lysates from different experimental groups of WT and *S1pr4*$^{+/-}$ mice. The adjusted *p*-values were reported using two-way ANOVA followed by Tukey's multiple comparison test between groups: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

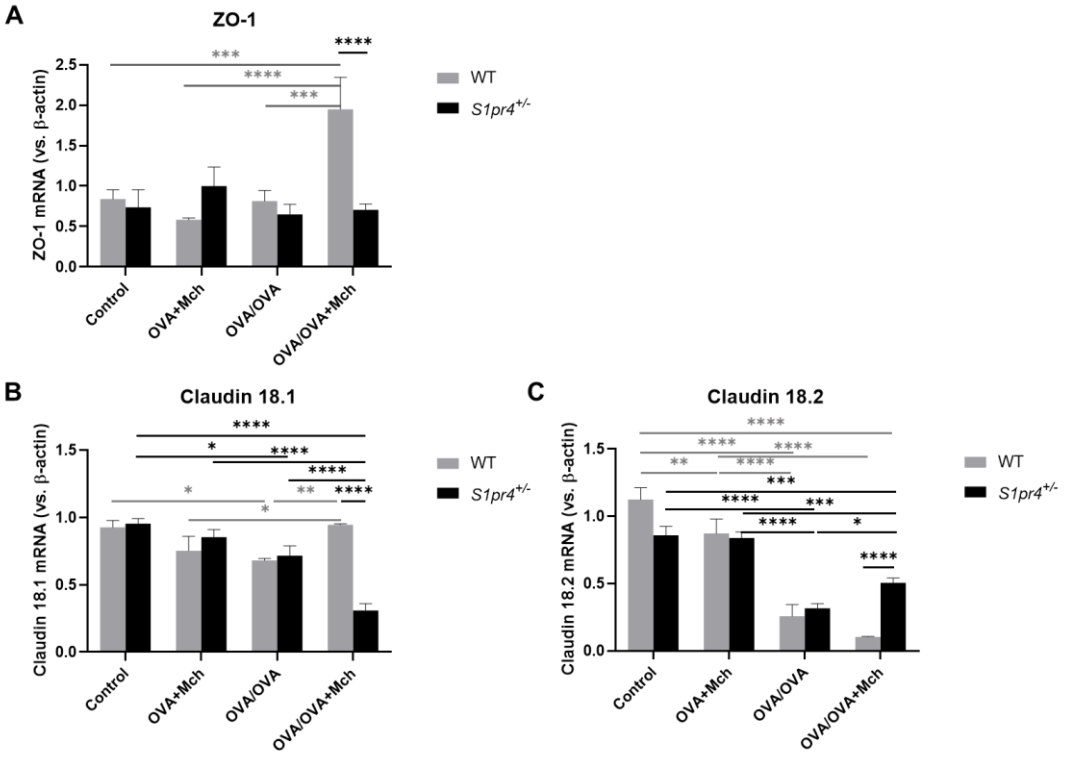


Figure 18. S1PR4 depletion affected the expression of tight junction genes in the OVA-Mch asthma remodeling model.

Relative mRNA expression of genes related to airway epithelial tight junctions, ZO-1 (A), claudin-18.1 (B), and claudin-18.2 (C) was measured by RT-qPCR in lung lysates from different experimental groups of WT and *S1pr4*$^{+/-}$ mice. The adjusted *p*-values were reported using two-way ANOVA followed by Tukey's multiple comparison test between groups: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

3.2.5. S1PR4 depletion affected EGF and WNT gene expression in the OVA-Mch asthma remodeling model

To elucidate the mechanism of S1PR in the OVA-Mch asthma airway remodeling model, bulk mRNA sequencing analysis was performed using lung lysates from WT and *S1pr4*$^{+/-}$ mice. Genes related to cell differentiation, extracellular matrix, and tight junctions that exhibit significant changes in the S1PR4-depleted OVA-Mch asthma remodeling model compared to WT mice in the same model were evaluated. The results were visualized using a heatmap, where upregulated genes were shown in red and downregulated genes in blue. S1PR4-depleted mice exhibited upregulation of genes related to mucins such as *Muc5ac*, *Muc5b*, *Muc4*, *Fcgbp*, and *Egf*, whereas *Cldn18*, *Wnt2*, and *Wnt7a* genes were downregulated (Fig 19A). Additionally, the scatter plot showing genes altered by more than 2-fold in the *S1pr4*$^{+/-}$ mice of OVA-Mch asthma remodeling model compared to WT mice of the same model revealed that S1PR4 depletion was significantly associated with increased expression of the *Egf* gene, while the expression of *Wnt2* and *Wnt7a* genes was decreased (Fig 19B).

