Suppression of the inflammatory response by triterpenes isolated from the mushroom *Ganoderma lucidum*

Shailesh Dudhgaonkar a, Anita Thyagarajan a, Daniel Sliva a,b,c,*

a Cancer Research Laboratory, Methodist Research Institute, 1800 N Capitol Ave, E504, Indianapolis, IN 46202, USA  
b Department of Medicine, School of Medicine, Indiana University, Indianapolis, IN, USA  
c Indiana University Simon Cancer Center, School of Medicine, Indiana University, Indianapolis, IN, USA

**A B S T R A C T**

*Ganoderma lucidum* is a popular medicinal mushroom, which has been used in the Traditional Chinese medicine for the prevention or treatment of a variety of diseases. In the present study we evaluated the anti-inflammatory effects of the triterpene extract from *G. lucidum* (GLT) in LPS-stimulated macrophages. Here we show that GLT markedly suppressed the secretion of inflammatory cytokine tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and inflammatory mediator nitric oxide (NO) and prostaglandin E2 (PGE2) from lipopolysaccharide (LPS)-stimulated murine RAW264.7 cells. GLT also down-regulated expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) in RAW264.7 cells. The anti-inflammatory effects of GLT were mediated by the inhibition of transcription factor NF-κB as demonstrated by decreased NF-κB-DNA binding activity, and the suppression of p65 phosphorylation in LPS-stimulated macrophages treated with GLT. Moreover, GLT inhibited LPS-dependent AP-1-DNA binding activity and down-regulated expression of MAP kinases as observed by the down-regulation of LPS-induced phosphorylation of ERK1/2 and JNK but not p38. *In vivo* experiments clearly demonstrated that GLT also inhibited the production of TNF-α and IL-6 in LPS-induced endotoxemic mice. Apart from its anti-inflammatory activity, GLT suppressed cell proliferation of RAW264.7 cells through cell cycle arrest at G0/G1-G2M, which was mediated by the down-regulation of expression of cell cycle regulatory proteins cyclin D1, CDK4 and cyclin B1, respectively. In conclusion, the anti-inflammatory and anti-proliferative effects of GLT on macrophages are mediated through the inhibition of NF-κB and AP-1 signaling pathways.

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1. Introduction

Inflammation is inherent to the pathogenesis of a variety of diseases. Macrophages are known to be one of the critical immune cells in the regulation of inflammatory responses. Activated macrophages secrete a number of different inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), reactive oxygen species (ROS), prostaglandin E2 (PGE2) and nitric oxide (NO) [1–3]. Excessive or unregulated production of these mediators has been implicated in mediating or exacerbating a number of diseases including rheumatoid arthritis, osteoarthritis, sepsis, chronic pulmonary inflammatory disease, Crohn’s disease, ulcerative colitis, and also carcinogenesis. Recently, it has been documented that inflammatory stimuli from macrophages promote tumor growth [4–6], whereas the depletion of macrophages reduced the tumor growth [6]. Clinical studies showed that increased macrophage density in tumor stroma has been shown to strongly correlate with poor prognosis in different types of solid tumors [7–9]. Thus, inhibition of activation and the proliferation of these inflammatory cells appear to be an important therapeutic target for small molecular drugs for the treatment of inflammatory diseases and cancer. In this regard, the evaluation of anti-inflammatory effects of various bioactive plant components has gained the widespread attention. One of these herbal medicinal plants, mushroom *Ganoderma lucidum*, is used commonly in the treatment of several diseases in Asian countries [10] and has been used as a home remedy in traditional Chinese medicine for over 2000 years [11]. Among the reported biological/pharmacological properties of *G. lucidum*, their anti-tumor activities are of particular interest. Investigations into the anticancer activity of *G. lucidum* have been performed in both *in vitro* and *in vivo* studies, supporting its application for cancer treatment and prevention [12,13]. Polysaccharides and triterpenes are two major categories of the bioactive ingredients from *G. lucidum* and it has been found previously that polysaccharides exert their effect mainly through an immune-modulatory mechanism [14,15] while triterpenes directly suppress growth and invasive behavior of cancer cells [10,16,17]. Although many
biological activities and pharmacological functions related to the *Ganoderma* polysaccharides and triterpenoids have been reported in the literature, the molecular mechanism of anti-inflammatory activity of *G. lucidum* triterpenes (GLT) is not well characterized. In order to extend the understanding of the anti-inflammatory actions of GLT, we investigated its effects in murine macrophage cell line (RAW264.7) stimulated with the Gram-negative bacterial cell wall component lipopolysaccharide (LPS). LPS acting via Toll-like receptor-4 is able to enhance the expression of a number of inflammatory genes such as TNF-α, IL-6, iNOS and COX-2, principally through the activation of a number of transcription factors including AP-1 and NF-κB [18]. The activity of AP-1 and NF-κB is regulated by members of the mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAP kinase and c-Jun N-terminal kinase (JNK), and the inhibitory kappa B kinases (IKKs) which regulate NF-κB translocation to the nucleus, respectively [18–22].

