ANTICANCER RESEARCH *40*: 5221-5227 (2020)
doi:10.21873/anticanres.14525

Gamma/Delta T-Cells Enhance Carboplatin-induced Cytotoxicity Towards Advanced Bladder Cancer Cells

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Abstract. *Background/Aim: Bladder cancer (BLCA, urothelial bladder cancer) is one of the most common malignancies with increasing incidence and mortality worldwide. Poor diagnosis and the limitation of treatment is still an unmet need in clinical practice. γδ T-Cells have been paid increasing attention because of their potent cytotoxicity against tumors. Herein, we investigated the cytolytic effect of γδ T-cells in combination with the chemotherapeutic drug, carboplatin, against BLCA cells. Materials and Methods: The standard protocol for the induction and expansion of peripheral blood mononuclear cell-derived γδ T-cells was a zoledronic acid/interleukin-2-based medium system for 2 weeks. The cytotoxicity of γδ T-cells with and without carboplatin against BLCA cells was examined. Results: After incubation, T-cell receptor-positive γδ T-cells showed a natural killer cell-like phenotypic characteristic and dose-dependently increased cytotoxicity against BLCA cells. Interestingly, we found that in advanced BLCA cells, which were more resistant to carboplatin, the cell viability was significantly ($p<0.05$) reduced in the presence of γδ T-cells. Conclusion: Our findings showed that γδ T-cell therapy has potent benefit in cancer treatment.*

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Key Words: Immunocellular therapy, gamma/delta T-cell, bladder cancer, chemotherapy, cytotoxicity.

γδ T-Cells are a distinct and promising population of lymphocytes that have been attracted interest due to their functions in many types of immune response (1). γδ T-Cells are immune cell types that express antigen receptors and lead to immune responses through producing cytokine or chemokine, inducing cell lysis, regulating stromal cell function, enhancing dendritic cell maturation, priming of αβ T-cells and producing IgE (2). The anticancer ability of γδ T-cells is characterized by the recognition of tumor cells through T-cell receptors (TCRs) or natural killer cell receptors that leads to direct cell lysis and the production of interferon-γ. The most well-known example is the use of *Bacille* Calmette–Guerin (BCG), a strain of *Mycobacterium bovis* for the immunotherapy of bladder cancer which activates tumour-specific cytotoxic lymphocytes, including γδ T-cells (3); however, the detailed mechanism involved remains incompletely understood.

Urothelial carcinoma of the bladder (BLCA) is one of the most malignant tumors with high incidence rates (4). Despite adverse responses and high rates of recurrence, chemotherapy remains the standard treatment for patients after surgery. To our best knowledge, there is no any study investigating the role of γδ T-cells in BLCA in the effects of chemotherapy. In this study, we successfully established a standard and clinical-grade cell culture formula composed of

zoledronic acid and interleukin 2 (IL2) to the hematopoietic cell culture medium in a Good Tissue Practice facility, and investigated the cytotoxic effects of γδ T-cells against carboplatin-resistant human BLCA cells.

Materials and Methods

Cell lines, reagents and chemicals. T24 human bladder carcinoma (BCRC No.60062) and BFTC905 human bladder papillary transitional cell carcinoma (BCRC No.60068) cell lines were purchased from Bioresources Collection and Research Center (Hsin Chu City, Taiwan, ROC). Ficoll®Paque Plus was purchased from GE Healthcare Life Sciences (Parramatta NSW, Australia). Carboplatin was purchased from Accela ChemBio. Inc. (San Diego, CA, USA). X-VIVO 15 hematopoietic cell medium was bought from Lonza (Basel, Switzerland). Human recombinant IL2 (Proleukin®) was purchased from Novartis (Taipei, Taiwan, ROC). Zoledronic acid (ZometaTM) was purchased from Yungshin Pharm Ind. Co. Ltd. (Taipei, Taiwan, ROC). Alamar Blue™ Cell Viability Reagent, McCoy's 5a (for T24 cells), RPMI 1640 (for BFTC 905 cells), L-glutamate, fetal bovine serum and TrypLE Express Enzyme solution were bought from Gibco (ThermoFisher Scientific Inc., Waltham, MA, USA). Human monoclonal antibody isotype fluorescein isothiocyanate (FITC)-conjugated IgG1, peridinin- hlorophyll-protein (PerCP)-conjugated IgG1, allophycocyanin (APC)-conjugated IgG1, CD3-PerCP, brilliant violet (BV) 421-conjugated TCRγδ, APC-conjugated natural killer group 2 member D (NKG2D), CD27-FITC, R-phyco-erythrin (PE)-cyanine 7 (Cy7)-conjugated CD45RA were all purchased from BD Biosciences (San Jose, CA, USA).

