Activation of Macrophages by the Components Produced from *Cordyceps militaris*

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ABSTRACT

Background: *Cordyceps militaris* have been reported to modify the immune and inflammatory responses both *in vivo* and *in vitro*. Macrophages play important roles in the innate immunity through the phagocytosis of antigens. This study examined the effects of *Cordyceps militaris* on the activation of murine macrophage RAW 264.7 cells and primary macrophages. **Methods:** The components contained in culture broth of *Cordyceps militaris* were purified by propyl alcohol extraction and HP 20 column chromatography to CMDB, CMDBW, CMDB5P, and CMDB25P. The amounts of nitric oxide (NO) were determined by using ELISA, Griess reagent respectively. The amounts of some cytokines were determined by using ELISA, western blot, and RT-PCR. The expression levels of cell surface molecules (ICAM-1, B7-1 and B7-2) were measured by flow cytometric analysis. **Results:** All the components of *Cordyceps militaris* produced significant amounts of NO. In particular, CMDB produced much more NO in RAW 264.7 cells and primary macrophages than other fractions of *Cordyceps militaris*. CMDB increased significantly the production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 dose-dependently in RAW 264.7 cells. Examination of the gene expression level also showed that the enhanced production of cytokines was correlated with the up-regulation of i-NOS expression, cycloxygenase (COX)-2 expression, IL-1β and IL-6 expression, and TNF-α expression on the expression of mRNAs by semi-quantitative RT-PCR. Western blot analysis also confirmed that CMDB enhances the expression level of these cytokines. **Conclusion:** These results show that CMDB stimulates the production of NO and pro-inflammatory cytokines and can also up-regulate the gene expression levels in macrophages. **(Immune Network 2007;7(2):57-65)**

Key Words: *Cordyceps militaris*, macrophage, nitric oxide, IL-1β, TNF-α, IL-6

Introduction

Cordyceps militaris is known as the Chinese caterpillar fungus because it is a parasitic organism that grows on a rare caterpillar, belongs to the Dong-Chong-Xia-Cao group in Chinese herbs. It is commonly used to replenish the kidney and soothe the lung for the treatment of fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthemia after severe illness, renal dysfunction, arrhythmias and other heart and liver diseases in the traditional systems of China and South East Asia (1). *Cordyceps militaris* was showed various properties such as anti-angiogenesis (2), anti-tumor and anti-diabetic (3), anti-mutagenic (4) and hypoglycemic effect (5).

Macrophage plays an important role in inflammatory responses by the production of cytokines, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, granulocyte/macrophage colony stimulating factor (GM-CSF), nitric oxide (NO), cycloxygenase (COX)-2 and other inflammatory mediators. TNF-α is a potential cancer therapeutic agent due to its ability to induce cancer cell death (6). TNF-α is the primary mediator of the systematic toxicity of endotoxemia (7,8).

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TNF-α also induces the secretion of cytokines such as IL-1, IL-6 and IL-10 and activates T cells and other inflammatory cells (9). NO is a pleiotropic biological molecule involved in a myriad of physiological and pathological processes such as regulation of blood pressure, neurotransmission, signal transduction, anti-microbial defense (10,11), immuno-modulation (12), cellular redox regulation (13,14), and apoptosis (15). COX-1 is constitutively expressed in most tissues and serves in general housekeeping functions. On the other hand, COX-2 is responsible for high-level production of prostanoids in response to proinflammatory agents, tumor promoters and growth factors (16). In recently study, it has been demonstrated that *Cordyceps sinensis* enhanced immunomodulatory effects (17). However, there are few studies about the immunomodulatory effects of *Cordyceps militaris*. In this study, we tested to determine whether CMDB enhanced immune response through the production of cytokines in murine macrophage RAW 264.7 cells.

Materials and Methods

Preparation of CMDB, CMDBW, CMDB5P, and CMDB25P. from Cordyceps militaris. Cordyceps militaris was maintained on potato dextrose agar plates and subcultured every month. The microorganism was transferred into the seed culture medium (potato dextrose broth) by punching out from the agar plate culture by cutter and cultured for 4 days in 500 ml-flask. The 400 ml of culture medium inoculated to jarferment containing 10 L water included 400 g draff and cultivated at 25°C for 1 week. The cultured mycelia filtrated and isolated with distilled deionized water, 5% propyl alcohol, and 25% propyl alcohol by using HP 20 column. The isolates were lyophilized to CMDB, CMDBW, CMDB5P, and CMDB25P.

Macrophage isolation and cell culture. Murine macrophage cell line RAW 264.7 (American Type Culture Collection, Manasa, VA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS, HyClone Labs, Logan, UT), and penicillin (100 U/ml)/ streptomycin (100 U/ml) (P/S). Female BALB/c mice (6~8 weeks; Orient Co.) were intraperitoneally injected with 1.5 ml of thioglycollate broth. Macrophages were collected from the peritoneal cavity after 4 days. Primary macrophages were cultured in six-well plates at 37°C in a humidified 5% CO_2 incubator under standard conditions. Supplements were purchased from Sigma Chemical Co (St. Louis, MO, USA).

