The Effect and Mechanism of Lipoxin A4 on Neutrophil Function in LPS-induced lung injury

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Abstract

Excessive inflammatory response caused by infiltration of a large number of neutrophils is one of the important features of acute lung injury(ALI)/acute respiratory distress syndrome (ARDS). Lipoxin A4 (LXA4) is an important endogenous mediator in the process of inflammation resolution, which has a strong role in promoting inflammation resolution. In this study, we examined the impact of LXA4 on the pulmonary inflmmatory response and the neutrophil function in a rat model of ARDS. Our results indicated that Exogenous administration of LXA4 could reduce the degree of lung injury in ARDS rats and inhibit the release of pro-inflammatory factors TNF- α and IL-1β in lung tissue homogenate. However, LXA4 has no lung protective effect on ARDS rats with neutrophil depletion in rats, nor can it inhibit the levels of pro-inflammatory factors TNF-α and IL-1β in lung tissue homogenate. LXA4 can inhibit the production of reactive oxygen species (ROS) and NETs in peripheral blood neutrophils of ARDS rats. At the same time, LXA4 can promote the phagocytosis of neutrophils in ARDS rats in vitro, and can also promote the apoptosis of neutrophils in ARDS rats and reduce neutrophil NETosis. In addition, the effect of LXA4 on the function of peripheral blood neutrophils in ARDS rats is mediated by its receptor ALX. LXA4 can inhibit the release of NE and MPO from neutrophils, thereby inhibiting the production of NETs, and the effect of LXA4 can be comparable to NEi and MPO inhibitors. In summary, these findings indicate that LXA4 has a protective effect on LPS-induced ARDS rats by affecting the function of neutrophils.

Summary sentence

LXA4 has protective effect on LPS induced ARDS rats by affecting the function of neutrophils.

1 Introduction

ARDS is a common clinical emergency. In recent years, although the research on the pathogenesis of ARDS has made great progress, there is still a lack of specific treatment. Therefore, it is urgent to study the pathogenesis and treatment of ARDS [1–4].

Neutrophils are the most abundant white blood cells in human circulation, participate in a variety of immune and inflammatory processes, and play a vital role in the process of immune defense against pathogenic microorganisms. When the body is invaded by foreign microorganisms, neutrophils are first recruited to the inflammatory injury site to play a defense function. Phagocytosis, degranulation and the formation of neutrophil extracellular traps (NETs) are three strategies to play the role of anti- infection.

In the process of ARDS disease, activated neutrophils play an important role in the clearance of ARDS inflammation by gathering from the peripheral circulation to the lung tissue, and eliminating potential harmful stimuli in ARDS patients [5]. However, neutrophils are also highly histotoxic cells. Excessive accumulation of neutrophils in pulmonary microcirculation, pulmonary interstitium and alveolar spaces in ARDS patients will cause neutrophil-mediated tissue damage [6]. Therefore, how to effectively promote the

antimicrobial activity of neutrophils and accelerate the resolution of dead neutrophils has always been our concern.

LXA4 is an important endogenous anti-inflammatory mediator produced in the process of inflammatory resolution, which can inhibit the recruitment of inflammatory cells, regulate the balance of pro-inflammatory / anti-inflammatory factors, limit inflammatory injury, and prevent injured tissue fibrosis [7–9]. The unique role of promoting timely apoptosis of neutrophils and improving the phagocytosis of macrophages to neutrophils to promote the timely regression of inflammation is regarded as the "stop signal" and "brake signal" of inflammatory response [10–14].

In this study, we used the ARDS rat model to further verify that LXA4 can promote the resolution of inflammation in ARDS rats by affecting the function of neutrophils.

2 Materials And Methods

2.1 Reagents

LXA4(Cayman Chemical Company), LPS (Escherichia coli serotype 055:B5), formyl methionyl leucyl phenylalanine (fMLP), interleukin-8 (IL-8), phorbol ester (PMA), cell chromatography C (Cytochrome C), superoxide dismutase (superoxide dismutase, SOD), Elastase, Hydroxyethylpiperazine Ethylsulfonic Acid (HEPES) and Emodin were obtained from Sigma-Aldrich (St Louis, MO, USA). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) ELISA kits were obtained from R&D Systems (Minneapolis, MN). MNase, RP-1 antibody (BD 550002), Anti-Myeloperoxidase antibody (abcam ab65871), SYTOX Green, and Annexin V-FITC were obtained from eBioscience (San Diego, CA). pHrodo Red E. coli (Cat.No.4615), pHrodo Green S.aureus (Cat. No. 4620) were obtained from Sartorius (Göttingen, Germany). RPMI 1640, fetal bovine serum (FBS), trypsin, and enzyme-free cell dissociation buffer were purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin in saline citrate buffer were from Invitrogen (Carlsbad, CA, USA). Other chemical reagents are of analytical grade.

2.2 Establishment of ALI rat model

Experiments were performed on adult male Sprague Dawley rats (250–300 g; Shanghai Experimental Animal Center of China). Rats were provided with water and food ad libitum. The use of animals in this study was approved by the Animal Studies Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Rats were weighed, given LPS (14 mg / kg) via tail vein for 6 h, intraperitoneally injected with 2% Pentobarbital Sodium (50 mg / kg) for anesthesia, fixed in supine position, disinfected with 70% alcohol at neck, haircut, skin incision, tracheal separation, intubation and small animal ventilator. Airway pressure was maintained at 8 – 10 cmH2O, respiratory rate was 35 – 45 BPM, positive end respiratory pressure was 1-2 cmH2O (1 cmH2O = 0.098 kPa) and respiratory ratio was 1:4. Mechanical ventilation for 1 h, 40% oxygen inhalation.

Criteria for successful modeling: Increased respiratory rate and respiratory distress. Cyanosis of the skin and mucous membrane of the lips. Upright hair and reduced activity. Visual observation of lung tissue swelling and bleeding spots on the surface after chest opening. PaO2 decreased.

Exclusion criteria: Although the rats have respiratory distress and other symptoms after the model is successfully prepared, rats with one of the following conditions are not included in this research: After opening the chest, the lung tissue is visually observed without congestion or swelling. Lung tissue section HE staining showed no pathological changes such as alveolar edema, structural damage, etc. Death before the observation time point. The death of rats in each experimental group due to various reasons should be supplemented by the principle of random sampling.

Rats were randomized into four groups (n=6): Control group(the same volume of saline was injected into caudal vein), LPS group(rats received LPS (14 mg/kg) through the tail vein. LPS was dissolved in 0.9% normal saline), LXA4 group(rats received LXA4 (200 ng/kg) via the tail vein injection 6 h after saline. LXA4 was dissolved in PBS) and LPS+ LXA4 group(rats received LXA4 (200 ng/kg) via the tail vein injection 6 h after LPS exposure. LXA4 was dissolved in PBS).

2.3 Pathological studies

Rats were anesthetized with chloral hydrate (7 ml/kg, intraperitoneally), intubated and connected to the animal ventilator (respiratory parameters are tidal volume: 10ml / kg and respiratory rate: 40-60 bpm) 6 hours after injection of LPS. After anesthesia and mechanical ventilation with pure oxygen for 1 hour, rats were killed by cutting off the abdominal aorta and bloodletting. Rats were subjected to thoracotomy and PBS (25 ml/min) was injected into the right ventricle to flush the pulmonary vessels. Finally, the right lower lung lobe of rats was cut and fixed in 4% paraformaldehyde for 24 hours at room temperature, and 4 μm sections were embedded in paraffin and stained with hematoxylin and eosin (H&E) for light microscopy analysis. The rest of the lung tissue was frozen in liquid nitrogen for 48 hours and stored in refrigerator at-80 ℃.

A semi-quantitative scoring system was adopted to evaluate lung injury, which included alveolar congestion, alveolar hemorrhaging, neutrophil infiltration or aggregation in the airspace or vessel wall, and alveolar wall/hyaline membrane thickness and inflammatory cell infiltration. The grading scale for the light microscopy pathologic findings was as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). The results were graded from 0 to 4 for each item, as described previously. The four variables were summed to represent the lung injury score (total score: 0–16).

2.4 Determination of inflammatory cytokines in lung homogenate by enzyme-linked immunosorbent assay (ELISA)

Part of the right lung from individual rats was homogenized and centrifuged, and the levels of TNF-α and IL-1βin the resulting tissue supernatants were determined using TNF-α and IL-1β ELISA kits.

After ultrasonic lysis of lung homogenate, the supernatant was obtained by centrifugation at 4 ℃ for 5000 r/min for 15 minutes. Follow the reagent instructions. 100 μL of standard or sample to be tested was added to each hole, and the reaction plate was fully mixed and placed at 37 ℃ for 30 minutes. Wash the reaction plate fully with washing solution for 4 times and print it on the filter paper for 6 times. 100 μL of enzyme-labeled antibody working solution was added to each well. Put the reaction plate at 37 ℃ for 30 minutes. The washing board is the same as before. 100 μL of substrate working solution was added to each hole and reacted in the dark at 37 ℃ for 15 minutes. Add 100 μL terminating liquid to each hole and mix well. The absorbance value of 450nm was measured by enzyme labeling instrument within 30 minutes.

2.5 Separation and of rat neutrophil

20 mL of heparinized fresh rat blood was treated with dextran to induce sedimentation of the red blood cells. Prepare a Percol gradient in a 15 mL Falcon Tube by first pipetting 5 mL 56% Percol, then put the sucker to the bottom of the tube, and slowly pipetting 2.5 mL 80% Percol to the bottom. Then carefully draw up the plasma and white blood cell suspension from the blood sample with a pipette and slowly layer them on top of the Percol gradient.4 ℃, 220g, centrifuge for 20 minutes, accelerate to 1, decelerate to 0, remove the top layer of serum, suck out the neutrophil layer, add PBS to wash twice, The cells were resuscitated with RPMI-1640 medium containing 5% FBS and then counted so that the cell concentration was 1×10^6/mL.

Neutrophils were divided into four groups: control group, LPS group(100 ng/ml), LXA4(100 nM), LPS+LXA4 group.

