Mogroside IIIE, a Novel Anti-Fibrotic Compound, Reduces Pulmonary Fibrosis through Toll-Like Receptor 4 Pathways

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ABSTRACT

Idiopathic pulmonary fibrosis is a progressive fibrotic lung disease, and eventually most patients develop respiratory failure with a median survival rate of 2 to 3 years after diagnosis due to the lack of clinically effective therapies. Mogroside IIIE (MGIIIE), a cucurbitane-type compound, was isolated from Siraitia grosvenorii. MGIIIE has shown the strongest inhibition of nitric oxide release, a crucial inflammatory factor, from lipopolysaccharide (LPS)-treated RAW264.7 cells compared with other mogrosides. In the pulmonary fibrosis mouse model induced by bleomycin, MGIIIE treatment attenuated pulmonary fibrosis, indicated as a reduction in myeloperoxidase activity, collagen deposition, and pathologic score. MGIIIE also significantly suppressed expression of several important fibrotic markers, e.g., α-smooth muscle actin, collagen I, transforming growth factor-β (TGF-β) signal, and metalloproteinases-9/tissue inhibitor of metalloproteinase-1. Furthermore, MGIIIE blocked transdifferentiation of lung resident fibroblasts into myofibroblast-like cells induced by TGF-β or LPS and subsequently inhibited collagen production in lung fibroblasts. These data indicate that MGIIIE is a potent inhibitor for pulmonary fibrosis. In vitro and in vivo mechanistic studies have shown that MGIIIE significantly decreased expression of toll-like receptor 4 (TLR4) and its downstream signals of myeloid differentiation factor 88 (MyD88)/mitogen-activated protein kinase (MAPK), an inflammatory signal essential for extracellular matrix (ECM) deposition in pulmonary fibroblasts. Taken together, these results demonstrate that MGIIIE significantly prevents pulmonary fibrosis by inhibiting pulmonary inflammation and ECM deposition through regulating TLR4/MyD88-MAPK signaling. Our study suggests that MGIIIE may have therapeutic potential for treating pulmonary fibrosis in clinical settings.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a severe lung disease, which is characterized by progressive and irreversible destruction of lung architecture caused by excessive extracellular matrix (ECM) deposition that ultimately leads to respiratory failure. The median survival time of patients with IPF is about 2 or 3 years due to limited clinical therapies (Gürbüzel et al., 2016). Extensive evidence indicates that dysfunctions of the resident fibroblasts by lung inflammation greatly contribute to the development of pulmonary fibrosis. Once fibroblasts become activated, they transform into α-smooth muscle actin (α-SMA)–expressing myofibroblasts that secrete ECM components leading to fibrotic lesions in the lung (Derynck and Zhang, 2003; Chen et al., 2012). A variety of cytokines and proteinases have been shown to be involved in the fibrotic process including transforming growth factor-β (TGF-β), myeloperoxidase (MPO), and metalloproteinases (MMPs) (Yoshimura et al., 2006; Bhattacharyya et al., 2013; Shi et al., 2014), which are considered as essential markers for pulmonary fibrosis. Recently, a crucial role of toll-like receptor 4 (TLR4) in fibroblasts during fibrogenesis has been highlighted (Poltorak et al., 1998; Jiang et al., 2005; Huebener and Schwabe, 2013). Pulmonary fibrosis induced by bleomycin, lipopolysaccharide (LPS), or radiation was significantly attenuated in TLR4 knockout mice (He et al., 2009, 2012; Bhattacharyya et al., 2013; Rhiieu et al., 2014). Moreover, it has been reported that TLR4 is required for fibroblast activation and collagen production as indicated by the fact that TGF-β failed to induce collagen production in TLR4 knockout fibroblasts (Bhattacharyya et al., 2013). Upregulated TLR4 in fibroblasts augmented TGF-β sensitivity and converted self-limited tissue dysfunctions to chronic fibrotic remodelling. This work was financially supported by the National Natural Science Foundation of China (81573553) to C.F., the National New Drug Innovation Major Project of China [2011ZX09307-002-02], the Priority Academic Program Development of Jiangsu Higher Education Institutions, and National Found for Fostering Talents of Basic Science [J1310032] in China; and the American Heart Association [12SDG9050018] to Y.G. in the United States.

