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The effect of alloferon on the enhancement of NK cell cytotoxicity against cancer *via* the up-regulation of perforin/granzyme B secretion

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ABSTRACT

Alloferon is a novel immunomodulatory peptide originally isolated from infected insects. It has anti-viral and anti-tumor effects *via* the activation of NK cells. However, specific mechanisms leading to NK cell activation and anti-tumor responses yet to be clarified. In this study, we demonstrate that alloferon increases killing activity of NK cells to cancer cells *via* the up-regulation of the expression of NK-activating receptors, 2B4. In addition, the production of IFN-γ and TNF-α and granule exocytosis from NK cells against cancer cell were increased by alloferon. Lastly, the anti-tumor effect of alloferon was confirmed *in vivo* to demonstrate effective retardation of tumor growth in the human-to-mouse xenograft model. All taken together, these results suggest that alloferon has anti-tumor effects through up-regulation of NK-activating receptor 2B4 and the enhancement of granule exocytosis from NK cells.

Introduction

NK cells are crucial component of the innate immune system and they can directly eliminate cancer cells or infected host cells. Since NK cells can target MHC class I-negative cells that escape recognition by cytotoxic T cells, they are very important in the immune surveillance of altered-self cells, such as virus-infected cells and cancer cells (Moretta et al. 1992; Lanier 1998a). In addition, they contribute to antigen-specific adaptive immunity by producing cytokines, such as IFN-γ, which promote Th1-type responses (Trinchieri 1995). NK cell-mediated cytotoxicity and cytokine secretion are controlled by multiple receptor–ligand interactions (Lanier 1998b). In particular, precise balance between stimulatory and inhibitory signals received from surface receptors is very crucial in this process (Long and Rajagopalan 2000; Moretta et al. 2000; Tomasello et al. 2000). NK cells induce death of their targets by either tumor necrosis factor family and death receptor/death ligand interaction or granule exocytosis (Kägi et al. 1994). Granule exocytosis is notable for the efficient killing of virus-infected cells or cancer cells (Russell and Ley 2002). Perforin, a putative pore-forming protein and granzymes are released from cytotoxic granules to induce target-cell death (Shi et al. 1992; Shresta et al. 1995).

Alloferon was originally isolated from the experimentally infected insect, a blow fly *Calliphora vicina* and consists of 13 amino acids: HGVSGHGQHGVHG. It has been demonstrated that alloferon stimulates the natural cytotoxicity of human peripheral blood lymphocytes and enhances anti-tumor and anti-viral activities through the induction of IFN synthesis (Chernysh et al. 2002). We have recently reported that alloferon has dual functions: one is direct inhibition of the replication of Kaposi's sarcoma-associated herpesvirus and the other is effective eradication of virus-infected cells through the activation of NK cells (Lee et al. 2011). And tumoristatic and tumoricidal activities of alloferon have been recently found in DBA/2 mice grafted with syngenic P388 murine leukemia cells (Chernysh et al. 2012). However, the anti-tumor effect of alloferon against human cancer cells through the activation of NK cells and its related mechanisms are still not reported. It is generally known that prostate cancer cell line, PC3 and colon cancer cell line, HCT116

Abbreviations: NK cells, natural killer cells; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; PBMC, peripheral blood mononuclear cell; CMA, concanamycin A; ELISA, enzyme-linked immunosorbent assay.

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are highly resistant to NK cell-mediated cytotoxicity (Romijn 1985; Raja Gabaglia et al. 2007; Moriwaki and Miyoshi 2010). In this study, we investigated the anti-tumor effects of alloferon on NK-resistant human cancer cells through the modulation of NK activity and its related mechanisms.

Materials and methods

NK cell isolation

Primary NK cells were isolated from peripheral blood mononuclear cells (PBMC) obtained from healthy individuals with density gradient centrifugation by using of Ficoll-Paque™ PLUS (Amersham Pharmacia Biotech, Piscataway, NJ, USA). NK cells were then purified from PBMC by MACS negative selection system using the NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of NK cells was determined by flow cytometry, after staining with FITC-conjugated anti-CD3 and CD16 Ab and PE-conjugated anti-CD56 Ab (Pharmingen, San Diego, CA, USA). Purified NK cells were used in the experiment after incubation in the absence or presence of 2 and 4 μg/ml of alloferon for the indicated time in each experiment. Alloferon was kindly provided by Allotech Co., Ltd., after it is synthesized by "Peptide synthesis Ltd." (Moscow, Russia) using solid-phase synthesis technique as described (Sidorova et al. 2006). The purity of the synthetic peptide measured by HPLC was more than 98%.

Cell lines

Human prostate cancer cell line, PC3 and colon cancer cell line, HCT116 were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in medium containing RPMI 1640, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen, Grand Island, NY, USA). All cells were maintained in a humidified 37 °C, 5% CO_2 incubator.

