

Cordycepin-enriched *Cordyceps militaris* induces immunomodulation and tumor growth delay in mouse-derived breast cancer

MIN-HO JEONG^{1,2}, CHANG-MIN LEE¹, SANG-WHA LEE^{1,2}, SU-YEONG SEO^{1,2}, MIN-JEONG SEO^{2,3},
BYOUNG-WON KANG², YONG-KEE JEONG^{2,3}, YOO-JIN CHOI⁴, KWANG-MO YANG⁴ and WOL-SOON JO⁴

¹Department of Microbiology, Dong-A University College of Medicine; ²Medi-Farm Industrialization Research Center and ³Department of Biotechnology, Dong-A University, Busan 604-714; ⁴Department of Research Center, Dong Nam Institute of Radiological and Medical Sciences, Busan 619-953, Republic of Korea

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Abstract. *Cordyceps militaris* (*C. militaris*) and its main functional component, cordycepin, has been shown to possess a number of pharmacological activities including immunological stimulation and antitumor effects. However, the pharmacological mechanisms of *C. militaris* on tumor immunity underlying its antitumor effect have yet to be elucidated. In the present study, we evaluated the antitumor and immunomodulatory effects of *C. militaris* on FM3A tumor-bearing C3H/He mice, comparing wild-type *C. militaris* and cordycepin-enriched *C. militaris* (JLM 0636). The concentration of cordycepin produced by crossbred JLM 0636 was 7.42 mg/g dry weight, which was 7-fold higher than that of wild-type *C. militaris*. Dietary administration of *C. militaris* revealed retardation of tumor growth as well as elongation of survival rates of tumor-bearing mice. This effect was more pronounced in JLM 0636. There was a cordycepin-dependent decrease in IL-2 and TGF- β secretion and an increase in IL-4 secretion without changes in the proliferative responses of concanavalin A-stimulated lymphocytes, which suggested that *C. militaris* feeding might induce changes in the subpopulations of tumor-derived T lymphocytes. CD4⁺CD25⁺ cell population was significantly reduced in the total splenocytes from JLM 0636-administered mice, while CD4⁺ T cell population remained unchanged. FoxP3⁺-expressing Treg cells among CD4⁺CD25⁺ population showed a similar pattern. On the contrary, CD8⁺ T cells as well as the IFN- γ expressing CD8⁺ T cells from tumor-bearing mice were significantly upregulated by the administration of

JLM 0636. These results demonstrated the suppressive role of JLM 0636 on the function of Treg cells contributing to tumor specific IFN- γ -expressing CD8⁺ T cell responses in tumor-bearing mice, which explained the underlying mechanism of the antitumor immunity of cordycepin. Therefore, cordycepin-enriched *C. militaris* is a promising candidate for an adjuvant in cancer immunotherapy.

Introduction

Cordyceps militaris (*C. militaris*) also known as the rare Chinese caterpillar fungus has been used in traditional medicine to maintain health and to treat numerous diseases associated with circulatory, respiratory, glandular and metabolic systems. Several studies demonstrated that a variety of chemical constituents in *C. militaris* are involved in these activities, with cordycepin, a nucleoside analog (3'-deoxyadenosine), considered to be one of the most important bioactive components mediating its beneficial effects (1,2). *C. militaris* has been shown to display anti-inflammatory (3), antidiabetic (4), and anti-infectious activities (5-7) conceivably being related to the immune response. Although it has also been considered to have antitumor activities (8-13), there is still considerable uncertainty about the effect on tumor immunity.

The recent advance in the mechanisms responsible for tumor progression have suggested the possibility of controlling tumor growth through chemotherapy-induced cancer cell destruction as well as by stimulating the antitumor immunity. Tumor-infiltrating lymphocytes (TILs) are seen as a reflection of a tumor-related immune response and are recognized as the principal effectors of the local antitumor immune response (14). During the neoplastic process, however, tumor cells acquire immunotolerance and cause an accumulation of immunosuppressive infiltrates in the tumor microenvironment (15). In our previous study, we evaluated the effect of cordycepin purified from *C. militaris* JLM 0636 strain on the production of cytokines in lipopolysaccharide-stimulated mouse splenocytes (16). Th1 cytokines such as IL-12 and IFN- γ , and Th2 cytokines such as IL-4 and IL-10, were significantly increased by cordycepin; however, IL-2 was

