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Rapamycin ameliorates lipopolysaccharide-induced acute lung injury by inhibiting IL-1β and IL-18 production

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ABSTRACT

Interleukin (IL)-1β and IL-18 play central and detrimental roles in the development of acute lung injury (ALI), and mammalian target of rapamycin (mTOR) is involved in regulating IL-1β and IL-18 production. However, it is not clear whether the mTOR specific inhibitor rapamycin can attenuate lipopolysaccharide (LPS)-induced ALI by modulating IL-1β and IL-18 production. In this study, we found that rapamycin ameliorated LPS-induced ALI by inhibiting NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome-mediated IL-1β and IL-18 secretion. Mechanistically, elevated autophagy and decreased nuclear factor (NF)-κB activation were associated with downregulated IL-1β and IL-18. Moreover, rapamycin reduced leukocyte infiltration in the lung tissue and bronchoalveolar lavage fluid (BALF), and contributed to the alleviation of LPS-induced ALI. Consistently, rapamycin also significantly inhibited IL-1β and IL-18 production by RAW264.7 cells *via* increased autophagy and decreased NF-κB signaling *in vitro*. Our results demonstrated that rapamycin protects mice against LPS-induced ALI partly by inhibiting the production and secretion of IL-1β and IL-18. mTOR and rapamycin might represent an appropriate therapeutic target and strategy for preventing ALI induced by LPS.

1. Introduction

Acute lung injury (ALI) is a severe clinical condition characterized by respiratory distress, refractory hypoxemia, and noncardiogenic pulmonary edema [1]. Despite significant advances in our understanding and management of ALI, the morbidity and mortality from ALI remain high [2]. Thus, further investigations of the pathogenesis of ALI and the development of better treatment strategies are indispensable.

Lipopolysaccharide (LPS), which is the major constituent of the cell wall of gram-negative bacteria, is widely used to simulate clinically relevant ALI caused by bacterial infections [3]. LPS is recognized by Toll-like receptor 4 of the innate immune cells, and induces their activation with elevated production of proinflammatory cytokines. Although this inflammatory response is essential for the eradication of infectious agents, an excessive and prolonged response can lead to lung injury, multiple organ failure, and even death. Interleukin (IL)-1β and IL-18, which are secreted following activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, are potential key cytokines that induce ALI after LPS challenge [4].

The NLRP3 inflammasome is a multiprotein complex consisting of the NLRP3, apoptosis-associated speck-like protein with CARD domain, and pro-caspase-1. It is a critical cytoplasmic pattern recognition receptor and can be activated by some pathogen-associated molecular patterns or damage-associated molecular patterns, such as bacteria, viruses, toxins and ATP. Once NLRP3 inflammasome is activated, pro-caspase-1 undergoes autoactivation and cleavage to produce an enzymatically mature caspase-1, which further cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, respectively [5,6]. Bioactive IL-1β and IL-18 can further amplify inflammatory responses and induce more proinflammatory cytokine and chemokine production, such as IL-1β, IL-18, IL-6, tumor necrosis factor (TNF)-α, and monocyte chemotactic protein (MCP)-1 [7]. Moreover, IL-1β has been shown to result in pulmonary edema by decreasing epithelial sodium channels [8], and IL-18 has been implicated in the development of acute respiratory distress

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syndrome (ARDS) [9]. High concentrations of IL-1β and IL-18 are associated with the risk of ARDS and indices of morbidity and mortality in critically ill patients [9–11]. Therefore, IL-1β and IL-18 are important therapeutic targets to reduce tissue damage and maintain immune homeostasis after LPS exposure.

Although the blocking of IL-1β or IL-18 partially attenuates lung injury in different mice models [12–14], several studies have suggested that the single inhibition of either IL-1β or IL-18 shows low effectiveness in ALI treatment. Blocking IL-1β with the IL-1R antagonist anakinra is insufficient to protect mice from LPS, cecal ligation and puncture or influenza virus-induced lung injury [15,16]. Moreover, most patients with ARDS did not benefit from anakinra treatment [17]. Furthermore, IL-18 neutralizing antibody failed to confer a complete survival benefit in mice treated with lethal LPS [16,18]. It is likely that the single blocking of IL-1β or IL-18 is not sufficient to inhibit the excessive immune responses; therefore, the dual inhibition of IL-1β and IL-18 might represent a better therapeutic strategy during LPS exposure. It has been shown that IL-18 neutralizing antibody combined with anakinra have additive benefits in protecting mice against lethal dose of LPS [16]. However, IL-18 inhibitors are not approved for clinical use [19]. Thus, common upstream targets which regulate both IL-1β and IL-18 production may be effective for the treatment of ALI.

Recent studies have shown that autophagy and nuclear factor (NF)-κB activity participate in IL-1β/IL-18 production and secretion. Deficiencies in autophagy-related proteins can induce significantly increased levels of IL-1β and IL-18, causing severe lung tissue damage [20]. In contrast, the induction of autophagy can suppress IL-1β and IL-18 secretion, limiting excessive inflammation [21]. Aberrant mammalian target of rapamycin (mTOR) activation can suppress autophagic activity [22]. As an inhibitor of mTOR, rapamycin is widely used as an autophagy inducer. Rapamycin can also effectively dampen the NF-κB signaling pathway [23,24]. Moreover, the NLRP3 inflammasome is a major downstream target of the mTOR signaling pathway. Previous studies have shown that mTOR inhibition by rapamycin could suppress the activity of NLRP3 inflammasome [5,6,25–27]. However, whether rapamycin modulates IL-1β and IL-18 production and ameliorates LPS-induced pathological damage has not yet been fully investigated.

Given these multiple functions of rapamycin, in the present study, its roles in the regulation of IL-1β/IL-18 production and the amelioration of LPS-induced ALI were evaluated.

2. Materials and methods

2.1. Mice

Female BALB/c mice, 6–8 weeks of age, were obtained from the Institute of Laboratory Animal Sciences, Beijing, China. The mice were kept on a 12:12 h light-dark cycle, with free access to food and water. All animal experiments were conducted in accordance with the guide for the care and use of laboratory animals, and were approved by the Animal Ethics Committee at Capital Medical University.

2.2. ALI mouse model

ALI was induced by the intratracheal administration of 1 mg/kg LPS (Cell Signaling Technology, MA) in 50 μl of PBS. Briefly, mice were anesthetized with pentobarbital. LPS was injected into the trachea using a microsyringe. After intratracheal instillation, the mice were kept vertical for 1 min to ensure the distribution of LPS in the lungs. The mock group was injected with PBS.