RAW 264.7 cell and primary macrophage treatment. RAW 264.7 cells (2×10^5 cells/well) treated with each component: LPS (50 ng/ml), CMDB and CMDBW (0.6, 1.2, 2.5, 5, 10 μg/ml), CMDB5P (6.25, 12.5, 25, 50, 100 μg/ml) and CMDB25P (12.5, 25, 50, 100, 200 μg/ml), and media only (DMEM-10). Primary macrophages (2×10^5 cells/well) were treated only CMDB (0.6, 1.2, 2.5, 5, 10 μg/ml)

Analysis of nitrite and nitrate. The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media by Griess reagent. To measure the concentration of NO produced in primary macrophages and murine macrophage RAW 264.7 cells used for this method. Nitrite accumulation was determined according to the method reported by Stuehr and Nathan. Four wells were used for each group. The plates were incubated for 22 h, and 100 μl of culture supernatant was transferred to a new 96-well flat plate. The transferred supernatant was combined with 100 μl Griess reagent (mixture of equal volume of 0.2% N-(1-naphthyl)ethylenediamine HCl in water and 2% sulfanilamide in 5% H_3PO_4) in a 96-well flat plate. Nitrite accumulation was measured by an ELISA reader at 540 nm. Standard calibration curves were prepared using sodium nitrite as the standard.

Measurement of pro-inflammatory cytokines. RAW 264.7 cells (5×10^5 cells/well) were cultured in 12-well flat plate. The cells were treated with various concentrations of each component; LPS (100 ng/ml), CMDB and CMDBW (0.6, 1.2, 2.5, 5, 10 μg/ml), CMDB5P (6.25, 12.5, 25, 50, 100 μg/ml), CMDB25P (12.5, 25, 50, 100, 200 μg/ml), and media only (DMEM-10). The plates were incubated at 37°C for 48 h in humidified 5% CO_2 incubator under standard conditions. The level of TNF-α, IL-1β and IL-6 in the supernatants of the culture medium were determined by TNF-α, IL-1β, and IL-6 ELISA kit (BD Biosciences, USA).

Western blot analysis. RAW 264.7 cells (5×10^6 cells/dish) were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (1% SDS, 1.0 mM sodium vanadate, 10 mM Tris-Cl buffer, pH 7.4) for 5 min. 44 μg of protein from the cell lysates was applied to 12% and 8% SDS-polyacrylamide gels and then

transferred to nitrocellulose membrane. The membranes were blocked with a solution containing 5% skim milk for 1 hr. They were then incubated with anti-TNF-α, anti-IL-1β, anti-IL-6, anti-i-NOS, and anti-COX-2 monoclonal antibody for 2 hrs and washed 3 times with PBS. After incubation with alkaline phosphatase-labeled secondary antibody for 2 hr, the band were visualized using western blot kit substrate for phosphatase (LumiGLO system, KPL, USA).

RNA isolation and reverse transcriptase-polymerase chain reaction analysis. Total RNA was prepared from RAW 264.7 cells using the RNeasy Mini kit (QIAGEN, USA). 1 μg of total RNA was converted to cDNA using M- MLV reverse transcriptase (Promega, USA), oligo (dT) 16 primer, 10 mM dNTP, 0.1 M DTT and 1 U RNase inhibitor. After mixture incubated by heating at 65°C for 5 min, 25°C for 10 min and 37°C for 60 min, and M-MLV reverse transcriptase was inactivated by heating at 72°C for 15 min. The polymerase chain reaction (PCR) was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 2.5 mM dNTPs with 5 units of *Taq* DNA polymerase and 10 pM of each primer set for IL-1β, TNF-α, IL-6, i-NOS, and COX-2 (Table I). The amplification was followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min. Final extension was performed at 72°C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The nucleotides sequence of each primers and the size of product are shown in Table I. β-actin was used as an internal control.

Table I. The sequences of primers used in RT-PCR analysis

Gene	Primer wequences
IL-1β	F5'-CAGGATGAGGACATGACACC-3'
	R5'-CTCTGCAGACTCAAACTCCAC-3'
iNOS	F5'-AGCTCCTCCCAGGACCACAC-3'
	R5'-ACGCTGAAGTACCTCATTGGC-3'
TNF-α	F5'-TTGACCTCAGCGCTGAGTTA-3'
	R5'-CCTGTAGCCCACGTCGTAGC-3'
COX-2	F5'-AAGAAGAAAGTTCATTCCTGATCCC-3'
	R5'-TGACTGTGGGAGGATACATCTCTC-3'
IL-6	F5'-GTACTCCAGAAGACCAGAGG-3'
	R5'-TGCTGGTGACAACCACGGCC-3'
β-actin	F5'-GTGGGCCGCCCTAGGACCAG-3'
	R5'-GGAGGAAGAGGATGCGGCAGT-3'

Flow cytometry. RAW 264.7 cells (1×10^6 cells/ml) were cultured in petri dishes. The cells were treated with various concentrations of CMDB (0.6, 1.2, 2.5, 5, 10 μg/ml). The dishes were incubated at 37°C for 24 h in humidified 5% CO_2 incubator under standard conditions. The cells washed with PBS. The washed cells blocked with staining buffer containing 10% normal mouse serum (NMS) for 20 min on ice. The blocked cells were incubated with ICAM-1, B7-1 and B7-2 antibody for 20 min on ice. The incubated cells were washed with staining buffer at 3 times. The washed cells fixed by 1% paraformaldehyde in PBS. The fixed cells were measured by flow cytometry (Beckman coulter).

