Sphingosine 1-Phosphate Elicits Proinflammatory Responses in ARPE-19 Cells

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PURPOSE. To investigate the effects of sphingosine 1-phosphate (S1P) on the production of inflammatory mediators and the signaling pathways involved in S1P-mediated production of cytokines by ARPE-19 cells.

METHODS. Expression of S1P receptors was examined using RT-PCR and real-time PCR. ARPE-19 cells were stimulated with S1P or TNF-α, and by coculturing with S1P in the presence or absence of pertussis toxin (PTX) or a series of kinase inhibitors. The induction of inflammatory cytokine production was determined by ELISA. Western blot analysis was used to detect the activation of signaling mediators and S1P receptor.

RESULTS. ARPE-19 cells express all the known receptors for S1P. Moreover, exogenously applied S1P induces ARPE-19 cell secretion of interleukin-8 (IL-8) in a dose- and time-dependent manner, but not IL-6 and monocyte chemotactic protein-1 (MCP-1). S1P-mediated IL-8 secretion is regulated by PTX, extracellular regulated protein kinases 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (MAPK). Interestingly, treatment of ARPE-19 cells with TNF-α increases S1P expression and correlates with the enhancement of S1P-induced IL-8 and IL-6 production.

CONCLUSIONS. This study demonstrates that S1P significantly promoted ARPE-19 cells to secrete inflammatory mediators, extracellular regulated protein kinases 1/2 (ERK1/2), p38 MAPK, and G protein-dependent pathways are important signaling components in S1P-mediated IL-8 secretion by ARPE-19 cells. Moreover, these results provide evidence that S1P stimulation of RPE cells may play a role in regulating leukocyte function during ocular inflammation. (Invest Ophthalmol Vis Sci. 2012; 53:8200–8207) DOI:10.1167/iovs.12-10965

Retinal pigment epithelial (RPE) cells form a blood-retina barrier that limits access of blood cells and proteins to the retina. There is evidence that RPE cell dysfunction plays a role in the pathogenesis of certain ocular diseases including age-related macular degeneration (AMD), proliferative vitreoretinopathy (PVR), and overtly inflammatory ocular diseases such as uveitis, disorders in which leukocyte and macrophage infiltration is often seen. Furthermore, this cell is a rich source of cytokines that may contribute to or limit pathologic processes. In response to IL-1 and other proinflammatory stimuli, RPE cells have been found to produce a variety of cytokines, including IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), and granulocyte-macrophage colony-stimulating factor (GMCSF), which may play important roles in ocular inflammation.

Sphingosine 1-phosphate (S1P) is a naturally occurring lipid produced by many different cell types and is present in circulating blood at a low micromolar concentration. A number of studies have clearly demonstrated that S1P can modulate multiple key biological processes, including cell proliferation, motility, differentiation, and survival, by acting either as an intracellular mediator or as ligand of at least five distinct G-protein–coupled receptors, termed S1P receptors (S1P1–5). S1P receptors (S1P1–5) are part of the G protein–coupled receptor (GPCR) superfamily and are widely expressed in most tissues at different levels, suggesting that this receptor influences many cellular processes. Recent studies have established the involvement of S1P in regulating intracellular events in cytokine signaling and in the induction of inflammatory mediators. S1P has been shown to exhibit proinflammatory effects by regulating interleukin-6 (IL-6) secretion in smooth muscle cells and osteoblasts, MCP-1 secretion in vein endothelial cells, and IL-8 secretion in bronchial epithelial cells and synoviocytes. Thus, it appears that extracellular S1P is a mediator of inflammation by regulating proinflammatory cytokine production.

There is evidence that S1P is present in primary human RPE, conjunctival fibroblasts, corneal fibroblasts, and rabbit vitreous fluid. Moreover, S1P has been shown to participate in the ocular fibrogenic cascade and to contribute to scar formation in ocular diseases. S1P stimulates neovascularization, whereas its selective binding with a monoclonal antibody significantly reduces the damage. Moreover, lipopolysaccharide-induced inflammation increases S1P levels in rat retina, promoting astrogliosis, suggesting that S1P may also participate in retinal inflammation. Control of S1P levels might therefore successfully be used for the treatment of several ocular diseases, such as AMD and diabetic retinopathy. Despite the increasing amount of evidence implicating S1P as a...
proinflammatory mediator, very few studies have assessed the role of S1P in ocular inflammation. In view of the important role of the RPE cell during ocular inflammation, we sought to fully characterize the role of the S1P/S1P receptor signaling pathway in RPE cell function.

