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Sphingosine-1-Phosphate Receptor-1 Promotes Environment-Mediated and Acquired Chemoresistance

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Abstract

Drug resistance is a major barrier for the development of effective and durable cancer therapies. Overcoming this challenge requires further defining the cellular and molecular mechanisms underlying drug resistance, both acquired and environment-mediated drug resistance (EMDR). Here, using neuroblastoma (NB), a childhood cancer with high incidence of recurrence due to resistance to chemotherapy, as a model we show that human bone marrow-mesenchymal stromal cells induce tumor expression of sphingosine-1-phosphate receptor-1 (S1PR1) leading to their resistance to chemotherapy. Targeting S1PR1 by shRNA markedly enhances etoposide-induced apoptosis in NB cells and abrogates EMDR, while over-expression of S1PR1 significantly protects NB cells from multidrug-induced apoptosis via activating JAK-STAT3 signaling. Elevated S1PR1 expression and STAT3 activation are also observed in human NB cells with acquired resistance to etoposide. We show *in vitro* and in human NB xenograft models that treatment with FTY720, an FDA-approved drug and antagonist of S1PR1, dramatically sensitizes drug-resistant cells to etoposide. In summary, we identify S1PR1 as a critical target for reducing both EMDR and acquired chemoresistance in NB.

Keywords

S1PR1; neuroblastoma; drug resistance; STAT3; tumor microenvironment

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Introduction

Acquired resistance by cancer cells to cytotoxic drug treatment develops over time as a result of sequential genetic and epigenetic changes (1–3). In addition to acquired resistance, dynamic signaling interactions between tumor cells and the tumor microenvironment (TME) induce an environment-mediated drug resistance (EMDR) phenotype in tumor cells (4). EMDR transiently protects tumor cells from therapy until more complex acquired drug resistance phenotypes can develop, resulting in a state of permanent resistance (4). It has also been shown that the bone marrow niche plays a particularly important role in EMDR through the presence of bone marrow mesenchymal stromal cells (BM-MSC) interacting with tumor cells, via contact-dependent and independent mechanisms (5). It has been demonstrated that aggressive drug-resistant tumor cells frequently induce the expression of high levels of growth factors and chemokines in the TME, which are able to activate growth signaling pathways, thereby providing a microenvironment favorable for tumors even in the presence of potent anti-cancer agents (6,7).

Accumulating evidence indicates that signal transducer and activator of transcription 3 (STAT3) is a determinant of chemoresistance and tumor recurrence in several types of solid malignancies including neuroblastoma (NB) (8–11). The upregulation of pro-survival and pro-proliferative genes by STAT3 enables cells to escape from conventional chemotherapies (12); while inactivation of tumor-suppressor genes such as p53, by STAT3, disrupts the sensitization of oncogenic stress in tumor cells (13). STAT3 has a role in driving EMDR development, by impacting multiple cell types in the microenvironment, including various immune subsets (suppressive T cells and myeloid cells), fibroblasts, and tumor vasculature. The dual role of STAT3 in tumor cells and in the microenvironment has led to multiple attempts to silence or inhibit STAT3 activity alone and in combination with other anti-cancer therapies (14,15).

Neuroblastoma (NB), a highly heterogeneous tumor, originating from peripheral sympathetic nervous system, accounts for 6%–10% of childhood cancers and 15% of all pediatric cancer deaths (16,17). In 70% of patient with metastatic disease, metastasis occurs in the bone marrow (18). Although chemotherapy drugs such as etoposide (VP16) have measurable effects against NB (19), development of drug resistance is the major cause of treatment failure (20). Several studies demonstrate that activation of STAT3 by IL-6 produced by BM-MSC plays a key role in EMDR in NB (10,21). Likewise, activation of STAT3 by G-CSF also directly negatively impacts chemosensitivity of NB cells (22). However, as a transcription factor, direct STAT3 targeting is difficult, especially for clinical translation. In addition, STAT3 is activated by multiple cytokines and chemokines, and blocking one pathway appears to be insufficient to effectively reverse STAT3-mediated drug resistance. Therefore, there is a strong need to identify additional druggable targets that are critical for STAT3 activation in cancer and its environment.

G protein-coupled receptors (GPCRs) are more amenable to small-molecule inhibition (23,24). Recent studies demonstrate that STAT3 upregulates expression of a specific GPCR, sphingosine-1-phosphate receptor 1 (S1PR1) (25,26). S1PR1, in turn, activates STAT3

through tyrosine kinase JAK2, thereby building an S1PR1/JAK2/STAT3 positive feedback loop for persistence STAT3 activation and tumor progression (25). S1PR1 transduces intracellular signals, leading to various biologic effects, including cell proliferation, suppression of apoptosis, and enhancement of cell survival (27,28). Furthermore, sphingosine-1-phosphate (S1P), which is the ligand for S1PR1 and synthesized by sphingosine kinases (SphKs), has been demonstrated to promote cancer growth, metastasis and drug resistance (29). Nevertheless, whether S1PR1-STAT3 pathway plays an important role in NB chemoresistance induced by the TME or acquired by prolonged drug exposure, and whether targeting S1PR1 may sensitize drug-resistant NB to chemotherapy remains unknown. In this study, we investigate the role of S1PR1-STAT3 in conferring drug resistance to NB cells, and whether targeting S1PR1 pathway using small-molecule drugs can chemo-sensitize drug-resistant tumors.

