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Nur77/NR4A1 Regulates Lung Macrophage Polarization in Repairing LPS-Induced Lung Damage in Acute Respiratory Distress Syndrome

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Abstract

Background: Acute respiratory distress syndrome (ARDS) is a life-threatening condition characterized by severe pulmonary inflammation and high mortality, with no effective pharmacological treatments. The orphan nuclear receptor Nur77/NR4A1 has been implicated in inflammation regulation, but its specific role in macrophage polarization and lung repair in ARDS remains unclear.

Objective: This study investigates the protective effects of Nur77/NR4A1 activation on macrophage polarization and lung tissue repair in a lipopolysaccharide (LPS)-induced ARDS mouse model.

Methods: A total of 144 SPF-grade C57BL/6 mice were randomly divided into four groups: NS, LPS, CsnB, and CsnB control. ARDS was induced by intratracheal instillation of LPS, and samples were collected at 4h, 12h, and 24h post-induction. Lung damage was assessed by wet/dry weight ratio, BALF protein concentration, TNF-α levels, histopathological scoring, and analysis of macrophage polarization markers using immunohistochemistry and Western blot.

Results: Compared to the LPS group, CsnB treatment significantly reduced lung injury, characterized by decreased wet/dry weight ratio, BALF protein levels, and TNF-α concentration, along with improved histological scores. Immunohistochemical and biochemical analyses showed that CsnB promoted M2 macrophage polarization, indicated by increased Arg-1 expression, and suppressed M1 markers such as iNOS. Additionally, CsnB intervention reduced IL-1β expression and accelerated tissue repair, mitigating inflammation over time.

Conclusion: Nur77/NR4A1 modulates macrophage polarization and reduces inflammatory injury, protecting against LPS-induced ARDS. These findings provide a promising therapeutic target for ARDS management.

Keywords: Nur77, NR4A1, Macrophages, Macrophage Polarization, Acute Respiratory Distress Syndrome

1. Introduction

With the in-depth study of the orphan nuclear receptor superfamily, its molecular biological network has been gradually established, and the mechanism of action in the pathophysiological process of inflammation-related lung diseases has been further understood. Studies have shown that the orphan nuclear receptor Nur77/NR4A1, as a member of this family, is involved in the pathophysiological process of acute respiratory distress syndrome (ARDS). Nur77/NR4A1 is rapidly induced and activated under a variety of physiological and pathological stimuli and has complex biological functions (L. Chen et al., 2020; Crean & Murphy, 2021; Maxwell & Muscat, 2006). Nur77 has multiple roles in the pathophysiology of disease: e.g., it resists apoptosis of pancreatic β-cells and is involved in glycogen synthesis (O et al., 2012). In inflammation, it affects the inflammatory process through multiple pathways (*Key Functions and Therapeutic Prospects of Nur77 in Inflammation Related Lung Diseases - PubMed*, n.d.). It inhibits endothelial-to-mesenchymal transition and attenuates cardiac fibrosis after Myocardial Infarction (*Nur77 Deficiency Exacerbates Cardiac Fibrosis after Myocardial Infarction by Promoting Endothelial-to-Mesenchymal Transition - PubMed*, n.d.). Nur77 is highly expressed in a variety of tumors and can act as a target protein for oncogenic proteins to promote tumor development (So et al., 2010). It plays a role in the pathology of neurodegenerative diseases such as Alzheimer's disease (Liu et al., 2021). ARDS is a common critical disease of the respiratory system. Its essence is a lung inflammatory response indirectly mediated by a variety of inflammatory cells (such as macrophages, neutrophils, alveolar epithelial cells, vascular endothelial cells and platelets) and cytokines. ARDS is also a pulmonary manifestation of systemic inflammatory response syndrome (SIRS), and its pathogenesis has not yet been fully elucidated. Currently, the treatment of ARDS mainly relies on non-drug intervention measures such as organ support, lung protective ventilation and fluid management, and lacks effective drug treatment(Matthay et al., 2017). The bottlenecks in drug development mainly include: (1) high heterogeneity of ARDS patients(Bos & Ware, 2022a): as a group of clinical syndromes, patients have significant differences in etiology, pathophysiological processes and clinical manifestations, which may lead to the failure of drug clinical trials; (2) patients have different responsiveness to drug treatment; (3) inappropriate targets may be selected. As a result, the mortality and morbidity of ARDS remain high. Studies have shown that macrophage polarization plays an important role in the repair of ARDS lung injury, and Nur77/NR4A1 can regulate the polarization of mouse bone marrow-derived macrophages (BMMs) and peritoneal macrophages (PMs), and has important functions in inflammatory response and tissue damage repair(Garabuczi et al., 2023a; Hanna et al., 2012; Murphy & Crean, 2015), but its role in lung macrophage polarization is still unclear. Studies have found that Nur77/NR4A1 downregulates endothelin-1 (ET-1) expression in LPS-induced ARDS rats and A549 cells by inhibiting NF-κB and p38MAPK signaling pathways, thereby exerting an organ protective effect (Y. Jiang et al., 2016). CsnB, an agonist of Nur77/NR4A1, can reduce the inflammatory response of neutrophils in LPS-induced ARDS rats by downregulating ET-1 expression, alleviating pathological damage to lung tissue, promote tissue repair, and improve hypoxemia, pulmonary edema, and liver and kidney dysfunction (Oita et al., 2009). Therefore, we speculate that in the pathophysiological process of ARDS, Nur77/NR4A1 may promote lung tissue damage and repair by regulating macrophage polarization. Exploring the regulatory mechanism of Nur77/NR4A1 in LPS-induced lung tissue damage

and repair in ARDS is expected to provide new potential targets for the treatment of ARDS.

2. Materials and Methods

2.1 Experimental animals

This study used 144 SPF C57BL/6 mice aged 6-8 weeks (either sex, weighing 18-25 g), provided by the Animal Experiment Center of Youjiang Medical College for Nationalities, license number: SCXK(Beijing) 2021-0006. All mice were housed in an SPF environment with a constant temperature (22 ± 2 °C) and constant humidity (50 ± 5%), with a 12-h light/dark cycle, standard laboratory feed and sterile water, free diet, and adaptive feeding for 1 week. The experimental operation strictly followed the animal ethics guidelines, and the experimental protocol was approved by the Animal Ethics Committee of Youjiang Medical College for Nationalities (approval number: 2022041901) to minimize animal suffering.

2.2 Experimental materials and equipment

The main reagents include: LPS (lipopolysaccharide, O111:B4): purchased from Sigma-Aldrich (USA). Nur77 agonist Cytosporone B (CsnB): purchased from MCE (China). Sulfonbutyl-β-cyclodextrin sodium: Solarbio (China). HE staining kit, ELISA kit (TNF-α, IL-10), immunohistochemistry kit, BCA protein quantification kit, etc., were all purchased from relevant Chinese companies. Primary and secondary antibodies, from Abcam, Proteintech, Wuhan Mitaka, etc.

The main equipment includes: Inverted fluorescence microscope (Zeiss, Axio Vert.A1), paraffin slicer (Leica, RM2235), tissue dehydrator (Thermo Fisher, Excelsior AS), microplate reader (BioTek, Synergy H1), etc.

2.3 Grouping and treatment

The experimental mice were randomly divided into the following 4 groups, with 36 mice in each group:

1. Control group (NS group): intratracheal instillation of equal volume of sterile saline (1 mg/mL).

2. Model group (LPS group): intratracheal instillation of LPS solution (1 mg/mL).

3. CsnB intervention group (CsnB group): intraperitoneal injection of CsnB (13 mg/kg, dissolved in sodium sulfobutyl-β-cyclodextrin) for 1 hour before intratracheal instillation of LPS (1 mg/mL).

4. CsnB cosolvent control group (CsnB control group): intratracheal instillation of LPS (1 mg/mL) after intraperitoneal injection of equal volume of cosolvent for 1 hour.

Mice in each group were sampled 4 hours, 12 hours, and 24 hours after intervention, and 12 mice were sampled at each time point.