In the present study, we evaluate the effect of GLT on RAW 264.7 macrophages. Here, we show that GLT inhibited production of several inflammatory mediators and induced cell cycle arrest. These results suggest that GLT can be used as an anti-inflammatory and anti-proliferative agent.

2. Materials and methods

2.1. Preparation and characterization of triterpene extract from *G. lucidum* (GLT)

*G. lucidum* triterpene extract (GLT) was obtained from Pharmanex (Provo, UT, USA). This extract (batch number: 050607, Shanghai R&D, Pharmanex) was prepared as follows: *G. lucidum* mushrooms were chopped and extracted with 95% EtOH under reflux. The ethanolic extract was concentrated to dryness under reduced pressure. HPLC analysis of the EtOH extract was performed on a C-18 column eluting with acetonitrile-0.1% aqueous trifluoroacetic acid (95/5, 50 min) at the flow rate of 1 ml/min. Lanostanoid triterpenes detected in the chromatogram were isolated and identified by NMR and MS spectrometry during the previously published studies [23–25]. In addition, we have recently developed LC/MS method for the identification of triterpenoid metabolic fingerprint for the in situ characterization of bioactive compounds in GLT and identified ganoderic acid A, F, H, Mh, S1, ganosporeric acid, lucidenic acid B, D, D1, E1, L, and methyl lucidenate G in GLT. Furthermore, we have quantified the amount of ganoderic acid A (3.88 μg/mg), H (1.74 μg/mg) and F (0.95 μg/mg) with available standards. In addition, GLT contains only triterpenes and polysaccharides were not detected in GLT. In this study, we used total triterpene extract GLT, which was dissolved in DMSO (Sigma, St. Louis, MO) at the concentration 40 μg/ml and stored at 4 °C. The presence of LPS in GLT was evaluated by the Limulus Ameobocyte Lysate assay (Lonza, Walkersville, MD). We detected a low level of LPS contamination corresponding to 0.1 pg LPS in the highest dose of GLT (100 μg/ml) in vitro or 9.1 pg LPS in the dose of GLT (12 mg/kg) in vivo which we used in our experiments, respectively. Therefore the levels of LPS were below our level for the stimulation of cells in vitro (1 μg/ml) or animals in vivo (100 μg/kg), respectively.

2.2. Cell culture

RAW264.7 cells, a murine macrophage cell line (American Type Culture Collection, Menassas, VA, U.S.A.), were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. RAW264.7 cells were plated at a density of 2–3 x 10⁶ and cultivated at 37 °C in a humidified atmosphere containing 5% CO₂. For all experiments, cells were grown to 80–90% confluence. RAW264.7 cells were stimulated with 1 μg/ml LPS (Escherichia coli 011: B4; Difco, Detroit, MI, U.S.A.). Cells were incubated with or without GLT in the serum free medium for 24 h and then exposed for another 24 h to LPS.

2.3. NO production

RAW264.7 cells were pretreated with GLT (0–50 μg/ml) for 24 h followed by an additional 24 h incubation with LPS (1 μg/ml). The level of TNF-α, IL-6 (Biolegends, San Diego, CA) and PGE2 (Cayman Chemical Company, Ann Arbor, MI) in the culture medium was measured by ELISA according to the manufacturer’s protocol.

2.4. Enzyme-linked immunoabsorbent assay (ELISA)

RAW264.7 cells were preincubated with 0–50 μg/ml of GLT for 24 h followed by an additional 24 h incubation with LPS (1 μg/ml). The level of TNF-α, IL-6 (Biolegends, San Diego, CA) and PGE2 (Cayman Chemical Company, Ann Arbor, MI) in the culture medium was measured by ELISA according to the manufacturer’s protocol.

2.5. IC₅₀ determination

The log dose of GLT was plotted vs. the percentage of inhibition, and regression analysis of log dose–response curve was used to calculate IC₅₀ by using GraphPad Prism 3.0 (GraphPad Software Inc., La Jolla, CA).

