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Cordycepin Suppresses MHC-restricted Antigen Presentation and Leads to Down-regulation of Inflammatory Responses in Antigen Presenting Cells

Seulmee Shin[1], Seulah Kim[1], Bobae Hyun[1], Aeri Lee[1], Sungwon Lee[1], Chan-Su Park[2], Hyunseok Kong[1], Youngcheon Song[1], Chong-Kil Lee[2], and Kyungjae Kim[1,*]

[1]College of Pharmacy, Sahmyook University, Seoul 139-742, Republic of Korea
[2]College of Pharmacy, Chungbuk National University, Cheongju 361-763, Republic of Korea

Abstract – *Cordyceps militaris*, a traditional medicinal mushroom, produces a component compound, cordycepin (3'-deoxyadenosine). Cordycepin has many pharmacological activities including immunological stimulating, anti-cancer, and anti-infection activities. However, the therapeutic mechanism has not yet been elucidated. In this study, we examined the effects of cordycepin on the antigen-presenting function of antigen-presenting cells (APCs). Dendritic cells (DCs) were cultured in the presence of cordycepin and then allowed to phagocytose microspheres containing ovalbumin (OVA). After washing and fixing, the efficacy of OVA peptide presentation by DCs was evaluated using CD8 and CD4 T cells. Also, we confirmed the protein levels of proinflammatory cytokines through RT-PCR and Western blot analysis. Cordycepin decreased both MHC class I and class II-restricted presentation of OVA and suppressed the expression of both MHC molecules and the phagocytic activity toward exogenous OVA. The class II-restricted OVA presentation-regulating activity of cordycepin was also confirmed using mice that had been injected with cordycepin followed by soluble OVA. Furthermore, cordycepin suppressed the mRNA and protein levels of iNOS, COX-2, pro-inflammatory cytokines in a concentration-dependent manner. These results provide an understanding of the mechanism of the T cell response-regulating activity of cordycepin through the inhibition of MHC-restricted antigen presentation in relation to its actions on APCs.
Keywords – Cordycepin, Inflammation, MHC-restricted antigen presentation, Antigen-presenting cells

Introduction

Cordycepin, also known as 3'-deoxyadenosine, is a bioactive compound present in fungi species belonging to the genus *Cordyceps* (Cunningham et al., 1950) and was first isolated from *Cordyceps militars*. *C. militaris* is commonly used in East Asian countries to maintain health and to prevent and treat a broad spectrum of illnesses including those of the circulatory, immune, respiratory, and glandular systems. Cordycepin has various biological effects including anti-fungal (Sugar and McCaffrey, 1998), anti-malarial (Trigg *et al*., 1971), anti-herpes (Julian-Ortiz *et al*., 1999), anti-tumorigenic, anti-leukemic (Deitch and Sawicki, 1979), anti-inflammatory (Shin *et al*., 2009; Shin *et al*., 2009), anti-diabetic (Yun *et al*., 2003; Lo *et al*., 2004; Kim *et al*., 2006) and anti-oxidant effects (Ramesh *et al*., 2012). Cordycepin also induces apoptosis in many cells and induces cell cycle arrest by targeting molecules and pathways (Lee *et al*., 2009; Lee *et al*., 2010; Lee *et al*., 2013). Even though cordycepin exhibits a number of pharmacological properties, further studies are needed to address these pharmacological differences and little known about its immune-regulating effects or its effects on antigen presentation in T cell responses based on the capability of antigen-presenting cells (APCs).

Antigen-presenting cells, especially dendritic cells (DCs), play a critical role in the initiation of immune responses and the induction of immune tolerance. DCs are the most important accessory cells for the activation of naïve T cells and the generation of primary T cell responses (Banchereau *et al*., 2000). They can acquire and process antigens in the periphery and then migrate to secondary lymphoid tissues where they prime primary T cell responses. The activation of T cells and subsequent generation of the effector function are dependent on MHC molecules, and modulation of MHC-restricted antigen processing pathways may provide novel pharmacological targets for the regulation of T cell responses. In fact, we previously showed that cyclosporin A (CsA) and tacrolimus

*Author for correspondence
Kyungjae Kim, College of Pharmacy, Sahmyook University, Seoul, Republic of Korea
Tel: +82-2-3399-1601; E-mail: kimkj@syu.ac.kr

inhibit both the class I and class II MHC-restricted antigen presentation pathways in DCs (Lee *et al*., 2005; Lee *et al*., 2007) and metformin, widely used for type 2 diabetes (T2D) therapy, suppresses MHC-restricted antigen presentation by inhibiting co-stimulatory factors and MHC molecules (Shin *et al*., 2013). Macrophage-colony stimulating factor (M-CSF) also enhances MHC-restricted antigen presentation (Han *et al*., 2005), and *Cordyceps militaris* water extract (CME) enhances MHC-restricted antigen presentation via the induced expression of MHC molecules and the production of cytokines (Shin *et al*., 2010). This suggests that modulation of the antigen-specific signal may be useful for therapeutic regulation of T cell responses.

