

Immune modulation of macrophage response by *Cordyceps militaris* extracts

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Research

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Abstract

Backgrounds: *Cordyceps militaris* is a well-known medicinal fungus. Cordycepin, a metabolite of this fungus, has strong biological activities against leukemia, oxidative stress, aging, tumors, and inflammation.

Methods: HPLC analysis was conducted to measure the content of cordycepin in the extract. Real time PCR was performed to evaluate the cytokines. Immunoactivity including the polarization, phagocytic activity and cellular differentiations were evaluated by flow cytometry.

Results: The yields of cordycepin and adenosine in the extract were 11.75 μg and 1.25 μg (per gram fresh mycelium), respectively. From measurements of the bioactivity in the extract, the levels of TNF- α and IL-1 β in macrophages treated with lipopolysaccharides (LPS) were found to be approximately 4 and 48 times higher than those in the control, as shown by qRT-PCR. However, cells treated with 1 $\mu\text{g}/\text{mL}$ of the extract showed 13 and 10-fold lower TNF- α and IL-1 β levels when compared to LPS-treated cells. This was corroborated by flow-cytometry, where their levels were 20 and 14 times lower, respectively. Addition of the extract to LPS-treated cells enhanced M2 polarization and attenuated M1 polarization. In addition, the extract also dose-dependently activated macrophage phagocytosis. Under treatment with the extract conditioned medium, DCs were strongly differentiated toward CD11b $^+$ and Xcr1 $^+$ cells as their density were 13.6 and 6.26 times higher than those in the LPS conditioned medium, respectively. Moreover, the number of T $_{\text{reg}}$ and NKT cells differentiated in the extract conditioned medium were increased about 1.67 and 6.73 times than those in the LPS conditioned medium, respectively.

Conclusions: These results suggest that the *C. militaris* hydrolytic extract has strong effects on the modulation of immune actors, such as macrophages and dendritic cells, under inflammatory stress.

1. Background

Cordyceps species have diverse biological activities and are well known in Chinese traditional medicine. The *Cordyceps* genus contains around 400 species worldwide. Among them, *Cordyceps militaris* and *Cordyceps sinensis* have been traditionally used since ancient times, and are distributed worldwide [1, 2]. Cordycepin, or 3'-deoxyadenosine, a functional compound found in some *Cordyceps*, is a precursor of polysaccharides, ergosterol, mannitol, and vitamins [3]. Cordycepin is not produced by all *Cordyceps* species; it was first discovered in 1951 by Professor Cunningham of the University of Glasgow, United Kingdom, in *C. militaris* [4]. The pharmacological functions of cordycepin are very diverse; it shows anti-cancer, anti-inflammatory, anti-viral, anti-leukemia, anti-tumor, anti-diabetic, and anti-obesity effects, and is known to modulate the human immune system [5–8]. Macrophages, derived from monocytes, play an important role in phagocytosis in disease lesions and act as anti-inflammatory antigen-presenting cells [9]. They also drive homeostasis as part of innate and acquired immunities. During inflammatory reactions, they also play a defensive role by producing nitric oxide (NO) and cytokines [10]. Lipopolysaccharides (LPS), major endotoxins, are derived from gram-negative bacterial cell walls [11].

Macrophages exposed to LPS produce inflammatory cytokines, including interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF), and polarize to M1 macrophages [12, 13]. Although previous studies have shown the anti-inflammatory effects of *C. militaris* extracts [5–8], there is no clear evidence whether these extracts could act on macrophages to modulate the immune response. The goal of this study was to quantify the 3'-deoxyadenosine levels in *C. militaris* and to evaluate the biological functions of *C. militaris* extracts in immunological modulation of LPS-treated macrophages.

2. Materials And Methods

2.1 Measurement of cordycepin by high-performance liquid chromatography (HPLC)

Cordycepin (3'-deoxyadenosine, $\geq 98.0\%$, HPLC grade, product number C3394-10MG, CAS Number 73-03-0) and adenosine ($\geq 99\%$, HPLC grade, P no. A9251-1G, CAS Number 58-61-7) were purchased from Sigma Aldrich (St. Louis, MO). They were used for HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific Inc, Germering, German) and the retention times for cordycepin and adenosine were 6.923 min and 5.687 min, while the linear equations for their standard curves were $y = 0.7745x - 0.0528$ ($r^2 = 0.9993$) and $y = 0.5606x - 0.1491$ ($r^2 = 0.9993$), respectively. The standard solutions of cordycepin and adenosine were injected into the HPLC instrument to evaluate the limits of detection (LOD) and quantification (LOQ), calculated by the formulae $LOD = 3\sigma/S$ and $LOQ = 10\sigma/S$ (σ : standard deviation of the response, S: slope of the calibration plot)

2.2 Extraction of cordycepin from Cordyceps militaris

Two grams of *C. militaris* powder (Damyang, Korea) was mixed with distilled water and treated at 85°C for 2.5 h, followed by ultrasonic extraction at 600 W for 35 min. The extract was filtered through a microporous membrane (0.22 μm , Merck, Darmstadt, Germany) and analyzed using HPLC to quantify cordycepin levels. Chromatographic separation was performed using the YMC-triart C18 column (TA12S05-2546WT, product number 97049-920) under the following conditions: mobile phase: methanol (15%) – water (85%); analytical time: 30 min; column temperature: 25°C; flow rate: 1.0 mL/min; UV detection wavelength: 260 nm; injection volume: 10 μL . The entire cycle was repeated thrice.

2.3 Cell culture

RAW 264.7 cells, a murine macrophage cell line, was maintained in a 5% CO₂ atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 $\mu\text{g}/\text{mL}$ penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. Cells were exposed to LPS for 12 h, and subsequently treated with *C. militaris* extracts (0.1, 0.25, 0.5, or 1 $\mu\text{g}/\text{mL}$) for 8 h. To analyze cellular differentiation, monocytes (JAWS \boxtimes ; ATCC CRL-11904) and T cells (TK-1; ATCC CRL-2396) were cultured with conditioned mediums derived from the supernatant of macrophages treated with *C. militaris* extracts.

