Original Article

Anti-tumor effects of DC-CIK cell in combination with PXT/DDP against ovarian cancer in mice

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Abstract: Ovarian cancer is one of the most common gynecological malignant tumors in women. The mortality of epithelial ovarian cancer accounted for the first place in various gynecological tumors. The aim of the present study was to explore the anti-effect of dendritic cell-cytokine induced killer cell (DC-CIK cell) in combination with paclitaxel/cisplatin (PXT/DDP) against ovarian cancer in mouse. We divided 30 mice into 6 groups by the different therapeutic approaches, which was respectively control group (A0), DC-CIK-1 group (A1), DC-CIK-2 group (A2), PXT/DDP group (B0), PXT/DDP+DC-CIK-1 group (B1) and PXT/DDP+DC-CIK-2 group (B2). Tumor tissues morphological changes were observed by hematoxylin and eosin (HE) staining. The method of Td mediated dUTP nick and labeling (TUNEL) was used to detect apoptosis of tumor cells. Natural kill cells (NK-cells), T lymphocytes (T-cells) and B lymphocytes (B-cells) were examined by flow cytometric analysis. The results showed that DC-CIK cell in combination with PXT/DDP significantly improved tumor morphology and promoted Skov-3 cells apoptosis. The combination therapy remarkably reduced microvessel density (MVD). The protein level of vascular endothelial growth factor (VEGF) was inhibited dramatically in combination therapy groups. NK cells, T cells and B cells were up-regulated significantly. These results suggested that DC-CIK cell in combination with PXT/DDP significantly suppressed the tumor growth through inhibition of angiogenesis, which could significantly improve immune functions by increasing NK cells, T cells and B cells number. It could serve as a new approach for ovarian cancer treatment.

Keywords: Ovarian cancer, anti-tumor, DC-CIK cell, PXT/DDP, vascular endothelial growth factor (VEGF)

Introduction

Ovarian cancer is one of the three malignant genital tumors, and it has the highest mortality [1]. Epithelial ovarian cancer (EOC) account for 85% to 90% of primary malignant ovarian neoplasms [2]. The five-year survival rate is low in advanced ovarian cancer, because of the symptom of ovarian cancer in early stage is not so special, which the disease is usually in advanced stage when it is diagnosed clearly [3]. The optimal treatment was operation, combined with chemotherapy and radiotherapy. This tumor has an aggressive clinical process, with a high tendency for both recurrence and metastasis, which were factors affecting the outcome of surgical treatment. Therefore chemotherapy still is main method to treat ovarian cancer [4, 5].

From the yew roots, bark extract paclitaxel, is an anti-angiogenic drug, and pairs of the most effective anti-cancer [6, 7]. DDP is platinum antineoplastic drug of the first generation, which is a broad spectrum anti-tumor drug, efficient to a series of tumors, but at the same time also has side effects. It is widely used to match with other anti-tumor drugs [8]. The combination of PXT and DDP was applied as the first-line drug of ovarian cancer, at the same time of killing malignant cells; it would also kill the normal cells, which was adverse to the prognosis and quality of patients [6-9]. The current treatment strategy focuses on finding a way to kill tumors while leave healthy cells alone. Therefore, at present, immune therapy become more and more important. With the development of tumor immunology and molecular biology, immunotherapy is breakthrough that stimulates immune cells to enhance their anti-cancer activity [10]. It reported that killing efficiency of CIK cells to tumor cells was higher than lymphokine-activated killer cells (LAK cells) and tumor infiltrating lymphocyte (TIL cells) [11]. Along with
the study of CIK cells further, studies have discovered that CIK cells co-cultured with DC cells have stronger anti-tumor activity [12, 13].

CIK cells have activity of anti-tumor and proliferative. More importantly, CD3+ and CD56+ expressed on CIK cells, which have advantages of MHC restriction [14]. DC cells are the most important antigen-presenting cells in vivo, which have unique immune function and play an important role in cancer suppression [15]. CIK cells co-cultured with DC cells can significantly increase antigen-presenting specificity of DC cells and costimulatory molecules, which can decrease cytotoxicity of CIK cells [16]. Therefore, combined therapy with DC cells and CIK cells can directly inhibit established tumor cells and efficiently induce the anti-tumor immunity of body.

