International Immunopharmacology 66 (2019) 224–235

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

S1P promotes inflammation-induced tube formation by HLECs via the S1PR1/NF-κB pathway

Zhi Zheng[1], Yong-Zhi Zeng[1], Kun Ren[1], Xiao Zhu, Ying Tan, Yi Li, Qian Li, Guang-Hui Yi*

Institute of Cardiovascular Disease, Key Laboratory for Atherosclerology of Hunan Province, University of South China, 28 W Changsheng Road, Hengyang, 421001, Hunan, China

ARTICLE INFO

Keywords:
Sphingosine-1-phosphate
Inflammation
Tumor necrosis factor-α
Interleukin-1β
Lymphangiogenesis

ABSTRACT

Inflammation-induced lymphangiogenesis is a widely accepted concept. However, most of the inflammatory factors and their related mechanisms have not been clarified. It has been reported that sphingosine-1-phosphate (S1P) is not only closely related to the chronic inflammatory process but also affects angiogenesis. Therefore, we investigated the inflammatory effects of S1P on human lymphatic endothelial cells (HLECs). Our results showed that S1P promotes tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) secretion in HLECs. We also confirmed that S1P-stimulated TNF-α and IL-1β secretion is mediated through S1P receptor 1 (S1PR1). Using TNF-α siRNA and IL-1β siRNA, we found that TNF-α and IL-1β play essential roles in S1P-induced HLEC proliferation, migration, and tube formation. S1P induces phosphorylation of NF-κB p65 and activation of NF-κB nuclear translocation. A S1PR1 antagonist (W146) and NF-κB inhibitor (BAY11-7082) inhibited S1P-induced TNF-α and IL-1β secretion and prevented NF-κB nuclear translocation. Taken together, the results demonstrated for the first time that S1P promotes the secretion of TNF-α and IL-1β in HLECs via S1PR1-mediated NF-κB signaling pathways, thus affecting lymphangiogenesis. The study provides a new strategy for finding treatments for lymphangiogenesis-related diseases.

1. Introduction

Inflammation-induced lymphangiogenesis is a widely accepted concept, but the mechanisms underlying their interaction have not been fully elucidated. In adults, the lymphatic system is stable and does not exhibit lymphangiogenesis [1]. However, lymphatic hyperplasia or dilatation occurs in the inflammatory process. In diabetic animal studies, lymphangiogenesis is present around islet cells, and this new lymphangiogenesis amplifies inflammation [2]. Atherosclerosis is a chronic inflammatory process. Early reports showed that lymphatic vessel density in the adventitia of the aorta increased with the degree of atherosclerotic lesions, but the exact mechanism remains unclear [3,4]. Regulation of lymphangiogenesis can be used as a treatment for inflammation. For example, in diabetes research, inhibition of lymphangiogenesis around islet cells reduces inflammation and preserves islets [2]. Similarly, blocking lymphangiogenesis prevents the spread of local tumor cells [5]. In recent years, researchers have also found that lymphatic vessels not only interfere with the inflammation in atherosclerosis but also participate in reverse transport of plaque cholesterol [6,7].

S1P is a bioactive phospholipid produced by activated platelets, blood cells, and vascular endothelial cells (ECs) [8]. Many physiological functions, such as cell proliferation, differentiation, migration, inflammatory cytokine secretion, and pro-angiogenesis activity, are achieved through highly specific and high affinity binding with S1P G protein-coupled receptors (S1PR1-5) [9–12]. S1P mainly exists in plasma (~1 μM) and lymph (~100 nM), and the concentration gradient of S1P in circulation is very important for regulating lymphocyte migration [13–15]. Most previous studies have linked lymphangiogenesis to the VEGF-C or VEGF-D/VEGFR3 axis, but lymphangiogenesis during inflammation is also controlled by other factors, such as IL-33, pannexin-1, and lysophosphatidic acid [15–17]. S1P is also closely related to angiogenesis during disease progression. In diabetes, advanced glycation end products are produced by chronic hyperglycemia, which can

* Corresponding author.
E-mail address: ghyi6108@163.com (G.-H. Yi).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.intimp.2018.11.032
Received 10 August 2018; Received in revised form 18 November 2018; Accepted 19 November 2018
Available online 23 November 2018

Table 1
Primer sequences for real-time PCR analysis.

Probe	Probe sequence
Human S1PR1	Forward 5′-AAATTCCACCGACCCATGTA-3′ Reverse 5′-AGTTATTGCTCCCGTTGTGG-3′
Human S1PR2	Forward 5′-GCCAATACCTTGCTCTCTGG-3′ Reverse 5′-AGGAGGCTGAAGACAGAGG-3′
Human S1PR3	Forward 5′-TGGTCATCTGCAGCTTCATC-3′ Reverse 5′-CGTCTTCTTGCCAGACATCA-3′
Human β-actin	Forward 5′-TCTACAATGAGCTGCGTGTG-3′ Reverse 5′-ATGGCTGGGGTGTTGAAG-3′

cause diabetic microvascular complications. Advanced glycation end products and hyperglycemia activate sphingosine kinase 1 (Sphk1) and increase the level of S1P in cells [18,19]. It is reported that S1P is closely related to the chronic inflammatory process in atherosclerosis, and S1P also induces activation of endothelial nitric oxide synthase (eNOS) and is responsible for vascular remodeling and angiogenesis [4,20].

This preliminary study confirmed that S1P promotes the secretion of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in HLECs. However, the secretion of inflammatory factors stimulated by S1P is mediated via S1PR1. In addition, using TNF-α siRNA and IL-1β siRNA, we found that TNF-α and IL-1β play essential roles in S1P-induced HLEC proliferation, migration, and tube formation. S1P induces phosphorylation of NF-κB p65 and activation nuclear translocation of NF-κB. A S1PR1 antagonist (W146) and NF-κB inhibitor (BAY11-7082) inhibited S1P-induced TNF-α and IL-1β secretion and prevented NF-κB nuclear translocation. In conclusion, our results suggest that S1P promotes the release of TNF-α and IL-1β through the S1PR1/NF-κB signaling pathway, thus affecting lymphangiogenesis. This study revealed that S1P proinflammatory affects lymphangiogenesis and provides new strategies for the treatment of diabetes, atherosclerosis, and lymphangiogenesis related diseases.

2. Materials and methods

2.1. Reagents and antibodies

DMEM/high glucose medium was obtained from Gibco (CA, USA). Fetal bovine serum (FBS) was obtained from EVERY GREEN (Zhejiang, China). S1P was obtained from Cayman (Ann Arbor, MI, USA). The Fingolimod (FTY720) and NF-κB inhibitor (BAY11-7082) was purchased from Selleck (Houston, TX, USA). The S1PR1 antagonist (W146), S1PR3 antagonist (CAY-10444), and S1PR1/3 antagonist (VPC23019) were purchased from APExBIO (Houston, TX, USA). IL-1β siRNA, TNF-α siRNA, S1PR1 siRNA, S1PR3 siRNA, and p-NF-κB P65 (p-P65) were acquired from Santa Cruz (Delaware Ave, CA, USA). Anti-Podoplanin, anti-TNF-α, anti-S1PR3, anti-NF-κB P65, anti-IL-1β, and anti-p-IκBα antibodies were purchased from Abcam (Catalog nos. ab77854, ab2105, ab108370, ab207297, ab6671, and ab92700; Cambridge, MA, USA). S1PR1, S1PR2, β-actin, and HRP Goat Anti-Rabbit IgG were purchased from Proteintech (Catalog nos. 55,133-1-AP, 21180-1-AP, 20536-1-AP, and SA00009-2; Chicago, IL, USA). Human TNF-α and IL-1β ELISA kits were obtained from Elabscience (Wuhan, Hubei, China). The reagents and antibodies used are of high quality and are commercially available.

