

Lipid Extract from Completely Sporoderm-broken Germinating *Ganoderma sinensis* Spores Elicits Potent Antitumor Immune Responses in Human Macrophages

Jing-Ping Zhang¹, Limin Zheng¹, Jiang-Hai Wang², Karl-Eric Magnusson³ and Xin Liu^{2*}

¹State Key Laboratory of Biocontrol, College of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, P. R. China

²Food Engineering Research Center of State Ministry of Education, Sun Yat-Sen University, Guangzhou 510275, P. R. China

³Division of Medical Microbiology, Faculty of Health Sciences, Linköping University, SE-581 85 Linköping, Sweden

Ganoderma sinensis has been used widely in Oriental countries for the prevention and treatment of various diseases including cancer. Previous studies have shown that the lipid extract from *Ganoderma* exhibits direct cytotoxicity against tumor cells. Here, it is reported that the lipid extract from germinating *G. sinensis* spores, at lower concentrations that have no direct tumoricidal activity, induce potent antitumor immune responses in human monocytes/macrophages. Upon stimulation with the lipid extract, monocytes/macrophages exhibited markedly increased production of proinflammatory cytokines and surface expression of costimulatory molecules. Conditioned medium from stimulated cells effectively suppressed the growth of tumor cells. Apparently, the lipid extract triggered macrophage activation via a mechanism different from that associated with LPS. Moreover, it was observed that the lipid extract could partially re-establish the antitumor activity of the immunosuppressive tumor-associated macrophages. These results indicated that in addition to its direct tumoricidal activity, the lipid extract from *G. sinensis* spores could exert antitumor activity by stimulating the activation of human monocytes/macrophages. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: antitumor immune response; human monocytes/macrophages; lipid extract; germination; *Ganoderma sinensis* spore.

INTRODUCTION

There is growing public interest in the use of complementary and alternative medicines for cancer prevention and treatment (Fugh-Berman, 2000; Cohen *et al.*, 2002). The medicinal mushroom *Ganoderma sinensis* has been used widely and praised in China and other Oriental countries, as herbal medicines to treat diverse ailments and chronic diseases (Lin, 2001). *Ganoderma* fruiting body has been utilized as traditional medicine for several thousand years, but its spore was consumed as health foods and herbal medicines only in the late 20th century (Liu *et al.*, 2002; Chan *et al.*, 2005). The evidence from both experimental and clinical studies indicates that *Ganoderma* and its spore extracts can enhance the activities of immune cells and inhibit the growth of tumors. Among the numerous bioactive com-

ponents identified from *G. sinensis*, polysaccharide and triterpenoid-enriched lipid are two major categories of active constituents (Lin, 2001).

The active constituents responsible for the antitumor and immunomodulating activities have been qualitatively described. In general, the polysaccharides from *Ganoderma* have potent immunomodulating effects by increasing the activities of lymphocytes, neutrophils and macrophages (M ϕ), and thus elicit antitumor immune responses (Wang *et al.*, 1997; Lin *et al.*, 2005; Hsu *et al.*, 2004). In contrast, the lipid extract could exert direct cytotoxicity against tumor cells by inducing cell cycle arrest and apoptosis, and has a stronger growth inhibition effect than the water extract (Wu *et al.*, 2001; Tang *et al.*, 2006). At present, no information is available about the immunomodulating effects of the lipid extract from *Ganoderma* spores on human immune cells.

Macrophages are essential components of host defense against tumors and infection, and act as both antigen presenting cells (APC) and effector cells. They are prominent in virtually all types of solid tumors. Macrophages in normal tissues exhibit spontaneous antitumor activity, whereas tumor-associated macrophages (TAM) exhibit a distinct phenotype that suppress antitumor immunity and promote tumor progression (Lewis and Pollard, 2006; Cheng *et al.*, 2007). Those findings agree with clinical studies showing that a high density of TAM is associated with a poor prognosis in most solid tumors (Budhu *et al.*, 2006). Although the precise underlying mechanisms are not yet clear, it is generally assumed that tumor-derived factors 'educate' the newly recruited

* Correspondence to: Professor X. Liu, Food Engineering Research Center of State Ministry of Education, Sun Yat-Sen University, Guangzhou 510275, P. R. China.

E-mail: lsslx@mail.sysu.edu.cn

Contract/grant sponsor: Scientific and Technological program of Guangdong Province; contract/grant number: 2004B30101008.

Contract/grant sponsor: Ministry of Education of China (20040558016), Asia-Swedish Research Partnership Programme; contract/grant number: VR-SIDA, 2004-4893.

