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Ganoderic acid C₁ isolated from the Anti-asthma Formula, ASHMI™ suppresses TNF- α production by mouse macrophages and peripheral blood mononuclear cells from asthma patients

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1. INTRODUCTION

Asthma is a global health concern, affecting 300 million people worldwide. In the US asthma affects 9.5% of children and 8.2% of adults (CDC, 2012). Current standard therapies

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Authors' disclosure

Dr. Xiu-Min Li shares the patent on ASHMI™ (PCT/US05/08600 for ASHMI™) with Herbal Spring LLC. The other authors have no financial interests to disclose.

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for asthma which include inhaled corticosteroids (ICS) and β_2 agonists, are sub-optimally effective in approximately 50% of patients, especially in children (Ducharme et al., 2010; Kelly and Fitzpatrick, 2011; Peters et al., 2007). Asthma is a heterogeneous chronic inflammatory disease, which is associated with inflammation mediated by Th2 cytokines, such as IL-4, IL-5 and IL-13, and inflammation mediated by other cytokines such as TNF- α . Unlike Th2 mediated eosinophil predominant inflammation, TNF- α mediated neutrophil and mixed eosinophil and neutrophil inflammation are generally insensitive to ICS. Effective therapies for these conditions are needed.

TNF- α is produced by many cell types, mainly macrophages and monocytes and lymphocytes, in response to lipopolysaccharide (LPS) and other stimuli (Berry et al., 2007; Kim et al., 2012). LPS induces TNF- α production through activation of transcription factors such as nuclear factor kappa-B (NF- κ B), activator protein 1 (AP-1) and mitogen-activated protein kinases (MAP kinases or MAPK) (Guha and Mackman, 2001). Increased levels of phosphorylated- I κ B- α (p-I κ B α) protein and p65 in cytosol and nuclear p-p65 indicate heightened NF- κ B activation (Ulevitch and Tobias, 1995). AP-1 is a heterodimeric protein constituted by members of the Jun and Fos families of DNA binding proteins. Many stimuli (like LPS) induce the binding of AP-1 to the promoter region of various genes that enhance the expression of inflammatory cytokines, like TNF- α (Chen et al., 2014). MAP kinases include extracellular-signal-regulated kinase 1/2 (ERK 1/2), p-38 and c-Jun N-terminal kinase (JNK). MAP kinase pathways activate NF- κ B and AP-1, which coordinate the induction of TNF- α gene expression (Chen et al., 2014).

Traditional Chinese medicines are increasingly used in the US by individuals with chronic inflammatory conditions including asthma. However, data on efficacy, safety, and mechanisms of action of natural products is limited. A major obstacle is the paucity of knowledge of characterized pharmacologically active compounds in complex herbal products. We previously investigated the anti-asthma herbal medicine intervention ASHMITM -extracts of *Ganoderma lucidum* (G. *lucidum*), *Sophora flavescens* Ait (S. *flavescens*) and *Glycyrrhiza uralensis* Fischer (G. *uralensis*) (Kelly-Pieper et al., 2009; Srivastava et al., 2014; Wen et al., 2005; Zhang et al., 2010). ASHMITM was reported to have beneficial effects and favorable safety in clinical studies (Kelly-Pieper et al., 2009; Wen et al., 2005). In addition to its long-lasting effectiveness in a model of eosinophil predominant asthma (Zhang et al., 2010), ASHMITM is also effective in a steroid resistant neutrophil predominant allergy asthma model (Srivastava et al., 2014). Therapeutic effects are associated with both reduced pro allergic Th-2 inflammation cytokine production, including IL-4, and non Th-2 inflammatory cytokines such as TNF- α . We previously demonstrated that 7, 4'-dihydroxyflavone is the most effective G. *uralensis* flavonoid inhibitor of pro-allergic chemokine production (Jayaprakasam et al., 2009) and the pro Th2 cytokine transcription factor GATA3 (Yang et al., 2013). However, the ASHMITM compound (s) responsible for inhibiting production of TNF- α , recently highlighted as important in steroid resistant asthma, was unknown.

