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Cell wall mannoprotein of *Candida albicans* polarizes macrophages and affects proliferation and apoptosis through activation of the Akt signal pathway

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ABSTRACT

Candida albicans is a commensal fungus that associates with human hosts. Under normal circumstances this interaction does not produce any severe life-threatening disease, as macrophages of the innate immune system will result in its clearance. However, disorders may arise in immunosuppressed individuals. To understand the bioactivity of *Candida albicans* cell wall polysaccharides, which represent an important component of its function, mannoprotein from this fungus was extracted, purified and analyzed. Mannoprotein with α-(1,2) and α-(1,6) linkages was investigated with use of HPLC and NMR. Co-incubation of mannoprotein with macrophages resulted in a mannoprotein with the potential to polarize macrophages to M1 and promote phagocytosis/microbial killing ability thus increasing the clearance of pathogens through Akt2. Moreover, mannoprotein within the cell wall promoted cell proliferation and inhibited apoptosis by activation of the Akt signaling pathway. Collectively, α-(1,6)(1,2)-mannoprotein, one of the five polysaccharides extracted from the cell wall of *Candida albicans*, demonstrates immune-enhancing effects by activation of the Akt signaling pathway. These findings provide important new insights into the biological effects of polysaccharides on macrophages. Such information can then serve as the foundation for the development of novel anti-fungal medications.

1. Introduction

Candida albicans is an opportunistic fungus that colonizes the human epidermis, digestive tract and other mucosal surfaces. *Candida albicans* are the most prevalent and most pathogenic of the commensal Candida species [1]. Ordinarily, this fungus does not result in any severe life threatening disease in healthy adults, but can do so in immunocompromised or immunosuppressive patients [2]. The majority of healthy humans develop a commensalism with *Candida albicans*, which depends on immune tolerance [3,4].

It is well known that macrophages act as immune surveillance cells to identify, phagocytosis invaders and recruit other immune effector cells [5,6]. However, cell wall composition and fungal morphology represent important fungal characteristics that can interfere with phagocytosis of host cells, leading to severe infectious processes [7]. With regard to *Candida albicans*, filamentation is considered an immune escape process resulting from the death of immunocytes [8]. The cell wall of *Candida albicans* mainly consists of polysaccharides such as chitin, cellulose, glucan and mannan [9]. The structure of polysaccharides within the fungal cell wall, such as O- and N-linked mannosides, has been shown to affect host reactions [10]. In addition to the dermatological changes and systemic infection, mannan can also induce hyperlipidemia by stimulating macrophages [11]. Results from previous studies have revealed that Dectin-2 and Dectin-3 play essential roles in recognizing cell wall mannan, specifically α-mannans, which can then lead to the activation of NF-κB [12]. Notably, Dectin-2 plays the primary role in directing the efficiency of anti-fungal function [13] and the binding of Dectin-2 and Dectin-3 into a heterodimer has been shown to be essential for the recognition and defense against *Candida albicans* [14]. Given the important roles played by Dectin-2 and Dectin-3, one goal of this study was to examine the effects of their activation.

As isolation, extraction and purification of cellular wall components serve as the foundation for exploring their roles in immune reactions [15], our initial approach was to obtain purified polysaccharides with defined molecular structures from the cell wall of *Candida albicans*. With this procedure it was possible to identify the specific

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polysaccharide which acts as an immunostimulant, thus enabling us to study its function as related to innate immunity. The significance of polysaccharides from *Candida albicans* is that they can facilitate the identification of signaling pathways and their downstream reactions as related to immunity. Furthermore, cell wall macrophages as the innate immunocytes, polarized to M1 in a pro-inflammatory state or M2 in an anti-inflammatory state, processes which are important for both the initiation and resolution of immune regulation. In specific, polarization to M1 enhances immune reactions and increases pathogen elimination.

In this report, we focused on the Akt pathway. This pathway is the one most frequently altered as related to cell cycles, cell apoptosis, metabolism and other processes [16]. Moreover, as it is mostly involved with malignant transformation of tumors, along with their growth, proliferation, and metastasis [17], it seemed that directing our efforts at investigating the effects of mannoprotein on the Akt pathway may provide some new perspectives on the mechanisms involved in these responses. However, polysaccharides also possess immunoenhancing functions that can be developed into vaccines, notably the live attenuated *Candida albicans* strain, protein vaccines and glycoconjugates vaccines [18]. Therefore, we adopted the α-(1,6)(1,2)-mannoprotein of *Candida albicans* as a stimulant to investigate its effects on the Akt signaling pathway. Taken together, results from this study provide new and important findings which can serve as a solid foundation for the development and production of novel molecular pharmaceuticals.

2. Materials and methods

2.1. Strains and culture media

Candida albicans (ATCC 11006) was obtained from the American Type Culture Collection (Rockville, MD). Strains were stored at −80 °C in 30% glycerol and 70% sabouraud dextrose broth (SDB). Yeasts were cultured at 25 °C on SDB (1% peptone, 2% dextrose). *Candida albicans* were subcultured 3 times prior to use. Pellets were collected after centrifugation (300 rcf for 10 min) and washed twice with PBS buffer.

2.2. Extraction of polysaccharides from Candida albicans

The alkali method was employed to extract polysaccharides from *Candida albicans*. In brief, *Candida albicans* were collected and freeze-dried to obtain a dried biomass. The biomass was extracted, neutralized and centrifuged into a crude polysaccharide sample. An insoluble fraction was formed in the retentate during dialysis. The precipitate was collected and freeze-dried to obtain CABII after centrifugation. CABII was dissolved in 15 ml 3% sodium hydroxide. The supernatant was collected and neutralized by adjusting the pH. A gelatinous insoluble mass was formed and freeze-dried into CABII-A and CABII-B.

The content of all fractions was determined with use of the phenol-sulfuric acid method through a DEAE-SepharoseFF column. Three fractions were obtained. High performance gel filtration chromatography (HPGPC) was then used to monitor the homogeneity and molecular weight of the polysaccharides. The molecular weights of fractions obtained were 33.9 kDa, 145 kDa, and 10.2 kDa.

The above fractions were completely hydrolyzed with 2 mol/l TFA into monosaccharides, which were derivatized with use of the PMP method and analyzed by high performance liquid chromatography (HPLC) equipped with a C^{18} reversed-phase column to determine the carbohydrate composition.

2.3. Cell culture

The murine macrophage cell line, Ana-1, was purchased from the Bena Culture Collection (Jiangsu, China) and maintained in a cell incubator at 37 °C, 5% CO_2, in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (penicillin −100 units/ml and streptomycin −100 mg/ml). After incubation, cells were seeded at a density of $1 * 10^5$/ml per well in six-well plates. Cultured cells determined as appropriate for use in the experiment were those that had once adhered to the culture plate and achieved a confluence of 70%–80%.

2.4. Experimental grouping and treatment of cells

For PCR and ELISA assays, macrophages were co-incubated with α-(1,6)(1,2)-mannoprotein for 4 h. The cells and their supernatant were then collected for use in future experiments. In order to investigate the effects of α-(1,6)(1,2)-mannoprotein on the expression of cell proliferation and apoptosis, related proteins and the activation of the Akt pathway in macrophages, cells were initially treated with varying concentrations (0, 30, 80, and 100 μg/ml) of mannoprotein for 6 h. Once having established the most suitable concentration of polysaccharide (100 μg/ml), cells were divided into four groups: 1) control, 2) α-(1,6)(1,2)-mannoprotein, 3) LY294002 and 4) α-(1,6)(1,2)-mannoprotein +LY294002. The Akt inhibitor, LY294002 (Cell signaling Technology, MA, USA), was used at a concentration of 50 μM for 40 min. Each group was cultured for 24 h prior to use in future tests.

2.5. Silencing of Akt1 and Akt2 expression via siRNA

Expressions of Akt1 and Akt2 were ablated with use of siRNA and transfected cells according to the manufacturer's protocol (GenePharma, Shanghai, China). The oligonucleotides of Akt1 were 5′-GCA CCU UUA UUG GCU ACA ATT-3′; 5′-UUG UAG CCA AUA AAG GUG CTT-3′; and oligonucleotides of Akt2 were 5′-GAU CUU UCA UUG GGU AUA ATT-3′; 5′-UUA UAC CCA AUG AAA GAU CTT-3′. Negative control cells were treated with the GenePharma Negative Control (5′-UUC UCC GAA CGU GUC ACG UTT-3′; 5′-ACG UGA CAC GUU CGG AGA ATT-3′). Transfection was performed using a GP-siRNA-Mate Plus Transfection Kit (GenePharma, Shanghai, China). The level of transfection efficiency was determined by the relative mRNA expressions of Akt1 and Akt2.

