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Research Article

The priming role of dendritic cells on the cancer cytotoxic effects of cytokine-induced killer cells

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ABSTRACT

Introduction: *In vitro* cultivation of DCs and cytokine-induced killer cells (CIK cells) — a special phenotype of T lymphocyte populations — for cancer treatment has gained significant research interest. The goal of this study is to understand whether the priming from DCs helps CIK cells to exert their toxic function and kill the cancer cells. **Methods**: In this research, DCs were differentiated from mononuclear cells in culture medium supplemented with Granulocyte-macrophage colony-stimulating factor (GM-CSF), and Interleukin-4 (IL-4), and were induced to mature with cancer cell antigens. Umbilical cord blood mononuclear cells were induced into CIK cells by Interferon-γ (IFN-γ), anti-CD3 antibody and IL-2. After 4-day exposure (with DC:CIK = 1:10), DCs and CIK cells interacted with each other. **Results**: Indeed, DCs interacted with and secreted cytokines that stimulated CIK cells to proliferate up to 133.7%. In addition, DC-CIK co-culture also stimulated strong expression of IFN-γ. The analysis of flow cytometry data indicated that DC-CIK co-culture highly expressed Granzyme B (70.47% $\pm$ 1.53, 4 times higher than MNCs, twice higher than CIK cells) and CD3+CD56+ markers (13.27% $\pm$ 2.73, 13 times higher than MNCs, twice higher than CIK cells). Particularly, DC-CIK co-culture had the most specific lethal effects on cancer cells after 72 hours. **Conclusion**: In conclusion, co-culture of DCs and CIK cells is capable of increasing the expression of CIK-specific characteristics and CIK toxicity on cancer cells.

Key words: co-culture, cytokine-induced killer cells (CIK cells), dendritic cells (DCs), umbilical cord blood mononuclear cells

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INTRODUCTION

Immunotherapy for cancer treatment has been extensively studied not only to improve the quantity of the immune cell mediators but also in quality (such as function) of these mediators to maximize the efficiency of immunotherapy[1,2]. It cannot be ignored that immune cell therapy plays a very important role in cancer treatment[3,4]. In general, the purpose of cancer treatments is to reduce tumor size and ultimately eliminate cancer cells[5]; when the tumor is not detectable anymore at the cellular level, this is evidence for successful therapy[6]. It is also the goal of immune cell therapy to demonstrate full and convincing ability to destroying the body's abnormal cells, including cancer cells[7]. Normal cells of the body accumulate mutations that cannot be recovered. In fact, some cancer cells arise from normal cells have a breakdown in the mechanism of self-control of cell growth, which can gradually lead to formation of tumors[8]. There is a disruption of the molecular balance between oncogenes and tumor-suppressor genes[9]. When this cell balance is disrupted, this can lead to an inbalance between cancer cells and immune cells[10]. The latter balance is considered the last and very important barrier that the body makes. If this barrier is properly maintained, cancer does not have a chance to progress and should degrade quickly and easily[11]. However, when this barrier becomes extremely fragile, cancer cells can pass the check-points, thereby allowing mutation cascades to take place, which can trigger uncontrolled proliferation[12]. Finally, the quantity of cancer cells becomes overwhelming to immune cells[13]. It is also because of the accumulation of many mutations that cancer cells easily transform their own characteristics, including dealing with the immune system[14]. When an inadequate amount of immune cells exists, cancer cells just keep evading from immune surveillance[15] until the quantity of cancer cells increases; these cells now can tolerate the immune system that was intended to engage or attack them[16].

However, there are still many opportunities to cure cancer. There have been many improvements in routine treatments, such as surgery, chemotherapy and radiotherapy for cancer- to increase the ability to eliminate tumor cells[17]. However, such conventional treatments are non-targeted treatments. For

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example, surgery is difficult to detect micro-tumors, while chemotherapy and radiation kill the proliferating cells, including normal cells of the body[18]. As a result, patients suffer from harmful side effects and disease easily recurs. As one of the novel approaches to find ways to destroy cancer cells effectively and overcome the limitations of routine therapies, immunotherapy has been studied extensively and has become prominent, achieving many encouraging results[19].

As mentioned above, the amount of immune cells capable of identifying and destroying cancer cells needs to be ensured and maintained[20]. This is difficult to achieve in patients with advanced disease or when they have undergone conventional therapy since as the disease progresses, the quantity of cancer cells completely overwhelms the immune cells[21]. In the latter situation, there are two unexpected outcomes which can occur: severely affected immune cells can't be recovered both in number and function, and cancer cells can survive after treatment (even acquiring strong resistance to the conventional methods)[22]. This also explains why there are many promising results. In general, though, the effectiveness of immunotherapy has not been as expected[23]. Part of this is due to the fact that the cells responsible for tissue and organ regeneration (stem cells) are negatively affected by chemotherapy and radiation, and the immune system is severely impaired[24].

A more appropriate approach towards cancer treatment is a combination of therapies that can combine widely used methods and/or incorporate novel, targeted ones[25]. The combination helps promote the advantages of each method as well as limit their deficiencies. In particular, the combination helps reduce the dose of chemotherapy and/or radiation therapy that is administered in patients[26]. The immediate benefit is to limit or prevent unwanted side effects. In the types of immune cells studied, two candidates emerged from both arms of the immune system: dendritic cells (DCs) from innate immunity[27] and cytokine-induced killer cells (CIK cells) from adaptive immunity[28].

Dendritic cells (DCs) are the most professional antigen-presenting cells (APC)[29]. APCs process protein into peptide fragments, which incorporate with major histocompatibility complex (MHC) and are presented to T cells[30]. A simultaneous secretion of co-stimulating factors are necessary for the recognition of antigen via the T-cell receptor (TCR)[31]. DCs are capable of activating both naive and memory T cells, while macrophages only present antigens to specific T cells, and B cells present antigens to helper T cells[32]. DCs have been considered to be the center of the immune system because they are capable of stimulating humoral and cellular immune responses[33]. In other words, both innate immunity (via activation of natural killer cells (NK cells), macrophages, and mast cells) and adaptive immunity are in play. DCs are a heterogeneous population of cells, possessing different markers and playing different roles in the immune response[34]. DCs are scattered throughout the covered surfaces of the body in the immature phenotype, ready to arrest foreign pathogens[35]. After capturing antigen, DCs perform the processing function and present the antigen to the cell surface, and they move to the T cell-rich region to present the antigen[36]. In vitro, DCs have been isolated and differentiated from bone marrow CD34+ cells, peripheral blood, and umbilical cord blood mononuclear CD14+ cells[37]. Hematopoietic stem cells have been cultured under the supplementation of stimulating factors, such as GM-CSF, IL-4 and Tumor necrosis factor-α (TNF-α), to differentiate into DCs[38]. After the antigen is processed, DCs rapidly move into secondary lymph nodes, presenting antigens to naive T cells to stimulate immune cells, including CD4+ T cells (TH1) and CD8+ T cells[39], to activate memory B cells and inactive B cells, NK and NKT cells[40].

Cytokine-induced killer (CIK) cells are a type of cytotoxic T-cells with the phenotype of both T lymphocytes and NK cells[41]. In 1990, Schmidt-Wolf and colleagues discovered that CIK cells, which exist in the form of motile cell populations, when they differentiated peripheral blood mononuclear cells with cytokines, such as interferon-gamma (IFN-γ), anti-CD3 mAb and interleukin-2 (IL-2)[42]. CIK cells are a heterogeneous cell population that is highly toxic to tumor cells both in vitro and in vivo, without being limited by MHC and which cause low graft reaction[43]. Their phenotypes include: CD56+CD3+, CD56+CD3- and CD56-CD3+[44]. CIK cell toxicity is closely related to increased expression of CD56+ and CD3+ markers[45]. CIK cells are capable of independent cytotoxicity and rapid growth in culture, making it easier to infuse initially than using T cells[46]. In co-culture, antigen-induced DCs is responsible for directing CIK cells to directly lyse tumor cells by secreting cytokines, such as TNF-α, IL-2, and IL-12[47].