2.6 Wright's staining

100 uL $1x10^6$/mL neutrophil suspension add to the slide slot of TD3 cell smear centrifuge, 3000 rpm / min, centrifuge for 5 minutes to prepare neutrophil smear. The smears were taken out, dried naturally, dripped with Wright's dye solution and stained for 3 minutes, so that the cell specimens were fixed by methanol in the dye solution. Then add the same amount of PH6.4 PBS solution, gently shake the slide, make it fully mixed with Wright dye, and dye 5min at room temperature. PBS was washed twice and the filter paper was used to dry the liquid on the slides. The morphology and purity of neutrophils were observed under microscope.

2.7 Cell Counting Kit-8 (CCK8 assay)

Neutrophils were isolated and adjusted concentration to 1×10^6/mL. Neutrophils were added to 96-well plate (100 μL per hole). Three multiple holes and blank control holes were set up at the same time (no cells). LXA4 was added into test wells as a stimulant. PBS was used as negative control. After 4 hours of LXA4 intervention, 10 μL of CCK-8 reagent was added into each well. The plate was cultured in 5% CO 2

incubator at 37 ℃ for 4 hours, the OD value of each well of wavelength 450nm was detected by enzyme labeling instrument. Cell inhibition rate (IC)can be calculated according to the formula:

Cell inhibition rate (IC) = [(control group OD value-experimental group OD value) / (control group OD value-zeroing group OD value) ×] 100%

2.8 Respiratory burst detection

The reactive oxygen species released by the activated inflammatory cells can reduce the membrane non-penetrating cytochrome C. The reduced cytochrome C has an absorption peak at 550 nm. Therefore, the amount of reduced cytochrome C is measured using a spectrophotometer. The amount of active oxygen produced can be inferred from this data. Set control group, LPS group and LXA4 groups, and the appropriate amount of LXA4 was added in each group, 100 μL of cytochrome C (1.5 mg/mL) and 100 μL neutrophils (2×10^7/mL) was then added; 10 μL SOD (5000 U/mL) was added, and the corresponding dose was added to the test group, equilibrated in a 5% CO_2 incubator at 37°C for 10min; 10 μL cytochalasin B (1mmol/L) was added to each group and after 3min, 10 μL fMLP (0.1mmol/L) was added for a total of 1 mL and each group was incubated in a 5% CO_2 incubator at 37°C for 30min; Each group was removed and centrifuged at 2000r/min for 10 min; Supernatant was collected and the OD value was measured with a spectrophotometer. Since the production of O_2^- and the decrease in cytochrome C are in a 1:1 mole stoichiometric relationship, the yield of O_2^- is easily calculated. The millimolar extinction coefficient of the 1 cm optical path is 21.1, and the amount of O_2^- produced by 2×10^6 / mL of cells in 1 ml of the solution with a diameter of 1 cm can be directly calculated according to the formula:

OD×47.4=nmol O_2^-/2×106cells/time unit test group O_2^- inhibition rate = (control O_2^- content-test group O_2^- content)/control group O_2^- content×100%.

2.9 Measuring ROS Production by Isolated Neutrophils

Following isolation, cells were resuspended at $1x10^6$/mL in HBSS (with Ca^{2+} and Mg^{2+}) (4.5 mL total) in 15 mL Falcon. 100 μL of neutrophils were added to each well of a 96-well plate. Cells were stimulated with IL-8, fMLP, and PMA. The concentration of IL-8, fMLP, and PMA was as shown in the following table for 1 hour. The Luminometer was set up and the ROS level was tested on the instrument.

	Dilution			Concentrations		
		Factor	Volumes	Stock	Working	Final (in cells)
Luminol		1:10	1 mL into 9 mL pH to 7.3	30 mM	3 mM	0.5 mM
IL8		1:625	1 µL into 624 µL	6.25 µM	10 nM	1.25 nM
fMLP		1:500	12 µL into 5922 µL	10 mM	20 µM	2.5 µM
PMA	A	1:800	1 µL into 799 µL	1620 µM (1mg/mL)	200 nM	25 nM
	B	1:10	55 µL into 495 µL			

2.10 Measuring Neutrophil NETs Production

Clear 96-well flat-bottomed plates were prepared, and 100 µL of neutrophils were added to the relevant wells. Lipopolysaccharide (LPS, 100 ng/mL), interleukin-8 (IL-8, 100 ng/mL), phorbol ester (PMA, 1.5 ng/mL) and N-formylthionyl-leucyl- Phenylalanine (fMLP, 1000 ng/mL) were used to treat the cells respectively. The control group was treated with an equal volume of medium. They were incubated for 3 h at 37°C in a 5% CO_2 incubator. SYTOX Green was diluted 1:500 (5 mM Stock; 1ul SYTOX Green into 499 µL PBS), and then stored in the dark. 20 µL of diluted SYTOX green was added to each well using a fresh tip for each well. 1 µL of MNase was added to each well using a fresh tip for each well. They were then incubated at room temp for 10 min in the dark. Samples were transferred to 0.5 mL micro-centrifuge tubes without any pipetting of the liquid up and down. They were immediately centrifuged at 5000 rpm for 10 min in the micro-centrifuge before 160 µL of the supernatant was removed and transferred to a black 96-well flat-bottomed plate. Fluorescence was measured immediately (Programme: Gen5; excitation 485nm, emission 528nm with optics position in top 50% of well with a 10-second ‘medium’ shake immediately prior to read).

2.11 Western blotting

Proteins were extracted using RIPA lysis buffer, and protein concentration was measured by a protein assay kit. 10% sodium-dodecyl sulphonate polyacrylamide gel was used to separate the protein. After primary and secondary antibodies cultured, the protein bands were measured by using a UVP gel imaging system.

2.12 Measuring the phagocytosis of Neutrophils

Neutrophils were isolated and adjusted concentration to 1×10^6/mL. Following LPS and LXA4 treatment, neutrophils were inoculated into 96-well plates at 100 µL/well. pHrodo Red E. coli and pHrodo Green S. aureus were added to neutrophils respectively to stimulate neutrophils for 30 min, 45 min, and 60 min. Neutrophils were incubated at 37℃ in a 5% CO_2 incubator in the dark, and then centrifuged at 250 g and 4℃ for 5 min to remove the supernatant. The cells were resuspended with 100 uL of 2% PBS/BSA, and

this was repeated twice before the cell suspension from each well was transferred into flow tubes. 100 μL of 2% PBS/BSA was added to each tube, gently mixed and placed on ice. Finally, the phagocytosis of neutrophils was measured using flow cytometry.

2.13 Measuring the rate of apoptotic Neutrophils

Neutrophils were isolated and inoculated into six-well plates at an adjusted concentration of 1 $\times 10^6$/mL. Neutrophils were divided into groups and treated for 4 h and 24 h. Cells were harvested as normal and cells were transferred to the appropriate FACS tubes. They were centrifuged at 600 g for 4 min before the supernatant was poured off. Cells were resuspended in 200 μL Annexin V buffer to wash the cells and then pelleted again. The cells were incubated in 100 μL Annexin V-FITC diluted 1:100 in Annexin V buffer for 15-20 min on ice and protected from the light. 200 μL Annexin V buffer was added to each tube. SYTOX was removed from the freezer and defrosted while being protected from the light. A SYTOX stock diluted 1:500 in Annexin V buffer was prepared. Immediately prior to running the sample on the CyAN, 30 μL of the SYTOX solution was added to each tube and they were vortexed well to mix. The FITC and Violet 1 channels on the FACS machine were used to measure.

2.14 Establishment ARDS rat model with neutrophil deficiency

Cyclophosphamide (CTX) is a kind of non-specific chemotherapeutic drug in cell cycle, which is widely used in clinic. It can kill the cells in each phase of the proliferation cycle and inhibit the number of leukocytes in bone marrow.

In this experiment, rats were injected intraperitoneally with cyclophosphamide (75 mg /kg) 4 days before and 1 day before the ARDS induced by LPS. And one day before ARDS rat model was prepared and one day after the model was prepared, the number of neutrophils in rat tail vein blood was less than 2×10^5/mL by using the blood cell count version technology, Therefore, the ARDS rat model with neutrophil deficiency was successfully prepared. Rats were randomized into three groups (n=6): Control group, LPS group, LPS+ LXA4 group.

2.15 Statistical analysis

The data represent the mean ± SEM. There were no missing, lost, or excluded data. Based on previous experience, no prior power analysis was conducted; all data were analyzed by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. All tests were two-sided, and significance was determined at the $P < 0.05$ level. Statistical analyses were performed using Prism 6.0 software (GraphPad Software, San Diego, CA).

3 Results

3.1 LXA4 protected lung tissues from LPS-induced ARDS rats

First, we evaluated the effect of LXA4 on LPS-induced ARDS rats. Control group revealed normal pulmonary histology (Fig. 1A). In contrast, lung tissues in the LPS group were significantly damaged, with interstitial edema, hemorrhaging, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitium and alveolar spaces, as evidenced by an increase in lung injury score (*P0.01*). Compared with the control group, all the morphologic changes observed were less pronounced in the LPS+LXA4 group. LXA4 significantly attenuated LPS-induced pathologic changes as shown by the decrease in lung injury score (*P0.01*)(Fig. 1B).

As expected, the concentrations of TNF-α and IL-1β in the lung tissue homogenate were significantly higher in the LPS group than in the control group. By comparison, the concentrations in the LXA4 groups were lower than in the LPS group (Fig. 1C-D).

3.2 100 nM LXA4 is the best effective concentration to stimulate neutrophils in rat peripheral blood

As shown in Fig. 2A, the structure of LXA4 shows that it belongs to the aromatic compound family. Pure rat neutrophils can be obtained by gradient centrifugation as mentioned above. Fig. 2B shows rat neutrophils after Wright's staining. The nucleus is blue-purple lobulated and the cytoplasm is light pink. The ratio of rat neutrophils was quantified, and the purity could reach 94.1% of the isolated cells. Subsequently, different concentrations of LXA4 were administrated to the neutrophils for 6 h. As shown in Fig. 2C, The LXA4 of 200 nM and 400 nM had certain cytotoxic effect on neutrophils, while the lower concentration of LXA4 had less cytotoxic effect on neutrophils. Therefore, we choose the LXA4 of 10 nM, 50 nM and 100 nM for further study.

