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Original Article

Combined induction with anti-PD-1 and anti-CTLA-4 antibodies provides synergistic antitumor effects in DC-CIK cells in renal carcinoma cell lines

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Abstract: Immune escape of cancer cells has become the main challenge in the immunocytotherapy field. In this study, we analyzed the cytotoxicity of DC-CIK cells induced by anti-PD-1 and anti-CTLA-4 antibodies in RCC cell lines. Flow cytometry analysis was performed to analyze the immune phenotypes of DC-CIK cells. Click-iT EdU assay was performed to analyze the proliferation of DC-CIK cells. ELISA analysis was performed to detect the expression of cytokines in DC-CIK cells. Compared with DC-CIK cells without any treatment, the growth inhibition rate was significantly higher in the other three groups. Moreover, combined induction with anti-PD-1 plus anti-CTLA-4 antibodies provides synergistic antitumor effects of DC-CIK cells in renal carcinoma cell lines. The combined treatment promoted DC-CIK cell proliferation and differentiation into $CD3^+CD56^+$ NKT cells and $CD3^+CD8^+$ CTL cells. Compared with the control group, combined treatment significantly up-regulated the secretion of immune-stimulatory cytokines, such as IFN-γ and TNF-α, and down-regulated the secretion of the immunosuppressive cytokine IL-10. Furthermore, the co-induction promoted the early activation of DC-CIK cells. These results indicated the co-induction with anti-PD-1 plus anti-CTLA-4 antibodies improved antitumor effects of DC-CIK cells by promoting proliferation, differentiation, and early activation and regulating the secretion of immune-stimulatory and suppressive cytokines in renal carcinoma cell lines.

Keywords: Anti-PD-1 antibody, anti-CTLA-4 antibody, dendritic cell-activated CIK (DC-CIK) cells, renal carcinoma cell

Introduction

Adoptive dendritic cell-activated cytokine-induced killer (DC-CIK) cells transfer, an adoptive immunotherapy, represents a promising non-toxic anticancer therapy [1-3]. DC-CIK cells are a heterogeneous subset of ex-vivo expanded T lymphocytes derived from human peripheral blood after in vitro expansion and activation with interferon-γ (IFN-γ), interleukin-2 (IL-2) and anti-CD3 antibody [4, 5]. There are two main populations that can be distinguished within in vitro expanded DC-CIK cells, one presenting a $CD3^+CD56^+$ phenotype while the other presents a $CD3^+CD56^-$ phenotype which includes $CD3^+CD4^+$ T helper cells and $CD3^+CD8^+$ cytotoxic T cell subpopulations [6, 7]. The NKT phenotype cell populations ($CD3^+CD56^+$) mediate potent MHC-unrestricted cytotoxicity against cancer cells, and can recognize and kill a variety of cancer cells without prior exposure or priming [8]. The subpopulation of $CD3^+CD8^+$ cytotoxic T cells activated by dendritic cells (DC) is extremely important due to the pivotal role they play in specific cellular immune responses. Recent clinical studies indicated that the adjuvant immunotherapy with DC-CIK cells might prevent recurrence and improve life quality and progression-free survival rates of cancer patients [9, 10]. However, due to immune resistance of cancer cells, the therapeutic activity of adoptive DC-CIK cells is not as efficient as anticipated. The possible explanation is immune resistance of cancer cells, mainly mediated by both the immune "checkpoint" programmed death-1 (PD-1) pathway and negative immune regulation of T cell surface transmembrane cytotoxic T-lymphocyte-associated

protein 4 (CTLA-4) receptor signaling pathway [11].

PD-1 is mainly expressed on T cells after activation and also present on natural killer (NK) cells, exhausted T cells, activated B cells, dendritic cells, and activated monocytes [12, 13]. Programmed death-ligand 1 (PD-L1), the principal ligand of PD-1, is mainly expressed in various tumors, such as cervical, colon, gastric, HBV-related HCC, HCC, melanoma, NSCLC, ovarian and RCC. PD-1 ligation via PD-L1 inhibits the signaling pathway of T-cell receptor (TCR) and the secretion of immune-stimulatory cytokines [13, 14], and increases the immunosuppressive cytokine IL-10 [15, 16]. Unlike PD-1, the primary role of CTLA-4 is to play an important role in governing early activation of naive and memory T cells. CTLA-4 in the surface of T cells is induced upon TCR binding, and its ligation decreases the activation, proliferation, and function of T-cells [17, 18]. CTLA-4 binds the antigen-presenting cell ligands (B7) as a co-stimulatory molecule responsible for delivering the second signal of T-cell activation [19, 20]. Large number of clinical studies has confirmed that anti-PD-1 and anti-CTLA-4 antibodies can effectively suppress immune escape of cancer cells. Different from radiation and chemotherapy drugs, the mechanism of immunotherapy is not directly to kill cancer cells but indirectly attack them by augmenting specific antitumor cells of DC-CIK cells. However, immune deficiency or low immunity in the cancer patients seriously limits the efficacy of Nivolumab (an anti-PD-1 monoclonal antibody) and Ipilimumab (an anti-CTLA-4 monoclonal antibody) [21, 22]. Moreover, the expensive price of immune preparation severely restricts the clinical application and promotion, especially in developing countries.