Materials and Methods

Cell lines and Reagents

Human NB cell lines CHLA-171, CHLA-255 and CHLA-255/FLuc transduced with a lentivirus carrying firefly luciferase (Fluc) were obtained from Dr. DeClerck (Children's Hospital Los Angeles, Los Angeles, CA; 2012). Cell line authentication was done by genotype analysis using AmpFISTR® Identifier® PCR Amplification Kit and Gene Mapper ID v3.2 (Applied Biosystems). Human BM-MSC were either purchased from ALLCells LLC or obtained from fresh bone marrow samples of patients with NB enrolled by the Children's Oncology Group from 2012–2015. Patient-derived MSC cells were characterized according to the criteria established by the International Society for Cell Therapy by flow cytometry for the expression of MSC markers, including CD44, CD73, CD90 and fibroblast activation protein alpha (FAP) and the absence of endothelial (CD31), hematopoietic precursor (CD34) and the myeloid (CD45) markers (30). Cells were also tested for their ability to differentiate into osteoblasts, adipocytes and chondrocytes when cultured in appropriate media. After receiving the cells, we grew the cells for minimum passages before freezing them in liquid nitrogen. The frozen cell lines were not further authenticated in our laboratory. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum except for BM-MSC from ALLCells, which were maintained in basal medium with growth supplements obtained from ALLCells. FTY720, etoposide, taxol, and topotecan were purchased from Sigma-Aldrich. S1P was purchased from Avanti Polar Lipids. W146 (trifluoroacetate salt) (31) and Stattic (32) were purchased from Santa Cruz. AZD1480 (33) was obtained from AstraZeneca and used at the indicated concentrations.

Generation of etoposide resistant cells

Etoposide-resistant NB sublines were developed by culturing parental CHLA-255 or CHLA-171 cells to increasing doses of etoposide (starting at 0.1 μ M). Resistant cells were selected stepwise in dose increments over a period of 6 months. Accordingly, CHLA-255 and CHLA-171 sublines were generated, which are resistant to 1 μ M and 0.5 μ M etoposide, respectively.

Gene expression and knockdown

Construction of MSCV-eGFP-*S1pr1*, cDNA encoding mouse *S1pr1* gene and production of retroviral supernatants were described previously (25). We generated S1PR1 knockdown in NB cells via two different shRNAs transductions: (#1) the shRNA part of pLKO.1 nontargeting shRNA control (NT) and S1PR1 shRNA lentiviral vectors (purchased from Sigma-Aldrich) were subcloned into GFP-tagging lentiviral vectors (eGFP-*fluc*_epHIV7). The production of lentivirus was performed as previously described (26). NB cells transduced with shRNA lentivirus (#1) were sorted based on their GFP expression (26). (#2) NB cells transduced with shRNA lentivirus (#2) (sc-37086-V) and control shRNA (sc-108080) (purchased from Santa Cruz Biotechnology) were selected with puromycin (1.75 µg/ml).

Preparation of conditioned medium and cell co-culture

Patient-derived BM-MSC cells were plated in a 10-cm tissue-culture dish in DMEM supplemented with 10% FBS, and allowed to grow until they reached approximately 80% confluence. The culture medium was changed to serum-free medium and 48 h later, medium was collected, 0.22 µm filtered to remove cell debris, aliquoted, and stored at -80°C as BM-MSC conditioned medium.

For conditioned medium exposure experiments, NB cells were plated in six-well culture plates with DMEM/10% FBS at a density of 4×10^5 cells per well. After 24 h, the cells were serum deprived for 2 h and then stimulated with 10% (v:v) BM-MSC conditioned medium mixed in serum free-medium for the indicated times.

In co-culture experiments, NB cells were added directly to BM-MSC cells or to the transwell inserts separated by 0.4-µm membrane (Costar; Corning) from BM-MSC cells. Controls were monocultures of BM-MSCs or NB cells alone.

Apoptosis assay

Cells were seeded in 12-well culture plates at a density of 2×10^5 cells per well. The following day, the cells were treated with indicated concentrations of etoposide and JAK inhibitor, AZD1480, for 24 h. After treatment, both detached and attached cells were collected, and apoptotic cells were detected by Annexin V Apoptosis Detection Kit according to the supplier's instruction (BD Biosciences). Viable and apoptotic cells were quantified by flow cytometry using the Accuri C6 or the BD LSR Fortessa flow cytometer.

MTS assay

Cells were seeded in 96-well plates at 6×10^3 cells per well. Cells were exposed to various concentrations of indicated agents or combination of drugs in the presence of 2% serum. Cell viability was measured after 24 h or 48 h using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions (Promega). IC₅₀ values were calculated using GraphPad Prism 6 software.

Caspase-3/7 activation assay

Quantification of caspase –3/7 activity was carried out using the Caspase Glo-3/7 assay kit according to the manufacturer's instruction (Promega).

