

11. Direct and Indirect Effect of TNFa and IFNg Toward Apoptosis in Breast Cancer Cells

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Direct and Indirect Effect of TNF α and IFN γ Toward Apoptosis in Breast Cancer CellsWahyu Widowati¹, Diana Krisanti Jasaputra¹, Sutiman Bambang Sumitro², Mochamad Aris Widodo³, Ervi Afifah⁴, Rizal Rizal⁴, Dwi Davidson Rihibiha⁴, Hanna Sari Widya Kusuma⁴, Harry Murti⁵, Indra Bachtiar⁵, Ahmad Faried⁶¹Medical Research Center, Faculty of Medicine, Maranatha Christian University, Bandung, West Java, Indonesia²Department of Biology, Faculty of Science, Brawijaya University, Malang, East Java, Indonesia³Pharmacology Laboratory, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia⁴Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung West Java, Indonesia⁵Stem Cell and Cancer Institute, Jakarta, Indonesia⁶Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, Indonesia1
Background: Breast cancer (BC) is the leading cause of death cancer in women. Cancer therapies using TNF α and IFN γ have been recently developed by direct effects and activation of immune responses. This study was performed to evaluate the effects of TNF α and IFN γ directly, and TNF α and IFN γ 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 TNF α and IFN γ in 175 and 350ng/mL, respectively. CM-hWJMSCs were produced by co-culture hWJMSCs and NK cells that secreted TNF α , IFN γ , 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:** TNF α and IFN γ at concentration of 350 ng/mL induced higher Bax expression compared to 175 ng/mL. TNF α and IFN γ 350 ng/mL, 175 ng/mL induced p53 expression, whilst TNF α and IFN γ at 350 ng/mL decreased Bcl-2 expression. Prf1, GzmB, TNF α and IFN γ -containing CM-hWJMSCs induced significantly apoptosis percentage, induced Bax expression, but did not effect p53, Bcl-2 expression.**Conclusion:** TNF α and IFN γ directly induce Bax, p53, decrease Bcl-2 gene expression. The Prf1, GzmB, TNF α , IFN γ -containing CM-hWJMSCs induce apoptosis and Bax expression.**Keywords:** breast cancer, Wharton's Jelly mesenchymal stem cells, TNF α , IFN γ 23
IntroductionBreast cancer (BC) is one of the most commonly oncologic diseases worldwide¹, the leading cause of cancer death amongwomen, 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 isDate of submission: October 12, 2017
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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 activity¹⁰, decreased cytotoxic functions¹¹⁻¹³, NK abnormalities¹², poor tumor infiltrate¹⁴⁻¹⁵, inefficient homing into malignant tissues¹². The promising cancer therapies are to stimulate NK cell functions, the combination NK cells with other anticancer agents¹⁶, NK cells respond to various cytokines, such as interleukin-15 (IL15), IL18¹⁷⁻¹⁹. 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 (TNF α), various effector molecules, such as interferon (IFN γ) 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}

TNF α 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 differentiation²⁸ and inhibition of tumour angiogenesis²⁹. My previous research resulted that TNF α and IFN γ 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 TNF α and IFN γ which indirectly effect of TNF α and IFN γ 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 TNF α , IFN γ

To determine the apoptosis induction ability of recombinant human TNF α (Biolegend 570106), recombinant human IFN γ (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 2x10⁶/well, 500 μ L RPMI (1640 Gibco 22400089), 10% FBS (Gibco 10270106), 1% Antibiotic Antimycotic (Gibco 1772653) and supplemented with TNF α (175, 350 ng/mL), IFN γ (175, 350 ng/mL) incubated in 37 $^{\circ}$ C, 5% CO₂ 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 $^{\circ}$ C for 5 minutes, then 42 $^{\circ}$ C for 30 minutes, and finally at 85 $^{\circ}$ 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 $^{\circ}$ C for 30 seconds, then 40 cycles of qPCR with denaturation for 5 seconds at 95 $^{\circ}$ C, annealing for 20 seconds at 58 $^{\circ}$ C, then elongation for 30 seconds at 72 $^{\circ}$ 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 CCTTCTACA ATG-3', reverse : 5'-AGC ACA GCC TGG ATA GCA ACG-3'), p53 (forward : 5' AGA GTC TAT AGG CCC A² 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 2×10^6 /well were grown and maintained in NK medium RPMI 1640 (Gibco 22400089), 20% FBS (Gibco 1027010⁴¹), 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% CO₂, 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 2×10^6 were cultured in minimum essential medium- α (α -MEM, Gibco 12561056), 10% FBS, 1%⁴ antibiotic Antimycotic. The hWJMSCs were incubated in a humidified atmosphere with 5% CO₂ at 37°C for 24 h. NK cells were adjusted to 1×10^5 for the ratio 1:1 (hWJMSCs : NK = 1:1; ³²WJMSCs : 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 NK⁴, IL15-NK, IL18-NK cells and hWJMSCs were incubated in a humidified atmosphere with 5% CO₂ 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 IFN γ , TNF α , Prf1, and GzmB.³¹⁻³²

