RESEARCH ARTICLE

MCBS
Mol Cell Biomed Sci. 2018; 2(2): 60-9
DOI: 10.21705/mcbs.v2i2.21

# Direct and Indirect Effect of TNFα and IFNγ Toward Apoptosis in Breast Cancer Cells

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**Background:** Breast cancer (BC) is the leading cause of death cancer in women. Cancer therapies using TNFα and IFNγ have been recently developed by direct effects and activation of immune responses. This study was performed to evaluate the effects of TNFα and IFNγ directly, and TNFα and IFNγ secreted by Conditioned Medium-human Wharton's Jelly Mesenchymal Stem Cells (CM-hWJMSCs) toward apoptosis of BC cells (MCF7).
**Materials and Methods:** BC cells were induced by TNFα and IFNγ in 175 and 350ng/mL, respectively. CM-hWJMSCs were produced by co-culture hWJMSCs and NK cells that secreted TNFα, IFNγ, perforin (Prf1), granzyme B (GzmB) for treating BC cells. The BC cells were treated with CM-hWJMSCs in 50%. The expression of apoptotic genes Bax, p53, and the anti-apoptotic gene Bcl-2 were determined using RT-PCR.
**Results:** TNFα and IFNγ at concentration of 350 ng/mL induced higher Bax expression compared to 175 ng/mL. TNFα and IFNγ 350 ng/mL, 175 ng/mL induced p53 expression, whilst TNFα and IFNγ at 350 ng/mL decreased Bcl-2 expression. Perf1, GzmB, TNFα and IFNγ-containing CM-hWJMSCs induced significantly apoptosis percentage, induced Bax expression, but did not effect p53, Bcl-2 expression.
**Conclusion:** TNFα and IFNγ directly induce Bax, p53, decrease Bcl-2 gene expression. The Prf1, GzmB, TNFα, IFNγ-containing CM-hWJMSCs induce apoptosis and Bax expression.

**Keywords:** breast cancer, Wharton's Jelly mesenchymal stem cells, TNFα, IFNγ

## Introduction

Breast cancer (BC) is one of the most commonly oncologic diseases worldwide[1], the leading cause of cancer death among women, with 882,900 cases diagnosed and 324,300 deaths in 2012, approximately 25% of cancer cases and 15% of cancer deaths among women.[2] The incidence BC worldwide will reach 3.2 million new cases per year by 2050.[3] BC is

Date of submission: October 12, 2017
Last Revised: December 15, 2017
Accepted for publication: January 11, 2018

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Cell and Biopharmaceutical Institute

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v2i2.21

the most diagnosed cancer and the leading cause of cancer deaths among women in the ASEAN region. The highest incidence rate per 100,000 is found in Singapore (59.9) and the lowest in Vietnam (15.6), Indonesia exhibits the highest mortality rate of 36.2 per 100,000 patients while the lowest is Singapore around 13.6 per 100,000 patients.[4]

BC treatments are chemotherapy, surgery, endocrinotherapy, radiotherapy and molecular-targeted therapy.[5] All therapies are expensive and low effectiveness.[4] The development of efficient and effective therapy is highly required, which can directly target both primary and metastatic side. Metastatic side that features invasives and escapes antitumor immunity.[6-9] Metastasis in BC is caused by an immunosurveillance deficiency, including an impairment of NK cell maturation, low NK cell count in peripheral blood mononuclear cells (PBMCs), low NK activity[10], decreased cytotoxic functions[11-13], NK abnormalities[12], poor tumor infiltrate[14-15], inefficient homing into malignant tissues[12]. The promising cancer therapies are to stimulate NK cell functions, the combination NK cells with other anticancer agents[16], NK cells respond to various cytokines, such as interleukin-15 (IL15), IL18[17-19]. NK cells suppress cancer by releasing cytoplasmic granules, perforin (Prf) and granzyme (Gzm), to trigger cells apoptosis.[20] Activated-NK cells secrete tumor necrosis factor (TNFα), various effector molecules, such as interferon (IFNγ) that can induce cancer cell apoptosis.[21]

Mesenchymal Stem Cells (MSCs) have ability the homing to tumors, these stem cells have been engineered as drug delivery system. The condition medium of human MSCs (hMSCs) have been proved to inhibit bladder cancer cells viability by secreting soluble factor which is involved in PTEN/PI3K/Akt.[22] Wharton's Jelly (WJ) is part of umbilical cord as one source of hMSCs has many advantages including low risk infection, non-carcinogenesis, multipotency and low immunogenicity.[23] The hWJMSCs have anticancer activity which mediate via cell-to-cell and/or non-cellular contact mechanism. The conditioned medium of hWJMSCs inhibit cancer proliferation.[24] Anticancer agents produced by engineered MSCs are: a). Immunostimulation such as chemokine C-X3-C motive ligand 1 (CX3CL1), IFN and interleukins (IL2, IL7, IL12), b). Pro-drug conversion such as cluster of differentiation (CD) and Herpes Simplex Virus Thymidine Kinase (HSV-tk), c). Apoptosis induction such as IL-8, Natural Killer 4 (NK4) and Tumour Necrosis Factor-related apoptosis inducing ligand (TRAIL).[21,25,26]

TNFα can induce apoptotic (caspase-dependent) or necrotic (caspase-independent) cell death *in vitro*, depending on the cell type used.[27] The IFNs mediate anticancer effect directly by modulating immunomodulatory response or directly by regulating tumour cell proliferation and differentiation[28] and inhibition of tumour angiogenesis[29]. My previous research resulted that TNFα and IFNγ have anticancer activities toward BC cells (T47D, MCF7), but it was non toxic toward hWJMSCs.[30]

This research was the continuing study to evaluate the directly effects of TNFα and IFNγ which indirectly effect of TNFα and IFNγ which secreted in CM co-culture hWJMSCs and NK cells toward apoptosis of BC cells (MCF7) and to increase the cytotoxic of NK cells was induced by interleukins (IL15, IL18).

## Materials and methods

### *Real-time PCR assay for the apoptotic induction of MCF7 cell line by TNFα, IFNγ*

To determine the apoptosis induction ability of recombinant human TNFα (Biolegend 570106), recombinant human IFNγ (Biolegend 570206) toward MCF7 (ATCC®HB22TM) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. The MCF7 cancer cells were cultured in density $2x10^6$/well, 500 µL RPMI (1640 Gibco 22400089), 10% FBS (Gibco 10270106), 1% Antibiotic Antimycotic (Gibco 1772653) and supplemented with TNFα (175, 350 ng/mL), IFNγ (175, 350 ng/mL) incubated in 370°C, 5% $CO_2$ for 24 h.[30]