PBMC isolation and cell culture. Health volunteers all gave their informed consent for blood apheresis approved by China Medical University Hospital. A venous blood sample of 5 ml was collected in a heparinized vial and mixed thoroughly. Four milliliters of density gradient solution was then prepared in a 15 ml sterile tube and 1 ml blood sample was carefully layered onto the density gradient solution and then centrifuged at 400 × *g* for 30 min at 20˚C. The buffy coat layer enriched in mononuclear cells was then carefully aspirated to another sterile tube. Phosphate-buffered saline (PBS) was then added to the tube and the cells were gently mixed and centrifuged at 400 × *g* for 10 min at 20˚C. After aspirating the supernatant, the cell pellet was suspended with X-VIVO 15 basal medium and transferred to a cell culture flask. The cells were cultured at 37˚C and 5% CO_2 in a humidified cell culture incubator.

γδ T-Cell induction and culture. On the first day of the experiment (day 0), the PBMCs were refreshed by X-VIVO 15 basal medium containing 5 μM zoledronic acid and 1,000 U/ml of IL2 for 3 days in cell culture. On the third day, the medium was refreshed with X-VIVO 15 basal medium containing 1,000 U/ml of IL2 thereafter. Fresh medium containing IL2 (1,000 U/ml) was added every 3 days until the expansion ended.

Cell-surface marker recognition for the assessment of γδ T-cells. After 14 days of activation, the γδ T-cells were harvested and then washed with PBS. The cell number was then adjusted and the cell viability was examined by trypan blue dye exclusion test and the number of cells was recorded. Furthermore, aliquots of γδ T-cells were placed into tubes and stained with NKG2D-APC, CD3-PerCP/TCRγδ-FITC and CD27-FITC/CD45RA-PE-Cy7 double-staining in order to determine the proportions of naïve ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$), effector memory ($CD27^-CD45RA^-$), terminally differentiated ($CD27^-CD45RA^+$) and exhausted ($CD27^+CD45RA^{high}$) T-cells. The fluorescence of the relative isotype antibodies served as a surrogate for background fluorescence. After 30 min of incubation at room temperature in the dark, the cells were then centrifuged at 300 × *g* for 10 min at 20˚C. The supernatant was aspirated directly and the cell pellet was then suspended and gently mixed with one ml of PBS. Finally, the cells were analyzed by an In Vitro Diagnosis-grade flow cytometer (FACSCantoTM II; BD Biosciences, San Jose, CA, USA). Over 20,000 cells/specimen were recorded for the analysis.

Culture of human bladder cancer cells. The T24 and BFTC905 human BLCA cell lines were cultured in complete medium (90% basal medium containing 1.5 g/l sodium bicarbonate plus 10% fetal bovine serum) in a humidified cell culture incubator at 37˚C and 5% CO_2. On the day before the experiment, the medium was removed and the cells were detached by TrypLE Express Enzyme solution and incubated for 5 min at 37˚C. Following, the cells were seeded into a multi-well plate or dish for further study, respectively. Furthermore, frozen medium containing 93% culture medium with 7% dimethyl sulfoxide was prepared for cell storage with liquid nitrogen.

