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Cytokine-induced Killer T Cells Enhance the Cytotoxicity Against Carboplatin-resistant Ovarian Cancer Cells

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Abstract. *Background/Aim: Ovarian cancer (OC) is typically diagnosed at an advanced stage with limitations for cure. Cytokine-induced killer (CIK) T cell therapy exerts significant cytotoxic effects against cancer cells and reduces the adverse effects of chemotherapy. Herein, we performed a flow cytometry-based method to evaluate the cytotoxicity of peripheral blood mononuclear cells-derived CIK cells against OC cells. Materials and Methods: The CIK cells were induced and expanded using an interferon-γ/IL-2-based xeno-free medium system. The cytotoxicity of CIK cells or carboplatin against OC cells was examined. Results: The CIK cells showed an NK-like phenotypic characteristic and dose-dependently increased cytotoxicity against OC cells. We found that the number of advanced OC cells, which were more resistant to carboplatin, was dramatically decreased by an additional one-shot CIK treatment. Conclusion: CIK cells have a potent cytotoxic ability that would be explored as an alternative strategy for cancer treatment in the near future.*

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Cytotoxic T lymphocytes comprise 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 cells, γδ T cells, and cytokine-induced killer (CIK) cells that have been developed for adoptive T cell therapies (ACT) (1). CIK cells are gaining increasing interest in immunocellular anti-cancer therapy since they can be conveniently expanded with a well-known standard protocol and require only qualified cytokines and medium to obtain very high amounts of CIK cells derived from autologous peripheral blood mononuclear cells (PBMC) (2).

CIK cells are a very promising cell population for ACT approaches. These cells exhibit a wide range of major histocompatibility complex (MHC)-unrestricted anti-tumor activity with confirmed survival benefits for hematological and advanced tumors (3-6). CIK cells are a combination of cells including T cells ($CD3^+/CD56^-$), NK-T cells ($CD3^+/CD56^+$), and NK cells ($CD3^-/CD56^+$). The optimization of the CIK induction protocol through the definition of a fixed schedule for the addition of IFN-γ, anti-CD3 antibody and IL-2, resulted in the expansion of CIK cells (7). The cytotoxic ability of CIK cells against cancer cells mainly depends on the engagement of NK Group 2 member D (NKG2D), a member of the C-type lectin-like receptor family, by NKG2D ligands on tumor cells and also on perforin-mediated pathways. Although the one-time CIK infusion did not increase the rate of full donor chimerism, strategies such as repeated infusion or genetic modification still merit exploration (8).

It has been reported that ovarian cancer (OC) is the fifth most common cause of cancer death in women (9). The low early detection rate results in lethality of ovarian cancer. It has also been revealed that around 75% of ovarian cancer patients are diagnosed at an advanced stage and have a poor prognosis without surgery and chemotherapy. The gold standard for the treatment of high-grade OC is surgical removal of tumor tissue which is followed by chemotherapy courses; unfortunately, around 70% of patients with advanced OC have recurrence within 3 years following first line chemotherapy (10).

In this study, we successfully performed a standard and clinical-grade cell culture formula composed of IFN-γ, IL-1α, anti-CD3 antibody and IL-2 to the hematopoietic cell culture medium in a Good Tissue Practice (GTP) facility, and investigated the cytotoxic effects of CIK cells against carboplatin-resistant human ovarian cancer cells.

Materials and Methods

Reagents and chemicals. Ficoll®Paque Plus was purchased from GE Healthcare Life Sciences (Parramatta NSW, Australia). X-VIVO 15 hematopoietic cell medium was from Lonza (Basel, Switzerland). Recombinant human interferon-gamma was purchased from CellGenix Inc. (Portsmouth, NH, USA). anti-CD3 antibody was purchased from TaKaRa Bio Inc. (Kusatsu, Shiga, Japan). Interleukin-1α was purchased from PeproTech Inc. (Rehovot, Israel). Recombinant human interleukin-2 (Proleukin®, NOVARTIS, Basel, Switzerland). OC-3-VGH (BCRC No.60599) and OC-117-VGH (BCRC No.60602) cell lines were all purchased from Bioresources collection and Research Center (Hsin Chu, Taiwan, ROC). DMEM/F12 and fetal bovine serum, TrypLE Express Enzyme solution was purchased from Gibco (ThermoFisher Scientific Inc., Waltham, MA, USA). isotype IgG1-FITC, isotype IgG1-APC, CD3-FITC mAb, CD56-APC mAb, Carboxyfluorescein diacetate succinimidyl ester (CFSE) and 7-aminoactinomycin D (7-AAD) dyes were all purchased from BD Biosciences (San Jose, CA, USA). In this study, all the cell culture materials were Good Manufacturing Practice (GMP)-grade.