2.4 ARDS model construction

Mice were anesthetized by by 100 mg/kg Ketamine + 10 mg/kg Xylazine, prepared a ratio of 10 mg/mL ketamine to 1 mg/mL xylazine, the mixture will be administered via intraperitoneal injection at a dosage of 0.1 ml/10 g body weight.. After anesthesia, the mice were fixed in supine position, the glottis was exposed, and endotracheal intubation was performed orally with a soft mouse endotracheal tube. Sterile LPS was aspirated and dripped into the mouse trachea at 1 mg/ml using a pipette. Mice in the NS group were dripped intratracheally with NS. After the instillation, the mouse chest was gently massaged several times to ensure even distribution of the liquid, and then the mouse was placed in a 37 °C incubator for warmth and returned to the cage after full awakening. Lung tissue and BALF were collected 4h, 12h, and 24h after the end of LPS instillation.

2.5 HE staining

HE staining was performed following standard histological protocols(Suvarna et al., 2019). Briefly, at each time point, mouse left lung tissues were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4 μm. Sections were stained with hematoxylin and eosin, dehydrated through graded ethanol, cleared in xylene, and sealed. Pathological changes were evaluated under light microscopy to assess inflammation and lung injury.

2.6 Mouse lung tissue W/D evaluation of pulmonary edema

At each time point, the left lung tissue of the mouse was taken, first washed with sterile PBS for a few seconds, then the water and blood on the surface of the tissue were gently absorbed with absorbent paper, and the wet weight was weighed. The tissue was placed in a 65 °C oven for 72 hours to constant weight and then the dry weight was weighed. The ratio of wet weight to dry weight (W/D) is used to evaluate the degree of pulmonary edema. A higher W/D ratio indicates more severe edema in the lung tissue.

2.7 Bronchoalveolar lavage fluid (BALF) total protein concentration detection

At each point, mice were anesthetized by intraperitoneal injection of 4% chloral hydrate (0.2 mL/20 g body weight). The mouse was fixed in a supine position, the trachea was exposed, and a PE-50 catheter was inserted into the tracheal bifurcation. The lungs were slowly lavaged with sterile PBS, 1 mL each time, for a total of 3 lavages, ensuring that the lavage fluid recovery rate was > 85%. After collecting the lavage fluid, centrifuge at 12000 r/min for 30 minutes at 4 °C, and take the supernatant for BCA method to determine the protein concentration. The specific steps are: according to the instructions of the BCA kit, mix the sample with the color working solution (reagent A and reagent B are mixed at a ratio of 50:1), add 200 μL to each well of the 96-well plate, incubate at 37 °C for 30 minutes, use an enzyme reader to measure the absorbance at 540-590 nm, and draw a standard curve to calculate the protein concentration.

2.8 ELISA detection of TNF-α and IL-10 levels in lung tissue

ELISA kits were used to detect TNF-α and IL-10 levels in lung tissue. Homogenized lung tissues were centrifuged (12,000 rpm, 20 min, 4°C), and supernatants were collected. Following the ELISA kit instructions, 50 μL of standards and samples were added to 96-well plates, followed by 50 μL of HRP-labeled antibody, and incubated at 37°C for 1 h. After washing five times, 50 μL of substrate solutions A and B were added, incubated in the dark at 37°C for 15 min, and stopped with 50 μL of stop solution. Absorbance was measured at 450 nm, and cytokine concentrations were calculated using a standard curve.

2.9 Immunohistochemistry (IHC) to detect the expression of iNOS, Arg-1 and RAGE

Immunohistochemistry (IHC) was performed to detect iNOS, Arg-1, and RAGE expressions. Paraffin sections were dewaxed in xylene, rehydrated through graded ethanol, and subjected to antigen retrieval in sodium citrate buffer (pH 6.0) using a microwave (15 min). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min, followed by blocking with normal goat serum for 30 min. Sections were incubated overnight at 4°C with primary antibodies (iNOS, 1:500, Arg-1, 1:400, RAGE, 1:300). After washing, biotinylated secondary antibodies were applied at 37 °C for 30 min. DAB was used for color development, and hematoxylin counterstaining was performed. Slides were dehydrated, cleared in xylene, and sealed for microscopic analysis.

2.10 Immunofluorescence (IF) detection of iNOS and Arg-1 expression

Lung tissue sections were baked at 65℃ for 2h, deparaffinized with xylene, hydrated with gradient ethanol and washed with ddH_2O. Microwave heat-induced antigen repair method was used, and the sections were boiled in citrate buffer at medium-high heat for 7.5 min and then cooled down. 4% paraformaldehyde fixation, 0.5% tralatone permeabilization membrane, and protein-free containment solution were used for closure. iNOS and Arg-1 primary antibodies were diluted at 1:500 and 1:400, respectively, incubated at 4℃ overnight, and rewarmed on the next day. 1:800 dilution of secondary antibody was incubated at room temperature for 1h, and DAPI was used for re-staining, and fluorescence sealing was performed after microscopic observation. Each step was washed thoroughly with PBS to ensure experimental standardization.

2.11 Western Blot detection of IL-1β and Arg-1 expression

Western blot analysis was conducted to detect IL-1β and Arg-1 expression. following standard protocols,lung tissues were homogenized in RIPA buffer with protease and phosphatase inhibitors, and supernatants were collected by centrifugation(12,000 rpm, 15 min, 4°C). Protein concentrations were determined using the BCA method, and 30 μg of protein per well was separated by SDS-PAGE (80 V for stacking gel, 120 V for separating gel). Protein were transferred to a PVDF membrane (300 mA, 90 min), blocked with 5% skim milk for 1 h, and incubated with primary antibodies (IL-1β, 1:1000; Arg-1, 1:2000) overnight at 4 °C. After washing with TBST, HRP-conjugated secondary antibodies were applied for 1 h at room temperature. Protein bands were visualized using ECL chemiluminescence and imaged with a gel documentation system.

3. Results

3.1 Histopathological changes in lung tissue of mice observed by HE staining

NS Group：At 4h, 12h and 24h, the lung tissue was structurally intact, with uniform alveolar intervals and no edema, hemorrhage or inflammatory cell infiltration;

LPS Group：4h the lung tissue structure is more or less intact, the alveolar septum is still homogeneous, without obvious edema, erythrocytes and inflammatory cell infiltration; 12h the lung tissue structure integrity is missing, the alveolar septum is thickened, most of the alveoli are collapsed, and some erythrocytes can be seen in the alveolar lumen, accompanied by edema fluid and inflammatory cell infiltration; 24h the lung tissue damage is obvious, the lung tissue is almost lack of integrity, the alveolar septum is thickened obviously, alveoli are collapsed, and erythrocytes can be seen in the alveolar lumen. Erythrocytes, a large amount of edema fluid and a large number of inflammatory cells infiltration, part of the hyaline membrane can be seen;

CsnB Group：Compared with the LPS group at 4h, 12h and 24h, the degree of lung injury was reduced, the lung tissue structure was still intact, the alveolar septum was slightly widened, and the erythrocytes, edema fluid and inflammatory cell infiltration were significantly reduced;

CsnB control Group: The degree of lung injury at each time period of 4h, 12h and 24h was similar to that of the LPS group at each time period(Figure 1).