Total RNA was isolated from MCF7 cells (AurumTM Total RNA Mini Kit, Bio-Rad 732-6820) based on the manufacturer's instructions. The total RNA yield was estimated spectrophotometrically at 260, 280 nm (Table 1). The RNA quality were confirmed via electrophoresis and measured its purity then RNA was reverse-transcribed into cDNA (iScript cDNA Synthesis Kit, 170-8841; Bio-Rad), for which the mixture was firstly incubated at 25°C for 5 minutes, then 42°C for 30 minutes, and finally at 85°C for 5 minutes. PCR amplification was performed using a PikoRealTM Real-Time PCR System (Thermo Scientific Inc.). The qPCR conditions were pre-denaturation at 95°C for 30 seconds, then 40 cycles of qPCR with denaturation for 5 seconds at 95°C, annealing for 20 seconds at 58°C, then elongation for 30 seconds at 72°C. As an internal control, β-actin was included as the house-keeping gene. The primers used for RT-PCR are summarized are : β-actin

(forward : 5'-TCT GGC ACC ACA CCT TCT ACA ATG-3', reverse : 5'-AGC ACA GCC TGG ATA GCA ACG-3'), p53 (forward : 5' AGA GTC TAT AGG CCC ACC CC 3', reverse : 5'-GCT CGA CGC TAG GAT CTG AC 3'), Bax (forward : 5'-TGC TTC AGG GTT TCA TCC AG 3', reverse : 5'-GGC GGC AAT CAT CCT CTG 3'), Bcl-2 (forward : 5'-GGT CAT GTG TGT GGA GAG CG -3', reverse : 5'-GGT GCC GGT TCA GGT ACT CA-3').

***Co-culture of hWJMSCs and ILs-NK cells for measuring the cytoplasmic granules and effector molecules***

NK92MI cells (ATCC® CRL2408™) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia at density $2x10^6$/well were grown and maintained in NK medium RPMI 1640 (Gibco 22400089), 20% FBS (Gibco 10270106), 1% Antibiotic Antimycotic (Gibco 1772653). The cells were treated with 5, 10 ng/mL of recombinant human IL15 (Biolegend 715902) or IL18 (GenScript Z031189) per 24 h and incubated at 5% $CO_2$, 37°C for 96 h, which resulted in IL15-induced NK (IL15-NK), IL18-induced NK (IL18-NK) cells.

The hWJMSCs were obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia at density $2x10^6$ were cultured in minimum essential medium-α (α-MEM, Gibco 12561056), 10% FBS, 1% Antibiotic Antimycotic. The hWJMSCs were incubated in a humidified atmosphere with 5% $CO_2$ at 37°C for 24 h. NK cells were adjusted to $1x10^5$ for the ratio 1:1 (hWJMSCs : NK = 1:1; hWJMSCs : IL15-NK = 1:1; hWJMSCs : IL18-NK = 1:1). NK cells were resuspended in 24-well plates containing hWJMSCs in NK medium (RPMI 1640, Gibco 22400089) and α-MEM medium at the ratio 1:1 (50%:50%) according the optimized research (data are not shown). The co-cultures of NK, IL15-NK, IL18-NK cells and hWJMSCs were incubated in a humidified atmosphere with 5% $CO_2$ at 37°C for 24 h. Cells and medium were centrifuged at 500 g for 4 mins, and the medium was preserved in -80 °C for the next assays such as for IFNγ, TNFα, Prf1, and GzmB.[31-32]

***Cytotoxic activity of CM from co-culture of hWJMSCs and ILs-NK toward MCF7***

The BC cell lines MCF7 ($5x10^3$/well) were cultured in complete medium which consisted of DMEM (Gibco 11995065), supplemented with 10% FBS and 1% Antibiotic Antimycotic. The cells were incubated in a humidified atmosphere, 5% $CO_2$, 37°C for 24 h.[33-35] Furthermore, the cells were treated with CM-(hWJMSCs+NK), CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18-NK) in 30% or 60% concentrations and were incubated for 24 h. The cell viability was assayed based on an optimized reagent containing resazurin, which was converted to fluorescent resorufin by viable cells that absorbed the light at 490 nm using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA).[36,24,30]

***Apoptotic activity of CM from co-culture of hWJMSCs and ILs-NK toward MCF7 using flowcytometer***

The BC cells with density $1x10^6$ cells/disk were cultured in complete medium (DMEM + 10% FBS + 1% Antibiotic Antimycotic). The cells were incubated in humidified atmosphere, 5% $CO_2$, 37°C for 24 h. The BC cells were treated using 50% CM of co-culture hWJMSCs and ILs-induced NK ((CM-(hWJMSCs), CM-(hWJMSCs+NK), CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18-NK)), the cells were incubated for 24 h. The MCF7 cells were washed using PBS and the cells were harvested and inactivated using 0.25% trypsin EDTA (Gibco, 25200072). The cells were centrifuged in 1600 rpm, and cells were counted using hemocytometer. The accounted cells $5x10^5$ - $1x10^6$ were added 500 µL Annexin V binding buffer (Miltenyi Biotec, 130-092-820), 5 µL Anti-FITC (Miltenyi Biotec, 130-048-701), 5 µL Propidium Iodide (Miltenyi Biotec, 130-093-233) furthermore the cells were incubated in darkness, 4°C and the apoptotic percentage of MCF7 cells were anayzed using MACSquant Analyzer 10 (Miltenyi Biotec).

***Apoptotic gene expression of CM from co-culture of hWJMSCs and ILs-NK toward MCF7 using RT-PCR***

The BC cells with density $1x10^6$ cells/disk were cultured in complete medium (DMEM + 10% FBS + 1% Antibiotic Antimycotic). The cells were incubated in humidified atmosphere, 5% $CO_2$, 37°C for 24 h. The BC cells were treated using 50% CM of co-culture hWJMSCs and ILs-induced NK ((CM-(hWJMSCs), CM-(hWJMSCs+NK) CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18-NK)), the cells were incubated for 24 h. The MCF7 cells were washed using PBS and the cells were harvested and inactivated using 0.25% trypsin EDTA (Gibco 25200072). Total RNA was isolated from MCF7 cells (AurumTM

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v2i2.21

Total RNA Mini Kit, Bio-Rad 732-6820) based on the manufacturer's instructions. The Bax, p53, Bcl-2 genes expression along with the constitutively expressed β-actin gene was analyzed using real-time quantitative polymerase chain reaction (qPCR).[37]

***Statistical analysis***

Statistical analysis was analyzed with Statistical Package for the Social Sciences (SPSS) statistics version 20.0 software. One-way analysis of variance (ANOVA) was conducted, followed by Tukey HSD post-hoc test and $p<0.05$ was considered to be significant.

## Results

***The effect directly TNFα, IFNγ toward apoptotic and antiapoptotic genes expression***

The previous research showed that TNFα, IFNγ inhibited breast cancer proliferation (MCF7) with median inhibitory concentration ($IC_{50}$) 0.36 μg/mL, 0.34 μg/mL.[30] This study was the continued-research to elucidate the apoptosis mechanism of TNFα, IFNγ in MCF7 cells. In order to determine the apoptotic inducing activity of TNFα, IFNγ toward MCF7 cells, 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), Bcl2-associated X protein (Bax). TNFα induced significantly the apoptotic genes both low and high concentrations (175, 350 ng/mL) toward Bax, p53 genes expression. TNFα 350 ng/mL was more active than 175 ng/mL to reduce Bcl-2 and induce Bax (Table1). IFNγ in both concentrations (175, 350 ng/mL) induced apoptotic genes, IFNγ 350 ng/mL was able to down regulate Bcl-2 (Table 1).

