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Different intensity of autophagy regulate interleukin-33 to control the uncontrolled inflammation of acute lung injury

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Abstract

Objectives Cytokines participate in the progression of acute respiratory distress syndrome (ARDS), and uncontrolled inflammation is a central issue of acute lung injury (ALI). Interleukin (IL)-33 is a nuclear protein that has been reported to have a proinflammatory role in ARDS. Studies have shown that excessive autophagy may lead to the increased mortality of patients with ARDS, while several investigations indicated that IL-33 and autophagy interact with one another. The present study sought to clarify the relation between autophagy and IL-33's proinflammatory role in ARDS.

Methods We built a lipopolysaccharide (LPS)-induced lung injury mouse model. To study the relationship between IL-33 and autophagy, mice were pretreated with rapamycin (RAPA; a promoter of autophagy) and 3-methyladenine (3-MA; an inhibitor of autophagy) prior to LPS administration. The expression of IL-33 in serum and bronchoalveolar lavage fluid (BALF) was measured. Immunohistochemistry of IL-33 in lung tissue was examined. Th1,Th2 cytokines/chemokine levels in serum and BALF were tested. Further, the severity of lung injury was evaluated. And the nuclear factor-kappa B (NF-κB)'s nuclear translocation in lung tissue was detected.

Results In comparison with the control group, the levels of IL-33 in serum and BALF were increased after LPS injection. Th1 cytokines/chemokine levels were significantly increased in serum and BALF, while Th2 cytokine levels changed only a little. The levels of IL-33 in serum and BALF of the RAPA group was significantly increased after LPS was injected as compared with the LPS group; additionally, the levels of IL-33 in serum and BALF of the 3-MA group was significantly reduced after LPS was injected as compared with the LPS group, and that lung injury was ameliorated after 3-MA pretreatment. Th1 cytokines and chemokines in both serum and BALF were also decreased in the 3-MA group. Furthermore, we found that the nuclear translocation of NF-κB increased after LPS administration, and NF-κB's nuclear translocation was significantly increased in comparison with the LPS group after RAPA pretreatment. In contrast, NF-κB's nuclear translocation decreased after 3-MA pretreatment as compared with the LPS group.

Conclusions These findings showed that autophagy might regulate IL-33 by activating or inhibiting NF-κB to control the uncontrolled inflammation of acute lung injury.

Keywords Acute respiratory distress syndrome · Interleukin-33 · Inflammation · Cytokine · NF-κB

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Introduction

Acute respiratory distress syndrome (ARDS), which has the high morbidity (10%) [1] and high mortality (45–57.8%) [2, 3] in the intensive care unit, is one of the most challenging clinical conditions to manage. Sepsis, pneumonia, and many other conditions are causes of ARDS [4]. The pathogenesis of ARDS is very intricate. At present, it is generally accepted that ARDS is characterized by rapid alveolar injury, inflammation, neutrophil accumulation, and cytokine induction, with an emphasis on the mechanisms of injury occurring to the lung endothelium and the alveolar epithelium. To date, many kind of molecular mechanisms have been determined to be involved in ARDS, but the uncontrolled inflammation remains a central issue of ALI. Thus, it is urgent to elucidate the possible molecular mechanisms and to develop new therapeutic target(s) to limit excessive inflammation.

Interleukin (IL)-33 is a new member of IL-1 family that is expressed in a variety of cells, especially barrier surface cells including airway epithelial cells, gastrointestinal epithelial cells, endothelial cells, and fibroblasts [5]. It was recently identified as the ligand for the orphan IL-1 receptor-like-1 (ST2) receptor [6, 7]. Additionally, ST2 is one of the members in the IL-1 receptor (IL-1R) family belonging to the Toll-like receptor (TLR)/IL-1R (TIR) superfamily [8]. Upon tissue/cell stress or damage, IL-33 is released from epithelial and endothelial cells to trigger an immune response [9–11]. With its dual functions, IL-33 can behave as both an inflammatory cytokine and a transcription factor [12]. Current studies have confirmed that IL-33 demonstrates a proinflammatory role in chronic obstructive pulmonary disease, allergic asthma, lung fibrosis, and pneumonia [13–16]. In ARDS, several reports have indicated that IL-33 might have a proinflammatory effect [12, 17, 18]. Our group has previously found that IL-33 only plays an inflammatory-promoting role in clinical and experiment pulmonary ARDS [19] and could regulate the expression of IL-33 via inhibited high-mobility group box-1 (HMGB1) to improve lung injury [18]. Thus, the regulation of IL-33 expression can be a potential target for the therapy of ARDS [18].

Recent research has suggested that lactic acid (LA) suppresses IL-33-mediated mast cell inflammatory responses [20] and that hypoxia-inducible factor (HIF)-1α and IL-33 form a regulatory circuit to perpetuate inflammation [21]. It can be seen that the expression and loop of regulating IL-33 are diverse. Autophagy is an essential process necessary to eliminate damaged proteins and organelles' toxicity. It also plays an important role in sustaining metabolism and assisting with the survival cells in starvation scenarios [22]. Because of oxidative stress, ARDS is often associated with high autophagy [23]. One study showed that excessive autophagy may lead to the increased mortality of patients with ARDS [24]. Several other reports suggested that IL-33 and autophagy have an interaction with one another [25, 26]. In the present study, we sought to clarify whether it is possible to control the expression of IL-33 by altering the excessive autophagy in ARDS.

