Aberrant Gi protein coupled receptor-mediated cell survival signaling in rheumatoid arthritis B cell lines

Shi-Yu Tan, Liqun Xiao, Xiujun Pi, and Joseph Holoshitz

Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0680, USA

TABLE OF CONTENTS

1. ABSTRACT

Sphingosine 1-phosphate (S1P) is a pleiotropic bioactive lipid that transmits potent signals through a family of G protein coupled receptors with resultant anti-apoptotic and pro-angiogenic effects. We have recently reported that lymphoblastoid B cell lines (LCLs) from rheumatoid arthritis (RA) patients are resistant to Fas-mediated cell death due to over-production of S1P, secondary to over-activity of sphingosine kinase-1 (SphK1). Here we investigated the signaling events that S1P triggers in those cells. Our results show that RA-derived LCLs display increased constitutive enzymatic activity of phosphatidylinositol 3-kinase (PI3K). Incubation of LCLs with a PI3K inhibitor wortmannin reversed PI3K over-activity and the resistance to Fas-mediated cell death. Incubation of RA LCLs with nanomolar concentration of S1P triggered exaggerated activation of both SphK and PI3K in RA LCLs compared to control cells. PI3K was mapped upstream of SphK, since wortmannin could block SphK activation by S1P. S1P signaling effect could be blocked by the G_i/G_0 protein inhibitor, pertussis toxin and by an inhibitor of S1P-receptor interaction, suramin. S1P receptor expression levels did not appear to be the cause of disparate S1P-triggered signaling, since LCLs from RA patients and their healthy twin controls did not show statistically significant differences in the expression levels of the five known S1P receptors, as determined by quantitative real time reverse transcription-polymerase chain reaction analyses. Thus, we conclude that Fas death signaling aberration in RA LCLs is caused by extracellular S1P, which triggers PI3K-dependent SphK over-activity through a G_i protein-coupled receptor-mediated signaling cascade.

2. INTRODUCTION

Sphingosine 1-phosphate (S1P) is a lysophospholipid that transmits potent signals through a family of G protein coupled receptors (GPCR), designated $S1P_1$-$S1P_5$ (reviewed in 1). S1P has been previously shown to be a mediator of a variety of cell functions, which are relevant to pannus formation: It inhibits apoptosis induced by Fas ligand (2), it affects lymphocyte trafficking into - and retention inside - secondary lymphoid organs (3, 4) and it enhances angiogenesis (5). It has been previously postulated that migration of cells from the peripheral blood into the synovial compartment, aberrant angiogenesis and impaired apoptosis may collaboratively contribute to formation and propagation of the rheumatoid pannus (6, 7). Thus, S1P could contribute to the pathogenesis of the RA pannus.

Over the past several years, there has been increasing interest in the role of B-lymphocytes in rheumatoid arthritis (RA). There is growing evidence that B-lymphocytes accumulate and mature in the inflamed synovium, where they can form ectopic germinal centers (8-10) and activate T cells (11). Additionally, experimental treatments with anti-CD20 antibodies have shown promise (12), and further support the growing consensus that B-lymphocytes play an important role in the pathogenesis of RA. The mechanism by which of B cells contribute to RA pathogenesis is incompletely understood and awaits functional studies. However, due to the scarcity of B cells in RA (13, 14), functional studies on primary B cells are technically challenging.

To overcome this technical hurdle, we have studied a more abundant source of cells: B lymphoblastoid

cell lines (LCLs). Using this approach, we have recently found that peripheral blood-derived LCLs form RA patients are resistant to Fas-mediated cell death due to constitutive over expression and over activity of sphingosine kinase-1 (SphK1) with resultant increased production of S1P (15). The aberration was disease specific, since LCLs from patients with other rheumatic and autoimmune diseases - as well as LCLs from paired healthy monozygotic (MZ) twins of RA patients - did not demonstrate it. The utility of LCLs as an investigative tool in RA was further supported by the finding of similar over-expression of SphK1 transcripts in the synovial tissues of RA patients (15). Consistent with these findings, another group has recently reported that RA synovial tissues, as well as *in vitro*-cultured synoviocytes, abundantly express $S1P_1$ receptors. Additionally, RA synoviocytes cultured in the presence of S1P proliferated and over-expressed cyclooxygenase-2 and prostaglandin E_2 (16). Thus, evidence for a potential role for S1P and Sphk1 in RA pathogenesis is beginning to emerge.

Given the fact that LCLs from RA patients demonstrate disease-specific S1P-mediated aberration (15), we have undertaken to investigate the signaling effects of S1P in these cells. Here we report that S1P activates survival signaling in RA LCLs through a GPCR-mediated pathway. S1P-triggered activation of RA LCLs involves enhanced activation of phosphatidylinositol 3-kinase (PI3K), which in turn activates SphK. Thus, these data suggest that S1P may aberrantly autoregulate its own production in RA LCLs, thereby potentially contributing to a self-perpetuating activation process.

3. MATERIALS AND METHODS

3.1. Study subjects

LCLs from a total of 36 individuals (17 patients with RA and 19 healthy individuals) were used in the present study. LCLs from 10 RA-discordant MZ twin pairs were used in some experiments. The demographics and clinical characteristics of the RA and the control groups have been described elsewhere (15).

3.2. Cells and culture conditions

LCLs were prepared from peripheral blood B cells using a standard EBV transformation technique (17). LCLs were cultured in supplemented RPMI 1640 containing 10% heat-inactivated FBS (Irvine Scientific, Santa Ana, CA) at a density of 0.5-1.0×10^6 cells/ml. All lines had been maintained in identical tissue culture conditions, cell density and viability. LCLs were kept in continuous long-term (2-6 months) culture, with no measurable change in their viability or functional properties over time. Aliquots of paired LCLs were periodically frozen. Those samples were occasionally used in rare instances of culture loss. In those instances, the most recently frozen samples of both the RA LCLs and the healthy control LCLs were thawed at the same time to assure identical tissue culture history.

3.3. Induction of apoptosis

Fas-mediated apoptosis was induced in LCLs by incubating 5×10^4 cells per well with 100 ng/ml of anti-Fas antibody CH-11 (IgM, purchased from Kamiya Biological Company) in 96 well microplates. In all experiments, control mouse monoclonal IgM antibody (TEPC-183, from Sigma) was used in equivalent concentrations. Cell death was determined at different time points, using the JAM assay (18) or a commercial MTS kit (Promega, Madison, WI). Microplate wells were read by ELISA plate reader at 490 nm.

