Alleviation of Experimental Septic Shock in Mice by Acidic Polysaccharide Isolated from the Medicinal Mushroom *Phellinus linteus*

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This study reports that acidic polysaccharide (PL) isolated from *Phellinus linteus* alleviated the septic shock induced by high dose lipopolysaccharide (LPS) injection in mice. To examine the origin of this effect, we investigated cytokine production in serum and the expression of MHC II in B cells and macrophages in areas of inflammation. Pretreatment with PL 24 h before LPS administration resulted in a significant inhibition of up to 68% of circulating tumor necrosis factor (TNF-α), a moderate reduction of 45% of interleukine (IL)-12 and 23% of IL-1β, but no significant reduction in IL-6. In addition, the expression of MHC II in B cells and macrophages was examined. Our results show that LPS-stimulated cytokine release and the level of MHC II can be modulated by *in vivo* administration of soluble PL in mice. The decrease of IL-1β, IL-12 and TNF-α in sera and the down-modulation of MHC II during septic shock may contribute to the long survival of mice by PL. Administration of PL in *in vivo* decreases IL-2, IFN-γ and TNF-α production in splenocytes and enhances spontaneous cell apoptosis in macrophages and lymphocytes stimulated with LPS in *vitro*. Thus, part of the anti-inflammatory effects of PL treatment in *in vivo* may result from the enhanced apoptosis of a portion of the activated macrophages and lymphocytes. The ability of PL to significantly reduce the TNF-α production indicates the potential of the polysaccharides in possible therapeutic strategies that are based on down-regulation of TNF-α.

Key words *Phellinus linteus*; septic shock; lipopolysaccharide (LPS); MHC II; macrophage; B cell

Sepsis caused by gram-negative bacilli or gram-positive cocci represents a major source of morbidity and mortality in medical facilities today.1,2) The reasons for the high incidence of bacterial sepsis are probably related to several key factors. Tumor necrosis factor-α (TNF-α) especially plays a major role in the pathogenesis of septic shock and related syndromes.3) Also, TNF-α, a pleiotropic polypeptide cytokine produced from macrophages and dendritic cells, plays a key role in the host immune response directed towards restricting the spread of pathogens. Certain infectious diseases can, however, induce exaggerated production of inflammatory cytokines, such as TNF-α, interleukine (IL)-1β, IL-6, and IL-12.4) Patients with septic shock show detectable levels of circulating TNF-α.5) Also, TNF-α has been shown to activate human immunodeficiency virus (HIV) mRNA,6) increase HIV replication,7) and enhance virus-mediated syncytium formation.8) Elevated TNF-α levels have also been observed in malaria patients,9) and antimalarial drugs used in cerebral malaria have been reported to reduce TNF-α levels.10) These observations provide the rationale for the development of potential therapeutic strategies based on the down-regulation of TNF-α and other inflammatory cytokines. Also, activation of cellular immune responses requires the expression of major histocompatibility complex (MHC) molecules in cells of the innate (macrophages) and adaptive (B cells) immune systems.11) In sepsis, the up-regulation of MHC II molecules and co-stimulatory molecules in macrophages and B cells is a hallmark of an overactivated immune system.12) In addition to the down-regulation of MHC II and co-stimulatory molecules on antigen-presenting cells are approved for use in diverse conditions such as endotoxic shock, immune deficiency, malarial infection, and inflammatory joint disease.13)

It is generally accepted that polysaccharides isolated from mushrooms and plants enhance various immune responses *in vivo* and *in vitro*.14,15) Also there are many reports of mushroom polysaccharide-induced nonspecific resistance against diverse microbial pathogens. These include protective effects against bacterial infections involved in sepsis, such as *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.16) Recently, some β-(1,3)-glucans that show potent immunomodulatory activity have been developed. Ami- nated β-(1,3)-glucans have been shown to induce resistance to bacterial infection17) and cause regression of solid tumors in mice.18) Curdlan, a β-(1,3)-glucan from *Aldagaliones faecalis* var. *myxogens*, sulfated to yield curdlan sulfate, has been shown to be a compound capable of inhibiting HIV infection *in vitro*,19) and is currently undergoing clinical trials.20) Lentinian is another β-(1,3)-glucan with β-(1,6)-glucopyranoside branches which is approved for clinical use as an adjunct cancer therapy.15) In addition, active polysaccharides isolated from the mushroom *Phellinus linteus* have been intensively investigated with regard to their immunological activities21) and the inhibition of tumors and metastasis.22)

