Original Paper

Sphingosine 1-Phosphate (S1P)/S1P Receptor_{2/3} Axis Promotes Inflammatory M1 Polarization of Bone Marrow-Derived Monocyte/Macrophage via G(α)_{i/o}/PI3K/JNK Pathway

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Key Words

Sphingosine 1-phosphate • Sphingosine 1-phosphate receptor • Inflammation • Bone marrow-derived monocyte/macrophage • Macrophage polarization • Signaling transduction

Abstract

Background/Aims: Macrophages, the most plastic cells in the haematopoietic system, are found in all tissues and show great functional heterogeneity. Sphingosine 1-phosphate (S1P)/S1P receptors (S1PRs) system is widely involved in the process of inflammatory disease, whereas little evidence concerning its role in functional macrophage polarization is available. Thus, the present study was designed to evaluate the effects of S1P/S1PRs on functional polarization of macrophage in mouse bone marrow (BM)-derived monocyte/macrophages (BMMs). **Methods:** For the detection of M1 macrophage markers, such as CD86, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1/ chemokine (C-C motif) ligand (CCL) 2, nitric oxide synthase (NOS) 2, and macrophage inflammatory protein (MIP)-1β, RT-qPCR and cytometric bead array (CBA) were performed in cultured primary BMMs after the treatment with selective S1PR_{2/3} antagonists or specific S1PRs siRNA. Western blotting and immunofluorescence were used for the detection of phosphorylation of JNK1/2. **Results:** BMMs expressed S1PR₁₋₃ and interestingly, S1PR_{2/3}, but not S1PR₁, mediates S1P-induced M1 macrophage polarization of BMMs as their siRNA or antagonists reduced M1 genes' expression. We found that PTX (inhibitor of G(α)_{i/o}), LY294002 (inhibitor of PI3K) or SP600125 (inhibitor of JNK1/2) prevented up-regulation of M1 genes expression mediated by S1P/S1PR_{2/3} signal, and S1P-induced JNK phosphorylation was inhibited by antagonists of S1PR_{2/3}, PTX or LY294002. **Conclusion:** Collectively, our results demonstrate that S1P/S1PR_{2/3} plays a key role in regulating M1 type polarization of BMMs and acts by activating G(α)_{i/o}/PI3K/JNK signaling pathway, with potential implications for new approaches to inflammatory liver disease therapy.

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Introduction

Macrophages, the most plastic cells in the haematopoietic system, are found in all tissues and show great functional heterogeneity [1]. They display an important role in innate and adaptive immune response. Functionally, macrophages can be classified in classically activated M1 macrophages and alternatively activated M2 macrophages [2, 3]. M1 macrophages express specific marker CD86 [4] as well as produce pro-inflammatory cytokines and chemokines such as tumor necrosis factor (TNF)- α , monocyte chemotactic protein (MCP)-1/ chemokine (C-C motif) ligand (CCL) 2, interleukin (IL)-1, IL-6, nitric oxide synthase (NOS) 2, macrophage inflammatory protein (MIP)-1 β [5, 6]. In contrast, M2 macrophages produce anti-inflammatory cytokine Arginase-1 and express special receptor CD163 and CD206 and play a crucial role in healing and tissue repair [7, 8]. It has been reported that macrophage polarization and function is largely controlled by chemokines, cytokines, and different varieties of ligands of G-protein-coupled receptor (GPCR) like lipopolysaccharide (LPS) [9-11].

Sphingosine 1-phosphate (S1P), one of the most important pleiotropic lysophospholipid mediators, is converted primarily from sphingosine by sphingosine kinase (SphK) and plays key roles in regulating the polarization of immune cells and secretion of inflammatory mediators [12, 13]. Recently, increasing evidence demonstrate that S1P is involved in the progression of various inflammatory-related diseases such as inflammatory bowel disease [14], inflammatory skin diseases [15] and rheumatoid arthritis [16]. Of note, S1P exerts different cellular functions via its receptors or as a cell second messenger [17]. Until now, studies have shown that most S1P biological functions were mediated by its receptors [18]. S1P receptors (S1PRs) are classified into 5 types, named S1PR₁₋₅, while S1PR₁₋₃ is widely expressed. These S1PRs bind to distinct heterotrimeric G-proteins and regulate different signaling pathways [19]. Our previous studies have shown that S1P/S1PR_{2/3} is involved in bile duct ligation (BDL)- or carbon tetrachloride (CCl₄)-induced liver injury [20]. However, the role of S1P/S1PRs in functional polarization of macrophage remains incompletely defined.

