

FTY720 and SEW2871 reverse the inhibitory effect of S1P on natural killer cell mediated lysis of K562 tumor cells and dendritic cells but not on cytokine release

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Abstract The aims of this study are to examine the effect of sphingosine 1-phosphate (S1P) on IL-2-activated natural killer (NK) cell lysis of K562 tumor cells and immature dendritic cells (iDCs), and to investigate the mechanisms involved in S1P activity. Our results show that S1P protected K562 cells or iDCs from NK cell lysis, which was reversed by FTY720 and SEW2871, the antagonists of S1P₁. S1P did not modulate the expression of NKG2D, NKp30, NKp44 or CD158 on the surface of NK cells, and neither affected the expression of CD80, CD83, or CD86 on the surface of DCs. In contrast, it increased the expression of HLA-I and HLA-E on DCs, an activity that was inhibited by FTY720 or SEW2871. Similarly, the inhibitory effect of S1P for NK cell lysis of K562 cells was directed toward S1P₁ expressed on the tumor cells but not on NK cells. Further analysis indicates that NK cells secreted various cytokines and chemokines with various intensities: (1) low (IL-4, IL-6, IL-12, TNF- α and MCP-1); (2) intermediate (IL-1 β , IL-10, TGF- β 1, and IL-17A); (3) high (IFN- γ , and MIP-1 α); and (4) very high (MIP-1 β). S1P significantly reduced the release of IL-17A and IFN- γ from NK cells, but this inhibition was S1P₁-independent. These results indicate that S1P is an anti-inflammatory molecule, and that S1P₁ is important for the interaction among NK cells and tumor cells or DCs leading to up-regulation of HLA-I and HLA-E on the surface of DCs, but not in S1P inhibition of the release of inflammatory cytokines from NK cells. Further, the results suggest that FTY720 and SEW2871 may

potentially be used as prophylactic and/or therapeutic drugs to treat cancer patients.

Keywords Natural killer cells · Dendritic cells · FTY720 · SEW2871 · Sphingosine 1-Phosphate · Tumor cells

Introduction

The major functions of natural killer (NK) cells are tumor rejection, inhibition of virally infected cells, and secretion of immunoregulatory cytokines [1]. Although NK cells are blood-born and primarily found in the blood circulation and the spleen, they migrate toward inflammatory or tumor growth sites in order to lyse infected or metastatic cells. Dendritic cells (DCs) are specialized antigen presenting cells which present antigens and consequently, activate T cells [2]. Human myeloid DCs are generated from monocytes exposed to growth factors such as IL-4 or type I IFN along with GM-CSF [3]. These are known as immature DCs (iDCs) which have certain phenotypes and are capable of capturing antigens, but have little ability to present these antigens. These cells mature upon binding bacterial and other pathogenic products, up-regulate the expression of co-stimulatory molecules CD80, CD83 and CD86, and become potent antigen presenting cells that promote T cell activation.

The serum factor sphingosine 1-phosphate (S1P) is released by platelets and constitutes a major part of serum and plasma [4, 5]. This lipid is pleiotropic which induces multiple biological activities in lymphoid and non-lymphoid cells. The receptors for S1P have been cloned, and are known as S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅ [6]. The drug FTY720 “fingolimod; 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol” is an immunosuppressive drug

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that proved to be powerful for suppressing allograft rejections during kidney transplantation and is currently evaluated for the treatment of multiple sclerosis patients [7]. It binds four out five S1P receptors, namely S1P₁, S1P₃, S1P₄ and S1P₅ [8]. It also induces the internalization of S1P₁, and consequently, inhibits S1P activity [7, 9].

Another drug known as SEW2871 “5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole” structurally unrelated to S1P, because it does not possess charged head groups, was described [10]. This drug has been found to be selective for S1P₁ and was used to dissect the differences among S1P₁ and S1P₃ [10, 11]. Also, SEW2871 was found to modulate the migration of DCs *in vitro* [11] and *in vivo* [12].

Nonetheless, the effects of FTY720 and SEW2871 on NK cell activities have not been extensively studied. Administration of FTY720 into kidney transplant patients did not affect the number of NK cells isolated from these patients [13]. In an animal transplant model, only high but not low doses of FTY720 prevented NK cell infiltration into allogeneic corneal grafts [14]. It was recently reported that S1P inhibits NK cell cytotoxicity of tumor cells [15], but the receptors involved in this activity are not known. In addition NK cells kill immature DCs [16, 17]. Therefore, we assumed that S1P which is constitutively present in the blood circulation and is secreted at high concentrations during inflammation may play a role in the cognate interaction among activated NK cells and DCs. This is particularly true when one considers that both activated NK cells and DCs express receptors for S1P, such as S1P₁, S1P₃, S1P₄, and S1P₅ [18–20]. To dissect the effects of S1P receptors involved in the interaction of NK cells with various target cells such as tumor cells or DCs, we used FTY720 and SEW2871 to determine the involvement of S1P₁ in NK cell lysis of these target cells.

Materials and methods

Reagents and antibodies

Sphingosine 1-phosphate (S1P) was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). SEW2871 was purchased from EMD Calbiochem (San Diego, CA, USA). FTY720 was a generous gift of Dr. Volker Brinkmann (Novartis Pharma AG, Basel, Switzerland).

Preparation of the cells

Buffy coats of healthy volunteers were obtained from the blood bank (Ullevål Hospital, Oslo, Norway). After histopaque (Sigma–Aldrich, Oslo, Norway) separation, the cells were purified using RosetteSep negative selection

human NK cell enrichment cocktail (StemCell Technologies SARL, Grenoble, France), which removes CD3, CD4, CD19, CD36, CD66b, and glycophorin A positive cells, leaving NK cells intact. These preparations were devoid of CD3⁺, CD14⁺ or CD19⁺ cells but were more than 95% positive for CD16 marker, as determined by flow cytometry. The cells were incubated at 1×10^6 cells/ml with 200 U/ml IL-2 at 37°C in a 5% CO₂ incubator for 5–7 days.

Human monocytes were isolated using RosetteSep human monocyte enrichment cocktail (Stem Cell Techniques). Most if not all of these cells were CD14⁺ as determined by flow cytometry, which were cultured at 1×10^6 cells/ml with 6 ng/ml IL-4 and 25 ng/ml rhGM-CSF (ImmunoTools, Friesoythe, Germany), for 5 days at 37°C to generate immature (i) DCs. Mature dendritic cells (mDCs) were generated by incubating iDCs with 1 µg/ml LPS (Sigma–Aldrich, Oslo, Norway) for 2 days, as previously described [19, 21].

NK cell cytotoxicity assay

This method has been recently described [17, 22]. The human myeloid leukemia K562 cells or human iDCs were used as targets. Target cells were incubated at 1×10^6 cells/ml with 5 µg/ml calcein-AM (Teflabs, Austin TX, USA) for 1 h at 37°C. Target cells (10,000/well) and effector cells were plated onto 96-well plates at the indicated E:T cell ratio in triplicate. To obtain total killing, target cells were incubated with 0.5% Triton-X, whereas total viability was obtained by incubating the cells with media only. The plates were spun down at 500 rpm for 5 min and incubated for 4 h at 37°C. After incubation, the cells were centrifuged, the supernatants removed and PBS added to each well. The fluorescence intensity of the calcein AM-loaded cells was measured in a BioTek FLX TBI plate reader (FLx 800, BioTek Instruments, Inc., Winooski, VM), using 485/528 nm fluorescence filters. The percentage of cytotoxicity was calculated according to the following formula: % Viability = fluorescence units (FU) of targets incubated with NK cells (experimental), minus FU of targets incubated with Triton-X (total lysis), divided by FU of targets incubated in media only (total viability), minus FU of targets incubated with Triton-X (total lysis). Percent cytotoxicity was then calculated as 100% minus % viability. In the figures, the 10:1 E:T cell ratio is shown, however, similar results were obtained using other E:T cell ratios.

