Topical Application of S1P$_2$ Antagonist JTE-013 Attenuates 2,4-Dinitrochlorobenzene-Induced Atopic Dermatitis in Mice

Jisoo Kang[1], Ju-Hyun Lee[2] and Dong-Soon Im[1,2,]*

[1]*Laboratory of Pharmacology, College of Pharmacy, Pusan National University, Busan 46241,*
[2]*Laboratory of Pharmacology, College of Pharmacy, and Department of Life and Nanopharmaceutical Sciences, Graduate School, Kyung Hee University, Seoul 02447, Republic of Korea*

Abstract

Sphingosine-1-phosphate (S1P) and its receptors have been implicated in atopic dermatitis. S1P$_2$ was found to function as a pro-allergic receptor, while its antagonist JTE-013 was found to suppress allergic asthma in mice. Topical application of JTE-013 has not been investigated in an *in vivo* model of atopic dermatitis. Therefore, the therapeutic potential of JTE-013 topical application was evaluated by the use of a 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis mouse model. DNCB-induced inflammation and mast cell accumulation in skin tissues were significantly suppressed by topical JTE-013 treatment in BALB/c mice. DNCB-induced increase of lymph nodes sizes and elevated inflammatory cytokines (IL-4, IL-13, IL-17, and IFN-γ) in lymph nodes were also significantly reduced by the JTE-013 treatment. Elevated serum levels of IgE were significantly suppressed by the topical treatment of JTE-013. In summary, the topical treatment of JTE-013 S1P$_2$ antagonist suppressed DNCB-induced atopic dermatitis symptoms and immune responses. These results suggested JTE-013 as a potential therapeutic agent for atopic dermatitis.

Key Words: Atopy, Dermatitis, Dendritic cell, Sphingosine 1-phosphate, S1P$_2$

INTRODUCTION

Sphingosine 1-phosphate (S1P) is an intercellular lipid mediator, which acts by binding to its specific G protein-coupled receptors in the plasma membrane, now referred to as S1P$_{1-5}$ (Park and Im, 2017). Sphingosine kinases 1 and 2 are responsible for S1P synthesis from sphingosine, while S1P lyase degrades S1P irreversibly to hexadecenal and ethanolamine phosphate (Spiegel and Milstien, 2007; Leong and Saba, 2010). S1P regulates cell differentiation, migration, survival and complex physiological processes (Proia and Hla, 2015; Park and Im, 2017). S1P and its receptors have also been implicated in the development of atopic dermatitis-like skin lesions in NC/Nga mice (Kohno *et al*., 2004; Bock *et al*., 2016) and animal skin allograft rejection (Yanagawa *et al*., 1999). In the skin lesions of dogs suffering from atopic dermatitis, S1P concentration was decreased, and S1P metabolism was enhanced (Baumer *et al*., 2011). Plasma S1P levels were elevated in patients severely affected with psoriasis, an inflammatory skin disorder (Checa *et al*., 2015; Mysliwiec *et al*., 2017). Therefore, S1P and its receptors have been implicated in skin disorders like atopic dermatitis.

Previously, S1P was found to act as a pro-allergic factor via S1P$_2$ in murine ovalbumin-induced allergic asthma model (Park and Im, 2019a). In addition, S1P modulates antigen capture by murine and human Langerhans cells via S1P$_2$ in the skin (Japtok *et al*., 2012; Bock *et al*., 2016). Therefore, chemical blockage of S1P$_2$ might be a therapeutic strategy for inflammatory skin disorders. However, systematic administration of S1P$_2$ antagonist can cause serious side effects, because S1P$_2$ expression is almost ubiquitous. In order to avoid systemic side effects of S1P$_2$ antagonist, topical application of S1P$_2$ antagonist in the skin must be tested to verify its efficacy in the skin. Therefore, the efficacy of the topical application of JTE-013, a specific S1P$_2$ antagonist, on murine 2,4-dinitro-chlorobenzene (DNCB)-induced atopic dermatitis model was investigated *in vivo* in this study.

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MATERIALS AND METHODS

Materials

JTE-013 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). 1-Chloro-2, 4-dinitrobenzene (DNCB) and olive oil were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Seven-week-old male BALB/c mice were purchased from Daehan Biolink (DBL; Seoul, Korea) and housed in the laboratory animal facility at Pusan National University. They were provided food and water *ad libitum*. The mice were housed in standard plastic cages (two mice per cage) 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 h and 19:00 h). There were likewise provided with standard laboratory food and water. The animal protocol used in this study was reviewed and approved by the Pusan National University–Institutional Animal Care Committee (PNU–IACUC) in accordance to procedure ethicality and scientific care.

