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Lucidenic acids-rich extract from antlered form of *Ganoderma lucidum* enhances TNFα induction in THP-1 monocytic cells possibly via its modulation of MAP kinases p38 and JNK

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ABSTRACT

The *Ganoderma lucidum* (*G. lucidum*) is one of the oriental fungi that has been reported to have immunomodulatory properties. Although effect of β-glucans from *G. lucidum* has been well documented, little is known about how other major bioactive components, the triterpenes, contribute to the immunomodulatory function of *G. lucidum*. Here, we showed that triterpenes-rich extract of antlered form of *G. lucidum* (*G. lucidum* AF) induces TNFα production in monocytic THP-1 cells. Furthermore, the extract also synergized with lipopolysaccharide (LPS) to induce TNFα production in THP-1 cells, suggesting an immunostimulatory role of triterpenes-rich extract of *G. lucidum* AF. Notably, the extract enhanced LPS-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK), while it suppressed LPS-induced phosphorylation of c-Jun N-terminal kinase (JNK) MAPK. p38 Inhibitor suppressed TNFα production, while JNK inhibitor enhanced TNFα production, implying that synergistic effect of the extract may work by modulating p38 and JNK MAPKs. Moreover, we found that the triterpenes-rich extract of *G. lucidum* AF contains high amounts of lucidenic acids. Lucidenic acid-A, -F and -D_2, which seem to dominantly exist in the extract, were purified from the triterpenes-rich extract. We also identified Lucidenic acid-A and -F as modulators of JNK and p38, respectively. Thus, our data demonstrate that lucidenic acids-rich extract from *G. lucidum* AF enhances LPS-induced immune responses in monocytic THP-1 cells possibly via the modulation of p38 and JNK MAPKs activation.

1. Introduction

The *Ganoderma lucidum* (*G. lucidum*) is one of the oriental fungi and is used as traditional Chinese medicine in many Asian countries such as China, Japan and Korea for thousands of years. Currently, the dried powder and aqueous/ethanol extracts of *G. lucidum* are used worldwide as dietary supplements [1]. *G. lucidum* has been reported to have a number of pharmacological effects including immunomodulation [2], anti-tumor [3], hepatoprotective [4], anti-diabetic properties [5], and so on. Among its many effects, immunomodulatory role seems to be one of the representative properties of *G. lucidum* because the anti-tumor effects can also be achieved by *G. lucidum*-induced immunostimulation [3,6].

There are different compounds with various pharmacological activities extracted from *G. lucidum* [7]. Although polysaccharides, especially β-glucans, and triterpenes are two of its major bioactive components in *G. lucidum*, only the immunomodulatory function of β-glucans has been well documented [8]. Increasing evidence suggests that β-glucans from *G. lucidum* may exert its immunomodulatory effects by promoting the function of antigen-presenting cells (APC) such as macrophages, dendritic cells, neutrophils, natural killer (NK) cells and lymphocytes [2]. On the other hand, *G. lucidum*-derived triterpenes, such as ganoderic acids, ganolucidic acids, ganolactone, lucidenic acids, methyl lucidenate and hydroxylucidenic acid, have also received wide attention in recent years due to pleiotropic functions [2,7,9]. These include inhibition of histamine release, inhibition of cholesterol synthesis, antihyper-

