Integrative Cancer Therapies
2018, Vol. 17(3) 674–683

DOI: 10.1177/1534735418762537
journals.sagepub.com/home/ict
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Antitumor and Immunomodulatory Activities of *Ganoderma lucidum* Polysaccharides in Glioma-Bearing Rats

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Abstract

Malignant gliomas are the most common brain tumors with high rates of recurrence and mortality. Novel approaches are in research, and immunotherapy emerges as a promising strategy. Recently, scientific attention has been focused on *Ganoderma lucidum* polysaccharides (GL-PS), one of the critical bioactive components of *G lucidum*, which have been recognized as a promising natural source of immunomodulatory and anticancer compounds. It remains unknown whether the GL-PS have any immunomodulatory and anticancer effects on brain glioma. This study was designed to identify and characterize the antitumor action and influence of immune system of GL-PS in glioma-bearing rats. Results showed that GL-PS increased the concentration of serum interleukin-2, tumor necrosis factor-α, and interferon-γ, and enhanced the cytotoxic activity of natural killer cells and T cells, promoting the functional maturation of dendritic cells, thus resulting in the inhibition of glioma growth and prolonged survival of rats. Therefore, GL-PS may be potentially useful as part of the treatment regimen to regulate host immune responses and increase the antitumor effects of immunotherapy for glioma.

Keywords

Ganoderma lucidum polysaccharides, glioma, antitumor, immunomodulation, tumor-bearing rat model

Submitted June 10, 2017; revised January 15, 2018; accepted February 3, 2018

Introduction

Malignant gliomas account for about 65% of all primary brain tumors.[1] Despite current multimodal therapies including surgery, radiation therapy, and chemotherapy, the prognosis for patients with this tumor remains poor. Unfortunately, the overall survival of glioblastoma patients at 2 years is dismal, at 27.2% even with a new, aggressive standard of care including upfront radiochemotherapy.[2] Adjuvant experimental therapies to follow surgical resection and radiochemotherapy are being explored, and among them are passive and active immunotherapies with cellular therapy, dendritic cells, or peptide-based vaccines, or combined approaches. How to improve a patient's immune function has been the focus of glioma immunotherapy in the last 20 years.[3]

Ganoderma lucidum has been widely used in some Asian countries, known as "Lingzhi" in China and "Reishi" in Japan, for more than 4000 years.[4] Following the views of ancient Chinese medical scholars, people have widely used *G lucidum* for the promotion of general health and longevity, as well as for the treatment of various human diseases such as bronchitis, gastric ulcer, hyperglycemia, hypertension, chronic hepatitis, hepatopathy, insomnia, nephritis, neurasthenia, inflammation, and cancer.[5,6] *Ganoderma lucidum* polysaccharides (GL-PS) are one of the main effective components isolated from *G lucidum*, and they have been demonstrated to have various biological activities, including immunomodulation and antitumor, anti-angiogenesis, anti-oxidation, anti-inflammatory, and hepatoprotective effects. It has been reported that GL-PS can inhibit

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tumor growth in different tumor cell-bearing mice (Ca755, s/c P388, S-180, EAC, and Heps) as well as reduce proliferation in a variety of tumor cell lines (MCF-7, PC-3, HCT-116, HepG2, HT29, and B16F10).[7-10] The in vivo antitumor activity of GL-PS may be mediated by different mechanisms, particularly the activation of the immune effector cells such as lymphocytes,[11,12] natural killer (NK) cells,[13,14] and macrophages.[11,15] However, the central nervous system is anatomically unique and immunologically specialized; as such, whether GL-PS has any effects on gliomas through antitumor action and influence of the immune response remains unknown. This study was designed to establish a glioblastoma rat model to investigate the hypothesis postulating that GL-PS affects glioma growth via immunomodulation and the regulation of antitumorigenicity.

Methods

Animals and Cell Lines

Male Fischer rats (F344 rats; 200-250 g) were purchased from Beijing Vitalrive Experimental Animal Technology Co Ltd (Beijing, China). Animals were housed in a colony room under controlled temperature (22°C), and a 12:12 light-dark cycle, with access to food and water ad libitum. All experiments involving animals were carried out in accordance with the institutional guidelines on the care and use of experimental animals.

RG2 glioma cells were purchased from the American type culture collection (Manassas, VA), and human myelogenous leukemia cells K562 were obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were maintained in RPMI 1640 medium (HyClone, Logan, UT) supplemented with 10% (v/v) fetal bovine serum (Life Technologies Co, Rochester, NY) and 100 units/mL penicillin and streptomycin (HyClone, Logan, UT). All cells were cultured at 37°C in a 5% CO_2 atmosphere.