2.4 qPCR analysis

Total RNA was extracted from cells using Ribospin mini kit (GeneAll, Seoul, Korea); then RNA samples were converted to cDNA using DiaStar™ 2X RT Pre-mix kit (Solgent, Daejeon, Korea) at 55 °C for 1 h and then at 95 °C for 5 min. Real time-PCR was performed using Topreal™ qPCR 2X PreMIX (Enzynomics; Daejeon, Korea) with the cDNA samples and analyzed using the StepOnePlus Real-Time PCR System instrument (Thermo Fisher Scientific Inc, Germering, German). TNF- α , IL-1 β , and b-actin primers (Table 1) were purchased from Bioneer (Seoul, Republic of Korea). PCR conditions were as follows: initial denaturation of 15 min at 95°C, followed by 35 cycles of 10 s at 95°C, 15 s at 60°C, and elongation of 30 s at 72°C. Data were analyzed using the StepOne Software V2.3.

2.5 Flow cytometric analysis

Cells treated with LPS and *C. militaris* extracts (0.1, 0.25, 0.5, and 1 $\mu\text{g}/\text{mL}$) were fixed with 2% paraformaldehyde for 2 h at 40° C [14]. Fixed cells were treated with Tween-20 (0.5%) and incubated with the specific probes (TNF- α and IL-1 β). Then, cells were analyzed using BD FACSCalibur (BD science, CA, USA). To analyze macrophage polarization, macrophages treated with 0.02% Tween-20 were incubated with the following probes: CXCL10 (Alexa fluor 647-5'-GCTTCCAAGGATGGACCACA-3') and CCL22 (Alexa fluor 670-5'-GAGATCTGTGCCGATCCCAG-3'). Stained cells were analyzed with a flow cytometer BD FACSCalibur and FlowJo 10.6.1 (BD science). To analyze cellular differentiation, monocytes and T cells were treated with fluorescence-conjugated antibodies including FITC-CD11b (BioLegend, San Diego, CA, USA), PE-Xcr1 (BioLegend), APC-CD304 (BioLegend), FITC-FoxP3 (BioLegend), and PE-NK1.1 (BioLegend).

2.6 Phagocytic activity

After treatment with LPS and *C. militaris* extracts, macrophages were exposed to fluorescence-labelled *E. coli* particles and phagocytosis was examined using Vybrant™ Phagocytosis Assay Kit (Thermo Fisher scientific Inc, Germering, Germany) for 2 h. Stained macrophages were analyzed by flow cytometry (BD calibur and FlowJo 10.6.1, BD science).

2.7 Statistical analysis

The data were analyzed by performing paired *t*-test and variance (ANOVA) using SPSS software V26.0 (IBM, New York, USA). *P* values (*P* < 0.05) were calculated using Turkey's method.

Table 1. Primers used for qPCR

Gene	Sequence	
β-actin	Forward	5'-GTG GGV CGC CCT AGG ACC AG-3'
	Reverse	5'-GGA GGA AGA GGA TGC GGC AGT-3'
TNF-α	Forward	5'-TTG ACC TCA GCG CTG AGT TA-3'
	Reverse	5'-CCT GTA GCC CAC GTC GTA GC-3'
IL-1β	Forward	5'-CAG GAT GAG GAC ATG ACA CC-3'
	Reverse	5'-CTC TGC AGA CTC AAA CTC CAC-3'

3. Results

3.1 Measurement of cordycepin levels in Cordyceps militaris extracts.

Cordycepin and adenosine levels were quantified by HPLC using appropriate standards. The total cordycepin and adenosine concentrations in the extracts were 0.47 ± 0.09 and 0.05 ± 0.01 µg/mL, respectively. Reported to the mycelium weight, the concentrations of cordycepin and adenosine in the fresh mycelium were 11.75 µg/g and 1.25 µg/g, respectively.

3.2 Effects of Cordyceps militaris extracts on the expression of IL-1β and TNF-α in macrophages

The mRNA levels of pro-inflammatory cytokines TNF-α and IL-1β were quantified in RAW 264.7 cells treated with LPS and extracts containing cordycepin. In macrophages stimulated only with LPS, the mRNA levels of TNF-α and IL-1β increased approximately 4- to 5-folds compared with the control (Fig. 2a). For macrophages treated with LPS and cordycepin extracts, the mRNA levels of TNF-α and IL-1β dramatically decreased, especially for 1 µg/mL of extract (Fig. 2a). Fluorescence in situ hybridization results also revealed that TNF-α and IL-1β were downregulated dose-dependently in macrophages treated with these extracts (Fig. 2b). Notably, treatment with 1 µg/mL cordycepin extract dramatically downregulated these cytokine levels (Fig. 2C3).

3.3 Polarization of macrophages

For cells not treated with the cordycepin extract, the number of M1 macrophages increased approximately four times following stimulation with LPS; however, under LPS treatment, the number of M2 macrophages decreased by approximately 60% compared to the control (Fig. 3). Surprisingly, when treated with *C. militaris* extracts, the number of M1 macrophages decreased and that of M2 macrophages

increased dose-dependently (Fig. 3). When 1 µg/mL extract was used, the number of M1 macrophages decreased approximately 4 times and the number of M2 macrophages increased approximately 9 times compared to the number observed following LPS treatment (Fig. 3).