Accumulating evidence showed that, among the complex array of the GPCR-activated intracellular signaling pathways, c-Jun N-terminal kinase (JNK), p38, extracellular regulated protein kinases (ERK), phosphatidylinositol 3-kinase (PI3K) and Rac1 are involved in macrophage polarization, thus contributing to inflammation [21]. For example, JNK activation is required for M1 macrophage polarization during obesity-induced liver inflammation, and JNK knock down mice abrogates M1-related inflammation and fibrosis [22]. Additionally, activation of p38 and JNK signaling induces increase of M1 genes expression upon Toll-like receptor (TLR) 4-induced inflammation [23, 24]. Moreover, M1 macrophage polarization can be induced by Forkhead box protein O1 (Fox O1) phosphorylation depended on PI3K and ERK signaling [25]. Another central regulator in cell polarization is Rac1, one of the important members of GTPase family, which is also involved in cell polarization for their switch role (GTP-bound for active) [26].

Our previous study revealed that hepatic S1P levels were significantly up-regulated at the early stage, and remained at a high level during with the progression of liver injury in human and mouse liver injury caused by multiple etiologies [27, 28]. Meanwhile, the analysis *in vivo* and *in vitro* suggested that bone marrow (BM)-derived monocytes/macrophages (BMMs) were recruited to the damaged liver [29, 30] and exposed to high levels of S1P in the hepatic microenvironment. Furthermore, it was also found that BMMs presented a pro-inflammatory property during the process [20]. Based on these previous *in vivo* and *in vitro* studies, we determined whether S1P is involved in the pro-inflammatory M1 polarization of BMMs, and identified the signaling pathway that mediates S1P-induced M1 polarization in BMMs. In the present study, we found that primary murine BMMs express S1PR₁, S1PR₂ and S1PR₃ and S1P can promote BMM polarization toward M1 phenotype through S1PR₂ and S1PR₃. S1PR_{2/3} mediated S1P-induced M1 polarization via G(α)_{i/o}/PI3K/JNK pathway in

BMMs. Our present work identified S1P/S1PR_{2/3} as an essential mediator in M1 macrophage polarization, which may present a novel therapeutic target in inflammatory disease.

Materials and Methods

Reagents

RPMI 1640 Medium was from Invitrogen (Grand Island, NY). Fetal bovine serum was from Biochrom (Berlin, Germany). PCR reagents were from Applied Biosystems (Foster City, CA). S1P and dihydro-S1P (H₂S1P) were from Biomol (Tebu, France). Lipopolysaccharide (LPS) was from Sigma-Aldrich (St. Louis, MO). S1PR₁ antagonist W146, S1PR₂ antagonist JTE-013 and S1PR₃ antagonist CAY-10444 were from Cayman Chemical (Ann Arbor, MI). Pertussis toxin (PTX, G(α)_{i/o} inhibitor), SB203580 (p38 inhibitor) and LY294002 (PI3K inhibitor), NSC 23766 (Rac1 inhibitor) were purchased from Tocris Bioscience (Bristol, UK). U0126 and SP600125 were purchased from Cell Signaling Technology (Beverly MA), SKI [2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole] (Calbiochem, Bad Soden, Germany).

Isolation and cultivation of mouse primary BMMs

BMMs are primary macrophage cells, derived from BM cells *in vitro* in the presence of growth factors. Macrophage colony-stimulating factor (M-CSF) is secreted by L929 cells and is used in the form of L929-conditioned medium. Firstly, L929 cells were obtained from the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO₂ at 37°C for 7 days (replacing culture medium at day 4). L929 cells were harvested using trypsin, and then implanted with 4.7×10⁵/bottle (BD Falcon) and cultured in the presence of DMEM-Gluta MAX medium. After 7 days, the medium was sterilized by filtrating through a 0.22 μ m filter and used as L929-conditioned medium.

Next, ICR mice at 4 weeks of age were sacrificed by cervical dislocation at the time of BM harvest. BM cells were extracted from the tibias and femurs by flushing with culture medium using a 25-gauge needle. The cells were then passed through a 70-mm nylon mesh (BD Biosciences, Franklin Lakes, NJ) and were washed three times with PBS containing 2% FBS. Extracted BM cells were implanted with 2.4×10⁷/100 mm culture dish (BD Falcon) and cultured for 7 days in the presence of L929-conditioned medium (replacing culture medium at day 3 and 5). After 7 days, Cells were serum starved for 12 hours prior to treatment with W146 (1 μ mol/L), JTE-013 (10 μ mol/L), CAY-10444 (10 μ mol/L), PTX (10 ng/mL), U0126 (10 μ mol/L), SB203580 (10 μ mol/L), SP600125 (50 μ mol/L), LY294002 (10 μ mol/L) or NSC23766 (100 μ mol/L) for additional 1 hour. Pretreated mouse primary BMMs were then allowed to S1P (1 μ mol/L) administration for another 6 hours.

