CORDYCEPS SINENSIS EXTRACT PROMOTES PHENOTYPIC AND FUNCTIONAL MATURATION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS

Chi-Jung Wei[1], Ming-Ling Kuo[2], Rong-Chi Yang[3], Hsueh-Ling Chang[1] and Chia-Jung Hsieh[1]

[1]Division of Gynecology/Pediatrics, Center for Chinese Medicine, Lin-Kou Chang Gung Memorial Hospital Taoyuan, Taiwan
[2]Department of Microbiology and Immunology, Graduate Institute of Basic Medical Science, Chang Gung University Taoyuan, Taiwan
[3]Department of Chinese Herbal Pharmacy, Lin-Ko Chang Gung Memorial Hospital Taoyuan, Taiwan
(Received 11th March 2005, accepted 2th May 2005)

Cordyceps sinensis (*C. sinensis*) is well known for its modulating effect on host immune system. As dendritic cells (DC) are professional antigen-presenting cells (APC) that play a key role in cancer immunotherapy, the maturation of DC is critical to the induction of T-cell responses. We investigated the effect of *C. sinensis* extract on the maturation of human monocyte-derived DC and the secretion of cytokines. Peripheral blood mononuclear cells (PBMC) from healthy donors were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 6 days, then stimulated by hot-water extract of *C. sinensis* for 2 days. Stimulation with *C. sinensis* extract increased cell surface expression, and the RMFI (ratio of mean fluorescence intensity) of CD80, CD83, CD86 and HLA-DR are 1.70 ± 0.32, 1.20 ± 0.01, 2.29 ± 0.29 and 1.44 ± 0.23 (n = 5, $p < 0.05$), respectively. Moreover, when co-cultured with allogeneic T cells, *C. sinensis* extract caused the significant production of IL-12 p40 (256.8 ± 185.4 pg/mL, control group is 98.2 ± 46.73 pg/mL) and IFN-γ (311.1 ± 156.4 pg/mL, control group is 22.5 ± 35.1 pg/mL). In conclusion, these findings suggest that *C. sinensis* extract is a potent stimulator of DC and may be related to modulation of Th1 cells functions.

Key words: Cordyceps sinensis, Dendritic cells, Co-stimulatory molecules, T cells, Herbal extract.

INTRODUCTION

Cordyceps sinensis (*C. sinensis*) is a parasitic organism that grows on *Hepialus armoricanus* until the caterpillar

Correspondence to: Chia-Jung Hsieh, M.D., Division of Gynecology/Pediatrics, Center for Chinese Medicine, Lin-Kou Chang Gung Memorial Hospital, Tel: 03-3196200 ext. 2611, Fax: 03-3298995, E-mail: hsiehcj@adm.cgmh.org.tw

dies and the fungus sprouts from the caterpillar's head. It is used as an important component in many Chinese prescriptions for thousands of years to restore energy and promote longevity.

There are many studies reported that *C. sinensis* has important biological activities in the modulating immune responses[1-3] and inhibiting tumor cell growth[4,5]. In host immune system, it reduced the PMN activation[6], enhanced the NK cell activities[7,8] and macrophage phagocytic function[9]. It further induced the expression of IL-2 and IFN-γ[10] with the protective effects against the immunological liver injury of mice[11]. In anti-tumor effect, an *in vivo* study revealed that the sarcoma 180, Lewis lung carcinoma, B16 melanoma[4] and liver carcinoma cells[12] in mice were significantly suppressed by the treatment of *C. sinensis*[13,14]. However, the mechanisms of the anti-tumor effect of *C. sinensis* have not yet been realized.

Dendritic cells (DC) are potent professional APCs that play an important role in the immune system, especially in mediating the activation of primary T lymphocytes[15]. The findings of the functions of DC have made it a preferred adjuvant for cancer immunotherapy today[16]. Immature DC (imDC) take up antigens at the entry sites of infectious pathogens and move into secondary lymphoid tissues. During the process, imDC convert into mature DC. After maturation, DC lose the endocytic capacity but enhance the expression of the co-stimulatory molecules (CD80 and CD86) and major histocompatibility complexes (MHC). The mature DC activates both the helper and cytotoxic T cells through MHC-peptide complexes, co-stimulatory factors, and multiple cytokines, such as IL-12 and IFN-γ. Then, the activated cytotoxic T cells have the killing effect on cancer cells. Therefore, the maturation stage of DC plays a key role in the initiation of immune response for clinical application in anti-tumor therapy[17].

