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Korean mistletoe lectin enhances natural killer cell cytotoxicity via upregulation of perforin expression

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Abstract

Background: Natural killer (NK) cells are crucial components of the innate immune system, providing the first line of defense against pathogens. In a previous study, we demonstrated prophylactic activity of water extract of Korean mistletoe (*Viscum album coloratum*) on tumor metastasis. However, the leading compound from water extract of Korean mistletoe was not clearly addressed.

Objective: The purpose of this research was mainly focused on addressing the effect of Korean mistletoe lectin (KMLC) on NK cell cytotoxicity, and the ability of cytokine secretion as well as its signal transduction, mitogen-activated protein kinase (MAPK) pathway.

Methods: KMLC was used to test NK cell-mediated cytotoxicity *in vitro* and *in vivo*. Non-isotope cytotoxicity assay (bis-N,N,N′,N′-tetraacetic acid (BATDA) release assay) was performed to test the cytotoxicity of NK cells against target tumor cells. Receptor expression was checked by flow cytometry analysis and MAPK signal molecules were analyzed by immunoblotting.

Results and Conclusions: KMLC at 200 ng/mL increased the cytotoxicity of NK92 cells by 35% compared with untreated cells. KMLC-treated (at 100 ng/mL) mice splenocytes showed a 20% increase in cytotoxic activity. Also, the B chain, one of the subchains of KMLC, increases perforin expression. We demonstrated that the signal transduction controlling NK cell cytotoxicity was mediated by upregulation of the NKG2D receptor and expression of a cytotoxic effector molecule. These results suggested that KMLC possessed immunological activity, mediated by NK cell activation.

Keywords: Korean mistletoe, lectin, natural killer cell, cytotoxicity, perforin.

Abbreviations: NK cell, natural killer cell; E:T, effectors to target; MAPK, mitogen-activated protein kinase.

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Introduction

For centuries, European mistletoe (EM; *Viscum album*) have been used as medicine.[1] Anti-tumor and immunomodulatory activities of EM extracts have been reported.[2] EM extracts have many biological effects such as enhancement of natural killer (NK) cell activity, immunoadjuvant activity, and antitum or activity.[3,4] It was reported that the antitumor activity of EM was tightly related to its enhancement of NK and lymphokine-activated killer cell activity and macrophage activation.[5] The active compound in EM extracts was determined to be lectin.[6] This consists of A and B subchains linked by disulfide bonds, and the amino acid sequence of lectin has been completely determined.[7]

Korean mistletoe (KM; *Viscum album coloratum*) is a therapeutic herb that has traditionally been used as a sedative. Recent reports have suggested that KM extracts have immunoadjuvant activity, enhancing cellular and humoral immunity and NK cell activity.[8] According to a recent study, KM was more cytotoxic than EM against various tumor cells.[9] Furthermore, KM extract was reported to have a prophylactic effect through its enhancement of NK cell function.[8] However, the major compound within KM was not examined, and the underlying cellular mechanisms influencing NK cell activity

were unclear.[8] Even though the lectin fraction of KM extract was shown to be one of the active ingredients with tumor-suppressing activity, the underlying cellular mechanisms involved have not been fully studied *in vitro*.

In order to examine the effects of KM extracts on NK cell cytotoxicity, we isolated KM lectin (KMLC) from water extracts of KM. Lectin is a major component of mistletoe extracts, and KMLC has different structural properties than EM lectin.[10] KMLC has various biological activities such as cytokine induction, mucosal immunity enhancement, antitumor activity, and immunoadjuvant activity.[11-14] It is composed of two subunits that are linked by a disulfide bond.[15] NK cells are innate immune lymphocytes that are classified as large granular immune cells; these lack surface immunoglobulin and CD3, while expressing CD16 and CD56.[16,17] NK cells mediate the killing of certain virus-infected and tumor cells, providing a first line of defense against pathogens.[18,19] Exocytosis of cytolytic granules containing granzyme-B and perforin is essential for NK cell cytotoxicity and represents their primary means of killing target cells.[20] It is well known that the expression of granzyme-B and perforin is regulated by mitogen activated protein kinase (MAPK) pathway phosphorylation and activation of this pathway in turn is regulated by phosphorylation of protein tyrosine kinase.[21,22]

The present study demonstrated that KMLC had antitumor activity via enhancement of perforin expression and NK cell cytotoxicity. KMLC activated NK92 cells, through effects on MAPK, increased perforin expression and augmented cytotoxic activity against K562 and Yac-1 tumor cell lines. Cytotoxic activity was measured using the bis(acetoxymethyl)-2,2′-6′,2″-terpyridine-6,6″-dicarboxylate (BATDA) release assay, which has various advantages over the chromium release assay.[23]

Materials and Methods
Cell culture and reagents
The NK92 tumor cell line was grown in alpha-minimal essential medium (MEM, GIBCO) containing 100 U/mL recombinant human interleukin-2 (IL-2; PeproTech), 12.5% fetal bovine serum (GIBCO), and 12.5% horse serum (GIBCO). The K562 erythroleukemia cell line and Yac-1 cell line were grown in Rosewell Park Memorial Institute (RPMI)-1640 (GIBCO) medium containing 10% fetal bovine serum.

Animals
Six-week-old specific pathogenic-free BALB/c male mice were purchased from the Dae-Han BioLink (DBL), Chungbuk, Korea. Mice were maintained in the Laboratory of Animal Experiment, Institute of Bioscience and Technology, Handong Global University. They were maintained 23 ± 2 °C with 55% ± 20% relative humidity and 12 hour day and night cycle. Water and diets were supplied *ad libitum*. Each group was composed of 5 mice, and KMLC was administered intraperitoneally (200 ng/head or 500 ng/head) as well as a Phosphate Buffered Saline (PBS) control as vehicle control 24 hours before sacrifice for analysis of granzyme-B and perforin expression in splenocytes. Freshly isolated splenocytes were washed in cold PBS twice and passed through a 40-μm cell strainer (Corning).