PBMC isolation and CIK expansion. Healthy volunteers signed in the informed consent form of blood apheresis approved by the China Medical University Hospital and provided blood specimens. The density gradient solution (Ficoll®Paque Plus) was heated to 20˚C prior to use and was well mixed before use. At total of 3-5 ml of human venous blood sample was collected in a heparinized vial and mixed thoroughly by gently inverting the tube several times. Density gradient solution (4 ml) was then prepared in a 15 ml sterile tube and the blood sample (1 ml) was carefully layered onto the density gradient solution. This was followed by centrifugation at 400×g for 30 min at 20˚C. The buffy coat layer of mononuclear cells was then carefully and immediately aspirated to a sterile tube. Phosphate-buffered saline (PBS) was then added to the buffy coat in the tube. The cells were gently mixed, centrifuged at 400×g for 10 min at 20˚C and the supernatant was aspirated. The cell pellet was suspended with X-VIVO 15 basal medium and transferred to a 75-T flask. The cells were cultured at 37˚C and 5% CO_2 in a humidified cell culture incubator.

CIK cell activation and expansion. On the first day of the experiment (Day 0), the PBMCs were cultured in fresh X-VIVO 15 basal medium containing 1,000 IU/ml of IFN-γ for 24 h. On Day 1, the medium was refreshed with X-VIVO 15 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. The medium was then replaced every three days. The medium was replaced with fresh X-VIVO 15 basal medium containing 1,000 U/ml of rh IL-2 on Day 7. The medium was replaced every three days until the end of the experiment (the total duration of incubation was two weeks).

CD marker recognition for the assessment of CIK cells. The CIK cells were washed with PBS. Following centrifugation at 300 × *g* for 10 min at 20˚C, the number of cells re-suspended in PBS was counted and their viability was examined by trypan blue dye exclusion test. Aliquots of CIK cells were placed into 1.5 ml tubes at a density of ~2-5×10^5 cells/1 ml PBS and stained with CD3-FITC or CD56-APC alone or in combination, respectively. The CIK cells were also stained with the corresponding isotype antibodies measure the background fluorescence. The CIK cells were gently mixed with the antibodies and the tubes were then allowed to stand for 30 min at room temperature in the dark. The tubes were then centrifuged at 300×g for 10 min at 20˚C. The supernatant was aspirated and the cell pellet was re-suspended with 1 ml of PBS. Following gentle mixing of the cells and the tubes were placed on the carousel of a flow cytometer (Canto II, BD Biosciences, San Jose, CA, USA). Following the specimens were examined sequentially and the data of over 10,000 cells/specimen were analyzed.

Culture and staining of human ovarian cancer cells. The OC-3 serous papillary adenocarcinoma (stage IIb) and OC-117 ovarian adenocarcinoma (stage IIIc) cells were cultured in complete media (90% DMEM/F12 with 1.5 g/l sodium bicarbonate + 10% fetal bovine serum) at a density of 0.5-1×10^6 cells in a cell culture flask at 37˚C and 5% CO_2. The culture media were removed, and the cells were washed with PBS one day prior to the experiment. The cells were then detached by TrypLE Express Enzyme solution and incubated for 5 min at 37˚C. The cells were suspended in PBS and mixed well gently. The cells were pelleted at 300 × *g* for 10 min, re-suspended in PBS and their concentration was adjusted to 1×10^6 cells/ml. Then, 0.5 μl of CFSE dye was added to 1 ml of OC cells suspension in a 15 ml sterile tube at a final concentration of 5 μM. The suspension was gently mixed, and the tube was then allowed to stand in a cell culture incubator at 37˚C and 5% CO_2 for 15 min. This was followed by the addition of 9 ml PBS and cells were pelleted at 300 × *g* for 10 min. The supernatant was decanted, and the cell pellet was then suspended in complete media. The cells (5×10^5 cells/well) were seeded into a 6-well plate and incubated in an incubator overnight.

