Specific and overlapping sphingosine-1-phosphate receptor functions in human synoviocytes: impact of TNF-α

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Abstract **Sphingosine-1-phosphate (S1P), via interaction with its G protein-coupled receptors, regulates various physiological and pathological responses. The present study investigated the role of S1P/S1P receptor signaling in several functional responses of human fibroblast-like synoviocytes (FLSs) that may contribute to the pathogenesis of rheumatoid arthritis (RA). We report that FLSs express the $S1P_1$, $S1P_2$, and $S1P_3$ receptors. Moreover, exogenously applied S1P induces FLS *1*) migration, *2*) secretion of inflammatory cytokines/chemokines, and *3*) protection from apoptosis. Using specific S1P receptor agonists/antagonists, we determined that S1P stimulates FLS migration through $S1P_1$ and $S1P_3$, induces cytokine/chemokine secretion through $S1P_2$ and $S1P_3$, and protects from cell apoptosis via $S1P_1$. The S1P-mediated cell motility and cytokine/chemokine secretion seem to be regulated by the p38 mitogen-activated protein kinase (MAPK), p42/44 MAPK, and Rho kinase signal transduction pathways. Interestingly, treatment of FLSs with tumor necrosis factor-α increases $S1P_3$ expression and correlates with the enhancement of S1P-induced cytokine/chemokine production. Our data suggest that $S1P_1$, $S1P_2$, and $S1P_3$ play essential roles in the pathogenesis of RA by modulating FLS migration, cytokine/chemokine production, and cell survival. Moreover, the cytokine-rich environment of the inflamed synovium may synergize with S1P signaling to exacerbate the clinical manifestations of this autoimmune disease.**—Zhao, C., M. J. Fernandes, M. Turgeon, S. Tancrède, J. Di Battista, P. E. Poubelle, and S. G. Bourgoin. **Specific and overlapping sphingosine-1-phosphate receptor functions in human synoviocytes: impact of TNF-α. *J. Lipid Res.* 2008.** 49: **2323–2337.**

Supplementary key words inflammation • G protein-coupled receptor • interleukin-8 • cell migration • apoptosis • mitogen-activated protein kinase • Rho kinase

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that primarily affects diarthroidal joints, leading to their progressive destruction. The disease is characterized by hyperplasia of fibroblast-like synoviocytes (FLSs) and a massive infiltration of inflammatory cells in the affected joint. FLSs comprise the synovial lining, a thin membrane in direct contact with cartilage and bone. In RA, FLSs play a key role in the pathogenesis (as reviewed in Ref. 1). FLSs increase in number, invade adjacent tissues, and produce proinflammatory cytokines, chemokines, and matrix metalloproteinases that promote inflammation and joint destruction (2). Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), appear to play a key role in the stimulation of FLSs toward this aggressive phenotype. There is mounting evidence, however, that the activation of FLSs can be maintained in the absence of inflammatory cytokines (3), suggesting the contribution of other proinflammatory molecules to the pathogenesis of RA.

Sphingosine-1-phosphate (S1P) is a biologically active sphingolipid that transmits potent signals through five G protein-coupled EDG (endothelial differentiation gene) receptors, namely, $S1P_1$/EDG-1, $S1P_2$/EDG-5, $S1P_3$/EDG-3, $S1P_4$/EDG-6, and $S1P_5$/EDG-8 (4). Among the five S1P receptors, $S1P_1$, $S1P_2$, and $S1P_3$ are widely expressed in various tissues, whereas the expression of $S1P_4$ and $S1P_5$ is mainly confined to cells of the immune system and nervous system, respectively (5). S1P receptors activate a variety of heterologous signaling pathways through coupling with

This project is supported by research grants from Canadian Institutes of Health Research and the Arthritis Society of Canada (S.G.B.) and the Canadian Arthritis Network Post-Doctoral Fellowship Award (C.Z.).

Manuscript received 17 March 2008 and in revised form 19 June 2008 and in re-revised form 18 July 2008.

Published, JLR Papers in Press, July 24, 2008.
DOI 10.1194/jlr.M800143-JLR200

Abbreviations: COX-2, cyclooxygenase-2; EDG, endothelial differentiation gene; FLS, fibroblast-like synoviocyte; GM-CSF, granulocyte-monocyte colony-stimulating factor; IL, interleukin; IP-10, interferon-γ-inducible protein 10; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; PGE_2, prostaglandin E_2; RA, rheumatoid arthritis; RANTES, regulated on activation normal T cells expressed and secreted; S1P, sphingosine-1-phosphate; SNP, sodium nitroprusside; TNF-α, tumor necrosis factor-α.

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multiple G proteins (G_q, $G_{i/o}$, and $G_{12/13}$) that regulate both physiological (cell growth, differentiation, migration, and survival) and pathological processes (angiogenesis, cancer, and autoimmunity) (as reviewed in Ref. 6).

Activated platelets are the main source of S1P (7). However, other cell types, such as neutrophils and mononuclear cells, also produce S1P (8, 9). S1P synthesis and secretion are stimulated by inflammatory mediators such as TNF-α (10). Virtually all cells that participate in immune responses express multiple S1P receptors (as reviewed in Ref. 11). Whereas S1P enhances the expression of inflammation-related genes (12), S1P signaling through the $S1P_1$ receptor also controls T cell migration and tissue distribution, as well as the initiation of early events in the differentiation of T cells into effector states (13).

Several lines of evidence strongly suggest that S1P contributes to the pathogenesis of RA. S1P is produced by neutrophils, the most abundant cell type in synovial fluids of RA patients. Also, TNF-α, a cytokine that plays a key role in the pathogenesis of RA, and other inflammatory mediators induce the synthesis and secretion of S1P. High levels of S1P have, indeed, been reported in the synovial fluid of patients with RA (14). Not only is the synthesis and secretion of S1P induced by proinflammatory mediators but, in turn, S1P also triggers the expression of inflammatory genes such as cyclooxygenase-2 (COX-2), and a superproduction of prostaglandin E_2 (PGE_2) by FLSs (14). Moreover, the expression of sphingosine kinase, the enzyme that generates S1P by phosphorylation of sphingosine (15), is significantly increased in the cells derived from RA B lymphoblastoid cells (16). These findings from in vitro experiments ought to be at least partly representative of the in vivo physiological function of S1P in the RA synovium.

Because FLS activation is partly cytokine-independent and S1P is known to induce gene expression in FLSs, we sought to fully characterize the role of the S1P/S1P receptor signaling pathway in FLS function. The objective of the present study was thus to determine whether the S1P/S1P receptor signaling pathway is involved in RA. Herein, we report that FLSs express the $S1P_1$, $S1P_2$, and $S1P_3$ receptors. We also provide evidence that these S1P receptors trigger specific and overlapping functional responses. $S1P_1$ was essential for the survival of FLSs, and $S1P_1$ and $S1P_3$ stimulated FLS migration. In contrast, the activation of $S1P_2$ and $S1P_3$ enhanced cytokine/chemokine secretion. In addition, $S1P_3$ expression by FLSs was enhanced by TNF-α, and S1P-mediated chemokine secretion was super-induced in TNF-α-primed FLSs. These results suggest that in addition to $S1P_1$, $S1P_2$ and $S1P_3$ may also play important roles in the pathogenesis of RA.

MATERIALS AND METHODS

Reagents

S1P was purchased from Biomol (Plymouth Meeting, PA). The specific $S1P_{1/3}$ receptor antagonist VPC23019 was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The $S1P_1$ agonist SEW2871, the $S1P_2$ antagonist JTE-013, and the $S1P_3$ antagonist CAY10444, were from Cayman Chemical (Ann Arbor, MI). TNF-α was from PeproTech, Inc. (Rocky Hill, NJ). Human IL-8 and IL-6 ELISA kits were purchased from BioSource International, Inc. (Camarillo, CA). The apoptosis assay kit (Annexin V-FITC) was from BD Pharmingen (Oakville, ON, Canada). The cell proliferation assay kit (CellTiter 96® Aqueous One Solution Cell Proliferation) was purchased from Promega (Madison, WI). Sodium nitroferricyanide (III) dihydrate [sodium nitroprusside (SNP)], and SYBR® Green JumpStart™ Ready Mix kit were obtained from Sigma (St. Louis, MO). TRIzol Reagent was from Invitrogen (Burlington, ON, Canada). Inhibitors of p42/44 mitogen-activated protein kinase (MAPK) PD98059, of p38 MAPK SB203580, and of Rho kinase Y27632 were purchased from Calbiochem (San Diego, CA). $S1P_1$ and $S1P_3$ antibodies were from Cayman Chemical. Antibodies to total and phosphorylated forms of p42/44 MAPK, of p38MAPK, and of JNK were purchased from Cell Signaling Technology (Beverly, MA). The human cytokine/chemokine LINCOplex kit was purchased from Linco Research (St. Charles, MO). Cell culture reagents were purchased from Wisent, Inc. (St-Bruno, QC, Canada).

Cell culture

Human primary FLSs were obtained from RA patients, who were diagnosed according to the criteria developed by the American College of Rheumatology (17) and were undergoing joint surgery on the knee or hip. Cells were maintained under standard conditions (37°C and 5% CO_2) and grown in DMEM supplemented with 10% FBS, penicillin (100 IU), and streptomycin (100 μM). Cells were used at passages 5 to 15.

Cell treatment

Semi-confluent cells were starved with serum-free medium for 24 h 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 in the details presented below.

