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Research Paper

Ganoderma lucidum polysaccharides antagonize the suppression on lymphocytes induced by culture supernatants of B16F10 melanoma cells

Li-Xin Sun[a,b], Zhi-Bin Lin[a], Xin-Suo Duan[b], Jie Lu[b], Zhi-Hua Ge[b], Xue-Jun Li[a], Min Li[a], En-Hong Xing[b], Jing Jia[b], Tian-Fei Lan[b] and Wei-Dong Li[a]

[a]Department of Pharmacology, Peking University Health Science Center, School of Basic Medical Sciences, Beijing and [b]Affiliated Hospital of Chengde Medical College, Chengde, Hebei Province, China

Abstract

Objectives Tumour cells produce factors such as interleukin 10 (IL-10), transforming growth factor β1 (TGF-β1) and vascular endothelial growth factor (VEGF) that suppress the function of immune cells or induce apoptosis of immune cells. One of the most important goals of tumour immunotherapy is to antagonize this suppression on immune cells. *Ganoderma lucidum* polysaccharides (*Gl*-PS) may have this potential. The purpose of this study was to determine the antagonistic effects of *Gl*-PS on the suppression induced by B16F10 melanoma cell culture supernatant (B16F10-CS) on lymphocytes.

Methods *Gl*-PS was used on lymphocytes incubated with B16F10-CS. Enzyme-linked immunosorbent assay was used to determine the levels of IL-10, TGF-β1 and VEGF in B16F10-CS. The MTT assay was used to determine the proliferation of lymphocytes. Immunocytochemistry and Western blot assay were used to determine perforin and granzyme B production in lymphocytes.

Key findings There were elevated levels of IL-10, TGF-β1 and VEGF in B16F10-CS. The lymphocyte proliferation, and perforin and granzyme B production in lymphocytes after induction with phytohemagglutinin, as well as lymphocyte proliferation in the mixed lymphocyte reaction, were suppressed by B16F10-CS. This suppression was fully or partially antagonized by *Gl*-PS.

Conclusions B16F10-CS suppressed lymphocyte proliferation and perforin and granzyme B production in lymphocytes after induction with phytohemagglutinin, as well as lymphocyte proliferation in the mixed lymphocyte reaction. This suppression may be associated with elevated levels of immunosuppressive IL-10, TGF-β1 and VEGF in B16F10-CS. *Gl*-PS had antagonistic effects on the immunosuppression induced by B16F10-CS, suggesting the potential for *Gl*-PS in cancer immunotherapy.

Keywords *Ganoderma lucidum* polysaccharides; granzyme B; lymphocyte; perforin; tumour

Introduction

A healthy immune system is necessary to control malignant disease. Various attempts to perform specific and nonspecific immunotherapy for human cancer in clinical trials have shown little or no success.[1] The reason is in part due the immune suppression directly or indirectly induced by tumour cells. Tumours have developed multiple strategies to evade the host immune system. They produce and release factors to suppress the function of immune cells or induce apoptosis of immune cells.[2] B16F10 is a melanoma cell line derived from the C57BL mouse strain. The immune suppressing factors produced and released by tumours are also found in B16F10 cells. Therefore, B16F10 cell culture supernatant (B16F10-CS), the conditioned medium, may induce suppression on lymphocytes as well. One of the most important goals of tumour immunotherapy is to antagonize the suppression by immune suppressing factors on immune cell function. *Ganoderma lucidum* polysaccharides (*Gl*-PS) may be one candidate to achieve this goal.

Gl-PS is one of the main effective components isolated from *Ganoderma lucidum* (Fr.) Krast (Ganodermataceae), a well-known medicinal mushroom widely used for centuries in

Correspondence: Wei-Dong Li, Department of Pharmacology, Peking University Health Science Center, School of Basic Medical Sciences, Beijing, 100191, China. E-mail: lwdpharma@126.com

China to promote longevity and improve vigour without significant adverse effects.[3,4] As with many natural products with immunomodulatory and antitumour effects,[5–9] *Gl*-PS has been extensively studied in recent decades and demonstrated to have various biological activities, including immunomodulatory and antitumour effects.[10] The immunomodulatory and antitumour effects of *Gl*-PS include: enhancement of cytokine production as well as dendritic cell maturation and function,[11] improvement in cytokine-induced killer cell function,[12] augmentation of cytotoxic T lymphocyte (CTL) function,[13] promotion of B16F10 cells to activate lymphocytes,[14] and inhibition of the growth of vascular endothelial cells and inhibition of the induction of vascular endothelial growth factor (VEGF) in human lung cancer cell.[15] In addition, *Gl*-PS can reverse multidrug resistance (MDR) by down-regulating the expression of MDR-1 and MDR-associated protein 1 (MRP1) in an adriamycin-resistant leukaemia cell line, K562/ADM.[16] *Gl*-PS also showed the effect of enhancement on function of immunological effector cells in immunosuppressed mice induced by cyclophosphamide.[17] Nanoparticles loaded with *Gl*-PS at 6 μg/ml and chitosan/sodium tripolyphosphate (mass ratio 5.5) showed significant cytotoxic effects on tumour cells and growth promotion effects on mouse spleen cells.[18] Treatment with *Gl*-PS alone induced proliferative responses on both THP-1 and U937 cells but only THP-1 transformed into typical dendritic cell morphology when stimulated with *Gl*-PS plus GM-CSF/IL-4, indicating that *Gl*-PS could induce selected monocytic leukaemic cell differentiation into dendritic cells with immunostimulatory function.[4] It was widely believed that the antitumour effects of *Gl*-PS were primarily achieved by boosting host immune function.[10] However, in addition to the effects of boosting host immune function and enhancing the function of immunological effector cells in immunosuppressed mice induced by cyclophosphamide, the antagonistic effect of *Gl*-PS against the suppression induced by immune suppressing factors on lymphocytes has yet to be demonstrated. The present study was designed to detect the antagonistic effects of *Gl*-PS against the suppression induced by B16F10-CS on lymphocytes.