Results

Effects of CMDB on NO production in RAW 264.7 cells. To examine the effects of *Cordyceps militaris* on NO production, RAW 264.7 cells were incubated with LPS and various *Cordyceps militaris* components. After overnight culture, the supernatants were collected, and the accumulated nitrite concentration was measured using Griess reagent. LPS was used as a positive control for macrophage activation. In RAW 264.7 cells, CMDB components increased NO production whereas macrophages did nearly release NO in response to medium alone (Fig. 1A). CMDB produced much more NO production than other components in RAW 264.7 cells. To examine whether the enhancive effects of CMDB on NO production were attributable to its influence on the expression of mRNAs, semi-quantitative RT-PCR experiments were performed for the iNOS mRNAs. Western blot analysis also confirmed that CMDB enhances the expression level of iNOS (Fig. 1B). As shown in Fig. 1C, the transcripts for iNOS were barely detectable in unstimulated RAW 264.7 cells. The transcripts of iNOS, however, were readily detectable when RAW 264.7 cells were stimulated with LPS. When the cells treated with various concentrations of CMDB, the amounts of iNOS transcripts were increased significantly in RAW 264.7 cells.

Effects of CMDB on cytokines production. To determine whether components of *Cordyceps militaris* had a direct effect on cytokine production, TNF-α, IL-1β and IL-6 secretion were measured in the RAW 264.7 cells using cytokine ELISA kits. TNF-α, IL-1β and IL-6

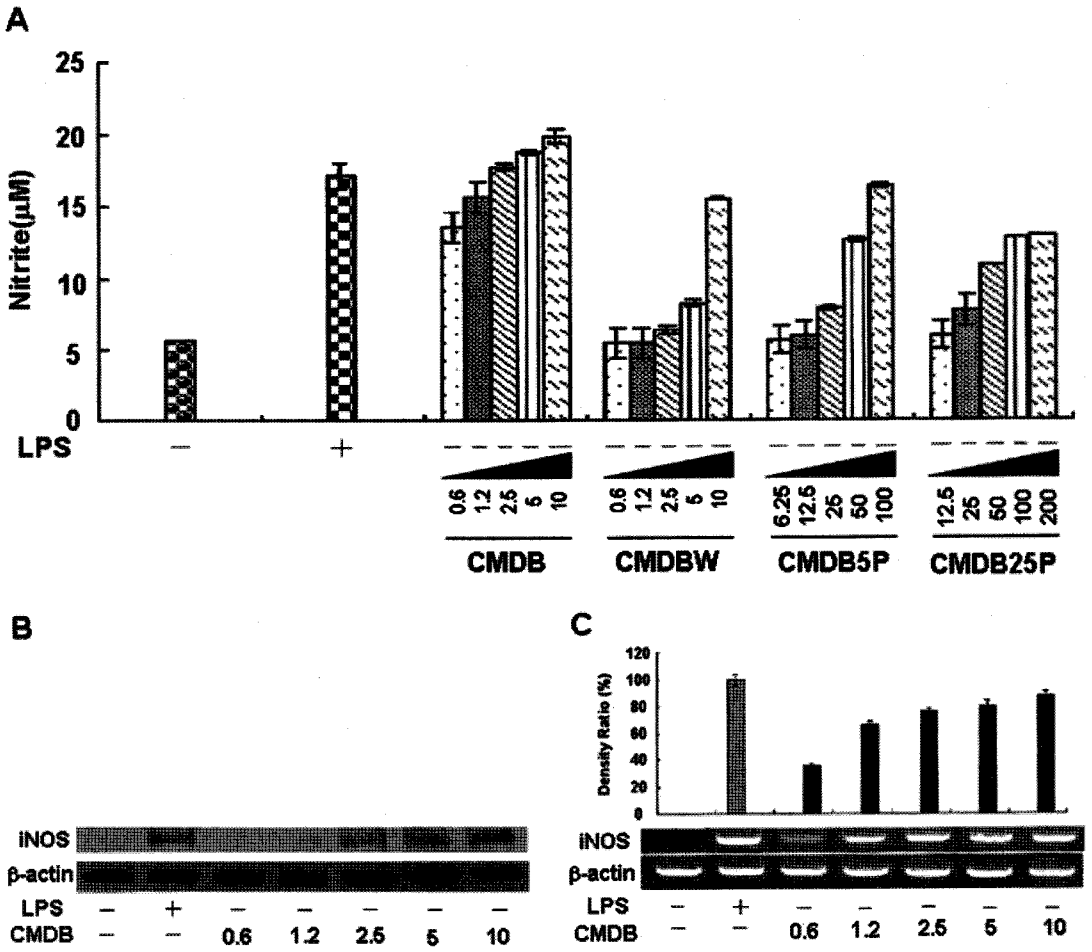

Figure 1. Effects of CMDB, CMDBW, CMDB5P, CMDB25P on NO production in RAW 264.7 cells and CMDB on iNOS expression. (A) RAW 264.7 cells (2×10^5 cells/well) treated different concentration of CMDB and CMDBW (0.6, 1.2, 2.5, 5, 10 μg/ml), CMDB5P (6.25, 12.5, 25, 50, 100 μg/ml), and CMDB25P (12.5, 25, 50, 100, 200 μg/ml). Nitrite concentrations in the culture media were determined using Griess reagent assay. (B) RAW 264.7 cells (5×10^6 cells/dish) treated different concentration of CMDB. The cells were lysed, and the lysates were analyzed by western blotting used anti-iNOS. (C) Total RNA was prepared and RT-PCR analysis. Densitometry data were preformed transilluminate. The results are reported as a mean±S.D. of three independent experiments.