To investigate the influence of *C. sinensis* extract on DC, we studied the phenotypic changes of DC from human peripheral monocytes and the production of cytokines after co-cultured with allogeneic T cells.

MATERIALS AND METHODS

Preparation of *C. Sinensis* Extract

Dried *C. sinensis*, including fruiting bodies and mycelia, was offered by pharmacy of Lin-Kou Chang Gung Memorial Hospital (Taoyuan, Taiwan). Fifty grams of *C. sinensis* was soaked in 500 mL of distilled water at room temperature, and then boiled for 2 hours. The supernatant was collected and the substance was boiled one more time. The combined supernatants were allowed to settle for 12 hours, filtered through a 0.22 μm filter, and then stored at 4 °C. Very low level of endotoxin was detected in the *C. sinensis* extract, *i.e.* below 0.03 EU/mL, when assayed with the Limulus amebocyte-lysate E-Toxate test (Sigma, St Louis, MO, USA) according to the manufacturer's instruction.

Generation and Culture of DC

Peripheral blood monocytic cells (PBMC) were isolated from fresh whole heparinized blood of different healthy

donors by Ficoll-Hypaque (1.077 g/mL, Gibco Invitrogen, Carlsbad, CA, USA) density gradient centrifugation in Lymphoprep. PBMC were re-suspended in RPMI 1640 medium and allowed to adhere in culture flasks for 2 hours at 37 °C. Nonadherent cells were removed by extensive washes and adherent monocytes were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 100 mM sodium pyruvate, 1% nonessential amino acids, and supplemented with GM-CSF (800U/mL, BD PharMingen, San Diago, CA, USA) and IL-4 (400U/mL, BD PharMingen). Because of the short half-life of the cytokines, GM-CSF and IL-4 were added one more time to the cultures at day 3 or day 4. The purity of the cell preparation was evaluated by flow cytometric analysis with anti-CD14 staining (> 80% viable monocytes).

In Vitro Activation of DC

Monocyte-derived DC were cultured at 1×10^6 cells/mL in 24-well plates either in medium alone or in the presence of 100 ng/mL LPS (from *Escherichia coli*, Sigma) or 500 μg/mL *C. sinensis* for 48 hours after 6 days of generation of the immature DC. Then, the cells were analyzed by flow cytometry for immunophenotypings of DC or addition of T cells for DC-T cell co-cultures.

Immunophenotyping of DC

Monocyte-derived DC were stained using FITC- or PE-labeled mAb specific for CD80, CD83, CD86, and HLA-DR (BD PharMingen). Briefly, 5×10^5 cells were incubated with the relevant mAbs or their isotype-matched controls for 20 min at 4 °C and washed. The fluorescent intensity was measured with a FACSCalibur flow cytometer (BD PharMingen) and analyzed with CELL Quest software. Results are expressed as percentage of positive cells and in terms of relative mean fluorescence intensity (RMFI), calculated as follows:

$$RMFI = (MFI_{treatment} - MFI_{control})/MFI_{control}$$

Cytokine Detection

PBMC were isolated from fresh heparinized whole blood of different healthy donors by density gradient centrifugation. PBMC were re-suspended in RPMI 1640 medium for 2 hours at 37 °C. Non-adherent cells were collected to use as the allogeneic T cells.

Allogeneic T cells 1×10^6/well were co-cultured with mature DC. After 5 days, the supernatants were collected. The cytokines, IL-12 and IFN-γ, in culture supernatants were determined by sandwich enzyme-linked immunosorbent assay (ELISA) (Pharmingen) according to the manufacturer's protocol.

Statistical Analysis

Data from the *C. sinensis*- or LPS-stimulated DC were compared with unstimulated DC, using unpaired or

paired Student *t* tests. A value of $p < 0.05$ is considered statistically significant.

RESULTS

C. Sinensis Extract Enhanced the Phenotypic Maturation of DC

To study the effect of *C. sinensis* extract on the maturation of DC, human monocytes derived DC were cultured with GM-CSF and IL-4 for 6 days, followed by another 2 days in the presence of *C. sinensis* (500 μg/mL). As a positive control, human monocytes were cultured with GM-CSF and IL-4 for 6 days, followed by another 2 days in the presence of lipopolysaccharide (LPS), a known activator of DC. The resulting population of immature DC and mature DC were analyzed by flow cytometry.