Real-time RT- PCR

Total RNA was prepared using RNeasy kit (Quiagen) following the manufacturer's instructions. cDNA was prepared using iScript cDNA Synthesis Kit (Bio-Rad), and real-time PCR reactions were performed using iQ SYBR Green supermix (Bio-Rad) on CFX Connect equipped (Bio-Rad). Primers were generated using SciTools (Integrated DNA Technologies) and PubMed blasted for gene and species specificity. Each primer set was validated using a standard curve across the dynamic range of interest with a single melting peak. GAPDH housekeeping gene was used as internal control to normalize mRNA expression.

Western Blotting

Whole cell lysates were prepared using RIPA lysis buffer containing 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.5% (v:v) NP-40, 1 mM NaF, 15% (v:v) glycerol, and 20 mM β -glycerophosphate. Protease inhibitor cocktail was added fresh to the lysis buffer (Complete-mini, Roche). The samples were separated in a 0.1 % (v:v) SDS, 12% (w:v) acrylamide gel and subsequently transferred to nitrocellulose membranes. After blocking with 5% (v:v) BSA in TBS containing 0.1% (v:v) Tween 20, the membranes were probed with primary antibodies at 4C overnight, followed by another incubation with horseradish peroxidase-conjugated linked secondary antibodies (Amersham). Rabbit polyclonal antibodies against pSTAT3 (Y705) (#9131), pJAK2 (#3771), cleaved PARP-1 (#9542), cleaved caspase-3 (9664), Bcl-xL (sc-8392), Bcl-2 (#2876) and survivin (#2808) were obtained from Cell Signaling Technology. Rabbit polyclonal antibodies against STAT3 (sc-482), S1PR1 (sc-25489), Sphk1 (sc-48825), and GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology, and mouse monoclonal antibody (mAb) against β -actin (A5441) was purchased from Sigma-Aldrich.

Immunofluorescence

Cells were plated onto coverslips in DMEM at ~70% confluence for 24 h. They were fixed with 4% (w:v) paraformaldehyde for 10 min, permeabilized by 0.1% (v:v) Triton X-100 for 5 min at room temperature, washed in PBS and blocked in PBS supplemented with 2% (w:v) BSA and 10% (v:v) goat serum. The cells were then incubated overnight with primary antibodies at 4C, followed by incubation for 1 h with fluorescence-labeled secondary antibody (Alexa Fluor 488 or Alexa Fluor 546, Invitrogen) and counterstained with Hoechst 33342 (Invitrogen). Slides were mounted and examined using the Zeiss LSM510 Meta inverted confocal microscope (Carl Zeiss).

For immunofluorescence staining, frozen sections (5 μ m) were fixed with 4% paraformaldehyde for 15 min, washed, and blocked with 10% goat serum in PBS for 30 min at room temperature. Sections were then incubated with CD31 (BD Biosciences), CD90 (THY1) (One World Lab) and CD44 (Cell Signaling Technology) antibodies in blocking solution at 4°C overnight. For visualization, sections were stained with fluorescence-labeled secondary antibodies (Alexa Fluor 488, Alexa Fluor 555 and Alexa Fluor 647, Invitrogen)

and counterstained with Hoechst 33342 (Invitrogen). Slides were mounted and examined using the Zeiss LSM5700 confocal microscope (Carl Zeiss).

Immunohistochemistry

Paraffin-embedded slides were deparaffinized, rehydrated and boiled in Antigen Unmasking Solution (Vector). Slides were incubated with 0.3% (v:v) H₂O₂ and then blocked with goat serum (DAKO) for 30 min at room temperature. Sections were stained overnight at 4°C with a cleaved caspase-3 antibody (Cell Signaling Technology). Biotinylated anti-rabbit secondary antibodies were added and incubated at room temperature for 1 hr. After 30 min with streptavidin-HRP, sections were stained with DAB (3, 3'-diaminobenzidine) substrate and counterstained with H&E and examined under Olympus AX70 automated upright microscope. For cleaved caspase-3 cell counting, 4–5 sections from each tumor sections were selected and processed with ImageJ software (NIH).

Xenograft tumor model

All animal research procedures were approved by the institutional animal care and use committee (IACUC) at City of Hope for assessment and accreditation of laboratory animal care, and were in accordance with NIH guidelines. In the first experiment, 5×10^6 CHLA-255/Fluc cells mixed with 0.8×10^5 BM-MSC were subcutaneously implanted in 8 week-old male NOD/SCID/interleukin 2 gamma-receptor-null (NSG) mice. When tumor volumes reached approximately 60 mm³, mice were randomly divided into 4 treatment groups: Group 1. control group (vehicle alone); Group 2. FTY720 alone (intraperitoneally (i.p.) injection at 5 mg/kg daily, 5 days a week for a total of 2 weeks); Group 3. etoposide alone (injected i.p. at 5 mg/kg on the first day of treatment and then administered 2 more times at 6-day interval, for a total of 3 doses), and Group 4. concurrent treatment of FTY720 and etoposide. The same treatment strategy was utilized for mice bearing CHLA-255 etoposide-resistant tumors. Tumor growth was evaluated twice per week and calculated with the following formula: Tumor volume = length $\times$ width²/2, where length represents the largest tumor diameter and width represents the perpendicular tumor diameter. The relative tumor volume (RTV) was calculated using the following formula: RTV = (tumor volume on measured day)/(tumor volume on day 0). Tumor were either frozen in liquid nitrogen or fixed in 4% (w:v) paraformaldehyde and embedded in paraffin for further analysis.

