Original Paper

Protection Against Lung Cancer Patient Plasma-Induced Lymphocyte Suppression by *Ganoderma Lucidum* Polysaccharides

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Key Words

Lung cancer • Lymphocyte activation • *Ganoderma lucidum* polysaccharides • CD69 • Perforin • Granzyme B

Abstract

Background/Aims: This study was conducted to determine the potential of *Ganoderma lucidum* polysaccharides (GL-PS) in protection against lung cancer patient plasma-induced suppression of lymphocytes. Lung cancer is a major cause of disease and loss of life in the United States and worldwide. Cancer cells release immunosuppressive mediators, such as PGE₂, TGF-β, IL-10, and VEGF, to inhibit the immune response to escape from immune surveillance. GL-PS has been shown to counteract this immune inhibition in an animal cell culture model, and thus to facilitate tumor control. The present study explored whether or not such an effect could also be demonstrated in human lung cancer patients. **Methods:** Immunofluorescence, flow cytometry, MTT, immunocytochemistry, and western blot analysis were used to assess lymphocyte activation with PHA. **Results:** The plasma of lung cancer patients suppressed proliferation, CD69 expression, and perforin and granzyme B production in lymphocytes upon activation by PHA, effects that were partially or fully reversed by GL-PS. **Conclusion:** Lung cancer patient plasma-induced suppression of lymphocyte activation by phytohemagglutinin may be antagonized fully or partially by GL-PS, an observation suggesting the potential of GL-PS in cancer therapy.

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Introduction

Cancer is a major public health problem in the United States and many other parts of the world. Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population aging and growth. Moreover, adoption of cancer-associated lifestyle choices, including smoking, physical inactivity, and “Westernized” diets is on the rise [1]. Lung cancer is the most commonly diagnosed cancer, the leading cause of cancer death in males, the fourth most commonly diagnosed cancer, and the second leading cause of cancer death in females in 2008 worldwide [1]. One in four deaths in the United States is currently due to cancer. In the United States, it is expected that lung cancer will be the second most commonly diagnosed cancer in 2013, as well as the leading cause of cancer deaths in both males and females [2].

Carcinogenesis and cancer progression are closely associated with the host immune system. An intact immune system is essential to prevent the development and progression of neoplastic cells in a process termed “immune surveillance,” during which the innate and adaptive immune systems closely cooperate; T cells in particular play an important role in detecting and eliminating tumor cells [3]. Despite ongoing surveillance by T cells and other components of the immune system, tumors develop despite the presence of an intact immune system and eventually become clinically detectable, indicating that tumors can escape from immune surveillance. Many tumors, including lung cancers, promote immune tolerance to escape host immune surveillance and facilitate tumor growth by utilizing numerous pathways, including the elaboration of immunosuppressive mediators, such as PGE₂, TGF- β , IL-10, and VEGF [4], which may directly or indirectly inhibit the immune response and hamper immunotherapy [5]. It has been demonstrated that the levels of PGE₂, TGF- β , IL-10, and VEGF in patients with lung cancer are increased [6-8]. Therefore, the plasma containing immunosuppressive factors in lung cancer patients may suppress the function of immune cells, while antagonism against this immunosuppression may facilitate tumor control.

Many natural products have been demonstrated with immunomodulatory and anti-tumor activities [9-15], including *Ganoderma lucidum* polysaccharides (*GI-PS*), important active constituents extracted from *G. lucidum*. *G. lucidum* is a medicinal mushroom widely used in traditional Chinese medicine for thousands of years to improve health and longevity [16]. *GI-PS* has been widely studied in recent decades for its anti-tumor activity, which is mainly achieved by boosting host immune function [17]. Dendritic cell maturation and function, cytokine production [18], cytotoxic T lymphocyte (CTL) function [19], and cytokine-induced killer cell (CIK) function [20] were promoted by *GI-PS*. *GI-PS* can also enhance the function of immunologic effector cells in immunosuppressed mice [21]. Multi-drug resistance (MDR) is reversed by *GI-PS* through down-regulation of the expression of MDR-1 and MDR-associated protein 1 (MRP1) in an adriamycin (ADM)-resistant leukemic cell line, K562/ADM [22], and also wound repair in intestinal epithelial cells was accelerated by *GI-PS* [23]. In addition, the growth of vascular endothelial cells, as well as the induction of vascular endothelial growth factor (VEGF) in human lung cancer cell, was inhibited by *GI-PS* [24]. *GI-PS* did not inhibit cell proliferation when it was added directly to PG human lung carcinoma cell cultures, while the serum derived from *GI-PS*-treated mice markedly inhibited PG cell proliferation [25], indicating that *GI-PS* had no direct cell toxicity in tumor cells, but promoted host anti-tumor immunity and anti-angiogenesis.