However, until recently, there has been little information available in the literature about the antitumor effect of combining adoptive cell immunotherapy with immunotherapy agents. In this study, we conducted this research to determine whether anti-PD-1 and anti-CTLA-4 antibodies could improve the antitumor effect of adoptive DC-CIK cells transfer and illustrate the possible mechanisms by which immunotherapy agents released the full potential of adoptive cell therapy. Our findings showed that the induction using anti-PD-1 plus anti-CTLA-4 antibodies could improve the antitumor effects of DC-CIK cells. This suggested that combining immunotherapy agents with DC-CIK cells held great promise in the treatment of patients with advanced stage renal carcinoma.

Materials and methods

Ethics statement

This study was performed in strict accordance with the laboratory guidelines of the Ministry of Science and Technology of the People's Republic of China. All procedures were approved by the Committee on the ethics of human and animal experiments of South China University of Technology.

Cell culture

The two human renal carcinoma cell (RCC) lines ACHN and 786 cells were obtained from Nanjing KeyGen Biotech Co, Ltd (Nanjing, China) and cultured in Dulbecco's modified Eagle's medium (GIBCO, USA) containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C.

Generation of DC-CIK cells

Generation of DC-CIK cells was performed as previously described [23]. Human peripheral blood mononuclear cells (PBMCs) were obtained from health donors with written informed consent. PBMCs (1×10^7 cells/mL) were incubated in GT-T551 serum-free medium in a humidified atmosphere of 5% CO_2 at 37°C for 2 h. The adherent cells were exposed to GT-T551 medium containing IL-4 (30 ng/mL) and GM-CSF (100 ng/mL) for 7 days. Then the DC cells were activated with renal carcinoma cell lysate (20 μg/mL) and continued to be cultured for 2 days. The non-adherent cells were harvested following by gentle washing with PBS. After that, the non-adherent cells were stimulated and activated by 1000 U/mL IFN-γ and 20 μg/mL anti-CD3 antibody at 37°C for 24 h and in the presence of 1000 U/mL IL-2 were continued to be induced CIK cells for 4-5 days. Subsequently, mature DCs were mixed with the CIK cells at a ratio of 1:100 and cultured for 2 days to generate DC-CIK cells. The phenotypes of DC-CIK cells were characterized by flow cytometry analysis.

This experiment is divided into four groups: DC-CIK cells (Control group), DC-CIK cells treated with anti-CTLA-4 antibodies (CTLA-4 group), DC-CIK cells treated with anti-PD-1 antibodies (PD-1 group) and DC-CIK cells treated with anti-PD-1 plus anti-CTLA-4 antibodies (PD-1 + CTLA-4 group).

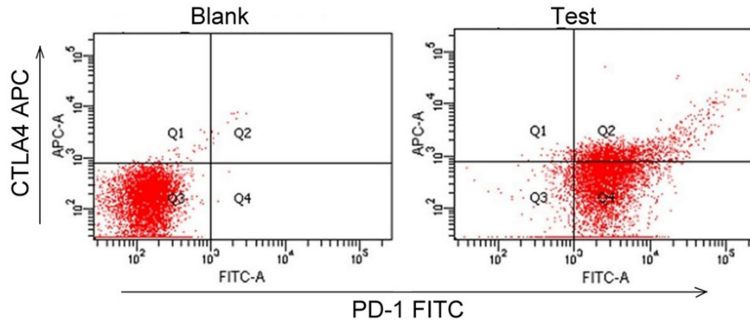

Figure 1. Flow cytometry analysis of PD-1 and CTLA-4 expression in DC-CIK cells. Representative data from at least three independent experiments are shown.

