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Protective effect of Ketone musk on LPS/ATP-induced pyroptosis in J774A.1 cells through suppressing NLRP3/GSDMD pathway

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ABSTRACT

Pyroptosis is a different type of proinflammatory and lytic mode of cell death from apoptosis and necrosis, which play a killer and cleaner to the pathogenic microorganisms as an immune response when the host is infected by pathogenic microorganisms. Ketone musk (KM) is a component of the native musk, which is widely used to medicine and chemical engineering. In this research, we studied whether KM can suppress the pyroptosis in J774A.1 cells induced by lipopoysaccharide (LPS)/Adenosine Triphosphate (ATP) stimulation. The results showed that KM increased the viability of LPS/ATP-stimulated cells, decreased the production of interleukin (IL)-1β/18, and suppressed the activation of caspased-1 and NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome and gasdermin D (GSDMD). Our present study demonstrated that KM inhibited LPS/ATP-induced pyroptosis and the release of IL-1β/18 in J774A.1 cells by inhibiting the activation of GSDMD and caspase-1 and the assembly of NLRP3 inflammasome. Our finding may be of significance on investigating that KM has a positive potential application in the treatment of pyroptosis-mediated diseases.

1. Introduction

Pyroptosis is an inflammatory form of programmed cell death featured as membranolysis and leakage of cytoplasm [1], discovered by Zychlinsky and Prevost in 1992 and then first named in 2001 for the Greek words 'pyro' (fire or fever) and 'ptosis' (to fall) [2,3]. Pyroptosis is an immune defense response initiated by the body after it senses the infection of pathogenic microorganisms, which plays a critical role to defend microbial products in the cytoplasm or irritants as crystalline substances, toxins, and extracellular ATP [4–6]. The characteristic of pyroptosis is that formation of micropores and vesicles on the cell membrane, accompanied with cells swelling and membrane rupture, secretion of pro-inflammatory cytokines, release of cytoplasmic components, which rapidly start the innate immunity and cause inflammatory reactions, and eventually induce osmotic disintegration of cells [1]. Activation of inflammatory caspases are necessary for pyroptosis [7]. Caspase-1 is one of the earliest known caspase-family members, which is activated by various inflammasome complexes after pathogen invasion, as an important pathway in the innate immunity. Aberrant or excessive activation of caspase-1 is associated with many diseases, including Human Immunodeficiency Virus (HIV), Alzheimer's, atheroma, diabetic cardiomyopathy, Parkinson's disease [8–12].

NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome is a multiprotein complex, including NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1, leads to recruitment and activation of procaspase-1. Inflammasomes

Abbreviations: KM, Ketone musk; ATP, Adenosine Triphosphate; LPS, lipopoysaccharide; NLRP3, NOD-like receptor pyrin domain-containing protein 3; GSDMD, gasdermin D; ASC, apoptosis-associated speck-like protein containing a CARD; PRRs, pattern recognition receptors; DAMPs, damage-associated molecular patterns; AIM2, absent in melanoma-2; NLRC4, NLR-family CARD-containing protein 4; IL, interleukin; LDH, lactate dehydrogenase; TCM, traditional Chinese medicine; ROS, reactive oxygen species; CARD, caspase recruitment domain

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are kinds of multiprotein complex assembled by pattern recognition receptors (PRRs) to engage innate immune defenses via scanning extracellular pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) [13,14]. Except for NLRP3, the inflammasomes recruited procaspase-1 include NLRP1, absent in melanoma-2 (AIM2, HIN-200 family member), NLR-family CARD-containing protein 4 (NLRC4, originally named as IPAF). The AIM2 inflammasome recruits procaspase-1 via adaptor protein ASC, the same as NLRP3 [13,15]. Inflammasomes control the activation of caspase-1, resulting in the secretion of pro-inflammatory cytokines interleukin (IL)-1β and IL-18, and inducing an inflammatory cell death [16]. IL-1β and IL-18, characterized cytokines in the IL-1 family, play significant roles in the innate immune defense with pro-inflammatory properties in acute or chronic inflammation [17].

In the last few years, several researchers identified a pore-forming protein gasdermin D (GSDMD), a substrate of both caspase-1 and caspase-4/5/11, which is the executor of inflammatory caspases-mediated pyroptosis [7,18,19]. GSDMD consists of two subunits including the N-terminal pore-forming domain (p30) and the C-terminal repressor domain (p20). Full-length GSDMD is inactive due to the inhibitory binding to GSDMD-N by its GSDMD-C domain [20]. Once the autoinhibited structure of GSDMD cleaved by caspase-1 or caspase4/5/11, the GSDMD-N domain interacts with the plasma membrane and 16 symmetric monomers oligomerize to form a gasdermin pore with the inner diameter of 10–15 nm [21]. Consequently, the mature IL-1β, IL-18 and lactate dehydrogenase (LDH) outflow through the gasdermin-pore, meanwhile the influx of water and Na^+ lead to the swelling of cells, which break oncotic pressure and rupture the cell membrane and then release more macromolecular cytoplasmic components, and eventually cause cell death by pyroptosis [22]. Recently, it has been found that the N-terminal of gasdermin family proteins have pore-forming function besides GSDMD [23].

Musk, a traditional Chinese medicine (TCM), has been widely used in treatment for various diseases in the past. Several studies showed that it has markedly effects on anti-myocardial ischemia [24], immunity-enhancing and anti-inflammatory [25], anti-cerebral ischemia [26] in clinical practice. Ketone musk (KM, Fig. 1A) is a synthetic succedaneum to take the place of the native musk. It is widely used to medicine and chemical engineering. In this study, we detected the effects of KM on pyroptosis induced by LPS/ATP in J774A.1 murine macrophages, and further investigated whether the mechanism is related to NLRP3/GSDMD pathway.

