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TITLE:

Isolation and Expansion of Cytotoxic Cytokine-induced Killer T Cells for Cancer Treatment

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SUMMARY:

Here, we present a protocol to perform the isolation and expansion of peripheral blood mononuclear cells-derived cytokine-induced $CD3^+CD56^+$ killer cells and illustrate their cytotoxicity effect against hematological and solid cancer cells by using an in vitro diagnosis flow cytometry system.

ABSTRACT:

Adoptive cellular immunotherapy focuses on restoring cancer recognition via the immune system and improves effective tumor cell killing. Cytokine-induced killer (CIK) T cell therapy has been reported to exert significant cytotoxic effects against cancer cells and to reduce the adverse effects of surgery, radiation, and chemotherapy in cancer treatments. CIK can be derived from peripheral blood mononuclear cells (PBMCs), bone marrow, and umbilical cord blood. CIK cells are a heterogeneous subpopulation of T cells with $CD3^+CD56^+$ and natural killer (NK) phenotypic characteristics that include major histocompatibility complex (MHC)-unrestricted antitumor activity. This study describes a qualified, clinically applicable, flow cytometry-based method for the quantification of the cytolytic capability of PBMC-derived CIK cells against hematological and solid cancer cells. In the cytolytic assay, CIK cells are co-incubated at different ratios with prestained target tumor cells. After the incubation period, the number of target cells are determined by a nucleic acid-binding stain to detect dead cells. This method is applicable to both research and diagnostic applications. CIK cells possess potent cytotoxicity that could be explored as an alternative strategy for cancer treatment upon its preclinical evaluation by a cytometer setup and tracking (CS & T)-based flow cytometry system.

INTRODUCTION:

Cytotoxic T lymphocytes are a specific immune effector cell population that mediates immune responses against cancer. Several effector cell populations including lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TILs), natural killer (NK) cells, γδ T cells, and cytokine-induced killer (CIK) cells have been developed for adoptive T cell therapy (ACT) purposes[1]. There is a growing interest in CIK cells, because they represent a mixture of cytokine-induced cytotoxic cell populations expanded from autologous peripheral blood mononuclear cells (PMBCs)[2].

The uncontrolled growth of lymphoid progenitor cells, myeloblasts, and lymphoblasts leads to three main types of blood cancers (i.e., leukemia, lymphoma, and myeloma), solid tumors, including carcinomas (e.g., lung cancer, gastric cancer, cervical cancer), and sarcomas, among other cancers[3]. CIK cells are a mixture of cell populations that exhibit a wide range of major histocompatibility complex (MHC)-unrestricted antitumor activity and thus hold promise for the treatment of hematological and advanced tumors[4–7]. CIK cells comprise a combination of

cells, including T cells ($CD3^+CD56^-$), NK-T cells ($CD3^+CD56^+$), and NK cells ($CD3^-CD56^+$). Optimization of the CIK induction protocol by use of a fixed schedule for the addition of IFN-γ, anti-CD3 antibody, and IL-2, results in the expansion of CIK cells[8]. The cytotoxic capability of CIK cells against cancer cells mainly depends on the engagement of NK group 2 member D (a member of the C-type lectin-like receptor family) NKG2D ligands on tumor cells, and on perforin-mediated pathways[9]. The results of a preclinical study revealed that IL-15-stimulated CIK cells induced potent cytotoxicity against primary and acute myeloid leukemia cell lines in vitro and exhibited a lower alloreactivity against normal PBMCs and fibroblasts[9]. Recently, the outcome of one-time healthy donor-derived CIK (1×10^8/kg $CD3^+$ cells) infusion as consolidation following nonmyeloablative allogeneic transplantation for myeloid neoplasms treatment in a phase II clinical study was published[10].

In the present study, we developed an optimized cell culture formula composed of IFN-γ, IL-1α, anti-CD3 antibody, and IL-2 added to the hematopoietic cell medium to increase CIK production, and investigated the cytotoxic effect of CIK cells against human chronic myeloid leukemia (K562) cells and ovarian cancer (OC-3) cells.

PROTOCOL:

The clinical protocol was performed and approved in accordance with the guidelines of the Institutional Review Board of the China Medical University and Hospital Research Ethics Committee. Peripheral blood specimens were harvested from healthy volunteers with their informed consent.

1. Preparation of materials

1.1. Store reagents, antibodies, and chemicals as shown in the Material Safety Data Sheet (MSDS). Dissolve the drugs or cytokines in solvents as stock solutions and then aliquot for storage at -20 °C or -80 °C.

NOTE: Detailed information for material preparation is noted in the **Table of Materials**.

2. PBMC isolation

2.1. Warm the density gradient solution (**Table of Materials**) to 18–20 °C before use. Invert the solution bottle several times to ensure thorough mixing.

2.2. Collect 3−5 mL of human venous blood sample in a heparinized vial and mix well by

gently inverting the tube several times.

2.3. Prepare 4 mL of density gradient solution in a 15 mL sterile tube.

2.4. Carefully layer 1 mL of the blood sample onto the density gradient solution.

2.5. Centrifuge at 400 x *g* for 30 min at 18–20 °C (turn off the break).

2.6. Carefully and immediately aspirate the buffy coat layer of mononuclear cells (about 1 mL) at once to avoid disturbing the layers to a sterile 15 mL tube using a 1 mL sterile pipette.

2.7. Add at least 3 volumes (~3 mL) of phosphate-buffered saline (PBS) to the buffy coat in the centrifuge tube. Suspend the cells by gently pipetting them up and down at least 3x with a sterile pipette.

2.8. Centrifuge at 400 x *g* for 10 min at 18–20 °C. Aspirate the supernatant.

2.9. Suspend the cell pellet with 5 mL of basal medium (**Table of Materials**) and transfer into a flask. Culture the cells in a cell culture incubator at 37 °C and 5% CO_2.

3. CIK induction and expansion

3.1. On Day 0, culture the PBMCs (1 x 10^6) in fresh basal medium containing 1,000 IU/mL of IFN-γ for 24 h in a humidified cell culture incubator at 37 °C and 5% CO_2.

3.2. On Day 1, refresh the medium with fresh basal medium containing 50 ng/mL of anti-CD3 antibody, 1 ng/mL of rh IL-1α, and 1,000 U/mL of rh IL-2. Refresh the medium every 3 days.

3.3. On Day 7, refresh the medium with fresh basal medium containing 1,000 U/mL of rh IL-2. Refresh the medium every 3 days until the end of cell expansion (Day 14).

4. Immunophenotyping for assessment of CIK cells

4.1. Wash the CIK cells with 10 mL of sterile PBS. Centrifuge for 10 min at 300 x *g* and 18–20 °C, aspirate the supernatant, and resuspend the cells with 10 mL of PBS. Count the cell number and test cell viability using the trypan blue exclusion assay.