Materials and Methods

Animals and drugs

Inbred male C57BL/6 (H-2^b) mice and BABL/c (H-2^d) mice (6–8 weeks old) were purchased from the Department of Experimental Animals, Health Science Centre, Peking University, Beijing, China. The use of mice was approved by the Ethics Committee of Peking University in biomedicine. As we previously described,[11] *Gl*-PS was isolated from the boiling water extract of the fruit bodies of *Gl* by ethanol precipitation and dialysis, followed by Sevag deproteination. The Sevag deproteination method has been reported previously.[19] In brief, the Sevag method used in this study was as follows: polysaccharides were dissolved in distilled water, mixed with 0.2 volumes of trichlormethane and 0.04 volumes of *n*-butyl alcohol, shaken for 30 min, followed by centrifugation and then the precipitate was removed. This procedure was repeatedly performed and discontinued when precipitate was no longer found at the interface between the water and trichlormethane. The molecular weight of the *Gl*-PS was 584 900, and the ratio of polysaccharides to peptides was 93.61 : 6.49%. The monosaccharides in the polysaccharides were determined by gas chromatography which showed that the polysaccharides were composed of D-rhamnose, D-xylose, D-fructose, D-galactose, D-mannose, D-glucose and uronic acid in a molar ratio of 0.793 : 0.964 : 2.944 : 0.167 : 0.389 : 7.94 : 0.33, with glycosidic linkages of major β-bonding with minor α-bonding. As determined by an Amino Acid Automatic Analyser (Hitachi L8500, Tokyo, Japan), the sixteen amino acid residues contained in the peptides were Asp, Thr, Ser, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Phe, Lys, His, Arg and Pro, with a mass ratio of 9.49 : 5.32 : 4.88 : 8.10 : 4.71 : 4.63 : 1.82 : 3.70 : 7.44 : 0.54 : 2.56 : 3.05 : 2.22 : 1.23 : 3.47 : 1.76. The prepared *Gl*-PS was identified by the wave spectrum. The *Gl*-PS, a water-soluble powder, was dissolved in B16F10-CS, filtered through a 0.22-μm filter and stored at 4°C before use.

Preparation of B16F10-CS

The B16F10-CS was prepared using B16F10 cells. Mouse B16F10 melanoma cells (H-2^b) were grown at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). B16F10 cells in 6-well culture plates (1×10^5 cells/well at the start) were cultured to 80% cell confluence. The RPMI 1640 medium was replaced with fresh medium and incubated for a further 8 h. The supernatants of the cultures (B16F10-CS) were harvested, filtered through a 0.22-μm filter and stored at 4°C. The fibroblast L929 cell culture supernatant (L929-CS) and the culture supernatant of fibroblast prepared from C57BL/6 mouse (FB-C57BL/6-CS) were also prepared as controls for the proliferation assay. The preparation of mouse fibroblast has been previously described.[20]

Detection of interleukin 10, transforming growth factor β1 and VEGF in B16F10-CS

The level of mouse interleukin 10 (IL-10), transforming growth factor β1 (TGF-β1) and VEGF in the B16F10-CS was determined by sandwich enzyme-linked immunosorbent assay (ELISA) kits (Beijing 4A Biotech Co. Ltd, Beijing, China) according to the manufacturer's protocol. In brief, microtiter plates were, respectively, coated with specific antibodies to capture IL-10, TGF-β1 or VEGF in the B16F10-CS. A second layer antibody was then added. Cytokine concentrations were determined with a standard curve derived from known amounts of the relevant cytokine using absorbance readings at 450 nm on a spectrophotometer (Bio-Rad, Hercules, CA, USA). The minimum detection level was 31.250 pg/ml for IL-10, 15.625 pg/ml for TGF-β1 and 15.625 pg/ml for VEGF.

Preparation of splenic mononuclear lymphocytes

The mice were killed after anaesthetizing with 3% sodium pentobarbital and the spleens were harvested and pressed through a 200-gauge stainless steel mesh. The splenocytes were suspended in RPMI 1640 medium and the mononuclear lymphocytes were isolated by a Ficoll-Urografin density gradient followed by two washes with RPMI 1640 medium. The

mononuclear lymphocytes were examined and counted under a light microscope. Few nonviable cells were found by the trypan blue exclusion test. Wright-Giemsa stain showed that the purification of the mononuclear lymphocytes exceeded 95%.

Assay of splenic mononuclear lymphocyte proliferation

Both splenic mononuclear lymphocytes of C57BL/6 mice and BABL/c mice were placed together into 96-well flat-bottomed microplates (1×10^6 cells/well for each of C57BL/6 mice and BABL/c mice) with B16F10-CS containing different concentrations of *Gl*-PS (0.2, 0.8, 3.2 and 12.8 μg/ml) in the wells, with a total volume of 200 μl/well for the mixed lymphocyte reaction (MLR), or 20 μg/well of phytohemagglutinin (PHA) instead of mononuclear lymphocytes of BABL/c mice for PHA induction. Two controls were set up. One was RPMI-1640 medium. The other was B16F10-CS without *Gl*-PS. To identify the contribution of the shortage of nutrition or the metabolism toxin in supernatants to the results, an additional experiment was set up to compare the RPMI-1640 medium with L929-CS and FB-C57BL/6-CS. Cell proliferation was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay after 72 h incubation.[21] MTT solution (Sigma, St Louis, MO, USA) (20 μl; 5 mg/ml) was added to each well. After 4 h incubation, the cells were lysed and the purple formazan crystals were solubilized with DMSO for detection at 490 nm. The absorbance was translated into lymphocyte proliferation ratio for comparison: lymphocyte proliferation ratio = (test absorbance/normal control absorbance) × 100%.

