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Sphingosine 1-phosphate induces Mcl-1 upregulation and protects multiple myeloma cells against apoptosis

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ABSTRACT

Sphingosine 1-phosphate (S1P) is a bioactive lysophospholipid which is known to induce diverse cellular responses through at least five G-protein-coupled receptors on various cell types. However, neither the distribution of S1P receptors nor the effects of S1P on multiple myeloma (MM) cells are fully understood. Here, we show that MM cells express the S1P receptors, S1P1, S1P2, and S1P3. Furthermore, S1P protects MM cells against Dex-induced apoptosis. Importantly, S1P upregulates Mcl-1 expression in a time- and concentration-dependent manner in human MM cell lines. Treatment of MM cells with pertussis toxin (PTX), a pan-S1P receptor inhibitor, results in blockage of S1P-induced upregulation of Mcl-1. These data demonstrate that S1P upregulates the expression of Mcl-1 and protects MM cells from Dex-induced apoptosis, providing the preclinical framework for novel therapeutics targeting at both Mcl-1 and/or S1P to improve the patient outcome in MM.

Multiple myeloma (MM) is a malignancy of terminally differentiated B cells. The proliferation and apoptosis of MM cells are tightly regulated by growth factors derived from bone marrow microenvironments, which directly interact with stromal cells and biomolecules from plasma. Sphingosine 1-phosphate (S1P) is a bioactive lysophospholipid mainly produced from activated platelets [1,2] and is physiologically detectable at concentrations of about 200 nM in human plasma and 500 nM in human serum [3]. It has been widely implicated in lymphocyte trafficking, inflammation, cardiovascular and neurological disorders [4–7]. S1P also acts as a tumorigenic and angiogenic growth factor in the tumor microenvironment to contribute to cancer progression. Thus, targeting S1P signaling has become a novel therapeutic strategy in the intervention of tumors [8–10]. Although S1P signaling has been well elucidated, its roles in tumorigenic and pathological progress of multiple myeloma remain unclear. Binding S1P with its receptors results in activation of multiple signaling, including Mitogen-Activated Protein Kinase (MAPK), Phosphoinositide-3 kinase (PI3K), and JAK/STAT-induced diverse processes such as cell migration, angiogenesis, vascular maturation, heart development, and neurite retraction. Given the fact that these signals also play important roles in the regulation of proliferation and survival of MM cells, we hypothesized that S1P signal might be involved in the regulation of MM cell survival. In MM, IL-6 activates the JAK/Stat-3 pathway leading to the upregulation of Mcl-1 expression [11,12]. In the present report, we explored whether S1P, like IL-6, can regulate Mcl-1 expression and thereby influence survival and proliferation of MM cells. Therefore, elucidation of mechanisms of S1P influencing survival of MM cells may provide the preclinical rationale for targeting Mcl-1 and S1P in novel therapeutics to improve a patient outcome in MM.

Materials and methods

Materials. S1P was purchased from Sigma-Aldrich (Sigma, St. Louis, MO). Anti-phospho-p44/42MAPK, anti-p44/42MAPK antibody, and PhosphoPlusStat3 (Tyr705) antibody kit were from Cell Signaling Technology (Beverly, MA). Anti-phospho-AKT, anti-T-AKT, and anti-actin were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rabbit anti-human Mcl-1 antibody was from R&D Systems Inc. (Minneapolis, MN). SEW2871, an S1P1-selective agonist [13], PD98059, AG490 and Wortmannin were from Calbiochem (San Diego, CA, USA). JTE-013, an S1P2-selective agonist [14], was obtained from Tocris Cookson Ltd (Bristol, UK). CAY10444, an S1P3-selective antagonist [15], was purchased from Cayman Chemical Co. (Ann Arbor, MI). S1P, SEW2871 and JTE-013 were completely dissolved in dimethylsulfoxide (DMSO), respectively, and CAY10444 was completely dissolved in dimethylformamide (DMF), then aliquoted and stored at −80 °C. Pertussis toxin (PTX) was from Biomol (Nottingham, UK). Trizol Reagent was from Invitrogen/Life Technologies (Grand Island, NY). Protein assay reagent was from Bio-Rad Laboratories (Hercules, CA).

Abbreviations: S1P, sphingosine-1-phosphate; MM, multiple myeloma; S1PR, S1P receptor; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; PI3K, Phosphoinositide-3 kinase; SPK, sphingosine kinase.

