Two-sided effect of *Cordyceps sinensis* on dendritic cells in different physiological stages

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Abstract: ***Cordyceps sinensis*** **(CS), a Chinese tonifying herb, has been widely used for centuries in Asian countries as a medicine and a health supplement. Although ample evidence indicates that CS can modulate immune responses, the functional effect of CS on dendritic cells (DCs) is still unclear. This study examines how CS affects human monocyte-derived DCs in two physiological states: naïve and LPS-induced inflammatory. Our experimental results demonstrate that CS acts as an activator and maturation inducer of immature DCs by stimulating the expression of costimulatory molecules and proinflammatory cytokines by DCs, enhancing the DC-induced, allogeneic T cell proliferation, and reducing the endocytic ability of DCs. In contrast, CS suppresses the LPS-induced, inflammatory response by decreasing the LPS-induced expression of costimulatory molecules and proinflammatory cytokines by DCs. CS also suppresses the LPS-induced, DC-elicited, allogeneic T cell proliferation and shifts the LPS-activated, DC-driven Th1 response toward a Th2 response. These results demonstrate that CS differentially regulates the DC activities according to the presence or absence of the inflammatory signs. Restated, with the lack of an ongoing inflammatory environment, CS primes DCs toward a Th1-type immunity, whereas in a potential inflammatory reaction, CS balances the over-reactivity of elicited Th1 immunity. This investigation illustrates the Yin-Yang balancing effects of CS as a medicine and a health supplement.** ***J. Leukoc. Biol.*** **85: 987–995; 2009.**

Key Words: *antigen-presenting cells · immune suppression · cell activation · inflammation*

INTRODUCTION

Cordyceps sinensis (CS), a complex of a parasitic fungus and its caterpillar host, is a renowned Chinese tonifying herb and has been documented in one of the most prestigious ancient Chinese pharmacopoeias [1, 2]. CS has been commonly used as an herbal medicine as well as a health supplement in China for over 2000 years [1–3]. A variety of pharmacological effects, such as anti-tumor [4, 5], immunomodulatory [6–8], anti-inflammatory [9–11], and anti-oxidant properties [12, 13], of CS has been reported. Furthermore, the extracts of wild CS and fermented mycelia of CS have been accepted recently as popular nutraceuticals in many Asian countries, such as China and Japan [14]. Studies have shown that CS contains various bioactive compounds, including cordycepin, adenosine, adenine, guanosine, ergosterol, uridine, uracil, hypoxanthine, mannitol, and polysaccharides [2, 15]. Multiple compound-based drugs may provide important combination therapies that simultaneously affect multiple pharmacological targets and provide clinical efficacy beyond the reach of single compound-based drugs [16].

Dendritic cells (DCs) are potent APCs that play a prominent role in the development of T cell immune responses [17, 18]. The development of DCs comprises two functional stages. In the immature stage, DCs are localized primarily in the peripheral tissues and participate in phagocytosis and antigen processing. Following the uptake of antigens, they migrate from the peripheral tissues to the lymphoid organs. During migration, DCs lose the capacity to capture antigens and mature to become potent antigen presenters to activate T cells [19]. The maturation of DCs is critical to induce antigen-specific T lymphocyte responses and to control the differentiation of T cells toward Th1 or Th2 immunity [20, 21]. Fully mature DCs exhibit high surface expression of MHC-II and costimulatory molecules, such as CD40, CD80, and CD86 [22, 23], but decrease the capacity to internalize antigens [24, 25]. Additionally, the up-regulation of CD83, a specific marker of DC maturation, occurs [26]. Among the various stimuli causing DCs to mature include proinflammatory cytokines, CD40 ligand (CD40L), and pathogens such as LPS, bacteria DNA, and an unmethylated DNA CpG motif [18].

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CS has been demonstrated to have immunomodulatory activity [6–8]. However, how CS affects APCs, especially DCs, has not been investigated. To examine the immunomodulatory activity of CS, human monocyte-derived immature DCs were used as a model for naïve-stage DCs, and LPS-activated DCs were used as a model for the inflammatory stage to investigate how CS affects DCs under different physiological conditions. Experimental results indicated that CS activates immature DCs and promotes the maturation of immature DCs by enhancing the expression of CD205, MHC-II, CD83, and costimulatory molecules (CD40, CD80, and CD86) by DCs and reducing the endocytic activity of DCs. CS promotes the production of cytokines by immature DCs and thereby drives the differentiation of T cells toward the Th1 type. On the other hand, CS suppresses the LPS-induced inflammatory response by decreasing the LPS-induced expression of CD205, MHC-II, CD83, and costimulatory molecules, inhibiting proinflammatory cytokine secretion by DCs, and reducing the LPS-activated DC-elicited allogeneic T cell proliferation. Moreover, CS shifts the LPS-activated DC-driven Th1 response toward a Th2 response. In summary, this investigation demonstrates that CS can exert differential immunomodulatory effects on DCs upon different physiological states. Results of this study support the role of the Yin-Yang nature of CS as a health supplement and a medicine.

MATERIALS AND METHODS

Reagents

The culture medium was RPMI 1640 (Gibco-BRL, Life Technologies, Paisley, UK), supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-BRL, Life Technologies), and 10% heat-inactivated FCS (Hyclone, Logan, UT, USA). Recombinant human (rh)GM-CSF, rhIL-4, and soluble (s)rhCD40L were purchased from PeproTech (Rocky Hill, NJ, USA). LPS (*Escherichia coli* serotype O55:B5), MTT, BSA, FITC-LPS, and FITC-dextran (40,000 Da) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A Limulus amoebocyte lysate (LAL) pyrochrome kit, purchased from Associates of Cape Cod (Falmouth, MA, USA), was used to determine the LPS content of CS extracts. FITC- or PE-conjugated anti-human mAb were used to detect CD11c (IgG1-12-0116), CD14 (IgG1-12-0149), CD40 (IgG1-11-0409), CD80 (IgG1-12-0809), CD83 (IgG1-11-0839), CD86 (IgG2b-12-0869), CD205 (IgG2b-11-2059), and HLA-DR (IgG2b-12-9956). All mAb and isotype control antibodies were purchased from eBioscience (San Diego, CA, USA). ELISA kits for detection of human TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-10, and IL-12p70 were purchased from eBioscience (San Diego, CA, USA). The NF-κB inhibitor 6-amino-4-(4-phenoxyphenylethylamino) quinazoline was purchased from Calbiochem (San Diego, CA, USA). Quantitative measurement of cell proliferation was made by using the BrdU ELISA kit, which was purchased from Roche Molecular Biochemicals (Mannheim, Germany). For HPLC fingerprint assay, adenine, adenosine, cordycepin, guanine, hypoxanthine, uracil, and uridine were purchased from Sigma Chemical Co.

Preparation of hot-water extracts of CS

To guarantee the quality of CS, the genetic variation was analyzed by DNA sequencing, which was performed as described in our previous studies [27, 28]. The hot-water extracts of CS were obtained as described in our previous study [29]. Briefly, CS samples were dried at 45°C in the dark to a constant weight and pulverized. The CS sample (2 g) was dissolved in 40 ml water (1:20, wt/vol), and hot-water extraction was performed at 90°C for 2 h. After centrifugation at 3000 g for 20 min, the supernatant was harvested and then sterilized by filtration through a 0.22-μm filter and stored at –20°C until used. To examine potential endotoxin contamination, CS extracts were measured by the LAL assay. Results indicated that the two batches of CS have undetectable levels (<0.05 endotoxin units/ml) of LPS (data not shown).