3.4. Caspase 3 activity assay

A Bio-Rad Apopain Assay Kit was used to determine caspase 3 activity. Two million cells were centrifuged for 5 min at 1,000 rpm. Supernatants were removed and pellets rinsed with PBS twice. 150 µl of a buffer [10 mM Hepes (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1mM PMSF, 10 µg/ml aprotinin] were added to cell pellets. Cell suspensions were vortexed gently, then frozen and thawed 4 times by transferring from liquid nitrogen to a 37°C water bath. The cell lysates were centrifuged for 60 min at 14,000 rpm. Protein concentration in the supernatants was adjusted to 1 mg/ml. Twenty-five µl of supernatants were incubated in 175 µl reaction buffer [10 mM PIPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, Z-DEVD-AFC 40 µg/ml] for 30 min in the fluorometer cuvettes at 25°C. Samples were read on VersaFluor™ fluorometer (Bio-Rad) at an excitation/emission wavelength of 360/520.

3.5. Determination of PI3K enzymatic activity

An Upstate Biotechnology protocol, based on previously described method (19) was used. Briefly, 2×10^7 cells were lysed in a buffer containing 1% NP 40 and 1 mM PMSF. The PI3K protein was immunoprecipitated with polyclonal rabbit anti-p85 PI3K antibody (Upstate Biotechnology) Protein A-coupled agarose. PI3K activity was determined by $[\gamma\text{-}^{32}P]$-ATP incorporation into PI-3-phosphate (PIP_3) following lipid extraction with chloroform/methanol and separation on thin layer chromatography plates (silica gel G from Analtech Inc. Newark, DE). Radioactive PIP_3 spots were quantified with a Molecular Dynamics PhosphorImager. Due to unavoidable variations in phosphoimaging signals between experiments, means and standard deviations cannot be confidently calculated in all cases. In those instances, a representative image, one out of 3-6 repetitions with the same pattern, is shown for illustrative purposes.

3.6. Radioligand binding studies

$[^{32}P]$-S1P binding studies were carried out as described (20). Briefly, 2×10^6 cells were washed twice with binding buffer (PBS containing 0.4% fatty acid-free bovine serum albumin). $[^{32}P]$-S1P was synthesized enzymatically as described (21), using NIH 3T3 cells as a source of sphingosine kinase. Cells were incubated with 5 nM $[^{32}P]$-S1P (200,000 cpm) in 200 µl binding buffer for 30 min at 4 °C in the presence or absence of 1 µM non-radioactive S1P or other ligands in 96-well plate. Cells were then washed three times with binding buffer. The cells were spotted on glass filters and radioactivity quantified by scintillation counting. Ligand binding experiments were performed in triplicates. Specific binding was calculated by subtracting

Table 1. Sequence of primers

Gene	Forward primer	Reverse primer	Probe(5'Fam+3'BHQ1)
$S1P_1$	TGC TGG GTC ATC TCC CTC AT	AGC GCA CTG ATG CAG TTC C	TGG GTG GCC TGC CTA TCA TGG G
$S1P_2$	CAC AAC AAC TCG GAG CGG T	ACA CGC TCA CCA CAA TCA CC	CAT GGC ACT GCT GCG GAC CG
$S1P_3$	TCC ATC ATC CTG TTG GCC A	TGA GCG GAC CAC GCA GTA G	CGT GGC CCT GTA CGT GCG CA
$S1P_4$	GAT GCG GGA GCC CCT G	GCA AGA CTG CAA CTT CAG ATG C	CCA GCA TCT CCA GCG TGC GG
$S1P_5$	TGG ACG CTT GCT CCA CTG T	AGC ACG CAG AAG AGC ACG T	TTG CCG CTC TAC GCC AAG GCC
β-actin	TGC GTG ACA TTA AGG AGA AG	GCT CGT AGC TCT TCT CCA	CAC GGC TGC TTC CAG CTC CTC

nonspecific binding cpm (residual counts in the presence of excess cold inhibitor) from the total binding cpm.

3.7. Determination of SphK enzymatic activity

Cells were washed twice with cold PBS, centrifuged, and resuspended in 200 μl of a 20 mM Tris buffer, pH 7.4, containing 20% (v/v) glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 μg/ml leupeptin and aprotinin, and 1 mM PMSF. Cells were lysed by freezing and thawing 4 times. Lysates were centrifuged for 30 min at 14,000 rpm/min. The buffer mixed with supernatant that contained 70-200 μg of protein to a final volume of 190 μl. The reactions were started by addition of 10 μl of $[\gamma-^{32}P]$-ATP (2 μCi, 20 mM) and sphingosine-BSA 50 μM final concentration containing 100 mM $MgCl_2$. Samples were incubated for 30 min at 37°C, and reactions were terminated by addition of 20 μl of 1N HCl. Lipids were extracted by addition of 0.8 ml of chloroform, methanol, and concentrated HCl (100:200:1, v/v/v). Phases were separated by addition of 240 μl of chloroform and 240 μl of 2 N KCl. The organic phase was evaporated under N_2 and resuspended in 50 μl of chloroform/methanol mixture (1:2). Lipids were separated by thin layer chromatography, using UNIPLATE^{TM} silica gel G (Analtech Inc. Newark, DE) with 1-butanol, methanol, acetic acid, and water (80:20:10:20,v/v/v/v) as solvent. The radioactive spots corresponding to authentic S1P were located by standard S1P, which was visualized by charring with cupric sulfate-phosphoric acid spray. Radioactive S1P spots were quantified with a Molecular Dynamics PhosphorImager. Due to unavoidable variations in phosphoimaging signals between experiments, means and standard deviations cannot be confidently calculated in all cases. In those instances, a representative image, one out of 3-6 repetitions with the same pattern, is shown for illustrative purposes.

3.8. Measurement of S1P receptor mRNA by real time RT-PCR

Total cellular RNA was extracted from LCLs using Trizol (Invitrogen). Briefly, approximately 1×10^7 cells were collected, centrifuged and lyzed with 2 ml of Trizol reagent. The cells were passed through a 22G 1-1/2 needle using a syringe to break up the cells. The extracted total RNA was finally collected with 50 μl of DEPC-treated water and stored at -70 °C. The concentration and purity of RNA was measured by absorbance at 260 nm and 280 nm. cDNA was synthesized from 1 μg total RNA by SuperScriptase (Invitrogen) provided with the First Strand Synthesis System using a GeneAmp 9600 system (PerkinElmer). The negative control was prepared using all reagents except the RNA sample/or without reverse

transcriptase. The primers specific for $S1P_1$-$S1P_5$ and β actin are shown in Table 1.