2.6. RNA extraction and quantitative real-time PCR

Total RNA was extracted using the Qiazol reagent (Qiagen, Germany). The quantity and quality of RNA were determined with use of a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA was generated with use of the Reverse Transcription Kit (Abcam, MA, USA) using 2 μg of RNA. Real-time PCR was performed using a 7900HT Fast Real-time PCR System (Applied Biosystems, CA, USA). Primers used for real-time PCR are listed in Table 1. Each reaction included 10 μl of 2xMix (Promega, USA), 2 μl of forward primer (10 μM), 2 μl of reverse primer (10 μM) and 1 μl of cDNA. The thermocycler protocol consisted of 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. GAPDH was used as a housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was used to evaluate relative expressions of mRNA (Applied Biosystems Prism7500, CA, USA). Primers involved in this study are listed in Table 1.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The supernatant (50 μl) of cultured cells subjected to different stimulatory treatments was then used to measure levels of TNFα, IL-1β, IL-12 and IL-10 as determined according to the instructions of the ELISA kit (Novus, CO, USA).

2.8. Assay of reactive oxygen species production

Cells were harvested and labeled with the ROS probe (Abcam, MA, USA) for analysis in FACS. After stimulation with mannoprotein, cells were collected and incubated in the dark. The culture medium was then removed; the cells were incubated in the dark with the redox-sensitive

Table 1
Primers used for real-time PCR in this study.

Genes	Forward primer sequence	Reverse primer sequence	Product
IL-1β	ACGGACCCCAAAAGATGAAG	TTCTCCACAGCCACAATGAG	139 bp
IL-10	AAGTGATGCCCCAGGCA	TCTCACCCAGGGAATTCAAA	66 bp
IL-12 p40	GACCATCACTGTCAAAGAGTTTCTAGAT	AGGAAAGTCTTGTTTTTGAAATTTTTTAA	153 bp
iNOS	CTGCAGTTGGATCAGGAACCTG	GGAGTAGCCTGTGTGCACCTGGAA	307 bp
ARG-1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC	250 bp
TNFα	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG	156 bp
CD206	CTCTGTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA	190 bp
Dectin-2	CACCAGTGAGCAGAACTGTGT	TGCCATTTGCCATTACCTTGTG	147 bp
Dectin-3	ACCCGACATCCCCAACTGAT	CTCTCGTCCAGCGTAAAAAGT	118 bp
Akt1	AGAATGGGCCACCGCATTCAG	TGTCACTGGGTGAGCCTGATGC	92 bp
Akt2	ATTCTACAACCAGGACCACGAG	CTGGGACCTCCGCCGAGCCTCT	147 bp
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226 bp

fluorescent dye (DCFH-DA, 10 μM) and maintained in a cell incubator for 30 min at 37 °C, 5% CO_2, in RRMI 1640 medium supplemented with antibiotics. Each sample was washed twice with cold PBS and the ROS level was measured by FACS and determined with use of mean fluorescent intensity (MFI).

2.9. Assay of nitric oxide production

Nitrite accumulation in the medium, which can serve as an indicator of NO production, was measured by following the kit protocol (Beyotime, China). Following stimulation with LPS (10 μg/ml) and mannoptotein for 24 h, the medium was centrifuged to isolate cells. Medium (50 μl) was added to 96-well plates followed first by Griess reagent I (50 μl) and then Griess reagent II (50 μl). Based on the standard curve, the nitrite production was measured with use of OD at 540 nm.

2.10. Phagocytosis and bacterial killing assay

Phagocytosis of macrophages resulting from the different experimental conditions was analyzed with use of FACS. Cells were pretreated with LPS (10 μg/ml) and mannoptotein for 24 h, and then incubated with 1 mg/ml FITC-dextran (Life, OR, USA) for 1 h at 37 °C, 5% CO_2 in the dark. After incubation, the cells were washed twice with PBS to remove any excess dextran and phagocytic capacity was then measured by FACS (BD Immunocytometry Systems, NJ, USA) and determined with use of fluorescent intensity (MFI).

To determine phagocytic and bactericidal function of macrophages, pretreated cells were co-incubated with 1×10^7 *Candida albicans* and *Staphylococcus epidermidis* (strain ATCC35984) for 1 h at 37 °C, 5% CO_2 to allow bacterial adhesion and colonization. After thoroughly washing with PBS, cells were used immediately or maintained in the incubator for 24 h, lysed by 0.1% Triton-X-100 and serially plated on LB agar. Colonies were counted after overnight culture at 30 °C. The survival rate percent served as a means to assess bacterial killing ability of cells.

2.11. Flow cytometry to detect macrophage polarization

Following the standard protocol of FACS Calibur flow cytometry (BD Immunocytometry Systems, NJ, USA), cells were stained using the following antibodies: anti-mouse MHC II, anti-mouse CD86, anti-mouse CD80, anti-mouse CD69, anti-mouse IL-10(Abcam, MA, USA) and mouse FCR blocking reagent (Miltenyi, USA).

For intracellular staining, cells were suspended in fixation buffer (eBioscience) at 4 °C for 30 min. Their pellets were resuspended in permeabilization buffer (eBioscience) for 10 min and then washed twice with permeabilization buffer. Cells were stained with anti-mouse CD206 (Abcam) and analyzed with use of FACS.

2.12. iCELLIGENCE system

The iCELLIGENCE system was employed to determine the effect of mannoprotein on macrophage proliferation. Microelectrode sensors located at the bottom of wells served as a means to sense cell adhesion while electrical impedance provided an index of the biological status of the cells in real time. It was then possible to evaluate the proliferation of cells as based on the normalized cell index (CI). Cells (60,000) were seeded into each well and incubated at 37 °C, 5% CO_2 for 16 h to achieve a logarithmic growth phase. Mannoprotein and LPS were added to each well for 48 h and cell proliferation was monitored with use of the iCELLIGENCE system as determined every 15 min.

2.13. Western blot analysis

Total proteins from macrophage cells were extracted in lysis buffer (Thermo Fisher Scientific, Rockford, IL) and quantified using the Bradford method. Proteins (40 μg) were separated by SDS–PAGE (12%). After transfer, the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were incubated overnight at 4 °C with the following antibodies: p-Akt (1:1000, Cell Signaling Technology), pan-Akt (1:2000, Cell Signaling Technology), Cyclin D1 (1:1000, Cell Signaling Technology), Bcl-2 (1:1000, Cell Signaling Technology), p27 (1:10,000, BD Biosciences), Cyclin A (1:1000, BD Biosciences), Cyclin B (1:2000, BD Biosciences) and GAPDH (1:2000, Santa Cruz Biotechnology). After incubation with peroxidase-coupled anti-mouse or rabbit IgG (1:2000, Santa Cruz Biotechnology) at 37 °C for 2 h, bound proteins were visualized using ECL (Thermo Fisher Scientific) and quantified using BioImaging Systems (UVP).

2.14. Cell cycle analysis

Cells treated with α-(1,6)(1,2)-mannoprotein were harvested, fixed using 75% ethanol and then stored at 4 °C for 24 h. Cells were then washed with PBS, incubated with 10 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO) at 37 °C for 20 min and stained with 20 μg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO). DNA content of the cells (10,000 cells/group) was quantified using fluorescence-activated cell sorting (FACS) (Calibur flow cytometer, BD Immunocytometry Systems, NJ, USA) [19].

2.15. Annexin V-binding assay for apoptosis

After treatment with α-(1,6)(1,2)-mannoprotein, the degree of apoptosis in macrophages was measured using an annexin V-binding assay kit (BD Pharmingen, Bedford, MA). Cells were trypsinized and washed with cold PBS, incubated with 5 μl of FITC Annexin V at room temperature in the dark and then analyzed within 1 h with use of flow cytometry (BD Immunocytometry Systems, NJ, USA).

2.16. Statistical analysis

The results are presented as means ± SD. Statistical analysis was performed with use of Prism version 7.0 software (GraphPad Software, San Diego, CA, USA). Data were analyzed using one- or two-way analysis of variance (ANOVA). When indicated, Dunnett's or Tukey's tests were used for multiple post-hoc comparisons. A P value < .05 was required for results to be considered as statistically significant.

