

Amelioration of Skewed Th1/Th2 Balance in Tumor-Bearing and Asthma-Induced Mice by Oral Administration of *Agaricus blazei* Extracts

Hiroaki Takimoto, Hanano Kato, Masahiro Kaneko, and
Yoshio Kumazawa

Department of Biosciences, School of Science and Graduate School of Science, Kitasato University, Sagamihara, Kanagawa, Japan

We showed in a previous study that hot-water extracts of *Agaricus blazei* (*Agaricus* extracts) had anti-tumor activity to Meth A fibrosarcoma, but it remains unclear whether the *Agaricus* extracts ameliorate the skewed balance of type-1 T helper (Th1) and type-2 T helper (Th2) cells. We examined whether *Agaricus* extracts effect the skewed Th1/Th2 balance in tumor-bearing and asthma-induced mice. When Meth A-bearing mice were given orally either *Agaricus* extracts or water once a day starting 5 days after tumor implantation, spleen T cells, prepared from tumor-bearing mice treated with *Agaricus* extracts, in response to anti-CD3 monoclonal antibody produced significantly higher levels of interferon γ (IFN- γ) than that of controls. The mRNA expression of IFN- γ -inducing protein 10 and the frequency of CD69⁺ or CD49d⁺ cells, among activated T cells infiltrated into tumors, significantly increased in *Agaricus*-treated mice, compared with those of tumor-controls. In asthma-induced mice, treatment with the *Agaricus* extracts caused significant downregulation of OVA-specific antibody responses of IgG1 and IgE but not of IgG2a, and significantly decreased total cell numbers, levels of interleukin 5, and eosinophil numbers in bronchial alveolar lavage fluids. IFN- γ production by anti-CD3-stimulated spleen cells, obtained from *Agaricus*-treated mice, significantly increased. Our results strongly suggest that oral administration of *Agaricus* extracts ameliorates the Th1/Th2 balance from the Th2-skewed conditions.

Keywords *Agaricus*, Th1/Th2, Asthma, Anti-Tumor, Eosinophils, IgE.

Address correspondence to Dr. Hiroaki Takimoto, Department of Biosciences, School of Science and Graduate School of Science, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan; E-mail: takimoto@sci.kitasato-u.ac.jp

INTRODUCTION

Recent studies have demonstrated that impairment of adoptive immunity usually occurs in patients with advanced cancer and their immunological conditions in terms of balance of type-1 T helper (Th1) and type-2 T helper (Th2) cells skew to the Th2 condition.^(1–5) In a previous study,⁽⁶⁾ we demonstrated that hot-water extracts from the fruiting bodies of the mushroom *Agaricus blazei* Murrill (*Agaricus* extracts) have a potent anti-tumor activity to Meth A fibrosarcoma in mice that received oral administration of the extracts once a day commencing from day 5 after tumor implantation. In addition, interferon γ (IFN- γ)-producing activity of spleen cells, prepared from the *Agaricus*-treated mice and stimulated with anti-CD3 monoclonal antibody (mAb) significantly increased. Since IFN- γ is one of the typical Th1 cytokines, these results suggest that *Agaricus* extracts act as an immunomodulator capable of ameliorating the Th2-skewed balance.

Type-I allergic symptoms, such as allergic rhinitis, asthma and systemic anaphylaxis, occur in individuals who present high levels of IgE to relevant allergens. Th2 cytokines, such as interleukin 4 (IL-4), IL-10, and IL-13, play important roles for driving IgE production via class switching of naïve B cells to IgE B cells.^(7–13) It is well documented that, in type-I allergic patients, secretion of high levels of IL-4 follows increased IgE antibody response, and the Th1/Th2 balance skews to the Th2 conditions.^(14–16) Although the development of drugs capable of normalizing Th1/Th2 imbalance and decreasing IgE antibody production is desirable, unfortunately, the creation of such orally administered medication, with lower side effects, has not yet occurred. In asthma patients, the accumulation of eosinophils in the bronchial region is caused by cytokines, chemokines and lipid mediators which are released by degranulation of mast cells given allergenic stimulation via Fc ϵ RI.⁽¹⁷⁾ In a murine asthma model, we found that *Agaricus* extracts downregulated serum IgE antibody response. In order to clarify why IgE antibody response is suppressed, we therefore investigated the immunomodulating effects of the extracts on production of IFN- γ and IL-4 and infiltration of eosinophils into the bronchial alveolar cavity.