Semi-quantitative RT-PCR and real-time PCR analyses

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNA (0.5–1 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 and PCR conditions are as follows: $S1P_1$ (778 bp product), sense, 5′-GGA-AGG-GAG-TAT-GTT-TGT-GGC-3′, antisense, 5′-TGA-CGT-TTC-CAG-AAG-ACA-TA-3′; $S1P_2$ (425 bp product), sense, 5′-AGT-GGC-CAT-TGC-CAA-GGT-CAA-G-3′, antisense, 5′-TAG-TGG-GCT-TTG-TAG-AGG-A-3′; $S1P_3$ (466 bp product), sense, 5′- AGG-GAG-GGC-AGT-ATG-TTC-G-3′, antisense, 5′-GCC-ACA-TCA-ATG-AGG-AAG-AGG-AT-3′; $S1P_4$ (672 bp product), sense, 5′-ATC-ACG-CTG-AGT-GAC-CTG-CTC-A-3′, antisense, 5′-TGC-GGA-AGG-AGT-AGA-TGA-3′; and $S1P_5$ (658 bp product), sense, 5′-CTA-CTG-TCG-GGG-CCG-CTC-AC-3′, antisense, 5′-CGG-TTG-GTG-AAC-GTG-TAG-ATG-A-3′. To ensure linear cDNA amplification, different amplifying cycles were tried. The experiments revealed linear amplification within 35 cycles. A total of 35 PCR cycles were run at 94°C (denaturation, 1 min), 63°C for $S1P_{1-3}$, 61°C for $S1P_{4-5}$ (annealing, 1 min), and 72°C (extension, 1 min). The ribosomal protein RPLP0 mRNA was used as an internal PCR control. RPLP0 (248 bp product) primer sequences are as follows: sense, 5′-GTT-GTA-GAT-GCT-GCC-ATT-G-3′, antisense, 5′-CCA-TGT-GAA-GTC-ACT-GTG-C-3′. The PCR products were subjected to electrophoresis on an 0.8% agarose gel and visualized by ethidium bromide staining. Densitometry analysis was used for band quantification using the software Alphamage

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2000. The results were expressed as a ratio of the band intensity relative to the corresponding RPLP0 band obtained by amplification of the same template cDNA. Semi-quantitative real-time PCR was also conducted to examine the mRNA expression of $S1P_{1-3}$ receptors. In real-time PCR experiments, we used the same primers as for RT-PCR to amplify $S1P_{1-3}$. The thermal cycling conditions were as follows: 95°C (initial denaturation, 3 min) followed by 40 cycles of 95°C (denaturation, 15 s), 63°C for $S1P_{1-3}$ (annealing, 20 s), and 72°C (extension, 20 s).

Western blot analyses

To monitor the activation of p42/44 MAPK, p38 MAPK, and JNK, cells were exposed to S1P (5 μM) for various times (5–30 min). Where indicated, cells were pretreated with PD98059 (25 μM), SB203580 (10 μM), and Y27632 (10 μM) for 30 min prior to S1P stimulation (5 μM, 15 min). Cells were lysed in boiling sample buffer [50 mM Tris/HCL (pH 6.8), 10% (v/v) glycerol, 50 mM DTT, 4% (v/v) SDS] and boiled for 7–10 min. Equal amounts of protein (50 μg) were separated by 10% SDS-PAGE.

For analysis of $S1P_1$/$S1P_3$ receptor expression, cell membrane fractions were prepared as described previously (18). Briefly, cells were treated with accutase, harvested in ice-cold KCl-Hepes relaxation buffer (50 mM Hepes, 100 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM PMSF, and 1 mM sodium orthovanadate, adjusted to pH 7.2), and sonicated for 20 s, and the samples were centrifuged for 7 min at 1,000 *g*. Unbroken cells and nuclei were discarded, and the supernatants were ultracentrifuged at 180,000 *g* for 45 min in a Beckman TL-100 ultracentrifuge. Membrane pellets were washed once and resuspended in a small volume of solubilization buffer containing 0.25 M Na_2HPO_4, 0.3 M NaCl, 2.5% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM PMSF, and samples were assayed for protein content with Pierce Coomassie Brilliant Blue Protein Assay reagent. To determine whether TNF-α can regulate the expression of $S1P_3$, cells were cultured for 24 h in the presence of TNF-α (100 ng/ml). Protein samples (150 μg) were separated by gradient (7.5–20%) SDS-PAGE.

Proteins were later transferred from polyacrylamide gel to methanol-soaked Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Primary antibody incubation was performed overnight at 4°C. The membranes were then washed three times and incubated with appropriate HRP-conjugated secondary antibodies at room temperature for 1 h. Membranes were washed three times, and antibody-antigen complexes were revealed using ECL® according to the manufacturer's instructions (Perkin Elmer Life Sciences, Boston, MA). The ECL signal was visualized by developing the signal on a film.

Wound-closing assay

Cells were plated at a concentration of 5×10^4 cells/ml in 12-well plates. After routine starvation, a plastic pipette tip (200 μl) was drawn across the center of the well to produce a clean wound area. Free cells were removed and the medium was replaced with serum-free medium containing S1P, selective agonists/antagonists of S1P receptors, and the specific inhibitors of p44/42 MAPK, p38 MAPK, and Rho kinase at indicated concentrations. Immediately following scratch wounding (0 h) and after incubation for 24 h, the wound-closing process was photographed with an inverted microscope (Nikon TE300). The cells that had migrated into the wound area were examined and monitored with MetaMorph software.

ELISA and Luminex 100 multiplex array assay

Cells plated at a concentration of 5×10^4 cells/ml in 24-well plates were stimulated with S1P or the $S1P_1$ agonist SEW2871 at indicated concentrations or times. Where indicated, cells were pretreated with the S1P receptor antagonists or the inhibitors of p42/44 MAPK, p38 MAPK, and Rho kinase for 30 min prior to stimulation with S1P. To evaluate the effect of TNF-α on S1P-mediated cytokine secretion, cells were pretreated with TNF-α for 2 h, 8 h, and 24 h and washed extensively prior to stimulation with S1P (5 μM, 24 h). Cell culture supernatants were collected and stored at −80°C until the cytokine ELISAs were performed. IL-8 and IL-6 in all samples were monitored in duplicate, according to the manufacturer's protocol. Optical densities were determined using a SoftMaxPro40 plate reader at 450 nm. The results were compared with a standard curve that was generated using known concentrations (pg/ml) of IL-8 and IL-6. The results were expressed in pg/ml.

TABLE 1. Cytokine/chemokine secretion in cell culture supernatants

	Treatment											
	Non-treated		S1P			TNF-α Priming			TNF-α Priming+S1P			
Cytokine/Chemokine	Mean	SD	Mean	SD	*P* Value vs. NT	Mean	SD	*P* Value vs. NT	Mean	SD	*P* Value vs. NT	
	pg/ml		*pg/ml*			*pg/ml*			*pg/ml*			
IL-1α	N/A		N/A			N/A			N/A			
IL-1β	N/A		N/A			N/A			N/A			
IL-8	N/A		260.7	30.96	[a]	90.98	22.77		1,306	219.9	[b]	
IL-15	N/A		N/A			N/A			N/A			
Eotaxin	N/A		N/A			N/A			N/A			
GM-CSF	N/A		N/A			N/A			N/A			
IP-10	N/A		N/A			N/A			250.1	54.97	[b]	
MCP-1	18.03	2.424	1,113	95.43	[b]	423.3	72.38	[a]	1,869	351.9	[b]	
MIP-1α	N/A		N/A			N/A			N/A			
RANTES	N/A		43.77	4.302		482.2	117.3		2,855	488.6	[b]	

IL, interleukin; GM-CSF, granulocyte-monocyte colony-stimulating factor; IP-10, interferon-γ-inducible protein 10; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; RANTES, regulated on activation normal T cells expressed and secreted; S1P, sphingosine-1-phosphate; N/A: not available (undetectable). Effect of TNF-α priming on S1P-induced cytokine/chemokine secretion. Cells were pretreated with TNF-α (100 ng/ml) for 8 h prior to stimulation with 5 μM S1P for 24 h as described in Materials and Methods. Cell culture supernatants were harvested to monitor cytokine/chemokine secretion using Luminex. Results are presented as means ± SD (n = 2). For statistical comparative analyses, we compared nontreated cells to S1P/TNF-α/TNF-α+S1P-treated cells, respectively.

[a] $P < 0.05$.

[b] $P < 0.01$.

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For the Luminex 100 multiplex array assay, cells were stimulated with S1P (5 μM, 24 h) with or without pretreatment of TNF-α (100 ng/ml, 8 h), and cell supernatants were collected for multiple cytokine and chemokine analyses. The multiplex bead-based sandwich immunoassay kit was used to measure the levels of IL-1α, IL-1β, IL-8, IL-15, Eotaxin, granulocyte-monocyte colony-stimulating factor (GM-CSF), interferon-γ-inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β, and regulated on activation normal T cells expressed and secreted (RANTES).

Apoptosis assay and proliferation assay

For the apoptosis assay, cells were stimulated with 5 μM S1P or 0.1–3 μM SEW2871 for 8 h prior to stimulation with 1 mM SNP for an additional 16 h. Cells were then harvested, and apoptosis was evaluated using Annexin V-FITC labeling according to the manufacturer's instructions. Cells were then analyzed by flow cytometry. For the proliferation assay, cells were cultured with or without S1P (1 nM–5 μM) in triplicate in 96-well microplates at a concentration of 2×10^5/ml in DMEM without serum. After 96 h, cell viability was assessed by measuring the conversion of the CellTiter 96® Aqueous One Solution Reagent [3-(4,5-dimethythizol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] to a water-soluble formazan product that absorbs light at 490 nm and is proportional to the number of live cells.

Statistical analysis

Unless otherwise stated, experiments were performed three times and results presented are expressed as mean values ± SE. In **Table 1** and Fig. 10C, results are expressed as mean values ±