Induction of atopic dermatitis in BALB/c mice and topical JTE-013 treatment

Following simple randomization procedure, 7-week old male BALB/c mice were divided into three groups (n=5): a vehicle-treated control group, a DNCB-treated mice group, and a JTE-013/DNCB-treated group. To induce experimental atopic dermatitis, ventral skin was shaved, and 300 μL of 1% DNCB in acetone/olive oil (3:1) was applied to the ventral skin on day 0. On day 7, mice were challenged again by the application of 200 μL of 0.3% DNCB to the ears every other day for up to 42 days. From day 20 until the completion of the experiment, the JTE-013/DNCB-treated group was topically treated with JTE-013 (200 μg/20 μL for each ear) every other day, which is an alternative to DNCB treatment days for up to 42 days. JTE-013 was dissolved in a mixture of acetone/olive oil (3:1). The mice were sacrificed on day 49.

Histologic analysis and mast cell count in the skin

After sacrifice on day 49, ear tissues were steeped in 10% formalin, dehydrated in 30% sucrose solution, embedded in O.C.T. compound, and sectioned in 8 μm thick segments. Sections were stained with hematoxylin and eosin (H&E) or with toluidine blue O (TBO). For H&E staining, sections were washed in distilled water and stained with hematoxylin solution for 15 s. After being rinsed with warm running tap water, sections were stained with eosin reagents for 10 s, followed by rinsing, dehydration, and coversliped with Permount.

For the detection of mast cells by TBO staining, sections were washed in distilled water and stained with toluidine blue solution for 2 min, then rinsed in distilled water, dehydrated, and coversliped with Permount.

Measurement of total serum IgE levels

Mouse IgE levels in the serum were determined by the use of ELISA kits (eBioscience, San Diego, CA, USA). Briefly, 96-well plates (Thermo Flsher Scientific, Waltham, MA, USA) were coated overnight at 4°C with capture antibodies for IgE. Following washing, plates were blocked for 2 h at room temperature with a blocking buffer. Serum was added to each well, and plates were 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, while 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.

Reverse transcriptase-PCR

To assess the expression levels of inflammatory markers in the lymph nodes of mice by RT-PCR, first-strand cDNA was first synthesized from total RNA isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA). Total RNAs were isolated from draining lymph nodes. Synthesized cDNA products, primers for each gene, and Promega Go-Taq DNA polymerase (Madison, WI, USA) were used for PCR. Specific primers and PCR conditions were previously described (Park and Im, 2019a). Aliquots (5 μL) were electrophoresed in 1.2% agarose gels and stained with StaySafeTM Nucleic Acid Gel Stain (Real Biotech Corporation, Taipei, Taiwan). Intensities of each PCR product were quantified by means of ImageJ software (NIH, Bethesda, MD, USA) and normalized with GAPDH levels.

Statistics

Results are expressed as the means ± standard error of the mean (SEM) of six evaluations for animal experiments. Statistical significances of differences were determined by the analysis of variance (ANOVA) and Tukey's multiple comparison test. Statistical significance was accepted for p values < 0.05. * indicates significance difference compared to the vehicle-treated group, or untreated controls, whereas # indicates significant difference compared to the DNCB-treated group. Analyses were performed through the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).
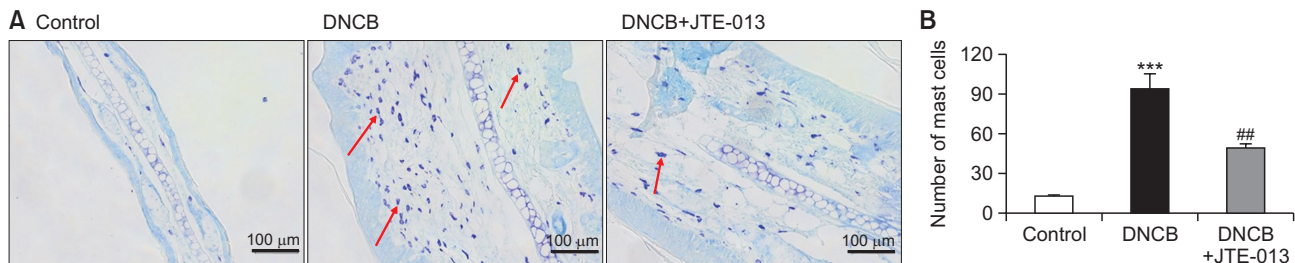
RESULTS