Contract/grant sponsor: Sun Yat-Sen University; contract/grant number: 2004-33000-1132091.

monocytes to take on an immunosuppressive phenotype and perform a protumor role (Kuang *et al.*, 2007). Interestingly, their antitumor activity can be re-established by local delivery of IL-12, and in that case a high density of TAM is correlated with a marked reduction in tumor growth (Watkins *et al.*, 2007). Such opposing effects of macrophages on tumor progression indicate that selective modulation of macrophage activity may serve as a novel strategy for cancer therapy.

In the present study, human monocytes/macrophages were used as a model system to investigate if and how the lipid extract from *G. sinensis* spores could modulate the antitumor immune responses. These results showed that the lipid extract, at lower concentrations that had no direct cytotoxicity against tumor cells, could elicit potent activation of human macrophages via a mechanism distinct from LPS stimulation. Moreover, evidence was provided that the lipid extract could partially restore the antitumor activity of the immunosuppressive TAM.

MATERIALS AND METHODS

Reagents. The antibodies and chemicals used and their sources were as follows: ELISA kits for human TNF- α , IL-10, IL-6 and IL-1 β , phycoerythrin (PE)-conjugated antibodies for human CD86 and HLA-DR, from eBioscience (San Diego, CA); cell isolation and tissue culture reagents, from Invitrogen (Grand Island, NY); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] stock, from Duchefa (Haarlem, The Netherlands). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated in the text.

Sample preparation and determination of total triterpenoid content. The high-quality fruiting bodies and spores of *G. sinensis* were collected from the log-cultivated *Ganoderma* base in a 1000-m high-alpine forested area in Fujian Province, southeastern China, which was established by the Food Engineering Research Center of the State Ministry of Education, Sun Yat-Sen University. The dried powder of *G. sinensis* fruiting bodies was incubated in hot water for 2 h. The solution was centrifuged, and the supernatant was concentrated and dried to powder. This hot water extract was GL1. The lipid was extracted from the completely sporoderm-broken germinating *G. sinensis* spores by supercritical CO₂ fluid extraction as described previously (Hsu *et al.*, 2001; Liu *et al.*, 2005, 2007). This lipid extract was GL6. Dressings were prepared by dissolving phosphatidylcholine (PC) (Central Soya Co., St. Louis, MO) in distilled water, and the solution was pre-emulsified in a blender for 1 min. Then GL1 and GL6 were emulsified in a Microfluidizer M-120E (Microfluidics Int. Corp, Newton, MA) at 1200 kPa and 50–55 °C (Christiansen *et al.*, 2004). The particle dimensions of two samples were in the range of 100–200 nm after emulsification. The yield of the GL1 and GL6 was 7.7% (w/w). GL6 mainly consists of more than 85% triglyceride, circa 2% ergosterol and 10% triterpenoid (Wang *et al.*, 2004; Yuan *et al.*, 2006; Liu *et al.*, 2007). The triglyceride in GL6 contains 18 fatty acids with the majority of C16:0, C16:1, C18:0, C18:1 and C18:2 (Liu *et al.*, 2007), and ergosterol

includes free ergosterol and ergosterol esters (Yuan *et al.*, 2006). Total triterpenoid content in GL6 was determined by measuring the absorbance at 548.1 nm using thin-layer chromatography-spectrophotometry and with ursolic acid as the standard (Wang *et al.*, 2004). The result showed that the total triterpenoid content was 10.28%.

Tumor cell lines and preparation of tumor culture supernatant (TSN). Human myeloid leukemia (U937) and hepatoma (HepG2) cell lines were obtained from the American Type Culture Collection. All cells were tested for mycoplasma contamination using single-step PCR method (Uphoff and Drexler, 2002), and grown in complete medium composed of RPMI 1640 (or DMEM) supplemented with 10% FCS. TSN was prepared by plating 5×10^6 tumor cells in 10 mL of complete medium in 100-mm dishes for 24 h, and thereafter changing the medium to complete DMEM medium supplemented with 10% human AB serum instead of FCS (Kuang *et al.*, 2007). After 2–3 days, the supernatant was harvested, centrifuged and stored in aliquots at –80 °C.

Generation of human monocytes, M ϕ and tumor-associated M ϕ (TAM). Human monocytes and macrophages were prepared from peripheral blood mononuclear cells (PBMC) as described previously (Zheng *et al.*, 2004). The cells in DMEM alone were plated at 4×10^6 /well in 24-well plates for 1.5 h, washed and then cultured in DMEM containing 10% human AB serum for 16 h to remove residual lymphocytes. Afterward, the monocytes in DMEM containing AB serum were cultured in the presence of 20% TSN or medium alone for 6–8 days to obtain TAM or macrophages, respectively (Kuang *et al.*, 2007).