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Cell lines and cell culture. Four human myeloma cell lines were used in this study: XG-7, Sko-007, U266, and RPMI-8226. XG-7 was a gift from Dr. Xue-Guang Zhang (Suzhou University, PR China). Sko-007 and U266 were kindly provided by Prof. Bei-Fen Shen (Beijing Institute of Basic Medical Sciences, Beijing, PR China). Sko-007, RPMI-8226, and U266 were cultured in RPMI 1640 (Sigma, Saint Louis, MO) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. XG-7 cells were cultured in RPMI 1640 containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, and 3 ng/ml of recombinant human IL-6 (rhIL-6) (PeproTech, Rocky Hill, NJ). Cells were passaged twice a week, and logarithmically growing cells were used for all experiments.

Purification of primary MM cells by CD138 microbeads. Bone marrow aspirates were obtained from MM patients, at diagnosis, with approval of the Local Research Ethics Committee and purified as described [16]. In brief, the mononuclear cell fraction was obtained by Ficoll–Paque density gradient centrifugation (GE Healthcare Biosciences AB, Uppsala, Sweden), labeled with magnetic-activated cell sorting (MACS) CD138 microbeads (clone B-B4, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and separated by MACS MS+ separation columns using a MultiMACS separator. Cells were labeled by CD138-phycoerythrin (PE) before and after separation to determine the purity.

RNA preparation and RT-PCR. Total RNA was prepared using the TRIzol method (Invitrogen Life Technologies) according to the manufacturer's protocol. RNA was reverse transcribed (SuperScript II reverse transcriptase; Invitrogen Life Technologies) using random primers. PCR was performed using Takara *Taq* (Takara Bio) polymerase/buffer system. PCR primers used were as follows: for S1P1 (sense) 5′ CCG CAA GAA CAT TTC CAA G 3′ and (antisense) 5′ ACC CAC CAA CAC CCG ACA C 3′; S1P2 (sense) 5′ CCT GCG GGA GCA TTA CCA 3′ and (antisense) 5′ CAC CTT ACG GCT GCT GGA C 3′; S1P3 (sense) 5′ AAG TTC CAC TCG GCA ATG TAC 3′ (antisense) 5′ GCA GCC AGC AGA CGA TAA A 3′; S1P4, (sense) 5′ TGC TGA AGA CGG TGC TGA TG 3′ and (antisense) 5′ GGG TGG TTA CCT CCT TGT CC 3′; β-actin (sense) 5′ GGG ACC TGA CTA ACT ACC TC 3′ and (antisense) 5′ CAG TGA TCT CCT TCT GCA TC 3′. β-Actin cDNA was used as an internal standard. The PCR consisted of 30 cycles (94 °C for 30 s, 57.5 °C for 1 min, and 72 °C for 1 min) after an initial denaturation step (95 °C for 1 min). PCR products were analyzed by electrophoresis on 2% agarose gels.

Cell lysis and Western blotting. Cells were washed twice with 1× PBS and suspended in lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 40 mM glycerophosphate, 125 μM Na_3VO_4, 50 mM NaF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 mM dithiothreitol). After 40 min on ice, lysates were cleared by centrifugation at 13,000g/min for 30 min at 4 °C. Forty-microgram samples of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. After blocking with 5% nonfat milk in Tris–buffered saline containing 0.1% Tween 20 (TBST), the nitrocellulose membrane was incubated with primary antibody in 5% bovine serum albumin in TBST overnight at 4 °C. The membrane was then washed three times with TBST and incubated with secondary antibody in 5% defatted milk in TBST. After washing with TBST five times, the membrane was developed with enhanced chemiluminescence (ECL, Amersham Pharmacia).

Apoptosis assay. Apoptotic cells were detected using FITC-conjugated Annexin-V (Annexin-V-FITC) (Caltag Laboratories, Burlingame, CA) and propidium iodide (PI). Cells were washed twice with cold PBS and resuspended in Annexin-V binding buffer, (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$) at a concentration of 1×10^7 cells/ml. One hundred microliters (1×10^6 cells) was added to a 5 ml culture tube, 5 μl of Annexin-V-FITC and 10 μl of PI were added, and the tube was gently vortexed and incubated for 15 min at room temperature in the dark. Binding buffer (400 μl) was then added to each tube and the cells were analyzed by flow cytometry (Becton Dickinson, Mountain View, CA, USA).