3.4 Phagocytic activity of macrophages

Changes in the phagocytic activity was not clear when macrophages were treated only with LPS; however, when cells were treated with LPS and cordycepin extracts, their phagocytic activity increased dose-dependently (Fig. 4a, b). Result of t-SNE showed that most cells had a weak phagocytic activity, but when treated with 1 µg/mL cordycepin extract, macrophages showed a strong phagocytic activity (Fig. 4c). When macrophages were treated with 0.1 µg/mL cordycepin extract, their phagocytic activity was dramatically stimulated compared to that of cells treated only with LPS. Moreover, when cells were treated with 1 µg/mL cordycepin extract, the phagocytic activity of macrophages increased 13 times compared to that observed following treatment with LPS only (Fig. 4d).

3.5 Differentiation patterns of monocytes and T lymphocytes

Although LPS blocked the differentiation of CD11b⁺ and Xcr1⁺ cells from monocytes, following treatment with the conditioned medium from macrophages treated with cordycepin and LPS, dendritic cells (DCs) were strongly differentiated toward CD11b⁺ and Xcr1⁺ cells as their densities increased 13.6 and 6.26 times, respectively, compared to those observed following treatment with conditioned medium from LPS-stimulated macrophages (Fig. 5). Compared to cordycepin, the extract conditioned medium slightly enhanced their differentiation from monocytes than differentiation by the cordycepin conditioned medium (Fig. 5). The conditioned medium from macrophages treated with LPS and cordycepin stimulated the differentiation of T cells to regulatory T cells (T_{reg}) and natural killer T (NKT) cells (Fig. 6). Notably, the differentiation of NKT cells increased by 6.75 times compared to that observed following treatment with conditioned medium from macrophages stimulated with LPS only (Fig. 6c, d). Moreover, although T cells were stressed with LPS, the differentiation of T_{reg} and NKT cells following treatment with conditioned medium from cordycepin-treated macrophages was increased about 1.67 and 6.73 times, respectively compared to that observed when treated with conditioned medium from LPS-stimulated macrophages (Fig. 6b, d).

4. Discussion

Cordyceps has been used as a health food because of its various effects of cleansing the blood, suppressing harmful bacteria and inflammation, and improving immunity [15]. Cordycepin, a functional substance of *Cordyceps sinensis*, was first found in *C. militaris* fruiting bodies. 3'-Deoxyadenosine has many biological activities such as anti-inflammatory, antioxidative and anti-aging, antitumor, anti-cancer, and anti-leukemia activities [5]. LPS, one of the components of the cell wall of gram-negative bacteria,

induces a strong inflammatory response through TLR4 (toll-like receptor 4) activation in host cells and sepsis. LPS is not only important for inflammatory reactions in host cells, but also plays a role in protecting bacterial cells from external toxic substances and antibiotics.

Total free amino acid, adenosine, and cordycepin contents were 83.35 mg/g, 0.24%, and 1.33%, respectively, in the *C. militaris* hydrolytic extract. The concentration of cordycepin was higher than that of adenosine [16]. The concentrations of adenosine and 3'-deoxyadenosine in the extracts were found to be 0.05 ± 0.01 $\mu\text{g/mL}$ and 0.47 ± 0.09 $\mu\text{g/mL}$, respectively, using HPLC. The cytotoxic concentrations of cordycepin in various cell lines ranged from 300 to 400 μM [17]. Although the extracts in this study contained low contents of cordycepin, the cytotoxic concentration of the extract was established at these low concentrations. These results suggested that the extract contained various compounds, and all these compounds, except cordycepin, could affect cellular apoptosis.

Recently, the relationship between inflammation and chronic disease has been reported. Although inflammation acts as a protective barrier against bacteria in the early stages of damage, it causes various chronic diseases such as obesity, diabetes, cancer, and brain and heart diseases [18]. Macrophages derived from monocyte precursors undergo specific differentiation depending on the local tissue environment. They respond to environmental cues within tissues such as cell damage, activated lymphocytes, or microbial products to differentiate into distinct functional phenotypes [19]. Macrophages are polarized to two phenotypes in response to various factors: classical macrophages, M1 and alternative macrophages, M2 [9, 17]. M1 macrophages are typically activated by IFN- γ or LPS and produce pro-inflammatory cytokines, IL-1 β and TNF- α , to kill pathogens. However, excessive production of M1 macrophages is associated with the onset of chronic inflammatory diseases [9]. M2 macrophages are selectively activated by exposure to specific cytokines such as IL-4, IL-10, or IL-13, and are involved in wound healing and tissue repair with anti-inflammatory activity [12, 13]. M1 and M2 cells secrete major cytokines, and the process of the production of these cytokines has the histopathological characteristics of various inflammation-related diseases. Although the M1 phenotype is essential for the immune system, prolonged M1 polarization leads to several diseases [20]. The M2 phenotype, on the other hand, attenuates inflammation through phagocytosis of a pathogen [9, 21]. In this study, the extracts dose-dependently suppressed the M1 polarization of macrophages but activated M2 polarization. Interestingly, qPCR showed that the levels of TNF- α and IL-1 β were notably downregulated in the macrophages at 1 $\mu\text{g/mL}$ of the extracts (Fig. 2). Various compounds in phytoextracts such as melatonin and L-3-n-butylphthalide from celery and polyphenols from cocoa have been shown to activate the M2 polarization of macrophages [22–24]. Flow-cytometry and qPCR analysis showed that the extracts significantly inhibited TNF- α and IL-1 β by suppression of M1 polarization in the macrophages. These results also suggested that the extracts strongly induced suppression of pro-inflammatory cytokines and activation of phagocytosis and contained compounds that activated M2 polarization.

Macrophages also showed enhanced phagocytic activity dose-dependently. As per the t-SNE results, macrophages exposed to 1 $\mu\text{g/mL}$ of the extracts formed populations with strong activity (Fig. 4c). This

result suggested that the extracts caused the activation of M2 polarization and phagocytic activity of M2 macrophages.

