Original Article

**Carthamus tinctorius Enhances the Antitumor Activity of Dendritic Cell Vaccines via Polarization toward Th1 Cytokines and Increase of Cytotoxic T Lymphocytes**

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**Carthamus tinctorius** (CT), also named safflower, is a traditional Chinese medicine widely used to improve blood circulation [1], extending the coagulation time in mice and exhibiting a significant antithrombotic effect [2]. However, CT is used not only for its traditional medicinal purposes but is also effective for treating breast cancer [3]. The oil extracted from the seed of CT is reported to contain alkane-6,8-diols, which have the activity to inhibit 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in two-stage carcinogenesis in mouse skin. In addition, N-feruloylserrotonin and N-(p-coumaroyl) serotonin strongly inhibit the melanin production of *Streptomyces bikiniensis* and B16 melanoma cells [4, 5]. These compounds are suggested to have potential antitumor effects. In addition, CT also is neuroprotective for cerebral ischemic injury *in vivo* and *in vitro* [2]. Recently, N-(p-coumaroyl)serotonin and N-(p-coumaroyl)tryptamine, active ingredients in CT, were shown to strongly inhibit the production of proinflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8 and TNF-α) from lipopolysaccharide-stimulated human monocytes [6]. Notably, extracts of CT exhibit a vast diversity of bioactivities, including immunomodulation, anti-infarction, antiallergic, anti-inflammatory and antiestrogenic effects, as well as functioning as a hemostatic agent to promote blood coagulation. Table 1 summarizes the diverse bioactivities of CT and delineates the therapeutic implications for this potent herbal medicine.

Dendritic cells (DCs) are professional antigen-presenting cells that stimulate immune responses by presenting endogenous or exogenous antigens to T-helper lymphocytes [31]. Immature DCs can be differentiated from monocytes and bone marrow progenitor cells by treatment with GM-CSF and IL-4 [32, 33], after which the cells gain strong phagocytic activity but not antigen presentation activity. Immature DCs are stimulated with maturation signals to express more surface immunological molecules (such as CD86, CD80,
Table 1: The biological activities and therapeutic indications for extracts of CT.

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MHC-I and MHC-II) for antigen presentation, leading to a strong immune response against foreign antigens [34]. TNF-α and CD40L can stimulate immature DCs to mature in vitro and may show potential for clinical use [35], suggesting that enhancement of DC maturation is required for improvement of DC immunotherapy. In the clinic, DC-based immunotherapies are being intensively studied for their application in the treatment of certain diseases, including cancer and infectious diseases [36–38]. Scientists are trying to improve the phagocytic activity or antigen presentation activity of DCs for use in immunotherapy [39, 40]. Certain herbal medicines can also improve DC function [41]. To date, CT has been used as a folk medicine in cancer adjuvant therapy, but its function has not been proven. In this study, we studied the immunomodulatory effects of CT on cytokine secretion and surface immunologic molecules of DCs in vitro and antitumor activities of CT-treated DCs in an animal model of breast cancer.
The splenocytes were homogenized from the spleen. Mouse splenic T lymphocytes were treated with various concentrations of CT extract (5, 10 and 20 μg/mL) at a stock concentration of 100 mg/mL for 24 h. The medium was collected to determine the concentrations of the cytokines IL-2, IFN-γ and IL-4. Cytokine levels were determined by the DuoSet ELISA kit as described in the manual.

2.3. Maturation of Mouse DCs by CT Extract. Bone marrow cells were taken from BALB/c mice and then suspended in RPMI-1640 medium containing 10% FBS. The cell suspension was allowed to stand at room temperature for 10 min to remove the cell clots, and then the cell suspension was centrifuged at 800 g for 5 min at 4°C and washed with PBS (pH 7.4) containing 0.5% FBS and 2 mM EDTA. The cells were allowed to attach to a 100-mm culture plate for 2 h and the floating cells were removed gently. The cells were then treated with GM-CSF (100 ng/mL) and IL-4 (100 ng/mL) at 37°C, 5% CO2 for 6 days, following the modifications of Zheng et al. [43]. After 6 days of differentiation, the DCs were treated with various concentrations of CT extract (5, 10 and 20 μg/mL of culture medium) for 72 h. After CT treatment, the DCs were collected for flow cytometric analysis of surface immunological molecules (CD80, CD86, MHC-I and MHC-II). In brief, the cells were stained with the specific FITC-conjugated antibody.