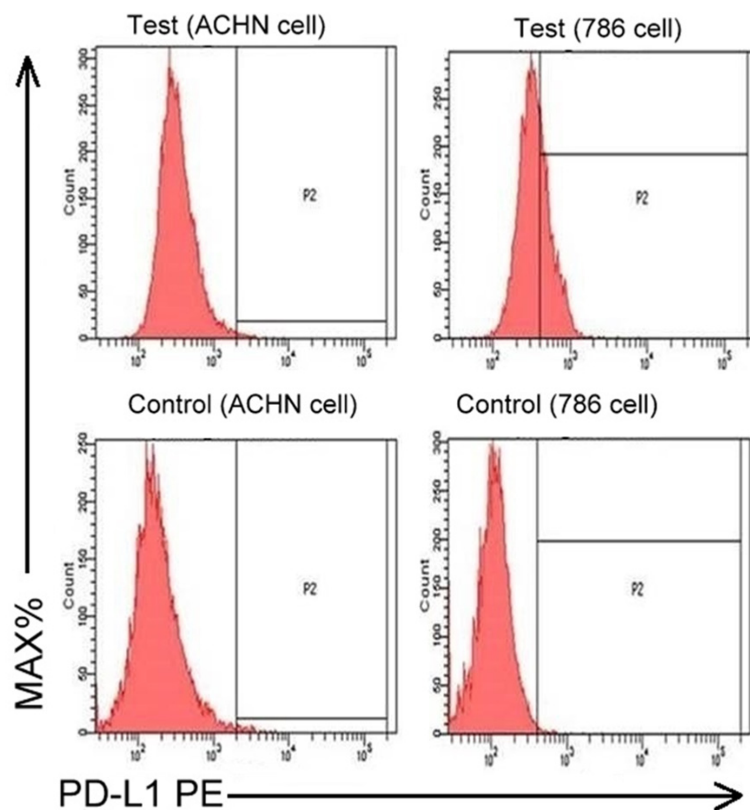

Figure 2. Flow cytometry analysis of PD-L1 expression in ACHN and 786 cells. Representative data from at least three independent experiments are shown.

Cytotoxicity of DC-CIK cells in different groups against the RCC cells

RCC cells at the logarithmic phase were collected and incubated in a 96-well plate at a concentration of 5 × 10^3 cells for 4 h. The RCC cells were reacted with 100 μL (10^4 cells) DC-CIK cells at an effector: target (E:T) cell ratio of 10:1 and cultured for 24 h. The RCC cells or each type of DC-CIK cells alone were used as negative controls. Following culture for 24 or 48 h, the 20 μL MTT (1 g/L) was added and the cells were continued to be cultured for 4 h. The supernatant was removed and 150 μL DMSO solution was added to each well, and then cells were slightly shaken for 15 min. The optical density value of each well was detected using Microplate Reader (BioTek, ELx-800) at a wave length of 490 nm. MTT assays were performed in six duplicates in three independent experiments.

Morphology

RCC cells at the logarithmic phase were collected and incubated in a 96-well plate at a concentration of 5 × 10^3 cells for 4 h. The tumor cells were reacted in triplicate with 10^4 DC-CIK cells induced with anti-CTLA-4 or anti-PD-1 antibodies at an estimated E:T ratio of 10:1, and cultured for 24 h. Subsequently, the morphology of tumor cells was examined by an inverted phase contrast microscope.

Click-iT EdU test

The Click-iT EdU assay was performed to analyze the proliferation of DC-CIK cells according to the manufacturer's instructions. DC-CIK cells were incubated with EdU for 12 h, and afterward images were used to determine percentages of EdU-labeled DC-CIK cells.

Flow cytometry and analysis

We analyzed the phenotype of DC-CIK by Flow cytometry analysis on FACSCAN using Cell

