Research Paper

Antrodia cinnamomea Galactomannan Elicits Immuno-stimulatory Activity Through Toll-like Receptor 4

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Abstract

Antrodia cinnamomea (*A. cinnamomea*) is a medicinal fungus used in traditional Chinese medicine to treat different kinds of ailments, including liver diseases, abdominal pain, drug intoxication, diarrhea, itchy skin, hypertension, and cancer. Polysaccharides have been identified as one of the major pharmacologically active ingredients present in *A. cinnamomea*. The present study aims to investigate the immunoenhancing activity of galactomannan isolated from *A. cinnamomea*. The cold water-soluble polysaccharide (galactomannan-repeated; MW>70 kDa; named ACP) of *A. cinnamomea* was isolated, and immunostimulatory properties were studied through different immune cell models including mouse macrophages and human dendritic cells. Through Toll-like receptor 4, ACP stimulated tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in J774A.1 mouse macrophages, mouse peritoneal macrophages and human dendritic cells. It was further identified that ACP elicited its immunostimulatory activity through protein kinase C-α (PKC-α) and mitogen activated protein kinases (MAPK) phosphorylation. Furthermore, ACP exerted the endotoxin tolerance-like effect through NF-κB inhibition. These findings demonstrate the potential of *A. cinnamomea* galactomannan as an immunostimulator or an adjuvant in immunotherapy and vaccination.

Key words: *Antrodia cinnamomea*, galactomannan, macrophages, dendritic cells, immunostimulation

Introduction

Medicinal mushrooms have been used as integral ingredients of dietary supplements in the last few decades. It is estimated that 80-85% of all medicinal mushrooms products are derived from fruiting bodies, which can either be artificially cultured or collected from nature [1]. The potent therapeutic activities of medicinal mushrooms, including chemopreventive and immunomodulatory activities, have garnered much attention [2-4].

Currently, important medicinal mushrooms used worldwide as dietary supplements, including *Antrodia cinnamomea*, *Cordyceps sinensis*, *Ganoderma lucidum*, *Phellinus igniarius*, *Polystictus versicolori*, *Agaricus blazei* Murill, *Coriolus versicolor*, and *Poria cocos* [5,6]. Molecules containing polysaccharides, such as the proteoglycans of *Ganoderma lucidum* [7], β-1,3-glucans and β-1,6-glucans of *Agaricus blazei* Murill [8,9], and protein-bound beta-glucan

polysaccharides (PSKs, known as Krestin in Japan) have known antitumor and immunological activities [10]. More specifically, the polysaccharides of *Ganoderma lucidum* have immunomodulatory, anti-angiogenic and cytotoxic effects [11], potential hypoglycemic and hypolipidemic activities [12], and anti-obesity and microbiota-modulating effects [13]. Lentinan of *Lentinus edodes* has antitumor, anti-virus, anti-bacteria, and anti-parasitic infections [14,15].

Antrodia cinnamomea, in the phylum Basidiomycota, grows as a parasitic fungus on *Cinnamomum kanehirai* and is a mushroom unique to Taiwan [16,17]. Historically, it has been used by Chinese ethnomedical practitioners to treat different kinds of ailments, including liver diseases, abdominal pain, drug intoxication, diarrhea, itchy skin, hypertension, and cancer [18-20]. Currently, it is considered a potent nutraceutical with very high economic value and demand. Based on its medicinal efficacy, the total market value of *A. cinnamomea* products, including raw fruiting bodies and health foods, is estimated to be more than US$100 million per year [16]. Hundreds of *A. cinnamomea* small molecules with different biological activities have been identified as active constituents [2]. The most important pharmacologically active constituents of *A. cinnamomea* are triterpenoids, steroids, benzoquinone derivatives, and polysaccharides [14]. Some crude polysaccharide fractions also have certain biological activities, including immunomodulatory, antitumor, antioxidant, and antiangiogenic activities [21-28]. The well-established chemical structures of *A. cinnamomea* polysaccharides were represented as fucosylated mannogalactan (PS) (400 kDa, major in galactose, minor in fucose and mannose, galactose: fucose: mannose in the ratio of 13: 4: 2) which showed antiangiogenic activity [27, 28] and galactomannan (ACP) (100 kDa, 75% mannose and 25% galactose) [29], with phagocytosis-enhancing activity. In the present study we investigated the immunostimulatory features of ACP, which is composed of octasaccharide repeating unit {→6)-D-Man*p*-(α1→2)-D-Man*p*-(α1→2) [D-Man*p*-(α1 → 3)-D-Man*p*-(α1 → 2)-D-Man*p*-(α1 → 6)-D-Gal*p*-(α1 → 6)]-D-Man*p*-(α1 → 6)-D-Gal*p*-(α1 →}[Figure 1].

The immune system is critical in maintaining human health in the face of infectious organisms and tumor cells [30]. Parts of this system, TNF-α and IL-6, are important pro-inflammatory cytokines produced mainly by activated macrophages, and they mediate multiple biological effects [31-33]. Thus, natural substances that can stimulate TNF-α/IL-6 have the potential to be adjuvants in immunotherapy and vaccination. Complex carbohydrates have attracted growing scientific interest over the past few decades for their immunoenhancing properties. However, their contribution as clinical adjuvants is still limited because of the structural complexity and the lack of studies on the structure-activity relationship and the underlying mechanisms of the biological activity of the individual polysaccharide components. In this study, we report the *in vitro* immunostimulatory potential of ACP on J774A.1 murine macrophage and human dendritic cell models as well as some signaling pathways involved in its mechanism of action, which deepens the current understanding of the immunomodulatory potential of this precious natural resource.

Materials and Methods

Materials

The procedures for the isolation of cold-water soluble polysaccharide and the structure elucidation of ACP were described in our recent publication [29].

