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Ganoderma lucidum polysaccharide peptide reduced the production of proinflammatory cytokines in activated rheumatoid synovial fibroblast

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Abstract The aim of the current study was to elucidate the potential therapeutic effect of *Ganoderma lucidum* polysaccharide peptide (GL-PP) in rheumatoid arthritis (RA). The effects of GL-PP on cell proliferation and cytokine production were studied in RA synovial fibroblasts (RASF). GL-PP significantly inhibited the proliferation of RASF. Following the incubation with GL-PP, production of interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 in RASF were significantly increased as expressed as percentage change from basal values. However, the actual effects were minimal due to the low basal values. When RASF were activated by IL-1β or lipopolysaccharides, IL-8 and MCP-1 production increased many folds. GL-PP significantly suppressed their productions. The inhibitory effects of GL-PP on cytokine production in RASF were at least in part, by inhibiting the nuclear factor-kappa B (NF-κB) transcription pathway. Our results demonstrated that GL-PP had the unique ability to modulate cytokine production in RASF and warrants further investigation into its mechanism of action.

Keywords *Ganoderma lucidum* · Rheumatoid arthritis · Synovial fibroblast · Cytokines · NF-κB · Inflammation

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Introduction

Rheumatoid arthritis (RA) is a prototypical chronic inflammatory disease manifested by progressive synovial joint inflammation and erosion of the subchondral bone. Abnormal proliferation of synovial fibroblasts and excessive secretion of proinflammatory cytokines such as interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1 by these cells account for many of the pathological changes seen in a RA joint. The severity of the joint inflammation fluctuates over time, and the outcome of the uncontrolled disease is progressive joint destruction, deformity, and disability. Efficacy of currently used antirheumatic therapy is often limited. Frequent problems are side effects, either cumulative or idiosyncratic, and high cost.

Ganoderma lucidum (Leyss, Ex Fr.), is a medicinal mushroom widely used in Traditional Chinese Medicine. *G. lucidum* has been used in various human diseases such as hepatitis, hypertension, arthritis, bronchitis, and tumorigenic diseases [1, 2]. Indeed, previous pre-clinical studies have shown *G. lucidum* have anti-inflammatory and analgesic effects [3]. *G. lucidum* has also been suggested to be effective in modulating immune functions [4]. In vitro studies have

shown *G. lucidum* can modulate peripheral mononuclear cell production of several cytokines, such as tumor necrosis factor (TNF)-α and ILs such as IL-1β, IL-2, and IL-6, which have all been associated with the pathogenesis of RA [4, 5]. The aim of this study was to investigate the effect of *G. lucidum* polysaccharide peptide on cytokine production in RA synovial fibroblasts (RASF). One of the main effective components of *G. lucidum* is the polysaccharide peptide fraction (GL-PP). A defined extract with known polysaccharide and peptide composition was used in this study.

Materials and methods

Synovial tissue and synovial cell isolation

RA patients, as defined by the American College of Rheumatology classification criteria for RA [6], were recruited from the rheumatology clinic of the Department of Medicine of Queen Mary Hospital. The synovial tissues were obtained from these patients at the time of surgery for total knee replacement. Isolation of synovial cells was carried out according to the method described by Harigai et al. [7], with some modifications. The isolated cells were used at passage 6 or above. At this time point, all cells had the appearance of fibroblasts.

Drug

G. lucidum was kindly provided by Prof. Lin Zhi-Bin, Department of Pharmacology, Peking University Health Science Center, School of Basic Medical Sciences, Beijing, China. *G. lucidum* was bag-cultured with the powder of *Dicranopteris dichotoma* (Thunb) Bernh, *Neyraudia reynaudiana* (kunth) Keng and *Pennisetum purpureum* Schumach. GL-PP was isolated from boiling water extract of the *G. lucidum* fruiting body. The hazel color powder contained a standardized polysaccharide peptide with an established average molecular weight of 5.13×10^5. The peptide portion contained 16 types of amino acids as follows: Asp 8.49, Thr 3.58, Ser 3.93, Glu 5.81, Gly 3.50, Ala 3.84, Cys 1.06, Val 2.68, Met 5.33, Iso-Leu 0.25, Leu 1.5, Phe 1.99, Lys 3.30, His 1.21, Arg 3.94, and Pro 1.22 (mg/g). The polysaccharide portion consists of fructose, galactose, glucose, rhamnose, xylose with the molecular ratios of 3.167: 0.556: 6.89: 0.549: 3.61 and linked together by β-glycosidic linkages [8].

