

Original Article

Effect of anti-asthma Chinese medicine Chuankezhi on the anti-tumor activity of cytokine-induced killer cells

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Abstract

Chuankezhi (CKZ), a new Chinese medicine, plays an important role in immunoregulation. Cytokine-induced killer (CIK) cells have been commonly used for immunotherapy in recent years. In this study, we aimed to investigate the immunoregulatory effect of CKZ on CIK cells. Peripheral blood monocytes were isolated from healthy donors, and CIK cells were generated by culturing monocytes with interferon-gamma (IFN- γ) and interleukin 2. Different concentrations of CKZ were added on day 2. After incubation for 14 days in culture, the antitumor effects of CIK cells were measured by cytotoxicity assay. Flow cytometry was used to explore the effect of CKZ on CIK cell immunophenotype, intracellular cytokine production, and apoptosis. The effect of CKZ on the antitumor activity of CIK cells in nude mice was also investigated. CKZ increased the percentage of CD3⁺CD56⁺ CIK cells but did not significantly change the percentage of CD4⁺, CD8⁺, or CD4⁺CD25⁺ CIK cells. CKZ-conditioned CIK cells showed a greater ability to kill tumor cells, as well as a higher frequency of IFN- γ and TNF- α production, compared with the CIK cells in the control group. CKZ also suppressed the apoptosis of CIK cells *in vitro*. Furthermore, CKZ combined with CIK cells had a stronger suppressive effect on tumor growth *in vivo* than the CIK, CKZ, or normal saline control groups. Our results indicate that CKZ enhances the antitumor activity of CIK cells and is a potential medicine for tumor immunotherapy.

Key words Chuankezhi, cytokine-induced killer cells, antitumor activity

Chuankezhi (CKZ) is a new Chinese medicine that is primarily composed of extracts of *Morinda officinalis* and *Epimedium*. *M. officinalis*, the dry root of plants of the Rubiaceae family^[1-3]. It contains several pharmacologically active ingredients, including onion wake compounds (such as rubiadin, rubiadin-1-methylether, 1-hydroxy-anthraquinone, 1-hydroxy-2-methylanthraquinone, 1,6-dihydroxy-2,4-dimethoxyanthraquinone, and phycion), iridoids (such as asperuloside, asperuloside acid, and desacetyl

asperuloside acid), organic acids, oligosaccharides, amino acids, and trace elements^[4]. The reported physiologic roles of *M. officinalis* are to improve the function of the cardiovascular system, regulate the function of the endocrine system, and enhance immunity^[4]. *Epimedium* is in the stems and leaves of Berberidaceae plants, and its major pharmacologically active ingredients include flavonoids possessing an 8-isoamyl alkenyl structure, alkaloids, and phenolic acid compounds^[5]. The reported physiologic role of *Epimedium* is to enhance immunity^[5]. CKZ was recently found to play an important role in relieving cough and asthma, and to have anti-allergy, anti-inflammatory, stress response, and immunoregulatory functions^[4]. Peripheral blood T lymphocytes of asthmatic patients who were treated with CKZ secreted more interferon-gamma (IFN- γ) and less interleukin-4 (IL-4)^[6]. In addition, CKZ was reported to up-regulate Th1 [IFN- γ , tumor necrosis factor (TNF)- α] and Th2 [interleukin (IL)-4, IL-6] cytokines secreted by peripheral blood monocytes from healthy individuals^[3]. In a clinical study, CKZ elevated immune function in patients with chronic obstructive pulmonary disease and was a safe and effective method for treating this condition^[7]. Similarly,

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CKZ improved endocrine and immune function and immune self-stabilization ability^[8].

Cytokine-induced killer (CIK) cells are cytotoxic lymphocytes generated by incubation of peripheral blood lymphocytes with anti-CD3 monoclonal antibody, IL-2, IL-1 β , and IFN- γ ^[9-13]. CIK cells possess a higher proliferation rate and an enhanced cytotoxicity compared with lymphokine-activated killer cells^[9,14,15]. The higher lytic cytotoxicity is mainly due to increased expansion of CD3⁺CD56⁺ cells^[16]. CIK cells lyse tumor cells in a non-major histocompatibility complex-restricted way^[14] and show antitumor activity *in vitro* and *in vivo*^[17-20]. More interestingly, CIK cells showed little or negligible cytotoxicity against normal tissues, including normal bone marrow^[21]. Moreover, CIK cells have also been tested in clinical trials for treatment of cancers such as hepatocellular carcinoma, renal cell carcinoma, malignant melanoma, and astrocytoma^[22,23]. Nevertheless, the functional role of CKZ in regulating CIK cells has not yet been determined.