LPS (from *Escherichia coli* 0111: B4), polymyxin B, peptidoglycan (PGN, from *Staphylococcus aureus*), mouse antibodies for mouse phospho-ERK1/2, phospho-JNK1/2, phospho-p38, and actin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for phospho-PKC-α/δ, IL-1β, COX-2, and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IL-1β, IL-6, TNF-α, and MCP-1 ELISA kits were purchased from R&D Systems (Minneapolis, MN).

Cell culture

Mouse J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT) and 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA) and then cultured at 37 °C in a 5% CO_2 incubator. All plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): ERK1 shRNA plasmid (sh-ERK1, sc-29308-SH), JNK1 shRNA plasmid (sh-JNK1, sc-29381-SH), p38α shRNA
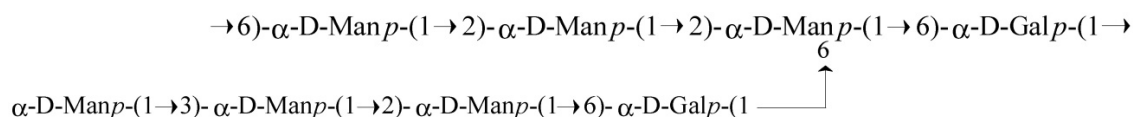
→6)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→6)-α-D-Gal*p*-(1→

6

↑

α-D-Man*p*-(1→3)- α-D-Man*p*-(1→2)- α-D-Man*p*-(1→6)- α-D-Gal*p*-(1 ———

Figure 1. Structure of the octasaccharide repeating unit of the *Antrodia cinnamomea* galactomannan (ACP).

plasmid (sh-p38α, sc-29434-SH), p38β shRNA plasmid (sh-p38β, sc-39117-SH), TLR2 shRNA plasmid (sh-TLR2, sc-40257-SH) and TLR4 shRNA plasmid (sh-TLR4, sc-40261-SH). NF-κB reporter cells, (J-Blue cells), were generated by stable transfection of J774A.1 macrophages with a secreted embryonic alkaline phosphatase (SEAP) reporter construct (pNiFty2-SEAP, Catalog number: pnifty2-seap, purchased from Invitrogen, Carlsbad, CA, USA) induced by NF-κB.

Isolation of mouse peritoneal macrophages

Male C57BL mouse was intraperitoneally injected with 2 mL of thioglycollate for 3 days. Peritoneal exudate cells (1×10^6/mL) isolated from the peritoneal fluids were cultured in 6-well plates for 2 h, and the non-adherent cells were removed through gentle washing 3 times with PBS. The adherent macrophages were stimulated as indicated.

Generation of Human Monocyte-derived Dendritic Cells

The detailed protocol for generating monocytes-derived dendritic cells (DC) was described in our previous study [34]. In brief, whole blood sample (250 mL) was obtained from one healthy volunteer. PBMCs were separated from the whole blood through Ficoll-Hypaque density gradient centrifugation, and then monocytes were isolated through the plastic adherence method. Immature dendritic cells were generated from the culture of the isolated monocytes (1×10^6/mL), which was treated for 6 days in 6-well plates with RPMI 1640 medium supplemented with 500 U/mL recombinant human GM-CSF and 1,000 U/mL recombinant human IL-4 (PeproTech, Rocky Hill, NJ). On days 2 and 4, half the medium was replaced with fresh medium containing recombinant human GM-CSF and recombinant human IL-4.

ELISA assay

Cells (2×10^5/well) were seeded in 24-well plates and treated as indicated. Secretion of TNF-α, IL-6, and IL-1β was measured by ELISA according to the manufacturer's protocol (R&D systems Minneapolis).

Western blot assay

After treatment, the cells were collected and lysed at 4 °C in lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The whole cell lysate was centrifuged at 4°C, and the supernatant was separated by SDS-PAGE and electrotransferred to a PVDF membrane (Millipore,

Billerica, MA). The membranes were incubated at 4 °C overnight in blocking buffer, and they were further incubated for 2 h at room temperature with specific primary antibody in blocking buffer. After three washes in PBS with 0.1% Tween 20, the membrane was incubated for 1 h at room temperature with HRP-conjugated secondary antibody in blocking buffer and developed through an enhanced chemiluminescence Western blot detection system (Millipore, Billerica, MA).

NF-κB reporter assay

J-Blue cells, J774A.1 macrophages stably expressing the gene for secreted embryonic alkaline phosphatase induced by NF-κB, were seeded in a 24-well plate at a density of 2×10^5 cells in 0.5 mL medium and grown overnight in a 5% CO_2 incubator at 37 °C. Cells were then treated with negative control, positive control, or ACP and incubated for another 24 h. The medium (20 μL) from treated J-Blue cells was mixed with 200 μL of QUANTI-Blue™ medium (Invitrogen, Carlsbad, CA) in 96-well plates and incubated at 37 °C for 15 min. The secreted embryonic alkaline phosphatase activity was assessed with a microplate absorbance reader measuring the optical density at 655 nm.

Statistical Analysis

All values are given as mean ± standard deviations (SDs). Data analysis involved one-way analysis of variance (ANOVA) with subsequent Scheffe's test.