Results and discussion

The previous reports have shown that the S1P receptor subtypes S1P1, S1P2, and S1P3 are ubiquitously expressed. By contrast, the expression of S1P4 is confined largely to lymphoid tissues and platelets, and that of S1P5 is restricted to the central nervous system [4,7,17]. These findings suggest a possibility that various effects of S1P may be attributed to the expression pattern of its receptors. In this report, therefore, we focused on multiple myeloma cells, characterized S1P receptor expression, and examined the biological effects of S1P on cultured multiple myeloma cells. The expression patterns of S1P receptor mRNA in primary MM and various MM cell lines were examined using RT-PCR analysis (Fig. 1A). S1P1, S1P2, and S1P3 were expressed in primary MM, XG-7, SKO-007, and RPMI-8226 cells. By contrast, U266 only expresses S1P3. No significant expression of either S1P4 or S1P5 was detected in any of the cells (data not shown). Given that the expression patterns of S1P receptors were similar between MM cell lines, XG-7 was selected for subsequent experiments. Additionally, we also evaluated the phosphorylation of extracellular signal-regulated kinase (ERK), AKT, and JAK-STAT3 by S1P, which were already reported to play key roles in multiple myeloma cell proliferation and apoptosis [18,19]. As demonstrated in Fig. 1B, S1P induces the rapid phosphorylation of MAPK (labeled p44/p42 MAPK in the figures), tyrosine phosphorylation of STAT3 and phosphorylation of AKT in XG-7 cells. These activations by S1P occurred as early as 15 min after adding it and were dose-dependent (Fig. 1B). To determine whether S1P stimulation requires Gi-protein-coupled receptor signaling to exert its effects on phosphorylation of MAPK, STAT3, and AKT, XG-7 cells were pre-treated with the Gi-protein uncoupling agent, pertussis toxin. As expected, PTX inhibited the S1P-driven MAPK, STAT3, and AKT phosphorylation. Out of the five members of the S1P receptor Edg family, XG-7 cells express Edg1/S1P1, Edg3/S1P3, and Edg5/S1P2 (Fig. 1A). We assessed the effects of the S1P1 agonist SEW2871, S1P2 antagonist JTE-013, and S1P3 antagonist CAY10444 on MAPK, STAT3, and AKT phosphorylation. It was found that JTE-013 (10 μM) pretreatment of XG-7 cells resulted in a complete inhibition of S1P-induced MAPK and AKT activation, and CAY10444 (10 μM) pretreatment of XG-7 cells resulted in a complete inhibition of S1P-induced STAT3 phosphorylation

