

## Effect of dietary supplementation with white button mushrooms on host resistance to influenza infection and immune function in mice

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### Abstract

Previously, we showed that mice fed white button mushrooms (WBM) had enhanced immune functions known to help the body's antiviral defence. In the present study, we tested whether WBM conferred protection against viral infection. Young (4-month-old) and old (22-month-old) C57BL/6 mice were fed a diet containing 0, 2 or 10% WBM powder for 8 weeks. Mice were then infected with influenza Puerto Rico/8/34 (H1N1), and killed at day 0 (uninfected), 2, 5 or 7 post-infection. The primary outcomes of the study were viral titre and body weight. Secondary outcomes were natural killer (NK) cell activity, lymphocyte proliferation and cytokine production. The results showed that WBM did not affect viral titre, nor did it prevent infection-induced weight loss. WBM supplementation was found to enhance NK cell activity in old mice and to increase interferon (IFN)- $\gamma$  production in young and old mice under naive (uninfected) conditions, but it had no such effect after infection. The lack of a mushroom supplementation effect on NK activity and concanavalin A-stimulated IFN- $\gamma$  production after infection may explain the immune system's failure to reduce viral load and weight loss in mice after influenza infection. WBM supplementation, however, did induce changes in other aspects of the immune response: it significantly increased the production of T-helper type 2 cytokines IL-4 and IL-10 in uninfected mice and pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  in infected mice. These mushroom-induced systemic changes, however, were not adequate to confer a protective effect against influenza infection.

**Key words:** Mushrooms: Influenza infection: Immune function: Natural killer cell activity: Cytokines: Lymphocytes

Immune function, including both innate and adaptive immune responses, is critical to preventing and controlling microbial infection<sup>(1–4)</sup> and neoplasia<sup>(5,6)</sup>. While there are limited strategies available to efficiently modulate the immune response, nutritional interventions that involve optimising the intake of essential nutrients and utilising promising functional foods have become an increasingly favoured approach to modulating immune cell function. Mushrooms have been shown to possess antitumour, antiviral and antibacterial properties, particularly because of their ability to modulate immune cell function<sup>(7–12)</sup>.

We previously showed that dietary supplementation with white button mushrooms (WBM) enhanced natural killer (NK) cell activity in a mouse model, partly through increasing interferon (IFN)- $\gamma$  and TNF- $\alpha$  production<sup>(13)</sup>. In another study<sup>(14)</sup>, we further demonstrated that *in vitro* supplementation with WBM also enhanced maturation and antigen-presenting function of bone marrow-derived dendritic cells, the most important antigen-presenting cells. These results suggest that WBM may enhance innate immune function (NK activity and antiviral cytokines) and promote the development of innate immunity into acquired (specific) immunity,

**Abbreviations:** Con A, concanavalin A; cpm, counts per min; IFN, interferon; LPS, lipopolysaccharide; NK, natural killer; RPMI, Roswell Park Memorial Institute; WBM, white button mushroom.

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i.e. the process of dendritic cells presenting an antigen to T cells to induce antigen-specific immunity. Both NK cells and T cells are critical in clearing microbial infection, particularly viral infection, and, thus, mushroom-induced enhancement of NK and T-cell function would have the potential to prevent viral infection and/or to expedite viral clearance post-infection. However, this speculated benefit from mushroom consumption needs to be substantiated in an infection model before any such claims can be made. Therefore, we conducted the present study employing a mouse model of influenza infection to test this hypothesis.

## Materials and methods

### *Animals and diets*

Specific pathogen-free male young (4-month-old) and old (22-month-old) C57BL/6JNIA mice were purchased from the National Institute on Aging colonies at Harlan Sprague Dawley, Inc. Fresh WBM were provided by Country Fresh Mushroom Company through the Mushroom Council. Mushroom stems were first cut at the level of fruit body (crown), and each mushroom was cut into quarters. The cut pieces of mushroom were freeze-dried for 5 d and then ground to a powder. The subsequent dried matter was about 7.5% of its original fresh weight. Mushroom powder was added at 0 (control), 2 or 10% (w/w) to an American Institute of Nutrition-93M diet (Research Diets, Inc.) and thoroughly mixed. These supplementation levels were chosen based on our previous study in which WBM were found to enhance NK cell activity<sup>(13)</sup>. These doses are also considered translationally relevant because they are achievable through dietary intake. The 2% dose for mice can be converted to a daily consumption of 2.2 g fresh mushrooms/kg body weight for humans by using isoenergetic calculation<sup>(15)</sup>, or about 150 g fresh mushrooms/d (two servings), for a person of 65–70 kg. However, the 10% dose can only be achieved by taking a concentrated supplement. A control mix was added to the control diet at 10%, and to the 2% mushroom diet at 8%, respectively, to equalise the levels of total energy and macronutrients in the experimental diets. The control mix was formulated to best match the mushroom powder in energy, total carbohydrates, dietary fibre and protein, as prepared by mixing 25% casein, 37.5% maize starch, and 37.5% cellulose. A group of ten mice for each age, diet and infection time point were individually housed in ventilated cages at constant room temperature (22°C) with a 12 h light–12 h dark cycle and allowed free access to water and the experimental or control diets for 8 weeks. Body weight was recorded at weeks 0 (start), 2, 4, 6 and 8 (end). All animal-related procedures were approved by the Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and conducted according to the Guide for the Care and Use of Laboratory Animals (1996).

### *Virus and infection*

Influenza A/Puerto Rico/8/34 (H1N1) (A/PR8) was kindly provided by Dr Barry Ritz, Drexel University. This H1N1 strain

of mouse-adapted influenza A was propagated in specific pathogen-free eggs (B&E Eggs), and cell-free supernatants were stored at  $-70^{\circ}\text{C}$  until use. After 8 weeks of WBM dietary supplementation, mice were anaesthetised with an intramuscular injection of a ketamine (100 mg/kg body weight)/xylazine (2 mg/kg body weight) solution, and each mouse was infected intranasally with sixty haemagglutinating units (HAU) of A/PR8 virus diluted in 0.05 ml PBS.