Immunoblot analysis

NK cells or K562 cells were lysed in ice-cold Nonidet P-40 lysis buffer containing 1% Nonidet P-40, 30 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate,

1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma–Aldrich Inc, Oslo, Norway). After constant agitation for 30 min at 4°C, the cell lysates were centrifuged for 15 min at 4°C, and then separated from the supernatants. Total protein concentration in the samples was measured using Bio-Rad Protein Assay (Bio-Rad, Uppsala, Sweden). Immunoblotting was performed as described [18, 19, 21]. In brief, the samples were run on SDS–PAGE Criterion gels and then electrotransferred to PVDF-membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skimmed milk (3% BSA for goat antibodies) in TBS for 1 h, and then incubated with 1:100 dilution of antibodies to S1P₁, S1P₂, S1P₃, S1P₄ and S1P₅ (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature or overnight at 4°C. The membranes were extensively washed in TBS supplemented with 0.05% Tween 20 and then incubated with HRP-conjugated secondary antibody (1:5,000) for 1 h at room temperature. The membranes were then washed and the proteins were detected with Supersignal (Pierce, Rockford, IL).

Flow cytometric analysis

Immature DCs were left intact or were incubated with 2 μM S1P, 10 nM FTY720, 10 nM SEW2871 or the combinations of S1P with these drugs for 4 h. As a control 1 μg/ml LPS was used. The cells were washed and incubated in a 96-well plate (v-bottom, 2 × 10⁵ cells per well), washed again and resuspended in PBS buffer containing 0.1% sodium azide. They were labeled with 1 μg/ml FITC-conjugated mouse anti-human CD80, 1 μg/ml FITC-conjugated mouse anti-human CD83, 1 μg/ml FITC-conjugated mouse anti-human CD86 (Becton–Dickinson Pharmingen, San Diego CA, USA), 1 μg/ml FITC-conjugated mouse anti-human HLA-class I, 1 μg/ml FITC-conjugated mouse anti-human HLA-DR, 1 μg/ml FITC-conjugated mouse anti-human HLA-E (ImmunoTools, Friesoythe, Germany), or 1 μg/ml FITC-conjugated mouse IgG (Becton–Dickinson Biosciences-Pharmingen, San Jose, CA, USA) as a control. The cells were washed twice, and examined in the flow cytometer (FACSCalibur, Becton–Dickinson Biosciences, San Jose, CA). Markers were set according to the isotype control FITC-conjugated mouse IgG.

To stain NK cells with antibodies for various NK cell activating receptors, they were either left untreated or incubated with 2 μM S1P for 4 h, washed and stained with 1 μg/ml PE-conjugated mouse anti-human NKp30 (CD337), 1 μg/ml PE-conjugated mouse anti-human NKp44 (CD336), 1 μg/ml PE-conjugated mouse anti-human NKG2D (CD314), or as a control 1 μg/ml PE-conjugated mouse IgG1 (all antibodies were from Becton–Dickinson Pharmingen), for 45 min at 4°C. NK cells were also stained with 1 μg/ml FITC-conjugated anti-killer inhibitory receptor

(KIR)/CD158 antibody which recognizes KIR2DL2, KIR2DL3, KIR2DS2 and KIR2DS4 (R&D Systems Europe Ltd, Abingdon, Oxon, UK), and as a control with FITC-conjugated mouse IgG (ImmunoTools, Friesoythe, Germany). The cells were washed twice, and examined in the flow cytometer. Markers were set according to the isotype control PE-conjugated or FITC-conjugated mouse IgG (Becton–Dickinson Biosciences-Pharmingen, San Jose, CA, USA).

Detection of cytokines and chemokines release utilizing the ELISArray kits

DCs or NK cells were incubated at a cell concentration of 1 × 10⁶ cells/ml with either media or with 2 μM S1P, 10 nM SEW2871, 10 nM FTY720 or their combinations. DCs were also incubated with 1 μg/ml LPS. After 24 h incubation, the cells were harvested and the cell suspensions were centrifuged at 1,000 × g for 10 min before the supernatants were collected. Detection of the levels of various cytokines and chemokines was carried utilizing the Multi-Analyte ELISArray Kit (SA Biosciences MD, USA) as described by the manufacturers' user manual. The kit analyzes the release of IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, TGF-β1, MCP-1, MIP-1α, and MIP-1β. Negative and positive controls supplied by the kits were also included.

Statistical analysis

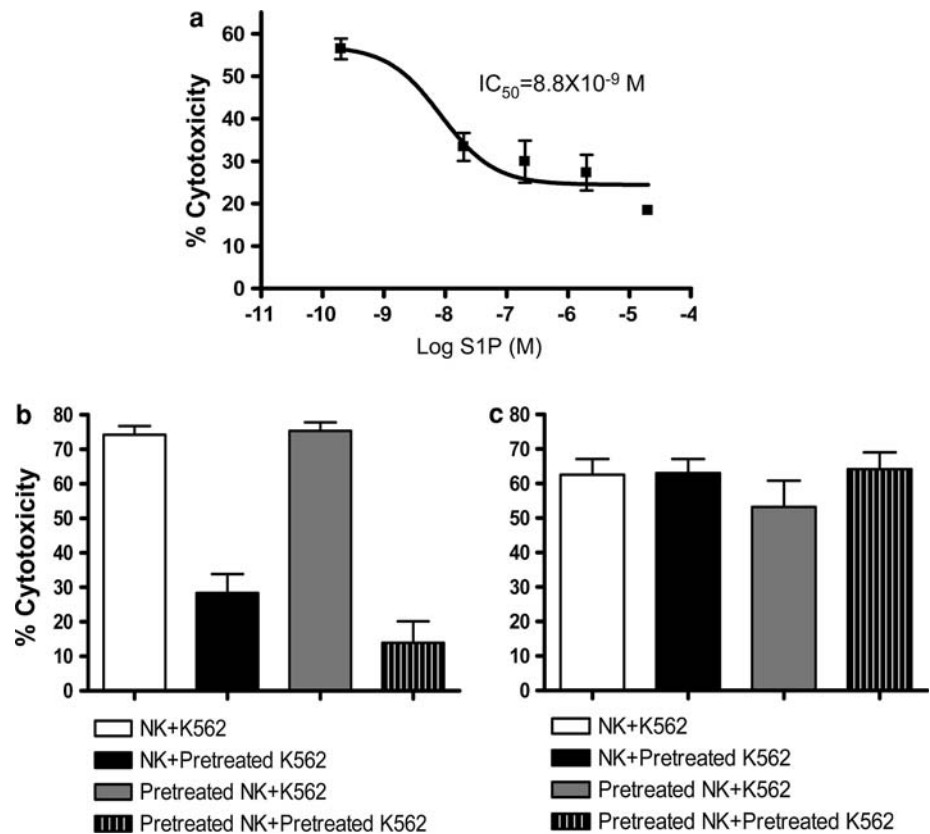
Significant values were determined using the two-tailed Student's *t* test. A *P* value <0.05 was considered to be statistically significant. Geometric mean of fluorescence intensity was calculated using a program provided with the FACSCalibur (BD CellQuest, Becton–Dickinson Biosciences-Pharmingen, San Jose, CA, USA). The IC₅₀ values were calculated using one-site competition of nonlinear regression curves (Graph Pad Prism, San Diego, CA, USA).

