

27-Jul-2017

Dear Dr Widowati:

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Thank you for your participation.

Sincerely,  
African Health Sciences Editorial Office

03-Nov-2019

Dear Dr Widowati:

It is a pleasure to accept your manuscript entitled "Effect of Interleukins (IL-2, IL-15, IL-18) on Receptors Activation and Cytotoxic Activity of Natural Killer Cells in Breast Cancer Cell" in its current form for publication in the African Health Sciences. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the African Health Sciences, we look forward to your continued contributions to the Journal.

Sincerely,  
Editor-in-Chief, African Health Sciences

Reviewer(s)' Comments to Author:  
Reviewer: 1

Comments to the Author  
authors increase quality of paper  
Authors accept all comments

29-Aug-2019

Dear Dr Widowati:

Manuscript ID WKRO-2018-05-0412 entitled "Effect of Interleukins (IL-2, IL-15, IL-18) on Receptors Activation and Cytotoxic Activity of Natural Killer Cells in Breast Cancer Cell" which you submitted to the African Health Sciences, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended publication, but also suggest some revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

To revise your manuscript, log into <https://mc.manuscriptcentral.com/mums-ahs> and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

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When submitting your revised manuscript, you will be able to respond to the comments made by the reviewer(s) in the space provided. You can use this space to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the reviewer(s).

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Because we are trying to facilitate timely publication of manuscripts submitted to the African Health Sciences, your revised manuscript should be uploaded as soon as possible. If it is not possible for you to submit your revision in a reasonable amount of time, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the African Health Sciences and I look forward to receiving your revision.

Sincerely,  
Editor-in-Chief, African Health Sciences

Reviewer(s)' Comments to Author:  
Reviewer: 1

Comments to the Author  
the work is potentially interesting  
there is a need for correction  
the abstract is not clear  
the methods are not clear

not clearly stated what was used for NK activity?

Target Breast Tumor Cells and Commercial NK Cells?

Usually when you write an NK cell test, you mean NK cells isolated from patients? is it not clear that lu cells are isolated from the patient at all?

a problem in explaining coculture with a tumor cell line!

it needs to be made clearer that it is a single cell line and not a tumor cell cocultured with NK cells (ie, with a commercial line) because this is how it is written, it is very confusing

therefore the NK cell commercial line is co-cultured with the MCF tumor cell line

i think it should have been written right at the beginning of the method and then added how NK cells were generated in the presence of cytokines

or say: the target cell was the MCF-7 cell line!

in the introduction add that it has been shown earlier that NK activity is reduced in patients with breast tumors due to cellular damage in the peripheral blood and add references: Association of NK cell dysfunction with changes in LDH characteristics of peripheral blood lymphocytes (PBL) in breast cancer patients. Breast Cancer Res Treat. 2001 Apr; 66 (3): 255-63.

The role of cytokines in the regulation of NK cells in the tumor environment. Cytokine. 2019 May; 117:30-40. doi: 10.1016/j.cyto.2019.02.001. Epub 2019 Feb 18. Review.

the authors wrote:

NK cells at a density of  $1 \times 10^4$  / well were grown in 96-well plates in NK medium.

what is NK medium? A regular medium in which cells or something else were grown?

Something like this does not exist in the literature

The authors described general data from the literature and referred to patients in the results section.

All this must be rejected because it is confusing

It is only necessary in the results section to describe the results of the own study. In the discussion, all the results obtained are compared with other studies.!

In the results section, the authors wrote some introduction with references. Any introduction that is written in all parts of the text: results; should be completely removed! In each part of the results it is necessary to start only with:

The results of this study show this and that .....

We obtained in this study.....

Results showing.....

In vitro treatment show.....

So, all parts of the results should be CHANGE

Reviewer: 2

Comments to the Author

I am reviewing a research article investigating the administration of cytokines to NK cell modulation as breast anticancer. This research still does not display good scientific quality and must be rewritten. Some important things that must be rewritten (major revision or rejection), include:

1. The background has not yet raised the scientific gap the importance of NK cell modulation in cancer. In addition, the reason for modulation using various cytokines is still unclear.
2. Results are still filled with theory, not yet displaying findings clearly and completely.
3. Discussion does not focus on discussing research findings. Too many theories that are not relevant

**Reviewer: 1**

Comments to the Author

the work is potentially interesting

there is a need for correction

the abstract is not clear

the methods are not clear

1. Not clearly stated what was used for NK activity?

**Answer: We using interleukins (IL-2, IL-15, and IL-18) to induce NK cells. This aim research to measure the activating and inhibiting receptors, proliferation activity of NK cells, and the cytotoxicity of NK cells.**

2. Target Breast Tumor Cells and Commercial NK Cells?

**Answer: This research we used Breast Cancer Cells MCF7 (ATCC® HTB22™) and NK Cells (ATCC® CRL2408™), both cells were not primary cells from patient. We have mentioned this used cells in method section.**

3. Usually when you write an NK cell test, you mean NK cells isolated from patients? is it not clear that NK cells are isolated from the patient at all?

**Answer : This NK cells was not primary cells but cell line from ATCC (ATCC® CRL2408™)**

4. a problem in explaining coculture with a tumor cell line!

it needs to be made clearer that it is a single cell line and not a tumor cell co-cultured with NK cells (ie, with a commercial line) because this is how it is written, it is very confusing therefore the NK cell commercial line is co-cultured with the MCF tumor cell line. I think it should have been written right at the beginning of the method and then added how NK cells were generated in the presence of cytokines or say: the target cell was the MCF-7 cell line.

**Answer: At the method section (*Co-culture of ILs-NK and breast cancer cells for measuring the cytoplasmic granules and effector molecules*), we have explained : NK cells were treated with 5 or 10 ng/ml of IL-2, IL-15, or IL-18 incubated at 5% CO<sub>2</sub>, 37°C for 24 h, which resulted in IL2-induced NK (IL2-NK), IL15-induced NK (IL15-NK), and IL18-induced NK**

(IL18-NK) cells. NK cells were adjusted to  $1 \times 10^5$  for the ratio 1:5 (MCF7:IL2-NK = 1:5; MCF7:IL15-NK = 1:5; MCF7:IL18-NK = 1:5) and the ratio 1:1 (MCF7:IL2-NK = 1:1; MCF7:IL15-NK = 1:1; MCF7:IL18-NK = 1:1 NK cells) and were resuspended in 24-well plates containing MCF7 with the NK medium and DMEM at the ratio 1:1 (50%:50%) according to the optimized research. The co-cultures of IL2-NK, IL15-NK, IL18-NK and MCF7 were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C for 96 h. Cells and medium were centrifuged at 500 g for 4 min, and the medium was preserved at -80°C for subsequent assays such as for IFN- $\gamma$ , TNF- $\alpha$ , PRF1, GzmB.