The LXA4 of 10 nM, 50 nM and 100 nM was used to act on neutrophils, and the reduction of cytochrome C was detected by spectrophotometer to infer the production of O_2 -. The results are shown in Fig. 2D: LXA4 inhibits the production of O_2-in a concentration-dependent manner. Fig. 2E showed that the concentration of ROS in the LPS group was significantly higher than that in the control group (*P<0.01*). Compared with LPS group, both 50 nM LXA4 and 100 nM LXA4 could reduce the production of ROS, and the effect of 100 nM LXA4 was stronger (*P<0.01*), while 10 nM LXA4 could not effectively reduce the production of ROS.

Therefore, we chose 100 nM as the best dose of LXA4 to stimulate peripheral blood neutrophils.

3.3 LXA4 inhibits the production of reactive oxygen species (ROS) of neutrophils

The expression of ROS in neutrophils was measured by Luminometer (Fig. 4A-B). According to the literature, we found that there are four groups of agents that can induce ROS production. The first group of priming agents is composed of physiological inflammatory agents, such as C5a, or formylated peptides/proteins such as fMLP. The second group of priming agents is composed of proinflammatory cytokines and adipokines, such as tumor necrosis factor (TNF-α), IL-8. The third group of priming agents

is composed of TLR agonists, such as lipopolysaccharide (LPS or endotoxin). The four group of priming agents is Phorbol ester (PMA). Therefore, we chose three agents IL-8、fMLP and PMA to stimulate neutrophils to produce ROS.

Neutrophils were isolated from peripheral blood of ARDS rats. The expression of reactive oxygen species (ROS) in neutrophils was detected after neutrophils were treated with IL-8, fMLP and PMA, respectively. The results showed that both fMLP and PMA could induce the production of reactive oxygen species (ROS) in peripheral blood neutrophils of ARDS rats, but the production of ROS in peripheral blood neutrophils in IL-8 stimulation group was not significantly different from that in control group (*P>0.05*), and the production of ROS in peripheral blood neutrophils after PMA stimulation was significantly higher than that in fMLP group (*P<0.01*) (Fig. 3A).It was found that PMA was the best at stimulating neutrophils to produce ROS (*P<0.01*) (Fig. 3A). Therefore, in the following experiments, PMA was used as an inducer to stimulate neutrophils to produce ROS.

Neutrophils were isolated from peripheral blood of control group and ARDS rats, and ROS content of neutrophils was detected after PMA intervention. The results showed that the content of ROS in ARDS rats without stimulation was higher than that in control rats (*P<0.01*). Moreover, PMA could stimulate not only the control rats, but also the production of ROS in peripheral blood neutrophils of ARDS rats (*P<0.01*). However, after PMA stimulation, the production of ROS in peripheral blood neutrophils of ARDS rats was significantly higher than that of the control rats (*P<0.01*) (Fig. 3B). These results indicate that the production of ROS in neutrophils is helpful to play a bactericidal role in the acute stage of ARDS inflammation.

Neutrophils were divided into four groups: (1) Control group; (2) LXA4 group; (3) BOC-2 group (LXA4 receptor antagonist); (4) LXA4+BOC2 group. The production of reactive oxygen species (ROS) was detected after PMA intervention. The results showed that compared with Control group, the production of ROS by neutrophils decreased significantly after the treatment of LXA4, and the inhibitory effect of LXA4 on the increase of ROS production was significantly weakened by BOC-2(Fig. 3C).

3.4 LXA4 reduces the release of NETs production of neutrophils

The literature also confirmed that four group of reagents that induce ROS production can also stimulate the production of NETs, so we also choose four agents LPS 、IL-8、fMLP and PMA to induce the production of NETs. The neutrophils were isolated from the peripheral blood of ARDS rats. The neutrophils were stimulated with IL-8 (100 ng/mL), LPS (100 ng/mL), fMLP (1000 ng/mL) and PMA (1.5 ng/mL) for 3 hours, and NETs was detected. The results showed that IL-8, LPS, fMLP and PMA could induce the production of NETs in peripheral blood neutrophils of ARDS rats (*P<0.05*), and the production of NETs in peripheral blood neutrophils after PMA stimulation was significantly higher than that in IL-8 group, LPS group and fMLP group (*P<0.01*), indicating that PMA has the best effect on NETs production in peripheral blood neutrophils. (Fig. 3D). As a result, PMA was used as the inducer.

Our study found that without stimulation, the content of NETs produced by ARDS rats was higher than that of the control rats (*P < 0.05*); PMA could stimulate not only the control rats, but also the production of ROS in neutrophils of ARDS rats (*P < 0.01*). However, after PMA stimulation, the production of NETs in peripheral blood neutrophils of ARDS rats was significantly more than that of the control rats (*P < 0.01*) (Fig. 3E), indicating that the production of NETs helps neutrophils play a bactericidal role in the acute phase of inflammation.

Neutrophils were isolated from peripheral blood of ARDS rats and divided into four groups: (1) Control group; (2) LXA4 group; (3) BOC-2 group (LXA4 receptor antagonist); (4) LXA4+BOC2 group. Neutrophils were stimulated with PMA (1.5 ng/mL) for 3 hours, and NETs was detected. The results showed that the production of NETs by peripheral blood neutrophils was significantly reduced after the treatment of LXA4 compared with Control group (*P<0 01*), and the effect of LXA4 on reducing NETs production was significantly inhibited by BOC-2 (*P<0 05*) (Fig. 3F).

3.5 LXA4 reduces the release of neutrophil elastase (NE) and Myeloperoxidase (MPO) production of neutrophils

As shown in Fig. 4A-B, the amount of NE released by neutrophils in the LPS group was significantly higher than that in control group after stimulation with fMLP(*P<0 05*). This suggests that neutrophils can directly resist infection by increasing the release of elastase. The addition of LXA4 can reduce the release of neutrophil NE (*P<0 01*), the same as NE inhibitor(NEi)(*P<0 01*).

Similar to the result in Fig. 4A-B, the result in Fig. 4C-D shows that the amount of myeloperoxidase (MPO) released by neutrophils in LPS group was significantly higher than that in control group after LPS stimulation (*P<0 05*). After adding MPO inhibitors(MPO inhibitor Ⅰ), the amount of MPO released by neutrophils decreased significantly(*P<0 05*), while exogenous LXA4 could reduce neutrophils to release MPO(*P<0 01*), the same as MPO inhibitor Ⅰ(*P<0 01*).

3.6 LXA4 reduces the production of NETs by inhibiting the release of neutrophils NE and MPO

Figure 4E shows that LPS can stimulate the release of NETs (*P<0.01*), and LXA4 can reduce the release of NETs (*P<0.01*) and is similar to NE inhibitors(NEi).

Figure 4F shows that LPS can stimulate the release of NETs (*P<0.01*), and LXA4 can reduce the release of NETs(P<0.01)and is similar to MPO inhibitor Ⅰ(*P<0.01*).

3.7 LXA4 promotes phagocytosis of neutrophils

Neutrophils were isolated from peripheral blood of control rats and ARDS rats. PE labeled E. coli (EC) and FITC labeled S. aureus (SA) were co cultured with neutrophils for 30 min, 45 min and 60 min. the phagocytosis rate of neutrophils was detected by flow cytometry. The results showed that the average fluorescence intensity of S. aureus phagocytosis of neutrophils in control rats was 2749.449 in 30 min ± 469.95 increased to 12305.01 for 60 minutes ± 1425.02. The average fluorescence intensity of neutrophil

phagocytosis of E. coli (EC) increased from 4159. 299 ± 357.72 increased to 7340.257 for 60 minutes ± 597.80. In ARDS rats, the average fluorescence intensity of S. aureus phagocytized by neutrophils decreased from 4979.964 ± 4336 to 24116.71 at 60 min ± 1377.896. The average fluorescence intensity of neutrophils phagocytizing E. coli increased from 6 392.38 to 6 392.38 in 30 min ± 910.25 to 12618.055 at 60 min ± 1303.405. It is suggested that the phagocytic ability of neutrophils to S. aureus(SA) and E. coli(EC) increases with time, and reaches the peak at 60 min. The phagocytic ability of neutrophils in peripheral blood of ARDS rats was higher than that of control rats at 30 min, 45 min and 60 min ($P < 0.05$), while the phagocytic ability of neutrophils in peripheral blood of ARDS rats was higher than that of control rats only at 45 min and 60 min ($P < 0.05$). There was no significant difference in phagocytic rate between the two groups at 30 min (Fig. 5B-D).

Neutrophils were isolated from peripheral blood of ARDS rats and divided into four groups: (1) Control group; (2) LXA4 group; (3) BOC-2 group (LXA4 receptor antagonist); (4) LXA4 + BOC-2 group, PE labeled E. coli (EC) and FITC labeled S. aureus (SA) were co-cultured with neutrophils for 30 min, 45 min and 60 min, and the phagocytic rate was detected by flow cytometry. The results showed that the phagocytosis of S. aureus (SA) and E. coli (EC) by neutrophils in LXA4 group was significantly higher than that in control group ($P < 0.05$); The effect of BOC-2 on neutrophil phagocytosis was significantly decreased ($P < 0.05$) (Fig. 5F-H).

3.8 LXA4 can effectively promote the apoptosis of neutrophils in ARDS rats and reduce the cell death caused by NETosis

Neutrophils were isolated from peripheral blood of control rats. After LXA4 100 nM treatment for 4 h and 24 h, the apoptosis of neutrophils was detected by flow cytometry. The results showed that: compared with control group, after LXA4 treatment for 4 h, the neutrophils in control rats's peripheral blood had the following characteristics: There was no significant difference in living cell, apoptosis cell, dead cells and necrosis cells ($P > 0.05$)(Fig. 6B). Compared with control group, LXA4 increased the number of living cells ($P < 0.05$), decreased the number of apoptotic cells ($P < 0.05$), and had no significant effect on dead cells and necrosis cells ($P > 0.05$) after LXA4 treated neutrophils of control rats for 24 hours (Fig. 6C). It is suggested that the survival rate of normal neutrophils in vitro will gradually decrease with the extension of time, and the survival rate of neutrophils in vitro is low at 24 h. LXA4 has a certain protective effect on normal neutrophils in healthy rats, and can appropriately prolong the life of neutrophils.