The present study found that G. *lucidum* extracts, but not those of S. *flavescens* or G. *uralensis* significantly suppressed murine macrophage TNF- α production; and that methylene chloride (MC) fraction of G. *lucidum* was the active fraction. Among the 15

bioactive triterpenoid compounds isolated from this fraction, only ganoderic acid C₁ (GAC₁) significantly suppressed TNF- α production by murine macrophages (RAW 264.7 cells). GAC₁ also non-toxically inhibited TNF- α production by PBMCs from asthma patients. GAC₁ inhibition of LPS stimulated macrophage TNF- α production was associated with suppression of NF- κ B, and partial suppression of the AP-1, and MAPK signaling pathways. Suppression of NF- κ B signaling was also found in asthma patient PBMCs. GAC₁ may prove to be a novel TNF- α inhibitor with application to TNF- α associated asthma and other disease therapy.

2. MATERIALS AND METHODS

2.1. Plant Materials

ASHMITM, *G. lucidum*, *S. flavescentis*, and *G. uralensis* aqueous extracts were manufactured by the Sino-Lion Pharmaceutical Company (a GMP certified facility in Weifang, China) as described previously (Kelly-Pieper et al., 2009; Zhang et al., 2010).

2.2. Extraction and isolation of compounds from *G. lucidum*

A dried aqueous extract of *G. lucidum* was dissolved in water and extracted with methylene chloride (MC). The MC layer and water residue layers after methylene chloride extraction were concentrated and dried under pressure to obtain two fractions: F1 (MC), and F2. The F1 fraction was further fractionated and purified using repeated silica gel, preparative HPLC, and Sephadex LH-20 column chromatography methods to obtain 15 pure compounds (**1–15**) (eTable 1).

2.3. Identification of compounds isolated from *G. lucidum*

¹H (300 MHz) and ¹³C (75 MHz) NMR were collected on a JEOL ECX-300 instrument. Samples were dissolved in DMSO-*d*₆, methanol-*d*₄, or chloroform-*d* containing TMS as an internal standard, and MS spectra were recorded on an Agilent 6130 single quadrupole LC MS. Analysis of ¹H-NMR and ¹³C-NMR spectra yielded characteristic signals for each compound as shown below. Ganoderiol F (**1**) (C₃₀H₄₆O₃) (DMSO-*d*₆): δ 6.46 (1H, d, *J* = 8.58 Hz, H-24), 5.64 (1H, s, H-7), 5.54 (1H, s, H-11), 4.80 (2H, m, H-26), 4.49 (2H, m, H-27). The ¹H-NMR spectrum of ganoderic acid α (**2**) (C₃₂H₄₆O₉) (chloroform-*d*) showed 8 methyl signals, including a signal at δ 2.22 (3H, s, COCH₃-12). Ganoderic acid V (**3**) (C₃₂H₄₈O₆) (chloroform-*d*): δ 6.17 (1H, s, H-24), 4.85 (1H, m, H-7), 4.48 (1H, m, H-15), 2.33 (3H, s, COCH₃-15). Ganoderic acid H (**4**) (C₃₂H₄₄O₉) (methanol-*d*₄): δ 3.23 (1H, m, H-3), 2.21 (3H, s, COCH₃-12). Ganoderic acid C₂ (**5**) (C₃₂H₄₆O₇) (methanol-*d*₄): δ 4.78 (1H, m, H-7), 4.57 (1H, m, H-15), 3.21 (1H, m, H-3). Ganolucidic acid A (**6**) (C₃₀H₄₄O₆) (methanol-*d*₄): δ 4.58 (1H, s, H-15). Ganolucidic acid E (**7**) (C₃₁H₄₆O₄) (chloroform-*d*): δ 4.78 (1H, m, H-15), 2.47 (3H, m, CH₃-26). The ¹H-NMR (DMSO-*d*₆) spectrum of Ganoderic acid C₁ (**8**) (C₃₀H₄₂O₇) showed a proton signal at δ 4.80 (1H, m, H-7). ¹³C-NMR (DMSO-*d*₆) spectra showed signals at δ 216.0 (C-15), 215.1 (C-3), 208.6 (C-23), 198.0 (C-11), 176.7 (C-26), 159.4 (C-9), 139.8 (C-8), and 64.8 (C-7). Ganoderenic acid A (**9**) (C₃₀H₄₂O₇) (methanol-*d*₄): δ 6.25 (1H, s, H-22), 4.65 (1H, s, H-7), 4.53 (1H, m, H-15). Ganolucidic acid D (**10**) (C₃₀H₄₄O₆) (DMSO-*d*₆): δ 5.32 (1H, s, H-24), 4.56 (1H, m, H-23). Ganoderic acid U (**11**) (C₃₀H₄₈O₄) (methanol-*d*₄): δ 4.87 (1H, s, H-7), 3.28 (1H, s, H-3).