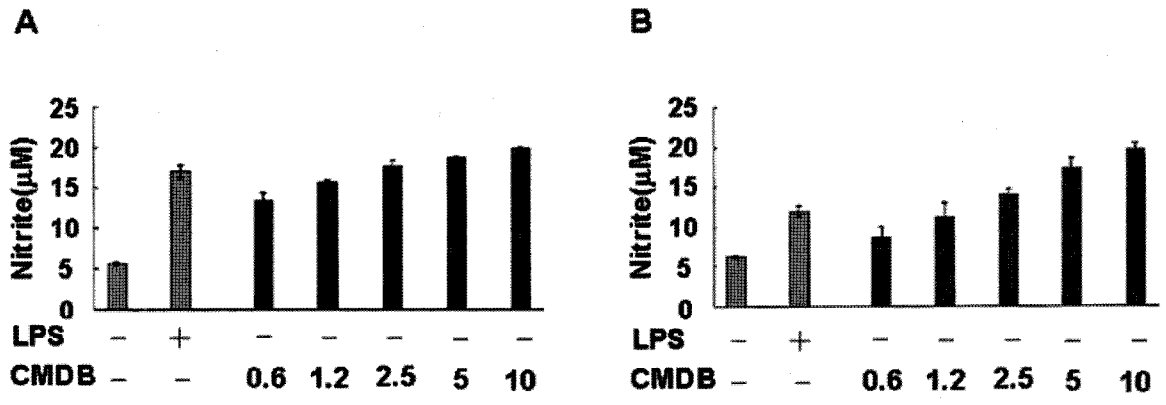

Figure 2. Effects of CMDB on NO production in RAW 264.7 cells and primary macrophages. (A) RAW 264.7 cells (2×10^5 cells/well) treated different concentration of CMDB (0.6, 1.2, 2.5, 5, 10 μg/ml). (B) The primary macrophages (4×10^5 cells/well) treated different concentration of CMDB (0.6, 1.2, 2.5, 5, 10 μg/ml). Nitrite concentrations in culture media were determined using Griess reagent assay. The results are reported as a mean±S.D. of three independent experiments.

