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Apoptotic Potential of Secretome from Interleukin-Induced Natural Killer Cells toward Breast Cancer Cell Line by Transwell Assay

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ABSTRACT

Breast cancer (BC) is the number one cause of deaths from cancer in women. Metastasis in BC is caused by immunosurveillance deficiency, including impairment of Natural Killer (NK) cell maturation, low NK activity, and decreasing cytotoxicity. This study was performed to improve activating receptors and cytotoxicity of NK cells using interleukin 15 (IL15) against BC cells. Human recombinant IL15 was used to induce NK cells. To evaluate the potential of IL15 in inducing NK cells, we measured the activating and inhibiting receptors (NKG2D, NKG2A), apoptotic potency of NK cells on BC cells (MCF7) using transwell assay. The IL15 inducer on the NK cell were measured NKG2D, NKG2A gene expression with quantitative polymerase chain reaction (qPCR), (GzmB) secretion using ELISA, apoptotic gene expression of MCF7 using qPCR. IL15 increased NKG2D expression 4.01-9.13%, but IL15 could not affect toward NKG2A expression on NK cells. IL15-activated NK cells, inhibited BC cells proliferation, induced apoptotic BC cells 25.89-32.19%, induced apoptotic genes of BC cells bax, p53. IL15 increase NK activating receptor (NKG2D), inhibit BC cells proliferation, induce apoptotic percentage and induce apoptotic gene expression.

1. Introduction

Breast cancer (BC) is one of the main causes of death in the world. In 2012, it caused around 8.2 million deaths (Ferlay *et al.* 2015). BC is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths (Tirona *et al.* 2010). Metastasis in BC is caused by deficient immunosurveillance, including impairment of Natural Killer (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 (Dewan *et al.* 2009), decreased cytotoxic function (Levy *et al.* 2011; Hwang *et al.* 2012), NK abnormalities (Levy *et al.* 2011), poor tumor infiltrate (Albertsson *et al.* 2003; Esendagli *et al.* 2008), low NK cell numbers in tumors due to their inefficient homing into malignant tissues (Levy *et al.* 2011), and defective expression of activating receptors such as NKG2D, overexpression of inhibitory receptors NKG2A, CD158a, CD158b (Levy *et al.* 2011).

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 (Cheng *et al.* 2013). NK cells are known as necessary effectors in suppressing cancer proliferation (Kelly *et al.* 2003; Smyth *et al.* 2005). Once NK cells recognize target cells such as cancer cells, they form an immunological synapse, and the secretory granules fuse with the presynaptic membrane and release perforin (Prf) and granzyme (Gzm) into the synaptic cleft. Released Prf provides transmembrane pores on the target cell

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and enables Gzm to diffuse into the cell. Gzm then initiates the apoptosis of the target cells, and the NK cells detach from the dying cells and can interact with other target cells to accomplish serial killing (Harada *et al.* 2017). Therefore, the focus of recent cancer therapy has been to promote and develop NK cells as drugs (Hwang *et al.* 2012), using NK effectors such as cytokines (Levy *et al.* 2011). This study was conducted to evaluate the effect of inducing Interleukin 15 (IL15) on NK cells toward the following: i) improvement of NK cell activating receptors (NKG2D), ii) inducement cytotoxic towards BC cells, iii) apoptotic inducer of NK cells, iv) apoptotic genes expression of BC cells, vii) morphology and density of MCF7 cells by transwell of NK and BC cells.

2. Materials and Methods

2.1. Culturing Breast Cancer and NK Cells

Breast cancer (MCF7) cells (ATCC® HTB-22™) obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia were cultured in RPMI 1640 (Gibco, 11875093) supplemented with 10% FBS (Gibco, 26140079) and 1% antibiotic/antimycotic. Meanwhile, NK92 cells (ATCC® CRL-2407™) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia were cultured in MEM-without nucleosides (Gibco, 12561056) supplemented with 12.5% FBS, 12.5% Horse Serum (Gibco, 16050122), Sodium bicarbonate (Merck, 106329), Myo-inositol (Sigma Aldrich, 17508), Folic Acid (Sigma Aldrich, F8758), IL-2 (BioLegend, 589106), 2-Mercaptoethanol (Gibco, 1628448), and 1% antibiotic/antimycotic (Gibco, 1772653). Cell cultures were maintained in T-75 flasks, at 37°C, 5% CO_2. Culture mediums were changed every three days and sub-cultured at 80% confluence (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.2. Determination of NKG2A and NKG2D Gene Expression

A suspension of NK92 cells (1 x 10^6 cells) were seeded onto T-25 Flask (TPP, 90026) and incubated for 24 hours at 37°C, 5% CO_2. Afterwards, the cells were induced by human recombinant IL15 (BioLegend, 715902) to reach final concentration at 5 ng/ml and 10 ng/ml and incubated for 24 hours at 37°C, 5% CO_2. The RNA isolation of NK92 was performed according to the protocol of RNA Isolation Kit (Bio-Rad, 732-6820). The concentration and purity of RNA of each sample was determined at 260/280 nm. The cDNA synthesis was performed using cDNA synthesis kit (Bio-rad, 170-8841) (Widowati *et al.* 2018; Afifah *et al.* 2019; Widowati *et al.* 2019). Primer sequences were used can be seen at Table 1.

2.3. Cytotoxic Assay

MCF7 and NK92 cells were seeded at a ratio 1:1 onto transwell 24 well-plate (Corning, 3396). MCF7 were seeded on the first day allowed to attach to the bottom chamber. Next day, NK92 induced by IL15 (0, 5, 10 ng/ml) were seeded to the upper chamber. The plate was incubated for 24 hours at 37°C, 5% CO_2. Hereinafter, the upper chamber and the medium were discarded. The cells were washed one time using PBS 1x. After that, 500 µl Presto Blue 1x (Invitrogen, A13262) were added to each well and incubated for 1 hour at 37°C, 5% CO_2. The solution was transferred to 96-well plate and the absorbance was read at 570 and 600 nm using spectrophotometer (Multiskan GO, ThermoScientific) (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.4. Apoptosis Analysis Assay by Flow Cytometry

MCF7 and NK92 cells were seeded at a ratio 1:1 onto 6 well/plate (Corning, 3412). On the first day, MCF7 were seeded at the bottom chamber and

Table 1. Activator, inhibitor receptor genes of NK Cells, RT PCR protocols

Gene symbols	Primer sequence (5' to 3') Upper strand:sense Lower strand:antisense	Product size (bp)	Annealing (°C)	Cycle	References
NKG2A	5'-CCAGAGAAGCTCATTGTTGG-3' 5'-CCAATCCATGAGGATGGT-3'	168	51	40x	Gen Bank:AF461812.1
NKG2D	5'-CTGGGAGATGAGTGAATTTCATA-3' 5'-GACTTCACCTTAAGTAAATC-3'	417	51	40x	Gen Bank:AF461811.1
GAPDH	5'-GGGCTGCTTTTAACTCTGGT-3' 5'-TGGCAGGTTTTTCTAGACGG-3'	702	51	40x	Sadeghi *et al.* 2015

plates were incubated for 24 hours at 37°C, 5% CO_2. On the next day, NK92 cells were added to the upper chamber. The NK92 cells were induced by IL15 (5 ng/ml and 10 ng/ml). The uninduced NK92 cells and MCF7 without transwell were also done as a control. The plate was then incubated for 24 hours at 37°C, 5% CO_2. Subsequently, MCF7 at the bottom chamber were collected and washed two times by FACS buffer (2% FBS in PBS 1x). Pellets were resuspended in 100 µl FACS buffer and 5 µl Annexin V (BioLegend, Part 79998), 10 µl PI (BioLegend, Part 79997) were added. The cells were incubated for 30 minutes in 4°C dark room. Following the 30 minutes incubation, samples were added 400 µl annexin V-binding buffer (BioLegend, 640194) and immediately analyzed by flow cytometry (MACSQuant™ Analyzer 10, Miltenyi Biotec) (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.5. Quantification of Granzyme-B Level

The quantitative determination of GzmB in the transwell-treatment, conditioned medium from NK cells from upper chamber and conditioned medium from MCF7 was performed using ELISA Kit Human Granyme-B (Human ELISA KIT, ElabSci E-EL-H1617) followed the manufactur protocol. Absorbance was read at 450 nm using spectrophotometer (Safta *et al.* 2015; Widowati *et al.* 2018, 2020).

2.6. Determination of Apoptosis Gene Expression

MCF7 cells had been transwelled with induced NK92 cells (IL15 with various concentration 0, 5, 10 ng/ml) for 24 h. Afterward, microscopic analysis was conducted under inverted phase-contrast microscope. Subsequently the MCF7 cells were collected to isolate the RNA. The RNA isolation of MCF7 was performed based on the protocol of RNA Isolation Kit (Bio-Rad, 732-6820). The concentration and purity of RNA of each sample was determined at 260/280 nm. The cDNA synthesis was performed using cDNA synthesis kit (Bio-rad, 170-8841) (Widowati *et al.* 2018; Afifah *et al.* 2019; Widowati *et al.* 2019). Primer sequences can be seen at Table 2.

2.7. Statistical Analysis

All the data are presented as the mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons with p-values less than 0.05 were considered significant.

3. Results

3.1. Effect of IL15 toward NK Cell Characteristics

To determine the effect of human recombinant IL15 toward NK receptors, we evaluated the NK receptors including gene expression of NKG2D and NKG2A (Figure 1). The data showed that IL15 (5, 10 ng/ml) significantly up-regulated NKG2D, meanwhile IL15 did not significantly down-regulate NKG2A.

Based on the data (Figure 1) shows that IL15 induced up-regulation of NKG2D gene expression, the highest NKG2D expression was IL15 (5 ng/ml) higher concentration of IL15 (10 ng/ml) lowered NKG2D expression. IL15 both concentrations (5, 10 ng/ml) didn't affect NKG2A expression of NK cells.

3.2. Cytotoxic Activity of IL15-Induced NK toward BC Cells

NK cells are involved in the elimination of tumor cells. To determine the effect of human recombinant IL15 on NK cells toward cytotoxic activity of BC cells. We determine the effect of IL15-induced NK toward MCF7 cells by transwell method. NK cells treated with IL15 at concentrations of 0, 5, 10 ng/ml (IL15-induced NK cells) against BC cell proliferation, we evaluated the inhibition of MCF7 proliferation (Figure 2).

Figure 2 shows that NK cells uninduced with IL15 had cytotoxic activity against MCF7 cells approximately 35.20% inhibitory growth cells. Both concentrations 5,

Table 2. Apoptotic primer for MCF7 cells, RT PCR protocols

Gene symbols	Primer sequence (5' to 3') Upper strand:sense Lower strand:antisense	Product size (bp)	Annealing (°C)	Cycle	References
p53	5'-AGAGTCTATAGGCCCACCCC-3' 5'-GCTCGACGCTAGGATCTGAC-3'	97	58	40x	Behbahani 2014; Widowati *et al.* 2019
bax	5'-TGCTTCAGGGTTTCATCCAG-3' 5'-GGCGGCAATCATCCTCTG-3'	169	58	40x	Wang *et al.* 2009; Widowati *et al.* 2019
bcl2	5'-GGTCATGTGTGTGGAGAGCG-3' 5'-GGTGCCGGTTCAGGTACTCA-3'	89	58	40x	Guan *et al.* 2018; Widowati *et al.* 2019
β-Actin	5'-TCTGGCACCACACCTTCTACAATG-3' 5'-AGCACAGCCTGGATAGCAACG-3'	166	85	40x	Han *et al.* 2018

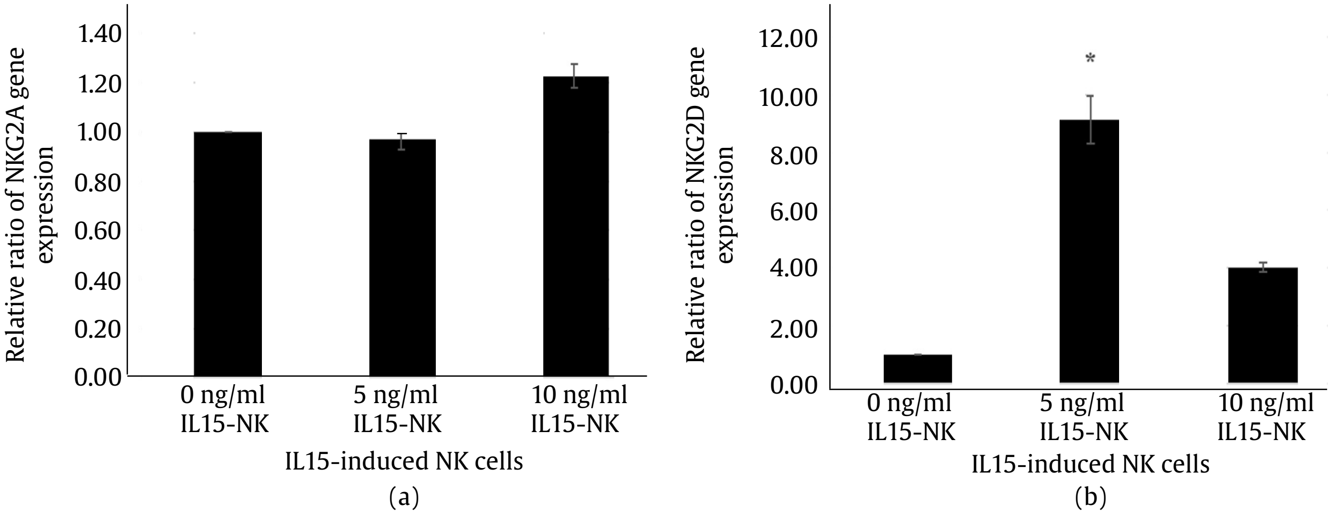

Figure 1. Effect IL15 inducer on NK cells toward NKG2A and NKG2D gene expression. The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) and non inducing of NK cells based on Tukey HSD post hoc test (p<0.05). (a) The IL15 didn't affect NKG2A gene expression. (b) The NKG2D expression of higher concentration (10 ng/ml IL15) was lower than 5 ng/ml IL15

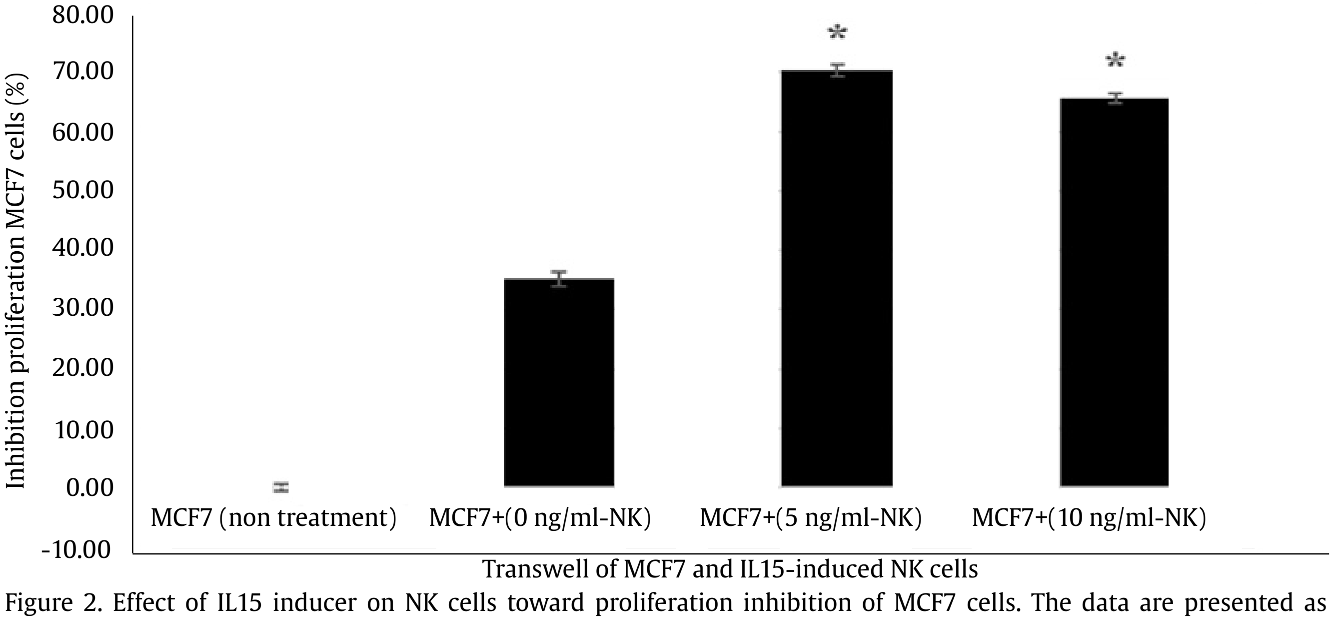

Figure 2. Effect of IL15 inducer on NK cells toward proliferation inhibition of MCF7 cells. The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) and non inducing of NK cells based on Tukey HSD post hoc test (p<0.05). Figure 2 shows that IL15 activated NK cells to inhibit MCF7 cell proliferation and increased anticancer activity NK cells against proliferation of MCF7 cells. The higher concentration (10 ng/ml) of IL15 was not significant difference compared to lower concentration of IL15 (5 ng/ml) against MCF7 cells proliferation

10 ng/ml of IL15 induced NK cells to inhibit MCF7 cells proliferation about 65.60-70.29%.