HPLC fingerprint analysis

The HPLC system consisted of a chromatography system (Waters Chromatography Division, Milford, MA, USA), including a series 600 controller, a series 717plus autosampler, and a series 996 photodiode-array detector, connected to an Inertsil ODS-3V column (5 μm, 4.6×250 mm), preceded by a 5-μm cartridge column (GL Sciences, Tokyo, Japan). Solvents that constituted the mobile phase were buffer A (20 mM Na_2HPO_4) and buffer B (methanol). The elution conditions applied were: 0–5 min, isocratic 5% buffer B; 5–35 min, linear gradient 5–40% buffer B; 35–40 min, isocratic 40% buffer B. The column was then reconditioned with 5% buffer B isocratic for 20 min. The flow rate was 0.6 ml/min, and the injection volume was 20 μl. The system was kept at 25°C, and the detection wavelength was set at 260 nm.

Generation of human monocyte-derived DCs

Fresh whole blood from normal volunteers was obtained from the Taiwan Blood Center. Human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Monocytes were purified by the plastic adherence method [30]. A total of 10^7 cells/well in six-well flat-bottom plates was incubated in RPMI-1640 culture medium. After 2 h incubation at 37°C in humidified air that contained 5% CO_2, nonadherent cells were removed by gentle washing, and plastic-adherent cells were used as monocytes. This monocyte population exhibited >90% CD14 positive staining, as revealed by flow cytometric analysis (data not shown). DCs were generated from monocytes that were cultured at 37°C in an incubator with 5% humidified CO_2 in RPMI-1640 culture medium that was supplemented with rhGM-CSF 500 U/ml and rhIL-4 1000 U/ml for 6 days. On Days 2 and 4, half of the medium was replaced with fresh medium that contained rhGM-CSF and rhIL-4. On Day 6, immature DCs were reseeded into a six-well culture plate at a total of 10^6 cells/well and treated with various concentrations of CS extracts (0, 0.25, 0.50, 0.75, and 1 μg/ml) in a culture for 2 days. LPS-activated DCs were generated from immature DCs by culturing for 2 days in the presence of 1 μg/ml LPS that contained various concentrations of CS extracts (0, 0.25, 0.50, 0.75, and 1 μg/ml). The viability of the cells under these treatments exceeded 90%, based on MTT assay (Supplemental Fig. 1), which were performed following the manufacturer's instructions (Sigma, St. Louis, MO, USA).

Phenotypic and functional characterization of DCs

In phenotypic analysis, DCs were washed using PBS that contained 2% FCS and then incubated with FITC- or PE-labeled mAb against human CD11c, CD14, CD40, CD80, CD83, CD86, CD205, HLA-DR, or isotype control antibodies for 40 min at 4°C. Cells were then washed twice using cold PBS that contained 2% FCS and analyzed by flow cytometry (Partec, Munster, Germany). A total of 2×10^4 cells was acquired in each analysis. Data were analyzed using WinMDI software (Scripps, La Jolla, CA, USA). CD14 is a representative monocyte marker and was not present on the DC surface (data not shown). All measurements were performed in triplicate.

Analysis of endocytotic activity

A method described previously [31] was used with slight modifications to measure the phagocytic activity of DCs. Briefly, DCs (5×10^5 cells) were resuspended in 100 μl PBS that contained 2% FCS and then incubated with FITC-dextran (0.1 mg/ml) at 37°C for 30 min. Following incubation, cells were washed four times in cold PBS that contained 2% FCS and analyzed by flow cytometry. As a control, cells cultured under each condition were maintained in the same solution for 30 min at 4°C. All measurements were performed in triplicate.

Allogeneic MLR assay

Responder cells (T cells) were obtained from allogeneic PBMCs by nylon wool column purification (Polysciences, Warrington, PA, USA). The purity of CD3-positive T cells was >85%, as determined by flow cytometry (data not shown). Immature DCs treated with CS, LPS, or the combination of LPS and CS for 2

days were used as stimulator cells. A total of 2×10^3 stimulator cells was cocultured with a graded ratio of responder cells (1:25, 1:50, 1:75, and 1:100) in 96-well flat-bottomed plates that contained 0.2 ml medium per well for 3 days. Allostimulatory activity was measured following the addition of 10 μM BrdU during the last 14 h of the 3-day culture period. The proliferative response was determined by measuring the incorporation of BrdU using a cell proliferation ELISA kit as described by the manufacturer's instructions (Roche Molecular Biochemicals). All measurements were performed in triplicate.

Measurement of cytokine secretion

Supernatants of DC cultures were harvested after 2 days of incubation with medium alone, CS, LPS, or the combination of LPS and CS. Human TNF-α, IL-1β, IL-6, and IL-12p70 levels in culture supernatants were measured using ELISA according to the manufacturer's instructions (eBioscience). T cells were cocultured with untreated, CS-treated, LPS-treated, or the combination of LPS and CS-treated DCs for 3 days. T cell differentiation was assessed by measuring the production of IFN-γ and IL-4 in the supernatant using ELISA. The levels of IFN-γ and IL-4 in medium were normalized to cell numbers at the end of the incubation period. Cell viability was measured by the MTT assay. All measurements were performed in triplicate.

Statistical analysis

All experiments were performed at least three times. The data are presented as the mean ± SD of the repeated experiments and analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). Comparisons between control and treatment groups were made using Student's *t*-test. Differences were regarded as statistically significant for *P* values under 5% ($P<0.05$) and 1% ($P<0.01$).

RESULTS

Genetic authentication of CS and chromatographic fingerprint

Quality control of traditional Chinese medicine (TCM) has been regarded as a key issue in ensuring safety and efficacy [15]. Therefore, selective and efficient analytical methods for quality assurance and authentication of the herbal material are required. Several recent phylogenetic studies have reported that the nucleotide sequences in the 18S ribosomal RNA (rRNA) gene can distinguish CS from other related *Cordyceps* species [27, 28]. To ensure the identity of the herbal materials used in this study, we examined the 18S rRNA gene of five different batches of CS samples by a DNA sequencing assay. Experimental results confirmed that CS samples used in this study are genuine CS, and the results were submitted to the European Molecular Biology Laboratory database. The accession numbers of five CS samples are FM164741, FM164742, FM164743, FM164744, and FM164745. Additionally, chromatographic fingerprinting can display the entire profiling pattern of an extracted sample and has been applied to evaluate the quality of traditional herb medicines. The U.S. Food and Drug Administration and the European Medicines Agency have stated that the appropriate chromatographic fingerprint should be applied to assess the consistency of botanical drugs [32]. Therefore, CS extracts were chemically fingerprinted by HPLC, demonstrating that the major components of CS are extracted (Supplemental Fig. 2).