Preparation of GL-PS

Purified GL-PS was kindly provided by Professor Shuqian Lin at the Fuzhou Institute of Green Valley Bio-Pharm Technology in China. As described previously,[16,17] GL-PS were isolated through boiling-water extraction of the fruit bodies of *G lucidum*, followed by ethanol precipitation, dialysis, and protein depletion using the Sevag method. The component sugars and molecular weight distributions of the glycopeptides were determined by gel permeation chromatography and high-performance liquid chromatography. The structures of the glycopeptides were detected by infrared absorption and ^{1}H and ^{13}C nuclear magnetic resonance(^{1}HNMR and ^{13}CNMR). The isolated GL-PS are peptide-bound polysaccharides and have a molecular weight of 584 900, with a ratio of polysaccharides to peptides of 93.61%:6.49%. The polysaccharides consist of d-rhamnose, d-xylose, d-fructose, d-galactose, d-mannose, d-glucose, and uronic acid, with a molar ratio of 0.793:0.964:2.944:0.167:0.389:7.94:0.33. The glycoside linkage was primarily in the β-form, with minor α-bonding. The peptides contained 16 amino acids (Asp, Thr, Ser, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Phe, Lys, His, Arg, and Pro). GL-PS, as a hazel powder, was dissolved in serum-free RPMI 1640 medium, then filtered through a 0.22 μm filter and stored at 4°C. Endotoxins in GL-PS samples were assayed under endotoxin-free experimental conditions using a limulus amebocyte lysate chromogenic assay kit (Beijing BXGK Technology Development Co, Ltd, Beijing, China). The quantity of endotoxin in GL-PS was less than 0.011 EU/mg, indicating that endotoxin contamination in GL-PS was negligible. GL-PS was further diluted to the indicated final concentration (5 mg/mL) prior to each assay.

Rat Models of RG2 Brain Tumor and Grouping

Rats were immobilized and anesthesia was induced and maintained with a 2% and 98% isoflurane and oxygen mixture. Next, rats were placed on a stereotaxic apparatus and the skull exposed. Rats were inoculated into 1 mm anterior and 2 mm lateral to the bregma on the right side of the head, with 1×10^5 RG2 glioma cells via a 10 μL gastight syringe and at a depth of 3 mm relative to the dural surface. After plugging the skull hole with wax, the skin was stitched and rats were put back into the cage.

Thirty-two glioma-bearing rats were randomly divided into 4 groups categorized according to the abdominal injection of 50, 100, and 200 mg/(kg d) GL-PS or equal volume of normal saline (NS). These groups are henceforth named GL-PS 50 mg/kg, GL-PS 100 mg/kg, GL-PS 200 mg/kg, and control group. Each group was exposed to the experimental treatment for 2 weeks followed by magnetic resonance imaging (MRI) examination. The animal survival time was also recorded.

Another 32 glioma-bearing rats were randomly divided into 4 groups and treated identically. After the 2-week treatment, the animals in this cohort were anesthetized and serum was collected through venous sinus of the eye-orbit. Removal of the spleen was also performed in order to isolate lymphocytes and NK cells.

MRI Experiments

All spectroscopic and imaging experiments were performed at 298 K on a Varian 7 T/160 mm animal MRI scanner with a 63/95 mm quadrature birdcage coil and a gradient strength up to 400 mT/m. The main magnetic field was shimmed to minimize field inhomogeneity artifacts and the radio frequency field (B1) was calibrated before experiments. Chloral hydrate solution (3.5 mL/kg) was injected into the

peritoneum of the rat for anesthesia, after which the animal was fixed on the retaining plate in prone position. Animals subsequently underwent head-first sequence scans in coronal T1WI, T2WI + FS, and axial T1WI, T2WI + FS positions, as well as enhanced scan. After injection of the contrast agent gadopentetate dimeglumine (0.2-0.3 mL/kg) into the tail vein, the rats were administered with coronal and axial T1WI + FS sequence scans under the following conditions: repetition time = 3 seconds, echo time = 20 minutes, field of view = 128 × 128 matrix, thickness = 1 mm, and the chemical shift = 0 Hz. Tumor volume was calculated as follows: tumor volume = length × (width^2)/2.[18]

Histopathological and Immunohistochemical Examinations of Tumor

Each rat's brain was removed at necropsy for histological and/or immunohistochemical examination. First, the rats were perfused with 500 mL of saline followed by 200 mL of 4% paraformaldehyde. Their brains were then removed and postfixed overnight in formalin. After dehydration in graded ethanol and turpentine, brain sections were embedded in paraffin. Slices were cut through the area of tumor implantation at a thickness of 20 μm, and the sections were either mounted on glass slides for routine histological staining with hematoxylin and eosin or floated in phosphate-buffered saline (PBS) for immunohistochemistry.

For the immunohistochemical analysis, sections were incubated overnight at 4°C with rat monoclonal antibodies directed against rat CD8 marker. After buffer washes, species-absorbed biotinylated goat anti-rat secondary antibody was applied to sections incubated with unlabeled primary antibody, followed by treatment with streptavidin horseradish peroxidase for 30 minutes at room temperature according to the manufacturer's protocol. Finally, immunoreactivity was visualized by incubation in a solution of 0.02% 3,3-diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in PBS at pH 7.6. Sections were counterstained with thionine and mounted and preserved on a glass slide. For each pair of rat brains analyzed (control compared with treated), all reactions were performed at the same time by using the same reagents. The CD8+ tumor infiltrating lymphocytes were manually counted within a square, 10 mm/10 units at high magnification (×400) using an eyepiece micrometer (Olympus, Tokyo, Japan), and 4 fields with the most abundant tumor infiltrating lymphocytes were selected.

Assay of Serum Interlukin-2 (IL-2), Tumor Necrosis Factor-α (TNF-α), and Interferon-γ (INF-γ)

Rats' serum IL-2, TNF-α, and INF-γ were measured using enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Norcross, GA) according to the instructions provided by the manufacturer of the corresponding kits.