In this study, we analyzed immunophenotypic changes and intracellular cytokine production of CIK cells cultured with or without CKZ. We also performed a cytotoxicity assay to evaluate the effect of CKZ on the antitumor activity of CIK cells and used an apoptosis assay to determine whether CKZ could prevent CIK cell apoptosis. Finally, we examined the role of CKZ in enhancing activity of CIK cells in suppressing tumorigenicity of tumor cells in mouse models.

Materials and Methods

Tumor cell lines and culture conditions

The hepatoblastoma cell line HepG2, the hepatocellular carcinoma cell line sk-Hep1, the lung cancer cell line A549, the renal carcinoma cell line ACHN, and the gastric carcinoma cell line SGC7901 were obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cells were incubated at 37°C in a humidified chamber containing 5% CO₂.

Preparation of CIK cells

Peripheral blood monocytes (PBMCs) were isolated from 50 mL samples of peripheral blood from healthy donors by Ficoll density-gradient centrifugation (GE Health, Piscataway, NJ, USA). The PBMCs were cultured in X-VIVO complete medium (Lonza, USA) supplemented with 1,000 U/mL IFN- γ and 160 U/mL gentamicin. After 24 h, 150 ng/mL mouse anti-human CD3 monoclonal antibody, 1,500 U/mL IL-2, and 10 ng/mL IL-1 α were added to CIK cells. CIK cells were divided into four groups: no CKZ (control), CKZ concentration 1 (1:100 dilution), CKZ concentration 2 (1:30 dilution), and CKZ concentration 3 (1:10 dilution). After 5 days of incubation, fresh medium (X-VIVO containing 1,000 U/mL IL-2 and CKZ) was added to the CIK cells, and after 14 days, the CIK cells were harvested.

Immunophenotypic analysis of CIK cells using flow cytometry

CIK cells were resuspended at 2×10^5 cells per 100 μ L of phosphate-buffered saline (PBS) and incubated for 30 min at 4°C with the following anti-human antibodies: PC5-conjugated anti-CD3, PC7-conjugated anti-CD4, FITC-conjugated anti-CD8, PE-conjugated anti-CD25, and FITC-conjugated anti-CD56 (BD Biosciences, USA). After three washes in cold PBS, cells were fixed with 4% paraformaldehyde. The cells were analyzed using a Cytomics™ FC500 Flow Cytometer (Beckman Coulter, USA). Data analysis was performed with CXP analysis software (Beckman Coulter, USA).

Analysis of intracellular cytokine expression using flow cytometry

CIK cells were collected and incubated at 37°C for 6 h in X-VIVO medium containing 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin (Sigma, USA). Brefeldin A (Sigma, USA), 10 ng/mL, was added for the final 5 h of incubation to block cytokine secretion. The cells were harvested, fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.1% saponin (Sigma, USA). Finally, the cells were labeled with APC-conjugated anti-IFN- γ , APC-conjugated anti-IL-4, and FITC-conjugated anti-TNF- α and analyzed by flow cytometry.

Cytotoxicity assay

Cytotoxicity assays were conducted using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit according to the manufacturer's protocol (Promega, USA). The four groups of CIK cells were used as effectors, and the targets were HepG2, A549, ACHN, and SGC7901 cells. The effector-to-target ratios were 3:1, 10:1, and 30:1.

Apoptosis assay

CIK cells that were cultured with different concentrations of CKZ were collected and washed twice in ice-cold PBS, resuspended in 400 μ L of 1 \times binding buffer, and incubated with annexin V-FITC (Bestbio, China) for 15 min at 4°C in the dark, according to the manufacturer's instructions. Then, the cells were incubated with propidium iodide (PI) for 5 min at 4°C in the dark. The cells were subsequently analyzed using a flow cytometer (Beckman Coulter, USA).

Tumorigenicity assays in nude mice

Female BALB/c athymic nude mice (4–5 weeks old) were obtained from the Medical Experimental Animal Center of Guangdong Province, China. The mice were randomly divided into four groups of 5 mice each: (1) normal saline (NS) control group with 2×10^6 sk-Hep1 cells suspended in 100 μ L normal saline and injected subcutaneously; (2) CKZ group with 2×10^6 sk-Hep1 cells suspended in 100 μ L CKZ and injected subcutaneously; (3) CIK group with

2×10^6 sk-Hep1 cells mixed with 1×10^7 CIK cells, suspended in 100 μ L normal saline, and then injected subcutaneously; and (4) CIK-CKZ group with 2×10^6 sk-Hep1 cells mixed with 1×10^7 CIK cells, suspended with 100 μ L CKZ, and then injected subcutaneously. Tumor size was monitored every 3 days by measuring length and width with calipers, and tumor volume was calculated as follows: $[\text{length} \times (\text{width})^2]/2$. Thirty days after inoculation, all mice were humanely killed, and the tumors were resected, photographed, and weighed. All experimental procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23, revised 1996) and the institutional ethical guidelines for animal experiments.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences, version 16.0 (SPSS Inc., Chicago, IL, USA). The results are presented as mean \pm standard deviation (SD) and analyzed using Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