ABBREVIATIONS: α-SMA, α-smooth muscle actin; Col I, collagen I; DMSO, dimethylsulfoxide; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; IL, interleukin; IPF, idiopathic pulmonary fibrosis; JNK, c-JUN N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MGIIIE, mogroside IIIE; MGV, mogroside V; MMP, metalloproteinase; MPO, myeloperoxidase; MyD88, myeloid differentiation factor 88; NO, nitric oxide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLF, primary lung fibroblast; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinase; TLR4, toll-like receptor 4.
repair into intractable fibrosis in bleomycin-induced pulmonary fibrosis (Bhattacharyya et al., 2013). In addition, downstream signals of TLR4, mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK), and p38, were significantly activated in lung tissue from IPF patients (Yoshida et al., 2002) and blockade of ERK, JNK and p38 reduced fibrosis in mouse models (Matsuoka et al., 2002; Madala et al., 2012; Vittal et al., 2013; van der Velden et al., 2016). Therefore, disruption of TLR4 overexpression in lung inflammation may represent a potential target for IPF prevention.

In China, the fruits of *Siraitia grosvenorii* (Cucurbitaceae), a famous traditional Chinese medicine, are used to treat dry cough, sore throat, extreme thirst, and constipation (Committee of National Pharmacopoeia, 2010; Chen et al., 2011). The active components of *Siraitia grosvenorii* are a group of cucurbitane-type triterpene glycosides named mogrosides. It has been reported that mogrosides have various bioactivities, including anti-inflammatory, anti-obesity, anti-diabetic, and anti-allergic effects as well as hepatoprotective effects (Chen et al., 2011; Di et al., 2011; Jin and Lee, 2012; Sun et al., 2012; Xiao et al., 2012; Xiao and Wang, 2013; Song et al., 2014). Recent research has shown that mogroside V (MGV) attenuated LPS-induced acute lung injury and inflammation (Suzuki et al., 2005; Shi et al., 2014; Xu et al., 2015); however, the anti-fibrotic effects of mogrosides on respiratory diseases have not been reported.

Mogroside IIIIE (MGIIIE) is a main component of the fruits of *S. grosvenorii* and has shown the strongest anti-inflammatory effects in LPS-induced macrophage activation in our screening experiment. Therefore, MGIIIE was selected to evaluate the anti-fibrotic effects in a mouse experimental model of pulmonary fibrosis. MGIIIE exhibited a significant anti-fibrotic activity in both in vitro and in vivo models. Mechanistic studies identified that the MGIIIE inhibition fibrosis may be dependent on TLR4-mediated MAPK signaling pathways.

**Materials and Methods**

**Chemicals and Reagents.** MGIIIE (purity above 99%), mogroside IVE (purity above 99%), siraitipenoside I (purity above 99%), and MGV (purity above 99%) were all purchased from Chengdu Biopurify Phytochemical Ltd. (Chengdu, China). The purity of these mogrosides has been tested by high-performance liquid chromatography. Bleomycin was obtained from Nippon Kayaku (Tokyo). Prednisone was obtained from Zhejiang Xianju Pharmaceutical Co., Ltd. (Xianju, China). Recombinant TGF-β1 was purchased from PeproTech Inc. (Rocky Hill, NJ). TAK-242 (a TLR4 inhibitor) was purchased from Merck Millipore Inc. (Billerica, MA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was obtained from Biosharp Technology Inc. (Hefei, China).

The MPO test kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against phospho-Smad2/3, p38/phospho-p38, ERK/phospho-ERK, and JNK/phospho-JNK were all obtained from Cell Signal Technology Inc. (Danvers, MA). Antibodies against β-actin, α-SMA, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, myeloid differentiation factor 88 (MyD88), TGF-β1, Smad2/3, and horseradish peroxidase-conjugated secondary antibody were all purchased from Bioworld Technology Inc. (Dublin, OH). Antibody against collagen I (Col I) was purchased from Wanleibo Technology Inc. (Shenyang, China). Antibody against TLR4 and secondary antibody Donkey anti-rabbit IgG H&L (Alexa Fluor 488) were obtained from Abcam Technology Inc. (Cambridge, United Kingdom).

**Animals.** Adult male C57BL/6 mice weighing between 20 and 2 g (6–8 weeks old) were purchased from the Comparative Medicine Center at Yangzhou University (Yangzhou, China). Care of the animals followed the recommended protocols of the General Recommendation and Provisions of the Chinese Experimental Animals Administration Legislation (Permit number SYXK2012-0035). The animal protocol was approved by the Institutional Ethical Committee of China Pharmaceutical University. Mice were housed in a climate-controlled room at 22°C ± 2°C and 50% ± 10% humidity with a 12-hour light/dark cycle, and given conventional feed and free access to drinking water.

