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Sphingosine-1-phosphate receptor-2 mediated NF κ B activation contributes to tumor necrosis factor- α induced VCAM-1 and ICAM-1 expression in endothelial cells

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Abstract

Sphingosine-1-phosphate (S1P) regulates a wide array of biological functions in endothelial cells. We previously showed that S1P receptor subtype 2 (S1P₂) is significantly up-regulated in the atherosclerotic endothelium (*J. Biol. Chem.* 283:30363, 2008). In this study, we investigated the roles of S1P₂-mediated signaling in the proinflammatory responses of endothelial cells. Treatment with tumor necrosis factor- α (TNF α), a proinflammatory cytokine, increased the expression of S1P₂ receptors in endothelial cells. TNF α treatment also enhanced sphingosine kinase 1 expression and increased S1P production. Pharmacological inhibition or knockdown of S1P₂ receptors completely abrogated the TNF α -induced VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) expression in endothelial cells. In contrast, pharmacological inhibition or knockdown of other S1P receptor subtypes had no effect on the TNF α -stimulated ICAM-1 and VCAM-1 expression. Moreover, ectopic expression of S1P₂ receptors increased VCAM-1 and ICAM-1 expression in endothelial cells in response to S1P stimulation. Mechanistically, we show that antagonizing S1P₂ signaling markedly inhibited the TNF α -stimulated NF κ B activation. Utilizing the NF κ B reporter luciferase assay, the S1P/S1P₂ signaling was shown to stimulate NF κ B activation. Moreover, the S1P/S1P₂-stimulated VCAM-1/

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ICAM-1 expression was completely abolished by the pharmacological inhibitor of NF- κ B. Collectively, our data suggest that TNF treatment activates autocrine S1P/S1P₂ signaling, which subsequently activates NF- κ B and leads to the proinflammatory responses in endothelial cells.

Keywords

sphingosine-1-phosphate; S1P family of G-protein coupled receptor; sphingolipids; sphingosine kinase; vasculature

1. Introduction

Atherosclerosis, a chronic inflammatory process, is characterized by the thickening of the arterial wall and is the primary cause of cardiovascular disease and cerebrovascular accident, two of the most common causes of illness and death worldwide [1]. An important risk factor for developing atherosclerosis is vascular inflammation, which involves the complex interactions of modified lipoproteins, monocyte-derived macrophages or foam cells, T lymphocytes, endothelial cells, and smooth muscle cells [2, 3].

Sphingosine-1-phosphate (S1P), a serum-borne bioactive lipid mediator synthesized by sphingosine kinase (SphK), regulates an array of biological activities in various cell types [4–7]. S1P can function either as an extracellular ligand or intracellular mediator [8–10]. When functioning as an extracellular ligand, S1P-regulated biological activities are mediated by the S1P family of G protein-coupled receptors (GPCRs) [8, 11, 12]. There are five identified members of the S1P receptor family: S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅ (previous nomenclature: EDG-1, -5, -3, -6, -8, respectively) [13]. S1P receptor subtypes couple to different G polypeptides to regulate distinct signaling pathways [14–16]. Various S1P receptors are expressed in distinct combinations in different cell types to produce the appropriate biological effects. For example, S1P₁, S1P₂, and S1P₃ receptors are expressed in endothelial cells (ECs) [17, 18]. The signaling pathways regulated by S1P₁ and S1P₃ are required for cellular chemotaxis, adherens junction assembly, endothelial morphogenesis, and angiogenic response [17, 19]. Moreover, the balance of S1P₁ and S1P₂ signaling contributes to vascular integrity *in vivo* [20]. Disturbance of this balance, *i.e.*, the up-regulation of S1P₂ signaling, may have functional implications in vascular dysfunctions such as endothelial senescence and atherosclerosis [18].

It has been shown that sphingosine kinase (SphK) plays a role in inflammatory disorders, such as rheumatoid arthritis [21, 22]. However, the molecular details of S1P signaling in the regulation of inflammation remain to be elucidated. Recent studies demonstrated that S1P₂ deficiency led to inhibition of macrophage proinflammatory responses and atherosclerosis in apoE-deficient mice [23]. Moreover, it was shown that S1P₃ mediates the chemotactic effect of S1P in macrophages *in vitro* and *in vivo*, and plays a causal role in atherosclerosis by causing inflammatory monocyte/macrophage recruitment and altering smooth muscle cell behavior [24]. These studies suggest that S1P signaling cascades play an important role in the development of atherosclerosis.

However, how S1P signaling contributes to atherosclerosis and vascular inflammation, particularly in the context of the endothelium, is poorly understood. We previously showed that senescence-associated endothelial dysfunctions can be attributed to the up-regulation of S1P₂ signaling [18]. We also found that S1P₂ receptors were markedly increased in atherosclerotic endothelium [18]. These results suggest that the up-regulated endothelial S1P₂ signaling may play a role in mediating endothelial proinflammatory responses and atherosclerosis development. Therefore, we examined roles of S1P signaling in ECs for

proinflammatory cytokine-induced endothelial inflammation in this study. We observed that TNF treatment increased the expression of S1P₂ receptors and production of S1P in ECs. More importantly, inhibition of endothelial S1P₂ signaling significantly diminished the TNF-induced activation of NF- κ B and expression of proinflammation-associated adhesion molecules (*i.e.* VCAM-1, ICAM-1) in ECs. Furthermore, S1P₂ signaling is capable of activating NF- κ B directly. Also, S1P stimulation increases the expression of VCAM-1/ICAM-1 in ECs, ectopically expressing S1P₂ receptors. Collectively, our results suggest that S1P₂ signaling plays an important role in proinflammatory cytokine induced endothelial inflammation.

