Investigation of the immunomodulatory activity of *Tricholoma matsutake* mycelium in cyclophosphamide-induced immunosuppressed mice

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Abstract. Tricholoma matsutake, a popular food and biopharmaceutical resource in Asia, possesses various pharmacological activities. Although T. matsutake mycelium (TM) may enhance immunity, previous studies, to the best of our knowledge, have been performed on normal animals or cells alone. The present study aimed to evaluate the immunomodulatory activity of TM at doses of 0.3, 1.0 and 2.0 g/kg in cyclophosphamide (CTX)-induced immunosuppressed mouse models. TM treatment for 2 weeks markedly improved the gain in bodyweight, increased organ indices, reduced hind paw swelling and positively regulated the cytotoxicity of natural killer cells and the proliferation of lymphocytes. These effects are similar to that of thymosin $\alpha 1$ (0.16 mg/kg) which served as the positive control. In CTX-induced immunosuppressed mice, TM demonstrated marked effects on the modulation of the production of immunoglobulin (Ig)G and IgA, and the levels of interleukin-2, 6, 10 and 12, interferon- α and γ and tumor necrosis factor- α in serum. Compared with CTX mice, the reduced activity of nuclear factor (NF)-KB in serum and spleen, and phosphorylation of inhibitor of NF- κ B kinase α/β in spleen were observed in TM-treated mice. Taken together, TM effectively improved immune function in immunosuppressed mice via modulation of ILs and inflammatory factors associated with the NF-kB signaling pathway.

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Introduction

Immunosuppression is observed in various pathophysiological conditions. The central role of the immune system is to protect the body and it comprises two parts; innate and adaptive immunity (1). During the immune response, intracellular signal transduction is responsible for modulating the production of pro-inflammatory and anti-inflammatory cytokines and other molecules. Nuclear factor (NF)- κ B is important for the immune response via the mediation of the transcription and production of interleukins (2). Due to the fact that NF- κ B signaling dictates the levels of cytokines, chemokines and other immune mediators, agents targeting NF- κ B-associated pathways may produce immunomodulatory effects (3,4).

Immunotoxicants are recognized as external factors that cause significant alterations in immune mechanisms in humans and other animals. Cyclophosphamide (CTX), used in chemotherapy for patients with cancer, causes immuno-suppression following a chronic treatment (5), and may also result in senescence by upsetting the oxidation-antioxidation equilibrium. All these effects of CTX may be associated with its modulation of NF- κ B activation (6).

A previous study was conducted to search for immunomodulatory agents in immunosuppressed and immunodeficient animal models (7). However, the drugs currently used in clinics, particularly single-component chemicals, are unilateral and exhibit various adverse effects including general malaise and neurotoxicity (8,9). Furthermore, single-component immunomodulatory drugs may modulate a target that does not meet the requirements of an immunosuppressive patient.

Natural products have become a focus in the search for efficient alternative agents. A previous study demonstrated that the *Cordyceps militaris* fruit body ameliorated membranous glomerulonephritis via the attenuation of oxidative stress and renal inflammation via the NF- κ B pathway (10). *Tricholoma matsutake*, a popular food and biopharmaceutical resource in Asia (11), possesses various pharmacological activities including immunomodulatory, antitumor and antioxidant properties (12,13). *T. matsutake* significantly enhances the immunity of normal mice (14). A study

performed in RAW264.7 cells identified that β -glucan derived from *T. matsutake* upregulated the phenotypic functions including cytokine expression levels via the modulation of multiple signaling pathways linked to NF- κ B activation (15). However, to the best of our knowledge, no studies have been performed on CTX-induced immunosuppressed mouse models to confirm the effects of *T. matsutake* mycelium (TM) obtained via submerged fermentation.

In the present study, the immunomodulatory and immuno-enhancing effects of TM were detected in a CTX-induced BALB/c mice model. The serum levels of immune factors and cytokines were determined via ELISA. To further analyze its underlying mechanisms, the expression levels of proteins associated with the NF- κ B pathway were detected using western blot analysis.

Materials and methods

TM preparation. TM was obtained by submerged fermentation (16) and administered directly to experimental mice.

Immunosuppressed mouse model establishment and drug treatment. A total of 72 six-week BALB/c mice (18-22 g; 1:1 male:female; SCXK (JI)-2014-0003; Changchun Yisi Experimental Animal Technology Co., Ltd., Changchun, China) were housed at 20°C with a 12 h light/dark cycle and fed autoclaved standard chow and water *ad libitum*.

