

NLRC3 as a Potential Therapeutic Target for Acute Lung Injury: Insights from Acacetin Studies

yingchou xiao

Fourth Military Medical University

bo zhang

Fourth Military Medical University

shiyuan hou

Fourth Military Medical University

xing shen

Fourth Military Medical University

xingan wu

Fourth Military Medical University

rongrong liu

Fourth Military Medical University

ying lu

luoying@fmmu.edu.cn

Fourth Military Medical University

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Abstract

Acute lung injury (ALI) is a severe condition characterized by respiratory insufficiency and tissue damage. Commonly caused by pneumonia and severe sepsis, which trigger an inflammatory response via Toll-like receptor 4 (TLR4) signaling activation. Nod-like receptor family CARD domain containing 3 (NLRC3), a member of the NLR family, modulates inflammation and immune responses by inhibiting NF- κ B, activation in response to TLR4 activation. Dysregulation of NLRC3 has been linked to increased susceptibility to inflammatory diseases. In the context of ALI, overexpression of NLRC3 reduces lung inflammation, while its silencing exacerbates inflammation. Acacetin, a flavonoid from *Agastache rugosa*, exhibits anti-inflammatory properties and has been suggested to involve NLRC3 in its mechanism. Silencing NLRC3 abolishes the protective effect of acacetin on LPS-induced inflammation in macrophages. Moreover, NLRC3 negatively regulates TLR4 signaling, which is involved in lipopolysaccharide (LPS)-induced inflammation. Acacetin has been reported to inhibit TLR4 signaling in various cell types. Thus, acacetin's anti-inflammatory effects may be partly mediated by its modulation of NLRC3 expression and function. In this study, our objective was to investigate the potential targets and functional mechanisms of acacetin in combating ALI. We employed molecular docking technology to anticipate and authenticate the interaction between acacetin and NLRC3. The findings were subsequently validated using an ALI model and LPS-induced macrophage model.

1. Introduction

Acute lung injury (ALI) is a severe clinical disease associated with significant morbidity and mortality. It manifests as acute hypoxic respiratory insufficiency, marked by widespread pulmonary interstitial and alveolar edema resulting from diverse direct and indirect injury factors [1]. Pneumonia and severe sepsis are the predominant factors leading to ALI in clinical practice [2, 3]. In sepsis, lipopolysaccharide (LPS) in bacterial endotoxin activates Toll like receptors 4 (TLR4) inflammatory signaling by binding directly to pattern recognition receptors on the immune cell membrane, resulting in an overwhelming surge of inflammatory factors that cause tissue and organ damage [4].

Acacetin (5,7-dihydroxy-40-methoxyflavone), a flavonoid derived from *Agastache rugosa*, exhibits diverse biological activities, including anti-oxidative, anti-inflammatory, and anticancer activities [5]. Studies have demonstrated that acacetin suppresses the production of pro-inflammatory mediators in LPS-induced macrophage and mitigates acute liver injury in mice by inhibiting the TLR4 signaling pathway [6]. Its anti-inflammatory and antioxidative effects suggest that it might be beneficial in the context of ALI [7–9]. Despite these findings, the underlying mechanisms remain to be explored. Therefore, this study aimed to investigate the effect of acacetin on LPS-induced ALI mice and its underlying mechanism.

The Nod-like receptor (NLR) family has multiple functions as signal sensors, including the regulation of inflammatory and the maintenance of homeostasis [10, 11]. As a member of NLR family, Nod-like receptor family CARD domain containing 3 (NLRC3) mainly exists in the cytoplasm and prevents the dysregulation of inflammatory response by acting as a checkpoint [12]. Prior researches have

demonstrated that NLRC3 plays a negative regulatory role in LPS-induced NF- κ B activation downstream of TLRs by interacting with TNF receptor-associated factor 6 (TRAF6) in macrophage [13, 14]. In the context of T cell immune response, NLRC3 directly affects T cell function and inhibit T cell activation by modulating the function of dendritic cells [15]. Dysregulation of NLRC3 is associated with increased susceptibility to of inflammatory and autoimmune diseases. NLRC3 is closely linked to the regulatory activity of pathogen recognition, exerting inhibitory effects on cell proliferation and pyroptosis, while promoting apoptosis. These cellular functions regulated by NLRC3 are particularly important in the onset and progression of a various of diseases, including infectious diseases, aseptic inflammatory diseases, and cancer [16–19]. Limited research has been conducted on the role of NLRC3 in ALI. Previously reports have indicated that overexpression of NLRC3 significantly reduces lung inflammation, while lentivirus-mediated silencing of NLRC3 exacerbates lung inflammation [20]. These fundings suggest that NLRC3 may mitigate sepsis-induced ALI by inhibiting suppressing inflammatory responses in lung tissues.

In summary, NLRC3 negatively regulates TLR4 signaling, which is a key pathway involved in LPS induced inflammation. Acacetin has been reported to inhibit TLR4 signaling in various cell types. Therefore, the anti-inflammatory effect of acacetin may be mediated, at least in part, through its ability to regulate NLRC3 expression and function. Further studies are needed to investigate the precise nature of the relationship between NLRC3 and acacetin in the context of ALI.

2. Methods

2.1 Animals

Wild type (WT) C57BL/6 mice were purchased from the Animal Center of the Fourth Military Medical University (China). NLRC3 knockout (NLRC3^{-/-}) mice were purchased from the Department of Microbiology of the Fourth Military Medical University. Both mice were male and weigh 18–22 g. All animal experimental procedures were approved by the Institutional Animal Ethics and Use Committee of the Fourth Military Medical University.

2.2 Survival studies

Mice were administered 50 mg/kg LPS (*Escherichia coli* lipopolysaccharide, O55:B5, Sigma, USA) along with varying doses of acacetin (40, 80 and 120 mg/kg, Amazigh Pharma, China) as a pretreatment through intraperitoneal injection. Record the mortality rate of each group of mice every 12 h for a duration of 3 days following the administration of LPS. WT mice were used for the experiment, with 20 mice in each group.

2.3 Modeling and grouping of ALI mice

WT and NLRC3^{-/-} mice were randomized into 5 groups, i.e., Control, LPS, LPS + A, LPS + NLRC3^{-/-}, LPS + A + NLRC3^{-/-} groups, each group contains 20 animals. LPS and acacetin were administered by intraperitoneal injection. Mice in the pretreatment group were injected with acacetin (80 mg/kg) once a

day for 3 consecutive days, and then were stimulated with LPS (10 mg/kg) for 24 h together with mice in the LPS group at the last administration.

2.4 Hematoxylin-eosin (HE) stains and inflammation score

After ALI modeling, the lung tissues of each group of mice were isolated and the right lung lobular was taken out (n = 8). The tissues were fixed by immersion in paraformaldehyde for at least 24 h before embedding in paraffin. The embedded tissues were sliced into sections and then stained with HE. Five visual fields of each section were observed under light microscope for histopathological analysis. The injury score was based on alveolar hemorrhage, mononuclear cell infiltration, hyaline membrane, alveolar wall thickening and alveolar structural destruction [21]. The severity of injury was expressed by score: 0 means no injury, 1 means moderate injury (range of 25%), 2 means intermediate injury (range of 25 to 50%), 3 means extensive injury (range of 50 to 75%), 4 means severe injury (range of 75%). The sum of the 5 parameters' s scores was used to compute the total score of injury.

2.5 Pulmonary edema measurement

To assess the severity of pulmonary edema in mice, we measured lung wet-dry (W/D) and lung-body (L/B) weight ratios. After ALI modeling, the body weight of each group of mice was measured (n = 8). The superior lobe of left lung was separated from the lung tissue and weighed wet weight, and then dried at 95 °C for 24 h. Finally, lung W/D and L/B weight ratio were calculated.

2.6 Immunofluorescence

The lung sections underwent three PBS rinses and subsequently permeabilized by immersion in Triton X-100. Sections were blocked with immunol staining blocking buffer (Beyotime, China) and subsequently incubated with primary antibodies (CD4, F4/80, Abcam, UK) at 4 °C overnight. Next, the sections were incubated with secondary fluorescent antibody (Abcam, UK) and DAPI (Beyotime, China) for 1 h and 30 min, respectively. Images were captured by a fluorescent microscope (Olympus, Japan) and results were analyzed in Caseviewer.

