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Rev-erbα can regulate the NF-κB/NALP3 pathway to modulate lipopolysaccharide-induced acute lung injury and inflammation

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ABSTRACT

Progressive lung injury and pulmonary inflammation can be induced by an intraperitoneal injection of lipopolysaccharide (LPS). Interleukin-1β (IL-1β) is a key pro-inflammatory cytokine that can further exaggerate inflammation, which is cleaved and activated by the NALP3 inflammasome. Although the nuclear receptor Rev-erbα attenuates the level of LPS-induced pulmonary inflammation, the mechanism remains unclear. In this study, we investigated the influence of LPS-induced production of IL-1β and Rev-erbα on the development of lung inflammation. Herein, we demonstrate that Rev-erbα reduces IL-1β production and lung injury following an intraperitoneal injection of LPS, which is dependent on the NF-κB/NALP3 pathway. Thus, Rev-erbα is able to decrease the extent of acute lung injury by regulating IL-1β production. This mechanism may represent a potential novel therapeutic approach for lung injury.

1. Introduction

Acute lung injury (ALI) is a severe complication that occurs following pulmonary inflammation and is associated with high mortality rates in the patient population [1]. Moreover, ALI is characterized as a severe acute inflammatory response that involves the production of multiple inflammatory mediators [2,3]. In particular, interleukin-1β (IL-1β) is a proinflammatory cytokine released by activated monocytes and macrophages that can further exaggerate an inflammatory response [4,5]. IL-1β activation typically requires two steps: 1) nuclear factor-kappa B (NF-κB) activation, which induces the synthesis of pro-IL-1β; and 2) caspase-1-mediated cleavage of the inactive pro-IL-1β into mature IL-1β, which occurs in a large multiprotein complex, termed the NALP3 inflammasome [6–8]. This inflammasome contains the Nod-like receptor (NLR) protein Nalp3 (also called cryopyrin or NLRP3), the adaptor (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain [ASC]), Cardinal and caspase-1, which is associated with the production of mature IL-1β [9,10]. Caspase-1 cleaves the precursor cytokine pro-IL-1β into its mature form, IL-1β [9,11]. The requirement of caspase-1 for the production of IL-1β is confirmed in Caspase-1-deficient mice, which exhibit a defect in mature IL-1β production following LPS stimulation, and subsequent resistance to endotoxic shock [12]. In addition, NF-κB is required to initiate the NALP3 inflammasome [13]. In short, both active NF-κB and caspase-1 are important for the production of active IL-1β by activated macrophages and monocytes [5].

Rev-erbα is an orphan nuclear receptor that plays a role in blocking proinflammatory signalling in macrophages [14,15], and is a negative regulator of NF-κB [16]. Therefore, we hypothesized that Rev-erbα is capable of regulating the NF-κB and NALP3 pathways to modulate pro-IL-1β and IL-1β production.

We analysed the association between the innate immune system and Rev-erbα using a mouse model of sepsis. Our results demonstrate that Rev-erbα can inhibit the caspase-1-dependent maturation of IL-1β by downregulating NF-κB. These findings suggest that Rev-erbα plays an important role in the initial inflammatory responses that alleviate lung injury.

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2. Materials and methods

2.1. Ethics statement

All animal experimental procedures were approved by ethics committee of Subei People's Hospital. All animal experiments were performed in accordance with Chinese Food and Drug Administration guidelines.

2.2. Mouse model of acute lung injury

Acute lung injury mouse model was prepared using Six-week-old C57BL/6 male mice (Centre of Comparative Medicine, Yangzhou University) weighing 18 to 22 g. Lung injury model was made by intraperitoneal injection of LPS into mice. Briefly, the mice ($n = 40$) were randomly divided into four groups and treated by: NS (control), 2 mg/kg of SR8278 (SR8278, antagonist of Rev-erbα, Sigma), 10 mg/kg of LPS, or 10 mg/kg of LPS and followed by 2 mg/kg of SR8278 after 2 h. The mice were sacrificed after 24 h of treatment, and BALFs and lung tissues were collected. For the survival experiment, the mice ($n = 32$) were also randomly divided into 4 groups, and the treatment protocol is the same with the above. The survival rate was monitored every 12 h. All animals were maintained in pathogen-free environment.