In the present study, we examined the effects of cordycepin on the *ex vivo* and *in vivo* function of APCs, exploring the modulation of T cell responses by cordycepin as an immune-regulating agent. We used OVA as an exogenous antigen in conjunction with cordycepin and then compared the change in cross-presentation of metformin-related DCs to that of a control group, along with the levels of MHC class I and II molecules. Also, we tested the role of cordycepin on the NF-κB-dependent inflammation cascades in lipopolysaccharide (LPS) - stimulated macrophages.

Experimental

Cells and reagents – The T cell hybridomas, CD8 OVA1.3 and DOBW, were kindly provided by Dr. Clifford V. Harding (Case Western Reserve University, Cleveland, OH) (Harding *et al*., 1991; Harding and Song, 1994). The DC cell line, DC2.4, was obtained from Dana-Farber Cancer Institute, Boston, MA, USA (Shen *et al*., 1997). Cordycepin was purchased from Sigma (St. Louis, MO, USA). The cell culture media DMEM, antibiotic-penicillin/streptomycin solution and fetal bovine serum (Hyclone, Logan, UT, USA) were used for the cell culture.

Preparation of peritoneal macrophages and cell culture – Peritoneal macrophages were elicited by injecting 1 ml of 3% thioglycollate in sterile water into the mouse peritoneum. After 4 days, the cells in the peritoneum were harvested by peritoneal lavage with ice-cold PBS. The red blood cells in the cell preparation were lysed by treatment with ACK lysis buffer (150 mM NH_4Cl, 1 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2 ~ 7.4) for 1 min. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), and 1% (v/v) penicillin (10,000 U/mL)/ streptomycin (10,000 U/mL) (P/S).

Isolation of total RNA and RT-PCR – Total RNA was extracted from peritoneal macrophages using the RNeasy Mini kit (QIAGEN, USA) in an RNase-free environment. The reverse transcription of 1 μg RNA was carried out using M-MLV reverse transcriptase (Promega, USA), oligo (dT) 16 primer, dNTP (0.5 μM) and 1 U RNase inhibitor. After incubation at 65 °C for 5 min and 37 °C for 60 min, M-MLV reverse transcriptase was inactivated by heating at 70 °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 i-NOS, IL-1β, IL-6, TNF-α, COX-2, and β-actin. The cDNA was amplified by 35 cycles of denaturing at 94 °C for 45 s, annealing at 62°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 1.5% agarose gels and stained with ethidium bromide. The primers used were 5' AGCTCCT CCCAGGACCACAC 3' (forward) and 5' ACGCTGAGT ACCTCATTGGC 3' (reverse) for iNOS, 5' CAGGATGA GACATGACACC 3' (forward) and 5' CTCTGCAGACT CAAACTCCAC 3' (reverse) for IL-1β, 5' GTACTCCAG AAGACCAGAGG 3' (forward) and 5' TGCTGGTGACA ACCACGGCC 3' (reverse) for IL-6, 5' TTGACCTCAG CGCTGAGTTG 3' (forward) and 5' CCTGTAGCCCAC GTCGTAGC 3' (reverse) for TNF-α, 5' AAGAAGAAAG TTCATTCCTGATCCC 3' (forward) and 5' TGACTGTG GGAGGATACATCTCTC 3' (reverse) for COX-2, and 5' GTGGGCCGCCCTAGGACCAG 3' (forward) and 5' GG AGGAAGAGGATGCGGCAGT 3' (reverse) for β-actin. β-actin was used as an internal control.

Western blot analysis – Peritoneal macrophages 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. 20 μg of protein from the cell lysates was applied to 8-12% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBST solution for 1 hr. They were then incubated with anti-IL-1β, anti-IL-6, anti-TNF-α, anti-i-NOS, or anti-COX-2 monoclonal antibody for 2 h and washed 3 times with PBST. After incubation with alkaline phosphatase-labeled secondary antibody for 2 h, the bands were visualized using a Western Blot Kit with alkaline phosphatase substrate (Vector, Burlingame, CA, USA.)