CKZ induced an immunophenotypic change in CIK cells

To evaluate the effects of CKZ on CIK cells, we first detected immunophenotypic changes using flow cytometry. CD3⁺CD56⁺ cells are the major effector population of CIK cells. Therefore, we tested the percentage of CD3⁺CD56⁺ CIK cells after exposure to CKZ. After 14 days in culture, CKZ increased the percentage of CD3⁺CD56⁺ CIK cells, and this effect increased as the concentration of CKZ increased (Figure 1). However, the percentages of other subgroups, including CD4⁺, CD8⁺, and CD4⁺CD25⁺ CIK cells, did not change significantly following CKZ treatment, compared with the control group (Figure 2). Because CD3 and CD56 are markers of differentiation, these results indicate that CKZ promoted CIK cell differentiation.

CKZ increased the intracellular cytokine production of CIK cells

To further assess the effect of CKZ on CIK cells, we performed intracellular staining for IFN- γ , IL-4, and TNF- α . As the concentration of CKZ increased, the percentage of IFN- γ ⁺ or TNF- α ⁺ CIK cells significantly increased (Figure 3). IL-4⁺ CIK cells were rare and did not significantly differ among groups (data not shown). These results indicate that CKZ further activated and enhanced the immunofunction of CIK cells.

CKZ enhanced the cytolytic activity of CIK cells against tumor cell lines *in vitro*

To evaluate the effect of CKZ on the antitumor activity of CIK cells, we employed a cytotoxicity assay. As shown in Figure 4, CIK

cells without CKZ treatment killed HepG2 hepatoma cells, A549 lung cancer cells, ACHN renal carcinoma cells, and SGC7901 gastric cancer cells in varying degrees. Adding CKZ further enhanced the cytotoxicity of CIK cells against these tumor cells, and this effect increased with increasing concentrations of CKZ. At the 1:10 dilution, CKZ-conditioned CIK cells showed significantly higher lysis of tumor cells compared with the control group when the ratio of effector cells to target cells was 30:1 [(62.5 \pm 0.7)% vs. (49.5 \pm 0.7)% for HepG2; (38.0 \pm 2.4)% vs. (12.0 \pm 1.5)% for A549; (79.0 \pm 0.6)% vs. (57.5 \pm 2.1)% for ACHN; and (36.0 \pm 1.4)% vs. (24.0 \pm 1.2)% for SGC7901]. These results suggest that CKZ enhanced the cytolytic activity of CIK cells.

CKZ treatment prevented CIK cell apoptosis *in vitro*

We explored whether CKZ could prevent the apoptosis of CIK cells by performing annexin V/PI double staining in the CKZ treatment groups and control group. As shown in Figure 5, the apoptosis rate of CIK cells in CKZ concentration groups 1, 2, and 3 after 14 days of culture was (7.0 \pm 0.2)%, (6.6 \pm 0.1)%, and (6.4 \pm 0.0)%, respectively, whereas the rate in the control group was (7.5 \pm 0.1)%. These results indicate that CKZ prevented apoptosis of CIK cells.

CKZ enhanced the antitumor activity of CIK cells *in vivo*

To assess the role of CKZ in enhancing the activity of CIK cells in suppressing the tumorigenic activity of HCC cell line, sk-Hep1 cells mixed with or without CIK cells were suspended in normal saline or CKZ and then injected subcutaneously into nude mice. The treatment with CIK alone inhibited tumor growth compared with the normal saline control, but treatment with CKZ alone did not delay tumor growth (Figure 6A). However, a significantly greater inhibition of tumor growth was observed after treatment that combined CIK and CKZ (Figure 6A). Furthermore, the mean tumor volume in the CIK-CKZ group at the end of observation was significantly smaller than that in the CIK, CKZ, or normal saline control groups (Figures 6A and 6B). Accordingly, the mean tumor weight in the CIK-CKZ group was markedly lower than that in the CIK, CKZ, or normal saline control groups (Figure 6C).

Discussion

Cancer is a major cause of human deaths. Even with aggressive treatments such as surgery, chemotherapy, and radiation, patients with cancer usually have a poor prognosis^[24]. Previous research has demonstrated that dysfunction in cellular immunity, which impacts proliferation and metastasis *in vivo*, is one of the reasons that malignant tumors are incurable^[25,26]. As a new potential approach, adoptive immunotherapy holds great promise for the treatment of solid tumors^[27].

