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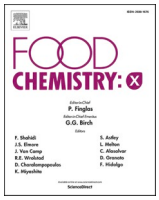

Immunoregulatory activity of a low-molecular-weight heteropolysaccharide from *Ganoderma leucocontextum* fruiting bodies *in vitro* and *in vivo*

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ABSTRACT

The chemical structure of GLP-1, a novel water-soluble heteropolysaccharide purified *Ganoderma leucocontextum* fruiting bodies, has been characterized in our previous study. This study aimed to investigate the immunostimulatory activity of GLP-1 *in vitro* and *in vivo* by using RAW264.7 macrophages and cyclophosphamide-induced immunosuppressed mice model. Results showed that GLP-1 was able to enhance phagocytic activity and promote the production of reactive oxygen species, nitric oxide, tumor necrosis factor-α, interleukin-6, and monocyte chemoattractant protein-1 in RAW264.7 macrophages. Moreover, GLP-1 could activate mitogen-activated protein kinase, phosphatidylinositol-3-kinase/protein kinase B, and nuclear factor-kappa B signaling pathways through toll-like receptor 2 and dectin-1 receptors. Furthermore, GLP-1 increased the thymus index, serum immunoglobulin levels, and percentage of $CD3^+$ T lymphocytes in cyclophosphamide-induced immunosuppressed mice. These results demonstrated that GLP-1 possessed significant immunostimulatory effects *in vivo* and *in vitro* and could be developed as an effective immunomodulator for application in functional foods.

1. Introduction

The suppressed immune system makes organisms more sensitive to pathogens. The innate immune response is the first step to recognize and defend infections, pollutants, and pathogens (Wang et al., 2019). Macrophages are central participants in the innate immune response to many physiological and pathological conditions. Activated macrophages participate in host defense through the phagocytosis of invading pathogens or the secretion of bioactive molecules, including pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, nitric oxide (NO), and reactive oxygen species (ROS). Therefore, the activation of macrophages is a promising approach to reinforce immunity (Bai et al., 2019).

In recent years, natural polysaccharides have been reported to possess remarkable pharmacological actions in the aspects of anti-diabetic (Fang et al., 2022), anti-tumor (Zhang et al., 2021), anti-photoaging (Yao et al., 2021), anti-oxidation (Zhou et al., 2020), immunoregulation (Su, Chen, Yang, & Cheung, 2021; Yu et al., 2019). Edible-medicinal mushroom is one of the most important resources for natural polysaccharides. Extensive studies have demonstrated that mushroom-derived polysaccharides exhibit diverse health-promoting effects, especially immunoregulation (Maity et al., 2021). They can

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exert immunomodulatory effects by enhancing macrophage phagocytosis, immune cells proliferation, and immune-related cytokine production (Wang et al., 2019). Lentinan (LNT), a β-1,3-glucan polysaccharide isolated from *Lentinula edodes*, has been applied as an immunomodulator in clinical practice (Ina et al., 2011).

Ganoderma species is a very famous medicinal mushroom in China and Japan (Ren, Zhang, & Zhang, 2021). Several studies have demonstrated that polysaccharides from *G. lucidum* (Li et al., 2020), *G. tsugae* (Gao, Chan, & Zhou, 2004), and *G. atrum* (Yu et al., 2015) possess immune regulatory functions. As a new species of *Ganoderma*, the biological activities of *G. leucocontextum* polysaccharides were few documented. Our previous study found that an α-1,4-glucan polysaccharide GLP-3 isolated from *G. leucocontextum* showed immunostimulatory activity in macrophages (Gao et al., 2020). Recently, our group purified a novel polysaccharide GLP-1 from *G. leucocontextum*, and further investigated its structural characteristics and antioxidant activity *in vitro* (Gao et al., 2021). We found that GLP-1 with molecular weight of 6.3 kDa was mainly composed of mannose, glucose, galactose, xylose, and arabinose. The backbone of GLP-1 contained (1 → 3)-β-Glc*p*, (1 → 4)-β-Glc*p*, and (1 → 6)-β-Gal*p* glycosidic linkages. The network structure of GLP-1 in aqueous solution was observed under atomic force microscopy. Ferreira et al. (2015) reviewed that the immunoregulatory activities of polysaccharides are closely related to their structural characterization. The monosaccharide composition, glycosidic linkage, molecular weight, and chain conformation of GLP-1 are different from those of GLP-3 (Gao et al., 2021; Gao et al., 2020). However, to our knowledge, there is no report on the macrophage activation of GLP-1 and its immunoregulatory effect *in vivo*. Therefore, the present study aimed to explore the underlying immunoregulatory mechanism of GLP-1 in RAW264.7 macrophages. Also, its immunoregulatory activity in immunosuppressed mice induced by cyclophosphamide (CTX) was further investigated. The results from this study might provide useful information for understanding the immunomodulatory activities of polysaccharides from *G. leucocontextum*.

2. Materials and methods

2.1. Materials

GLP-1 used in this study was prepared according to the reported method in our previous research (Gao et al., 2021). RAW264.7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Lipopolysaccharides (LPS), CTX, polymyxin B (PMB), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). LNT (98%) was obtained from Shanghai Yuanye Bio-Technology Co. Ltd. (Shanghai, China). Fetal bovine serum (FBS), NuPAGE Bis-Tris gels, Dulbecco's modified Eagle's medium (DMEM), and TRIzol reagent were purchased from Thermo Scientific (Rockford, IL, USA). The cell counting kit-8 (CCK-8), SP600125, U0126, SB203580, LY294002, and BAY 11-7082 were obtained from MCE (Monmouth Junction, NJ, USA). The p-IKKα/β, p-Akt, p-NF-κB p65, NF-κB p65, p-JNK, JNK, p-p38, p38, p-IκBα, IκBα, GAPDH, and Lamin B1 antibodies were obtained from CST (Boston, MA, USA). The p-ERK, ERK, Akt, and IKKα/β antibodies were obtained from Affinity Biosciences (Cincinnati, OH, USA). The toll-like receptor 4 (TLR4) and TLR2 antibodies were purchased from Abcam (Cambridge, MA, USA). The dectin-1 antibody was purchased from GeneTex (Irvine, CA, USA). The ROS assay kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). First Strand cDNA Synthesis Kit and SYBR Green qPCR Master Mix were purchased from Servicebio Technology Co. Ltd. (Wuhan, China). Monocyte chemoattractant protein-1 (MCP-1), TNF-α, and IL-6 ELISA kits were obtained from NeoBioScience Technology Co. Ltd. (Shenzhen, China).

2.2. Cell culture

RAW264.7 macrophages were incubated in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin in a humidified atmosphere at 37 °C with 5% CO_2.

2.3. Cell viability

The cell viability was determined by the CCK-8 method as described in our previous study (Gao et al., 2020). RAW264.7 (1×10^5 cells/well, 200 μL) were seeded on a 96-well microplate for 24 h. Subsequently, cells were treated with 200 μL of GLP-1 (2.5, 10, and 40 μg/mL) or LPS (100 ng/mL) dissolved in DMEM medium for another 24 h. Then 200 μL of diluted CCK-8 solution was added to each well. After 1 h incubation, the absorbance at 450 nm was measured using a microplate reader. The experiment was conducted with four replicates.

2.4. Detection of endotoxin contamination

The PMB was used to examine whether endotoxin contamination presented in GLP-1 according to a previous method (Morrison & Jacobs, 1976). RAW264.7 cells (1×10^5 cells/well) were seeded on a 96-well microplate and cultured at 37 °C for 24 h. GLP-1 (40 μg/mL) and LPS (100 ng/mL) were pre-treated with or without PMB (10, 20, and 40 μg/mL) for 2 h at 37 °C and then used to stimulate RAW264.7 cells for another 24 h. The NO content in the cell supernatant was measured by Griess reagent (Gao et al., 2017). The experiment was performed with three replicates.

2.5. Phagocytic capacity assay

The phagocytic capacity was determined by a Vybrant phagocytosis assay kit (Molecular Probes, Carlsbad, CA, USA) according to a method described previously (Lee et al., 2015). RAW264.7 cells (1×10^6 cells/well) were seeded on 60 mm^2 culture dishes and stimulated with different concentrations of GLP-1 or LPS (100 ng/mL) for 24 h. Afterward, the FITC-labeled *E. coli* was added and the plate was incubated at 37 °C. After 2 h incubation, the supernatant was discarded, and the cells were washed three times with DPBS. The phagocytic capacity of RAW264.7 cells was analyzed by CytoFLEX flow cytometry (Beckman Coulter, Brea, CA, USA) and an Axio Observer A1 fluorescence microscope (Carl Zeiss, Jena, Germany). The experiment was conducted with three replicates.

