

Influence of autologous dendritic cells on cytokine-induced killer cell proliferation, cell phenotype and antitumor activity *in vitro*

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Abstract. Dendritic cell (DCs) are essential antigen processing and presentation cells that play a key role in the immune response. In this study, DCs were co-cultured with cytokine-induced killer cells (DC-CIKs) *in vitro* to detect changes in cell proliferation, cell phenotype and cell cytotoxicity. The results revealed that the DCs were suitable for co-culture with CIKs at day 7, and that cell quantity of DC-CIKs was lower than that of CIKs until day 11, but it was significantly improved to 1.17-fold that of CIKs at day 13. Flow cytometry was used to detect the cell phenotype of CIKs and DC-CIKs. Compared with CIKs at day 13, the percentage of CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ and CD3⁺CD56⁺ T cells in DC-CIKs was significantly improved 1.02, 1.79, 1.26 and 2.44-fold, respectively. In addition, trypan blue staining analysis demonstrated that the cell viability of CIKs and DC-CIKs was 96% and 98%, respectively. Furthermore, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis verified that CIK and DC-CIK cytotoxicity in Hela cells was 58% and 80%, respectively, with a significant difference. Taken together, our results indicate that the cell proliferation, cell phenotype and antitumor activity of CIKs were all enhanced following co-culture with DCs *in vitro*. These results are likely to be useful for DC-CIK application in antitumor therapies.

Introduction

Cellular immunotherapy is a novel treatment for tumors following chemotherapy and hematopoietic stem cell transplantation (1). It aims to stimulate the immune system of patients to triggered an anti-tumor immune response, eventually enhancing the body's natural abilities to recognize and

kill cancer cells (2). Additional research found that cellular immunotherapy based in different immune cells had various anti-tumor efficacy (3). Among the numerous types of immune cells, dendritic cells (DCs) and cytokine-induced killer cells (CIKs) are extensively used in the clinic (2,4,5); the former are highly specialized antigen-presenting cells (APC) (6) and the latter have a broad spectrum in the killing of tumor cells (7).

Clinical studies have demonstrated that specifically designed DC-targeted cancer cell vaccines have different clinical benefits (8-10). Frank *et al* demonstrated that patients who received dendritic cell vaccines generated by the adherence method demonstrated increased T cell proliferation in response to the vaccination (11). Zhu *et al* noted that DC vaccines and CIK therapy could induce an immune response against advanced colorectal cancer, thereby improving quality of life and prolonging overall survival (12). A large clinical study demonstrated that the antitumor response of CIKs could be influenced by DCs *in vivo* (1,4,12), but the influence of DCs on CIKs cultured *in vitro* was unclear.

In this study, data analysis revealed that the highest amplification fold of CIKs occurred on day 7. Further study revealed that the DC-CIK cell quantity, partial cell phenotype and cell cytotoxicity were significantly upregulated compared with CIKs. The results are likely to be useful for DC-CIK application and development in antitumor therapies.

Materials and methods

Ethics and consent. Peripheral blood was donated from volunteers after receiving informed consent, and the study was approved by the ethics committee of the Second Affiliated Hospital of Nanhua University, Hengyang, China.

CIK culture. Lymphocytes were separated and cultured in accordance with the studies of Pan *et al* (13) and Laport *et al* (14), with certain modifications. Peripheral blood was mixed 1:1 (V:V) with 0.9% physiological saline and used for Ficoll density gradient separation (LymphoPrep, PAA, Cölbe, Germany). Following centrifugation at 1,800 rpm for 20 min, the leukocyte layer was collected in fresh tubes. These cells were then washed twice with 0.9% physiological saline at 1,500 rpm for 7 min. Next, the lymphocyte was cultured in GT-T551 medium (Takara Biotechnology Co., Ltd., Dalian,

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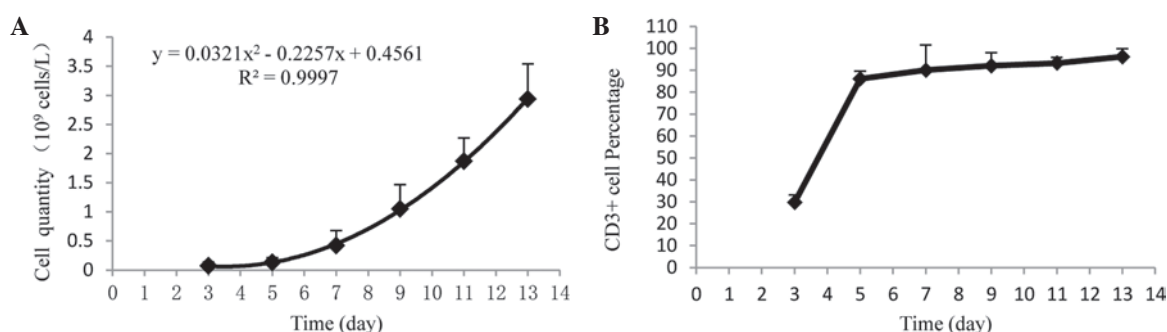