CIK cells are a heterogeneous subset of T lymphocytes whose biological features—easy expansion, reduced allo-reactivity, and MHC-unrestricted tumor-killing—make them appealing for adoptive

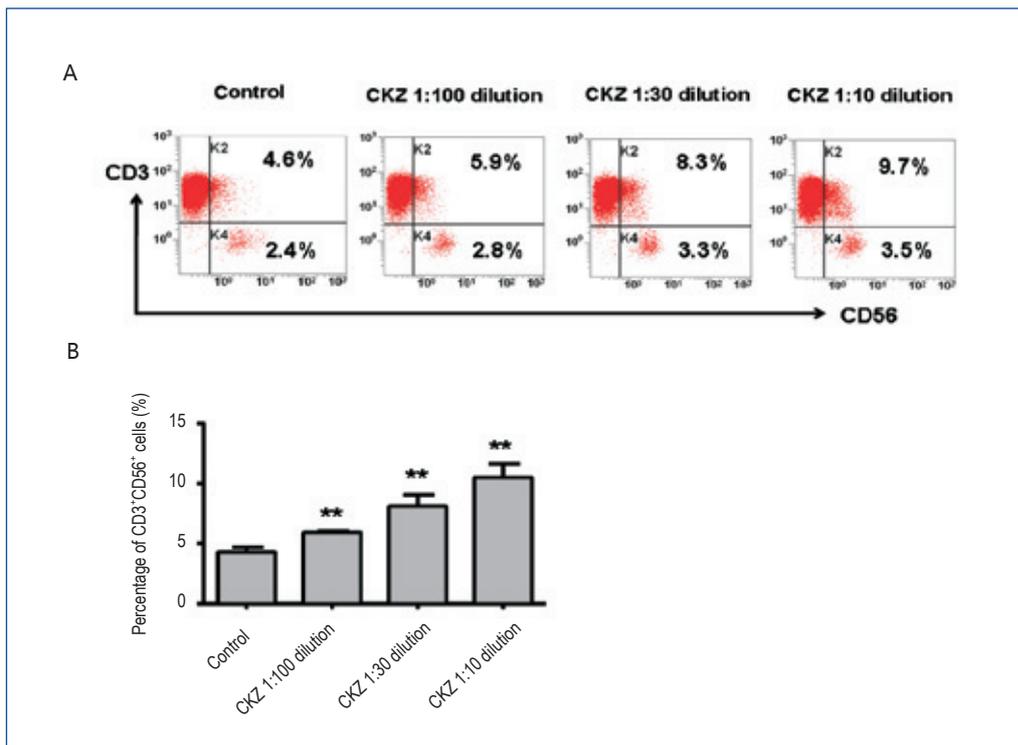


Figure 1. Chuankezhi (CKZ) increased the percentage of CD3⁺CD56⁺ subgroup of cytokine-induced killer (CIK) cells. CIK cells were harvested after 14 days of culture and then analyzed with flow cytometry. A, dot plots show the percentage of CD3⁺CD56⁺ cells after exposure to CKZ or no exposure to CKZ (control). B, the corresponding percentage of CD3⁺CD56⁺ cells is shown in the bar graph. Columns: mean of three experiments; bars: standard deviation (SD). ***P* < 0.01 versus control group.