4.2. Aliquot the CIK cells into six sterile 1.5 mL tubes at a density of ~5–10 x 10^5 cells/mL PBS.

Label and treat as follows: Tube 1, Blank (no antibody); Tube 2, add 20 µL of isotype IgG1-FITC; Tube 3, add 20 µL of isotype IgG1-APC mAbs; Tube 4, add 20 µL of CD3-FITC; Tube 5, add 20 µL of CD56-APC mAbs; and Tube 6, add 20 µL of CD3-FITC and 20 µL of CD56-APC mAbs.

4.3. Gently mix the CIK cells with the antibodies by gently pipetting them up and down at least 3x with a 1 mL sterile pipette, and then incubate for 30 min at room temperature in the dark.

4.4. Centrifuge the tubes for 10 min at 300 x *g* and 18–20 °C. Aspirate the supernatant and suspend the cell pellet once with 1 mL of PBS. Gently pipet them up and down at least 3x with a 1 mL sterile pipette.

4.5. Repeat step 4.4.

4.6. Leave the tubes in the dark before flow cytometric analysis.

5. CD marker recognition

5.1. Transfer the cell suspension to a sterile 5 mL polystyrene round bottom tube with a cell strainer cap (100 µm mesh) by gently pipetting through the cap. Put the tubes on the carousel in order.

5.2. Open the flow cytometry analysis software and create an experimental folder. Click the **New Specimen** button to add a specimen and tube to the experiment and name the tubes as follows: Tube 1, Blank; Tube 2, Isotype IgG1-CD3; Tube 3, Isotype IgG1-CD56; Tube 4, CD3; Tube 5, CD56; Tube 6, CD3CD56.

5.3. Create a scatter gating system for the CIK cell populations (**Figure 2A**).

5.3.1. Select Tube 1 (Blank) and click on the **Dot Plot** button to create an FSC-A/SSC-A plot. Draw a rectangle gate over the entire cell population with an FSC-A threshold >5 x 10^4 to exclude cell debris.

5.3.2. Select the **SSC-A/SSC-H** parameter for the new dot plot and draw a polygon gate around all single cells. Select the **Count/FITC (CD3)** and **Count/APC (CD56)** parameter for the new histogram plot, respectively. Select the **FITC (CD3)/APC (CD56)** parameter for the new dot plot and draw a four quadrant gate to define the four subpopulations.

5.3.3. Record the data from 20,000 single cells in each specimen. Click the **Load Sample** button to analyze the Blank control sample first. Identify the whole CIK cell population by using the CD56 and CD3 channel parameters.

5.4. Repeat step 5.3 for the investigation of all specimens.

5.5. Open the files containing the statistical values of the individual specimen to analyze CIK cell populations and reprint them into analysis files.

6. Culturing and staining of human chronic myeloid leukemia K562 cells and ovarian cancer OC-3 cells

6.1. K562 cells

6.1.1. Culture K562 cells in complete media (RPMI basal medium containing 10% fetal bovine serum [FBS] and 50 U/mL antibiotics and adjust glucose to 4.5 g/L) at a density of 0.5−1 x 10^6 cells/mL in a cell culture flask and incubate in a humidified incubator at 37 °C and 5% CO_2.

6.1.2. Transfer the culture media containing the K562 cells into 50 mL sterile tubes and pellet the cells at 300 x *g* for 10 min at 18−20 °C on the day of the experiment.

6.1.3. Aspirate the supernatant, resuspend the cells in 5 mL of sterile PBS, and mix well gently.

6.1.4. Pellet the cells at 300 x *g* for 10 min. Aspirate the supernatant, resuspend the cells in PBS, and adjust the K562 cells to a concentration of 0.5-1 x 10^6 cells/mL.

6.1.5. Add 0.5 µL of CFSE dye to the 1 mL of K562 cell suspension in a 15 mL sterile tube at a final concentration of 5 µM. Gently mix the suspension by pipetting up and down at least 3x.

6.1.6. Leave the tube in a cell culture incubator at 37 °C and 5% CO_2 for 10–15 min.

6.1.7. Add 9 mL of PBS to the tube and pellet the cells at 300 x *g* for 10 min. Decant the supernatant and then suspend the cell pellet in 10 mL of complete media. Transfer the cell suspension to a cell culture flask and place in the incubator.

6.2. OC-3 cells

6.2.1. Culture OC-3 cells in complete media (DMEM/F12 medium containing 10% FBS and 50 U/mL antibiotics) at a density of 0.5–1 x 10^6 cells in a cell culture flask at 37 °C and 5% CO_2.

6.2.2. Aspirate the culture media and wash the cells with PBS 1 day before the experiment.

6.2.3. Detach the cells by adding 1 mL of cell dissociation enzyme solution (**Table of Materials**) and incubate for 5 min at 37 °C.

6.2.4. Suspend the cells by adding 5 mL of PBS and mix well gently. Pellet the cells at 300 x *g* for 10 min and aspirate the supernatant. Resuspend the cells in PBS and adjust the cells to a concentration of 0.5–1 x 10^6 cells/mL.

6.2.5. Add 0.5 µL of CFSE dye to 1 mL of the OC-3 cell suspension in a 15 mL sterile tube at a final concentration of 5 µM. Gently mix the suspension by pipetting up and down at least 3x.

6.2.6. Leave the tube in a cell culture incubator at 37 °C and 5% CO_2 for 10–15 min.

6.2.7. Add 9 mL of PBS to the tube and pellet the cells at 300 x *g* for 10 min. Decant the supernatant and then suspend the cell pellet with complete media. Seed 5 x 10^5 cells/well into a 6 well plate and incubate in a humidified incubator at 37 °C and 5% CO_2 overnight.

7. Cytotoxic assay

7.1. Coculture of CIK and K562 cells (CIK-K562)

7.1.1. Count the K562 cells from step 6.1.7 and test the cell viability by trypan blue exclusion assay. Add 1 mL of K562 cells to each well in a 6 well plate at a density of 5 x 10^5/mL.

7.1.2. Add 1 mL of basal medium with or without CIK cells from step 3.4 to the 6 well plate from step 7.1.1 as follows: Well 1 = Blank, K562 cells alone (5 x 10^5); Well 2 = CFSE-stained K562 cells alone (5 x 10^5); Well 3 = CIK cells (E [effector], 25 x 10^5) + CFSE-stained K562 cells (T [target], 5 x 10^5); Well 4 = CIK cells (E, 50 x 10^5) + CFSE-stained K562 cells (T, 5 x 10^5).

7.1.3. Mix the cell suspensions by gently pipetting them up and down at least 3x. Place the plate in the incubator for 24 h.