3.3. Effect of IL15-Induced NK Cells toward Apoptosis of MCF7 Cells

NK cells require effectors to activate NK cells; thus, this study was conducted to evaluate the effect of IL15 toward apoptosis of MCF7 cells by transwell method. To determine the effect of human recombinant IL15 on NK cells toward apoptotic percentage of MCF7 cells, we evaluated apoptotic percentage of MCF7 cells (Figures 3). We used IL15 at levels of 0 (uninduced), 5 and 10 ng/ml. The data (Figure 3) show that IL15 increased apoptotic percentage of MCF7, the highest apoptotic potency was NK induced by 5 ng/ml, higher concentration of IL15 (10 ng/ml) lower apoptotic percentage than NK induced by 5 ng/ml.

Based on the apoptotic percentage of MCF7 cells were treated by IL15-induced NK cells using transwell method (Figure 3), shows that NK cells uninduced with IL15 was very low apoptotic potency (3.98%), IL15 activated NK cells and increased apoptotic potency against MCF7 cells, the best IL15 concentration was 5 ng/ml, higher concentration of IL15 significantly lowered apoptotic percentage of MCF7 cells.

3.4. Effect of IL15-Induced NK Cells toward Granzyme Level in Transwell of MCF7 and NK Cells

We measured NK cells were activated using IL15 (0, 5, 10 ng/ml). The cell ratios (MCF7: NK cells=1:1) were assessed for the ability to secrete GzmB. The effects of IL15-induced NK (IL15-NK) transwelled with MCF7 cells, which made NK released cytoplasmic granules (GzmB) can be seen in Figure 4.

The GzmB level secreted by NK cells which co-cultured with MCF7 cells using transwell method (Figure 5). NK cells uninduced by IL15 secreted GzmB in low level (77.06 pg/ml), inducing IL15 (5, 10 ng/ml) increased secretion of GzmB, the highest GzmB level was secreted by NK cells which induced by IL15 (5 ng/

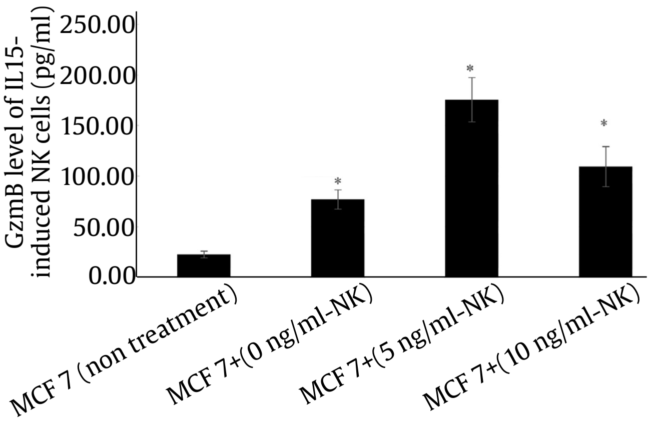

Figure 4. Effect of IL15 inducer on NK cells toward GzmB level of transwell MCF7 and NK cells. The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment. The asterisks symbol (*) present significant differences among concentrations of inducer IL15 (0, 5, 10 ng/ml) compared to control (MCF7 non treatment) toward GzmB level

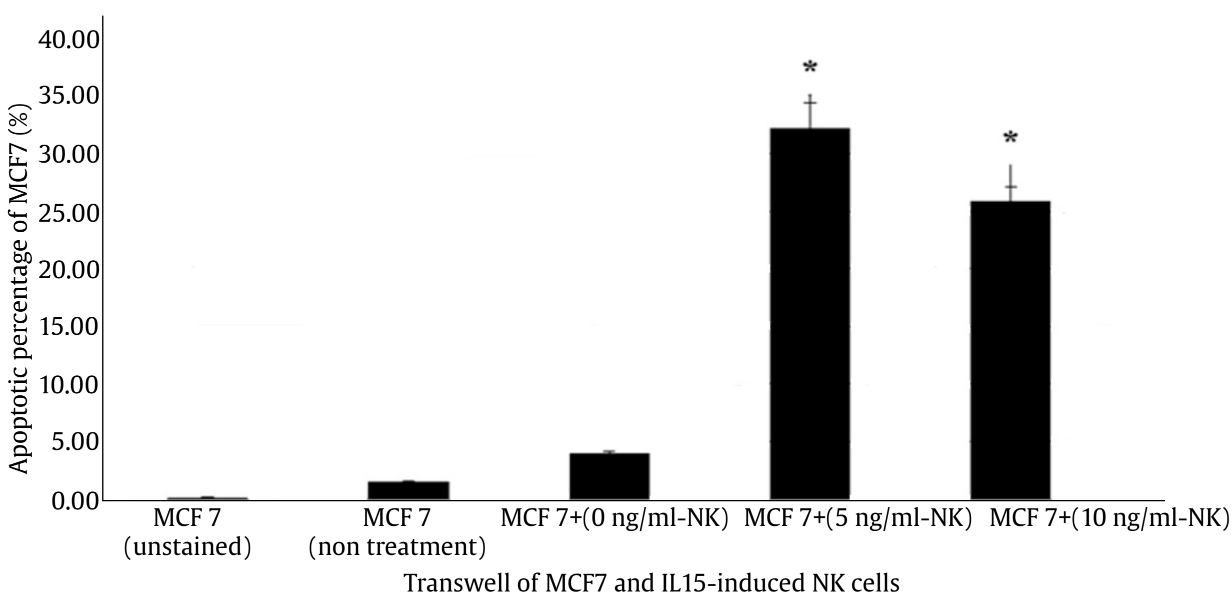

Figure 3. Effect IL15 inducer on NK cells toward apoptotic percentage of MCF7 cells. The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment. The asterisks symbol (*) present significant differences among concentrations of IL15 inducer (0, 5, 10 ng/ml) compared to control (MCF7 non treatment) toward apoptotic percentage of MCF7

ml), meanwhile higher IL15 concentration (10 ng/ml) lowered GzmB level.

3.5. Effect IL15-Induced NK Cells toward Apoptotic and Antiapoptotic Genes Expression of MCF7

This study was the continued-research to elucidate the apoptosis mechanism of IL15-induced NK cells toward MCF7 cells, in order to determine the apoptotic inducing activity of IL15-induced NK cells toward MCF cells by transwell assay, the expression of apoptotic genes was determined by qPCR. We measured the expression of proapoptotic genes, specifically p53, b-cell cll/lymphoma 2 (bcl-2), bcl2-associated x protein (bax). The p53, bax and bcl2 genes expression of MCF7 can be seen at Figure 5.

NK cells induced by IL15 significantly increased the apoptotic genes both low and high concentrations (5, 10 ng/ml) toward p53, bax genes expression (Figure 5a and 5b) but IL15-induced NK didn't affect bcl2 expression (Figure 5c). The highest p53 expression was MCF7 treated with 5 ng/ml IL15-induced NK cells, the higher concentration

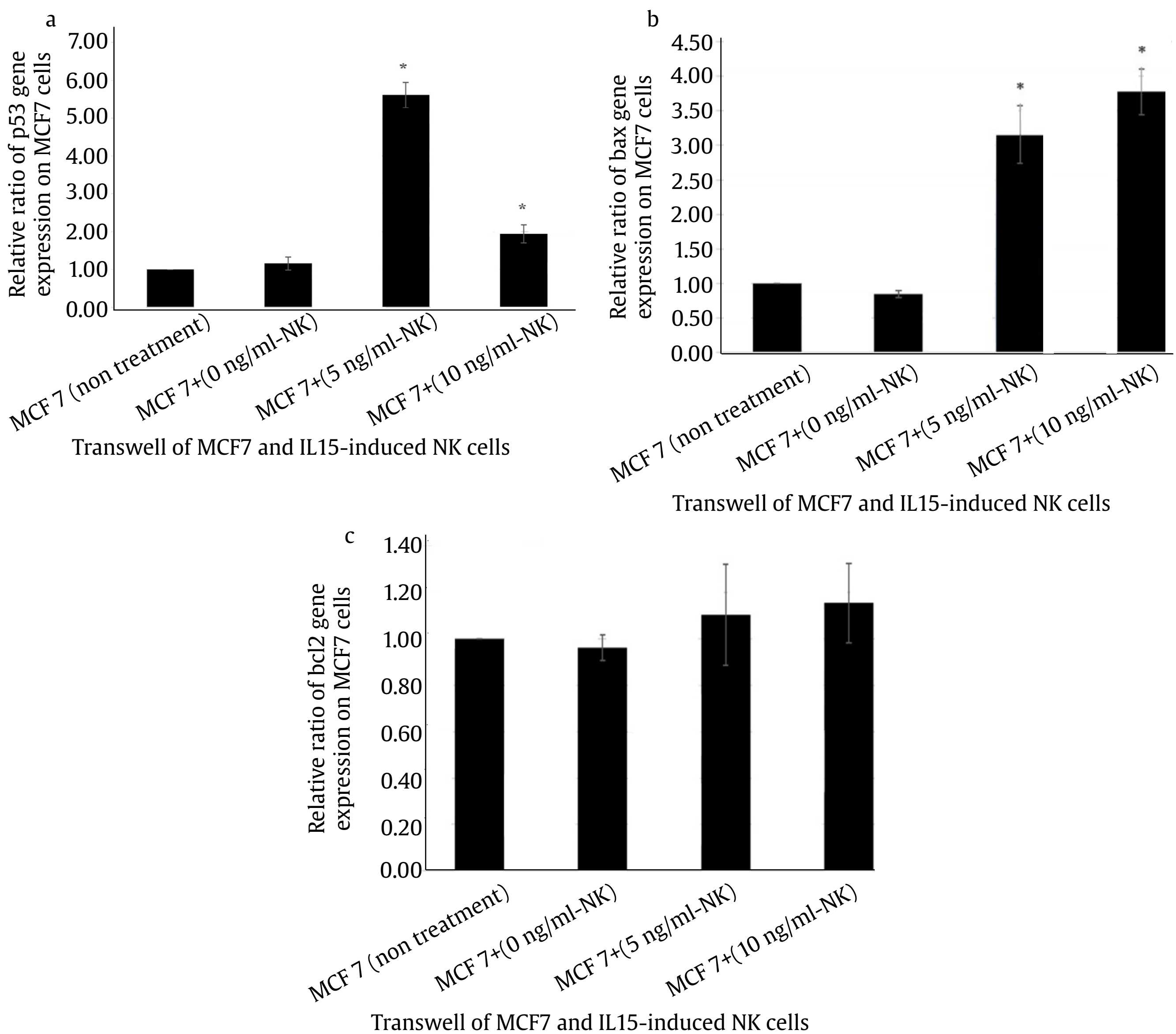

Figure 5. Effect IL15 inducer on NK cells toward p53, bax and bcl2 gene expression of MCF7 cells. The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) compred control (MCF7 non treatment) based on Tukey HSD post hoc test ($p<0.05$). Figure 5 shows that IL15 (5, 10 ng/ml) up-regulated gene expression of p53 (Figure 5a), bax (Figure 5b), but didnt affected bcl2 gene expression (Figure 5c)

of IL15 (10 ng/ml) was significantly lower p53 expression (Figure 5a). The bax gene expression of MCF7 cells treated with 10 ng/ml IL15-induced NK cells was unsignificantly difference compared to 5 ng/ml IL15-induced NK cells.

3.6. Effect IL15-Induced NK Cells against MCF7 Cells Morphology

This study was performed by investigating the effect of IL15-induced NK cells on viability, density, morphology of MCF7 cells (Figure 6). The

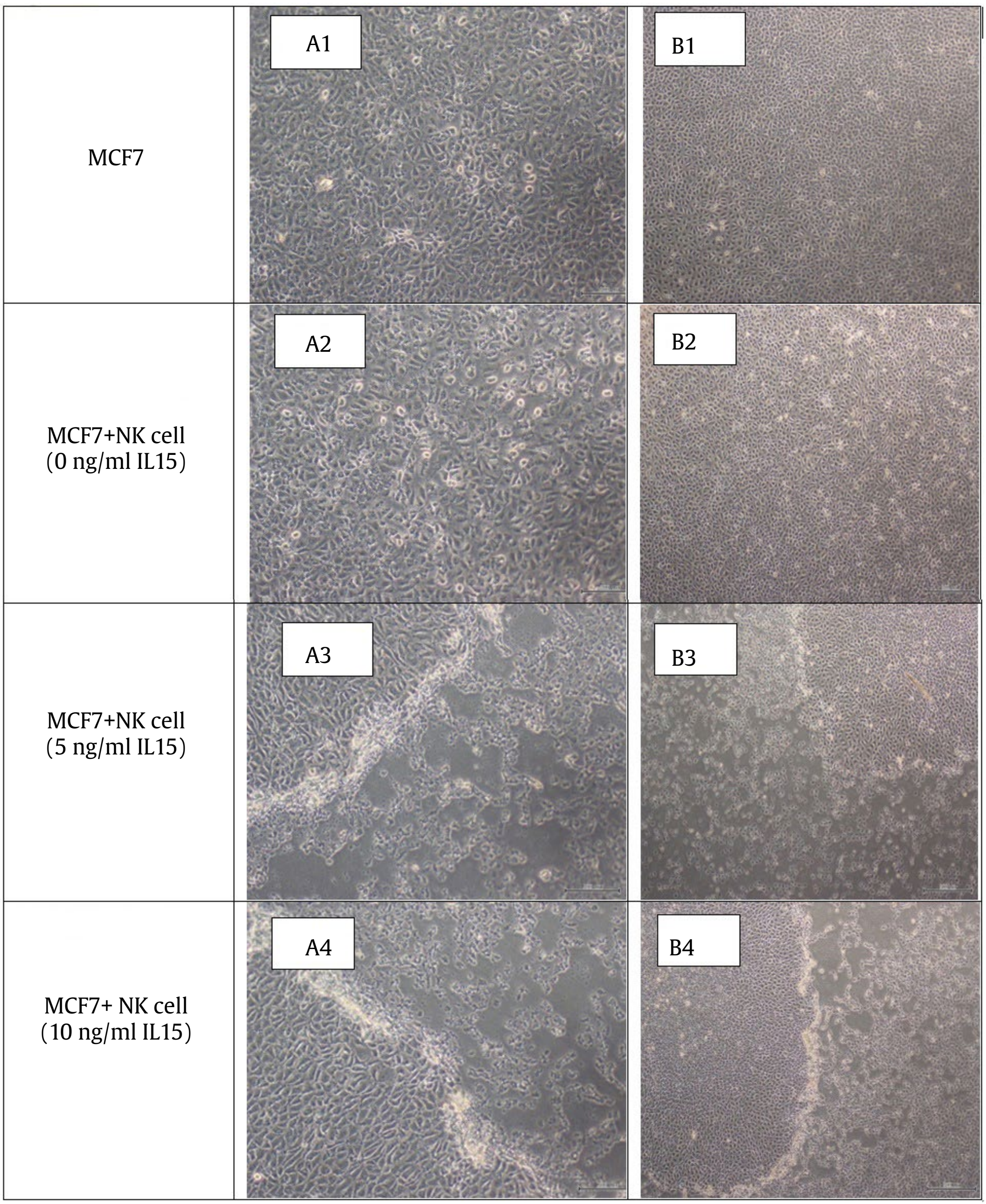

Figure 6. Morphological analysis using phase-contrast microscope at ×100 magnification (A1, A2, A3, A4) and x40 magnification (B1, B2, B3, B4). Different concentration of IL15 as inducer for NK cells decreased the survival rate of MCF7 cells

result demonstrates that NK cells induced dose-dependent reduction in viability of MCF7 cells.

Based on the morphological MCF7 cells treated with NK cells using transwell assay shows that NK cells lower the viability and density BC cells. The highest viability and density cells was MCF7 (control), the lowest viability, density cells was MCF7 treated by IL15 (5 ng/ml) as NK cells inducer.

4. Discussion

NK cell abnormalities in cancer patients such as imbalance of immune status, inclined to immunosuppression, decreased in NK cell numbers, decreased cytotoxicity, declined activiating receptor (NKG2D, NKG2C, NKp30, NKp46) and inclined inhibitory receptor (NKG2A, CD158, CD158a). Recently has been developed the immunotherapy using activated-NK cells (Mamessier *et al.* 2011). IL2, IL12, IL15, and IL18, 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 (Konjevic *et al.* 2016). Cytokines such as interleukins (IL2) can be used to increase the anticancer potency of NK cells (Widowati *et al.* 2019). Various types of stimulation have been reported to enable NK cells to achieve their full effector potential, such as IL15, IL12, IL18, IL21 (Lucas *et al.* 2007; Guia *et al.* 2008; Chaix *et al.* 2008; Brehm *et al.* 2011). Based on the result (Figure 1), IL15 induced activating receptor NKG2D, this result was validated with previous research that IL2 and IL15 induce the expression of KIRs and activating receptors (NKG2D and NKp44) on NK cell surface (Rham *et al.* 2007; Boieri *et al.* 2017). This result was in line with previous research that metastatic melanoma (MM) patients exhibited decreased CD161 and NKG2D (Konjevi'c *et al.* 2009). NK cells treated with IL15 up-regulated the expression of NK receptors, including Nkp30, Nkp46, NKG2C, and NKG2D (Szczepanski *et al.* 2010). IL15 didn't decline the inhibitory receptor (NKG2A), this result was not consistent with previous research that IL15+IL12 was significantly decline the NKG2A of HIV-infected individuals (Parasa *et al.* 2012).

