Pro-Inflammatory Role of S1P₃ 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 S1P₁ and S1P₂ on cell motility have been investigated. However, the function of S1P₃ has been poorly investigated. In this study, the roles of S1P₃ on inflammatory response were investigated in primary peritoneal macrophages. S1P₃ receptor was induced along with sphingosine kinase 1 by stimulation of lipopolysaccharide (LPS). LPS treatment induced inflammatory genes, such as iNOS, COX-2, IL-1β, IL-6 and TNF-α. TY52156, an antagonist of S1P₃, 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 S1P₃ induction in the inflammatory conditions and its pro-inflammatory roles. Targeting S1P₃ might be a strategy for regulating inflammatory diseases.

Key Words: S1P, S1P₃, Macrophage, Inflammation, Caspase 1, GPCR

INTRODUCTION

Sphingosine 1-phosphate (S1P) is a specific ligand for five G protein-coupled receptors, S1P₁-₅ (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, PGE₂, 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 caused pro-inflammatory responses; for examples, S1P increases COX-2, iNOS, PGE₂, 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 caused pro-inflammatory responses; for examples, S1P increases COX-2, iNOS, PGE₂, 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).

Murine RAW264.7 macrophages, bone marrow-derived monocytes, human peripheral monocytes, and human alveolar macrophages have been reported to express S1P₁ and S1P₂ (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 S1P₁ specific agonist, indicating a pivotal role of S1P₁ in S1P₁-dependent activation of anti-inflammatory macrophages (Hughes et al., 2008). S1P₁ 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 S1P₁ receptor activation on macrophages (Hughes et al., 2008). The inhibitory role of S1P₂ in macrophage recruitment during inflammation (Michaud et al., 2010) and the pro-atherogenic role of S1P₂ signaling in the plaque macrophage have been reported (Skoura et al., 2011). Therefore, although anti-inflammatory function of S1P₁ has been reported, specific S1P receptors involved in the pro-inflammatory function of S1P have not been fully elucidated. Especially, the function of S1P₃ in macrophages has not been studied well. Therefore, we hypothesized that S1P₃ is a pro-inflammatory S1P receptor in macrophages and investigated the roles of S1P₃ on inflammatory response.
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% CO2 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 Transcription System (Promega, Madison, 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 a 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’), iNOS (sense 5’-ACC TAC CAC ACC GTA AGG AGA GAG GC-3’, antisense 5’-GCC ATG GAC TCT CTT CTG GA-3’), COX-2 (sense 5’-CCG TGG GGA ATG TAT GAG CA-3’, antisense 5’-CCA GGT CCT CGC TTA TGA TCT G-3’), and GAPDH (sense 5’-ACC ACC ATG GAG AAG GC-3’, antisense 5’-GCC ATG GAC TGT GAT CAT GA-3’) were used and annealing was undertaken at 57°C. INOS (sense 5’-ACC TAG CAC ACC CGA GAT GGC GAG-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 CAG AGA TA-3’, antisense 5’-GCT ACA CAG GGG TTT CTG GA-3’), SphK2 (sense 5’-ACC ACC ATG GAG GC-3’, antisense 5’-GCC ATG GAC TGT GAT CAT GA-3’) were used and annealing was performed at 60°C and over 30 amplification cycles. For S1P1, (sense 5’-CAC CCG CCC ATG TAC TAT-3’, antisense 5’-GCA GCC CAC ATC TAA CAG-3’), S1P2 (sense 5’-CAT GGG CCG CTT ATC CTC A-3’, antisense 5’-CAG TGC ACG GGA GTT AAG GA-3’), S1P3 (sense 5’-GGG AGG GCA GTG TGT TCG TA-3’, antisense 5’-GGA GCC CGC AAC AGA TAA G-3’),1, 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). Concent-
The concentration 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.
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 S1P3 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 S1P1 and S1P2. We confirmed an LPS-induced increase of SphK1 expression as well as the presence of S1P3 mRNA and its simultaneous induction in mouse peritoneal macrophages after LPS treatment (Fig. 1). Because S1P3 has been poorly investigated in inflammatory responses compared to S1P1 and S1P2, we studied the function of S1P3 by using an S1P3 antagonist, TY52156 (Murakami et al., 2010).

