Sphingosine 1-Phosphate Receptor 4 Promotes Nonalcoholic Steatohepatitis by Activating NLRP3 Inflammasome

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SUMMARY
Type 4 sphingosine-1-phosphate receptor, which mediates the activation of the Nod-like receptor (NLR) family pyrin domain containing 3 inflammasome, emerges as a new therapeutic target for nonalcoholic steatohepatitis. We developed a selective functional antagonist for type 4 sphingosine-1-phosphate receptor (SLB736), which protected mice against nonalcoholic steatohepatitis and fibrosis.

BACKGROUND & AIMS: Sphingosine 1-phosphate receptors (S1PRs) are a group of G-protein-coupled receptors that confer a broad range of functional effects in chronic inflammatory and metabolic diseases. S1PRs also may mediate the development of nonalcoholic steatohepatitis (NASH), but the specific subtypes involved and the mechanism of action are unclear.

METHODS: We investigated which type of S1PR isoforms is activated in various murine models of NASH. The mechanism of action of S1PR4 was examined in hepatic macrophages isolated...
from high-fat, high-cholesterol diet (HFHCD)-fed mice. We developed a selective S1PR4 functional antagonist by screening the fngolimod (2-amino-2-[2-(4-n-octylphenyl)ethyl]-1,3-propanediol hydrochloride)-like sphingolipid-focused library.

**RESULTS:** The livers of various mouse models of NASH as well as hepatic macrophages showed high expression of S1pr4. Moreover, in a cohort of NASH patients, expression of S1pr4 was 6-fold higher than those of healthy controls. S1pr4+/− mice were protected from HFHCD-induced NASH and hepatic fibrosis without changes in steatosis. S1pr4 depletion in hepatic macrophages inhibited lipopolysaccharide-mediated Ca2+ release and deactivated the nod-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome. S1P increased the expression of S1pr4 in hepatic macrophages and activated NLRP3 inflammasome through inositol trisphosphate–inositol trisphosphate–receptor–dependent [Ca2+] signaling. To further clarify the biological function of S1PR4, we developed SLB736, a novel selective functional antagonist of S1PR4. Similar to S1pr4+/− mice, administration of SLB736 to HFHCD-fed mice prevented the development of NASH and hepatic fibrosis, but not steatosis, by deactivating the NLRP3 inflammasome.

**CONCLUSIONS:** S1PR4 may be a new therapeutic target for NASH that mediates the activation of NLRP3 inflammasome in hepatic macrophages. ([Cell Mol Gastroenterol Hepatol 2022;13:925–947; https://doi.org/10.1016/j.jcmgh.2021.12.002])

**Keywords:** Hepatic Macrophages; Ca2+; Functional Antagonist; S1P.

Nonalcoholic fatty liver disease (NAFLD) has become a major health issue worldwide. Approximately 10%-20% of patients with NAFLD develop nonalcoholic steatohepatitis (NASH), an advanced stage of NAFLD that subsequently may progress to liver cirrhosis and hepatocellular carcinoma.

The mechanism by which simple steatosis progresses to NASH and liver fibrosis is not completely understood, and an effective treatment for halting the progression of NASH has yet to be discovered. Lipotoxic hepatocyte death may be the primary lesion that causes liver inflammation and fibrosis. Damage-associated molecular patterns released from dying hepatocytes may activate hepatic macrophages, and secretion of proinflammatory and fibrogenic cytokines from macrophages promotes hepatic stellate cell (HSC) activation.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that influences a wide range of important cellular processes by activating 5 G-protein–coupled receptors (S1PR1–5). Receptor-mediated S1P signaling has become an attractive therapeutic target in several diseases such as chronic inflammatory disease, autoimmunity, cancer, and metabolic disease. In the liver, S1PR2 participates in cholestasis-induced liver injury and in chronic liver damage of different etiologies, including bile duct ligation, as well as in methionine–choline–deficient diet (MCDD) and high-fat diet (HFD) feeding, or carbon tetrachloride–mediated liver injury and fibrosis. S1PR1 and S1PR3 are involved in HSC motility and activation and play a crucial role in the angiogenic process required for fibrosis development. Targeting S1PRs was shown to be a promising strategy for treating NASH after the recent preclinical success of 2-amino-2-[2-(4-n-octylphenyl)ethyl]-1,3-propanediol hydrochloride (fngolimod; FTY720), a drug developed for multiple sclerosis. FTY720, a nonselective modulator of S1PRs (S1PR1, 3, 4, and 5), has been shown to prevent the development of alcoholic liver disease, NAFLD, and NASH in murine models. However, the widespread use of FTY720 in NASH has been hampered by its lymphopenic effects.

In the present study, we identified S1PR4 as a novel player in the pathogenesis of NASH by examining various murine models of NASH, as well as a cohort of NASH patients. We found that S1pr4 expression was signi¢cantly higher in the liver of various diet-induced murine models of NASH. S1pr4 heterozygous knockout (S1pr4+/−) mice were protected from high-fat, high-cholesterol diet (HFHCD)-induced NASH and hepatic fibrosis by showing minimal activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome in hepatic macrophages. To provide further insights into the biological role of S1PR4, we developed and characterized SLB736 as a S1PR4-selective modulator, which acted as a functional antagonist of S1PR4. SLB736 was effective in preventing the development of NASH and fibrosis via inhibiting the activation of the NLRP3 inflammasome in hepatic macrophages. Collectively, our results suggest that S1PR4 is a potential target for the treatment of NASH and hepatic fibrosis.

**Results**

**S1PR4 Expression Is Increased in the Liver of Diet-Induced Murine Models of NASH and in NASH Patients**

We first investigated which type of S1PR isoform is activated in murine models of NASH. HFHCD feeding is one of the animal models that closely resembles the clinical characteristics of NASH. We also used the MCDD, Western diet (WD), and choline-deficient, L-amino acid-defined (CDA)-HFD. Interestingly, S1pr4 was the only...
isoform that consistently showed increased messenger RNA (mRNA) expression in the livers of mice fed HFHCD, MCDD, WD, or CDA+HFD; the expression of S1pr1 and S1pr2 were increased only in mice fed a WD, and S1pr3 expression was increased only in mice fed HFHCD, MCDD, or WD (Figure 1A). To validate the clinical relevance of the earlier-described findings, we examined the S1PR4 expression in the livers of patients with NASH/cirrhosis undergoing liver transplantation (n = 9). Surgical specimens of the donor livers were used as controls (n = 10). All data are shown as means ± SEM. Data were analyzed by Student two-tailed unpaired t test. *P < .05, **P < .01, ***P < .001, and ****P < .0001. CON, control; Rel., relative.

