

RESEARCH PAPER

Blockage of sphingosine-1-phosphate receptor 2 attenuates allergic asthma in mice

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Background and Purpose: Sphingosine-1-phosphate 2 (S1P₂) receptors have been implicated in degranulation of mast cells. However, functions of S1P₂ receptors have not been investigated in an in vivo model of allergic asthma.

Experimental Approach: Using an ovalbumin (OVA)-induced asthma model, the function of S1P₂ receptors was evaluated in S1P₂-deficient mice or in mice treated with JTE-013, a selective S1P₂ antagonist. Bone marrow-derived dendritic cells (BMDCs) were used to investigate the roles of S1P₂ receptors in dendritic cell maturation and migration.

Key Results: Eosinophil accumulation and elevated Th2 cytokine levels in broncho-alveolar lavage fluid and inflamed lung tissues were strongly inhibited by administration of JTE-013 before OVA sensitization, before OVA challenge, and before both events. In S1P₂-deficient mice, allergic responses were significantly lower than in wild-type mice. LPS- and OVA-induced maturation of BMDCs was significantly blunted in dendritic cells from S1P₂-deficient mice and by treatment with JTE-013. Migrations of immature and mature BMDCs were also dependent on S1P₂ receptors. It was found that OVA-challenged mice into which in vitro OVA primed BMDCs from S1P₂-deficient mice were adoptively transferred, had less severe asthma responses than OVA-challenged mice into which OVA-primed BMDCs from wild-type mice were adoptively transferred.

Conclusions and Implications: Pro-allergic functions of S1P₂ receptors were elucidated in a murine asthma model. S1P₂ receptors were involved not only in maturation and migration of dendritic cells in the sensitization phase but also in mast cell degranulation in the challenge phase. These results suggest S1P₂ receptor as a therapeutic target for allergic asthma.

1 | INTRODUCTION

The pathogenesis of asthma is associated with initial sensitization to environmental antigens and subsequent repeated exposure to these antigens. Antigen-presenting dendritic cells and Th2 lymphocytes play

important roles in this sensitization process. Exposure to environmental antigens induces inflammatory reactions in the airway, which are characterized by the activation of mast cells and eosinophils (Jolly, Rosenfeldt, Milstien, & Spiegel, 2002).

Genome-wide association studies have identified ORMDL3, which could affect asthma through inhibition of sphingolipid synthesis (Worgall, 2017). Non-coding RNAs (miRNAs and long non-coding RNAs) have been shown to play an important role in allergic diseases and bronchial asthma; moreover, miRNAs target components of the **sphingosine 1-phosphate** (S1P) signalling pathway (Saluja, Kumar,

Abbreviations: S1P, sphingosine 1-phosphate; S1P₂, sphingosine 1-phosphate receptor type 2 (EDG5); OVA, ovalbumin; BMDC, bone marrow-derived dendritic cell; BALF, bronchoalveolar lavage fluid; WT, wild-type; KO, knockout; imDC, immature dendritic cell; mDC, mature dendritic cell

Jain, Goel, & Jain, 2017). In asthmatic patients, S1P levels in lung bronchoalveolar lavage fluid (BALF) are significantly increased 1 to 2 days after antigen challenge (Ammit et al., 2001). Antigen-induced aggregation of IgE antibody on mast cells elicits multiple biochemical events, including activation of sphingosine kinase (Choi, Kim, & Kinet, 1996; Ryu, Lee, Suk, Park, & Choi, 2009), which leads to the generation of S1P in mast cells (Jolly et al., 2004; Prieschl, Csonga, Novotny, Kikuchi, & Baumruker, 1999). S1P is a specific ligand for five GPCRs, S1P₁₋₅ (Moolenaar & Hla, 2012).

The involvement of both S1P₁ and S1P₂ receptors in asthma has been studied. Stimulation of S1P₁ receptors inhibits airway inflammation, whereas S1P-induced degranulation of rodent and human mast cells is mediated through S1P₂ receptors (Oskeritzian et al., 2010; Prieschl et al., 1999). However, there have been few preclinical studies on the role of S1P and S1P₂ receptors in allergic responses. A mast cell-dependent model of passive systemic anaphylaxis was used to evaluate the function of S1P₂ receptors in mast cells (Oskeritzian et al., 2010). Also stimulation of the S1P₂ receptor was found to regulate anaphylaxis-induced hypotension, the elimination of histamine from the circulation, and duration of anaphylactic shock (Olivera et al., 2010).

Although the role of the S1P₂ receptor in mast cell functions has been elucidated, little is known about how this receptor executes its functions in allergic asthma in vivo. In the present study, we used a murine ovalbumin (OVA)-induced asthma model to investigate the role of S1P₂ receptors in vivo and examined the overall allergic responses in S1P₂-deficient mice and in JTE-013 (a specific S1P₂ antagonist)-pretreated mice.

2 | METHODS

2.1 | Animals

Three S1P₂ heterozygous mice were kindly provided by Richard Proia at NIH (Kono et al., 2007). They were of mixed C57BL/6 (RRID:IMSR_JAX:00664) and 129Sv (RRID:IMSR_APB:4898) background. They had been backcrossed to Balb/c (RRID:IMSR_TAC:balb) mice for eight generations. S1P₂ wild-type (WT) littermates and knockout (KO) mice were housed in the Laboratory Animal facility at Pusan National University. The mice were housed, two per cage, in standard plastic cages with sawdust as bedding and maintained under controlled conditions of temperature at 22–24°C, humidity at 60 ± 5%, and alternating light/dark cycles (lights were on between 7:00 and 19:00 hr) and provided with standard laboratory chow and water ad libitum. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*. The animal protocol used in this study was reviewed and approved by the Pusan National University–Institutional Animal Care Committee (PNU-IACUC) with respect to procedure ethics and scientific care (Approval Number PNU-2016-1131).

2.2 | Induction of asthma in Balb/c mice and JTE-013 administration

Following a simple randomization procedure, 6-week-old female WT Balb/c mice were randomly assigned to one of five treatment groups ($n = 6$): PBS-injected control group, OVA-injected asthma group, OVA-injected plus JTE-013 asthma groups (3 mg·kg⁻¹; Figure 1a). The OVA-injected asthma group was injected with vehicle (15 µl DMSO in 300 µl PBS for a 20 g mouse). JTE-013 was administered by i.p. injection 30 min before challenge (JTE 1 group), before sensitization (JTE 2 group), or before both sensitization and challenge (JTE 3 group). In addition, 6-week-old female S1P₂ WT and KO Balb/c mice ($n = 6$) were assigned to the following four groups: S1P₂ WT or KO PBS-injected control groups and S1P₂ WT or KO OVA-injected groups. Asthma was induced by i.p. injection of 50 µg OVA and 1 mg aluminium hydroxide on experimental Days 0 and 14 (sensitization). From Day 28, the mice were exposed to nebulized OVA (1% in PBS) for 20 min for three consecutive days (challenge; Aoki et al., 2010). On Day 32, the mice were killed by CO₂ inhalation, and lung and BALF were collected for subsequent experiments (Figure 1a; Lee et al., 2013). Experiments were conducted more than three times for each group to obtain a group size of six mice, because age- and gender-matched WT and KO mice numbers were not available all at once and varied depending on birth rate.

2.3 | Histological analysis of the lung and cell count in BALF

The extracted lungs were fixed in 10% formalin and embedded in paraffin and sectioned (4 µm). The sections were stained with periodic acid-Schiff (PAS, ab150680, Abcam) for 15 min, rinsed with running tap water, and stained with haematoxylin for 90 s. They were then rinsed, dehydrated, and cover-slipped (Aoki, Mogi, & Okajima, 2014). Lung inflammation was scored by three independent, blinded investigators, and degrees of peribronchial and perivascular inflammation were evaluated using a subjective 0–3 point scale, as previously described (Braber, Henricks, Nijkamp, Kraneveld, & Folkerts, 2010; Kwak et al., 2003; Tournoy, Kips, Schou, & Pauwels, 2000). A value of 0 was assigned when no inflammation was detectable, 1 was adjudged for occasional cuffing with inflammatory cells, 2 when most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and 3 was given when most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells. Total lung inflammation was defined as the average of the lung inflammation scores. Lung sections per mouse were scored, and inflammation scores were expressed as a mean value (Braber et al., 2010). To obtain total cell counts in BALF samples, cells were stained with Wright's stain and then counted under a light microscope. For differential cell counts, cells were attached to a glass slide using a cytospin and stained with May–Grünwald–Giemsa stain. Cells were identified as macrophages, eosinophils, and lymphocytes using their cellular and nuclear morphologies (Figure 1b,c). In the