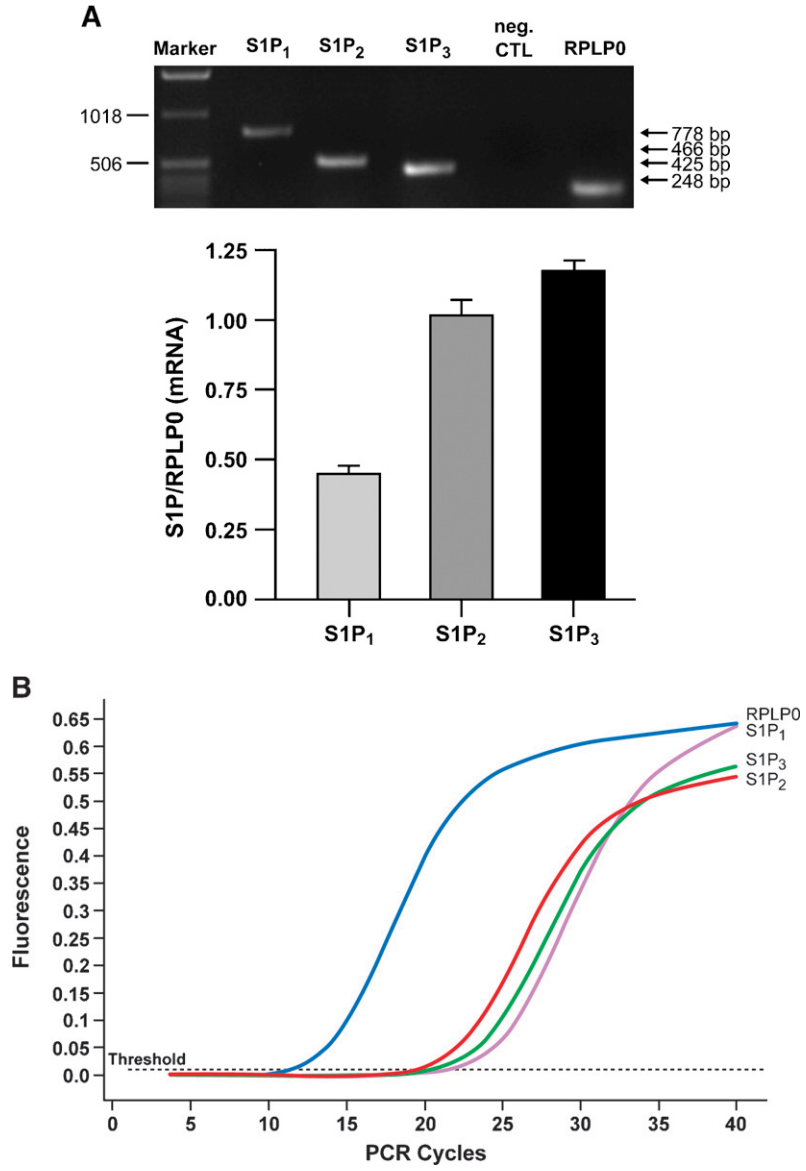

Fig. 1. Expression of sphingosine-1-phosphate$_1$ ($S1P_1$), $S1P_2$, and $S1P_3$ mRNA in human fibroblast-like synoviocytes (FLSs). Semi-quantitative RT-PCR (A) and real-time PCR (B) analyses of $S1P_1$, $S1P_2$, and $S1P_3$ mRNA expression in FLSs. Total RNA from resting-state FLSs was extracted for RT-PCR or real-time PCR. The reaction performed without oligonucleotide primers was used as a negative control, and RPLP0 was used as an internal control. Data shown are representative of three separate experiments and are expressed as means ± SE.

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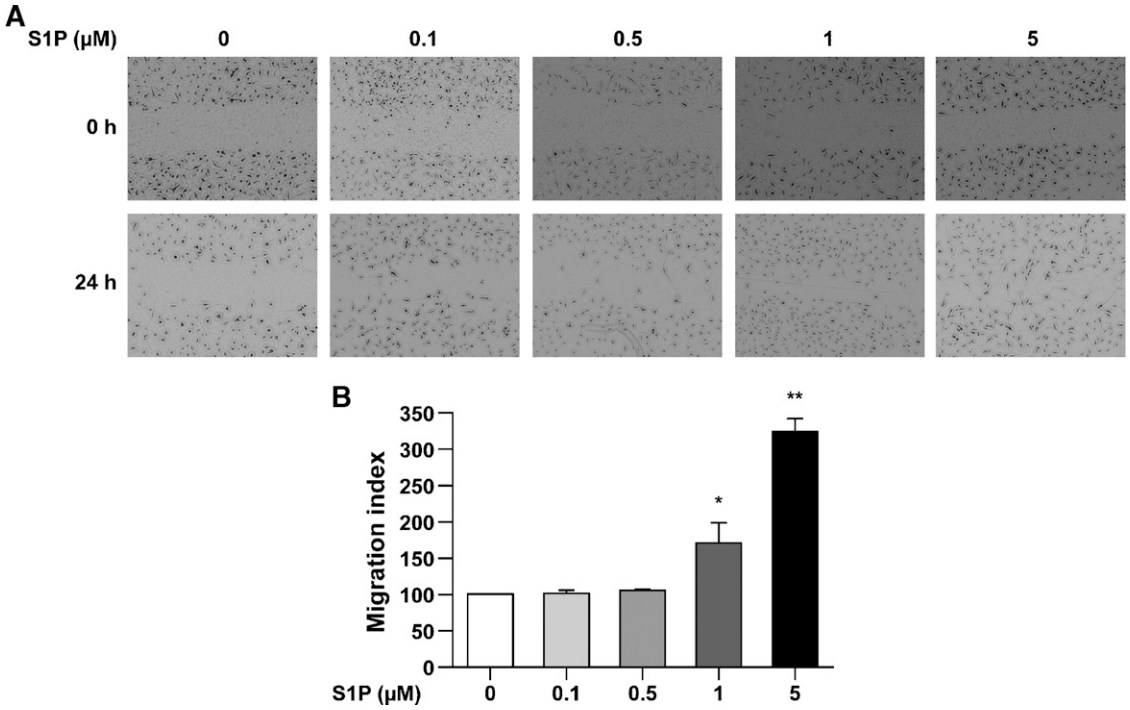

Fig. 2. Stimulation of human FLS motility by S1P. A clean wound area was made on a monolayer of FLSs. After free cells were removed, the wound was allowed to close for 24 h in serum-free medium containing S1P (0.1–5 μM). The wound-closing process was photographed at 0 h and at 24 h (A). The data shown are representative of three separate experiments. The migration index corresponds to the percentage of cells that migrated in the presence of S1P over that of nontreated cells (B). For statistical comparative analyses, we compared cells treated with and without S1P. Data shown are means ± SE of three independent experiments. $*P < 0.05$; $**P < 0.01$.

SD. Statistical significance of the difference between treated and untreated samples was determined by *t*-test (two-tailed *P* value). For the dose response and time course studies, statistical significance between untreated samples and samples treated with S1P, $S1P_1$ receptor agonist, TNF-α, and kinase inhibitors (dose response experiments) and between samples treated at 0 h with those treated at indicated time points (time course study) was determined by ANOVA, Dunnett's Multiple Comparison Test. Calculations were made with the Prism 4.0 software. *P* values less than 0.05 were considered statistically significant.

RESULTS

$S1P_1$, $S1P_2$, and $S1P_3$ receptor expression in human FLSs

Because the biological activity of S1P is via interaction with specific cell surface receptors, we examined whether human FLSs express all the known receptors for S1P. The expression of $S1P_1$, $S1P_2$, and $S1P_3$ receptor transcripts in FLSs was investigated by using both semi-quantitative RT-PCR (**Fig. 1A**) and real-time PCR (Fig. 1B). Oligonucleotide primers designed for $S1P_4$ and $S1P_5$ did not yield a detectable signal in human FLSs but amplified the expected 672 bp ($S1P_4$) and 658 bp ($S1P_5$) bands in human monocytes that were used as positive controls (data not shown).

To confirm the expression of these receptors at the protein level, Western blot analysis was performed using purified membrane fractions of FLSs. The only antibodies available for Western blot analysis are those for the $S1P_1$ and $S1P_3$ receptors. As a positive control, cDNAs coding for the hemagglutinin (HA)-tagged $S1P_1$ and $S1P_3$ receptors were expressed in CHO cells. As shown in Fig. 10C, an expression of $S1P_3$ (47 kD) was detected in FLSs. $S1P_1$ expression, however, was lower than the threshold for detection by the $S1P_1$ antibody (data not shown).

Induction of human FLS migration by S1P via $S1P_1$ and/or $S1P_3$ receptors

S1P is known to induce cell migration. We therefore investigated whether S1P could enhance the migration of FLSs using a wound-closing assay. As shown in **Fig. 2**, cell migration was stimulated by S1P at a concentration of 1 μM or 5 μM, by 1.7 ± 0.3-fold ($P < 0.05$) and 3.2 ± 0.2-fold ($P < 0.01$) above the nontreated control, respectively.

Because FLSs express the $S1P_1$, $S1P_2$, and $S1P_3$ receptors, the contribution of each of these receptors to S1P-mediated cell motility was then examined using selective S1P receptor agonists/antagonists (**Table 2**). The $S1P_1$-

TABLE 2. S1P receptor agonists/antagonists

Name	Receptor	Agonists/antagonists
SEW2871	$S1P_1$	Agonist
VPC23019	$S1P_{1/3}$	Antagonist
JTE-013	$S1P_2$	Antagonist
CAY10444	$S1P_3$	Antagonist

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specific agonist SEW2871 stimulated FLS migration in a concentration-dependent manner (**Fig. 3A**). SEW2871 at 1 μM, 5 μM, and 10 μM increased cell motility by 1.8 ± 0.01-fold, 3.3 ± 0.3-fold ($P < 0.01$), and 3.5 ± 0.4-fold ($P < 0.01$) when compared with nonstimulated FLSs, respectively. The specific $S1P_{1/3}$ antagonist VPC23019 and the $S1P_3$ antagonist CAY10444 decreased S1P-induced cell motility by 90.8 ± 9.3% ($P < 0.01$) and 84.4 ± 4.7% ($P < 0.01$), respectively (Fig. 3B). In contrast, the $S1P_2$ antagonist JTE-013 had no significant effect on cell motility induced by S1P ($P = 0.74$; Fig. 3B). It should be noted that the S1P receptor agonists/antagonists used throughout our study did not affect cell viability, as measured using Annexin V/PI staining (data not shown).