5. The target cell was the MCF-7 cell line!

**Answer :** The target research was to improve NK cells including increase NK cells proliferation, increase cytotoxic function. This research we used Interleukins (IL2, IL15, IL18) to induce NK cells. The activated NK cells was confirm by NK cell receptors (CD107a, CD314), Cell viability, effector molecules (IFN- $\gamma$ , TNF- $\alpha$ ). The activated NK cells was used to inhibit breast cancer proliferation (MCF7 cells) by co-culture method. The growth inhibition breast cancer cells was confirmed with assay of IFN- $\gamma$ , TNF- $\alpha$ , PRF1

6. In the introduction add that it has been shown earlier that NK activity is reduced in patients with breast tumors due to cellular damage in the peripheral blood and add references: Association of NK cell dysfunction with changes in LDH characteristics of peripheral blood lymphocytes (PBL) in breast cancer patients. Breast Cancer Res Treat. 2001 Apr; 66 (3): 255-63. The role of cytokines in the regulation of NK cells in the tumor environment. Cytokine. 2019 May; 117:30-40. doi: 10.1016/j.cyto.2019.02.001. Epub 2019 Feb 18. Review.

**Answer:** We have mentioned in the Introduction section that NK cells from cancer patients : impairment of NK cell maturation, low NK cell counts in peripheral blood mononuclear cells (PBMCs), significantly lower NK activity in patients with breast cancer than in healthy individuals [7], decreased cytotoxic function [8,9], NK abnormalities [8], poor tumor infiltrate [10,11], low NK cell numbers in tumors due to their inefficient homing into malignant tissues [8], defective expression of activating receptors such as NKG2D, NKG2C, NKp30, NKp46, CD161, CD56<sup>dim</sup>, CD16, DNAM-1, and CD69 and high immunosuppression, namely overexpression, of inhibitory receptors CD158a, CD158b, and NKG2A [8].

7. the authors wrote:

NK cells at a density of  $1 \times 10^4$  / well were grown in 96-well plates in NK medium.

what is NK medium? A regular medium in which cells or something else were grown? Something like this does not exist in the literature.

A medium containing RPMI 1640 (Gibco 22400089), 10% FBS (Gibco 10270106), and 1% antibiotic/antimycotic (Gibco 1772653).

**Answer:** We are using NK medium with composition of MEM- $\alpha$ , 1,5g/L Sodium bicarbonate, 0,2 mM Myo-Inositol, 0,1 mM 2-Mercaptoethanol, 0,02 mM Folic Acid, 100 – 200 U/ml recombinant IL-2, 12,5% Horse Serum, 12,5% Fetal Bovine Serum, 1% Antibiotic-antimycotic, 1% Amphoterycin B, and 0,1% Gentamicin.

7. The authors described general data from the literature and referred to patients in the results section.

All this must be rejected because it is confusing

It is only necessary in the results section to describe the results of the own study. In the discussion, all the results obtained are compared with other studies.!

**Answer:** The literature in the results has been removed.

8. In the results section, the authors wrote some introduction with references. Any introduction that is written in all parts of the text: results; should be completely removed! In each part of the results it is necessary to start only with:

The results of this study show this and that .....

We obtained in this study.....

Results showing.....

In vitro treatment show.....

So, all parts of the results should be CHANGE

**Answer:** The theory or introduction in the results has been removed.

## **Reviewer: 2**

Comments to the Author

I am reviewing a research article investigating the administration of cytokines to NK cell modulation as breast anticancer. This research still does not display good scientific quality and must be rewritten. Some important things that must be rewritten (major revision or rejection), include:

1. The background has not yet raised the scientific gap the importance of NK cell modulation in cancer. In addition, the reason for modulation using various cytokines is still unclear.

**Answer:** The reason has been presence in introduction line numbers 38-47

2. Results are still filled with theory, not yet displaying findings clearly and completely.

**Answer:** The theory in the results has been removed.

3. Discussion does not focus on discussing research findings. Too many theories that are not relevant

### Preview (WKR0-2018-05-0412.R1)

**From:** benidictmak@gmail.com

**To:** wahyu\_w60@yahoo.com

**CC:**

**Subject:** African Health Sciences - Decision on Manuscript ID WKR0-2018-05-0412.R1

**Body:** 03-Nov-2019

Dear Dr Widowati:

It is a pleasure to accept your manuscript entitled "Effect of Interleukins (IL-2, IL-15, IL-18) on Receptors Activation and Cytotoxic Activity of Natural Killer Cells in Breast Cancer Cell" in its current form for publication in the African Health Sciences. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the African Health Sciences, we look forward to your continued contributions to the Journal.

Sincerely,  
Editor-in-Chief, African Health Sciences

Reviewer(s)' Comments to Author:  
Reviewer: 1

Comments to the Author  
authors increase quality of paper  
Authors accept all comments

**Date Sent:** 03-Nov-2019

# Effect of interleukins (IL-2, IL-15, IL-18) on receptors activation and cytotoxic activity of natural killer cells in breast cancer cell

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Tjandrawati Mozeft<sup>4</sup>, Rizal Rizal<sup>5</sup>, Hanna Sari W. Kusuma<sup>5</sup>, Dian R. Laksmitawati<sup>6</sup>,  
Harry Murti<sup>7</sup>, Indra Bachtiar<sup>7</sup>, Ahmad Faried<sup>8</sup>

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## Abstract

**Introduction:** Breast cancer is one of the leading cause of cancer deaths in women. Metastasis in BC is caused by immunosurveillance deficiency, such NK cell maturation, low NK activity and decreasing cytotoxicity. This study was performed to improve activating receptors and cytotoxicity of NK cells using interleukins (ILs).

**Methods:** Human recombinant IL-2, -15, and -18 were used to induce NK cells. We measured the activating and inhibiting receptors, proliferation activity of NK cells, and the cytotoxicity of NK cells on BC cells (MCF7). The effects of ILs were tested on the NK cell receptors CD314, CD158a and CD107a with flowcytometry, proliferation at various incubation times with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and concentrations of TNF- $\alpha$  and IFN- $\gamma$  by NK cells with ELISA.

**Results:** ILs increased NK cell receptor levels (CD314, CD158a, and CD107a) at 24 hours of incubation. ILs increased NK cell viability, which increased with longer incubation. Moreover, ILs-induced NK cells inhibited proliferation in MCF7 cells, as well as increased TNF- $\alpha$ , IFN- $\gamma$ , PRF1 and GzmB secretion.

**Conclusion:** IL-2, IL-15, and IL-18 improved activating receptors and proliferation of NK cells. IL-induced NK cells increased TNF- $\alpha$ , IFN- $\gamma$ , **perforin, and granzyme** secretion and cytotoxic activity on BC cells. High NK cell numbers increased BC cell growth inhibition.