Correspondence to: Dr Wol-Soon Jo, Department of Research Center, Dong Nam Institute of Radiological and Medical Sciences, Jwadong-gil 40, Jangan-eup, Gijang-gun, Busan 619-953, Republic of Korea
E-mail: sailorjo@dirams.re.kr

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rather decreased with time, indicating that cordycepin might induce change in the regulatory subpopulation among CD4⁺ T lymphocytes (Treg cells). Treg cell is one of the most potent and well-studied suppressive phenotypes found in the tumor microenvironment, constitutively expressing high levels of CD25, CTLA-4, GITR, and a transcriptional factor forkhead box P3 (Foxp3) (17-19). They play an important role in immune evasion mechanisms employed by tumors which may differentiate, expand, recruit and activate them and potentially abrogate antitumor immunity. Indeed, higher numbers of Treg cells are associated with malignancy progression in various solid tumors (lung, breast and pancreas) as well as hematological malignancies. The increased populations of CD4⁺CD25⁺ Treg cells appear to correlate with a poorer survival for several types of cancer (20).

In the present study, we demonstrated that *C. militaris* induced the immunomodulatory and antitumor properties controlled by the suppression of Treg cell population, which made peritumoral microenvironment unfavorable to tumors and eventually resulted in growth inhibition of tumor cells. We also showed that these effects were dependent on the cordycepin content in *C. militaris* by comparing wild-type *C. militaris* and cordycepin-enriched *C. militaris* JLM 0636 strain.

Materials and methods

Preparation of *C. militaris* and quantitative analysis of cordycepin. *C. militaris* used in the present study was supplied by Chungwon-Industrial Farm (Gimhae, Korea) which had constructed *C. militaris* JLM 0636 strain by single spore fusion of various strains of *C. militaris*. For the preparation of *C. militaris* contained food, the powder of dried fruiting bodies was replaced with cornstarch 3% in the basal food (casein 20%, cornstarch 15%, sucrose 44.5%, cellulose 5%, corn oil 10%, AIN-93M-MX mineral mixture 4%, AIN-93-VX vitamin mixture 1%, choline bitartrate 0.2% and DL-methionine 0.3%). For the preparation of *C. militaris* contained water, the powder of dried fruiting bodies was extracted with distilled deionized water for 3 h at 121°C, the insoluble materials were removed by centrifugation at 10,000 g for 30 min and filtration with 0.45 µm membrane filter, and the resulting water extract was added to the standard water to 1%. Cordycepin content in the water extract was analyzed by high performance liquid chromatography (PerkinElmer 200 Series System; PerkinElmer, Waltham, MA, USA) using a Bridge C18 column (4.6 x 250 mm, 3.5 mm; Waters, Taunton, MA, USA) and UV Detector (260 nm). The mobile phase was 0.01 M phosphate buffer, pH 6.5 and linear gradient with MeOH: 0-15% for 10 min, 15-10% for 5 min, 10-20% for 2 min and 20% for 12 min. The column oven was kept at 40°C, and the flow rate was 5 ml/min. The cordycepin content was calculated from the peak area shown in the standard curve of commercial cordycepin (Sigma, St. Louis, MO, USA).

Animal and experimental design. Female C3H/He mice, 6 weeks of age, were obtained from Central Laboratory Animal Inc., (Seoul, Korea) and housed at the animal maintenance facility of the Clinical Research Center of Dong-A University Hospital. All animals were maintained in specific pathogen-

free conditions according to Good Laboratory Practices OECD guidelines. All animal procedures were performed according to approved protocols (Approval number; DIACUC-09-24) from the Institutional Animal Care and Use Committee (IACUC) of Dong-A University and in accordance with recommendations for the proper use and care of laboratory animals. Animals were allowed free access to basal food and standard water for 2 week before the experiment. Animals were then randomly divided into four experimental groups: normal mice group fed with basal food and standard water (normal), tumor-bearing mice group fed with basal food and standard water (control), tumor-bearing mice group fed with wild-type *C. militaris* contained food and water (CMloC), tumor-bearing mice group fed with *C. militaris* JLM 0636 strain contained food and water (CMhiC). FM3A murine breast cancer cells originated from the mammary gland of the C3H/He mouse and cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) at 37°C in a humidified 5% CO₂ atmosphere. Cells in logarithmic growth phase (2x10⁶ cells/50 µl saline) were inoculated subcutaneously on the right flank of female C3H/He mice. Mice were monitored daily for signs of toxicity and survival, and tumor volumes were measured twice weekly with a caliper. Tumor volumes were calculated as (width)² x length x 0.52. When the tumor-bearing mice were sacrificed at day 30, upon reaching >100 mm³ of tumor volume, the spleen was aseptically removed. Single cell suspension was prepared by gently teasing the cells through sterile stainless steel screen and the erythrocytes were lysed at room temperature using ACK lysis buffer (NH₄C₁, KHCO₃ and Na₂EDTA). The isolated splenocytes were suspended in complete medium (RPMI-1640 supplemented with 10% FBS, 50 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin) and cultured at 37°C in a humidified 5% CO₂ atmosphere.

