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Modulation of T cell response by *Phellinus linteus*

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***Phellinus linteus*, a species of mushroom, has been shown to contribute to health benefits, such as anti-inflammatory activity and immunomodulatory efficacy. The aim of this study was to analyze the most effective constituents of *P. linteus* fermented broths, polysaccharides, and to evaluate their immunoregulatory effects on T cells. Four fermented broths (PL1–4) and the dialyzate medium (MD) were prepared from *P. linteus* mycelia, and the polysaccharide contents of each were analyzed. The *P. linteus* samples were tested for biological activity in the regulation of T cell activation. In T cells, the production of mitogen-induced interleukin (IL)-2 and cell cycle progression were dose-responsively inhibited by PL3 and MD, primarily through cell-cycle arrest in S phase. PL3 broth, which contained large quantities of polysaccharides, significantly decreased the ratio of interferon-gamma (IFN-γ) to interleukin 4 (IL-4) in T cells. Thus, *P. linteus* fermented broths produced additive effects on the regulation of the Th1/Th2 balance and show promise for the development of immunomodulatory therapeutics.**

[**Key words:** Cell cycle; Cytokine; *Phellinus linteus*; Polysaccharide; Th1/Th2 balance]

Traditional Chinese herbal medicines have been used for over a thousand years in East Asia, and have recently attracted much attention in Western countries. Mushrooms, as important ingredients in traditional Chinese medicines, have been shown to provide health benefits, including anti-inflammatory and immunomodulatory activities (1,2). *Phellinus linteus*, a species of mushroom in the family *Hymenochaetaceae* in the class *Basidiomycetes*, is indigenous to tropical South America, Africa, and East Asia, has attracted increasing attention in the last decade (2).

P. linteus has been used for its medicinal effects in the treatment of allergies, diabetes, gastroenteric dysfunction, and hemorrhage (2,3), and it shows the potential for development of anticancer therapy (4,5). In addition to fruiting body extracts of *P. linteus*, polysaccharides have shown to possess anti-oxidative and anti-inflammatory properties (6). Moreover, polysaccharides isolated from *P. linteus* have been demonstrated to inhibit the production of inflammatory cytokines in T helper 1 (Th1) cells and thus to attenuate the progression of autoimmune diabetes in non-obese diabetic mice (7). These findings indicate that *P. linteus* has multiple applications in medicinal treatments.

Dysregulation of cytokine production by Th1 versus Th2 cells and the pro-inflammatory/anti-inflammatory balance may lead to human diseases (8). Our recent study demonstrated that *P. linteus* fermented broths modulated innate immunity by suppressing inflammatory responses in macrophages (9). However, no detailed study has been published to date on the regulation of T cells by *P. linteus* fermented broths, or exploring their relevance for clinical applications. In this study, several *P. linteus* fermented broths were prepared and their biological activity for the regulation of cell cycle progression in T cells was evaluated. Additionally, their regulatory effect on the cytokine production of T cells and the Th1/Th2 balance were investigated.

MATERIALS AND METHODS

***P. linteus* mycelial materials, preparation, and purification** *P. linteus* mycelial fermented broths were prepared by Yushen Biotechnology Co., Ltd (Taichung, Taiwan). Briefly, four different compositions of broths (PL1-4) were prepared to culture *P. linteus* as described by our previous study (9). The mycelia were incubated in broth (pH5.2) at 28°C for 10 days with 125 rpm continues shaking. The cultured broths were prepared by filtrations. The dialyzate medium (MD) which was prepared from PL1 culture broth and dialyzed against de-ionized water at 4°C for 72 h by using dialysis tubing with a molecular weight cutoff of 12,000–14,000. The broth filtrates and dialyzate were lyophilized for experimental use.

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TABLE 1. Contents of polysaccharides in *Phellinus linteus* fermented broths.

Tested sample[a]	Polysaccharides (mg/L)
PL1	103
PL2	82
PL3	172
PL4	115
MD	98

[a] The preparation of *P. linteus* fermented broths (PL1-4) and dialysate medium (MD) were described in Materials and methods.