Results

S1P inhibits NK cell lysis of K562 cells via S1P₁

It was recently reported that S1P inhibits NK cytotoxicity of melanoma cell lines as well as the Burkitt's lymphoma B cell line RAJI [15]. To determine whether this effect of S1P may be exerted on NK cell cytotoxicity of the universally used NK cell-sensitive tumor target, i.e., K562 cells, we incubated various concentrations of S1P with the mixtures of NK and K562 cells. The results indicate that S1P inhibited NK cell lysis of K562 cells with an IC₅₀ effect of 8.8 nM

Fig. 1 Effect of S1P on NK cell lysis of K562 tumor cells. K562 cells were incubated with NK cells in the presence of different concentrations of S1P in the 4-h NK cell cytotoxicity assay. The IC_{50} effect of S1P is also shown (a). NK cells or K562 cells were pretreated with 2 μ M S1P for 4 h. Pretreated K562 cells were added to either untreated or pretreated NK cells in the 4-h NK cell assay. Similarly, pretreated NK cells were added to untreated or pretreated K562 cells (b). Panel c is similar to panel b, except that K562 cells or NK cells were pretreated with 2 μ M S1P for 24 h prior to adding into the 4-h cytotoxicity assay. Mean \pm SEM of 3 separate experiments. The cytotoxicity of untreated NK cell lysis of untreated K562 cells is shown in white columns of panels b and c



(Fig. 1a). Next, we pretreated NK cells or K562 with 2 μ M S1P for 4 or 24 h and then incubated these cells together. The results indicate that pretreatment of K562 for 4 h with S1P significantly protected these cells from lysis by NK cells ($P < 0.002$, as compared to untreated NK cells incubated with untreated K562, Fig. 1b). However, pretreatment of NK cells for 4 h with 2 μ M S1P did not inhibit the ability of these cells to lyse K562 cells (Fig. 1b). Incubating K562 cells pretreated with S1P with S1P-pretreated NK cells for 4 h also inhibited the cytotoxicity of K562 ($P < 0.0001$). In contrast, no effect was observed on NK cell lysis of K562 cells when either cell type was pretreated with 2 μ M S1P for 24 h (Fig. 1c). These results suggest that S1P activity is directed toward K562 and not NK cells during the 4-h incubation period.

To clarify the roles of S1P receptors important in NK cell lysis of K562, we determined the expression of S1P receptors in these cell types. The results demonstrate that K562 expressed S1P₁, S1P₂, S1P₃ and S1P₅ but not S1P₄ (Fig. 2a). NK cells on the other hand, expressed S1P₁, S1P₃, S1P₄ and S1P₅ but not S1P₂ (Fig. 2b). In order to determine if S1P₁ is involved in S1P inhibition of NK cell lysis of K562 cells, we used FTY720 and SEW2871. The cultures of K562 and NK cells were incubated in the presence of 2 μ M S1P and increasing concentrations of FTY720 or SEW2871. Addition of various concentrations of FTY720 blocked the inhibitory effect of S1P with an

IC_{50} value calculated at 3.3×10^{-11} M (Fig. 2c). SEW2871 also reversed the inhibitory effect of S1P with an estimated IC_{50} of 6.2×10^{-11} M (Fig. 2d).

S1P inhibits NK cell lysis of immature dendritic cells (iDCs)

To demonstrate whether the effect of S1P might be directed toward other NK target cells, we used immature DCs. Because both DCs and NK cells express receptors for S1P [18–20], and in order to demonstrate whether the effect of S1P is exerted on DCs or NK cells, we pretreated either cell type with 2 μ M S1P for 4 h and then added these cells together. The results revealed that pretreatment of DCs with S1P for 4 h rendered these cells resistant to lysis by NK cells ($P < 0.005$, as compared to the lysis of untreated cells; Fig. 3a). In contrast, when NK cells were pretreated with S1P and then added to untreated DCs, no effect on the cytotoxicity was observed. However, when both cell types were pretreated with S1P and then added together, NK cell killing of DCs was significantly inhibited ($P < 0.002$, as compared to the lysis of untreated iDCs by untreated NK cells; Fig. 3a). These results suggest that S1P affects DCs and not NK cells.

To further demonstrate the activity of S1P, we pretreated monocyte-derived iDC with various concentrations of S1P for various periods of time prior to their incubation with

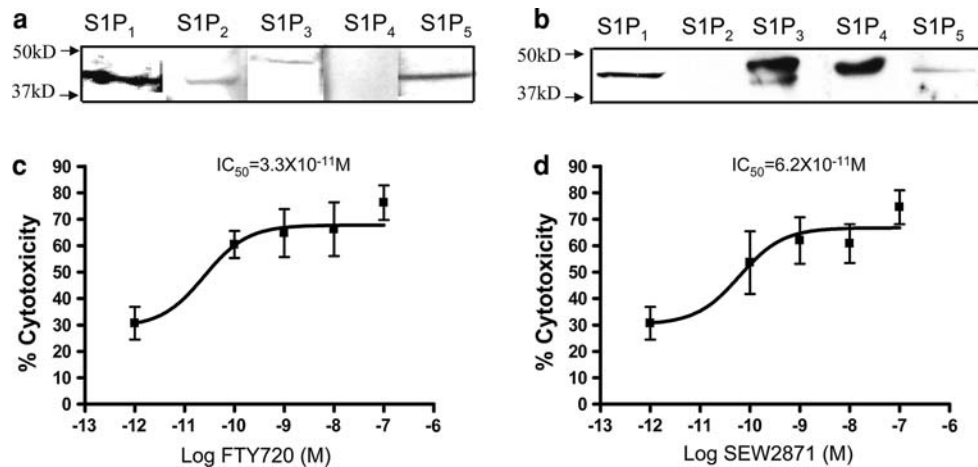


Fig. 2 S1P₁ mediates S1P inhibition of NK cell lysis of K562 cells. Expression of receptors for S1P in K562 cells is shown by immunoblot analysis (a). Expression of receptors for S1P in NK cells is shown using immunoblot analysis (b). K562 cells were incubated with NK cells in the presence of 2 μ M S1P and increasing concentrations of

FTY720 in the 4-h NK cytotoxicity assay (c). The IC₅₀ effect of FTY720 on S1P inhibition is shown. Panel d is similar to panel c except that SEW2871 was used instead of FTY720. Mean \pm SEM of 4 separate experiments