2.6. Measurement of intracellular ROS

The intracellular ROS level of RAW264.7 cells was measured by the fluorescence probe DCFH-DA (Ren, Lin, Alim, Zheng, & Yang, 2017). After 24 h incubation on 60 mm^2 culture dishes, RAW264.7 cells were treated with various concentrations of GLP-1 or LPS for another 24 h. The DCFH-DA solution (15 μM) dissolved in DMEM medium was added into each culture dish and cells were further incubated in dark for 30 min at 37 °C. Subsequently, cells were washed three times with serum-free DEME medium to remove the excess DCFH-DA. Finally, the ROS production of RAW264.7 macrophages was measured by a flow cytometer and a fluorescence microscope. The experiment was performed with three replicates.

2.7. Measurement of NO, TNF-α, IL-6, and MCP-1

RAW264.7 cells (1×10^5 cells/well) were plated on a 96-well microplate and treated with various concentrations of GLP-1 or LPS for 24 h. The NO, IL-6, TNF-α, and MCP-1 contents in cell culture supernatants were determined using Griess reagent and ELISA kits according to the manufacturer's instructions.

The signaling pathways that participated in the macrophage

activation induced by GLP-1 were further investigated. RAW264.7 cells were pre-treated with p38 MAPK inhibitor SB203580 (30 μM), ERK inhibitor U0126 (30 μM), JNK inhibitor SP600125 (30 μM), PI3K inhibitor LY294002 (30 μM), and NF-κB inhibitor BAY 11–7082 (10 μM) for 1 h and then co-cultured with GLP-1 (40 μg/mL) for another 24 h. Subsequently, the NO, TNF-α, and IL-6 contents were measured with three replicates as mentioned above.

2.8. Real-time polymerase chain reaction (RT-PCR) analysis

The mRNA expression levels of inducible nitric oxide synthase (iNOS), IL-6, TNF-α, and MCP-1 were analyzed by the quantitative RT-PCR method. RAW264.7 cells in 60 mm^2 culture dishes were treated with various concentrations of GLP-1 or LPS for 24 h. The total RNA was extracted by TRIzol reagent, and reverse transcription was performed using First Strand cDNA Synthesis Kit according to the manufacturer's instructions. The primer sequences of each target gene were listed in Table S1. The RT-PCR was performed as the procedure described in SYBR Green qPCR Master Mix using the ABI 7500 system (Applied Biosystem, Carlsbad, CA, USA). The relative mRNA expression levels of each target gene were quantified by the method of $2^{-\Delta\Delta Ct}$. β-Actin was used as an internal control to normalize the results with three replicates.

2.9. Western blot analysis

The protein expression levels were analyzed by western blot according to a previous study (Ren et al., 2017). RAW264.7 cells were seeded on 60 mm^2 culture dishes for 24 h and then treated with different concentrations of GLP-1 or LPS. After 30 min, the proteins were prepared according to our previous research (Gao et al., 2020). The denatured proteins were separated by NuPAGE Bis-Tris gels and transferred to a polyvinylidene fluoride membrane. After blocking with 5% non-fat milk in TBST at room temperature for 1 h, the membranes were incubated with primary antibodies (p-IKKα/β, IKKα/β, p-Akt, Akt, p-NF-κB p65, NF-κB p65, p-IκBα, IκBα, p-JNK, JNK, p-ERK, ERK, p-p38, p38, GAPDH, and Lamin B1) overnight at 4 °C. Subsequently, the membranes were incubated with the secondary antibody at room temperature for 1 h. Finally, the signal was visualized by using Omega Lum G imaging system. Similar experiments were repeated three times.

2.10. Pattern recognition receptors (PRRs) blocking experiments

The effects of membrane receptors on the NO and IL-6 production were investigated as described by Lee et al. (2015). RAW264.7 cells (1×10^5 cells/well) were pre-treated with 10 μg/mL of blocking antibody (TLR2, TLR4, or dectin-1) for 1 h. Subsequently, the cells were co-cultured with 40 μg/mL of GLP-1 for another 24 h. The NO and IL-6 contents were measured with three replicates as described in Section 2.7.

2.11. Animals and experimental design

Fouty-eight female BALB/c mice (6–8 weeks old, 18–20 g) were purchased from Zhuhai BesTest Bio-Tech Co. Ltd. (Zhuhai, China, certificate number: SCXK(Yue)2020–0051). All mice were maintained under specific pathogen-free conditions of temperature (20 ± 3 °C), humidity (50 ± 5%), and light (12 h light–dark cycle). All experimental procedures were approved by the Guangdong Institute of Microbiology Laboratory Animal Ethics Committee.

After 7-day acclimatization, the mice were randomly divided into 6 groups (8 mice per group), including normal group (NG), model group (MG), low-dose group (GLP-1-L), medium-dose group (GLP-1-M), high-dose group (GLP-1-H), and positive group (LNT). CTX (8 mg/mL) was dissolved in 0.9% physiological saline. GLP-1 (4, 8, and 16 mg/mL) and LNT (8 mg/mL) were dissolved in double-distilled water. The NG group was treated once daily with 0.9% physiological saline and the other groups were intraperitoneally injected with CTX (80 mg/kg/d) for three consecutive days. For the following 10 days, the NG and MG groups were orally administrated with 0.9% physiological saline. The GLP-1-L, GLP-1-M, and GLP-1-H groups were orally administered with GLP-1 at doses of 40, 80, and 160 mg/kg/d, respectively. The positive group was orally administered with LNT at a dose of 80 mg/kg/d. The liquid volume (0.2 mL/20 g) of intraperitoneal injection and gavage were adjusted according to the body weight of each mouse. Twenty-four hours after the last drug administration, the blood samples were collected by enucleating eyeball and all animals were sacrificed by cervical dislocation to harvest spleen and thymus. The body weights of mice were recorded during the experimental process. The spleen and thymus indices were calculated using the following formula: index (mg/g) = (weight of spleen or thymus)/body weight. The result of each group was obtained from eight mice.

2.12. Measurement of serum immunoglobulins

After centrifugation at 4 °C for 15 min (3000 r/min), serum was obtained from the blood sample. The level of immunoglobulin M (IgM) was determined by the immunity transmission turbidity method using a BS-480 automatic biochemistry analyzer (Mindray, Shenzhen, China). The level of immunoglobulin G (IgG) was measured by the commercial ELISA kit (Cusabio Biotech, Wuhan, China). All procedures were performed with eight replicates according to the manufacturer's instructions.

2.13. Preparation of spleen splenocytes and determination of T-lymphocyte subsets

The *T*-lymphocyte subsets were analyzed by flow cytometry as described by Tang et al. (2019) with some modifications. The harvested spleens were gently ground in RPMI 1640 medium and filtrated through a 200-mesh sieve. The cells were lysed with erythrocyte lysing solution (MultiSciences Biotech, Hangzhou, China) for 10 min to remove red blood cells. After centrifugation at 1000 r/min for 5 min, the cells were washed and resuspended in RPMI 1640 medium. The obtained single-splenocyte suspensions were stained with 5 μL of anti-mouse CD3ε-FITC/CD4-PE/CD8α-PerCP-Cy5.5 antibody (MultiSciences Biotech, Hangzhou, China) for 15 min at room temperature in dark. The lymphocytes were washed with 2 mL of staining buffer to remove the excess antibody and then resuspended for flow cytometry analysis. The experiment was conducted with six replicates.

2.14. Statistical analysis

The obtained data were presented as mean ± standard deviation (SD) and analyzed by SAS 9.2 software (Cary, NC, USA). Significant differences between the groups were evaluated using ANOVA and Duncan's multiple-range test. Different letters represent significant difference ($p < 0.05$).

3. Results

3.1. GLP-1 promoted cell proliferation

In the present study, RAW264.7 cells were used to investigate the immunostimulatory effect of GLP-1 *in vitro*. After treatment with GLP-1 (2.5–40 μg/mL) for 24 h, the cell viability was measured using the CCK-8 assay. As shown in Fig. 1A, compared with the control group (100%), GLP-1 at 2.5–40 μg/mL increased the relative viability of cells to 104.0%, 108.2%, and 114.2%, respectively, indicating the absence of cytotoxic effects. Besides, GLP-1 significantly promoted the proliferation of RAW264.7 cells at 10 and 40 μg/mL.