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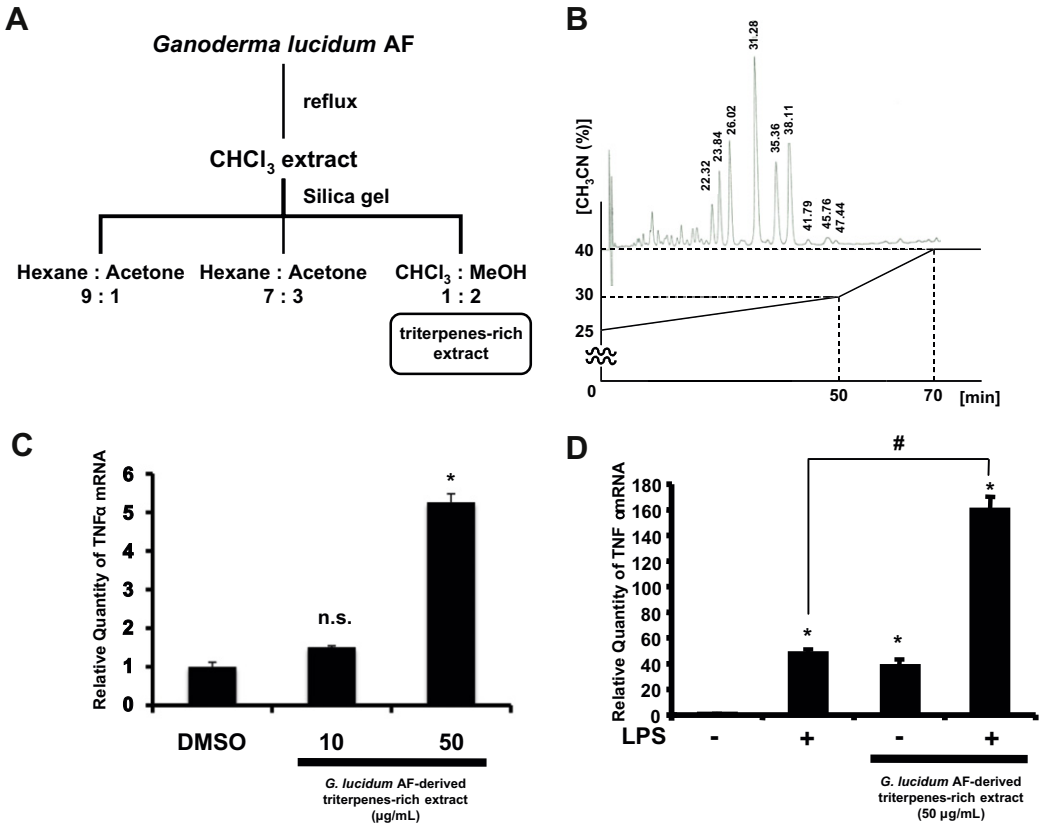

Fig. 1. Extraction of *G. lucidum* AF-derived triterpenes-rich extract and its role in an immunoregulation in both LPS-treated and non-treated monocytic THP-1 cells. (A) Procedure of the extraction of triterpenes-rich fraction from *G. lucidum* AF. (B) HPLC chromatogram of triterpenes-rich extract from *G. lucidum* AF. Column: TSK gel ODS-80TM (150 × 4.6 mm id); Mobile phase: 2% AcOH/H_2O—CH_3CN (0 min, 75:25; 50 min, 70:30; 70 min, 60:40); Flow rate: 1.0 mL/min; Operating temperature: 40 °C; Detector: UV 250 nm. (C, D) THP-1 cells were incubated with the extract at indicated doses without (C) or with (D) 100 ng/mL LPS for 4 h and then quantitative real-time RT-PCR was performed using isolated RNA to determine the level of TNFα mRNA. TNFα mRNA levels were normalized to the level of ribosomal RNA 18 subunit (18srRNA). Results represent the mean ± S.E.M. performed in triplicate. ANOVA with the Tukey–Kramer procedure was used to identify the differences of TNFα expression between DMSO-treated and the extract-treated cells (C) and between non-treated and other cells (D) (*$P < 0.05$, n.s., not significant). ANOVA with the Tukey–Kramer procedure was used to identify the differences of TNFα expression between LPS-treated and LPS + extract-treated cells (D) (# $P < 0.05$).

tensive, anti-tumor and anti-human immunodeficiency virus (HIV) [10]. Whether *G. lucidum*-derived triterpenes exert immunomodulatory effects has yet to be investigated.

The antlered form of *G. lucidum* (*G. lucidum* AF, *rokkaku-reishi* in Japanese) is a variant type of *G. lucidum* rarely found in nature. Because of the development of efficient cultivation system, *G. lucidum* AF is now available in China, Korea and Japan. Growing evidence points out that *G. lucidum* AF may have much stronger pharmacological properties than normal *G. lucidum* since the number of triterpenes in *G. lucidum* AF is much larger [11,12]. In the present study, to better understand the potential application of triterpenes fraction in *G. lucidum* AF, we sought to evaluate whether *G. lucidum* AF-derived triterpenes have immunomodulatory function *in vitro*. We first obtained β-glucans-free $CHCl_3$ extract from *G. lucidum* AF and further identified the triterpenes-rich extract to be an inducer of TNFα production in both LPS-treated and non-treated monocytic THP-1 cells. In addition, the extract-dependent enhancement of p38 activation and/or inhibition of c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) are likely important for immunostimulatory function of the extract. Finally, we found that the triterpenes-rich extract of *G. lucidum* AF contains high amounts of lucidenic acids and Lucidenic acid-A and -F, purified from the extract, as modulators of JNK and p38, respectively, to exert immunostimulatory function.