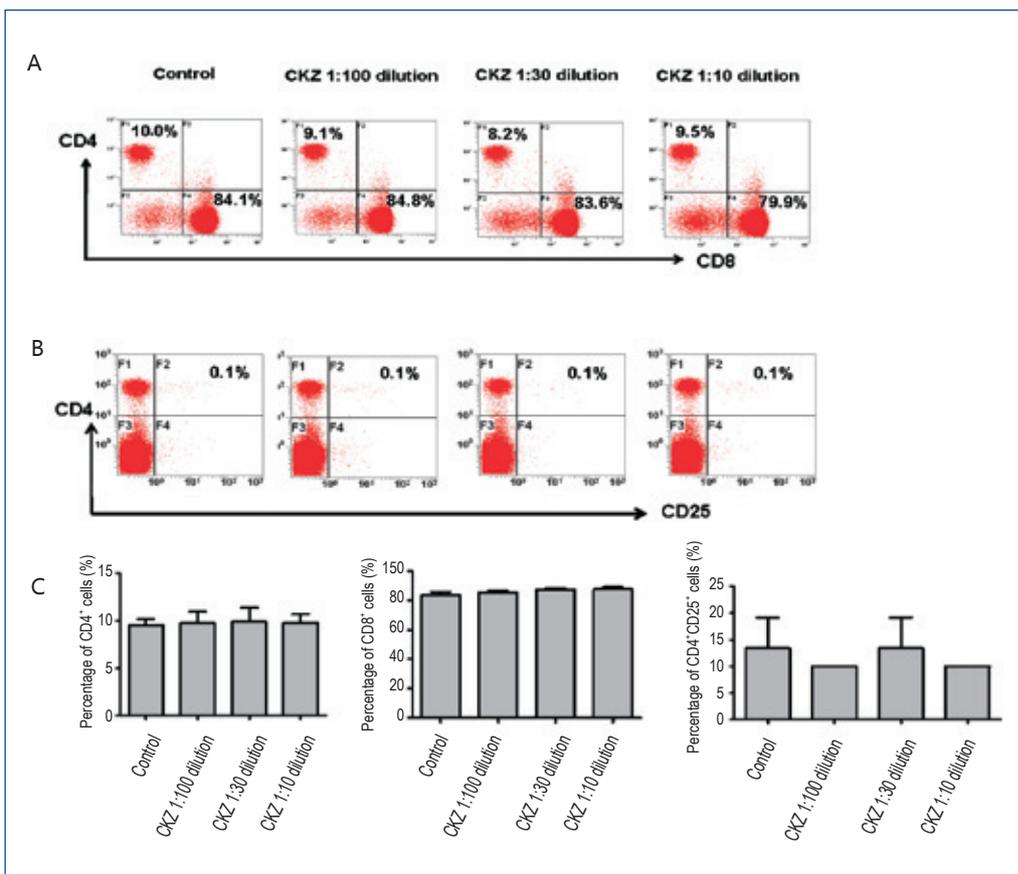


Figure 2. CKZ had no effect on the percentages of CD4⁺, CD8⁺, and CD4⁺CD25⁺ subgroups of CIK cells. After 14 days of culture, CIK cells were harvested and analyzed with flow cytometry. Dot plots show the percentages of CD4⁺ and CD8⁺ CIK cells (A) and the percentage of CD4⁺CD25⁺ CIK cells (B) after CKZ treatment or no CKZ treatment (control). C, the percentages of CD4⁺, CD8⁺, and CD4⁺CD25⁺ CIK cells are shown in the bar graphs. Columns: mean of three experiments; bars: SD.