Real-time PCR was carried out with 2x Platinum Quantitative PCR SuperMix-UDG (Invitrogen) in a total volume of 50 μl containing 8 μl (2 μl for β actin) of cDNA, 0.4 μM of each PCR primer, 0.2 μM of Taqman probe with passive reference, and 25 μl PCR master mix. S1P receptor genes and β actin gene were amplified in separate tubes in duplicate. The amplification parameters were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence emission from individual PCR tubes at each cycle was monitored in an iCycler system (Bio-rad). The cycle of threshold (Ct) values were collected corresponding to the PCR cycle number at which the fluorescence was detectable above an arbitrary threshold, based on baseline data within cycles 3 to 15. The arbitrary threshold was decided to ensure the Ct values were obtained in the exponential phase of PCR where there are no rate-limiting components. The Ct value for S1P receptor minus Ct value for β actin was designated as delta Ct value.

3.9. Statistical analysis

Unless stated otherwise, Student's *t* test was used to determine statistical significance. Mann Whitney test was used for nonparametric values. Statistical significance was achieved at $p < 0.05$ (marked with an asterisk). Calculations of IC_{50} and EC_{50} were performed using PRISM 3.0 software (GraphPad Software, San Diego, California).

4. RESULTS

We have recently identified SphK1-mediated resistance to Fas-mediated death signaling in RA LCLs (15). Since much like SphK (2), PI3K is known to enhance cell survival and its activity is regulated by serum growth factors (22-24), we have undertaken to determine whether PI3K is involved in the observed SphK1-mediated death signaling aberration in RA LCLs. In order to minimize inter-individual variations, we have compared pairs of RA disease-discordant MZ twins. Those individuals differ in the presence or absence of RA, but are genetically identical. Figure 1A shows PI3K activity in cell lysates from LCLs of a representative pair of RA-discordant MZ twins. As can be seen, constitutive PI3K enzymatic activity in the RA twin was over 3-fold higher than that of the healthy twin. Similar results were found in 7 out 10 pairs tested (mean PI3K activity in RA twins was 2.6±0.45-fold higher then their respective healthy co-twins). In two pairs, PI3K activity was equal and in only one of the 10 pairs tested, the RA twin cells had lower PI3K enzymatic
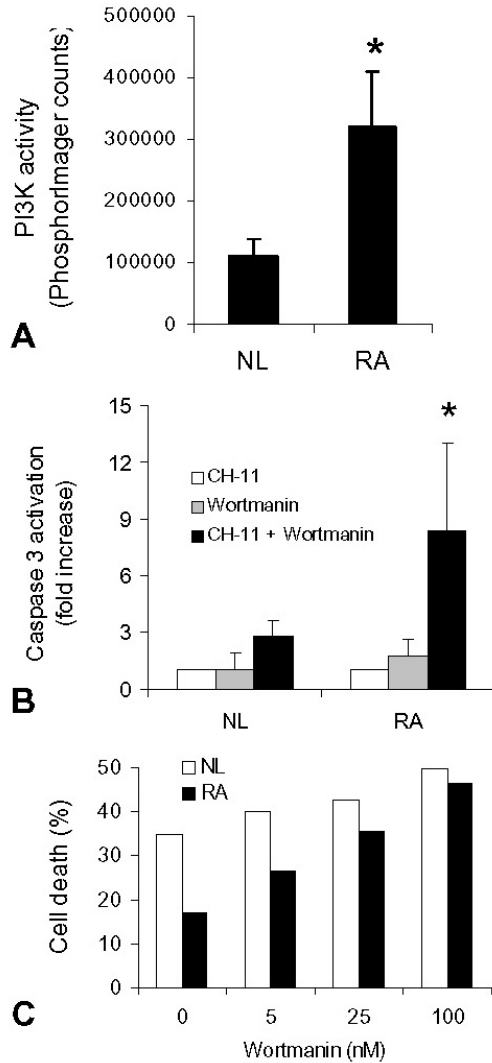
Figure 1. Role of PI3K. A, Comparison of PI3K activity in a representative pair of RA-discordant MZ twins. The activity was determined in LCLs from a healthy twin (left) and his RA-afflicted MZ twin (right). As can be seen, the RA twin had over 3-fold higher constitutive PI3K activity. Six additional twin pairs showed the same pattern. B, Reversal of resistance to Fas-mediated caspase 3 activation in RA cells with wortmannin. LCLs from 10 RA patients and their 10 healthy MZ twins were tested for Fas-mediated caspase 3 activation with either anti-Fas antibody CH-11, 200 ng/ml, alone (white bars), wortmannin, 100 nM alone (hatched bars) or a combination of the two (black bars). C, Reversal of resistance to Fas-mediated apoptosis by wortmannin. Fas-mediated cell death at 20 h post stimulation was determined using the JAM assay in LCLs of a RA-discordant MZ twin pair. LCLs from the RA twin (black bars) or the healthy twin (white bars) were incubated with different concentrations of wortmannin, in the presence of 100 ng/ml of anti-Fas antibody CH-11. As can be seen, wortmannin increased Fas-mediated apoptosis in the RA twin in a dose-dependent manner to near normal levels. NL, normal; RA, rheumatoid arthritis

activity than the healthy co-twin ($p < 0.05$ by Mann Whitney).

Treatment of cells with a specific PI3K inhibitor wortmannin enhanced Fas-mediated caspase 3 activation (Figure 1B) and reversed the resistance to Fas-mediated apoptosis (Figure 1C) in RA cells. As shown in Figure 1B, the effect of wortmannin on Fas-mediated caspase 3 activation was markedly higher in the RA-twins group, compared to that of the healthy-twins group. Within individual pairs, wortmannin effect was higher in the RA twin in 7 of the 10 cases, was equal in two, and was lower in only one pair ($p < 0.05$ by Mann Whitney). Taken together, these data suggest that higher constitutive PI3K activity is found in the majority of RA LCLs. That activity contributes to the resistance of these cells to Fas-mediated death signaling.

RT-PCR analysis using pairs of primers for the four known PI3K isozymes α,β,γ and δ, as well as Western blot analysis with anti-PI3K antibodies were performed. No differences in PI3K expression levels could be detected (data not shown). The higher activity of PI3K in RA cells could not be attributed to a mutated enzyme either, since cDNA sequencing of the invariable p85 regulatory subunit as well as the α,β,γ and δ p110 catalytic subunits did not reveal differences between the healthy and RA twins (data not shown). These findings suggest that activation by an upstream regulatory mechanism, rather than PI3K abundance or mutations contribute to its higher enzymatic activity in RA cells.

