Sphingosine-1-phosphate inhibits the cytotoxic activity of NK cells via G_s protein-mediated signalling

MARIANA LAGADARI[1], KATJA LEHMANN[1], MIRJANA ZIEMER[1], KRISZTINA TRUTA-FELES[1], LUCIANA BEROD[1,3], MARCO IDZKO[4], DAGMAR BARZ[2], THOMAS KAMRADT[3], AZZAM A. MAGHAZACHI[5] and JOHANNES NORGAUER[1]

[1]Department of Dermatology, [2]Institute of Transfusion Medicine, [3]Institute of Immunology, Friedrich-Schiller University, Jena; [4]Department of Pneumology, Albert-Ludwigs University, Freiburg, Germany; [5]Department of Physiology, University of Oslo, Norway

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Abstract. Sphingosine-1-phosphate (S1P) is a bioactive phospholipid that transmits signals through G-protein-coupled receptors to control cellular differentiation, survival, and several functions of immune cells. S1P is a chemoattractant for NK cells, which are critical members of the immunological tumor surveillance machinery. In this study we analyzed the influence of S1P on the interaction of NK cells with tumor cells such as the human melanoma cell line Hs294T and the Burkitt's lymphoma cell line Raji. We found that S1P inhibited the cytotoxic activity of NK cells. Analysis of signal transduction pathways revealed that S1P induced common signalling pathways of chemotaxins such as G_i protein-dependent actin reorganization and activation of the phosphatidylinositol 3-kinase (PI3K) dependent signal molecules, protein kinase B (PKB/Akt) and glycogen synthase kinase-3ß (GSK-3ß). In contrast to most chemotaxins, S1P is also able to activate G_s-dependent signalling molecules. This signalling cascade involves increase of cAMP levels and protein kinase A (PKA) activation. Additionally, blocking the regulatory subunits of PKA I abrogated the inhibitory effect of S1P, whereas the catalytic subunits were not involved. Our data indicate that S1P may contributes to the tumor escape from NK cell-dependent immunological surveillance machinery.

Introduction

The bioactive sphingosine-1-phosphate (S1P) has been implicated as a mediator of different cellular functions. S1P exerts most of its actions as a specific ligand for G protein-coupled receptors which regulate cytoskeletal rearrangements, cellular movement, angiogenesis, vascular maturation and immune functions (1). In recent years several investigations pointed out that many functional aspects of the melanoma cells are regulated by microenvironment interactions. The relevance of autocrine secreted S1P in the growth response and metastatic process of melanoma cells has been demonstrated (1,2). Moreover, S1P-neutralizing monoclonal antibodies remarkably retard progression of deadly and multiresistant cancers such as malignant melanoma as well as other types of cancers in murine xenograft and allograft models (3).

Natural killer (NK) cells are able to attack abnormal cells such as virally infected cells or transformed tumor cells. The cytotoxic activity of NK cells is actively inhibited by receptors that recognize major histocompatibility complex (MHC) class I molecules. However, NK-activating receptors such as NKG2D also participate in the control of cytotoxic activity (4,5). Moreover, enhanced cytotoxic activity after stimulation with chemotaxins for NK cells such as macrophage-inflammatory protein-1ß (MIP-1ß/CCL1), thymus and activation-regulated chemokine (TARC/CCL17), regulated upon activation normal T-cell expressed and secreted (RANTES/CCL5) and monocyte chemoattractant protein 1 (MCP-1/CCL-2) is well known (6,7). Therefore, these findings implicate a regulative role in the functional balance between cytotoxicity inhibitory and activating receptors by chemotaxins. The above mentioned chemotaxins bind to specific pertussis toxin-sensitive G_i protein-coupled receptors linked to activation of phospholipase C and phosphatidylinositol 3,4,5 triphosphate (PIP3)-generating type IB phosphatidylinositol 3-kinase.

Besides the classical chemokines, small phospholipids such as S1P have chemotactic activity for NK cells (8). Meanwhile, five different high-affinity S1P-specific plasma membrane G protein-coupled receptors have been identified (S1P1-S1P5). The expression of three different S1P receptors namely S1P-1, -4, and -5 has been described in NK cells (8). Recently, NK cell trafficking in mice has been attributed to the G_i protein-coupled S1P5 (9). Moreover, S1P-mediated chemotaxis of human NK cells was inhibited by pertussis toxin, implicating involvement of S1P receptors and G_i proteins

Correspondence to: Dr Johannes Norgauer, Department of Dermatology, Friedrich-Schiller University Jena, Erfurterstrasse 35, D-07740 Jena, Germany
E-mail: johannes.norgauer@med.uni-jena.de

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in humans. However, in other cell types, pertussis toxin-insensitive responses have been described after stimulation with S1P (10-12). Currently the knowledge on S1P-induced responses in NK cells apart G_i protein-mediated processes is very limited.

In order to understand better the role of S1P in cross-talk between NK cells and tumor cells, we further characterized the influence of S1P in this context. Surprisingly, we found that S1P enhanced intracellular cAMP, activated protein kinase A and inhibited the cytotoxicity of NK cells.