2. Materials and Methods

2.1 Reagents

Sphingosine-1-phosphate was purchased from Biomol; JTE-013 and VPC23019 were from Avanti. BAY 11-7085 was from Calbiochem EMD. Mouse-derived S1P₁ monoclonal antibody (E1-49) was generated by conventional hybridoma technology using bacteria expressed S1P₁ as an immunogen [25, 26]. E1-49 was shown to specifically react with the human S1P₁ receptor by immunostaining, immunoblotting, and immunoprecipitation [20, 25, 26]. Polyclonal anti-S1P₂ and anti-S1P₃ were from Cayman Chemical. Monoclonal Anti-human ICAM-1/CD54 antibody was purchased from R&D Systems. Polyclonal rabbit anti-VCAM-1 (H-276), phospho-I κ B p65 (Ser32) (14D4) rabbit mAb, and polyclonal rabbit anti-actin (H-300) were purchased from Santa Cruz. Rabbit polyclonal anti-phospho-I κ B p65 was purchased from Millipore. Peroxidase-conjugated sheep anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG were from MP Biomedicals and Pierce Biotechnology, respectively. Alexa conjugated secondary antibodies were from Molecular Probes. C17-sphingosine, C17-sphingosine-1-phosphate and D7-sphingosine were purchased from Avanti. Other reagents, unless specified, were purchased from Sigma.

2.2 Cell lines and culture conditions

Human umbilical vein endothelial cells (HUVECs, Clonetics, Cc-2517, passage 4–12) were cultured in M199 medium (Mediatech) supplemented with 10% fetal bovine serum (FBS, Hyclone) and heparin-stabilized endothelial cell growth factor as we previously described [18]. Chinese hamster ovary (CHO) cells were cultured in RPMI1640 (HyClone) with 10% FBS.

2.3 RT-PCR and real-time PCR

Total RNA was isolated from cultured cells and RT-PCR was performed as we described previously [27]. PCR primer pairs used were: human S1P₁: sense, 5'-GCACC AACCC CATCA TTTAC-3', anti-sense, 5'-TTGTC CCCTT CGTCT TTCTG-3'; human S1P₂: sense, 5'-CAAGT TCCAC TCGGC AATGT-3', anti-sense, 5'-CAGGA GGCTG AAGAC AGAGG-3'; human S1P₃: sense, 5'-TCAGG GAGGG CAGTA TGTTC-3', anti-sense, 5'-GAGTA GAGGG GCAGG ATGGT-3'; human ICAM-1: sense, 5'-GGCTG GAGCT GTTTG AGAAC-3', anti-sense, 5'-CCTCT GGCTT CGTCA GAAT C-3'; human VCAM-1: sense, 5'-GGTGC TGCAA GTCAA TGAGA-3', anti-sense, 5'-AAGAT GGTGC TGATC CTTGG-3'; human sphingosine kinase 1 (SphK1): sense, 5'-GAGGC TACAC AGGGG CTT-3', anti-sense, 5'-TTTGG CTGAG GCTGA AAT-3'; human SphK2: sense, 5'-GCACA GCAAC AGTGA GCA-3', anti-sense, 5'-GAGCC TGAGT GAGTG GGA-3'; human GAPDH: sense, 5'-GAAGG TGAAG GTCGG AGTC-3', anti-sense, 5'-GAAGA TGGTG ATGGG ATTTC-3'. For real-time PCR quantitation, 50 ng of reversely transcribed cDNAs were amplified with the ABI 7500 system (Applied Biosystems) in the presence of TaqMan DNA polymerase. The sense and antisense primers used to detect the gene expression of S1P receptor subtypes and GAPDH were purchased

from Applied Biosystems. The sense and antisense primers of VCAM-1, ICAM-1, SphK1, and SphK2 were purchased from GeneScript. The qPCR reaction was performed by using a universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions.

2.4 Sphingosine kinase activity assay by LC-MS/MS

HUVECs were washed with ice cold PBS and scraped in 200 μ l SK buffer (20 mM Tris buffer, pH 7.4, 20% glycerol, 1 mM β -mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 1 mM PMSF) containing 1x Protease inhibitor cocktail (Calbiochem). Subsequently, cells were disrupted by sonication (three times, 5 second each) at 4°C using a Vibra Cell ultrasonic processor (Sonics & Materials), followed by centrifugation at 12,000g for 10 min at 4°C. The protein concentration of the resulting supernatant was determined by a bicinchoninic acid protein assay kit (Thermo Scientific) with BSA as the standard. The enzymatic activity of sphingosine kinase (SphK) was then measured as described previously [28] with minor modifications. Briefly, 60 μ g of proteins were used in the enzyme assay. The reaction was initiated by adding 5 μ l of 200 μ M C17-sphingosine (dissolved in 5% Triton X-100) and 5 μ l of 20 mM ATP containing MgCl_2 (200 mM) in a final volume of 100 μ l. After incubation at 37°C for 20 min, the reaction was terminated with 10 μ l of 1 M HCl, and then 100 ng of D7-sphingosine was added as an internal standard. After vigorous vortexing for 10 seconds, samples stood at room temperature for 5 min, and then 223 μ l of methanol were added. The mixture was vigorously vortexed for 10 seconds and stood at room temperature for 5 min. After centrifuging at 12,000g for 5 min at 4°C, samples were transferred to a new 1.5 ml Eppendorf tube, vacuum-dried, and then reconstituted in 100 μ l of the Mobile Phase solvent A (2 mM ammonium formate in methanol with 0.2% formic acid). Samples were filtered through 0.45 μ m nylon filters directly into auto sampler vials for LC-MS/MS analysis.