Determination of polysaccharides in culture broths To determine the concentrations of polysaccharides in the samples, the broth filtrates and dialyzate were precipitated in four volumes of 95% (v/v) ethanol and incubated at 4°C for 24 h, then centrifuge at 13,000 ×*g* for 15 min. The pellet was suspended in 1 M NaOH at 60°C for 1 h, and total polysaccharide was then determined by phenol–sulfuric acid method (10).

Cell culture Murine lymphoma cell line, EL-4 cells (TIB-39) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), and incubated at 37°C in 5% CO_2 humidified air atmosphere.

Cytotoxicity assay The mitochondrial respiration-dependent MTT assay was used to determine the cytotoxicity of *P. linteus* fermented extracts on EL-4 cells. MTT (0.1 mg in PBS) was added into each well and incubated at 37°C for 3 h. The MTT formazan crystals [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] were dissolved in 100 μl DMSO, and the mean OD of each well was used for assessing the cell viability and expressed as percentage of control.

Analysis of cell cycle EL-4 cells were cultured with the assigned concentrations of *P. linteus* fermented extracts for 24 h. The cells were washed with PBS and fixed in 75% alcohol at −20°C for 2 h. The prepared cells were stained with 20 μg/μl propidium iodide containing 0.1% Triton X-100 and 0.1 mM EDTA for 30 min at 4°C, and then analyzed by a FACS Calibur cytometer (Becton–Dickinson, San Jose, CA, USA) as described previously (11). The data were analyzed using Cell Quest software WinMDI (Verity Software House, Topsham, ME, USA).

Determination of cytokine secretions EL-4 cells were cultured with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) or various concentrations of *P. linteus* fermented extracts for 24 h. The culture media were centrifuged at 13,000 ×*g* for 10 min, and culture supernatant were collected for further analysis. The levels of IL-2, IL-4, and INF-γ in supernatants were then analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instruction.

Reverse transcription and real-time quantitative PCR Total RNA from EL-4 cells was isolated by using TRIzol (Invitrogen, Carlsbad, CA, USA), as described previously with a slight modification (12). Briefly, total RNA (1 μg) was reverse-transcribed into cDNA by using oligo(dT) primers and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). The RNA expression levels of T-bet and GATA-3 were determined by reverse transcription quantitative PCR (RT-qPCR), using StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers used corresponded to T-bet (forward: 5′-TGCCAGGGAACCGCTTATAG-3′; and reverse: 5′-AACTTCCTGGCGCATCCA-3′), GATA-3 (forward: 5′-GGTGGACGTACTTTTTAACATCGA-3′; and reverse: 5′-CGTAGCCCTGACGGAGTTTC-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH: forward, 5′-ATGTGTCCGTCGTGGATCTGA-3′; and reverse: 5′-CCTGCTTCACCACCTTCTTGA-3′) were synthesized by Invitrogen. After pre-incubation at 95°C for 20 s, PCR was performed with 40 cycles of 95°C for 18 s and 60°C for 30 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (denoted as [C_T]).

Statistical analysis All data are presented as mean ± standard deviation of triplicate experiments. Statistical significance analysis was performed using Student's *t*-test; a *P* value <0.05 was considered significant.

RESULTS

Suppression of mitogen-induced cell cycle progression in T cells by *P. linteus* *P. linteus* fermented broths were prepared and characterized as described in our previous study (9). The polysaccharide contents of each *P. linteus* fermented broth were analyzed. The PL1, PL2, PL3, PL4, and MD samples contained 82 mg/L, 103 mg/L, 172 mg/L, 115 mg/L, and 98 mg/L of polysaccharides, respectively (Table 1).