Cytotoxic assay of γδ T-cells against BLCA cells in combination with chemotherapeutic. In the beginning of this study, BLCA cells (target cells, T) were co-cultured with or without γδ T-cells (effector cells, E) at a ratio of E/T=0:1, 2.5:1, 5:1 and 10:1 for 24 h. At the end of incubation, cell viability was analyzed. At the same time, BLCA cells were treated with carboplatin (0-100 μM) for 24 h, and then the cells were washed with PBS to remove the drug. The culture medium was replaced with γδ T-cell-containing medium (E/T=5:1) and the cells were incubated for a further 24 h and then cell viability was examined. The cell viability assay was performed using AlamarTM Blue. The cells were treated with AlamarTM Blue Reagent (1/10 fold dilution from stock solution by cell culture basal medium) directly and incubated for 3 h, and then the fluorescence intensity was examined at excitation wavelength 544 nm and the emission at 590 nm by an enzyme-linked immunosorbent assay reader (SpectraMax® M2e; Molecular Devices, San Jose, CA, USA). The cell viability was then expressed as: (fluorescence of treated cells − fluorescence of the control)/fluorescence of the control) ×100%.

Statistical analysis. All data were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). They were expressed as the mean±SE of the mean, and represented at least three independent experiments. Differences between two groups were analyzed using one-way ANOVA, followed by a *post hoc* analysis. A *p*-value of less than 0.05 was set as statistically significant.

Results

Activation and expansion of γδ T-cells. The induction and expansion of γδ T-cells were based on a standard protocol, in which PBMCs were induced with zoledronic acid/IL2 first, followed by the long-term supplement of IL2 until the end of expansion. Figure 1A shows the morphological transformation from PBMCs to γδ T-cells during the 14 days of activation and expansion. The expansion rate for three healthy donors was about 10-fold (Figure 1B).