Treatment of human monocytes/M ϕ with GL6 or LPS. Human monocytes/macrophages were incubated in the medium alone, GL6 or LPS for the indicated time periods. Where indicated, the cells were preincubated with 10 μ g/mL polymyxin B for 30 min before stimulation. The culture supernatants (conditioned medium, CM) were centrifuged to remove particulate debris and stored in aliquots at –80 °C. The cells were collected for flow cytometric analysis of CD86 and HLA-DR surface expression.

Flow cytometric analysis. Cells were stained with PE-conjugated antibodies against CD86 or HLA-DR and then analysed by flow cytometry (FACS VantageSE, BD Immunocytometry Systems, Mountain View, CA) using CellQuest software.

Apoptosis of tumor cells was quantified by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated annexin V (R&D Systems, Abingdon, UK) according to the instructions provided by the manufacturer. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with annexin V and PI before analysis. The binding of annexin V-FITC and PI to the cells was measured by flow cytometry as described (Zheng *et al.*, 2004).

ELISA. Cytokine concentrations were determined by ELISA kits according to the instructions provided by the manufacturer.

Cell proliferation assay. Tumor cells (U937 or HepG2 cells) were seeded at 2×10^4 cells/well in 96-well plates, and then cultured with conditioned medium, GLs, or medium alone for the indicated times. The growth inhibition on tumor cells was determined by MTT assay. The inhibitory rate was calculated according to the following formula: inhibition (%) = $(1 - \text{absorbance of experimental group} / \text{absorbance of control group}) \times 100\%$.

Statistical analysis. The data were expressed as mean \pm SEM. Statistical significance was determined by Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Lipid extract (GL6) from *G. sinensis* spores directly suppressed the proliferation of tumor cells

In initial experiments, leukemic U937 and hepatoma HepG2 cells were used to examine the growth inhibitory activities of the lipid extract (GL6) from *G. sinensis* spores and the hot-water extract (GL1) from fruiting bodies of *G. sinensis*. Cells were exposed to incremental concentrations (0–3 mg/mL) of GL6 and GL1 for 24 h and the growth of cells was determined by MTT assay. The results showed that GL6 effectively inhibited the proliferation of tumor cells in a dose- and time-dependent manner (Fig. 1). The growth inhibitory effects of GL6 were similar for both cell lines, reaching over 80% inhibition at a concentration of 0.3 mg/mL (Fig. 1A). Flow cytometry using FITC-conjugated annexin V revealed that exposure to GL6 triggered rapid apoptosis of U937 cells, which was positively correlated with exposure time (Fig. 1B). Morphological examination of GL6-treated U937 or HepG2 cells showed typical

apoptotic changes in these cells, including decreased cell volume and chromatin condensation (data not shown).

Low concentrations of GL6 elicited potent antitumor immune responses in human monocytes

The antitumor effect of polysaccharides could be mediated by cytokines released from activated immune cells (Wang *et al.*, 1997). To find out whether the same is true for the lipid extract from *G. sinensis* spores, the effect of GL6 on the activation of human monocytes was studied. The results showed that exposure of monocytes to GL6 elicited a dose-dependent cell activation that included a markedly increased production of IL-1 β , IL-6 and TNF- α as well as upregulated surface expression of CD86 and HLA-DR. A relatively low concentration (10–30 $\mu\text{g/mL}$) of GL6 was sufficient to trigger the cell activation (Fig. 2A, 2B). Similarly, exposure of macrophages to GL6 also elicited a dose- and time-dependent cell activation. It was rapid, with the production of TNF- α by macrophages reaching a maximum within 8 h (data not shown).

The cytokines released by stimulated monocytes, such as TNF- α , may possess a cytotoxic effect against tumor cells (Urban *et al.*, 1986; Li *et al.*, 2005). To test this possibility, human monocytes were stimulated with various concentrations of GL6 for 3 days and the culture supernatants (conditioned medium, CM) were collected. As shown in Fig. 2C, GL6-mo-CM from GL6-stimulated monocytes effectively inhibited the proliferation of U937 cells in a dose-dependent fashion. In contrast, GL6 alone (100 $\mu\text{g/mL}$) or mo-CM from unstimulated monocytes had no suppressive effects on the growth of U937 cells. These results indicated that the lipid extract from germinating *G. sinensis* spores, at lower concentrations that had no direct cytotoxic effects on tumor cells, could induce potent antitumor immune responses in human monocytes.