The expression level of CD83, a maturation marker for DC, as expressed by RMFI (ratio of mean fluorescence intensity) on monocyte-derived DC after cultured without stimulus, and with LPS and *C. sinensis* extract are 1, 1.84 ± 0.32, 1.21 ± 0.01, respectively. The expression levels of CD80, CD86 and HLA-DR as expressed by RMFI were also examined. The data showed that the *C. sinensis* extract up-regulated the expression levels of CD83, HLA-DR and co-stimulatory molecules CD80 and CD86 (Table 1). An increase in the expression of various markers also occurred after treating cells with LPS. Typical data of phenotypes are shown in Fig. 1. This indicated that the DC are activated by *C. sinensis* extract.

C. Sinensis Extract Induced IL12-p40 and IFN-γ Production after DC-T Cells Cocultures

Next, we evaluated the function of mature DC activated by *C. sinensis* extract. Immature DC from PBMC cultured with GM-CSF and IL-4 for 6 days, then added *C. sinensis* extract, LPS, or without stimulus. On day 8, allogeneic T cells were added at stimulator/responder ratio of 1:10 and co-cultured with DC for 5 days. The cell-free supernatants were collected and the cytokines were measured by ELISA.

C. sinensis-primed mature DC co-cultured with allogeneic T cells secreted much more IL-12 p40 (256 ± 185.43 pg/mL) when compared with the control group (98.22 ± 46.73 pg/mL). IFN-γ secreted by *C. sinensis*-primed mature DC

Table 1. Effects of *C. sinensis* extract on DC phenotypes

	CD80	CD83	CD86	HLA-DR
Control	1	1	1	1
LPS	2.60 ± 0.43*	1.84 ± 0.32*	2.70 ± 1.11	1.74 ± 0.21*
C. sinensis	1.70 ± 0.32*	1.20 ± 0.01*	2.29 ± 0.29*	1.44 ± 0.23*

After stimulation of immature DC with *C. sinensis* extract (500 μg/mL), LPS (100 ng/mL), or vehicle control for 48 hours, the fluorescent intensity of CD80, CD83, CD86 and HLA-DR were measured by flow cytometry. Data are shown as ratio of mean fluorescent intensity (RMFI) ± SD of 5 independent experiments. *$p < 0.05$ compared with control group.

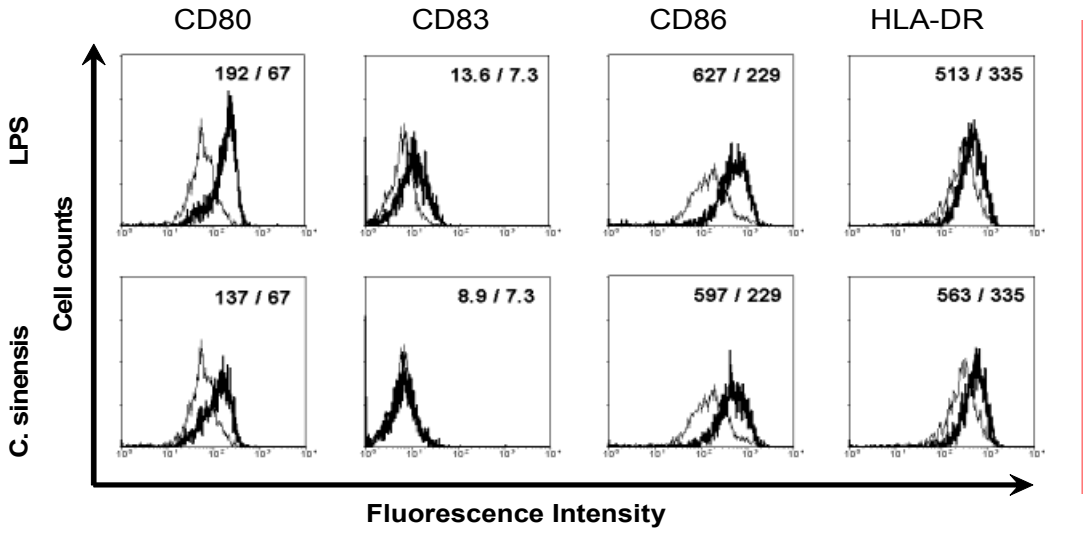

Fig. 1. Phenotype of mature DC differentiated with LPS and *C. sinensis* extract. The immature DC from PBMC were cultured with GM-CSF (800 U/mL) and IL-4 (400 U/mL) for 6 days, then blank (thin-lined histogram), *C. sinensis* extract 500 μg/mL and LPS (thick-lined histograms) were cultured for another 2 days. The cell surface phenotype of CD80, CD83, CD86 and HLA-DR were measured by flow cytometry. One representative example of 5 experiments is shown.