2.3. Rapamycin treatment in mice

Rapamycin was dissolved in DMSO and then diluted with PBS to a final concentration of DMSO of 1.8% [28]. Mice were intraperitoneally injected with rapamycin (5 mg/kg body weight dissolved in 1.8% DMSO). The mock group was administered with an equal volume of 1.8% DMSO. After 24 h, the mice received a second injection of rapamycin or 1.8% DMSO, followed by the intratracheal administration of either PBS or LPS [28,29]. Six randomly selected mice in each group were sacrificed 24 h after LPS exposure, and lung and bronchoalveolar lavage fluid (BALF) samples were collected for further analyses.

2.4. Cell culture

RAW264.7 cells were obtained from American Type Culture Collection (ATCC, VA), and were cultured in DMEM (Life Technologies, CA) supplemented with 10% fetal bovine serum (Life Technologies, CA), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Life Technologies, CA) in 5% CO_2 at 37 °C.

2.5. Cell intervention

RAW264.7 cells were pretreated with rapamycin (50 nM) for 1 h and then stimulated with LPS (200 ng/ml) for 6 h. After that, cells were incubated with rapamycin (50 nM) for 18 h and lysed for further analysis. The cell culture supernatant was collected by centrifugation at 1500 rpm for 10 min at 4 °C, and stored at −80 °C for further analysis.

2.6. Histological assessment

The lung samples were immediately fixed with 10% formalin, and embedded in paraffin. The sections (4 μm thickness) were sliced for hematoxylin and eosin staining, and visualized using an Olympus inverted microscope (Olympus BX53, Tokyo, Japan).

2.7. Lung injury score

Slides were randomized, read blindly, and then scored using a semiquantitative scoring system as described previously [30]. Edema, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, atelectasis, necrosis, and hyaline membrane formation were each scored on a 0- to 4-point scale: no injury = score of 0; injury in 25% of the field = score of 1; injury in 50% of the field = score of 2; injury in 75% of the field = score of 3; and injury throughout the field = score of 4. Results were confirmed by an experienced and qualified pathologist.

2.8. Analysis of BALF

The lungs were lavaged with 0.5 ml of PBS four times. The BALF was centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant was collected and stored at −80 °C for further analyses. The cells were resuspended in 1 ml of PBS and the total cell numbers were counted using a hemocytometer. Cytospin samples were prepared by centrifuging the suspensions (200 μl) at 350 rpm for 10 min. The ratio and numbers of neutrophils, macrophages and lymphocytes were determined by counting at least 300 leukocytes after Giemsa staining (Solarbio Science & Technology Co., Ltd., Beijing, China) according to manufacturer's instructions.

2.9. Western blot analysis

Protein samples were extracted from lung tissues or RAW264.7 cells using RIPA buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) with protease and phosphatase inhibitor cocktails (Abcam, Cambridge, UK). Equal amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline (TBST) at room temperature for 1 h, the membranes were incubated with primary antibodies against pro-IL-1β (1:1000, Abcam, Cambridge, UK), IL-1β (1:500, Abcam, Cambridge, UK), IL-18 (1:1000, Abcam, Cambridge, UK), NLRP3 (1:500, Adipogen, CA), LC3 I/II (1:500, Cell

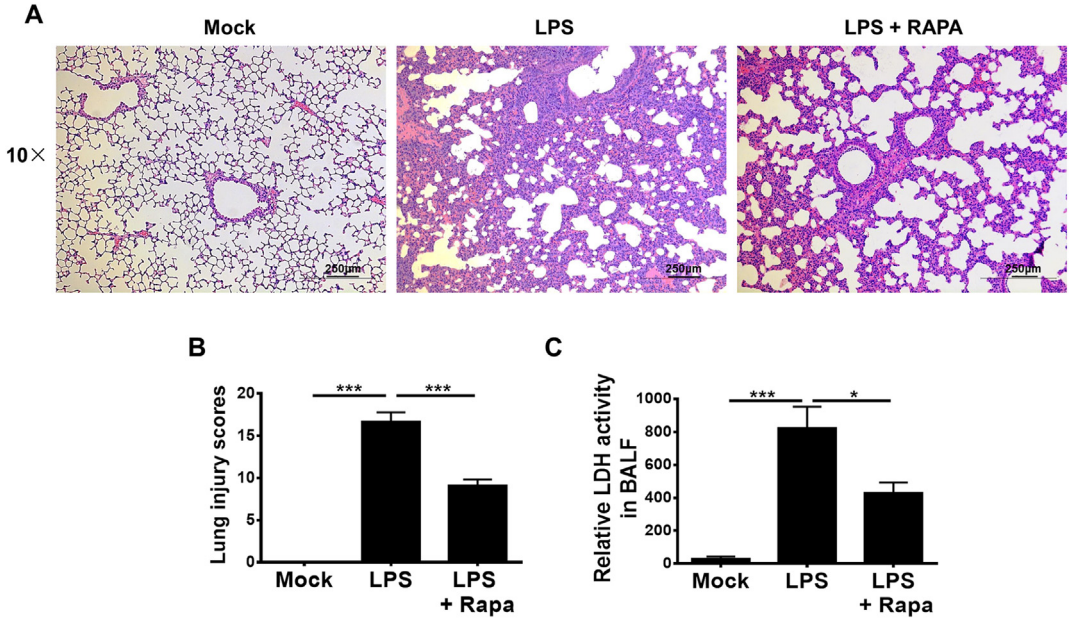

Fig. 1. Rapamycin alleviated LPS-induced lung injury. (A) Representative images of lung tissue injury assessed by hematoxylin and eosin staining. (B) Semiquantitative histological scoring of lung injury. This experiment was performed in a blinded manner. (C) LDH activity in BALF from different treatment groups. Data are representative of two independent experiments and presented as means ± SEM ($n = 6$ for each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Signaling Technology, MA), mTOR (1:1000, Cell Signaling Technology, MA), p-mTOR (1:500, Cell Signaling Technology, MA), S6RP (1:1000, Cell Signaling Technology, MA), p-S6RP (1:500, Cell Signaling Technology, MA), p65 (1:1000, Cell Signaling Technology, MA), p-p65 (1:500, Cell Signaling Technology, MA) or β-actin (1:5000, Abcam, Cambridge, UK) overnight at 4 °C, respectively. After washing, the membranes were incubated with corresponding HRP-coupled secondary antibodies for 1 h at room temperature. Protein bands were detected using the ECL SuperSignal reagent (Millipore, MA), and a densitometric analysis was performed using Image J software (version 1.51w, National Institutes of Health, MD).