These two types of cells receive a lot of attention because they can be easily obtained from differentiating mononuclear cells in cord blood[48], which could be of great application significance when we can easily

isolate, proliferate and select them, then infuse functional cells back into patients with the goal of killing cancer cells[49]. There are many studies that demonstrate the ability of DCs and CIK cells in vitro[50 51]. However, the treatment effect is low if only DCs were infused into patients when the immune system no longer has enough functional cells to destroy cancer, or if only CIK cells were infused (without previous priming). Thus, the time to recognize cancer cells is delayed, which results in the uncontrollable incident when tumor mass becomes significant. The combination of DCs and CIK cells helps to limit the mentioned disadvantages, and DCs can present cancer cell antigens to CIK cells by hundred-fold increase[52]. After being administered into the patient's body, these cells help find and carry out the mechanism of poisoning of cancer cells without harming normal cells.

The goal of this study is to understand whether priming from DCs can help CIK cells to express their toxic function, and kill the cancer cells. The results from this study are clear evidence that the adequate combination helps the immune system to effectively identify and destroy cancer cells, and thus DC-CIK cell mixture is a potential platform choice for cancer immunotherapy.

MATERIALS AND METHODS

Human materials

Cord blood samples were collected from three healthy pregnant women at the Van Hanh Hospital following consent from donors. The collection procedure and usage of these blood samples were approved by the hospital ethical committee. Breast cancer cells (VNBRCA) and human fibroblasts (hF) were provided from the biological bank of Stem Cell Institute (VNUHCM University of Science). These cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS) and 1x Antibiotic-Antimycotic (Gibco, Carlsbad, CA).

Method to produce cancer antigen

Confluent cancer cells were trypsinized and pelleted, then suspended in 1ml of PBS. Membrane breaking was conducted by quick freeze-thaw method: -196^{0}C in liquid nitrogen, 2 minutes → 37^{0}C, 2 minutes 30 seconds → vortex 30 seconds; this process was replicated 5 times. Samples were centrifuged at 13000 rpm, 5 minutes, 4^{0}C. The suspension was collected, and the antigen concentration was quantified by Bradford method.

Bradford method

To determine the amount of protein in the sample, a known standard protein curve was made that showed the correlation between concentration and absorption value at 595 nm (OD595). A common standard protein solution is bovine serum albumin (BSA). After adding the dye to the protein solution, the color will appear within 2 minutes and last up to 1 hour. The optical density measurement was performed with a spectrophotometer (DTX 880, Beckman Coulter).

Standard BSA protein (0.1mg/ml) was made. Protein (antigen) samples were tested by diluting with distilled water (diluted 100 times). Bradford solution was diluted 2.5-fold with distilled water. A standard BSA curve was made: 0, 20, and 40-100 µl of standard BSA solution (0.1 mg/ml) was aliquoted into each well and distilled water added to 100 µl. Antigen samples (100 µl each) were added into the other wells then 100 µl of Bradford solution was added to each well. The blank well contained 200 µl of distilled water. The wells were shaken for 5 minutes at room temperature. Optical density (OD) at 595 nm wavelength was measured. From the measurement results, a standard protein curve was created for the relation between protein concentration and OD595 values. The value of the antigen concentration to be measured was extrapolated.

Isolate umbilical cord blood mononuclear cells

Based on the difference in density of blood cells, granulocytes and erythrocytes were separated from mononuclear cells. Granulocytes and erythrocytes have a higher density at osmotic pressure of Ficoll, and are deposited through the Ficoll layer during centrifugation. Mononuclear cells with a lower density are in the middle of the plasma-Ficoll layer. Mononuclear cells can be easily collected, then washed to remove platelets, Ficoll and plasma.

The following is the step-wise procedure for collecting mononuclear cells:

Aliquot blood from blood collection bags into 50 ml centrifuge tube. Dilute blood with sterile PBS at a ratio of 1: 1. Add 15 ml Ficoll straight to the bottom of a 50-ml centrifuge tube. Add 30 ml diluted blood on the Ficoll layer. Avoid disturbance between Ficoll and blood, and create clear layer. Centrifuge at a speed of 400 g, 30 minutes, 25^{0}C. Remove the above plasma layer without affecting the interface between the plasma-Ficoll. Transfer the mononuclear cells at the plasma-Ficoll interface into another centrifuge tube, wash with sterile PBS (mixed at a ratio

of 1: 1), and centrifuge at 800 g, 10 minutes. Remove the supernatant, collect cell pellet, and suspend with 5 ml red blood cell lysis buffer for 5 minutes at room temperature. Add PBS to 20 ml, centrifuge at a speed of 300 g, 6 minutes. Repeat once. Suspend cell pellet with 5 ml of basic culture medium and transfer to sterile culture flask. Incubate in a 37^0C, 5% CO2 incubator. After 2 hours, transfer the cell suspension to another culture flask and continue incubating at 37^0C, 5% CO2 in the incubator. Perform 2 more times to get MNCs, and differentiate into DCs. For the last step, take the cell suspension to differentiate to CIK cells. The determination of MNC cell count was done by Trypan blue staining and marker expression of MNC sample was tested at the end of the experiment.

Differentiation of cord blood cord mononuclear cells into DC and CIK cells

Differentiation of DCs

Mononuclear blood cells could be obtained from peripheral blood or umbilical cord blood. In this study, DC were induced to mature from cord blood mononuclear cells by a 10-day procedure.

Phase 1, day D1: obtained from the attached mononuclear cells in culture flask. Induction of mononuclear cells by CM1 medium (containing 40 ng/ml IL-4 and 50 ng/ml GM-CSF). Refresh the culture medium every 3 days.

Phase 2, day D7: Determine cell density and conduct maturation of immature DC (iDCs) with antigen (Ag) lysates with concentration of 50 µg/ml medium.

Phase 3, day D10: mature DCs were obtained. DC cell density was evaluated to determine the amount of cells needed to perform DC-CIK co-culture.

Phase 4, day D14: DC samples cultured in CM1 medium were collected and used in MTT assay (group of DC+CIK individual cell experiments).

Evaluation of cell growth was done by determining the number of cells obtained on day D10 and day D14 by Trypan blue staining.

Differentiation of CIK cells

In this study, we isolated MNCs on day D0, then cultured them, and induced and differentiated them into CIK cells for 14 days the following procedure: MNCs were cultured in RPMI-1640, 10% FBS, and 1% antibiotic. On day D0, MNCs were induced with IFN-γ 1000 U/ml, and on D1 they were induced with 50 ng/ml anti-CD3 Ab and 1000 U/ml IL-2. The medium was refreshed with 1000 U/ml IL-2 every 3 days.

DC-CIK Co-culture

In co-culture, DCs and CIKs can directly or indirectly interact using physical or chemical barriers (e.g. EDTA in the culture medium). In this experiment, DC-CIK co-culture was in RPMI-1640, supplemented with IL-2 (1000 U/ml). The ratio used in this experiment was DC:CIK = 1:10, in which DCs were previously induced to mature before co-culture.