S1P-mediated production of IL-8 and IL-6 through $S1P_2$ and $S1P_3$ in human FLSs

Although S1P was recently shown to induce the expression of inflammatory genes in FLSs such as COX-2 (14), the release of proinflammatory chemokines in response

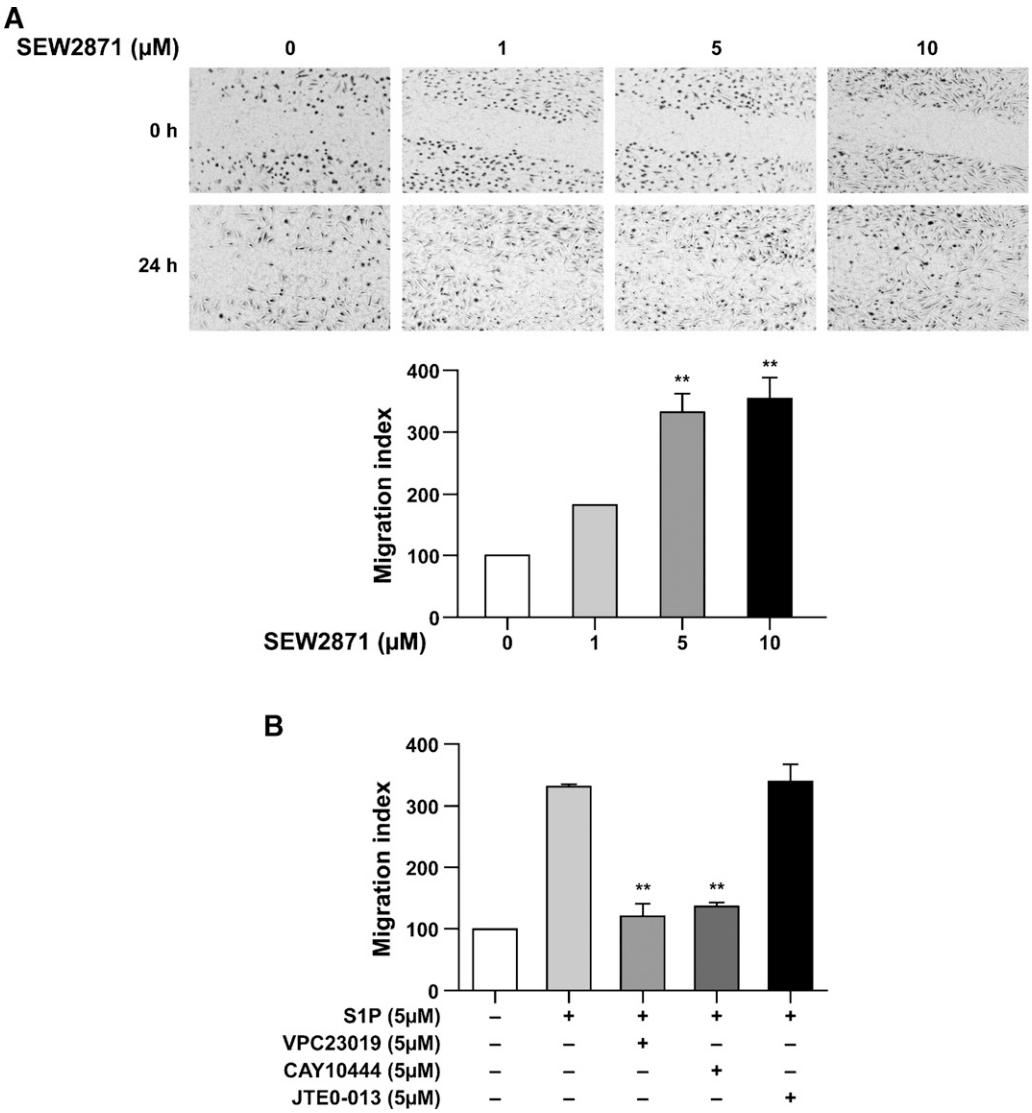

Fig. 3. Effect of selective S1P receptor agonists/antagonists on FLS motility. A: Effect of $S1P_1$ agonist SEW2871 on cell motility. After scratching the monolayer and removing free cells, the wound was allowed to close for 24 h in serum-free medium containing SEW2871 (1–10 μM). The wound-closing process was photographed at 0 h and at 24 h (upper panels). The data shown are representative of three separate experiments. Migrated cell numbers were expressed as percentage of nontreated cells (lower panel). For statistical comparative analyses, we compared cells treated with and without SEW2871. B: Effect of the $S1P_{1/3}$ antagonist VPC23019, $S1P_2$ antagonist JTE-013, and $S1P_3$ antagonist CAY10444 on cell motility. The wound was allowed to close for 24 h in serum-free medium containing S1P (5 μM) in the presence or absence of VPC23019 (5 μM), JTE-013 (5 μM), and CAY10444 (5 μM). Migrated cell numbers were expressed as percentage of nontreated cells. For statistical comparative analyses, samples treated with S1P were compared with those treated with S1P+VPC23019/JTE-013/CAY10444. Data shown are means ± SE of three independent experiments. $^{**}P < 0.01$.

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to stimulation of FLSs with S1P has not yet been investigated. We chose to investigate IL-8 and IL-6 because IL-8 is one of the most abundant chemokines in RA synovial tissues and both IL-8 and IL-6 are potent chemoattractants that are involved in the recruitment of inflammatory cells into the synovium (19). As shown in **Fig. 4**, a significant release of IL-8 (Fig. 4A) was detected with 2–5 μM S1P ($P < 0.01$ for both 2 μM and 5 μM S1P vs. nontreated FLSs). S1P-mediated IL-8 (Fig. 4C) secretion increased in a linear manner up to 48 h, the last time point tested. S1P also stimulated IL-6 secretion in a concentration- and time-dependent manner (Fig. 4B, D).

The contribution of each S1P receptor in S1P-mediated IL-8 and IL-6 production was then evaluated using specific S1P receptor agonists/antagonists. Interestingly, the $S1P_1$ agonist SEW2871 had no significant stimulatory effect on IL-8 and IL-6 secretion by FLSs (data not shown). In contrast, the $S1P_{1/3}$ antagonist VPC23019, the $S1P_2$ antagonist JTE-013, and the $S1P_3$ antagonist CAY10444 significantly decreased S1P-induced IL-8 and IL-6 secretion (**Fig. 5**). S1P-induced IL-8 secretion was inhibited by 58 ± 9% with VPC23019 ($P < 0.01$), 85 ± 3% with JTE-013 ($P < 0.01$), and 82 ± 6% with CAY10444 ($P < 0.01$) (Fig. 5A). VPC23019, JTE-013, and CAY10444 also inhibited S1P-induced IL-6 secretion by 76 ± 0.6% ($P < 0.01$), 69 ± 3% ($P < 0.05$), and 71 ± 2% ($P < 0.01$), respectively (Fig. 5B). Taken together, the data suggest a role for $S1P_2$ and $S1P_3$ but not $S1P_1$ in S1P-mediated cytokine/chemokine synthesis.

Protective effect of S1P on SNP-mediated FLS apoptosis

Abnormal proliferation and resistance to apoptosis is believed to contribute to both chronic inflammation and hyperplasia of the lining layer (20). Therefore, the next series of experiments was designed to examine the regulation of FLS proliferation and survival by S1P. Under routine serum starvation conditions, no increase in cell proliferation was observed following stimulation with S1P (1 nM–5 μM) for up to 96 h (data not shown). In contrast, FLSs incubated with 5 μM S1P for 8 h reduced FLS apoptosis, induced by the NO donor (SNP), by 28 ± 0.7% ($P < 0.001$; **Fig. 6A**). NO was chosen as a pro-apoptotic agent owing to its overproduction in the RA joint and its ability to induce apoptosis of FLSs in vitro (21, 22). Whereas the protective effect of S1P on SNP-mediated FLS apoptosis was not blocked by a pretreatment of the cells with the $S1P_2$ antagonist JTE-013 and the $S1P_3$ antagonist CAY10444 (data not shown), the $S1P_1$ agonist SEW2871 significantly reduced SNP-mediated FLS cell apoptosis in a concentration-dependent manner (Fig. 6B). SEW2871 at 0.3 μM, 1 μM, and 3 μM reduced SNP-

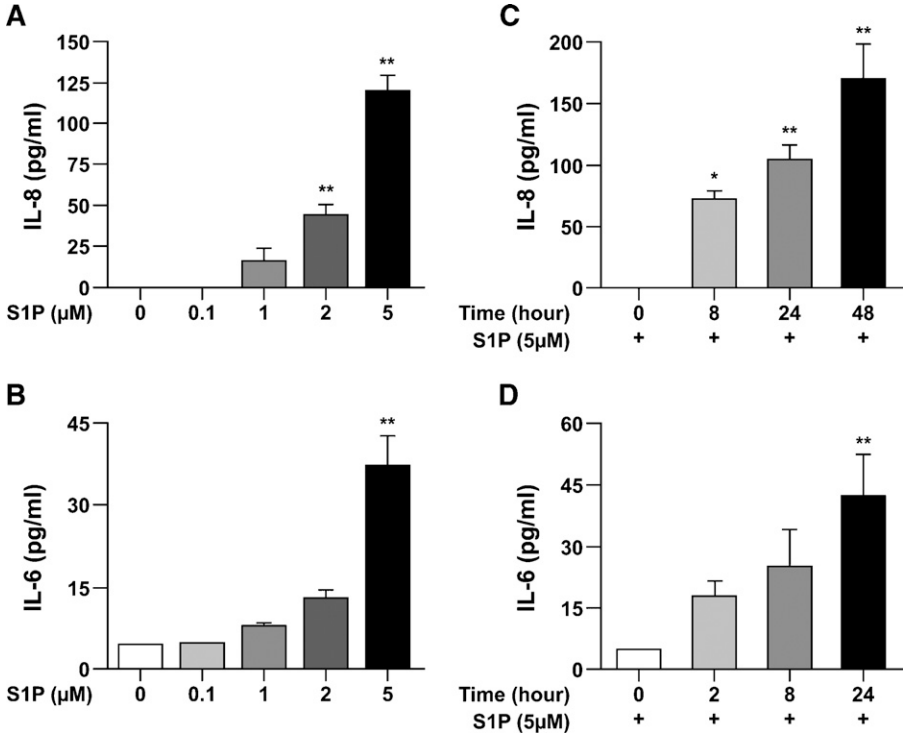

Fig. 4. S1P-mediated interleukin-8 (IL-8) and IL-6 secretion. A, B: Dose response effect of S1P on IL-8/IL-6 secretion. FLSs were treated with the indicated concentrations of S1P for 24 h before collecting supernatants for cytokine quantification. C, D: Kinetics of IL-8/IL-6 secretion. Cells were incubated with S1P (5 μM) for the indicated times, and the cell culture supernatants were collected for the ELISA assay. Experiments were repeated three times, and the results are displayed as mean value ± SE. For statistical analyses, samples incubated with diluents were compared with those treated with S1P at the indicated concentrations (A, B), or times (C, D). *$P < 0.05$; **$P < 0.01$.