2.7 Isolation and culture of bone marrow derived macrophages (BMDMs)

WT and NLRC3^{-/-} mice aged 3–5 months were selected, and the cells in bone marrow were isolated and collected [22]. The cells derived from bone marrow were treated with erythrocyte lysis buffer and suspended in 2 ml of PBS. Next, cells were transferred into RPMI1640 supplemented with 15% fetal bovine serum (Gibco, USA) and 10 ng/ml of m-CSF (Proteintech, China). The suspension was transferred to a 6-wells plates after 24 h. The growth and differentiation of BMDMs lasted more than 7 d, and fresh medium should be replaced at days 2, 4, and 6.

2.8 Cell viability measurement

BMDMs with good growth were inoculated into 96-well plates with approximately $1-2 \times 10^4$ cells per well. Subsequently, different concentrations of acacetin (1, 50, 100, 150 µg/ml) were added to stimulated

BMDMs. After 12 h, the viability of BMDMs were detected by cell counting kit-8 assay (CCK-8, Beyotime, China).

2.9 Cell treatment and grouping

WT and NLRC3^{-/-} BMDMs were randomized into 5 groups, i.e., Control, LPS, LPS + A, LPS + NLRC3^{-/-}, LPS + A + NLRC3^{-/-} groups. LPS (100 ng/ml) was added to the culture medium of BMDMs in LPS-treated group. The BMDMs medium in acacetin pretreatment group was pretreated with acacetin (100 µg/ml) for 1h, and followed by LPS (100 ng/ml). Cells and cell culture supernatant were collected after 1, 6 and 12 h of LPS administration.

2.10 Cytokines measurement

The collected BMDMs culture supernatant was centrifuged and aspirated. Then, the concentration of TNF-α, IL-1β, and IL-6 in the supernatant were detected by enzyme linked immunosorbent assay (ELISA) kits (R&D, USA). Instructions for use provided by manufacturer.

2.11 mRNA expression

RNA was extracted and purified from the collected lung tissues and BMDMs by RNA extraction kit (Beyotime, China). The concentration of RNA was measured and diluted, and cDNA was subsequently prepared by reverse transcription using the SweScript RT cDNA synthesis kit (Servicebio, China). TB green PCR kit (Servicebio, China) and samples were mixed. Quantitative real-time PCR (qRT-PCR) was performed using an Biosystems PCR machine (Bio-rad, USA). The relative expression of each gene was calculated using the 2^{-ΔΔCt} method. Expression was normalized to the mean expression of all control individuals.

The following primer were used: β-actin, 5'-CACGATGGAGGGGCCGGAATCATC-3' (forward) and 5' -TAAAGACCTCTATGCCAACACAGT-3' (reverse); NLRC3, 5'-CAGATTGGTAACAAAGGAGCCA-3' (forward) and 5'-CGTTCGGTTTATCTTCAGAGCA-3' (reverse).

2.12 Western blot

Total protein was extracted from lung tissue and BMDMs. Subsequently, the protein concentration was determined with BCA assay kit (Beyotime, China). 50 µg of sample was added to 10% SDS polyacrylamide gel and separated by electrophoresis for 1 h. Protein was subsequently transferred to polyvinylidene fluoride membranes and blocked with western blot blocking buffer (Beyotime, China). Membranes were incubated with the corresponding primary antibodies (NLRC3, IL-1β, IL-18, pNF-κB p65, NF-κB p65, β-actin, Abcam, UK) at 4 °C overnight, and finally with the secondary antibody (Abcam, UK) for 1 h. Detection was performed using the chemiluminescence system (Chemiscope, China).

2.13 Molecular docking

The binding site between acacetin and NLRC3 was identified using Molecular Operating Environment (MOE, version 2022) software. The proper structure of NLRC3 was selected from the Uniprot (Uniprot ID:

Q7RTR2) and the 3D structure of acacetin was constructed in MOE. Subsequently, the structure and energy of the protein and compound were optimized by MOE. The compound acacetin with the highest scoring data was matched with the target, and the sites were selected as Asp222 and Tyr213. The results were presented as docking score table and combination model diagram [23].

2.14 Statistical analyses

Data were presented as mean $\pm$ SEM, and statistical analysis was performed using the GraphPad prism software 9.0. Significant differences between groups were determined by one-way analysis of variance (ANOVA). Survival rate was analyzed by Kaplan-Meier method, and differences between groups were compared by log rank test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Acacetin has a protective effect on mice given lethal doses of LPS

To evaluate the protective impact of acacetin in septic mice, mice were administered LPS after three times of acacetin pretreatment. As illustrated in Fig. 1, the survival rate of mice pretreated with 80 mg/kg or 120 mg/kg of acacetin for 3 days had a significantly increase compared with the LPS group, with no discernible difference between the two doses. In contrast, lower doses (40 mg/kg) did not reduce mortality. These findings indicated that pretreatment with acacetin could effectively shield mice with endotoxemia from death in a dose-dependent manner. Consequently, 80 mg/kg was chosen for further investigation.

3.2 Acacetin ameliorated LPS-induced lung histopathologic changes

To study the impact of acacetin on LPS-induced ALI in mice, we initially assessed histopathological changes through HE staining. The control group exhibited normal pulmonary alveolar morphology, while the LPS-treated group showed evident pathological alterations, including alveolar congestion and edema, inflammatory cell accumulation, thickened alveolar walls, and hyaline membrane formation. In contrast to the LPS group, the acacetin pretreatment group demonstrated significantly attenuated lung structural damage (Fig. 2A). These observations were consistent with the lung injury scores (Fig. 2B).

3.3 Acacetin alleviates pulmonary edema induced by LPS

To explore the protective impact of acacetin against pulmonary edema, we assessed lung edema as an indicator of LPS-induced ALI resulting from alterations in barrier permeability [24]. Our findings revealed a notable increase in lung W/D and L/B weight ratio following LPS administration compared to the control

group. However, pretreatment of acacetin prior to LPS significantly mitigated the rise in lung W/D and L/B weight ratios (Fig. 2C and D).

3.4 Acacetin down-regulated inflammatory mediators in lung tissue of ALI mice

LPS serves as the primary stimulator of the inflammatory response in sepsis, triggering the synthesis of inflammatory cytokines including IL-1 β and IL-18 [25]. To explore the influence of acacetin on LPS-induced inflammation, we detected the content of inflammatory cytokines in the lung tissues of mice within each experimental group. Subsequent to LPS administration, a notable increase in the content of IL-1 β and IL-18 was observed compared with the control group. Nevertheless, acacetin pretreatment effectively inhibited the generation of these inflammatory cytokines (Fig. 2E).

3.5 Acacetin attenuated NF- κ B activation in lung tissue of ALI mice

NF- κ B serves as a key transcription factor, which influences the production of inflammatory mediators in the pathogenesis of ALI [26]. Consequently, our study delved into the influence of acacetin on NF- κ B activation in the lung tissues of ALI mice. Following LPS stimulation, there was a notable increase in the phosphorylation of p65 in lung tissues compared to the control group. However, acacetin pretreatment effectively mitigated this increase, as depicted in Fig. 3A.

3.6 Acacetin up-regulated NLRC3 in lung tissues of ALI mice

NLRC3 is well known as a suppressor of NF- κ B activation and a negative regulator of inflammation. We then examined the potential of acacetin to up-regulate NLRC3 expression and thereby inhibit NF- κ B signaling in lung tissues. Following LPS administration, the mRNA and protein levels of NLRC3 decreased in lung tissues. However, pretreatment with acacetin significantly restored NLRC3 expression (Fig. 3B and C).