Cytotoxic assay of co-culture of CIK-OC cells. A total of 1 ml of X-VIVO 15 basal media with or without CIK cells was added to a 6-well plate. CFSE-stained OC cells (target, T) were co-cultured with or without CIK cells (effector, E) at a ratio of E/T=0:1, 5:1 and 10:1. Unstained CFSE OC cells or unstained CIK cells represented the original status of cells. The floating cells were gently mixed and the plate was placed in an incubator and incubated for 24 h.

7-AAD dye staining. At the end of the experiment, all the cells in each group were harvested. The cell suspension was transferred into

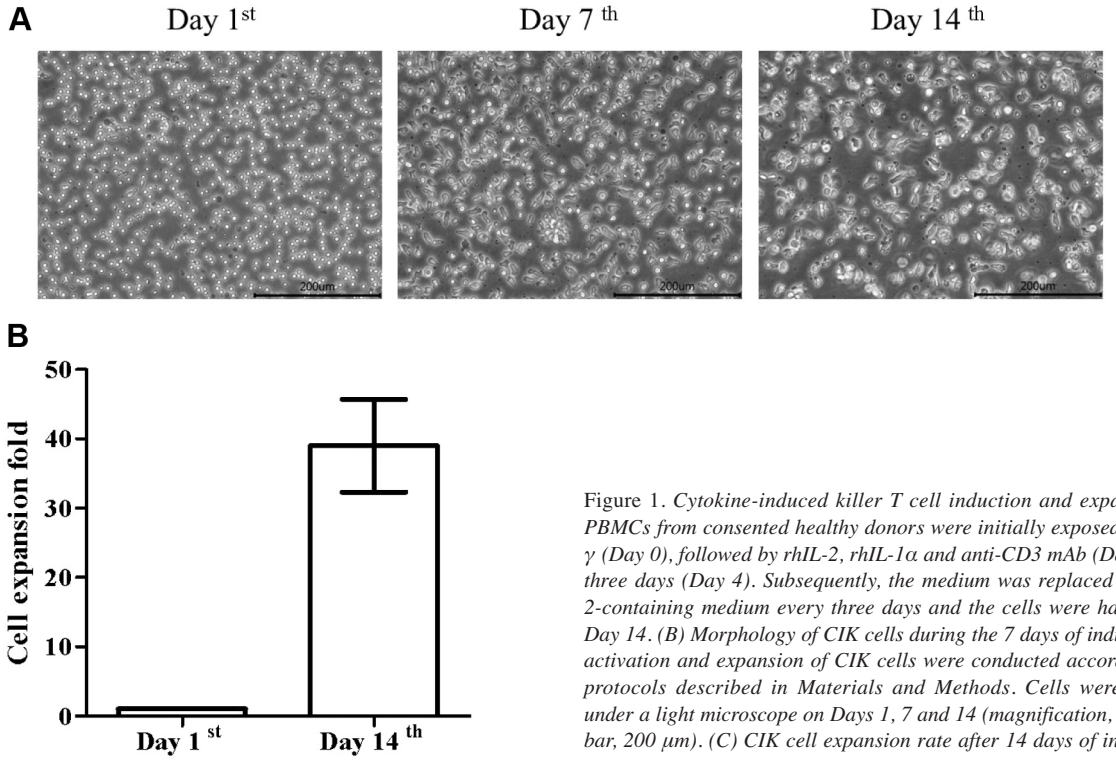

Figure 1. *Cytokine-induced killer T cell induction and expansion. (A) PBMCs from consented healthy donors were initially exposed to rhIFN-γ (Day 0), followed by rhIL-2, rhIL-1α and anti-CD3 mAb (Day 1) every three days (Day 4). Subsequently, the medium was replaced with rhIL-2-containing medium every three days and the cells were harvested on Day 14. (B) Morphology of CIK cells during the 7 days of induction. The activation and expansion of CIK cells were conducted according to the protocols described in Materials and Methods. Cells were observed under a light microscope on Days 1, 7 and 14 (magnification, ×40; scale bar, 200 μm). (C) CIK cell expansion rate after 14 days of incubation.*

a 15 ml tube. The well was then washed with PBS, and the PBS was collected to the same tube followed by the addition of TrypLE solution and incubation for 5 min at 37˚C. Then, the tubes were centrifuged at 300 × *g* for 10 min and the cells were re-suspended in 1 ml of PBS. The cells were then pelleted at 300 × *g* for 10 min and re-suspended in 100 μl of PBS. A total of 5 μl of 7-AAD dye (stock, 50 ng/μl) was then added to the cell suspension and the cells were gently mixed. Following a 10 min incubation, the cell suspension was mixed and transferred to a sterile 5 ml polystyrene round-bottom tube with cell-strainer cap. The tubes were placed on the carousel in order of precedence. Then, the cells were analyzed by the flow cytometer and dead cells were identified by using the CFSE and 7-AAD channel parameters. Data from >10,000 $CFSE^+$ cells in each specimen were recorded.