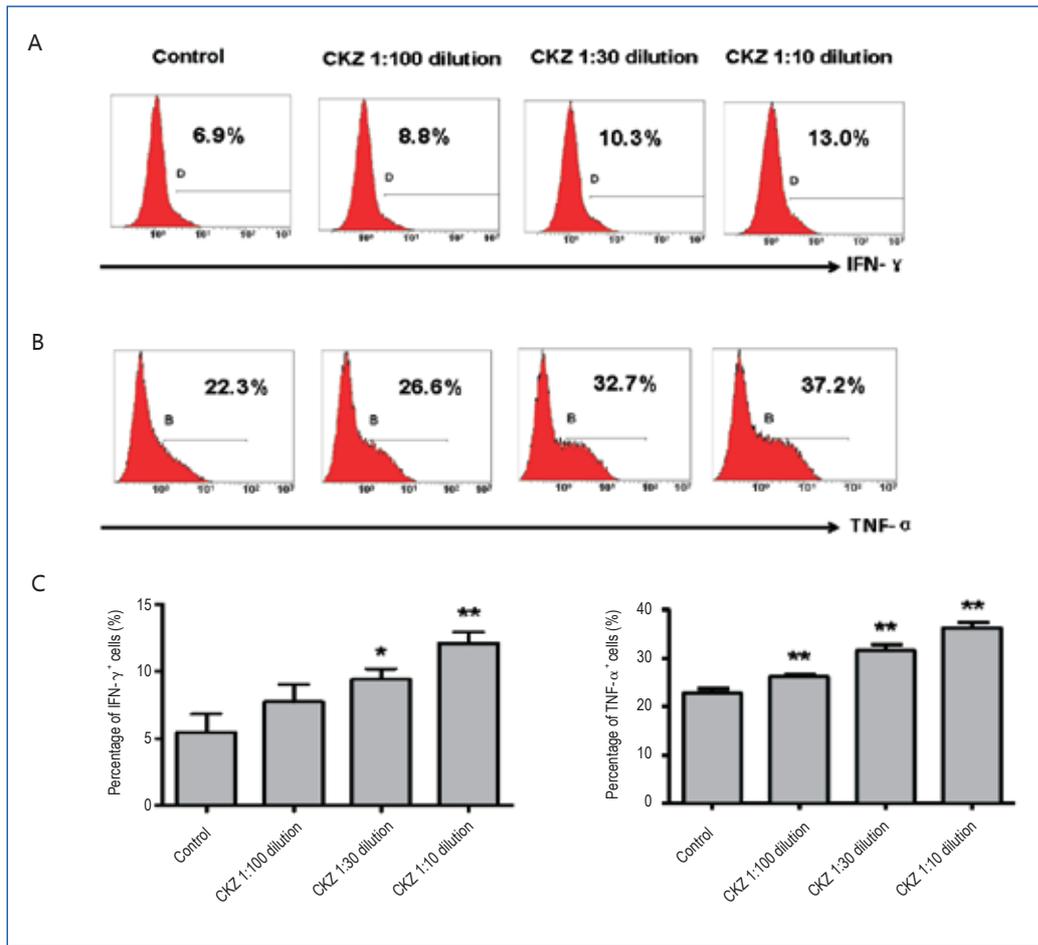


Figure 3. CKZ increased the intracellular cytokine (IFN- γ or TNF- α) production of CIK cells. After 14 days of culture, the CIK cells were harvested and analyzed with flow cytometry. Histograms show the percentage of IFN- γ ⁺ CIK cells (A) and the percentage of TNF- α ⁺ CIK cells (B) after exposure to CKZ or no exposure to CKZ (control). C, the bar graphs show the percentage of IFN- γ ⁺ or TNF- α ⁺ CIK cells. Columns: mean of three experiments; bars: SD. * $P < 0.05$, ** $P < 0.01$ versus control group.

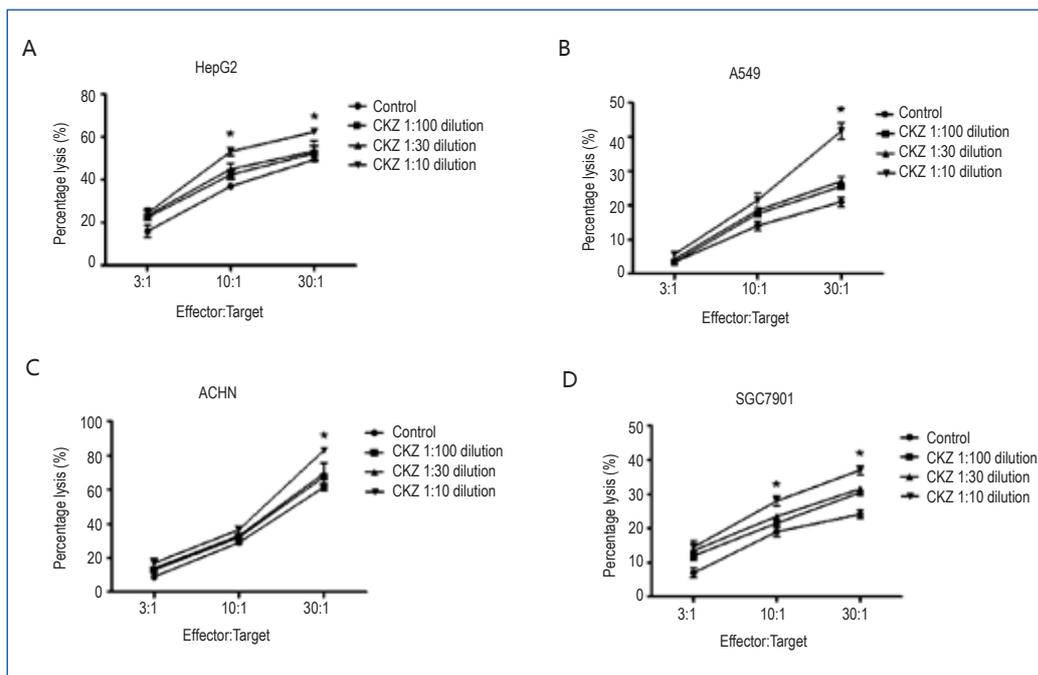


Figure 4. CKZ enhanced the cytolytic activity of CIK cells against tumor cell lines *in vitro*. After 14 days of culture, CIK cells were harvested and analyzed with a cytotoxicity assay. CIK cells treated with CKZ or not treated with CKZ (control) had antitumor effects on four types of tumor cells: A, HepG2; B, A549; C, ACHN; D, SGC7901. * $P < 0.05$ versus control group.