2.10. RT-PCR

Total RNA from lungs or RAW264.7 cells was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). The quality of the RNA was assessed with the Nano-Drop 2000 Spectrophotometer (Thermo Scientific, MA) according to the manufacturer's instructions. Complementary DNA was synthesized using PrimeScript™ RT Master Mix (Perfect Real Time) (TaKaRa, Japan). Real-time PCR (RT-PCR) was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Japan) and the ABI 7500 Real Time PCR System (Applied Biosystems, MA). Relative gene expression was calculated using the comparative Ct (ΔΔ Ct) method, with GAPDH as a reference gene. The following primers were used:

IL-1β, forward, 5′-GGTGTGTGACGTTCCCATTA-3′, reverse, 5′-GGCCACAGGTATTTTGTCGT-3′; *IL-18*, forward, 5′-ACAGGCCTGACATCTTCTGC-3′, reverse, 5′-CCTTGAAGTTGACGCAAGAGT-3′; *TNF-α*, forward, 5′-ACTGAACTTCGGGGTGATCG-3′, reverse, 5′-ACAGGCTTGTCACTCGAATTTTG-3′; *MCP-1*, forward, 5′-CCCCAAGAAGGAATGGGTCC-3′, reverse, 5′-GGTTGTGGAAAAGGTAGTGG-3′; *IL-6*, forward, 5′-TGTTCTCTGGGAAATCGTGGA-3′, reverse, 5′-TGCAAGTGCATCATCGTTGTTC-3′; *GAPDH*, forward, 5′-ATTCCACCCATGGCAAATTC-3′, reverse, 5′-CGCTCCTGGAAGATGGTGAT-3′.

2.11. Cytokine analysis

The concentrations of IL-1β and IL-18 in the BALF and cell culture supernatant were measured using IL-1β and IL-18 ELISA Kits (Abcam, Cambridge, UK) according to the manufacturer's instructions. Protein concentrations in the BALF samples were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China).

2.12. Measurement of lactate dehydrogenase (LDH) and myeloperoxidase (MPO) activity

The activity of LDH was measured using the Lactate dehydrogenase assay kit (Nanjing jiancheng bioengineering institute, Jiangsu, China). The activity of MPO was measured using the Myeloperoxidase assay kit (Nanjing jiancheng bioengineering institute, Jiangsu, China).

2.13. Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc., CA) was used for statistical analyses. Data are presented as means ± SEM. The statistical significance between two groups was assessed by Student's *t*-tests. For comparisons of more than two groups, significance was determined by one-way ANOVA. A two-sided p-value < 0.05 was considered statistically significant.

3. Results

3.1. Rapamycin alleviated LPS-induced lung injury

For *in vivo* experiments, preliminary studies were performed to assess whether rapamycin (5 mg/kg) alone had any effect on lung. As shown in Supplementary Fig. 1, rapamycin only treatment group did not show lung tissue injury. There was no difference in lung injury score between mock group and rapamycin alone treatment group (Sup. 1A and B). Meanwhile, total numbers of leukocytes in BALF in rapamycin only treatment group were also similar with that in mock group (Sup. 1C and D). Furthermore, the expression of NLRP3 and secretion of IL-1β/IL-18 were not obviously depressed by rapamycin in lungs of LPS-untreated mice (Sup. 1E). Due to the lower activity of mTOR and NF-κB signaling pathway in lung of normal mice, there were slightly further suppressed by rapamycin. In addition, the level of autophagy was increased after rapamycin treatment (Sup. 1F).