Figure 1. Proliferation activity analysis of cytokine-induced killer cells (CIKs). (A) Change in cell quantity at day 3, 5, 7, 9, 11 and 13. (B) Expression of CD3⁺ T cells in CIKs at day 3, 5, 7, 9, 11 and 13. Bars represent means \pm standard deviation (n=5).

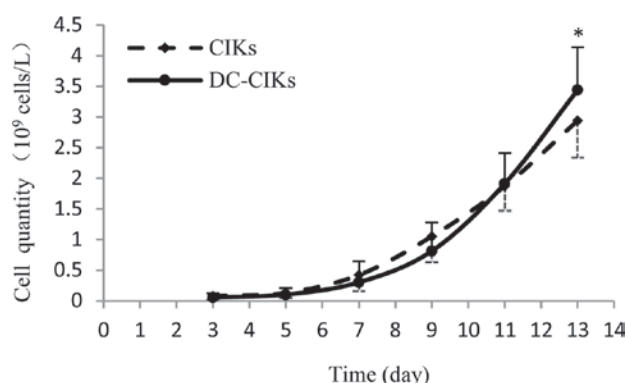


Figure 2. Detection of cell quantity in cytokine-induced killer cells (CIKs) and dendritic cell (DC)-CIKs at various times. Cell quantity was measured at day 3, 5, 7, 9, 11 and 13, and the DCs were co-cultured with CIKs on day 7. Bars represent means \pm standard deviation (n=5). *P<0.05.

China) with 1,000 U/ml γ -interferon (Beijing Biocoen Biotechnology Co., Ltd., Beijing, China). Ten percent autologous plasma was added on day 0, then 50 μ g/ml CD3 monoclonal antibody (Skoda Biotechnology Co., Ltd., Beijing, China) and 100 U/ml interleukin 1 α (IL-1 α ; PeproTech, Suzhou, China) were added on day 1, and 1,000 U/ml rhIL-2 (SL-PHARM, Beijing, China) and 2% autologous plasma was included in the medium from day 1 onward with the concentration calculated by a poch-100i hematology analyzer (Sysmex, Milton Keynes, UK). The cells were cultured at 37°C in 5% CO₂ until day 13.

DC-CIK culture. The DC cells were cultured *in vitro* according to the studies of Miao *et al* and Pan *et al* with certain modifications (15,16). The lymphocyte separated from the peripheral blood was resuspended with 20 ml GT-T551 medium, and cultured for 3 h at 37°C in 5% CO₂. Finally, the adhered and suspended cells were separated and cultured as mononuclear cells and CIK cells, respectively. The mononuclear cells were cultured with 20 ml AIM-V medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% autologous plasma, GM-CSF (0.2 μ g/ml, Beijing Biocoen Biotechnology Co., Ltd, Beijing, China) and IL-4 (1 μ g/ml, CELLBO Biotechnology Co., Ltd, Wuxi, China). Half of the medium was replaced with fresh medium supplemented with cytokines on day 3, and TNF- α (0.2 μ g/ml, Beijing Biocoen Biotechnology Co., Ltd) was added on day 5 to induce maturation of the DCs. On day 7, the DCs were collected and co-cultured with CIK at 37°C in 5% CO₂ until day 13.