Cell Isolation

The rat's spleen was homogenized using a tissue grinder and rinsed with PBS (pH 7.4). The suspension was isolated by layering over Ficoll-paque with the specific density for rat's mononuclear cells and centrifugation at 500*g* for 20 minutes. The isolated mononuclear cells were washed twice with RPMI medium to remove residual Ficoll-paque solution. Then, NK cells were isolated by rat spleen NK cell separation kit (Sangon Biotech (Shanghai) Co, Ltd, Shanghai, China) and were subsequently used for cytotoxicity assay of NK cells. The mononuclear cells isolated by rat lymphocyte Ficoll-paque were used for assay of the lymphocyte proliferative response. In the experiments, NK cells and lymphocytes were cultured in complete RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and streptomycin. All cells were cultured at 37°C in a 5% CO_2 atmosphere.

Cytotoxic Activity of NK Cells

Cytotoxicity was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Fitchburg, WI), according to the manufacturer's instructions. In brief, NK cells were isolated as mentioned above and used as effectors in the cytotoxicity assays against K562 or RG2 cells. The cytotoxicity capacity of NK cells was assessed by plating cells for 4 hours at different effector/target ratios. Cytotoxicity rate was calculated according to the following formula: % Cytotoxicity = ([Experimental − Effector Spontaneous − Target Spontaneous]/[Target Maximum − Target Spontaneous]) × 100%.

Lymphocyte Proliferative Response in Tumor-Bearing Rat

T-lymphocyte proliferation was tested by flow cytometry analysis by CD3-PE antigen labeling combined with 5-ethynyl-29-deoxyuridine (EdU; RiboBio, Guangzhou, China) incorporation. EdU is a thymidine analog whose incorporation can be used to label cells undergoing DNA replication.[19] Rats were injected intraperitoneally with 5 mg/kg of EdU and spleen cells, which were separated 48 hours after injection. The percentage of CD3+ cells incorporating EdU was evaluated using the Cytomic FC 500 flow cytometer and CXP 2.1 software (Beckman Coulter, Krefeld, Germany).

Assays of Monocyte-Derived DC Maturity (Mo-DC)

Rat's Mo-DCs were prepared, as previously described with some modifications.[20] Briefly, 2 to 3 mL heparinized blood was obtained by orbital venous plexus puncture. Peripheral blood mononuclear cells were separated by centrifugation on a density gradient using Ficoll-paque PREMIUM 1.084

sterile solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and placed into culture for 2 hours in 6-well plates in complete RPMI 1640 medium. Nonadherent cells were removed and adherent cells were cultured in medium containing rat recombinant granulocyte-macrophage colony-stimulating factor and IL-4 (PeproTech, Rocky Hill, NJ) at a concentration of 10 ng/mL. At day 6, the cultured cells were treated with 6.25, 12.5, and 25 μg/mL GL-PS or NS for 24 hours. At day 7, the nonadherent cells and supernatant were extracted for flow cytometry and ELISA assay.

The harvested cells were washed with cold buffer (PBS containing 2% FCS and 0.1% sodium azide), incubated in cold buffer, and subsequently stained with FITC- or PE-labeled mAbs (anti-rat MHC-II, CD40, CD80, CD86, or relevant isotype controls; eBioscience, San Diego, CA) based on the manufacturer's instructions. The DC surface marker expression was analyzed using the Cytomic FC 500 flow cytometer and CXP 2.1 software. The levels of IL-12p70 and IL-10 in the culture supernatant extracted from DC cell culture were assayed with ELISA kits, per the manufacturer's instructions.

Statistical Analysis

All data were expressed as the mean ± SD. Data analyses were performed with SPSS16.0. The median survival time was analyzed using Kaplan-Meier and log-rank tests. Other data were analyzed by 1-way ANOVA. Differences between groups were considered statistically significant at $P < .05$.

Results

Tumor Size and Survival Time

To study the general effect of GL-PS on the glioma-bearing rat, we recorded the tumor size after 2 weeks of treatment as well as the survival time. Different groups had different tumor sizes and survival times. As shown in Figure 1, the median tumor size of the GL-PS 50 mg/kg, 100 mg/kg, 200 mg/kg, and control groups were 101.93 ± 53.58, 113.56 ± 39.76, 161.28 ± 56.69, and 162.99 ± 48.34 mm^3, respectively, and the median survival times were 27.67 ± 2.87, 31.78 ± 6.38, 27.33 ± 4.97, and 24.44 ± 2.55 days, respectively. Compared with the NS group, the GL-PS 50 mg/kg and 100 mg/kg groups showed a significant decrease in the tumor size and increase in the median survival time. However, there was no significant difference in tumor size or survival time between the GL-PS 200 mg/kg and control group.

Determination of CD8+ Tumor Infiltrating Cytotoxic T-Lymphocytes (CTL)

In many solid tumors, including glioma, the presence of $CD8^+$ CTL is associated with a better prognosis.[21,22] Three weeks after tumor implantation, we examined the presence of CD8+ CTL in all rat tumors. Rats treated with GL-PS displayed significantly increased CD8+ CTL compared with those NS treated (Figure 2).

Serum IL-2, TNF-α, and INF-γ

To examine the effects of GL-PS on the immune function of glioma-bearing rats, we measured the serum IL-2, TNF-α, and INF-γ levels using the appropriate ELISA kit. As shown in Table 1, the concentrations of IL-2, TNF-α, and INF-γ were elevated significantly in the GL-PS groups compared with the control group.