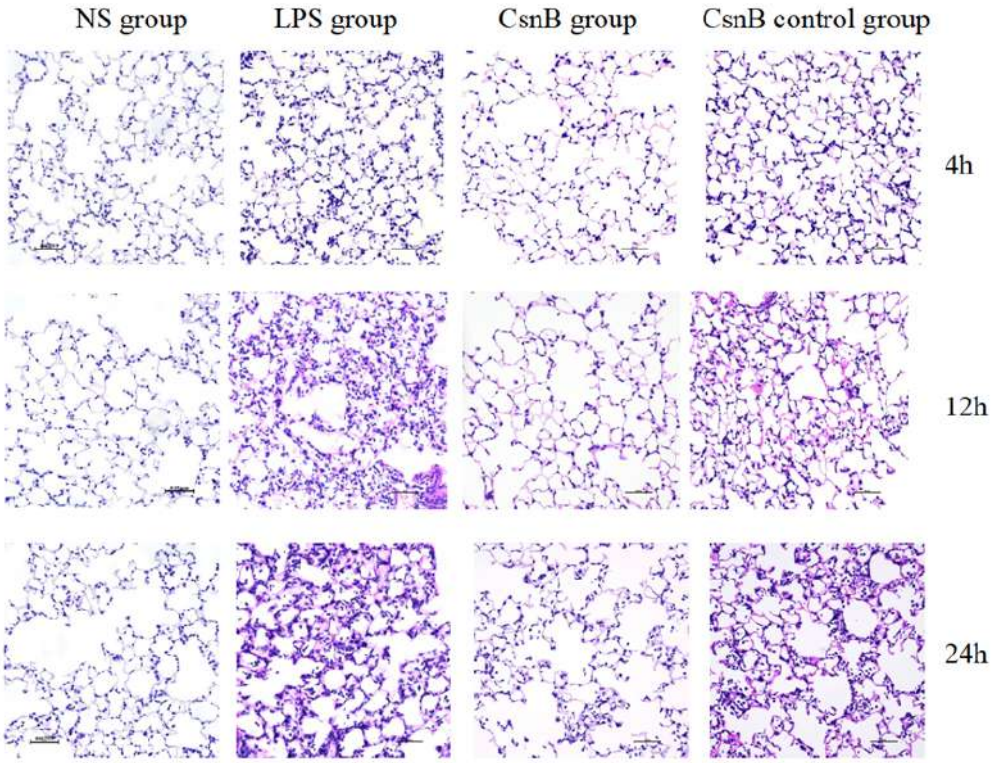

Figure 1: Pathological changes in the lung tissues (400×)Bars, 50μm.

3.2 Comparison of W/D in mouse lung tissue

The lung tissue W/D ratios in the 4h LPS group (4.406±0.349), 12h LPS group (5.279±0.349), 24h LPS group (6.657±1.129), 4h CsnB control group (4.328±0.175), 12h CsnB control group (5.499±0.510), 24h CsnB control group (5.507±0.129) lung tissue W/D ratios were significantly higher than those of the corresponding 4h NS (3.221±0.388), 12h NS group (2.725±0.527), and 24h NS group (3.266±0.851), respectively (p<0.05); and the 4h CsnB group (3.969±0.149) after CsnB intervention, 12h CsnB group (4.667±0.333), and 24h CsnB group (4.691±0.626) W/D ratios were decreased compared with the CsnB control group in the corresponding time periods (p < 0.05). It was similar to the trend of pathological dynamics of lung injury (Figure 2).

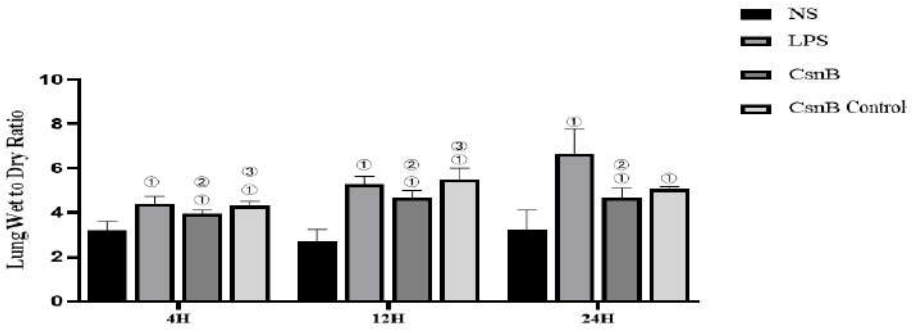

Figure 2: The Lung Tissue Wet/Dry Ratio

Note: ① p<0.05 compared with NS group, ② p<0.05 compared with LPS group ③ p<0.05 compared with CsnB group.

3.3 Comparison of total protein content in mouse BALF

After 4h of LPS infusion, the protein content in BALF among the four groups of mice did not change significantly, and after 12h of LPS infusion, the protein content in BALF of mice in the LPS group (642.200±195.615) and the CsnB control group (569.584±37.471) was elevated compared with that in the NS group (265.713±100.835) (p<0.01).), after CsnB intervention, 12h protein content in BALF of CsnB group (376.048±138.501) was significantly lower than that of LPS group and CsnB control group (p<0.05); after 24h of LPS titration, the protein content in BALF of LPS group (752.634±160.995), CsnB control group (810.603±161.744) was elevated (p<0.01) compared to NS group (231.734±59.968), and after CsnB intervention, the protein content in BALF of 24h CsnB group (387.487±142.940) was significantly reduced (p<0.05) compared to LPS group and CsnB control group(Figure 3).

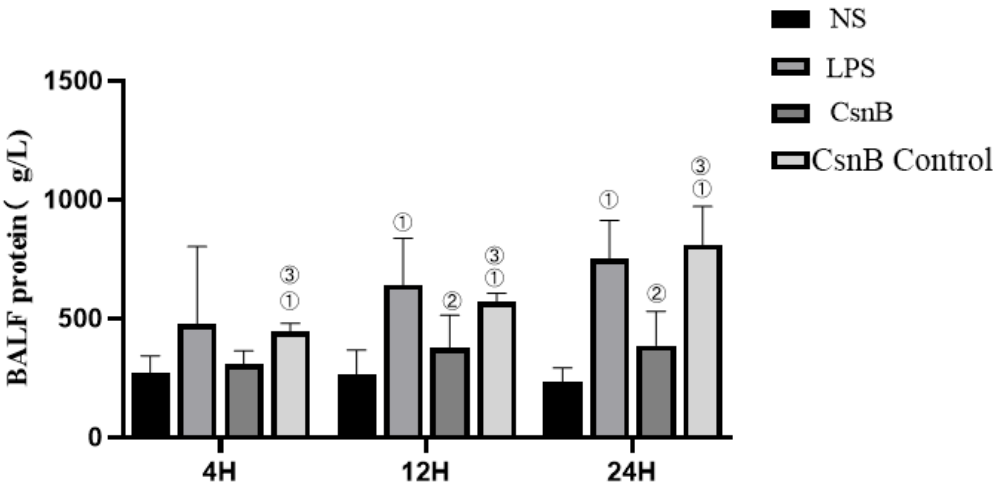

Figure 3: The Protein Levels in The BALF

Note: ① p<0.05 compared with the NS group, ② p<0.05 compared with the LPS group ③ p<0.05 compared with the CsnB group.

3.4 ELISA for inflammatory factor levels in lung tissue

After 4h of LPS titration, compared with the NS group, the lung tissue TNF-α content in the LPS group (509.755±55.582) and the CsnB control group (617.225±31.883) mice was elevated compared with that in the NS group (345.827±29.909) (p<0.05); after CsnB intervention, the lung tissue TNF-α content in the CsnB group (271.129± 35.651) mice lung tissue TNF-α content was significantly lower (p < 0.05) compared with the LPS group and CsnB control group; after 12 h of LPS titration, compared with the NS group, the lung tissue TNF-α content in the LPS group (926.039 ± 39.245) and the CsnB control group (966.301 ± 52.414) mice lung tissue was significantly higher (p < 0.05) than that in the NS group (356.383±23.144) was elevated (p<0.05), and after CsnB intervention, the lung tissue TNF-α content of mice in CsnB

group (336.782±39.531) was significantly lower than that in the LPS group and CsnB control group (p<0.05); and after 24h of LPS titration, the LPS group (1241.704±70.581), CsnB control group (1506.663±100.362) mice had elevated protein content in BALF compared with NS group (382.503±22.119) (p<0.01), and after CsnB intervention, the protein content in BALF of CsnB group (419.531±36.544) at 24h was significantly lower than that of the LPS group and the CsnB control group (p < 0.05)(Figure 4).