Several studies in vitro have documented that DC vaccines is therapeutic for ovarian cancer [17, 18]. Previous studies in vitro have already confirmed that CIK cell show obviously killing effect on ovarian cancer cells [19]. It reported that the immunotherapy of DC-CIK has made some progress on the treatment of ovarian cancer, which is definite curative effect to other tumors in clinical application [19-21]. The anti-tumor effect of DC-CIK has been clearly affirmed.

Growth of tumors are dependent on neovascularization, therefore, the inhibition of angiogenesis has broad applied in anti-cancer treatment prospectively [22]. VEGF is important angiogenic regulatory factor, which is essential for tumor growth. By specific inhibiting the neovascularization to prevent the tumors forming and growth, and it does not influence body’s normal organism function [23]. It reported that VEGF can determine the detection of biological behavior of cancer, which be used as evaluation of prognosis [22, 23]. Therefore, it is important to explore the effect of DC-CIK cell in combination with PXT/DDP against VEGF.

Materials and methods

Animal and tumor model

Female C57BL/6 mice were purchased at 6-8 weeks of age, weighing 20-22 g, from the lab animal center of 301 Hospital (Beijing, China). Animal experiments were conducted accordance with the local ethics committee of lab animal center (Hubei Arts and Science University, China), which follows the China Public Health Service’s guide for care and use of animals.

ID-8 cell were generated from MOSEC ovarian cancer cell lines of C57BL/6 mice (Zhejiang Tianhang Biological Technology Co., Ltd., Zhejiang, China), which was cultured in RPMI 1640 containing obtained 5% fetal calf serum, insulin-transferrin-selenium and 0.5% penicillin/streptomycin (Gibco Life Technologies, Carlsbad, CA, USA), in a 5% CO₂ atmosphere and incubated at 37°C. The backs of the mice were completely shaved, and a subcutaneous (SC) injection that contained 3×10⁶ ID-8 cell was administered to left shoulder. Mice were selected for tumor model when the diameter of the tumor ranged between 5-7 mm and was approximately round in shape.

Grouping and treatment

A total of 30 mice, with ID-8, were randomly divided into for six groups (n=5 per group), control group (A0): tumor mice were injected by cauda vein with 0.1 mL physiological saline once a week for four weeks, DC-CIK-1 group (A1): 8×10⁷ DC-CIK cells were intraperitoneally with sterile syringes in tumor mice once a day for consecutive 3 days on 1st week after injecting physiological saline on the first day. DC-CIK-2 group (A2): 8×10⁷ DC-CIK cells were intraperitoneally with sterile syringes in tumor mice once a day for consecutive 3 days on 1st and 3rd week after injecting physiological saline on the first day. PXT/DDP group (B0): tumor mice were injected by cauda vein with 0.1 mL PTX (5 mg/kg)+DDP (3 mg/kg) once a week for four weeks. PXT/DDP+DC-CIK-1 group (B1): 8×10⁷ DC-CIK cells were intraperitoneally with sterile syringes in tumor mice once a day for consecutive 3 days on 1st and 3rd week after injecting physiological saline on the first day. PXT/DDP+DC-CIK-2 group (B2): 8×10⁷ DC-CIK cells were intraperitoneally with sterile syringes in tumor mice once a day for consecutive 3 days on 1st and 3rd week after injecting PXT/DDP.

Executing animal and taking material

Whole blood was collected 2 weeks later after treatment ended from the orbit, which was stored in 2 mL centrifuge tube with heparin and conserved in the refrigerator at 4°C. The mice were sacrificed by cervical dislocation and were...
immediately immersed in the volume fraction of 75% ethanol for 5 min. The spleen was removed under sterile surgical conditions, and tumors were completely stripped for wet tumor weight, which were used for follow-up experiment.