**RAW 264.7 Culture and Nitric Oxide (NO) Measurement.** The RAW 264.7 cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). RAW264.7 cells were maintained in GIBCO Dulbecco’s modified Eagle’s medium (Grand Island, NY) supplemented with Hyclone 10% fetal bovine serum (Thermo Scientific, Waltham, Massachusetts), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C, with 95% humidity and 5% carbon dioxide. For treatment, RAW264.7 cells were detached and seeded in 96-well plates at 4 × 10^3 cells per well. When reaching a confluence of 70%–80%, cells were incubated with LPS (5 μg/mL), chemicals, or controls [phosphate-buffered saline (PBS) or dimethylsulfoxide (DMSO)] for 24 hours, and 50 μL cell medium was used for NO measurement according to a previous published procedure (Fan et al., 2013). Briefly, 50 μL of Griess reagent (Beyotime, Shanghai) was mixed with 50 μL culture medium. After standing for 5 minutes, the optical density of the mixture was measured by a microplate reader at a 540 nm wavelength. The concentration of nitrite was determined by a standard curve using sodium nitrite.

**Experimental Model of Bleomycin-Induced Pulmonary Fibrosis in Mice.** The method of bleomycin-induced pulmonary fibrosis has been previously described (You et al., 2015). Briefly, after 1 week of acclimation, animals were divided randomly into six groups and anesthetized by intraperitoneal injection of chloral hydrate solution (4%, 10 ml/kg), followed by intratracheal instillation of bleomycin (5 mg/kg) or saline as the control. Seven days after bleomycin administration, MGIIIE-H (20 mg/kg), MGIIIE-M (10 mg/kg), and MGIIIE-L (1 mg/kg) were orally administered to mice once a day for 14 consecutive days. Prednisone (6.5 mg/kg) was used as the positive drug. The control groups were given the same volume of 0.9% sterilized saline. On day 21, mice were euthanized by excessive intraperitoneal injection of chloral hydrate. Lung tissue was excised for pulmonary index measurement (lung weight/body weight; mg/g). The left lower lobes were fixed in 10% formalin for histopathological examination, and the rest of the lung tissue was stored at ~80°C.

**Histologic Analysis.** The lung tissue samples were fixed with 10% formalin and embedded in paraffin, sectioned, and stained with H&E or Masson trichrome. Pathologic evaluation was conducted by experienced pathologists in a single-blind procedure. The results were scored in accordance with a previously reported method (Szapiel et al., 1979), and score numbers 0–3 corresponded to grades of −, +, ++, and ++++, respectively.

**MPO and Interleukin (IL)-1β Assays.** MPO was an indicator of polymorphonuclear leukocyte accumulation. The method of MPO assay has been previously described (Shi et al., 2014). Briefly, 21 days after bleomycin treatment, right lungs were excised and homogenized in extraction buffer. After centrifuging, the homogenate and reaction mixture was used for NO measurement according to a previous published procedure (Fan et al., 2013). Briefly, 50 μL of Griess reagent (Beyotime, Shanghai) was mixed with 50 μL culture medium. After standing for 5 minutes, the optical density of the mixture was measured by a microplate reader at a 540 nm wavelength. The concentration of nitrite was determined by a standard curve using sodium nitrite.

**Isolation and Culture of Mouse Primary Lung Fibroblasts (PLFs).** The mouse PLFs were isolated as described previously, with some modifications (Bruce and Honaker, 1998). Briefly, lung tissue
collected from C57/B6 mice were minced into 1- to 2-mm³ pieces and digested with trypsin for 15 minutes at 37°C. The digested cell suspensions were collected and cultured with Dulbecco’s modified Eagle’s complete medium and incubated at 37°C in an atmosphere of 5% CO₂ in air overnight. After cells attached, the medium was changed the next day. The percentage of fibroblast was identified as 95% by morphology under a microscope.

**Cell Treatment.** For TGF-β1-treated mouse PLFs, cells were detached with 0.25% trypsinization and seeded in 96-well plates at 4 × 10⁴ cells per well or 6-well plates at 2 × 10⁵ cells per well. Cells were treated with TGF-β1 (10 ng/ml), MGIIIE (10 μM), or controls (PBS or DMSO) for 48 hours. For LPS-treated mouse PLFs, cells were treated with LPS (5 μg/ml), MGIIIE (10 μM), or controls (PBS or DMSO) for 48 hours. In some experiments, cells were pretreated with or without the TLR4 inhibitor, TAK-242 (10 nM), for 1.5 hours and subsequently incubated with or without LPS (5 μg/ml), MGIIIE (10 μM), or controls (PBS or DMSO) for 48 hours.

**Cell Viability Assay.** Cell viability was assayed with the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Briefly, cells were seeded in 96-well plates at 4 × 10⁴ cells per well and 6-well plates at 2 × 10⁵ cells per well. Cells were treated with TGF-β1 (10 ng/ml), MGIIIE (10 μM), or controls (PBS or DMSO) for 48 hours. For LPS-treated mouse PLFs, cells were treated with LPS (5 μg/ml), MGIIIE (10 μM), or controls (PBS or DMSO) for 48 hours. In some experiments, cells were pretreated with or without the TLR4 inhibitor, TAK-242 (10 nM), for 1.5 hours and subsequently incubated with or without LPS (5 μg/ml), MGIIIE (10 μM), or controls (PBS or DMSO) for 48 hours.