After centrifugation at 400 x *g* for 10 minutes, erythrocytes were eliminated by red blood cell (RBC) specific lysis buffer (Calbiochem) 5 minute in room temperature. After washing once again, the collected pellet was resuspended in 40 mL cold PBS, then used intracellular staining after counts with a hemocytometer.

Extraction of KMLC
KMLC was prepared as described previously.[10] Briefly, the chopped leaves of KM were homogenized in 10 volumes (weight/volume) of distilled water for 30 seconds. The homogenates were then stirred overnight at 4 °C. The insoluble materials were removed by centrifugation at 15,000 x *g* for 30 minutes at 4 °C, and the supernatant was passed through a 0.45-μm filter (Millipore, Massachusetts, USA) followed by a 0.2-μm filter (Millipore, Massachusetts, USA). The final filtered supernatant was loaded onto a Sepharose 4B column (GE Healthcare, Sweden), which had been treated with 0.2 M HCl for 2.5 hours. The bound material (KMLC) was eluted using lactose buffer (0.1 M lactose in 0.01 M sodium phosphate and 0.14 M NaCl, pH 7.3). The fractions containing KMLC were pooled, dialyzed against distilled water, and freeze-dried. For mouse experiments, KMLC was dissolved in PBS, the concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific), and stored at 4 °C prior to use.

Preparation of KMLC B chain
The separation of the KMLC B chain has been reported previously.[24] In brief, KMLC was dissolved in PBS with 5% β-mercaptoethanol (Sigma, Missouri, USA) and incubated overnight at 25 °C. It was then loaded onto a lactose affinity column (Sigma, Missouri, USA) that had previously been equilibrated with the same buffer. The unbound fraction was eluted with the same buffer, and the bound fraction was eluted with PBS containing 0.1 M lactose (Sigma, Missouri, USA). The fractions containing the B chain were then pooled, dialyzed against distilled water, and freeze-dried.

BATDA release cytotoxicity assay
The NK cell cytotoxicity assay was performed as described previously.[25] Briefly the target cells, the human leukemia K562 and Yac-1 cell lines, were labeled with BATDA complex (Thermo Fisher) and co-cultured with the NK92 cell line or with splenocytes as effector cells. The target cells (5×10^3 cells/well) were cultured in round-bottomed microwell plates with various concentrations of effector cells in duplicate under standard culture conditions for 4 hours. Spontaneous BATDA release wells were composed of target cells and 100 μL of normal medium and the maximum release wells contained target cells with 100 μL of Triton X-100 lysis buffer. After the 4-hour incubation, 20 μL of the supernatant was transferred to a flat-bottomed plate and Europium solution was added. Finally, fluorescence was measured in a time-resolved fluorometer (PerkinElmer VICTOR 2D). To check reproducibility, all experiments were performed at least 3 times.

The percentage of specific lysis was calculated using the following formula:

specific BATDA release = (experimental count – spontaneous count/maximum count – spontaneous count) × 100.

Intracellular staining and flow cytometry

NK92 cells were stained with antibodies detecting granzyme-B and perforin, and splenocytes were stained for surface CD3 and NK1.1 and intracellular granzyme-B and perforin, as described. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 monoclonal antibody (mAb) (BD, California, USA), FITC-conjugated anti-human Gzmb mAb (BD), Phycoerythrin (PE)-conjugated anti-human Prf mAb (BD), PE-conjugated anti-mouse Gzmb mAb (BD), and PE-conjugated anti-mouse Prf mAb (e-Bioscience) were used. Samples were then acquired on a flow cytometer and analyzed. Isotype controls were used to set quadrant gates. In a separate study, NK92 cells were co-cultured in 6-well culture plates (Falcon) with various concentrations of KMLC or B chain for a range of time periods. After 24 hours, NK92 cells were stained for surface NKp44 and NKG2D using specific mAbs (Biolegend, California, USA) conjugated with FITC and PE. Samples were acquired on a flow cytometer (Beckman Coulter, California, USA) and analyzed. To check reproducibility, all experiments were performed at least 3 times.

Preparation of protein lysates and immunoblots

Protein lysates were prepared from KMLC-activated spleen and NK92 cell lines using a lysis buffer (Intron, South Korea). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Massachusetts, USA). Activation of MAPK was detected using p-Extracellular signal-Regulated Kinase (ERK) (#4370, Cell signaling, Massachusetts, USA) and p-JNK (#9255, Cell signaling, Massachusetts, USA) mAbs diluted 1:1000.