***The effect of ILs-NK cells on TNFα, IFNγ, Prf and Gzm levels in hWJMSCs and NK cells***

*In vitro* study proved that CM of hWJMSCs have the ability to inhibit various cancer proliferation[24], but MSCs are able to suppress the proliferation and function of immune system cells, influence cytotoxic activities of NK cells[38-40], TNFα, IFNγ secretion[41]. Our study try to improve the NK cells cytotoxic, increase TNFα and IFNγ secretion to induce cancer cell cytolysis[42], release cytotoxic granules namely Gzm and Prf[43-44]. We measured the cytolotic secretion of co-culture hWJMSCs and ILs-induced NK cells including TNFα, IFNγ, Gzm and Prf (Table 2).

hWJMSCs secreted TNFα, IFNγ in low level, NK cells secreted in higher level than hWJMSCs. NK cells did not secrete Gzm, Prf and but it secreted in co-culture with hWJMSCs. Interleukins (IL15, IL18) induced-NK cells to secrete TNFα, IFNγ, Gzm and Prf significantly. IL18 was more active to induce NK cells in secreting TNFα, IFNγ, Gzm and Prf.

***Effect CM coculture hWJMSCs and NK cells towards cytotoxic effect of MCF7***

CM-hWJMSCs have been known to exhibit anticancer activities, which contains micro-particles that mediate therapeutic effects against cancer.[45] MSCs release the

**Table 1. Effect directly TNF-α, IFN-γ toward apoptotic gene expression in MCF7 cells.**

| Treatment | RNA Purity (260/280 nm) | Bax | p53 | Bcl-2 |
|---|---|---|---|---|
| **1 TNFα on MCF7** | | | | |
| Control (MCF7 untreated) | 2.4288±0.4768 | 1.00±0.00 $^{a}$ | 1.00±0.00 $^{a}$ | 1.00±0.00 $^{b}$ |
| TNFα 350 ng/mL | 2.3493±0.2650 | 23.62±1.43 $^{c}$ | 2.94±0.31 $^{b}$ | 0.33±0.08 $^{a}$ |
| TNFα 175 ng/mL | 2.5902±0.3898 | 5.29±0.93 $^{b}$ | 2.17±0.56 $^{b}$ | 1.28±0.31 $^{b}$ |
| **2 IFNγ on MCF7** | | | | |
| Control (MCF7 untreated) | 2.4288±0.4768 | 1.00±0.00 $^{a}$ | 1.00±0.00 $^{a}$ | 1.00±0.00 $^{b}$ |
| IFNγ 350 ng/mL | 2.6019±0.4678 | 34.79±4.35 $^{b}$ | 4.17±1.06 $^{b}$ | 0.18±0.04 $^{a}$ |
| IFNγ 175 ng/mL | 2.2870±0.3335 | 3.49±0.35 $^{a}$ | 2.83±0.62 $^{b}$ | 1.07±0.16 $^{b}$ |

The data are presented as mean±standard deviation. The gene expression were measured in triplicate for each sample. Different superscripts in the same column (a,b,c) for each effect of TNFα or IFNγ on MCF7 indicate significant differences among the means of groups (concentrations of TNFα or IFNγ ) based on Tukey HSD post-hoc comparisons ($p< 0.05$).

**Tabel 2. Effect interleukins (IL-15, IL18)-induced NK cells toward TNFα, IFNγ, perforin, granzyme level in co-culture hWJMSCs and NK cells.**

| Conditioned Medium of Co-culture hWJMSCs and ILs-induced NK Cells | Level of CM Secretome | | | |
|---|---|---|---|---|
| | IFNγ (pg/mL) | TNFα (pg/mL) | Prf1 (pg/mL) | GzmB (pg/mL) |
| CM-hWJMSCs | 0.94±0.19 [a] | 2.64±0.47 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] |
| CM-NK cells | 8.06±1.90 [a] | 8.49±1.60 [a] | 50.00±10.00 [a] | 6.67±0.58 [a] |
| CM co-culture hWJMSCs + NK (1:1) | 7.72±2.52 [a] | 5.07±0.25 [a] | 50.00±20.00 [a] | 6.00±2.00 [a] |
| CM co-culture hWJMSCs + IL15-NK (1:1) | 8.17±2.60 [a] | 18.17±3.45 [b] | 120.00±30.00 [b] | 33.00±7.21 [b] |
| CM co-culture hWJMSCs + IL18-NK (1:1) | 19.72±5.52 [b] | 17.06±1.48 [b] | 140.00±50.00 [b] | 31.00±9.64 [b] |

The data are presented as mean±standard deviation. The level of secretome were measured in triplicate for each sample. Different superscripts in the same column (a,b) of TNFα, IFNγ, granzyme and perforin among single cells (hWJMSCs, NK cells) and co-culture cells (hWJMSCs and ILs-induced NK cells, hWJMSCs and NK cells) indicated significant differences based on Tukey HSD post-hoc comparisons ($p< 0.05$)

complex factor which are able to reduce the proliferation of glioma, melanoma, lung cancer, hepatoma, and breast cancer cells.[46-49] CM-hWJMSCs can weaken the immuno-modulatary system, NK cells, lower cytotoxic effect. To activate hWJMSCs and NK cells are required to increase cytotoxic effect toward cancer cells. To activate the anticancer potential by utilizing cytokines, such as interleukins, may directly boost the anticancer property of NK cells and indirectly CM-hWJMSCs. Conditioned medium of coculture hWJMSCs and ILs-induced NK cells for treatment toward MCF7 cells. The cells were treated CM-(hWJMSCs+NK), CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18-NK) in 30% or 60% concentrations and were incubated for 24 h (Table 3).

***Effect CM from co-culture of hWJMSCs and ILs-NK towards Apoptotic activity of MCF7***

MSCs secrete secretome such as chemokins, cytokines, growth factor as bioactive and nutrition[50], these secretome are important to preserve the homeostasis, cross talk with stromal cells (fibroblast, endothel cell, macrophage). The soluble factor of CM affect stem cells fate by *in vitro* assay.[51] The released soluble factor of MSCs affect brain cancer cells, melanoma, lung cancer by *in viv*o assay[46], CM-MSCs reduce NFkB secretion of hepatome, breast cancer cells and inhibit cells proliferation by *in vitro* assay[49].