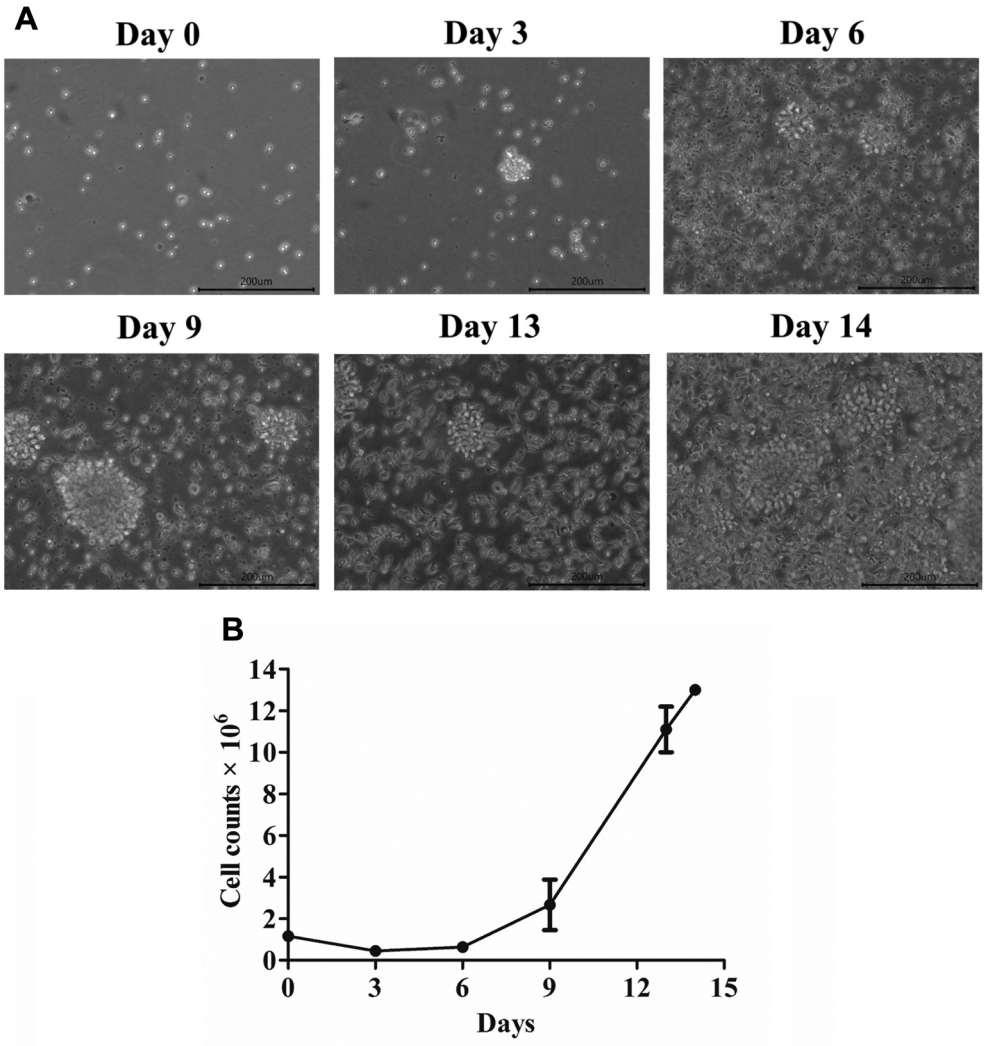

Figure 1. *Activation and expansion of peripheral mononuclear cell (PBMC)-derived γδ T-cells (GDT). PBMCs were harvested from the whole blood of volunteers. On day 0, the cells were exposed to zoledronic acid (5 μM) and recombinant human interleukin 2 (rhIL-2, 1,000 U/ml) for 24 h. Subsequently, the medium was replaced with rhIL2-containing medium (1,000 U/ml) every 3 days and the cells were harvested on day 14th. A Morphology of PBMCs in their expansion into γδ T-cells during 14 days of induction. Cells were observed under a light microscope on days 1, 7 and 14, respectively (scale bar=200 μm). B: Fold expansion of γδ T-cells after 14 days of incubation.*

$CD3^+$/TCR γδ T-cells with NK-like phenomenon. After induction and expansion, representative results of enhanced $CD3^+TCR\gamma\delta^+$ and the switch of CD27/CD45RA γδ T-cells from three healthy donors is illustrated in Figure 2A and B. Most notably, the proportion of $CD27^-CD45RA^-$ effector memory γδ T cells was less than 10% in original PBMCs and then increased to over 80% after the induction. Furthermore, we analyzed NKG2D on γδ T-cells. Figure 2C shows that the expression of NKG2D was highly promoted on the surface of γδ T-cells. The average proportion of $CD3^+$ $TCR\gamma\delta^+$ γδ T-cells was about 20% within the total cell population, while it was about 2.5% in the PBMCs (Figure 2D).

Cytotoxic effect of γδ T-cells against BLCA cells. In the cytolytic study, the BLCA cells were co-cultured with various amount of γδ T-cells for 24 h, respectively. Figure 3A shows the morphology of cell damage by the treatment of γδ T-cells. Figure 3B shows the statistical analysis of γδ T-cells against BLCA cells (E/T=0:1, 2.5:1, 5:1 and 10:1) following 24 h of incubation. We found that γδ T-cells were both sensitized to BFTC905 and T24 cells.

Cytotoxic effect of chemotherapeutic drug on BLCA cells. We evaluated the chemotherapeutic effect of carboplatin on bladder cancer cells. BLCA cells were treated with carboplatin for 24 h. Figure 4 shows that T24 cells were