**Keywords:** Activator; breast cancer; interleukins; natural killer; receptor.

**DOI:**

**Cite as:**

## Introduction

Breast cancer (BC) is the leading cause of cancer deaths in women<sup>1</sup>, accounting for approximately 43.3%<sup>2</sup>. Mor-

talidity of most patients with solid tumors is due to metastatic spread to other organs<sup>1</sup>. Metastasis occurs when tumor cells acquire invasive features<sup>3</sup> and the ability to escape from antitumor immunity<sup>4,5</sup>. Defects in antitumor immunity may also facilitate BC occurrence<sup>6</sup>. Metastasis in BC is caused by deficient immunosurveillance, including impairment of NK cell maturation, low NK cell counts in peripheral blood mononuclear cells (PBMCs), significantly lower NK activity in patients with BC than in healthy individuals<sup>7</sup>, decreased cytotoxic function<sup>8,9</sup>, NK abnormalities<sup>8</sup>, poor tumor infiltrate<sup>10,11</sup>, low NK cell numbers in tumors due to

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their inefficient homing into malignant tissues<sup>8</sup>, defective expression of activating receptors such as NK-G2D, NKG2C, NKp30, NKp46, CD161, CD56dim, CD16, DNAM-1, and CD69 and high immunosuppression, namely overexpression, of inhibitory receptors CD158a, CD158b, and NKG2A<sup>8</sup>. One cancer therapy is to stimulate NK cell function and combine them with other agents to boost anti-cancer activity<sup>12</sup>. NK cells as immunomodulators can be activated using interleukin into lymphokine-activated killer cells (LAK). NK cells respond to a variety of cytokines, such as IL-2, IL-12, IL-15, IL-18, IL-21, and Type I Interferons (IFNs), discretely or in combination with each other or with other modulators<sup>13</sup>, and tumor necrosis factor (TNF)<sup>14</sup>, which increases their cytolytic, secretory and anti-cancer functions<sup>15</sup>. Through its interaction with NK cells, IL-2 treatment was related with favourable result in various cancer type, making it the first effective immunotherapy for human cancer<sup>16</sup>.

Immunotherapy using NK cells can be used to obtain the large and sufficient numbers of functional NK cells necessary for clinical therapy. The number, purity and state of NK cell proliferation and activation are key factors in immunotherapy<sup>17</sup>. NK cells are known as necessary effectors in suppressing cancer proliferation<sup>18</sup>. Therefore, the focus of recent cancer therapy has been to promote and develop NK cells as drugs<sup>9</sup>, using NK effectors such as cytokines<sup>8</sup>.

This study was conducted to evaluate the effect of inducing interleukins (IL-2, IL-5, and IL-18) on NK cells toward the following: i) improvement of NK cell activating receptors, including CD314, CD158d and CD107a; ii) increased proliferation of NK cells; iii) NK cell secretion of factors such as IFN- $\gamma$  and TNF- $\alpha$ ; iv) secretion of IFN- $\gamma$ , TNF- $\alpha$ , perforin (PRF1) and granzyme B (GzmB) co-cultured NK and BC cells inhibition of BC cell proliferation.

## Materials and methods

### Induction of NK cells using interleukins (IL-2, IL-15, IL-18)

NK92MI cells (ATCC® CRL2408™) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia, at a density of  $2 \times 10^6$ /well, were grown and maintained in medium containing RPMI 1640 (Gibco 22400089), 10% FBS (Gibco 10270106), and 1% antibiotic/antimycotic (Gibco 1772653). Cells were treated with 5 or 10 ng/ml of IL-2

(Biolegend 589106), IL-15 (Biolegend 715902) or IL-18 (GenScript Z031189) incubated at 5% CO<sub>2</sub>, 37°C for 24 h, which resulted in IL2-induced NK (IL2-NK), IL15-induced NK (IL15-NK), and IL18-induced NK (IL18-NK) cells, respectively. The IL-NK cells were used for assays such as measuring NK cell receptors, NK cell viability, cytotoxic activity toward BC cells by co-culture, and effector molecule secretion by NK cells (IFN $\gamma$ , TNF $\alpha$ , PRF1, and GzmB levels).

### Detection of NK cell receptors using fluorescence activated cell sorting (FACS)

NK92 cells with a density of  $5 \times 10^5$ /well were cultured in complete medium containing RPMI 1640, 10% FBS, and 1% antibiotic/antimycotic incubated at 5% CO<sub>2</sub>, 37 °C for 24 h. The NK cells were induced with IL-2, IL-15, and IL-18 (10 ng/ml) and incubated for 24 h. The medium was collected, and  $1 \times 10^5$  -  $1 \times 10^6$  NK cells were added to 500 ml of FACS Buffer (PBS + 2% FBS). Furthermore, NK receptors were stained with the surface markers CD314 (Ms anti-human, Invitrogen 521927), CD107a (Ms anti-human, Invitrogen 519297), and CD158d (Ms Anti-human, 523653) in single and multiple staining, followed by incubation in a dark room at 4 °C for 30 min. The cells were washed twice using FACS Buffer. NK receptors were stained with CD314, CD158d, or CD107a antibody conjugated with PE. The receptors were analyzed by flow cytometry with a MACSQuant® Analyzer. The experiments and measurement of surface markers were performed in triplicate<sup>19,20</sup>.

### Cell viability of ILs-induced NK (ILs-NK)

The NK cells at a density of  $1 \times 10^4$ /well were grown in 96-well plates in NK medium. The cells were induced directly with 5 or 10 ng/ml of IL-2, IL-15, or IL-18 daily. Furthermore, cells were incubated at 5% CO<sub>2</sub> and 37°C, in a humidified atmosphere for 24, 48, 72, or 96 h<sup>20</sup>. To determine the those above IL-NK viability per day, cell viability was assayed based on an optimized reagent containing resazurin converted to fluorescent resorufin by viable cells that absorbs light at 490 nm using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS; Promega G3580)<sup>21,22</sup>.

### Effector molecules of ILs-induced NK cells

ILs-induced NK cells at a density of  $1 \times 10^5$  cells/well were grown and maintained in NK medium and incubated at 5% CO<sub>2</sub>, 37 °C for 24 h. Cells and medium

were centrifuged at 500 g for 4 min, and the culture supernatant was preserved at -80°C for subsequent assays, such as for IFN- $\gamma$  and TNF- $\alpha$ <sup>22,23</sup>. IFN- $\gamma$  secretion from NK cells was measured using a commercial enzyme-linked immunosorbent assay kit (ElabSci E-EL-H0109) and the TNF $\alpha$  secretion from NK cells using a human ELISA Kit (ElabSci E-EL-H0109) following the manufacturer's recommendation.