3.7 NLRC3 deficiency inhibited the protective effect of acacetin against ALI

To investigate the association between acacetin and NLRC3, we conducted additional animal experiments to evaluate its anti-inflammatory effects in vivo. Histopathological analysis revealed that NLRC3^{-/-} mice showed increased severity of inflammatory changes and pulmonary edema after LPS administration, compared to WT mice. However, pretreating NLRC3^{-/-} mice with acacetin did not lead to a reduction in the W/D and L/B weight ratios, resulting in continued severe damage compared to the LPS + A group (Fig. 4A-D). We then examined the content of inflammatory cytokines and the activation of NF- κ B in lung tissues of NLRC3^{-/-} mice. Following LPS administration, the content of IL-1 β and IL-18 in lung tissues were obviously higher than those in WT mice, and the phosphorylation of p65 was also increased.

However, pretreatment of NLRC3^{-/-} mice with acacetin did not reduce the content of IL-18 or inhibit the phosphorylation of p65. In addition, the content of inflammatory cytokines was increased and the activation of NF-κB was enhanced compared with the LPS + A group (Fig. 4E-F).

3.8 Docking studies between acacetin and NLRC3

Our results showed that acacetin might represent a valuable therapeutic approach for ALI by up-regulating NLRC3. Therefore, we used the protein structure of NLRC3 as the target for fine molecular docking with the compound acacetin. The interaction diagram between acacetin and NLRC3 (Fig. 5A) and the 3D binding mode diagram (Fig. 5B) showed that this compound formed a hydrogen bond with Asp222 and formed an aromatic ring stacking interaction with Tyr413. In addition, the amino acids Ala147, Phe453, Ala330, Leu456 around the binding site constructed a strong hydrophobic force. This indicated a tight binding between acacetin and NLRC3.

3.9 Acacetin affected the recruitment of immune cells through NLRC3

Previous studies have demonstrated NLRC3's role in suppressing macrophage and T cell activation. To investigate the acacetin's potential impact on immune cell recruitment via NLRC3, immunofluorescence staining was conducted on CD4⁺T cells and macrophages in lungs. As illustrated in Fig. 6, a marked increase in CD4 and F4/80-positive cells as observed in lung tissues after LPS administration compared to the control group. However, acacetin pretreatment resulted in a reduction in the number of these immune cells. In NLRC3^{-/-} mice, a significant increase was observed only in the macrophages within the lung tissue following LPS administration (Fig. B). Notably, acacetin pretreatment did not induce any significant difference in the number of these immune cells in lung tissues but exhibited an increase compared with LPS + A group (Fig. 6A,B).

3.10 Acacetin pretreatment inhibited the production of inflammatory cytokines by BMDMs

Previous research indicated that acacetin did not inhibit the substantial accumulation of macrophages in the lungs of NLRC3^{-/-} mice during LPS-induced ALI. Given the close association between macrophages and the excessive inflammatory response in ALI, it was imperative to further evaluate the impact of acacetin on inflammatory cytokines production. Additional in vitro studies using BMDMs were conducted to assess this effect. The effect of acacetin concentration on BMDMs viability was tested by CCK-8. Our results demonstrated that acacetin exhibited non-toxic properties at concentrations up to 100 µg/ml (Fig. 7A). Consequently, the concentration of 100 µg/ml of acacetin was selected for further investigation.

Upon LPS administration, the concentrations of TNF-α, IL-1β and IL-6 in BMDMs medium were increased at 1, 6 and 12 h compared with the control group. However, acacetin pretreatment notably decreased their

concentration. When NLRC3^{-/-} BMDMs were exposed with LPS, the concentration of TNF-α and IL-6 in culture medium was significantly higher than WT BMDMs at each time points. Intriguingly, acacetin pretreatment was not effectively reducing the concentration of these inflammatory cytokines, which were higher than those in the LPS + A group (Fig. 7B, C and D).

3.11 Acacetin attenuated NF-κB activation in LPS-stimulated BMDMs

Our previous study found that acacetin could inhibit the activation of NF-κB in the lung tissues of ALI mice induced by LPS. NF-κB has been an important signaling pathway in macrophages that regulates inflammatory responses, so we evaluated the activation of NF-κB in BMDMs. The phosphorylation of p65 in BMDMs was increased at 1, 6 and 12 h after LPS administration, while pretreatment with acacetin significantly reduced the phosphorylation of p65 at 6 h and 12 h. When NLRC3^{-/-} BMDMs were administered with LPS, the phosphorylation of p65 was higher than that in WT BMDMs at each time points. However, acacetin pretreatment could not effectively reduce the phosphorylation of p65 at 6 h and 12 h, and they were significantly higher than those in LPS + A group at each time point (Fig. 8A, B and C).

3.12 Acacetin up-regulated the expression of NLRC3 in BMDMs

According to our previous study, acacetin pretreatment restored the expression of NLRC3 inhibited by LPS in mice lung tissue. To further assess the effect of acacetin on NLRC3 expression in vitro, we measured the expression of NLRC3 in BMDMs. LPS administration decreased the mRNA and protein levels of NLRC3 in BMDMs at 6 h and 12 h. Notably, acacetin pretreatment restored the expression of NLRC3 in the BMDMs from LPS group at the same time points (Fig. 9A and B). Based on the animal and cellular experiments, we hypothesized that acacetin may inhibit NF-κB activation by promoting NLRC3 expression in macrophages, reducing cell aggregation and reducing the synthesis of inflammatory cytokines.

4. Discussion

Our study found that acacetin prevented LPS-induced ALI and reduced mortality in mice. Further, we firstly showed that acacetin regulated NLRC3-NF-κB-associated pathway and reduced macrophage infiltration in lung. This novel information partially clarified the mechanism that acacetin against LPS-induced ALI were related to the suppression of macrophage infiltration activity, which was possibly related to the up-regulation of NLRC3 and the inhibition of NF-κB activation.

As a natural flavonoid, acacetin possesses anti-infectious, anti-inflammation, anti-cancer and antioxidant properties. Among them its prominent role in anti-infectious and anti-inflammation have received extensive attention from scholars in recent years [27, 28]. Earlier reports suggest that acacetin inhibits the growth of various gram-negative bacterium and gram-positive bacterium [29–31]. LPS, as the principal constituent of endotoxins, is an important pathogenic substance in the infection of gram-negative

bacterial infections. LPS causes systemic inflammation response by penetrating into the blood stream, which can lead to endotoxic shock and multiple organ failure in severe cases [32]. Lung is one of the most vulnerable organs in endotoxemia, and LPS induced-acute respiratory distress syndrome (ARDS) is the major cause of death. Therefore, LPS modeling is a frequently method in ALI experimental researches [33].

In this study, we induced ALI by intraperitoneal injection of LPS. The histopathological alterations of the lungs, the ratio of W/D and L/B, as well as the expression of pro-inflammatory factors were seen to demonstrate morphological changes in lung tissue, edema, and inflammation. This was done to explore the preventive effect of acacetin on ALI caused by LPS. We found that both 80 mg/kg and 120 mg/kg of acacetin provided significant protection against ALI lethality. However, 40 mg/kg of acacetin administered 1 h before LPS did not have any advantage in survival, indicating that a dose-dependent antiendotoxin effect of acacetin. Histopathologic results showed that the lung injury in acacetin-pretreated group was significantly less than that in LPS group. In addition, pretreatment with acacetin also reduced the W/D and L/B weight ratios, as well as the content of IL-1 β and IL-18, which were increased by LPS administration.

The excessive inflammatory response is an important pathological mechanism in the occurrence and development of ALI [34]. The NLRs affects the occurrence and development of inflammatory response. Some family members are known to form multiprotein complexes, initiate inflammatory signaling cascades, secrete pro-inflammatory mediators, such as IL-1 β , IL-6, IL-18 and TNF- α [35]. NLRC3 is a novel pattern recognition receptors which is expressed in various immune cells and acts as a negative modulator for signaling pathways triggered by TLRs [36]. Additionally, researches have indicated that NLRC3 can inhibit the activation of NF- κ B by associating with TRAF6 in immune cells that are activated [13, 14, 37, 38].

