ORIGINAL ARTICLE

Interleukin-27 Disrupts the Crosstalk of Apoptotic Activities between 4T1 Breast Cancer Cells and M2 Macrophages

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ABSTRACT

Introduction: Cytokine immunotherapy such as Interleukin-27 (IL-27) has been foreseen as a promising alternative anti-cancer treatment. Thus, this study aimed to investigate whether IL-27 gene therapy regulates crosstalk between breast cancer cells and macrophages in the sense of pro-apoptotic activities. **Methods:** This study has led to the development of recombinant pcDNA3.4-IL27. The recombinant pcDNA3.4-IL27 was transfected into 4T1 murine mammary carcinoma cells alone and co-culture of 4T1 with M2 macrophages. The successful expression of IL-27 in the cells were determine through the immunofluorescence staining and detection of CD206, M2 macrophages marker. Apoptotic effects of pcDNA3.4-IL27 were assessed through MTT assay, Annexin V flow cytometer analysis, and AO/PI dual staining. **Results:** Our findings shows that pcDNA3.4-IL27 has the ability to induce apoptosis in both of the cell group and performs better in the co-culture of 4T1 with M2 macrophages compared to 4T1 cells alone. PcDNA3.4-IL27 induced apoptosis through the altered cell morphology and reduction in the number of viable cells. **Conclusion:** These data demonstrate that pcDNA3.4-IL27 has the ability to induce apoptosis in both 4T1 cell alone and co-cultured 4T1 with M2 macrophages. Thus, could serve as a potential anti cancer candidate against breast cancer.
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Keywords: Breast cancer; Interleukin-27 (IL-27); pcDNA3.4-IL27; apoptosis; M2 macrophages

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INTRODUCTION

Breast cancer is one of the commonly diagnosed and one of the leading causes of cancer-related mortality in the modern world. Global cancer statistics 2020 has recorded more than 2.2 million of new cases with 684,996 mortality (1). In Malaysia, there were 5,410 new cases reported with 2,572 of cancer mortality in 2012 were recorded (2). National Cancer Registry of Malaysia, 2016 reported 21,634 breast cancer cases between 2012 and 2016 about 18% higher cases recorded in the 2007 - 2011(3). Existing drugs and treatments available have difficulties in combating cancer mainly due to the high probability of non-specific targets of the treatment, resistance to the anti-cancer drugs, and risk of the emerging side effects (4,5).

Tumor-associated macrophages (TAMs) are one of the significant populaces in the breast tumor microenvironment that releases cytokines, chemokines and signalling cues that can either promote or inhibit the tumor progression. Different stimuli activates different TAMs phenotypes (classically activated M1 macrophage or alternatively activated M2 macrophages) that response differently in the tumor microenvironment (6, 7). A multitude of evidence has shown that TAMs may represent up to 50% of the tumor microenvironment of an established tumor, usually resembles the M2 macrophages (6,7,8). M2 macrophages are known as the “repair” macrophage that releases cytokines to encourage the activity of T-helper 2. The activation of T-helper 2 contributes to cell proliferation, hence favor tumor development and aggressiveness. Another player that is also present in the tumor environment is the M1 macrophage that promotes the T helper 1

and produces pro-inflammatory cytokines that induce tumor eradication. During the early stages of tumor development, M1 macrophages are found infiltrating the tumor microenvironment that promotes the anti-tumor responses. However, these immune responses shifted to the anti-inflammatory responses influenced by the activation of M2 macrophages, during the later stages of tumor development (8, 9, 10). The activated M2 macrophages secretes anti- inflammatory cytokines, IL-10 and TGFβ that impede the effect of CD8+ cytotoxic T lymphocytes against cancer cells and encourage tumor progression. (11). The plasticity of macrophages in a tumor microenviroment is regulated by various microenvironmental cues such as cytokines, chemokines, and growth factors from the surrounding (12). Although studies have found that M1 macrophages have pro-inflammatory properties, the ratio of M2 macrophages infiltrating in the tumor microenvironment is higher than the M1 macrophages during the tumor development (8, 12). The M2 macrophage subtypes are activated by distinct triggering stimuli. M2 macrophages can be further divided into M2a, M2b, M2c, and M2d . The M2a subtype is activated by cytokine IL-4 and IL-13 that promotes cell proliferation and growth factors. The activation of TLRs ligand and immune complexes converts macrophages to M2b subtype. IL-10 and glucocoticoids induces the M2c subtype that responsible for tissue repair and remodeling(12, 13). Previous study found that a higher percentage of M2 found in later stages of breast cancer in patients which resulted in an increased IL-10 cytokine release in the microenvironment that may promote cancer progression (13). The high expression of IL-10 in the microenvironment induces the production of IL-4 and IL-13 that encourages the cell proliferation(14). Therefore, a therapy targeting these immunosuppressive and tumor-promoting activities to treat cancer may be beneficial.

