Enhancement of Extracts from *Celastrus orbiculatus* on Maturation and Function of Dendritic Cells *in vitro* and *in vivo*

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Abstract: **Objective** To examine the immunoregulation of *Celastrus orbiculatus* extracts (COE), a traditional Chinese medicine, on maturation and function of dendritic cells (DCs) *in vitro* and *in vivo*. **Methods** *In vitro*, after treated with COE in different nontoxic concentrations (0, 10, 20, 40, 80, and 160 μg/mL) for 5 d, the surface immunological molecules and cytokine secretion of mice bone marrow-derived DCs in response to COE were analyzed by flow cytometric analysis (FACS) and enzyme linked immunosorbent assay (ELISA), respectively. *In vivo*, mouse hepatoma cells (Hepa1-6, 1×10^6) were injected sc and were treated with different dosages of COE (10, 20 or 40 mg/kg/d). Effects on tumor growth were determined by tumor volume and histology analysis after 28 d administration of COE. The relative proportions of mature DCs and $CD8^+$ T cells were measured in mononuclear cells that had been isolated from spleen by FACS. **Results** COE stimulated IL-2 and IFN-γ secretion of DCs, simultaneously enhanced the maturation of DCs by enhancing immunological molecule (CD40, CD80, CD86, $H\text{-}2K^b$, and $I\text{-}A^b$) expression in a dose-dependent manner. Furthermore, the chemotactic responses of DCs were significantly higher in COE-treated than untreated DCs, in association with higher chemokine receptor 7 expression. Furthermore, COE increased DCs produce IFN-γ and IL-2 in a dose-dependent manner when the concentration of COE less than 40 μg/mL, decreased DCs produce IL-10 and IL-4 also in a dose-dependent manner. In *in vivo* studies, COE can not only suppress growth of malignant hepatocellular carcinomas but also stimulate maturation of DCs, associated with strongly enhanced $CD8^+$ CTL responses. **Conclusion** These data provide new insight into the mechanism of action of COE and indicate that the stimulation of maturation and function of DCs by COE contributes to its immunoregulatory effects.

Key words: antitumor; *Celastrus orbiculatus*; dendritic cell; immunoregulation
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Introduction

Celastrus orbiculatus Thunb. has been used for thousands of years in China as a remedy against arthritis and other inflammatory diseases (Jin *et al*, 2002). At the laboratory level, a variety of biological effects of *C. orbiculatus* extracts (COE) have been observed including anti-proliferation, anti-angiogenesis, and apoptosis induction, and their potent antitumor activity with low adverse effects *in vitro* and *in vivo* as well (Mao *et al*, 2006; Wang, 2007; Zhang *et al*, 2006; Yang *et al*, 2009). Previously, we have shown that one of the possible mechanisms of COE anti-angiogenesis is the inhibition of vascular endothelial growth factor (VEGF) expression of tumor cells (Qian and Liu, 2010). VEGF is produced in large amounts by most tumors and its production is closely associated with a poor prognosis (Ellis and Fidler, 1996). Recently VEGF was identified as one of the factors responsible for defective dendritic cells (DCs) maturation (Gabrilovich *et al*, 1996). DCs are the most potent and professional antigen presenting cells that determine either T helper lymphocytes type1 (Th1) or Th2 polarization of naive T

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cells, and they have been a promising tool for cancer immunotherapy (Shibata *et al*, 2006). The immature state of DCs is known to be appropriate for antigen processing, and in turn, they must be matured to fully activated DCs, which express high levels of cell surface MHC-antigen complex and costimulatory molecules, for sufficiently productive immunological response (Banchereau and Steinman. 1998).

Here we investigated whether COE would promote maturation and function of DCs. To answer this question, we studied the effects of COE on DCs development, maturation, and cytokines production. It was found that COE not only had dramatic effects on DCs development and maturation, but also significantly affected DCs function of immunoregulation. The results of our experiments suggest that COE may exert potential *in vitro* effects relevant for treating many inflammation-related diseases, such as atherosclerosis, rheumatoid arthritis, and cancers.