CS promotes the phenotypic maturation of immature DCs

To investigate the effect of CS on DC maturation, human monocytes were cultured with GM-CSF and IL-4 for 6 days, followed by another 2 days in the presence of various concentrations (0, 0.5, and 1 μg/ml) of CS extracts or LPS (1 μg/ml), which is a known activator of DCs, as a positive control. The expression of surface molecules (CD11c, CD205, CD40, CD80, CD83, CD86, and MHC-II) and endocytic activity were examined by flow cytometry. Experimental results indicated that CS increases the expression of CD11c, CD205, CD40, CD80, CD83, CD86, and MHC-II molecules by immature DCs in a dose-dependent manner (**Fig. 1A**). This effect closely resembles that of LPS. To gain further insight into the effect of CS on DC maturation, we examined whether CS affects the endocytic activity of immature DCs. Our results demonstrated that CS, like LPS, decreases the endocytic capacity of DCs (Fig. 1A). This indicates that immature DCs can be activated and matured by CS.

CS induces the production of TNF-α, IL-1β, IL-6, and IL-12p70 by immature DCs

DC-secreted cytokines play a pivotal role in the immune response and the interaction between DCs and T cells [18, 33]. The release of IL-12 by DCs drives the differentiation of naïve T cells toward the IFN-γ-producing Th1 phenotype [34]. Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, play a key role in inducing innate and acquired immune responses [35]. In this study, cytokine levels in the supernatant of DCs following the addition of various doses of CS were measured by ELISA. According to Figure 1B, CS enhances the production of TNF-α, IL-1β, IL-6, and IL-12p70 by immature DCs in a CS dose-dependent manner.

CS enhances the allogeneic T cell proliferation and promotes Th1 response by immature DCs

Mature DCs have a greater capacity to induce the proliferation of allogeneic T cells than immature DCs do [19]. The above results demonstrate that CS not only induces the expression of cell surface molecules such as MHC-II and the costimulatory molecules (CD40, CD80, and CD86) of immature DCs but also enhances the secretion of IL-12 and proinflammatory cytokines by immature DCs. To determine whether changes in surface molecule expression and cytokine secretion by DCs sufficiently alter their ability to activate naïve T cells, immature DCs were treated with CS or LPS for 2 days and then cocultured with allogeneic T cells. The MLR assay revealed that CS enhances the proliferation of allogeneic T cells by immature DCs (**Fig. 2A**).

To further examine how CS-treated DCs affect the differentiation of T cells, we analyzed cultured supernatants from allogeneic MLR experiments by ELISA. As shown in Figure 2B, the CS-treated DCs increase the production of IFN-γ by T cells, but the treatment does not increase the secretion of IL-4 by T cells significantly (Fig. 2B). The results indicate that CS induces the proliferation of T cells and promotes a Th1 response.

CS inhibits the maturation of LPS-activated DCs

The above experiments demonstrate that CS can promote DC maturation, enhance the production of cytokines by DCs, in-

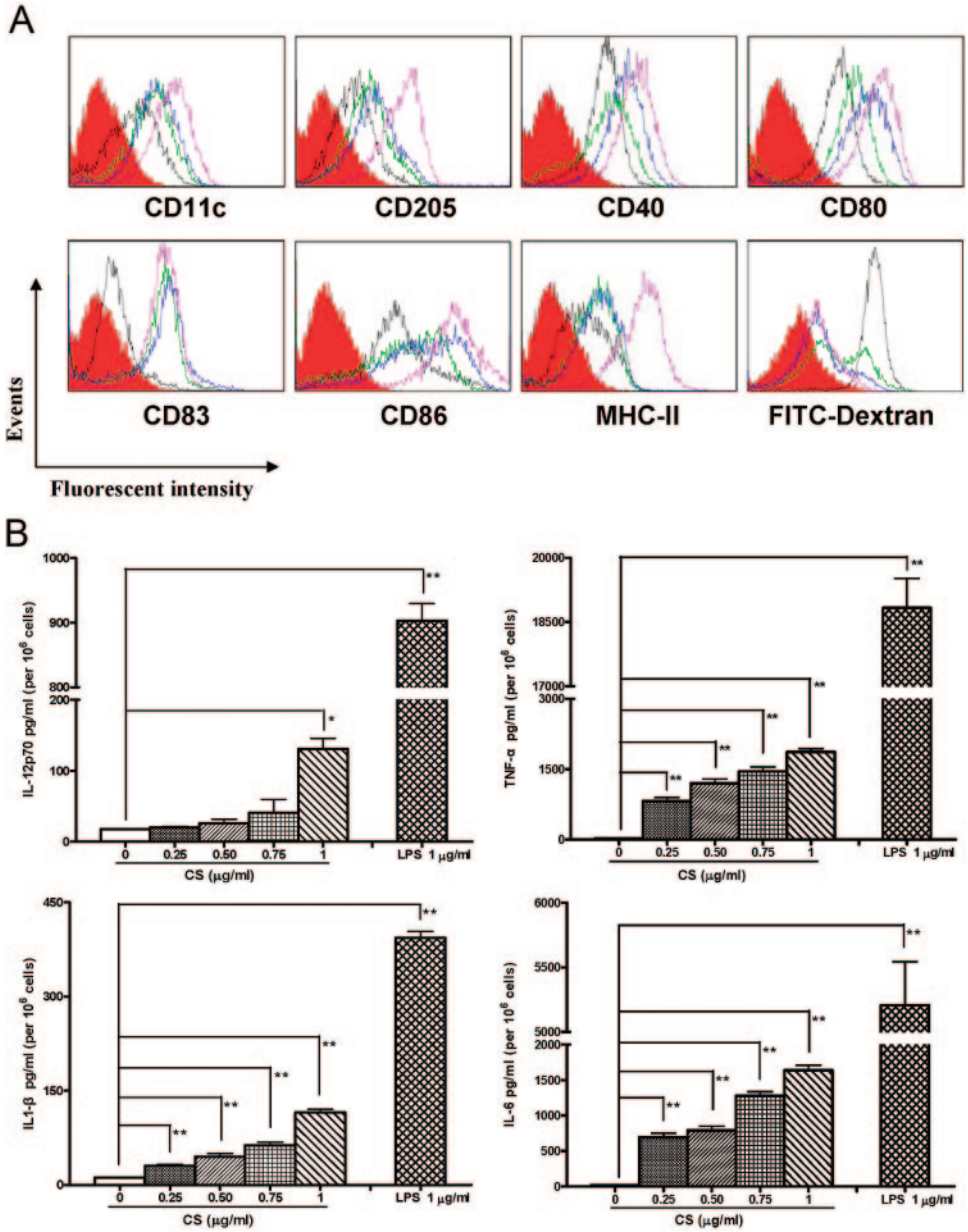

Fig. 1. CS-induced DC maturation and the production of cytokines by immature DCs. (A) Phenotypic characteristics and endocytic capacity of immature DCs treated with CS. Cell surface expression was analyzed by flow cytometry on DCs treated with various concentrations of CS (0 μg/ml, black line; 0.5 μg/ml, green line; or 1 μg/ml, blue line) or LPS 1 μg/ml (purple line) as a positive control. The red histograms represent staining with corresponding isotype control mAb. Endocytic capacity was determined by the endocytosis of FITC-dextran followed by flow cytometry. Immature DCs were treated with various concentrations of CS (0 μg/ml, black line; 0.5 μg/ml, green line; or 1 μg/ml, blue line) or LPS 1 μg/ml (purple line) as a positive control for 2 days. Cells were collected and then incubated with FITC-dextran at 37°C for 30 min or at 4°C as a control (red histogram). The results are representative of three independent experiments. (B) The effects of CS on cytokine productions of immature DCs. Immature DCs were treated with various concentrations of CS (0, 0.25, 0.50, 0.75, or 1 μg/ml) or LPS 1 μg/ml as a positive control for 2 days. Culture supernatants were collected and the amounts of IL-12p70, TNF-α, IL-1β, and IL-6 were measured by ELISA. Data are presented as mean ± SD from three independent experiments. Statistical significance for the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: *, $P < 0.05$; **, $P < 0.01$.