On D10, DCs and CIK cells were collected from culture, and cell density was determined with Trypan blue staining. DC-CIK co-culture was done at a ratio of 1:10 in RPMI-1640 medium, supplemented with IL-2 (1,000 U/ml). Proliferation of the mixture was evaluated after 4 days (D10-D14). The typical phenotypic expression of CIK cells (e.g. for Granzyme B and CD3+CD56+ markers) was evaluated in the co-culture by flow cytometry.

Evaluation of CIK gene expression after 4 days of co-culture

At day D14, cells in the culture plates were collected.

Acquisition of total RNA using easy-BLUE^{TM}

The protocol was as follows:

Collect $5x10^5$ cells in each group to harvest total RNA. Add 500 µl easy-BLUE ^{TM}, and vigorously vortex to completely dissolve cell pellet. Add 200 µl Chloroform and vigorously vortex. Centrifuge 13,000 rpm for 10 minutes. Gently aspirate the supernatant layer into a new 1.5 mL centrifuge tube, avoiding disturbance of the middle protein layer. Add isopropanol to the tube at the same volume. Incubate for 10 minutes at 4^0C and then centrifuge at 13,000 rpm for 10 minutes. Discard the supernatant and dry the pellet. Add 1 ml of 70% Ethanol, invert the tube few times, and centrifuge at 10,000 rpm for 5 minutes. Discard the supernatant and dry the pellet. Then, dissolve RNA pellet in 20-30 µl DEPC water. Finally, use 6 µl RNA solution to measure OD (determination of total RNA concentration) and perform electrophoresis to determine RNA quality after separation. hF cell RNA was isolated for the control group.

RT-PCR

The brightness of RT-PCR products on the electrophoresis was analyzed by ImageJ software (NIH, USA) and GraphPad Prism (GraphPad Software, San Diego, CA).

Reactive ingredients	Volume
2x PCR One Step Mix	12.5 µl
Forward primer (10µM)	0.75 µl
Reverse primer (10µM)	0.75 µl
20x RTase	1.25 µl
Template RNA	Te µl (160 ng/µl reaction)
dH2O	9.75-Te µl
Total volume	25 µl

Table 1: Primers used in the study

Primer	Primer sequence	Pairing temperature	Melting temperature	Product size (bp)
GAPDH	F: GGGAGCCAAAAGGGTCATCA R: TGATGGCATGGACTGTGGTC	51.8 ^{o}C	54.36 ^{o}C 56.50 ^{o}C	203
IFN-γ	F: TGGTTGTCCTGCCTGCAATA R: TAGGTTGGCTGCCTAGTTGG	55.5 ^{o}C	59.60 ^{o}C 59.38 ^{o}C	277
TNF-α	F: CCAGGCAGGTTCTCTTCCTC R: GGGTTTGCTACAACATGGGC	58.6 ^{o}C	59.75 ^{o}C 59.75 ^{o}C	355
IL-2	F:AGTAACCTCAACTCCTGCCAC R: TGTGAGCATCCTGGTGAGTT	60.2 ^{o}C	59.65 ^{o}C 58.94 ^{o}C	300

Table 2: Reaction cycle

Number of cycles	Temperature	Time	
1	45 ^{o}C	10 minutes	cDNA reverse transcription
1	95 ^{o}C	2 minutes	Activate polymerase
40	95 ^{o}C 60 ^{o}C 72 ^{o}C	10 seconds 10 seconds 30 seconds	cDNA denaturation Pairing primers on cDNA Multiply product

Flow cytometry

Antibodies for flow cytometry were the following: anti-Granzyme B antibody-phycoerythrin (PE) (Life Technologies, Waltham, MA, USA), anti-CD3 monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX), IgG2a-fluorescein isothiocyanate (FITC) (Sigma Aldrich, St. Louis, MO), anti-CD56 antibody-allophycocyanin (APC) (Life Technologies), and anti-CD56 (Santa Cruz Biotechnology) and IgG1-fluorescent peridinin-chlorophyll protein (PER-CP) (Santa Cruz Biotechnology).

Cells were fixed in 4% paraformaldehyde solution and stored at 4^0C. Cells were divided into 3 tubes for analysis: (1) Unlabelled - No staining, (2) Surface marker: CD3-FITC, CD56-APC, and (3) Intracellular marker: Granzyme B-PE. For intracellular marker, permeabilization was carried out by adding ice-cold FCM Permeabilization buffer solution onto cell pellet while vortexing. The sample was shook for 5 minutes at room temperature, and then centrifuged at 2000 rpm, 5 minutes. PBS wash was done to remove the buffer solution.

Here is the stepwise protocol for FCM:

Add 2 µl of fluorescent antibody to each test tube accordingly. Add 100 µl of cell suspension. Vortex and incubate at 4^0C, 30 minutes. Wash with PBS to remove excess antibodies. After centrifugation, resuspend with 500 µl 1% PFA solution. Samples were analyzed by FacsCalibur (BD Biosciences, San Jose, CA). Results were analyzed by CellQuest Pro software (BD Biosciences, San Jose, CA). The graph was drawn with GraphPad Prism (BD Biosciences, San Jose, CA).

MTT method

On D13, target cells were seeded into 96-well plate with density of 2000 cells/100 µl of RPMI-1640

medium/well. Cells were divided into 3 experimental groups as follows: group A (culture medium), group B (hF cells), and group C (VNBRCA cells). On D14, cells were seed into each well (20,000 cells/100 µl RPMI-1640, with IL-2 at 1000 U/ml) at a target cell: effector cell ratio of 1:10. Effector cells were divided into 3 groups (DC-CIK, DC+CIK, and CIK). (1) Group DC-CIK: DC-CIK cells co-cultured from D10. (2) Group DC+CIK: DC and CIK are collected after 14 days of culture, and DC:CIK ratio = 1:10 (3). Group CIK: CIK cells were assessed after 14 days of culture.

On D16/D17, after 48/72 hours of seeding effector cells, MTT measurements were done: The brief protocol included adding 20 µl of 0.5 mg/ml MTT solution to each well, and the plate was shook at 115 rpm/5 minutes at room temperature. After 3.5 hours, the formation of MTT crystals was observed with a microscope. After 4 hours, all of the solution in the wells was removed to be measured. Then, 200 µl of DMSO solution was added to wash the MTT precipitate. OD measurement at wavelength 570 nm was done to determine the amount of formazan crystals formed, or the number of cells alive after 48/72 hours exposure to effector cells. Determination of the cytotoxicity of the effector cells corresponding to the determination of the target cell ratio (VNBRCA, hF) was destroyed after the time of exposure with the effector cell (%):

$$\text{Cell death rate} = \left(1 - \frac{OD\ target\ cell - effector\ cell - OD\ effector\ cell}{OD\ target\ cell}\right) \times 100$$

The optical density value measures absorption at wavelength of λ570nm. Target cells: VNBRCA cells or hF cells. Effector cells: DC-CIK, DC+CIK cells, and CIK cells.

RESULTS

Protein concentration determination by Bradford method

The standard protein curve is a linear line between protein concentration and OD value measured at a wavelength of λ595nm (OD595). Protein concentration value can be easily determined by the equation: y = 1.8479x + 0.5878, where x is the protein concentration (mg/ml), and y is the value at OD595.

Based on the linear equation, we deduced that the antigen concentration obtained after VNBC cancer cell lysis. Antigen concentration (from the results) was 1.97 mg/ml.

Differentiation of DCs, CIK cells and DC-CIK co-culture

Differentiation of DCs

After 24 hours of primary culture, mononuclear cells were differentiated into immature DCs in CM1 medium supplemented with 50 ng/ml GM-CSF and 40 ng/ml IL -4. After 4 days, when observing the cells culture under a microscope, a group of dendritic cells which attached on the surface of flask appeared; another group of cells attached but did not yet grow branched projections. The remaining component of the culture was floating cells (**Figure 3**A). After 7 days, immature DC candidate cells were induced to mature in CM1 medium supplemented with VNBRCA antigen at a concentration of 50 µg/ml (**Figure 3**B).