Mouse models of liver injury

Adult ICR mice received intraperitoneal injections of 1 μ L/g body weight of a CCl₄/olive oil (OO) mixture, 1:9 v/v, twice per week. SKI (0.4 mg/kg B.W.) was administered intraperitoneally one day before CCl₄ or OO treatment and twice per week for 4 weeks (n=6 per group). The mice were sacrificed at 4 weeks of CCl₄ treatment, always on the day after the last injection. All animal work was conformed to the Ethics Committee of Capital Medical University and in accordance with the approved guidelines (specific institutional approval number of animal experiment: AEEI-2014-131).

Cell migration assay

Serum-starved BMMs were treated with vehicle, LPS or IL-4 six hours, respectively. These cells were then seeded to the upper chamber, and allowed to migration for another 4 hours in the presence of 1 μ mol/L S1P. The chambers were incubated at 37 °C in 5% CO₂. Cells migrated to the lower surface of the filter were hematoxylin stained and quantified by cell counting. The migration index was defined as the number of cells in the lower chamber under the tested condition divided by the number of cells under control.

RT-qPCR

Total RNA was extracted from cells or liver frozen specimen using RNeasy mini kit (Qiagen, Hilden, Germany) and the quantity and purity of RNA was determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using oligo (dT) and M-MLV reverse transcriptase (Invitrogen). RT-qPCR was performed using TaqMan® Universal PCR Master Mix or SYBR Green qPCR Master Mix on the ABI 7300 TH Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All RT-qPCR assays and independent experiments were performed in triplicate respectively. Relative mRNA expression normalized to the house-keeping gene 18S rRNA was carried out using $2^{-\Delta\Delta Ct}$ method. All primers were synthesized by Biotech (Beijing, China). Primers used for RT-qPCR were as follows:

Mouse 18S rRNA: sense, 5'-GTAACCCGTTGAACCCATT-3'
antisense, 5'-CCATCCAATCGGTAGTAGCG-3'
Mouse TNF- α : sense, 5'-GGCAGGTTCTGTCCCTTCA-3'
antisense, 5'-CTGTGCTCATGGTGTCTTTTCTG-3'
Mouse MCP-1/CCL2: sense, 5'-TCTGGCCCTGCTGTTTCA-3'
antisense, 5'-GGATCATCTTGCTGGTGAATGA-3'
Mouse NOS2: sense, 5'-TGACGGCAAACATGACTTCAG-3'
antisense, 5'-GGTGCCATCGGGCATCT-3'
Mouse MIP-1 β : sense, 5'-CCAGCTCTGTGCAAACCTAACC-3'
antisense, 5'-GCCACGAGCAAGAGGAGAGA-3'
Mouse CD86: sense, 5'-TCCAAGTTTTTGGGCAATGTC-3'
antisense, 5'-CCTATGAGTGTGCACTGAGTTAAACA-3'
Mouse Arginase-1: sense, 5'-GTCTGGCAGTTGGAAGCATCT-3'
antisense, 5'-GCATCCACCAAATGACACA-3'
Mouse CD163: sense, 5'-CAGGTGTTATCTGCTCCGAGTTC-3'
antisense, 5'-CCCCATGTACCATTTGTAACAACT-3'
Mouse CD206: sense, 5'-GGTGAAGAAGAAGTAGCCT-3'
antisense, 5'-GAAGGGTCAGTCTGTGTTTG-3'

Immunofluorescence staining

BMMS (5, 000) were plated in the well of 96-well plates (Corning, NY) and treated with chemicals after overnight attachment. The cells were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.5% Triton X-100 in PBS (Amresco, OH) for 15 min. After blocked with 2% BSA (Roche, Switzerland), they were incubated with anti-S1PR₁, anti-S1PR₂ or anti-S1PR₃ rabbit polyclonal antibody (1:100, Santa Cruz Biotechnology, CA), anti-phospho-JNK1/2 rabbit polyclonal antibodies (1:100, Cell Signaling Technology, Beverly, MA), respectively, FITC-conjugate affinity-purified goat-anti-rabbit IgG (1:100, Jackson ImmunoResearch, PA) was used as secondary antibodies. At last, nuclei were incubated with 10 μ g/mL DAPI for 5 min.

Western blot analysis

Protein samples were separated on 12% SDS-PAGE, transferred onto a nitrocellulose membrane (Millipore, Massachusetts, USA) and blocked with 5% (w/v) bovine serum albumin (BSA) in TBS pH 7.4. Then, the membrane was incubated with rabbit anti-JNK1/2 (1:1, 000; Cell Signaling Technology, Beverly, MA), rabbit anti-phospho-JNK1/2 (1:1, 000; Cell Signaling Technology, Beverly, MA) or rabbit anti- β -actin antibody (1:1, 000, Abcam, UK), followed by incubation with IRDye™ 800-conjugated goat anti-rabbit IgG (1:10, 000, LI-COR Biosciences, Lincoln, NE) as secondary antibodies. Protein level was visualized by the LI-COR Odyssey® Imaging System and assessed by Odyssey® software (LI-COR Biosciences, Lincoln, NE), respectively.

