



RESEARCH ARTICLE

# Sesamolin promotes cytolysis and migration activity of natural killer cells via dendritic cells

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**Abstract** The defense mechanism of the immune system is based on the interaction of many kinds of leukocytes. Among them, dendritic cells (DCs) control most immune responses. In our previous study, sesamolin was shown to create an optimal environment for natural killer (NK) cells to kill cancer cells. Here we attempted to demonstrate how sesamolin influences DCs to promote the killing and migration activity of NK cells. We co-cultured DCs and NK cells and analyzed the communication between them. NK cells co-cultured with 5 µg/ml sesamolin-treated mature dendritic cells (mDCs) had better cytolytic activity than did NK cells or mDCs co-cultured NK cells. Moreover, the migration of NK cells toward mDCs was enhanced compared to immature dendritic cells (iDCs). The migration of NK cells stimulated by mDCs was stronger after sesamolin activation of the mDCs. Altogether, this study demonstrated that sesamolin activated NK cells by modulating the differentiation and activation of DCs.

**Keywords** Sesamolin · Natural killer cells · Dendritic cells · Interleukin-2 · Interleukin-15 · MIP-1α

## Introduction

Numerous reports have documented that dendritic cells (DCs) are the most important antigen-presenting cells (APCs) for T-cell activation. Peripheral resident DCs,

however, are the least efficient at performing immunological functions and, therefore, must be activated and differentiated further by signals in the microenvironmental to exert their full effects. Mature and activated DCs are distinguishable from immature DCs (iDCs) because they express high levels of MHC class II molecules and co-stimulatory molecules (B7-1, B7-2, CD40, and CD54) (Pulendran et al. 2008). Natural killer (NK) cells are included in the lymphoid lineage but can be distinguished from other lymphocytes by cell-surface phenotypes, such as CD19 (B lymphocyte marker) and CD3 (T lymphocyte marker). As typical markers, human NK cells express CD56 and CD16 and mouse NK cells express NK1.1 and DX5 (Bernardini et al. 2008).

Dendritic cells and natural killer cells play crucial roles in the innate, as well as the adaptive, immune response (Andoniou et al. 2005). In recent years, the functional interactions between DCs and NK cells have been widely examined and evidence of interactions between these two cells have been demonstrated by several studies (Fernandez et al. 1999). DCs stimulate the release of cytokines by NK cells (mainly interferon (IFN)-γ) and increase the cytolytic activity of NK cells. Most of the research on NK and DC interactions has been based on DCs derived from peripheral blood or bone marrow (BM) cells, which are generated after a number of days of culture with various combinations of cytokines. The subtype of ex vivo-generated DCs can change depending on the combination of cytokines. Therefore, NK and DC interactions have been insufficiently investigated so far (Gerosa et al. 2005; Della Chiesa et al. 2006; Perrot et al. 2010). These studies reported that both myeloid and plasmacytoid DCs were capable of activating NK cells in response to virus.

Sesame (*Sesamum indicum*, Linn, Pedaliaceae) is an important seed from which oil has been extracted since ancient times. The primary nutrients of sesame include fat,

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carbohydrates, and protein (McKevith 2005; Jeng and Hou 2005). Although it is a minor component, sesame also contains unique lignans, such as sesamolins (Lim et al. 2007).

In a previous study, we reported that cancer-cell lines can be classified into sensitive and non-sensitive cell lines according to their NK-cell cytotoxic activity (Kim and Lee 2015). Among those non-sensitive to NK-cell cytotoxic activity, Raji cells (Burkitt's lymphoma cells) became more susceptible to NK cells when treated with sesamolins. Moreover, the recognition of cancer cells by NK cells increased when they were treated with sesamolins (Lee and Lee 2018). Based on our preliminary results, we suggest that sesamolins has potential as a tumor therapeutic agent. However, the influence of sesamolins on how NK cells prevent and treat cancer in mixed conditions with other leukocytes has yet to be demonstrated. Therefore, this study was designed to demonstrate the influence of sesamolins-activated DCs on the bio-performance of NK cells.

## Materials and methods

### Reagents

Sesamolins was purchased from Nagara Science (Tokyo, Japan) and anti-mouse monoclonal antibody (mAb) specific for CCR5 was purchased from BioLegend (San Diego, ca., USA). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Generation of mouse DCs

DCs were generated from BM cells obtained from eight-week-old C57/BL6 mice (Dooyeol Biotech Lab, Republic of Korea). BM cells were cultured in 90 mm diameter cell culture dishes in DC medium (Dulbecco's Modified Eagle's Medium (DMEM), Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA), 100 U/ml penicillin/100 µg/ml streptomycin (Gibco BRL), 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN, USA), and 2 ng/ml interleukin (IL)-4 (R&D Systems). On culture day 8, loosely adherent cells were harvested and used as iDCs. DC maturation was induced by stimulation of the iDCs with 1 µg/ml lipopolysaccharide (LPS) for 24 h. We confirmed the characteristics of the immature and mature DCs by cell-surface phenotypes (CD11c, MHC class II (I-A[b]), CD80 and CD86) (Fig. 1a). The iDCs revealed the characteristics of general iDCs by positive staining with CD11c, MHC class II, CD80, and CD86. The expression of these markers was increased by stimulation with LPS.

### Preparation of naïve NK cells

NK cells were isolated from mice (C57/BL6) spleens. The spleen was flushed with phosphate-buffered saline, the aggregated tissues were removed by passage through a nylon filter (70 µm pore size), and NK cells were isolated from the splenocytes by immunomagnetic bead selection according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The mice were housed at the Chungbuk National University Animal Care Unit, maintained at 24 °C and fed a normal diet. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Chungbuk National University (Permit Number: CBNU-201704-BR-434-01, Korea).

### Cell viability

Cell viability was measured using the WST-8 based colorimetric assay (Dojindo, Japan). The cells were plated at  $5 \times 10^4$  cells/well in a 96-well plate (flat bottom) for 24 h. WST-8 reagent was added to the cells, followed by incubation at 37 °C and 5% CO<sub>2</sub> for 3 h. Cell viability was measured at 450 nm by a spectrophotometer (Bio-Rad 680, Bio-Rad, Hercules, ca., USA).