duce the proliferation of allogeneic T cells, and drive Th1 polarization. These results indicate that CS can potentially activate Th1 immunity through the activation of DCs. However, several studies also reported that CS has anti-inflammatory activity [9–11]. Whether CS has an anti-inflammatory effect on DCs was examined further. As is well known, LPS is an inducer of inflammation and can promote the maturation of DCs [36–38]. The immature DCs generated from human monocytes were treated with LPS (1 μg/ml) and various concentrations of CS extracts (0, 0.5, and 1 μg/ml) for 2 days. The expression of the surface molecules (CD11c, CD205, CD40, CD80, CD83, CD86, and MHC-II) and their endocytic activity were assayed by flow cytometry. **Figure 3A** shows that CS suppresses the expression of CD11c, CD205, CD40, CD80, CD83, CD86, and MHC-II by LPS-activated DCs, but it does not affect the endocytic activity of LPS-activated DCs (Fig. 3A). Similar effects on CD86 expression were also found when CS was added 1 day after LPS stimulation (Supplemental Fig. 3A). These results indicate that CS interferes with LPS-activated DC maturation.

CS reduces the secretion of TNF-α, IL-1β, IL-6, and IL-12p70 by LPS-activated DCs

Whether CS affects the production of cytokines by LPS-activated DCs was examined further by determining the concentration of cytokines in the supernatant of DCs treated with LPS (1 μg/ml) and various concentrations of CS extracts (0, 0.5, and 1 μg/ml) for 2 days. Figure 3B shows that CS inhibits the production of TNF-α, IL-1β, IL-6, and IL-12p70 by LPS-activated DCs in a CS dose-dependent manner. The effects on TNF-α and IL-6 production were also found when CS was added 1 day after LPS stimulation (Supplemental Fig. 3B). These findings suggest that CS can act as an anti-inflammatory agent by decreasing the production of proinflammatory cytokines by LPS-activated DCs.

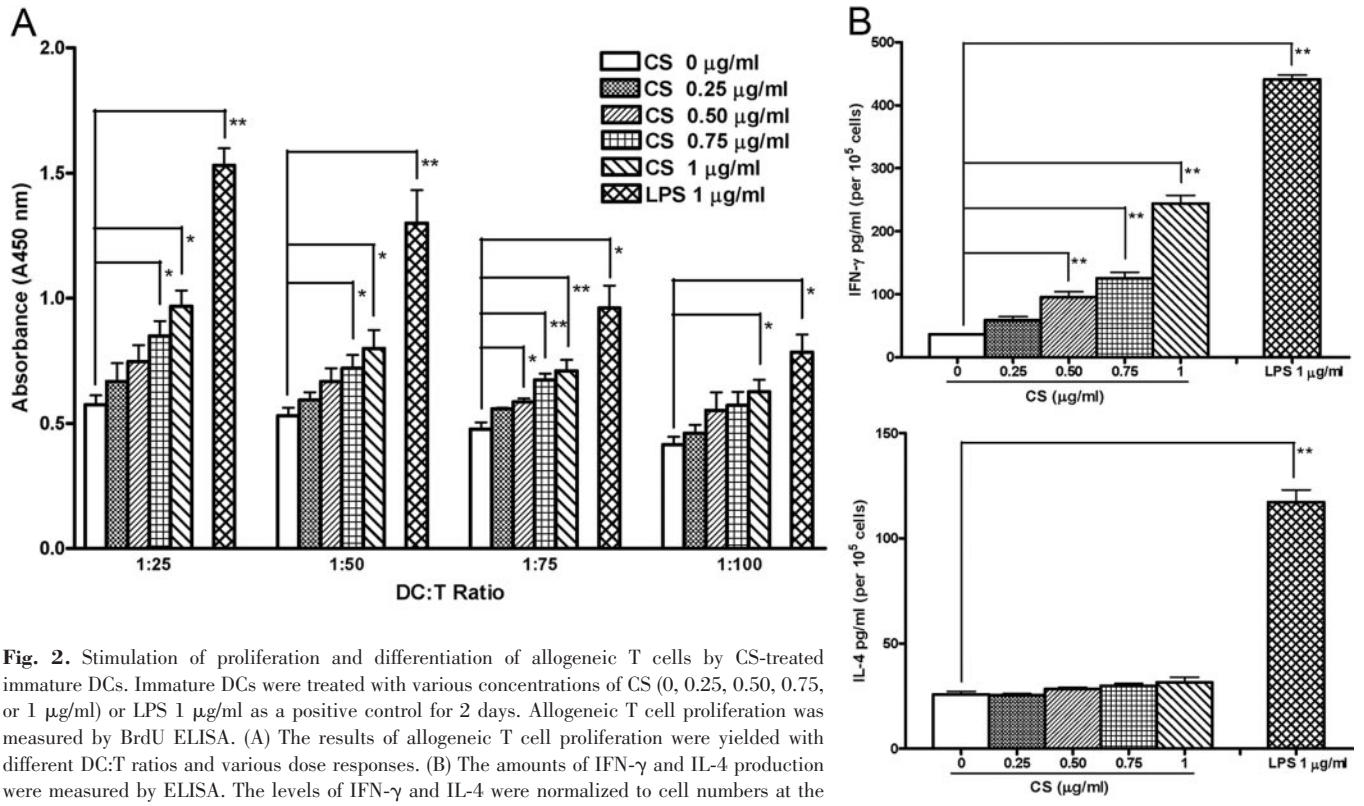

Fig. 2. Stimulation of proliferation and differentiation of allogeneic T cells by CS-treated immature DCs. Immature DCs were treated with various concentrations of CS (0, 0.25, 0.50, 0.75, or 1 μg/ml) or LPS 1 μg/ml as a positive control for 2 days. Allogeneic T cell proliferation was measured by BrdU ELISA. (A) The results of allogeneic T cell proliferation were yielded with different DC:T ratios and various dose responses. (B) The amounts of IFN-γ and IL-4 production were measured by ELISA. The levels of IFN-γ and IL-4 were normalized to cell numbers at the end of the incubation period. Data are presented as the mean ± SD from three independent experiments. Statistical significance for the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: *, $P < 0.05$; **, $P < 0.01$.

CS suppresses the LPS-induced, allogeneic T cell proliferation and shifts the LPS-induced Th1 response toward the Th2 response

The above results indicate that CS inhibits the maturation and cytokine productions of LPS-activated DCs. This study examined further whether CS affects the T cell activation by LPS-activated DCs. The immature DCs generated from human monocytes were treated with LPS (1 μg/ml) and various concentrations of CS extracts (0, 0.5, and 1 μg/ml) for 2 days. The DCs were then collected to be cocultured with allogeneic T cells for MLR assay. **Figure 4A** shows that CS suppresses the LPS-induced DC-elicited allogeneic T cell proliferation. Moreover, how CS affects the differentiation of T cells by LPS-activated DCs was examined further. Cultured supernatants from allogeneic MLR experiments were analyzed by ELISA. Experimental results indicate that the IFN-γ production by T cells is lower when treatment involves LPS and CS than the treatment with LPS alone (Fig. 4B). However, treatment with LPS and CS yields a greater production of IL-4 than treatment with LPS alone does (Fig. 4B). From these results, we infer that CS suppresses the proliferation of LPS-activated DC-elicited allogeneic T cells and shifts the LPS-activated DC-promoted Th1 response toward a Th2 response.