Immunocytochemistry

Splenic mononuclear lymphocytes of C57BL/6 mice were placed into 96-well flat-bottomed microplates (1×10^6 cells/well) with B16F10-CS containing different concentrations of *Gl*-PS (0.2, 0.8, 3.2 and 12.8 μg/ml) and 20 μg/well of PHA; the total volume of each well was 200 μl. Both RPMI-1640 medium and B16F10-CS without *Gl*-PS were used as controls. After 72 h incubation, splenic mononuclear lymphocytes were smeared on slides and fixed with cold acetone for 5 min. The endogenous peroxidase activity was quenched with 3% hydrogen peroxide. After blocking with 10% normal serum, goat polyclonal primary antibody against granzyme B or perforin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at a 1 : 50 dilution and incubated overnight at 4°C. The next day, the horseradish peroxidase labelled secondary antibody was applied for 1 h and staining was finalized with a diaminobenzidine solution to detect the target antigen. Slides were extensively washed with phosphate-buffered saline between the different stages and counterstained with hematoxylin before mounting. Slides were examined under a light microscope. Replacement of primary antibody with phosphate-buffered saline was used as a negative control.

Sodium dodecyl sulfate-polyacrylamide electrophoresis and Western blot assay

The protein levels of granzyme B and perforin expressed in splenic mononuclear lymphocytes after stimulation with PHA for 72 h were determined by Western blot assay. The levels of total protein extracted from the splenic mononuclear lymphocytes were determined with the Bradford assay. Equal amounts of protein (50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were subsequently pre-blocked in Tris-buffered saline containing 5% non-fat milk powder and then incubated with goat polyclonal antigranzyme B or antiperforin antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1 : 100, followed by peroxidase-conjugated rabbit anti-goat IgG antibody. The antigen–antibody complex was visualized with Western blotting luminol reagent (Santa Cruz Biotechnology, Inc.). The bands were quantified with a Gel Doc 2000 system and Quantity One software (Bio-Rad) and expressed as a ratio (granzyme B *vs* β-actin, perforin *vs* β-actin) followed by standardization with the ratio in normal control as 1.

Statistical analysis

The results, except immunocytochemistry, are expressed as the mean ± SD of quadruplicate experiments in the Western blot assay and ELISA, or eight experiments in the cell proliferation assay with MTT, and statistical comparison between the experimental groups and the control was performed using one-way analysis of variance followed by the Dunnett's *t*-test. Values of $P < 0.05$ were considered significant.

Results

Suppression by B16F10-CS and antagonism by *Gl*-PS of splenic mononuclear lymphocyte proliferation induced by PHA

The function of the lymphocytes can be demonstrated by the proliferation induced by mitogens such as PHA in the lymphocytes. However, inhibition of lymphocyte proliferation may result from a shortage of nutrition or the metabolism toxin in supernatants. An additional experiment was therefore performed which showed that there were no significant differences among the RPMI-1640 medium, L929-CS and FB-C57BL/6-CS (data not shown). Compared with RPMI-1640 medium (with neither B16F10-CS nor *Gl*-PS, control, 100%), 72 h after induction with PHA, the proliferation ratio in B16F10-CS wells (without *Gl*-PS) was markedly reduced ($P < 0.05$), whereas in wells with any concentration of *Gl*-PS, the reduction of the proliferation ratio was significantly antagonized (all $P < 0.05$; Figure 1).

Suppression by B16F10-CS and antagonism by *Gl*-PS on splenic mononuclear lymphocyte proliferation in the MLR

The lymphocyte proliferation in the MLR can also demonstrate the function of lymphocytes. In the MLR, lymphocytes from C57BL/6 mice and BABL/c mice were used. Again, given that the inhibition of lymphocyte proliferation may result from a shortage of nutrition or the metabolism toxin in the supernatants, an additional experiment was performed, which showed that there were no significant differences among the RPMI-1640 medium, L929-CS and FB-C57BL/6-CS (data not shown). Regarding the proliferation ratio in the MLR in wells with RPMI-1640 medium (with neither

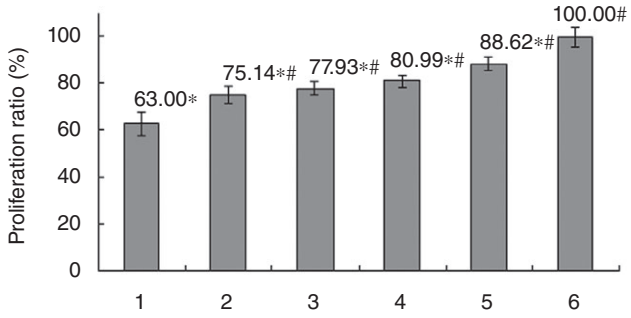

Figure 1 Suppression by B16F10-CS and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on splenic mononuclear lymphocyte proliferation induced by phytohemagglutinin (PHA). After 72 h incubation, the proliferative responses of splenic mononuclear lymphocytes to PHA were measured by MTT assay. 1, Control wells containing B16F10-CS; 2, wells containing B16F10-CS and 0.2 μg/ml of *Gl*-PS; 3, wells containing B16F10-CS and 0.8 μg/ml of *Gl*-PS; 4, wells containing B16F10-CS and 3.2 μg/ml of *Gl*-PS; 5, wells containing B16F10-CS and 12.8 μg/ml of *Gl*-PS; 6, control wells containing neither B16F10-CS nor *Gl*-PS. Error bars indicate the SD. $^{*}P < 0.05$, significantly different compared with the RPMI 1640 medium control (with neither B16F10-CS nor *Gl*-PS); $^{\#}P < 0.05$, significantly different compared with the B16F10-CS control (without *Gl*-PS); one-way analysis of variance followed by Dunnett's *t*-test.