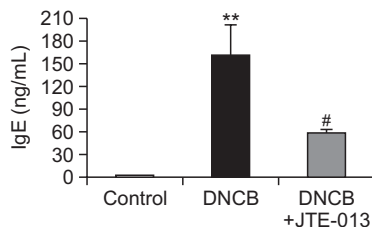### Topical JTE-013 treatment inhibited DNCB-induced atopic dermatitis in the ears

In order to identify the therapeutic potential of JTE-013 topical application, BALB/c mice were treated with JTE-013 after the induction of atopic dermatitis (Fig. 1A). To induce experimental atopic dermatitis, 300 μL of 1% DNCB in acetone/olive oil (3:1) was applied to shaved ventral skin on day 0, and mice were challenged again through the application of 200 μL of 0.3% DNCB to the ears from day 7 up to day 49 every other day (Fig. 1A). JTE-013 (200 μg/20 μL for each ear) was topically applied to the ears from day 20 up to day 49 every other day, which is an alternative to DNCB treatment days. Symptoms of DNCB-induced atopic dermatitis, such as edema, erythema, and cracking of the skin, were observed in the exposed ears (Fig. 1B). Topical JTE-013 treatment reduced these symptoms as shown in Fig. 1B. In addition, H&E staining confirmed that DNCB induced extensive hypertrophy of epidermis due to hyperkeratosis, as well as infiltration of immune cells was increased in DNCB-treated group compared to the control group (Fig. 1C). Topical JTE-013 treatment significantly reduced the DNCB-induced hypertrophy of the epidermis, as well as DNCB-induced immune cell infiltration to the ears (Fig. 1C).
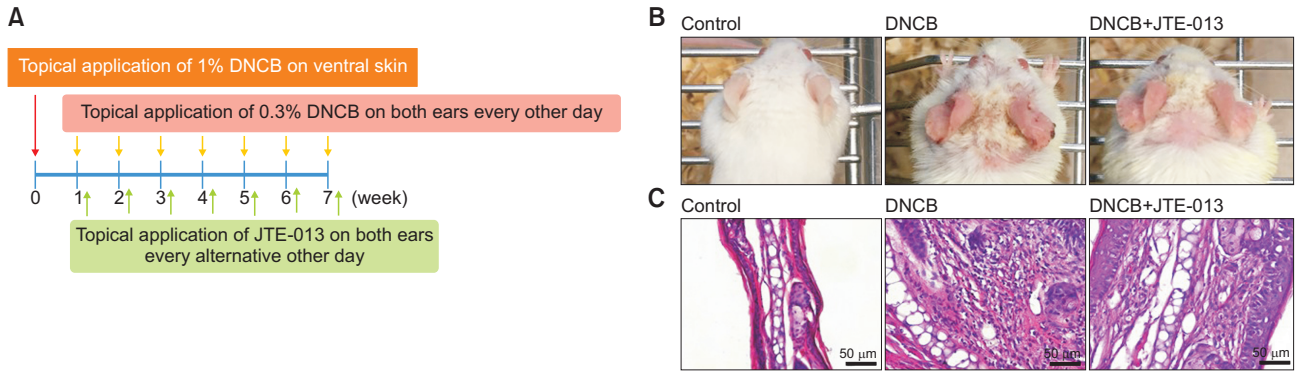
Fig. 1. Effect of topical JTE-013 treatment on DNCB-induced atopic dermatitis in ears. (A) Experimental protocol. A murine model of DNCB-induced atopic dermatitis is established by DNCB sensitization on day 0, as well as repeated DNCB challenges on days 7-49 to induce atopic dermatitis. BALB/c mice were treated with either vehicle or DNCB, while JTE-013 was topically applied on the skin on alternative days to DNCB challenge days. (B) Macroscopic view of the ears. (C) H&E staining of ear tissue sections.