7.2. Coculture of CIK and OC-3 cells (CIK-OC-3)

7.2.1. Add 1 mL of basal medium with or without CIK cells from step 3.4 to the 6 well plate from step 6.2.7 as follows: Well 1 = Blank, OC-3 cells alone (5 x 10^5); Well 2 = CFSE-stained OC-3 cells alone (5 x 10^5); Well 3 = CIK cells (E, 25 x 10^5) + CFSE-stained OC-3 cells (T, 5 x 10^5); Well 4 = CIK cells (E, 50 x 10^5) + CFSE-stained OC-3 cells (T, 5 x 10^5).

7.2.2. Mix the cell suspensions by gently pipetting them up and down at least 3x. Put the plate in the incubator for 24 h.

7.3. 7-Aminoactinomycin D (7-AAD) dye staining

7.3.1. Harvest the CIK-K562 cell suspension from step 7.1.3 directly into a 15 mL sterile tube.

7.3.2. Harvest both the suspension and adherent cells from the CIK-OC-3 groups from step 7.2.2.

7.3.2.1. Transfer the cell suspension to a 15 mL sterile tube. Wash the well with 1 mL of sterile PBS, collect the PBS, and add to the tube. Add 0.5 mL of cell dissociation enzyme solution, and incubate for 5 min at 37 °C.

7.3.2.2. Add 1 mL of the solution from the same tube to the corresponding well and gently mix the cells by pipetting them up and down at least 3x with a 1 mL sterile pipette. Collect all the cells in the same tube.

7.3.3. Centrifuge at 300 x *g* for 10 min, aspirate the supernatant, and resuspend the cells in 1 mL of sterile PBS. Pellet the cells at 300 x *g* for 10 min, aspirate the supernatant, and resuspend cells in 100 µL of sterile PBS.

7.3.4. Add 5 µL of 7-AAD dye (50 ng/µL stock) to the cell suspension. Gently mix the cells by pipetting them up and down at least 3x with a 1 mL sterile pipette. Incubate for 10 min and leave in the dark before analysis.

7.4. Cytolytic capability assay

7.4.1. Mix the cell suspension from step 7.3.4 and repeat steps 5.1 and 5.2 once.

7.4.2. Click the **New Specimen** button to add a specimen and tube to the experiment and

name the tubes as follows: Tube 1, K562 (or OC-3) cells only; Tube 2, CFSE-stained K562 (or OC-3) cells only; Tube 3, E:T = 5:1; Tube 4, E:T = 10:1.

7.4.3. Create a Scatter Gating System for the cytolytic assay (**Figure 3A**).

7.4.3.1. Select Tube 1 and click on the **Dot Plot** button to create an FSC-A/SSC-A plot. Draw a rectangle gate over all events with an FSC-A threshold >5 x 10^4 to exclude cell debris.

7.4.3.2. Select the **SSC-A/CFSE** parameter for the new dot plot. Select the **7-AAD/CFSE** parameter for the new dot plot and draw a four-quadrant gate to define the four subpopulations.

7.4.3.3. Click the **Load Sample** button to analyze the blank control sample first.

7.4.3.4. Adjust the voltage of SSC-A and FSC-A. Identify the dead cell population by using the CFSE and 7-AAD channel parameters. Record the data from >20,000 $CFSE^+$ cells in each specimen.

7.4.4. Repeat section 7.4.6 for the investigation of all specimens.

7.4.5. Open the files containing the statistical values of each individual specimen to analyze the non-viable cell populations and export the data into analysis files.

REPRESENTATIVE RESULTS:

The purpose of the present protocol is to isolate and expand cytokine-induced killer (CIK) T cells from peripheral blood monocytes and evaluate the cytotoxic effect of CIK against hematological malignancy and solid cancer cells, respectively. The induction of CIK was identified by the CD3/CD56 recognition. **Figure 1A** shows the protocol for CIK induction and expansion. The representative results of the gating strategy for analyzing the subpopulation of $CD3^+CD56^+$ T cells from healthy donors is illustrated in **Figure 1B**. **Figure 1C** shows the statistical analysis of the CIK proportion from three individuals.

Figure 2A shows that the $CD3^+CD56^+$ cell proportion (0.65% for the original PBMC, left lower panel and 27.4% for the CIK cells harvested on Day 14^{th}, right lower panel) significantly increased after 14 days of expansion. In our culture system, the CIK cells yielded about half a hundred-fold changes compared to the original number of PBMCs (**Figure 2B**).

Figure 3 shows the cytotoxic effect of CIK against human chronic myeloid leukemia K562

cells and human ovarian cancer OC-3 cells. K562 or OC-3 cells (target, T) were stained with a non-fluorescent dye (CFSE), which was cleaved by intracellular esterases within viable cells and then became a highly fluorescent dye. In the cytotoxic coculture study, CFSE-stained K562 or OC-3 cells were cotreated with CIK cells for 24 h. At the end of incubation, the total cells were harvested and stained with 7-AAD dye, which is a nucleic acid-binding dye that is used as a viability probe for dead cell exclusion. The size and granularity of the CIK and $CFSE^+$ cells are illustrated in **Figure 3A**. The CFSE-stained K562 cells (target cells, T) were co-treated with CIK cells (effector cells, E) at a ratio of E/T = 0:1, 5:1, and 10:1, respectively. The $7\text{-}AAD^+$ cells of $CFSE^+$ K562 cells were all evaluated. The statistical results were from three independent experiments. Basal lysis means the percentage of cell death in the absence of effector cells (E:T = 0:1). **Figure 3B** shows the obvious cytotoxicity of CIK against OC-3 cells (E:T = 10:1) following 24 h of incubation.

FIGURE LEGENDS:

Figure 1: Flow chart of cytokine-induced killer cells induction and expansion. (**A**) PBMCs from consented healthy donors were initially exposed to rhIFN-γ (Day 0), followed by rhIL-2, rhIL-1α, and anti-CD3 mAb (Day 1) every 3 days (Day 4). Subsequently, the medium was refreshed with rhIL-2-containing medium every 3 days and the cells were harvested on Day 14. (**B**) Morphology of CIK cells during 7 days of induction. The activation and expansion of CIK cells were conducted as described in the protocol. Cells were observed under a light microscope on Days 1, 5, and 7, respectively (magnification = 40x, scale bar = 200 μm). (**C**) Cell counts were performed weekly.

Figure 2: The proportion of $CD3^+CD56^+$ T cells from a representative PBMC sample. (**A**) Lymphocytes were recognized by specific size and granularity. Selected single cell population for analysis by flow cytometry. (**B**) Statistical analysis of CIK expansion efficacy from three healthy donors was conducted using a t-test (*, $p < 0.01$).