Proliferation assay

RASF were plated in a 96-well microplate at a density of 1×10^4 cells/well in Dulbecco's Modified Eagle Medium (DMEM) (Gibco; Carlsbad, California, USA) medium supplemented with 1% fetal bovine serum (FBS) (Gibco; Carlsbad, California, USA), 10 μg/ml gentamicin (Gibco; Carlsbad, California, USA) and 2.5 μg/ml amphotercin B (Roche; Basel, Switzerland) for overnight to ensure uniform initial adherent cell numbers. Experiments were initiated by replacement with fresh DMEM medium supplemented with 10% FBS. GL-PP at different concentrations (0–250 μg/ml) was added to the culture on day 3 of the 5-day incubation period. The morphological changes of the cells were observed under phase-contrast microscopy. The effect of GL-PP on RASF proliferation was estimated by MTT (methyl thiazolyl tetrazolium) cell proliferation kit I (Roche; Basel, Switzerland). Percentage of change was calculated by [(number of treated cells – number of untreated cells)/number of untreated cells] $\times$ 100.

Cytokine assay

RASF were plated in a 24-well plate (1×10^5 cells/well) in DMEM medium supplemented with 1% FBS, gentamicin (10 μg/ml), and amphotercin B (2.5 μg/ml) for overnight to ensure uniform initial adherent cell numbers. After 1 day, 500 μl of fresh medium (DMEM supplemented with 1% FBS) with or without IL-1β (1 ng/ml) or lipopolysaccharides (LPS, from *E. coli*) (5 μg/ml) and various concentrations of GL-PP (0–250 μg/ml) were added at the same time. The synovial fibroblasts were incubated for another 24 h. The supernatants were collected and stored at –70°C. The supernatant cytokine levels of IL-1β, IL-6, IL-8, IL-10, MCP-1, and TNF-α were determined with Enzyme-Linked Immunosorbent Assay (ELISA) kits according to the manufacturers' directions (Pharmingen; San Jose, California, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

To determine the concentration-dependent effects of GL-PP on IL-1β induced nuclear factor-kappa B (NF-κB) gene expression, RASF were plated in a 12-well plate at a density of 2.0×10^5 cells/well in DMEM medium supplemented with 1% FBS, gentamicin (10 μg/ml) and amphotercin B (2.5 μg/ml) for overnight. GL-PP at various concentrations (0–250 μg/ml) and with or without IL-1β (1 ng/ml) were added to RASF for 4 h (pre-determined optimal time) before

total RNA extraction. The total RNA from the synovial fibroblasts was extracted using the RNEASY MINI KIT (QIAGEN; Hilden, Germany) according to the instructions given by the supplier. Purified total RNA was reverse transcribed using random hexamer priming on a 20 μl total volume scale as described in the Advantage® RT-for-PCR kit (Clontech Laboratories Inc.; Mountain View, California, USA) and the cDNA were stored at –70°C. Amplification of cDNA was carried out by PCR using p50 primers (5′ CAC CTA GCT GCC AAA GAA GG 3′, 5′ AGG CTC AAA GTT CTC CAC CA 3′) and p65 primers (5′ TCA ATG GCT ACA CAG GAC CA 3′, 5′ CAC TGT CAC CTG GAA GCA GA 3′) as described [9]. A 3:7 ratio of 18S primers to competimers primers (user menu, Ambion; Woodward Austin, TX, USA) was used as control to correct the values of p50 and p65 expression as described [10]. The size of the PCR products for p50, p65, and 18S primers was expected to be a 399, 308 and 495 bp fragment, respectively. PCR products were analyzed on a 2% agarose gel electrophoresis in 0.5× Tris/Boric acid/EDTA buffer (TBE), containing ethidium bromide at 120 V for 60 min. The bands were visualized on a Chemi Genius^2 Bio Imaging System and analyzed by Gene Snap 6.0026 (Syngene; Cambridge, UK). mRNA expression of 18S, a housekeeping gene, was used as internal standard. p65/18 S and p50/18 S ratio were used to determine the expression so as to adjust for minor experimental error in RT-PCR.

