


The Possible Role of PD-I Protein in *Ganoderma lucidum*-Mediated Immunomodulation and Cancer Treatment

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Abstract

Background: *Ganoderma lucidum* has been used in Chinese medicine for thousands years to improve health and to promote longevity. One important function of *G lucidum* is to modulate the immune system. However, the underlying mechanism is not well understood. Programmed cell death protein 1 (PD-I) is a cell surface protein present in certain immune cells (eg, B- and Tcells) and plays an important role in modulating the immune response. The role of PD-I protein in *G lucidum*-mediated immunomodulation is unknown. **Methods:** Cultured human Blymphocytes and extract prepared from *G lucidum* spores (GLE) were used to determine PD-I protein in *G lucidum*-mediated immunomodulation. Both western blotting and immunofluorescence (IF) microscopy assays were used to determine the effect of GLE treatment on PD-I protein expression. A reverse transcription-based quantitative polymerase chain reaction (real-time PCR) assay was used to determine the effect of GLE on transcription of *pdcd-1* gene. **Results:** Both our western blotting and IF staining results demonstrated great reduction in PD-I protein and in proportion of PD-I⁺ cells in these B-lymphocytes. Our real-time PCR results indicated that this PD-I protein reduction was not caused by a transcriptional inhibition of the gene. In addition, our western blotting study further revealed that the GLE treatment caused an increase in expression of CCL5 chemokine in the cultured B-lymphocytes. **Conclusions:** PD-I protein is an important target of *G lucidum*-mediated immunomodulation. *G lucidum* and its bioactive compounds can be developed into novel immunomodulators for prevention and treatment of cancer and many other diseases.

Keywords

Ganoderma lucidum, *Ganoderma lucidum* spore extract, GLE, immunomodulation, PD-I, B-lymphocytes, protein reduction, transcription inhibition, novel immunomodulators

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Introduction

Fungi have been used in traditional Chinese medicine for thousands of years to prevent and treat a variety of diseases including allergies, arthritis, hypertension, hepatitis, and inflammation, among others.^{1–8} *Ganoderma lucidum*, which also is known as Lingzhi (in China) or Reishi (in Korea and Japan), is a well-known medicinal fungus that has been used to improve health and to promote longevity.^{1,2,4} Recent studies demonstrated the effects of *G lucidum* on

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immunomodulation and antitumor properties,⁹⁻¹⁵ although the underlying molecular mechanisms are not fully understood.

Many bioactive compounds are carried by *G lucidum*.^{2,3,7,13,16} Based on their chemical structures, these compounds can be categorized into 2 major groups: triterpenoids and polysaccharides.^{2,5-7,11-15} There are over 150 different triterpenoid products, including ganoderic acids and ganoderma alcohols (Ganoderiols), identified in *G lucidum*.^{13,17} The polysaccharides carried by *G lucidum* contain many different sugar compositions (eg, glucose, mannose, galactose, and glucuronic acid) and are generally in the range of 13 kDa to 23 kDa.^{11,15,18} It is generally believed that the polysaccharides function as immunomodulators, whereas the triterpenoids inhibit cancer cell proliferation and metastasis.^{3,10} Both fruit body and spores of *G lucidum* carry polysaccharides and triterpenoids, although high levels of these contents are found mostly in the spores of *G lucidum*. The contents of spores cannot be easily released because of a 2-layer wall structure unless the wall structure is removed or punctured.

Programmed cell death protein 1, also known as PD-1 and CD279, is a cell membrane protein encoded by *pdc1-1* gene.¹⁹ PD-1 protein is expressed in many types of immune cells, including B- and T-lymphocytes.²⁰⁻²⁶ PD-1 protein interacts with programmed death ligand 1 (PD-L1), which is expressed in dendritic cells, macrophages, and many types of cancer cells, to regulate the immune system.²⁰⁻²⁶ The interaction of PD-1 with PD-L1 inhibits T-cell proliferation, suppresses T-cell inflammatory activity, and prevents autoimmune diseases.²⁰⁻²⁸ However, this interaction also prevents the immune system from killing tumor cells, leading to cancer development. Therefore, strategies to block the PD-1/PD-L1 interaction have attracted great attention in cancer prevention and treatment. Antibodies that target PD-1 and PD-L1 have been used in treating many types of cancer.²⁹⁻³³ However, many challenges exist in applying the antibody-based immunotherapy to cancer treatment.³⁴ Developing alternative approaches or identifying new compounds that can more effectively disrupt the PD-1/PD-L1 interactions, therefore, would be important for PD-1/PD-L1-based cancer therapy in the future.

In our recent studies, we investigated the possible role of PD-1 protein in *G lucidum*-mediated immunomodulation. Using extract prepared from wall-broken *G lucidum* spores (GLE), the results of our western blotting demonstrated that treatment of GM00130 and GM02248 human B-lymphocytes with GLE caused a great reduction of PD-1 protein in these cells. The results of our immunofluorescence (IF) microscopy study revealed that the GLE treatment caused a reduction of PD-1⁺ cells in the cultured GM00130 B-lymphocytes. However, the results of our real-time polymerase chain reaction (PCR) studies indicated that the transcription level of *pdc1-1* gene was not inhibited by the GLE treatment in these

cells, which rules out the possibility of transcriptional inhibition of *pdc1-1* gene for GLE-mediated reduction of PD-1 protein. In addition, the results of our western blotting studies revealed that the GLE treatment caused an increase of chemokine CCL5 in both GM00130 and GM02248 human B-lymphocytes. These results suggest that reducing PD-1 protein is an important mechanism for *G lucidum*-mediated immunomodulation, and the GLE-mediated PD-1 protein reduction is not caused by a transcriptional inhibition of the *pdc1-1* gene. These results further suggest that *G lucidum* and its contents can be developed into novel immunomodulators to target the PD-1 protein for treatment of cancer and many other diseases.

Materials and Methods

Cells, Chemicals, and Oligonucleotides Used in the Study

The GM00130 and GM02248 cells were purchased from the Coriell Institute for Medical Research (Camden, NJ). Both GM00130 and GM02248 cells were Epstein-Barr virus-transformed human B-lymphocytes. The GM00130 B-lymphocytes were derived from a normal individual, whereas the GM02248 B-lymphocytes were derived from a patient who carried a mutant XPC protein, which is required for nucleotide excision repair process.³⁵⁻³⁷ Both GM00130 and GM02248 B-lymphocytes were maintained in RPMI1640 medium supplemented with 15% fetal bovine serum at 37°C with 5% CO₂.

The phorbol 12-myristate 13-acetate (PMA) was purchased from the Sigma Aldrich Inc (St. Louis, MO). The ionomycin (Io) was purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals used in this study were purchased from the Sigma Aldrich Inc. The PMA was prepared as 5 mg/mL stock in dimethyl sulfoxide and stored at -80°C. The Io was purchased as 10 mM stock in ethanol and stored at -80°C. The Io was diluted to 1 mM in 1× phosphate-buffered saline (PBS) and used for cell treatment immediately.

The oligonucleotides used in this study were synthesized by Midland Certified Reagent Company (Midland, TX), and the sequences of these oligonucleotides are listed in Table 1.