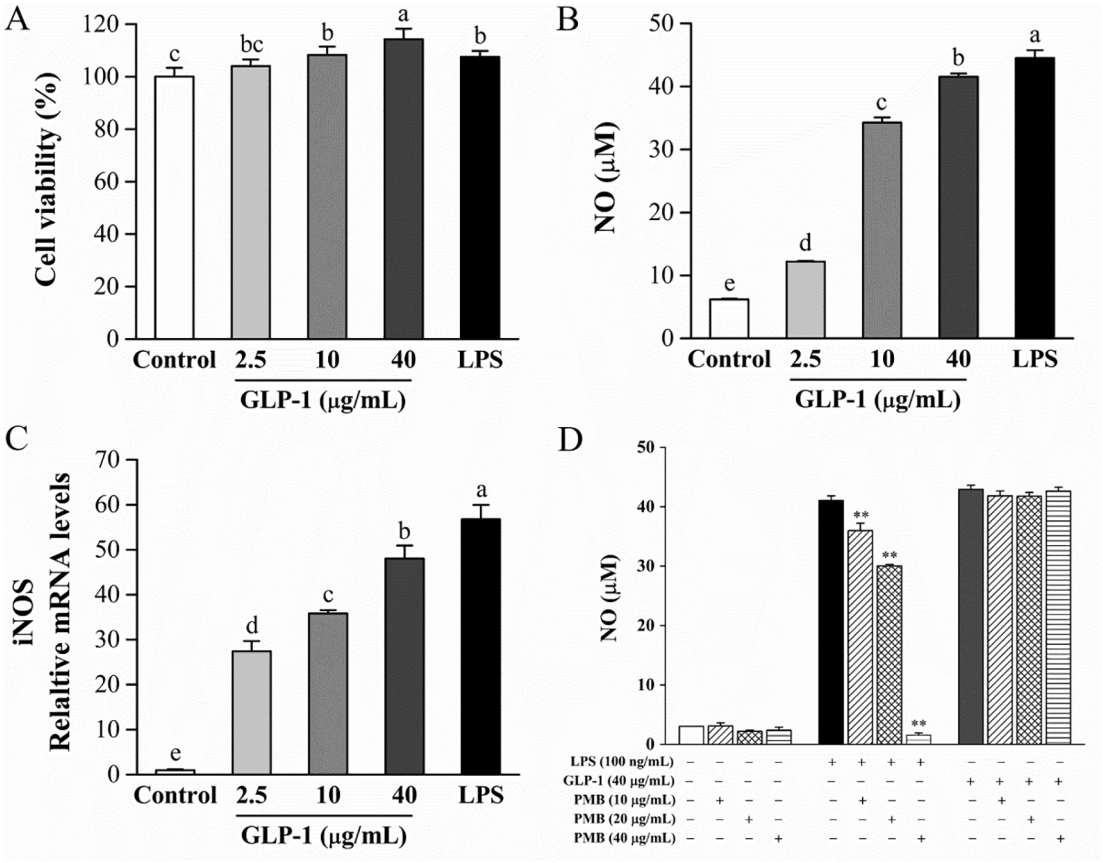

Fig. 1. Effects of GLP-1 on the cell viability (A), NO production (B), and iNOS mRNA expression level (C) in RAW264.7 macrophages; Effect of PMB treatment on NO production induced by GLP-1 or LPS (D). Data are shown as mean ± SD (n = 4). $^{**}p < 0.01$ vs. the LPS treated group.

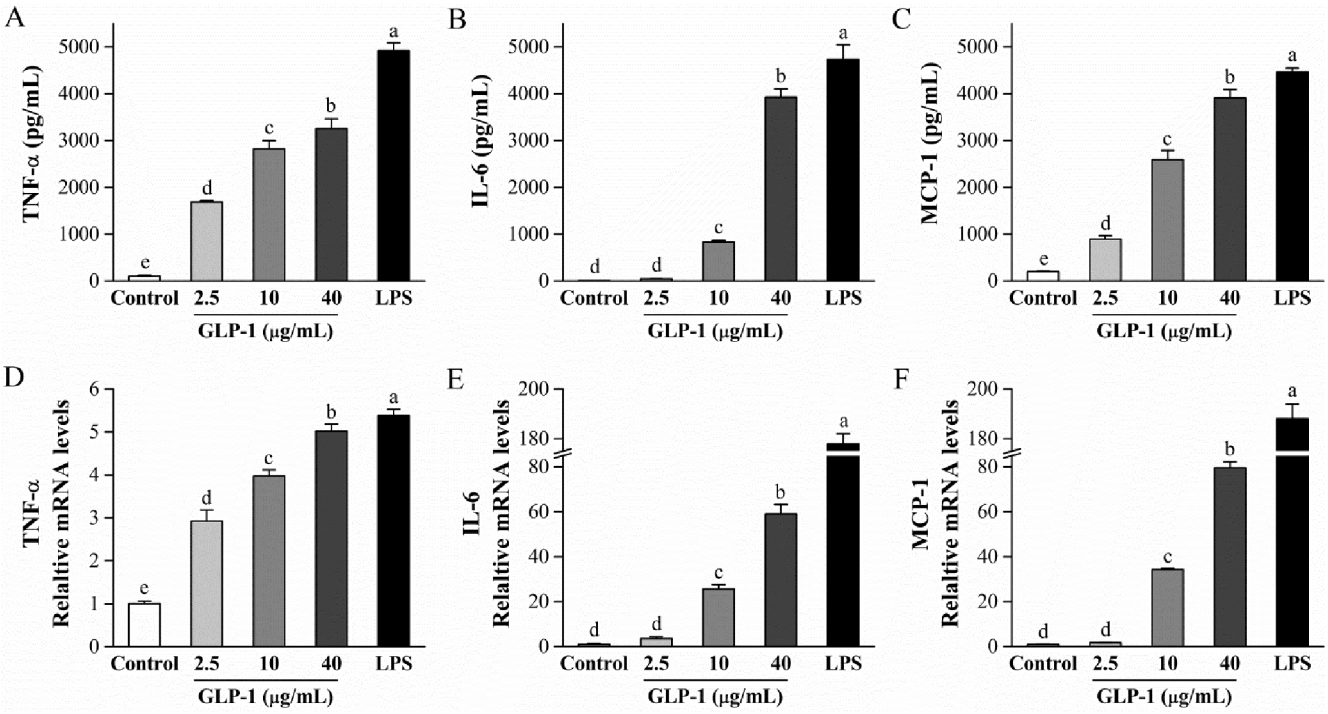

Fig. 2. Effects of GLP-1 on the secretion of TNF-α (A), IL-6 (B), and MCP-1 (C), and their mRNA expression levels (D-F) in RAW264.7 macrophages. Data are shown as mean ± SD (n = 3).

3.2. GLP-1 promoted NO production and iNOS mRNA expression

NO synthesized by iNOS is considered to be a crucial marker of macrophage activation (Zhou et al., 2020). In this study, the NO content in the cell culture supernatant was measured by Griess reagent. The result showed that, compared with the control group (6.2 μM), GLP-1 at 2.5–40 μg/mL remarkably increased the concentration of NO to 12.2, 34.3, and 41.5 μM, respectively. (Fig. 1B). Meanwhile, the effect of GLP-1 on iNOS mRNA expression was further analyzed by RT-PCR assay. As shown in Fig. 1C, the iNOS mRNA expression levels of GLP-1 at 2.5–40 μg/mL were 27.4, 35.9, and 48.0-fold higher than that of the control group, respectively. These results suggested that GLP-1 could promote the secretion of NO by up-regulating the transcriptional level of iNOS. To exclude the endotoxin contamination in GLP-1, RAW264.7 cells were treated with GLP-1 or LPS in the presence or absence of PMB. Fig. 1D showed that PMB treatment (10–40 μg/mL) remarkably inhibited LPS-induced NO production in RAW264.7 cells, whereas no significant difference was observed in GLP-1. These results verified that GLP-1 was free of LPS contamination.