Our results showed that LPS could induce the activation of NF- κ B in lung tissue and macrophages, but acacetin pretreatment could effectively inhibit its activation. These indicated that acacetin could inhibit the production of IL-1 β and IL-18, upregulate NLRC3 and inhibit the activation of NF- κ B in vivo and in vitro. However, our understanding of the mechanism of acacetin in the prevention and treatment of ALI was still limited, and whether acacetin up-regulated the NLRC3 to decrease LPS-induced ALI has yet to be elucidated. To explore whether acacetin alleviates ALI directly by regulating NLRC3, we investigated its influence of acacetin on acute lung injury in WT and NLRC3^{-/-} mice. Our results demonstrated that NLRC3^{-/-} mice had a stronger response to LPS-induced ALI than WT mice, including histological alterations, lung edema, and the release of inflammatory cytokines. The protective effect of acacetin in septic NLRC3^{-/-} mice was strikingly different from that of their WT counterparts. In NLRC3^{-/-} mice, the protective effect of acacetin was largely lost.

Previous studies have demonstrated that NLRC3 in macrophages and T cells inhibits NF- κ B activation by attenuating k63 ubiquitination of TRAF6 [14, 37]. We found a significant difference in the number of macrophages in lung tissue of ALI mice between WT and NLRC3^{-/-} groups, but it is not known whether

acacetin attenuates macrophage hyperactivation by the same mechanism. Therefore, we used primary BMDMs from NLRC3^{-/-} mice for validation. In vitro, acacetin inhibits NF-κB signaling by up-regulating NLRC3 and limiting immune response of macrophages. After knocking out NLRC3, the inhibitory effect of acacetin on LPS-treated macrophage overactivation was almost abolished. These results indicate that acacetin may avoid excessive immune activation of macrophages by up-regulating NLRC3.

Although the current study demonstrated that acacetin could up-regulate NLRC3 expression, inhibit macrophage overactivation, and improve the survival rate of mice in LPS-induced ALI group without obvious effects on control group. However, studies have shown that high NLRC3 expression is associated with the defective monocyte/macrophage glycolysis, resulting in immunosuppression [39]. It is too early to conclude that NLRC3 overexpression has a protective effect on ALI patients with sepsis. Further study is still required to unveil the potential mechanisms.

5. Conclusion

In conclusion, we showed that the effects of acacetin on ALI was at least due to its inhibiting NF-κB activation via up-regulating NLRC3 expression. The present study suggested that acacetin, as a natural flavonoid compound widely used drug, may serve as a new specific and attractive therapy for ALI.

Declarations

Funfing

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Compliance with ethical standards

Ethics statement. The experimental procedures was approved by the Institutional Animal Ethics and Use Committee of the Fourth Military Medical University.

Conflict of Interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

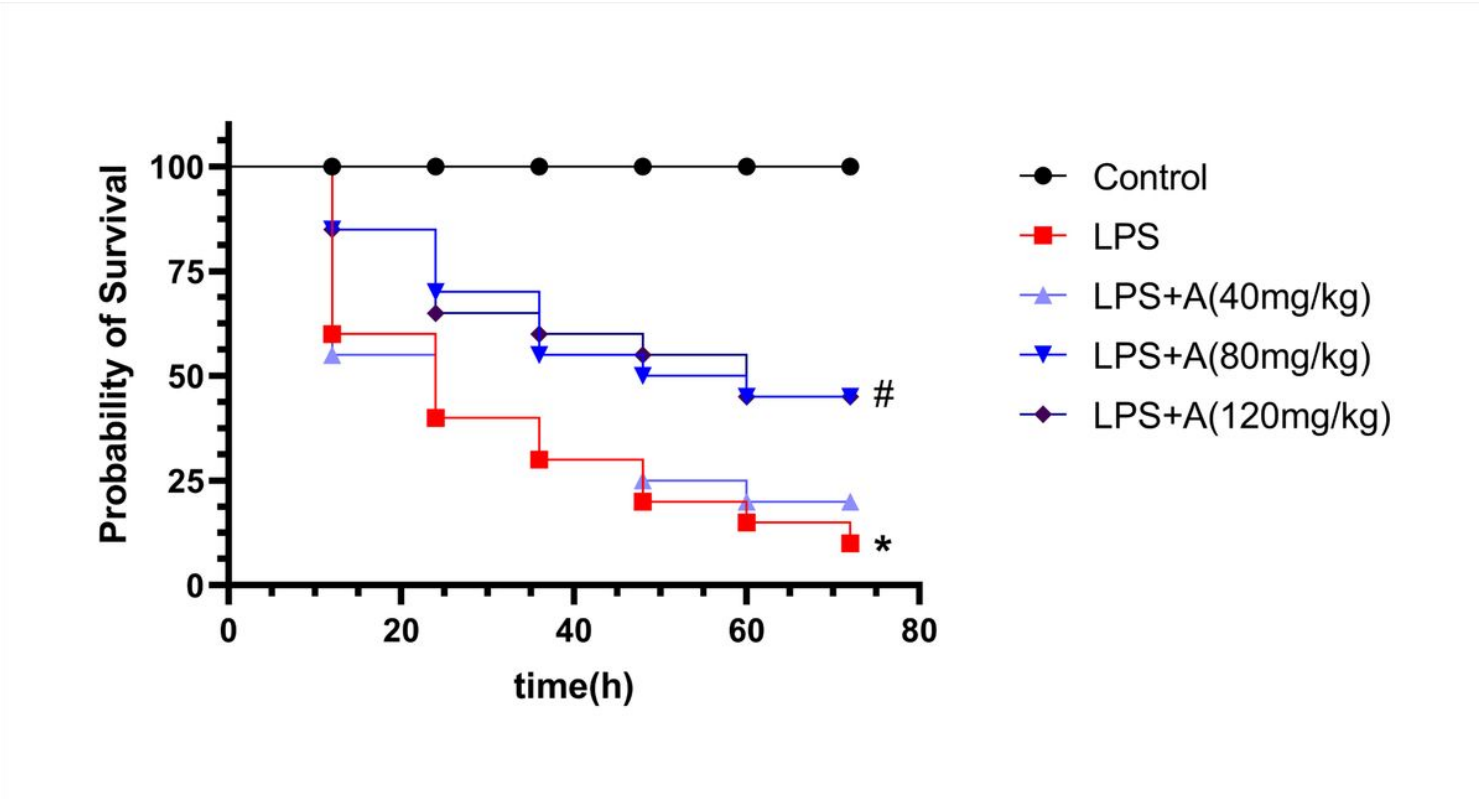


Figure 1

Effect of acacetin on LPS-induced mortality in mice. Mice were challenged by 50mg/kg LPS to induce ALI with or without acacetin (40 mg/kg, 80 mg/kg or 120 mg/kg) pretreatment 1 h before LPS was given. Survival was observed every 12 h within 72 h and the probability of survival was expressed as Kaplan-Meier survival curves (n = 20). * P < 0.05 vs. Control group, # P < 0.05 vs. LPS group.

