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Research Article

Ganoderic Acids in *Ganoderma Lucidum*-Mediated PD-1 Reduction and Immunoregulation

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Abstract

Ganoderma lucidum (G. lucidum), also called Lingzhi (China) or Reishi, (Japan and Korea) is a medicinal fungus that has been used in many Asian countries for centuries to treat various illness and to promote healthy life. Enhancing immune response is one of the well-known benefits of *G. lucidum* consumption. The results of our previous study demonstrated that treatment of cultured human B lymphocytes with extract prepared from spores of *G. lucidum* (GLE) caused a significant reduction of PD-1 protein in the cultured B lymphocytes. Many bioactive compounds, including polysaccharides and triterpenoids, are contained in both fruit body and spores of *G. lucidum*. In this work, we investigated the role of purified ganoderic acids, including ganoderic acid A (GAA), ganoderic acid B (GAB), ganoderic acid D (GAD), and ganoderic acid F (GAF), in reducing PD- 1 protein in cultured cell systems. Using both GM02248 human B-lymphocytes and Jurkat human T-lymphoma cells, the results of our immunoblotting studies demonstrated that treatment with these individual ganoderic acids caused significant reductions of PD-1 protein in both GM02248 and Jurkat cells. The results of our immunofluorescence (IF)-based microscopy study further confirmed the effects of individual ganoderic acids in reducing PD-1 protein in Jurkat cells. The results of our cell proliferation studies revealed that both GM02248 and Jurkat cells were well tolerant to these individual ganoderic acids and no significant change in the rate of cell proliferation was observed in these cells with the presence of individual ganoderic acids at concentrations as high as 100 μ M. In addition, the results of our real time PCR studies indicated that treatment with these individual ganoderic acids did not cause significant decrease in transcription of the *pdcd-1* gene in either GM02248 or Jurkat cells. These results suggest that ganoderic acids play a key role in *G. lucidum*-mediated PD-1 protein reduction. Considering the important function of PD-1 protein in modulating immune response and in treating many diseases, especially in cancer treatment, these results suggest that ganoderic acids could be developed as novel immunomodulating drugs for treatment of cancer and many other diseases.

Introduction

Ganoderma lucidum (*G. lucidum*), also known as Lingzhi or Reishi, is a medicinal fungus that has been used for centuries in China and other Asian countries (*e.g.,* Korea and Japan) to treat various illness, including chronic bronchitis, inflammation, hyperlipidemia, hypertension, neurasthenia, hepatitis, and leukopenia [1-7]. *G. lucidum* has also been used to improve health and to promote longevity [2,4,7,8]. One of the well-known benefits of *G. lucidum* consumption is its ability to improve immune response [9,10]. The underlying mechanism, however, is not fully understood. Determining the molecular mechanism of *G. lucidum*-mediated immune modulation, and identifying its responsible compounds, therefore, would be important in understanding the G lucidum-mediated immune response and developing *G. lucidum*-based therapeutics for treatment of many disease conditions.

Many important bioactive compounds are contained in the fruit body and spores of *G. lucidum* [1-7]. Triterpenoids and polysaccharides are two of the most important pharmacological compounds carried in *G. lucidum*. There are over 150 different triterpenoid compounds identified in *G. lucidum* [1,4,7]. Recent studies suggest that triterpenoids of *G. lucidum* possess activities against cancer cell proliferation/metastasis [11-15] whereas the polysaccharides of *G. lucidum* function as immunomodulators [16-19]. The results of our previous studies demonstrated that extract prepared from the spores of *G. lucidum* (GLE) caused significant reduction of PD-1 protein, an important immunomodulator that plays a critical role in immune response [20-24] and in cancer treatment [25-32], in cultured human B-lymphocytes [33]. In this work, we studied the role of purified triterpenoids in *G. lucidum*-mediated PD-1 protein reduction. Using purified ganoderic acids, including ganoderic acid A (GAA), ganoderic acid B (GAB), ganoderic acid D (GAD), and ganoderic acid F (GAF), the results of our immunoblotting studies demonstrated that treatments of GM02248 human B-lymphocytes and Jurkat human T-lymphoma cells with these ganoderic acids caused significant reduction of the PD-1 protein in these cells. The results of our immunofluorescence (IF) microscopy studies confirmed the effects of purified GAF on reducing PD-1 protein in Jurkat cells. The results of our cell toxicity studies revealed that both GM02248 and Jurkat cells were well tolerant to the presence of purified ganoderic acids, with no significant difference in the rate of cell proliferation was observed when these cells were treated with individual ganoderic acids at concentrations as high as 100 μM. The results of our reverse transcription-based qPCR (real time PCR) study indicated that the presence of purified ganoderic acids (100 μM) did not inhibit transcription of the *pdcd-1* gene in these cells, suggesting that a transcriptional inhibition of PD-1 gene was not a mechanism for these ganoderic acids-mediated PD-1 protein reductions. Taking together, these results strongly suggest that ganoderic acids play a key role in *G. lucidum*-mediated PD-1 protein reduction and ganoderic acids could be developed as novel immunomodulating drugs for treatment of cancer and many other immune-related diseases via targeting the PD-1 protein of immune cells.