Recent advances in cancer research have emerged the idea of combining cytokine and immunotherapy as a tool for cancer therapy. Cytokines are a small messenger molecule that communicates among the immune system to generate responses to target antigens (15). Interleukin-27 (IL-27) has been reported to have anti-tumor properties in various tumor models since 2004 through several mechanisms such as triggering immune responses and inhibition of cell proliferation and survival (16, 17) . IL-27 belongs to the IL-12 family and has been reported to have insignificant toxicity in preclinical trials (14). IL-27 is a heterodimeric cytokine composed of p28 and Epstein-Barr virus-induced gene (EBI3) that signals through receptors IL-27Rα (WSX1) and GP130 chains that trigger the Janus kinase (JAK) signal transducer and transcription (STAT) pathway (14, 18, 19). Recent studies have revealed that IL-27 impedes the polarization of M2 macrophages which indirectly inhibits cancer cell proliferation and migration (14). Thus, this present study aims to assess the anti-cancer potential of recombinant pcDNA3.4-IL27 against 4T1 murine mammary carcinoma and co-culture of 4T1 with M2 macrophages.

MATERIALS AND METHODS

Cell lines

The 4T1 murine mammary carcinoma and murine macrophage RAW264.7 cell lines used were purchased from the American Type Culture Collection (ATCC, USA). Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin in a 25cm2 flask. The cells were grown in a humidified incubator at 37 °C with 5% CO2. The cells were passaged upon reaching 70-90% of cell confluency.

Reverse transcription PCR (RT-PCR)

Reverse transcription polymerase chain reaction of CD206 (NM_008625.2) and IL-27 gene (NM_145636.1). This assay performed to confirm the successful polarization of M2 macrophages and the expression of IL-27 in cells. The RT-PCR reaction mixture are as follows: DNA template (cDNA), forward primer, reverse primer, One Taq polymerase Master Mix (New England Biolabs, USA), and sterile water was added to make up to 25 µl of total volume. The annealing temperature used was 57.45ºC. The sequences for forward and reverse primer used are 5'-GAGGCTTCTCCTGCTTCTGG-3' (Forward primer), 5'-GAATCTGACACCCAGCGGAA-3' (Reverse primer) and for IL-27 gene primer used are forward primer 5'-GAATTCATGGGCCAGGTGACAGGAG-3' and reverse primer 5'-GCGGCCGCTCGAGTTAGGAATCCCA-3'.

Polarization of RAW 264.7

For M2 differentiation, the RAW264.7 cell was polarized by using the M2 macrophage inducers IL-4 (2 µg/mL) and IL-13 (0.1 µg/mL). Briefly, RAW264.7 cells were seeded in a 6-well plate at a concentration of $2x10^5$ in a complete DMEM media. After overnight incubation, IL-4 and IL-13 cytokines at final concentration 50 ng/mL respectively were added into the complete DMEM culture media. The cells were then incubated for another 48 hours to induce the alternatively activated M2 macrophage cells.

Co-culture of 4T1 cancer cells with M2 polarized macrophages

For co-culture experiments, 4T1 cells were added into the M2 polarized cells in the same 6-well-plate at a concentration of $4x10^4$ and incubated overnight before further analysis.

Construction of Plasmids and Treatment

IL-27 gene sequences obtained from NCBI Genbank (reference number: NM_145636.1) gene were amplified, purifed and cloned into pcDNA3.4 vector

using the TOPO Cloning reaction kit (Invitrogen,USA). As treatment, a concentration of $2x10^4$ of 4T1 and co-cultured cells was seeded in a 24-well culture plate for 24 hours. Once the culture becomes 70-80% confluent, the cells were transfected by Calphos transfection reagent (Takara Bio Inc, USA) according to the manufacturer's instructions. Both cell groups (4T1 alone; Co-culture of 4T1 with M2 macrophages) transfected with pcDNA3.4-IL27. Empty pcDNA3.4 was constructed to serve as control in the experiment.

