ORIGINAL RESEARCH

Immunostimulating Properties of Two Different β–glucans Isolated from Maitake Mushrooms *(Grifola frondosa)*

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ABSTRACT

β-glucans have been extensively studied for their immunological and pharmacological effects. The number of individual glucans is almost as great as the number of sources used for isolation. Not surprisingly, mushrooms are one of the prime sources of (1-3)-β-D-glucans, but their activities are not always described consistently. In addition, the most commonly used route of administration is injection, which is less convenient for clinical practice.

In this paper, we compared the immunostimulating properties of two different glucans from Maitake mushrooms with lentinan, a standard, well-researched mushroom-derived glucan. A number of major immunological parameters were tested – phagocytosis, NK cell activity, expression of surface markers, cytokine secretion and apoptosis. Our study showed not only have these glucans significantly increased all tested characteristics, but they also have similar, and in some tests even higher activity than lentinan, are active at lower doses and can be administered orally with no loss of activity. Therefore, this report represents evidence that Maitake-derived supplements taken orally can stimulate the defense systems.

Key Words: glucan, phagocytosis, cytokines, mushrooms.

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INTRODUCTION

Polysaccharides in general have a long history as immunomodulators, and interest in them rose particularly after experiments showed that zymosan stimulates macrophages via activation of the complement system.[1] Various types of β-glucans can be isolated from numerous sources, the major ones being yeast, mushrooms and seaweed. Despite the fact that the number of individual β–glucans is almost as great as the number of sources used for its isolation, and despite the enormous numbers of studies performed all over the world, it is still impossible to say that only one particular glucan is the optimal immunomodulator. As with all natural products, there are considerable variations not only among individual β–glucans, but also among individual batches. In view of the ever increasing popularity of glucans as immunomodulators, the functional comparison of new glucans is therefore more important than ever.

Thus far, the immunostimulating effects of β–glucans have been demonstrated in every single animal species tested, from earthworms[2] to humans. This supports not only the conclusion that β–glucans are active over the broad spectrum of biological species, but also that they represent one of the first immunostimulants active across the evolutionary spectrum.

Soluble fungal β–glucans such as schizophyllan and lentinan have been used for tumor immunotherapy in Japan for the past 25 years.[3,4] These polysaccharides promote natural host defense mechanisms and boost specific tumor immunity. For some time, investigators have failed to define the exact mechanisms of action. Only after identification of CR3 (CD11b/CD18) molecule as the principal receptor for β–glucans of leukocytes[5] was it possible to determine the cellular basis for their action and to design more rational approaches for their potential use in immunotherapy. However, not all cellular and molecular mechanisms responsible for glucan effects on the immune system are completely understood.

In addition to the well-studied mushroom-derived glucans such as lentinan,[6] numerous additional glucans have been isolated from various mushrooms. Some glucans, such as β–glucans from *Phytophthora parasitica,* were merely described without any biological tests.[7] Many of them, however, have very interesting properties, including the strong antitumor activity of β–glucan from *Glomerella cingulata*[8] or potentiation of TNF by scleroglucan.[9] As some of the published reports described rather confusing data, we decided to evaluate the immunostimulating effects of two mushroom-derived glucans and to compared them to the standard glucan, lentinan.

MATERIAL AND METHODS

Animals

Female, 6-to-10-week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO_2 asphyxiation.

Materials

RPMI 1640 medium, sodium citrate, dextran, Ficoll-Hypaque, antibiotics, sodium azide, bovine serum albumine (BSA), Wright stain, Limulus lysate test E-Toxate, and Concanavalin A were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was purchased from Hyclone Laboratories (Logan, UT), and CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (Promega, Madison, WI).

Antibodies

For fluorescence staining, the following antibodies have been employed: anti-mouse CD4, CD8, and CD19, conjugated with FITC were purchased from Biosource (Camarillo, CA).