Statistical analysis

Results were obtained from duplicate or triplicate determination depending on the experimental protocol. Due to individual variations, results of cytokine production are expressed as percentage change against control. Statistical analysis was performed by a paired sample *t*-test for comparison of the effects of GL-PP against control medium. Data are expressed as mean ± SD and *n* represents the number of patient samples tested. Differences between samples with *P*-values of <0.05 were considered statistically significant.

Results

Inhibition of the hyperactive proliferation of RASF is an important target in RA therapy. RASF cultured in the presence of GL-PP showed a small but significant reduction in proliferation (Fig. 1).

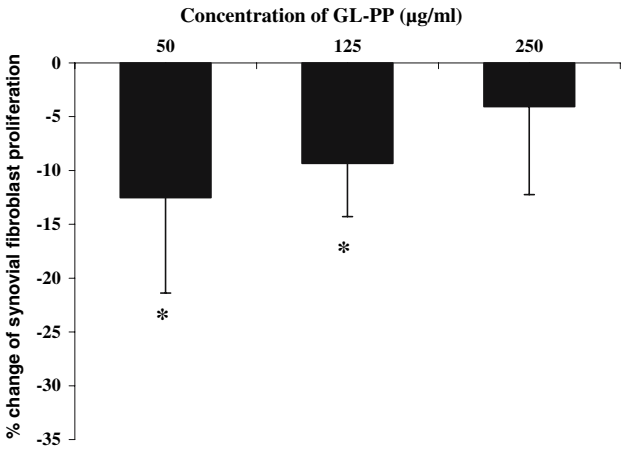

Fig. 1 Effect of GL-PP on proliferation of RASF. GL-PP was added to the culture on day 3 of the 5-day incubation period. Proliferation was determined by the MTT. Data are expressed as the mean ± S.D. of the tested samples. The significance was evaluated by a paired sample *t*-test against the control culture in the absence of GL-PP. $P < 0.05$ was considered statistically significant. ($n = 8$). * = $P < 0.05$

The effects of GL-PP on RASF IL-1β, IL-6, IL-8, IL-10, MCP-1, and TNF-α production were investigated. Results showed that GL-PP did not have any effect on the production of IL-1β, IL-10, and TNF-α (data not shown). However, there was a significant increase in IL-6 and MCP-1 production by RASF following incubation with GL-PP (Fig. 2). While these

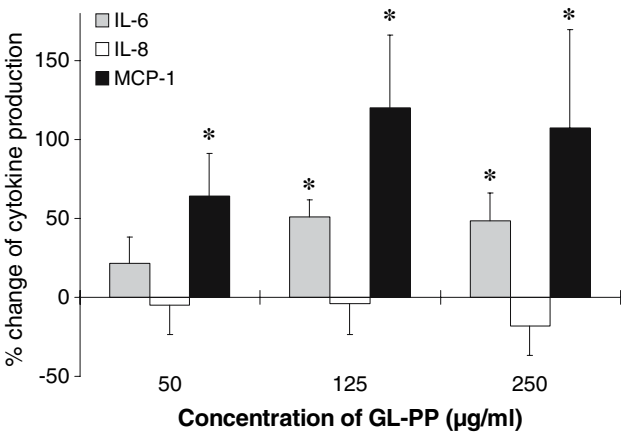

Fig. 2 Effects of GL-PP on cytokine production by RASF. The cultured cells were incubated with 0–250 μg/ml of GL-PP for 24 h. After incubation, the supernatants were collected and the cytokine levels of IL-6, IL-8, and MCP-1 in the supernatant were measured by ELISA. Data are represented as percentage change against control culture in the absence of GL-PP. The amounts of IL-6, IL-8, and MCP-1 in the control medium detected at 24 h were 0.85 ± 0.08, 1.05 ± 0.72, and 4.70 ± 2.55 ng/ml, respectively. Data are expressed as the mean ± S.D. of the tested samples. The significance was evaluated by a paired sample *t*-test against the control culture in the absence of GL-PP. $P < 0.05$ was considered statistically significant. ($n = 4$). * = $P < 0.05$

effects were statistically significant in terms of percentage change, the actual increase in the amount of cytokines produced was minimal because of very low baseline levels of IL-6 and MCP-1.