**Real-Time Quantitative Polymerase Chain Reaction (PCR) Analysis.** Total RNA was extracted from the lung tissue using Trizol reagent (Invitrogen Life Technologies Carlsbad, CA), reverse-transcribed to cDNA using the TransScript first-Strand cDNA Synthesis kit (TOYOBO, Osaka) and stored at −80°C until reverse transcription. The relative gene expression was quantified by quantitative PCR using SYBR Premix Ex Taq (TaKaRa, Dalian, China) in StepOne Real-Time PCR (Life Technologies, Carlsbad, CA.). In each reaction, 0.5 μg of total RNA was reverse transcribed before the following PCR conditions: 94°C for 2 minutes followed by 40 cycles at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with final extension at 72°C for 10 minutes. The sequences (5'-3') for the primers are given in Table 1 and the data were quantified using the comparative Ct (ΔCt) method and are presented as mean ratio to β-actin.

**Immunohistochemistry and Immunofluorescence Stainings.** For immunohistochemistry staining, the paraffin sections (5 μm) were incubated with 3% H₂O₂ to eliminate endogenous peroxidase. After antigen retrieval by heating, nonspecific binding sites were blocked with 5% skim milk in PBS for 1 hour. The sections were incubated with primary antibody and then incubated with secondary antibodies conjugated with horseradish peroxidase. Color was visualized by incubated section with diaminobenzidine. Images were acquired under an Olympus BX55 microscope (Olympus, Tokyo, Japan).

For immunofluorescence staining, cell were fixed with 4% paraformaldehyde/PBS for 15 minutes, followed by incubation with 0.3% Triton X-100/PBS for 10 minutes and 2% bovine serum albumin/PBS block solution for 2 hours, and then probed with primary antibody overnight at 4°C. Alexa Fluor 488-conjugated secondary antibodies were used to apply the signal. 4', 6-diamidino-2-phenylindole was used to stain nuclei. Images were obtained by fluorescent microscopy (Olympus IX53) or confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany).

**Western Blot Analysis.** Total proteins extracted from lung homogenate or cell lysate were lysed in ice-cold radioimmunoprecipitation assay lysis buffer containing 1:100 dilution of phenylmethanesulfonyl fluoride (Beyotime, China). Protein concentrations were determined using BCA Protein Assay Kit (Beyotime). After boiling for 10 minutes, equal amounts of the protein (50 μg/lane) were separated by SDS-PAGE gel and transferred to polyvinylidene fluoride membranes (Merck Millipore) that were probed with primary antibodies overnight at 4°C and horseradish peroxidase-labeled secondary antibodies at 25°C for 2 hours and the bands were visualized using superenhanced chemiluminescence detection reagent (Beyotime).

**Statistical Analysis.** All data are presented as mean ± S.D. from at least three independent experiments. One-way analysis of variance and two-tailed Student’s t test were used to analyze differences among different groups (GraphPad Prism software version 5.0). Values of *P* < 0.05 are regarded as statistically significant.

### Results

**Anti-Inflammatory Activity Screening of Mogrosides on LPS-Activated RAW264.7 Cells.** MGV, siameroside I, mogroside IVE, and MGIIIE are major mogrosides from the fruits of Cucurbitaceae (Fig. 1A). It has been reported that MGV has anti-inflammatory activity in lung inflammation (Shi et al., 2014). Using the LPS-stimulated RAW264.7 mouse macrophages activation model, we screened the anti-inflammatory effects of these four mogrosides. No cell toxicity was observed in all four mogroside-treated groups (Fig. 1B). After cells were treated with LPS (5 μg/ml) for 24 hours, NO production was increased approximately 3-fold. All four mogrosides significantly inhibited NO production induced by LPS in a dose-dependent manner. MGIIIE had the lowest IC₅₀ value (10.22 μM) compared with the other mogrosides (Fig. 1C), indicating the strongest anti-inflammatory effect of MGIIIE. Therefore, MGIIIE was selected for further in vivo studies.