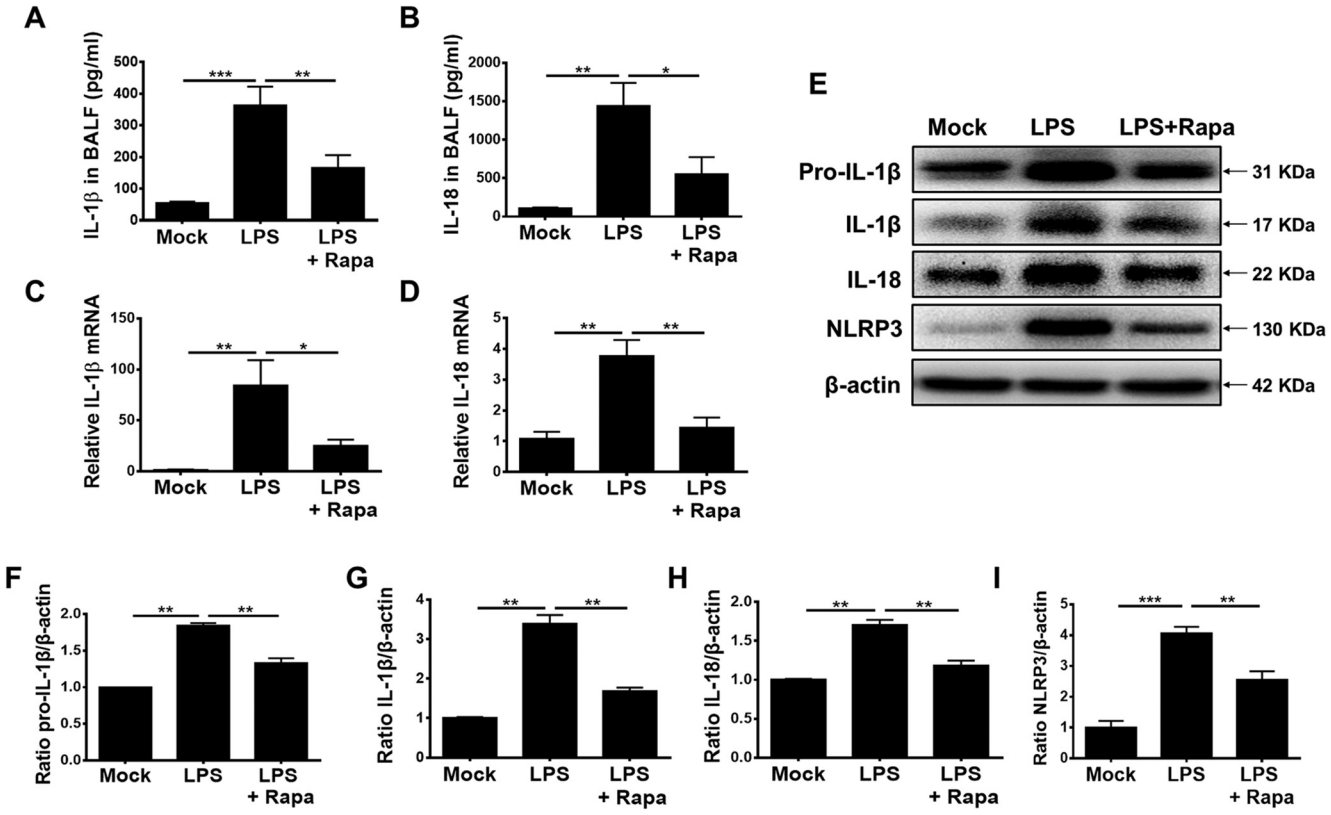

Fig. 2. Rapamycin inhibited the secretion of IL-1β and IL-18, and the expression of NLRP3. The concentrations of (A) IL-1β and (B) IL-18 in BALF. Real-time PCR analysis of (C) IL-1β and (D) IL-18 levels in lung tissues. (E) Western blot analysis of pro-IL-1β, IL-1β, IL-18 and NLRP3 expression in lung tissues. Expression of (F) pro-IL-1β, (G) IL-1β, (H) IL-18 and (I) NLRP3 relative to β-actin. Data are representative of two independent experiments and presented as means ± SEM ($n = 6$ for each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In subsequent studies, a mice model of LPS-induced ALI was established. Histopathology assessments revealed that LPS caused extensive lung damage, characterized by widespread lung inflammation and necrosis of the bronchiolar walls. However, pulmonary histopathological injury was significantly relieved by rapamycin (Fig. 1A, B). In addition, LDH activity in BALF, an indicator of pathological damage to the lungs, also significantly decreased after rapamycin treatment (Fig. 1C).

3.2. Rapamycin inhibited the secretion of IL-1β and IL-18, and the expression of NLRP3

Considering the critical roles of IL-1β and IL-18 in LPS-induced ALI, the mRNA and protein levels of IL-1β and IL-18 were detected in the control and rapamycin treatment groups. As shown in Fig. 2A and B, the high concentrations of IL-1β and IL-18 induced by LPS in BALF were inhibited by rapamycin. Moreover, the mRNA and protein levels of IL-1β and IL-18 in the lung tissues were significantly suppressed by rapamycin (Fig. 2C–H).

To further confirm the impact of rapamycin on NLRP3 inflammasome activation, the protein expression of NLRP3 was measured *in vivo*. As shown in Fig. 2E and I, LPS significantly increased the expression of NLRP3 in mouse lungs, whereas rapamycin significantly suppressed NLRP3 expression. These results suggest that rapamycin alleviates LPS-induced ALI by repressing the NLRP3 inflammasome-mediated secretion of IL-1β and IL-18.

3.3. Rapamycin decreased IL-1β and IL-18 production by enhancing autophagy and reducing NF-κB activation

To further investigate the mechanisms by which rapamycin decreased IL-1β and IL-18 production in the LPS-induced ALI model, mTOR activity was assayed after rapamycin treatment. As shown in Fig. 3A and B, the phosphorylation levels of mTOR and the downstream target S6RP were significantly increased in lung tissues after LPS exposure, while rapamycin treatment efficiently reduced the phosphorylation of mTOR and S6RP.

As mTOR is a major negative regulator of autophagy [22], we evaluated whether mTOR inhibition by rapamycin can increase autophagy. Western blot analysis revealed that the level of the autophagy-related hallmark ratio of LC3-II/LC3-I increased in lung tissues after LPS infection. Moreover, mTOR inhibition by rapamycin further increased the ratio of LC3-II/LC3-I, which might be correlated with the decreased IL-1β and IL-18 production (Fig. 3C, D). These findings imply that the inhibition of LPS-induced mTOR activation by rapamycin decreases IL-1β and IL-18 production by inducing autophagy.

NF-κB is the crucial transcription factor that induces the production of IL-1β and IL-18 [31]. Previous studies have reported that rapamycin modulates NF-κB activity by mTOR inhibition [24,32]. Therefore, we evaluated whether the decreases in IL-1β and IL-18 production were mediated by downregulated NF-κB activation after rapamycin treatment. LPS activated NF-κB signaling, as indicated by the increased expression of p-p65 in lung tissues. However, the increase in p-p65 was significantly reduced by rapamycin (Fig. 3E, F). Furthermore, NF-κB-induced increases in other genes, such as *IL-6, TNF-α,* and *MCP-1*, were also downregulated by rapamycin (Fig. 3G-I). These results suggest that rapamycin decreases IL-1β and IL-18 production by inhibiting NF-κB activation.

