

Bif-1 inhibits activation of inflammasome through autophagy regulatory mechanism

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Abstract. Inflammasome activation is a crucial mechanism in inflammatory responses. Bax-interacting factor 1 (Bif-1) is required for the normal formation of autophagosomes, but its ability to exert an inflammatory regulatory effect remains unclear. The aim of the present study was to explore the role of Bif-1 in inflammation, possibly mediated through autophagy regulation. Using a lipopolysaccharide (LPS)/adenosine triphosphate (ATP)-induced inflammatory model in J774A.1 cells, the effect of Bif-1 on inflammasome activation and the underlying mechanisms involving autophagy regulation were investigated. Elevated levels of NLR family pyrin domain containing protein 3 inflammasome and interleukin-1 β (IL-1 β) proteins were observed in J774A.1 cells after LPS/ATP induction. Furthermore, Bif-1 and autophagy activity were significantly upregulated in inflammatory cells. Inhibition of autophagy resulted in inflammasome activation. Silencing Bif-1 expression significantly upregulated IL-1 β levels and inhibited autophagy activity, suggesting a potential anti-inflammatory role of Bif-1 mediated by autophagy. Additionally, inhibition of the nuclear factor- κ B

(NF- κ B) signaling pathway downregulated Bif-1 and inhibited autophagy activity, highlighting the importance of NF- κ B in the regulation of Bif-1 and autophagy. In summary, the current study revealed that Bif-1 is a critical anti-inflammatory factor against inflammasome activation mediated by a mechanism of autophagy regulation, indicating its potential as a therapeutic target for inflammatory regulation.

Introduction

Inflammatory reactions are the key to the occurrence and treatment of various diseases, such as sepsis and cardiovascular disorders (1,2). Among the complex mechanisms of the inflammation process, there is an important mechanism related to the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome, the activation of which often acts as the key mechanism in the inflammatory response (3). Signal 1 is triggered by the activation of pathogen-associated molecular patterns (PAMPs). This activation leads to the upregulation of NLRP3 inflammasome transcription, which is dependent on the nuclear factor- κ B (NF- κ B) pathway (4). NF- κ B is a family of transcription factors involved in regulating various biological processes, including inflammasome activation and cell survival (5). When inflammatory signals are activated, the phosphorylated form of p65 is released and enters the nucleus, binds to target genes and regulates their transcriptional activities, thus regulating the extent and duration of the inflammatory response (6). Signal 1 and 2 can be triggered by PAMPs; signal 2 is also regulated by other damage-associated molecular patterns (DAMPs) such as adenosine triphosphate (ATP), a classical DAMP, typically leading to the initiation of various upstream signaling events (7). During the inflammation process, the activated NLRP3 inflammasome recruits the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) to facilitate the nucleation of ASC filaments, which serves as a platform for the recruitment and activation of caspase-1 (8). This activation of caspase-1 leads to the processing and release of proinflammatory cytokines

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and can trigger pyroptotic cell death (9). Upon activation, caspase-1 cleaves pro-interleukin (IL)-1 β and pro-IL-18, leading to the generation of their active forms (10). The active form of IL-1 β plays a critical role in promoting inflammation and regulating cellular immune responses by recruiting and activating immune cells (11).

Autophagy maintains the homeostasis of the intracellular environment by removing damaged organelles and metabolites, thereby inhibiting the onset and progression of inflammation (12,13). Autophagy can inhibit inflammasome formation and IL-1 β production by degrading components of the NLRP3 inflammasome, such as NLRP3, ASC and pro-IL-1 β (14,15). Lipopolysaccharide (LPS) treatment stimulates toll-like receptor 4 on the surface of macrophages, which in turn activates autophagy-related signaling pathways (16). p62, a classical autophagy receptor, is involved in autophagosome formation (17). In addition, the conversion of microtubule-associated protein 1 light chain 3 (LC3) to the LC3-II form has been widely used to assess autophagic activity (18).

Bax-interacting factor 1 (Bif-1), also known as SH3GLB1 or lipophilic enzyme B1, is present in the cytoplasm, and is a key protein in the process of autophagy and autophagosome formation (19-21). The loss of Bif-1 may impede the endogenous apoptotic pathway while facilitating tumor development (22,23). Bif-1 exerts inhibitory effects on the production of mitochondrial and glycolytic ATP, and its downregulation contributes to the proliferation of melanoma cells (24). However, there is also contradictory evidence that Bif-1 is involved in tumor formation to a certain extent (25). In addition, studies have shown that Bif-1 is crucial for maintaining the morphology and function of mitochondria (26,27). However, the specific mechanism and the role of Bif-1 in the development of inflammation is still unclear.

In the present study, the critical role of Bif-1-mediated regulation of autophagy in inflammasome activation and inflammatory factor release was revealed. These findings provide new insights in the understanding of the regulatory mechanisms of the inflammatory response.

Materials and methods

Cell culture. J77A4A.1 (cat. no. CL-0370) were provided by Procell Life Science & Technology Co., Ltd. The cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Premium; PAN-Biotech GmbH) and 1% penicillin-streptomycin antibiotic (Gibco; Thermo Fisher Scientific, Inc.) at 37°C. To induce inflammation, cells were first primed with LPS (500 ng/ml; Sigma-Aldrich; Merck KGaA) for 6 h at 37°C. All inhibitors used in the present study were added at the same time as LPS. Specifically, the following inhibitors were employed: MLN120B (1 μ M; Sigma-Aldrich; Merck KGaA) and MRT68921 (0.25 μ M; MedChemExpress). Subsequently, cells were stimulated with ATP (5 mM; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C.

After concentration in ultrafiltration centrifuge tubes (3 kDa; Merck KGaA), the supernatant was collected. Cells were washed twice with ice-cold phosphate-buffered saline (PBS; Sangon Biotech Co., Ltd.). For RNA extraction, 1 ml

RNA isolation reagent (TRIzol[®]; Invitrogen; Thermo Fisher Scientific, Inc.) was added to the cells. For protein extraction, 70 μ l lysis buffer [50 mM Tris (Sangon Biotech Co., Ltd.), 150 mM NaCl (Sangon Biotech Co., Ltd.), 0.1% (w/v) sodium dodecyl sulfate (Sangon Biotech Co., Ltd.), 1% Triton X-100 (Sangon Biotech Co., Ltd.) and one tablet/50 ml Protease Inhibitor Cocktail (Roche Diagnostics); pH 8.0] was added.

