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Pro-Inflammatory Role of $S1P_3$ in Macrophages

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Abstract

Sphingosine kinase 1 and its product, sphingosine 1-phosphate (S1P), as well as their receptors, have been implicated in inflammatory responses. The functions of receptors $S1P_1$ and $S1P_2$ on cell motility have been investigated. However, the function of $S1P_3$ has been poorly investigated. In this study, the roles of $S1P_3$ on inflammatory response were investigated in primary peritoneal macrophages. $S1P_3$ receptor was induced along with sphingosine kinase 1 by stimulation of lipopolysaccharide (LPS). LPS treatment induced inflammatory genes, such iNOS, COX-2, IL-1β, IL-6 and TNF-α. TY52156, an antagonist of $S1P_3$ suppressed the induction of inflammatory genes in a concentration dependent manner. Suppression of iNOS and COX-2 induction was further confirmed by western blotting and NO measurement. Suppression of IL-1β induction was also confirmed by western blotting and ELISA. Caspase 1, which is responsible for IL-1β production, was similarly induced by LPS and suppressed by TY52156. Therefore, we have shown $S1P_3$ induction in the inflammatory conditions and its pro-inflammatory roles. Targeting $S1P_3$ might be a strategy for regulating inflammatory diseases.

Key Words: S1P, $S1P_3$, Macrophage, Inflammation, Caspase 1, GPCR

INTRODUCTION

Sphingosine 1-phosphate (S1P) is a specific ligand for five G protein-coupled receptors, $S1P_{1-5}$ (Park and Im, 2017). In response to inflammatory stimuli, up-regulation or activation of sphingosine kinase 1 (SphK1) and increased generation of its product, S1P, have been observed in many cell types, including RAW264.7 macrophages and microglia (Xia *et al*., 1998; Pettus *et al*., 2003; Hammad *et al*., 2008; Jin *et al*., 2018). S1P caused pro-inflammatory responses; for examples, S1P increases COX-2, iNOS, PGE_2 levels, interleukin-1β (IL-1β) as well as tumor necrosis factor-α (TNF-α) in several cell types, including murine peritoneal macrophages (Lee *et al*., 2002; Pettus *et al*., 2003; Hammad *et al*., 2008; Muller *et al*., 2017). On the contrary, S1P was shown to block lipopolysaccharide (LPS)-dependent stimulation of NF-κB activation and NO production, implying that S1P exerts an anti-inflammatory role (Hughes *et al*., 2008).

Murine RAW264.7 macrophages, bone marrow-derived monocytes, human peripheral monocytes, and human alveolar macrophages have been reported to express $S1P_1$ and $S1P_2$ (Lee *et al*., 2002; Hughes *et al*., 2008; Ishii *et al*., 2009). LPS-induced TNF-α, MCP-1, and IL-12 secretion were suppressed in murine peritoneal macrophages pre-incubated with a $S1P_1$ specific agonist, indicating a pivotal role of $S1P_1$ in S1P-dependent activation of anti-inflammatory macrophages (Hughes *et al*., 2008). $S1P_1$ signaling reciprocally up-regulated IL-6 gene expression in primary mouse macrophages in a JAK2-dependent manner (Zhao *et al*., 2018). S1P increases arginase I activity and inhibits LPS-induced inducible NO synthase activity in LPS-treated macrophages, again through $S1P_1$ receptor activation on macrophages (Hughes *et al*., 2008). The inhibitory role of $S1P_2$ in macrophage recruitment during inflammation (Michaud *et al*., 2010) and the pro-atherogenic role of $S1P_2$ signaling in the plaque macrophage have been reported (Skoura *et al*., 2011). Therefore, although anti-inflammatory function of $S1P_1$ has been reported, specific S1P receptors involved in the pro-inflammatory function of S1P have not been fully elucidated. Especially, the function of $S1P_3$ in macrophages has not been studied well. Therefore, we hypothesized that $S1P_3$ is a pro-inflammatory S1P receptor in macrophages and investigated the roles of $S1P_3$ on inflammatory response.