2. Materials and methods

2.1. Reagents

Ketone musk (KM, (CH3)3CC6 (NO2)2(CH3)2COCH3, 98% purity) was obtained from Chengdu Herbpurify Co., Ltd. (Chengdu, China). Lipopolysaccharide and Adenosine 5′-triphosphate disodium salt hydrate (A6419) were purchased from Sigma-Aldrich (St Louis, MO). SYBR Green was obtained from Bio Rad (California, USA). Primary antibody sources were as follows: antibodies against NLRP3 (D4D8T) and ASC (D2W8U) (Cell Signaling Technology, Boston, MA, USA); antibody against caspase-1(ab179515); antibody against GSDMD (Santa Cruz Biotechnology, Delaware, USA); antibody against β-actin (ZENBIO, Chengdu, China). Goat anti-rabbit antibody and goat anti-mouse antibody obtained from Merck (Darmstadt, Germany) for western blot.

2.2. Cell culture and treatment

J774A.1 cell line was obtained from Kunming cell bank of Type Culture Collection Chinese Academy of Sciences (Yunnan, China). Cells were cultured in high glucose- Dulbecco's Modified Eagle Medium (DMEM) (Corning, USA) supplemented with 10% fetal bovine serum (Gibco, Australia) and 100 U/ml penicillin, 100 μg/ml streptomycin (HyClone, USA) in incubator with 5% CO_2 at 37 °C. For the measurement of cytotoxic effect of KM, J774A.1 cells were treated with KM (0–80 μg/ml) for 18 h. Except that, cells pretreated with KM (10, 20, 40 μg/ml) for 12 h, then stimulated with 100 ng/ml LPS for 4 h, after that treated with 5 mM ATP for 30 min.

2.3. Cell viability assay

Cell viability was detected using enhanced cell counting kit-8 (Beyotime Biotechnology, Shanghai, China). J774A.1 cells (5×10^4/well) were cultured in 96-well plate treated with KM (10–40 μg/ml) for 18 h, or treated with100 ng/ml LPS for 4 h followed by treatment with KM for 12 h, thereafter stimulated 5 mM ATP for 30 min. After that, 10 μl CCK-8 working Solution was added to each well and incubated for 2 h at 37 °C, the optical density of each well was measured at 450 nm using a microplate reader.

2.4. LDH assay

LDH activity in cell supernatants was assessed by CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, USA). Briefly, J774A.1 (5×10^4/well) were planted in 96-well plate, and followed by the treatment mentioned above. The maximum LDH release control were added with 10 μl 10× lysis solution 45 min before adding the working solution. Then 50 μl supernatants was transferred to a new 96-well plate, and 50 μl working solution was added to each well, incubated for 30 min at room temperature avoiding light. Thereafter, added 50 μl stop solution and recorded the absorbance at 490 nm or 492 nm.

2.5. Enzyme-linked immunosorbent assay

IL-1β and IL-18 levels in cell culture media were detected using

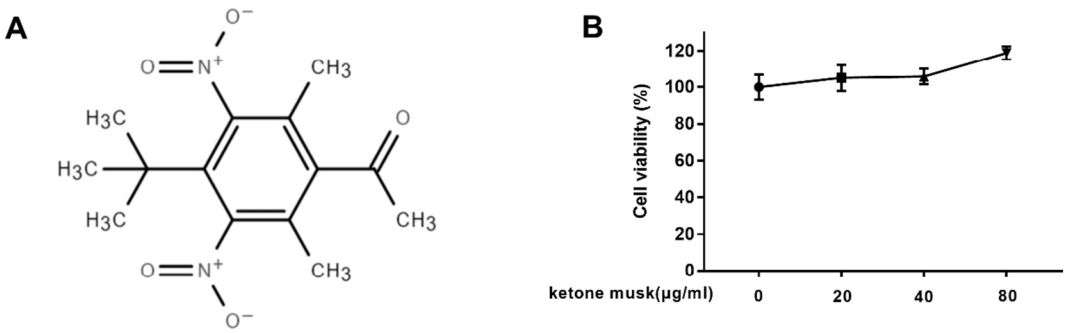

Fig. 1. The cytotoxicity effect of KM on J774A.1s. A. Chemical structure of KM; B. J774A.1 cells were treated with KM (0–80 μg/ml) for 18 h, and then CCK-8 assay was used to measure the cell viability (n = 5).

Table 1
Primers used in real time-PCR.

Genes	Primers for real time-PCR	
	Forward (5′–3′)	Reverse (5′–3′)
Caspase-1	TGGCAGGAATTCTGGAGCTT	CTTGAGGGTCCCAGTCAGTC
NLRP3	AGCCTTCCAGGATCCTCTTC	CTTGGGCAGCAGTTTCTTTC
ASC	TCCACCTGGCATTTCTTTGC	AGATAGGCTGTGCAAGGGTG
GSDMD	GGGGACTGCTGGGATGAAAA	GGAACAGGGAGGCATAGAGC
IL-1β	CTG CTT CCA AAC CTT TGA CC	AGC TTC TCC ACA GCC ACA AT
IL-18	AAGACTCTTGCGTCAACTTCAAGGA	AGTCGGCCAAAGTTGTCTGATTC
GAPDH	TGATGGGTGTGAACCACGAG	AGTGATGGCATGGACTGTGG

ELISA kits (MultiSciences Biotechnology, Hangzhou, China) according to the manufacturer's instructions.