Statistical Analyses

Data were analyzed by Student t-test using GraphPad Prism software. *p* values < 0.05 were considered statistically significant.

Results

BM-MSC upregulates S1PR1 in NB cells leading to drug resistance

To investigate the role of S1PR1 in EMDR, we collected conditioned medium (CM) derived from primary cultures of BM-MSC obtained from patients with NB (BM-MSC) and tested its effects on expression of S1PR1 in CHLA-255 NB cells. We found that CM upregulated S1PR1 expression as well as phosphorylation of its downstream signaling molecules, JAK2 and STAT3, in CHLA-255 cells (Fig. 1A-left panel). An increase in the levels of S1PR1

following stimulation with CM was also confirmed by immunofluorescence staining (Fig. 1A-right panel). To assess the role of S1PR1 in drug resistance, S1PR1 was silenced in CHLA-255 cells by 2 different shRNAs, resulting in significant inhibition (88% in shRNA #1 and 85% in shRNA #2) of STAT3 activity (phosphorylated STAT3, pSTAT3) (Fig. 1B), and a 2.5 fold increase in etoposide-induced apoptosis, as compared to non-targeting shRNA controls (NT shRNA) (Fig. 1C). Silencing S1PR1 also increased etoposide-induced apoptosis in transwell co-cultures of CHLA-255 and BM-MSC cells (Fig. 1D), emphasizing an important role of S1PR1 in EMDR in NB. To further confirm that the increase in STAT3 phosphorylation is directly linked to increased S1PR1 expression, the levels of pSTAT3 were evaluated in the presence of BM-MSC CM in NB cells stably expressing S1PR1 shRNA and NT shRNA (Fig. 1E). Indeed, pSTAT3 was significantly inhibited (35% after 20 min and 42% after overnight (ON) stimulation in S1PR1 shRNA cells in the presence of BM-MSC CM as compared to NT shRNA cells. We further demonstrated that not only BM-MSC CM but also co-culturing CHLA-255 cells with BM-MSC upregulated S1PR1 expression, STAT3 activation, and protected NB cells from etoposide-induced apoptosis (Supplemental Fig. 1A, B).

S1PR1 in NB tumor cells promotes chemoresistance and STAT3 activation

To further confirm the importance of S1PR1 in drug resistance, we transduced CHLA-255 and CHLA-171 NB cell lines with MSCV-*S1pr1*-expressing retrovirus. Overexpression of *S1pr1* led to increased levels of pSTAT3 in both cell lines as detected by western blotting (Fig. 2A). Furthermore, overexpression of *S1pr1* caused resistance to etoposide in both CHLA-255 and CHLA-171 NB cells (Fig. 2B, C; Supplemental Fig. 2A, B). Cross-resistance of *S1pr1* transfectants to another chemotherapeutic agent, topotecan (topoisomerase I inhibitor), was also observed (Fig. 2B, C). Furthermore, *S1pr1* overexpression reduced etoposide treatment-associated expression of the apoptosis markers, cleaved caspase-3 and cleaved PARP-1 (Fig. 2D, Supplemental Fig. 2C).

Our previous work showed that JAK2 is critical for S1PR1-induced STAT3 activation and tumor survival (25). We next tested the effects of the JAK inhibitor, AZD1480, on S1PR1-mediated NB-drug resistance, and found that JAK signaling blockade abrogated *S1pr1*-mediated protection of CHLA-171 NB cells against etoposide-induced apoptosis (Supplemental Fig. 2D). Collectively these data thus far demonstrate a key role for S1PR1 in regulating EMDR, and show that JAK/STAT3 signaling is an important driver of S1PR1-induced EMDR in NB cells.

FTY720 inhibits STAT3 activity and sensitizes NB cells to etoposide

We next examined the effects of FTY720, a small-molecule antagonist of S1PR1, on STAT3 activation in NB cells induced by BM-MSC. CHLA-255 cells were pretreated with FTY720 for 2 h and then stimulated with 10% CM from BM-MSC for 20 min. This experiment demonstrated that BM-MSC CM-induced STAT3 activation was inhibited by treatment with FTY720 (Fig. 3A). Furthermore, FTY720 (at 10 μ M) decreased the levels of pSTAT3 in control and *S1pr1* overexpressing CHLA-255 cells by 4.3 and 5.2 fold, respectively (Fig. 3B). The increase in cleaved caspase-3 protein levels following FTY720 treatment was observed in control cells, which was even more pronounced in *S1pr1* overexpressing cells.