Activation of PI3K is inducible by many growth factors and cytokines through a variety of receptor-mediated mechanisms (reviewed in 25). S1P has been previously shown to activate PI3K through the S1P receptors and to inhibit apoptosis (1, 2, 26). Based on our finding that RA LCLs produce higher levels of S1P (15) and because S1P is abundant in serum (27, 28), we examined whether it could preferentially activate PI3K in RA LCLs.

As shown in Fig 2, incubation of cells for 1 min with nanomolar concentrations of S1P ($EC_{50} \approx 50$ nM) produced potent activation of PI3K in the RA twin LCLs, but not in the normal twin LCLs (Figure 2A). The activation was ligand-specific, as a structurally related control sphingolipid, *N, N*-dimethylsphingosine, did not activate the enzyme (Figure 2B). To assess whether S1P-triggered PI3K activation involves a receptor-mediated mechanism, we used suramin, a compound that blocks S1P receptor/ligand interactions. As can be seen in Figure 2C, suramin inhibited activation of PI3K by S1P in a dose-dependent manner, with an $IC_{50} \approx 7\mu M$, suggesting the involvement of S1P-binding receptors. Additionally, pertussis toxin (PTX), a G_i/G_o-coupled receptor inhibitor, blocked serum-induced PI3K hyperactivity (Figure 2D), as well as S1P-induced PI3K activation (Figure 2E), and reversed resistance of RA LCLs to Fas-mediated cell death (Figure 2F).
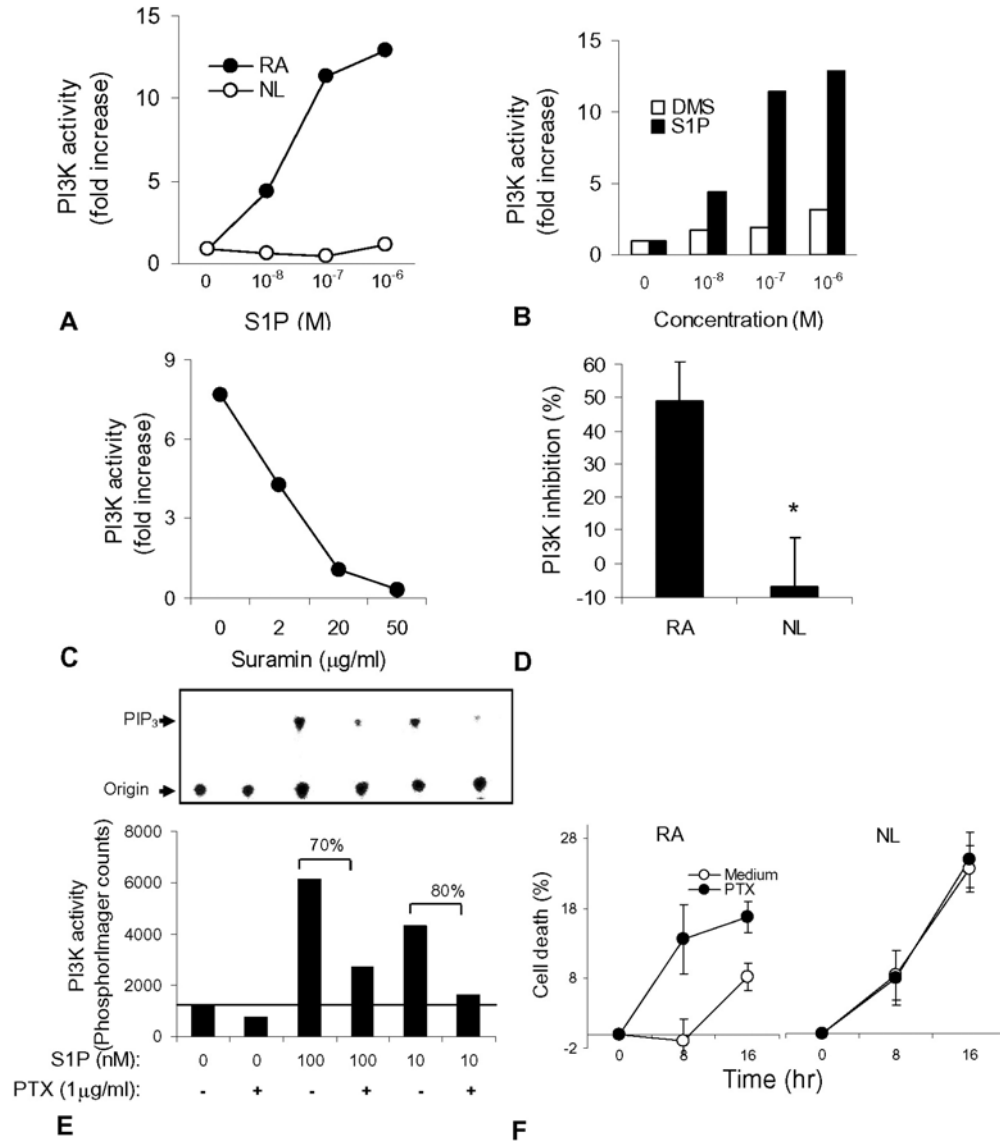
Figure 2. Involvement of a G_i protein-coupled receptor. A, Activation of PI3K by extracellular S1P. Serum-starved LCLs from an RA patient (black circles) and his healthy MZ twin (white circles) were incubated for 1 min with various concentrations of S1P in serum-free medium and PI3K activity was determined. B, Ligand specificity of PI3K activation. RA twin LCLs cells were incubated with various concentrations of N,N-dimethylsphingosine (DMS, white bars) or S1P (black bars) for 1 min in serum-free conditions. PI3K activity was determined as above. C, Inhibition of extracellular S1P-mediated PI3K activation by suramin. RA LCLs were stimulated for 1 min with 100 nM S1P in serum-free conditions in the presence of various concentrations of suramin. PI3K activity was determined as above. D, Inhibition of constitutive PI3K activity in RA cells by PTX. Serum-grown LCLs from 5 patients with RA (left) and 5 healthy controls (right) were pre-incubated for 1 h with or without 1 µg/ml of PTX. PI3K activity was determined and percent inhibition of PI3K activity by PTX was calculated. As can be seen, PTX suppressed PI3K activity in RA cells, but not in the normal controls ($p < 0.05$), suggesting that constitutive PI3K activities in RA and control cells are regulated by different mechanisms. E, Inhibition of S1P-induced PI3K activation by PTX. RA LCLs were pre-incubated for 1 h with or without 1µg/ml of PTX, followed by 1 min stimulation with various concentrations of S1P and PI3K activity was then measured. The upper panel shows the PIP$_3$ spots in a thin layer chromatogram and the lower panel shows PhosphorImager quantification of those spots. The horizontal line marks the basal activity and the numbers inside the lower panel represent the calculated inhibition of PI3K activity by PTX. As can be seen PTX produced a significant inhibition of extracellular S1P-induced PI3K activation. F, Reversal of Fas-resistance in RA cells by PTX. LCLs from six RA patients (left) and eight healthy controls (right) were pre-incubated for 1 h with (black circles) or without (white circles) PTX, 1 µg/ml. At the end of incubation, Fas-mediated cell death was determined by the MTS method. As can be seen, pretreatment with PTX reversed RA cells resistance to Fas-mediated killing but had no effect on normal cells. NL, normal; RA, rheumatoid arthritis.
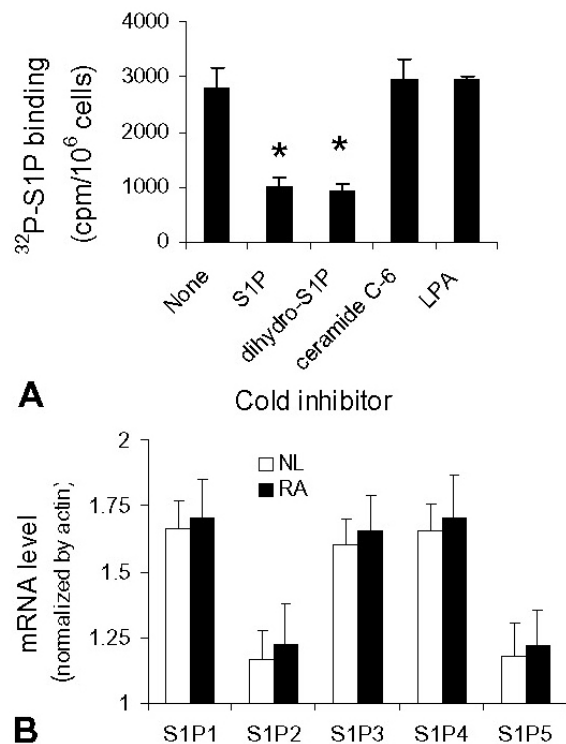
Figure 3. Involvement of S1P receptors. A, Ligand binding specificity. S1P binding was determined using 5 nM $[^{32}P]$-S1P in a representative RA LCL. The inhibitory effect of 10 μM unlabeled ligands was measured. Results are presented as cpm/10^6 cells ± SD in triplicate experiments. B, Real time RT-PCR-based mRNA quantification of the five S1P receptors in 3 RA twins (black bars) and their normal co-twins (white bars). Results were normalized to β actin. LPA, lysophosphatidic acid.