Immunofluorescence staining

Immunofluorescence staining was performed to detect the specific target of IL-27 antigens. IL-27 Antibody(C-8) Alexa Fluor®594 was purchased from Santa Cruz Biotechnology, USA. 4T1 and co-culture of 4T1 and M2 macrophage cells were cultured in a culture dish at 37° C overnight. The cells were washed briefly with 0.1% PBS-BSA and fixed in ice-cold acetone for 2 minutes. The cells were then incubated for 30 minutes with 5% PBS-BSA. Next, the cells were rinsed with three changes of 0.1% PBS-BSA for 5 minutes each. Followed by the incubation of the cells with anti-IL-27 primary antibody conjugated to a fluorophore (Alexa Fluor 594) for 90 minutes at room temperature. After incubation, the cells were rinsed again with three changes of 0.1% PBS-BSA for 5 minutes each. The cells were then examined using a fluorescence microscope (Nikon FC-35DX, Japan).

Cell Viability Assay

To assess the cell viability after transfection, 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) dye reduction was performed according to the protocols described in (20, 21) with minor modifications. Approximately $2x10^5$ of cells were seeded in a 24 well plate and maintained in DMEM medium for 24 hours. The cells were then transfected with pcDNA3.4-IL27 and empty pcDNA3.4 with different plasmid concentrations (0-3000 ng/µl). After 48 hours of transfection, the cells were incubated with 50µl of MTT reagent (5mg/mL) for 4 hours in the dark, at 37 °C, 5% CO2 environment. Then dimethyl sulfoxide (DMSO) was added to the wells to solubilize the tetrazolium salt. Once the tetrazolium salt was dissolved, the plate was measured at 570 nm wavelength using ELISA (Bio-Tek Instrument, USA). The untransfected cells were used as a control. The cell viability was compared to the control cells which was known to be 100%. The percentage of cell viability was calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{\text{OD sample}}{\text{OD control}} \times 100\%$$

The 50% inhibitory concentration of the cell growth (IC50) was achieved by plotting the dose-response curve. The IC50 values and concentrations were used throughout the study as a cytotoxicity parameter.

Acridine Orange/ Propidium Iodide Dual Staining assay (AO/PI)

The apoptotic-related changes in the cell morphology were visualized by the AO/PI dual staining assay. This assay was carried out according to the protocol described by (21). Briefly, both 4T1 cells alone and co-culture of 4T1 with M2 macrophages were seeded in culture dishes. The culture dishes were then incubated in the dark with 5% CO2. After overnight, the cells were transfected with pcDNA3.4-IL27 and empty pcDNA3.4. The cells were then incubated in a 5% CO2 humidified incubator. Afterward, the old medium was discarded and rinsed twice with PBS. Next, 20µl of Acridine Orange / Propidium Iodide staining solution was dispensed into the culture dishes. The cells were then visualized by using a fluorescent microscope equipped with a Nikon camera (Nikon FC-35DX, Nikon, Tokyo, Japan) with filter range of 450- 490 nm. The green fluorescent colour indicates the viable cells with intact membrane and nuclei, orangy-yellow fluorescent coloured cells with distinct features of disrupted membrane and nuclei were counted as apoptotic cells. Meanwhile, red fluorescent colours with complete loss of membrane integrity were counted as necrotic cells.The percentage of viable and apoptotic cells were calculated.

Flow cytometry analysis

Flow cytometry analysis was conducted to determine the events of cell apoptosis. This analysis was performed by using Enzo GFP certified apoptosis/necrosis detection kit (Enzo Life Sciences Inc, USA). This assay was carried out according to the manufacturer's protocol. The number of cells prepared was between the range of 2×10^5 and 1×10^6 cells for the experiment. Both 4T1 cells and co-culture of 4T1 with M2 macrophage cells were harvested via trypsinization. The cells were then spun for 5 min at 400 xg at room temperature. The supernatants were carefully removed and re-suspended in PBS. The pellet was then washed in 5.0 mL cold PBS. The cells were then spun again for 5 min at 400 x g at room temperature. The supernatant was carefully removed. The pellet was then re-suspended in 500 uL Dual Detection Reagent. The cells were incubated for 15 min in the dark. The samples were dyed by FITC (viable cell), annexin V-Cyanine 3 (apoptosis cell) and far red (necrotic cell) and were examine via flow cytometry using a 488 nm laser together with the FL 1, FL 2 and FL 3 channel for FITC, apoptosis and necrosis detection, respectively. The percentage of viable and apoptotic cells were calculated.