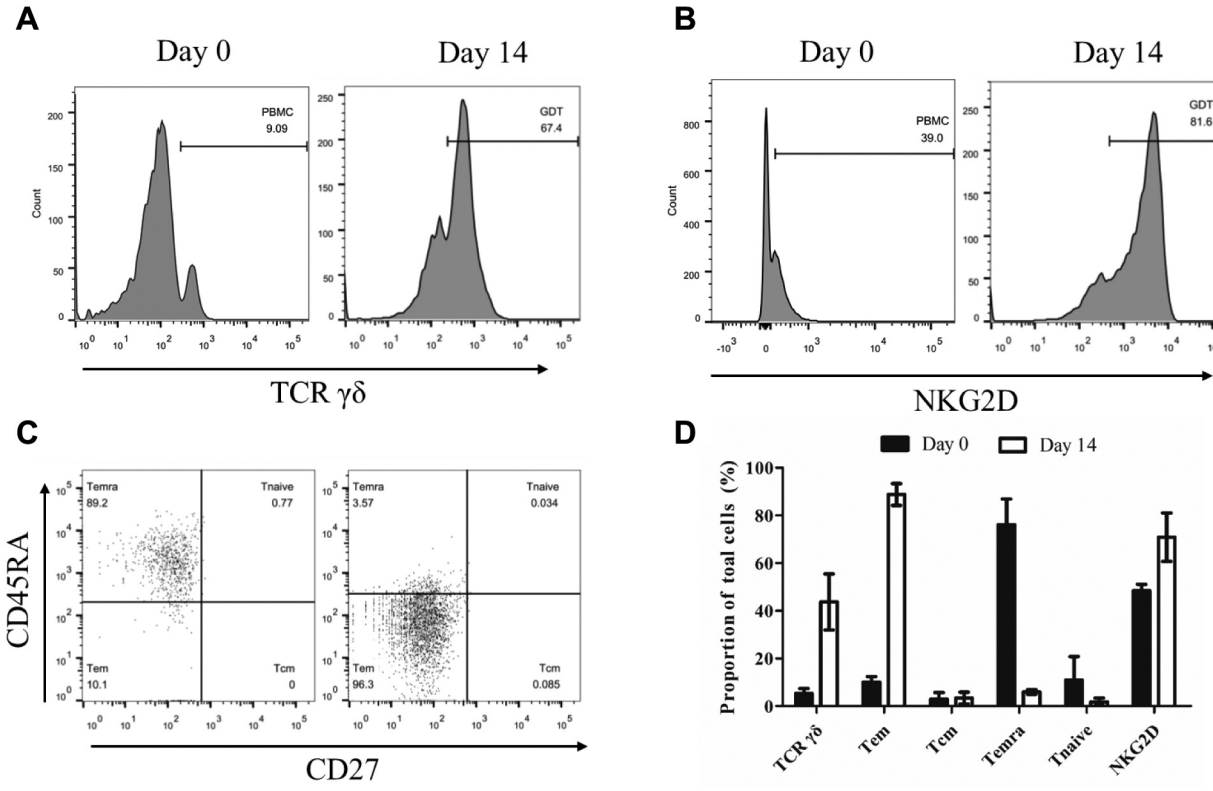

Figure 2. *Expansion of γδ T-cells with effector memory and natural killer cell (NK)-like phenomenon. After induction, the phenotype of γδ T-cells was analyzed. Illustrations of CD3+ T-cell receptor (TCR)+ (A), CD27/CD45RA (B) and natural killer group 2 member D (NKG2D)+ (C) cells in peripheral mononuclear cells (PBMCs) (left panel) and γδ T-cells (right panel) before and after the induction. D: Statistical analysis of the proportion of NKG2D and each sub-population of cells in PBMCs and γδ T-cells. Tnaive: Naïve-cells (CD27+CD45RA+); Tcm: central memory T-cells (CD27+CD45RA−); Tem: effector memory (CD27−CD45RA−); Temra: terminally differentiated T-cells (CD27−CD45RA+).*

much more resistant to carboplatin (half-maximal inhibitory concentration ≈400 μM for T24 cells *vs*. 80 μM for BFTC905 cells in a 24 h incubation).

γδ T-cells promote chemotherapeutic effect against BLCA cells. We further investigated the effect of γδ T-cells in combination with carboplatin. BLCA cells were treated with carboplatin for 24 h, then the medium was replaced with γδ T-cell-containing medium for another 24 h. As compared with the 24-h treatment with carboplatin alone, cytotoxicity was significantly ($p<0.05$) increased when the cells were treated with low-dosage carboplatin for 24 h first and then treated with the γδ T-cells for another 24 h (Figure 5).