Flow cytometry

Cells were stained with monoclonal antibodies on ice in 12x75 mm glass tubes using standard techniques. Pellets of $5x10^5$ cells were incubated with 10 µl of FITC-labeled antibodies (1 to 20 µg/ml in PBS) for 30 minutes on ice. After washing with cold PBS, the cells were resuspended in PBS containing 1% BSA and 10 mM sodium azide. Flow cytometry was performed with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer and the data from over 10,000 cell samples were analyzed.

Cell lines

Human cell lines ZR-75-1, PC-3, SW900 and K562 were purchased from ATCC. The cells were maintained in RMPI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and antibiotics.

β -1,3 glucans

Two different soluble β–glucans were isolated from Maitake mushrooms *(Grifola frondosa)* according to manufacturer's specification. Briefly, MaitakeGold 404 was produced under a patented method (US Patent 5,854,404). The product, a glucan/protein complex, is derived by thermally extracting the fruit body of Maitake with water under pressure at 100°C or more for 30 minutes to an hour. After that, alcohol is added to the extract at a final concentration of 20% to 60% by volume to remove floating material by filtration. The resulting extract is concentrated under heating to remove residual alcohol. The product, a hygroscopic powder in shades of brown is soluble in water, alkaline solutions, and dimethyl sulphoxide, and has a molecular weight around 1,000 kD.

Maitake Pro D Fraction is prepared as follows: Fruit bodies of Maitake are treated with hot water, and the water-soluble fraction is then saturated with ethyl alcohol. The resulting precipitate is then treated with acetic acid and alkalic material. The extract is acid-insoluble, alkali-soluble, with a molecular weight around 1 ,000 kD.[10]

MDF glucan is a highly-purified β–glucan.[11] MTG glucan is a β–glucan-protein complex.[12] MDF used in our experiments was Grifton-Pro Maitake D Fraction manufactured by Maitake Products, Inc. The MTG used in our experiments was MaitakeGold 404 purchased from Tradeworks Group, Inc. (Brattleboro, VT).

The soluble mushroom-derived β–glucan, lentinan (MW app. 1,000 kD), was obtained from the Developmental Therapeutic Program, Division of Cancer Treatment, Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD), and served as a control.

All media and buffers were tested for endotoxin contaminations and shown to contain >0.1 ng/ml of endotoxin using the *Limulus* lysate test (E-Toxate). The glucans were administered either by ip. injection or via intragastric tube (100 µg/day).

Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier.[13] Briefly: peritoneal cells were incubated with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; $5x10^8$/ml). The test tubes were incubated at 37° C for 60 min with intermittent

shaking. Smears were stained with Wright stain. The cells with three and more HEMA particles were considered positive. The same smears were also used for evaluation of cell types.

In vitro cytotoxicity assay

Spleen cells were isolated from the spleens of mice by standard methods. Cell suspension was generated by pressing minced spleen against the bottom of a petri dish containing PBS. After elimination of erythrocytes by 10-second incubation in distilled water and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by trypan blue exclusion, and only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10^6/ml; 0.1 ml/well) in V-shaped 96-well microplates were incubated with various glucans (2 μg/ml) for 30 min at 37° C and then washed three times with RPMI 1640 medium. After washing, 50 μl of target cell line K562 (three different concentrations of target cells were used: the final effector-target ratio was 10:1, 50:1, and 100:1) were added. After spinning the plates at 250x g for 5 min, the plates were incubated for 4 hrs at 37°C. The cytotoxic activity of cells was determined by the use of CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions. Briefly, 10 μl of lysis solution was added into appropriate control wells 45 min before the end of incubation. The next step was to spin the plates at 250x g for 5 min., followed by transferring 50 μl of supernatant into flat-bottomed, 96-well microplates. After 50 μl of reconstituted substrate was added into each well, plates were covered and incubated for 30 min. at room temperature in the dark. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:

Percent-specific killing (% cytotoxicity) =100x [(OD_{492} experimental - OD_{492} spontaneous) divided (OD_{492} maximum - OD_{492} spontaneous)] as described in the manufacturer's instructions, where spontaneous release was target cells incubated with medium alone, and maximum release was that obtained from target cells lysed with the solution provided in the kit.