Materials and methods

Reagents

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 4 (IL-4), and lipopolysaccharide (LPS) were purchased from Sigma (Saint Louis, MO). Antimouse CD11c MicroBeads (Hamster IgG) was purchased from Miltenyi Biotec (CA, USA). Cisplatin was from Changzhou Pharmaceutical Co. (Changzhou, China). Antimouse and isotype control antibodies, including anti-CD80 (B7-1) [Armenian hamster IgG, phycoerythrin (PE), 16-10A1 clone], anti-CD86 (B7-2) [Rat IgG2a, fluorescein isothiocyanate (FITC), GL1], anti-H-2K^b (MHC-I) (Mouse IgG1, PE, 25-D1.16), anti-I-A^b (MHC-II) (Rat IgG2b, FITC, M5/114.15.2), anti-CD3e (Armenian hamster IgG, FITC, 145-2C11), anti-CD8a (Rat IgG2a, PE, 53-6.7), anti-CD4 (Rat IgG2b, PE, GK1.5), and anti-CCR7 (Rat IgG2a, PE, 4B12) were from eBiosciences (San Diego, CA).

Preparation and characterization of the COE

The stems of *C. orbiculatus* were purchased from Guangzhou Zhixin Pharmaceutical Co., Ltd. (Guangzhou, China), and their identity was confirmed by Prof. QIN Min-jian, a plant taxonomist at Department of Resources Science of Chinese Medicines, China Pharmaceutical University, Nanjing, China. The dried stems of *C. orbiculatus* were minced using a grinder and the powder (15 kg) was extracted with 95% ethanol (150 L) for 3 h, this procedure was repeated three times. The combined extract was filtered and evaporated to dryness using rotary evaporator. A membrane pump was used to evacuate the extract in order to remove the residual solvent. The ethanol extract (900 g) was subsequently partitioned between ligarine (0.5 L × 3) and water (0.5 L). The aqueous layer was further partitioned by ethyl acetate (0.5 L × 3). The final ethyl acetate extract was condensed by rotary evaporator and finally lyophilized into powder (250 g), stored at 4℃. The preparation and characterization of COE were kindly provided by Prof. WANG Qiang, Department of Chinese Materia Medica Analysis, China Pharmaceutical University, Nanjing, China. 1 g extract equivalently 60 g crude drug (Zan *et al*, 2007). The resultant micropowder was diluted in DMSO (Sigma, St Louis, MO) to the required concentrations and filtered before use. The final DMSO concentration did not exceed 0.1% throughout this research. In the study, the COE concentration was expressed as the amount of COE per milliliter of media bathing the cells (μg/mL).

DCs isolation and purification

DCs were generated according to Inaba *et al* (1992). Briefly, bone marrow cells were harvested from the tibias and femurs of six- to eight-week-old female C57BL/6 mice and suspended in PBS (pH 7.4) containing 0.5% FBS and 2 mmol/L EDTA prior to the isolation of CD11c-positive (CD11c$^+$) cells. CD11c$^+$ cells were isolated using magnetic microbeads (Miltenyi Biotec Inc, CA, USA) as described in the manual. Briefly, 1×10^7 cells were added to 20 mL anti-CD11c$^+$ 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 CD11c-negative cells were eluted with PBS buffer in the presence of a magnetic field. The CD11c$^+$ cells were then eluted in the absence of a magnetic field before being differentiated to DCs by adding GM-CSF and IL-4 according to the manner described in "DCs culture and COE treatment".

DCs culture and COE treatment

The CD11c$^+$-derived DCs were cultured at a density of 1×10^7/well in 6-well plates (Falcon) in G4 medium (RPMI 1640 containing 10% fetal bovine serum [FBS], 2 mmol/L glutamine, 25 mmol/L HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 100 U/mL penicillin, 100 μg/mL streptomycin, 50 ng/mL recombinant

murine [rm] GM-CSF, and 50 ng/mL rmIL-4) at 37 ℃ in a humidified 5% CO_2 atmosphere for 7 d. The cultures were fed with the fresh cytokine-containing medium every 2 or 3 d. To induce DCs maturation, immature DCs were cultured in the same cytokine cocktails with the addition of LPS (10 ng/mL) for 2 d (Chen *et al*, 2005). For the COE treatment groups, various concentrations of COE (10, 20, 40, 80 and 160 μg/mL) were added to the cell suspension at day 2 of culture and washed thoroughly at day 7, and cells were cultured with G4 medium with LPS but without COE at day 8 and 9. The conditioned medium was determined by enzyme-linked immunosorbent assay (ELISA) for IL-2, IL-4, IL-10, and interferon-γ (IFN-γ) production. The DCs were collected for flow cytometric analysis of surface immunological molecules (CD40, CD80, CD86, H-2K^b, and I-A^b).