Results

Effect of KMLC on NK92 cell and splenocyte cytotoxicity

To explore the effect of KMLC on NK cell activity, we examined whether KMLC enhanced NK92 cell and splenocyte cytotoxicity. Initially, we determined the direct cytotoxicity of a range of KMLC concentrations up to 200 ng/mL on NK92 cells. No significant effect of KMLC on viability of the NK92 cell line was observed at concentrations below 200 ng/mL (**Figure 1A**). The NK cell-mediated cytotoxicity assay was performed using K562 as the target cells for NK92 cells and Yac-1 cells for splenocytes. The NK92 cells primed with 10-100 μg/mL of KMLC were co-incubated 4 hours with the target cells at the indicated effector cell to target cell ratio (E:T ratio). As shown in **Figure 1B and 1C**, the cytolytic activities of both NK92 cells and splenocytes were enhanced by treatment with KMLC in a dose-dependent manner. At a KMLC concentration of 100 ng/mL, activity was increased by 40% compared with that of the untreated cells at the same E:T ratio (25:1) in NK92 cells (**Figure 1B**). In addition, treatment of splenocytes with 100 ng/mL KMLC produced a 25% increase in cytotoxic activity compared with that of the untreated cells at the same E:T ratio (250:1; **Figure 1C**).
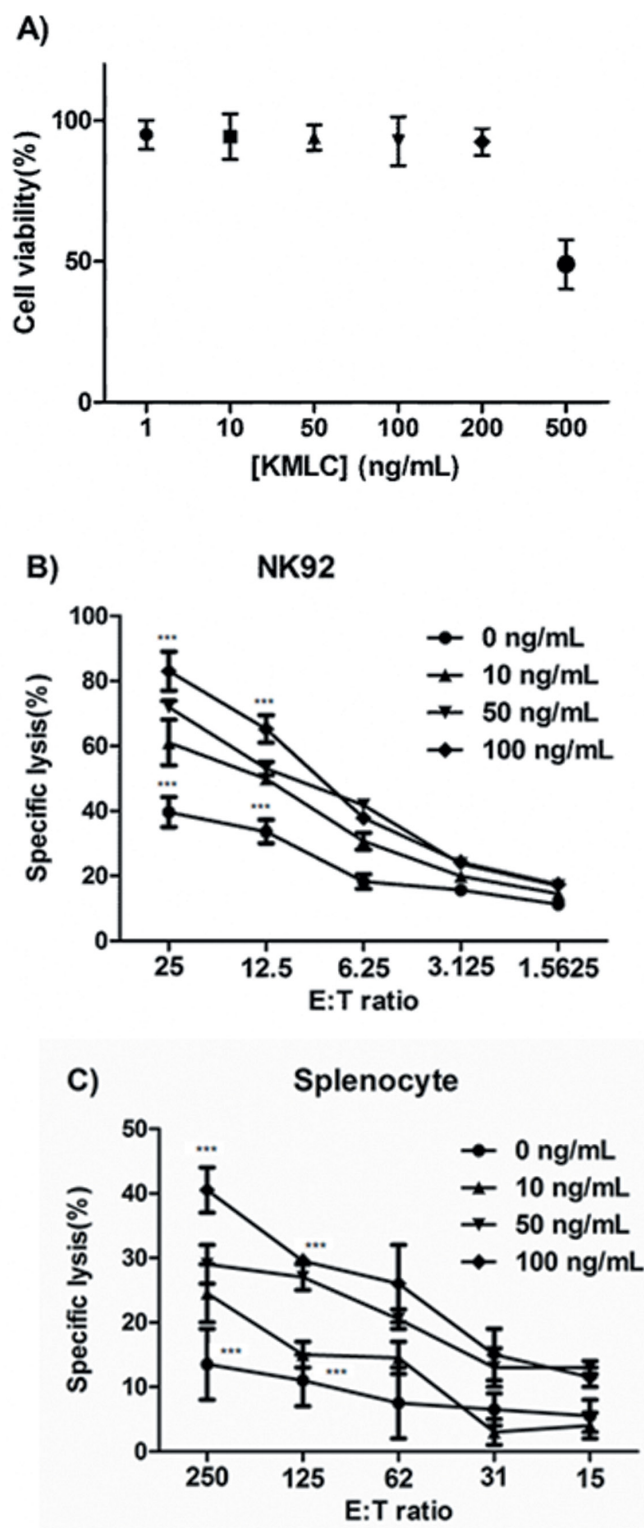