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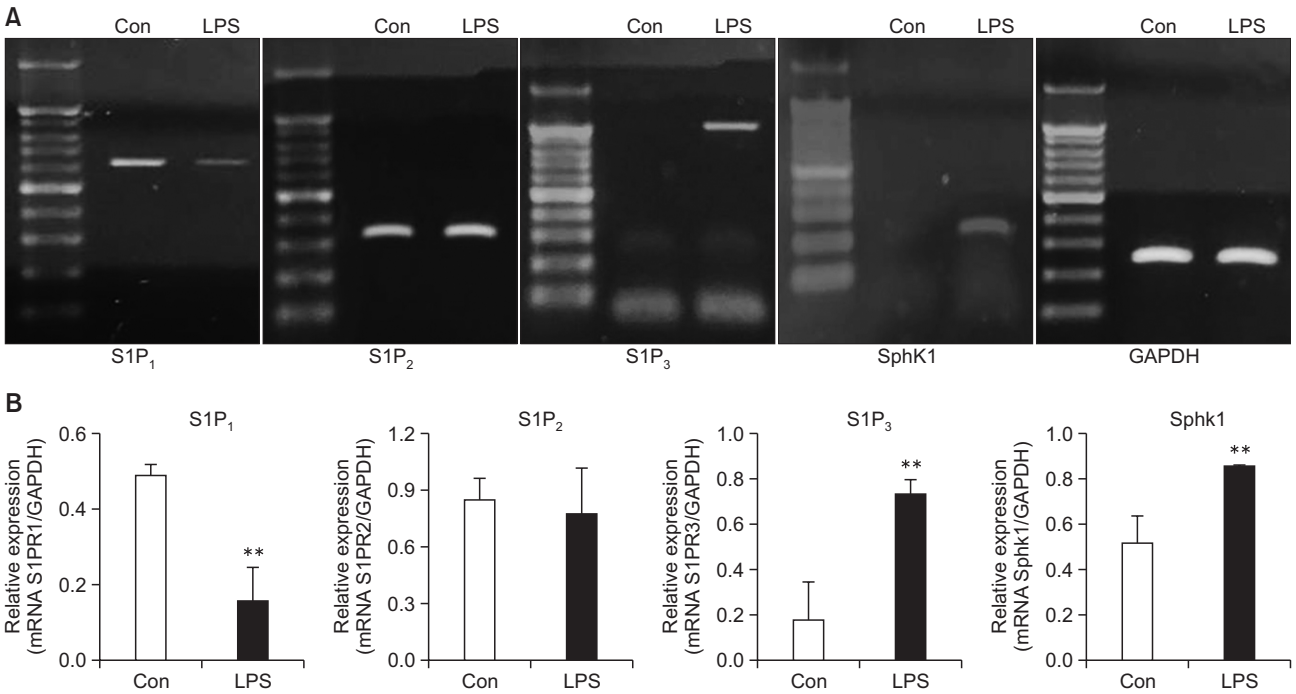

Fig. 1. Changes in the expression of $S1P_3$ and SphK1 caused by LPS stimulation. (A) Mouse peritoneal macrophages were treated with vehicle or LPS 10 ng/mL for 5 h, and RT-PCR was then performed to detect the expression of the $S1P_1$, $S1P_2$, $S1P_3$, and SphK1 genes. The data shown are representatives of three independent experiments. (B) Histogram of quantitated mRNA expression. Results are the mean ± standard deviation of three independent experiments. Statistically significant at $^{**}p<0.01$ level vs. the vehicle-treated macrophages.

MATERIALS AND METHODS

Materials

LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). TY52156 was purchased from Tocris Bioscience (cat. 5328, Bristol, UK).

Isolation and culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated from the peritoneal cavity of C57BL/6 mice treated with 3% thioglycollate at 4 days after treatment and cultured at 37°C in a 5% CO_2 humidified incubator. Isolated macrophages were maintained in RPMI1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate for 18 h, and then incubated in 0.5% FBS-containing media for 24 h. Samples for RNA or protein analysis were collected at 5 h or 24 h after treatment with LPS (10 ng/ml or 100 ng/ml), respectively. TY52156 was added 1 h before LPS treatment.

Reverse Transcription-PCR

Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined by a Nanodrop ND-1000 spectrophotometer. One microgram of RNA was transcribed by using a ImProm-II Reverse Transicription System (Promega, Madision, WI, USA) in accordance with the manufacturer's protocol. First-strand cDNA was synthesized from isolated total RNA using Trizol reagent (Invitrogen). Synthesized cDNA products and primers for each gene were used for PCR, which was conducted using Go-Taq DNA polymerase (Promega). Specific primers for IL-6 (sense 5'-CCG GAG AGG AGA CTT CAC AG-3', antisense 5'-TGG TCT TGG TCC TTA GCC AC-3'), IL-1β (sense 5'-GGA GAA GCT GTG GCA GCT A-3', antisense 5'-GCT GAT GTA CCA GTT GGG GA-3') were used to amplify gene fragments. PCR was performed over 27 amplification cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s) in an Mastcycler gradient PCR machine (Eppendorf, Hamburg, Germany). Specific primers for TNF-α (sense 5'-CAA AGA AAG CCG CCT CAA AC-3', antisense 5'-TTC ACA GAG AGG GTC ACA GC-3') were used and annealing was performed at 57°C. iNOS (sense 5'-ACC TAC CAC ACC CGA GAT GGC CAG-3', antisense 5'-AGG ATG TCC TGA ACA TAG ACC TTG GG-3'), COX-2 (sense 5'-CCG TGG GGA ATG TAT GAG CA-3', antisense 5'-CCA GGT CCT CGC TTA TGA TCT G-3'), SphK1 (sense 5'-TCC TGG AGG AGG CAG AGA TA-3', antisense 5'-GCT ACA CAG GGG TTT CTG GA-3') and GAPDH (sense 5'-TTC ACC ACC ATG GAG AAG GC-3', antisense 5'-GGC ATG GAC TGT GGT CAT GA-3') were used and annealing was performed at 60°C and over 30 amplification cycles. For $S1P_1$ (sense 5'-CAC CGG CCC ATG TAC TAT-3', antisense 5'-GCA GCC CAC ATC TAA CAG-3'), $S1P_2$ (sense 5'-CAT GGG CGG CTT ATA CTC A-3', antisense 5'-CAC TGC ACG GGA GTT AAG GA-3'), $S1P_3$ (sense 5'-GGG AGG GCA GTA TGT TCG TA-3', antisense 5'-GGA GCC CGC AAC AGA TAA G-3'), annealing was undertaken at 57°C. Aliquots (5 μl) were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