Next, we assessed whether FTY720 treatment enhances the anti-tumor effects of etoposide on NB cells. We selected a concentration of etoposide (0.1 μ M) that did not alter cell growth after 48 h to assess FTY720 effect. *In vitro* cell viability assays indicated that FTY720 sensitized CHLA-255 cells to etoposide (Fig. 3C). Furthermore, western blot analysis demonstrated that FTY720 (5 μ M) and etoposide (1 μ M) combinatorial treatments for 24 h also effectively down-regulated pSTAT3 and Bcl- χ L levels, and induced the cleavage of apoptotic proteins, caspase-3 and PARP-1 (Fig. 3D).

We further examined the effects of FTY720 on NB etoposide resistance in a human NB xenograft model in NSG mice by co-injecting human BM-MSC and CHLA-255 NB cells. To detect the presence of BM-MSCs in the tumor stroma we used immunofluorescence staining for hBM-MSCs markers (Fig. 3E). Both FTY720 and etoposide inhibited tumor growth, but combination of the two compounds inhibited tumor growth more significantly than each compound alone (Fig. 3F). Furthermore, the combinatorial treatment resulted in a significant increase in apoptotic cells within the tumor as shown by the increase presence of apoptotic cells positive for cleaved caspase-3 (Fig. 3G).

The S1PR1/JAK2/STAT3 pathway is over-activated in acquired drug-resistance

Our data so far suggest that overexpression of S1PR1 in tumor cells by the TME is a mechanism that contributes to transient EMDR. We next asked whether S1PR1 signaling pathway could also contribute to acquired and permanent resistance. We generated etoposide-resistant human NB cells by exposing CHLA-255 and CHLA-171 parental cell lines to increased concentrations of etoposide over seven months. The acquisition of stable resistance in NB cells that grew in the presence of etoposide was demonstrated by MTS assay performed in the absence of CM from BM-MSC (Fig. 4A). Etoposide resistance in CHLA-255 and CHLA-171 cells cultured in the presence of etoposide increased by 35-fold (IC_{50} 0.58 μ M vs. 20.28 μ M) and 40-fold (IC_{50} 0.29 μ M vs. 11.58 μ M), respectively (Fig. 4A, Supplemental Fig. 3A). Interestingly, cross-resistance with the tubulin inhibitor taxol (paclitaxel) was also observed in etoposide-resistant CHLA-255 cells (IC_{50} 0.3 μ M vs. 1.52 μ M, Supplemental Fig. 4A).

The resistance to etoposide-induced apoptosis was also demonstrated by staining cells with Annexin V/PI. The percentage of apoptotic cells after etoposide treatment was 4.5- and 2.5-fold higher in the CHLA-255 and CHLA-171 sensitive cells than in the resistant cells, respectively (Fig. 4B). Additionally, etoposide-induced caspase-3/7 activity was enhanced 4-fold in sensitive cells, but not in the resistant cells, at 0.5 μ M etoposide compared to vehicle-treated CHLA-255 cells (Fig. 4C). Importantly, we show that etoposide-resistant NB cells had highly elevated S1PR1 and pSTAT3 levels as compared to the sensitive cells. In addition, cleavage of PARP-1 was detected in the sensitive NB cells but not in the resistant cells, upon exposure to etoposide (Fig. 4D). Using confocal fluorescence microscopy we confirmed a 5- and 2-fold increase in S1PR1 expression in the etoposide-resistant CHLA-255 and CHLA-171 cells as compared to the sensitive cells, respectively (Fig. 4E, Supplemental Fig. 3B). Since JAK2 is activated by S1PR1 (25), and we demonstrated that inhibition of JAK sensitized S1PR1 overexpressing NB cells to etoposide (Supplemental Fig. 2D), we tested the potential of the JAK inhibitor, AZD1480, in re-sensitizing resistant

cells to etoposide. Our results showed that blocking JAK signaling in the drug-resistant NB cells also sensitized them to etoposide treatment, as shown by Annexin V analysis (Supplemental Fig. 4B). In addition, treatment of CHLA-255 cells with the small-molecule inhibitor of STAT3 activation, Stattic, suppressed the proliferation of the drug-sensitive and drug resistant NB cells (Supplemental Fig. 5A). Furthermore, Stattic prevented STAT3 activation by BM-MSC CM (Supplemental Fig. 5B), and restored etoposide-induced apoptosis (Supplemental Fig. 5C).

FTY720 inhibits STAT3 activity and restores sensitivity to etoposide-resistant NB cells *in vitro* and *in vivo*

Since inhibition of S1PR1 by FTY720 reduced STAT3 activation and increased etoposide-induced apoptosis in NB cells exposed to BM-MSC CM or overexpressing *S1pr1* (Fig. 3A–D), we next explored whether FTY720 could restore sensitivity to etoposide in NB cells with acquired resistance to the drug. We found that FTY720 reduced pSTAT3 levels and decreased the expression of Bcl-2 and survivin in a dose-dependent manner not only in the sensitive NB cells but also in etoposide-resistant NB cells (Fig. 5A). We further confirmed these findings by using a competitive S1PR1 antagonist, W146, which also decreased STAT3 activation in etoposide-resistant NB cells induced by BM-MSC CM (Supplemental Fig. 5D). In addition, using non-cytotoxic doses of FTY720 (2.5 μ M), we observed that it sensitized drug-resistant NB cells to etoposide (Fig. 5B). Furthermore, combinatorial treatments with FTY720 and etoposide resulted in an increase in cleavage of caspase-3 and PARP-1, as well as inhibition of pSTAT3 and Bcl-2, compared to either agent alone, in both sensitive and resistant cells (Fig. 5C). Finally, we evaluated whether FTY720 could sensitize drug-resistant NB cells to etoposide in a human NB xenograft model. This experiment demonstrated that although etoposide and FTY720 monotherapy failed to effectively control the growth of drug-resistant NB tumors, a significant growth inhibition was observed when FTY720 was added to etoposide treatment (Fig. 5D). Consistently, the combination of FTY720 and etoposide caused an increased apoptotic response, as shown by an increase in cells positive for cleaved caspase-3 in tumors (Fig. 5E).