2. Materials and methods

2.1. Plant materials and isolation procedure

The powder of *G. lucidum* AF was kindly provided by REFARMER Co., Ltd. (Kumamoto, Japan). The voucher specimen (No. 201004016) was deposited in Department of Natural Medicines, Faculty of Medical & Pharmaceutical Sciences, Kumamoto University (Kumamoto, Japan). The powder of *G. lucidum* AF (40 g) was extracted with $CHCl_3$ (400 ml, 5 times) under reflux for 2 h, and the extract was concentrated *in vacuo* to afford residues (1.2 g). The residues were loaded onto a silica gel column (150 g) and eluted with Hexane–Acetone (9:1, v/v; 1.0 L), Hexane–Acetone (7:3, v/v; 1.0 L), $CHCl_3$-MeOH (1:2, v/v; 0.75 L), respectively. The $CHCl_3$—MeOH (1:2, v/v) eluate fraction (0.58 g) is the triterpenes-rich extract of *G. lucidum* AF. A part of the extract was dissolved in DMSO to a 10 mg/ml stock solution. To purify Lucidenic acid-A, -F and -D_2, the $CHCl_3$ extract of *G. lucidum* AF (0.52 g) was subjected to Chromatorex ODS (130 g) chromatography eluted with CH_3CN-H_2O (1:3, v/v; 2.0 L), CH_3CN—H_2O (3:7, v/v; 1.0 L), CH_3CN-H_2O (2:3, v/v; 1.0 L) and analyzed by HPLC to partition ten fractions. A part of major fraction containing lucidenic acids (52.5 mg) was chromatographed on a preparative HPLC (C18 reversed-phase column: Cosmosil 5C18-AR II, 250 × 10.0 mm id) eluted with 2% acetic acid in CH_3CN/H_2O (3:7, v/v) (5.0 ml/min),

obtaining pure Lucidenic acid-A (20.0 mg, t*R* = 33.4 min), -F (10.0 mg, t*R* = 38.4 min) and -D_2 (3.5 mg, t*R* = 43.0 min). The structures were identified on the basis of their NMR spectra and MS data [13–16].

2.2. Reagents and antibodies

Lipopolysaccharide (LPS), SB203580 and polymyxin B were purchased from Sigma (St. Louis, MO). SP600125 was purchased from Biomol (Plymouth Meeting, PA). PD98059 was purchased from Calbiochem (La Jolla, CA). Anti-phospho-JNK (Thr183/Tyr185), anti-JNK, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho IκBα (Ser32) and anti-IκBα antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin antibody was purchased from Santa Cruz Technology (Santa Cruz, CA).

2.3. Cell culture and treatment

Monocytic THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml of Penicillin and 100 μg/ml of Streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 °C. For the experiments in reagents-treated THP-1 cells, no toxicity was observed (data not shown). The $CHCl_3$ extract of *G. lucidum* AF or the three lucidenic acids purified from the extract (Lucidenic acid-A, -F and -D_2) were added to cells with or without 100 ng/ml of LPS at the indicated time and concentration.

2.4. Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR for human TNFα and 18s ribosomal RNA (18srRNA) was performed as previously described [17].

2.5. SDS–PAGE, Western blotting

Confluent (90–100%) cells grown on 60-mm dishes were used to collect the cell lysates and are subjected to SDS–PAGE, followed by Western blotting as described earlier [18].

2.6. Statistical analysis

For statistical analysis, the data were analyzed by one-way ANOVA with Tukey–Kramer multiple comparison test or Student's *t*-test (JMP software, SAS Institute, NC, USA) as indicated in each figure legend.

3. Results

3.1. G. lucidum AF-derived $CHCl_3$ extract up-regulates TNFα gene expression in both LPS-treated and non-treated monocytic THP-1 cells

To determine whether *G. lucidum*-derived triterpenes contribute to immunomodulatory function, we first sought to obtain the triterpenes-rich extract from *G. lucidum* AF. The crude $CHCl_3$ extract of *G. lucidum* AF powder was subjected to silica gel column and $CHCl_3$—MeOH was used for elution of the triterpenes-rich extract (Fig. 1A), followed by an observation of HPLC patterns of the