Neutrophils were isolated from peripheral blood of ARDS rats. After LXA4 100 nM treatment for 4 hours, the apoptosis of neutrophils was detected by flow cytometry. The results showed that compared with the untreated group, the number of living cells in LXA4 group decreased ($P < 0.05$), and the number of apoptotic cells increased ($P < 0.05$), There was no significant difference between dead cell and necrosis cell ($P > 0.05$) (Fig. 6E). It is suggested that most of the neutrophils isolated from ARDS rats are activated neutrophils, which have strong bactericidal function. After 4 hours of treatment with LXA4, the living neutrophils can be significantly reduced, and the apoptotic neutrophils can be promoted, so as to avoid the damage of excessive inflammatory reaction to the body.

After treatment with LXA4 100 nM for 24 hours, the results showed that compared with the control group, the number of living cells decreased (*P < 0.05*), the number of early apoptotic cells increased significantly (*P < 0.05*), and the number of dead cells decreased significantly (*P < 0.05*) (Fig. 6F). The survival rate of neutrophils isolated from peripheral blood of ARDS rats was still about 25% after 24 hours of culture in vitro. It indicated that the survival time of activated neutrophils was significantly longer than that of normal neutrophils. However, the survival rate of activated neutrophils could be reduced by LXA4 treatment, and the apoptosis was significantly increased and the dead cell was significantly reduced, These results suggest that LXA4 can not only effectively promote the apoptosis of activated neutrophils, but also reduce the cell death caused by NETosis, which is helpful to the resolution of inflammation.

Neutrophils of ARDS rats were divided into four groups: (1) control group; (2) LXA4 group; (3) BOC-2 group; (4) LXA4 + BOC-2 group, the apoptosis of neutrophils was detected by flow cytometry. The results showed that: compared with the untreated group, the number of apoptotic neutrophils of ARDS rats treated with LXA4 for 24 h was significantly increased (*P < 0.01*), but BOC-2 could significantly inhibit the effect of LXA4 on promoting neutrophil apoptosis (*P < 0.01*)(Fig. 6H). LXA4 inhibited dead cell significantly (*P < 0.01*). BOC-2 also inhibited the effect of LXA4 on reducing dead cell (P < 0.01) (Fig. 6I). The results showed that LXA4 promoted the apoptosis of neutrophils in ARDS rats cultured for 24 hours in vitro, and the effect of reducing dead cell induced by NETosis was mediated by ALX receptor.

3.9 LXA4 has no protective effect on ARDS rats with neutrophil deficiency

On the second day after receiving cyclophosphamide immunosuppression, the rats showed poor mental state, reduced diet and activity, gray and yellow hair, and the above performance was aggravated after injection of LPS.

From the H&E staining samples of rat lung tissue, we can see that the lung histology of the control group is normal (Fig. 7A). In LPS group, the lung tissue injury was markedly damaged, with interstitial edema, hemorrhaging, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitium and alveolar spaces, as evidenced by an increase in lung injury score (*P□0.01*). In LPS+LXA4 group, the histomorphology of lung tissue was similar to that of LPS, and the injury was severe (Fig. 7B). Emo could not reduce the concentration of TNF-α and IL-1β in lung tissue homogenate of LPS group. (Fig. 7C-D).

4 Discussion

ARDS is a rapid non-cardiogenic bilateral lung infiltration syndrome characterized by alveolar vascular injury, neutrophil infiltration and accompanied by the release of pro-inflammatory factors [15]. Neutrophils and macrophages play an important role in the process of lung injury, in which neutrophils are the first line of defense against the invasion of pathogens [16]. Macrophages play a key role in the subsequent clearance of apoptotic neutrophils and promoting the regression of inflammation. This study focuses on the effect of LXA4 on the function of neutrophils in ARDS, so as to explore the anti-inflammatory mechanism of LXA4.

Neutrophils are the largest number of white blood cells in human circulation, and they play an important role in the process of immune defense against pathogenic microorganisms [17]. As effector cells of the innate immune system, neutrophils participate in a variety of immune and inflammatory processes and play an important role in coordinating the overall immune and inflammatory response. In the last 5 years, there have been many reports on the anti-inflammatory effects of LXA4 [18–19]. As the first kind of regression medium, LXA4 has a strong anti-inflammatory and regression effect [7]. Recently, some studies have shown that LXA4 and synthetic analogues can protect tissues from acute and chronic inflammation. Its mechanism includes down-regulation of proinflammatory cytokines and chemokines (such as IL-1β and TNF- α), inhibition of the activation of major pro-inflammatory pathways, and increase the release of proinflammatory cytokines (such as IL-10) [18]. Therefore, LXA4 is one of the earliest endogenous lipid mediators, which is used to inhibit the aggregation of neutrophils, inhibit inflammation, and promote the resolution of inflammation [19].

In this study, we have identified a unique role for LXA4 related to the function of neutrophils in LPS-induced inflammatory lung injury. Our data clearly demonstrate that Exogenous administration of LXA4 can reduce the lung injury score of ARDS rats induced by LPS. Our experimental results also confirm that treatment with LXA4 inhibits plasma inflammatory cytokines, such as TNF-α and IL-1β. Decreased plasma TNF-α and IL-1β levels in LXA4-treated rats are consistent with the paradigm that LXA4 reduces the inflammatory response following ARDS, in addition to ameliorating the severity of disease. This suggests the potential of LXA4 as an effective lung protective therapeutic agent.

We used percoll density gradient centrifugation to extract neutrophils from peripheral blood of patients with ARDS. The purity of neutrophils was (97.5 ± 2.3)%, and the cell viability was more than 96% by trypan blue staining.

Neutrophils are over-activated during ARDS, which releases neutrophil respiratory burst and ROS to damage the surrounding tissues. Priming of the neutrophil ROS production is believed to be involved in many inflammatory diseases, such as acute respiratory distress syndrome (ARDS), rheumatoid arthritis (RA), atherosclerosis, ischemia-induced tissue injury, hypertension, diabetes, kidney disease, and sepsis [20]. Our experimental results supported this conclusion. In this study, we evaluated the initiation of respiratory burst of suspended neutrophils in vitro using a superoxide dismutase (SOD)-inhibited cytochrome c reduction test, and luminol-amplified chemiluminescence to measure ROS [21]. The results showed that IL-8, fMLP and PMA could stimulate the production of reactive oxygen species (ROS) in neutrophils, and PMA was the best. The ROS production of neutrophils in ARDS rats without stimulation was higher than that in control group. PMA could not only stimulate the ROS production of neutrophils in control group, but also in the peripheral blood of ARDS rats. However, after PMA stimulation, the ROS production of neutrophils in the peripheral blood of ARDS rats was significantly higher than that in the control group. After LXA4 treatment, the production of ROS in peripheral blood neutrophils of ARDS rats was significantly reduced. The inhibition effect of LXA4 on the production of ROS was significantly weakened by the addition of LXA4 receptor antagonist BOC-2, which indicated that LXA4 could reduce the

damage of peripheral blood neutrophils to the surrounding tissues in the process of sterilization by combining with ALX receptor.

NETs is a kind of outer network structure, which is composed of complex DNA triple network structure. It contains major proteins, elastase (NE), myeloperoxidase (MPO), cathepsin G and other antibacterial proteases. This is a special bactericidal mechanism of neutrophils [22]. In the process of inflammation, NETs is a double-edged sword. Studies have shown that a large number of extensive NETs can damage epithelial cells and endothelial cells, leading to the spread of inflammation [23]. However, a large number of studies have shown that in the acute stage of inflammation, NETs can not only capture microorganisms, but also limit the spread of cytotoxic antibacterial proteins, reduce the damage of host tissue, and play an important role in inhibiting the spread of inflammation [24]. In this study, we isolated neutrophils from peripheral blood of ARDS rats and stimulated neutrophils with IL-8, LPS, fMLP and PMA respectively. We found that IL-8, LPS, fMLP and PMA could induce the production of nets in peripheral blood neutrophils of ARDS rats in vitro, but PMA had the best effect. Therefore, we chose PMA as the stimulant of nets in subsequent experiments. The effect of PMA on promoting the production of neutrophil NETs is more obvious in peripheral blood neutrophils of ARDS rats, which indicates that the production of NETs helps neutrophils to play a bactericidal role in the acute phase of inflammation. The results showed that LXA4 could reduce the NETs function in ARDS rats mediated by binding to ALX receptor.

Neutrophil elastase (NE), a serine protease secreted by polymorphonuclear neutrophils (PMNs), plays an important role in many physiological and pathological processes such as the various inflammatory reactions, adult respiratory distress syndrome, and acute lung injury [25]. When exposed to various inflammatory factors, neutrophils release NE via degranulation, which is then involved in the inflammatory response. Some studies believe that NE is a destructive elastase that attacks the extracellular matrix and modulates in□ammation and tissue remodeling. Its involvement may be direct (tissue damage) or indirect (pro-in□ammatory or pro-apoptotic) [26–28]. However, it is undeniable that NE is a necessary condition for neutrophil migration to inflammatory sites, and the proteolytic activity of NE contributes to the body's defence against infectious agents by promoting the destruction of pathogenic bacteria [29–30]. Our results show that LXA4 can reduce NE release induced by fMLP. To analyze the reasons, we consider that the time point selected in this study is 6 hours after the successful establishment of ARDS rats model induced by LPS. This is the peak of inflammation, a large number of neutrophils have already migrated to the site of inflammation and have played an effective bactericidal function. The subsequent period of inflammation subsides. So it is very important to reduce the damage of NE to surrounding tissues. At this time, LXA4 reduces the release of NE, which is beneficial to reduce NE damage to surrounding tissues.