Figure 1. Hepatic S1pr4 expression is uniquely increased in the liver of murine NASH models. (A) Diet was administered to mice for 12 weeks for HFHCD, 8 weeks for MCDD, 16 weeks for WD, and 6 weeks for CDA+HFD. (A) Hepatic S1pr mRNA expression in HFHCD-, MCDD-, WD-, and CDA+HFD–induced dietary models of NASH (n = 6). (B) Hepatic mRNA expression levels of S1PR4 in the liver samples of patients with NASH/cirrhosis undergoing liver transplantation (n = 9). Surgical specimens of the donor livers were used as controls (n = 10). All data are shown as means ± SEM. Data were analyzed by Student two-tailed unpaired t test. *P < .05, **P < .01, ***P < .001, and ****P < .0001. CON, control; Rel., relative.

S1PR4 Is the Key Mediator of NASH Development

We thus tested the possible involvement of S1PR4 in the development of NASH by using genetic modulation. HFHCD-fed heterozygous S1pr4 knockout mice (S1pr4<sup>+/−</sup> mice) showed significantly lower degrees of hepatic inflammation and fibrosis compared with HFHCD-fed wild-type (WT) mice (Figure 2A and B). However, the degree of hepatic steatosis was similar regardless of the S1pr4 genotype (Figure 2C and D). In addition, we observed that the expression levels of both S1pr4 and genes involved in inflammation (ie, Tnf-α and Mcp-1) were higher in mice fed HFHCD for 4 and 12 weeks than in control mice. On the other hand, these changes were decreased significantly in the livers of S1pr4<sup>−/−</sup> mice (Figure 2D and E). In addition, S1pr4 knockdown ameliorated HFHCD-induced liver fibrosis and reduced the expression of Tgf-β, α-Sma, and Col3a1 (Figure 2F). Collectively, these data indicate that S1PR4 is a critical mediator of the development of NASH.

NLRP3 Deficiency Prevents Diet-Induced NASH and Fibrosis, but Not Hepatic Steatosis

NLRP3 inflammasome is involved in the pathogenesis of various inflammatory and metabolic diseases including
S1PR4 Is Necessary for Activation of the NLRP3 Inflammasome in Hepatic Macrophages

We next evaluated the role of the NLRP3 inflammasome in the S1pr4−/− mice. Compared with HHFCD-fed WT mice, HHFCD-fed S1pr4−/− mice showed significantly decreased NLRP3 inflammasome markers, such as Nlrp3 and Il-1β in the liver (Figure 4A). S1PR4 was reported to be expressed specifically in myeloid cells such as dendritic cells and macrophages1,2,3,; however, its role in the pathogenesis of NASH is largely unknown. To address which cell types are responsible for the up-regulation of S1PR4, we examined the expression levels of S1pr4 in the liver, primary hepatocytes, hepatic macrophages, and HSCs. Significant increases in the expression of S1pr4 by HHFCD were not observed in hepatocytes and HSCs (Figure 4B); in contrast, S1pr4 expression was significantly higher in hepatic macrophages isolated from HHFCD-fed mice for 4 weeks than those isolated from control mice (Figure 4C). We next examined whether S1pr4 expression is increased in resident macrophages in other tissues. Spleen and bone marrow (BM) macrophages isolated from HHFCD-fed mice showed similar S1pr4 mRNA levels compared with those of control-fed mice (Figure 4C). These data suggest that the increase of S1PR4 from hepatic macrophages is an important event in the development of hepatic inflammation and fibrosis.

To explore the relationship between high S1pr4 expression in hepatic macrophages and NLRP3 inflammasome, we isolated hepatic macrophages from S1pr4−/− mice. Hepatic macrophages from S1pr4−/− mice had a significantly lower degree of lipopolysaccharide (LPS)- and adenosine triphosphate (ATP)-induced increases in IL-1β production (Figure 4D). These results suggest that S1PR4 is necessary for NLRP3 inflammasome activation in hepatic macrophages. The activation of NLRP3 inflammasome is achieved through 2 sequential steps: signal 1 (priming) and signal 2 (activation)3: signal 1 is provided by microbial molecules or endogenous cytokines and leads to the up-regulation of NLRP3 and IL-1β through the activation of the transcription factor nuclear factor-κB (NF-κB), and signal 2 is triggered by ATP, pore-forming toxins, viral RNA, and particulate matters.

Interestingly, LPS-induced increases in Nlrp3 and Il-1β were significantly nullified in S1pr4−/− hepatic macrophages (Figure 4E). In addition, the phosphorylation of NF-κB in LPS-primed S1pr4−/− hepatic macrophages was decreased.

| Table 1. Clinical and Analytical Characteristics of NASH Patients Undergoing Liver Transplantation and Controls (Donors) |
|---------------------------------------------------------------|
| Human samples | Age, y | Sex, M/F | Weight, kg | BMI, kg/m² | AST, IU/L | ALT, IU/L | GGT, IU/L | ALP, IU/L | Bilirubin, mg/dL | INR | Total cholesterol, mg/dL | TG, mg/dL |
| Donor 1 | 42 | M | 90 | 27.8 | 30 | 30 | 30 | NA | NA | 1.0 | NA | NA |
| Donor 2 | 70 | F | NA | NA | 58 | 25 | 18 | NA | NA | 1.1 | NA | NA |
| Donor 3 | 67 | M | 85 | 29.4 | NA | NA | NA | NA | NA | NA | NA | NA |
| Donor 4 | 76 | M | 75 | 27.5 | 20 | 35 | 21 | NA | NA | 1 | NA | NA |
| Donor 5 | 62 | M | 80 | 26.4 | 117 | 66 | NA | NA | NA | NA | NA | NA |
| Donor 6 | 75 | F | 78 | 28.7 | 19 | 16 | 11 | NA | NA | NA | NA | NA |
| Donor 7 | 77 | M | 70 | 27.3 | 14 | 15 | 27 | NA | NA | NA | NA | NA |
| Donor 8 | 28 | M | 58 | 17.9 | 50 | 30 | 23 | NA | NA | 1.0 | NA | NA |
| Donor 9 | 58 | M | 90 | 31.1 | 5 | 23 | 36 | NA | NA | NA | NA | NA |
| Donor 10 | 74 | M | 82 | 26.8 | 9 | 12 | 40 | NA | NA | 1.0 | NA | NA |
| Patient 1 | 46 | M | 78 | 26.1 | NA | 76 | 735 | 298 | 1.2 | 4.68 | 192 | 212 |
| Patient 2 | 57 | M | 93 | 28.7 | 20 | 13 | 23 | 203 | 1.3 | 2 | 58 | 45 |
| Patient 3 | 56 | F | 69 | 27.3 | 18 | 6 | 42 | 116 | 0.6 | 1.36 | 183 | 172 |
| Patient 4 | 66 | M | 77 | 24.9 | 14 | 1 | 22 | 84 | 0.6 | 1.2 | NA | NA |
| Patient 5 | 62 | F | 80 | 27.1 | 56 | 26 | 154 | 158 | 1.2 | 1.2 | 183 | 180 |
| Patient 6 | 60 | F | 100 | 35.9 | 45 | 25 | 183 | 152 | 1.4 | 1.4 | 79 | 70 |
| Patient 7 | 67 | M | 95 | 33.7 | 37 | 20 | 76 | 199 | 3.2 | 1.4 | 59 | 47 |
| Patient 8 | 58 | F | 95 | 33.3 | 27 | 11 | 27 | 92 | 3.7 | 1.9 | 39 | 29 |
| Patient 9 | 64 | F | 79 | 32.9 | 105 | 68 | 20 | 270 | 5.3 | 2.1 | 140 | 53 |