Materials and Methods

Cells, chemicals, and oligonucleotides used in this study. The GM02248 human B- lymphocytes were purchased from the Coriell Institute for Medical Research (Camden, NJ). The Jurkat human T-lymphoma cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Both GM02248 and Jurkat cells were maintained in RPMI1640 medium supplemented with 10mM HEPES (pH7.6) and 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Aldrich Inc. (St. Louis, MO). The ionomycin (Io) was purchased from Cayman Chemical Company (Ann Arbor, MI). The ganoderic acid A (GAA) and ganoderic acid D (GAD) were purchased from Sigma Aldrich Inc. The ganoderic acid B (GAB) and ganoderic acid F (GAF) were purchased from the Cayman Chemical Company. The PMA was prepared as 10mg/ml stock in dimethyl sulfoxide (DMSO) and stored at the -80°C freezer. The Io was purchased as 10mM ethanol stock and stored at the -80°C freezer. The individual ganoderic acids were prepared as 100mM stock in 95% ethanol and stored at the -80°C freezer. The oligonucleotides used in this study were synthesized by Midland Certified Reagent Company (Midland, TX) and the sequences of these oligonucleotides were listed in Table 1.

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

Name of the Primer	Sequences of the primer
pdcd-1 forward primer	5'-CAGCCTGGTGCTGCTAGTCTG-3'
pdcd-1 reverse primer	5'-GTCCACAGAGAACACAGGCAC-3'
CCL5 forward primer	5'-CTGCGCTCCTGCATCTGCCTC-3'
CCL5 reverse primer	5'-GAGTTGATGTACTCCCGAAC-3'
IL-6 forward primer	5'-CATCCTCGACGGCATCTCAG-3'
IL-6 reverse primer	5'-CTGCCAGTGCCTCTTTGCTG-3'
TNF forward Primer	5'-GGACCTCTCTCTAATCAGCC-3'
TNF reverse Primer	5'-CGGTTCAGCCACTGGAGCTG-3'

Cell proliferation assay

Both GM02248 and Jurkat cells were diluted in the cell growth medium to a density of ~5x10^5 cells/ml in T-25 flasks. Some cells were treated with individual ganoderic acids at indicated concentrations (0, 10 μM, 50μM, or 100 μM) by adding the ganoderic acid stock solution directly into the cultured cells. All cells were cultured in cell culture incubator at 37°C with 5% CO2. The cell density was determined for each treatment at the time points 0 hr, 24 hrs, 48 hrs, 72 hrs, and 96 hrs following the ganoderic acid treatment using an Invitrogen's Countess automated cell counter. The cell growth curve was established for both GM02248 and Jurkat cells using the cell density data.

PD-1 induction and ganoderic acid treatment

Both cultured GM02248 and Jurkat cells were treated with PMA (50ng/ml) and Ionomycin (1 μM) for 24 hours to induce expression of PD- 1 protein. Some of the PD-1 inducing cells were then treated with individual ganoderic acid (100 μM) for 48 hours. Both untreated and ganoderic acid-treated cells were harvested. Some of the harvested cells were lysed in RIPA cell lysis buffer and the cell lysates were used in immunoblotting assay to determine the levels

of desired target proteins. The remaining cells were used for isolation of total RNA using a RNeasy Mini Kit (Qiagen Inc. Valencia, CA). The RNA samples were used in a reverse transcription-based qPCR (real time PCR) assay to determine the mRNA levels of desired target genes.

Immunoblotting assay

Cell lysates prepared from both untreated and ganoderic acid-treated GM02248 and Jurkat cells were used in immunoblotting assays to determine the levels of desired target proteins in each cell lysate. The immunoblotting assay was performed using SDS-PAGE with 10% gel and 15 μg total protein/lane was used. After transferring the proteins to polyvinylidene difluoride (PVDF) membranes, the membranes were first hybridized with a primary antibody that targeted individual target proteins and then hybridized with an ECL- conjugated second antibody that targeted the primary antibody. The primary antibodies used in this study included goat anti-human PD-1 antibody (AF1086, R &D System), mouse anti-human CD4 antibody (MT-310, Santa Cruz Biotechnology), mouse anti-human CDK-7 antibody (C-4, Santa Cruz Biotechnology), mouse anti-human CCL5 (RANTES) antibody (C-12, Santa Cruz Biotechnology), mouse anti-human XPC antibody (D-10, Santa Cruz Biotechnology), mouse anti- human μ -Actin antibody (C-2, Santa Cruz Biotechnology), and mouse anti-human GAPDH antibody (6C5, Santa Cruz Biotechnology). The protein level of μ -Actin was used as a protein loading control of GM02248 cells and the protein level of GAPDH was used as protein loading control of Jurkat cells. Quantification of the protein band was conducted using ImageJ software (https://imagej.nih.gov/ij/).

Immunofluorescence (IF) microscopy assay

For IF microscopy study, both untreated and GAF-treated Jurkat human T-lymphoma cells were fixed in 1xPBS containing 4% formaldehyde for 15 minutes at room temperature. After incubating the cells in a blocking buffer (1X PBS, 5% normal serum, 0.3% Triton X-100) for 30 minutes at room temperature to block any unspecific binding, the Jurkat cells were then incubated in flow cytometry staining buffer (1xPBS, 1% bovine serum albumin, and 0.1% sodium azide) containing APC-labeled mouse anti-human PD-1 antibody (eBioJ105, Invitrogen) for 1 hour at room temperature. The nuclei of the Jurkat cells were counterstained with a DAPI reagent. The PD-1$^+$ cells were visualized by a Zeiss LSM 780 Fluorescence microscope using red light (630nm), and the nuclei of cells were visualized using blue light (480nm). A total of 100-250 cells were counted for each sample, and the percentage of PD-1$^+$ cells were determined for each treatment.