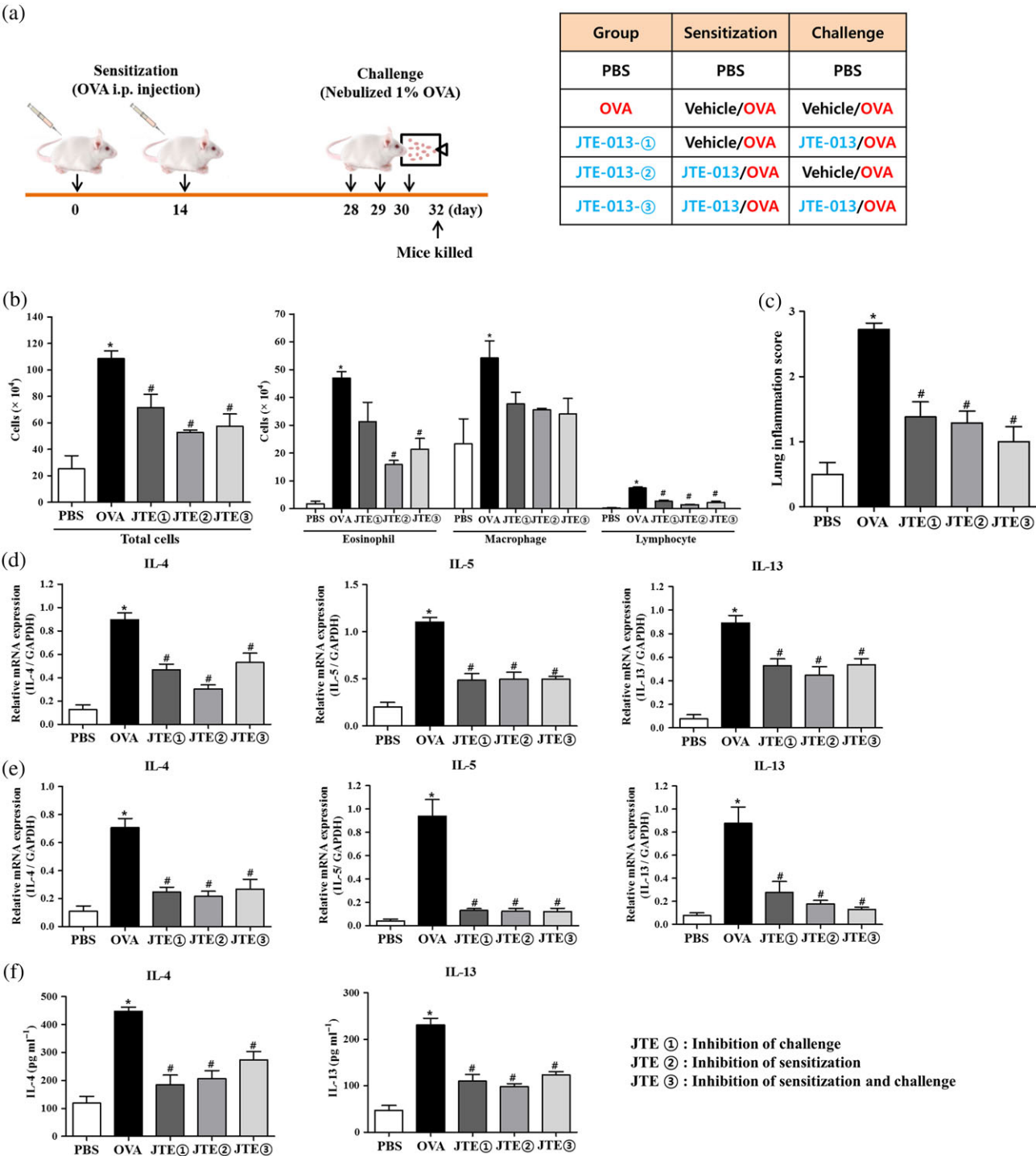


FIGURE 1 Experimental protocol for the induction of allergic asthma and effect of S1P₂ antagonist treatment on the features of asthma. (a) Experimental protocol. A murine model of OVA-induced asthma is established through OVA sensitization by i.p. injection on Days 0 and 14, as well as through repeated intratracheal OVA challenges on Days 28–30 to induce allergic asthma. JTE-013 (a S1P₂ antagonist) was administered by i.p. injection 30 min before OVA sensitization or aerosol challenge. S1P₂ WT litter mates were divided into five experimental groups for the S1P₂ antagonist study according to the time of JTE-013 injection. Mice in the OVA group were treated with vehicle. (b) Cell counts in BALF. (c) Degrees of peribronchial inflammation and perivascular inflammation in PAS-stained sections were evaluated using a subjective 4-point scoring system as described in 2. (d and e) RT-PCR analysis of Th2 cytokines (IL-4, IL-5, and IL-13) was performed using mRNA isolated from mouse lung tissues (d) or inflammatory cells in BALFs (e). mRNA levels were also quantified as ratios of GAPDH mRNA levels. (f) Protein levels of IL-4 and IL-13 in BALF. BALFs were assayed for cytokine production using IL-4 and IL-13 ELISA assay kits. Results are presented as means $\pm$ SEM ($n = 6$)

PAS/haematoxylin stained sections (Figure 1d), eosinophils appear as small, navy blue dots, as indicated by the red arrows. Secreted and stored mucins are stained purple with PAS. Dark violet-stained cells surrounded the bronchioles in the OVA group, as indicated by the green arrows.

2.4 | Reverse transcriptase-PCR

To assess the expression of asthmatic markers in Th2 cells by RT-PCR, first-strand cDNA was first synthesized from total RNA isolated using Trizol reagent (Invitrogen, Waltham, MA). Total RNAs were isolated from BALF-associated cells and lung tissues. Synthesized cDNA products, primers for each gene, and Promega Go-Taq DNA polymerase (Madison, WI) were used for PCR. Specific primers and PCR conditions were as previously described (Lee, Kang, Park, Huang, & Im, 2016; Park et al., 2014). Aliquots (7 μ l) were electrophoresed in 1.2% agarose gels and stained with StaySafe™ Nucleic Acid Gel Stain (Real Biotech Corporation, Taipei, Taiwan; Kang et al., 2014). The intensities of each PCR product were quantified by using ImageJ (RRID:SCR_003070, <https://imagej.net/>) software (NIH, Bethesda, MD, USA) and normalized with GAPDH.

2.5 | Measurements of cytokines (IL-4 and IL-13)

Cytokine levels in BALF were determined using ELISA kits (eBioscience, San Diego, CA), as previously described (Park et al., 2014).

2.6 | Generation and cell culture of bone marrow-derived dendritic cells

Dendritic cells were generated from the bone marrow cells of female S1P₂ WT or KO mice, as previously described by Lutz et al. (1999). In brief, bone marrow cells were flushed from the femurs and tibias using sterile PBS and placed in RPMI 1640 medium containing 10% FBS, 100 μ g·ml⁻¹ penicillin, 50 μ g·ml⁻¹ streptomycin, recombinant mouse GM-CSF (20 ng·ml⁻¹), recombinant mouse IL-4 (20 ng·ml⁻¹), and 50 μ M 2-mercaptoethanol. On Day 3, fresh medium containing these cytokines was added. On Day 7, bone marrow-derived dendritic cells (BMDCs) were collected, and CD11c⁺ cells were sorted using anti-mouse CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.7 | Chemotaxis assay

Chemotaxis was analysed by measuring the number of cells migrating through a polycarbonate filter (5 μ m pore size; Corning, Omnilab, Bremen, Germany) in 24-well Transwell chambers. After being isolated, CD11c⁺ BMDCs were seeded in six-well plates containing RPMI 1640/0.5% FBS and then matured for 24 hr with 1 μ g·ml⁻¹ LPS. Immature dendritic cells (imDCs) and mature dendritic cells (mDCs) were subjected to Boyden chamber experiments to investigate their migration to defined DC chemokines (imDC: CXCL12

100 ng·ml⁻¹; mDC: CCL9 100 ng·ml⁻¹). ImDCs or mDCs (2×10^6 cells·ml⁻¹) were placed in upper chambers (100 μ l per well) and chemokines in RPMI 1640/0.5% FBS in lower chambers (500 μ l per well). After an incubation time of 24 hr, numbers of migrated cells were counted by flow cytometry (Ceballos et al., 2007; Gollmann et al., 2008).

2.8 | Flow cytometry

BMDCs were seeded in RPMI 1640 containing 0.5% FBS into six-well plates and cultured with the indicated concentrations of JTE-013 or vehicle for 1 hr prior to LPS treatment (1 μ g·ml⁻¹). After 24 hr of incubation, cells were collected, washed in PBS twice, incubated in FITC-conjugated anti-MHCII antibody, APC-conjugated anti-CD86 antibody, FITC-conjugated anti-CD80 antibody, or PE-conjugated anti-CD40 antibody (eBioscience), diluted 1:1,000 in 3% BSA in PBS for 15 min, and then washed twice in PBS containing 3% BSA. Cells were analysed using a BD Accuri™ C6 Flow Cytometer (BD, Franklin Lakes, NJ).

2.9 | Adoptive transfer experiment with BMDCs

BMDCs isolated from female S1P₂ WT or KO mice were treated with OVA (100 μ g·ml⁻¹) for 24 hr. Under anaesthesia by i.p. injection of tribromoethanol 300 mg/kg, 1×10^6 PBS-pulsed or OVA-pulsed WT or S1P₂ KO BMDCs (1×10^6) in 50 μ l of PBS were instilled into naïve WT female mice through the trachea, under fiberoptic illumination. Ten days later, mice were exposed to aerosolized OVA (1% in PBS) for 20 min·day⁻¹ for three consecutive days. Next, 48 hr after the last challenge, BALF and lung tissues were obtained to confirm asthmatic responses, as previously described (Figure 2e; Miyahara et al., 2008).