Fig. 2. Effect of topical JTE-013 treatment on DNCB-induced mast cell accumulation in ears. (A) TBO staining of ear samples. Red arrows indicate several mast cells. (B) Number of mast cells. Results are presented as means ± SEM (n=5). ***$p<0.001$ *vs.* the vehicle-treated group, ##$p<0.01$ *vs.* the DNCB-treated group.

Fig. 3. Effect of topical JTE-013 treatment on DNCB-induced increases in serum IgE levels. Serum IgE levels. Results are presented as means ± SEM (n=5). **$p<0.01$ *vs.* the vehicle-treated group, #$p<0.05$ *vs.* the DNCB-treated group.

Topical JTE-013 treatment inhibited DNCB-induced infiltration of mast cells in the ears

TBO staining was employed to confirm increased infiltration of mast cells into the dermis (Fig. 2A). Mast cells were shown as small, red-purple dots (Fig. 2A). Not only increased infiltration of mast cells, but also hypertrophy, was observed in the DNCB-treated atopic group (Fig. 2A). In sections from mice treated with DNCB plus topical JTE-013, however, fewer stained mast cells were observed than in sections from mice treated only with DNCB (Fig. 2A). The numbers of mast cells were counted semi-quantitatively. As shown in Fig. 2B, the number of mast cells in the dermis was increased by 628% in the DNCB group than in the control group, while topical JTE-

013 treatment significantly reduced DNCB-induced increase of mast cell infiltration into the dermis by 55% (Fig. 2B).

Topical JTE-013 treatment reduced DNCB-induced increases in serum IgE levels

Serum IgE levels were measured to determine the immunological effects of DNCB and topical JTE-013 treatment. Hyperproduction of IgE was observed in the sera of DNCB-treated mice, while topical JTE-013 treatment significantly reduced the elevated serum IgE level (Fig. 3). The IgE level in DNCB-treated mice was increased by 2750% compared to the control mice, while topical JTE-013 treatment significantly reduced the DNCB-induced increase of IgE level by 65% (Fig. 3).

Topical JTE-013 treatment reduced DNCB-induced atopic responses in lymph nodes

Lymph node sizes were evaluated in drained lymph nodes. Lymph nodes are important components of the lymphatic system, and are filled with B cells and T cells. When an infection or an allergic reaction occurs, lymph nodes undergo swelling. The lymph nodes from DNCB-treated mice were swollen compared to those from the control mice, and lymph node weight was increased by 600% (Fig. 4). Topical JTE-013 treatment significantly reduced the DNCB-induced increase in lymph node weight by 32% (Fig. 4).

Topical JTE-013 treatment reduced DNCB-induced increases in cytokine levels in the lymph nodes

Atopic dermatitis is believed to be regulated not only by Th2 but also by Th17 and Th1 responses (Koga *et al*., 2008; Kim *et al*., 2014; Muraro *et al*., 2016). Thus, the Th2, Th17, and Th1 responses were investigated by the measurement of IL-4 and IL-13 for Th2, IL-17A for Th17, and INF-γ for Th1 in the lymph nodes. The messenger RNA levels of each cytokine were significantly increased in lymph nodes, following the application of DNCB (Fig. 5). The increases for IL-4, IL-13, IL-17A, and IFN-γ were 965%, 360%, 849%, and 657%, respectively, compared to the control. Topical JTE-013 treatment significantly reduced DNCB-induced increases in the Th2 cytokines IL-4 and IL-13 (Fig. 5A, 5B). Also, the levels of the Th17 and Th1 cytokines IL-17A and INF-γ were significantly reduced by topical JTE-013 treatment (Fig. 5C, 5D). Topical JTE-013 treatment significantly reduced the DNCB-induced increases in IL-4, IL-13, IL-17A, and INF-γ by 48, 54, 63, and 51%, respectively (Fig. 5).

DISCUSSION

Based on the pro-allergic functions of S1P$_2$ in allergic asthma, topical JTE-013 treatment in this study was investigated in the murine DNCB-induced atopic dermatitis model. Topical treatment of JTE-013 reduced not only atopic responses in the skin but also immune responses in the lymph nodes and serum. This study shows a therapeutic possibility of JTE-013 S1P$_2$ antagonist. Because topical JTE-013 treatment was undertaken after the induction of atopic dermatitis, JTE-013 or S1P$_2$ antagonists have strong therapeutic significance. Furthermore, topical application could avoid the possible side effects of systemic JTE-013 administration. In addition, this study supports the previous finding of pro-allergic functions of S1P$_2$ (Park and Im, 2019a) and also pro-inflammatory functions of S1P$_2$ (Park and Im, 2019b). Therefore, this finding proves a new direction for S1P$_2$ receptor therapeutics.