Flow cytometry analysis. Following culture of CIKs and DC-CIKs for 13 days, 1 ml cell suspension was collected and centrifugated at 1,000 rpm for 10 min, then the precipitate was resuspended in 1 ml 0.9% physiological saline, centrifugated at 1,000 rpm for 10 min, then the precipitate was resuspended with 150 μ l 0.9% physiological saline, and divided into two groups. APC mouse IgG1 (5 μ l), FITC mouse IgG2 α (5 μ l), PE mouse IgG1 (5 μ l) and PerCP-CyTM5.5 mouse IgG1 (1 μ l) were added to one group to form the isotype control, and FITC mouse anti-human CD3 (5 μ l), PE mouse anti-human CD4 (5 μ l), PerCP-CyTM5.5 mouse anti-human CD8 (1 μ l) and APC mouse anti-human CD56 (5 μ l) were added to the second group to form the experimental group. The two groups were all incubated for 15 min at room temperature, then resuspended with 1 ml 0.9% physiological saline, and centrifugated at 1,000 rpm for 10 min. Finally, the precipitate was resuspended with 0.2 ml 0.9% physiological saline, and prepared for analysis using a BD Accuri C6 flow cytometer (BD Biosciences, Shanghai, China).

Cell viability. Following the culture of CIKs and DC-CIKs for 13 days, 1 ml cell suspension was collected and centrifugated at 1,000 rpm for 10 min, then the precipitate was resuspended in 1 ml 0.9% physiological saline and centrifugated at 1,000 rpm for 10 min. Next, the precipitate was resuspended and diluted with physiological saline to 1 \times 10⁵ cells/ml, then the cell suspension was mixed with 0.4% trypan blue at 9:1 (V:V), and analyzed by Countstar (Inno-Alliance Biotech, Shanghai, China) within 3 min.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. Hela cells as target cells were obtained at logarithmic growth phase and the concentration was adjusted to 1 \times 10⁵ cells/ml. CIKs or DC-CIKs cultured for 13 days were used as effector cells, and then mixed with target cells at a proportion of 50:1 (effector cells to target cells). CIK or DC-CIK culture medium (10 ml) was collected, and following centrifugation at 1,000 rpm for 10 min, the precipitate was resuspended with GT-T551 containing 2% autologous plasma and diluted to 5 \times 10⁶ cells/ml. The cells were divided into three groups: the effector-target group comprised 100 μ l effector cells and target cells, respectively; the effector cells group comprised 100 μ l effector cells and GT-T551 culture medium; and the target cells group comprised 100 μ l target cells and GT-T551 culture medium. All groups were cultured at 37°C in