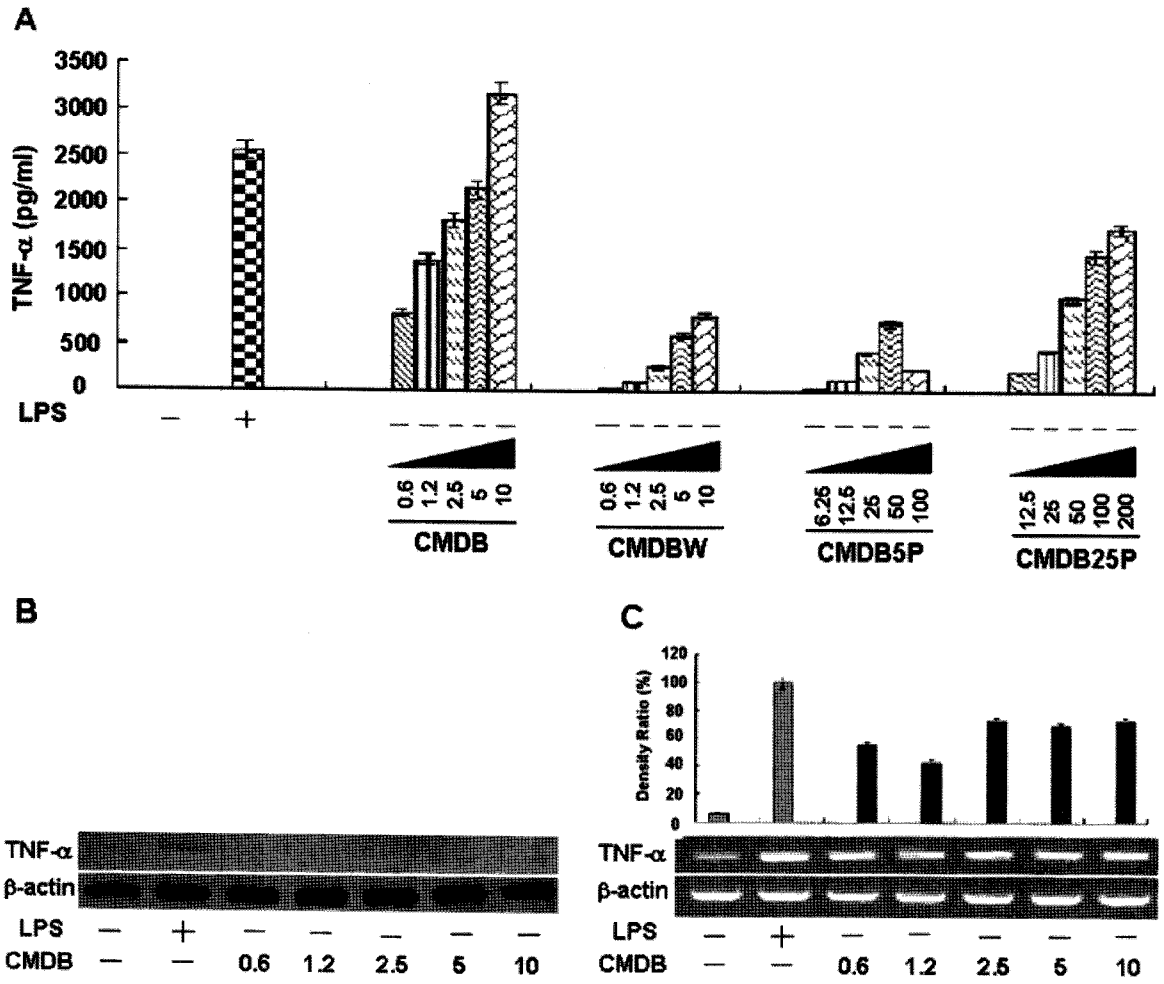

Figure 3. Effects of CMDB, CMDBW, CMDB5P, CMDB25P on TNF-α production in RAW 264.7 cells and CMDB on gene expression. The cultures treated different concentration of CMDB and CMDBW (0.6, 1.2, 2.5, 5, 10 μg/ml), CMDB5P (6.25, 12.5, 25, 50, 100 μg/ml), CMDB25P (12.5, 25, 50, 100, 200 μg/ml). The supernatant collected and extracellular level of TNF-α was measured in culture media using TNF-α ELISA kits. (B) RAW 264.7 cells (5×10^6 cells/dish) treated different concentration of CMDB. The cells were lysed, and the lysates were analyzed by western blotting used anti-TNF-α. (C) Total RNA was prepared and RT-PCR analysis. Densitometry data were preformed transilluminate. The results are reported as a mean±S.D. of three independent experiments.

are the major pro-inflammatory cytokines that are produced by macrophages. As shown in Fig. 3A, 4A, and 5A, components of *Cordyceps militaris* increased cytokines production in a dose-dependant manner. We also determined the intracellular levels of the same cytokines by western blot analysis, again showing that CMDB increased the cellular levels of TNF-α, IL-1β, and IL-6 in dose-dependent manner (Fig. 3B, 4B, and 5B). We next examined whether CMDB dose-dependently activated the mRNA levels of the pro-inflammatory cytokines by RT-PCR analysis (Fig. 3C, 4C, and 5C).

Effect of CMDB on COX-2 expression. CMDB dose-dependently increased the intracellular levels of COX-2 by western blot analysis (Fig. 6A). We also determined the mRNA levels of COX-2 in RAW 264.7 cells by RT-PCR, again showing that CMDB activated the cellular levels of COX-2 in a dose dependent manner (Fig. 6B).

Effect of CMDB on expression of co-stimulatory molecules. Since the adhesion molecules play an important role in the macrophage activation process. The RAW 264.7 cell surface expression of ICAM-1 (CD54), B7-1 (CD80), and B7-2 (CD86) was examined by flow cytometry. As shown in Fig. 7, CMDB was enhanced cell surface molecules (ICAM-1, B7-1, and B7-2) in a dose-dependent manner. The cells treated with high concentration of CMDB (10 μg/ml) appeared more