Cell culture

Mouse hepatoma (Hepa1-6) cells were obtained from the Cell Bank of Chinese Academy of Sciences Shanghai Institute of Cell Biology (Shanghai, China). Hepa1-6 cells were maintained in RPMI 1640 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS, 2 mmol/L glutamine, 25 mmol/L HEPES, Penicillin (100 units/mL), and streptomycin (100 μg/mL) and incubated at 37 ℃ in a humidified atmosphere with 5% CO_2.

Flow cytometric analysis

Flow cytometric analysis was performed on FACScan (BD Biosciences, Mountain View, CA). Briefly, cells (5×10^5) were first incubated with anti-mouse CD16/32 mAb in 4 ℃ for 10 min to block surface Fc receptors, washed with 0.5% bovine serum albumin (BSA)-PBS, and then incubated with fluorochrome-conjugated mAb (CD40, CD80, CD86, H-2K^b, and I-A^b) in 4 ℃ for 30 min. Appropriate immunoglobulin isotype controls were used for phenotype analysis. Data were analyzed using WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, CA). And results were expressed as mean fluorescence intensity (MFI).

Mixed lymphocyte reaction (MLR) analysis

Mixed lymphocyte reaction was performed according to Kakazu *et al* (2007). The CD11c$^+$-derived DCs isolated by magnetic microbeads were cultured at a density of 1.0×10^5 cells/well in 96-well round-bottom plates (Falcon) containing 200 μL of various concentrations of COE in G4 medium for the generation of immature DCs. On day 5, immature DCs were induced to mature using 10 ng/mL LPS for 24 h. On day 6, the allostimulatory capacity of 5.0×10^4 irradiated DCs (3000 rad) was tested in a one-way MLR with normal, allogeneic T lymphocytes (isolated from spleen: 1.0×10^5 cells/well) in triplicate. Co-cultured cells were maintained for 4 d at 37 ℃ in a 5% CO_2 humidified atmosphere. The proliferation rate of the cells was measured using an MTS assay (CellTiter 96 aqueous one-solution cell proliferation assay; Promega). Forty microliters of CellTiter 96 aqueous one-solution were added to each well. After 2 h of incubation, the UV absorbance of the solution was measured at a wavelength of 490 nm.

Cytokine production of cultured DCs

CD11c$^+$-derived DCs were cultured in G4 medium with various concentrations of COE (0, 10, 20, 40, 80, and 160 μg/mL) for 7 d. After 7 d of differentiation, the DCs (1×10^6 cells/mL) were pulsed with 10 ng/mL LPS for an additional 2 d. The culture mediums were harvested and the concentration of murine IL-2, IL-4, IL-10, and IFN-γ were measured by quantitative sandwich ELISA using ELISA kit (R&D Systems) according to the instructions of manufacturer.

Apoptotic assay

Alexa Fluor 488 annexin V/propidium iodide (PI) Apoptosis Kit (Invitrogen) was used to measure the potential cytotoxic effect of COE on the cultured DCs. Briefly, mouse bone marrow-derived DCs were cultured in G4 medium with various concentrations of COE (0, 10, 20, 40, 80, and 160 μg/mL) for 7 d. The cells were harvested and doubly labeled with Alexa Fluor 488-conjugated annexin V and propidium iodide (PI) according to the instructions provided by the manufacturer. The uptake of Annexin V and PI were detected using FACScan, and data were analyzed using Cell Quest software.

***In vivo* studies**

Five-week-old female C57BL/6 mice (H-2^b) were purchased from the Comparative Medicine Laboratory Animal Center of Yangzhou University (Jiangsu, China). They were maintained under specific pathogenfree conditions. Animal care was provided in accordance with the procedures outlined by Yangzhou University Guide for the Care and Use of Laboratory. On day 0, Hepa1-6 cells (1×10^6) suspended in 0.1 mL of serum-free RPMI 1640 were inoculated sc in the left flank of each

mouse. Mice were randomly assigned to six groups (ten mice per group) as follows: untreated control, solvent vehicle control (DMSO), cisplatintreated group, and different dosages COE-treated groups (10, 20 or 40 mg/kg/d). On day 14 after inoculation, the animals received 200 μL of a vehicle (1% DMSO and 99% PBS) or COE at different dosages (10, 20 or 40 mg/kg/d) by gavage in 200 μL PBS containing 1% DMSO. In the cisplatin-treated group, mice were ip injected with 1 mg/kg/d of cisplatin. Subcutaneous tumors were measured twice per week using calipers and their volumes were calculated using a standard formula (width^2 × length × 0.5). Body weight was measured weekly. Mice received 28 doses and 24 h after the last dose, they were sacrificed and the tumors were removed and weighed. The spleens and peripheral blood of each mouse were homogenized into a single cell suspension for flow cytometric analysis.

