# Activation of Selective Transcription Factors and Cytokines by Water-Soluble Extract from *Lentinus lepideus*

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**We isolated a water-soluble extract, PG101, from cultured mycelia of *Lentinus lepideus*. Treatment of human peripheral blood mononuclear cells (PBMCs) with PG101 increased levels of TNF-α, IL-1β, IL-10, and IL-12 by 100- to 1000-fold, whereas GM-CSF and IL-18 were activated by an order of magnitude. On the contrary, IFN-γ and IL-4 were not affected. The response to PG101 occurred in a dose- and time-dependent manner. From the human PBMCs treated with PG101, TNF-α was a first cytokine to be activated, detectable at 2 hr post-treatment followed by IL-1β at 6 hr post-treatment. IL-12 and IL-10 were the next to follow. GM-CSF and IL-18 both showed significant increases 24 hr after treatment. When PBMCs were sorted into various cell types, monocyte/macrophages, but not T and B cells, were the major target cell type responsive to PG101. Consistent with this result, the profile of cytokine expression upon PG101 treatment was comparable between PBMCs and a human promonocytic cell line (U937), whereas cell lines of T cell and myeloid origins did not respond to PG101. Data from a transient transfection assay involving specific reporter plasmids indicated that cellular transcription factor such as NF-κB, but not AP-1, was highly activated by PG101. Results from a gel retardation assay and the experiment involving a specific NF-κB inhibitor confirmed the involvement of NF-κB. Despite its significant biological effect on various cytokines, PG101 remained nontoxic in both rats and PBMCs even at a biological concentration approximately 20 times greater. PG101 demonstrates great potential as a therapeutic immune modulator.** Exp Biol Med 228:749–758, 2003

**Key words:** *Lentinus lepideus*; PG101; immune modulator; monocyte/macrophage; cytokines; transcription factor; NF-κB

Mycelia or fruit bodies of many varieties of mushrooms have been revealed to contain biological response modifiers (BRMs). Indeed, various extracts from medicinal mushrooms have been reported to exhibit antiviral, antibiotic, anti-inflammatory, hypoglycemic, and hypotensive activities (1–6). The most widely known effects of fungal compounds are shown by BRMs isolated from *Ganoderma lucidum*, *Lentinus edodes* (Shiitake), and *Grifola frondosa* (Maitake), which contain antitumor and immune modulating activities. For example, oral intake of powdered fruit bodies of Shiitake has been found to be effective in inhibiting carcinoma growth in C3H/He mice (7). It has been reported that the cytotoxic activity of natural killer (NK) and lymphokine-activated killer (LAK) cells was significantly increased by Shiitake feeding (7, 8). In mice treated with an immunosuppressive carcinogen, administration of a mushroom-enriched diet containing shiitake, maitake, and oyster mushrooms restored the normal level of the chemotactic activity of macrophages and the capability of lymphocytes to proliferate in response to mitogen (9). A number of such BRMs have been identified in numerous other mushroom species.

Polysaccharide BRMs, which are mostly branched (1→3)-β-D-glucans, have been reported to contain potent antitumor and immune modulating activities by interacting with various immune cells. These BRMs contain numerous biological activities, including induction of hematopoiesis, activation of cytokine system, inhibition of tumor cell growth, and induction of resistance to viral and bacterial infection (10, 11). In particular, soluble glucans such as lentinan from *L. edodes* and schizophyllan from *Schizophillium commune* have been used for cancer immunotherapy in Japan for more

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This work was supported in part by grants from the Molecular Biology and Pharmacology Departments of the Oriental Medicine (MOM) program of PanGenomics, Co. Ltd., by the IVI-Affiliated Laboratory program (01-1-1), and by the Korea Health 21 R&D project, Ministry of Health and Welfare, Republic of Korea (02-PJ-PG11-VN01-SV04-0016).

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Received June 3, 2002.
Accepted January 24, 2003.

1535-3702/03/2286-0749$15.00

than 15 years. Clinical studies have revealed that lentinan, administrated i.v. or i.p., is effective in prolonging the survival of patients, particularly with gastric and colorectal cancer, and schizophyllan, administered by intratumoral or i.v. injection, has therapeutic effects in patients with cervical carcinoma (12, 13). It has been demonstrated that levels of IL-1 and TNF-α are increased in both mouse and human macrophages upon treatment with lentinan (14–16), subsequently augmenting T cell response to IL-2 and activating LAK and NK cells (17). It has been also reported that when schizophyllan is injected intratumorally to cervical cancers, there is a significant infiltration of Langerhans cells and T cells (18).

We have screened a variety of mushroom extracts for the possible presence of immune modulating activities. During this study, we previously found that extracts from *Lentinus lepideus* contain strong anti-cancer activity and that water-soluble glycan from *L. lepideus* induces B cell proliferation in the mouse spleen cells (19). To explore the possibility of developing this fungal extract into a therapeutically viable BRM, we tested the total water-soluble extract, PG101, from this mushroom in human PBMCs. A water-soluble fraction was found to increase production of TNF-α, IL-1β, IL-10, IL-12, granulocyte/macrophage-colony stimulating factor (GM-CSF) and IL-18 in a dose- and time-dependent manner. There was a specific sequence in responses of various cytokines. The major target cells appeared to be macrophages or related cells. Data from various experiments, including transient transfection with reporter plasmids, gel retardation assay, and the use of a specific inhibitor all indicated that activation of cytokines is achieved mainly by controlling the cellular transcription factor NF-κB.