### Co-culture of ILs-NK and breast cancer cells for measuring the cytoplasmic granules and effector molecules

The NK cells were treated with 5 or 10 ng/ml of IL-2, IL-15, or IL-18 incubated at 5% CO<sub>2</sub>, 37°C for 24 h, which resulted in IL2-induced NK (IL2-NK), IL15-induced NK (IL15-NK), and IL18-induced NK (IL18-NK) cells. The MCF7 cells at a density of 1x10<sup>6</sup> cells/well (DMEM+20% FBS+1% antibiotic/antimycotic) were cultured in a 24-well plate. The cells were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C for 24 h. NK cells were adjusted to 1x10<sup>5</sup> for the ratio 1:5 (MCF7:IL2-NK = 1:5; MCF7:IL15-NK = 1:5; MCF7:IL18-NK = 1:5) and the ratio 1:1 (MCF7:IL2-NK = 1:1; MCF7:IL15-NK = 1:1; MCF7:IL18-NK = 1:1 NK cells) and were resuspended in 24-well plates containing MCF7 with the NK medium and DMEM at the ratio 1:1 (50%:50%) according to the optimized research (data not shown). The co-cultures of IL2-NK, IL15-NK, IL18-NK and MCF7 were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C for 96 h. Cells and medium were centrifuged at 500 g for 4 min, and the medium was preserved at -80°C for subsequent assays such as for IFN- $\gamma$  (Human ELISA KIT, Biolegend 430104), TNF- $\alpha$  (Human ELISA KIT, Biolegend 430204), PRF1 (Human ELISA KIT, ElabSci E-EL-H1123) and GzmB (Human ELISA KIT, ElabSci E-EL-H1617)<sup>23,24</sup>.

#### *Growth inhibition of ILs-NK cells on breast cancer cells*

This research method was adopted and modified based on the previous research by Parihar et al. (2002) and Lu et al. (2008)<sup>22,23</sup>. NK cells at a density of 1x10<sup>6</sup>/well were grown and maintained in NK medium, and cells were treated with 5 and 10 ng/ml of IL-2, IL-15, or IL-18 daily and incubated at 5% CO<sub>2</sub>, 37°C for 96 h, resulting in IL-induced NK cells. The BC cell line MCF7 (ATCC® HTB22™) was obtained from Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung, Indonesia, plated at a density of

1x10<sup>4</sup>/well and cultured on 96-well plates in 100  $\mu$ l of complete medium (Dulbecco's Modified Eagle's Medium (DMEM high glucose; Gibco 11995065), 10% FBS, 1% antibiotic/antimycotic) and incubated at 5% CO<sub>2</sub>, 37°C for 24 h. The following day, culture supernatant was aspirated, and the cells were washed with PBS (Invitrogen 1740576) and replaced with complete medium (DMEM + 1% antibiotic/antimycotic, 10% FBS). Cells were incubated at 5% CO<sub>2</sub>, 37°C for 24 h. For the BC cells treated with NK cells in the number-dependent experiment, the ratios of BC cells to IL-induced NK cells were 1 : 5 (10,000 : 50,000), 1 : 2 (10,000 : 20,000), 1 : 1 (10,000 : 10,000) and 2 : 1 (10,000 : 5,000). The medium comparison of cocultured cells was based on a preliminary study, and the best medium comparison of NK medium and BC medium was 1:1. The controls were the single cultures of NK92MI or MCF7. The co-cultured NK and BC cells were incubated at 5% CO<sub>2</sub>, 37°C for 24 h, 48 h, 76 h, or 96 h. To determine the growth inhibition or cytotoxic effects of NK cells toward BC cells using MTS assays, 20  $\mu$ l of reagent cell titer 96 ® Aqueous one solution assay (Promega G3580) was added to each well and incubated for 3 h. The cell absorbance was measured at 490 nm wavelength (Multiskan Go, Thermo Scientific, USA). The inhibitory effect of IL-NK cells toward BC cell viability was calculated according to the following formula<sup>23</sup>:

$$\text{Growth Inhibition (\%)} = \frac{[(\text{Abs Cancer} - \text{Abs Cancer} + \text{Abs NK}) + \text{Abs NK}]}{\text{Abs Cancer}} \times 100\%$$

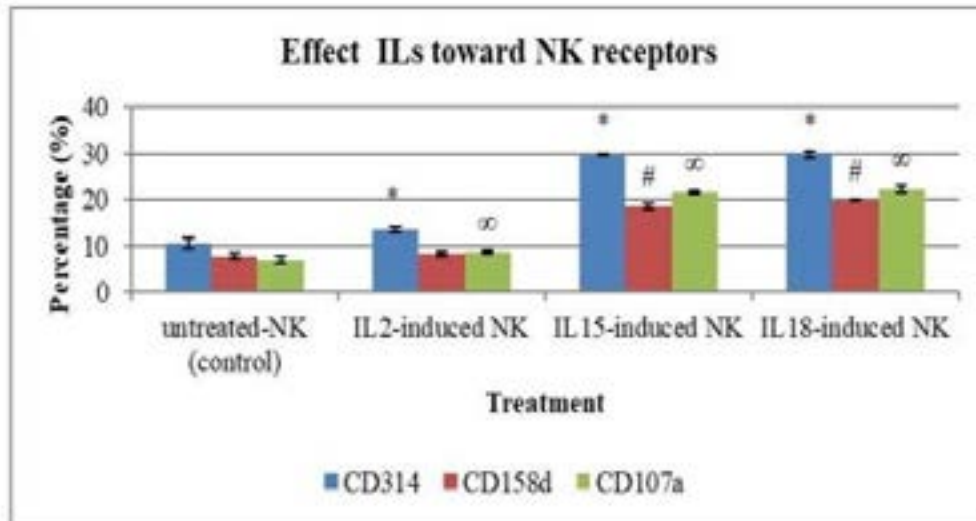
### Statistical analysis

Statistical analysis was conducted using SPSS software (version 16.0). Data were presented as Mean $\pm$ Standard Deviation. Significant differences among treatments were determined using the one-way Analysis of variance (ANOVA) and  $p < 0.05$  were considered as statistically significant, along with Tukey honestly significant difference post hoc test and 95% confidence interval.