Reverse phase HPLC was performed using BDS HYPERSIL C8 columns (100 $\times$ 2.1 mm, 2.4 μ m, Thermo Scientific) and gradient elution on Waters Alliance 2695 system (Waters Corporation). The mobile phase consisted of methanol, water, and ammonium formate. Solvent A was 2 mM ammonium formate in methanol with 0.2% formic acid. The column was equilibrated with solvent A for 5 min. Samples were injected using the autosampler (an integral part of the Waters Alliance 2695 system) maintained at $10 \pm 2^\circ\text{C}$. The injection volumes were 80 μ l for each sample. A complete injection of each sample took 7 min including column equilibration. The flow rate was 0.3 ml/min. The HPLC eluent was directly introduced to QuattroLC mass spectrometer (Micromass-Waters, MA), equipped with an electrospray ion source that was used for ESI-MS/MS. The ESI-MS/MS experiments for the quantitation of sphingolipids were carried out in the positive ion mode with ESI needle voltage, 2.8 kV; source block temperature, 120°C; desolvation temperature, 350°C; desolvation gas flow, 540 l/h; nebulizer gas flow, 80 l/h; and the collision gas pressure was 3.2×10^{-4} bar. Cone voltage and collision energy for each MRM transition were optimized. Chromatographic data were analyzed by the Quanlynx module of the Masslynx software (Waters Corporation, MA) to integrate the chromatograms for each MRM transition.

2.5 Measuring endogenous SphK1 and SphK2 activity in HUVEC cells

The assay of SphK1 and SphK2 activity was essentially performed as reported by Billich, *et al* [29], with slight modifications. Briefly, cells were washed with 1x PBS and resuspended in ice-cold SK lysis buffer (20 mM Tris-HCl, pH 7.4, 20% glycerol, 1 mM β -mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 40 mM β -glycerophosphate, and 0.5 mM 4-deoxy pyridoxine) containing Complete™ protease inhibitors cocktail (Roche Applied Science). Cells were homogenized with 20 passages

through a 26 gauge needle. Lysates were cleared by centrifugation then protein concentrations were determined using Bradford assay. Enzyme activity was measured using 20 µg of total protein in total volumes of 100 µl with 20 µM sphingosine (prepared as a complex with fatty acidfree bovine serum albumin), 1 mM ATP and 2 µCi of ³³P-ATP as substrates. For SphK1 activity, the reactions were carried out in 50 mM HEPES (pH 7.4), 0.5% Triton X-100, 15 mM MgCl₂, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, and 0.5 mM 4-deoxypyridoxine. For SphK2 activity, the buffer was altered to favor the SphK2 isoform (50 mM HEPES (pH 7.4), 15 mM MgCl₂, 1 M KCl, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, and 0.5 mM 4-deoxypyridoxine). Reactions were started by the addition of protein and incubated at 37°C for 2 hours. Reactions were stopped by addition of 700 µl cold chloroform/methanol/HCl (100:200:1, v/v). Phases were broken by addition of 400 µl 1M KCl and 400 µl chloroform followed by extensive vortexing and centrifugation at 2000g for 10 minutes. The organic phase was removed to a clean glass tube, dried under a stream of nitrogen, then resuspended in 100 µl chloroform. The entire reaction was applied to a thin layer chromatography plate (Whatman, Silica Gel 60) and developed using butanol/acetic acid/water (60:20:20, v/v). Radiolabeled S1P was visualized and quantified using a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA) and identified by co-migration with a known standard.

2.6 Western-blot analysis

Cells were collected in ice-cold PBS using cell scrapers followed by centrifugation (250g, 5 min). Cell extracts were prepared in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Calbiochem) with constant agitation at 4°C for 30 min. After centrifugation at 15,000g for 20 min, supernatant was collected and protein concentration was measured using a bicinchoninic acid protein assay kit with BSA as standard. 50 µg of protein extracts were dissolved in 2x Laemmli sample buffer, heated at 95°C for 5 min, and resolved on a 10% SDS-PAGE gel. After electrophoresis, gels were transferred to nitrocellulose membranes. Subsequently, membranes were blocked in 5% non-fat dry milk (Lab Scientific) in TBST buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl and 0.05% Tween-20). Membranes were washed and incubated with the indicated primary antibodies on a rotary shaker at 4°C overnight. The blots were then incubated with peroxidase-conjugated second antibody for 1 hour at room temperature, and developed with enhanced chem-luminescence reagent (Amersham).

2.7 Adenoviral transduction

Confluent HUVECs were transduced with various concentrations (2, 20 and 200 multiplicity of infection, m.o.i.) of adenoviral particles carrying S1P₂ or -galactosidase vectors for 16 hrs as we previously described [18, 19]. After wash, cells were stimulated with S1P (200 nM) for 4 hours, and RNAs were prepared for real-time PCR analysis.

2.8 Confocal microscopy analysis

HUVECs were cultured in glass bottom culture dishes (Mat Tech Corporation) for three days. Cells were washed and starved in plain M199 medium for 2 hours. After treatment with TNF and/or JTE-013 for half hour, cells were fixed with 4% paraformaldehyde for 30 min, and permeabilized with Triton X-100 (0.05 %) for 30 min. Cultures were then incubated with primary antibody for overnight. Subsequently, cells were incubated with Alexa conjugated secondary antibody at room temperature for 1 hour, and analyzed with Leica TCS SP5 confocal microscopy.