## Materials and Methods

**Preparation of PG101.** *L. lepideus* was obtained from the Korea Forestry Research Institutes. The mycelia of *L. lepideus*, originally stored in agar medium, were grown in a liquid medium containing glucose, peptone, yeast extract, $KH_2PO_4$, $K_2HPO_4$, and $MgSO_47H_2O$ with an aeration at pH 5.5. Mycelia were harvested by filtration, washed several times with distilled water, and dried at 56°C overnight. Typically, 14 g of dried mycelia was obtained from 4 liters of liquid culture. Dried mycelia (14 g) were extracted with 500 ml of hot water twice for 3 hr each. The two water-soluble fractions were combined, centrifuged, and filtered through ADVANTEC filter paper, No. 2 (Toyo, Tokyo, Japan). The water-soluble fraction was obtained by centrifugation after filtration. The supernatant was concentrated by a rotary evaporation. The brown powder, named PG101, was obtained by freeze-drying of the concentrates. The yield of obtaining PG101 from dried mycelia was approximately 14%.

**Limulus Test.** Endotoxin was assayed under endotoxin-free experimental conditions using a Limulus Amebocytes Lysate (LAL) Pyrogen kit (BioWhittaker, Walkersville, MD). Experiments were performed according to the manufacturer's protocol. Briefly, 100 μl of standard reagent, PG101, or phosphate-buffered saline (PBS) was mixed with 100 μl of LAL reagent and was incubated for 1 hr at 37°C. Each tube was then examined for gelation. The quantity of endotoxin in PG101 was less than 0.015 EU/mg.

**HPLC Analysis.** PG101 and monosaccharide mixture (Gal, Glc, Xyl, Man, Fuc, GalNAc, GlcNAc, and ManNAc) were appropriately diluted with distilled water, and 50 μl of diluents were concentrated and dried in the GlycoTAG vial. Samples were hydrolyzed with 4 *N* HCl/4 *M* trifluoroacetic acid at 100°C for 4 hr, and 100 p*M* of pyridyl amino acid (PA) was then added. PA derivatives were analyzed using an HPLC system with PALPAK Type A column. The quantities of each monosaccharide were calculated by a peak height method.

**Protein Content Analysis.** Protein content was assayed with DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Experiments were performed according to the manufacturer's protocol. Briefly, a standard dilution of bovine serum albumin (Bio-Rad Laboratories) and an appropriate dilution of PG101 were prepared. Five microliters of standard or PG101 sample was mixed with reagent A in the kit. Two hundred microliters of reagent B was added to each mixture and mixed thoroughly. After 15 min, absorbance was measured at 750 nm and the protein content of PG101 was calculated from the standard curve.

**Preparation of PBMCs and Isolation of Different Cell Types.** Blood samples were obtained from healthy volunteers (three donors for each experiment) and were treated with EDTA as an anticoagulant. PBMCs were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) gradient centrifugation. Approximately 1 × $10^7$ cells were incubated with CD11b/Mac-1 or CD19 (BD PharMingen, San Diego, CA) antibody at 0°C for 40 min. Cells were placed in the dishes coated with anti-mouse IgG (10 μg/ml) for 1 hr at 4°C. Cells were then washed with PBS containing 1% fetal bovine serum (FBS), and attached cells were collected by scrapping. For isolation of T cells, PBMCs were incubated with microbeads conjugated with mouse monoclonal antibody to human CD3 (Miltenyi Biotech, Heidelberg, Germany) for 30 min at 4°C. After incubation, the mixture was passed through a magnetic column according to the manufacture's instruction. The column was washed with PBS containing 1% albumin, and bound cells were eluted with the same buffer. Isolated cells were analyzed by FACS, and cells showing over 80% purity for a given antibody were used for the experiments. Cells were incubated with PG101 at concentrations ranging from 0.1 to 100 μg/ml at 37°C under an atmosphere containing 5% $CO_2$. Lipopolysaccharides (LPS), zymosan A, and pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO).

**Cell Culture.** Human erythroblastic leukemia cell line K562, human T cell line Jurkat, human embryonic kidney cells 293, and human promonocytic cell line U937 were obtained from the American Type Culture Collection (Rockville, MD). Each cell line was maintained with DMEM or RPMI 1640 medium supplemented with 200 μg/ml streptomycin and 120 μg/ml penicillin G (Sigma) containing 10% FBS (Invitrogen, Carlsbad, CA). To test effects of PG101, cell lines were incubated with 250 μg/ml PG101 at 37°C under an atmosphere containing 5% $CO_2$.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT) Assays.** Human fibrosarcoma cell line HT1080 and human promonocytic cell line U937 were obtained from the American Type Culture Collection, and PBMCs (for isolation procedure, see below) were placed at 5000 cells per well in a 96-well plate. Cells were grown in the presence of PG101 at concentrations ranging from 5 to 5000 μg/ml at 37°C in a 5% $CO_2$ incubator. After 96 hr of incubation with PG101, viable cells were stained with MTT (5 mg/ml) for 30 min. The medium was then removed and the formazan crystals produced were dissolved by the addition of 200 μl of dimethyl sulfoxide. Absorbance was measured at 540 nm using an ELISA microplate reader. The $IC_{50}$ value was defined as the drug concentration that resulted in a 50% reduction in cell number compared with untreated controls. These values were derived from semilog plots of percentage viability (% = absorbance of treated sample/absorbance of untreated control × 100) versus drug concentration.