DISCUSSION

Chinese herbal medicine has become increasingly popular worldwide in health promotion and adjuvant therapy [6, 29, 39]. The advantage of natural products with multiple pharmacological ingredients may provide more clinical effectiveness with fewer side-effects than a single compound-based drug [16]. This study elucidates the immunomodulatory effects of CS on DCs in two different physiological stages: naïve and LPS-induced inflammatory. When immature DCs were used, CS effects were similar to that of LPS on immature DCs. CS and LPS increase the surface expression of CD11c, CD205, CD40, CD80, CD83, CD86, and MHC-II by DCs and reduce the endocytic activity of immature DCs. These changes point toward the role of CS in promoting DC maturation. Functionally, CS enhances the production of cytokines by DCs, including the Th1-related IL-12p70 and proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6. Although CS has similar effects as LPS on immature DCs, CS-induced maturation appeared much less complete than that observed after the stimulation of LPS. This reflects the nature that CS, unlike LPS as a pathogen, is a modulating agent. This finding is in part consistent with some known CS functions in other immune cells, such as the enhanced production of IFN, IL-1, and TNF by rat Kupffer cells [40] and increased production of IL-6 by human or murine macrophages [8, 41]. Moreover, CS can increase the numbers of Th cells in peripheral blood and spleen [42]. In addition, we demonstrated that CS-primed DCs induce the proliferation of allogeneic T cells and promote the Th1 polarization. Those results indicate that CS can potentially modulate DCs toward cell-mediated immunity via the production of cytokines and the promotion of the Th1 response.

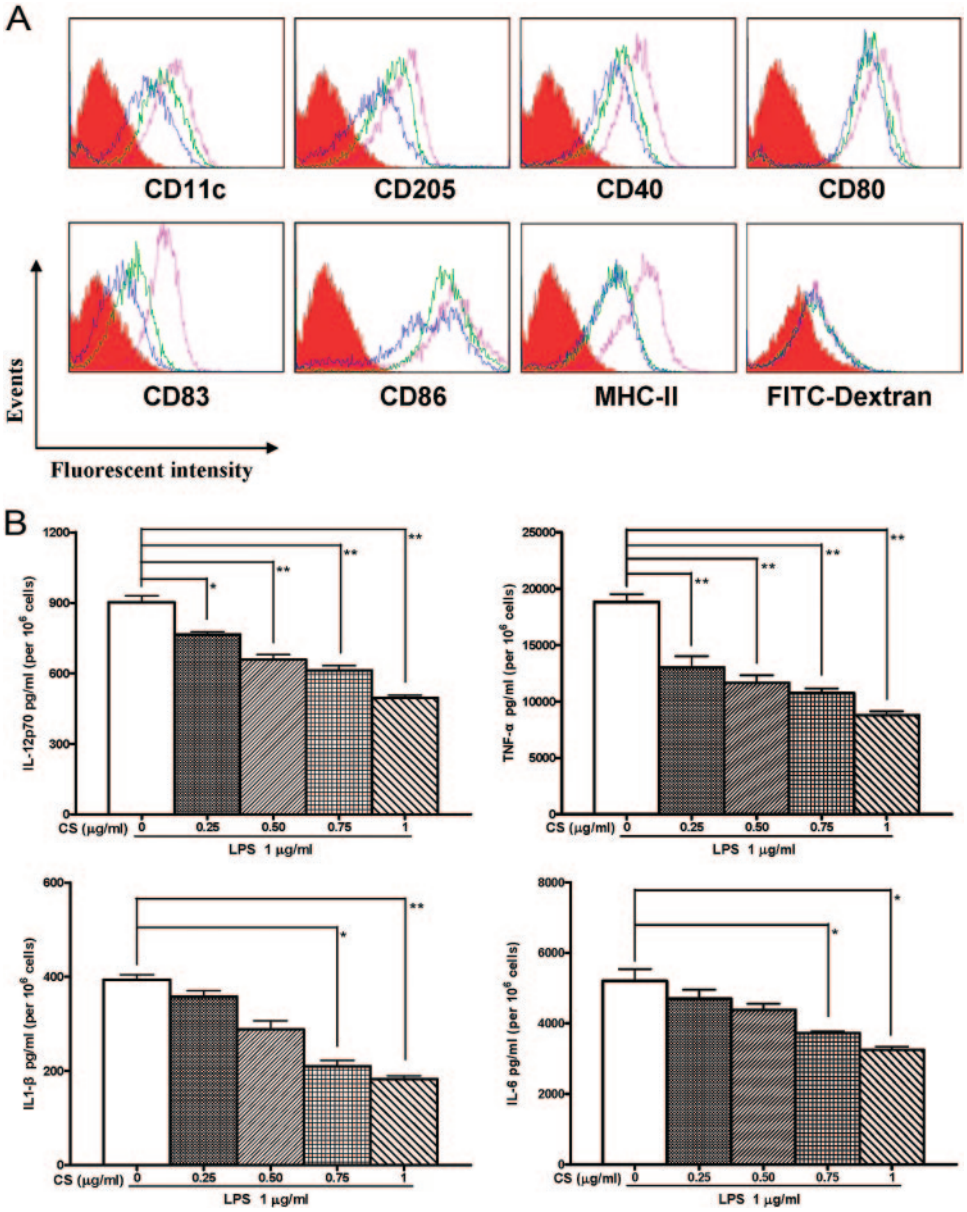

Fig. 3. CS suppressed the expression of phenotypic characteristics and the production of cytokines by LPS-activated DCs. (A) Phenotypic characteristics and endocytic capacity of LPS-activated DCs treated with CS. Cell surface expression was analyzed by flow cytometry on DCs treated with LPS 1 μg/ml and various concentrations of CS (0 μg/ml, purple line; 0.5 μg/ml, green line; or 1 μg/ml, blue line) for 2 days. The red histograms represent staining with corresponding isotype control mAb. Endocytic capacity was determined by the endocytosis of FITC-dextran followed by flow cytometry. Immature DCs were treated with LPS 1 μg/ml and various concentrations of CS (0 μg/ml, purple line; 0.5 μg/ml, green line; or 1 μg/ml, blue line) for 2 days. Cells were collected and then incubated with FITC-dextran at 37°C for 30 min or at 4°C as a control (red histogram). The results are representative of three independent experiments. (B) The effects of CS on cytokine productions of LPS-activated DCs. Immature DCs were treated with LPS 1 μg/ml and various concentrations of CS (0, 0.25, 0.50, 0.75, or 1 μg/ml) for 2 days. Culture supernatants were collected and the amounts of IL-12p70, TNF-α, IL-1β, and IL-6 were measured by ELISA. Data are presented as mean ± SD from three independent experiments. Statistical significance for the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: *, $P < 0.05$; **, $P < 0.01$.