Ligand-specificity studies showed that S1P binding could be inhibited by excess of unlabeled S1P and dihydro S1P, but not by lysophosphatidic acid or ceramide C6 (Figure 3A), indicating that S1P-specific receptors are involved in S1P binding to LCLs. S1P transmembrane signaling is transmitted by a family of five GPCRs, designated $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$, respectively (1). In order to determine whether enhanced S1P signaling in RA LCLs might be due to greater abundance of S1P receptors, we quantified mRNA expression levels of these 5 receptors in three pairs of disease discordant MZ twins using a real time RT-PCR method. As can be seen (Figure 3B), very similar mRNA expression levels of the five S1P receptors were found in the normal and the RA co-twins. Thus, enhanced S1P-triggered signaling in RA LCLs is not due to increased abundance of any of the 5 known S1P receptors.

Finally, experiments were carried out to determine the signaling relationship between the PI3K and the SphK pathways. To that end, SphK enzymatic activity was first determined in cells in the presence or absence of extracellular S1P. As can be seen, stimulation of RA cells with S1P induced SphK activation, which reached a plateau at 60 min (Figure 4A), compared to peak PI3K stimulation by S1P, which could be seen within 1-10 min (Figure 2). This result suggests that PI3K may be located in an upstream position relative to SphK. Indeed, pre-incubation with the PI3K inhibitor wortmannin suppressed constitutive SphK activity in LCLs from RA patients, but not in control lines (Figure 4B, $p < 0.05$). Thus, we conclude that SphK hyperactivity in RA cells is attributable to an aberrant upstream control by PI3K.

5. DISCUSSION

Due to their scarcity in RA, functional studies involving primary B lymphocyte have been technically challenging. In order to gain insights into the role of these cells in the disease, we have used here LCLs. We have identified and mapped an aberrant pathway in RA LCLs and obtained evidence that in these cells, S1P triggers signaling through a GPCR of the G_i subtype, thereby activating PI3K, which in turn activates SphK. As a result, RA LCLs fail to efficiently propagate Fas-death signaling.

Given the central role of B cells in RA, the findings reported here could provide new insights into candidate pathogenic mechanisms in this disease. It should be cautioned, however, that while B cells are unequivocally involved in the pathogenesis of RA, it is presently unknown to what extent the *in vitro* immortalized LCLs faithfully represent the B cell population that contributes to disease pathogenesis in RA. Noteworthy, however, in two recent studies (15, 29), in which we analyzed synovial tissues and LCLs from RA patients and controls, we have demonstrated that peripheral blood-derived LCLs display disease-specific functional aberrations and they over-express many RA-relevant gene products, which are also over-expressed in RA synovial tissues, including in synovial B cells. Therefore, although their relevance to primary B cells remains to be explored, investigating the biology of LCLs may be a useful tool in the research of RA pathogenesis.