The experimental protocol was approved by the Animal Ethics Committee of Jilin University (Guangdong, China). A total of 60 mice were intraperitoneally injected with 80 mg/kg CTX for 3 days. Following confirmation of immunosuppression, the CTX-injected mice were separated randomly into 5 groups (n=12), and treated with saline (CTX mice) and 0.3, 1.0 and 2.0 g/kg TM (TM + CTX mice) orally once a day for 2 weeks. The positive control mice were intraperitoneally injected with 0.16 mg/kg thymosin $\alpha 1$ (T $\alpha 1$ + CTX group) twice a week for 2 weeks. Another 12 mice were treated with normal saline during the whole experimental process and served as control (CTRL). All mice were weighed at day 0, 3, 10 and 17, and sacrificed at the end of the experiment by injection with 200 mg/kg pentobarbital. The spleen and thymus were extracted and weighed, and the organ index calculated.

Delayed type hypersensitivity (DTH) test. Following 2 week's treatment, mice were sensitized with 0.2 ml of 2% (v/v) sheep red blood cells (SRBC; Yuanye Bio-Technology Co., Ltd., Shanghai, China) for 4 days via intraperitoneal injection, and then the thickness of hind paw was measured using a Vernier caliper. Previously sensitized mice were again challenged with 20 μ l 20% (v/v) SRBC which was injected into the measured hind paw. The thickness of the hind paw was recorded at 24 and 48 h. The swelling of the hind paw was calculated as the different thickness prior to and following 20% SRBC injection.

Cytotoxic activity assay of natural killer (NK) cells. Spleen cell suspension (1x10⁶; 100 μ l) from each mouse, was produced as in a previous study (17), was seeded into a 96-well cell culture plate with 1x10⁵ of YAC-1 cells (cat. no. TCM28; Type Culture Collection of the Chinese Academy of Sciences,

Shanghai, China) (effector-to-target ratio 10:1). For a control group, 100 μ l RPMI-1640 medium was used in place of the spleen cell suspension. The cells were incubated under a humidified atmosphere containing 5/95% CO₂/air at 37°C for 4 h. Following centrifugation at 150 x g for 10 min at room temperature, the lactate dehydrogenase concentration in medium was detected using an *in vitro* Toxicology Assay kit (cat. no. TOX1; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocols. The killing rate of NK cells vs. YAC-1 cells was calculated as:

Killing rates
$$(\%) = \left(1 - \frac{(OD_{e+t} - OD_e)}{OD_t}\right) \times 100\%$$

Where OD is optical density; OD_{e+t} is the average OD_{490} of six wells for NK and YAC-1 cells; OD_e is the average OD_{490} of six wells for NK effector control; and OD_t is the average OD_{490} of six wells for YAC-1 target control (18).

Lymphocyte proliferation assay. Spleen cells $(1x10^6/100 \ \mu l)$ from each mouse were seeded into a 96-well plate and mixed with 10 μ l RPMI1640 (control group) or 10 μ l 200 μ g/ml concanavalin (ConA; Yuanye Bio-Technology Co., Ltd.), and then incubated under a humidified atmosphere containing 5%/95% CO₂/air at 37°C for 48 h. As in a previous study (19), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich; Merck KGaA) analysis was performed to detect cell viability. The stimulation index (SI) of T lymphocyte transformation was calculated as: SI=OD_{test}/OD_{control}.

Where OD_{test} is the average OD_{490} of three wells for T lymphocytes and ConA and $OD_{control}$ is the average OD_{490} of three wells for T lymphocytes only.

ELISA assay. Following the last treatment, blood was sampled from the caudal vein and the serum levels of immunoglobulin (Ig)G (cat. no. 40157) and IgA (cat. no. 40156), cytokines interleukin (IL)-2 (cat. no. 42903), IL-6 (cat. no. 42899), IL-10 (cat. no. 42912) and 12 (cat. no. 42910), interferon (IFN)-α (cat. no. 43102) and IFN-γ (cat. no. 42918), tumor necrosis factor (TNF)-α (cat. no. 42868)] and NF-κB (cat. no. 43059) were investigated via ELISA using commercial kits (Yuanye Bio-Technology Co., Ltd) according to the manufacturer's protocol.