NK cells have been used in clinical studies in order to treat various malignancies (Harada *et al.* 2017), NK cells abnormalities in cancer patients such as low NK cells counts in PBMC, decreased cytotoxicity (Levy *et al.* 2011). NK cells inhibited MCF7 cells proliferation, IL15 activated NK cells to inhibit MCF7 cells proliferation, this result was consistent with previous research that NK cells control tumor growth and metastasis diffusion *in vivo* (Zamai *et al.* 2007). The low NK cell numbers in tumors due to their inefficient homing into malignant tissues (Levy *et al.* 2011). Decreasing NK cell numbers are observed in peripheral blood (PB) of cancer patients; therefore, NK cells decrease in tumor infiltrate (Levy *et al.* 2011). The activity and numbers of NK cells need to be enhanced for better efficacy (Mandal and Viswanathan 2002). NK cell infiltration in solid tumors was associated with a better prognosis (Villegas *et al.* 2002). IL-15 is essential for NK cell survival, differentiation, and proliferation (Anton *et al.* 2015). Improving NK cells cytotoxicity used ILs (IL15, IL18), the result showed that ILs increased TNFα, IFNγ secretion by NK cells (Widowati *et al.* 2020)

IL15 induces optimal production of IFN-γ from NK cells, subsequently induce apoptosis of the NK cells toward cancer cells (Ross and Caligiuri 1997; Widowati *et al.* 2020). The human MHC class I-negative of small cell lung cancer cell line (N592) genetically engineered to secrete IL-15, N592/IL-15, showed a reduced tumor growth rate (Orengo *et al.* 2003). IL15 and IL12 induces optimal production of IFN-γ from NK cells (Ross and Caligiuri 1997). IFNγ inhibited BC proliferation (MCF7) with median inhibitory 0.34 µg/ml (Widowati *et al.* 2016). IFNγ against MCF7 cells exhibited that the cytokines decreased the cell viability in a dose dependent manner (Widowati *et al.* 2016).

NK secrete GzmB, IL15-activated NK cells up-regulated GzmB secretion. This result was validated previous research that activated NK cells released higher levels of IFN-γ, TNF-α, Prf1, and GzmB compared to non-induced NK cells. IL2, IL15, and IL18 increased the secretion of IFN-γ, TNF-α, Prf1, and GzmB by co-culture cells (Widowati *et al.* 2020). IL15 in NK cell controls as well survival of mature NK cells in the periphery (Marçais *et al.* 2013; Widowati *et al.* 2020), mediated by up-regulation of anti-apoptotic bcl2 family members and down regulation of apoptotic (Marçais *et al.* 2013). The production of TNF-α, IFN-γ, Prf1, GzmB increased when the ratio of NK cells and hWJMSCs was high (Widowati *et al.* 2019). NK cells activity were controlled by cytokine and ILs (IL2, IL12, IL15, IL18) (Domaica *et al.* 2012). NK cells eliminate malignantly transformed cells principally by releasing the contents of cytotoxic granules into the immune synapse formed with their target cell (Lieberman 2003). The granule mediators of target cell lysis are serine proteases, known as Gzm, which induce programmed cell death (Safta *et al.* 2015; Harada *et al.* 2017). Cytotoxicity of NK cells is executed mainly through the granule exocytosis pathway by releasing Prf1 and GzmB into the immunological synapse after the conjugate formation with targets (Lieberman 2003). Human GzmB preferentially induces target cell apoptosis, induces a rapid accumulation of the tumor-suppressor protein p53 within target cells (Safta *et al.* 2015). Human GzmB-induced p53 accumulates on target cell mitochondria where it interacts with the prosurvival protein bcl2. This interaction allows the release of the proapoptotic protein bax from its inhibitory interaction with bcl2 (Safta *et al.* 2015). Several proteins that are involved in GzmB-induced apoptosis, including casp-9 and -3, bim, bid, bak, bax, and xiap. GzmB induced apoptosis cancer cells by involving induction of p53 tumor suppressor gene (Meslin *et al.* 2007).

IL15 inhibit MCF7 proliferation, induced apoptotic BC cells, this result also supported by MCF7 morphology, BC cells appear low density and viability. This result was supported by previous research that ILs (IL1, IL2, IL15) and CD28, serve as co-stimulatory factors, enhancing IFN-γ production by NK cells, as well as NK cell proliferation and cytotoxicity (Hunter *et al.* 1997; Cui *et al.* 2016). ILs (IL12, IL18) stimulate NK cell lines, increasing the secretion of IFN-γ (Wang *et al.* 2012). IL12 and IL18 are critical regulators that activate NK cells via the production of cytokines and direct lysis of target cells (Vivier *et al.* 2011). IL15 activate, increase NK proliferation (Widowati *et al.* 2020). Activated NK cells induce IFN-γ, TNF-α, Prf1, GzmB secretion and inhibit BC cells proliferation on co-cultured of MCF7 and NK cells (Widowati *et al.* 2020).

The proposed mechanism of our research which IL15 activated NK cells including increased NK cells number, up regulated activating receptor (NKG2D) but IL15 didn't influnce inhibitor receptor (NKG2A), improved Prf, Gzm secretion. IL15-activated NK induced apoptosis of BC cells through increased p53, bax gene expression and inhibited BC cells proliferation. For the detail mechanism can be seen at Figure 7.

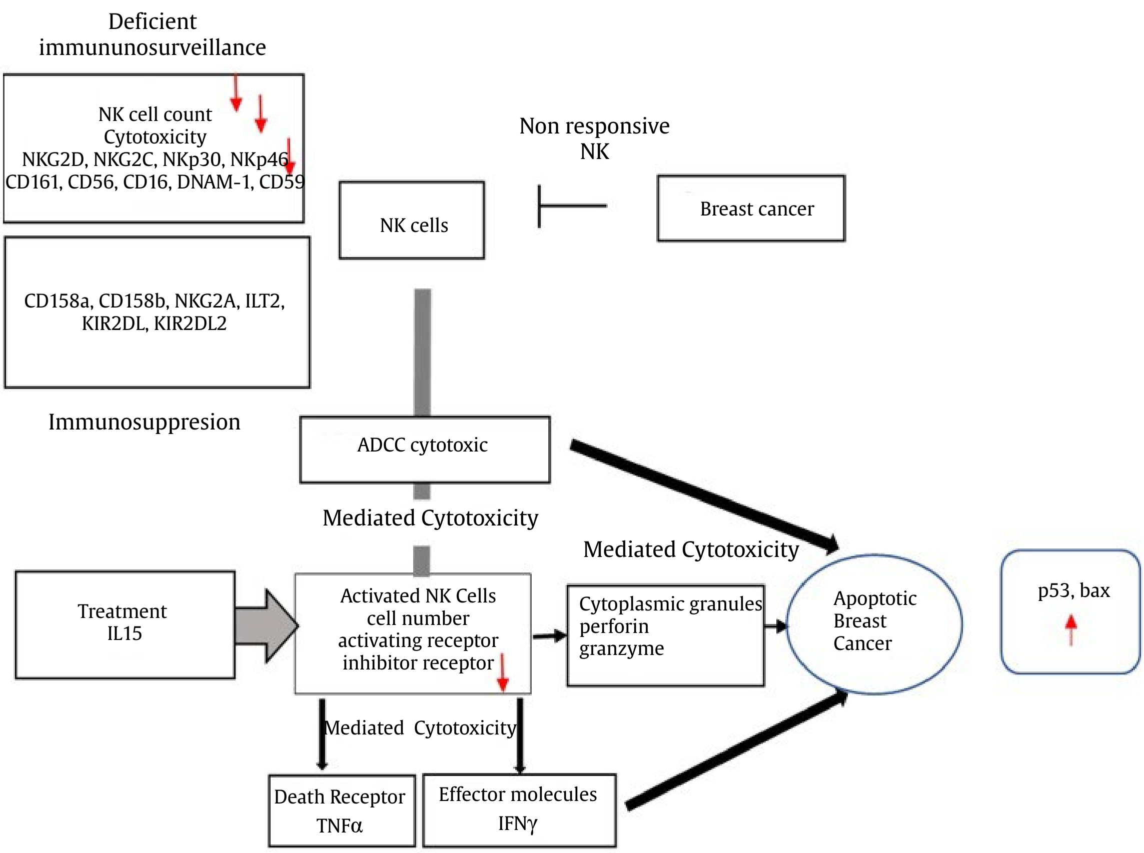

Figure 7. Proposed mechanism of activated NK cells to inhibit and kill breast cancer cells. NK cells abnormalities in breast cancer patients. The imbalance of immune status, which defficient immunosurveillance including low NK cell number in PBMC, poor tumor inflitrate to cancer cells, decreased cytotoxicity, defective expression of activating receptor (NKG2D). IL15 activated NK cells to kill target cancer cells through: cytoplasmic granules release (Prf, GzmB), death receptor-induced apoptosis (TNFα), effector molecules production (IFNγ) and released antibody-dependent cellular cytotoxicity (ADCC). p53, bax induced apoptotic BC cells

5. Conclusion

IL15 improve, activate NK cells resulted in activating receptors (NKG2D), increasing GzmB secretion and cytotoxic activity on BC cells, inducing apoptotic genes bax, p53 expression and induce apoptotic cells on transwell assay.

Acknowledgements

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Apoptotic Potential of Secretome from Interleukin-Induced Natural Killer Cells toward Breast Cancer Cell Lines by Transwell Assay

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ABSTRACT

Breast cancer (BC) is the number one cause of deaths from cancer in women. Metastasis in BC is caused by immunosurveillance deficiency, including impairment of NK cell maturation, low NK activity, and decreasing cytotoxicity. This study was performed to improve activating receptors and cytotoxicity of NK cells using interleukin (IL15) against BC cells. Human recombinant IL15 was used to induce NK cells. To evaluate the potential of IL15 in inducing NK cells, we measured the activating and inhibiting receptors (NKG2D, NKG2A), apoptotic potency of NK cells on BC cells (MCF7) using transwell assay. The IL15 inducer on the NK cell were measured NKG2D, NKG2A gene expression with quantitative polymerase chain reaction (qPCR), the apoptotic potency of NK toward BC cells using flowcytometer, granzyme B (GzmB) secretion, apoptotic gene expression of MCF7 cells. IL15 increased NKG2D expression 4.01-9.13%, but IL15 could not affect toward NKG2A expression on NK cells. IL15-activated NK cells, inhibited BC cells proliferation, induced apoptotic BC cells 25.89-32.19%, induced genes apoptotic of BC cells bax, p53. IL15 increase NK activating receptor (NKG2D), inhibit BC cells proliferation, induce apoptotic percentage and induce apoptotic gene expression.

1. Introduction

Breast cancer (BC) is one of the main causes of death in the world. In 2012, it caused around 8.2 million deaths (Ferlay *et al.* 2015). BC is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths (Tirona *et al.* 2010). Metastasis in BC is caused by deficient immunosurveillance, including impairment of Natural Killer (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 (Dewan *et al.* 2009), decreased cytotoxic function (Levy *et al.* 2011; Hwang *et al.* 2012), NK abnormalities (Levy *et al.* 2011), poor tumor infiltrate (Albertsson *et al.* 2003; Esendagli *et al.* 2008), low NK cell numbers in tumors due to their inefficient homing into malignant tissues (Levy *et al.* 2011), and defective expression of activating receptors such as NKG2D, overexpression of inhibitory receptors NKG2A, CD158a, CD158b (Levy *et al.* 2011).

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 (Cheng *et al.* 2013). NK cells are known as necessary effectors in suppressing cancer proliferation (Kelly *et al.* 2003; Smyth *et al.* 2005). Once NK cells recognize target cells such as cancer cells, they form an immunological synapse, and the secretory granules fuse with the presynaptic membrane and release perforin (Prf) and granzyme (Gzm) into the synaptic cleft. Released Prf provides transmembrane pores on the target cell and enables Gzm to diffuse into the cell. Gzm then initiates the apoptosis of the target cells, and the NK cells detach from the dying cells and can interact with other target cells to accomplish serial killing (Harada

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et al. 2017). Therefore, the focus of recent cancer therapy has been to promote and develop NK cells as drugs (Hwang *et al.* 2012), using NK effectors such as cytokines (Levy *et al.* 2011). This study was conducted to evaluate the effect of inducing interleukin (IL15) on NK cells toward the following: i) improvement of NK cell activating receptors (NKG2D), ii) induced cytotoxic towards BC cells, iii) apoptotic inducer of NK cells, iv) genes apoptotic expression toward BC cells, vii) morphology and density of MCF7 cells by transwell of NK and BC cells.

2. Materials and Methods

2.1. Culturing Breast Cancer and NK Cells

Breast cancer (MCF7) cells (ATCC® HTB-22™) obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia were cultured in RPMI 1640 (Gibco, 11875093) supplemented with 10% FBS (Gibco, 26140079) and 1% antibiotic/antimycotic. Meanwhile, NK92 cells (ATCC® CRL-2407™) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia were cultured in MEM-without nucleosides (Gibco, 12561056) supplemented with 12.5% FBS, 12.5% Horse Serum (Gibco, 16050122), Sodium bicarbonate (Merck, 106329), Myo-inositol (Sigma Aldrich, 17508), Folic Acid (Sigma Aldrich, F8758), IL-2 (BioLegend, 589106), 2-Mercaptoethanol (Gibco, 1628448), and 1% antibiotic/antimycotic (Gibco, 1772653). Cell cultures were maintained in T-75 flasks, at 37°C, 5% CO_2. Culture mediums were changed every three days and sub-cultured at 80% confluence (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.2. Determination of NKG2A and NKG2D Gene Expression

A suspension of NK92 cells (1 x 10^6 cells) were seeded onto T-25 Flask (TPP, 90026) and incubated for 24 hours at 37°C, 5% CO_2. Afterwards, the cells were induced by human recombinant IL15 (BioLegend, 715902) to reach final concentration at 5 ng/ml and 10 ng/ml and incubated for 24 hours at 37°C, 5% CO_2. The RNA isolation of NK-92 was performed according to the protocol of RNA Isolation Kit (Bio-Rad, 732-6820). The concentration and purity of RNA of each sample was determined at 260/280 nm. The cDNA synthesis was performed using cDNA synthesis kit (Bio-rad, 170-8841) (Widowati *et al.* 2018; Afifah *et al.* 2019; Widowati *et al.* 2019). Primer sequences used were can be seen at Table 1.

2.3. Cytotoxic Assay

A suspension of MCF7 and NK92 cells were seeded at a ratio 1:1 onto transwell 24 well-plate (Corning 3396). MCF7 were seeded on the first day allowed to attach to the bottom chamber. Next day, NK-92 induced by IL15 (0, 5, 10 ng/ml) (BioLegend, 715902) were seeded to the upper chamber. The plate was incubated for 24 hours at 37°C, 5% CO_2. Hereinafter, the upper chamber and the medium were discarded. The cells were washed one time using PBS 1x. After that, 500 µl Presto Blue 1x (Invitrogen, A13262) were added to each well and incubated for 1 hour at 37°C, 5% CO_2. The solution was transferred to 96-well plate and the absorbance was read at 570 and 600 nm using spectrophotometer (Multiskan GO, ThermoScientific) (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.4. Apoptosis Analysis Assay by Flow Cytometry

MCF7 and NK92 cells were seeded at a ratio 1:1 onto 6 well/plate (Corning, 3412). On the first day, MCF7 were seeded at the bottom chamber and plates were incubated for 24 hours at 37°C, 5% CO_2. On the next day, NK92 cells were added to the upper chamber. The NK92 cells were induced by IL-15 (5 ng/ml and 10 n/ml) (BioLegend, 715902). The uninduced NK92 cells and MCF7 without transwell were also done as a control. The plate was then incubated for 24 hours at 37°C, 5% CO_2. Subsequently, MCF-7 at the bottom chamber were collected and washed two times by FACS buffer (2% FBS in PBS 1x). Pellets were resuspended in 100 µl FACS buffer and 5 µl Annexin V (BioLegend, Part 79998), 10 µl PI (BioLegend, Part 79997) were

Table 1. Activator, inhibitor receptor genes of NK Cells, RT PCR protocols

Gene symbols	Primer sequence (5' to 3') Upper strand:sense Lower strand:antisense	Product size (bp)	Annealing (°C)	Cycle	References
NKG2A	5'-CCAGAGAAGCTCATTGTTGG-3' 5'-CCAATCCATGAGGATGGT-3'	168	51	40x	Gen Bank:AF461812.1
NKG2D	5'-CTGGGAGATGAGTGAATTTCATA-3' 5'-GACTTCACCTTAAGTAAATC-3'	417	51	40x	Gen Bank:AF461811.1
GAPDH	5'-GGGCTGCTTTTAACTCTGGT-3' 5'-TGGCAGGTTTTTCTAGACGG-3'	702	51	40x	Sadeghi *et al.* 2015

added. The cells were incubated for 30 minutes in 4°C dark room. Following the 30 minutes incubation, samples were added 400 µl annexin V-binding buffer (BioLegend, 640194) and immediately analyzed by flow cytometry (MACSQuant™ Analyzer 10, Miltenyi Biotec) (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.5. Quantification of Granzyme-B Level

The quantitative determination of GzmB in the transwell-treatment, conditioned medium from NK cells from upper chamber and condioned medium from MCF7 was performed using ELISA Kit Human Granyme-B (BioLegend, 439207) followed the manufactur protocol. Absorbance was read at 450 nm and 570 nm using spectrophotometer (Safta *et al.* 2015; Widowati *et al.* 2018).