^{51}Cr release assay

NK cells were cultured in the absence or presence of alloferon (2 and 4 μg/ml) at 37 °C in 5% CO_2 for 6 and 12 h. And then cells were washed twice with PBS and used as effectors for ^{51}Cr release assay. Target cells, PC3 and HCT116 (1×10^6) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 37 °C in 5% CO_2 for 1 h. Effector cells were distributed on a U-bottom 96-well plate and mixed with ^{51}Cr-labeled target cells (1×10^4 cells/well) at effector-to-target (E:T) ratios 30:1. After further incubation for 4 h, the supernatants were harvested and radioactivity was measured by an automated gamma counter. Maximum release of ^{51}Cr was obtained by lysis of the target cells in 2% NP-40 (Sigma–Aldrich, St. Louis, MI, USA) and spontaneous release of ^{51}Cr was determined in wells containing labeled target cells incubated with medium alone. Results are shown as percent specific release: ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100. To analyze whether granule exocytosis is involved in the killing of cancer cells by alloferon-treated NK cells, assay was performed after NK cells were pre-treated with 10 nM of concanamycin A (Calbiochem, San Diego, CA, USA), inhibitor of granzyme/perforin secretion from NK cells, for 2 h.

Flow cytometric analysis

NK cells were cultured in the absence or presence of alloferon (2 and 4 μg/ml) at 37 °C in 5% CO_2 for 12 h. Cells were washed twice with PBS and then Fc receptors on NK cells were blocked with Fc blocking reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). And cells were stained with FITC-conjugated anti-2B4 (CD244) Ab, APC-conjugated anti-NKG2D Ab (Becton Dickinson, Mountain View, CA, USA) and FITC-conjugated anti-CD94 and KIR Ab (R&D systems, Minneapolis, MN, USA) on ice for 30 min. After washing twice with FACS buffer, the expression of NK-activating and inhibitory receptors was analyzed by FACS Calibur (BD Biosciences). FlowJo software (Tree Star, Ashland, OR) was used for data analysis. Results are shown either as staining histograms (*X*-axis represents fluorescence intensity and *Y*-axis represents cell counts) or as the geometric mean fluorescence intensity (MFI) of the stained populations.

Cytokine ELISA

NK cells were cultured in the absence or presence of alloferon (2 and 4 μg/ml) at 37 °C in 5% CO_2 for 6 and 12 h. After washing twice with PBS, cells were distributed on a U-bottom 96-well plate and mixed with target cells (PC3) at effector-to-target (E:T) ratio of 30:1. Cells were further incubated for 4 h, the supernatants were harvested. *In vivo* production of IFN-γ and TNF-α in tumor-bearing *BALB/c* nude mice upon the administration of alloferon was also analyzed. Briefly, mice were intraperitoneally injected with PBS or alloferon (50 μg/ea) every day from the day of tumor inoculation. After 4 weeks, blood was collected from the intra-orbital plexus of heparinized capillary tube and the plasma was obtained by centrifugation. IFN-γ and TNF-α in the supernatants or plasma were measured by ELISA kit (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

CD107a mobilization assay and granzyme B ELISA

NK cells were cultured in the absence or presence of alloferon (2 and 4 μg/ml) at 37 °C in 5% CO_2 for 6 and 12 h. After washing twice with PBS, cells were distributed on a U-bottom 96-well plate and mixed with target cells (PC3) at effector-to-target (E:T) ratio of 30:1. And then FITC-conjugated anti-CD107a Ab (Becton Dickinson, Mountain View, CA, USA) was added to the mixture of effector and target cell. Cells were incubated for 1 h at 37 °C in 5% CO_2 incubator, followed by an additional 3 h in the presence of monensin that is a protein transport inhibitor commonly used to enhance intracellular cytokine staining signals by blocking transport processes during cell activation (Sigma–Aldrich, St. Louis, MI, USA). At the end of the incubation period, the cells were harvested and stained with PE-conjugated anti-CD56 Ab. Results were read on a FACS Caliber (Becton Dickinson, Mountain View, CA, USA). The supernatants of the cultures were also collected and granzyme B was assayed using Granzyme B ELISA kit (Bender Medsystems, Burlingame, CA, USA) according to the manufacturer's instructions.

Tumor xenograft in immunodeficient mice

Six-week-old female *BALB/c* nude mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Non-obese diabetic/severe combined immunodeficiency/interleukin-2 receptor gamma chain-deficient (NOD/SCID/IL-2Rγ(−/−)) mice 9–10 weeks of age were kindly provided by Prof. Dong-Sup Lee, Transplantation Research Institute in Seoul National University Hospital (Seoul, Korea). NOD/SCID/IL-2Rγ(−/−) mice were produced by intercrossing the NOD/SCID and IL-2Rγ-deficient mice in NOD background. Genotypes of offsprings were evaluated by PCR method. All the animals were maintained in specific pathogen free condition at the animal facility of center for animal resources and development (CARD) in the Seoul National University Medical College. 9–10 were used for the experiment. The animal protocol for

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experiments was reviewed and approved by Ethics Committee of the Seoul National University. Human colon cancer cell line, HCT116 was subcutaneously injected into the left flank of *BALB/c* nude mice (5×10^5 cells/ea) and NOD/SCID/IL-2Rγ(−/−) mice (2×10^5 cells/ea) after avertin/metofane anesthesia. Alloferon (50 μg/ea) was injected intraperitoneally into the mice every day from the day of tumor inoculation. Tumor sizes were measured every other day and the tumor volume was calculated with the following formula: tumor volume = ½ × (length × width × height).