2.6. Real-time reverse transcription-PCR

J774A.1 cells (1×10^6/well) were planted in 6-well plate for treatment mentioned above. Total-RNA was isolated with using TRI-Reagent (Invitrogen, Grand Island, USA). cDNA was synthesized using the TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR kit (Beijing, China), and real-time polymerase chain reactions were performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, Canada). The mRNA expressions of caspase-1, NLRP3, ASC, GSDMD, IL-1β and IL-18 were performed using a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad, Hercules, Canada) for quantitative RT-PCR. The primer sequences used in this study were listed in Table 1. All data were normalized to the mRNA expression level of GAPDH.

2.7. Hochest33342 and PI double staining

Hochest33342 and propidium iodide (PI) double staining were detected using Apoptosis and Necrosis assay Kit (Beyotime Biotechnology, Shanghai, China). J774A.1 cells (5×10^5/well) were cultured in 12-well plate for treatment mentioned above. Cells were washed twice by ice-cold PBS, then cell nuclei were revealed by Hoechst 33342 staining (5 μg/ml; staining for all cells) and PI (2 μg/ml; staining for membrane-damaged cells) at 4 °C for 20 min. After that, cells were washed once by ice-cold PBS and observed under a fluorescent microscope (OLYMPUS, Tokyo, Japan).

2.8. Western blot assay

Total proteins from J774A.1 cells were extracted using lysing buffer (Cell Signaling Technology, Boston, MA, USA). Protein concentration was measured by using BCA Protein Assay Kit (Beyotime, Jiangsu, China). Equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto 0.22 μm PVDF membranes (Millipore Corporation, Billerica, USA). Membranes were blocked with 5% skim milk for 1 h at room temperature with sustained shaking. Thereafter, membranes were incubated with antibody of anti-Caspase-1 (1:1000 dilution), anti-NLRP3 (1:1000 dilution), anti-ASC (1:1000 dilution), anti-GSDMD (1:200 dilution), anti-β-actin (1:8000 dilution) at 4 °C overnight. Subsequently, membranes were incubated with secondary antibodies for 1 h at room temperature with sustained shaking. Protein bands were visualized by ECL reagent (Bio-Rad, Hercules, Canada). The densities of the bands were assessed by image-pro plus and normalized to the β-actin signals.

2.9. Statistical analysis

All the results were presented as mean ± SD, and all the data were measured by three independent experiments. The statistical significances of the data were determined using analysis of variance (ANOVA; SPSSv22.0, IBM, USA). $p < .05$ was considered to be significant.

3. Results

3.1. The cytotoxic effect of KM on J774A.1 cells

The cytotoxicity of KM on J774A.1 cells was evaluated by using CCK-8 assay. Cells were pretreated with KM (0–80 μg/ml) for 18 h, cytotoxicity testing was then performed. The data in (Fig. 1B) showed that KM had no significant cytotoxicity to the J774A.1 cells ($p > .05$) during 18 h treatment at the concentration ranges from 0 to 80 μg/ml.

3.2. KM alleviated the mortality of LPS/ATP-induced J774A.1 cells

To confirm the influence of KM to the mortality of LPS/ATP-induced J774A.1 cells, levels of cell viability were measured by CCK-8 assay. LPS and ATP significantly inhibited the cell viability compared to the controls, and pretreatment with KM greatly upregulated the viability in a dose-dependent manner (Fig. 2A). The cell morphology was observed under a microscope (40×), and picked a same size in each group at random. Cells morphology particularly including membrane integrality were prominently injured by LPS and ATP, and pretreatment with KM has a significant protection compared to the LPS/ATP group (Fig. 2C).

In order to further evaluate the influence of membrane integrality on LPS/ATP- challenged J774A.1 cells that pretreated with KM, cytotoxicity was measured by LDH release assay. Similarly, the LPS/ATP-induced LDH release was significant increase compared to the negative control, which only significantly inhibited by pretreatment with 40 μg/ml KM (Fig. 2B). In addition, cellular membrane integrality was detected by Hochest33342 and PI double staining. We observed the ratio of membrane-damaged cells (PI permeable, red) to the total cells (Hochest33342, blue) that was induced by LPS and ATP treatment was observably increased compared to negative control, and pretreatment with KM greatly downregulated the ratio in a dose-dependent manner (Fig. 2D). The results in Fig. 2 suggested that KM (10–40 μg/ml) could markedly inhibit the mortality of LPS/ATP-challenged J774A.1 cells.

3.3. KM suppressed LPS/ATP-induced production of IL-1β and IL-18

IL-1β and IL-18 are two landmark pro-inflammatory cytokines which were cleaved by caspase-1 in pyroptosis [2]. Release of IL-1β and IL-18 occurs as a consequence of caspase-1 and 'inflammasome' activation [27]. To confirm that KM is able to inhibit the level of IL-1β and IL-18 in LPS/ATP-challenged J774A.1 cells, we analyzed them by ELISA in the cell culture supernatant, and KM could greatly down-regulate the levels as predicted (Fig. 3A, C). We further examined whether this effect was due to the inhibitory effect of KM on the level of mRNA expression of IL-1β and IL-18 induced by LPS and ATP. Similarly, KM inhibited the levels of mRNA expression on LPS/ATP-induced J774A.1 cells in a dose-dependent manner (Fig. 3B, D). The data in Fig. 3 showed that KM (10–40 μg/ml) could distinctly suppress the production of IL-1β and IL-