2.6. Determination of Apoptosis Gene Expression

MCF7 cells had been transwelled with induced NK92 cells (IL15 with various concentration 0, 5, 10 ng/ml) for 24 h. Afterward, microscopic analysis was conducted under inverted phase-contrast microscope. Subsequently the MCF7 cells were collected to isolate the RNA. The RNA isolation of MCF7 was performed based on the protocol of RNA Isolation Kit (Bio-Rad, 732-6820). The concentration and purity of RNA of each sample was determined at 260/280 nm. The cDNA synthesis was performed using cDNA synthesis kit (Bio-rad, 170-8841) (Widowati et al. 2018; Afifah et al., 2018; Widowati et al., 2019). Primer sequences can be seen at Table 2.

2.7. Statistical Analysis

All the data are presented as the mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons with p-values less than 0.05 were considered significant.

3. Results

3.1. Effect of IL15 toward NK Cell Characteristics

To determine the effect of human recombinant IL15 toward NK receptors, we evaluated the NK receptors including gene expression of NKG2D and NKG2A (Figure 1). The data showed that IL15 (5, 10 ng/mL) significantly up-regulated NKG2D, meanwhile IL15 did not significantly down-regulate NKG2A.

Based on the data (Figure 1) shows that IL15 induced up-regulation of NKG2D gene expression, the highest NKG2D expression was IL15 (5 ng/ml), higher concentartion of IL15 (10 ng/mL) lowered NKG2D expression. IL15 both concentrations (5, 10 ng/mL) didn't affected NKG2A expression of NK cells.

3.2. Cytotoxic Activity of IL15-Induced NK toward BC Cells

NK cells are involved in the elimination of tumor cells. To determine the effect of human recombinant IL15 on NK cells toward NK cells cytotoxic activity toward BC cells, we determine the effect ofIL15-induced NK toward MCF7 cells by transwell method. NK cells treated with IL5 at concentrations of 0, 5, 10 ng/mL (IL5-induced NK cells) against BC cellproliferation, we evaluated the inhibition of MCF7 proliferation (Figure 2).

Figure 2 shows that NK cells uninduced with IL15 had cytotoxic activity against MCF7 cells approximatelly 35.20% inhibitory growth cells. Both concentrations 5, 10 ng/mL of IL15 induced NK cells to inhibit MCF7 cells proliferation about 65.60-70.29%.

Table 2. Apoptotic primer for MCF7 Cells, RT PCR protocols

Gene symbols	Primer sequence (5' to 3') Upper strand:sense Lower strand:antisense	Product size (bp)	Annealing (°C)	Cycle	References
p53	5'-AGAGTCTATAGGCCCACCCC-3' 5'-GCTCGACGCTAGGATCTGAC-3'	97	58	40x	Behbahani 2014; Widowati et al. 2019
bax	5'-TGCTTCAGGGTTTCATCCAG-3' 5'-GGCGGCAATCATCCTCTG-3'	169	58	40x	Wang *et al.* 2009 Widowati et al.2019
bcl2	5'-GGTCATGTGTGTGGAGAGCG-3' 5'-GGTGCCGGTTCAGGTACTCA-3'	89	58	40x	Guan *et al.* 2018 Widowati et al. 2019
β-Actin	5'-TCTGGCACCACACCTTCTACAATG-3' 5'-AGCACAGCCTGGATAGCAACG-3'	166	85	40x	Han *et al.* 2018

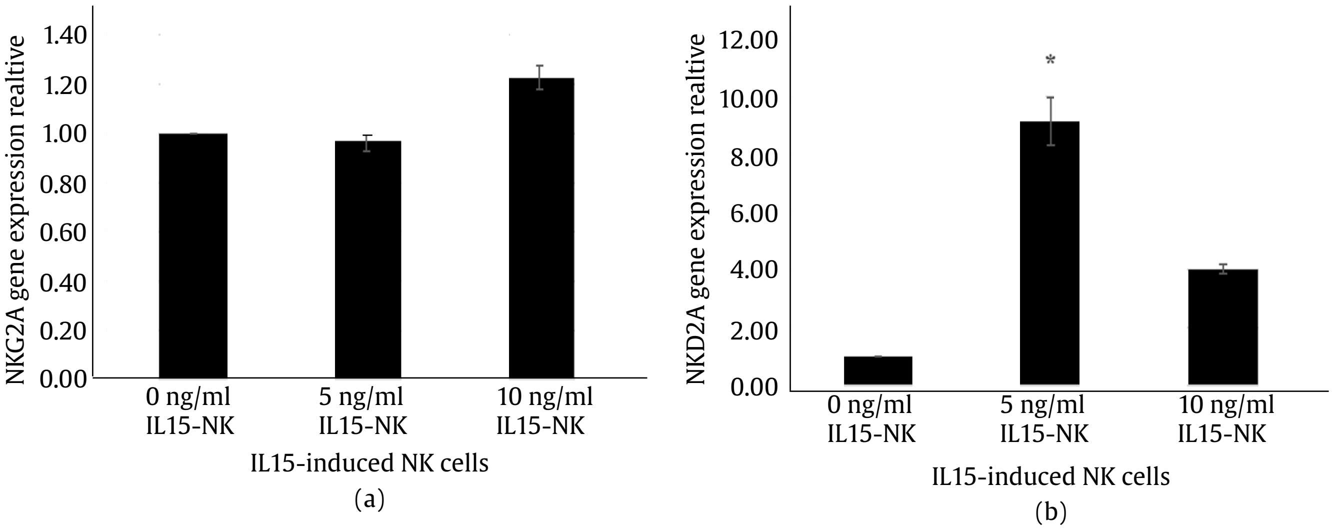

Figure 1. Effect IL15 inducer on NK cells toward NKG2A and NKG2D gene expression. The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) and non inducing of NK cells based on Tukey HSD post hoc test (p<0.05). (a) Shows that IL15 didn't affect NKG2A gene expression, (b) The NKG2D expression of higher concentration (10 ng/ml IL15) was lower than 5 ng/ml IL15

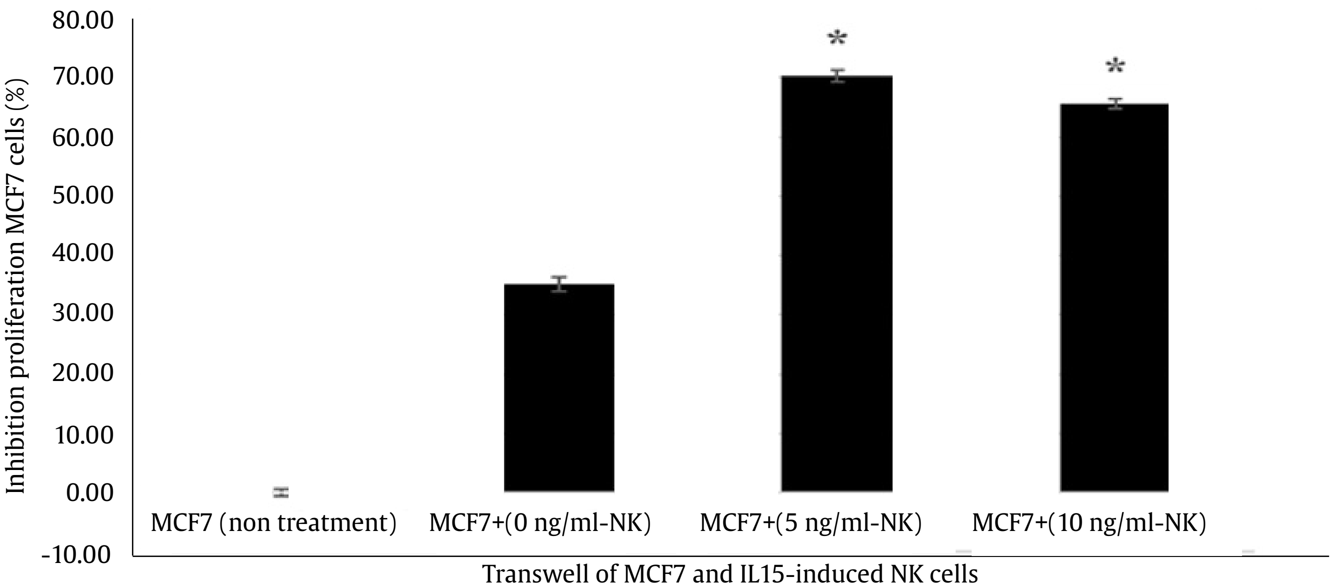

Figure 2. Effect of IL15 inducer on NK cells toward proliferation inhibition of MCF7 cells. The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) and non inducing of NK cells based on Tukey HSD post hoc test (p<0.05). Figure 2 shows that IL15 activated NK cells to inhibit MCF7 cell proliferation and increased anticancer activity NK cells against proliferation of MCF7 cells. The higher concentration (10 ng/ml) of IL15 was not significant difference compared to lower concentration of IL15 (5 ng/ml) against MCF7 cells proliferation

3.3. Effect of IL15-Induced NK Cells toward Apoptosis of MCF7 Cells

NK cells require effectors to activate NK cells; thus, this study was conducted to evaluate the effect of IL15 toward apoptosis of MCF7 cells by transwell method. To determine the effect of human recombinant IL15 on NK cells toward apoptotic percentage of MCF7 cells, we evaluated apoptotic percentage of MCF7 cells (Figures 3). We used IL15 at levels of 0 (uninduced), 5 and 10 ng/ml. The data (Figure 3) show that IL15 increased apoptotic percentage of MCF7, the highest apoptotic potentcy was NK induced by 5 ng/ml, higher concentration of IL15 (10 ng/ml) decreased apoptotic percentage.

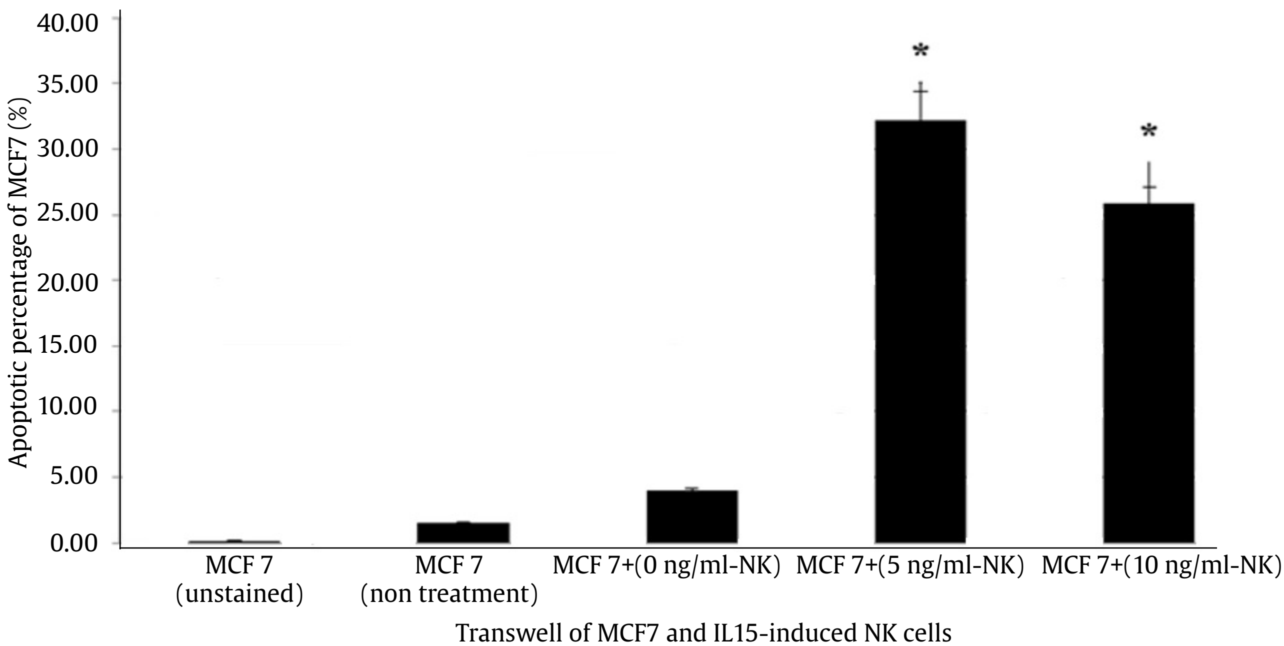

Figure 3. Effect IL15 inducer on NK cells toward apoptotic percentage of MCF7 cells. The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment. The asetrisks symbol (*) present significant differences among concentrations of IL15 inducer (0, 5, 10 ng/ml) compared to control (MCF7 non treatment) toward apoptotic percentage of MCF7

Based on the apoptotic percentage of MCF7 cells were treated by IL15-induced NK cells using transwell method (Figure 3), shows that NK cells uninduced with IL15 was very low apoptotic potency (3.98%), IL15 activated NK cells and increased apoptotic potentcy against MCF7 cells, the best IL15 concentration was 5 ng/ml, higher concentration of IL15 significantly lowered apoptotic percentage of MCF7 cells.

3.4. Effect of IL15-Induced NK Cells toward Granzyme Level in Transwell of MCF7 and NK Cells

We measured NK cells were activated using IL15 (0, 5, 10 ng/ml). The cell ratios (MCF7: NK cells=1:1) were assessed for the ability to secrete GzmB. The effects of IL15-induced NK (IL15-NK) transwelled with MCF7 cells, which made NK released cytoplasmic granules (GzmB) can be seen in Figure 4.

The GzmB level secreted by NK cells which co-cultured with MCF7 cells using transwell method (Figure 5). NK cells uninduced by IL15 secreted GzmB in low level (77.06 pg/ml), inducing IL15 (5, 10 ng/ml) increased secretion of GzmB, the highest GzmB level was secreted by NK cells which induced by IL15 (5 ng/ml), meanwhile higher IL15 concentration (10 ng/ml) lowered GzmB level.

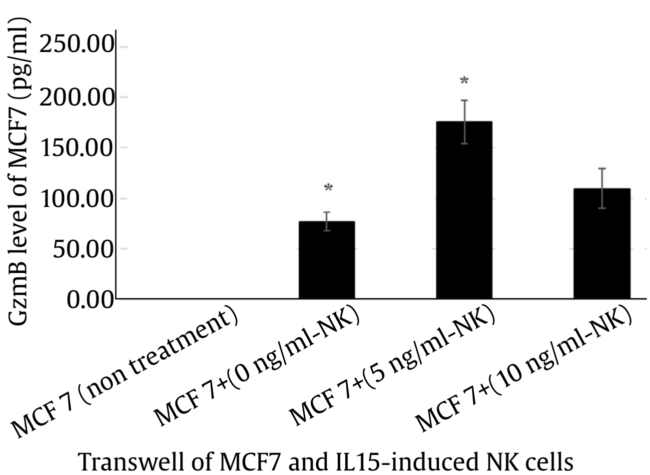

Figure 5. Effect of IL15 inducer on NK cells toward GzmB level of transwell MCF7 and NK cells. The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment. The asetrisks symbol (*) present significant differences among concentrations of inducer IL15 (0, 5, 10 ng/ml) compared to control (MCF7 non treatmnet) toward GzmB level

3.5. Effect IL15-Induced NK Cells toward Apoptotic and Antiapoptotic Genes Expression of MCF7 and NK Cells

This study was the continued-research to elucidate the apoptosis mechanism of IL15-induced NK cells toward MCF7 cells, in order to determine the apoptotic inducing activity of IL15-induced NK cells toward MCF cells by transwell assay, the expression of apoptotic genes was determined by RT-PCR. We measured the expression of proapoptotic genes, specifically p53, b-cell cll/ lymphoma 2 (bcl2), bcl2-associated x protein (bax). The p53, bax and bcl2 genes expression of MCF7 can be seen at Figure 6.

NK cells induced by IL15 significantly increased the apoptotic genes both low and high concentrations (5, 10 ng/mL) toward bax, p53 genes expression (Figure 5A, 5B) but IL15-induced NK didn't affect bcl2 expression (Figure 5C). The highest p53 expression was MCF7 treated with 5 ng/mL IL15-induced NK cells, the higher concentration of IL15 (10 ng/mL) was significantly lower p53 expression (Figure 5A). The bax gene expression by MCF7 cells treated with 10 g/mL IL15-induced NK cells was unsignificantly difference compared to 5 ng/mL IL15-induced NK cells.

3.6. Effect IL15-Induced NK Cells against MCF7 Cells Morphology

This study was performed by investigating the effect of IL15-induced NK cells on viability, density, morphology of MCF7 cells (Figure 6). The result demonstrates that NK cells induced dose-dependent reduction in viability of MCF7 cells.

Based on the morphological MCF7 cells treated with NK cells using transwell assay shows that NK cells lower the viability and density BC cells. The highest viability and density cells was MCF7 (control), the lowest viability, density cells was MCF7 treated by IL15 (5 ng/ml) as NK cells inducer.