Our previous studies with the B16F10 melanoma cell line demonstrated that in addition to the effects on host immune function, *GI-PS* also acts on tumor cells to enhance the expression of MHC class I and co-stimulatory molecules [26] which may at least partially contribute to promotion of lymphocyte activation [27] and induction of stronger anti-tumor cytotoxicity in CTLs with granzyme B and perforin *in vitro* accompanied by a reduction in the incidence of tumorigenesis and prolonged latency *in vivo* [28]. In addition, suppression of lymphocytes as well as macrophages caused by B16F10 cell culture supernatants containing immunosuppressive factors, such as TGF- β , IL-10, and VEGF, was antagonized by *GI-PS* [29-30].

31]. Lymphocyte proliferation, and perforin and granzyme B production in lymphocytes after induction with phytohemagglutinin (PHA), as well as lymphocyte proliferation in the mixed lymphocyte reaction, were suppressed by B16F10 cell culture supernatants in which there were elevated levels of IL-10, TGF- β 1, and VEGF, but the suppression was fully or partially antagonized by *GI-PS* [29]. However, the antagonism was only studied in an animal cell culture model of melanoma, and it is important to know what is in the plasma of cancer patients, especially patients with other cancers, such as lung cancer, because the constituents may be more complex in cancer patients than in an animal cell culture model, and there are many differences between human beings and other animals. The ultimate purpose of researching the antagonistic effects of *GI-PS* against immunosuppression by cancer cells is to benefit cancer control in cancer patients by antagonism with *GI-PS* or similar agents against immunosuppression by cancer cells. Therefore, although a study using an animal cell culture model is useful and beneficial, a study with cancer patients is necessary, which may be helpful to understand the effects of the antagonism with *GI-PS* against immunosuppression by cancer cells from cancer patients, such as lung cancer. The plasma of lung cancer patients may also contain immunosuppressive factors with immunosuppressive effects on lymphocytes and *GI-PS* may antagonize these immunosuppressive effects as well, which remains to be demonstrated. The present study was designed to detect the suppressive effect of lung cancer patient plasma on lymphocytes and the antagonistic effect of *GI-PS* against this suppression.

Materials and Methods

Preparation of the plasma and lymphocytes

Venous blood was collected with informed consent from 12 lung cancer patients (9 males and 3 females; age range, 48-79 years; average age, 56.5 years) who were cared for at The Affiliated Hospital of Chengde Medical College (Chengde, Hebei province, China) before any therapies, including surgery, chemotherapy, and radiotherapy. After centrifugation, the plasma was isolated from the venous blood and stored at -80°C , while the remainder of the venous blood was re-suspended with normal saline followed by Ficoll-Paque gradient centrifugation to collect human peripheral blood mononuclear lymphocytes. The lymphocytes were adjusted to $2.5 \times 10^6/\text{ml}$ in RPMI-1640. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the hospital. All of the subjects provided written informed consent before participating in the study.

Preparation of GI-PS

GI-PS, isolated from the boiling water extract of the fruit bodies of *G. lucidum* by ethanol precipitation and dialysis, followed by Sevag deproteinization, was kindly provided by the Fuzhou Institute of Green Valley Bio-Pharm Technology [21]. As we previously described [29], *GI-PS* is a glycopeptide with a molecular weight of 584,900; the ratio of polysaccharides-to-peptides was 93.61%:6.49%. The polysaccharides consisted of d-rhamnose, d-xylose, d-fructose, d-galactose, d-mannose, and d-glucose with a molar ratio of 0.793:0.964:2.944:0.167:0.389:7.94, and linked together by β -glycosidic linkages. Sixteen amino acid contained in the peptides were as follows: Asp; Thr; Ser; Glu; Gly; Ala; Cys; Val; Met; Ile; Leu; Phe; Lys; His; Arg; and Pro. The *GI-PS* was identified by the wave spectrum and dissolved in serum-free RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA), then filtered through a $0.22\text{-}\mu\text{m}$ filter and stored at 4°C before each assay.