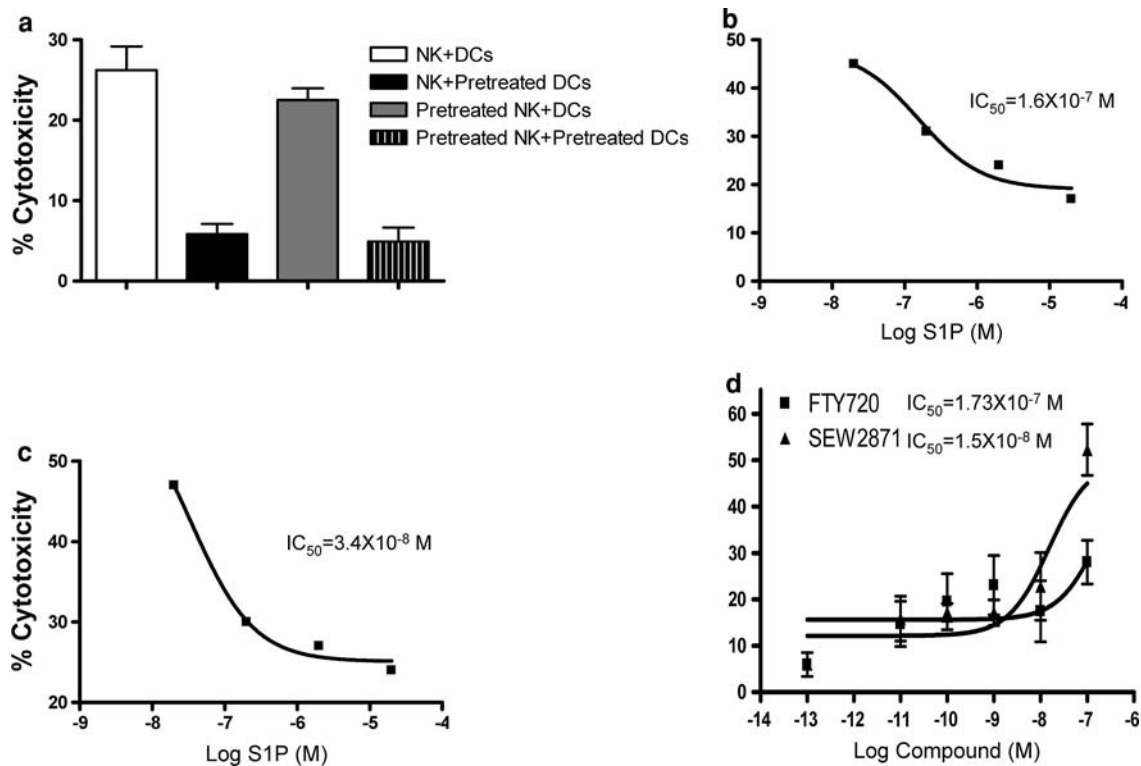


Fig. 3 Effect of S1P on NK cell lysis of DCs. Untreated NK cells were added to untreated autologous iDCs, untreated NK cells were added to autologous iDCs pretreated with 2 μ M S1P for 4 h, NK cells pretreated with 2 μ M S1P for 4 h were added to untreated autologous iDCs, or S1P-pretreated NK cells were added to S1P-pretreated autologous iDCs. These cells were cultured together for an additional 4 h before percent cytotoxicity was measured (a). Results show the mean \pm SEM of 4 experiments. NK cells were incubated with autologous iDCs

pretreated with various concentrations of S1P for 4 h before culturing with NK cells in the 4 h cytotoxicity assay (b). Panel c is similar to panel b except that allogeneic iDCs were used as targets. The IC₅₀ value of the effect of S1P on NK cell lysis is shown. NK cells were incubated with autologous iDCs in the presence of 2 μ M S1P and increasing concentrations of FTY720 or SEW2871. IC₅₀ values of FTY720 and SEW2871 on S1P inhibition of NK cell lysis of autologous iDCs are shown (d)

NK cells. Four hours incubation of autologous or allogeneic iDCs with 0.2–20 μ M of S1P significantly protected these cells from NK cell lysis. The IC₅₀ values of S1P were

calculated at 160 nM for autologous iDCs (Fig. 3b), and 34 nM for allogeneic iDCs (Fig. 3c). Next, we wished to determine the receptor involved in this activity of S1P

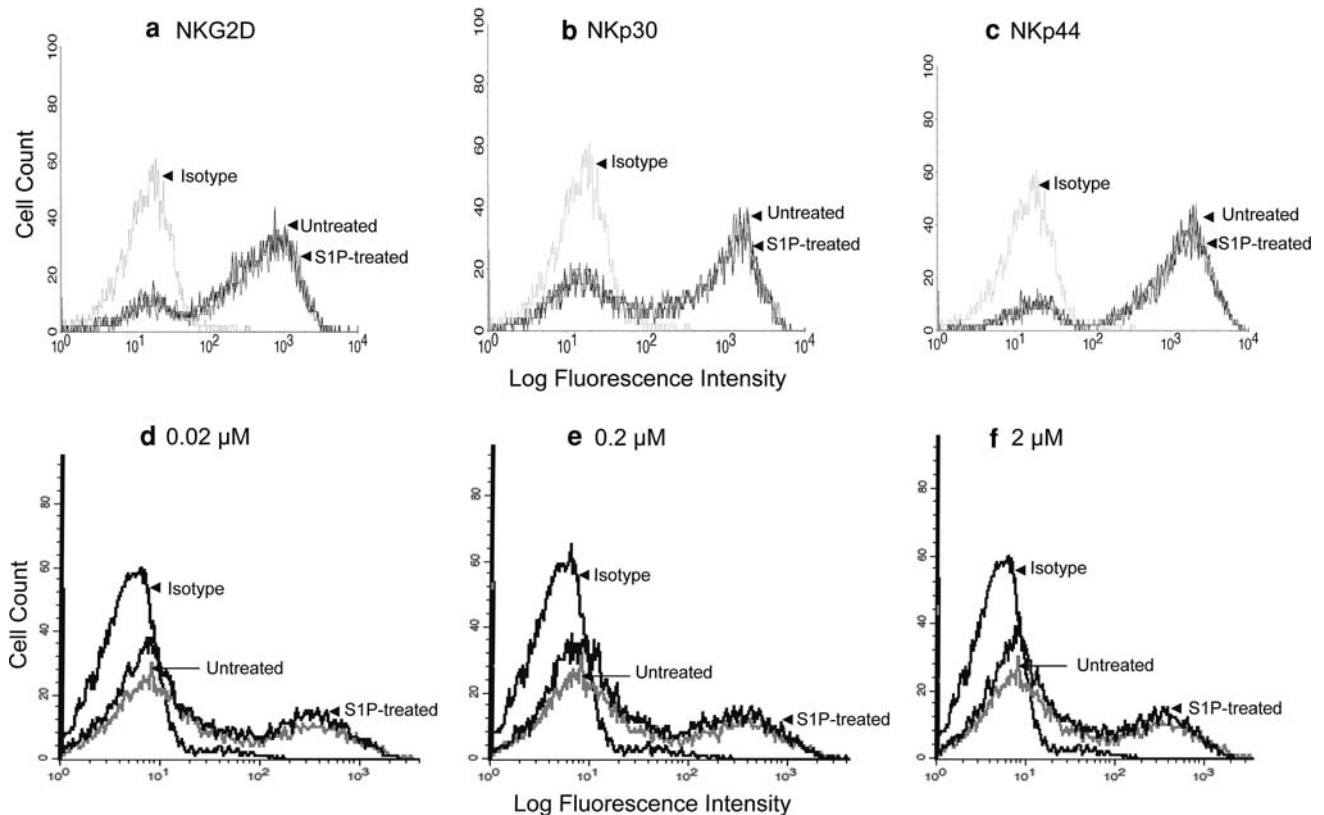


Fig. 4 Expression of NK cytotoxicity or inhibitory receptors on NK cells before and after incubation with S1P. IL-2-activated NK cells were either left untreated or were incubated with 2 μ M S1P for 4 h. These cells were washed and then examined for the expression of activating receptors NKG2D (a), NKp30 (b) or NKp44 (c). Staining with

PE-conjugated isotype control (mouse IgG) is also shown. Panels d–f show expression of KIR/CD158 after incubation with 0.02, 0.2 or 2 μ M S1P for 4 h, respectively. The expressions of CD158 on S1P-untreated NK cells as well as staining with isotype FITC-conjugated IgG are also shown

using the drugs FTY720 and SEW2871. We observed that the inhibitory effect of S1P was reversed by various concentrations of FTY720 or SEW2871, with an IC_{50} effect of 173 or 15 nM, respectively (Fig. 3d).

