J. Microbiol. Biotechnol. (2013), **23**(2), 156–160
http://dx.doi.org/10.4014/jmb.1211.11032
First published online January 8, 2013
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of Cordycepin on the Expression of the Inflammatory Cytokines TNF-alpha, IL-6, and IL-17A in C57BL/6 Mice

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Received: November 14, 2012 / Revised: November 30, 2012 / Accepted: December 1, 2012

Culture supernatants of splenocytes from C57BL/6 mice were exposed to 0.3, 1.0, and 3.0 μg/ml cordycepin plus 3.0 μg/ml lipopolysaccharide (LPS) to investigate the effects of cordycepin (3'-deoxyadenosine) on the production of inflammatory cytokines. Co-administration of 3.0 μg/ml cordycepin with LPS in cultured murine spleen cells significantly diminished the expression of the inflammatory cytokines tumor necrosis factor-α and interleukin-6 (IL-6) in a time-dependent manner. Expression of the inflammatory cytokine IL-17A was substantially down-regulated in a time- dependent manner at all cordycepin concentrations. These findings suggest that cordycepin down-regulates the immediate hypersensitivity reaction stimulated by LPS.

Key words: Cordycepin, IL-17A, IL-6, splenocytes, TNF-α

Cordyceps militaris is a traditional medicinal mushroom with a variety of benefits in the human body such as antitumor [30], antimutagenic [6], antiangiogenesis [31], and hypoglycemic effects [7]. Cordycepin has many pharmacological activities including immune stimulating, anticancer, antivirus, and anti-infection activities [18, 27]. A variety of chemical constituents, including polysaccharides and glycoproteins, are involved in these activities [3, 14, 27, 29]. A nucleoside analog (3'-deoxyadenosine), cordycepin, which was first reported as a metabolite isolated from *C. militaris* culture broth, is the major active constituent of the strain [8]. Cordycepin has antitumor, antimetastatic, antibacterial, antiviral, immunomodulatory, and anti-inflammatory activities [12, 16, 19, 28, 32]. Cytokines are signaling molecules involved in host defense, growth, and repair processes within injured tissues [5, 24, 25]. Tumor necrosis factor-alpha (TNF-α) is an inflammatory cytokine associated with systemic inflammation and stimulates the acute phase reaction. TNF-α is mainly produced by activated macrophages (M1). The primary role of an endogenous pyrogen, TNF-α, is to regulate immune cells. TNF-α can induce fever, apoptotic cell death, and sepsis through the production of interleukin-1 (IL-1), IL-6, and other inflammatory cytokines. TNF-α also inhibits tumorigenesis and viral replication [20]. IL-6 is an interleukin that performs pro-inflammatory and anti-inflammatory functions, and is secreted to stimulate T cell and macrophage immune responses. IL-6 is one of the most important mediators inducing fever and the acute phase response [26], which is involved in many diseases such as diabetes [22], atherosclerosis [13], depression [4], Alzheimer's disease [17], systemic lupus erythematosus [15], prostate cancer [4], and rheumatoid arthritis [21]. IL-17A is also a pro-inflammatory cytokine produced by activated T cells and regulates the activities of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases. This cytokine stimulates IL-6 and cyclooxygenase-2 expression. High levels of IL-17A are involved in chronic inflammatory diseases, including rheumatoid arthritis, psoriasis, and multiple sclerosis [2]. However, the effect of cordycepin on inflammatory cytokines in mouse splenocytes has not been reported. We investigated the changes in the expression of inflammatory cytokines,

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including TNF-α, IL-6, and IL-17A, after cordycepin treatment. We also observed the changes in the expression of cytokines resulting from concomitant treatment with cordycepin and lipopolysaccharide (LPS) as a cellular stimulant. Results were expressed as the mean or the mean ± standard deviation, and the significance of differences observed between experimental groups and the control was evaluated using Student's *t* test. *P* values less than 0.05 were regarded as significant.