S1P has been previously shown to activate PI3K through the S1P family of receptors and to inhibit apoptosis (1, 2, 26). It is an abundant bioactive lipid with concentrations in normal human serum at the μM range (27, 28). Therefore, the activation of the aberrant pathway at nanomolar concentrations, shown here, is in agreement with its reported biological activity and within the physiologic concentration range of S1P. In view of the findings reported here, our prior findings of SphK1 over-expression in RA synovial tissues (15), and in agreement with a recent report by others (16), it is conceivable that extracellular S1P acts in RA as an autocrine growth and survival factor, which is produced and used by synovial cells in a self-perpetuating fashion. Alternatively, S1P produced by platelets (30), which are abundantly present in the inflamed joint (31), could act in a paracrine fashion. It is of interest that S1P and its prototypic receptor, $S1P_1$, have been shown to play a role in angiogenesis (5), a process which has long been recognized as a key feature in early pannus pathology (32). Thus, the proposed role of
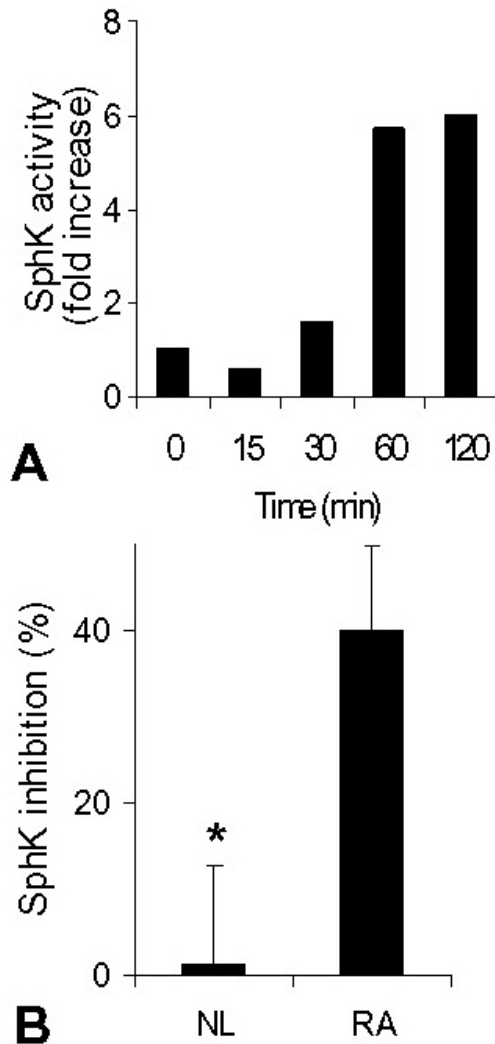
Figure 4. Cross talk between the PI3K and SphK pathways. A, Induction of SphK activity by extracellular S1P in RA cells. RA LCLs were incubated with 100 nM S1P in serum-free conditions. SphK enzymatic activity was determined at different time-points thereafter. Results are presented as fold-increase of SphK enzymatic activity over basal activity in a representative experiment. B, Inhibition of constitutive SphK activity by wortmannin. SphK activity was determined in LCLs from 7 RA patients (right) and 9 normal controls (left), which had been pre-incubated overnight with or without 20 nM of wortmannin. Results are shown as percent inhibition of SphK activity. *, $p < 0.05$. NL, normal; RA, rheumatoid arthritis.

extracellular S1P in RA pathogenesis is consistent with several lines of evidence.

We demonstrate here that S1P-triggered activation of PI3K and, subsequently, activation of SphK, is transmitted by an S1P surface receptor. The S1P receptor family consists of 5 members, designated $S1P_1$-$S1P_5$. Although originally identified in endothelial cells, their tissue distribution is actually much wider, and includes

leukocytes, the cardiovascular system, central nervous system, gastrointestinal tract, gonadal tissue and placenta (reviewed in 1, 33). Although the precise identity of the receptor(s) used by S1P in RA LCLs is unknown, the inhibitory effect of PTX suggests that either $S1P_1$ (34), $S1P_4$ (35) or $S1P_5$ (36), known to couple to G_i protein, are involved in S1P binding, since the two other known high-affinity S1P receptors, $S1P_2$ and $S1P_3$ have been previously shown to couple to G_q and G_{13} proteins (37, 38). Among the three PTX-sensitive S1P receptors, either $S1P_1$ or $S1P_4$ are more likely to be involved, since $S1P_5$ is expressed mostly in the brain (39). However, we cannot rule out the possibility that more than one receptor type - or a novel member of the S1P receptor family - is involved.

Our findings indicate that activation of PI3K-dependent survival signaling is mediated through a G_i protein-coupled receptor mechanism in RA cells. Pre-treatment with PTX or wortmannin restored Fas death signaling in those cells. PI3K has been implicated previously in experimental autoimmune models (40, 41). Transgenic mice expressing a constitutively active PI3K form in T cells have been reported to display a lymphoproliferative disorder and autoimmune glomerulonephritis (40). Another study reported impaired Fas death signaling with resultant lymphoid proliferation and autoimmunity in $Pten^{+/-}$ heterozygous mice, presumably due to increased cellular PIP_3, which is a product of PI3K and a substrate of the PTEN phosphatase (41). It has been recently shown that PI3K-deficient mice are resistant to induction of experimental models of arthritis (42). In humans, recent data suggests that PI3K may be playing an important pathogenic role in human RA as well. PI3K has been found to be a key signaling element in synovial fibroblast proliferation (43), overproduction of the proinflammatory cytokine IL-17 (44) and soluble E selectin-induced angiogenesis (45), activities, which have all been implicated in the pathogenesis of RA.

We show here that in RA LCLs, S1P could activate both PI3K and SphK, and SphK activity could be inhibited by wortmannin. These findings suggest a signaling relationship between the two pathways, where SphK is subjected to an upstream activation by PI3K. SphK and PI3K have been previously noticed to share several functional properties. Both enzymes can be activated by serum-derived lipid growth factors (46, 47) and both can inhibit apoptosis (1, 22). Therefore, a hierarchical relationship between the two enzymes is a plausible scenario. Physiologic activation of SphK by an upstream PI3K has been previously documented in the signaling pathway of hepatocyte growth factor in endothelial cells (48). However, to the best of our knowledge, such hierarchical cross talk between the PI3K and SphK pathways has been neither shown previously in lymphocytes, nor has it been implicated in any pathologic condition. We have previously shown that S1P-mediated survival signaling is specific for RA, while other autoimmune and arthritis conditions do not exhibit this abnormality (15). When taken together, those findings, and our present findings showing that signaling modulation by S1P, wortmannin and PTX is selectively affecting cells of
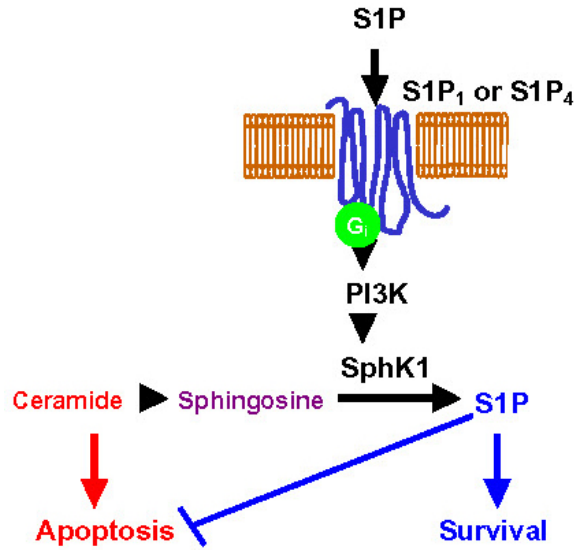
Figure 5. Model of signaling events in RA. Extracellular S1P activates G_i protein-coupled receptors in either an autocrine or paracrine fashion. The identity of the specific S1P receptors is yet to be determined. The receptors shown here are depicted for illustrative purpose. As a result of a signaling defect, activation of those receptors in RA cells lead to aberrant activation of PI3K, with resultant excessive activation of SphK and generation of higher intracellular levels of S1P, which inhibit Fas-mediated apoptosis.