Preparation of *G lucidum* Spore Extract

The wall-broken *G lucidum* spores powder was obtained from Longevity Valley Pharmaceuticals Inc (People's Republic of China). The GLE was prepared as follows: the *G lucidum* spore powder was weighed and resuspended in 10 times 95% ethanol (w/v) and incubated at 85°C for 2 hours. The supernatant was collected after centrifugation (10000 rpm × 10 minutes at 4°C). The pellets were

Table 1. Primers Used in the Real-Time PCR Study.

<i>pdc-1</i> forward primer	5'-CAGCCTGGTGCTGCTAGTCTG-3'
<i>pdc-1</i> reverse primer	5'-GTCCACAGAGAACACAGGCAC-3'
<i>PD-L1</i> forward primer	5'-GTGTACCGCTGCATGATCAG-3'
<i>PD-L1</i> reverse primer	5'-GGTAGCCCTCAGCCTGACATG-3'
<i>Bcl_{XL}</i> forward primer	5'-GAGGTGATCCCCATGGCAGC-3'
<i>Bcl_{XL}</i> reverse primer	5'-CCAAGCTGCGATCCGACTCAC-3'
<i>CCL5</i> forward primer	5'-CTGCGCTCCTGCATCTGCCTC-3'
<i>CCL5</i> reverse primer	5'-GAGTTGATGTACTCCCGAAC-3'
<i>p16</i> forward primer	5'-GAGCCTTCGGCTGACTGGCTG-3'
<i>p16</i> reverse primer	5'-CCGTGGAGCAGCAGCAGCTC-3'
<i>p53</i> forward primer	5'-AGCAGTCACAGCACATGACG-3'
<i>p53</i> reverse primer	5'-CACTCGGATAAGATGCTGAG-3'

Abbreviation: PCR, polymerase chain reaction.

resuspended into another 10 times 95% ethanol (w/v) and incubated at 85°C for 2 hours. The supernatant was collected by centrifugation. The supernatants were combined, filtered through a 0.22 μm filter, and dried in CentriVap concentrator (Labconco). The dried pellets were weighted and resolved into 1 × PBS at a concentration of 100 mg/mL and filtered through a 0.22 μm filter. The solution was designated as GLE and stored at -80°C for further use.

Inducing Expression of PD-1 Protein

The cultured GM00130 and GM02248 B-lymphocytes were treated with both PMA (50 ng/mL) and Io (1 μM) by adding the stock solutions directly to the culture medium and incubated at 37°C cell culture incubator for 48 hours. Levels of *pdc-1* mRNA and PD-1 protein were determined by reverse transcription-based quantitative PCR (qPCR, real-time PCR) and western blotting, respectively.

GLE Treatment

The cultured GM00130 and GM02248 human B-lymphocytes were treated with PMA/Io for 24 hours to induce expression of the PD-1 protein. The PMA/Io-treated cells were then treated with GLE by adding the GLE directly into the cell growth medium. For the real-time PCR study, cells were collected 24 hours after the GLE treatment and total RNA was isolated from the harvested cells using the RNeasy Mini Kit (Qiagen Inc, Valencia, CA). For the immunoblotting study, cells were collected 48 hours after the GLE treatment and lysed in RIPA cell lysis buffer.

Cell Growth Curve Assay

For the cell growth study, both GM00130 and GM02248 human B-lymphocytes were treated with GLE at indicated concentrations (0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL) in

the presence or absence of PMA (50 ng/mL)/Io (1 μM). The cell density was determined for each treatment at various time points after the GLE treatment (days 0, 1, 2, 3, and 4) using Invitrogen's Countess automated cell counter. The cell growth study was performed in 3 independent experiments, and the cell growth curve was determined for each treatment using a GraphPad Prism software (La Jolla, CA).

Fluorescence-Activated Cell Sorting (FACS) Assay

For FACS study, cells were collected 48 hours after the GLE treatment. Cells were washed once in 10 mL 1 × PBS/1% bovine serum albumin buffer and then resuspended into 100 μL flow cytometry staining buffer (1 × PBS, 1% bovine serum albumin, and 0.1% sodium azide). The cells were stained with allophycocyanin (APC)-labeled mouse anti-human CD279 (PD-1) antibody (MIH4, eBioscience) by mixing the cells with 5 μL APC-labeled PD-1 antibody and incubating on ice for 30 minutes. The cells were washed 3 times in flow cytometry staining buffer (1 mL × 3) to remove any free PD-1 antibody. The cells were finally resuspended in 1 mL flow cytometry staining buffer and counterstained with a DAPI reagent for visualizing nuclei of live cells. The cells were analyzed by flow cytometry to determine the percentage of PD-1⁺ cell population in each treatment.

Immunofluorescence Microscopy Assay

For IF microscopy study, the GM00130 human B-lymphocytes were stained with APC-labeled mouse anti-human CD279 (PD-1) antibody and resuspended into 1 mL flow cytometry staining buffer. The nuclei of cells were counterstained with a DAPI reagent. Twenty microliters of cells was placed onto Invitrogen's Countess cell counting chamber slide. The PD-1⁺ cells were visualized by a Zeiss LSM 780 fluorescence microscope using red light (630 nm), and the nuclei of cells were visualized by blue

light (480 nm). A total of 250 to 300 cells were counted for each treatment, and the percentage of PD-1⁺ cells were determined for each treatment.

Reverse Transcription-Based qPCR (Real-Time PCR) Assay

RNA isolated from both untreated and treated GM00130 and GM02248 human B-lymphocytes were used in the real-time PCR assay. A reverse transcription reaction was used to generate cDNA from the RNA samples using a High-Capacity cDNA Reverse transcription Kit (Applied Biosystems Inc, Foster City, CA). The cDNAs were then used as templates in a qPCR protocol to determine the mRNA levels of *pdcf-1*, *PD-L1*, *Bcl_{XL}*, *CCL5*, *p16*, and *p53* using a Power Sybr Green PCR Master Mix (Applied Biosystems Inc) with primers designed to bind to the coding region sequences of these genes. The mRNA level of β -actin was also determined from each cDNA sample and used as an internal control for the real-time PCR study. The mRNA level of β -actin in the untreated GM00130 B-lymphocytes was used as a standard, and the levels of *pdcf-1*, *PD-L1*, *Bcl_{XL}*, *CCL5*, *p16*, and *p53* mRNAs in each RNA sample were calculated as fold change compared with that of the untreated GM00130 B-lymphocytes. Triplicates were used for each sample in the real-time PCR study, and 3 independent experiments were done for the real-time PCR study. The mRNA level of each target gene was calculated as mean fold + standard deviation (SD) to that of the GM00130 B-lymphocytes. Statistical analysis was done to determine if any significant difference in the level of mRNA exists for each tested gene between untreated and treated cells for GM00130 and GM02248 B-lymphocytes (* $P \leq .05$; ** $P \leq .01$).