The activated-NK cells significantly increase secretion of IFNγ, TNFα, chemokins, cytokin playing role immune respond, eliminate cancer cells[16,52]. We measured

**Table 3. Effect cytotoxic of CM co-culture hWJMSCs and NK cells toward MCF7.**

| CM of Co-culture of hWJMSCs and ILs-activated NK Cells | Proliferation Inhibition Toward MCF7 Cells (%) | |
|---|---|---|
| | Concentration 30% of CM Co-culture (hWJMSCs+ ILs-NK) | Concentration 60% of CM Co-culture (hWJMSCs+ ILs-NK) |
| Non-CM (MCF7 cells only) | 0.00±6.61 [a] | 0.00±6.61 [a] |
| CM-hWJMSCs | 32.55±6.57 [b] | 48.00±8.70 [b] |
| CM-hWJMSCs + NK (1:1) | 40.44±4.02 [b] | 51.86±3.17 [bc] |
| CM-hWJMSCs + IL15-NK (1:1) | 56.82±7.66 [c] | 60.55±4.91 [bc] |
| CM-hWJMSCs + IL18-NK (1:1) | 58.91±3.17 [c] | 64.49±1.25 [c] |

The data are presented as mean±standard deviation. The proliferation inhibition were measured in triplicate for each sample. Different superscripts letter in the same column (a,b,c) of among 30% CM concentration of coculture hWJMSCs, NK cells and different superscript letter culture in the same column (a,b,bc,c) of among 60% CM concentration of coculture hWJMSCs, NK cells indicated significant differences based on Tukey HSD post-hoc comparisons ($p< 0.05$)

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v2i2.21

the CM from co-culture of hWJMSCs and ILs-NK (with concentrations 50%) towards apoptotic activity in MCF7 cells. CM-(hWJMSCs+ILs-NK) induced apoptosis of BC cells (Table 4, Figure 2).

Table 4 showed that CM from coculture hWJMSCs and NK cells which were activated by ILs 10 ng/mL (IL15, IL18) induced apoptosis, reduced live cells, necrosis in MCF7 cells. The acivated NK cells were more active to induce apoptosis and kill BC cells compared to NK cells without activating or CM-hWJMSCs. IL15 and IL18 activate NK cells to induce apoptosis MCF7 cells, IL18 was more active to induce NK cells compared to IL15.

***Effect CM from co-culture of hWJMSCs and ILs-NK towards pro- and anti-apoptotic gene***

The progressive BC cells involved disfunction NK cells, furthermore cancer therapy needs to improve NK cells cytotoxicity, inhibit lossing antitumor immune system.[53] CM-hMSCs significantly inhibited proliferation, induced apoptosis, significantly upregulated the apoptotic genes of both Casp3 and Casp9, significantly downregulated the antiapoptotic genes such as SURVIVIN and XIAP, induced and completed differentiation in human U251 cell line.[6] We measured the proapoptotic and antiapoptotic gen expression namely Bax, p53 and Bcl-2 (Table 5.)

**Table 4. Effect CM from co-culture of hWJMSCs and ILs-NK towards Apoptotic activity of MCF7.**

| CM-(hWJMSCs + ILs-NK) | Early Apoptosis (%) | Live cells (%) | Necrosis (%) | Dead cells (%) |
|---|---|---|---|---|
| Non-CM (MCF7 cells only) | 4.51±0.78 [a] | 87.10±1.56 [c] | 6.74±0.29 [b] | 1.65±0.78 [a] |
| CM-hWJMSCs | 33.11±0.92 [b] | 49.30±1.01 [b] | 6.70±0.28 [b] | 10.88±0.15 [d] |
| CM-(hWJMSCs+NK) | 34.66±1.93 [b] | 53.15±3.75 [b] | 4.87±0.22 [a] | 7.32±1.74 [c] |
| CM-(hWJMSCs+IL15-NK) | 38.16±2.14 [c] | 38.99±1.48 [a] | 15.29±0.89 [c] | 7.55±0.42 [c] |
| CM-(hWJMSCs+IL18-NK) | 47.03±0.54 [d] | 42.95±1.12 [a] | 4.96±0.33 [a] | 5.06±0.80 [b] |

The data are presented as mean±standard deviation. The apoptosis, live cells, necrosis, dead cells were measured in triplicate for each sample. Different superscripts letter in the same column (a,b,c,d,e) of apoptosis among CM concentration of co-culture hWJMSCs and ILs-NK cells and different superscript letter in the same column (a,b,c) of live cells among CM concentration of co-culture hWJMSCs and NK cells, different superscript letter in the same column (a,b,c) of necrosis cells among CM concentration of co-culture hWJMSCs and NK cells , different superscript letter in the same column (a,b,c,d) of necrosis cells among CM concentration of co-culture hWJMSCs and NK cells, different superscript letter in the same column (a,b,c,d) of dead cells among CM concentration of co-culture hWJMSCs and NK cells indicated significant differences based on Tukey HSD post-hoc comparisons ($p< 0.05$)

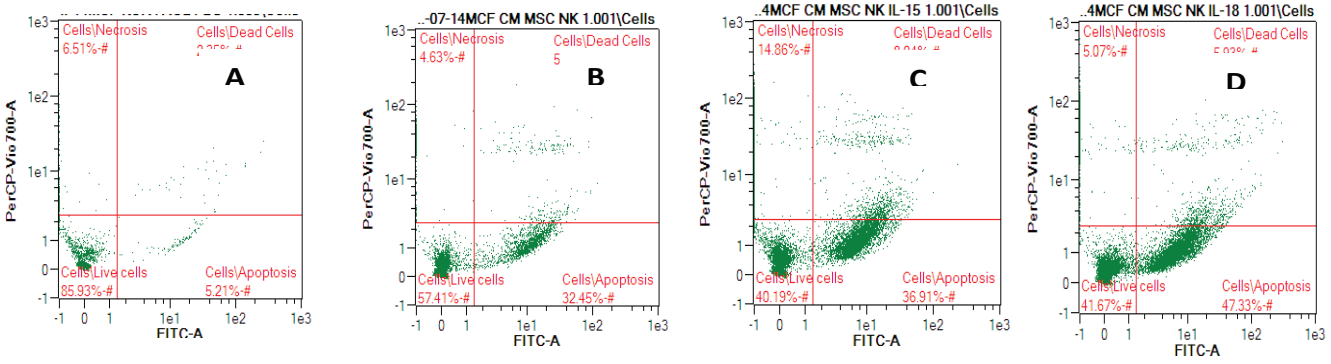


**Figure 1. Effect CM-coculture hWJMSCs and ILs-activated NK cells toward apoptosis inducing activity of BC cells.** Percetage Apoptosis of BC cells treated with CM co-culture of hWJMSCs and ILs-activated NK cells were done in triplicate using flowcytometry. A: Control (MCF7 cell untreated, live cells 85.93%, necrosis 6.51%, dead cells 2.35%, apoptosis 5.21%), B: MCF7 treated with CM (hWJMSCs+NK) (live cells 57.41%, necrosis 4.63%, dead cells 5.51%, apoptosis 32.45%), C: MCF7 treated with CM (hWJMSCs+IL15-NK) (live cells 40.19%, necrosis 14.86%, dead cells 8.04%, apoptosis 36.91%), D: MCF7 treated with CM (hWJMSCs+IL18-NK) (live cells 41.67%, necrosis 5.07%, dead cells 5.93%, apoptosis 47.33%)