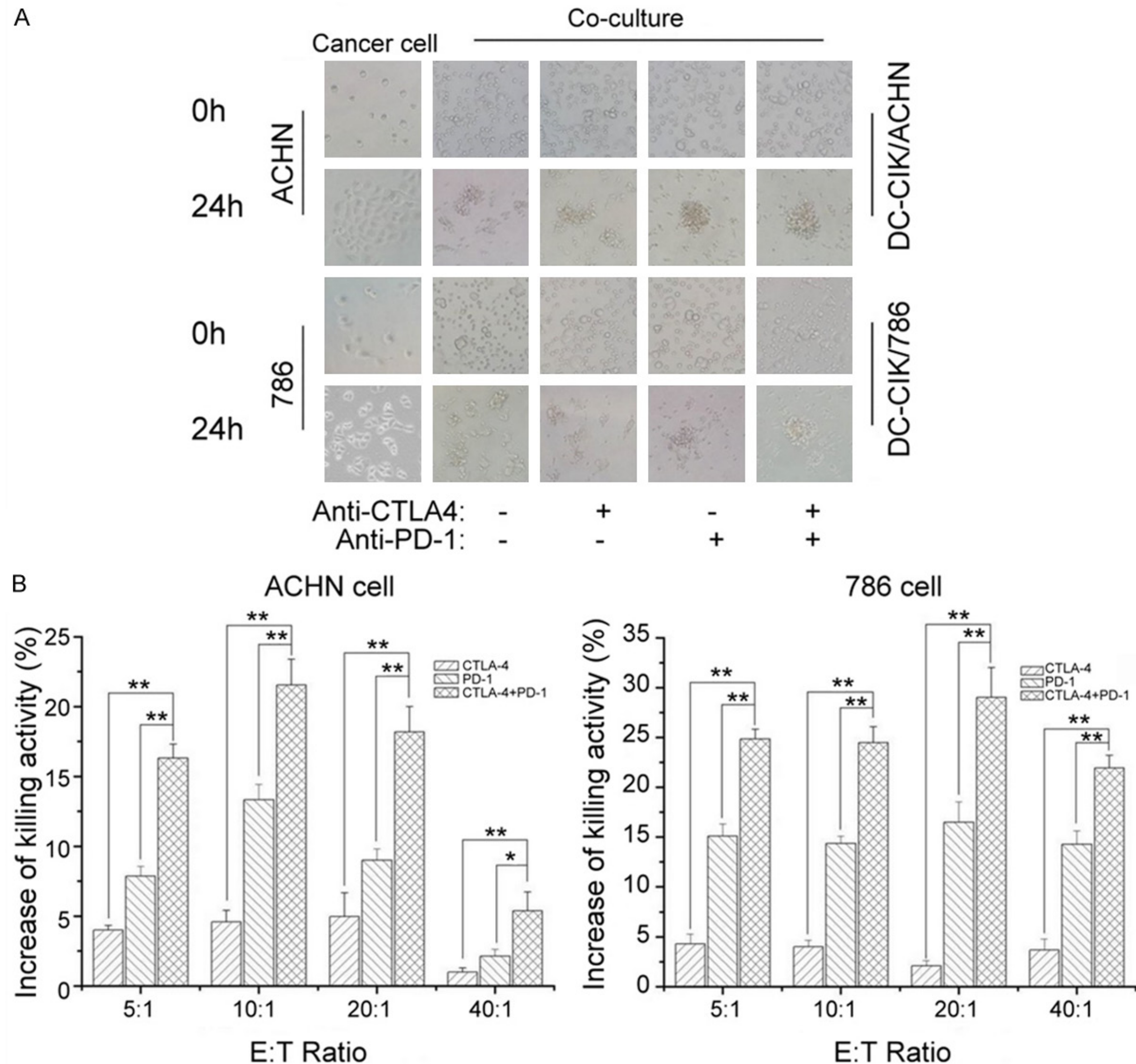

Figure 3. Cytotoxic analysis of DC-CIK cells against RCC cells in vitro. A. The cytotoxicity of DC-CIK cells against the monolayer tumor cells at 24 h post-interaction (Magnification, × 200); B. The cytotoxicity analysis of DC-CIK cells treated with anti-PD-1 or anti-CTLA-4 individually and in combination in RCC cells. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments, each of which was performed in triplicate. $*P < 0.05$, $**P < 0.01$.

Quest software (BD Bioscience). Data analysis was obtained to determine through Flow Jo software (Treestar).

Cytokine assay

Culture supernatants were collected from DC-CIK cells at 24 h after induction with induction with anti-PD-1 or anti-CTLA-4 antibodies, and concentrations of IL-2, IL-10, IFN-γ and TNF-α were respectively measured by sandwich enzyme-linked immunosorbent assay (ELISA) using four specific monoclonal antibodies according to the manufacturer's instructions (BD Biosciences, San Jose, CA).

Statistical analysis

All graphs were generated using Prism 4 (GraphPad Software, Inc.). Quantifiable determinations were expressed as means ± SEM of the indicated number. The significance of differences was evaluated using one-way ANOVA using SPSS 18.0. The Student-Newman-Keuls post-hoc test was performed when more than two conditions were evaluated. Significant