Western Blotting Assay

The immunoblotting study was performed using SDS-PAGE with 10% or 12% gel, and 15 μ g total protein/lane was used for each cell lysate. After transferring the proteins to polyvinylidene difluoride membranes, the membranes were hybridized with antibodies against PD-1 (AF1086, R & D System), RANTES (CCL5; C-12, Santa Cruz Biotechnology, Dallas, TX), CD19 (PDR134, Santa Cruz Biotechnology), AKT1 (B1, Santa Cruz Biotechnology), or β -actin (C4, Santa Cruz Biotechnology). Quantification of the proteins bands on X-ray films was done using an ImageJ software (<https://imagej.nih.gov/ij/>).

Data Analysis

All data were presented as mean + SD. Statistically significant differences were determined using a Student's *t* test with 95% confidence interval (CI). The data were obtained from at least 3 independent experiments.

Results

The Effect of GLE Treatment on Proliferation of GM00130 and GM02248 Human B-Lymphocytes

Many studies have demonstrated the effects of *G lucidum* on immunomodulation.^{10,11,15} Therefore, we first determined the effect of GLE on proliferation of immune cells. Since our work was focused on determining the role of PD-1 protein in *G lucidum*-mediated immunomodulation and results of studies have demonstrated expression of the PD-1 protein in various types of immune cells, including B- and T-cells,²⁰⁻²⁶ we chose both GM00130 and GM02248 human B-lymphocytes in this study as a model. We also used both PMA and Io to induce expression of PD-1 in cultured B-lymphocytes in this work because the work of others has demonstrated the induced expression of PD-1 in B- and T-cells by PMA/Io.³⁸⁻⁴⁰

Both GM00130 and GM02248 B-lymphocytes were treated with GLE at various concentrations (0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL) by adding the GLE directly into the cell growth medium. At various time points (days 0, 1, 2, 3, and 4), cell density was determined and the cell growth curve was determined for each treatment (Figure 1). The results of our cell proliferation studies indicated that the cell growth of both GM00130 and GM02248 human B-lymphocytes were not greatly affected by the GLE at concentrations as high as 0.5 mg/mL (Figure 1). At 1 mg/mL GLE concentration, however, some clear inhibitory effects were observed in proliferation of these cells (Figure 1). Therefore, the 0.5 mg/mL GLE was chosen for our PD-1 protein study.

The GLE Treatment Caused Reduction of the PD-1 Protein in Both GM00130 and GM02248 Human B-Lymphocytes

To study a mechanism through which the presence of GLE causes immunomodulation, we focused on determining the role of PD-1 protein on this action because of its important function in immunomodulation.²⁰⁻²⁶ The clinical implication of PD-1/PD-L1-based immunotherapy in cancer treatment²⁹⁻³³ also prompted us in studying the role of PD-1 in *G lucidum*-mediated immunomodulation.

Both GM00130 and GM02248 human B-lymphocytes were treated with PMA/Io to induce expression of PD-1 protein in these cells. The cells were then treated with GLE (0.5 mg/mL) for 48 hours. The cells were harvested and lysed in RIPA cell lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM Tris, pH 7.4). The level of PD-1 protein was determined for each cell lysate by an immunoblotting assay (Figure 2). As controls, the protein levels of CD19, which is a cell membrane protein expressed on B-lymphocytes, and AKT1, which is an intracellular

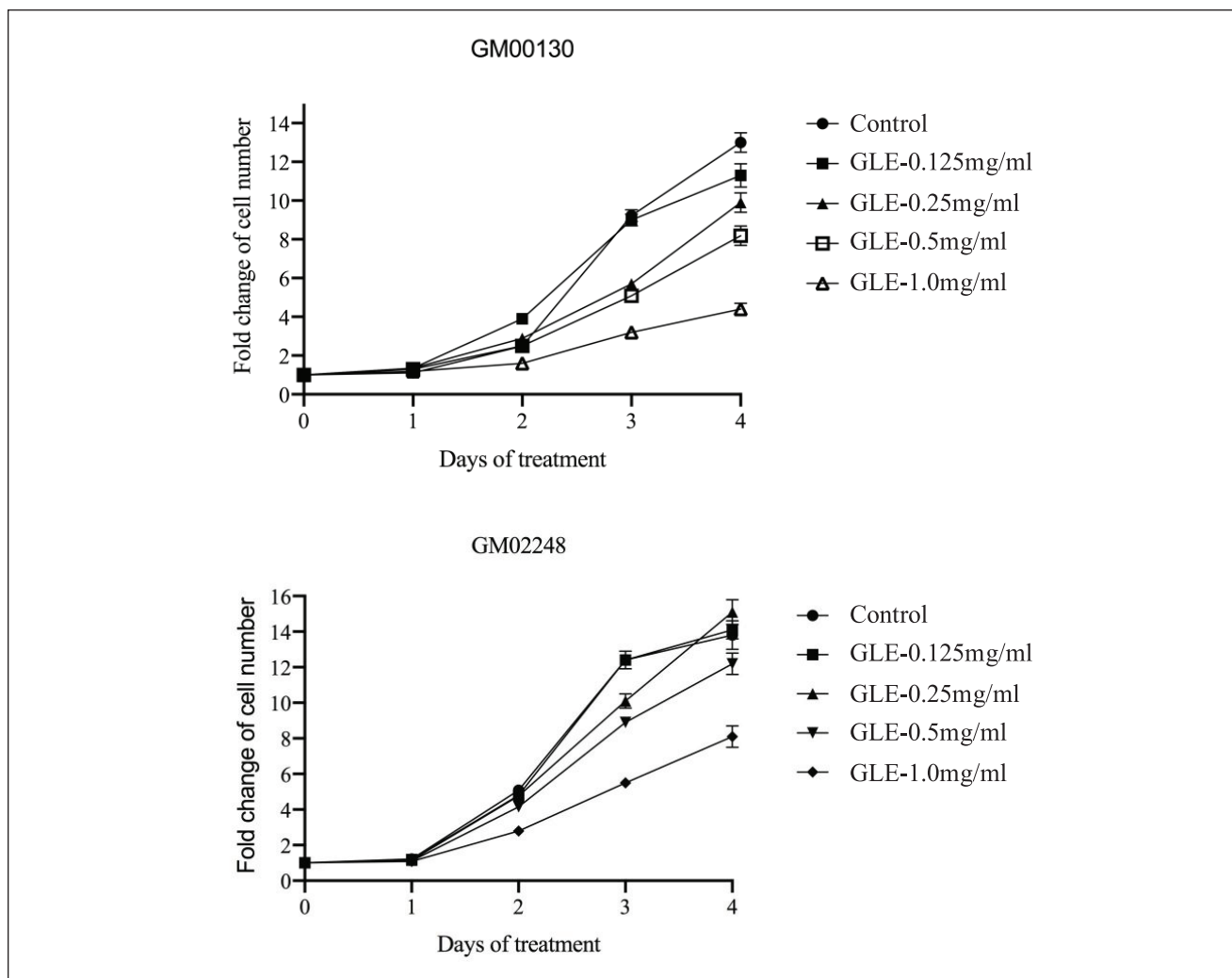


Figure 1. The growth curve of GM00130 and GM02248 human B-lymphocytes in the presence of various concentrations of GLE. Cells were treated with GLE at indicated concentration by adding the GLE directly into the cell growth medium. The cell density was determined at various time points after the GLE treatment (days 0, 1, 2, 3, and 4), and the cell growth curve was generated from the cell density obtained from this study (mean \pm SD). The results were based on 3 independent experiments. The cell growth curve was generated by GraphPad Prism software (La Jolla, CA).