Discussion

Heterogeneous superficial BLCAs represent about 70% of all bladder carcinomas and are treated with transurethral resection followed by intravesical administration of chemotherapeutic agents (5). It has been suggested that smoking, infection with *Schistosoma haematobium*, genetic instability and other risk factors are the major causes for bladder cancer progression. Although cisplatin is preferred for the treatment of metastatic urothelial carcinoma as compared with carboplatin-based regimens, patients often did not receive cisplatin due to the limitation of age or smoking history, or other factors (6, 7). It has been suggested that patients with intermediate-risk or high-risk disease can be treated with intravesical BCG, but unfortunately, despite this, many of these patients will still suffer tumor recurrence (8). Thus, transplantation of immune cells directly might be an alternative strategy for the treatment of patients with high-grade malignancies. In this article, we described a standard protocol for the induction and expansion of γδ T-cells derived from peripheral blood monocytes from healthy volunteers in a Good Tissue Practice-certified facility using Good Manufacturing Practice-compliant cell culture protocol, and evaluated the cytotoxic effects of γδ T-cells against advanced BLCA cells. This was a practicable and convenient protocol for the isolation, activation and expansion of cytotoxic γδ T-cells derived from whole blood samples of donors. γδ T-cells can be induced and expanded

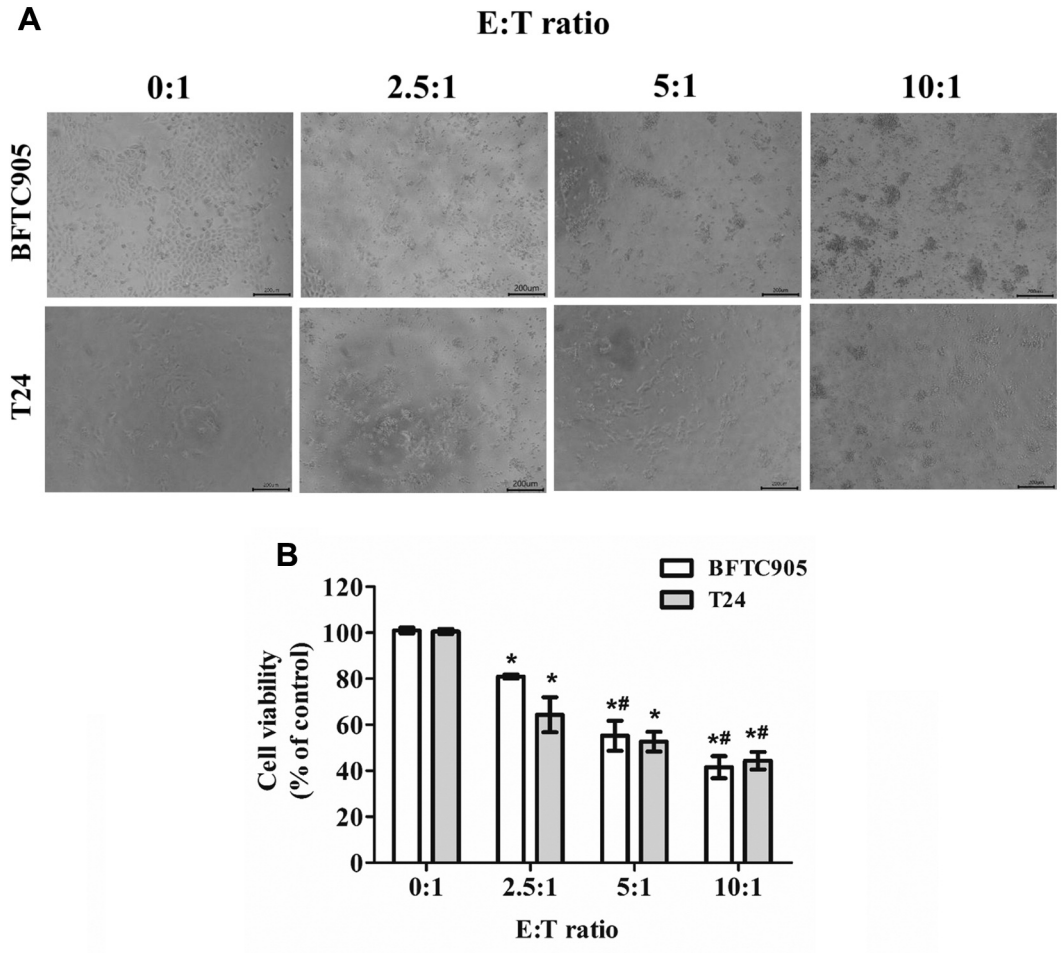

Figure 3. *Cytolytic effects of γδ T-cells on human bladder cancer cells. A: Morphology of BFTC905 and T24 cells treated with γδ T-cells at different effector:target ratios (E:T). B: Statistical analysis of the viability of BFTC905 and T24 cells co-cultured with γδ T-cells from three independent experiments. Significantly different from the *control group (E:T ratio=0:1) and #between treated cell lines at p<0.05.*

using a qualified cytokine- and serum-free medium for use in further clinical treatment (9). It has been shown that the proportion and expansion efficacy of γδ T-cells are dependent on the physiological status of donors (10).