**Measurement of Cytokine Levels.** Levels of TNF-α (detection range: 15.6-1000 pg/ml), IFN-γ (25.6-1000 pg/ml), IL-1β (10.24-400 pg/ml), IL-4 (10.24-400 pg/ml), IL-10 (15.36-600 pg/ml), IL-12 (25.6-1000 pg/ml), GM-CSF (15.36-600 pg/ml), and IL-18 (25.6-1000 pg/ml) were measured using commercially available ELISA kits according to the manufacturer's instruction. ELISA kits for the first seven cytokines were purchased from Endogen (Woburn, MA), and the level of IL-18 was measured using the kit from Medical and Biological Laboratories (Nagoya, Japan). The supernatants from the cell culture were tested for their cytokine contents in duplicate or triplicate from three independent experiments.

**DNA Transfection.** Reporter plasmids containing binding sites for NF-κB, AP-1, and CRE (cyclic AMP response element) were purchased from Stratagene (La Jolla, CA). These plasmids contain the luciferase-coding sequence as a reporter gene and a synthetic promoter drives gene expression that consists of a minimum general transcription machinery and multiple copies of short nucleotide sequences to which respective transcription factors bind. The coding sequence for luciferase is located downstream from this control region and its expression is dependent upon the presence of respective transcription factors. These reporter plasmids have long been used to study the cellular factors and have been well documented (20–22). Activation of transcription factors was assayed by transiently transfecting 293 cells with reporter plasmids (2 μg) together with 0.5 μg of β-galactosidase plasmid (Invitrogen) using the calcium phosphates method (23). Six hours after transfection, cells were treated with 250 μg/ml PG101 for 18 to 24 hr. Cell lysates were prepared and assayed for luciferase activity using the Luciferase Reporter kit (Promega, Madison, WI) and Reporter Microplate Luminometer (Turner Instruments, Sunnyvale, CA).

**Electromobility Shift Assay (EMSA).** For NF-κB, U937 cells were cultured at 5 × $10^5$/ml for 16 to 20 hr and were stimulated with PG101. Nuclear extracts were prepared as before (24). The reaction mixture contained, in a final volume of 20 μl, 3 μg of poly (dI-dC), 0.3 ng of radioactive double-stranded NF-κB probe (5′- ATCCTCC GCTGGGGACTTTCCAGGGAGGA-3′), and 3 μg of nuclear extracts in binding buffer (10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 1 m*M* DTT, 5% glycerol, 2 μg of bovine serum albumin, and 3 m*M* GTP). After incubation for 20 min at room temperature, the reaction was analyzed on a low-ionic-strength 4% polyacrylamide gel and run with running buffer (6.7 m*M* Tris-HCl, pH 7.5, 3.3 m*M* sodium acetate, pH 7.0, and 1 m*M* EDTA, pH 7.5). The specificity of the retarded complexes was confirmed by competition with 40-fold excess of cold wild-type or mutant oligonucleotide (5′-GATCCTCCGCTCT CGACTTTCCAGG GAGGA-3′).

**Statistical Analysis.** Data are expressed as means ± SD. Statistical significances for protein levels and luciferase activity were determined using a Student's *t* test.

## Results

**Characterization of PG101.** We previously reported that the acid-polysaccharide fraction from cultured mycelia of *L. lepideus* activated B cells from mouse spleen (19). To test effects of this fungal extract on human cells, we initially chose to use the entire water-soluble fraction, PG101, prepared by treating cultured mycelia with boiling water for 3 hr twice as indicated in "Materials and Methods." This total fraction contained 72.4% polysaccharides, 26.24% proteins, and 1.36% hexosamine, suggesting that PG101 might consist of protein-bound polysaccharides. The HPLC analysis showed that the monosaccharide moiety consisted of 55.6% glucose, 18.5% mannose, and 25.9% galactose, indicating that PG101 includes heteroglycan (Fig. 1).

To examine the toxicity of PG101, MTT assay was performed on various human cells. For example, the level of PG101 showing 50% growth inhibition ($IC_{50}$) after 96 hr of incubation was 976 μg/ml for HT1080, 1951 μg/ml for U937, and 703 μg/ml for PBMCs. In addition, using a rat model, a single dose of 4 g of PG101/kg body weight showed no apparent clinical signs or notable changes in mortality, body weight, or gross findings at necropsy.

**Effects of PG101 on Cytokine Production in Human PBMCs.** We examined the effects of PG101 on human PBMCs. Cells were treated with varying concentrations of PG101. Bacterial LPS was used as a positive control at the concentration of 10 ng/ml. The level of cytokines in