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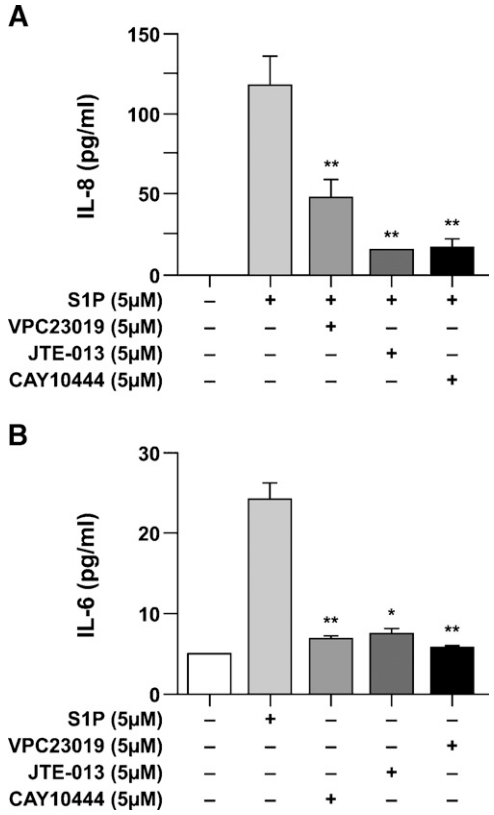

Fig. 5. Effect of selective S1P receptor antagonists on S1P-induced IL-8 and IL-6 secretion. Cells were incubated with or without 5 μM VPC23019, JTE-013, and CAY10444 for 30 min prior to stimulation with S1P (5 μM) for 24 h. Cell culture supernatants were collected and IL-8 (A) and IL-6 (B) were quantified by ELISA. The results are displayed as mean value ± SE (n = 3). For statistical comparative analyses, we compared the samples stimulated with S1P to those treated with S1P+VPC23019/JTE-013/CAY10444. *$P < 0.05$; **$P < 0.01$.

mediated cell apoptosis by 14.8 ± 6.7% ($P < 0.05$), 47.6 ± 2.9% ($P < 0.01$), and 61.3 ± 7.3% ($P < 0.01$), respectively. The data suggest that the $S1P_1$ receptor protects FLSs from SNP-induced apoptosis.

Analysis of downstream signaling pathways coupled to S1P functional responses

Activation of S1P receptors induces a range of downstream signaling cascades. These include MAPK activation and activation of the small GTPases Rho, Rac and Ras, to name a few (23). The pathways and the functional responses are dependent on several variables that include receptor usage, receptor expression level and cell type. We therefore explored the involvement of MAPK and Rho kinase pathways in S1P-mediated functional responses. We first assessed the activation state of p42/44 MAPK, p38 MAPK, and JNK. S1P enhanced the phosphorylation of p38 and p42/44 MAPK (**Fig. 7**). Phosphorylation of p38 (Fig. 7A) and p42/44 MAPK (Fig. 7B) peaked at 5 min and 15 min, respectively. No phosphorylation of JNK was detected following stimulation with S1P for up to 30 min (data not shown). As expected, SB203580 blocked S1P-induced phosphorylation of p38 MAPK (Fig. 7C) but not that of p42/44 MAPK (Fig. 7D). In unstimulated RA synovial cells from RA patients, p42/44 MAPK was phosphorylated (24, 25). PD98059 similarly attenuated basal and S1P-induced phosphorylation of p42/44 MAPK (Fig. 7D; $P < 0.05$ for S1P vs. S1P+PD98059) but had no significant effect on S1P-induced phosphorylation of p38 MAPK (Fig. 7C). Interestingly, the Rho kinase inhibitor Y27632 significantly diminished S1P-induced activation of p38 MAPK (Fig. 7E) but had no effect on S1P-mediated activation of p42/44 MAPK (Fig. 7F). The data suggest that p38 MAPK may act, at least partly, downstream of Rho/Rho kinase to promote S1P-induced IL-8 secretion and cell motility.

The following experiments addressed the involvement of p38 and p42/44 MAPK and Rho kinase in S1P-induced cell motility. As shown in **Fig. 8**, all three inhibitors attenuated S1P-induced cell migration. PD98059 at 25 μM decreased S1P-induced cell motility by 53.1 ± 2.6% ($P < 0.01$) (Fig. 8A). SB203580 at 3 μM and 10 μM decreased S1P-mediated cell migration by 35.5 ± 4.3% ($P < 0.01$) and 79.6 ± 1.6% ($P < 0.01$), respectively (Fig. 8B). Y27632 at 1 μM and 10 μM also reduced S1P-mediated cell migration

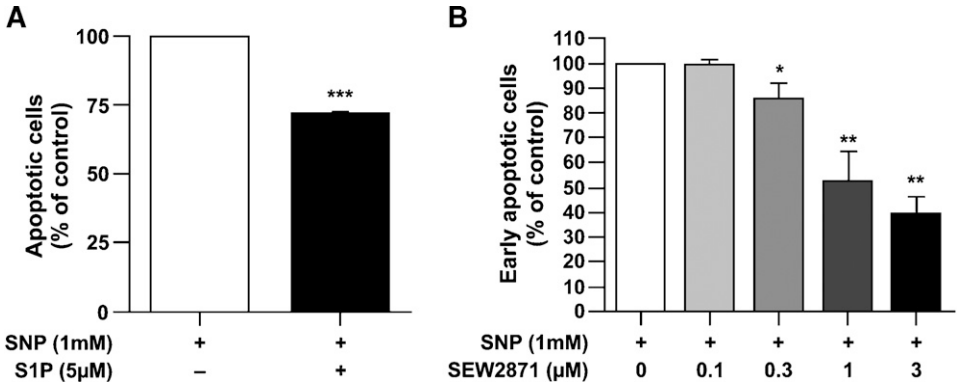

Fig. 6. Protection of FLSs from apoptosis by S1P and the $S1P_1$ agonist SEW2871. Human FLSs were pretreated for 8 h with 5 μM S1P (A) or the indicated concentrations of the $S1P_1$ receptor agonist SEW2871 (B) prior to the addition of 1 mM sodium nitroprusside (SNP) for 16 h. Apoptotic cells were analyzed by Annexin V-propidium iodide (PI) labeling. Total apoptotic cells represent the cells that were Annexin V+/PI+ and Annexin V+/PI−. Early apoptotic cells correspond to the percentage of cells that were Annexin V+/PI−. The results are displayed as mean values ± SE (n = 3). For statistical comparative analyses, we compared samples incubated with SNP to those treated with SNP+S1P/SEW2871. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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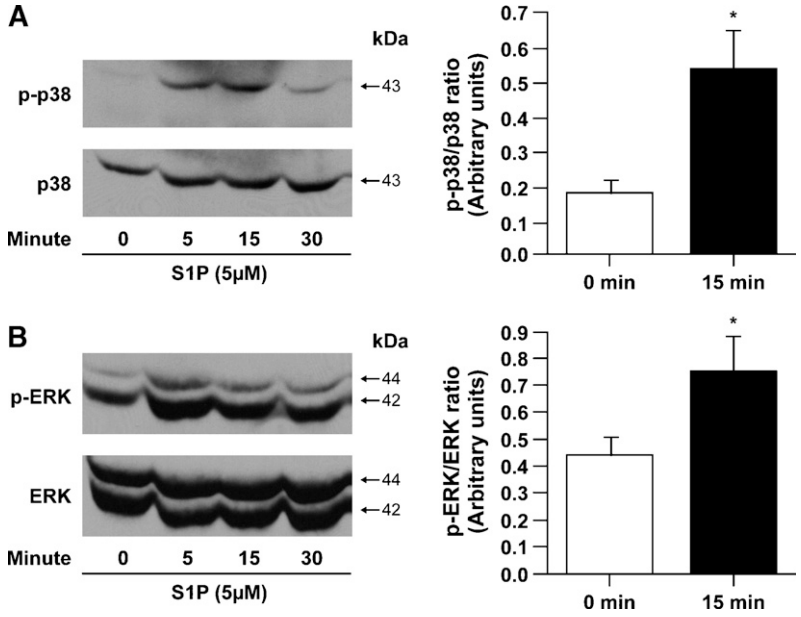

Fig. 7. S1P-induced activation of p42/44 mitogen-activated protein kinase (MAPK) and p38 MAPK. A: Effect of S1P on the activation of p38 MAPK. Cells were stimulated with S1P (5 μM) for up to 30 min, and cell lysates were analyzed by Western blot analysis. Blot shown is a representative example from three independent experiments with similar results. Bands corresponding to phosphorylated p38 (15 min time point) were quantified densitometrically, and were normalized with respect to the total amounts of p38 (n = 4). Data are shown as the ratio of arbitrary units for the phosphorylated p38 to total p38. B: Effect of S1P on the activation of p42/44 MAPK. Cells were stimulated with S1P (5 μM) for up to 30 min, and cell lysates were analyzed by Western blot analysis. Blot shown is a representative example from three independent experiments with similar results. Bands corresponding to phosphorylated p42/44 (15 min time point) were quantified densitometrically, and were normalized with respect to the total amounts of p42/44 (n = 4). Data are shown as the ratio of arbitrary units for the phosphorylated p42/44 to total p42/44. C–F: Effect of p42/44 MAPK, p38 MAPK, and Rho kinase inhibitors on S1P-induced activation of p42/44 MAPK and p38 MAPK. Cells were pretreated with 25 μM PD98059 (C, D), 10 μM SB203580 (C, D), and 10 μM Y27632 (E, F) for 30 min prior to stimulation with 5 μM S1P for 15 min. Cell lysates were analyzed by Western blot analysis. Data shown are representative of three separate experiments and are expressed as means ± SE. In panel D, bands corresponding to phosphorylated p42/44 were quantified densitometrically, and were normalized with respect to the total amounts of p42/44. For statistical comparative analyses, we compared nontreated samples to those treated with S1P ($^{*}P < 0.05$), and samples treated with S1P to those treated with S1P+PD98059 ($^{\dagger}P < 0.05$).

by 61.8 ± 2.4% ($P < 0.01$) and 71.6 ± 6.6% ($P < 0.01$), respectively (Fig. 8C).

Regarding cytokine production, the inhibitor of p42/44 MAPK PD98059 had a marginally inhibitory effect on S1P-induced IL-8 (**Fig. 9A**) and IL-6 secretion (Fig. 9D). On the one hand, 3 μM and 10 μM SB203580 reduced S1P-mediated IL-8 secretion by 38.7 ± 4.0% ($P < 0.01$; Fig. 9B) and 40.2 ± 3.6% ($P < 0.01$; Fig. 9B) and that of IL-6 by 38.1 ± 6.9% ($P < 0.01$; Fig. 9E) and 42 ± 4.1% ($P < 0.01$; Fig. 9E), respectively. On the other hand, Y27632 reduced S1P-mediated IL-8 and IL-6 secretion in a dose-dependent manner. At a higher concentration (10 μM), Y27632 reduced S1P-mediated IL-8 and IL-6 secretion by 96.9 ± 0.2% ($P < 0.01$; Fig. 9C) and 83.6 ± 1.7% ($P < 0.01$; Fig. 9F), respectively. It is worth noting that the MAPK and Rho kinase inhibitors used throughout our study did not affect cell viability, as measured using Annexin V/PI staining (data not shown).