S1P does not influence the expression of NK activating or inhibitory receptors

The above results suggest that S1P does not influence NK cell cytotoxicity via interaction with $S1P_1$ in these cells. To further prove this point, we examined the surface expression of the C-type lectin NKG2D, or the natural cytotoxicity receptors NKp30 and NKp44 expressed on NK cells after incubation with S1P. In three separate experiments, addition of 2 μ M S1P to NK cells did not significantly affect the percentages of cells expressing these receptors, or the mean fluorescence intensity (MFI) of these receptors as measured by flow cytometric analysis (Fig. 4a–c). Hence, there was no difference in the expression of NKG2D, NKp30 or NKp44 molecules after incubating NK cells for 4 h with S1P as compared to cells incubated with media only. A reciprocal experiment was done in which the effect of S1P on NK cells expressing the inhibitory KIR/CD158

receptors was done. The results indicate that 0.02, 0.2 or 2 μ M S1P did not increase the percentages of cells expressing CD158 or affect the MFI of CD158 when compared to cells untreated with the lipid (Fig. 4d–f).

Effect of S1P on the expression of co-stimulatory molecules in DCs

To characterize the molecules present in DCs that could be involved in the inhibitory activity of S1P, we first investigated the expression of co-stimulatory molecules because these are important in regulating NK cell lysis of DCs [17]. Hence, iDCs were incubated with 2 μ M S1P alone or in the presence of 10 nM SEW2871 or 10 nM FTY720, whereas 1 μ g/ml LPS was used as a positive control. The results demonstrate that S1P did not affect the percentages of cells expression of CD80, CD83 or CD86 (Fig. 5a, b, and c) or their mean fluorescence intensity “MFI” (Fig. 5d–f). Similarly, the combination of S1P with SEW2871 or FTY720 did not affect the expression of these molecules on the surface of iDCs (Fig. 5). In addition, 10 nM SEW2871 or FTY720 when incubated alone exerted no effect on the expression of co-stimulatory molecules. In contrast,

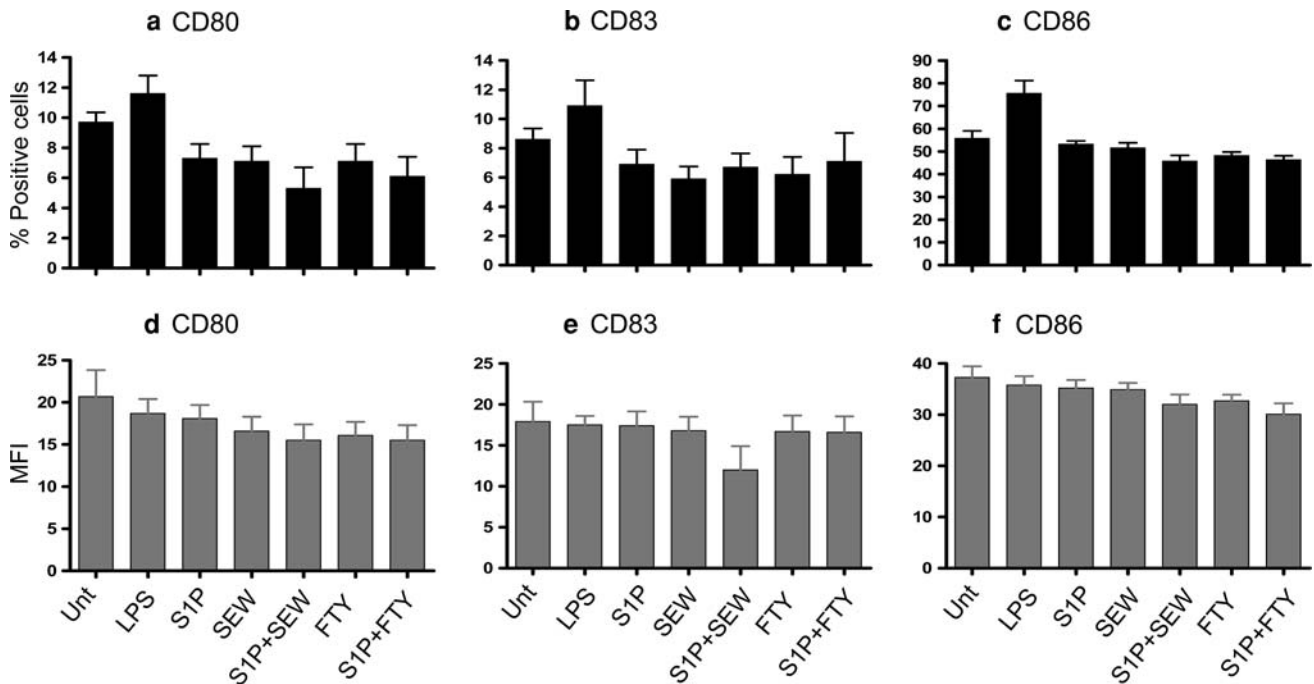


Fig. 5 Expression of co-stimulatory molecules and their mean fluorescence intensity on immature dendritic cells before and after incubation with S1P. Immature DCs were either left untreated or were incubated with 2 μ M S1P, 10 nM FTY720, 10 nM SEW2871 or their combinations for 4 h. These cells were washed and then examined for

the expression CD80, CD83 and CD86. Mean \pm SEM of percentages of positive cells isolated from three different donors (a–c). Mean \pm SEM of mean fluorescence intensity (MFI) of positive cells isolated from three different donors (d–f)

incubating iDCs with LPS for 4 h increased the percentages of cells expressing CD80, CD83, or CD86 molecules (Fig. 5).

S1P increases the expression of MHC class I molecules on the surface of iDCs

MHC molecules play important roles in NK cell recognition of target cells [1]. Therefore, it was imperative to examine the expression of these molecules on the surface of iDCs which are targets for NK cells. After 4 h incubation, S1P as well as LPS and both 10 nM SEW2871 and 10 nM FTY720, or the combinations of S1P plus SEW2871 or FTY720 increased the percentages of iDCs expressing HLA-DR molecule which was significant when the experiments were done with three different donors ($P < 0.05$ for all drugs when compared to untreated cells; Fig. 6a). Only LPS significantly increased the MFI of HLA-DR on iDCs ($P < 0.05$ as compared to untreated cells collected from three donors; Fig. 6d). Intriguingly, S1P significantly increased the percentages of iDCs expressing HLA-I ($P < 0.01$ as compared to untreated cells; Fig. 6b), or the MFI of HLA-I ($P < 0.01$; Fig. 6e). FTY720 reversed the increased expression of HLA-I induced by S1P ($P < 0.04$) for both the percentages of cells and the MFI, upon comparing the effect of S1P to the effect of combining S1P with FTY720 (Fig. 6b, e, respectively). Similarly, SEW2871

inhibited the enhancing effect of S1P on the percentages of iDCs expressing HLA-I ($P < 0.04$ when the effect of S1P was compared to the effect of combining S1P with SEW2871; Fig. 6b), or the MFI of HLA-I ($P < 0.04$; Fig. 6e).

Further, S1P increased the expression of HLA-E on the surface of iDCs ($P < 0.03$ for both the percentages of cells and the MFI expression when compared to untreated cells, Fig. 6c, f, respectively). FTY720 reversed this effect of S1P ($P < 0.05$ for both percentages of cells and MFI of receptor expression, when the expression is compared between cells incubated with S1P and those incubated with S1P plus FTY720; Fig. 6c, f). Similarly, SEW2871 reversed the enhancing activity of S1P by inhibiting the increase in the MFI of HLA-E induced by S1P ($P < 0.05$; Fig. 6f), but has no effect on cell percentages (Fig. 6c). There was some increase in the expression of HLA-E after incubation with FTY720 or SEW2871 alone, but this was not statistically significant.

