The Anti-Tumorigenic Mushroom Agaricus blazei Murill Enhances IL-1β Production and Activates the NLRP3 Inflammasome in Human Macrophages

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Abstract

Agaricus blazei Murill (AbM) has been reported to possess immune activity against tumors and infections through stimulation of mononuclear phagocytes. Recently, AbM extract was shown to induce the production of the pro-inflammatory cytokine, interleukin-1β (IL-1β), in human monocytes. IL-1β is a key pro-inflammatory cytokine produced by activated macrophages and monocytes and its secretion is strictly controlled by the inflammasome. The purpose of this study is to investigate the effect of AbM water extracts on the regulation of IL-1β production and activation of the NLRP3 inflammasome in human THP-1 macrophages. The NLRP3 inflammasome consists of an NLRP3 receptor, an adaptor protein called ASC, and the inflammatory protease, caspase-1. Typically, stimulation of immune cells with microbial products results in production of pro-IL-1β, but a second stress-related signal activates the inflammasome and caspase-1, leading to processing and secretion of IL-1β. Our results show that AbM enhances transcription of IL-1β and triggers NLRP3 inflammasome-mediated IL-1β secretion in human THP-1 macrophages. AbM-mediated IL-1β secretion was markedly reduced in macrophages deficient in NLRP3 and ASC, demonstrating that the NLRP3 inflammasome is essential for AbM-induced IL-1β secretion. In addition, caspase-1 was activated and involved in proteolytic cleavage and secretion of IL-1β in AbM-treated macrophages. AbM-mediated IL-1β secretion also decreased in cells treated with cathepsin B inhibitor, suggesting that AbM can induce the release of cathepsin B. Furthermore, our data show that AbM-induced inflammasome activation requires the release of ATP, binding of extracellular ATP to the purinergic receptor P2X7, the generation of reactive oxygen species, and efflux of potassium. Taken together, these findings reveal that AbM activates the NLRP3 inflammasome via multiple mechanisms, resulting in the secretion of IL-1β.

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Introduction

The medicinal mushroom Agaricus blazei Murill (AbM), a member of the Basidiomycetes family, is an edible mushroom that grows wildly in the coastal Piedade area of São Paulo, Brazil. It has recently received great attention in folk medicine due to its use in the prevention of a variety of diseases, including cancer, chronic hepatitis, diabetes, arteriosclerosis, and hyperlipidaemia [1]. The mushroom grows wildly in the coastal Piedade area of São Paulo, Brazil. It has recently received great attention in folk medicine due to its use in the prevention of a variety of diseases, including cancer, chronic hepatitis, diabetes, arteriosclerosis, and hyperlipidaemia [1].

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activation. Taken together, we found that the NLRP3 inflammasome and caspase-1

strate that incubation of THP-1 macrophages with AbM extracts

stimulation of IL-1

was recently shown to form a cytoplasmic complex known as the

inflammasome, which is the most fully characterized

inflammasome and is responsible for activation of caspase-1 (also

converting enzyme or ICE) by a vast number of pathogens and stress- or damage-related stimuli [21,22]. Upon

recruits an adaptor, apoptosis-associated speck-like protein (ASC) containing a C-terminal caspase recruitment domain (CARD), which in turn, recruits procaspase-1 to form a multi-protein complex leading to the activation of caspase-1 via autoprocessing [23,24]. Caspase-1 activation is required for proteolytic cleavage of pro-IL-1β, leading to the secretion of biologically active IL-1β [25]. The NLRP3 inflammasome has been also shown to be activated by endogenous or exogenous stimuli, such as ATP, monosodium urate (MSU) crystals, cholesterol crystals, UVB irradiation, pathogen-derived nucleic acids, silica, asbestos, and amyloid-β [26–34]. A broad array of mediators have been reported to activate the NLRP3 inflammasome. Thus, elevated reactive oxygen species (ROS) levels, depletion of intracellular potassium (K⁺), disruption of lysosomal membrane leading to the release of the cysteine protease cathepsin B, and apoptosis can modulate NLRP3 inflammasome activation [26,30,35,36]. In addition, the NLRP3 inflammasome can be activated by the “danger signal”, extracellular ATP, through stimulation of the purinergic P2X7 receptor (P2X7R), which serves as an ATP-gated cation channel and rapidly causes collapse of ionic gradients by allowing K⁺ efflux [31,37,38].