Cytotoxic Activity of NK cells

NK cells are capable of killing a wide range of cancer cells and are thus a promising tool for cell therapy in cancer. We analyzed whether GL-PS can enhance the cytotoxic activity of NK cells. As shown in Figure 3, compared with the control group, the splenic NK cytotoxic activity of GL-PS group was significantly increased. Moreover, the effect was dose-dependent.

Lymphocyte Proliferative Response

To determine the effect of GL-PS on lymphocytes in the tumor model, lymphocyte proliferation was detected by flow cytometry analysis via labeling of the CD3-PE antigen combined with EdU incorporation. As shown in Figure 4, spleen lymphocyte proliferation rates of the GL-PS groups were higher than that of control group, and highest in GL-PS 100 mg/kg group.

Maturity of Monocyte-Derived DC

In order to define the maturity of Mo-DCs that was induced by the GL-PS, we checked the surface expression of antigen presentation molecules and costimulation molecules that Mo-DCs normally express. In Figure 5, we found that the GL-PS treated DCs expressed relatively higher levels of MHC-II, CD40, CD80, and CD86 when compared with untreated DCs.

We then measured IL-10, IL-12p70 by ELISA of supernatants from cultured Mo-DCs isolated from the different treatment groups. As shown in Figure 6, Mo-DCs of the control group showed little secretion of IL-10 and IL-12p70, while GL-PS groups produced a significant increase in the secretion of IL-10 and IL-12p70.

The purity of each population used in the current study was confirmed by multicolor flow cytometry where more than 85% of the Mo-DC populations bound to the CD103 mAb (data not shown).

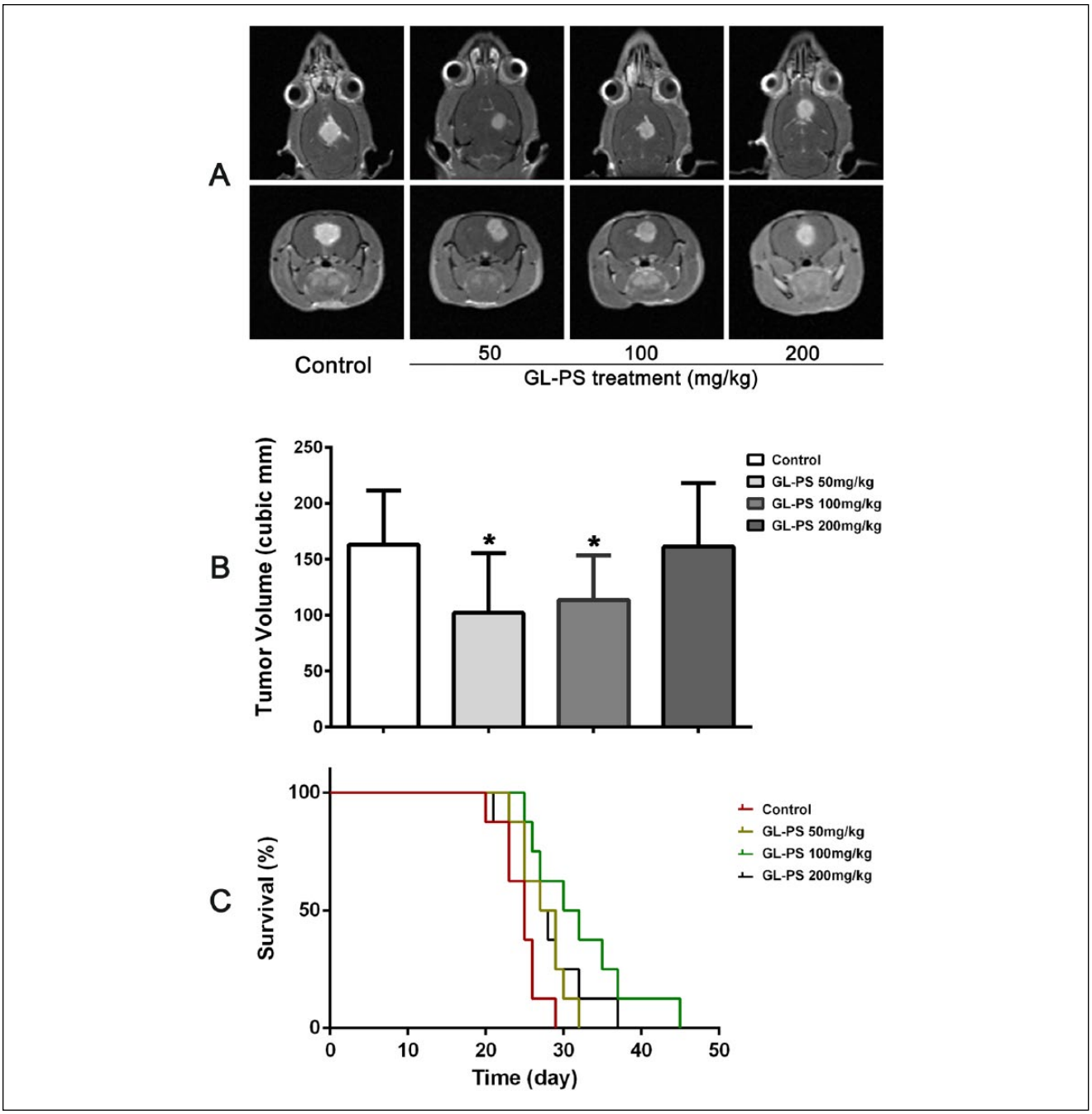

Figure 1. The tumor size and survival curve in the glioma-bearing model (n = 8). (A) Each rat underwent MRI after the 2-week treatment and the maximum length of the tumor was calculated. (B) MRI scan showed the brain tumor size in GL-PS 50 mg/kg and 100 mg/kg groups was significantly less than the control group (*$P < .05$). There was no statistical difference between the GL-PS 200 mg/kg group and control group. (C) After the 2-week treatment, the survival time was recorded. The survival curve showed the curve of the control group dropped faster than other groups. There was a statistical difference between GL-PS 50 mg/kg and 100 mg/kg groups and the control group ($P < .05$), but no difference between the GL-PS 200 mg/kg group and control group.