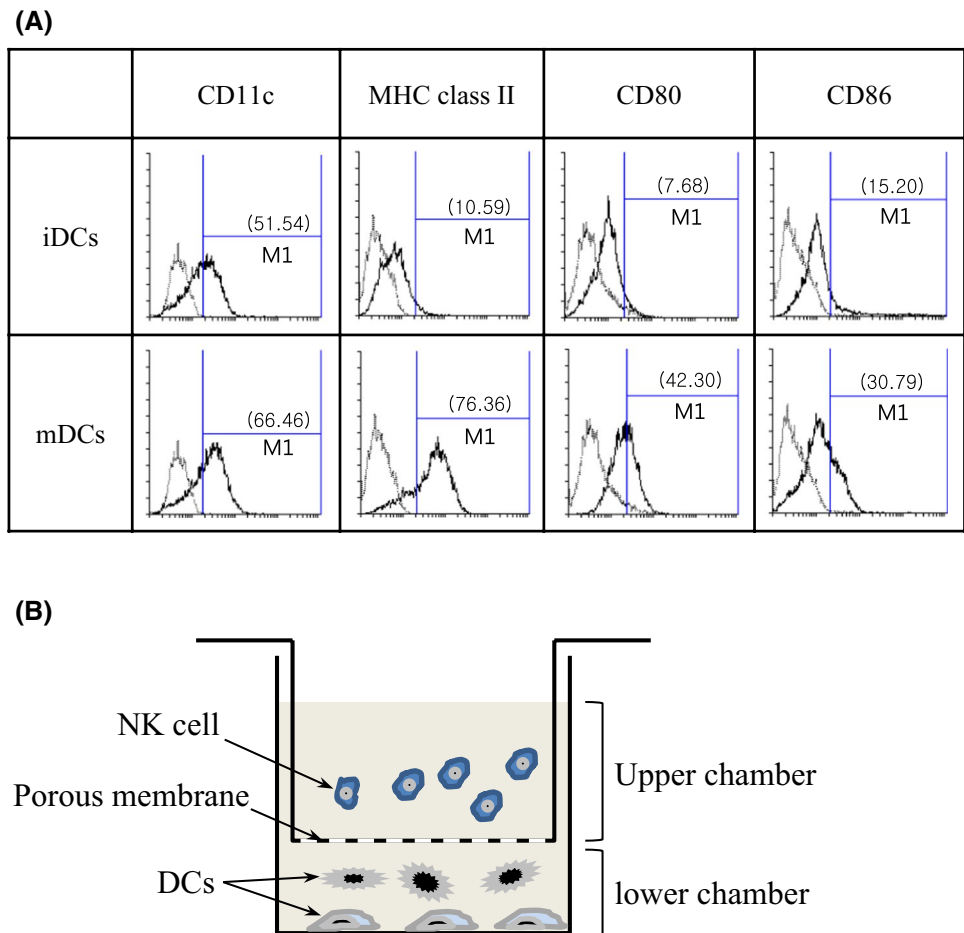
### Phenotypic analysis

Phenotypic analyses were performed as described by Kim and Lee (2015). Briefly, the cells were stained with fluorescence-labeled antibodies, which identified DC surface markers, and then were analyzed by a FACSCalibur (Becton Dickinson, ca., USA). The antibodies obtained from Becton Dickinson included FITC-CD11c, FITC-MHC class II (I-A[b]), PE-CD80, FITC-CD86, PE-CD49b, FITC-NKp46, and PE-NKG2D, along with isotype control antibodies.

### IL-2, IL-15, MIP-1α, MIP-1β, RANTES and IP-10 measurements by ELISA

Purified DCs were cultured in 24-well culture plates at  $1 \times 10^5$  cells per well in a final volume of 1 ml and stimulated with sesamolins, LPS, or LPS + sesamolins. The culture supernatants were harvested at designated time points and subjected to enzyme-linked immunosorbent assays (ELISA; R&D Systems). IL-2, IL-15, macrophage inflammatory proteins (MIP)-1α, MIP-1β, regulated on activation normal T-cell expressed and secreted (RANTES) protein, and IFN-γ-induced protein 10 (IP-10) in the conditioned supernatants obtained from the DC cell cultures were measured by ELISA according to the manufacturer's instructions.

**Fig. 1** Preparations of DCs and the trans-well chamber system. **a** Confirmation of DC phenotypes. Mouse BM cells were cultured in GM-CSF and IL-4 supplemented medium for 8 days and these cells were stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) for 24 h. Cells before (iDCs) and after (mDCs) LPS stimulation were used for flow cytometry analysis. The expression levels of CD11c, MHC class II, CD80, and CD86 are represented by the percentage of cells in the M1 gate. The experiment was repeated three times and similar results were obtained each time. **b** Illustration of the co-culture and chemotaxis assay system using a trans-well chamber. This system was made of two chambers separated by a trans-well chamber. The DCs were placed in the lower compartment of the well chamber and the NK cells were placed in the upper compartment of the well chamber



### NK cell and DC co-culture system

NK cells were co-cultured with DCs in a transwell chamber (0.4  $\mu\text{m}$  pore size, Millipore, Billerica, MA, USA) system in RPMI medium (Gibco BRL) supplemented with 10% FBS and penicillin (100 U/ml)/streptomycin (100  $\mu\text{g}/\text{ml}$ ) (Fig. 1b). The NK cells were placed in the upper compartment (well) and DCs (iDCs and mDCs) were in the lower compartment of the transwell plate, as described previously (Lee et al. 2015). Briefly, the cell density ratio of NK cells to DCs was 1:3. DCs were plated at a density of  $9 \times 10^5$  cells/300  $\mu\text{L}$  in a 24-well plate. Culture inserts containing  $3 \times 10^5$  cells/300  $\mu\text{L}$  NK cells were placed in three of the 24 wells (one insert per well). A 0.4- $\mu\text{m}$  pore size membrane was placed in the well to permit the transmission of only soluble elements and prevent direct interaction between the NK cells and the DCs.

### NK cell migration assay

The NK cells were harvested, washed, and suspended at  $1 \times 10^6$  cells/ml in chemotaxis media (CM: RPMI 1640, 0.5% BSA, 25 mM HEPES pH 7.4). NK cell suspension

(300  $\mu\text{L}$ ) was added to the upper compartment of a transwell chamber (3  $\mu\text{m}$  pore size, Millipore) and 300  $\mu\text{L}$  of chemotaxis media in the presence or absence of chemoattractant (DC culture supernatant or DCs (sesamolin-stimulated DCs ( $3 \times 10^6$  cells/ml)) was added to the lower compartment. For the blocking assay, CCR5 mAb (CCR5 mAb) (10  $\mu\text{g}/\text{ml}$ ) was mixed with the NK cells for 15 min before they were added to the upper compartment. The transwell plate was covered and incubated for 8 h at 37  $^\circ\text{C}$  and 5%  $\text{CO}_2$ . We removed the transwell chamber and the migrated cells in the lower compartment were harvested for flow cytometry analysis with FITC-conjugated NK1.1 mAb. The ratio of migrated NK cells was calculated as follows: ratio of migrated NK cells = [number of NK cells in lower compartment of experiment group]/[number of spontaneously migrated NK cells in lower chamber compartment]. The NK cells, which migrated toward the CM, were considered spontaneously migrated NK cells.

### NK cell cytotoxicity assay

We confirmed the cytolytic capacity of the NK cells by lactate dehydrogenase (LDH) assays (Takara, Japan).