### *Lung viral titre*

Lung viral titre was determined using the Madin–Darby canine kidney assay in which the serially diluted supernatants from lung homogenates were used to infect Madin–Darby canine kidney cells (American Type Culture Collection), as described previously<sup>(16)</sup>. Briefly, the plates were incubated at  $37^{\circ}\text{C}$  for 24 h, and the medium was replaced with Dulbecco's modified Eagle's medium (Sigma) containing amphotericin B, gentamicin and 0.0002% trypsin (Worthington). After 72 h incubation, 50  $\mu\text{l}$  of 0.5% fresh chicken erythrocyte suspension (Sigma) was added. The viral titres were then determined based on the presence or absence of haemagglutination. The 50% tissue culture infectious dose was calculated by the method of Reed & Muench<sup>(17)</sup>.

### *Lung and spleen cell isolation*

After mice were euthanised with  $\text{CO}_2$  asphyxiation, lungs and spleens were aseptically removed. Lungs were minced and incubated at  $37^{\circ}\text{C}$  for 1.5 h in Roswell Park Memorial Institute (RPMI)-1640 (Biowhittaker) medium containing 5% fetal bovine serum, 3 mg collagenase A/ml and 80 units DNase/ml (Roche). The digested lung samples were passed through a 30  $\mu\text{m}$  cell strainer and centrifuged at 500 g for 5 min. Supernatants were divided into aliquots and stored at  $-70^{\circ}\text{C}$  for subsequent analysis of virus titres and cytokines. To obtain lung mononuclear cells, cell pellets were resuspended in RPMI-1640 medium, layered on Histopaque-1083 (Sigma), and then subjected to density gradient centrifugation at 1400 g for 20 min. Single-cell suspensions from spleens were prepared by gently disrupting spleens between two sterile, frosted glass slides. After erythrocytes were lysed using Gey's reagent, splenocytes were washed twice with complete RPMI-1640 medium, i.e. supplemented with 25 mM-HEPES/l (Gibco Invitrogen), 2 mM-glutamine/l (Gibco),  $1 \times 10^2$  units penicillin/ml and 100  $\mu\text{g}$  streptomycin/ml (Gibco), and viability was determined by Trypan Blue exclusion. Cells from each tissue were resuspended in complete RPMI-1640 medium containing 5% fetal bovine serum at the appropriate density for use in subsequent assays. All the experiments were conducted under the following conditions:  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  and 95% humidity.

### *Natural killer cell cytotoxicity*

NK cell activity was assessed using the radioisotope  $^{51}\text{Cr}$  release assay. YAC-1 cells, a murine lymphoma cell line

purchased from the American Type Culture Collection, were used as target cells in the NK activity assay. These cells are sensitive to the action of NK cells and are the most commonly used target cells in the NK activity assay. YAC-1 cells were incubated with  $^{51}\text{Cr}$  (925 kBq/ $1 \times 10^6$  cells; Perkin Elmer) for 90 min at 37°C. Labelled YAC-1 cells were added to appropriately diluted mononuclear cells from lungs or splenocytes in a round-bottomed ninety-six-well plate (Nunc) for final effector:target cell ratios of 10:1, 25:1, 50:1 and 100:1. The co-cultured cells were incubated in triplicate for 4 h at 37°C. Spontaneous release was measured as the amount of  $^{51}\text{Cr}$  released by target cells alone, and maximum release was measured by the amount of  $^{51}\text{Cr}$  released after the addition of a 5% Triton X solution. The supernatant was collected after brief centrifugation (250 g, 1 min) and counted in a Cobra II gamma counter (Packard Instrument) for radioactivity as counts per min (cpm). NK cell activity was expressed as specific lysis (%): (sample cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)  $\times$  100.

#### Lymphocyte proliferation

Splenocytes ( $1 \times 10^5$  cells/well) in ninety-six-well flat-bottom plates (Becton Dickinson Labware) were stimulated with the T-cell mitogens concanavalin A (Con A; Sigma) at 0.5, 1.5 or 3  $\mu\text{g}/\text{ml}$ , phytohaemagglutinin (Difco Laboratories) at 2, 5 or 20  $\mu\text{g}/\text{ml}$ , or immobilised anti-CD3 (5  $\mu\text{g}/\text{ml}$ ) plus soluble anti-CD28 (1  $\mu\text{g}/\text{ml}$ ) (anti-CD3/CD28) for 72 h. Cultures were pulsed with 18.5 kBq [ $^3\text{H}$ ]thymidine (Perkin Elmer) during the final 4 h of incubation. The cells were harvested onto glass fibre filter mats (Wallac) by a Tomtec harvester (Wallac), and cell proliferation was quantified as the amount of [ $^3\text{H}$ ]thymidine incorporation into DNA as determined by liquid scintillation counting in a 1205 Betaplate counter (Wallac). The counter had an efficiency of more than 50% for  $^3\text{H}$ . Data are expressed as cpm.

#### Cytokine production

For IL-2, IFN- $\gamma$ , IL-4 and IL-10 production, splenocytes were cultured at  $5 \times 10^6$  cells/well in the presence of Con A (5  $\mu\text{g}/\text{ml}$ ) or anti-CD3/CD28 in twenty-four-well culture plates (Becton Dickinson Labware) for 48 h. For IL-6, TNF- $\alpha$  and IL-1 $\beta$  production, splenocytes were stimulated with lipopolysaccharide (LPS, 0111:B4; Sigma) at 1  $\mu\text{g}/\text{ml}$  and cultured for 24 h; this LPS stimulation condition is optimal in inducing inflammatory response of splenocytes and has been used in our previous studies<sup>(13,18)</sup>. Cell-free supernatants were collected and stored at  $-70^\circ\text{C}$ . Supernatants collected from collagenase-digested lung tissue were used to determine local cytokine production in response to infection. All of the cytokines were measured using ELISA following the standard protocols from the manufacturers. Reagents for IL-1 $\beta$  were from R&D System, and reagents for all the other cytokines were from BD Pharmingen.