protein expressed in most cell types including B cells, were also determined from the cell lysates (Figure 2). Low levels of PD-1 protein were detected from both GM00130 and GM02248 human B-lymphocytes (Figure 2A and B). The treatment of PMA/Io increased the PD-1 protein level to 11.9-fold in GM00130 and 12.9 fold in GM02248 B-lymphocytes (Figure 2B and D). The treatment of GM00130 and GM02248 human B-lymphocytes with GLE, however, greatly reduced the level of PD-1 protein in the PMA/Io-treated cells to 0.14 fold and 1.71 fold to that of the untreated GM00130 and GM02248 B-lymphocytes, respectively (Figure 2B and D). As controls, the protein levels of CD19 and AKT1 were not affected by the GLE treatment in these cells (Figure 2A and C). (The Supplement Figure 1 contains more controls, including cells treated with DMSO

alone as a negative control). These results clearly demonstrated that PD-1 protein was an important target for GLE treatment and the GLE treatment caused great reduction of the PD-1 protein in the human B-lymphocytes.

The GLE Treatment Resulted in a Decrease of PD-1⁺ Cells in GM00130 Human B-Lymphocytes

The results of our immunoblotting studies demonstrated the greatly reduced levels of PD-1 protein in the GLE-treated GM00130 and GM02248 human B-lymphocytes. To further evaluate the effect of GLE treatment on expression of PD-1 protein, we determined the status of PD-1⁺ cells in both untreated and GLE-treated GM00130 human B-lymphocytes.

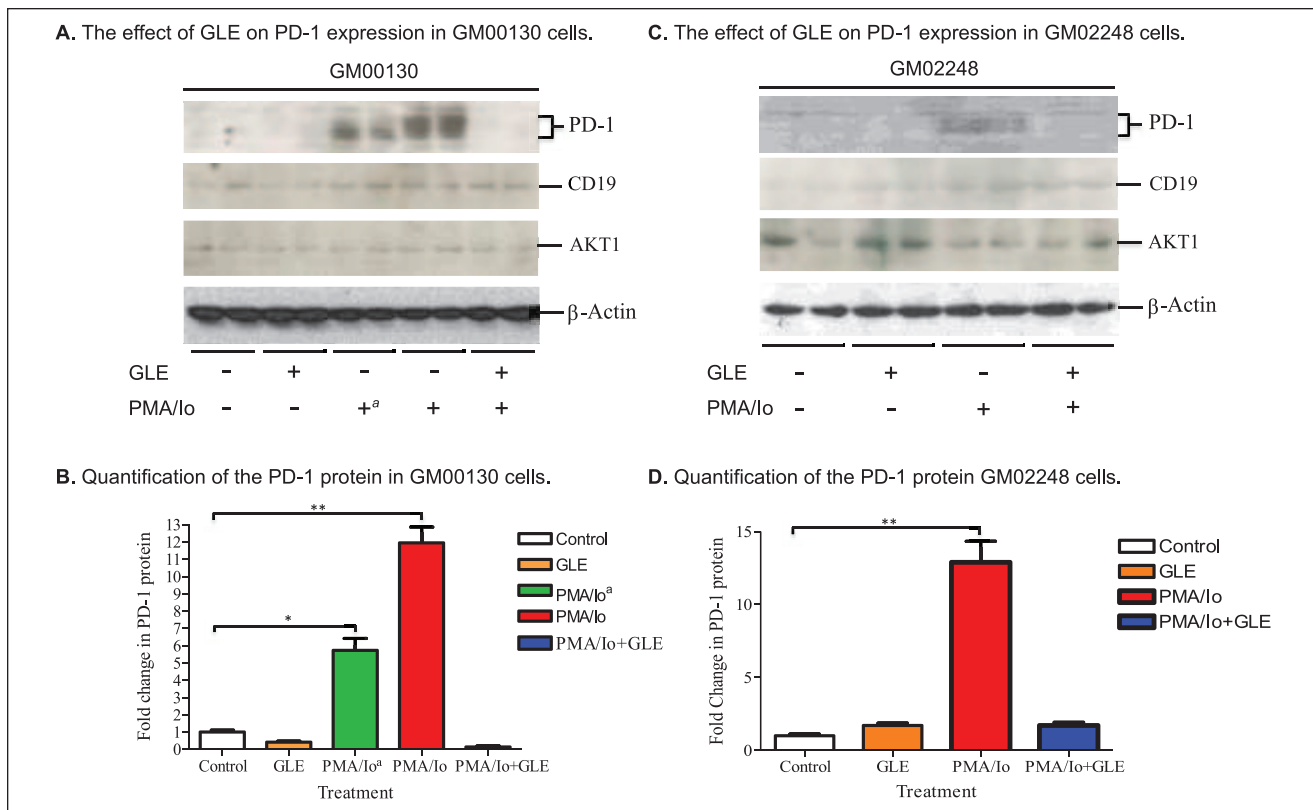


Figure 2. The effect of GLE on expression of PD-1 protein in both GM00130 and GM02248 human B-lymphocytes. The cells were first treated with PMA/Io for 24 hours. Some of the cells were then treated with GLE (0.5 mg/mL) for 48 hours. Cells lysates prepared from the cells (15 μ g protein/lane) were analyzed by western blotting. Quantification of the PD-1 protein was the result of 4 independent western blots (* $P \leq .05$; ** $P \leq .01$).

The effect of GLE treatment on the number of PD-1⁺ cells was first determined by a FACS assay. The GM00130 human B-lymphocytes were treated with GLE in the presence or absence PMA/Io for 48 hours. The cells were harvested and stained with an APC-labeled mouse anti-human PD-1 antibody. The cells were analyzed by a FACS assay to determine the proportion of PD-1⁺ cells in each treatment (Figure 3). A low percentage of PD-1⁺ cells (11.3%) was detected in the cultured GM00130 human B-lymphocytes in our FACS assay (Figure 3A). When treated with PMA/Io, the PD-1⁺ cells were increased to 24.4% (Figure 3B). The GLE treatment decreased the PD-1⁺ cells from 11.3% to 8.54% in cultured GM00130 cells and from 24.4% to 21.7% in PMA/Io-treated GM00130 cells, respectively (Figure 3C vs A and D vs B).

Although some decrease of PD-1⁺ cells was observed in the GLE-treated GM00130 B-lymphocytes in our FACS study, this rate of decrease was not as great as we anticipated based on our western blot results. To further evaluate the effect of GLE treatment on the number of PD-1⁺ cells, we also performed an IF microscopy assay to determine the effect of GLE treatment on the number of PD-1⁺ cells using the cells prepared for our FACS assay (Figure 4 and Table 2).

The results of our IF microscopy study revealed that 3.7% of PD-1⁺ cells exist in the cultured GM00130 human B-lymphocytes (Figure 4 and Table 2). The PMA/Io treatment increased the PD-1⁺ cells to 19.2% in the cultured GM00130 human B-lymphocytes (Figure 4 and Table 2). When treated with GLE, however, the percentage of PD-1⁺ cells was decreased to 1.8% and 4.5% in GM00130 and PMA/Io-treated GM00130 B-lymphocytes, respectively (Figure 4 and Table 2). The results of this IF microscopy analysis clearly demonstrated that the GLE treatment caused a reduction of PD-1⁺ cells in the GM00130 human B-lymphocytes.