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; GGT, γ-glutamyltransferase; INR, international normalized ratio; M, male; NA, not applicable; TG, triglyceride.
suggesting that S1PR4 activates the NLRP3 inflammasome from signal 1. Considering that S1PR4 proteins were reduced markedly in the livers of S1pr4\(^{+/−}\) mice compared with wild-type mice (Figure 2B), we further investigated the inhibitory effects of an S1pr4-targeting lentivirus-mediated short hairpin RNA (shRNA) on the inflammasome activation in the hepatic macrophages. When compared with the nontargeting control shRNA, S1pr4 shRNA significantly reduced S1pr4 mRNA in a dose-dependent manner (Figure 4G). S1pr4 silencing significantly diminished the LPS-induced increases in Nlrp3 and IL-1\(β\) in a dose-dependent manner (Figure 4H). In addition, increases in IL-1\(β\) concentration induced by LPS and ATP were abrogated by S1pr4 silencing (Figure 4I).

**S1PR4-Dependent Calcium Release From Endoplasmic Reticulum Plays a Pivotal Role in the NLRP3 Inflammasome Activation in Hepatic Macrophages**

Intracellular ions such as K\(^+\), Ca\(^{++}\), and Cl\(^−\) have significant roles in the activation of the NLRP3 inflammasome.\(^{34}\)

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**Figure 2.** (See previous page). **S1PR4 is a critical mediator of hepatic inflammation and fibrosis.** S1pr4\(^{+/−}\) mice were fed a chow diet or HFHCD for 12 weeks. (A) Representative H&E and Masson’s trichrome staining of the livers of WT and Nlrp3\(^{−/−}\) mice fed HFHCD for 12 weeks. Scale bar: 50 \(\mu\)m. (B) Relative mRNA expression levels of the genes associated with inflammation and fibrosis in the liver (\(n = 4\)). (C) TG levels in the livers of WT mice fed chow or HFHCD, and Nlrp3\(^{−/−}\) mice fed HFHCD (\(n = 4\)). All data are shown as means \(±\) SEM. (B and C) Data were analyzed by one-way analysis of variance with Bonferroni correction. \(*P < .05, **P < .0005, and ****P < .0001. MW, molecular weight.

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**Figure 3.** Nlrp3\(^{−/−}\) mice are protected from the development of NASH. (A) Representative H&E and Masson’s trichrome staining of the livers of WT and Nlrp3\(^{−/−}\) mice fed HFHCD for 12 weeks. Scale bar: 50 \(\mu\)m. (B) Relative mRNA expression levels of the genes associated with inflammation and fibrosis in the liver (\(n = 4\)). (C) TG levels in the livers of WT mice fed chow or HFHCD, and Nlrp3\(^{−/−}\) mice fed HFHCD (\(n = 4\)). All data are shown as means \(±\) SEM. (B and C) Data were analyzed by one-way analysis of variance with Bonferroni correction. \(*P < .05, **P < .0005, and ****P < .0001. MW, molecular weight.
Among them, intracellular Ca\(^{2+}\) signaling plays one of the major roles in the activation of NLPR3 inflammasomes.\(^{15}\) Accordingly, treatment of hepatic macrophages with the [Ca\(^{2+}\)] chelator 1,2-Bis(2-aminophenoxy)ethane-N,N’,N’’,N’’’-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM; A1076, sigma-aldrich, St. Louis, MO), significantly decreased the IL-1\(\beta\) production in response to LPS and ATP stimulation, as well as the LPS-induced increases in the expression of Nlrp3 and IL-1\(\beta\) (Figure 5A–C).

A previous study indicated that phospholipase C (PLC)-dependent changes in [Ca\(^{2+}\)] are the downstream signaling of S1PR4.\(^{36}\) Activation of PLC triggers the release of inositol trisphosphate (IP\(_3\)) from phosphatidylinositol 4, 5-bisphosphate, and [Ca\(^{2+}\)] is released to the cytosol when IP\(_3\) interacts with IP\(_3\) receptor (IP\(_3\)R) located at the endoplasmic reticulum (ER) membrane.\(^{37}\) In our experimental setting, treatment with the PLC inhibitor U73122 (1-[6-(1H-pyrrole-2,5-dione) or IP\(_3\)R inhibitors Xestospongin C (Xes-c) and 2-Aminoethyl diphenylborinate (2-APB) significantly decreased the LPS-mediated increases in the expression levels of Nlrp3 and IL-1\(\beta\) (Figure 5A, D, and E), as well as the production of IL-1\(\beta\) in response to LPS and ATP stimulation (Figure 5F). Taken together, these results indicate that increases in [Ca\(^{2+}\)] release from the ER through the PLC/IP\(_3\)R axis play an important role in the activation of the NLRP3 inflammasome in hepatic macrophages.\(^{35}\)

The LPS-induced increase in the level of IP\(_3\), the product of PLC,\(^{38}\) was decreased significantly in S1pr4\(^{-/-}\) cells (Figure 5G). Consistently, measurement of [Ca\(^{2+}\)] showed that LPS treatment in hepatic macrophages induced a robust increase in [Ca\(^{2+}\)] (Figure 5H), which is in line with previously reported data.\(^{39}\) On the other hand, LPS-induced [Ca\(^{2+}\)] release was decreased significantly in S1pr4\(^{-/-}\) hepatic macrophages (Figure 5H). These results collectively indicate that S1PR4 is required for the calcium signaling associated with the NLRP3 inflammasome activation.