Materials and methods

Culture medium and reagents. rHu IL-2 (Proleukine) was from Chiron (Chiron GmbH, Ratingen, Germany); mouse anti-CD3 and mouse anti-CD4 antibodies were purchased from Immunotools (Friesoythe, Germany); S1P (sphingosine 1-phosphate), myristoylated PKI_{14-22}, cholera toxin and pertussis toxin were purchased from Sigma-Aldrich (Taufkirchen, Germany); anti-human phospho-Akt/protein kinase B (Ser473) (pAkt), Akt and phospho-glycogen synthase kinase-3ß (Ser9) (pGSK-3ß) were purchased from Cell Signalling Technology (New England Biolabs GmbH, Frankfurt am Main, Germany); anti-actin was purchased from Santa Cruz Biotechnology (Heidelberg, Germany); anti-GSK-3ß was from BD Transduction Laboratories (Heidelberg, Germany); Rp-8-Br-cAMPS was from Axxora Platform (Lörrach, Germany); NK cell culture medium consisted of RPMI-1640 supplemented with 10% human AB serum, 10 U/ml penicillin, 10 U/ml streptomycin and 1 mM L-glutamine (Promocell, Heidelberg, Germany).

Cell lines. The A2058, SKMel23 and HS294T human melanoma cell lines and the human Burkitt's lymphoma cell line Raji, were maintained at 37˚C in a 5% CO_2 incubator in RPMI-1640 supplemented with 10% fetal bovine serum, 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine (Promocell).

Preparation of IL-2-activated NK cells (NK). The use of human cells was approved by the Research Ethics Board of the University of Jena. For each single experiment IL-2-activated NK cells were isolated from blood of volunteers. IL-2-activated NK cells were prepared by adherence to plastic flasks of nylon wool column-nonadherent cells in the presence of 1000 IU/ml rHu IL-2. After 24 h non-adherent cells were removed. Cells were grown for an additional 7-10 days as described by Inngjerdingen *et al* (13). Thereafter, the cell suspension was depleted of T cells by binding to mouse anti-human CD3 and anti-human CD4 using anti-mouse M-450 Dynabeads (Dynal, Oslo, Norway). This procedure resulted in a purity >95% of NK cell subsets expressing CD56.

Analysis of filamentous F-actin content. The intracellular f-actin content was analyzed by flow cytometry with NBD-phallacidin staining. Briefly, aliquots of stimulated cell suspension (10^6 NK/ml) were withdrawn at the indicated time intervals from a 37˚C incubator. Equal volumes of cells (50 µl) were fixed in a 7.4% formaldehyde buffer and mixed with the staining mixture containing 7.4% formaldehyde, 0.33 µM NBD-phallacidin (MoBiTec, Göttingen, Germany) and 1 mg/ml lysophosphatidylcholine (Sigma-Aldrich). The fluorescence intensity was measured by flow cytometry.

In vitro cytotoxicity assay. Cytotoxicity was determined with a standard ^{51}Cr release assay. Target cells were labelled at 37˚C for 1 h with 100 µCi $Na_2\ ^{51}CrO_4$ (Amersham, Freiburg, Germany). Cells were washed and resuspended at cell density of $1x10^6$ cells/ml with RPMI-1640 2% lipid-free albumin medium. Effector and target cells in different ratios (10:1, 5:1 and 2.5:1) were placed into individual wells of 96-well plates in a total volume of 200 µl at 37˚C for 4 h. After incubation, 100 µl from the supernatants were mixed with MicroScint-40 cocktail (Perkin-Elmer, Jügesheim, Germany) and analyzed with a gamma counter (Topcount™, Packard Instruments). The maximum or spontaneous release was defined as counts from samples incubated with 2% Triton-X or medium alone, respectively. The percentage of specific cytotoxicity was calculated as the measure of NK cell function against each target. Percentage specific lysis was calculated using the following formula:

$$\frac{\text{(experimental release - spontaneous release)}}{\text{(maximum release - spontaneous release}} \times 100$$

Measurements of cAMP levels. Cells ($1x10^6$/ml) were stimulated with S1P and after the indicated time-points cells were lysed by addition of 100 µl lysis buffer. The amount of intracellular cAMP in the NK cell preparation was determined using a cAMP assay kit (R&D, Wiesbaden-Nordenstadt, Germany).

Protein kinase A (PKA) activity assay. The StressXpress nonradiactive PKA activity kit (Biomol, Hamburg, Germany) was used to test the PKA activity in NK cell lysates. A specific synthetic peptide is used as a substrate for PKA and a polyclonal antibody recognizes the phosphorylated form of the substrate. Briefly, NK cells ($5x10^6$ per sample) were treated with S1P for the indicated time-points. Thereafter, cells were lysed in lysis buffer, centrifuged at 10,000 rpm for 15 min, and supernatants were frozen at - 80˚C. A microtiter plate was soaked with 50 µl of kinase assay dilution buffer. After a washing step 30 µl (corresponding to 10 µg of whole cell lysate) of each sample was added. The reaction was initiated by addition of 10 µl ATP. The plate was incubated for 90 min at 30˚C and phosphospecific substrate antibody was added. The level of antibody binding was estimated using secondary anti-rabbit IgG-horseradish peroxidase conjugate and corresponding substrate. Absorbance was determined using a microplate reader set at a wavelength of 450 nm. Kinase activity in cell lysates was calculated as follows:

$$\frac{\text{mean absorbance (sample) - mean absorbance (negative control)}}{\text{protein in cell lysate}}$$

Immunoblot analysis. Immunoblotting was performed by running the samples on SDS-PAGE gels (20 µg protein/lane)