Activated macrophages modulated the differentiation of dendritic and T cells. First, activated macrophages strongly increased the number of CD11b⁺ and Xcr1⁺ DCs (Fig. 5). From some reports [25–27], pre-conventional dendritic cells (pre-cDC) among DC precursors were differentiated by interferon regulatory factor 4 (IRF4), macrophage colony-stimulating factor receptor (M-CSFR), and granulocyte-macrophage colony-stimulating factor receptor (GM-CSF). In addition, IRF4 induces down-regulation of pro-inflammatory cytokines in leukocytes [27]. Xcr1⁺ DCs enhance T cell survival and activation [28], while CD11b⁺ DCs are involved in antigen presentation to CD4⁺ cells associated with the induction of Th2 or Th17 cells [26, 29]. These results suggest that the cordycepin extract activates macrophages to synthesize suppressors of pro-inflammatory signals and enhances DC differentiation.

Besides stimulating CD304⁺FoxP3⁺ T_{reg} cells, the number of CD304⁺NK1.1⁺ NKT following incubation with conditioned medium from macrophages treated with the extract and LPS was 6.73 times higher than that observed when conditioned medium from LPS-stimulated macrophages was used. FoxP3⁺ T_{reg} cells are known as homeostatic modulators of the immune system [30]. T_{reg} cells play important roles in various immune systems, including hypersensitivity suppression, pathogenic immunopathology, and autoimmune disease suppression [31]. These results show that the extract activates macrophages to enhance the differentiation of CD304⁺NK1.1⁺ NKT and suppress the inflammatory response induced by LPS. Moreover, although the *C. militaris* extract 1 µg/mL contained lower quantities of cordycepin than did the conditional medium from cordycepin-treated macrophages, cell differentiation was enhanced 1.6 times by the extract conditioned medium compared to the cordycepin conditioned medium. These results suggest that the extract contained other bioactive compounds in addition to cordycepin that stimulated the differentiation of immune cells. Moreover, the extract is more effective to activate an immune response when compared to cordycepin.

5. Conclusions

Consequently, the *C. militaris* hydrolytic extract aids in the prevention and resolution of inflammation under LPS stress through four functions. First, the extract modulates macrophage polarization to achieve prolonged production of macrophages with the M2 phenotype. Second, the extract suppresses the secretion of pro-inflammatory cytokines from macrophages. Third, the extract enhances the phagocytic activity of macrophages. Fourth, the extract enhances differentiation of DCs, T_{reg} cells, and NKT for suppression of inflammation. Therefore, the *C. militaris* hydrolytic extract has strong effects on the modulation of immune actors, such as macrophages and dendritic cells, under inflammatory stress.

List Of Abbreviations

NO: nitric oxide; LPS: Lipopolysaccharides; TNF: tumor necrosis factor; HPLC: high-performance liquid chromatography; LOD: limits of detection; LOQ: limits of quantification; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; ANOVA: analyzed by performing paired t-test and variance; T_{reg}: regulatory T cells; NKT: natural killer T cells; TLR4: toll-like receptor 4; pre-cDC: pre-conventional dendritic cells; IRF4: interferon regulatory factor 4; M-CSFR: macrophage colony-stimulating factor receptor; GM-CSF: granulocyte-macrophage colony-stimulating factor receptor.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Seoyoon Choi, Ahran Song and Yoonjin Park: conceived this study and designed the experiments. **Boyong Kim and Seung Gwan Lee:** conducted the experiments. **Yoonjin Park , Jinkwan Kim:** analysed the results. **Boyong Kim:** contributed in writing manuscript.