**MGIIIE Protects against Bleomycin-Induced Pulmonary Fibrosis in Mice.** Bleomycin is a commonly used agent to induce an experimental IPF model in mice, which is characterized by activated myofibroblasts that contribute to the exuberant fibrosis (Bhattacharyya et al., 2013). In this model, 7–9 days is the switching point from lung inflammation to the fibrotic phase. In the present study, experimental pulmonary fibrosis was induced by a single intratracheal instillation of bleomycin (5 mg/kg). Three doses of MGIIIE, MGIIIE-L (1 mg/kg/d), MGIIIE-M (10 mg/kg/d), and MGIIIE-H (20 mg/kg/d) were orally administered for 14 days, starting 7 days after bleomycin administration. As a positive control, prednisone, a synthetic corticosteroid drug for IPF treatment in

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Product Size (Base Pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-α-SMA</td>
<td>CCA CGA AAC CAC CTA TAA CAG C</td>
<td>GGA AGG TAG ACA GCG AAG CC</td>
<td>236</td>
</tr>
<tr>
<td>M-Col I</td>
<td>CTG ACT AGA AGA GCG GAG AG</td>
<td>CGG CTG AGT AGG GAA CAC AC</td>
<td>116</td>
</tr>
<tr>
<td>M-TGF-β1</td>
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<td>TGC GAC CCA CTT GAT AGA CG</td>
<td>286</td>
</tr>
<tr>
<td>M-TLR4</td>
<td>ACA CTT TAT TCA GAG CCG TGT TTG</td>
<td>CAG GTGCAA GTT GCC GTT TC</td>
<td>297</td>
</tr>
<tr>
<td>M-MyD88</td>
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<td>GAC TGG TGT CAA GGG TGG TGA T</td>
<td>195</td>
</tr>
<tr>
<td>M-actin</td>
<td>CTG AGA GGG AAA TCG TGC TTG</td>
<td>CCA CGA GAT TCC ATA CCC AAG A</td>
<td>208</td>
</tr>
</tbody>
</table>
the clinical setting was administrated into mice after bleomycin injection. After bleomycin treatment, MGIIIE-M and MGIIIE-H significantly protected mice from weight loss and death even better than prednisone treatment (Fig. 2, A and B). Histologic changes in the lung including edema, alveolar wall thickening, and neutrophil infiltration in the lung parenchyma were observed in mice after bleomycin treatment. MGIIIE markedly inhibited these lung damages as shown in Fig. 2, C–F. Furthermore, we compared several inflammatory factor levels in mice in these groups. Both MPO activity and cytokine IL-1β were elevated in the bleomycin-treated group; however, they were greatly reduced in the MGIIIE-treated group (Fig. 2, G and H), indicating an anti-inflammatory effect of MGIIIE in bleomycin-induced lung inflammation. These data demonstrated that MGIIIE significantly attenuated lung inflammation and fibrosis in bleomycin-induced pulmonary fibrosis.

**Fibrotic Markers Are Altered by MGIIIE during Bleomycin-Induced Pulmonary Fibrosis.** Hydroxyproline, α-SMA, Col I, and MMP-9/TIMP-1 ratios are major fibrotic markers during pulmonary fibrosis (Yoshimura et al., 2006; Todd et al., 2012; Li et al., 2015). Hydroxyproline and Col I indicate collagen production in tissue (Li et al., 2015). α-SMA expression indicates a myofibroblast transformation after fibroblast activation. The ratio of protease MMP-9/TIMP-1 is essential for ECM remodeling (You et al., 2015). The TGF-β/Smad2/3 signal is a major profibrotic factor that is responsible for switching the inflammatory phase to the fibrotic phase in lung fibrosis (Yang et al., 2012; Bhattacharyya et al., 2013). To investigate whether MGIIIE has an anti-fibrotic effect in bleomycin-induced pulmonary fibrosis, we measured these markers in fibrotic lung tissue in the MGIIIE-M- and MGIIIE-H-treated groups. As shown in Fig. 3, bleomycin significantly increased hydroxyproline level, α-SMA expression, and Col I content, and decreased the MMP-9/TIMP-1 ratio; however, both MGIIIE-M and MGIIIE-H treatment reduced the magnitude of all of these changes at both the protein and mRNA level. In addition, 21 days after bleomycin injection, TGF-β and phosphorylation of its downstream signals in Smad2/3 were markedly increased in mice.
MGIIIE treatment attenuated bleomycin-induced pulmonary fibrosis in mice. Mice were given an intragastric administration of MGIIIE (1, 10, or 20 mg/kg, respectively) and prednisone (6.5 mg/kg) daily 7 days after an intratracheal instillation of bleomycin, and mice were killed 21 days after bleomycin injection. The body weight (A), survival rate (B), and pulmonary index (F) were determined, and the representative images of H&E staining and Masson’s trichrome staining (C) of lung sections in mice as well as comparison of the inflammation score (D) and fibrosis score (E) among the experimental groups are shown. Data are expressed as mean ± S.D. (n = 10). The MPO activity and IL-1β level were detected in the lung tissue (G and H). Data are expressed as mean ± S.D. (n = 5). ***P < 0.001 versus control group. **P < 0.01, ***P < 0.001 versus model group.
MGIIIE significantly inhibited TGF-β/Smad2/3 pathway activation as shown in Fig. 4. These data suggest that MGIIIE protects against lung fibrosis through regulating multiple fibrotic factors.