Western blot analysis. Collected spleen samples were homogenized with RIPA buffer containing protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (all from Sigma-Aldrich; Merck KGaA). Following determination of the total protein concentration, 30 μ g protein was separated with 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (0.45 μ m, Bio Basic, Inc., Markham, ON, Canada). The transferred membrane was blocked through being incubated with 5% bull serum albumin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 3 h at room temperature, and then incubated with primary antibodies phosphorylated (P)-inhibitor of NF- κ B kinase (IKK) α/β (ab195907; Abcam, Cambridge, UK), total (T)-IKK α/β (ab178870; Abcam) and glyceraldehyde-3-phosphate dehydrogenase (ab8245; Abcam) all at 1:1,000 dilution at 4°C overnight. The membranes were then treated with horseradish peroxidase-conjugated goat anti-rabbit secondary

Characteristic	CTX, mg/kg		TM, g/kg		Tal, mg/kg	
	CTRL	80	0.3	1.0	2.0	0.16
Body weight, g						
Day 0	22.3±0.7	22.2±0.6	22.2±0.5	21.7±0.5	21.8±0.5	21.7±0.5
Day 3	22.6±0.5	19.1±0.7ª	19.2±0.6	19.1±0.7	19.5±0.7	19.3±0.8
Day 10	23.6±1.7	18.4 ± 2.2^{a}	18.7±2.1	20.4 ± 2.1^{d}	19.8±1.6	20.9 ± 1.3^{d}
Day 17	24.3 ± 2.1	20.3±2.3ª	21.8±1.3	22.1 ± 1.3^{d}	22.3 ± 2.0^{d}	23.7 ± 1.4^{d}
Organ index, mg/10 g						
Spleen	1.13±0.08	0.62 ± 0.05^{b}	0.99±0.08°	0.89±0.05 ^e	1.04±0.07 ^e	1.27 ± 0.06^{f}
Thymus	6.1±0.3	4.9 ± 0.2^{b}	5.7 ± 0.3^{d}	8.2 ± 0.4^{f}	7.9±0.6 ^e	6.9±0.4 ^e
Swelling of hind paw, mm						
24 h	0.48±0.02	0.62±0.03 ^b	0.59±0.03	0.58±0.03	0.53 ± 0.02^{d}	0.52 ± 0.03^{d}
48 h	0.29±0.02	0.51±0.02°	0.43 ± 0.01^{d}	0.37±0.03 ^e	0.39±0.03 ^e	0.34 ± 0.02^{f}

Table I. Regulatory effects of Ta1 and TM on body weight, organ indexes and paw swelling.

Bodyweight changes were monitored during the whole experimental period. Following 2-week TM and T α 1 treatment, spleen and thymus indices were examined. The delayed type hypersensitivity test was performed, and swelling of hind paw was determined following 24 h and 48 h 20% sheep red blood cells injection. Data are expressed as the mean ± standard error (n=12). *P<0.05, *P<0.01 and *P<0.001 vs. CTRL group, dP<0.05, *P<0.01 and *P<0.001 vs. model group. TM, *Tricholoma matsutake* mycelium; T α 1, thymosin α 1; CTX, cyclophosphamide; CTRL, control.

antibody (sc-3836; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted 1:2,000 (IH-0011; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). Bands were visualized with an ECL detector (GE Healthcare Life Sciences, Chalfont, UK) and quantified by ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All values were expressed as the mean \pm standard error. A one-way analysis of variance was used to detect statistical significance followed by Dunn's test using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of TM on bodyweight, organ index and swelling of hind paw. Suppressed growth rate, low organ indexes of thymus and spleen, and a high degree of hind paw swelling were observed in CTX mice (P<0.01; Table I). Comparatively, T α 1 and TM improved gain in body weight, enhanced thymus and spleen indexes, and suppressed the swelling of the hind paw (P<0.05; Table I).

TM enhances the cytotoxicity of NK cells and the proliferation of lymphocytes. The cytotoxicity of NK cells, the first defense against tumor cells, was investigated. Compared with CTX mice, TM enhanced the activity of NK cells $\leq 14.2\%$ (P<0.05; Fig. 1A); meanwhile, Ta1 enhanced the activity of NK cells to 14.8% (P<0.05; Fig. 1A).

The lymphocyte proliferative response is responsible for immune function. Compared with CTX mice, TM and Ta1 $\,$

enhanced the stimulation index by $\sim 20\%$ following 2 weeks of treatment (P<0.05; Fig. 1B).