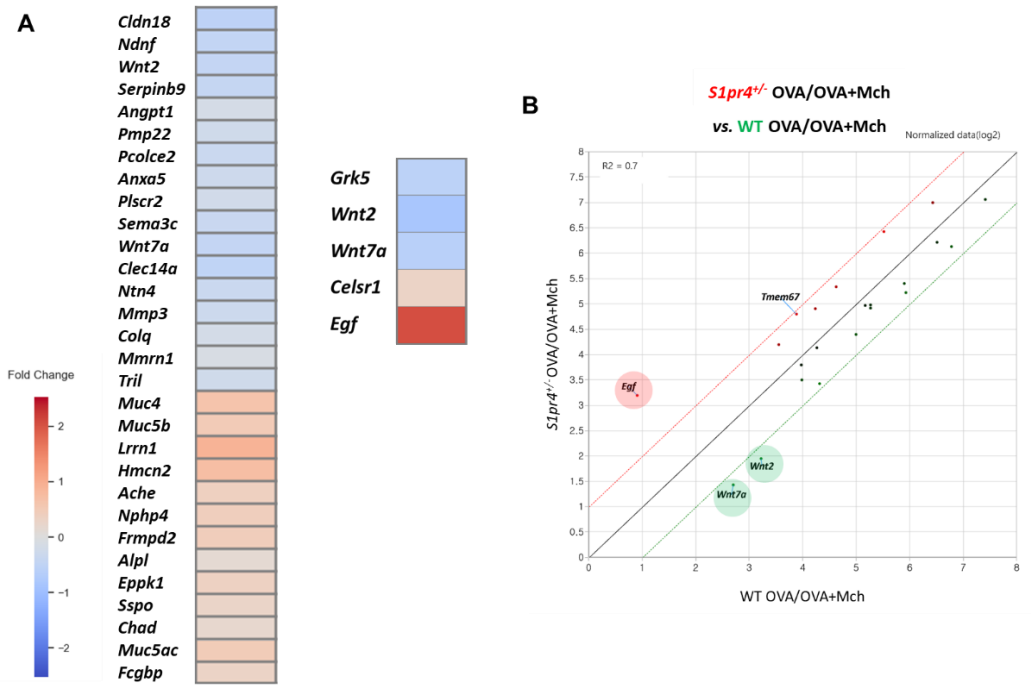


Figure 19. S1PR4 depletion affected EGF and WNT gene expression in the OVA-Mch asthma remodeling model.

Bulk mRNA sequencing analysis was performed using lung lysates from WT and *S1pr4*$^{+/-}$ mice subjected to the OVA-Mch asthma remodeling model. Significant genes related to cell differentiation, extracellular matrix, and tight junction were reported in a heatmap, with upregulated genes shown in red and downregulated genes in blue (A). The scatter plot showed genes upregulated by more than 2-fold in red, and downregulated gene in green (B). Both compared the results of *S1pr4*$^{+/-}$ mice to those of WT mice in the OVA-Mch asthma remodeling model.

4. Discussion

The effects of S1PR4 on modulating asthma inflammation have previously been reported in different types of airway inflammation models, while the specific role of S1PR4 in goblet cell hyperplasia and airway remodeling of asthma remains unclear. In the present study, the depletion of S1PR4 contributed to enhanced mucin production and collagen deposition in lung, tracheal, and bronchial tissues, which also exhibited airway structural changes in micro-CT imaging, potentially worsening airway remodeling in a murine inflammation- and bronchoconstriction-driven asthma remodeling model. Specifically, mucin-related genes, such as MUC5AC, MUC5B, and SPDEF, in the lungs, along with increased IL-13 secretion in the S1PR4-depleted airway supported the role of S1PR4 in regulating goblet cell hyperplasia.