Statistical analysis

Data were expressed as mean ± SD of each group in independent experiments. For comparison of three or more groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test. A value of $P < 0.05$ was considered statistically significant. Statistical tests were carried out using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

Results

Alloferon enhances NK cell cytotoxicity against prostate cancer cell line, PC3 and colon cancer cell line, HCT116

To determine the effects of alloferon on the cytotoxic activity of human NK cells cytotoxicity against tumor, we isolated NK cells from the peripheral blood of healthy volunteers. We confirmed that the purified NK cells were $CD3^-CD16^{bright}CD56^{dim}$ that is conventional phenotype of cytotoxic NK cells (Supplementary Fig. 1). NK cells were stimulated with 2 and 4 μg/ml of alloferon for 6, 9, and 12 h, and then co-cultured with ^{51}Cr-labeled human prostate cancer cell line, PC3. As shown in Fig. 1A and B, alloferon increases NK cell cytotoxicity to PC3 in a time and dose-dependent manner of the treatment of alloferon, when it compared with NK cells that are cultured without alloferon. However, we could not find stimulatory effect of alloferon on NK cells at less than 2 μg/ml (data not shown). The effect of alloferon on the up-regulation of NK cell cytotoxicity

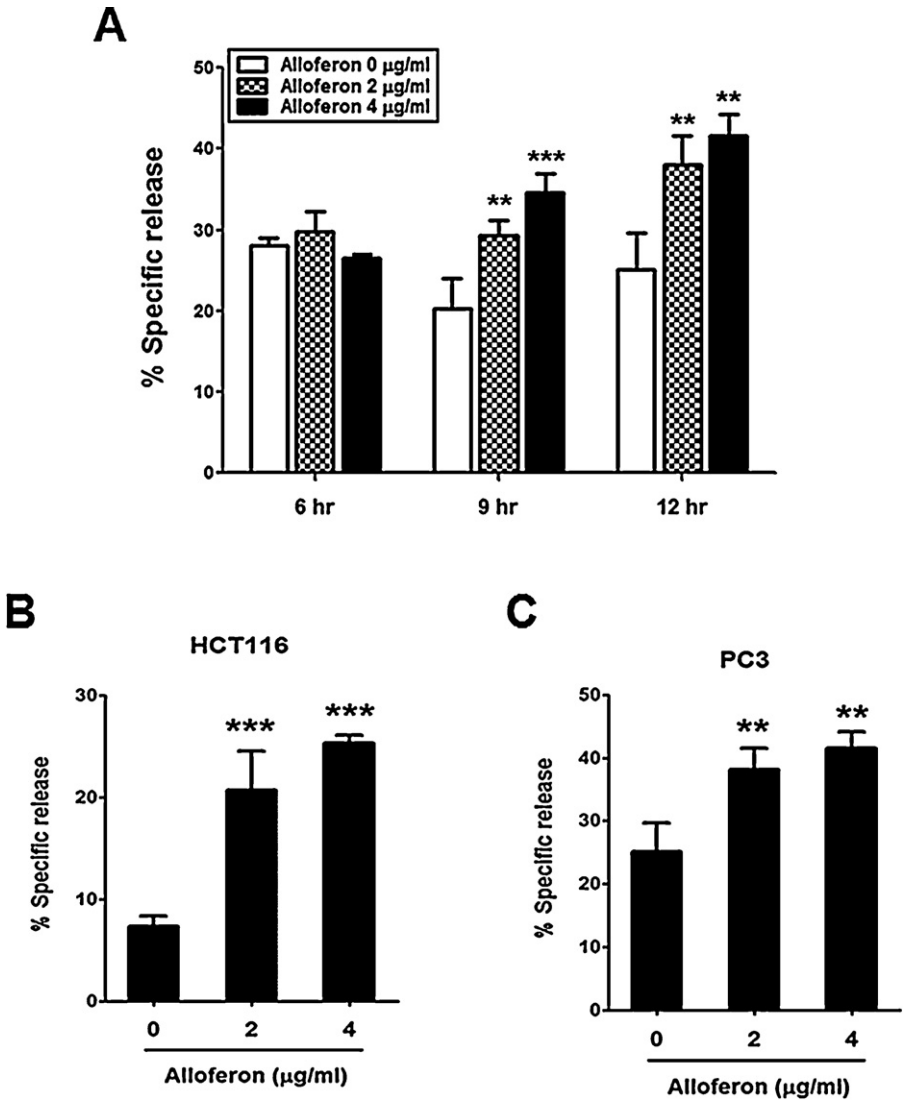

Fig. 1. Increase of NK cell-mediated cytotoxicity to cancer cells by alloferon. (A) Freshly isolated human NK cells were incubated with various doses of alloferon (2 and 4 μg) for 6, 9, 12 h and co-cultured with ^{51}Cr-labeled PC3 for 4 h. Culture supernatants were harvested and radioactivity was measured with an automated gamma counter. (B and C) Freshly isolated human NK cells were incubated with various doses of alloferon (2 and 4 μg) for 12 h and co-cultured with ^{51}Cr-labeled (B) HCT116 and (C) PC3 for 4 h. Culture supernatants were harvested and its radio activity was measured with an automated gamma counter. Each performed in triplicate, and *P*-Value is <0.01 (**) or 0.001 (***), when it compared with control.