Regulation of $S1P_3$ receptor expression by TNF-α

Given that TNF-α is a key inflammatory molecule in RA (26), we analyzed the expression profile of S1P receptors in response to TNF-α by RT-PCR, real-time PCR, and Western blot analyses. Treatment of FLSs with TNF-α upregulated the expression of $S1P_3$ mRNA (**Fig. 10A, B**) in a concentration-dependent manner. A 3.1 ± 0.5-fold ($P < 0.01$) increase in $S1P_3$ expression was observed with 100 ng/ml of TNF-α, the highest concentration tested. Under the same conditions, $S1P_1$ and $S1P_2$ expression was not altered by TNF-α (data not shown). Upregulation by TNF-α of $S1P_3$ expression at the protein level (1.8 ± 0.5-fold; n = 2) was confirmed by Western blot (Fig. 10C).

Effect of TNF-α on S1P-induced cytokine/chemokine secretion in human FLS

The next series of experiments was designed to examine the effect of a proinflammatory environment, established by a pretreatment of FLSs with TNF-α, on S1P-induced secretion of cytokines/chemokines that is relevant to RA pathogenesis (27). Starved FLSs were treated with S1P (5 μM) for 24 h with or without a pretreatment of TNF-α (100 ng/ml) for 8 h. Cell culture supernatants were analyzed using a Luminex 100 array assay (Table 1). Among the 11 cytokines/chemokines tested, IL-1α, IL-1β, IL-15,

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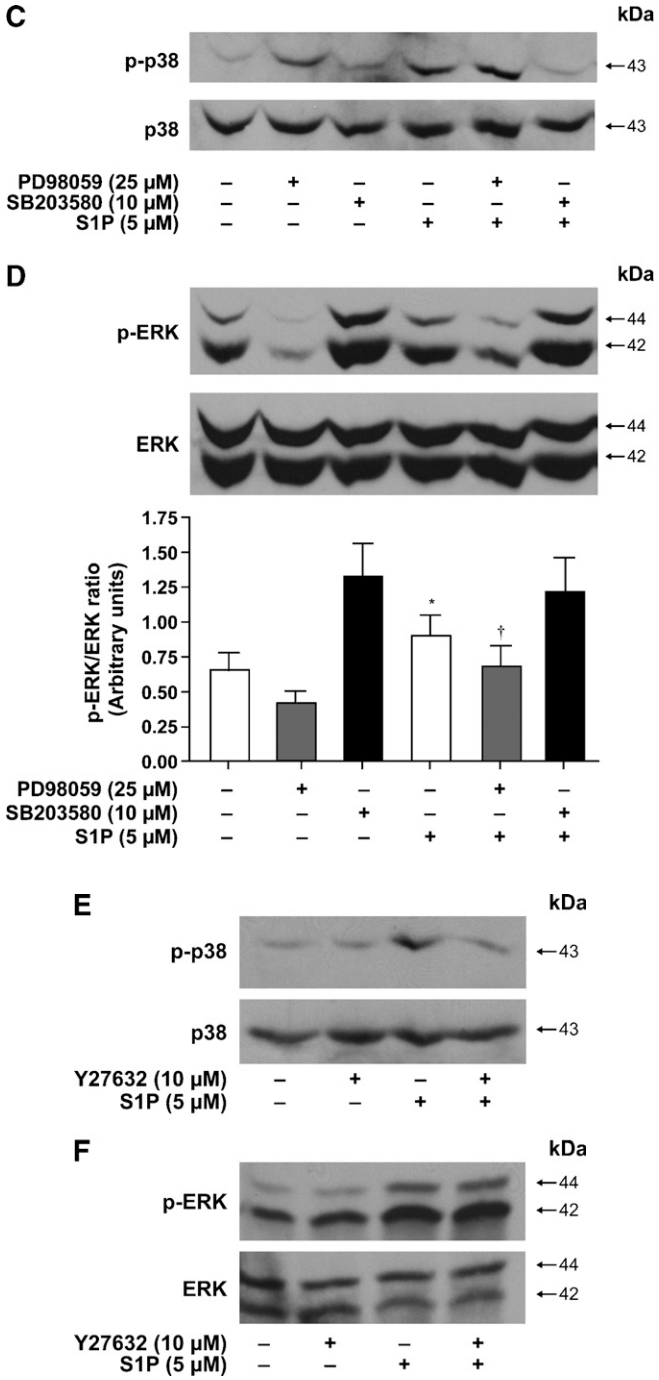

Fig. 7.—*Continued.*

Eotaxin, GM-CSF, MIP-1α, and MIP-1β were not secreted in response to S1P, TNF-α, or their combination. In contrast, significant amounts of IL-8, MCP-1, and RANTES were secreted following stimulation with S1P and, most importantly, S1P-mediated cytokine synthesis was strongly enhanced in TNF-α-primed FLSs. The release of IL-8, MCP-1, and RANTES was increased 5.0 ± 0.3 ($P < 0.01$)-, 1.7 ± 0.2 ($P < 0.05$)-, and 66 ± 6.4 ($P < 0.01$)-fold, respectively, in TNF-α-primed and S1P-stimulated samples as compared with unprimed cells stimulated with S1P. Moreover, S1P or TNF-α priming did not stimulate the secretion of IP-10, but their combination resulted in a significant secretion of this chemokine.

As shown in **Fig. 11A**, after priming FLSs with TNF-α for 2 h, 8 h, and 24 h, S1P-induced IL-8 secretion was superinduced. To determine the relevance of the S1P receptor (s) to this process, FLSs were treated with the $S1P_{1/3}$ receptor antagonist VPC23019. VPC23019 had no effect on the release of IL-8 by TNF-α-primed FLSs, but almost completely inhibited the enhanced secretion of cytokine induced by S1P in these cells (Fig. 11B). S1P-mediated super-production of IL-8 in TNF-α-primed cells was also completely abolished by a selective $S1P_3$ antagonist, CAY10444 (Fig. 11C). The data suggest that TNF-α enhances S1P receptor ($S1P_3$) expression and $S1P_3$-dependent responses in human FLSs.

DISCUSSION

S1P has been previously implicated in RA owing to the expression of the $S1P_1$ and $S1P_3$ receptors in the synovium of RA patients (14). Moreover, S1P was shown to potentiate cytokine-induced COX-2 expression and PGE_2 production (14). S1P, however, mediates a wide variety of cellular responses, including cell migration, apoptosis, and cell proliferation and differentiation. The effect of S1P on these cellular processes in FLSs has not been investigated. Herein, we report that S1P stimulates FLS migration, and cytokine/chemokine synthesis, and that S1P protects FLSs from apoptosis. The S1P receptors mediating these effects were also identified. Moreover, we provide direct evidence for the modulation of S1P-induced cytokine secretion by TNF-α. Our expression analysis also revealed that FLSs not only express the $S1P_1$ and $S1P_3$ receptors but also express $S1P_2$.

The observation that S1P induces FLS migration is significant in the context of RA because one of the hallmarks of RA is synovial hyperplasia. This pro-migratory effect of S1P was mimicked by the $S1P_1$-specific agonist SEW2871 but abolished by the $S1P_{1/3}$ antagonist VPC23019 or the $S1P_3$ selective antagonist CAY10444. The results suggest a prevailing role for $S1P_1$ and $S1P_3$ receptors in this process. These observations are in agreement with previous reports that identified a role for $S1P_1$ and $S1P_3$ in the migratory response to S1P in other cell types (28–30). Because FLSs express $S1P_2$ and because this receptor is known to decrease growth factor-mediated cell migration (31), we hypothesized that blocking this receptor would increase cell migration. Our results reveal, however, that blocking the $S1P_2$ receptor with the selective antagonist JTE-013 had no significant effect on S1P-induced FLS migration. These data suggest that the activation of $S1P_2$ by S1P does not act as a negative regulator of S1P-induced migration in human FLSs. Whether $S1P_2$ can counteract growth factor-mediated or CC or CXC chemokine-mediated migration of FLSs remains to be elucidated.

A key feature of RA is the infiltration of immune cells such as neutrophils, monocytes, and T lymphocytes into the joints due to the large amount of CC and CXC chemokines (32–34) produced by activated cells of the synovial

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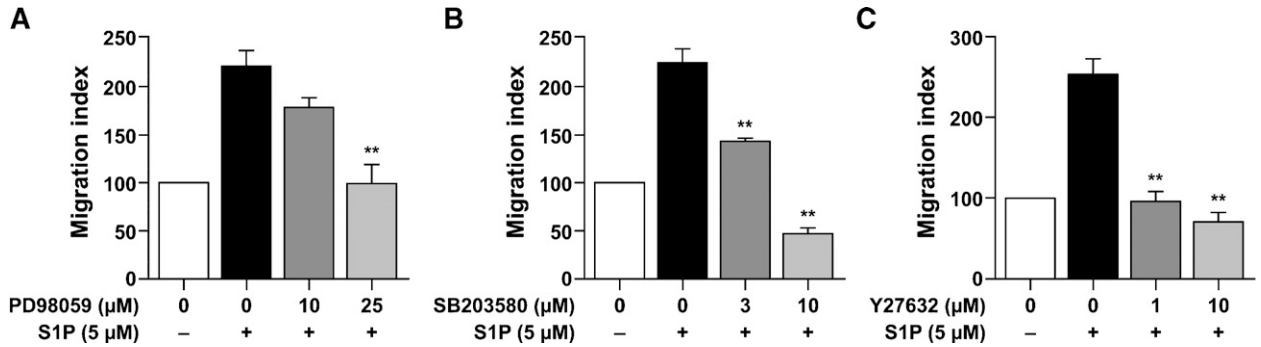

Fig. 8. MAPKs and Rho kinase regulation on S1P-induced FLS migration. After scratching the monolayer and removing free cells, the wound was allowed to close for 24 h in serum-free medium containing 5 μM S1P, with or without PD98059 (A), SB203580 (B), or Y27632 (C), at the indicated concentrations. Migrated cell numbers were expressed as percentage of nontreated cells. Data shown are means ± SE of three independent experiments. For statistical comparative analyses, the samples treated with S1P and S1P+inhibitors were compared. $^{**}P < 0.01$.

lining. In particular, IL-8 exhibits selective chemotactic activity for neutrophils, whereas MCP-1, MIP-1α, MIP-1β, and RANTES primarily attract monocytes (19). To gain insight into additional molecular mechanisms by which S1P may contribute to RA pathogenesis, we profiled the secretion of inflammatory CC and CXC chemokines by FLSs stimulated with S1P. FLSs do not secret detectable levels of cytokines or chemokines, except for low amounts of MCP-1 in the resting state. Upon treatment with S1P, the secretion of IL-8, IL-6, MCP-1, and RANTES was strongly induced, thereby suggesting that S1P can contribute to and/or amplify the secretion of chemokines by cells of the inflamed synovium. Because immune cells express a wide repertoire of chemokine receptors, including IL-8, MCP-1, SDF-1α, IP-10, and RANTES (35), our results suggest that S1P-mediated cytokine secretion may contribute to the recruitment and retention of inflammatory cells in RA. Chemokines such as MCP-1, SDF-1α, IP-10, and RANTES enhance the migration and proliferation of FLSs and upregulate matrix metalloproteinase production by FLSs (27), indicating a direct role for FLSs in the destructive phase of RA beyond the regulation of immune cell trafficking.