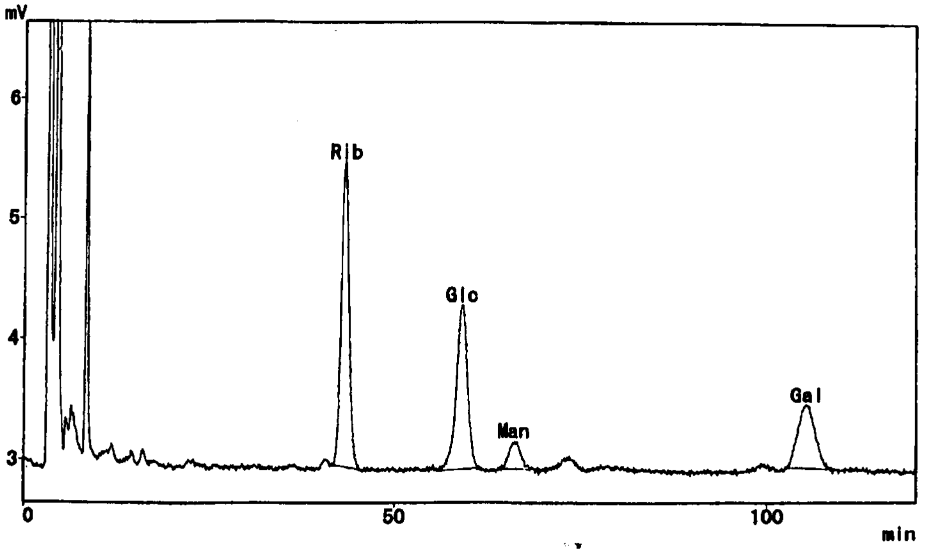


**Figure 1.** HPLC analysis of monosaccharide moiety of PG101. PG101 and a monosaccharide mixture (Gal, Glc, Xyl, Man, Fuc, GalNAc, GlcNAc, and ManNAc) were hydrolyzed with 4 *N* HCl/4 *M* trifluoroacetic acid at 100°C for 4 hr and 100 p*M* PA was then added. PA derivatives were analyzed using a HPLC system with PALPAK Type A column. The quantities of each monosaccharide were calculated using a peak height method.

the culture supernatant was measured by commercially available ELISA kits. Table I summarizes the results obtained using 10 μg/ml PG101, the concentration that produces the maximal effect in most cytokines (see below). Background levels of many cytokines were very low, but treatment with PG101 dramatically increased levels of a small number of cytokines. Tested cytokines could be classified into four categories, depending on the magnitude of effects of PG101 on these cellular proteins. The first type of cytokines (Type I) is those whose levels are increased by more than three orders of magnitudes by PG101. They are TNF-α and IL-1β. It is worth noting that the effect of PG101 on IL-1β and TNF-α is significantly higher than that of LPS at any given concentration, whereas it is comparable in other cytokines. The second type (Type II) is IL-10 and IL-12, whose levels are increased on average by two orders of magnitude. GM-CSF and IL-18 belong to Type III, whose levels are increased by less than 10-fold upon PG101 treatment. These cytokines are characterized by significantly high background levels present before PG101 treatment. Lastly, there were cytokines such as IFN-γ and IL-4 that did not respond to PG101 even at high concentrations. These data indicated that PG101 was able to increase the level of a few selective cytokines (Table I). It must be noted that the above classification could change to some extent depending on the source of PBMCs. For example, in PBMCs from certain individuals, IL-12 was more sensitive to PG101 to the extent that it could be categorized as a Type I instead of a Type II protein. However, the overall pattern of cytokine responsiveness to PG101 was quite reproducible among samples taken from different people or from the same individual at different times.

All affected cytokines responded to PG101 in a dose-dependent manner (Fig. 2). For Type I and Type II cytokines, levels were increased in a logarithmic manner up to 1 μg/ml PG101. At higher concentrations, the effects of PG101 became less pronounced. Levels of Type I cytokines reached a peak at 100 μg/ml PG101, whereas the highest levels of Type II proteins were achieved at 10 μg/ml PG101. The response of Type III molecules was characteristically slow and was highest at 10 μg/ml.

Effects of PG101 (at 10 μg/ml) was also time depen-

**Table I.** Effects of PG101 on Cytokine Production

| Type | Cytokines | Fold induction |  | Amount of cytokines PG101 (10 μg/ml) |
|---|---|---|---|---|
|  |  | LPS (10 ng/ml) | PG101 (10 μg/ml) |  |
| Type I | TNF-α | 915 ± 61 | 2461 ± 1501 | 3982 ± 617 |
|  | IL-1β | 97 ± 2 | 1238 ± 426 | 1321 ± 449 |
| Type II | IL-10 | 201 ± 78 | 220 ± 4.5 | 252 ± 41 |
|  | IL-12 | 136 ± 2 | 334 ± 158 | 1772 ± 299 |
| Type III | GM-CSF | 7 ± 0.3 | 18.9 ± 14 | 262 ± 150 |
|  | IL-18 | 4 ± 0.6 | 6.2 ± 2.3 | 14 ± 0.4 |
| Type IV | IFN-γ | – | – | – |
|  | IL-4 | – | – | – |

*Note.* PBMCs were treated with 10 μg/ml PG101 and 10 ng/ml LPS, and the level of respective cytokines was measured by ELISA after 24 hr. As a control, untreated cells were also grown with PBS for 24 hr. Fold induction was calculated by the level of cytokines of treated cells by that of untreated control at 24 hr. Actual amounts of cytokines were presented as picograms per milliliter. Experiments were performed more than three to five times for each cytokine. Data are presented as means ± SD.