2.10 | Statistics

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology2018 (Curtis et al., 2018). Results are expressed as the means $\pm$ SEM of five or six determinations for animal experiments and as the means $\pm$ SEMs of five determinations for BMDC experiments. Statistical significances of differences were determined by ANOVA and Tukey's multiple comparison test. Statistical significance was accepted for *P* values <0.05. * indicates significance difference compared to the PBS-treated group or untreated controls; # indicates significant difference compared to the OVA-treated group or LPS-treated group. Analyses were performed using the GraphPad Prism (RRID:SCR_002798, <http://www.graphpad.com/>) software (GraphPad Software, Inc., La Jolla, CA).

2.11 | Materials

S1P was purchased from Avanti Polar Lipids (Alabaster, AL). JTE-013 was purchased from Cayman Chemicals (Ann Arbor, MI), and

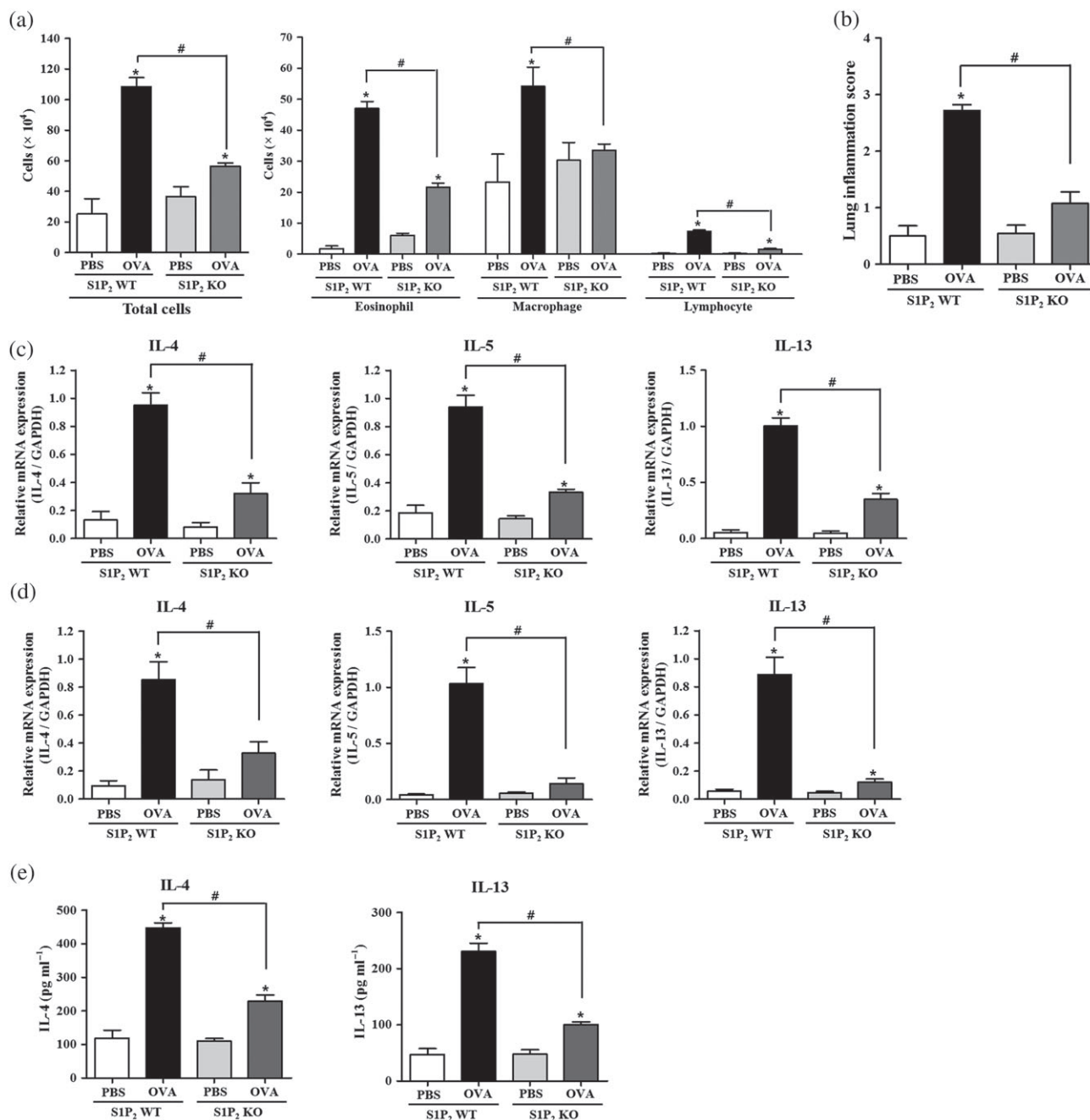


FIGURE 2 Effect of S1P₂ deficiency on OVA-induced allergic responses. S1P₂ WT and KO mice were treated with PBS or OVA. (a) May–Grünwald–Giemsa staining was used to obtain differential cell counts in BALF. (b) Degrees of peribronchial and perivascular inflammation in PAS-stained sections were evaluated using a 4-point scoring system as described in 2. (c and d) RT-PCR analyses for Th2 cytokines (IL-4, IL-5, and IL-13) were performed using mRNA isolated from mouse lung tissues (c) or inflammatory cells in BALFs (d). mRNA levels were also expressed as ratios versus GAPDH mRNA. (e) Protein levels of IL-4 and IL-13 in BALF. BALFs were assayed for cytokine production using IL-4 and IL-13 ELISA assay kits. Results are presented as means $\pm$ SEM ($n = 6$)

OVA (Sigma A5503, grade V), aluminium hydroxide, monoclonal anti-dinitrophenyl specific mouse IgE (Cat# D8406, RRID: AB_259249), human dinitrophenyl albumin, 4-nitrophenyl *N*-acetyl- β -D-glucosaminide, and 2-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant mouse GM-CSF and IL-4 were purchased from Shenandoah Biotechnology (Warminster, PA, USA).

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

3.1 | JTE-013 inhibited asthma-induced immune responses in BALF and lungs

The S1P₂ receptor has been suggested to mediate mast cell degranulation (Olivera, Dillahun, & Rivera, 2013; Oskeritzian et al., 2010). To confirm the *in vitro* inhibitory effect of JTE-013 on antigen-induced degranulation in mast cells, we utilized a murine model of OVA-induced asthma. JTE-013 was administered at three different times (refer to Figure 1a): before challenge (JTE 1 group), before sensitization (JTE 2 group), or before both sensitization and challenge (JTE 3 group).

JTE-013 treatment significantly inhibited cell recruitment to the airway lumen in the three JTE-013 groups (Figures 1b and S1A). It also significantly reduced the numbers of eosinophils and lymphocytes (Figure 1b). On the basis of the number of eosinophils, JTE-013 administration in the OVA sensitization phase was found to be more effective than JTE-013 administration during OVA challenge (Figure 1b).

Histological analyses on PAS/haematoxylin stained lungs indicated eosinophils (small blue stainings indicated by red arrows) accumulated densely around bronchioles in the OVA group, but this eosinophil accumulation was lower in the OVA + JTE-013 groups (Figure S1B). Mucous glycoproteins (mucins) produced by goblet cells were stained purple, as indicated by green arrows in Figure S1B. Staining around bronchioles was greater in the OVA group than in the PBS group, indicating more mucin production and goblet cell hyperplasia (Figure S1B). In contrast, staining was lower in the JTE-013 treated groups than in the OVA group, suggesting suppressed mucin production (Figure S1B). In summary, the increased accumulation of eosinophils and mucin production in the lungs of OVA-induced asthmatic mice were strongly reduced by treatment with JTE-013 (Figure S1B).

Degrees of lung inflammation were also analysed as previously described (Kwak et al., 2003; Tournoy et al., 2000). Mean inflammation score in the OVA-treated group was 2.6, which was significantly higher than that in the PBS group (0.5), and treatment with JTE-013 reduced the inflammation score to approximately 1.2 (Figure 1C).

Furthermore, airway hyperresponsiveness, one of the cardinal features of asthma, was measured by using whole-body plethysmography 48 hr after the final challenge. An increase in enhanced pause (Penh) in response to increasing doses of **methacholine** was assessed as an index of airway contraction. OVA sensitization/challenge clearly increased Penh in response to methacholine; however, JTE-013 treatment reduced airway hyperresponsiveness, as shown in Figure S3A. Total serum levels of IgE were markedly increased in the OVA-sensitized and OVA-challenged mice, and JTE-013 treatment attenuated this OVA-induced increase in serum IgE levels, as presented in Figure S3B. Moreover, we measured the number of T cells in draining lymph nodes, as shown in Figure S3C. The number of IL-4⁺ T cells in the JTE-2 group was low but not in the JTE-1 and JTE-3 groups. We

think this is not due to the presence of the S1P₂ receptor in T cells, because S1P₂ receptors are not expressed in the T cell lineage (Sawicka et al., 2003).