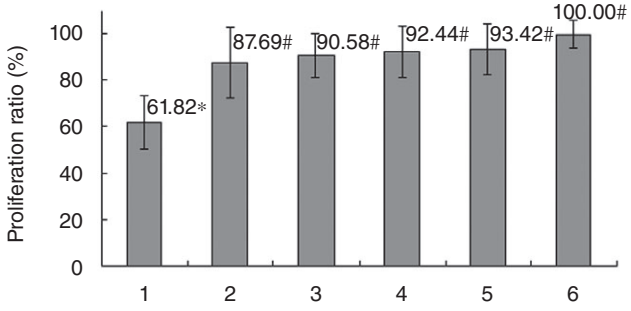

Figure 2 Suppression by B16F10-CS and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on splenic mononuclear lymphocyte proliferation in the mixed lymphocyte reaction (MLR). After 72 h incubation, the proliferative responses of splenic mononuclear lymphocytes in MLR were measured by MTT assay. 1, Control wells containing B16F10-CS; 2, wells containing B16F10-CS and 0.2 μg/ml of *Gl*-PS; 3, wells containing B16F10-CS and 0.8 μg/ml of *Gl*-PS; 4, wells containing B16F10-CS and 3.2 μg/ml of *Gl*-PS; 5, wells containing B16F10-CS and 12.8 μg/ml of *Gl*-PS; 6, control wells containing neither B16F10-CS nor *Gl*-PS. Error bars indicate the SD. $^{*}P < 0.05$, significantly different compared with the RPMI 1640 medium control (with neither B16F10-CS nor *Gl*-PS); $^{\#}P < 0.05$, significantly different compared with the B16F10-CS control (without *Gl*-PS); one-way analysis of variance followed by Dunnett's *t*-test.

B16F10-CS nor *Gl*-PS, control, 100%), the proliferation ratio in MLR was markedly reduced after 72 h in wells with B16F10-CS (without *Gl*-PS) ($P < 0.05$), whereas in wells with any concentration of *Gl*-PS, the reduction of the proliferation ratio was significantly antagonized (all $P < 0.05$; Figure 2).

Suppression by B16F10-CS and antagonism by *Gl*-PS on granzyme B in splenic mononuclear lymphocytes induced by PHA

The granule-mediated pathway is the most important cell death pathway, killing target cells in a cell contact manner. Granzyme B is one of the important molecules in the granule-mediated pathway. The granzyme B induced by mitogens such as PHA in splenic mononuclear lymphocytes is closely associated with the function of lymphocytes to kill target cells. It was shown by immunocytochemistry that the granzyme B in mononuclear lymphocytes was markedly reduced after 72 h induction by PHA in B16F10-CS wells (without *Gl*-PS), when compared with the RPMI 1640 medium wells (with neither B16F10-CS nor *Gl*-PS), while *Gl*-PS in the wells antagonized the reduction of the granzyme B (Figure 3). The Western blot assay also showed that the granzyme B in mononuclear lymphocytes induced by PHA was significantly reduced after 72 h incubation with B16F10-CS, when compared with the RPMI 1640 medium control (with neither B16F10-CS nor *Gl*-PS) wells ($P < 0.05$), while 3.2 μg/ml and 12.8 μg/ml of *Gl*-PS in the wells significantly antagonized the reduction of the granzyme B (both $P < 0.05$; Figure 4).

Suppression by B16F10-CS and antagonism by *Gl*-PS on perforin in splenic mononuclear lymphocytes induced by PHA

Perforin is another important molecule in the granule-mediated pathway. The perforin in splenic mononuclear lymphocytes induced by mitogens such as PHA is closely associated with the function of lymphocytes to kill target cells. It was shown by immunocytochemistry that the perforin in mononuclear lymphocytes induced by PHA was markedly reduced after 72 h in B16F10-CS wells (without *Gl*-PS), when compared with the RPMI 1640 medium control wells (with neither B16F10-CS nor *Gl*-PS), while *Gl*-PS in the wells antagonized the reduction of the perforin (Figure 5). Western blot assay also showed that the perforin in mononuclear lymphocytes induced by PHA was markedly reduced after 72 h in B16F10-CS wells (without *Gl*-PS), when compared with the RPMI 1640 medium control (with neither B16F10-CS nor *Gl*-PS) wells ($P < 0.05$), while any concentration of *Gl*-PS significantly antagonized the reduction of perforin (all $P < 0.05$; Figure 6).

Levels of IL-10, TGF-β1 and VEGF in B16F10-CS

Some soluble immunosuppressive cytokines are commonly secreted by many cancer cells. Among these cytokines, IL-10, TGF-β1 and VEGF were detected in the B16F10-CS by ELISA. It was shown that the levels of IL-10, TGF-β1 and VEGF in the B16F10-CS were all significantly higher than those in the RPMI 1640 medium control (with neither B16F10-CS nor *Gl*-PS) (all $P < 0.05$, Figures 7–9). In contrast, there were no significant differences among the RPMI-1640 medium, L929-CS and FB-C57BL/6-CS (all $P > 0.05$; Figures 7–9), except for IL-10 in L929-CS, which was significantly higher than that in RPMI-1640 medium ($P < 0.05$; Figure 7) but not lower than that in B16F10-CS ($P > 0.05$; Figure 7).