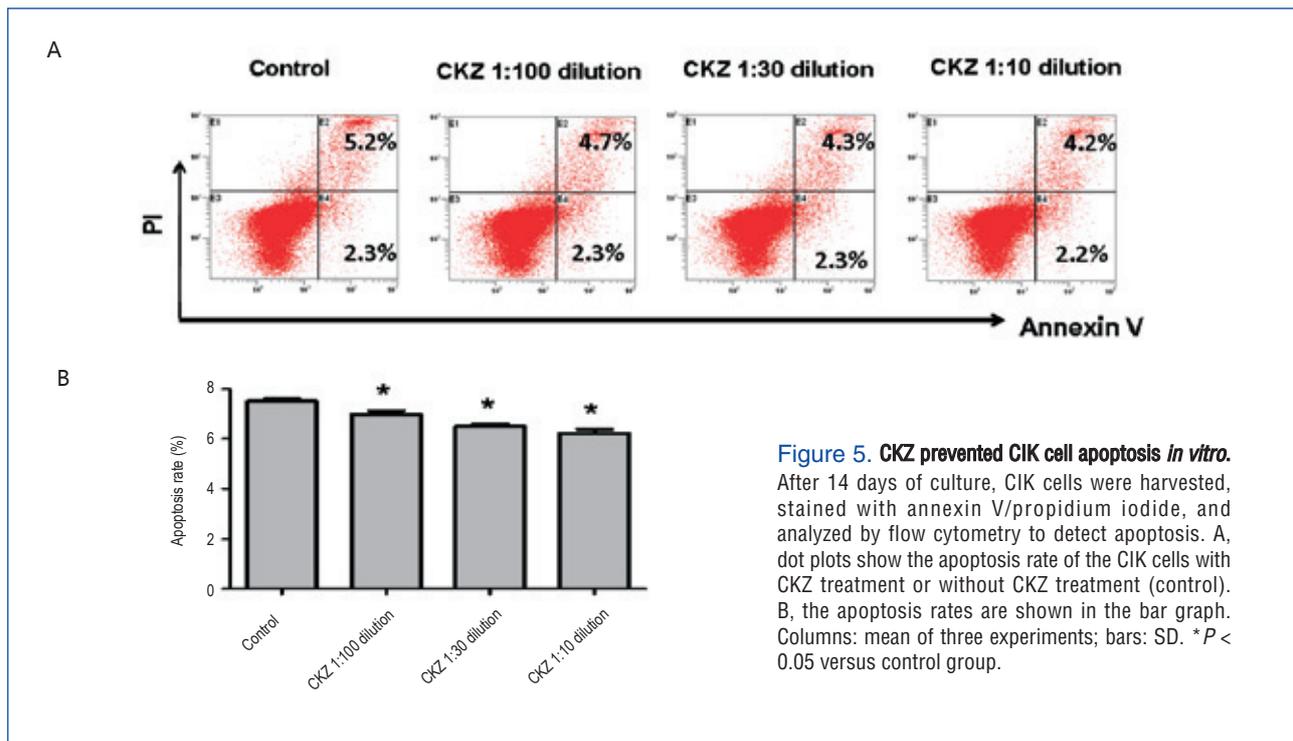


Figure 5. CKZ prevented CIK cell apoptosis *in vitro*. After 14 days of culture, CIK cells were harvested, stained with annexin V/propidium iodide, and analyzed by flow cytometry to detect apoptosis. A, dot plots show the apoptosis rate of the CIK cells with CKZ treatment or without CKZ treatment (control). B, the apoptosis rates are shown in the bar graph. Columns: mean of three experiments; bars: SD. * $P < 0.05$ versus control group.