Asthmatic airway remodeling is differentiated from lung tissue remodeling in COPD and idiopathic pulmonary fibrosis (IPF) in terms of the main sites of remodeling, such as large, medium, small airways, and lung parenchyma, as well as the specific mechanisms of remodeling.[56] Goblet cell metaplasia and hyperplasia, key features of airway remodeling in asthma, are characterized by the overexpression of MUC5AC, which increases gel viscoelasticity and tethers mucin to goblet cells.[14] Asthma is characterized by airway hyperresponsiveness, a condition where airways constrict excessively and easily in response to non-specific and innocuous stimuli, in addition to chronic airway inflammation. In fact, several in vitro and in vivo studies have reported that not only Th2 inflammatory cells and

mediators, but also the mechanical stress from bronchoconstriction, independently contribute to goblet cell hyperplasia and mucus hypersecretion.[11,17,18,43]

Mouse models of acute allergic asthma typically involve acute systemic sensitization with an allergen in the presence of an adjuvant, followed by subsequent allergen challenges via the airway, over a total period of 3-4 weeks.[57] Acute asthma models show highly exacerbated perivascular inflammation while exhibiting only mild changes in subepithelial fibrosis and goblet cell hyperplasia, thus having significant limitations for investigating the pathogenesis in chronic asthma pateints.[58,59] In contrast, chronic allergic asthma models that involve repeated low level allergen exposure to the airways for periods of up to 12 weeks better replicate features of airway remodeling in human asthma, including goblet cell hyperplasia, dense collagen deposition, and an increase in airway smooth muscle mass. Furthermore, our experimental model built upon a chronic asthma model with repeated and prolonged methacholine inhalation, integrating chronic airway inflammation and repetitive bronchoconstriction.[57,58] Methacholine, a cholinergic agonist, is commonly used to diagnose bronchial hyperreactivity in asthma by mimicking acetylcholine and inducing bronchoconstriction through binding to muscarinic M3 receptors on airway smooth muscle.[60] The broncho-constrictive effect of inhaled methacholine was demonstrated by a dose-dependent increase in airway resistance and functional residual capacity (FRC) in rats.[61] Methacholine inhalation after sensitization and challenge with OVA led to prolonged airway

inflammation, enhanced goblet cell hyperplasia, and airway smooth muscle thickening.[62] This suggests that bronchoconstriction, in addition to airway inflammation, plays a role in the chronicity and airway remodeling of asthma. In the current study, the model was exposed to a lower dose of methacholine inhalation for a prolonged period, along with a low-dose OVA challenge following OVA sensitization, compared to previous studies.[55,62] This model was characterized by exacerbated mucus secretion from hyperplastic goblet cells and mucous cells in the airway tissues, accompanied by hyperexpression of mucus genes. However, collagen deposition and subepithelial fibrosis in the lungs and airways were comparable to those of the conventional OVA-induced chronic asthma model.

Several studies have reported that S1P levels are elevated in the blood and BAL fluid of asthma patients.[46,47,51] However, in those studies, the specific mechanism of action through the responsive receptors of S1P were not examined in detail. Although additional evidence is needed to determine whether it works the same way in asthma, a study on S1P's effect on human platelet function revealed a biphasic, concentration-dependent response according to the S1PR subtypes.[41] Low concentrations of S1P enhanced platelet function via S1PR1, while high concentrations of S1P inhibited it through S1PR4 and S1PR5, suggesting endogenous mechanisms in controlling platelet activation.

S1PR4, also known as endothelial differentiation G-protein-coupled receptor 6 (EDG6), has been observed at considerable levels in human adult and fetal spleen, adult peripheral

leukocytes, and adult lung tissue.[40] We investigated the role of S1PR4 in the experimental model of OVA and methacholine-induced asthma remodeling using *S1pr4* heterozygous mice. In this study, the depletion of S1PR4 in experimental mice had significant effects on exacerbated mucin production with increased expression of the mucin genes, MUC5AC and MUC5B, along with the SPDEF gene, in the OVA-Mch asthma remodeling model compared to the conventional OVA inflammation model. In the BAL fluid analysis, the levels of IL-13 were observed to increase most significantly in the airway remodeled group of S1PR4-depleted mice.