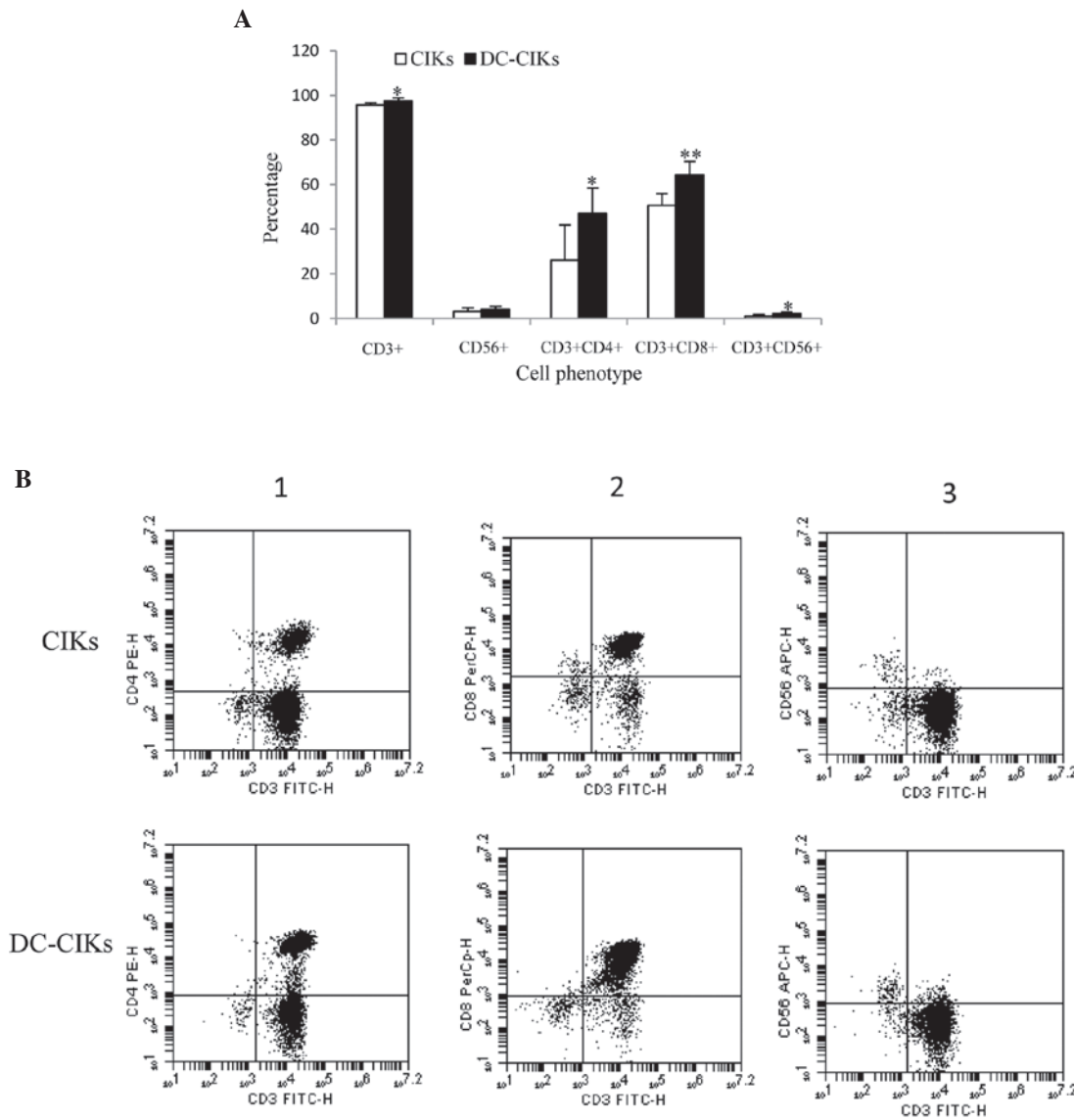


Figure 3. Flow cytometry analysis of the cell phenotype in cytokine-induced killer cells (CIKs) and dendritic cell (DC)-CIKs. (A) Bar graph demonstrating percentage of different cell phenotypes between CIKs and DC-CIKs cultured at day 13. (B) Percentage of different phenotype T cells measured by flow cytometry. Lanes 1-3 represent CD3⁺CD4⁺, CD3⁺CD8⁺ and CD3⁺CD56⁺ phenotype, respectively. Bars represent means \pm standard deviation (n=5). *P<0.05; **P<0.01.

5% CO₂ for 24 h. There were five parallel tubes in every group. Ten microliters MTT (5 mg/ml) was added and cultured at 37°C in 5% CO₂ for 4 h. Following centrifugation at 2,000 rpm for 5 min, the precipitate was dissolved in 100 μ l dimethyl sulfoxide, agitated for 15 min, and analyzed at an optical density (OD) of 490 nm. The killing rate was calculated as follows:

$$\text{Rate} = [1 - (\text{OD}_{\text{effector-target cell well}} - \text{OD}_{\text{effector cell well}}) / \text{OD}_{\text{target cell well}}] \times 100\%.$$

Results

Effect of DCs on CIK cell quantity. To determine the appropriate co-culture time of DCs and CIKs, the cell proliferation of CIKs was analyzed. From day 7, the CIKs were in a period of rapid proliferation (Fig. 1A), and the percentage of CD3⁺ T cells was over 90% (Fig. 1B). Of these, DCs were co-cultured with CIKs for 7 days. As shown in Fig. 2, the

DC-CIKs were in rapid proliferation on day 7, but the cell quantity was lower than that of CIKs until day 11 and the DC-CIK quantity was significantly (1.17-fold) greater than that of CIKs on day 13.

Difference in cell phenotype between CIKs and DC-CIKs. Next, cell phenotype was analyzed by flow cytometry. As Fig. 3A reveals, the expression of CD3⁺, CD56⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ and CD3⁺CD56⁺ T cells in DC-CIKs was higher than that in CIKs, and the expression of CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ and CD3⁺CD56⁺ T cells was significantly upregulated 1.02, 1.79, 1.26 and 2.44-fold, respectively.

Cell viability and cell cytotoxicity. For further analysis of the influence of DCs on CIKs *in vitro*, cell viability analysis and MTT were used. The CIK and DC-CIK cell viability was 96 and 98%, respectively (Fig. 4), and there was no significant difference between CIKs and DC-CIKs. In addition, MTT results revealed that the CIK and DC-CIK cell cytotoxicity