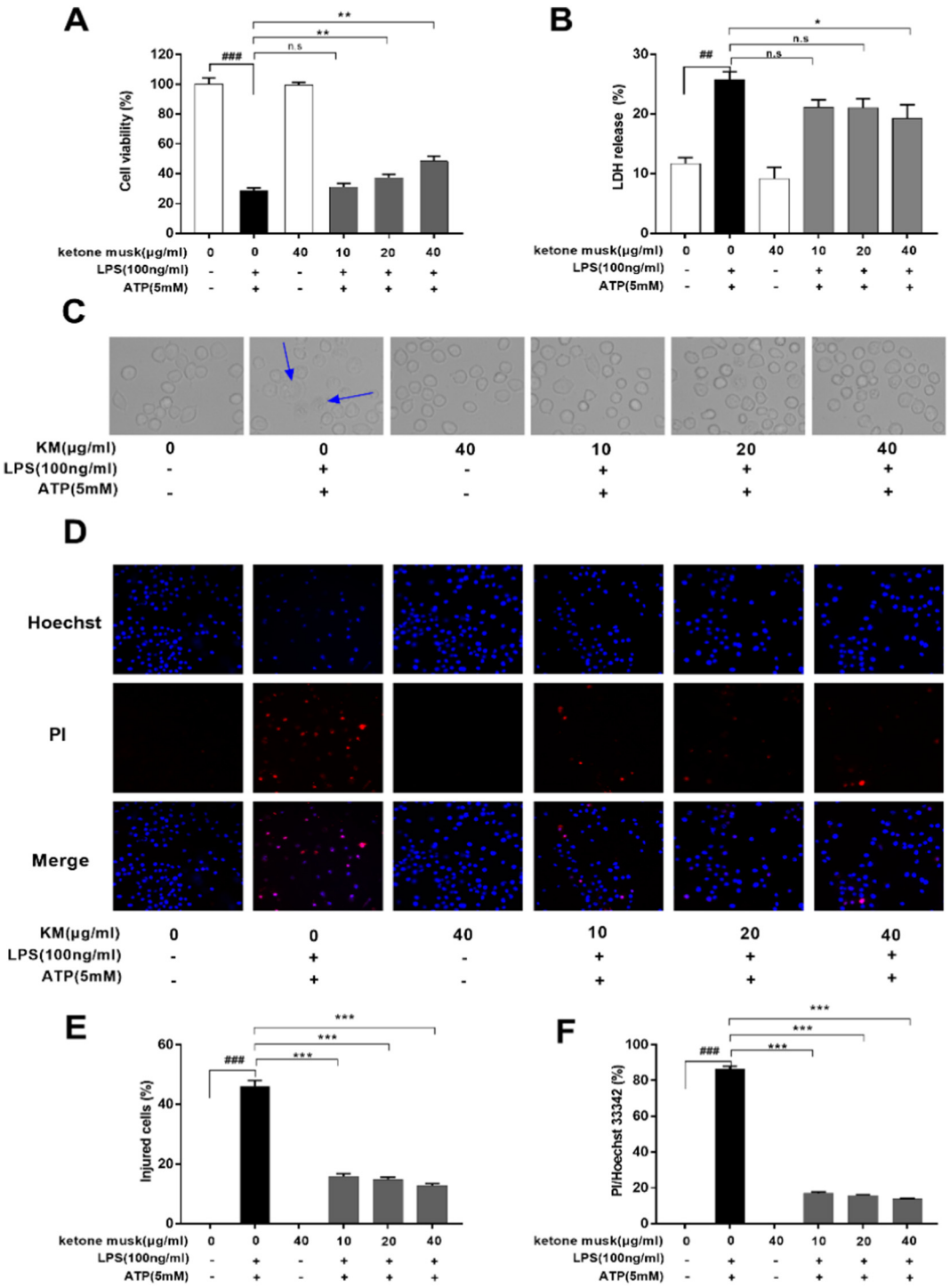

Fig. 2. KM reduced the J774A.1 cells mortality induced by LPS/ATP. The cells were treated with 100 ng/ml LPS for 4 h followed by pretreatment with or not KM (10, 20, 40 μg/ml) for 12 h, and then 30 min of incubation with 5 mM ATP. A. cell viability measured by CCK-8 assay; B. Cytotoxicity was measured by lactate dehydrogenase (LDH) release assay. C. The cell morphology was observed by microscope (40×), and picked a same size in each group at random. D. Hochest33342 and PI double staining images were captured by fluorescence microscopy (20×), merged with bright-field images. One set of representative images of three independent experiments are shown (C, D). Results are shown as mean ± SD (n = 5). E. The rates of injured cells in panel C were calculated. F. The rates of PI/Hochest33342 in panel D were calculated. $^{*}p < .05$, $^{**}p < .01$; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$.

18 on LPS/ATP-induced J774A.1 cells.