Ganoderic acid J (**12**) ($C_{30}H_{42}O_7$) (DMSO- d_6): δ 4.57 (1H, s, H-15). Ganoderic acid A (**13**) ($C_{30}H_{44}O_7$) (DMSO- d_6): δ 4.76 (1H, s, 7-H), and 4.60 (1H, s, 15-H). Ganoderic acid K (**14**) ($C_{30}H_{44}O_7$) (DMSO- d_6): δ 3.19 (1H, s, 3 H) and 4.53 (1H, s, 15-H). Ganoderenic acid D (**15**) ($C_{30}H_{40}O_7$) (methanol- d_4): δ 4.48 (1H, s, H-7).

2.4. RAW 264.7 cell culture

RAW 264.7 cells, a murine macrophage cell line, purchased from American Type Collection Culture (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin. To determine potential inhibitory effects of herbs and compounds on LPS-stimulated TNF- α production, linearly growing cells were detached using a cell scraper, and 5×10^5 cells per well were seeded in 24-well culture plates. Cells were pretreated with herbal extracts or individual compounds for 24 hours followed by stimulation with 1.0 μ g/mL LPS (*Escherichia coli* 0111:B4, Sigma Aldrich, St. Louis, MO) for another 24 hours. Supernatants were harvested for measurement of TNF- α . Compounds were dissolved in DMSO. The concentration of DMSO in all cultures, including medium alone and LPS alone cultures, was 0.1%. Cell viability was determined using trypan blue dye exclusion and calculating the ratio of viable cells to total cells.

2.5. Annexin V apoptosis assay and MTT cell proliferation assay

Annexin V assay was performed according to the manufacturer's instructions (BD Biosciences Annexin V-FITC Apoptosis Detection Kit). Cells were washed twice with PBS and then exposed to annexin V-FITC and propidium iodide (PI) in binding buffer for 15 minutes in the dark at room temperature. Analyses were performed using a FACScan (Becton Dickinson) emitting an excitation laser light at 488 nm. Fluorescence signals were detected at 518 and 620 nm for FITC and PI detection, respectively. Data were analyzed using CELLQuest (Becton Dickinson) software. For each analysis, 10,000 events were recorded.

MTT Cell proliferation was measured by MTT assay as previously described (Liu et al., 2015)(Deng et al., 2013). Briefly, 100 μ L of Raw264.7 cells at a concentration of 2×10^5 /mL were added to a 96-well tissue culture plate and allowed to adhere. After 30 minutes, the cells were treated with GAC₁ at various concentrations. After 24 hours, 50 μ L of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 2.0 mg/mL in culture medium was added and incubated for 3 hours at 37°C in a humidified, 5% CO₂/95% air, water-jacketed incubator. The supernatant was then discarded and replaced with 200 μ L of DMSO and the plate was placed on an orbital shaker at room temperature. After 30 minutes absorbance was read at 570 nm using a Vmax Kinetic ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

2.6. TNF- α levels measurement

TNF- α levels were measured by ELISA according to the manufacturer's protocol (BD Biosciences, San Diego, CA). All experiments were replicated at least three times with duplicate ELISA measurement of each sample.

2.7. Human Subjects

Human studies were approved by the Institutional Review Board of the Icahn School of Medicine at Mount Sinai. Blood samples were taken from 14 physician-diagnosed asthma patients (age: 25.3 ± 8.7 yrs; Male/Female: 6/8), who had not taking oral steroids for at least one month. All patients signed informed consent.

2.8. PBMC isolation and cell culture

Eleven asthma patients PBMCs were isolated by Ficoll Hypaque (Thermo Fisher Scientific, Piscataway, NJ) density-gradient centrifugation at 1800 RPM for 30 minutes, and washed 3 times in physiological buffer solution (PBS). To assess the effect of GAC₁ on TNF- α production, purified PBMCs (2×10^6 /well) were cultured in RPMI 1640 supplemented with 25 mM Hepes 10% (v/v) heat-inactivated FBS, 60 mg/L (100U/mL) penicillin, 100 μ g/mL streptomycin and 0.29 g/L L-glutamine in 24-well plates with or without GAC₁ (20 μ g/mL) for 24 hours. LPS (2 μ g/mL) was added and culture conditions maintained for another 24 hours. (Preliminary studies showed that LPS at 2 μ g/mL is optimal for PBMC stimulation). At the end of incubation, supernatants were harvested for TNF- α measurement. Cell viability was determined by trypan blue dye exclusion, and the ratio of viable cells to total cells was calculated. To investigate the possible underlying mechanisms, PBMC (2×10^5) samples from additional 3 asthma patients were cultured in a 96-well plate overnight in serum free medium with or without GAC₁ (20 μ g/mL). Cells were stimulated with LPS (2 μ g/mL) for 30 minutes. Whole cell proteins were extracted from three asthma patients PBMCs.