2.2. Mice and treatments

Male apoE$^{-/-}$ mice on a C57BL/6 background at 8 weeks of age were purchased from Nanjing CAVENS Biotechnology Co, Ltd. and were housed at a constant temperature of 24–26 °C with 50–60% humidity. Mice were randomized into the following three groups: Normal diet (n = 5), High-fat diet (HFD, n = 8), and HFD + FTY720 (n = 8). The HFD contained 0.5% sodium cholate, 2% cholesterol, 4% whole milk powder, and 10% fatty oil. When the HFD started, FTY720 was administered to apoE$^{-/-}$ mice in the drinking water (10 mg/L for a dose of 1.25 mg/kg/d). After 10 weeks, the mice were sacrificed and the aorta and carotid artery were collected to measure the number of adventitial lymphatic vessels. All animal work was conformed to the Animal Ethics Committee of University of South China and in accordance with the approved guidelines.

2.3. Cell culture

Primary human lymphatic endothelial cells (HLECs) were obtained from PromoCell (Heidelberg, Germany). HLECs were cultured in DMEM supplemented with 10% FBS (37 °C, 5% CO_2), and fresh medium was changed daily. Cells were used for experiments between passage 3 and 8. The HLECs were starved for 10 h in DMEM containing 1% FBS before treatment. Then, treatment with an antagonist for 30 min or siRNA was treated for 24 h. The corresponding concentration of S1P was then added and finally the cells and medium were harvested for Western blotting, Immunofluorescence, ELISA, and RT-PCR analyses.

2.4. Cell proliferation, migration, and tube formation assays

For HLEC proliferation assays, cells were collected, and 100 μl of cell suspension was added to each well of a 96-well plate. The plates were placed in an incubator (5% CO_2, 37 °C) for 24 h until the cells were spread over the bottom of the wells. Then, various test substances were added to the culture plate and incubated with the cells in an incubator for 24 h. Finally, 10 μl of Cell Counting Kit-8 (Solarbio, Beijing, China) solution was added to each well, and the plate was incubated for 4 h. Then, the absorbance was measured at 490 nm with a microplate reader.

Scratch wounds were produced on a single-layer HLEC surface at 100% confluency. Images of the HLEC migration process were captured at 0 and 24 h using a fluorescence microscope (IX70; Olympus, Tokyo) [21]. The scratch wound area was calculated using ImageJ software version 1.38.

For the tube formation assay, 100 μl of Matrigel (BD Biosciences) was pipetted into a 96-well plate, and then, the plate was placed in an incubator for 30 min. Cells were treated with different factors and then collected into a suspension. Finally, approximately 6×10^3 HLECs in 100 μl of DMEM were placed on the Matrigel layer in each well. After incubation at 37 °C for 12 h, tube formation was observed using phase contrast microscopy.

2.5. Blocking S1P receptors and NF-κB

When the HLEC confluence is about 70%, the cells were pretreated with S1P antagonist or NF-kB inhibitor for 30 min, then the corresponding concentration of S1P was added. Finally, cells and supernatants were collected for Western blot and ELISA analysis.

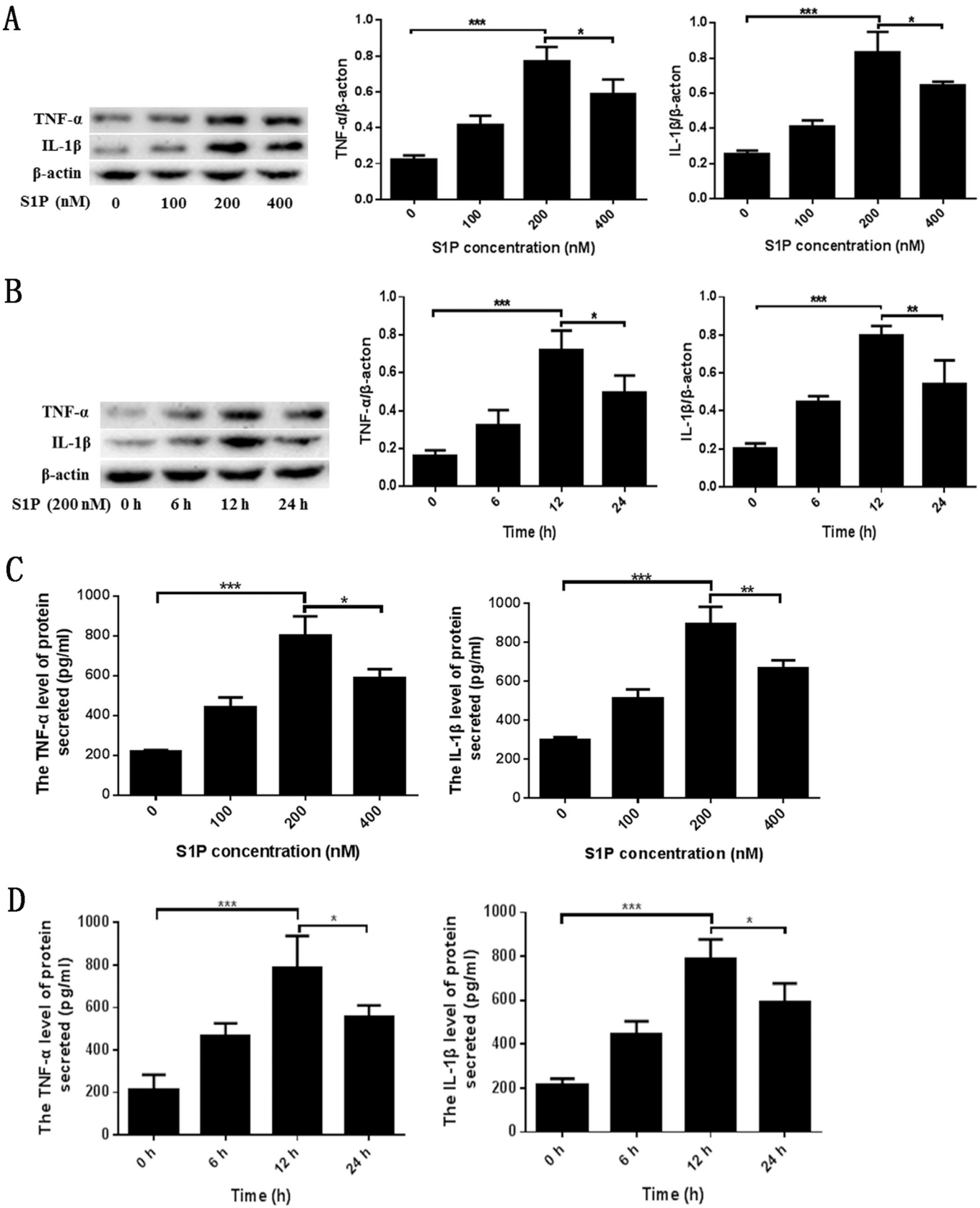


Fig. 1. S1P promotes the secretion of TNF-α and IL-1β in HLECs. (A) HLECs were incubated with different concentrations of S1P (0, 100, 200, or 400 nM) for 24 h. TNF-α and IL-1β protein levels were detected by Western blot. (B) HLECs were cocultured with S1P (200 nM) for 0, 6, 12, or 24 h, and the levels of TNF-α and IL-1β were detected by Western blotting. (C) HLECs were incubated with S1P (0, 100, 200, or 400 nM) for 12 h, and then, TNF-α and IL-1β secretion levels were analyzed via ELISA. (D) HLECs were incubated with S1P (200 nM) for 0, 6, 12, or 24 h, and then, TNF-α and IL-1β secretion levels were analyzed via ELISA. *P < 0. 05, **P < 0.01, and ***P < 0.001; n = 5.