**Tabel 5. Effect CM from co-culture of hWJMSCs and ILs-NK towards pro-apoptotic and anti-apoptotic genes on MCF7 cells.**

| Conditioned Medium CM-(hWJMSCs + ILs-NK) | RNA Purity (260/280 nm) | Bax | p53 | Bcl-2 |
|---|---|---|---|---|
| Non-CM (MCF7 cells only) | 2.2368±0.2639 | 1.00±0.00 [a] | 1.00±0.00 [a] | 1.00±0.00 |
| CM-hWJMSCs | 2.6868±0.4326 | 1.36±0.36 [a] | 1.23±0.25 [a] | 0.93±0.09 |
| CM-(hWJMSCs+NK) | 2.4541±0.3385 | 1.52±0.17 [a] | 1.47±0.15 [a] | 0.96±0.19 |
| CM-(hWJMSCs+IL15-NK) | 2.7321±0.1301 | 1.61±0.27 [a] | 2.03±0.36 [a] | 0.98±0.10 |
| CM-(hWJMSCs+IL18-NK) | 2.3336±0.3337 | 2.56±0.40 [b] | 3.70±0.95 [b] | 0.89±0.24 |

The data are presented as mean±standard deviation. The proapoptotic (Bax, p53) and antiapoptotic genes (BCL2) were measured in triplicate for each sample. Different superscripts letter in the same column of BAX, TP53 gene expression (a,b) among CM concentration of co-culture hWJMSCs and ILs-NK cells indicated significant differences based on Tukey HSD post-hoc comparisons ($p< 0.05$).

## Discussions

The effect of TNFα and IFNγ against MCF7 cells exhibited that the cytokines decreased the cell viability in a dose dependent manner. The $IC_{50}$ value of TNFα and IFNγ against BC cell lines were found 242.77-266.88 ng/mL for T47D and 295-03-364.78 ng/mL for MCF7.[30] Concentration 350 ng/mL of TNF-α and IFN-γ was more active to increase Bax expression compared to 175 ng/mL but there was not significant difference between 350 ng/mL and 175 ng/mL in p53 gene expression. Concentration 350 ng/mL of TNFα, IFNγ reduced Bcl-2 expression, 175 ng/mL of TNFα, IFNγ could not reduce Bcl-2 expression. TNFα-induced apoptosis in BC cells through Bcl-2 in low expression.[54] IFNγ increased Bax level.[55] p53 and Bax may be the targets for the IFNγ based chemo-immunotherapy of the chemotherapy-resistant cancers.[55] This study was contradictory with previous reserach that Bcl-2 expression inhibited TNFα-induced apoptosis in MCF7 cells[56], overexpression of Bcl-2 and Bcl-XL was correlated with an increased resistance to TNFα-induced apoptosis[57]. Insulin-Like Growth Factor- Binding Protein-3 (IGFBP-3) expression is up-regulated in response to TNFα, with some evidence that it may mediate the inhibitory effects of TNFα in TNF-sensitive BC cell lines[58]. IGFBP-3 and -5 translocate to the nucleus in BC cells[59,60], they can transcriptionally modulate the expression of apoptotic genes such as bax and Bcl-2[61,62]. It has been reported that p53 can induce apoptosis through a transcription-independent pathway, promote cell death by binding to Bcl-xL, contribute to TNFα-induced apoptosis in retinoblastoma fibroblasts.[63] IFNγ inhibits growth human pancreatic carcinoma cell lines (AsPc-1, Capan-1, and Capan-2), induced DNA fragmentation and poly (ADP ribose) polymerase (PARP) cleavage and increase antiproliferative activity in pancreatic cancer cells due to apoptotic induction.[64] ILs-activated NK cells release cytoplasmic granules (Prf1, Gzm), detah receptor (FASL, TRAIL, TNFα), effector molecules (IFNγ, NO) which kill target tumor cells.[43]

CM-hWJMSCs contains various growth factors, cytokines, chemokines, and tissue regenerative agents, therapeutic potency[65], CM-hWJMSCs inhibit proliferation of cancer cell lines including HeLa, SKOV3, HepG2, PC3, HSC3[24], A549, HT29, MCF7[66]. MSCs have side effect to suppress the proliferation and function of immune system cells, influence cytotoxic activities of NK cells[38-40], suppress TNFα, IFNγ secretion[41]. Our research to improve NK cells cytotoxicity using ILs (IL15, IL18), the result showed that ILs increased TNFα, IFNγ secretion by NK cells or co-culture ILs-NK and hWJMSCs. ILs increased GzmB and Prf1 secretion by co-culture NK cells and hWJMSCs (Table 2). NK cells activity were controlled by cytokine and ILs (IL2, IL12, IL15, IL18) and IFNs.[67] IL-15 induces NK cells viability and proliferations, inhibits antiapoptosis through inhibition of gene expression Bim, Noxa and induces Mcl-1.[68] IL15 triggers NK cells activity and proliferation.[43] IL12 induced IFNγ secretion by NK cells dose dependent manner.[69] Co-culture of NK cells and human Adipocyte Stem Cells (hASCs) secretes 40±32.5 pg/mL IFNγ but co-culture NK cells uninduced IL2 and hASCs did not secrete IFNγ (DellaRosa, 2012). hMSCs increase IFNγ secretion by NK cells induced IL12/IL18. MSCs modulate the cells

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v2i2.21

cross talk the IL12R/STAT4 pathway.[70] IL12, IL18 induce NK cells to release IFNγ.[70] Co-culture hWJMSCs and NK cells could help NK cells to release TNFα, IFNγ, Prf1, Gzm (Table 2) and IL15, IL18-induced NK cells increased the secretion of secretome (Table 2). This results were in line with cytotoxic effect of hWJMSCs-CM through apoptosis pathway to kill cancer cells (Table 3, 4). IL18 was most active to induce NK cells in producing TNFα, IFN-γ, Prf1, Gzm and furthermore CM-( hWJMSCs+IL18-NK) was the most active to induce apoptosis in BC cells (Table 3, 4). This result was validated with previous research that IL2 is capable to restore the cytotoxicity and granular content of exhausted NK cells.[71] 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.[72] Strategies to improve NK cell activity, growth, development and differentiation for tumor immunotherapy are actively using IL2, IL15.[73-75] Prf-mediated cytotoxicity used for direct killing by NK cells is more important than indirect killing by secretion of death-inducing ligands by NK cells.[76] Gzm B binds to the target cell surface in the concentration-dependent and saturable manners and enters the cells via endocytosis.[77] GzmB primarily induces apoptosis via the intrinsic mitochondrial pathway by either cleaving Bid or activating Bim leading to the activation of Bak/Bax and subsequent generation of active Casp-3.[78] CM of co-culture hWJMSCs and NK cells secreted GzmB which induced apoptosis (Table 4), induced Bax gene expression (Table 5), this results was consistent with previous research that the several proteins that are involved in gzmB-induced apoptosis, including casp-9 and -3, Bim, Bid, Bak, Bax, and XIAP and Bcl-2 was not detected. Gzm B induced apoptosis cancer cells by inlvolving induction of p53 tumor suppressor gene.[79] Prf1 induced cell growth inhibition and cell death, apoptosis, chromosome condensation and DNA fragmentation, increased Casp-3 activity, and the release of apoptosis inducing factor (AIF) and cytochrome c from the mitochondria toward cancer cells (Hep G2, SK-BR-3, HeLa).[54]

## Conclusion

TNFα and IFNγ induce Bax, p53, decrease Bcl-2 gene expression directly. CM of coculture hWJMSCs and ILs-activated NK increase secretion of TNFα, IFNγ, Prf1, GzmB furthermore inhibit breast cancer cells proliferation, induce apoptosis and increase Bax expression.