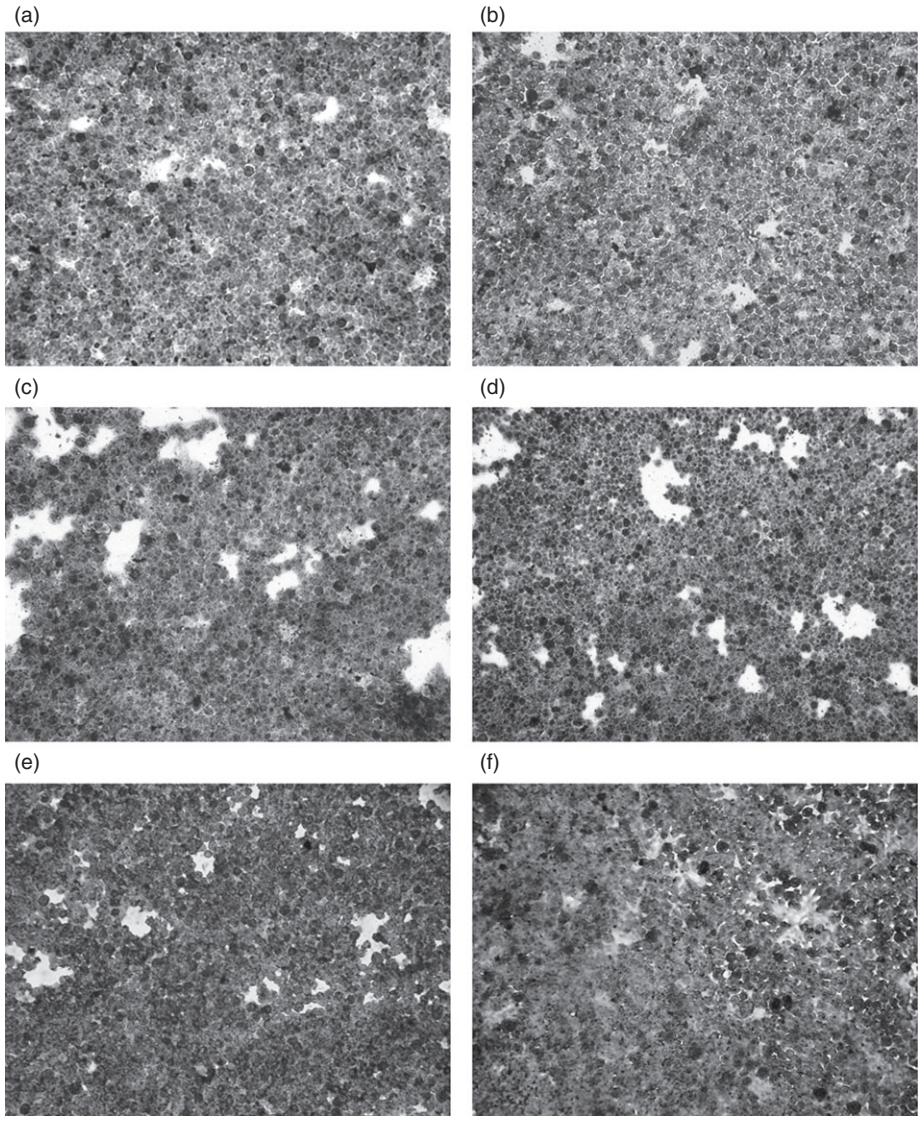

Figure 3 Suppression by B16F10-CS and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on granzyme B induced by phytohemagglutinin (PHA) in splenic mononuclear lymphocytes measured by immunocytochemistry. After induction by PHA and incubation for 72 h, the granzyme B in splenic mononuclear lymphocytes was measured by immunocytochemistry. (a) Control well containing B16F10-CS. (b) Well containing B16F10-CS and 0.2 μg/ml of *Gl*-PS. (c) Well containing B16F10-CS and 0.8 μg/ml of *Gl*-PS. (d) Well containing B16F10-CS and 3.2 μg/ml of *Gl*-PS. (e) Well containing B16F10-CS and 12.8 μg/ml of *Gl*-PS. (f) Control containing neither B16F10 cell culture supernatant nor *Gl*-PS.

Discussion

It is known that cell-mediated innate and adaptive immunity have the potential to prevent outgrowth of primary tumours and reject transplanted tumours.[22,23] However, the immune system often fails to prevent tumours or to limit their spread, although activated immune cells often exist in cancer patients.[24] Established tumours must have strategies to inhibit or evade the immune system. Part of the immunosuppressive strategies in tumours is attributed to the production and secretion of soluble factors suppressing the functions of immune cells or inducing the apoptosis of immune cells.[25] Common immunosuppressive factors produced by tumours include IL-10, TGF-β, VEGF and prostaglandins, which may directly or indirectly inhibit the immune response and hamper immunotherapy.[26]

There are two types of immune response: cell-mediated immunity and humoral immunity.[27] Cell-mediated immunity is associated with Th1 $CD4^+$ T lymphocytes. Humoral immunity is associated with Th2 $CD4^+$ T lymphocytes. Two different $CD4^+$ T lymphocytes produce different cytokines. Cytokines produced by Th1 $CD4^+$ T lymphocytes are commonly IL-2, interferon γ and tumour necrosis factor α. The common cytokines produced by Th2 $CD4^+$ T lymphocytes are IL-4, IL-6 and IL-10.[28] The Th1 response leads to tumour rejection because Th1 pathways typically produce some