CD69 expression assay by immunofluorescence staining and flow cytometry

CD69 expression on the surface of lymphocytes was determined by immunofluorescence staining and flow cytometry. Lymphocytes ($1 \times 10^6/\text{well}$) were cultured in RPMI-1640, containing $40\text{ }\mu\text{l}$ of plasma, $2\text{ }\mu\text{l}$ of PHA (final concentration, $100\text{ }\mu\text{g}/\text{ml}$), and different concentrations of *GI-PS* in 96-well culture plates for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 . Plasma from healthy donors was used as a control. Cells were harvested and washed with ice cold fluorescence activated cell sorter (FACS) buffer (PBS containing 2% FBS and 0.1% sodium azide). The same buffer was used for the incubation with antibodies, as well as all washes. Cells were adjusted to $1 \times 10^7/\text{ml}$ and non-conjugated isotype IgG1 κ was used to block non-specific antibody binding. After washing three times, fluorescein isothiocyanate (FITC)-conjugated

anti-CD69 (BD Biosciences, San Jose, CA, USA) antibody were added to the cells and the samples were left at 4°C for 45 min in the dark. The CD69 expression on the cell surface was determined by flow cytometric analysis. Fluorescence profiles were generated on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Histogram and density plots were generated by CellQuest software (Becton Dickinson). Dead cells and debris were gated out.

Lymphocyte proliferation assay

Cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [32]. The lymphocytes (1×10^6 /well) were cultured in RPMI-1640, containing 40 μ l of plasma and 2 μ l of PHA (final concentration, 100 μ g/ml) and different concentrations of *GI-PS* in 96-well culture plates at 37°C in a humidified atmosphere containing 5% CO₂. Plasma from healthy donors was used as a control. After a 68-h incubation, MTT solution (20 μ l [5 mg/ml]; Sigma, St. Louis, MO, USA) was added to each well and followed by an additional 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 a lymphocyte proliferation ratio for comparison, as follows: lymphocyte proliferation ratio = (test absorbance/normal control absorbance) $\times$ 100%.

Immunocytochemistry

After incubation (1×10^6 cells/well) in RPMI-1640 containing 40 μ l of plasma, 2 μ l of PHA (final concentration, 100 μ g/ml), and different concentrations of *GI-PS* (total volume, 200 μ l/well) in 96-well culture plates for 48 h at 37°C in a humidified atmosphere containing 5% CO₂, the lymphocytes were aspirated from plated cells, which were then rinsed three times with PBS. Cells were smeared on slides, then allowed to air dry for 30 min and fixed with cold acetone for 5 min. Plasma from healthy donors was used as a control. The endogenous peroxidase activity was quenched with 3% hydrogen peroxide and the non-specific binding sites were blocked with 10% normal serum. After PBS washing, primary antibody (purified goat anti-granzyme B or goat anti-perforin antibody, 1:50 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added to the cells and incubated overnight at 4°C followed by three rinses with wash buffer. The expression of granzyme B and perforin was visualized with a horseradish peroxidase-labelled rabbit anti-goat secondary antibody for 2 h at 37°C, and finalized with a diaminobenzidine solution. Coverslips were applied to the slides with mounting medium after counterstaining with hematoxylin and observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Western blot analysis

Western blot analysis was used to determine the protein levels of granzyme B and perforin expressed in the mononuclear lymphocytes after stimulation with PHA and treatment with plasma of lung cancer patients and *GI-PS* for 48 h. After treatment, cells were collected and washed once with 1 $\times$ PBS, lysed in lysis buffer on ice for 30 min, and briefly vortexed, followed by centrifugation at 12,000 rpm for 20 min and collection of the supernatant. Protein concentrations were determined. Equal amounts of cell lysate were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis and the separated proteins were then electrotransferred to polyvinylidene fluoride (PVDF) membranes. After exposure to the antibodies and horseradish peroxidase-labelled secondary antibodies, specific proteins were detected using western blotting luminol reagent (Santa Cruz Biotechnology, Inc.). The band intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analyses

All data, except immunocytochemistry data, were presented as the means with standard deviations (SD). One-way analysis of variance (ANOVA), followed by Dunnett's *t*-test, were used to compare the differences between different groups. Statistical significance was considered at a *p*-value <0.05.