2.4. Isolation of CD117+ Bone Marrow Cells. Bone marrow cells were collected as above and suspended in PBS (pH 7.4) containing 0.5% FBS and 2 mM EDTA prior to the isolation of CD117+ cells. CD117+ cells were isolated using magnetic-activated cell sorting (Miltenyi Biotec Inc, CA, USA) as described in the manual. Briefly, 1 × 10^6 cells were added to 20 μl anti-CD117+ provided by the kit for 15 min and then washed with PBS to remove excess unbound antibodies. The treated cells were loaded on a column, from which the CD117+ cells were eluted with PBS buffer in the presence of a magnetic field. The CD117+ cells were then eluted in the absence of a magnetic field and were then differentiated to DCs by adding GM-CSF and IL-4 as described above.

2.5. Generation of CD117+ Derived DCs. The CD117+ cells were cultured at a density of 6.25 × 10^5/well of a 6-well plate in RPMI-1640 medium containing 10% FBS, 1.5 mg/mL sodium bicarbonate, 0.1 μmol/mL nonessential amino acids, 1 μmol/mL sodium pyruvate, 100 U/mL penicillin G and 100 μg/mL streptomycin supplemented with GM-CSF (100 ng/mL) and IL-4 (100 ng/mL) at 37°C, 5% CO2 for 6 days. Medium containing GM-CSF and IL-4 was refreshed every 2 days.

2.6. Assessment of Cytokines Released from JC Lysate-Pulsed CT-Treated CD117+ Derived DCs. After differentiation of CD117+ cells to DCs for 6 days, DCs were pulsed with JC-lysate and treated with or without CT extract (5, 10 and 20 μg/mL) for an additional 3 days. The conditioned medium was collected for determination of the concentrations of IL-2, IL-10, TNF-α and IL-1β [44].

2.7. Assessment of Immunological Molecules of JC Lysate-Pulsed CT-Treated CD117+ Derived DCs. After differentiation of CD117+ cells to DCs for 6 days, DCs were pulsed with...
2.8. Preparation of DC Vaccine and JC Tumor Animal Experiment. CD117+ cells were cultured in differentiation medium (GM-CSF and IL-4) for 6 days followed by cultivation in fresh RPMI medium in the presence or absence of CT extract for 24 h. The cells were then scraped and collected for surface molecule analysis (CD80, CD86, MHC-I and MHC-II) using flow cytometry.

BALB/c female mice (4–6 weeks) were purchased from the National Laboratory Animal Center (Taiwan, ROC). JC cells (3 × 10^6) were subcutaneously inoculated in the flank of BALB/c mice. Twenty-four tumor-bearing mice were grouped (n = 8/group) as follows: control, untreated DC group, DC vaccine group and CT-treated groups. In the DC vaccine group and CT-treated DC vaccine group, mice were injected intraperitoneally with the JC-pulsed DC cell suspension (1.7 × 10^6 per 0.2 mL PBS) and the CT-treated JC-pulsed DC cell suspension (1.7 × 10^6 per 0.2 mL PBS) on day 13, respectively. The tumor growth of JC tumor-bearing mice was measured by a caliper every 4–6 days and calculated using the equation, tumor weight (mg) = length (mm) × width (mm^2)/2, until the tumor reached 2 × 10^3 mm^3. On day 32, mice were euthanized by CO2, and the spleens of each mouse were homogenized into a single cell suspension pulsed with or without CT-treated DCs to measure Th1- and Th2-related cytokines. The splenocytes were also re-stimulated with the previous corresponding JC-pulsed DC vaccine and CT-treated JC-pulsed DC vaccine for flow cytometric analysis of cytotoxic T lymphocytes. All mice received humane care, and the study protocol followed the guidelines of the Institutional Animal Care and Use Committees of the Development Center for Biotechnology (accredited by AAALAC).

2.9. Effects of CT-Treated DCs on the Cytokine Secretion and Cytotoxic T Lymphocytes of Mouse Splenocytes. DC vaccine was prepared as described above and cultured in AIM-V medium (Gibco, USA), and splenic T lymphocytes were taken from the normal and the JC tumor-bearing mice and cultured in RPMI-1640 medium (Gibco, USA). In brief, splenic T lymphocytes were partially purified using a nylon wool column [46]. Different cytokines in response to CT extract (20 μg/mL)-treated DCs were determined when DCs (2 × 10^6) were pulsed with splenocytes from tumor-bearing mice at a cell:cell ratio of 1:10, 1:20 and 1:40 for 48 h at 37°C, 5% CO2. After cocultivation of splenocytes and DCs, the medium was collected and stored at −20°C prior to cytokine analysis. Cytokines were analyzed by ELISA as described above. The splenocyte culture was continued for an additional 72 h and then subjected to flow cytometric analysis, in which specific T lymphocytes were gated by staining with FITC-anti-CD3 and PE-anti-CD8.