2.9. Western-blot analysis

RAW 264.7 cells (5×10^5 /mL) were pretreated with GAC₁ (0, 2.5, 10, 20, 40 μ g/mL) for 24 hours, then stimulated with LPS (1 μ g/mL) for 30min. Purified asthma patient PBMCs (2×10^6 /mL) were pretreated with GAC₁ (20 μ g/mL) for 24 hours then stimulated for 30 min with LPS (2 μ g/mL). Nuclear and whole cell protein were extracted from RAW cells and asthma patient PBMCs (Active Motif nuclear extract kit, Active Motif, Carlsbad, CA). Whole cell and nuclear extract protein concentrations were determined according to the manufacturer's protocol (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad laboratories, Hercules, CA), and proteins were stored at -80°C .

Proteins (20–40 μ g) were separated by SDS-PAGE and transferred to nitrocellulose transfer membranes. The membranes were blocked with 5% bovine serum albumin in TBS-Tween 20 solution and were incubated with the following antibodies: anti ERK1/2 (rabbit, 1:1000), p-ERK1/2 (mouse, 1:1000), JNK (rabbit, 1:1000), p-JNK (mouse, 1:1000), p38 (mouse, 1:1000), p-p38 (mouse, 1:1000), p-p65 (rabbit, 1:1000), p65 (rabbit, 1:1000), c-Jun (rabbit, 1:1000), c-Fos (rabbit, 1:1000), p-I κ B (mouse, 1:1000), β -actin (rabbit, 1:5000) and histone-H3 (mouse, 1:1000) overnight at 4°C . After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin diluted 1:3000 in TBS-Tween 20 for 1 hour at room temperature with continuous agitation. Reactive bands were visualized using Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, South Plainfield, NJ) according to the manufacturer's protocol.

2.10. Statistical and data analysis

All statistical analyses were performed using Graphpad Prism4 software (GraphPad Software, Inc, La Jolla, CA). Differences between multiple groups and control were analyzed by one-way ANOVA followed by Dunnett's test. Differences between 2 treatments of cells from individual patients were analyzed by paired t test, other dual comparisons by unpaired t tests. A p-value 0.05 was considered to be statistically significant. Percent inhibition was calculated by comparing values obtained by ELISA to respective controls. Data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA, USA) and expressed as percent inhibition, as in our previous publication (Jayaprakasam et al., 2013).

3. RESULTS

3.1. The ASHMITM constituent *G. lucidum* extract, but not *S. flavescentis* or *G. uralensis* extracts significantly suppressed macrophage TNF- α production

To identify the herb(s) in ASHMITM responsible for TNF- α suppression, we compared the effects of 12.5, 25, 50, and 100 μ g/mL ASHMITM, *G. lucidum*, *S. flavescentis*, and *G. uralensis* extracts on RAW 264.7 cell TNF- α production. As expected, LPS stimulation (LPS alone) markedly increased TNF- α production ($p < 0.05$ vs medium). ASHMITM and *G. lucidum* at 50 and 100 μ g/mL concentration significantly suppressed LPS stimulated TNF- α production ($p < 0.05$ vs LPS alone group, Fig. 1A). *S. flavescentis* and *G. uralensis* had no significant inhibitory effect at any tested concentration. We found that *G. lucidum* was more potent than *S. flavescentis* or *G. uralensis* in suppression of macrophage TNF- α production. Calculated IC₅₀ values of ASHMITM, *G. lucidum*, and *G. uralensis* were 56.1, 33.8, and 88.9 μ g/mL, respectively (no IC₅₀ value of *S. flavescentis* was obtained, Fig. 1B).

3.2. The *G. lucidum* methylene chloride fraction suppressed macrophage TNF- α production

Of the two extraction fractions obtained, the triterpenoid-enriched MC fraction (F1) but not the water residue fraction (F2), significantly inhibited LPS-stimulated TNF- α production at 25 μ g/ml (equivalent to 250 μ g/mL *G. lucidum* aqueous extract) (eResult 1 and eFig. 1). Therefore, the triterpenoid-enriched MC fraction was used to isolate compounds with anti-TNF- α actions.