Discussion

Even with new aggressive standards of care including upfront radiochemotherapy, the overall survival of glioblastoma patients at 2 years remains poor, at 27.2%.[2] Adjuvant experimental therapies to follow surgical resection and radiochemotherapy are being explored, among them passive and active immunotherapies.[3] Although initial clinical studies centered on DC-based immunotherapy resulted in very limited success, they have prompted many new studies

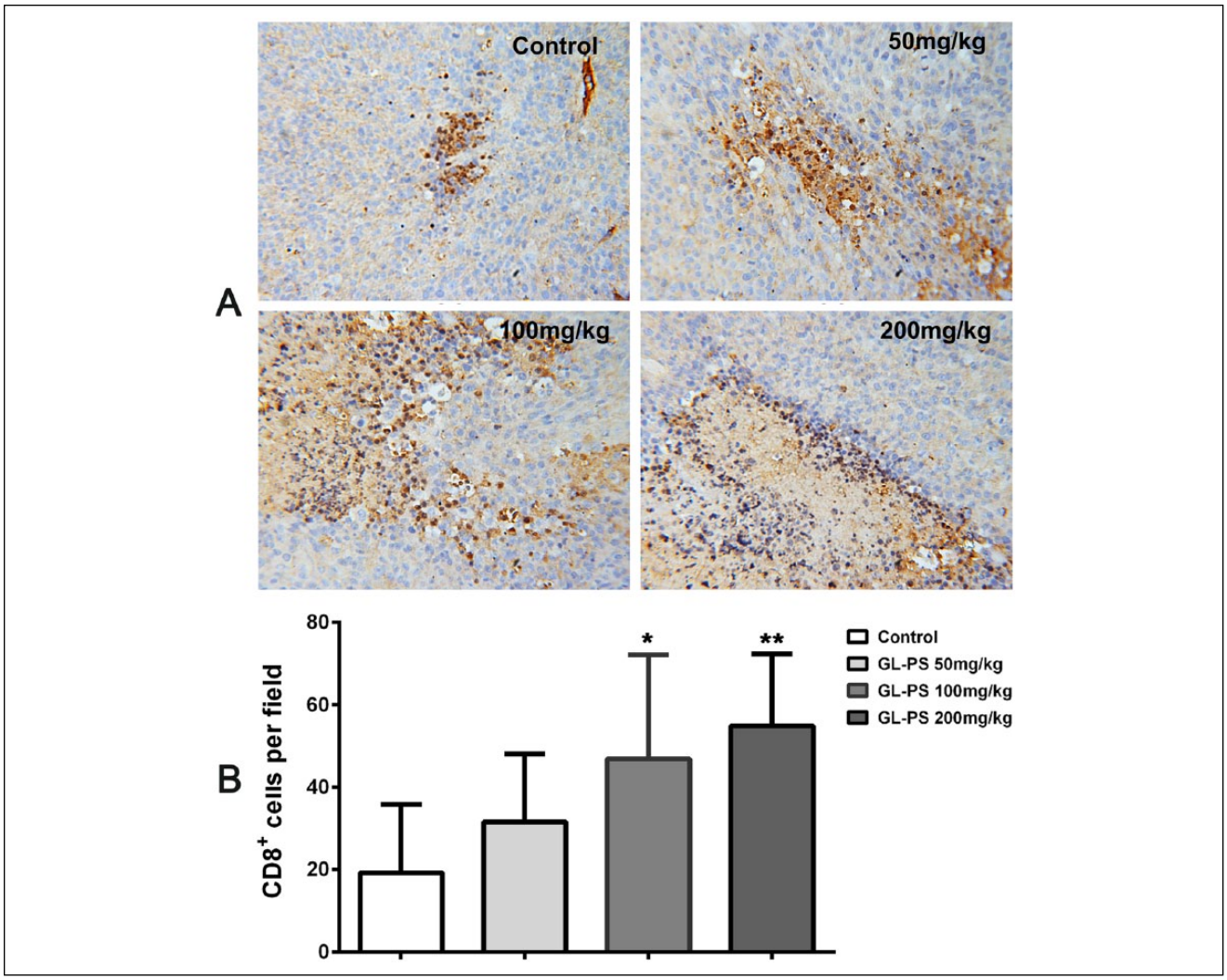

Figure 2. Intratumoral infiltration of CD8+ cytotoxic T lymphocytes (n = 8). (A) Twenty-one days after tumor implantation, CD8+ cytotoxic T-cells (brown color, original magnification, ×400) were present throughout the tumors of all groups. (B) The number of CD8+ T-cell infiltration was dramatically higher in tumors of GL-PS 100 mg/kg and 200 mg/kg groups compared with the control group (**$P < .01$, *$P < .05$).

