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Sphingosine kinase inhibitor suppresses IL-18-induced interferon-gamma production through inhibition of p38 MAPK activation in human NK cells

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ABSTRACT

Natural killer (NK) cells play an important role in the innate immune response. Interleukin-18 (IL-18) is a well-known interferon-gamma (IFN-γ inducing factor, which stimulates immune response in NK and T cells. Sphingosine kinase (SPHK) catalyzes the formation of sphingosine 1-phosphate (S1P), which acts as a second messenger to function as an anti-apoptotic factor and proliferation stimulator of immune cells. In this study, to elucidate whether SPHK is involved in IL-18-induced IFN-γ production, we measured IL-18-induced IFN-γ production after pre-treatment with SPHK inhibitor (SKI) in NK-92MI cells. We found that IL-18-induced IFN-γ expression was blocked by SKI pre-treatment in both mRNA and protein levels. In addition, the increased IFN-γ production by stimulation with IL-18 is mediated through both SPHK and p38 MAPK. To determine the upstream signals of SKI and p38 MAPK in IL-18-induced IFN-γ production, phosphorylation levels of p38 MAPK was measured after SKI pre-treatment. As a result, inhibition of SPHK by SKI blocked phosphorylation of p38 MAPK, showing that SPHK activation by IL-18 is an upstream signal of p38 MAPK activation. Inhibition of SPHK by SKI also inhibited IL-18-induced IFN-γ production in human primary NK cells. In conclusion, SPHK activation is an essential factor for IL-18-induced IFN-γ production via p38 MAPK.

Natural killer (NK) cells are $CD3^{-}CD14^{-}CD56^{+}$ cells containing a lot of large granules and play an important role in innate immune response [1]. They induce spontaneous killing against transformed or virus-infected cells through Fas/Fas ligand system, antibody dependent cell-mediated cytotoxicity, and granule exocytosis [2]. In particular, they are important sources of various cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) in early response against viral and bacterial infection. These cytokines enhance cell-mediated immune response including T and NK cell-mediated immunity by altering expression of co-stimulatory molecules and natural cytotoxicity receptors (NCRs) [3].

Interleukin-18 (IL-18) is known as an IL-1 family member and is expressed in a 24 kDa inactive protein [4]. It becomes biologically functional after proteolytic cleavage by IL-1β converting enzyme to the 18 kDa form and stimulates proliferation and cytolytic activity of T and NK cells with IL-2 or IL-12 [5]. IL-18 is also known as a stimulator of IFN-γ production through the mitogen-activated protein kinase (MAPK) signaling pathway containing activation of stress kinase p38 (p38 MAPK) and extracellular regulated kinase (ERK) [4].

Sphingosine kinase (SPHK) has been demonstrated as a key enzyme that phosphorylates sphingosine to sphingosin-1-phosphate (S1P) in sphingolipid metabolism. It is activated by extracellular stimulators such as platelet-derived growth factor (PDGF), IgE, and TNF-α via ERK1/2-mediated phosphorylation at Ser225 of SPHK and increases intracellular S1P concentration and decreases its substrate, sphingosine [6]. S1P, the last product of sphingolipid metabolism, is a well-known lipid second messenger that is implicated in various biological processes, including survival, proliferation, and migration [7]. It also has a function as an extracellular ligand of its specific G-protein coupled receptors, designated $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ [8]. In immune cells, increased S1P by activation of SPHK triggers enhancement of intracellular Ca^{2+} concentration, which induces activation of immune cells [9]. Additionally, activation of SPHK and intracellular/extracellular S1P inhibits apoptosis in immune cells. SPHK activation inhibits Fas- and ceramide-induced

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apoptosis in T cells, and intracellular/extracellular S1P prevents apoptosis through activation of ERK1/2 MAPK pathway and inhibits ceramide-induced JNK activation [10]. S1P also acts as a chemoattractant through interaction with S1P specific receptors on immune cells such as lymphocytes and dendritic cells to regulate migration and trafficking of immune cells [11]. In $S1P_1$ deficient mice, circulating T cells and B cells are absent and most thymocytes exist in the thymus. In addition, FTY720, which is structurally similar to sphingosine and when phosphorylated acts as an agonist of S1P receptors consisting $S1P_1$–$S1P_5$, also suppresses T and B cell egress from lymphoid organs [12]. Of particular importance, SPHK and S1P play an important role in cytokine generation in immune cells. The interaction with S1P and S1P receptor enhances IL-4 production in $CD4^+$ T cells, and sphingosine kinase inhibitor blocks production of IL-12 in dendritic cells [13]. However, the effect of SPHK on cytokine production in NK cells has not been elucidated.