Terminal deoxynucleotidyl transferase-mediated nick end labeling assay

For the histologic TUNEL staining of the Hepa1-6 tumor tissues (sc Hepa1-6 tumors with or without COE treatment), the tumors were harvested and fixed with 10% neutral formalin. Paraffin-embedded sections (7 μm per section) of tumors were stained by using In Situ Cell Death Detection kit. After dewaxing, rehydration, and proteinase digestion, the slides with tumor tissues were incubated with 100 μL TUNEL reaction mixture and covered with lid for 60 min at 37 ℃ in a humidified atmosphere in the dark. Finally, slides were washed thrice with 1 × PBS, mounted, and visualized under an optical microscope.

Statistical analysis

All experiments were performed at least 3 times and the results of a representative experiment presented. Statistical significance was analyzed by Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Results

COE enhanced differentiation and maturation of DCs

CD11c$^+$-derived immature DCs were cultured in G4 medium with various concentrations of COE (0, 10, 20, 40, 80, and 160 μg/mL) for 7 d. On day 8, immature DCs were induced to mature using 10 ng/mL LPS for another 3 d. Then, DCs survival after exposure to COE was monitored by FACS analysis using Annexin V/PI staining according to Nicoletti *et al* (1991). Results obtained indicated COE did not induce apoptotic DCs death. Therefore, the COE concentrations varying from 0–160 μg/mL were used as optimal concentrations for the subsequent experiments.

The expression of cell surface costimulatory molecules CD40, CD80, CD86, MHC-I (H-2K^b), and MHC-II (I-A^b) were measured using the flow cytometry. In comparison to the untreated culture, as shown in Fig. 1 (A–E), COE-treated DCs showed increased expression of all tested surface molecules in a dose-dependent manner, as indicated by mean fluorescence intensity of DCs. These results strongly support the view that COE is a potent stimulator of DCs maturation based on promotion of maturation markers.

COE enhanced the chemotactic response of LPS-stimulated DCs

A previous report suggested that dendritic cell migration to peripheral lymph node is sensitization of the CCR7 chemokine system (Randolph *et al*, 1998). To determine whether the COE influenced the chemotactic response of DCs, the CCR7 expression was analyzed by FACS. As in Fig. 1F, FACS analysis showed that following treatment with COE (day 2–7), LPS-stimulated DCs to express elevated levels of CCR7. Therefore, COE treatment not only phenoltypically and functionally stimulated DCs maturation, but also enhanced their chemokine receptor expression.

COE modulated DCs allostimulatory capacity

Based on the results that DCs differentiation and maturation were stimulated by the COE, we hypothesized that the concentration of COE could influence the function of DCs. The most characteristic functional feature that discriminates DCs from other APCs is their ability to induce T-cell proliferative responses, as determined by allostimulatory assays (Chen *et al*, 2005). To examine whether the addition of COE enhanced the function of DCs, we cultured DCs under various media that contained 0–160 μg/mL COE, and evaluated the allostimulatory capacity of the DCs. Table 1 shows that the addition of COE strongly increased allogeneic T-cell proliferation of DCs in a dose-dependent manner. Therefore, COE treatment not only enhanced the phenotypic maturation of DCs, but also reinforced their function.