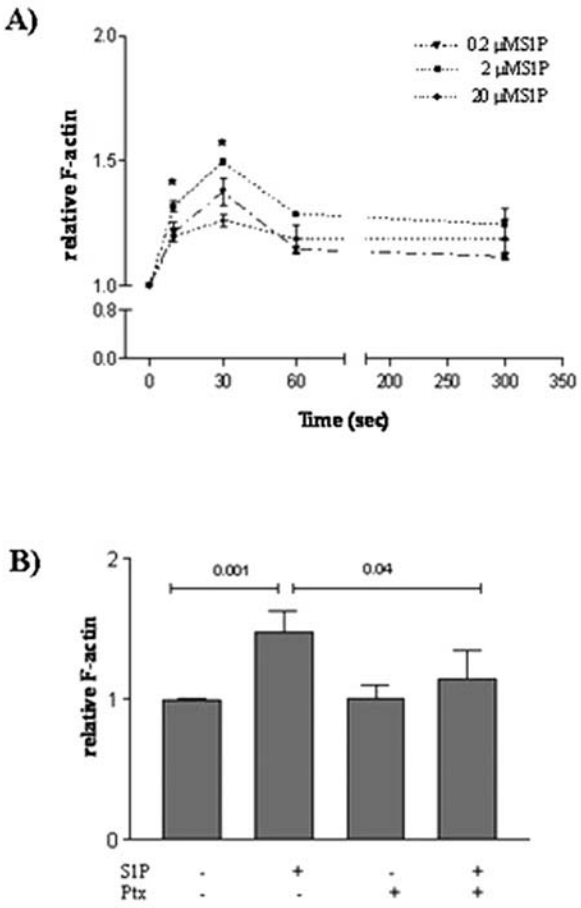

Figure 1. Effect of S1P on the actin polymerization in NK cells. (A) Cells were stimulated with S1P 2, 0.2 or 0.02 μM. The relative intracellular F-actin content was determined at the indicated time-points by flow cytometry. At 10 and 30 sec S1P stimulation had significantly increased fluorescence compared to the control (p=0.014, 0.0048 and 0.03 for 10 sec and p=0.01, 0.0001 and 0.02 for 30 sec). Data are means of three experiments from different donors ± SEM. (B) Cells were incubated without or with 100 ng/ml pertussis toxin (Ptx) for 1 h at 37˚C. Thereafter cells were stimulated without or with 2 μM S1P and actin polymerization was analyzed. After 30-sec stimulation, S1P significantly increased the actin polymerization (p=0.001 for 2 μM of S1P). Pretreatment with pertussis toxin completely inhibited the S1P-induced actin response (p=0.04). Data are means of three experiments from different donors ± SEM.

and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h at room temperature, and then incubated with the first antibody (1:2000) overnight at 4˚C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10000 dilution) for 1 h at room temperature. Proteins were detected by ECL (Amersham).

Statistical analysis. Significant values were determined by using the two-tailed Student's t-test.

Results

The effect of S1P on the actin network of NK cells was analyzed by flow cytometry. A rapid and concentration-dependent polymerization of actin molecules is shown in Fig. 1. S1P caused a rapid polymerization of the actin molecules within 30 sec with a 50-60% increase in the F-actin content (Fig. 1A). The response was concentration-dependent and maximal effect was observed after stimulation with 2 μM S1P. To investigate the involvement of G_i proteins in this response, NK cells were incubated with G_i protein-inactivating pertussis toxin (Ptx). As shown in Fig. 1B pretreatment of NK cells with pertussis toxin completely inhibited the S1P-induced actin response. As a control to exclude toxicity by pretreatment with the toxin, cells were stimulated with ionomycin and Ca^{2+}-transients were monitored in Fura-2-loaded cells. Pertussis toxin did not influence Ca^{2+}-transients triggered by this agent (data not shown).

Intracellular signalling pathways induced by chemotaxins such as CCL5/RANTES stimulate phosphatidylinositol 3-kinase in leukocytes (14,15). To study intracellular signal transduction events downstream of phosphatidylinositol 3-kinase, we analyzed the activation and/or phosphorylation of protein kinase B/Akt and glycogen synthase kinase-3ß by Western blotting. NK cells were treated with 2 μM S1P for the indicated time-points (Fig. 2A-C). Probes were analyzed with antibodies against phospho-Akt/protein kinase B (Ser473) (pAkt) and phospho-glycogen synthase kinase-3ß (Ser9) (pGSK-3ß). The membranes were stripped and reprobed with anti-actin, anti-Akt (data not shown) and anti-GSK-3ß (data not shown) to assure comparable amounts of the analyzed proteins in all samples. Basal levels of pAkt, pGSK-3ß activation were observed in S1P-unstimulated NK cells. Exposure of NK cells to S1P induced a further transient enhancement of pAkt (Fig. 2B) and pGSK-3ß (Fig. 2C). Quantification of the luminescence indicated that S1P enhanced pAkt and pGSK-3ß within 60 sec by ~50 or 75%, respectively. In addition, short incubation of NK cells with the phosphatidyl-inositol 3-kinase inhibitor wortmannin reduced the levels of pAkt (Fig. 2B) and pGSK-3ß (Fig. 2C) in S1P-unstimulated NK-cells and inhibited S1P-induced pAkt and pGSK-3ß responses. Moreover, pretreatment of NK cells with pertussis toxin did not influence the basal levels of pAkt and pGSK-3ß in unstimulated NK cells, but inhibited the S1P-induced enhancement (Fig. 2D and E).