![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<0.01 level vs. the vehicle-treated macrophages, and at ***p<0.001 vs. the LPS-treated macrophages.](image)

Fig. 4.

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![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 representative 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<0.01 level vs. the vehicle-treated macrophages, and at ***p<0.001 vs. the LPS-treated macrophages.](image)

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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 suppressed by treatment with TY52156 (Fig. 3C).

The inhibitory effect of TY52156 on COX-2 mRNA expression was also further studied at protein level. As shown in Fig. 4, TY52156 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 S1P3. Induction of SphK1 may increase production of S1P, which activates S1P3, thereby amplifying inflammatory response. In order to confirm this signaling route, we applied dimethylsphingosine (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 S1P3, reversed DMS-induced gene suppression (Fig. 8), implying that the induction of SphK1 and its product, S1P3, and the action of S1P on S1P3 sequentially mediated the pro-inflammatory response of LPS in macrophages (Fig. 9).

DISCUSSION

In this study, we found that LPS induced S1P3 expression in murine macrophages. Previously, expression of S1P3 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, S1P3 was induced during mac-
In this study, we observed a pro-inflammatory function of S1P3, which was supported by the result of a previous study that bone marrow-derived S1P3-deficient macrophages produced low MCP-1 in response to LPS stimulation (Keul et al., 2011). Previously, the functions of S1P3 have been reported in macrophage migration. S1P exerts a potent migratory action in bone marrow-derived macrophages via S1P2 and S1P3 in cholestatic liver injury (Yang et al., 2015). In vitro, S1P induces a chemotactic action in wild-type peritoneal macrophages, but not in S1P3-deficient peritoneal macrophages (Keul et al., 2011). S1P3 mediates the chemotactic effect of S1P in macro-

**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 vs. the vehicle-treated macrophages, at **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<0.01 vs. the vehicle-treated macrophages, at **p<0.01 vs. the LPS/DMS-treated macrophages.

We observed that S1P3 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 PGE2 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 S1P3 was reported as the pro-inflammatory receptor in this study (Fig. 9). On the basis of the results of this study, S1P3-targeting therapeutics may be beneficial as an anti-inflammatory therapy.
Hughes, J. E., Srinivasan, S., Lynch, K. R., Proia, R. L., Ferdek, P. and Hedrick, C. C. (2008) Sphingosine-1-phosphate induces an anti-inflammatory phenotype in macrophages. *Circ. Res.* **102**, 950-958.

Ishii, M., Egen, J. G., Klauschen, F., Meier-Schellersheim, M., Saeki, Y., Vacher, J., Proia, R. L., and Germain, R. N. (2009) Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature* **458**, 524-528.

Jin, J., Lu, Z., Li, Y., Ru, J. H., Lopes-Virella, M. F. and Huang, Y. (2018) LPS and palmitate synergistically stimulate sphingosine kinase 1 and increase sphingosine 1 phosphate in RAW264.7 macrophages. *J. Leukoc. Biol.* **104**, 843-853.

Kang, S., Park, S. J., Lee, A. Y., Huang, J., Chung, H. Y. and Im, D. S. (2018) Ginsenoside Rg3 promotes inflammation resolution through M2 macrophage polarization. *J. Ginseng Res.* **42**, 68-74.

Keul, P., Lucke, S., von Wnuck Lipinski, K., Bode, C., Graier, M., Heusch, G. and Levkau, B. (2011) Sphingosine-1-phosphate receptor 3 promotes recruitment of monocyte/macrophages in inflammation and atherosclerosis. *Circ. Res.* **108**, 314-323.

Lee, H., Liao, J. J., Graier, M., Huang, M. C. and Goetzl, E. J. (2002) Lysophospholipid regulation of mononuclear phagocytes. *Biochim. Biophys. Acta* **1582**, 175-177.

Michaud, J., Im, D. S. and Hia, T. (2010) Inhibitory role of sphingosine 1-phosphate receptor 2 in macrophage recruitment during inflammation. *J. Immunol.* **184**, 1475-1483.

Muller, J., von Bernstorff, W., Heidecke, C. D. and Schulze, T. (2017) Differential S1P receptor profiles on M1- and M2-polarized macrophages affect macrophage cytokine cytokine production and migration. *BioMed Res. Int.* **2017**, 7564621.