As the synovial fibroblasts used in the experiment were at passage 6 or above, the cells would have become inactive. Activated RASF may be a better model to study the effects of GL-PP on rheumatoid synovial fibroblast cytokine release. The effects of GL-PP on IL-6, IL-8, and MCP-1 production were therefore investigated in IL-1β activated RASF. The effects of IL-1β were maximal at 1 ng/ml (data not shown). This concentration was used in subsequent studies. The production of IL-6, IL-8 and MCP-1 in RASF was up-regulated by 350-, 107- and 14-folds by IL-1β, respectively. Our findings are in agreement with previous studies that IL-1β is one of the major physiological stimulants for IL-6, IL-8, and MCP-1 synthesis [11, 12]. Interestingly, RASF cultured in the presence of GL-PP showed a significant reduction in IL-8 and MCP-1 production in response to IL-1β when compared with cultures without GL-PP (Fig. 3).

LPS was used to evaluate whether the inhibitory effects of GL-PP in IL-1β induced IL-8 and MCP-1 production were stimulant specific. Our previous study has shown that the stimulatory effects of LPS on RASF peaked at 5 μg/ml (data not shown). RASF were stimulated by LPS at 5 μg/ml and incubated with

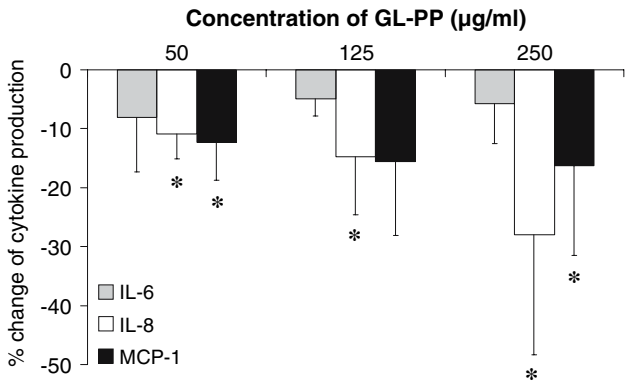

Fig. 3 Effects of GL-PP on cytokine production by IL-1β stimulated RASF. The cultured cells were incubated with 1 ng/ml of IL-1β and 0–250 μg/ml of GL-PP for 24 h. After incubation, the supernatants were collected and the concentrations of IL-6, IL-8, and MCP-1 in the supernatant were measured by ELISA. Data are represented as percentage change against control culture (stimulated with IL-1β) in the absence of GL-PP. The amounts of IL-6, IL-8, and MCP-1 in the control medium (stimulated with IL-1β) detected at 24 h were 295.9 ± 218.2, 112.6 ± 83.8, and 63.2 ± 21.5 ng/ml, respectively. Data are expressed as the mean ± S.D. of the tested samples. The significance was evaluated by a paired sample *t*-test against the control culture in the absence of GL-PP. $P < 0.05$ was considered statistically significant. ($n = 6$). * = $P < 0.05$