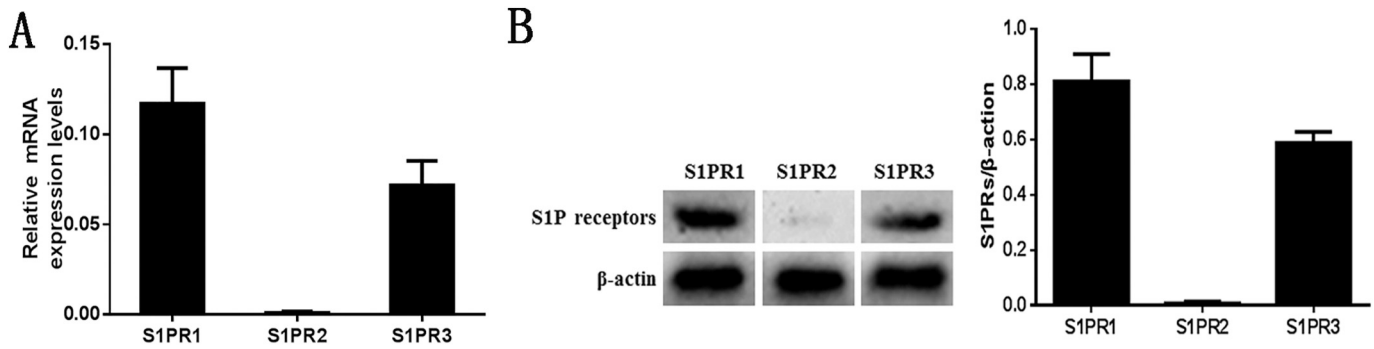


Fig. 2. The expression levels of three S1P receptors in HLECs. (A) The expression of S1PR1, S1PR2, and S1PR3 in HLECs was measured by RT-PCR. (B) The expression of S1PR1, S1PR2, and S1PR3 in HLECs was analyzed by Western blotting.

2.6. Cell transfection with siRNA and Real-time PCR assay

When the cell confluence reached 30%, non-targeted control or targeted siRNAs were transfected into HLECs using DharmaFECT 1 Transfection Reagent (Baomanbio, Shanghai, China). The GFP-reporter plasmid detects the efficiency of siRNA transfection. The effects of S1P on HLECs were observed 24 h after siRNA transfection.

Total RNA from HLECs was extracted using Trizol reagent (Thermo Fisher Scientific, MA, USA) according to the reagent product instructions. 1 μg of total RNA was reverse transcribed using a GoldScript one-step RT-PCR kit (Invitrogen, CA, USA), and the obtained cDNA was used as a PCR template, and mRNA expression level was measured using a quantitative PCR kit (Thermo Fisher Scientific, MA, USA). β-Actin served as a reference. Design primers are shown in Table 1.

2.7. Western blot analysis

Cytoplasmic proteins and nuclear proteins were extracted from HLECs according to the instructions provided in NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, MA, USA). The protein concentration in the samples was calculated using a BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) prior to Western blotting. The proteins to be tested were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Sigma-Aldrich, MO, USA). The PVDF membranes were blocked with 5% nonfat milk for 3 h and the membranes were incubated with the corresponding antibody overnight at 4 °C. The PVDF membranes were incubated with goat anti-rabbit lgG for 2 h at room temperature. Target proteins were visualized using an eECL Western Blot Kit (CWBIO, Beijing, China) with an Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE). Finally, the optical density scan of ImageJ software version 1.38 image processing software was used for quantification.

2.8. ELISA

The supernatant of HLECs were harvested after treatment and used in ELISA experiment. Inflammatory cytokines were detected using ELISA kits (Elabscience, Hubei, China) according to the manufacturer's instructions.

2.9. Immunohistochemistry

Immunohistochemical detection of the lymphatic vessel marker podoplanin (D2–40) in frozen mouse aorta sections was carried out according to the immunohistochemical reagent specifications (CWBIO, Beijing, China).

2.10. Immunofluorescence

After washing the treated HLECs with phosphate-buffered saline (PBS), they were fixed with 4% paraformaldehyde for 20 min and washed 3 times with PBS. Then the cells were permeated with 0.1% Triton X-100 (Thermo Fisher Scientific, MA, USA) for 20 min, and the non-specific antigen-binding sites were blocked with 10% BSA for 1 h. HLECs were incubated with anti-NF-κB antibody overnight at 4 °C, then the cells were washed 3 times with PBS. Cells were incubated with CY3-conjugated AffiniPure goat anti-rabbit lgG for 1 h at room temperature. After the cells were washed with PBS, the nuclei were counterstained with DAPI solution (Thermo Fisher Scientific, MA, USA) for 15 min. The cells were washed with PBS and the results were analyzed under a fluorescence microscope (IX70; Olympus, Tokyo).

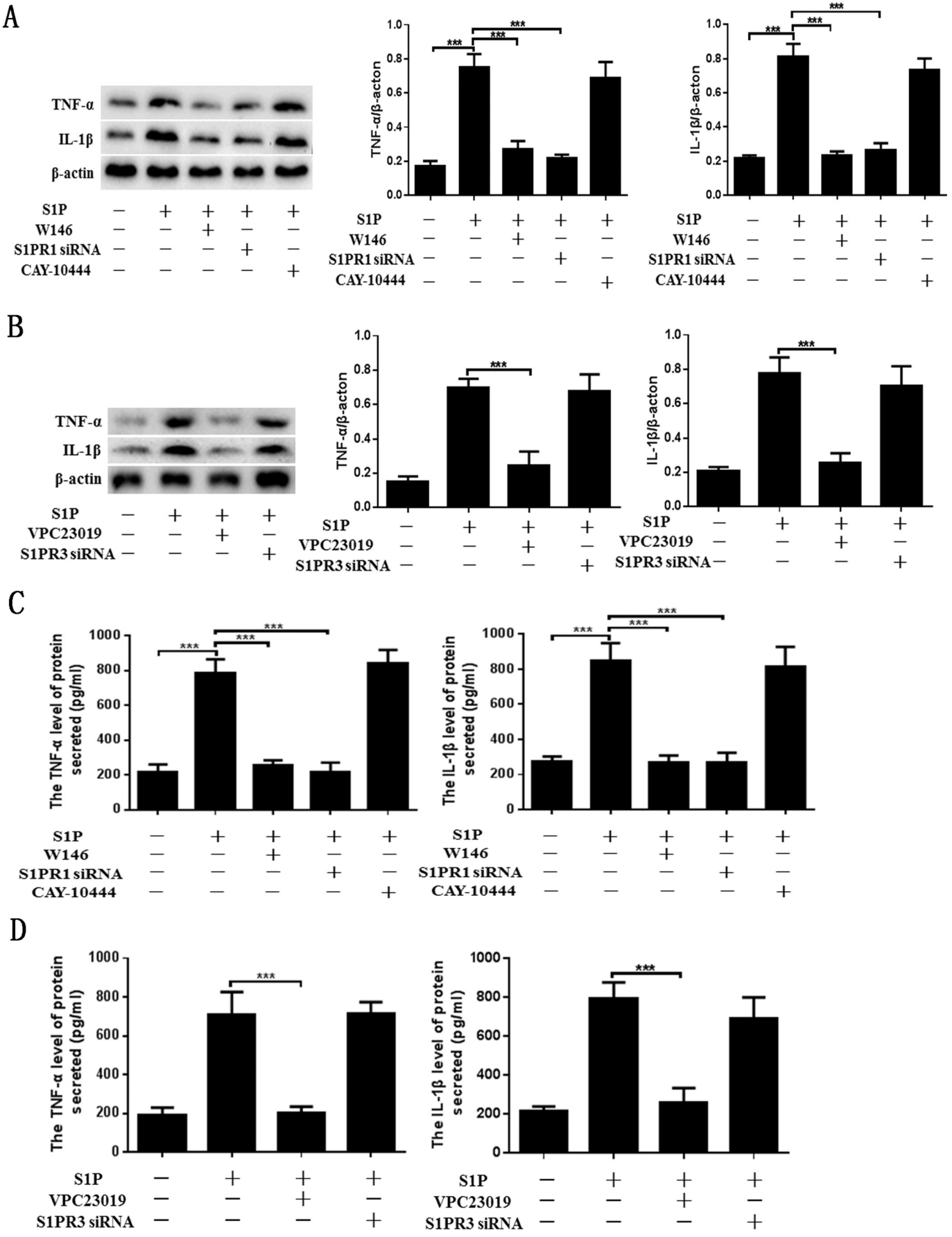
2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 6.02 (San Diego, CA). The data are reported as the mean ± SD. Analysis of differences between groups were performed using one-way ANOVA and Tukey. A $P < 0.05$ was considered significant.