3.3. GLP-1 induced cytokines secretion and mRNA expression

Apart from NO, the pro-inflammatory cytokines (TNF-α, IL-6, MCP-1, etc.) can stimulate the innate immune responses (Gao et al., 2020). To further investigate the macrophage activation of GLP-1, the secretion of TNF-α, IL-6, and MCP-1, and their mRNA expression levels in RAW264.7 cells were analyzed by ELISA and RT-PCR. As presented in Fig. 2A-C, GLP-1 at 2.5–40 μg/mL induced the secretion of TNF-α, IL-6, and MCP-1 in a dose-dependent manner. Compared with the control group, the concentration of TNF-α, IL-6, and MCP-1 in RAW264.7 cells treated with 40 μg/mL of GLP-1 were dramatically increased from 105.0 to 3255.9 pg/mL, 8.3 to 3925.3 pg/mL, and 202.6 to 3913.5 pg/mL, respectively. Similar results were observed in the up-regulation of their mRNA expression levels (Fig. 2D-F). When RAW264.7 cells were exposed to 40 μg/mL of GLP-1, the mRNA expression levels of TNF-α, IL-6, and MCP-1 were increased to 5.0, 59.0, and 79.6-fold, respectively. These results indicated that GLP-1 could mediate cytokines secretion at the transcriptional level.

3.4. GLP-1 increased phagocytic activity and ROS generation

The phagocytic response, a distinguishing characteristic of macrophage activation, is indispensable for host defense (West, Shadel, & Ghosh, 2011). To investigate whether GLP-1 enabled to increase the phagocytic activity of macrophages, the internalization of FITC-labeled *E. coli* by RAW264.7 cells was analyzed by flow cytometry and fluorescence microscopy. Flow cytometry analysis showed that GLP-1 (2.5–40 μg/mL) significantly increased the uptake of FITC-labeled *E. coli* to 1.1, 1.3, and 1.4-fold of the control group, respectively. (Fig. 3A), which was supported by the result of fluorescence microscopy (Fig. 3B). These results confirmed that GLP-1 could improve the phagocytosis of RAW264.7 macrophages.

Phagocytosis is associated with the production of ROS (West et al., 2011). The intracellular ROS level in RAW264.7 macrophages stimulated by GLP-1 was analyzed by the DCFH-DA staining method. Flow cytometry analysis showed that GLP-1 at 2.5–40 μg/mL remarkedly

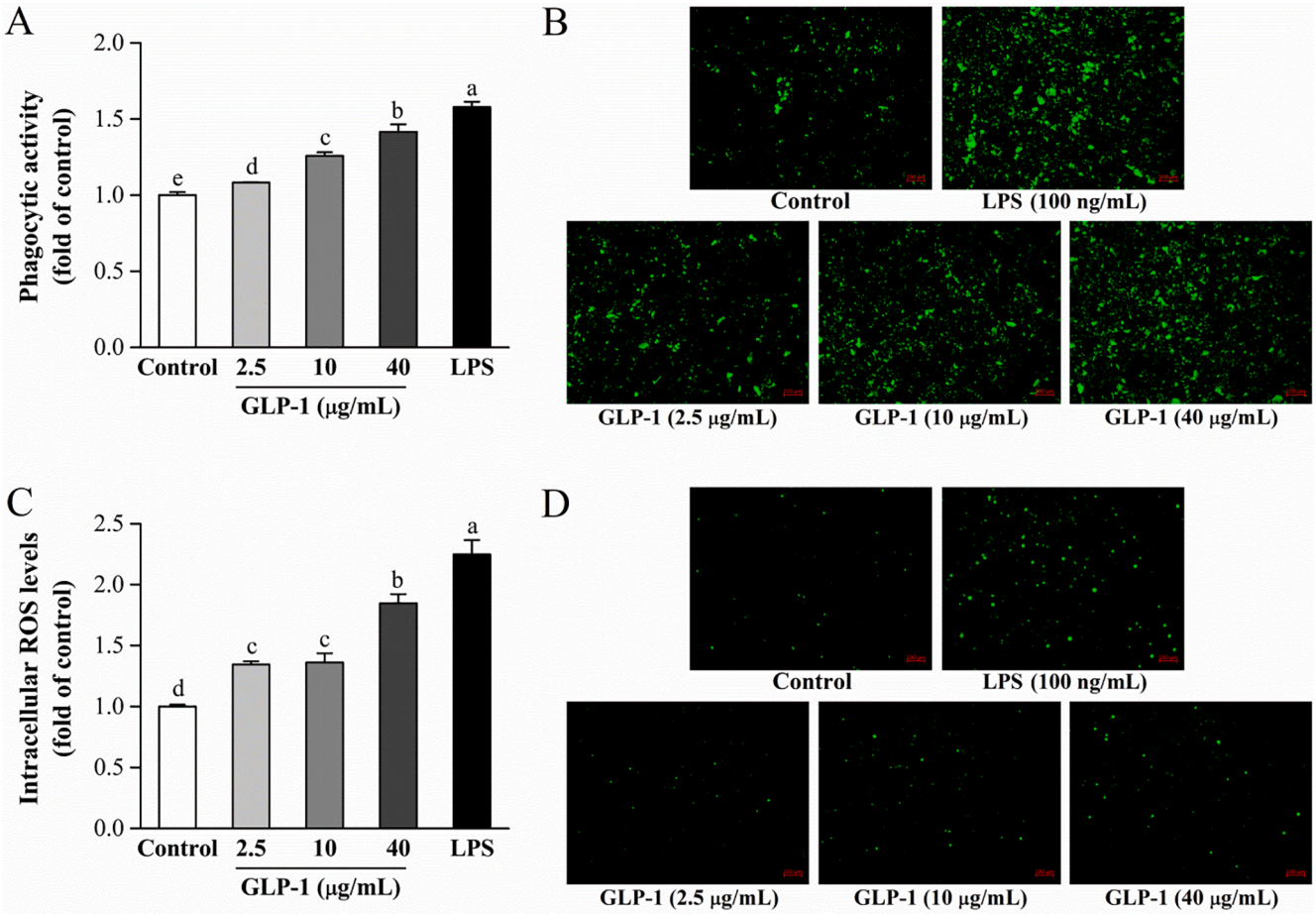

Fig. 3. Effect of GLP-1 on the phagocytic capacity of FITC-labeled *E. coli* measured by flow cytometry (A) and representative fluorescence microscopic images (B); Effect of GLP-1 on the ROS production measured by flow cytometry (C) and representative fluorescence microscopic images (D). Data are shown as mean ± SD (n = 3).

increased the ROS production to 1.3, 1.3, and 1.9-fold of the control group, respectively. (Fig. 3C). The consistent result was observed under fluorescence microscopy (Fig. 3D). These data indicated that GLP-1 promoted the production of ROS was probably involved in the enhancement of phagocytosis in macrophages.

3.5. GLP-1 activated mitogen-activated protein kinase (MAPKs) and phosphatidylinositol-3-kinase (PI3K)/ protein kinase B (Akt) signaling pathways

MAPKs and PI3K/Akt signaling pathways have been reported to regulate important immune functions (Meng et al., 2018). To explore whether MAPKs and PI3K/Akt signaling pathways were involved in