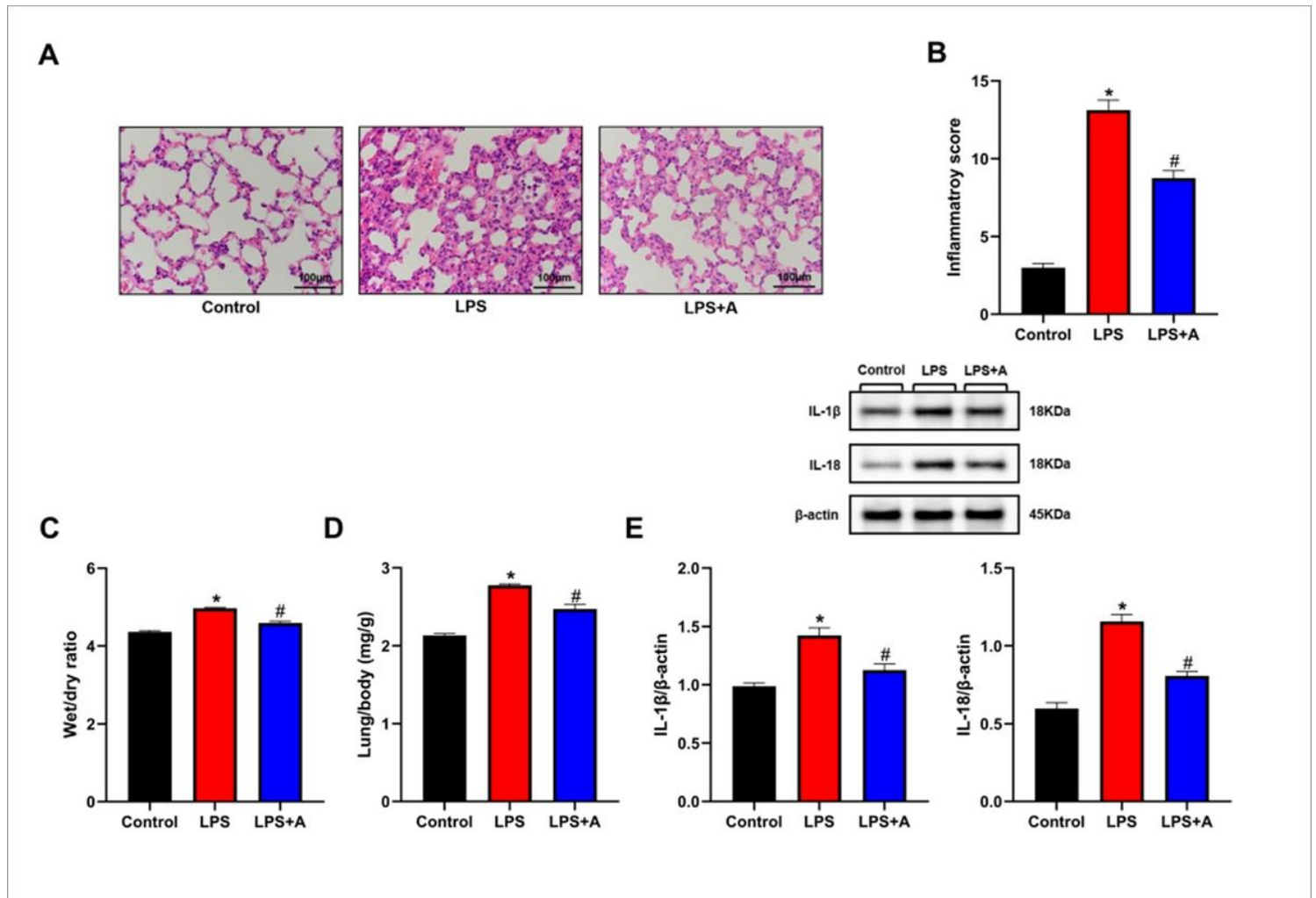


Figure 2

Pretreatment of acacetin ameliorated LPS-induced lung injury in mice. After pretreatment with acacetin (80 mg/kg), mice were stimulated with LPS (10 mg/kg) for 24 h to observe the pathological changes of lungs and detect the content of inflammatory cytokines in tissues (A): HE staining of lung sections, the scale bar is 100 μm. (B): Histopathological damage score statistics (n = 8). (C): Lung W/D ratios (n = 8). (D): L/B weight ratios (n = 8). (E): Western blots showed the protein expression of IL-1β and IL-18 in mice lung tissues (n = 5). Data are expressed as mean ± SEM, with statistical significance denoted as * P < 0.05 vs. Control group, # P < 0.05 vs. LPS group.

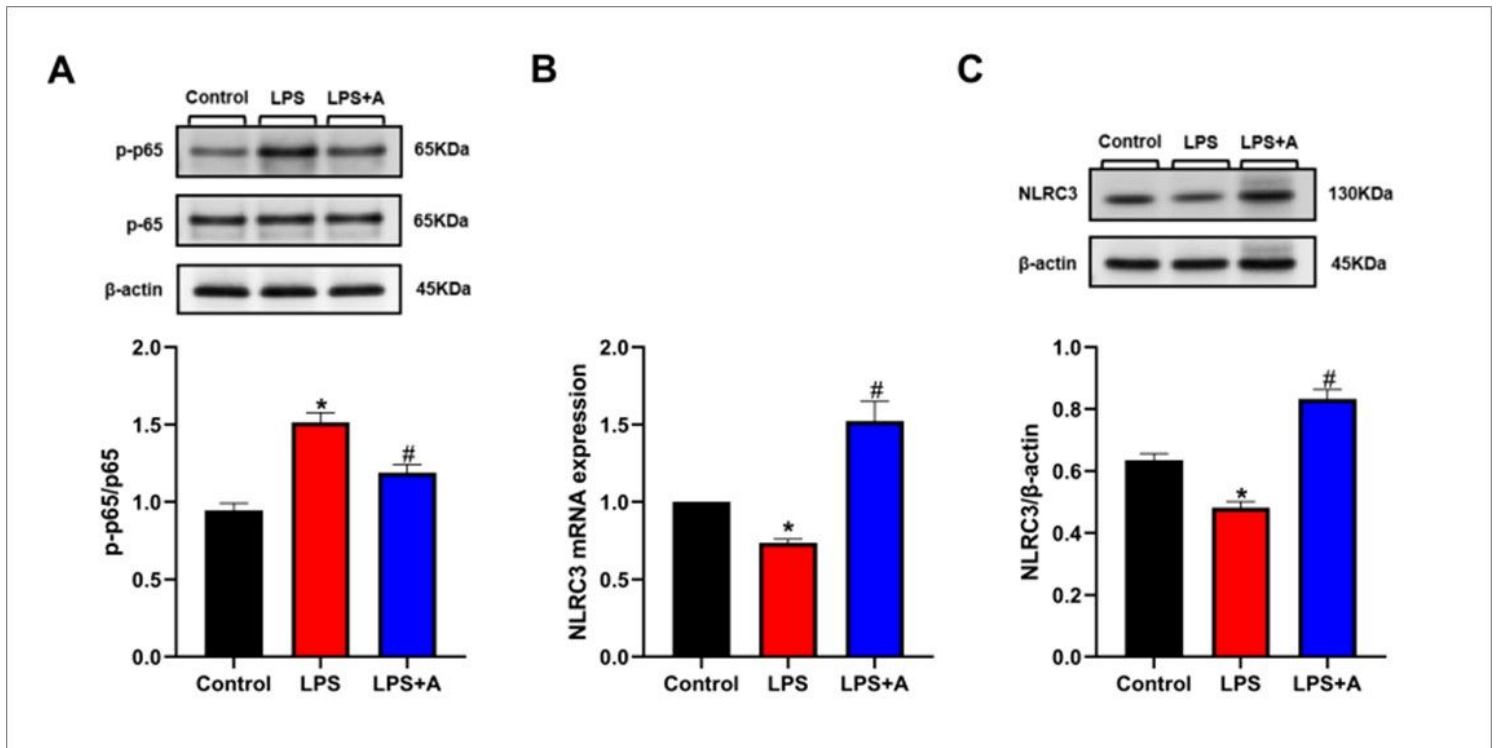


Figure 3

Acacetin pretreatment promoted the expression of NLRC3 in lung tissue of ALI mice, while the activation of NF-κB was inhibited. (A): The protein expression of total and phosphorylated NF-κB p65 in mice lung tissues (n = 5). (B): qRT-PCR showed the mRNA expression of NLRC3 in lung tissues (n = 3). (C): The protein expression of NLRC3 in mice lung tissues (n = 5). Data are expressed as mean ± SEM, with statistical significance denoted as * P < 0.05 vs. Control group, # P < 0.05 vs. LPS group.