**MGIIIE downregulates TLR4/MyD88-MAPK Signals during Pulmonary Fibrosis.** It is well documented that LPS binding to TLR4 induces MyD88-dependent pathways and that TLR4 is responsible for the initiation of pulmonary inflammation and fibrosis after acute and chronic lung injury, which is integrally involved in the TGF-β1 pathway in a feed-forward loop and results in increased matrix production (Willis et al., 2005; Yang et al., 2012). To investigate whether MGIIIE regulates pulmonary fibrosis through the TLR4 receptor, we measured TLR4 and its downstream signaling in the MGIIIE-M- and MGIIIE-H-treated groups. The results from both immunohistochemistry staining and western blotting showed that TLR4 expression was upregulated in lung tissue after bleomycin treatment; however, both MGIIIE middle and high doses (10 and 20 mg/kg) significantly inhibited TLR4 expression as shown by the lighter brown color in lung tissue compared with the model group (see Fig. 5, A and B). Consistently, TLR4 and MyD88 mRNA and protein levels were significantly downregulated after treatment with MGIIIE compared with the bleomycin group (Fig. 5, C–G), indicating that MGIIIE may regulate TLR4 expression during lung inflammation and fibrosis. In addition, we measured the activity of the MAPK family, JNK, ERK, and p38, the downstream signals of TLR4/MyD88 activation. It has been reported that JNK, ERK, and p38 play critical roles in the development of lung fibrosis (Matsuoka et al., 2002; Yoshida

![Figure 3](https://i.imgur.com/3Q5G5F5.png)

**Fig. 3.** MGIIIE treatment decreased the hydroxyproline (HYP) and collagen content and other fibrotic markers in bleomycin-damaged lung tissues in mice. Mice were given an intragastric administration of MGIIIE (10 and 20 mg/kg, respectively) and prednisone (6.5 mg/kg) daily 7 days after an intratracheal instillation of bleomycin, and mice were killed 21 days after bleomycin injection. (A) The HYP content in the lung tissues was detected. The mRNA expressions of α-SMA (B) and Col I (C) in the lung tissues were detected by real-time PCR analysis. (D–I) The protein expressions of α-SMA, Col I, MMP-9, and TIMP-1, and the ratio of MMP-9/TIMP-1 in the lung tissues were detected by western blot analysis. Data are expressed as mean ± S.D. (n = 3–5). ***P < 0.001 versus control group. *P < 0.05, **P < 0.01, ***P < 0.001 versus model group.
In our results, phosphorylation of JNK, ERK, and p38 were significantly enhanced after bleomycin injection; however, their activation was inhibited by MGIIIE-H and MGIIIE-M, and to a similar extent to by prednisone (Fig. 5, H and I). These data indicated that MGIIIE suppressed both TLR4/MyD88 expression and its downstream MAPK signal activation, which may contribute to its anti-fibrotic effects in pulmonary fibrosis.

**MGIIIE Suppressed ECM Deposition and the TLR4/MyD88 MAPK Signaling Pathway in Activated Lung Fibroblasts Induced by TGF-β.** Pulmonary fibroblasts transform into myofibroblast-like cells and are one of the key steps in initiation of pulmonary fibrosis. Myofibroblasts are the major source of ECM production in the fibrotic lung (Hay et al., 1991). To further investigate the mechanism underlying the MGIIIE inhibitory role on fibrosis in lung, PLFs were isolated from mice and subjected to TGF-β challenge to induce fibrosis in the in vitro model, which is characterized as myofibroblast transformation and excessive collagen production in fibroblasts. First, our data showed that cell viability of fibroblasts was not affected by MGIIIE (0.1–10 μM) (Fig. 6A). Second, our data showed that α-SMA and Col I expressions were significantly reduced (almost to an extent similar to the control group), confirming the anti-fibrotic role of MGIIIE in vitro (Fig. 6, B–D). Moreover, both western blotting and immunofluorescence staining show that TLR4/MyD88 and its downstream MAPK cascades upregulated by TGF-β are significantly inhibited by MGIIIE (Fig. 6, B and E–I). Consistent with the data obtained in vivo, these data confirmed that MGIIIE inhibits fibroblast activation and ECM deposition and its anti-fibrotic effect may act through regulating TLR4/MyD88 signaling.