3.3. Isolation and identification of *G. lucidum* ganoderic acids in the MC fraction

Fifteen compounds were isolated and purified from the MC fraction. These compounds were identified by NMR and LC-MS analysis and comparison of the spectroscopic data with those reported in the literature to be ganoderiol F (**1**) (el Mekawy et al., 1998), ganoderic acid α (**2**) (el Mekawy et al., 1998), ganoderic acid V (**3**) (Qiao et al., 2005), ganoderic acid H (**4**) (el Mekawy et al., 1998), ganoderic acid C₂ (**5**) (Yue et al., 2010), ganolucidic acid A (**6**) (Min et al., 2000), ganolucidic acid E (**7**) (Qiao et al., 2005), ganoderic acid C₁ (**8**) (Seo et al., 2009), ganoderenic acid A (**9**) (Wang and Liu, 2008), ganolucidic acid D (**10**) (Min et al., 2000), ganoderic acid U (**11**) (Chen and Yu, 1990), ganoderic acid J (**12**) (Chen and Yu, 1990), ganoderic acid A (**13**) (el Mekawy et al., 1998), ganoderic acid K (**14**) (Yue et al., 2010), and ganoderenic acid D (**15**) (Wang and Liu, 2008). 3D-HPLC analysis of aqueous

extract of *G. lucidum* was measured with peaks identified with corresponding *G. lucidum* triterpenoids isolated (**1–15**) by comparing the maximum absorption and retention time (eFig. 2).

3.4. Effect of GAC₁ and other *Ganoderma* triterpenoids on LPS stimulated TNF- α production

Potential inhibition of TNF- α production by each compound were tested at concentrations of 10 and 20 $\mu\text{g/mL}$ as shown in Fig. 2A. The percent inhibition of compounds **8**, **11**, **12**, **13** and **14** was comparable, with compound **8** (GAC₁) being more effective at 20 $\mu\text{g/mL}$. At 10 $\mu\text{g/mL}$, only compound **8** produced >30 % inhibition.

We next tested GAC₁ at concentrations ranging from 2.5–40 $\mu\text{g/mL}$. GAC₁ suppression of TNF- α was concentration dependent and significant suppression was observed at as low as 10 $\mu\text{g/mL}$ (Fig. 2B). The calculated IC₅₀ value (concentration producing 50% inhibition) was 24.5 $\mu\text{g/mL}$ (47.7 μM). Cell viability was unaffected at any concentration tested by trypan blue staining assay. The percentages of PI (+) and Annexin V (+) cells were all less than 5% at all dose (Fig. 2C, D), considered no-to minimal toxic (Yang et al., 2014) (Yamauchi et al., 2004). GAC₁ suppressed up to 60% LPS stimulated TNF- α secretion by RAW 264.7 cells at 40 $\mu\text{g/mL}$, exhibiting 5% PI(+)/Annexin V(+) cells. This demonstrated that suppression of TNF- α production was not due to apoptosis.

3.5. GAC₁ inhibited TNF- α production without affecting cell proliferation

We next tested the effect of simultaneous GAC₁ and LPS co-incubation on TNF- α production. RAW264.7 cells were simultaneously treated with GAC₁ at various concentrations (0, 2.5, 5, 10, 20 and 40 $\mu\text{g/mL}$) and LPS (1 $\mu\text{g/mL}$) for 24 hours. As shown in Fig 3A, LPS stimulation (LPS alone) markedly increased TNF- α production ($p < 0.05$ vs medium). Co-administration of GAC₁ at 20 and 40 $\mu\text{g/mL}$ concentrations significantly suppressed LPS stimulated TNF- α production ($p < 0.05$ vs LPS alone group). MTT assays showed no effects on proliferation (Fig. 3B).

We also investigated if GAC₁ induced overall suppression of constitutive TNF- α production. To this end, RAW264.7 cells were treated with GAC₁ at various concentrations (0, 2.5, 5, 10, 20 and 40 $\mu\text{g/mL}$) for 48 hours. No reduction of GAC₁ TNF- α production was detected, and 20 and 40 $\mu\text{g/mL}$ concentrations slightly increased production (eFig. 3).

3.6. GAC₁ inhibited TNF- α production by PBMCs from asthma patients

Based on the findings that GAC₁ non toxically inhibited RAW cells LPS stimulated TNF- α production at IC₅₀ approximately 20 $\mu\text{g/mL}$, we next investigated if this effect would be reproduced in asthma patient PBMCs. LPS stimulation significantly increased TNF- α production by PBMCs from asthma patients ($n=11$, $p < 0.05$, LPS alone vs medium). Pretreatment with 20 $\mu\text{g/mL}$ GAC₁ significantly inhibited TNF- α production ($p < 0.05$ vs LPS alone, Fig. 4A). GAC₁ did not affect the percent of viable cells (Fig. 4B).