2.9 NFκB reporter driven luciferase assay

CHO cells were transiently transfected with pNF-κB-MetLuc (Clontech) together with *pcDNA3.1*, *pcDNA3.1* vector carrying S1P₁ (*pcDNA3.1-S1P₁*), S1P₂ (*pcDNA3.1-S1P₂*), or S1P₃ (*pcDNA3.1-S1P₃*) construct by using the Mirus 2130 transfection kit (Mirus). Cells were serum-starved for 24 hours, followed by stimulation with or without S1P (200 nM) at 37°C for 6 hours. Luciferase activity was measured with a Luminometer (TECAN) [30].

3. Results

3.1 TNFα treatment enhances the expression of S1P₂, VCAM-1, and ICAM-1, and activates SphK1 in endothelial cells

Our previous study showed that S1P₂ was markedly increased in the lesion region of the atherosclerotic endothelium [18]. To investigate the role of S1P receptor subtypes in endothelial inflammation, cultured endothelial cells were treated with proinflammatory cytokine TNF-α for 4 hours. The expression of S1P receptor subtypes was assessed by real-time PCR. As shown in Fig. 1A, S1P₂ expression was significantly elevated by 2.3 fold at messenger RNA level ($p < 0.01$, *t*-test). The expression of S1P₁ and S1P₃ was not altered by TNF-α treatment. S1P₄ and S1P₅ receptors were not detected in HUVECs with or without TNF-α treatment (data not shown). Also, TNF-α treatment increased S1P₂ proteins by 3.2 fold ($p < 0.05$, *t*-test) (Fig. 1B). In contrast, TNF-α did not alter the protein levels of S1P₁ receptors. S1P₃ proteins were not detected in the presence or absence of TNF-α treatment by Western-blotting analysis (data not shown). In addition, the expression of S1P₂ increased temporally in TNF-α-treated HUVECs (Fig. 1C). S1P₂ was up-regulated by ~2 fold at 2 hours, peaked (~4 fold) at 24 hours, and plateaued up to 72 hours following TNF-α treatment (Fig. 1C). In contrast, the level of S1P₁ and S1P₃ did not change with TNF-α treatment over this 72 hrs period (Fig. 1D).

VCAM-1 (vascular cell adhesion molecule 1) expression is enhanced upon TNF-α treatment [31, 32]. Therefore, we examined TNF-α-regulated cell adhesion molecules in HUVECs. We focused on the expression of VCAM-1 and ICAM-1 (intercellular adhesion molecule 1), two critical adhesion molecules which play important roles in mediating proinflammatory responses of ECs. Both VCAM-1 and ICAM-1 were significantly induced in HUVECs after 4 hours of TNF-α treatment (Supplemental Figure 1A and 1B). Furthermore, TNF-α treatment increased VCAM-1 and ICAM-1 expression in a time-dependent manner in HUVECs (Supplemental Figure 1C and 1D). Both VCAM-1 and ICAM-1 induction apexed after 24 hours with TNF-α treatment.

It was shown that TNF-α treatment induced SphK activation and S1P generation in ECs [33]. Treatment with N, N-dimethylsphingosine, an inhibitor of SphK, profoundly inhibited TNF-α-induced VCAM-1 expression [33], suggesting that SphK activation and S1P production play a key role in the TNF-α-induced VCAM-1 expression. However, the molecular details of the TNF-α/SphK/S1P signaling in VCAM-1 induction remain elusive. Initially, cellular extracts, prepared from HUVECs treated in the absence or presence of TNF-α for varying amounts of time, were incubated with the C17-sphingosine, followed by measuring the biosynthesis of C17-S1P with the LC-MS/MS mass spectrometry. As shown in Fig. 2A, S1P concentration was significantly increased following 1 hour of TNF-α exposure. The level of C17-S1P significantly increased in a time-dependent manner in response to TNF-α treatment. Also, levels of S1P were significantly increased in culture media of TNF-α-treated ECs (Fig. 2B). These results indicate that TNF-α treatment activates SphK and increases S1P secretion in endothelial cells.

There are two isoforms of sphingosine kinases (SphK), SphK1 and SphK2, which are responsible for S1P biosynthesis. Next, we determined which SphK isoform contributed to

S1P production in endothelial cells following TNF treatment. Real-time PCR analysis showed that there was a significant increase of SphK1 expression (~ 1.6 fold; $p < 0.05$, t -test) in HUVECs after TNF treatment (Fig. 2C). In contrast, there is no alteration in SphK2 expression in HUVECs following TNF treatment. The enzymatic activity of SphK1 and SphK2 can be differentially measured in assay buffer containing Triton X-100 and KCl, respectively [29]. Using the differential measurement of enzymatic activity, we observed that TNF treatment activated SphK1, but not SphK2, in ECs (Fig. 2D). These data together strongly suggest that SphK1 contributes to the TNF -increased S1P production in endothelial cells.

Collectively, these results indicate that TNF treatment enhances the expression of S1P₂, VCAM-1, and ICAM-1 in endothelial cells. Moreover, TNF activates SphK1, leading to the increase of S1P production.

3.2 S1P₂ signaling contributes to TNF α -induced VCAM-1 and ICAM-1 up-regulation in ECs

Next, we utilized JTE-013, a well characterized pharmacological antagonist of S1P₂ receptors [34, 35] to investigate whether S1P₂ signaling contributes to the TNF -mediated up-regulation of VCAM-1 and ICAM-1 in ECs. TNF stimulated up-regulation of VCAM-1 mRNA was almost completely inhibited in the presence of JTE-013, shown by real-time PCR (Fig. 3A) and RT-PCR (Fig. 3B). TNF induced VCAM-1 polypeptides were diminished by approximately 45% in the presence of JTE-013 (Fig. 3C). Similarly, JTE-013 significantly abolished TNF up-regulated ICAM-1 at both mRNA (Fig. 3D, 3E) and protein levels (Fig. 3F). In a control, treatment of VPC23019, an antagonist specific for both S1P₁ and S1P₃ receptors [36], had no effect on TNF -induced expression of VCAM-1 and ICAM-1 (Supplemental Figure 2A and 2B). Together, these data suggest that signaling cascades transduced by S1P₂, not S1P₁ or S1P₃, contribute to TNF -induced VCAM-1 and ICAM-1 expression in ECs.