Effect of S1P on the release of inflammatory cytokines and chemokines from DCs

With the exception of enhancing the release of IL-2 or IFN- γ from human T cells [21, 23], or IL-6 and IL-8 from maturing DCs [19], the effect of S1P on the release of inflammatory cytokines and chemokines by other cell types

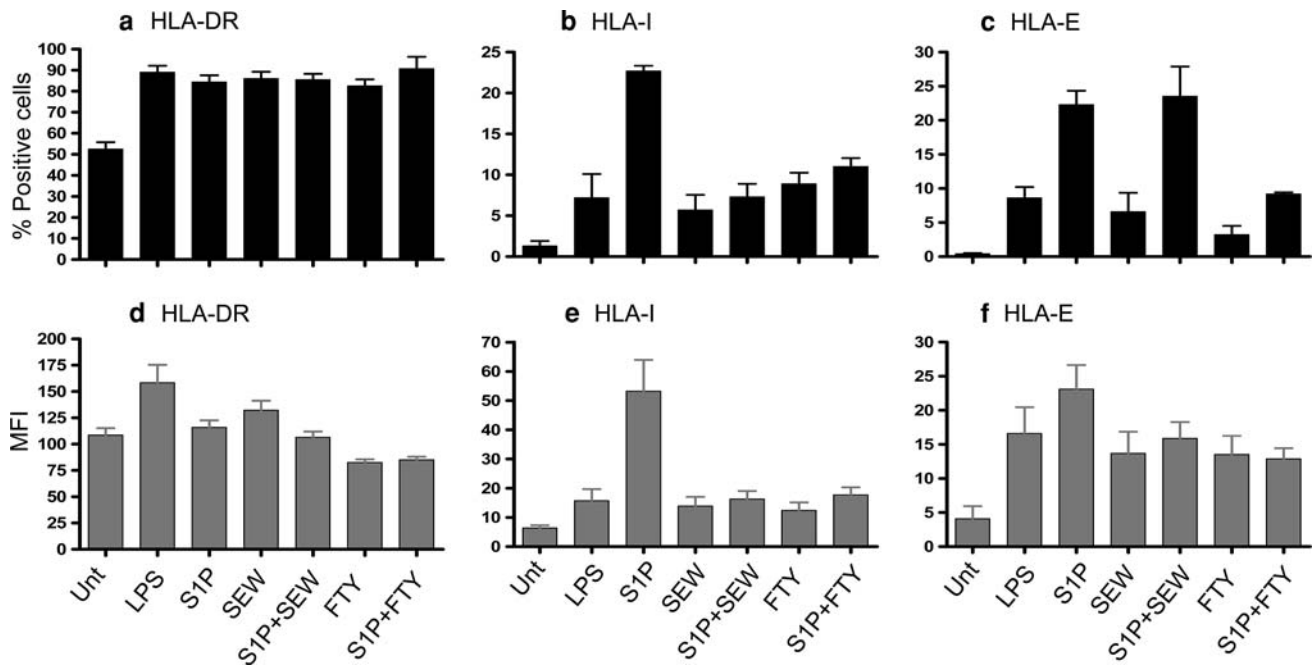


Fig. 6 Expression of MHC class I and II molecules and their mean fluorescence intensity (MFI) on iDCs before and after incubation with S1P. Immature DCs were either left untreated or were incubated with 2 μ M S1P, 10 nM FTY720, 10 nM SEW2871 or their combinations for 4 h. These cells were washed and then examined for the expression

of HLA-DR, HLA-I and HLA-E. Mean \pm SEM of percentages of positive cells isolated from three different donors (a–c). Mean \pm SEM of mean fluorescence intensity (MFI) of positive cells isolated from three different donors (d–f)

is not known. Here, we investigated whether addition of S1P to human immature DCs might affect the release of these cytokines. As a control LPS was used to induce the release of inflammatory cytokines and chemokines from these cells. The results demonstrate that LPS induced DCs to secrete IL-1 β , IL-6, IL-10, TNF- α , MIP-1 α and MIP-1 β ($P < 0.05$, 0.013, 0.008, 0.004, 0.03 and 0.05, respectively) as compared to DCs alone; Fig. 7). However, we could not observe any effect on the release of the cytokines and chemokines examined after incubating iDCs with S1P (Fig. 7). Similarly, addition of FTY720 or SEW2871 alone or in combination with S1P did not affect the release of IL-1 β , IL-4, IL-6, IL-10, IL-12, IL-17, IFN- γ , TNF- α , TGF- β 1, MCP-1, MIP- α or MIP-1 β (Fig. 7).

Effect of S1P on the release of inflammatory cytokines and chemokines from NK cells

To further demonstrate the effects of S1P on the release of inflammatory cytokines and chemokines, we examined whether the addition of this lipid might influence the release of these molecules by NK cells. Results generated from 4–5 different experiments shown in Fig. 8 demonstrate that NK cells secrete various cytokines and chemokines with different intensity. Based on the optical density (OD) values, this secretion can be divided into 4 categories: (1) Low secretion where the OD is below 0.5. Cytokines

and chemokines included in this category are IL-4, IL-6, IL-12, TNF- α , and MCP-1 (ODs 0.3 ± 0.08 , 0.4 ± 0.4 , 0.4 ± 0.1 , 0.4 ± 0.1 and 0.4 ± 0.02 , respectively). (2) Intermediate secretion where the ODs are between 0.5 and 1. Included in this category are IL-1 β , IL-10, TGF- β 1, and IL-17A (ODs 0.5 ± 0.08 , 0.5 ± 0.06 , 0.6 ± 0.1 and 0.8 ± 0.1 , respectively). (3) High secretion where the ODs are between 1 and 2. Cytokines and chemokines included in this category are IFN- γ and MIP-1 α (ODs 1.5 ± 0.1 and 1.5 ± 0.3 , respectively). (4) Very high secretion where the OD is above 2. In the latest category only MIP-1 β is included (OD 3.8 ± 0.1). S1P did not affect the release of IL-1 β , IL-4, IL-6, IL-10, IL-12, TNF- α , TGF- β 1, MIP-1 α , or MIP-1 β . However, it significantly reduced the release of IL-17A, and robustly inhibited IFN- γ secretion after incubating NK cells with S1P ($P < 0.04$ and $P < 0.0005$, respectively) as compared to NK cells not incubated with S1P alone). The inhibitory effect of S1P was not reversed by FTY720 or SEW2871, suggesting that this activity of S1P is mediated by receptors other than S1P $_1$.

Discussion

We provide novel evidence showing that S1P inhibits NK cell lysis of immature monocyte-derived DCs, regardless whether NK cells engage autologous or allogeneic DCs.