Generation of DCs from bone marrow cells – Total bone marrow cells obtained from the femurs of Balb/c

mice were cultured in 6-well plates (5×10^6/well) in culture medium supplemented with 400 U/ml recombinant mouse GM-CSF (CreaGene, Korea). At days 3 and 4 from the initiation of the culture, the nonadherent cells were discarded after gentle shaking and replacing the culture medium with fresh medium containing cytokines. The DCs were harvested by gentle pipetting on day 5.

Preparation of OVA-nanospheres – Nanospheres containing OVA were prepared using a homogenization/solvent evaporation method, as described previously, with 400 μl of OVA-containing water (50 mg/ml OVA) and 2 ml of ethyl acetate containing Polylactic-Co-Glycolic Acid (PLGA, 100 mg/ml, Sigma-Aldrich) (Lee *et al*., 2010). Fluorescein isothiocyanate (FITC)-containing PLGA-nanospheres were prepared by adding FITC to the ethyl acetate phase together with PLGA. The OVA content was determined using a micro-bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) after lysing the nanospheres in lysis buffer containing 0.1% SDS and 0.1 N NaOH.

MHC class I-restricted presentation assay of exogenous OVA – DCs were cultured in the presence of different concentrations of cordycepin for 2 h in 96-well plates (1×10^5 cells/ml) and then combined with OVA-microspheres (50 μg/ml). After 6-h incubation at 37 ºC, the cells were washed twice with pre-warmed PBS and then fixed with ice-cold 1% paraformaldehyde for 5 min at room temperature. The plate was washed and CD8OVA1.3 cells (1×10^5 cells/ml) were added. After overnight incubation at 37 ºC, the plate was centrifuged at 1800 rpm; the culture supernatant was collected and assayed for IL-2 content using an IL-2 ELISA kit (eBioscience, San Jose, CA, USA).

MHC class II-restricted presentation assay of exogenous OVA – DCs and peritoneal macrophages were cultured in the presence of different concentrations of cordycepin for 2 h in 96-well plates (1×10^5 cells/ml) and then combined with OVA-microspheres (50 μg/ml). After 6-h incubation at 37 ºC, the plate was washed twice with pre-warmed PBS and then fixed with ice-cold 1% paraformaldehyde for 5 min at room temperature. The plate was washed and DOBW cells (1×10^5 cells/ml) were added. After overnight incubation at 37 ºC, the plate was centrifuged at 1800 rpm; the culture supernatant was collected and assayed for IL-2 content using an IL-2 ELISA kit.

Phagocytosis assay – DCs were cultured in the presence of different concentrations of cordycepin for 2 h in 6-well plates (2×10^6 cells/well) and then combined with microspheres (average diameter, 300 nm) containing both OVA and FITC. After 2 h, unphagocytosed microspheres were removed by washing with pre-warmed PBS. The plate was chilled on ice for 20 min, and the cells remaining on the bottom of the plate wells were subsequently harvested by rough pipetting with cold PBS, washed with 10 ml of cold PBS to remove the extra-particular OVA, and then fixed with 1% paraformaldehyde. Flow cytometric analysis was performed on a FACS Canto flow cytometer (Beckman Coulter, Brea, CA, USA).

Phenotype analysis – DCs were cultured in the presence of different concentrations of cordycepin for 2 h in 6-well plates (2×10^6 cells/well). The plates were chilled on ice for 20 min, and the cells remaining on the bottom of the plate wells were subsequently harvested by rough pipetting with cold PBS. The cells were stained with monoclonal antibodies recognizing murine cell surface molecules after blocking with FcR-binding anti-CD16/CD32 monoclonal antibody, and flow cytometry analysis was performed on a FACS Canto flow cytometer. The monoclonal antibodies, anti-H-$2K^b$ and anti-I-A^b, and isotype-matched control antibodies were purchased from BD Biosciences (San Jose, CA, USA).