MATERIALS AND METHODS

Mice

Female BALB/c mice, obtained from Clea Japan Inc. (Tokyo, Japan), SLC Inc. (Hamamatsu, Japan) and the animal facility of the Kitasato University School of Science, were used at 7 weeks of age. All mice were housed under specific pathogen free conditions in the animal facility of the Kitasato University School of Science and fed autoclaved food and water. Approval of the Animal Use Committee of the Kitasato University School of Science was obtained prior to initiating the experiments.

Agaricus Extracts

A commercially available Kohjusen, which contains only the hot-water extracts of *A. blazei* Murrill, was obtained from Asuke Pharmaceutical Co. (Suwa, Nagano, Japan) and freeze-dried by ourselves. The content of β -glucan, estimated by a colorimetric analysis using the G-test (Seikagaku Corp., Tokyo, Japan), was 7.2 μ g/ml. The freeze-dried material was dissolved in distilled water before use and orally administered once a day to each mouse at a dose of 350 mg in a volume of 0.5 ml.

Tumor Implantation and Measurement of Size and Weight of Tumors

Mice received intradermal implantation of 1×10^6 Meth A cells. The tumor size at indicated time points was measured using a micrometer and expressed as a value multiplying the short length and the long length. The tumor weight was estimated on days 15 and 21. Meth A-bearing mice were orally given *Agaricus* extracts once a day starting 5 days after tumor implantation.

Preparation of Tumor-Infiltrating Cells

Tumors, removed from a group of 5 mice 10 days after implantation, were pooled, minced by scissors, and treated with 10 ml of 1 mg/ml collagenase (type IV, Sigma Chemical Co., St. Louis, MO, USA) and 30 μ g/ml DNase I (Sigma) 37 °C for 1 h. After passing through 200 mesh filters and centrifugation, cells were suspended in 45% Percoll (Amersham Biosciences, Uppsala, Sweden) and layered on 67.5% Percoll. After centrifugation at $850 \times g$ for 30 min, cells present in the interlayer were collected as tumor-infiltrating cells.

Immunization for Asthma Induction

An experimental asthma in mice was produced by a modified method of Eum *et al.*⁽¹⁸⁾ Mice were immunized subcutaneously with 100 μ g ovalbumin (OVA, Grade III, Sigma), precipitated with aluminum hydroxide (Wako Pure Chemical Ind., Osaka, Japan), twice on days 0 and 7. From day 7 after the secondary antigen stimulation, mice received intranasal stimulation with 100 μ g OVA in a volume of 20 μ l for three successive days (days 14, 15, and 16 after primary immunization). Mice orally received *Agaricus* extracts once a day from day 0 to day 17 after primary immunization.

Preparation of Bronchial Alveolar Lavage Fluids (BALF)

Following deep anesthetization with diethyl ether, blood was taken from the mice by cardiac puncture. Aliquots of 0.5 ml phosphate buffered saline (PBS) containing 2.7 mM EDTA were instilled through a catheter tube and

lavage fluid removed from the bronchial alveolar; this exercise was repeated twice. Both collections of fluids were mixed and the total number of cells was counted. The fluids were centrifuged at $1,500 \times g$ for 3 min. Supernatants were recovered to estimate IL-5 levels and cells were smeared to stain with Diff-Quik (Sysmex Co., Kobe, Japan). Differential cell counts were determined from more than 600 cells per each specimen by a light microscopy.

Preparation of Lung Interstitial T Cells

After removal of bronchoalveolar lavage fluid and drainage of blood from the heart with 3 ml of PBS, lungs were removed from mice, cut into small pieces and treated with 1 mg/ml collagenase (typeIV) at 37 °C for 1 h. After passing through 200 mesh filters and centrifugation, cells were suspended in 45% Percoll and layered on 67.5% Percoll. After centrifugation at $850 \times g$ for 30 min, cells present in the interlayer were collected as lung interstitial T cells.