Cytokine production assay. The isolated splenocytes were cultured with concanavalin A (ConA, 5 µg) for 24 h at 10⁷ cells/ml in serum-free RPMI medium containing 200 µg/ml BSA. IL-2, IL-4 and TGF-β concentrations were determined in supernatant using enzyme-linked immunosorbent assay kits (ELISA; BD Biosciences Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions.

Lymphocyte proliferation assay. Lymphocyte proliferation was determined by BrdU (5-bromo-2-deoxyuridine) incorporation assay using ConA-stimulated cell suspension at 5x10⁵ cells/well in flat-bottom 96-well microculture plates. The cells were cultured for 48 h and further incubated for 24 h in the presence of 10 µl of the BrdU solution in RPMI medium (1:100 diluent). The BrdU incorporation was measured by using the Cell Proliferation ELISA BrdU kit (Roche Diagnostics-Applied Science, Mannheim, Germany), following the supplier's specifications.

Lymphocyte subpopulation analysis. Lymphocyte subpopulations among splenocytes were determined by flow cytometric analysis. The freshly prepared splenocytes were washed three times with ice cold PBS-containing 0.1% FBS and then

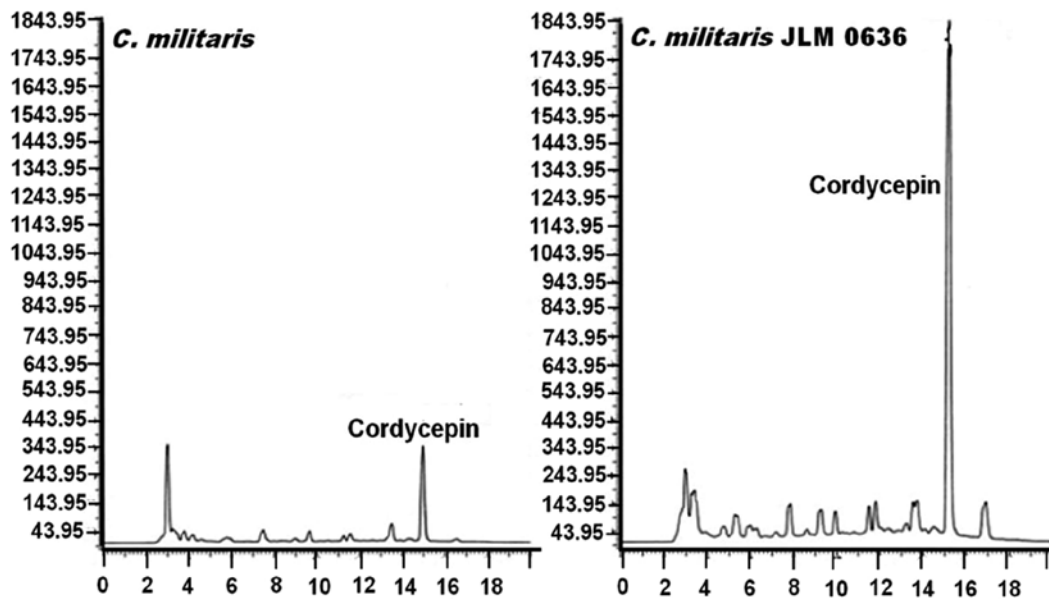


Figure 1. HPLC chromatogram of hot water extracts from wild-type *C. militaris* and *C. militaris* JLM 0636 strain.