### Results

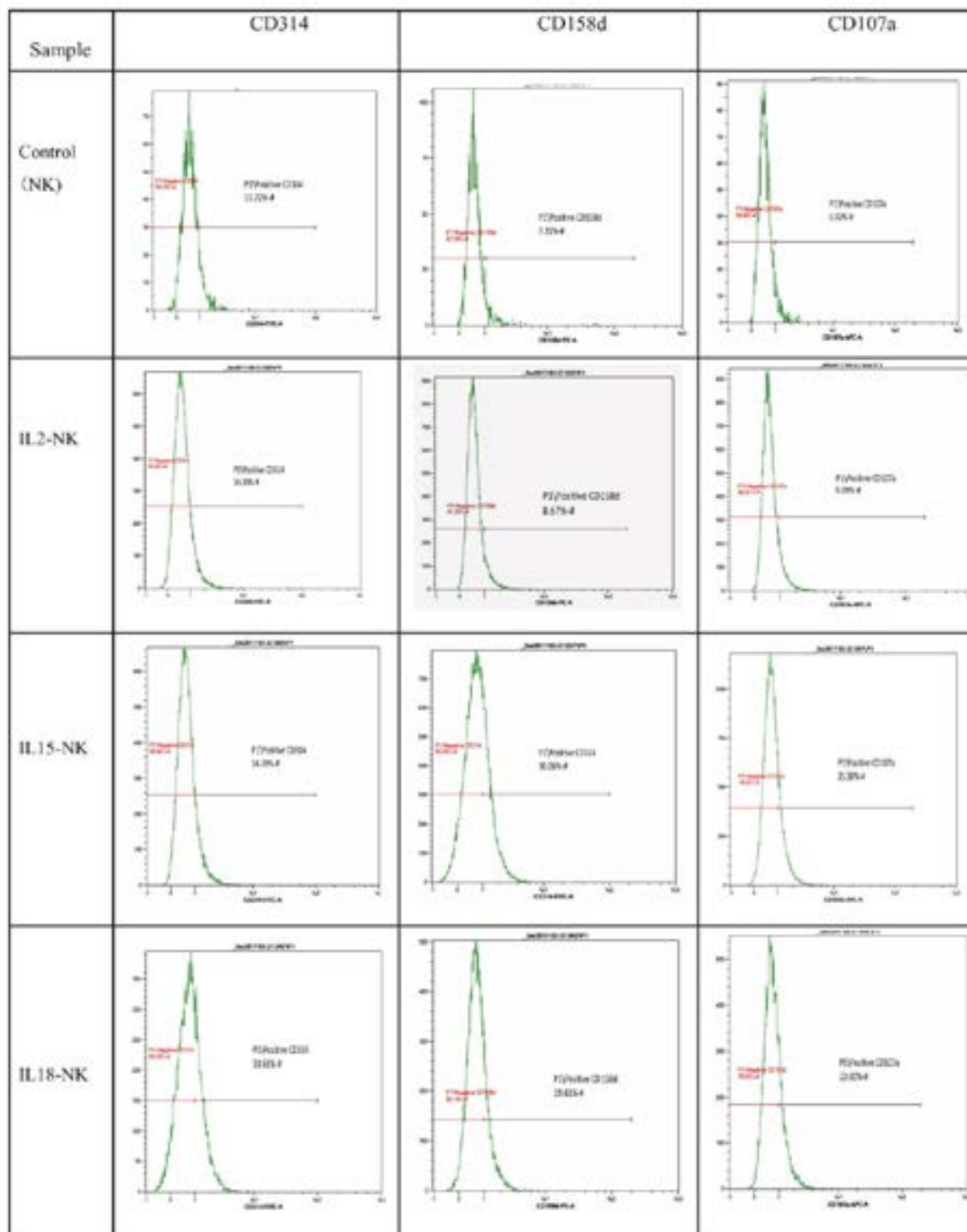
#### Effect of ILs toward NK cell characteristics

To determine the effect of human recombinant ILs toward NK receptors, we evaluated the NK receptors including CD314, CD107a and CD158d (Figures 1-2). The data showed that ILs (IL-2, IL-15, and IL-18) significantly up-regulated CD314 and CD107 and that IL-15 and IL-18 significantly up-regulated CD158d, but IL-2 did not significantly up-regulate CD158d.



**Figure 1.** Effect of 10 ng/ml ILs (IL-2, IL-15, and IL-18) toward NK cell receptors (CD314, CD158d, and CD107a) for 24 h incubation.

\*The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment, symbol \*present significant differences among inducer (IL2-induced NK, IL15-induced NK, IL18-induced NK) compared to control (untreated NK cells) toward CD314 expression, symbol # present significant differences among inducer (IL2-induced NK, IL15-induced NK, IL18-induced NK) compared to control (untreated NK cells) toward CD158d expression, symbol ∞ present significant differences among inducer (IL2-induced NK, IL15-induced NK, IL18-induced NK) compared to control (non-induced NK cells) toward CD107a expression.



**Figure 2.** Effect of ILs (IL-2, IL-15, and IL-18) toward dot blot of NK receptors

\*The dot blot of NK receptors (CD314, CD158d, and CD107a) are represented of triplication treatment

### Effect of ILs toward NK cell proliferation

NK cells require effectors to activate NK cells; thus, this study was conducted to evaluate the effect of ILs (IL-2, IL-15, and IL-18) toward NK cell numbers. We used ILs at levels of 5 and 10 ng/ml, and NK cell proliferation was calculated at various incubation times (24,

48, 72, and 96 h; Table 1). The data show (Table 1) that ILs (IL-2, IL-15, and IL-18) increased NK cell proliferation and that proliferation was lower at 24 h of incubation compared to 48 h, 72 h, and 96 h of incubation. A higher concentration (10 ng/ml) of ILs resulted in higher proliferation compared to 5 ng/ml.

**Table 1.** Effect ILs (IL-2, IL-15, IL-18) toward NK cells viability (%)

Concentrations (ILs) for inducing NK cells	NK cells viability (%)			
	Incubation time (24 h)	Incubation time (48 h)	Incubation time (72 h)	Incubation time (96 h)
Control (non-induced NK)	100.00±13.16 <sup>abA</sup>	100.00±4.17 <sup>abA</sup>	100.00±0.46 <sup>aA</sup>	100.00±6.15 <sup>aA</sup>
IL2-NK (5 ng/ml)	104.14±12.49 <sup>abA</sup>	109.66±9.12 <sup>abcAB</sup>	134.50±16.67 <sup>bcBC</sup>	143.13±3.20 <sup>bcC</sup>
IL2-NK (10 ng/ml)	122.67±9.36 <sup>ba</sup>	129.12±0.39 <sup>aA</sup>	152.69±11.63 <sup>cB</sup>	153.16±4.21 <sup>cB</sup>
IL15-NK (5 ng/ml)	79.36±12.27 <sup>aA</sup>	124.92±0.72 <sup>bcB</sup>	138.27±1.14 <sup>bcB</sup>	141.88±9.35 <sup>bcB</sup>
IL15-NK (10 ng/ml)	109.29±23.55 <sup>abA</sup>	134.62±5.64 <sup>cAB</sup>	140.77±0.44 <sup>bcAB</sup>	153.37±1.73 <sup>cB</sup>
IL18-NK (5 ng/ml)	81.19±17.92 <sup>abA</sup>	87.14±8.76 <sup>aA</sup>	120.36±6.64 <sup>abB</sup>	128.88±5.53 <sup>abB</sup>
IL18-NK (10 ng/ml)	104.81±11.12 <sup>abA</sup>	115.75±21.71 <sup>bcAB</sup>	155.28±7.59 <sup>cC</sup>	142.31±7259 <sup>bcC</sup>

\*This research was performed 3 replication for NK cells viability. Data of NK cells viability (%) are presented as mean ±standard deviation, different small letters in the same column (among concentrations of ILs (IL-2, IL-15, IL-18) treatment in each periodic incubations (day 1, 2, 3, 4). Different capital letters in the same row (among periodic incubations in each ILs treatment), were significantly different at  $p < 0.05$  (Tukey honestly significant difference post hoc test)