The cytokines of Th2 cells play key roles in the pathogenesis of asthma (Fish, Donaldson, Goldman, Williams, & Kasaian, 2005; Locksley, 2010). In particular, IL-4, **IL-5**, and **IL-13** are associated with eosinophil recruitment and activation, goblet cell metaplasia, mucus hyper-secretion in epithelial cells, and smooth muscle proliferation (Fish et al., 2005; Locksley, 2010). Therefore, changes in the mRNA levels of IL-4, IL-5, and IL-13 in lung tissues and in BALF immune cells were assessed. As shown in Figure 1d,e, the mRNA levels of IL-4, IL-5, and IL-13 were elevated in the lung tissues and BALF immune cells of the OVA group versus those of the PBS control, but these elevations were inhibited by JTE-013 in all three JTE-013-treated groups (before challenge, before sensitization, and before both sensitization and challenge).

In order to confirm the effect of JTE-013 on the expressions of Th2 cytokines, the protein levels of IL-4 and IL-13 in BALF were measured by ELISA. As shown in Figure 1f, IL-4 and IL-13 levels were significantly higher in the OVA group than in the PBS control group (Figure 1f), but these increases were significantly inhibited by JTE-013 in all three JTE-013-treated groups (Figure 1f).

3.2 | Asthma-induced immune responses in BALF and lungs were suppressed in S1P₂ KO mice

S1P₂ KO mice were used to further examine the effects of JTE-013 on OVA-induced allergic asthma. The number of eosinophils, macrophages, and lymphocytes in BALF were lower in S1P₂ KO mice than in WT mice (Figures S2A and 2a). Eosinophil accumulation around bronchioles was lower in S1P₂ KO mice than in WT mice (Figure S2B), and mucin staining was significantly less intense in S1P₂ KO mice than in WT mice. In addition, the mean inflammatory score in OVA-treated WT mice was 2.6, whereas it was only 1.5 in S1P₂ KO mice (Figure 2b).

As shown in Figure 2c,d, the mRNA levels of IL-4, IL-5, and IL-13 were elevated in the lung tissues and BALF immune cells of OVA-treated WT mice, but these elevations were markedly lower than those of S1P₂ KO mice. Furthermore, as shown in Figure 2e, increased protein levels of IL-4 and IL-13 in OVA-treated WT mice were significantly blunted in OVA-treated S1P₂ KO mice (Figure 2e).

3.3 | Effects of JTE-013 and S1P₂ deficiency on the maturation and migration of BMDCs

The suppressive effects of JTE-013 and S1P₂ deficiency were expected because the S1P₂ receptor has been known to have a function in mast cell degranulation. However, when JTE-013 was administered before sensitization, suppressive effects of JTE-013 were unexpectedly observed, suggesting the S1P₂ receptor in dendritic cells

is involved in the sensitization phase. Thus, we focused on the function of S1P₂ receptors in dendritic cells, which are antigen-presenting cells (Ueno, Schmitt, Palucka, & Banchereau, 2010). When subjected danger signals, such as those elicited by pathogens, dendritic cells undergo a maturation process (Ueno et al., 2010) that entails cytokine production, changes in chemokine receptor expression, and increases in the membrane expression of major histocompatibility complexes and costimulatory molecules (Ueno et al., 2010). As shown in Figure 3a, LPS treatment induced maturation of the BMDCs supported by concentration-dependent increases in the surface expression of maturation marker proteins, namely, MHCII, CD86, CD80, and CD40. In the presence of JTE-013, this up-regulation of maturation marker proteins was blunted (Figure 3a), as was also observed in BMDCs from S1P₂ KO mice (Figure 3b). Maturation was also confirmed by determining changes in the expression of cytokines. As shown in Figures 3c,d, treatment with LPS or OVA increased the expressions of IL-6, IL-12, TNF- α , IL-23, and IL-17 (Figure 3c,d). However, in the presence of JTE-013, these increases were inhibited in a concentration-dependent manner (Figure 3c,d), and similar effects were also observed in BMDCs from S1P₂ KO mice (Figure 3e,f). In summary, the results strongly suggested that the S1P₂ receptor plays an important role in dendritic cell maturation after antigen challenge (Figure 3e,f).

In addition to maturation, migration of dendritic cells is very important for presenting antigen to naïve T cells in lymph nodes and subsequent activation of T cells. Thus, we assessed the migration abilities of imDCs and mDCs to CXCL-12 (also known as SDF-1 α) and CCL19 (also known as MIP-3b), respectively (Figure 3g). The migrations of imDCs and mDCs were inhibited by JTE-013 in a concentration-dependent manner. This was also confirmed in dendritic cells from S1P₂-deficient mice (Figure 3h). The migratory responses of imDCs and mDCs from S1P₂-deficient mice were significantly blunted compared with those from WT mice (Figure 3h).

3.4 | In vivo verification of the functions of S1P₂ receptors in dendritic cells in allergic asthma

In order to verify the function of S1P₂ receptors in DCs, an adoptive transfer experiment was conducted as shown in Figure 4a (Miyahara et al., 2008). In the adoptive transfer experiment, WT mice were exposed to OVA 10 days after instillation of *in vitro* OVA primed BMDCs from S1P₂-WT or S1P₂-deficient mice. Total cell counts and numbers of eosinophils and macrophages in BALF were significantly lower in the S1P₂ KO DC-instilled mice than in the WT DC-instilled mice (Figure 4b,c). Furthermore, fewer eosinophils around bronchioles were observed in the S1P₂ KO DC-instilled mice than in the WT DC-instilled mice, and increased mucin staining in WT DC-instilled mice was significantly attenuated in the S1P₂ KO DC-instilled mice (Figure 4d). Mean inflammatory score in the WT DC-instilled mice was 2.1; however, it was only 1.3 in the S1P₂ KO DC-instilled mice, which constituted a significant reduction (Figure 4e).

As shown in Figure 4f,g, elevations in the mRNA levels of IL-4, IL-5, and IL-13 mRNAs in the lung tissues and BALF immune cells of WT DC-instilled mice were lower in the S1P₂ KO DC-instilled mice (Figure 4f,g). Similar results were obtained from the adoptive transfer experiment with JTE-013-treated DCs (Figure S5).

4 | DISCUSSION

In the present study, using an S1P₂ antagonist, JTE-013, and S1P₂-deficient mice, two main findings were obtained in OVA-induced allergic asthma. Firstly, the S1P₂ receptor functions to exacerbate allergic asthma responses *in vivo*; this was previously observed using JTE-013 by Terashita et al. (2016), implying that blockade of the S1P₂ receptor offers a potential strategy for the treatment of allergic asthma. Secondly, the S1P₂ receptor is involved in dendritic cell maturation and migration in the antigen sensitization phase, which is a novel finding. Previously, S1P was shown to inhibit LPS-induced secretion of IL-12 and TNF- α in a dose-dependent manner and to up-regulate release of IL-10 in human mature dendritic cells (Idzko et al., 2002). However, the involvement of specific S1P receptors for S1P-induced release of Th2 cytokines from mature dendritic cells were not investigated previously (Idzko et al., 2002). Idzko et al. reported that local application of FTY720 (S1P receptor agonist) to the lung attenuates experimental asthma by inhibiting lung DC migration to the mediastinal lymph nodes, consequently preventing the formation of allergen-specific Th2 cells (Idzko et al., 2006). Because FTY720-phosphate, a metabolite of FTY720, is an agonist of S1P_{1,3-5} receptors and a functional antagonist of the S1P₁ receptor (Brinkmann et al., 2002; Brinkmann, Cyster, & Hla, 2004), this result could not directly elucidate the specific S1P receptor that functions to inhibit DC migration. In contrast, Maeda et al. reported the S1P induces the migration of mature DC in mice, not that of immature DC, and also reported the importance of the S1P₃ rather than the S1P₁ receptor for S1P-induced DC migration (Maeda et al., 2007). In the present study, for the first time, the functions of S1P₂ receptors in the maturation and migration of dendritic cells were elucidated using JTE-013 and S1P₂-deficient mice. Therefore, S1P by itself could induce chemotaxis of mature DCs through S1P₃ receptors, but S1P₂ receptor activation could positively influence the cytokine-induced migration of mature and immature DCs.