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

The BC cell lines MCF7 ³¹ 10^3 /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% CO₂, 37°C for 24 h.³³⁻³⁵ Furthermore, the cells were treated with CM-(hWJMSCs+NK), CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18³⁰) 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 resorin⁹ 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 1×10^6 cells/disk were cultured in complete medium (DMEM + 10% FBS + 1% Antibiotic Antimycotic).⁴ The cells were incubated in humidified atmosphere, 5% CO₂, 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-(¹⁹MSCs+IL15-NK), CM-(hWJMSCs+IL18-NK)), the cells were incubated for ¹⁸ 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 5×10^5 - 1×10^6 were added 500 μ L Annexin V binding buffer (Miltenyi Biotec, 130-092-820), 5 μ L Anti-FITC (Miltenyi Biotec, 130-048-701), 5 μ L Propidium Iodide (Miltenyi Biotec, 130-093-233) furthermore the cells were incubated in darkness, 4°C and the apoptotic percentage of MCF7 cells were analyzed 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 1×10^6 cells/disk were cultured in complete medium (DMEM + 10% FBS + 1% Antibiotic Antimycotic).⁴ The cells were incubated in humidified atmosphere, 5% CO₂, 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-(¹⁹MSCs+IL15-NK), CM-(hWJMSCs+IL18-NK)), the cells were incubated for ¹⁸ 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

12 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

15 **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 TNF α , IFN γ toward apoptotic and antiapoptotic genes expression

The previous research showed that TNF α , IFN γ inhibited breast cancer proliferation (MCF7) with median inhibitory concentration (IC₅₀) 0.36 μ g/mL, 0.34 μ g/mL.³⁰ This study was the continued-research to elucidate the apoptosis mechanism of TNF α , IFN γ in MCF7 cells. In order to determine the apoptotic inducing activity of TNF α , IFN γ 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). TNF α induced significantly the apoptotic genes both low and high concentrations (175, 350 ng/mL) toward Bax, p53 genes expression. TNF α 350 ng/mL was more active than

175 ng/mL to reduce Bcl-2 and induce Bax (Table 1). IFN γ in both concentrations (175, 350 ng/mL) induced apoptotic genes, IFN γ 350 ng/mL was able to down regulate Bcl-2 (Table 1).

The effect of ILs-NK cells on TNF α , IFN γ , Prf and Gzm levels in hWJMSCs and NK cells

In vitro study proved that CM of hWJMSCs has the ability to inhibit various cancer proliferation²⁴, but MSCs are able to suppress the proliferation and function of immune system cells, influence cytotoxic activities of NK cells³⁸⁻⁴⁰, TNF α , IFN γ secretion⁴¹. Our study try to improve the NK cells cytotoxic, increase TNF α and IFN γ secretion to induce cancer cell cytolysis⁴², release cytotoxic granules namely Gzm and Prf³³⁻⁴⁴. We measured the cytotoxic secretion of co-culture hWJMSCs and ILs-induced NK cells including TNF α , IFN γ , Gzm and Prf (Table 2).

hWJMSCs secreted TNF α , IFN γ 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 TNF α , IFN γ , Gzm and Prf significantly. IL18 was more active to induce NK cells in secreting TNF α , IFN γ , 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 \pm 0.4768	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^b
TNF α 350 ng/mL	2.3493 \pm 0.2650	23.62 \pm 1.43 ^c	2.94 \pm 0.31 ^b	0.33 \pm 0.08 ^a
TNF α 175 ng/mL	2.5902 \pm 0.3898	5.29 \pm 0.93 ^b	2.17 \pm 0.56 ^b	1.28 \pm 0.31 ^b
2 IFNγ on MCF7				
Control (MCF7 untreated)	2.4288 \pm 0.4768	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^b
IFN γ 350 ng/mL	2.6019 \pm 0.4678	34.79 \pm 4.35 ^b	4.17 \pm 1.06 ^b	0.18 \pm 0.04 ^a
IFN γ 175 ng/mL	2.2870 \pm 0.3335	3.49 \pm 0.35 ^a	2.83 \pm 0.62 ^b	1.07 \pm 0.16 ^b

The data are presented as mean \pm 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 TNF α or IFN γ on MCF7 indicate significant differences among the means of groups (concentrations of TNF α or IFN γ) based on Tukey HSD post-hoc comparisons ($p < 0.05$).