Results and Discussion

ACP induces TNF-α and IL-6 secretion in mouse macrophages and human monocyte-derived dendritic cells

TNF-α and IL-6 are important pro-inflammatory cytokines produced mainly by activated macrophages and dendritic cells; they mediate multiple biological effects, including the activation of immune responses [31-33]. To detect the effect of ACP on macrophage activation, we quantified TNF-α and IL-6 levels in the conditioned medium of ACP-stimulated murine J774A.1 macrophages through ELISA. As shown in Figure 2A, 0.25, 0.45, and 1.3 ng/mL of TNF-α were detected in the conditioned medium of 10, 30, and 100 μg/ml ACP-treated J774A.1 macrophages, respectively. Relatively, the concentration of lipopolysaccharide (1 μg/mL LPS from *E. coli*) induced TNF-α level was 33 ng/mL.
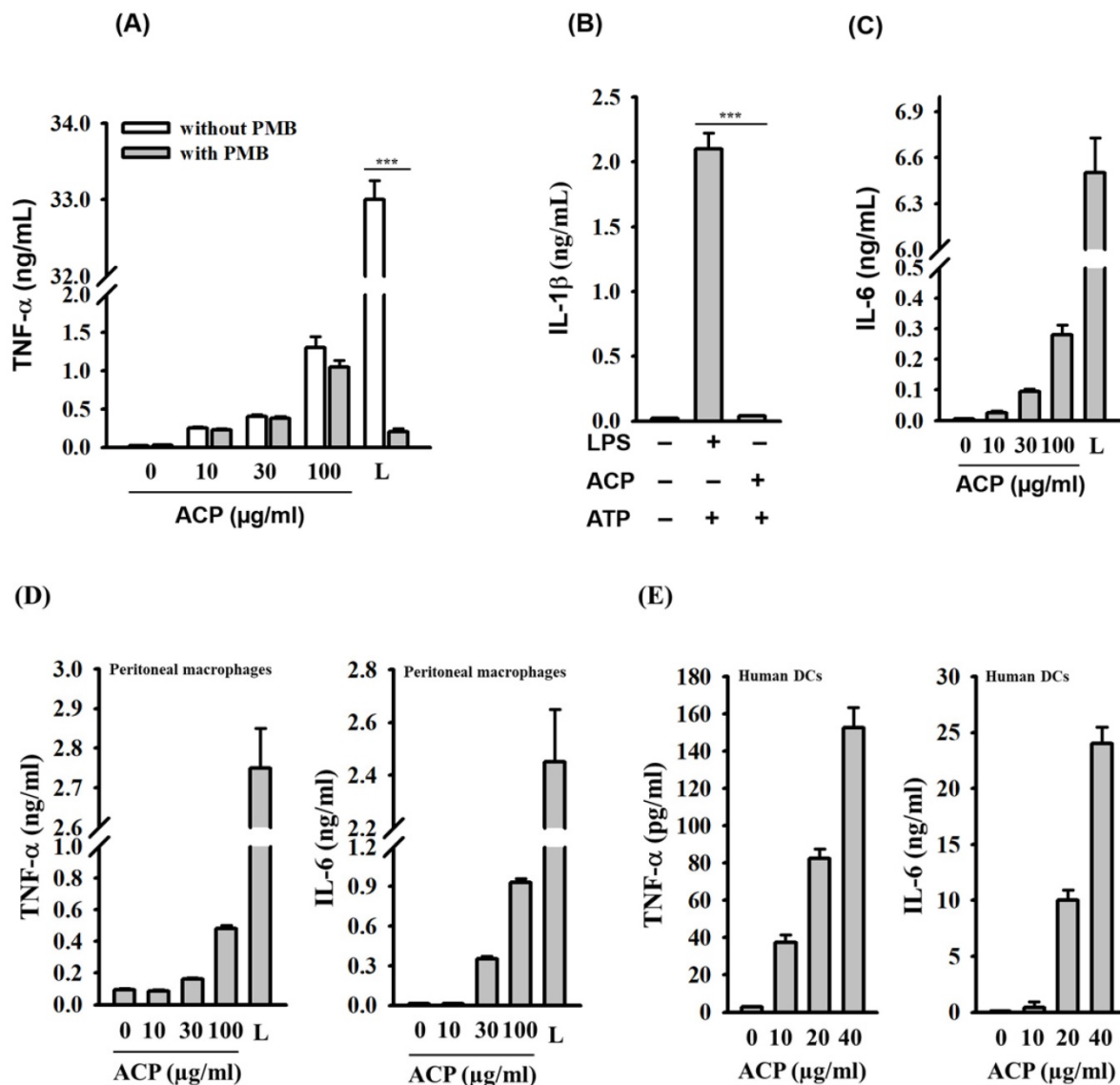
Figure 2. ACP induces cytokine expression in macrophages and dendritic cells. (A) J774A.1 macrophages were incubated for 30 min with or without polymyxin B (PMB) (10 µg/mL) and then for 24 h with or without ACP or LPS (1 µg/mL). The levels of TNF-α in the culture medium were measured by ELISA. **(B)** J774A.1 macrophages were incubated for 5.5 h with or without ACP (100 µg/mL) or LPS (1 µg/mL) and then for 30 min with or without ATP (5 mM). The levels of IL-1β in the culture medium were measured by ELISA. **(C)** J774A.1 macrophages were incubated for 24 h with or without ACP or LPS (1 µg/mL). The levels of IL-6 in the culture medium were measured by ELISA. **(D)** Mouse peritoneal macrophages were incubated for 24 h with or without ACP or LPS (1 µg/mL). The levels of TNF-α and IL-6 in the culture medium were measured by ELISA. **(E)** Human monocyte-derived dendritic cells were incubated for 24 h with or without ACP or LPS (1 µg/mL). The levels of TNF- α and IL-6 in the culture medium were measured by ELISA. The data are expressed as the means ± SD of three separate experiments. *** indicates a significant difference at the level of *p*< 0.001. L: LPS
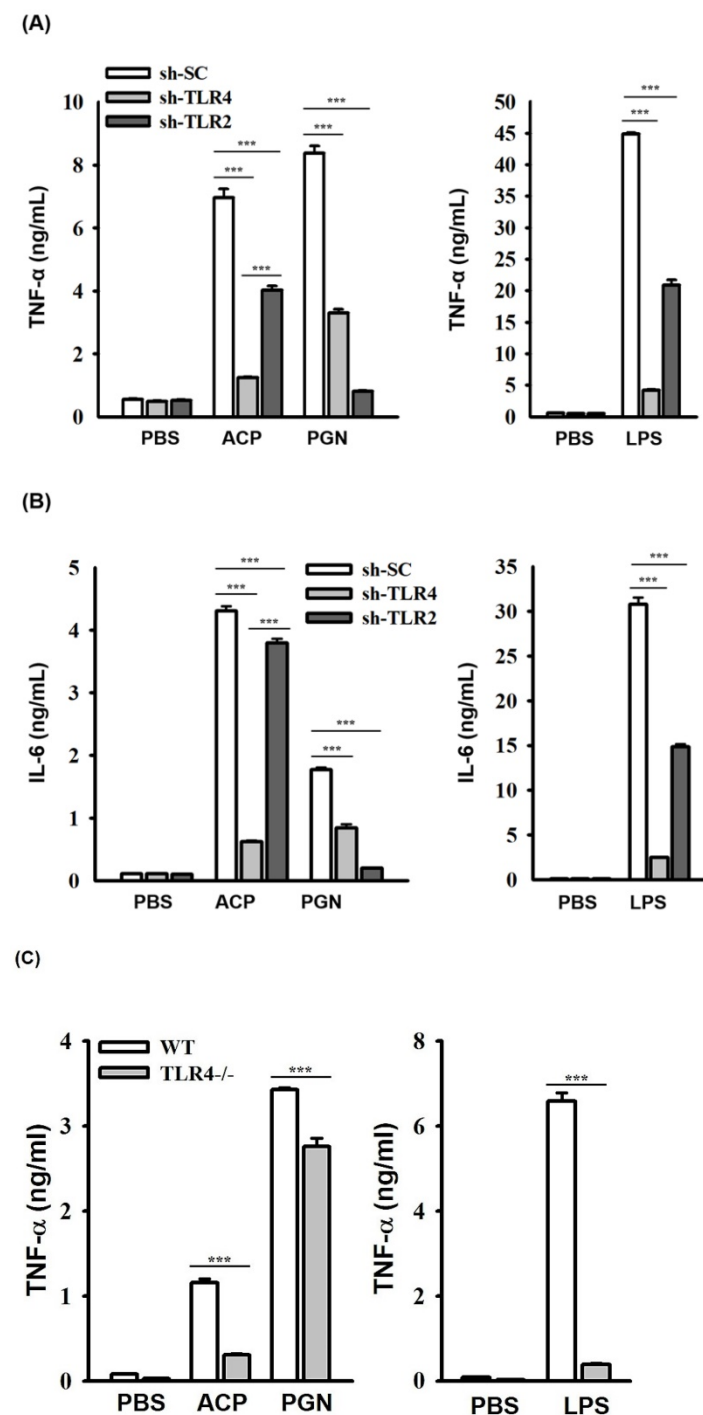
Lipopolysaccharide (LPS), a potent macrophage stimulator, is often presented as a contaminant in natural polysaccharides. Therefore, it is extremely important to avoid false positive responses caused by such contaminations. The effect polymyxin B (PMB), an LPS-sequestering agent, on ACP- and LPS-induced TNF-α levels in J774A.1 macrophages was tested to rule out the possibility of endotoxin contamination in ACP. As shown in Figure 2A, ACP-induced TNF-α secretion was not reduced by PMB, but PMB significantly inhibited the LPS-induced TNF-α secretion. In addition, NLRP3 inflammasome, a protein complex composed of NLRP3, ASC and caspase-1, which regulates IL-1β secretion, was