Target cells ( $6 \times 10^4$ ) and effector cells ( $1.8 \times 10^5$ ) were plated into 96-cell plates and incubated for 4 h at 37 °C and 5% CO<sub>2</sub>. The assay plate was centrifuged ( $250 \times g$  for 10 min) and the supernatant was transferred to a 96-well plate and mixed with 100  $\mu$ l of substrate. After 30 min incubation at 24 °C, the absorbance was measured at 490 nm. The percentage of cytotoxicity was calculated as follows: cytotoxicity (%) = [(A – low control)/(high control – low control)]  $\times$  100, where A = [effector–target cell mix] – [effector–cell control]. Low control measured the spontaneous LDH release, which was the level of LDH activity released from the target cells, and high control measured the maximum level of releasable LDH from the target cells, which was the maximum level of LDH release induced by the addition of Triton X-100. The effector-cell control measured the spontaneous LDH release, which was the level of LDH activity released from the effector cells. The low and effector-cell control groups were treated with DMSO, the solvent used for sesamol.

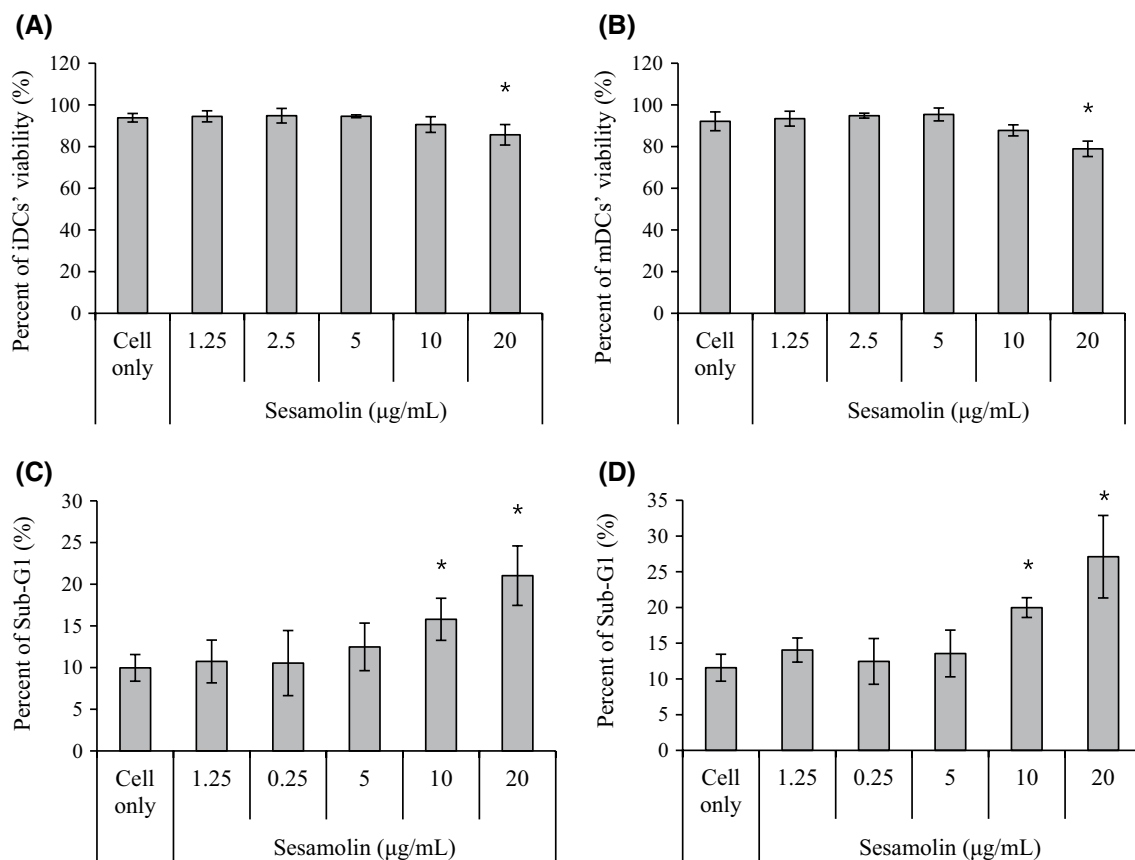
## Statistics

The results are expressed as means  $\pm$  SDs (standard deviations). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Statistical significance of the between-group comparisons is indicated by \* or # in the figures.

## Results

### Cell viability of sesamol-treated DCs

Initial experiments were conducted to determine the cell viability of sesamol-treated iDCs and mDCs over a broad concentration range of sesamol (1.25–20  $\mu$ g/ml) using the WST-8 assay. As shown in Fig. 2, sesamol affected the cell viability of the iDCs (A) and mDCs (B). The viability of the iDCs and mDCs was decreased by 15 and 22%, respectively, by treatment with 20  $\mu$ g/ml sesamol compared to untreated iDCs. Propidium iodide (PI) staining was performed under



**Fig. 2** Effects of sesamol on DC viability. iDCs (a) and mDCs (b) were treated with sesamol for 24 h and cell viability was measured using the WST-8 assay. The DNA fragmentation of iDCs (c) and mDCs (d) was measured by PI staining analysis. The results are presented as the percentage of sub-G<sub>1</sub> cells. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean  $\pm$  SD. \* $p$  < 0.05 compared to the cell-only control

the same conditions. As shown in Fig. 2c and d, a high percentage of DNA fragmented cells was observed at high concentrations (10 and 20  $\mu\text{g/ml}$ ) of sesamol. Both the iDCs (Fig. 2c) and mDCs (Fig. 2d) cells showed a low population of sub-G1 cells in the concentration range of 1.25–5  $\mu\text{g/ml}$ . These results demonstrated that treatment with sesamol did not have cytotoxic or pro-apoptotic effects on iDCs and mDCs at concentrations under 5  $\mu\text{g/ml}$ .