Reverse transcription-based qPCR (Real-time PCR) assay

Total RNA isolated from both untreated and treated GM02248 and Jurkat cells 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 several genes, including *pdcd-1*, *CCL5*, *IL-6*, and *TNF*, 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 gene was also determined from each RNA sample and used as an internal control for the real-time PCR study. The mRNA level of β-actin gene in the untreated GM02248 and Jurkat cells was used as a standard, and the levels of *pdcd-1*, *CCL5*, *IL-6*, and TNF mRNAs in each RNA sample were calculated as fold change to that of the untreated GM02248 and Jurkat cells, respectively. Triplicates were used for each sample in the real-time PCR study, and 3 independent experiments were conducted for the real-time PCR study. The mRNA level of each target gene was calculated as mean fold ± standard deviation (S.D.) to that of the GM02248 or Jurkat cells. Statistical analysis was used to determine if any significant difference in the level of mRNA exists for tested gene between untreated and treated cells for GM02248 and Jurkat cells (*$p \leq 0.05$; **$p \leq 0.01$).

Data Analysis

All data were presented as mean ± SD. Statistically significant differences were determined by a GraphPad Prism software (La Jolla, CA) using a student's t test with 95% confidence interval (CI). The data were obtained from at least 3 independent experiments.

Results

The effects of ganoderic acids on GM02248 and Jurkat cell proliferation

To study the role of ganoderic acids in *G. lucidum*-mediated PD-1 protein reduction, we first determined the effects of purified ganoderic acids on cell proliferation. Both GM02248 and Jurkat cells were treated with individual ganoderic acids at indicated concentrations (0, 10 μM, 50 μM, and 100 μM) by adding the ganoderic acid stock solution directly into the cell growth medium. The cells were cultured in the cell culture incubator at 37°C with 5% CO_2 supplement. The cell density was determined for both GM02248 and Jurkat cells in each treatment 24 hrs, 48 hrs, 72hrs, and 96 hrs after initiation of the ganoderic acid treatment. The cell growth curve was established for both GM02248 and Jurkat cells at each concentration using the cell density data (Figure 1). The results of our cell proliferation studies revealed that both GM02248 and Jurkat cells were well-tolerance to these purified individual ganoderic acids and no significant difference in cell proliferation was observed with the presence of individual ganoderic acids at concentrations as high as 100 μM (Figure 1).

The effects of ganoderic acids on reducing PD-1 protein in GM02248 and Jurkat cells

The results of our recent study have demonstrated the effect of extract prepared from spores of *G. lucidum* (GLE) on reducing PD-1 protein in the cultured human B-lymphocytes [33]. The GLE used in that study was prepared as an ethanol extract, which contained many different bioactive compounds of *G. lucidum*. To identify the compounds that are responsible for the GLE-mediated PD-1 protein reduction, we further determined the effects of ganoderic acids on reducing PD-1 protein in the cultured GM02248 and Jurkat cells

using purified ganoderic acid A (GAA), ganoderic acid B (GAB), ganoderic acid D (GAD), and ganoderic acid F (GAF). Both GM02248 and Jurkat cells were first treated with PMA/ionomycin to induce expression of PD-1 protein. The PD-1 expressing cells were then treated with individual ganoderic acids at 100 μ M for 48 hours. Both untreated and ganoderic acid-treated cells were harvested and the cell lysates were analyzed by immunoblotting assay to determine the protein level of PD-1 in each cell lysate (Figure 2). In addition, the protein levels of several other proteins, including CCL5, CD4, CPK7, XPC, GAPDH, and μ -Actin were also determined from the GM02248 or Jurkat cell lysates (Figure 2).