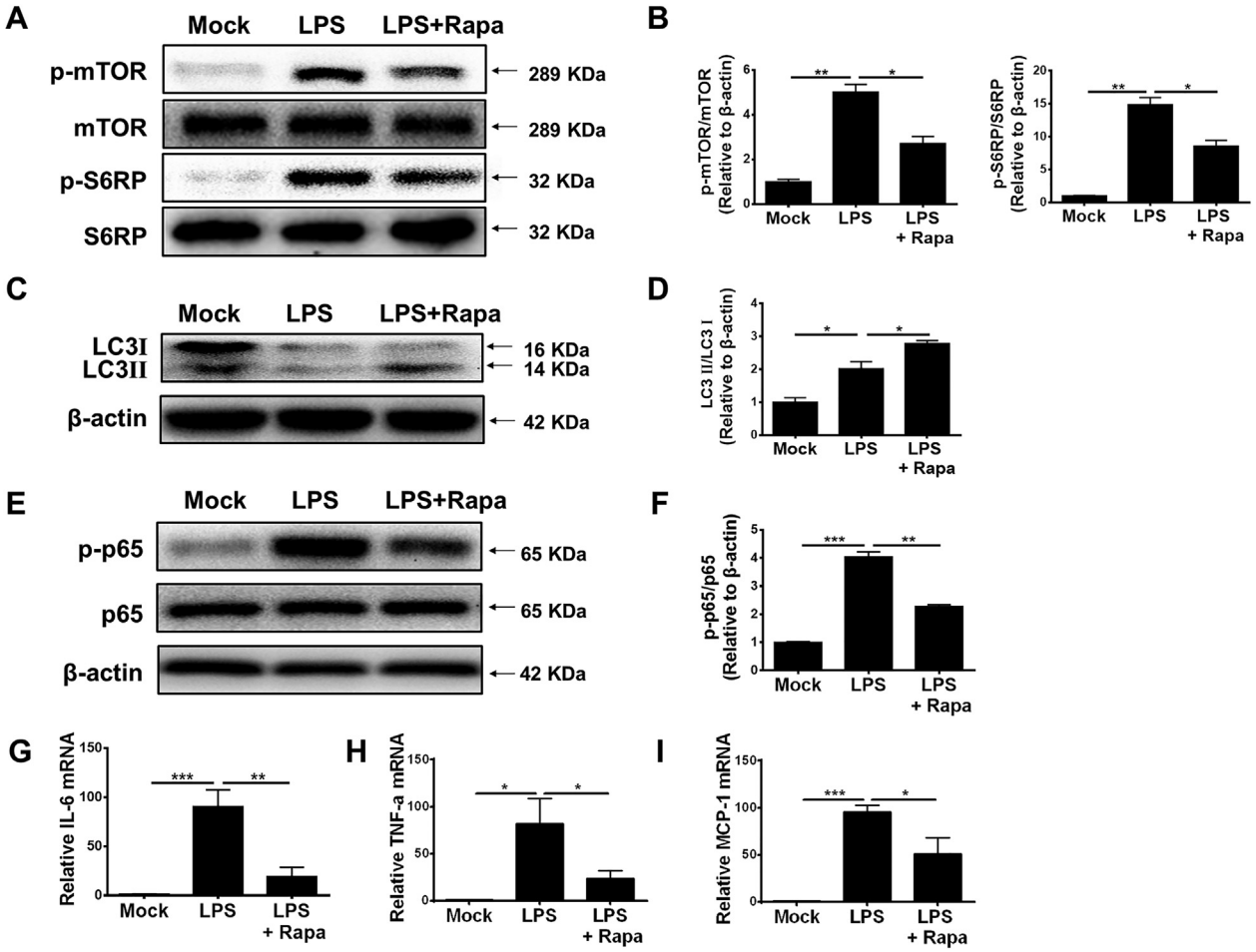

Fig. 3. Rapamycin decreased IL-1β and IL-18 production by enhancing autophagy and reducing NF-κB activation. (A) Western blot analysis of p-mTOR, mTOR, p-S6RP and S6RP in lung tissues from different treatment groups. (B) Expression of p-mTOR relative to mTOR, and p-S6RP relative to S6RP. (C) Western blot analysis of LC3 I/II in lung tissues. (D) Expression of LC3-II relative to LC3-I. (E) Western blot analysis of p-p65 and p65 in lung tissues. (F) Expression of p-p65 relative to p65. Real-time PCR analysis of (G) IL-6, (H) TNF-α and (I) MCP-1 in lung tissues. Data are representative of two independent experiments and presented as means ± SEM ($n = 6$ for each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.4. Rapamycin inhibited inflammatory cell infiltration in the lung tissue and BALF

LPS-induced chemokine production can recruit inflammatory cells to the lung. The effect of rapamycin on inflammatory cell infiltration after LPS exposure was evaluated. As shown in Fig. 4A and B, LPS caused excessive inflammatory cell infiltration to the lung tissue and BALF, whereas rapamycin treatment reduced infiltration.

To further evaluate the effect of rapamycin on immune cells, the total number of leukocytes and the numbers of different cell types in BALF were also examined. As shown in Fig. 5A–D, the rapamycin treatment group showed significantly lower total number of leukocytes, as well as neutrophils, macrophages and lymphocytes in BALF than the LPS group. MPO activity, an indicator of neutrophil infiltration in the lung, was also dramatically decreased after rapamycin treatment (Fig. 5E).

3.5. Rapamycin suppressed LPS-induced IL-1β and IL-18 production in macrophages in vitro

Lung macrophages play key roles in the pathogenesis of ALI. Previous studies have indicated that the production of IL-1β and IL-18 by macrophages is essential for initiating and driving inflammatory responses [5,33].

For *in vitro* experiments, as shown in Supplementary Fig. 2 and Ko's reports [25], the activation of mTOR and NF-κB, the expression of NLRP3 and secretion of IL-1β/IL-18 were slightly suppressed by rapamycin. Rapamycin (50 nM) only treatment did not exhibit any cytotoxic effects on the Raw264.7 cells [25].

The downregulation of IL-1β and IL-18 levels by rapamycin in macrophages was further confirmed *in vitro*. As shown in Fig. 6A, elevated mTOR activity (including increased p-mTOR and p-S6RP levels) induced by LPS was reversed by rapamycin in RAW264.7 cells. Similarly, LPS caused high expression of IL-1β and IL-18 in whole-cell extracts and cell culture supernatants, whereas rapamycin significantly suppressed IL-1β and IL-18 production and secretion (Fig. 6B–D). The protein expression of NLRP3 in whole-cell extracts also decreased after rapamycin administration (Fig. 6B).

3.6. The decreased IL-1β and IL-18 production was associated with autophagy and NF-κB activity in vitro

Mechanistically, further increased ratio of LC3-II/LC3-I and decreased p-p65 in response to rapamycin might explain the decreased IL-1β and IL-18 levels (Fig. 7A–C). Moreover, rapamycin obviously reduced the mRNA levels of NF-κB-induced proinflammatory mediators, including *IL-6*, *TNF-α* and *MCP-1* (Fig. 7D–F). These results suggest that rapamycin inhibits IL-1β and IL-18 secretion in lung macrophages by inducing autophagy and inhibiting NF-κB activity.