### S1P Activates the NLRP3 Inflammasome by the S1PR4/PLC/IP\(_3\) Axis

We examined the possible role of the S1P/S1PR4 axis in the activation of the NLRP3 inflammasome. S1P has been shown to contribute to nonalcoholic fatty liver disease and liver fibrosis.\(^{10,40}\) Sphingosine kinases (SKs; eg, SK1 and SK2) catalyze the formation of S1P from the precursor sphingosine.\(^{8}\) Interestingly, expression of Sk1 was increased markedly in the livers but not in hepatocytes of HFD-fed mice (Figure 6A and B). On the other hand, S1P mRNA expression was increased significantly in hepatic macrophages, whereas the macrophages isolated from the spleen and BM were not affected significantly by HFD feeding (Figure 6C). In contrast to Sk1, Sk2 expression was not increased in the livers of HFD-fed mice (Figure 6A). These data suggest that increased S1P levels in the hepatic macrophages may induce hepatic inflammation. To further explore the role of S1P on the activation of the NLRP3 inflammasome, hepatic macrophages were treated with S1P. S1P significantly increased the expression level of S1pr4 in hepatic macrophages (Figure 6D). S1P also increased the expression levels of Nlrp3 and IL-1\(\beta\) significantly, an effect that was dampened in S1pr4\(^{-/-}\) hepatic macrophages (Figure 6D). S1P also stimulated the phosphorylation of NF-\(\kappa\)B in hepatic macrophages, and this was reduced in S1pr4\(^{-/-}\) hepatic macrophages (Figure 6E). Pretreatment with BAPTA-AM, U73122, XestC, or 2-APB significantly reduced the S1P-mediated induction of Nlrp3 and IL-1\(\beta\) expression (Figure 6F). These results suggest that extracellular S1P may act as a modulator of the NLRP3 inflammasome in hepatic macrophages through the PLC/IP\(_3\)/IP\(_3\)R signaling axis.

### Development of a Novel Functional Antagonist for S1PR4

To further clarify the biological function of S1PR4, we developed a chemical probe that selectively modulates S1PR4. We envisioned that introducing additional heteroatoms into the structure of FTY720 could change the selectivity for the S1P subtype by referring to the X-ray crystal structure of S1PR1\(^{41}\) and previous structural studies of S1PR4.\(^{42}\) Accordingly, we designed and synthesized several types of heteroatom-containing analogues of FTY720. Among the designed analogues, a triazole-containing compound SLB736 (Figure 7A) showed a selective agonistic activity against S1PR4 (EDG6; endothelial differentiation G-protein coupled receptor 6) based on \(\beta\)-arrestin recruitment assay (Figure 7B). The unique action of the currently used drugs targeting S1PRs, including

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**Figure 4.** (See previous page.) S1PR4 depletion decreases NLRP3 inflammasome activation in hepatic macrophages. (A) Relative hepatic mRNA expression levels of the genes associated with the components of NLRP3 inflammasome (\(n = 6\)). S1pr4\(^{-/-}\) mice were fed chow or HFD for 4 or 12 weeks. (B) mRNA expression of S1pr4 in primary hepatocytes and HSCs from mice fed chow diet or HFD for 4 weeks (\(n = 4\)). (C) mRNA expression of S1pr4 in macrophages isolated from the liver, spleen, and BM. S1pr4\(^{-/-}\) mice were fed chow or HFD for 4 weeks (\(n = 4\)). (D–F) S1pr4 depletion decreases NLRP3 inflammasome activation in hepatic macrophages. (D) Hepatic macrophages isolated from S1pr4\(^{-/-}\) mice were stimulated with LPS (100 ng/mL) for 3 hours followed by ATP (1 mmol/L) for 30 minutes. Cell culture media were collected and IL-1\(\beta\) levels were measured by ELISA (\(n = 4\)). (E) Relative mRNA expression levels of Nlrp3 and IL-1\(\beta\) (\(n = 4\)) and (F) representative Western blots of NF-\(\kappa\)B phosphorylation (pNF-\(\kappa\)B) and corresponding quantification (\(n = 3\)). S1pr4\(^{-/-}\) hepatic macrophages were stimulated with LPS (100 ng/mL) for (E) 3 hours or (F) 30 minutes. (G–I) Dose-dependent effect of S1pr4 shRNA transfection on the NLRP3 inflammasome in hepatic macrophages. Relative mRNA expression levels of (G) S1pr4 and (H) Nlrp3 and IL-1\(\beta\) are shown. (I) IL-1\(\beta\) in the cell culture media after transfection of normal hepatic macrophages with the indicated multiplicity of infection (MOI) of S1pr4 shRNA. Hepatic macrophages were infected with lentiviral vectors coding S1pr4 shRNA at an MOI of 0, 10, and 50. After 48 hours, hepatic macrophages were stimulated with (G and H) LPS (100 ng/mL) for 3 hours, (I) followed by stimulation with ATP (1 mmol/L) for 30 minutes. All data are shown as means ± SEM. (A and F–I) Data were analyzed by one-way analysis of variance with Bonferroni correction. (B–E) Data were analyzed by Student two-tailed unpaired t test. *\(P < .05\), **\(P < .01\), ***\(P < .001\), and ****\(P < .0001\). Con, control; MW, molecular weight.
SLB736 Prevents the Development of NASH and Fibrosis

Administration of SLB736 to HFD-fed mice prevented the development of NASH and hepatic fibrosis (Figure 8A and B). Conversely, liver TG content was not altered significantly after SLB736 administration (Figure 8C), suggesting that SLB736 treatment prevents diet-induced liver injury, inflammation, and fibrosis, but not steatosis.

Interestingly, the administration of SLB736 did not reduce the number of lymphocytes, which is a well-known adverse effect of FTY720 through its effect on S1PR1 (Figure 8D). Although treatment with SLB736 did not significantly reduce the diet-induced increases in the mRNA level of S1pr4 (Figure 8E), the protein level of S1PR4 was decreased significantly upon treatment with SLB736 (Figure 8F), thus signifying that SLB736 carries functional antagonistic roles on S1PR4 in vivo. Whether SLB736 promotes S1PR4 protein instability or degradation remains to be established.

We further investigated whether SLB736 shows a similar preventive effect in other diet-induced murine models of NASH. We found that similar to HFD-fed mice, mice fed MCDD or CDA+HFD developed NASH along with increases in the expression of inflammation and inflammasome markers, which effectively were nullified by the administration of SLB736 (Figure 9A–D). To further show the therapeutic effect of SLB736, we administered SLB736 to mice fed MCDD for 4 weeks, a time point at which hepatic steatosis was evident (Figure 9E). Administration of SLB736 for 4 weeks ameliorated NASH and fibrosis in these mice (Figure 9F). Thus, SLB736 was effective in preventing the development of NASH in different nutritional models.

SLB736 Suppresses the NLRP3 Inflammasome in Hepatic Macrophages

We next checked the effect of SLB736 on the NLRP3 inflammasome in hepatic macrophages. As expected, SLB736 decreased the production of IL-1β in response to LPS and ATP, and the expression of Nlrp3 and Il-1β in response to LPS (Figure 10A and B). Consistently, LPS-induced increase in the level of IP3 (IP-one), the product of PLC, was decreased significantly in cells treated with SLB736 (Figure 10C). Pretreatment with SLB736 inhibited the LPS-induced [Ca2+] release in hepatic macrophages (Figure 10D). These data suggest that SLB736 acts as a functional antagonist of S1PR4, thereby leading to the inactivation of NLRP3 inflammasome and prevention of NASH development.