To elucidate the assumption about IL-33 and excessive autophagy, we established an LPS-airway instillation-induced ALI model, used rapamycin (RAPA) and 3-methyladenine (3-MA) to change the intensity of excessive autophagy, detected and analyzed the changes of IL-33, and then measured the other inflammatory factors. Meanwhile, we detected nuclear factor-kappa B (NF-κB) after pretreatment by RAPA and 3-MA so as to determine the correlations with IL-33.

Materials and methods

Study population

Seventeen adult patients with pulmonary ARDS were recruited from the ICU of the First Affiliated Hospital of Chongqing Medical University from July 2018 to September 2018. ARDS diagnosis was based on the Berlin standard. Study patients were admitted to the ICU while in the acute phase of the disease (onset within 24 h). Patients with massive transfusion or hemofiltration within the preceding 24 h, immunosuppressive or immune-enhancing therapy, or chronic lung diseases were excluded. Control samples were obtained from healthy donors ($n = 15$). The study protocol was approved by the Clinical Research Ethics Committee of the University and informed consent was obtained from all participants.

Human serum cytokine measurements

The levels of IL-33 in the serum of ARDS patients were measured by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA).And the level of IL-1β, IL-2, IL-6, IL-10 and TNF-α in the serum sent to the first affiliated hospital of Chongqing Medical University clinical testing center for determination.

Mice

We purchased C57BL/6 male mice (aged 6–8 weeks) from the Laboratory Animal Center of Chongqing Medical University in Chongqing, China and placed them into a specific pathogen-free environment. Fifty mice were randomized into 10 different groups: 5 mice in the control group; 15 mice in three different time point (6 h, 12 h, 24 h) LPS administration groups; 15 mice in three different time point (6 h, 12 h, 24 h) LPS administration with RAPA preprocessed groups; and 15 mice in three different time point (6 h, 12 h, 24 h) LPS administration with 3-MA preprocessed groups. All environment conditions were set according to the national standards. All experiments were done in accordance with the guidelines of the Institutional Animal Care and Use Committee at Chongqing Medical University.

Animal model of LPS-induced acute lung injury

We screened mice with similar weights, anesthetized each with an intraperitoneal injection of chloral hydrate (3.5%), and gave each mouse about 50 µg of LPS (055:B5: 1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) [19]. Part of each mouse was pretreated with rapamycin or 3-MA a half-hour before LPS injection. The mice were injected with anesthetic until death, at which point we obtained blood, bronchoalveolar lavage fluid (BALF), and lung tissue. We subsequently centrifuged the blood (coagulated for 1 h at room temperature) and BALF at 1500×*g* at 4 °C for 10 min. All serum and BALF were frozen at − 80 °C.

Treatment by RAPA and 3-MA

The experimental mice was treated intraperitoneally with 4 mg/kg of RAPA [27] [dimethylsulfoxide (DMSO)-dissolved; Sigma, USA] and 15 mg/kg 3-MA [28] (PBS-dissolved; Sigma, USA) one half-hour prior to intratracheal administration of LPS. The mice were killed by anesthesia at 6 h, 12 h, and 24 h. Blood, BALF, and lung tissues were collected for further analysis.

Histopathology

The right lungs of the mice were fixed in formalin for 24 h and then sent to the tissue embryo laboratory, where each lung sample was cut in 4 µm thick sections. The lung sample sections were subsequently stained with hematoxylin and eosin. Lung injury scores were estimated by the method of Mikawa, with a higher score indicating a more severe injury.

Mice serum and BALF cytokines measurement

The levels of IL-33 in the serum and BALF were measured by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA), while the levels of CXCL-1, interferon (INF)-γ, tumor necrosis factor (TNF)-α, IL-10, IL-13, IL-17A, IL-1β, IL-27, IL-4, IL-6, and CXCL-5 in the serum and BALF were measured by multifactor detection kit.

Western blot

Western blot analysis was performed as described previously. In brief, lung tissues were homogenized in the presence of a protease inhibitor and phosphatase inhibitor and then protein concentrations were detected using a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). Tissue lysates were boiled at 98 °C for 15 min in lithium dodecyl sulfate (LDS) buffer (Beyotime, Shanghai, China), and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before blotting onto a polyvinylidene difluoride membrane. After blocking for 1 h, the membranes were then incubated overnight at 4 °C with an anti-P62 antibody (diluted at 1:2000), anti-LC3 antibody (diluted at 1:1500), anti-p-P65 antibody (diluted at 1:500), or β-actin antibody (diluted at 1:1000). The densitometric analyses of bands were performed by the use of an Imager system (BIO-RAD). All experiments were repeated three times using different samples.

Immunohistochemistry

Xylene was used to dewax the lung sections, which were then put on gradient alcohol to rehydration. Then, 3% H_2O_2 was used to block the endogenous peroxidase activity for 10 min, while 0.3% Triton X-100 (50 µL) was applied for 15 min to rupture the cell membrane. Subsequently, the sections were incubated in normal goat serum. Goat anti-mouse IL-33 polyclonal antibody was then added (15 µg/mL dilution, R&D Systems, Minneapolis, MN, USA) on the section and incubation was performed overnight at 4 °C in a humid chamber. The sections were washed and streptavidin–peroxidase complex was added (Zhongshan, Beijing, China). Diaminobenzidine (DAB) was used to prompt the IL-33 to be visualized, while hematoxylin was used to dye the cell nucleus. Dehydration with gradient alcohol was completed and the section in was placed xylene for 10 min for differentiation, after which point, neutral gum sealing of the piece was performed.