2.6. Preparation of nuclear and whole cell extract

RAW264.7 cells were pretreated with GLT (0–100 μg/ml) and stimulated with LPS (1 μg/ml) at indicated times. Whole cell extracts were prepared as described [26]. Nuclear extracts were isolated from cells resuspended with 0.5 ml of ice-cold RSB lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 1 mM DTT, and protease inhibitor cocktail Complete™), by dounce homogenization. The nuclei were collected by centrifugation and the nuclear pellet was resuspended in 3 volumes of buffer C (20% glycerol, 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 100 μM Na₃VO₄ and protease inhibitor cocktail Complete™), incubated for 30 min on ice, and the final supernatant (nuclear extract) collected by centrifugation. The protein concentration in whole cell and nuclear extract was determined according to the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA).

2.7. Western blot analysis

Western blot analysis was performed as previously described [27]. Briefly, total cell lysates were separated by SDS-PAGE and were transferred to a PVDF membrane. The membrane was blocked with 5% bovine serum albumin in TBS-Tween 20 solution and was further incubated with the corresponding antibody: COX-2 (mouse, 1:1000; BD Biosciences, San Jose, CA), ERK1/2 (rabbit, 1:1000), p-ERK1/2 (mouse, 1:1000), JNK (rabbit, 1:1000), p-JNK (mouse, 1:1000), p38 (rabbit, 1:1000), p-p38 (mouse, 1:1000), p-p65 (rabbit, 1:1000) (Cell Signaling, Danvers, MA), INOS (mouse, 1:500), c-Jun (rabbit, 1:500), c-Fos (rabbit, 1:500), p65 (rabbit, 1:1000), cyclin D1 (rabbit, 1:1000), cyclin B1 (rabbit, 1:1000), CDK4 (rabbit, 1:1000), and GAPDH-HRP (goat, 1:10,000) (Santa Cruz Biotechnology, Santa Cruz, CA). Reactive bands were visualized with HRP-coupled respective secondary antibody (1:1000 or 1:2000) via an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, UK) according to the manufacturer’s procedures.
2.8. Electrophoretic mobility shift assay (EMSA)

Oligonucleotide probes containing consensus sequences for AP-1 and NF-κB binding sites were purchased from Promega (Madison, WI, USA). EMSA for AP-1 and NF-κB was performed as previously described [27]. EMSA for AP-1 and NF-κB was carried out in gel shift binding 5× buffer (Promega, Madison, WI, USA) with 5 μg of nuclear protein on ice for 20 min. One μl (70,000 cpm) of 32P-AP-1 or 32P-NF-κB probe was added to the reaction mixture and incubated for another 20 min at room temperature. The reaction mixture was then separated on a 4.5% PAGE gel in 0.25× Tris/borate/EDTA (TBE) buffer. The specificity was confirmed by competitive EMSA with cold AP-1 or NF-κB oligonucleotide or unrelated DNA (URL).

2.9. Cell proliferation assays (MTT and BrdU assays)

The inhibition of cell proliferation was measured by using the MTT assay kit (Roche, Indianapolis, IN), respectively. For MTT assay, RAW264.7 cells were collected, and 2.5×10^3 cells were dispensed into each well of a 96-well culture plate in 100 μL DMEM for 0-24 h, with control cells having 100 μL DMEM for 0–24 h, with control cells having ≤0.15% DMSO. The cells were then incubated with 10 μM of BrdU (Roche) in 100 μL of the media for another 2 h, followed by immunostaining with anti-BrdU monoclonal antibody conjugated with peroxidase (Roche) for 30 min. BrdU incorporation was evaluated by the absorbance at 370 nm minus that at 492 nm.

2.10. Cell cycle analysis

The effect of GLT on cell cycle distribution was determined by flow cytometric analysis of DNA content of nuclei of cells following staining with propidium iodide. RAW264.7 cells (7×10^5 cells/ml) were seeded in 100 mm² tissue culture dish, and allowed to attach overnight. The medium was replaced with fresh serum free medium containing GLT, and the cells were incubated for another 24 h at 37 °C. The cells were harvested by scraping, washed with PBS and fixed in 70% ethanol for 1 h at 4 °C, and treated with 20 mg/ml of RNaseA for 2 h at 37 °C. Finally, the cells were then stained with 50 μl of propidium iodide solution (4.975 ml of 3.8 mM sodium citrate solution + 25 μl of 10 mg/ml of propidium iodide) for 30 min on ice, and analyzed using a FACStar® flow cytometer (Becton-Dickinson, San Jose, CA, USA) as previously described [28].