2.3. Cell culture

The RAW 264.7 cells were supplied by the Cellular Immunity Laboratory of the Medical College, Yangzhou University. The cells were cultured in a humidified cell incubator with 5% CO2 and 37 °C. DMEM-F12 medium (HyClone) is supplemented with 1% penicillin/streptomycin (GIBCO-BRL) and 10% fetal bovine serum (FBS, GIBCO-BRL). RAW 264.7 cells were planted into 6-well culture plates at a concentration of 1 × 106 cells/ml. Raw cells were first treated by ATP (5 μM), meanwhile, treated with NS (control), GSK 4112 (20 μM), LPS (1 μg/mL) for 24 h, GSK 4112 (20 μM) for 6 h followed by LPS (1 μg/mL), SR 8278 (20 μM), or SR 8278 (20 μM) for 6 h followed by LPS (1 μg/mL).

2.4. Cell viability assay

The viability of RAW 264.7 cells was determined using a methyl thiazol tetrazolium (MTT) assays. Briefly, RAW 264.7 cells were seeded into a 96-well plate and treated with NS (control), GSK 4112 (20 μM), LPS (1 μg/ml) for 24 h, or GSK 4112 (20 μM) for 6 h followed by LPS (1 μg/ml) for the MTT assay.

2.5. Wet-to-dry weight ratios

An intraperitoneal injection of 5% chloral hydrate (0.1 mL/10 g) was delivered to anesthetize the mice, and the lungs were surgically removed. The upper left lung was ligated, excised, weighed, and then dried at 80 °C for 72 h. Finally, we calculated the wet-to-dry weight ratio of the upper left lung.

2.6. Histopathological analysis of the lungs of mice

The mice that were not used for bronchoalveolar lavage fluid (BALF) collection were sacrificed and the lungs were removed for HE (hematoxylin-eosin) staining using a standard method. The extent of injury was determined using the Murakami technique [17].

2.7. Cytokine measurements

The level of pro-IL-1β and IL-1β in the BALF and supernatants of RAW 264.7 cells were measured using a commercial ELISA kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions.

2.8. Western blot analysis

The protein concentration of each sample was assayed using a BCA protein assay kit (Beyotime, China) and standardized to BSA according to the manufacturer's protocol. Equal amounts of protein were resolved by 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with TBST containing 5% non-fat dried milk for 1 h at room temperature. Then, the membranes were incubated overnight with primary antibodies at 4 °C and with secondary antibodies in TBST at room temperature for 1 h. Rabbit anti- NALP3 (1:1000, Beyotime, China), Rabbit Anti-ASC Polyclonal Antibody (1:1000, Absin, China), Rabbit anti- caspase-1 (1:1000, Beyotime, China), Rabbit anti-NF-κB p65 (1:1000, Beyotime, China), Rabbit anti-NF-κB p-p65 (1:1000, Beyotime, China), Rabbit anti- IκB (1:1000, Beyotime, China), and Rabbit anti-p-IκB (1:500, Jianglai, China) were used as the primary antibodies to detect the target proteins.

2.9. Immunofluorescence confocal microscopy

RAW 264.7 cells were harvested and fixed in 4% paraformaldehyde for 20 min, and the cells were treated with Alexa Fluor 555-conjugated donkey anti-rabbit IgG (Abcam) for confocal microscopy.

2.10. Bronchoalveolar lavage for the total cell counts

An intratracheal injection of 0.8 mL PBS followed by gental aspiration three times was performed to obtain the BALF. After centrifuging at 1200 × g for 10 min at 4 °C, the cell pellets were resuspended in 10 μL of PBS and the total number of cells was counted. The cells were enumerated in a double-blind manner using a haemocytometer following Giemsa staining.

2.11. Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared using a nuclear extract kit (Thermo Fisher Scientific, USA). The EMSA method was used to characterize the binding activity of the NF-κB transcription factor in nuclear extracts using the DIG Gel Shift Kit as described in our previous paper.

2.12. Statistical analysis

All data are presented as the mean ± S.E.M. A one-way analysis of variance (ANOVA) was used to compare the differences between groups using SPSS 17.0 (SPSS Inc., Chicago, IL). A threshold value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Pre-treatment with the Rev-erbα antagonist, SR8278, exacerbates LPS-induced lung permeability in mice

During lung injury, there is increased water content in the lungs, and thus, the degree of injury is generally judged by the W/D ratio of the lung. Fig. 1A shows that the lung W/D ratio significantly increased following LPS challenge compared to that of the control group ($P < 0.001$). There was a significant increase in the lung W/D ratio following the administration of SR8278 (2 mg/kg) + LPS ($P = 0.004$, vs. the LPS group). However, compared with the control treatment, a single dose of SR8278 did not have an effect.