3. Results

3.1. S1P promotes TNF-α and IL-1β secretion in HLECs

S1P is a biologically active lipid closely related to inflammation [19,20]. To investigate the inflammatory effects of S1P on HLECs, cells were starved for 10 h and then incubated with different concentrations of S1P (0, 100, 200, or 400 nM) for 24 h. ELISA kits were used to measure the secretion of TNF-α and IL-1β. In addition, the protein levels of TNF-α and IL-1β were determined by Western blotting. As shown in Fig. 1 AC, S1P promoted an increase in the protein levels of TNF-α and IL-1β, and the maximum effect was observed at an S1P



(caption on next page)

Fig. 3. S1P-induced release of TNF-α and IL-1β from HLECs occurs through an S1PR1-mediated mechanism. (A) HLECs were treated with W146 (Ki = 18 nM), S1PR1 siRNA, or CAY-10444 (Ki = 10 μM) and then incubated with S1P or blank vector for 12 h. The levels of TNF-α and IL-1β were detected via Western blot. (B) HLECs were treated with VPC23019 (EC50 = 13 nM) or S1PR3 siRNA, TNF-α and IL-1β levels were detected via Western blot. (C–D) After treatment with different inhibitors and siRNA, the HLEC secretion levels of TNF-α and IL-1β in culture supernatants were analyzed via ELISA. Representative data from at least 3 independent experiments are shown. *P < 0.05, **P < 0.01, and ***P < 0.001.

concentration of 200 nM. Subsequently, HLECs were treated with S1P (200 nM) for 0, 6, 12, and 24 h to determine whether S1P promoted TNF-α and IL-1β release in a time-dependent manner (Fig. 1BD). The results suggested that S1P promotes the secretion of TNF-α and IL-1β in HLECs in a dose- and time-dependent manner. Therefore, in the following experiments, we used 200 nM S1P treatment for 12 h to determine the effect on TNF-α and IL-1β release from HLECs.

3.2. S1P induced the secretion of TNF-α and IL-1β from HLECs through S1PR1 binding

Studies have shown that S1P receptors (S1PR1-5) are distributed differently in vivo, S1PR1-3 are expressed in many tissues, while S1PR4/S1PR5 are mainly expressed in the immune system and central nervous system [22]. To investigate the mechanism by which S1P promotes the secretion of inflammatory factors in HLECs, we performed RT-PCR and Western blotting to detect the expression pattern of S1P receptors (S1PR1-3) in HLECs. The results showed that HLECs mainly expressed S1PR1 and S1PR3 but rarely expressed S1PR2 (Fig. 2AB).

Next, we explored whether S1P promotes the secretion of inflammatory factors in HLECs through binding to S1P receptors. Pretreatment of HLECs with W146 (S1PR1 antagonist) and VPC23019 (S1PR1/S1PR3 antagonist) significantly inhibited S1P-induced TNF-α and IL-1β release, but the release of TNF-α and IL-1β was not significantly changed by CAY-10444 (S1PR3 antagonist), as shown in Fig. 3. S1PR1 siRNA also inhibited S1P-induced secretion of TNF-α and IL-1β from HLECs (Fig. 3AC). These results suggest that the secretion of TNF-α and IL-1β from HLECs induced by S1P is mediated by S1PR1.

3.3. TNF-α and IL-1β play an essential role in S1P-induced tube formation by HLECs

Inflammatory factors are closely involved in lymphangiogenesis. Studies have shown that IL-1β overexpression in mouse airways promotes lymphangiogenesis [23], and abnormal expression of TNF-α has also been found during lymphangiectasis [24]. To explore the significance of S1P-induced upregulation of TNF-α and IL-1β in HLECs, we used siRNA to silence the expression of TNF-α and IL-1β in HLECs. HLECs were transfected with a GFP reporter plasmid, and the efficiency of transfection with siRNA was detected by GFP fluorescence, which was found to be 90%. ELISA showed that TNF-α siRNA and IL-1β siRNA inhibited the expression of endogenous TNF-α and IL-1β at the protein level in HLECs induced by S1P. The results indicate that TNF-α siRNA and IL-1β siRNA partially inhibited S1P-induced HLEC proliferation (Fig. 4AB), migration (Fig. 4C), and tube formation (Fig. 4D) compared with control cells transfected with a non-targeted siRNA. Altogether, these data support a significant functional role for TNF-α and IL-1β in S1P-induced tube formation by HLECs.

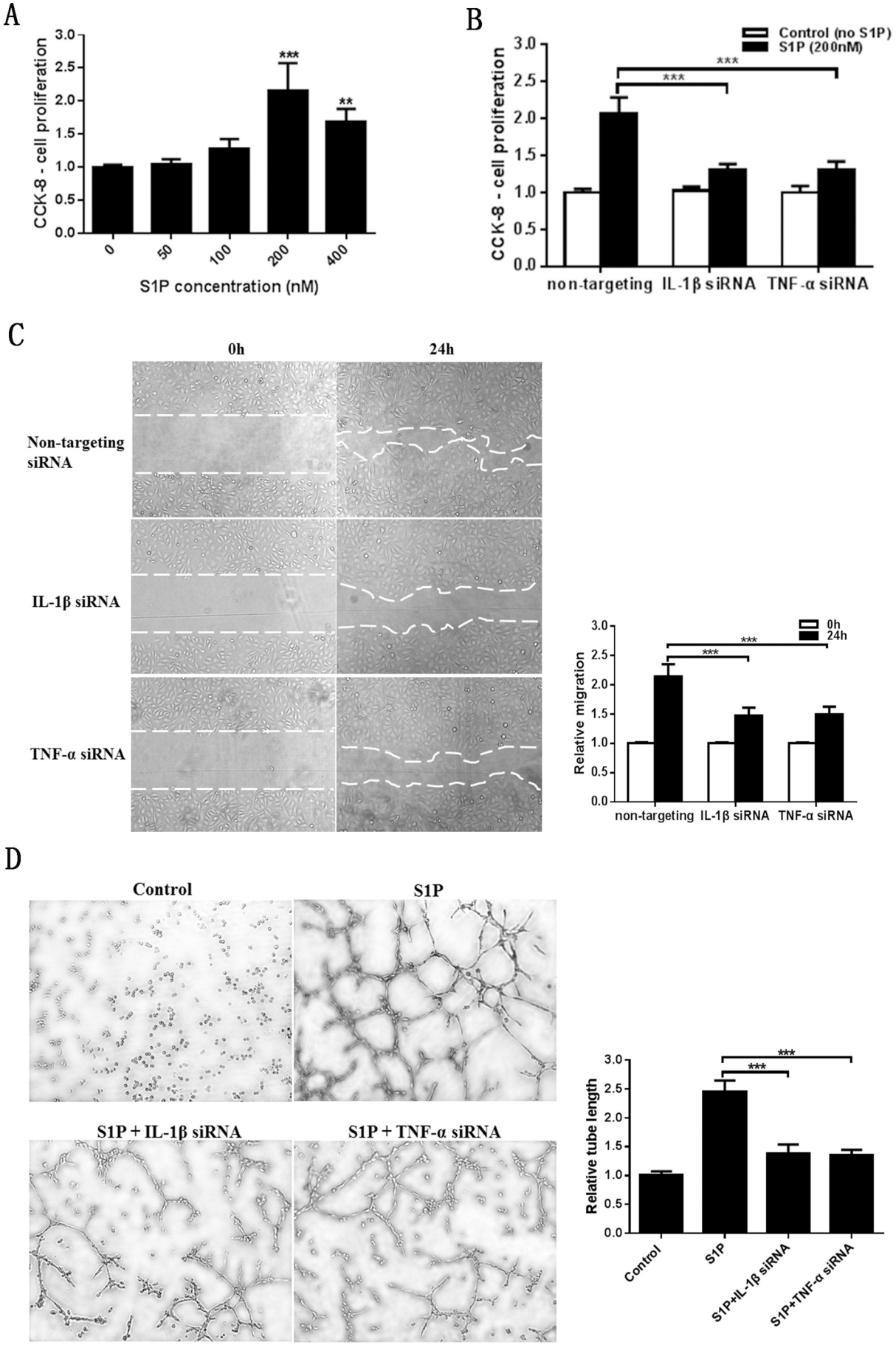
3.4. NF-κB is the key to S1P/S1PR1 proinflammation-induced HLEC tube formation