3.7. GAC₁ inhibited LPS-stimulated p-I κ B α , p-NF- κ B expression, and AP-1 and MAPK activation in a murine macrophages and LPS-stimulated p-NF- κ B expression in asthma patient PBMCs

RAW 264.7 cells were pretreated with GAC₁ (0, 10, and 20 μ g/mL) and stimulated with LPS (1 μ g/mL). Total protein extract measurements showed that 10 and 20 μ g/mL GAC₁ pretreatment reduced LPS-induced RAW cell p-I κ B α and NF- κ B (p65) expression, 20 μ g/mL being more effective (Fig. 5A). GAC₁ pretreatment also reduced nuclear p-p65 levels in a concentration-dependent manner (Fig. 5B). In addition, GAC₁ inhibited, AP-1 c-Jun, but not c-Fos expression. GAC₁ also inhibited JNK and ERK1, ERK2 activation (JNK and ERK1/2 phosphorylation). p38 and p-p38 expression levels were not affected by GAC₁ treatment (Fig. 5C). These data showed that GAC₁ inhibited NF- κ B pathway and partial AP-1 and MAPK family activation.

LPS stimulation also significantly increased PBMC NF- κ B activation (p-p65 expression) ($p < 0.05$, $n = 3$). 20 μ g/mL GAC₁ nearly abrogated this activation as demonstrated by western blot of NF- κ B (p-p65) expression, Fig. 5E, F).

4. DISCUSSION

Asthma is a heterogeneous airway disease. Most asthma studies have focused on Th2 cytokine-driven allergic inflammation. More recently, excessive TNF- α production and increased NF- κ B expression have been demonstrated to be associated with steroid resistant asthma (Berry et al., 2007; Ito et al., 2008). At present, there is no safe and effective treatment for this type of asthma. Monoclonal antibodies against TNF- α , TNF- α receptors, and TNF- α receptor constructs are currently used to treat certain TNF- α associated disorders, such as rheumatoid arthritis (RA) and Crohn's disease (Gardam et al., 2003; Scheinfeld, 2004). Although a few preliminary studies of small numbers of asthma patients found improvement in lung function, airway hyperresponsiveness, quality-of-life and exacerbation rate following treatment with anti-TNF therapy, such therapy for asthma is limited because of inconsistent findings of efficacy and concern about serious adverse effects (Berry et al., 2007; Brightling et al., 2008).

ASHMITM was previously shown to suppress TNF- α production in a murine asthma model and in cultured macrophages without toxicity (Brown LL et al., 2009; Srivastava et al., 2014). As a first attempt to identify TNF- α inhibitory compounds present in ASHMITM, we compared potential anti-TNF- α effects of extracts of the three herbs in ASHMITM, and found that *G. lucidum* was more potent than *G. uralensis* in suppressing macrophage TNF- α production. *S. flavescens* had negligible inhibitory effect on macrophage TNF- α production. We therefore, focused on identifying *G. lucidum* active compounds that inhibit TNF- α production using RAW 264.7 cells as a screening tool. We found that the non polar methylene chloride triterpenoid enriched fraction (F1(MC)), but not the water-soluble, residue after organic solvent extraction (F2), significantly suppressed TNF- α production. Triterpenes and polysaccharides are the predominant biologically active compounds in *G. lucidum*. (Boh et al., 2007) These two classes of compounds have different effects on immune responses. *G. lucidum* polysaccharides increase pro-inflammatory cytokine production including TNF- α , IFN- γ and IL-6 production (Habijanec et al., 2015; Pan et al.,

2013), whereas a triterpene-rich extract suppressed inflammatory cytokines (Dudhgaonkar et al., 2009). Why F2 did not show any effect on LPS-stimulated TNF- α production in our study is unknown, but it might be because F2, which was the water-soluble polysaccharides containing fraction after organic solvent extraction, contained other water-soluble compounds that counteracted the polysaccharide stimulation. Since our goal is to identify compounds that inhibit excessive LPS stimulated TNF- α production, we focused on the triterpene rich MC fraction, and isolated and identified *G. lucidum* triterpenoid compounds. Among the 15 isolated compounds, GAC₁ showed greatest inhibition of TNF- α production, and in a dose dependent, non-cytotoxic manner. It is also worth noting that the exact extent to which GAC₁ contributes to the effect of *G. lucidum* MC fraction inhibition of TNF- α production by RAW 264.7 cell TNF- α inhibition is unknown. Several other compounds isolated from the *G. lucidum* MC fraction including ganoderic acids U, J, A and K produced more than 30% inhibition of TNF- α production at 20 μ g/mL concentration. These compounds may act additively and/or synergistically with GAC₁. Further investigation is required.