Herein, we report that ARPE-19 cells express the S1P<sub>1-4</sub> receptors. We also provide evidence that S1P markedly stimulated IL-8 secretion in a pertussis toxin (PTX)-sensitive manner, as well as the elucidation role of extracellular regulated protein kinases 1/2 (ERK1/2) and p38 MAPK (mitogen-activated protein kinase) in S1P-induced IL-8 production. In addition, S1P<sub>2</sub> expression by ARPE-19 cells was enhanced by TNF-α, and S1P-mediated cytokine secretion was super-induced in TNF-α-primed ARPE-19 cells. These results suggest that S1P effects on RPE cells may be important in regulating inflammatory responses in the eye.

Materials and Methods

Cell Culture

Human ARPE-19 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and at passages 16 to 20 were used for the experiment described here. The cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained under standard cell culture conditions at 37°C in humidified air with 5% CO₂. Corning flasks were used with 1 × 10<sup>6</sup> cells/flask, resulting in completely confluent (≈2 × 10<sup>6</sup> cells/flask) cultures in 4 days.

Cell Treatment

Completely confluent cells were starved with serum-free medium for 24 hours before treatment. At the moment of cell treatment, the culture medium was replaced with fresh serum-free medium containing various concentrations of the tested compounds as indicated below.

Semi-quantitative RT-PCR and Real-Time PCR Analyses

Total cellular RNA was extracted from ARPE-19 cells using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). RNA (1 μg) was reverse transcribed using random priming and the Superscript II Reverse Transcriptase system (Invitrogen). Oligonucleotides used as primers were designed to amplify specific cDNA sequences. Primer sequences are listed in the Table. PCR reactions were performed for 35 cycles. A total of 35 PCR cycles were run at 94°C with denaturation at 94°C for 1 minute, annealing at 65°C for S1P<sub>1-3</sub> and 61°C for S1P<sub>4-5</sub> for 1 minute, and finally extension at 72°C for 1 minute. PCR products were separated by a 2% agarose gel and photographed by laser densitometry scanning. In real-time PCR experiments, we used the same primers as for RTPCR to amplify S1P<sub>1-5</sub>. The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 63°C for S1P<sub>1-3</sub> and 61°C for S1P<sub>4-5</sub> for 20 seconds, and finally extension at 72°C extension for 20 seconds. The S1P<sub>1-5</sub> PCR products were normalized in relation to the β-actin internal control. All experiments were repeated independently on four different occasions.

Enzyme-Linked Immunosorbent Assay

ARPE-19 cells plated at a concentration of 1 × 10<sup>6</sup> cells/mL in six-well plates were incubated at S1P at indicated concentrations or times. Where indicated, cells were pretreated with an inhibitor of ERK1/2 (PD98059 at indicated dose for 1 hour), an inhibitor of p38 MAPK (SB203580 at indicated dose for 1 hour), or PTX (100 ng/mL for 24 hours) prior to stimulation with S1P (5 μM for 24 hours). To detect the effect of TNF-α on S1P-mediated cytokine secretion, cells were pretreated with TNF-α for 24 hours and washed extensively prior to stimulation with S1P (5 μM for 24 hours). Cell culture supernatants were centrifuged (1000 × g for 5 min) to remove particulates and stored at −80°C until the cytokine ELISAs were performed. IL-8, IL-6, and MCP-1 were measured using human ELISA development kits (Duoset; R&D Systems, Minneapolis, MN). Each stimulation experiment was performed in quadruplicate.

Western Blot Analysis

To monitor the activation of ERK1/2, p38 MAPK, and C-Jun N-terminal Kinases (JNK), ARPE-19 cells were exposed to S1P (5 μM) for various times (5, 15, 30, 60 minutes). Where indicated, cells were pretreated with PD98059 (25 μM for 1 hour) or SB203580 (10 μM for 1 hour) prior to S1P stimulation (5 μM for 15 minutes). To determine whether TNF-α can regulate the expression of S1P<sub>3</sub> receptor, cells were cultured for 24 hours in the presence of TNF-α (10 ng/mL). Cells were lysed in boiling sample buffer (50 mM Tris/Cl [pH 6.8], 10% [v/v] glycerol, 50 mM dithiothreitol (DTT), 4% [v/v] SDS) and boiled for 7 to 10 minutes. Equal amounts of protein (40 μg) were separated by 10% SDS-PAGE.

Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked by 5% skim milk or 5% BSA at 37°C for 2 hours, and incubated with the primary phosphorylated or total antibodies against ERK1/2, p38 MAPK, JNK (Cell Signaling Technology, Beverly, MA) and S1P<sub>3</sub> (Epitomics, Inc., Burlingame, CA) at 4°C for 16 hours, followed by a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. The membranes were washed three additional times with Tris-Buffered saline and Tween 20 (TBST), and antibody-antigen complexes were revealed using an enhanced chemiluminescent (ECL) technique, according to the manufacturer’s instructions. Quantification was performed by computer with commercial software (ImageQuant; Molecular Dynamics, Sunnyvale, CA). Each stimulation experiment was performed in quadruplicate.

Statistical Analyses

One-way ANOVA and paired sample t-test were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL). Data are expressed as mean values ± SD. A value of P < 0.05 was considered significant.

### Table. Primers for PCR

<table>
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<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
<th>Product</th>
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<tr>
<td>S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>TGGCTGGCTACATCCCTCAT</td>
<td>AGCGCAGTGTGAGCTTCC</td>
<td>101 bp</td>
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<tr>
<td>S1P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>GACCAACTCAGGGAGCAGG</td>
<td>ACACGCTCACACACCACTAC</td>
<td>142 bp</td>
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<tr>
<td>S1P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>TCCATCATCTGGTGTCGGA</td>
<td>TACGGGACACCGCGATTAG</td>
<td>120 bp</td>
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<tr>
<td>S1P&lt;sub&gt;4&lt;/sub&gt;</td>
<td>GATGGGGAGCCCCCTG</td>
<td>GCACGACTCTGCACACACATGC</td>
<td>141 bp</td>
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<tr>
<td>S1P&lt;sub&gt;5&lt;/sub&gt;</td>
<td>TGACGGCGTCCTACGTG</td>
<td>AGCCCGGACAGGACTCAG</td>
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<tr>
<td>β-actin</td>
<td>GATGCGAAGGAGATCAGT</td>
<td>CGATCCACCGGATCTTG</td>
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bp, base pair.
RESULTS

S1P1–5 Receptor Expression in ARPE-19 Cells

To determine whether human ARPE-19 cells express all the known receptors for S1P, the mRNA expression of S1P1–5 receptors was investigated using both semiquantitative RT-PCR (Fig. 1A) and real-time PCR (Fig. 1B). As shown in Figure 1A, RT-PCR analysis confirmed the mRNA expression of S1P1–5 receptors in ARPE-19 cells. S1P3 was the most highly expressed, with lesser amounts of S1P1, S1P2, S1P4, and S1P5 (Fig. 1B).

Influence of S1P on the Production of Inflammatory Mediators

To determine the influence of S1P on the production of inflammatory mediators by ARPE-19 cells, the amount of IL-8, IL-6, and MCP-1 produced by these cells was assayed. The results showed that S1P significantly enhanced the level of IL-8, but did not influence the constitutive production of IL-6 and MCP-1 (data not shown) by these cells. As shown in Figure 2A, a significantly enhanced level of IL-8 was detected following stimulation with 0.1 to 5 \(\mu\)M S1P (\(P < 0.001\) for both 1 \(\mu\)M and 5 \(\mu\)M S1P versus nontreated ARPE-19 cells). ARPE-19 cell treatment with S1P (5 \(\mu\)M) resulted in a time-dependent IL-8 increase (Fig. 2B), which became apparent when cells had been incubated in the presence of 5 \(\mu\)M S1P for 8 hours.