Quantitative real-time PCR

TRIzol Reagent (Takara, Dalian, China) was used to isolate lung tissues' total RNA. The Prime Script RT Reagent kit was employed to construct first-stand complementary DNA (cDNA)-isolated total RNA. The cDNA were used as templates to run for polymerase chain reaction (RCR). SYBR Premix Ix Taq II (Takara, Dalian, China) was used to quantify the target gene in the Bio-Rad CFX-96 Real-Time System (Bio-Rad, Hercules, CA, USA). The following were our primers: LC3, forward 5′ AAGACCAAGTTCCTGGTGCC 3′ and reverse 5′ CCAGGAGGAAGAAGGCTTGG 3′; NF-κB, forward 5′ CGACGTATTGCTGTGCCTTC 3′ and reverse 5′ TGAGATCTGCCCAGGTGGTAA 3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′ AGGCCGGTGCTGAGTATGTC 3′ and reverse 5′ TGCCTG CTTCACCACCTTCT 3′. First, for 30 s at 95 °C, cDNA was kept in a despiralization state, which was then followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. GAPDH was used as an internal control for normalization. The wild-type mice were used to represent the baseline value.

Immunofluorescence

The lung frozen sections were recovered by PBS, 0.3% Triton X-100 (50 μL) was administered for 15 min to rupture cell membranes, sodium citrate solution was used to do antigen retrieval, and then normal goat serum was used to incubate the section. Rabbit antimouse NF-κB polyclonal antibody (15 μg/mL dilution; Sigma, USA) were added to the section and incubation was performed overnight at 4 °C in a humid chamber. The section was subsequently kept for 1 h at room temperature and the goat anti-rabbit antibody, which contained fluorescein isothiocyanate (FITC), was added for 1 h at room temperature. 4′,6-Diamidino-2-phenylindole (DAPI) was used to dye the cell nuclei. Neutral gum sealing was performed.

Statistical analyses

All data are represented in the format of mean ± standard error of the mean. A Student's *t* test or two-way analysis of variance followed by an LSD multiple comparison test was used for data analysis. SPSS 19.0 (IBM Corp., Armonk, NY, USA) was used for the statistical analyses and $P < 0.05$ was considered to be statistically significant.

Result

Concentration of IL-33 in patients with pulmonary ARDS and relationship between serum IL-33 and other cytokines in ARDS

We first compared the concentrations of serum IL-33 in healthy controls and pulmonary ARDS patients. The serum IL-33 levels in patients were significantly higher than those in controls(Fig. 1a, $P < 0.01$). In addition, as others have reported a variety of inflammatory mediators involved in initiating, amplifying, and maintaining inflammation during ARDS, we therefore measured serum indice including TNF-α, IL-1β, IL-2, IL-6 and IL-10,to determine their correlation with IL-33.The level of IL-33 in serum was positively and significantly correlated with TNF-α, IL-1β, IL-6 and IL-2 (Fig. 1b–e, $P < 0.01$), but negatively correlated with IL-10 during ARDS(Fig. 1f, $P < 0.01$).