stained with appropriately diluted labeled rat anti-mouse antibodies [anti-CD4 FITC and anti-CD8 PE (BD Biosciences Pharmingen); anti-CD25 PERCP-Cy5.5 (eBioscience) and isotype controls (BD Biosciences Pharmingen)] at 0.1-0.5 $\mu\text{g}/\text{ml}$ for 40 min on ice. For the identification of intracellular IFN- γ expression in CD8⁺ T cells, the freshly prepared cells were stimulated with PMA (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma) for 2 h and further incubated with protein transport inhibitor brefeldin A (10 $\mu\text{g}/\text{ml}$; Sigma) for 2 h to accumulate for intracellular cytokines. At the end of the incubation, Fc Block (BD Biosciences Pharmingen) was added for 15 min at 4°C, and standard surface staining procedures were performed with anti-CD8 PE. The cells were then fixed, permeabilized and stained with anti-IFN- γ FITC (BD Biosciences Pharmingen). Following staining, cells were washed and analyzed immediately with an FC500 flow cytometer (BD FACSAria; BD Biosciences). Two color flow cytometric analyses were performed and data represent 50,000 events unless otherwise noted. The phenotype of Treg cells (CD4⁺CD25⁺FoxP3⁺) was evaluated by three-color fluorescence flow cytometric analysis following standard surface staining procedures combined with intracellular FoxP3 staining method. Intracellular detection of FoxP3 was performed by using PE anti-mouse FoxP3 staining buffer set purchased from eBioscience. Briefly, the cells stained with anti-CD4-FITC and anti-CD25-PERCP-Cy5.5 were washed, fixed and permeabilized according to the manufacturer's instructions, and then incubated with PE-conjugated rat anti-mouse FoxP3 Ab for 40 min on ice.

Statistical analysis. All statistical analyses were performed using commercially available statistical software (SPSS, Inc., Chicago, IL, USA). Results are expressed as means \pm standard deviation (SD), and numerical data were analyzed by Student's t-test or one-way analysis of variance (ANOVA). For all analyses, a difference was considered statistically significant at $P < 0.05$.

Results

Induction of immunomodulation by cordycepin-enriched *C. militaris*. For the determination of cordycepin content, hot water extract was prepared and subjected to HPLC analysis (Fig. 1). The concentration of cordycepin contained in *C. militaris* JLM 0636 strain (7.42 mg/g dry weight) was 7-fold higher than that of wild-type *C. militaris* (1.21 mg/g dry weight), which was calculated from the peak area shown in the standard curve of commercial cordycepin. To examine whether *C. militaris* could lead modulation of tumor-derived T cells, ConA-stimulated lymphocyte proliferative response was tested by BrdU incorporation assay. The ConA response of splenocytes from tumor-bearing mice was generally lower than that from normal mice and was not significantly altered by the feeding of *C. militaris* (Fig. 2). Having demonstrated the presence of cytokines for the development of effector T cells and Treg cells, the cultured supernatants of ConA-stimulated lymphocytes were analyzed for the production of IL-2, IL-4 and TGF- β . Tumor-bearing mice fed with *C. militaris* showed cordycepin-dependent decrease in IL-2 and TGF- β production and increase in IL-4 production (Fig. 2), which indicated that *C. militaris* may induce changes in the subpopulations of tumor-derived T lymphocytes. Thus, immunofluorescent analysis for cell surface markers using flow cytometric analysis was performed to investigate T lymphocyte subpopulations. The average frequencies of CD4⁺ T cells among total splenocytes from tumor-bearing mice groups were generally lower than the normal mice group and were not significantly altered by the differences in the feeding conditions (data not shown). The frequency of CD8⁺ T cells from the control tumor-bearing mice group was also decreased compared with the normal mice group; however, *C. militaris* feeding prominently recovered the frequencies of CD8⁺ T cells particularly in the cordycepin-enriched *C. militaris* JLM 0636 fed group (Fig. 4).