Statistical analysis

All the experiments were conducted in three independent experiments. The data obtained were expressed as the means ± standard error of the mean (S.E.M). The analysis was performed with one-way analysis of variance (ANOVA) and the group means were compared by

Tukey's post hoc test. Values of P <0.05 were considered as statistically significant

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RESULTS

Polarization of RAW264.7 cells into M2 macrophages

To imitate the real physiological setting in a tumor microenvironment, RAW264.7 Murine monocyte cells were polarized into M2 macrophages and co-cultured with 4T1 cells. M2 macrophage inducers (IL-4 and IL-13 cytokines) were used to polarize the RAW264.7 cells to M2 macrophage. The successful polarization was visualized by the morphological changes of the cells after the introduction of the inducers. The RAW264.7 cells were small, circular, and uniform morphology. Alteration of the cell morphology was observed after the addition of (IL-4 and IL-13). The cells showed elongated, spindle-like shaped cells (22, 23, 24) (Fig. 1 A-B). The polarization was verified using M2 macrophage marker, CD206 (mannose receptor c-type 1), an amplicon at the specific size of 291 bp was obtained on the gel electrophoresis (Fig. 1 C).

Construct, treatment and expression of IL-27 in breast cancer cells

The recombinant plasmid, pcDNA3.4-IL27 was amplified by the colony PCR, was digested with restriction enzymes (single and double digestions), and DNA sequencing. In principle, the expression of IL-27 in the transfected cells was verified through semi-quantitative RT-PCR, and immunofluorescence staining. Semi quantitative RT-PCR exhibited the presence of IL-27 expression in the cells. A single amplicon (IL-27) was observed in the total RNA extracted from 4T1 cells transfected with pcDNA3.4-IL27 and no similar results were observed from the total RNA extracted from the empty pcDNA3.4 transfected 4T1 cells. This indicates the absence of IL-27 gene in the cells. Similarly, pcDNA3.4-IL27 transfected M2 macrophage cells displayed a band at expected size of 731bp. The findings demonstrated that both 4T1 and M2 macrophages cells are able to uptake the pcDNA3.4-IL27.

The successful expression of IL-27 in the cells was determined by immunofluorescence staining. Fig. 2 A-B shows that IL-27 was expressed in the 4T1 cells and co-cultured of 4T1 with M2 macrophages transfected with pcDNA3.4-IL27. Control and empty pcDNA3.4 transfected cells were included as comparison. Thus, it can be deduced that IL-27 was expressed and was found to be primarily localized in the cell cytoplasm of the cells transfected with pcDNA3.4-IL27.

Reduction of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed as a preliminary screening procedure to determine the cell viability after transfection with pcDNA3.4-IL27. Fig. 3 A- B showed different plasmid concentrations (0-3000 ng/µl) were tested against the 4T1 cell and co-culture of 4T1 with M2 macrophages. The IC50 obtained for 4T1 cells is 2200 ±1.23 ng/µL of which pcDNA3.4-IL27 reduced 50% of the cells at 24 hours post-transfection.Whereas the IC50 of 2500 ±1.18 ng/µl, was obtained for pcDNA3.4-IL27 transfected co-culture of 4T1 and M2 macrophage cells. On the contrary, empty pcDNA3.4 showed less than 20% of cell reduction for both cell groups. The cell viability of control and empty pcDNA3.4 transfected cells remained high.

IL-27 induces apoptosis in breast cancer cells

The morphology and cell death mode were microscopically visualized through the acridine orange/ propidium iodide (AO/PI) dual-staining assay. This assay was employed to examine the apoptosis-associated morphological changes of the cell membranes and nucleus. As depicted in Fig. 4 A-B, pcDNA3.4-IL27 induced the apoptotic-related changes in cell