Determination of Antibody Levels by ELISA

At 2 days after the final antigen stimulation, serum samples were prepared from blood collected by cardiac puncture following deep anesthetization. To determine levels of total IgG, IgG1, and IgG2a, aliquots (50 μ l) of 10 μ g/ml OVA were added to 96-well microplates and left to stand overnight for coating. After washing with 0.05% Tween 20 in PBS, wells were blocked with 200 μ l of 1% BSA for 1 h. Serum samples were diluted in a ratio of 1:100 for determining levels of total IgG and IgG1 or 1:10 for IgG2a and then serially diluted. For detection, alkaline phosphatase (AP)-conjugated rat anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA, USA), AP-conjugated rat anti-mouse IgG1 (Zymed) and AP-conjugated rabbit anti-mouse IgG2a (Zymed) were used. In order to determine serum IgE levels, wells were coated with anti-mouse IgE mAb (BD Pharmingen, San Diego, CA, USA), and washed and blocked as described previously. Serum samples (1:5 diluted) were diluted serially. For IgE determination, biotinylated OVA and streptavidin-conjugated horseradish peroxidase (HRP, Zymed) were used.

Determination of IFN- γ , IL-4, and IL-5 Levels by ELISA

For IFN- γ and IL-4 production, spleen cells (1×10^6 /mL) were prepared from Meth A-bearing and asthma mice and stimulated with anti-CD3 mAb (145-2C11) for 3 days. IFN- γ levels in culture supernatants were measured by ELISA as described previously.⁽¹⁹⁾ Levels of IL-4 in culture supernatants and IL-5 in BALF were measured by ELISA kits (BD Bioscience, San Diego, CA, USA) following the manufacturer's instructions.

RT-PCR

RNA (1 µg), obtained from cells infiltrating into Meth A-tumors 10 days after Meth A-implantation, was reverse-transcribed with Superscript II (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions with random primers (Takara Bio Inc., Shiga, Japan). The primer pairs for PCR with KOD DNA polymerase (Toyobo Co., Osaka, Japan) were used as follows: HPRT (forward: GTT TGT TGT TGG ATA TGC CCT TGA C, reverse: GGG GAC GCA ACT GAC ATT TCT A, 266 base pairs (bp)) and IP-10 (forward: CGC ACC TCC ACA TAG CTT ACA G, reverse: CCT ATC CTG CCC ACG TGT TGA G, 431 bp). The cDNA samples were amplified by PCR for 35 cycles at 94 °C (15 s), 60 °C (30 s) and 68 °C (1 min). The resultant products were analyzed by agarose gel electrophoresis.

Flow Cytometry

Tumor-infiltrating cells were treated with anti-FcγR mAb (2.4G2), and stained with a combination of three mAbs such as fluorescent isothiocyanate (FITC)-labeled anti-CD69 mAb, FITC-labeled anti-CD8 mAb, phycoerythrin (PE)-labeled anti-CD4 mAb, biotinylated anti-CD8 mAb, and biotinylated anti-CD49d mAb. All mAbs were obtained from BD Pharmingen (San Diego, CA). Cells were further stained with Cy-chrome bound streptavidin (BD Pharmingen). The phenotypes of T cells infiltrating to tumors was analyzed by flow cytometry with EPICS ELITE (Beckman Coulter, Hialeah, FL, USA).

Statistics

The statistical significance of differences between groups was determined by Student's *t*-test. A *P* value of less than 0.05 after comparison with the control group was considered significant.

RESULTS

Confirmation of Anti-Tumor Activity of *Agaricus* Extracts Used

In a previous study,⁽⁶⁾ we demonstrated that *Agaricus* extracts had a potent anti-tumor activity in Meth A-bearing mice. There was a strong possibility that the activity of extracts from the *Agaricus* mushroom appeared inconstant with each experimental preparation. We therefore created a new preparation of *Agaricus* extracts which contained 7.2 µg/ml of β-glucan to ensure more stable anti-tumor activity. Meth A-bearing BALB/c mice received, once a day, oral administration of either 15 g/kg of the *Agaricus* extracts or distilled water (controls) in a volume of 0.5 ml starting 5 days

after tumor implantation until the end of the experiment. The extracts exhibited a significant anti-tumor activity ($P < 0.01$) on inhibition of tumor growth (Figure 1A), and a significant decrease in tumor weight ($P < 0.01$) (Figure 1B).