activated by LPS in the presence of ATP. However, ACP was not able to activate the NLRP3 inflammasome (Figure 2B). These results clearly demonstrated that ACP was not contaminated with LPS. ACP also induced IL-6 secretion in a dose-dependent fashion in J774A.1 macrophages (Figure 2C). The cytokine induction ability of ACP in macrophages was further confirmed through the measurement of TNF-α and IL-6 secretion in mouse primary peritoneal macrophages upon ACP treatment. We observed that ACP induced TNF-α and IL-6 secretion in mouse primary peritoneal macrophages in a concentration-dependent manner (Figure 2D). Furthermore, there was a

dose-dependent increase of TNF-α and IL-6 levels in the ACP-treated human primary monocyte-derived dendritic cells (Figure 2E). These results indicate that

Figure 3. ACP induces cytokine expression through TLR4. (A, B) J774A.1 macrophages stably transfected with a control shRNA plasmid (sh-SC), a TLR4 shRNA plasmid (sh-TLR4), and a TLR2 shRNA plasmid (sh-TLR2) were incubated for 24 h with or without ACP (100 μg/mL), PGN (10 μg/mL) or LPS (1 μg/mL). The levels of TNF-α (**A**) and IL-6 (**B**) in the culture medium were measured by ELISA. (**C, D**) Mouse peritoneal macrophages isolated from wild-type (WT) and TLR4 knockout (TLR4-/-) mice were incubated for 24 h with or without ACP (100 μg/mL), PGN (10 μg/mL) or LPS (1 μg/mL). The levels of TNF-α (**C**) and IL-6 (**D**) in the culture medium were measured by ELISA. (**E**) J774A.1 macrophages were incubated for 30 min with or without ACP (100 μg/mL) and then for 24 h with or without LPS (1 μg/mL). The levels of TNF-α in the culture medium were measured by ELISA. The data are expressed as the means ± SD of three separate experiments. *** indicates a significant difference at the level of *p*< 0.001.

ACP is able to activate an immune response to induce cytokine secretion in both macrophages and dendritic cells.