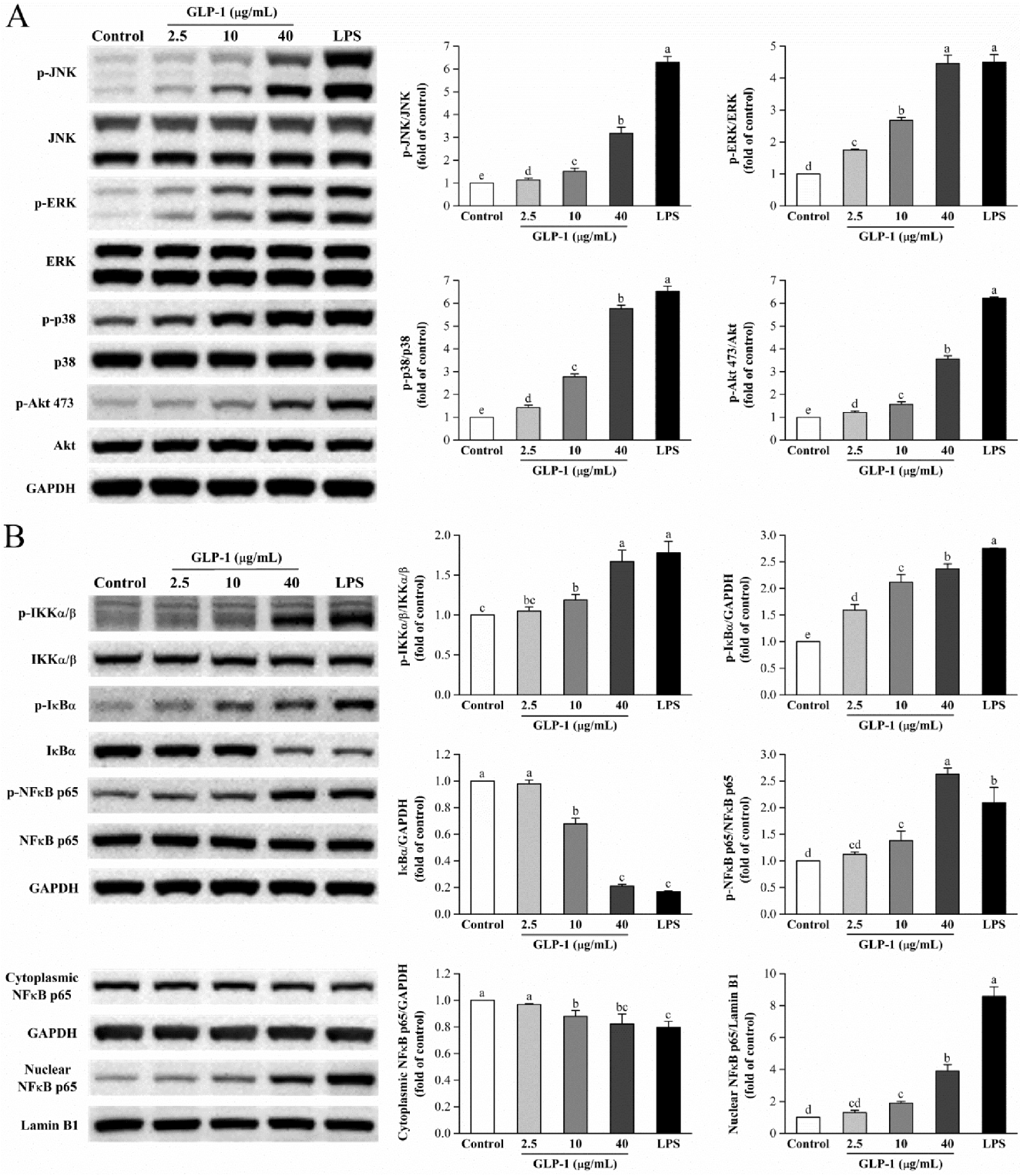

Fig. 4. Effects of GLP-1 on the expression levels of p-IKKα/β, p-IκBα, IκBα, p-NF-κB p65, and cytoplasmic and nuclear NF-κB p65 in RAW264.7 macrophages (A). Effects of GLP-1 on the expression levels of p-JNK, p-ERK, p-p38, and p-Akt in RAW264.7 macrophages (B). Data are shown as mean ± SD (n = 3).

macrophage activation by GLP-1, the phosphorylated levels of JNK, ERK, p38, and Akt were detected by western blot analysis. As shown in Fig. 4A, GLP-1 at 2.5–40 μg/mL significantly up-regulated the phosphorylation of JNK, ERK, p38, and Akt in a dose-dependent manner. After exposure to 40 μg/mL of GLP-1, the phosphorylated levels of JNK, ERK, p38, and Akt were increased to 3.2, 4.5, 5.8, and 3.6-fold, respectively. Furthermore, pre-treatment with four types of specific inhibitors (SP600125, U0126, SB203580, and LY294002) remarkedly suppressed the secretion of NO, TNF-α, and IL-6 induced by GLP-1 in RAW264.7 cells (Fig. 5A-C). These results suggested that MAPKs and PI3K/Akt signaling pathways participated in macrophage activation by GLP-1.

3.6. GLP-1 activated nuclear factor-kappa B (NF-κB) signaling pathway

The transcription factor NF-κB family plays an important role in modulating the mRNA expression of various immune genes (Gao et al., 2022; Wang et al., 2019). Thus, whether GLP-1 mediated macrophage activation through the NF-κB signaling pathway was investigated. As shown in Fig. 4B, GLP-1 (2.5–40 μg/mL) increased the phosphorylated levels of IKKα/β, NF-κB p65, and IκBα, and reduced the expression level of IκBα in a dose-dependent manner. The protein expression levels of p-IKKα/β, p-NF-κB p65, p-IκBα, and IκBα were 1.7, 2.6, 2.4, and 0.2-fold of the control group after exposure to 40 μg/mL of GLP-1, respectively. Additionally, NF-κB p65 in the cytosol was significantly translocated into the nucleus after exposure to 10 and 40 μg/mL of GLP-1. Moreover, pre-treatment with the NF-κB inhibitor (BAY 11–7082) obviously decreased NO (by 65.7%), TNF-α (by 20.0%), and IL-6 (by 53.9%) secretion stimulated by GLP-1 in RAW264.7 macrophages (Fig. 5A-C). These findings indicated that NF-κB signaling pathway was involved in macrophage activation by GLP-1.

3.7. Recognition of GLP-1 by TLR2 and dectin-1 receptors

Increasing evidence confirms that macrophage activation by polysaccharides largely depends on the recognition of PRRs (Ellefsen, Wold, Wilkins, Rise, & Samuelsen, 2021; Lee et al., 2015). In this study, we investigated the roles of TLR2, TLR4, and dectin-1 receptors on the immunoregulatory activity of GLP-1. As shown in Fig. S1, pre-treatment with TLR2 and dectin-1 antibodies significantly decreased NO (by 17.8% and 12.9%) and IL-6 (by 39.8% and 34.1%) release from RAW264.7 cells compared with the GLP-1-treated group. No reduction in the secretion of NO and IL-6 was observed in RAW264.7 cells pre-treated with TLR4 antibody. These results implied that GLP-1 was able to activate macrophages by interacting with TLR2 and dectin-1 receptors.

3.8. Immunomodulatory activity of GLP-1 in CTX-induced immunosuppressed mice

CTX, a commonly immunosuppressive agent, has been widely used to establish the immunosuppressed animal model (Yu et al., 2019). Hence, we further investigated the immunoregulatory effect of GLP-1 on CTX-induced immunosuppressed mice *in vivo*. Compared with the NG group, the body weights of mice were significantly decreased after intraperitoneal injection with CTX for three consecutive days. For the next 7 days, the GLP-1-M and GLP-1-H groups obviously reversed the body weight loss caused by CTX (Fig. S2). Compared with the MG group, the GLP-1-M and GLP-1-H groups significantly increased the thymus index by 65.4% and 76.0%, respectively (Table 1). In contrast, the spleen index of the MG group and all GLP-1-treated groups was higher than that of the NG group.

The serum immunoglobulins are indispensable indicators for humoral immunity (Li et al., 2020). As shown in Table 1, treatment with CTX significantly decreased the contents of IgM and IgG in serum. Consistent with the thymus index, the dose-dependent increases in IgM and IgG were observed after administration with GLP-1. Compared with the MG group, the GLP-1-H groups remarkably increased IgM and IgG by 32.5% and 60.6%, respectively. These data indicated that GLP-1 could recover the suppression of humoral immunity induced by CTX.

Table 1
Effects of GLP-1 on immune organs indices, serum immunoglobulin levels, and spleen T lymphocyte subsets in CTX-treated mice.

Group	Spleen index (mg/g)	Thymus index (mg/g)	IgM (μg/mL)	IgG (μg/mL)	$CD3^+$ (%)	$CD4^+/CD8^+$
NG	3.43 ± 0.39	2.10 ± 0.44	214.77 ± 19.81	22.28 ± 3.69	26.15 ± 1.33	2.78 ± 0.17
MG	4.46 ± 0.49$^{\Delta\Delta}$	1.04 ± 0.44$^{\Delta\Delta}$	162.78 ± 7.62$^{\Delta\Delta}$	13.97 ± 3.92$^{\Delta\Delta}$	10.22 ± 1.81$^{\Delta\Delta}$	2.74 ± 0.40
GLP-1-L (40 mg/kg)	4.49 ± 0.57	1.47 ± 0.45	175.92 ± 20.44	19.46 ± 4.91	16.45 ± 3.17**	2.76 ± 0.61
GLP-1-M (80 mg/kg)	4.11 ± 0.86	1.72 ± 0.46*	205.31 ± 17.22**	20.50 ± 3.61*	23.42 ± 2.93**	2.42 ± 0.25
GLP-1-H (160 mg/kg)	5.06 ± 0.77	1.83 ± 0.29**	215.69 ± 27.24**	22.44 ± 2.89**	16.48 ± 4.35**	2.44 ± 0.35
LNT (80 mg/kg)	4.91 ± 0.54	1.84 ± 0.66*	203.39 ± 31.06**	20.58 ± 2.73**	18.25 ± 3.08**	2.93 ± 0.44

Data are shown as mean ± SD (n = 6 ~ 8). $^{\Delta\Delta}p < 0.01$ vs. the NG group; $^*p < 0.05$ and $^{**}p < 0.01$ vs. the MG group.