Cytokine evaluation

BALB/c mice were intraperitoneally injected with various doses of tested glucans. Control mice obtained PBS only. After various time intervals, the mice were sacrificed and blood was collected in Eppendorf tubes. Subsequently, the serum was prepared, collected and stored at –80°C for no more than 1 week.

The levels of TNF-α and IL-1 in serum samples were evaluated using a commercial kit OptEIA Mouse TNF-? (Mono/Mono) and OptEIA Mouse IL-1 Sets (Pharmingen, San Diego, CA) according to the manufacturer's instructions. The optical density was determined using a STL ELISA reader at 450 nm with a correction at 570 nm. Data were calculated from the standard curve prepared by the automated data reduction using linear regression analysis. A standard curve was run with each assay.

For evaluation of IL-2, we incubated purified spleen cells (2x10^6/ml in RPMI 1640 medium with 5% FCS) in wells of a 24-well tissue culture plate. After addition of 1 μg of Concanavalin A into positive-control wells, cells were incubated for 72 hr in a humidified incubator (37°C, 5% CO_2). At the endpoint of incubation, supernatants were collected, filtered through 0.45 μm filter and tested for the presence of IL-2 (Vetvicka et al., 2002). Levels of the IL-2 were measured using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

Apoptosis

Apoptosis was evaluated using the APO-BRDU kit (BD Biosciences, San Diego, CA) according to the manufacturer's instruction with the use of a flow cytometer equipped with a 488 nm laser.

Statistics

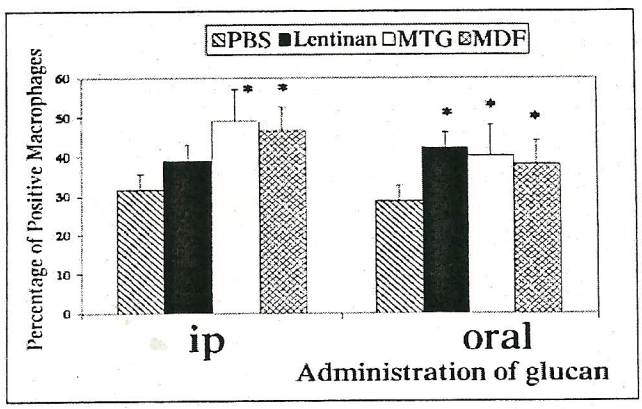
Student's t-test was used to statistically analyze the data.

RESULTS

Phagocytosis

It is well established that β–glucans strongly influence the phagocytic activity of professional phagocytes. At the same time, however, it is always important to start with phagocytosis when a new glucan is being evaluated. First we compared the effects of MTG and MDF β–glucans on phagocytosis of synthetic HEMA microspheres by peripheral macrophages (Figure 1). When the glucans were administered intraperitoneally, both tested glucans stimulated the phagocytosis more than lentinan used as a control. On the

Figure 1. Effect of an administration of different glucan (100 μg/mouse) samples on peritoneal macrophage phagocytosis. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level.



other hand, after oral administration (14 days of intragastric delivery of (100 μg of glucan/mouse) we found no differences between lentinan and our β–glucans, but all three types of glucan showed significant stimulation of phagocytosis.

In the next step we compared the effects of these glucans on phagocytosis by peripheral blood leukocytes. Results summarized in Figure 2 showed significant stimulation of monocytes by both MTG and MDF, whereas significant stimulation of neutrophils was achieved only after injection with lentinan (similar level of stimulation by MTG was not significant due to higher variation among samples). These experiments were followed up by evaluation of dose-dependence. Six different doses (from 1.8 to 250 μg/mouse) were used. Lentinan was active only in three higher doses, MTG caused increase in numbers of phagocytosing monocytes even at low 2.6 μg concentration, whereas MDF was effective only at the two higher concentrations (Figure 3).