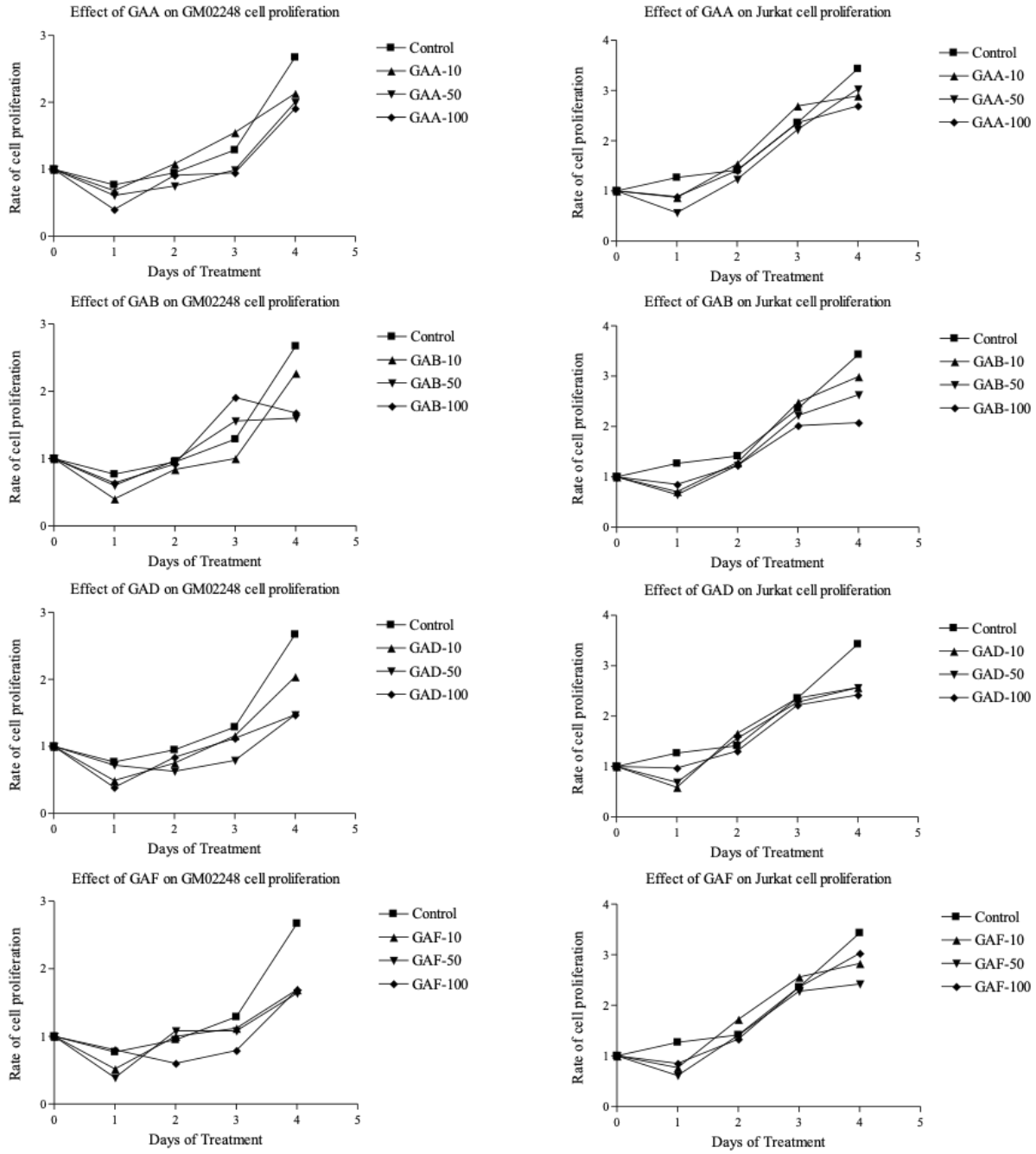

Figure 1: The effect of ganoderic acids on proliferation of GM02248 and Jurkat cells. Cells were diluted in cell growth medium to a density of ~5x10^5 cells/ml. Cells were then treated with individual ganoderic acids at indicated concentration by adding the ganoderic acid stock solution directly into the cell growth medium. The cells were incubated in cell culture incubator at 37oC with 5% CO_2. The cell density was determined at various time points after initiation of the ganoderic acid treatment (0, 24 hr, 48hr, 72 hr, and 96 hr). The cell growth curve was generated using the cell density data obtained from this study (mean + S.D). The results were based on 3 independent experiments. The cell growth curve was generated by GraphPad Prism software (La Jolla, CA).