Results

CD69 expression on mononuclear lymphocytes

As shown by flow cytometry, after a 24-h incubation with lung cancer patient plasma, upon PHA stimulation *in vitro*, CD69 expression on mononuclear lymphocytes was inhibited

Fig. 1. Suppression by lung cancer patient plasma and antagonism by *Ganoderma lucidum* polysaccharides (*GI-PS*) on CD69 expression in mononuclear lymphocytes induced by phytohemagglutinin (PHA). After a 24-h incubation, CD69 expression on mononuclear lymphocytes following stimulation with PHA was measured by flow cytometry assay. 1, Control wells containing lung cancer patient plasma; 2, wells containing lung cancer patient plasma and 0.2 $\mu\text{g/ml}$ of *GI-PS*; 3, wells containing lung cancer patient plasma and 0.8 $\mu\text{g/ml}$ of *GI-PS*; 4, wells containing lung cancer patient plasma and 3.2 $\mu\text{g/ml}$ of *GI-PS*; 5, wells containing lung cancer patient plasma and 12.8 $\mu\text{g/ml}$ of *GI-PS*; 6, control wells containing healthy donor plasma without *GI-PS*. Error bars indicate the SD. * $p < 0.05$, significantly different compared with healthy donor control (without lung cancer patient plasma or *GI-PS*); # $p < 0.05$, significantly different compared with lung cancer patient plasma control (without *GI-PS*); one-way analysis of variance followed by Dunnett's *t*-test.

markedly compared with controls ($p < 0.05$), while *GI-PS* at concentrations of 3.2 and 12.8 $\mu\text{g/ml}$ significantly antagonized this inhibition (both $p < 0.05$, Fig. 1).

Proliferation in mononuclear lymphocytes

The MTT assay showed that after a 72-h incubation with lung cancer patient plasma, upon PHA stimulation *in vitro*, proliferation in mononuclear lymphocytes was inhibited markedly compared with their controls ($p < 0.05$), while *GI-PS* at any concentration used in this study significantly antagonized this inhibition (all $p < 0.05$, Fig. 2).

Fig. 2. Suppression by lung cancer patient plasma and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on mononuclear lymphocyte proliferation induced by phytohemagglutinin (PHA). After a 72-h incubation, the proliferative responses of the mononuclear lymphocytes to PHA were measured by a MTT assay. 1, Control wells containing lung cancer patient plasma; 2, wells containing lung cancer patient plasma and 0.2 $\mu\text{g/ml}$ of *Gl*-PS; 3, wells containing lung cancer patient plasma and 0.8 $\mu\text{g/ml}$ of *Gl*-PS; 4, wells containing lung cancer patient plasma and 3.2 $\mu\text{g/ml}$ of *Gl*-PS; 5, wells containing lung cancer patient plasma and 12.8 $\mu\text{g/ml}$ of *Gl*-PS; 6, control wells containing healthy donor plasma without *Gl*-PS. Error bars indicate the SD. * $p < 0.05$, significantly different compared with healthy donor control (without lung cancer patient plasma or *Gl*-PS); # $p < 0.05$, significantly different compared with lung cancer patient plasma control (without *Gl*-PS); one-way analysis of variance followed by Dunnett's *t*-test.

Fig. 3. Western blot assay showing suppression by lung cancer patient plasma and antagonism by *Ganoderma lucidum* polysaccharides (*Gl*-PS) on perforin expression on mononuclear lymphocytes induced by phytohemagglutinin (PHA). After a 48-h incubation, perforin expression on mononuclear lymphocytes to PHA was determined by western blot analysis. 1, Control wells containing lung cancer patient plasma; 2, wells containing lung cancer patient plasma and 0.2 $\mu\text{g/ml}$ of *Gl*-PS; 3, wells containing lung cancer patient plasma and 0.8 $\mu\text{g/ml}$ of *Gl*-PS; 4, wells containing lung cancer patient plasma and 3.2 $\mu\text{g/ml}$ of *Gl*-PS; 5, wells containing lung cancer patient plasma and 12.8 $\mu\text{g/ml}$ of *Gl*-PS; 6, control wells containing healthy donor plasma without *Gl*-PS. Error bars indicate the SD. * $p < 0.05$, significantly different compared with healthy donor control (without lung cancer patient plasma or *Gl*-PS); # $p < 0.05$, significantly different compared with lung cancer patient plasma control (without *Gl*-PS); one-way analysis of variance followed by Dunnett's *t*-test.

Perforin expression in mononuclear lymphocytes

After a 48-h incubation with lung cancer patient plasma, upon PHA stimulation *in vitro*, the expression of perforin in mononuclear lymphocytes, as detected by western blot analysis, was inhibited markedly compared with controls ($p < 0.05$), while *Gl*-PS at any concentration used in this study significantly antagonized this inhibition (all $p < 0.05$, Fig. 3). The immunocytochemistry data showed similar results (Fig. 4).