It is confirmed that NE and MPO are involved in the generation of NETs. Neutrophil chromatin densification requires neutrophil elastase (NE), NE deficiency mice unable to form NETs and show immunodeficiency; the formation of NETs also requires myeloperoxidase (MPO) to act on histones in the

transitional nucleus, and hypochlorite, a product produced by MPO, is necessary for the release of NETs, so patients without MPO activity can't produce NETs. We treated neutrophils with NE inhibitor (NEi) and MPO inhibitor(MPO inhibitor□), as shown in Fig. 4B□E, the amount of NE released by LPS group was significantly higher than that of control group after stimulation with fMLP. This indicated that neutrophils increased the release of NE, on the one hand, directly antagonized infection, on the other hand, increased the production of NETs and enhanced bactericidal function. After adding NEi, the release of NE from neutrophils was significantly reduced. And LXA4 also reduces the release of NE from neutrophils and is similar to NE inhibitors(NEi). As shown in Fig. 4C□F, the amount of MPO released by neutrophils in LPS group was significantly higher than that in control group. However, the amount of MPO released by neutrophils was significantly reduced after the addition of MPO inhibitor □. The addition of LXA4 could reduce the release of MPO and is similar to MPO inhibitor □. These results indicate that after the peak period of inflammation, LXA4 can reduce the production of NETs by inhibiting the release of NE and MPO from neutrophils, thereby reducing the damage to surrounding tissues caused by excessive activated neutrophils, which is conducive to the regression of inflammation.

During the inflammatory injury, neutrophils gather at the injury site and play a phagocytic role, which is an important mechanism to kill pathogenic microorganisms. Our study found that the phagocytic ability of neutrophils to S. aureus(SA) and E. coli(EC) increased with time, reaching the peak at 60 minutes. At 45 minutes and 60 minutes, the phagocytic ability of neutrophils in ARDS rats was higher than that in healthy control group, but there was no significant difference between the two groups at 30 minutes. The ability of phagocytosis of S. aureus (SA) and E. coli (EC) by neutrophils in LXA4 group was significantly higher than that in control group. The effect of BOC-2 on phagocytosis of neutrophils in LXA4 group was significantly reduced, On the other hand, it is confirmed that LXA4 can increase the bactericidal function of peripheral blood neutrophils in ARDS rats by binding with ALX receptor.

As we all know, the life span of neutrophils is very short, mainly because with the passage of time, neutrophils in the circulatory system will undergo apoptosis. The resolution of acute inflammation requires macrophages to phagocytize apoptotic neutrophils. Delayed apoptosis of activated neutrophils can lead to persistent acute pneumonia and eventually develop into ARDS. The results in Fig. 5A-D of this study suggest that: as the survival rate of normal neutrophils in vitro will gradually decrease with the extension of time, the survival rate of neutrophils in vitro will be lower at 24 hours, and LXA4 has a certain protective effect on normal neutrophils in healthy rats, which can appropriately prolong the life of neutrophils. However, most of the neutrophils isolated from ARDS rats are activated neutrophils. These neutrophils have strong bactericidal function. After 4 hours of LXA4 treatment, the survival rate of these neutrophils can be significantly reduced, and their apoptosis can be increased, so as to avoid the damage of excessive inflammatory reaction to the body. The results in Fig. 5A-D also indicate that the survival rate of neutrophils isolated from peripheral blood of ARDS rats can still reach about 25% after 24 hours of culture in vitro, which indicates that the survival time of activated neutrophils in vitro is significantly longer than that of normal neutrophils, and LXA4 treatment can reduce the survival rate of activated neutrophils and significantly promote their apoptosis, LXA4 can not only effectively promote the apoptosis of activated neutrophils, but also reduce the cell death caused by NETosis, which is helpful to

the resolution of inflammation. Our results showed that that LXA4 promotes the apoptosis of neutrophils in peripheral blood of ARDS rats cultured in vitro for 24 hours, and the effect of reducing neutrophil NETosis death is mediated by ALX receptor.

Finally, we used cyclophosphamide to inhibit circulating neutrophils in rats, and made a rat model of ARDS with neutrophil deficiency. The results showed that LXA4 could not reduce the pulmonary inflammatory infiltration and lung injury score in the ARDS rats with neutrophil deficiency(Fig. 7A-B), nor could it reduce the levels of inflammatory factors TNF-α and IL-1β in lung homogenate(Fig. 7C-D). It is further confirmed that LXA4 had a protective effect on ARDS rats by affecting the function of neutropenia.

In summary, this study demonstrates that LXA4 alleviates lung injury and reduces the release of inflammatory cytokines in ARDS rats induced by LPS, but has no protective effect on ARDS rats with neutrophil deficiency. Moreover, LXA4 also down-regulated neutrophil respiratory burst and the production of ROS and NETs in neutrophils of ARDS rats, thus reducing the damage of neutrophils to the surrounding tissues. LXA4 can also up-regulate the ability of neutrophils to phagocytize bacteria, thereby enhancing the bactericidal ability of neutrophils. In addition, LXA4 can promote the apoptosis of neutrophils and accelerate the resolution of inflammation. Our findings reveal a novel mechanism for LXA4 to attenuate the inflammatory reaction and shows that LXA4 could be exploited therapeutically for ARDS.

5 Conclusion

LXA4 has a protection effect on LPS-induced ARDS rats. It can alleviate lung injury and reduces the release of inflammatory cytokines, by affecting the various function of neutrophils.

Abbreviations

LXA4
Lipoxin A4
ALI
Acute lung injury
ARDS
acute respiratory distress syndrome
Emo
Emodin
LPS
lipopolysaccharide
TNF-α
tumor necrosis factor-α
IL-1β
Interleukin-1β

NETs
neutrophil extracellular traps
ROS
reactive oxygen species
fMLP
formyl methionyl leucyl phenylalanine
IL-8
interleukin-8
PMA
phorbol ester
Cytochrome C
cell chromatography C
SOD
superoxide dismutase
HEPES
Hydroxyethylpiperazine Ethylsulfonic Acid
FBS
fetal bovine serum
ELISA
enzyme-linked immunosorbent assay
CCK8
Cell Counting Kit-8
CTX
Cyclophosphamide
MPO
myeloperoxidase
RA
rheumatoid arthritis.

Declarations

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Availability of data and material: All data generated or analyzed during this study are included in this published article and are available from the corresponding author upon request.

Authors' contributions: The authors contributed in the following manner: study concept and design: all authors; acquisition of data: Wenhao Pan; analysis and interpretation of data: Qichao Xu; and drafting of

the manuscript and approval of the final version: Hongxia Mei.

Ethics approval: All animals received care in compliance with the Principles of Laboratory Animal Care and National standards.

Consent to Participate: Not applicable.

Consent to Publication: Not applicable.

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Figures

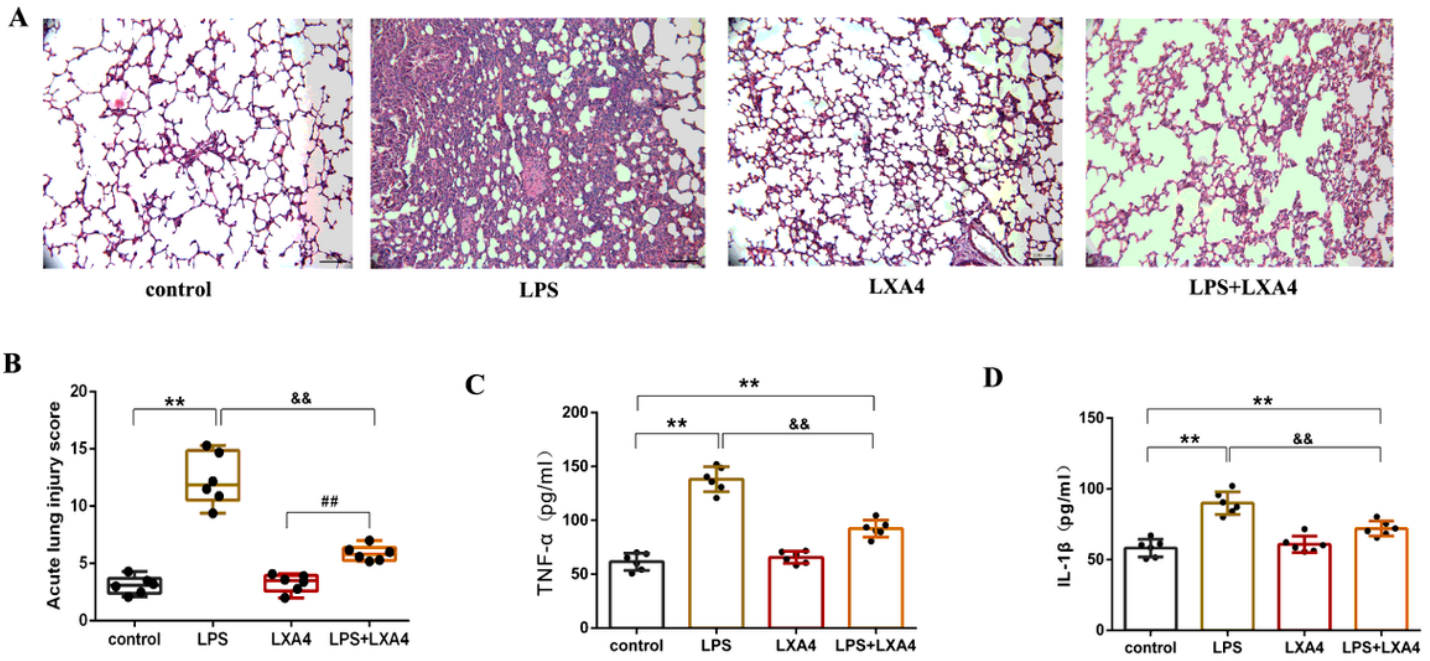

Figure 1

LXA4 protected lung tissues in LPS-induced ARDS rats. (A) The lung tissues were obtained immediately after exsanguination (6 h after LPS), and the effect of LXA4 was assessed histologically in H&E-stained sections (original magnification ×200). (B) Lung injury scores were recorded from 0 to 16 according to the criteria described in Materials and Methods.The data are presented as the mean ± SEM. n = 6. **P0.01 versus control group; &&P0.01 versus LPS group. (C) The lung tissue homogenate TNF-α protein expression.The data are presented as the mean ±SEM. n = 6. **P0.01 versus control group; &&P0.01 versus LPS group. (D) the lung tissue homogenate IL-1βprotein expression. The data are presented as the mean ±SEM. n = 6. **P0.01 versus control group; &&P0.01 versus LPS group.