Animal experiments. Male NIH Swiss mice, aged 4 weeks, with an average weight of ~18 g, were obtained from Guangdong Medical Laboratory Animal Center (derived from National Institutes of Health). They were housed in controlled conditions with a constant temperature of 22 \pm 2°C, humidity of 60 \pm 5% and a 12 h dark/light cycle. The study strictly adhered to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The protocol was approved by The Bioethics Committee of the Shenzhen International Graduate School, Tsinghua University [Shenzhen, China; Ethical issue (2020) No. 9]. The mice were divided into either the control group or the LPS (10 mg/kg i.p.; Sigma-Aldrich; Merck KGaA) group, with each group consisting of three mice. The mice had *ad libitum* access to food and water throughout the experiment.

After a 4 h period of LPS induction, the mice were anesthetized with an intraperitoneal injection of a 10% (w/v) urethane solution (10 ml/kg; dissolved in normal saline; Sangon Biotech Co., Ltd.), equivalent to a dose of 1,000 mg/kg. Blood samples were collected and stored at -80°C for further research. Finally, the mice were euthanized by cervical dislocation.

siRNA transfection. siRNA transfection was performed as described in our previous study, and successful knockdown was confirmed by western blotting analysis (28). J77A.1 cells were cultured in 6-well plates until they reached 50% confluence. For every well, a transfection mixture was prepared, consisting of 250 μ l transfection reagent [5 μ l Lipofectamine[®] 3000 (Thermo Fisher Scientific, Inc.) and 245 μ l Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.)], and 250 μ l siRNA solution (100 nM; si-Sh3glb1; siB119191002-1-5; Guangzhou RiboBio Co., Ltd.; diluted with Opti-MEM). The siRNA sequence targeting Bif-1 was as follows: 5'-GGGCAAGGTGCCAATTACCTACTTA-3'. For the siRNA negative control, the sequence used was: 5'-CAGUACUUUUGUGUAGUACAA-3' (100 nM; Guangzhou RiboBio Co., Ltd.; diluted with Opti-MEM). These mixed solutions were incubated for 20 min at room temperature. Next, cells were washed twice with PBS, and 1.5 ml Opti-MEM along with the mixed solution were added to every well. A total of 24 h after transfection, cells were primed with LPS (500 ng/ml) for 6 h, followed by stimulation with 5 mM ATP for 30 min. The incubations were performed at a constant temperature of 37°C to maintain physiological conditions.

Western blotting. The Bradford assay was performed using the Bradford protein concentration determination kit (cat. no. P0006; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. For each lane, an equal mass of protein (25 μ g) was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (12.5%; Epizyme Biomedical Technology Co., Ltd.). The protein samples

were subsequently transferred to nitrocellulose membranes (BioTrace, Ltd.). Membranes were blocked using a solution of 5% (w/v) non-fat milk (Anchor Ltd.) dissolved in tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h at room temperature. After blocking, the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies were dissolved in 1.5% bovine serum albumin (BioFroxx; neoFroxx GmbH). Following three washes with TBST, the membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature and then washed again with TBST. To visualize the protein bands, an enhanced chemiluminescence solution (Thermo Fisher Scientific, Inc.) was used. Using ImageJ (version 1.45; National Institutes of Health), the relative grey density values of the protein bands were quantified and subsequently normalized to the density of β -actin. The specific primary antibodies used in the present study included anti- β -actin (1:50,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA), anti-Bif-1 (1:1,000; cat. no. 4467S; Cell Signaling Technology, Inc.), anti-NF- κ B p65 (1:2,000; cat. no. 6956S; Cell Signaling Technology, Inc.), anti-phospho-NF- κ B p65 (1:2,000; cat. no. 3033S; Cell Signaling Technology, Inc.), anti-NLRP3 (1:1,000; cat. no. 15101S; Cell Signaling Technology, Inc.), anti-IL-1 β (1:1,000; cat. no. 12242S; Cell Signaling Technology, Inc.), anti-p62 (1:2,000; cat. no. 88588S; Cell Signaling Technology, Inc.) and anti-LC3B (1:2,000; cat. no. A19665; ABclonal Biotech Co., Ltd.). The secondary antibodies used included goat polyclonal antibody to rabbit IgG H&L HRP (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) or goat polyclonal antibody to mouse IgG H&L HRP (1:5,000; cat. no. 32230; Thermo Fisher Scientific, Inc.).

ELISA. The supernatants from cell culture were collected and preserved at a temperature of -80°C. Subsequently, they were analyzed for IL-1 β (cat. no. KET7005; Abbkine Scientific Co., Ltd.) levels following the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). RNAiso Plus (Takara Bio USA, Inc.) was used to extract total RNA from J774A.1 cells, following the manufacturer's instructions. The concentration of the extracted RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, cDNA was synthesized using Evo M-MLV RT Premix (cat. no. AG11706; Accurate Biology). The kit included the reverse transcriptase enzyme, buffer, dNTPs, and primers necessary for cDNA synthesis. The reverse transcription protocol followed the manufacturer's instructions for the Evo M-MLV RT Premix kit (Hunan Accurate Bio-Medical Co., Ltd.), including temperature specifications. cDNA was quantitatively analyzed using the SYBR Green Premix Pro Taq HS qPCR Kit (Hunan Accurate Bio-Medical Co., Ltd.). Using the $2^{-\Delta\Delta C_q}$ method, the relative gene expression was calculated with β -actin as the reference gene (29). The parameters required for denaturation, annealing and extension were as follows: 95°C for 30 sec, 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The sequences of the primers used were: 5'-GCA ACTGTTCTCTGAACCTCAACT-3' and 5'-ATCTTTTGGGGT CCGTCAACT-3' for IL-1 β ; 5'-ATTACCCGCCGAGAAAG G-3' and 5'-TCGCAGCAAAGATCCACACAG-3' for Nlrp3; and 5'-GGCTGTATTCCCCTCCATCG-3' and 5'-CCAGTT GGTAACAATGCCATGT-3' for β -actin.