MUC5AC and MUC5B are two major secretory and gel-forming mucins in the respiratory tract.[16] SPDEF is a key transcriptional factor in goblet cell differentiation and mucin production. Deletion of *Spdef* results in the absence of goblet cells, while expression of *Spdef* in the respiratory epithelium causes goblet cell hyperplasia and induces mucin production in the murine airway.[63,64] Mucus hypersecretion is known to be correlated with an increase of eosinophils and Th2 cytokines such as IL-4, IL-5, IL-9, or IL-13. However, mucus hypersecretion and goblet cell hyperplasia were still induced in mice deficient in eosinophils, IL-4, or IL-5, but not in the absence of IL-13 or IL-4Rα, a shared receptor for both IL-4 and IL-13.[16] In addition, other Th2 cytokines involved in the development of mucus hypersecretion under allergic airway inflammation were dependent on the IL-13-mediated pathways in airway epithelial cells, indicating the fundamental role of IL-13.[65] Specifically, the induction of

SPDEF and goblet cell hyperplasia has been reported to be dependent on IL-13 and STAT-6 in mice models.[64,66] In a previous study, S1P administration to mice significantly increased mucus production and AHR, along with elevated levels of prostaglandin D2, IgE, IL-4, and IL-13 in the lungs.[49] However, the relationship between S1PR4 and IL-13 has not been elucidated to date. Our study suggests that S1PR4 might be related to the negative regulation of S1P, and that the increase in S1P due to S1PR4 deficiency could have influenced the upregulation of IL-13.

Through the analysis of mRNA sequencing data, we confirmed that S1PR4-depletion in the OVA-Mch asthma remodeling model enhanced the mucin-related genes, such as *Muc5ac*, *Muc5b*, *Muc4*, and *Fcgbp*. Furthermore, we uncovered evidence that could help elucidate the mechanism by which S1PR4 influences mucous cell hyperplasia in asthma. Specifically, S1PR4 depletion was significantly associated with an increase in the expression of the *Egf* gene and a decrease in the expression of the *Wnt2* and *Wnt7a* genes. EGF and EGFR signaling have been reported to be required for mucus production in both constitutive and IL-13-induced conditions.[18] Upregulated EGF due to S1PR4 depletion can enhance the signaling of goblet cell differentiation and hyperplasia through increased gene expression of MUC5AC. On the other hand, Kim et al. reported that WNT signaling restricted goblet cell differentiation and mucus hypersecretion. In this study, mice with a mutation in *Ryk* gene, which functions as a WNT coreceptor, exhibited decreased gene expression of the *Wnt2* and *Wnt7a*. The mutant

mice showed goblet cell hyperplasia in the airway epithelium, with decreased expression of the *Muc5ac*, *Muc5b* and *Foxa3* genes.[67] Our study suggests that S1PR4 plays a role in regulating EGF and WNT signaling in goblet cell hyperplasia, although additional mechanistic studies are needed to confirm this.

The tight junction protein, ZO-1, was observed to decrease in various types of experimental asthma models, including those induced by house dust mite (HDM) or OVA, across different types of inflammation such as eosinophilic, neutrophilic, and mixed granulocytic.[68,69] However, contrary to expectations, the expression of ZO-1 showed a further increase in the asthma airway remodeling model in the present study. Hints on this phenomenon can be found in a paper that studied the effects on pulmonary epithelial cells using an in vitro system designed to replicate mechanical stresses. In that study, applying mechanical stress with a fluid-filled, parallel-plate flow chamber resulted in significantly higher basolateral surface stresses, where tight junction proteins like ZO-1 were concentrated, accompanied by increased paracellular permeability.[70] Therefore, it is possible that uneven mechanical stress caused by methacholine inhalation actually increased the expression of ZO-1, which can also be interpreted as a component of airway remodeling. However, S1PR4-depleted mice did not show differences in each experimental model compared to control mice, therefore further study is needed to confirm the role of S1PR4 in ZO-1 and other tight junction proteins.