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Figures

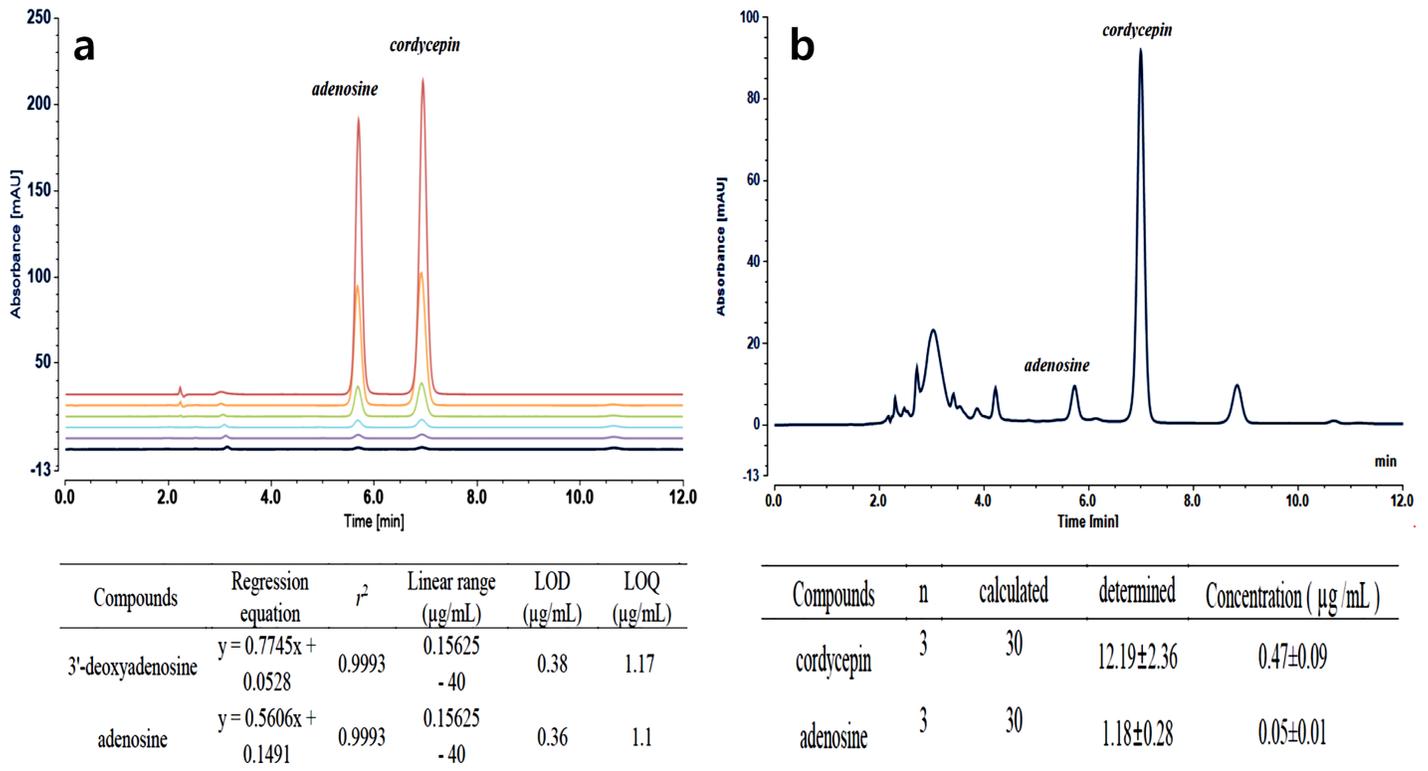


Figure 1

Determination of the cordycepin content in *Cordyceps militaris* extracts by HPLC Panel a shows the overlay chromatogram of various concentrations of the cordycepin and adenosine standards. Panel b showed the concentration of cordycepin in *Cordyceps militaris* extracts. ($P < 0.05$)

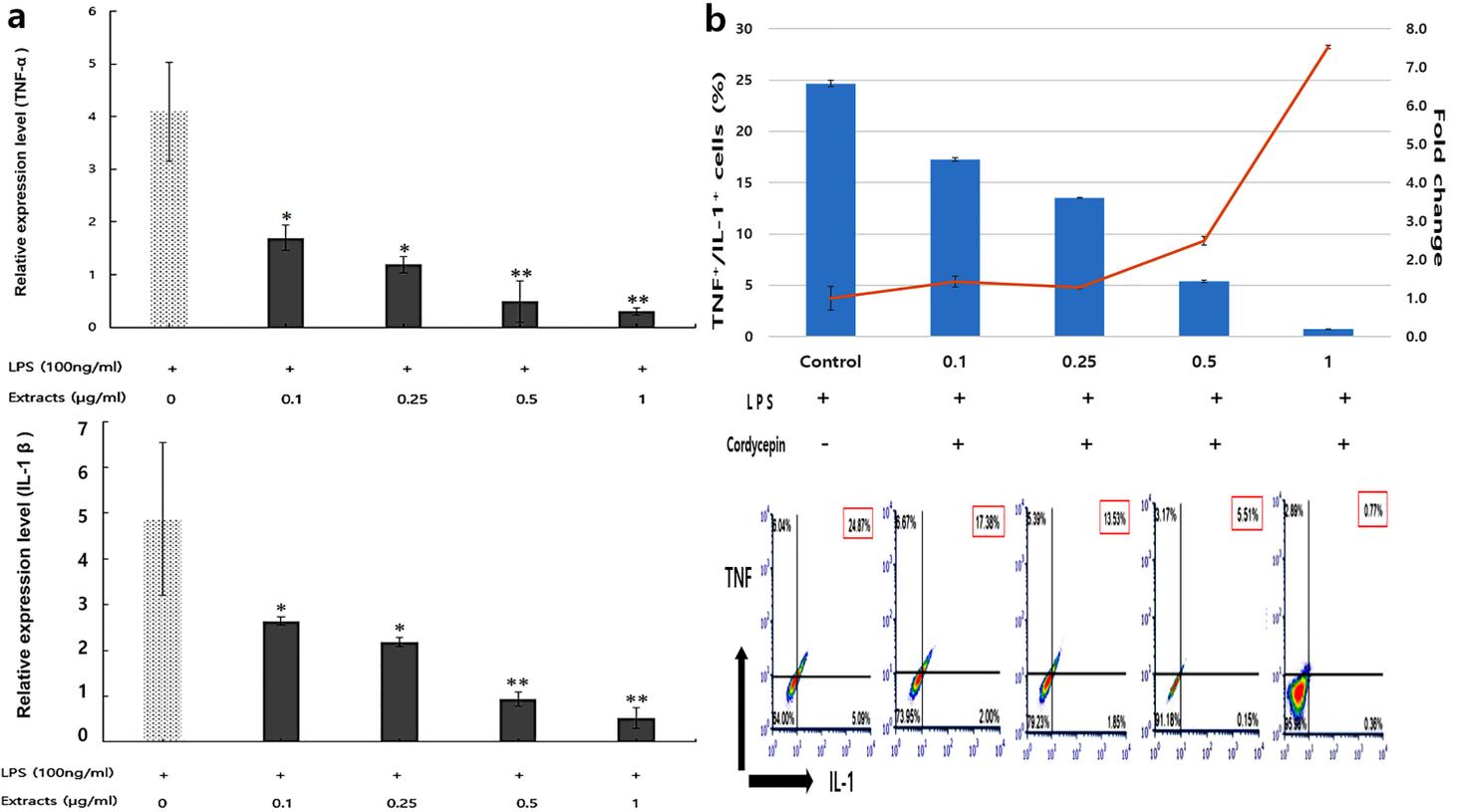


Figure 2

Expression levels of IL-1 β and TNF-α from macrophages activated by *Cordyceps militaris* extracts under LPS stress. Expression levels of IL-1 β and TNF-α from macrophages using qPCR and FACS have been shown in the a and b panels, respectively. The bar graphs shown in the b panel are the relative fold changes of double positive cells by the doses of the extract under LPS stress. (P < 0.05)