Immunofluorescence and confocal assay. The immunofluorescence assay in J774A.1 cells was performed following a previously described protocol (30). First, circular transparent glass slides (diameter, 10 mm) were placed at the bottom of a 6-well plate. A total of 2.5×10^5 cells/well (2 ml cell culture medium) were added to the surface of glass slides (cat. no. 12-545-83; Fisherbrand™ microscope cover glass; Thermo Fisher Scientific, Inc.) in a 6-well plate. The cells were cultured in fresh medium for 12 h and then stimulated with the corresponding drugs.

The immobilized cells on the slides were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were then washed with PBS three times and incubated with 0.1% triton in PBS for 15 min. After three washes with PBS, cells were blocked with 3% bovine serum albumin (cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.) in PBS for 1 h at room temperature. Next, cells were incubated with anti-LC3B (cat. no. A19665; ABclonal Biotech Co., Ltd.; 1:200) in PBS for 1 h at 37°C and washed with PBS three times.

The cells were incubated with goat anti-rabbit IgG H&L (1:1,000; cat. no. ab150077; Alexa Fluor® 488; Abcam) in PBS for 1 h at 37°C. The samples were sealed with Antifade mounting medium with DAPI (cat. no. P0131; Beyotime Institute of Biotechnology). The fluorescence signal was acquired using Nikon Confocal A1R system (Nikon Corporation).

Statistical analysis. The data are presented as mean \pm SD of three independent experiments. Statistical comparisons between two groups were performed using one-way ANOVA followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses and calculations were performed using GraphPad Prism (version 9.5; Dotmatics).

Results

Inflammasome is activated by the NF- κ B pathway in LPS/ATP-induced J774A.1 cells. Previous studies have shown that LPS/ATP treatment can induce an inflammatory response (31,32). The inflammatory response is activated when the NLRP3 inflammasome senses signals from pathogenic microorganisms, cellular stress and injury. The transcription of inflammation-related genes and cellular immune responses is regulated by the NF- κ B signaling pathway (33).

First, using the LPS/ATP-treated J774A.1 cell model, the levels of NLRP3 and IL-1 β proteins were measured in cell lysates (Fig. 1A). The levels of NLRP3 and IL-1 β proteins measured using western blotting were significantly increased in LPS/ATP-induced J774A.1 cells. Also, the level of IL-1 β in the cell medium of LPS/ATP-induced J774A.1 cells was significantly increased as indicated by the results generated using ELISA (Fig. 1D), suggesting that LPS/ATP treatment can contribute to the activation of the NLRP3 inflammasome and lead to the release of IL-1 β . This result is consistent with previous studies and supports the mechanism by which LPS/ATP treatment triggers an inflammatory response (34,35).

Secondly, there was a significant increase in the phosphorylation level of p65 in J774A.1 cells (Fig. 1B). Phosphorylated

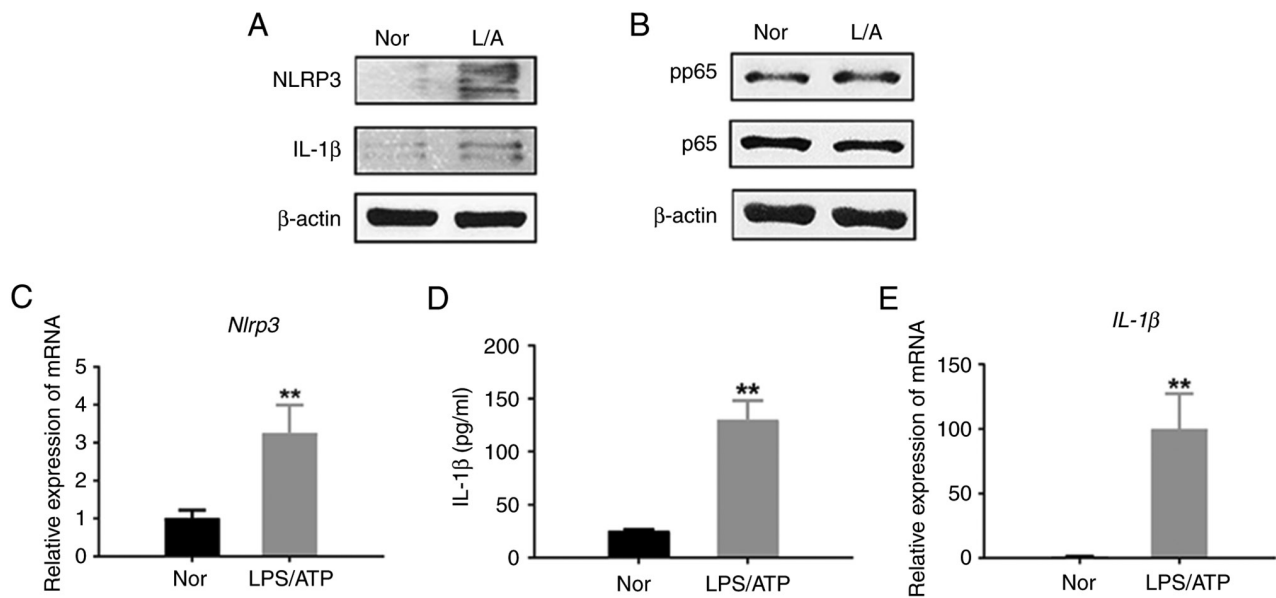


Figure 1. Inflammasome is activated by the NF- κ B pathway in LPS/ATP-induced J774A.1 cells. Phosphorylation of p65 in J774A.1 cells was significantly increased, and the NF- κ B signaling pathway was activated. The protein levels of (A) NLRP3, IL-1 β and (B) p-p65 in J774A.1 cells were measured using western blotting. The plasma levels of (D) IL-1 β were measured using ELISA. The mRNA level of (C) NLRP3 and (E) IL-1 β in J774A.1 cells was quantified using quantitative PCR. Data are expressed as mean \pm SD (n=3). $P < 0.05$ was considered to indicate a statistically significant difference (** $P < 0.01$ vs. Nor). Nor, normal control group; LPS/ATP (L/A), LPS/ATP group; P, phosphorylated; LPS, lipopolysaccharide; ATP, adenosine triphosphate; NF- κ B, nuclear factor- κ B; NLRP3, NLR family pyrin domain containing protein 3; IL, interleukin.