Discussion

S1PR4 is expressed specifically in myeloid cells such as dendritic cells and macrophages. S1PR4 also is required for the differentiation of plasmacytoid dendritic cells and regulates the production of interferon-α thereof. However, compared with other S1PRs, our knowledge of the physiological relevance of S1PR4 has been modest.

We found that the S1PR4 expression was significantly higher in the livers of both diet-induced NASH animal models.
models and patients with NASH undergoing liver transplantation. This is in line with a previous study that reported the up-regulation of S1PR4 in human samples of liver cirrhosis. In addition, our data showed that genetic depletion of S1PR4 protected the mice against hepatic inflammation and fibrosis. Hepatocyte death and inflammation are the critical trigger of NASH, which sequentially activates HSCs. Interestingly, S1pr4 expression was induced significantly by HHFCD feeding in hepatic macrophages, whereas it was not detected in hepatocytes and HSCs. A recent study reported that S1PR2 is involved in the NLRP3 inflammasome activation in hepatic macrophages during chronic liver injury. In the present study, we found that S1PR4 in hepatic macrophages also plays an important role in the pathogenesis of NASH by activating the NLRP3 inflammasome.

As a major reservoir of intracellular [Ca++]+, the ER plays a critical role in the regulation of intracellular [Ca++]+ regulation. Activation of the IP3R, a Ca+++-release channel on the ER surface, is triggered by IP3, a product of PLC-mediated phosphatidylinositol 4, 5-bisphosphate cleavage. We found that LPS sequentially activated PLC and IP3R in hepatic macrophages to increase [Ca++]+. To activate the NLRP3 inflammasome, and that this reaction was abrogated by genetic depletion of S1pr4 or treatment with SLB736. In addition to LPS, we found that S1P also can activate the NLRP3 inflammasome. Interestingly, expression of Sk1 but not Sk2 was induced selectively in hepatic macrophages by HHFCD feeding, and S1P increased the expression of S1pr4 in hepatic macrophages. Accordingly, a previous study showed that the overloading of saturated fatty acids induces Sk1 in hepatocytes to initiate proinflammatory signaling. On the other hand, in HHFCD-fed mice, Sk1 expression was induced in hepatic macrophages, but not in hepatocytes. We thus suggest that S1P produced by SK1 from hepatic macrophages induces S1PR4 in a paracrine manner to activate the NLRP3 inflammasome (Figure 11).

In our study, we used mice with heterozygous mutation of S1PR4 considering that homozygous mutation of S1PR4 leads to embryonic lethality. Interestingly, some studies reported the use of adult S1pr4 homozygote knockout mice. Among them, 1 study used S1pr4 knockout mice that were bred in a BALB/C background, whereas our mice were bred in a C57BL/6J background. Therefore, it may be possible to study the effects of S1pr4 homozygous knockout in NASH by using BALB/C mice, albeit the results may have limited implications owing to the distinct genetic and immunologic characteristics of BALB/C mice.

Nevertheless, our study showed the importance of S1PR4 in NASH because the deletion of 1 copy of the S1PR4 gene was effective in preventing hepatic inflammation and fibrosis in nutritional NASH. We also studied the effect of S1PR4 modulation by shRNA-induced knockdown, which resulted in dose-dependent protection against LPS- or ATP-induced inflammasome activation in primary hepatic macrophages. Our findings should be confirmed in future studies using WT, S1pr4+/−, and S1pr4−/− mice to determine whether S1PR4 confers a protective effect on hepatic inflammation and fibrosis in a dose-dependent manner in vivo.

An important highlight of the present study was the design and synthesis of heteroatom-containing FTY720 analogues with the aim of obtaining high S1PR4 activity and subtype selectivity. Among the synthesized analogues, SLB736 showed an excellent subtype selectivity and functional antagonism for S1PR4. The administration of SLB736 prevented the development of NASH and fibrosis. Interestingly, SLB736 did not affect the expression of S1PR4 at the mRNA level, although the protein levels of S1PR4 in the liver were decreased significantly by SLB736. Notably, SLB736 did not induce lymphopenia, a potentially serious side effect of FTY720 arising from its action on S1PR1, highlighting the relevance of SLB736 as a novel therapy for NASH.

An interesting finding of our study was that both S1pr4 heterozygous knockout and SLB736 treatment did not affect the degree of hepatic steatosis in HHFCD-fed mice, while effectively preventing diet-induced liver injury, inflammation, and fibrosis. This may be explained by the fact that the primary effect of SLB736 is on NLRP3, which is dominant in hepatic macrophages. Accordingly, similar findings also were observed in Nlrp3 knockout mice.

NAFLD occurs mostly in obese individuals, and insulin resistance and deregulation of lipid metabolism increase the risk of NAFLD and NASH. Although lifestyle modification is the first-line treatment for patients with NASH, it usually is unsuccessful. Therefore, many agents for the treatment of NASH by targeting different pathways are under development, and several compounds have shown promising histologic results in phase IIa studies. However, it was pointed out that histologic NASH is not an independent predictor of long-term mortality and that the stage of fibrosis is the only robust and independent predictor of liver-related mortality. In this regard, targeting NLRP3 inflammasome activation, which plays a central role in hepatic inflammation and fibrosis, increasingly is recognized as a promising strategy for developing an efficient therapy against NASH. In accordance, our study showed that

Figure 6. (See previous page.) S1P activates NLRP3 inflammasome through S1PR4. (A) Sk1 and Sk2 mRNA expression in the livers of mice fed HHFCD for 12 weeks (n = 4). (B and C) Sk1 mRNA expression in (B) primary hepatocytes and (C) in macrophages from mice fed HHFCD for 4 weeks (n = 4–5). Macrophages were isolated from the liver, spleen, and BM. (D and E) S1pr4 knockout decreases the S1P–S1PR4–NLRP3–inflammasome activation. (D) The mRNA expression of S1pr4, Nlrp3, and Il-1β. Serum-starved S1pr4−/− hepatic macrophages were treated with S1P (1 μmol/L) for 2 hours (n = 4). (E) Representative Western blots of NF-kB phosphorylation (pNF-kB) and corresponding quantification (n = 3). S1pr4−/− hepatic macrophages were treated with S1P (1 μmol/L) for 30 minutes. (F) mRNA expression of Nlrp3 and Il-1β. Serum-starved hepatic macrophages were treated with BAPTA-AM (10 μmol/L), U73122 (10 μmol/L), Xes C (5 μmol/L), or 2-APB (100 μmol/L), and treated with S1P (1 μmol/L) (n = 4). All data are shown as means ± SEM. (A–D) Data were analyzed by Student two-tailed unpaired t test. (E and F) Data were analyzed by one-way analysis of variance followed by Bonferroni correction. **P < .01, ***P < .001, and ****P < .0001. Con, control; MW, molecular weight.
SLB736 was effective in preventing the development of NASH and fibrosis by deactivating the NLRP3 inflammasome.