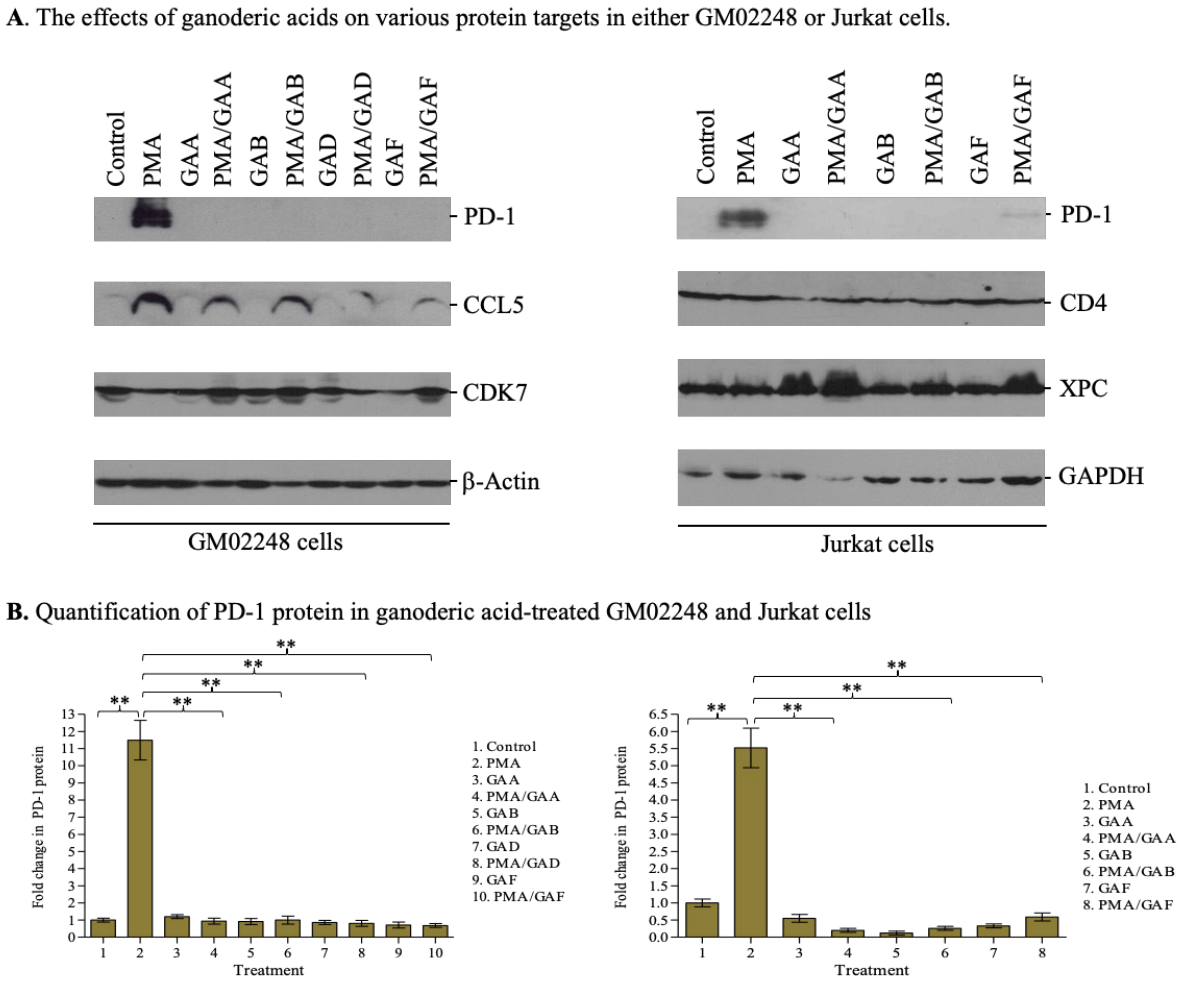

Figure 2: The effect of ganoderic acid treatment on levels of PD-1 protein in both GM02248 and Jurkat cells. (A) The effects of ganoderic acids on various protein targets in either GM02248 or Jurkat cells. (B) Quantification of PD-1 protein in individual ganoderic acid-treated GM02248 and Jurkat cells. The results were from 4 independent western blots (*$p < 0.05$; ** $p < 0.01$)

The protein level of μ-Actin and GAPDH were used as protein loading controls of GM02248 and Jurkat cells respectively for our immunoblotting studies. The results of our immunoblotting study indicated that the PMA/Ionomycin treatment caused 11.5 folds increase of PD-1 protein in GM02248 cells when compared to that of the untreated GM02248 cells (Figure 2). In the presence of GAA, GAB, GAD, and GAF, however, the levels of PD-1 protein in the PMA/ionomycin-treated GM02248 cells were reduced to only 94%, 100%, 80%, and 68% of the untreated GM02248 cells, respectively (Figure 2). Similarly, the PMA/Ionomycin treatment increased the PD-1 protein level by 5.5 folds in the Jurkat cells (Figure 2). In the presence of GAA, GAB, and GAF, however, the PD-1 protein level in the PMA/Ionomycin-treated Jurkat cells were reduced to 20%, 26%, and 59% of the untreated Jurkat cells (Figure 2). As controls, the levels of other tested proteins, including CCL5, CD4, CDK7, and XPC, were not significantly affected by the presence of ganoderic acid in GM02248 or Jurkat cells (Figure 2).

To confirm the results obtained from our immunoblotting study, we also performed an immunofluorescence (IF)-based microscopy study to determine the percentage of PD-1$^+$ cells in both untreated and ganoderic acid-treated Jurkat cells. The Jurkat cells were treated with individual ganoderic acids (100 μM) in the presence or absence of PMA/ionomycin for 48 hours. Both untreated and ganoderic acid-treated Jurkat cells were then fixed by formaldehyde, stained with an APC-labeled PD-1 antibody, and counter-stained with a DAPI reagent. The percentage of PD- 1$^+$ cells were determined for each treatment (Figure 3 and Table 2). The results of our IF-based microscopy study revealed that 21.2% of untreated Jurkat cells expressed low levels of PD-1 protein (Figure 3 and Table 2).