3.4. KM suppressed LPS/ATP-induced caspase-1 activation

Caspase-1 is the crucial inflammatory caspase in canonical inflammasome-mediated pyroptosis [1], which could accelerate the maturation and release of cytokines pro-IL-1β and IL-18 [28]. Caspase-1 plays an anther vital rule that cleaves GSDMD from its autoinhibited structure in pyroptosis [18]. To define that KM is able to inhibit caspase-1 activation in LPS/ATP-induced J774A.1 cells, we examined the total protein and the protein released in the medium. The results (Fig. 4A–C) showed that caspase-1p10 domain significantly increased induced by LPS and ATP, and pretreatment with KM greatly down-regulated the content of caspase-1 p10 in a dose-dependent manner in

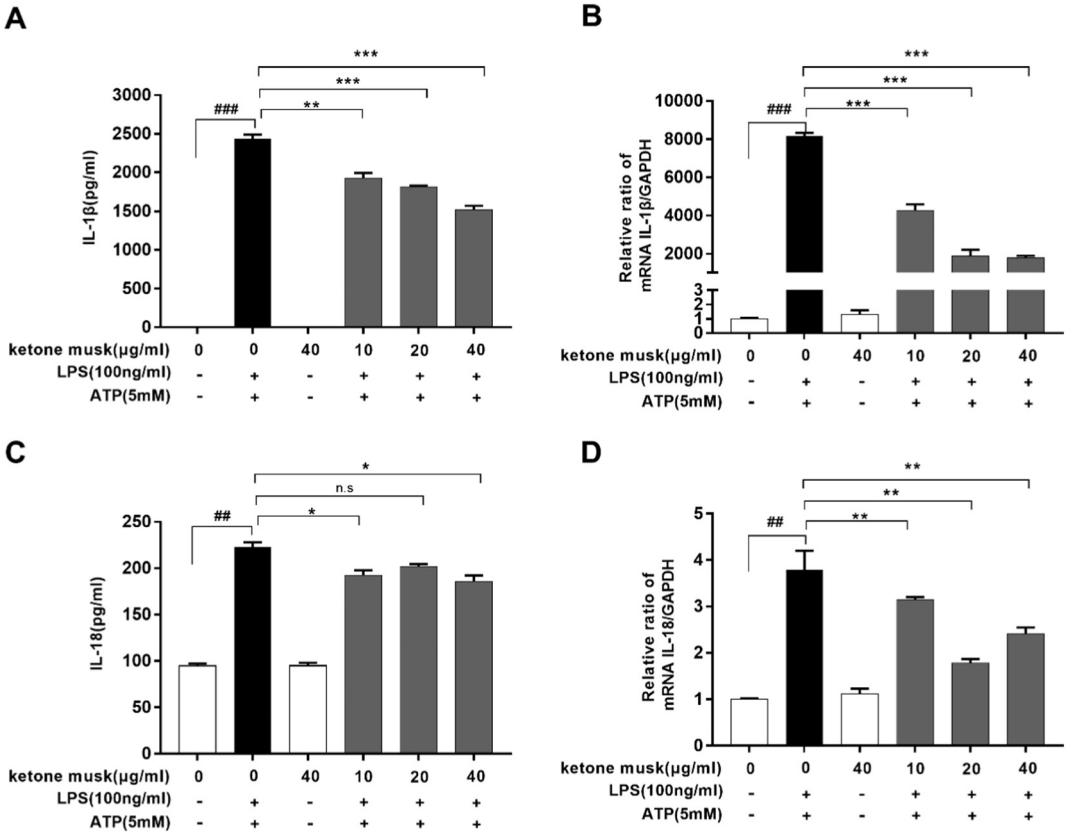

Fig. 3. KM suppressed the production of IL-1β and IL-18 in LPS/ATP-induced J774A.1 cells. The cells were treated with 100 ng/ml LPS for 4 h followed by pretreatment with KM (10, 20, 40 μg/ml) for 12 h, and then 30 min of incubation with 5 mM ATP. A, C. The levels of IL-1β and IL-18 in cell culture medium were measured by ELISA. B, D. The mRNA levels of IL-1β and IL-18 were detected by qPCR. Results are shown as mean ± SD (n = 4). $^{*}p < .05$, $^{**}p < .01$; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$.

medium, and 40 μg/ml KM significantly suppressed the expression of caspase-1p10 in total protein. In addition, we further measured the level of mRNA expression of caspase-1 which manifested the similar result (Fig. 4D). The data in Fig. 4 indicated that KM (10–40 μg/ml) could obviously suppress LPS/ATP-induced caspase-1 activation.

3.5. KM suppressed LPS/ATP-induced NLRP3 inflammasome activation but ASC

The NLRP3 inflammasome is one of the caspase-1-activating complexes, which include NLRP3, ASC, and procaspase-1 [13]. To explore whether the activation of caspase-1 is required for the NLRP3 inflammasome, we evaluated the expression of NLRP3 and ASC in total protein. As shown in Fig. 5A–B, the expression of NLRP3 induced by LPS and ATP, which significantly increased compared to negative control, was effectively attenuated with KM-pretreatment. We further examined the level of mRNA expression of NLRP3, and detected the similar result (Fig. 5C). Interestingly, the expression of ASC detected in total protein has no significant effect in the absence or presence of stimulating with LPS and ATP (Fig. 5D). The data in Fig. 5 indicated that KM (10–40 μg/ml) could suppress LPS/ATP-induced NLRP3 inflammasome activation but ASC.

3.6. KM suppressed LPS/ATP-induced GSDMD activation

GSDMD has been reported to be an executor of pyroptosis in canonical inflammasome or noncanonical inflammasome signaling [29], which was separated into an N-terminal ‘p30’ or ‘GSDMD-N’ domain, and a C-terminal ‘p20’ or ‘GSDMD-C’ domain by inflammasome caspases [18,19], and the GSDMD N-terminal ‘p30’ formed pores in membranes [30]. To define that KM is able to inhibit GSDMD activation in LPS/ATP-induced J774A.1 cells, we measured the expression of GSDMD in total protein and the mRNA expression of GSDMD. As shown in Fig. 6A, B, only pretreatment with 40 μg/ml KM could reduce the expression of GSDMDp30 induced by LPS and ATP similarly as capsase-1p10 in total protein. While 10–40 μg/ml KM had a significant effect on the LPS/ATP induced upregulation of mRNA expression of GSDMD (Fig. 6C).