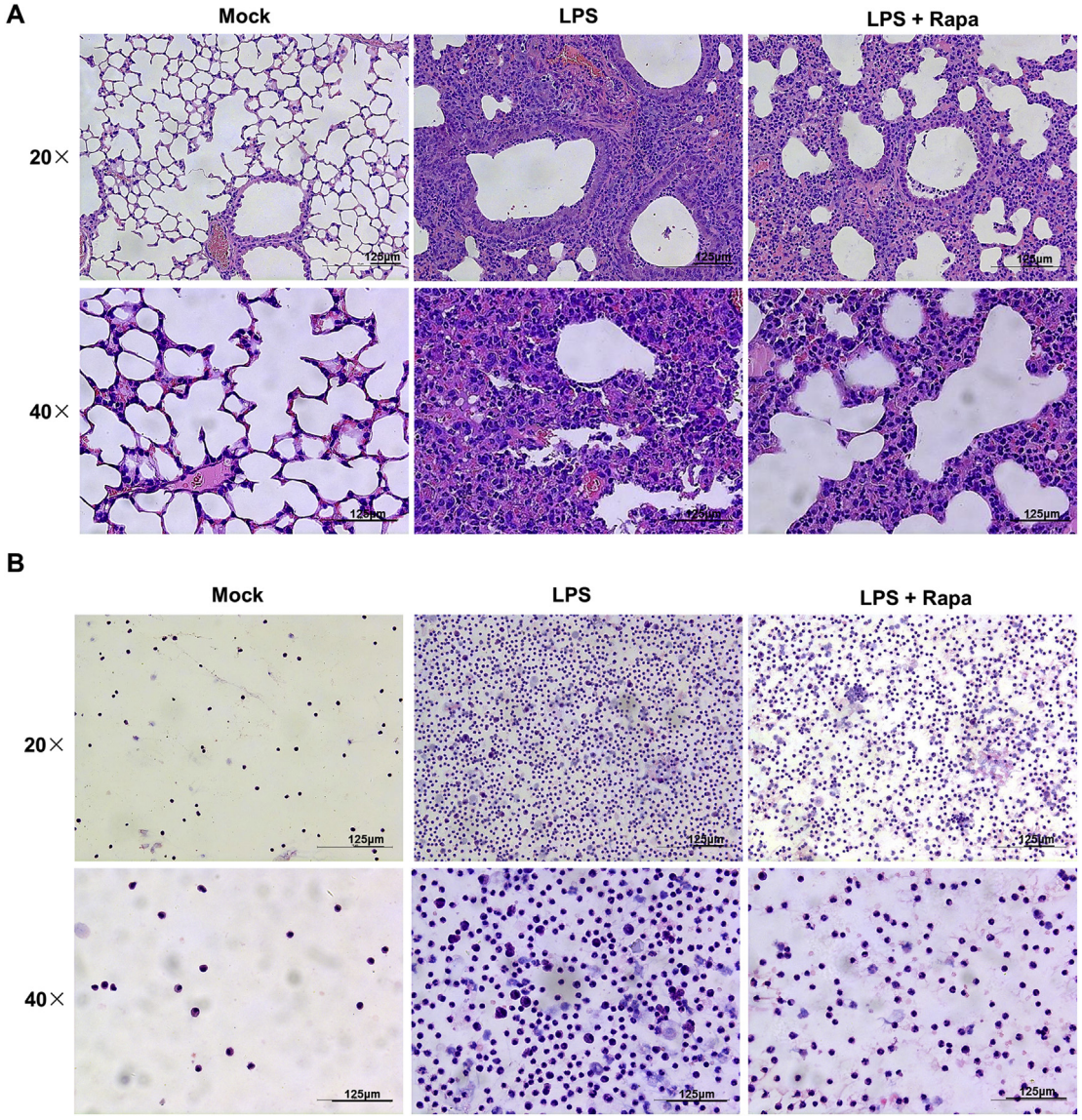

Fig. 4. Rapamycin inhibited inflammatory cell infiltration in lung tissues and BALF. (A) Histopathological examination of inflammatory cell infiltration by hematoxylin and eosin staining in lung tissues. (B) Representative images of Giemsa-stained cytospin of BALF.

4. Discussion

Pulmonary inflammation and ALI caused by bacterial infection have high morbidity and mortality. LPS-induced ALI is a common model used to simulate bacterial infection-induced ALI. Our present findings demonstrated that rapamycin could ameliorate LPS-induced ALI by depressing NLRP3 inflammasome mediated-IL-1β and IL-18 secretion. The enhanced autophagy and inhibited NF-κB activity by rapamycin were associated with suppressed IL-1β and IL-18 production. In addition, rapamycin reduced inflammatory cell infiltration into the lung and BALF. Consistently, rapamycin inhibited LPS-induced IL-1β and IL-18 secretion by macrophages *via* enhanced autophagy and inhibited NF-κB activity *in vitro*.

IL-1β and IL-18 belong to the IL-1 cytokine family [34]. Previous studies have demonstrated that IL-1β and IL-18 have synergistic detrimental effects on LPS-induced mortality. Only the dual neutralization of IL-1β and IL-18 confers complete protection against LPS-induced lethality. Inhibition of either of them merely results in partial protection [16]. Consistent with data from the mice model, IL-1R antagonist anakinra shows slightly survival benefits in patients with ARDS [17]. These results suggest that the blocking of both IL-1β and IL-18 production/secretion or their upstream signaling pathways might be effective for the treatment of ALI/ARDS. NLRP3 inflammasome can mediate the maturation and secretion of IL-1β and IL-18. However, the specific NLRP3 inhibitor is not available for clinical use [19,35,36]. Therefore, finding the Food and Drug Administration approved-drug to target IL-1β and IL-18 might be a better strategy to treat ALI.

The mTOR inhibitor rapamycin, a natural product with potent immunosuppressant property, is widely used in organ transplant patients. It has been shown that mTOR can actively suppress autophagy, which is one of predominant mechanisms to modulate IL-1β and IL-18 production [22]. Our data showed that mTOR was activated after LPS challenge and subsequently increased IL-1β and IL-18 production in mouse lung tissues. Rapamycin could inhibit IL-1β and IL-18 production by increasing autophagy activity (Fig. 3). Consistent with our results, previous studies have also demonstrated that the induction of autophagy limits IL-1β and IL-18 secretion by removing damaged mitochondria and preventing mtROS release [37,38]. Furthermore, autophagy had been shown to be involved in elimination of pro-IL-1β molecules [39]. Similarly, the ratio of LC3-II/LC3-I was up-regulated in LPS-infected RAW264.7 cells, and rapamycin suppressed IL-1β and IL-18 production by inducing autophagy *in vitro* (Fig. 7). The reason why