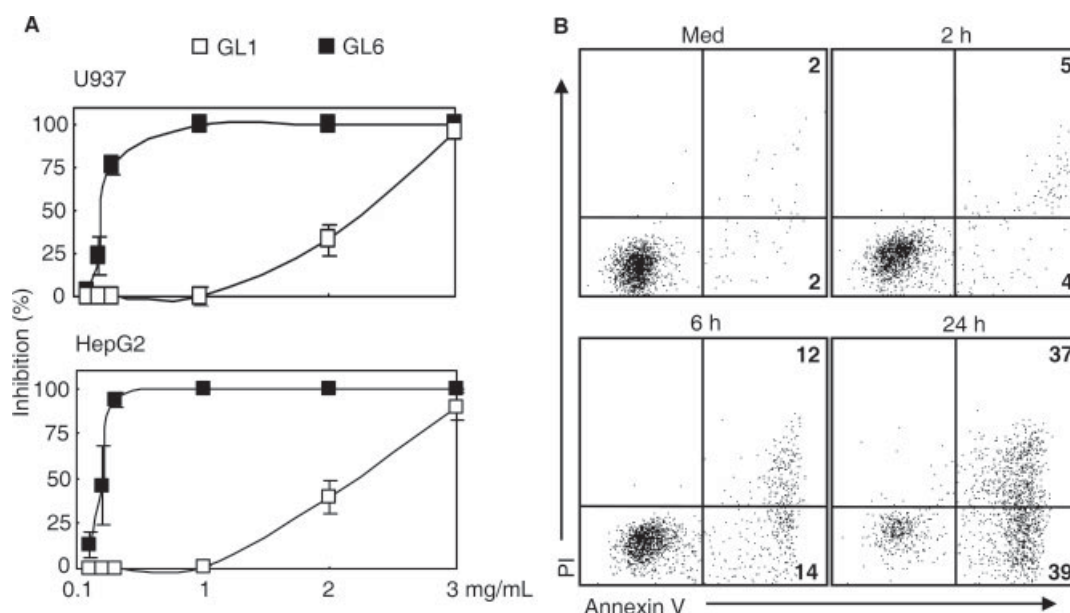


Figure 1. Effects of GL1 and GL6 on the proliferation (A) and apoptosis (B) of tumor cells. (A) U937 or HepG2 cells were cultured with GL1 or GL6 for 24 h. Cell viability was determined by MTT assay. The data represent the mean \pm SEM of six experiments. (B) U937 cells were cultured in the medium alone (Med) or with 0.3 mg/mL GL6 for 1 ~ 24 h. Apoptosis was quantified by flow cytometric analysis of binding of annexin V-FITC and PI to cells. The relative distribution of cells manifesting early (annexin⁺PI⁻) and late (annexin⁺PI⁺) apoptosis is illustrated (percentage); for clarity, the fluorescence profiles of 25% of the analysed events are shown.

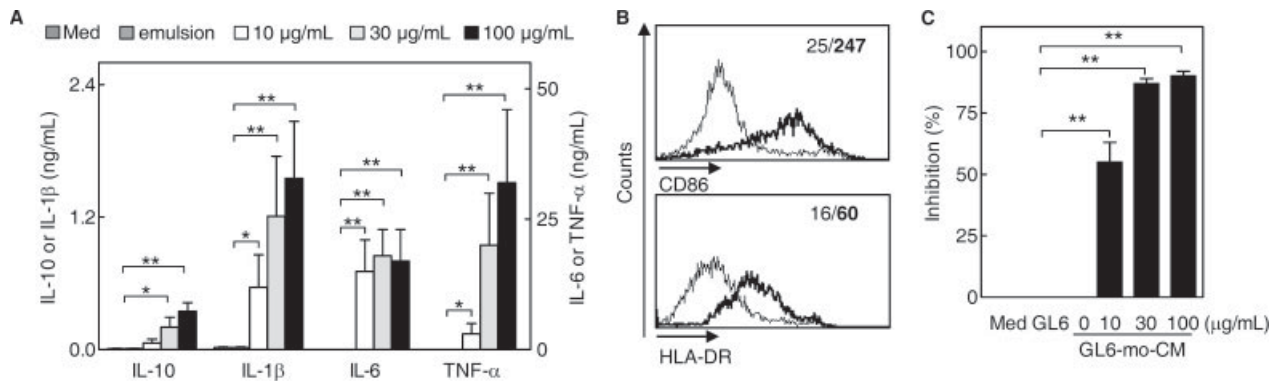


Figure 2. Lower doses of GL6 elicited antitumor immune responses in human monocytes. (A and B) Monocytes were cultured with medium alone, emulsion or GL6 for 24 h. Production of cytokines and expression of surface markers were determined by ELISA and flow cytometry, respectively. The histograms in B are representative of three experiments, and the mean fluorescence intensities (MFI) for GL6-treated cells are shown in boldface. (C) U937 cells were cultured for 3 days in medium alone, 100 μg/mL GL6 or with 50% conditioned medium from various concentrations of GL6-stimulated monocytes (GL6-mo-CM). Cell viability was determined by MTT assay. The data represent the mean ± SEM of four experiments. * $p < 0.05$, ** $p < 0.01$.