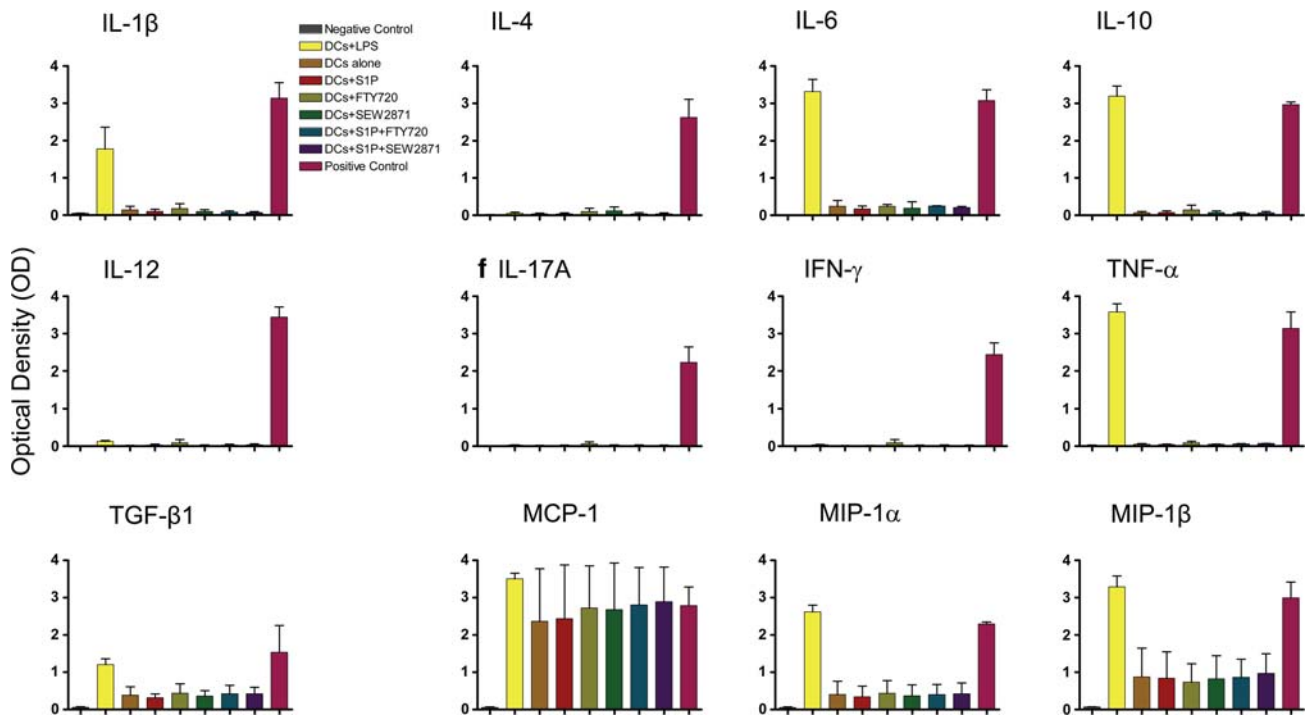


Fig. 7 Effect of S1P, FTY720, SEW2871 and their combinations on the release of inflammatory cytokines and chemokines by DCs. iDCs were incubated with media (DCs alone), 1 μg/ml LPS, 2 μM S1P, 10 nM FTY720, 10 nM SEW2871 or the combinations of S1P plus

FTY720 or SEW2871 for 24 h. Supernatants were collected and examined for the release of various cytokines and chemokines. Negative and positive controls supplied by the manufacturer were also included. Results shown are from two different experiments

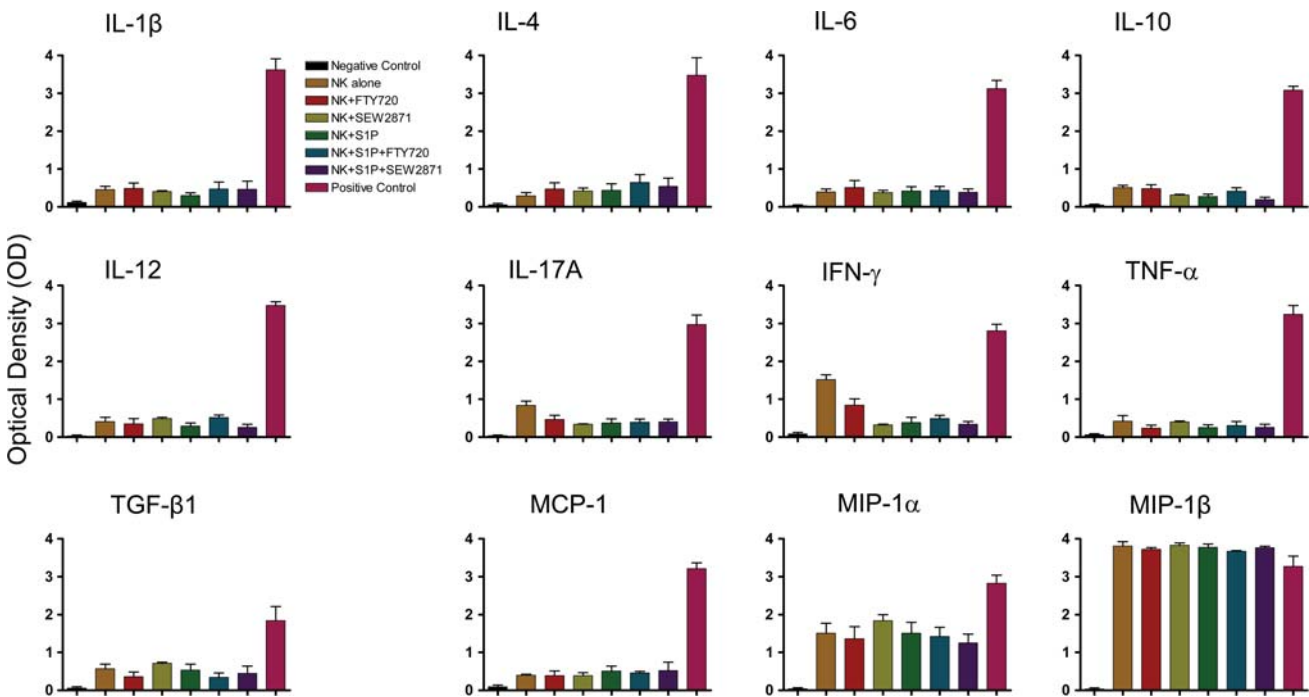


Fig. 8 Effect of S1P, FTY720, SEW2871 and their combinations on the release of inflammatory cytokines and chemokines from IL-2-activated NK cells. These cells were incubated with media (NK alone), 2 μM S1P, 10 nM FTY720, 10 nM SEW2871 or the combinations of

S1P plus FTY720 or SEW2871 for 24 h. Supernatants were collected and examined for the release of various cytokines and chemokines. Negative and positive controls supplied by the manufacturer were also shown. Results shown are mean ± SEM of 4–5 different experiments

The results showing that lipid mediators released by inflammatory cells protect iDCs from NK cell lysis suggest that lysolipids exert an important control mechanism for the immune system during inflammation, supporting recent findings [15, 24]. Based on these findings, it is difficult to envisage how NK cells might kill iDCs in situ where lysolipids accumulate.

Although S1P is a potent stimulus for T cell migration both in vivo and in vitro, this molecule was found to inhibit T cell proliferation [21, 25]. Because S1P is also a chemoattractant for NK cells [18], and DCs [20], it was surprising to discover that it inhibits NK cell lysis of iDCs. This might be a mechanism developed by the system in order to allow iDCs to mature and then capture and present antigens to T cells. Protection of iDCs could represent part of a “bystander” effect in which lysis of these cells may be prevented by locally destroyed unhealthy cells which secrete this lipid. In addition, S1P protects tumor cells from lysis by NK cells ([15], and this report). It remains to be examined how chemotactic factors such as S1P inhibit the cytotoxicity of anti-tumor effector cells. It is plausible that tumor cells secreting this lipid may recruit NK cells and at the same time inhibits their cytolytic function, an effect that is beneficial for the survival of tumor cells. It may also explain how tumor cells can survive and avoid destruction by NK cells in situ. In this regard another lysophospholipid, i.e., lysophosphatidic acid (LPA) also protected tumor cell from lysis by NK cells [24]. The ability of FTY720 and SEW2871 to reverse the inhibitory effect of S1P should be explored to evaluate these molecules as potential anti-tumor drugs.