Male C57BL/6 mice (6–8 weeks old) were purchased from Daehan BioLink (Eumseong, Chungbuk, Korea). All procedures involving animals were approved by the Animal Care Committee of Kosin Medical School, Busan, Korea. Cordycepin was purified from *Cordyceps militaris* [11] and used in this study. LPS purified from *Escherichia coli* (serotype O26:B6) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse enzyme-linked immunosorbant assay kits were purchased from Biolegend (San Diego, CA, USA) and used to measure TNF-α, IL-6, and IL-17A expression. Splenocytes were isolated from each animal by aseptically removing the spleens from mice. Mouse spleens were homogenized in tissue culture medium (Celox Laboratories Inc., Hopkins, MN, USA) and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphothericin B, and 10% heat-inactivated fetal bovine serum (FBS). Cultured spleen cells were adjusted to 1.5×10^6 cells/ml using RPMI 1640 medium with 10% FBS, and the adjusted suspensions were distributed into 24-well tissue culture plates (Costar, Cambridge, MA, USA) at 1 ml/well. The cells were treated with 0.3, 1.0, and 3.0 μg/ml cordycepin and incubated at 37°C in a humidified atmosphere of 5% CO_2. Concomitant treatment with cordycepin and LPS was also conducted. Cells were treated with 0.01% DMSO as the control. The cells were incubated for 6, 24, 48, and 72 h before collection. The suspensions were then subjected to an initial centrifugation at 300 ×*g* for 10 min and a secondary centrifugation at 1,000 ×*g* for 30 min. The supernatants were stored at −70°C until the cytokine expression analysis. We measured TNF-α, IL-6, and IL-17A expression in samples collected from 6, 24, and 48, and 72 h treatments. Capture antibodies for TNF-α, IL-6, and IL-17A were diluted with coating buffer, loaded on 96-well plates at 100 μl/well, and incubated at 4°C overnight. The wells were washed twice with washing buffer, and 200 μl of the assay diluent was added to each well. The plates were incubated at room temperature for 1 h. The wells were washed again twice with washing buffer, and 100 μl of the sample was added to each well. The plates were then incubated at room temperature for 2 h. After washing twice, 100 μl of the detection antibody for the appropriate cytokine was added to each well and the plates were incubated at room temperature for 1 h. The wells were then

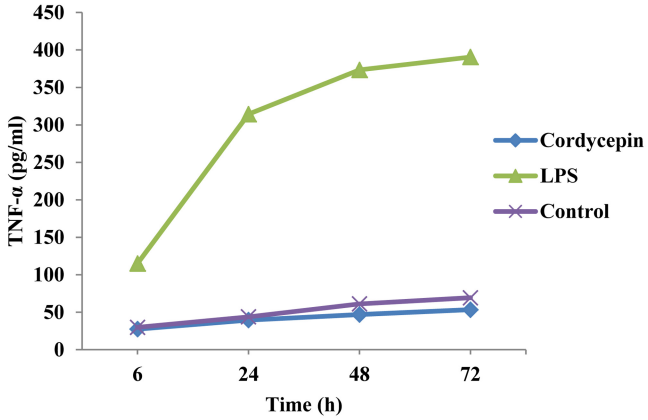

Fig. 1. Production of TNF-α in mouse splenocytes exposed to cordycepin.
Splenocytes were cultivated with cordycepin for 24, 48, or 72 h. Data are the mean ± SE (standard errors) of three independent experiments performed in duplicate. *$p < 0.05$ compared with the corresponding control (cultivated splenocytes).

washed twice with the washing buffer, 100 μl of avidin-horseradish peroxidase was added to each well, and the plates were incubated at room temperature for 30 min. One hundred microliters of substrate fluid containing tetramethylbenzidine was then added and the plates were incubated at room temperature for 20 min. One hundred microliters of a stop solution was added to the stop reaction, and the optical density was measured at 450 nm using a microplate reader (Model 550 microplate reader; Bio-Rad, Richmond, VA, USA).

Murine splenocyte suspensions were treated with 1.0 μg/ml cordycepin for 72 h to observe the effect of cordycepin on TNF-α expression. As shown in Fig. 1, the treatment of cultured spleen cells with cordycepin alone had no significant effect on TNF-α expression compared with the vehicle control. The treatment of LPS significantly up-regulated TNF-α expression in a time-dependent manner. This result suggests that cordycepin does not play a significant role in inducing TNF-α expression in normal murine spleen cells. We also observed changes in IL-6 and IL-17A expression after treating murine splenocyte suspensions with 0.3, 1.0, and 3.0 μg/ml cordycepin for 72 h. IL-6 expression in the cells was not significantly affected by the cordycepin treatment (Fig. 2A), whereas the expression of IL-17A was down-regulated in accordance with the cordycepin concentrations (Fig. 2B).