Previously, the importance of S1P and S1P receptors has been shown in several animal studies. For example, antigen-induced airway inflammation and increased cell number in BALF were significantly augmented by an intranasal administration of S1P (Chiba, Suzuki, et al., 2010). In antigen-induced airway inflammation, inhalation of sphingosine kinase inhibitors, dimethylsphingosine, or SK-I resulted in decreased S1P concentrations in BALF, which returned to basal levels, accompanied by decreased eosinophil infiltration and peroxidase activity (Nishiuma et al., 2008). Furthermore, a role for sphingosine kinase 1 and S1P in allergen-induced airway inflammation was suggested by using sphingosine kinase 1-deficient mice and subjecting them to acute and chronic allergen exposure (Haberberger et al.,

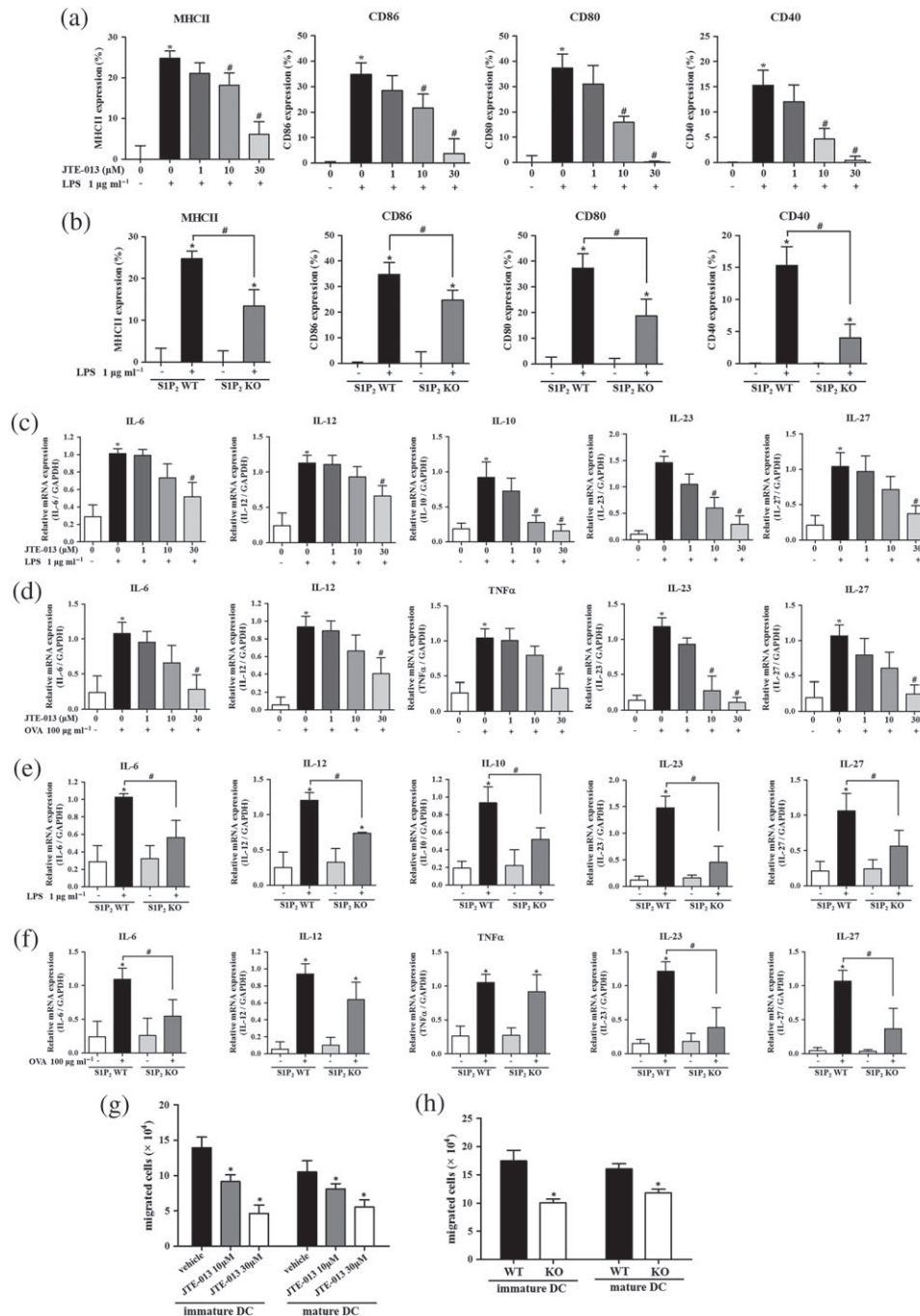


FIGURE 3 Inhibitory effects of S1P₂ antagonist and S1P₂ deficiency on DC maturation and migration. (a) Immature S1P₂ WT DCs were pretreated with vehicle or the indicated concentrations of JTE-013 1 hr before LPS administration and matured in the presence of LPS 1 μg·ml⁻¹ for 24 hr. Degrees of maturation were determined by flow cytometry. Graphs show the expression levels of MHC class II, CD86, CD80, and CD40 in samples matured under identical conditions. (b) Immature S1P₂ WT DCs or KO DCs were treated with LPS 1 μg·ml⁻¹ for 24 hr. Degrees of maturation were determined by flow cytometry. (c) DCs were pre-incubated with the indicated concentrations of JTE-013 for 1 hr and then stimulated with LPS for 24 hr. The mRNA expressions of cytokines (IL-6, IL-10, IL-12, IL-23, IL-27, and TNF-α) were determined by RT-PCR. (d) S1P₂ WT DCs were pre-incubated with the indicated concentrations of JTE-013 for 1 hr and then treated with OVA 100 μg·ml⁻¹ for 24 hr. RT-PCR was performed to determine the levels of cytokines related to DC maturation, namely, the levels of IL-6, IL-12, IL-23, IL-27, and TNF-α. (e) S1P₂ WT and KO DCs were treated with LPS for 24 hr, and mRNA levels were assayed by RT-PCR. (f) S1P₂ WT and KO DCs were treated with OVA for 24 hr, and the expression levels of cytokines were determined by RT-PCR using GAPDH as an internal reference. The results shown are from five independent experiments and are presented as means ± SEM (n = 5). The effect of JTE-013 (g) or S1P₂ deficiency (h) on the migration of immature and mature DCs (imDC and mDCs); imDC and mDCs derived from bone marrow cells of S1P₂ WT and KO mice were subjected to migration assays. DCs were pretreated with vehicle or JTE-013 and then subjected to chemotaxis assays using CXCL12 (100 ng·ml⁻¹ for imDC) or CCL9 (100 ng·ml⁻¹ for mDC) as attractants. Numbers of migrating imDCs and mDCs were determined by flow cytometry. The results shown are from five independent experiments and are presented as means ± SEM (n = 5).