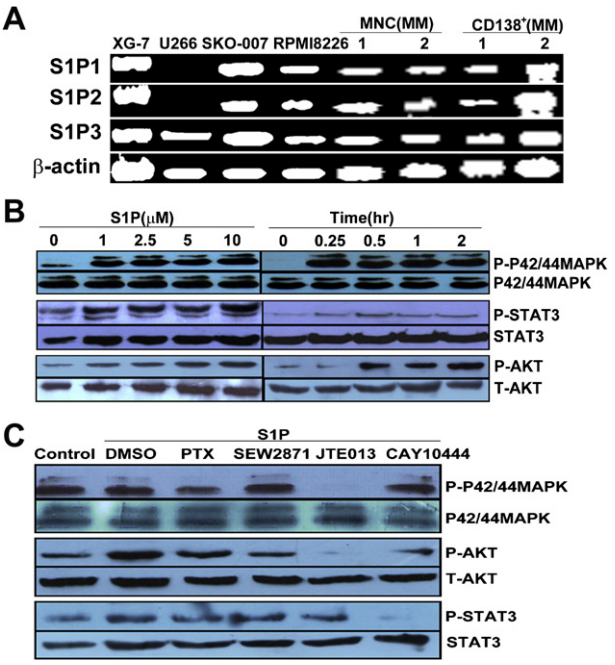

Fig. 1. S1P stimulation of MAPK, STAT3, and AKT phosphorylation. Edg5/S1P2 receptor is necessary for the regulation of MAPK and AKT phosphorylation, and Edg3/S1P3 receptor is necessary for the regulation of STAT3 phosphorylation. (A) Expression of S1P receptors in primary MM and MM cell lines was analyzed by RT-PCR. β-actin was used as an internal reference. (B) Western blot analysis to detect phosphorylated MAPK, STAT3, and Akt. Cells were treated with 1 μM of S1P for indicated time or with different concentrations of S1P for 30 min and the cell lysates were assessed for MAPK, STAT3, and Akt activation. S1P modulated MAPK, STAT3, and Akt activation of signaling. The same blot was stripped and reprobed with an antibody to MAPK, STAT3 and Akt, respectively, as a control of protein loading. The Western blot shown is a representative example of at least three independent experiments. (C) S1P receptor subtype-dependent MAPK, Akt, and JAK/STAT3 activation by S1P. XG-7 cells were pre-treated with 200 ng/ml PTX for 2 h or either control media (0.01% DMSO) on the indicated concentrations of SEW2871 (10 μM), JTE-013 (10 μM), and CAY10444 (10 μM) for 30 min, respectively. Then cells were washed with PBS and added with 1 μM S1P for 30 min. Cells were lysed, electrophoresed, and immunoblotted with anti-phospho-p42/44 MAPK (p-p42/44 MAPK) or MAPK (p42/44 MAPK), phospho-specific Akt S473 (p-AKT) or Akt (total Akt), phospho-STAT3 (p-STAT3) or STAT3 antibodies. The Western blot here shown is a representative example of at least three independent experiments.

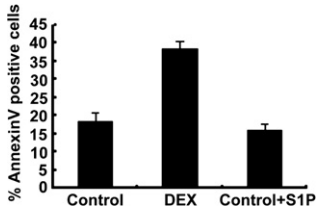

Fig. 2. S1P protects MM cells from Dex-induced apoptosis. XG-7 cells were treated with 10 μM Dex in 6-well plates for 24 h in RPMI 1640, in the presence or absence of S1P (1 μM). Cells were evaluated for apoptosis using Annexin-V staining. The percentages of apoptotic cells were determined by flow cytometry (Becton Dickinson, Mountain View, CA, USA). The data shown represent average values (±SD) derived from three independent experiments. All data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. Statistical significance was defined as *P*-values $\leqslant 0.05$ for all tests.

(Fig. 1C). These results clearly indicate that S1P stimulates MAPK, STAT3, and AKT phosphorylation through S1P receptor subtypes S1P2 and S1P3, respectively.

Evidence for the apoptotic protective effects of S1P in MM cells was investigated using XG-7 cells treated with 10 μM Dex for 24 h and analyzed following Annexin-V-FITC and PI double staining. XG-7 cells responded to Dex with an increase of 38.43% shown by Annexin-V-FITC staining. Basal XG-7 cell apoptosis determined after serum deprivation for 24 h in the absence of Dex showed a basal 18.47% apoptosis in XG-7 cells. In the presence of 1 μM S1P, XG-7 cells displayed significantly less Annexin-V-FITC$^+$ staining (15.81%) than the cells treated with Dex alone. The percentages of apoptotic cells were determined by flow cytometry. The data represent average values (±SD) derived from three independent experiments (Fig. 2). Since both S1P and IL-6 promote MM cell proliferation, and IL-6 upregulates Mcl-1 expression, it was subsequently explored whether S1P can also upregulate Mcl-1 expression. XG-7 cells were starved overnight in RPMI1640 with 0.5% FBS, followed by culture in the absence or presence of 1 μM of S1P for different times (0, 0.25, 0.5, 1, and 2 h). Cells were lysed and Mcl-1 expression was determined by Western blot. Time- and dose-dependent Mcl-1 upregulation by S1P was observed in XG-7 cells (Fig. 3A and B): time course of experiments shows that S1P-triggered upregulation of Mcl-1 is transient, peaking at 0.5 h. Moreover, Mcl-1 expression was upregulated at 1μM and peaked at 5 μM S1P.