***In vivo* antigen presentation assay** – Peritoneal macrophages were elicited by injecting 1 ml of 3% thioglycollate in PBS into the mouse peritoneum. After 4 days, cordycepin (2 mg/kg) was injected subcutaneously. Two hours later, soluble OVA (2 mg/mouse) was injected into the peritoneum. Cordycepin (2 mg/kg) was again injected subcutaneously 2 h after injecting the soluble OVA. The peritoneal macrophages were harvested from the peritoneum 2 h after the second injection of cordycepin and washed. The class II MHC-complexed OVA peptide quantities were then assessed by IL-2 secretion assays after culturing the paraformaldehyde-fixed peritoneal macrophages with DOBW cells (1×10^5/well) for 18 h.

Statistical analysis – The statistical significance of the difference between the control and treatment groups was assessed using one-way ANOVA followed by the Student's t-test.

Result and Discussion

This study shows that short-term exposure to cordycepin decreases immune responses and the ability of professional APCs to present MHC class I- and MHC class II-restricted exogenous OVA both *ex vivo* and *in vivo*. Cordycepin decreases phagocytosis of exogenous antigen and suppresses MHC molecules that prime the T cell responses, leading to inhibited secretion of pro-inflammatory cytokines.

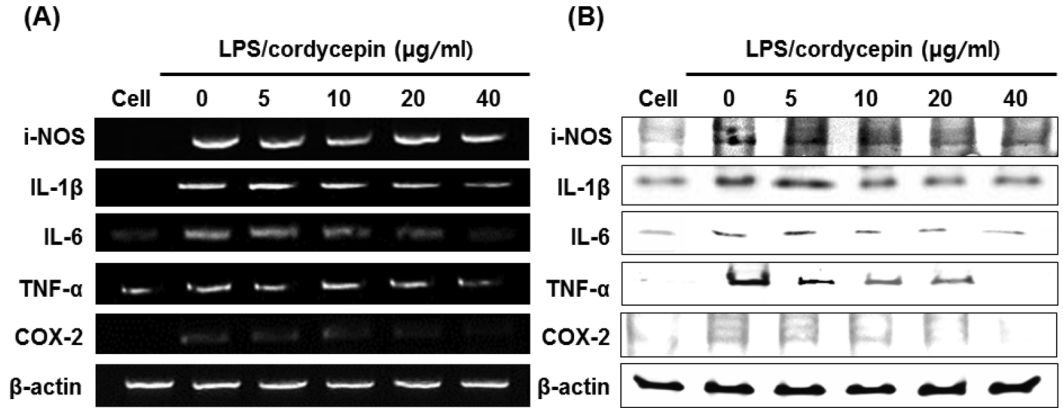

Fig. 1. Effect of cordycepin on the expression of cytokines in macrophages. Levels of i-NOS, IL-1β, IL-6, TNF-α and COX-2 gene and protein in peritoneal macrophages. Cells were incubated with various concentrations of cordycepin in the presence of LPS (100 ng/mL) for 24 hrs. (A) The mRNA levels of cytokines were determined by RT-PCR analysis, and (B) protein (20 μg) from each sample was resolved in 8-12% SDS-PAGE and then analyzed by Western blotting. β-actin was used as a control.

Cordyceps militarys (CM) is known as the rare Chinese caterpillar fungus and has benefits to the human body including the circulatory, respiratory, and glandular systems. Cordycepin is a major component of CM and a derivative of the nucleoside adenosine only differing from the latter by the absence of oxygen in the 3' position of its ribose entity. Cordycepin has been studied for anti-fungal (Sugar and McCaffrey, 1998), anti-inflammatory (Shin *et al*., 2009; Shin *et al*., 2009), and anti-diabetic effects (Yun *et al*., 2003; Lo *et al*., 2004; Kim *et al*., 2006). Cordycepin could affect many cellular immune reactions mediated by T cells. Even though cordycepin demonstrates a number of pharmacological properties, further studies are necessary to address these pharmacological differences.

To further investigate to role of cordycepin in inflammation, peritoneal macrophages were treated with LPS (100 ng/mL) or cordycepin (5~40 μg/mL) for 24 h. As shown in Fig. 1A, cordycepin suppressed mRNA levels of i-NOS, IL-1β, IL-6, TNF-α, and COX-2 in a dose-dependent manner. We also determined the intracellular levels of pro-inflammatory cytokines by Western blot analysis, showing that cordycepin decreased the cellular levels of i-NOS, IL-1β, IL-6, TNF-α, and COX-2 in a dose-dependent manner (Fig. 1B).