Figure 2. Effect of an ip. administration of different glucan (100 μg/mouse) samples on phagocytosis by peripheral blood granulocytes and monocytes. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level.

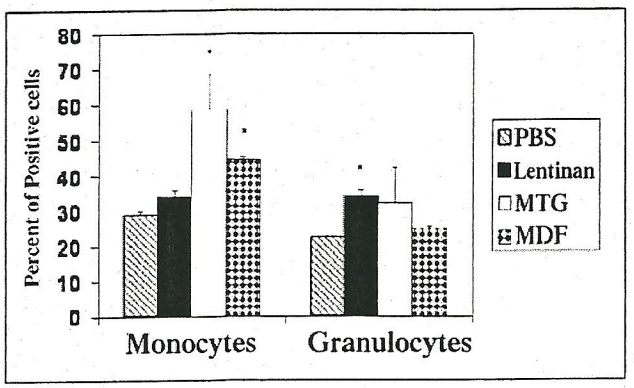
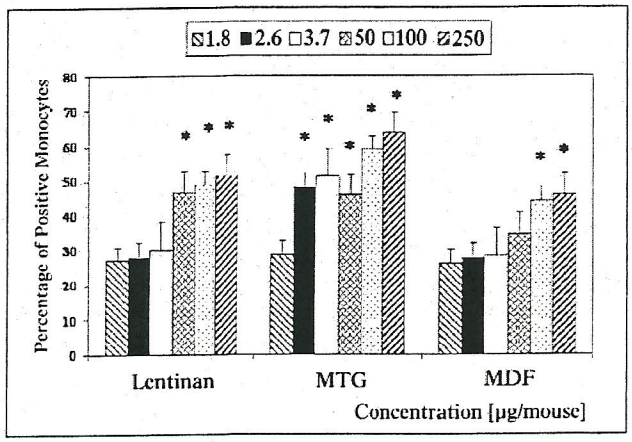


Figure 3. Potentiation of phagocytosis of synthetic microspheres by different doses of ip-injected glucans. Monocytes with three and more HEMA particles were considered positive. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level.



MEMBRANE MARKERS

We then evaluated the effects of MTG and MDF on expression of several membrane markers. Twenty four hours after an ip. injection of either MTG, MDF, or lentinan, spleen cells were isolated and the surface expression of CD4, CD8, and CD19 was evaluated by flow cytometer. The results summarized in Figure 4 show that the application of both MTG and MDF significantly increase the number of CD4-positive T helper cells in the spleen.

Cytokines

Next, we compared the effects of a single intraperitoneal injection of MTG, MDF, or lentinan on systemic *in*

Figure 4. Effect of ip. injection of 100 μg of tested glucans on the expression of CD4, CD8, and CD19 markers by spleen cells. The cells from three donors at each time interval were examined and the results given represent the means ± SD. *Represents significant differences between control (PBS and samples) at P ≤ 0.05 level.