### Influence of sesamol on NK cell activity via DCs

Generally, immature and mature DCs influence the killing activity of NK cells via membrane-bound and secreted proteins, such as MHC class I and cytokines (Fernandez et al. 1999). Therefore, we analyzed the influence of DCs on the killing activity of NK cells using transwell chambers (0.4  $\mu\text{m}$ ). iDCs or mDCs were incubated with various concentrations of sesamol for 24 h, then co-cultured with NK cells in a microenvironment that was constructed using a transwell chamber in a 24-well plate (Table 1). NK cells, which were co-cultured with DCs, were used to measure the killing activity of Yac-1. As shown in Fig. 3a, control NK cells, which were not co-cultured with DCs, exhibited basal cytolytic activity (30–35%) against Yac-1. NK cells that were cultured in a transwell with iDCs exhibited cytolytic activity toward Yac-1 like that of the control NK cells. Moreover, NK cells that were co-cultured with sesamol-treated iDCs also exhibited cytolytic activity like that of the control NK cells. However, NK cells that were cultured in a transwell with mDCs exhibited much higher cytolytic activity than did the control NK cells. Moreover, NK cells that were co-cultured with 5  $\mu\text{g/ml}$  sesamol-treated mDCs (mDCs-S5) exhibited higher cytolytic activity than did non-co-cultured NK cells or NK cells co-cultured with mDCs. Although not significant, the NK cells co-cultured with mDC-S2.5 (mDCs, which was stimulated with 2.5  $\mu\text{g/ml}$  sesamol) also exhibited higher cytolytic activity than did the non-co-cultured NK cells. These results demonstrate that sesamol affected the cytolytic activity of NK cells via mDCs

### Change in phenotypic maturation of NK cells by co-culture with mDCs

To determine whether the effect of sesamol-treated DCs on the cytolytic activity of NK cells was related to the modulation of cell-surface molecules on the NK cells, we examined the expression levels of NK cell markers CD49b, Nkp46, and NKG2D via flow cytometry. As shown in Fig. 3b, the general NK cell marker, CD49b, was expressed similarly in control NK cells, mDC co-cultured NK cells, and sesamol (5  $\mu\text{g/ml}$ )-stimulated mDC (mDC-S5) co-cultured NK cells. However, the activation markers of NK cells, Nkp46 and

NKG2D, were elevated by co-culture with sesamol-treated mDCs. Nkp46 positive NK cells were increased almost 3.8-fold by co-culture with sesamol-treated mDC compared to NK cells that were co-cultured with mDCs. NKG2D positive NK cells were increased almost 1.6-fold by co-culture with sesamol-treated mDCs (mDC-S5) compared to NK cells that were co-cultured with mDCs. These results indicated that NK-cell activation markers were slightly elevated by co-culturing with mDCs. Furthermore, the induction of NK cell activation markers by sesamol-treated mDCs was higher than that in unstimulated mDCs.

### Sesamol induces the production of NK cell activation cytokines from DCs

We next sought to further characterize the NK cell activation effect of sesamol-treated iDCs (Fig. 4a, b) and mDCs (Fig. 4c, d) by subjecting the conditioned supernatants obtained from iDCs and mDCs cultures to ELISA cytokine assays for IL-2 and IL-15, which are well known for their ability to promote the cytolytic activity of NK cells. As shown in Fig. 4a, the IL-2 production of iDCs was significantly increased by DCs' maturation with LPS treatment. Although the amount of IL-2 produced was not more than that of the mDCs, sesamol-treated iDCs produced more IL-2 than did untreated iDCs. Like IL-2, mDCs produced more IL-15 than did iDCs (Fig. 4b). Moreover, iDCs produced more IL-15 after sesamol stimulation

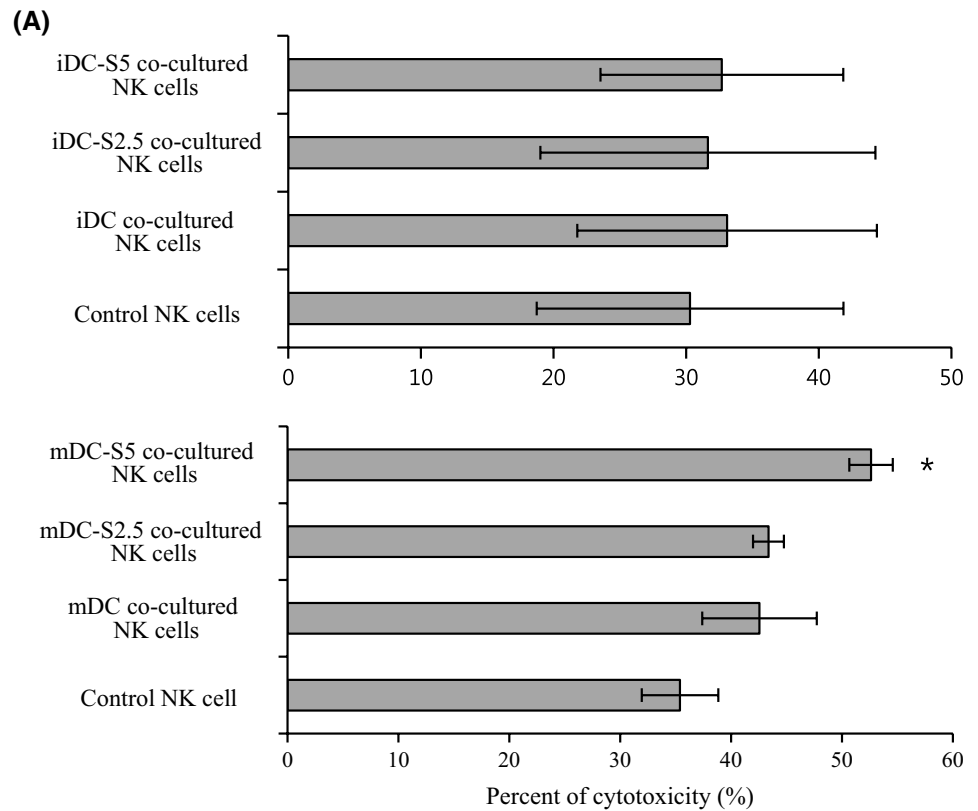
As shown in Fig. 4c, the IL-2 production by mDCs was increased by stimulation with sesamol in a dose-dependent manner. However, IL-15 production by the mDCs was not increased, even after stimulation with the highest concentration of sesamol (Fig. 4d).