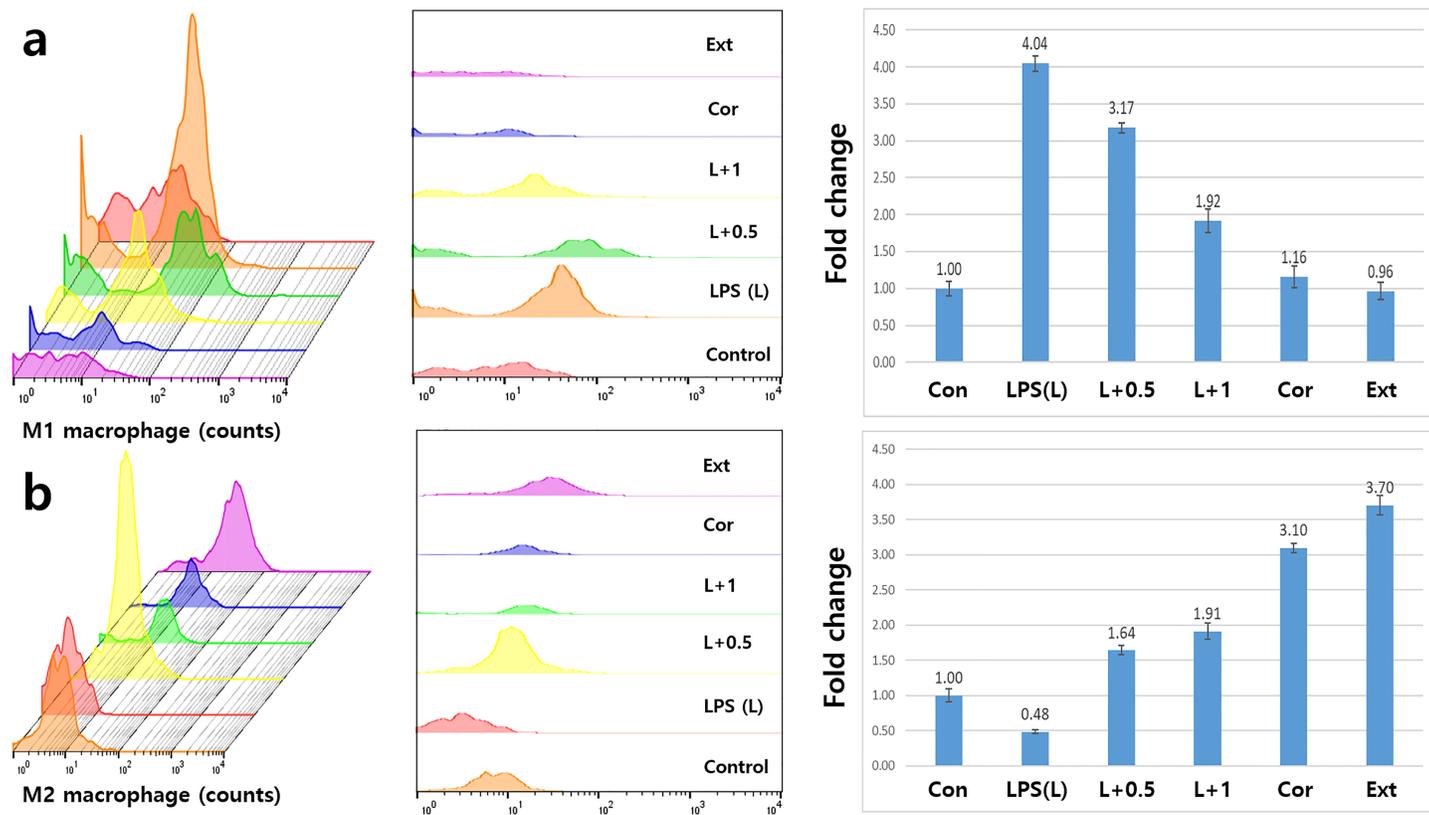


Figure 3

Polarization patterns of macrophages by *Cordyceps militaris* extracts under LPS stress Fluorescence in situ hybridization (FISH) results measured by flow cytometry. Changes in M1 and M2 polarization have been shown in panel a and b, respectively. Bar graphs show the relative fold changes for M1 and M2 polarization by the doses of the extract under LPS stress. ($P < 0.05$)