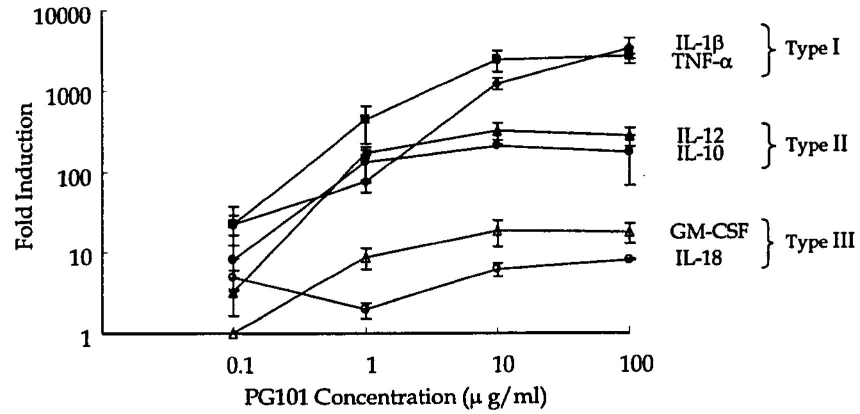
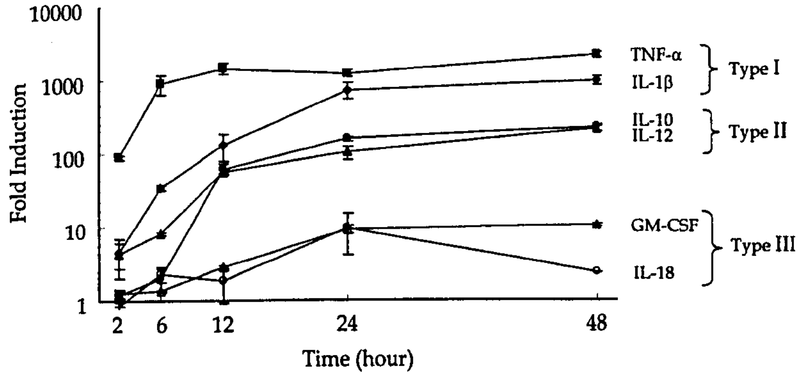


**Figure 2.** Effects of PG101 on cytokine production in human PBMCs. PBMCs were treated with PG101 at varying concentrations for 24 hr and supernatants were removed to measure the level of respective cytokines. Fold induction of respective cytokines were calculated at each concentration and plotted against the concentration of PG101 in a log-log format. Experiments were performed three times, and the results are presented as means ± SD.



| Type | Cytokines | Time (hour) | | | | |
|---|---|---|---|---|---|---|
| | | 2 hr | 6 hr | 12 hr | 24 hr | 48 hr |
| Type I | TNF-α | 91 ± 13 | 926 ±532 | 1469 ± 515 | 1258 ± 271 | 2275 ± 446 |
| | IL-1β | 4.4 ± 4.8 | 35 ± 4.7 | 135 ± 108 | 755 ± 370 | 1005 ± 218 |
| Type II | IL-10 | 1.2 ± 0.29 | 2.0 ± 1.4 | 63 ± 19 | 166 ± 23 | 231 ± 41 |
| | IL-12 | 4.3 ± 3.3 | 8.0 ± 0.48 | 58.5 ± 11 | 108 ± 47 | 227 ± 34 |
| Type III | GM-CSF | 1.2 ± 0.35 | 1.3 ± 0.05 | 2.7 ± 0.28 | 9.2 ± 2.4 | 10 ± 0.86 |
| | IL-18 | 0.85 ± 0.2 | 2.3 ± 0.5 | 1.8 ± 1.8 | 9.6 ± 11 | 2.4 ± 0.02 |

**Figure 3.** Kinetics of cytokine production upon treatment with PG101. PBMCs were treated with 10 μg/ml PG101 and culture supernatants were taken to measure the level of cytokines. Fold induction was calculated at each time point and plotted against time in a semi-logarithmic manner. The table displays the actual number, and shadowed boxes represent over 10-fold induction in cytokine production. Experiments were performed three times, and the results are presented as means ± SD.

dent and reached a peak at 12 to 24 hr after treatment in most cases (Fig. 3). From the human PBMCs treated with PG101, TNF-α was the first detectable cytokine. Only 2 hr after treatment of PBMCs with 10 μg/ml of PG101, the level of TNF-α was increased by 100-fold. The response of IL-1β was slower than that of TNF-α. Its level remained unchanged during the first 2 hr, but increased by approximately 40-fold at 6 hr post-treatment. The 50-fold induction of PG101 on Type II cytokines was found at 12 hr after treatment. It is interesting to note that response of IL-12 was detectable as early as 2 hr, but only at a level of 4-fold. The 10-fold activation of Type III cytokines, GM-CSF and IL-18, was found at 24 hr after PG101 treatment. In summary, when 10-fold activation was used as a criterion, the sequence of activation of cytokines was (TNF-α → IL-1β → (IL-10, IL-12) → (GM-CSF, IL-18).

**Major Target Cell Type for PG101.** To determine the cell type affected by PG101, PBMCs were sorted into macrophages, T cells, and B cells by a panning method using antibodies specific to each cell type (see "Materials and Methods"). Neither B cells nor T cells responded to PG101 in terms of cytokine production (Table II). In contrast, CD11b/Mac-1-positive cells, which are thought to contain a predominantly monocyte/macrophage lineage, responded to PG101 in a manner similar to PBMCs, suggesting that macrophages might be the major cell target of this fungal extract.