In terms of airway inflammation, mice lacking S1PR4 exhibited decreased infiltration of inflammatory cells, such as eosinophils, in the standard allergic airway inflammation group, whereas no significant change was observed in the OVA-Mch asthma airway remodeling model. The dose of intranasal OVA challenge (20 μg) used in this model was lower than the 50 μg dose used in the previous study that demonstrated the role of S1PR4 in allergic airway inflammation.[55] Accordingly, the inflammatory features such as cell numbers in BAL fluid and the extent and degree of cellular infiltration in lung tissue were relatively attenuated compared to those in the previous model. However, it is uncertain whether the lack of a notable effect on airway inflammation in the airway remodeling group is caused by the weakened effect of S1PR4 on inflammation as airway remodeling worsens, or by insufficient induction of airway inflammation. Wang et al. demonstrated that S1PR4 deficiency predominantly affected neutrophilic airway inflammation conducted by OVA and LPS-induced asthma model, in contrast to an eosinophilic inflammation model.[51] Therefore, additional experiments are needed to determine whether S1PR4 has a major role in neutrophilic airway inflammation or established airway remodeling more than in eosinophilic airway inflammation.

This study has several limitations. Firstly, the suitability of the OVA-Mch asthma remodeling model to accurately represent the distinctive features of goblet cell hyperplasia in asthma remains uncertain, as it has not been extensively compared under various experimental conditions. Further studies are required to examine the role of S1PR4 in various settings that

enhance the mucin-hypersecretory phenotype. Such settings encompass heightened airway inflammation, elevated dosages of inhaled methacholine, or neutrophilic asthmatic inflammation. Second, we subjected mice to repeated methacholine inhalation to mimic the mechanical stress resulting from recurrent bronchospasm. However, the actual elevation in airway resistance was not quantified in our study. Consequently, it remains uncertain whether the observed outcomes are caused by the mechanical stress itself or by the pharmacological effects of methacholine. Additionally, the levels of S1P were not measured in this study, and therefore, we were unable to clearly explain the mechanism of action between S1P and S1PR4. Lastly, the model was not fully investigated for other hallmarks of asthmatic airway remodeling, such as basement membrane thickening, subepithelial fibrosis, and increased smooth muscle hyperplasia. Therefore, it is limited in comprehensively describing the complete spectrum of asthmatic airway remodeling.

Nevertheless, our study added the knowledge about potential role of S1PR4 on regulating the goblet hyperplasia and mucin production through assessing the effect of S1PR4 depletion in the conceptual inflammatory- and bronchoconstriction-driven asthmatic airway remodeling model. These findings suggest that S1PR4 may be a potential therapeutic target in the treatment of asthma-related goblet cell hyperplasia and mucous hypersecretion.

5. References

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Table S1. The primers used for each target gene

Primer name	Sequences (Mer)
S1PR4	F: CTGGGGCAATCTCAACTGGT (20) R: CTGCCACAGCATATCTCCGT (20)
B-actin	F: AGTACTTGCGCTCAGGAGGA (20) R: GACCTCTATGCCAACACAGT (20)
MUC5AC	F: TGTTCCTCACCCTCAAGGGCATGT (24) R: TGCACACCCGCCTGGTATGTC (21)
MUC5B	F: GAAACTGGAGCTGGGCTCTGGT (22) R: CTCTCTGACTGTCTCCGGTGAGTTC (25)
SPDEF	F: CACGTTGGATGAGCACTCGCTA (22) R: AGCCACTTCTGCACGTTACCAG (22)
ZO-1	F: TGCCATTACACGGTCCTCTG (20) R: GGTTCTGCCTCATCATTTCCTC (23)
Occludin	F: CTACTCCTCCAATGGCAAA (19) R: CTCTTGCCCTTTCCTGCTTT (20)
Claudin-3	F: GCACCCACCAAGATCCTCTA (20) R: TCGTCTGTCACCATCTGGAA (20)
Claudin-4	F: CGCTACTCTTGCCATTACG (19) R: ACTCAGCACACCATGACTTG (20)
Claudin-5	F: TAACCTGAAAGGGCAGCTGGAGAAAC (26) R: AGGTCCAGGCTAAGTCCTTTGGTTCAGTAG (30)
Claudin-18 1.2A	F: AGTATGAAGGGCTCTGGAGGAGTTG (25) R: AGAACAATGCCCACGATCATCAG (23)
Claudin-18 2.1A	F: GTATTCAACTACCAAGGGCTATGGCGTTC (29) R: ATCATCAGGGCTCGTACAGCTTGC (24)
N-cadherin	F: TCCTTGTTCGGCTATGTGTC (20) R: GGCATGCACCTAAGAATCAG (21)
E-cadherin	F: TGAAACGGCGGGATAAAGAG (20) R: GGCTCCACAGTATCTGGTTG (20)