Water extracts of AbM were previously shown to induce the production of cytokine IL-1β in human monocyteic THP-1 cells; however, the molecular basis of IL-1β was not characterized. The aim of this study is therefore to investigate whether AbM stimulation of the secretion of IL-1β in macrophages involves caspase-1 or inflammasome activation. In the present report, we demonstrate that incubation of THP-1 macrophages with AbM extracts results in NLRP3 inflammasome-dependent activation of caspase-1 and secretion of mature IL-1β. We also investigated the molecular mechanisms involved in inflammasome and caspase-1 activation. Taken together, we found that the NLRP3 inflammasome plays an important role in regulating AbM-induced IL-1β secretion via multiple mechanisms.

**Results**

**AbM extract activates pro-IL-1β expression and induces the secretion of IL-1β in human THP-1 macrophages**

The effects of AbM extract on the viability of human THP-1 macrophages were first studied. THP-1 macrophages were treated with AbM extract at concentrations increasing from 0.1 to 5% for 24 h at 37°C. After 24 h of incubation, the viability of THP-1 macrophages was assessed by the MTT assay. As compared to untreated control cells, the cell viability of THP-1 macrophages was slightly reduced by treatment with higher concentrations of AbM extract (Fig. 1). In addition, no striking morphological changes in the macrophages were observed during the course of this experiment.

AbM extract was further examined for its ability to induce the synthesis or causing the secretion of IL-1β. The macrophages were therefore treated with increasing concentrations of AbM extract for 24 h. After AbM treatment, the cells were collected and mRNA expression levels of IL-1β were analyzed by RT-PCR. The AbM extract can strongly induce expression of the IL-1β gene (Figs. 2A and 2B).

The ELISA assay was used for analyzing whether AbM extract can induce secretion of the mature form of IL-1β from macrophages. Thus, the cells were treated with AbM extract (0.1–5%) for 24 h, and IL-1β was measured in the cell culture supernatants. The results show that AbM extract induced dose-dependent secretion of mature IL-1β (Fig. 2C). In order to confirm the production and processing of pro-IL-1β in the cells and also verify the secretion of mature IL-1β into the cell culture supernatants, cells treated with the AbM extract for 24 h were characterized by Western blot analysis. AbM extract enhanced proteolytic cleavage of pro-IL-1β in the cells and also induced the appearance of mature IL-1β in the cell culture supernatants (Fig. 2D). These data show that AbM extract triggers both the transcription and the secretion of IL-1β in human THP-1 macrophages.

The secretion of IL-1β induced by AbM extract is caspase-1 dependent

Inflammasome activation results in the recruitment and activation of caspase-1. Caspase-1 is the key enzyme involved in the processing of pro-IL-1β to form the biologically active IL-1β [39]. To characterize the activation of caspase-1, cells were treated with AbM extract for 24 h, and the presence of the inactive procaspase-1 and activated caspase-1 (p20) was measured in cell lysates by Western blot analysis. As shown in Fig. 3A, AbM extract enhanced the formation of caspase-1 p20 in human macrophages.

![Figure 1. Absence of toxicity of the AbM extract on THP-1 macrophages.](image-url)
This result was confirmed by measuring the presence of caspase-1 p20 subunits secreted into the supernatants of the AbM-treated macrophages, as detected by ELISA (Fig. 3B). The secretion of activated caspase-1 was significantly increased in the supernatants of the AbM-stimulated macrophages, indicating that AbM extract can induce the proteolytic processing of caspase-1.

In order to determine whether AbM-induced IL-1β secretion required caspase-1-mediated processing of pro-IL-1β, the macrophages were pretreated for 30 min with a specific caspase-1 inhibitor (Z-YVAD-fmk) and, subsequently, were treated with the AbM extract. The protein expression of procaspase-1 and caspase-1 in caspase-1 inhibitor-treated macrophages was determined by Western blot analysis. Caspase-1 activation was reduced dramatically in the caspase-1 inhibitor-treated cells as seen in Fig. 3C. IL-1β secretion induced by AbM extract was also significantly decreased when macrophages were pretreated with caspase-1 inhibitor (Fig. 3D). Thus, we conclude that IL-1β secretion of AbM extract is mediated through caspase-activation.