Chemotaxins play a significant role in the cytolytic responses of NK cells. An enhanced cytotoxic response has been described after NK cells exposure to chemotaxins such as CCL5/RANTES (16). We analyzed the effects of CCL5/RANTES and S1P on the cytotoxic activity of NK cells against the human Burkitt's lymphoma cell line Raji. In well accordance with the literature, CCL5/RANTES increased the cytotoxic activity of NK cells in a concentration dependent manner by up to 40% (Fig. 3A). In contrast to CCL5/RANTES, S1P inhibited the cytotoxic activity of NK cells against Raji by 40-50% (Fig. 3B). To exclude the possibility that the interaction of S1P with the Raji target cell might cause a cell type-specific inhibitory effect on cytotoxicity a second set of experiments with the human melanoma cell line Hs294T was performed. Indeed, S1P significantly inhibited in a concentration-dependent manner the cytotoxic activity of NK cells (Fig. 3C). In addition, similar inhibitory effects on NK cell cytotoxicity have been also observed at various effector:target (E:T) ratios (10:1, 5:1 and 2.5:1) and with the A2058 and SK-Mel 23 human melanoma cell lines (data not shown). These findings indicate that chemotaxins differentially influence the cytotoxic activity of NK cells.

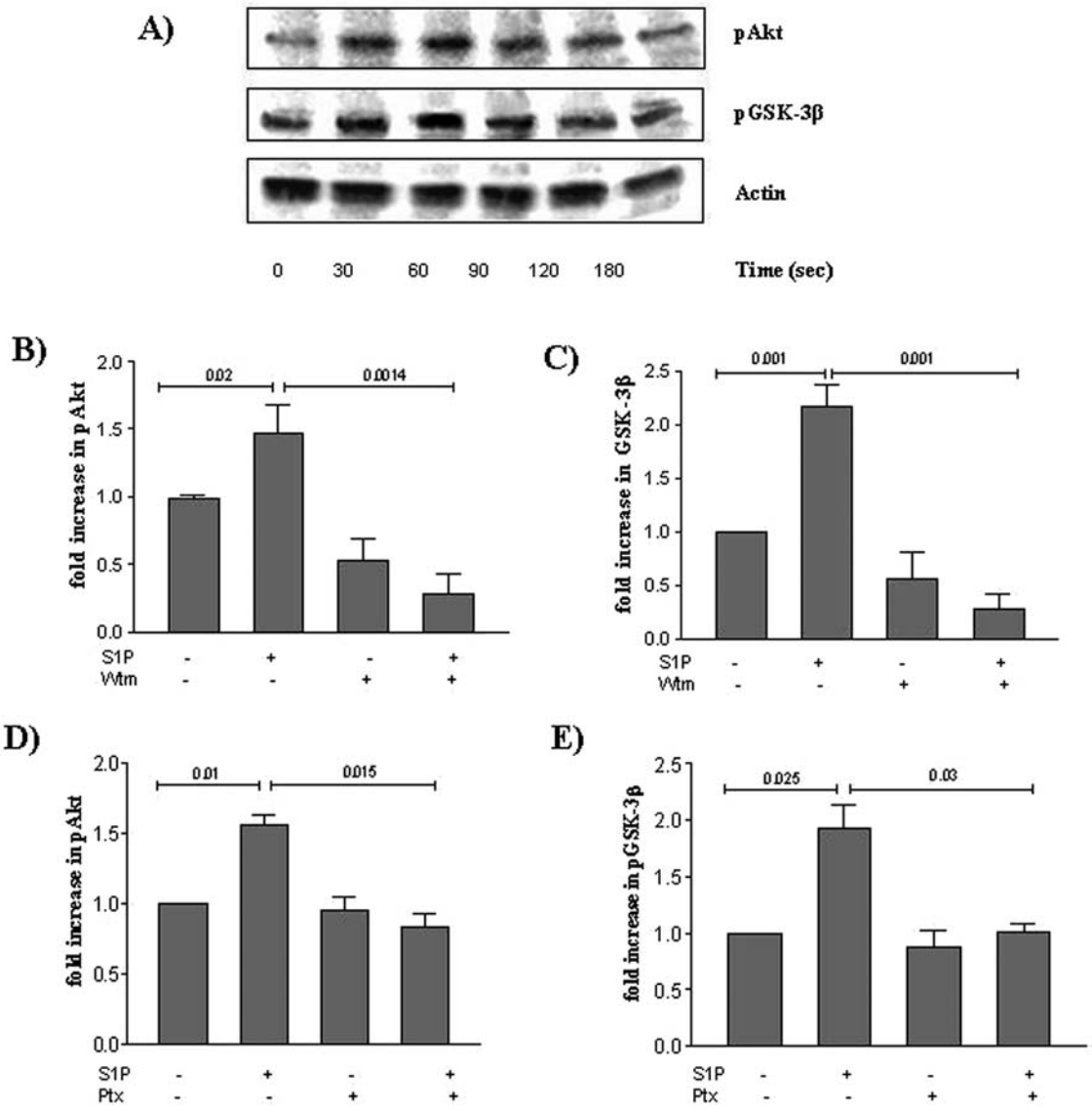

Figure 2. S1P signalling pathway in NK cells. (A) NK cells were stimulated at 37˚C with 2 μM S1P for 30, 60, 90, 120 and 180 sec. After lysis the samples were analyzed for pAkt, pGSK-3ß as well as actin by Western blotting. Representative blot from three independent experiments with identical results is shown. The quantification of the luminescence indicated that S1P significantly enhanced pAkt and pGSK-3ß within 60 sec (p=0.01 for pAkt and p=0.02 for pGSK-3ß). (B and C) Wortmannin (Wtm) pretreatment significantly reduced the levels of pAkt and pGSK-3ß in S1P-unstimulated NK cells and inhibited S1P-induced pAkt (p=0.0014) and pGSK-3ß (p=0.0012) responses. (D and E) NK cells were incubated with or without 100 ng/ml pertussis toxin (Ptx) for 1 h at 37˚C. Thereafter cells were stimulated with or without 2 μM S1P. The effect of Ptx on S1P-induced responses to pAkt and pGSK-3ß at 30 sec was analyzed. Pretreatment with Ptx significantly inhibited the S1P-induced enhancement of pAkt and pGSK-3ß (p=0.002 and 0.02 respectively). Data are means ± SEM of three experiments with NK cells isolated from different donor/s.