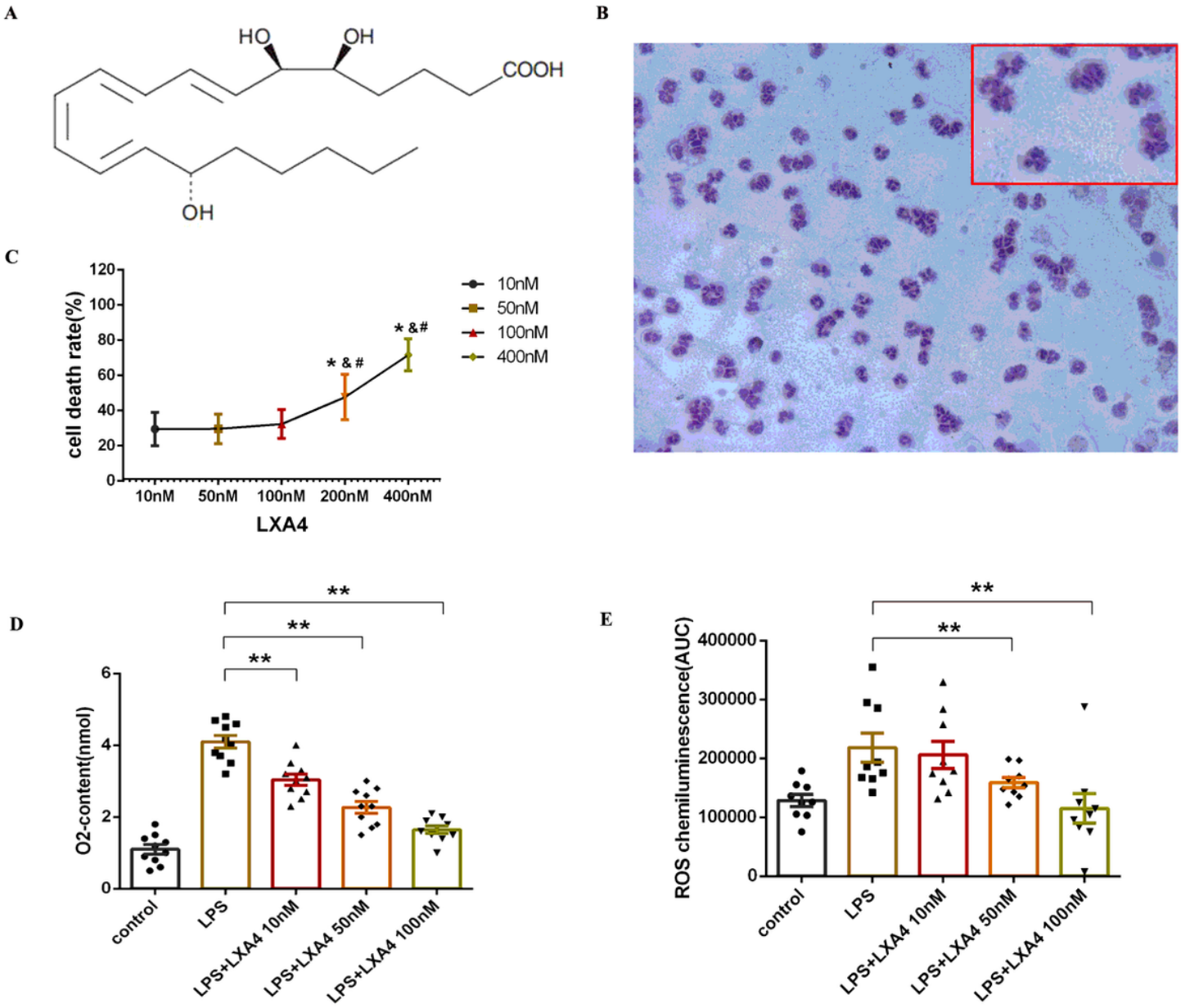

Figure 2

100 nM LXA4 is the best effective concentration to stimulate neutrophils in peripheral blood of rats (A) Schematic diagram of LXA4; (B) Wright's staining of rat neutrophils.The data are presented as the mean ±SEM. n = 10. *P0.05 10nM vs 200nM400nM&P 0.05 50nM vs 200nM400nM#P0.05 100nM vs 200nM400nM (C) CCK8 assay. The effect of different concentrations of LXA4 on neutrophil Cytotoxicity in rats was examined. The data are presented as the mean ±SEM. n = 10. *P0.05 10 nM vs 200 nM400 nM&P0.05 50 nM vs 200 nM400 nM#P0.05 100 nM vs 200 nM400 nM (D) Effects of different concentrations of LXA4 on respiratory burst of neutrophils. (The data are presented as the mean ±SEM. n =10. **P<0.01 LPS vs LPS+LXA4 10 nM, LPS+LXA4 50 nM, LPS+LXA4 100 nM) (E) Effects of different concentrations of LXA4 on ROS of neutrophils. (The data are presented as the mean ±SEM. n = 10. *P<0.01 LPS vs LPS+LXA4 50 nM, **P < 0.01 LPS vs LPS+LXA4 100 nM)

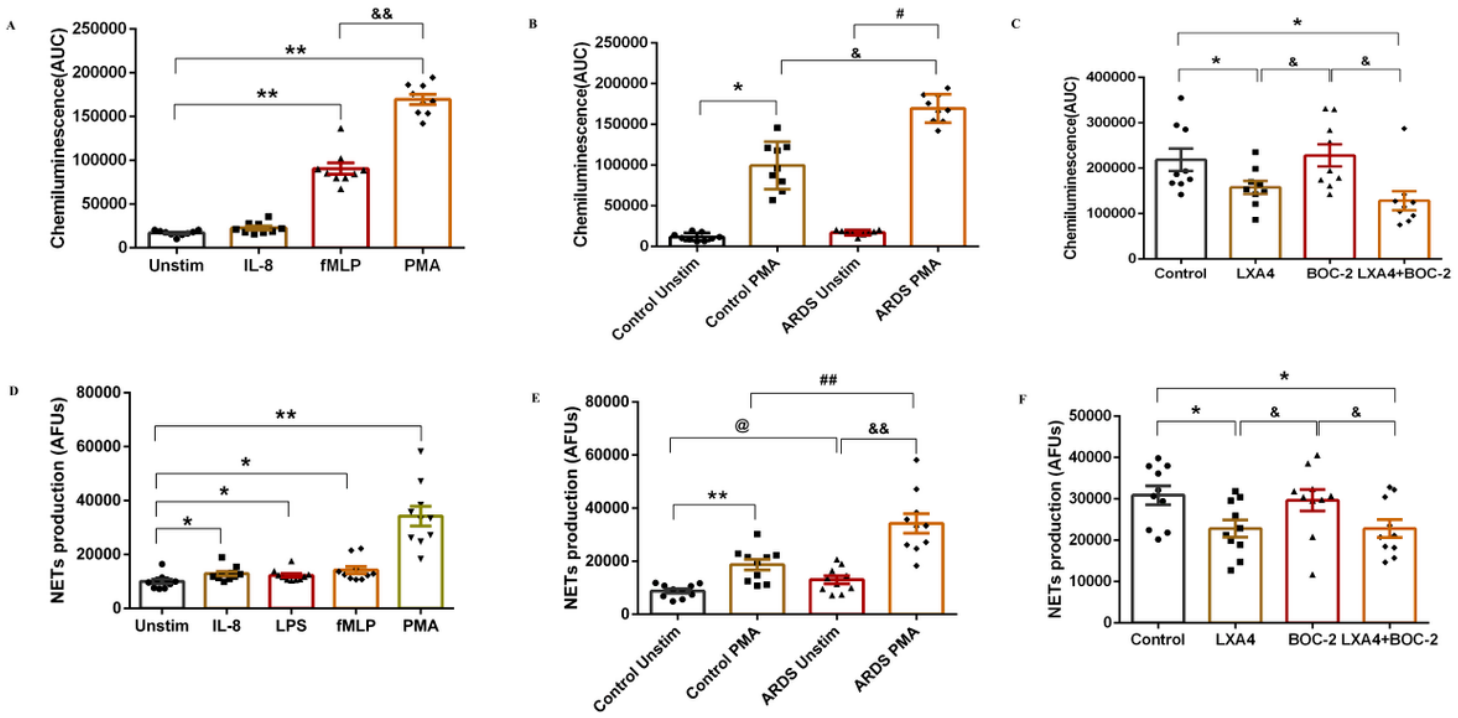

Figure 3

Effect of LXA4 on ROS production and NETs production of neutrophil. (A) Comparison of the ROS level produced of neutrophils after stimulation with IL-8, fMLP, and PMA. The data are presented as the mean ± SEM. n =10.**P0.01 Unstim vs fMLPPMA, &&P0.01 fMLP vs PMA (B) Comparison of ROS production of neutrophils between control rats and ARDS rats. The data are presented as the mean ± SEM. n =10. *P0.05 Control Unstim vs Control PMA#P0.05 ARDS Unstim vs ARDS PMA &P0.05 Control PMA vs ARDS PMA (C) LXA4 inhibits the production of ROS in peripheral blood neutrophils of ARDS rats. The data are presented as the mean ± SEM. n =10.*P0.05 Control vs LXA4LXA4+BOC-2&P 0.05BOC-2 vs LXA4LXA4+BOC-2 (D) Comparison of NETs production of neutrophils after stimulation with various stimulants. The data are presented as the mean ± SEM. n =10.*P0.05 Unstim vs IL-8LPS fMLP**P0.01 Unstim vs PMA (E) Comparison of NETS production of neutrophils between control rats and ARDS rats. The data are presented as the mean ± SEM. n =10. **P0.01 Control Unstim vs Healthy PMA&&P0.01 ARDS Unstim vs ARDS PMA ##P0.01 Control PMA vs ARDS PMA@P0.05 Control Unstim vs ARDS Unstim (F) LXA4 reduces the production of NETs in neutrophils of ARDS rats in vitro by binding to ALX receptorThe data are presented as the mean ± SEM. n =10. *P0.05 Control vs LXA4 LXA4+BOC-2 &P0.05 BOC-2 vs LXA4LXA4+BOC-2