3. Results

3.1. Extraction and purification of α-(1,6)(1,2)-mannoprotein

The precipitant of *Candida albicans* was fractionated and purified by ultrafiltration, anion-exchange chromatography and gel filtration chromatography. CABI-A consisted of mannan and a small amount of glucose. As based upon ^{1}H NMR spectrum, the following signal identifications were achieved: δ5.29–4.92 = H-1 of mannan, δ78.63–78.47 = C2, δ 68.77–66.07 = glycosyl substitution at C6 and δ4.43 = β-anomeric configuration. On the basis of above results, the product obtained was considered as α-(1,6)(1,2)-mannan with minor amounts of β-glucan (Fig. 1a).

^{1}H and ^{13}C NMR spectrum of CABI-B1 were similar to that of CABI-A, with the only difference being that CABI-A contained a small amount of protein. Therefore, CABI-B1 as a α-(1,6)(1,2)-mannoprotein was used for investigation in this study (Fig. 1b).

Due to the low content of CABI-B2, it was not possible to perform NMR analysis. As based upon monosaccharide composition analysis, CABI-B2 was found to be similar to CABI-B1 with the only difference being a higher level of protein in the CABI-B2.

CABII-A contained only glucose, with a chemical shift at C1 of δ103.5–103.7 indicating a β-anomeric configuration monosaccharide composition. The signal at δ 86.59 indicated a glycosyl substitution at C3. Thus, CABII-A was a water-insoluble β-(1,3)-glucan (Fig. 1c).

3.2. α-(1,6)(1,2)-Mannoprotein promotes M1 macrophage polarization

To examine the immunofunction of mannoprotein in macrophage polarization, cells were co-incubated with α-(1,6)(1,2)-mannoprotein for 4 h. Alterations resulting from mannoprotein, with LPS as a positive control and PBS as a negative control were then compared. With use of RT-qPCR analysis, we observed significant increases in iNOS, IL-1β, IL-12p40 and TNFα, all of which are markers of M1 polarization. In addition, sharp declines in Arg-1, IL-10 and CD206 were obtained, which are markers of M2 polarization (Fig. 2a–c) [20]. Under normal conditions, M1-like macrophages possess higher levels of cytokines, such as TNFα, IL-1β and IL-6 [20], while M2-associated cytokines, such as IL-10, are not elevated [21]. Results from the ELISA assay substantiated

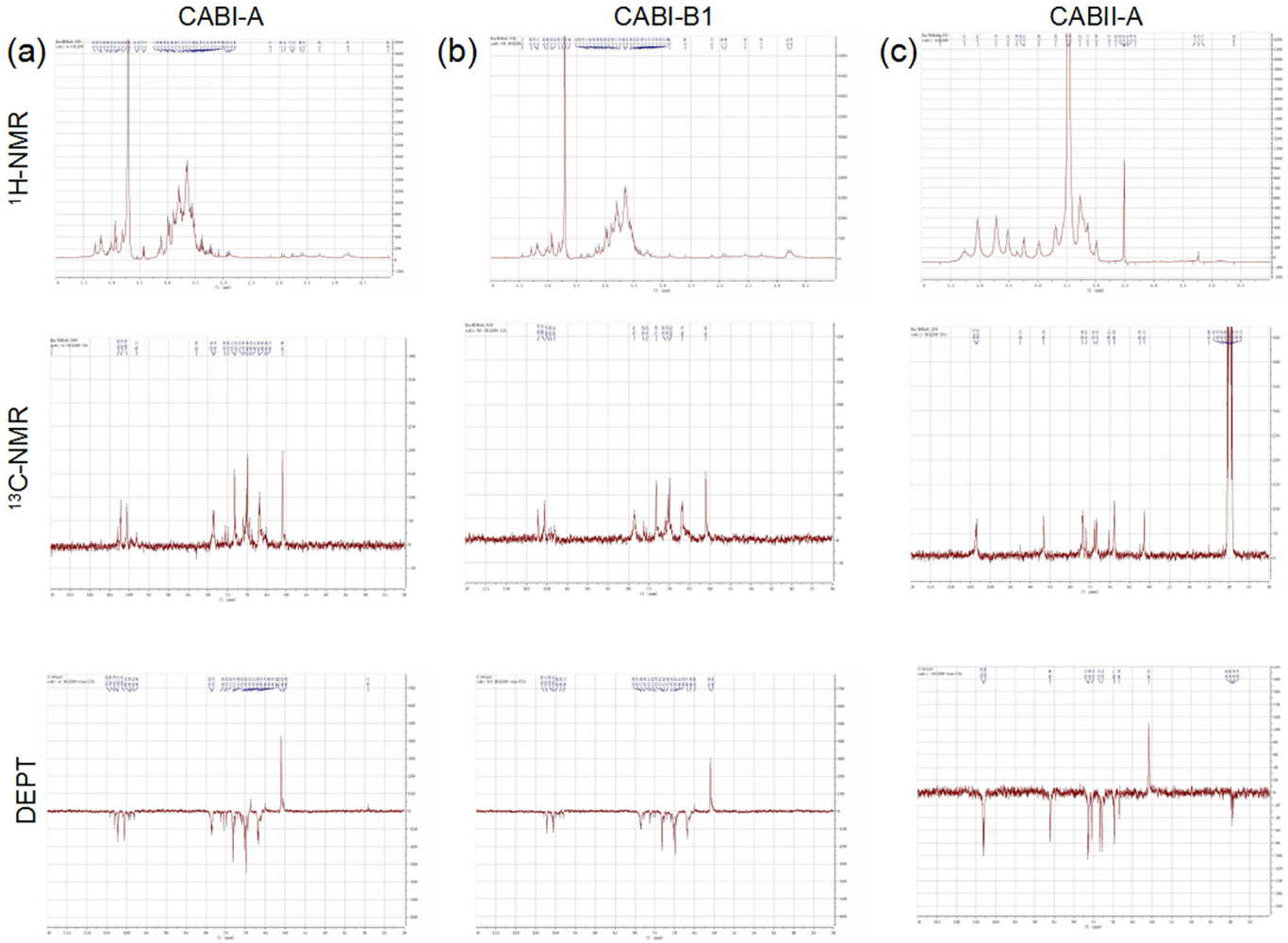

Fig. 1. Proton nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) data for α-(1,6)(1,2)-mannoprotein extracted from *Candida albicans*.
(a) CABI-A is α-(1,6)(1,2)-mannan with minor amounts of β-glucan.
(b) CABI-B1 is α-(1,6)(1,2)-mannoprotein.
(c) CABII-A is water-insoluble β-(1,3)-glucan.