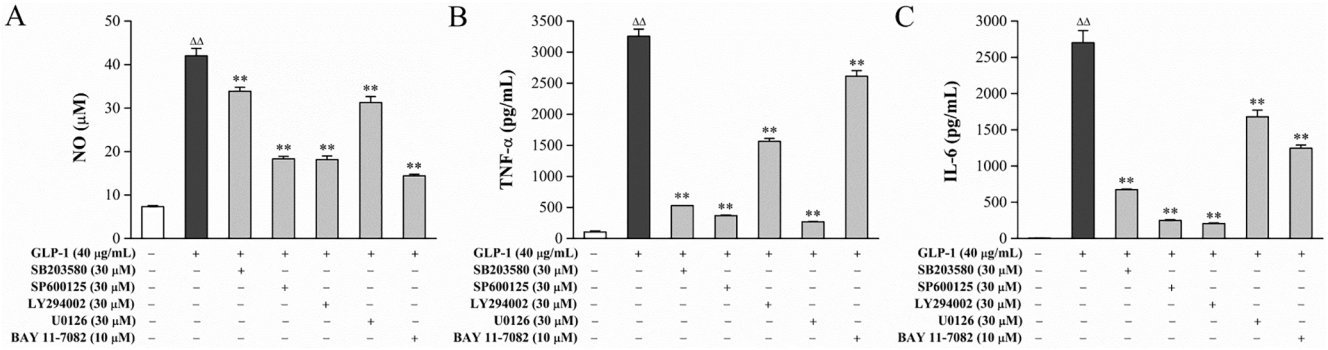

Fig. 5. Effects of SP600125 (JNK inhibitor), U0126 (ERK inhibitor), SB203580 (p38 inhibitor), LY294002 (PI3K inhibitor), BAY 11-7082 (NF-κB inhibitor) on GLP-1-induced NO (A), TNF-α (B), and IL-6 (C) secretion in RAW264.7 macrophages. Data are shown as mean ± SD (n = 3). $^{**}p < 0.01$ vs. the GLP-1 treated group.

To further explore the effect of GLP-1 on cellular immunity, the percentage of $CD3^+$ and the proportion of $CD4^+/CD8^+$ T lymphocytes in the spleen were analyzed by flow cytometry. As presented in Table 1, treatment with CTX sharply reduced the percentage of $CD3^+$ T lymphocytes in the spleen. Compared with the MG group, the GLP-1-L, GLP-1-M, and GLP-1-H groups significantly increased the percentage of $CD3^+$ T lymphocytes by 61.0%, 129.2%, and 61.3%, respectively. However, all GLP-1-treated groups did not significantly adjust the ratio of $CD4^+/CD8^+$ T lymphocytes. LNT was used as the positive control in this study. It needed to point out that the immunoregulatory effects of GLP-1-M and GLP-1-H groups on body weight, immune organs indices, serum immunoglobulins, and spleen *T*-lymphocyte subsets were comparable to those of the LNT group.

4. Discussion

Immunostimulation is considered as a crucial strategy for improving the host defense in hypoimmunity populations (Lee et al., 2015). Polysaccharides are the main components of mushrooms with lower toxicity and stronger immunostimulatory activity (Maity et al., 2021). *Ganoderma* species is commonly consumed in Asia and its polysaccharides can exert considerable effects on the host immune system (Ren et al., 2021). *G. leucocontextum*, a new species of *Ganoderma*, was discovered by Professor Li in Southwestern China in 2014 (Li et al., 2015). Our group purified an α-1,4-glucan polysaccharide (GLP-3) with molecular weight of 159.7 kDa from *G. leucocontextum* for the first time (Gao et al., 2020). GLP-3 at 50–200 μg/mL could exert immunomodulatory activity through enhancing the phagocytic capacity and the production of NO, cytokines, and chemokines in RAW264.7 macrophages. Interestingly, GLP-1 exhibited stronger immunomodulatory effects than GLP-3 according to the results obtained from this study. GLP-1 mainly consisted of glucose (60.9%), galactose (12.0%), xylose (8.6%), arabinose (7.5%), and mannose (7.0%), while GLP-3 was mainly composed of glucose (92.7%). The complicated monosaccharide composition of GLP-1 made it have more branches of residues units, which was associated with high immunostimulatory activity (Ferreira et al., 2015). Yu et al. (2017) found that polysaccharides from American ginseng with both α- and β-configurations showed higher immunostimulatory activities than those with α-configuration only. Despite both GLP-1 and GLP-3 simultaneously had α- and β-configurations, the backbone of GLP-1 was linked with β-type glycosidic bonds including (1 → 3)-β-Glc*p*, (1 → 4)-β-Glc*p*, and (1 → 6)-β-Gal*p*, while GLP-3 only contained (1 → 4)-α-Glc*p* glycosidic bond in the backbone. The molecular weight is very important for the immunostimulatory activities of polysaccharides. Although it is not an exclusive property, the differences in molecular weight are intrinsically related to other structural features. The molecular weight of GLP-1 was 6.3 kDa, which is much lower than that of GLP-3 (159.7 kDa). Tabarsa, You, Dabaghian, and Surayot (2018) reported that the lower molecular weight of polysaccharide fraction from *Ulva intestinalis* exhibited better immunomodulatory activity, which is consistent with our results. Therefore, the difference in immunomodulation between GLP-1 and GLP-3 was probably attributed to their diverse monosaccharide composition, glycosidic linkage, and molecular weight.

Macrophage activation is regarded as one of the most important events in the immune response (Lee et al., 2015). Activated macrophages can phagocytize invading pathogens and microbes by promoting the production of NO, ROS, and cytokines (TNF-α, IL-6, IL-1β, MCP-1, etc.) (Cui et al., 2021). Several studies have proved that polysaccharides from *Ganoderma* can exert immunomodulatory activities through activating macrophages (Ren et al., 2021). In this study, GLP-1 was found to improve phagocytic capacity and increase the production of NO, ROS, and cytokines (TNF-α, IL-6, and MCP-1) in RAW264.7 cells. GLP-1 also up-regulated the mRNA expression of iNOS, TNF-α, IL-6, and MCP-1. The MAPKs (ERKs, JNKs, p38 MAPK, etc.) and PI3K/Akt signaling pathways have been reported to participate in macrophage activation. NF-κB, a ubiquitous transcription factor, is indispensable for the modulation of various cellular genes involved in immune responses (Ren et al., 2017). Previous studies showed that fungal polysaccharides from *G. atrum* (Yu et al., 2012) and *Flammulina velutipes* (Meng et al., 2018) could activate macrophage through MAPKs, PI3K/Akt, and NF-κB pathways. Moreover, Yu et al. (2012) and Meng et al. (2018) found that the activation of NF-κB transcription factor was dependent on MAPKs and PI3K/Akt pathways. Our results showed that GLP-1 increased the phosphorylation of JNK, ERK, p38, Akt, IKKα/β, NF-κB p65, and IκBα, and induced the nuclear translocation of NF-κB p65 in RAW264.7 cells. Moreover, specific inhibitors of MAPKs, PI3K/Akt, and NF-κB could significantly inhibit NO, TNF-α, and IL-6 production. These results indicated that the immunostimulatory activity of GLP-1 was involved in MAPKs, PI3K/Akt, and NF-κB signaling pathways.

The macrophage activation begins with the recognition of PRRs (TLR2, TLR4, dectin-1, etc.) on the cell surfaces, followed by activating the intracellular signaling pathways (Deng, Fu, Shang, Chen, & Xu, 2018). Several studies have demonstrated that polysaccharides can activate macrophages by binding to TLR2, TLR4, and dectin-1 receptors (Guo et al., 2009; Wang et al., 2013; Lee et al., 2015). In the present study, we found that TLR2 and dectin-1 antibodies could reduce the NO and IL-6 secretion induced by GLP-1. Therefore, GLP-1 mediated macrophage activation was partly attributed to its interaction with TLR2 and dectin-1 receptors. Guo et al. (2009) found that a β-glucan polysaccharide from the spores of *G. lucidum* exhibited immunostimulatory effects through binding to the dectin-1 receptor, but not TLR4. Lee et al. (2015) reported that a polysaccharide with β-linkage configuration from *Cordyceps militaris* culture broth could activate macrophages *via* TLR2, TLR4, and dectin-1 receptors. So we concluded that the (1 → 3)-β-Glc*p*, (1 → 4)-β-Glc*p*, and (1 → 6)-β-Gal*p* glycosidic linkages in the backbone of GLP-1 were associated with the recognition of TLR2 and dectin-1 receptors.