## Acknowledgement

We gratefully acknowledge the financial support of Hibah Kompetensi 2017, Insinas Individu Pratama from Directorate General of Higher Education, Ministry of Research, Technology and Higher Education of the Republic of Indonesia (IPPK/E/E4/2017, 1598/K4/KM/2017). This study was supported by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for laboratory facilities and research methodology. We are thankful to Hana Sari W Kusuma, Hayatun Nufus, Annisa Amalia, from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for their valuable assistance.

## References

1. Muti P. The Role of Endogenous Hormones in the Etiology and Prevention of Breast Cancer: The Epidemiological Evidence. In: Senn HJ, Morant R, editors. Tumor Prevention and Genetics III. Recent Results in Cancer Research, Vol 166. Berlin: Springer, 2005. p. 245-56.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global Cancer Statistics, 2012. CA Cancer J Clin. 2015; 65(2): 87-108.
3. Tao Z, Shi A, Lu C, Song T, Zhang Z, Zhao J. Breast Cancer: Epidemiology and Etiology. Cell Biochem Biophys. 2015; 72(2): 333-8.
4. Kimman M, Norman R, Jan S, Kingston D, Woodward M. The Burden of Cancer in Member Countries of the Association of Southeast Asian Nations (ASEAN). Asian Pac J Cancer Prev. 2012; 13(2): 411-21.
5. Yu LY, Tang J, CongMin Z, WenJing Z, Han Y, MuPeng L. New Immunotherapy Strategies in Breast Cancer. Int J Environ Res Public Health. 2017; 14(1): 68.
6. Yang ZS, Tang XJ, Guo XR, Zou DD, Sn XY, Feng JB. Cancer Cell-Oriented Migration of Mesenchymal Stem Cells Engineered with an Anticancer Gene (PTEN): An Imaging Demonstration. Onco Targets Ther. 2014; 7: 441-6.
7. Joyce JA, Pollard JW. Microenvironmental Regulation of Metastasis. Nat Rev Cancer. 2009; 9(4): 239-52.
8. Kute TE, Savage L, Stehle JR, Kim-Shapiro JW, Blanks MJ, Wood J, *et al*. Breast Tumor Cells Isolated from In Vitro Resistance to Trastuzumab Remain Sensitive to Trastuzumab Anti-Tumor Effects In Vivo and to ADCC Killing. Cancer Immunol Immunother. 2009; 58(11): 1887-96.
9. Smyth MJ, Dunn GP, Schreiber RD. Cancer Immunosurveillance and Immunoediting: The Roles of Immunity in Suppressing Tumor Development and Shaping Tumor Immunogenicity. Adv Immunol. 2006; 90: 1-50.
10. Dewan MZ, Takada M, Terunuma H, Deng X, Ahmed S, Yamamoto N, *et al.* Natural Killer Activity of Peripheralblood Mononuclear Cells in Breast Cancer Patients. Biomed Pharmacother. 2009; 63: 703-6.
11. Caras I, Grigorescu A, Stavaru C, Radu DL, Mogos I, Szegli G. Evidence for Immune Defects in Breast and Lung Cancer Patients. Cancer Immunol Immunother. 2004; 53(12): 1146-52.