2.10. Statistical Analysis. Data were analyzed by one-way ANOVA using SPSS statistical software followed by the Dunnett’s test for multiple comparisons between each group. A P-value < .05 was considered to indicate a significant difference.

3. Results

3.1. IFN-γ and IL-10 Secretion from Mouse Splenic T Lymphocytes. To determine the effect of CT extract on the stimulation of mouse resting T lymphocytes, the splenic T lymphocytes were partially purified and treated with various concentrations of CT extract for 24 h. The supernatants were collected and evaluated for the levels of IL-2, IFN-γ and IL-4 and IL-10. Figure 1 shows that CT extract stimulated the production of IL-10 > IFN-γ in a dose-dependent manner (Figure 1). However, no such stimulation was observed for IL-2 and IL-4.

3.2. Expression of Immunological Molecules in the Immature DCs. To determine whether the CT extract stimulated the maturation of DCs, mouse bone marrow cells were isolated and differentiated into immature DCs by adding GM-CSF and IL-4 for 6 days. The immature DCs were treated with various concentrations of CT extract (5, 10 and 20 μg/mL) for 48 h. Figure 2 shows that CT extract promoted the expression of DC surface markers (CD86 < MHC-I = MHC-II = CD80) in a dose-dependent manner (Figure 2).

3.3. Enhancement of Maturation of DCs by CT Extract. To study whether the pattern of cytokine secretion was altered in DCs stimulated by CT extract in the presence of tumor
antigens, the immature DCs were pulsed with JC tumor lysate along with different concentrations of CT extract (5, 10 and 20 μg/mL) for 48 h. The conditioned medium was analyzed for IL-2, IL-10, TNF-α and IL-1β by ELISA and the DCs were subjected to analysis of surface immunological molecules (CD80, CD86, MHC-I and MHC-II). The results showed that CT extract increased TNF-α and IL-1β levels in a dose-dependent manner (Figure 3). However, the level of IL-2 was reduced when DCs were pulsed with tumor lysate. IL-10 decreased with an increase in the concentration of CT extract, suggesting that CT extract might increase the secretion of TNF-α and IL-1β to enhance the maturation of immature DCs. Treatment with CT extract maintained the high profile of cell surface markers, such as CD80, CD86, MHC-I and MHC-II on the CD117⁺-derived CDs in the presence of tumor antigen (Figure 4).

3.4. Inhibition of Tumor Growth by CT Treated-DC Vaccine. To study the antitumor activity of CT-treated DC vaccine, BALB/c mice were inoculated with JC tumor cells and challenged with a single dose of CT-treated DC vaccine. The JC tumor-bearing mice were challenged with DC vaccine and CT-treated DC vaccine on day 13, when tumor reached the size of 5 mm (length) × 5 mm (width). Results showed that CT-treated DC vaccine and DC vaccine decreased the tumor mass by 32.5% and 17.2%, respectively, compared to the untreated control (Figure 5). This result suggested that CT might increase the antitumor activity of the DC vaccine.
3.5. Polarization of Cytokine Secretion to Th1 Response. To determine the Th1 and Th2 cytokine secretion in DC vaccine treatment, the tumor-bearing mice that had been challenged with DC vaccine were sacrificed and their splenocytes subjected to cytokine analysis, in which those cells were re-stimulated with the previous corresponding DC vaccine or CT-treated DC vaccine ex vivo. Splenocytes of the CT/DC vaccine compared with those of the CT-untreated DC-vaccine showed increased or identical production of IL-2, IL-10 and IFN-γ. However, IL-4 production was notably decreased in splenocytes of mice treated with the CT/DC vaccine (Figure 6). The secretion of cytokines was not increased in the sham splenocytes when those cells pulsed with DC vaccine or CT-treated DC vaccine with lack of JC antigen. This result suggested that either the DC vaccine or CT-treated DC vaccine elicited antitumor activity through a Th1 response, and that CT extract might also enhance the immune response of the DC vaccine.