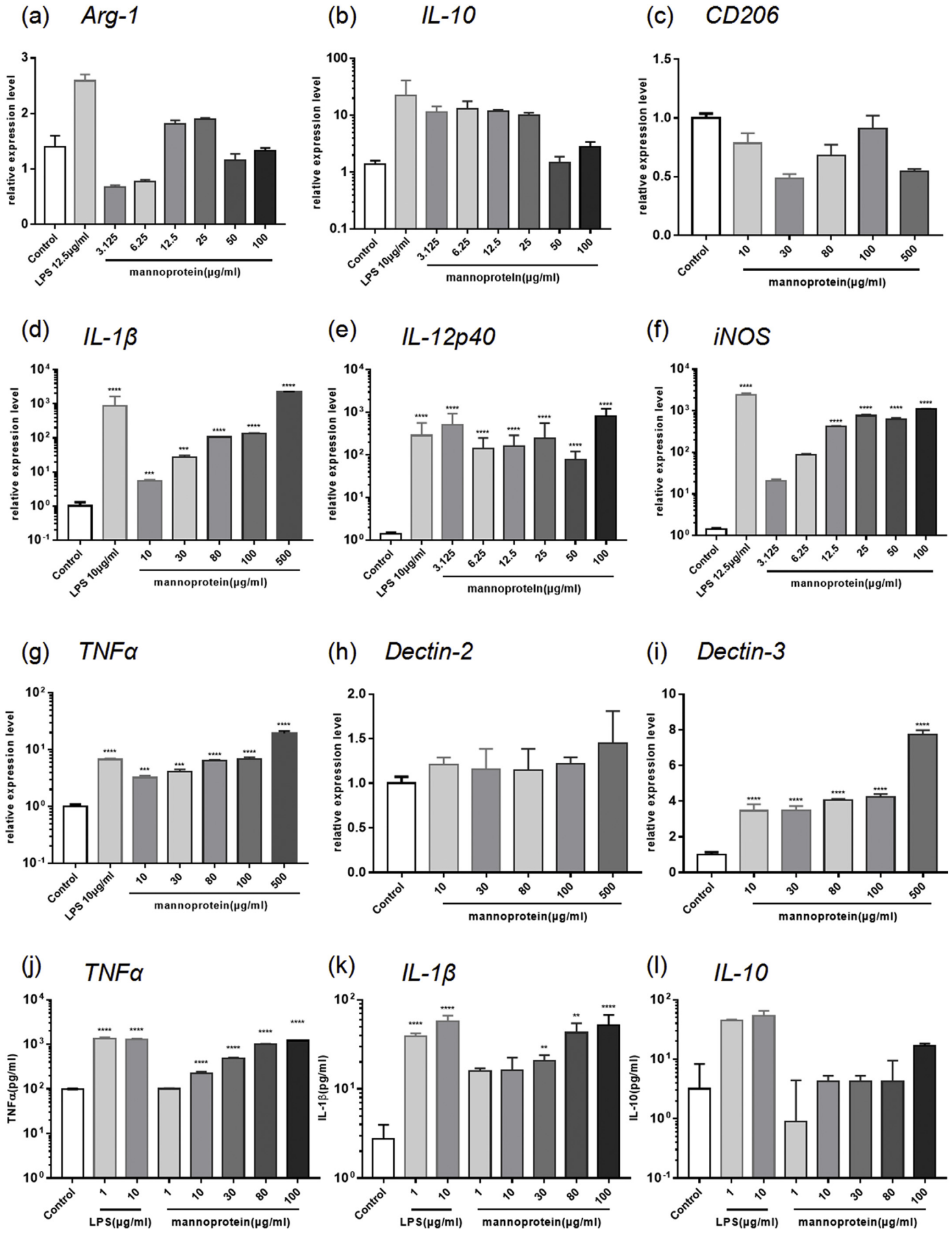

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Fig. 2. Effects of α-(1,6)(1,2)-mannoprotein on macrophages. Ana-1 murine macrophages were co-incubated with mannoprotein for 4 h, cell pellets and supernatants were then collected for analysis.
(a–i) Total RNA was extracted and expressions of iNOS, IL-1β, IL-12p40, Arg-1, IL-10, CD206, TNFα, Dectin-2 and Dectin-3 were measured with use of real-time PCR. (j–l) TNFα, IL-1β and IL-10 were quantified with use of ELISA.
Results were analyzed by Graphpad Prism 7.0 with use of a one way ANOVA. Results are the means and S.D. of three independent experiments *: $P < .05$,**: $P < .01$, ***: $P < .001$, ****: $P < .0001$.

these PCR results that mannoprotein was capable of promoting macrophages to M1 polarization (Fig. 2d–g). In order to examine the roles of Dectin-2 and Dectin-3, RT-qPCR analysis was employed to assess the expression of these two factors which are essential for the recognition of α-mannans in *Candida albicans* [14]. While the expression of Dectin-3 was elevated as related to mannoprotein, no obvious increase in Dectin-2 expression was observed (Fig. 2h, i). The unresponsiveness of Dectin-2 is inconsistent with the concept of α-mannans. This result may be related primarily with differences in molecular structure and the activation of Dectin-3 could lead to downstream reactions of the NF-κB signal pathway [12].

3.3. Activation of the Akt signaling pathway in macrophages leads to alterations in cell proliferation, cell cycles and apoptosis after stimulation with α-(1,6)(1,2)-mannoprotein

Activation of the Akt signaling pathway can result in an increase of key marker proteins involved with changes in downstream factors relevant to cell proliferation and apoptosis. Results obtained from western blotting showed that α-(1,6)(1,2)-mannoprotein increases Akt phosphorylation levels in a concentration-dependent manner. Such results indicate that α-(1,6)(1,2)-mannoprotein activated the Akt signaling pathway in macrophages. Moreover, up-regulated expressions of proliferative proteins and anti-apoptosis proteins, such as Cyclin D1 and Bcl-2, along with suppression in the expression of anti-proliferative proteins(p27) were observed in response to α-(1,6)(1,2)-mannoprotein. Therefore, α-(1,6)(1,2)-mannoprotein of *Candida albicans* promoted cell proliferation and inhibited cell apoptosis (Fig. 3a).

In order to further verify the role of this Akt signaling pathway activation, we tested the effects of a commonly used PI3K-Akt pathway inhibitor, LY294002. This agent is also a potent broad-spectrum inhibitor of PI3K [17], which can inhibit the phosphorylation of Akt and block three different isoforms of Akt [22]. We confirmed that LY294002 suppressed activation of the Akt signaling pathway which led to a decrease in the phosphorylation of Akt, and, of particular relevance to the present investigation, inhibited the pro-proliferative and anti-apoptosis effects of α-(1,6)(1,2)-mannoprotein (Fig. 3b).

Activation of the Akt signaling pathway represents an important component in cell cycle progression. For this reason, we sought to determine whether α-(1,6)(1,2)-mannoprotein influences cell cycle processes in macrophages. To accomplish this goal the iCELLIGENCE system was utilized to assess these cell cycles after 16 h of co-incubation and analysis after stimulation with varying concentrations for 48 h. The adhesion rate of cells was reflected in the rate of cell proliferation as achieved with use of the iCELLIGENCE system [23]. As LPS can promote cell proliferation, it was used as a positive control in this iCELLIGENCE system [24]. The DNA synthesis phase was found to accumulate in a dose-dependent manner. However, the G2/M population of macrophages was not dramatically altered (Fig. 3d). These results indicate that α-(1,6)(1,2)-mannoprotein promoted cell cycle progression. Furthermore, with application of the Akt signaling pathway inhibitor, LY294002, a reversal in cell cycle progression was observed in response to α-(1,6)(1,2)-mannoprotein (Fig. 3e).

As an approach to evaluate the anti-apoptotic effects of α-(1,6)(1,2)-mannoprotein, we utilized flow cytometric analysis with propidium iodide and annexin V-fluorescein isothiocyanate. Surprisingly, we found that α-(1,6)(1,2)-mannoprotein decreased the percent of annexin V-positive macrophages, which equalled the degree of apoptotic death in a concentration gradient (Fig. 3f). Next, we sought to examine this anti-apoptotic effect under conditions of inhibiting the Akt signaling pathway. While treatment with α-(1,6)(1,2)-mannoprotein alone decreased the percent of apoptotic cells, the combination of LY294002 with α-(1,6)(1,2)-mannoprotein significantly increased the number of apoptotic macrophages. Results obtained from flow cytometry substantiated that α-(1,6)(1,2)-mannoprotein acts on the Akt signaling pathway to produce this decrease in macrophage apoptosis (Fig. 3g).

3.4. Mannoprotein regulates ROS and NO production of macrophages through the Akt signal pathway

The pro-inflammatory effects of macrophages are related to the production of ROS and NO which can lead to microbial killing. M1-like macrophages possess high levels of expression of the enzyme, inducible nitric oxide synthase (iNOS), which is required to generate nitric oxide. As results from previous studies have revealed that the Akt signal pathway is involved in macrophage polarization [25], an understanding of the mechanisms through which mannoprotein regulates macrophage polarization via Akt signaling would provide important new insights into the microbial killing effect of mannoprotein. In our present study, levels of NO production were determined with use of the Griess reagent [26] while that of ROS production by DCF flurorescence [27]. We found that macrophages produced increased amounts of ROS (Fig. 4a–b) and NO (Fig. 4c) and showed an increased ability to eliminate pathogens in response to mannoprotein. Moreover, these effects of mannoprotein were decreased under conditions of treatment with the Akt signal pathway inhibitor, LY294002.

3.5. Mannoprotein increases macrophage phagocytosis and microbial killing through the Akt signal pathway

A well-known function of macrophages is the devouring and eliminating of pathogens [22]. The fact that mannoprotein, as part of the cell wall of *Candida albicans* [28], can play a role in regulating pathogen survival in macrophages prompted our investigation of this process. In this experiment, dextran was used as a phagocytic marker [29] and *Candida albicans* and *Staphylococcus epidermidis* were used as target pathogens [30]. Our results demonstrated that macrophages stimulated by mannoprotein showed increased rates of phagocytosis (Fig. 5a–c) and microbial killing ability (Fig. 5d) as compared with that obtained in the control group. Moreover, this enhancement involved activation of the Akt signal pathway.