### Change in phenotypic maturation of DCs by sesamol

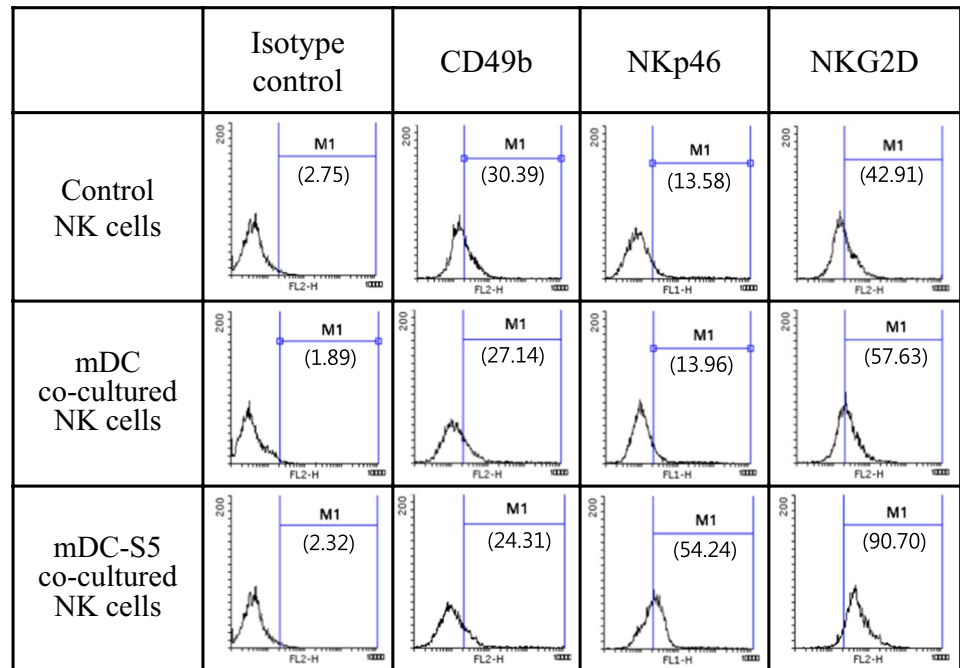
To determine whether the modulation of the cytolytic activity of NK cells by sesamol-treated DCs was related to the degree of DC maturation, flow cytometry was used to examine the expression levels of the maturation markers of the DC, such as CD11c, MHC class II (I-A[b]), CD80 and CD86. As shown in Table 2 and 3% of iDCs basally expressed CD11c and this was increased almost twofold by 5  $\mu\text{g/ml}$  sesamol (S5) stimulation. Moreover, when iDCs were treated with 5  $\mu\text{g/ml}$  sesamol (iDC+S5), there was a greater increase in MHC class II (5.8-fold), CD80 (3.7-fold), and CD86 (2.8-fold) positive cells compared to untreated iDCs.

To demonstrate the sesamol influence on mDCs, iDCs were stimulated with 1  $\mu\text{g/ml}$  of LPS for 24 h and then stimulated mDCs with sesamol for 24 h (Table 3). The expression of CD11c on mDCs was increased almost 1.5-fold by 5  $\mu\text{g/ml}$  sesamol (S5) stimulation. The expression

**Fig. 3** Sesamolins effect on the cytolytic activity of NK cells via DCs. **a** Effect of sesamolins on NK cell cytotoxicity mediated by DCs. NK cells were prepared by incubating with DCs or sesamolins-treated DCs for 24 h and then the cytolytic activity of the NK cells against Yac-1 was measured by LDH assay. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean  $\pm$  SD. \* $p < 0.05$  compared to the NK cell control. **b** Analysis of NK cell phenotypes. The expression of CD49b, NKp46, and NKG2D on NK cells was measured on control NK cells, mDCs co-cultured NK cells, and sesamolins-treated mDCs co-cultured NK cells. The expression levels are represented by the percentage of cells in the M1 gate. All the experiments were repeated three times and representative data are presented



**(B)**



of antigen-presenting related molecules on the surface of the mDCs was also increased by treatment with sesamolins. Specifically, the expressions of MHC class II and CD80 were increased 1.2-fold and 1.9-fold, respectively, by treatment with 5  $\mu$ g/ml sesamolins (mDCs + S5).

**Sesamolins increases NK cells' migration toward DCs**

To further demonstrate the influence of sesamolins on NK cells via DCs, NK cell migration through the transwell chambers (3  $\mu$ m) was examined. For these experiments,

**Table 1** Design of co-culture systems to demonstrate the effect of DCs on NK cell killing activity

Group	Upper well/lower well
1	NK cells/media
2	NK cells/iDC
3	NK cells/2.5 µg/ml sesamol-stimulated iDCs (iDC-S2.5)
4	NK cells/5 µg/ml sesamol-stimulated iDCs (iDC-S5)
5	NK cells/mDC
6	NK cells/2.5 µg/ml sesamol-stimulated mDCs (mDC-S2.5)
7	NK cells/5 µg/ml sesamol-stimulated mDCs (mDC-S5)

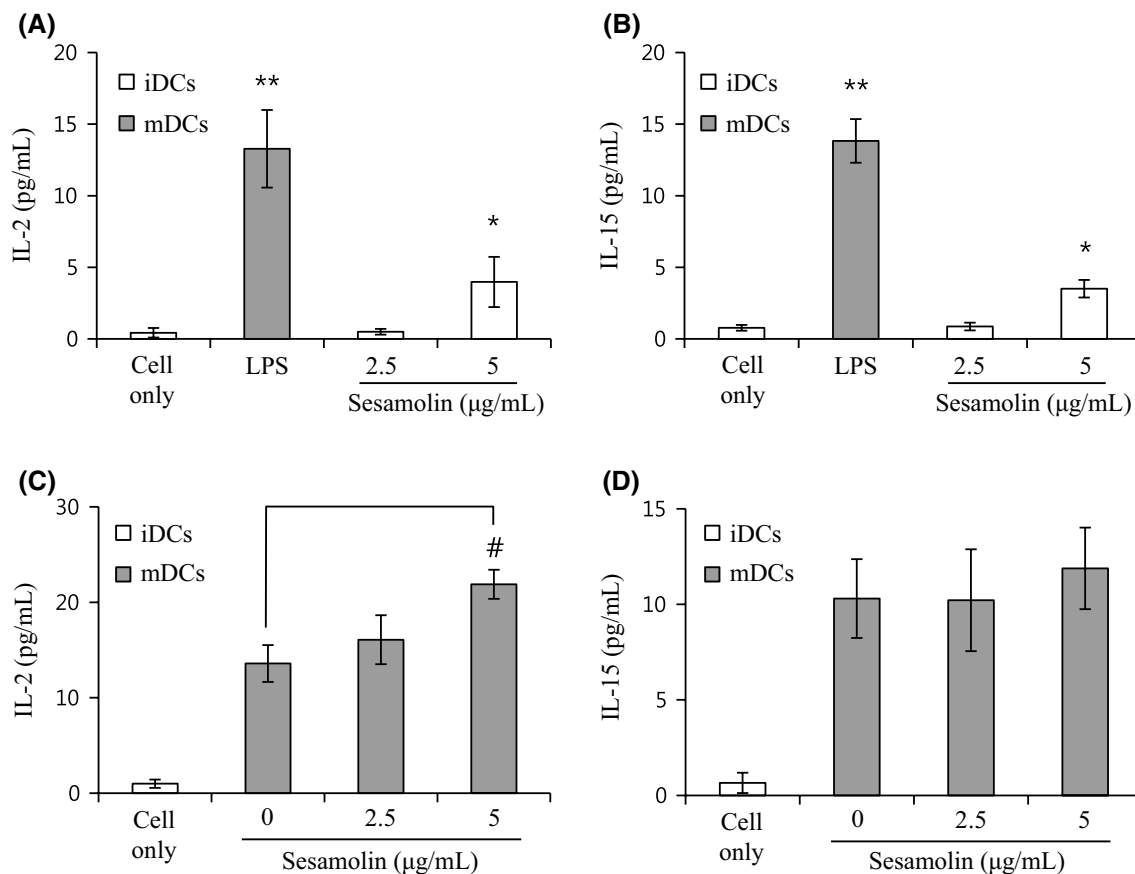
non-adherent and loosely adherent DCs and supernatant were used as iDCs and iDC supernatant. The iDCs were stimulated with LPS (1 µg/ml) for 24 h and used as the mDCs. To measure NK-cell migration, we inserted NK cells in the upper compartment of a well chamber ('Upper well' in the figures). DC supernatants, DCs (iDCs and mDCs), and sesamol-stimulated DCs were positioned in the lower compartment of the well chamber ('Lower well' in the figures).