In the present study we analyzed the effects of the *in vivo* administration of soluble acidic polysaccharide (PL), isolated from oriental medicinal mushroom *P. linteus*, on circulating proinflammatory cytokines such as IL-1, IL-12 and TNF-α, and interferon (IFN)-γ, production and the expression of MHC II in endotoxin-stimulated peritoneal B cells and macrophages. PL suppressed IL-2, IL-6, IFN-γ and TNF-α in splenocytes and down-regulated MHC II expression in B cells and macrophages. Also, PL administration prolonged the survival of septic shock mice. Therefore, we suggest that *in vivo* treatment with PL not only suppresses the circulating cytokine levels, but also may prolong the survival of these...
MATERIALS AND METHODS

Mice and Septic Shock Induction Eight- to twelve-week-old female BALB/c mice were used. These mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). The animal care and experimental procedures conformed to the regulations of the Pusan National University Animal Care and Use Committee. The model for LPS-induced septic shock syndrome in BALB/c mice was established with the intraperitoneal (i.p.) injection of 8 mg/kg of *E. coli* 026:B6 LPS (Sigma, St. Louis, MO, U.S.A.). The mice were treated with PL (100 mg/kg) 24 h before LPS injection. After 24 h of LPS injection, the mice were exsanguinated from the orbital sinus and sacrificed for analysis of cellular surface markers.

Characterization of *P. linteus* Polysaccharide The active PL was isolated from *P. linteus* using ethanol precipitation methods, followed by DEAE-cellulose and gel filtration chromatography.25) According to profiles of the gel filtration, the molecular weight of PL was estimated to be approximately 150 kDa. The main components of PL are polysaccharides that consist of mannose, glucose and galactose. Endotoxin was assayed under endotoxin-free experimental conditions using a *Limulus* Amebocytes Lysate (LAL) Pyrogen Kit (BioWhittaker, Walkersville, MD, U.S.A.). Experiments were conducted according to the manufacturer’s protocol. Briefly, 100 μl of standards, PL or controls were mixed with 100 μl of LAL reagent and incubated for 1 h at 37°C. Each tube was then examined for gelation. The quantity of endotoxin in PL was ≤0.01 ng/ml.

Cytokine Analysis A standard enzyme linked-immunosorbent assay (ELISA) method was used for a cytokine assay with sera and culture supernatants. Briefly, a 96-well Immuno-Maxisorp Plate (Nunc, Roskilde, Denmark) was coated with murine anti-IL-1β, anti-IL-2, anti-IL-6, anti-IL-12, anti-IFN-γ and anti-TNF-α (R&D Systems, Minneapolis, MN, U.S.A.) overnight at 4°C and blocked with 10% PBS in PBS. Sample sera were diluted at a ratio of 1/10000 and incubated for 2 h at 37°C. After washing, bound Ab isotypes were detected with a biotin-conjugated anti-rat antibody. Thereafter, the plates were washed and incubated with 100 μl of 2,2-azinodi(3-ethylbenzthiazoline sulfonate) substrate (ABTS; Boheringer Mannheim, Indianapolis, IN, U.S.A.) at 1 mg/ml and read at 405 nm. A parallel experiment was performed in vitro in the culture supernatant of splenocytes from mice with PL treatment in vivo for 24 h before PBS (negative control), LPS or PL in vitro.

Monoclonal Antibodies (mAbs) To evaluate the expression of co-stimulatory and adhesion molecules, the following mAbs were used against murine cell surface markers: PE-conjugated anti-MHC II, anti-CD25, anti-CD49 and anti-NK1.1, (2) FITC-conjugated anti-CD3 and anti-CD19. All antibodies were purchased from PharMingen (San Diego, CA, U.S.A.) and used according to the manufacturer’s instructions.

MHC II Expression of Peritoneal Macrophages and B Cells CD45R/B220^+^ B cells were purified from peritoneal exudate cells by positive selection using a magnetic cell separator (MACS; Miltenyi Biotec, Germany) according to the manufacturer’s instructions. Purity was assessed by FACS analysis using a PE-labeled anti-CD19 mAb (PharMingen, San Diego, CA, U.S.A.) at >95%. Macrophages were allowed to adhere for 3 h at 37°C in a 5% CO₂ humidified atmosphere. The purity of macrophages was determined, by the examination of cytoplasm stained with acridine orange using a fluorescence microscope, to be >95%. To analyze MHC II expression, the cells were initially incubated for 10 min at 4°C with Fc-Block (PharMingen, San Diego, CA, U.S.A.) to avoid nonspecific binding. The cells were then incubated with mAbs (1 μg/1×10⁶ cells) for 30 min at 4°C in the dark. The fluorescence was controlled by unrelated antibodies labeled with fluorochromes. The cells were washed with 2% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) in PBS by centrifugation at 400×g and fixed with 1% (v/v) paraformaldehyde in PBS. In a parallel experiment, splenocytes were stained with anti-CD3, anti-CD19, anti-CD25, and anti-CD49.