Granzyme B expression in mononuclear lymphocytes

The expression of granzyme B in mononuclear lymphocytes after a 48-h incubation with lung cancer patient plasma, as detected by western blot analysis, upon PHA stimulation *in vitro*, was inhibited markedly compared with controls ($p < 0.05$), while *Gl*-PS at concentrations of 0.8, 3.2, and 12.8 $\mu\text{g/ml}$ significantly antagonized this inhibition (all $p < 0.05$, Fig. 5). The immunocytochemistry data showed similar results (Fig. 6).

Discussion

Lung cancer is a major cause of disease burden and loss of life in the US and worldwide [33, 34]. It is estimated by the American Cancer Society that there will be 228,190 new cases

Fig. 4. Immunocytochemistry showing suppression by lung cancer patient plasma and antagonism by *Ganoderma lucidum* polysaccharides (*GI-PS*) on perforin expression on mononuclear lymphocytes induced by phytohemagglutinin (PHA). After a 48-h incubation, perforin expression on the mononuclear lymphocytes to PHA was measured by immunocytochemistry. 1, Control wells containing lung cancer patient plasma; 2, wells containing lung cancer patient plasma and 0.2 $\mu\text{g/ml}$ of *GI-PS*; 3, wells containing lung cancer patient plasma and 0.8 $\mu\text{g/ml}$ of *GI-PS*; 4, wells containing lung cancer patient plasma and 3.2 $\mu\text{g/ml}$ of *GI-PS*; 5, wells containing lung cancer patient plasma and 12.8 $\mu\text{g/ml}$ of *GI-PS*; 6, control wells containing healthy donor plasma without *GI-PS*.

Fig. 5. Western blot analysis showing suppression by lung cancer patient plasma and antagonism by *Ganoderma lucidum* polysaccharides (*GI-PS*) on granzyme B expression on mononuclear lymphocytes induced by phytohemagglutinin (PHA). After a 48-h incubation, granzyme B expression on mononuclear lymphocytes to PHA was determined by western blot analysis. 1, Control wells containing lung cancer patient plasma; 2, wells containing lung cancer patient plasma and 0.2 $\mu\text{g/ml}$ of *GI-PS*; 3, wells containing lung cancer patient plasma and 0.8 $\mu\text{g/ml}$ of *GI-PS*; 4, wells containing lung cancer patient plasma and 3.2 $\mu\text{g/ml}$ of *GI-PS*; 5, wells containing lung cancer patient plasma and 12.8 $\mu\text{g/ml}$ of *GI-PS*; 6, control wells containing healthy donor plasma without *GI-PS*. Error bars indicate the SD. * $p < 0.05$, significantly different compared with healthy donor control (without lung cancer patient plasma or *GI-PS*); # $p < 0.05$, significantly different compared with lung cancer patient plasma control (without *GI-PS*); one-way analysis of variance followed by Dunnett's *t*-test.

of lung cancer and 159,480 deaths attributed to lung cancer in 2013. [2] In the US, more people die of lung cancer than colon, breast, and prostate cancers combined. Only 16% of lung cancers are diagnosed when the disease is still localized, while the majority of lung cancers are diagnosed at later stages, which results in a high mortality rate. The initiation

Fig. 6. Immunocytochemistry showing suppression by lung cancer patient plasma and antagonism by *Ganoderma lucidum* polysaccharides (*GI-PS*) on granzyme B expression on mononuclear lymphocytes induced by phytohemagglutinin (PHA). After a 48-h incubation, granzyme B expression on mononuclear lymphocytes to PHA was measured by immunocytochemistry. 1, Control wells containing lung cancer patient plasma; 2, wells containing lung cancer patient plasma and 0.2 $\mu\text{g/ml}$ of *GI-PS*; 3, wells containing lung cancer patient plasma and 0.8 $\mu\text{g/ml}$ of *GI-PS*; 4, wells containing lung cancer patient plasma and 3.2 $\mu\text{g/ml}$ of *GI-PS*; 5, wells containing lung cancer patient plasma and 12.8 $\mu\text{g/ml}$ of *GI-PS*; 6, control wells containing healthy donor plasma without *GI-PS*.

and progression of the lung cancer is associated with many factors, including immunity, cytogenetics [35], epithelial-mesenchymal transition [36], and sunlight [37]. Although the immune system with the potential to destroy tumor cells serves as the final natural defense against tumor development, tumor cells can escape from the host immune system by numerous mechanisms which suppress anti-tumor immune function [38]. Tumors possess both direct and indirect mechanisms by which immune function is suppressed; one of the direct mechanisms is the secretion of immune suppressive products, such as PGE₂, VEGF, IL-10, and TGF- β [39]. Therefore, the plasma of lung cancer patients may contain immune suppressive products and suppress immune function in lymphocytes.