co-cultured with allogeneic T cells are also significantly higher than those of control group (Fig. 2). These results indicated that the *C. sinensis*-primed mature DC possessed functional effects.

DISCUSSION

In this study, we analyzed the effects of *C. sinensis* extract on human monocyte-derived DC. In culture, DC exists in two phenotypically and functionally distinct states, immature and mature. Immature cells are adept at endocytosis and express relatively low levels of surface MHC class I, MHC class II (in human that is HLA-DR) and co-stimulatory molecules (CD80 and CD86). The differentiation of immature DC into mature DC is important to the immune response and in clinical application. During differentiation, DC up-regulate the expression of MHC class I, MHC class II and co-stimulatory molecules to increase their efficiency as antigen presenting cells. Here we have demonstrated that the *C. sinensis* extract is a potent maturation factor for human monocyte-derived DC by up-regulating the expressions of CD80, CD83, CD86 and HLA-DR. Besides, we had tested three concentrations of *C. sinensis* extract, 100, 300, and 500 μg/mL (data not showed). The concentration-dependent effect by *C. Sinensis* was noticed with in these concentrations. The maximum effects were observed at both 500 and 300 μg/mL. This may be caused by individual differences from donors.

A key advance in dendritic-cell immunotherapy might provide the relevant signals to induce T cell differentiation and activation that are either not produced appropriately or down-regulated by the tumor. IL-12 plays an important role

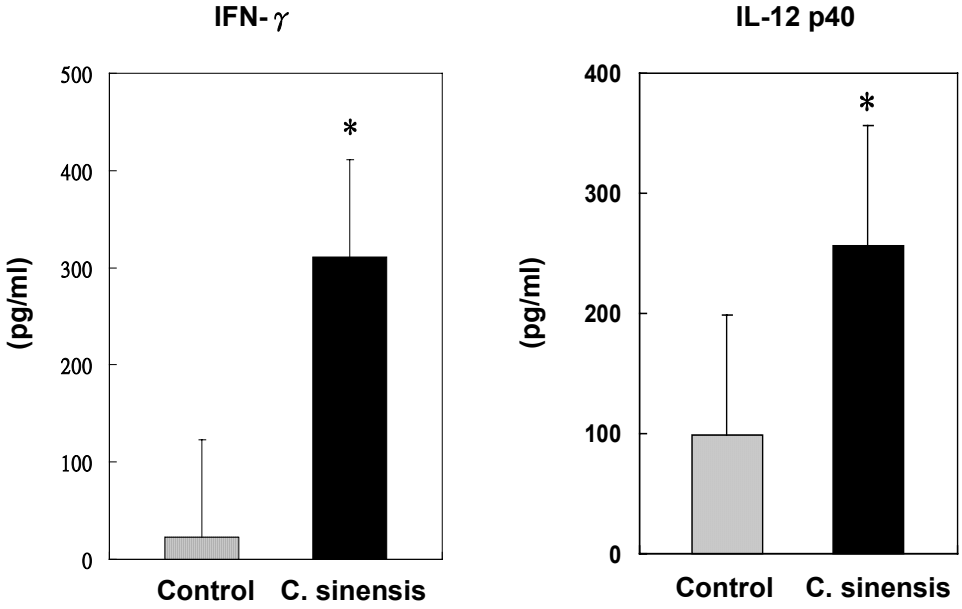

Fig. 2. Cytokine production in supernatant of allogeneic T cells stimulated with or without *C. sinensis*-primed mature DC. The production of IL-12 p40 (256.8 ± 185.43 pg/mL) and IFN-γ (311.1 ± 46.73 pg/mL) from *C. sinensis*-primed mature DC are significantly higher than those of the control group. * $p < 0.05$ compared with the control group, n = 3.