After 3 days of antigen exposure (D10), DCs had a marked morphological change, which is a sign of maturity. The group of half-adhered and suspended cells was much higher than in the previous period (data not shown) and DCs have fewer dendrites. The change of immature and mature DC morphology partly aids in seeing the effects of antigens added to the culture medium (**Figure 3**C and D).

Differentiation of CIK cells

MNCs obtained in the last transfer were used to induce CIK with primary culture medium supplemented with IFN-γ (1000 U/ml). After 24 hours, 50 ng/ml anti-CD3 Ab and 1000 U/ml IL-2 were added to the CIK culture medium. On D14, a homogeneous CIK population was obtained; the cells had a rounded morphology and showed strong proliferative capability.

DC-CIK cell co-culture

On D10, a co-culture of DC and CIK cells was initiated, at a 1:10 ratio (DC:CIK) in RPMI-1640 medium supplemented with 1000 U/ml IL-2.

The proliferation of DCs, CIK cells, and DC-CIK during culture

From D10-D14, the density of the cell populations differentiated from cord blood mononuclear cells (DCs, CIK cells and DC-CIK cells) was checked (**Figure 6**).

The rate of cell proliferation on day D14 compared with day D10 is determined by the formula:

$$\mathrm{H}(\%) = \frac{cell\ quantity\ at\ D14 - cell\ quantity\ at\ D10}{cell\ quantity\ at\ D10} \times 100$$

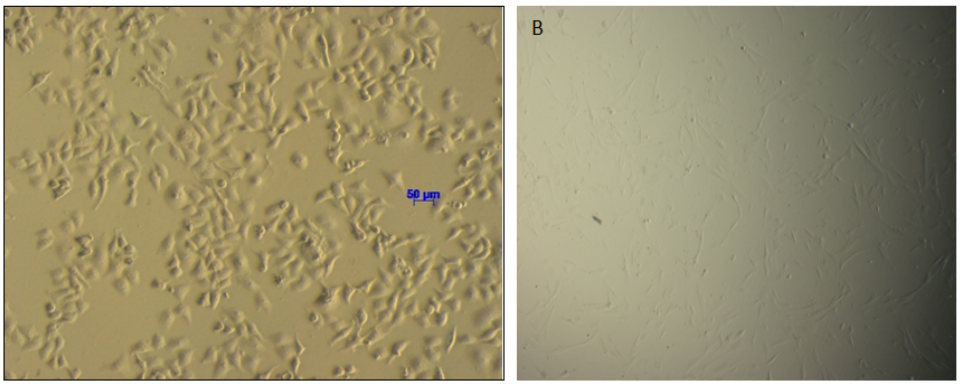

Figure 1: **VNBCA(A) and hF (B) in complete DMEM/F12 medium.** Breast cancer cells (VNBRCA) have a typical epithelial form, and human fibroblasts (hF) are elongated shape. Both cell types grow fast in culture, and the mediumare refresh every 2 days until cells get confluency.

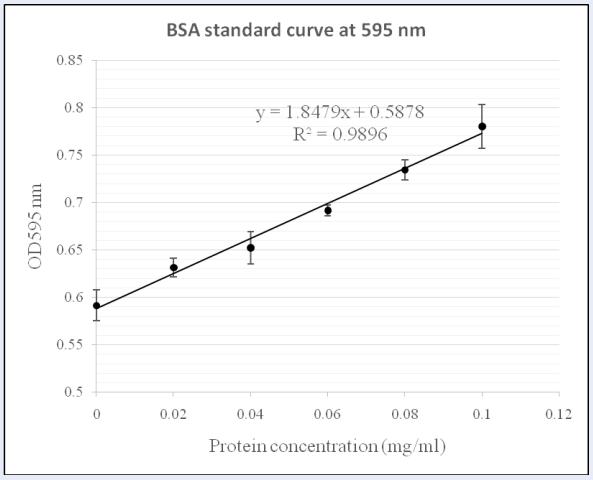

Figure 2: **Standard BSA curve at 595 nm wavelength**. Bovine serum albumin (BSA) standard protein curve was made that showed the correlation between concentration and absorption value at 595 nm (OD595). Data shown as mean ± SD of triplicate wells. Theoptical density measurement was performed with a spectrophotometer. Confluent cancer cells were used to produce protein mixture by quick freeze-thaw method. The value of the antigen concentration to be measured was extrapolated.

The rate of cell proliferation after 4 days of culture (D10-D14) of the groups differed significantly ($p<0.05$). In particular, CIK cells had the fastest growth rate (average of 133.88%), DC-CIK co-culture had slower growth rate (average 33.7%), and DCs no long proliferate (growth rate<0). The results of growth rate reflect the physiological state of the cell. In culture on D14, DCs had been induced to mature and were dying, CIK cells had a rapid growth rate during the culture period of 14-21 days. The proliferation in the DC-CIK group reflects the effectiveness of the DC-CIK co-culture. After exposure time (4 days), DCs and CIK cells interacted with each other. Thus, DCs are capable of presenting antigens to CIK cells and secreting cytokines that stimulate CIK cells to proliferate.

Gene expression

RNA was harvested with high purity and without rupture.

In the three samples of gene expression analysis, the results showed that the DC-CIK co-culture stimulated a strong expression of IFN-γ in comparison with the CIK alone ($p <0.0001$). There were no differences be-

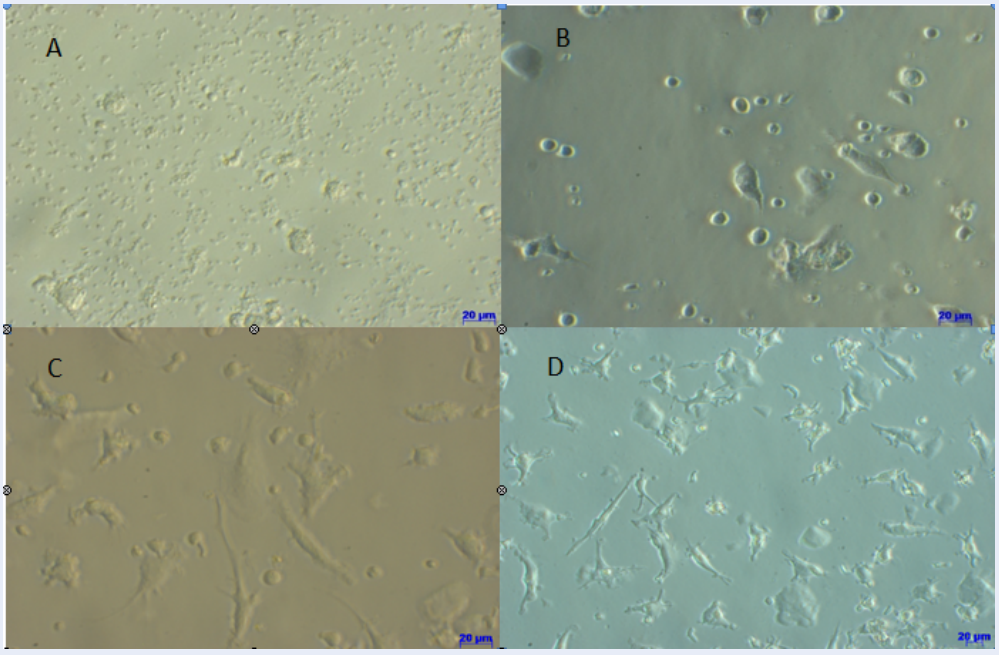

Figure 3: **DC phenotype during culture. DCs at immature stage: DCs on D4 (A) and D7 (B); DCs at mature stage: DCs day on D10 (C) and day D14 (D).** The cell morphological changes can easily be differentiated between two stages, which suggests that antigens have a significant impact on DC characteristics. After antigen induction, DCs are fully capable of activating lymphocytes to function to destroy cells carrying that antigen.