Figure 3. Cytotoxic effects of CIK cells against human chronic myeloid leukemia K562 and human ovarian cancer OC-3 cells. (**A**) Following coculture with the CIK cells for 24 h, K562 target cells were recognized and gated based on the staining of CFSE dye. Quadrant illustration of the total cell population under the selected 7-AAD/CFSE parameter and the cumulative cytotoxicity of CIK cells at the indicated E:T ratio. (**B**) The cytotoxic effect of CIK cells against OC-3 cells at a E:T = 10:1 ratio.

DISCUSSION:

The described method is a fast, convenient, and reliable protocol for the isolation and expansion of cytotoxic cytokine-induced killer (CIK) T cells from whole blood samples of

healthy donors. It also shows the cytotoxic effect of CIK against leukemia (K562) and ovarian cancer cells (OC-3) using a flow cytometry setup and tracking (CS & T) system. CIK cells can be induced and expanded in good manufactory practices (GMP) conditions by using GMP-grade cytokines and serum-free medium for further clinical infusion[11]. However, the efficacy of CIK induction and expansion exhibits individual differences[12–14]. Moreover, safety is the advantage of the infusion of patient-derived CIK cells for cancer cell therapy. It has been reported that CIK cells exert cytolytic effects on epithelial solid cancer cells mostly in a NKG2D-dependent manner. In hematological cancer cells, blocking NKG2D with a specific antibody significantly inhibits CIK-induced cytotoxicity against NKG2D-low K562 cells; however, this treatment does not have any effect on HL-60 cells lacking NKG2D[15]. Furthermore, CIK cells exhibit less cell-killing activity against K562 cells as compared to $CD8^+$ CIK cells[16]. In this study, we found that CIK exhibited a greater cytotoxic potential against ovarian cancer OC-3 cells compared to leukemia K562 cells. These data suggest that the exact molecular mechanisms through which CIK effectors kill tumor cells are not yet clear.

Tracking target cell viability and evaluating the cytotoxic potential of effector cells using flow cytometry has become a standard and conventional method for clinical examination[17]. It is has been suggested that a negative effect is observed on the cell viability and the expression of activation markers, such as the $CD3^+$ population in CFSE-stained lymphocytes with flow cytometry[18,19]. Thus, staining the target cancer cells is a more effective strategy for evaluating the cytotoxic effects of primary CIK cells. IFN-γ, OKT3, and IL-2 are major cytokines or stimulators for CIK differentiation and proliferation. Furthermore, other factors such as thymoglobulin, IL-1α, IL-10, IL-15, are also stimulators. Currently, human serum, human platelet-rich plasma, and even fetal bovine serum are used as medium supplements that can enhance the proliferation of CIK cells. Although serum or plasma are enriched with nutrients and growth factors, the addition of allogeneic animal products presents source, batch, and lot variations that result in experimental variability, and inevitably disconcert studies with therapeutic outcomes for cultured cells. In this study, we used a commercially available serum-free, albumin-free, and xeno-free GMP-grade media supplemented with clinical-grade cytokines to successfully culture the CIK cells. The disadvantage of using xeno-free or allogeneic-free supplements is that they reduce the efficacy of cell proliferation.

The two-color cell tracking methods provided in the protocols independently calculated viable or dead effector target cells in a direct cytotoxicity assay. In our gating strategies, $CFSE^+$ target cells can be obviously distinguished from CIK effector cells (**Figure 3**). Most importantly, the process of CIK induction and expansion must be qualified and show high viability. For further multiple-dosage infusions, the condition of cryopreservation, and the viability and the cytotoxicity after thawing, are other critical challenges. The actual ratio of

specific lysis equals the proportion of 100 x (%Sample lysis - %Basal lysis)/(100 - %Basal lysis). In contrast to other studies[18,20], it is recommended that all target cells be investigated to reveal the exact and actual cytotoxicity of CIK cells.

In conclusion, the protocol described in this study is designed to increase the number of PBMC-derived CIK cells from healthy donors and to evaluate their cytotoxic functions against cancer cells with two-color photoactivatable probes for selective tracking of target cells by a flow cytometry with an in vitro diagnostic (IVD) system.

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DISCLOSURES:

The authors declare no competing conflicts of financial interest.