6. Korean abstract

천식의 점액 과다분비 조절에서 S1PR4 (sphingosine-1-phosphate receptor 4)의 역할

천식 기도개형의 주요 특징 중 하나인 기도상피의 술잔세포 과증식은 과도한 점액 생성 및 기도 막힘을 유발하여 천식의 증상과 폐 기능을 악화시키며, 이는 특히 심각한 급성 악화 및 치료 저항성 천식 표현형에서 두드러진다. 그러나, 현재의 천식 치료제들은 술잔세포 과증식 및 과도한 점액분비 조절에 제한적인 효과만을 나타내고 있어 천식 환자의 점액 생성 조절을 표적으로 하는 치료제 개발이 시급하다.

스핑고신-1-인산염 (sphingosine-1-phosphate, S1P)은 스핑고지질의 생리 활성 대사 산물로 세포 생존, 증식, 이동, 및 혈관 신생 등 다양한 세포 과정에서 중요한 역할을 한다. 천식 환자에서 정상대조군에 비해 S1P 가 증가되었으며, S1P 의 증가는 천식의 중증도에 비례하였다. S1P 수용체 4 (S1PR4)는 면역 세포와 폐에서 특이적으로 발현되는 S1P 수용체로서 알레르기성 기도 염증과의 연관성이 보고되었으며, 특히 호중구성 천식 및 점액 생성 호흡기질환에서 S1PR4 의 발현이 감소되었다. 그러나, 천식의 점액 과다분비 및 기도 개형에서의 S1PR4 의 역할은 아직 밝혀져 있지 않다. 이에 이 연구는 S1PR4 경로가 천식의 점액 과다분비에 미치는 영향과 기전을 규명하고자 하였다.

먼저, 이를 위해 Ovalbumin (OVA) 감작 후 OVA 유발과 동시에 메타콜린을 흡입시켜 기도 염증 및 기관지 수축 유발 천식기도개형 마우스 모델을 확립하였다. 이 마우스 모델은 기존 OVA 유발 천식 모델과 비교하여 기도 내 점액의 생성과 콜라겐 침착이

악화된 표현형을 나타냈다. 그런 다음, 기도 점액세포의 증식에서 S1PR4 의 역할을 규명하기 위해 이형 접합성 S1PR4 knockout 마우스를 적용하여 모델을 생성하였다. S1PR4 가 결핍된 기도개형 마우스모델에서는 폐 조직, 기관, 및 기관지 조직에서 점액 생성과 콜라겐 침착이 더욱 악화되었으며, 마이크로 CT 스캔을 통해 S1PR4 고갈에 의해 기관지 주위 반점이 증가되고, 심한 기도 폐쇄가 동반된 것을 확인하였다.

S1PR4 의 결핍은 기도개형이 형성된 마우스의 폐에서 점액 유전자인 MUC5AC 및 MUC5B 와 점액 유전자의 발현을 조절하는 SPDEF 의 발현을 유의하게 증가시켰다. 또한, S1PR4 의 결핍은 EGF 유전자 발현의 증가와 WNT2 및 WNT7a 유전자 발현의 감소와 유의한 연관성을 보였다.

이러한 연구결과는 S1PR4 경로가 천식의 기도개형 과정에서 술잔세포의 증식 및 점액 생성을 조절하는 데에 중요한 역할을 한다는 것을 시사한다. 따라서, 천식의 기도개형 과정에서 S1PR4 발현을 높게 유지하는 것은 천식관련 술잔세포의 과증식 및 과도한 점액분비를 조절하기 위한 잠재적인 치료 접근방법이 될 수 있을 것이다.

핵심어: S1PR4, 천식, 기도개형, 술잔세포 과증식, 점액 과다분비