DCs play a key role in the initiation of primary immune responses, and the activation of even naïve T cells in primary responses has been explained by their ability to express high levels of MHC molecules (Banchereau *et al*., 2000). Furthermore, DCs can acquire and process antigens in the periphery and migrate to secondary lymphoid tissues where they prime primary T cell responses. The classical paradigm of antigen presentation by APCs is that endogenous antigens are presented via MHC class I molecules to CD8 T cells, whereas exogenous antigens are presented via CD4 T cells (Guermonprez *et al*., 2002). However, APCs process exogenous antigens for presentation by MHC class I molecules to CD8 T cells. This process, termed cross presentation, may be a mechanism by which naïve T cells can be primed to antigens that are present in nonprofessional APCs (Bevan *et al*., 1976; Harding 1995). The activation of T cells are dependent on at least two signals delivered an antigen-specific signal delivered via an MHC/peptide structure and a co-stimulatory signal delivered via B7 family by APCs. Antigenic stimulation of T cells in the absence of co-stimulation leads to the development of functional unresponsiveness T cells (Boussiotis *et al*., 1996; Greenfield *et al*., 1998). Since T cells can only recognize antigens presented on MHC molecules, the impact of the inhibition of MHC-restricted antigen presentation must be far-reaching.

Since T cells can only recognize the antigens presented on MHC molecules, modulators of the MHC-restricted antigen processing pathways could be novel pharmacological agents for regulating the T cell responses. The results presented in this paper demonstrate that short-term exposure of DCs to cordycepin suppressed both MHC class I and II-restricted antigen presentation capabilities of DCs. Based on the importance of DCs in the initiation of T cell responses and the current findings, which demonstrated that short-term exposure to cordycepin inhibited MHC-restricted antigen presentation, we speculate that the therapeutic efficacy of cordycepin is due, at least in part, to the enhancement of the antigen presenting capability of DCs.

The effects of cordycepin on the MHC class I-restricted presentation of exogenous OVA were examined using DC2.4 and BM-DCs. As shown in Fig. 2A, treatment of

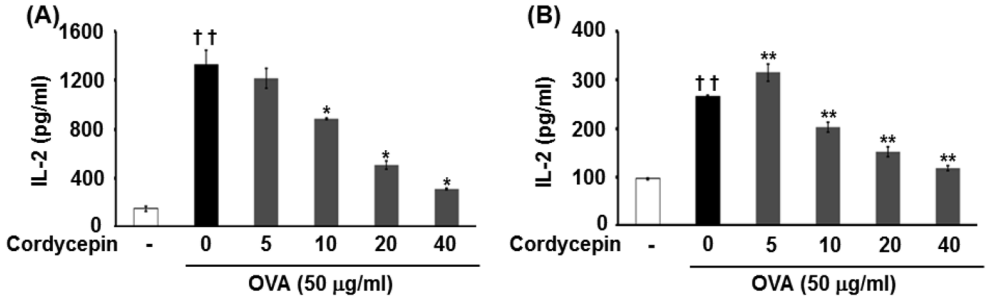

Fig. 2. Effects of cordycepin on the cross-presentation of exogenous OVA. (A) DC2.4 cells and (B) BM-DCs were cultured with the indicated amounts of cordycepin for 2 h, followed by the addition of OVA-nanospheres. After 6-h incubation, the cells were washed and fixed; the amounts of OVA peptides presented on MHC class I molecules were assessed using OVA-specific CD8 T cell hybridoma, CD8OVA1.3. The amounts of IL-2 produced from OVA-specific CD8 T cells were assayed by a commercial IL-2 ELISA kit. $^{\dagger\dagger}p < 0.01$ vs. untreated group based on Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. OVA only based on Student's t-test.