Table 1. Effect of GL-PS Treatment on Serum IL-2, TNF-α, and INF-γ Levels (n = 8)[a].

Group	IL-2 (pg/mL)	TNF-α (pg/mL)	INF-γ (pg/mL)
Control	10.73 ± 1.42	123.38 ± 29.62	13.58 ± 2.62
GL-PS 50 mg/kg	19.25 ± 1.60[b]	181.02 ± 29.76[c]	26.21 ± 3.67[b]
GL-PS 100 mg/kg	20.15 ± 4.35[b]	212.50 ± 42.65[c]	36.94 ± 3.60[b]
GL-PS 200 mg/kg	21.49 ± 5.31[b]	247.80 ± 41.78[b]	33.49 ± 11.26[b]

Abbreviations: GL-PS, *Ganoderma lucidum* polysaccharides; IL-2, interlukin-2; TNF-α, tumor necrosis factor-α; INF-γ, interferon-γ.
[a]Data presented as mean ± SD.
[b]$P < .01$, [c]$P < .05$, compared with control group.

exploring strategies to induce a more robust antitumor immune response. The relation between the occurrence, growth, and decline of tumor and immune states is an essential problem in tumor immunology. The discovery and identification of novel antitumor drugs that enhance immune function has become an important goal of immunopharmacological studies.

As a traditional Chinese herb, *G. lucidum* has been extensively used for the improvement of the immune activity of patients treated by chemical or radiation therapy, and

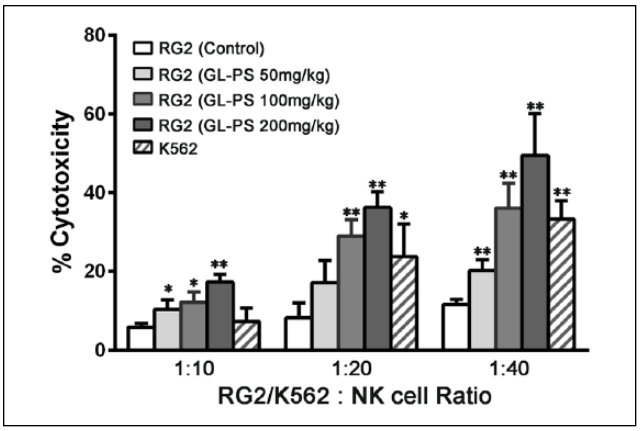

Figure 3. Effect of GL-PS on splenic NK cell cytotoxic activity (n = 4). Splenic NK cells were isolated from the control and GL-PS group rats. The cytotoxic activity of NK cells against RG2 or K562 cells was evaluated at varying effector-to-target ratios, and co-cultures were assessed for lactate dehydrogenase (LDH) release. The percentage of LDH released reflected NK cytotoxic activity. Data presented as mean ± SD of 6 rats. $^{**}P < .01$, $^{*}P < .05$, with correction for multiple comparisons.

many studies have demonstrated the immunomodulatory and antitumor capacity of GL-PS.[23-25] However, it remains unclear whether GL-PS has any effects on glioma cells through its antitumor action or influence of the immune system. In this study, we demonstrate the significant antitumor effect of GL-PS and its immunomodulatory action in a glioma-bearing rat model.

In this study, an appropriate concentration of GL-PS (50 or 100 mg/kg) could significantly slow tumorigenesis and increase the survival time in a glioma-bearing rat model. Interestingly, GL-PS 200 mg/kg did not result in improved tumor size control or survival benefits, and the concentration of GL-PS 100 mg/kg appeared as the optimal concentration. This suggests that anticancer activity of GL-PS may be attributed to modulation of the immune response of the host rather than direct cytotoxicity to cancer cells. The higher concentrations of the GL-PS may cause heavy infiltration by immune and inflammatory cells, not only CD8+ T lymphocytes but also the immunosuppressive cells (eg, Treg, TAM), leading to an initial increase in tumor burden.

T cells, including CD8+ CTL, play a prominent role in protective immunity. Some studies found that GL-PS can significantly increase the lymphocyte proliferation and the cytotoxic activity of CTL in vitro.[26,27] Our study showed that GL-PS could significantly promote spleen T-lymphocyte proliferation in tumor-bearing rats, as well as increase CTL infiltration in tumors, suggesting that GL-PS could enhance cellular immunity and improve the immune system.

NK cells are essential effector cells of the innate immune system, which rapidly recognize and eliminate microbial pathogens and abnormal cells. Activated NK cells have been applied with a notable success in the therapy of certain cancers.[28] Some studies found that GL-PS could enhance the cytotoxicity of splenic NK cells in tumor-bearing mice.[29,30] In our study, the same effects of GL-PS on NK cells occurred in glioma-bearing rats.

Since IL-2, TNF-α, and INF-γ play prominent roles in the development of both the immune and antitumor responses,[31-34] we also investigated the effects of GL-PS on these cytokines in glioma-bearing rats. Our study showed that the mean serum concentration of IL-2, TNF-α, and INF-γ apparently increased in GL-PS-treated groups. This result indicated that IL-2, TNF-α, and INF-γ may mediate the effects of GL-PS on the immune system, contributing to the antitumor mechanism of GL-PS in vivo.