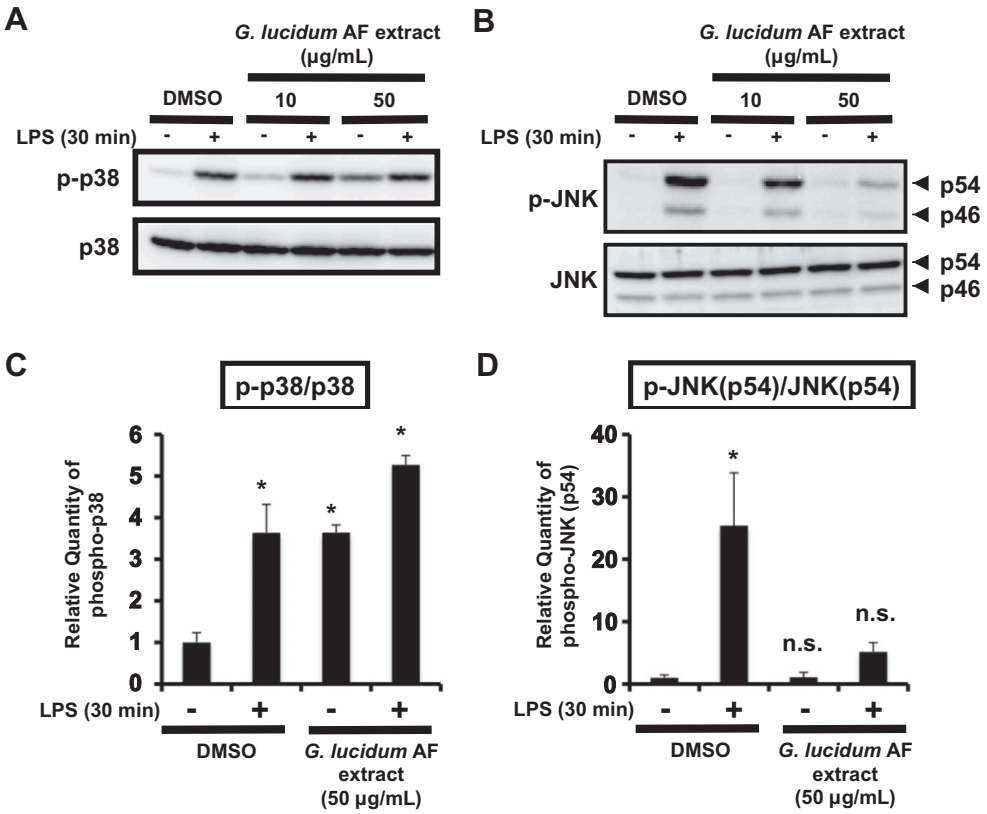

Fig. 2. The *G. lucidum* AF-derived extract induces p38 phosphorylation in both LPS-treated and non-treated cells, but suppresses LPS-induced phosphorylation of JNK. (A, B) THP-1 cells were incubated with the *G. lucidum* AF-derived extract at indicated doses without or with 100 ng/mL LPS for 30 min. Cells were harvested and cell lysates were subjected to Western blotting to detect phosphorylation states of p38 (A) and JNK (B). Band intensities were measured using Image Gauge software and phosphorylation ratio was expressed as p-p38/p38 (C) and p-JNK (p54)/JNK (p54) (D). Results represent the mean ± S.E.M. performed in triplicate. ANOVA with the Tukey–Kramer procedure was used to identify the differences of phosphorylation ratio between non-treated and other cells (*$P < 0.05$, n.s., not significant).

extract (Fig. 1B). To elucidate whether the extract has immunomodulatory roles in human immune cells, the mRNA expression level of TNFα in human monocytic THP-1 cells was analyzed using real-time quantitative RT-PCR. As shown in Fig. 1C, after the treatment of THP-1 cells with the triterpenes-rich extract, TNFα mRNA expression was increased. Significant increase was observed at 50 μg/mL of the extract in THP-1 cells. To exclude the possible contamination of endotoxin-like molecules in the extract, similar experiment was also performed in polymyxin B-treated THP-1 cells [19]. There was no obvious change observed in the mRNA level of TNFα in both polymyxin B-treated and non-treated THP-1 cells (Supplementary Fig. 1). Next, to investigate the modulatory roles of the extract during infection signaling, effect on the LPS-induced TNFα up-regulation in THP-1 cells was determined. Significant synergistic induction of TNFα gene expression was observed with the combination of the extract and bacterial lipopolysaccharide (LPS), a strong inducer of TNFα expression in monocytic cells (Fig. 1D), suggesting an immunostimulatory role of triterpenes acids-rich extract of *G. lucidum* AF.