In the present study, extracellular S1P partially inhibited the appearance of apoptotic Dex-induced MM cells. The effective S1P concentrations in the present study (1 μM) are within the physiological range found in blood (0.4–1.5 μM) [2]. The effects of extracellular S1P are largely mediated by a receptor transduction

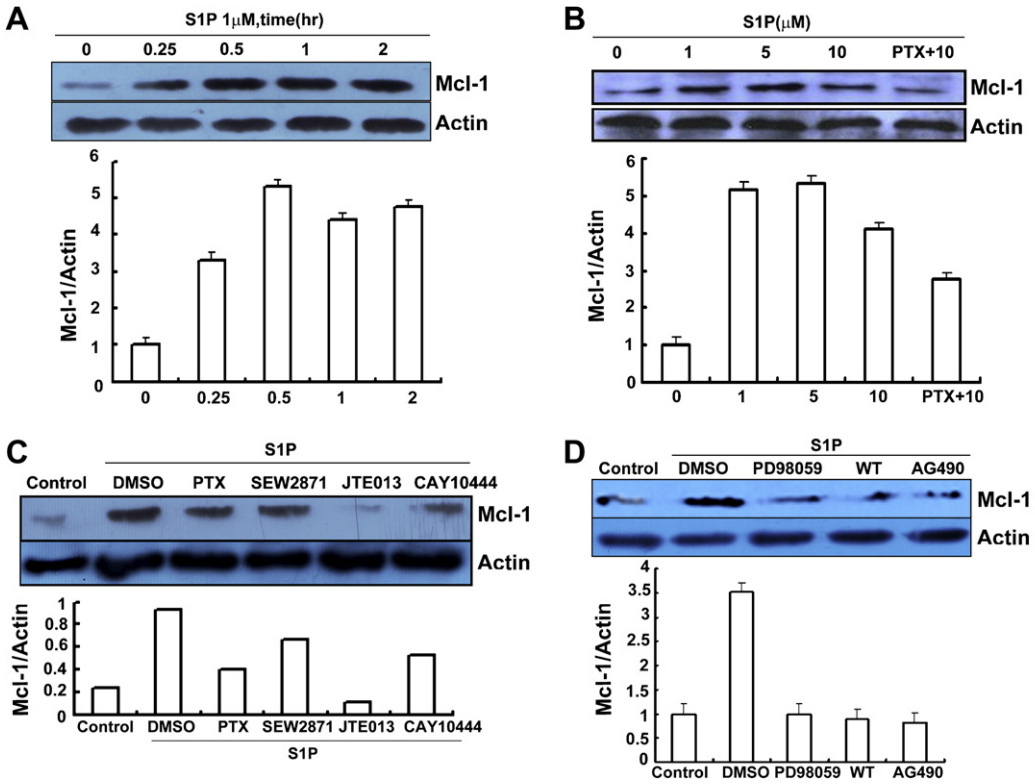

Fig. 3. S1P triggers time- and dose-dependent Mcl-1 expression in XG-7 cells, which is specifically inhibited by JTE-013. (A) S1P triggers time-dependent modulation of Mc-1 expression in XG-7 cells. XG-7 cells were culture overnight in RPMI 1640 supplemented with 0.5% FBS, followed by culture with S1P (1 μM) for different times (0, 0.25, 0.5, 1, and 2 h). Cells were lysed and Mcl-1 expression was determined by Western blot. Actin served as a loading control. Time course of experiments shows that S1P-triggered upregulation of Mcl-1 is transient, peaking at 0.5 h. Actin served as a loading control. (B) Concentration-dependent Mcl-1 upregulation by S1P in MM cells. XG-7 cells were starved overnight in RPMI 1640 with 0.5% FBS, followed by culture with various concentrations of S1P (1, 5, and 10 μM), and dose-dependent Mcl-1 upregulation by S1P was observed in XG-7 cells after 6 h stimulation. Mcl-1 expression was upregulated at 1 μM and peaked at 5 μM S1P. (C) S1P upregulates Mcl-1 expression through S1P receptor subtypes S1P2 and S1P3 in MM cells. XG-7 cells were pre-treated with the Gi-protein uncoupling agent, pertussis toxin 200 ng/ml for 2 h or SEW2871, an S1P1-selective agonist, JTE-013, an S1P2-selective agonist, CAY10444, an S1P3-selective antagonist, respectively. Then cells were washed with cold PBS then added with 1 μM S1P for 2 h. PTX treatment significantly attenuated the upregulation effect of S1P on Mcl-1 expression. JTE-013 and CAY10444 inhibited the S1P-driven increase in Mcl-1 remarkably. Cell lysates (30 μg in each lane) were analyzed by Western blot with Mcl-1 and actin, Actin was used as loading controls. (D) PD98059, Wortmannin, and AG490 partly inhibit S1P-induced the upregulation of Mcl-1 expression. Regulation of Mcl-1 expression was analyzed using inhibitors of the Ras/ERK, PI3K/AKT or Jak/STAT pathways. XG-7 cells were pre-treated with either control media (0.01% DMSO), or PD98059 (50 μM), Wortmannin (WT) (10 μM) and AG490 (100 μM) for 30 min before treatment with 1 μM S1P for 2 h. Cell lysates were collected and analyzed for Mcl-1 and actin by Western blotting. The Western blot analysis shown is a representative example of at least three independent experiments. Densities of signals were determined by densitometry and shown relative to control arbitrarily normalized to 1.