12. Levy EM, Roberti MP, Mordoh J. Natural Killer Cells in Human Cancer: from Biological Functions to Clinical Applications. J Biomed Biotechnology. 2011; 2011: 1-11.
13. Hwang YJ, Kim J, Park DS. Study on The Immunomodulation Effect of Isodon japonicus Extract via Splenocyte Function and NK Anti-Tumor Activity. Int J Mol Sci. 2012; 13: 4880-88.
14. Esendagli G, Bruderek K, Goldmann T, Busche A, Branscheid D, Vollmer E. Malignant and Non-malignant Lung Tissue Areas are Differentially Populated by Natural Killer Cells and Regulatory T Cells in Non-small Cell Lung Cancer. Lung Cancer. 2008; 59(1): 32-40.
15. Albertsson PA, Basse PH, Hokland M, Goldfarb RH, Nagelkerke JF, Nannmark U. NK Cells and The Tumour Microenvironment: Implications for NK-cell Function and Anti-tumour Activity. Trends Immunolog. 2003; 24(11): 603-9.
16. James AM, Cohen AD, Campbell KS. Combination Immune Therapies to Enhance Anti-tumor Responses by NK cells. Frontiers Immunolog. 2013; 4(481): 1-12.
17. Carson WE, Fehniger TA, Haldar S, Eckhert K, Lindemann MJ, Lai CF. A Potential Role for Interleukin-15 in the Regulation of Human Natural Killer Cell Survival. J Clin Invest. 1997; 99(5): 937-43.
18. Zwirner NW, Domaica CI. Cytokine Regulation of Natural Killer Cell Effector Functions. Biofactors. 2010; 36(4): 274-28.
19. Domaica CI, Fuertes MB, Lin S. Human Natural Killer Cell Maturation Defect Supports in vivo CD56 Bright to CD56 Dim Lineage Development. Cell Transplant. 2015; 24: 339-47.
20. Shah K. Mesenchymal Stem Cells Engineered for Cancer Therapy. Adv Drug Deliv Rev. 2012; 64(8): 739-48.
21. Menon LG, Picinich S, Koneru R. Differential Gene Expression Associated with Migration of Mesenchymal Stem Cells to Conditioned Medium from Tumor Cells or Bone Marrow Cells. Stem Cells. 2007; 25(2): 520-8.
22. Yu X, Boxing S, Peng G, Zicheng W, Sen L, Bingwei H, *et al.* Human Adipose Derived Stem Cells Induced Cell Apoptosis and S Phase Arrest in Bladder Tumor. Stem Cell Int. 2015; 2015: 1-12.
23. Ding D, Chang Y, Shyu W, Lin S. Human Umbilical Cord Mesenchymal Stem Cells: A New Era for Stem Cell Therapy. Cell Transplant. 2015; 24(3): 339-47.
24. Widowati W, Wijaya L, Murti H, Widyastuti H, Agustina D, Laksmitawati DR. Conditioned Medium from Normoxia (WJMSCs-norCM) and Hypoxia-treated WJMSCs (WJMSCs-hypoCM) in Inhibiting Cancer Cell Proliferation. Biomarkers Gen Med. 2015; 7(1): 8-17.
25. Loebinger MR, Janes SM. Stem Cells as Vectors for Antitumour Therapy. Thorax. 2010; 65(4): 362-9.
26. Sun B, Roh KH, Park JR. Therapeutic Potential of Mesenchymal Stromal Cells in A Mouse Breast Cancer Metastasis Model. Cytother. 2009; 11(3): 289-98.
27. Ghavami S, Eshraghi M, Kadkhoda K, Mutawe MM, Maddika S, Bay GH, *et al*. Role of BNIP3 in TNF-induced Cell Death — TNF Upregulates BNIP3 Expression. Biochimica et Biophysica Acta. 2009; 1793(3): 546-60.
28. Chawla-Sarkar M, Leaman DW, Borden EC. Preferential Induction of Apoptosis by Interferon (IFN)-beta Compared with IFN-alpha2: Correlation with TRAIL/Apo2L Induction in Melanoma Cell Lines. Clin Cancer Res. 2001; 7(6): 1821-31.
29. Ikeda H, Old LJ, Schreiber RD. The Roles of IFN γ in Protection Against Tumor Development and Cancer Immunoediting. Cytokine Growth Factor Rev. 2002; 13(2): 95-109.
30. Widowati W, Murti H, Jasaputra DK, Sumitro SB, Widodo MA, Fauziah N, *et al.* Selective Cytotoxic Potential of IFN-γ and TNF-α on Breast Cancer Cell Lines (T47D and MCF7). Asian J Cell Biol. 2016; 11(1): 1-12.
31. Lu YR, Yuan Y, Wang XJ, Wei LL, Chen YN, Cong C, *et al.* The Growth Inhibitory Effect of Mesenchymal Stem Cells on Tumor Cells in vitro and in vivo. Cancer Biol Ther. 2008; 7(2): 245-51.
32. Jewett A, Tseng HC. Tumor Induced Inactivation of Natural Killer Cell Cytotoxic Function; Implication in Growth, Expansion and Differentiation of Cancer Stem Cells. J Cancer. 2011; 2: 443-57.
33. Parihar R, Dierksheide J, Hu Y, Carson WE. IL-12 Enhances the Natural Killer Cell Cytokine Response to Ab-coated Tumor Cells. J Clin Invest. 2002; 110(7): 983-92.
34. Widowati W, Mozef T, Risdian C, Yelliantty Y. Anticancer and Free Radical Scavenging Potency of Catharanthus roseus, Dendrophthoe petandra, Piper betle and Curcuma mangga Extracts in Breast Cancer Cell Lines. Oxid Antioxid Med Sci. 2013; 2(2): 137-42.
35. Widowati W, Wijaya L, Wargasetia TL, Bachtiar I, Yelliantty Y, Laksmitawati DR. Antioxidant, Anticancer, and Apoptosis-inducing Effects of Piper extracts in HeLa Cells. J Exp Integr Med. 2013; 3(3): 225-230.
36. Kalaszczynska I, Ferdyn K. Wharton's Jelly derived Mesenchymal Stem Cells: Future of Regenerative Medicine? Recent Findings and Clinical Significance. Biomed Res Int. 2015; 2015: 1-13.
37. Hidayat M, Prahastuti S, Fauziah N, Maesaroh M, Balqis B, Widowati W. Modulation of Adipogenesis-related Gene Expression by Ethanol Extracts of Detam 1 Soybean and Jati belanda Leaf in 3T3-L1 Cells. Bangladesh J Pharmacol. 2016; 11: 697-702.
38. Aggarwal S, Pittenger MF. Human Mesenchymal Stem Cells Modulate Allogeneic Immune Cell Responses. Blood. 2005; 105(4): 1815-22.
39. Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Characterization of the Optimal Culture Conditions for Clinical Scale Production of Human Mesenchymal Stem Cells. Stem Cells. 2006; 24(2): 462-71.
40. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, *et al*. Role for Interferon-gamma in the Immunomodulatory Activity of Human Bone Marrow Mesenchymal Stem Cells. Stem Cells. 2006; 24(2): 386-98.
41. Kovach TK, Dighe AS, Lobo PI, Cui Q. Interactions between MSCs and Immune Cells: Implications for Bone Healing. J Immunol Res. 2015; 2015: 752510.
42. Wang Y, Wang X, Zhao H, Liang B, Du Q. Clusterin Confers Resistance to TNF-alpha-induced Apoptosis in Breast Cancer Cells through NF-kappaB Activation and Bcl-2 Overexpression. J Chemother. 2012; 24(6): 348-57.
43. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK Cell-based Immunotherapy for Malignant Diseases. Cell Mol Immunol. 2013; 10(3): 230-52.
44. Reefman E, Kay JG, Wood SM, Offenhäuser C, Brown DL, Roy S, *et al.* Cytokine Secretion is Distinct from Secretion of Cytotoxic Granules in NK Cells. J Immunol. 2010; 184(9): 4852-62.
45. Kelly JM, Darcy PK, Markby JL, Godfrey DI, Takeda K, Yagita H. Induction of Tumor-specific T Cell Memory by NK Cell-mediated Tumor Rejection. Nat Immunol. 2002; 3(1): 83-90.
46. Maestroni GJ, Hertens E, Galli P. Factors from Nonmacrophage Bone Marrow Stromal Cells Inhibit Lewis Lung Carcinoma and B16 Melanoma Growth in Mice. Cell Mol Life Sci. 1999; 55(4): 663-7.