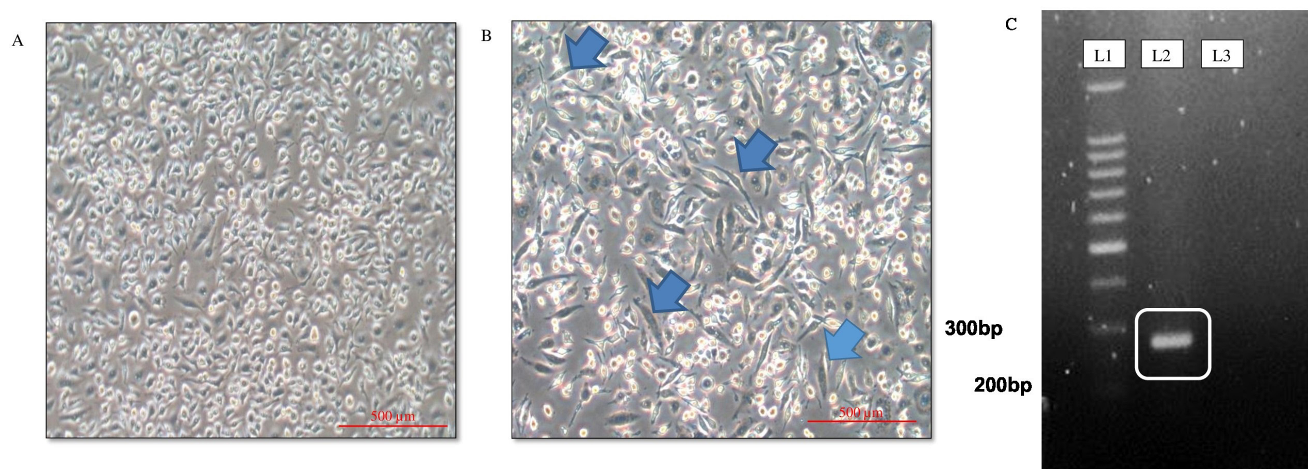

Figure 1: Representative photomicrographs of RAW264.7 cells. (A) Morphology of RAW264.7 before the addition of M2 macrophages inducing cytokines. (B) Morphology of RAW264.7 after 30 hours of the addition of M2 macrophages inducing cytokine in the complete DMEM growth medium. (Magnification of objective 100x). The blue arrows show the morphological changes of the cells. (C) Gel electrophoresis profile of RT-PCR product for CD206 marker. The lane 1(L1): 100 bp ladder; lane 2(L2): CD206 expression of polarized RAW264.7and lane 3(L3): CD206 expression of non-polarized RAW264.7.

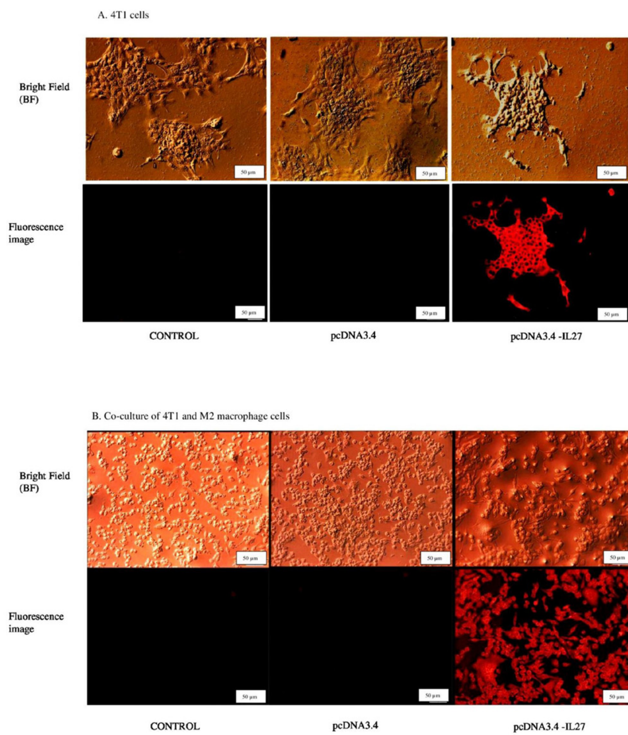

Figure 2: Representative photomicrographs of immunofluorescence staining of IL-27 (A) 4T1 cells alone. (B) Co-culture of 4T1 with M2 macrophages. The figures showed the different intensity of the staining indicates the expression of IL-27 in both cell groups. (Magnification of objective 20x; scale bar represents 50 µm).

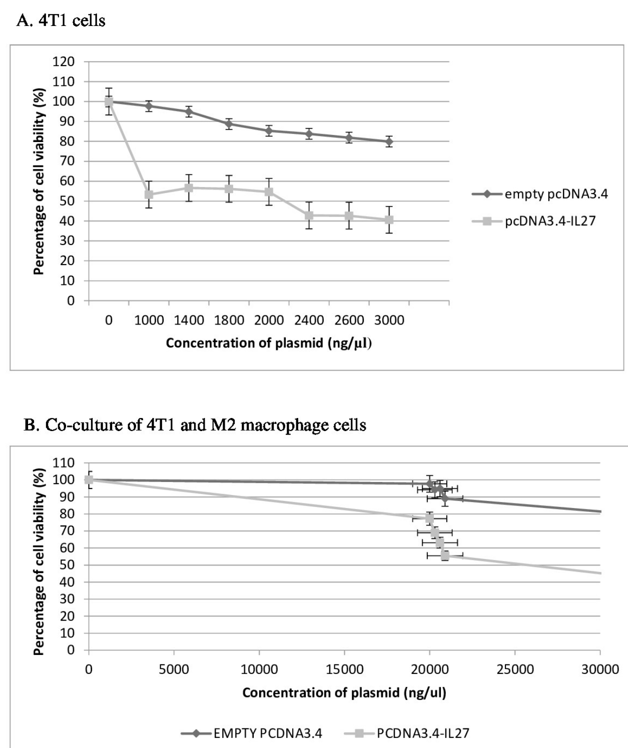

Figure 3: The dose-response curve of pcDNA3.4-IL27 (A) 4T1 cells and (B) co-culture of 4T1 with M2 macrophage cells after transfection. Data were expressed as a means of triplicates experiments.