**MGIIIE Inhibited Collagen Production Mainly through TLR4/MyD88 MAPK Signaling in Activated Fibroblasts Induced by LPS.** LPS-induced fibrosis in lung fibroblasts is another well-established in vitro model for pulmonary fibrosis. We investigated the anti-fibrotic role of MGIIIE in the LPS model as well. Similarly, fibroblasts were incubated with LPS in the presence or absence of MGIIIE and α-SMA and Col I contents were measured by western blotting. Consistently, MGIIIE exhibited an anti-fibrotic effect as shown by the much lower α-SMA and Col I contents compared with the control group (Fig. 7, C, E, and F). To test whether or not MGIIIE acts mainly through TLR4 pathway, mouse PLFs in some groups were cotreated with MGIIIE and TAK-242, a specific antagonist of TLR4. Single addition of TAK-242 or...
the combination with MGIIIE showed no cell toxicity in mouse PLFs (Fig. 7, A and B). LPS induced a robust expression of TLR4 in fibroblasts; however, TAK-242 significantly blocked LPS-induced fibroblast activation and collagen production, indicating LPS-induced fibrosis is TLR4 dependent (Fig. 7, C–F). When cotreated with TAK-242 in fibroblasts, MGIIIE failed to further reduce α-SMA and Col I contents compared with the MGIIIE-only group (Fig. 7, C–F), indicating MGIIIE may inhibit fibrosis mainly through TLR4 signals. Taken together, these data demonstrate that MGIIIE also inhibited fibroblast activation and collagen production induced by LPS and its inhibitory effect may be through downregulating the TLR4/MyD88-MAPKs signaling pathways.

**Discussion**

The fruit of *S. grosvenorii* Swingle (Cucurbitaceae) is a famous traditional Chinese herb used in the treatment of dry cough, sore throat, extreme thirst, and constipation (Committee of National Pharmacopoeia, 2010). A previous study has shown that MGV has protective effects on LPS-induced acute lung injury (Shi et al., 2014). MGIIIE was one of cucurbitane-type triterpene...
glycosides (mogrosides) isolated from the fruits of *S. grosvenorii* (Xu et al., 2015). MGIIIE showed stronger inhibitory effects on maltase than MGV (Suzuki et al., 2005); however, no studies have investigated the effect of MGIIIE in fibrosis. In this study, using both in vitro and in vivo models, we demonstrated for the first time that MGIIIE significantly reduced fibrosis in the bleomycin-induced pulmonary fibrosis mouse model and its anti-fibrotic effect may be due to its inhibition of fibroblast

Fig. 6. MGIIIE reduces ECM deposition and TLR4/MyD88-MAPK signaling pathways in mouse PLFs induced by TGF-β1. Mouse PLFs were treated with or without TGF-β1 (10 ng/ml) in the absence or presence of MGIIIE (10 μM) for 48 hours. (A) Cytotoxicity of MGIIIE on mouse PLFs was detected by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (B–H) Protein expression of α-SMA, Col I, TLR4, MyD88, and MAPK in mouse PLFs; (I) Representative image of protein expression of TLR4 and α-SMA by immunofluorescence staining. Data are expressed as mean ± S.D. (n = 3–5). *P < 0.05, **P < 0.01, ***P < 0.001.
activation and ECM deposition through downregulating the TLR4 signal in fibroblasts.

IPF is a chronic, progressive, and irreversible lung injury. There are no clinically effective therapies (Hashimoto et al., 2004). Bleomycin-induced pulmonary fibrosis in mouse is a frequently used mouse model to study the pathogenesis of pulmonary fibrosis. After bleomycin administration, the expression of inflammatory cytokines elevates quickly and returns to background levels 9 days after injection, while profibrotic gene expression peaks between 7 and 14 days and remains at a high level up to 21 days, suggesting a switch between inflammation and fibrosis in this interval (Chaudhary et al., 2006). Therefore, the time point for MGIIIE administration to investigate its anti-fibrotic effect was chosen to be 7 days. Our data have shown that MGIIIE is a potent inhibitor for pulmonary fibrosis in the bleomycin model.

In IPF, the predominant cell types are the fibroblasts and myofibroblasts. Inflammatory damage to the affected epithelium results in the secretion of growth factors, which stimulate the underlying fibroblast transformation to myofibroblasts. Myofibroblasts secrete growth factors and ECM components and contribute to increased collagen deposition, which eventually leads to fibrosis (Bocchino et al., 2010). Many inflammatory cytokines and profibrotic factors are involved in the fibrosis process, among which IL-1β, MPO, TGF-β, MMP-9/TIMP, α-SMA, and Col I are the most important players. IL-1β