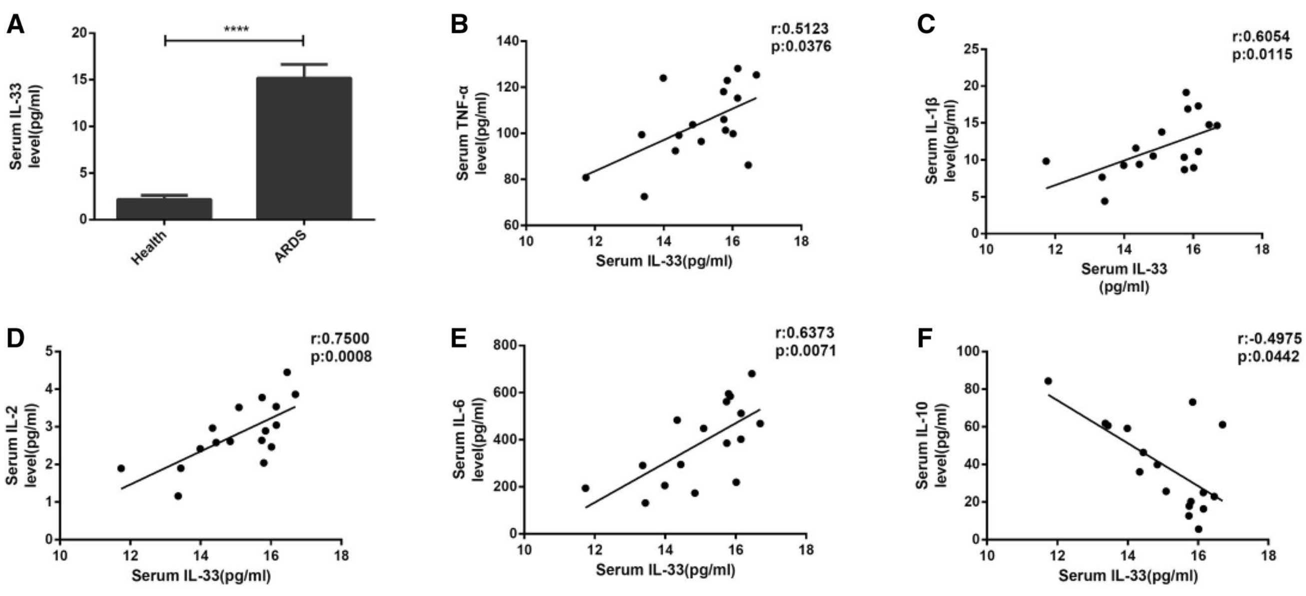


Fig. 1 IL-33 was elevated in pulmonary ARDS patients compared to health people and was correlated with immune mediators but negatively associated with IL-10. **a** IL-35 from healthy donors and pulmonary ARDS patients. Each sample was tested in duplicate and concentrations were determined from a standard curve (****$P < 0.0001$). **b**–**f** IL-33 was positively and significantly correlated with immune mediators, but negatively associated with IL-10 when compared between healthy people and ARDS patients

Effects of RAPA or 3-MA on changes of histopathology in ALI models

As compared with control group, lung injury was much more serious in the LPS group, congestion and hemorrhage of alveoli got much heavy after was LPS injected, while the alveolar walls were thick and inflammation cell infiltration was present in the pulmonary alveolus and mesenchyme. After RAPA pretreatment, lung inflammation infiltrate increased and the alveoli congestion and hemorrhage became much more serious as compared with in the LPS group, with the lung injury score rising significantly. The lung inflammation infiltrate decreased, the alveoli congestion and hemorrhage lessened, and the lung injury score was smaller after 3-MA pretreatment than in the LPS group (Fig. 2a, b).The level of IL-33 on BALF and serum was measured by ELISA kit. The results showed that IL-33's level on the serum and BALF was changed in the LPS, RAPA, and 3-MA groups. After RAPA pretreatment, IL-33's level in the serum and BALF was increased after LPS was injected as compared with the LPS group and decreased with 3-MA pretreatment (Fig. 2c, d). Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) data showed that IL-33 messenger RNA (mRNA) in the lungs increased after LPS which compared with the control group, but after pretreatment with 3-MA its level was significant decreased. Meanwhile, IL-33 mRNA in the lungs increased much more than LPS group which treat with RAPA (Fig. 2e).We also examined the IL-33 protein levels in the lung tissues after LPS administration and treatment with anti-IL-33 antibody by immunohistochemistry staining. The results showed that the IL-33 detected by immunohistochemistry was significantly increased in the lung tissues after LPS administration in comparison with in the control mice. IL-33 protein levels in lung tissues were significantly increased following RAPA pretreatment, but decreased following 3-MA pretreatment (Fig. 2f, g).

The levels of the other cytokines and chemokines after the pretreatment of RAPA or 3-MA in ALI models

Concentrations of cytokines and chemokines (including CXCL-1, CXCL-5,TNF-α, IL-10, IL-17A, IL-6, IL-13 and IL-4) in the serum and BALF of mice before LPS were compared between the mice pretreated with either RAPA or 3-MA in ARDS models. In the ARDS models, promoter of autophagy RAPA upregulated the production of proinflammatory cytokines include CXCL-1, CXCL-5,TNF-α, IL-10, IL-17A and inhibitor of autophagy 3-MA can downregulate the above proinflammatory cytokines during LPS-induced ARDS models, However, they have no significant effect on cytokines such as IL-4 and IL-13 (Fig. 3).