TM upregulates Ig secretion. Ig is responsible for identification and neutralization of foreign substances (20). CTX injection significantly suppressed IgG and IgA levels, which had been successfully enhanced by 2 weeks of TM treatment (P<0.05; Fig. 2). Unlike TM, T α 1 upregulated only IgA levels in CTX mice (P<0.05; Fig. 2A).

Effects of TM on the secretion of immune and inflammatory factors. IL serves important roles in immune function by mediating the activation and proliferation of immune cells (21). In CTX mice, low levels of IL-2 and IL-12 and high levels of IL-6 and IL-10 were noted (P<0.05; Fig. 3). TM reversed these abnormal changes (P<0.05, Fig. 3). Unlike TM, T α 1 demonstrated no significant effect on IL-10 level (P<0.05, Fig. 3C).

IFN is associated with immunostimulatory and immunomodulatory effects. CTX resulted in a significant reduction on serum levels of IFN- α and IFN- γ , and an increase of TNF- α level (P<0.05; Fig. 4A-C). T α 1 reversed these abnormal changes in immunosuppressed mice with the exception of IFN- α (P<0.05, Fig. 4B and C). Compared with CTX mice, TM enhanced by 55.4 and 20.9% respectively serum levels of IFN- α (P<0.001, Fig. 4A) and IFN- γ (P<0.01, Fig. 4B), and reduced TNF- α level by 15.9% (P<0.05, Fig. 4C).

TM regulated NF-κB signaling pathway. Due to the central function served by NF-κB during inflammation development, the effects of TM on the NF-κB signaling pathway were investigated. TM significantly reduced the activities of NF-κB in serum compared with CTX mice (P<0.05; Fig. 5A). Furthermore, TM strongly reduced the activations of NF-κB



Figure 1. The effects of TM on (A) NK cell activity and (B) ConA-induced T cell proliferation in CTX-induced immunosuppressed mice. Data are expressed as the mean \pm standard error (n=12). ^{*P*}P<0.05 vs. CTRL mice and ^{*}P<0.05 vs. CTX mice. TM, *Tricholoma matsutake* mycelium; NK, natural killer; ConA, concanavalin; CTX, cyclophosphamide; CTRL, control; Ta1, thymosin α 1.



Figure 2. TM enhanced serum levels of (A) IgG and (B) IgA in CTX-induced immunosuppressed mice. Data are expressed as the mean \pm standard error (n=12). [#]P<0.05 vs. CTRL mice and ^{*}P<0.05 vs. CTX mice. TM, *T. matsutake* mycelium; Ig, immunoglobulin; CTX, cyclophosphamide; CTRL, control; Ta1, thymosin a1.



Figure 3. TM regulated serum levels of (A) IL-2, (B) IL-6, (C) IL-10 and (D) IL-12 in CTX-induced immunosuppressed mice. Data are expressed as the mean \pm standard error (n=12). $^{e}P<0.05$ and $^{#i}P<0.01$ vs. CTRL mice, $^{*}P<0.05$ and $^{**}P<0.01$ vs. CTX mice. TM, *Tricholoma matsutake* mycelium; IL, interleukin; CTX, cyclophosphamide; CTRL, control; Ta1, thymosin a1.







Figure 4. TM regulated serum levels of (A) IFN- α , (B) IFN- γ and (C) TNF- α in CTX-induced immunosuppressed mice. Data are expressed as the mean \pm standard error (n=12). *P<0.05 and ***P<0.001 vs. CTRL mice, *P<0.05, **P<0.01 and ***P<0.001 vs. CTX mice. TM, *Tricholoma matsutake* mycelium; IFN, interferon; TNF, tumor necrosis factor; CTX, cyclophosphamide; CTRL, control; T α 1, thymosin α 1.

and IKK α/β in the spleens of CTX-induced immunosuppressed mice (P<0.05; Fig. 5B). Similarly, T α 1 demonstrated the same regulatory effects as TM (P<0.05, Fig. 5B).