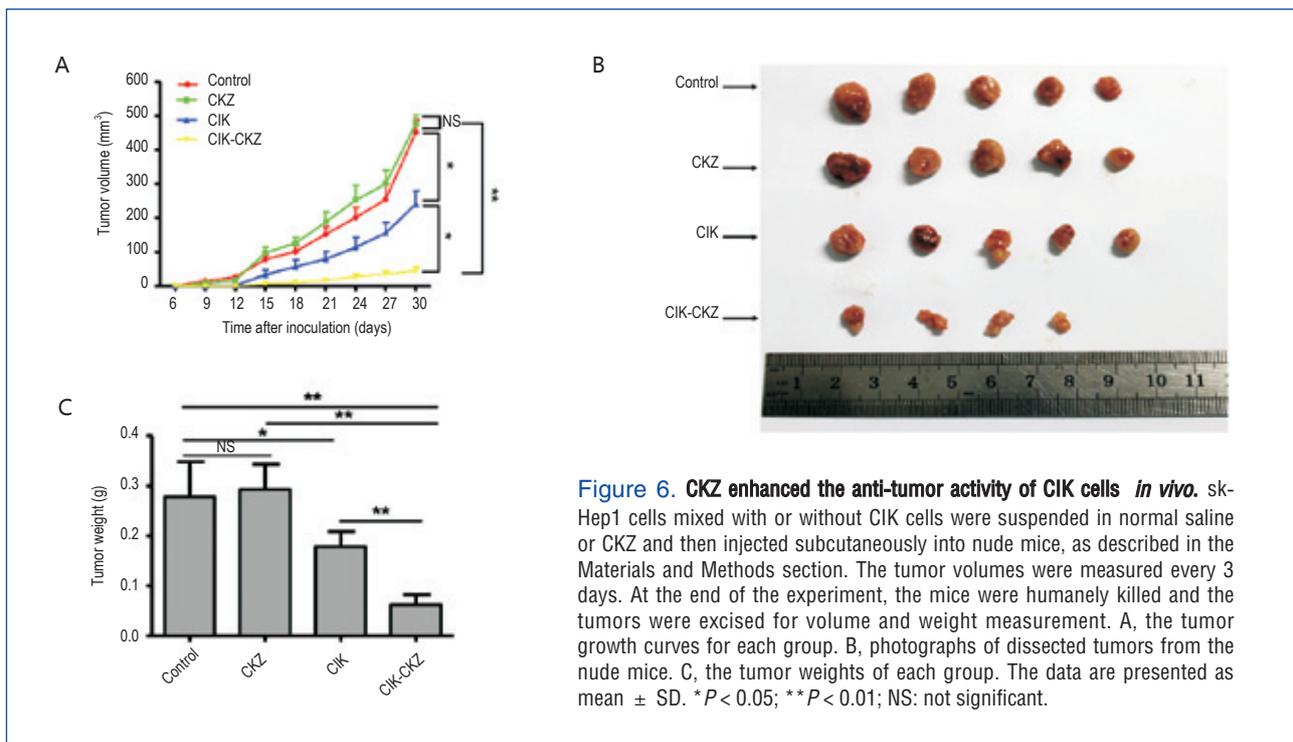


Figure 6. CKZ enhanced the anti-tumor activity of CIK cells *in vivo*. sk-Hep1 cells mixed with or without CIK cells were suspended in normal saline or CKZ and then injected subcutaneously into nude mice, as described in the Materials and Methods section. The tumor volumes were measured every 3 days. At the end of the experiment, the mice were humanely killed and the tumors were excised for volume and weight measurement. A, the tumor growth curves for each group. B, photographs of dissected tumors from the nude mice. C, the tumor weights of each group. The data are presented as mean \pm SD. * $P < 0.05$; ** $P < 0.01$; NS: not significant.

immunotherapy^[28,29]. The antitumor activity of CIK cells is mainly associated with the CD3⁺CD56⁺ fraction, not the CD8⁺ fraction that comprises the highest percentage^[30]. Therefore, increasing the

percentage of CD3⁺CD56⁺ cells and promoting CIK cells to secrete high levels of IFN- γ and TNF- α are important in elevating the anti-tumor activity of CIK cells.

In the present study, we reported the effect of CKZ, a new Chinese medicine, on the immunophenotype and intracellular cytokine production of CIK cells. CKZ increased the percentage of CD3⁺CD56⁺ CIK cells in the culture, which suggests that CKZ treatment may selectively enhance effector cells among CIK cells. Furthermore, CKZ treatment significantly enhanced the IFN- γ and TNF- α secretion levels of CIK cells. Consistent with our results, Zhao *et al.*^[3] found that CKZ up-regulated IFN- γ and TNF- α production of PBMCs *in vitro*. Wan *et al.*^[6] also found that peripheral blood T lymphocytes of asthmatic patients secreted more IFN- γ and less IL-4 after treatment with CKZ *in vivo*. Thus, CKZ may enhance the function of immuno-effector cells by up-regulating inflammatory cytokine production.

In line with the above findings, a cytotoxicity assay further confirmed that CKZ strengthened the tumor-killing abilities of CIK cells. In parallel experiments, we also found that CKZ combined with CIK cells had a stronger suppressive effect on tumor growth than the CIK, CKZ, or normal saline control groups in mouse models. We think this may be due to the increase in the percentage of CD3⁺CD56⁺ subgroup cells and in the production level of inflammatory cytokines. Shi *et al.*^[31] also found that CKZ treatment delayed cancer progression and induced disease stabilization in patients with advanced lung cancer. The possible reason may be improvement

of the ratio of natural killer cells in blood after CKZ treatment. Thus, CKZ is expected to help to enhance the body's antitumor ability by increasing the number of immuno-killer cells.

Our results also revealed that CKZ suppressed CIK cell apoptosis, indicating that CKZ maintained better viability of CIK cells. Thus, adoptive transfer of CIK cells combined with a CKZ regimen may point to improved treatment effects for cancer patients.

In conclusion, our data show that CKZ holds promise for enhancing the antitumor activity of CIK cells. Further investigation should be done to evaluate the efficacy of this drug in clinical applications.

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