3.2. G. lucidum AF-derived triterpenes-rich extract enhance LPS-dependent p38 activation, but suppresses LPS-dependent JNK activation in THP-1 cells

Having demonstrated that LPS-induced TNFα mRNA is up-regulated in monocytic THP-1 cells by the *G. lucidum* AF-derived triterpenes-rich extract, still unknown is how the extract affects the LPS signaling in the cells. Since LPS-dependent MAPKs signaling pathway has been shown to modulate the expression levels of downstream genes, we first confirmed which MAPKs signaling pathways are activated in LPS-treated THP-1 cells. As shown in Supplementary Fig. 2A, LPS strongly phosphorylates both p38 and JNK but not ERK pathways. To understand the role of these signaling pathways in the regulation of LPS-dependent TNFα induction in THP-1 cells, we evaluated the effect of SB203580 and SP600125, specific inhibitors of p38 and JNK, respectively [20,21]. SB203580 significantly suppressed, but SP600125 enhanced LPS-dependent TNFα induction (Supplementary Fig. 2B and C). These data suggest that p38 is positively while JNK is negatively involved in LPS-dependent signaling pathway for the TNFα induction in THP-1 cells. Notably, western blotting analysis using phospho-specific antibodies showed that the extract induced p38 phosphorylation in both LPS-treated and non-treated cells but suppressed LPS-induced phosphorylation of JNK (Fig. 2A and B). The same experiments were repeated and significant changes of phosphorylation states of p38 and JNK were also confirmed in THP-1 cells treated with 50 μg/mL of the triterpenes-rich extract (Fig. 3C and D). These results imply that synergistic effect of the extract on TNF up-regulation may work by modulating p38 and JNK MAPKs.

3.3. Purification of lucidenic acids from G. lucidum AF-derived triterpenes-rich extract and their roles in immunomodulation

Although HPLC chart (Fig. 1B) showed several strong peaks from the total 160.3 mg of the extract, 82.78% (132.7 mg) of the extract was eluted around the peaks with a retention time between 31 and 38 min (Fig. 3A), suggesting that these three peaks could be the dominant components in the extract and that an extraction of