2.11. Induction of systemic inflammation

Female Balb/C (8–9-week-old) mice, weighing 18–20 g, were procured and maintained at our animal house facility. Systemic inflammation was induced by a single intraperitoneal injection of LPS (0.1 mg/kg) in saline [29]. To examine whether GLT has protective effects on endotoxin shock, mice were divided into three groups. Mice in the first group were kept untreated (n = 6) while animals from 2nd and 3rd group were treated with LPS plus appropriate volume of vehicle (Cremophore: ethanol: DPBS; n = 6), and LPS plus GLT (12 mg/kg; n = 6), respectively. GLT was administered intraperitoneally 1 h before LPS administration. 90 min after LPS administration, blood sample was collected by orbital puncture, and plasma was separated by centrifugation at 7000 rpm for 10 min at 4 °C for the determination of TNF-α, and IL-6 levels by ELISA as described above. The animal studies have been reviewed and approved by the Animal Research Committee at the Methodist Research Institute, Clarian Health, Inc.

3. Results

3.1. GLT suppresses LPS-dependent induction of pro-inflammatory cytokines and mediators in RAW264.7 cells

In order to determine whether GLT possessed anti-inflammatory activity, we evaluated whether GLT affects the production of pro-inflammatory cytokines from RAW264.7 macrophages exposed to LPS. RAW264.7 cells were pretreated for 24 h with GLT followed by stimulation with 1 μg/ml LPS for additional 24 h. As seen in Fig. 1A, GLT at doses 10–50 μg/ml markedly inhibited TNF-α production from RAW264.7 cells (IC50 = 15.1 μg/ml). In addition, GLT also suppressed IL-6 production from LPS-stimulated RAW264.7 cells (Fig. 1B, IC50 = 14.4 μg/ml). Finally, the concentration 100 μg/ml of GLT did not affect the morphology and did not demonstrate any sign of toxicity in RAW264.7 cells (not shown).

Next we evaluated whether GLT could also inhibit the production of inflammatory mediators PGE2 and NO. RAW264.7 cells were treated with GLT and LPS as described above, and the production of PGE2 and nitrite accumulation was examined. As seen in Fig. 2, GLT decreased the secretion of LPS-induced PGE2 and NO in a dose-
dependent manner with the IC50 values 28.2 μg/ml (PGE2) and 11.4 μg/ml (NO). Since the expression of PGE2 and NO is controlled by enzymes COX-2 and iNOS, respectively, we evaluated if GLT directly modulates the levels of COX-2 and iNOS in RAW264.7 macrophages. Therefore, cell extracts were prepared from the RAW264.7 cells treated with GLT and LPS as described above, and these cell extracts were subjected to Western blot analysis with anti-COX-2 and anti-iNOS antibody, respectively. As seen in Fig. 2C, LPS-induced expression of COX-2 and iNOS in RAW264.7 cells, whereas GLT pretreatment markedly suppressed this LPS-dependent expression of both COX-2 and iNOS in macrophages. In conclusion, these results are consistent with the profile of the inhibitory effect of GLT on the LPS-dependent release of PGE2 and NO from RAW264.7 cells.

3.2. Effect of GLT on LPS-inducible NF-κB activation

The expression of COX-2 and iNOS is controlled by the transcription factor NF-κB, which is activated by LPS or other inflammatory stimuli in cells [30]. Because, as we show above, GLT suppressed LPS-dependent expression of COX-2 and iNOS we were interested whether GLT also modulates the activity of NF-κB in LPS-challenged macrophages. First, gel shift analysis was conducted to determine whether GLT changed NF-κB DNA-binding activity in RAW264.7 cells. As seen in Fig. 3A, the challenge of cells with 1 μg/ml of LPS for 30 min increased the binding activity of nuclear extracts to the NF-κB DNA consensus sequence, and the treatment of macrophages with 30 and 50 μg/ml of GLT for 48 h prior to the addition of LPS significantly inhibited LPS-induced increase in the band intensity of NF-κB binding. Second, since the phosphorylation and translocation of p65 subunit of NF-κB from cytosol to nucleus is the major component in NF-κB activation, we determined the effect of GLT on p65. Pretreatment of RAW264.7 cells with GLT significantly inhibited the LPS-induced phosphorylation of p65 as well as the total expression of p65 (Fig. 3B). To confirm these findings we also checked the nuclear level of phosphorylated p65. As seen in Fig. 3C, we observed that the nuclear level of phosphorylated p65 was significantly reduced in GLT-treated RAW264.7 cells as compared to control. Thus, GLT inhibition of LPS-dependent activation NF-κB is mediated by the suppression of phosphorylation and expression of p65 resulting in the decreased nuclear translocation of p65.