4. Discussion

As a special form of cell vital movement, cell death is necessary for normal tissues and pathological tissues to maintain normal physiological functions and morphology, and is also the main cause of clinical diseases and serious pathological injuries. Pyroptosis is a different type of proinflammatory and lytic mode of cell death from apoptosis and necrosis, which play a killer and cleaner to the pathogenic microorganisms as an immune response when the host is infected by pathogenic microorganisms. However, excessive or abnormal activation of pyroptosis is associated with a lot of infectious diseases [5], neurodegenerative diseases [9] and autoimmune diseases [11]. Therefore, it is necessary to develop a new therapeutic medicine to alleviate the activation of pyroptosis.

In recent years, a series of studies demonstrated the GSDMD-N domain, which is cleaved by inflammatory caspases, is the executor of pyroptosis [18,19,29,30]. Caspase-1 is the most deeply investigated caspase-family protein in pyroptosis in murine and human, besides activating GSDMD, another key rule is that regulate and control the maturation and release of pro-IL-1β/18 [28]. In this study, we emphatically detected the NLRP3 inflammasome and GSDMD, which are

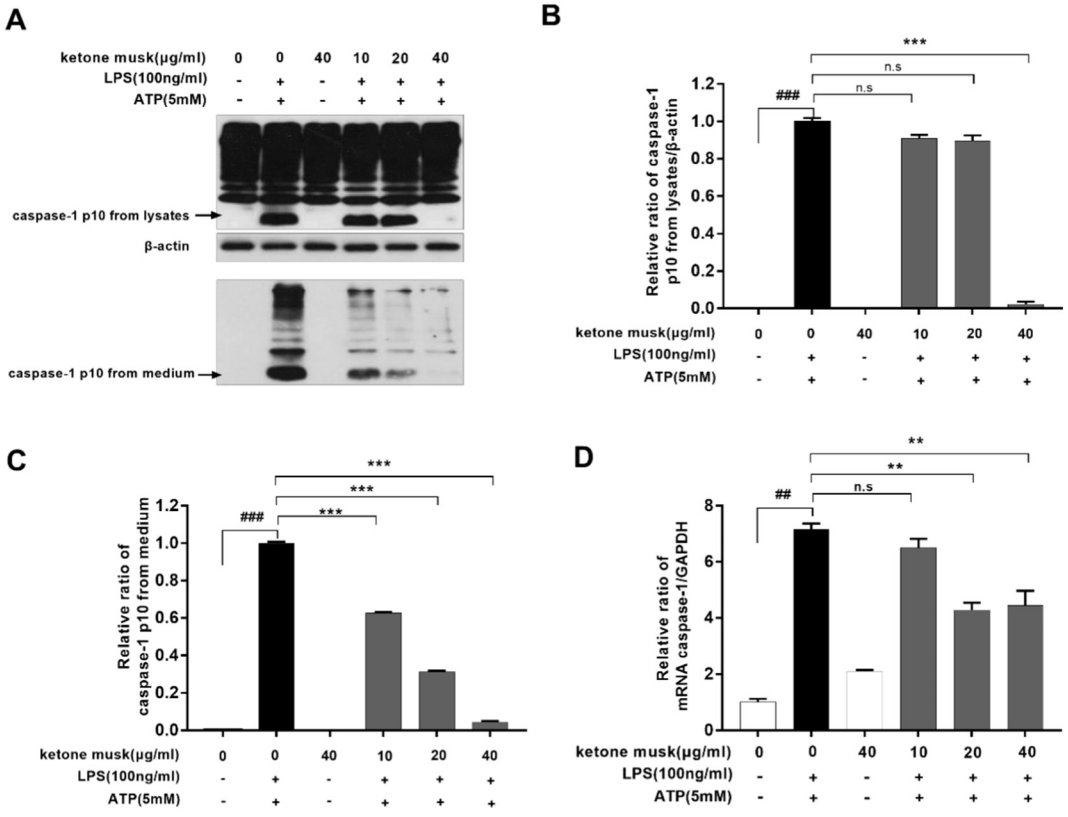

Fig. 4. KM suppressed LPS/ATP-induced caspase-1 activation. The cells were treated with 100 ng/ml LPS for 4 h followed by pretreatment with KM (10, 20, 40 μg/ml) for 12 h, and then 30 min of incubation with 5 mM ATP. A-C. The expression of caspase-1 in total protein and released in the medium were measured by western blot. D. The mRNA levels of caspase-1 were detected by qPCR. Results are shown as mean ± SD (n = 3). $^{*}p < .05$, $^{**}p < .01$; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$.