Statistical analysis. Three independent experiments with n≥3 were performed in each case. Values are presented as the mean±standard error of the mean. Statistically significant differences between the treatment group and control group were assessed by one-way analysis of variance followed by Tukey's multiple-comparisons test using Prism 6.1d (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Induction and expansion of CIK cells. The activation and expansion of CIK cells were based on a gold protocol where PBMCs were induced with IFN-γ followed by treatment with anti-CD3 antibody, IL-1a as well as IL-2, and consecutive culture in the presence of IL-2 to the end of expansion. Figure 1A shows the morphological changes from PBMCs to the CIK cells during the 14 days of induction and expansion. On average, over thirty-fold expansion rate was obtained in PBMCs from three healthy individuals (Figure 1B).

$CD3^+/CD56^+$ CIK cells with NK-like phenotype. After induction and expansion, the proportion of $CD3^+CD56^+$ T cells from three healthy donors is illustrated in Figure 2A. The average proportion of $CD3^+CD56^+$ T cells was about 20% within the total cell population (Figure 2B). Furthermore, we examined the expression of the NK group 2 member D ligand family on the CIK cells. Figure 2C shows that NKG2D, DNAM1, and NKp30 were all highly expressed on the surface of CIK cells and the statistical analysis of the proportion from three individuals (Figure 2D).

Cytotoxic effect of CIK cells. Figure 3 shows the cytotoxic effects of CIK cells against human ovarian cancer cells. In the cytotoxic experiments, the $CFSE^+$ OC-117 or OC-3 cells were co-cultured with CIK cells at various ratios for 24 h. Then, the floating and adherent cells were harvested and stained with the 7-AAD dye. The sub-population of 7-AAD+ cells in the $CFSE^+$ OC cells was evaluated. The data are presented as the percentage of cell death in each group. Figure 3A shows the morphology of cells following treatment with CIK cells. Figure 3B shows the statistical analysis of the effect of CIK