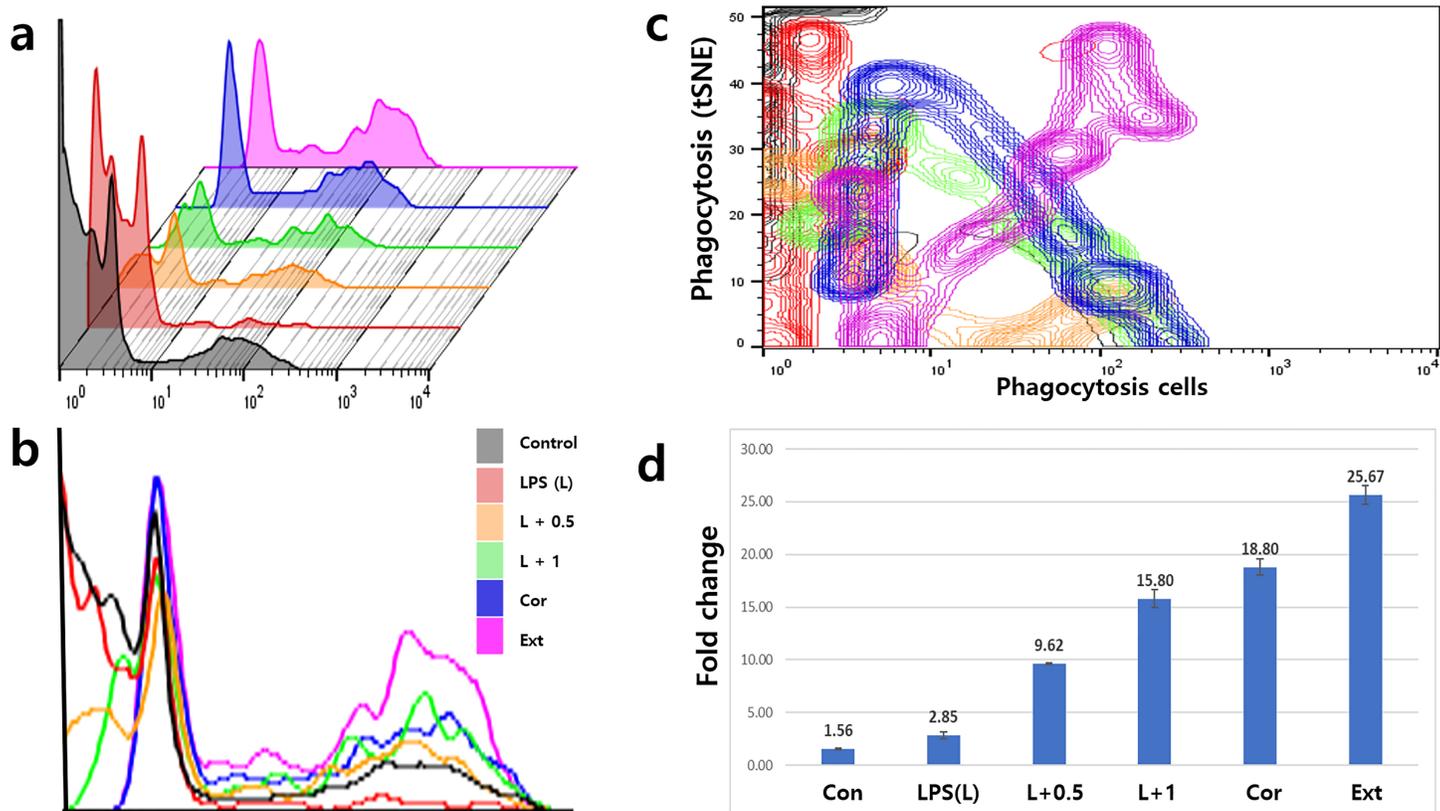


Figure 4

Activation of phagocytosis by *Cordyceps militaris* extracts in macrophages under LPS stress The phagocytosed macrophages were counted by flowcytometry and were analyzed by FlowJo software 10.6.1. The a and b panels show histograms of activation of phagocytosis by *Cordyceps militaris* extracts, and the c panel shows the visualizing datum using t-SNE for the phagocytosis. Panel d shows the relative fold changes for phagocytic activation by serial doses of the extract under LPS stress. ($P < 0.05$)