It was recently shown that JTE-013 can also function as an antagonist of S1P₄ receptors [37]. Because S1P₄ receptors were not detected in HUVECs treated with or without TNF , the effects of JTE-013 on inhibition of TNF -induced VCAM-1/ICAM-1 expression (Fig. 3) are most likely mediated by antagonizing S1P₂ receptors in ECs. Therefore, HUVECs stably transduced with Lentiviral particles expressing sh-S1P₁ or sh-S1P₂ [18, 26, 38] were used to further validate the role of S1P₂ in TNF -induced proinflammatory responses. The specificity and efficacy of knocking-down S1P₁ or S1P₂ receptors in those ECs stably transduced with sh-S1P₁ or sh-S1P₂ was shown in our previous studies [18, 26, 38]. Also, the efficacy of knocking-down S1P₁ or S1P₂ receptors in current study was shown in Fig. 4A. Subsequently, sh-S1P₁ or sh-S1P₂ stably transduced ECs were treated with and without TNF . The induction of S1P₂, VCAM-1 and ICAM-1 were measured using real-time PCR. As shown in Fig. 4B–D, the TNF -stimulated expression of S1P₂, VCAM-1 and ICAM-1 was completely inhibited in HUVECs stably transduced with Lentiviral particles carrying the sh-S1P₂ vector. In contrast, stable transduction with Lentiviral particles carrying sh-S1P₁ had no effect on TNF -stimulated S1P₂, VCAM-1 and ICAM-1 expression in HUVECs. Collectively, our findings indicate that S1P₂ receptors play a key role in the TNF -induced VCAM-1 and ICAM-1 expression in ECs.

3.3 S1P₂-NF κ B signaling contributes to TNF α -induced VCAM-1 and ICAM-1 expression

It has been well demonstrated that NF κ B plays a critical role in the TNF -stimulated proinflammatory responses [39–43]. We next examined whether S1P₂ signaling contributes to the TNF -induced NF κ B activation in ECs. Chinese hamster ovary (CHO) cells were transiently transfected with a NF κ B reporter driven Luciferase vector [30], together with *pcDNA3.1* control vector, *pcDNA3.1-S1P₁*, *pcDNA3.1-S1P₂*, or *pcDNA3.1-S1P₃* vectors,

followed by stimulation with or without S1P. As shown in Fig. 5A, S1P-S1P₂ signaling was able to activate NF- κ B. In contrast, cells transfected with the *pcDNA3.1* control vector, *pcDNA3.1-S1P₁*, or *pcDNA3.1-S1P₃* were incapable of inducing NF- κ B activation.

Subsequently, we investigated the role of S1P₂ signaling in TNF- α -stimulated NF- κ B activation in ECs. As shown in Fig. 5B, TNF- α treatment resulted in a markedly increased phosphorylation of I- κ B polypeptides, an indicator of NF- κ B activation [39–43]. Importantly, TNF- α -induced I- κ B phosphorylation was diminished by 0.53 ± 0.11 fold ($p < 0.05$, *t*-test) in the presence of JTE-013 (Fig. 5B). In a time course study, we observed that JTE-013 inhibited 67% of TNF- α -stimulated I- κ B phosphorylation as early as 30 min after TNF- α addition (Fig. 5C). In addition, TNF- α treatment markedly induced the nuclear translocation of NF- κ B polypeptides (Fig. 5D), another indicator of NF- κ B activation [39–43]. Similarly, TNF- α -enhanced nuclear translocation of NF- κ B was significantly inhibited in the presence of JTE-013 (Fig. 5D). Quantitative analysis showed that JTE-013 inhibited $69\% \pm 5.8\%$ ($n = 5$ microscopic fields; $p < 0.01$, *t*-test) of the TNF- α -induced nuclear translocation of NF- κ B polypeptides.

Under normal culture condition, HUVECs (passage 3–12) express low level of S1P₂ receptors [17, 18]. Therefore, we ectopically expressed S1P₂ in HUVECs, and determined whether S1P₂ *per se* was able to induce VCAM-1 and ICAM-1 expression following S1P stimulation. HUVECs were transduced with various concentrations (0 – 200 multiplicity of infection, m.o.i.) of adenoviral particles carrying the S1P₂ construct as we previously described [18, 19]. Subsequently, levels of VCAM-1 and ICAM-1 were measured in transduced cells following S1P stimulation. In agreement with our previous studies [18, 19], transduction with adenoviral particles carrying S1P₂ vector specifically increased the amount of S1P₂ receptors in HUVECs (Fig. 6A). ECs ectopically expressing S1P₂ receptors dose-dependently induced VCAM-1 (Fig. 6B) and ICAM-1 (Fig. 6C) expression following S1P stimulation, indicating that S1P-S1P₂ signaling can sufficiently induce VCAM-1 and ICAM-1 expression in ECs. In contrast, S1P treatment was unable to enhance the expression of VCAM-1 or ICAM-1 in ECs transduced with -galactosidase control particles (Fig. 6B–C). Because ECs express endogenous S1P₁ and S1P₃ receptors, this result suggests that S1P-S1P₁ and S1P-S1P₃ signaling were unable to enhance VCAM-1/ICAM-1 expression in ECs.

