Immunomodulating Activities of β-Glucan of *Ganoderma lucidum* by Binding to Dectin-1 Receptor

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Abstract: β-Glucan is a glucose polymer that contains β-(1, 3), β-(1, 4) and β-(1, 6) linkages. It is found exclusively in fungal and bacterial cell walls, and is not present in animals. β-Glucans are recognized by the innate immune system. Dendritic cells (DC) or macrophages possess a pattern recognition molecule (PRM) for binding β-glucan in a pathogen-associated molecular pattern (PAMP). Recently, a β-glucan receptor was cloned from DC and designated as dectin-1, which belongs to the type II C-type lectin family. In our macrophage cell line culture system, dectin-1 mRNA was detected in RAW264.7 cells by reverse transcription-polymerase chain reaction (RT-PCR). Dectin-1 was also detected in the murine spleen, thymus, lung and intestines. Treatment of RAW264.7 cells with β-glucans of *Ganoderma lucidum* (GLG) resulted in increased expression of IL-6 and TNF-a in the presence of LPS. However, GLG alone did not increase IL-6 or TNF-a. These results seem to indicate dectin-1 receptor cooperates with dectin-1 and CD14 for activation of signal transduction.

Key words: Immunomodulation, β–glucan, *Ganoderma lucidum*, dectin-1 receptor

1 Introduction

β-(1,3)-D-Glucans are glucose polymers found in the cell walls of fungi and bacteria and their conserved structures can be considered as classical pathogen-associated molecular patterns (PAMPs).[1] They are also known as biological response modifiers and have a variety of effects on the immune system, including antitumor[2] and anti-infective activities against fungi,[3] bacteria,[4] viruses,[5] and protozoa.[6] Many β-glucans have immuno-pharmacological activity,[7] although the activity varies with each glucan.[8] The differences in activity are at least partially dependent on their structural parameters, such as solubility, molecular mass, degree of branching and conformation.[9-11] β-Glucans possess anti-infective and anti-tumorigenic properties that stem from their ability to activate leukocytes, stimulating their phagocytic activity, the production of reactive oxygen intermediates, inflammatory mediators and cytokines, including TNF-α.[2, 12] Some data also suggested that β-glucans could promote T cell-specific responses, perhaps through triggering the secretion of IFN-γ, IL-6, IL-8, and IL-12 from macrophages,[13] neutrophils,[14] and NK cells[15] to kill sensitive tumor cells.
Dectin-1 is a β-glucan receptor, a Ca^{2+}-dependent lectin-like receptor, whose gene is located on mouse chromosome 6 and human chromosome 12. The dectin-1 mRNA is highly expressed in dendritic cells.[16] The presence of dectin-1 on dendritic cells or macrophages has been controversial. Dectin-1, as a β-glucan receptor from murine macrophages, possessed a single C-type lectin-like carbohydrate recognition domain (CRD) connected to the transmembrane regions by a stalk and a cytoplasmic tail possessing immunoreceptor tyrosine-based activation motif (ITAM).[17] Murine and human dectin-1 protein has 244 and 247 amino acids (33-43 kDa glycoprotein), respectively. Dectin-1 that was found to be widely expressed in mouse and human tissues acted as a pattern recognition receptor by recognizing a variety of carbohydrates containing β-1,3- and/or β-1,6-glucan linkages, intact *Saccharomyces cerevisiae* and *Candida albicans*.[18] In addition, the receptor could bind to T-lymphocytes[17] but at a site distinct from that which recognized β-glucans.[18]

The aim of the present investigation was to study the effect of a β-glucan alone, or in combination with LPS, on cytokine mRNA production activity by RT-PCR in β-glucan treated murine macrophage RAW264.7 cells.

2 Materials and Methods

2.1 Reagents

β-Glucans of *Saccharomyces cerevisiae* (SCG) and laminarin from *Laminaria digitata* and LPS were purchased from Sigma-Aldrich Co. (St. Louis, USA). β-Glucan of *Ganoderma lucidum* (GLG) was partially purified from the fruit bodies. Phycoerythrin hamster anti-mouse TNF receptor type II (p75) monoclonal antibody (mAb) was purchased from BD Biosciences Pharmingen (San Diego, USA). PCR primers were synthesized by Takara-Korea Biomedicals Inc. (Seoul, Korea). Two-step RNA PCR kit was purchased from Takara Biomedicals (Japan). DMEM, RPMI, penicillin-streptomycin and TRIzol reagent were purchased from Gibco-BRL (Grand Island, NY, USA). AMV reverse transcriptase was purchased from Promega (USA).

2.2 Cell culture

Mouse monocyte-macrophage RAW264.7 (ATCC, Rockville, MD) cells were maintained in DMEM medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% heat inactivated fetal bovine serum (JRH Biosciences Co., Lenexa, USA), penicillin (100 μg/ml)-streptomycin (100 unit/ml) (Life Technologies, Inc.) and incubated at 37°C in 5% CO_2.