Studies using S1P receptor agonists/antagonists revealed that $S1P_2$ and $S1P_3$ are probably involved in S1P-

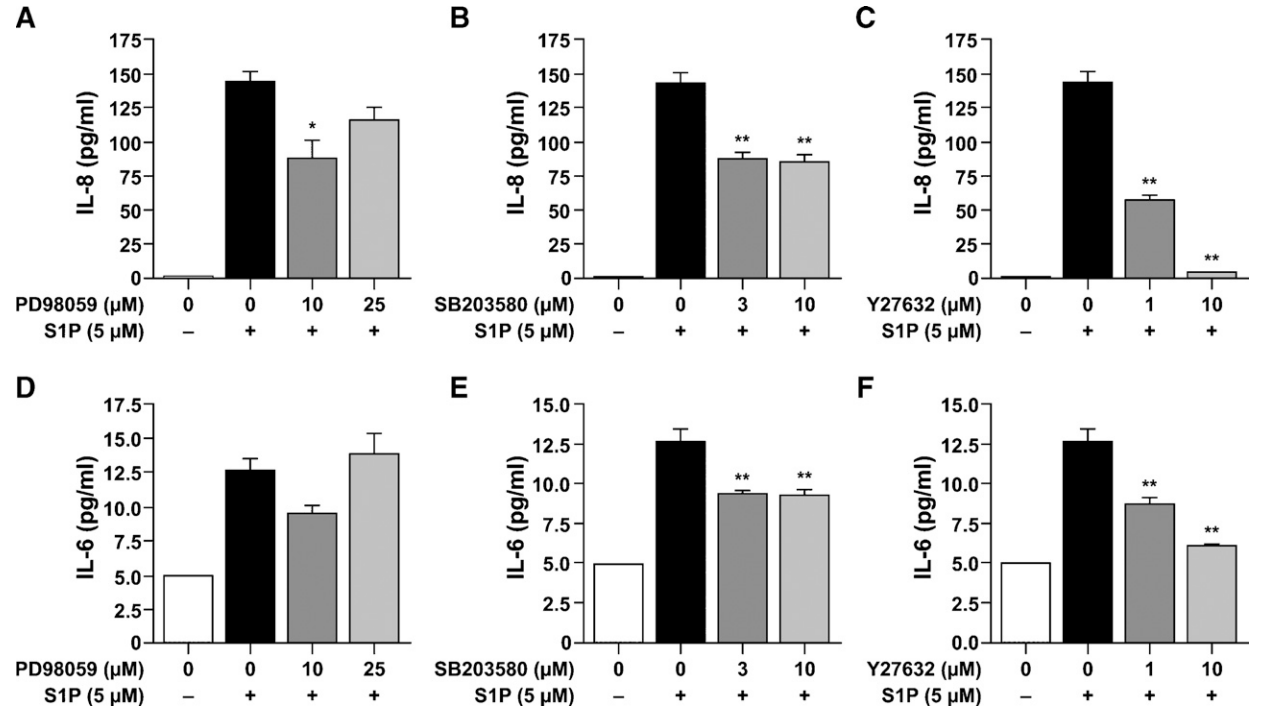

Fig. 9. Role of p42/44 MAPK, p38 MAPK, and Rho kinase in S1P-induced cytokine secretion. Cells were treated with S1P (5 μM) for 24 h, in the presence or absence of PD98059 (A, D), SB203580 (B, E), or Y27632 (C, F), at the indicated concentrations. Secreted IL-8 (A–C) and IL-6 (D–F) were quantified by ELISA. Data shown are means ± SE of three independent experiments. For statistical comparative analyses, the samples incubated with S1P and S1P+inhibitors were compared. $^{*}P < 0.05$; $^{**}P < 0.01$.

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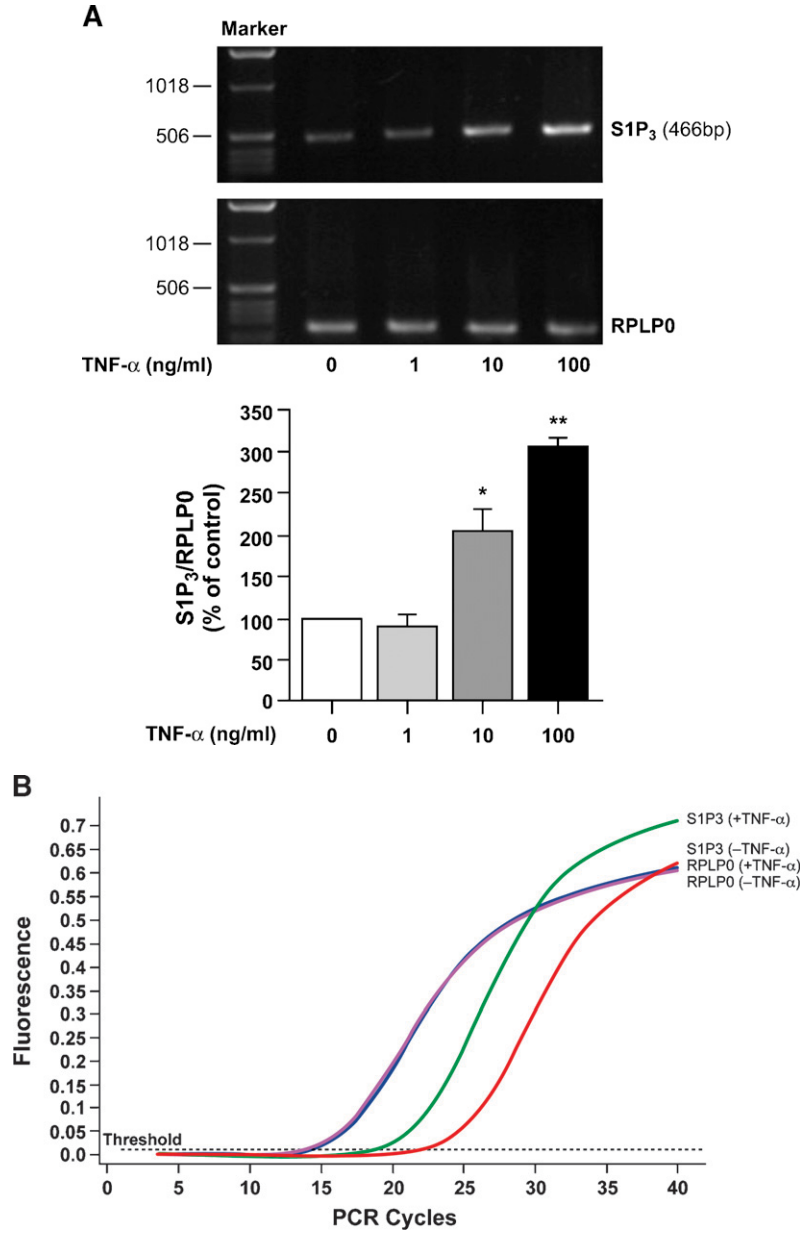

Fig. 10. Regulation of $S1P_3$ receptor expression by tumor necrosis factor-α (TNF-α). A: Concentration-dependent response of $S1P_3$ mRNA expression to TNF-α (RT-PCR). Cells were treated with TNF-α (2 h, at indicated concentrations) prior to RNA extraction and RT-PCR analyses. Results are presented as a representative agarose gel (upper panels) and/or as ratios (means ± SE, n = 3) of $S1P_3$ and RPLP0 (lower panels). B: Real-time PCR analysis of $S1P_3$ mRNA expression upon TNF-α treatment. Cells were incubated with TNF-α (100 ng/ml) for 2 h prior to RNA extraction and real-time PCR analyses. C: Western blot analysis of $S1P_3$ protein expression upon TNF-α treatment. Cells were incubated with or without TNF-α (100 ng/ml) for 24 h. Cell lysates were subjected to 7.5–20% gradient SDS/PAGE, and samples were probed with antibodies to $S1P_3$, HA-tag, and flotillin. Blot shown is a representative example from two independent experiments with similar results (upper panel). Bands corresponding to $S1P_3$ were quantified densitometrically, and their levels were normalized with respect to flotillin. Data are shown as the ratio of arbitrary units for $S1P_3$ to flotillin and as means ± SD (n = 2, lower panel). For statistical comparative analysis, we compared nontreated cells to those treated with TNF-α. $^*P < 0.05$; $^{**}P < 0.01$.

mediated cytokine/chemokine secretion in FLSs. The ability of the $S1P_1$ agonist SEW2871 to induce cell migration but not cytokine/chemokine secretion implies that S1P-driven FLS migration is independent of and not secondary to synthesis of CC or CXC chemokines (27).