GL6 was different from LPS in triggering macrophage activation

To rule out the possible LPS contamination in the preparation of GL6, a series of experiments was conducted, which showed different results between GL6 and LPS regarding their effects on macrophage activation. As shown in Fig. 3A, GL6 stimulated TNF-α production was inhibited by approximately 40% by the addition of polymyxin B, whereas in parallel experiments the activity of LPS was completely blocked by polymyxin

B, implying different signaling pathways responsible for GL6 and LPS.

It is known that LPS can induce rapid tolerance in macrophages, or the down-regulation of endotoxin-driven cell responses following a first exposure to LPS (Medvedev *et al.*, 2000). Therefore, macrophages were incubated with GL6 or LPS for 24 h, washed and re-stimulated with GL6 or LPS for another 24 h. Pre-exposure to LPS rendered macrophages refractory to subsequent LPS stimulation. In contrast, macrophages that had been pretreated with GL6 not only retained

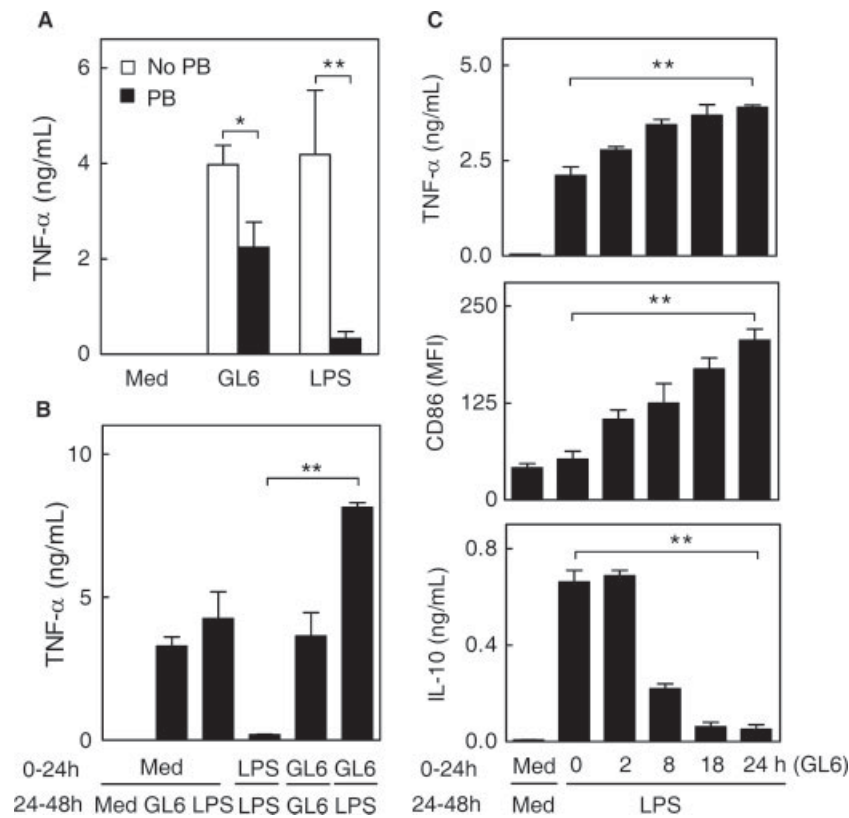


Figure 3. GL6 was different from LPS in triggering macrophage (Mφ) activation. (A) Mφ were preincubated with or without 10 μg/mL polymyxin B (PB) for 30 min, and then stimulated with 100 μg/mL GL6 or 10 ng/mL LPS for 24 h. TNF-α concentration was measured by ELISA. (B and C) Mφ were pre-exposed to 30 μg/mL GL6, 10 ng/mL LPS or medium alone for 24 h (B) or the indicated periods of time (C). Thereafter, the cells were washed twice and re-stimulated with GL6 or LPS for 24 h. Expression of CD86 and production of TNF-α and IL-10 were measured by flow cytometry and ELISA, respectively. The data represent the mean ± SEM of four experiments. * $p < 0.05$, ** $p < 0.01$.

their capacity to respond to the secondary GL6 stimulation, but also exhibited an augmented response to the subsequent LPS challenge (Fig. 3B).