RA patients, but not cells from their respective healthy MZ twins or normal controls, strongly suggest that the signaling hierarchy between PI3K and SphK observed here represents RA-specific signaling aberration.

6. CONCLUSIONS

We have mapped the signaling pathway, which aberrantly activates SphK-dependent cell survival in RA LCLs. A model of the signaling events, which lead to impaired Fas-mediated cell death in RA, is schematized in Figure 5. We propose that further characterization of the molecular basis of the aberration described here in primary B cells could pave the way to identification of novel interventional approaches in RA.

7. ACKNOWLEDGEMENTS

8. REFERENCES

1. Rosen H, & E J. Goetzl: Sphingosine 1-phosphate and its receptors: An autocrine and paracrine network. *Nat Rev Immunol* 5, 560-570 (2005)

2. Cuvillier O, G. Pirianov, B. Kleuser, P.G. Vanek, O.A. Coso, J.S. Gutkind, & S. Spiegel: Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* 381, 800-803 (1996)

3. Cinamon G, M. Matloubian, M. J. Lesneski, Y. Xu, C. Low, T. Lu, P.L. Richard, & C. G. Jason: Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol* 5, 713-720 (2004)

4. Cyster J.G: Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Ann Rev Immunol* 23, 127-159.(2005)

5. Lee M.J, S. Thangada, K.P. Claffey, N. Ancellin, C.H. Liu, M. Kluk, M. Volpi, R.J. Sha'afi, & T. Hla: Vascular endothelial cell adherence junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* 99, 301-312 (1999)

6. Liu H, & R.M. Pope: Apoptosis in rheumatoid arthritis: friend or foe. *Rheum Dis Clin North Am* 30, 603-625 (2004)

7. Szekanecz Z, L. Gaspar, & A.E. Koch: Angiogenesis in rheumatoid arthritis. *Frontiers Biosci* 10, 1739-1753 (2005)

8. Dechanet J, P. Merville, I. Durand, J. Banchereau, & P. Miosec: The ability of synoviocytes to support terminal differentiation of activated B cells may explain plasma cell accumulation in rheumatoid synovium. *J Clin Invest* 95, 456-463 (1995)

9. Hayashida K, Y. Shimaoka, T. Ochi, & P. E. Lipsky: Rheumatoid arthritis synovial stromal cells inhibit apoptosis and up-regulate Bcl-x_L expression by B cells in a CD49/CD29-CD106-dependent mechanism. *J Immunol* 164, 1110-1116 (2000)

10. Magalhaes R, P. Stiehl, L. Morawietz, C. Berek, & V. Krenn: Morphological and molecular pathology of the B cell response in synovitis of rheumatoid arthritis. *Virchows Arch* 441, 15-427 (2002)

11. Takemura S, P.A. Klimiuk, A. Braun, J.J. Goronzy, & C.M.Weyand: T cell activation in rheumatoid synovium is B cell dependent. *J Immunol* 167, 4710-4718 (2001)

12. Edwards J.C.W, L. Szczepanski, & J. Szechinski, A. Fillipowicz-Sosnowska, P.E. Emery, D.R. Close, R.M. Stevens, & T. Shaw: Efficacy of B-cell-targeted therapy with Rituximab in patients with rheumatoid arthritis. *N Eng J Med* 350, 2572-2581 (2004)

13. Wagner U, S. Kaltenhauser, M. Pierer, B. Wilke, & S. Arnold: B lymphocytopenia in rheumatoid arthritis is associated with the DRB1 shared epitope and increased acute phase response. *Arthritis Res* 4:R1, (2002)

14. Symmons D.P, M. Farr, M. Salmon, & P.A. Bacon: Lymphopenia in rheumatoid arthritis. *J R Soc Med* 82, 462-463 (1989)

15. Pi X, S-Y. Tan, M. Hayes, L. Xiao, J.A. Shayman, & J. Holoshitz: Sphingosine kinase 1 – mediated inhibition of Fas death signaling in rheumatoid arthritis B lymphoblastoid cells. *Arthritis Rheum* 54, 754-764 (2006)

16. Kitano M, T. Hla, M. Sekiguchi, Y. Kawahito, R. Yoshimura, K. Miyazawa, T. Iwasaki & H. Sano: Sphingosine 1-phosphate/sphingosine 1-phosphate receptor 1 signaling in rheumatoid synovium. *Arthritis Rheum* 54, 742-753 (2006)

17. Tosato G: Generation of Epstein-Barr virus (EBV)-immortalized B cell lines. In: Coligan JE, Kruisbeek AM,

Margulies DH, Shevach EM, & Strober W, editors. *Current protocols in immunology.* 7.22.1-7.22.3 New York, John Wiley & Sons, Inc. (1988)

18. Matzinger P: The JAM test: A simple assay for DNA fragmentation and cell death. *J. Immunol Methods* 1991, 145:185-192.

19. Record RD, L.L. Froelich, C.J. Vlahos, & B.L. Blazer-Yost. Phosphatidylinositol 3-kinase activation is required for insulin-stimulated sodium transport in A6 cells. *A J Physiol* 274, E611-617 (1998)

20. Van Brocklyn J.R, M.J. Lee, R. Menzzeleev, A. Olivera, L. Edsall, O. Cuvillier, D.M. Thomas, P.J. P. Coopman, S. Thangada, C, H. Liu, T. Hla, & S. Spiegel: Dual actions of sphingosine-1-phosphate: Extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J Cell Biol* 142, 229-240 (1998)

21. Olivera A, J. Rosenthal, & S. Spiegel: Sphingosine kinase from Swiss 3T3 fibroblasts: a convenient assay for the measurement of intracellular levels of free sphingoid bases. *Anal Biochem* 223, 306-312 (1994)

22. Radeff-Huang J, T.M. Seasholtz, R.G. Matteo, & J.H. Brown: G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *J Cell Biochem* 92, 949-966 (2004)

23. Franke TF, D.R. Kaplan, & L.C. Cantley: PI3K: downstream AKTion blocks apoptosis. *Cell* 88, 435-437 (1997)

24. Wymann M.P, & L. Pirola: Structure and function of phosphoinositide 3-kinases *Biochim Biophys Acta* 1436, 127-150 (1998)