A previous study revealed the strong induction effect of the high molecular weight (> 100 kDa) polysaccharide fraction of *A. cinnamomea* has on dendritic cells in secreting TNF-α, IL-6, and IL-12 [23]. In a similar vein, the polysaccharides of *A. cinnamomea* were reported to have various other biological activities related to immunity modulation, including anti-HepG2 [17,35,36], anti-tumors [23], IL-6, TNF-α, IL-10, and monocyte chemotactic protein-1, all of which decrease the inflammatory mediator expression at the location of injury and in circulation [37] and suppress angiogenesis [26,27,38]. The high molecular weight (MW > 100 kDa) polysaccharide fraction was the most effective in inhibiting vascular endothelial growth factor, it increased the levels of IL-12 and IFN-γ secretion of mononuclear cells [39]. The immunomodulatory effect of the polysaccharides from *A. cinnamomea* mycelia induced Th1-type cytokines (IFN-γ, TNF-α) that are crucial for both cell-mediated and humoral immunity [40]. Even though aforementioned polysaccharides from *A. cinnamomea* have shown immunostimulatory effects, certain *Antrodia* polysaccharides have also shown antiinflammatory activities. For example, Antrodan, a 442 kD glycoprotein purified from *A. cinnamomea* that contains a particularly high content of uronic acid, is reported to have a potent suppressive effect on the lipopolysaccharide-induced inflammatory responses in the Raw 264.7 cell line [38], anti-metastatic effects in lung carcinoma cells [25], and hepatoprotective activity [41].

ACP induces cytokine expression mainly through TLR4

The roles of TLRs including TLR2 and TLR4, in polysaccharides-mediated cytokine induction have been elucidated [7, 42-47]; however, the roles of TLRs playing in recognizing the immunological polysaccharides of *A. cinnamomea* were less studied. We observed that the secretion of ACP-induced TNF-α (Figure 3A) and IL-6 (Figure 3B) was significantly reduced in J774A.1 macrophages stably transfected with shRNA plasmids targeting TLR4 (sh-TLR4), compared with that in the cells stably transfected with a control shRNA plasmid encoding a scrambled shRNA sequence (sh-SC). These results indicated that TLR4 is one of the putative receptors for ACP. Notably, ACP- and

LPS-induced TNF-α and IL-6 secretions were also reduced in sh-TLR2 cells. Some mannose-containing glycoconjugates/polysaccharides have been reported to be recognized by TLR-2 and TLR-4 [48,49]. During the isolation procedure of ACP, the sample was heat-denatured and treated with protease K to digest the protein part in the sample completely that the contamination of the fungal peptidoglycan would be prevented. We believed that the reduced secretions of TNF-α and IL-6 in ACP-treated sh-TLR2 cells were not due to peptidoglycan contamination, but TLR2 may partially contribute to the cytokine induction activity of ACP. The more detailed investigation are required, including defined oligosaccharides as ligands for TLR2 and TLR4 activation and binding.

To confirm the role of TLR4 played in ACP-mediated cytokine secretion, we compared the cytokine induction activity of ACP in primary peritoneal macrophages isolated from wild-type (WT) mice with those from TLR4-deficient (TLR4-/-) mice. The secretion of ACP- and LPS-induced TNF-α (Figure 3C) and IL-6 (Figure 3D) in peritoneal macrophages from TLR4-/- mice was significantly lower than that in peritoneal macrophages from WT mice, whereas the secretion of peptidoglycan, a TLR2 ligand-induced TNF-α and IL-6 was not impaired in peritoneal macrophages from TLR4-/- mice. These results indicated that TLR4 functions as a major putative receptor for ACP. In addition, ACP pretreatment did not significantly reduce the secretion of LPS-induced TNF-α, and that indicated ACP's inability to significantly compete with LPS in binding to TLR4 (Figure 3E). These results indicated that both ACP and LPS could bind to TLR4 and might have different TLR4 interaction sites respectively. Recent studies on the adjuvant effects, hot water-extracted *A. cinnamomea* high-molecule-weight polysaccharides (greater than 100 kDa) working on dendritic cell activation and maturation [23] revealed to the TLR2/TLR4 and NF-κB/MAPK signaling pathways, induction of T cell activation, and Th1 differentiation. A lot of TLR4-related polysaccharide ligands have been identified, with varying molecular weights and different monosaccharide compositions. Additionally, homoglycans, which contain glucan, galactan, and mannan, are well-known TLR4 ligands [50]. Glucose, mannose, galactose, fucose, and sialic acid have been identified as the possible key "pharmacophore" of TLR4/MD-2 polysaccharide ligands, and they may participate in enhancing ligand interaction with TLR4/MD-2 [50]. Furthermore, α-(1-3), α-(1-4), β-(1-3), and β-(1-4) glycosidic linkages containing polysaccharides are more frequently reported as the TLR4 ligands [50].

ACP induces cytokine secretion through MAPK

Activation of TLR4 results in the activation of downstream mitogen-activated protein kinases (MAPK, including ERK1/2, JNK1/2, and p38) and leads to the production of proinflammatory mediators [47]. To examine whether the secretion of ACP-induced TNF-α and IL-6 was associated with MAPK signaling pathways, we measured the phosphorylation levels of ERK1/2, JNK1/2, and p38 in ACP-activated J774A.1 macrophages through Western blot analysis, and the results revealed that phosphorylation levels of ERK1/2, JNK1/2, and p38 were increased by ACP compared with those in control cells (Figure 4A). The effect ACP on TNF-α and IL-6 secretion in J774A.1 macrophages, which are stably transfected with shRNA plasmids targeting ERK1 (sh-ERK1), JNK1 (sh-JNK1), p38α (sh-p38α), and p38β (sh-p38β), was investigated to elucidate the role MAPK plays in the regulation of cytokine secretion in ACP-activated macrophages. Significantly lower TNF-α (Figure 4B) and IL-6 (Figure 4C) secretion was observed in ACP-treated sh-ERK1, sh-JNK1, and sh-p38α cells compared with that in cells stably transfected with a control shRNA plasmid encoding a scrambled shRNA sequence (sh-SC). However, no significant difference was detected between sh-p38β and sh-SC cells in ACP-induced TNF-α and IL-6 secretion (Figures 4B and 4C), and that indicated the mediation of ERK1, JNK1 and p38α in ACP-induced cytokine secretion.