Among the cells that migrated toward the lower wells, the NK1.1 positive cells were assumed to be NK cells.

When the iDC and mDC supernatants ('iDC sup' and 'mDC sup' in the figure) were placed in the lower well chambers, the mDC sup induced more NK-cell migration than did the iDC sup (Fig. 5a). Interestingly, when the cells (iDCs or mDCs) were placed in the lower well chambers, NK cells showed more migration than when supernatant was placed in the lower well chambers. Moreover, NK cells showed more migration toward mDCs than toward iDCs.

Next, the NK cell migration effect on sesamol-treated mDCs was characterized. To measure NK cell migration, NK cells were inserted in the upper well and sesamol-stimulated mDCs were inserted in the lower well. As shown in Fig. 5b, the chemotactic activity of the NK cells toward mDC was stronger after sesamol treatment. The migration activity of the NK cells toward mDCs was increased depending on the treatment concentration of sesamol.

We also observed changes in the migration activity of NK cells after the direct treatment with sesamol. To measure NK cell migration, we inserted NK cells (NK) or



**Fig. 4** Effect of sesamol on IL-2 and IL-15 production of DCs. The conditioned supernatants obtained from iDCs and mDCs cultures were used for ELISA assays for IL-2 and IL-15. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01 compared to the supernatant of cells only. # $p$  < 0.05 compared to the supernatant of sesamol-untreated mDCs

**Table 2** Expression of DC cell markers of iDCs after treatment with sesamolins

Markers	Cells		
	Percent M1-gated		
	iDCs	iDCs + S 2.5	iDCs + S 5
CD11c	51.54	92.45	99.91
MHC class II	10.59	12.03	61.47
CD80	7.68	32.89	28.79
CD86	15.20	14.29	43.44

All experiments were repeated three times and representative data are presented

**Table 3** Expression of DC cell markers of mDCs after treatment with sesamolins

CellsMarkers	Percent M1-gated		
	mDCs		
	mDCs	mDCs + S 2.5	mDCs + S 5
CD11c	66.46	66.26	98.66
MHC class II	76.36	78.68	95.80
CD80	42.30	66.47	83.78
CD86	30.79	22.22	37.21

All experiments were repeated three times and representative data are presented

sesamolins-treated NK cells (NK + S2.5) in the upper well. mDCs or sesamolins-treated mDCs were inserted in the lower well. As shown in Fig. 5c, the migration activity of NK cells was not changed by direct treatment with sesamolins.

### Sesamolins increases the production of chemokines from DCs

Many of the cytokines secreted from DCs are chemokines, which are known to increase leukocyte migration. Therefore, we sought to characterize the mechanism of sesamolins by subjecting the conditioned supernatants obtained from DC cultures to chemokine ELISAs for MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, and IP-10, which are representative chemokines that promote the migration of NK cells. As shown in Fig. 5d, mDCs produced almost 11-fold more MIP-1 $\alpha$ , 6-fold more MIP-1 $\beta$ , 16-fold more RANTES, and 15-fold more IP-10 than did the iDCs. Moreover, the secretion of MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES was increased after stimulation with sesamolins (S). Sesamolins-treated mDCs produced almost twice as much MIP-1 $\alpha$ , twice as much MIP-1 $\beta$ , and 1.5-fold more RANTES as did untreated mDCs. However, IP-10 production was not increased by sesamolins stimulation of the mDCs. These results demonstrate that sesamolins stimulated the production of CCR5 ligands, such as MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES.

### NK cells migration blocking assay

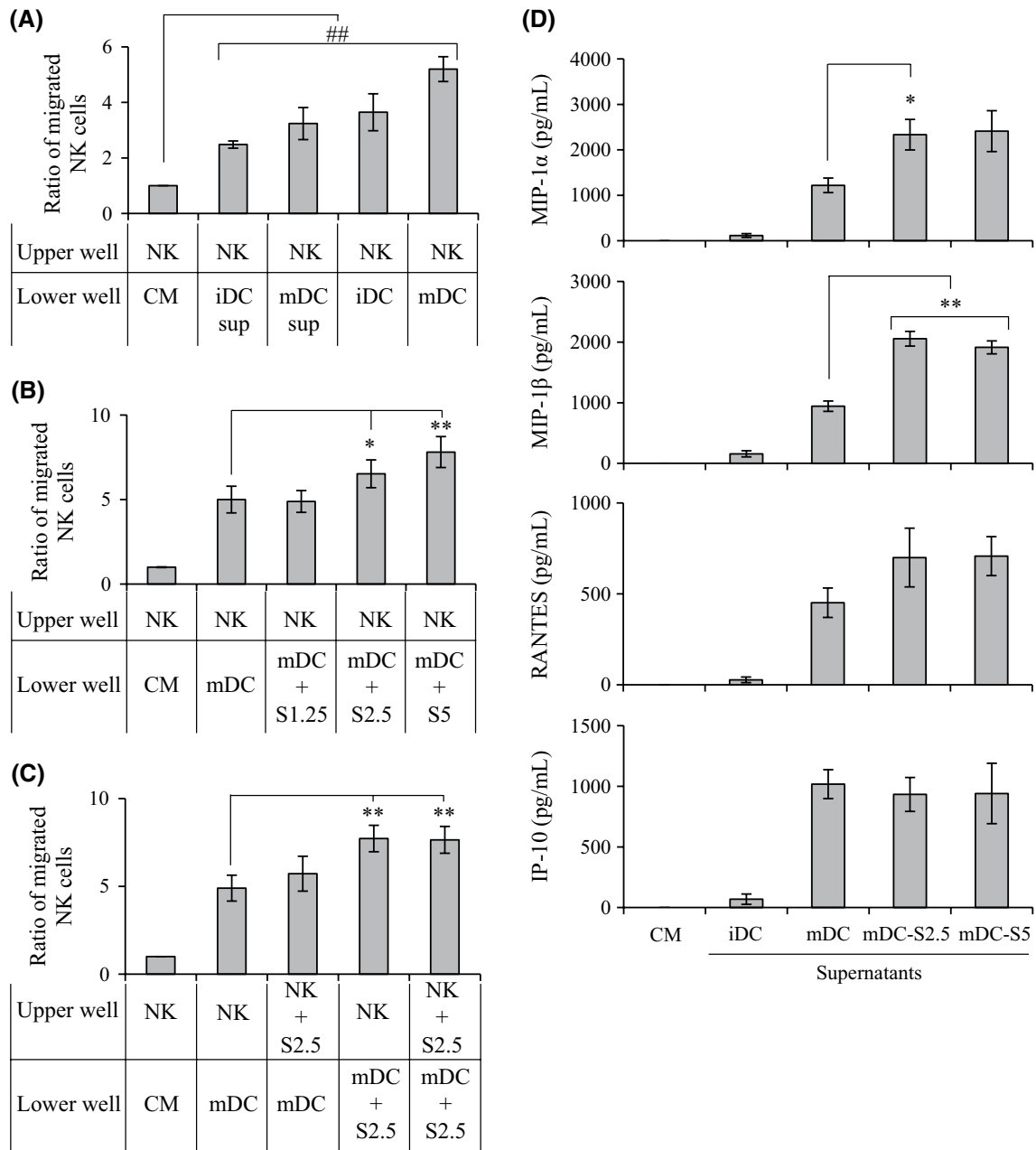
To confirm whether NK cell migration was induced by CCR5 ligands, we used a neutralizing antibody against CCR5 (purified anti-mouse CD195 mAb) to specifically block the response to CCR5. We pretreated NK cells with isotype or CCR5 mAb for 15 min and then used these cells for a migration assay. As shown in Fig. 6, migration of the NK cells was stimulated by 10 ng/ml MIP-1 $\alpha$ . However, that migration was attenuated by pre-treatment with CCR5 mAb. Although it was not statistically significant, the migration of NK cells toward mDCs was also inhibited by pre-incubation with CCR5 mAb. However, the migration of NK cells toward 2.5  $\mu$ g/ml sesamolins (S2.5)-stimulated mDCs (mDC + S2.5) was significantly decreased by the CCR5 mAb.