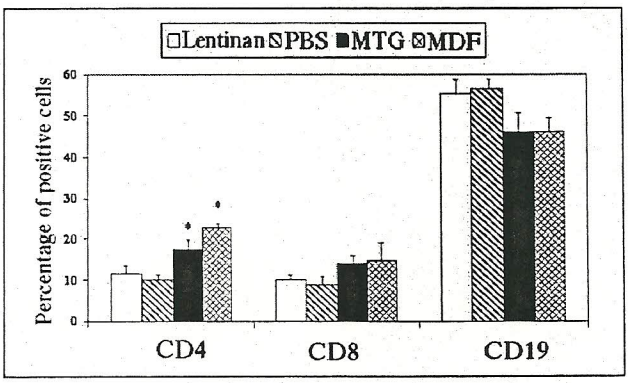
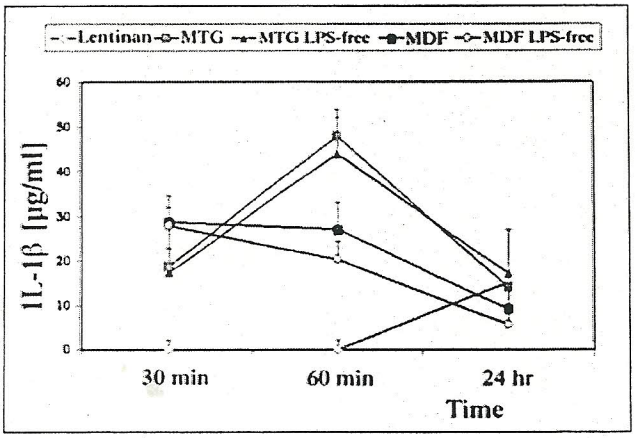


Figure 5. Stimulation of IL-1β secretion in peripheral blood by different types of glucan. Each value represents the mean ± SD. As the control values (PBS) were always zero, each value represents significant differences at P ≤0.05 level.



vivo release of two different cytokines, IL-1β and TNF-α. Peripheral blood was isolated at three different intervals after the injection and the serum obtained was stored at –80°C for no more than 1 week. The data summarized in Figure 5 shows that MTG and MDF caused significant elevation in levels of IL-1β in every tested interval. These elevated levels were demonstrated as early as 30 minutes and as late as 24 hours after application. Lentinan-caused increase could be observed only after 24 hrs. For both MTG and MDF glucans, the highest concentration of IL-1β was found 60 minutes after injection. There is a possibility that contamination of samples with LPS is causing the measured effects, but we also used LPS-free samples with virtually identical results.

A different situation was observed in the case of TNF-α, where only the MTG showed significant effects at the 60-minute interval. And again, LPS-free samples yielded similar data as normal samples (Figure 6). Several individual doses of all three glucans have been used, but the most pronounced effects were found with 100 μg doses.

Evidence of the immunomodulating activity was also demonstrated through effects on the production of IL-2 by spleen cells (Figure 7). The production of IL-2 was measured after a 72 hr *in vitro* incubation of spleen cells isolated from control and glucan-administered animals. Injection of lentinan and MDF resulted in strong stimulation of IL-2 production, which was even significantly higher than control stimulation by Concanavalin A. The effects of MTG were slightly lower than control Con-A-stimulated levels, but still almost 60 times higher than the level where only PBS was used (63 pg of IL2/ml).

Figure 6. Stimulation of TNF-α secretion in peripheral blood by different types of glucan. Each value represents the mean ± SD. As the control values (PBS) were always zero, each value represents significant differences at P ≤0.05 level.

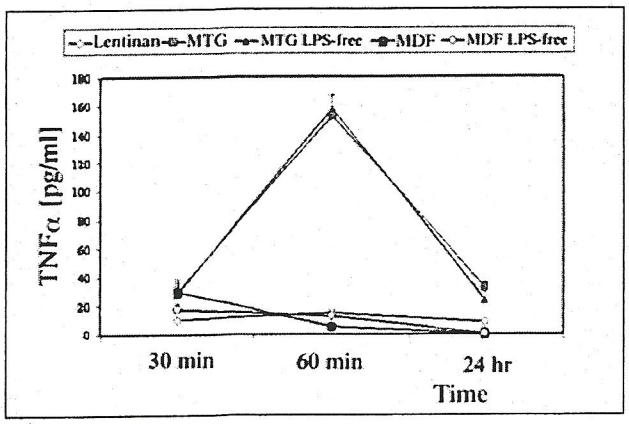
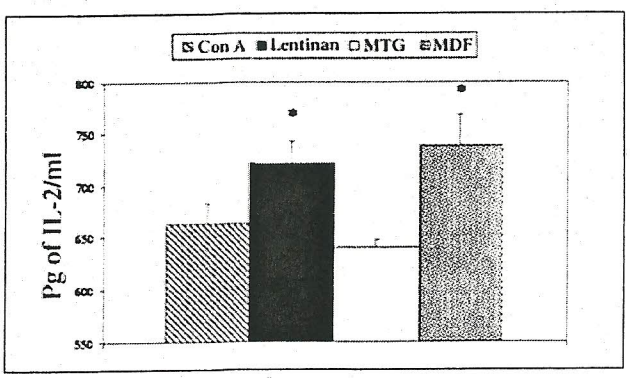