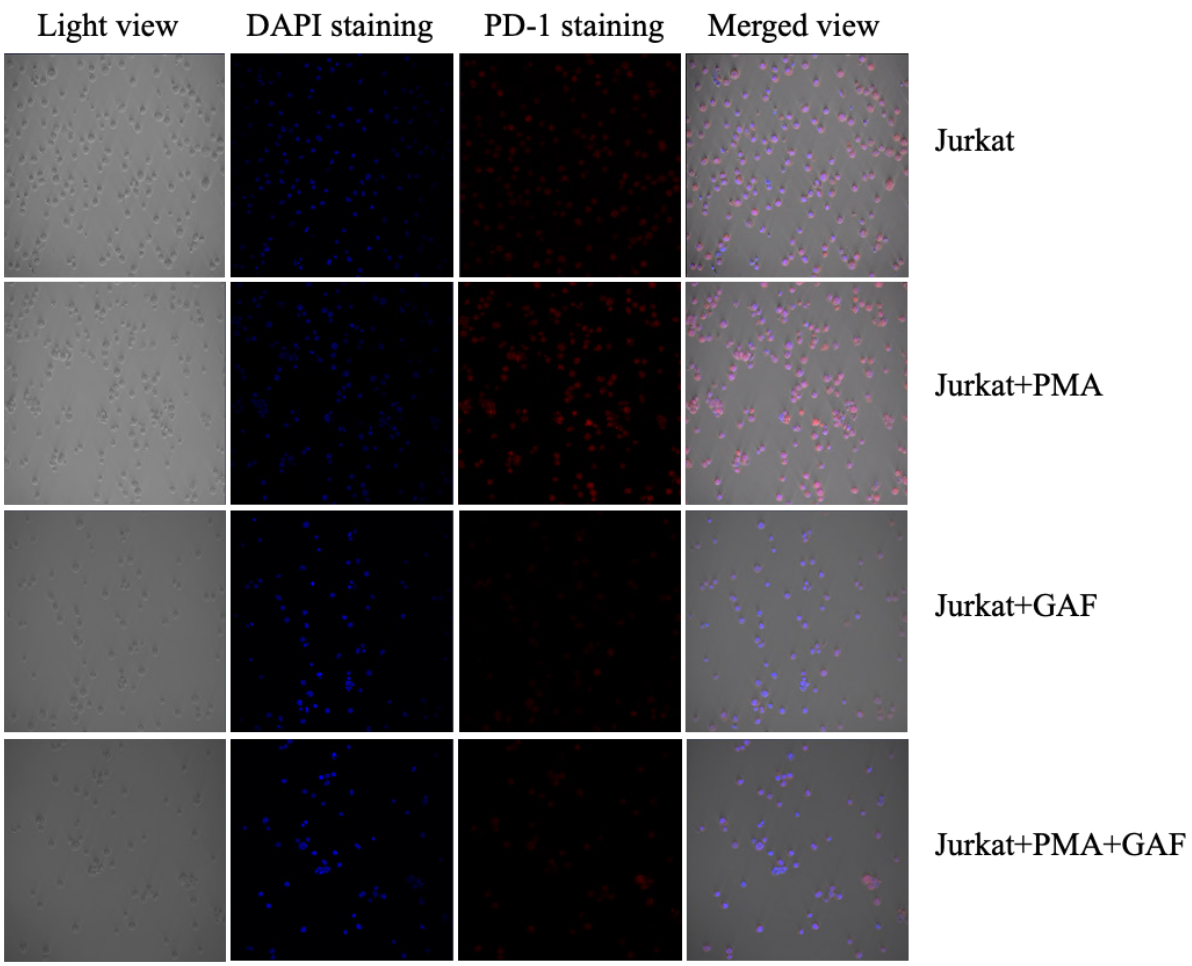

Figure 3: The effect of ganoderic acid F (GAF) on expression of PD-1 protein in Jurkat cells as determined by immunofluorescence (IF)-based microscopy assay. The Jurkat cells were treated with 100 μM GAF in the absence or presence of PMA (50ng/ml)/Ionomycin (1 μM) at 37oC for 48 hours. The cells were fixed in 4% formaldehyde, stained with an APC-labeled mouse anti-human 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 by the Zeiss LSM 780 fluorescence microscope using a blue light (480 nm).

Table 2: The effect of ganoderic acid treatment on PD-1$^+$ cells in the cultured Jurkat cells.

Treatment	No. of PD-1$^-$ cells	No. of PD-1$^+$ cells	Total No. of cells	% of PD-1$^+$cells
Control	149	40	189	21.2
PMA	12	201	213	94.3
GAA	104	31	135	23.0
PMA + GAA	124	38	162	23.5
GAB	103	29	132	22.0
PMA + GAB	83	28	111	25.2
GAF	91	23	114	20.2
PMA + GAF	95	26	121	21.5

(The number of PD-1$^+$ and PD-1- cells were counted from 3 views/treatment).

The PMA/Ionomycin treatment increased the PD-1$^+$ cells to 94.3% (Figure 3 and Table 2). Treatment with 100 μM GAA, GAB, or GAF, however, decreased the percentage of PD-1$^+$ cells to 23.5%, 25.2%, and 21.5% respectively for the PMA/Ionomycin- treated Jurkat cells (Figure 3 and Table 2). The presence of purified individual ganoderic acids has insignificant effect to the percentage of PD-1$^+$ Jurkat cells (23% in the GAA-treated Jurkat cells, 22.0% in the GAB-treated Jurkat cells, and 20.2% in the GAF-treated Jurkat cells vs 21.2% in the untreated Jurkat cells) (Figure 3 and Table 2). This result, in combination with our immunoblotting results, clearly demonstrated that the presences of these purified ganoderic acids caused significant reduction of the PD-1 protein in both GM02248 and Jurkat cells, suggesting the key role of ganoderic acids in *G. lucidum*-mediated PD-1 protein reduction.

The effects of ganoderic acids on transcriptions of immune-responsive genes in GM02248 and Jurkat cells