Mediation of S1P-Enhanced IL-8 Production via PTX-Sensitive Pathway

It has been reported that S1P receptors couple to PTX-sensitive G proteins of the \(G_{i/0}\) family. To study whether the signal transduction involves coupling to \(G_{i}\) proteins, we next examined the IL-8 response in the presence or absence of PTX. ARPE-19 cells were preincubated with 100 ng/mL PTX for 24 hours. After rigorous washing, the cells were stimulated with 5 \(\mu\)M S1P. After 24 hours, IL-8 was measured in culture supernatants. As shown in Figure 3, PTX pretreatment for 24 hours effectively blocked (90.3 \(\pm\) 6.1\%) S1P-enhanced IL-8 secretion by ARPE-19 cells. These results indicate that S1P enhancement of IL-8 production by ARPE-19 cells depends on a \(G_{i}\) pathway.
Signaling Pathways Involved in the Biological Response to S1P

We next examined the ability of S1P to activate ERK1/2, p38 MAPK, and JNK signaling pathways and their involvement in the biological response to S1P. S1P enhanced the phosphorylation of ERK1/2 and p38 MAPK (Fig. 4). As shown in Figures 4A and 4B, enhanced phosphorylation of ERK1/2 (2.24 ± 0.1-fold) was detected within 5 minutes of exposure to S1P, while maximum phosphorylation (4.45 ± 0.3-fold) occurred at 15 minutes. The response was sustained up to 30 minutes and declined at 60 minutes. Similarly, S1P-activated p38 MAPK
(2.94 ± 0.1-fold) occurred within 5 minutes and peaked at 5 minutes. The response was sustained up to 30 minutes and attenuated at 60 minutes (Figs. 4C, 4D). No phosphorylation of JNK was detected following stimulation with S1P for up to 60 minutes (data not shown). These results indicated that S1P-induced IL-8 secretion was mediated through ERK1/2 and p38 MAPK pathway in ARPE-19 cells, but not JNK.

To further examine the signaling pathways involved in the activation of ERK1/2 and p38 MAPK, ARPE-19 cells were pretreated with 10 μM PD98059 (ERK1/2 inhibitor) or 25 μM SB203580 (p38 MAPK inhibitor). As expected, PD98059 blocked S1P-induced phosphorylation of ERK1/2 (Fig. 4E), and SB203580 similarly attenuated S1P-induced phosphorylation of p38 MAPK (Fig. 4F).

Treatment of ARPE-19 cells with PD98059 (ERK1/2 inhibitor) 1 hour before the S1P addition completely abolished S1P-induced release of IL-8 (Fig. 5A). The difference in the amount of IL-8 in medium between cells treated with and without PD98059 (positive control) was very significant; 10 μM and 25 μM PD98059 reduced S1P-mediated IL-8 secretion by 44.0 ± 3.2% (P = 0.003) and 72.5 ± 4.7% (n = 4, P < 0.001), respectively. The level of IL-8 in medium from PD98059 (25 μM)- and S1P-treated cells was slightly greater than that of negative controls (cells cultured without S1P, P < 0.001), indicating that the p38 inhibitor (SB203580) slightly inhibited the S1P-induced increase of the release of IL-8 by ARPE-19 cells (Fig. 5B).

Role of TNF-α in Regulation of S1P Receptor Expression

TNF-α is an important inflammatory molecule in multiple ocular disorders including AMD,②② uveitis,②③ and PVR.②④ The following experiments were designed to detect the expression of S1P receptors in response to TNF-α by real-time PCR and Western blot. Treatment of ARPE-19 cells with TNF-α (1, 10, and 100 ng/mL) upregulated the expression of S1P3 mRNA (Fig. 6A). A 2.03 ± 0.3-fold (P = 0.008) enhancement in S1P3 expression was observed with 10 ng/mL TNF-α. S1P1, S1P2, S1P4, and S1P5 expression was not upregulated by TNF-α under the same conditions (data not shown). Enhancement of S1P3 receptor expression at the protein level was identified by
Western blot (Fig. 6B). Our findings indicate that only S1P3 receptor expression is upregulated in TNF-α-activated ARPE-19 cells, whereas S1P5 receptor expression was observed with 10 ng/mL TNF-α.