### Effect of ILs toward NK cell secretion of IFN- $\gamma$ and TNF- $\alpha$

In this study, we evaluated the effect of 10 ng/ml ILs (IL-2, IL-15, and IL-18) toward IFN- $\gamma$  and TNF- $\alpha$  secretion by NK cells for 24 h (Figure 3). Figure 3 shows that IL-2, IL-15, and IL-18 significantly increased IFN- $\gamma$  and TNF- $\alpha$  compared to controls (untreated NK cells). Effect of ILs toward IFN- $\gamma$ , TNF- $\alpha$ , **perforin**, and **granzyme** in co-cultured breast cancer and NK cells

This study was aimed to measure the cytokines including IFN- $\gamma$ , TNF- $\alpha$ , and cytolytic enzymes such as PRF1 and GzmB, which are secreted by co-cultured MCF7 and NK cells (Table 2). The data in Table 2, show that

MCF7 cells secreted low levels of PRF1 (20.00 pg/ml) and GzmB (13.33 pg/ml) and produced low levels of IFN- $\gamma$  (1.83 pg/ml) and TNF- $\alpha$  (1.98 pg/ml). Co-culture of MCF7 and non-activated NK cells increased IFN- $\gamma$ , TNF- $\alpha$ , PRF1, and Gzm. Higher NK cell numbers in co-cultured cells increased the cytokines and cytolytic enzyme secretion of both non-activated and activated NK cells. IL-15 and IL-18 were the best effectors to induce NK cells to secrete IFN- $\gamma$ , TNF- $\alpha$ , PRF1, and GzmB. The highest levels of IFN- $\gamma$ , TNF- $\alpha$ , PRF1, and GzmB in co-cultured MCF7 and NK cells were secreted from co-cultured MCF7 and IL18-NK cells at a ratio of 1:5.

**Table 2.** Effect ILs toward cytokines, cytolytic enzyme of co-culture breast cancer and NK cells

Samples (Co-culture MCF7+NK) in various ratio	Level of cytokines, cytolytic enzyme			
	IFN- $\gamma$ (pg/ml)	TNF- $\alpha$ (pg/ml)	PRF1 (pg/ml)	GzmB (pg/ml)
Control (MCF7)	1.83±0.33 <sup>a</sup>	1.98±0.24 <sup>a</sup>	20.00±10.00 <sup>a</sup>	13.33±4.51 <sup>a</sup>
MCF7 + NK(1:1)	14.50±3.93 <sup>b</sup>	5.24±0.28 <sup>a</sup>	40.00±10.00 <sup>ab</sup>	29.00±4.36 <sup>b</sup>
MCF7 + NK(1:5)	32.28±1.35 <sup>c</sup>	13.85±2.79 <sup>b</sup>	110.00±10.00 <sup>b</sup>	55.67±12.50 <sup>b</sup>
MCF7 + IL2-NK(1:1)	17.39±3.56 <sup>b</sup>	15.04±0.21 <sup>bc</sup>	160.00±40.00 <sup>cd</sup>	87.00±11.79 <sup>c</sup>
MCF7 + IL2-NK (1:5)	35.72±0.38 <sup>c</sup>	37.44±1.47 <sup>d</sup>	230.00±40.00 <sup>de</sup>	149.00±7.21 <sup>d</sup>
MCF7 + IL15-NK (1:1)	18.28±5.74 <sup>b</sup>	19.03±1.66 <sup>bc</sup>	150.00±20.00 <sup>cd</sup>	88.67±11.68 <sup>c</sup>
MCF7 + IL15-NK (1:5)	59.28±1.90 <sup>d</sup>	42.21±4.23 <sup>d</sup>	250.00±30.00 <sup>de</sup>	140.67±12.86 <sup>d</sup>
MCF7 + IL18-NK (1:1)	17.72±3.37 <sup>b</sup>	20.44±2.37 <sup>c</sup>	170.00±40.00 <sup>cd</sup>	96.00±1.73 <sup>c</sup>
MCF7 + IL18-NK (1:5)	60.17±3.71 <sup>d</sup>	60.56±3.15 <sup>c</sup>	320.00±70.00 <sup>f</sup>	149.67±11.93 <sup>d</sup>

\*This research was performed 3 replication for IFN- $\gamma$ , TNF- $\alpha$ , PRF1, GzmB. Data are presented as mean ±standard deviation. Different superscript small letters (<sup>a,b,c,d</sup>) in the same column (among ratio of MCF7 and ILs-induced NK cells in IFN- $\gamma$  level), different superscript small letters (<sup>a,b,c,d</sup>) in the same column (among ratio of MCF7 and ILs-induced NK cells in TNF- $\alpha$  level), different superscript small letters (<sup>a,ab,b,cd,de,f</sup>) in the same column (among ratio of MCF7 and ILs-induced NK cells in PRF1 level), different superscript small letters (<sup>a,b,c,d</sup>) in the same column (among ratio of MCF7 and ILs-induced NK cells in GzmB level), were significantly different at  $p < 0.05$  (Tukey honestly significant difference post hoc test).



## Effect of ILs toward BC proliferation in co-cultured BC and NK cells

To investigate NK cells as immunomodulators to kill and inhibit BC cell proliferation, we performed this research using co-cultured MCF7 and NK cells at various ratios. The effect of NK cells at various ratios can be seen in Table 3. The data in Table 3, showed that the ratio of BC and NK cells determined cancer cell proliferation, and the NK cell number affected cytotoxic

potency on cancer cells. The growth inhibition among the MCF7 : NK ratio was significantly different. Higher NK cell numbers were more toxic than lower NK cell numbers, and the lowest NK cell number demonstrated the lowest growth inhibition on MCF7. The highest growth inhibition of NK cells was obtained with the highest NK numbers, with the MCF7: NK ratio of 1:5. The IL inducers (IL-2, IL-15, and IL-18) did not exhibit significant differences in the inhibition of cell proliferation.