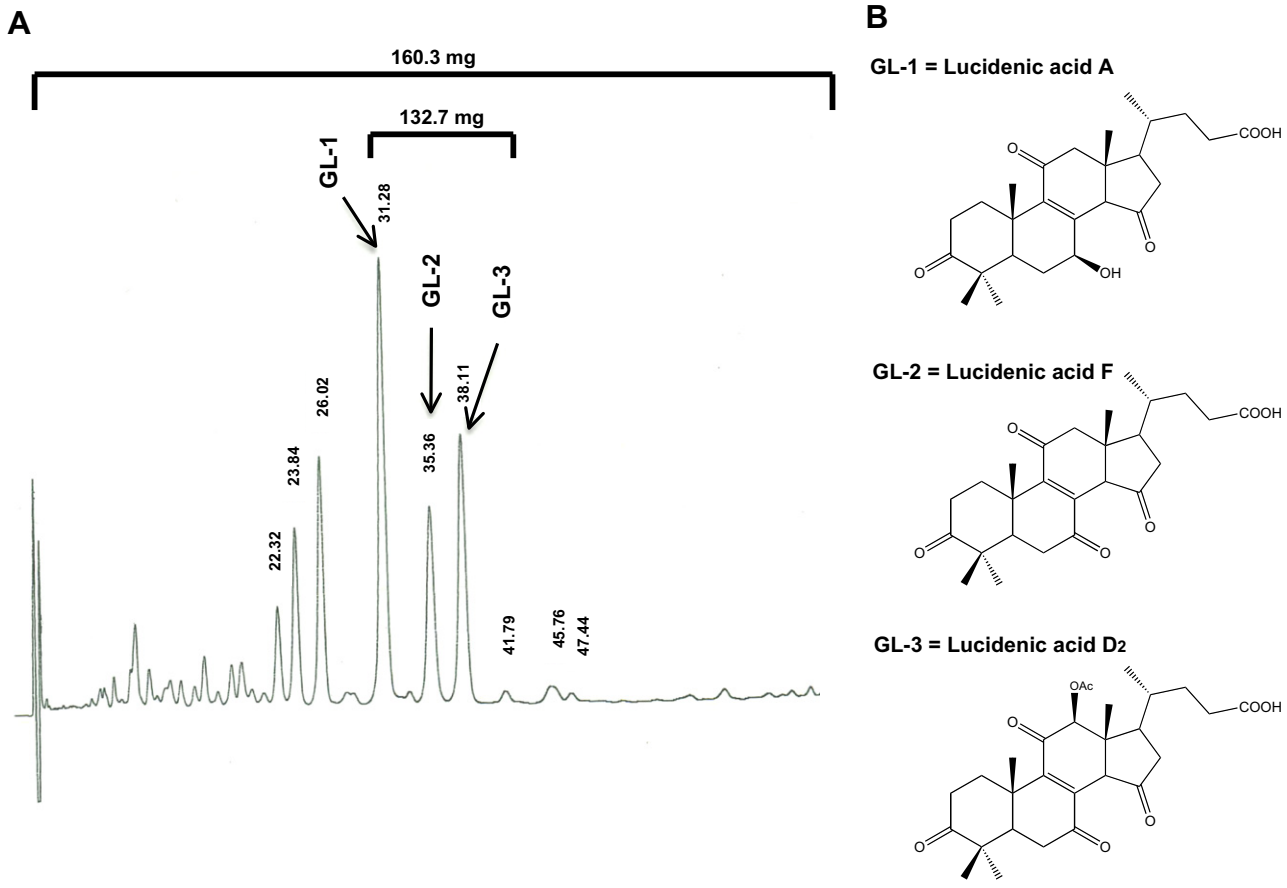

Fig. 3. Purification of lucidenic acids from *G. lucidum* AF-derived triterpenes-rich extract. (A) HPLC chromatogram of triterpenes-rich extract from *G. lucidum* AF. Total 160.3 mg of the extract was subjected to HPLC chromatogram and 132.7 mg of the extract was eluted around the peaks with a retention time between 31 and 38 min as indicated in the figure. (B) Lucidenic acids isolated from *G. lucidum* AF. GL-1 = Lucidenic acid-A; GL-2 = Lucidenic acid-F; GL-3 = Lucidenic acid-D_2.