One of the characteristics of lung injury is the infiltration of inflammatory cells, such as macrophages and neutrophils [18]. Therefore, we counted the total number of cells, alveolar macrophages and neutrophils in BALF. LPS treatment caused a marked increase in the total

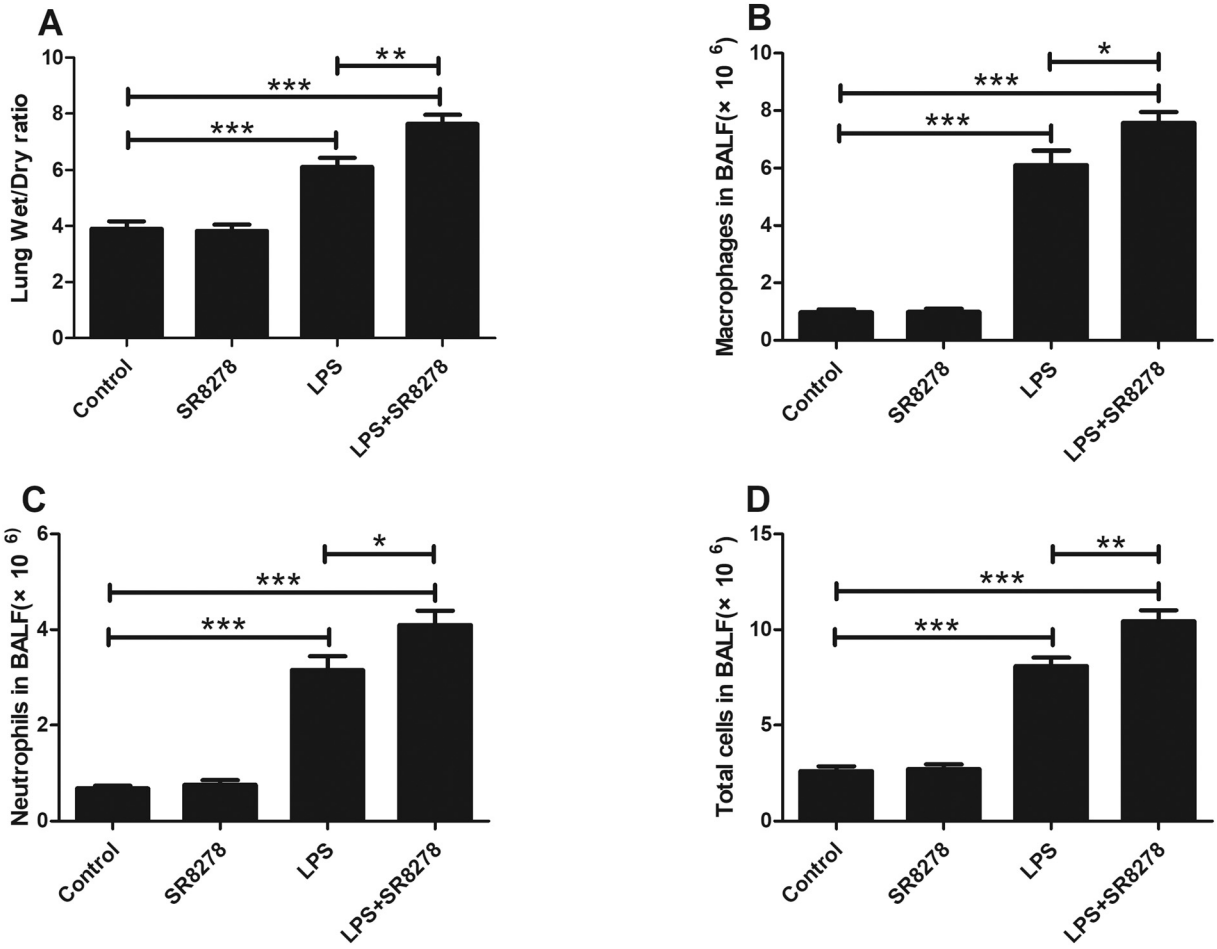

Fig. 1. The effect of SR8278 on the wet/dry ratio of the lung and number of cells in the BALF associated with LPS-induced ALI in mice. (A) Lung wet/dry ratio. (B) Number of alveolar macrophages in the BALF. (C) Number of neutrophils in the BALF. (D) Number of total cells in the BALF. The data are presented as the mean ± S.E.M. ($n = 10$); *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