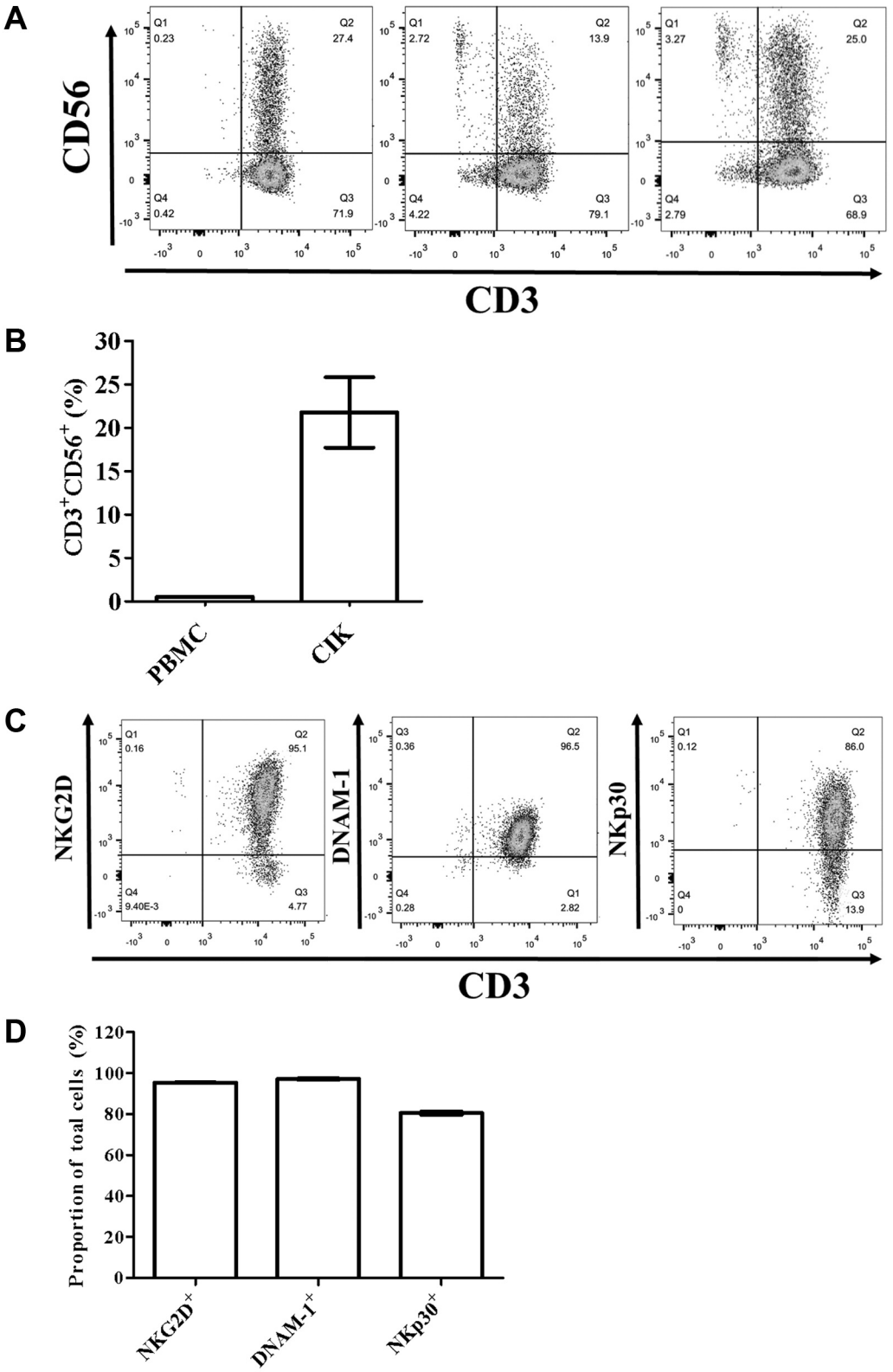

Figure 2. *The proportion of CD3+CD56+ cells and NK-like phenotype of CIK cells. Flow cytometric analysis of CD3+CD56+ (A and B) T cells and the expression of NKG2D, DNAM-1 and NKp30 (C and D) in CIK cells derived from the PBMCs of three healthy donors. Data were analyzed using the Student t-test (*p<0.01).*