3.6. α-(1,6)(1,2)-Mannoprotein enhances the M1-like macrophage marker through the Akt signal pathway

To further examine these effects of mannoprotein, immunofluorescence was used to determine the phenotype of macrophages. Under normal conditions, CD86, CD80, MHC II and CD69 serve as specific markers for the M1 phenotype [31,32] while CD206 and IL-10 are specific markers for the M2 phenotype [33]. Mannoprotein treatment enhanced the expressions of CD86, CD80, MHC II and CD69 in macrophages (Fig. 6a–d), but expressions of CD206 and IL-10 were not substantially altered (Fig. 6e–f). With the addition of the Akt signal pathway inhibitor, LY294002, the effect of macrophage polarization to M1 was diminished. Although, cells stained with IL-10 and CD206 showed a slight increase of MFI after co-incubation with mannoprotein,

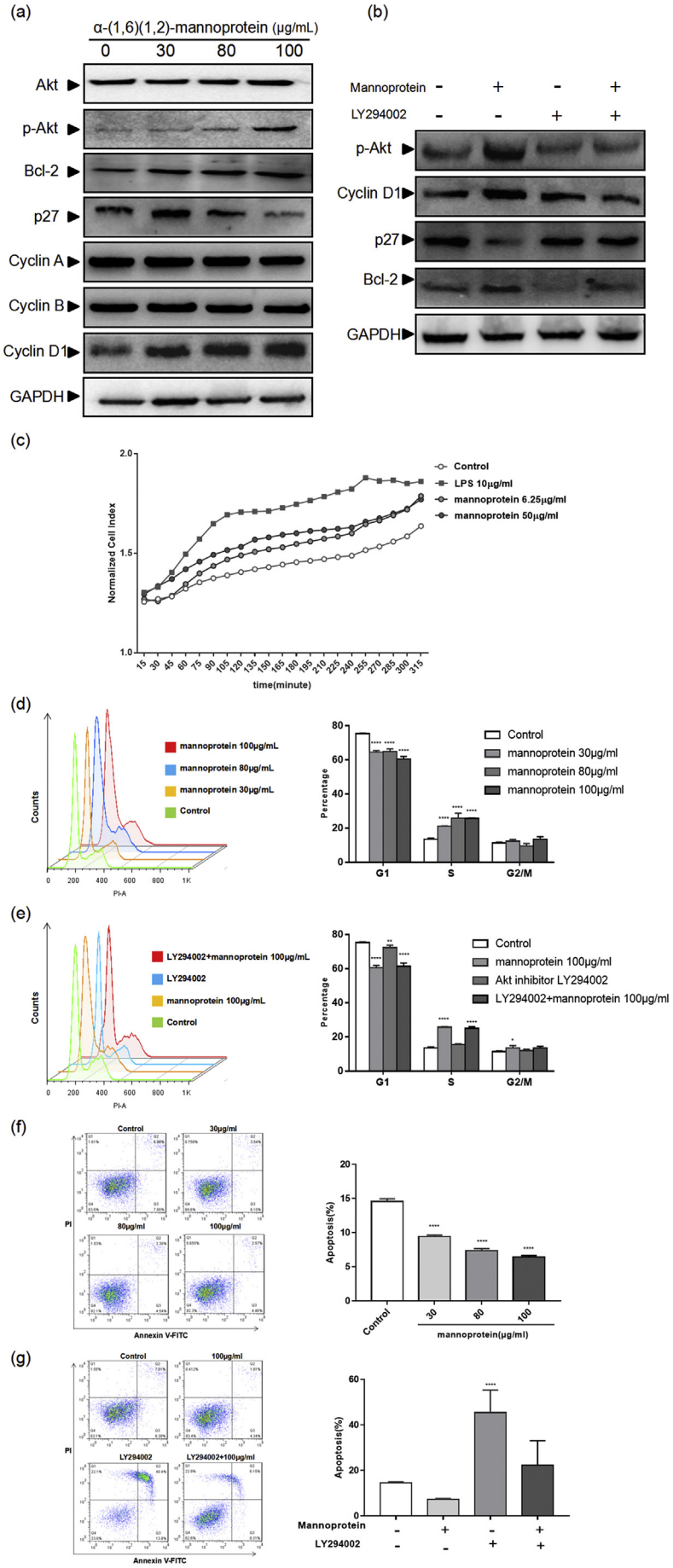

Fig. 3. α-(1,6)(1,2)-Mannoprotein induced proliferation, cell cycles and anti-apoptosis resulting from activation of the Akt signal pathway.

(a, b) Cells were co-incubated with α-(1,6)(1,2)-mannoprotein (0, 30, 80 or 100 μg/ml) for 6 h. The results indicated that mannoprotein promoted macrophage proliferation and inhibited apoptosis through Akt pathway activation. Application of the Akt inhibitor, LY294002, suppressed Akt activation, cellular proliferation, and apoptosis induced by α-(1,6)(1,2)-mannoprotein.

(c) ICELLIGENCE system detected the proliferation of macrophages which was increased in response to mannoprotein.

(d, e) Flow cytometric results revealed a dose-dependent accumulation of the S phase in response to mannoprotein. Application of the Akt inhibitor, LY294002, suppressed this stimulating effect of mannoprotein. The percent of macrophages in S phase decreased in groups treated with mannoprotein and LY294002 as compared to that of mannoprotein alone.

(f, g) The percent of apoptotic macrophages in groups co-incubated with mannoprotein decreased significantly as compared to the control group. Application of the Akt inhibitor, LY294002, inhibited these effects of mannoprotein.

Results are the means and S.D. of three independent experiments *: $P < .05$,**: $P < .01$, ***: $P < .001$, ****: $P < .0001$.