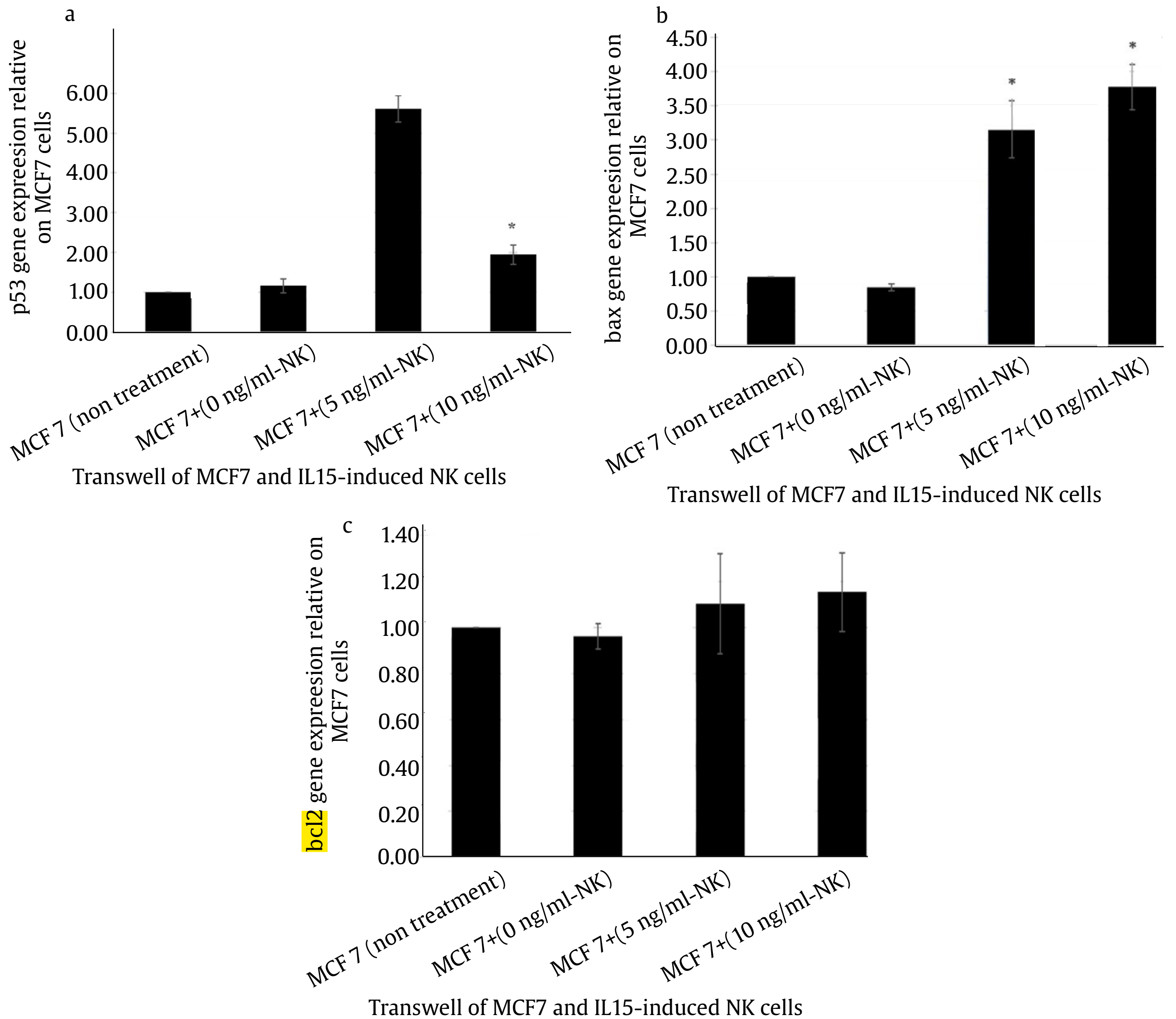

Figure 5. Effect IL15 inducer on NK cells toward bax, p53 and bcl2 gene expression of MCF7 cells. The data are presented as histogram among treatments, this research was conducted intriplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) compred to non inducing of NK cells based on Tukey HSD post hoc test ($p<0.05$). Figure 5 shows that IL15 (5, 10 ng/ml) up-regulated gene expression of p53 (Figure 5a), bax (Figure 5b) but didn't affected bcl2 gene expresion (Figure 5c)

4. Discussion

NK cell abnormalities in cancer patients such as imbalance of immune status, inclined to immunosuppression, decreased in NK cell numbers, decreased cytotoxicity, declined activiting receptor (NKG2D, NKG2C, NKp30, NKp46) and inclined increasing inhibitory receptor (NKG2A, CD158, CD158a). Recently has been developed the immunotherapy using activated-NK cells (Mamessier *et al.* 2011). IL2, IL12, IL15, and IL18, 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 (Konjevic *et al.* 2016). Cytokines such as interleukins (IL2) can be used to increase the anticancer potency of NK cells (Widowati *et al.* 2019). Various types of stimulation have been reported to enable NK cells to achieve their full effector potential, such as IL15, IL12, IL18, IL21 (Lucas *et al.* 2007; Guia *et al.* 2008; Chaix *et al.* 2008; Brehm *et al.* 2011). Based on the result (Figure 1), IL15 induced activating receptor NKG2D, this result was validated

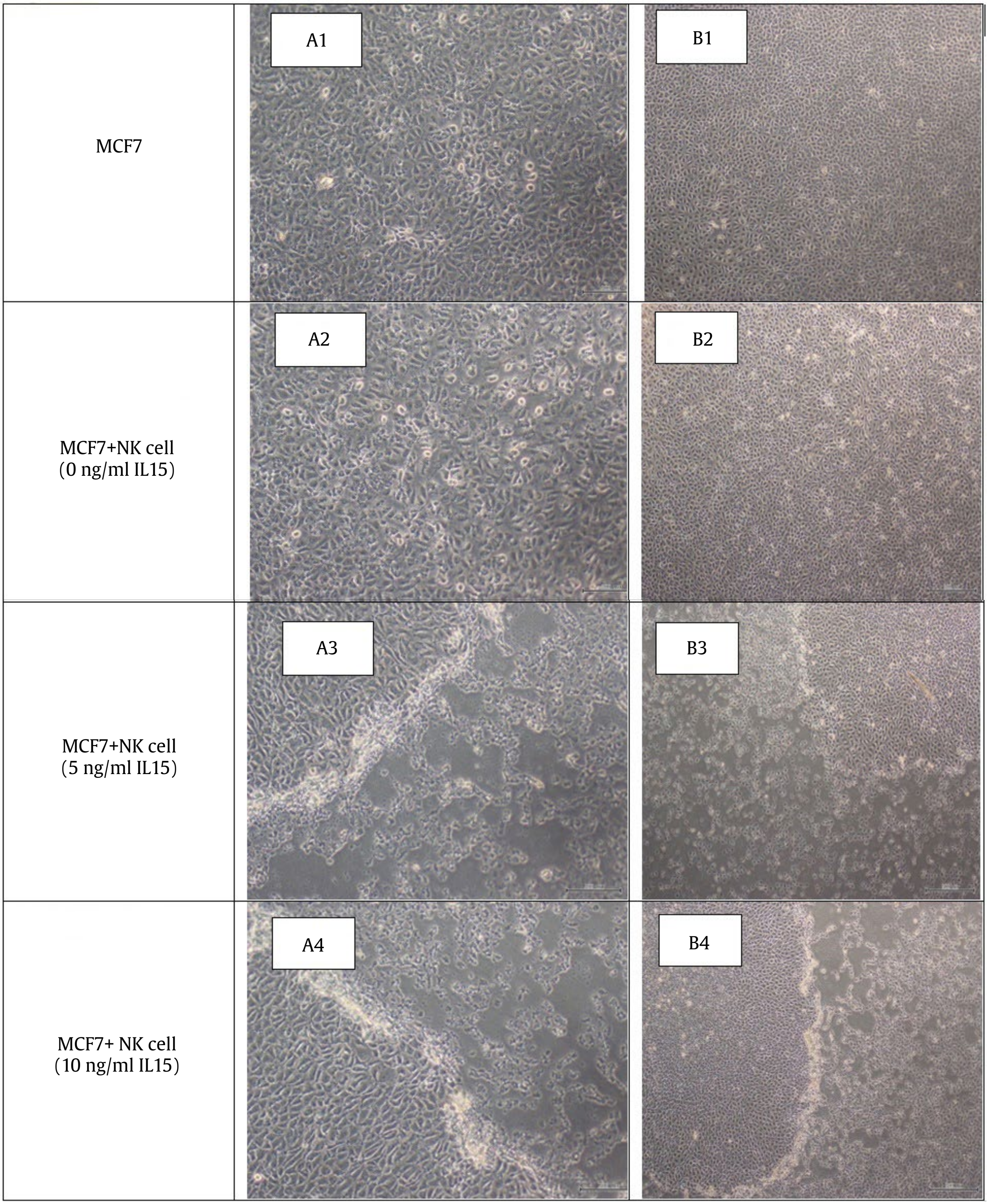

Figure 6. Morphological analysis using phase-contrast microscope at ×100 magnification (A-1, A-2, A-3, A-4) and x40 magnification, (B-1, B-2, B-3, B-4). Different concentration of IL15 as inducer for NK cells decreased the survival rate of MCF7 cells

with previous research that IL2 and IL15 induce the expression of KIRs and activating receptors (NKG2D and NKp44) on NK cell surface (Rham *et al.* 2007; Boieri *et al.* 2017). This result was in line with previous research that metastatic melanoma (MM) patients exhibited decreased CD161 and NKG2D (Konjevi'c *et al.* 2009). NK cells treated with IL15 up-regulated the expression of NK receptors, including Nkp30, Nkp46, NKG2C, and NKG2D (Szczepanski *et al.* 2010). IL15 didn't decline the inhibitory receptor (NKG2A), this result was not consistent with previous research that IL15+IL12 was significantly decline the NKG2A of HIV-infected individuals (Parasa *et al.* 2012).

NK cells have been used in clinical studies in order to treat various malignancies (Harada *et al.* 2017), NK cells abnormalities in cancer patients such as low NK cells counts in PBMC, decreased cytotoxicity (Levy *et al.* 2011). NK cells inhibited MCF7 cells proliferation, IL15 activated NK cells to inhibit MCF7 cells proliferation, this result was consistent with previous research that NK cells control tumor growth and metastasis diffusion *in vivo* (Zamai *et al.* 2007). The low NK cell numbers in tumors due to their inefficient homing into malignant tissues (Levy *et al.* 2011). Decreasing NK cell numbers are observed in PB of cancer patients; therefore, NK cells decrease in tumor infiltrate (Levy *et al.* 2011). The activity and numbers of NK cells need to be enhanced for better efficacy (Mandal and Viswanathan 2002). NK cell infiltration in solid tumors was associated with a better prognosis (Villegas *et al.* 2002). IL-15 is essential for NK cell survival, differentiation, and proliferation (Anton *et al.* 2015). Improving NK cells cytotoxicity used ILs (IL15, IL18), the result showed that ILs increased TNFα, IFNγ secretion by NK cells (Widowati *et al.* 2020)

IL15 induces optimal production of IFN-γ from NK cells, subsequently induce apoptosis of the NK cells (Ross and Caligiuri 1997; Widowati *et al.* 2020). The human MHC class I-negative small cell lung cancer cell line (N592) genetically engineered to secrete IL-15, N592/IL-15, showed a reduced tumor growth rate (Orengo *et al.* 2003). IL15 and IL12 induces optimal production of IFN-γ NK cells (Ross and Caligiuri 1997). IFNγ inhibited BC proliferation (MCF7) with median inhibitory 0.34 µg/ml (Widowati *et al.* 2016). IFNγ against MCF7 cells exhibited that the cytokines decreased the cell viability in a dose dependent manner (Widowati *et al.* 2016).

NK secrete GzmB, IL15-activated NK cells up-regulated GzmB secretion. This result was validated previous research that activated NK cells released higher levels of IFN-γ, TNF-α, Prf1, and GzmB compared to non-induced NK cells. IL2, IL15, and IL18 increased the secretion of IFN-γ, TNF-α, Prf1, and GzmB by co-culture cells (Widowati *et al.* 2020). IL15 in NK cell controls as well survival of mature NK cells in the periphery (Marçais *et al.* 2013; Widowati *et al.* 2020), mediated by up-regulation of anti-apoptotic bcl2 family members and down regulation of apoptotic (Marçais *et al.* 2013). The production of TNF-α, IFN-γ, Prf1, GzmB increased when the ratio of NK cells and hWJMSCs was high (Widowati *et al.* 2019). NK cells activity were controlled by cytokine and ILs (IL2, IL12, IL15, IL18) (Domaica *et al.* 2012). NK cells eliminate malignantly transformed cells principally by releasing the contents of cytotoxic granules into the immune synapse formed with their target cell (Lieberman 2003). The granule mediators of target cell lysis are serine proteases, known as Gzm, which induce programmed cell death (Safta *et al.* 2015; Harada *et al.* 2017). Cytotoxicity of NK cells is executed mainly through the granule exocytosis pathway by releasing Prf1 and GzmB into the immunological synapse after the conjugate formation with targets (Lieberman 2003). Human GzmB preferentially induced target cell apoptosis, induces a rapid accumulationof the tumor- suppressor protein p53 within target cells (Safta *et al.* 2015). Human GzmB-induced p53 accumulates on target cell mitochondria where it interacts with the prosurvival protein Bcl2. This interaction allows the release of the proapoptotic protein Bax from its inhibitory interaction with Bcl-2 (Safta *et al.* 2015). several proteins that are involved in GzmB-induced apoptosis, including Casp-9 and -3, Bim, Bid, Bak, Bax, and Xiap. GzmB induced apoptosis cancer cells by involving induction of p53 tumor suppressor gene (Meslin *et al.* 2007).

IL15 inhibit MCF7 proliferation, induced apoptotic BC cells, this result also supported by MCF7 morphology, BC cells appear low density and viability. This result was supported by previous research that ILs (IL1, IL2, IL15) and CD28, serve as co-stimulatory factors, enhancing IFN-γ production by NK cells, as well as NK cell proliferation and cytotoxicity (Hunter *et al.* 1997; Cui *et al.* 2016). ILs (IL12, IL18) stimulate NK cell lines, increasing the secretion of IFN-γ (Wang *et al.* 2012). IL12 and IL18 are critical regulators that activate NK cells via the production of cytokines and direct lysis of target cells (Vivier *et al.* 2011). IL15 activate, increase NK proliferation (Widowati *et al.* 2020). Activated NK cells induce IFN-γ, TNF-α, Prf1, GzmB secretion and inhibit BC cells proliferation on co-cultured of MCF7 and NK cells (Widowati *et al.* 2020).

The proposed mechanism of our research which IL15 activated NK cells including increased NK cells number, up regulated activating receptor (NKG2D) but IL15 didn't influnce inhibitor receptor (NKG2A), improved perforin, granzyme secretion. IL15-activated NK induced apoptosis of BC cells through increased p53, bax gene expression and inhibited BC cells proliferation. For the detail mechanism can be seen at Figure 8.

5. Conclusion

IL15 improve, activate NK cells resulted in activating receptors (NKG2D), increasing GzmB secretion and cytotoxic activity on BC cells, inducing apoptotic genes bax, p53 expression and induce apoptotic cells on transwell assay.