Figure 1. KMLC enhanced NK cell cytotoxic activity. (A) Viability of NK92 cells treated with KMLC. NK92 cells (B) or splenocytes (C) were stimulated with the indicated concentrations of KMLC and co-cultured with K562 cells for 4 h at the indicated E:T ratios. Cytotoxic activity was performed by BATDA release assay.
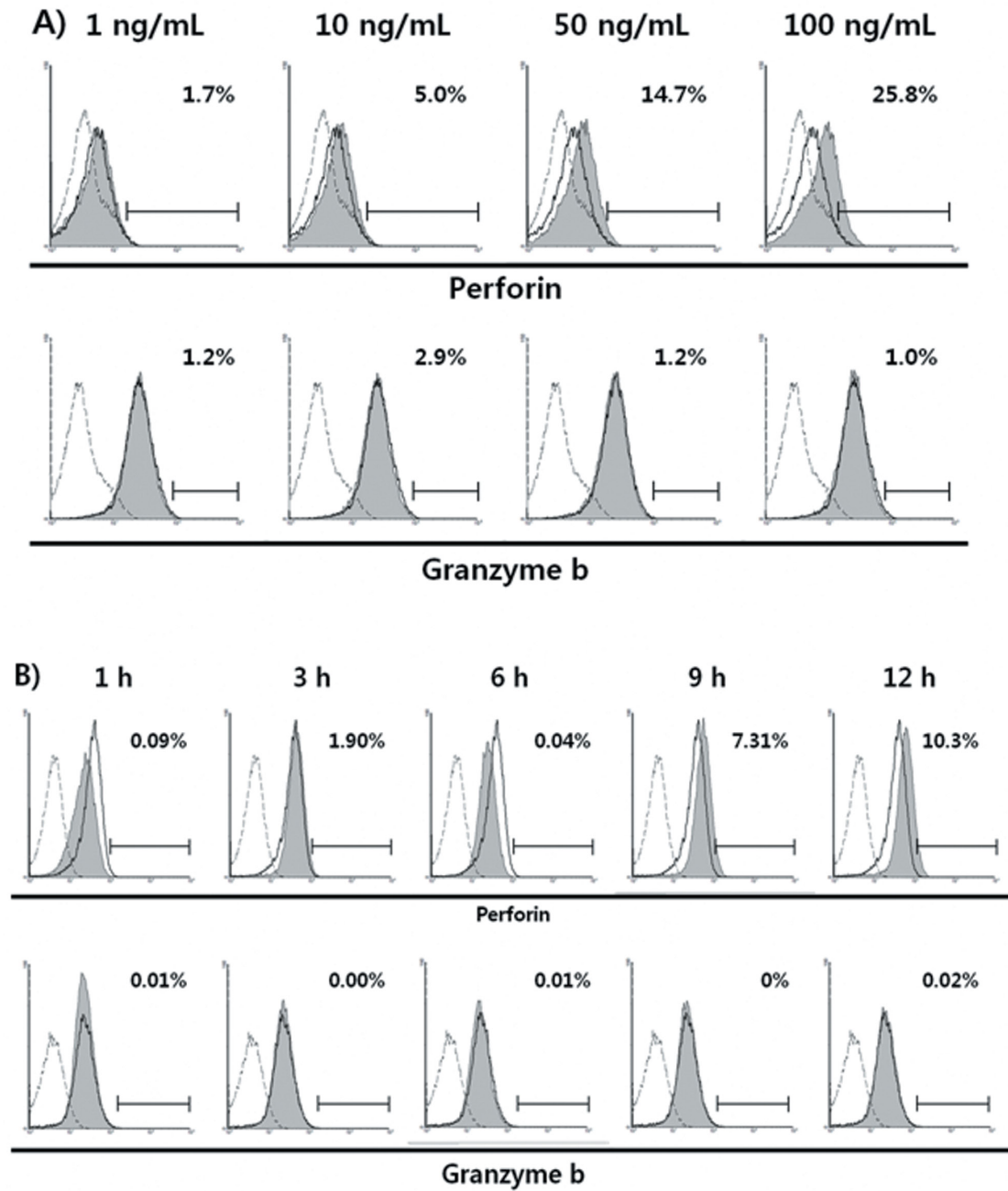
Figure 2. KMLC increased perforin expression in NK92 cells. 1×10^6 NK92 cells were treated with KMLC using the indicated doses and time periods. Representative flow cytometry histogram plots were shown for perforin and granzyme-B expression. The expression of granzyme-B (upper panels) and perforin (bottom panels) is shown at a range of KMLC concentrations (A) and times (B). Dash lines and bold lines indicate non-treatment and isotype control staining of the cells. Values of the fold increase with respect to isotype control are reported.

Expression of perforin and granzyme-B in the NK92 cell line

NK and T cell cytotoxicity is mediated by expression of cytolytic granules containing granzyme-B and perforin.[16] Therefore, we examined the effect of KMLC on perforin and granzyme-B expression in the NK92 cell line. NK92 cells were primed with KMLC at various concentrations for a range of time periods, and the expression of perforin and granzyme-B was assessed by flow cytometry. NK92 cells were also pretreated to examine time-dependent effects. As shown in **Figure 2**, KMLC treatment increased perforin expression in a dose- and time-dependent manner. At a KMLC concentration

of 100 ng/mL, the perforin expression level was increased by 25% compared with that observed in the untreated cells. However, no significant upregulation of granzyme-B was detected in KMLC-treated cells (**Figure 2A and 2B**).