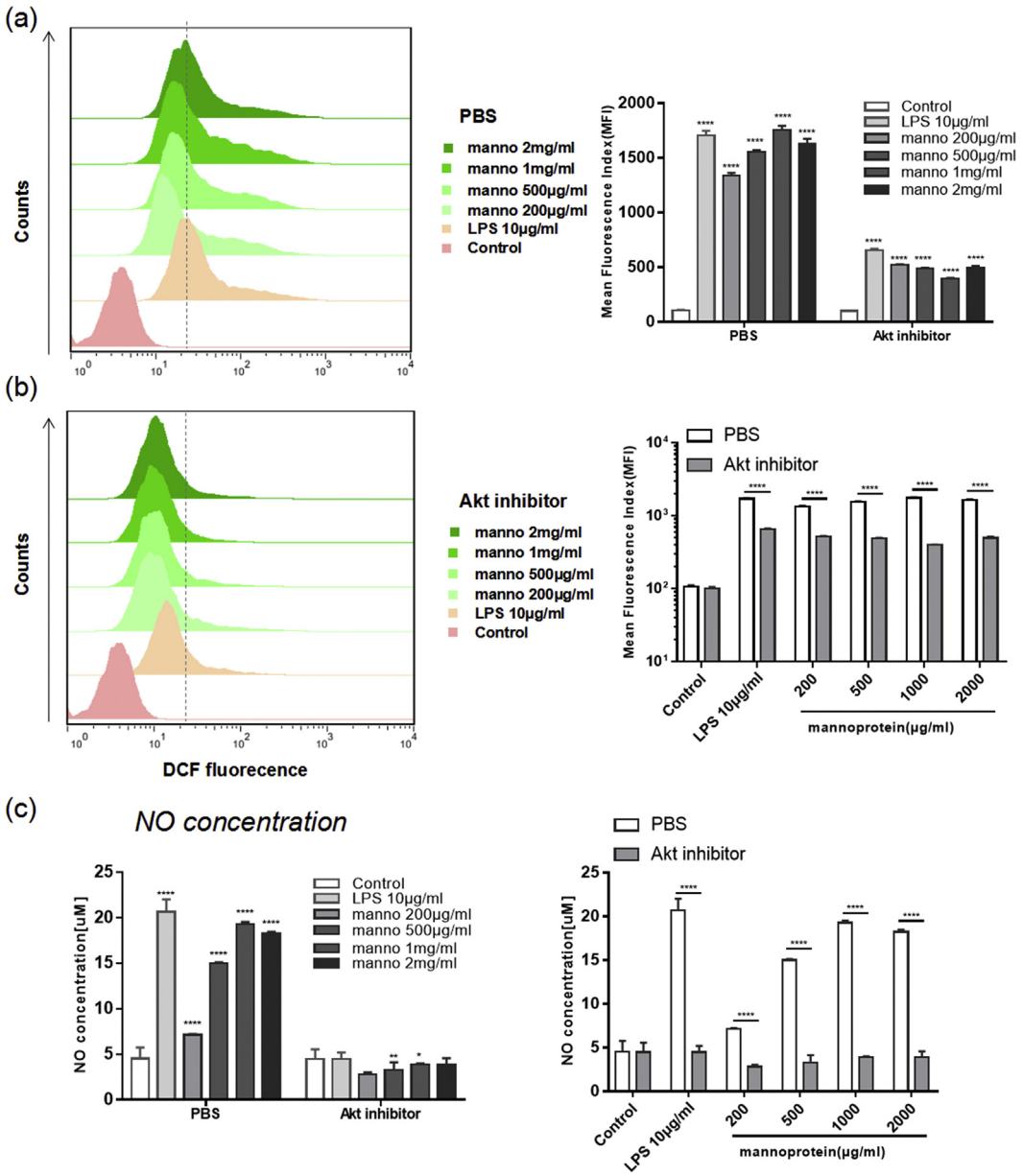

Fig. 4. α-(1,6)(1,2)-Mannoprotein induced macrophages to produce ROS and NO through the Akt signal pathway.
(a, b) α-(1,6)(1,2)-Mannoprotein increased levels of intracellular ROS production of macrophages in a dose-dependent manner. Application of the Akt signal pathway inhibitor, LY294002, into co-incubated cultures decreased ROS production.
(c) NO production was elevated by mannoprotein, whereas the Akt signal pathway inhibitor, LY294002, inhibited this effect of mannoprotein.
Results are the means and S.D. of three independent experiments *: $P < .05$,**: $P < .01$, ***: $P < .001$, ****: $P < .0001$.

no obvious differences were observed between the PBS and LY294002 group.

3.7. α-(1,6)(1,2)-Mannoprotein induces macrophages to M1 through Akt2, inhibits apoptosis by Akt1, and promotes cell cycle activity by the joint effects of Akt1 and Akt2

The main target of α-(1,6)(1,2)-mannoprotein on macrophages involves Akt and the individual isoforms of Akt, which include Akt1, Akt2 and Akt3, may play independent roles in these reactions. Based on the previous results, Akt1 ablation was shown to result in the M1 phenotype, while Akt2 ablation results in the M2 phenotype [34]. To examine the roles of Akt1 and Akt2 in the polarization of macrophages as induced by α-(1,6)(1,2)-mannoprotein, the effects of their depletion were assessed as performed by siRNA and confirmed by RT-PCR (Fig. 7e, f). As described above, mannoprotein can induce the phosphorylation of Akt to activate the Akt signal pathway. However, we found that downregulation of Akt2, but not Akt1, abolished Akt phosphorylation without influencing the overall expression of Akt (Fig. 7a). This result suggested that mannoprotein stimulated Akt2 phosphorylation to polarize M1. Associated with this Akt2 depletion was an elevation of Arg-1 expression (Fig. 7h), an elevation that was decreased in the presence of mannoprotein. Moreover, the expression of TNFα was decreased in the Akt2 depletion group. Results of the ELISA assay for TNFα, IL-1β, IL-12 and IL-10 (Fig. 7k–n) confirmed that mannoprotein polarized macrophages to M1 by Akt2, while depletion of Akt2 was diminished when co-incubated with mannoprotein. Interestingly, Akt1 depletion of macrophages can produce an increase in NO as compared to that of Akt2 depletion under the influence of mannoprotein (Fig. 7g).

In addition, the effects of mannoprotein on cell cycle and apoptosis

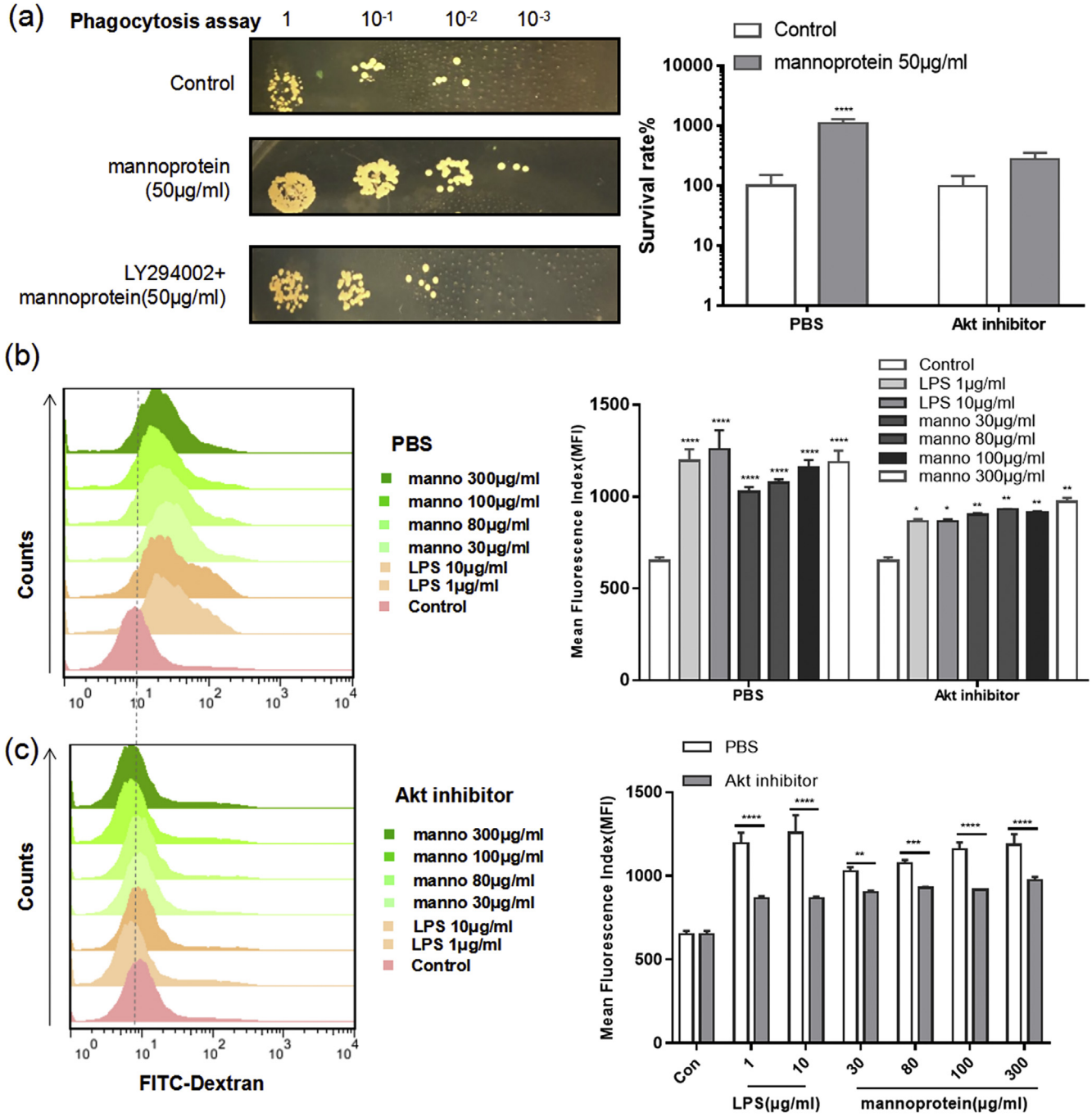

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Fig. 5. α-(1,6)(1,2)-Mannoprotein promoted phagocytosis and microbial killing ability of macrophages through the Akt signal pathway.
(a) *Candida albicans* were seeded into each well after treatment with the Akt signal pathway inhibitor, LY294002, and mannoprotein. With serial dilution on plates, the colony-forming unit provided an index of intracellular phagocytosis.
(b, c) Dextran was used to confirm the above results, with phagocytic capacity being measured with use of MFI flow cytometry. Phagocytosis was increased in a dose-dependent manner in response to stimulation with mannoprotein. Application of LY294002 into cell cultures suppressed phagocytosis induced by mannoprotein but failed to influence basal phagocytosis.
(d)*Staphylococcus epidermidis* was used to detect bactericidal ability. Survival rates provided an index of remaining intracellular pathogens. Macrophages pretreated with mannoprotein exerted stronger bactericidal ability, whereas those supplemented with LY294002 restored bacterial killing abilities to that observed in basal conditions.
Results are the means and S.D. of three independent experiments *: $P < .05$, **: $P < .01$, ***: $P < .001$, ****: $P < .0001$.

warranted investigation, especially the roles of Akt1 and Akt2. This follows, as Akt1 has been described as a key factor in cell apoptosis [35] and Akt2 serves as a factor which facilitates cell cycles [35]. Apoptosis of macrophages was examined with use of flow cytometry and the results showed that an increase of apoptosis was observed in response to Akt1 depletion (Fig. 7b), while the addition of mannoprotein slightly decreased this apoptosis (Fig. 7b). This finding suggests that Akt1 may be a predominant isoform in the regulation of apoptosis. Moreover, depletion of Akt1, as well as Akt2, resulted in G1 cell cycle arrest (Fig. 7c). However, in the presence of mannoprotein a slight reduction of G1 cells along with a slight increase of S phase cells was observed (Fig. 7d). The increase in apoptosis in response to Akt1 depletion demonstrates that Akt1 represents a critical factor responsible for apoptosis; while the G1 cell cycle arrest observed in response to both Akt1 and Akt2 suggest they may play joint roles in cell cycles. Notably, mannoprotein treatment partially rescues these apoptotic cells and promotes cell cycles under conditions of Akt1 or Akt2 depletion.