**Materials and Methods**

All authors had access to the study data and reviewed and approved the final manuscript.
Figure 8. SLB736 treatment prevents HFHCD-induced NASH. (A) Representative H&E, Masson’s trichrome, and Sirius Red staining of the livers. Scale bars: 50 μm. SLB736 (1 mg/kg/d) was administered for 12 weeks in HFHCD-fed mice. (B) Relative mRNA expression levels of the genes associated with inflammation (Tnf-α, Mcp-1) and fibrosis (Tgf-β, α-Sma, Col3α1) (n = 5). (C) Liver TG contents (n = 4). (D) Lymphocyte counts in the blood. FTY720 or SLB736 (1 mg/kg/d) were administered for 12 weeks in HFHCD-fed mice (n = 4). (E and F) mRNA expression of (E) S1pr4 and (F) representative Western blots of S1PR4 and corresponding quantification in the liver of HFHCD-fed mice treated with SLB736 (n = 4). All data are shown as means ± SEM. (B–F) Data were analyzed by one-way analysis of variance followed by Bonferroni correction. *P < .05, **P < .01, and ****P < .0001. Con, control; MW, molecular weight.
Mice and Diet

Mice were housed at ambient temperature (22°C ± 1°C) with a 12:12-hour light-dark cycle and free access to water and food. All animal use and experiment protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences (Seoul, Korea).

Eight-week-old male C57BL/6J mice were fed either normal chow diet (12% energy from fat), CDA + HFD containing 60 kcal fat and 0.1% methionine (A06071302; Research Diets, New Brunswick, NJ) for 6 weeks, MCDD (Dyets, Inc, Bethlehem, PA) for 8 weeks, or HFHCD (60% energy from fat and 2.5% cholesterol; Dyets, Inc) for 12 weeks. In another group, mice were fed a WD (TD.120330, 0.2% cholesterol + 22% hydrogenated vegetable oil, Envigo RMS, Inc, Indianapolis, IN) supplemented with high-fructose syrup in the drinking water for 16 weeks. After the indicated time of diet feeding, mice were fasted for 5 hours in the morning before they were killed.

*S1pr4*^+/-^ mice were purchased from Jackson Laboratories (mouse strain 005799; Bar Harbor, ME). Eight-week-old male *S1pr4*^+/-^ mice and their littermate controls (*S1pr4*^+/+^) were fed either a normal chow diet or HFHCD for 4 or 12 weeks. Considering the description on the MGI (mouse genome informatics) website (1333809) on the...
embryonic lethality of the homozygous mutation of the S1pr4 gene, we used heterozygote mice for this study and observed the deletion of 1 copy of the S1pr4 gene. Nrp3 knockout mice were generated using the transcription activator-like effector nuclease method.57

**Human Liver Samples**

Human liver samples were obtained from the liver explants of donors and recipients diagnosed with NASH/liver cirrhosis undergoing liver transplantation at the Liver Transplantation Unit of the Hospital Clinic (Barcelona, Spain). Healthy liver tissues were obtained from the surgical specimens of donor livers used for transplantation. Biopsy of the resected livers from the recipients was performed immediately after the hepatectomy and the samples were fixed in formalin for histologic examination. The clinical data of the patients are presented in Table 1. The protocol was approved by the Hospital Clinic/University of Barcelona Ethics Committee (HCB/2012/8011) of the Hospital Clinic (Barcelona, Spain).

**Isolation of Hepatocytes and Hepatic Macrophages**

Hepatocytes and hepatic macrophages were isolated from mice by collagenase digestion, gradient centrifugation, and selective adherence,58 with modifications. Briefly, the mice were anesthetized and the peritoneal cavity was opened; the livers were perfused with Ca²⁺ and Mg²⁺-free

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**Table 2. Reverse-Transcription PCR Primers**

| Primer   | Forward                        | Reverse                           |
|----------|--------------------------------|-----------------------------------|
| Tbp      | CTTCAACCAATGACTCTCTATGAC       | CAAGTTTACAGCCAGATTACAC           |
| 18S rRNA | GTAACCGTGGAAACCCATT            | CCATCACAATGGTAGAATG             |
| Tnf-α    | GAGAAGTCAACCTCTCTCTCTG         | GAAGACTCTCCCCAGGTATATG         |
| Mcp-1    | ACGCATGGAATCTCAAGAGAC          | AGATAGCAATGGGCTGAGC           |
| Tgf-β    | TATAGCAACATCTTGCGC             | CCGTATTGCTGCTCTCTTG             |
| α-Sma    | ACTGGAGACGAGATGGAAAG            | GTGAGGCCTGGCTCTGGA             |
| Col3a1   | GGGTTTCCCTGTCTCTAAAG           | CCTGTTGTCCTTTTCTCC             |
| Nlrp3    | ATTACCCGCGGAGAAAGG            | TCGCAGCAAGATCCCCCAACAG         |
| Il-1β    | TTCCTGAAGTGAGGACC             | TGAGTTGACTGCTGCTG             |
| S1pr1    | ATGGGCCTGACTAGATCC            | CGATGTCACTTGGCTCTG             |
| S1pr2    | ATGGGCCTGTTAATGCAAGAG         | GGGGACGACAGATGGATGATG         |
| S1pr3    | ACTTCGGGGAACACATTAGAT         | CAAGACGAAGATGGCAGATGATG        |
| S1pr4    | GGCTGTACTACGGCTGCTG            | AGCAGACTGAAAGGTGATGCA           |
| S1pr5    | GCTTGTTGGTGCTTGAG             | GCAGCTACAGATGGTCCAGGAGCATG    |
| Sk1      | CCACTCGAGAACCCTCTG             | ACCTGCTGAGACAGCGATG          |
| Sk2      | AGAGGAGGACCAATG               | CAGAGGAGGACCAATG               |

Reverse-transcription PCR primers (human)

| S1PR4    | GAGCCTGGGTCTACTATTGCC          | CCTCCCGTAGGAAACACTG           |
| ACTB     | TGTTGATGAGGAGGTTTATGAGT       | AACCAATACACCTAATCTCCCTTTA    |