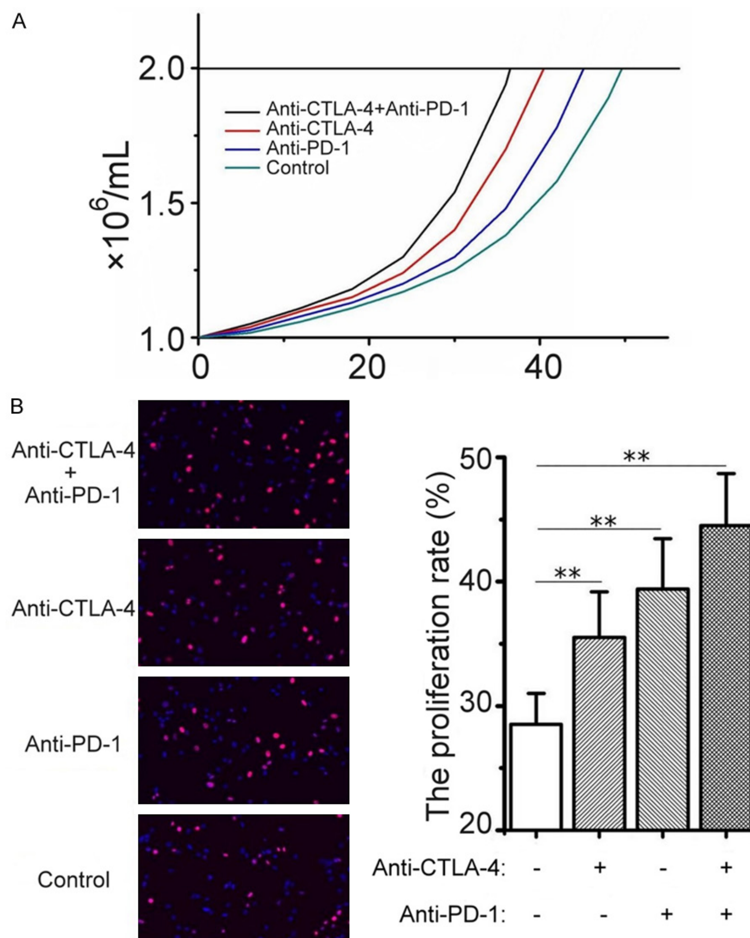

Figure 4. Proliferation analysis of DC-CIK cells treated with anti-PD-1 plus anti-CTLA-4 antibodies. A. Total cell number assays. Cells were seeded in both full-serum (10%) and total cell number counted every 12 hours. B. EdU incorporation assays showed a statistically significant increase in proliferation of DC-CIK cells treated with anti-PD-1 plus anti-CTLA-4 antibody. Results represent mean ± SEM. $^{**}P < 0.01$. Statistical significance was assessed by one-way ANOVA followed by the Student-Newman-Keuls post-hoc test ($P < 0.05$ was considered as significant).

changes were indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$.

Results

Characterization of immune cells

The results of phenotypic analysis showed the purity of DC-CIK cells was more than 90% and the majority of DC-CIK cells were $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$. These data was consistent with previous reports [24]. After 24 h incubation with anti-PD-1 and CTLA-4 antibodies, the percentage of PD-1 and CTLA-4 double-positive DC-CIK cells was 13.20% ± 1.24%, the percentage of PD-1 or CTLA-4 single positive cells were 97.23% ± 3.14% and 13.47% ± 1.31%, respectively (**Figure 1**).

Expression of PD-L1 in RCC cells by flow cytometry analysis

The results of flow cytometry analysis showed that the percentage of PD-L1 positive cells in 786 cells was significantly higher than that of ACHN cells, the percentage of PD-L1 positive cells was 48.23% ± 3.00% and 0.70% ± 0.25%, respectively, as shown in **Figure 2**.

Cytotoxicity of DC-CIK cells treated with anti-PD-1 or anti-CTLA-4 antibody in RCC cells

The MTT assays revealed that with the increase of E:T ratio, the growth inhibition rate of RCC cells was significantly enhanced. No matter the ratios of E:T, the co-incubation with anti-PD-1 plus anti-CTLA-4 antibodies significantly enhanced the growth inhibition for ACHN and 786 cells. Following 24 h treatment, the growth inhibition rate of RCC cells that were reacted with co-induced DC-CIK cells was significantly higher than that reacted with DC-CIK cells treated anti-PD-1 or anti-CTLA-4 antibody alone ($P < 0.01$, **Figure 3**). All of the q values were greater than 1.20 after 24 h, suggesting that combined treatment results in a synergistic effect. Compared with that of anti-CTLA-4 antibody, the antitumor effect of DC-CIK treated with anti-PD-1 antibody was more pronounced, especially for PD-L1 positive 786 cells.