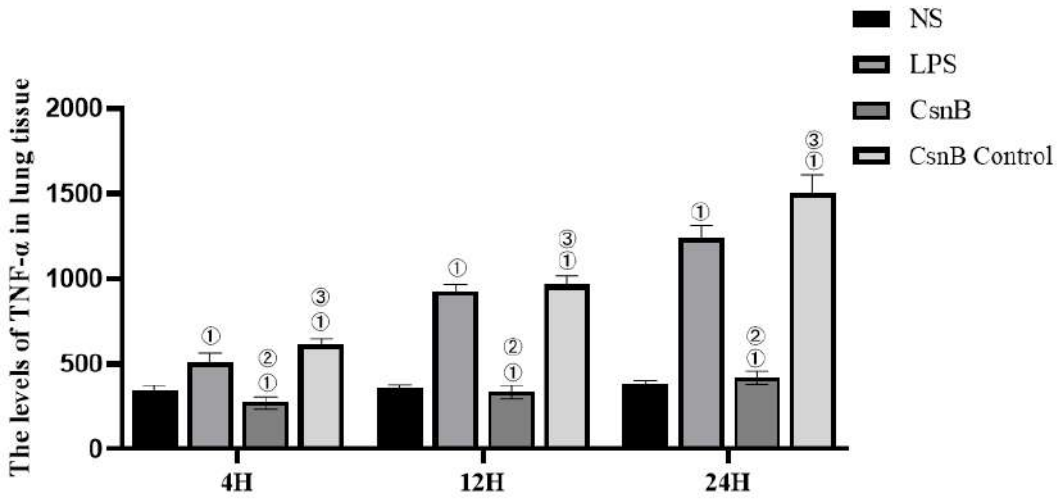

Figure 4: The Levels Of TNF-A In Lung Tissue.

Note: ① p<0.05 compared with the NS group, ② p<0.05 compared with the LPS group ③ p<0.05 compared with the CsnB group.

3.5 Immunohistochemical (IHC) detection of RAGE expression in lung tissue

After 4h, 12h and 24h of LPS titration, the RAGE expression levels of LPS group, CsnB group and -dependent(Figure 5).

CsnB control group were significantly higher than those of NS group at each time period (p<0.01), which were significantly lower than those of LPS group after CsnB intervention at each time period (p<0.01). The expression levels of RAGE in the LPS group and the CsnB control group at each time period were time

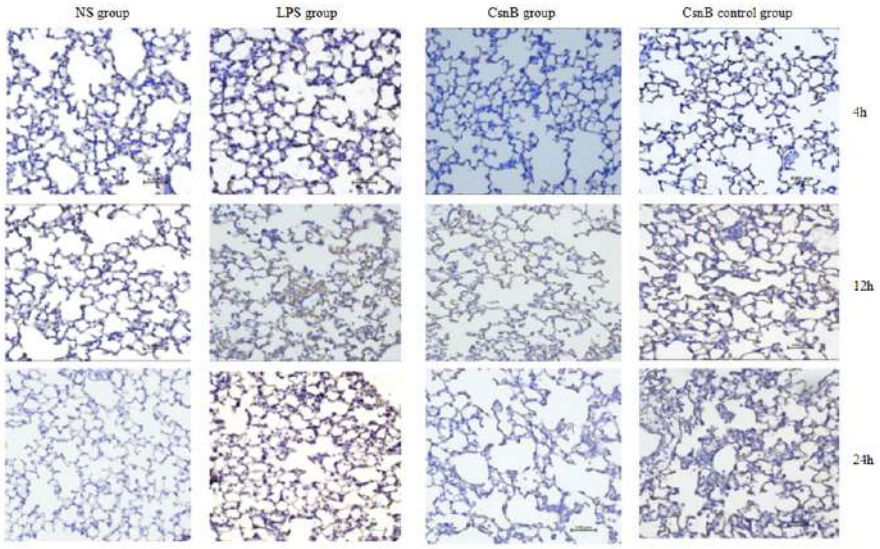

Figure 5: Expression of RAGE, a marker of acute lung injury in mice (400×) Bars, 50μm.

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3.6 Detection of iNOS and Arg-1 expression in lung tissues by immunohistochemistry (IHC)

Macrophage polarization plays an important role in the repair of lung tissue injury in ARDS. In this experiment, we observed the expression of M1-type macrophage marker iNOS and M2-type macrophage marker Arg-1 in lung tissues at 4h, 12h and 24h by immunohistochemistry(Figure 6, Figure 7).

In 4h, 12h, 24h ARDS model, compared with NS group and CsnB group, LPS group and CsnB control group presented more positive signals of iNOS and Arg-1, but their positive signals were positively expressed in airway epithelial cells, alveolar epithelial cells, and alveolar macrophages, and their expression lacked specificity.

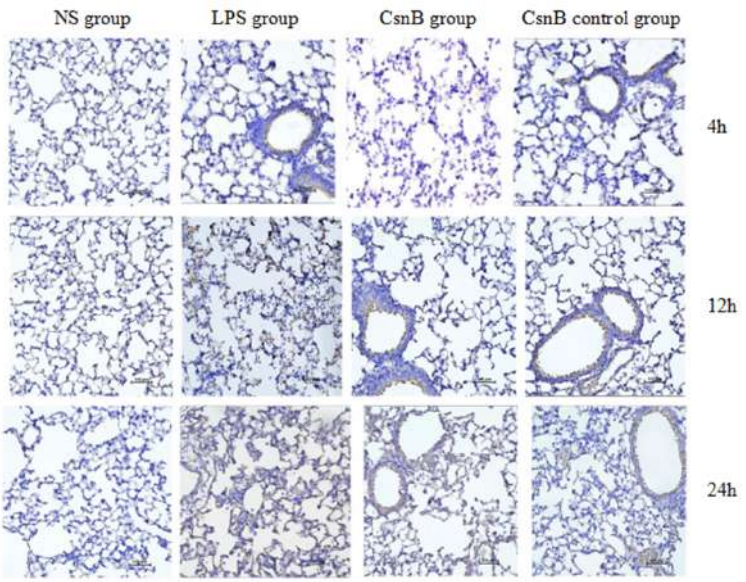


Figure 6: Expression of Inos on M1 Alveolar Macrophages in Lung Tissue (400×) Bars, 50μm.

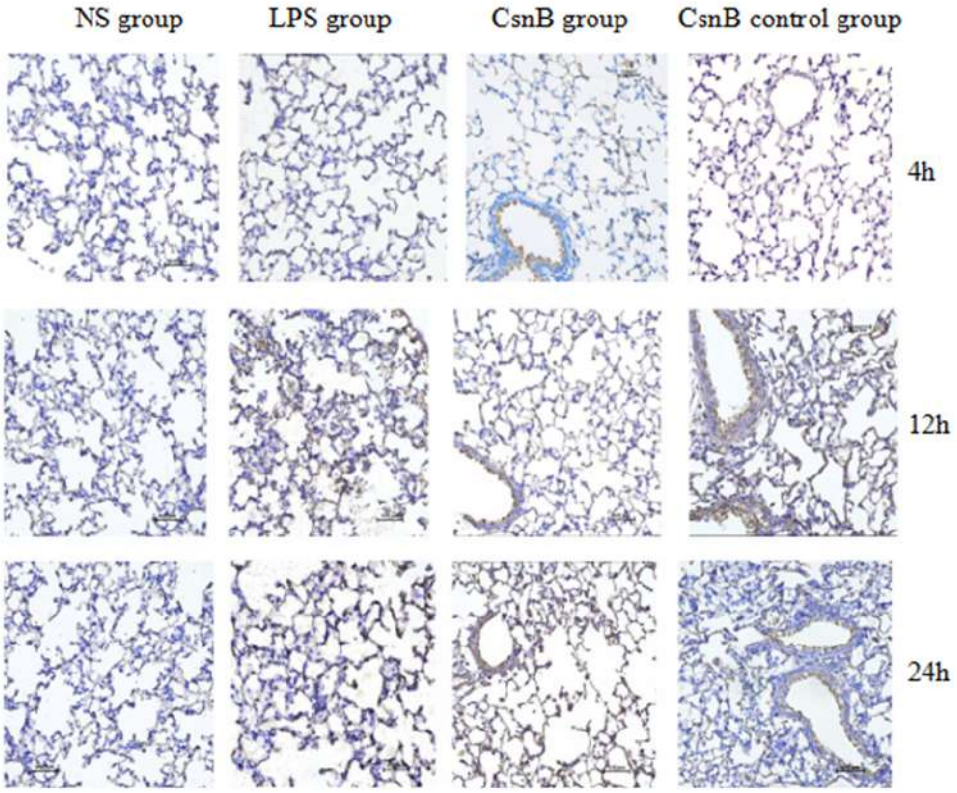
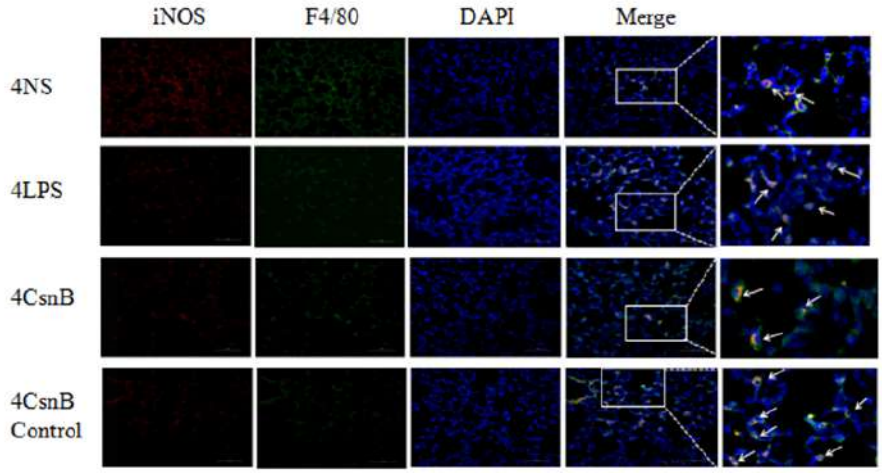