**In vitro differentiation of DCs from bone marrow (BM) precursors**

BM-derived DCs were generated from primary cultures of femoral marrow obtained from 6-7-week-old female C57BL/6 mice. The mice were sacrificed by cervical dislocation and were immediately immersed in the volume fraction of 75% ethanol for 5 min, as described above. The DCs were derived from BM as previously described [24]. Briefly, the BM cells were depleted of lymphocytes. The remaining cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco), 1% HEPES buffer, 1% penicillin-streptomycin, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine, 2-mercaptoethanol, recombinant murine granulocyte-macrophage colony-stimulating factor (mGM-CSF; 20 ng/mL; R&D Systems, Inc., Minneapolis, MA, USA) and recombinant murine interleukin 4 (mIL-4; 10 ng/mL; R&D Systems). The media was changed every two days, and fresh complete RPMI medium containing mGM-CSF and mIL-4 was applied each time. On the 7th day, tumor necrosis factor-α (TNF-α; 10 ng/ml; R&D Systems) was added to the media.

**In vitro differentiation of CIK from spleen precursors**

The mice were sacrificed by cervical dislocation and were immediately immersed in the volume fraction of 75% ethanol for 5 min. The spleen was removed under sterile surgical conditions and the spleen cells were isolated. The cells were washed twice with RPMI 1640 containing 1% fetal bovine serum (FBS; Gibco), 1% HEPES buffer, 1% penicillin-streptomycin, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine, 2-mercaptoethanol, recombinant murine granulocyte-macrophage colony-stimulating factor (mGM-CSF; 20 ng/mL; R&D Systems, Inc., Minneapolis, MA, USA) and recombinant murine interleukin 4 (mIL-4; 10 ng/mL; R&D Systems). The media was changed every two days, and fresh complete RPMI medium containing mGM-CSF and mIL-4 was applied each time. On the 7th day, tumor necrosis factor-α (TNF-α; 10 ng/ml; R&D Systems) was added to the media.

**Flow cytometry analysis**

Evaluation of cell count was performed by using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA). Briefly, cells were suspended in 500 μL binding buffer (0.01 M Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) with a mixture of 5 μL FITC Annexin V (1 μg/mL) and 5 μl PI (2 μg/mL) to incubate for 15 min. The samples were analyzed using a BD FACS Canto II flow cytometer (San Diego, CA, USA).

**HE staining**

Thin slices of tumor tissue for all cases received in histopathology unit were fixed in 4% formaldehyde solution (pH 7.0) for periods not exceeding 24 h. The tissues were processed routinely for paraffin embedding, and 4 μm-thick sections were cut and placed on glass slides. Tissue samples were stained with hematoxylin and eosin, and the two experienced pathologist determined the histological type.

**Microvessel density**

Microvessel density (MVD) in the tumor stroma was measured to quantitatively assess angiogenesis [25]. CD34 is usually used as an indicator of angiogenesis, as CD34 is a marker of undifferentiated endothelial cells, which are added after 24 h. Fresh medium and IL-2 were added to each well every two days.

**DC cells and co-culture with CIK cells**

Subsequent to five days of culture, the DC cells were incubated at 37°C in a 5% CO₂ atmosphere for 48 h. On day nine, these two groups of cells were co-cultured with CIK cells, which had been cultured for seven days, at a cell ratio of 1:5, for 48 h.

**Immunophenotype analysis of DC-CIK cells**

After 7 days of culture, DC-CIK cells were harvested, washed and centrifuged. Cell density was adjusted to 1×10⁷ cells/mL. The DC-CIK cells were blocked with 2% human immunoglobulins (2 μL/100 μL cells) for 15 min. Cells were then washed, centrifuged and re-suspended in 1 mL PAB before subjecting them to flow cytometry.
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found in the new blood vessels of malignant tumors. High density of undifferentiated blood vessels indicates a worse prognosis [26]. MVD was quantified by measuring the number of

Figure 1. Immunophenotype analysis of DC-CIK cells. The cellular purity of DC and CIK cells were detected with flow cytometer before and after sorting, which were more than 80%. DC and CIK cells were co-cultured, cell density was adjust to $1 \times 10^7$ cells/mL. By means of flow cytometry, the proportion of CD3+/CD56+ in the DC-CIK population was 30.13%.