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Figure 1-revised

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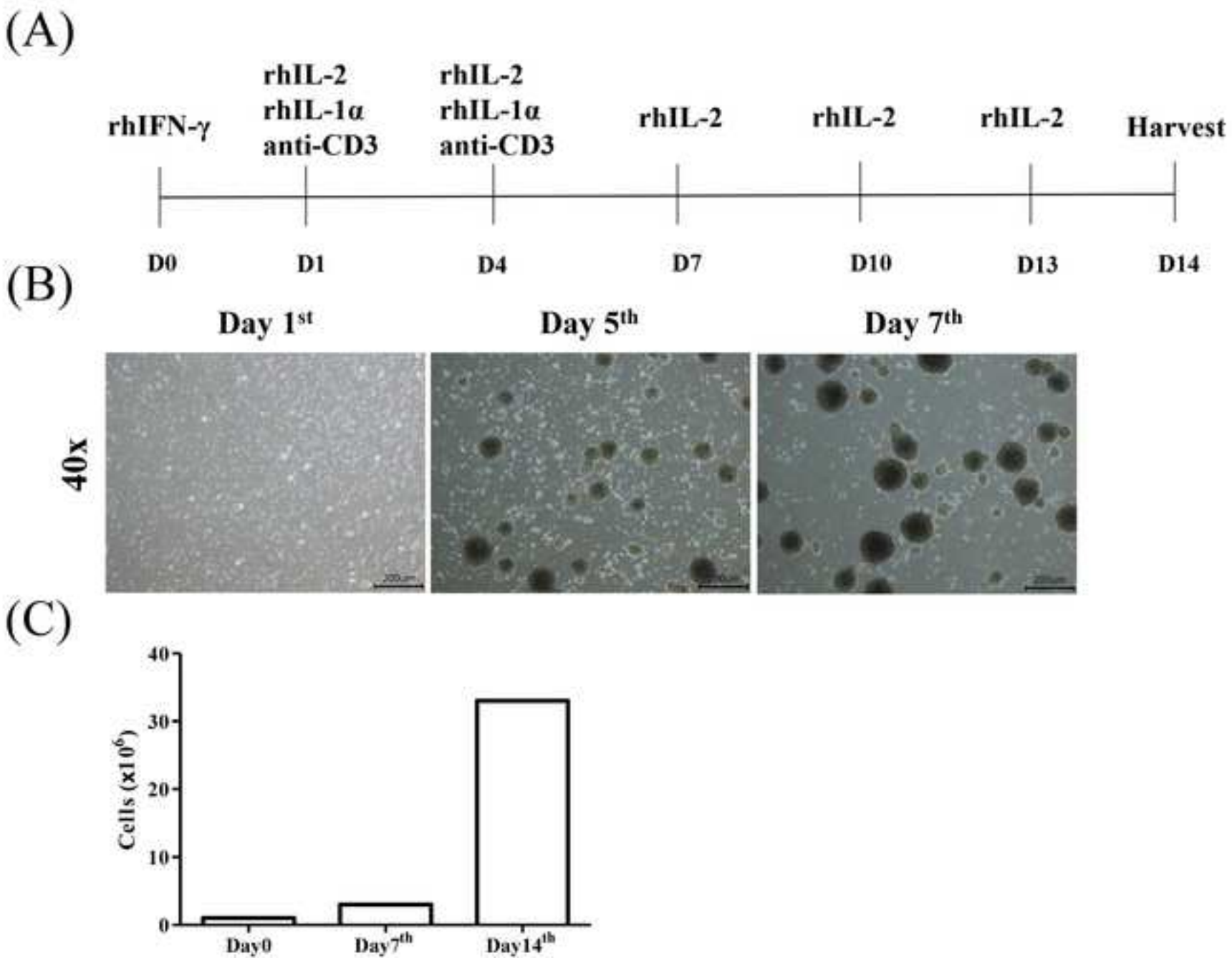
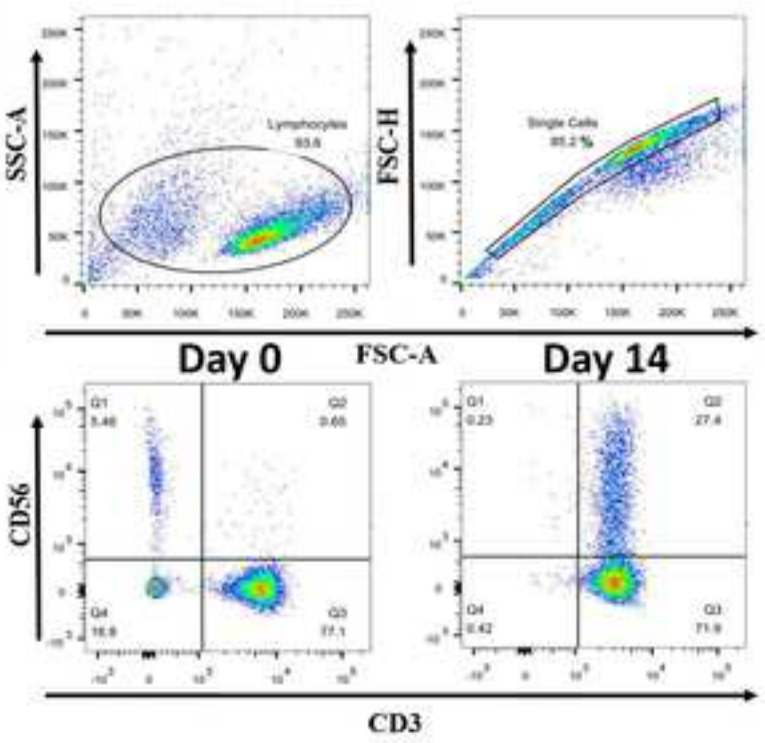


Figure 2-revised

Click here to access/download;Figure;Fig 2-R1.jpg

(A)



(B)

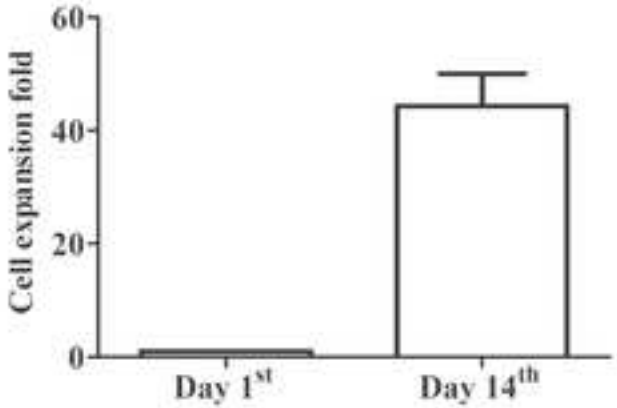
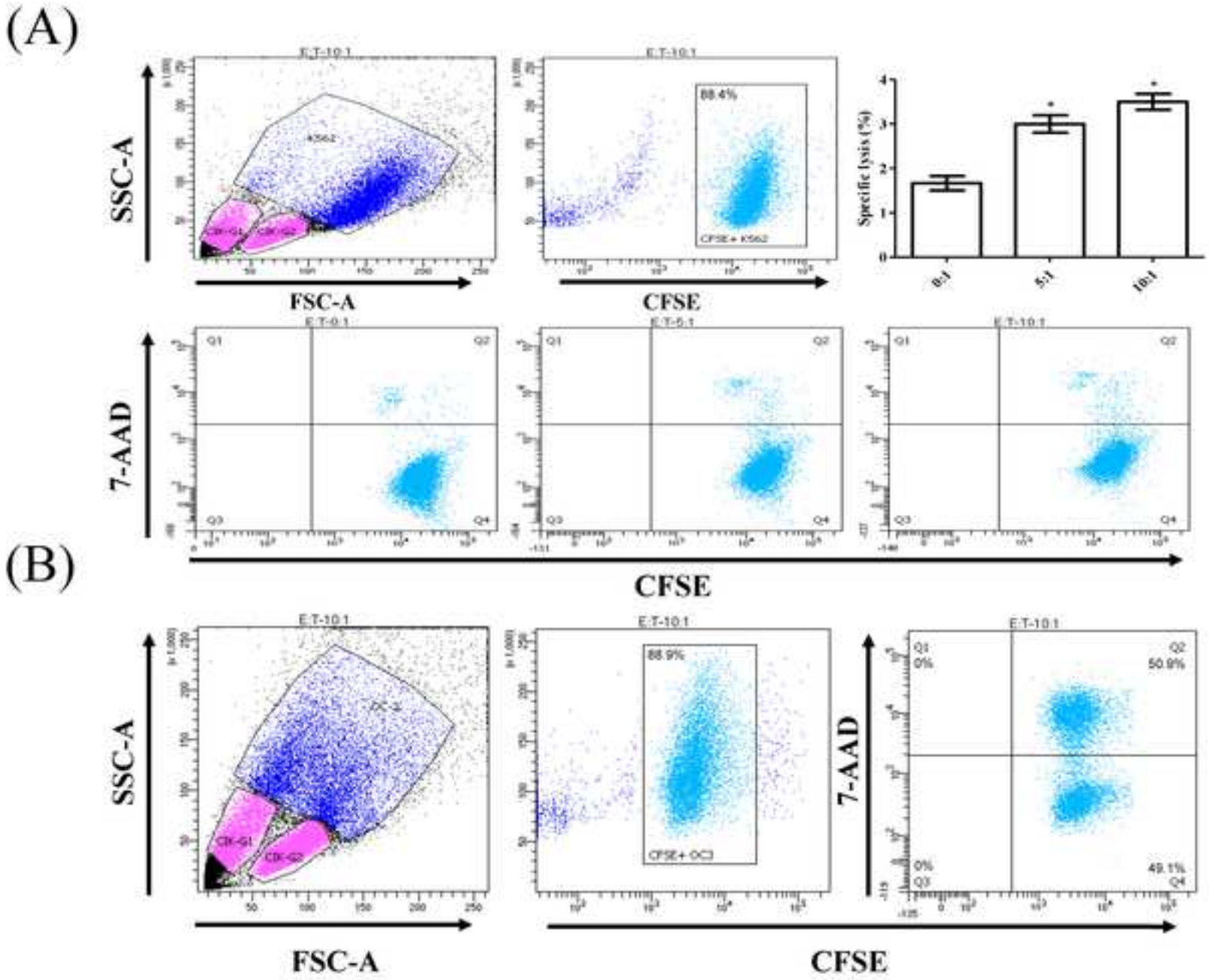