Western blotting

Macrophages were harvested and resuspended in RIPA lysis buffer (GenDEPOT, Baker, Carlsbad, CA, USA). Concen-

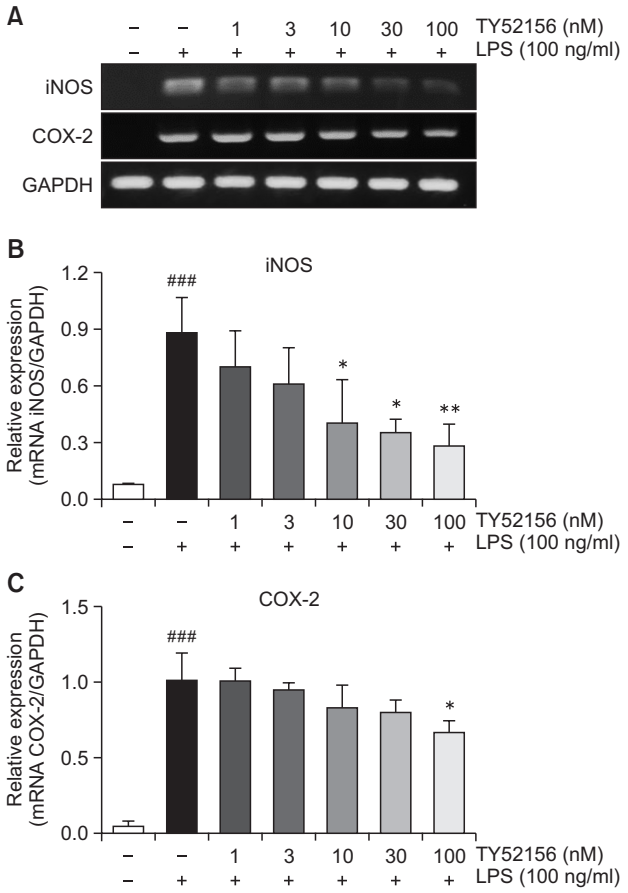

Fig. 2. Effect of TY52156 on the mRNA expressions of iNOS and COX-2 in macrophages. (A) Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with vehicle or LPS 10 ng/mL for 5 h, and RT-PCR was performed to detect the expression of the pro-inflammatory genes iNOS and COX-2. The data shown are representatives of three independent experiments. (B, C) Histograms of quantitated mRNA expressions of iNOS and COX-2. Results are the means ± standard deviation of three independent experiments. Statistically significant at $^{###}p<0.001$ level vs. the vehicle-treated macrophages, and at $^{*}p<0.05$ and $^{**}p<0.01$ vs. the the LPS-treated macrophages.