Crosstalk between NB cells and BM-MSC cells upregulates Sphk1

Since sphingosine kinases are critical for production of S1P, the ligand for S1PR1 (34), we next assessed whether Sphk1 protein expression level was upregulated in co-culture of NB cells with BM-MSC. Indeed, we found that Sphk1 was highly expressed at the protein level in contact-dependent co-culture of NB cells with BM-MSC cells as compared to mono-cultures (Supplemental Fig. 6A). Furthermore, Sphk1 mRNA levels were also upregulated in contact-dependent co-culture of NB cells with BM-MSC as compared to combined mono-cultures (Supplemental Fig. 6B). Moreover, co-culture of CHLA-255 cells with BM-MSC enhanced Stat3 (1.85-fold), Il-6 (1.7-fold) and Bcl-2 (2.8-fold) mRNA levels as compared to combined mono-cultures (Supplemental Fig. 6B). Upregulation of Sphk1 was also observed in *S1pr1*-overexpressing CHLA-255 cells and etoposide-resistant NB cells (Supplemental Fig. 6C). These data suggest that the crosstalk between the hBM-MSC cells and NB cells can increase Sphk1 production, which in turn produces more S1P in the tumor microenvironment to activate S1PR1-STAT3 pathway in NB cells (Supplemental Fig. 6D).

Discussion

Acquisition of drug resistance through interactions between tumor cells and the TME as well as by prolonged drug treatment remains a major clinical challenge in cancer therapy (2,4). An increasing number of studies have reported that multiple cells in the TME, such as BM-MSC, tumor-associated macrophages (TAM) and tumor associated fibroblasts, promote tumor progression as well as drug resistance (11,35–37). In the case of NB, it has been reported that STAT3 activation by IL-6 secreted by TAM or BM-MSC is a critical signaling molecule for EMDR (10).

However, because STAT3 activation occurs through multiple mechanisms other than IL-6/IL-6R interaction, blocking IL-6 or IL6 receptor is unlikely to be effective, as recently confirmed by the lack of efficacy of IL-6 inhibitors in clinical trials (38). Our previous studies have shown that although IL-6-induced STAT3 activation is often transient, S1PR1 persistently activates STAT3 (25). Although the NB cell lines we studied here do not exhibit high levels of S1PR1 *in vitro*, we show that BM-MSC cells are able to induce S1PR1 expression in NB cells. Our earlier studies indicated that IL-6 secreted by tumor-associated immune cells can activate STAT3, which in turn upregulates S1PR1 expression (25,39). In support of a critical role of S1PR1 in mediating chemoresistance, we showed that enforced expression of S1PR1 in NB cells conferred resistance to chemotherapeutic agents. Conversely, silencing S1PR1 in tumor cells sensitized them to chemotherapy. Furthermore, knockdown of S1PR1 in NB cells significantly reduced the ability of BM-MSC to promote their survival when exposed to etoposide.

In addition to EMDR, NB tumor cells frequently become resistant to cytotoxic drugs because of intrinsic alterations in the drug targets and/or activation of pro-survival pathways. By generating two multiple-drug resistant NB cell lines in the presence of etoposide, we provide evidence that overexpression of S1PR1 and subsequent STAT3 activity are part of the selection process leading to acquired and permanent drug resistance. These findings strongly suggest that S1PR1 is a major pathway not only in transient and reversible EMDR, but also in permanent acquired drug resistance. Additionally, in NB cells co-cultured with BM-MSC cells, as well as in resistant and S1PR1 overexpressing NB cells, we observed an increase in the levels of the S1P-generating enzyme, SPHK1. Upregulation of SPHK1 was shown to increase the production of S1P, and is correlated with poor cancer prognosis (40). Furthermore, we have demonstrated increased expression of anti-apoptotic gene, Bcl-2, in co-culture conditions and in resistant cells, potentially aiding in resistance to chemotherapy.

Our recent studies demonstrated that S1PR1-mediated persistent activation of STAT3 requires tyrosine kinase JAK2 (25). Supporting an important role of JAK2 in mediating S1PR1-induced chemoresistance, we found that the small-molecule JAK inhibitor (AZD1480) overrides the protective effect of S1PR1 and induce apoptosis in NB cells in the presence of etoposide. Several JAK inhibitors are currently being evaluated in clinical trials in patients with myeloproliferative disorders and solid tumors (41,42). In the case of NB, AZD1480 was shown to be effective in inhibiting tumor growth in pre-clinical studies (43). Our results suggest that JAK inhibitors can also overcome NB drug resistance mediated through S1PR1/STAT3 signaling.