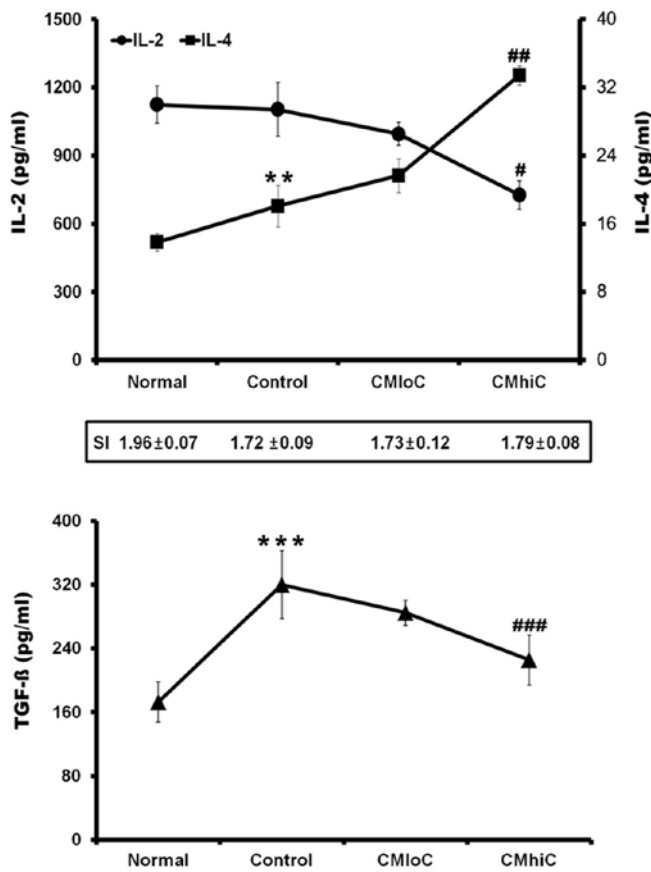


Figure 2. Cordycepin-enriched *C. militaris* induces modulation of tumor-derived T cells. C3H/He female mice were inoculated s.c. with 2×10^6 FM3A tumor cells in $50 \mu\text{l}$ saline. Splenocytes were obtained at 30 days after inoculation of tumor cells and cultured with ConA-stimulation ($5 \mu\text{g}/\text{ml}$). The concentrations of IL-2, IL-4 and TGF- β were analyzed in the supernatants which were cultured for 24 h at 10^7 cells/ml. Stimulation index (SI) mean ratio of ConA-stimulated/spontaneous proliferation determined by BrdU incorporation assay using cell suspension at 5×10^5 cells/well. Data are presented as means \pm SD of 5 mice/group. ****P<0.05 as compared with the tumor-free normal mice. #####P<0.05 as compared with the control tumor-bearing mice.

Suppression of Treg cells by cordycepin-enriched C. militaris.

To investigate the effect of *C. militaris* on Treg cell population, CD4⁺CD25⁺ cell proportions were determined by flow cytometric analysis. Based on the changes in the average frequency of CD4⁺ T cells between the normal and tumor-bearing mice groups, comparison of CD4⁺CD25⁺ to CD4⁺ cell ratio was better to discriminate the differences between groups. The higher ratio of the control tumor-bearing mice group compared to the normal mice group was significantly decreased in the cordycepin-enriched *C. militaris* JLM 0636 fed group (Fig. 3). The expression of a specific Treg cell marker, FoxP3, was further investigated to confirm that the reduced CD4⁺CD25⁺ cells were true Treg cells. The proportions of FoxP3⁺ cells among CD4⁺CD25⁺ cells showed similar but more prominent changes between the experimental groups (Fig. 3). On the other hand, FoxP3⁺ cells among CD4⁺CD25⁻ cells were almost unchanged at minimal level (data not shown).

Enhancement of IFN-γ-expressing CD8 T cells by cordycepin-enriched C. militaris. To investigate whether *C. militaris* could

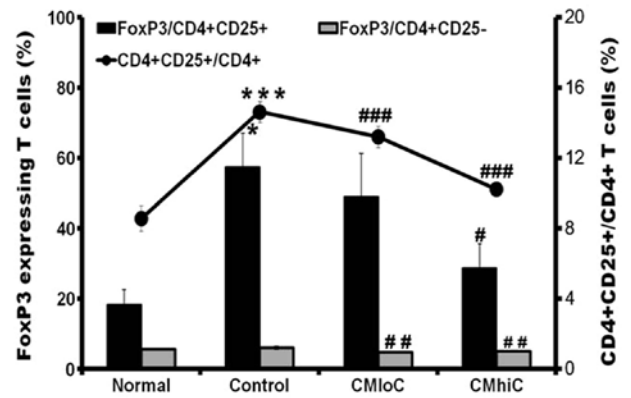


Figure 3. Cordycepin-enriched *C. militaris* suppresses Treg cells in tumor-bearing mice. C3H/He female mice were inoculated s.c. with 2×10^6 FM3A tumor cells in $50 \mu\text{l}$ saline. Splenocytes were obtained at 30 days after inoculation of tumor cells. The cells were analyzed by flow cytometry for the expressions of CD4, CD25 and intracellular FoxP3 and expressed as percent gated cells of CD4⁺CD25⁺ cells among CD4⁺ T cells and FoxP3 expressing cells within CD4⁺CD25⁺ cells. Data are reported as the means \pm SD of 5 mice/group. ****P<0.05 as compared with the tumor-free normal mice. #####P<0.05 as compared with the control tumor-bearing mice.