On the other hand, when DCs were stimulated by LPS toward an inflammatory stage, the effect of CS on LPS-activated DCs differs from that on immature DCs. Our results demonstrate that CS reduces the expression of CD11c, CD205, CD40, CD80, CD83, CD86, and MHC-II by LPS-activated DCs. Furthermore, CS suppresses the production of Th1-related IL-12p70 and proinflammatory cytokines TNF-α, IL-1β, and IL-6 by LPS-activated DCs. The functional assays also demonstrate that CS reduces the capacity of LPS-activated DCs on stimulating allogeneic T cell proliferation and skews the Th1 reaction toward a Th2 response by decreasing IFN-γ production and increasing IL-4 production. The fact that CS retards LPS-activated DC activation indicates that CS has anti-LPS-elicited inflammatory activity. A similar immunomodulatory effect of CS has been reported in that CS recovers the balance on the Th1:Th2 ratio in patients with *Condyloma acuminatum* [43] and down-regulates the expression of inflammation-related genes in the rat kidney following ischemia/reperfusion [9].

Among the several compounds in the CS extracts include cordycepin, adenosine, guanosine, ergosterol, uridine, mannitol, and polysaccharides [15]. According to a recent study, polysaccharide purified from a Chinese herb *Ganoderma lucidum* induces the activation and maturation of human monocyte-derived DCs via the NF-κB and p38 MAPK pathway by TLR-4 [25], which resembles the effects of LPS on DC maturation [44]. The NF-κB pathway has been considered as a common pathway for many activators leading to DC maturation [45]; our data (Supplemental Fig. 4) add that the NF-κB pathway is also involved in CS action on DC maturation. Furthermore, Jordan et al. [41] demonstrate that CS activates murine macrophages to produce inflammatory cytokines via the MAPK pathway by TLR-2 and TLR-4 using TLR-2-, MYD88-, and TLR-4-deficient mice. These observations suggest that although CS was used as a sole stimulating agent, its acting mechanism resembles LPS action by affecting NF-κB, p38 MAPK, and the JNK signaling pathway via TLR. Although the

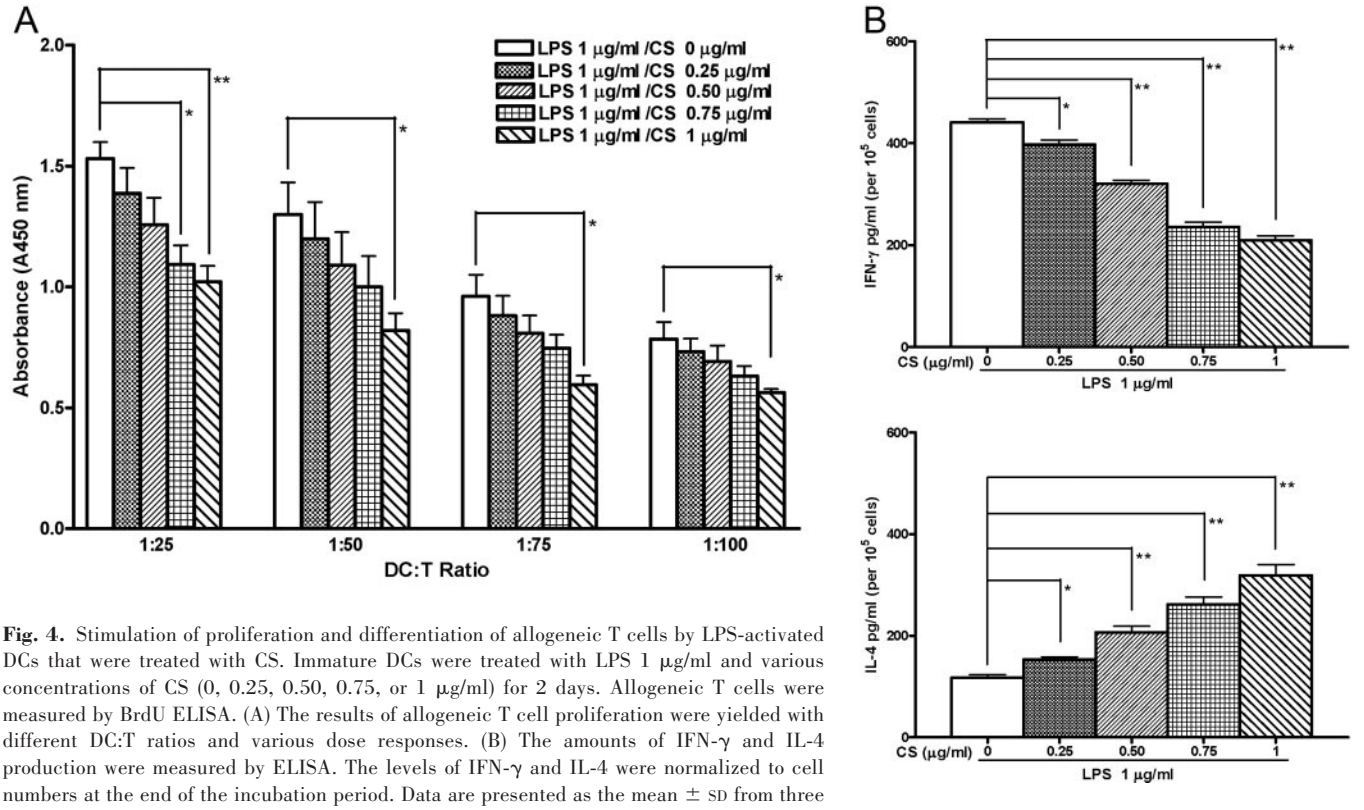

Fig. 4. Stimulation of proliferation and differentiation of allogeneic T cells by LPS-activated DCs that were treated with CS. Immature DCs were treated with LPS 1 μg/ml and various concentrations of CS (0, 0.25, 0.50, 0.75, or 1 μg/ml) for 2 days. Allogeneic T cells were measured by BrdU ELISA. (A) The results of allogeneic T cell proliferation were yielded with different DC:T ratios and various dose responses. (B) The amounts of IFN-γ and IL-4 production were measured by ELISA. The levels of IFN-γ and IL-4 were normalized to cell numbers at the end of the incubation period. Data are presented as the mean ± SD from three independent experiments. Statistical significance for the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: *, $P < 0.05$; **, $P < 0.01$.

CS sample used in this study ruled out bacterial endotoxin contamination, we can't exclude the possibility that the CS samples used in this study contain other minor TLR ligands. On the other hand, we found that CS suppresses LPS-induced DCs maturation and inflammatory response but does not inhibit sCD40L-induced DCs maturation (Supplemental Fig. 5). This indicates that CS has specific effects on the LPS-involved signaling pathway. A question derived appears that CS may compete the binding of LPS on the TLR-4 receptor or neutralize LPS in culture medium. We can exclude the possibility, as we found that CS does not affect FITC-conjugated LPS binding to DCs (Supplemental Fig. 6), and CS has similar effects when CS was added 1 day after LPS stimulation (Supplemental Fig. 3). Suppressive effects of CS on LPS-activated DCs still remain unclear. However, according to a previous study, cordycepin, a component of CS, inhibits LPS-induced NO production and the expression of inducible NO synthesis and cycloxygenase-2 genes by Raw 264.7 macrophages via the suppression of NF-κB activation, Akt, and p38 phosphorylation [10]. We speculate that the multiple components of CS extracts account largely for the differential effects of CS on immature DCs and LPS-activated DCs. Moreover, recent studies have demonstrated that the differential binding to the DC-specific intercellular adhesion molecule-grabbing nonintegrin (SIGN) and TLR results in immune activation or suppression [46, 47]. More than a pathogen receptor, DC-SIGN also controls immunoregulation that shifts the Th1 to the Th2 response by altering the TLR-mediated activation of DCs [48]. Thus, the differential effects of CS on DCs in the absence or presence of LPS are likely owing to the alteration of DC-SIGN-dominant versus TLR-dominant pathways.