Because NK cells and dendritic cells express multiple S1P receptors [18–21], it was important to distinguish the involvement of these receptors in regard to the inhibitory effect of S1P. For this purpose we used two agonists/antagonists directed toward S1P receptors, namely, FTY720 and SEW2871. No discriminatory effects of FTY720 and FTY720-phosphorylated form were observed in terms of their activity on DCs and T cell functions [26]. Similar findings were observed when this drug was used to induce pulmonary endothelial cell barrier enhancement [27]. In addition, FTY720 is converted to FTY-phosphorylated form by sphingosine kinase secreted by most cell types [28]. Therefore, we used the unphosphorylated form of FTY720 in our assay. Both FTY720 and SEW2871 reversed the inhibitory effects of S1P on NK cell lysis of DCs with about tenfold higher activity for SEW2871 than FTY720, suggesting that S1P₁ mediates important regulatory function in the interaction among NK cells and DCs, and that SEW2871 inhibits this activity by antagonizing S1P₁. Finally, the effect of S1P seems to be exerted on S1P₁ expressed on DCs but not on NK cells.

Next, we sought to determine the cellular mechanisms important for S1P-induced inhibition of NK cell lysis of DCs. Hence, we took in consideration that multiple mechanisms might be involved in this activity of S1P. Therefore, we examined the effect of S1P on the followings: (1) the expression of NK cell receptors on the surface of NK cells; (2) the expression of co-stimulatory molecules on the surface of DCs; (3) the expression of MHC class I and class II molecules on the surface of DCs; (4) the release of inflammatory cytokines and chemokines by DCs; and (5) the release of inflammatory cytokines and chemokines by NK cells.

Incubating NK cells with S1P did not modulate the percentages of NK cells expressing NKG2D, NKp30 or NKp44. This observation suggests that S1P inhibition of NK cell lysis of DCs is not due to down-regulating the expression of NK cytotoxicity receptors. Reciprocally, S1P did not affect the expression of inhibitory receptors on NK cells. There are multiple killer inhibitory receptors (KIR) expressed on NK cells, collectively known as CD158 [29]. These receptors ensure that under normal conditions NK cells are inhibited upon ligating self-MHC molecules, which guards against autoimmunity. Hence, Ig inhibitory receptors bind class MHC class I gene products [1]. Anti-CD158 used in this study recognizes four of the killer inhibitory receptors, and in particular KIR2DL2, KIR2DL3, KIR2DS2 and KIR2DS4, but it does not recognize KIR2DL1, KIR2DL4, KIR3DL1 or KIR3DL2. Our results suggest that at least KIR2DL2, KIR2DL3, KIR2DS2 and KIR2DS4 are not targets for S1P inhibitory activity.

In addition to Ig-like receptors, the C-type lectin-like domain containing inhibitory receptors include the heterodimer of CD94/NKG2A which recognizes gene products of HLA-E [30]. HLA-E has a limited polymorphism since it binds peptides of the leader sequence of most HLA-class I molecules and is then transported to the cell membrane. Therefore, HLA-E is ubiquitously expressed in most tissues. Here, we examined the expression of MHC class I gene products on the surface of iDCs which are targets for NK cell mediated cytotoxicity. Our results clearly show that S1P increased the expression of HLA-class I molecule as well as HLA-E in these cells. These results might explain how S1P protects iDCs from NK cell cytolysis. In accordance with this, both FTY720 and SEW2871 reversed this activity of S1P, i.e., these drugs reduced the increased expression of HLA-I and HLA-E molecules induced by S1P on the surface of iDCs. These results provide an insight into the activity of these antagonists in reversing the protection of iDCs lysis by NK cells. Of note, incubating K562 with S1P did not up-regulate the expression of HLA-I or HLA-E on the surface of these cells, suggesting that the mechanisms of inhibition are different among iDCs and

tumor cells. It remains to be seen whether S1P might down-regulate the expression of ligands recognizing NK cytotoxicity receptors on the surface of K562 cells. Because the nature of these ligands is not clearly established, they were not examined in this study. Alternatively, S1P might up-regulate other molecules on tumor cells that might inhibit NK cell lysis of K562 cells. These issues are currently under investigation. In addition, S1P, FTY720, SEW2871 or the combinations of S1P with these drugs did not affect the expression of co-stimulatory molecules CD80, CD83 or CD86 expressed on the surface of DCs.

Further analysis demonstrates that S1P did not affect the release of the inflammatory cytokines IL-1 β , IL-4, IL-6, IL-10, IL-12, IL-17, IFN- γ , TNF- α , and TGF- β 1, or the inflammatory chemokines MCP-1, MIP-1 α , and MIP-1 β from DCs. Addition of FTY720 or SEW2871 to S1P also did not influence these cytokines and chemokines secreted by DCs. Different results were observed when NK cells were used. We observed that NK cells released inflammatory cytokines and chemokines with different intensity. Hence, these cells secreted low amounts of IL-4, IL-6, IL-12, TNF- α , and MCP-1, intermediate amounts of IL-1 β , IL-10, TGF- β and IL-17A, high amounts of IFN- γ and MIP-1 α , and very high amount of MIP-1 β . A surprising finding is the ability of IL-2-activated NK cells to secrete IL-17 since this is an inflammatory cytokine involved in autoimmune reactions [31]. However, this result supports those showing that IL-17 secreting cells express the NK cell marker CD161 [32, 33]. Whether NK cells that secrete IL17A represent a unique subset is not clear at the present time, since our activated NK cell preparation contains all subsets of NK cells including CD16⁻, CD16⁺ as well as other unique subpopulations of NK cells. Also, the finding showing that activated NK cells secreted MIP-1 α and MIP-1 β strongly suggests that these cells recruit other cell types; hence, they are important players in inflammation. Interestingly, S1P inhibited the release of the inflammatory cytokines IFN- γ and IL-17A by NK cells indicating that S1P performs important anti-inflammatory functions. Neither FTY720 nor SEW2871 reversed this effect of S1P suggesting that S1P inhibits the release of inflammatory cytokines from NK cells via S1P₁-independent pathway.

In summary, the results shown here indicate that S1P protects K562 tumor cells and monocyte-derived iDCs from lysis by NK cells. Furthermore, S1P₁ antagonists reversed the inhibitory effects of S1P for DCs and tumor cells. In both cases, it is the S1P₁ expressed on the target cells and not NK cells that is involved in this activity. This could be due to the fact that although immunoblot analysis showed that S1P₁ is expressed in IL-2-activated NK cell, only about 10% of these cells express this receptor, whereas more than 50% of K562 cells or iDCs express S1P₁ (data not shown). Hence, FTY720 and SEW2871 act

as reverse agonists for S1P inhibitory effects, indicating that S1P₁ is important for NK cell cytolysis of target cells. In contrast, S1P₁ is not involved in S1P effect on the secretion of inflammatory cytokines by NK cells. These results differentiate among NK cell cytotoxicity and cytokine release and clearly indicate that S1P₁ is only involved in the cognate interaction among NK cells and tumor cells or iDCs. It is worth mentioning that S1P₅ was found to be important in mediating NK cell chemotaxis [34]. Therefore, there is selectivity in the differential functionality of S1P receptors expressed in NK cells. Our results also suggest that lysophospholipids such as S1P present in many food products and are secreted physiologically by many blood cell types may protect tumor cells from lysis by NK cells, and that caution should be considered when these lipids are considered as therapeutic modalities or used as food additives. It is anticipated that FTY720 and SEW2871 might also be utilized in certain cases to allow anti-tumor effector cells to recognize and destroy cancerous cells. Hence, these molecules should be evaluated as prophylactic as well as therapeutic drugs for cancer.

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