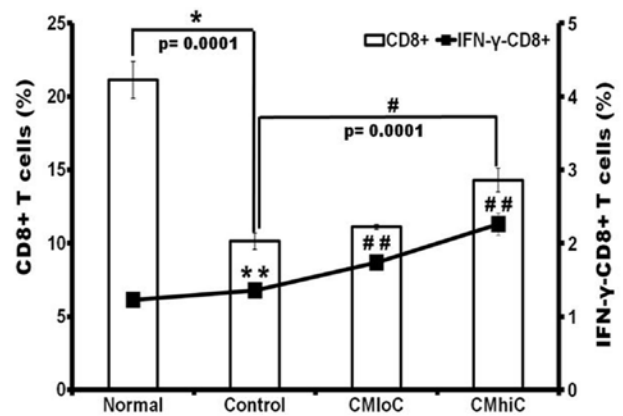


Figure 4. Cordycepin-enriched *C. militaris* enhances IFN- γ -expressing CD8 T cells in tumor-bearing mice. C3H/He female mice were inoculated s.c. with 2×10^6 FM3A tumor cells in $50 \mu\text{l}$ saline. Splenocytes were obtained at 30 days after inoculation of tumor cells. The cells were analyzed by flow cytometry for the expressions of CD8 and intracellular IFN- γ and expressed as percent gated cells of CD8⁺ cells and IFN- γ -expressing CD8⁺ T cells. Data are reported as the means \pm SD of 5 mice/group. Student's t-test P<0.05 for tumor-free normal mice vs. control (* and **) and Dunnett's multiple comparison posttest P<0.05 for control vs. CMloC or CMhiC (# and ##).

lead to enhanced effector T cells involved in antitumor immunity in tumor-bearing mice, IFN- γ -expressing CD8⁺ T cells were examined by flow cytometric analysis. The number and percentage of IFN- γ -expressing CD8⁺ T cells in tumor-bearing mice were significantly increased in the experimental groups fed with *C. militaris*, compared to those in the normal mice (Fig. 4). The degree of increase was much more pronounced in the mice group fed with the *C. militaris* JLM 0636 strain, which was in accordance with the recovery of the frequencies of CD8⁺ T cells. This result indicates that *C. militaris* enhanced IFN- γ secretion and effector T cell function, which may suggest unfavorable microenvironmental change to tumor and induction of effective antitumor immunity.