Increased IFN- γ Production by Spleen Cells from *Agaricus*-Treated Tumor-Bearing Mice

In order to determine cytokine levels produced by T cells of tumor-bearing mice, spleen cells were removed from mice 20 days after Meth A-implantation, and stimulated *in vitro* with anti-CD3 mAb for 3 days. Treatment with the *Agaricus* extracts significantly enhanced IFN- γ producing activity of spleen T cells, compared with those of tumor controls (Figure 2A). However, no decrease in IL-4 levels was observed (Figure 2B).

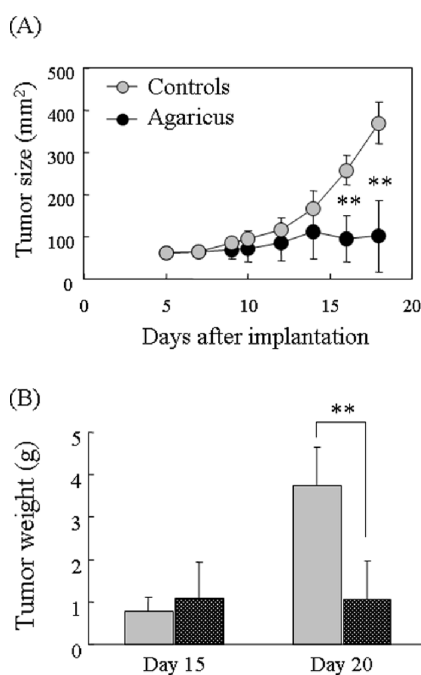


Figure 1: Tumor regression by orally administered *Agaricus* extracts BALB/c mice are orally given *Agaricus* extracts (closed) or water (open) every day starting 5 days after implantation of 1×10^6 Meth A cells into the right flank. (A), tumor size was estimated three times per week. Results show arithmetic mean $\pm$ SD of 5 mice per group, calculated by multiplying the short length (mm) and the long length (mm). (B), tumor weight (g) was estimated on days 15 and 20 after tumor implantation. Data represents arithmetic mean $\pm$ SD of 5 mice per group. **: $P < 0.01$ (by Student's *t*-test, vs. tumor controls).

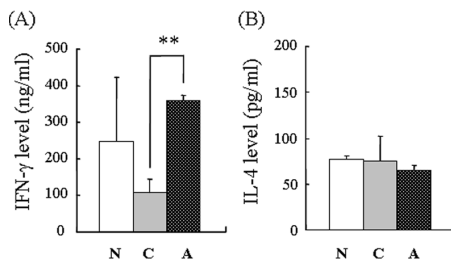


Figure 2: Augmentation of IFN- γ production by *Agaricus* extracts. Spleen cells, prepared from Meth A-bearing BALB/c mice that had been treated with or without *Agaricus* extracts, were stimulated in triplicate with anti-murine CD3 mAb coated on wells for 3 days. IFN- γ (A) and IL-4 (B) levels in supernatants were determined by ELISA. Results represent arithmetic mean $\pm$ SD calculated from individual supernatants of 5 mice per group. Both levels in supernatants stimulated with medium were non-detectable levels. N: untreated controls; C: tumor-controls; and A: tumor+*Agaricus*-treated group. **: $P < 0.01$ (by Student's t -test, vs. tumor controls).

Upregulation of IFN- γ -Inducible Protein (IP-10) mRNA Expression in *Agaricus*-Treated Mice

IP-10 is a chemokine which belongs to the CXC family.^(20,21) Upregulation of IP-10 levels in tumors should depend on an increase of IFN- γ levels. Therefore, expression of IP-10 mRNA in infiltrated cells into tumors was analyzed by RT-PCR. Infiltrated cells were prepared from mice 10 days after tumor implantation. Increased expression of IP-10 mRNA was observed in cells prepared from *Agaricus*-treated mice, compared with that of control tumor-bearing mice (Figure 3). These results suggest that oral administration of the *Agaricus* extracts can ameliorate the skewed Th1/Th2 balance in tumor-bearing mice.

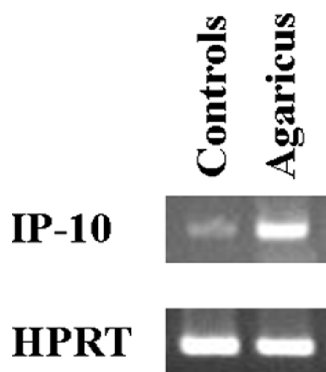


Figure 3: Increased expression of IP-10 mRNA in cells infiltrated into tumors by *Agaricus* extracts. Infiltrated cells were separated from tumors of 5 mice per group on day 10 after Meth A implantation. Expression of IP-10 mRNA was analyzed by RT-PCR.