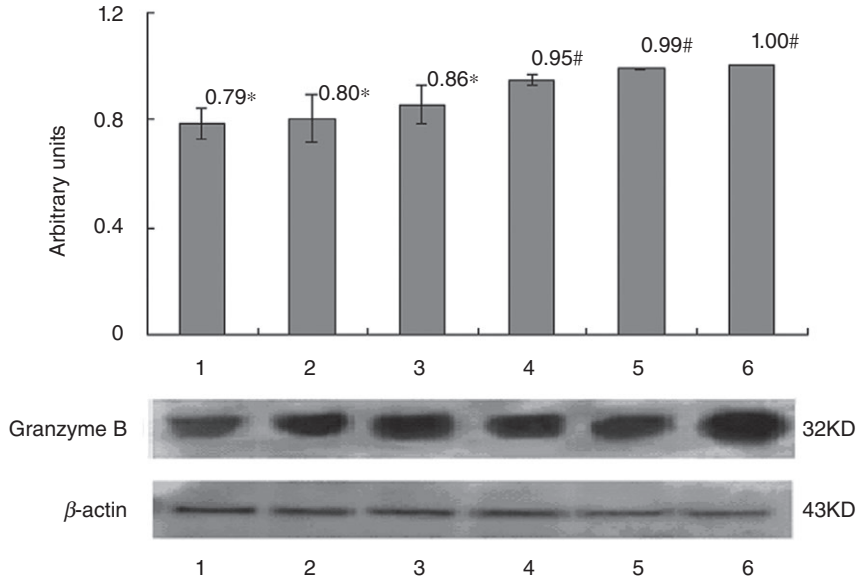

Figure 4 Suppression by B16F10-CS and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on granzyme B induced by phytohemagglutinin (PHA) in splenic mononuclear lymphocytes measured by Western blot assay. After 72 h incubation, the granzyme B in splenic mononuclear lymphocytes was measured by Western blot assay. 1, Control wells containing B16F10-CS; 2, wells containing B16F10-CS and 0.2 μg/ml of *Gl*-PS; 3, wells containing B16F10-CS and 0.8 μg/ml of *Gl*-PS; 4, wells containing B16F10-CS and 3.2 μg/ml of *Gl*-PS; 5, wells containing B16F10-CS and 12.8 μg/ml of *Gl*-PS; 6, control wells containing neither B16F10-CS nor *Gl*-PS. Error bars indicate the SD. $^{*}P < 0.05$, significantly different compared with the RPMI 1640 medium control (with neither B16F10-CS nor *Gl*-PS); $^{\#}P < 0.05$, significantly different compared with the B16F10-CS control (without *Gl*-PS); one-way analysis of variance followed by Dunnett's *t*-test.

effector cells to attack cancer cells and generally defend against tumours. These effector cells are CTL, natural killer cells, macrophages and monocytes.[29] In contrast, the Th2 response prevents tumour rejection.[30,31] Almost all malignancies are associated with suppression of cell-mediated immunity.[28]

Many malignant diseases overexpress IL-10. These malignant diseases include melanoma,[32] basal cell and squamous carcinoma,[33] renal cell carcinoma[34] and colorectal cancer.[35–38] IL-10 is a Th2 type pleiotropic cytokine.[37] It limits and ultimately terminates inflammatory responses[39] and suppresses Th1 clone stimulation by Langerhans cells.[40] It prevents Th1 cells from producing Th1 cytokines.[41] It inhibits antigen presenting cells such as Langerhans cells from expressing B7 molecules as well as presenting antigen to Th1 cells.[40] It mediates tumour cell resistance to CTL killing,[42,43] suppresses CTL development and blocks the differentiation of Th0 cells to Th1 cells.[44] It may also downregulate the function of TAP (transporter associated with antigen processing) molecules and the expression of major histocompatibility complex class I molecules on target cells, thereby suppressing T cell-mediated immunity.[45]

Malignant cells often secrete large amounts of TGF-β.[46] TGF-β may also be released from the extracellular matrix within and around the tumour. It may be secreted by mesenchymal cells, resident leukocytes, or by monocytes and macrophages recruited to the tumour.[47–50] TGF-β acts on non-transformed cells in the tumour mass as well as distal cells in the host to suppress antitumour immune responses. TGF-β creates an environment of immune tolerance. TGF-β is a prominent family of cytokines with multifunctions that regulate cell proliferation, differentiation and extracellular matrix production.[51,52] It is essential for the development and maintenance of a multicellular organism. TGF-β has been recognized to play a wide range of roles in cancer.[53–55] Although the promoter regions of genes encoding three mammalian TGF-β (TGF-β1, 2, 3) show little similarity in sequence, their protein products are functionally very similar.[56] It has been reported that TGF-β1 exerts an immunosuppressive effect on all cells in the immune system.[57–59] It can act on T lymphocytes, which could develop into effector ($CD8^+$, CTL) or helper ($CD4^+$, Th1 or Th2) cells. Among the several cytokines reported to influence differentiation of naive T cells, TGF-β1 is the most effective inhibitor of the maturation of naive T cells. It inhibits the differentiation of $CD8^+$ T cells to CTL and $CD4^+$ T cells to the Th1 or Th2 phenotype.[60] Blocking TGF-β1 signalling in immune cells promotes an antitumour response. Both thymoma and melanoma-derived cell lines were eradicated in animals expressing a dominant negative type II TGF-β receptor under the control of a T-specific promoter.[60]

In addition to IL-10 and TGF-β, VEGF is also produced by nearly all tumour cells[61] and the levels of VEGF in the serum of cancer patients are often elevated.[62] The abnormal production of VEGF can stimulate the proliferation of endothelial cells and play an important role in angiogenesis in tumours which facilitates the growth of tumours.[63] It has been reported that VEGF is not only important for tumour vascularization but is also a key factor in inhibiting recognition and destruction of tumour cells by the immune system.[64]