#### Percentages of natural killer cells in lung and spleen cells

The percentages of NK cells in lung and spleen cells were determined using fluorescence-activated cell sorter analysis. Lung cells and splenocytes ( $1 \times 10^6$  per sample) were stained with fluorescein isothiocyanate-conjugated anti-NK1.1 (NK cells), or the isotype control. Anti-CD16/32 (Fc block) were used to block non-specific binding. All the antibodies were from BD Pharmingen. Stained cells were analysed on a FACS-Calibur (BD Biosciences), and the results were analysed using the software Summit 4.0 (DakoCytomation).

#### Statistical analysis

All results are expressed as means with their standard errors. Statistical analysis was performed using a SYSTAT statistical package (SYSTAT 10, 2000; Systat Software). Statistically significant differences were determined by ANOVA for overall effect of age, infection or diet (mushroom), followed by Fisher's least significance difference *post hoc* test for individual comparisons. Significance was set at  $P < 0.05$ .

## Results

### Body weight

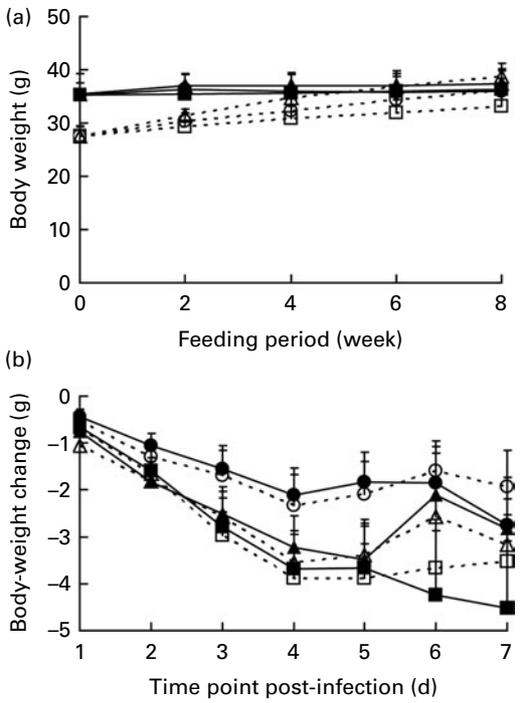
Body weight was recorded during the 8-week feeding period to monitor the general health of mice before and after infection as a marker for the impact of WBM supplementation on the severity of influenza infection. Old mice maintained and young mice gradually gained weight during the 8-week feeding period. The mushroom diet did not cause weight change in old mice; however, weight gain in young mice was highest for mice fed the 2% mushroom diet, intermediate for those fed the control diet and least for those fed the 10% mushroom diet. ANOVA showed an overall diet effect on weight gain in young mice. Young mice fed 2% mushroom before infection had a higher weight gain, while those fed 10% mushroom had a lower weight gain compared with young mice fed the control diet (Fig. 1(a)). After infection, mice fed mushroom had a greater overall weight loss compared with mice fed the control diet. This effect was statistically significant when young and old mice were pooled but insignificant when the results from young and old mice were considered separately (Fig. 1(b)).

### Viral titre

There was no significant difference between young and old mice in viral titre levels at days 2, 5 and 7 post-infection. Mushroom supplementation at either level (2 or 10%) had no significant effect on lung viral titre in mice of either age group (Fig. 2).

### Natural killer activity and percentage of natural killer cells in total lung and spleen cells

NK activity was determined in cells isolated from lungs (Fig. 3(a)–(c)) and spleen (Fig. 3(d)–(g)). ANOVA indicated



**Fig. 1.** Body weight of young (Y) and old (O) mice fed mushrooms (a) before infection and (b) weight loss after infection. Values are means, with their standard errors represented by vertical bars ((a)  $n$  18 per group, (b)  $n$  8 per group). C, control diet; 2% M, diet containing 2% mushroom powder (w/w); 10% M, diet containing 10% mushroom powder (w/w).  $\circ$ —, Y, C;  $\Delta$ —, Y, 2% M;  $\square$ —, Y, 10% M;  $\bullet$ —, O, C;  $\blacktriangle$ —, O, 2% M;  $\blacksquare$ —, O, 10% M.

an age effect and a time effect in both tissues, i.e. old mice had lower NK activity compared with young mice; influenza infection induced a higher NK activity. Mushroom supplementation enhanced NK activity in uninfected mice (day 0) but not in infected mice (days 2, 5 and 7). In both lung and spleen cells from uninfected old mice fed the 10% mushroom diet, NK activity was significantly higher compared with those cells from uninfected old mice fed the control or 2% mushroom diet. However, mushroom supplementation did not significantly affect the NK activity in either lungs or spleen of young mice or virus-infected old mice. ANOVA showed an overall age effect and time effect, but not an interaction between age and time, for the percentage of NK cells in total lung cells (Fig. 4(a)) and total spleen cells (Fig. 4(b)). These results indicated that old mice had a lower percentage of NK cells compared with young mice, and infection induced an increase in the percentage of NK cells irrespective of the age. However, mushroom supplementation did not affect this pattern.

*Cytokine production in spleen cells*

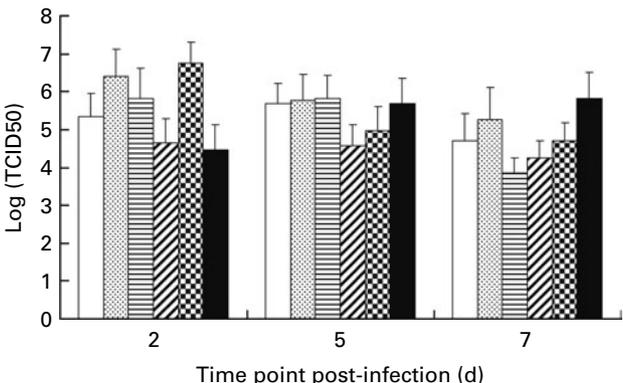
**Interferon- $\gamma$ .** IFN- $\gamma$ , produced predominantly by T cells and NK cells, is an important cytokine in antiviral defence because it can stimulate NK activity and macrophage activation. In our previous study, we found that mushroom supplementation increased IFN- $\gamma$  production, which was correlated with increased NK activity<sup>(13)</sup>. Consistent with our previous study,

we found that old mice produced significantly more IFN- $\gamma$  than young mice under naive (uninfected) conditions. After influenza infection, young mice increased their IFN- $\gamma$  production more dramatically than old mice, so that IFN- $\gamma$  levels were comparable between young and old mice. We used the T-cell mitogen Con A (Fig. 5(a)) or T-cell receptor antibody (anti-CD3) plus co-stimulation (anti-CD28) (Fig. 5(b)) to stimulate spleen cells for cytokine production. With Con A stimulation, spleen cells from young and old mice fed 10% mushroom produced more IFN- $\gamma$  compared with those from the control mice. However, this mushroom-induced change in IFN- $\gamma$  production was not observed in the infected mice.