Chemotaxin receptors in leukocytes usually mediate their action by interaction with G_i protein-coupled receptors causing Ca^{2+}-transients, actin reorganization and phosphatidylinositol-3 kinase activation (17,18). In contrast to the G_i protein-coupling CCL5/RANTES receptors, signal coupling of the different types of S1P receptors is pleiotrophic including coupling to G_s proteins, which activate adenylylcyclase. Thus, we analyzed the influence of S1P on cAMP-levels in NK cells. After 4-min stimulation of NK cells with S1P a significant and concentration-dependent increase of cAMP was observed (Fig. 4A). Moreover, pretreatment of NK cells with 0.5 μg/ml G_s protein-activating cholera toxin caused an enhancement of intracellular cAMP levels in S1P-unstimulated NK cells and exposure of cholera toxin-pretreated NK cells to S1P further increased the cAMP levels. In contrast, pretreatment of NK cells with pertussis toxin neither influenced the cAMP-levels nor inhibited S1P-mediated increase in the cAMP response (Fig. 4B). We also examined NK cell cytotoxicity after pretreatment with cholera toxin and pertussis toxin. Pertussis toxin did not influence the cytotoxic activity in S1P-unstimulated or S1P-treated NK cells. In contrast, cholera toxin pretreatment inhibited the cytotoxic activity of S1P-unstimulated NK cells. The inhibitory effect of cholera toxin was further enhanced by S1P (Fig. 4C).

Signalling by cAMP leads to the immediate activation of protein kinase A (PKA). This results in the release of two catalytic subunits and two regulatory subunits. The catalytic subunits are able to phosphorylate serine and threonine

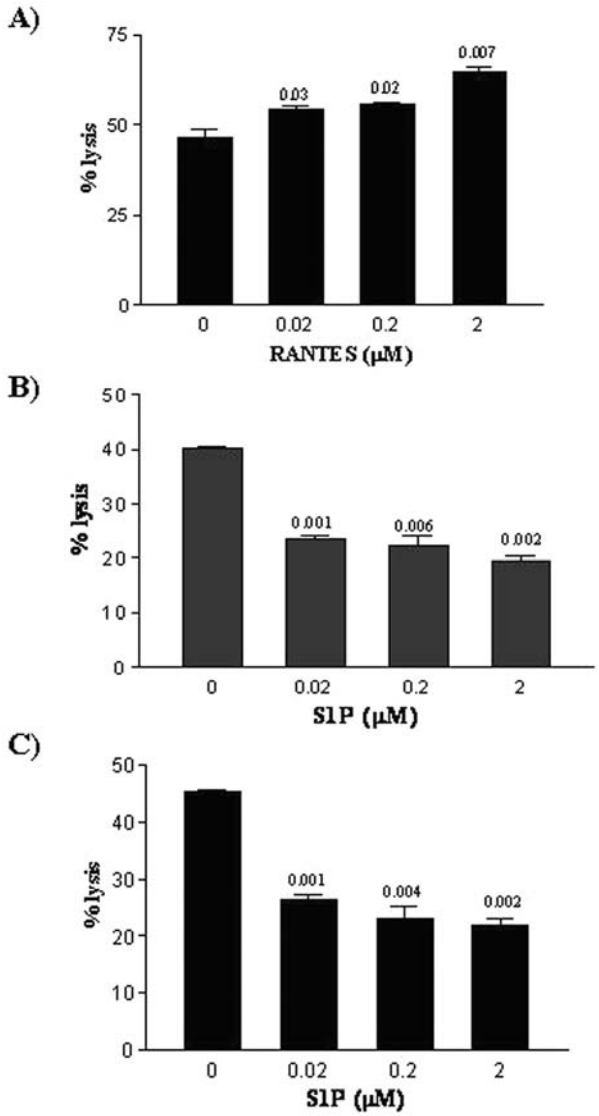

Figure 3. Chemotaxins differentially influence the cytotoxic activity of NK cells. NK cells were mixed with ^{51}Cr-labelled Raji at E:T ratio 2.5:1 in the presence of the indicated concentrations of (A) RANTES and (B) S1P. After 4-h incubation, the percentage of ^{51}Cr release was determined. 2, 0.2 and 0.02 μM RANTES significantly (p=0.007, 0.02 and 0.03) increased the NK cell cytotoxicity whereas 2, 0.2 and 0.02 μM S1P inhibited the NK cell lysis of Raji (p=0.002, 0.006 and 0.001) (C) The effect of S1P was also tested with the human melanoma cell line Hs294T as a target. S1P inhibited NK cytotoxicity against Hs294T cells (p=0.005, 0.002 and 0.006). Data are from one representative experiment. The experiment was repeated three times with NK cells isolated from different donors.