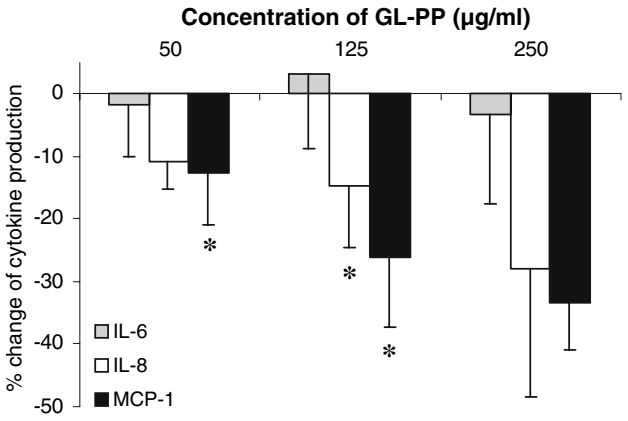

Fig. 4 Effects of GL-PP on cytokine production by LPS stimulated RASF. The cultured cells were incubated with 5 μg/ml of LPS and 0–250 μg/ml of GL-PP for 24 h. After incubation, the supernatants were collected and the concentrations of IL-6, IL-8 and MCP-1 in the supernatant were measured by ELISA. Data are represented as percentage change against control culture (stimulated with LPS) in the absence of GL-PP. The amounts of IL-6, IL-8, and MCP-1 in the control medium (stimulated with LPS) detected at 24 h were 1340.7 ± 1628.5, 21.6 ± 27.3, and 68.72 ± 26.9 ng/ml, respectively. Data are expressed as the mean ± S.D. of the tested samples. The significance was evaluated by a paired sample *t*-test against the control culture in the absence of GL-PP. $P < 0.05$ was considered statistically significant. ($n = 5$). * = $P < 0.05$

GL-PP (0–250 μg/ml) for 24 h. Figure 4 shows similar inhibitory effects of GL-PP on production of these cytokines in LPS stimulated RASF as in Fig. 3. This implies that the inhibitory effect of GL-PP on activated RASF was not specific for IL-1β stimulation.

Stimulation of RASF by LPS and IL-1β can be mediated through a common pathway such as NF-κB with activation of p65 and p50 mRNA expression. The inhibitory effects of GL-PP may involve p65 and p50 mRNA expression. GL-PP alone produced a slight increase in both p65 and p50 mRNA expression. However, after IL-1β stimulation, GL-PP had inhibitory effects on p65 and p50 mRNA expression (Figs. 5, 6). These findings imply that GL-PP might suppress NF-κB activation resulting in the inhibition of IL-8 and MCP-1 production in activated RASF.

Discussion

The present study demonstrated a unique property of *G. lucidum* in the modulation of immune responses in RASF. GL-PP increased basal IL-8 and MCP-1 production in RASF. When RASF were activated, the increased production of IL-8 and MCP-1 were suppressed by GL-PP. This inhibitory effect of GL-PP on

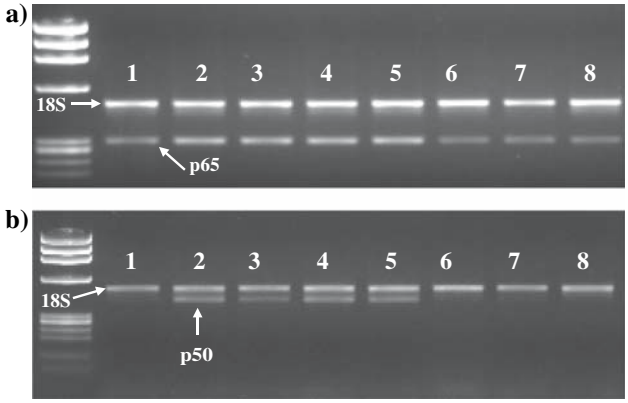

Fig. 5 Effects of GL-PP on NF-κB p65 and p50 mRNA expression of RASF. The RT-PCR products of (**a**) p65 and (**b**) p50 were separated by a 2% agarose gel and stained with ethidium bromide. 18S was used as internal control to correct the value of p65 and p50 expression. The size of the PCR products for (**a**) p65, (**b**) p50 and 18S primers was expected to be a 308, 399, and 495 bp fragments, respectively. 1 = medium, 2 = IL-1β, 3 = IL-1β + GL-PP 50 μg/ml, 4 = IL-1β + GL-PP 125 μg/ml, 5 = IL-1β + GL-PP 250 μg/ml, 6 = GL-PP 50 μg/ml, 7 = GL-PP 125 μg/ml, 8 = GL-PP 250 μg/ml