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**Figure 11. Conceptual model showing the role of the SK1/S1PR4 axis in the pathogenesis of NASH.** S1P produced by SK1 from hepatic macrophages induces S1PR4 in a paracrine manner, which is necessary for the [Ca²⁺]-dependent priming of the NLRP3 inflamma-some. ASC, Apoptosis-associated speck-like protein containing a C-terminal caspase-recruitment domain; DAG, diacylglycerol; Sph, sphingosine.
Hank’s balanced salt solution (LB 003-04; Welgene, Daegu, Korea) containing collagenase (17101-015; Gibco, Carlsbad, CA) and trypsin inhibitor (T2011; Sigma-Aldrich, St. Louis, MO). The digested livers were removed and placed in 60-mm Petri dishes. The livers were frittered with forceps in RPMI1640 (LM 011-01; Welgene) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (16000-044; Gibco). The cell suspensions were filtered through a sterile Falcon 100-

μm nylon cell strainer (352360; Corning Inc, NY) to remove undigested tissues and connective tissues. The cells were centrifuged at 50 × g for 3 minutes at room temperature. For isolation of hepatocytes, the pellet was resuspended in Dulbecco’s modified Eagle medium with 36% Percoll (Sigma-Aldrich), and then centrifuged at 50 × g for 10 minutes at room temperature. The pellet then was washed 2 times with phosphate-buffered saline (PBS). Finally, the hepatocytes were plated on collagen-coated tissue culture dishes in Dulbecco’s modified Eagle medium cell culture medium supplemented with 10% FBS and 1% penicillin/streptomycin solution, and kept in a humidified medium supplemented with 10% FBS and 1% penicillin/streptomycin solution, and kept in a humidi-

Table 3. Antibodies

| Antibodies          | Cat#   | RRID:AB           |
|---------------------|--------|-------------------|
| NF-κB               | Cell Signaling Technology | 6956, RRID:AB_10828935 |
| Phospho-NF-κB       | Cell Signaling Technology | 3033, RRID:AB_331284 |
| S1PR4               | Novus  |                   |
| β-actin             | Sigma-Aldrich                  | A5441, RRID:AB_476744 |
| HRP goat anti-mouse IgG | BioLegend                      | 405306, RRID:AB_315009 |
| HRP donkey anti-rabbit IgG | BioLegend                  | 406401, RRID:AB_2099388 |
| F4/80               | Invitrogen                     | 12-4801-82, RRID:AB_465923 |
| CD45.2              | Invitrogen                     | 45-0454-82, RRID:AB_953590 |

HRP, horseradish peroxidase.

Isolation of Macrophages From the Spleen and BM

Spleens were excised and digested for 30 minutes with collagenase (Sigma-Aldrich) at 37°C while shaking. Cell suspensions were filtered through a 70-μm sieve and centrifuged at 450 × g for 5 minutes. Femurs were collected in RPMI, and bone marrow cells were flushed from the femurs and then depleted of red blood cells using red blood cell lysis buffer (R7757; Sigma-Aldrich). For macrophage staining, cells were incubated with Fc (fragment crystallizable) Block (101302; BioLegend, San Diego, CA) for 10 minutes in ice, and then washed and stained with anti-F4/80 antibody (12-4801-82; Invitrogen) and anti-CD45.2 antibody (45-0454-82; Invitrogen) for 30 minutes on ice in the dark. Macrophages were sorted as live CD45.2 and F4/80 double-positive cells into RPMI supplemented with 20% FBS using FACS Aria2 (BD Bioscience).

Primary HSC Isolation

Selective macrophage depletion was achieved with a single intraperitoneal injection of clodronate (20 mg/mL) according to the manufacturer’s instructions (FormuMax Scientific, Inc, Sunnyvale, CA). After 24 hours, primary HSCs were isolated using the same protocol used in the isolation of hepatic macrophages, and the cell fraction between the upper layer and the 20% OptiPrep gradient were collected without contamination from the pellets. After centrifugation, the cells were seeded into culture plates in Dulbecco’s modified Eagle medium containing fetal serum at 37°C. The culture medium was changed and the RNA was isolated from primary HSCs.

Histologic Analysis

Liver tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Serial sections (5-μm
Liver TG Contents

TG content in the livers was determined in duplicate using the triglyceride kit (GPO-Trinder; Sigma-Aldrich).

Real-Time Polymerase Chain Reaction Analysis

Total RNA isolated from each sample was reverse-transcribed and the target complementary DNA levels were quantified by real-time polymerase chain reaction analysis using gene-specific primers (Table 2). Total RNA was isolated using TRIzol (Invitrogen), and 1 µg of each sample was reverse-transcribed with random primers using the Reverse Aid M-MuLV Reverse-Transcription Kit (Fermentas, Amherst, NY). The relative expression levels of each gene were normalized to that of 18S rRNA, Tbp, or ACTB.

Western Blot Analysis

Cell and liver samples were homogenized in lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L KCl, 4 mmol/L EDTA, 4 mmol/L ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and 1% NP-40 containing protease (04693132001; Roche, Carlsbad, CA) and phosphatase (04906837001; Roche) inhibitor mixture tablets) at 4°C for 30 minutes. The resulting protein (40–50 µg) was subjected to immunoblotting with primary antibodies: antibodies against phosphorylated NF-κB and NF-κB were purchased from Cell Signaling (Danvers, MA). Anti-S1PR4 antibody was purchased from Novus (Centennial, CO). β-actin (A5441; Sigma-Aldrich) was used as housekeeping control (Table 3). The signal intensities of protein bands were quantified and normalized to the volume of each cell. The absolute intensity for all cells in a field at different time points was obtained, and normalized to t = 0 to calculate the fold increases in intensity. Data are shown as the relative intensity of cells in a field.

IL-1β Measurement

Mouse IL-1β in cell culture supernatants was measured using the mouse IL-1β/IL1F2 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (DY401; R&D Systems, Minneapolis, MN).

Lentiviral-Silencing S1pr4

The shRNA sequences for S1pr4 were as follows: Forward, 5′- GCC TGC TGA ACA TCA CAC TGA TCA AGA GTC AGT GTG ATG TTC AGC AGG CTT TTT TG-3′; Reverse: 5′- CAA AAA AGC AGC CTG AAC ATC ACA CTG ACT CTT GAT CAG TGT GAT GTT CAG CAG GC-3′. S1pr4 shRNA was subcloned into the pCDH-MCS lentiviral vector (CD513B-1; System Biosciences, Mountain View, CA) and the plasmids were transfected in Lenti-X 293T cells (632180; Clontech, Mountain View, CA), along with the packaging plasmids pMDLg/pRRE (12251; Addgene, Cambridge, MA) and pRSV-Rev (12253; Addgene) and the envelope plasmid pCMV-VSV-G (8454, Addgene) using Lipofectamine 3000 (L30000015; Invitrogen). Hepatic macrophages were infected with lentiviral vectors coding S1pr4 shRNA for 12 hours at a range of multiplicity of infection (ie, 0, 10, and 50). The medium was changed after 12 hours.