**Effect of TNF-α on S1P-Induced Inflammatory Mediator Secretion**

To detect the role of TNF-α in S1P-induced inflammatory mediator production, ARPE-19 cells were primed with TNF-α. Starved ARPE-19 cells were treated with S1P (5 μM) for 24 hours with or without pretreatment with TNF-α (10 ng/mL) for 24 hours. Among the three cytokines tested, MCP-1 secretion was not enhanced in the presence of S1P (data not shown). As shown in Figure 7A, in contrast, the amount of IL-8 that was secreted following stimulation with S1P and, most importantly, S1P-mediated cytokine synthesis was significantly enhanced in TNF-α-primed ARPE-19 cells. The release of IL-8 was increased 1.41-fold \( (P = 0.001) \) in TNF-α-primed and S1P-stimulated samples as compared with unprimed cells stimulated with S1P. Moreover, S1P alone did not stimulate the secretion of IL-6; but in combination with TNF-α, it resulted in a significant enhanced secretion of this cytokine \( (P < 0.001) \) (Fig. 7B). The data suggest that the proinflammatory environment potentiates some of the functional outcomes of the S1P/S1P receptor signaling pathway in ARPE-19 cells.

**DISCUSSION**

The present study shows that ARPE-19 cells express all the known receptors for S1P. Among five subtypes of G protein-coupled S1P receptors, that is, S1P1, S1P2, S1P3, S1P4, and S1P5, we demonstrated abundant expression of S1P3 mRNA, whereas S1P4 mRNA was barely detectable. In the presence of its ligand S1P, ARPE-19 cells were readily stimulated to release IL-8. We furthermore demonstrated that the ERK1/2 and p38 MAPK pathways are involved in S1P-induced IL-8 release by these cells. Pretreatment of ARPE-19 cells with TNF-α increased S1P3 receptor expression, enhanced S1P-induced IL-8 production, and also stimulated the constitutive release of IL-6. Taken together, these data indicate that S1P can augment local inflammatory reactions mediated by RPE cells.

S1P, a potent bioactive sphingolipid, mediates a number of cellular responses, including cell proliferation, chemotaxis, and angiogenesis. S1P binds to five related GPCRs, termed S1P1-5, S1P3, S1P2, and S1P5 receptors are widely expressed in the immune, cardiovascular, and central nervous systems, with S1P1 being the dominant receptor also on lymphocytes/leukocytes. S1P3 is specifically expressed in lymphoid tissue, and S1P5 is present in spleen and white matter tracts of the central nervous system. The major source of S1P is that produced from sphingosine through the action of sphingosine kinase (SphK1–2). Inflammatory mediators, such as C5a and TNF-α, have been found to stimulate the synthesis and secretion of S1P upon activation of sphingosine kinase. Moreover, a reservoir of S1P is stored in and potentially released from red blood cells, platelets, and mast cells. While human choroidal and retinal cells express SphK1, as a potential source for S1P in the posterior segment of the eye, we suggest that the RPE layer could be the major source of S1P in inflamed eyes.

Although S1P signaling has been characterized in multiple tissue and organ systems, very few studies have investigated whether S1P has the potential to be a mediator of cellular signaling and function in the eye. Recent findings that S1P can promote RPE cell proliferation and that it stimulates choroidal and retinal neovascularization prompted us to examine whether S1P could also play a role in ocular inflammation.

RPE cells are multifunctional cells that are uniquely positioned within the eye. They have properties similar to those of macrophages, among which are the capacity to phagocytose and the ability to generate several cytokines including IL-6, IL-8, and MCP-1. IL-8 and MCP-1 are known to have strong chemotactic activity for polymorphonuclear cells (PMN) and monocytes/macrophages, respectively. Since the infiltration of both cell types is commonly seen in many ocular diseases, these cytokines may play important roles in the initial stage of inflammation. IL-6 is an important mediator of the acute-phase response and possesses biological functions that support host immune reactions. There is evidence that IL-6, IL-8, and MCP-1 play an important role in the pathogenesis of retinal diseases such as AMD, PVR, and uveitis.

Virtually all cells that participate in immune responses express multiple S1P receptors. A recent study showed that S1P signaling through the S1P1 receptor can control T-cell migration and tissue distribution, as well as the initiation of early events in the differentiation of T cells into effector cells. In experimental autoimmune uveoretinitis (EAU), controlling S1P1 activation via fingolimod blocks both T-cell entry and requisite signals for recruitment to the tissue, and thus also...
blocks subsequent mononuclear cell activation within the tissue.\textsuperscript{17,18}