TLR2 has been proven to be involved in the activation of myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway *via* the Toll/IL-1R domain-containing adaptor protein (TIRAP). MyD88 can associate with IL-1R-associated kinases (IRAK), leading to the sequential activation of TNF receptor-associated factor 6 (TRAF6). Subsequently, TRAF6 forms a complex with transforming-growth-factor-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), and TAB2, promoting the activation of TAK1. TAK1, in turn, phosphorylates both MAPKs (JNKs, ERKs, p38 MAPK, etc.) and the IκB kinase (IKK) complex, which consists of IKK-α, IKK-β, and IKK-γ. The IKK complex then phosphorylates IκB and leads to its degradation and translocation of NF-κB into the nucleus, which induces the expression of target genes (Lee & Kim, 2007). The PI3K/Akt signaling pathway also seems to be activated upon TLR2 stimulation by forming a PI3K-MyD88 complex. This leads to the activation of Akt, which triggers the phosphorylation of NF-κB (Akira & Takeda, 2004). Dectin-1 can recruit spleen tyrosine kinase (Syk), and in turn, activates the caspase recruitment domain protein 9 (CARD9)-B cell lymphoma 10 (BCL10)-mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) signalosome (Lee & Kim, 2007). Dectin-1 stimulation also can promote phagocytosis. Based on these discussions and our results, the possible immunomodulatory molecular mechanism of GLP-1 in RAW264.7 macrophage is shown in Fig. S3.

The immune system, which consists of innate and adaptive immunity, can control and adjust autoimmune responses and maintain homeostasis (Yu et al., 2019). Macrophages activation is belonged to innate immunity, while immune organs, immunoglobulins in serum, and *T*-lymphocytes in the spleen are important components for adaptive immunity (Tang et al., 2019). The spleen and thymus are critical immune organs that play central roles in the modulation of nonspecific immunity (Yu et al., 2019). IgM and IgG, secreted by B cells, are two key effector molecules in the humoral immune response. IgM exhibits a strong anti-infectious effect, while IgG participates in the phagocytosis of monocytes (Zhang et al., 2021). T lymphocytes are important immune cells involved in the regulation of cellular immunity. $CD3^+$ marked cells

reflect the total level of mature T lymphocytes. $CD4^+$ and $CD8^+$ marked cells represent helper and cytotoxic T lymphocytes, respectively (Huang, Shen, Yu, Liu, & Xie, 2020). In our study, GLP-1 increased the thymus index, IgM and IgG levels, and percentage of $CD3^+$ T lymphocytes in CTX-induced immunosuppressed mice. These results suggested that GLP-1 could enhance adaptive immunity *in vivo*. However, the ratio of $CD4^+/CD8^+$ T lymphocytes in all groups showed no significant differences, which is similar to the results reported by Li et al. (2020). Additionally, intraperitoneal injection with CTX increased the spleen index. This phenomenon is possibly related to the activation of extramedullary hematopoiesis induced by CTX according to the previous literature (Li et al., 2020).

In conclusion, we found that TLR2 and dectin-1 were two major receptors responsible for the interaction of GLP-1 and RAW264.7 macrophages. Moreover, GLP-1 could enhance the phagocytic capacity of macrophages and promote the production of NO, ROS, and cytokines (TNF-α, IL-6, and MCP-1) through MAPKs, PI3K/Akt, and NF-κB signaling pathways. Furthermore, GLP-1 could partly improve the immune function in the CTX-induced immunosuppressed mice. We have elucidated the molecular mechanism related to the immunostimulatory effect of GLP-1 *in vitro*. However, the immune enhancement mechanism of GLP-1 *in vivo* will be further conducted in our future studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100321.

References:

Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology, 4*(7), 499–511. https://doi.org/10.1038/nri1391

Bai, Y., Jiang, Y., Liu, T., Li, F.u., Zhang, J., Luo, Y., … Hu, W. (2019). Xinjiang herbal tea exerts immunomodulatory activity *via* TLR2/4-mediated MAPK signaling pathways in RAW264.7 cells and prevents cyclophosphamide-induced immunosuppression in mice. *Journal of Ethnopharmacology, 228*, 179–187. https://doi.org/10.1016/j.jep.2018.09.032

Cui, L., Chen, L., Yang, G., Li, Y., Qiao, Z., Liu, Y., … Sun, L. (2021). Structural characterization and immunomodulatory activity of a heterogalactan from *Panax ginseng* flowers. *Food Research International, 140*, 109859. https://doi.org/10.1016/j.foodres.2020.109859

Deng, C., Fu, H., Shang, J., Chen, J., & Xu, X. (2018). Dectin-1 mediates the immunoenhancement effect of the polysaccharide from *Dictyophora indusiata*. *International Journal of Biological Macromolecules, 109*, 369–374. https://doi.org/10.1016/j.ijbiomac.2017.12.113

Ellefsen, C. F., Wold, C. W., Wilkins, A. L., Rise, F., & Samuelsen, A. B. C. (2021). Water-soluble polysaccharides from *Pleurotus eryngii* fruiting bodies, their activity and affinity for Toll-like receptor 2 and dectin-1. *Carbohydrate Polymers, 264*, 117991. https://doi.org/10.1016/j.carbpol.2021.117991

Fang, J., Lin, Y., Xie, H., Farag, M. A., Feng, S., Li, J., & Shao, P. (2022). *Dendrobium officinale* leaf polysaccharides ameliorated hyperglycemia and promoted gut bacterial associated SCFAs to alleviate type 2 diabetes in adult mice. *Food Chemistry: X, 13*, Article 100207. https://doi.org/10.1016/j.fochx.2022.100207

Ferreira, S. S., Passos, C. P., Madureira, P., Vilanova, M., & Coimbra, M. A. (2015). Structure function relationships of immunostimulatory polysaccharides: A review. *Carbohydrate Polymers, 132*, 378–396. https://doi.org/10.1016/j.carbpol.2015.05.079

Gao, X., Lin, X., Li, X., Zhang, Y., Chen, Z., & Li, B. (2017). Cellular antioxidant, methylglyoxal trapping, and anti-inflammatory activities of cocoa tea (*Camellia ptilophylla* Chang). *Food & Function, 8*(8), 2836–2846. https://doi.org/10.1039/c7fo00368d

Gao, X., Qi, J., Ho, C.-T., Li, B., Mu, J., Zhang, Y., … Xie, Y. (2020). Structural characterization and immunomodulatory activity of a water-soluble polysaccharide from *Ganoderma leucocontextum* fruiting bodies. *Carbohydrate Polymers, 249*, 116874. https://doi.org/10.1016/j.carbpol.2020.116874

Gao, X., Qi, J., Ho, C.-T., Li, B., Xie, Y., Chen, S., … Wu, Q. (2021). Purification, physicochemical properties, and antioxidant activities of two low-molecular-weight polysaccharides from *Ganoderma leucocontextum* fruiting bodies. *Antioxidants, 10*(7), 1145. https://doi.org/10.3390/antiox10071145

Gao, X., Zeng, R., Ho, C.-T., Li, B., Chen, S., Xiao, C., … Wu, Q. (2022). Preparation, chemical structure, and immunostimulatory activity of a water-soluble heteropolysaccharide from *Suillus granulatus* fruiting bodies. *Food Chemistry: X, 13*, 100211. https://doi.org/10.1016/j.fochx.2022.100211

Gao, Y., Chan, E., & Zhou, S. (2004). Immunomodulating activities of *Ganoderma*, a mushroom with medicinal properties. *Food Reviews International, 20*(2), 123–161. https://doi.org/10.1081/FRI-120037158

Guo, L., Xie, J., Ruan, Y., Zhou, L., Zhu, H., Yun, X., … Gu, J. (2009). Characterization and immunostimulatory activity of a polysaccharide from the spores of *Ganoderma lucidum*. *International Immunopharmacology, 9*(10), 1175–1182. https://doi.org/10.1016/j.intimp.2009.06.005

Huang, R., Shen, M., Yu, Y., Liu, X., & Xie, J. (2020). Physicochemical characterization and immunomodulatory activity of sulfated Chinese yam polysaccharide. *International Journal of Biological Macromolecules, 165*, 635–644. https://doi.org/10.1016/j.ijbiomac.2020.09.213