Figure 7: Expression of Arg-1 on M1 alveolar macrophages in lung tissue (400×)Bars, 50μm.

3.7 Detection of iNOS and Arg-1 expression in alveolar macrophages by immunofluorescence (IF) assay

Macrophage polarization plays an important role in the repair of lung tissue injury in ARDS. In this experiment, we assessed the polarization status of the M1-type macrophage marker iNOS and the M2-type macrophage marker Arg-1 in the repair of lung injury at 4h, 12h, and 24h by immunofluorescence using F4/80 labeling of macrophages in lung tissue (Figure 8, Figure 9).



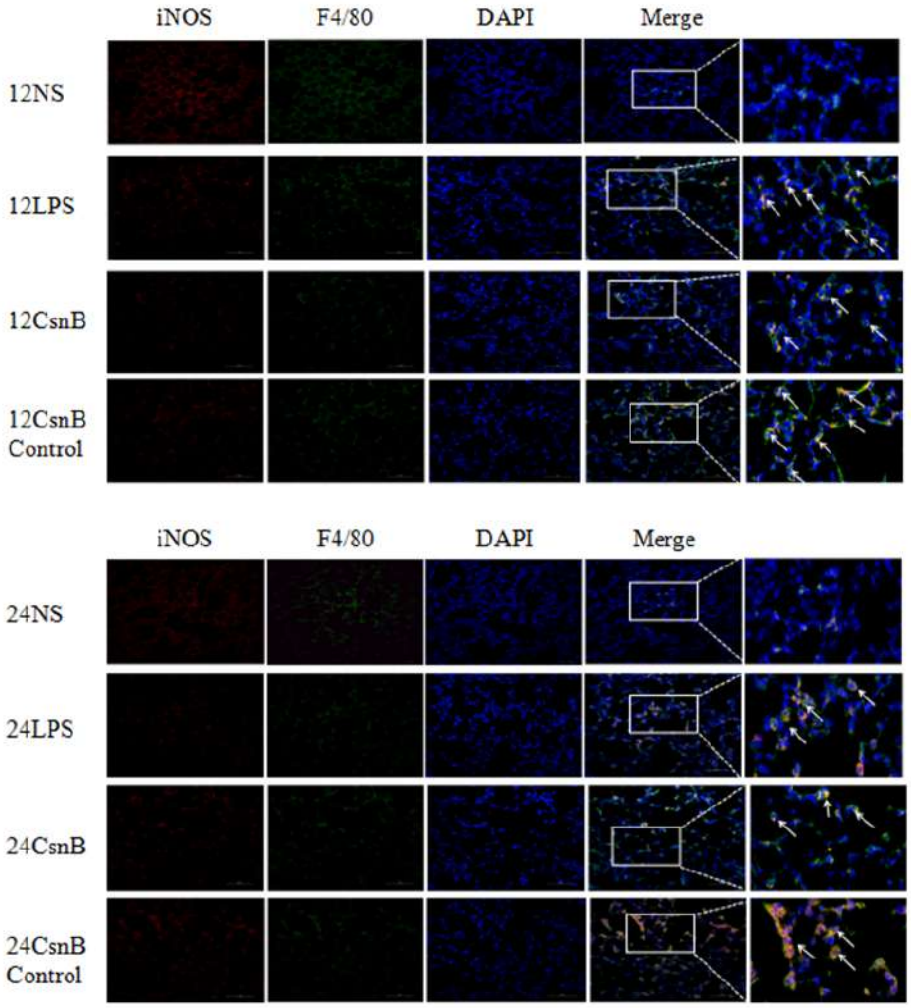


Figure 8: Expression of iNOS on M1 alveolar macrophages in mouse tissue (400×) Bars, 50μm.