in inducting maturation of Th1 cells from naïve Th0 cells and a key cytokine in anti-tumoral responses[18-20]. IL-12 also acts on NK cell, Th1 cells and CD8+ T cell to stimulate IFN-γ production and induces cytolytic activity[21]. Therefore, IFN-γ is not only a mediator of innate immunity. In adaptive immunity, IFN-γ produced by T cells most enhanced by IL-12 possesses important immune functions. The sequence of reactions involving IL-12 and IFN-γ is central to cell-mediated immunity against intracellular microbes. In addition, the Th1 cells that produce IFN-γ have been shown to exert a powerful anti-tuomr effect[22]. Compared with the control group, we have demonstrated that the production of IL-12 p40 and IFN-γ by DC stimulated with *C. sinensis* extract is significantly promoted. Although the precise source of IFN-γ secretion remains unclear, it seems that the *C. sinensis* extract initiate the production of IL-12 from DC and then direct the T cells to proliferation. However, whether it helps Th0 cells differentiation into Th1 cells or not, has not yet been convinced. It needs to have further research.

In conclusion, our data demonstrated that the *C. sinensis* extract used in our study influences the maturation of human monocyte-derived DC. Moreover, *C. sinensis*-primed mature DC displayed increased production of IL-12 and IFN-γ when co-cultured with allogeneic T cells. It indicates that the *C. sinensis* extract may help Th cells to differentiate into Th1 cells although we need more data to prove. These observations suggest that the natural herb, *C. sinensis*, can be applied as a promising adjuvant in immunotherapy.

ACKNOWLEDGEMENTS

This study was supported by a grant from Chang Gung Memorial Hospital, grant number CMRPG33132.

REFERENCES

1. Muller WEG, Weiler BE, Charubala R, Pfleriderer W, Lserman L, Sobol RW, Suhadolnik RJ and Schroder HC. Cordycepin analogues of 2′5′-oligoadenylate inhibit human immunodeficiency virus infection via inhibition of reverse transcriptase. Biochemistry 30: 2027-2033, 1991.
2. Kuo YC, Tsai WJ, Wang JY, Chang SC, Lin CY, Shiao MS. Regulation of bronchoalveolar lavage fluids cell function by the immunomodulatory agents from *Cordyceps sinensis*. Life Sci. 68: 1067-1082, 2001
3. Kong XH, Xiao ZX, Wagn H, Chu JH, Yin XJ. Study on the immunity of culture *Cordyceps sinensis* nutritional liquid. J Capital Univ. Med. Sci. 3: 168-169, 1999.
4. Nakamura K, Yamaguchi Y, Kagota S, Kwon YM, Shinozuka K, Kunitomo M. Inhibitory effect of *Cordyceps sinensis* on spontaneous liver metastasis of Lewis lung carcinoma and B16 Melanoma cells in syngeneic mice. Jpn J Pharmacol. 79: 335-341, 1999.
5. Chen YJ, Shiao MS, Lee SS, Wang SY. Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. Life Sci. 60: 2349-2359, 1997.
6. Lin YG, Chen UL, Wu YH, Chen YP, Yang XH. Influence of *Cordyceps* on PMN activation of human race. Shanghai J. Trad. Chinese Med. 37: 49-51, 2003.
7. 仇志根、林梓、錢龍華，冬蟲夏草對白血病人 NK 和 LAK 細胞活性的影響，Shanghai mian yi xue za zhi. 14: 30-31, 1994。
8. Zeng XK, Tang Y, Yuan SR. Effect of CS and CN80-2 on T lymphocytes subsets and natral killer cell activities. Pharmacol. Clinics Chin. Materia Med. 16: 21-23, 2003.
9. Wang GW, Tang SC, Chen XW. Effects of *Cordyceps sinensis* extracts on macrophage phagocytic function in mice. J. Shanxi Med. Univ. 31: 20-21, 2000.
10. Qiu DK, Xiao SD, Zeng MD. Effects of *Cordyceps* polysaccharides on the production of IL-2 and IFN-γ and the Expression of IL-2R in human peripheral blood lymphocytes. Shanghai mian yi xue za zhi 15: 321-323, 1995.
11. Zeng XK, Tang Y, Yuan SR. The protective effects of CS and CD80-2 against the immunological liver injury in mice. Chin. Pharm. J. 36: 161-164, 2001.
12. Liu MG, Tao LI, Liang XQ, et.al. Analysis of effects of aweto against planted tumor of liver carcinoma ascitic strain on juvenile mice of different sexes. Guangxi yi ke da xue xue bao. 18: 21-23, 2001.
13. Li SP, Ji H,Li P, Dong TX, Zhan HQ, Li WQ. Advances in studies on anti-tumor activity of *Cordyceps sinensis*. Zhong cao yao 32: 373-375, 2001.
14. Bok JW, Lermer L, Chilton J, Klingeman HG, Towers GH. Antitumor sterols from the mycelia of *Cordyceps sinensis*. Phytochemistry 51: 891-898, 1999.
15. Turley SJ, Inaba K, Garrett WS, Ebersold M, Unternaehrer J, Steinman RM, Mellman I. Transport of peptide-