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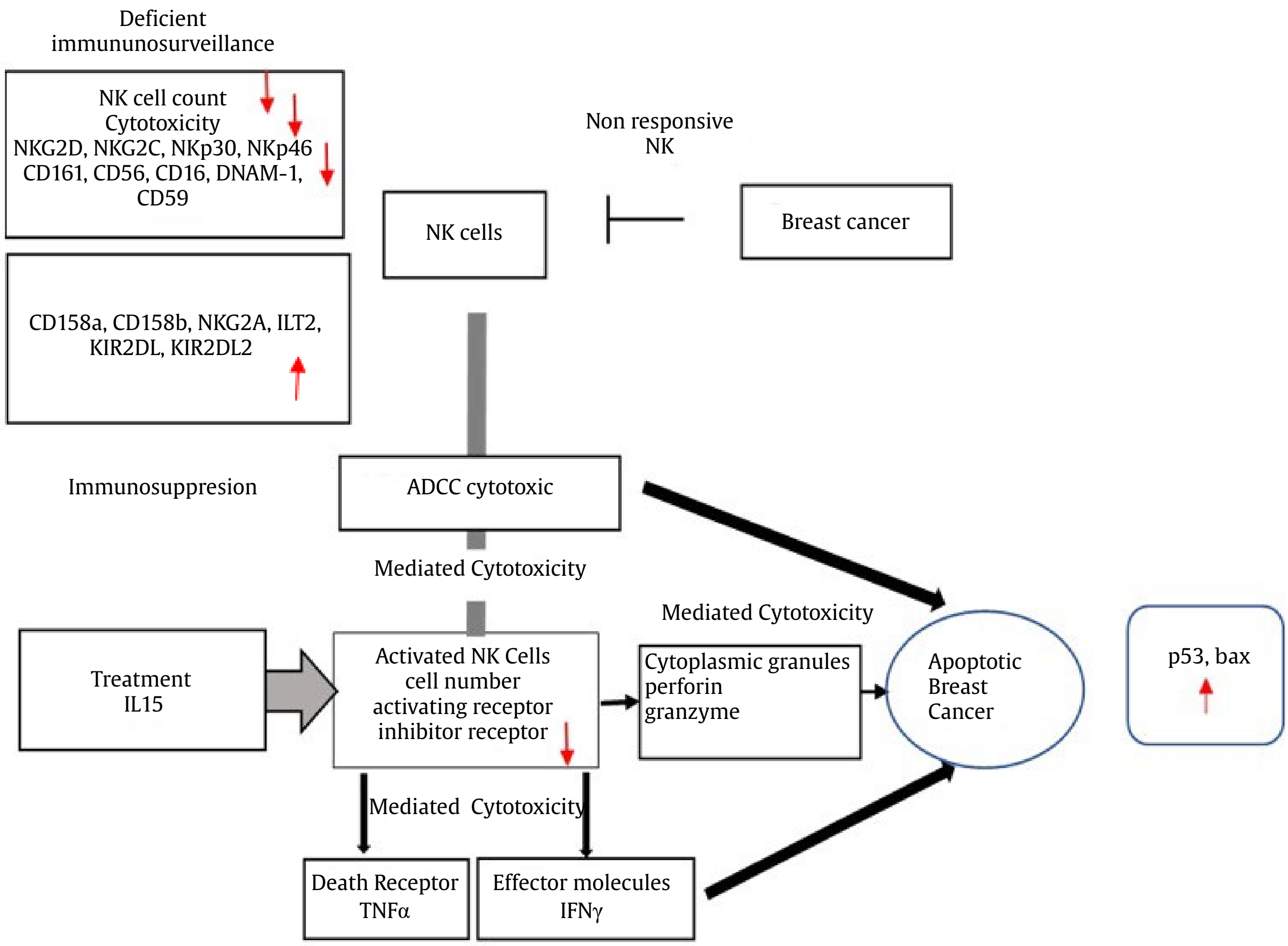

Figure 7. Proposed mechanism of activated NK cells to inhibit and kill breast cancer cells. NK cells abnormalities in breast cancer patients. The imbalance of immune status, which defficient immunosurveillance including low NK cell number in PBMC, poor tumor inflitrate to cancer cells, decreased cytotoxicity, defective expression of activating receptor (NKG2D). IL15 activated NK cells to kill traget cancer cells through: cytoplasmic granules release (Prf, GzmB), death receptor-induced apoptosis (TNFα), effector molecules production (IFNγ) and released antibody-dependent cellular cytotoxicity (ADCC). The genes of p53, bax induced apoptotic BC cells

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Apoptotic Potential of Secretome from Interleukin-Induced Natural Killer Cells toward Breast Cancer Cell Lines by Transwell Assay

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ABSTRACT

Breast cancer (BC) is the number one cause of deaths from cancer in women. Metastasis in BC is caused by immunosurveillance deficiency, including impairment of NK cell maturation, low NK activity, and decreasing cytotoxicity. This study was performed to improve activating receptors and cytotoxicity of NK cells using interleukin (IL15) against BC cells. Human recombinant IL15 was used to induce NK cells. To evaluate the potential of IL15 in inducing NK cells, we measured the activating and inhibiting receptors (NKG2D, NKG2A), apoptotic potency of NK cells on BC cells (MCF7) using transwell assay. The IL15 inducer on the NK cell were measured NKG2D, NKG2A gene expression with quantitative polymerase chain reaction (qPCR), the apoptotic potency of NK toward BC cells using flowcytometer, granzyme B (GzmB) secretion, apoptotic gene expression of MCF7 cells. IL15 increased NKG2D expression 4.01-9.13%, but IL15 could not affect toward NKG2A expression on NK cells. IL15-activated NK cells, inhibited BC cells proliferation, induced apoptotic BC cells 25.89-32.19%, induced genes apoptotic of BC cells bax, p53. IL15 increase NK activating receptor (NKG2D), inhibit BC cells proliferation, induce apoptotic percentage and induce apoptotic gene expression.

1. Introduction

Breast cancer (BC) is one of the main causes of death in the world. In 2012, it caused around 8.2 million deaths (Ferlay *et al.* 2015). BC is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths (Tirona *et al.* 2010). Metastasis in BC is caused by deficient immunosurveillance, including impairment of Natural Killer (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 (Dewan *et al.* 2009), decreased cytotoxic function (Levy *et al.* 2011; Hwang *et al.* 2012), NK abnormalities (Levy *et al.* 2011), poor tumor infiltrate (Albertsson *et al.* 2003; Esendagli *et al.* 2008), low NK cell numbers in tumors due to their inefficient homing into malignant tissues (Levy *et al.* 2011), and defective expression of activating receptors such as NKG2D, overexpression of inhibitory receptors NKG2A, CD158a, CD158b (Levy *et al.* 2011).

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 (Cheng *et al.* 2013). NK cells are known as necessary effectors in suppressing cancer proliferation (Kelly *et al.* 2003; Smyth *et al.* 2005). Once NK cells recognize target cells such as cancer cells, they form an immunological synapse, and the secretory granules fuse with the presynaptic membrane and release perforin (Prf) and granzyme (Gzm) into the synaptic cleft. Released Prf provides transmembrane pores on the target cell

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and enables Gzm to diffuse into the cell. Gzm then initiates the apoptosis of the target cells, and the NK cells detach from the dying cells and can interact with other target cells to accomplish serial killing (Harada *et al.* 2017). Therefore, the focus of recent cancer therapy has been to promote and develop NK cells as drugs (Hwang *et al.* 2012), using NK effectors such as cytokines (Levy *et al.* 2011). This study was conducted to evaluate the effect of inducing interleukin (IL15) on NK cells toward the following: i) improvement of NK cell activating receptors (NKG2D), ii) induced cytotoxic towards BC cells, iii) apoptotic inducer of NK cells, iv) genes apoptotic expression toward BC cells, vii) morphology and density of MCF7 cells by transwell of NK and BC cells.

2. Materials and Methods

2.1. Culturing Breast Cancer and NK Cells

Breast cancer (MCF7) cells (ATCC® HTB-22™) obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia were cultured in RPMI 1640 (Gibco, 11875093) supplemented with 10% FBS (Gibco, 26140079) and 1% antibiotic/antimycotic. Meanwhile, NK92 cells (ATCC® CRL-2407™) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia were cultured in MEM-without nucleosides (Gibco, 12561056) supplemented with 12.5% FBS, 12.5% Horse Serum (Gibco, 16050122), Sodium bicarbonate (Merck, 106329), Myo-inositol (Sigma Aldrich, 17508), Folic Acid (Sigma Aldrich, F8758), IL-2 (BioLegend, 589106), 2-Mercaptoethanol (Gibco, 1628448), and 1% antibiotic/antimycotic (Gibco, 1772653). Cell cultures were maintained in T-75 flasks, at 37°C, 5% CO_2. Culture mediums were changed every three days and sub-cultured at 80% confluence (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.2. Determination of NKG2A and NKG2D Gene Expression

A suspension of NK92 cells (1 x 10^6 cells) were seeded onto T-25 Flask (TPP, 90026) and incubated for 24 hours at 37°C, 5% CO_2. Afterwards, the cells were induced by human recombinant IL15 (BioLegend, 715902) to reach final concentration at 5 ng/ml and 10 ng/ml and incubated for 24 hours at 37°C, 5% CO_2. The RNA isolation of NK92 was performed according to the protocol of RNA Isolation Kit (Bio-Rad, 732-6820). The concentration and purity of RNA of each sample was determined at 260/280 nm. The cDNA synthesis was performed using cDNA synthesis kit (Bio-rad, 170-8841) (Widowati *et al.* 2018; Afifah *et al.* 2019; Widowati *et al.* 2019). Primer sequences used were can be seen at Table 1.

2.3. Cytotoxic Assay

MCF7 and NK92 cells were seeded at a ratio 1:1 onto transwell 24 well-plate (Corning 3396). MCF7 were seeded on the first day allowed to attach to the bottom chamber. Next day, NK92 induced by IL15 (0, 5, 10 ng/ml) (BioLegend, 715902) were seeded to the upper chamber. The plate was incubated for 24 hours at 37°C, 5% CO_2. Hereinafter, the upper chamber and the medium were discarded. The cells were washed one time using PBS 1x. After that, 500 µl Presto Blue 1x (Invitrogen, A13262) were added to each well and incubated for 1 hour at 37°C, 5% CO_2. The solution was transferred to 96-well plate and the absorbance was read at 570 and 600 nm using spectrophotometer (Multiskan GO, ThermoScientific) (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.4. Apoptosis Analysis Assay by Flow Cytometry

MCF7 and NK92 cells were seeded at a ratio 1:1 onto 6 well/plate (Corning, 3412). On the first day, MCF7 were seeded at the bottom chamber and plates

Table 1. Activator, inhibitor receptor genes of NK Cells, RT PCR protocols

Gene symbols	Primer sequence (5' to 3') Upper strand:sense Lower strand:antisense	Product size (bp)	Annealing (°C)	Cycle	References
NKG2A	5'-CCAGAGAAGCTCATTGTTGG-3' 5'-CCAATCCATGAGGATGGT-3'	168	51	40x	Gen Bank:AF461812.1
NKG2D	5'-CTGGGAGATGAGTGAATTTCATA-3' 5'-GACTTCACCTTAAGTAAATC-3'	417	51	40x	Gen Bank:AF461811.1
GAPDH	5'-GGGCTGCTTTTAACTCTGGT-3' 5'-TGGCAGGTTTTTCTAGACGG-3'	702	51	40x	Sadeghi *et al.* 2015

were incubated for 24 hours at 37°C, 5% CO_2. On the next day, NK92 cells were added to the upper chamber. The NK92 cells were induced by IL-15 (5 ng/ml and 10 n/ml) (BioLegend, 715902) The uninduced NK92 cells and MCF7 without transwell were also done as a control. The plate was then incubated for 24 hours at 37°C, 5% CO_2. Subsequently, MCF7 at the bottom chamber were collected and washed two times by FACS buffer (2% FBS in PBS 1x). Pellets were resuspended in 100 µl FACS buffer and 5 µl Annexin V (BioLegend, Part 79998), 10 µl PI (BioLegend, Part 79997) were added. The cells were incubated for 30 minutes in 4°C dark room. Following the 30 minutes incubation, samples were added 400 µl annexin V-binding buffer (BioLegend, 640194) and immediately analyzed by flow cytometry (MACSQuant™ Analyzer 10, Miltenyi Biotec) (Widowati *et al.* 2018; Widowati *et al.* 2019).

2.5. Quantification of Granzyme-B Level

The quantitative determination of GzmB in the transwell-treatment, conditioned medium from NK cells from upper chamber and condioned medium from MCF7 was performed using ELISA Kit Human Granyme-B (BioLegend, 439207) followed the manufactur protocol. Absorbance was read at 450 nm and 570 nm using spectrophotometer (Safta *et al.* 2015; Widowati *et al.* 2018).

2.6. Determination of Apoptosis Gene Expression

MCF7 cells had been transwelled with induced NK92 cells (IL15 with various concentration 0, 5, 10 ng/ml) for 24 h. Afterward, microscopic analysis was conducted under inverted phase-contrast microscope. Subsequently the MCF7 cells were collected to isolate the RNA. The RNA isolation of MCF7 was performed based on the protocol of RNA Isolation Kit (Bio-Rad, 732-6820). The concentration and purity of RNA of each sample was determined at 260/280 nm. The cDNA synthesis was performed using cDNA synthesis kit (Bio-rad, 170-8841) (Widowati *et al.* 2018; Afifah *et al.* 2019; Widowati *et al.* 2019). Primer sequences can be seen at Table 2.

2.7. Statistical Analysis

All the data are presented as the mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons with p-values less than 0.05 were considered significant.

3. Results

3.1. Effect of IL15 toward NK Cell Characteristics

To determine the effect of human recombinant IL15 toward NK receptors, we evaluated the NK receptors including gene expression of NKG2D and NKG2A (Figure 1). The data showed that IL15 (5, 10 ng/ml) significantly up-regulated NKG2D, meanwhile IL15 did not significantly down-regulate NKG2A.

Based on the data (Figure 1) shows that IL15 induced up-regulation of NKG2D gene expression, the highest NKG2D expression was IL15 (5 ng/ml), higher concentartion of IL15 (10 ng/ml) lowered NKG2D expression. IL15 both concentrations (5, 10 ng/ml) didn't affected NKG2A expression of NK cells.

3.2. Cytotoxic Activity of IL15-Induced NK toward BC Cells

NK cells are involved in the elimination of tumor cells. To determine the effect of human recombinant IL15 on NK cells toward NK cells cytotoxic activity toward BC cells, we determine the effect of IL15-induced NK toward MCF7 cells by transwell method. NK cells treated with IL5 at concentrations of 0, 5, 10 ng/ml (IL5-induced NK cells) against BC cellproliferation, we evaluated the inhibition of MCF7 proliferation (Figure 2).

Table 2. Apoptotic primer for MCF7 Cells, RT PCR protocols

Gene symbols	Primer sequence (5' to 3') Upper strand:sense Lower strand:antisense	Product size (bp)	Annealing (°C)	Cycle	References
p53	5'-AGAGTCTATAGGCCCACCCC-3' 5'-GCTCGACGCTAGGATCTGAC-3'	97	58	40x	Behbahani 2014; Widowati *et al.* 2019
bax	5'-TGCTTCAGGGTTTCATCCAG-3' 5'-GGCGGCAATCATCCTCTG-3'	169	58	40x	Wang *et al.* 2009; Widowati *et al.* 2019
bcl2	5'-GGTCATGTGTGTGGAGAGCG-3' 5'-GGTGCCGGTTCAGGTACTCA-3'	89	58	40x	Guan *et al.* 2018; Widowati *et al.* 2019
β-Actin	5'-TCTGGCACCACACCTTCTACAATG-3' 5'-AGCACAGCCTGGATAGCAACG-3'	166	85	40x	Han *et al.* 2018

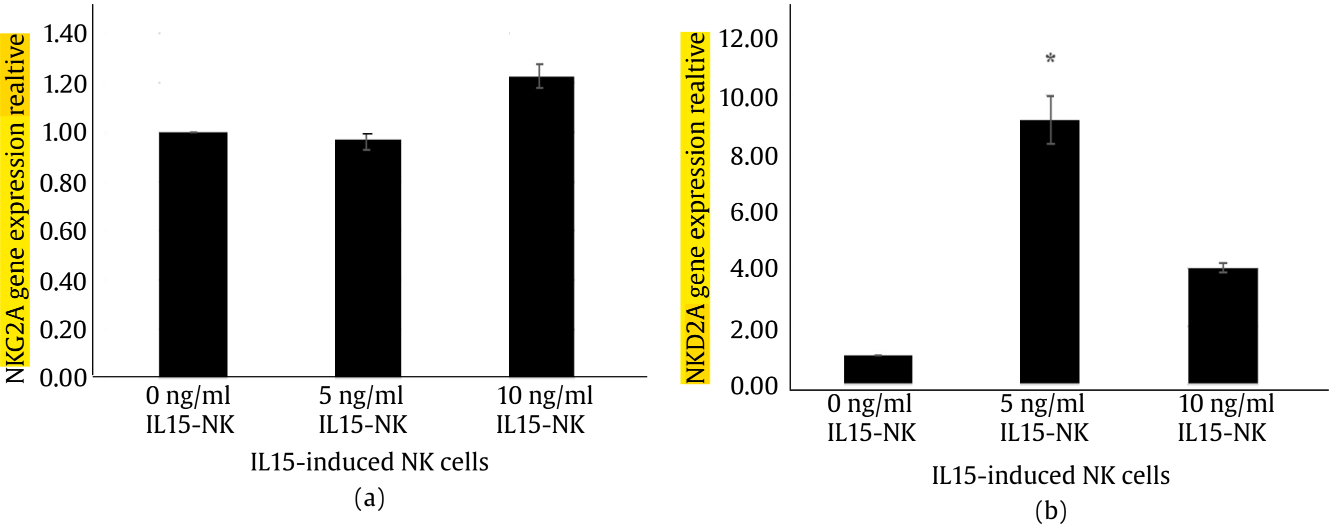

Figure 1. Effect IL15 inducer on NK cells toward NKG2A and NKG2D gene expression. The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) and non inducing of NK cells based on Tukey HSD post hoc test (p<0.05). (a) Shows that IL15 didn't affect NKG2A gene expression. (b) The NKG2D expression of higher concentration (10 ng/ml IL15) was lower than 5 ng/ml IL15

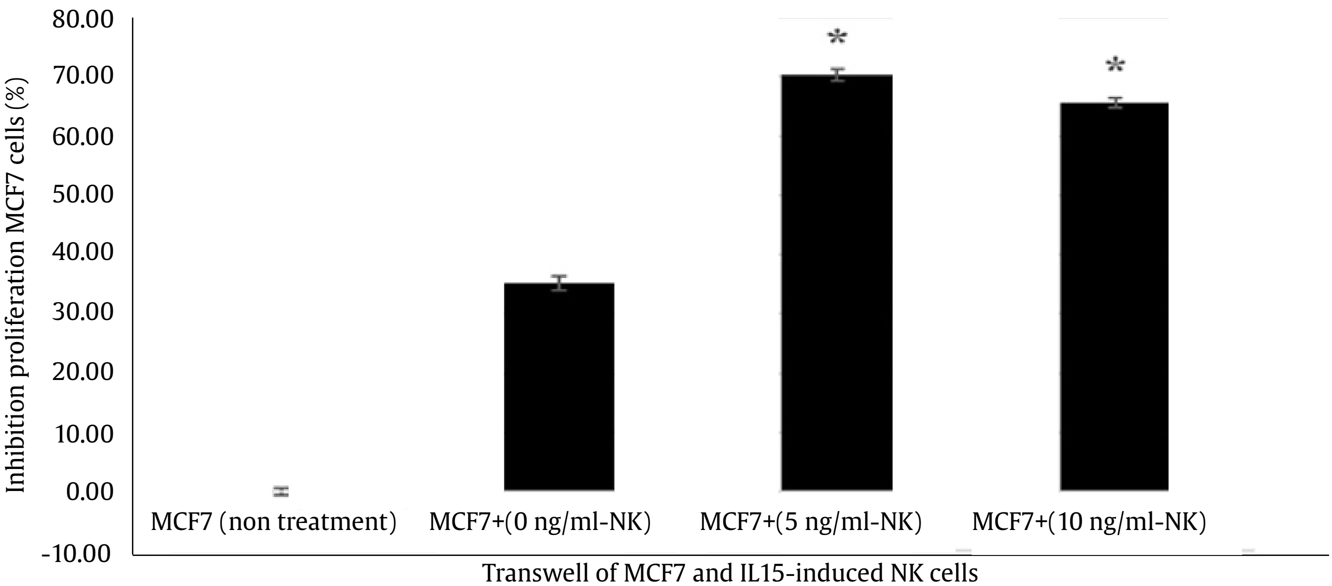

Figure 2. Effect of IL15 inducer on NK cells toward proliferation inhibition of MCF7 cells. The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) and non inducing of NK cells based on Tukey HSD post hoc test (p<0.05). Figure 2 shows that IL15 activated NK cells to inhibit MCF7 cell proliferation and increased anticancer activity NK cells against proliferation of MCF7 cells. The higher concentration (10 ng/ml) of IL15 was not significant difference compared to lower concentration of IL15 (5 ng/ml) against MCF7 cells proliferation

Figure 2 shows that NK cells uninduced with IL15 had cytotoxic activity against MCF7 cells approximatelly 35.20% inhibitory growth cells. Both concentrations 5, 10 ng/ml of IL15 induced NK cells to inhibit MCF7 cells proliferation about 65.60-70.29%.