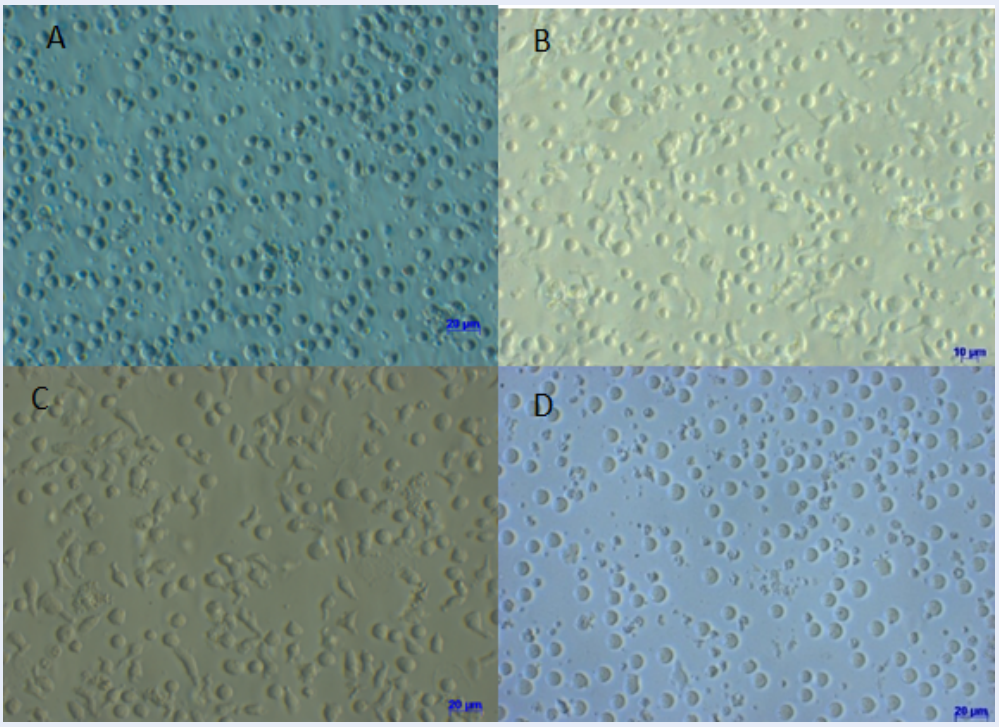

Figure 4: **CIK cell morphology during culture. MNCs on day D0 (A), CIK cells on D7 (B), CIK cells on D10 (C), and CIK cells on D14 (D).** CIK cells gradually proliferate without any changes in morphology

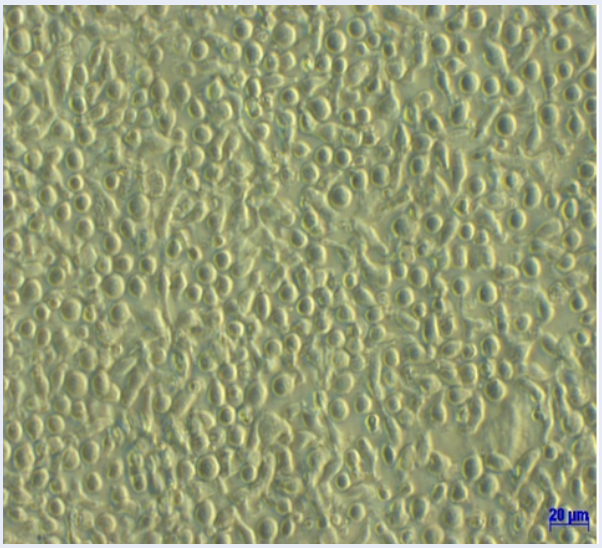

Figure 5: **DC-CIK cell co-culture on D14**. After co-culture period, it is easy to see that the cell mixture has strong proliferation capacity which occupies the entire culture flask surface into many cell layers (the spherical cells cover the cell layer below), which shows that there is an interaction between the two cell types keeps them dividing.

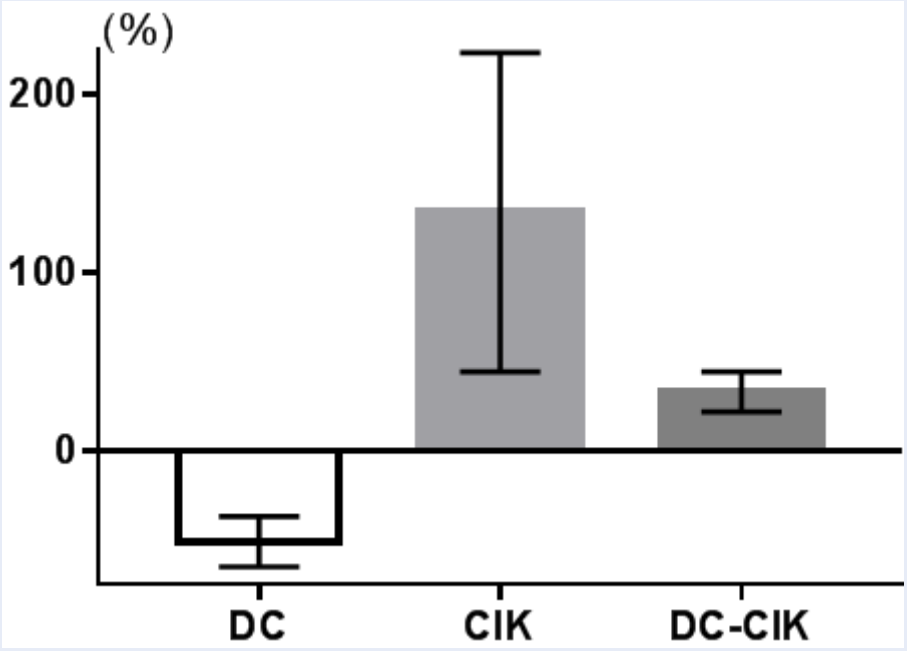

Figure 6: **Cell growth rate after 4 days (D10-D14).** CIK cells grow fastest (average of 133.88%), DC-CIK co-culture had slower growth rate (average 33.7%), and DCs no longer proliferate.

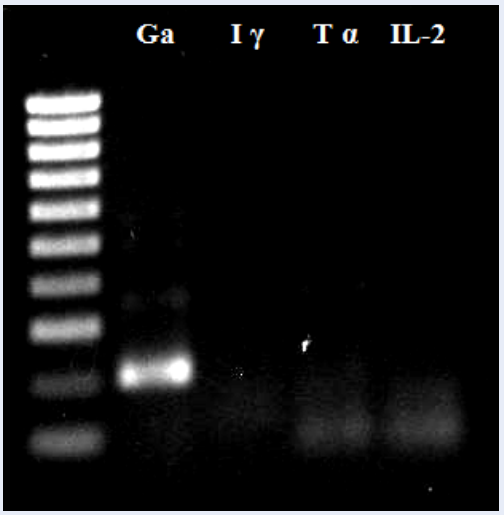

Figure 7: **Gene expression on hF cells**. hF cells do not express IFN-γ (I γ), TNF-α (T α),and IL-2 genes. Ga refers to GAPDH.