Fig. 2. Effect of GLT on LPS-induced PGE2, NO, COX-2 and iNOS expression in RAW264.7 cells. (A) PGE2 and (B) NO secretions were determined in cell culture media from RAW264.7 cells treated with GLT and LPS as described in Fig. 1. Each value is the mean ± S.D. of two independent experiments, repeated minimally twice. Statistical analysis was performed using Student’s t-test, *p < 0.005. (C) Expression of COX-2 and iNOS was determined in cell lysates from RAW264.7 cells pretreated with GLT (0, 30, 50 μg/ml) for 36 h followed by the LPS (1 μg/ml) treatment for additional 12 h by Western blot analysis as described in Materials and methods. The equal protein loading was verified with anti-GADPH antibody. The results are representative of three separate experiments.

Fig. 3. Effect of GLT on NF-κB. (A) RAW264.7 cells were pretreated with GLT (0, 30, 50 μg/ml) for 48 h followed by the LPS (1 μg/ml) treatment for 30 min. Nuclear extracts were prepared and subjected to gel shift analysis with [32P]-labeled NF-κB probe as described in Materials and methods. The specificity was confirmed by the competitive gel shift with cold NF-κB or unrelated DNA (URL). The data are representative of three separate experiments. (B) RAW264.7 cells were treated with GLT and LPS as described in A, and the expression of p-p65 and p65 in whole cell extracts evaluated by Western blot analysis as described in Materials and methods. The equal protein loading was verified with anti-GADPH antibody. The results are representative of three separate experiments. (C) RAW264.7 cells were treated with GLT and LPS as described in A, and the nuclear translocation of p-p65 evaluated in nuclear extracts by Western blot analysis as described in Materials and methods. The results are representative of three separate experiments.
3.3. Effect of GLT on LPS-stimulated AP-1 activation

In addition to the activation of NF-κB, transcription factor AP-1 also plays a crucial role in the regulation of inflammatory response [31]. Therefore, we investigated whether GLT modulates AP-1 activity in macrophages exposed to LPS. As seen in Fig. 4A, LPS treatment increased binding activity of nuclear extracts to the AP-1 DNA consensus sequence, whereas GLT suppressed this LPS-dependent binding activity of AP-1 in a dose-dependent manner. The activation of AP-1 can be preceded by the up-regulation and phosphorylation of its AP-1 subunits c-Jun and c-Fos [32]. To assess whether GLT could directly affect these AP-1 subunits, RAW264.7 cells were treated with LPS and the protein level of c-Jun and c-Fos was evaluated by Western blot analysis. Thus, LPS markedly up-regulated the expression of c-Jun, whereas the treatment of RAW264.7 cells with 30 and 50 μg/ml of GLT inhibited this LPS-dependent expression of c-Jun (Fig. 4B). On the other hand, no significant changes were observed in the level of c-Fos in cells treated with LPS in the absence or presence of GLT (Fig. 4B).

3.4. Effect of GLT on the MAPK activities

The MAP kinases ERK1/2, JNK and p38 are induced by LPS and can therefore regulate the activity of AP-1 and NF-κB [33]. To investigate whether GLT inhibits the LPS-induced activation of MAP kinases, we examined the effect of GLT on the LPS-induced phosphorylation of ERK1/2, JNK and p38 in RAW264.7 cells. As expected, LPS treatment induced phosphorylation of ERK1/2, JNK and p38 (Fig. 5). Moreover, GLT suppressed LPS-induced phosphorylation of ERK1/2 and JNK, whereas GLT did not affect the activity of LPS-dependent induction of p38 phosphorylation (Fig. 5). In conclusion, GLT specifically modulates the activity of ERK1/2 and JNK in activated macrophages.

3.5. Effect of GLT on systemic inflammation in mice

After establishing the potent in vitro anti-inflammatory activity of GLT we further evaluated its in vivo efficacy in a mouse model of LPS-induced endotoxemia. Intraperitoneal injection of LPS (0.1 mg/kg) caused the significant elevation of plasma TNF-α at 90 min after the LPS challenge, whereas the pretreatment of the mice with 12 mg/kg of GLT significantly decreased the plasma TNF-α level (Fig. 6A). In addition, we have found the same pattern in the suppression of LPS-induced IL-6 levels in mice pretreated with GLT (Fig. 6B). Therefore, our data suggest that GLT possesses anti-inflammatory activity in vivo.
3.6. Effect of GLT on RAW264.7 cell proliferation

In addition to the anti-inflammatory effect on macrophages, GLT can also directly affect their growth. Thus, the exponentially grown RAW264.7 cells were treated with GLT (0–100 μg/ml) for 24 h and the cell proliferation was determined as described in Materials and methods. As seen in Fig. 7A, GLT markedly suppressed the proliferation of RAW264.7 cells in a dose-dependent manner with the IC₅₀ value 14.8 μg/ml. As mentioned above GLT (100 μg/ml) did not change the morphology or viability of RAW264.7 cells, suggesting that GLT has a cytostatic effect. To further confirm that GLT suppresses cell proliferation, RAW264.7 cells were treated with GLT (0, 50, 100 μg/ml) for 1, 2, 4, 8, 16 and 24 h and BrdU incorporation assay was performed. Therefore, we also observed dose- and time-dependent inhibition of proliferation of RAW264.7 macrophages by GLT (Fig. 7B).