AbM-induced secretion of IL-1β is dependent upon the NLRP3 inflammasome

The NLRP3 inflammasome consists of the NLRP3 receptor and an adaptor ASC, which are required for caspase-1 activation. To evaluate whether the AbM extract can activate the NLRP3 inflammasome, the role of either NLRP3 or ASC was determined by gene silencing experiments with NLRP3 or ASC shRNA in the human THP-1 macrophages. The mRNA expression level of inflammasome components was significantly reduced in comparison with nontarget shRNA, which was measured by RT-PCR (Fig. 4A). Protein depletion was also determined by Western blot analysis (Fig. 4B). The ELISA assay and Western blot analysis showed that depletion of either NLRP3 or ASC led to a significant decrease of IL-1β secretion and processing after 24 h treatment of macrophages with AbM extract (Figs. 4C and 4D). Taken together, these results demonstrate that the NLRP3 inflammasome is required for optimal AbM-induced IL-1β secretion.

Inhibition of cathepsin B decreases AbM-induced secretion of IL-1β

Phagocytosis of MSU, silica crystals, aluminum salts, or fibrillar amyloid-β has been shown to induce lysosomal damage and the release of cathepsin B into the cytosol, leading to activation of the NLRP3 inflammasome and IL-1β secretion [30,40]. Since Hentze et al., (2003) and Vancompernolle et al., (1998), had also shown that the lysosomal protease cathepsin B can activate caspase-1 [41,42], we evaluated whether AbM-induced IL-1β secretion is
dependent on cathepsin B. Thus, THP-1 macrophages were either untreated (control) or pretreated with cathepsin B inhibitor (10 μM CA-074-Me) for 30 min and, subsequently, treated with AbM extract at concentrations of 0.1–5% for 24 h. As seen in Fig. 5A, inhibition of cathepsin B with CA-074-Me leads to a partial but significant reduction of AbM-induced IL-1β release, implying that the effect of AbM treatment is mediated at least partially by lysosomal disruption.

Potassium efflux contributes to AbM-induced IL-1β secretion

Lowering the intracellular concentration of potassium (K⁺) is crucial for activation of the NLRP3 inflammasome in response to a variety of stimuli [35]. In order to examine whether K⁺ efflux affects AbM-induced IL-1β secretion, THP-1 macrophages were untreated or pretreated with extracellular KCl at concentrations of either 1 mM or 5 mM for 30 min and, subsequently, treated with AbM extract at concentrations of 0.1–5% for 24 h. As seen in Fig. 5B, increasing the extracellular KCl concentration could significantly reduce AbM-induced IL-1β secretion. Our results thus suggest that K⁺ efflux is needed for AbM-mediated NLRP3 inflammasome activation in macrophages.

Inhibition of reactive oxygen species (ROS) decreases AbM-induced secretion of IL-1β

ATP and particulate activators such as asbestos and silica have been shown to trigger ROS production. The ROS production was required for downstream NLRP3 inflammasome-dependent caspase-1 activation [26,43,44]. Based on these previous studies, we first measured ROS production in THP-1 macrophages after exposure to AbM extract at concentrations of 0.1–5% using a commercially available ROS detection kit. A dose-dependent relationship was observed when ROS production in THP-1 macrophages was increased as increment in AbM concentration (Fig. 6A). We also analyzed whether ROS production was required for AbM-induced IL-1β secretion. THP-1 macrophages were untreated or pretreated with ROS inhibitors-APDC (50 μM) or BHA (10 μM) for 30 min and, subsequently, treated with AbM extract at concentrations of 0.1–5% for 24 h. As shown in Fig. 6B, the ROS inhibitors significantly reduced AbM-mediated IL-1β secretion.
secretion in macrophages. These results suggest that AbM stimulates production of physiological levels of ROS, which result in IL-1β secretion.

The release of ATP and binding of ATP to P2X7 are involved in AbM-induced IL-1β secretion

Ligation of the purinergic receptor P2X7 by extracellular ATP can stimulate NLRP3 inflammasome and caspase-1 activation the secretion of IL-1β [45,46]. To evaluate whether the effect of AbM on IL-1β secretion is mediated through P2X7, we pretreated macrophages with the P2X7 antagonist, oxidized ATP (oATP), for 30 min, and then treated with AbM extract at concentrations of 0.1–5% for 24 h. As shown in Fig. 7A, preincubation with oATP significantly reduced the AbM-induced secretion of IL-1β, suggesting that P2X7-R signaling is involved in AbM-induced inflammasome activation. As this result also suggested that AbM may stimulate ATP release from macrophages, we also determined the possible involvement of ATP release in AbM-induced IL-1β secretion by using apyrase, an enzyme that can rapidly hydrolyze extracellular ATP. In fact, apyrase significantly reduced AbM-induced IL-1β secretion in macrophages (Fig. 7B). Collectively, these results indicate that AbM-induced IL-1β secretion is dependent on ATP release and binding of extracellular ATP to P2X7.