DCs are the most efficient and critical of all antigen-presenting cells and are important for the initiation of the primary immune response of both helper and CTLs. DC functions are closely related to their maturation and activation state. Immature DCs have a high capability for antigen capture and processing, though their capacity to activate the naive T cells is weak. Fully mature DCs show a high surface expression of MHC class II and costimulatory molecules (CD40, CD80, and CD86) but a decreased capacity to internalize antigens.[35] Fully mature DCs can also secrete IL-12 and IL-10. In immune responses, IL-12 plays a central role as a link between the innate and adaptive immune systems.[36] Thus, IL-12 induces and promotes the production of IFN-γ by T cells and NK cells. In addition, IL-12 polarizes the immune system toward a primary T helper cell type 1 (Th1) response. IL-10 is a pleiotropic cytokine, which can act in a feedback regulatory role in mature DCs.[37,38] Although the reverse relationship between the IL-10 and IL-12 production by activated DCs has been suggested,[39,40] the mechanisms behind this relationship are not well understood. A few studies also showed that activated DCs can simultaneously increase the production of IL-12p70 and IL-10.[41,42]

Therefore, it is very important for antitumor agents to stimulate DCs to differentiate from immature to mature stages. Some studies have found that GL-PS can promote the phenotypic and functional maturation of DCs from a variety of sources.[43-45] Our study showed that GL-PS also could promote the phenotypic and functional maturation of rats' Mo-DCs as defined by their enhanced expression of cell-surface expression of MHC-II, CD40, CD80, and CD86 as well as secrete cytokine IL-10 and IL-12p70.

In this study, there was no linear dose-effect relationship between the GL-PS concentration and the inhibitory effects of GL-PS on glioma and its immunomodulatory activity on the glioma-bearing rat. The effect of GL-PS at the concentration of 100 mg/kg appeared to be optimal compared with the 50 mg/kg and 200 mg/kg dosages. Some researchers have postulated that the key to regulate the immune function by polysaccharide was the level of state in the body, not the

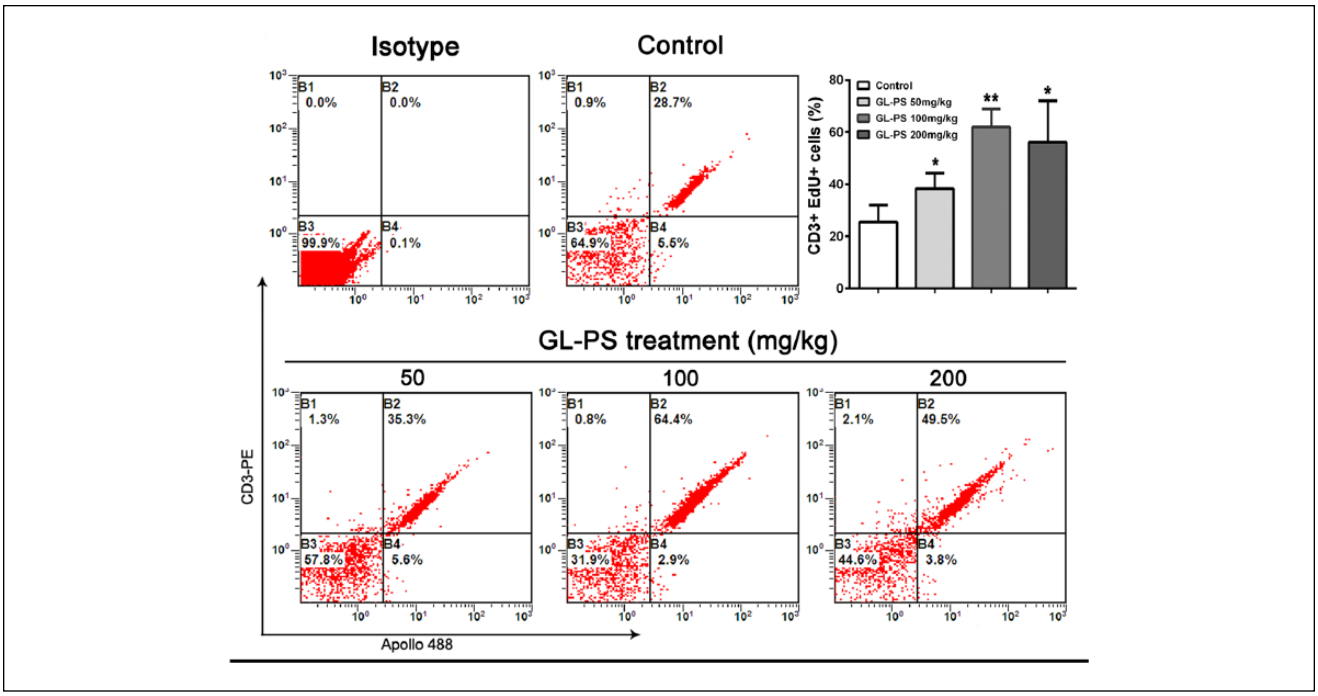

Figure 4. Cell proliferation detected by EdU incorporation (n = 4). Twenty-one days after tumor implantation, rats were injected intraperitoneally with 5 mg/kg of EdU and spleen cells were separated 48 hours after injection. After staining by Apollo 488 azide and CD3-PE antigen, the cells were washed twice with phosphate-buffered saline and 0.05% saponin before flow cytometry analysis. The numbers in the upper right quadrant indicate the percentages of EdU+ cells among CD3+ lymphocytes (***P* < .01, **P* < .05).