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v2i2.21

47. Nakamura K, Ito Y, Kawano Y, Kurozumi K, Kobune M, Tsuda H. Antitumor Effect of Genetically Engineered Mesenchymal Stem Cells in a Rat Glioma Model. Gene Ther. 2004; 11(14): 1155-64.
48. Qiao C, Xu W, Zhu W, Hu J, Qian H, Yin Q. Human Mesenchymal Stem Cells Isolated from the Umbilical Cord. Cell Biol Int. 2008; 32(1): 8-15.
49. Qiao L, Xu Z, Zhao T, Zhao Z, Shi M, Zhao RC. Suppression of Tumorigenesis by Human mesenchymal Stem Cells in a Hepatoma Model. Cell Res. 2008; 18(4): 500-7.
50. Feng DQ, Zhou Y, Ling B. Effects of The Conditioned Medium of Mesenchymal Stem Cells on Mouse Oocyte Activation and Development. Braz J Med Biol Res. 2009; 42(6): 506-14.
51. Mintz PJ, Huang KW, Reebye V. Exploiting Human CD34+ Stem Cell–conditioned Medium for Tissue Repair. Mol Ther. 2013; 22(1): 149-59.
52. Lakshmikanth T, Johansson MH. Current Perspectives on Immunomodulation of NK Cells in Melanoma. n.p; INTECH Open Access Publisher; 2011.
53. Mamessier E, Sylvain A, Thibult ML, Houvenaeghel G, Jacquemier J, Castellano R, *et al.* Human Breast Cancer Cells Enhance Self Tolerance by Promoting Evasion from NK Cell Antitumor Immunity. J Clin Inves. 2011; 121(9): 3609.
54. Wang LF, Wang F, Li JT, Wen WH, Zhao J, Jia LT, *et al.* Ectopically Expressed Perforin-1 is Proapoptotic in Tumor Cell Lines by Increasing Caspase-3 Activity and The Nuclear Translocation of Cytochrome c. PLoS ONE. 2012; 7(7): 1-11.
55. Jiang MC, Luo SF, Li LT, Lin CC, Du SY, Lin CY, *et al.* Synergic CSE1L/CAS, TNFR-1, and p53 Apoptotic Pathways in Combined Interferon-gamma/Adriamycin-induced Apoptosis of Hep G2 Hepatoma Cells. J Exp Clin Cancer Res. 2007; 26(1): 91-9.
56. Vanhaesebroeck B, Reed JC, De Valck D, Grooten J, Miyashita T, Tanaka S, *et al.* Effect of bcl-2 proto-oncogene Expression on Cellular Sensitivity to Tumor Necrosis Factor-mediated Cytotoxicity. Oncogene. 1993; 8(4): 1075-81.
57. Jaattela M, Benedict M, Tewari M, Shayman JA, Dixit VM. Bcl-x and Bcl-2 Inhibit TNF and Fas-induced Apoptosis and Activation of Phospholipase A2 in Breast Carcinoma Cells. Oncogene. 1995; 10(12): 2297-305.
58. Rozen F, Zhang J, Pollak M. Antiproliferative Action of Tumor Necrosis Factor-α on MCF-7 Breast Cancer Cells is Associated with Increased Insulin-like Growth Factor Binding Protein-3 Accumulation. Int J Oncol. 1998; 13(4): 865-9.
59. Schedlich LJ, Young TY, Firth SM, Baxter RC. Insulin-like Growth Factorbinding Protein (IGFBP)-3 and IGFBP-5 Share a Common Nuclear Transport Pathway in T47D Human Breast Carcinoma Cells. J Biol Chem. 1998; 273(29): 18347-52.
60. Schedlich LJ, Le Page SL, Firth S, Briggs LJ, Jans DA, Baxter RC. Nuclear Import of Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) and IGFBP-5 is Mediated by the Importin Subunit. J Biol Chem. 2000; 275(31): 23462-70.
61. Butt AJ, Fraley KA, Firth SM, Baxter RC. Insulin-like Growth Factor Binding Protein-3-induced Growth Inhibition and Apoptosis Do Not Require Cell-surface Binding and Nuclear Translocation in Human Breast Cancer Cells. Endocrinolog. 2002; 143: 2693-9.
62. Butt AJ, Dickson KA, McDougall F, Baxter RC. Insulin-like Growth Factor Binding Protein-5 Inhibits the Growth of Human Breast Cancer Cells in vitro and in vivo. J Biol Chem. 2003; 278(32): 29676-85.
63. Chau BN, Chen TT, Wan YY, DeGregori J, Wang J. Tumor Necrosis Factor Alpha-induced Apoptosis Requires p73 and c-ABL Activation Downstream of RB Degradation. Mol Cell Biol. 2004; 24(1): 4438-47.
64. Detjen KM, Farwig K, Welzel M, Wiedenmann B, Rosewicz S. Interferon ã Inhibits Growth of Human Pancreatic Carcinoma Cells via Caspase-1 Dependent Induction of Apoptosis. Gut. 2001; 49(2): 251-62.
65. Pawitan JA. Prospect of Stem Cell Conditioned Medium in Regenerative Medicine. Biomed Res Int. 2014; 2014: 1-14.
66. Yang ZS, Tang XJ, Guo XR, Zou DD, Sn XY, Feng JB. Cancer Cell-Oriented Migration of Mesenchymal Stem Cells Engineered with an Anticancer Gene (PTEN): An Imaging Demonstration. Onco Targets Ther. 2014; 7: 441-6.
67. Domaica CI, Fuertes MB, Uriarte I. Human Natural Killer Cell Maturation Defect Supports in vivo CD56 Bright to CD56 Dim Lineage Development. PLoS ONE. 2012; 7(12): 1-12.
68. Huntington ND, Legrand N, Alves NL. IL-15 Trans-presentation Promotes Human NK Cell Development and Differentiation in vivo. J Exp Med. 2009; 206(1): 25-34.
69. Yu TK, Caudal EG, Smid C. Ay IL-2 Activation of NK Cells: Involvement of MKK1/2/ERK but Not p38 Kinase Pathway. J Immunol. 2000; 164(12): 6244-51.
70. Thomas H, Jäger H, Mauel K. Interaction with Mesenchymal Stem Cells Provokes Natural Killer Cells for Enhanced IL-12/IL-18-induced Interferon-Gamma Secretion. Mediators Inflam. 2014; 2014:1-11.
71. Bhat R, Watzl C. Serial Killing of Tumor Cells by Human Natural Killer Cells – Enhancement by Therapeutic Antibodies. PLoS ONE. 2007; 2(3):1-7.
72. Lieberman J. The ABCs of Granule-mediated Cytotoxicity: New Weapons in the Arsenal. Nat Rev Immunol. 2003; 3(5) p. 361-70.
73. Waldmann TA, Dubois S, Tagaya Y. Contrasting Roles of IL-2 and IL-15 in the Life and Death of Lymphocytes: Implications for Immunotherapy. Immunity. 2001; 14(2): 105-10.
74. Gluck WL, Hurst D, Yuen A, Levine AM, Dayton MA. Phase I Studies of Interleukin (IL)-2 and Rituximab in B-cell Non-hodgkin's Lymphoma: IL-2 Mediated Natural Killer Cell Expansion Correlations with Clinical Response. Clin Cancer Res. 2004; 10(7): 2253-64.
75. Waldmann TA. The Biology of Interleukin-2 and Interleukin-15: Implications for Cancer Therapy and Vaccine Design. Nat Rev Immunol. 2006; 6(8): 595-601.
76. Brodbeck T, Nehmann N, Bethge A, Wedemann G, Schumacher U. Perforin-dependent Direct Cytotoxicity in Natural Killer Cells Induces Considerable Knockdown of Spontaneous Lung Metastases and Computer Modelling-Proven Tumor Cell Dormancy in A HT29 Human Colon Cancer Xenograft Mouse Model. Molecular Cancer. 2014; 13(244): 1-11.
77. Rousalova I, Krepela E. Granzyme B-induced Apoptosis in Cancer Cells and Its Regulation. Int J Oncolog. 2010; 37(6): 1361-78.
78. Catalán E, Jaime-Sánchez P, Aguiló N. Mouse Cytotoxic T Cell-Derived Granzyme B Activates the Mitochondrial Cell Death Pathway in a Bim-dependent Fashion. J Biol Chem. 2015; 290(11): 6868-77.
79. Meslin F, Thiery J, Richon C, Jalil A, Chouaib S. Granzyme B-induced Cell Death Involves Induction of p53 Tumor Suppressor Gene and Its Activation in Tumor Target Cells. J Biol Chem. 2007; 282(45): 32991-9.