Importantly, the effectiveness and safety of GAC₁ suppression of TNF- α was also demonstrated in human PBMC from asthma patients. Although various *G. lucidum* triterpenoids have been isolated, most studies focused on their anti-cancer (Li et al., 2013; Yao et al., 2012) and anti HIV properties (Akbar and Yam, 2011; el Mekawy et al., 1998). No previous study determined inhibition of TNF- α production by GAC₁ or any other *G. lucidum* triterpenoid compounds in PBMCs from asthma patients.

LPS induces TNF- α through the activation of several intracellular signaling pathways including NF- κ B, AP-1 and MAP kinases. GAC₁ reduction of p-I κ B levels in total cell protein and p-p65 in nuclear protein demonstrated that it inhibited the NF- κ B signaling pathway. It also partially suppressed the AP-1 family as shown by reduction of c-Jun, but not c-fos expression, and the MAPK pathway as shown by suppression of p-ERK1/2 and p-JNK, but not p-p38 expression. Therefore, in addition to the effect of GAC₁ on NF- κ B, GAC₁ suppression of TNF- α may also be partially attributed to suppression of MAPK and AP-1. Because of the limited availability of human asthma patient PBMCs, we focused on the NF- κ B pathway, and demonstrated that GAC₁ also suppressed p-p65 expression. These findings demonstrate that non-toxic suppression of TNF- α production by GAC₁ is due, at least in part, to suppression of NF- κ B signaling pathway.

TNF- α is a key cytokine involved in the immediate host defense against invading microorganisms prior to activation of the adaptive immune system (Medzhitov and Janeway, Jr., 2000). It also inhibits tumorigenesis and viral replication (Kumar et al., 2013; Landskron et al., 2014). However, excessive levels of TNF- α have been implicated in mediating or exacerbating a number of refractory inflammatory diseases including Crohn's disease (CD) (Brynskov et al., 2002), rheumatoid arthritis (Choy and Panayi, 2001), and asthma (Brightling et al., 2008). Anti-TNF- α medications, including receptor constructs and monoclonal antibodies against TNF- α , are currently used to treat inflammatory bowel disease and arthritis, and are being explored as treatments for recalcitrant asthma. Unfortunately, adverse effects, including lymphoma and infections have been associated with current TNF blockers (Scheinfeld, 2004). Therefore developing safe and effective

natural TNF- α inhibitors may be of value in treating excessive TNF- α associated condition. If GAC₁ would affect cancer and infections is unknown, but previous publication showed that GAC₁ exhibits anti- HIV-1 (el Mekawy et al., 1998) and anti-cancer cell activities properties (Gao et al., 2002). We found that GAC₁ suppression of TNF- α production is not via induction of cell death or apoptosis, but due, at least partially, to modulation of cell signaling pathways. In addition, we found that GAC₁ does not cause overall TNF- α production suppression. RAW 264.7 cells constitutively produce low levels of TNF- α . We found that GAC₁ co-incubation at 20 and 40 μ g/mL slightly but significantly increased constitutive TNF- α production. This effect is similar to the *G. lucidum* polysaccharide immune stimulatory effect on non LPS stimulated cells. (Habijanec et al., 2015; Pan et al., 2013). Taken together, GAC₁ treatment may be of less concern regarding adverse effect than current anti-TNF- α therapy. The precise mechanisms underlying GAC₁ regulatory effect on TNF- α and other cytokines are unknown and require further investigation.