A phase I trial on the feasibility and safety of γδ T-cell therapy against advanced solid tumors including breast cancer, melanoma and colon cancer, was carried out (11). It was suggested that the therapeutic benefits of γδ T-cell therapy appeared when used in combination with other therapies. A phase I/II clinical trial of transferring γδ T-cells to patients with advanced renal cell carcinoma was conducted (12). The well-tolerated transfer of γδ T-cells in combination with zoledronic acid as well as IL-2 was accompanied with the better treatment responses. Recently, the clinical outcome from a case study for the treatment of cholangiocarcinoma by allogenic Vγ9Vδ2 T-cells was reported (13). About 4×10^8 γδ T-cells derived from a healthy donor were adoptively transferred to the patient, and after eight γδ T-cell treatments, the tumor activity was dramatically reduced and the patient's lifespan was prolonged. Most importantly, as compared with chemotherapy, severe adverse effects were not observed in patients treated with γδ T-cell therapy (14). Patients with more peripheral γδ T-cells had a better clinical outcome. Literature has discussed the cytotoxic effects of natural killer cells, cytokine-induced killer cells and γδ T-cells against several hematological malignancies (15). It was shown that γδ T-cells had the best proliferative capability. Regretfully, there is less evidence about the cytotoxic capability of the different types of T-cells against solid tumors. Recently, we found the cytotoxic effects of cytokine-induced killer T-cells to be enhanced against carbolatin-resistant ovarian cancer (16). In the present study, we found that the original γδ T-cells in the PBMCs from our donors almost displayed a

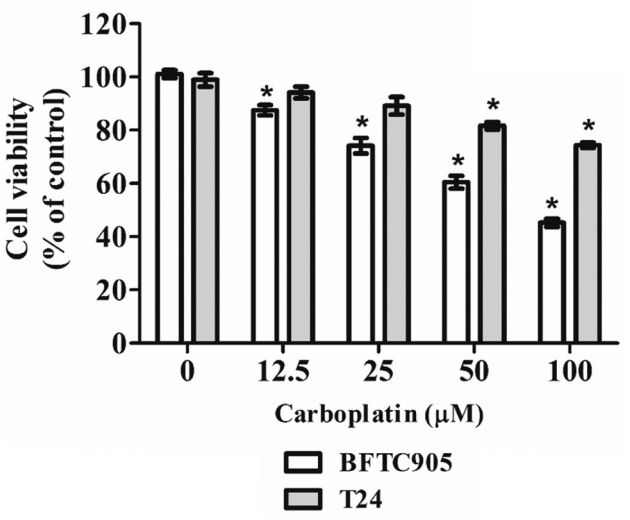

Figure 4. *Cytotoxic effects of the therapeutic drug carboplatin on bladder cancer cells. BFTC905 and T24 cells were treated with the indicated concentrations of carboplatin for 24 h. At the end of the experiment, the cell viability was tested directly by adding diluted Alamar BlueTM reagent to the cells. After a 3 h of incubation, the fluorescence intensity was examined at 544 nm of excitation and at 590 nm of emission by an enzyme-linked immunosorbent assay reader. *Significantly different from the control group (0 μM) at p<0.05.*