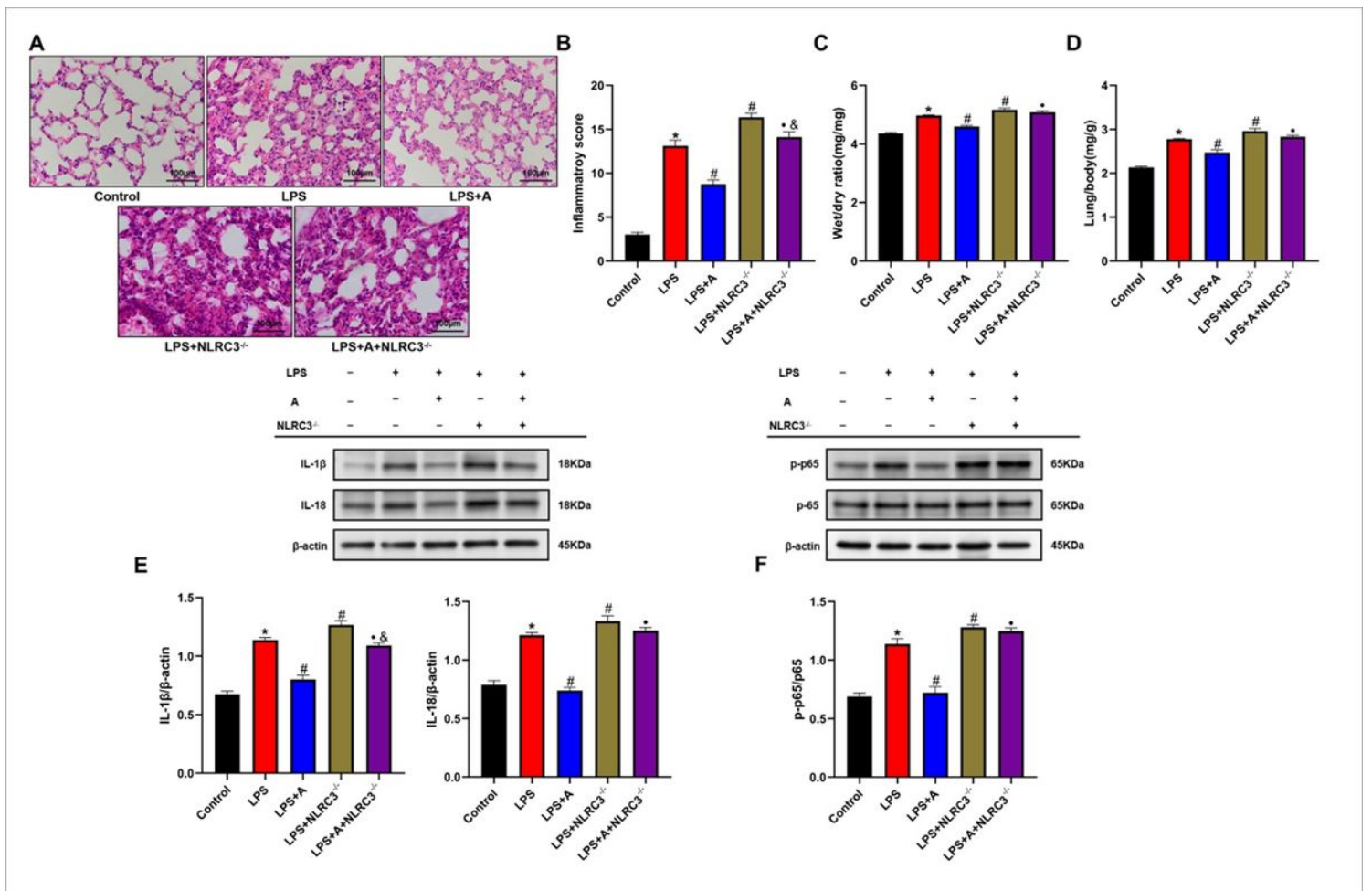


Figure 4

The absence of NLRC3 in mice interfered with the preventive and therapeutic effects of acacetin on ALI. (A): HE staining of lung sections, the scale bar is 100 μ m. (B): Histopathological damage score statistics (n = 8). (C): Lung W/D ratios (n = 8). (D): L/B weight ratios (n = 8). (E): Western blots showed the protein expression of IL-1 β and IL-18 in mice lung tissues (n = 5). (F): The protein expression of total and phosphorylated NF- κ B p65 in mice lung tissues (n = 5). Data are expressed as mean $\pm$ SEM, with statistical significance denoted as * P<0.05 vs.Control group, # P < 0.05 vs. LPS group, ● P < 0.05 vs. LPS+A, & P < 0.05 vs. LPS+NLRC3^{-/-}.

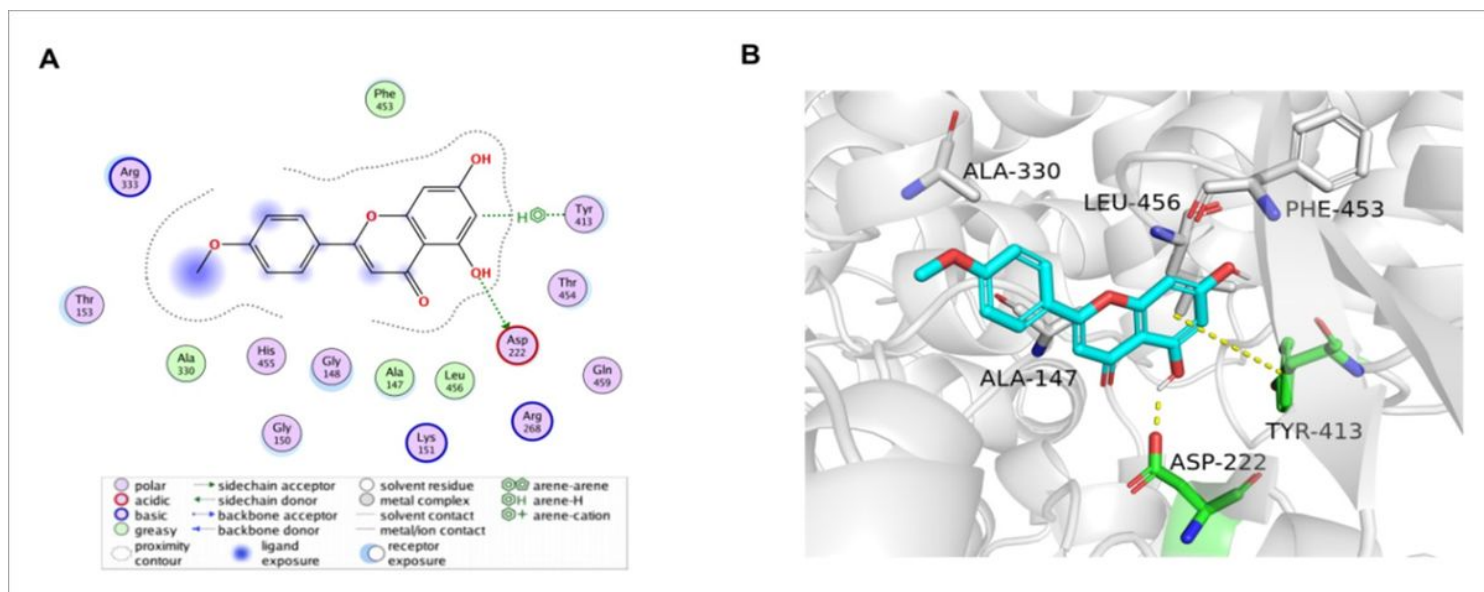


Figure 5

Interaction forces analysis (A) and binding pattern analysis (B) between compound acacetin and NLRC3.