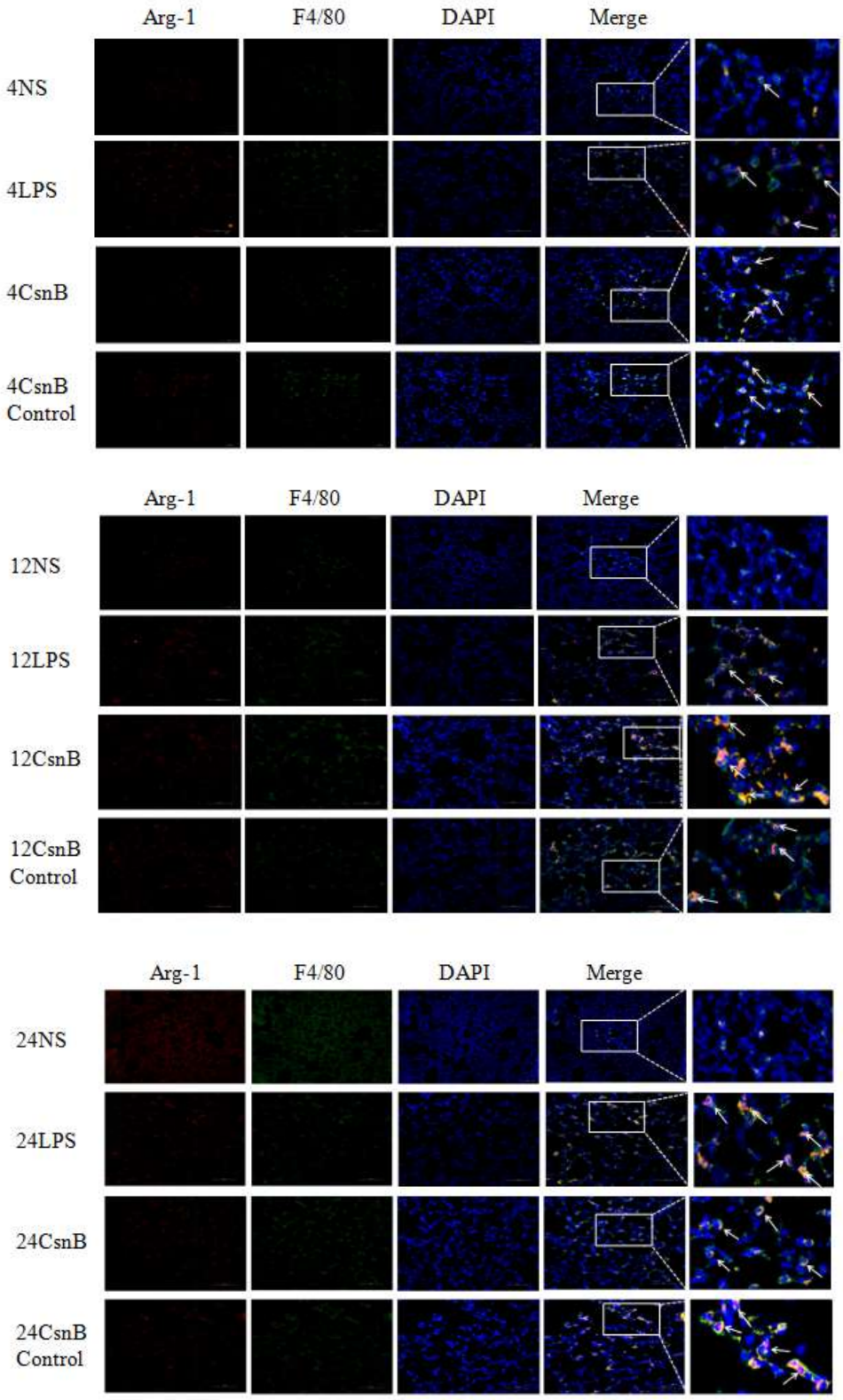


Figure 9: Expression of Arg-1 on M1 alveolar macrophages in lung tissue (400×) Bars, 50μm.

It can be observed that the expression levels of M1-type macrophage marker iNOS in the 4h, 12h and 24h NS groups were less than those in the corresponding 4h, 12h and 24h LPS groups, and the expression levels of M1-type macrophage marker iNOS gradually increased with the progression of lung injury, suggesting that the macrophages had initiated the inflammatory network and started to polarize towards M1-type macrophages at 4h after the establishment of the lipopolysaccharide-induced mouse ARDS model. At the same time, it can be observed that 4h, 12h and 24h after the intervention of Nur77 agonist CsnB, the CsnB group was reduced compared with the corresponding time period of the LPS group and the CsnB control group, suggesting that the Nur77 agonist CsnB can reduce the macrophage polarization to the M1-type macrophage, and improve the lung tissue injury in order to play a protective role.

At 4h after the establishment of the mouse ARDS model, the M2-type macrophage surface marker Arg-1 was slightly expressed in the LPS group, the CsnB group and the CsnB control group compared with that in the NS group, but there was no significant difference; at 12h after the progression of the lung injury, it could be observed that the M2-type macrophage surface marker Arg-1 was slightly expressed in the LPS group and the CsnB control group, but the expression level increased in the CsnB group, increased in the CsnB group, suggesting that after intervention with the Nur77 agonist CsnB, compared with the decrease in the expression level of the M1-type macrophage marker iNOS, the resident macrophages or M1-type macrophages had begun to polarize to M2-type macrophages by 12 h of lung injury progression, suggesting that the Nur77 agonist CsnB may regulate the polarization of macrophages to M2-type macrophages, to Reversing the anti-inflammatory microenvironment and attenuating lung injury; at 24h of lung injury progression, it could be observed that the M2-type macrophage surface marker Arg-1 was highly expressed in the LPS group, CsnB group, and the CsnB control group, compared with the expression level of iNOS, a M1-type macrophage marker, in the corresponding groups at 24h, suggesting that without the intervention of the Nur77 agonist CsnB, the The ratio of M1 macrophages to M2-type macrophages in the pathological process of lung injury began to reach equilibrium in the inflammatory microenvironment at 24h, whereas after the intervention of the Nur77 agonist CsnB, an elevated level of Arg-1, a surface marker for M2-type macrophages, was observed in the same group as in the 12h CsnB group.

3.8 Detection of IL-1β and Arg-1 protein expression levels in lung tissues by Western blot method

The expression levels of M1-type macrophage marker IL-1β and M2-type macrophage marker Arg-1 in lung tissues were detected by Western blot at 4h, 12h and 24h after the establishment of mouse ARDS model. The results of the experiment showed that the protein level of M1-type macrophage marker IL-1β in the LPS group and CsnB control group was higher than that in the NS group at 4h, 12h and 24h, while its expression was significantly reduced after the application of the Nur77 agonist CsnB intervention; there was no significant expression of the protein level of the M2-type macrophage marker Arg-1 in the groups at 4h; and the results of the 12h showed that the M2-type macrophage marker Arg-1 was highly expressed in the CsnB group. macrophage marker Arg-1 protein level; while NS group, LPS group and CsnB control group had no significant expression; 24h results showed that LPS group, CsnB group and CsnB control group had significant expression (Figure 10).

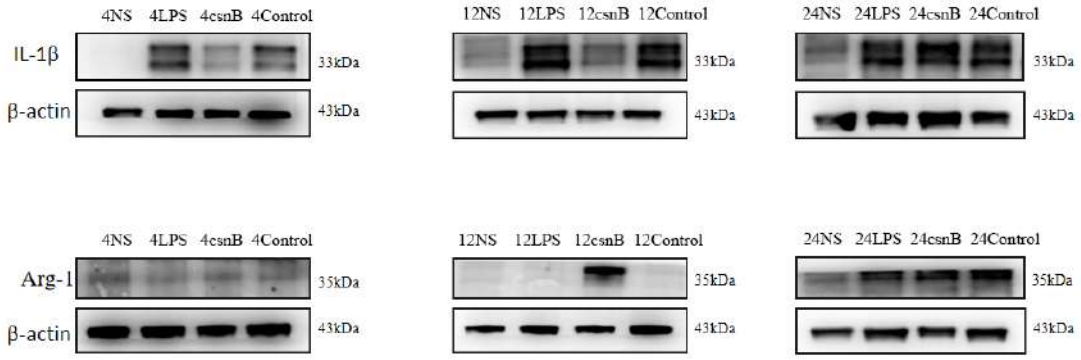


Figure 10: Levels of IL-1β and Arg-1 in lung tissue of mice (400×) Bars, 50μm.

4. Discussion

1 Current Status of ARDS and Treatment Strategies

Acute Respiratory Distress Syndrome (ARDS) continues to be a major challenge in the field of critical care and is characterized by high morbidity and mortality rates (Gorman et al., 2022). This life-threatening disease is characterized by decreased pulmonary compliance, refractory hypoxemia, and respiratory distress (Gorman et al., 2022), Caused by widespread inflammation of the lungs. ARDS is driven by an inflammatory process involving multiple cell types, including macrophages, neutrophils, alveolar epithelial cells, and vascular endothelial cells, which together amplify lung injury. Damage to lung microvascular endothelial cell and alveolar epithelial cells increases permeability, leading to leakage of protein-rich fluid into the alveolar spaces, causing pulmonary edema and hyaline membranes formation, a hallmark features of ARDS(Meyer et al., 2021).Bacterial and viral pneumonias are the main causes of ARDS, and endotoxemia due to Gram-negative bacterial infections is also a common trigger(Dai et al., 2015). Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a potent inducer of the innate immune response (Bertani & Ruiz, 2018), it activates immune cells and perpetuates the inflammatory cascade that characterizes ARDS. Pathologic specimens from patients with sepsis-associated ARDS frequently show extensive alveolar injury, and studies have shown that lung epithelial and endothelial injury is the underlying cause (Gong et al., 2022). The pathophysiology of ARDS is complex, involving activation and dysregulation of multiple overlapping and interacting pathways of lung and systemic injury, inflammation, and coagulation (Bos & Ware, 2022b). Despite numerous studies, the mortality rate for ARDS is still about 40% (Vellingiri et al., 2020). Currently recommended treatments to reduce ARDS morbidity and mortality remain limited and include small tidal volume mechanical ventilation, prone ventilation, and more recently ECMO rescue therapy in extreme cases(G et al., 2018), There is still no effective targeted drug therapy (Huang et al., 2024) that can ultimately change the course of the disease, and treatment remains supportive, emphasizing lung-protective ventilation, fluid management, and organ support.