p65 is one of the hallmarks of NF- κ B signaling pathway activation. Its activation significantly increased the mRNA levels of NLRP3 and IL-1 β in J774A.1 cells as indicated by qPCR (Fig. 1C-E). Therefore, the results of the present study suggested that LPS/ATP treatment triggered the activation of the NF- κ B signaling pathway, which may lead to increased transcription of inflammation-related genes.

si-Bif-1 significantly upregulates the IL-1 β level in supernatants and lysates. Bif-1 is a key factor in regulating autophagy. It was shown that Bif-1 was significantly increased in LPS/ATP-induced J774A.1 cells (Fig. 2A). The NIH mice were treated with LPS, as described in Fig. S1, and an increase in Bif-1 levels in peripheral blood mononuclear cells was observed. However, using si-Bif-1 in LPS/ATP-induced J774A.1 cells significantly increased IL-1 β levels in supernatants and whole-cell lysates (Fig. 2B-D). It seemed that Bif-1 exerted an anti-inflammatory effect during inflammation activation. However, the mechanisms involved in the anti-inflammatory effects of Bif-1 remain unclear.

Autophagy inhibitors increased the levels of inflammatory cytokines in LPS/ATP-induced J774A.1 cells. Autophagy involves the maintenance of normal cellular function and biological homeostasis through the breakdown and removal of damaged or unwanted cellular components (36). An increasing number of studies indicated that autophagy is also related to the regulation of inflammation (37,38).

It was shown that treatment with LPS/ATP significantly increased autophagy activity in J774A.1 cells, suggesting that LPS and ATP may influence cellular functions and physiological processes by regulating the autophagy pathway (Fig. 3A). The upregulation of p62 protein levels is shown in Fig. 3A.

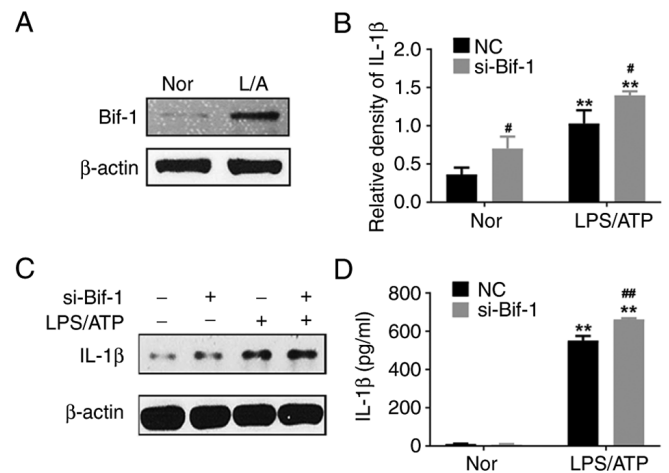


Figure 2. si-Bif-1 significantly upregulated the IL-1 β level in supernatants and lysates. In LPS/ATP-induced J774A.1 cells, Bif-1 was significantly increased. si-Bif-1 significantly upregulated IL-1 β in the supernatant and lysate, which indicated that Bif-1 plays a role in anti-inflammatory reactions. Protein levels of (A) Bif-1 and (B and C) IL-1 β in J774A.1 cells were measured using western blotting. The plasma levels of (D) IL-1 β were measured by ELISA. Data are presented as mean \pm SD (n=3). $P < 0.05$ was considered to indicate a statistically significant difference (** $P < 0.01$ vs. Nor; # $P < 0.05$, ## $P < 0.01$ vs. NC). Nor, normal control group; LPS/ATP (L/A), LPS/ATP group; si, silencer; LPS, lipopolysaccharide; ATP, adenosine triphosphate; IL, interleukin; Bif-1, Bax-interacting factor 1; NC, negative control.

The LPS/ATP-induced inflammatory model is associated with NF- κ B activation and activation of NF- κ B can directly upregulate the level of p62 (30). This upregulation of p62 may enhance intracellular autophagy, which is triggered by factors such as oxidative stress and protein aggregation in the condition of inflammasome activation.