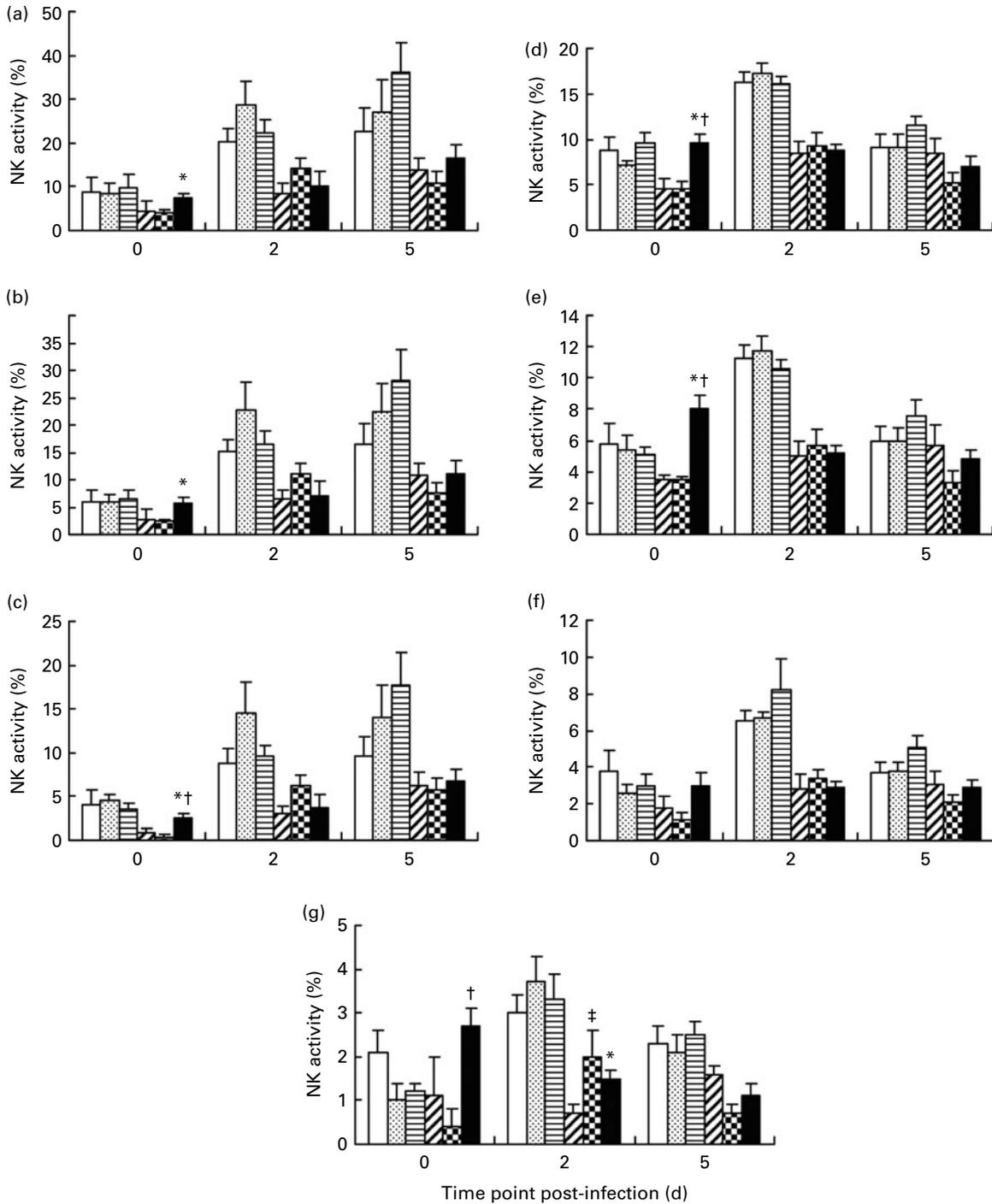
**IL-2.** IL-2, a T-helper type 1 cytokine, is critical for T-cell expansion and T-cell-mediated function. Declined production of IL-2 with ageing is a hallmark of immune senescence. In the present study, as expected, both Con A- and anti-CD3/CD28-stimulated IL-2 production were lower in old mice compared with young mice (Fig. 5(c) and (d)). Influenza infection significantly inhibited anti-CD3/CD28-stimulated IL-2 production in mice of both ages, which was not influenced by mushroom supplementation (Fig. 5(d)) in young mice. However, Con A-stimulated IL-2 production was increased in both uninfected and infected (day 7 post-infection) old mice fed 2% mushroom (Fig. 5(c)).

**IL-4.** IL-4, a T-helper type 2 cytokine, is primarily involved in the regulation of humoral immune function and allergy response. Con A-stimulated IL-4 production was low or undetectable. Anti-CD3/CD28-stimulated IL-4 production was comparable between young and old mice and was not significantly changed after viral infection. The only diet-induced change was that uninfected old mice fed 2% mushroom had a higher IL-4 production compared with uninfected old mice fed the control diet (data not shown).