The treatment with anti-PD-1 plus anti-CTLA-4 antibodies promotes the proliferation and differentiation of DC-CIK cells

By cell number analysis we found the treatment with anti-PD-1 plus anti-CTLA-4 antibod-

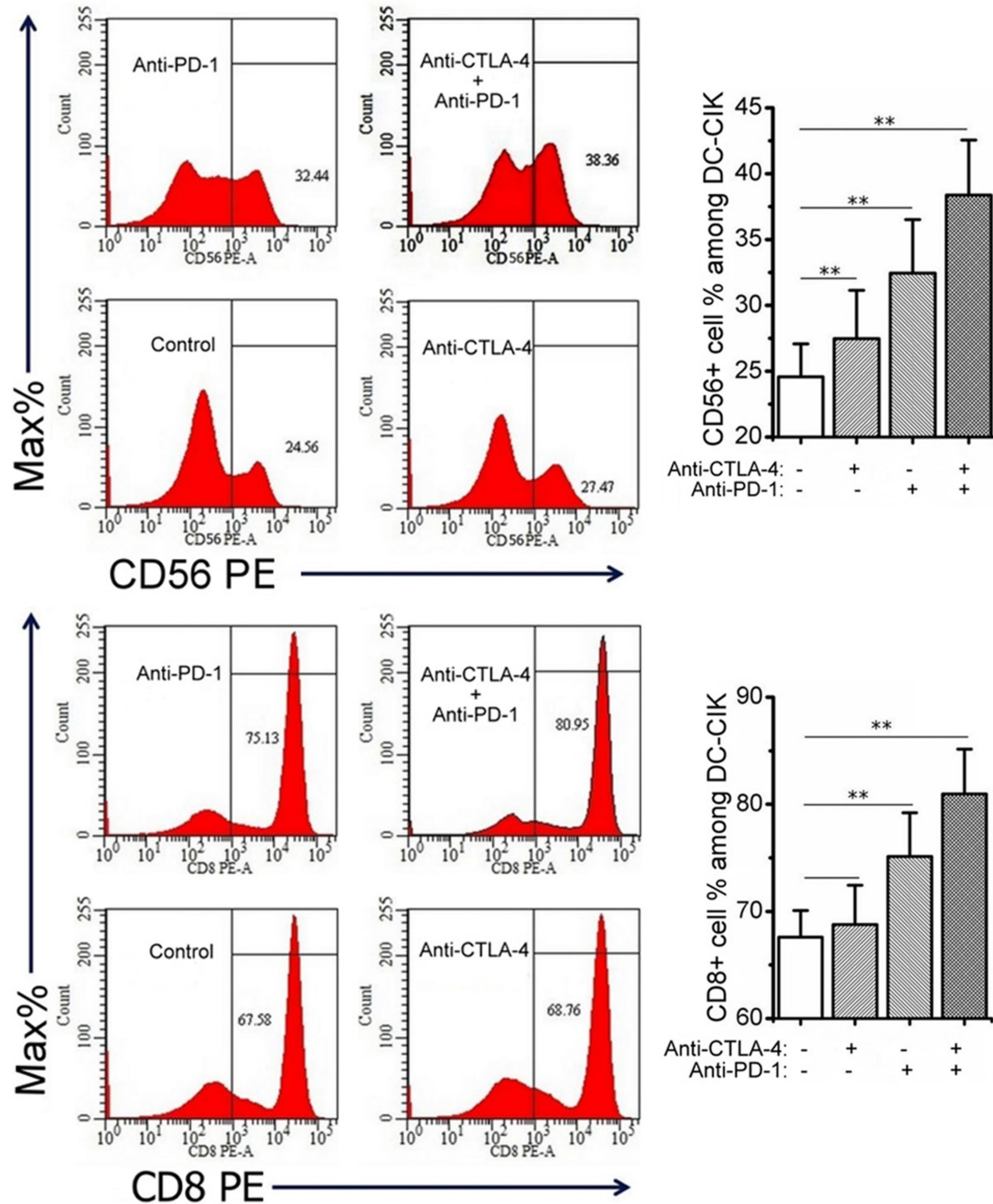

Figure 5. The treatment with anti-PD-1 plus anti-CTLA-4 antibodies promotes the differentiation of DC-CIK cells into $CD3^+CD56^+$ NKT cells and $CD3^+CD8^+$ CTL cells. Results represent mean ± SEM. $^{**}P < 0.01$. Statistical significance was assessed by one-way ANOVA followed by the Student-Newman-Keuls post-hoc test ($P < 0.05$ was considered as significant).

ies promoted the proliferation of DC-CIK cells and increased total cell number in vivo. The combined treatment increased DC-CIK cell number by 1.48-fold after 48 h, as shown in **Figure 4**. Furthermore, the proliferation rate of DC-CIK cells in combined treatment group was