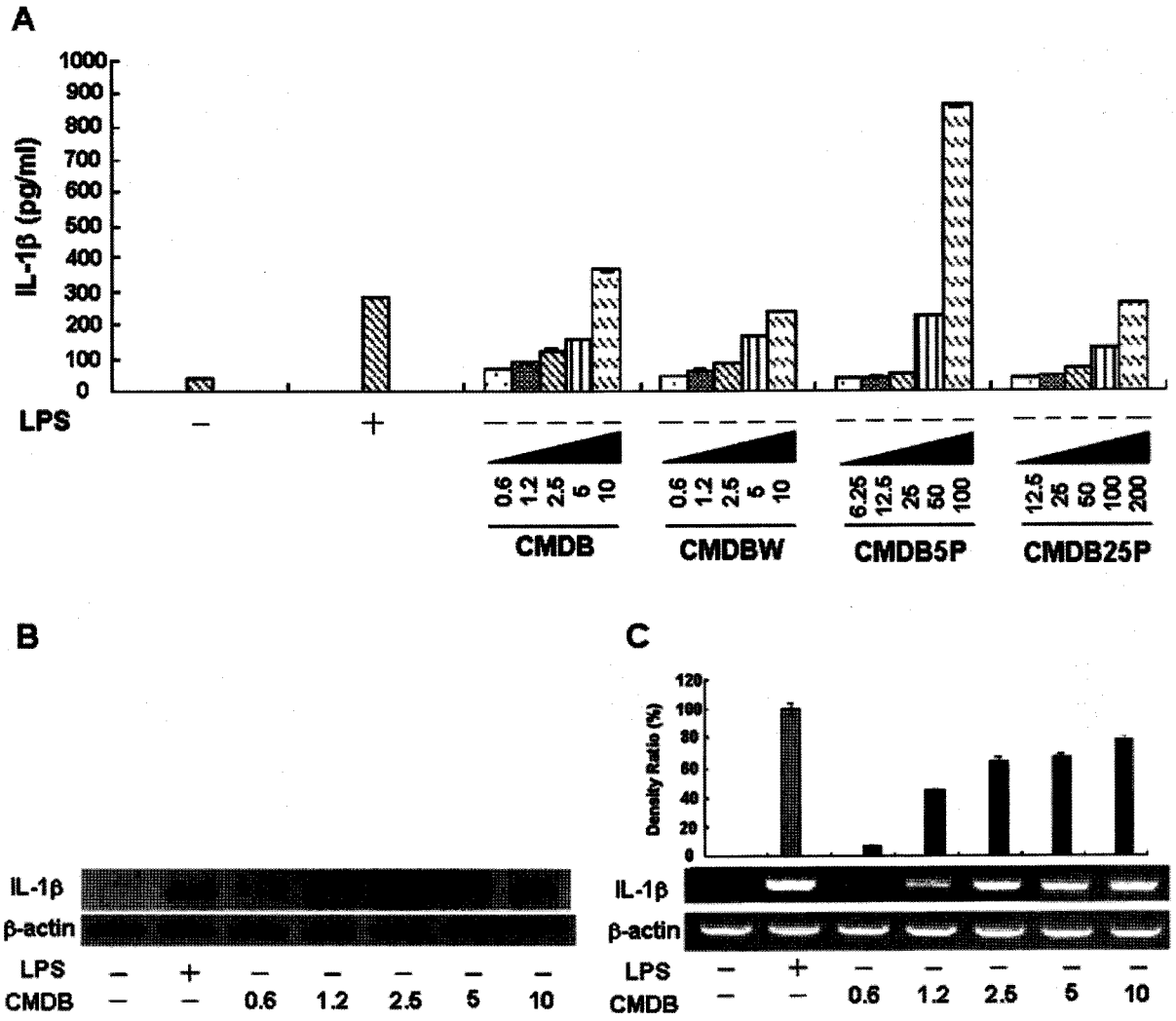

Figure 4. Effects of CMDB, CMDBW, CMDB5P, CMDB25P on IL-1 β production in RAW 264.7 cells and CMDB on gene expression. The cultures treated different concentration of CMDB and CMDBW (0.6, 1.2, 2.5, 5, 10 μg/ml), CMDB5P (6.25, 12.5, 25, 50, 100 μg/ml), CMDB25P (12.5, 25, 50, 100, 200 μg/ml). The supernatant collected and extracellular level of IL-1 β was measured in culture media using IL-1 β ELISA kits. (B) RAW 264.7 cells (5×10^6 cells/dish) treated different concentration of CMDB. The cells were lysed, and the lysates were analyzed by western blotting used anti-IL-1 β. (C) Total RNA was prepared and RT-PCR analysis. Densitometry data were preformed transillumination. The results are reported as a mean±S.D. of three independent experiments.

powerful revelation than these treated with LPS.

Discussion

Activated macrophages fuse their lysosomes more efficiently to phagosomes, exposing intercellular or recently ingested extracellular microbes to a variety of microbicidal lysosomal enzymes. Activated macrophages also produce nitric oxide (NO), tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, and COX-2. NO is a multi-functional mediator which plays an important role in regulating various biological function *in vivo*. NO production by iNOS in macrophages is essential for the defense mechanisms against microorganisms and tumor cells (18,19). As shown in Fig. 1A, all components of *Cordyceps militaris* increased NO productions in RAW 264.7 cells. In particular, *Cordyceps militaris* draff broth (CMDB) produced much more NO in RAW 264.7 cells and primary macrophages than the other components of *Cordyceps militaris* (Fig. 1, 2). CMDB was also enhanced on expression levels of mRNAs and iNOS mRNAs (Fig. 1B, C).

Cytokines are local protein mediators that are now known to be involved in almost all important biological processes, including cell growth and activation,