Expression of perforin and granzyme-B in splenocytes

To explore whether *in vivo* treatment with KMLC also induced perforin expression, we assessed perforin expression in splenocytes of mice injected with KMLC. Intraperitoneal injection of KMLC enhanced the perforin-containing NK cell population (CD3-NK1.1+Prf+) in splenocytes in a
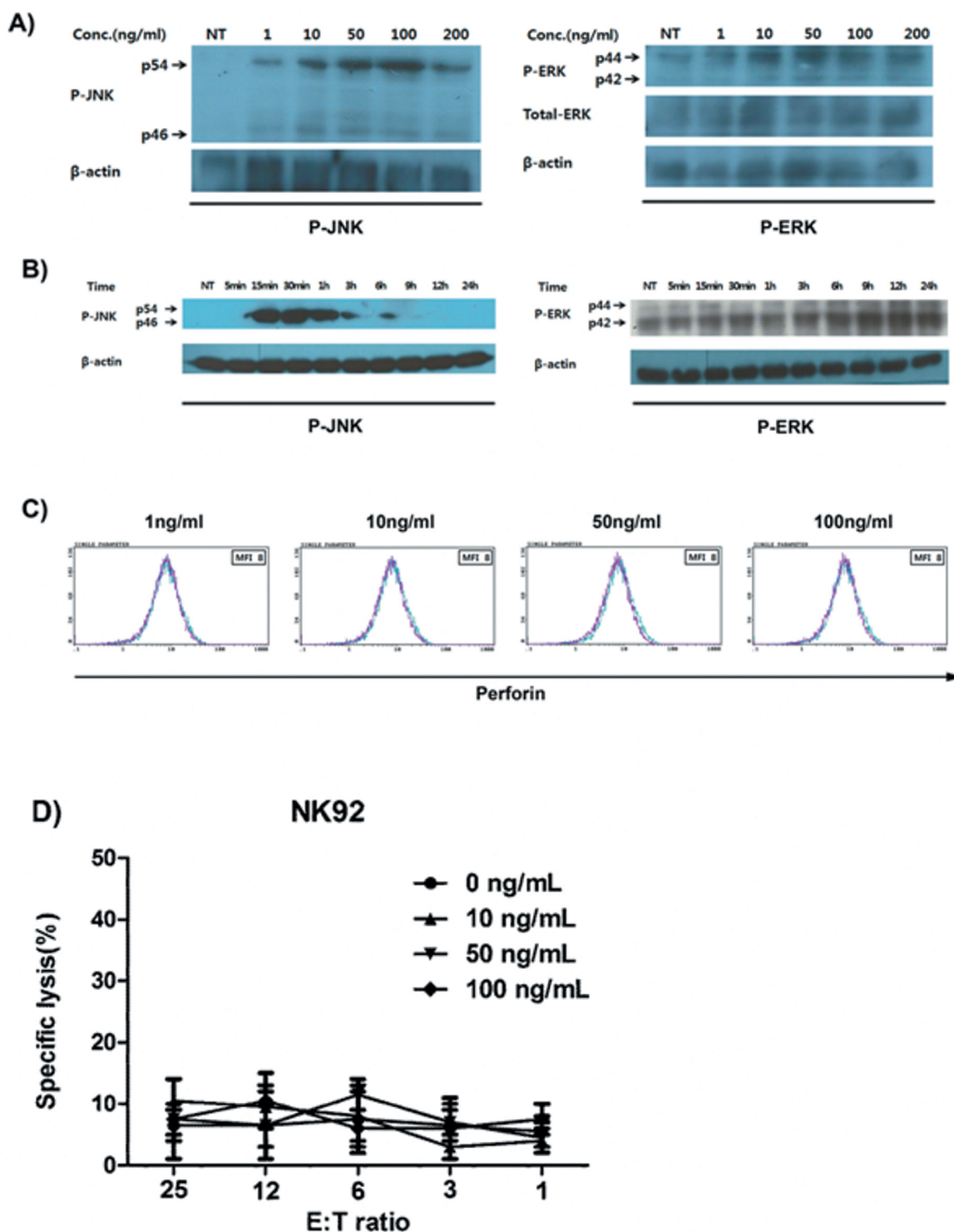
Figure 3. KMLC enhanced perforin expression via the MAPK pathway. 1×10^6 NK92 cells were pretreated with KMLC at the indicated doses and times. (A, B) Phosphorylated JNK and ERK were detected by western blot. β-actin was used as the loading control. (C) The effect of SP600125 (20 nM) on perforin expression of NK92 cells. (D) BATDA release assay was performed with treatment of 20nM SP600125 to NK92 cell line against K562 target cells.

dose-dependent manner. However, no significant upregulation of granzyme-B was detected following KMLC treatment.

KMLC upregulated phosphorylation of JNK and ERK

MAPK signaling has been shown to be associated with NK cell cytotoxicity.[22] Therefore, we assessed whether KMLC activated the MAPK signaling pathway in the NK92 cells.

MAPK phosphorylation was examined following treatment with KMLC at various concentrations for a range of time periods. As shown in **Figure 3A and 3B**, KMLC treatment increased the level of phosphorylated JNK in 30 minutes. However, altered ERK1/2 phosphorylation was not detected following KMLC treatment. To address whether the activity of KMLC on NK cytotoxicity and perforin expression was

dependent on JNK activation, SP600125, a JNK inhibitor, was added to the NK92 cell line prior to measuring their cytotoxic activity towards target cells and their perforin expression. As shown in **Figure 3C**, no difference in perforin expression was detected in cells exposed to various concentrations of KMLC, and NK cell cytotoxicity against K562 target cells was decreased by KMLC treatment (**Figure 3D**). These data suggested that JNK must be a crucial kinase involved in the regulation of NK
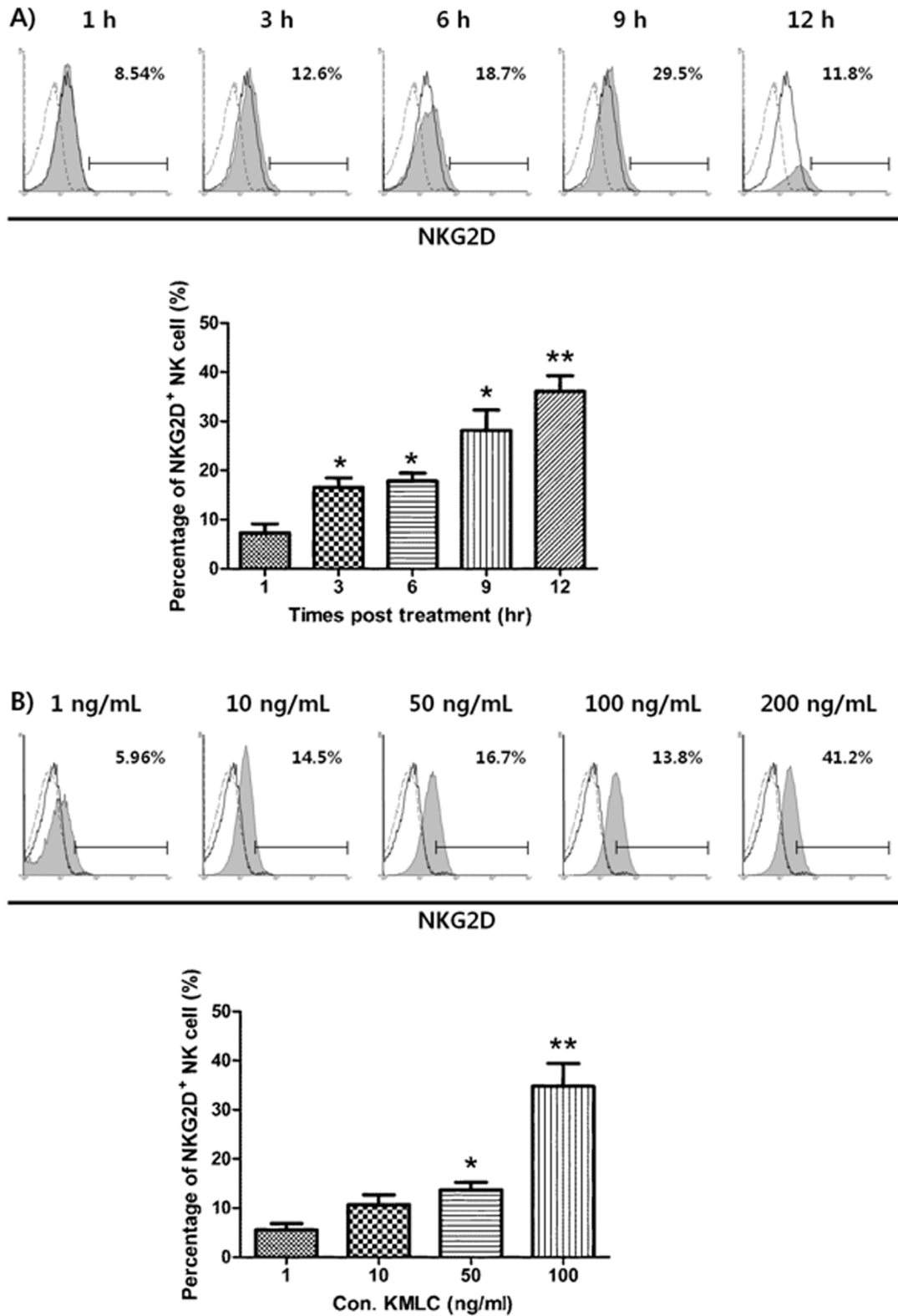