In most pharmacological studies, *N,N*-dimethylsphingosine (DMS), D,L-*threo*-dihydrosphingosine, and *N,N,N*-trimethylsphingosine are usually used as sphingosine kinase inhibitors to confirm the effect of SPHK on cellular function and for therapeutic usage on various disease. However, these inhibitors are not specific for SPHK. They block not only SPHK but also several protein kinases such as protein kinase C (PKC) [14]. Recently, specific inhibitors of SPHK were discovered (Compound I–V) by chemical library screening. It has been reported that compound II is most effective for SPHK inhibition among five discovered inhibitors [15]. In this study, to determine the effect of SPHK on IFN-γ production in NK cells, we used compound II as a sphingosine kinase inhibitor (SKI). Our data confirmed that SKI and SB203580, inhibitor of p38 MAPK, suppress IL-18-induced IFN-γ production in human NK cells. In addition, SKI blocked IL-18-induced p38 MAPK phosphorylation in human NK cells. These findings suggest that SPHK may be an important regulator of IL-18-induced IFN-γ production through phosphorylation of p38 MAPK.

Materials and methods

Cell lines and culture. The NK-92MI cells were purchased from the American Type Culture Collection. NK-92MI cells were cultured in alpha modified minimum essential medium Eagle (Gibco-BRL) supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, and 20 % heat- inactivated fetal bovine serum (Gibco-BRL). Cells were maintained without recombinant IL-2 because they were obtained by transfection of NK-92 cells with human IL-2 cDNA by retroviral vector [16]. These cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 and maintained.

Apoptosis measurement (Annexin-V/7-AAD staining). The NK-92MI cells were incubated with or without human recombinant IL-18 for 24 h after pre-treatment with SKI (Calbiochem) for 2 h. After incubation, cells were washed with PBS twice and then resuspended in Annexin-V binding buffer. The cells were incubated with 5 μL of FITC-conjugated Annexin-V and 3 μL of 7-AAD (BD Pharmingen) for 15 min at room temperature and measured by flow cytometry. Cell viability and number were measured using trypan blue.

RT-PCR and real-time PCR for analysis of human IFN-γ mRNA. NK-92MI cells were pre-treated with 10 μg/mL of SKI for 2 h and then incubated with 100 ng/mL of human recombinant IL-18 for 6 h after washing with PBS. Total RNA was isolated from NK-92MI cells using Trizol (Invitrogen), and cDNA was made using a cDNA synthesis kit (Promega). The cDNAs were used as template for PCR amplification with human IFN-γ primers. For real-time PCR analysis, the SYBR Green RT-PCR kit (Takara) was used and the thermal cyclings were performed on a Corbett Research RotorGene PCR machine.

Isolation of human primary NK cells. Human primary NK cells were isolated from different healthy donors using RosetteSep™ NK cell enrichment cocktail (Stem Cell Technology). The blood was incubated with antibody cocktail and then diluted with an equal volume of PBS containing 2% of FBS. Diluted blood was laid on the top of Ficoll–Hypaque (Sigma). After centrifugation for 20 min, enriched cells were removed from the Ficoll–Hypaque and washed three times with PBS containing 2% FBS.

Enzyme linked immunosorbent assay (ELISA) for detection of IFN-γ. Human primary NK cells and NK-92MI cells were pre-treated with or without 10 μg/ml of SKI for 2 h, and then stimulated with 1 ng/mL of IL-2, 100 ng/mL of IL-18, or a combination of IL-2 and IL-18 for 24 h. After stimulation, supernatant from each cell group was collected for measurement of IFN-γ concentration. The concentration of IFN-γ in culture supernatant was measured using human IFN-γ ELISA kit (R&D system).