For further confirmation, human cell lines of various origins were used. They include U937, Jurkat, and K562 that are human promonocytic, T lymphoid, and multipotent erythroblastic leukemia lines, respectively. Cells were treated with 250 μg/ml PG101 for 24 hr and levels of

**Table II.** Effects of PG101 on Isolated Cell Types in Human PBMC

| Type | Cytokines | Cell types | | | |
|---|---|---|---|---|---|
| | | PBMCs | Macrophage ($CD11b^+$) | B cell ($CD19^+$) | T cell ($CD3^+$) |
| Type I | TNF-α | +++ | +++ | – | – |
| | IL-1β | +++ | ++ | – | – |
| Type II | IL-10 | ++ | – | – | – |
| | IL-12 | ++ | + | +/– | – |
| Type III | GM-CSF | + | +++ | – | – |
| | IL-18 | + | – | – | – |
| Type IV | IFN-γ | – | – | – | – |
| | IL-4 | – | – | – | – |

*Note.* Total PBMCs ($1 \times 10^6$) and isolated primary cells ($1 \times 10^5$) were treated with PG101 at 10 μg/ml for 24 hr. Supernatants were taken to measure the level of cytokine. Because the number of isolated cells were 10-fold less than that of total PBMCs, the level of cytokine in purified population was recalculated to that per cell. Untreated control cells were also grown for 24 hr. Fold induction was calculated by dividing the level of each cytokine of treated by untreated cells at 24 hr. Fold induction is indicated as follows: +++, > 500 fold; ++, 50–500 fold; +, 10 fold; –, no stimulation. Experiments were performed more than two times for each cytokine. Data are presented as means ± SD.

cytokines were measured. Jurkat and K562 lines were hardly affected, if at all, by PG101. In contrast, the response of U937 was similar to that of PBMC or primary monocytes/macrophages (Table III). These data further confirmed that cells of a macrophage lineage are the major targets of PG101.

**PG101 Regulates Cytokine Expression by Controlling NF-κB.** In an effort to understand the molecular mechanism(s) underlying activation of various cytokines by PG101, we first tested effects of PG101 on cellular transcription factors. This was because they are known to play key roles in regulating cytokine gene expression, while multiple effects of PG101 could be most readily interpreted if this fungal extract controls transcription factors. Structures of promoters of cytokine genes are shown in Figure 4. NF-κB-binding sequences are the most frequently found element, present in four promoters except for those of IL-10 and IL-18 (25–30). AP-1-binding sites are also present in promoters of TNF-α, IL-1β, and GM-CSF (31–33). Promoters of TNF-α, IL-1β, and IL-12 contain the nucleotide sequences that can interact with CREB (34–36). Based on this observation, the effects of PG101 on three transcription factors were determined, first by transient transfection assays involving reporter plasmids. Treatment with PG101 increased the level of luciferase activity by 60-fold when the NF-κB reporter plasmid used (Fig. 5). The level of luciferase activity from a CREB reporter plasmid was also increased, but by less than 5-fold. PG101 did not have any significant effect on AP-1 (Fig. 5).

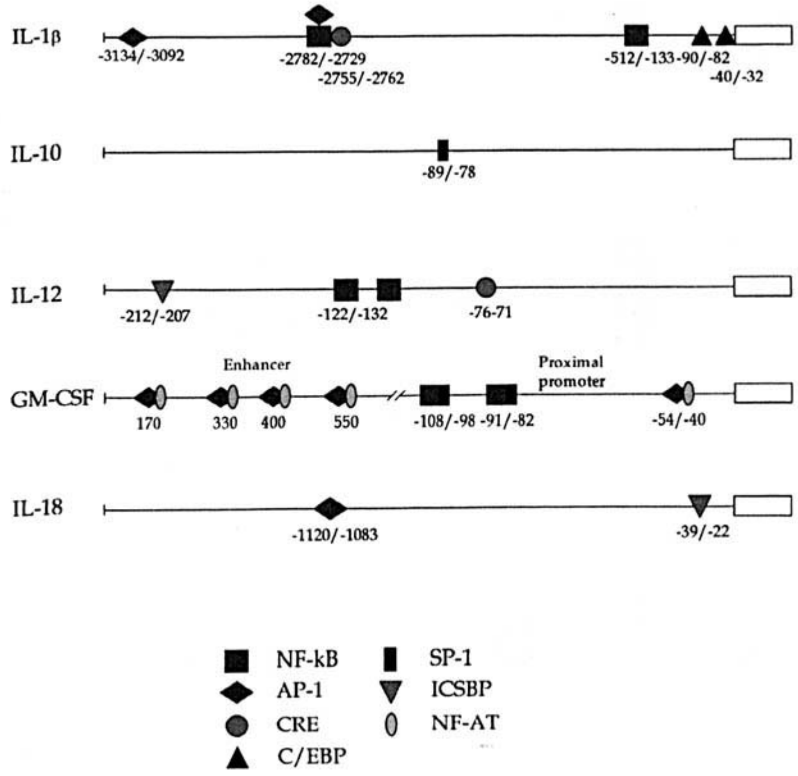
**Table III.** Effects of PG101 on Various Cell Lines

| Type | Cytokines | Cell lines | | |
|---|---|---|---|---|
| | | U937 | K562 | Jurkat |
| Type I | TNF-α | ++ | – | + |
| | IL-1β | +++ | – | – |
| Type II | IL-10 | ++ | – | – |
| | IL-12 | ++ | – | – |
| Type III | GM-CSF | ++ | ++ | – |

*Note.* U937, K562, and Jurkat cell lines were treated with PG101 at 10 μg/ml for 24 hr. Supernatants were taken to measure the level of cytokine. Untreated control cells were also grown for 24 hr. Fold induction was calculated by dividing the level of each cytokine of treated by untreated cells at 24 hr. Fold induction is indicated as follows: +++, > 500 fold; ++, 50–500 fold; +, 10 fold; –, no stimulation. Experiments were performed more than two times for each cytokine. Data are presented as means ± SD.