4. Discussion

Skin, a large organ of the integumentary system, is a complex environment colonized by a variety of microorganisms such as viruses, bacteria and fungi. Healthy individuals are able to maintain a homeostatic microbiome, but individuals with immunodefects can be significantly compromised when infected with *Candida albicans* [18]. Once infection commences, the innate immune system defends against the invasion by recognizing the pathogen-associated molecular pattern. Macrophages then play an important role in preventing infection by phagocytosis and the recruitment of other immunocytes.

Results from previous studies had indicated that heat-killed *Candida albicans* exert effects upon macrophage polarization. Some components as extracted from yeast can promote the production of pro-inflammatory cytokines [36] or lead to immune tolerance [37]; and dendritic cells induced by N-linked mannan of *Candida albicans* have been shown to produce pro-inflammatory cytokines [38]. In reviewing the literature on *Candida albicans*, it was revealed that the cell wall was comprised of two layers, with the outer surface containing mannoprotein and the inner surface chitin and β-glucan. With regard to chitin, its effects on macrophages had revealed mechanisms involved with immune tolerance [39], while Dectin-2 and Dectin-3 represent predominant factors necessary for the recognition of α-mannans [40]. These findings indicate that an understanding of the relationship between cell wall components and immune reactions will be critical for the development of novel pharmaceuticals for clinical use.

As one approach to address this issue, here we extracted and purified cell wall components of *Candida albicans*. The successful extraction of pure cell wall components and revelation of their carbohydrate composition represent a fundamental component for conducting subsequent functional analysis. From our initial experiments, we extracted and identified five unique polysaccharides and chose α-(1,6)(1,2)-mannoprotein as our main research target due to its purity and stability.

We first examined the effects of structural polysaccharides of *Candida albicans* on macrophages. Our results revealed that macrophages induced by mannoprotein could polarize to M1 through the activation of the Akt signaling pathway (Fig. 8). In this way, we confirmed that mannoprotein has the potential to polarize macrophages to M1. Induced macrophages resulted in elevated amounts of phagocytosis and microbial killing ability with higher levels of NO and ROS production. All phenotypes listed above could increase pathogen clearance. The PI3K-Akt signal pathway is mainly involved in cell cycles and apoptosis, and the signals resulting from this pathway as mediated from multiple receptors can regulate macrophage responses [25]. In addition, transcriptome and proteome of α-(1,6)(1,2)-mannoprotein on macrophages showed an enrichment in the PI3K-Akt signal pathway (data not shown).

Here, we described some of the alterations in expression levels of proteins and changes in cell cycle and cell apoptosis as related to α-(1,6)(1,2)-mannoprotein. In specific, we demonstrate for the first time that α-(1,6)(1,2)-mannoprotein activates the Akt signaling pathway. This activation was indicated by the expression of symbolic proteins and their downstream proteins. Some of the notable effects we observed in response to α-(1,6)(1,2)-mannoprotein include, increased the phosphorylation levels of the Akt pathway, up-regulation of the pro-proliferative relative protein (Cyclin-D1) and anti-apoptotic protein (Bcl-2), and down-regulation of the anti-proliferative protein (*p*27). An overexpression of p-Akt, Cyclin-D1 and Bcl-2 is associated with an increase in cell proliferation and accumulation and a concomitant decrease in apoptosis. Taken together, our results strongly indicate that α-(1,6)(1,2)-mannoprotein has immune-enhancing effects through activation of the Akt signaling pathway.

Activation of Akt signaling is a ubiquitous component of cell survival, proliferation and apoptosis. This pathway also modulates cell cycles and cell growth. Results from our flow cytometric assay suggest that α-(1,6)(1,2)-mannoprotein influenced DNA synthesis by increasing the percent of cells in the S phase and cell apoptosis in a dose-dependent manner. With the application of the Akt inhibitor, LY294002, we confirmed that α-(1,6)(1,2)-mannoprotein polarized macrophages to M1, promoted cell proliferation and inhibited cell apoptosis through the Akt signaling pathway.

Findings from previous studies have shown that Akt1 deletion resulted in M1 polarization, while Akt2 deletion promoted M2 polarization [34]. Cells transfected with Akt1-siRNA and Akt2-siRNA resulted in opposite phenotypes when treated with mannoprotein. Depletion of Akt2 resulted in low expressions of M1 markers, but high expressions of M2 markers in macrophages, while Akt1 ablation produced massive cell apoptosis. Downregulation of both Akt1 and Akt2 led to G1 cell cycle arrest (Fig. 7). While these effects may be cell line specific, the results of our study revealed that these actions of α-(1,6)(1,2)-mannoprotein on macrophages involve an activation of Akt, but on different isoforms of Akt.

We are currently working on experiments to provide further evidence in support of the findings presented in this report. For example, we have tested the time-dependent effects of an intracutaneous injection of mannoprotein in mice. Our initial histopathological analysis revealed that a significant activation of macrophages was obtained in response to this treatment (data not shown). Additional work within our laboratory will include an examination of the differences and similarities between each polysaccharide on other cells as well as determinations of the detailed molecular mechanisms and phenomena related to polysaccharide induction through Akt signaling.