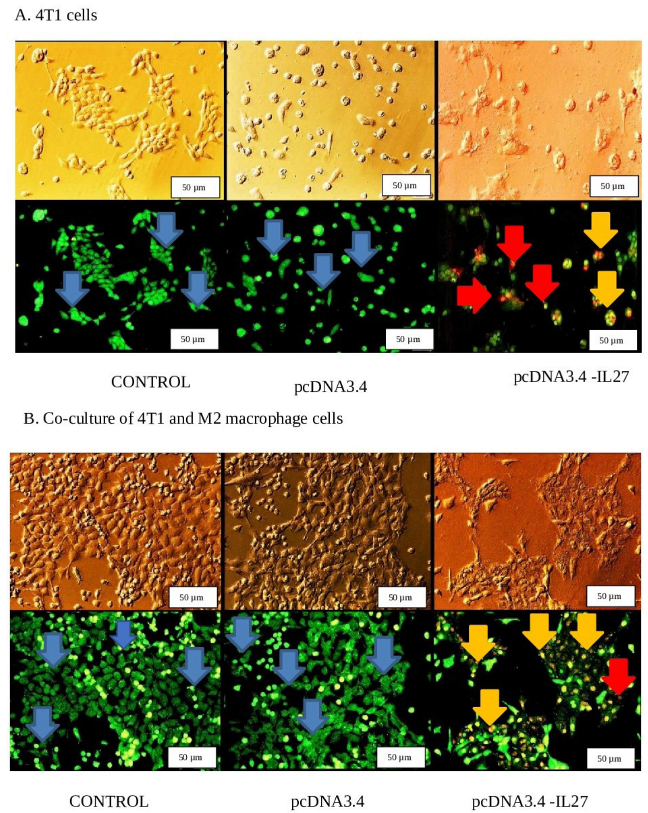

Figure 4: Cells stained with dual staining AO/PI upon transfection with pcDNA3.4-IL27 and empty pcDNA3.4. Representative photomicrographs of stained cells with AO/PI in (A) 4T1 cells alone and (B) co-culture of 4T1 and M2 macrophages cells. (Magnification of objective 100x; scale bar represents 50µm). The cells were transfected with IC50 of pcDNA3.4-IL27. The blue arrows in the figures represent healthy viable cells, red arrows represent late apoptosis stage in cells, and yellow arrows represent early apoptosis stage in cells. Cell quantification of viable, early, and late apoptosis of 4T1 (C) and co-cultured cells (D).The analysis was performed in triplicates. Non-transfected cells were used as control. All data are expressed as mean ± SD.* $p < 0.05$ compared to corresponding controls.

morphology in both 4T1 cells alone and co-culture of 4T1 with M2 macrophages. The photomicrograph of pcDNA3.4-IL27 transfected cells exhibited noticeable cell population which consists of bright green, orange, and reddish orange-stained cells as results of the intercalation of AO and PI within a cell indicating viable, early and late apoptosis respectively. The control and empty pcDNA3.4 transfected cell populations appeared in bright green, large with intact nuclear structures. In comparison, pcDNA3.4-IL27 transfected 4T1 cells and co-culture of 4T1 with M2 macrophages exhibited a bright orange stain with distinct membrane disintegration and condensed nuclear structures. The bar chart revealed that almost 100% of viable cells were prominent in control and empty pcDNA3.4 transfected cells for both 4T1 cells and co-culture cells. Upon transfection of pcDNA3.4-IL27 into both 4T1 and co-cultured cells, the percentage of the viable cells

remarkably decreased to less than 15% with an increase in the apoptotic cell population observed.

Flow cytometry analysis was performed to further enumerate the percentage of viable, early and late apoptotic and necrotic cells after the introduction of pcDNA3.4-IL27 into the cells. The dye used in Annexin V assay detects the externalization of the phosphatidylserine in both 4T1 cells alone and co-cultured 4T1 with M2 macrophages cells. Fig. 5 A-B showed the percentage of viable and apoptotic cells. Fig. 5 A-B showed a notable difference in the viable cell percentage of over 90% in control and empty pcDNA3.4 transfected cells in comparison to pcDNA3.4-IL27 transfected cells which are about 56% and 40% in 4T1 cells and co-culture of 4T1 with M2 macrophages, respectively. The data also showed that higher percentage of early cell apoptosis was observed in co-cultured 4T1 with M2 macrophage (34.8%) compared to 4T1 cells alone (5.62%). This data suggested that IL-27 could induce apoptosis in both of the 4T1 cells and co-culture 4T1 with M2 macrophages. The apoptosis process is more prominent in the co-cultured cells compared to 4T1 cells alone.