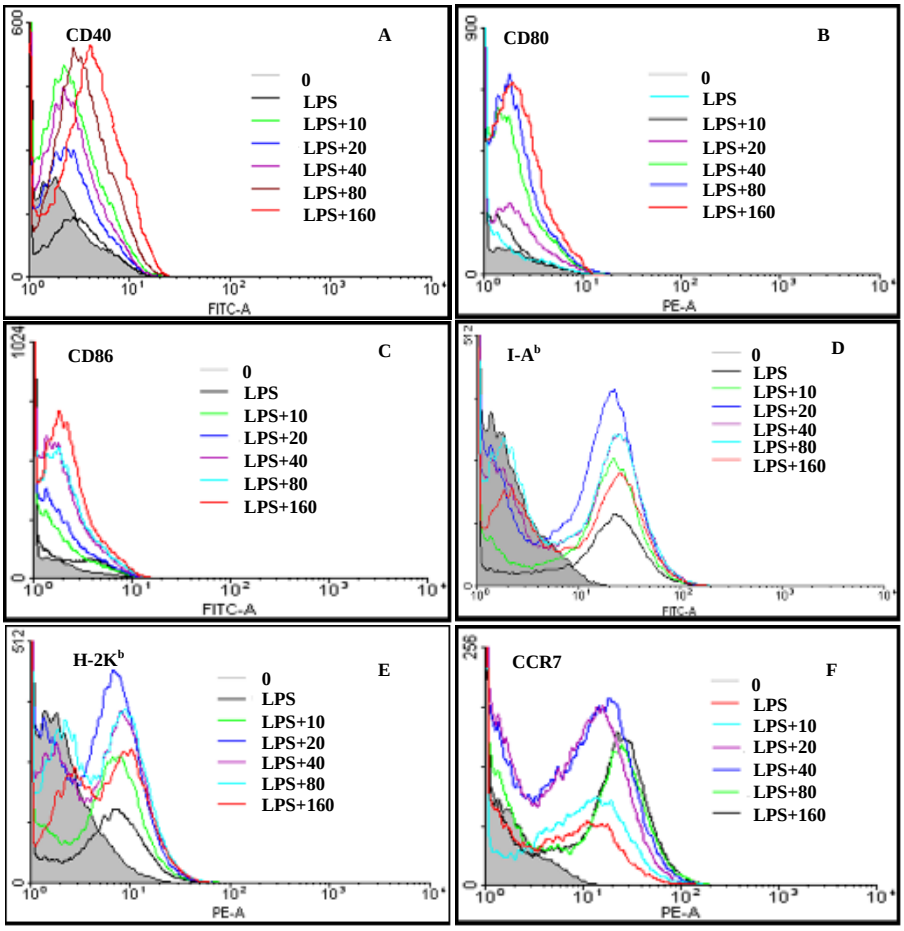

Fig. 1 Promotion of maturation and chemotactic response of DCs by COE

$CD11c^+$-derived DCs were cultured with 50 ng·mL^{-1} GM-CSF and 50 ng·mL^{-1} IL-4 in the presence of 10 ng·mL^{-1} LPS and various concentrations (10, 20, 40, 80, and 160 μg·mL^{-1}) of COE (open profiles) or medium alone. Cells were stained with the designated mAb and analyzed by FACS. COE-treated DCs showed increased expression of costimulatory molecules (A, B, C), MHC molecules (D, E), and CCR7 chemokine (F) in a dose-dependent manner. A typical experiment from at least 5 independent experiments with similar results is shown

Table 1 Effects of COE on DCs allostimulatory capacity

Group	Dose / (μg·mL^{-1})	Proliferation of T lymphocytes / %
DC	—	23.7 ± 2.4
DC + LPS	—	42.4 ± 5.1
DC + LPS + COE	10	45.0 ± 1.3
	20	58.3 ± 6.2
	40	63.6 ± 6.1
	80	78.1 ± 7.3*
	160	105.0 ± 5.7**

Values are $\bar{x} \pm s$ in each group ($n = 4$)

$^*P < 0.05$ $^{**}P < 0.01$ *vs* DC + LPS group

COE induced the production of Th1 cytokines in DCs

Upon stimulation, DCs increasingly produced cytokines and chemokines that modulated T helper lymphocytes (Th) and linked the innate and adaptive immunity (Tian *et al*, 2007). Mosmann and Sad (1996) believed Th was important for both cell-mediated and humoral immunity. The particular type of immune response is determined by the differentiation of precursor naive Th0 cells into Th1 or Th2 cells, a process which is dependent upon local cytokine concentrations, antigen load, and mode of antigen presentation. Each cell subset secreted a particular array of cytokines which further augment the differentiation into that subset (O'Garra and Arai, 2000). Th1 cells produce predominantly IFN-γ and IL-2, while Th2 cells secrete predominantly IL-4 and IL-10.

Thus, we determined the impact of COE treatment on cytokine production patterns in DCs. Bone marrow-derived immature DCs cultured in G4 medium with various concentrations of COE (0, 10, 20, 40, 80, and 160 μg/mL) for 7 d. On day 8, immature DCs were induced to mature using 10 ng/mL LPS for another 72 h. The conditioned medium was analyzed for IL-2, IL-10, IFN-γ, and IL-4 by ELISA. The results showed that COE increased DCs produce IFN-γ and IL-2 in a dose-dependent manner when the concentration of COE was less than 40 μg/mL, simultaneously decreased DCs

produce IL-10 and IL-4 also in a dose-dependent manner (Table 2). Taken together, these results suggested that COE enhanced cell-mediated immune response through polarization of cytokine secretion to Th1 response.