Results

Sphingosine kinase inhibitor suppresses IL-18-induced IFN-γ production in human NK cell line, NK-92MI

SPHK phosphorylates sphingosine to S1P, which is known to correlate with cell proliferation and apoptosis in most immune cells [6]. As shown in Table 1, when NK-92MI cells were stimulated with IL-18, SPHK1 (∼2.72-fold) and IFN-γ (∼8.59-fold) increased, but SPHK2 levels did not change. This indicates that IL-18 is able to regulate SPHK1. Therefore, we predicted that SPHK is an important mediator in IL-18-induced IFN-γ production and decided to use SKI to show the effect of SPHK in IL-18-induced IFN-γ production. First, we measured the effect of SKI on cell death to find the optimal concentration of SKI for further experiments that confirm the effect of SKI on IFN-γ production in NK cells. NK-92MI cells were pretreated with various concentration of SKI (0–40 μg/mL) for 2 h and then incubated with or without human recombinant IL-18 for 24 h. We decided to use 10 μg/mL of SKI for further experiments to confirm whether SKI affects IL-18-induced IFN-γ production in NK cells (Fig. 1). NK-92MI cells were stimulated with IL-18 with or without pretreatment of SKI. IL-18 enhanced IFN-γ mRNA expression markedly, and this effect was blocked by SKI (Fig. 2A). This effect was also confirmed by quantitative real-time RT-PCR. As shown Fig. 2B, IFN-γ expression was elevated ∼4.7-fold, and SKI inhibited IL-18-induced IFN-γ expression completely (Fig. 2B). Enhanced IFN-γ secretion was also antagonized by SKI in a dose dependent manner (Fig. 2C). These data demonstrate that SKI inhibits IL-18-induced IFN-γ production in human NK-92MI cells at both mRNA and protein levels, implying that IL-18 stimulates IFN-γ production via sphingosine kinase activation.

Sphingosine kinase inhibitor blocks phosphorylation of p38 MAPK by IL-18 in NK cells

It has been reported that IL-18 stimulates IFN-γ production through phosphorylation of p38 MAPK in NK cells [4]. In addition, p38 MAPK is a downstream signaling molecule of SPHK. In inflammation response, SPHK activation-induced phosphorylation of p38 MAPK [17–19]. To investigate whether p38 MAPK is actually associated with IL-18-induced IFN-γ production via SPHK activation, we performed an inhibitor assay. NK-92MI cells were pre-treated with 10 μg/mL of SKI, 20 μM of SB203580 (p38 MAPK inhibitor), or both SKI and SB203580, and then stimulated

Table 1
Increased SPHK1 expression by IL-18 in NK cells

Ratio	Gene	GenBank No.
8.59	Interferon gamma	NM_000619
2.73	Sphingosine kinase 1	NM_021972
1.16	Sphingosine kinase 2	NM_020126

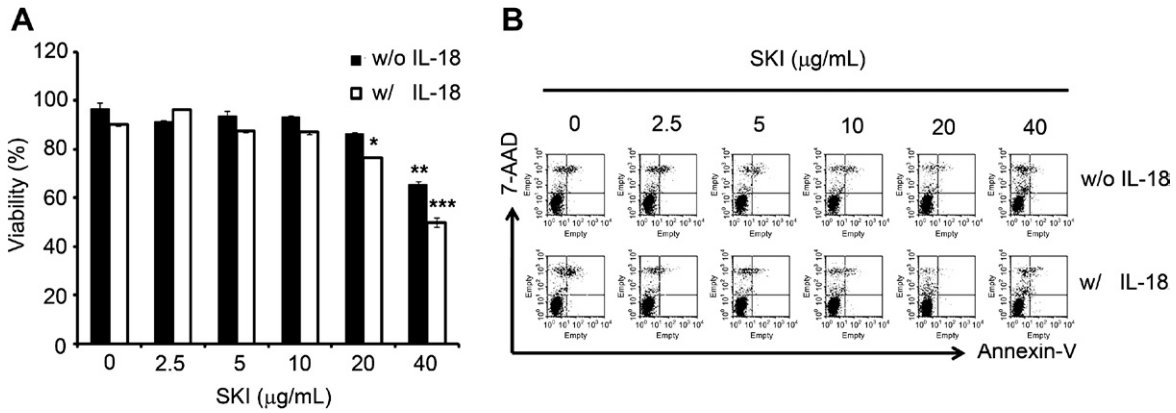

Fig. 1. Ten microgram per milliliter of SKI is optimal concentration to determine the effect of SKI on cytokine production of NK-92MI cells. NK-92MI cells were pre-treated with various concentrations of SKI (2.5, 5, 10, 20 or 40 μg/mL) or DMSO (solvent control; 0 μg/mL of SKI) for 2 h, then cells were incubated with or without 100 ng/mL of human recombinant IL-18 for 24 h. After incubation (A) cell viability was measured using trypan blue (black bar; incubation without IL-18, white bar; incubation with IL-18). These data are representative of three independent experiments; *bars*, mean ± SD $^{*}p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.002$ *versus* control (0 μg/mL of SKI). And (B) apoptosis induction was measured by Annexin-V and 7-AAD staining. These data are representative of three independent experiments.