**Table 3.** Effect ILs-induced NK cells and NK cells number toward breast cancer cells growth inhibition

ILs-induced NK cells (ILs-NK)	Comparison breast cancer (MCF7) and NK cell			
	(1:5)	(1:2)	(1:1)	(2:1)
Control (non-induced NK)	85.01±2.65 <sup>ad</sup>	65.16±4.21 <sup>ac</sup>	42.19±5.60 <sup>ab</sup>	16.79±1.08 <sup>aA</sup>
IL2-NK (5 ng/ml)	101.42±1.26 <sup>ad</sup>	78.22±1.91 <sup>ac</sup>	65.02±2.23 <sup>ab</sup>	35.71±0.72 <sup>bcA</sup>
IL2-NK (10 ng/ml)	98.79±4.20 <sup>ad</sup>	77.74±9.39 <sup>ac</sup>	58.72±1.86 <sup>ab</sup>	37.64±1.70 <sup>cA</sup>
IL15-NK (5 ng/ml)	86.75±10.58 <sup>ac</sup>	66.22±4.10 <sup>ab</sup>	44.68±7.10 <sup>aA</sup>	34.65±4.75 <sup>bcA</sup>
IL15-NK (10 ng/ml)	86.83±13.86 <sup>ac</sup>	69.64±8.53 <sup>abc</sup>	47.03±11.93 <sup>aAB</sup>	37.83±8.25 <sup>cA</sup>
IL18-NK (5 ng/ml)	87.58±1.98 <sup>ab</sup>	77.78±4.79 <sup>ab</sup>	47.66±17.77 <sup>aA</sup>	25.28±3.28 <sup>abA</sup>
IL18-NK (10 ng/ml)	86.01±1.52 <sup>ad</sup>	67.62±6.79 <sup>ac</sup>	52.45±1.08 <sup>ab</sup>	30.04±1.75 <sup>bcA</sup>

\* This research was performed 3 replication. The data of cells inhibition (%) are presented as mean ±standard deviation, different small letters in the same column (among concentrations of ILs (IL-2, IL-15, IL-18) treatment on comparison MCF7 and NK cell (1:5, 1:2, 1:1, 2:1), different capital letters in the same row (among ratio MCF7 and NK cells) in each ILs concentrations.

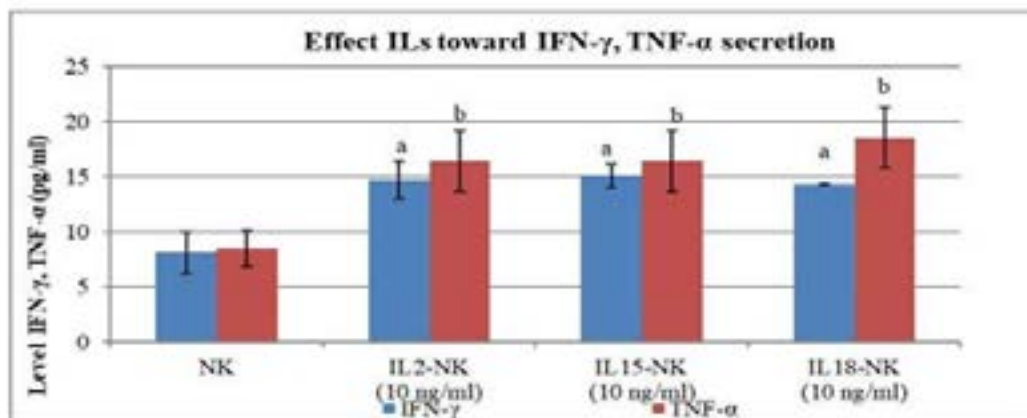
## Discussion

Induction using ILs (IL-2, IL-15, and IL-18) in NK cells showed that ILs increased activating receptors in NK-92 cells, namely NKG2D/CD334, CD158d, and CD107. This result was in line with previous research that metastatic melanoma (MM) patients exhibited decreased CD161 and NKG2D<sup>24</sup>. The expression of the activating NK cell receptors was significantly decreased in acute myeloid leukemia (AML) patients compared to healthy donors<sup>25</sup>. The biological functions of NK cells are tightly regulated during their interaction with cytokines such as IL-12, IL-15, and IL-18, and the consequences of this interaction are manifested in NK cells<sup>26</sup>. NK cells treated with IL-15 up-regulated the expression of NK receptors, including Nkp30, Nkp46, NKG2C, and NKG2D<sup>25</sup>. This up-regulation is associated with a concomitant increase in the NK cell activity<sup>27</sup>. Human NK cell memory-like responses have been directly demonstrated in vitro following cytokine activation

with IL-12, IL-15, and IL-18. A brief (16-hour) pre-activation with IL-12, IL-15, and IL-18, followed by rest in vitro for 1–6 weeks, resulted in enhanced functionality, including IFN- $\gamma$  production following re-stimulation with cytokines, or exposure to leukemia targets<sup>28,29</sup>. IL-2, IL-12, IL-15 and IL-18, applied systemically and for ex vivo activation and expansion of NK cells, have improved NK cell antitumor activity by increasing the expression of NK cell activating receptors and by inducing cytotoxic effector molecules<sup>30</sup>. IL-2 significantly increased KIR2DL1, KIR2DL2, and KIR3DL2 receptors on their surface by 30% vs. 22%, 37% vs. 30%, and 24% vs. 14%, respectively<sup>31</sup>. IL-12 alone or in combination with IL-18 significantly induced NK cell activity and CD107a degranulation marker expression in MM<sup>32</sup>. Resting NK cells typically express very low or undetectable levels of KIR2DL4 on their cell surfaces. Activation of NK cells with IL-2 and feeder cells resulted in a transient increase in the level of cell surface KIR2DL4<sup>33</sup>.

ILs increased NK proliferation, and a longer periodic incubation significantly increased NK viability (Figure 3). These data were validated with previous research showing that IL15 efficiently triggered the activation and proliferation of NK and CD8+ T cells<sup>34,35</sup>. NK cell activity is regulated by cytokines such as IL-2, IL-12, IL-15, IL-18 and type I interferons (IFNs)<sup>36,37</sup>. NK cells treated with IL2 (IL2-NK) induced the proliferation of injected NK cells in the lung and increased the overall

survival of mice with osteosarcoma lung metastasis<sup>38</sup>. The NK cell expansion has been attempted using cytokines such as IL-2 and IL-15<sup>39</sup>, which are required for both NK cell maturation and survival<sup>40</sup>. IL-2 is the main cytokine that stimulates NK cell activation and proliferation<sup>41</sup>. NK cells also exhibit memory-like properties, and cytokine-induced memory-like (CIML) NK cells are generated via brief pre-activation with IL-12, IL-15, and IL-18 and later exhibited enhanced functionality upon re-stimulation<sup>42</sup>.



**Figure 3.** Effect of ILs (IL-2, IL-15, and IL-18) toward IFN-γ and TNF-α secretion by NK cells.

\*The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment, NK cells were induced ILs 10 ng/ml, incubation for 24 h, symbol (a) present significant differences among inducer (IL-2 10 ng/ml, IL-15 10 ng/ml, IL-18 10 ng/ml) compared to control (non-induced NK cells) toward secretion of IFN-γ, symbol (b) present significant differences among inducer (IL-2 10 ng/ml, IL-15 10 ng/ml, IL-18 10 ng/ml) compared to control (non-induced NK cells) toward secretion of TNF-α.