It has been shown that NF-κB plays an important role in angiogenesis and the secretion of proinflammatory cytokines [25,26]. Therefore, we evaluated whether the NF-κB signaling pathway is involved in the S1P/S1PR1-mediated proinflammatory effects on HLECs. HLECs were incubated with different concentrations of S1P for 12 h. Then, Western blotting was performed to quantitate IκBα, P65, p-IκBα, and p-P65 protein levels. The nuclear translocation of NF-κB was detected by Immunofluorescence. As shown in Fig. 5A, S1P significantly increased IκBα and P65 phosphorylation levels compared with the control group. Additionally, W146 and S1PR1 siRNA blocked phosphorylation of P65 in S1P-treated cells (Fig. 5B). These results suggest that S1P promotion phosphorylation of P65 is mediated through S1PR1. To further verify whether the NF-κB pathway is involved in the release of TNF-α and IL-1β, we used W146 and BAY11-7082 to treat HLECs and detect the secretion levels of TNF-α and IL-1β. The results showed that the secretion of TNF-α and IL-1β from HLECs was significantly lower than that from control group (Fig. 5CD), and the nuclear translocation of NF-κB was also decreased (Fig. 5E).

Subsequently, to further demonstrate that S1P promotes inflammation-induced HLEC migration and tube formation via the S1PR1/NF-κB pathway, we treated cells with an W146 or BAY11-7082 and found that S1P-induced HLEC migration and tube formation were inhibited (Fig. 6AB). Taken together, these data suggest that NF-κB plays an indispensable role in S1P proinflammation-induced lymphangiogenesis.

3.5. S1P affects lymphangiogenesis in the adventitia of atherosclerotic lesions in apoE$^{-/-}$ mice

Since S1P was found to stimulate HLEC tube formation in vitro, we performed in vivo studies to determine the activity of S1P in lymphangiogenesis. We fed a high-fat diet of apoE$^{-/-}$ mice with oral or non-oral FTY720 (1.25 mg/kg/d). FTY720 is an analog of S1P that competes with S1P for binding to S1PR1 and thus interferes with S1PR1 signaling. After 10 weeks, the mice were sacrificed, the aorta were removed for Immunohistochemical analysis. The results showed that the number of adventitial lymphatic vessels in the aorta was significantly lower in the treatment group than in the HFD group (Fig. 7AB). These findings indicate that S1P is an effective lymphangiogenesis lipid molecule in vivo.



(caption on next page)

Fig. 4. TNF-α and IL-1β play an important role in S1P-induced HLEC tube formation. (A-B) TNF-α siRNA or IL-1β siRNA partially blocked S1P-induced proliferation of HLECs, as demonstrated with a CCK-8 assay at 24 h (*P < 0.01 and **P < 0.001 vs. the non-S1P-treated controls). (C) Transfection with TNF-α siRNA or IL-1β siRNA partially blocked S1P-induced HLEC migration at 24 h. (D) TNF-α siRNA or IL-1β siRNA partially blocked S1P-induced tube formation by HLECs on Matrigel at 12 h, Original magnification: X 20 (C); X 10 (D). Scale bars 100 μm. The data are expressed as the means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

4. Discussion

Inflammatory factors are closely related to lymphangiogenesis [1,7]. In this study, we found that S1P promotes the release of TNF-α and IL-1β in HLECs. HLECs mainly express the S1PR1 and S1PR3 receptors, and S1P promotes the release of TNF-α and IL-1β through S1PR1. However, previous studies have not shown a proinflammatory effect of S1P in lymphangiogenesis. By transfecting cells with TNF-α siRNA and IL-1β siRNA, we found that TNF-α and IL-1β play important roles in the proliferation, migration, and tube formation of HLECs. Finally, we found that S1P proinflammatory-induced lymphangiogenesis is achieved through the S1PR1/NF-κB pathway.

Since the identification of S1P as a functional ligand for S1P receptors, the immunomodulatory and inflammatory effects of S1P have been well studied [27]. In the process of inflammation associated diseases, the level of S1P was changed in different immune environments, resulting in significant changes in cell behavior. For example, spondylarthritis (SpA) is a chronic inflammatory joint disease, and Bougault et al. [28] showed a significant increase in serum S1P levels in SpA patients and that the metabolic pathway of S1P is involved in the mineralization capacity of osteoblasts and chondrocytes, possibly providing a therapeutic target for SpA. Keul et al. [29] used the S1P analog FTY720 to treat apoE$^{-/-}$ mice with atherosclerotic lesions and found that aortic plaques in the treated group were reduced compared with those in the control group. S1P is closely associated with inflammatory disease, but it is unclear whether S1P plays a role in inflammation-related lymphangiogenesis.

Because the concentration of S1P in normal lymph is ~100 nM, we treated HLECs with different concentrations of S1P and detected the levels of the inflammatory factors TNF-α and IL-1β using Western blotting and ELISA, and found that the levels of TNF-α and IL-1β were significantly elevated (Fig. 1). Many cell physiological functions are mediated by S1P and highly specific S1P receptors. Therefore, we detected the expression pattern of S1PR1, S1PR2, and S1PR3 in HLECs. We found that HLECs mainly expressed S1PR1 and S1PR3 (Fig. 2AB), which was consistent with the results of Yoon et al. [30]. To explore the mechanism by which S1P promotes the secretion of TNF-α and IL-1β in HLECs, we used S1P receptor-specific antagonists to treat cells and found that S1PR1 antagonists but not S1PR3 antagonists reduced S1P-induced TNF-α and IL-1β secretion levels (Fig. 3), and transfection with S1PR1 siRNA produced similar results (Fig. 3AC). These results suggest that the secretion of TNF-α and IL-1β by HLECs induced by S1P is mediated via S1PR1.