ACP induces cytokine secretion through PKC

Protein kinase C family proteins have been extensively studied for their mediation in immunomodulatory signal transduction and immune cell regulation processes. The potential of polysaccharide-based prebiotics in strengthening the intestinal epithelial barrier through PKC-δ signaling has been recently reported [51]. Further activation of PKC-α and δ in macrophages by fungal polysaccharides (*Ganoderma lucidum*) [7,42], exopolysaccharides from algae (*Chlorella pyrenoidosa*) [44], and capsular polysaccharides from bacteria (*Klebsiella pneumoniae*) [45] has also been reported. Therefore, in our study, we examined the possible involvement of protein kinase C (PKC) in ACP-induced TNF-α secretion. As revealed through the Western blot analysis, phosphorylation levels of PKC-α and PKC-δ were increased in ACP-activated J774A.1 macrophages compared with those in the ACP untreated cells (Figure 5A). The roles of PKC-α and PKC-δ playing in the regulation of TNF-α secretion in ACP-activated macrophages were
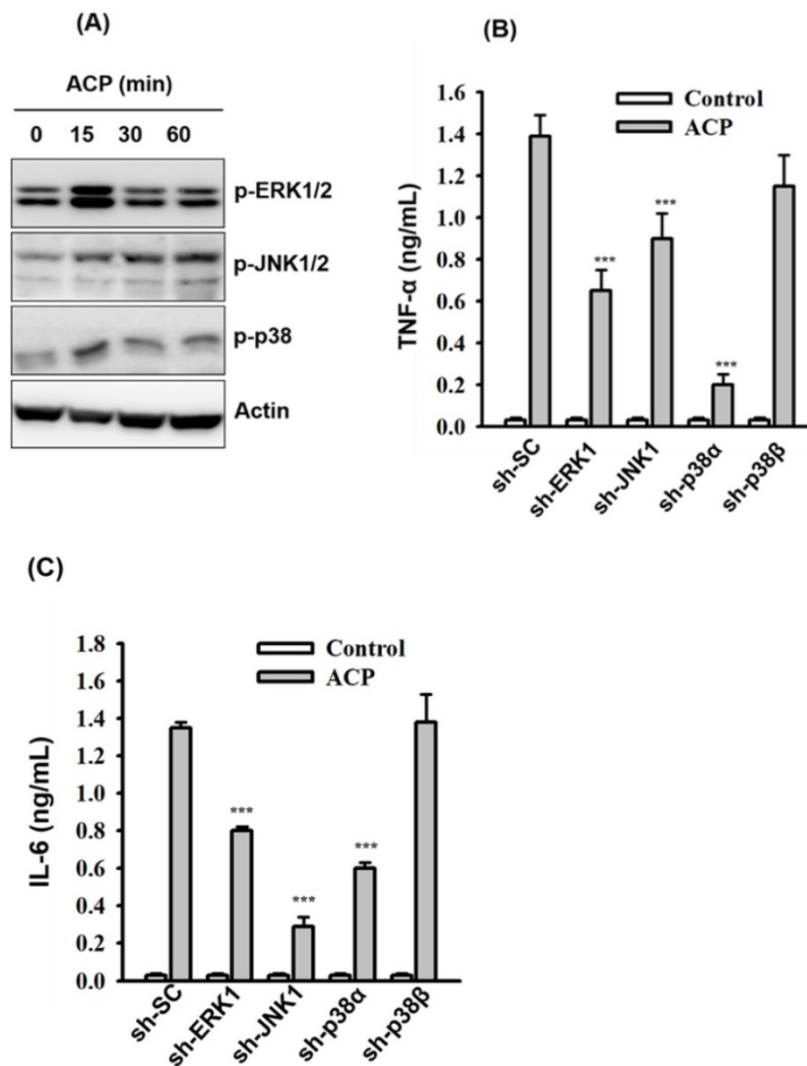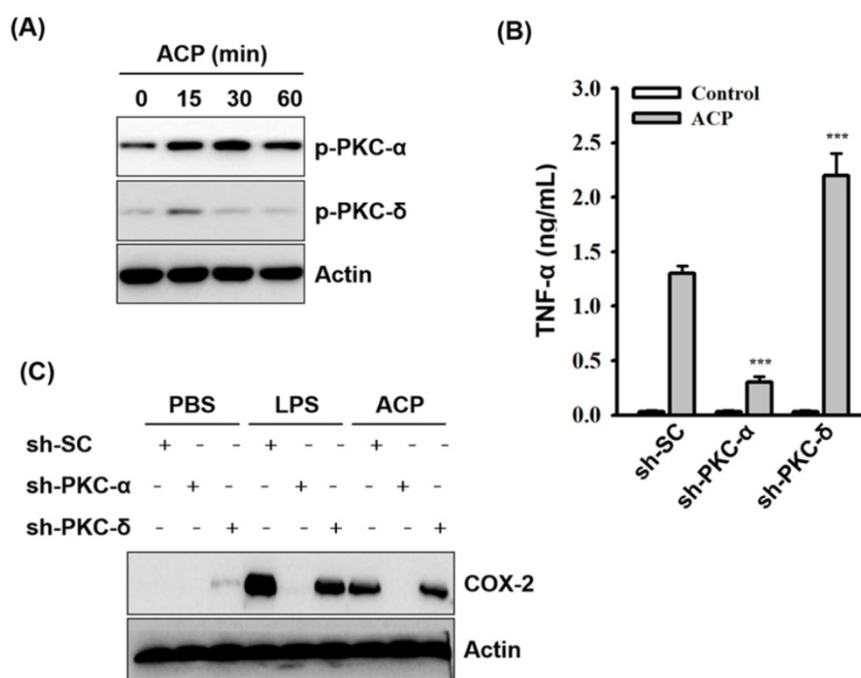
Figure 4. ACP induces cytokine expression through MAPK. (A) J774A.1 macrophages were incubated for 0-60 min with or without ACP (100 μg/mL). The phosphorylation levels of ERK1/2, JNK1/2 and p38 were assayed by Western blot. **(B, C)** J774A.1 macrophages stably transfected with a control shRNA plasmid (sh-SC), a ERK1 shRNA plasmid (sh-ERK1), a JNK1 shRNA plasmid (sh-TLR2), a p38α shRNA plasmid (sh-p38α), and a p38β shRNA plasmid (sh-p38β) were incubated for 24 h with or without ACP (100 μg/mL). The levels of TNF-α **(B)** and IL-6 **(C)** in the culture medium were measured by ELISA. The Western blot results are representative of those obtained in three different experiments. The ELISA data are expressed as the means ± SD of three separate experiments. *** indicates a significant difference at the level of $p < 0.001$.