**IL-10.** IL-10, a T-helper type 2 and also a regulatory T-cell cytokine, plays a key role in maintaining tolerance and preventing excessive or inappropriate T-cell function. While



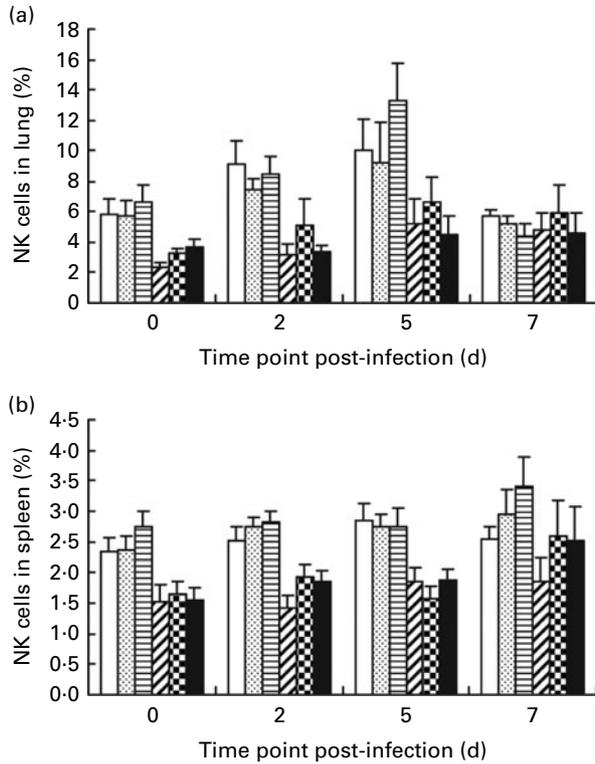
**Fig. 2.** Effect of mushroom supplementation on lung viral titre. At days 0, 2, 5 and 7 post-infection, lungs were collected and homogenised. The titres of virus in lung homogenates were measured using the Madin–Darby canine kidney assay and expressed as the 50% tissue culture infectious dose (TCID50). Values are means, with their standard errors represented by vertical bars ( $n$  8 or 9 per group). Y, young; O, old; C, control diet; 2% M, diet containing 2% mushroom powder (w/w); 10% M, diet containing 10% mushroom powder (w/w).  $\square$ , Y, C;  $\boxplus$ , Y, 2% M;  $\boxminus$ , Y, 10% M;  $\square$ , O, C;  $\blacksquare$ , O, 2% M;  $\blacksquare$ , O, 10% M.



**Fig. 3.** Effect of mushroom supplementation on the natural killer (NK) activity of lung and spleen cells from young (Y) and old (O) mice infected with influenza. At days 0, 2 and 5 post-infection, lung and spleen cells were isolated and incubated with  $^{51}\text{Cr}$ -labelled YAC-1 cells (NK target cells) at the ratios as indicated. NK activity was determined as described in the Materials and methods section. Lung cells:target cells at a ratio of (a) 50:1, (b) 25:1 and (c) 10:1. Spleen cells: target cells at a ratio of (d) 100:1, (e) 50:1, (f) 25:1 and (g) 10:1. ANOVA indicated an overall age and time effect for NK activity in both lung and spleen cells at all effector:target cell ratios. Values are means, with their standard errors represented by vertical bars ( $n$  7–9 per group). C, control diet; 2% M, diet containing 2% mushroom powder (w/w); 10% M, diet containing 10% mushroom powder (w/w). \* Mean values were significantly different between the 10% mushroom group and the control group ( $P < 0.05$ ). † Mean values were significantly different between the 10% mushroom group and the 2% mushroom group ( $P < 0.05$ ). ‡ Mean values were significantly different between the 2% mushroom group and the control group ( $P < 0.05$ ). □, Y, C; ▤, Y, 2% M; ▥, Y, 10% M; ▦, O, C; ▧, O, 2% M; ▨, O, 10% M.

IL-10 is important in preventing autoimmune and inflammatory disorders, it also causes immunosuppression and impairs antiviral defence. In the present study, we found no significant age-related difference in its production. Viral infection

up-regulated IL-10 production at later time points (days 5 and 7), probably in response to the mice's elevated inflammation state. Mushroom supplementation increased IL-10 production in only uninfected old mice, i.e. Con A-stimulated



**Fig. 4.** Percentage of natural killer (NK) cells in (a) lung and (b) spleen cells. Isolated lung and spleen cells were stained with fluorescein isothiocyanate-conjugated anti-NK1.1 (NK cells) and percentage of NK cells was determined by flow cytometry. Values are means, with their standard errors represented by vertical bars ( $n$  7–9 per group). Y, young; O, old; C, control diet; 2% M, diet containing 2% mushroom powder (w/w); 10% M, diet containing 10% mushroom powder (w/w). ANOVA indicated an overall age and time effect for the percentage of NK cells in both lung and spleen cells. □, Y, C; ▤, Y, 2% M; ▥, Y, 10% M; ▦, O, C; ▧, O, 2% M; ▨, O, 10% M.

IL-10 production in 10% mushroom-fed mice (Fig. 5(e)), and anti-CD3/CD28-stimulated IL-10 production in both 2 and 10% mushroom-fed mice (Fig. 5(f)). Since mushroom supplementation did not affect IL-10 production in the infected mice, it is unlikely that the observed absence of a protective effect with mushroom supplementation against influenza infection was due to the mushroom-induced increase in IL-10 production.

**Pro-inflammatory cytokines.** Pro-inflammatory cytokines usually increase after infection. On the one hand, they help mobilise and activate immune cells to fight pathogens; conversely, the resulting inflammation often causes tissue damage and severe symptoms. We selected hallmark pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  as the secondary outcomes. We measured these cytokines under two conditions: infection-induced production in lung cells (local response) and bacterial endotoxin LPS-stimulated *ex vivo* production in spleen cells (systemic response). Infection induced an increase in lung cells for all these pro-inflammatory cytokines over the time course and did not reach a plateau up to day 7 post-infection. Influenza-induced production of IL-1 $\beta$  and TNF- $\alpha$  but not IL-6 was more dramatic in old mice than in young mice, and none of them was affected by mushroom supplementation (data not shown).