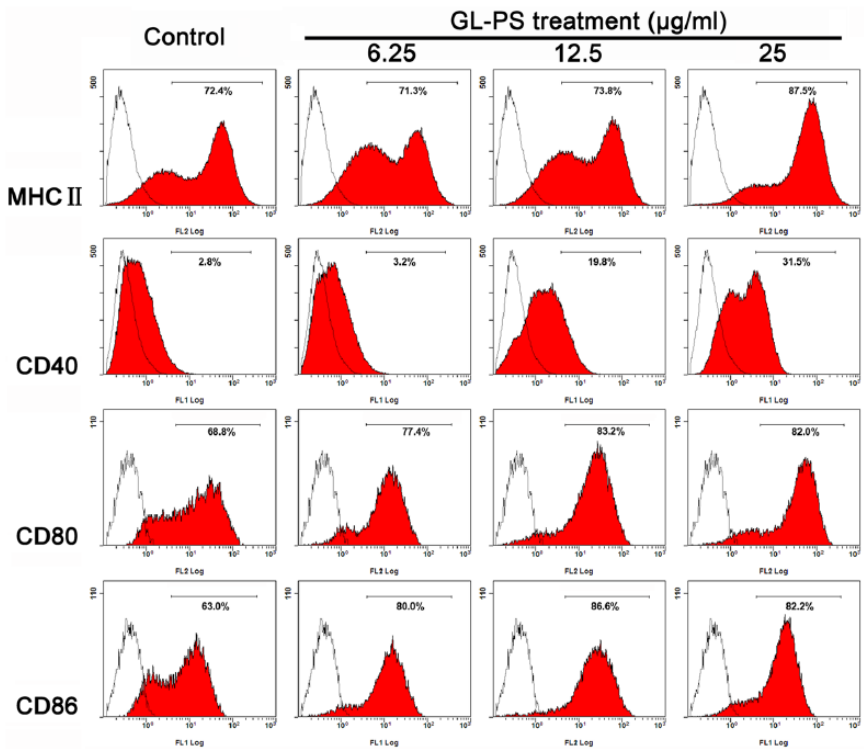

Figure 5. Expression of the key DC surface markers MHC-II, CD40, CD80, and CD86. After treatment with GL-PS for 24 hours, the mature DCs' upregulation of MHC-II and CD40 were dose-dependent and expressed higher levels of CD80 and CD86 versus that observed in the control group, especially in the GL-PS 12.5 μg/mL group. Tests were performed in triplicate.

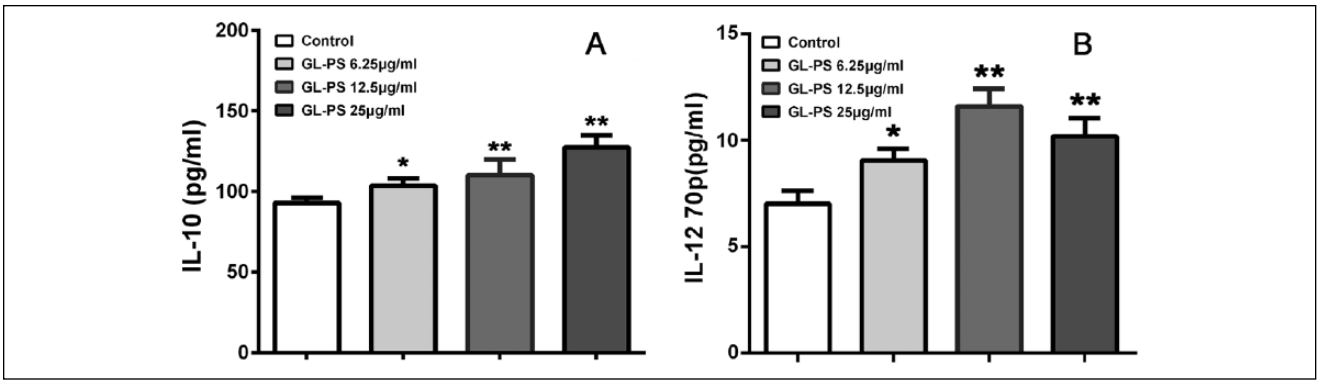

Figure 6. The production of IL-10 and IL-12p70 by the DCs after treatment with GL-PS. On GL-PS treatment, DCs secreted higher levels of IL-10 (A) and IL-12p70 (B) versus that observed in the control group. $^{**}P < .01$, $^{*}P < .05$, with correction for multiple comparisons. Tests were performed in triplicate.

dose. The immune level can be normalized by polysaccharides during the autoimmunity process and not in a dose-dependent manner.[46] Normalization in these cases was achieved by integral harmonic function in which the network of immune-neuroendocrine interactions was the priority.[47]

Conclusion

In summary, we found that GL-PS could increase the concentration of serum IL-2, TNF-α, and INF-γ and enhance the cytotoxic activity of NK cells and T cells in glioma-bearing rats. Moreover, GL-PS was able to promote the functional maturation of DCs, and have a role in inhibiting tumor growth and prolong the survival time of glioma-bearing rats. Our findings thus suggested that GL-PS may be a potential part of the treatment regimen in immunotherapy for glioma.

Acknowledgments

GL-PS was kindly provided by Professor Shuqian Lin at the Fuzhou Institute of Green Valley Bio-Pharm Technology, Fujian, China.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by China National Engineering Research Center of Juncao Technology (Grant No. JCJJ14008) and the Medical Innovation Project of Fujian Province (Grant No. 2016-CX-18).

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