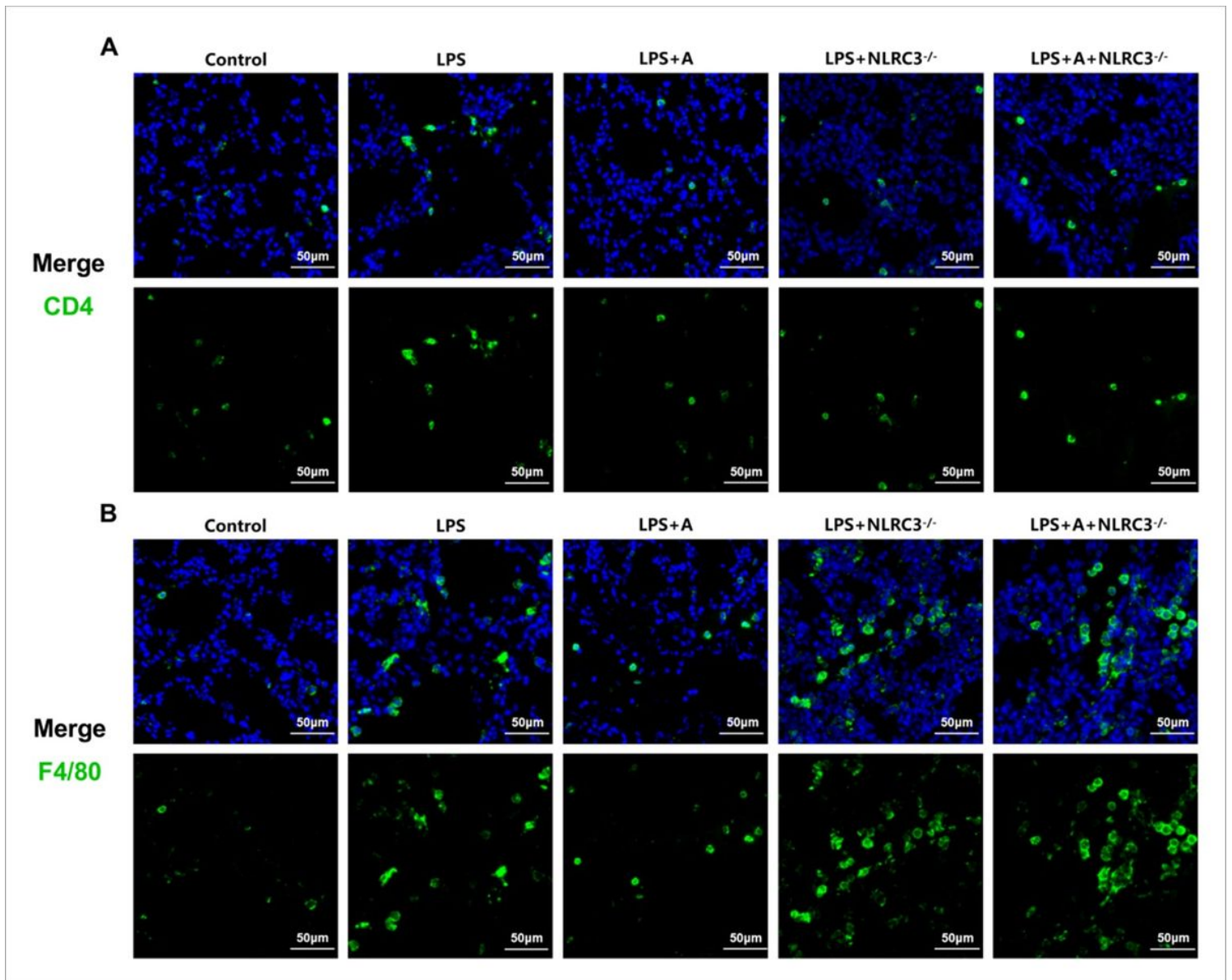


Figure 6

Acacetin affected the recruitment of immune cells in the lungs of ALI mice through NLRC3. Fluorescence green staining of CD4 (A) and F4/80 (B). The blue nuclear staining was activated by using DAPI staining solution with a scale bar of 50 μ m.

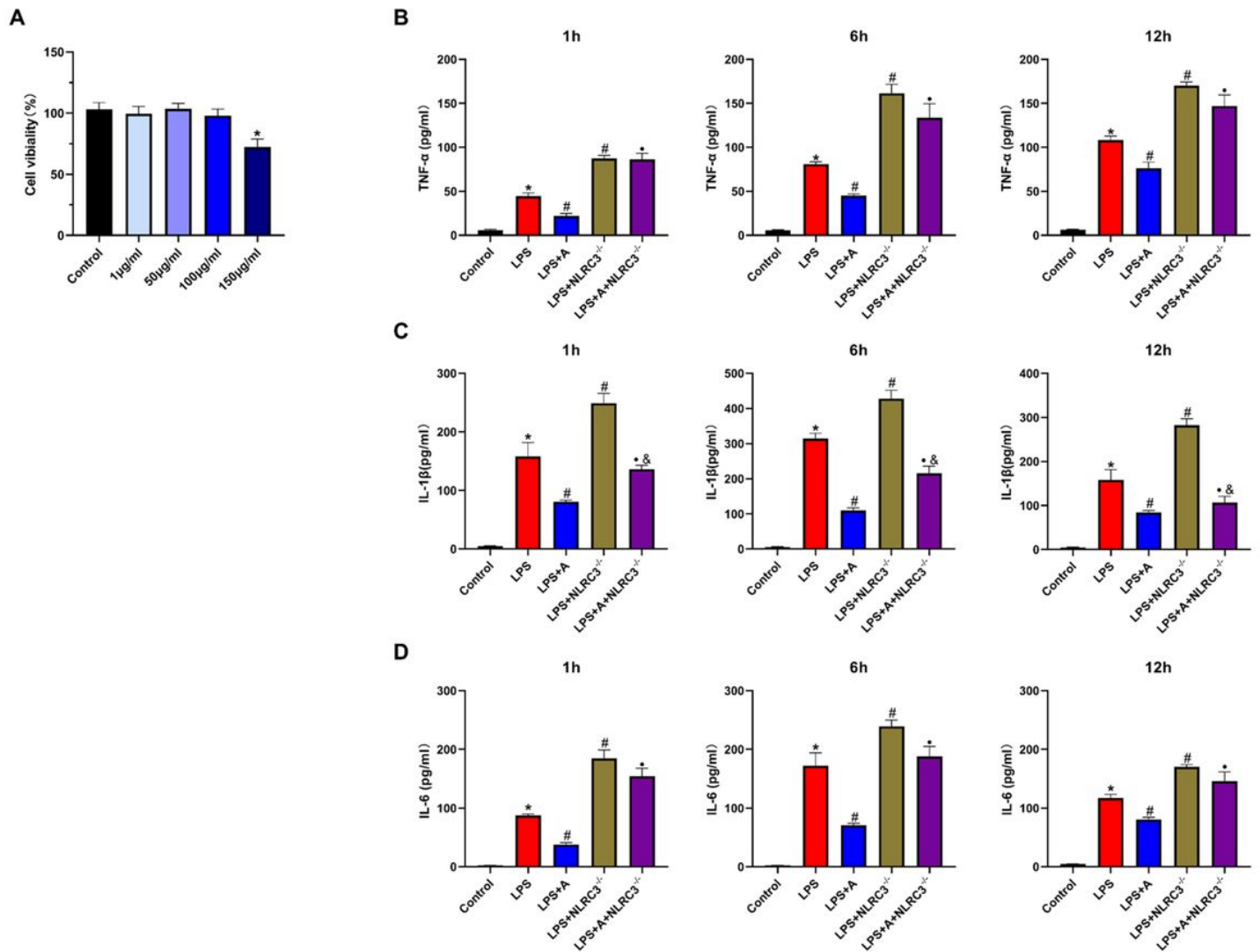


Figure 7

Pretreatment of BMDMs with acacetin can reduce LPS-induced inflammatory cytokines production. (A): Effect of different doses of acacetin (1, 50, 100, 150 μg/ml) on BMDMs viability (n = 5). (B-D): The concentrations of TNF-α (B), IL-1β (C), and IL-6 (D) in BMDMs culture medium were measured by ELISA at 1, 6 and 12 h (n = 6). Data are expressed as mean ± SEM, with statistical significance denoted as * P < 0.05 vs. Control group, # P < 0.05 vs. LPS group, ● P < 0.05 vs. LPS+A, & P < 0.05 vs. LPS+NLRC3^{-/-}.