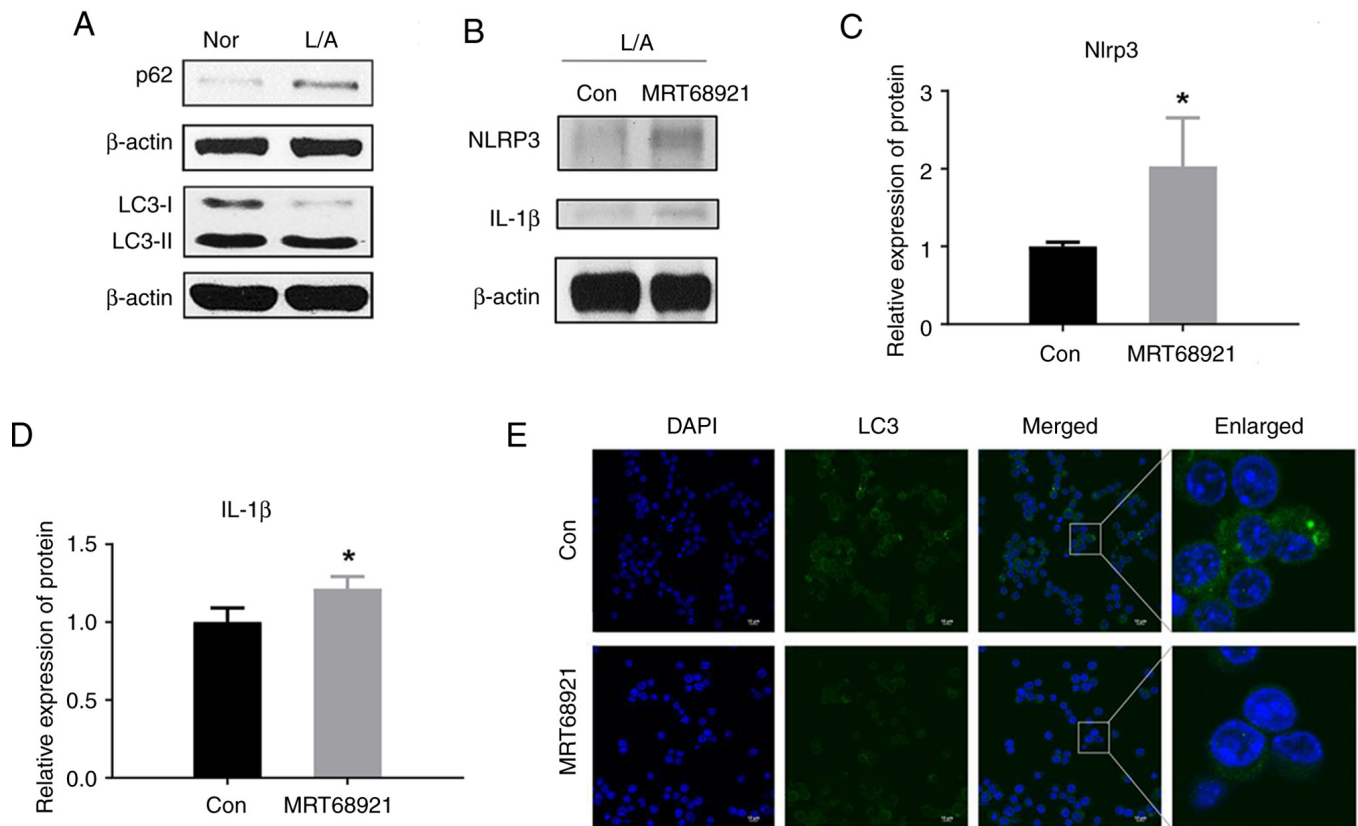


Figure 3. Effect of autophagy inhibitor on inflammatory factor levels in LPS/ATP-induced J774A.1 cells. J774A.1 cells were treated with LPS (500 ng/ml) for 6 h, followed by stimulation with ATP (5 mM) for 30 min, in the presence or absence of MRT68921 (0.25 μ M). Protein levels of (A) p62, LC3-II, LC3-I and (B) NLRP3, IL-1 β in J774A.1 cells were measured using western blotting. Protein levels of (B-D) NLRP3 and IL-1 β were analyzed. (E) J774A.1 cell monolayers stained with LC3 (green) and DAPI (blue) fluorescence. Representative images from three independent experiments are shown. Scale bar, 10 μ m (magnification, x600). Data are expressed as mean \pm SD (n=3). $P < 0.05$ was considered to indicate a statistically significant difference ($P < 0.05$ vs. Con). Nor, normal control group with neither LPS nor ATP; LPS/ATP (L/A), LPS/ATP group; Con, control group treated with LPS/ATP; MRT68921, LPS/ATP group treated with MRT68921 (0.25 μ M); LPS, lipopolysaccharide; ATP, adenosine triphosphate; IL, interleukin; LC, light chain; NLRP3, NLR family pyrin domain containing protein 3.

Meanwhile, the use of the autophagy inhibitor MRT68921, a ULK1 inhibitor, increased LPS/ATP-induced inflammatory cytokine levels (Fig. 3B-D). The serine/threonine-protein kinase ULK1 is a key molecule in the initiation of autophagy (39). Treatment with the autophagy inhibitor MRT68921 led to a reduction in the overall fluorescence of LC3 (Fig. 3E), indicating successful inhibition of autophagy. This implies that autophagy may play a protective role in suppressing inflammatory responses, and that autophagy inhibition may lead to increased inflammatory responses. These results provide insights about the relationship of autophagy with inflammatory regulation.

Based on these results, it was hypothesized that Bif-1 might exert an anti-inflammatory effect through a mechanism of autophagy regulation since Bif-1 is also an important autophagy regulation factor.

Use of si-Bif-1 significantly inhibits autophagy. Simultaneously, it was shown that the autophagy process was inhibited when the protein expression of Bif-1 was inhibited using si-Bif-1 (Fig. 4A and B). This suggested that Bif-1 plays an important role in the regulation of autophagy in inflammasome-activated cells, and its inhibition may lead to decreased autophagosome formation and degradation activity of inflammatory factors.

The level of p62 protein was increased after using si-Bif-1 (Fig. 4C). This was attributed to the fact that the decreased autophagic activity resulted in the inability of autophagosomes to efficiently degrade p62 protein, which led to its accumulation in cells. In the case of si-Bif-1 treatment, the action of inhibiting Bif-1 likely disrupts the degradation pathway of p62, thereby resulting in its upregulation.

It was also shown that the LC3-II/LC3-I ratio was decreased (Fig. 4D). LC3 is a key protein in the autophagy process, which undergoes phosphorylation and lipolysis to form LC3-I (40). Therefore, decreased autophagic activity leads to a decrease in the relative level of LC3-II. A decrease in the overall fluorescence of LC3 was observed when Bif-1 protein expression was inhibited using si-Bif-1 (Fig. 4E). This finding further supported the notion that Bif-1 plays a crucial role in the regulation of autophagy in inflammasome-activated cells, and its inhibition leads to the suppression of autophagy.

These results provided insights about the importance of Bif-1 in the regulation of autophagy and lay the foundation for in-depth exploration of the mechanism of Bif-1 in autophagy regulation.