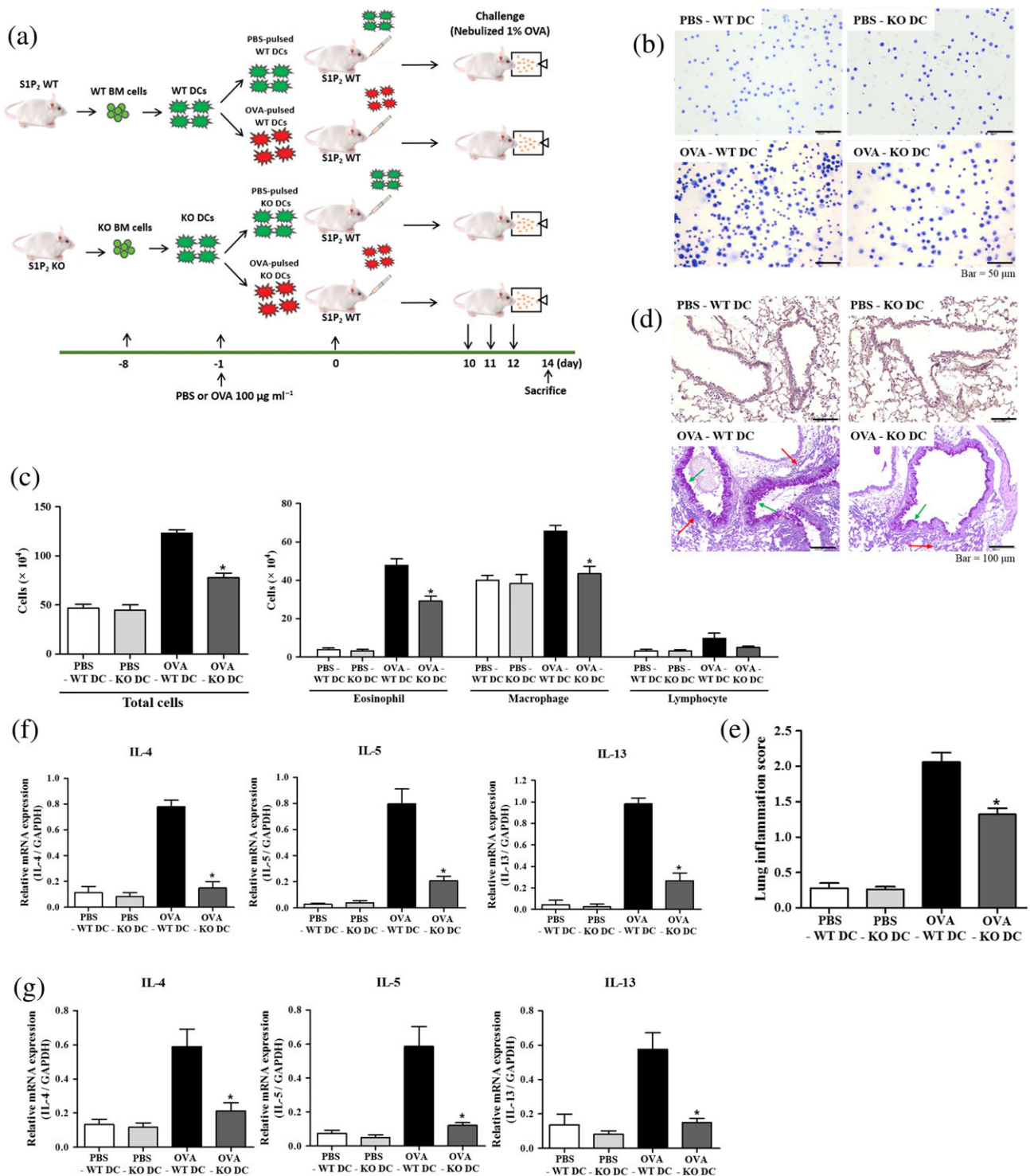


FIGURE 4 Intratracheal administration of S1P₂-deficient DCs lowered eosinophilia to a greater extent than administration of WT DCs. (a) Protocol for the adoptive transfer experiment. The protocol used for the OVA-pulsed DC adoptive transfer experiment. BMDCs from S1P₂ WT or KO mice were pulsed with PBS or 100 µg ml⁻¹ of OVA for 24 hr. The next day, PBS- or OVA-pulsed BMDCs isolated from S1P₂ WT or KO mice were administered intratracheally to WT mice. Ten days later, the mice were challenged with OVA inhalation on Days 10–12. On Day 14, lung tissues and BALF were collected. (b and c) Total and differential cell counts in BALF were determined by May–Grünwald–Giemsa staining. (d) Lung tissue sections were stained with PAS/haematoxylin for histological analysis. (e) Degrees of peribronchial and perivascular inflammation in PAS-stained sections were evaluated using a 4-point scale as described in 2. (f and g) RT-PCR analyses for Th2 cytokines (IL-4, IL-5, and IL-13) were performed using mRNA isolated from immune cells in BALFs (f) or lung tissues (g). mRNA levels were expressed as ratios of GAPDH mRNA levels. The values shown are means ± SEM (*n* = 5)

2009). Intranasal delivery of sphingosine kinase 1 inhibitor (SK1-I) significantly reduces mast cell-dependent allergic inflammation, such as airway hyperresponsiveness and increased eosinophils and cytokines in BALF (Price et al., 2013). However, systemic treatment with the sphingosine kinase inhibitor SKI-II had no effect on antigen-induced inflammatory signs, such as increases in cell counts, IL-4, and IL-13 in BALFs and changes in airway histology (Chiba, Takeuchi, Sakai, & Misawa, 2010), although antigen exposure-induced bronchial smooth muscle hyperresponsiveness was ameliorated by treatment with SKI-II (Chiba, Takeuchi, et al., 2010). In OVA-sensitized mice, the S1P and sphingosine kinase pathway triggers airway hyperresponsiveness. In the whole lung system, S1P increases airway resistance only in OVA-sensitized mice (Roviezzo et al., 2007). Therefore, S1P exacerbates airway inflammation, but inhibition of sphingosine kinases is not consistently effective as an inhibitor of airway inflammation, although it is effective at reducing airway hyperresponsiveness. Therefore, instead of inhibiting sphingosine kinase, targeting a specific S1P receptor might be more beneficial for treating allergic airway inflammation. The identification of the S1P₂ receptor as a molecular target of allergic asthma in the present study may serve as a base for the development of drugs targeting S1P₂ receptors to clinically improve allergic responses.

The in vivo anti-allergic effects of JTE-013 and S1P₂ deficiency may be caused not only by inhibition of the functions of S1P₂ receptors in dendritic cells and mast cells but also partly by inhibition of S1P₂ receptors in other cell types. In previous studies, treatment with JTE-013 attenuated IgE-stimulated anaphylactic responses and pulmonary oedema in mice (Oskeritzian et al., 2010). JTE-013 has been reported to inhibit S1P-induced fibroblast chemotaxis (Hashimoto et al., 2008). It also blocks S1P-induced inhibition of migration and Rac1-dependent signalling pathway in human bronchial smooth muscle cells (Kawata et al., 2005). S1P modulates airway smooth muscle cell function, including pro-inflammatory cytokine production and cell growth, in human airway smooth muscle cultures (Ammit et al., 2001). These findings suggest that antagonism of S1P₂ receptors on the vasculature, fibroblasts, and smooth muscle cells in a direct or indirect manner may also contribute to the anti-allergic effects of JTE-013 and S1P₂ deficiency in airway allergic asthma. In summary, using an animal model, we showed that the S1P₂ receptor could be a therapeutic target for allergic asthma and elucidated novel functions of S1P₂ receptors in dendritic cells.

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AUTHOR CONTRIBUTIONS

S.J.P. and D.S.I. designed the experiments. S.J.P. performed the experiments and analysed the data. S.J.P. and D.S.I. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis, Immunoblotting and Immunochemistry](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplemental Figures Legends

Supplemental Figure 1. Effect of S1P₂ antagonist treatment on the features of asthma

- (A) May-Grünwald-Giemsa staining was used to obtain differential cell counts in BALF.
- (B) PAS-stained sections of lung tissues. Panels showing the PAS/hematoxylin-stained sections of lung tissues in the 5 experimental groups.

Supplemental Figure 2. Effect of S1P₂ deficiency on OVA-induced allergic responses

- (A) May-Grünwald-Giemsa staining was used to obtain differential cell counts in BALF.
- (B) PAS-stained sections of lung tissues. Panels showing the PAS/hematoxylin-stained sections of lung tissues in the 5 experimental groups.

Supplemental Figure 3. Effect of S1P₂ antagonist treatment on the lung function, serum IgE levels, and numbers of T cells

- (A) Airway hyperresponsiveness. (B) Serum IgE levels. (C) Percentage of IL-4⁺ T cells in draining lymph nodes. Results are presented as means $\pm$ SEM (n = 5).

Supplemental Figure 4. Expression of S1P receptors in BMDC

RT-PCR analysis of S1P receptor (S1P₁₋₅) expression was performed on BMDCs. The left lane shows DNA ladder.

S1P₁ F CACCGGCCCATGTACTAT, S1P₁ R GCAGCCCACATCTAACAG

S1P₂ F CATGGGCGGCTTATACTCA, S1P₂ R CACTGCACGGGAGTTAAGGA

S1P₃ F GGGAGGGCAGTATGTTTCGTA, S1P₃ R GGAGCCCGCAACAGATAAG

S1P₄ F TGGAGGGCTAGGGATGCT, S1P₄ R GGCCCAGACATTAGAACCAA

S1P₅ F CCTTGGCAGCCTCACTTTG, S1P₅ R GCCCGCACCTGACAGTAAAT

Supplemental Figure 5. The intratracheal administration of JTE-013/OVA-treated DCs lowered eosinophilia than the administration of OVA-treated DCs.

BMDCs from S1P₂ WT mice were pulsed with PBS, 100 µg/ml of OVA for 24 h, or JTE-013 10 µM for 1 h and 100 µg/ml of OVA for 24 h. The next day, PBS-, OVA-, JTE/OVE-pulsed BMDCs isolated from S1P₂ WT were administered intratracheally to WT mice. Ten days later, mice were challenged by OVA inhalation on 10-12 days. On day 14, lung tissues and BALF were collected. (A) Images of BALF cells. (B and C) Total and differential cell counts in BALF were determined by May-Grünwald-Giemsa staining. (D) Degrees of peribronchial and perivascular inflammations in PAS-stained sections were evaluated using a 4-point scale as described in Materials and Methods. (E) Serum IgE levels. (F and G) RT-PCR analyses for Th2 cytokines (IL-4, IL-5, and IL-13) were performed using mRNA isolated from immune cells in BALFs (F) or lung tissues (G). mRNA levels were expressed as ratios of GAPDH mRNA levels. The values shown are means ± SEMs (n = 5).

Supplemental Methods

Measurements of immunoglobulin (IgE)

Mouse IgE levels in the BALF were determined using ELISA kits (eBioscience, San Diego, CA). Briefly, 96-well plates (NUNC, USA) were coated overnight at 4°C with capture antibodies for IgE. Following washing, plates were blocked for 2 h at room temperature with blocking buffer, BALF supernatants were added to each well, and incubated for 2 h at room temperature. Pre-titrated, biotin-conjugated detection antibodies for IgE were then added and incubated for 1 h at room temperature, pre-titrated streptavidin-HRP was added for 30 min at room temperature, and plates were incubated with substrate solution for 15 min at room temperature. Stop solution was then added and absorbance was read at 450 nm.

Measurement of airway hyperresponsiveness (AHR)

Twenty-four hours after the last OVA challenge, the AHR was assessed in conscious, unrestrained mice using noninvasive whole-body plethysmography (Allmedicus, Seoul, Korea). In brief, the mice were placed in the plethysmography chamber and exposed to increasing concentrations of aerosolized methacholine at 0, 12.5, 25, and 50 mg/ml for 5 min using ultrasonic nebulizers. The enhanced pause (Penh) was measured for 3 min after each methacholine aerosol in the plethysmography chamber during inspiration and expiration combined with the time of each phase and calculated during each 3 min interval were then averaged.