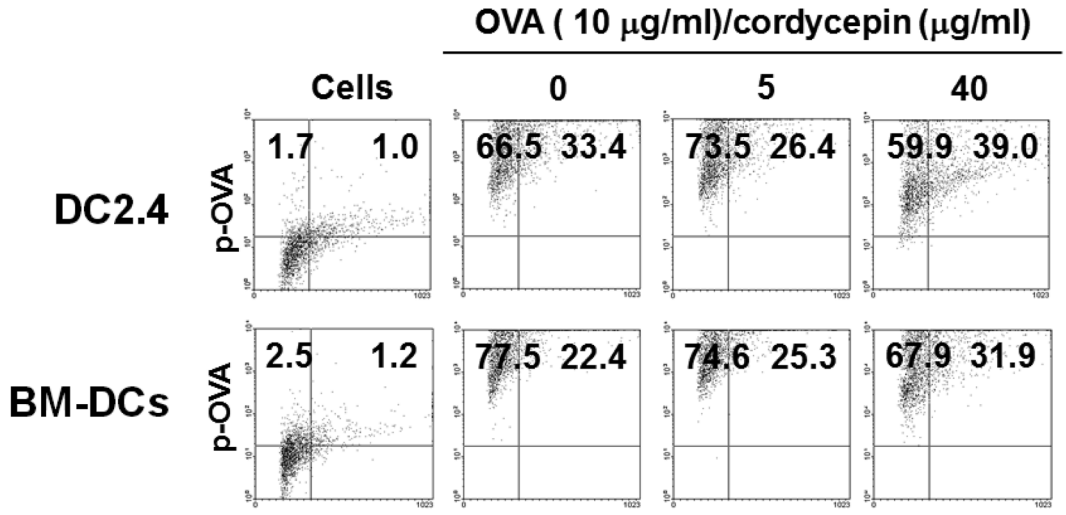

Fig. 3. Effects of cordycepin on the MHC class II-restricted presentation of exogenous OVA. (A) BM-DCs and (B) peritoneal macrophages were cultured with the indicated amounts of cordycepin, washed and fixed, and the amounts of OVA peptides presented on MHC class II molecules were assessed using the OVA-specific CD4 T cell hybridoma, DOBW. The amounts of IL-2 produced from OVA-specific CD4 T cells were assayed by a commercial IL-2 ELISA kit. $^{\dagger\dagger}p < 0.01$ vs. untreated group based on Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. OVA only based on Student's t-test.

DC2.4 with cordycepin dose-dependently suppressed MHC class I-restricted OVA. The effects of cordycepin on the cross-presentation of exogenous OVA were also examined in BM-DCs. The treatment of BM-DCs with cordycepin also decreased the level of MHC class I-restricted OVA peptide presentation (Fig. 2B). Also, The effects of cordycepin on the class II presentation of exogenous OVA in both BM-DCs and peritoneal macrophages were examined. As shown in Fig. 3A, MHC class II-restricted presentation of exogenous OVA was decreased by cordycepin in BM-DCs. The effects of cordycepin on the MHC class II presentation of exogenous OVA were also examined in peritoneal macrophages isolated from the thioglycollate-elicited mouse peritoneum. The treatment of peritoneal macrophages with cordycepin also decreased the level of MHC class II-restricted OVA peptide presentation (Fig. 3B). Thus, cordycepin suppressed exogenous OVA presentation in both MHC class I- and MHC class II-restricted presentation.

To prove that the antigen presentation was not changed by the effects of cordycepin on the phagocytic activity of DCs, the results show that the MHC-restricted antigen presentation-decreasing effect of cordycepin is not due to the inhibition of the phagocytic activity of DCs (Fig. 4), but cordycepin decreased the expression of MHC class I ($H\text{-}2K^b$) and II ($I\text{-}A^b$) molecules (Fig. 5) . Furthermore, we determined the *in vivo* relevance of the MHC class II-restricted antigen presentation-increasing effect of cordycepin was examined in mice. As shown in Fig. 6, cordycepin decreased the MHC class II-restricted OVA