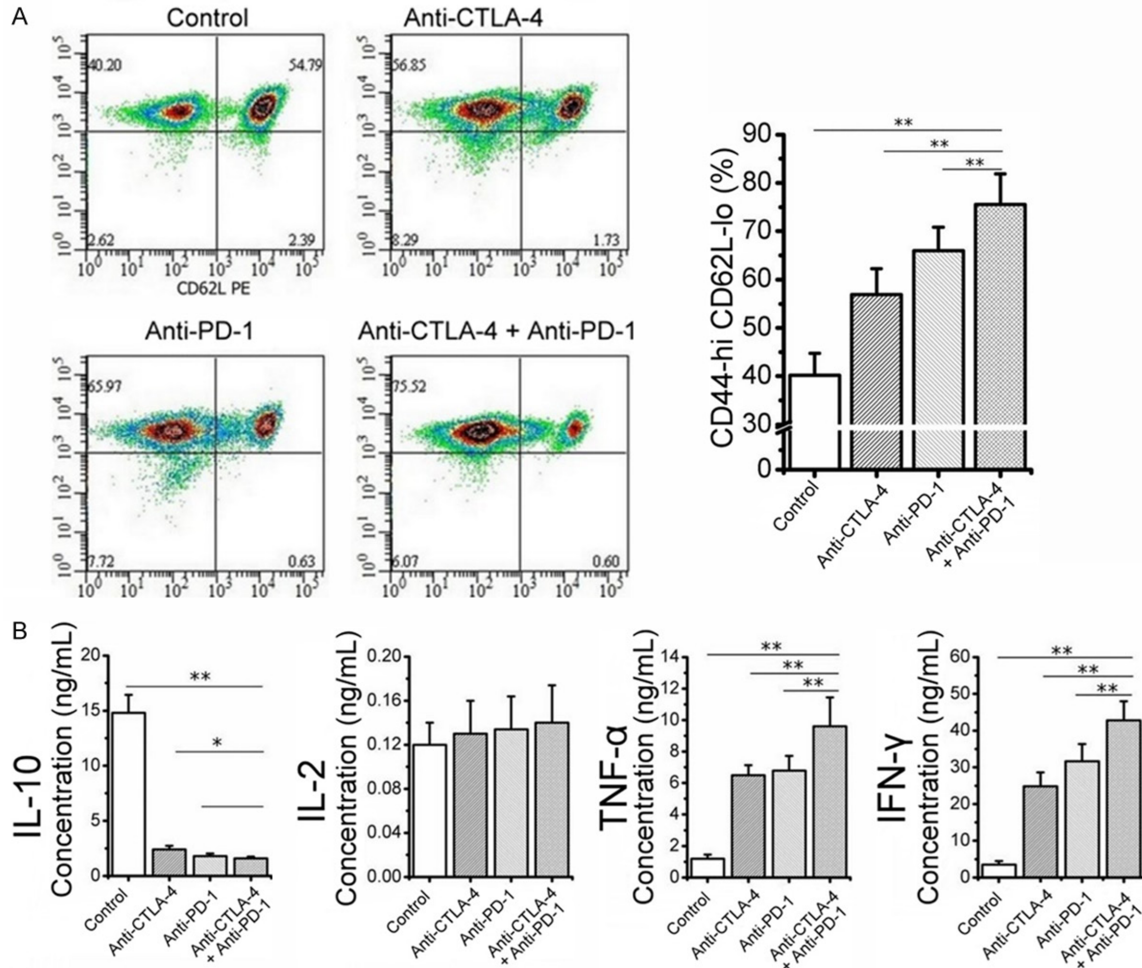

Figure 6. The effect of anti-PD-1 and anti-CTLA-4 antibodies on early activation markers of TCR and secretion of immune-stimulatory/suppressive cytokines in DC-CIK cells. A. The treatment with anti-PD-1 and anti-CTLA-4 antibodies promoted early activity of the DC-CIK cells. B. Culture supernatants were collected at 24 h, and the level of IL-10, IL-2, TNF-α and IFN-γ were analyzed by ELISA. For all conditions, supernatant from six duplicated wells was pooled for ELISA analysis and shown as means ± SEM. Shown are representative results from at least three experiments.

significantly increased and was significantly higher than the other three groups (**Figure 4**, $P < 0.01$).