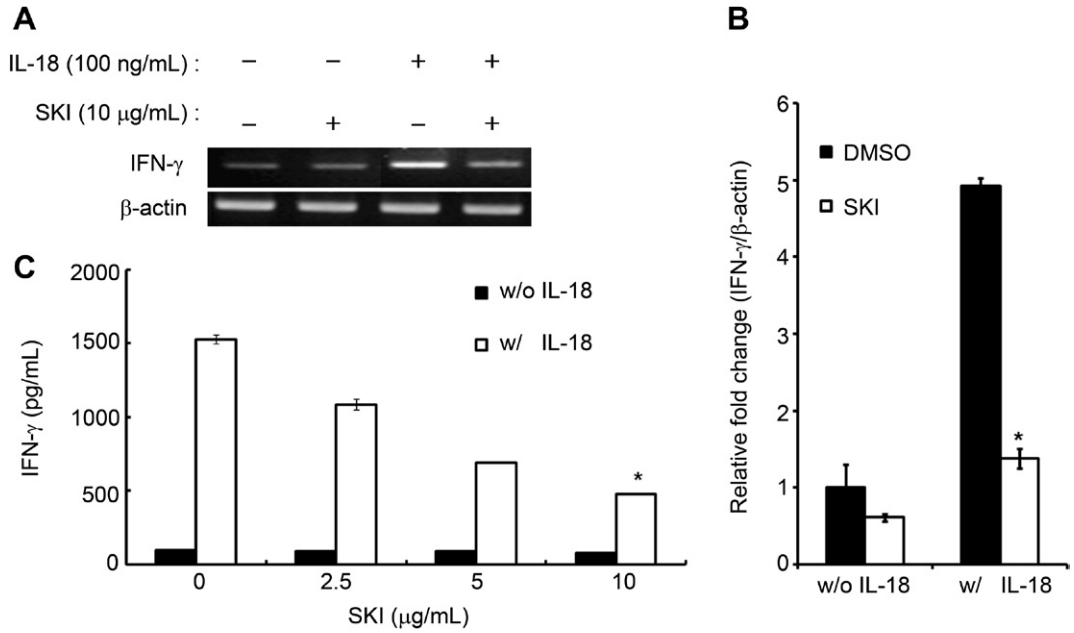

Fig. 2. Sphingosine kinase inhibitor suppresses IL-18-induced IFN-γ production in human NK cell line, NK-92MI. NK-92MI cells were pre-treated with 10 μg/mL of SKI or DMSO (solvent control) for 2 h, then cells were incubated with or without 100 ng/ml of human recombinant IL-18. After 6 h, the cells were harvested, and total RNA from each group was used for cDNA synthesis followed by PCR. (A) RT-PCR. Electrophoresis was performed on 1.5% agarose gel. (B) Real-time RT-PCR (black bar; pre-treatment with DMSO, white bar; pre-treatment with SKI). This data is representative of three independent experiments; *bars*, mean ± SD $^{*}p < 0.02$ vs DMSO control. (C) Dose titration of SKI. NK-92MI cells were pre-treated with various concentrations of SKI (2.5, 5, and 10 μg/mL) or DMSO, and incubated with or without 100 ng/mL of human recombinant IL-18 for 24 h. After incubation time, the cultured supernatants were collected, and the secreted IFN-γ concentration was determined by ELISA (black bar; incubation without IL-18, white bar; incubation with IL-18). This data is representative of three independent experiments; *bars*, mean ± SD $^{*}p < 0.02$ vs DMSO control.