To confirm that NF-κB was indeed highly activated by PG101, a gel retardation assay was performed. Nuclear extracts were prepared from human cells, either before or after PG101 treatment, and were incubated with a double-stranded $[^{32}P]$-labeled oligonucleotide probe, corresponding to the −86 to −116 region of the HIV LTR, which has already been shown to interact with NF-κB. Untreated cells showed a low basal level of NF-κB activity (Fig. 6). However, treatment of cells with PG101 significantly increased the amount of electrophoretically retarded DNA-protein complex (Fig. 6, Lane 3). This complex was specific to NF-κB because they were effectively competed by the unlabelled wild-type -κB sequence, but not by a mutant oligonucleotide, which was identical to the wild-type probe except for three nucleotide changes (Fig. 6, Lanes 4 and 5).

For further confirmation on the involvement of NF-κB in PG101-mediated activation of cytokines, it was also tested whether cytokine activation by PG101 could be suppressed by PDTC, which specifically inhibits the activity of NF-κB (37). Consistent with data from transient transfection and gel retardation assays, levels of respective cytokines were completely suppressed by PDTC at 100 μ*M* concentration (which does not show any cytotoxicity; Fig. 7). It is interesting to note that activation of IL-10 and IL-18 was also inhibited by PDTC, although its promoter dose not contain the NF-κB sites. It is possible that PG101 controls IL-10 and IL-18 by the indirect use of NF-κB. Whatever the actual mechanism, these cumulative data clearly indicated that PG101 regulates the expression of affected cytokines by controlling cellular transcription factor NF-κB.



**igure 4.** Schematic representation of the transcrip- ion factor binding sites in various cytokine promoters. arious nucleotide sequences that bind to transcrip- ion factors are shown.

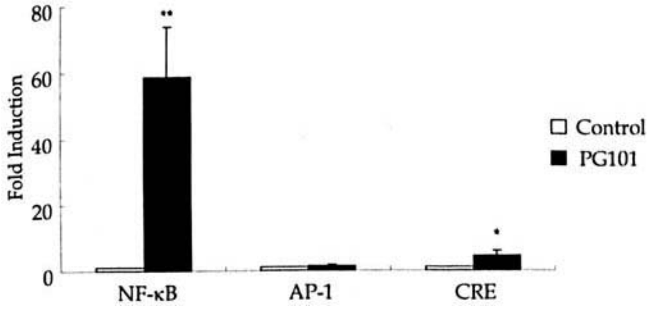


**Figure 5.** Activation of transcription factors by PG101 stimulation. 293 cells were transfected with 2 μg of reporter plasmids (Luc-NF-κB, Luc-CREB, and Luc-AP-1) and a control plasmid expressing lacZ by calcium phosphate method. Six hours later, PG101 was treated at 250 μg/ml for 24 hr. Cells were harvested and assayed for luciferase and β-galactosidase activities. The level of luciferase activity was normalized by transfection efficiency using the level of β-galactosidase activity. Fold induction was calculated by comparing PG101-treated cells with untreated cells. Data are expressed as means ± SD in three separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs control (Student's *t* test)

## Discussion

PG101 appears to be a potent immune modulator. This water soluble extract from an edible mushroom, *L. lepideus*, could significantly increase the production of several cytokines known to be involved in controlling various types of

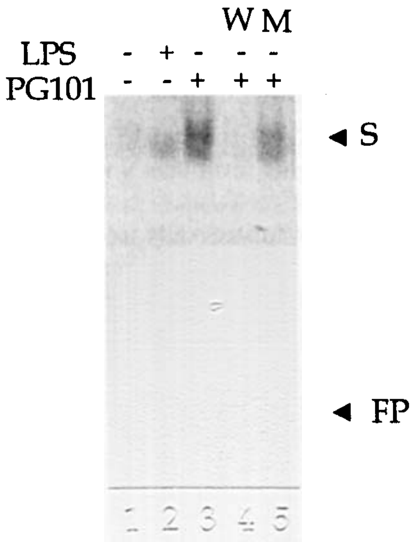


**Figure 6.** Induction of NF-κB-binding activity by PG101 in human monocytic cells. EMSA was performed with [$^{32}$P]-labeled NF-κB oligonucleotide. Nuclear extract prepared from U937 either untreated or stimulated with PG101 were incubated with a labeled probe. Specificities of DNA-protein complexes were verified by competition analysis, which involved incubating the protein from Lane 3 with unlabelled wild-type (W) or mutated (M) oligonucleotide (Lanes 4 and 5). S, specific DNA-protein complex; FP, free probe.