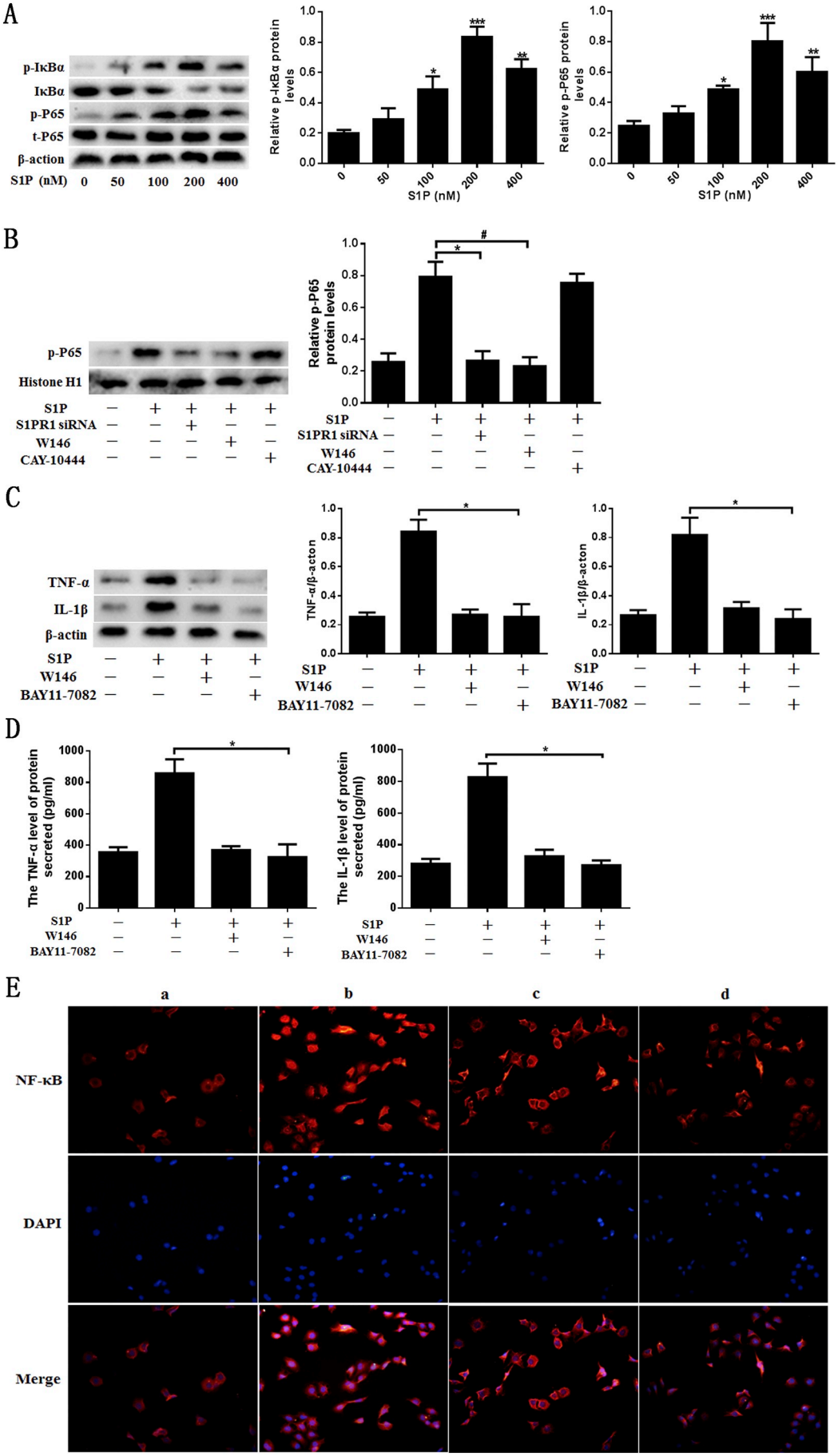
Studies have shown that IL-1β overexpression in mouse airways promotes lymphangiogenesis [23], and abnormal expression of TNF-α has been observed during lymphangiectasis [24]. Therefore, we determined the role of these inflammatory factors in HLEC proliferation, migration, and tube formation by transfecting HLECs with TNF-α siRNA and IL-1β siRNA. The results showed that TNF-α and IL-1β play an indispensable role in tube formation by S1P-treated HLECs (Fig. 4). Previous studies have shown that S1P has a potential pro-angiogenesis role [31], and thus, we supplemented the effect of S1P on EC tube formation. We found that S1P also promoted tube formation by ECs, but when S1PR1 was blocked, we found that the tube formation by ECs was not completely blocked, and thus other S1P receptors may be involved (Supplementary Fig. 1).

NF-κB controls the expression of proinflammatory cytokines, growth factors, adhesion molecules, and inducible enzymes involved in cell proliferation, apoptosis, inflammation, and tumor microangiogenesis [32]. Aspirin inhibits components of lymphangiogenesis and lymphatic vessel remodeling by inhibiting the NF-κB pathway in HLECs [33]. Therefore, we investigated the effect of S1P on NF-κB pathway and found that S1P significantly increased the phosphorylation levels of IκBα and P65 (Fig. 5A). In addition, the phosphorylation level of p65 in HLECs induced by S1P was decreased by W146 or S1PR1 siRNA (Fig. 5B). To investigate whether inflammatory factor secretion are associated with NF-κB, we used NF-κB inhibitors and found that TNF-α and IL-1β secretion was reduced in S1P-treated HLECs (Fig. 5CD). Similarly, S1P-induced nuclear translocation of NF-κB was blocked by NF-κB inhibitors, as shown in Fig. 5E. To further confirm that NF-κB drives S1P-induced TNF-α and IL-1β release, affecting lymphangiogenesis, we applied NF-κB inhibitors and found that S1P promotes HLEC migration and tube formation through the S1PR1/NF-κB pathway (Fig. 6AB).

S1P is closely related to chronic inflammatory lesions of atherosclerosis, in order to investigate whether S1P has an effect on adventitial lymphangiogenesis. We interfere with S1P receptors signaling by FTY720, a drug approved by the U.S. Food and Drug Administration. FTY720 is an analog of S1P that competes with S1P for binding to S1PR1 and thus interferes with S1PR1 signaling [34]. We used FTY720 to feed apoE$^{-/-}$ mice and found that the number of adventitial lymphatic vessels in aortas from the treatment group was significantly lower than that in aortas from the HFD group (Fig. 7). The results indicate that S1P plays a certain role in the formation of adventitial lymphatic vessels in atherosclerotic lesions. However, we have not found a more suitable method for the detection of inflammatory factors in the adventitia region, which may be the content of our next research.

Overall, the data presented here reveal a link between S1P, S1PR1, NF-κB, IL-1β, TNF-α, and lymphangiogenesis, suggesting that S1P may be a therapeutic target for the intervention of lymphangiogenesis under inflammatory conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.032.



(caption on next page)

Fig. 5. Effect of S1P on NF-κB phosphorylation in HLECs. (A) HLECs were treated with different concentrations of S1P, and the relative levels of IκBα, p-IκBα, P65, and p-P65 were determined by Western blot, (*P < 0.05, **P < 0.01, and ***P < 0.001 vs. the Control group). (B) Prior to pretreatment of HLECs with S1P, cells were treated with an W146, CAY-10444, or S1PR1 siRNA. The protein levels of p-P65 and Histone H1 were examined by Western blot. (C-D) The levels of TNF-α and IL-1β were measured by Western blot and ELISA. (E) Immunofluorescence assays were used to detect the effect of S1P on nuclear translocation of NF-κB p65 protein in HLECs. **a** Control group; **b** S1P group; **c** S1P + W146 group; **d** S1P + BAY11-7082 group. NF-κB stain red; DAPI stain blue; the merged image indicates colocalization of NF-κB and DAPI. Original magnification: X 20 (E). Scale bar = 100 μm. The values represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