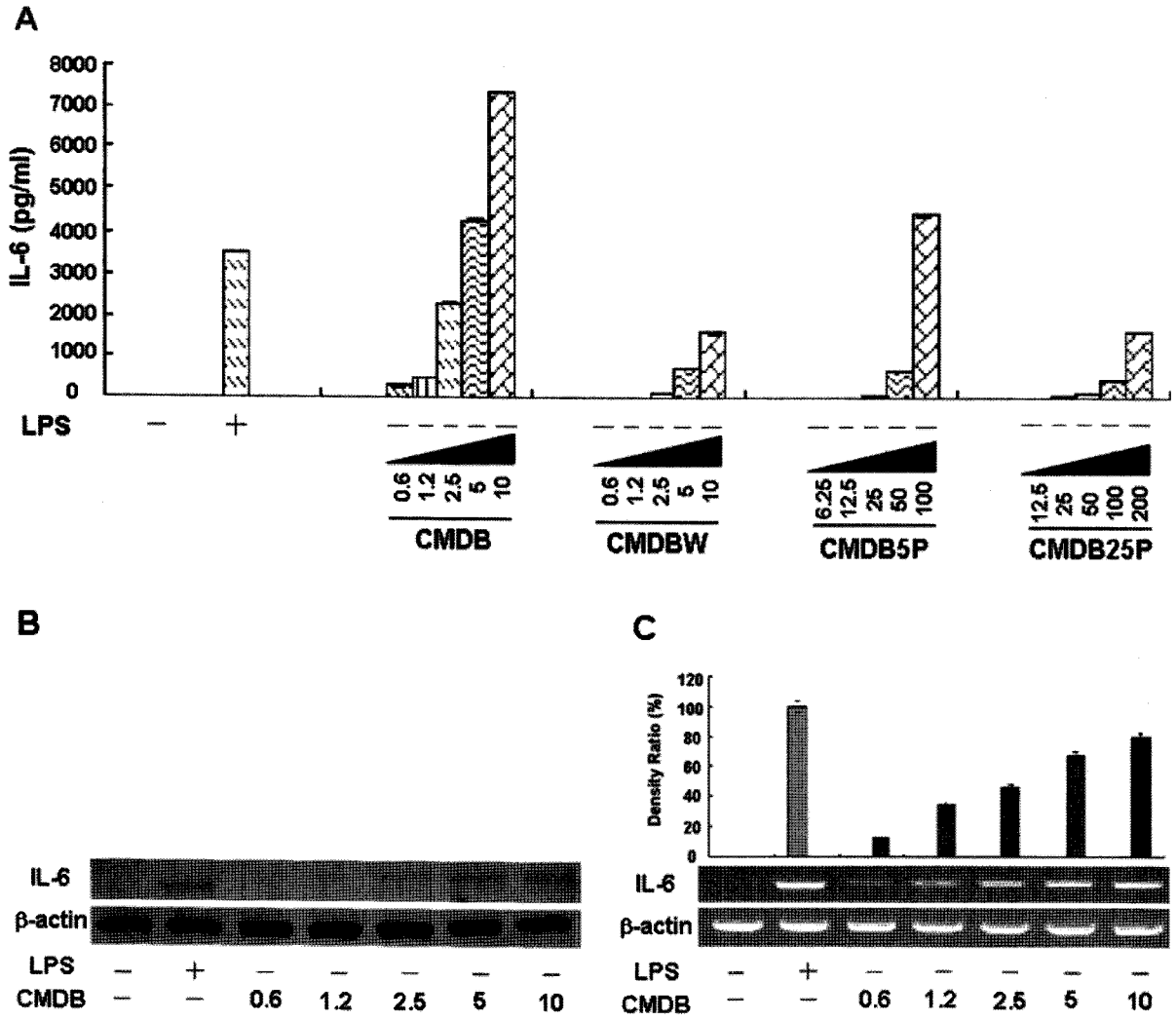

Figure 5. Effects of CMDB, CMDBW, CMDB5P, CMDB25P on IL-6 production in RAW 264.7 cells and CMDB on gene expression. The cultures treated different concentration of CMDB and CMDBW (0.6, 1.2, 2.5, 5, 10 μg/ml), CMDB5P (6.25, 12.5, 25, 50, 100 μg/ml), CMDB25P (12.5, 25, 50, 100, 200 μg/ml). The supernatant collected and extracellular level of IL-6 was measured in culture media using IL-6 ELISA kits. (B) RAW 264.7 cells (5×10^6 cells/dish) treated different concentration of CMDB. The cells were lysed, and the lysates were analyzed by western blotting used anti-IL-6. (C) Total RNA was prepared and RT-PCR analysis. Densitometry data were preformed transillumination. The results are reported as a mean±S.D. of three independent experiments.

inflammation, immunity, and differentiation. All components of *Cordyceps militaris* dose-dependently enhanced TNF-α, IL-1β, and IL-6 productions in RAW 264.7 cells. Interestingly, CMDB increased much more TNF-α, IL-1β, IL-6 productions and expression of mRNAs in RAW 264.7 cells than the other components of *Cordyceps militaris*. CMDB was also enhanced COX-2 production on expression levels of mRNAs.

The intercellular adhesion molecules (ICAMs), ICAM-1, ICAM-2, and ICAM-3 are cell-surface ligands for the leukocyte integrins. They are crucial in the binding of lymphocytes and other leukocytes to certain cells, including antigen-presenting cells (APCs) and endothelial cells. The B7 family plays important roles by co-stimulatory factor in APCs. The CD24 was combined with B7 family aid in APCs. As shown in Fig. 7, CMDB increased cell surface molecules (ICAM-1, B7-1, and B7-2) in RAW 264.7 cells. *Cordyceps militaris* is one of the well-known medicinal entomopathogenic fungi, and has been widely used for the treatment of various diseases. However, it's pharmacological and biochemical actions have not been clearly elucidated.

In summary, these results suggest that CMDB has immunostimulatory effects on macrophages through the enhanced production of cytokines and other bio-