2.3 RNA isolation

Total RNA was extracted from the cultured RAW264.7 cells or mouse tissues with TRIzol reagent according to the manufacturer's instructions. The cells were lysed by adding 1 ml of TRIzol reagent to a 6-well plate and passing the cell lysate several times through a pipette. Mouse tissues were lysed by adding 1 ml of TRIzol reagent and homogenized in a homogenizer. The cell lysates were incubated for 5 minutes at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added and the lysates were shaken vigorously for 15 seconds and incubated at room temperature (RT) for 2 to 3 minutes. Centrifugation was performed at 12,000 × g for 15 minutes at 2 to 8°C. The aqueous phase was transferred to a fresh tube and RNA was precipitated by addition of isopropyl alcohol. The RNAs were incubated at RT for 10 minutes and centrifuged at 12,000 × g for 10 minutes at 2 to 8°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. They were mixed by vortexing and centrifuged at 7,500 × g for 5 minutes at 2 to 8°C. The RNA pellet was briefly dried, dissolved in RNase-free water, and stored at -70°C until used.

2.4 Reverse transcription-polymerase chain reaction (RT-PCR)

A two-step RNA PCR kit (Takara Biomedicals, Japan) was used for the reverse transcription from RNA to cDNA using AMV reverse transcriptase and subsequently amplified by utilizing AMV-optimized *Taq* DNA polymerase. The PCR primers were designed on the basis of the sequence of mouse dectin-1 (MD-1, AF262985), TNF-α (NM-013693), and IL-6 (X54542) (Table 1).

Table 1. Primer sequences for RT-PCR analysis

Gene	Sequence
MD-1F (Forward)	5'-GCCCTGTGAAGCAATGAAATATC-3'
MD-1R (Reverse)	5'-CACATACATTTACAGTTCCTTCTCACAGA-3'
TNF-α F (Forward)	5'-CGGGATCCATGAGCACAGAAAGCAT-3'
TNF-α R (Reverse)	5'-CCCAAGCTTTCACAGAGCAATGACTCC-3'
IL-6-F (Forward)	5'-ATGAAGTTCCTCTCTGCAAG-3'
IL-6-R (Reverse)	5'-GGTTTGCCGAGTACATCTCA-3'

The RT-PCR program consisted of 30 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 59, 65 and 55°C for annealing MD-1, TNF-a and IL-6, respectively, and polymerized for 1 minute at 72°C. The PCR products were identified by 2 % agarose gel electrophoresis.

2.5 Flow cytometry

Cells were washed twice with PBS, and then re-suspended in PBS at a concentration of 1×10^6 cells/ml. One hundred µl of cell suspension was transferred to a 1.5 ml centrifuge tube. Cells were then incubated at room temperature in the dark for 15 minutes with 5 µl of 1 µg/5 µl phycoerythrin hamster anti-mouse TNF receptor type II (p75) mAb. After adding binding buffer or PBS (400 µl), cells were analyzed by flow cytometry with FACSCalibur™ (Becton Dickinson Co., USA). The percentage of positive cells was determined from cells that bound TNF receptor-PE. At least 5000 cells were analyzed for each sample.

2.6 Statistics

All statistical analyses were performed using Sigma-Stat 3.0 (Statsoft, USA). Data were analyzed by one-way ANOVA (analysis of variance) followed by Holm-Sidak method. P-values <0.05 were considered significant.

3 Results

3.1 Expression of dectin-1 α and β mRNA in RAW264.7 macrophage and mouse organs

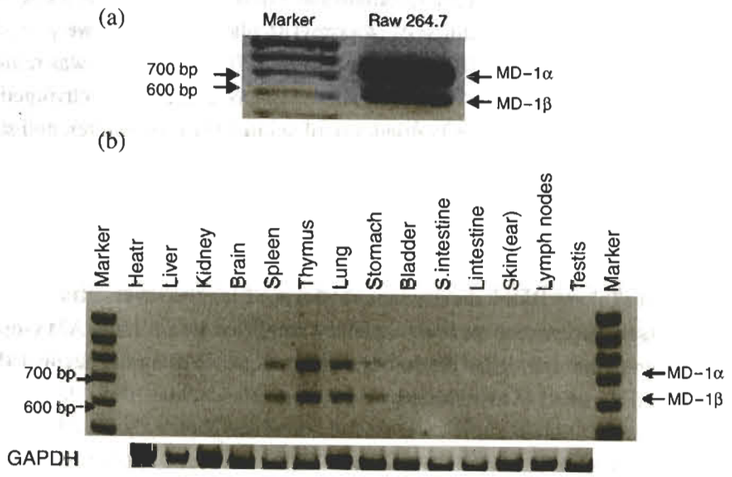

Figure 1. RT-PCR expression analysis of the murine dectin-1 (MD-1) a and β with primers that could detect the full length (MD-1α) and splice variant (MD-1β)