Figure 3-revised

Click here to access/download;Figure;Fig 3-R1.jpg



Name of Material/Equipment	Company
7-Amino Actinomycin D	BD
APC Mouse Anti-Human CD56 antibody	BD
APC Mouse IgG1, κ Isotype Control	BD
BD FACSCanto II Flow Cytometer	BD
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	BD
D-(+)-Glucose solution	SIGMA
Dulbecco's Modified Eagle Medium/F12	HyClone
Fetal bovine serum	HyClone
Ficoll-Paque Plus	GE Healthcare Life Sciences
FITC Mouse Anti-Human CD3 antibody	BD
FITC Mouse IgG1, κ Isotype Control	BD
Human anti-CD3 mAb	TaKaRa
Penicillin-Streptomycin	Gibco
Proleukin	NOVARTIS
Recombinant Human Interferon-gamma	CellGenix
Recombinant Human Interleukin-1 alpha	PEPROTECH
RPMI1640 medium	Gibco
Sigma 3-18K Centrifuge	Sigma
TrypLE Express Enzyme	Gibco
X-VIVO 15 medium	Lonza

Catalog Number	Clone name
559925	
555518	B159
555751	MOPC-21
338962	
565082	
G8644	
SH30023.02	
SH30084.03	
71101700-EK	
555332	UCHT1
555748	MOPC-21
T210	OKT3
15140122	
1425-050	
200-01A	
11875-085	
10295	
12605028	
04-418Q	

Comments/Description

SN: R33896202856
Reconstritution of CFSE dye (500 mg) with 90 mL of DMSO
For K562 cell culture. Add 12.5 mL to 500 mL of complete medium
Basal medium for OC-3 cell culture
For K562 and OC-3 cell culture. Complete medium contains 10% of FBS
Density gradient solution

Add 2.5 mL of stock (1 mg/1 mL) to 50 mL of Induction medium. Storage stock at -80 °C
Add 5 mL of stock (10,000 U/mL) to 500 mL of complete medium. Storage stock at 4 °C.
Reconstitution of Proleukin Powder ($22x10^6$ IU) with 1.2 mL of sterile water and add 2.7 mL to 50 mL
Reconstitution of rh IFN-g ($5x10^5$ IU/50 µg) with 200 µL of sterile water and add 20 mL to 50 mL of Inc
Reconstitution rh IL-1α (10 µg) with 1 mL of sterile water and add 5 mL to 50 mL of Induction mediur
Basal medium for K562 cell culture. Storage stock at 4 °C

Cell dissociation enzyme; For deattachment of adheren cells. Storage at room temperature
Basal medium for PBMC and CIK cells. Storage at 4 °C

. of Induction medium. Storage stock at -20 °C

luction medium. Storage stock at -20 °C

n. Storage stock at -20 °C

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Ans: Thank you for your suggestion.

2. Please revise lines 90-92, 128-130, 231-233 to avoid textual overlap with previously published work.
Ans: These sentences had been revised.

3. Authors and affiliations: Please provide an email address for each author.
Ans: The email for each author had been added.

4. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: “Here, we present a protocol to …”
Ans: The section Summary had been added.

5. Abstract: Please revise to focus on the method being presented rather than the results of a specific experiment. Include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.
Ans: The abstract was revised.

6. Keywords: Please provide at least 6 keywords or phrases.
Ans: The Keywords had been revised.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.
Ans: The Protocol had been revised.

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before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Ficoll-Paque™, GE Healthcare Life Sciences, Gibco™, BD Horizon™, Thermo Fisher Scientific, CellGenix, Peperotech, etc.
Ans: It had been revised.

9. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.”
Ans: It had been revise.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.
Ans: It had been revised.

11. Line 185: Please specify temperature.
Ans: It had been stated.

12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Ans: It had been revised.

13. Please include single line spacing between each numbered step or note in the protocol.
Ans: It had been revised.

14. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be

filmed. Our scriptwriters will derive the video script directly from the highlighted text.
Ans: It had been highlighted.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
Ans: It had been highlighted.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Ans: It had been highlighted.

17. Discussion: As we are a methods-based journal, please discuss critical steps in the protocol, modifications and troubleshooting of the method, and limitations of the method.
Ans: It had been revised.

18. Figure 1: Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.
Ans: Fig 1 was revised.

19. Figure 2: Please define error bars in the figure legend.
Ans: It had been revised.

20. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.
Ans: Table was been revised.

21. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.
Ans: It had been revised.

22. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
Ans: It had been added.

23. References: Please do not abbreviate journal titles; use full journal name.
Ans: It had been revised.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This is an interesting manuscript on the generation of CIK cells using a modified protocol.

Major comments:

1. The authors should state in the Abstract why they modified the CIK cell protocol and which were the major modifications.
Ans: We thank reviewer's comment. The aim of this study is to provide a standard operation procedure for technicians or clinicians who will participate cell therapy for cancer treatment. By using commercial GMP-grade and xeno-free reagents, we can successfully isolation and expansion the cytotoxic CIK cells.

2. The original report of Schmidt-Wolf et al., J Exp Med 1991 should be referenced.
Ans: We thank reviewer's suggestion. The reference had been cited.

3. The IRCC report of Schmeel et al. should be added, J Cancer Res Clin Oncol. 2015 May;141(5):839-49.
Ans: We thank reviewer's suggestion. The reference had been cited.

Reviewer #2:

Manuscript Summary:

In this study, the authors revealed the fundamental skills of isolation and expansion of cytokine-induced killer (CIK) cells and showed the cytotoxic effect of CIK against human chronic myeloid leukemia cells. However, there are two major issues should be disclosed:

Major Concerns:

1. In addition to the liquid tumor, could the expanded CIK cells target solid cancer cells?
Ans: Thank reviewer's suggestion. We also found that cultured CIK cells showed cytotoxic effect against OC-3 ovarian cancer cells (as shown in Fig. 3C), and other researchers has also suggested that CIK cells act as a feasible adoptive immunotherapy for the treatment of lung cancer (Ref. 13).