Measurement of cytokine and chemokine by CBA

BMMS (500, 000 cells) were lysed in 40 μ L lysis buffer. TNF- α and MCP-1/CCL2 in homogenates were detected using cytometric bead array (CBA) mouse TNF- α Flex Set (Catalog No. 558299, BD Biosciences) and mouse MCP-1/CCL2 Flex Set (Catalog No. 558342, BD Biosciences) and then analyzed by fluorescence-activated cell sorting.

RNAi *in vitro*

siRNA sequence targeting specifically mouse S1PR₁₋₃ was purchased (L-051684-00, L-063765-00, L-040959-00; Dharmacon, Lafayette, CO). Forty to fifty percent confluent BMMs were prepared. Transient transfection of siRNA (40 nmol/L) was used by employing Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Control cells were treated with RNAi negative control duplexes (scramble siRNA, 40 nmol/L). After 24 hours, cells were used to fulfill further experiments.

Statistical analysis

The results are expressed as mean ± SEM. Statistical significance was assessed by Student's t-test or one-way analysis of variance (ANOVA) for analysis of variance when appropriate. $P < 0.05$ was considered to be significant. All results were verified in at least three independent experiments.

Results

S1P promotes M1 macrophage polarization in BMMs

To assess the efficiency and capacity of the BMM polarization *in vitro*, we treated primary mouse BMMs with LPS, a potent inducer of M1 macrophage polarization [31]. RT-qPCR analysis showed that inflammatory cytokine expressions including TNF- α , MCP-1/CCL2, NOS2, MIP-1 β and CD86 were significantly increased in BMMs upon LPS (10 ng/mL) challenge (Fig. 1A). In contrast, the mRNA expression of Arginase-1 was obviously up-regulated in BMMs treated with IL-4 (5 ng/mL) (Fig. 1B), indicating that LPS and IL-4 can be effectively used to trigger M1 BMMs and M2 BMMs, respectively.

We next employed the BMMs to determine the effect of S1P on macrophage polarization *in vitro*. The mRNA expression of M1 markers (TNF- α , MCP-1/CCL2, NOS2, MIP-1 β and CD86) in BMMs in response to S1P at different time was determined. BMMs were treated with 1 μ mol/L S1P and collected after 0, 2, 4, 6, 12, 18, and 24 hours. RT-qPCR analysis demonstrated that the mRNA expression of M1 markers including TNF- α , MCP-1/CCL2, NOS2, MIP-1 β and CD86 were elevated at 4 hours then reached to the topmost level (~ 30.8-fold, ~ 27.6-fold, ~ 135.5-fold, ~ 25.4-fold, and ~ 4.4-fold, respectively) at 6 h (Fig. 1C-G). However, S1P had no effects on M2 marker Arginase-1 mRNA expression (Fig. 1H). Moreover, the mRNA expression of CD163 and CD206 were also unchanged after treatment with S1P (Fig. 1I). These results indicate that M1, but not M2 macrophage polarization was induced by S1P in BMMs.

Antagonism of S1PR_{2/3} inhibits S1P-induced BMMs polarization toward M1 phenotype

To further assess whether S1P triggered BMM polarized toward M1 phenotype via its cell surface receptors, we performed the same experiment using H₂S1P, a structural analog of S1P that is only able to exert its biological functions through a surface bound S1PRs. As shown in Fig. 2A, the effect of S1P on TNF- α mRNA expression was completely mimicked by H₂S1P. These results indicate that S1P induces BMM polarized toward M1 type via its cell surface receptors. We next used immunofluorescence assays to determine the expression of S1PR₁, S1PR₂ or S1PR₃ in BMMs. As expected, the immunostaining for these receptors were detected (Fig. 2B). To further determine which receptors mediate the S1P-induced M1 macrophage polarization, we next employed pharmacological antagonist of S1PRs and detected the expression of M1 markers with RT-qPCR and CBA. As shown in Fig. 2C, treatment of BMMs with S1P lead to up-regulation of mRNA expression of M1 markers, including TNF- α and MCP-1/CCL2, and these increases were abrogated by specific S1PR₂ antagonist JTE-013 (10 μ mol/L) or S1PR₃ antagonist CAY-10444 (10 μ mol/L). However, the mRNA expression of TNF- α and MCP-1/CCL2 were unchanged after specific S1PR₁ antagonist W146 (1 μ mol/L) pretreatment. Moreover, the protein level of these M1 markers (TNF- α , and MCP-1/CCL2) was significantly decreased by JTE-013 or CAY-10444 after S1P stimulation, as analyzed by CBA (Fig. 2D).