Figure 4. NKG2D receptor expression in NK92 cells was increased by KMLC. The levels of NKG2D and NKp44 were analyzed by flow cytometry. Representative flow cytometry histogram plots showing the fluorescence intensity of the FITC-conjugated anti-NKG2D monoclonal antibody (A and B) and PE-conjugated anti-NKp44 monoclonal antibody (C and D), compared with isotype controls. Values of the respective MFI fold increase are reported. *P < 0.05.
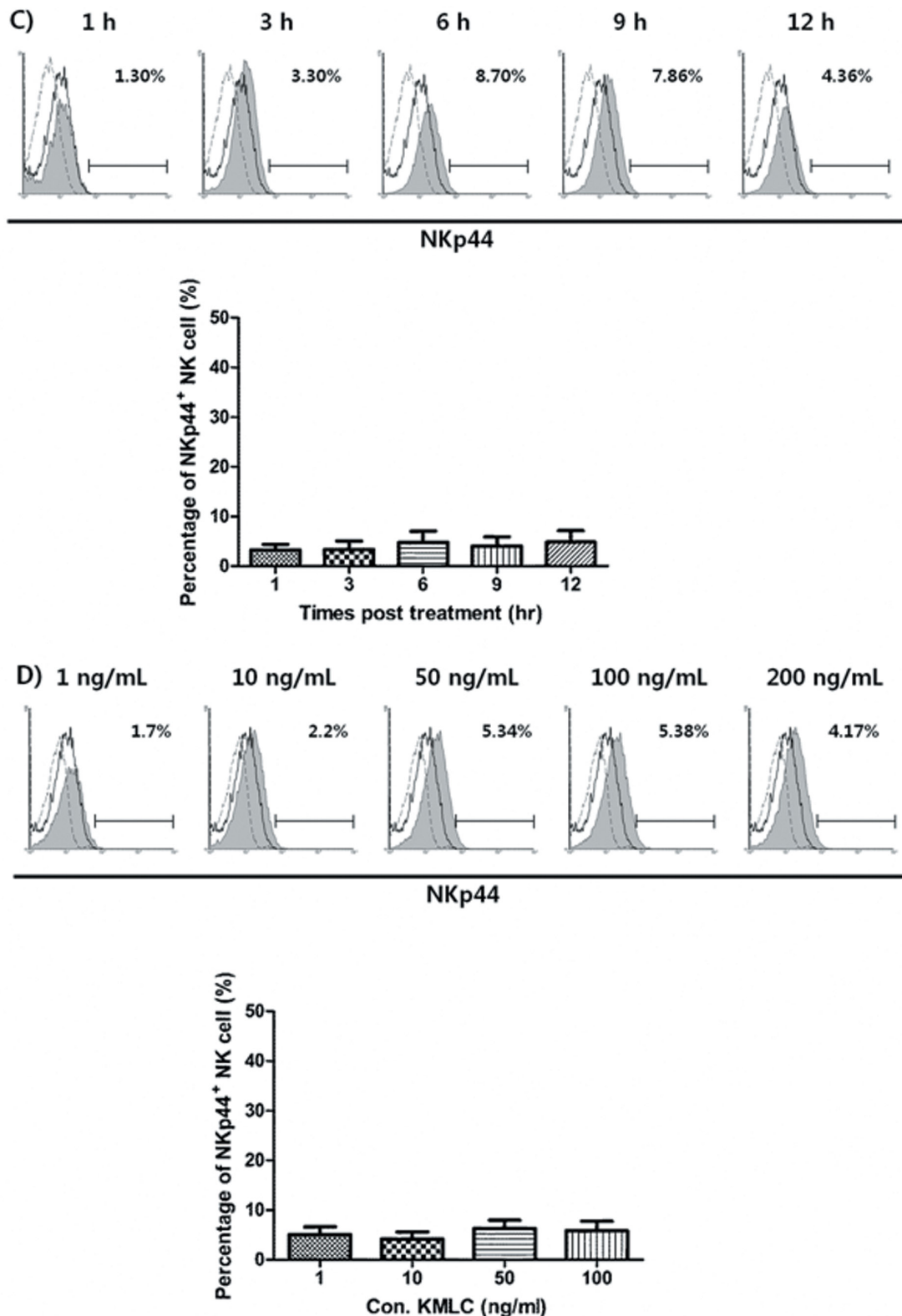
Figure 4. (Continued) NKG2D receptor expression in NK92 cells was increased by KMLC.