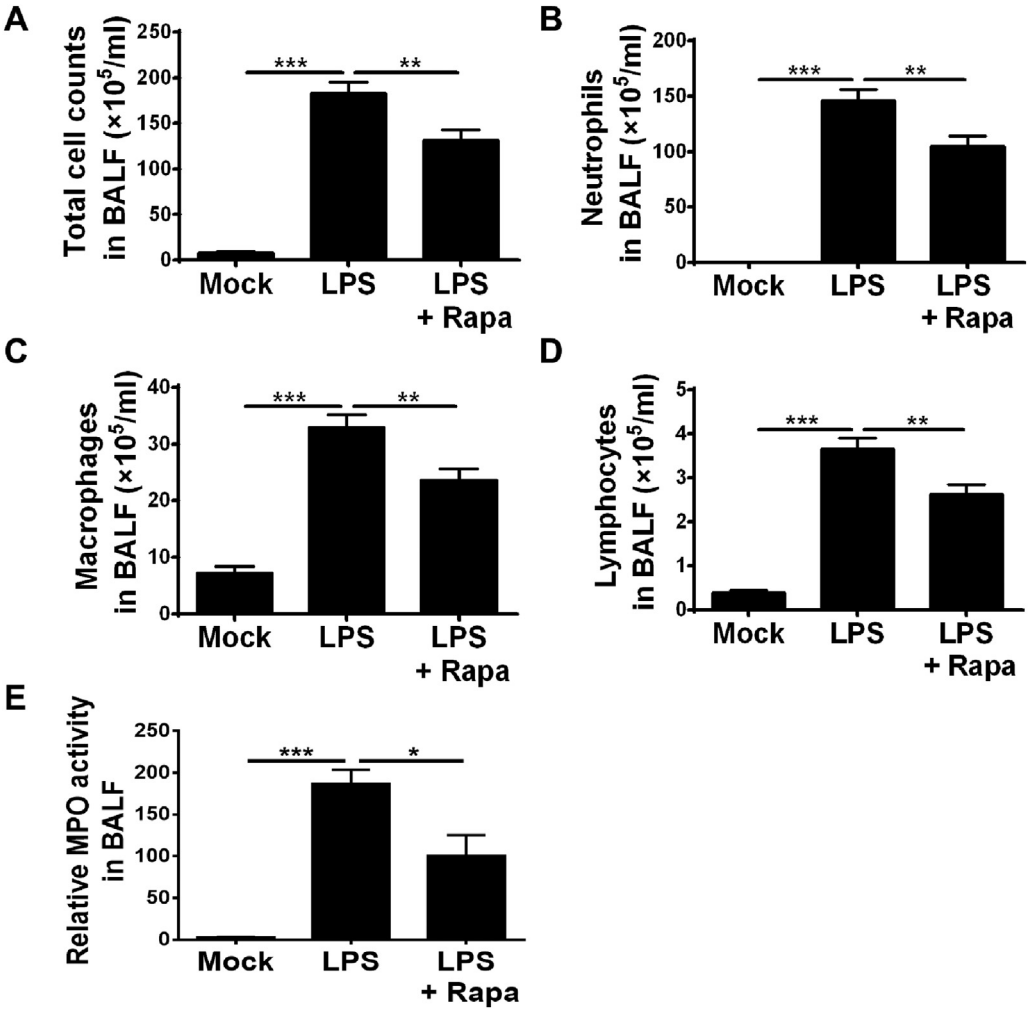

Fig. 5. Rapamycin inhibited inflammatory cell infiltration in the lung tissue and BALF. (A) Numbers of total cells, (B) neutrophils, (C) macrophages and (D) lymphocytes in BALF. (E) MPO activity in BALF. Data are representative of two independent experiments and presented as means ± SEM ($n = 6$ for each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

autophagy was increased after activation of mTOR may be the mTOR is not the sole regulator of autophagy. For instance, glutamine-derived ammonia can activate autophagy in an mTOR-independent pathway [40]. Despite our research showing that LPS triggered autophagy, increased production of IL-1β and IL-18 were still found in mice and RAW264.7 cells. Moreover, with further increased LC3-II, rapamycin could significantly decrease IL-1β and IL-18 production. Therefore, our results suggested that autophagy induction by rapamycin contributes to the decreased production of IL-1β and IL-18 after LPS exposure.

mTOR can also regulate NF-κB activity [23,24]. NF-κB is an important transcription factor in inflammatory responses and increases the levels of NF-κB p65-dependent proinflammatory cytokines and chemokines, such as IL-1β, IL-18, IL-6, TNF-α and MCP-1. In our study, the activation of NF-κB was correlated with mTOR activity. Rapamycin decreased the phosphorylation of p65, resulting in reduced levels of inflammatory cytokines and chemokines, including IL-1β, IL-18, IL-6, TNF-α and MCP-1, in both the lung tissues and macrophages. Similar to our findings, Lorne et al. also found that mTOR activation increases the expression of proinflammatory mediators *via* the enhanced phosphorylation of p65, whereas mTOR inhibition by rapamycin significantly attenuated lung inflammation in mice [29]. In addition, Dan et al. reported that rapamycin controls NF-κB activity by inhibiting inhibitor of nuclear factor kappa-B kinase (IKK) activation [41].

Excessive inflammatory cell infiltration is also involved in the pathogenesis of LPS-induced ALI. Our results demonstrated that rapamycin could reduce inflammatory cell infiltration into the lung and BALF. Lorne and Hu et al. also demonstrated that rapamycin treatment decreased total cell counts and the number of neutrophils in BALF after LPS infection [29,42]. However, Jill et al. have found that rapamycin augments LPS-induced lung injury *via* STAT1-related apoptosis [43]. Possible explanations for this discrepancy include: 1. differences in the dose of LPS; 2. differences in the dose and timing of rapamycin administration. The detailed mechanisms as why rapamycin can be either protective or deleterious in ALI need further investigation.

In conclusion, our present study demonstrates that mTOR inhibition by rapamycin protects mice against LPS-induced lung injury by downregulating IL-1β and IL-18 production and suppressing immune cell infiltration. Enhanced autophagy and downregulated NF-κB activity induced by rapamycin may be correlated with decreased IL-1β and IL-18 production. Thus, rapamycin might represent a better therapeutic strategy for preventing ALI induced by bacterial infections.

Conflicts of interest

All authors declare no conflicts of interest associated with this manuscript.