Cell Viability Assay Cell viability was measured by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Splenocytes (5×10⁶ cells/ml) from mice stimulated with PL for 24 h were cultured in 96-well plates and incubated with vehicle, PL, or LPS. After treatment for 72 h, the cells were washed twice with PBS and incubated with 110 μl of 0.5 mg/ml MTT for 3 h at 37°C. The medium was discarded and 100 μl acidic isopropanol (0.04 N HCl) was then added. After 20 min incubation, a microplate reader was read the optical density at 570 nm.

Statistical Analysis Unless otherwise indicated, the results are expressed as the mean±S.D. of data obtained from triplicate experiments. Statistical analysis was performed by a paired t-test. Differences of *p*<0.05 were considered statistically significant.

RESULTS

**PL Prolongs the Survival of Septic Shock Mice** For the induction of sepsis, 8-week-old female BALB/c mice (*n* = 5) were injected i.p. with 8 mg/kg of LPS. To analyze the effect of PL on the LPS-induced septic shock mice, they were pretreated i.p. with 100 mg/kg of PL 24 h before the LPS administration. The control group was treated with PBS in the absence of PL or LPS. The effects of PL on the survival rate of septic shock mice with LPS are shown in Fig. 1. PL prolonged the survival rate in sepsis. During the treatment with PL alone, no life-threatening toxicity was detected (data not shown). These results indicate that treatment with PL prolongs survival in septic mice.

**PL Inhibits LPS-Induced Cytokine Production** To gain insight into the scope of cytokine production in this system, we compared the cytokine concentration of sera in vivo pretreated with PL to that of sera from PL-ununtreated animals. The results presented in Fig. 2 show a marked production of TNF-α in septic mice compared with that of control mice. In contrast, mice treated with PL 24 h before LPS treatment showed statistically significant inhibition (68%, *p*<0.05 vs. LPS-stimulated mice) of TNF-α production. IL-12 and IL-1β production was similarly inhibited. Interestingly, there were no significant differences in IL-6 production between the PL-pretreated group and the PL-ununtreated group.
PL Decreases the Expression of MHC II Molecules in Peritoneal Macrophages and B Cells  To evaluate the expression of MHC II in macrophages and B cells, cells were isolated from endotoxin-shock mice with or without PL administration. Representative FACS histograms in Fig. 3A show the up-regulation of MHC II in macrophages and B cells during endotoxin-shock mice, but not in PL-administered mice. A significant difference was observed in the percentage of MHC II+ peritoneal macrophages and B cells isolated from the PL-treated or un-treated septic shock mice (Fig. 3B). The cells of the PL-administered mice showed similar MHC II expression in comparison with that of the control mice. But the MHC II expression level was diminished in the cells from the PL-treated mice represented by the mean fluorescence intensity (MFI) (Fig. 3C), in comparison to that observed in cells from the control cells. The above data indicate that decreases in MHC II expression induce blocking of the secondary signal into T cells. Thus, T-cell-mediated immunity may be lessened in septic shock animals pretreated with PL.

Expression of Surface Molecules in Splenocytes of Septic Mice  In order to determine whether PL influences surface molecule expression in splenic T and B cells, the expression of CD25 and CD49 were analyzed. FACS analysis showed that the PL-treated septic mice showed similar patterns to the PBS-treated mice in T and B cell populations, as well as CD25 and CD49 expression. However, only LPS-treated mice showed a comparable increase in CD3+ and CD3+/CD49+ populations (Fig. 4). These effects may be related in augmentation of the CD3+ population in spleen through the activation of macrophages and B cells in areas of inflammation. A previous study has also demonstrated that inhibition of septic shock in mice was induced by augmentation action of the NKT cell population in the spleens of experimentally-induced septic shock mice. So, we investigated whether NK1.1+CD3+ (NKT) cells were related to the suppression of endotoxin-shock in the spleens of PL-treated septic mice. However, we showed that PL has no effect on NKT cell population (data not shown). These results suggest that PL alleviates septic shock irrespective to NKT cell activation.

PL-Pretreatment Decreases Endotoxin-Induced Cytokine Release in Splenocytes  To investigate the effects of in vivo PL-pretreatment on splenocyte cytokine production, we treated mice with PL 24 h before LPS injection. As shown in Fig. 5, LPS-stimulated (in vitro) splenocytes from PL-pretreated (in vivo for 24 h) mice showed significant differences in cytokine production in comparison with just PL-treated cells (in vitro). Interestingly, in the splenocytes isolated from PL-pretreated mice, IL-2, IL-6, IFN-γ and TNF-α production was significantly lower in comparison with that of mice treated with LPS in vitro (Fig. 5). Thus, in vivo pre-
treatment with PL for 24 h may suppress the magnitude of lymphocyte cytokine production in the case of stimulation with LPS \textit{in vitro}. These results, consistent with the serum cytokine level (Fig. 2), show that \textit{in vivo} treatment with PL also suppresses the magnitude of LPS-stimulated cytokine production.