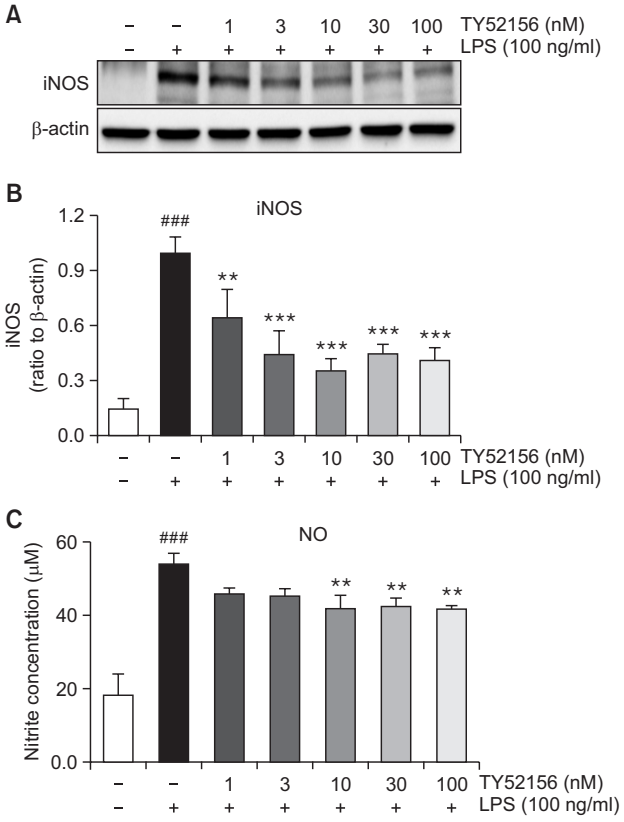

Fig. 3. Effect of TY52156 on iNOS protein expression and NO production in macrophages. Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with LPS 100 ng/mL for 24 or 48 h. Western blotting was conducted on cell lysates. The data shown in (A) are representative of three independent experiments. Relative protein levels of iNOS versus β-actin are presented as histograms (B). LPS-induced productions of nitrite (C) was measured. TY52156 inhibited nitrite production in a concentration-dependent manner (C). Results are the means ± standard deviation of three independent experiments. Statistically significant at $^{###}p<0.001$ level vs. the vehicle-treated macrophages, and at $^{**}p<0.01$ and $^{***}p<0.001$ vs. the LPS-treated macrophages.

tration of proteins was determined using a BCA protein assay (ThermoScientific, Rockford, IL, USA). Proteins (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to a nitrocellulose paper, which was incubated with specific primary antibodies recognizing β-actin, COX-2, iNOS, IL-1β, and caspase 1, and then incubated HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). Signals were developed using an enhanced chemiluminescence system (Pierce Biotechnology Inc., Rockford, IL, USA).

Nitrites measurement

NO production was estimated by measuring the amount of nitrite (a stable metabolite of NO) in medium using Griess reagent, as previously described (Kang *et al*., 2018). Cells were pretreated with different concentrations of TY52156 for 1 h and subsequently stimulated with LPS (100 ng/ml) for 48 h. Nitrite concentrations in the medium was determined using a Griess Reagent System (Promega).

ELISA

Ninety-six-well plates (NUNC) were coated with capture antibodies overnight at 4°C (eBioscience, San Diego, CA, USA, IL-1β cat 14-7012-85). The plates were washed, and then blocked with blocking buffer for 1 h at room temperature. Standard dilutions of cytokines were prepared and added to the wells with supernatants. The plates were incubated for 2 h at room temperature with shaking, and then washed five times. Biotinylated detection antibody (eBioscience, IL-1β cat 13-7112-81) was then added to the wells, which were incubated for 1 h at room temperature with shaking. The plates were then washed five times and Avidin-HRP was added for 30 min at room temperature with shaking. The plates were then washed fourteen times and incubated with substrate solution for 15 min at room temperature. Stop solution (eBioscience) was then added and absorbance was read at 450 nM.

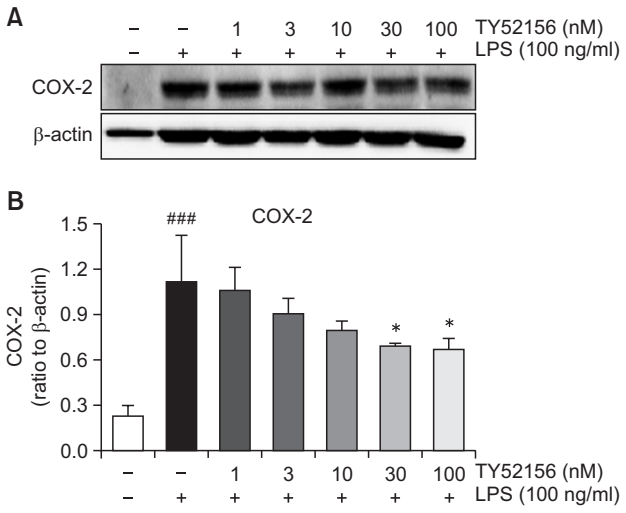

Fig. 4. Effect of TY52156 on the protein expression of COX-2 in macrophages. Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with LPS 100 ng/mL for 24 h. Western blotting was conducted on cell lysates. The data shown in (A) are representative of three independent experiments. Relative protein levels of COX-2 versus β-actin are presented as histograms (B). Results are the means ± standard deviation of three independent experiments. Statistically significant at $^{###}p<0.001$ level vs. the vehicle-treated macrophages, and at $^{*}p<0.05$ vs. the LPS-treated macrophages.

Animals

Eight- to ten-week-old male C57BL/6 (19-22 g) mice were purchased from Daehan Biolink (DBL; Seoul, Korea), housed in a Laboratory Animal facility at Pusan National University (Busan, Korea), and provided food and water *ad libitum*. The animal protocol used in this study was reviewed and approved beforehand by the Pusan National University-Institutional Animal Care Committee (PNU-IACUC) with respect to ethics and scientific care.

Statistics

All results were expressed as mean ± standard deviation. The data were analyzed by one-way ANOVA. A p-value<0.05 was considered statistically significant.