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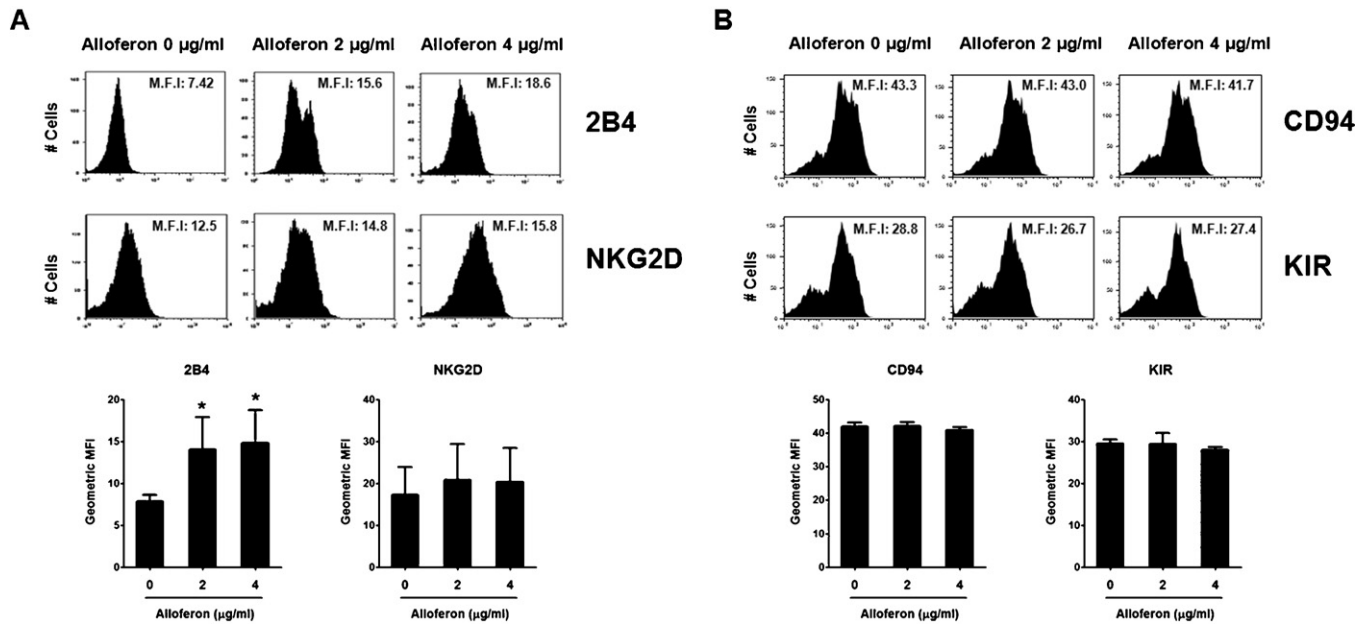

Fig. 2. Changes of NK-activating and inhibitory receptors expression by alloferon. Human NK cells were incubated with 2 and 4 μg of alloferon for 12 h. Cells were stained with FITC-conjugated anti-2B4 (CD244) Ab, APC-conjugated anti-NKG2D Ab (Becton Dickinson, Mountain View, CA, USA) and FITC-conjugated anti-CD94 and KIR Ab (R&D systems, Minneapolis, MN, USA) as described in *Materials and methods*. (A) The expression of activating receptor, 2B4 and NKG2D. (B) The expression of inhibitory receptor, CD94 and KIR. The Results are shown as the geometric mean fluorescence intensity (MFI) of the stained populations. *P*-Value is <0.05 (*), when it compared with control.