Intracellular IP-One Measurement

PLC activity was tested with the IP-one ELISA (721P1PEA; Cisbio, Bedford, MA), in which hepatic macrophages were stimulated with LPS or S1P and then the cell culture medium was replaced with fresh medium. Intracellular IP-one, a surrogate measure for the level of inositol triphosphate, was measured after treatment with LiCl (50 mmol/L) to prevent the degradation of IP-one into myo-inositol. The level of inositol triphosphate in cell lysates was measured using an ELISA.

Calcium Analysis by Confocal Microscopy

Hepatic macrophages were plated on a 35-mm imaging dish (81156; Ibidi, Gräfelfing, Germany) at a density of 0.1 x 10^6 cells and incubated with Fluo-4/FM (F36206; Invitrogen). Images of untreated cells were acquired at t = 0, and the cells were treated with 1 µg/mL LPS or 1 mmol/L ATP in RPMI1640. The cells were imaged for 5 minutes at 5-second intervals on a Zeiss LSM780 Confocal Imaging System (Carl Zeiss, Oberkochen, Germany) using the 488-nm laser and emission in the range of 500–600 nm. The images were analyzed using Zen 2012 SP5 software (Carl Zeiss, Oberkochen, Germany) by creating surfaces to encompass the volume of each cell. The absolute intensity for all cells in a field at different time points was obtained, and normalized to t = 0 to calculate the fold increases in intensity. Data are shown as the relative intensity of cells in a field.

Reagents for Calcium Signaling

BAPTA-AM (A1076;Sigma-Aldrich), U73122 (D9754; Sigma-Aldrich), 2-APB (D9754; Sigma-Aldrich), and Xes-c (X2628; Sigma-Aldrich) were used for detecting calcium signaling.

S1P Treatment in Hepatic Macrophages

Hepatic macrophages were serum-starved for 6 hours and then stimulated with 1 µmol/L S1P (S9666; Sigma-Aldrich) for 2 hours.

Synthesis of SLB736

The chemical and spectroscopic data are as follows: melting point 130 °C; 1H NMR (400 MHz, CD3OD) δ 8.35 (s, 1H), 4.53 (t, J = 7.2 Hz, 2H), 3.69 (s, 4H), 2.97 (td, J = 4.3, 8.0 Hz, 2H), 2.11 (td, J = 4.3, 8.0 Hz, 2H), 2.00–1.95 (m, 2H), 1.35–1.28 (m, 14H), 0.89 (t, J = 6.8 Hz, 3H), 13C NMR (100 MHz, CD3OD) δ 146.5, 127.3, 63.0 (2C), 62.6, 54.3, 33.8, 32.0, 31.5, 31.4, 31.3, 31.2, 30.8, 28.1, 24.5, 19.9, 15.2; IR (neat) ʋmax = 3180, 2918, 2851, 2421, 1599, 1454, 1080, 1063, 958, and 715 (cm−1); HRMS (FAB) calculated for C17H35N4O2 [M–Cl]− 327.2760, found 327.2762. (NMR: nuclear magnetic resonance; s: singlet; d: doublet; t: triplet; dd: triplet of doublets; m: multiplet; H: proton; J: coupling constant; C: carbon; IR: infrared spectroscopy; ʋmax: lambda max).
To two-sided sample sizes and were determined on the basis of comparisons. All statistical tests were conducted according to the Bonferroni correction was applied for post hoc analysis of the multiple group comparisons. Bonferroni groups, and one-way analysis of variance was used to compare variables among multiple groups. Bonferroni analysis was performed using IBM SPSS Statistics for Windows version 22.0 (IBM Corp, Armonk, NY) or GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

**Treatment With SLB736 or FTY720 In Vivo**

Mice were administered SLB736, FTY720 (1 mg/kg body weight each), or vehicle (0.9% NaCl) via oral gavage every day for 5 d/wk for the indicated periods. After the indicated period of treatment, the mice were fasted overnight and killed. The liver tissues were quickly removed and kept frozen at -70°C for subsequent analysis.

**SLB736 Treatment In Vitro**

Hepatic macrophages were treated with chemicals at the indicated doses or sterile water (control) for 2 hours. After washing twice with PBS, the cells were stimulated with LPS (L2880; Sigma-Aldrich) at a concentration of 100 ng/mL for 3 hours, and then 1 mmol/L ATP (A6419; Sigma-Aldrich) was added for 30 minutes.

**Determination of S1PR4 Localization in a C6 Glioma Cell Line**

Stable C6 glioma cells expressing EGFP-conjugated S1PR4 were prepared by infection with retrovirus bearing S1PR4-EGFP fusion construct (kindly provided by Dr Jerold Chun at Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA). S1PR4 internalization and recycling were assessed as previously described. In brief, cells were plated on poly-L-lysine (100 μg/mL)-coated coverslips, cultivated, serum-deprived, and then used for experiments. The cells were treated with vehicle (0.1% fatty acid-free bovine serum albumin), S1P, FTY720-P, or SLB736 for 0.5 hours; in some cases, the cells were washed and further incubated in the presence of cycloheximide (5 μg/mL) for 2 or 4 hours. At the end of each experiment, the cells were fixed in 4% paraformaldehyde and mounted with Vectashield (H-1000; Vector Laboratories, Burlingame, CA). S1PR4 localization in cells was assessed by detecting the EGFP signal using laser scanning confocal microscopy (Eclipse A1++; Nikon, Tokyo, Japan).

**S1PR β-Arrestin Assay**

β-arrestin recruitment assays for S1PR activity were performed by DiscoveRx (Fremont, CA).

**Blood Lymphocyte Measurement**

Blood lymphocytes were counted using an automated hematology analyzer (ADVIA 2120i 53; Siemens Healthcare Diagnostics, Tarrytown, NY).

**Statistical Analysis**

Data are expressed as means ± SEM. Unpaired two-tailed Student t tests were used to compare variables between groups, and one-way analysis of variance was used to compare variables among multiple groups. Bonferroni correction was applied for post hoc analysis of the multiple comparisons. All statistical tests were conducted according to two-sided sample sizes and were determined on the basis of previous experiments that used similar methodologies. For all experiments, the stated replicates are biological replicates. Statistical analysis and graphing were performed using IBM SPSS Statistics for Windows version 22.0 (IBM Corp, Armonk, NY) or GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

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Conflicts of interest
These authors disclose the following: Eun Hye Koh, Sanghee Kim, and Ki-Up Lee have filed a provisional patent application in the Korea patent office (10-2017-00040139: composition for preventing and treating nonalcoholic steatohepatitis by targeting S1PR4). The remaining authors disclose no conflict.

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