Activation of Infiltrated T Cells into Tumors in *Agaricus*-Treated Mice

Infiltration of activated T cells into tumors is a pivotal step in the exclusion of tumors. To test whether expression of CD69, an activation marker, upregulates on both CD4⁺ and CD8⁺ T cells, infiltrated cells into tumors were prepared from mice 10 days after implantation and analyzed by a flow cytometer. Treatment with the *Agaricus* extracts increased CD69 expression on both CD4⁺ and CD8⁺ T cells (upper panels of Figure 4). Expression of CD49d, a homing receptor to the inflammation site, on T cells, tended to increase in both T cells of *Agaricus*-treated mice (lower panels of Figure 4). These results suggest that the *Agaricus* extracts activate T cells for tumor rejection.

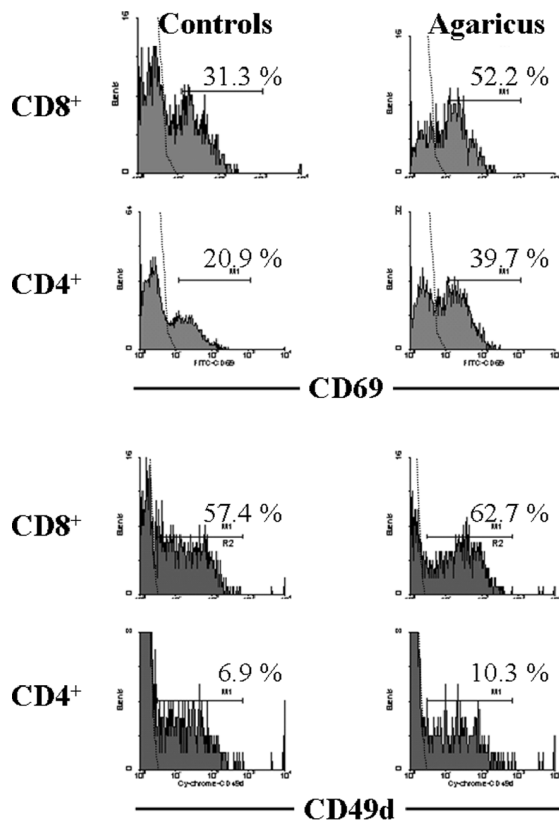


Figure 4: Activation of T cells infiltrated into tumors by *Agaricus* extracts. Infiltrated cells were separated from tumors of 5 mice per group on day 10 after Meth A implantation. Expression of CD69 and CD49d on CD4⁺ or CD8⁺ T cells was analyzed by flow cytometry. Isotype antibodies corresponding to anti-CD69 mAb or anti-CD49d mAb were used as negative controls (dotted line).

Downregulation of IgG1 and IgE Antibody Responses by Treatment with *Agaricus* Extracts

In order to evaluate whether the *Agaricus* extracts downregulate antibody responses to OVA in a murine asthma model, OVA-primed mice orally received either *Agaricus* extracts or water (controls) from day 0 to day 17 after primary immunization. Serum levels of total IgG, especially of IgG1 and IgE, against OVA were significantly suppressed by treatment with the *Agaricus* extracts, when compared with those of controls (Figure 5). No significant increase in serum IgG2a levels, IFN- γ -dependent production, was detected in *Agaricus*-treated mice. This result suggests that the extracts downregulate Th2-dependent antibody responses.

Decrease of Eosinophils Numbers in BALF of *Agaricus*-Treated Mice

In the asthma model, antigen stimulation via the nasal route caused an accumulation of inflammatory cells including eosinophils in the bronchial region. Inflammatory cells consisted of 70% macrophages, 20% neutrophils, 10% lymphocytes, and 10% eosinophils in BALF in the asthma controls. In order to determine whether the extracts reduce allergic conditions, differential cell counting revealed decreases in total cell numbers and eosinophil numbers in BALF of *Agaricus*-treated mice ($P < 0.01$), compared with those of controls (Figure 6A).