Figure 2. Effect of DC-CIK combined with PTX/DDP on tumor tissue. A0 as comparison group; A1-B2 as treatment groups; A: Shows the photos of each group’s solid tumor; B And C: Respectively is tumor weight and tumor volume of each group in line chart; D And E: Respectively is histogram of tumor inhibition rate and growth rate. Data were presented as mean ± SD, n=5, *$P<0.05$, **$P<0.01$, †$P<0.05$, ‡$P<0.01$, §$P<0.05$, §§$P<0.01$, ^$P<0.05$, ^^$P<0.01$, compared with A0 or B0 group.
CD34-positive endothelial cells in the tumors. Five random fields per tumor sample, at 200 magnifications, were captured. MVD was reported as the average number for each group [25].

TUNEL staining

Paraffin-embedded ID-8 tumor sections (6 μm) were stained for apoptosis cells were detected by a terminal deoxynucleotidyl tranferase-mediated dUDP-nick-end labelling (TUNEL) apoptosis assay kit (Roche, Indianapolis, IN, USA), as previously described [20]. Nuclei were stained using 4,6-diamidino-2-phenylindole. In brief, images were captured by digital imaging (×200 magnification) sequentially over the entire cut surface of tumor tissue. Each image was divided into 192 squares by a grid. A square without a PTC was considered positive for loss. Images of β-catenin staining were captured in 10 random medullar fields per mice, and the mean area of positive staining was quantified. Images were obtained by Nikon microscopy (Nikon, Tokyo, Japan) and processed by NIS-Elements software.

Western blot

Tumor tissue homogenate was prepared were lysed with RIPA buffer (1% SDS 10%, 1% NP40, 0.5% de Sodium deoxycholate) plus protease inhibitors. The homogenate was centrifuged at 12000 rpm for 15 min at 4°C and the supernatant (30-50 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a nitrocellulose NC membrane (Millipore, Shanghai, China), then detected with VEGF protein. Protein loading was estimated using mouse anti-GAPDH monoclonal antibody. Blots were visualized using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China). Antibodies were purchased from Cell Signaling Technology, Abcam, Santa Cruz.

Statistical analyses

Data are expressed as mean ± standard error. Statistical analysis was performed using IBM SPSS 19 software (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used to determine the significant differences of the tumor growth rates, tumor inhibition rates and lymphocyte count between control and treatment groups. TUNEL and CD34 measurements were analyzed with the unpaired Student’s T-test. All tests performed were 2-sided. A p-value <0.05 is considered to be statistically significant.

Results

Immunophenotype analysis of DC-CIK cells

The cellular purity of DC and CIK cells were detected with flow cytometer before and after sorting, which were more than 80% (Figure 1).
DC and CIK cells were co-cultured by 1:5, cell density was adjusted to $1 \times 10^7$ cells/mL and re-suspended in 1 mL PAB. Flow cytometry tests expression of CD3+/CD56+ in co-culture of DC and CIK cells. The proportion of CD3+/CD56+ in the DC-CIK population was 30.13% (Figure 1).