number of cells in the BALF ($P < 0.001$, vs. the control group), as illustrated in Fig. 1D. However, treatment with SR8278 (2 mg/kg) + LPS significantly increased the number of total cells ($P = 0.006$, vs. LPS group), as shown in Fig. 1D. A similar trend was also observed for the number macrophages and neutrophils in the BALF (Fig. 1B and C). These results demonstrate that SR8278 + LPS could significantly increase LPS-induced lung vascular permeability and inflammatory cells in mice with ALI.

3.2. Effects of pre-treatment with the Rev-erbα antagonist, SR8278, on ALI-induced histopathological changes in the lung of mice

To determine the effect of Rev-erbα on the severity of LPS-induced lung injury we performed a histopathological analysis of the lungs with a light microscope (Fig. 2). Fig. 2A is the normal lung tissue and there is no change in SR8278 alone group (Fig. 2B). Fig. 2C shows that the LPS group exhibited significant pathological changes, including thickening of the alveolar walls, infiltration of inflammatory cells and congestion; however, Fig. 2D shows that the LPS-induced histopathological changes were significantly exacerbated by SR8278 (2 mg/kg) treatment. The Murakami technique was used to grade the degree of lung injury as an indication of the responses to these changes (Fig. 2E). These data indicate that the changes in the group treated with 2 mg/kg SR8278 + LPS were more obvious compared with LPS group ($P = 0.037$).

3.3. Gene expression of Rev-erbα under treatment of Rev-erbα antagonist, SR8278, in the lung of mice

As a specific antagonist of Rev-erbα, SR8278 was used to decrease Rev-erbα expression. Western Blotting was then used to quantify the level of Rev-erbα expression in the lung (Fig. 3A–B). Fig. 3 indicated that Rev-erbα expression was suppressed in the group treated with LPS ($P < 0.001$, vs. the control group). Treatment with SR8278 (2 mg/kg) significantly decreased Rev-erbα expression in the lung tissues ($P = 0.004$, vs. the control group). The expression of Rev-erbα in lung tissues after treatment with SR8278 (2 mg/kg) + LPS was the lowest among the four groups ($P = 0.037$, vs. the LPS group).

3.4. Toxic effects of the Rev-erbα agonist, GSK4112, on cell viability in vitro

To assess whether an effective concentration of GSK4112 is cytotoxic, we treated RAW 264.7 cells with 20 μM GSK4112 with or without LPS (1 μg/mL). After 18 h, cell viability was measured using an MTT assay. Fig. 3C showed that GSK4112 caused no toxicity in RAW 264.7 cells ($P = 0.088$, vs. the control group).

3.5. The effects of Rev-erbα on LPS-mediated pro-IL-1β and IL-1β production

Inflammation is an important pathological characteristic of ALI [20]. In this study, the level of IL-1β in the BALF and cell supernatant