RESULTS

Induction of $S1P_3$ in peritoneal macrophages

Previously, LPS, a cell wall component of Gram-negative bacteria, was shown to induce the expression and activation of SphK1 in RAW264.7 macrophages. In addition, its product, S1P, has been well studied in relation with the functions of $S1P_1$ and $S1P_2$. We confirmed an LPS-induced increase of SphK1 expression as well as the presence of $S1P_3$ mRNA and its simultaneous induction in mouse peritoneal macrophages after LPS treatment (Fig. 1). Because $S1P_3$ has been poorly investigated in inflammatory responses compared to $S1P_1$ and $S1P_2$, we studied the function of $S1P_3$ by using an $S1P_3$ antagonist, TY52156 (Murakami *et al*., 2010).

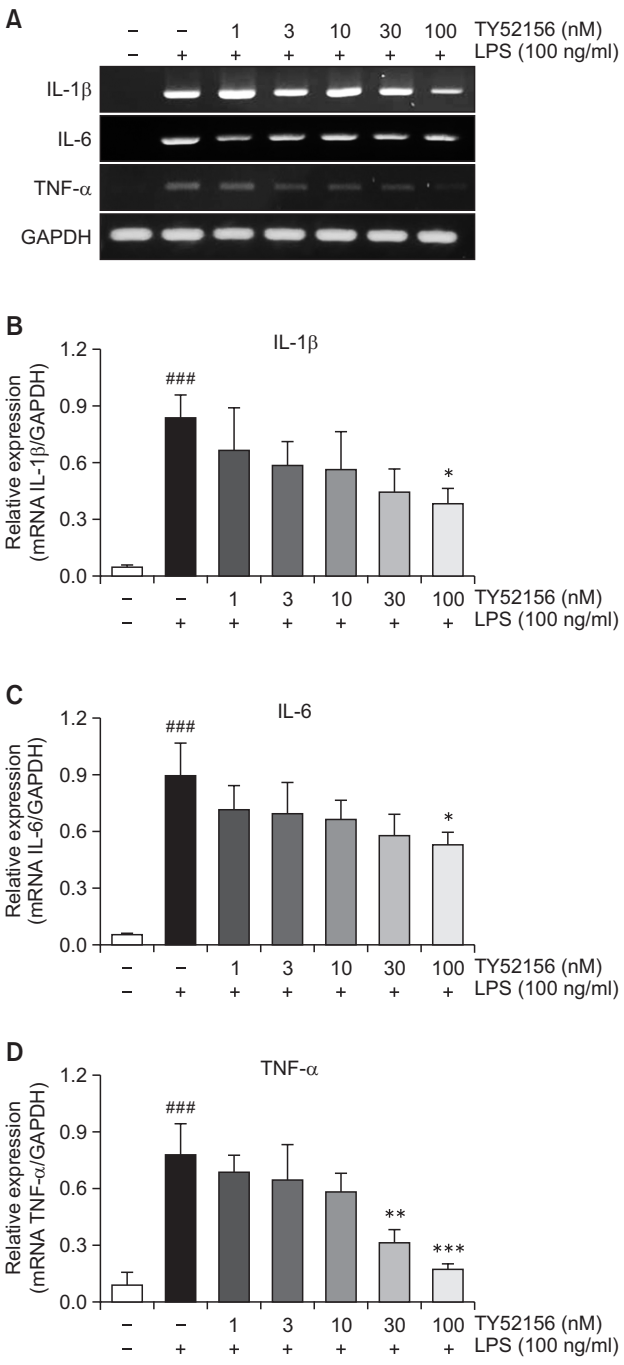

Fig. 5. Effect of TY52156 on the expression of IL-1β, IL-6, and TNF-α in macrophages. (A) Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with vehicle or LPS 10 ng/mL for 5 h, and RT-PCR was performed to detect the expression of the pro-inflammatory genes, IL-1β, IL-6, and TNF-α. The data shown are representatives of three independent experiments. (B-D) Histograms of quantitated mRNA expression of IL-1β, IL-6, and TNF-α. Results are the means ± standard deviation of three independent experiments. Statistically significant at $^{###}p<0.001$ level vs. the vehicle-treated macrophages, and at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. the LPS-treated macrophages.