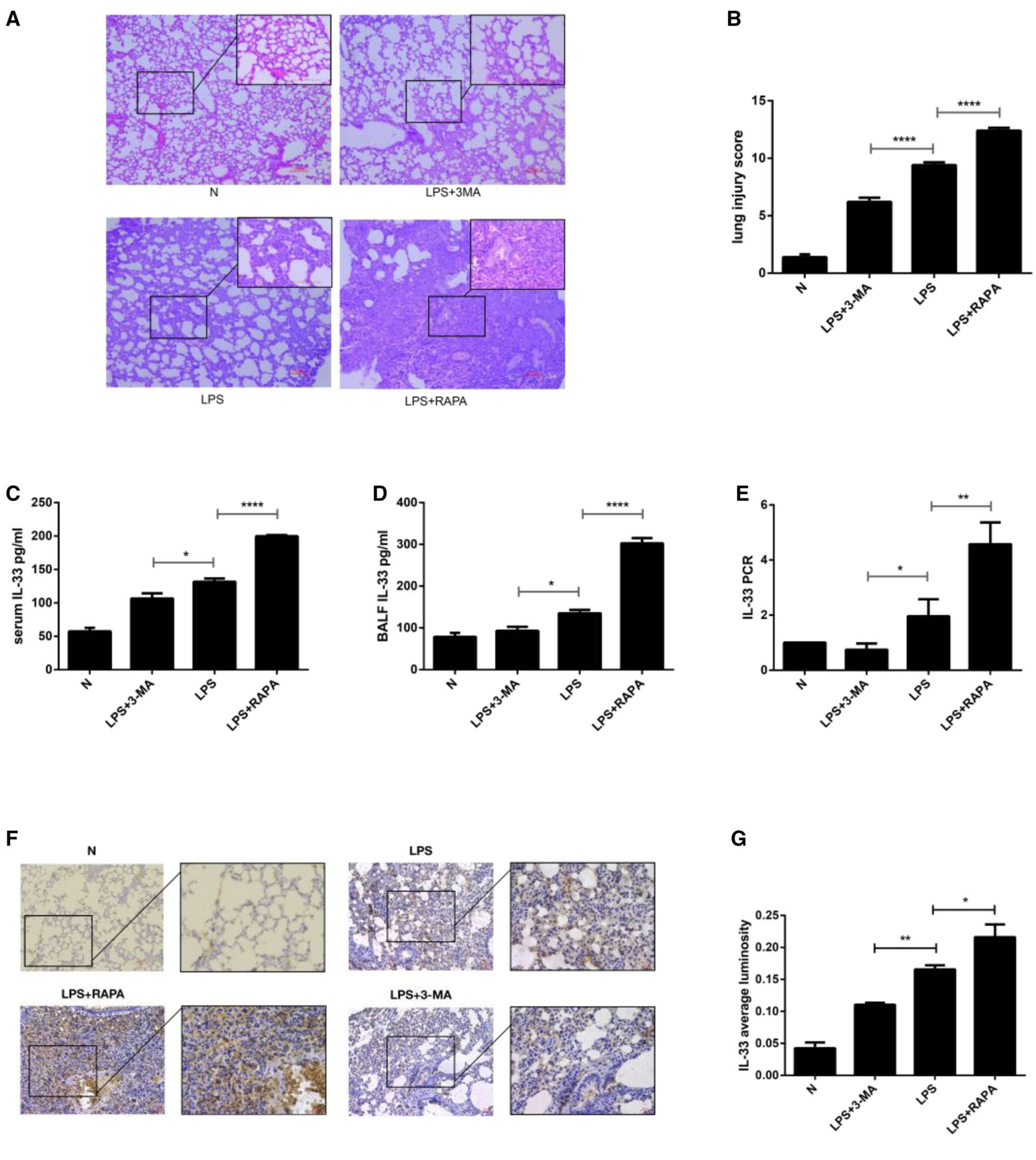
LC3II/LC3I and *P62* protein levels in lung were different and the nuclear translocation of NF-κB was changed after the pretreatment of RAPA or 3-MA in ALI models

Western blot data show that *LC3II/LC3I* and *P62* did what was expected in LPS-intranasal instillation ARDS. The ratio of *LC3II/LC3I* in the lung increased after LPS administration. As compared with in the LPS group, The ratio of *LC3II/LC3I* was higher in lung tissue after RAPA treatment, but lower after 3-MA treatment. The expression of *P62* in lung tissue is contrary to the above results (Fig. 4a–c). Furthermore we measured the nuclear translocation of NF-κB by immunofluorescence. NF-κB's nuclear translocation was increased after LPS administration. After RAPA pretreatment, NF-κB's nuclear translocation was increased as compared with LPS group. In contrast, NF-κB's nuclear translocation was decreased after 3-MA pretreatment as compared with that of the LPS group (Fig. 4d, e). Next we further measured the expression of p-P65 in the lung tissue using western blot. The results showed that, after RAPA pretreatment, p-P65 protein expression was higher, but was lower after 3-MA pretreatment as compared with the LPS group (Fig. 4f, g).

Discussion

Since the first description of ARDS in 1967, considerable progress has been made in understanding the pathophysiology and pathogenesis of the syndrome [29–31]. Uncontrolled inflammation plays a central role in ALI, and understanding how this is regulated is important for the development of new therapies to limit excessive inflammation. The latest view is that inflammation of ARDS should be looked as a complex system, which is controlled by scale-free networks of genome-wide molecular interaction with hubs (e.g., NF-κB, C/EBPβ, and ATF3) [32]. Additionally, several regulatory circuits, including the resolution of inflammation, the inflammasome, apoptosis, and autophagy, play a complex role during pulmonary inflammation [33].

Autophagy is an essential process to eliminate damaged proteins and organelles' toxicity. It is associated with inflammatory diseases and organ dysfunction. Both the concerted activation of Nrf2 [34] and regulation by parkin [35] represent ways in which autophagy regulates the inflammatory response in ALI. Additionally, excessive autophagy may stimulate the strong inflammatory response observed in the development of LPS-induced ALI [36]. Regulating autophagy can be seen as another preventive and therapeutic intervention to ameliorate ALI/ARDS. However, if the damage is too serious, autophagy may become another damage factor leading to cell death [37]. In this report, we found



that the pathological changes of the RAPA group seen in the lungs, including increased congestion and hemorrhage, inflammation cell infiltration, and alveolar wall thickness, were heavier than in the LPS group (Fig. 1c). RAPA, which was a pharmacological drug developed from filamentous bacteria [38], can regulate inflammatory factor (e.g., IFN-γ, IL-4) expression and modulate the interaction between gδ T-cells and dendritic cells (DCs) in ALI [39]. On the other hand, because of the ability to sense metabolic, nutrient, and hormonal signals, the mammalian target of RAPA (mTOR) signaling pathway plays an important role in transmitting autophagic stimuli [40]. As such, RAPA, an autophagy activator, can upregulate the autophagy in cells and accelerate disease progression [41]. Since RAPA may be linked with lung toxicity [42], we examined the ratio of *LC3II*/*LC3I* and *P62* protein to demonstrate the autophagy as activated