Cell Viability of Splenic Macrophages and Lymphocytes by PL in Septic Shock Mice To investigate the effects on cell viability, macrophages and lymphocytes isolated from the splenocytes of PL-treated mice showed slightly decreases in the percentage of cell viability compared with untreated cells (Fig. 6). A significant decrease in cell viability was observed \textit{in vitro} LPS-stimulated cells isolated from PL-treated mice. Macrophages isolated from PL-treated mice showed a marked decrease in cell viability compared to that of lymphocytes isolated from the same mice. With this data, we addressed the fact that the \textit{in vivo} administration of PL enhanced the spontaneous cell apoptosis of macrophages and lymphocytes in a septic shock model. Thus, some of the anti-inflammatory effects of PL treatment \textit{in vivo} may result from the enhanced apoptosis of a portion of the activated macrophages and lymphocytes.

Fig. 3. MHC II Expression in the Peritoneal Macrophages and B Cells from Mice Treated with LPS (8 mg/kg) in the Absence or Presence of PL (100 mg/kg)
(A) Representative histograms of MHC II were depicted in untreated control (thin line) or PBS (top panel)/LPS (intermediate panel)/LPS+PL (bottom panel). The percentage (B) and MFI (C) was represented.

Fig. 4. Expression of CD25 and CD49 of T or B Cells after PL Treatment for 24 h Prior to LPS Administration
Female BALB/c mice were intraperitoneally injected with 8 mg/kg LPS after 24 h in the absence or presence of 100 mg/kg. PL was administered intraperitoneally before 24 h of LPS-administration. The cells were analyzed by flow cytometry.
DISCUSSION

There are many reports of mushroom polysaccharides inducing nonspecific resistance against diverse microbial pathogens in animals
as well as humans. However, the mechanism of the modulation of the immune responses by immunomodulators, such as mushroom polysaccharides, especially β-glucan, is not well understood. Considering the main role that proinflammatory cytokine production plays in the pathogenesis of septic shock, we have examined how the in vivo administration of PL can modulate circulating cytokine responses in LPS-treated mice. The present study shows that pretreatment with PL can significantly down-regulate proinflammatory cytokines, especially TNF-α and IL-12 production, in LPS-treated mice. The major outcome of exposure to LPS is the production of a cascade of proinflammatory cytokines, where TNF-α can regulate the production of IL-1β. Evidence for the involvement of TNF-α in diverse conditions such as endotoxic shock, immune deficiency, inflammatory joint disease, allograft rejection and cachexia has also been steadily accumulating during the post decade. These observations further suggest that PL may be able to inhibit bacterial septic shock by suppressing TNF-α production in areas of inflammation.

Also, in sepsis, the up-regulation of MHC II in macrophages and B cells is a hallmark of an overactivated immune system, so we investigated the expression of MHC II molecules in peritoneal macrophages and B cells. The down-regulation of MHC II, both in macrophages and B cells, may be beneficial in controlling not only acute inflammatory responses, but likely also chronic inflammatory immune reactions. This suggests that PL may also be effective in the prevention and possibly the suppression of bacterial septic shock. Secondarily, VLA-4 was suppressed in PL-treated peritoneal T cells (data not shown). Also, NK1.1+ CD3+ cells (NKT cells) are believed to be involved in immune responses ranging from the suppression of autoimmunity to tumor rejection, and Khan and coworkers were reported that splenic NKT cells were activated in LPS-treated mice. However, we did not find any significant differences in splenic NK or NKT cell populations (data not shown). These cells are quite selectively located in the liver, and their involvement in a septic shock model may not be obvious.

Jimenez et al. recently reported that delayed apoptosis contributed to postoperative systemic inflammatory response syndrome. It was reported that treatment with β-(1,6)-branched β-(1,3)-glucan modulated endotoxin- and enterotoxin-induced cytokine release and apoptosis in vivo. We found that the in vivo administration of PL enhanced spontaneous cell cytotoxicity in macrophages and lymphocytes in our septic shock model. Thus, part of the anti-inflammatory effects of PL treatment in vivo may result, to a certain extent, from the enhanced apoptosis of the activated macrophages.
and lymphocytes.

Taken together, our results demonstrate that cytokine release induced by LPS stimulation can be manipulated by the in vivo administration of PL in a septic shock mouse model. These results may contribute to the possible practical application of PL in reducing the severity of septic shock, especially in situations where application of anti-cytokine or immunosuppressive drug treatment may exacerbate systemic infection or worsen the outcome in a patient with sepsis.37,38)

munosuppressive drug treatment may exacerbate systemic infection or worsen the outcome in a patient with sepsis.37,38) We have yet to be elucidated the exact mechanisms involved in down-regulation of TNF production by PL.

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REFERENCES