Fig. 1. S1P induces BM cells polarized toward M1 type *in vitro*. (A) The mRNA expression of M1 markers (TNF- α , MCP-1/CCL2, NOS2, MIP-1 β and CD86) in BM cells treated with 10 ng/mL LPS 6 hours. (B) The mRNA expression of M2 marker Arginase-1 in BM cells treated with 5 ng/mL IL-4 six hours. The mRNA expression of TNF- α (C), MCP-1/CCL2 (D), NOS2 (E), MIP-1 β (F), CD86 (G) and Arginase-1 (H), in BM cells treated with S1P for 0 to 24 hours. (I) The mRNA expression of CD163 and CD206 in BM cells treated with 1 μ mol/L S1P at different time. Data are presented as mean $\pm$ SEM; n=6 per group; * P <0.05 compared with control group.

Fig. 2. S1P-induced M1 polarization is prevented by S1PR_{2/3} antagonists in BMs. (A) The mRNA expression of TNF- α in BMs treated with S1P or H₂S1P (0 to 1 $\mu\text{mol/L}$) for 6 hours. Stock solutions of S1P and H₂S1P were dissolved in methanol at -80°C. Dilutions of S1P and H₂S1P were freshly prepared after evaporation of methanol by resuspension in 0.4% fatty acid-free BSA and sonication. (B) Representative images of immunofluorescence staining for S1PR₁, S1PR₂ and S1PR₃ (green) were shown in BMs. (C) The mRNA expression of TNF- α and MCP-1/CCL2 in BMs from vehicle-, JTE-013-, and CAY10444-treated group after 1 $\mu\text{mol/L}$ S1P administration. (D) The protein expression of TNF- α and MCP-1/CCL2 were measured by CBA in BMs from vehicle-, JTE-013-, and CAY10444-treated group after 1 $\mu\text{mol/L}$ S1P administration. (E) Quantification in CBA. Data are presented as mean $\pm$ SEM; n=6 per group; * P <0.05 compared with control group, # P <0.05 compared with the same treated group without antagonists. BSA, bovine serum albumin.

Fig. 3. Knockdown of S1PR_{2/3} restrains S1P-induced M1 polarization in BMMs. (A) Efficiency of knockdown of S1PR₁₋₃ mRNA by S1PR₁-, S1PR₂-, or S1PR₃-siRNA transfection in BMMs. (B) The mRNA expression of TNF-α and MCP-1/CCL2 in BMMs from scramble siRNA-, S1PR₁₋₃-siRNA-treated group after 1 μmol/L S1P administration. (C) The protein expression of TNF-α and MCP-1/CCL2 was measured by CBA in BMMs from scramble siRNA-, S1PR₁₋₃-siRNA-treated group after 1 μmol/L S1P administration. (D) Quantification in CBA. Data are presented as mean ± SEM; n=6 per group; *P<0.05 compared with scramble group, #P<0.05 compared with the same treated group without siRNA.

Genetic ablation of S1PR_{2/3} inhibits S1P-induced BMMs polarization toward M1 phenotype

To further evaluate the significant role of S1PRs, individual S1PRs was knocked down by specific siRNA. We first confirmed that genetic ablation of S1PR₁, S1PR₂ or S1PR₃ effectively silenced the corresponding target gene at the mRNA level and their mRNA expression was reduced by 87%, 82%, and 86%, respectively in mouse BMMs (Fig. 3A). S1PR₁, S1PR₂ or S1PR₃ siRNA *in vitro* did not alter the expression of other S1PRs, which confirmed the specificity of these siRNAs (data not shown). Furthermore, silencing S1PR_{2/3} prevented the mRNA and protein expression of TNF- α and MCP-1/CCL2 induced by S1P administration (Fig. 3B-D). These results further confirm that S1PR_{2/3}, but not S1PR₁ mediates the S1P-induced BMM polarized toward M1 phenotype *in vitro*.

G(α)_{i/o} PI3K and JNK signal mediates the effect of S1P on M1 polarization in BMMs

As a G protein-coupled receptor, the biological function of S1P/S1PRs depends on multiple signaling pathways, such as ERK, p38 and JNK signaling pathways [22, 32-35]. Our previous study indicated that S1P activated PI3K and Rac1 to promote macrophage migration. To identify the responsible signal pathways for M1 polarization of BMMs regulated by S1P, pharmacological inhibitors of signal transduction, including U0126 (ERK inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK1/2 inhibitor), LY294002 (PI3K inhibitor) and NSC23766 (Rac1 inhibitor) were applied. The S1P-induced increase of TNF- α , and MCP-1/CCL2 protein level were only lessened by LY294002 and SP600125 in mouse primary BMMs (Fig. 4 A-B) whereas U0126, SB203580, and NSC23766 had no effect on S1P-induced M1 markers expression (Fig. 4 A-B), indicating that PI3K and JNK signal, but not ERK, p38, Rac1 signal, might be necessary for S1P-induced M1 polarization of BMMs. Moreover, PTX (G(α)_{i/o} protein inhibitor) pre-treatment also inhibited S1P-induced M1 markers (TNF- α and MCP-1/CCL2) mRNA expression (Fig. 4 A-B). Furthermore, PTX, LY294002 and SP600125 pretreatment also decreased the up-regulation of protein levels of TNF- α and MCP-1/CCL2 in BMMs treated with S1P, as detected by CBA (Fig. 5 A-C). These results indicate that G(α)_{i/o} PI3K and JNK signals involves in S1P-induced BMMs polarization toward M1 phenotype.