elucidated in J774A.1 macrophages, which are stably transfected with shRNA plasmids targeting PKC-α (sh-PKC-α) and PKC-δ (sh-PKC-δ). We found that TNF-α secretion in ACP-activated sh-PKC-α cells was significantly lower than that in the cells stably transfected with a control shRNA plasmid encoding a scrambled shRNA sequence (sh-SC) (Figure 5B). In contrast, there was a significantly higher amount of TNF-α detected in ACP-treated sh-PKC-δ cells than that in the control sh-SC cells (Figure 5B). Additionally, COX-2 expression was also upregulated in ACP treated sh-SC cells and PKC-δ cells, but it was down-regulated in sh-PKC-α cells (Figure 5C). These results indicate that ACP induces TNF-α secretion and COX-2 expression through PKC-α. However, PKC-δ negatively regulates the ACP-induced TNF-α secretion.

ACP induces endotoxin tolerance

Primary exposure of cells to LPS leads to a reduced responsiveness to a second LPS challenge, a phenomenon known as endotoxin tolerance. Endotoxin tolerance is characterized as a global down-regulation of inflammatory gene expression [52], and it augments the bacterial clearance and improves survival rate of mice with sepsis [53]. Although ACP induces an increase in TNF-α and IL-6 secretion in macrophages, pre-incubation of J774A.1 macrophages with ACP for 24 h produced an endotoxin tolerance-like phenomenon, a markedly attenuated response to LPS stimulation with decreased TNF-α and IL-6 secretion (Figure 6A). The endotoxin tolerance-like phenomenon induced by ACP was also confirmed in mouse primary peritoneal macrophages (Figure 6B). The endotoxin tolerance-

Figure 5. ACP induces cytokine expression through PKC-α. (**A**) J774A.1 macrophages were incubated for 0-60 min with or without ACP (100 μg/mL). The phosphorylation levels of PKC-α and PKC-δ were assayed by Western blot. (**B**) J774A.1 macrophages stably transfected with a control shRNA plasmid (sh-SC), a PKC-α shRNA plasmid (sh-PKC-α), and a PKC-δ shRNA plasmid (sh-PKC-δ) were incubated for 24 h with or without ACP (100 μg/mL). The levels of TNF-α in the culture medium were measured by ELISA. (**C**) sh-SC, sh-PKC- α, and sh-PKC-δ cells were incubated for 24 h with or without ACP (100 μg/mL) or LPS (1 μg/mL). The expression levels of COX-2 were assayed by Western blot. The Western blot results are representative of those obtained in three different experiments. The ELISA data are expressed as the means ± SD of three separate experiments. *** indicates a significant difference at the level of *p*< 0.001 compared to ACP-treated sh-SC cells.

like phenomenon induced by ACP might result from the reduced NF-κB activation (Figure 6C), rather than from the phosphorylation levels of MAPKs (Figure 6D). Based on our results, it is clear that the immunostimulatory activity of ACP is much lower than that of LPS. Induction of LPS tolerance shows the ability of ACP to attenuate high inflammatory responses caused by endotoxins. These results suggested that ACP, without causing severe inflammation, was able to enhance immunity by activating macrophages.

Conclusion

Over several decades, the complex carbohydrates of mushrooms have attracted growing scientific interest for their immunoenhancing properties. However, their contribution as clinical adjuvants is still limited because of the structural complexity and the lack of studies on the structure-activity relationship and the underlying mechanisms of the biological activity of individual polysaccharide components. The present study reports the immunostimulatory activity of *Antrodia cinnamomea* galactomannan (ACP) in mouse macrophages and human monocyte derived dendritic cells. We identified TLR4 as the putative receptor for ACP binding and some signaling pathways involved

in ACP-induced macrophage activation to secrete proinflammatory cytokines, TNF-α, and IL-6. Furthermore, the ability of ACP to exert an endotoxin tolerance-like effect on murine macrophages was demonstrated *in vitro*. We previously explored the detailed structure of ACP and its ability to enhance phagocytosis and bactericidal activity of murine macrophages [29]. In that study, we further reported that ACP pretreatment enhances the immune responses against invading bacteria in the early stage of infection but attenuates the risk of severe inflammation in the latter stages by lowering the secretion of pro-inflammatory cytokines. Considering the above facts and our current data including an endotoxin tolerance-like effect, we suggest ACP as a strong but harmless candidate for the development of new carbohydrate-based nutraceutical supplements and adjuvants in immunotherapy for a broad range of diseases.