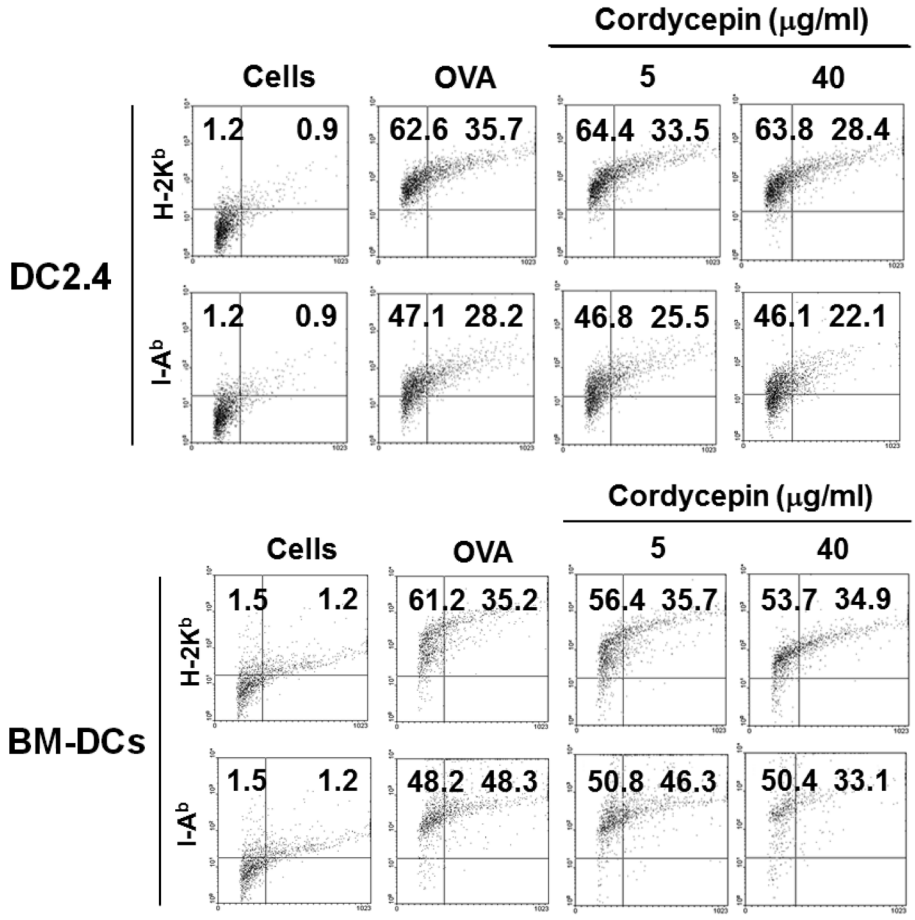

Fig. 4. Effects of cordycepin on the phagocytic activity. DC2.4 cells and BM-DCs were incubated with cordycepin for 2 h, and nanospheres containing both OVA and FITC were added. After 2-h incubation, unphagocytosed microspheres were washed, and the cells were harvested by gentle pipetting and then analyzed by flow cytometry.

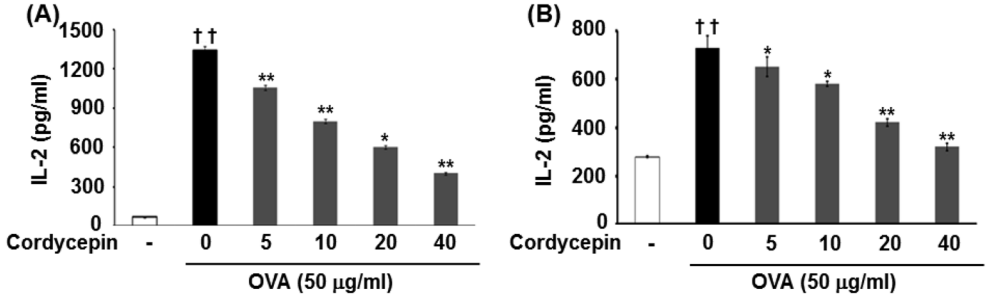

Fig. 5. Effects of cordycepin on the expression of MHC molecules. (A) DC2.4 cells and (B) BM-DCs were incubated with cordycepin (5 or 40 μg/ml) for 2 h and harvested by gentle pipetting. The expression levels of class I and class II MHC molecules were assessed using anti-H-2K^b and anti-I-A^b monoclonal antibodies.

peptide presentation in peritoneal macrophages. These results show that cordycepin suppresses the MHC class II-restricted exogenous antigen presentation *in vivo.*

In all experiments, this is the first report concerning the effects of cordycepin on APCs. In this study, we have shown that cordycepin suppressed protein and mRNA expression of pro-inflammatory cytokines and inhibited MHC-restricted exogenous antigen-presenting capability. Furthermore, cordycepin suppressed MHC class I and class II-restricted exogenous antigen-presenting capability of DCs, leading to reduced expression of total MHC molecules. We speculate that cordycepin would have some therapeutic efficacy as an immune-regulating agent for immunodeficiency diseases.