An increase of IL-5 levels in the bronchial region caused an accumulation of eosinophils which play a crucial role for late inflammatory responses and

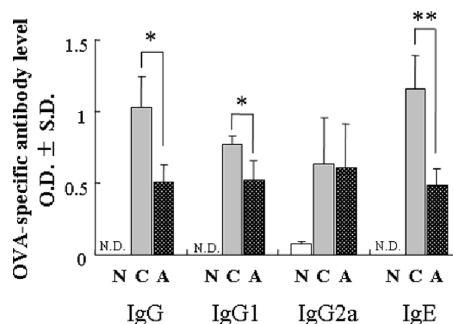


Figure 5: Downregulation of OVA-specific IgG1 and IgE antibodies by *Agaricus* extracts. Samples were prepared from 5 mice per group 18 days after primary immunization with OVA. Serum levels of total IgG (1:400 dilution), IgG1 (from 1:200 dilution), IgG2a (1:5 dilution) and IgE (1:10 dilution) against OVA were determined by ELISA. Data represents arithmetic mean $\pm$ SD of 5–6 mice per group. *: $P < 0.05$, **: $P < 0.01$ (by Student's t -test, vs. controls). ND: not detected. N: untreated controls; C: asthma controls; and A: *Agaricus*-treated. *: $P < 0.05$, **: $P < 0.01$ (by Student's t -test, vs. tumor controls).

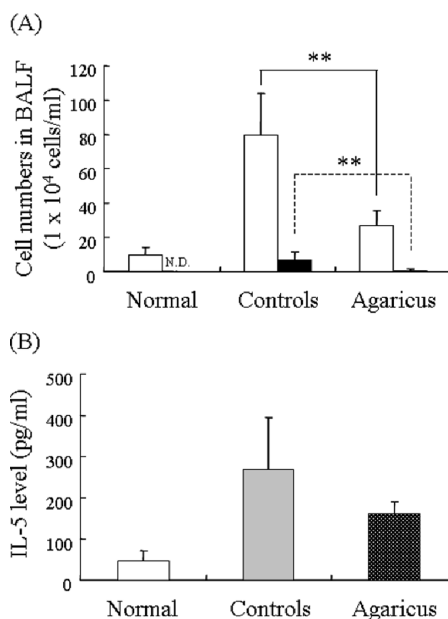


Figure 6: Decreases of total cell numbers and eosinophil numbers and IL-5 in BALF by *Agaricus* extracts. BALF samples were obtained from lungs of 5 mice per group 18 days after primary immunization. (A), numbers of total cells and eosinophils were differentially counted. □: total cells ■: eosinophils **: $P < 0.01$. (B), supernatants were prepared from BALF by centrifugation at 3,000 rpm for 3 min. Levels of IL-5 were determined by ELISA.

tissue damage. IL-5 levels in BALF from asthma mice treated with *Agaricus* extracts were lower than those in control mice, but were not significant vs. control mice (Figure 6B).

Increases of IFN- γ Production and Decreases of IL-4 Production in Asthma-Induced Mice Treated with *Agaricus* Extracts

In the asthma model, Th2-skewed conditions are shown by downregulation of Th1-cytokine IFN- γ production and upregulation of Th2-cytokine IL-4 production. To test whether the *Agaricus* extracts upregulates IFN- γ production and downregulate IL-4 production, spleen and lung interstitial T cells, prepared from mice 18 days after primary immunization, were stimulated *in vitro* with anti-CD3 mAb for 3 days. Spleen T cells of *Agaricus*-treated mice showed significantly higher levels of IFN- γ production than those of controls (Figure 7A). Lung interstitial T cells showed similar increased patterns in IFN- γ production but were not significant (Figure 7B).

With regard to IL-4 production, spleen T cells of *Agaricus*-treated mice had significantly lower levels of IL-4 production, compared with those of controls (Figure 7A). Lung interstitial T cells showed similar decreased patterns