In contrast to the induction of FLS migration and cytokine/chemokine secretion by S1P, this bioactive lipid had no effect on FLS proliferation (data not shown). Our observations differ from those of Kitano et al. (14), who reported that S1P induces FLS proliferation. The discrepancy between these

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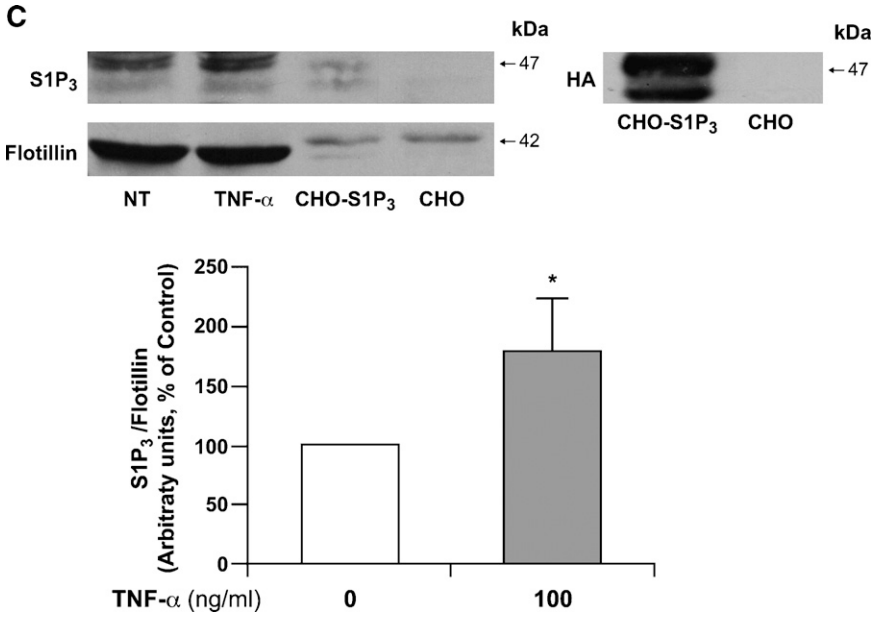

Fig. 10.—*Continued.*

two observations may be partly explained by the different experimental conditions. Kitano et al. performed their experiments in the presence of serum as opposed to our serum-starved culture of FLSs during the proliferation assay.

Because proliferation is scarce in RA synovium (36), dysregulation of apoptosis has been proposed to explain synovial hyperplasia (37). We also provide direct evidence for the inhibition of FLS apoptosis by S1P. Indeed, S1P appears capable of increasing cell survival and inhibiting apoptosis of various other cell types (38–40), including B lymphoblastoid cells derived from patients with RA (16).

TNF-α plays a key role in the pathogenesis of RA. Khoa et al. (41) reported that TNF-α can cross-talk with the G protein-coupled receptor adenosine A_{2A} receptor. Because S1P receptors are coupled to G proteins, we sought to determine whether the cross-talk between TNF-α and G protein-coupled receptors is a general phenomenon. We provide direct evidence for the significant enhancement of S1P-induced cytokine/chemokine secretion, including IL-6, IL-8, MCP-1, and RANTES in FLSs primed with TNF-α. Moreover, S1P and TNF-α alone were not able to stimulate IP-10 secretion. Their combination, however, resulted in an impressive induction of IP-10 production, suggestive of cross-talk between TNF-α and S1P receptors. These observations are in agreement with the findings of Kitano et al. (14), who reported that S1P and TNF-α signaling pathways synergize. The increase in S1P-induced cytokine secretion by TNF-α can be partly explained by the induction of $S1P_3$ expression by TNF-α. Although a large number of genes upregulated by TNF-α have been identified in FLSs (26), this is the first report of the regulation of $S1P_3$ receptor expression by TNF-α, raising the possibility of a causal relationship between enhanced expression of the $S1P_3$ receptor and production of chemokines by S1P following priming of FLSs with TNF-α. Indeed, the $S1P_{1/3}$ receptor antagonist VPC23019 and the selective $S1P_3$ antagonist CAY10444 did not reduce TNF-α-induced cytokine secretion but totally blocked that induced by S1P in TNF-α-primed FLSs. Together, these observations suggest that the proinflammatory environment potentiates some of the functional outcomes of the S1P/S1P receptor signaling pathway.

To identify the signaling molecules involved in the functional responses of FLSs to S1P, the activation of the classical signaling proteins involved in G protein-coupled receptor signaling was investigated. We suggest that the main pathway that regulates S1P-induced IL-8 and IL-6 secretion is Rho/Rho kinase related, and that p38 MAPK is also involved in this process. On the other hand, p42/44 MAPK, p38 MAPK and Rho kinase, but not JNK, are all involved in S1P-induced FLS motility. The results suggest that the coupling of S1P receptors to various heterotrimeric G proteins and, consequently, distinct downstream signaling pathways lead to downstream pathological phenomena, such as FLS invasion into cartilage and bone and recruitment of immune cells into the inflamed synovium. We previously reported that a lysophospholipid that is structurally related to S1P, lysophosphatidic acid (LPA), can also stimulate FLS cytokine secretion and migration by interacting with its cognate receptors (42). The functional responses induced by LPA, however, differ from those induced by S1P in that LPA-induced cytokine synthesis is strongly dependent on p42/44 MAPK activity, whereas signal transduction through p42/44 MAPK is dispensable for LPA-mediated FLS migration. As reported previously (24, 25), p42/44 MAPK is slightly phosphorylated in synovial cells from RA patients, even in the absence of stimulation. Basal levels of p42/44 MAPK may result from autocrine activation by unknown stimulatory factor(s) released by synoviocytes in culture.

In summary, the present study provides evidence that S1P/S1P receptor signaling may contribute to RA pathogen-

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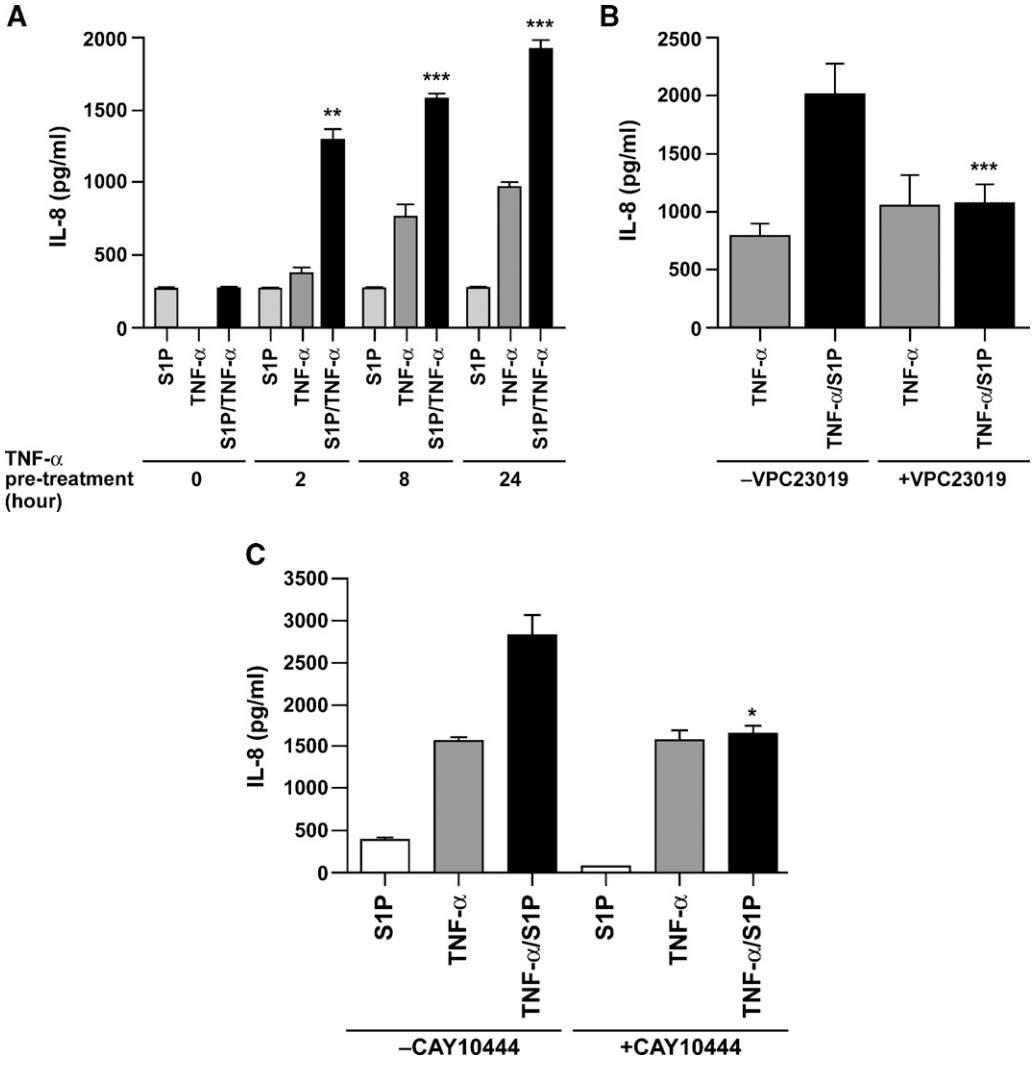

Fig. 11. Super-induction of S1P-induced IL-8 secretion by cell priming with TNF-α. A: Kinetics of TNF-α pretreatment on S1P-induced IL-8 secretion. Cells were pretreated with TNF-α (100 ng/ml) for different time lengths, as indicated, prior to stimulation with 5 μM S1P for 24 h. Cell culture supernatants were harvested for IL-8 measurement. Results are presented as means ± SE (n = 3). For statistical comparative analyses, we compared the samples incubated with S1P to those treated with S1P+TNF-α. $^{**}P < 0.01$; $^{***}P < 0.001$. B, C: Effect of $S1P_{1/3}$ antagonist VPC23019 and $S1P_3$ antagonist CAY10444 on S1P-induced super-production of IL-8 after TNF-α priming. Cells were stimulated with TNF-α (100 ng/ml) for 8 h before stimulation with S1P (5 μM) for another 24 h in the presence/absence of 5 μM VPC23019 and CAY10444. The results are presented as means ± SE (n = 3). For statistical comparative analyses, we compared the samples treated with TNF-α+S1P to those incubated with TNF-α+S1P+VPC23019/CAY10444. $^{*}P < 0.05$; $^{***}P < 0.001$.

esis by stimulating FLS migration and cytokine/chemokine secretion and by inhibiting apoptosis. Although S1P receptors exert receptor subtype-specific responses, we also demonstrated a redundancy of function of the three S1P receptors expressed by FLSs. Furthermore, our data suggest that upregulation of $S1P_3$ receptor expression and enhanced S1P-induced cytokine secretion by TNF-α-primed FLSs may amplify the inflammatory process in RA. Moreover, our results suggest a role for S1P/S1P receptor signaling in the development and progression of RA, and also suggest that $S1P_3$ may represent a critical player in the events that take place in the RA synovium. Thus, subtype-specific antagonists for S1P receptors could be novel therapeutic modalities for limiting inflammation in the destructive phase of RA.

The authors would like to express thanks for the use of the Bioimaging Platform of the Centre de Recherche en Infectiologie, Centre de Recherche du CHUQ-CHUL, for Luminex analysis.

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