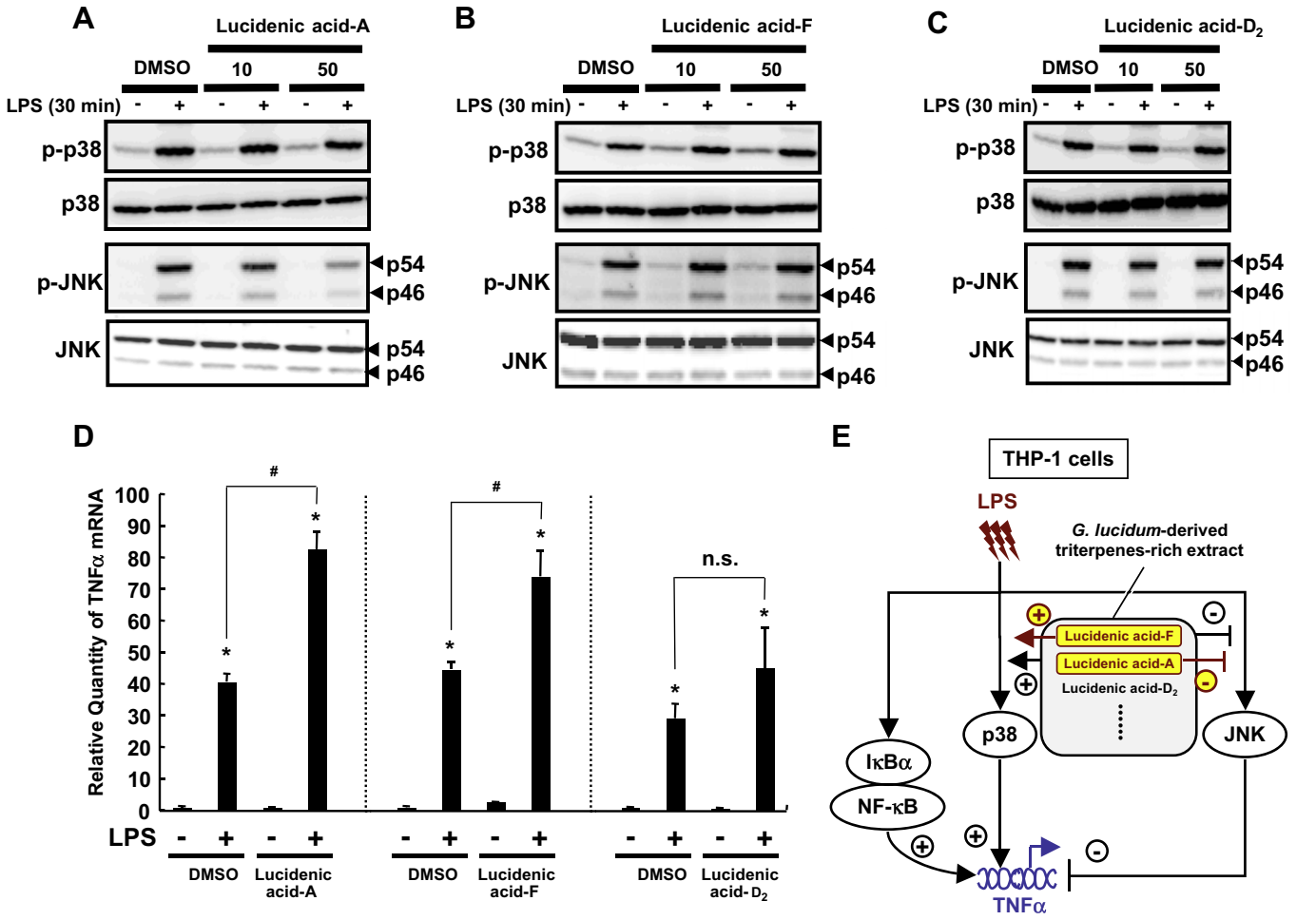

Fig. 4. Effects of Lucidenic acids purified from the *G. lucidum* AF-derived extract on LPS-dependent p38 and JNK signaling pathways. (A–C) Lucidenic acid-A inhibits LPS-dependent JNK phosphorylation in THP-1 cells and Lucidenic acid-F induces p38 phosphorylation in both LPS-treated and non-treated THP-1 cells. THP-1 cells were incubated with Lucidenic acid-A (A), -F (B) and D_2 (C) at indicated doses without or with 100 ng/mL LPS for 30 min. Cells were harvested and cell lysates were subjected to Western blotting to detect phosphorylation states of p38 and JNK. (D) Lucidenic acid-A and -F synergize with LPS to induce TNFα mRNA expression in THP-1 cells. THP-1 cells were incubated with 50 μg/mL of Lucidenic acid-A, -F and -D_2 without or with 100 ng/mL LPS for 4 h and then quantitative real-time RT-PCR was performed. TNFα mRNA levels were normalized to the level of 18srRNA. Results represent the mean ± S.E.M. performed in triplicate. ANOVA with the Tukey–Kramer procedure was used to identify the differences of TNFα expression between non-treated and LPS-treated cells ($^*P < 0.05$) and between LPS-treated and LPS + lucidenic acids-treated cells ($\#P < 0.05$, n.s., not significant). (E) Schematic mechanism of immunomodulation by the *G. lucidum* AF-derived extract and related compounds in THP-1 cells. LPS up-regulates TNFα expression in THP-1 cells via a positive IκBα-dependent NF-κB and p38 pathways and likely a negative JNK pathway. The *G. lucidum* AF-derived extract enhance LPS-induced TNFα up-regulation likely via a positive cross-talk with p38 pathway and/or a negative cross-talk with the inhibitory JNK pathway. Furthermore, Lucidenic acid-A and -F derived from the extract may explain, at least in part, the immunostimulatory function of the lucidenic acids-rich extract from *G. lucidum* AF.

G. lucidum AF with $CHCl_3$ affords high amounts of lucidenic acids. We next purified the components that correspond to these three peaks and they were designated GL-1, GL-2 and GL-3 (Fig. 3A). After the structure determination, GL-1, GL-2 and GL-3 were identified as Lucidenic acid-A, -F and -D_2, respectively (Fig. 3B).

Finally, to clarify if purified lucidenic acids *per se* have an immunostimulatory function, we treated THP-1 cells with each Lucidenic acid with or without LPS. Interestingly, Lucidenic acid-A strongly suppressed LPS-induced JNK activation (Fig. 4A). The inhibitory effect on JNK activation seemed to be specific since p38 activation was not affected by Lucidenic acid-A in this condition. On the other hand, specific activation of p38, but not JNK, was observed upon treatment with Lucidenic acid-F in LPS-treated and non-treated THP-1 cells (Fig. 4B). Neither LPS-induced activation of p38 nor JNK was affected by the treatment with Lucidenic acid-D_2 (Fig. 4C). Consistent with these observations, both Lucidenic acid-A and -F, but not -D_2, significantly enhanced LPS-induced TNFα mRNA up-regulation (Fig. 4D). Especially, Lucidenic acid-F, which activates p38 phosphorylation (Fig. 4B, 10 and 50 μg/mL without LPS), up-regulated TNFα mRNA expression without LPS (Fig. 4D). These results prove that Lucidenic acid-A and -F exert immunostimulatory function possibly via modulation of JNK and activation of p38, respectively. Overall, our data may support the idea that Lucidenic acid-A and -F are, at least in part, crucial for the immunostimulatory function of lucidenic acids-containing triterpenes-rich extract from *G. lucidum* AF.