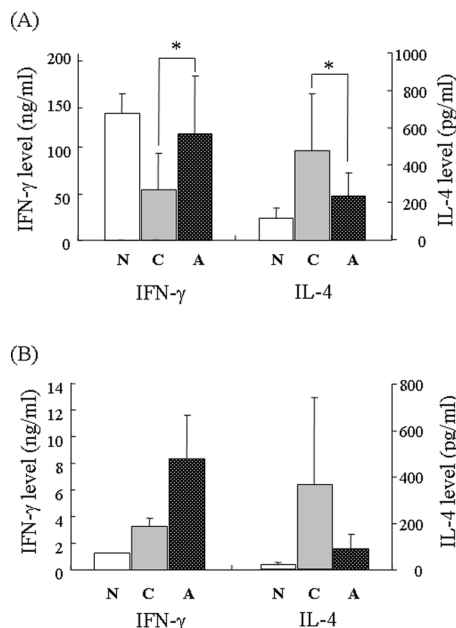


Figure 7: Modulation of IFN- γ and IL-4 production by anti-CD3-activated T cells from asthma-induced mice by *Agaricus* extracts. Spleen (A) and lung interstitial T cells (B) were obtained from 5–6 mice per group 18 days after primary immunization, and stimulated with anti-CD3 mAb for 3 days. Supernatants were obtained by centrifugation at 3,000 rpm for 3 min. IFN- γ and IL-4 levels were determined by ELISA. N: untreated controls; C: asthma controls; and A: *Agaricus*-treated. *: $P < 0.05$ (by Student's t -test, vs. tumor controls).

in IL-4 production but were not significant (Figure 7B). Taken together, these two results suggest *Agaricus* extracts can normalize from the Th2-skewed conditions in pulmonary regions.

DISCUSSION

This is the first study to investigate whether the effects of oral administration of hot-water extracts from the mushroom (*Agaricus blazei* Murrill), with anti-tumor activity, can ameliorate the skewed Th1/Th2 balance in an experimental asthma model. In a previous study,⁽⁶⁾ we demonstrated that treatment with the *Agaricus* extracts enhanced not only natural killer (NK) activity in innate immunity but also induction of antigen-specific cytotoxic T lymphocytes in adoptive immunity. This result suggested that there was a strong possibility the *Agaricus* extracts could modulate T cell functions, especially cytokine production by Th1 and Th2 cells, in not only tumor-bearing mice but also asthma-induced mice. In fact, upregulation of IFN- γ response was induced by treatment with the *Agaricus* extracts in both experimental

systems (Figures 2 and 7). In contrast, downregulation of IL-4 levels occurred in asthma-induced mice treated with the *Agaricus* extracts. Class-switching of naïve B cells to IgG1 or IgE B cells needs the assistance of IL-4 and other Th2 cytokines.^(13,14,22–25) The present study showed that OVA-specific IgG1 and IgE antibody responses significantly reduced in *Agaricus*-treated mice (Figure 5). These results suggest that oral administration of the *Agaricus* extracts is one of the most effective methods for the prevention of allergy development.

Cross-linking of antigen with IgE antibodies bound to FcεRI, expressed on mast cells, triggers degranulation and causes allergic reactions to release a variety of mediators such as enzymes (tryptase, chymase, cathepsin G, and carboxypeptidase), toxic mediators (histamine and heparin), cytokines (IL-3, IL-4, IL-5, IL-13, GM-CSF, and TNF-α), and lipid mediators (leukotrienes C4 and D4, platelet-activating factor).^(26–30) Th2-cytokine IL-5, released from mast cells, promotes eosinophils proliferation and their activation.⁽³¹⁾ The accumulation of eosinophils in the lung is closely related to the late-phase reaction in asthmatic response.⁽³²⁾ Therefore, downregulation of IL-5 levels and suppression of eosinophils accumulation would be beneficial for prevention of asthma provocation. Although oral administration of the *Agaricus* extracts resulted in downregulation of IL-5 levels and eosinophils numbers in bronchial lesion, accumulation of eosinophils is also stimulated by IL-13, eotaxin and prostaglandin D2. Therefore, it still remain unclear whether the *Agaricus* extracts activate these chemotactic factors to accumulate eosinophils into bronchial lesion. These results indicate that the extracts ameliorate not only Th2-skewed antibody responses but also reduction of mast cell-mediated anaphylactic reactions.

Among the components of *Agaricus* extracts, active components have not yet been identified. There is no published data to suggest that β-glucans, separated from different materials, have anti-allergic activity, although they have potent immunostimulating activities such as anti-tumor and adjuvant activities. Therefore, anti-allergic activity of the *Agaricus* extracts could not be due to the action of β-glucan in the extracts. We consider further empirical studies are necessary in order to identify the unknown active component(s) present in the *Agaricus* extracts.

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