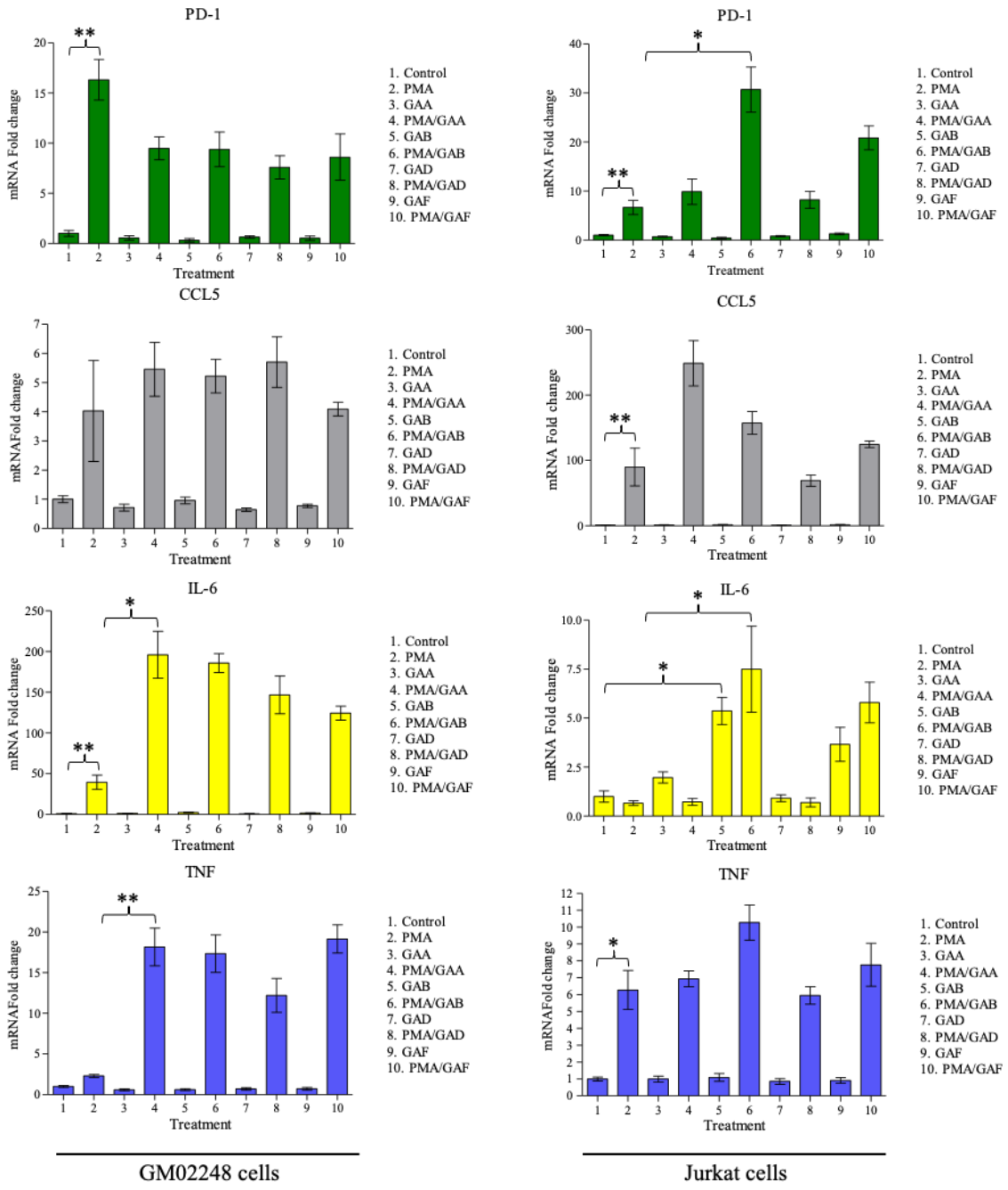

Figure 4: The effect of ganoderic acid treatment on transcriptions of pdcd-1, CCL5, Il-6, and TNF genes in both GM02248 and Jurkat cells. Both GM02248 and Jurkat cells were treated with 100 μM of individual ganoderic acids in the absence or presence of PMA (50ng/ml)/ Ionomycin (1 μM) for 48 hours. Total RNA isolated from both untreated and treated cells were analyzed by a reverse transcription-based qPCR (real time PCR) assay to determine the mRNA levels of the *pdcd-1*, *CCL5*, *IL-6*, and *TNF* genes in each RNA sample. The mRNA level of beta-Actin gene was used as an internal control for the real time PCR assay.

The results of our immunoblotting and IF-based microscopy studies have demonstrated the effects of purified ganoderic acids on reducing PD-1 protein in both GM02248 and Jurkat cells. To determine if a transcriptional inhibition played a role for the purified ganoderic acid-mediated PD-1 protein reduction, we further determined the mRNA levels of pdcd-1 gene from the RNA samples isolated from these cells using a reverse transcription-based qPCR (real- time PCR) study. As controls, several immune responsive genes, including *CCL5*, *IL-6*, and *TNF*, were also determined from these RNA samples by the real time PCR assay. Total RNA isolated from both untreated and ganoderic acid-treated GM02248 and Jurkat cells were analyzed by the real time PCR assay to determine the mRNA levels of *pdcd-1, CCL5*, *IL-6*, and *TNF* genes (Figure 4). The results of our real time PCR study revealed that the PMA/Ionomycin treatment increased transcription of the *pdcd-1* mRNA to 16.3 folds in GM02248 cells (Figure 4).

In the presence of 100mM GAA, GAB, GAD, and GAF, the *pdcd-1* mRNA levels in the PMA/Ionomycin-treated GM02248 cells were 9.5 folds, 9.4 folds, 7.6 folds, and 8.6 folds to that of the untreated GM02248 cells, respectively (Figure 4). The treatment with GAA, GAB, GAD, or GAF alone did not cause meaningful change in the *pdcd-1* mRNA level of GM02248 cells (Figure 4). Similarly, the PMA/Ionomycin treatment increased the *pdcd-1* mRNA level by 6.6 folds in Jurkat cells (Figure 4); in the presence of 100 μM GAA, GAB, GAD, and GAF, however, the *pdcd-1* mRNA levels in the PMA/Ionomycin-treated Jurkat cells were increased to 9.9 folds, 30.7 folds, 8.2 folds, and 20.8 folds, respectively (Figure 4). The presence of GAA, GAB, GAD, or GAF also did not cause meaningful change in the *pdcd-1* mRNA level in the Jurkat cells (Figure 4). The presence of the purified ganoderic acids also caused increases in transcriptions some but not all of the tested immune related genes (Figure 4). For example, the presence of GAA, GAB, GAD, and GAF clearly caused some more increases in the mRNA levels of *IL-6* and *TNF* genes in the GM02248 cells (Figure 4). In the Jurkat cells, however, only the presence of GAB and GAF but not GAA or GAD caused more increases in the mRNA level of *IL-6* gene (Figure 4). This result suggests that a transcriptional inhibition is not a mechanism for ganoderic acid-mediated PD-1 protein reduction in these cells. In addition, this result also suggests that different ganoderic acid might have different effect in transcriptions of certain immune related genes.