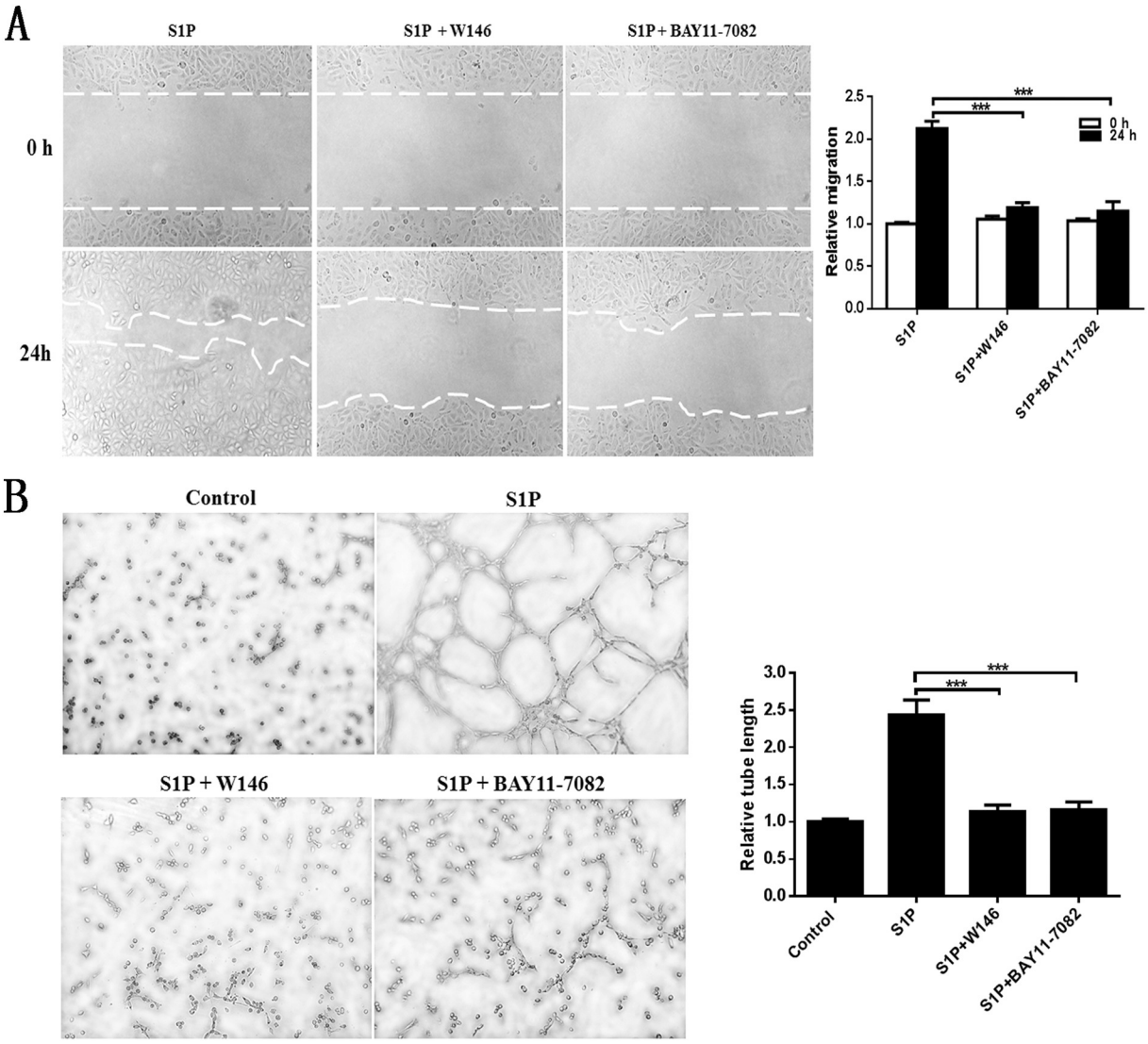


Fig. 6. Effects of NF-κB inhibitors on HLEC tube formation induced by S1P. (A) W146 or BAY11-7082 treatment of HLECs blocked S1P-induced HLEC migration. (B) W146 or BAY11-7082 blocked S1P-induced HLEC tube formation on Matrigel. Cell migration and tube formation were observed using a phase contrast microscope. Original magnification: X 20 (A); X 10 (B). Scale bars 100 μm. *P < 0.05, **P < 0.01, and ***P < 0.001.

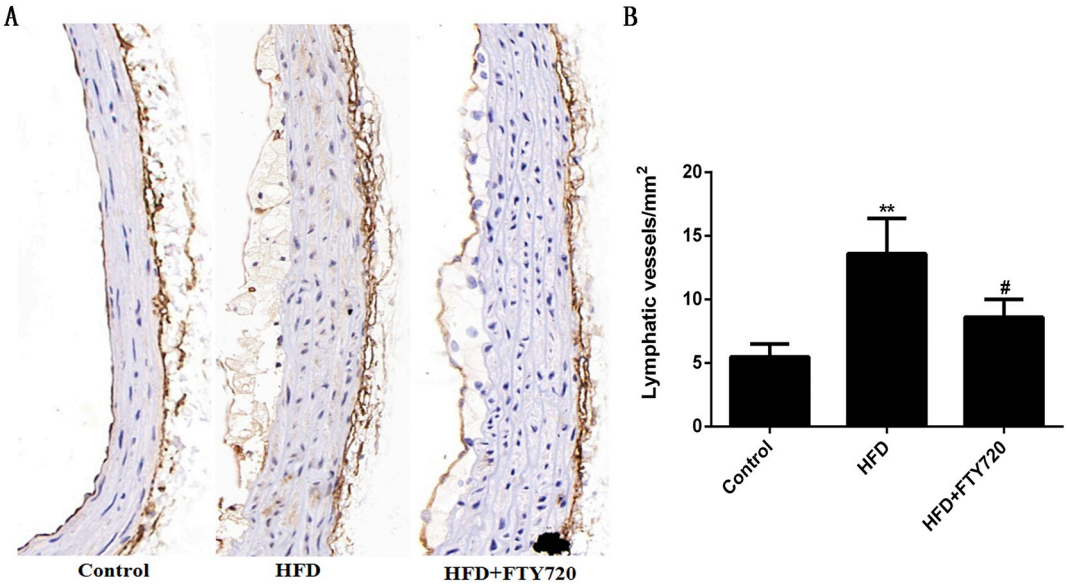


Fig. 7. Effect of the S1P analog FTY720 on the number of aortic adventitial lymphatic vessels in apoE$^{-/-}$ mice. (A–B) Immunohistochemical staining of podoplanin^{+} (D2–40) lymph capillaries (brown) in the adventitia of apoE$^{-/-}$ mice aortic atherosclerotic lesions. Original magnification: X 20. Scale bars 50 μm. **P < 0.01 vs. the Control group. $^{\#}$P < 0.05 vs. the HFD group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

The study was fiscally supported by the National Natural Science Foundation of China (grant nos. 81770490) and the Construct Program of the Key Discipline in Hunan Province (Basic Medicine Sciences in University of South China).

Conflict of interest

The authors declare no conflict of interests.