Finally, we utilized Bay 11–7085, a specific inhibitor of NF- κ B [44], to examine whether S1P/S1P₂ stimulated VCAM-1 and ICAM-1 expression requires NF- κ B activity. HUVECs were transduced with 20 m.o.i adenoviral particles carrying S1P₂ receptors, and stimulated with or without S1P in the presence or absence of Bay 11–7085. As shown in Fig. 7, the S1P/S1P₂-enhanced VCAM-1 and ICAM-1 expression was completely inhibited by Bay 11–7085. This result suggests that the endothelial S1P/S1P₂ signaling induced VCAM-1 and ICAM-1 expression requires NF- κ B activity.

4. Discussion

Endothelial inflammation is an important risk factor in the initiation and development of atherosclerosis [2, 3]. Therefore, the specific signaling cascades that regulate endothelial inflammation, and the patho-physiological outcomes mediated by these endothelial proinflammatory signals are important questions in vascular biology. TNF- α is a critical proinflammatory cytokine, and its inhibition is currently being used to treat various inflammatory and autoimmune disorders such as rheumatoid arthritis, Crohn's disease, and psoriasis [45]. In present study, cultured ECs were treated with TNF- α to investigate role of sphingolipids in endothelial inflammation. We observed that TNF- α treatment significantly increased S1P₂ receptors (Fig. 1). Moreover, we previously reported that S1P₂ receptors are markedly increased in atherosclerotic endothelium [18]. These data together suggest that

endothelial S1P₂-mediated signaling may have a critical role in endothelial inflammation and atherosclerosis.

VCAM-1 and ICAM-1, adhesive proteins on the endothelium surface, are induced by proinflammatory cytokines [3, 44, 46]. ICAM-1 and VCAM-1 are required for monocyte adhesion to the endothelium and the subsequent extravasation of monocytes into the sub-endothelial space, initiating the atherosclerotic process. Therefore, we investigated the role of S1P₂ signaling in TNF α -induced VCAM-1/ICAM-1 expression in ECs. Several lines of evidence strongly suggest that S1P₂ signaling indeed plays an important role in TNF α -enhanced VCAM-1/ICAM-1 expression in ECs. Firstly, JTE-013, a well-characterized antagonist of S1P₂ [34, 35], significantly inhibited TNF α -enhanced VCAM-1/ICAM-1 expression at both the mRNA and protein levels (Fig. 3). Moreover, specific knockdown of endothelial S1P₂ receptors diminished the TNF α -induced expression of ICAM-1 and VCAM-1 in ECs (Fig. 4). These results demonstrate that S1P₂ receptors play a key role in TNF α -induced ICAM-1 and VCAM-1 expression in ECs.

Secondly, it has been demonstrated that TNF α -induced VCAM-1/ICAM-1 requires NF κ B activity [3, 34, 40, 46]. Treatment with S1P₂ specific antagonist JTE-013 significantly inhibited TNF α -regulated I κ B phosphorylation and NF κ B nuclear translocation (Fig. 5), suggesting that S1P₂ is a critical regulator in TNF α -mediated NF κ B activation. Moreover, utilizing the NF κ B reporter driven luciferase assay, we present direct evidence showing that S1P₂ signaling is capable of inducing NF κ B activation (Fig. 5A). Also, we showed that TNF α treatment activated SphK1 and increased S1P production in ECs (Fig. 2). Together, these results suggest that TNF α activates the autocrine feedforward reaction of S1P/S1P₂ signaling, which contributes to the TNF α -induced NF κ B activation and ICAM-1/VCAM-1 expression in ECs. Finally, HUVECs express low level of S1P₂ receptors [17, 18]. Ectopic expression of S1P₂ by adenoviral particles dose-dependently induced VCAM-1/ICAM-1 expression following S1P stimulation (Fig. 6), without prior TNF α treatment. As a control, S1P was unable to stimulate the expression of VCAM-1/ICAM-1 in HUVECs transduced with adenoviral particles carrying the β -galactosidase vector (Fig. 6). Moreover, NF κ B activity was essentially required for the S1P/S1P₂ mediated VCAM-1/ICAM-1 expression as Bay 11-7085, a specific NF κ B inhibitor, completely abolished S1P/S1P₂ mediated VCAM-1/ICAM-1 expression (Fig. 7).

It should be noted that VCAM-1 and ICAM-1 were up-regulated approximately 20- and 8-fold, respectively, after TNF α treatment for 4 hours (Supplemental Figure 1). Transduction with 20 m.o.i. of adenoviral particles carrying S1P₂ resulted in ~ 2–3 fold increase in the amount of S1P₂ (Fig. 6A), which is comparable to the increase of S1P₂ induced by 4-hr TNF α treatment. However, transduction with 20 m.o.i. of S1P₂ adenoviral particles only resulted in 3- and 5-fold increase of VCAM-1 and ICAM-1, respectively, following S1P stimulation (Fig. 6B, 6C). The reduced VCAM-1/ICAM-1 up-regulation can be explained by the deleterious effects of adenoviral transduction, because the basal level of VCAM-1/ICAM-1 expression is significantly diminished in HUVECs transduced with 20 m.o.i. of control viral particles (β -galactosidase transduction, 0 vs 20 m.o.i., Fig. 6B, 6C). However, it is also possible that the reduced induction of VCAM-1/ICAM-1 is due to the fact that the S1P₂ signaling is a critical regulator, but not the sole regulator, contributing to TNF α -stimulated VCAM-1/ICAM-1 expression in ECs. This notion is supported by the observation that JTE-013 treatment significantly, but not completely, abolished TNF α -enhanced expression of VCAM-1/ICAM-1 at the mRNA and protein levels (Fig. 3), I κ B phosphorylation (Fig. 5B–C), and NF κ B nuclear translocation (69% $\pm$ 5.8% of inhibition) (Fig. 5D).