The GLE Treatment Resulted in an Increase in Expression of CCL5 Protein in Both GM00130 and GM02248 Human B-Lymphocytes

The results of both our immunoblotting and IF microscopy studies demonstrated the effect of GLE on reducing PD-1 protein in both GM00130 and GM02248 human B-lymphocytes. To further understand the effect of *G lucidum*-mediated immunomodulation, we determined the level of chemokine C-C motif ligand 5 (CCL5) in both

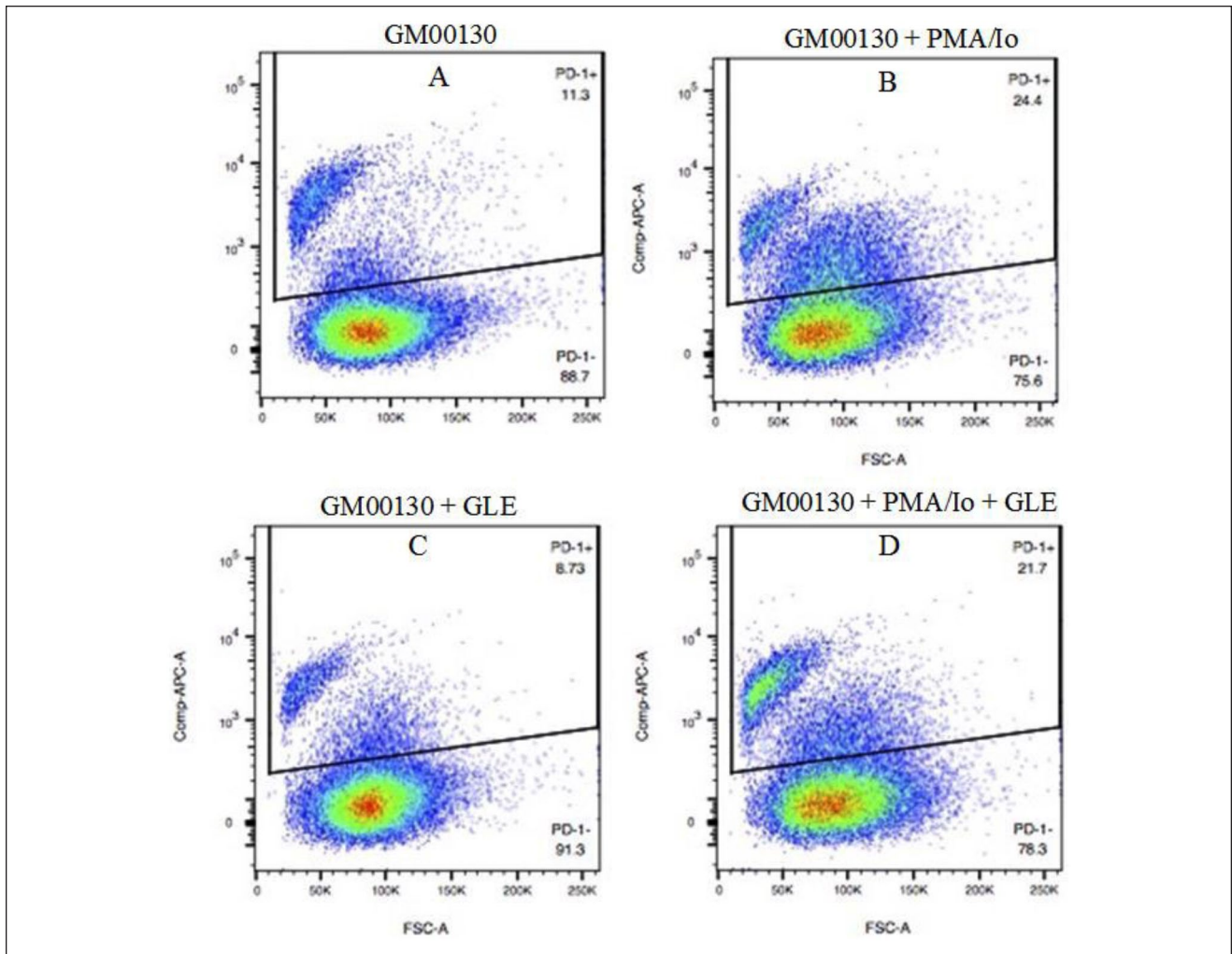


Figure 3. The effect of GLE on PD-1⁺ cells in GM00130 human B-lymphocytes by FACS. The GM00130 human B-lymphocytes were treated with GLE (0.5 mg/mL) in the absence or presence of PMA (50 ng/mL)/Io (1 μ M) at 37°C in cell culture incubator for 48 hours. The cells were harvested and stained with APC-labeled mouse antihuman PD-1 antibody (MIH4, eBioscience) and counterstained with a DAPI reagent. The cells were analyzed by FACS to determine the percentage of PD-1⁺ cells in each treatment. The flow cytometry data were from 3 independent experiments.

untreated and GLE-treated GM00130 and GM02248 human B-lymphocytes by western blotting assay. CCL5 is a chemokine that plays a very important role in recruiting immune cells (eg, T-cells and eosinophils) to tumors and in proliferating immune cells.⁴¹⁻⁴⁴ Recent work of others demonstrated that blockade of PD-1 protein with a PD-1 antibody resulted in an increase in expression of CCL5 and enhanced migration of T-cells to tumors.^{42,44}

The results of our western blotting revealed that the CCL5 chemokine was expressed at very low levels in both untreated GM00130 and GM02248 human B-lymphocytes (Figure 5A/B and C/D). The PMA/Io treatment had little effect on CCL5 protein expression in both GM00130 and GM02248 human B-lymphocytes (Figure 5A/B and C/D). In the presence of GLE, however, the level of CCL5 was

significantly more increased, especially in the cells that were treated with both PMA/Io and GLE (Figure 5A/B and C/D). This result suggests that the GLE treatment caused significantly more increase of the CCL5 chemokine in both GM00130 and GM02248 human B-lymphocytes.