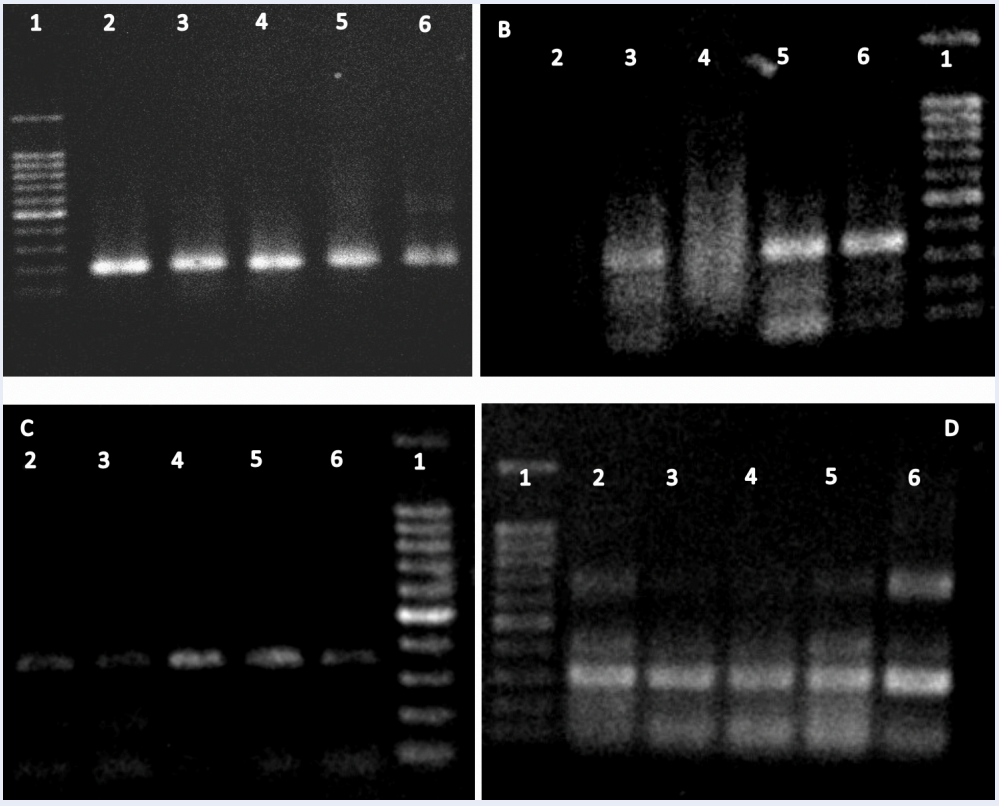

Figure 8: **Results of gene expression of GAPDH (A), IFN-γ (B), TNF-α (C), IL-2 (D).** Lane 1 is 100bp ladder; lane 2, 3, 4, 5 & 6 correspond to CIK cells, DC-CIK cells, and DC-CIK in culture medium added with EDTA at concentration of 0.08, 0.04 and 0.02 mg/m, respectively. EDTA was added to interfere cell-cell interaction, apparently at high concentrations of EDTA (0.08 mg/ml), cells do not express IFN gene.

tween TNF-α and IL-2 gene expression between those groups.

Evaluation of marker expression in DC-CIK co-culture and CIK cell populations

The expression of intracellular marker (Granzyme B) and surface marker ($CD3^+CD56^+$) among the groups differed significantly (**Figure 9**). For Granzyme B, MNCs showed the lowest expression (18.74% ± 10.92), while DC-CIK co-culture showed the strongest (70.47% ± 1.53, 4 times higher than MNCs). The lowest expression of Granzyme B was observed for CIK cells (42.67% ± 7.78, 2.28 times greater than MNCs). For CD3+CD56+, the co-culture showed the most expression (13.27% ± 2.73), which is 13 times higher than that for MNCs (1.02% ± 0.11). Note that CIK cells expressed lower (6.82% ± 2.42, nearly 6.67 times that of MNCs). The results showed that co-culture expressed the strongest expression, which is better than CIK group. This proves that co-culture facilitates DCs and CIK cells to interact with each other, increasing the CIK cell toxicity after exposure to DCs induced with antigen. Therefore, the analysis results of flow cytometry prove that DC-CIK co-culture has the effect of increasing the ability of CIK to induce tumor cell cytoxicity.

Evaluation of the ability to cause VNBRCA cell death of DC-CIK co-culture

Experiments were conducted on two cell lines (VNBRCA and hF) with 3 cell effector cell groups, namely the co-culture (DC-CIK), individual (DC+CIK), and CIK, which assessed the ability to target cancer cells through the priming of DCs in DC-CIK and DC+CIK groups, compared to the CIK group. The time of contact with target cells in 48 hours (for sample 1 and sample 2) was evaluated, as well at 72 hours (for samples 3 and 4). The results of VNBRCA and hF cytotoxic assessment are expressed by the ratio of dead target cells when contacting with effector cells. After 48 hours of exposure with effector cells, in sample 1, effector cells do not affect fibroblasts (hF cells) but cause cell death on VNBRCA breast cancer cells (**Figure 10**).

In terms of VNBRCA cell death, cells in the individual culture group (DC+CIK) gave the best removal efficiency (41.21% ± 1.02), which was 3.73 times more than the DC-CIK group (11.06% ± 0.54), while the CIK group was lowest (20.72% ± 1.11), and was 1.83 times greater than the DC-CIK group (**Figure 10**).

In sample 2, effector cells did not affect fibroblasts but caused cell death on VNBRCA breast cancer cells (**Figure 11**).

In considering VNBRCA cell death, cells in DC+CIK and CIK groups gave better killing efficiency than the co-culture group (6.59 % ± 0.22). The rate of cell death induced on VNBRCA cells did not differ between the 2 groups of effectors: DC+CIK cells (23.63% ± 0.94) and CIK cells (24.50% ± 1.78), ($p = 0.0603 > 0.05$, **Figure 11**). Thus, after 48 hours, effector cells do not have toxic effects on hF cells but are capable of lethality on VNBRCA cells, show since the group of effector DC+CIK cells showed toxic effects more significantly than the 2 groups of CIK and DC-CIK.

After 72-hour exposure, in sample 3, the effector cells of DC+CIK and CIK group caused toxic effects on both hF and VNBRCA target cells (**Figure 12**).

On VNBRCA cells, the effector cells of DC+CIK cells had the highest cell death effect (91.09% ± 4.17), which was 1.6 times higher than the DC-CIK group (57.24% ± 3.2). CIK cells do not make deadly effects on VNBRCA cells (**Figure 12**). In sample 4, DC+CIK effector cells caused toxicity on both target cell lines, while DC-CIK cells had the most effective VNBRCA cytotoxicity.

For hF cells, the groups of effector DC-CIK and CIK cells did not kill target cells, but DC+CIK cells had toxic effects on cell death at the rate of 31.71% ± 2.65. For VNBRCA cells, the lowest rate of cell death was in the CIK group (20.58% ± 3.24), DC+CIK group had the highest cell death rate (53% ± 6.01, 2.58 times more than CIK), and the DC-CIK group had the lowest toxic effect (25.01% ± 1.94, 1.22 times higher than CIK cells) (**Figure 13**). After 72 hours, DC+CIK cell group had the highest VNBRCA cytotoxic effect but also killed hF cells. Therefore, DC-CIK co-culture induced optimal cytotoxic effects after 72 hours of exposure to target cells.