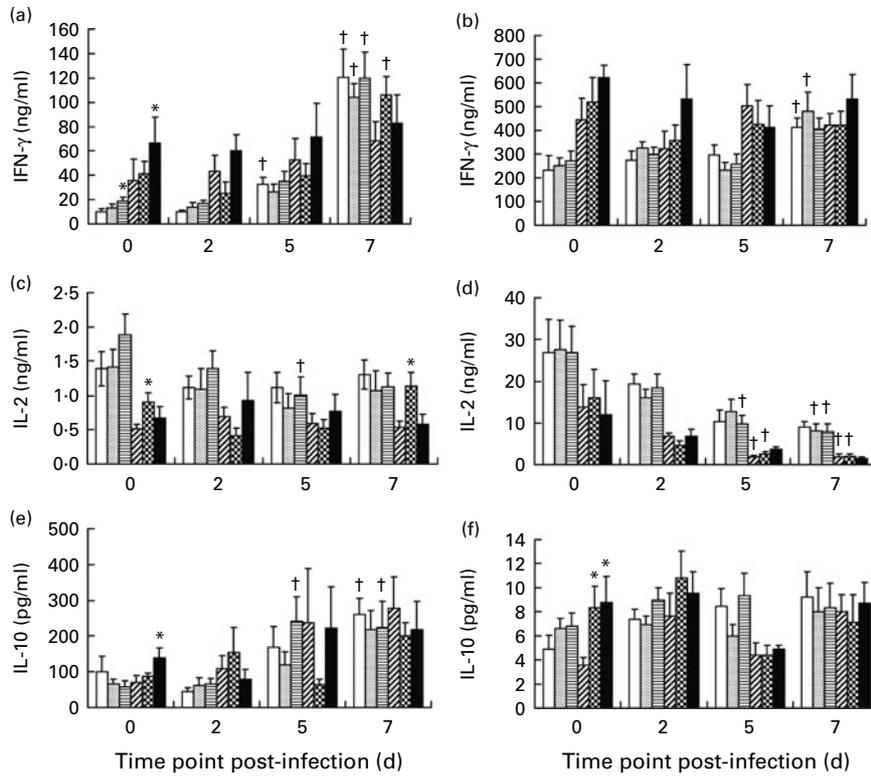
As to the production of these cytokines by LPS-stimulated spleen cells, IL-1 $\beta$  levels were comparable in young and old mice, and not significantly affected by the viral infection. While mushroom supplementation did not affect IL-1 $\beta$  production in the uninfected mice, young mice fed 10% mushroom at day 5 post-infection and old mice fed 2% mushroom at day 2 post-infection had significantly higher IL-1 $\beta$  production compared with their respective controls (Fig. 6(a)). IL-6 production was not different between the young and old mice, nor was it affected by the infection. An increase in IL-6 production was observed in old mice fed 10% mushroom at day 2 post-infection (data not shown). While there were no observed age-related changes in TNF- $\alpha$  production, the infection appeared to up-regulate TNF- $\alpha$  production. Significant changes related to mushroom supplementation were found in the infected mice as demonstrated by a higher TNF- $\alpha$  production in 2% mushroom-fed old mice at day 2 post-infection and 10% mushroom-fed young mice at day 5 post-infection (Fig. 6(b)).

### Lymphocyte proliferation

Upon stimulation, the ability to quickly expand their numbers is an important indicator of immune cell function. As shown in Fig. 7, there was an overall significant age and infection effect. Lymphocyte proliferation levels were significantly lower in old mice compared with young mice, and viral infection suppressed lymphocyte proliferation in both age groups at day 2. Mushroom supplementation did not have a significant effect on lymphocyte proliferation. In the present study, we induced lymphocyte proliferation with the T-cell mitogens Con A, phytohaemagglutinin and anti-CD3/CD28 at different concentrations, and only presented here the results generated with the optimal level of Con A (1.5  $\mu$ g/ml) as representatives (Fig. 7). Similar results were observed under other stimulation conditions (data not shown).

### Discussion

Studies have shown that a variety of mushrooms, their extracts and isolated components are capable of modulating some aspects of immune function<sup>(7,8)</sup>. Since immune response is the body's most important defence against microbial infection, it has been implied that consumption of certain mushrooms could have a protective effect against viral infection by modulating the body's immune system. The body's antiviral defence relies on a coordinated immune response involving both innate and adaptive arms. NK cells are known to be an important component of the body's first line of defence against viruses owing to their cytotoxic activity and robust production of inflammatory cytokines including IFN- $\gamma$  and TNF- $\alpha$ . In our previous study<sup>(13)</sup>, we found that dietary supplementation with WBM enhanced NK activity and production of the cytokines IFN- $\gamma$  and TNF- $\alpha$ . Therefore, we conducted the present study utilising an animal model of influenza infection to test the hypothesis that supplementation with WBM would enhance resistance to viral infections. However, the results do not support our hypothesis, as mushroom supplementation