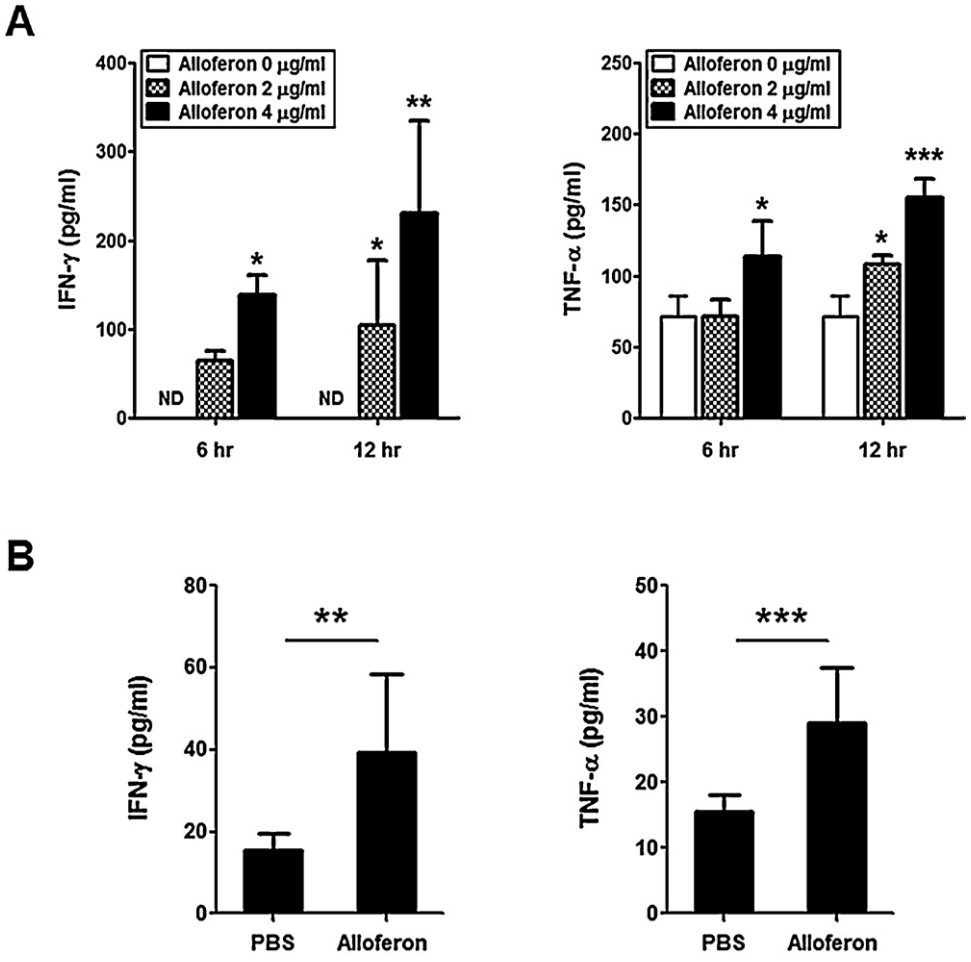

Fig. 3. Cytokine production of NK cells by the treatment of alloferon. (A) After NK cells were cultured in the absence or presence of alloferon (2 and 4 μg/ml) 6 and 12 h, and then mixed with target cells at effector-to-target (E:T) ratio of 30:1. Cells were further incubated for 4 h, the supernatants were harvested. IFN-γ and TNF-α were assayed using IFN-γ and TNF-α ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. (B) Blood was collected from the intra-orbital plexus of tumor-bearing nude mice with a heparinized capillary tube. And then plasma was separated and used to measure *in vivo* production of IFN-γ and TNF-α upon the administration of alloferon as described in *Materials and methods*. *P*-Value is <0.05 (*) or 0.01 (**) or 0.001 (***) when compared with control.

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was also confirmed by using human colon cancer cell line, HCT116 (Fig. 1C).

Alloferon induces expression of NK cell-activating receptors, 2B4

It is generally known that cytotoxic activity of NK cells are tightly regulated by the balance between the expression of activating and inhibitory receptors on its surfaces (Moretta et al. 2000; Tomasello et al. 2000). Therefore, we investigated whether alloferon could modulate the expression of activating receptors, such as 2B4 and NKG2D, and inhibitory receptors, KIR and CD94. As a result, the expression of 2B4 is remarkably increased by alloferon, while NKG2D was slightly increased (Fig. 2A). In addition, there were no significant changes on the expression of inhibitory receptors, CD94 and KIR (Fig. 2B).

Alloferon enhances the production of IFN-γ and TNF-α from NK cells

2B4 is involved not only in the up-regulation of NK cell-mediated cytotoxicity, but also in the induction of IFN-γ secretion (Nakajima et al. 1999; Chuang et al. 2001). In addition, the effector function of NK cells against cancer cells is mediated by the production of IFN-γ and TNF-α. Based on the result regarding the increase of NK cytotoxicity and the up-regulation of 2B4 expression, we examined whether the production of IFN-γ and TNF-α from NK cells is also increased by the treatment of alloferon. As shown in Fig. 3A, the production of IFN-γ and TNF-α from alloferon-treated NK cells was increased in a time- and dose-dependent manner, when it compared with NK cells that are cultured without alloferon. Next, we investigated *in vivo* effects of alloferon on the production of both cytokines in tumour-bearing mice. We found notable increase of IFN-γ and TNF-α production by the administration of alloferon (Fig. 3B).