Table 2 Concentrations of secreted cytokines were determined by ELISA (pg·mL^{-1})

Group	Dose / (μg·mL^{-1})	IL-2	IFN-γ	IL-10	IL-4
DC		34.3 ± 12.5	193.8 ± 27.2	35.3 ± 13.4	38.3 ± 10.4
DC + LPS		112.8 ± 16.1	745.3 ± 18.5	349.7 ± 17.3	558.9 ± 11.7
DC + LPS + COE	10	176.1 ± 13.9*	891.7 ± 15.2	301.0 ± 15.9	445.6 ± 17.8
	20	184.2 ± 13.1*	983.9 ± 25.9*	233.2 ± 24.7*	424.5 ± 15.6
	40	208.0 ± 12.3**	1060.2 ± 43.8**	185.7 ± 11.3*	324.6 ± 25.2*
	80	116.3 ± 18.2	883.4 ± 30.7	157.6 ± 21.8**	179.8 ± 17.0**
	160	128.6 ± 15.2	706.2 ± 22.3	145.5 ± 12.2**	53.2 ± 16.3**

Values are $\bar{x} \pm s$ in each group ($n = 4$) $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* DC + LPS group

Oral COE attenuates growth of solid hepatic carcinoma in mice

To verify COE antitumor activity, *in vivo* animal experiments were carried out. Hepa1-6 cells were derived from a C57BL/6 mouse hepatoma origin and represent advanced tumors. The effect of COE on solid hepatic carcinoma growth *in vivo* was tested in C57BL/6 mice with a Hepa1-6 tumor implanted subcutaneously. Treatment started after hepatic carcinoma implant had established a palpable (0.4 cm diameter) tumor, and herbal extract was administrated by gavage once daily at doses of 10, 20, and 40 mg/kg body weight. We monitored the solid hepatic carcinoma growth by measuring tumor size and the tumor mass at the end of herbal treatment, both data revealed that oral intake of COE significantly retarded solid hepatic carcinoma growth (Fig. 2). Endpoint tumor mass showed dose-dependent growth suppression by COE, wherein the mean tumor masses of 10, 20, and 40 mg/kg/d groups were (0.832 ± 0.085) g, (0.693 ± 0.008) g, and (0.512 ± 0.021) g compared with (1.423 ± 0.031) g of the solvent vehicle control (DMSO) group (Fig. 2B). During the 28-day treatment period, mice did not exhibit any symptoms of toxicity such as loss of appetite, decreased locomotion, or any other apparent sign of illness.

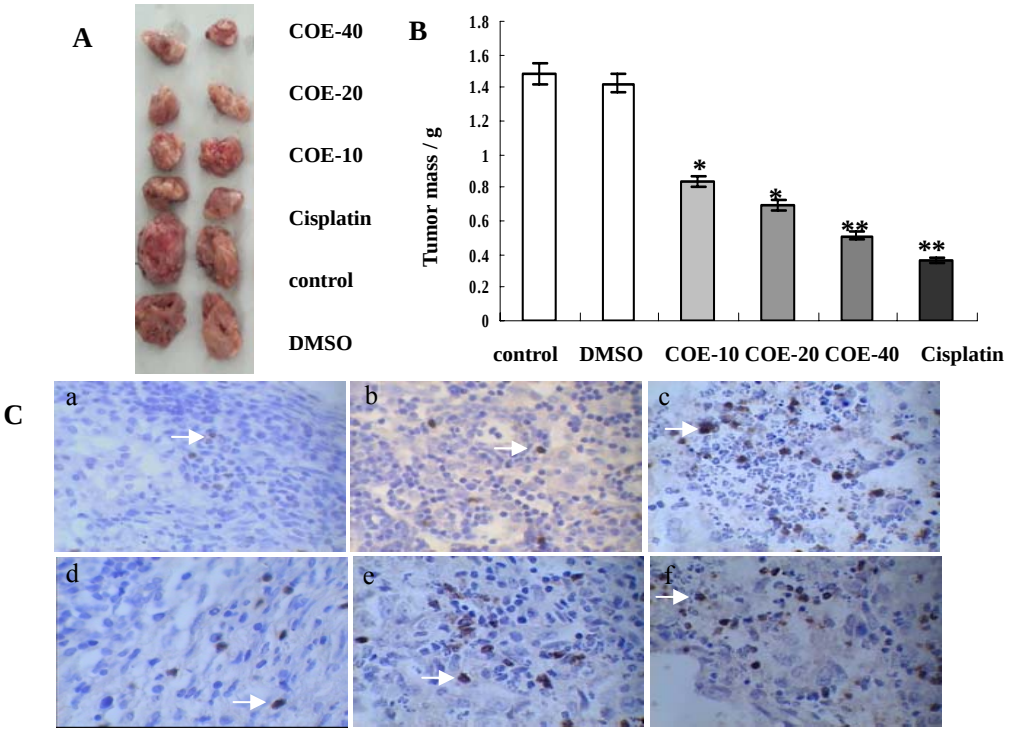

Fig. 2 COE attenuates tumor volume of Hepa1-6 engrafted in the subcutis of C57BL/6 mice