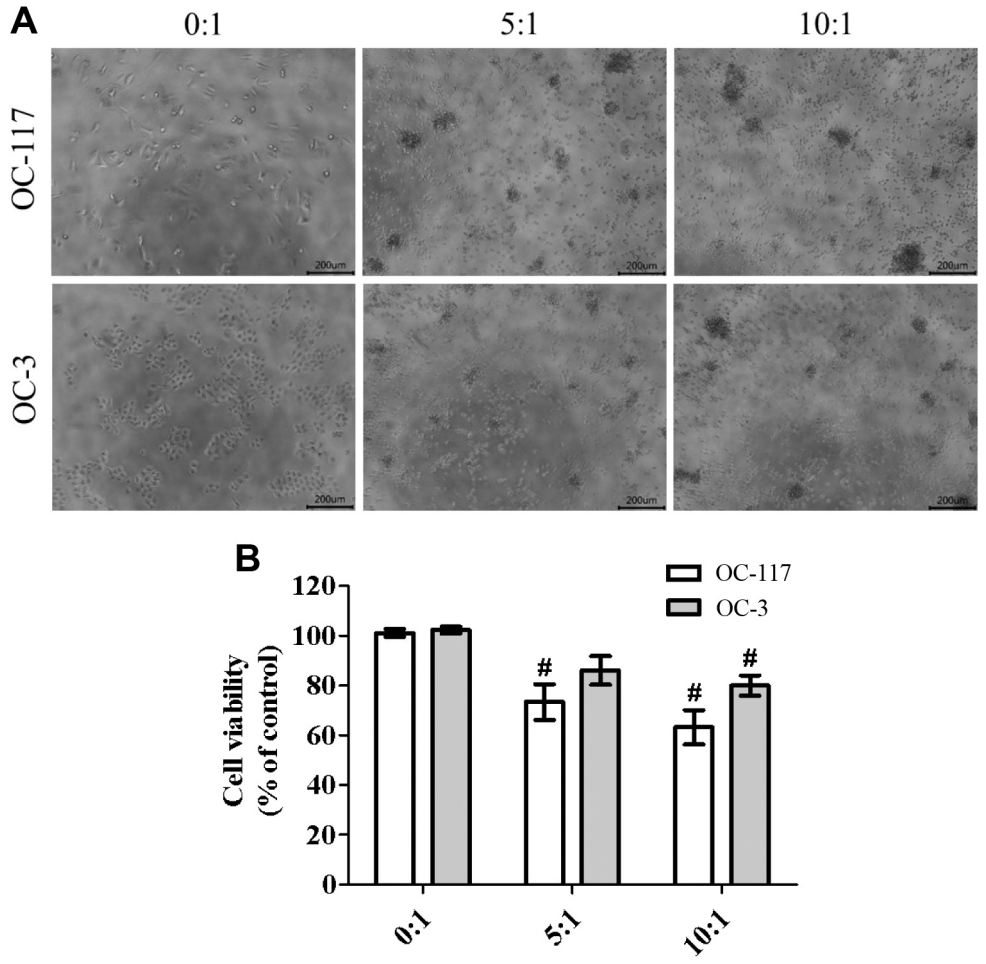

Figure 3. *Cytotoxic effects of CIK cells against human ovarian cancer cells. (A) Morphology of OC-117 and OC-3 cells (T) treated with CIK cells (E) at various ratios (E:T=0:1, 5:1 and10:1, respectively). (B) Statistical analysis of cell viability of OC-117 and OC-3 cells and the cumulative cytotoxicity of CIK cells at the indicated E:T ratios. #Significant difference between control group (E:T=0:1) and the indicated group of each cell line.*

on OC-3 cells (E/T=0:1, 5:1 and 10:1) following 24 h of incubation. We found that CIK cells were more effective towards OC-117 cells than OC-3 cells.

Cytotoxic effect of chemotherapeutic agent on OC cells. We also evaluated the chemotherapeutic effect of carboplatin on OC cells. OC-117 and OC-3 cells were treated with various concentrations of carboplatin for 24 and 48 h. Figure 4A and B show that OC-117 cells were much more resistant to carboplatin (48 h IC_{50}=140 μM for OC-117 *vs*. 19 μM for OC-3 in the 48-h treatment).

CIK cells enhance the chemotherapeutic effect against OC cells. We further investigated the effect of CIK cells on the cytotoxic effect of carboplatin on OC cells. The experimental design was as follows: 1) OC-117 and OC-3 cells were treated with indicated concentration of carboplatin for 48 h. 2) The OC cells were treated with indicated concentrations of carboplatin for 24 h and then the media was refreshed and the cells were co-treated with the CIK cells for another 24 h. As compared with the 48-h carboplatin treatment, the cytotoxicity was significantly increased if the cells were treated with carboplatin for 24 h and then with the CIK cells for another 24 h (Figure 5).

Discussion

In the present study, we applied a standard protocol for the activation and expansion of cytokine-induced killer (CIK) T cells from peripheral blood monocytes in GTP-grade facility by using GMP-grade cell culture formula, and evaluated the cytotoxic effects of CIK cells against ovarian cancer cell lines. This is a practiced and reliable protocol for the isolation, induction and expansion of cytotoxic CIK T cells from whole