We then analyzed whether *P. linteus* fermented broths have cytotoxic effects on T cells. Cell viability testing was performed after

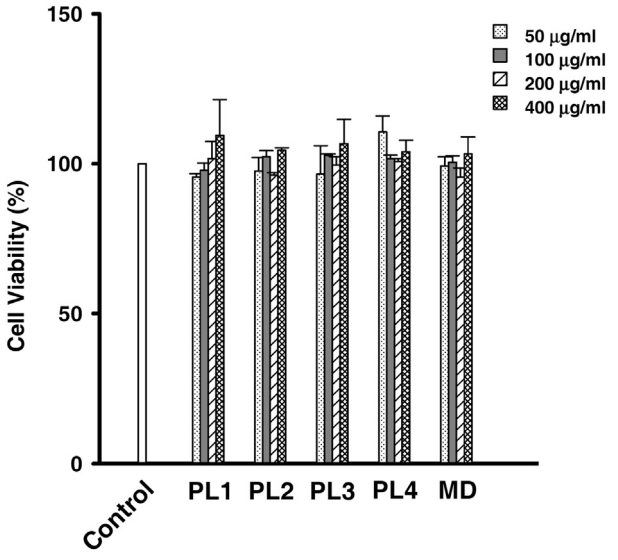

FIG. 1. *Phellinus linteus* fermented broths did not influence the proliferation of T cells. EL-4 cells were incubated with *P. linteus* fermented broths at the indicated concentrations (50, 100, 200, 400 μg/ml) for 24 h. The culture supernatants were collected and the effect of each tested sample on the proliferation of EL-4 cells was assessed by MTT assay. The data showed that there was no loss of cell viability in EL-4 cells during the incubation period. The results are shown as means ± standard deviations for three independent experiments.

EL-4 cells were incubated with various concentrations (50–400 μg/ml) of *P. linteus* fermented broths for 24 h. As shown in Fig. 1, the *P. linteus* fermented broths showed no cytotoxic effects on the cells. Therefore, a maximal concentration (400 μg/ml) of *P. linteus* fermented broth was used to perform the following experiments.

To analyze whether the growth inhibition of T cells in response to *P. linteus* fermented broths was associated with inhibition of cell activation, cell proliferation and cell cycle progression were analyzed in EL-4 cells following treatment with phorbol 12-myristate 13-acetate (PMA), a potent mitogen that activates T cells (13). As shown in Supplementary Fig. S1, following treatment of cells with PMA, cell proliferation increased. However, in cells treated with PL2, PL3, PL4, and MD, PMA-induced cell proliferation was significantly inhibited. We then investigated whether *P. linteus* fermented broths affect cell cycle progression. In the absence of treatment with PMA, a large proportion of the cells were found to be in G1 phase (Table 2). With PMA treatment, the proportion of cells in G1 phase was significantly suppressed. Noticeably, PMA-induced cell cycle progression was inhibited by PL3 and MD, mostly through arrest of cells in the S phase.

Inhibitory effects of *P. linteus* on mitogen-induced IL-2 production in T cells To determine whether the modulation of cell cycle progression in T cells by *P. linteus* was associated with cytokine stimulation, the production of IL-2 was measured. As shown in Fig. 2, PMA enhanced IL-2 production in T cells to a

TABLE 2. Attenuation of cell cycle progression in T cells by *Phellinus linteus* fermented broths.

Tested sample	G1	S	G2/M
Control	58.36 ± 1.5	16.18 ± 2.6	24.19 ± 1.6
PMA	43.74 ± 2.9	20.71 ± 1.1	31.30 ± 0.6
PL1	45.49 ± 1.0	19.98 ± 0.5	31.45 ± 0.7
PL2	45.16 ± 1.9	21.98 ± 1.1	30.06 ± 1.5
PL3	45.55 ± 0.1	25.00 ± 0.4[a]	27.37 ± 1.8[a]
PL4	59.48 ± 0.8[a]	17.41 ± 2.1	22.25 ± 1.1[a]
MD	57.60 ± 1.6[a]	23.28 ± 2.2[a]	20.11 ± 3.9[a]

[a] $P < 0.05$ compared with PMA treatment.