with IL-18. As expected, SKI and SB203580 both blocked IL-18-induced IFN-γ production in NK cells (Fig. 3A). Interestingly, the effect of co-treatment with SKI and SB203580 was similar to the effect seen with SKI or SB203580 alone, indicating that p38 MAPK is involved in IL-18-enhanced IFN-γ production via SPHK activation as an upstream signal or downstream signal of SPHK. To determine whether phosphorylation of p38 MAPK is an upstream or a downstream signal of SPHK activation in IL-18-induced IFN-γ production of NK cells, we examined phosphorylation of p38 MAPK by SKI using Western blot analysis. NK-92MI cells were pre-treated with or without 10 μg/mL of SKI for 2 h and then stimulated with 100 ng/mL of IL-18 for 0, 10, 20, or 30 min. Fig. 3B shows that IL-18-activated phosphorylation of p38 MAPK at 10 min, but phosphorylation of p38 MAPK was suppressed by SKI at all other incubation times. This result means that SKI blocks phosphorylation p38 MAPK, implying that phosphorylation of p38 MAPK functions as a downstream signal for SPHK activation in IL-18-induced IFN-γ production of NK cells.

Sphingosine kinase inhibitor suppresses IFN-γ production in primary NK cells

Previous data has shown that SKI suppresses IL-18-induced IFN-γ production in human NK cell line, NK-92MI. To further confirm this, we isolated human primary NK cells from blood of healthy donors. As expected, IL-18 increased IFN-γ production in primary NK cells, and IL-2 and IL-18 synergistically stimulated IFN-γ production in primary NK cells. Similar to previous data using human NK cell line, these effects were blocked by SKI pre-treatment (Fig. 4).

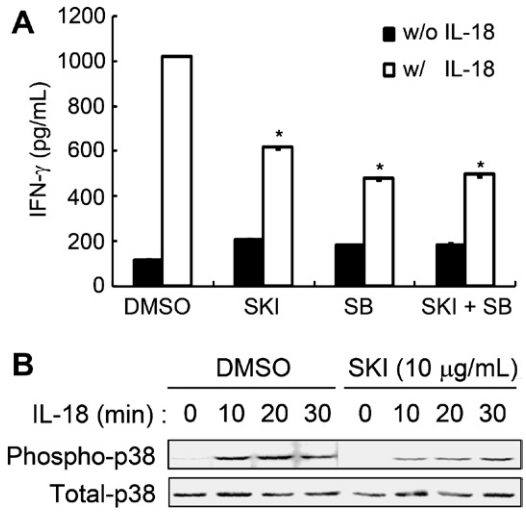

Fig. 3. Sphingosine kinase inhibitor blocks phosphorylation of p38 MAPK by IL-18 in human NK cell. (A) Inhibitor assay. NK-92MI cells were pre-treated with DMSO (solvent control), 10 μg/ml of SKI, 20 μM of SB203580, or SKI plus SB203580, then cells were incubated with or without 100 ng/ml of human recombinant IL-18. After incubation for 24 h, the cultured supernatants were collected, and the secreted IFN-γ concentration was determined by ELISA (black bar; incubation without IL-18, white bar; incubation with IL-18). This data is representative of three independent experiments; *bars*, mean ± SD $^{*}p < 0.02$ vs DMSO control. (B) Immunoblotting of p38 MAPK phosphorylation. NK-92MI cells were cultured in media containing 2% FBS. After 24 h, cells were pre-treated with DMSO or 10 μg/mL of SKI for 2 h, and then stimulated with 100 ng/ml of human recombinant IL-18 for 0, 10, 20, or 30 min and then collected. The harvested cells were lysed, and cytosolic protein was extracted. Proteins from each group were resolved by SDS–PAGE and immunoblotted with anti-phospho-p38 MAPK or anti-p38 MAPK. This data is representative of three independent experiments.