Previously, the topical administration of S1P diminished imiquimod-induced psoriasis symptoms, such as, ear swelling and epidermal thickness in the ear (Schaper *et al*., 2013), while topical application of S1P and FTY720, an S1P modulator, attenuated allergic contact dermatitis reactions (Nakashima *et al*., 2008; Reines *et al*., 2009). Also, S1P receptors have been implicated in the development of atopic dermatitis-like skin lesions in NC/Nga mice, because FTY720 prevented spontaneous dermatitis and impaired initiation of contact hypersensitivity (Kohno *et al*., 2004; Nakashima *et al*., 2008; Tsuji *et al*., 2012, 2016). However, specific S1P receptors have not been investigated in skin disorders.

The *in vivo* anti-atopic effects of topical JTE-013 treatment may be caused by the inhibition of S1P$_2$ in mast cells and dendritic cells (Oskeritzian *et al*., 2010; Japtok *et al*., 2012; Olivera *et al*., 2013; Bock *et al*., 2016; Park and Im, 2019a), because S1P$_2$ has been proven to function as an activator of mast cell degranulation and dendritic cell maturation and migration (Prieschl *et al*., 1999; Oskeritzian *et al*., 2010; Park and Im, 2019a). However, the inhibition of S1P$_2$ in other cell types such as keratinocytes also could contribute to the effect of JTE-013 (Kim *et al*., 2004; Jeong *et al*., 2015). In summary, the present *in vivo* efficacy of topical JTE-013 treatment could be due to the following: 1) the suppression of mast cell degranulation, 2) the suppression of dendritic cell maturation and migration, 3) the suppression of inflammatory cytokines (IL-4, IL-13, IL-17A, and INF-γ) production, and 4) the reduced accumulation of mast cells in the dermis. The effects of topical JTE-013 treatment on other cell types are currently unknown and should be elucidated in the near future.
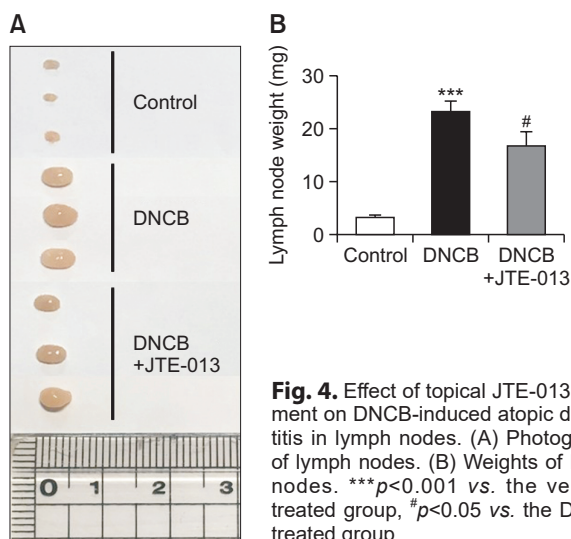
Fig. 4. Effect of topical JTE-013 treatment on DNCB-induced atopic dermatitis in lymph nodes. (A) Photographs of lymph nodes. (B) Weights of lymph nodes. ****p*<0.001 *vs.* the vehicle-treated group, #*p*<0.05 *vs.* the DNCB-treated group.
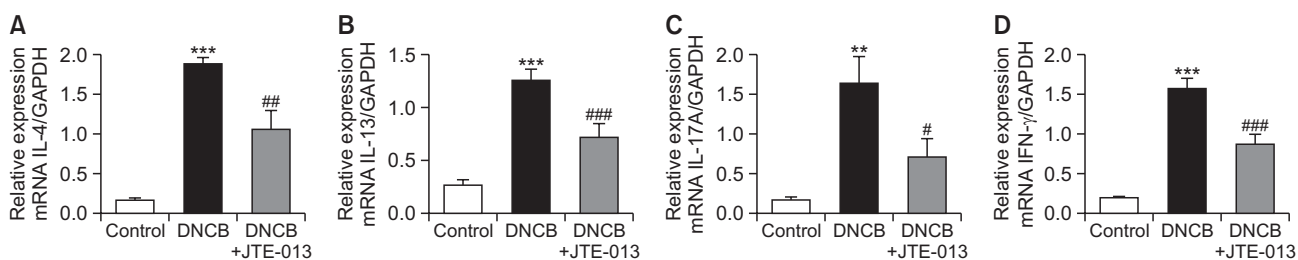
Fig. 5. Effect of topical JTE-013 treatment on DNCB-induced increases in cytokine levels in lymph nodes. RT-PCR analyses for Th2 cytokines (IL-4 and IL-13), a Th17 cytokine (IL-17A), and a Th1 cytokine (IFN-γ). (A) IL-4, (B) IL-13, (C) IL-17A, and (D) IFN-γ. Results are presented as means ± SEM (n=5). ***p*<0.01, ****p*<0.001 *vs.* the vehicle-treated group, #*p*<0.05, ##*p*<0.01, ###*p*<0.001 *vs.* the DNCB-treated group.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