TNF α has been shown to activate SphK in a variety of cell types. TNF α mediated SphK activation, which produces S1P, has been suggested to regulate various cellular functions like myogenesis in C2C12 myoblasts [47], proliferation in glioblastoma cells [48], and inhibition of apoptosis in ECs and hepatocytes [33, 49, 50]. Treatment with N, N-dimethylsphingosine, an inhibitor of SphK, profoundly inhibited TNF α -induced VCAM-1 expression [33], suggesting that SphK activation plays a key role in the TNF α -induced VCAM-1 expression. In accordance with previous studies, we observed that TNF α treatment activated SphK and increased S1P production in ECs (Fig. 2A, 2B). However, we further showed that TNF α activates SphK1, and not SphK2, in ECs (Fig. 2C, 2D). Importantly, in contrast to previous studies, we demonstrate that TNF α treatment significantly enhanced the expression of S1P₂ in ECs (Fig. 1), and the S1P₂-mediated signaling plays a critical role in regulating the TNF α -stimulated VCAM-1/ICAM-1 expression. Collectively, these data suggest that treatment with proinflammatory cytokine TNF α enhances the amount of the ligand S1P and the receptor S1P₂ simultaneously. TNF α -enhanced S1P/S1P₂ signaling thus constitutes a novel “switch” of inflammation by activating NF κ B and subsequently stimulating the expression of VCAM-1/ICAM-1, critical to the initiation of inflammation in endothelial cells.

5. Conclusion

TNF α treatment increased the expression of S1P₂ receptors and production of S1P in ECs. Inhibition of endothelial S1P₂ signaling significantly diminished the TNF α -induced activation of NF κ B and expression of proinflammation-associated adhesion molecules in ECs. Our results highlight a novel role of S1P₂ signaling in the proinflammatory responses of the endothelium. Moreover, it was demonstrated that S1P₂ signaling plays an important role in the retention and pro-inflammation of macrophages present in the atherosclerotic plaque [23]. Therefore, targeting S1P₂ signaling may provide a novel therapeutic utility for the treatment of vascular pro-inflammatory diseases such as atherosclerosis, *via* the inhibition of endothelial pro-inflammation as well as the extravasation/inflammation of monocytic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

S1P	sphingosine-1-phosphate
GPCR	G-protein coupled receptor
S1P₁₋₅	high affinity GPCRs for S1P
TNF	tumor necrosis factor-
VCAM-1	vascular cell adhesion molecule 1
ICAM-1	intercellular adhesion molecule 1
SphK	sphingosine kinase
HUVEC	human umbilical vein endothelial cells

Ecs cultured endothelial cells

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Highlights

- TNF treatment induces S1P₂ expression in endothelial cells.
- Ectopic expression of S1P₂ induces VCAM-1/ICAM-1 expression following S1P treatment in endothelial cells.
- S1P/S1P₂ signaling activates NF- κ B.
- NF- κ B inhibition abrogates S1P/S1P₂-induced ICAM-1/VCAM-1 expression.
- Inhibition of S1P₂ signaling diminishes TNF- α -induced NF- κ B activation and VCAM-1/ICAM-1 expression in endothelial cells.

Fig. 1. TNF induces S1P₂ expression in ECs

A: HUVECs were treated with TNF α (10 ng/ml) for 4 hours at 37°C. Subsequently, levels of S1P receptor subtypes were measured by real-time PCR (**A**) or Western-blot analysis (**B**). Note that S1P₂ was significantly up-regulated after TNF α treatment (**, $p < 0.01$, t -test). +Ve, positive control lysates: ECs were transduced with adenoviral particles carrying S1P₁ or S1P₂ construct. *, non-specifically immune-reactive protein bands. **C** and **D**: HUVECs were treated with TNF α (10 ng/ml) at 37°C for various durations. The expression of S1P₁, S1P₂, and S1P₃ was quantitated by real-time PCR. Note that TNF α treatment up-regulated S1P₂ temporally. The data shows the mean $\pm$ S.D. of triplicate determinations, which are normalized to endogenous GAPDH levels, of a representative experiment that had been repeated three times with similar results.

Fig. 2. TNF α activates SphK1 and increases S1P production in ECs

A: HUVECs were treated with TNF α (10 ng/ml) for indicated times. Cell lysates were incubated with the C17-sphingosine, an analogue of sphingosine that contains 17 carbons in its backbone (there are 18 carbons in the natural sphingosine). The relative quantities of C17-S1P, an indicator of the enzymatic activity of SphK as it converts C17-sphingosine to C17-S1P, were measured by LC-MS/MS as described in the Materials and Methods section. *, $p < 0.05$, t -test ($n = 3$), TNF α treated vs untreated (Ctrl, control). **B:** ECs were treated with or without TNF α . Cultural media were measured for S1P quantity by LC-MS/MS (**, $p < 0.01$, $n = 3$). **C:** TNF α induced SphK1 expression in HUVECs. Cells were treated with TNF α (10 ng/ml) for 4 hour at 37°C. Subsequently, levels of SphK1 and SphK2 were determined by real-time PCR ($n = 3$). **D:** TNF α activated SphK1 in ECs. Cell lysates were differentially measured for the enzymatic activity of SphK1 and SphK2 as described [29]. Note that TNF α treatment significantly activated SphK1, but not SphK2, in ECs ($n = 12$; $p < 0.01$, t -test).