Murakami, A., Takasugi, H., Ohnuma, S., Koide, Y., Sakurai, A., Takeda, S., Hasegawa, T., Sasamori, J., Konno, T., Hayashi, K., Watanabe, Y., Mor, K., Sato, Y., Takahashi, A., Mochizuki, N. and Takakura, N. (2010) Sphingosine 1-phosphate (S1P) regulates vascular contraction via S1P1 receptor: investigation based on a new S1P1 receptor antagonist. *Mol. Pharmacol.* **77**, 704-713.

Nayak, D., Huo, Y., Kwang, W. X., Pushparaj, P. N., Kumar, S. D., Ling, E. A. and Dheen, S. T. (2010) Sphingosine kinase 1 regulates the expression of proinflammatory cytokines and nitric oxide in activated microglia. *Neuroscience* **166**, 132-144.

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

Pettus, B. J., Bielawski, J., Porcelli, A. M., Reames, D. L., Johnson, K. R., Morrow, J., Chaffant, C. E., Obeid, L. M. and Hannun, Y. A. (2003) The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J.* **17**, 1411-1421.

Skoura, A., Michaud, J., Im, D. S., Thangada, S., Xiong, Y., Smith, J. D. and Hia, T. (2011) Sphingosine-1-phosphate receptor-2 function in myeloid cells regulates vascular inflammation and atherosclerosis. *Atherosclerosis. Thromb. Vasc. Biol.* **31**, 81-85.

Snider, A. J., Orr Gandy, K. A. and Obeid, L. M. (2010) Sphingosine kinase role in regulation of bioactive sphingolipid mediators in inflammation. *Biochimie* **92**, 707-715.

Takakuwa, N., Ohkura, S., Takashima, S., Ohtani, K., Okamoto, Y., Tanaka, T., Hirano, K., Usui, S., Wang, F., Du, W., Yoshioka, K., Banno, Y., Sasaki, M., Ichi, I., Okamura, M., Sugimoto, N., Mizugishi, K., Nakamura, Y., Ishii, I., Takamakura, M., Kaneko, S., Kojo, S., Satouchi, K., Mitumori, K., Chun, J. and Takakuwa, Y. (2010) S1P1-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species. *Cardiovasc. Res.* **85**, 484-493.

Thielmeier, G., Schmidt, C., Herrmann, J., Keul, P., Schafer, M., Herrodt, I., Marschner, J., Larmann, J., Herrmann, S., Stumphmann, J., Schober, O., Hildebrand, R., Schulz, R., Heusch, G., Haude, M., von Wnuck Lipinski, K., Herzog, C., Schmitz, M., Erbel, R., Chun, J. and Levkau, B. (2006) High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P1 lysophospholipid receptor. *Circulation* **114**, 1403-1409.

Van Doorn, R., Van Horsens, J., Verzijl, D., Witte, M., Ronken, E., Van Het Hof, B., Lakenman, K., Dijkstra, C. D., Van Der Valk, P., Reijerkerk, A., Alewinse, A. E., Peters, S. L. and De Vries, H. E. (2010) Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. *Glia* **58**, 1465-1476.
Wu, Y. P., Mizugishi, K., Bektas, M., Sandhoff, R. and Proia, R. L. (2008) Sphingosine kinase 1/S1P receptor signaling axis controls glial proliferation in mice with Sandhoff disease. *Hum. Mol. Genet.* **17**, 2257-2264.

Xia, P., Gamble, J. R., Rye, K. A., Wang, L., Hii, C. S., Cockerill, P., Khew-Goodall, Y., Bert, A. G., Barter, P. J. and Vadas, M. A. (1998) Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14196-14201.

Yang, L., Han, Z., Tian, L., Mai, P., Zhang, Y., Wang, L. and Li, L. (2015) Sphingosine 1-phosphate receptor 2 and 3 mediate bone marrow-derived monocyte/macrophage motility in cholestatic liver injury in mice. *Sci. Rep.* **5**, 13423.

Zhao, S., Adebiyi, M. G., Zhang, Y., Couturier, J. P., Fan, X., Zhang, H., Kellems, R. E., Lewis, D. E. and Xia, Y. (2018) Sphingosine-1-phosphate receptor 1 mediates elevated IL-6 signaling to promote chronic inflammation and multitissue damage in sickle cell disease. *FASEB J.* **32**, 2855-2865.