(a) Expression of MD-1 α and β mRNAs in RAW264.7 murine macrophage cell line.
(b) Expression of MD-1 α and β mRNAs in the murine thymus, lung, spleen, stomach and intestine isolated from ICR mice. RT-PCR for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) served as the control.
To detect dectin-1 expression, we investigated RAW264.7 macrophage cell line by RT-PCR using MD-1 primers. Murine dectin-1 primers could detect both the full length, 757 bp (MD-1α), and spliced variant isoform, 620 bp (MD-1β). The MD-1 a-receptor is known to possess a single D-type lectin-like domain, transmembrane region, stalk region, and cytoplasmic tail containing an ITAM whereas the MD-1 β-isoform has a deleted stalk region. RT-PCR analysis showed that both dectin-1 α and β mRNAs were expressed in RAW264.7 murine macrophage cell line (Fig. 1a). Then, we further analysed the expression of dectin-1 α and β mRNAs in various organs of the mouse by RT-PCR analysis. Both dectin-1 α and β mRNAs were also expressed in various organs such as the thymus, lung, spleen, stomach and intestine isolated from ICR mice (Fig. 1β). Dectin-1 α and β were widely expressed in a variety of lymphoid organs with strong expressions in the thymus, spleen, and digestive organs such as the stomach and intestine.

3.2 GLG increased expression of TNF-α and IL-6 in LPS-treated macrophage

By stimulating macrophages together with LPS, β-glucan as a PAMP could activate macrophages and induce production of various cytokines. We investigated the effect of β-glucan on RAW264.7 macrophages in the absence or presence of LPS for 12 hrs. Expression of TNF-α and IL-6 mRNA was analyzed by RT-PCR. Treatment of the cells with LPS alone induced weak TNF-α. A concentration of 100 µg/ml GLG alone induced much stronger induction of TNF-α than LPS alone. At 300 µg/ml GLG, TNF-α was induced much stronger than by 100 µl/ml GLG indicating concentration dependent induction of TNF-α in macrophages by β-glucan treatment. When GLG was added to the LPS containing culture system, TNF-α was increased much more strongly than the culture system without LPS (Fig. 2a). For the IL-6 expression analysis, RAW264.7 cells were treated with β-glucans of SCG, GLG and laminarin in the absence or presence of LPS for 12 hrs. All three β-glucans did not induce IL-6. However, they strongly induced IL-6 expression in the presence of LPS. Among the β-glucans, GLG showed the strongest synergistic effect on the expression of IL-6 with LPS (Fig. 2b).

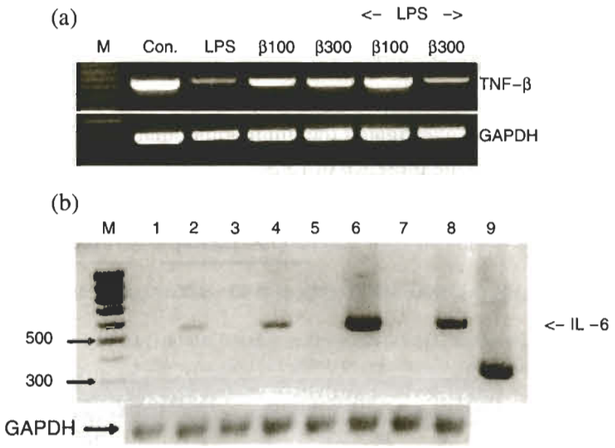

Figure 2. β-Glucan induced TNF-α and IL-6 mRNAs expression in macrophages

RAW264.7 cells were treated with 100 µg/ml β-glucan in the absence or presence of LPS (100 ng/ml) for 12 hrs. (a) RT-PCR analysis for TNF-α expression by treating RAW264.7 cells with 100 µg/ml and 300 µg/ml of GLG in the absence or presence of LPS for 12 hrs. (b) RT-PCR analysis for IL-6 expression by treating RAW264. 7 cells with 100 µg/ml of GLG. M is size marker; lane 1: control; lane 2: LPS (100 ng/ml), lane 3: SCG (100 µg/

ml), lane 4: SCG (100 μg/ml) plus LPS (100 ng/ml), lane 5: GLG (100 μg/ml), lane 6: GLG (100 μg/ml) plus LPS (100 ng/ml), lane 7: laminarin (100 μg/ml), lane 8: laminarin (100 μg/ml) plus LPS (100 ng/ml); lane 9: IL-6 positive control. GAPDH was used as control in RT-PCR.