**DC-CIK combined with PTX/DDP exhibited significant anti-tumor effects**

All of tumors were tidily arranged on a white sheet of paper according to different groups, which could be see directly (Figure 2A). The results showed that tumor weight of A0, A1 and A2 groups respectively were (91.8 ± 4.55), (63.21 ± 4.72) and (50.4 ± 4.46) mg, and tumor weight of B0, B1 and B2 groups respectively was (84.02 ± 3.67), (45.6 ± 2.97) and (38.1 ± 2.24) mg, the tumor weight of B2 group was decreased notably than other groups ($P<0.05$) (Figure 2B). The tumor inhibition rate of A (1-2) and B (0-2) groups were increased significantly than A0 group ($P<0.05$), and results showed that the tumor inhibition rate of A1, A2, B0, B1 and B2 groups respectively was 31.07 ± 2.77%, 45.08 ± 2.81%, 8.41 ± 2.95%, 50.25 ± 1.85%, 58.47 ± 3.95% (Figure 2D). It was showed in Figure 2C, the tumor volume of B0, B1 and B2 groups was substantially below tumor volume in the A0, A1 and A2 groups ($P<0.05$), the tumor volume of A0, A1, A2, B0, B1 and B2, in order, was (1.75 ± 0.12), (1.24 ± 0.09), (0.86 ± 0.07), (1.34 ± 0.76), (0.64 ± 0.07) and (0.52 ± 0.11) cm$^3$. The A (0, 1 and 2) and B (0, 1 and 2) groups respectively were gradually to reduce tumor growth rate, the tumor growth rate of B2 group was the lowest in all groups, which was 26.2 ± 1.24 cm$^3$ per day.

Figure 4. Effect of DC-CIK combined with PTX/DDP on tumor angiogenesis. A: Shows the immunohistochemical demonstration of microvessel density using anti-CD34 antibody in a tumor (×200). A0 as comparison group; A1-B2 as treatment groups; Tumor tissue shows high microvessel density in A0 group, but B1 and B2 groups show low microvessel density. B: Is histogram of tumor microvessel density. C: Shows the effect DC-CIK combined with PTX/DDP on the expression of VEGF, which were detected the expression by western blot. The expression of VEGF shows high level in A0 group, but B1 and B2 groups show low levels of expression. GAPDH was also detected as the control of sample loading. D: Is histogram of protein level. Data were presented as mean ± SD, n=5, *$P<0.05$, **$P<0.01$, $^*P<0.05$, $^{#*}P<0.01$, $^{#P}<0.05$, $^{##}P<0.01$, $^\Delta P<0.05$, $^{##\Delta}P<0.01$, $^\wedge P<0.05$, $^{##\wedge}P<0.01$, compared with A0 or B0 group.
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The morphological changes of tumor tissue by HE staining were shown in Figure 3, and results showed that intercellular substance was not clearly in A0 group, and morphological changes including chromatic agglutination and karyopyknosis could be observed in some of the cells under microscope. Intercellular substance was clearly visible in B2 group, and cells arranged in neat rows.

DC-CIK combined with PTX/DDP exhibited significant anti-angiogenesis effects

In Figure 4, the quantity of MVD with CD34 marks increased significantly and stained stronger in A0 group (Figure 4A). Staining in tumor decreased both in the B1 and B2 group, compared with that of the control group (Figure 4B) \((P<0.05)\). The MVD of B2 group was far less than A0 group, which was 1.83 ± 0.75 mm². The MVD of A0, A1, A2, B0 and B1 groups respectively was \((25.83 ± 1.53), (12.17 ± 1.74), (8.51 ± 1.05), (20.50 ± 1.47)\) and \((5.0 ± 1.57)\) mm² (Figure 4B).

DC-CIK combined with PTX/DDP inhibited the expression of VEGF

VEGF was closely associated with tumor angiogenesis. Therefore, it was detected the expression by western blot. Compared with the A0 group, VEGF expression was significantly suppressed in A1, A2, B0, B1 and B2 groups \((P<0.05)\) (Figure 4C and 4D). The expression of VEGF in A0, A1, A2, B0, B1 and B2, in order, was \(0.51 ± 0.02, 0.31 ± 0.02, 0.15 ± 0.02, 0.41 ± 0.03, 0.15 ± 0.02\) and \(0.07 ± 0.02\) (Figure 4D).

DC-CIK combined with PTX/DDP significantly promoted apoptosis in tumor cells

The apoptosis level of tumor cells was evaluated by TUNEL. The results showed that apoptosis level of tumor cells in A1 and A2 groups were significantly increased than A0 group, and The apoptosis level of tumor cells in B1 and B2 group far more than A0 and B0 groups \((P<0.05)\) (Figure 5). The apoptosis index of A (0-2) and B (0-2) was \(9.11 ± 1.02\), \(23.02 ± 2.61\), \(31.67 ± 3.04\), \(12.67 ± 1.08\), \(38.77 ± 1.51\) and \(50.01 ± 2.57\), in that order (Figure 5B).