FACS analysis of Th2 cell quantifications in draining lymph node

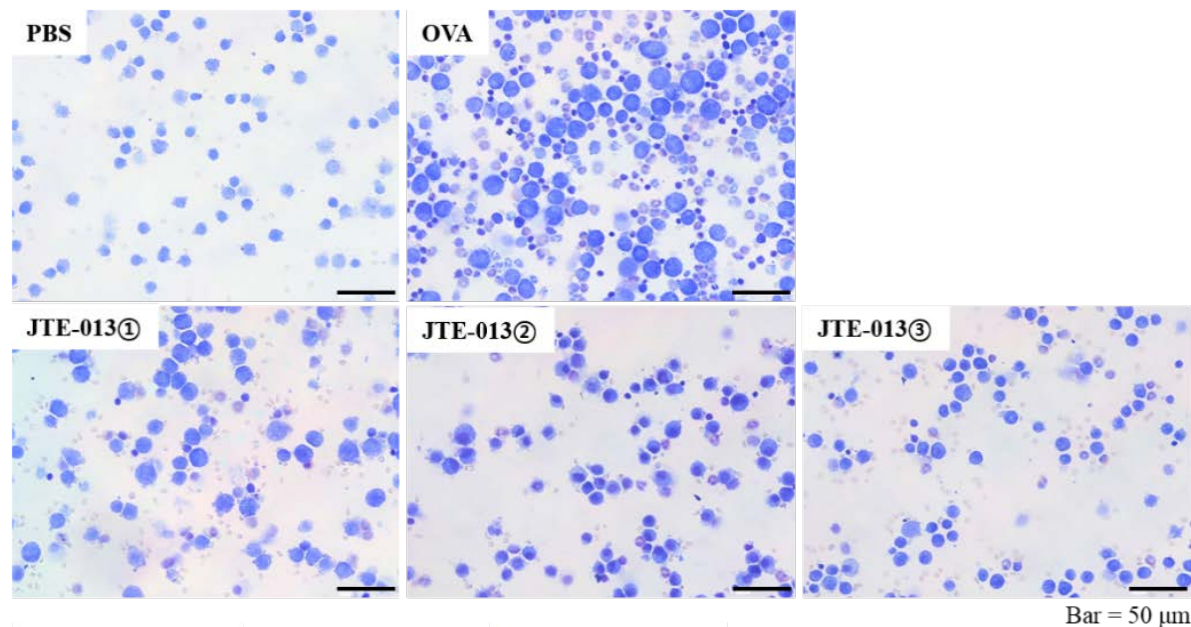
Cells isolated from the draining lymph nodes were stained with FITC-labeled anti-IL-4 antibody in accordance with the manufacturer's recommendations (eBioscience, San Diego, CA). The cells were permeabilized using Intracellular Fixation & Permeabilization buffer set (eBioscience, San Diego, CA). After permeabilization, the cells were stained with anti-IL-4-FITC. Cells were analyzed using a BD Accuri™ C6 Flow Cytometer (BD, Franklin Lakes, NJ, USA).

Adoptive Transfer Experiment with BMDCs

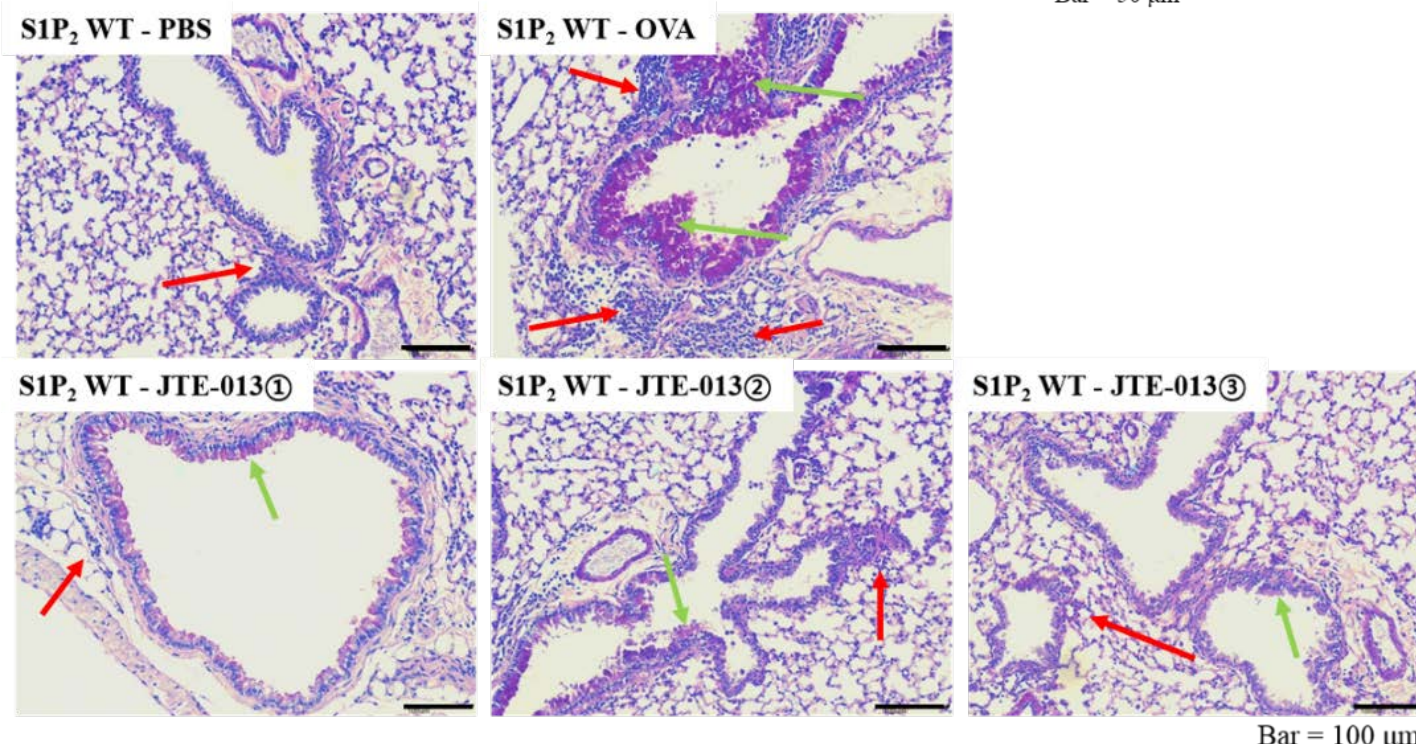
For adoptive transfer experiments, BMDCs isolated from S1P₂ WT mice were pretreated with vehicle or JTE-013 (10 μ M) for 1 h and treated OVA (100 μ g/ml) for 24 h. PBS-pulsed BMDCs, OVA-pulsed BMDCs, and JTE-013/OVA-pulsed BMDCs (1×10^6) in 50 μ l of PBS were instilled into S1P₂ WT mice through trachea, respectively. Ten days later, mice were exposed to aerosolized OVA (1% in PBS) for 20 min/day for three consecutive days, and 48 h after last challenge, BALF and lung tissues were obtained to confirm asthmatic responses, as previously described.