4. Discussion

Growing attention has been focused on many biological and pharmacological activities of *G. lucidum*, especially *G. lucidum* AF, because of the complexity and diversity of its chemical composition [11,12]. Among many bioactive components, triterpenes isolated from *G. lucidum* or *G. lucidum* AF have been suggested to be of great importance because of their significant role in the regulation of biological activities [2,7,9]. The studies presented here are the first to show that triterpenes from $CHCl_3$ extract of *G. lucidum* AF, which in turn was identified as the lucidenic acids-rich extract,

induces the TNFα mRNA expression in both LPS-treated and non-treated monocytic THP-1 cells. This does not seem to be due to the contamination of endotoxin-like molecules based on the experiment performed with polymyxin B (Supplementary Fig. 1). Moreover, we isolated three lucidenic acids of high purity, which include Lucidenic acid-A, -F and -D_2, and demonstrate that Lucidenic acid-A and -F may mimic, at least in part, the immunostimulatory function of the *G. lucidum* AF-derived extract. These may exclude the existence of possible contamination of other bioactive non-triterpenes.

It has been widely accepted that p38 pathway is positively involved in the regulation of LPS-dependent genes expression [22–24]. On the other hand, although LPS has been shown to activate JNK signaling pathway, its role in the regulation of LPS-dependent gene expression is still controversial [24–26]. Despite the reports that identify JNK as a positive regulator [24,27,28], the present study indicates that JNK is negatively involved in LPS-dependent TNFα induction (Supplementary Fig. 2C). This is similar to what was observed for the regulation of LPS-dependent IL-12 induction in macrophages [25]. In the experiment by Utsugi, et al., SP600125 was also used in THP-1 cells to determine the role of JNK as we have done in this study. Notably, JNK has been shown to have multiple isoforms (e.g., JNK1α1, JNK1β1, JNK2α1 and JNK2β1 for p46; JNK1α2, JNK1β2, JNK2α2, JNK2β2 and JNK3α2 for p54) whose functions, expression levels and patterns are likely to depend on cell type and extracellular stimulus [29,30]. This aspect may need to be considered when assessing the role of JNK under each condition. Although further studies are needed, our findings may have unique implication in terms of the function of JNK.

It is still unclear whether signaling pathways other than p38 and JNK MAPKs are also targeted by the *G. lucidum* AF-derived triterpenes. To further determine potential target signaling pathways, we checked LPS-dependent NF-κB signaling pathway, a major pathway positively involved in LPS-dependent gene up-regulation [24]. As shown in Supplementary Fig. 3, LPS induced IκBα phosphorylation and degradation, a marker of the activation of NF-κB signaling pathway [31]. However, this was not affected by the *G. lucidum* AF-derived ectract, sugesting that NF-κB signaling pathway is not targeted by the extract. There is still a possibility of the existence of other molecules which can be targeted by the extract in the cells.

Finally, whether Lucidenic acid-A and -F are the only components that can affect LPS signaling is an important issue. We identify Lucidenic acid-A and -F as possible bioactive compounds in our system. There may still be some possibility of other triterpenes which can induce immunostimulation via targeting cellular signaling molecules although our identified three lucidenic acids seem to be the dominant components in the extract. Further study would be continued to isolate bioactive triterpenes from the extract.

Overall, our data uniquely identify p38 and JNK MAPKs, which seem to play antagonistic roles in regulating the LPS signaling that lead to the induction of TNFα mRNA in THP-1 cells, as the targets of the *G. lucidum* AF-derived lucidenic acids-rich extract (Fig. 4E). Because most of the previous reports has suggested that ganoderic acids are the dominant factors that can be extracted from from *G. lucidum*, and none of the reports has shown lucidenic acids as key components of *G. lucidum* or *G. lucidum* AF for an immunostimulatory function, our study may have significant implications for understanding pleiotropicity of *G. lucidum* AF function.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.108.

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