In conclusion, we for the first time, demonstrated that GAC₁ isolated from *G. lucidum*, an ASHMITM herbal constituent, inhibited TNF- α production by LPS stimulated murine macrophages and asthma patients PBMCs; and that this effect was associated with suppression of the NF- κ B signaling pathway and in part suppression of AP1 and MAPK pathways. GAC₁ may have potential as a novel therapy for treatment of TNF- α associated asthma and other inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Ganoderic acid C₁ (*G. lucidum*) inhibits LPS-evoked TNF- α produced by macrophages
- Ganoderic acid C₁ reduces LPS-evoked TNF- α produced by PBMCs from asthma patients
- Ganoderic acid C₁ inhibition of TNF- α production is associated with decreased NF- κ B

Fig. 1. Effect of ASHMITM and extracts of its constituent herbs on TNF- α production

(A) RAW 264.7 cells were cultured with or without extracts for 24 hours, and then cultured in the presence of 1 μ g/mL LPS for 24 hours. TNF- α levels in cell culture supernatants were determined by ELISA. Data are expressed as mean $\pm$ S.D. of three independent experiments. #, $p < 0.05$ vs medium alone culture; ** $p < 0.01$ vs. LPS stimulated, but untreated (LPS alone) culture. (B) Percent inhibition was calculated in relation to LPS stimulated untreated cells.

Fig. 2. Effect of 15 compounds isolated from *G. lucidum* on TNF- α production

(A). RAW 264.7 cells were pre-incubated with or without isolated *G. lucidum* triterpenoids (10 and 20 μ g/mL) for 24 hours, followed by addition of LPS (1 μ g/mL) and culture for additional 24 hours. TNF- α levels in cell culture supernatants were measured by ELISA. Data are expressed as percent inhibition compared with LPS only group. (B). Concentration dependent GAC₁ inhibition of TNF- α production by LPS-stimulated RAW 264.7 cells. (C). Cell viability of GAC₁-treated and LPS-stimulated macrophages. Data are expressed as mean $\pm$ S.D. of 3–6 independent experiments. ** p <0.01, * p <0.05 Vs LPS alone. (D). Cells were exposed to 0, 10, 20, 40 μ g/mL GAC₁ for 24 hours, followed by addition of LPS (1.0 μ g/mL) and cultured for an additional 24 hours. Cells were then collected and subjected to Annexin V-FITC/PI staining and analysis by flow cytometry.

Fig. 3. GAC₁ inhibited TNF-α production when simultaneously co-incubated with LPS (A). RAW 264.7 cells were treated by simultaneous co-incubation with various concentrations of GAC₁ and LPS (1.0 μg/mL) for 24 hours. TNF-α levels in cell culture supernatants were determined by ELISA. Data are expressed as mean ± S.D. of 3–6 independent experiments. **p<0.01, *p<0.05 vs medium and LPS control groups. (B). RAW264.7 cells were treated with 0, 2.5, 5, 10, 20 and 40 μg/mL GAC₁ for 24 hours then subjected to MTT cell proliferation assay. Cell proliferation percentage was calculated by comparison with the medium alone group, which was set at 100%.

Fig. 4. GAC₁ inhibited TNF-α production by PBMCs from asthma patients

Purified PBMCs were treated with or without GAC₁ (20 μg/mL) for 24 hours. LPS (2 μg/mL) was added and culture conditions maintained for additional 24 hours. Supernatants were harvested and TNF-α levels were determined by ELISA. (A). GAC₁ (20 μg/mL) inhibition of TNF-α production by LPS-stimulated PBMCs from asthma patients. (B). PBMC viability after treatment with GAC₁ (20 μg/mL). * p<0.05, paired t-test, (n=11).

Fig. 5. GAC₁ regulation of NF-κB, AP-1 and MAPK

RAW 264.7 macrophages were pretreated with GAC₁ (0, 10, or 20 μg/mL) for 24 hours, followed by stimulation with LPS (1 μg/mL) for 30 minutes. (A). p65, and p-IκBα expression were evaluated by Western-blot analysis of whole cell extracts. (B). Nuclear translocation of p-p65 was evaluated by Western-blot analysis of nuclear extracts. Equal protein loading was verified with Histone-H3 antibody. (C). c-Fos, c-Jun, ERK1/2, p-ERK1/2, JNK, p-JNK, p38, and p-p38 were evaluated by Western-blot analysis of whole cell extracts. Results are representative of three independent experiments. Purified PBMCs were pretreated with GAC₁ (20 μg/mL) for 24 hours, followed by stimulation with LPS (2 μg/mL) for 30 minutes. (D). Representative Western blotting analysis of NF-κB (p-p65) expression in PBMCs cultured in medium alone, stimulated with LPS, with and without GAC₁ treatment. GAPDH expression was used as control. (E). Quantitation of Western blotting analysis of p-NF-κB (p-p65) expression by PBMCs cultured in medium alone, stimulated with LPS, and stimulated and treated with GAC₁ ($p < 0.05$ Vs LPS alone, Mean $\pm$ S.D., $n=3$).