We showed that the receptors S1P\textsubscript{1}, S1P\textsubscript{3}, S1P\textsubscript{4}, and S1P\textsubscript{5}, but mainly S1P\textsubscript{3}, could be detected in human ARPE-19 cells. These observations explain the effects of S1P on ARPE-19 cells. S1P significantly stimulates ARPE-19 cells to secrete IL-8, but did not influence the constitutive production of IL-6 and MCP-1. We investigated whether SIP-enhanced IL-8 production by ARPE-19 cells used S1P/S1P receptor signaling pathways. Lysosphospholipid receptors exhibit differential coupling to heterotrimeric G proteins. For instance, S1P\textsubscript{1} and S1P\textsubscript{4} couple directly to the G\textsubscript{12/13} pathway, whereas S1P\textsubscript{2} and S1P\textsubscript{3} stimulate the G\textsubscript{o}, G\textsubscript{q}, and G\textsubscript{12/13} pathways.\textsuperscript{43} S1P\textsubscript{3} activates both G\textsubscript{o} and G\textsubscript{12/13} proteins.\textsuperscript{34} In this study, we showed that 100 ng/mL PTX pretreatment of RPE cells significantly prevented S1P-induced IL-8 secretion, so we can affirm that S1P-induced IL-8 production is dependent on G\textsubscript{i} protein-mediated signaling response, which is probably mediated by the interaction with S1P\textsubscript{3}.

TNF-\alpha plays a key role in the pathogenesis of many ocular diseases. Recently it was reported that TNF-\alpha can cross-talk with the G protein-coupled receptor adenosine A\textsubscript{2A} receptor.\textsuperscript{45} Because S1P receptors are coupled to G proteins, we tried to determine whether the cross-talk between TNF-\alpha and G protein-coupled receptors is present in ARPE-19 cells. We detected a significant enhancement of S1P-induced cytokine secretion, including IL-6 and IL-8, in ARPE-19 cells pretreated with TNF-\alpha. However, the amount of MCP-1 expression was not increased when cells were treated with S1P and TNF-\alpha. These results are in accordance with previous research,\textsuperscript{46} which showed that S1P and TNF-\alpha signaling pathway synergize. The enhancement in S1P-induced cytokine secretion by TNF-\alpha can be partly explained by regulation of the expression of the S1P\textsubscript{3} receptor by TNF-\alpha. Although many genes regulated by TNF-\alpha have been confirmed in ARPE-19 cells, this is the first demonstration of regulation of S1P\textsubscript{3} receptor expression by TNF-\alpha, proposing the possibility of a causal connection between enhanced increased S1P\textsubscript{3} receptor expression and cytokine production by S1P pretreatment of ARPE-19 cells with TNF-\alpha.

SIP has been reported to act as an important mediator through activation of MAPK cascades in different cell types.\textsuperscript{47} This family mainly consists of ERK1/2, JNK, and p38 kinase pathways. Therefore, we investigated the role of ERK1/2, JNK, and p38 MAPK involved in IL-8 expression in RPE cells. In this study, we found that S1P induces phosphorylation of ERK1/2 and p38 MAPK in ARPE-19 cells, but not JNK. On the other hand, our results also demonstrated that activation of ERK1/2 and p38 MAPK was necessary for S1P-induced IL-8 expression in RPE cells. PD98059 and SB203580, selective inhibitors of ERK1/2 and p38 MAPK, significantly attenuated S1P-induced IL-8 secretion and ERK1/2 and p38 MAPK phosphorylation in RPE cells. In addition, an ERK1/2 inhibitor could completely abolish the S1P-induced increase of IL-8 release by RPE cells, indicating that the stimulation of IL-8 production in ARPE-19 cells by S1P mainly occurs through the activation of the ERK1/2 signaling pathway, which is consistent with previous studies on other cell types.\textsuperscript{48}

It is worthwhile to point out that there are some limitations of our study. Our experiments were performed with the ARPE-19 cell line, and we did not investigate whether there was a different effect of S1P on cytokine production using other types of RPE cells such as fetal or primary cultures of these cells.

In conclusion, we have reported that IL-8 production is significantly induced by S1P in human ARPE-19 cells and that this effect is mediated by ERK1/2 and p38 MAPK-dependent signal transduction. The present study expands earlier studies on the role of S1P in ocular disease and provides a rationale for the use of inhibitors of this pathway to control intraocular inflammation.

**Acknowledgments**

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**References**