Figure 7. Stimulation of IL-2 secretion by splenocytes by different types of glucan. *Represents significant differences between control (PBS and samples) at P ≤ 0.05 level.



Cytotoxicity

Next, for evaluation of the effects on NK cells, human K562 cells were incubated with mouse spleen cells stimulated by either by our glucan samples or lentinan (Figure 8). A short treatment of glucans was adequate to cause significant enhancement of cytotoxicity at the higher E:T ratio.

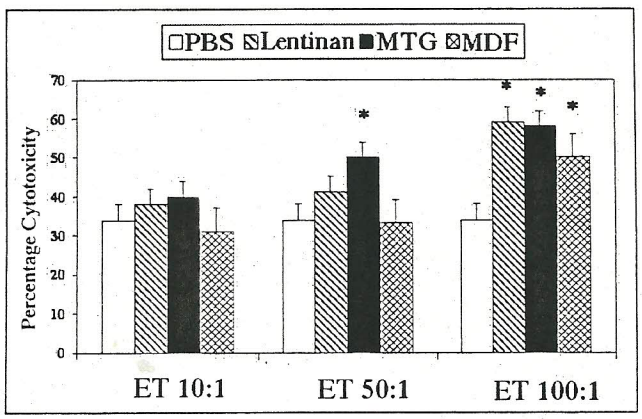
Apoptosis

We also measured the effects of our samples on induction of apoptosis. We decided to use three different and well-established human cancer cell lines: ZR-75-1 (breast cancer), PC-3 (prostate cancer), and SW900 (lung cancer). We used these lines to represent three different types of human cancer. However, using the APO/BRDU test, no apoptotic cells were observed (data not shown).

DISCUSSION

β–glucans, originally recognized as potent stimulants of the reticuloendothelial system,[1] are commonly considered typical representatives of substances that achieve their resistance-enhancing effects by stimulation of macrophages.[14] More recent discoveries of the interaction of immunocytes with β–glucans have demonstrated that complement recep-

Figure 8. Effects of tested glucans on NK cell cytotoxicity of K562 cells. Different ratios of NK cells to target cells were tested for cytotoxicity in the presence or absence of β-glucans for 30 minutes at 37°C. The data points shown are mean values from three experiments. The differences were significant at P≤0.05 level at all three effector to target cell ratios.



tor type 2 (CR3; CD11b/CD18) is primarily responsible for both the binding and biological effects of β–glucans.[5,15,16] Subsequent experiments helped to show that glucan used orally has similar biological effects to injected glucans,[17] and that the mechanisms responsible for glucan-mediated killing of tumor targets is via interaction of anti-tumor antibodies with glucan-activated cells.[18] The results of these experiments changed our view of glucans from merely nonspecific immunostimulators to natural agents specifically affecting immune reactions.

Despite our knowledge of the mechanisms of interactions between glucan and mammalian cells, and despite a vast number of individually described β–glucans, consensus on which glucan is best suited for stimulation of immunity remains lacking.

Most β–glucans are derived from fungi and have a backbone structure of linear β–1,3-linked D-glucose molecules (β1,3-D-glucan) with β-1,6-linked side chains of β1,3-D-glucan of varying sizes occurring at different intervals.[19] However, contradictory data and conclusions exist on the effects of molecular weight, degree of branching, conformation and polymer charge on biological activities.[9,19] Therefore, the quest for the biologically most active β–glucan continues.