Kinetic experiments revealed that pretreatment with GL6 for 8 h was sufficient to enhance the LPS-stimulated macrophage activation, characterized by the increased TNF- α production and CD86 expression with reduced IL-10 secretion (Fig. 3C). These results clearly indicated that GL6 triggered macrophage activation via a mechanism different from that associated with LPS.

GL6 could partially restore TNF- α production by tumor-associated macrophages

Macrophages in most solid tumors exhibit a distinct phenotype that suppress antitumor immunity and promote tumor progression (Lewis and Pollard, 2006; Cheng *et al.*, 2007; Budhu *et al.*, 2006; Biswas *et al.*, 2006; Kuang *et al.*, 2007). It was shown recently that exposure to the culture supernatants from solid tumors redirected monocytes to develop into immunosuppressive macrophages with key functional properties of TAM (Kuang *et al.*, 2007). Upon stimulation with LPS, these macrophages produced significant amounts of IL-10, but not TNF- α . However, exposure of these immunosuppressive macrophages to GL6 could partially restore their capacity to produce TNF- α . Moreover, GL6 synergistically increased the LPS-stimulated release of TNF- α coincided with reduced production of IL-10 from both normal and immunosuppressive macrophages (Fig. 4).

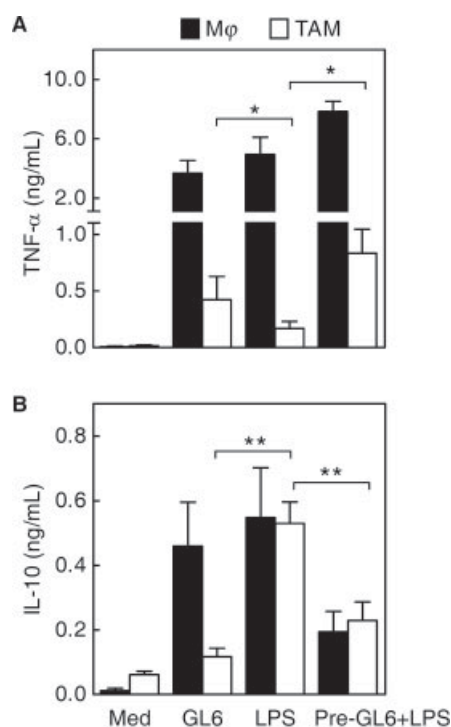


Figure 4. GL6 could partially restore the capacity of tumor-associated macrophages (TAM) to produce TNF- α . Human monocytes were cultured in medium alone or with 20% culture supernatant from HepG2 for 6 days to obtain M ϕ or TAM. Thereafter, the cells were left untreated or exposed to 30 μ g/mL GL6 for 24 h, washed and stimulated with 10 ng/mL LPS or 30 μ g/mL GL6 for additional 24 h. Production of TNF- α (A) and IL-10 (B) were assayed by ELISA. Values represent the mean \pm SEM of six separate experiments. * p < 0.05, ** p < 0.01.

DISCUSSION

There appears to be exceptional and growing public enthusiasm for herbal medicines, especially amongst patients with cancer and chronic infections. This has recently begun to be matched by increasing scientific attention (Fugh-Berman, 2000; Cohen *et al.*, 2002). The present study showed that in addition to the direct cytotoxic effect on tumor cells, the lipid extract from germinating *G. sinensis* spores induced potent antitumor immune responses in human monocytes and macrophages. Apparently, this lipid extract activated macrophages via a mechanism different from that associated with LPS. Moreover, evidence is provided that the lipid extract could partially restore the antitumor activity of the immunosuppressive TAM.

Although previous studies in animal models have shown that the lipid constituents of *G. lucidum* are able to suppress tumor growth *in vivo* (Liu *et al.*, 2002), the underlying mechanisms have not been elucidated clearly. It has been suggested that the antitumor effect of the lipids might be mediated by its direct cytotoxicity against tumor cells. In agreement with previous reports (Wu *et al.*, 2001; Tang *et al.*, 2006), it is noted that the lipid extract can inhibit the proliferation and trigger the apoptosis of tumor cells. However, the lipid extract, at relatively lower concentrations that have no direct tumoricidal activity, stimulates human monocytes to release significant amounts of pro-inflammatory cytokines, including TNF- α , IL-10 and IL-6, and up-regulate their surface expression of molecules associated with antigen presentation (CD86 and HLA-DR). In addition, the conditioned medium from the lipid-stimulated monocytes effectively suppress the tumor cell growth. Therefore, the lipid extract can exert their antitumor activity by regulating the activation of human monocytes.