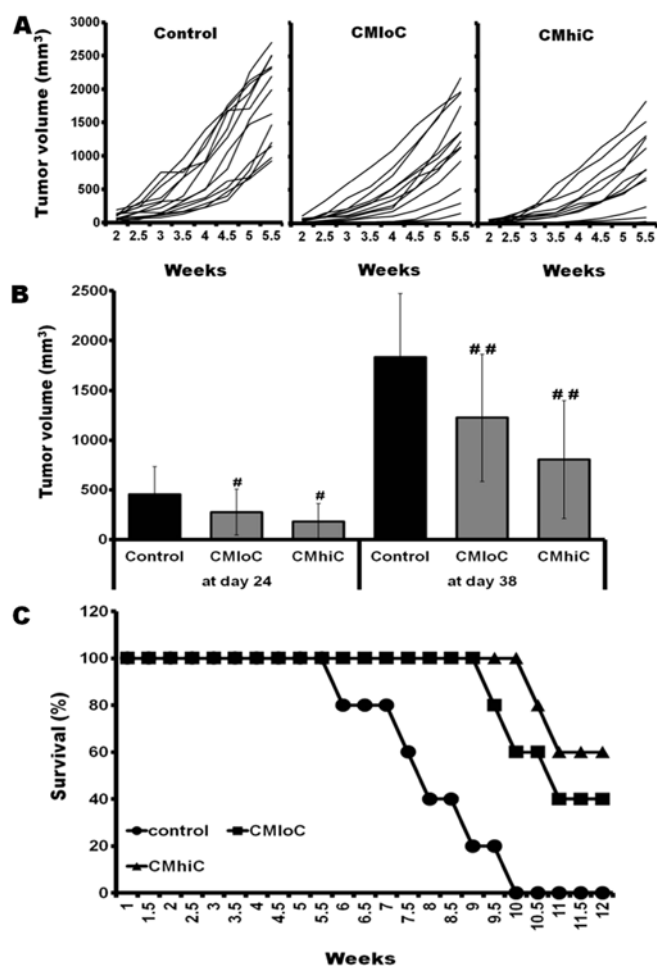


Figure 5. Antitumor activity of *C. militaris*. C3H/He mice of each experimental group were inoculated subcutaneously with FM3A cells (2×10^6 cells/ $50 \mu\text{l}$ saline) on the right flank. (A) Tumor size was assessed once a week, and data are reported as tumor growth curve of individual mice in the relevant group. (B) Tumor growth delay compared with control was significantly increased by *C. militaris* powder and water extract contained diet at day 38. Data are reported as the means \pm SD of 5 mice/group. Dunnett's multiple comparison posttest $P < 0.05$ for control vs. CMloC or CMhiC (# and ##). (C) Survival rate was recorded as the percentage of surviving mice in each experimental group.

Tumor growth delay by cordycepin-enriched *C. militaris*. To determine the ability of *C. militaris* to induce a protective antitumor activity *in vivo*, mice were fed systematically with *C. militaris* powder and water extract contained diet during which mice were challenged subcutaneously with FM3A murine breast cancer cells. When mice were fed with *C. militaris*, tumor formations were delayed and the survival rates were prolonged compared with control mice (Fig. 5). The mice group fed with *C. militaris* JLM 0636 strain showed much slower tumor growth and survived for longer time periods than the mice group fed with wild-type *C. militaris* (Fig. 5). *C. militaris* at the dose used was not toxic to the animals as we observed no differences in body weight gain, food intake, water consumption and behaviors between experimental groups until visible tumor formation. Although these parameters of tumor-bearing mice were gradually decreased with the lapse of time compared with the normal mice, there were no significant differences between control and *C. militaris*-fed mice (data not shown).

Discussion

There has been considerable interest in cordycepin as a potential cancer chemotherapeutic agent. Due to the absence of oxygen in the 30 position of its ribose moiety, the incorporation of cordycepin during RNA synthesis results in termination of chain elongation. This activity has been well described *in vitro* with purified RNA polymerases and poly (A) polymerases from a number of organisms, including yeast and mammals (21,22). However, cordycepin is not a potent cytotoxic compound when compared to other chemotherapeutic drugs, and the chemical compounds including cordycepin contained in *C. militaris* have been known to mediate unique properties related to the immunity. As tumor growth can be controlled by destroying cancer cells as well as by stimulating antitumor immunity, it is necessary to explore the role of *C. militaris* in immune response underlying antitumor activity. For this purpose, we constructed *C. militaris* JLM 0636 strain with 7-fold higher cordycepin content than wild-type *C. militaris* and fed each containing food and water to mice inoculated with FM3A tumor cells. The results presented in the present study showed that the feeding of cordycepin-enriched *C. militaris* JLM 0636 strain for 30 days had a considerable effect on *in vivo* growth and survival rate of an FM3A breast tumor in CH3/He mice (Fig. 5), which demonstrated that it may be one of the most appropriate candidate antitumor substances.

The antitumor effect can be explained to the end-result of several interactions involving cytokines and immune cells, provided by the stimulatory or suppressive effects on the antitumor immunity (23). The lack of an effective antitumor immune response in most cancer patients with advanced disease would simply depend on the prevalence of immunosuppressive mechanisms with respect to the immunostimulatory ones. The suppression of the antitumor immune response is mediated by several cytokines, and the various endogenous suppressive factors would exert their inhibitory immune effect through a common end-mechanism, consisting of the generation of a subtype of T helper lymphocytes (Treg cell), which at present seems to constitute the main mechanism responsible for cancer-related immunosuppressive status (24).

In the present study, ConA-stimulated lymphocyte proliferative response of splenocytes from tumor-bearing mice, which was lower than that of normal mice, was unaltered by the feeding of *C. militaris* (Fig. 2). However, it is noteworthy that IL-2, IL-4 and TGF- β secretion of ConA-stimulated lymphocytes from tumor-bearing mice was modulated in a cordycepin-dependent manner by the feeding of *C. militaris* (Fig. 2). *C. militaris* also induced changes in the subpopulations of tumor-derived T lymphocytes. The average frequency of CD4⁺ T cell population among total splenocytes from tumor-bearing mice, which was lower than that from normal mice, was not significantly altered by the feeding of *C. militaris*. However, CD4⁺CD25⁺ cell population from the tumor-bearing mice, which was higher than that in the normal mice, exhibited a cordycepin-dependent decrease, and this decreasing trend in the CD4⁺CD25⁺ cell population became more definite when it was illustrated in terms of the CD4⁺CD25⁺ to CD4⁺ cell ratio (Fig. 3). The regulatory subpopulation among CD4⁺ cells constitutively expressing high levels of CD25 (Treg cells) is one of the most potent and well-studied suppressive