Compared with the control group, the proportion of $CD3^+CD56^+$ NKT cells and $CD3^+CD8^+$ CTL cells significantly increased in the co-induction group ($P < 0.01$) and was significantly higher than that treated with anti-PD-1 or anti-CTLA-4 antibodies alone ($P < 0.01$, **Figure 5**). However, there was no significant difference in $CD3^+CD4^+$ T cells. These results indicated the combined treatment in vitro before the infusion had little effect on the expression of $CD3^+CD4^+$ T cells, but significantly increased the production of NKT and CTL cells.

Effect of anti-PD-1 and anti-CTLA-4 antibodies on early activation markers of TCR and secretion of immune-stimulatory and suppressive cytokines in DC-CIK cells

To further understand the mechanism of immunotherapy agent-mediated antitumor effect, the early activation markers of TCR were assessed in different groups. Consistent with the positive effect on cell proliferation, there was a global progression on the expression of the early activation markers CD44 and CD62L (**Figure 6A**, $P < 0.01$).

Culture supernatants were collected from DC-CIK stimulated with anti-PD-1 or anti-CTLA-4 antibodies after 24 h. The ELISA analy-

sis showed that compared with the control group, combined treatment significantly up-regulated the secretion of immune-stimulatory cytokines, IFN-γ and TNF-α, and reduced the production of the immunosuppressive cytokine IL-10. However, there was no significant difference in secretion of IL-2 (**Figure 6B**, $*P < 0.05$, $**P < 0.01$).

Discussion

This study contributes to understanding the molecular mechanisms of immunotherapy agent-mediated antitumor effect of DC-CIK cells on RCC cells. The combination of anti-PD-1 and anti-CTLA-4 antibodies provides synergistic antitumor effects of DC-CIK cells on RCC cells. The co-induction with anti-PD-1 plus anti-CTLA-4 antibodies promoted DC-CIK cell proliferation and differentiation into $CD3^+CD56^+$ NKT cells and $CD3^+CD8^+$ CTL cells which are the main effector cells of DC-CIK cells. In addition, the combined treatment up-regulated the secretion of immune-stimulatory cytokines and decreased the production of immunosuppressive cytokines in DC-CIK cells. As predicted, the co-induction promoted the early activation of DC-CIK cells. These results indicated the combined treatment provided synergistic antitumor effects of DC-CIK cells by promoting proliferation, differentiation, and early activation and regulating the secretion of immune-stimulatory and -suppressive cytokines in RCC cells.

Recently, many clinical studies have confirmed the antibody against the above receptor can effectively suppress immune escape of tumor cells. Nivolumab can abrogate the interaction of PD-1 with PD-L1 to modulate the antitumor immune response [31]. In vitro, the addition of Nivolumab to cultures resulted in improved T-cell proliferation, increased absolute numbers of T cells, augmented type 1 and 2 cytokine production, and enhanced cytotoxic T lymphocyte recognition and lysis of cancer targets [32]. Moreover, the administration of anti-CTLA-4 antibody to mice demonstrated that the treatment could mediate the regression of multiple implantable and spontaneous tumor types [33-35]. In light of these preclinical findings, a large number of agents were developed for use in humans, the most notable being the fully human antibody, Ipilimumab [36]. Ipilimumab demonstrated activity against various tumor types and safety studies [37, 38]. These checkpoint-blocking antibodies have demonstrated clinical activity in a variety of tumor types, such as NSCLC, RCC and melanoma. As novel antitumor agents, they have a distinct antitumor activity, underscoring their unique mechanism of activity.

Immunotherapy agents indirectly function by enhancing their killing activity of specific antitumor cells, which is different from radiation and chemotherapy drugs [12-15]. But immune deficiency or low immunity in the cancer patients seriously deduces the efficacy of immunotherapy. Administration of DC-CIK cell therapy to patients increases the number of specific antitumor cells and enhances immunity [39, 40]. The antitumor activity of DC-CIK cells is mostly owing to the high proliferative potential of $CD3^+CD8^+$ CTL cells and $CD3^+CD56^+$ NKT cells, which increased about 1,000 fold [23, 24]. The DC-CIK cells induced by anti-PD-1 and anti-CTLA-4 antibodies attack cancer cells by suppression of cancer immune escape. Moreover, compared with direct infusion of immunotherapy agents in vivo, smaller doses of therapy agents reduce the cost for application in cancer patients.

In summary, this study showed for the first time that the combination of anti-PD-1 and anti-CTLA-4 antibodies provides synergistic antitumor effects of DC-CIK cells in RCC cells and promotes DC-CIK cell proliferation, differentiation, and early activation. Our results would be helpful for further studies to elucidate the molecular functions by which anti-PD-1 and anti-CTLA-4 antibodies enhance antitumor effects of DC-CIK cells in RCC cells.

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Disclosure of conflict of interest

None.

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