3.3. Effect of IL15-Induced NK Cells toward Apoptosis of MCF7 Cells

NK cells require effectors to activate NK cells; thus, this study was conducted to evaluate the effect of IL15 toward apoptosis of MCF7 cells by transwell method. To determine the effect of human recombinant IL15 on NK cells toward apoptotic percentage of MCF7 cells, we evaluated apoptotic percentage of MCF7 cells (Figures 3 and 4). We used IL15 at levels of 0 (uninduced), 5 and 10 ng/ml. The data (Figure 3 and 4) show that IL15 increased apoptotic percentage of MCF7, the highest apoptotic potentcy was NK induced by 5 ng/ml, higher concentration of IL15 (10 ng/ml) decreased apoptotic percentage.

Based on the apoptotic percentage of MCF7 cells were treated by IL15-induced NK cells using transwell method (Figure 3), shows that NK cells uninduced with IL15 was very low apoptotic potency (3.98%), IL15 activated NK cells and increased apoptotic potentcy against MCF7 cells, the best IL15 concentration was 5 ng/ml, higher concentration of IL15 significantly lowered apoptotic percentage of MCF7 cells.

3.4. Effect of IL15-Induced NK Cells toward Granzyme Level in Transwell of MCF7 and NK Cells

We measured NK cells were activated using IL15 (0, 5, 10 ng/ml). The cell ratios (MCF7: NK cells=1:1) were assessed for the ability to secrete GzmB. The effects

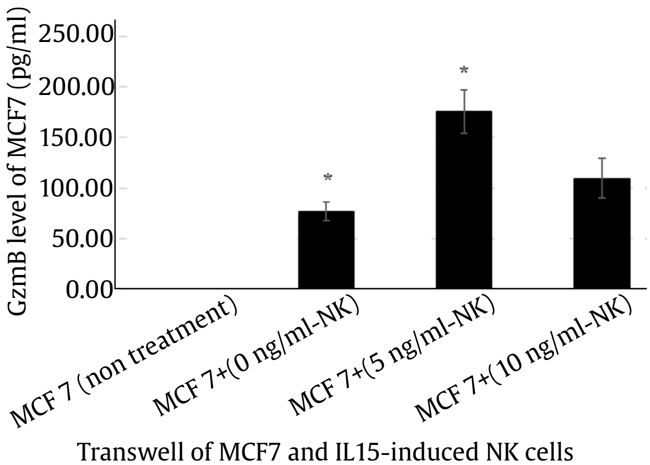

Figure 4. Effect of IL15 inducer on NK cells toward GzmB level of transwell MCF7 and NK cells. The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment. The asetrisks symbol (*) present significant differences among concentrations of inducer IL15 (0, 5, 10 ng/ml) compared to control (MCF7 non treatmnet) toward GzmB level

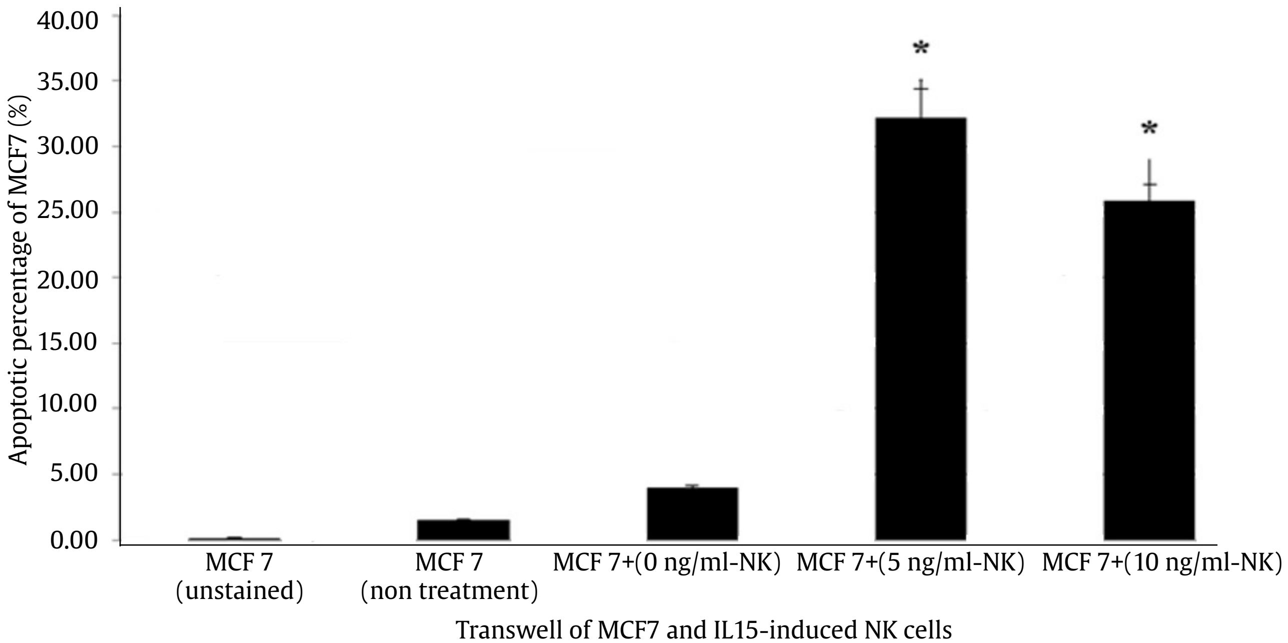

Figure 3. Effect IL15 inducer on NK cells toward apoptotic percentage of MCF7 cells. The data are presented as histogram among treatment, this research was conducted in triplicate for each treatment. The asetrisks symbol (*) present significant differences among concentrations of IL15 inducer (0, 5, 10 ng/ml) compared to control (MCF7 non treatment) toward apoptotic percentage of MCF7

of IL15-induced NK (IL15-NK) transwelled with MCF7 cells, which made NK released cytoplasmic granules (GzmB) can be seen in Figure 5.

The GzmB level secreted by NK cells which co-cultured with MCF7 cells using transwell method (Figure 5). NK cells uninduced by IL15 secreted GzmB in low level (77.06 pg/ml), inducing IL15 (5, 10 ng/ml) increased secretion of GzmB, the highest GzmB level was secreted by NK cells which induced by IL15 (5 ng/ml), meanwhile higher IL15 concentration (10 ng/ml) lowered GzmB level.

3.5. Effect IL15-Induced NK Cells toward Apoptotic and Antiapoptotic Genes Expression of MCF7 and NK Cells

This study was the continued-research to elucidate the apoptosis mechanism of IL15-induced NK cells toward MCF7 cells, in order to determine the apoptotic inducing activity of IL15-induced NK cells toward MCF cells by transwell assay, the expression of apoptotic genes was determined by RT-PCR. We measured the expression of proapoptotic genes, specifically p53, b-cell cll/ lymphoma 2 (bcl-2),

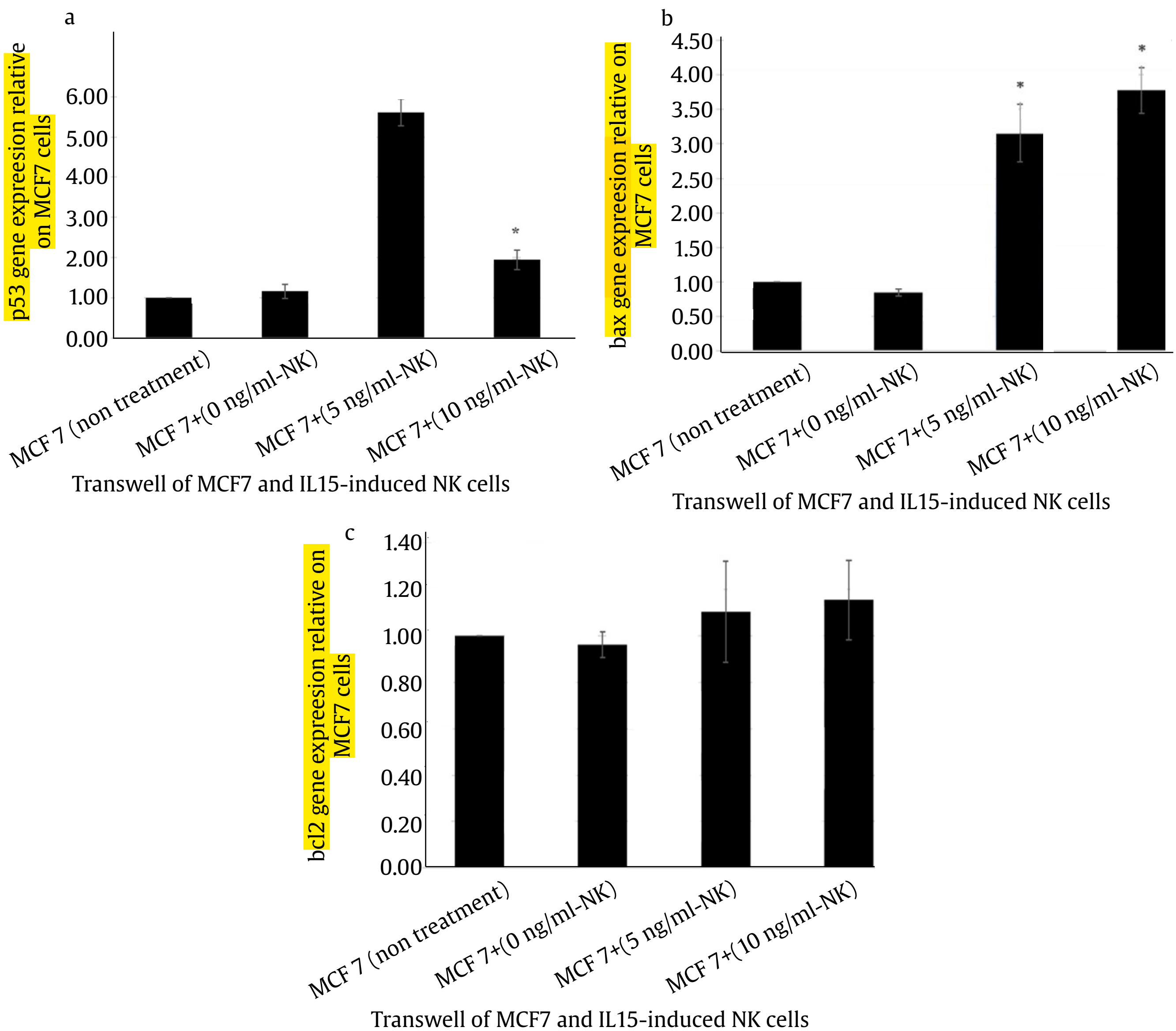

Figure 5. Effect IL15 inducer on NK cells toward bax, p53 and bcl2 gene expression of MCF7 cells. The data are presented as histogram among treatments, this research was conducted intriplicate for each treatment. The asterisks symbol (*) presents significant difference between (inducing IL15 5 ng/ml, inducing IL15 10 ng/ml) compred to non inducing of NK cells based on Tukey HSD post hoc test ($p<0.05$). Figure 6a shows that IL15 (5, 10 ng/ml) up-regulated gene expression of p53 (Figure 6a), bax (Figure 6b) but didn't affected bcl2 gene expresion (Figure 6c)

bcl2-associated x protein (bax). The p53, bax and bcl2 genes expression of MCF7 can be seen at Figure 6.

NK cells induced by IL15 significantly increased the apoptotic genes both low and high concentrations (5, 10 ng/ml) toward bax, p53 genes

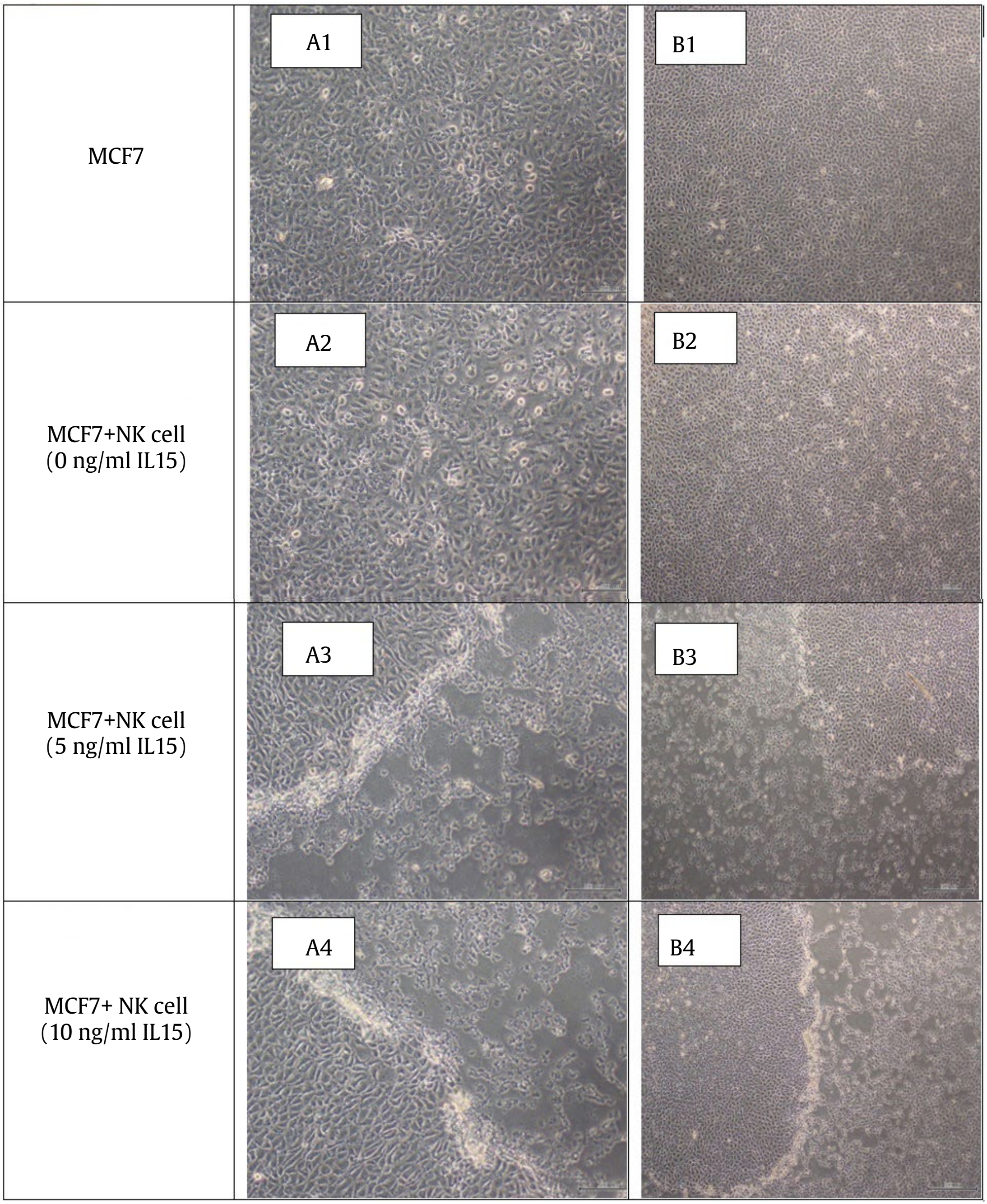

Figure 6. Morphological analysis using phase-contrast microscope at ×100 magnification (A-1, A-2, A-3, A-4) and x40 magnification, (B-1, B-2, B-3, B-4). Different concentration of IL15 as inducer for NK cells decreased the survival rate of MCF7 cells

expression (Figure 6a and b) but IL15-induced NK didn't affect bcl2 expression (Figure 6c). The highest p53 expression was MCF7 treated with 5 ng/ml IL15-induced NK cells, the higher concentration of IL15 (10 ng/ml) was significantly lower p53 expression (Figure 6a). The bax gene expression by MCF7 cells treated with 10 ng/ml IL15-induced NK cells was unsignificantly difference compared to 5 ng/ml IL15-induced NK cells.