Alloferon up-regulates granule exocytosis from NK cells

It is well-known that granule exocytosis is important in the elimination of virus-infected cells and cancer cells by NK cells (Shi et al. 1992; Shresta et al. 1995). In addition, it is known that the interaction between 2B4 and its ligand, CD48 increases granule exocytosis from NK cells (Garni-Wagner et al. 1993; Nakajima et al. 1999). When NK cells were activated by virus-infected cells or cancer cells, lytic granules move to the site of interaction with the target cell and merge with the plasma membrane. In the meantime, the lytic contents of granules including granzymes and perforin are released, and the CD107a is temporarily appeared on the cell surface. Therefore, the increased CD107a expression reflects the

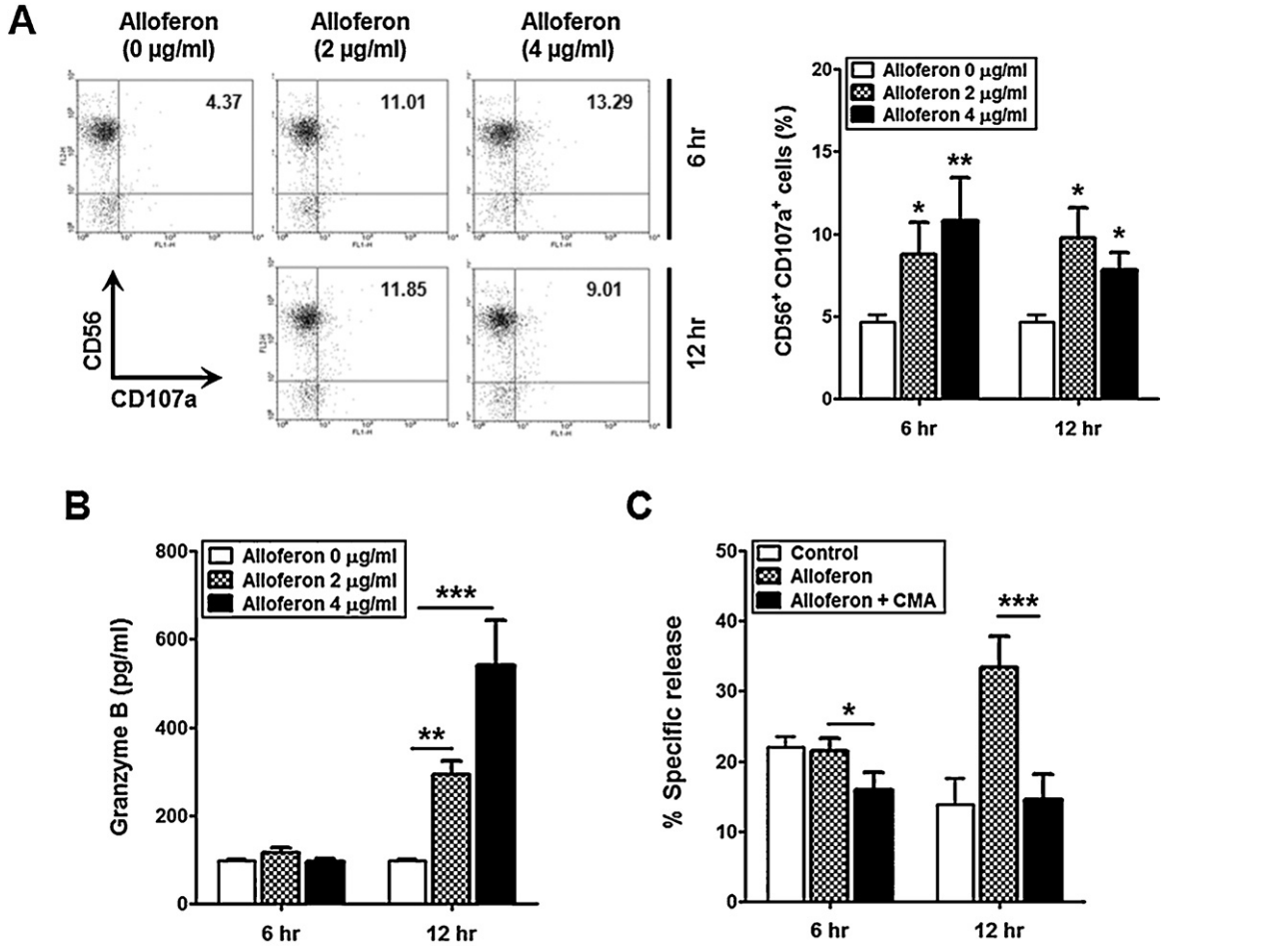

Fig. 4. The involvement of granule exocytosis on the increased cellular cytotoxicity of alloferon-treated NK cells. (A) NK cells were incubated with various doses of alloferon (2 and 4 μg) for 6 and 12 h and co-cultured with PC3. Transient expression of CD107a on NK cells was examined by flow cytometry as described in *Materials and methods*. (B) Granzyme B production from NK cells against cancer cells was measured by ELISA. *P*-value is <0.01 (**) or 0.001 (***) when compared with control. Results are representative of three independent experiments, and each performed in triplicates. (C) Alloferon (4 μg/ml) treated NK cells were cultured with ^{51}Cr-labeled PC3 in the presence or absence of 10 nM of concanamycin A for 4 h. The cytolytic activity of NK cell exposed to alloferon was measures as % specific release of ^{51}Cr from target cells. Results are representative of three independent experiments and shown as percent specific release. Each performed in triplicate. *P*-Value is 0.05 (*) or 0.001 (***) when compared with control.