cell cytotoxicity via perforin expression.

KMLC upregulated the expression of NKG2D and NKp44 in the NK92 cell line

NKG2D is a C-type lectin-like receptor and its activation triggers perforin expression and NK cell cytotoxicity.[26] NKp44 is a human natural cytotoxicity receptor (NCR) family receptor

classified as part of the immunoglobulin superfamily, which is composed of NKp44, NKp46, and NKp30.[27] These receptors have been reported to be upstream of the MAPK pathway,[28] and we, therefore, examined whether KMLC enhanced the expression of NKG2D or NKp44. KMLC treatment enhanced the expression of NKG2D in a dose- and time-dependent manner (**Figure 4A and 4B**). KMLC enhanced NKG2D expression
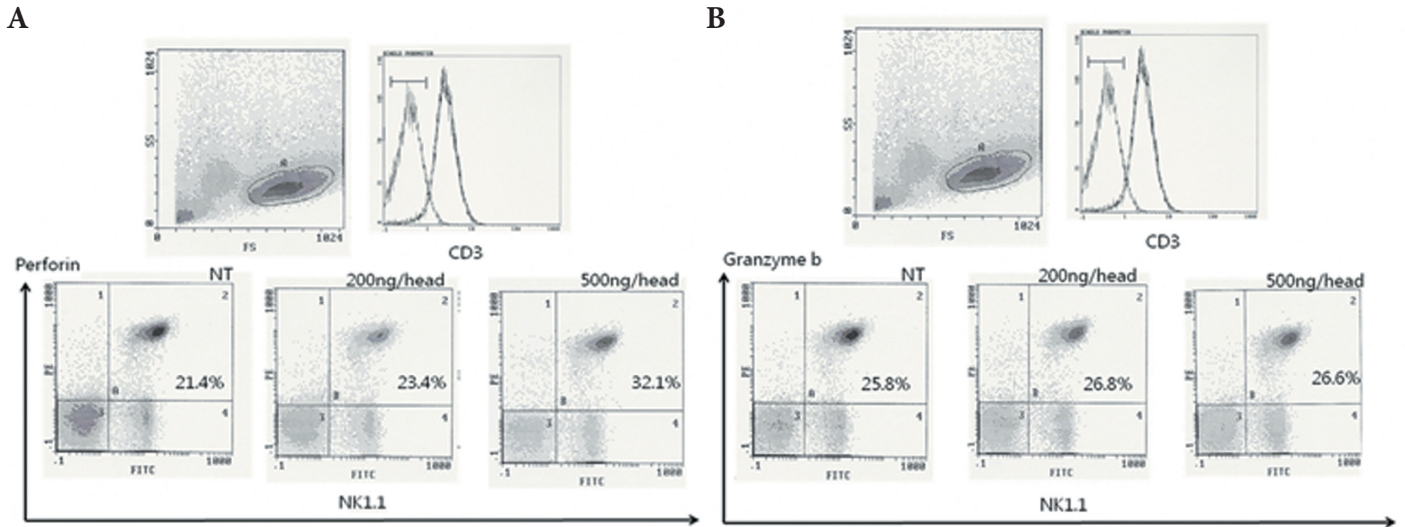
A

B

Figure S1. KMLC upregulated perforin expression in splenocyte. After BALB/c mice were injected with a range of KMLC concentrations, the expression of perforin and granzyme-B were analyzed in their splenocytes; (A) % population of perforin-positive cells in splenocytes; (B) % populaion of granzyme-B-positive cells in splenocytes.
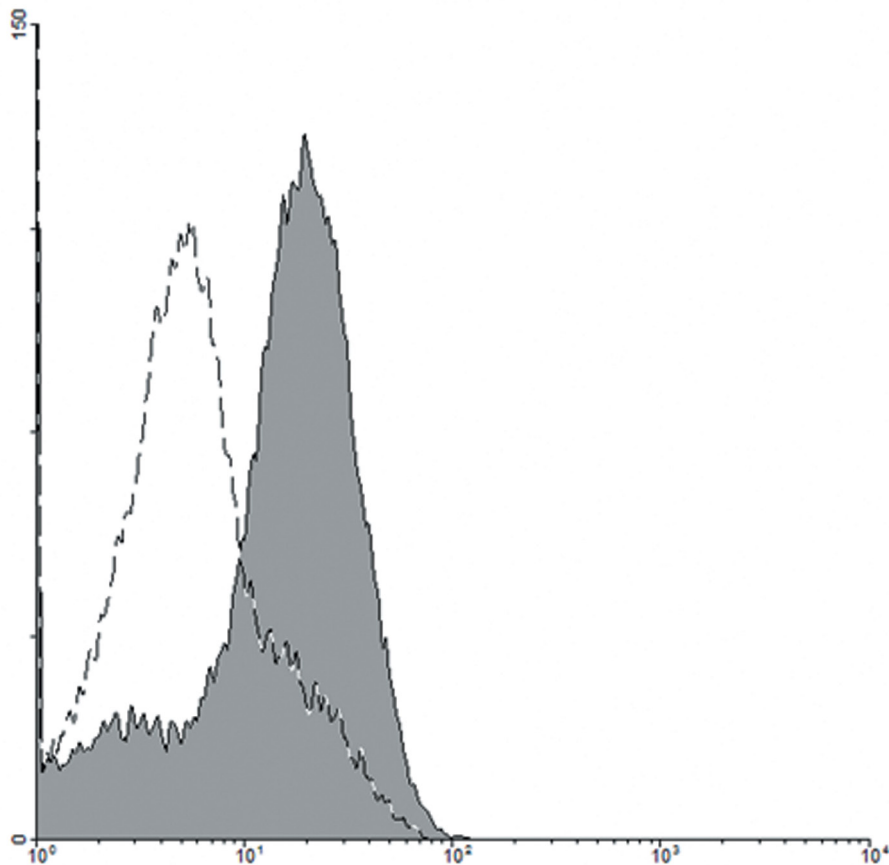