The GLE Treatment Did Not Inhibit Transcription of pdcd-1 Gene in Either GM00130 or GM02248 Human B-Lymphocytes

The results of our immunoblotting studies demonstrated the effect of GLE treatment on reducing expression of PD-1 protein in both cultured GM00130 and GM02248 human B-lymphocytes. To define a mechanism through which the GLE treatment causes decreased PD-1 protein in these

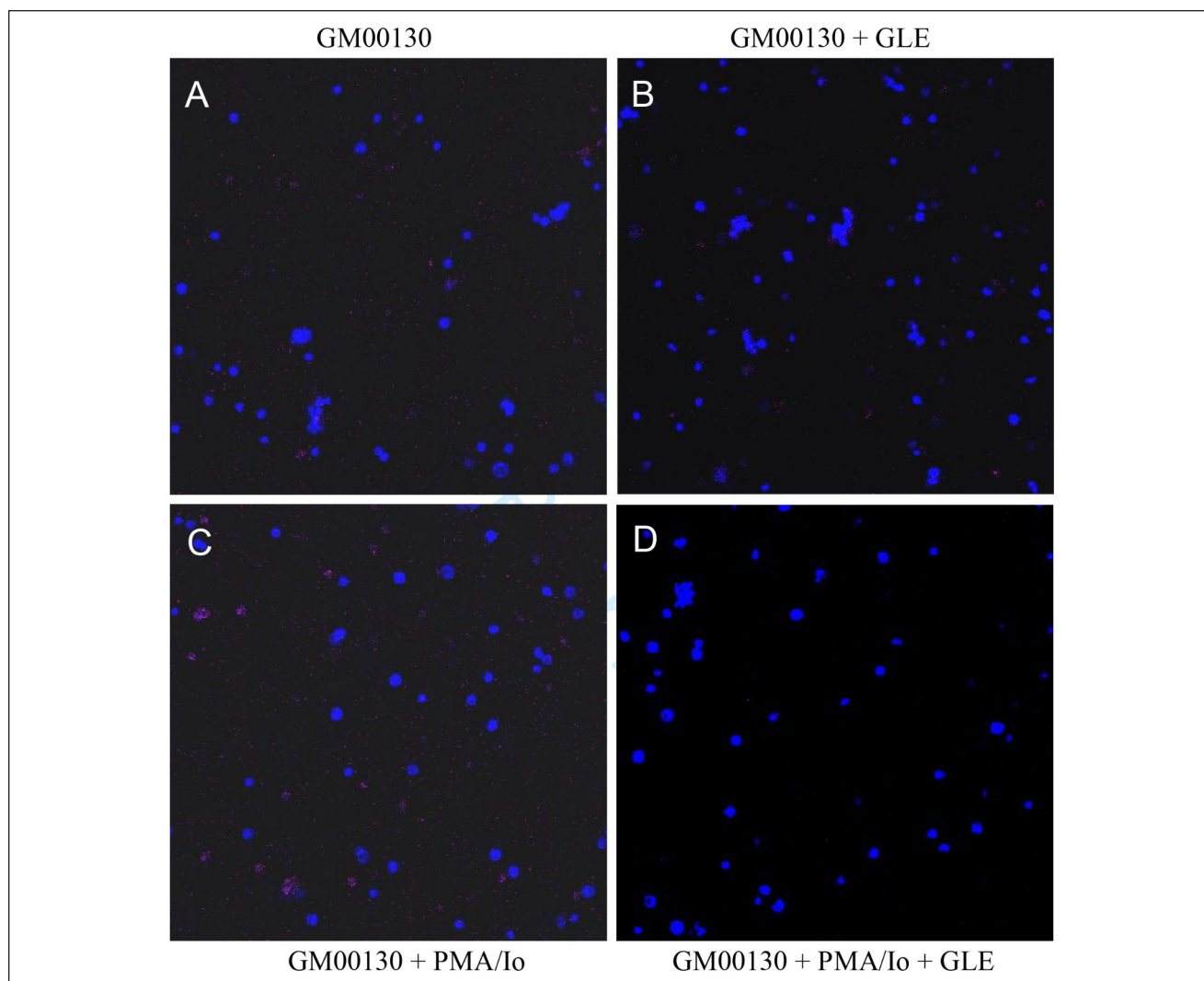


Figure 4. The effect of GLE on PD-1⁺ cells in GM00130 human B-lymphocytes by immunofluorescence (IF) microscopy assay. The GM00130 human B-lymphocytes were treated with GLE (0.5 mg/mL) in the absence or presence of PMA (50 ng/mL)/Io (1 μ M) at 37°C for 48 hours. The cells were harvested, stained with an APC-labeled mouse antihuman PD-1 antibody, and counterstained with a DAPI reagent. The PD-1⁺ cells were visualized by a Zeiss LSM 780 fluorescence microscope using red light (630 nm). The nuclei of cells were visualized under the fluorescence microscope using a blue light (480 nm). The IF microscopy study was done in 3 independent experiments.

Table 2. The Effect of GLE Treatment on PD-1⁺ Cells in the Cultured GM00130 Human B-Lymphocytes. The Number of Both PD-1⁺ and PD-1⁻ Cells Were Counted From 3 Views/Reactions.

Treatment	No. of PD-1 ⁻ Cells	No. of PD-1 ⁺ Cells	Total No. of Cells	% of PD-1 ⁺ Cells
Control	258	10	268	3.7%
PMA/Io	232	55	287	19.2%
GLE	219	4	223	1.8%
PMA/Io + GLE	276	13	289	4.5%

Abbreviations: GLE, *Ganoderma lucidum* spores extract; PMA, phorbol 12-myristate 13-acetate; Io, ionomycin.