![Graph](image)

**Fig. 7.** GLT inhibits proliferation of RAW264.7 cells. (A) RAW264.7 cells were treated with GLT (0–100 μg/ml) for 24 h and cell proliferation determined by the MTT assay as described in Materials and methods. The data are mean ± S.D. from three independent experiments. Statistical analysis was performed using Student’s t-test, *p* < 0.001. (B) RAW264.7 cells were treated with GLT (0, 50 and 100 μg/ml) for 1, 2, 4, 8, 16 and 24 h and cell proliferation determined by the BrdU assay as described in Materials and methods. The data are mean ± S.D. (n = 3). Similar data were obtained in additional two independent experiments. Statistical analysis was performed using Student’s t-test, *p* < 0.05. (C) RAW264.7 cells were treated with GLT (0, 50, 100 μg/ml) for 24 h, and the expression of cyclin D1, CDK4 and cyclin B1 in whole cell extracts was evaluated by Western blot analysis as described in Materials and methods. The equal protein loading was verified with anti-GADPH antibody. The results are representative of three separate experiments.

In addition, we used flow cytometry to evaluate whether GLT modulates cell cycle progression and population distribution of RAW264.7 cells. In the present study, we used 50 and 100 μg/ml concentrations of GLT to explore its inhibitory activity on cell cycle distribution and its related proteins, because we observed more than 50% inhibition in cell proliferation at these two concentrations. Notably, the cell cycle analysis of cells treated with GLT for 24 h demonstrated significant G0/G1 and G0/G1→G2/M arrest at 50 and 100 μg/ml of GLT treatment, respectively (Table 1). D-type cyclins are induced in resting cells after growth factor stimulation [34] and are expressed throughout G1 phase and contribute to G1-S transition [35]. Conversely, cyclin B accumulation peaks at G2-M phase and modulates the catalytic activity of cdc2, which is restricted to mitosis [36]. Therefore, we investigated whether the effects induced by GLT were at the level of G1-S transition-related protein (i.e., cyclin D1) and/or at the level of G2-M transition-related protein (i.e., cyclin B1) in RAW264.7 cells. As seen in Fig. 7C, the expression pattern of cyclin D1 was significantly down-regulated in cells treated with 50 and 100 μg/ml of GLT for 24 h. Interestingly, the expression of cyclin B1 in cells treated with 100 μg/ml of GLT but not 50 μg/ml of GLT was significantly down-regulated during the same period. Further experiments demonstrated that significant decrease in cyclin D1 expression observed in GLT-treated cells was accompanied by substantial reduction in the amount of cdk4 that associates with cyclin D1 (Fig. 7C).

### 4. Discussion

Macrophages play an important role in both host-defense mechanism and inflammation [37,38] and the overproduction of inflammatory mediators by macrophages has been implicated in several inflammatory diseases and cancer [39]. In this study, we show that GLT (triterpene extract from the medicinal mushroom *G. lucidum*) markedly suppressed the inflammatory response in LPS-activated murine macrophages. Specifically, GLT suppressed LPS-dependent secretion of TNF-α, IL-6, NO and PGE₂ from RAW264.7 cells. The inhibition of production of NO and PGE₂ by GLT was mediated through the down-regulation of expression of iNOS and COX-2, respectively. Moreover, GLT inhibited LPS-dependent induction of NF-κB as well as expression, phosphorylation and nuclear translocation of p65 NF-κB subunit. GLT also suppressed LPS-induced activation of AP-1 and down-regulated expression of AP-1 subunit c-Jun. Finally, GLT inhibited LPS-dependent phosphorylation of ERK1/2 and JNK kinases demonstrating its potency to suppress the key molecules responsible in the inflammatory response (Fig. 8).

Given that the NF-κB/AP-1 axes are crucial for the inflammatory response leading to the production of TNF-α, IL-6, NO and PGE₂ and other inflammatory mediators from activated macrophages [40–42], the suppression of NF-κB/AP-1 signaling has a potential therapeutic effect. Indeed the pivotal role of NF-κB and IL-6 was recently confirmed by the successful therapeutic use of inhibitors of these cytokines [43]. Moreover, here we show the inhibition of blood levels of circulating TNF-α and IL-6 in LPS-challenged mice treated with GLT, which would further suggest the possible therapeutic use of GLT. Nevertheless, this hypothesis will be tested in our future studies.