Discussion

AbM is thought to exert its effect mainly through modulation of the immune system, and to promote strong pro-inflammatory responses through macrophages, neutrophils and lymphocytes. In fact, modulation of the immune system has been reported to be the predominant mechanism behind the protective effect of AbM against tumor development and bacterial infection in murine models [7,12,47–49]. Previous reports have established that the expression of IL-1β mRNA was augmented by AbM extract in mouse peritoneal macrophages upon oral administration [50], and that AbM extract also upregulates the genes for IL-1β and caspase-1 in THP-1 cells [15,16]. However, the ability of AbM to...
stimulate IL-1β secretion or caspase-1 activation was not investigated.

Proteolytic processing and the secretion of the mature IL-1β require the activity of caspase-1, which depends on the assembly of an inflammasome [51]. Our studies show that caspase-1 plays a major role in AbM-induced IL-1β production by macrophages. We further demonstrated that AbM-mediated caspase-1 activation...

Figure 5. Cathepsin B and potassium efflux were required for AbM-induced inflammasome activation. Cells were untreated or pretreated for 30 min with the cathepsin B inhibitor, CA-074-Me (10 μM) (A), or with extracellular potassium chloride (1 mM or 5 mM) (B). Cells were then incubated with AbM at concentrations of 0.1−5% for 24 h, IL-1β production was measured by ELISA. Data are presented as means ± SE of 3 experiments performed in duplicate. *P<0.05 versus cathepsin B inhibitor-treated cells. **P<0.05 versus 1 mM KCl-treated cells. ***P<0.05 versus 5 mM KCl-treated cells. doi:10.1371/journal.pone.0041383.g005

Figure 6. Inflammasome activation by AbM depended upon ROS production. (A) Cells were incubated with AbM at concentrations of 0.1−5% for 24 h, and ROS production were measured with the total ROS detection kit that finally detected by the fluorescence microplate reader. Pyocyanin (200 μM), a ROS inducer, induces the formation of ROS. (B) Cells were untreated or pretreated for 30 min with ROS inhibitor, APDC (50 μM) or BHA (10 μM), and then cells were incubated with concentrations of AbM at 0.1−5% for 24 h. IL-1β production was measured by ELISA. Data are presented as means ± SE of 3 experiments performed in duplicate. #P<0.05 versus untreated cells. *P<0.05 versus ROS inhibitor-treated cells.

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30 min with P2X7 antagonist, oATP (250 μM) (A), or the extracellular ATP-hydrolyzing enzyme, apyrase (1 U/ml) (B); and cells were then incubated with AbM at concentrations of 0.1–5% for 24 h. IL-1β production was measured by ELISA. Data are presented as means ± SE of 3 experiments performed in duplicate. *P<0.05 versus oATP inhibitor-treated cells. #P<0.05 versus apyrase-treated cells.

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exogenous ATP, which activated the P2X7-R [58]. The role of the P2X7-R in mediating caspase-1 activation was also demonstrated by the inability of peritoneal macrophages from P2X7-R-deficient mice to generate mature IL-1β in response to ATP [62]. These observations suggest that AbM extract treatment may stimulate the release of ATP, which activates the P2X7-R through an autocrine loop, promoting inflammasome activation and IL-1β secretion.

In conclusion, our results demonstrated that AbM activated the NLRP3 inflammasome, causing caspase-1-dependent IL-1β secretion in human macrophages (Fig. 8). AbM-induced IL-1β secretion also depended upon ATP release, binding of extracellular ATP to the P2X7-R, the release of active cathepsin B from lysosomes, K+ efflux, and ROS production. The production and secretion of pro-inflammatory cytokine IL-1β is crucial for stimulating innate immune responses and recruiting the phagocytic cells to defend against tumors and bacterial infection. Therefore, the findings of this study suggest that previous effects of AbM that were observed against tumors and infection may be attributed to the ability of AbM to both enhance IL-1β transcription and stimulate NLRP3 inflammasome-dependent caspase-1 activation.