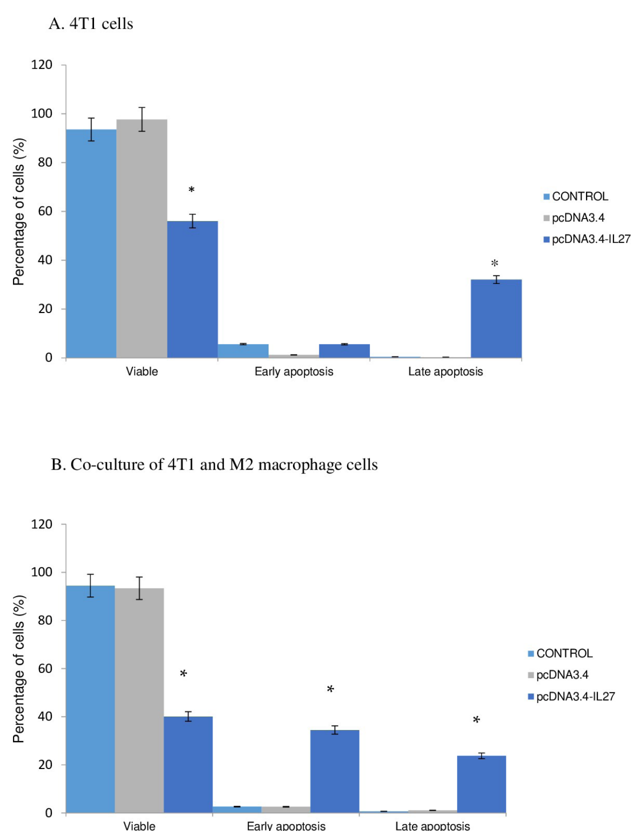

Figure 5: Detection of externalization of phosphatidylserine by Annexin V-FITC by flow cytometry. The percentage of cell population consists of viable, early and late apoptosis after transfection (A) 4T1cells and (B) co-cultured 4T1 with M2 macrophages. Non-transfected cells were used as control. The analysis was performed in triplicates. The data obtained were expressed as mean ± standard error mean (S.E.M). * p < 0.05 compared with corresponding controls.

DISCUSSION

The influence of the immune system players with cancer cells in a tumor microenvironment has been extensively studied. Tumor-associated macrophages (TAMs) are one of the main players in a tumor microenvironment that may present up to 50% of the tumor mass. These macrophages usually resemble the M2 macrophage phenotype that is responsible for the release of cytokines that promotes tumor progression and aggressiveness (6, 7). Thus, a combination of cytokines and immunotherapy has emerged as a tool for breast cancer treatment as evidenced by promising results in numerous preclinical studies (24). Several studies have been conducted suggesting IL-27 as an antitumor or anticancer by promoting immune responses like T-helper 1 activities and suppress T-helper 2 activities which indirectly inhibits the activities of M2 macrophage (14, 25). Functional signalling of IL-27 is through the WSX1 receptor and GP130 subunits together. The lack of either of the subunits attenuates the IL-27 signals (26). WSX1 is a widely studied receptor expressed in various cell types including macrophages and epithelial cells. Previous study has confirmed that WSX1 receptor expressed 13- to 78-fold higher in cancer cells compared to normal epithelial cells. Thus, the effect of IL-27 cytokine in normal cells may not be as significant as compared to the cells that expressed more of the WSX receptor (26, 27). To date, there are no studies that have been reported investigating the effects of recombinant pcDNA3.4-IL27 towards the co-culture of M2 macrophages with breast cancer cell lines. In the present study, a recombinant of pcDNA3.4-IL27 was constructed and employed to examine the apoptotic potentials of IL-27 against 4T1 cells alone and co-culture of 4T1 with M2 macrophages in vitro. 4T1 cells have been extensively utilized as a syngeneic breast tumor model for clinical grade IV human breast tumors. This cell line has several qualities as a human mammary cancer model which is easily transplanted into the mammary gland , highly tumorigenic and invasive and easily metastasized to distant organs (28). In this experiment, RAW264.7 cells were polarized into M2 macrophages by the influences of M2-inducing cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13) (22, 23). RAW264.7 cells showed an altered morphology after being stimulated with IL4/IL13 as illustrated by previous studies (22, 23). These polarized M2 macrophages also expressed the signature M2 macrophage marker, CD206 (29). The high level of IL-4/IL-13 in the tumor microenvironment skewed the RAW264.7 cells towards the tumor promoting M2 macrophages. This plays a pivotal role in the activation of the STAT6 pathway which is critically involved in several chronic diseases such as cancer. Thus, the 4T1 cells were co-cultured with M2 macrophages to mimic the real scenario in the tumor microenvironment. Based on previous studies conducted, the population of M2 macrophages in the tumor microenvironment is high, thus anti-cancer regimens that target M2 macrophages

could be a promising anti- cancer therapy (17, 30).