In summary, for the hF cells, cells of DC+CIK and CIK groups had the effect of causing cell death after 72-hour exposure, in which the cell death rate of DC+CIK group was high (38.24% ± 7.69 on average). Meanwhile, the DC-CIK group does not cause hF target cell death after both time points. For VNBRCA cells, all 3 groups of the effector cells have a lethal effect on cancer cells, in which DC+CIK group gave the highest effect. The lethality of the DC-CIK group increased after 72-hour exposure with the target cell. Therefore, DC-CIK co-culture has the most specific ability to eliminate VNBRCA breast cancer cells with only causing lethal effects on cancer cells yet without causing toxicity on fibroblasts (hF). The optimal toxicity of this group on VNBRCA occurs after 72 hours.

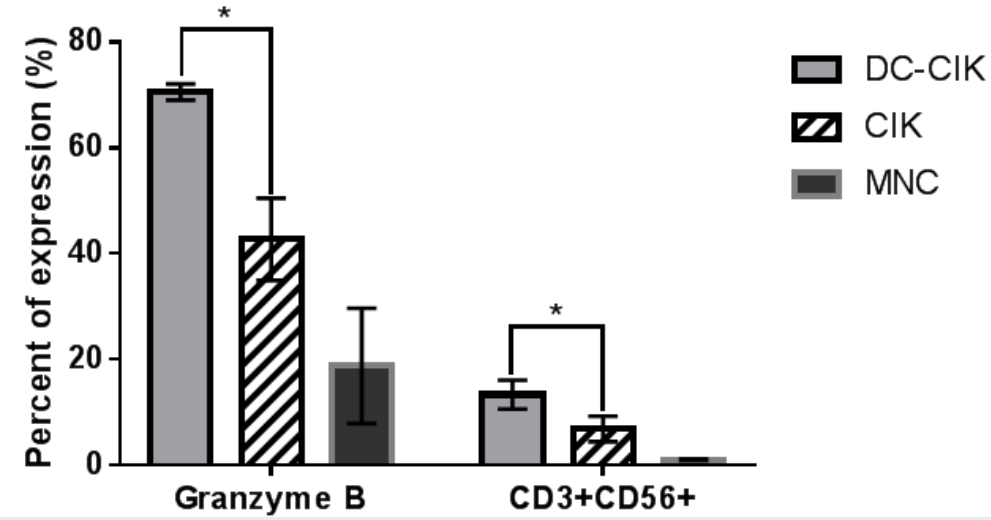

Figure 9: **Expression of CIK-specific cell marker**. MNCs express Granzyme B lowest, while DC-CIK co-culture showed the strongest expression. The co-culture also showed the most CD3+CD56+expression. Data shown as mean ± SD; * p<0.05

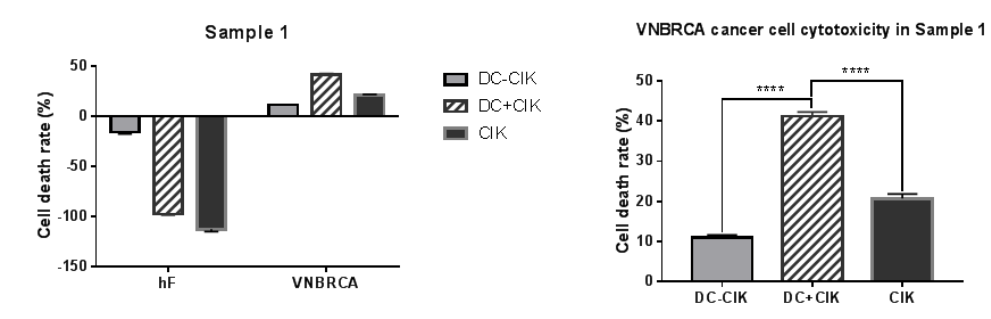

Figure 10: **Percentage of dead cells after 48 hours of exposure to effector cells (%) of sample 1.** Effector cells do not affect fibroblasts (hF cells) but cause cell death on VNBRCA breast cancer cells. Data shown as mean ± SD; **** p<0.0001.

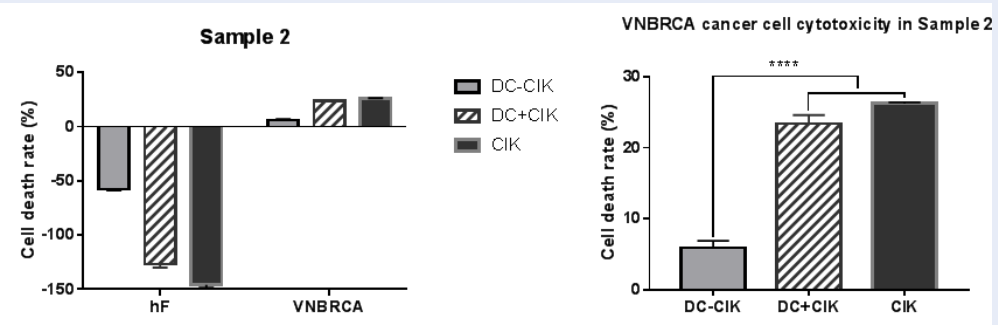

Figure 11: **Percentage of dead cells after 48 hours of exposure of sample 2 to effector cells (%).** Effector cells do not affect fibroblasts (hF cells) but cause cell death on VNBRCA breast cancer cells. Data shown as mean ± SD; **** p<0.0001.

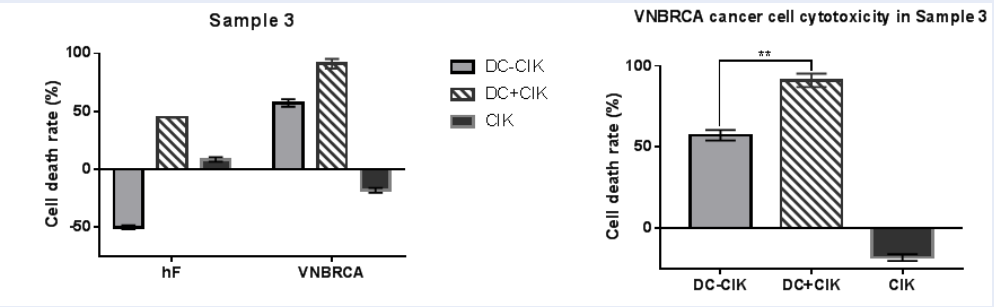

Figure 12: **Percentage of dead cells after 48 hours of exposure to effector cells (%) of sample 3**. The effector cells of DC+CIK and CIK group caused toxic effects on hF normal cell, CIK cells even do not make deadly effects on VNBRCA cells. Data shown as mean ±SD; ** p<0.005.

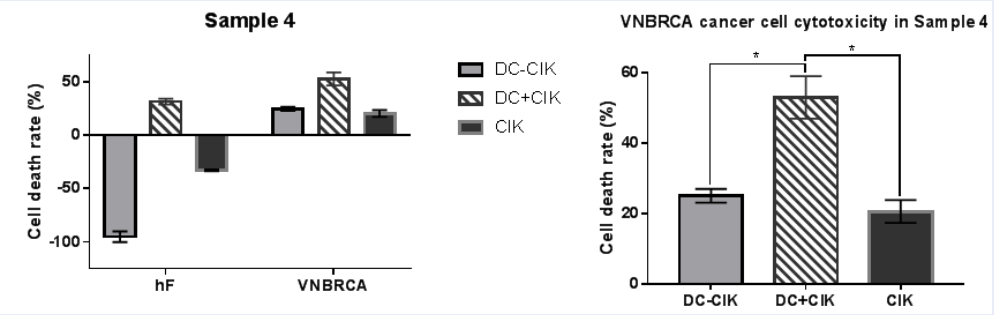

Figure 13: **Percentage of dead cells after 72 hours of exposure of sample 4 to effector cells (%).** DC+CIK effector cells caused toxicity on both target cell lines, while DC-CIK cells had the most effective VNBRCA cytotoxicity. Data shown as mean ± SD; * p<0.05.