TCM, in contrast to Western medicine, uses decoctions of herb mixtures that are typically customized for patients. The healing philosophy of TCM relies on the concept of restoring and maintaining the internal balance of Yin and Yang. As a result, TCM is recognized as more suitable for disease prevention and health promotion [49]. The Chinese Yin and Yang concept represents the dialectic elements in nature, such as light versus dark, hot versus cold, or weak versus strong. This concept is used extensively to describe the balance of immunoregulation [50–52]. According to the Yin-Yang theory in Chinese medicine, all herbs can be classified as Yin or Yang in nature. CS is a unique, tonifying herb in Chinese medicine that possesses "Yin-nourishing" and "Yang-invigorating". Recent studies have demonstrated that the pharmacological bases of the Yin-nourishing and Yang-invigorating characteristics of CS are anti-oxidation and oxidation activities, respectively [53, 54]. Although the Yin-Yang theory is the basic concept of TCM, the pharmacological foundation of the Yin-nourishing and Yang-invigorating of the immune function by CS still remains unclear. This study demonstrates that CS can potentially enhance the immunity of DCs by generating proinflammatory cytokines, enhancing the reactivity of allogeneic T cells, and promoting Th1 polarization. On the other hand, CS retards the overactive immunity of LPS on DCs by suppressing LPS-induced proinflammatory cytokine productions, reducing LPS-induced DC reactivity on allogeneic T cells, and shifting a LPS-elicited DC-driven Th1 response toward a Th2 response.

In summary, to our knowledge, this investigation is the first to demonstrate the ability of CS to differentially modulate the activities of different stages of DCs, which is potentially critical in balancing the control of the homeostatic steady-state of host immunity. Our results demonstrate that CS poses seesaw-like balance, by which it boosts a weak immune system under normal circumstances and suppresses overactive immune responses. These suggest that CS, as a health supplement and medicine, has a Yin-Yang balancing act in immunomodulation through enhancing host immunity and suppressing overactive immune reactions.

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REFERENCES

1. Zhu, J. S., Halpern, G. M., Jones, K. (1998) The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis*: part I. *J. Altern. Complement. Med.* **4,** 289–303.
2. Zhu, J. S., Halpern, G. M., Jones, K. (1998) The scientific rediscovery of a precious ancient Chinese herbal regimen: *Cordyceps sinensis*: part II. *J. Altern. Complement. Med.* **4,** 429–457.
3. Buenz, E. J., Bauer, B. A., Osmundson, T. W., Motley, T. J. (2005) The traditional Chinese medicine *Cordyceps sinensis* and its effects on apoptotic homeostasis. *J. Ethnopharmacol.* **96,** 19–29.
4. Kuo, Y. C., Lin, C. Y., Tsai, W. J., Wu, C. L., Chen, C. F., Shiao, M. S. (1994) Growth inhibitors against tumor cells in *Cordyceps sinensis* other than cordycepin and polysaccharides. *Cancer Invest.* **12,** 611–615.
5. Chen, Y. J., Shiao, M. S., Lee, S. S., Wang, S. Y. (1997) Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. *Life Sci.* **60,** 2349–2359.
6. Kuo, Y. C., Tsai, W. J., Shiao, M. S., Chen, C. F., Lin, C. Y. (1996) *Cordyceps sinensis* as an immunomodulatory agent. *Am. J. Chin. Med.* **24,** 111–125.
7. Kuo, Y. C., Tsai, W. J., Wang, J. Y., Chang, S. C., Lin, C. Y., Shiao, M. S. (2001) Regulation of bronchoalveolar lavage fluids cell function by the immunomodulatory agents from *Cordyceps sinensis*. *Life Sci.* **68,** 1067–1082.
8. Koh, J. H., Yu, K. W., Suh, H. J., Choi, Y. M., Ahn, T. S. (2002) Activation of macrophages and the intestinal immune system by an orally administered decoction from cultured mycelia of *Cordyceps sinensis*. *Biosci. Biotechnol. Biochem.* **66,** 407–411.
9. Shahed, A. R., Kim, S. I., Shoskes, D. A. (2001) Down-regulation of apoptotic and inflammatory genes by *Cordyceps sinensis* extract in rat kidney following ischemia/reperfusion. *Transplant. Proc.* **33,** 2986–2987.
10. Kim, H. G., Shrestha, B., Lim, S. Y., Yoon, D. H., Chang, W. C., Shin, D. J., Han, S. K., Park, S. M., Park, J. H., Park, H. I., Sung, J. M., Jang, Y., Chung, N., Hwang, K. C., Kim, T. W. (2006) *Cordycepin* inhibits lipopolysaccharide-induced inflammation by the suppression of NF-κB through Akt and p38 inhibition in RAW 264.7 macrophage cells. *Eur. J. Pharmacol.* **545,** 192–199.
11. Rao, Y. K., Fang, S. H., Tzeng, Y. M. (2007) Evaluation of the anti-inflammatory and anti-proliferation tumoral cells activities of *Antrodia camphorata*, *Cordyceps sinensis*, and *Cinnamomum osmophloeum* bark extracts. *J. Ethnopharmacol.* **114,** 78–85.
12. Yamaguchi, Y., Kagota, S., Nakamura, K., Shinozuka, K., Kunitomo, M. (2000) Antioxidant activity of the extracts from fruiting bodies of cultured *Cordyceps sinensis*. *Phytother. Res.* **14,** 647–649.
13. Tsai, C. H., Stern, A., Chiou, J. F., Chern, C. L., Liu, T. Z. (2001) Rapid and specific detection of hydroxyl radical using an ultraweak chemiluminescence analyzer and a low-level chemiluminescence emitter: application to hydroxyl radical-scavenging ability of aqueous extracts of food constituents. *J. Agric. Food Chem.* **49,** 2137–2141.
14. Hsu, T. H., Shiao, L. H., Hsieh, C., Chang, D. M. (2002) A comparison of the chemical composition and bioactive ingredients of the Chinese medicinal mushroom DongChongXiaCao, its counterfeit and mimic, and fermented mycelium of *Cordyceps sinensis*. *Food Chem.* **78,** 463–469.
15. Li, S. P., Yang, F. Q., Tsim, K. W. (2006) Quality control of *Cordyceps sinensis*, a valued traditional Chinese medicine. *J. Pharm. Biomed. Anal.* **41,** 1571–1584.
16. Schmidt, B. M., Ribnicky, D. M., Lipsky, P. E., Raskin, I. (2007) Revisiting the ancient concept of botanical therapeutics. *Nat. Chem. Biol.* **3,** 360–366.
17. Banchereau, J., Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
18. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., Palucka, K. (2000) Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18,** 767–811.
19. Cella, M., Sallusto, F., Lanzavecchia, A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* **9,** 10–16.
20. Seder, R. A., Paul, W. E. (1994) Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu. Rev. Immunol.* **12,** 635–673.
21. Abbas, A. K., Murphy, K. M., Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383,** 787–793.
22. Chambers, C. A., Allison, J. P. (1999) Costimulatory regulation of T cell function. *Curr. Opin. Cell Biol.* **11,** 203–210.
23. Quah, B. J., O'Neill, H. C. (2005) Maturation of function in dendritic cells for tolerance and immunity. *J. Cell. Mol. Med.* **9,** 643–654.
24. Cella, M., Engering, A., Pinet, V., Pieters, J., Lanzavecchia, A. (1997) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388,** 782–787.
25. Lin, Y. L., Liang, Y. C., Lee, S. S., Chiang, B. L. (2005) Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-κB and p38 mitogen-activated protein kinase pathways. *J. Leukoc. Biol.* **78,** 533–543.
26. Reddy, A., Sapp, M., Feldman, M., Subklewe, M., Bhardwaj, N. (1997) A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. *Blood* **90,** 3640–3646.
27. Chen, C. S., Hseu, R. S. (1999) Differentiation of *Cordyceps sinensis* (Berk.) Sacc. specimen using restriction fragment length polymorphism of 18S rRNA gene. *J. Chin. Agric. Chem. Soc.* **37,** 533–545.
28. Chen, C. S., Hseu, R. S. (2002) Differentiation of *Cordyceps sinensis* (Berk.) Sacc. with 18S rRNA gene sequences. *Taiwanese J. Agric. Chem. Food Sci.* **40,** 219–225.
29. Liu, W. C., Wang, S. C., Tsai, M. L., Chen, M. C., Wang, Y. C., Hong, J. H., McBride, W. H., Chiang, C. S. (2006) Protection against radiation-induced bone marrow and intestinal injuries by *Cordyceps sinensis*, a Chinese herbal medicine. *Radiat. Res.* **166,** 900–907.
30. Thurner, B., Roder, C., Dieckmann, D., Heuer, M., Kruse, M., Glaser, A., Keikavoussi, P., Kampgen, E., Bender, A., Schuler, G. (1999) Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J. Immunol. Methods* **223,** 1–15.
31. Duperrier, K., Eljaafari, A., Dezutter-Dambuyant, C., Bardin, C., Jacquet, C., Yoneda, K., Schmitt, D., Gebuhrer, L., Rigal, D. (2000) Distinct subsets of dendritic cells resembling dermal DCs can be generated in vitro from monocytes, in the presence of different serum supplements. *J. Immunol. Methods* **238,** 119–131.
32. Liu, M., Li, Y. G., Zhang, F., Yang, L., Chou, G. X., Wang, Z. T., Hu, Z. B. (2007) Chromatographic fingerprinting analysis of Danshen root (Salvia miltiorrhiza Radix et Rhizoma) and its preparations using high performance liquid chromatography with diode array detection and electrospray mass spectrometry (HPLC-DAD-ESI/MS). *J. Sep. Sci.* **30,** 2256–2267.
33. Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., Amigorena, S. (2002) Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* **20,** 621–667.
34. Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S., Adorini, L., Gubler, U., Presky, D. H. (1998) The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu. Rev. Immunol.* **16,** 495–521.
35. Dinarello, C. A. (1996) Biologic basis for interleukin-1 in disease. *Blood* **87,** 2095–2147.
36. Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F., Austyn, J. M. (1995) Dendritic cell loss from nonlymphoid tissues after

systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J. Exp. Med.* **181,** 2237–2247.
37. De Jong, E. C., Vieira, P. L., Kalinski, P., Kapsenberg, M. L. (1999) Corticosteroids inhibit the production of inflammatory mediators in immature monocyte-derived DC and induce the development of tolerogenic DC3. *J. Leukoc. Biol.* **66,** 201–204.
38. Kelleher, M., Beverley, P. C. (2001) Lipopolysaccharide modulation of dendritic cells is insufficient to mature dendritic cells to generate CTLs from naive polyclonal CD8+ T cells in vitro, whereas CD40 ligation is essential. *J. Immunol.* **167,** 6247–6255.
39. Liu, W. C., Chuang, W. L., Tsai, M. L., Hong, J. H., McBride, W. H., Chiang, C. S. (2008) *Cordyceps sinensis* health supplement enhances recovery from taxol-induced leukopenia. *Exp. Biol. Med. (Maywood)* **233,** 447–455.
40. Liu, P., Zhu, J., Huang, Y., Liu, C. (1996) Influence of *Cordyceps sinensis* (Berk.) Sacc. and rat serum containing same medicine on IL-1, IFN and TNF produced by rat Kupffer cells. *Zhongguo Zhong Yao Za Zhi* **21,** 367–369.
41. Jordan, J. L., Sullivan, A. M., Lee, T. D. (2008) Immune activation by a sterile aqueous extract of *Cordyceps sinensis*: mechanism of action. *Immunopharmacol. Immunotoxicol.* **30,** 53–70.
42. Chen, G. Z., Chen, G. L., Sun, T., Hsieh, G. C., Henshall, J. M. (1991) Effects of *Cordyceps sinensis* on murine T lymphocyte subsets. *Chin. Med. J. (Engl.)* **104,** 4–8.
43. Gao, Q., Wu, G., He, D. (2000) Effect of *Cordyceps sinensis* on the Th1/Th2 cytokines in patients with *Condyloma acuminatum*. *Zhong Yao Cai* **23,** 402–404.
44. O'Neill, L. A. (2002) Toll-like receptor signal transduction and the tailoring of innate immunity: a role for Mal? *Trends Immunol.* **23,** 296–300.
45. Koski, G. K., Lyakh, L. A., Cohen, P. A., Rice, N. R. (2001) CD14+ monocytes as dendritic cell precursors: diverse maturation-inducing pathways lead to common activation of NF-κB/RelB. *Crit. Rev. Immunol.* **21,** 179–189.
46. Geijtenbeek, T.B., van Vliet, S.J., Engering, A., 't Hart, B. A., van Kooyk, Y. (2004) Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu. Rev. Immunol.* **22,** 33–54.
47. Zhou, T., Chen, Y., Hao, L., Zhang, Y. (2006) DC-SIGN and immunoregulation. *Cell. Mol. Immunol.* **3,** 279–283.
48. van Kooyk, Y., Geijtenbeek, T. B. (2003) DC-SIGN: escape mechanism for pathogens. *Nat. Rev. Immunol.* **3,** 697–709.
49. Yuan, R., Lin, Y. (2000) Traditional Chinese medicine: an approach to scientific proof and clinical validation. *Pharmacol. Ther.* **86,** 191–198.
50. Mills, R., Bhatt, D. L. (2004) The Yin and Yang of arterial inflammation. *J. Am. Coll. Cardiol.* **44,** 50–52.
51. 't Hart, B. A., van Kooyk, Y. (2004) Yin-Yang regulation of autoimmunity by DCs. *Trends Immunol.* **25,** 353–359.
52. Zhang, J. (2007) Yin and Yang interplay of IFN-γ in inflammation and autoimmune disease. *J. Clin. Invest.* **117,** 871–873.
53. Ko, K. M., Mak, D. H., Chiu, P. Y., Poon, M. K. (2004) Pharmacological basis of "Yang-invigoration" in Chinese medicine. *Trends Pharmacol. Sci.* **25,** 3–6.
54. Szeto, Y. T., Benzie, I. F. (2006) Is the Yin-Yang nature of Chinese herbal medicine equivalent to antioxidation-oxidation? *J. Ethnopharmacol.* **108,** 361–366.