Supplemental Figure 1

A

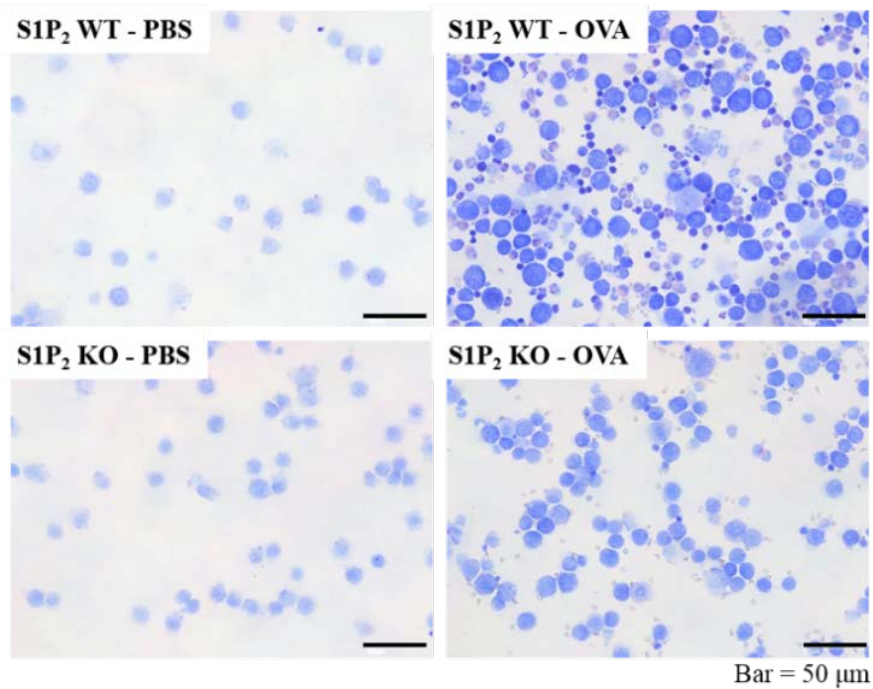


B

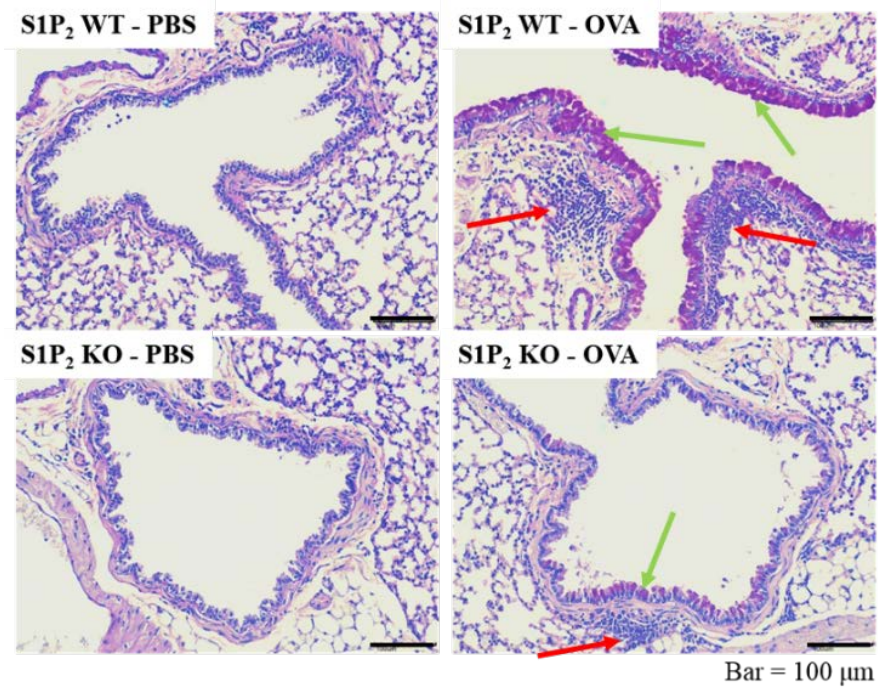


Supplemental Figure 2

A

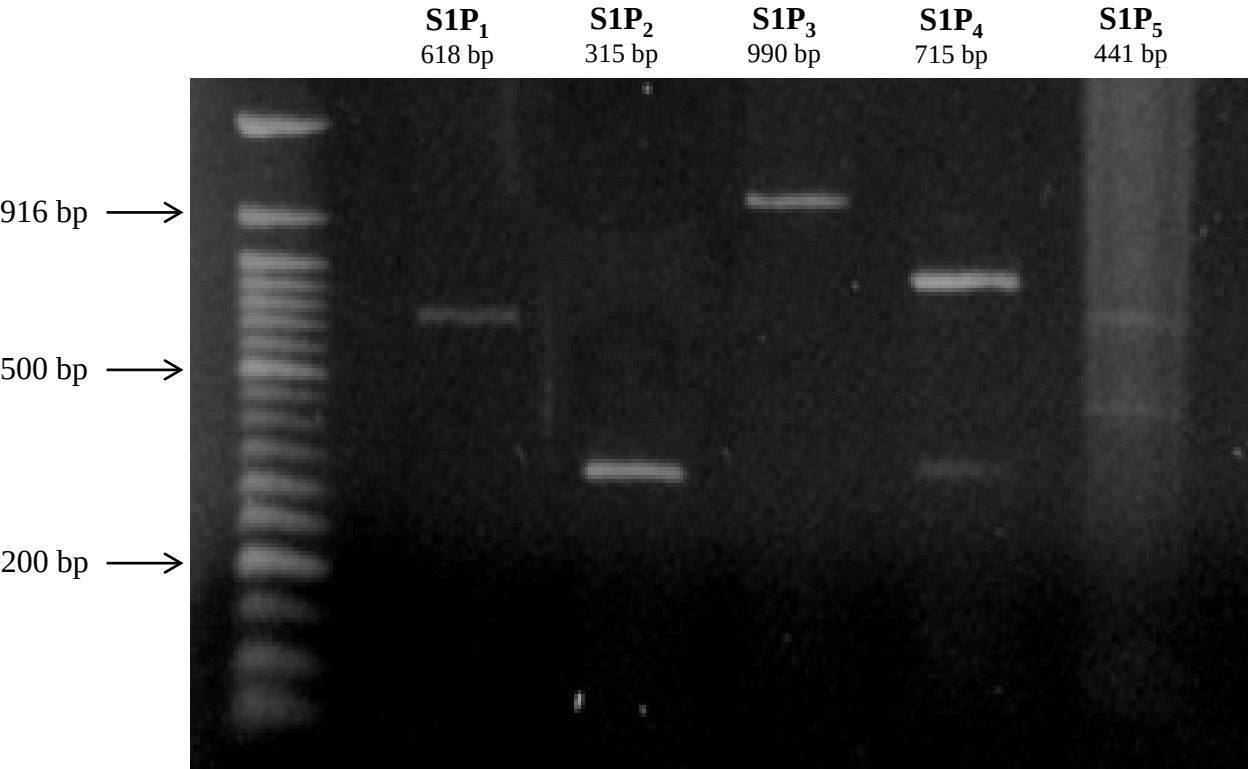


B

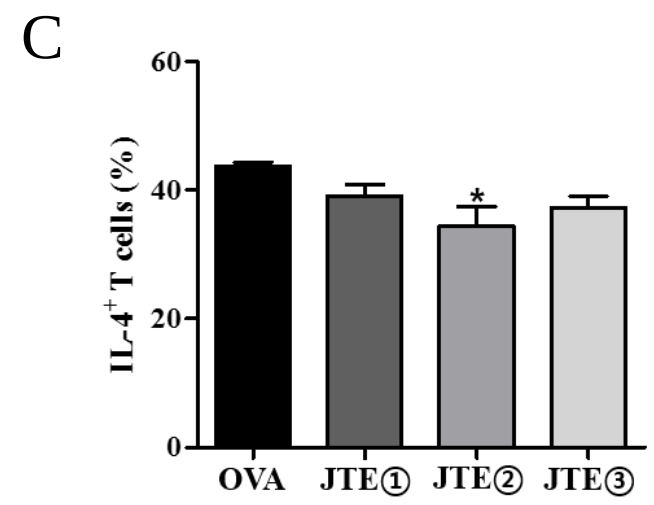
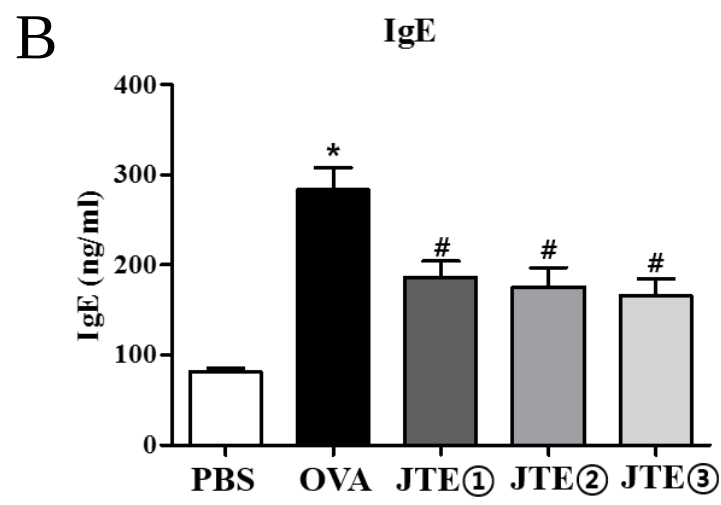
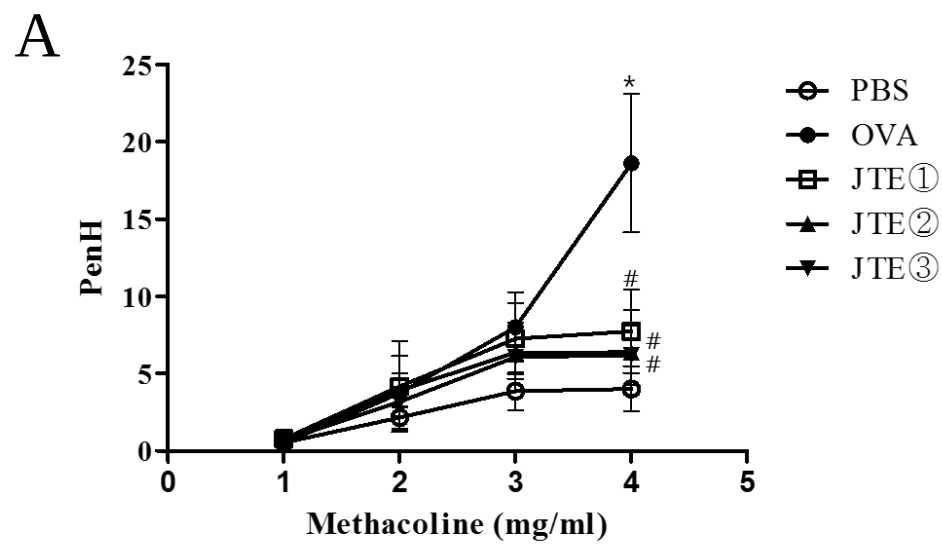


Supplemental Figure 3

S1P₂ WT BMDC



Supplemental Figure 4



Supplemental Figure 5