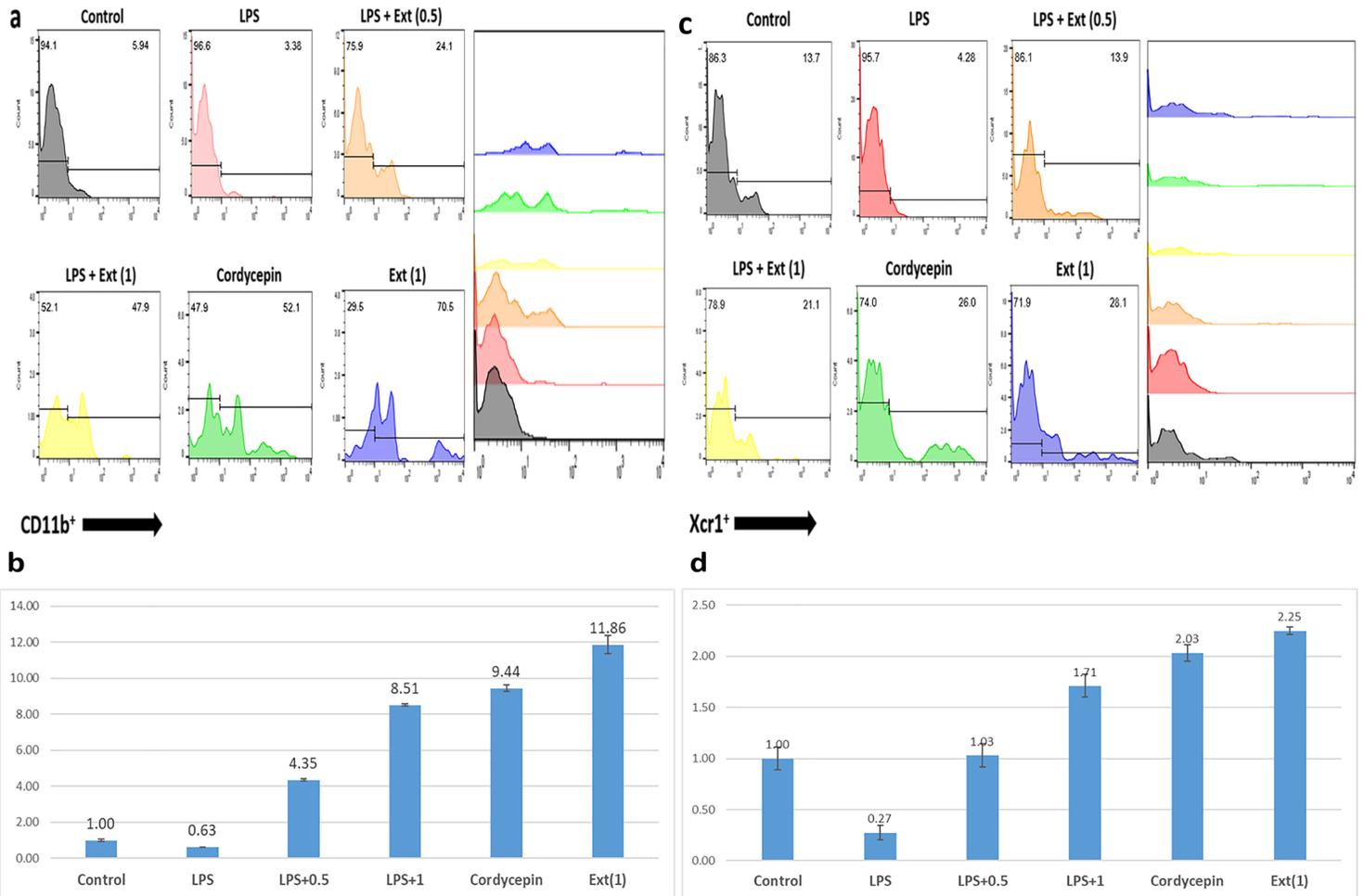


Figure 5

Dendritic differentiation by activated macrophages in the extract conditioned medium Differentiated dendritic cells were counted by flow cytometry and were analyzed using the FlowJo software 10.6.1. Panels a and b show the density of CD11b⁺ cells under various conditions as follows: LPS only, extract 0.5 μg/mL and 1 μg/mL (Ext 0.5, 1) following LPS treatment, cordycepin and extract. Panels c and d show the density of Xcr1⁺ cells under the same conditions. (P < 0.05)

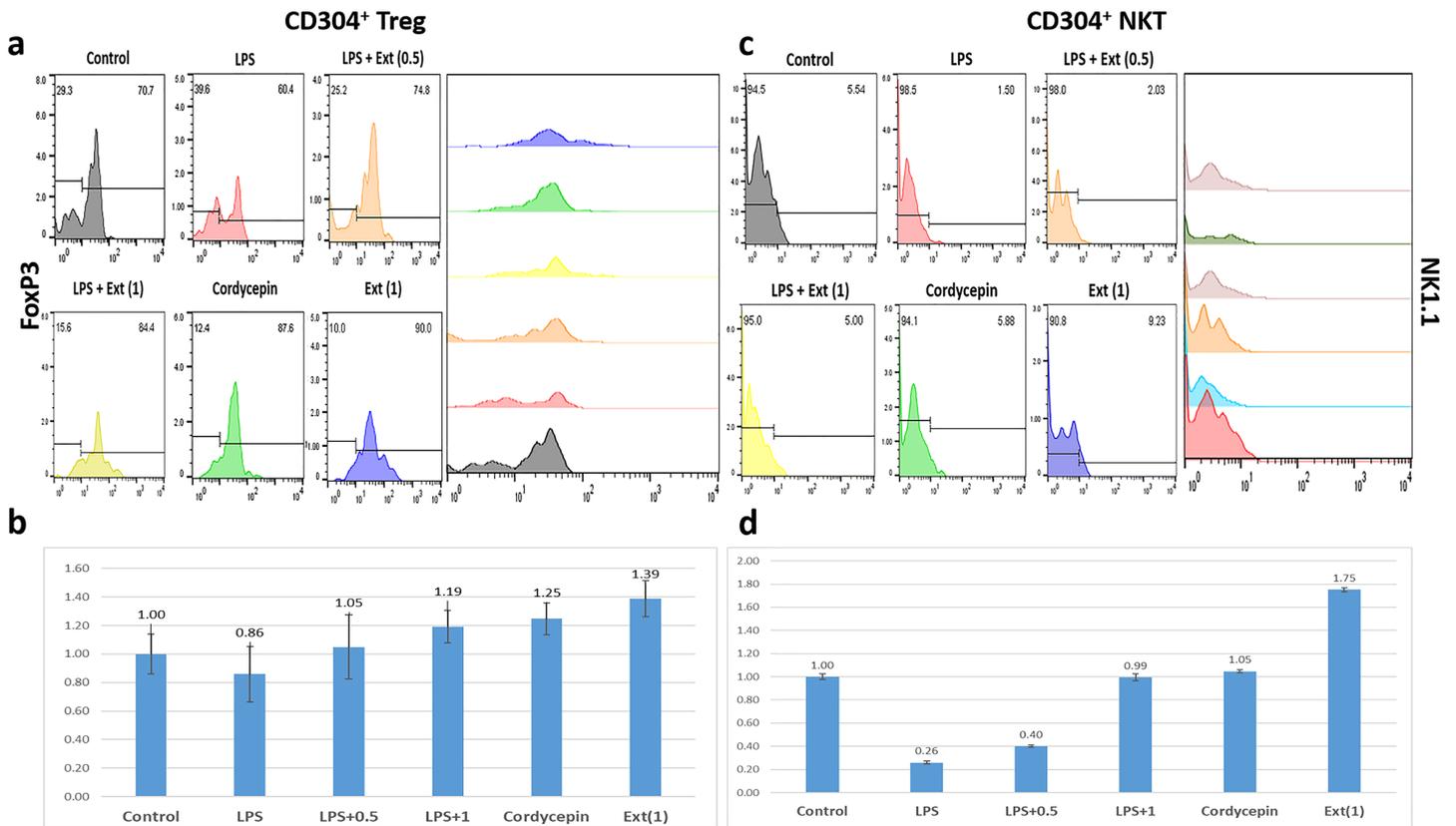


Figure 6

Lymphocyte differentiation by activated macrophages in the extract conditioned medium Differentiated lymphocytes were counted by flowcytometry and were analyzed using the FlowJo software 10.6.1. Panels a and b show the density of CD304⁺FoxP3⁺ cells under various conditions as follows: LPS only, extract 0.5 μ g/mL and 1 μ g/mL (Ext 0.5, 1) following LPS treatment, cordycepin and extract. Panels c and d show the density of CD304⁺NK1.1⁺ cells under the same conditions. ($P < 0.05$)