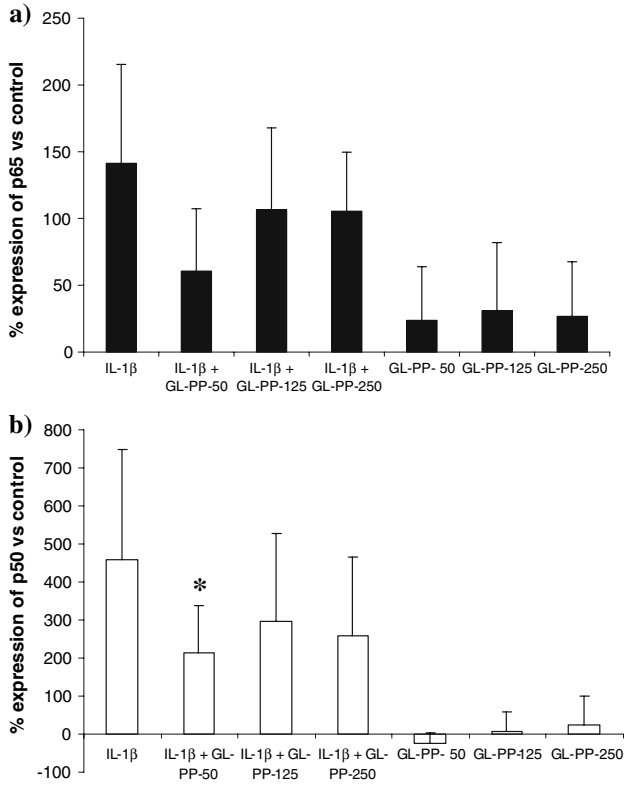

Fig. 6 Effects of GL-PP on NF-κB p65 (**a**) and p50 (**b**) mRNA expression of RASF. RASF were incubated with GL-PP at various concentrations (0–250 μg/ml) with or without IL-1β (1 ng/ml) stimulation. Data are expressed as the mean ± S.D. of the tested samples. The significance was evaluated by a paired sample *t*-test against the control culture in the absence of GL-PP and IL-1β. The significance was also evaluated by a paired sample *t*-test against the control culture in the presence of IL-1β and without GL-PP. ($n = 7$). * = $P < 0.05$ GL-PP-50 = GL-PP 50 μg/ml

cytokine production was mediated by inhibition of the NF-κB transcription pathway.

Previous studies indicated that 60–90% of RA patients used complementary and alternative medicine [13]. Many RA patients have claimed *G. lucidum* can to some extent alleviate the symptoms of their arthritis condition. Furthermore, traditional Chinese medicine doctors widely use *G. lucidum* to treat inflammatory diseases. This growing interest implicates the need for investigation of the efficacy of complementary and alternative medicine in RA patients.

G. lucidum, a medicinal mushroom, has been used as a traditional Chinese medicine for more than 5000 years [14]. Many biological effects of *G. lucidum* have been reported. Most of them were related to changes in immune functions. It has anti-allergy properties and inhibits histamine release from mast cells [15], and reduces the production of immunoglobulin G antibodies [16]. *G. lucidum* also possesses inhibitory effects on platelets [17] and mononuclear cells [18]. A recent study carried out by Lai et al. [14] showed *G. tsugae*, which consists of similar components of *G. lucidum* had beneficial therapeutic effects on a mouse model of systemic lupus erythematosus, a multisystem autoimmune disease. The authors found that *G. tsugae* improved the survival rate of lupus mice and suggested a potential usage of *Ganoderma* in autoimmune disease. In contrast, Wang and his colleagues [5] showed that *G. lucidum* could be used to potentiate IL-1β, IL-6, and TNF-α production by human monocyte-macrophages cultured with polysaccharides from *G. lucidum* (100 μg/ml) and the production of IL-6 was 29-fold higher. If the same effects were found in RASF, it would be of disadvantage to RA.

In the present study, we observed that GL-PP could significantly increase the production of IL-6 and MCP-1 (Fig. 2) when compared with controls. This suggests that GL-PP has stimulatory effects on both IL-6 and MCP-1 production in RASF. Although the effect was statistically significant in terms of percentage change, it should be noted that the actual stimulatory effect was minimal. For example, the production of MCP-1 following stimulation by GL-PP (50 μg/ml) and IL-1β (1 ng/ml) after 24 h incubation were 2.78 and 58.71 ng/ml, respectively. In addition, the stimulatory effect of GL-PP on RASF on IL-6 production was milder than with other cell types. We observed that GL-PP at 125 μg/ml increased IL-6 production by 1.6-fold in RASF while Wang et al. [5] found that *G. lucidum*

polysaccharide at 100 μg/ml increased the production of IL-6 in human monocyte-macrophages by 29-fold. Furthermore, our results showed GL-PP did not stimulate IL-1β, IL-8, IL-10, and TNF-α production in RASF. This is in accordance with previous studies which showed IL-10 was mainly secreted by B cell [19], T cell [20], and monocyte [21]. This result also confirms the findings of Schwachula et al. [22] that RASF did not express mRNA of TNF-α spontaneously and IL-1β is produced mainly by macrophages [23, 24]. This implies that *G. lucidum* have different effects on different cell types.