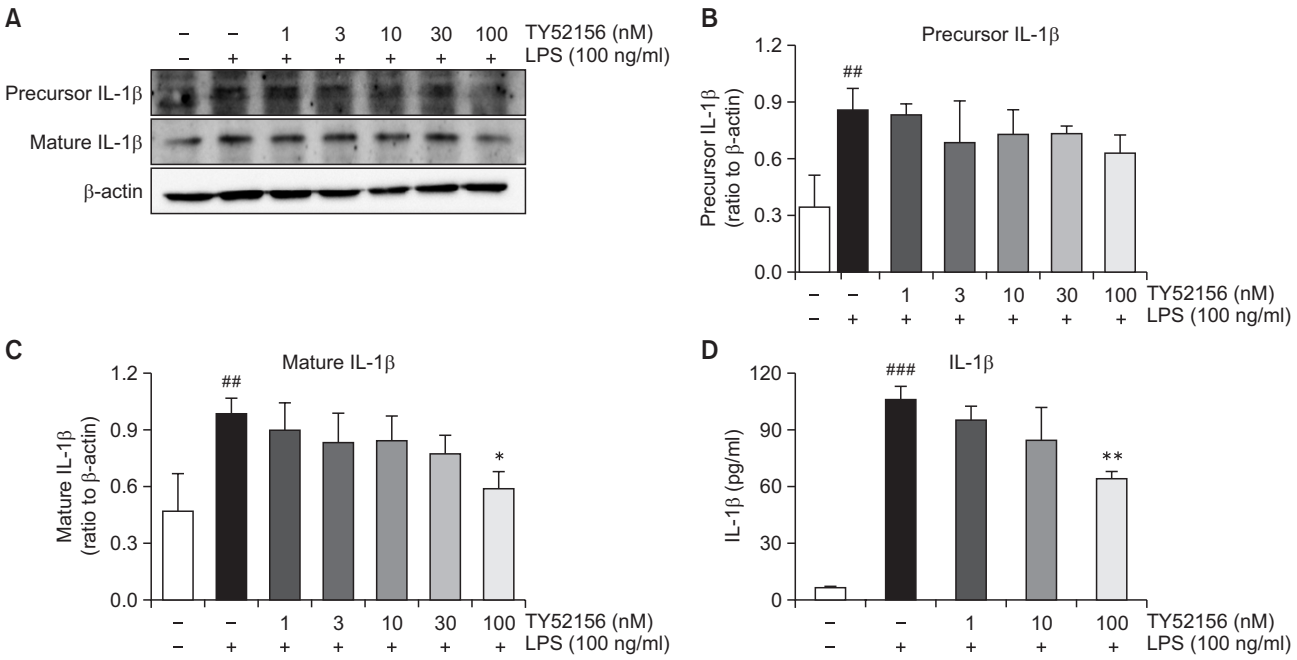

Fig. 6. Effect of TY52156 on IL-1β protein expression and IL-1β production in macrophages. Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with LPS 100 ng/mL for 24 h. Western blotting was conducted on cell lysates. The data shown in (A) are representatives of three independent experiments. Relative protein levels of IL-1β versus β-actin are presented as histograms (B, C). LPS-induced production of IL-1β in the media was measured by ELISA (D). Results are the means ± standard deviation of three independent experiments. Statistically significant at $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. the vehicle-treated macrophages, and at $^{*}p<0.05$ and $^{**}p<0.01$ vs. the LPS-treated macrophages.

TY52156 inhibits induction of COX-2 and iNOS in peritoneal macrophages

In the presence of 10 ng/ml LPS, expression of pro-inflammatory genes, iNOS and COX-2, was increased, and the induction of iNOS and COX-2 expressions at mRNA level was significantly inhibited by TY52156 in a concentration-dependent manner (Fig. 2).

The inhibitory effect of TY52156 on iNOS and COX-2 was studied at protein level. In the presence of 100 ng/ml LPS, western samples were made. The protein expression of iNOS was significantly suppressed by treatment with TY52156 (Fig. 3A, 3B). Also, the level of NO, the product of iNOS, was significantly decreased (Fig. 3C).

The inhibitory effect of TY52156 on COX-2 mRNA expression was also further studied at protein level. As shown in Fig. 4, TY51256 strongly inhibited the induction of COX-2 at protein level in a concentration-dependent manner (Fig. 4).

TY52156 inhibits induction of IL-1β in peritoneal macrophages

Treatment of TY52156 also inhibited LPS-induced induction of other inflammatory genes of IL-1β, IL-6 and TNF-α (Fig. 5). Because treatment of TY52156 inhibited LPS-induced induction of the IL-1β gene (Fig. 5), the protein level of IL-1β was examined by western blotting and ELISA (Fig. 6). TY52156 inhibited induction of preform and mature form IL-1β. ELISA result showed significant inhibition on IL-1β secretion in the media (Fig. 6D).

TY52156 inhibits caspase 1 expression in peritoneal macrophages

Furthermore, we evaluated the expression of caspase 1, which converts the preform IL-1β to mature IL-1β. Caspase 1 expression was induced by LPS and suppressed by treatment of TY52156 (Fig. 7).