We also tested the ability of a sphingosine analogue, FTY720, to block S1PR1 signaling thereby sensitizing NB cells, especially drug-resistant tumor cells, to chemotherapy. FTY720, which is phosphorylated by sphingosine kinase (SK)2 (44), interacts with S1PR1 at high affinity, leading to S1PR1 endocytosis and degradation *in vitro* (45) and *in vivo* (46,47). In addition to various types of cancer cells tested with FTY720 (48), it has also been demonstrated to inhibit NB tumor growth and enhance the anti-tumor effects of topotecan (49). Here we show that S1PR1 inhibition with FTY720 enhanced the anti-tumoral effects of etoposide in chemo-resistant tumors.

It is noted that the therapeutic FTY720 doses (of 5–10 mg/kg) that we and others have used in animal cancer models (50,51) are higher (by fold 10) than that used in models of cerebral ischemia (52,53) or multiple sclerosis (54). FTY720 treatment is associated with adverse effects that include headache, influenza-like illness, fatigue, gastrointestinal dysfunction, hemorrhaging focal encephalitis as well as transient bradycardia, and skin cancer (55). On the other hand, in our human NB xenograft model we did not observe severe side-effects associated treatment with FTY720, which is consistent with several previous studies (48).

In this study, we demonstrate that S1PR1 upregulation contributes not only to transient drug resistance through EMDR but also to drug resistance acquired by prolonged drug exposure. Inhibition of the S1PR1 pathway with either a JAK inhibitor or an S1PR1 antagonist blocks STAT3 activation and sensitize NB cells to chemotherapeutic agents. The S1PR1/JAK/STAT3 signaling axis has also been found to be operative in the development of breast cancer (25), lymphoma (26), and colon cancer (34), and our data now support the role of this signaling pathway in chemotherapeutic resistance. Importantly, we demonstrate that S1PR1-STAT3 pathway is crucial not only for acquired drug resistance but also EMDR in NB. Overall, our results suggest that pharmacological inhibition of S1PR1 in combination with chemotherapeutic agents may be a promising strategy to manage chemoresistance in NB and potentially in other cancers.

Supplementary Material

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Figure 1. S1PR1 is upregulated in NB cells in the presence of BM-MS-CM and inhibits etoposide-induced apoptosis

(A) Western blotting showing expression of indicated proteins in CHLA-255 cells after 24 h stimulation with BM-MS-CM. Data are representative of 3 independent experiments with similar results. Right panel, immunofluorescent images showing S1PR1 expression and nuclei in CHLA-255 cells stimulated for 1.5 h with BM-MS-CM. Shown are representative images of 3 independent experiments. Scale bar, 10 μ m. (B) pSTAT3 protein levels in CHLA-255 cells transduced with lentiviral vectors containing nontargeting (NT) shRNA control or two *S1PR1* shRNA variants (#1 and #2). Data are representative of 3 independent experiments with similar results. (C) Flow cytometry analysis of apoptosis in CHLA-255

cells expressing the indicated shRNAs after treatment with etoposide (1 μ M) for 24 h. **(D)** Representative Annexin V staining by flow cytometry of CHLA-255 cells expressing NT or *S1PR1* shRNA co-cultured with BM-MSC followed by exposure to 1 μ M etoposide. Lower panel, the relative apoptotic index of the NB cells calculated as the percentage of the apoptotic cells co-cultured with BM-MSC relative to the percentage of the apoptotic cells without co-culture. In panel C and D, data represent mean $\pm$ SEM from 3 independent experiments. **(E)** Western blotting shows pSTAT3 and STAT3 expression levels in CHLA-255 cells stably expressing NT or *S1PR1* shRNA (#2) stimulated with BM-MSC CM for 20 min or overnight (ON). Data are representative of 3 independent experiments with similar results. In all western blots, band densitometry was normalized to GAPDH or b-actin using ImageJ software and the values are indicated below the gel figures.

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Figure 2. S1PR1 promotes STAT3 activation and chemoresistance in NB cells

(A) Western blotting showing S1PR1, pSTAT3 and STAT3 expression levels in control (vector alone) and MSCV-*S1pr1*-overexpressing CHLA-255 and CHLA-171 cells. Data are representative of 3 independent experiments with similar results. (B) Flow cytometry analysis of control and *S1pr1* overexpressing CHLA-255 cells treated with 1 μ M etoposide or 0.05 μ M topotecan for 24 h, and then stained with Annexin V. (C) Graph showing percentages of Annexin-V-positive apoptotic cells in control and *S1pr1* overexpressing NB cells treated with indicated drugs. Data represent mean $\pm$ SEM of 3 independent experiments. (D) Western blotting of uncleaved and cleaved PARP-1 and cleaved caspase-3 in control and *S1pr1* overexpressing CHLA-255 cells following exposure to the indicated concentrations of etoposide for 24 h.