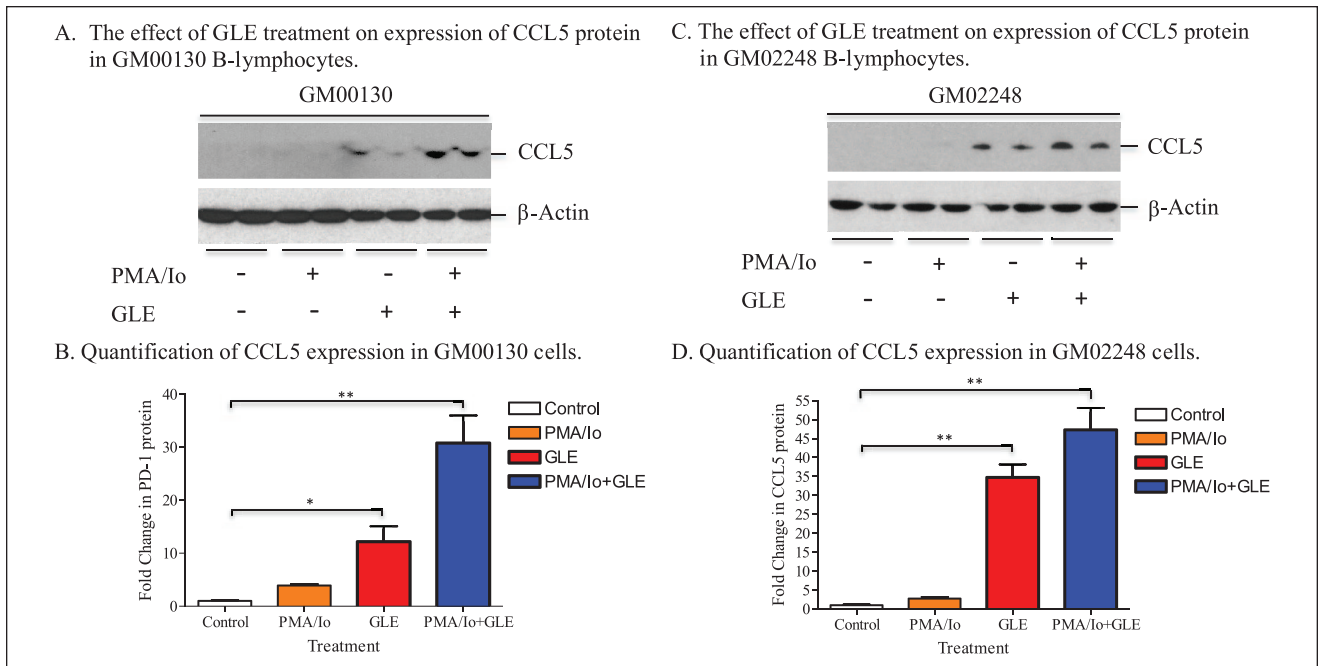


Figure 5. The effect of GLE on expression of CCL5 protein in both GM00130 and GM02248 human B-lymphocytes. Both GM00130 and GM02248 human B-lymphocytes were treated with GLE (0.5 mg/mL) in the presence or absence of PMA (50 ng/mL)/Io (1 μ M) at 37°C cell culture incubator for 48 hours. The cells were collected, and 15 μ g total protein/lane was used in the western blotting with 13% gel. The CCL5 (C-12) antibody was purchased from Santa Cruz Biotechnologies Inc. Quantification of the CCL5 protein was the result of 4 independent western blots (* $P \leq .05$; ** $P \leq .01$).

cells, we further performed a reverse transcription-based qPCR (real-time PCR) study to determine the mRNA level of *pdc1-1* genes, which encodes the PD-1 protein, in both untreated and GLE-treated GM00130 and GM02248 human B-lymphocytes (Figure 6). As controls, the mRNA levels of several genes, including *Bcl_{XL}*, *CCL5*, *p16*, *p53*, and *PD-L1*, were also determined from the RNA samples by the real-time PCR assay (Figure 6). The results of our real-time PCR study indicated that the PMA/Io treatment caused a moderate increase of *pdc1-1* mRNA in both GM00130 and GM02248 human B-lymphocytes (Figure 6). Interestingly, the GLE treatment caused significant more increases in the level of *pdc1-1* mRNA in both GM00130 and GM02248 human B-lymphocytes (Figure 6), and the combinational treatment of both PMA/Io and GLE caused even more increases of the *pdc1-1* mRNA in both GM00130 and GM02248 human B-lymphocytes (Figure 6). The mRNA levels of the *Bcl_{XL}*, *CCL5*, *p16*, *p53*, and *PD-L1* genes were also significantly more increased in the GLE-treated GM00130 and GM02248 B-lymphocytes than those untreated or PMA/Io-treated GM00130 or GM02248 B-lymphocytes (Figure 6). These results strongly suggest that transcriptional inhibition of the *pdc1-1* gene was not a mechanism for GLE-mediated PD-1 reduction in GM00130 or GM2248 human B-lymphocytes.

Discussion

Ganoderma lucidum has been used in traditional Chinese medicine for thousands of years to promote better health and to treat various diseases.^{1-8,45,46} One important benefit of *G lucidum* consumption is its ability to modulate the immune system, which enhances the immune response to fight against development of many diseases.¹⁰⁻¹⁵ Although many studies have been done in determining the medicinal effects of *G lucidum*, the mechanism that leads to the immunomodulating effect of *G lucidum* is largely unknown. In this work, we focused on determining the role of PD-1 protein in GLE-mediated immunomodulation using cultured human B-lymphocytes as a model. The results of our immunoblotting studies demonstrated that the GLE treatment caused a great reduction of the PD-1 protein in both GM00130 and GM02248 human B-lymphocytes. The results of our FACS and IF microscopic studies revealed that the GLE treatment led to a decrease in PD-1⁺ cells in GM00130 human B-lymphocytes. However, the results of our real-time PCR studies indicated that the GLE treatment did not inhibit transcription of the *pdc1-1* gene in either GM00130 or GM02248 human B-lymphocytes, eliminating the possible role of transcriptional inhibition for GLE-mediated PD-1 protein reduction in these human

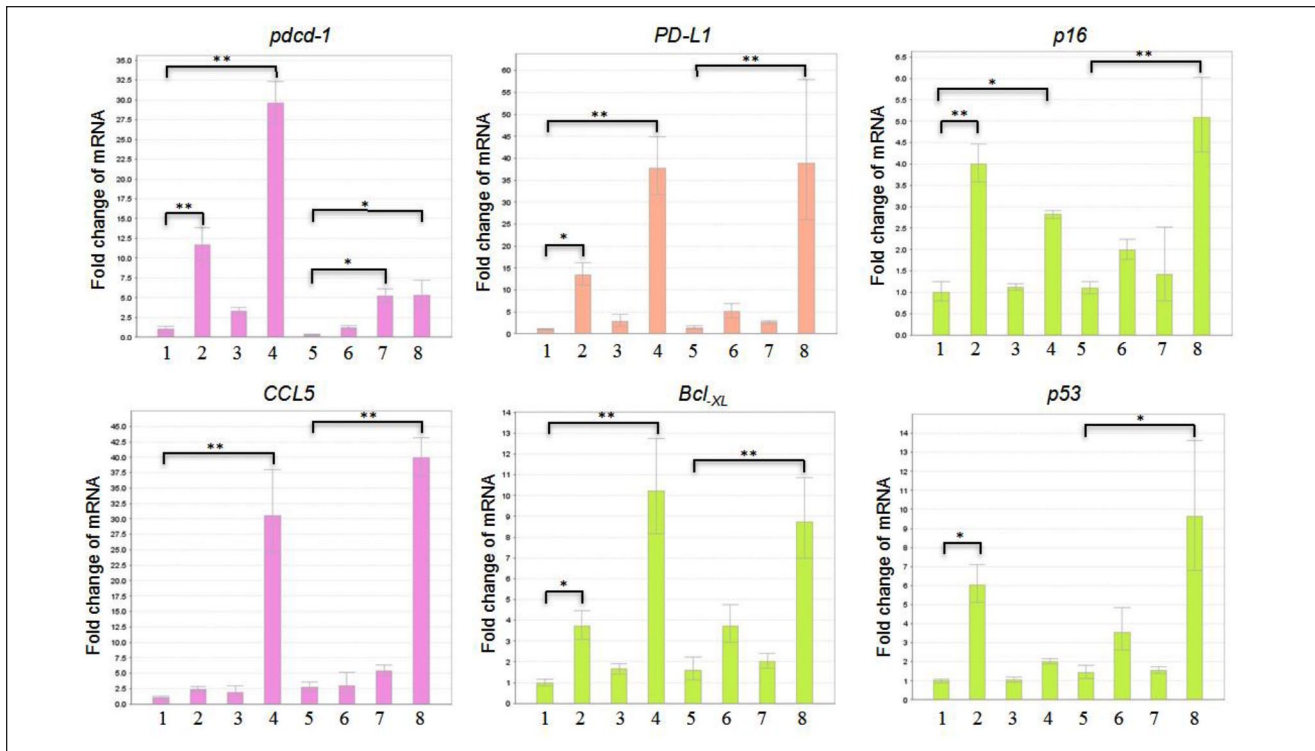


Figure 6. The effect of GLE on transcriptions of *pdcd-1*, *Bcl_{XL}*, *CCL5*, *p16*, *p53*, and *PD-L1* genes in both GM00130 and GM02248 human B-lymphocytes. The cells were treated with GLE (0.5 mg/mL) in the absence or presence of PMA (50 ng/mL)/lo (1 μ M) for 24 hours, and total RNA was isolated. A reverse transcription-based quantitative PCR (real-time PCR) assay was performed to determine the mRNA levels of the indicated genes in each RNA sample, and 40 ng total RNA/reaction was used in the real-time PCR study. (1) Untreated GM00130 cells; (2) GLE-treated GM00130 cells; (3) PMA/lo-treated GM00130 cells; (4) PMA/lo + GLE-treated GM00130 cells; (5) untreated GM02248 cells; (6) GLE-treated GM02248 cells; (7) PMA/lo-treated GM02248 cells; (8) PMA/lo + GLE-treated GM02248 cells (* $P \leq .05$; ** $P \leq .01$).