We further analyzed the changes in TNF-α, IL-6, and IL-17A expression after treating the murine splenocyte suspensions with 0.3, 1.0, and 3.0 μg/ml cordycepin plus 3.0 μg/ml LPS for 72 h. Co-administration of 3.0 μg/ml cordycepin with LPS significantly diminished the TNF-α expression in a time-dependent manner, whereas 0.3 and 1.0 μg/ml cordycepin did not affect TNF-α expression

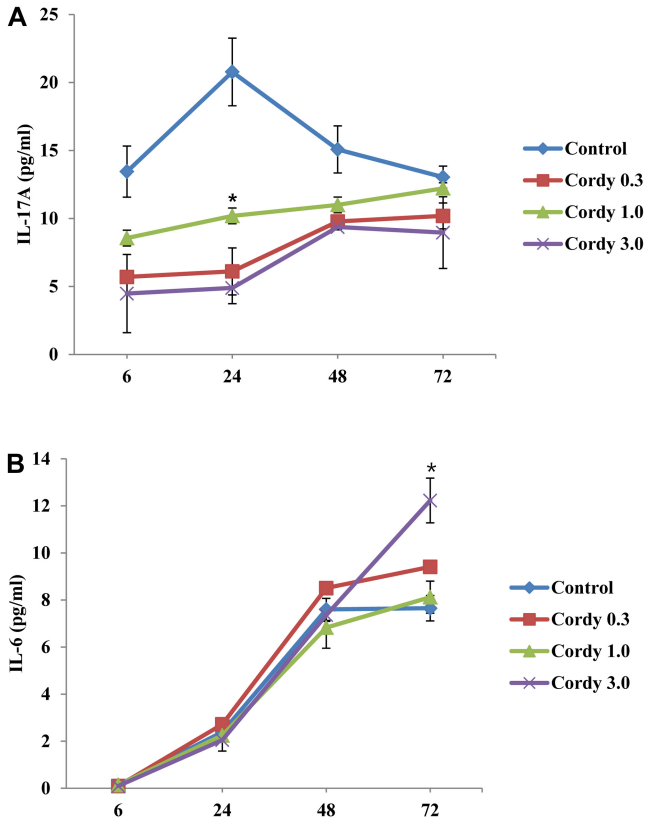

Fig. 2. Production of IL-6 (**A**) and IL-17A (**B**) in mouse splenocytes exposed to cordycepin.
Splenocytes were cultured with 0.3, 1.0, or 3.0 μg/ml cordycepin for 6, 24, 48, or 72 h. Data are the mean ± SE (standard errors) of three independent experiments performed in duplicate. *$p < 0.05$ compared with the corresponding control (cultivated splenocytes).

(Fig. 3A). The IL-6 expression was also significantly down-regulated in a time-dependent manner by concomitant administration of 3.0 μg/ml cordycepin and LPS. However, the 0.3 and 1.0 μg/ml cordycepin plus LPS treatment did not reduce IL-6 expression (Fig. 3B). Compared with the control, the expression of IL-17A was down-regulated by the simultaneous administration of cordycepin and LPS (Fig. 3C).

TNF-α, IL-6, and IL-17A are inflammatory cytokines that cause fever, inflammation, tissue damage, and/or even shock. Inhibiting these inflammatory cytokines is effective for preventing graft rejection and treating inflammatory colitis [21]. Increases in adipose tissue abundance can induce the expression of certain cytokines, such as TNF-α and IL-6, leading to the disruption of insulin signaling [2]. The anti- and pro-inflammatory cytokine IL-6 is involved in the acute reactions and is abundant in the synovial fluid of patients with rheumatic arthritis and in muscle tissues (specifically inflammatory cells, endodermal cells, or muscle cells within tissues) of patients with myositis syndrome and/or those who express multiple cytokines [9, 15, 17].