DISCUSSION

Our body is capable of stimulating immune responses to remove abnormal cells (cancer cells)[53]. The use of the immune system to eliminate cancer cells in the body is well known as immunotherapy for cancer treatment[54]. One of the main methods used is immune cell therapy[55]. Immune cells have been cultured in vitro in which they are induced to strongly identify tumor cells before being infused into cancer patients[56]. In vitro cultivation of DCs and Cytokine-induced Killer cells (CIK cells) - a special phenotype of T lymphocyte populations- for cancer treatment has gained increasing research interests[57].

Mononuclear cells were collected and cultured in primary culture supplemented with 10% serum (FBS) to enhance selection of cells capable of adhering to the surface of the flask; this helps to maximize the cells with good adhesion, creating a good cell source for DC differentiation[58]. In addition, FBS supplementation is also convenient for transferring the cell suspension afterwards and removing red blood cells about 2-3 hours later. According to Steinman and Cohn (1973), the ability of mononuclear cell adhesion decreases with time in the primary medium, so that the transferring creates space for the best mononuclear cell adhesion[59]. It is very important to remove red blood cells from the first stage of isolation[60]. If it has been done without caution, mononuclear cell samples have an abundance of both platelets and erythrocytes, affecting the effectiveness of the subsequent lysis process. This makes it difficult to determine the number of cells or containment of noise signal via interference marker analysis. Mononuclear cell population with the best adhesion capacity obtained during the first transfer was used as cell source for DCs differentiation[61]. The cell population in the final transfer was used to induce into CIK cells because these cells are non-adherent cells.

DCs are one of the professional antigen presenting cells (APC) of the immune system[62]. DCs are able of capturing and presenting foreign antigens to T lymphocytes, thereby activating naive T cells into antigen-specific cytotoxic T cells[63]. On the other hand, activated T-cells secrete cytokines to stimulate the proliferation of DCs[64]. The co-culture of T-cells and

antigen-primed DCs will stimulate the proliferation and toxicity of T cells. *In vitro*, CD14+ mononuclear cells can be induced and differentiated into DC with the presence of GM-CSF, IL-4, and TNF-α (tumor necrosis factor-α)[65]. GM-CSF is believed to ensure the existence and differentiation of monocytes in vitro, while IL-4 has been shown to inhibit the differentiation into macrophages[66]. TNF-α also helps to produce high levels of IL-12p70, thereby enhancing the ability to activate TH1 and CTLs[67]. In studies using DC in cancer therapy, replacing necrosis factor TNF-α by tumor antigen is increasingly concerning but markedly effective, especially in *in vivo* and clinical trials[68]. Quick Freeze-Thaw is a common physical method normally used for mammal cell lysis[69]. This method relies on mechanical impact directly on cell membrane by forming ice crystals during the freezing causing tearing, breaking cell membrane[70]. Cells will be cleaved into smaller peptides[71]. Cancer antigens are biological agents induced dendritic cell maturity as a physiological condition in the body[72]. The use of antigens derived from tumor cell lysis to induce DC maturation is necessary to provide strong antitumor effect in both *in vitro* and *in vivo*[73]. After being exposed to antigens, DCs reduce adhesion and are able to migrate to lymph nodes due to secretion of cytokines, such as TNF-α, IL-1 β[74]. In particular, TNF-α is the stimulus for differentiating immature DCs into mature DCs[75]. The change of DC cell morphology pre- and post-antigen exposure (or immaturity and maturity) is clearly demonstrated by observations on D7, D10 and D14 (**Figure 3**B, C, and D). Immature DCs have distinctly long dendrites and better adhesion than mature DCs[76].

Mononuclear cells isolated from peripheral blood may be induced into CIK cells by IFN-γ, anti-CD3 antibody, and IL-2[77]. CIK cells have T lymphocytes and natural killer (NK) cell characteristics, and contain heterogeneous phenotypic features (*e.g.* CD3-CD56+, CD3+CD56-, and CD3+CD56+)[78]. CIK cells have pre-eminent traits, such as rapid proliferation, toxicity to cancer cells regardless of HLA and low rejection ability[79]. CIK cells are capable of rapid growth during the 2-3 week culture period, and CIK cells can grow up to a few hundred fold[80]. Control of IFN-γ induction before supplementation with IL-2 and anti-CD3 antibody is crucial in creating cytotoxicity[81]. Specifically, IFN-γ activates monocytes to provide an important signal in CD56+ T-cell proliferation[82]. The addition of IL-2 and anti-CD3 then mainly provide mitotic signals[83]. CIK is a round-shape, floating cell population, not adhering in culture (**Figure 4**B, C and D).

In our body, cell populations cannot function independently, but have an interaction with other cell populations to coexist[84]. Cell co-culture is one of the methods to determine the interplay between populations, which can stimulate or inhibit each other[85]. Co-culturing DCs and T cells to stimulate the body's immune response to pathogens as well as cancer cells has yielded positive results[86]. However, the method also has certain limitations such as weak T-cell vitality, low quantity of T-cells isolated from patients, and cultured T-cell characteristic alteration. All of these factors increase in the risk of T-cell rejection out of the patient's body[87]. Bearing the characteristics of CD3+ T lymphocytes, CIK cells are able to identify the antigen presented on DCs[88]. Co-culture of DCs and CIK cells is capable of increasing the expression of CIK-specific characteristics and CIK toxicity on cancer cells[89]. The ratio of DC-CIK co-culture is as diverse as 1: 3, 1: 5, 1:10, and 1:20, according to previous studies[90,91]. The ratio used in this experiment was DC:CIK = 1:10. After exposure time (4 days), DCs and CIK cells interacted with each other, and DCs interacted with and secreted cytokines that stimulate CIK cells to proliferate (**Figure 6**). In addition, DC-CIK co-culture also stimulated strong expression of IFN-γ (**Figure 8**B). The analysis results of flow cytometry demonstrate that DC-CIK co-culture highly express Granzyme B and CD3+CD56+ markers (**Figure 9**). Specifically, DC-CIK co-culture has the most specific lethal effects on cancer cells after 72 hours.

CONCLUSION

The combination of DCs with CIK cells- with a function to determine the target cancer cells- is a potential combined strategy with high efficiency and safety compared to traditional therapies, such as chemotherapy and radiotherapy, which cause systemic immunodeficiency.

ABBREVIATIONS

Ag: antigen
APC: allophycocyanin
APC: Antigen-presenting cell
BSA: bovine serum albumin
CIK: Cytokine-induced killer cell
DC: Dendritic cell
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
GM-CSF: Granulocyte-macrophage colony-stimulating factor
hF: human fibroblasts
iDC: immature DC

IFN-γ: Interferon-γ
IL-2: Interleukin-2
IL-4: Interleukin-4
MHC: Major histocompatibility complex
MNC: Mononuclear cell
NK cell: Natural killer cell
PE: phycoerythrin
PER-CP: peridinin-chlorophyll
TCR: T-cell receptor
TNF-α: Tumor necrosis factor-α
VNBRCA: Breast cancer cell

COMPETING INTERESTS

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

BTV and PVP designed the study. BTV, NATT, TTN and PML carried out study on differentiation of mononuclear cells and acquired results. BTV, QNTD and HTL evaluated of effector cells after co-culture. BTV and PVP analyzed data. BTV and PVP wrote the paper. PVP edited all the figures. All authors read and approved the final manuscript.

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