S1P/S1PR_{2/3}-mediated the M1 polarization depends on the G(α)_{i/o}/PI3K/JNK signal pathway

As we found that JNK and PI3K pathways were involved in S1P-induced M1 polarization, we next investigated whether these pathways are influenced by S1P/S1PR_{2/3}. Western blotting assay revealed that S1P alone induced a significant increase in the protein level of phospho-JNK1/2 (~3.5 fold) and kept longer excitation of JNK1/2 (Fig. 6A). To further confirm it, immunofluorescence assays were used to identify the phosphorylation of JNK1/2 in BMMs. As expected, the immunostaining for phospho-JNK1/2 was clearly detected (Fig. 6B). Pretreatment with PTX, S1PR antagonists JTE-013 or CAY-10444 prevented the increase of phospho-JNK1/2, respectively (Fig. 6A). Moreover, we found that PI3K inhibitor LY294002 can prevent S1P-induced phospho-JNK1/2 (Fig. 6A), implying JNK as an obligatory downstream target of PI3K pathway in S1P-reduced BMMs polarized toward M1 phenotype. Altogether, these results show that G(α)_{i/o} protein coupled with S1PR_{2/3} mediates S1P-induced M1 macrophage polarization through PI3K/JNK signal pathway.

Fig. 4. $G(\alpha)_{1/o}$ protein, PI3K and JNK signaling pathways involves in S1P/S1PR_{2/3}-mediated M1 polarization of BMMs. Serum-starved cells were pretreated for 1 hour with $G(\alpha)_{1/o}$ inhibitor PTX, ERK inhibitor U0126, p38 inhibitor SB203580, JNK inhibitor SP600125, PI3K inhibitor LY294002 or Rac1 inhibitor NSC27366. Pretreated mouse primary BMMs were then allowed to 1 μ mol/L S1P treatment for another 6 h. The mRNA expression of TNF- α (A) and MCP-1/CCL2 (B) in BMMs were detected by RT-qPCR. (C) The effect of S1P on cell migration of LPS- (M1 BMMs) or IL-4-treated BMMs (M2 BMMs), respectively. (D) The effect of S1P inhibitor, SKI [2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole], on macrophages compartment in mouse liver injury induced by carbon tetrachloride (CCl₄). The mRNA expression of TNF- α , MCP-1 and F4/80 in the liver were measured by RT-qPCR. The ratio of TNF- α / F4/80 or MCP-1/ F4/80 expression was then used to represent the level of inflammatory cytokines per macrophage. Data are presented as the mean $\pm$ SEM. * P <0.05, compared with vehicle. # P <0.05 compared with the same treated group without inhibitors (A-B) or CCl₄-treated mice without SKI administration (D).

Fig. 5. $G(\alpha)_{i/o}$ protein, PI3K, and JNK signaling pathways involves in S1P/S1PR_{2/3}-induced M1 polarization of BMMs. BMMs were exposed to S1P (1 μ mol/L) for 6 hours with $G(\alpha)_{i/o}$ inhibitor PTX (A), PI3K inhibitor LY294002 (B) or JNK inhibitor SP600125 (C) pretreatment, and then the protein expression of TNF- α , and MCP-1/CCL2 were determined by CBA. Data are presented as the mean $\pm$ SEM; n=6 per group; * P <0.05 compared with control group, # P <0.05 compared with the same treated group without inhibitors.

Fig. 6. G protein coupled with S1PR_{2/3} mediates S1P-induced M1 polarization of BMMs through PI3K/JNK signaling pathway. (A) The phosphorylation of JNK was determined by Western blot in BMMs treated with S1P for 6 hours. Serum-starved cells were pretreated for 1 h with G(α)_{1/10} inhibitor PTX, S1PR₂ antagonist JTE-013, S1PR₃ antagonist CAY10444, PI3K inhibitor LY294002. Pretreated mouse primary BMMs were then allowed to 1 μ mol/L S1P treatment for another 6 hours. phospho-JNK were determined by Western blot. (B) Representative images of immunofluorescence staining for phospho-JNK (green) were shown in BMMs. (C) Scheme of G(α)_{1/10}-coupled with S1PR_{2/3} mediated BMMs polarized toward M1 phenotype through PI3K/JNK signaling pathway. Data are presented as the mean $\pm$ SEM; n=3 per group in (A), n=6 per group in (B); * P <0.05 compared with control group, # P <0.05 compared with the same treated group without inhibitors or antagonists. min, minute; h, hour.