2. As the authors mentioned that there're about 30% to 60% of CD3+CD56+ cells identified as CIK cells. Did the remaining cells show the cytotoxic capability on cancer cells? Should we exclude the non-CD3+CD56+ cells in such kind of immunotherapy to reduce side effects?
Ans: We thank reviewer's comment. Since CIK cells are a combination of cells including T cells (CD3+CD56−), NK-T cells (CD3+CD56+), and NK cells (CD3-CD56+) that all possess cytotoxic potential against cancer cells. Thus, in our opinion we should infusion all of the induced-CIK cells in a well concerned manner for patient treatment.

Reviewer #3:

Manuscript Summary:
The current article described an improved method for the isolation and expansion of cytokine-induced killer T cells (CIK).The article will interest readers from cell-based immunotherapy. However, there are certain parts of the article that needs to be clarified to ensure its scientific accuracy and consistency. Below are line by line corrections that the authors need to address before the current manuscript can be considered for publication.

Major Concerns:

1. In line 193, the expansion period for CIK cells is 21 days, but in line 346 and

Fig. 1 legend (line 450) it is 14 days. The exact time for CIK expansion should be confirmed.
Ans: We thank reviewer's comment. It was a typo since the total duration of CIK expansion is 14 days as shown in Fig.1A.

2. The CIK expansion protocol used in this paper is similar with conventional methods[ref 1,2]. The author should make a comparison with conventional ones to show the differences and advantages of current process (e.g. the effect of any single cytokines/reagents/unique steps...), at least in the discussion section.
Ans: We thank reviewer's comment. To our knowledge, IFN-γ, OKT3 and IL-2 are major cytokines or stimulators for CIK differentiation and proliferation. Besides, other factors such as thymoglobulin, IL-1a, IL-10, IL-15, etc. Furthermore, human serum, human platelet-rich plasma, even fetal bovine serum are used as medium supplements that could enhance the proliferation of CIK cells. Here we used a commercially available serum-free, albumin-free, and xeno free GMP-grade media supplemented with clinical-grade cytokines to successfully culture the CIK cells. Although serum or plasma are enriched with nutrients and growth factors, the addition of allogeneic animal products presents source, batch and lot variations that result in experimental variability, and inevitably disconcert studies with therapeutic outcomes for cultured cells. These sentences had been added in the Discussion section in line 1184-1195.

3. One critical concern is that: in Fig. 3A, the gating strategy may not be appropriate. e.g. the blue colored population on the left also included dead CIK cells rather than the dead target cells only. The authors might need to re-analyze this result.
Ans: We thank reviewer's comment. Fig. 3 was revised.

4. More papers on the method of CIK expansion should be cited in the introduction section.
Ans: We thank reviewer's comment. Some references were cited in the Introduction section.

5. The authors should provide high-quality pictures for the figures instead of current ones.
Ans: We thank reviewer's comment. High-quality pictures were provided.

Minor Concerns:

Line 47: this should be: polyclonal CD3+CD56+ T cells
Ans: We thank reviewer’s comment. It had been revised.

Line 50: correct to "following a modified"
Ans: We thank reviewer’s comment. It had been revised.

Lines 51-52: this should be "Obviously, cytokines-induction for CIK cell expansion for two weeks showed an increased number of CD3+CD56+ cells".
Ans: We thank reviewer’s comment. It had been revised.
.
Line 53: change CIK harbored NK-like phenomenon to "CIK harbored NK cell-like phenomenon "
Ans: We thank reviewer’s comment. It had been revised.

Lines 56-57: Conclusion should be rephrased: "These data suggest that CIK should possess potent cytotoxicity that could be explored as an alternative strategy for cancer treatment upon its preclinical evaluation".
Ans: We thank reviewer’s comment. It had been revised.

Line 83: remove "that"
Ans: We thank reviewer’s comment. It had been removed.

Line 86-87: this should be rephrased: "and they require quality grade cytokines and medium to obtain"
Ans: We thank reviewer’s comment. It had been revised.

Line 98-99: this also needs to be clarified. Change to: "NK Group 2 member D (a member of the C-type lectin-like receptor family) NKG2D ligands on tumor cells"
Ans: We thank reviewer’s comment. It had been revised.

Line 115: Put K562 in bracket:"leukemia (K562) cells"
Ans: We thank reviewer’s comment. It had been revised.

Line 164: interphase between plasma. This is an incomplete description of interphase. It should read as thus

"interphase between plasma and Ficoll-Paque layer (contains mononuclear cells with other slowly sedimenting particles (e.g. platelets) with low density)"
Ans: We thank reviewer’s comment. The protocol for PBMC isolation had been revised.

Line 172: with 5 mL of PBMC were maintained a flask containing.Authors need to clarify this part for readers and accuracy.
"do the authors mean to say "Suspend the cell pellet in 5ml PBS or suspend cell pellet (containing PBMC) in RPMI 1640 complete medium?"
Ans: We thank reviewer’s comment. It had been revised.

Line 199: Aliquot the CIK cells into sex sterile.This should be checked and changed
"Aliquot the CIK cells into six sterile 1.5 mL tube at a density of"
Ans: We thank reviewer’s comment. It was a typo and had been revised.

Lines 201, 202, 203 and 204: Authors should clearly state how each of the six tubes (mentionedin line 199) containing the CIK cells were stained with the relevant antibodies e.g. Tube1 - blank (no antibody is added), Tube2- add 20 µl of isotype IgG1-APC mAbs, Tube3- add .....and soon on.
Ans: We thank reviewer’s comment. It had been revised.

Line 220: with 1 mL of sterile pipette.Do the authors mean PBS or pipette?
"1 mL of sterile PBS"
Ans: We thank reviewer’s comment. It had been revised.

Line 222:Transfer the suspension. Change this to
"Transfer the cell suspension"
Ans: We thank reviewer’s comment. It had been revised.

Lines 229, 230, 231 and 232: Authors should ensure consistency with CD marker labeling on the tubes 1-6 with regards recognition on the flow cytometry (see line 203 and 204).
Ans: We thank reviewer’s reminding. It had been confirmed.

Line 286:plate as following. Change to
"plate as follows"
Ans: We thank reviewer’s comment. It had been revised.

Line 306: 1 mL of sterile pipette and gently pipetting them up and down. I think the Authors meant to say "1ml of sterile PBS and by gently pipetting up and down"
Ans: We thank reviewer’s comment. It had been revised.

Line 308: Transfer the suspension. Change to
"Transfer the cell suspension"
Ans: We thank reviewer’s comment. It had been revised.