Materials and Methods

Chemicals and reagents

Apyrase, butylated hydroxyanisole (BHA), DMSO, potassium chloride (KCl), oxidized ATP (oATP) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Human caspase-1 inhibitor (Z-YVAD-fmk), and (2R, 4R)-4-Aminopyrrolidine-2,4-dicarboxylic acid (APDC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cathepsin B inhibitor (CA-074-Me) was purchased from Calbiochem (La Jolla, CA). Cell culture medium (RPMI 1640), FBS, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY). For Western blot analysis, the antibodies against IL-1β was from Cell Signaling Technology (Beverly, MA); against ASC and pro-IL-1β, from Santa Cruz Biotechnology; against NLRP3, from Sigma-Aldrich; against caspase-1, from Millipore (Billerica, MA); and against β-actin, from Novus Biologicals (Littleton, CO). The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs (Santa Cruz Biotechnology).

Preparation of AbM water extract

Water extracts of AbM were obtained from Chang Gung Biotechnology (Taipei, Taiwan). This mushroom extract is a commercial product and components of extract were partially released due to business confidential. The AbM mycelia powder contains per 100 g the following constituents: fat 0.48±0.12 g, sodium 66±16.5 mg, carbohydrate 49.6±12.4 g, protein 19.7±4.9 g, ash 6.48±1.62 g, sugars <16 g, ergosterol >0.039 g, β-glucan >7.62 g, water-soluble polysaccharide >11 g, and moisture <5%. Briefly, the AbM water extract was first prepared by adding 400 g of AbM mycelia powder and 10 liters of distilled water into New Brunswick Scientific BioFlo 4500 fermentor (Edison, NJ) and stirred at 121°C for 30 min with a speed of 150 rpm. The AbM mycelia extract was cooled to room temperature and subsequently, centrifuged at 4500 rpm for 30 min. The supernatants were collected and concentrated to a final volume of 2 liters by Buchi R220 vacuum concentrator.

Figure 8. Schematic model for AbM-induced NLRP3 inflammasome activation and IL-1β secretion in human macrophages. AbM treatment resulted in ATP release and autocrine P2X7-R activation, followed by release of cathepsin B from lysosomes, efflux of K+, and production of ROS. The multiple downstream pathways activated the NLRP3 inflammasome, triggering caspase-1 activation and the secretion of IL-1β.

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(Zurich, Switzerland) at 65°C. The AbM extract was finally subjected to sterilize at 121°C for 20 min by a 0.45 μm filter (Millipore), and store in dark glass bottles at −20°C until use. The amount per milliliter of the AbM extract was water-soluble polysaccharide 76 mg, adenosine 2 mg, protein 21 mg, carbohydrate 30 mg, and ash 0 mg.

Cell culture and treatments
THP-1 (American Type Culture Collection, TIB-202), a human acute monocytic leukemia cell line, were cultured in RPMI 1640 medium supplemented with 10% (v/v) heated-inactivated PBS and 100 units/ml penicillin and 100 μg/ml streptomycin. THP-1 cells were incubated at 37°C in a 5% CO2 incubator with saturated humidity. For experiments, cells were plated in 6-well plates at 2×10^6 cells per well. The cells were differentiated to adherent macrophages by overnight culture in complete medium supplemented with 500 ng/ml PMA, and then with fresh complete medium for an additional 2 days. THP-1 derived macrophages were treated with AbM at extract concentrations from 0.1 to 5% and incubated for 24 h. Cell culture supernatants were harvested as previously described. Levels of secreted IL-1β were measured using sandwich enzyme immuno assays (ELISA). Commercially available ELISA kits for human IL-1β, NLRP3 and β-actin were purchased from R&D Systems (Minneapolis, MN), and were performed according to the manufacturer’s instructions.

Cell viability assay
Cell viability was determined using a commercial MTT based in vitro toxicology assay Kit (Sigma-Aldrich), which detects viable cells colorimetrically based on the purple formazan compound produced by the viable cells. THP-1 cells were initially seeded in 96-well plates (1×10^5 cells/well) for 24 h. For macrophage differentiation, cells were treated and incubated with PMA in the same manner as described above. Cell media were replaced by complete media containing different concentrations of AbM extract in a range from 0.1 to 5% and then incubated for 24 h. After incubation, 10 μl of MTT (5 mg/ml) were added to each well, and the plates were incubated at 37°C for 4 h. After incubation, each well was followed by eluting and dissolving the precipitate with 100 μl of the MTT solubilization solution. Cell viability was obtained by calculating absorption values at 570 nm using a VersaMax microplate ELISA reader (Sunyvale, CA). All treated samples and controls were tested in triplicate.