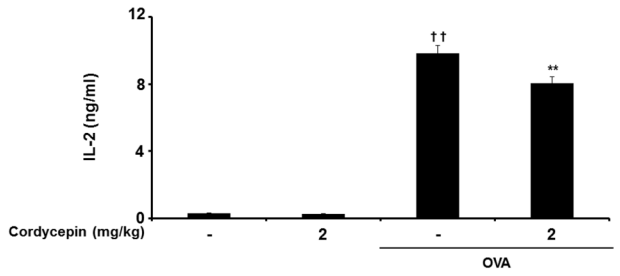

Fig. 6. Cordycepin decreases the MHC class II-restricted presentation of exogenous OVA in peritoneal macrophages *in vivo*. The peritoneal macrophages were elicited by injecting thioglycollate in PBS into the mouse peritoneum. After 4 days, cordycepin (2 mg/kg) was injected subcutaneously. Two hours later, soluble OVA (2 mg/mouse) was injected into the peritoneum. Cordycepin (2 mg/kg) was again injected subcutaneously 2 h after the injection of soluble OVA. The peritoneal macrophages were harvested from the peritoneum 2 h after the second injection of cordycepin, washed, and class II MHC-complexed OVA peptide quantities were then assessed by IL-2 secretion assays after culturing the paraformaldehyde-fixed peritoneal macrophages with DOBW cells for 18 h. $^{\dagger\dagger}p < 0.01$ vs. untreated group based on Student's t-test. $^{**}p < 0.01$ vs. OVA only based on Student's t-test.

Acknowledgments

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Supplementary Data

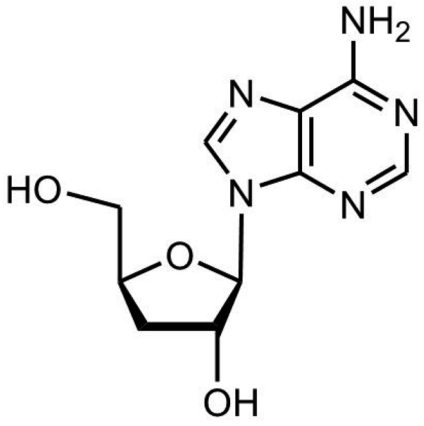

Fig. 1. Structure of cordycepin.

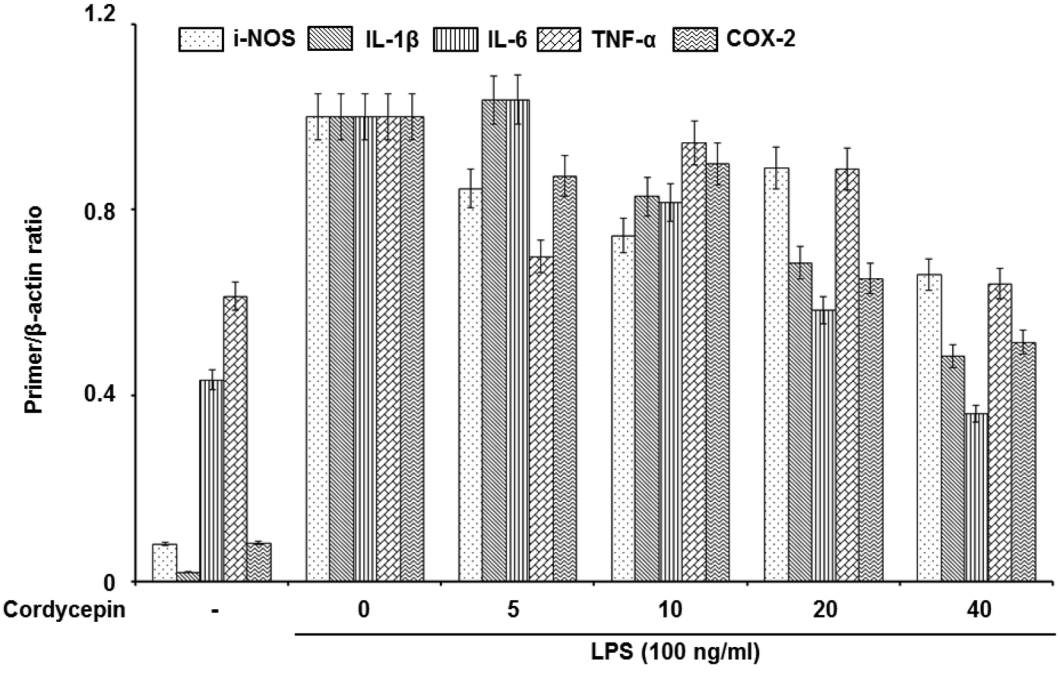

Fig. 2. Effect of cordycepin on the expression of cytokines in macrophages. Levels of i-NOS, IL-1β, IL-6, TNF-α and COX-2 gene and protein in peritoneal macrophages. Cells were incubated with various concentrations of cordycepin in the presence of LPS (100 ng/mL) for 24 hrs. The mRNA levels of cytokines were determined by RT-PCR analysis. β-actin was used as a control.