residues on specific substrates. Hence, the influence of S1P on PKA activity in NK cells was analyzed. Fig. 5A shows that S1P treatment increased PKA activity in NK cells after 4-min stimulation. In order to identify the subunit of PKA modulating cytotoxic activity, experiments were performed with two different PKA inhibitors. The myristoylated peptide PKI_{14-22} (PKI peptide) is a specific blocker of the catalytic activity of PKA and Rp-8-Br-cAMPS binds to the regulatory subunit I preventing the PKA I holoenzyme dissociation (19). Pretreatment with PKI peptide neither affected the cytotoxic activity of unstimulated NK cells nor influenced the S1P-induced inhibition suggesting that the inhibitory effect of S1P is not mediated via the catalytic subunits of PKA (Fig. 5B). In contrast, pretreatment of NK cells with Rp-8-Br-cAMPS completely reversed the inhibitory effect of S1P (Fig. 5C). These findings would indicate that the inhibitory effect of S1P on cytotoxic activity of NK cell is mediated by the regulatory subunits of PKA.

Discussion

Sphingosine-1-phosphate is a mediator of cellular functions playing an important role for calcium homeostasis, cell growth and suppression of apoptosis (1). S1P levels in cells and tissues are low and tightly regulated. S1P is produced by phosphorylation of sphingosine by two sphingosine kinase isoenzymes. Sphingosine kinase 1 is elevated in a variety of solid tumors, and inhibitors of sphingosine kinase 1 reduce gastric and mammary adenocarcinoma tumor growth in mice (20). Neutralizing S1P with monoclonal antibodies effectively retarded progression of deadly and multiresistant cancers such as malignant melanoma, in murine xenograft and allograft models (3). Moreover S1P is able to stimulate chemotaxis in NK cells (8). Sphingosine-1-phosphate receptors-1, -4, and -5 are expressed in freshly isolated NK cells as well as in IL-2 activated NK cells. No up- or down-regulation of the S1P receptors is found upon stimulation of NK cell with IL-2 (3). Recently, it was shown that S1P-5 regulates the trafficking and the tissue distribution of NK cells *in vivo* in steady-state and inflammatory situations (9). Natural killer (NK) cells are thought to be specialized leukocytes able to attack abnormal cells, thus contributing to the control of tumor growth. However, histological investigations and clinical studies have shown that primary melanoma and metastasis can actually co-exist with effector cells of the immune system (e.g., dendritic cells, cytotoxic T cells and NK) (21). In order to better understand the interaction between leukocytes and tumor cells, biological functions and signal transduction mechanisms of S1P in NK cells were further studied.

Here we show that S1P induced a transient reorganization of the motility-associated actin network and activated several PI-3-kinase-dependent signal molecules such as Akt and GSK-3ß. In addition, we demonstrate that these signalling pathways induced by S1P were inhibited by pertussis toxin, which inactivates heterotrimeric G_i-proteins by ADP-ribosylation (22). This set of data is in accordance with signalling pathways induced by chemotaxins such chemokines, complement fragment C5a and N-formylpeptides in leukocytes. These receptors predominately activate G_i protein-coupled receptors. Previous reports have shown an enhanced cytotoxicity of NK cells after stimulation with chemotaxins such as RANTES/CCL5, MIP-1ß/CCL1, TARC/CCL17 and MCP-1/CCL-2 (6,7). Surprisingly, we found that CCL5/RANTES and S1P influenced the cytotoxicity of NK cells differently. S1P inhibited the cytotoxic activity of NK cells against the human Burkitt's lymphoma cell line Raji and melanoma cell lines. Similar inhibitory effects on NK cell cytotoxicity have been observed with freshly isolated NK cells (data not shown). Chemokine receptors predominantly couple to G_i proteins and mediate their biological activity via activation of phospholipase C and phosphatidylinositol 3-kinase-γ.