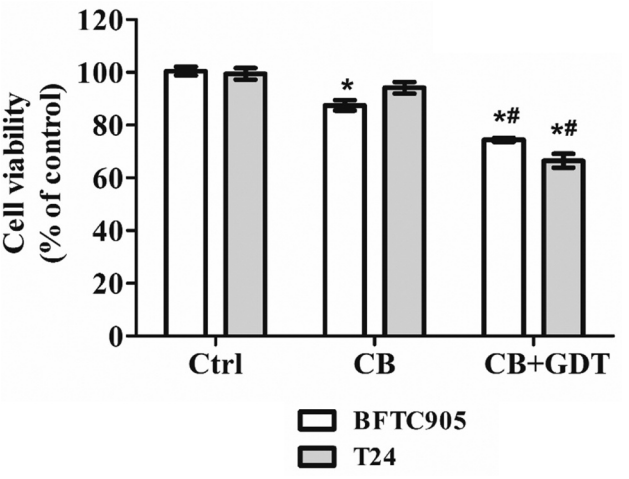

Figure 5. *γδ T-cells promote carboplatin-mediated cytotoxicity in bladder cancer cells. BFTC905 and T24 cells were treated with 12.5 μM carboplatin (CB) for 24 h, followed by washing twice and replacement with γδ T-cell-containing medium (GDT) (effector:target=5:1) for another 24 h of incubation. Finally, the cell viability was evaluated. Significantly different from the *control group (Ctrl) and #between cell lines at p<0.05.*

terminally differentiated ($CD27^{-}CD45RA^{+}$) phenotype, while after activation, the phenotype of γδ T-cells was switched to effector memory T-cells ($CD27^{-}CD45RA^{-}$), which showed significant cytotoxicity against BLCA cells and enhanced the therapeutic effects of carboplatin. Most importantly, it is suggested that the higher proportion of non-$CD27^{-}CD45RA^{hi}$ γδ T-cells should lead to successful expansion efficacy in large-scale cell culture for clinical treatment (10).

In the present study, we found that γδ T-cells showed cytotoxic potential against advanced BLCA cells. We also found that the cytotoxic effect was significantly ($p<0.05$) enhanced when the BLCA cells were treated with carboplatin first and then co-cultured with γδ T-cells. This study shows that chemotherapy in combination with γδ T-cell therapy might reduce adverse responses to chemotherapy by allowing the dosage and the duration of chemotherapy to reduced, and may enhance the therapeutic effect. Previously, a meta-analysis study demonstrated that γδ T-cell-based immunocellular therapy promoted overall survival and acted as adjuvant to conventional therapies for the treatment of patients with chemo-/radioresistant or recurrent disease (17). Interestingly, it has been reported that administration of immune checkpoint inhibitors against programmed cell death protein 1 restored the cytotoxicity of γδ T-cells in combination with histone deacetylase inhibitors towards oral, colonic and B lymphoblast cancer cells (18).

Conclusion

We describe a protocol designed to amplify γδ T-cells from whole blood in a clinical-grade facility and demonstrated their cytotoxic capability against malignant BLCA cells. These findings suggest that γδ T-cell therapy might have benefits for treatment of advanced BLCA in combination with standard chemotherapy after surgery. However, pre-clinical *in vivo* study should be carried out to investigate the physiological effects of γδ T-cell therapy.

Conflicts of Interest

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

Authors' Contributions

SCC, DYC and PHS contributed to the experimental design and prepared the article. 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 article. All Authors read and approved the final article.

Acknowledgements

This work was supported by the Department of Medical Research, China Medical University Hospital (grant nos. DMR-107-153 and

DMR-CELL-1809) and Taipei Municipal WanFang Hospital (grant no.109-wf-eva-28).

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Received July 23, 2020
Revised July 29, 2020
Accepted July 30, 2020