Over the years, the standard administration of recombinant cytokines have several drawbacks which are the high toxicity of the therapeutic doses and low efficacy particularly the residual microscopic tumors that were unable to be reached by the standard treatment. Thus, researchers found that utilizing the cytokine genes to modified cells might be a promising approach against cancer (31). In this study, a plasmid based vector harboring the IL-27 gene was transferred into 4T1 cells and co-cultured with M2 macrophages cells. The results showed that the expression of IL-27 gene was upregulated in pcDNA3.4-IL27 transfected 4T1 and co-cultured cells. The expression of IL-27 in the cells were validated by immunofluorescence staining. In this experiment, the IL-27 antibody conjugated with Alexa Fluor 594 was employed to the pcDNA3.4-IL27 transfected and non-transfected cells. It can be concluded that the IL-27 protein was found to be expressed more in the cytoplasm of the cell that has been transfected with pcDNA3.4-IL27 as illustrated in Fig. 3.

The ability to induce cell apoptosis has been the ultimate goal for researchers in the development of cancer treatment. In this present study, the cell viability after the transfection with pcDNA3.4-IL27 were determined through the cell viability (MTT) assay. The data revealed that the IC50 value of the co-culture of 4T1 with M2 macrophages is higher compared to 4T1 cells alone after the tranfection with pcDNA3.4-IL27. The apoptotic effects of IL27 were further investigated through the microscopic visualization of the cellular changes with the use of AO/PI dual staining. This staining enables distinguishing between viable, apoptotic and necrotic cells and distinct apoptotic characteristics of the cells such as blebbing of the membrane, condensed chromatin and cell shrinkage (20,21). The number of viable cells were reduced in the 4T1 cells and co-cultured cells transfected with pcDNA3.4-IL27 as compared to the control and empty pcDNA3.4 transfected cells. The viable, apoptotic, and necrotic cells after the introduction of the pcDNA3.4-IL27 gene were also further quantified by flow cytometry analysis. Flow cytometry is a sensitive fluorescent detection method of the distribution of viable, apoptotic, and necrotic cells (32). The cells were quantitated by the separated population that resulted from the binding of Annexin V to the phospholipid component of the cell membrane called phosphatidylserine (PS). The Annexin V stain is only able to bind the externalized PS to the outer leaflet from the plasma membrane. This is due to the disintegration of the plasma membrane which is one of the characteristics of an apoptotic cell (20, 33). This analysis revealed that the pcDNA3.4-IL27 transfected cells demostrated higher apoptotic cells compared to cells transfected with empty pcDNA3.4 and control cells. Similar to previous findings, the cancer cells that were treated with IL-27 demonstrated cell apoptotic events in human melanomas and pancreatic cancer cells (17, 18). These data are consistent with the previous studies that conclude that M2 macrophages influence the progression of cancer (8, 11, 34) . According to a study conducted by Chiba et al, 2013 on human melanomas cells, IL-27 was found to complement the expression of toll-like receptor 3 (TLR3) and TNF-related apoptosis-inducing ligand (TRAIL) induces cell apoptosis (17). The enhancement of TLR3 and TRAIL expression plays a critical role in the early activation of NK cells and activates the caspase apoptotic pathways such as caspase-3 and caspase-8 (35). It is probable that IL-27 induces cell apoptosis in breast cancer cells through the TRAIL, TLR3, caspase 3 and 8 pathways.

Collectively, the results revealed that pcDNA3.4-IL27 is more potent in the co-culture of 4T1 with M2 macrophages. This is because the alternatively activated M2 macrophages have demonstrated an upregulated WSX-1 expression, which resulted in increased IL-27 induced signals (tumor-killing activities) (26, 27).

CONCLUSION

In summary, pcDNA3.4-IL27 was successfully developed. The recombinant pcDNA3.4-IL27 induced cell apoptosis in both 4T1 cells alone and co-cultured 4T1 with M2 macrophages. This study also has demonstrated that pcDNA3.4-IL27 induces higher percentage of cell apoptosis in co-cultured 4T1 with M2 macrophages compared to 4T1 cells alone. These data provided an insight that IL-27 could be a potential anti-cancer treatment.

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