On the basis of these findings, we hypothesized that LPS stimulation may induce not only inflammatory genes, such as, iNOS, caspase 1, and IL-1β, but also SphK1 and $S1P_3$. Induction of SphK1 may increase production of S1P, which activates $S1P_3$, thereby amplifying inflammatory response. In order to confirm this signaling route, we applied dimethylsphingoinse (DMS), a specific inhibitor of SphK1. As shown in Fig. 8, in the presence of DMS, LPS induced less expression of caspase 1. However, treatment with CYM5541, a specific agonist of $S1P_3$, reversed DMS-induced gene suppression (Fig. 8), implying that the induction of SphK1 and its product, S1P, and the action of S1P on $S1P_3$ sequentially mediated the pro-inflammatory response of LPS in macrophages (Fig. 9).

DISCUSSION

In this study, we found that LPS induced $S1P_3$ expression in murine macrophages. Previously, expression of $S1P_3$ was reported to be very low in macrophages (Lee *et al*., 2002). However, its expression was confirmed by immunofluorescence, RT-PCR and western blotting in human monocytes/macrophages and murine bone marrow-derived macrophages (Duong *et al*., 2004; Durafourt *et al*., 2011; Yang *et al*., 2015; Muller *et al*., 2017). In addition, $S1P_3$ was induced during mac-

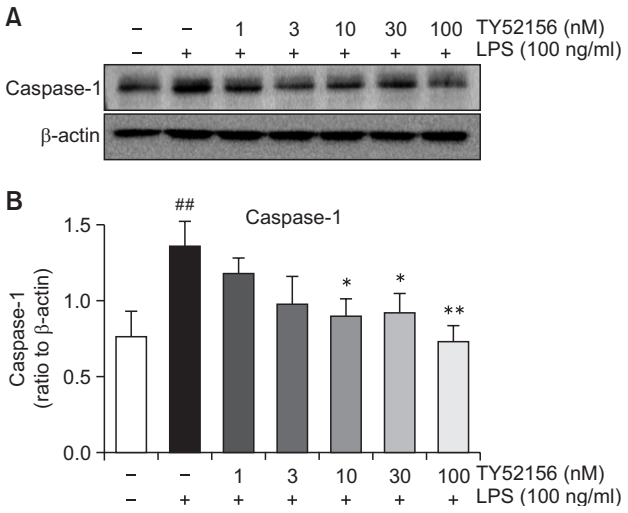

Fig. 7. Effect of TY52156 on the protein expression of caspase-1 in macrophages. Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with LPS 100 ng/mL for 24 h. Western blotting was conducted on cell lysates. The data shown in (A) are representatives of three independent experiments. Relative protein levels of caspase-1 versus β-actin are presented as histograms (B). Results are the means ± standard deviation of three independent experiments. Statistically significant at $^{\#\#}p$<0.01 level vs. the vehicle-treated macrophages, and at $^{*}p$<0.05 and $^{**}p$<0.01 vs. the LPS-treated macrophages.

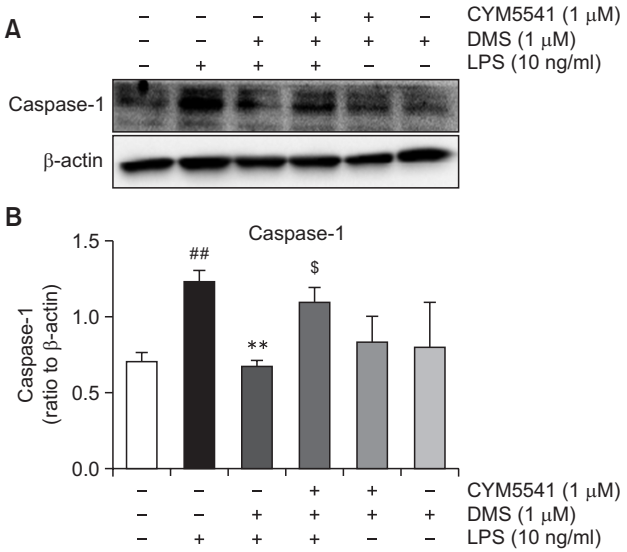

Fig. 8. Effect of dimethylsphingosine and CYM5541 on the protein expression of caspase-1 in macrophages. Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with LPS 100 ng/mL for 24 h. Western blotting was conducted on cell lysates. The data shown in (A) are representative of three independent experiments. Relative protein levels of caspase-1 versus β-actin are presented as histograms (B). Results are the means ± standard deviation of three independent experiments. Statistically significant at $^{\#\#}p$<0.001 level vs. the vehicle-treated macrophages, at $^{**}p$<0.01 vs. the LPS-treated macrophages, at $^{\$}p$<0.05 vs. the LPS/ DMS-treated macrophages.

rophage differentiation from human monocytes (Duong *et al*., 2004). Moreover, stimulation of $S1P_3$ by FTY-720 reduced monocyte recruitment to atherosclerotic lesions (Theilmeier *et al*., 2006). Furthermore, TNF-α increased the mRNA of both $S1P_1$ and $S1P_3$ in human astrocytes and LPS upregulated the expression of $S1P_1$ and $S1P_3$ in human gingival epithelial cells (Eskan *et al*., 2008; Van Doorn *et al*., 2010). Strong increases in $S1P_1$ and $S1P_3$ expression in reactive astrocytes were detected in active and inactive chronic multiple sclerosis lesions (Van Doorn *et al*., 2010). Upregulation of $S1P_3$ in astrocytes was also previously reported in a mouse model of Sandhoff disease, a prototypical neuronopathic lysosomal storage disorder (Wu *et al*., 2008). Therefore, induction of $S1P_3$ has been reported in many cell types.