References

[1] L.C. Dieterich, C.D. Seidel, Lymphatic vessels: new targets for the treatment of inflammatory diseases, Angiogenesis 17 (2014) 359–371.
[2] J.P. Scallan, M.A. Hill, Lymphatic vascular integrity is disrupted in type 2 diabetes due to impaired nitric oxide signaling, Cardiovasc. Res. 107 (2015) 89–97.
[3] K. Alitalo, T. Tammela, T.V. Petrova, Lymphangiogenesis in development and human disease, Nature 438 (2005) 946–953.
[4] G.M. Lemole, The role of lymphstasis in atherogenesis, Ann. Thorac. Surg. 31 (1981) 290–293.
[5] R. Paduch, The role of lymphangiogenesis and angiogenesis in tumor metastasis, Cell. Oncol. (Dordr.) 39 (5) (2016) 397–410.
[6] C. Martel, G.J. Randolph, Atherosclerosis and transit of HDL through the lymphatic vasculature, Curr. Atheroscler. Rep. 15 (2013) 354.
[7] H. Kim, R.P. Kataru, G.Y. Koh, Inflammation-associated lymphangiogenesis: a double-edged sword? J. Clin. Invest. 124 (2014) 936–942.
[8] F. Tukijan, M. Chandrakanthan, The signalling roles of sphingosine-1-phosphate derived from red blood cells and platelets, Br. J. Pharmacol. 175 (2018) 3741–3746.
[9] S. Chawla, S. Saxena, Differential modulation of S1PR(1-5) and specific activities of SphK and nSMase in pulmonary and cerebral tissues of rats exposed to hypobaric hypoxia, Lipids 50 (2015) 39–48.
[10] V. Limaye, P. Xia, C. Hahn, M. Smith, M.A. Vadas, S.M. Pitson, J.R. Gamble, Chronic increases in sphingosine kinase-1 activity induce a proinflammatory, pro-angiogenic phenotype in endothelial cells, Cell. Mol. Biol. Lett. 14 (2009) 424–441.
[11] S. Ito, S. Iwaki, R. Kondo, M. Satoh, K. Iwabuchi, R. Ohkawa, Y. Mishima, Y. Yatomi, T. Furumoto, H. Tsutsui, S. Fujii, TNF-α production in NKT cell hybridoma is regulated by sphingosine-1-phosphate: implications for inflammation in atherosclerosis, Coron. Artery Dis. 25 (4) (2014) 311–320.
[12] S.N. Syed, M. Jung, A. Weigert, S1P provokes tumor lymphangiogenesis via macrophage-derived mediators such as IL-1 or lipocalin-2, Mediat. Inflamm. 2017 (2017) 7510496.
[13] E. Moritz, D. Wegner, S. Groß, M. Bahls, M. Dörr, S.B. Felix, T. Ittermann, S. Oswald, M. Nauck, N. Friedrich, R.H. Böger, G. Daum, Reference intervals for serum sphingosine-1-phosphate in the population-based study of health in pomerania, Clin. Chim. Acta 468 (2017) 25–31.
[14] S. Fukuhara, S. Simmons, S. Kawamura, A. Inoue, Y. Orba, T. Tokudome, Y. Sunden, Y. Arai, K. Moriwaki, J. Ishida, A. Uemura, H. Kiyonari, T. Abe, A. Fukamizu, M. Hirashima, H. Sawa, J. Aoki, M. Ishii, N. Mochizuki, The sphingosine-1-phosphate transporter spns2 expressed on endothelial cells regulates lymphocyte trafficking in mice, J. Clin. Invest. 122 (2012) 1416–1426.
[15] L. Han, M. Zhang, X. Liang, Interleukin-33 promotes inflammation-induced lymphangiogenesis via ST2/TRAF6-mediated Akt/eNOS/NO signalling pathway, Sci. Rep. 7 (1) (2017) 10602.
[16] J. Boucher, C. Simonneau, G. Denet, J. Clarhaut, A.C. Balandre, M. Mesnil, L. Cronier, A. Monvoisin, Pannexin-1 in human lymphatic endothelial cells regulates lymphangiogenesis, Int. J. Mol. Sci. 19 (6) (2018).
[17] H. Mu, T.L. Calderone, M.A. Davies, V.G. Prieto, H. Wang, G.B. Mills, M. Bar-Eli, J.E. Gershenwald, Lysophosphatidic acid induces lymphangiogenesis and IL-8 production in vitro in human lymphatic endothelial cells, Am. J. Pathol. 180 (5) (2012) 2170–2181.
[18] K. Geoffroy, L. Troncy, N. Wiernsperger, M. Lagarde, S.E. Bawab, Glomerular proliferation during early stages of diabetic nephropathy is associated with local increase of sphingosine-1-phosphate levels, FEBS Lett. 579 (2005) 1249–1254.
[19] L. Wang, X.P. Xing, A. Holmes, C. Wadham, J.R. Gamble, M.A. Vadas, P. Xia, Activation of the sphingosine kinase-signaling pathway by high glucose mediates the proinflammatory phenotype of endothelial cells, Circ. Res. 97 (9) (2005) 891–899.
[20] C. Barthomeuf, Inhibition of S1P-induced angiogenesis, metastasis and inflammation by dietary polyphenols, Free Radic. Biol. Med. 42 (2007) 312–313.
[21] H. Mu, R. Ohashi, H. Yang, X. Wang, M. Li, P. Lin, Q. Yao, C. Chen, Thymosin beta10 inhibits cell migration and capillary-like tube formation of human coronary artery endothelial cells, Cell Motil. Cytoskeleton 63 (2006) 222–230.
[22] A. Vestri, F. Pierucci, A. Frati, L. Monaco, E. Meacci, Sphingosine 1-phosphate receptors: do they have a therapeutic potential in cardiac fibrosis? Front. Pharmacol. 8 (2017) 296.
[23] P. Baluk, A. Hogmalm, M. Bry, K. Alitalo, K. Bry, D.M. McDonald, Transgenic overexpression of interleukin-1β induces persistent lymphangiogenesis but not angiogenesis in mouse airways, Am. J. Pathol. 182 (2013) 1434–1447.
[24] H. Hong, L. Jiang, Y. Lin, C. He, G. Zhu, Q. Du, X. Wang, F. She, Y. Chen, TNF-alpha promotes lymphangiogenesis and lymphatic metastasis of gallbladder cancer through the ERK1/2/AP-1/VEGF-D pathway, BMC Cancer 16 (2016) 240.
[25] T. MaruYama, The nuclear IκB family of proteins controls gene regulation and immune homeostasis, Int. Immunopharmacol. 28 (2015) 836–840.
[26] Z.B. Xia, F.R. Meng, Y.X. Fang, X. Wu, C.W. Zhang, Y. Liu, D. Liu, G.Q. Li, F.B. Feng, H.Y. Qiu, Inhibition of NF-κB signaling pathway induces apoptosis and suppresses proliferation and angiogenesis of human fibroblast-like synovial cells in rheumatoid arthritis, Medicine (Baltimore) 97 (2018) e10920.
[27] M. Nagahashi, A. Yamada, E. Katsuta, T. Aoyagi, W.C. Huang, K.P. Terracina, N.C. Hait, J.C. Allegood, J. Tsuchida, K. Yuza, M. Nakajima, Targeting the Sphk1/S1P/S1PR1 axis that links obesity, chronic inflammation, and breast cancer metastasis, Cancer Res. 78 (7) (2018) 1713–1725.
[28] C. Bougault, A. El Jamal, A. Briolay, S. Mebarek, M.A. Boutet, T. Garraud, B. Le Goff, F. Blanchard, D. Magne, L. Brizuela, Involvement of sphingosine kinase/sphingosine 1-phosphate metabolic pathway in spondyloarthritis, Bone 103 (2017) 150–158.
[29] P. Keul, M. Tölle, S. Lucke, K. von Wnuck Lipinski, G. Heusch, M. Schuchardt, M. van der Giet, B. Levkau, The sphingosine-1-phosphate analogue FTY720 reduces atherosclerosis in apolipoprotein E-deficient mice, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 607–613.
[30] C.M. Yoon, B.S. Hong, H.G. Moon, S. Lim, P.G. Suh, Y.K. Kim, C.B. Chae, Y.S. Gho, Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/

PLC/Ca2+ signaling pathways, Blood 112 (2008) 1129–1138.
[31] M. Nagahashi, S. Ramachandran, E.Y. Kim, J.C. Allegood, O.M. Rashid, A. Yamada, R. Zhao, S. Milstien, H. Zhou, S. Spiegel, K. Takabe, Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis, Cancer Res. 72 (2012) 726–735.
[32] B. Miraghazadeh, M.C. Cook, Nuclear factor-kappab in autoimmunity: man and mouse, Front. Immunol. 9 (2018) 613.
[33] O. Prangsaengtong, P. Jantaree, K. Lirdprapamongkol, L. Ngiwsara, J. Svasti, K. Koizumi, Aspirin suppresses components of lymphangiogenesis and lymphatic vessel remodeling by inhibiting the NF-κB/VCAM-1 pathway in human lymphatic endothelial cells, Vasc. Med. 23 (3) (2018) 201–211.
[34] S. Zhao, M.G. Adebiyi, Y. Zhang, J.P. Couturier, X. Fan, H. Zhang, R.E. Kellems, D.E. Lewis, Y. Xia, Sphingosine-1-phosphate receptor 1 mediates elevated IL-6 signaling to promote chronic inflammation and multitissue damage in sickle cell disease, FASEB J. 32 (2018) 2855–2865.