Subcutaneous Hepa1-6 tumor xenografts were established in mice treated with COE *via* gavage once daily for 28 d. A: representative tumor photographs of solvent vehicle control (DMSO), untreated control, cisplatin-treated group, and different dosages COE-treated groups (10, 20 or 40 mg·kg^{-1}·d^{-1}) at day 28 (down to up). B: tumor mass of COE-treated groups *vs* control groups. Bars, $\bar{x} \pm s$ ($n = 10$). $^{*}P \le 0.05$ $^{**}P \le 0.01$ (*t* test). C: Representative photographs of sections of the untreated control (a), solvent vehicle (DMSO) control (b), cisplatin-treated group (c), and different dosages COE-treated groups (10, 20 or 40 mg·kg^{-1}·d^{-1}, d, e, and f). Hepa1-6 tumors DNA fragmentation of apoptosis cell with TUNEL staining. TUNEL-positive cells stain brown (white arrow)

COE increased proportions of $CD11c^+CD11b^+$ DCs and $CD8^+$ T cells in the spleen of tumor bearing mice

To determine which DC subset is more abundant during induction of oral COE, the relative proportions of $CD11c^+CD11b^+$ DCs and $CD8^+$ T cells were measured in mononuclear cells that had been isolated from spleen after 28 d administration of COE. The proportions of $CD11c^+CD11b^+$ DCs and $CD8^+$ T cells were higher in COE-treated mice than in solvent vehicle control mice (Fig. 3). These results indicate that administration of COE increases mature-type $CD11c^+CD11b^+$ DCs, and $CD8^+$ T cells are the predominant effector cells in antitumor immunity for hepatic carcinoma.

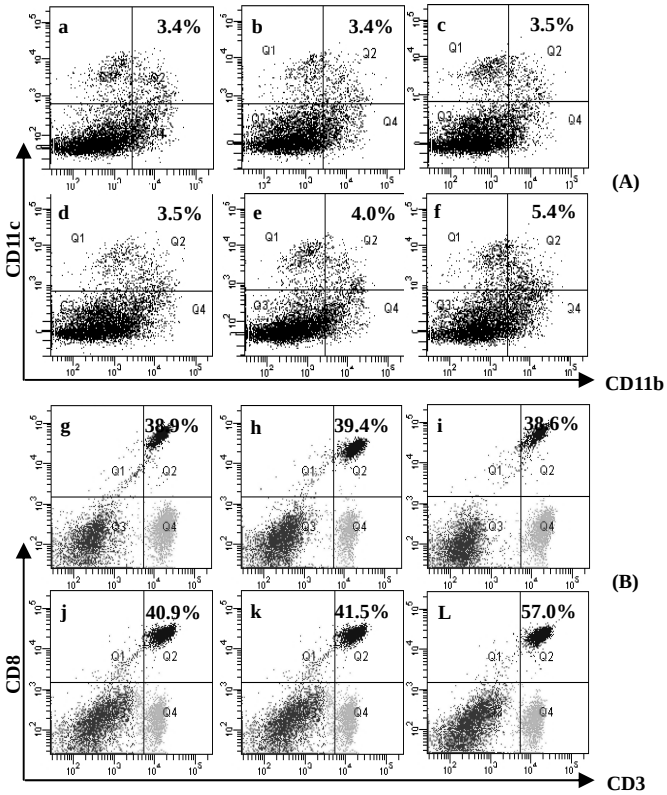

Fig. 3 Increased proportion of $CD11c^+CD11b^+$ DCs (A) and $CD8^+$ T cells (B) in tumor bearing C57BL/6 mice

Mononuclear cells were isolated from spleen. The proportions of $CD11c^+CD11b^+$ DCs and $CD8^+$ T cells were determined using flow cytometry. One representative experiment of three is shown

a and g: representative photographs of untreated control

b and h: solvent vehicle (DMSO) control

c and I: cisplatin-treated group

d and j: 10 $mg \cdot kg^{-1} \cdot d^{-1}$ COE-treated group

e and k: 20 $mg \cdot kg^{-1} \cdot d^{-1}$ COE-treated group

f and L: 40 $mg \cdot kg^{-1} \cdot d^{-1}$ COE-treated group

Discussion

DCs are potent professional antigen-presenting cells able to induce primary immune responses (Steinman, 1991). Immunization of mice with tumor vaccines plus DCs or tumor antigen-pulsed DCs are effective for inducing systemic protective antitumor immunity (Ashley *et al*, 1997).