Ina, K., Furuta, R., Kataoka, T., Kayukawa, S., Yoshida, T., Miwa, T., … Takeuchi, Y. (2011). Lentinan prolonged survival in patients with gastric cancer receiving S-1-based chemotherapy. *World journal of clinical oncology, 2*(10), 339–343. https://doi.org/10.5306/wjco.v2.i10.339

Lee, J. S., Kwon, D. S., Lee, K. R., Park, J. M., Ha, S., & Hong, E. K. (2015). Mechanism of macrophage activation induced by polysaccharide from *Cordyceps militaris* culture broth. *Carbohydrate Polymers, 120*, 29–37. https://doi.org/10.1016/j.carbpol.2014.11.059

Lee, M. S., & Kim, Y.-J. (2007). Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annual Review of Biochemistry, 76*(1), 447–480. https://doi.org/10.1146/annurev.biochem.76.060605.122847

Li, J., Gu, F., Cai, C., Hu, M., Fan, L., Hao, J., & Yu, G. (2020). Purification, structural characterization, and immunomodulatory activity of the polysaccharides from *Ganoderma lucidum*. *International Journal of Biological Macromolecules, 143*, 806–813. https://doi.org/10.1016/j.ijbiomac.2019.09.141

Li, M.-Z., Huang, X.-J., Hu, J.-L., Cui, S. W., Xie, M.-Y., & Nie, S.-P. (2020). The protective effects against cyclophosphamide (CTX)-induced immunosuppression of three glucomannans. *Food Hydrocolloids, 100*, 105445. https://doi.org/10.1016/j.foodhyd.2019.105445

Li, T., Hu, H., Deng, W., Wu, S., Wang, D., & Tsering, T. (2015). *Ganoderma leucocontextum*, a new member of the *G. lucidum* complex from southwestern China. *Mycoscience, 56*(1), 81–85. https://doi.org/10.1016/j.myc.2014.03.005

Maity, P., Sen, I. K., Chakraborty, I., Mondal, S., Bar, H., Bhanja, S. K., … Maity, G. N. (2021). Biologically active polysaccharide from edible mushrooms: A review. *International Journal of Biological Macromolecules, 172*, 408–417. https://doi.org/10.1016/j.ijbiomac.2021.01.081

Meng, Y., Yan, J., Yang, G., Han, Z., Tai, G., Cheng, H., & Zhou, Y. (2018). Structural characterization and macrophage activation of a hetero-galactan isolated from *Flammulina velutipes*. *Carbohydrate Polymers, 183*, 207–218. https://doi.org/10.1016/j.carbpol.2017.12.017

Morrison, D. C., & Jacobs, D. M. (1976). Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry, 13*(10), 813–818. https://doi.org/10.1016/0019-2791(76)90181-6

Ren, D., Lin, D., Alim, A., Zheng, Q., & Yang, X. (2017). Chemical characterization of a novel polysaccharide ASKP-1 from *Artemisia sphaerocephala* Krasch seed and its macrophage activation *via* MAPK, PI3k/Akt and NF-kappa B signaling pathways in RAW264.7 cells. *Food & Function, 8*(3), 1299–1312. https://doi.org/10.1039/c6fo01699e

Ren, L., Zhang, J., & Zhang, T. (2021). Immunomodulatory activities of polysaccharides from *Ganoderma* on immune effector cells. *Food Chemistry, 340*, 127933. https://doi.org/10.1016/j.foodchem.2020.127933

Su, Y., Chen, L., Yang, F., & Cheung, P. C. K. (2021). Beta-D-glucan-based drug delivery system and its potential application in targeting tumor associated macrophages. *Carbohydrate Polymers, 253*, 117258. https://doi.org/10.1016/j.carbpol.2020.117258

Tabarsa, M., You, S., Dabaghian, E. H., & Surayot, U. (2018). Water-soluble polysaccharides from *Ulva intestinalis*: Molecular properties, structural elucidation and immunomodulatory activities. *Journal of Food and Drug Analysis, 26*(2), 599–608. https://doi.org/10.1016/j.jfda.2017.07.016

Tang, J., Zhen, H., Wang, N., Yan, Q., Jing, H., & Jiang, Z. (2019). Curdlan oligosaccharides having higher immunostimulatory activity than curdlan in mice treated with cyclophosphamide. *Carbohydrate Polymers, 207*, 131–142. https://doi.org/10.1016/j.carbpol.2018.10.120

Wang, H., Xu, L., Yu, M., Wang, Y., Jiang, T., Yang, S., & Lv, Z. (2019). Glycosaminoglycan from *Apostichopus japonicus* induces immunomodulatory activity in cyclophosphamide-treated mice and in macrophages. *International Journal of Biological Macromolecules, 130*, 229–237. https://doi.org/10.1016/j.ijbiomac.2019.02.093

Wang, Y., Fang, J., Ni, X., Li, J., Liu, Q., Dong, Q., … Ding, K. (2013). Inducement of cytokine release by GFPBW2, a novel polysaccharide from fruit bodies of *Grifola frondosa*, through Dectin-1 in macrophages. *Journal of Agricultural and Food Chemistry, 61*(47), 11400–11409. https://doi.org/10.1021/jf4029915

West, A. P., Shadel, G. S., & Ghosh, S. (2011). Mitochondria in innate immune responses. *Nature Reviews Immunology, 11*(6), 389–402. https://doi.org/10.1038/nri2975

Yao, W., Chen, X., Li, X., Chang, S., Zhao, M., & You, L. (2021). Current trends in the anti-photoaging activities and mechanisms of dietary non-starch polysaccharides from natural resources. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2021.1939263

Yu, F., Zhang, Z., Ye, S., Hong, X., Jin, H., Huang, F., Yang, Z., Tang, Y., Chen, Y., & Ding, G. (2019). Immunoenhancement effects of pentadecapeptide derived from *Cyclina sinensis* on immune-deficient mice induced by cyclophosphamide. *Journal of Functional Foods, 60*, 103408. https://doi.org/10.1016/j.jff.2019.06.010

Yu, Q., Nie, S., Wang, J., Huang, D., Li, W., & Xie, M. (2015). Signaling pathway involved in the immunomodulatory effect of *Ganoderma atrum* polysaccharide in spleen lymphocytes. *Journal of Agricultural and Food Chemistry, 63*(10), 2734–2740. https://doi.org/10.1021/acs.jafc.5b00028

Yu, Q., Nie, S., Wang, J., Yin, P., Li, W., & Xie, M. (2012). Polysaccharide from *Ganoderma atrum* induces tumor necrosis factor-alpha secretion *via* phosphoinositide 3-kinase/Akt, mitogen-activated protein kinase and nuclear factor-kappa B signaling pathways in RAW264.7 cells. *International Immunopharmacology, 14*(4), 362–368. https://doi.org/10.1016/j.intimp.2012.09.005

Yu, X., Liu, Y., Wu, X., Liu, L., Fu, W., & Song, D. (2017). Isolation, purification, characterization and immunostimulatory activity of polysaccharides derived from American ginseng. *Carbohydrate Polymers, 156*, 9–18. https://doi.org/10.1016/j.carbpol.2016.08.092

Yu, Y., Zhang, Y., Hu, C., Zou, X., Lin, Y., Xia, Y., & You, L. (2019). Chemistry and immunostimulatory activity of a polysaccharide from *Undaria pinnatifida*. *Food and Chemical Toxicology, 128*, 119–128. https://doi.org/10.1016/j.fct.2019.03.042

Zhang, J., Gao, S., Li, H., Cao, M., Li, W., & Liu, X. (2021). Immunomodulatory effects of selenium-enriched peptides from soybean in cyclophosphamide-induced immunosuppressed mice. *Food Science & Nutrition, 9*(11), 6322–6334. https://doi.org/10.1002/fsn3.v9.1110.1002/fsn3.2594

Zhang, S., Hu, J., Sun, Y., Tan, H., Yin, J., Geng, F., & Nie, S. (2021). Review of structure and bioactivity of the *Plantago* (Plantaginaceae) polysaccharides. *Food Chemistry: X, 12*, 100158. https://doi.org/10.1016/j.fochx.2021.100158

Zhou, W., Zhao, Y., Yan, Y., Mi, J., Lu, L., Luo, Q., … Cao, Y. (2020). Antioxidant and immunomodulatory activities in *vitro* of polysaccharides from bee collected pollen of Chinese wolfberry. *International Journal of Biological Macromolecules, 163*, 190–199. https://doi.org/10.1016/j.ijbiomac.2020.06.244