The macrophage-stimulating effect of the lipid extract is not due to LPS contamination. This conclusion is based on the following observations: (1) Treatment with polymyxin B completely abolished the LPS-mediated macrophage activation, but had less pronouncedly inhibitory effect on the GL6-stimulated cell responses. (2) Pre-exposure of macrophages to LPS induced rapid tolerance to subsequent LPS stimulation. In contrast, macrophages that had been pretreated with GL6 retained their capacity to respond to the secondary GL6 stimulation. (3) Pre-exposure to GL6 could significantly attenuate the IL-10 production but enhance TNF- α production and CD86 expression in LPS-stimulated macrophages. These results clearly indicate that the lipid extract from *G. sinensis* spores is different from LPS in triggering macrophage activation.

Unlike the macrophages in normal or inflamed tissues that exhibit the spontaneous antitumor activity, TAM display a distinct immunosuppressive phenotype with poor antigen presenting capacity (Lewis and Pollard, 2006; Cheng *et al.*, 2007; Budhu *et al.*, 2006; Biswas *et al.*, 2006; Kuang *et al.*, 2007). It was shown recently that exposure to tumor culture supernatants compel monocytes to develop into immunosuppressive macrophages. These macrophages released significant amounts of IL-10, but not TNF- α and IL-12, and became refractory to further LPS or IFN- γ stimulation (Kuang *et al.*, 2007). By using this model, it was found that the

lipid extract could partially restore the capacity of macrophages to produce the pro-inflammatory cytokine TNF- α . Interestingly, pretreatment with the lipid extract attenuated the release of IL-10 in both normal macrophages and TAM (Fig. 4). IL-10 is a strict suppressor of various stages of the immune responses and represents one of the most potent immunosuppressive cytokines present at the tumor site (Sica *et al.*, 2000; Sakamoto *et al.*, 2006; Katakura *et al.*, 2004). Through its effects on antigen-presenting cells, IL-10 can block the development of a Th1 response, which is thought to be the most effective for the eradication of malignant cells, thereby favoring the development of a Th2 response (Moore *et al.*, 2001). Therefore, the reduced IL-10 production by macrophages that had been pre-exposed to the lipid extract might help to subvert the 'tolerizing' conditions and to restore the antitumor response in patients.

Macrophages constitute a major component of the leukocyte infiltrate in virtually all types of tumors and serve as a marker of poor prognosis in most cases (Lewis and Pollard, 2006; Cheng *et al.*, 2007; Budhu *et al.*, 2006; Biswas *et al.*, 2006; Kuang *et al.*, 2007). However, recent studies have shown that selective modulation of macro-

phage phenotype, e.g. by delivery of IL-12, can re-establish the antitumor activity of macrophages both *in vitro* and *in vivo* (Watkins *et al.*, 2007). In that case, a high density of TAM is correlated with a marked reduction in tumor growth studies. Such opposing effects of macrophages on tumor progression indicate that macrophages might serve as a novel target for cancer therapy. The present study showed that the lipid extract from *G. sinensis* spores could exert antitumor activities by their direct tumoricidal effects (at higher doses) or indirectly via activating monocytes/macrophages (at relatively lower concentrations). The lipid extract is an effective stimulator for both normal macrophages and immunosuppressive TAM, and therefore may have the potential to serve as a novel anticancer agent.

Acknowledgements

This work is supported by the Outstanding Young Scientist Fund from the NSFC (30425025), Scientific and Technological program of Guangdong Province (2004B30101008), Ministry of Education of China (20040558016), Asia-Swedish Research Partnership Programme (VR-SIDA, 2004-4893) and the fund from the Sun Yat-Sen University (2004-33000-1132091).