COE is a potent immunomodulator isolated from *C. orbiculatus*. The present study, for the first time, clearly demonstrates that COE promoted the phenotypic and functional maturation, cytokine production, and chemotactic response of DCs *in vitro* and *in vivo*.

In vitro assays, we first found that various concentrations of COE enhanced the expression of costimulatory molecules (CD40, CD80, and CD86) on DCs in a dose-dependent manner (Fig. 1A–C). Optimal activation of T cells requires that DCs undergo a process of maturation resulting in the increased expression of costimulatory molecules, such as CD86, and CD80, and the production of cytokines (Pejawar *et al*, 2005). Both CD80 and CD86 bind CD28 on T cells and provide the second signal required for the activation and expansion in response to antigen (Fields *et al*, 1998; Kaye, 1995). This study supported our hypothesis that COE influenced the differentiation and the maturation of DCs. Moreover, this phenomenon was accompanied by an increase in the expression of MHC-I and II molecules (Fig. 1D–E), and suggested that COE may improve DCs function of antigen-presenting. In addition, DCs that were exposed to COE showed strengthened migratory capacity, which was the result of a reinforced expression of the corresponding receptor CCR7 (Fig. 1F).

We next showed that the addition of COE increased the IFN-γ and IL-2 production by DCs when the concentration of COE less than 40 μg/mL, and decreased the IL-10 and IL-4 production in a dose-dependent manner (Table 2). IFN-γ and IL-2 are involved in the differentiation of naive T cells into Th1 cells, which are important in the resistance to foreign pathogens. IL-10 and IL-4 are secreted predominantly by Th2 cells and favour humoral immunity. Our results raised the possibility that elevating the extracellular COE concentration could modulate Th1/Th2 differentiation. A study by Chaudhry *et al* (2006) showed that natural killer dendritic cells (NKDCs) are the major IFN-γ-producing dendritic cell subtype and may play a significant role in the host antitumor response. Unlike the chemotherapy which causes severe side effects during treatment, the

relevance of this unusual feature of DCs should be further explored in light of these potential therapeutic applications (Chauvin and Josien, 2008).

Recent studies showed that a number of transcription factors play important roles in regulation of Th1/Th2 cytokines including nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB), and interferon regulatory factor-1 (IRF1) (Li-Weber and Krammer, 2003). However, the mechanisms of COE affecting Th1/Th2 differentiation are not clearly known.

It is well known that tumor-bearing hosts suffer from strong immunosuppression. Many factors (e.g. regulatory T cells, suppressor cells, down-modulation of MHC molecules, over-production of immuno-suppressive factors, down-modulation of IL-2 response and IL-2 production, and poor DC function at the tumor site) explain the decreased immune responses in tumor-bearing hosts (Gabrilovich *et al*, 1997; Sakaguchi *et al*, 2001; Whiteside, 1999). Therefore, it is essential to overcome this strong immunosuppression to initiate tumor-specific immune responses in tumor-bearing hosts (Steinman and Dhodapkar, 2001; Perales *et al*, 2002). As shown in Fig. 2, COE reduced tumor volume, specifically increased proportions of $CD11c^+CD11b^+$ DCs and $CD8^+$ T cells in the spleen of tumor bearing mice. These results suggest that COE could not only inhibit HCC tumor cell growth and induce HCC tumor cell apoptosis, but also stimulate DCs maturation and T cells activation.

In conclusion, pronounced antitumor activities *in vitro* and *in vivo* suggest that COE has potent anticancer effects and enhance DCs function by inducing DCs migration and their capacity for stimulation of lymphocytes. The results of anti-HCC treatment with COE are significant, providing new hope for effective chemotherapy for such malignant hepatocellular carcinomas. Isolating the specific active compounds of COE and examining their mechanisms of action are in process; This work may lead to new therapeutic options and improve understanding of the interaction of phytochemicals in hepatic cancer cells.

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