phenotypes found in the tumor microenvironment. Indeed, IL-2, IL-4 and TGF- β are essential for naturally occurring Treg cell homeostasis and activation. Treg cells are refractory to TCR-induced proliferation (25,26) and depend on IL-2 for survival (27,28). The signaling via the high-affinity IL-2R complex, in combination with TCR engagement, is essential for Treg cell proliferation as well as the acquisition of their potent suppressive function (28). IL-2 leads to phosphorylation and activation of STAT5 and, consequently, binding to the FoxP3 promoter, resulting in enhanced FoxP3 expression (29). TGF- β induces CD4⁺CD25⁺ T-cell proliferation (30,31) and promotes the differentiation of CD4⁺CD25⁺ Treg cells as a costimulator of FoxP3 expression (32). By contrast, IL-4 inhibits TGF- β -induced Foxp3 expression and thus suppresses the new generation of Foxp3⁺ Treg cells (33). It may be assumed that *C. militaris* inhibited IL-2 and TGF- β secretion and promoted IL-4 secretion of tumor-derived T lymphocytes, which downregulated CD25⁺-expressing CD4⁺ T cells more sensitively. As the transcriptional factor FoxP3 is crucial in the developmental and functional factors expressed in Treg cells and is regarded as a useful intracellular Treg cell marker, we investigated the expression of FoxP3 to more clearly define Treg cells. FoxP3 was expressed mainly in the CD4⁺CD25⁺ cell population and was extensively increased in cells from tumor-bearing mice. Moreover, CD4⁺CD25⁺FoxP3⁺ cells were decreased significantly after the feeding of *C. militaris* in a cordycepin-dependent manner (Fig. 3).

Tumor-infiltrating lymphocytes are seen as a reflection of a tumor-related immune response and are recognized as the principal effectors of a local antitumor immune response, particularly IFN- γ -producing CD8⁺ T cells (cytotoxic T lymphocytes; CTL) which kill tumor cells and impede tumor growth (14). As shown in the results of our present study, the average frequency of CD8⁺ T cell population among total splenocytes from the tumor-bearing mice, which was lower than that from normal mice, was significantly increased by the feeding of *C. militaris*. The IFN- γ -producing CD8⁺ T cell population from tumor-bearing mice also showed a cordycepin-dependent increase (Fig. 4). IFN- γ plays a key role in tumor surveillance and immunoediting and block TGF- β -mediated Treg cell differentiation (34), while Treg cells suppress tumor specific CD8⁺ T cell cytotoxicity through TGF- β signals *in vivo* (35). Although the exact mechanism of enhanced IFN- γ expression in CD8⁺ cells by the feeding of *C. militaris* remains unclear, it may be related, in part, to *C. militaris*-induced Treg cell depletion in tumor-bearing mice.

In conclusion, the present study revealed that cordycepin-enriched *C. militaris* effectively suppressed the Treg cell population and enhanced IFN- γ production of CD8⁺ T cells in FM3A breast cancer cell-bearing C3H/He mice. Furthermore, another advantage of cordycepin-enriched *C. militaris* involves the fact that the CD4⁺ cell pool did not decrease, which may include tumor-specific CD4⁺ cells involved in antitumor immunity. Based on the results of our present and previous studies, cordycepin-enriched *C. militaris* itself could be applied as an adequate principal therapeutic agent, facilitating the immune system against pre-established cancer and inducing apoptosis of cancer cells. As it is an ideal conditioning strategy to make the peritumoral microenvironment unfavorable to tumor merely by manipulating Treg cells without harming the host

tissue, cordycepin-enriched *C. militaris* may be applied as an adequate therapeutic agent against the tumor, facilitating antitumor immunity via Treg cell depletion and CTL enhancement. Furthermore, cordycepin-enriched *C. militaris*-induced antitumor immunity may play a factor via a bystander effect in eradicating chemotherapy or radiotherapy-resistant cancer cells and may synergistically act when treated with dendritic cell vaccine.

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