Most of the β–glucans with high activity have been isolated from the Basidiomycetes, and not surprisingly, the two mushroom glucans most often studied, lentinan and schizophyllan, both came from the Basidiomycete family. The comparison of the biological and antitumor properties of these β–glucans can be found in Borchers et al.[20]

Due to the significant differences in activities among various glucans isolated from numerous sources, it is imperative to evaluate the biological properties of any glucan before making suggestions for use in clinical practice. This investigation focused on the biological activities of two individual glucans, MTG and MDF, isolated from Maitake mushrooms *(Grifola frondosa)*.

In the present study we demonstrated that the Maitake-derived glucans are functionally similar not only to lentinan, often considered a standard β–glucan, but also to other glucans.[21] The evaluation of the biological activities started, as usual, by effects on phagocytosis. Using the synthetic microspheres as prey, we took advantage of the unique properties of these microspheres, which have an extremely low negative charge and thus no nonspecific adherence to the cell membrane.[22] First we measured the phagocytosis of peripheral blood cells, and later the peritoneal cells. In both cases, we found a significant increase in the number of phagocytosis monocytes and macrophages after both MDF and MTG. When we compared the dose-dependence of the increased phagocytosis, the surprising result was that MTG was effective even in very small doses (2.6 and 3.7 μg/mouse). More importantly, the oral administration of our samples showed similar stimulation of phagocytosis.

The evaluation of the effects of Maitake glucans on expression of cell surface markers showed that both glucans caused a significant increase in the number of CD4-positive cells after the 24 hr interval. The rest, i.e., the numbers of CD8 and CD19 cells, were unchanged. The data on lentinan did not differ from PBS control, which is in agreement with previously described findings.[21]

In addition to the direct stimulation of cells involved in immune reactions, the immunostimulating effects of natural immunomodulators such as glucans are indirectly caused by potentiation of synthesis and subsequent release of numerous cytokines. Individual glucans significantly differ in their effects upon cytokine synthesis.[23-26] The only glucan found so far with either *in vivo* or *in vitro* stimulation of cytokines, is betafectin.[27]

We found only minor effects of tested glucans on TNF-α production, with only MTG having significant effects. When IL-1β was tested, both glucans showed significant stimulation even 30 minutes after injection, and these effects lasted for the whole tested interval. On the other hand, all samples strongly stimulated the production of IL-2, which is comparable to the earlier finding using yeast-derived glucans.[28] As cytokine production is extremely sensitive to the presence of LPS, contamination with LPS might mask or even overcome the real effects of any immunomodulator. Therefore we used LPS-free samples (depleted by addition of 10 μg/ml of polymixin B) in parallel to the regular glucan samples. In both cases, there were no differences between normal and LPS-free samples. Due to these results, we used only regular samples in all other experiments.

As the last set of experiments we evaluated the effects of glucan injection on NK cell activity. Using a human K562 cell as a model, we found that in higher E:T ratios, the 4 hr incubation of spleen cells with glucan samples caused significant potentiation of natural killer cells. When compared to lentinan, MTG was effective at lower ratio.

The sporadic data about glucan and apoptosis are confusing. While some authors found induction of apoptosis,[10,29] others found no effects.[30] Our data using breast, lung and prostate cancer cells showed absolutely no effects of tested samples (data not shown).

Our current paper clearly demonstrates that Maitake-derived glucans act via the same mechanisms as yeast-derived glucans, and that they are, in many cases, even more biologically and immunologically active. When compared to lentinan, Maitake-derived glucans (MDF and MTG in particular) showed a higher stimulation of defense reactions. Another important conclusion is the fact that these glucans do not lose their biological activities when delivered orally. And finally, this report represents a proof that orally-taken Maitake-derived supplements can stimulate the defense systems.

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