REFERENCES

Baumer, W., Rossbach, K., Mischke, R., Reines, I., Langbein-Detsch, I., Luth, A. and Kleuser, B. (2011) Decreased concentration and enhanced metabolism of sphingosine-1-phosphate in lesional skin of dogs with atopic dermatitis: disturbed sphingosine-1-phosphate homeostasis in atopic dermatitis. *J. Invest. Dermatol.* **131**, 266-268.

Bock, S., Pfalzgraff, A. and Weindl, G. (2016) Sphingosine 1-phospate differentially modulates maturation and function of human Langerhans-like cells. *J. Dermatol. Sci.* **82**, 9-17.

Checa, A., Xu, N., Sar, D. G., Haeggstrom, J. Z., Stahle, M. and Wheelock, C. E. (2015) Circulating levels of sphingosine-1-phosphate are elevated in severe, but not mild psoriasis and are unresponsive to anti-TNF-a treatment. *Sci. Rep.* **5**, 12017.

Japtok, L., Schaper, K., Baumer, W., Radeke, H. H., Jeong, S. K. and Kleuser, B. (2012) Sphingosine 1-phosphate modulates antigen capture by murine Langerhans cells via the S1P2 receptor subtype. *PLoS ONE* **7**, e49427.

Jeong, S. K., Kim, Y. I., Shin, K. O., Kim, B. W., Lee, S. H., Jeon, J. E., Kim, H. J., Lee, Y. M., Mauro, T. M., Elias, P. M., Uchida, Y. and Park, K. (2015) Sphingosine kinase 1 activation enhances epidermal innate immunity through sphingosine-1-phosphate stimulation of cathelicidin production. *J. Dermatol. Sci.* **79**, 229-234.

Kim, D. S., Kim, S. Y., Kleuser, B., Schafer-Korting, M., Kim, K. H. and Park, K. C. (2004) Sphingosine-1-phosphate inhibits human keratinocyte proliferation via Akt/protein kinase B inactivation. *Cell. Signal.* **16**, 89-95.

Kim, J. Y., Jeong, M. S., Park, M. K., Lee, M. K. and Seo, S. J. (2014) Time-dependent progression from the acute to chronic phases in atopic dermatitis induced by epicutaneous allergen stimulation in NC/Nga mice. *Exp. Dermatol.* **23**, 53-57.

Koga, C., Kabashima, K., Shiraishi, N., Kobayashi, M. and Tokura, Y. (2008) Possible pathogenic role of Th17 cells for atopic dermatitis. *J. Invest. Dermatol.* **128**, 2625-2630.

Kohno, T., Tsuji, T., Hirayama, K., Watabe, K., Matsumoto, A., Kohno, T. and Fujita, T. (2004) A novel immunomodulator, FTY720, prevents spontaneous dermatitis in NC/Nga mice. *Biol. Pharm. Bull.* **27**, 1392-1396.

Leong, W. I. and Saba, J. D. (2010) S1P metabolism in cancer and other pathological conditions. *Biochimie* **92**, 716-723.

Muraro, A., Lemanske, R. F., Jr., Hellings, P. W., Akdis, C. A., Bieber, T., Casale, T. B., Jutel, M., Ong, P. Y., Poulsen, L. K., Schmid-Grendelmeier, P., Simon, H. U., Seys, S. F. and Agache, I. (2016) Precision medicine in patients with allergic diseases: airway diseases and atopic dermatitis-PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology. *J. Allergy Clin. Immunol.* **137**, 1347-1358.