![Fig. 7. MGIIIE inhibited fibrosis induced by LPS mainly through regulating TLR4 expression. The mouse lung fibroblasts were pretreated with or without TAK-242 (10 nM) for 1.5 hours and subsequently in the absence or presence of MGIIIE (10 μM) and LPS (5 μg/ml) for 48 hours. (A and B) Effects of TAK-242 and MGIIIE on mouse lung fibroblast proliferation were measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. (C–F) Expressions of TLR4, α-SMA, and Col I in mouse lung fibroblasts by western blot analysis. Data are expressed as mean ± S.D. (n = 3–5). *P < 0.05, **P < 0.01, ***P < 0.001. Nonsignificant (NS).]
and MPO are the proinflammatory factors mediating lung inflammation after bleomycin injection. TGF-β is a major inducer of fibroblast activation to myofibroblast (Wynn, 2007). MMP-9 causes collagen degradation, while TIMPs play a pivotal role in control of MMP catalytic activity in fibrogenesis (Chen et al., 2012). The ratio of MMP-9/TIMP inversely controls ECM deposition in lung fibrogenesis (You et al., 2015). α-SMA is a marker for myofibroblasts and Col I is the hallmark for fibrosis (Li et al., 2015). In our study, MGIIIE markedly decreased activation, confirming the essential role of TLR4 in fibroblast activation (including JNK, ERK, and p38). In addition, the TLR4-bleomycin-induced pulmonary fibrosis mouse model, our data showed that MGIIIE inhibited MPO and IL-1β levels induced by bleomycin, indicating that MGIIIE may have anti-inflammatory effects, which may contribute to lung fibrosis attenuation. In the future, the anti-inflammatory effects of MGIIIE including inflammatory cytokine expression and neutrophil or macrophage infiltration will be evaluated at earlier time points (1–6 days after bleomycin injection) during the inflammation phase.

It is well documented that LPS or TGF-β can induce fibroblast activation and collagen deposition in cultured lung fibroblasts (Bhattacharyya et al., 2013). In the cell model of transdifferentiation of quiescent fibroblasts into myofibroblasts with TGF-β1 induction, mouse PLFs acquired a mesenchymal phenotype and higher α-SMA expression (Sun et al., 2015). LPS induces fibrosis by increasing the rapid release of proinflammatory cytokines, which activates fibroblasts as well (Synenki et al., 2007; Krebs et al., 2015). Using these two in vitro fibrosis models, our data showed that MGIIIE significantly inhibited fibroblast activation and collagen production in mouse PLFs induced by LPS or TGF-β and confirmed the anti-fibrotic effects of MGIIIE in lung fibrosis.

TLR4 has been shown to be essential in the protective immunity against infection during the pathogenesis of fibrosis (Jiang et al., 2005). MyD88 serves as a key TLR4 adaptor protein, linking the receptors to downstream kinases, and deficiency of MyD88 protects mice from inflammation and fibrosis after bleomycin treatment, indicating that TLR4/MyD88 may be a specific target in inflammatory responses (Gasse et al., 2007; Salaun et al., 2007). In addition, extensive evidence has shown that downstream signals of TLR4, JNK, ERK, and p38 are robustly increased in IPF patients and pharmacological inhibition of these three MAPKs significantly reduced fibrosis in mice and patients (Matsuoka et al., 2002; Yoshida et al., 2002; Moran, 2011; Madala et al., 2012; Vittal et al., 2013; van der Velden et al., 2016). Inhibited TGF-β1 and pulmonary fibrosis was observed in JNK knockout mice, indicating JNK is required for fibrosis development (Alcorn et al., 2009). PD98059, a highly selective inhibitor of ERK activation ameliorated lung injury and pulmonary fibrosis (Galuppo et al., 2011), and a p38 inhibitor has been approved for IPF treatment in Europe (Moran, 2011). In the bleomycin-induced pulmonary fibrosis mouse model, our data showed that MGIIIE significantly decreased expressions of TLR4/MyD88, as well as phosphorylations of MAPKs (including JNK, ERK, and p38). In addition, the TLR4-specific inhibitor TAK-242 blocked LPS-induced fibroblast activation, confirming the essential role of TLR4 in fibroblast activation. Consistently, MGIIIE markedly decreased TLR4/MyD88 and collagen synthesis induced by LPS or TGF-β, suggesting that MGIIIE may inhibit fibrosis through regulating the TLR4 signaling. Moreover, MGIIIE failed to induce further reduction of collagen production after TAK-242 pretreatment on fibroblasts, implicating that MGIIIE may inhibit fibrosis mainly through TLR4 expression; however, more direct evidence is needed before drawing this conclusion. Currently, our laboratory is investigating whether TLR4 is required for anti-fibrotic effects of MGIIIE by performing rescue experiments using a lentiviral-TLR4 overexpression system in mouse PLFs.

In summary, the present study demonstrated that MGIIIE protected against bleomycin-induced pulmonary fibrosis both in vitro and in vivo and its anti-fibrotic effect may be due to inhibiting fibroblast activation and collagen deposition regulated by TLR4 signals. These findings provide new insights into understanding the bioactivity of MGIIIE. MGIIIE may have therapeutic potential for the treatment of pulmonary fibrosis in clinical applications.

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Authorship Contributions

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