Fig. 3. JTE-013, a S1P₂ antagonist, inhibits TNF-induced VCAM-1 and ICAM-1 expression
 HUVECs were treated with or without TNF (10 ng/ml), in the presence or absence of JTE-013 (5 μ M) at 37°C for 4 hours. **A:** Real-time measurement of VCAM-1 mRNA. **B:** VCAM-1 expression was determined by semi-quantitative RT-PCR. The PCR products were resolved in agarose gel (*upper panel*). Note that TNF-induced VCAM-1 expression was inhibited by S1P₂ antagonist JTE-013. **C:** Western-blot analysis showed that JTE-013 inhibited the induction of VCAM-1 polypeptides following TNF treatment. **D, E, and F:** ICAM-1 expression was measured by qPCR, semi-quantitative RT-PCR, and Western-blot, respectively. Note that JTE-013 inhibited TNF-enhanced ICAM-1 expression. *Lower panels* in (**C**) and (**F**), immunoreactivity intensity was measured by NIH Image J software, and normalized to actin. The fold increase represents the mean $\pm$ S.D. of triplicate determinations. **, $p < 0.01$, *, $p < 0.05$, t -test.

Fig. 4. Knockdown of S1P₂ receptors abolishes TNF up-regulated S1P₂, VCAM-1 and ICAM-1 HUVECs stably transduced with Lentiviral particles expressing sh-S1P₁ or sh-S1P₂ [18, 26, 38] were treated with or without TNF (10 ng/ml) at 37°C for 24 hours. **A:** Efficacy of S1P₁ and S1P₂ knockdown in ECs transduced with sh-S1P₁ and sh-S1P₂, respectively. qPCR quantitation of levels of S1P₂ (**B**), VCAM-1 (**C**), and ICAM-1 (**D**) in sh-S1P₁ or sh-S1P₂ transduced cells, treated with or without TNF. Data are mean $\pm$ S. D. of triplicate determinants. **, $p < 0.01$, t -test. NS, not statistically significant.

Fig. 5. S1P₂ signaling contributes to TNF α -mediated NF κ B activation in ECs

A: S1P/S1P₂ signaling activates NF κ B reporter-driven luciferase. CHO cells were transfected with *pNF κ B-MetLuc* (Clontech) together with *pcDNA3.1*, *pcDNA3.1-S1P₁*, *pcDNA3.1-S1P₂*, or *pcDNA3.1-S1P₃* vectors. Subsequently, cells were treated with or without S1P (200 nM), and luciferase activity was measured according to the manufacturer's instructions. Data represent mean $\pm$ S.D. of triplicate determinations from a representative experiment, which was repeated three times with similar results. **, $p < 0.01$, t -test. **B:** S1P₂ signaling contributes to TNF α -induced phosphorylation of I κ B polypeptides. HUVECs were treated with or without TNF α (10 ng/ml), in the presence or absence of JTE-013 (5 μ M). Extracts were immunoblotted with phospho-I κ B antibody (Santa Cruz). The membrane was stripped and reprobed with β -actin antibody (Santa Cruz) as a loading control. *Lower panel*, intensity of immunoreactivity for phospho-I κ B was measured by NIH Image J, and normalized to actin. Data shown are mean $\pm$ S.D. of triplicate determination. **, $p < 0.01$; *, $p < 0.05$; t -test. **C:** HUVECs were treated with TNF α for indicated times, in the presence or absence of JTE-013. Extracts were immunoblotted with

phospho-I κ B antibody. *Lower panel*, intensity of immunoreactivity for phospho-I κ B was measured by NIH Image J, and normalized to actin. *D*: JTE-013 inhibits TNF α -induced nuclear translocation of NF κ B. HUVECs were stimulated with or without TNF α (10 ng/ml) in the presence or absence of JTE-013 (5 μ M) for 0.5 hours at 37°C. After fixation, cells were immunostained with rabbit monoclonal anti-NF κ B p65 (Santa Cruz), and analyzed with Leica TCS SP5 confocal microscopy. *Panel a*, control without treatment; *panel b*, TNF α alone; *panel c*, TNF α + JTE-013, and *panel d*, JTE-013 alone. Note that TNF α -induced NF κ B nuclear translocation was significantly inhibited by co-treatment with JTE-013 (69% $\pm$ 5.8% inhibition, 5 microscopic fields, $p < 0.01$, t -test).

Fig. 6. S1P/S1P₂ signaling enhances the expression of VCAM-1 and ICAM-1 in ECs
 Confluent HUVECs were transduced with various concentrations of adenoviral particles carrying S1P₂ or control β -galactosidase vectors (0, 2, 20, 200 m.o.i) for 16 hours. Following stimulation with S1P (200 nM) for 4 hours, the expression of (A) S1P₂, (B) VCAM-1, and (C) ICAM-1 was quantitated by real-time PCR. Data represent mean $\pm$ S.D. of triplicate determinations. *, $p < 0.05$ (t -test).

Fig. 7. S1P/S1P₂ induced VCAM-1 and ICAM-1 expression requires NF- κ B activity

Confluent HUVECs were transduced with adenoviral particles carrying S1P₂ vectors (20 m.o.i) for 16 hours. Subsequently, the media was removed and 2 ml of plain M199 was added. Cells were then pre-treated with or without Bay 11-7085 (10 μ M) for 1 hour, followed by stimulating in the presence or absence of S1P (200 nM) for additional 4 hours. The expression of VCAM-1 (A) and ICAM-1 (B) was determined by qPCR. **, $p < 0.01$; NS, not statistically significant.