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exocytosis of granzymes and perforin (Alter et al. 2004). As we expected, CD107a expression was increased by the treatment with alloferon (Fig. 4A). In addition, we found that granzyme B secretion was definitely increased from alloferon-treated NK cells in a dose- and a time-dependent manner, when it compared with NK cells that are cultured without alloferon. To investigate whether the enhanced NK cell cytotoxicity by alloferon is mediated by granule exocytosis, we performed inhibition assay by using of concanamycin A (CMA), which chelates with calcium and blocks polymerization of perforin. When alloferon-treated NK cells were incubated with ^{51}Cr-labeled cancer cells in the presence of CMA, its increased killing activity by alloferon was completely blocked (Fig. 4C).

Alloferon treatment suppresses in vivo tumor growth in immunodeficient mice

To evaluate the effect of alloferon on the growth of cancer cells *in vivo*, 5×10^5 of cancer cells were subcutaneously injected on the left flank of nude mice. From the day of tumor inoculation, 50 μg of alloferon per each mouse was intraperitoneally injected every day for 4 weeks. As shown in Fig. 5A, tumor growth in PBS-injected nude mice was extensively increased, but it was completely suppressed by the injection of alloferon (Fig. 5A). In nude mice, NK cells normally exist, but T cells are deficient. Therefore, it seems that the suppression of *in vivo* tumor growth by alloferon is mediated by the activation of NK cells. In addition, we did the same experiment on NOD-SCID IL2Rγ(−/−) mice, which have defect on immune cells including NK cells. *In vivo* tumor growth in alloferon-injected NOD-SCID IL2Rγ(−/−) mice was not the same as in PBS-injected NOD-SCID IL2Rγ(−/−) mice. Nevertheless, we observed tumor growth in alloferon-injected NOD-SCID IL2Rγ(−/−) mice at 13 days after injection of cancer cells (Fig. 5B).

Discussion

NK cells are important cellular components for eliminating virus-infected or tumor cells and for regulating downstream adaptive immune responses. For this reason, NK cell-based immunotherapy has been extensively studied. The most common NK cell applied to immunotherapy of cancer is the lymphokine-activated killer (LAK) cell (Rosenberg et al. 1987). It is known that LAK cell is induced by the treatment of several kinds of cytokines and shows an increased cellular cytotoxicity against cancer (Rosenberg et al. 1987; Hsu et al. 1996). IL-2, -12, -15, -18 and -21 are commonly used cytokines to increase cellular cytotoxicity of NK cells (Fehniger et al. 1999; Lauwerys et al. 2002; Parrish-Novak et al. 2001). In fact, these cytokines effectively increase the antitumor effect of NK cells *in vitro*, but there are some restrictions of direct application in cancer patients, for example, dosages and combinations of cytokines. To overcome the restriction of *in vivo* application, there have been extensive studies on the adaptive transfer of activated NK cells in cancer patients, after activation and expansion of autologous NK cells by the treatment with cytokines described above *in vitro* (Igarashi et al. 2004; Miller et al. 2005). It is reported that adaptive transfer of NK cells show efficient therapeutic effect on some tumor cells, but its application is still limited. So people think the natural substances, such as polyphenols, vitamins and herbal extracts, could be effectively used for cancer therapy because they act as a direct cytotoxicity against cancer and have an effect on the enhancement of anticancer immune response. In fact, some natural substances, such as vitamin C and resveratrol, show the potent effect on the induction of the anticancer effect of NK cells (Siegel and Morton 1983; Lu and Chen 2010). However, the major problem is that they have inconsistent *in vivo* and *in vitro* effects. For this reason, alloferon can be a useful anti-cancer agent as it has consistent *in vivo* and *in vitro* anti-cancer effects.

We have recently reported that alloferon has significant anti-inflammatory effect not only on UVB-induced inflammation in the normal human keratinocyte cell line, HaCaT, but also on mouse skin. We also reported that alloferon has no toxicity to normal cells and does not affect the growth of normal cells (Kim et al. 2012). However, it suppresses the proliferation of virus-infected cells and shows an cytotoxic effect on tumor cells (Lee et al. 2011; Chernysh et al. 2012). The anti-tumor effect of alloferon was firstly reported by using of K562, one of the most sensitive cell line to NK cytotoxicity, or murine leukemia cell line, p388 (Chernysh et al. 2002, 2012). However, the anti-tumor effect of alloferon on NK-resistant human cancer cells is not examined yet. It is known that prostate cancer cell line, PC3 is highly resistant to NK cell cytotoxicity. Because MICA/B, ligand for NK activating receptor NKG2D, is not expressed, but MHC class I, ligand for NK inhibitory receptor KIR (killer Ig-like receptor), is highly expressed on PC3 (Romijn 1985; Raja Gabaglia et al. 2007). It is also known that HCT116 escapes from NK-cell mediated immune surveillance due to the deficiency of fucosylation, which comprises the transfer of a fucose residue to oligosaccharides and proteins (Moriwaki and Miyoshi 2010). Therefore, we could not find remarkable increase of the specific killing of PC3 and HCT116 by alloferon-treated NK cells at E:T ratio 2.5:1, 5:1 and 10:1 (Supplementary Fig. 2). However, we found the increase of the specific killing at 30:1 and it is also remarkably increased by the treatment of alloferon (Fig. 1B). It suggests that alloferon might be useful as an immune-modulating reagent to increase NK cell cytotoxicity to NK-resistant human cancer cells.