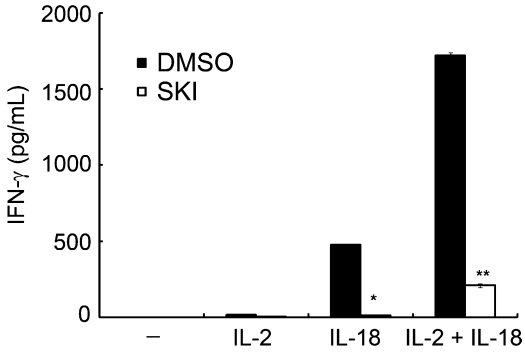

Fig. 4. Sphingosine kinase inhibitor suppresses IFN-γ production in primary human NK cells. Human NK cell were isolated from healthy donors using RosetteSep™. Isolated NK cells were pre-incubated with DMSO or 10 μg/ml of SKI for 2 h, and then cells were stimulated with or without 10 ng/ml of IL-2, 100 ng/ml of IL-18, or IL-2 plus IL-18 for 24 h. After incubation time, the cultured supernatants were collected, and the secreted IFN-γ concentration was determined by ELISA (black bar; pre-treatment with DMSO, white bar; pre-treatment with SKI). This data is representative of three independent experiments; *bars*, mean ± SD $^{*}p < 0.02$, $^{**}p < 0.002$ vs DMSO control.

These data indicate that IL-18 stimulates IFN-γ production through SPHK activation in human primary NK cells.

Discussion

SPHK has an important role in survival, proliferation, and trafficking of various immune cells such as dendritic cells and T cells [10]. In addition, it also regulates cytokine production including IL-4 and IL-12 in immune cells [13]. In this study, we focused on the effect of SPHK in cytokine production in NK cells, and our data demonstrated that SKI blocks IL-18-induced IFN-γ production. This indicates that SPHK may be necessary for IL-18-induced IFN-γ production. In fact, SKI blocks production of IL-12 in dendritic cells and production of IFN-γ in T cells [20] suggesting that IFN-γ production requires SPHK activation in both T cells and NK cells, and SPHK activation may be a common pathway in IFN-γ production by various immune cells. Our data also suggests that IL-18 may directly or indirectly regulate SPHK activation. However, IL-18 may stimulate both SPHK1 and SPHK2 because blocking SKI inhibits both SPHK1 and SPHK2. Two isoforms of SPHK, SPHK1 and SPHK2, have five conserved domains and unique catalytic domains [21]. In particular, there is a great difference in expression pattern between SPHK1 and SPHK2. SPHK1 is mainly expressed in lung, spleen, kidney, and blood, but SPHK2 is generally detected in liver, kidney, brain, and heart [22]. This indicates that SPHK1 has more important functions than SPHK2 in blood. In mast cells, SPHK1 has a more important role in degranulation and migration, although both SPHK1 and SPHK2 function as stimulators of TNF-α secretion. In our microarray data, IL-18 increased SPHK1 mRNA expression but not SPHK2 mRNA expression in human NK cell line. Therefore, we supposed that IL-18 may regulate SPHK1 activation but not SPHK2, and that SPHK1 may be one signaling molecule in IL-18-induced IFN-γ production in NK cells.

Activation of p38 MAPK is a major event in IL-18-induced IFN-γ production in NK cells and T cells [12], and our data demonstrated that SKI blocks phosphorylation of p38 MAPK. In dendritic cells, SKI also suppresses p38 MAPK [20], signifying that p38 MAPK may be a major signal molecule target of SPHK. From these data, we concluded that p38 MAPK is a main target molecule of SPHK in IL-18-induced IFN-γ production, and ERK1/2 is independent of IL-18-induced SPHK activation.

Because MAPKs play an important role as signal transduction mediators, and are required for a variety of growth factor and cytokine-induced signal transduction for migration, survival, or proliferation [23], there is a possibility that the regulation of p38 MAPK by SPHK mediates cell migration ability. Actually, in dendritic cells, inhibition of SPHK by SKI reduced dendritic cell migration through blocking of p38 MAPK activation. At that time, SKI also reduced chemokine receptor, CCR7, an important mediator of cell migration [20]. This implies that SPHK activation is required for NK cell migration since CCR7 is also an important mediator of migration in NK cells and IL-18 induces $CD83^{+}CCR7^{+}$ NK helper cells [24]. Additionally, IL-18 has a role as a cytolytic stimulator in NK cells and stimulates p38 MAPK. P38 MAPK is a known regulator of Toll-like receptor 3-mediated cytotoxicity in NK cells [25]. From these facts, we hypothesize that IL-18-induced SPHK activation controls NK cytolytic activity.

Although SPHK has been demonstrated as an important regulator of biological processes in most cells, the specific function and mechanism in NK cells was unclear. Our results demonstrate that SPHK activation may be a key process in IL-18-induced cytokine production and activation of p38 MAPK signal pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.091.

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