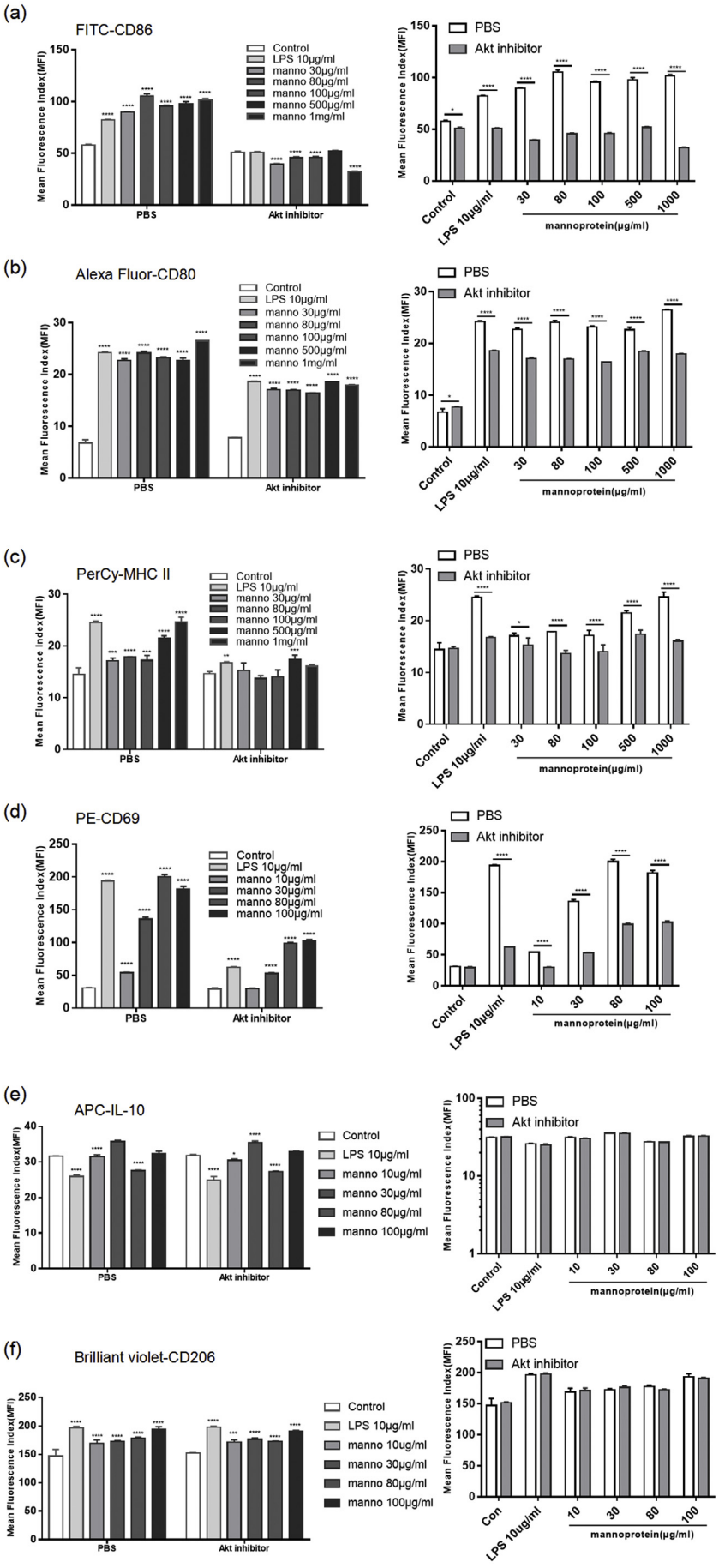

Fig. 6. α-(1,6)(1,2)-Mannoprotein induced macrophages to M1 polarization through the Akt signal pathway.
(a, b, c and d) The M1 macrophage markers CD86, CD80, MHC II and CD69 were highly expressed in response to mannoprotein. Expressions of above markers induced by mannoprotein were suppressed following treatment with the Akt signal pathway inhibitor, LY294002.
(e, f) IL-10 and CD206 was slightly increased in response to mannoprotein treatment. Expressions of IL-10 and CD206 within macrophages were not significantly influenced following inhibition of the Akt signal pathway with LY294002.
Results are the means and S.D. of three independent experiments *: $P < .05$, **: $P < .01$, ***: $P < .001$, ****: $P < .0001$.

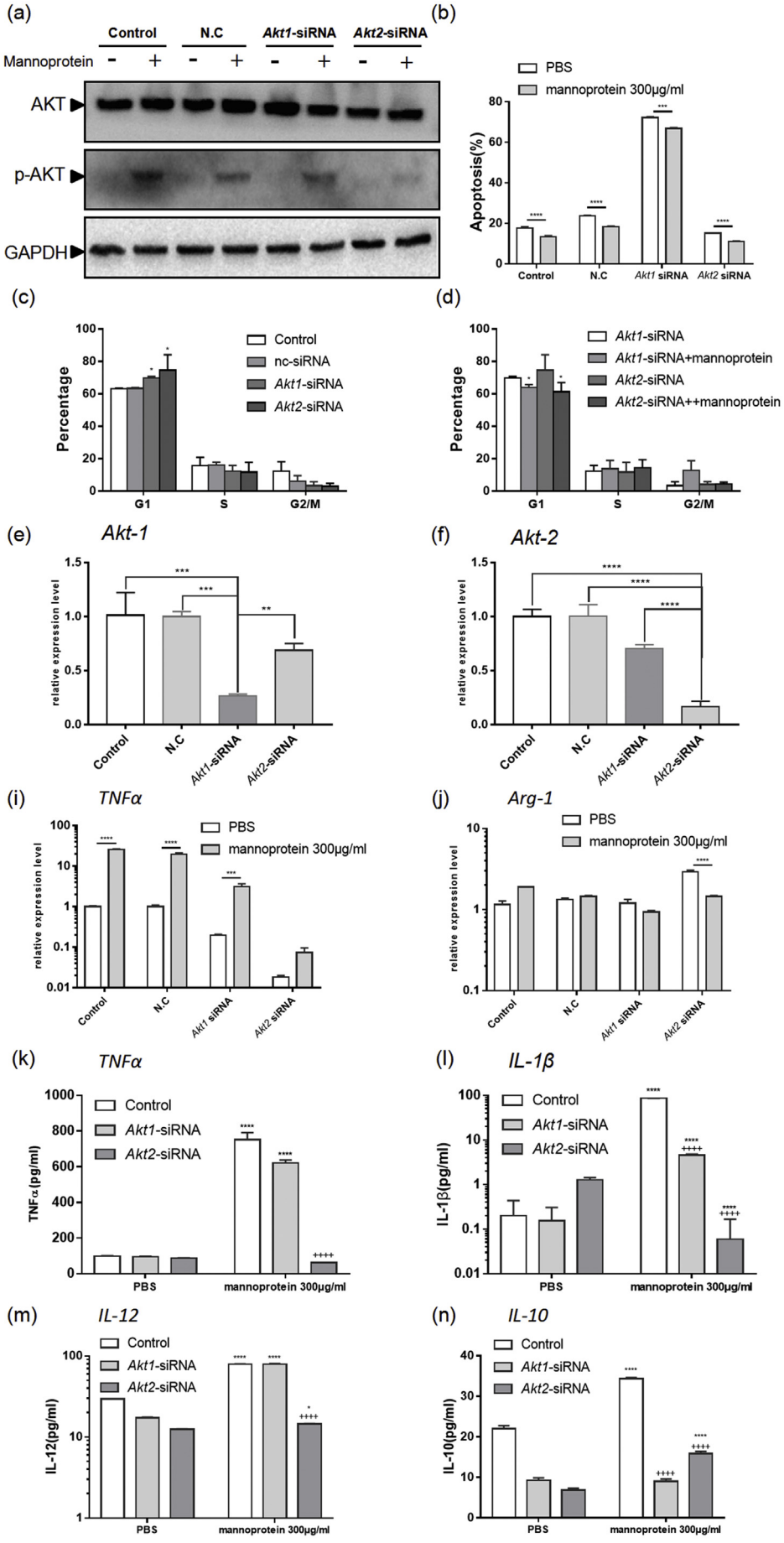

Fig. 7. α-(1,6)(1,2)-Mannoprotein polarized macrophages through Akt2 activation while effects upon apoptosis and cell cycles were exerted through Akt1 and Akt2 activation.
(a) Western blot analysis of phosphorylated Akt with or without mannoprotein treatment. Cells were initially transfected with siRNA for 48 h, then co-incubated with or without mannoprotein for 6 h. Downregulation of Akt2 abolished Akt phosphorylation which would be induced with mannoprotein.
(b, c, d) Apoptosis and cell cycles were analyzed with use of flow cytometry. Ablation of Akt1 resulted in massive apoptosis of macrophages, while downregulation of Akt1 and Akt2 led to G1 cell cycle arrest. Addition of mannoprotein partially rescues cells from these effects.
(e, f) Transfection efficiency of nc-siRNA, Akt1-siRNA, or Akt2-siRNA was confirmed by RT-PCR assay of mRNA relative expression.
(g–n) Akt1 depletion resulted in a M1 phenotype. NO production, TNFα expression and the secretion of TNFα, IL-1β and IL-12 were all significantly increased in response to mannoprotein. Akt2 depletion resulted in a M2 phenotype. Expression of Arg-1 and secretion of IL-10 were substantially elevated following downregulation of Akt2.
Results are the means and S.D. of three independent experiments *: $P < .05$, **: $P < .01$, ***: $P < .001$, ****: $P < .0001$ versus two groups; $^{\#\#\#}P < .001$, $^{\#\#\#\#}P < .0001$ within group.

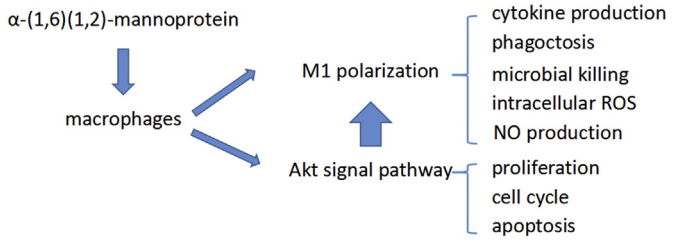

Fig. 8. Schematic presentation of mechanisms of *Candida albicans* α-(1,6)(1,2)-mannoprotein regulation of macrophage polarization and activation of the Akt signal pathway.

In summary, our current results demonstrate some of the immune-enhancing effects of *Candida albicans* polysaccharides. Such information provides a foundation for the development and design of novel molecular pharmaceuticals for the treatment of fungal infection and stimulation of immune responses.

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Conflict of interest

The authors report no conflicts of interest.

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