Discussion

Our previous studies demonstrated the effect of extract prepared from the spores of *G. lucidum* (GLE) on reducing PD-1 protein of cultured human B-lymphocytes [33]. Given the important roles of PD-1 protein in modulating immune response [21-25] and in cancer immunotherapy [26-32], it would be necessary to identify the bioactive compounds that are responsible for *G. lucidum*-mediated PD-1 protein reduction so that *G. lucidum*-based therapeutics can be developed for treatment of cancer and other immune-related diseases. In this study, we focused on determining the role of purified ganoderic acids, including GAA, GAB, GAD, and GAF, in reducing PD-1 protein of cultured human B and T-lymphocytes. The results of our immunoblotting study clearly demonstrated the effects of these purified ganoderic acids on reducing PD-1 protein in both GM02248 human B lymphocytes and Jurkat human lymphoma cells. The results of our IF-based microscopy study further revealed that the presence of GAF reduced the percentage of PD-1$^+$ Jurkat cells. In addition, the results of our cell proliferation studies indicated that the presence of ganoderic acids did not significantly affect the rate of cell proliferation at concentrations as high as 100 μ M. These results suggest that ganoderic acids play a key role in *G. lucidum*-mediated PD-1 protein reduction in these cells and contribute significantly to *G. lucidum*-mediated immune response. Considering the important function of PD-1 protein in immunomodulation [21-25] and in treatment of many diseases [26-32], these results suggest that ganoderic acids of *G. lucidum* could be developed as novel immunomodulating drugs for treatment of cancer and many other diseases via targeting the PD-1 protein of immune cells.

The results of our immunoblotting studies revealed that the presence of the tested ganoderic acids, including GAA, GAB, GAD, and GAF, reduced the level of PD-1 protein in the cultured GM02248 and Jurkat cells (Figure 2). Therefore, it is highly likely that the triterpenoids carried in *G. lucidum* play a key role in *G. lucidum*-mediated PD-1 protein reduction as observed in our previous study [33]. There are over 150 triterpenoids identified in the *G. lucidum* and many of them possess very different chemical structures [1,4,5,7]. The ganoderic acids used in this study possess similar chemical structures. Although the results obtained from this work demonstrated the effect of these ganoderic acids in reducing PD-1 protein in the cultured human cell models, it is unclear if other triterpenoids with different chemical structures could also to reduce the PD-1 protein in these cells. It would be necessary to determine the effects of those triterpenoids on reducing PD-1 protein in immune cells so that the mechanisms of *G. lucidum*-mediated immunomodulation and immunoresponse could be better understood and the *G. lucidum*-based therapeutics can be developed for more elective treatment of cancer and many other diseases. The results obtained from our studies clearly suggest that the ganoderic acids of *G. lucidum* play a key role in *G. lucidum*-mediated PD-1 reduction. However, it is well known that many different compounds, including polysaccharides and peptidoglycans, are also contained in the *G. lucidum* [1-7]. Works of others have indicated the modulation effects of polysaccharides in modulating immune response [16-19]. The results of our study, however, indicated that the presence of polysaccharides did not cause reduction of PD-1 protein in the cultured human B or T cells (data not provided). Therefore, the polysaccharides of *G. lucidum* must use a different mechanism to modulate immune response. In fact, works of others suggested that the polysaccharide might modulate immune response through the generation of reactive oxygen species (ROS), the secretion of cytokines, cell proliferation, or the phagocytic activity of macrophages [51]. Therefore, it is likely that the immune response observed in the *G. lucidum* is a combined effects of all these bioactive compounds through different mechanisms and targets.

The results of our real time PCR studies indicated that the presence of purified individual ganoderic acids did not inhibit transcription of the *pdcd-1* gene in these cell models; however, the results of our immunoblotting studies clearly demonstrated the effect of

ganoderic acids in reducing the level of PD-1 protein in the cultured GM02248 and Jurkat cells. Therefore, a transcriptional inhibition of *pdcd-1* gene is unlikely a mechanism for ganoderic acid-mediated PD- 1 protein reduction; therefore, other mechanisms, such as PD-1 protein degradation, must be involved in ganoderic acid-mediated PD-1 protein reduction in these cells. Further studying the underlying mechanism would be necessary in understanding the *G. lucidum*-mediated immune responses and in developing *G. lucidum*-based therapeutics for treatment of many diseases. Interestingly, the results of our real time PCR study suggest that different ganoderic acids might possess different effects in transcriptions of specific genes, especially the immune-related genes. Given the important roles of these immune-related proteins in treatment of cancer and other diseases, it would be important to determine the effects of individual ganoderic acids in transcriptions of important immune-related genes so that the ganoderic acids that can most effectively modulate immune response will be identified and used for treatment of cancer and other immune-related diseases.

Significant efforts have been devoted for novel therapies that target the PD-1/PD L-1 pathway for cancer treatment [26-32]. The antibody-based immunotherapies that target either PD-1 or PD L-1 have been developed and used successfully in the treatment of many cancer types [26- 32]. However, many adverse effects are associated with the antibody-based immunotherapies [36,37]. Ganoderic acids have some clear advantages over antibody-based immunotherapy: the size of ganoderic acid is small (~520 Da), the stability of ganoderic acid is very high, and the toxicity of ganoderic acid is very low [42-46]. If the effect of ganoderic acids in reducing PD-1 protein observed in our cell model system could be confirmed in animal model and in human study, ganoderic acids-based small molecule immunomodulating drugs could be easily developed and used in treatment of cancer and other immune-related diseases.

Conclusion

In conclusion, the results of our studies clearly demonstrated that the effects of purified ganoderic acids, including GAA, GAB, GAD, and GAF, in reducing PD-1 protein level in the cultured GM02248 and Jurkat cells model systems. Therefore, the ganoderic acids of *G. lucidum* play a key role in *G. lucidum*-mediated PD-1 reduction. Considering the important function of PD- 1 protein in modulating immune response and in cancer immunotherapy, and the clear advantages of ganoderic acids over antibody-based immunotherapy, our studies suggest that ganoderic acids of *G. lucidum* could be developed into novel small molecule immunomodulating drugs for treatment of cancer and many immune-related diseases.

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Author Contributions

GW designed and carried out most of the studies. GW also drafted the manuscript. FC was involved in western blotting studies. ZL, JZ, and XX were involved in the design of the study. All authors read and approved the final manuscript.

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