◂**Fig. 2** Pretreatment with RAPA or 3-MA in LPS-induced ARDS. IL-33 expression differed in lungs, serum and BALF of different groups at 24 h. Lungs from each experimental group at 24 h were processed for histological examination after haematoxylin and eosin staining. C57BL/5 mice (5/group) were subjected to LPS, RAPA or 3-MA group. **a**, **c** Were representative dot plots shown. **a**, **b** Lung injury in RAPA groups showed thickened alveolar wall, alveolar hemorrhage, (and) collapse, inflammatory cell in filtration and lung cores were more severe than in the LPS groups. And lung injury in 3-MA groups showed thickened alveolar wall, alveolar hemorrhage and collapse, inflammatory cell in filtration and lung cores were less severe than in the 3-MA groups. **c**, **d** IL-33 in serum and BALF using ELISA. After RAPA pretreatment its level in the serum and BALF were increased after LPS injected compared with LPS group. After 3-MA pretreatment, the level of IL-33 in the serum and BALF were significantly less than the LPS group. **e** IL-33 mRNA levels in the lungs were measured with qRT-PCR. Relative expression levels of the genes were expressed with the GAPDH housekeeping gene as an internal reference. **f**, **g** Immunohistochemistry assessment and colorimetric detection with DAN (brown stain) was used to determine the expression of IL-33 in lung tissues at 24 h after pretreatment with LPS, LPS + RAPA and LPS + 3-MA. The figure is presented at a magnification of ×200 and ×400. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, by the one-way ANOVA followed by LSD multiple comparisons test, compared with mice treated with LPS group

by RAPA (Fig. 4) in the same period. In contrast, research has shown that autophagy inhibitor 3-MA protects against endothelial cell barrier dysfunction in ALI [43]. We found that the 3-MA group had a lower degree of lung injury at 24 h as compared with the LPS group (Fig. 1a, b), accompanied by the reduction of autophagy (Fig. 4). It prompted the idea that excessive autophagy cannot bring a benefit outcome to ALI.

Cytokines of the IL-1 family (e.g., IL-1α, IL-1β, IL-18, IL-36, and IL-38) play major roles in inflammatory, infectious, and autoimmune diseases [44–49]. IL-33 is a chromatin-associated nuclear cytokine from the IL-1 family. The IL-33/ST2 axis plays an important role, which mediates pulmonary inflammation and immune system-related disorders [17, 50]. The neutrophil recruitment, proapoptotic, and inflammatory effects of IL-33 could regulate the development of lung injury [12, 50, 51]. Recently, IL-33 was found to have an inflammatory-promoting effect in pulmonary ARDS/ALI [12, 18, 19]. So, IL-33 has the potential to become a new target for the treatment of ALI. Another study showed that IL-33 plays a role in tissue integrity and aging through the regulation of autophagy [25]. Additionally, it provides potential neuroprotection through suppressing autophagy and NF-κB-mediated inflammatory pathways [52]. At this point, whether autophagy may also be a factor to regulate IL-33 expression in ALI is a significant question. Our study showed that after the treatment of LPS was an key point regarding the changing of IL-33 expression in the serum and BALF. As compared with in the LPS group, the levels of IL-33 (serum and BALF) were increased in RAPA group but decreased in 3-MA group (Fig. 2a, b). However, the IL-33 in the lung tissue as measured by immunohistochemistry was higher in the RAPA group than in the 3-MA group. Epithelial cells were seen as a source of alarming cytokines [53]. IL-33 is a cytokine that is released from epithelial and endothelial cells at barrier surfaces to operate as an alarmin upon tissue stress or damage [11, 54–56]. It was shown to play a role in the initiation of Th2 differentiation and in the maintenance and restimulation of Th2 memory cells [53, 57]. Changes of autophagy caused by RAPA or 3-MA elicited the “alarmin” of IL-33. Subsequently, such caused the changes of other cytokines and chemokines (Fig. 3). However, excessive autophagy caused severe damage to the effector cells. Thus, cytokines and chemokines, except for CXCL5, gradually decline over time. Since this ALI model was established by LPS tracheal instillation, CXCL5 plays a role in chemotaxis and the activation of neutrophils [58, 59]. The role of 3-MA pretreatment has been previously considered. The corresponding result is the delay of uncontrolled inflammation, which then continued to stimulate the production of IL-33. Our results suggested that the early intensity of autophagy can affect the extent of uncontrolled inflammation and lung injury in ALI by altering IL-33 expression.

NF-κB is a protein complex that controls the transcription of DNA, cytokine production, and cell survival. It is activated in the lungs [60] and plays a key role in regulating the immune response to inflammation in ARDS. Because binding sequences for NF-κB are present in the promoter/enhancer sequences of multiple proinflammatory cytokines, the activation of NF-κB contributes to the increased expression of multiple cytokines in ARDS [61, 62]. Downregulation of the expression of NF-κB can lead to a decrease in Beclin-1 transcription and inhibition of autophagy [36]. However, autophagy can regulate injury, repair, and regeneration of tissue by suppressing NF-κB activation in the inflammatory environment [63, 64]. In our study, the nuclear translocation of NF-κB (p65) occurred by excessive autophagy (RAPA group) and was inhibited by reducing autophagy (3-MA group). However, this does not exactly match the change of IL-33 expression. As such, these findings mean that the regulatory role of autophagy on IL-33 is in part a result of NF-κB-mediated inflammatory pathways.