DC-CIK combined with PTX/DDP regulated expression of immunocyte

The percents of T-cells, B-cells and NK-cells were analyzed by Flow cytometry. The percentage of T-cells showed a significant rise in A1, A2, B0, B1 and B2 groups compared with A0 group \((P<0.05)\) (Figure 6). The percents of T-cells in B2 group was much higher than in the other group \((P<0.05)\), which was \(30.29 ± 0.89\%\) (Figure 6B). The rest of the group, in order, was \(8.01 ± 0.89\), \(20.61 ± 0.86\), \(22.47 ± 1.16\), \(9.67 ± 0.63\) and \(27.20 ± 1.91\%\) (Figure 6B). The percents of B-cells were significantly increased in B1 and B2 groups.
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**Figure 6.** Effect of DC-CIK combined with PTX/DDP on T-cells. Peripheral blood was taken to detect immune function of BALB/C mice with flow cytometry. The percentage of T-cells was determined by flow cytometry image pattern analysis instrument. A: Shows the automatic image of T-cells by flow cytometry. B: Is histogram of T-cells percent, which groups were higher in B1 and B2. Data were presented as mean ± SD, *P<0.05, **P<0.01, *P<0.05, **P<0.01, ΔP<0.05, ΔΔP<0.01, ^P<0.05, ^^P<0.01, compared with A0 or B0 group.

**Figure 7.** Effect of DC-CIK combined with PTX/DDP on B-cells. Taking peripheral blood to detect immune function of BALB/C mice with flow cytometry. The percentage of B-cells was determined by flow type cytopath and image pattern analysis instrument. A: Shows the automatic image of B-cells by flow cytometry. B: Is histogram of B-cells percent, which groups were higher in B1 and B2. Data were presented as mean ± SD, *P<0.05, **P<0.01, *P<0.05, **P<0.01, ΔP<0.05, ΔΔP<0.01, ^P<0.05, ^^P<0.01, compared with A0 or B0 group.

compared with A0 group (P<0.05) (Figure 7). There was no obvious difference in percentage between B1 and B2 groups, the six groups were, in order, A0, A1, A2, B0, B1 and B2 groups, which was 8.05 ± 0.38%, 18.77 ± 0.42%, 19.17 ± 0.60%, 8.93 ± 0.38%, 28.06 ± 0.74% and 28.80 ± 0.82% (Figure 7B). The percents of NK-cells, in A0, A1, A2, B0, B1 and B2 groups, was 2.59 ± 0.44%, 4.57 ± 0.49%, 7.38 ± 0.51%, 3.37 ± 0.45%, 8.32 ± 0.32% and 9.28
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The percentage of NK-cells showed a significant rise in B1 and B2 groups compared with A0 group (\(P<0.05\)) (Figure 8A and 8B).

**Discussion**

EOC is a high malignancy with insidious onset, difficulty of early diagnosis, invasive fast-growing, high recurrence rate and fatality [2], which poses a threat to the health and life of women. The combination chemotherapy of platinum-based is still the first-line chemotherapeutic regimen, the emergence of drug-resistant during therapy greatly increases the risk of a poor clinical outcome, including death [27, 28]. The cancer patients’ body immunity obviously decline, which have high drug resistance than healthy patients [29]. Therefore, tumor’s biotherapy presented by adoptive cellular immunotherapy (ACI/AIT) has become the fourth choice of cancer therapy following operation, radiotherapy and chemotherapy in recent years, which can enhance treatment outcomes, consolidate the effect and reduce recrudescent rate [30-32]. According to the results, the cellular purity of DC and CIK cells were more than 80% by flow cytometer (Figure 1), which provided the comparatively accurate data for subsequent experiments. The measurements of A1, A2 and B0 groups indicated a significant decline in tumor volume. The efficiency of inhibiting tumor in B1 and B2 groups was better than the other group. In Figure 2, both immuno-therapy group (A1) and chemotherapy alone group (B0) could be significant effect on the growth of tumors, tumor inhibitory rate of chemotherapy groups (B1 and B2) were more high than A1 and B0 group, which showed that combination therapies provide better outcomes. DC-CIK and PXT/DDP had synergistic. We compared and analyzed the results of HE staining and TUNEL, the shape of tumors in B1 and B2 groups was better than A1, A2 and B0 groups, but, the degree of apoptosis in B1 and B2 groups was higher (Figures 3 and 5). It showed that DC-CIK cell in combination with PXT/DDP have anti-tumor effects of promoting tumor cells apoptosis and inhibiting tumor growth. Results from our laboratory suggested that the combination of DC-CIK and PXT/DDP exert the synthesize action in suppressing the growth of tumors.