In our study, SphK1 and $S1P_3$ were concomitantly upregulated, but $S1P_1$ was downregulated. Similarly, concomitant regulation of SphK1 and $S1P_3$ was shown to play a pivotal role in murine cardiac fibrosis and in the transdifferentiation of myoblasts into myofibroblasts (Cencetti *et al*., 2010; Takuwa *et al*., 2010). Furthermore, SphK1 and $S1P_3$ were functionally upregulated in astrocytes under pro-inflammatory conditions (Fischer *et al*., 2011).

In this study, we observed a pro-inflammatory function of $S1P_3$, which was supported by the result of a previous study that bone marrow-derived $S1P_3$-deficient macrophages produced low MCP-1 in response to LPS stimulation (Keul *et al*., 2011). Previously, the functions of $S1P_3$ have been reported in macrophage migration. S1P exerts a potent migratory action in bone marrow-derived macrophages via $S1P_2$ and $S1P_3$ in cholestatic liver injury (Yang *et al*., 2015). *In vitro*, S1P induces a chemotactic action in wild-type peritoneal macrophages, but not in $S1P_3$-deficient peritoneal macrophages (Keul *et al*., 2011). $S1P_3$ mediates the chemotactic effect of S1P in macrophages *in vitro* and *in vivo*, and plays a causal role in atherosclerosis by promoting inflammatory recruitment of monocyte/ macrophage (Keul *et al*., 2011). This was also supported by a finding that S1P, a constituent of HDL, acutely protects the heart against ischemia/reperfusion injury *in vivo* via an $S1P_3$-mediated and NO-dependent pathway (Theilmeier *et al*., 2006).

We observed that $S1P_3$ mediated iNOS, COX-2, IL-1β, IL-6, and TNF-α expression under LPS stimulation (Fig. 9). The pro-inflammatory action of S1P has previously been reported in several cell types, including macrophages. For examples, SphK1 activation by LPS or cytokines, including TNF-α and IL-1β, has been observed (Xia *et al*., 1998; Pettus *et al*., 2003; Billich *et al*., 2005; Hammad *et al*., 2008; Nayak *et al*., 2010; Snider *et al*., 2010). Moreover, S1P caused increases in COX-2 and PGE_2 levels in several cell types (Pettus *et al*., 2003; Hammad *et al*., 2008). S1P increases the expression of iNOS under M2-polarizing conditions (Muller *et al*., 2017). Furthermore, S1P induces the release of IL-1β and TNF-α from murine peritoneal macrophages (Lee *et al*., 2002). Therefore, S1P has been reported to play crucial roles in pro-inflammatory responses. However, a specific S1P receptor for the responses has not been elucidated, and for the first time $S1P_3$ was reported as the pro-inflammatory receptor in this study (Fig. 9). On the basis of the results of this study, $S1P_3$ targeting therapeutics may be beneficial as an anti-inflammatory therapy.

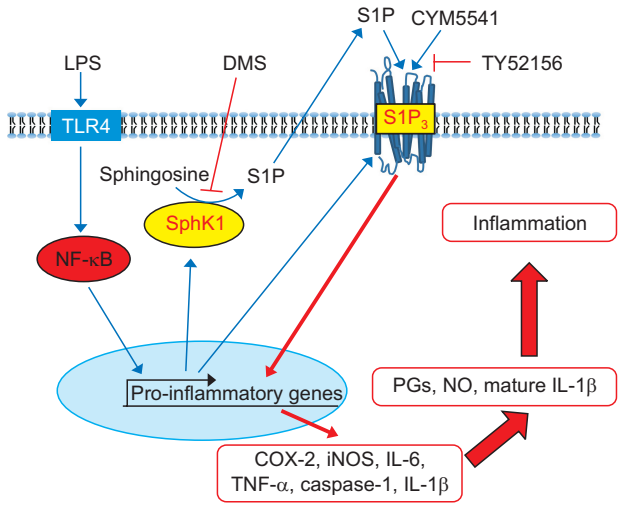

Fig. 9. Proposed signaling of pro-inflammatory S1P and $S1P_3$ in peritoneal macrophages. LPS activates NF-κB and induces induction of SphK1 and $S1P_3$ expression in macrophages. S1P produced by SphK1 activates pro-inflammatory signaling through $S1P_3$, resulting in increased productions of prostaglandins (PGs), nitric oxides, and mature IL-1β.

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