B-lymphocytes. In addition, the results of our immunoblotting studies further revealed that the GLE treatment caused a great increase of the CCL5 chemokine in both GM00130 and GM02248 human B-lymphocytes. Considering the important function of PD-1 protein in immunomodulation, these results suggest that reducing PD-1 protein is an important mechanism for *G lucidum*-mediated immunomodulation.

The results of our western blotting studies demonstrated that the GLE treatment caused a large decrease in the levels of PD-1 protein in cultured GM00130 and GM02248 human B-lymphocytes. However, the results of our real-time PCR studies revealed that the GLE treatment did not cause any decrease in transcription of the *pdcd-1* gene in these cells. Therefore, transcriptional inhibition of the *pdcd-1* gene is not a likely mechanism for GLE-mediated reduction of the PD-1 protein in these human B-lymphocytes. The mechanism that leads to a reduced PD-1 protein in the GLE-treated GM00130 and GM02248 human B-lymphocytes is unknown. However, the work of a recent report suggests that ubiquitination of PD-1 protein is an important mechanism for modulating the anticancer immunity of T-cells.⁴⁷ It

is possible that the *G lucidum* modulates the immune system (eg, T- and B-lymphocytes) through ubiquitination and subsequent proteasomal degradation of the PD-1 protein. Although GLE might cause PD-1 protein reduction through enhancing membrane internalization and subsequent ubiquitination of the PD-1 protein, the results of our western blotting study on CD19 are strongly against this possibility since the levels of CD19 B-cell membrane protein remains unchanged under the GLE treatment. Another possible mechanism is that certain compound(s) contained in the GLE may selectively bind to the PD-1 protein, which cause conformation change of the protein and lead to ubiquitination and degradation of the PD-1 protein. Further studies need to determine if ubiquitination indeed occurs in the GLE-treated GM00130 and GM02248 human B-lymphocytes, and the compound(s) that selectively bind to the PD-1 protein also need to be identified.

The results of our real-time PCR studies revealed that the GLE treatment did not inhibit transcription of the *pdcd-1* gene but, instead, caused increased transcriptions of the *pdcd-1* gene in both GM00130 and GM02248 human B-lymphocytes. Interestingly, increased transcriptions were

also observed for several other tested genes, including *Bcl_{XL}*, *CCL5*, *p16*, *p53*, and PD-L1, when the GM00130 and GM02248 human B-lymphocytes were treated with GLE in the absence or presence of PMA/Io. Although the mechanisms that lead to increased transcriptions of these genes are unknown, it is possible that some compounds contained in the GLE may possess activities to induce global gene transcription. Therefore, further studies are needed to identify the compounds of the GLE that cause increased transcriptions of these genes. Future studies also need to identify all the genes affected by the GLE treatment.

The protein-protein interaction of PD-1 with PD-L1 prevents T-cells from killing tumor cells by inhibiting T-cells from producing cytokines and chemokines that help recruit immune cells (eg, T-lymphocytes) to the tumor sites and kill tumor cells.^{41,42,44} Works of others demonstrate that blockade of PD-1 protein with PD-1 antibodies increases expression of the *CCL5* chemokine and enhances recruitment of T-lymphocytes to tumor sites.^{42,44} The results of our studies also revealed that the GLE treatment caused an increase in expression of the *CCL5* chemokine. Therefore, *G lucidum* may modulate immune response by reducing PD-1 protein and enhancing immune response (eg, infiltration of T-cells) to fight disease development such as tumor growth. Therefore, *G lucidum* and its bioactive compounds could be developed into novel immunomodulators for both prevention and treatment of many diseases.

It is well known that the interaction of PD-1 and PD-L1 causes T-cell exhaustion and inhibits T-cell proliferation, resulting in an increased risk in cancer development.^{26,34,48,49} Clinical studies have also demonstrated that many types of cancer cells express high level of PD-L1 protein.^{26,49} Therefore, blockade of PD-1/PD-L1 interaction provides an attractive strategy in cancer treatment. Antibodies that target PD-1 and PD-L1 have been used in immunotherapy to treat many types of cancer.²⁹⁻³³ However, the PD-1/PD-L1 antibody-based immunotherapy also faces many challenges.³⁴ Other alternative approaches that can effectively disrupt the PD-1/PD-L1 interaction need to be explored. Small molecule immunomodulators that can disrupt the PD-1/PD-L1 interactions have attracted great attention in cancer drug design and development.⁵⁰⁻⁵³ Although many clinical trials are currently ongoing, no small molecule immunomodulator drugs that target the PD-1 protein are currently used in clinical application.⁵³ The results of our studies demonstrate that the GLE selectively reduces PD-1 protein in the cultured GM00130 and GM02248 human B-lymphocytes. Therefore, *G lucidum* and its bioactive contents could be developed into novel immunomodulators to target the PD-1 protein for treatment of many diseases, including cancer. Future studies need to determine if the results obtained in the cultured human B-lymphocytes can be achieved in animal studies and, more important, in human clinical studies.

In this work, we used 95% ethanol extract of GLE in our studies. The 95% ethanol extract of *G lucidum* was used in many studies.⁵⁴⁻⁵⁷ The 95% ethanol GLE is known to contain higher levels of triterpenoids than water GLE of *G lucidum*.⁵⁴⁻⁵⁷ Therefore, the observed effect of GLE on reducing PD-1 protein is likely caused by triterpenoids, although the contribution of polysaccharides on reducing PD-1 protein level could not be excluded in this work. Further identifying the compounds of GLE that are responsible for GLE-mediated PD-1 protein reduction would be important in understanding the mechanisms of *G lucidum*-mediated immunomodulation and anticancer activities.

In conclusion, the results of our studies suggest that reducing PD-1 protein is an important mechanism for *G lucidum*-mediated immunomodulation, and this reduction was not caused by a transcriptional inhibition of the *pdccl-1* gene. In addition, the results of our studies further demonstrate that the GLE treatment causes an increase in expression of chemokine *CCL5*. Therefore, *G lucidum* and its bioactive compounds can be developed into novel immunomodulators to target the PD-1 protein for prevention and treatment of many diseases, including cancer.

Authors' Note

The data generated or analyzed during this study are included in the published article.

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Author Contributions

GW designed and carried out most of the studies. GW also drafted the manuscript. LW was involved in manuscript preparation. JZ provided the wall-broken *Ganoderma lucidum* spores. XX was involved in design of the study. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

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Supplemental Material

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