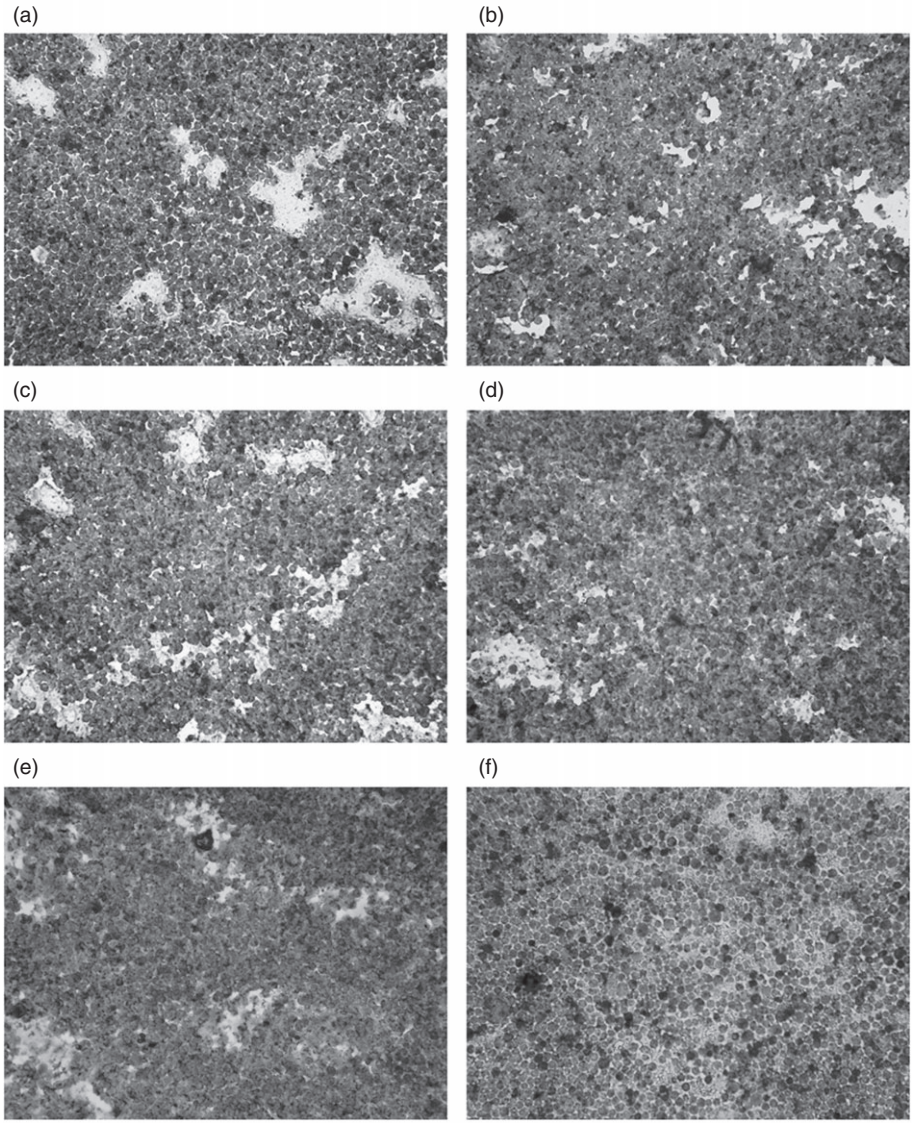

Figure 5 Suppression by B16F10-CS and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on perforin induced by phytohemagglutinin (PHA) in splenic mononuclear lymphocytes measured by immunocytochemistry. After induction by PHA and incubation for 72 h, the perforin in splenic mononuclear lymphocytes was measured by immunocytochemistry. (a) Control well containing B16F10-CS. (b) Well containing B16F10-CS and 0.2 μg/ml of *Gl*-PS. (c) Well containing B16F10-CS and 0.8 μg/ml of *Gl*-PS. (d) Well containing B16F10-CS and 3.2 μg/ml of *Gl*-PS. (e) Well containing B16F10-CS and 12.8 μg/ml of *Gl*-PS. (f) Control well containing neither B16F10-CS nor *Gl*-PS.

Continuous infusion of VEGF results in the inhibition of dendritic cell function, alterations in lymphocyte numbers, and the accumulation of immature myeloid cells and granulocytes,[65,66] indicating that the immune function is impaired by VEGF.

Another immunosuppressive cytokine commonly produced and released by tumour cells is prostaglandin E2, a lipid mediator with important immunomodulatory properties. Prostaglandin E2 has the effect of downregulating Th1 cytokines (tumour necrosis factor α, interferon γ and IL-2)[67] and upregulating Th2 cytokines (IL-4, IL-10 and IL-6).[68–70] Moreover, prostaglandin E2 can inhibit dendritic cell differentiation and T cell proliferation, and suppress the antitumour activity of natural killer cells and macrophages.[71,72] Thus, the effects of prostaglandin E2 on the immune system may allow neoplastic cells to evade attack by the immune system.[73]

B16F10 cells are melanoma cells derived from the C57BL mouse strain that induce immune suppression as well. Of the four soluble immunosuppressive factors commonly secreted by most tumours outlined above, three of them, IL-10, TGF-β1 and VEGF, were detected in B16F10-CS in this study. All three immunosuppressive factors were higher in the B16F10-CS than in RPMI-1640 medium, as well as L929-CS and FB-C57BL/6-CS, except for IL-10 in L929-CS which was not significantly lower than it in B16F10-CS, while there were no significant differences among the RPMI-1640

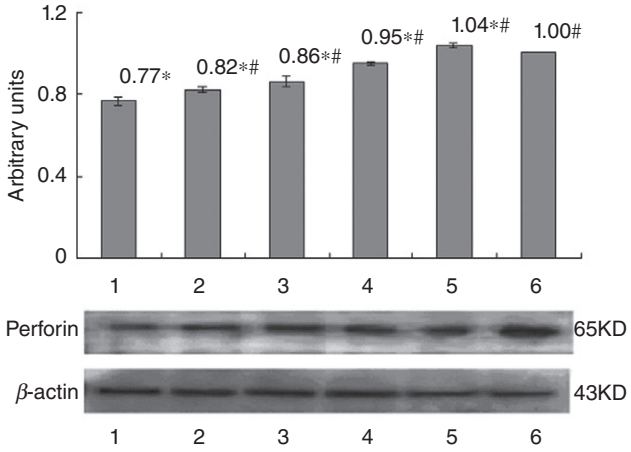

Figure 6 Suppression by B16F10-CS and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on perforin induced by phytohemagglutinin (PHA) in splenic mononuclear lymphocytes measured by Western blot assay. After 72 h incubation, the perforin in splenic mononuclear lymphocytes was measured by Western blot assay. 1, Control wells containing B16F10-CS; 2, wells containing B16F10-CS and 0.2 μg/ml of *Gl*-PS; 3, wells containing B16F10-CS and 0.8 μg/ml of *Gl*-PS; 4, wells containing B16F10-CS and 3.2 μg/ml of *Gl*-PS; 5, wells containing B16F10-CS and 12.8 μg/ml of *Gl*-PS; 6, control wells containing neither B16F10-CS nor *Gl*-PS. Error bars indicate the SD. $^{*}P < 0.05$, significantly different compared with the RPMI 1640 medium control (with neither B16F10-CS nor *Gl*-PS); $^{\#}P < 0.05$, significantly different compared with the B16F10-CS control (without *Gl*-PS); one-way analysis of variance followed by Dunnett's *t*-test.