Figure S2. KMLC B chain increased perforin expression in NK92 cells. 1×10^6 NK92 cells were treated with KMLC B chain. Representative flow cytometry histogram plot was shown for perforin expression. The expression of perforin is shown at 20ng/mL of B chain concentration. Dash line indicates non-treatment and filled area is a sample-treated cells.

by more than 20% at 9 hours and showed a 35% increase in expression in the 200 ng/mL-treated group compared with the untreated group. However, there was no effect on NKp44 expression. These data suggested that KMLC had a potential role as a NK cell enhancer via modulation of expression of the NKG2D surface receptor, but not of the NKp44 NCR family.

Expression of perforin by KMLC B chain treatment in the NK92 cell line

KMLC B chain is a subchain of KMLC, and it has immunoadjuvant activity with low cytotoxicity.[24] Therefore, we examined the effect of KMLC B chain on perforin expression in the NK92 cell line. NK92 cells were primed with KMLC B

chain, and the expression of perforin was assessed by flow cytometry. As shown in **Figure S2**, treatment with KMLC B chain increases perforin expression (**Figure S2**).

At a KMLC concentration of 100 ng/mL, the perforin expression level was increased by 25% compared to that observed in the untreated cells. However, no significant upregulation of granzyme-B was detected in KMLC-treated cells (**Figure 2A and 2B**).

Discussion

The investigation of cancer treatments involving improvement of host defense systems is a novel approach.[29] The important immune cell in host defense is the NK cell, which kills tumor cells and virus-infected cells.[16] NK cell therapy is an innovative and well- researched method. Normally, IL-2 is used for activation of NK or T cells isolated from cancer patients.[30] It improves their killing effects and helps to treat the illness. However, this therapy has limitations for the patient, because of the side effects of IL-2. To solve this problem, scientists have aimed to discover new natural compounds with fewer side effects.[21] In a previous study, we showed that natural KM extract stimulated NK cell-mediated cytotoxicity in a mouse model.[8] This finding showed that KM extract might represent a potential natural compound for cancer treatment.

The present study examined the mechanisms of NK cell cytotoxicity involving the activating receptor and signaling molecules downstream of the NKG2D receptor. KMLC appeared to activate NKG2D, leading to the activation of JNK phosphorylation. Finally, this resulted in upregulation of perforin expression, which improved the targeting of cancer cells.

In this study, we clearly showed that KMLC promoted perforin-mediated NK cell cytotoxicity via increased MAPK pathway (JNK) phosphorylation. KMLC enhanced NK92 cell cytotoxicity against the K562 human leukemia cell line, which is major histocompatibility complex (MHC) class I-deleted. The cytotoxicity was measured by a BATDA release assay that is a more specific and rapid method than the traditional[51] Chromium (Cr) release assay.

Interestingly, only NKG2D was upregulated when co-cultured with KMLC *in vitro*.[26] The most effective NK cell activating receptor, NKG2D, is closely related to NK cell cytotoxicity against cancer and virus-infected cells. However, no difference was observed in the expression of the NKp44 receptor, which is classified as a member of the Ig superfamily, along with NKp44, NKp46, and NKp30.[27] This may be due to the NCR receptor binding affinity for viral glycoproteins.[31] In addition, perforin expression was increased following exposure to KMLC. Resveratrol also upregulates perforin protein in NK cells and activates the MAPK pathway by NKG2D activation.[21] Although the phenomenon results in demonstrable cytotoxicity in mice and cell lines, the signaling and downstream mechanisms involved in NK cell cytotoxicity are still unclear. We will approach that which is the NK cell surface receptor with KMLC, binding affinity, and other intracellular signal molecules of NK cells upstream of JNK.

KMLC is composed of A and B subchains.[7] The B chain binds to carbohydrates on the cell surface receptor, and

the A chain hinders translation and induces apoptosis as a ribosome-inactivating protein (RIP).[7] In previous studies, the B chain was shown to have a variety of immunological activities with low cytotoxicity.[32] We isolated the B chain and examined its immunomodulatory activity on NK cell cytotoxicity. These findings indicated that the B chain was the major active compound involved in modulation of NK cell cytotoxicity. Future studies will aim to identify the optimal mixture and concentration of KMLC and B chain for the development of a new natural anti-cancer drug.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

Ethics approval

All of the animal experiments were approved by the Ethics Review Committee of the Handong Global University.

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