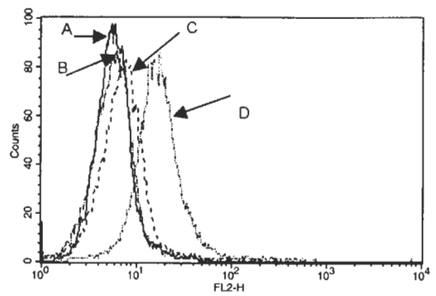

Figure 3. FACS analysis of TNF receptor type II induced by LPS and β-glucan
RAW264.7 cells were treated with (B) LPS (100 ng/ml), (C) GLG (100 μg/ml), and (D) LPS (100 ng/ml) plus GLG (100 μg/ml) for 6 hrs. (A) is autofluorescence of the cell

3.3 Effect Ca^{2+} on binding of β-glucan to dectin-1

β-Glucan binds to dectin-1 in the presence of Ca^{2+}. To investigate Ca^{++}-dependent binding of β-glucan to dectin-1, our system was treated with EDTA, a Ca^{++}-chelator. RAW264.7 cells were treated with GLG or laminarin for 12 hrs in the presence of 10 nM EDTA and/or LPS. Treatment with laminarin or GLG in the presence of LPS led to the expression of IL-6. However, addition of EDTA to this system abolished IL-6 mRNA expression (Fig. 4) indicating a Ca^{++} requirement for binding of β-glucan to dectin-1.

4 Discussion

Glucans are a heterogeneous groups of glucose polymers found in the cell walls of plants, bacteria and fungi. β-Glucan has immuno-pharmacological activity,[7] and various biological activities including anti-tumor activity.[19] Czop & Kay[20] found a β-glucan receptor, and suggested that β-glucan was triggering immune cell activation by β-glucan receptor. Therefore, we investigated the immunomodulating gene expression by treatment with various β-glucans in macrophages in the presence of LPS.

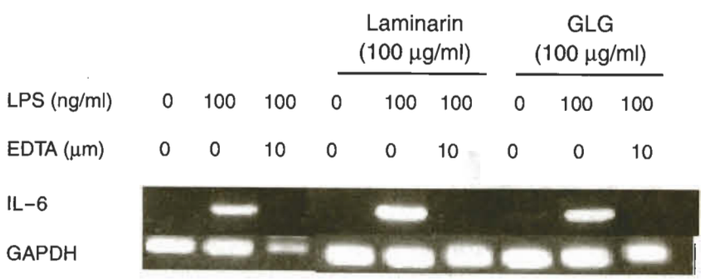

Figure 4. Effect of EDTA in IL-6 mRNA induction by β-glucan
RAW264.7 cells were treated with LPS (100 ng/ml), EDTA (10 uM), laminarin (100 μg/ml) and GLG (100 μg/ml) for 12 hrs. GAPDH served as control.

Expression of dectin-1 was not clear among immune cells. Ariizumi et al.[17] identified a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. Taylor et al.[21] found that the β-glucan receptor,

dectin-1, is widely expressed on the surface of dendritic cells as well as monocytes, macrophages and neutrophils. To identify dectin-1 expression on the macrophages among immune cells, we examined RAW264.7 murine macrophages. The RT-PCR analysis led to the detection of dectin-1 α and β in the murine macrophage RAW264.7 cell line. Dectin-1 α and β were also detected among the various lymphoid organs of mouse, the thymus, lung, spleen, stomach and intestine. The detection of dectin-1 seems to be derived from tissue macrophages/dendritic cells in the thymus or spleen, alveolar macrophages in lung, and macrophages of Peyer's patches in the intestine. We have examined the influence of β-glucans on cytokine production and activation of the immune system. When treated with b-glucan and LPS, induction of gene expression of TNF-α and IL-6 was confirmed by RT-PCR analysis. Induction of TNF-receptor type II expression was confirmed by FACS analysis. It was suggested that immunomodulation with β-glucan might stimulate expression of cytokine genes. IL-6 expression was abolished by EDTA in β-glucan and LPS treated RAW264.7 cell line, indicating that β-glucan binds to dectin-1 in a Ca^{++}-dependent manner. These results showed that β-glucan have immuno-pharmacological activity.[7] Some data also suggested that b-glucans could promote T cell-specific responses, perhaps through triggering the secretion of IFN-γ, IL-6, IL-8, and IL-12 from macrophages,[13] neutrophils,[14] and NK cells[15] to kill sensitive tumor cells. Exposure to b-glucan induced production of a cascade of pro-inflammatory cytokines, where TNF-α can regulate the production of IL-6.[22]

In conclusion, our results demonstrate that β-glucan and LPS induce various changes typical for immune modulating genes such as TNF-α, TNF receptor and IL-6. Based on this result, β-glucan might be a promising agent because it is safe and potent in anti-tumor efficacy.

Acknowledgements

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