RA is an autoimmune disease characterized by chronic inflammation and hyper-proliferation of the synovial lining. The inflamed RA joint is abundant of proinflammatory cytokines, such as IL-1β and TNF-α. Enhanced levels of a number of proinflammatory cytokines up-regulate the disease process leading to eicosanoid formation, matrix degradation, bone resorption and synovial proliferation in the joint [25–27]. The significant inhibitory effect of GL-PP on the proliferation of RASF (Fig. 1) suggested a potential benefit on RA therapy. In order to mimic the actual situation in RA joints, IL-1β was chosen to stimulate the RASF and further experiments were carried out to evaluate the ability of GL-PP to counteract the effects of IL-1β stimulation. It was found that GL-PP significantly inhibited IL-1β stimulated IL-8 and MCP-1 production in a dose-dependent manner (Fig. 3). LPS was also used to evaluate whether the inhibitory effects were specific for IL-1β stimulation. Similar results were obtained (Fig. 4). This indicates that the inhibitory effects were not stimulus-specific and might be due to the immunoregulatory effects of GL-PP on stimulated RASF. A cell viability test was performed to evaluate whether the reduction in the cytokine production was due to cell killing. No significant cell death was found at the tested concentrations (50–250 μg/ml) of GL-PP (data not shown). This immunomodulatory effect of *Ganoderma* is in accordance with Gao et al.'s study [28]. The authors showed water soluble polysaccharides of *Ganoderma tsugae* mycelium significantly up regulated TNF-α production in THP-1 cells without stimulants or at a low dose of LPS and Phorbol Myristate Acetate (PMA), whereas down regulated high-dose of LPS- and PMA-induced IL-1α or TNF-α mRNA and their protein production in THP-1 cells.

Activation of NF-κB is the major contributor to IL-6, IL-8, and MCP-1 secretion by RASF following IL-1β stimulation [12, 29]. p65 and p50 are the subunits of NF-κB and widely expressed in various cell types. The mRNA expression of these subunits can reflect the activity of NF-κB. Our results show that GL-PP significantly inhibited IL-1β stimulated p50 expression and probably had an inhibitory effect on the expression of p65 (Figs. 5, 6).

IL-8 and MCP-1 are major mediators of inflammation and cause joint damage indirectly. The reduction of inflammatory chemokines IL-8 and MCP-1 by fibroblasts may reduce the stimulus for leukocyte recruitment and activation. Thus, inhibition of chemokine production forms the basis of some currently used RA drugs. For example, methotrexate inhibits the production and activity of IL-1β, IL-6, and IL-8. Both gold salts and dexamethasone inhibit IL-8 and MCP-1 production [30]. Therefore, the inhibitory effects of GL-PP on IL-1β and LPS stimulated production of IL-8 and MCP-1 may form the basis of the therapeutic use of *Ganoderma* products on RA. Although GL-PP caused a small increase in IL-8 and MCP-1 production in RASF under basal condition, this may not be applicable in the case for RA patients since the fibroblasts in the synovial joints will be activated to various extents by the presence of proinflammatory cytokines.

In conclusion, *G. lucidum* significantly inhibited the proliferation of RASF and IL-1β or LPS induced IL-8 and MCP-1 production. The inhibitory effects of *G. lucidum* on IL-1β induced IL-8 and MCP-1 productions by RASF are at least in part, due to the inhibition of the NF-κB transcription pathway. This pilot study has demonstrated potential beneficial effects of *G. lucidum* in RA therapy. In vivo and clinical studies are required to further evaluate the potential use of *G. lucidum* in RA patients.

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