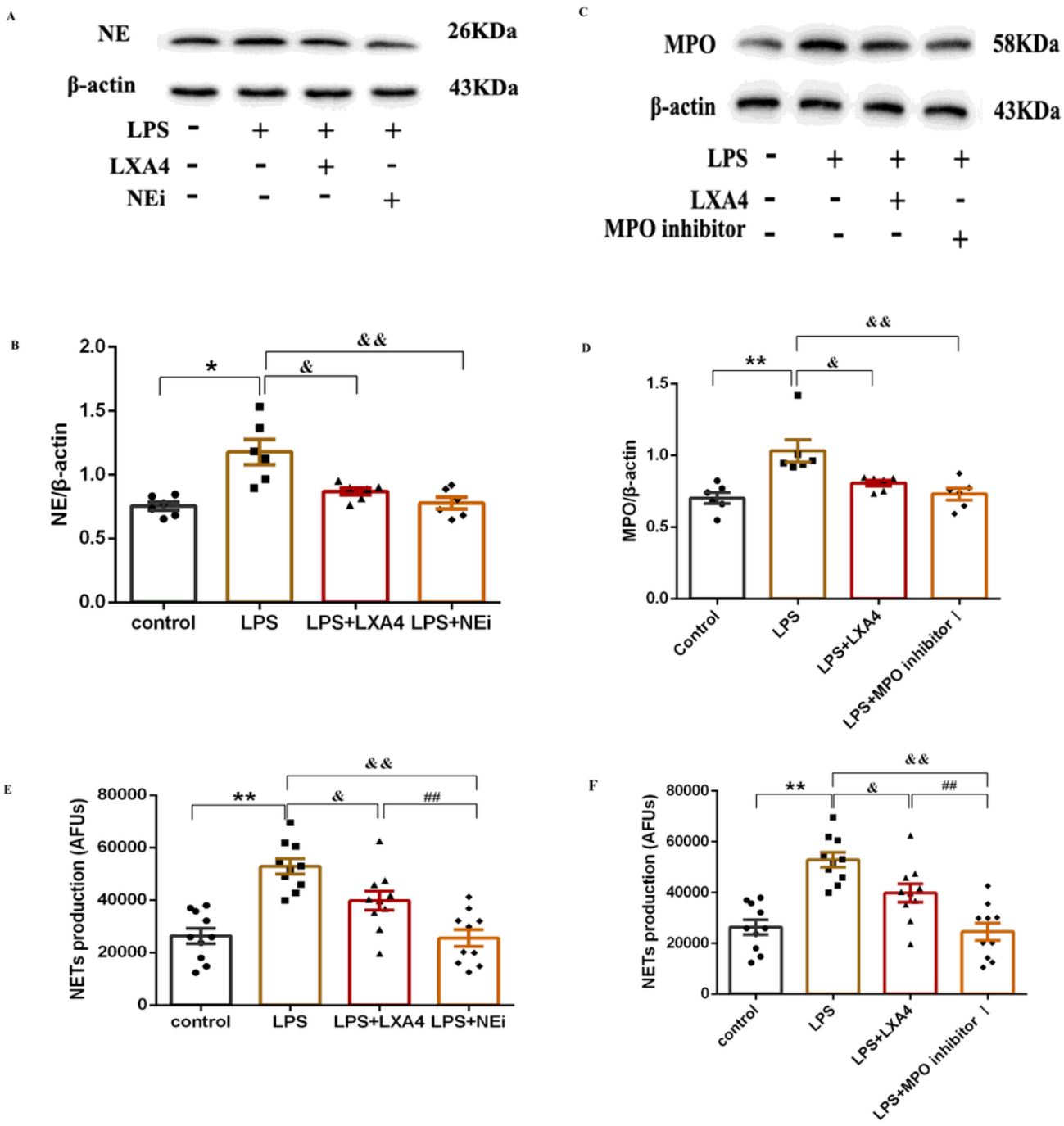

Figure 4

LXA4 reduces the production of NETs by repressing the release of NE and MPO from neutrophils. (A) Western blot analysis of NE in peripheral blood neutrophils. (B) LXA4 reduces the release of NE from neutrophils and is similar to NE inhibitors（NEi）.　The data are presented as the mean ± SEM. n =10. *P＜0.05Control vs LPS；&P＜0.05 LPS vs LPS+LXA4；&&P＜0.01 LPS vs LPS+NEi； (C) Western blot analysis of MPO in peripheral blood neutrophils. (D) LXA4 reduces the release of MPO from neutrophils and is similar to MPO inhibitors（MPO inhibitor）.　The data are presented as the mean ± SEM. n =10. **P＜0.01 Control vs LPS； &P＜0.05 LPS vs LPS+LXA4；&&P＜0.01 LPS vs LPS+ MPO inhibitor； (E) Both LXA4 and NE

inhibitors（NEi） can reduce the release of neutrophil NETs. （The data are presented as the mean ± SEM. n =10.**P＜0.01 Control vs LPS；&P＜0.05 LPS vs LPS+LXA4；&&P＜0.01 LPS vs LPS+NEi；##P＜0.01 LPS+LXA4 vs LPS+NEi） (F) Both LXA4 and MPO inhibitors（MPO inhibitor） can reduce the release of neutrophil NETs.（The data are presented as the mean ± SEM. n =10.**P＜0.01 Control vs LPS；&P＜0.05 LPS vs LPS+LXA4；&&P＜0.01 LPS vs LPS+ MPO inhibitor； ##P＜0.01 LPS+LXA4 vs LPS+ MPO inhibitor ）

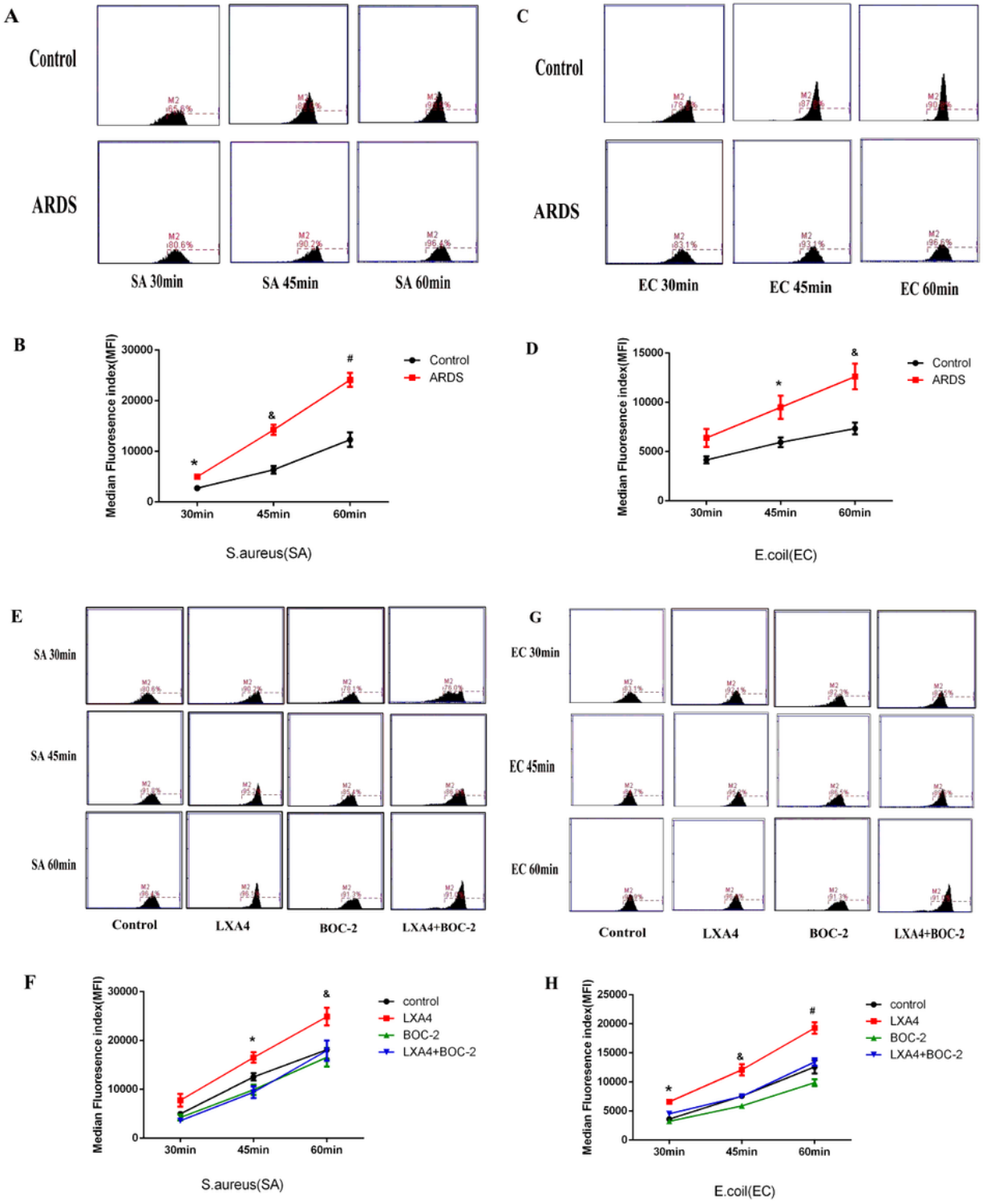

Figure 5

LXA4 enhances phagocytosis of neutrophils in ARDS rats by binding to ALX receptor (A-B) Comparison of the phagocytosis of S. aureus (SA) between control rats and ARDS rats. The data are presented as the mean ± SEM. n =10.*P0.05 SA ARDS 30 min vs SA Control 30 min&P0.05 SA ARDS 45 min vs SA Control 45 min#P0.05 SA ARDS 60 min vs SA Control 60 min (C-D) Comparison of the phagocytosis of E.coli (EC) between control rats and ARDS rats. The data are presented as the mean ± SEM. n =10. *P 0.05 ECARDS 45 min vs EC Control 45 min&P0.05 EC ARDS 60 min vs EC Control 60 min (E-F) LXA4 enhances phagocytosis S. aureus (SA) of neutrophils in ARDS rats by binding to ALX receptorThe data are presented as the mean ± SEM. n =10.*P0.05 SA LXA4 45 min vs SA Control 45 minSA BOC-2 45 minSA LXA4+BOC-2 45 min&P0.05 SA LXA4 60 min vs SA Control 60 minSA BOC-2 60 minSA LXA4+BOC-2 60 min (G-H) LXA4 enhances phagocytosis E.coliECof neutrophils in ARDS rats by binding to ALX receptorThe data are presented as the mean ± SEM. n =10.*P0.05 SA LXA4 45 min vs SA Control 45 minSA BOC-2 45 minSA LXA4+BOC-2 45 min&P0.05 SA LXA4 60 min vs SA Control 60 minSA BOC-2 60 minSA LXA4+BOC-2 60 min