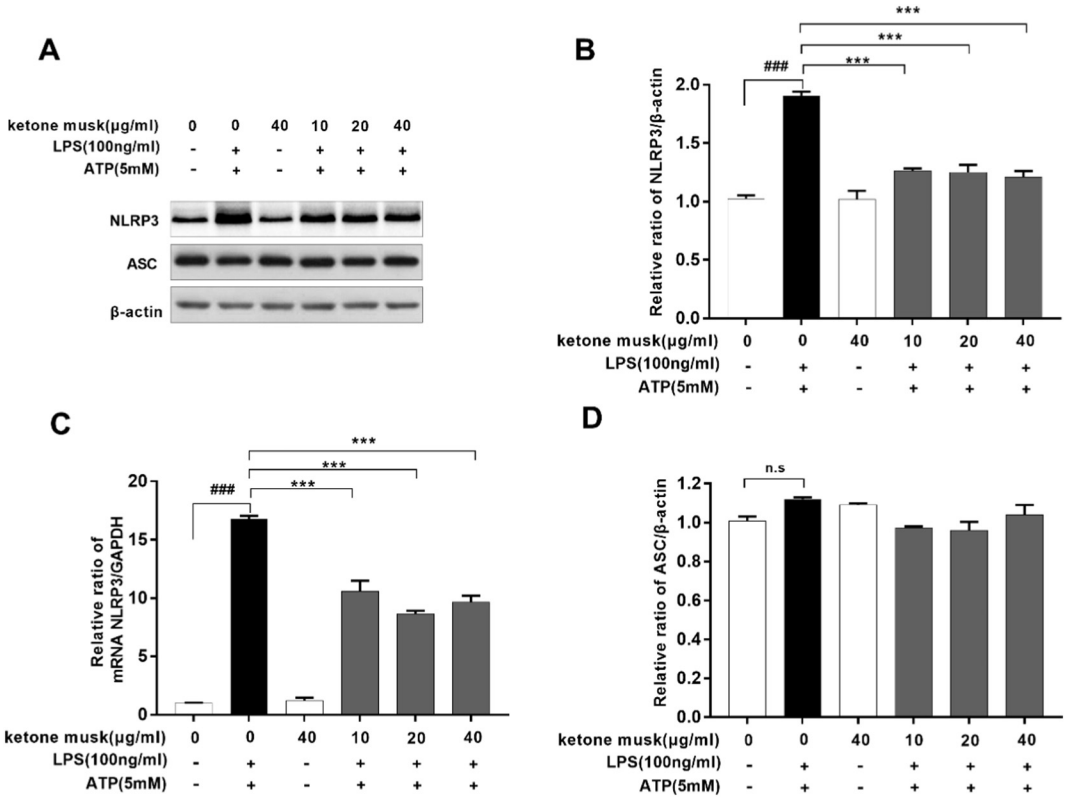

Fig. 5. KM suppressed LPS/ATP-induced NLRP3 inflammasome activation but ASC. The cells were treated with 100 ng/ml LPS for 4 h followed by pretreatment with KM (10, 20, 40 μg/ml) for 12 h, and then 30 min of incubation with 5 mM ATP. A, B, D. The expression of NLRP3 and ASC in total protein were measured by western blot. C. The mRNA levels of NLRP3 were detected by qPCR. Results are shown as mean ± SD (n = 3). $^{*}p < .05$, $^{**}p < .01$; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$.

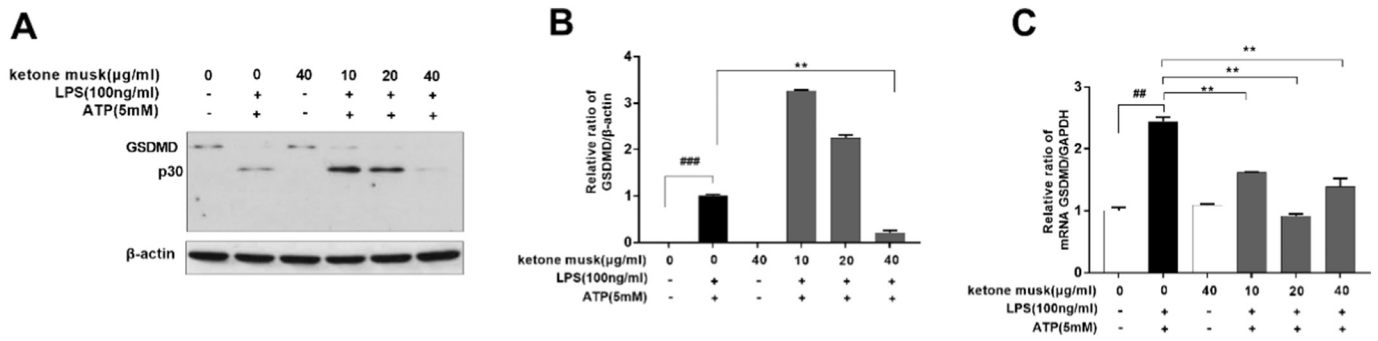

Fig. 6. KM suppressed LPS/ATP-induced GSDMD activation. The cells were treated with 100 ng/ml LPS for 4 h followed by pretreatment with KM (10, 20, 40 μg/ml) for 12 h, and then 30 min shown of incubation with 5 mM ATP. A, B. The expression of GSDMD in total protein were measured by western blot. C. The mRNA levels of GSDMD were detected by qPCR. Results are shown as mean ± SD (n = 3). $^{*}p < .05$, $^{**}p < .01$; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$.