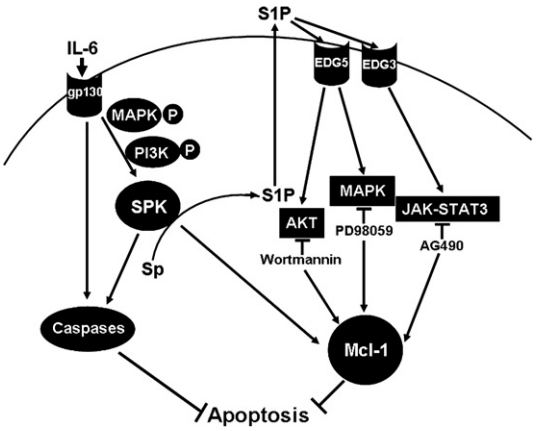

Fig. 4. The signal of SPK-S1P regulation Mcl-1 in MM cells. In previous report [16], we show that IL-6 activates SPK in MM cells, which mediates the suppressive effects of IL-6 on MM cell apoptosis. In this research work, we elucidate that S1P stimulates MAPK, STAT3, and AKT phosphorylation and upregulates Mcl-1 expression through S1PR subtypes S1P2 and S1P3 in MM cells.

mechanism. To determine whether S1P stimulation required Gi-protein-coupled receptor signaling to exert its effect on Mcl-1 expression, XG-7 cells were pre-treated with the Gi-protein uncoupling agent, PTX or SEW2871, a S1P1-selective agonist, JTE-013, a S1P2-selective agonist, CAY10444, a S1P3-selective antagonist, respectively. Then cells were washed with cold PBS and added with S1P. As demonstrated in Fig. 3C, the upregulation effect of S1P on Mcl-1 expression was significantly attenuated by PTX treatment. JTE-013 and CAY10444 inhibited the S1P-driven increase in Mcl-1 remarkably. Taken together, these results indicated the evidence that S1P signaling via S1P receptor subtypes S1P2 and S1P3 participates in Mcl-1 upregulation.

The regulation of Mcl-1 gene expression has been investigated in a number of other experimental systems. In different cell lineages, the Ras/MAPK, PI3K/AKT, and Jak/STAT3 pathways have been implicated in the positive regulation of Mcl-1 expression [20,21]. Therefore, we examined if the effects of inhibitors of these pathways on S1P can induce the upregulation of Mcl-1 expression. XG-7 cells were cultured in RPMI1640 plus S1P (1 μM) after pre-treating with the addition of either PD98059 (MEK1/2 inhibitor), Wortmannin (PI3K inhibitor) or AG490 (a putative Jak2 inhibitor) for the indicated time periods, after which the phosphorylation status of target proteins and the expression levels of Mcl-1 were determined. As shown in Fig. 3d, PD98059, Wortmannin, and AG490 could partly abolish the S1P-induced upregulation of Mcl-1 expression, respectively.

In summary, our present report shows that S1P protects MM cells against Dex-induced apoptosis. Indeed, S1P upregulates Mcl-1 expression in MM cells, thereby mediating, at least in part, its anti-apoptotic capacity. S1P receptor ligand binding initiates a signal transduction mechanism that protects MM cells from Dex-induced apoptosis (Fig. 4). Previously, we detected that IL-6 activates SPK activity in MM cells. The increase in endogenous S1P production may be a survival response of the cells to IL-6 challenge [16]. The protective effects of extracellular S1P against Dex-induced apoptosis suggest that S1P in blood has potential protective effects in vivo in MM patients.

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