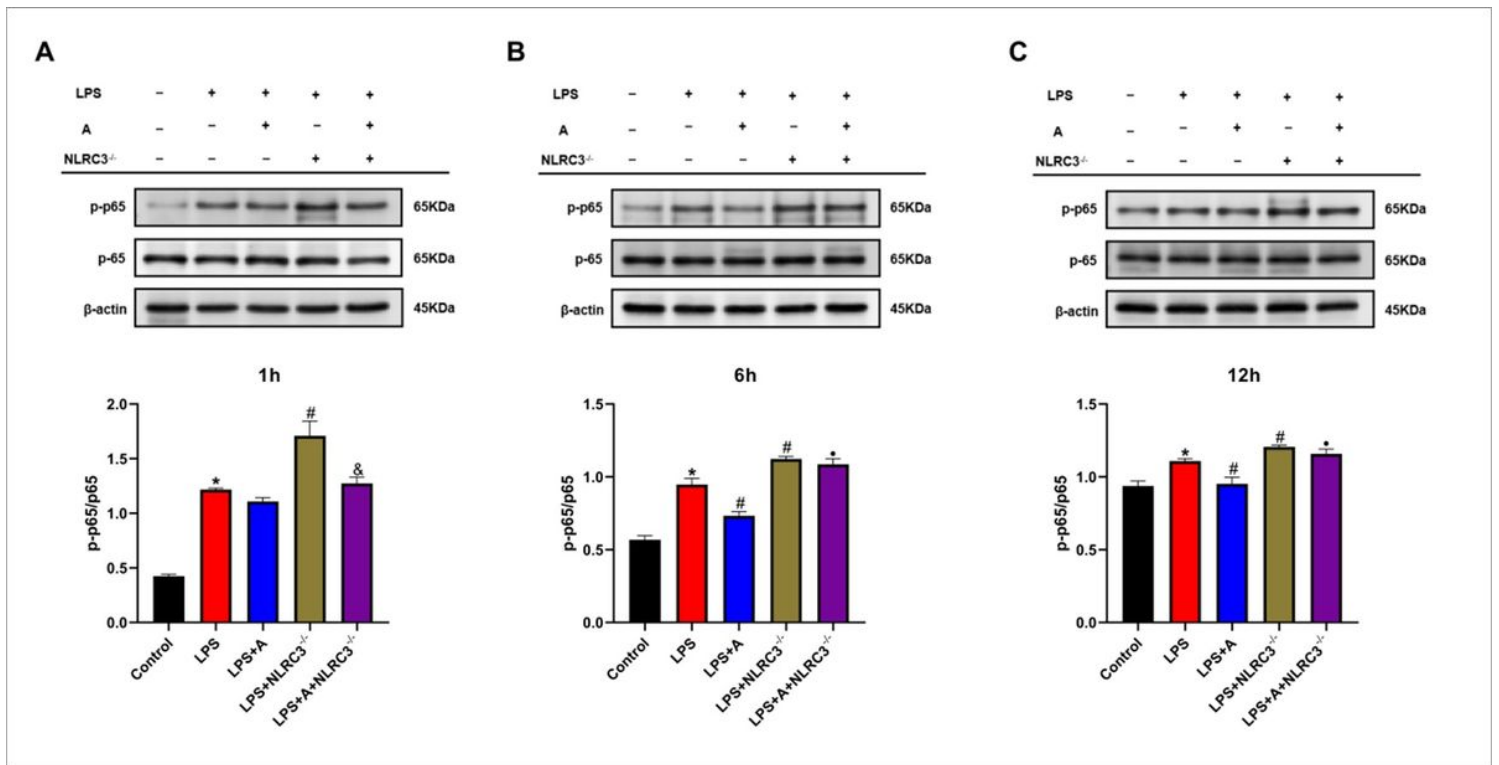


Figure 8

Effect of acacetin pretreatment on the activation of NF- κ B in macrophages. (A-C) The protein expression of total and phosphorylated NF- κ B p65 in BMDMs at 1, 6 and 12 h (n = 5). Data are expressed as mean $\pm$ SEM, with statistical significance denoted as * P < 0.05 vs. Control group, # P < 0.05 vs. LPS group, ● P < 0.05 vs. LPS+A, & P < 0.05 vs. LPS+NLRC3^{-/-}.

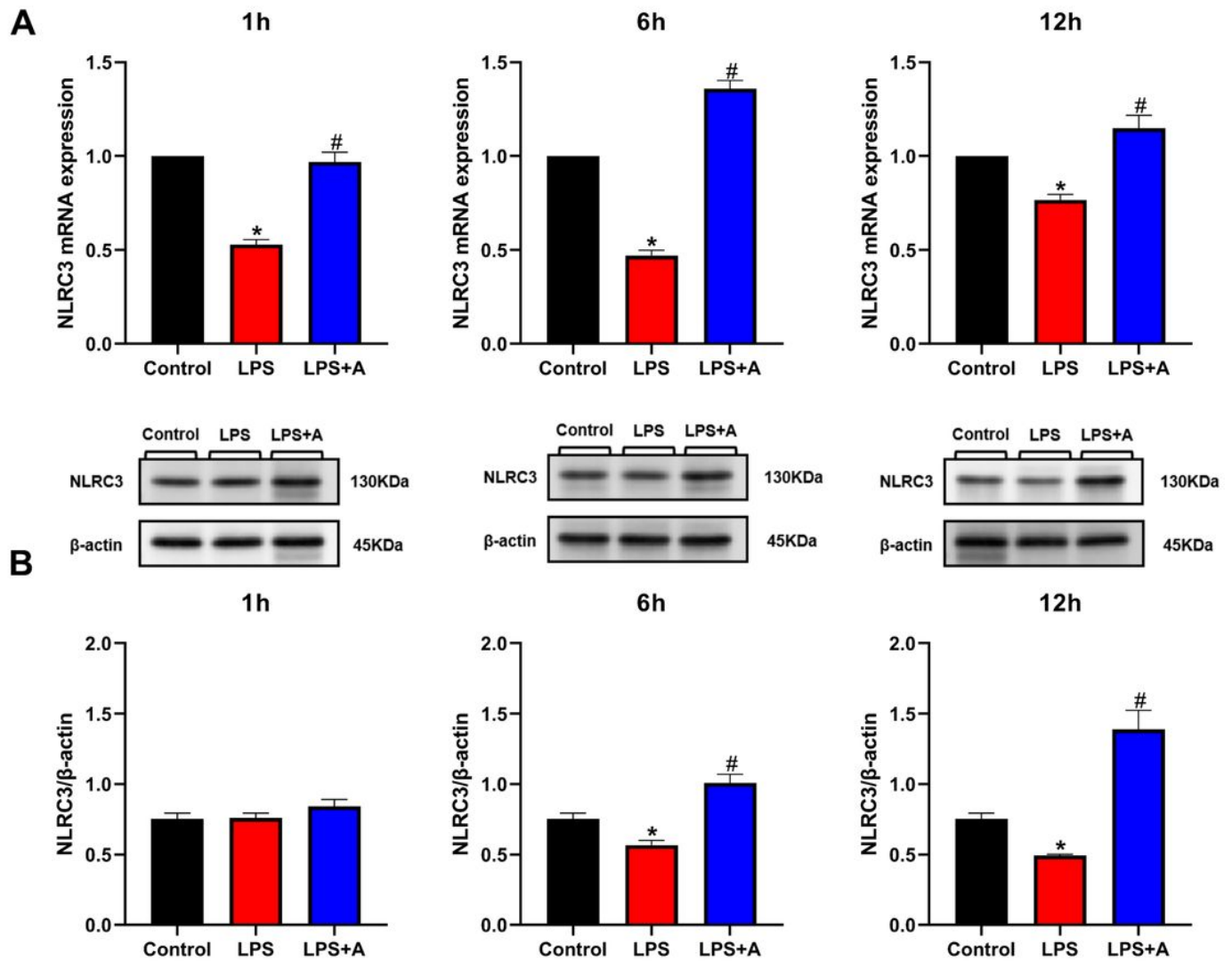


Figure 9

Acacetin pretreatment promoted the expressions of NLRC3 in BMDMs. (A): The mRNA expression of NLRC3 in BMDMs at 1, 6 and 12 h (n = 3). (B): The protein expression of total and phosphorylated NF-κB p65 in BMDMs at 1, 6 and 12 h (n = 5). Data are expressed as mean ± SEM, with statistical significance denoted as * P < 0.05 vs. Control group, # P < 0.05 vs. LPS group.