### Table 1

Effect of GLT on cell cycle distribution.

<table>
<thead>
<tr>
<th>GLT [μg/ml]</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>71.2±0.35</td>
<td>23.4±0.21</td>
<td>5.5±0.11</td>
</tr>
<tr>
<td>50</td>
<td>82.6±3.08*</td>
<td>12.1±4.56</td>
<td>5.3±1.4</td>
</tr>
<tr>
<td>100</td>
<td>77.8±2.17*</td>
<td>12.4±1.51</td>
<td>9.9±0.66*</td>
</tr>
</tbody>
</table>

Cell cycle distribution G0/G1, S, and G2/M in % after 24 h.

Statistical significance *p* < 0.05 for cells at G0/G1 phase (50 and 100 μg/ml GLT) and G2/M (100 μg/ml GLT) vs. control (0 μg/ml GLT) from the three experiments.
Our data, demonstrating the suppression of the inflammatory response in macrophages by GLT through the inhibition of NF-κB/AP-1 signaling, are in agreement with other studies describing the anti-inflammatory activity of other natural triterpenes. For example, ginsenoside Rh1, triterpene isolated from *Panax ginseng*, inhibited iNOS and COX-2 expressions and the activation of NF-κB in RAW264.7 cells [44]. Extract from *Glossogyne tenuifolia*, containing oleanolic acid, down-regulated the expression of iNOS and COX-2, suppressed the release of TNF-α and IL-6, and suppressed the LPS-dependent activation of NF-κB in RAW264.7 cells [45]. Laxifolone A, a triterpene isolated from *Euonymus laxiflorus*, suppressed the expression of iNOS through the inhibition of NF-κB, which was mediated by the inhibition of nuclear translocation of p65 in stimulated macrophages [46]. Momordin I, structurally related oleanolic acid glycoside isolated from *Ampelopsis radix*, markedly suppressed the activity of NF-κB and AP-1 in activated macrophages [47]. Celastrol, isolated from *Celastrus orbiculatus*, prevented LPS-induced mRNA expression of iNOS and TNF-α, and production of NO and TNF-α in LPS-stimulated RAW264.7 cells [48]. Interestingly, celastrol suppressed NF-κB DNA-binding activity without affecting DNA-binding activity of AP-1 [48]. On the other hand, ginsenoside Rg3 induced the production of NO and expression of iNOS together with the induction of NF-κB, in RAW264.7 cells [49]. In addition, ginsenoside Rg3 induced the expression of COX-2 and production of PGE2 through the activation of transcription factors C/EBP and CREB, but not p65 [50]. Finally, ursolic acid induced the activation of MEK1/2 and ERK1/2 in RAW264.7 macrophages [51]. Nevertheless, in our study, we demonstrate, for the first time, the molecular mechanism responsible for the anti-inflammatory activities of mushroom triterpene extract GLT.

In addition to its anti-inflammatory effect, GLT also suppress the proliferation of RAW264.7 cells through the cell cycle arrest in G0/G1 and G2/M phases. Interestingly, different *G. lucidum* triterpenes demonstrated specific cell cycle arrest in particular cell lines. Therefore, uncharacterized ganoderic acid and ganoderiol F induced cell cycle arrest at G1 phase in hepatoma cells [52,53], whereas ganoderic acid T induced cell cycle arrest at G1 phase in lung cancer cells [54], and *Ganoderma* triterpenes arrested cervical cancer cells at G0/G1 [55]. On the other hand, triterpene enriched extract from *G. lucidum* arrested hepatoma cells at G2 phase [56], and ganoderic acid D induced cell cycle arrest of cervical cancer cells at G2/M [57].