25. Donahue AC, & D.A. Fruman: PI3K signaling controls cell fate at many points in B lymphocyte development and activation. *Semin. Cell. Dev. Biol*. 15, 183-97 (2004)

26. Crey A, Q. Chen, K. Callon, X. Xu, I.R. Reid, & J. Cornish: The phospholipids sphingosine-1-phosphate and lysophosphatidic acid prevent apoptosis in osteoblastic cells via a signaling pathway involving G_i proteins and phosphatidylinositol-3 kinase. *Endocrinol* 143, 4755–4763 (2002)

27. Yatomi Y, Y. Igarashi, L. Yang, N. Hisano, R.Qi, N. Asazuma, K. Satoh, Y. Ozaki, & S. Kume: Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J Biochem* 121, 969-973 (19970

28. Okajima F: Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator? *Biochim Biophys Acta* 1582, 132-137 (2002)

29. Haas C, C.J. Creighton, X. Pi, I. Maine, E.A. Koch, G.K. III Haines, S. Ling, A.M. Chinnaiyan, & J. Holoshitz: Identification of genes modulated in rheumatoid arthritis using cDNA microarray analysis of disease-discordant monozygotic twin cells. *Arthritis Rheum.* 54, 2047-2060, (2006)

30. Yatomi Y, F. Ruan, S. Hakomori, & Y. Igarashi: Sphingosine 1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood* 86, 193-202 (1995)

31. Endresen G.K, & O. Førre: Human platelets in synovial fluid: A focus on the effects of growth factors on the inflammatory responses in rheumatoid arthritis. *Clin Exp Rheumatol* 10, 181-187 (1992)

32. Firestein G.S: Evolving concepts of rheumatoid arthritis. *Nature* 423, 356-61 (2003)

33. Anliker B, & J. Chun: Cell surface receptors in lysophospholipid signaling. *Cell Dev Biol* 2004, 15:457-65.

34. Lee M.J, M. Evans, & T. Hla: The inducible G protein-coupled receptor edg-1 signals via the G_i mitogen-activated protein kinase pathway. *J Biol Chem* 271, 11272-11279 (1996)

35. Van Brocklyn J.R, M.H. Graler, G. Bernhardt, T.P. Hobson, M. Lipp, & S. Spiegel: Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. *Blood* 95, 2624-2629 (2000)

36. Im D.S, C.E. Heise, N. Ancellin, B.F. O'Dowd, G. Shei, R.P. Heqavens, M.R. Rigby, T. Hla, S. Mandala, G. McAlister, S.R. George, & K.R. Lynch: Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J Biol Chem* 275, 14281-14286 (2000)

37. Ancellin, N, & T. Hla: Differential pharmacological properties and signal transduction of sphingosine-1-phosphate receptors EDG-1, EDG-3 and EDG-5. *J Biol Chem* 274, 18997–19002 (1999)

38. Windh R.T, M.J. Lee, T. Hla, S. An, a. J. Barr, & D.R. Manning: Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3 and H218/Edg-5 to the Gz, Gq and G12 families of heterotrimeric G proteins. *J Biol Chem* 274, 27351–27357 (1999)

39. Jaillard C, S. Harrison, B. Stankoff, M.S. Aigrot, A.R. Calver, G. Dudd, F.S. Walsh, M.N. Pangalos, N. Arimura, K. Kaibuchi, B. Zalc, & C. Lubetzki: Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. *J Neurosci* 25, 1459-1469 (2005)

40. Borlado L.R, C. Redondo, B. Alvarez, C. Jimenez, L.M. Criado, J. Flores, M.A. R. Marcos, C. Martinez-A, D. Balomenus, & A.C. Carrera: Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation *in vivo*. *FASEB J* 14, 8950-8953 (2000)

41. Di Cristofano A, P. Kotsi, Y.F. Peng, C. Cordon-Cardo, K.B. Elkon, & P.P. Pandolfi: Impaired Fas response and autoimmunity in $Pten^{+/-}$ mice. *Science* 285, 2122-2125 (1999)

42. Camps M, T. Ruckle, H. Ji, V. Ardissone, F. Rintelen, J. Shaw, C. Ferrandi, C. Chabert, C. Gillieron, B. Francon, T. Martin, D. Gretener, D. Perrin, D. Leroy, P.A. Vitte, E. Hirsch, M.P. Wymann, R. Cirillo, M.K. Schwarz, & C. Rommel: Blockade of PI3Kγ suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat Med* 11, 924-925 (2005)

43. Morel J, R. Audo, M. Hahne, & B. Combe: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces rheumatoid arthritis synovial fibroblast proliferation through mitogen-activated protein kinases and phosphatidylinositol 3-kinase/Akt. *J Biol Chem* 280, 15709-15718 (2005)

44. Kim K.W, M.L. Cho, M.K. Park, C.H. Yoon, S.H. Park, S.H. Lee & H.Y. Kim: Increased interleukin-17 production via a phosphoinositide 3-kinase/Akt and nuclear factor kappaB-dependent pathway in patients with rheumatoid arthritis. *Arthritis Res Ther* 7, R139-148 (2005)

45. Kumar P, M.A. Amin, L.A. Harlow, P.J. Polverini, & A.E. Koch: Src and phosphatidylinositol 3-kinase mediate

soluble E-selectin-induced angiogenesis. *Blood* 10, 3960-3968 (2003)

46. Sato K, H. Tomura, Y. Igarashi, M. Ui & F. Okajima: Exogenous sphingosine 1-phosphate induces neurite retraction possibly through a cell surface receptor in PC12 cells. *Biochem Biophys Res Comm* 240, 329-334 (1997)

47. Auge N, M. Nikolova-Karakashian, S. Carpenter, S. Parthasarathy, A. Ngre-Salvayre, R. Salvayre, A. H. Merrill, Jr. & T. Lavade: Role of sphingosine 1-phosphate in the mitogenesis induced by oxidized low-density lipoprotein in smooth muscle cells via activation of sphingomyelinase, ceramidase and sphingosine kinase. *J Biol Chem* 274, 21533-21538 (1999)

48. Duan H-F, C.T. Wu, Y-L. Hua, H-J Liu, Q-W Zhang, X-X Jia, Z-Z Lu & L-S. Wang: Sphingosine kinase activation regulates hepatocyte growth factor induced migration of endothelial cells. *Exp Cell Res* 298, 593-601 (2004)

Send correspondance to: Joseph Holoshitz, M.D. 5520D MSRB1, University of Michigan Medical Center, Ann Arbor, Michigan, 48109-0680, USA, Tel: 734-764-5470, Fax: 734-763-4151, E-mail: jholo@umich.edu