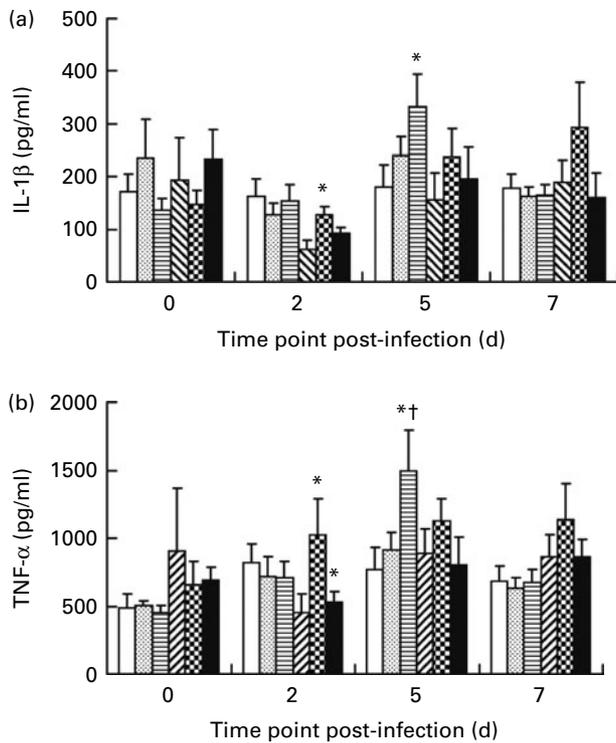


**Fig. 5.** T-cell cytokine production by concanavalin A (Con A)- or anti-CD3/CD28-stimulated splenocytes from young (Y) and old (O) mice infected with influenza. Splenocytes were cultured in the presence of Con A (5 µg/ml) or immobilised anti-CD3 (5 µg/ml)/soluble anti-CD28 (1 µg/ml) for 48 h. Supernatants were collected to measure cytokine concentrations using ELISA. (a, c, e) Con A-stimulated samples for interferon (IFN)-γ, IL-2 and IL-10. (b, d, f) Anti-CD3/CD28-stimulated samples for IFN-γ, IL-2 and IL-10. Values are means, with their standard errors represented by vertical bars (*n* 7–9 per group). C, control diet; 2% M, diet containing 2% mushroom powder (w/w); 10% M, diet containing 10% mushroom powder (w/w). \*Mean values were significantly different from mice fed the control diet of the same age and at the same post-infection day (*P* < 0.05). †Mean values were significantly different from day 0 of the same age and diet group (*P* < 0.05). □, Y, C; ▤, Y, 2% M; ▥, Y, 10% M; ▦, O, C; ▧, O, 2% M; ▨, O, 10% M.

did not have an effect on viral titre nor did it prevent weight loss after influenza infection, the two primary outcomes of the present study.

NK activity as measured in the present study is mainly determined by the number (or percentage) of NK cells in the cell mixture (lung or spleen cells), and/or by the cytolytic activity per NK cell. We found a lower NK activity together with a similarly smaller percentage of NK cells in total lung and spleen cells from old mice compared with those from young mice, suggesting that reduced total NK cells contribute to the lower NK activity seen in old mice. Consistent with our previous results<sup>(13)</sup>, WBM-induced enhancement in NK activity was mainly due to an increase in cytolytic activity rather than the number of total NK cells because the percentage of NK cells was not affected by the diet. In the present study, the lack of a protective effect of WBM against influenza infection does not seem to be due to the absence of a positive effect of WBM on the immune response, which is important for the host's antiviral defence; in fact, we did observe that uninfected, young and old mice fed WBM had a significantly higher production of IFN-γ, and uninfected old mice fed 10% WBM also had a higher NK activity, compared with those fed the control diet. While these results partially reproduce the findings from our previous study, it remains more

important to learn whether and how an intervention affects the host's immune response after a real pathological challenge such as infection, instead of the mimetic stimuli widely used in a majority of studies. In the present study, influenza infection induced more IFN-γ production as well as higher NK activity, both locally (in lung cells) and systemically (in spleen cells) in young mice than in old mice. However, WBM had no effect on the NK activity and IFN-γ production in infected mice at any post-infection time point. These results indicate that an elevated basal level in NK activity and IFN-γ may not be sufficient or even relevant to provide effective protection against viral infections in this animal model. One reason might be that infection-induced, antiviral responses such as increased NK activity and IFN-γ production were not further potentiated by WBM. IL-10 is an important cytokine for maintaining immune tolerance in order to control autoimmune and inflammatory disorders. It has been shown to inhibit the T-cell production of IFN-γ after antigen presentation<sup>(19)</sup> and T-cell response to antigens by reducing IL-2 production and IL-2 receptor expression<sup>(20,21)</sup>. While IL-10 may help reduce immunopathology, it may impair the host's ability to clear pathogens. In the present study, we found that WBM supplementation increased IL-10 production in the uninfected mice. Influenza infection induced an up-regulation in IL-10



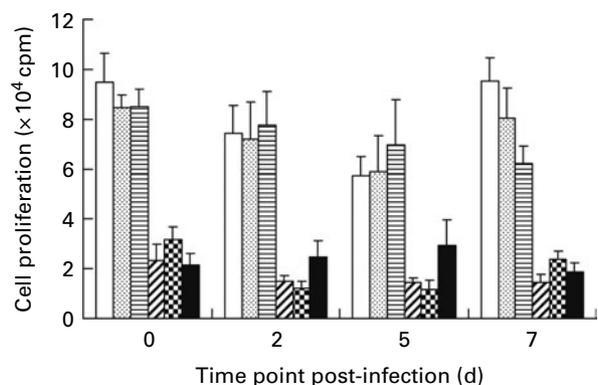
**Fig. 6.** Pro-inflammatory cytokine (a) IL-1 $\beta$  and (b) TNF- $\alpha$  production by lipopolysaccharide (LPS)-stimulated splenocytes from young (Y) and old (O) mice infected with influenza. Splenocytes were cultured in the presence of LPS (1  $\mu$ g/ml) for 24 h. Supernatants were collected to measure cytokine concentrations using ELISA. Values are means, with their standard errors represented by vertical bars ( $n$  7–9 per group). C, control diet; 2% M, diet containing 2% mushroom powder (w/w); 10% M, diet containing 10% mushroom powder (w/w). \* Mean values were significantly different from mice fed the control diet of the same age and at the same post-infection day ( $P$  < 0.05). † Mean values were significantly different from day 0 of the same age and diet group ( $P$  < 0.05). □, Y, C; ▤, Y, 2% M; ▥, Y, 10% M; ▦, O, C; ▧, O, 2% M; ▨, O, 10% M.

production, but WBM had no effect on IL-10 production in the infected mice. We also measured IL-4 because it has been reported to delay viral clearance probably through inhibiting cytotoxic T-lymphocyte activity and IFN- $\gamma$  production<sup>(22)</sup>. In the present study, neither infection nor WBM supplementation had an effect on IL-4 production. We found that infection did not induce increased IL-4 production, and WBM had no effect on its production in the infected mice. Therefore, the lack of a mushroom effect on viral clearance is not likely to be related to either IL-10 or IL-4, the cytokines that compromise the host's antiviral response.