REFERENCES

- Biswas SK, Gangi L, Paul S *et al.* 2006. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood* **107**: 2112–2122.
- Budhu A, Forgues M, Ye QH *et al.* 2006. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell* **10**: 99–111.
- Chan WK, Lam DT, Law HK *et al.* 2005. *Ganoderma lucidum* mycelium and spore extracts as natural adjuvants for immunotherapy. *J Altern Complement Med* **11**: 1047–1057.
- Cheng J, Huo DH, Kuang DM, Yang J, Zheng L, Zhuang SM. 2007. Human macrophages promote the motility and invasiveness of osteopontin-knockdown tumor cells. *Cancer Res* **67**: 5141–5147.
- Christiansen KF, Vegarud G, Langsrud T, Ellekjaer MR, Egeland B. 2004. Hydrolyzed whey proteins as emulsifiers and stabilizers in high-pressure processed dressings. *Food Hydrocolloids* **18**: 757–767.
- Cohen I, Tagliaferri M, Tripathy D. 2002. Traditional Chinese medicine in the treatment of breast cancer. *Semin Oncol* **29**: 563–574.
- Fugh-Berman A. 2000. Herb–drug interactions. *Lancet* **355**: 134–138.
- Hsu HY, Hua KF, Lin CC, Lin CH, Hsu J, Wong CH. 2004. Extract of Reishi polysaccharides induces cytokine expression via TLR4-modulated protein kinase signaling pathways. *J Immunol* **173**: 5989–5999.
- Hsu RC, Lin BH, Chen CW. 2001. The study of supercritical carbon dioxide extraction for *Ganoderma lucidum*. *Ind Eng Chem Res* **40**: 4478–4481.
- Katakura T, Miyazaki M, Kobayashi M, Herndon DN, Suzuki F. 2004. CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. *J Immunol* **172**: 1407–1413.
- Kuang DM, Wu Y, Chen N, Cheng J, Zhuang SM, Zheng L. 2007. Tumor-derived hyaluronan induces formation of immunosuppressive macrophages through transient early activation of monocytes. *Blood* **110**: 587–595.
- Lewis CE, Pollard JW. 2006. Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66**: 605–612.
- Li CY, Huang Q, Kung HF. 2005. Cytokine and immuno-gene therapy for solid tumors. *Cell Mol Immunol* **2**: 81–91.
- Lin ZB. 2001. *Modern Research of Ganoderma*, 2nd edn. Beijing Medical University Press: Beijing.
- Lin YL, Liang YC, Lee SS, Chiang BL. 2005. Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathways. *J Leukocyte Biol* **78**: 533–543.
- Liu X, Wang JH, Yuan JP. 2005. Pharmacological and anti-tumor activities of ganoderma spores processed by top-down approaches. *J Nanosci Nanotech* **5**: 2001–2013.
- Liu X, Xu SP, Wang JH *et al.* 2007. Characterization of ganoderma spore lipid by stable carbon isotope analysis: implications for authentication. *Anal Bioanal Chem* **388**: 723–731.
- Liu X, Yuan JP, Chung CK, Chen XJ. 2002. Antitumor activity of the sporoderm-broken germinating spores of *Ganoderma lucidum*. *Cancer Lett* **182**: 155–161.
- Medvedev AE, Kopydlowski KM, Vogel SN. 2000. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *J Immunol* **164**: 5564–5574.
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**: 683–765.
- Sakamoto T, Saito H, Tatebe S *et al.* 2006. Interleukin-10 expression significantly correlates with minor CD8+ T-cell infiltration and high microvessel density in patients with gastric cancer. *Int J Cancer* **118**: 1909–1914.
- Sica A, Saccani A, Bottazzi B *et al.* 2000. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *J Immunol* **164**: 762–767.
- Tang W, Liu JW, Zhao WM, Wei DZ, Zhong JJ. 2006. Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. *Life Sci* **80**: 205–211.
- Uphoff CC, Drexler HG. 2002. Detection of mycoplasma in leukemia-lymphoma cell lines using polymerase chain reaction. *Leukemia* **16**: 289–293.
- Urban JL, Shepard HM, Rothstein JL, Sugarman BJ, Schreiber H. 1986. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc Natl Acad Sci USA* **83**: 5233–5237.

- Wang SY, Hsu ML, Hsu HC *et al.* 1997. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *Int J Cancer* **70**: 699–705.
- Wang JH, Yuan JP, Xu SP, Liu X. 2004. Determination of triterpenoids in ganoderma spore lipids by the TLC-spectrophotometry method. *J Chin Inst Food Sci Technol* **4**: 76–79.
- Watkins SK, Egilmez NK, Suttles J, Stout RD. 2007. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages *in vitro* and *in vivo*. *J Immunol* **178**: 1357–1362.
- Wu TS, Shi LS, Kuo SC. 2001. Cytotoxicity of *Ganoderma lucidum* triterpenes. *J Nat Prod* **64**: 1121–1122.
- Yuan JP, Wang JH, Liu X, Kuang HC, Huang XN. 2006. Determination of ergosterol in ganoderma spore lipid from the germinating spores of *Ganoderma lucidum* by high-performance liquid chromatography. *J Agric Food Chem* **54**: 6172–6176.
- Zheng L, He M, Long M, Blomgran R, Stendahl O. 2004. Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages. *J Immunol* **173**: 6319–6326.