Mechanical ventilation, while essential to the treatment of ARDS, carries inherent risks. Prolonged mechanical ventilation can lead to ventilator-induced lung injury (VILI) and increase the risk of secondary infections and fibrosis (P et al., 2015). Fluid management strategies to optimize oxygenation and minimize edema shown efficacy in improving oxygenation and reducing duration of mechanical ventilation, but do not significantly reducing mortality (Pan et al., 2018). In recent years, it has been shown that mesenchymal stem cells (MSCs) are safe for the treatment of ARDS and can significantly reduce mortality and improve certain clinical symptoms, while potentially attenuating the inflammatory response of ARDS to some extent (Wang et al., 2023). However, the treatment is expensive and still in the research stage, and its efficacy and safety need to be further confirmed by more high-quality and large-scale clinical trials. Therefore, ARDS remains an urgent and unsolved global health issue that requires innovative approaches to improve patient prognosis (Qadir et al., 2024; Ramji et al., 2023).

2 The Role of Orphan Nuclear Receptor Nur77/NR4A1 in Inflammation Modulation

The orphan nuclear receptor family includes three members: Nur77 (NR4A1), Nurr1 (NR4A2), and NOR1 (NR4A3) (L et al., 2020). Nur77/NR4A1, as a member of the nuclear receptor superfamily, is an early response gene that regulates the expression of multiple target genes. After being rapidly induced under a variety of physiological and pathological stimuli, it directly regulates downstream genes including cell metabolism, cell cycle, apoptosis, and inflammation (Deng et al., 2022; Ipseiz et al., 2014). It is expressed in the lungs, liver, skeletal muscle, adipose tissue, heart, brain, kidneys, and T cells (Pearen & Muscat, 2010), and participates in their energy conversion, energy metabolism, and pathophysiological processes. Research data show that Nur77/NR4A1 is highly expressed in many inflammation-related lung diseases, such as bronchial asthma, acute lung injury, and pulmonary fibrosis, and inhibits inflammatory responses by inhibiting NF-κB signaling, indicating that Nur77/NR4A1 plays a certain role in the occurrence and development of lung diseases (Asoka Banno et al., 2019).

important role in inflammatory response and tissue repair, but its role in lung macrophage polarization is still unclear. Macrophage polarization is a precisely regulated process involving signaling pathways such as JNK, PI3K/Akt, Notch, JAK/STAT, TLR4/NF-κB, and TGF-β (Zhou et al., 2014). Among them, TLR activation of NF-κB under LPS stimulation can regulate macrophage polarization to M1 or M2 in different pathological environments (Sawoo et al., 2021; Sica & Mantovani, 2012). Studies have shown that in the ARDS rat model, Nur77 inhibits NF-κB and p38MAPK, reduces the expression of ET-1, reduces lung pathological damage, improves oxygenation,

3 Role of Nur77/NR4A1 in Regulating Macrophage Polarization During ARDS

The pathogenesis of ARDS involves a variety of effector cells and target cells, among which macrophages, as the first line of defense against external pathogenic microorganisms, play an important role in lung injury and repair. In the exudative phase of ARDS, lung macrophages polarize to M1 type, release proinflammatory factors, form an inflammatory microenvironment and induce lung inflammatory response; while in the proliferation and fibrosis phases, macrophages polarize from M1 type to M2 type, reduce lung tissue damage and play a protective role. However, excessive M2 polarization may lead to lung tissue fibrosis and pathological fibrosis proliferation (X. Chen et al., 2020). Therefore, inhibiting excessive macrophage inflammatory response may become a new target for the treatment of inflammatory diseases (Sinitski et al., 2019). Studies have shown that Nur77/NR4A1 can regulate the polarization of mouse bone marrow-derived macrophages (BMMs) and peritoneal macrophages (PMs) and plays an

reduces neutrophil inflammation, promotes pulmonary edema absorption and lung injury repair. Cytosporone B, as an agonist of Nur77, also showed similar effects. In addition, Nur77 deficiency can promote macrophage differentiation to M1, and participate in the induction of macrophage apoptosis (Garabuczi et al., 2023b; *Nur77 Deficiency Exacerbates Macrophage NLRP3 Inflammasome-Mediated Inflammation and Accelerates Atherosclerosis - PubMed*, n.d.). Therefore, Nur77 may regulate lung injury repair by regulating the polarization of lung macrophages (Figure 11).

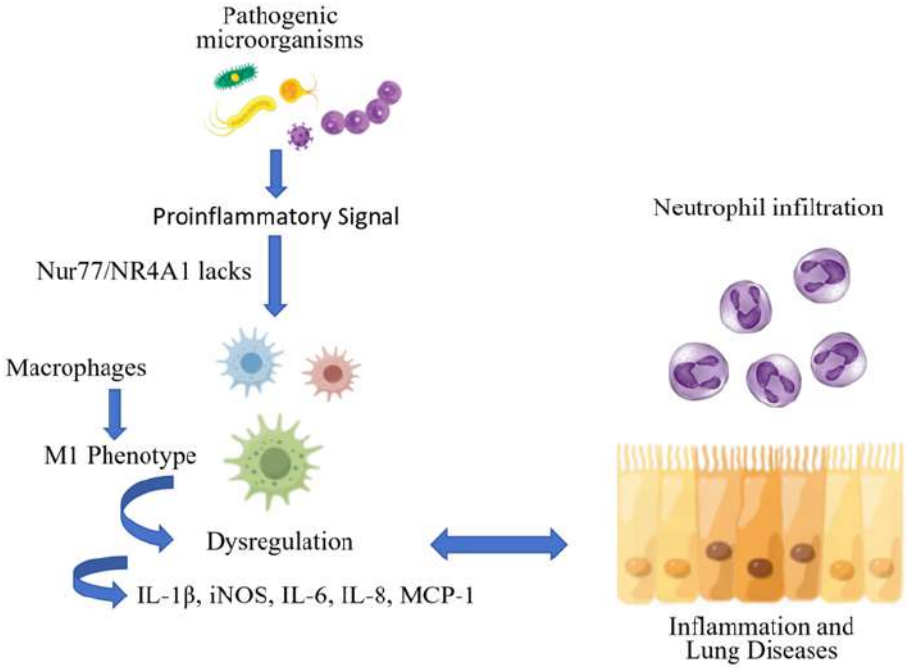


Figure 11: Diagram of macrophage differentiation towards pro-inflammatory M1 phenotype due to Nur77 deficiency.

Macrophages are a widely distributed natural immune cell that differentiates from monocytes after passing through blood vessels. They play an important role in organ development, host defense, inflammation and cellular homeostasis (Mosser & Edwards, 2008). Under different stimuli, macrophages can be polarized into classical activation (M1) or alternative activation (M2). M1 macrophages engulf pathogens in the early stage of inflammation, while M2 macrophages promote tissue repair in the late stage of inflammation damage. Identifying the polarization state of macrophages and converting them from M1 to M2 is expected to become a new strategy for the treatment of acute and chronic inflammatory diseases.

The pathological process of ARDS is divided into exudative phases, proliferative phase and fibrotic phase. Macrophages play different roles in different stages according to the microenvironment. In the first 72 hours of the exudative phase of ARDS, MCP-1 is released at the inflammatory site to recruit monocytes/macrophages, polarize into M1 macrophages, release pro-inflammatory factors such as IL-1β, IL-6 and IL-8, and form an inflammatory microenvironment to eliminate pathogens (Arora et al., 2018). After establishing (*Epigenetic Control of Macrophage Polarisation and Soluble Mediator Gene Expression during Inflammation - PMC*, n.d.; Qin et al., 2012). Chronic airway inflammation and airway remodeling are common features of lung diseases such as chronic obstructive pulmonary disease and bronchial asthma, which are caused by the interaction of inflammatory cells and cytokines. Macrophages play a key role in this pathological process (Figure 12). Inhibiting the release of inflammatory cells and cytokines can reduce airway the ARDS mouse model in this experiment, HE showed that lung tissue damage was not obvious within 4 hours, but at 12 hours and 24 hours, lung tissue showed significant damage, including alveolar collapse, septal thickening, edema fluid and inflammatory cell infiltration, and hyaline membrane formation. After intervention with the Nur77 agonist CsnB, lung damage was significantly alleviated.