It is widely acknowledged that angiogenesis is essential for solid tumor growth, which is a crucial process as it furnishes tumor cell with enough nutrients and oxygen, and MVD may serve as significant quantitative indicators in tumor angiogenesis [33]. VEGF is a positive regulator factor of angiogenesis, which plays a
critical role in tumor-associate angiogenesis and has become the new targets of anti-tumor therapy [34, 35]. Low dose continuous chemotherapy could be widely used for clinical treatment, killing tumor cells while inhibiting tumor angiogenesis in microenvironment [36]. We aimed at choking off a tumor’s blood supply by inhibiting the expression of angiogenic factors. The MVD and expression of VEGF were significantly reduced in the A1, A2, B0, B1 and B2 groups. The monotherapy of DC-CIK and PXT/DDP could reduce MVD and inhibit the expression of VEGF. There were also studies that DC vaccination could enhance antiangiogenesis by inhibiting the expression of VEGF, CIK cells could decrease serum VEGF levels in patients with POEMS syndrome [37, 38]; it suggested that DC and CIK cell inhibit angiogenesis. Speculation that DC-CIK would affect angiogenesis, therefore, the combination therapy was superior to single therapy. The experimental results were consistent with that of the speculation (Figure 4). In B1 and B2 groups, tumor volume and MVD were the lowest (Figures 2 and 4). Thus we concluded that DC-CIK cell in combination with PXT/DDP can change the local tumor microenvironment via inhibiting the expression of VEGF.

CIK and DC cell were concerned as two important parts in tumor immunotherapy, CIK cell could kill tumor cells, and DC could activate adaptive immunity by recognition pathogen, at the same time of killing malignant cells, they would also protect normal cells [39-41]. Lymphocyte is vital cell components of body’s immune response, which can be divided into T-cells, B-cells and NK-cells [42-44]. It can steadily maintain the internal environment and prolong the years of life of patients as well as the restoration of organ function, which is crucial for tumor treatment [42, 44]. In our results, the percentage of lymphocyte was significantly up-regulated in treatment groups, and percentage of lymphocyte in B1 and B2 groups treated with DC-CIK cell in combination with PXT/DDP were higher than the other groups (Figures 6-8). The B2 group was two separate treated with DC-CIK cell in combination with PXT/DDP, which has better regulating results. In A1 and A2 groups, the percentage of lymphocyte was higher than B0 group, which showed that DC-CIK cell can up-regulate the count of T-cells, B-cells and NK-cells. It indicated the immuno-microenvironment was improved. Previous experiments had demonstrated that the anti-tumor activity of DC-CIK is more stronger [43]. This could be because chemotherapy stimulate the widely release of tumor antigens, and then immunocyte exert antigen presentation for cancer, it showed that DC-CIK cell in combination with PXT/DDP enhanced immune response by increasing count of T-cells, B-cells and NK-cells.

In conclusion, DC-CIK cell in combination with PXT/DDP can choke off tumor angiogenic by suppressing the expression of VEGF and so further inhibit the growth of soil tumor, which also can strengthen the immunity via p-regulating the count of T-cells, B-cells and NK-cells. DC-CIK cell in combination with PXT/DDP have more better of anti-tumor effect than the monotherapy by inhibiting the expression of VEGF and enhancing the immunological protection.

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Disclosure of conflict of interest

None.

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