Table 2. Effect interleukins (IL-15, IL18)-induced NK cells toward TNF α , IFN γ , 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			
	IFN γ (pg/mL)	TNF α (pg/mL)	Prf1 (pg/mL)	GzmB (pg/mL)
CM-hWJMSCs	0.94 \pm 0.19 ^a	2.64 \pm 0.47 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a
CM-NK cells	8.06 \pm 1.90 ^a	8.49 \pm 1.60 ^a	50.00 \pm 10.00 ^a	6.67 \pm 0.58 ^a
CM co-culture hWJMSCs + NK (1:1)	7.72 \pm 2.52 ^a	5.07 \pm 0.25 ^a	50.00 \pm 20.00 ^a	6.00 \pm 2.00 ^a
CM co-culture hWJMSCs + IL15-NK (1:1)	8.17 \pm 2.60 ^a	18.17 \pm 3.45 ^b	120.00 \pm 30.00 ^b	33.00 \pm 7.21 ^b
CM co-culture hWJMSCs + IL18-NK (1:1)	19.72 \pm 5.52 ^b	17.06 \pm 1.48 ^b	140.00 \pm 50.00 ^b	31.00 \pm 9.64 ^b

The data are presented as mean \pm standard deviation. The level of secretome were measured in triplicate for each sample. Different superscripts in the same column (a,b) of TNF α , IFN γ , 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 immunomodulatory 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 nutrition⁵⁰, 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 vivo* assay⁴⁶, CM-MSCs reduce NFkB secretion of hepatome, breast cancer cells and inhibit cells proliferation by *in vitro* assay⁴⁹.

The activated-NK cells significantly increase secretion of IFN γ , TNF α , 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 \pm 6.61 ^a	0.00 \pm 6.61 ^a
CM-hWJMSCs	32.55 \pm 6.57 ^b	48.00 \pm 8.70 ^b
CM-hWJMSCs + NK (1:1)	40.44 \pm 4.02 ^b	51.86 \pm 3.17 ^{bc}
CM-hWJMSCs + IL15-NK (1:1)	56.82 \pm 7.66 ^c	60.55 \pm 4.91 ^{bc}
CM-hWJMSCs + IL18-NK (1:1)	58.91 \pm 3.17 ^c	64.49 \pm 1.25 ^c

The data are presented as mean \pm 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 ($p < 0.05$)

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 activated 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 losing 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 gene 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,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 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 ($p < 0.05$)

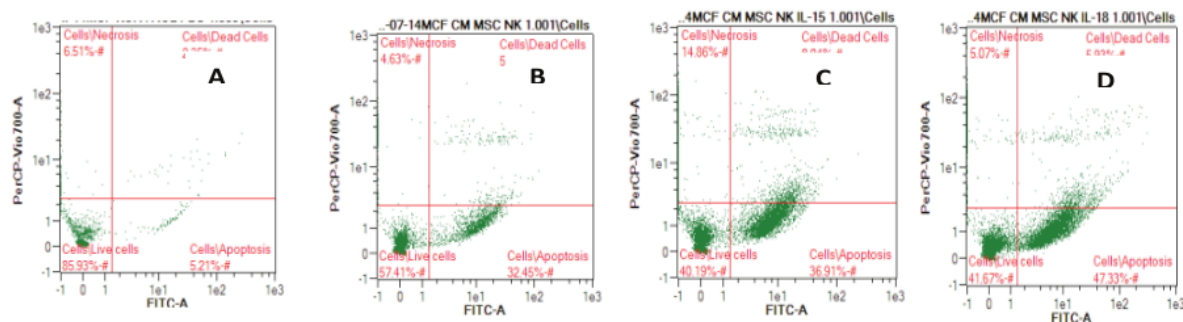


Figure 1. Effect CM-co-culture hWJMSCs and ILs-activated NK cells toward apoptosis inducing activity of BC cells. Percentage Apoptosis of BC cells treated with CM co-culture of hWJMSCs and ILs-activated NK cells were done in triplicate using flowcytometry. A: Control (MCF7 cell untreated, live cells 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%)