Discussion

In the present study, we reveal that S1PR_{2/3}, but not S1PR₁, mediates S1P-induced M1 macrophage polarization in BMMs. We also provide evidence that S1PR_{2/3} plays a crucial role in the expression of M1 markers *in vitro*. Inhibition of S1PR_{2/3} decreases BMM polarization toward M1 phenotype through G(α)_{i/o}/PI3K/JNK signaling pathway (Fig. 6C).

BMMs are commonly used and well defined source of macrophages in experimental study [3]. Our previous studies have shown that recruitment of BMMs in the injured liver could aggravate liver inflammation and thus played a crucial role in BDL- or CCl₄-induced liver disease [20, 29, 30]. Among a variety of factors that mediate switch BMMs to pro-inflammatory M1 phenotype, S1P is probably one of the most potent regulatory molecules [36]. S1P has been implicated in many key cellular processes, including cell proliferation, differentiation, migration and survival [37]. Besides, S1P acts as a crucial inflammatory mediator and can be released in response to inflammation and following injury [36, 38]. Recent studies showed that S1P levels was markedly increased in acutely inflamed tissues and released by apoptotic cells [39]. Moreover, S1P level in serum was regarded as a prognostic factor [40]. Thus, S1P is a promising candidate as a regulator of the different variety of inflammatory disease. S1P has been implicated in the regulation of macrophage functions, including migration and polarization. However, although BMMs are known to have unique characteristics, the roles of S1P signaling in the regulation of macrophage polarization during liver injured are poorly understood. Our preceding study has showed that blockade of S1PR_{2/3} decreased liver inflammation and fibrosis by preventing BMM migration [20]. In the current study, we thus investigated the effects of S1P on BMM further, focusing on BMM polarization. Our *in vitro* studies demonstrated that S1P promoted inflammatory M1 polarization via S1PR_{2/3}/G(α)_{i/o}/PI3K/JNK pathway in BMMs. Furthermore, we revealed that CCl₄ administration apparently increased M1 markers expression, and these increases were inhibited by SphK inhibitor, SKI [2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole], in mouse injured liver (Fig. 4D). These findings further support previous studies demonstrating a crucial role of BMMs in liver inflammation [41]. Emerging data suggest that recruitment of BM-derived cells following liver injury promotes liver inflammation [20, 42]. Therefore, further studies will be needed to better explore which BM-derived cells infiltrates the inflamed liver during pathology.

Biological functions of S1P are mainly mediated by its cell surface receptors [19]. For example, recent studies showed that S1PR₂ inhibited chemoattractant-induced motility in murine primary macrophages *in vitro* and may limit macrophage infiltration into the inflamed peritoneum [13]. In addition, a recent report showed that S1PR₂ induced the cAMP/protein kinase pathway to inhibit macrophage motility *in vitro* [43]. An earlier study has found that S1PR₂ was needed for TLR4-induced the release of pro-inflammatory cytokines such as IL-1 β and IL-18 [44]. Furthermore, during thioglycollate-induced sterile peritonitis, the elevated S1P production in the inflamed peritoneum induces an inflammatory response in the injured site, which in turn stimulates the recruitment of various types of inflammatory cells by promoting S1PR₃ expression [45]. Blockade of S1PR₃ by antagonist or knockout limits the accumulation of peritoneal macrophage cells in the inflamed site *in vivo* [45]. Our previous studies have shown that the BMMs played a crucial role in BDL- or CCl₄-induced liver disease [20, 29]. Inhibition of S1PR_{2/3} depressed M1 mobility and thus attenuated the inflammation and liver fibrosis in injured liver [20]. These above results have showed significant contributions of S1PRs on macrophages, but which S1P receptor subtype mediated M1 polarization remains unclear. A key finding of the present study is that S1PR_{2/3} is involved in M1 polarization in BMMs. These data are in agreement with recent studies highlighting a central role of S1P-induced immune cell polarization in many different cellular types including T cell, macrophage, and mast cell [46], further proving that S1P production is necessary for M1 polarization. Many studies have reported that polarization of immune cells appear to be mediated via the binding of S1P to S1P receptors [37]. However, S1P signaling is complex and may have both pro- and anti- inflammatory properties, especially for BMMs. In an earlier study, Park et al. showed that S1P stimulated M2 phenotype polarization of

macrophages via IL-4 [10]. The reason might be that distinct macrophages were used, BMMs were obtained from ICR mice in the present experiments, while peritoneal macrophages from C57BL/6 mice in the study conducted by Park et al. Furthermore, owing to their different origins, BMMs and peritoneal macrophages may have distinct gene expression profile which causes a different response to S1P. The reagents of inducing macrophage polarization were also different, S1P was used alone in the current experiments, but S1P was used in conjunction with LPS in the study conducted by Park et al. Furthermore, S1P induced macrophage toward anti-inflammatory phenotype in tumor environment [46]. We reason that the roles of S1P on functional phenotype of macrophage might be diverse depending on the expression of different S1P receptors in different tissue, as well as on their different inflammatory microenvironment.