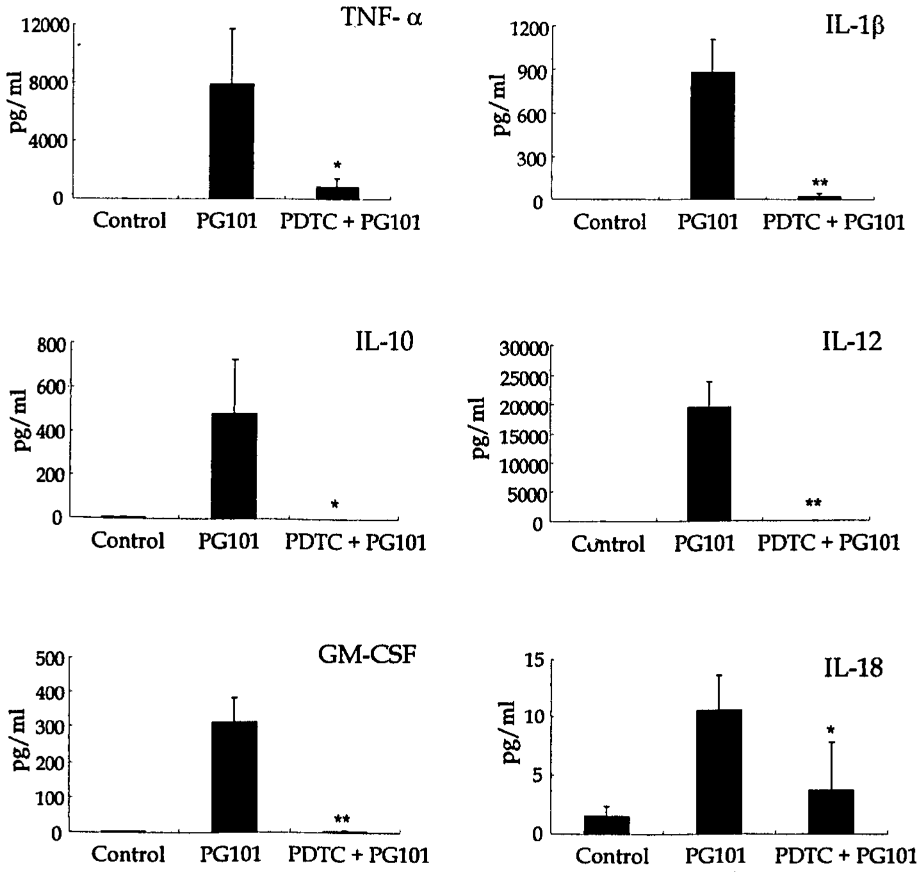


**Figure 7.** Involvement of NF-κB in PG101-induced cytokine expression. Human PBMCs were pretreated with 100 μ*M* PDTC for 1 hr and were then treated with PG101 for 24 hr. Supernatants were removed for detection of cytokine levels using ELISA. Cell numbers were determined to ensure that the equal cell number per well was sampled. Data are expressed as means ± SD in three independent experiments. ELISA readings are expressed in picograms per milliliter. $^*P < 0.05$; $^{**}P < 0.01$ vs PG101-treated group (Student's *t* test).

immune cells. The effect was prominent in Type I and II cytokines (TNF-α, IL-1β, IL-10, and IL-12). The responses were dose dependent and showed distinctive time kinetics. When a 10-fold induction was used as a basis, it appears that TNF-α was the first detectable cytokine, and next in a sequential manner was IL-1β→ (IL-10, IL-12)→ (GM-CSF, IL-18). Despite its role as a potent immune modulator, PG101 was not cytotoxic in various human cells, including PBMCs. Our preliminary data from animal experiments also indicated that PG101 is not toxic at 4 g/kg in rat, which is 20- to 80-fold higher level than a biologically effective concentration.

Macrophages or related cells as defined by CD11b/Mac-1-positive cells seem to be the major target of PG101. When PBMCs were fractionated by a panning method, cells of monocytes/macrophages lineage, but not T and B cells, responded to PG101. Consistent with this result, the human cell line of monocyte origin, but not those of T cell and B cell lineages, showed the cytokine expression profile similar to PBMCs or primary macrophages after treatment with PG101. It should be noted that macrophages are indeed known to produce TNF-α, IL-1β, IL-10, IL-12, GM-CSF, and IL-18 (30, 38–40).

The roles of each cytokines affected by PG101 are distinct and multifunctional. Inter-regulation of cytokines is a crucial aspect of immune regulation. TNF-α and IL-1β exert multiple pro-inflammatory effects on a broad range of cell types and play essential homeostatic roles in an inflammatory process by regulating other cytokines (41). IL-12 activates macrophages and also generates $T_H1$ and cytotoxic T cells, whereas it suppresses IgG and IgE production (42). IL-18 augments NK cell activity in synergy with IL-12, resulting in the induction of IFN-γ and $T_H1$ development (43). GM-CSF has been well known to stimulate hematopoiesis. This cytokine has the potential ability to enhance antitumor immunity by augmentation of tumor antigen presentation (44). IL-10 is well known for its inhibitory effect on TNF-α and inflammatory reactions (45, 46). IL-10 mediates downregulation of $T_H1$ response by inhibiting the production of IL-12 in macrophages and also protects the host from the harmful effects of prolonged inflammatory response (46).

Cellular transcription factor NF-κB appears to play a key role in PG101-mediated activation in a selected cell. It is well known that NF-κB is required for optimal expression of many cytokines, including TNF-α, IL-1β, IL-12, and other colony stimulating factors. NF-κB is usually induced by various stimulators. In responses, NF-κB interacts with the basal transcription apparatus as well as many other cellular factors to coordinate gene expression from various promoters (47). Based on our results, we propose that PG101 interacts with macrophages or related cells, somehow activates NF-κB, and sets off a series of reactions producing a variety of cytokines in a sequential manner. PG101 contains many features ideal for a therapeutic and safe BRM. It was isolated from the mycelia of an edible mushroom and showed almost no cytotoxicity in culture cells and little toxic effect in the rat model. Selective cell types are affected to produce specific cytokines that play important roles in the regulation of various immune pathways. Considering the type of affected cytokines, it is possible that PG101 could be used to enhance the immune system in immunosuppressed or immunocompromised individuals or to control the hematopoiesis of specific cell types or lineages. In summary, PG101 demonstrates a great promise as an immune modulator.

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