ELISA
THP-1 macrophages (2×10^6 cells/well) in 6-well culture plates were treated with AbM extracts in 1 ml of complete medium for 24 h. Cell culture supernatants were harvested as previously described. Levels of secreted IL-1β and activated caspase-1 in cell culture supernatants were measured using sandwich enzyme immuno assays (ELISA). Commercially available ELISA kits for human IL-1β and caspase-1 were purchased from R&D Systems (Minneapolis, MN), and were performed according to the manufacturer’s instructions.

Measurement of ROS production
Total ROS/Superoxide detection kit (Enzo Life sciences, Farmingdale, NY) was used for assessment of ROS production in THP-1 macrophages. Briefly, cells were first seeded (1×10^5 cells/well) in 96-well culture plates for 24 h. For macrophage differentiation, cells were treated and incubated with PMA in the same manner as described above. Cell media were replaced by complete media containing different concentrations of AbM extract (0.1 to 5%) and then incubated for 24 h. In addition, cells were treated with ROS inducer pyocanin (200 μM), a positive control, at 37°C for 30 min. After treatment, cells were washed with 200 μl of 1× wash buffer and loaded with 100 μl of ROS/Superoxide detection reagents, and then incubated at 37°C for 1 h. Read the plates using a VersaMax microplate ELISA reader (Sunyvale, CA) at 520 nm after excitation at 488 nm. The increase in relative fluorescence intensity (RFI) was used to determine intracellular ROS production.

Western blot analysis
Twenty-four hours after AbM extract treatment, cell extracts and cell culture supernatants were analyzed by Western blot analysis. The AbM-treated cells were collected and washed twice with PBS before incubation in RIPA lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA (Millipore) and complete protease inhibitor cocktail (Roche, Mannheim, Germany) on ice for 30 min. Cell suspensions were then centrifuged at 15,000×g, 4°C for 30 min, and the supernatants of cell suspensions were collected and stored at −80°C. Total protein concentration in samples was determined using the Bio-Rad Bradford assay (Hercules, CA). Protein profiles were separated by electrophoresis in 10 to 15% SDS-polyacrylamide gels and transferred onto Millipore PVDF membranes, and specific proteins were detected by the appropriate primary and secondary antibodies before visualization using an enhanced chemiluminescence detection kit (Millipore).

RNA isolation and RT-PCR
Total RNA was extracted from THP-1 cells using total RNA mini kit, and RNA extraction was followed by the Geneaid’s instruction (Taipei, Taiwan). Two micrograms of RNA were reversely transcribed reaction volumes of 20 μl which contains an oligo (dT) primer (Invitrogen, Carlsbad, CA) and the M-MLV reverse transcriptase (Promega, Madison, WI). The cDNA for ASC, IL-1β, NLRP3 and β-actin were amplified by PCR with specific primers: ASC forward primer 5’-ATCAGGCACGCTGTCGTCTT-3’, and reverse primer 5’-GGTTTGATGACCCCTTCCCGATAAG-3’; IL-1β forward primer 5’-AAAACGCTTGGTGATGTCTG-3’, and reverse primer 5’-GGTTACACGGCAGGAGG-3’; NLRP3 forward primer 5’-CTCTTGATGAGGCCCCAG-3’, and reverse primer 5’-GGACCAAATGGAAGAAGG-3’; and β-actin forward primer 5’-GGAGACCCTTCACCAAGCCGACCC-3’, and reverse primer 5’-GGATCTTCATGAGTACTGACAG-3’. The PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining on an image system.

Generation of THP-1 cells stably expressing shRNA
THP-1 cells stably expressing short hairpin RNA (shRNA) against ASC, NLRP3, and nontarget control were obtained as previously described [63]. ASC, NLRP3, and nontarget control knockdown THP-1 cells were confirmed by RT-PCR and Western blot analysis. For experiments, cells were either untreated or treated with AbM extract in the same manner as previously described. Cell culture supernatants were harvested and stored at −80°C for cytokine assays and Western blot analysis.

Statistical analysis
Triplicate data in each experiment were presented as mean ± SE. Mean comparisons between AbM extract-untreated control cells and treated cells were analyzed using Student’s t-test. P values below 0.05 were considered statistically significant.
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Author Contributions

Conceived and designed the experiments: TTH DMY YHW YFK TYW CYW CCL. Contributed reagents/materials/analysis tools: TTH YFW YFK YCW CYW CCL. Wrote the paper: TTH DMY YHW.

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Conceived and designed the experiments: TTH DMY YHW YFK TYW CYW CCL. Contributed reagents/materials/analysis tools: TTH YFW YFK YCW CYW CCL. Wrote the paper: TTH DMY YHW.
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