Figure 3. FTY720 inhibits STAT3 activity and sensitizes NB cells to etoposide

(A) Western blotting showing the effects of FTY720 on pSTAT3 in CHLA-255 cells. CHLA-255 cells were serum starved for 2 h, treated for 2 h with 10 μ M FTY720, and followed by 20 min incubation with BM-MSC CM (10%). (B) Western blotting comparing the indicated protein levels in control or *S1pr1* overexpressing CHLA-255 cells treated with indicated concentrations of FTY720. (C) Effects of etoposide or FTY720 alone or in combination on the viability of CHLA-255 cells by MTS assay following 48 h treatment. Data represent mean $\pm$ SEM percentage of viable cells from 4 independent experiments. (D) Western blotting of indicated proteins in CHLA-255 cells treated with 5 μ M FTY720 or 1

μM etoposide alone or in combination for 24 h. In panel A, B and D, data are representative of 2–3 independent experiments with similar results. **(E)** Immunofluorescence staining of CHLA-255+hBM-MSCs tumors, collected at the end of the *in vivo* experiment, with anti-CD90 (green), anti-CD44 (red) and anti-CD31 (purple) antibodies. Zoomed inset highlights cells expressing MSC markers, CD90⁺/CD44⁺/CD31[−] (20× magnification). **(F)** Relative tumor volume of NB xenografts in mice treated with vehicle (n =4), FTY720 alone (n = 6), etoposide alone (n = 5), or FTY720 plus etoposide (n = 6). Mice were treated with 5 mg/kg FTY720 daily, 5 days a week for a total of 2 weeks, 5 mg/kg etoposide on the first day of treatment and then 2 more times at 6-day interval (for a total of 3 doses), or a combination of FTY720 and etoposide. **(G)** Representative immunohistochemistry images for cleaved caspase-3 staining in tumor sections (20× magnification). Right panel, quantification of cleaved caspase-3-positive cells in tumor tissue. Data were analyzed by ImageJ software from 3–4 fields examined in 3 sections for each tumor.

Figure 4. The S1PR1/JAK2/STAT3 pathway is over-activated in acquired drug-resistance
(A) Dose dependent cell viability curve of CHLA-255 etoposide-sensitive and etoposide-resistant NB cells treated with etoposide for 24 h. The IC_{50} values were determined using GraphPad Prism 6 software. **(B)** Flow cytometry analysis of apoptosis by Annexin-V staining of the CHLA-255 and CHLA-171 etoposide-sensitive and etoposide-resistant NB cells after treatment with etoposide (1 μ M) for 24 h. Data represent mean $\pm$ SEM percentage of Annexin-V positive cells of 4 independent experiments, each performed in duplicate. **(C)** Caspase-3/7 activation in CHLA-255 etoposide-sensitive and resistant cells responding to etoposide treatment. **(D)** Western blotting of S1PR1, full-length PARP-1 and its cleavage

fragment, pSTAT3 and total STAT3 in CHLA-255 and CHLA-171 sensitive (S) and resistant (R) cells following exposure to etoposide for 24 h. Data are representative of 3 independent experiments with similar results. (E) Immunofluorescent (red) staining of S1PR1 in CHLA-255 sensitive and resistant cells by confocal microscopy. Nuclei were stained with Hoechst 33342. Images are representative of 3 independent experiments. Scale bar, 10 μ m. Right panel, quantitation of fluorescence of immunostained cells using ImageJ. The data represent the mean + SEM total cellular fluorescence value (in pixels) from 20–25 cells analyzed.

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Figure 5. FTY720 inhibits STAT3 and sensitizes etoposide-resistant NB cells *in vitro* and *in vivo*
(A) Western blotting comparing the indicated protein amounts in CHLA-255 sensitive or resistant cells treated with indicated concentrations of FTY720 for 4 h. GAPDH was used as loading control. Data are representative of 2 independent experiments with similar results.
(B) MTS assay to measure effects of treatment (48 h) with etoposide or FTY720 alone or in combination on the viability of CHLA-255 resistant cells. Data represent mean $\pm$ SEM percentage of viable cells from 4 independent experiments. **(C)** Western blotting of indicated protein levels from CHLA-255 etoposide sensitive and resistant NB cells following treatment with FTY720 at indicated concentrations or 0.5 μ M etoposide alone or in

combination for 24 h. **(D)** Relative tumor volume of etoposide resistant NB xenografts in mice treated with vehicle (n= 5), FTY720 alone (n= 5), etoposide alone (n= 6), or FTY720 plus etoposide (n= 6), ** $p < 0.01$ and * $p < 0.05$ versus FTY720-treated mice. ns= non significant. Mice were treated with 5 mg/kg FTY720 daily, 5 days a week for a total of 2 weeks, 5 mg/kg etoposide on the first day of treatment and then 2 more times at 6-day interval (for a total of 3 doses), or a combination of FTY720 and etoposide. **(E)** Representative immunohistochemistry images for cleaved caspase-3 staining in tumor tissue (20× magnification). Right panel, quantification of cleaved caspase-3-positive cells in tumor tissue. Data were analyzed by ImageJ software and represent the mean + SEM number of positive cells from 3–4 fields in 3 sections of each tumor.

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