NF- κ B inhibitor significantly inhibited the increase of Bif-1 and autophagy in J774A.1 cells. There are two signaling

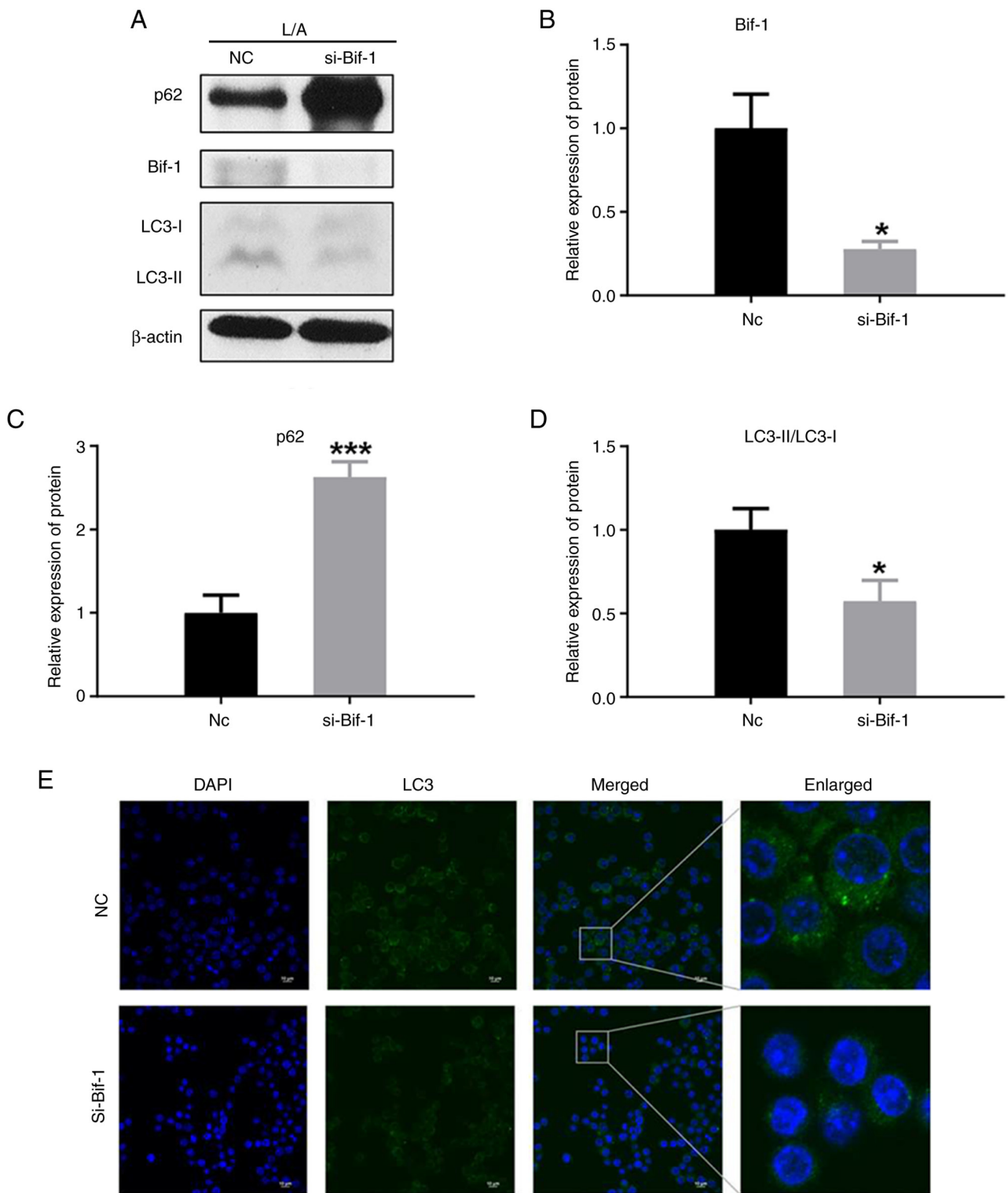


Figure 4. Use of si-Bif-1 significantly inhibited autophagy. (A-E) J774A.1 cells were transfected with si-Bif-1 or si-NC using Lipofectamine® 3000. A total of 24 h after transfection, cells were primed with LPS (500 ng/ml) for 6 h, then stimulated with 5 mM ATP for 30 min. Protein expression of (A) Bif-1, p62, LC3-II and LC3-I was investigated using western blotting. The relative protein levels of (B) Bif-1, (C) p62 and (D) LC3-II/I were analyzed using ImageJ (version 1.45; National Institutes of Health). (E) J774A.1 cell monolayers stained with LC3 (green) and DAPI (blue) fluorescence. Representative images from three independent experiments are shown. Scale bar, 10 μ m (magnification, x600). Data are expressed as mean \pm SD (n=3). $P < 0.05$ was considered to indicate a statistically significant difference (* $P < 0.05$, *** $P < 0.001$ vs. NC). LPS/ATP (L/A), LPS/ATP group; NC, negative control group treated with LPS/ATP; si-Bif-1, group treated with si-Bif-1 and LPS/ATP; si, silencer; LPS, lipopolysaccharide; ATP, adenosine triphosphate; IL, interleukin; Bif-1, Bax-interacting factor 1; Nc, negative control; LC, light chain.

pathways that activate NF- κ B, and both cascades involve the activation of an I κ B kinase (IKK) complex. MLN120B was used to block IKK β phosphorylation to clarify the regulatory

role of NF- κ B signaling on Bif-1. Bif-1 protein levels were significantly downregulated in the MLN120B-treated group under LPS/ATP stimulation (Fig. 5A and B).