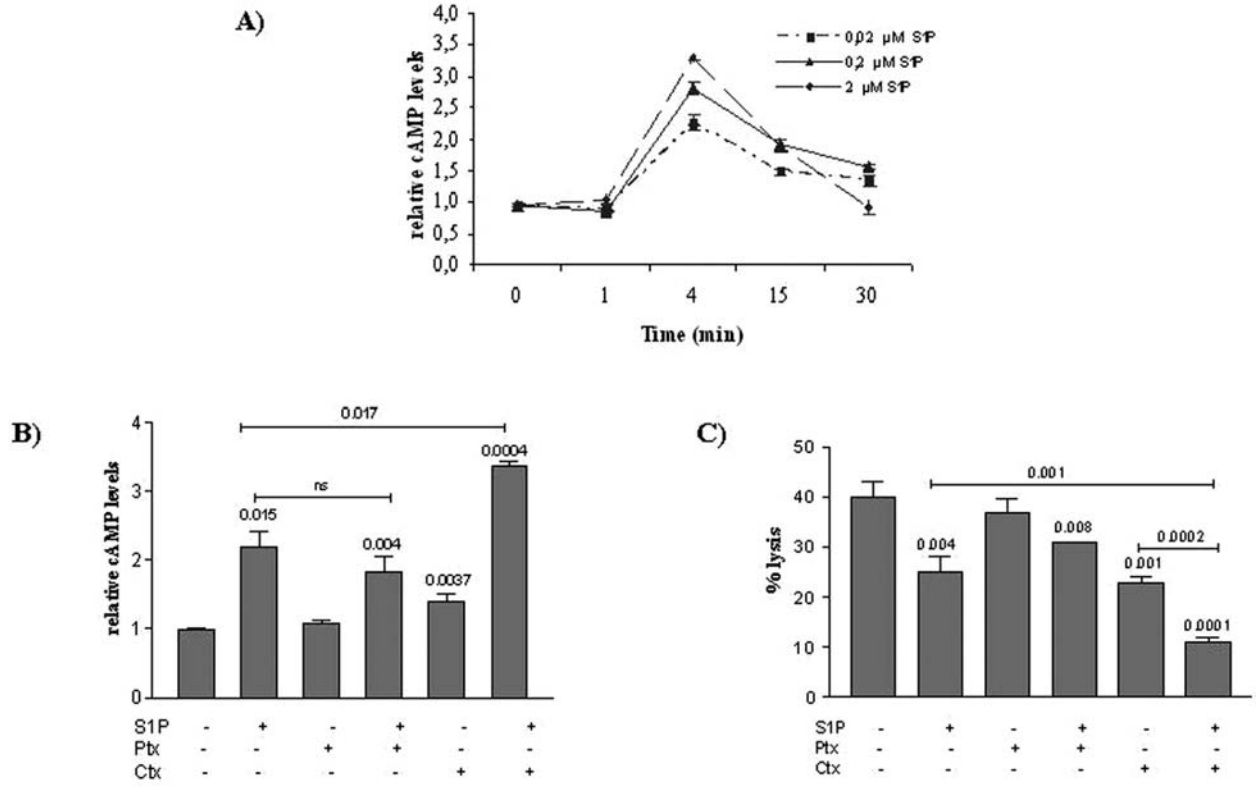

Figure 4. S1P increases cAMP levels in NK cells. (A) The intracellular cAMP levels were measured with cAMP direct immunoassay kit. NK cells were stimulated for 1, 4, 15 and 30 min with the indicated concentrations of S1P. S1P stimulation significantly increased cAMP levels in the NK cell. S1P treatment showed maximal enhancement at 4 min [p=0.012 (2 μM S1P), 0.01 (0.2 μM S1P), 0.02 and (0.02 μM S1P)]. Data are means ± SEM of three experiments with NK cells isolated from different donors. (B) Cells were preincubated with pertussis toxin (100 ng/ml) or cholera toxin (0.5 μg/ml) for 60 min at 37˚C and then cAMP levels were measured after 4-min stimulation without and with 2 μM S1P. Pretreatment with Ptx showed no significant differences on S1P-unstimulated cell or with S1P-mediated increase in cAMP levels. In contrast, cholera toxin (Ctx) significantly (p=0.0037) enhanced the intracellular cAMP levels in unstimulated NK cells and further increased cAMP in S1P stimulated NK cells (p=0.0004). Ctx petreatment of NK cells exposed to S1P further enhanced intracellular cAMP levels compared to S1P-stimulated NK cells with a p=0.017 for 2 μM S1P. Data are means ± SEM of three experiments with NK cells isolated from different donors (C) In addition, the cytotoxic activity was also analyzed after pretreatment of NK cells with Ctx (0.5 μg/ml) or Ptx (100 ng/ml) for 60 min at 37˚C. Pretreatment with pertussis toxin did not influence the cytotoxic activity in either unstimulated or S1P-treated NK cells. In contrast, cholera toxin pretreatment inhibited the cytotoxic activity of unstimulated NK cells (p=0.001). The inhibitory effect of the cholera toxin on treated cells was further enhanced by S1P stimulation (p=0.0001 compared to untreated cells and p=0.001 compared to S1P-stimulated NK cells). Data are from one representative experiment. The experiments were repeated three times with NK cells isolated from different donors.

Heterologous expression studies showed that receptors for S1P couple to the pertussis toxin-sensitive G_i as well as to pertussis toxin-insensitive G proteins (8). Moreover, studies with different cell types indicated that S1P is able to induce G_i protein-independent activation pathways (23,24).

Indeed, we showed here that S1P is able to enhance cAMP-levels in NK cells and it stimulates activation of cAMP-dependent protein kinase A. These data indicate that S1P behaves differently than classical chemotaxins such as CC-chemokines. S1P is able to stimulate G_i as well as G_s protein-dependent signalling pathways in NK cells. Previously it has been reported that the adenylyl cyclase-activating cholera toxin inhibits the cytotoxic activity of NK cells (24). Moreover reduced cytotoxic activity has been reported after exposure of NK cells to G_s protein-stimulating ligands like adenosine, prostaglandin E_2 and prostaglandin D_2 (25-27). Therefore, our data are consistent with the concept that cAMP regulates crucial steps in cytotoxic response machinery of NK cells. Additionally, the idea of modulation of cytotoxicity in NK cells by G_s protein-dependent signal pathways was further corroborated here using protein kinase A inhibitors. Currently two different isoenzymes of the protein kinase A are known and both are expressed in NK cells (28,29). Although the catalytic subunits of both PKA types are similar, the regulatory subunits may have distinctive functions. We found that blocking the catalytic subunit with PKI peptide does not abrogate the inhibitory effect of S1P, but blocking the release of the regulatory subunits RI by Rp-8-Br-cAMP prevented S1P-mediated modulation of the cytotoxicity. Our data therefore extend and confirm previous reports using the cAMP-inducing agent adenosine in murine and rat NK cells (28,30). Therefore one could assume that the regulatory subunits I have signalling functions in NK cells. In this context it is worth mentioning that mice with a selective knockout of genes encoding regulatory subunits show distinctive phenotypic changes (29). Regulatory subunit $I\alpha^{-/-}$ mice present early embryonic lethality, whereas regulatory subunit RIIα knockout mice show no detectable abnormality in spite of a reduction in PKA activity. In addition, regulatory subunit $I\beta^{-/-}$ mice results in hippocampal alterations reduced inflammatory