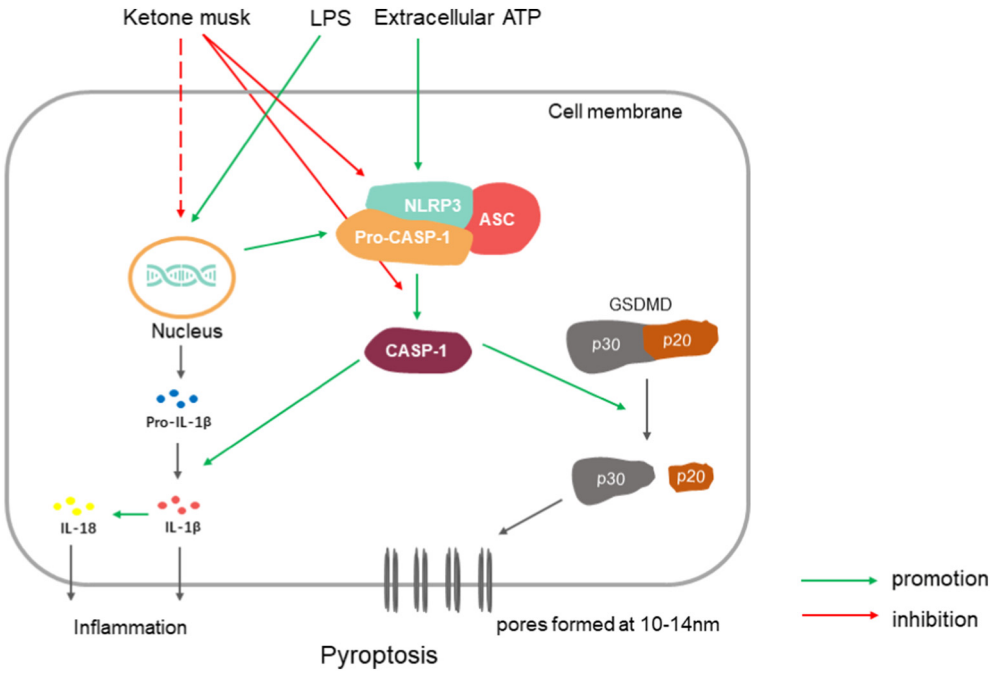

Fig. 7. Diagram representing the putative mechanism by which Ketone musk inhibited LPS/ATP-induced pyroptosis.

the key proteins in pyroptosis. Our data showed that KM suppressed the activation of NLRP3 inflammasome and GSDME in response to LPS/ATP triggering, thereby alleviated pyroptosis and release of IL-1β/18 in J774A.1 cells, suggesting that KM has a positive potential application in the treatment of some diseases which relate to pyroptosis.

Inflammasome activation is a crucial defense mechanism against microbial infection in the innate immune response [14], through the recruitment and activation of caspase-1 and IL-1β. Our current understanding of the mechanisms of NLRP3 inflammasome need priming and activation two signals. Firstly, microbial or endogenous molecules leads to upregulate the transcription and translation of NLRP3 and pro-IL-1β through the activation of the transcription factor nuclear factor-kappa B (NF-κB); and then the second signal molecules include ATP, pore-forming toxins, viral RNA, and particulate matter and reactive oxygen species (ROS) induce the activation of the NLRP3 inflammasome [31]. For example, in the case of bacterial infection, aside from the activation of NF-κB, host monocytes/macrophages or bacteria can release ATP into the extracellular environment, extracellular ATP induce intracellular K^+ efflux by binding to purine energy receptor (P2X7R) on the cell membrane, and result in activation of NLRP3 inflammasome [32].

In our study, LPS and ATP have succeeded in inducing the activation of NLRP3 inflammasome in J774A.1 cells, which was markedly down-regulated by pretreatment with KM (Fig. 5). While the expression of ASC in total protein has no significant difference in this study that in keeping with some previous studies using the same experimental treatment [33,34]. The adaptor protein ASC consists of a pyrin domain (PYD, binding NLRP3) and a caspase recruitment domain (CARD, binding procaspase-1). Recruitment of NLPR3 and procaspase-1 by the inflammasomes formed by the assembly of ASC dimers [35]. Hence, the further detection of the effect of KM on LPS/ATP-induced ASC may consider from the aspects of ASC dimers or protein-protein interaction.

In addition, our data showed that 10–40 μg/ml KM could suppress the mortality of J774A.1 cells (Fig. 2) and release of IL-1β/18 (Fig. 3) in a dose-dependent manner induced by LPS and ATP. Nevertheless, the expression of caspase-1p10 and GSDMDp30 in total protein had a significant down-regulation only upon 40 μg/ml KM pretreatment. Moreover, pretreatment of 10–40 μg/ml KM significantly suppressed the activation of NLRP3 induced by LPS and ATP (Fig. 5). Apparently, the dose-dependency does not seem to be consistent. In fact, the three proteins caspase-1, NLPR3 and ASC must be assembled into NLRP3 inflammasome in order to play a further role. Therefore, from the above results, only the effect of KM on NLRP3 protein is significant, which is consistent with the phenotype of pyroptosis including the mortality and

release of IL-1β/18 in the preceding results.

Our results also showed that KM at concentrations of 10 and 20 μg/ml potentiated GSDMD protein expression. According to a previous literature [36], synthetic KM strongly induced the apoptosis of cancer cells. Notably, synthetic KM exerted significant effects under low concentration, which indicate that it may increase the activity of caspase-3. Based on this reported, we speculated that KM might also increase the activity of caspase-1. Therefore, without decreasing the expression of caspase-1, the increase of enzyme activity of caspase-1 can cleave GSDMD into more GSDMDp30. We think that only a relatively high concentration of KM can ensure that it plays a role in inhibiting the pyroptosis. In addition, although GSDMD is the main executor of inflammatory caspases-mediated pyroptosis, we further speculated that other members of the gasdermin family may contribute to pyroptosis as well, such as GSDME, because recent study demonstrated that GSDME is also an executioner of pyroptosis [37]. KM also has the potential to inhibit GSDME. We will thoroughly explore the relationship between KM and GSDMD and GSDME in the following work.

In summary, our present study demonstrated that KM inhibited LPS/ATP-induced pyroptosis and the release of IL-1β/18 in J774A.1 cells by inhibiting the activation of GSDMD and caspase-1 and the assembly of NLRP3 inflammasome (Fig. 7). Our finding may be of significance on investigating that KM has a positive potential application in the treatment of pyroptosis-mediated diseases.

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Disclosure statement

The authors declare no conflict of interest.

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