The experiment used iNOS and IL-1β as M1 macrophage markers, and Arg-1 as M2 markers, and their expression was detected by immunohistochemistry, immunofluorescence and Western blot. Immunofluorescence results showed that the expression of F4/80 and iNOS in lung tissues in the LPS group and CsnB control group

was higher than that in the NS group in a time-dependent manner; after CsnB intervention, the expression of F4/80 and iNOS was significantly reduced. Western blot results showed that the expression level of IL-1β in lung tissues in the LPS group and CsnB control group was higher than that in the NS group and was significantly reduced after CsnB intervention. The results showed that lung injury in the ARDS mouse model began at 4 hours, and the expression of early M1 macrophage markers increased, indicating that alveolar macrophages polarized to the M1 type and initiated an inflammatory response.

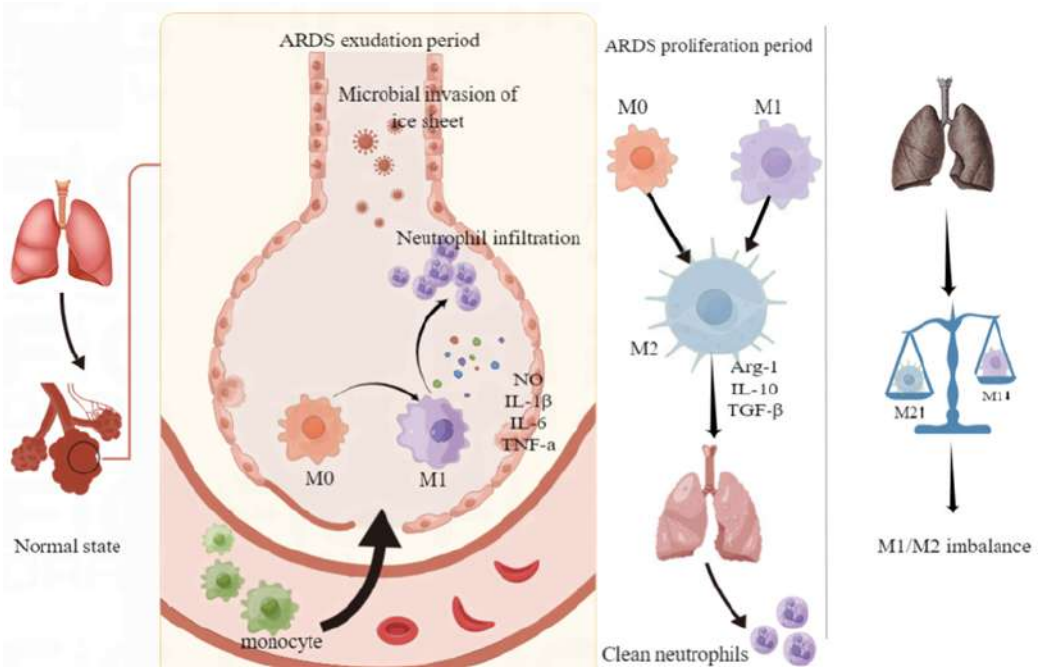


Figure 12: Schematic diagram of macrophages participating in the pathological process of ARDS

After the exudative phase of ARDS, the proliferation phase begins as early as 72 hours, during which the resident and recruited macrophages transform from M1 to M2 phenotype, inhibiting the expression of proinflammatory factors, clearing apoptotic neutrophils and cell debris, alleviating lung inflammation, and promoting lung tissue repair. In addition to regulating the levels of proinflammatory and anti-inflammatory factors, M2 macrophages can also activate anti-inflammatory signaling pathways and coordinate the termination of anti-inflammatory effects (Anderson & Mosser, 2002). Ten days after the onset of ARDS, obvious fibrosis appeared in the lung tissue, and M2 macrophages released anti-inflammatory factors such as IL-10 and TGF-β to counteract the inflammatory process induced by Th1 cytokines,promote inflammation subsidence and tissue repair. However, M2-type macrophages may also exacerbate pulmonary fibrosis by over-secreting cytokines such as TGF-β1[(Cheng et al., 2021)]. At the three points set up in this experiment, CsnB intervention significantly alleviated lung injury, inhibited M1 proinflammatory macrophage polarization, and promoted M2 macrophage polarization at 12h and 24h in the ARDS model, improving the inflammatory microenvironment, indicating that Nur77 may repair lung injury by inhibiting M1 polarization and increasing M2 polarization, reversing the anti-inflammatory microenvironment, and inhibiting downstream inflammatory responses. However, the ARDS model time point in this experiment was only up to 24h, and the role of M2 polarization of macrophages in the proliferation and fibrosis stages still needs further study.

Pulmonary macrophage polarization is a specific phenotype formed by macrophages in different pathophysiological states and inflammatory microenvironments, exerting anti-inflammatory or proinflammatory responses. According to the literature, regulating M1 polarization to M2 polarization may become a new treatment strategy for inflammatory diseases (P. Jiang & Li, 2022; Peng et al., 2023). Pulmonary macrophage polarization plays an important role in the

pathophysiological process of ARDS, and M2 macrophages exert anti-inflammatory and repair functions. The results of this experiment show that Nur77 can regulate macrophage polarization to M2 and inhibit M1 macrophages, playing an anti-inflammatory and repair role in ARDS. By regulating the polarization state of macrophages to reduce excessive inflammatory response and lung damage, and balance the process of pulmonary fibrosis and repair, it is a potential new therapeutic target for ARDS. Further investigation into the mechanisms by which Nur77 regulates macrophage activation and polarization will remain a central focus of future research. Additionally, a deeper understanding of the interactions between macrophages and other cell types, such as epithelial cells, fibroblasts, and endothelial cells, is essential. Exploring how the metabolic state of macrophages influences their function will also be critical. Targeting the regulation of macrophage polarization and their cellular origins holds significant promise for developing novel therapeutic strategies to treat pulmonary fibrosis(Cheng et al., 2021).

5 Disadvantages and prospects

The sample size of this experiment at three time points is small, and the sample size needs to be increased in the future to further verify and explore whether Nur77/NR4A1 can regulate pulmonary macrophage polarization and improve lung tissue damage and repair in mice with lipopolysaccharide-induced acute respiratory distress syndrome (ARDS).

The subjects of this experiment were wild-type mice, and the lipopolysaccharide-induced ARDS model group and the Nur77 agonist CsnB intervention group were mainly observed. If conditions permit, a lipopolysaccharide-induced Nur77 gene-deficient ARDS model should be established to further evaluate whether Nur77 gene deficiency regulates macrophage polarization in the pathological process of lung injury in ARDS mice.

In the natural pathological progression of ARDS, the transformation of M1 macrophages to M2 macrophages dominates the proliferation and fibrosis stages. If the macrophage polarization state and lung injury/repair process of ARDS mice at 3d, 5d, 7d, 14d, and 28d can be evaluated in the future, the research data will be more convincing.

Conclusion

This study established an ARDS mouse model using tracheal instillation of lipopolysaccharide (LPS), followed by lung tissue assessment at 4-, 12-, and 24-hours post-injury. The pathological progression, confirmed by wet/dry ratios, BALF protein concentration, HE stains, and inflammatory cytokine levels, indicated successful modeling of ARDS. The findings revealed significant lung injury at all observed time points, which was alleviated by treatment with the Nur77 agonist CsnB. In particular, M1 macrophages predominated during the early injury phases, indicating an ongoing exudative phase up to 24 hours. Moreover, the transition from M0/M1 to M2 macrophages observed at 12 and 24 hours suggest that Nur77 may facilitate early lung repair by promoting M2 macrophage polarization. These findings underscore Nur77's potential role in modulating macrophage function during the acute stages of ARDS, providing a basis for further exploration of its role during the proliferative and fibrotic phases of ARDS. Future studies are needed to clarify these mechanisms and evaluate Nur77's therapeutic potential in lung injury management.

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