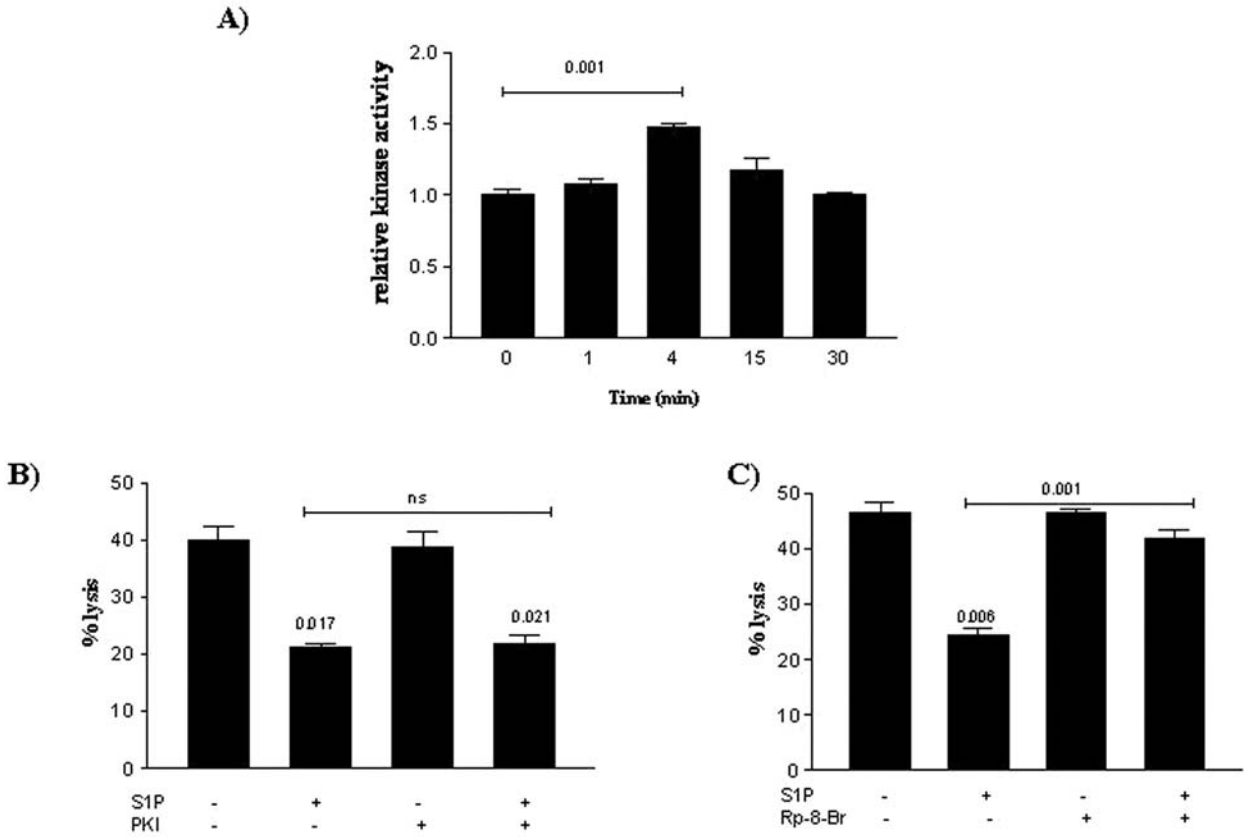

Figure 5. S1P-induced activation of PKA in NK cells. (A) NK cells were treated with S1P (2 μM) for 1, 4, 15 and 30 min. PKA activity in cell lysates was tested using StressXpress nonradiactive PKA activity kit. S1P significantly (p=0.001) increased PKA activity. Data are means ± SEM of three experiments with NK cells isolated from different donors. (B) Effect of PKI peptide (1 μM); and (C) Rp-8-Br-cAMP (1 mM) on the lytic activity of NK cells. NK cells were preincubated for 30 min with PKA inhibitors before target cells and S1P were added. Pretreatment with PKI peptide neither affected the cytotoxic activity of unstimulated NK cells nor influenced the S1P-induced inhibition. In contrast, pretreating NK cells with Rp-8-Br-cAMPS abrogated the inhibitory effect of the S1P (p=0.001). Data are means ± SEM of three experiments with cells from different donors.

responses and nociceptive pain without changes in a total PKA activity (29,30).

In conclusion, we have shown here that the S1P inhibits the cytotoxicity of NK cells enhancing cAMP levels and activating protein kinase A. In recent years several functions of S1P in respect to the growth response and metastatic process of melanoma cells have been demonstrated. Based on these data it can also be assumed that S1P may contribute to the escape of tumor cells from NK cells-dependent immunological surveillance machinery.

Acknowledgments

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