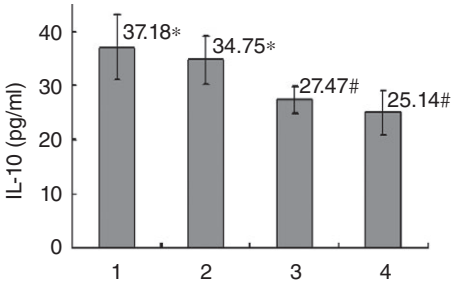

Figure 7 Level of interleukin 10 (IL-10) in B16F10-CS. After 8 h incubation, the B16F10-CS as well as L929-CS and FB-C57BL/6-CS were harvested and the level of IL-10 was determined by enzyme-linked immunosorbent assay. 1, B16F10-CS; 2, L929-CS; 3, FB-C57BL/6-CS; 4, RPMI 1640 medium. Error bars indicate the SD. $^{*}P < 0.05$, significantly different compared with the RPMI 1640 medium control; $^{\#}P < 0.05$, significantly different compared with the B16F10-CS control; one-way analysis of variance followed by Dunnett's *t*-test.

medium, L929-CS and FB-C57BL/6-CS, except for IL-10 in L929-CS which was significantly higher than in RPMI-1640 medium. Combined with the suppressive effects of B16F10-CS on lymphocytes, we conclude that IL-10, TGF-β and VEGF may play a role in the suppression of lymphocytes by B16F10-CS.

Lymphocytes play important role in antitumour immunity. Immune effector cells execute their function to mediate target cell death through two effector pathways: the granule-mediated pathway and the death receptor-mediated pathway.[74,75] In the granule-mediated pathway, perforin is released upon contact with a susceptible target cell and polymerized into the target cell membrane forming pores,[76] which facilitate granzyme to enter the cytosol of the target cell.[77] Granzymes, of which granzyme B is the most potent member, are serine proteases capable of cleaving proteins on the carboxy side of aspartate residues.[78] The subsequent translocation of pro-apoptotic granzymes into the cytosol provides these proteases with access to numerous protein substrates that promote apoptosis after cleavage. Suppression of perforin and granzyme B leads to the suppression of the cytotoxicity of lymphocytes against target cells. It was shown in this study that perforin and granzyme B were suppressed in the B16F10-CS after induction with PHA. The suppression is most probably associated with the elevated levels of IL-10, TGF-β and VEGF in B16F10-CS.

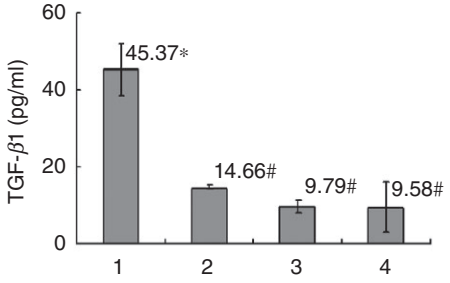

Figure 8 Level of transforming growth factor β1 (TGF-β1) in B16F10-CS. After 8 h incubation, the B16F10-CS as well as L929-CS and FB-C57BL/6-CS were harvested and the level of TGF-β1 was determined by enzyme-linked immunosorbent assay. 1, B16F10-CS; 2, L929-CS; 3, FB-C57BL/6-CS; 4, RPMI 1640 medium. Error bars indicate the SD. $^{*}P < 0.05$, significantly different compared with the RPMI 1640 medium control; $^{\#}P < 0.05$, significantly different compared with the B16F10-CS control; one-way analysis of variance followed by Dunnett's *t*-test.

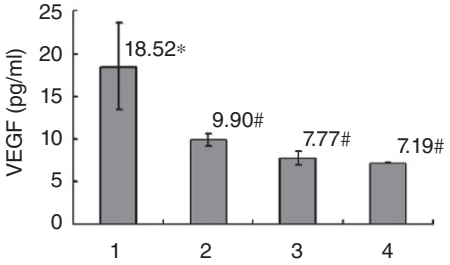

Figure 9 Level of vascular endothelial growth factor (VEGF) in B16F10-CS. After 8 h incubation, the B16F10-CS as well as L929-CS and FB-C57BL/6-CS were harvested and the level of VEGF was determined by enzyme-linked immunosorbent assay. 1, B16F10-CS; 2, L929-CS; 3, FB-C57BL/6-CS; 4, RPMI 1640 medium. Error bars indicate the SD. $^{*}P < 0.05$, significantly different compared with the RPMI 1640 medium control; $^{\#}P < 0.05$, significantly different compared with the B16F10-CS control; one-way analysis of variance followed by Dunnett's *t*-test.

Conclusions

Lymphocyte proliferation, and the level of perforin and granzyme B induced by mitogens in lymphocytes, as well as

lymphocyte proliferation in the MLR, can demonstrate the function of lymphocytes. Antagonism of the immunosuppression by tumour cells, and thus antagonism of the suppression of lymphocyte function against target cells, is one of the most important goals of immunotherapy. It was shown in this study that B16F10-CS had elevated levels of IL-10, TGF-β and VEGF and suppressed lymphocyte proliferation, and granzyme B and perforin production in lymphocytes after induction with PHA, as well as proliferation in the MLR. *Gl*-PS fully or partially antagonized this suppression, suggesting the potential for *Gl*-PS in cancer immunotherapy.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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