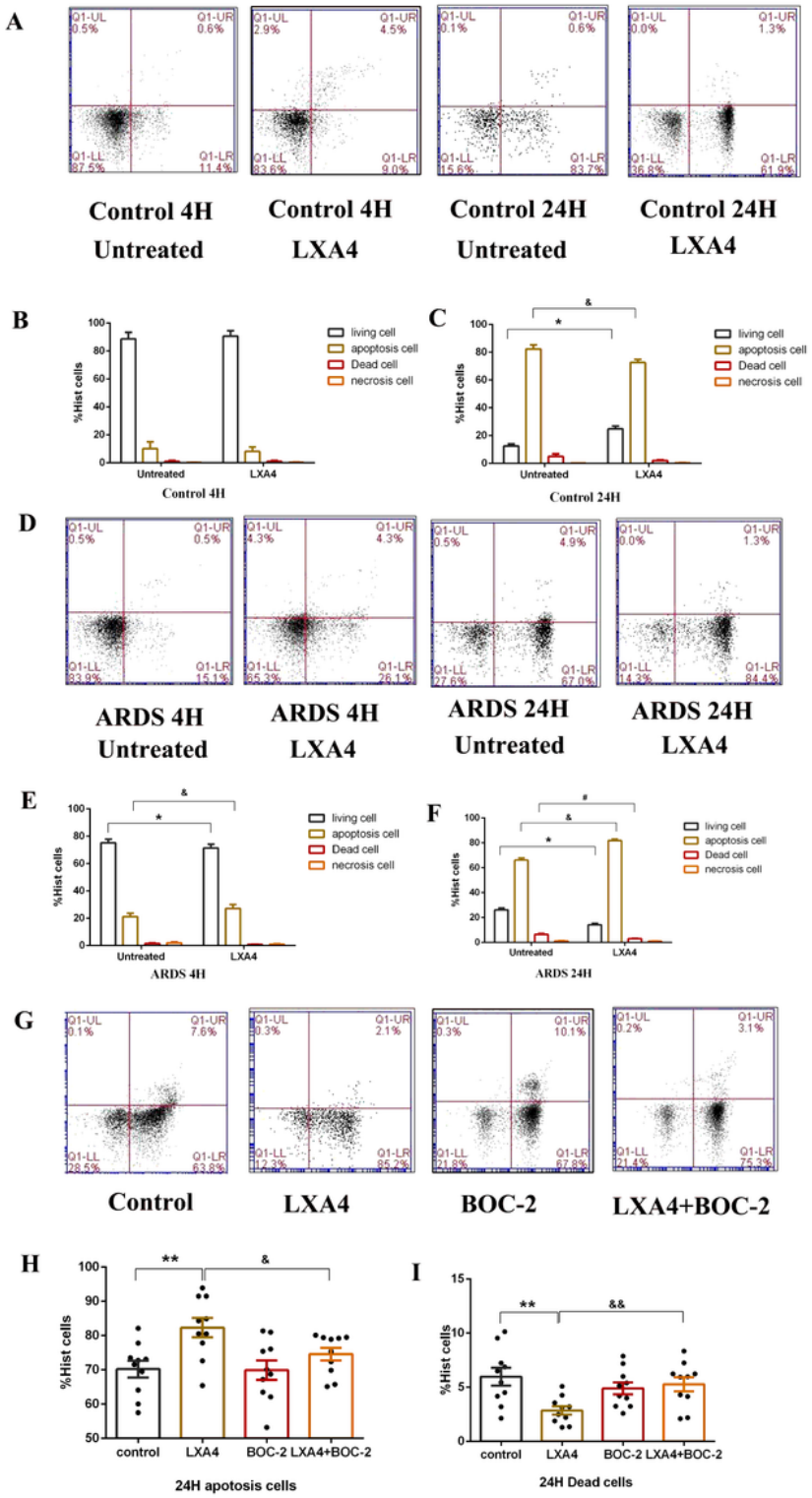

Figure 6

Effects of LXA4 on neutrophil apoptosis and NETosis in ARDS rats (A-C) The effect of LXA4 on neutrophil apoptosis in control rats was detected by flow cytometry.The data are presented as the mean ± SEM. n =10. *P0.05 living cell :control vs LXA4&P0.05 apoptosis cell: LXA4 vs Control (D-F) The effect of LXA4 on neutrophil apoptosis in ARDS rats was detected by flow cytometry.The data are presented as the mean ± SEM. n =10. **P0.05 living cell :control vs LXA4 &P0.05 apoptosis cell: LXA4 vs Control#P

0.05 Dead cell :LXA4 vs Control (G-I) Effects of LXA4 on neutrophil apoptosis and NETosis in ARDS rats.The data are presented as the mean ± SEM. n =10. **P0.01 control vs LXA4&P0.05 LXA4 vs LXA4+BOC-2, &&P0.01 LXA4 vs LXA4+BOC-2n=10

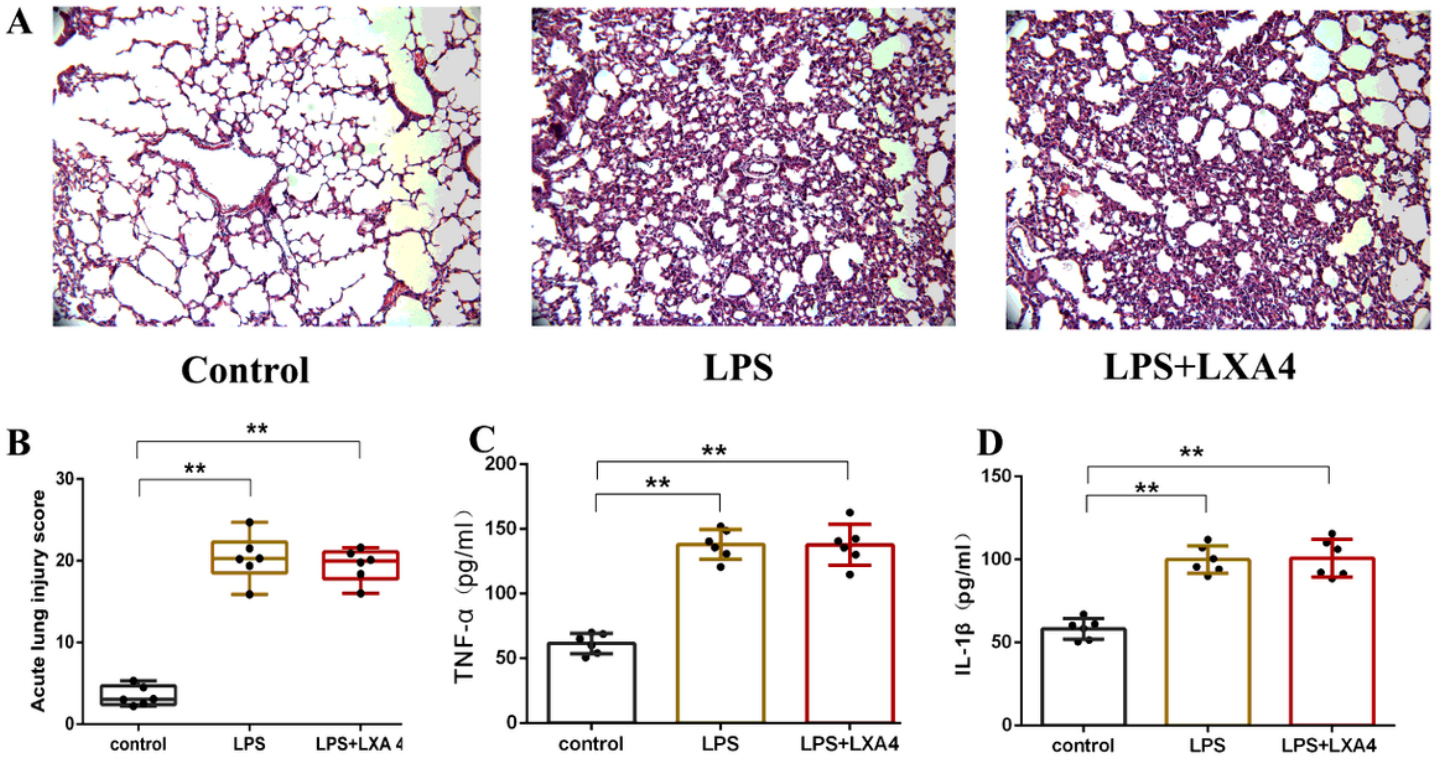

Figure 7

LXA4 has no protective effect on the rat model of acute lung injury with neutrophil deficiency. (A) The lung tissues were obtained immediately after exsanguination (6 h after LPS), and the effect of LXA4 100 ng/kg was assessed histologically in H&E-stained sections (original magnification ×200). (B) Lung injury scores. The data are presented as the mean ± SEM. n =6. **P0.01 Control vs LPS and LPS+ LXA4 (C) The lung tissue homogenate TNF-α protein expression. the data are presented as the mean ± SEM. n =6. **P0.01 Control vs LPS and LPS+LXA4 (D) the lung tissue homogenate IL-1β protein expression. the data are presented as the mean ± SEM. n =6. **P0.01 Control vs LPS and LPS+LXA4