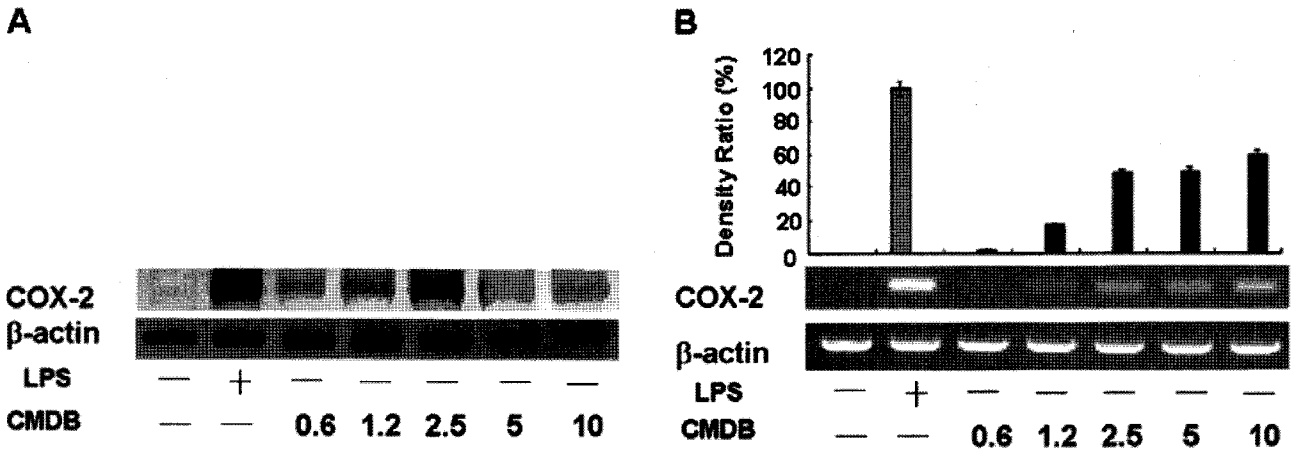

Figure 6. Effect of CMDB on COX-2 production. (A) Western blot analysis and (B) RT-PCR analysis. (A) RAW 264.7 cells (5×10^6 cells/dish) treated different concentration of CMDB (0.6, 1.2, 2.5, 5, 10 μg/ml). The cells were lysed, and the lysates were analyzed by western blotting used anti-COX-2. (B) Total RNA was prepared and RT-PCR analysis. Densitometry data were preformed transillumination. The results are reported as a mean±S.D. of three independent experiments.

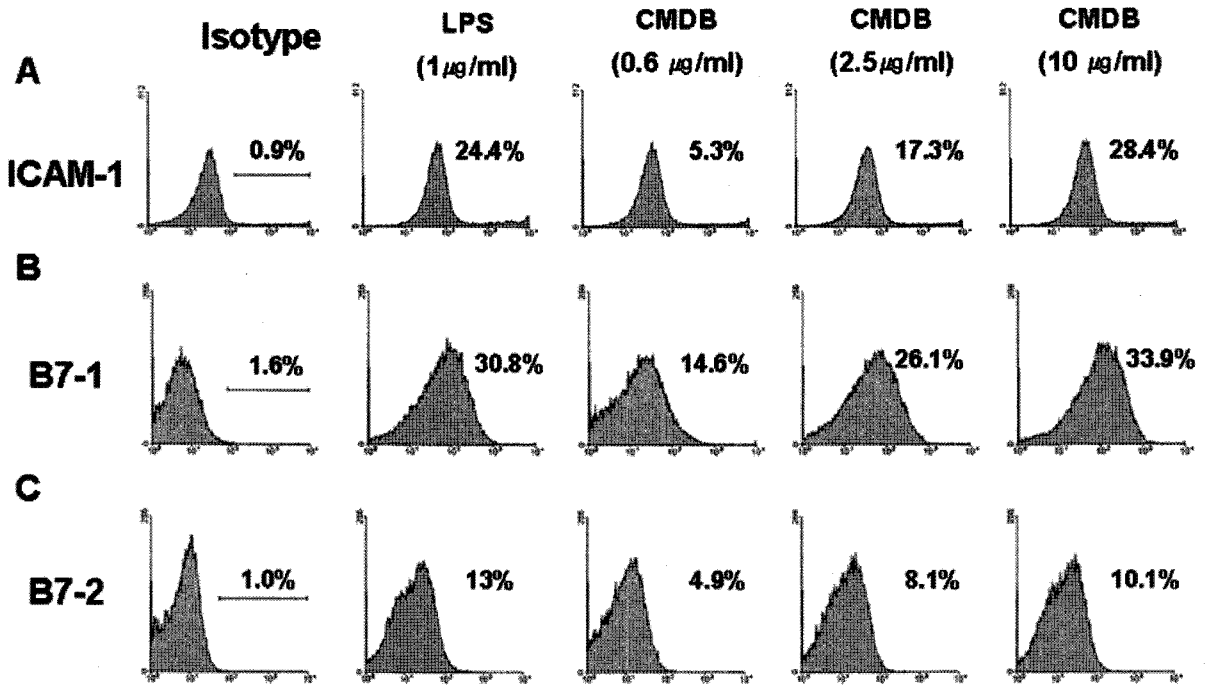

Figure 7. Effects of CMDB on ICAM-1 and B7-1, B7-2 expressions. The RAW 264.7 cells were cultured in the presence of LPS (1 μg/ml), CMDB (0.6, 1.2, 2.5, 5, 10 μg/ml) for 24 hours. The surface ICAM-1 (A), and B7-1 (B), B7-2 (C) molecules were labeled with either anti-ICAM-1, anti-B7-1, B7-2 and the cell were stained using anti-V β8.1+8.2-FITC, anti-V β2-PE, anti-V β2-FITC (shaded histogram), which served as an isotype control for the nonspecific binding.

active substances as well as the increased expression of co-stimulatory and adhesion molecules.

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