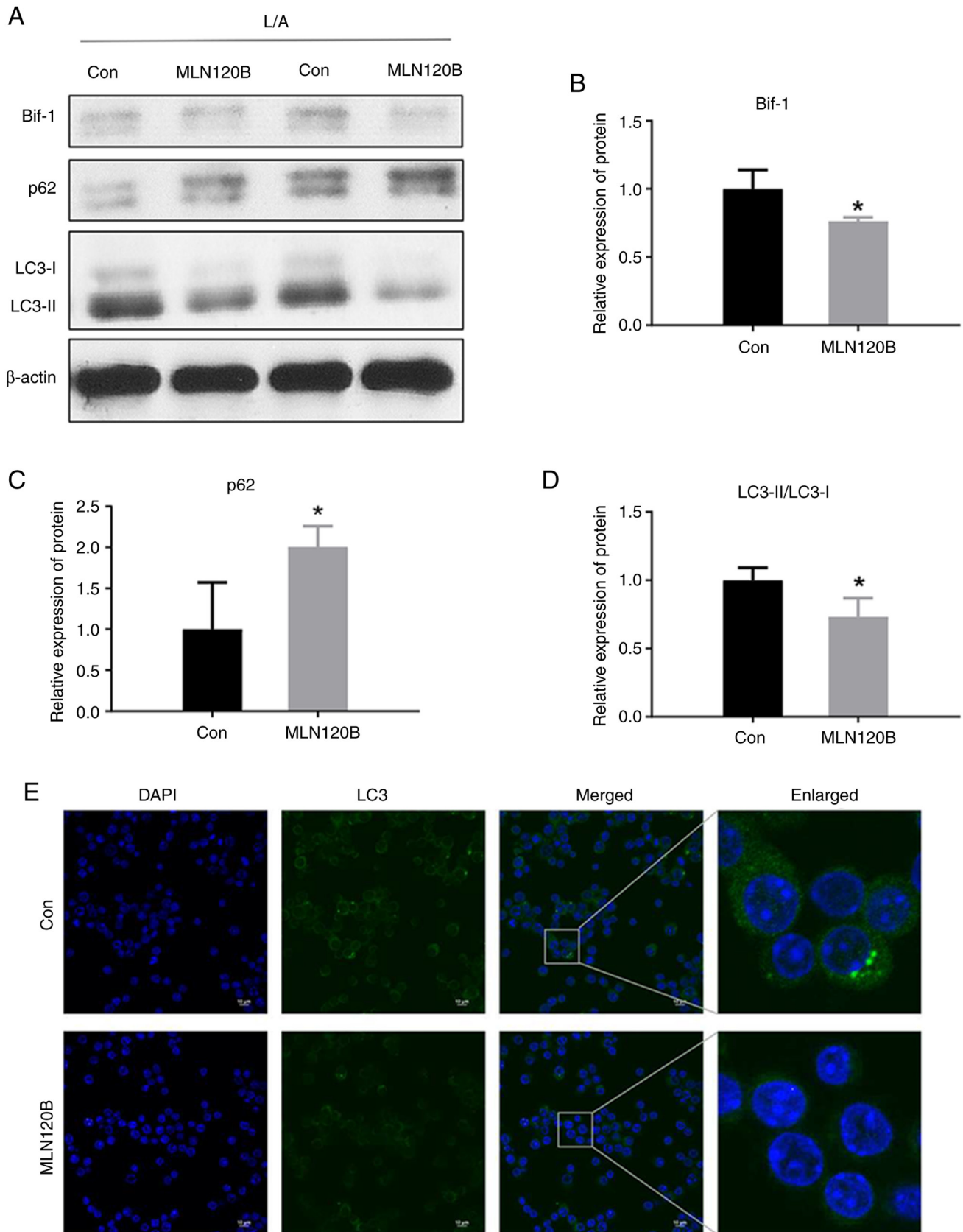


Figure 5. NF- κ B inhibitor significantly inhibited the increase of Bif-1 and autophagy in J774A.1 cells. (A-D) J774A.1 cells were treated with LPS (500 ng/ml) for 6 h in the presence or absence of MLN120B (1 μ M), then stimulated with 5 mM ATP for 30 min. Protein expression of (A) Bif-1, p62, LC3-II and LC3-I was investigated using western blotting. The relative protein levels of (B) Bif-1, (C) p62 and (D) LC3-II/I were analyzed using ImageJ (version 1.45; National Institutes of Health). (E) J774A.1 cell monolayers stained with LC3 (green) and DAPI (blue) fluorescence. Representative images from three independent experiments are shown. Scale bar, 10 μ m (magnification, x600). Data are expressed as mean \pm SD (n=3). P<0.05 was considered to indicate a statistically significant difference (*P<0.05 vs. Con). LPS/ATP (L/A), LPS/ATP group; Con, control group treated with LPS/ATP; MLN120B, LPS/ATP group treated with MLN120B (1 μ M); LPS, lipopolysaccharide; ATP, adenosine triphosphate; IL, interleukin; Bif-1, Bax-interacting factor 1; Con, control; LC, light chain; NF- κ B, nuclear factor- κ B.

Meanwhile, the LC3-II protein level was downregulated in the MLN120B-treated group, and the p62 protein level was also affected (Fig. 5C and D). When treated with MLN120B, a decrease in the overall fluorescence of LC3 was observed, indicative of autophagy inhibition (Fig. 5E). These results further supported the importance of Bif-1 in the regulation of autophagy. Notably, total p62 protein level was increased after MLN120B treatment in the present study. The increased level of p62 is determined by numerous factors. NF- κ B as a key factor can increase p62 and then enhance an early-stage autophagy and theoretically inhibition of NF- κ B should decrease p62 (41). However, p62 can be degraded by a late-stage autophagy-lysosomal pathway and a significant inhibition of the pathway might result in the accumulation of intracellular p62 (42). Here, it seemed that p62 was mainly determined by MLN120B-induced autophagy inhibition instead of NF- κ B inactivation. Further mechanisms should be investigated in the future. Despite this, these results provided a basis for further investigation of the relationship between NF- κ B and Bif-1-mediated autophagy.

Discussion

The inflammatory response plays a key role in the development of a number of diseases. The regulation of the inflammatory response is important for the prevention and treatment of diseases (43). IL-1 β is an important inflammatory mediator (44), the overproduction of which is closely associated with the development of a variety of inflammation-related diseases, and its inhibition has been shown to have therapeutic benefits in treating a range of severe but relatively rare inherited inflammatory diseases, such as cryopyrin-associated periodic syndromes and familial Mediterranean fever (45,46). IL-1 β can activate the NF- κ B signaling pathway, which in turn regulates the expression of a range of inflammation-related genes, including inflammatory mediators, cell adhesion molecules and inflammatory signaling molecules (47).