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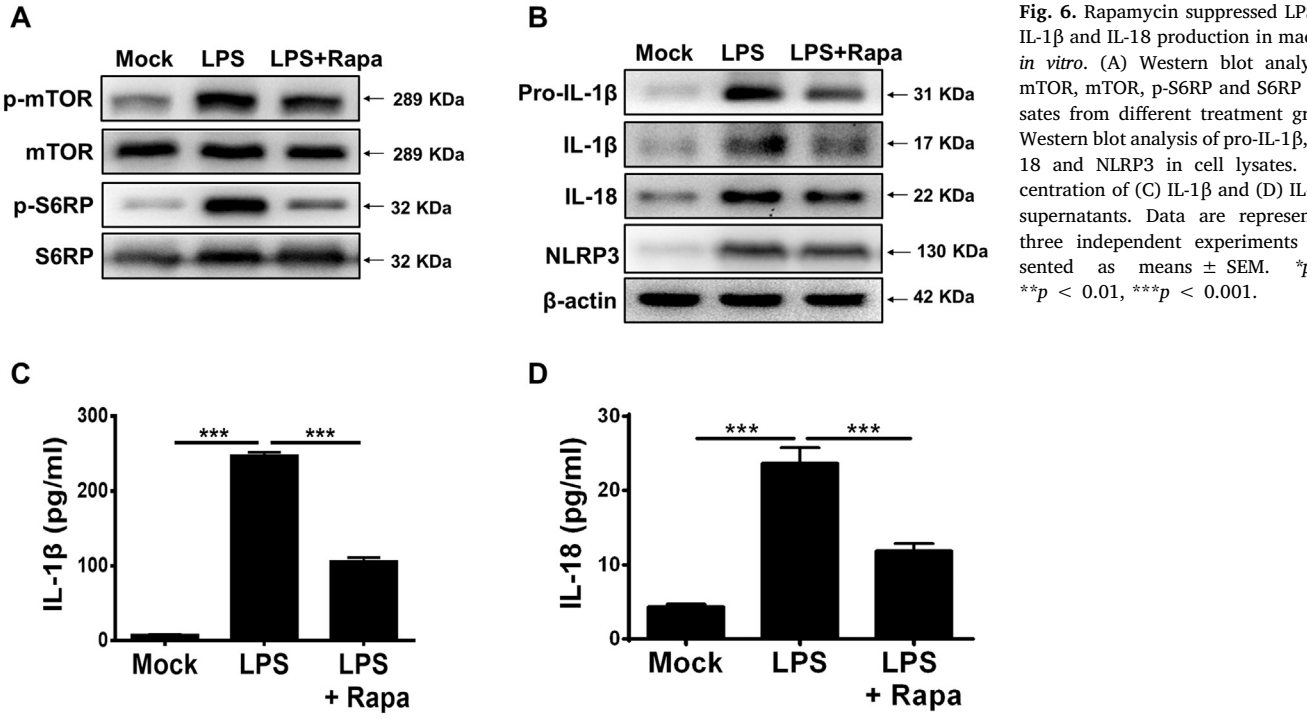

Fig. 6. Rapamycin suppressed LPS-induced IL-1β and IL-18 production in macrophages *in vitro*. (A) Western blot analysis of p-mTOR, mTOR, p-S6RP and S6RP in cell lysates from different treatment groups. (B) Western blot analysis of pro-IL-1β, IL-1β, IL-18 and NLRP3 in cell lysates. The concentration of (C) IL-1β and (D) IL-18 in the supernatants. Data are representative of three independent experiments and presented as means ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

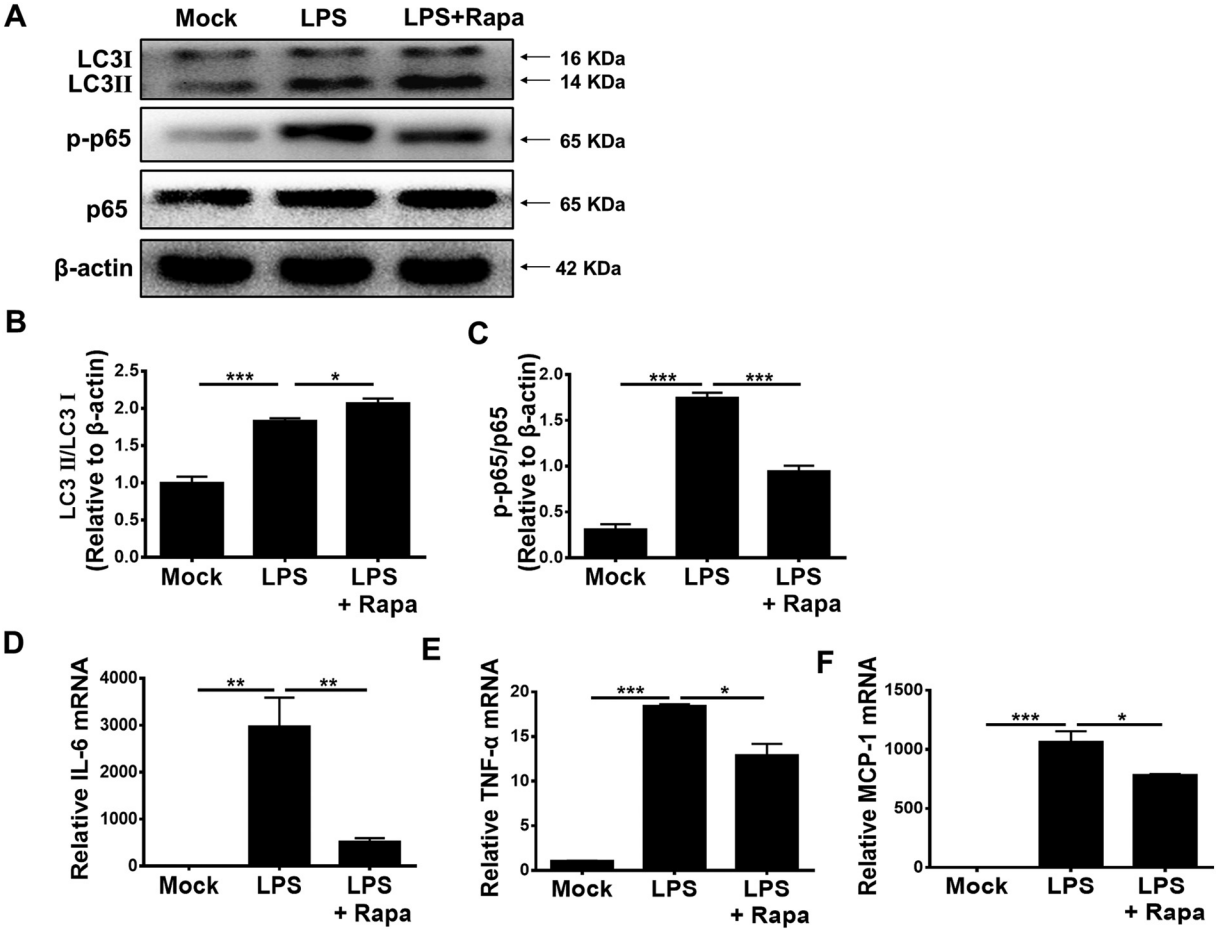

Fig. 7. The decreased IL-1β and IL-18 production was associated with autophagy and NF-κB activity *in vitro*. (A) Western blot analysis of LC3 I/II, p-p65 and p65 in cell lysates. Expression of (B) LC3-II relative to LC3-I, and (C) p-p65 relative to p65. Real-time PCR analysis of (D) IL-6, (E) TNF-α and (F) MCP-1 in cells from different treatment groups. Data are representative of three independent experiments and presented as means ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.017.

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