### Discussion

DCs are a major conductor of cellular immune responses by the expression of MHC and lymphocyte costimulatory molecules and the secretion of cytokines, such as IL-2, IL-12, IL-15, type I, and type II interferons. Immature DCs can acquire antigens in the peripheries, and process the antigens to load into MHC molecules during the period of migration toward secondary lymphoid tissue. Although the antigen acquisition and migration abilities are reduced, DCs become able to present antigens after maturation (Steinman 1991; Pulendran et al. 2008; Legitimo et al. 2014). DCs include multiple subsets with diverse morphologic, phenotypic, and functional characteristics. Although many subsets have been reported, two main classes, myeloid DCs and plasmacytoid DCs, similar in both mice and humans, are accepted (Pulendran et al. 2008). Myeloid DCs, called classical DCs, express CD11c<sup>+</sup> and are considered to be principal producers of type I IFNs. The cells used in this study were considered to be bone marrow-derived common myeloid DCs. Plasmacytoid DCs are considered to originate from CD34<sup>+</sup> bone-marrow precursors and express mainly CD123 and indoleamine 2,3-dioxygenase (Koido et al. 2014).

Generally, DCs activate helper and cytotoxic T lymphocytes to defeat invasive pathogens and stimulate the overall immune system. However, the proper interaction between DCs and NK cells plays a more crucial role in initiating immune responses against pathogens. This cross-talk activates both DCs and NK cells and affects the subsequent innate and adaptive immune responses by inducing the development of helper and cytotoxic T lymphocytes. (Andrews et al. 2003; Kassim et al. 2006; Lucas et al. 2007). The effect of DCs on the activity of NK cells was confirmed in the case of viral infections or cancer development (Fernandez et al. 1999).

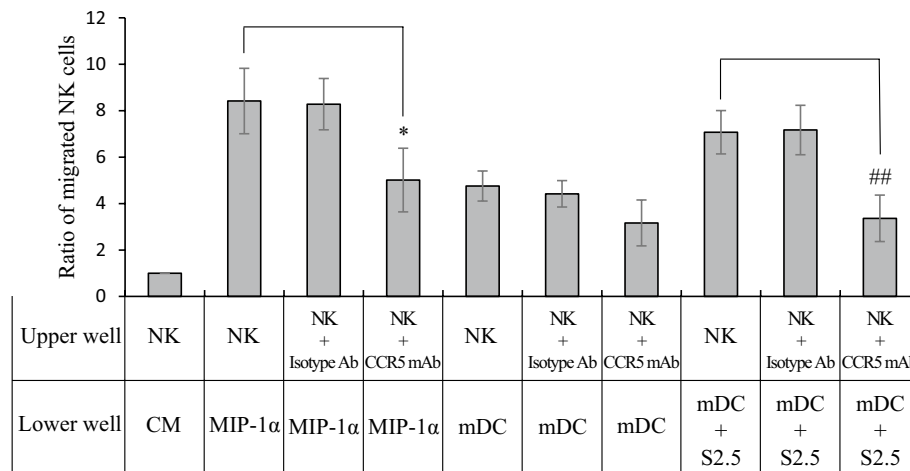




**Fig. 5** Analysis of sesamol effect on NK cell migration toward DCs. In all migration assays (a–c), NK cells or 2.5  $\mu\text{g/ml}$  (S2.5) sesamol-treated NK cells were inserted in the upper compartment (well) of a trans-well chamber. **a** Chemotaxis media (CM), supernatants of DCs and DCs (iDCs or mDCs) were inserted in the lower compartment (well). **b** and **c** CM, mDCs and 2.5  $\mu\text{g/ml}$  (S2.5) or 5  $\mu\text{g/ml}$  (S5) sesamol-treated mDCs were inserted in the lower compartment of the trans-well chamber. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean  $\pm$  SD.  $^{##}p < 0.01$  compared to the ratio of migrated NK cells toward CM.  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  compared to the ratio of migrated NK cells toward mDCs. **d** The production of chemokines from DCs, which affect NK cell migration, were analyzed by ELISA assays. iDCs and mDCs were cultured in the absence or presence of 2.5  $\mu\text{g/ml}$  (S2.5) or 5  $\mu\text{g/ml}$  (S5) sesamol for 24 h. The conditioned supernatants obtained from DCs culture were used for ELISA assays. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean  $\pm$  SD.  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  compared to the mDC supernatant

In general, mouse bone marrow-derived iDCs are stimulated to mature by TLR4 ligands (LPS) or inflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$ ). The most notable changes in DCs after maturation are the development of antigen-presenting capability and the secretion of NK-cell-related cytokines,

IL-2, IL-12, and IL-15 (Granucci et al. 2001, 2003). In vivo, IL-2 produced by endotoxin-stimulated mDCs induced NK-cell activation, such as cytolytic activity and IFN- $\gamma$  secretion, as a paracrine effect (Granucci et al. 2004). Interestingly, Sauma and colleagues reported that IL-4, which was