As the complexity of M1 polarization, it is crucial to understand the molecular signaling cascades in the regulation process of BMM polarization by S1P. S1PRs are seven-transmembrane G-protein-coupled receptors, which are linked predominantly to $G(\alpha)_{i/o}$ protein and thus generate a various biological action [47]. A review showed activations of S1PRs induced complex signal transduction such as PI3K and mitogen activated protein kinase (MAPK) [47, 48]. Here we show that inhibition of $G(\alpha)_{i/o}$ protein signal by PTX reduces S1P/S1PR_{2/3}-mediated M1 polarization of BMMs. The result demonstrated that the signal of S1P/S1PR_{2/3} coupling $G(\alpha)_{i/o}$ protein triggered the release of macrophages pro-inflammatory cytokines, and promoted BMMs polarized toward M1 phenotype. Studies have shown that JNK1/2 was important for pro-inflammatory macrophage polarization [22, 25] and PI3K has been shown to be related to polarization of various cell types [49]. Here we revealed that S1P/S1PR_{2/3}-induced increase of M1 markers is abrogated by specific $G(\alpha)_{i/o}$, PI3K or JNK inhibitors, indicating that PI3K/JNK signaling pathway might be involved in M1 phenotype polarization of macrophage. Inhibition of S1PR_{2/3}, $G(\alpha)_{i/o}$ or PI3K can prevent the phospho-JNK induced by S1P. The result above suggested that JNK may function as an downstream activator of $G(\alpha)_{i/o}$ and PI3K. Our previous study also has reported that S1P activated PI3K and Rac1 to promote macrophage migration [20]. In this study the expression of S1PRs in BMM was already shown and a down-regulation of pro-inflammatory cytokines including MCP-1, IL-12 and IL-6 was shown to occur by the use of S1PR antagonists in the context of liver injury that could be related to a reduced infiltration of macrophages into the injured liver [20]. To further determine whether M1 polarization in BMMs associated with higher mobility, we performed the experiments to determine the effect of S1P on the mobility of M1, M2 macrophages. The analysis on cell migration demonstrated that S1P did not affect the mobility of macrophages in different polarization states (Fig. 4C), indicating that the increase in S1P-induced M1 phenotype was due to macrophage polarization. Further studies will be needed to illustrate the exact molecular mechanisms by which S1P/S1PR_{2/3} mediates M1 polarization in BMMs. Moreover, although we have carefully verified that S1P/S1PR_{2/3} mediated M1 polarization in BMMs, in the present study, however, mainly employed an *in vitro* model that might not completely reflect a real impact on macrophage biology, the effect of S1PR_{2/3} on M1 polarization of BMMs in the progression of liver inflammation remains unclear. The future experiments may need to be designed to better explore whether S1PR_{2/3} inhibition is associated to reduction of M1 genes in injured liver.

Conclusion

In summary, the results of this study provide the genetic and pharmacologic evidence to support the hypothesis that S1PR₂ and S1PR₃ pathway play a crucial role in BMM polarized toward M1 phenotype in a positive manner. This work identifies new clues to the molecular mechanisms underlying the production of M1 genes elicited by $G(\alpha)_{i/o}$ coupled with S1PR_{2/3} and PI3K/JNK signal pathway. These results expand our understanding of S1PR_{2/3} and the regulation of M1 genes expression. Recently, M1 macrophages have been regarded as a therapeutic target for inflammatory disease treatment. Our results reveal the possibility

that blockade of S1PR_{2/3} can inhibit M1 genes' expression and thus might regulate the inflammatory processes, which finally serve as a therapeutic target.

Abbreviations

S1P (sphingosine 1-phosphate); S1PR (S1P receptor); SphK (sphingosine kinase); BM (bone marrow); BMM (bone marrow-derived monocyte/macrophage); GPCR (G-protein-coupled receptor); TNF- α (tumor necrosis factor- α); MCP-1 (monocyte chemotactic protein-1); CCL2 (chemokine (C-C motif) ligand 2); NOS2 (nitric oxide synthase 2); MIP-1 β (macrophage inflammatory protein-1 β); JNK (c-Jun N-terminal kinase); PTX (pertussis toxin); ERK (extracellular regulated protein kinases); siRNA (small interfering RNA); CBA (cytometric bead array); LPS (lipopolysaccharide).

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Disclosure Statement

The authors declared that they have no conflicts of interest related to this work.

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