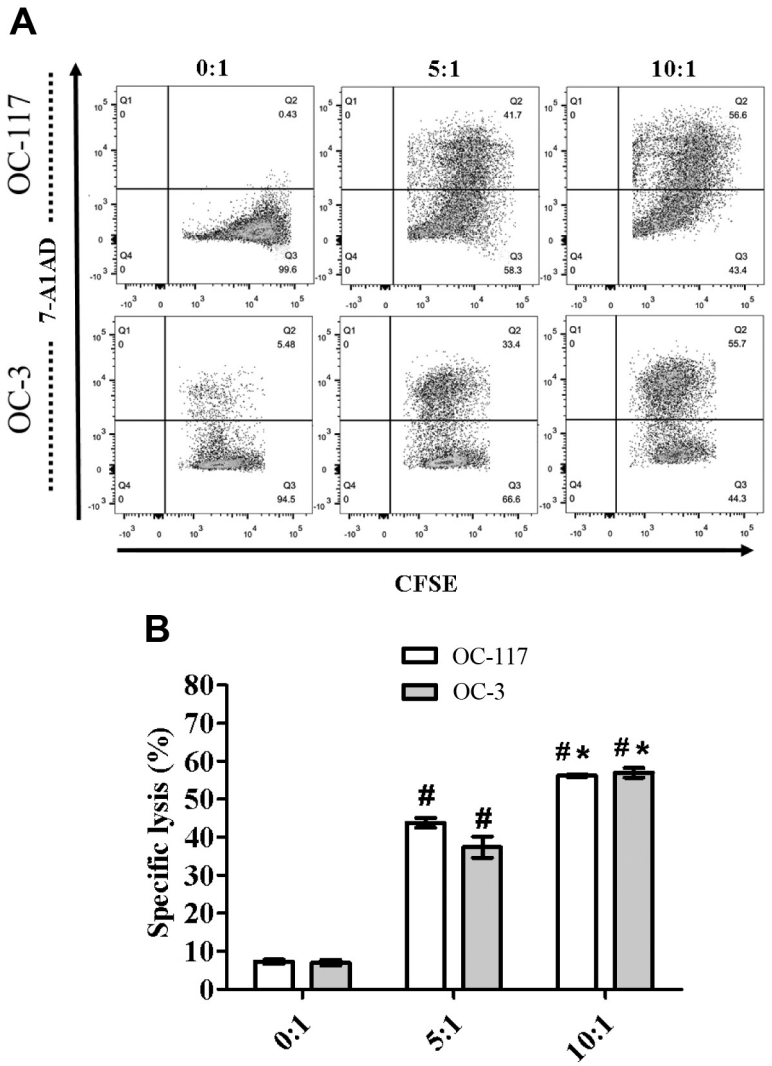

Figure 4. *Cytolytic activity of CIK cells on ovarian cancer target cells analyzed by the CFSE/7-AAD assay. CFSE-stained OC cells (T) were co-treated with CIK cells (E) at various E:T ratios for 24 h. The fluent and adherent cells were all harvested and stained with 7-AAD after washing with PBS. (A) Quantile illustration of CIK-induced cytotoxicity on CFSE$^+$-OC cells and statistical analysis of the specific lysis percentages (B). Significance ($p<0.05$) as compared with E:T=0:1 group (#) and as compared with E:T=5:1 group (*).*

blood samples of healthy donors even from patients. It has been reported that chemotherapy would decrease the number of immune cells (11, 12). Thus, allogeneic transplantation of immune cells derived from healthy donors would be an alternative strategy for the treatment of patients with lower numbers of immune cells due to chemotherapy. CIK cells can be activated and expanded by using qualified cytokines and serum-free medium for further clinical treatment (13). Although the efficacy of CIK induction and expansion exhibits individual differences (12-14), the safety is still a primary concern of the infusion of CIK cells for cancer cell therapy. IFN-γ, anti-CD3 antibody and IL-2 are the major cytokines or activators for CIK induction and proliferation. It has been reported that other factors such as thymoglobulin, IL-1α, IL-10, IL-15, *etc*. are also acting as stimulators. Recently, a study has revealed that IL-15 improved the clinical manufacturing of CIK cells (15). Recently, the outcome of allogeneic CIK cell infusion for the treatment of hematologic malignancies in a clinical study was disclosed (16). The cytotoxicity of PBMCs and isolated NK cells was significantly increased compared with the baseline in patients with non-small cell lung cancer (NSCLC) after two weeks of CIK cell therapy (17). In this study, we successfully expanded allogeneic CIK cells by using a commercially available xeno-free GMP-grade media supplemented with clinical-grade cytokines and the overall procedure was performed in a qualified GTP facility. The advantage of using