**Fig. 6** Blocking assay of NK cell migration. NK cells were pretreated in the absence or presence of CCR5 mAb for 15 min and then these cells were used for the migration assay. Chemotaxis media (CM), MIP-1 $\alpha$  (10 ng/ml), mDCs, and 2.5  $\mu$ g/ml (S2.5) sesamololn-treated mDCs were inserted in the lower compartment of the well chamber as chemoattractants. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean  $\pm$  SD. \* $p$  < 0.05 compared to the ratio of migrated NK cells toward MIP-1 $\alpha$ . ## $p$  < 0.01 compared to the ratio of migrated NK cell toward 2.5  $\mu$ g/ml sesamololn-treated mDCs

included in the medium for DC differentiation, blocked the IL-2 production by DCs (Sauma et al. 2004). In contrast, many previous reports suggested that DCs, which were differentiated with GM-CSF and IL-4 from BM cells, could produce IL-2 (Xu et al. 2007). Our results also suggested that GM-CSF/IL-4 DCs could produce IL-2. This disparity may be due to differences in the IL-4 concentration and the protocol used to generate DCs from BM cells.

IL-15, which is also produced by DCs, is considered to be an important cytokine for NK-cell activation. In particular, IL-15 exists in two forms, one is a secreted form and the other is a membrane-bound form. When NK-cell activity is required, IL-15 is upregulated and performs a critical role in stimulating NK-cell cytolytic activity. (Koka et al. 2004). IL-15 can induce the proliferation and activation of NK cells and triggers NK cell immunity (Biron et al. 1999). Specifically, Langerhans cells, DCs of the skin, use IL-15 as the main activator of the survival and cytotoxicity of NK cells (Munz et al. 2005). In this study, we found that sesamololn induced the immune response of DCs, such as IL-2 and IL-15 production, which suggests that sesamololn might be involved in NK–DC cross-talk.

NK cells express  $\beta$  and  $\gamma$  chains of the IL-2 receptor (IL-2R) in a constitutive manner (Rebollo et al. 1996). Although the  $\beta\gamma$  dimer of the IL-2R is a receptor that binds weakly to IL-2, it can induce the activity of NK cells. The binding of IL-2R $\beta\gamma$  and IL-2 leads to the production of the  $\alpha$  chain of IL-2R and the newly synthesized  $\alpha$  chain binds IL-2R $\beta\gamma$  to form an  $\alpha\beta\gamma$  heterotrimer with a high affinity for IL-2 (Minami and Taniguchi 1995; Karnitz and Abraham 1996). Functional or structural deficiency of the  $\gamma$  or  $\beta$  chain of the IL-2R causes an absence of NK cell

activity (Noguchi et al. 1993; Tanaka et al. 1993). These results indicate that  $\beta\gamma$  chains of the IL-2R are more than just receptors of IL-2. Although IL-15 and IL-2 have no sequence homology, they share the  $\beta$  and  $\gamma$  chains as a receptor. However, IL-15R and IL-2R have their own  $\alpha$  chain that provides specificity to the receptor (Carson et al. 1995; Waldmann and Tagaya 1999). These two cytokines have many similar immunological effects in that they increase the immunoglobulin secretion and proliferation of B lymphocytes and the cytotoxicity and proliferation of T and NK cells (Armitage et al. 1995; Carson and Caligiuri 1996; Seder 1996; Trentin et al. 1997). These similarities in action are considered to be caused by the sharing of  $\beta$  and  $\gamma$  chains of the receptors and IL-2 and IL-15 even evoke similar intracellular signaling pathways. In this study, we found that sesamololn induced IL-2 and IL-15, which influenced NK-cell activities.

Understanding NK-cell migration mechanisms from the BM can facilitate the development of NK cells for cancer immunotherapeutic applications (Ljunggren and Malmberg 2007). NK cells are scattered in several organs, such as the liver, spleen, and lung, as well as the BM, during the steady-state (Takeda et al. 2005; Hayakawa et al. 2006; Vosshenrich et al. 2006). To reach the target organs, NK cells should express several kinds of chemotactic receptors, including CXCR3, CXCR4, CCR1, and CCR5, depending on the surrounding environment, and react to the corresponding ligands (Bernardini et al. 2008). Interestingly, the interaction between CXCR4 and SDF-1 $\alpha$  keeps NK cells in the BM, whereas CCR5 and the MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES receptors engage in NK-cell migration to peripheral organs and tissue from the BM.

A critical role for CCR5 ligands, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES, which mediate NK-cell migration to the spleen, liver, and lungs, has been revealed as protection against viruses and bacteria (Salazar-Mather et al. 1998; Shang et al. 2000; Zeng et al. 2003). In a melanoma model, CCR5 ligands produced by mDCs aroused an immune response involving NK cells and T lymphocytes to cause tumor regression. In the same report, CpG-activated DCs induced the migration of NK cells to the site of a tumor via CCR5 ligand (MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES) production (Jiang et al. 2004; Martin-Fontecha et al. 2004; Liu et al. 2008). However, the independent migration of NK cells via CXCR3 and CCR5 from the BM to peripheral tissues was also observed in a hepatitis animal model, even if NK cells were recruited to the liver in a MIP-1 $\alpha$ -dependent manner (Wald et al. 2006). Although this result apparently disagrees with our result, that MIP-1 $\alpha$  recruits NK cells via CCR5, it should be noted that in a disease context, the other MIP-1 $\alpha$  receptor, CCR1, may also be expressed on NK cells and mediate MIP-1 $\alpha$  responses.

Sesamolol is present in a negligible amount, but is a representative lignan component of sesame oil (Lim et al. 2007) and has been reported to have some biological effects, such as neuroprotective and anti-lipogenesis effects. In a previous report, we verified that sesamolol influenced both NK and cancer cells to increase the sensitivity of tumor cells to NK-cell cytolytic activity (Kim and Lee 2015). These reports demonstrated that the increased susceptibility of cancer cells to NK-cell lysis was caused by activation of a MAP kinase signaling pathway in cancer cells and NK cells (Biron et al. 1999). These results led us to a tentative conclusion about the anticancer effect of sesamolol. However, the influence of sesamolol on NK-cell activity to treat cancer has yet to be demonstrated in mixed conditions with other leukocytes. Therefore, we tested the indirect activity of sesamolol on NK cells via DCs and found that sesamolol increased the activity of NK cells via DCs.

In summary, our results demonstrated the effects of sesamolol on NK cells via DCs. DCs matured by sesamolol secreted IL-2 and IL-15 to increase the cytolytic capacity of NK cells. Moreover, sesamolol-treated DCs vigorously attracted NK cell homing compared to unstimulated DCs via CCR5 ligands. Although our results lack the biochemical analyses needed to suggest it as an anti-cancer compound, we suggest that sesamolol is a potential candidate for cancer therapy.

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## Compliance with ethical standards

**Conflict of interest** The author declares no conflict of interest.

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