MHC class II complexes in developing dendritic cells. Science 288: 522-527, 2000.

16. Nestle FO, Banchereau J, Hart D. Dendritic cells: On the move from bench to bedside. Nat. Med. 7: 761-765, 2001.
17. Tsuyoshi T, Yuji T, Mie N, Takeshi A, Shigeru C, Takeo J, Yoichi S, Hisamaru H. Dendritic cell vaccination for patients with chronic myelogenous leukemia. Leuk. Res. 27: 795-802, 2003.
18. Trinchieri G, Scott P. The role of interleukin 12 in the immune response, disease and therapy. Immunol. Today 15: 460-463, 1994.
19. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. 16: 495-521, 1998.
20. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260: 547-549, 1993.
21. Abul K. Abbas, Andrew H. Lichtman, Cellular and molecular immunology, 5th edition. W.B. Saunders Company, pp 260-270.
22. Shurin MR, Lu L, Kalinski P, Stewart-Akers AM, Lotze MT. Th1/Th2 balance in cancer, transplantation and pregnancy. Springer Semin Immunopathol. 21: 339-359, 1999.

J Chin Med 16(1): 47-55, 2005

冬蟲夏草提取液促進人類單核球轉化之樹突狀細胞的成熟與功能

魏麒容[1]　郭敏玲[2]　楊榮季[3]　張雪玲[1]　謝佳蓉[1]

[1]林口長庚紀念醫院，中醫醫院婦兒科

[2]長庚大學，基礎醫學研究所，微生物免疫學組

[3]林口長庚紀念醫院，中醫藥劑部

桃園，台灣

(2005 年 3 月 11 日受理，2005 年 5 月 2 日接受刊載)

冬蟲夏草（*Cordyceps sinensis, C. sinensis*）為大家熟知可以調節人體免疫系統的中草藥。樹突狀細胞（dendritic cells，DC）是很重要的抗原呈現細胞（antigen presenting cell），其成熟所引發 T 細胞的反應，在癌症的免疫治療上扮演了很重要的角色。我們研究冬蟲夏草提取液是否影響取自人類週邊血液單核球（peripheral blood monocyte，PBMC）所培養之樹突狀細胞的成熟，並且測量細胞激素的分泌。來自健康捐贈者血液的單核球與顆粒球–巨噬細胞群落刺激因子（GM-CSF）和白細胞介素 4（IL-4）共同培養 6 天，然後加入熱水提取的冬蟲夏草培養 2 天。經冬蟲夏草提取物的刺激，細胞表面分子 CD80、CD83、CD86 和 HLA-DR 相較於控制組皆有明顯的增加，其 RMFI（ratio of mean fluorescence intensity）分別為 1.70 ± 0.32，1.20 ± 0.01，2.29 ± 0.29 和 1.44 ± 0.23（n = 5, $p < 0.05$）。此外，與異體 T 細胞共同培養，冬蟲夏草提取物亦顯著地增加 IL-12 p40（256.8 ± 185.4 pg/mL，控制組為 98.2 ± 46.73 pg/mL）和 IFN-γ（311.1 ± 156.4 pg/mL，控制組為 22.5 ± 35.1 pg/mL）的製造。這些發現顯示，冬蟲夏草提取物對於人類樹突狀細胞來說是很有潛力的刺激物，且其作用可能與調節 Th1 細胞的功能有關。

關鍵詞：冬蟲夏草，樹突狀細胞，協同刺激分子，T 細胞，中藥。

聯絡人：謝佳蓉，林口長庚醫院中醫分院婦兒科，電話：03-3196200 ext. 2611，傳眞：03-3298995，E-mail: hsiehcj@adm.cgmh.org.tw