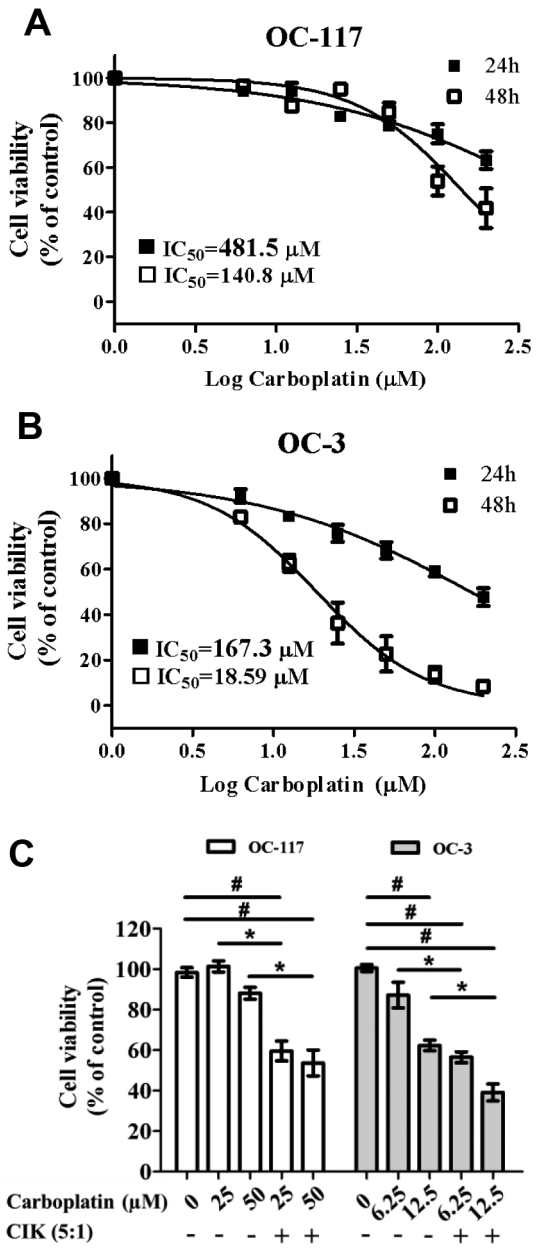

Figure 5. *CIK cells enhance the cytotoxicity in carboplatin-resistant OC cells. OC-3 and OC-117 cells were treated with various concentrations of carboplatin for 24 and 48 h and then cell viability was evaluated (A and B). (C) OC-3 and OC-117 were treated with indicated concentrations of carboplatin for 24 h, and then the cells were co-treated with CIK (E:T=5:1) for another 24 h after the refresh of medium. The symbols represent the significance of differences (p<0.05) between groups.*

xeno-free or allogeneic-free supplements is that it reduces the risk of cell contamination. Although serum or plasma are enriched with nutrients and growth factors, batch and lot variations that result in experimental variability are still an unsolvable cell culture problem.

In this preclinical study, we also demonstrated the cytotoxic effects of CIK cells using flow cytometry setup and tracking system. This system will help clinical technicians to monitor the quality and performance of *in vitro* diagnosis-grade flow cytometer. The two-color cell tracking methods provided in the protocols independently analyzed viable or dead effector and target cells. In this study, we found that CIK cells exhibited a greater cytotoxic potential against high-grade OC-117 cells as compared to low-grade OC-3 cells. Interestingly, we also found that the cytotoxic effects would be improved if the OC cells were treated with carboplatin first and then co-cultured with CIK cells instead of carboplatin alone. Recently, a meta-analysis study revealed that CIK cells plus radiotherapy can improve the clinical response, overall survival and progression free survival in lung cancer and might have lower risk of side effects (18). Thus, CIK cells really play pharmacological roles for cancer immunotherapy (19). In addition, the combination of immune blockers such as PD-1 inhibition led to an improvement of cytotoxicity of CIK cell therapy (20). In comparison with the convenient of the CIK therapy, the effect of cytokines used for *in vitro* differentiation on downstream T cell responses and the sources of antigens are the two major critical issues in dendritic cell vaccine therapy (21). Furthermore, the cytotoxic ability of CIK cells against hematologic malignancies was greater than that of gamma-delta T cells (22). These results suggested that the underlying molecular mechanisms through which CIK cell effectors target tumor cells are still unclear, while the combination therapy with carboplatin and CIK may reduce chemotherapy-induced adverse effects by curtailing the courses.

Conclusion

Taken together, the protocol described in this study was designed to amplify PBMC-derived CIK cells from healthy donors in clinical-grade facilities and to evaluate their cytotoxic actions against cancer cells with two-color photoactivatable probes to selectively track target cells by a flow cytometry with *In Vitro* Diagnosis-clinic system.

Conflicts of Interest

The Authors declare that they have no conflicts of interest to declare in relation to this study.

Authors' Contributions

SCC, DYC and PHS contributed to the conception and design of the study and prepared the manuscript. YP, YHC and PHS performed the experiments and data analysis. LML, YCW and JWP reviewed the literature and interpreted the results. CHH and PHS revised the manuscript. All Authors read and approved the final manuscript.

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