3.6. Increased Antitumor Activity Is Mediated by Cytotoxic T Lymphocytes. To determine whether cytotoxic T lymphocytes were increased in the CT-treated DC vaccine, the splenocytes of vaccine-challenged mice were re-stimulated with the previous corresponding DC vaccine or CT-treated DC vaccine. The JC tumor lysate-pulsed DC vaccine stimulated 30.1% of cytotoxic T lymphocytes and the JC tumor lysate-pulsed CT-treated DC vaccine stimulated 35.6% of cytotoxic T lymphocytes (Figure 7), indicating that CT-treated JC tumor lysate-pulsed DC vaccine could increase more cytotoxic T lymphocytes against tumors than DC vaccine without CT treatment. Additionally, the cytotoxic T lymphocytes of sham mice were only stimulated 27.6% with CT-treated DC vaccine in the absence of JC tumor antigens. This result was consistent with the decrease in tumor weight shown in Figure 5.

4. Discussion

DCs are professional antigen-presenting cells that have been enlisted for use in vaccines against cancer. DCs can acquire tumor antigens by cocultivation with tumor cells or pulsation with tumor lysates to become activated. After maturation, the DCs present the tumor antigens to lymphocytes and trigger the immune response cascade. In this study, CT stimulated splenic T lymphocytes to secrete IFN-γ and IL-10, suggesting that CT can immunomodulate T lymphocyte function. Heightened expressions of CD80, CD86, MHC-I and MHC-II imply that CT stimulates maturation of antigen-presenting cells; moreover, an increase in the expression of MHC-I and II molecules, together with an increase in CD8-positive T cells, suggests that HLA-mediated presentation of tumor antigens accelerates after treatment with CT extract. CT also improved the antitumor activity of the DC vaccine as evidenced by a reduction in tumor weight. The ex vivo analysis of cytokine secretion and lymphocyte population suggested that CT polarizes the immune response toward the Th1 pathway by increasing the secretions of IL-2 and IFN-γ, but not IL-4, and consequently produces more cytotoxic T lymphocytes to elicit antitumor activity than DC vaccine without CT treatment. It has been reported that stimulated DCs secrete TNF-α, IL-1β and IL-10 during the
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Figure 6: Secretion of cytokines from mouse splenocytes re-stimulated with CT extract-treated DC vaccine ex vivo. Spleens from sham (sham-spl) and tumor-bearing (JC-spl) BALB/c mice challenged with DC vaccine or CT-treated DC vaccine were taken and homogenized into single cell suspensions. The splenocytes were cocultured with DC vaccine or CT-treated DC at cell:cell ratios of 10:1, 20:1 and 40:1 for 48 h. The concentrations of secreted cytokines were determined by ELISA as described in Section 2.

maturation process and then polarize the T lymphocytes toward the Th1 pathway [47], which is consistent with our observations shown in Figure 3.

DCs are widely distributed in the human body with different morphologies, such as Kupffer cells in liver [48] and Langerhans cells in the skin [49]. Unlike chemotherapy which produces severe side effects during treatment, the use of DCs provides an alternative strategy against tumors [50]. The functions of DCs are improved in such as the maturation, antigen presentation and regulatory cytokine secretion, which has survival benefits in cancer patients [51]. Although the CT-treated DC vaccine was intraperitoneally injected to activate immunity in this study, oral administration of CT extract might promote the recognition of antigens and facilitate antigen presentation via intestinal DCs, and thus this attractive approach is worthy of further investigation. However, many traditional medicinal herbals have being studied for immunomodulatory activities, such as *Melilotus suaveolens* Ledeb [52] and *Tanacetum parthenium* [53]. In literatures, both herbal plants were found to demonstrate their anti-inflammation activities in a monocytic cell-based assay system and exhibited effects on regulating the production of chemokines. Due to these recent studies, *M. suaveolens* Ledeb [52] and *T. parthenium* have been
implicated to exhibit potential therapeutic benefits in treating many inflammation-related diseases, such as cancers, atherosclerosis and rheumatoid arthritis. Therefore, herbs in treating cancers might have activities via the modulation of cytokine profiles. In this article, our results suggest that CT could promote immunity through the activation of DCs per se that do not alter the cytokine secretion during immune responses of tumor lysate-pulsed DCs toward the Th1 pathway.

References