In summary, the interaction between autophagy and IL-33 is a complex network. The relationship between them is different in diverse diseases [52] or different phases of inflammation. This report can prove that too strong or too weak autophagy in ALI/ARDS can affect the expression of IL-33,

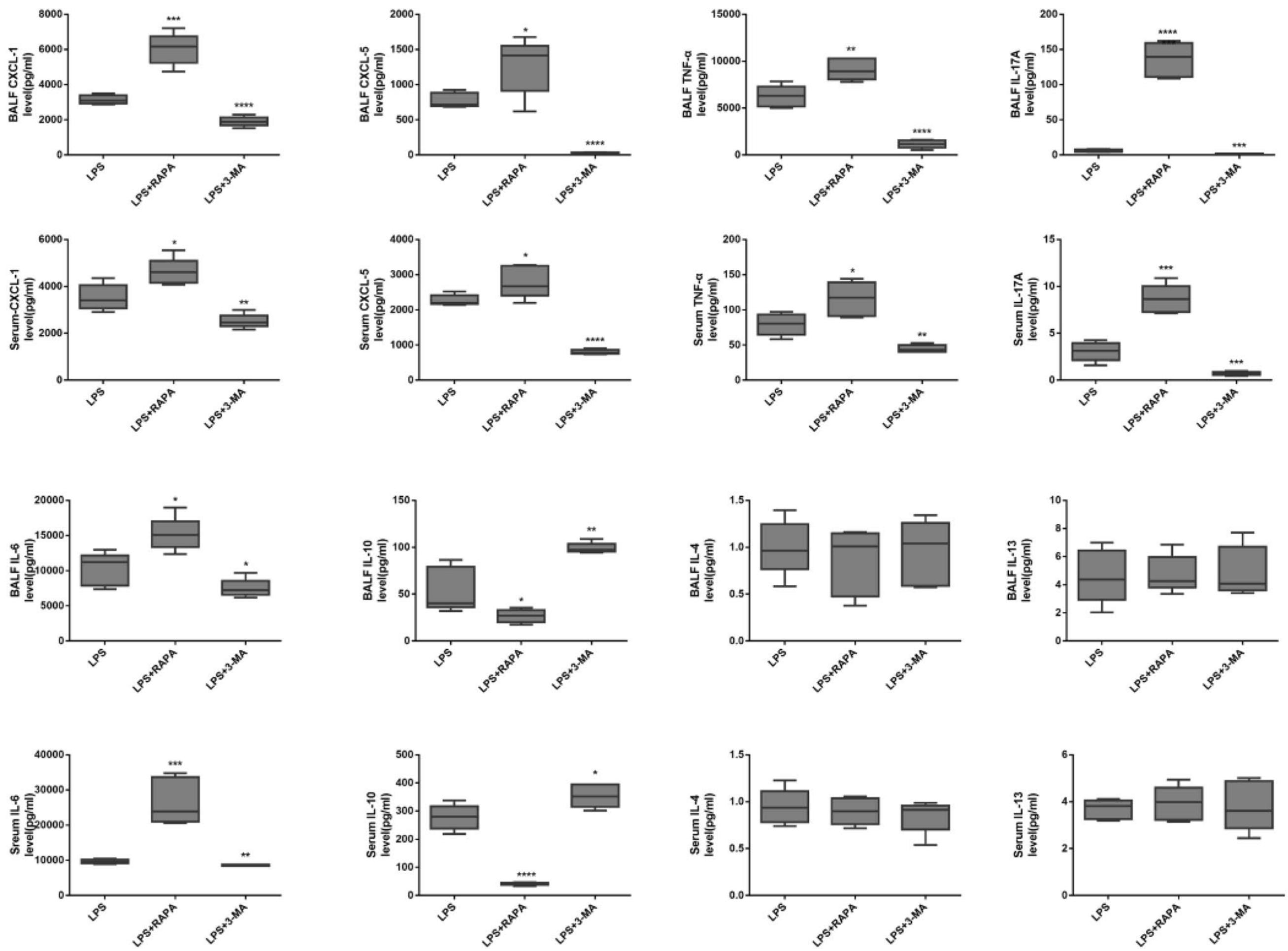


Fig. 3 Promoter of autophagy RAPA upregulated the production of proinflammatory cytokines and inhibitor of autophagy 3-MA downregulated proinflammatory cytokines during LPS-induced ARDS models, However, they have no significant effect on cytokines such as IL-4 and IL-13.Cytokine and chemokine concentrations in bronchoalveolar lavage fluid (BALF), and blood specimens from five mice treated with or without RAPA or 3-MA were determined by mice cytokine/chemokine magnetic bead panel kit assays 24 h after LPS-induced ARDS. $*P<0.05$, $**\ P<0.01$, $***P<0.001$, $****P<0.0001$, by the one-way ANOVA followed by LSD multiple comparisons test, compared with mice treated with LPS group. ARDS featured increased CXCL-1, CXCL-5, TNF-α, IL-6 and IL-17A and reduced IL-10 after giving autophagy enhancer RAPA in BALF and serum. And after the administration of the autophagy inhibitor 3-MA, the above cytokines decreased significantly

uncontrolled inflammation, and lung injury, which may be related to cell necrosis (caused by excessive autophagy) or clear obstacles of necrotic cells (caused by insufficiency of autophagy).