Lines 366, 367 and 368: There seems to be an oversight of the main aim of this experiment. Lines 366 - 368 should be rewritten to capture the main goal of the experiment

This can read like this "The described method is a fast, convenient and reliable protocol for the isolation and expansion of cytotoxic cytokine-induced killer (CIK) T cell from whole blood sample of healthy donors. It also shows the cytotoxic effect of CIK against K562 cells using flow cytometry setup and tracking (CS & T) system"
Ans: We thank reviewer’s comment. It had been revised.

Line 369, 370, 371: This part needs to be rephrased for clarity. See below

"CIK cells can be induced and expanded in good manufacturing practices (GMP) conditions by using GMP-grade cytokines and serum-free medium for further clinical infusion.
Ans: We thank reviewer’s comment. It had been revised.

Line 373: become a standard.This should be changed to
"became a standard"
Ans: We thank reviewer’s comment. It had been revised.

Line 365: The discussion part of the current manuscript needs to be expatiated by the authors. This should cover how the current method is superior to common or other existing methods.
Ans: We thank reviewer’s comment. It had been revised.

References:

1 Introna, M. et al. Rapid and massive expansion of cord blood-derived cytokine-induced killer cells: an innovative proposal for the treatment of leukemia relapse after cord blood transplantation. Bone marrow transplantation38, 621-627, doi:10.1038/sj.bmt.1705503 (2006).
2 Schmidt-Wolf, I. G., Negrin, R. S., Kiem, H. P., Blume, K. G. & Weissman, I. L. Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. The Journal of experimental medicine174, 139-149, doi:10.1084/jem.174.1.139 (1991).

Reviewer #4:

Manuscript Summary:
Authors present a new protocol to expand NKT cells for therapeutic purpose. PBMCs from volunteers were cultured in a modified CIK expansion protocol. Cytokines induced CIK cell expansion for two weeks showed an increase in the number of CD3+CD56+ cells and cytotoxic cell death of K562 targets.

Major Concerns:
poor English and at times difficult to comprehend. Lack of detail on cytokines and concentrations; functional assays for NK cell such as cytokine production is missing.
Ans: We thank reviewer’s comment. The manuscript had been re-organized. The aim of the present study is to develop a standardized protocol for clinicians and technicians to perform the CIK induction and expansion by GMP-grade culture system, and investigate the cytotoxic capability of activated-CIK cells against tumor cells by a cytometer setup and tracking (CS&T)-based flow cytometry system. The cytokine production of activated NK or CIK cells had been shown in the previous studies (Ref. 14-17).

Reviewer #5:

Manuscript Summary:
This is a manuscript to provide details for expanding CIK cells for cancer immunotherapy. The paper is of general interest but should be revised prior to

acceptance.

Major Concerns:

1) Details in the abstract should be modified to reflect the nature of CIK cells. Polyclonal CD3+CD56+ T cells is incorrect as it is stated later in the introduction that these cells are a mixture of NKT, NK, etc expressing CD3 and/or CD56 from peripheral blood. This should be corrected to be aligned with the statement in line 93-94 that CIK cells are a combination of cells including T cells, NK-T cells and NK cells.
Ans: We thank reviewer's comment. It had been revised.

2) The introduction should be expanded to include additional information such as the type of hematologic malignancies and solid tumors, differences in function from other cell types. This section is repetitive as written and contains general rather than specific information.

3) line 101 is repeat of general statement on line 92.
Ans: We thank reviewer's comment. It had been revised.

4) K562 cells express receptors other than NKG2D. Please provide data showing that anti-NKG2D blocking antibody or other control method reverses the cytotoxicity.
Ans: We thank reviewer's comment. Previously, it had been reported that CIK induced cytolytic effects on epithelial solid cancer cells mostly via NKG2D-dependent manner, while in haematological cancer cells, blocking NKG2D by specific antibody significantly inhibited CIK-induced cytotoxicity against NKG2D-low K562 cells but no any effect on NKG2D-lack HL-60 cells. Furthermore, it had been shown that CIK cells showed less cell killing activity against K562 cells as compared to CD8+ CIK cells. These data suggested that the exact molecular mechanism by which CIK effectors kill tumor cells is not clearly. These sentences had been added into the Discussion on line 1169-1178.

5) From step 7 on line 161 to step 8 on line 165, is there a step to remove the "light yellow plasma" prior to acquisition of the buffy coat layer?
Ans: We thank reviewer's comment. The procedure for **PBMC isolation** had

been revised.

6) step 12 should be clarified to include a rest period or not prior to addition of the cytokine-containing media. The cell pellet is resuspended in media and then in step 13 the media is refreshed every 3 days. Exactly where is the Day 0 X-VIVO 15 basal medium added containing IFNg? Is this immediately added, or is there a period of rest? Please insert a step where the CIK cytokines are added and then the CIK induction and expansion cytokines (section 3) 182 can provide the details of the cytokines as it is stated currently.
Ans: We thank reviewer's comment. It had been revised. Actually, the original procedure for **PBMC isolation and culture** includes Step 12 and Step 13 without any addition of cytokines. In procedure of CIK induction and expansion we refresh the medium with IFN-r-containing basal medium on Day 0.

7) please clarify labeling for figure 2A. Is the left bottom panel Day 0 and right bottom panel Day 15? Label on the figures for clarity.
Ans: We thank reviewer's comment. Fig 2 had been revised.

8) Figure 3 should be clarified. Why is the gate shifting upwards after 7-AAD staining with higher E:T ratios. What is the geometric mean of this population? Labeling should be more clear.
Ans: We thank reviewer's comment. All the Figs had been revised.

Minor Concerns:
Modify line 53 - CIK harbored NK-like phenomenon to CIK possess NK-like cytotoxic functions.
Ans: We thank reviewer's comment. It had been revised.

Consider restructure of lines 84-88 to the following: "There is a growing interest in CIK cells as they represent a mixture of cytokine-induced cytotoxic cell populations expanded from autologous peripheral blood mononuclear cells (PMBC)."
Ans: We thank reviewer's comment. It had been revised.

In line 104 - what is the meaning of presence of IL-15, slightly .....What is "slightly"?
Ans: We thank reviewer's comment. It had been revised.

Line 156 - correct to heparinized
Ans: We thank reviewer’s correction. It was a typo and had been revise.

Line 199 - correct "sex" should this be six?
Ans: We thank reviewer’s correction. It was a typo and had been revise.

Throughout, please provide catalog numbers and clone names for the antibodies and other reagents.
Ans: We thank reviewer’s comment. Please find them in the Materials File.

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Author(s): Chih-Hao Hsiao, Ya-Hui Chiu, Shao-Chih Chiu, Dee-Yang Chao, Liang-Ming Lee, Yu-Ching Wen, Jia-Tse Lu, Ping-Hsiao Shih

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