Mysliwiec, H., Baran, A., Harasim-Symbor, E., Choromanska, B., Mysliwiec, P., Milewska, A. J., Chabowski, A. and Flisiak, I. (2017) Increase in circulating sphingosine-1-phosphate and decrease in ceramide levels in psoriatic patients. *Arch. Dermatol. Res.* **309**, 79-86.

Nakashima, D., Kabashima, K., Sakabe, J., Sugita, K., Kobayashi, T., Yoshiki, R. and Tokura, Y. (2008) Impaired initiation of contact hypersensitivity by FTY720. *J. Invest. Dermatol.* **128**, 2833-2841.

Olivera, A., Dillahunt, S. E. and Rivera, J. (2013) Interrogation of sphingosine-1-phosphate receptor 2 function *in vivo* reveals a prominent role in the recovery from IgE and IgG-mediated anaphylaxis with minimal effect on its onset. *Immunol. Lett.* **150**, 89-96.

Oskeritzian, C. A., Price, M. M., Hait, N. C., Kapitonov, D., Falanga, Y. T., Morales, J. K., Ryan, J. J., Milstien, S. and Spiegel, S. (2010) Essential roles of sphingosine-1-phosphate receptor 2 in human mast cell activation, anaphylaxis, and pulmonary edema. *J. Exp. Med.* **207**, 465-474.

Park, S. J. and Im, D. S. (2017) Sphingosine 1-phosphate receptor modulators and drug discovery. *Biomol. Ther. (Seoul)* **25**, 80-90.

Park, S. J. and Im, D. S. (2019a) Blockage of sphingosine-1-phosphate receptor 2 attenuates allergic asthma in mice. *Br. J. Pharmacol.* **176**, 938-949.

Park, S. J. and Im, D. S. (2019b) Deficiency of sphingosine-1-phosphate receptor 2 (S1P$_2$) attenuates bleomycin-induced pulmonary fibrosis. *Biomol. Ther. (Seoul)* **27**, 318-326.

Prieschl, E. E., Csonga, R., Novotny, V., Kikuchi, G. E. and Baumruker, T. (1999) The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fc epsilon receptor I triggering. *J. Exp. Med.* **190**, 1-8.

Proia, R. L. and Hla, T. (2015) Emerging biology of sphingosine-1-phosphate: its role in pathogenesis and therapy. *J. Clin. Invest.* **125**, 1379-1387.

Reines, I., Kietzmann, M., Mischke, R., Tschernig, T., Luth, A., Kleuser, B. and Baumer, W. (2009) Topical application of sphingosine-1-phosphate and FTY720 attenuate allergic contact dermatitis reaction through inhibition of dendritic cell migration. *J. Invest. Dermatol.* **129**, 1954-1962.

Schaper, K., Dickhaut, J., Japtok, L., Kietzmann, M., Mischke, R., Kleuser, B. and Baumer, W. (2013) Sphingosine-1-phosphate exhibits anti-proliferative and anti-inflammatory effects in mouse models of psoriasis. *J. Dermatol. Sci.* **71**, 29-36.

Spiegel, S. and Milstien, S. (2007) Functions of the multifaceted family of sphingosine kinases and some close relatives. *J. Biol. Chem.* **282**, 2125-2129.

Tsuji, T., Okuno, S., Kuroda, A., Hamazaki, J., Chikami, T., Sakurai, S., Yoshida, Y., Banno, R., Fujita, T. and Kohno, T. (2016) Therapeutic approach to mite-induced intractable dermatitis using novel immunomodulator FTY720 ointment (fingolimod) in NC/Nga mice. *Allergol. Int.* **65**, 172-179.

Tsuji, T., Yoshida, Y., Iwatsuki, R., Inoue, M., Fujita, T. and Kohno, T. (2012) Therapeutic approach to steroid-resistant dermatitis using novel immunomodulator FTY720 (Fingolimod) in combination with betamethasone ointment in NC/Nga mice. *Biol. Pharm. Bull.* **35**, 1314-1319.

Yanagawa, Y., Hoshino, Y., Kataoka, H., Kawaguchi, T., Ohtsuki, M., Sugahara, K. and Chiba, K. (1999) FTY720, a novel immunosuppressant, prolongs rat skin allograft survival by decreasing T-cell infiltration into grafts. *Transplant. Proc.* **31**, 1227-1229.