Table 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 \pm 0.2639	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^a	1.00 \pm 0.00
CM-hWJMSCs	2.6868 \pm 0.4326	1.36 \pm 0.36 ^a	1.23 \pm 0.25 ^a	0.93 \pm 0.09
CM-(hWJMSCs+NK)	2.4541 \pm 0.3385	1.52 \pm 0.17 ^a	1.47 \pm 0.15 ^a	0.96 \pm 0.19
CM-(hWJMSCs+IL15-NK)	2.7321 \pm 0.1301	1.61 \pm 0.27 ^a	2.03 \pm 0.36 ^a	0.98 \pm 0.10
CM-(hWJMSCs+IL18-NK)	2.3336 \pm 0.3337	2.56 \pm 0.40 ^b	3.70 \pm 0.95 ^b	0.89 \pm 0.24

The data are presented as mean \pm 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 TNF α and IFN γ against MCF7 cells exhibited that the cytokines decreased the cell viability in a dose dependent manner. The IC₅₀ value of TNF α and IFN γ 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 as 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 TNF α , IFN γ reduced Bcl-2 expression, 175 ng/mL of TNF α , IFN γ could not reduce Bcl-2 expression. TNF α -induced apoptosis in BC cells through Bcl-2 in low expression.⁵⁴ IFN γ increased Bax level.⁵⁵ p53 and Bax may be the targets for the IFN γ based chemo-immunotherapy of the chemotherapy-resistant cancers.⁵⁵ This study was contradictory with previous research that Bcl-2 expression inhibited TNF α -induced apoptosis in MCF7 cells⁵⁶, overexpression of Bcl-2 and Bcl-XL was correlated with increased resistance to TNF α -induced apoptosis.⁵⁷ Insulin-Like Growth Factor- Binding Protein-3 (IGFBP-3) expression is up-regulated in response to TNF α , with some evidence that it may mediate the inhibitory effects of TNF α in TNF-sensitive BC cell lines.⁵⁸ IGFBP-3 and -5 translocate to the nucleus in BC cells^{59,60}, they can transcriptionally modulate 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 TNF α -induced apoptosis in retinoblastoma fibroblasts.⁶³ IFN γ 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 (Prfl, Gzm), death receptor (FASL, TRAIL, TNF α), effector molecules (IFN γ , NO) which kill target tumor cells.⁴³

CM-hWJMSCs contains various growth factors, cytokines, chemokines, and tissue regenerative agents, therapeutic potency⁶⁵, CM-hWJMSCs inhibit proliferation of cancer cell lines including HeLa, SKOV3, HepG2, PC3, HSC3²⁴, A549, HT29, MCF7⁶⁶. MSCs have side effect to suppress the proliferation and function of immune system cells, influence cytotoxic activities of NK cells³⁸⁻⁴⁰, suppress TNF α , IFN γ secretion⁴¹. Our research to improve NK cells cytotoxicity using ILs (IL15, IL18), the result showed that ILs increased TNF α , IFN γ secretion by NK cells or co-culture ILs-NK and hWJMSCs. ILs increased GzmB and Prfl 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 IFN γ secretion by NK cells dose dependent manner.⁶⁹ Co-culture of NK cells and human Adipocyte Stem Cells (hASCs) secretes 40 \pm 32.5 pg/mL IFN γ but co-culture NK cells uninduced IL2 and hASCs did not secrete IFN γ (DellaRosa, 2012). hMSCs increase IFN γ secretion by NK cells induced IL12/IL18. MSCs modulate the cells

cross talk the IL12R/STAT4 pathway.⁷⁰ IL12, IL18 induce NK cells to release IFN γ .⁷⁰ Co-culture hWJMSCs and NK cells could help NK cells to release TNF α , IFN γ , 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 TNF α , IFN- γ , Prf1, Gzm and furthermore CM-(hWJMSCs+IL18-NK) was the 27st active to induce apoptosis in BC cells (Table 3, 4). This res⁶ 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 gr⁶ule exocytosis pathway by releasing Prf1 and GzmB into the immunological synapse after the conjuga⁴⁴ formation with targets.⁷² Strategies to improve NK cell activity, growth, development and differentiation for tu¹³or immunotherapy are actively using IL2, IL15.⁷³⁻⁷⁵ Prf-mediated cytotoxicity used for direct killing by NK cells is more important than indirect killing by¹⁷ retention of death-inducing ligands by NK cells.⁷⁶ Gzm B binds to the target cell surface in the concentration-dependent and⁷ 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 involv⁸ 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) and⁴³ochrome c from the mitochondria toward cancer cells (Hep G2, SK-BR-3, HeLa).⁵⁴

Conclusion

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

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