Interleukin activation of NK cells increased the proliferation and number of NK cells, and longer incubation of NK cells further enhanced proliferation. This result was validated with previous research showing that aerosol IL-2 augments the efficiency of NK cell therapy. Aerosol IL-2 increased organ-specific migration and NK cell expansion in the lung, the number of NK cells in the individual tumor nodules, and tumor cell killing<sup>43</sup>. Aerosol IL-2 increases lung NK cell numbers by stimulating local NK cell proliferation. Aerosol IL2's effect on NK cell proliferation is organ-specific, which makes it ideal for the specific targeting of lung metastasis<sup>38,43</sup>. In vitro studies with IL-2 to support proliferation and in vivo adoptive transfers into murine cytomegalovirus-infected mice demonstrated that NK cell proliferation increased<sup>44</sup>. Interleukin 15 (IL-15) promotes the survival of NK cells by preventing apoptosis. IL-15 suppressed apoptosis by limiting Bim expression through the kinases Erk1 and Erk2 and mechanisms dependent on the transcription factor Foxo3a, while promoting ex-

pression of Mcl-1, which was necessary and sufficient for the survival of NK cells<sup>45</sup>. NK cells are dramatically reduced in patient carrying mutations, IL-15 may regulate human NK cell development<sup>46,47</sup>, and human IL15 (hIL15) induces survival and proliferation of mouse NK cells<sup>48</sup>. IL-18 promotes NK cell proliferation and activation, alleviating IL18-induced FasL expression and activation of Fas-mediated death signaling and increasing anti-apoptosis molecules (Bcl-XL)<sup>49</sup>.

Interleukins (IL2, IL15, and IL18) triggered cytokine (IFN-γ and TNF-α) secretion by NK cells. This result was supported by previous research demonstrating that NK cells can be activated by various stimuli, including cytokines such as IL-2, IL-12, IL-15, and IL-18<sup>50</sup>. Upon cytokine stimulation, NK cells become LAK cells that proliferate, produce cytokines, and up regulate effector molecules such as adhesion molecules, NKp44, PRF, Gzm, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL)<sup>51,52</sup>. Other factors,

such as TNF- $\alpha$ , IL-1, IL-2, IL-15 and CD28, serve as co-stimulatory factors, enhancing IFN- $\gamma$  production by NK cells, as well as NK cell proliferation and cytotoxicity<sup>53,54</sup>. IL-12 and IL-18 stimulate NK cell lines, increasing the secretion of IFN- $\gamma$ <sup>55</sup>. IL-12 and IL-18 are critical regulators that activate NK cells via the production of cytokines and direct lysis of target cells<sup>56</sup>. Two of the primary cytokines released by activated NK cells are IFN- $\gamma$  and TNF- $\alpha$ <sup>57</sup>. Activated NK cells are potent producers of numerous cytokines, including IFN- $\gamma$ , TNF- $\alpha$  and numerous chemokines<sup>56</sup>.

Co-cultured BC cells (MCF7) and activated NK cells released higher levels of IFN- $\gamma$ , TNF- $\alpha$ , PRF1, and GzmB compared to non-induced NK cells. Interleukins (IL-2, IL-15, and IL-18) increased the secretion of IFN- $\gamma$ , TNF- $\alpha$ , PRF1, and GzmB; this result was in line with previous research that demonstrated an increase in the production of IFN- $\gamma$  and TNF- $\alpha$  in CD107a of co-cultured NK-92 cells with cancer cells (HeLa, SiHa, and C-33A) pre-treated with the antitumor HO-1 inhibitor (SnPP). Antitumor agents increased IFN- $\gamma$  and TNF- $\alpha$  in co-cultured NK cells and cancer cells<sup>19</sup>. Expression of apoptosis-related proteins such as cleaved caspase-3 and Bax was increased in cancer cells co-cultured with NK-92 cells<sup>20</sup>. NK cells kill cancer cells by the release of cytoplasmic granules that contain a number of proteins, such as PRF1 and Gzm, which lyse target cells<sup>20</sup>. NK cells release the membrane-disrupting protein, PRF1, and proteolytic serine proteases, Gzm, from secretory granules<sup>57</sup>. The secretory pathways in NK cells ultimately regulate the separate lytic and regulatory capacity of these cells at the frontline of antitumor responses<sup>58</sup>. When K562 target cells and NK cells were co-cultured at ratios of 5:1 or 1:1, after 60 min, NK cells produced the cytokines IFN- $\gamma$  and TNF- $\alpha$ <sup>58</sup>.

NK cells have anticancer potency, and higher NK cell numbers significantly increased cytotoxic activity (Table 3). These data were verified by previous studies showing that these cells are major effector cells of innate immunity and are generally thought to play a fundamental role in antitumor responses<sup>8,59</sup>. NK cells control tumor growth and metastasis diffusion *in vivo*<sup>50</sup>. There are low NK cell numbers in tumors due to their inefficient homing into malignant tissues<sup>8</sup>. Decreasing NK cell numbers are observed in PB of cancer patients; therefore, NK cells decrease in tumor infiltrate<sup>8</sup>. The activity and numbers of NK cells need to be enhanced for better efficacy<sup>60</sup>. NK cell infiltration in solid tumors was associated with a better prognosis<sup>61</sup>. NK cells

need to be isolated and expanded in sufficient numbers for them to act as effector cells<sup>60</sup>. The comparison of effector and tumor determined NK cell cytotoxicity. A previous study showed that the lowest ratio (1.25:1) of effector and cervical epidermoid carcinoma (CaSki), effector and grade II, human cervix squamous cell carcinoma (SiHa) resulted in the highest viable cell target, while the highest ratio (20:1) resulted in the lowest viability or highest inhibition of target cells<sup>62</sup>. In mice lacking IL-12 and IL-18 cytokines, the cytolytic activity of the NK cells is further impaired, indicating that the cytolytic activity of NK cells is synergized by IL-12 and IL-18 *in vivo*<sup>19</sup>. The NK cells kill cancer cells through at least three mechanisms. NK cells can use the PRF1/Gzm-containing granule exocytosis pathway, the nitric oxide (NO) pathway and the death receptor-ligand pathway. The PRF1/Gzm pathway is the principle pathway by which NK cells kill cancer cells<sup>63</sup>.

## Conclusion

IL-2, IL-15, and IL-18 improved activating receptors and proliferation of NK cells. IL-induced NK cells increased TNF- $\alpha$ , IFN- $\gamma$ , PRF1, and GzmB secretion and cytotoxic activity on BC cells. High NK cell numbers increased BC cell growth inhibition.

## Acknowledgments

This study was supported by the Grants-in-Aid from Penelitian Dasar Unggulan Perguruan Tinggi (2018-2020) and Bantuan Seminar Luar Negeri 2018, the Ministry of Research, Technology and Higher Education of the Republic of Indonesia. The authors like to thank to Yukko Arinta, Annisa Amalia, Rismawati Laila Q, Fajar Sukma Perdana, Annisa Arlisyah, Jenifer Kiem Aviani and Ika Adhani Sholihah from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia for their technical assistants.

## Conflict of interest

None.

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