Discussion

The investigation of nutritional immunology from food and its components have become an important focus of research (22). Nutritional research focuses on dietary factors to reduce the risk of infection in animal models and in immune-compromised hosts (23). Among functional natural foods, *T. matsutake* is well known due to its various biological activities. Although studies have demonstrated that TM may regulate immunity,

Figure 5. TM demonstrated significant effects on the activities of (A) NF- κ B in serum and (B) NF- κ B and IKK α/β in spleen of CTX-induced immunosuppressed mice. Quantification data of the expression levels of P-NF- κ B and P-IKK α/β were normalized by corresponding total protein levels respectively. Data are expressed as the mean \pm standard error (n=12) and analyzed using a one-way analysis of variance. *P<0.05, **P<0.01 and ***P<0.001 vs. CTRL mice, *P<0.05, **P<0.01 and ***P<0.001 vs. CTX mice. TM, *Tricholoma matsutake* mycelium; NF, nuclear factor; CTX, cyclophosphamide; P-, phosphorylated; IKK, I κ B kinase; CTRL, control; T α 1, thymosin α 1; T-, total.

the experiments were only performed in normal animals or cells (14,15). A previous study identified that TM exerted an anti-fatigue activity via the antioxidant pathway in normal Kunming mice (16). As CTX has been widely used for clinical regimes including modulation of autoimmune response, CTX-induced immunosuppressed mouse/rat models can be used to evaluated candidates with immune regulatory properties (24). TM markedly reversed CTX-induced abnormal changes in body weight and organ indexes, reduced hind paw swelling, and regulated the cytotoxicity of NK cells and the proliferation of lymphocytes. The cytotoxicity of NK cells, the DTH test and T lymphocyte proliferation are considered important assessment criteria of immunity in an animal (25). When an antigen is exposed in humoral immune response, opsonophagocytosis is mediated by IgA and IgG which are evidently enhanced among total Igs (26). In children with recurrent infections, bacterial antigen lysate significantly improved IgG and IgA levels in serum to enhance their immunity (27). In the present study, TM successfully improved immunity in CTX-induced immunosuppressed BALB/c mice as indicated by its regulation of IgA and IgG levels, and other standard criteria of immunity.

The immune response is a multi-step process and associated with various transcription molecules which may target the levels of cytokines. Certain mediators directly influence the immunity of organs. IL serves important roles during immune response by activating immune cells, as IL-2 overexpression activates CD4+ T cells. IL-10 and IL-12, which were regulated by TM in CTX mice, are involved in the proliferation of immune effector cells and contribute to activating B lymphocytes (28). As a pro-inflammatory cytokine, the damaging and inflammatory effects of TNF- α have been clearly demonstrated in neuropathology (29), and may cause overexpression of IL-6 (30). It has been observed that TM enhanced the serum levels of IFN- α and IFN- γ in CTX mice, which may further regulate the secretion of interleukins (31). Based the data of the present and previous studies, TM regulates the levels of IFN-y and IL-12, promoting transformation of T helper (Th)0 cells into Th1. This process forms a positive feedback system of IFN-y secretion, in addition to promoting cytotoxic lymphocyte formation (21). It may further explain the immune regulatory activities of TM during the present study. However, TM contains multi-effective components, which may show 'systemic targets' including regulation of pro-inflammatory and anti-inflammatory factors. These synergistic effects on immunosuppression may suggest fewer adverse effects, which is also an advantage of natural products.

NF-kB is recognized as a central transcription mediator which regulates the generation and amplification of inflammatory cytokines. Based on its pivotal role in the modulation of effector molecules, its activity in serum and the spleen was evaluated and identified as being markedly reduced following TM treatment in CTX mice. The activation of IkB leads to its ubiquitination and proteasomal degradation. IKKα/β impels the ubiquitination of $I\kappa B$ and further facilitates the releases of a subunit of NF-kB from IkB, which allows the subsequent translocation of NF- κ B to the nucleus to bind to target genes (29). Suppressed NF-kB activation significantly reduces the expression levels of pro-inflammatory cytokines (32). As reported recently, daphnetin inhibits pro-inflammatory responses by modulating the IKK/IkB signaling pathway (33). Collectively, NF-KB may contribute to TM-mediated immunomodulatory activity in CTX-induced immunosuppressed model; however, further experiments are required.

TM enhances the immunity of CTX-induced immunosuppressed mice by increasing the function and activation of immune cells, and by promoting the secretion of immunomodulatory molecules, which may be associated with the NF- κ B signaling pathway. Although the present data are insufficient to elucidate the effect of TM on immune-related disease prior to its possible application in the clinic, and further studies are required, the present study confirmed the immunomodulatory effects of TM *in vivo*.

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