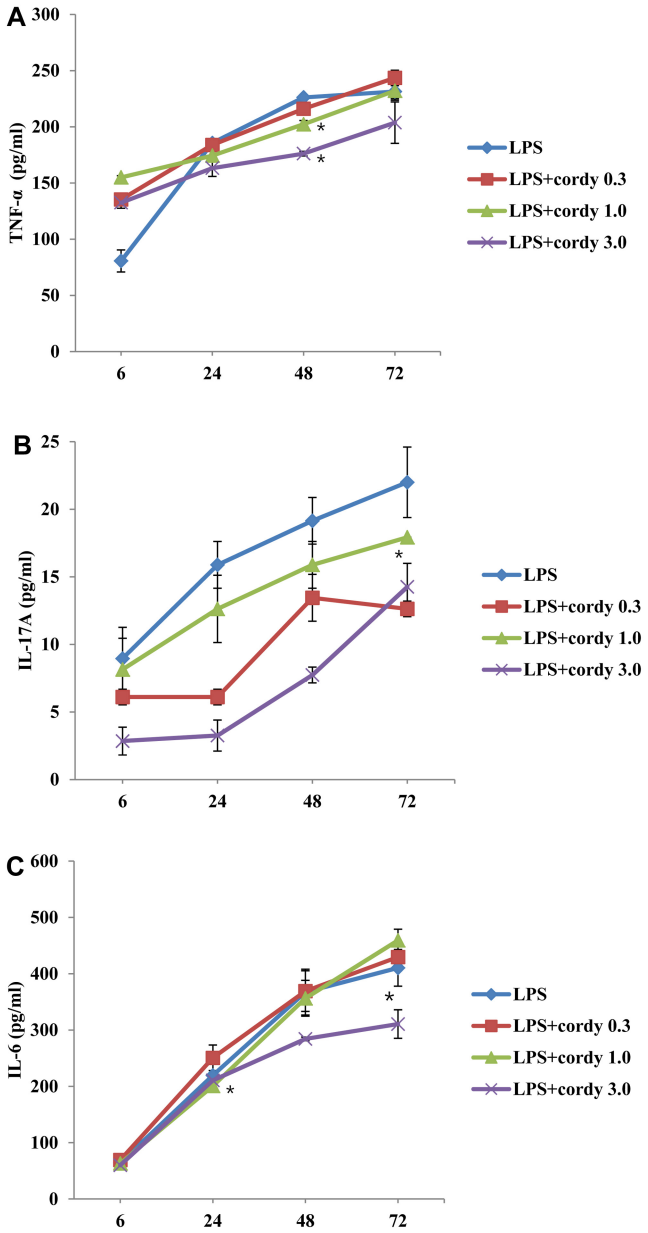

Fig. 3. Production of TNF-α (**A**), IL-6 (**B**), and IL-17A (**C**) in mouse splenocytes exposed to cordycepin and LPS.
Splenocytes were cultured with LPS plus 0.3, 1.0, or 3.0 μg/ml cordycepin for 6, 24, 48, or 72 h. Data are the mean ± SE (standard errors) of three independent experiments performed in duplicate. *$p < 0.05$ compared with the corresponding control (cultivated splenocytes).

These studies suggest that a higher expression of TNF-α, IL-6, and IL-17A in murine splenocytes may be involved in activating T helper (Th) cells.

In summary, our results demonstrate that cordycepin significantly down-regulated TNF-α, IL-6, and IL-17A expression when LPS was used as the stimulant. Concomitant administration of cordycepin and the cellular stimulant (LPS) to suspensions of normal murine spleen

cells led to down-regulation of TNF-α, IL-6, and IL-17A. These results suggest that cordycepin attenuates the inflammatory reaction by reducing the expression of the inflammatory cytokines involved in mediating allergic reactions. In other words, the inflammatory responses involve the down-regulation of TNF-α, IL-6A, and IL-17A, preventing cells from an allergic response and various manifestations of allergic inflammation. Thus, cordycepin can have putative therapeutic applications in chronic allergic diseases, such as Th1 inflammatory disorders, rheumatoid arthritis, colitis (inflammatory bowel disease/Crohn's disease), and psoriasis [1, 9, 10, 23].

Acknowledgment

This study was supported by the Dong-A University Research Fund, Korea.

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