Abbreviations

ACP: *Antrodia cinnamomea* polysaccharide; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; IL-1β: interleukin-1β; LPS: lipopolysaccharide; MAPKs: mitogen activated protein kinase; PKC: protein kinase C; COX-2: cycloxygenase-2; TLR4: toll-like receptor 4; NF-κB: nuclear factor-κB.

(A)

(B)

(C)   **(D)**
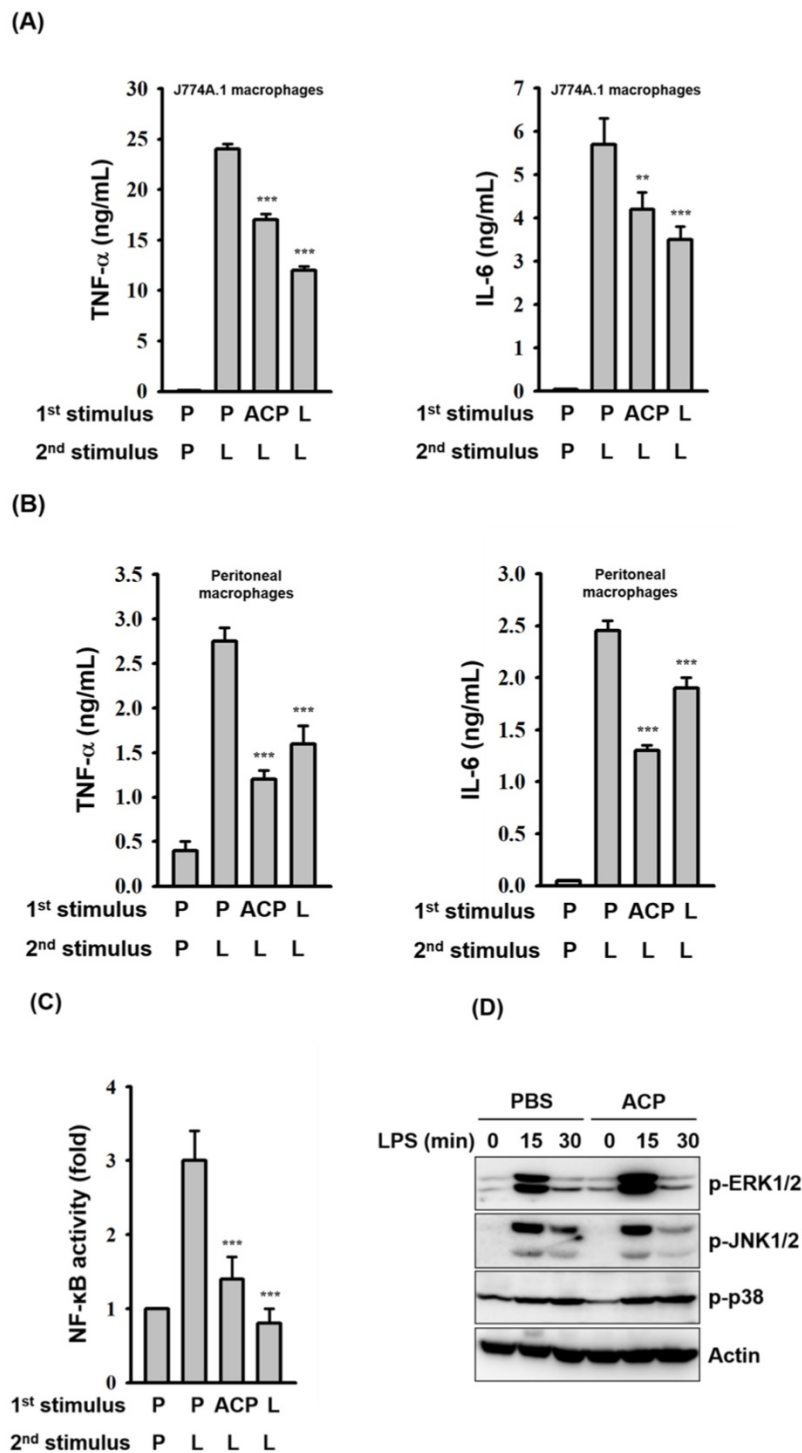
Figure 6. ACP pretreatment induces LPS tolerance. (A, B) J774A.1 macrophages (**A**) and mouse peritoneal macrophages (**B**) were incubated for 24 h with or without ACP (100 μg/mL) or LPS (0.1 μg/mL)(1st stimulus). After washing, cell cultures were changed for fresh medium and then were incubated for 24 h with or without LPS (1 μg/mL)(2nd stimulus). The levels of TNF-α and IL-6 in the culture medium were measured by ELISA. (**C**) J-Blue cells were incubated for 24 h with or without ACP (100 μg/mL) or LPS (0.1 μg/mL)(1st stimulus). After washing, cell cultures were changed for fresh medium and then were incubated for 24 h with or without LPS (1 μg/mL)(2nd stimulus). The activation levels of NF-κB were measured by an NF-κB reporter assay. (D) J774A.1 macrophages were incubated for 24 h with or without ACP (100 μg/mL). After washing, cell cultures were changed for fresh medium and then were incubated for 0-30 min with or without LPS (1 μg/mL). The phosphorylation levels of ERK1/2, JNK1/2 and p38 were assayed by Western blot. The Western blot results are representative of those obtained in three different experiments. The data are expressed as the means ± SD of three separate experiments. *** indicates a significant difference at the level of *p*< 0.001, compared to 1st P/2nd L group. P: PBS; L: LPS.

Competing Interests

The authors have declared that no competing interest exists.

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