Conclusion

The autophagy status and the expression of IL-33 have corresponding changes after the pretreatment RAPA and 3-MA in ALI. Furthermore, excessive autophagy can aggravate the severity degree of lung injury and uncontrolled inflammation related with high IL-33 expression due to active NF-κB.

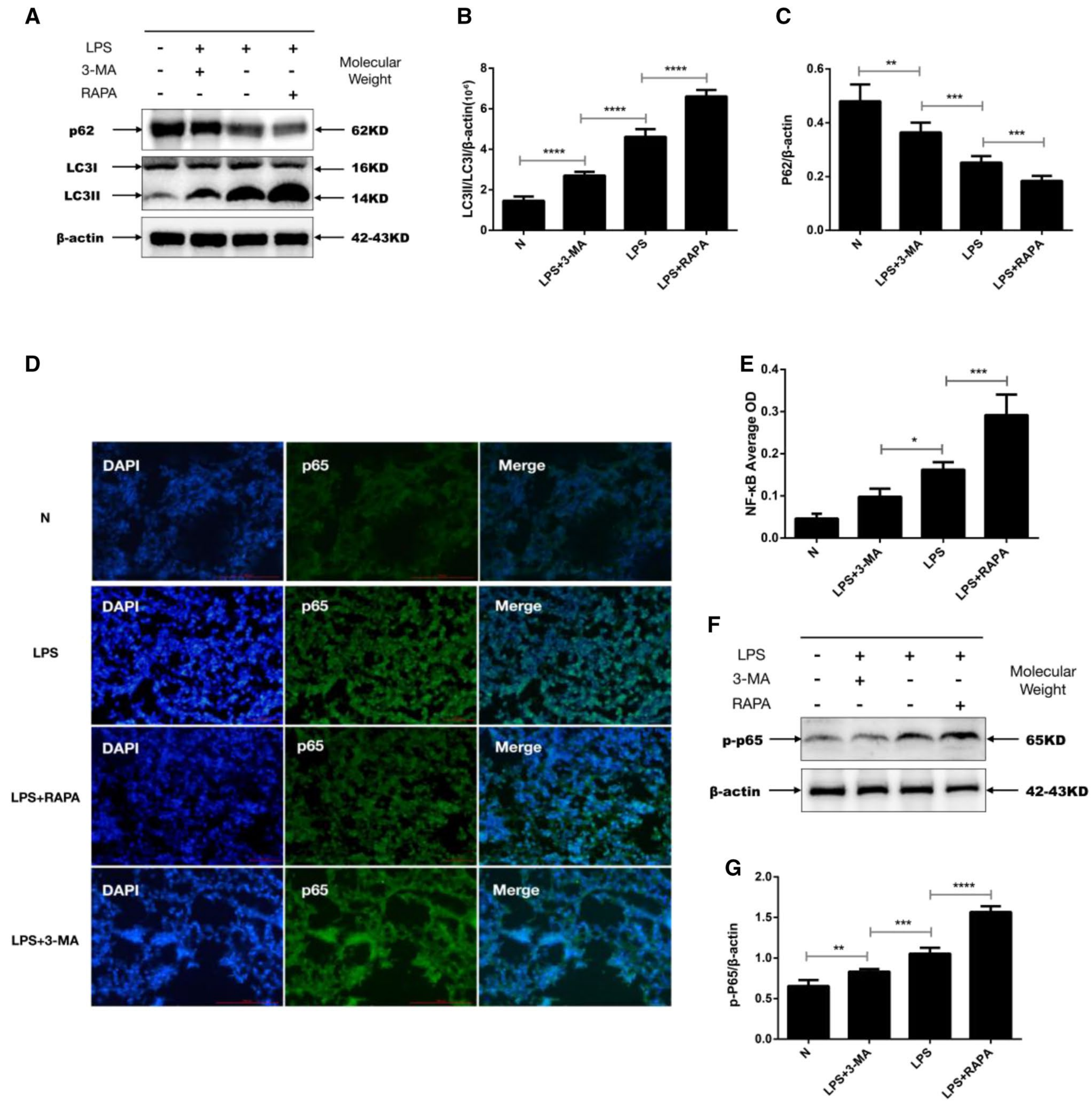


Fig. 4 The expression of *LC3II/LC3I,P62* protein and NF-κB in lung were different after the pretreatment of RAPA or 3-MA pretreatment in ARDS models. *LC3II/LC3I,P62* and *p-P65* protein expression in lung were determined by western blot and NF-κB expression was determined by immunofluorescence at 24-h point. C57BL/5 mice (5/group) were subjected to LPS, RAPA or 3-MA group. **a**, **d**, **f** Were representative dot plots shown. **b** The ratio of *LC3II/LC3I* was significantly higher than LPS group after RAPA treatment, but lower after 3-MA treatment. **c** P62 protein expression was significantly higher than LPS group after 3-MA treatment, but lower after RAPA treatment. **d**–**g** Immunofluorescence and *p-P65* protein detection display that after RAPA pretreatment NF-κB expression in lung tissue is higher, but is lower after 3-MA pretreatment than LPS group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, by the one-way ANOVA followed by LSD multiple comparisons test, compared with mice treated with LPS group

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