Our data demonstrate that GLT at the concentration of 50 μg/ml induces cell cycle arrest at G0/G1, whereas 100 μg/ml of GLT induces cell cycle arrest at both G0/G1 and G2/M phases. Although a cell arrest in the different stages of cell cycle by the same treatment at different concentrations was unexpected, we evaluated whether this effect of GLT is associated with the cell cycle regulatory proteins. Generally, entry into the S phase (essential for DNA synthesis) is blocked by G0/G1 check point while entry in to the mitosis is blocked by G2/M checkpoint mechanisms. G0/G1 transition is modulated by cdk4/cyclin D1 kinase complex whereas the regulation of G2/M transition is dependent on the activation of cdk1/cyclin B1 [58,59]. In agreement with our data from cell cycle analysis, 50 and 100 μg/ml of GLT down-regulated the expression of cyclin D1 and cdk4 (G0/G1), whereas the expression of cyclin B1 (G2/M) was down-regulated only at 100 μg/ml of GLT (Fig. 7). However, it is possible to hypothesize that, in addition to the first cell arrest at G0/G1 (through the down-regulation of expression of cyclin D1 and cdk4), another cell arrest at G2/M (through the down-regulation of cyclin B expression) can be induced by the higher concentration of GLT. For example, the expression of cyclin D1 is controlled by NF-κB and AP-1 [60,61], whereas cyclin B1 expression is controlled “only” by AP-1 [62]. Since 50 μg/ml of GLT suppressed both (constitutive, LPS-independent) NF-κB and AP-1 (Figs. 3A and 4A), this concentration of GLT was able to down-regulate the expression of cyclin D1, but was not sufficient for the down-regulation of the expression of cyclin B1 (Fig. 7C). Alternatively, GLT can also regulate the expression of cyclin D1 and
cycillin B1 by other mechanisms. Nevertheless, our data clearly demonstrate that GLT suppresses the proliferation of RAW264.7 macrophages, which could contribute to its anti-inflammatory properties.

The majority of studies describing the effect of G. lucidum or its compounds on the immune system demonstrated the activation of macrophages, neutrophils, dendritic cells, natural killer cells and T- and B-lymphocytes. For example, G. lucidum polysaccharides induced cytokine expression via Toll-like receptor (TLR)-4 in macrophages and dendritic cells, whereas immunoglobulin (Ig) production was mediated through TLR-4/TLR-2 in B-lymphocytes. Collectively, this polysaccharide-dependent expression of cytokines and Ig played a signaling role leading to the activation of MAPKs and NF-κB. In addition, an immunomodulatory protein, purified from G. lucidum, stimulated the production of IL-1β, IL-2, TNF-α and IFN-γ by human peripheral blood lymphocytes, and modulated protein tyrosine kinase (PTK) and protein kinase C (PKC) signaling to produce IL-2 in human T cells. Interestingly, only one study describes an induction of the immune response by G. lucidum triterpenes, ganoderic acid Me, as demonstrated by the increased expression of IL-2 and IFN-γ in mice. On the other hand, extract of G. lucidum containing triterpenes or isolated triterpenes (ganoderic acid A, F, DM, T-Q, lucidic acid A, B, D2, E2, F, methyl lucidenate A, D2, E2, Q and 20-hydroxylucidenic acid N) suppressed ear-edema inflammation in laboratory animals. In isolated G. lucidum triterpenes (ganoderic acid A, C1, C2, E, F, DM, T-Q, ganodermanon-diol, ganloactone, methyl ganofuran, ganoderic acid A, B, C, D2, E2, F, N, P, methyl lucidenate A, C, D2, E2, F, L, P, Q, 20(21)-dehydroelucidic acid A, methyl 20(21)-dehydroelucidic acid A, fangistol, 5,6-dihydroergosterol, ergosterol, ergosterol peroxde, 9(11)-dehydroergosterol peroxide and demethylcinnisterol A3) demonstrated the inhibitory effects on the induction of Epstein–Barr virus early antigen (EBV-EA). G. lucidum triterpenes increased the inhibition of NF-κB activity, and they are currently used in the form of dietary supplements, GLT with limited, nevertheless, important experiments. On the other hand, in our study we use G. lucidum triterpene extract (GLT) containing ganoderic acid A, F, F, Me, S1, ganosperic acid, lucidic acid B, D, D1, E1, L, and methyl lucidenate G and we have quantified the amount of ganoderic acid A (3.88 μg/gM), H (1.74 μg/gM) and F (0.95 μg/gM) with available standards. Therefore, the use of GLT allowed us to elucidate the molecular mechanism responsible for the inhibition of inflammatory response by G. lucidum triterpenes. Because extracts from G. lucidum have been used in Traditional Chinese Medicine for thousands of years and they are currently used in the form of dietary supplements, GLT with the characterized triterpenes, can be considered as a natural product with anti-inflammatory properties.

In summary, our results demonstrate that GLT exerts anti-inflammatory and anti-proliferative effects, which are mediated through the inhibition of NF-κB and AP-1 signaling in LPS-activated macrophages, finally resulting in the suppression of the production of pro-inflammatory mediators. More importantly, GLT was found to have an anti-inflammatory effect in the LPS-induced endotoxemia model in mice, one of the well-established acute inflammatory models in vivo. Our findings showing inhibition by GLT of pro-inflammatory cytokines may help to understand the in vivo anti-inflammatory potential of GLT and also to develop GLT as an anti-inflammatory and anti-proliferative agent.

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**References**

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