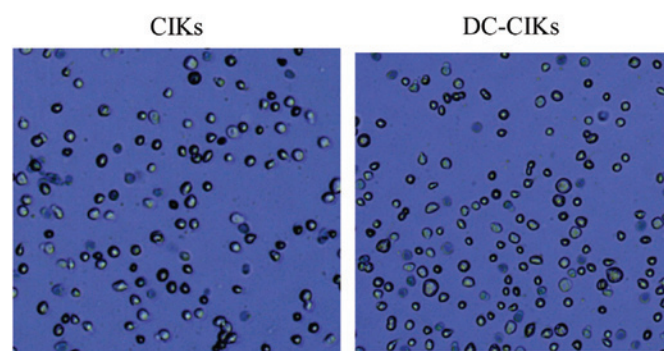


Figure 4. Cell viability analysis. Cells (900 μ l cell suspension) were dyed with 100 μ l 0.4% trypan blue at room temperature for 3 min, then a 10 μ l sample was analyzed by Countstar. Those with bright color are living cells. CIKs, cytokine-induced killer cells; DC, dendritic cells.

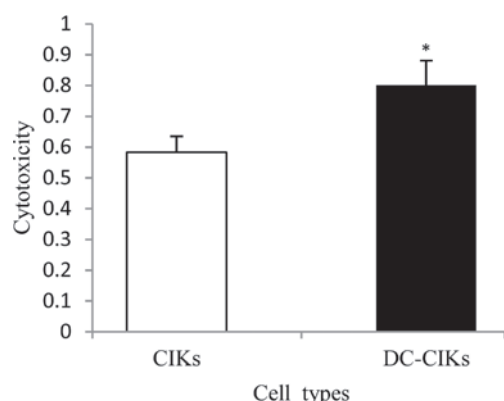


Figure 5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis of cytotoxicity of cytokine-induced killer cells (CIKs) and dendritic cell (DC)-CIKs in Hela cells. Bars represent means \pm standard deviation (n=5). *P<0.05.

in Hela cells was 58 and 80%, respectively, with a significant difference (Fig. 5).

Discussion

DCs, known to be the most powerful APCs, play a significant role in immune response regulation, induce primary immune responses, and potentiate the effector functions of previously primed T lymphocytes (17-19). Usually, tumor patients have low immune function, which leads to tumor cells escaping autoimmune response. CIK cells are a subset of natural killer T lymphocytes (NKTs) that are type II NKT cells (20) with enhanced tumor cell lytic activity (21), higher proliferation rate (22), and relatively lower toxicity (23). Due to these characteristics, CIKs are extensively used in antitumor therapy in clinics (24-26).

In this study, the influence of DCs on CIKs was analyzed. The results revealed that DCs were suitable for co-culture with CIKs on day 7 (Fig. 1), and that the cell quantity of DC-CIKs was significantly improved compared with that of CIKs on day 13 (Fig. 2). Notably, the cell proliferation quantity is related to the cytotoxicity of CIKs to tumors (27,28). Therefore, these results strongly suggest that the antitumor activity of DC-CIKs is enhanced compared with that of CIKs.

Furthermore, the difference in cell phenotype between CIKs and DC-CIKs was analyzed by flow cytometry. The results revealed that CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ and CD3⁺CD56⁺ T cells were significantly increased (1.02, 1.79, 1.26 and 2.44-fold, respectively) in DC-CIKs compared with CIKs (Fig. 3). The CD3⁺ phenotype is a characteristic of T cells (29). CD3⁺CD4⁺ T cells may induce differentiation into Th1 or Th2 cells by DCs, which secrete IFN- γ or IL-4, IL-10, and IL-13 (18). CD3⁺CD8⁺ T cells play an essential role in the immune response against cancers (30). CD3⁺CD56⁺ T cells are a subset of type II NKT cells with non-major histocompatibility complex-restricted tumor-killing activity (20,31). Collectively, these findings revealed that DC-CIKs were more effective as antitumor agents than CIKs.

To investigate the cytotoxicity of CIKs and DC-CIKs in Hela cells, cell viability analysis and MTT were used. As a result, the DC-CIK and CIK cell viability was 96% and 98%, respectively (Fig. 4). MTT revealed that the CIK and DC-CIK cytotoxicity was 58% and 80%, respectively, with a significant difference (Fig. 5). This is similar to the finding that CIK cytotoxicity was significantly enhanced using the MTT method (1,32). Thus, it may be assumed that DCs are beneficial to the enhancement of CIK cytotoxicity.

In conclusion, this study is likely to be useful in the application of DC-CIKs in antitumor therapy in the clinic. It describes for the first time how DCs co-cultured with CIKs are of benefit for the improvement of the CIK cell proliferation, cell phenotype and antitumor activity *in vitro*.

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