CD69 is one of the earliest proteins expressed after leukocyte activation; CD69 is essential in the control of innate and adaptive immune responses [40]. CD69 is a widely expressed type II transmembrane glycoprotein related to the C-type animal lectins. CD69 exhibits regulated expression on a variety of cells of the hematopoietic lineage, including lymphocytes, and activation of T lymphocytes results in the induced expression of CD69 at the cell surface, while cross-linking of CD69 by specific antibodies leads to the activation of cells bearing this receptor and the induction of effector functions [41]. As one of the earliest cell surface antigens expressed by T cells following activation, CD69 is detectable within 1 h of ligation of the T cell receptor/CD3 complex, and once expressed, CD69 acts as a co-stimulatory molecule for T cell activation and proliferation [42]. In the current study, it was shown that CD69 expression on lymphocytes after stimulation with PHA was suppressed by lung cancer patient plasma, while this suppression was antagonized by *GI-PS*, indicating that lung cancer patient plasma may suppress lymphocyte activation, but *GI-PS* may antagonize this suppression.

The process of lymphocyte activation is very tightly regulated, resulting in the production of cytokines, as well as clonal expansion and differentiation of effector lymphocytes [43], accompanied by a cascade of signaling events that ultimately results in the induction of IL-2 gene expression, cell-cycle entry, proliferation, and T cell effector functions [44]. Therefore, the lymphocyte proliferation induced by mitogens, such as PHA, can disclose the activation of lymphocytes. It was shown in the current study that lymphocyte proliferation after stimulation with PHA was suppressed by lung cancer patient plasma, while this suppression was antagonized by *GI-PS*, indicating also that lung cancer patient plasma may suppress lymphocyte activation, while *GI-PS* may antagonize this suppression.

Cytotoxic lymphocytes rapidly respond and destroy malignant cells and cells infected with intracellular pathogens. Cellular apoptosis induced by cytotoxic lymphocytes is mainly mediated by two pathways: granule exocytosis employs the secretory granules of these lymphocytes; and signaling through death receptors of the TNF- α R superfamily, especially FasL. Cytotoxic lymphocytes are armed with granules that are released in the granule-exocytosis pathway to kill tumor and virus-infected cells. These granules include the pore-forming protein perforin (pfp) and a family of serine proteases (granzymes) that cleave and activate effector molecules within the target cell [45]. Perforin and granzyme B are two of the first proteins isolated from these granules. Perforin plays a central role in apoptosis induced by granzymes [46]. While perforin facilitates the entry of granzymes into a target cell, the latter initiate distinct apoptotic routes [47]. In the secretory granule-mediated cell death pathway, which is the key mechanism for elimination of virus-infected and transformed target cells by cytotoxic lymphocytes, the formation of the immunologic synapse between an effector and a target cell leads to exocytic trafficking of the secretory granules and the release of their contents, which include pro-apoptotic serine proteases, granzymes, and pore-forming perforin into the synapse. In the synapse, perforin polymerizes and forms a transmembrane pore that allows the delivery of granzymes into the cytosol, where various apoptotic death pathways are initiated [48]. Therefore, perforin and granzyme B in the lymphocytes induced by mitogens, such as PHA, are closely associated with the function of cytotoxic lymphocytes. It was shown in the current study that the expression of perforin and granzyme B in lymphocytes after stimulation with PHA is suppressed by lung cancer patient suppression, while this suppression was antagonized by *GI-PS*, which may further indicate that lung cancer patient plasma may suppress lymphocyte function, while *GI-PS* may antagonize this suppression.

Considering that the most well-known and best-characterized tumor-derived immunosuppressive molecules are IL-10, TGF- β , VEGF [49], and PGE2 [50], the most likely mechanism by which *GI-PS* antagonizes the immunosuppression by lung cancer patient plasma may involve the immunosuppressive molecules in the plasma derived from tumors. To achieve this antagonism, there are different ways to promote the degradation of immunosuppressive molecules and inhibition of the receptors and signaling pathways. The definite mechanisms underlying lung cancer patient plasma may be demonstrated in the future.

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Conflict of Interest

The authors report no declarations of interest.

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