Bioactive polysaccharides in mushrooms, such as  $\beta$ -glucans, belong to pathogen-associated molecular patterns (mushrooms as fungi), which can be recognised by the corresponding pattern recognition receptors expressed on the membrane of immune cells. Thus, WBM may stimulate immune cells and affect cytokine production through this mechanism. However, it did not appear that dietary WBM stimulated cytokine production because: (1) there was no difference in unstimulated cytokine production in the infected lung cells between the diet groups and (2) there was no detectable level of cytokines in unstimulated splenocytes. The difference in cytokine

production induced by mushroom supplementation was only found in Con A-, or LPS-stimulated cells. This is consistent with our previous study<sup>(13)</sup>, in which dietary WBM enhanced Con A- or phytohaemagglutinin-induced IFN- $\gamma$  production. We speculate that immune cells might have been primed in the body by exposing to bioactive mushroom components in the gut and/or systemically and primed cells would respond differently upon stimulation in *in vitro* cultures.

Although there is still a lack of consistency in the literature, our previous studies have shown that compared with young animals, aged animals have higher viral titres and delayed viral clearance after influenza infection, which may primarily reflect an age-related decline in immune response<sup>(16,23)</sup>. In the present study, however, we did not observe a significant difference between the young and old mice in either viral titre or weight loss. The lack of age-related differences in resistance to the viral infection cannot be explained by the immune response because we found significantly lower NK activity (in both lungs and spleens), T-cell proliferation and IL-2 production in both uninfected and infected old mice compared with young mice under the same conditions. Alternatively, one possible factor may be that a different influenza A strain was used in our previous studies (Port Chalmers/1/73, H3N2) *v.* the present study (PR/8, H1N1). The latter strain may be relatively more virulent because, in general, the titres were higher than those observed in our previous studies<sup>(16,23)</sup>. Further, at day 7 post-infection, at which time titres were found to be significantly lower than their peaks in previous studies, the titre levels were not different from those seen at days 2 and 5 in the present study. Thus, the viral infection might have overwhelmed any age- or mushroom-related differences. For the most part, influenza infection suppressed the T-cell-mediated response as demonstrated by a lower T-cell proliferation at day 2 post-infection,



**Fig. 7.** Effect of mushroom supplementation on cell proliferation by concanavalin A (Con A)-stimulated splenocytes. Splenocytes were isolated from each mouse at different time points and stimulated with Con A (1.5  $\mu$ g/ml) for 72 h and cell proliferation was measured by [<sup>3</sup>H]thymidine incorporation. Values are means, with their standard errors represented by vertical bars ( $n$  7–10 per group). Y, young; O, old; C, control diet; 2% M, diet containing 2% mushroom powder (w/w); 10% M, diet containing 10% mushroom powder (w/w). ANOVA revealed an overall significant age and infection effect: old mice had lower levels of lymphocyte proliferation; the infection induced a suppressed lymphocyte proliferation. cpm, Counts per min. □, Y, C; ▤, Y, 2% M; ▥, Y, 10% M; ▦, O, C; ▧, O, 2% M; ▨, O, 10% M.

and a larger inhibition with time in anti-CD3/CD28-stimulated IL-2 production in both young and old mice, compared with before infection. WBM supplementation at 2% resulted in a higher Con A-stimulated IL-2 production in the uninfected mice, an observation consistent with our previous study<sup>(13)</sup>. Additionally, we found an enhanced Con A-stimulated IL-2 production in mice fed 2% WBM at day 7 post-infection as well. These results suggest that WBM may moderately enhance innate immunity as well as T-cell-mediated function, but this effect was not adequate to have an impact on resistance to influenza infection with the strain and the dose used in the present study. Nevertheless, we cannot rule out the possibility that mushroom supplementation may be beneficial against infection with a less virulent strain or at lower infective doses of the strain used in the present study.

Influenza infection induces robust production of pro-inflammatory cytokines. These cytokines not only facilitate the maturation and function of innate immune cells but also help the development of an influenza-specific, cell-mediated immune response. However, these cytokines also contribute to tissue damage and clinical symptoms of the disease. In particular, TNF- $\alpha$  has been shown to be responsible for influenza-induced anorexia and body-weight loss<sup>(24,25)</sup>; we have previously reported a positive correlation between weight loss and lung TNF- $\alpha$  and IL-6 levels<sup>(26)</sup>. In the present study, we measured the production of the prominent pro-inflammatory cytokines, IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , in both lung homogenates (local, at the infection site) and spleen (systemic). In general, WBM supplementation did not affect the production of these cytokines in the uninfected mice. In the infected mice, however, cytokine production was enhanced by WBM at some, but not all, combinations of supplementation level, age and days post-infection. Taken together, it is possible that the altered production of pro-inflammatory cytokines might be associated with higher weight loss in WBM-fed mice.

The diet-associated difference in weight gain of young mice before infection was unexpected because in our previous study, we used the same conditions in terms of the strain and age of mice, source and preparation of mushrooms, and feeding period, and we did not see any difference in food intake and body weight. A 3 d diet intake sampling (data not shown) during the late feeding period showed that both young control and young 2% mushroom groups had similar food intake (4.0 g/d) while the young 10% mushroom group had slightly lower intake (3.75 g/d). The old mice in all diet groups had an average intake at 3.5 g/d. It appeared that young mice might be sensitive to the taste of mushroom so that when fed a high level of mushroom (10%), they consumed less diet and thus gained less weight. Nevertheless, given the fact that WBM supplementation did not show a protective effect (regardless of animal age and mushroom dose) but still affected some immune response parameters similarly as we observed in our previous study, the small difference in weight gain in young mice should not affect our conclusions drawn by taking together all data in the present study.

In summary, the results from the present study suggest that while WBM supplementation moderately enhances innate and cell-mediated immune response, this effect does not afford

adequate protection to the host against the strain and infection dose of influenza used in the present study. It is possible that the infection overwhelmed the antiviral immune response within the time frame, diminishing the impact of WBM-induced differences in immune response and thus, its impact on influenza infection. Future research should consider using different doses/strains of influenza or other types of microbes to prove or rule out the protective effect of mushroom supplementation against influenza and other types of microbes.

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