The balance between the expression of activating and inhibitory receptor on NK cells is a critical factor to kill the cancer cells. Therefore, the increased expression of activating receptor on NK cells implies the up-regulation of NK cell cytotoxicity against cancer

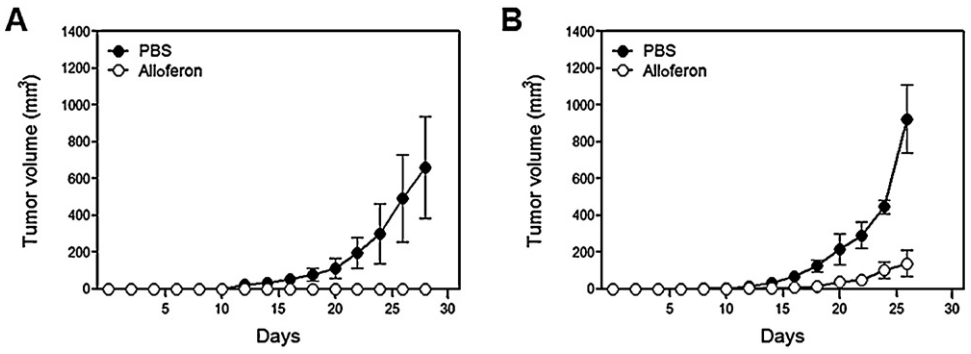

Fig. 5. Effect of alloferon on tumor growth in nude mice and NOD/SCID/IL-2Rγ(−/−) mice. HCT116 cells were inoculated subcutaneously into the left flanks of (A) 6 weeks old female nude mice and (B) 9 weeks old male NOD/SCID/IL-2Rγ(−/−) mice. From the day of tumor inoculation, PBS or alloferon (50 μg/ea) was intraperitoneally injected every day. Tumor sizes were measured every other day. Six mice were used for each test groups and tumor volume is the mean volume ± SD.

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cells. Among the various kinds of NK activating receptors, 2B4 is known that it increases NK cell cytotoxicity through the induction of IFN-γ production and granule exocytosis after ligation with its ligand, CD48 (Garni-Wagner et al. 1993; Nakajima et al. 1999). As shown in Fig. 2, the expression of 2B4 was increased on alloferon-treated NK cells, but there was no remarkable changes on the expression of inhibitory receptors. We also investigated whether alloferon could increase the susceptibility of HCT116 and PC3 to NK cells *via* the changes on the expression of MHC class I, ligand for inhibitory receptor, KIR, and CD48, ligand for activating receptor, 2B4 on the both of cancer cell lines. However, we could not find any changes on the expression of MHC I and CD48 (data not shown). In addition, we have shown that the IFN-γ production and granule exocytosis from alloferon-treated NK cells were increased, when they were cultured with cancer cells (Figs. 2 and 4). Moreover, the increased production of IFN-γ and TNF-α was also observed in the plasma from tumor-bearing athymic nude mice (Fig. 2). Therefore, the anti-tumor activity of alloferon is mediated by the up-regulation of 2B4 expression followed by the increase of IFN-γ and TNF-α production and granule exocytosis.

The general characteristic of nude mice is the deficiency of T cells, but NK cells are normally existed (Povlsen et al. 1973). When alloferon was administrated to tumor inoculated nude mice, *in vivo* tumor growth in was completely inhibited (Fig. 5A). It is generally known that NOD-SCID IL2Rγ(−/−) mice are deficient of T cells, B cells, and especially NK cells (Ito et al. 2002; Shultz et al. 2005). When we considered the defect of NK cells in NOD-SCID IL2Rγ(−/−) mice, we expected that there was no difference of tumor growth in PBS-injected NOD-SCID IL2Rγ(−/−) mice and alloferon-injected NOD-SCID IL2Rγ(−/−) mice. However, we found the delay on tumor growth in alloferon-injected NOD-SCID IL2Rγ(−/−) mice. Since Chenysh et al. reported that alloferon monotherapy demonstrates moderate tumoristatic and tumoricidal activities comparable with low dose chemotherapy (Chernysh et al. 2002), delayed tumor growth in alloferon-injected NOD-SCID IL2Rγ(−/−) mice might be caused by direct cytotoxicity of alloferon on cancer cells. Therefore, it seems that the delay of tumor growth in alloferon-injected NOD-SCID IL2Rγ(−/−) mice at 13 day after tumor inoculation is due to the defect of NK cells. Taken together, alloferon could be used as an immune-modulating agent for cancer therapy *via* the up-regulation of NK cells-mediated cytotoxicity.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2012.12.002.

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