3.6. Effect IL15-Induced NK Cells against MCF7 Cells Morphology

This study was performed by investigating the effect of IL15-induced NK cells on viability, density, morphology of MCF7 cells (Figure 7). The result demonstrates that NK cells induced dose-dependent reduction in viability of MCF7 cells.

Based on the morphological MCF7 cells treated with NK cells using transwell assay shows that NK cells lower the viability and density BC cells. The highest viability and density cells was MCF7 (control), the lowest viability, density cells was MCF7 treated by IL15 (5 ng/ml) as NK cells inducer.

4. Discussion

NK cell abnormalities in cancer patients such as imbalance of immune status, inclined to immunosuppression, decreased in NK cell numbers, decreased cytotoxicity, declined activiating receptor (NKG2D, NKG2C, NKp30, NKp46) and inclined inhibitory receptor (NKG2A, CD158, CD158a). Recently has been developed the immunotherapy using activated-NK cells (Mamessier *et al.* 2011).

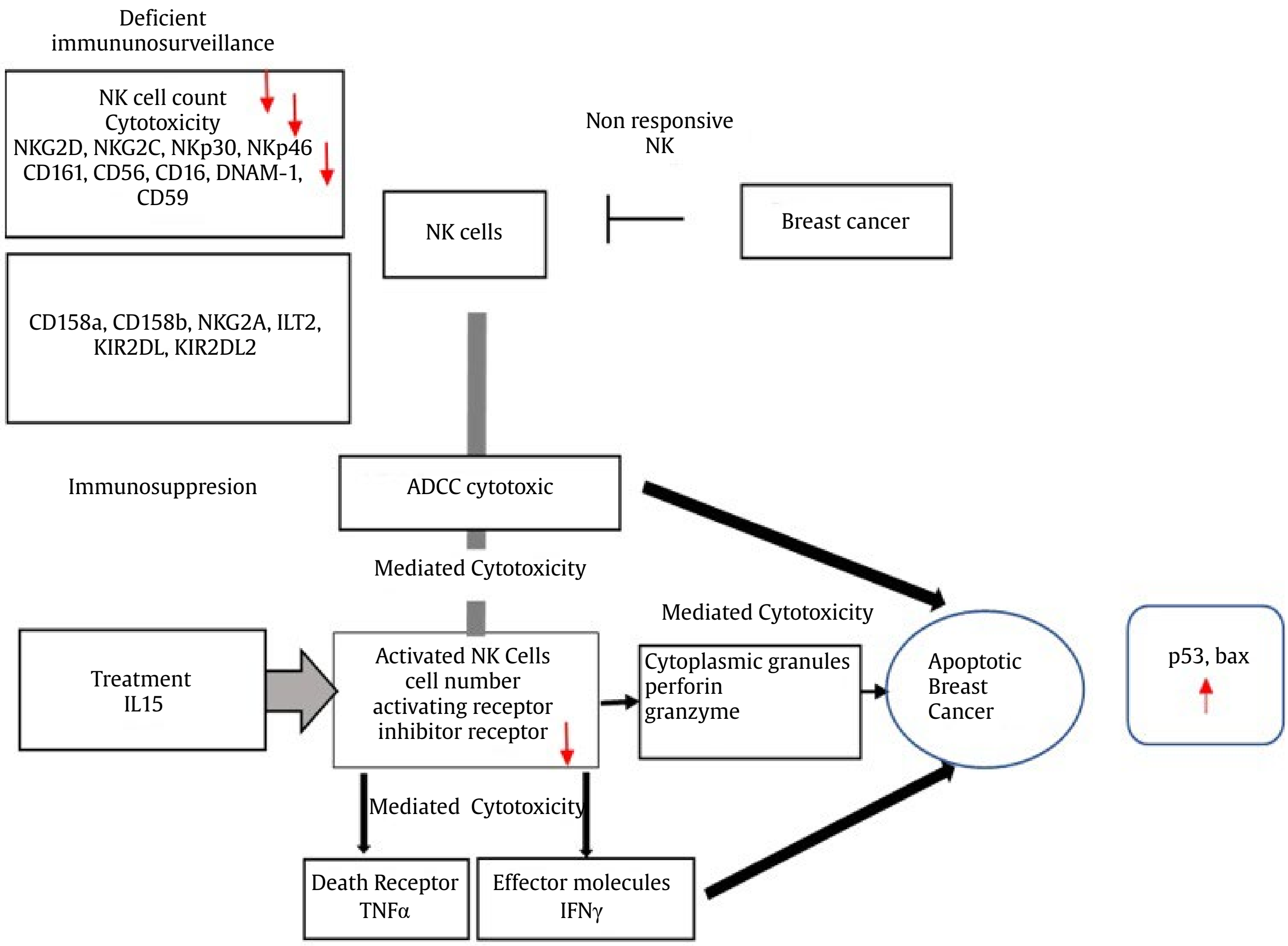

Figure 7. Proposed mechanism of activated NK cells to inhibit and kill breast cancer cells. NK cells abnormalities in breast cancer patients. The imbalance of immune status, which defficient immunosurveillance including low NK cell number in PBMC, poor tumor inflitrate to cancer cells, decreased cytotoxicity, defective expression of activating receptor (NKG2D). IL15 activated NK cells to kill traget cancer cells through: cytoplasmic granules release (Prf, GzmB), death receptor-induced apoptosis (TNFα), effector molecules production (IFNγ) and released antibody-dependent cellular cytotoxicity (ADCC). p53, bax induced apoptotic BC cells

IL2, IL12, IL15, and IL18, 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 (Konjevic *et al.* 2016). Cytokines such as interleukins (IL2) can be used to increase the anticancer potency of NK cells (Widowati *et al.* 2019). Various types of stimulation have been reported to enable NK cells to achieve their full effector potential, such as IL15, IL12, IL18, IL21 (Lucas *et al.* 2007; Guia *et al.* 2008; Chaix *et al.* 2008; Brehm *et al.* 2011). Based on the result (Figure 1), IL15 induced activating receptor NKG2D, this result was validated with previous research that IL2 and IL15 induce the expression of KIRs and activating receptors (NKG2D and NKp44) on NK cell surface (Rham *et al.* 2007; Boieri *et al.* 2017). This result was in line with previous research that metastatic melanoma (MM) patients exhibited decreased CD161 and NKG2D (Konjevi'c *et al.* 2009). NK cells treated with IL15 up-regulated the expression of NK receptors, including Nkp30, Nkp46, NKG2C, and NKG2D (Szczepanski *et al.* 2010). IL15 didn't decline the inhibitory receptor (NKG2A), this result was not consistent with previous research that IL15+IL12 was significantly decline the NKG2A of HIV-infected individuals (Parasa *et al.* 2012).

NK cells have been used in clinical studies in order to treat various malignancies (Harada *et al.* 2017), NK cells abnormalities in cancer patients such as low NK cells counts in PBMC, decreased cytotoxicity (Levy *et al.* 2011). NK cells inhibited MCF7 cells proliferation, IL15 activated NK cells to inhibit MCF7 cells proliferation, this result was consistent with previous research that NK cells control tumor growth and metastasis diffusion *in vivo* (Zamai *et al.* 2007). The low NK cell numbers in tumors due to their inefficient homing into malignant tissues (Levy *et al.* 2011). Decreasing NK cell numbers are observed in PB of cancer patients; therefore, NK cells decrease in tumor infiltrate (Levy *et al.* 2011). The activity and numbers of NK cells need to be enhanced for better efficacy (Mandal and Viswanathan 2002). NK cell infiltration in solid tumors was associated with a better prognosis (Villegas *et al.* 2002). IL-15 is essential for NK cell survival, differentiation, and proliferation (Anton *et al.* 2015). Improving NK cells cytotoxicity used ILs (IL15, IL18), the result showed that ILs increased TNFα, IFNγ secretion by NK cells (Widowati *et al.* 2020)

IL15 induces optimal production of IFN-γ from NK cells, subsequently induce apoptosis of the NK cells (Ross and Caligiuri 1997; Widowati *et al.* 2020). The human MHC class I-negative small cell lung cancer cell line (N592) genetically engineered to secrete IL-15, N592/IL-15, showed a reduced tumor growth rate (Orengo *et al.* 2003). IL15 and IL12 induces optimal production of IFN-γNK cells (Ross and Caligiuri 1997). IFNγ inhibited BC proliferation (MCF7) with median inhibitory 0.34 µg/ml (Widowati *et al.* 2016). IFNγ against MCF7 cells exhibited that the cytokines decreased the cell viability in a dose dependent manner (Widowati *et al.* 2016).

NK secrete GzmB, IL15-activated NK cells up-regulated GzmB secretion. This result was validated previous research that activated NK cells released higher levels of IFN-γ, TNF-α, Prf1, and GzmB compared to non-induced NK cells. IL2, IL15, and IL18 increased the secretion of IFN-γ, TNF-α, Prf1, and GzmB by co-culture cells (Widowati *et al.* 2020). IL15 in NK cell controls as well survival of mature NK cells in the periphery (Marçais *et al.* 2013; Widowati *et al.* 2020), mediated by up-regulation of anti-apoptotic Bcl2 family members and down regulation of apoptotic (Marçais *et al.* 2013). The production of TNF-α, IFN-γ, Prf1, GzmB increased when the ratio of NK cells and hWJMSCs was high (Widowati *et al.* 2019). NK cells activity were controlled by cytokine and ILs (IL2, IL12, IL15, IL18) (Domaica *et al.* 2012). NK cells eliminate malignantly transformed cells principally by releasing the contents of cytotoxic granules into the immune synapse formed with their target cell (Lieberman 2003). The granule mediators of target cell lysis are serine proteases, known as Gzm, which induce programmed cell death (Safta *et al.* 2015; Harada *et al.* 2017). Cytotoxicity of NK cells is executed mainly through the granule exocytosis pathway by releasing Prf1 and GzmB into the immunological synapse after the conjugate formation with targets (Lieberman 2003). Human GzmB preferentially induces target cell apoptosis, induces a rapid accumulation of the tumor-suppressor protein p53 within target cells (Safta *et al.* 2015). Human GzmB-induced p53 accumulates on target cell mitochondria where it interacts with the prosurvival protein Bcl2. This interaction allows the release of the proapoptotic protein Bax from its inhibitory interaction with Bcl2 (Safta *et al.* 2015). Several proteins that are involved in GzmB-induced apoptosis, including casp-9 and -3, Bim, Bid, Bak, Bax, and Xiap. GzmB induced apoptosis cancer cells by involving induction of p53 tumor suppressor gene (Meslin *et al.* 2007).

IL15 inhibit MCF7 proliferation, induced apoptotic BC cells, this result also supported by MCF7 morphology, BC cells appear low density and viability. This result was supported by previous research that ILs (IL1, IL2, IL15) and CD28, serve as co-stimulatory factors, enhancing IFN-γ production by NK cells, as well as NK cell proliferation and cytotoxicity (Hunter *et al.* 1997; Cui *et al.* 2016). ILs (IL12, IL18) stimulate NK cell lines, increasing the secretion of IFN-γ (Wang *et al.* 2012). IL12 and IL18 are critical regulators that activate NK cells via the production of cytokines and direct lysis of target cells (Vivier *et al.* 2011). IL15 activate, increase NK proliferation (Widowati *et al.* 2020). Activated NK cells induce IFN-γ, TNF-α, Prf1, GzmB secretion and inhibit BC cells proliferation on co-cultured of MCF7 and NK cells (Widowati *et al.* 2020).

The proposed mechanism of our research which IL15 activated NK cells including increased NK cells number, up regulated activating receptor (NKG2D) but IL15 didn't influnce inhibitor receptor (NKG2A), improved perforin, granzyme secretion. IL15-activated NK induced apoptosis of BC cells through increased p53, bax gene expression and inhibited BC cells proliferation. For the detail mechanism can be seen at Figure 7.

5. Conclusion

IL15 improve, activate NK cells resulted in activating receptors (NKG2D), increasing GzmB secretion and cytotoxic activity on BC cells, inducing apoptotic genes bax, p53 expression and induce apoptotic cells on transwell assay.

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Judul Karya Ilmiah (Artikel) : Apoptotic Potential of Secretome from Interleukin-Induced Natural Killer Cells toward Breast Cancer Cell Line by Transwell Assay

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No	Komponen Yang Dinilai		Nilai Maksimal JURNAL ILMIAH: Internasional / Bereputasi [√]	Nasional Terakreditasi [ ]	Nasional ***) [ ]	Nilai Akhir Yang Diperoleh *)
a.	Kelengkapan unsur isi karya	(10%)	4			3,7
b.	Ruang lingkup dan kedalaman pembahasan	(30%)	12			11,9
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Jurnal ini sudah sesuai dengan kaidah penulisan dan sesuai antara unsur dan isinya

b. Ruang lingkup & kedalaman pembahasan
Ruang lingkup bahasan sudah memadai dan ada kedalaman dalam pembahasannya dan juga analisisnya.

c. Kecukupan & kemutakhiran data serta metodologi
secara umum metodologi masih terbatas dan belum kecepatnya

d. Kelengkapan unsur dan kualitas penerbit
Kualitas penerbit sudah memenuhi reputasi yang baik

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Secara umum belum ditemukan adanya unsur indikasi plagiasi.
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REVIEWER 2

(Prof. Dr. Ermi Girsang, M. Kes)
NIK : 0117057501
UNIVERSITAS PRIMA INDONESIA

LEMBAR HASIL PENILAIAN
SEJAWAT SEBIDANG atau *PEER REVIEW*

KARYA ILMIAH : JURNAL ILMIAH

Judul Karya Ilmiah (Artikel)	:	Apoptotic Potential of Secretome from Interleukin-Induced Natural Killer Cells toward Breast Cancer Cell Line by Transwell Assay
Jumlah Penulis	:	10
Nama-nama Penulis	:	**Wahyu Widowati**, Diana Krisanti Jasaputra, Teresa Liliana Wargasetia, The Fransiska Eltania, Alya Mardhotillah Azizah, Mawar Subangkit, I Nyoman Ehrich Lister, Chrismis Novalinda Ginting, Ermi Girsang, Ahmad Faried
Status Penulis	:	Penulis Pertama / ~~Penulis ke ....~~ / Penulis Korespondensi **)
Identitas Jurnal Ilmiah	:	a. Nama jurnal : Hayati Journal of Biosciences
		b. Nomor ISSN : 1978-3019
		c. Vol., No., Bulan, Tahun : Vol. 27, No. 3, Juni, 2020
		d. Penerbit : Institut Pertanian Bogor
		e. DOI Artikel (jika ada) : 10.4308/hjb.27.3.186
		f. Alamat Web Jurnal : https://journal.ipb.ac.id/index.php/hayati
		g. Terindeks di : Scopus Q2, SJR 0.31

Kategori Publikasi Jurnal Ilmiah: [√] ~~Jurnal Ilmiah Internasional~~ / Internasional Bereputasi **)
(beri tanda √ yang dipilih)
[ ] Jurnal Ilmiah Nasional Terakreditasi
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a.	Kelengkapan unsur isi karya	(10%)	4			3,7
b.	Ruang lingkup dan kedalaman pembahasan	(30%)	12			11,7
c.	Kecukupan dan kemutakhiran data/ informasi dan metodologi	(30%)	12			11,6
d.	Kelengkapan unsur dan kualitas penerbitan	(30%)	12			11,6
	Total	**100%**	**40**			38,6

Catatan Penilaian ARTIKEL oleh Reviewer :

a. Kelengkapan dan kesesuaian unsur
Paper ditulis dengan baik, unsur lengkap. hasil sesuai dengan judul penelitian, runtun mengikuti kaidah penulisan karya ilmiah internasional bereputasi

Jurnal ini sudah sesuai dengan kaidah penulisan dan sesuai antara unsur dan isinya

b. Ruang lingkup & kedalaman pembahasan
Data penelitian meliputi data potensi sekretom dari sel NK yang diinduksi Interleukin dapat meningkatkan penghambatan proliferasi sel kanker payudara secara in vitro.

Ruang lingkup bahasan sudah memadai dan ada kedalaman dalam pembahasannya dan juga analisinya

c. Kecukupan & kemutakhiran data serta metodologi Sumber pustaka mutakhir, metode penelitian kokultur sel NK dan sel kanker payudara untuk mengukur aktivitas sekretom sel NK yang diinduksi IL-15 menunjukkan IL-15 meningkatkan ekspresi gen antikanker sehingga menghambat proliferasi sel kanker

Secara umum metodologi masih terbatas dan belum sesempurnanya

d. Kelengkapan unsur dan kualitas penerbit

Hayati J. Biosci terindeks scopus Q2, SJR 0,31 Penerbit IPB.

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