As an intracellular degradation mechanism, autophagic degradation involves a variety of pathophysiological processes, and it is generally considered to be a cytoprotective mechanism (48-50). Autophagy inhibits inflammasome formation and IL-1 β production by degrading components of the NLRP3 inflammasome (37). Autophagosome is a key structure in the autophagy process, and its formation is the initiation stage of the autophagy process (51). Beclin-1 interacts with multiple cofactors such as Bif-1, Rubicon, Ambra1 and survivin, promoting the formation of the Beclin-1-Vps34-Vps15 complex (52). This complex triggers the cascade of autophagy reactions (53). Bif-1 plays an important regulatory role as a key protein in the autophagy process (19). In the present study, it was shown that the use of si-Bif-1 significantly inhibited autophagy. When Bif-1 expression is inhibited, autophagosome formation may be affected, thereby inhibiting autophagy. In addition, Bif-1 inhibition may also affect the expression of autophagy-related proteins, such as autophagy-related 5 and autophagy-related 9 (22). The functions of these proteins are closely related to the different steps of autophagy, and therefore the relationship between Bif-1 inhibition and autophagy needs to be investigated further.

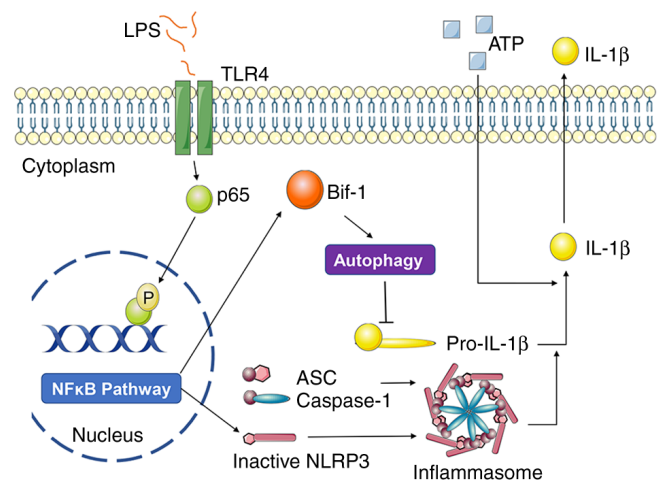


Figure 6. Schematic depicting that Bif-1 inhibits activation of inflammasome through an autophagy regulatory mechanism. Bif-1, Bax-interacting factor 1; ASC, apoptosis-associated speck-like protein containing a CARD; NLRP3, NLR family pyrin domain containing protein 3; TLR, toll-like receptor; NF- κ B, nuclear factor- κ B; LPS, lipopolysaccharide; ATP, adenosine triphosphate; IL, interleukin.

In the current study, it was also shown that the expression of Bif-1 may be regulated by the NF- κ B signaling pathway. It is hypothesized that there might be NF- κ B binding sites in the promoter region of the Bif-1 gene, which allow NF- κ B to directly or indirectly regulate the transcription of Bif-1. Alternatively, NF- κ B signaling pathway may also be able to affect the expression of Bif-1 by regulating the activity of other transcription factors. Additional research is required to investigate these hypotheses. In the present study, a decrease of LC3 levels caused by MLN120B, an inhibitor of NF- κ B signaling pathway, was observed, similar to what occurred with si-Bif-1. It seemed that both MLN120B and si-Bif-1 could inhibit autophagy. Since the NF- κ B signaling pathway can affect Bif-1 and Bif-1 can affect autophagy, the results indicated that the NF- κ B signaling pathway may affect autophagy through Bif-1. However, exact molecular mechanism should be investigated in the future.

Notably, in the present study, p62 levels were increased in a number of conditions, such as inflammasome activation induced by LPS/ATP, autophagy inhibition by si-Bif-1 and inhibition of the NF- κ B signaling pathway by MLN120B. It seemed that p62 was affected by numerous separate factors as aforementioned. On one hand, as an autophagy adapter, increased levels of p62 facilitate the clearance of harmful protein aggregates and regulate oxidative stress, thereby maintaining cellular homeostasis. Furthermore, p62 can interact with inflammatory signaling molecules, modulating the activity of inflammatory signaling pathways (54). As aforementioned, the activation of the NF- κ B signaling pathway in the condition of inflammation can upregulate p62 level (30). On the other hand, autophagic flux can affect p62 levels. Autophagic flux involves three steps: Nucleation, elongation and fusion (55). The initial nucleation of autophagic vesicles involves the class III phosphatidylinositol 3-kinase complex, comprising the kinase, Beclin-1 and UV radiation resistance-associated gene. Subsequently, LC3 undergoes lipidation, converting from the LC3 I to the LC3 II form.

Finally, mature autophagosomes directly fuse with lysosomes or fuse with endosomes before being transported to lysosomes. Substrates within autolysosomes are then degraded and released into the cytoplasm. P62 localizes to autophagosomes through its interaction with LC3 and undergoes continuous degradation by the autophagy-lysosome system. Consequently, defective autophagy leads to the accumulation of p62 (56). Therefore, it seemed that early-stage autophagy activation and late-stage autophagy inhibition could increase p62 levels. It is clear that the use of MLN120B inhibited the NF- κ B signaling pathway, however, the levels of p62 were likely still increased as a number of factors, other than NF- κ B, contribute to the levels of p62.

The findings of the present study revealed the critical role of Bif-1 in the activation of the inflammasome. This finding is important for an in-depth understanding of the mechanisms of inflammatory response regulation. By revealing the key role of Bif-1 in the autophagy process, new insights were introduced into the understanding of the mechanisms of autophagy and inflammation. Further studies on the interactions of Bif-1 with other key proteins such as Beclin-1 and its regulatory mechanisms in inflammation and autophagy-related diseases will enable a deeper understanding of these complex biological processes. Further studies could explore the expression of Bif-1 in disease models and the relationship with inflammation- and autophagy-related signaling pathways to deepen the understanding of its mechanism of action.

In conclusion, the findings of the present study revealed the mechanism used by Bif-1 to regulate inflammasome activation (Fig. 6), which provided new insights for elucidating the regulatory mechanism of inflammasome activation and provided the theoretical basis for the development of new drug targets.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Data acquisition, processing and validation was performed by YZ, WS, LW and WX; the study investigation was carried out by YZ, WS, YN, HZ and LL; project administration was carried out by YZ, LW and WX; YZ, WS and WX wrote the original draft of the manuscript; YZ, LW and WX reviewed and edited the manuscript. YZ and WS confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal study was reviewed and approved by The Bioethics Committee of Shenzhen International Graduate School, Tsinghua University [Shenzhen, China; approval no. (2020) No. 9].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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