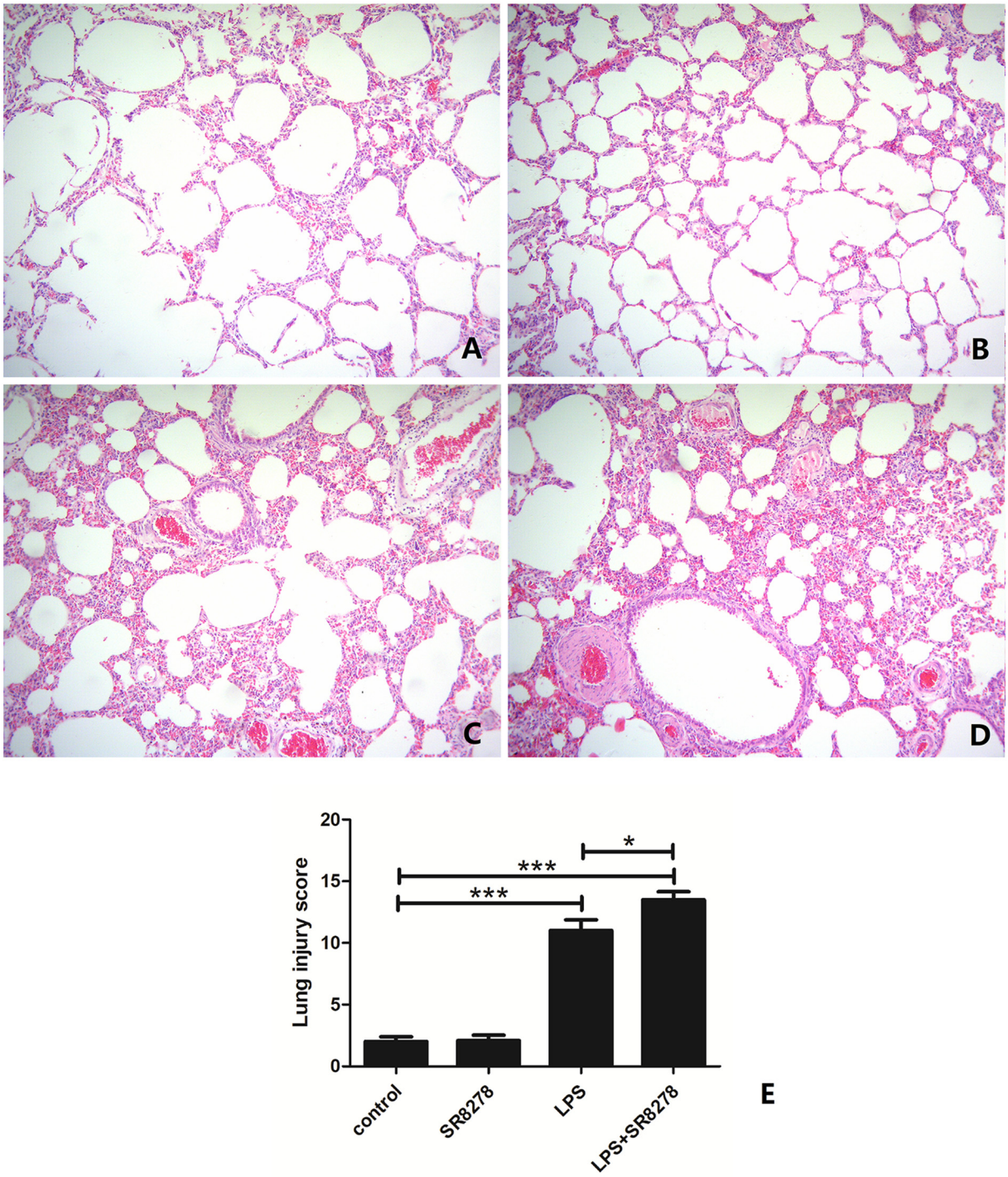

Fig. 2. Effect of SR8278 on changes in the lung morphology of mice with LPS-induced ALI. Tissue sections were analysed via light microscopy (magnification: ×200). (A) Control group; (B) SR8278 group; (C) LPS group; (D) LPS + SR8278 group. (E) The Murakami technique was used to pathologically score the lung tissue samples. The data are expressed as the mean ± S.E.M. ($n = 10$); *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

was determined. The results showed that IL-1β levels in the BALF and cell supernatant of RAW 246.7 cells were significantly higher than those in the control group ($P < 0.001$) following LPS (Fig. 4A-B). Moreover, treatment with 2 mg/kg SR8278 + LPS substantially enhanced this effect ($P = 0.009$), and treatment with GSK4112 + LPS ($P = 0.007$) substantially inhibited this effect in vitro (Fig. 4B). We also detected pro-IL-1β expression in the BALF and RAW 264.7 cells, and found that the trend was consistent with the expression of IL-1β (Fig. 4C–F).

3.6. Rev-erbα inhibits lipopolysaccharide (LPS)-induced NALP3 activation in vitro

IL-1β is regulated by the activation of caspase-1, which cleaves pro-IL-1β into mature bioactive IL-1β. This process is dependent on the NALP3 inflammasome. The NALP3 inflammasome contains the NLR protein Nalp3, the adaptor ASC and caspase-1. Protein expression was detected using Western blotting (Fig. 5A). We observed that NALP3, ASC, and p20 active caspase-1 fragments in RAW 264.7 cells were elevated 24 h after LPS treatment compared with the control group

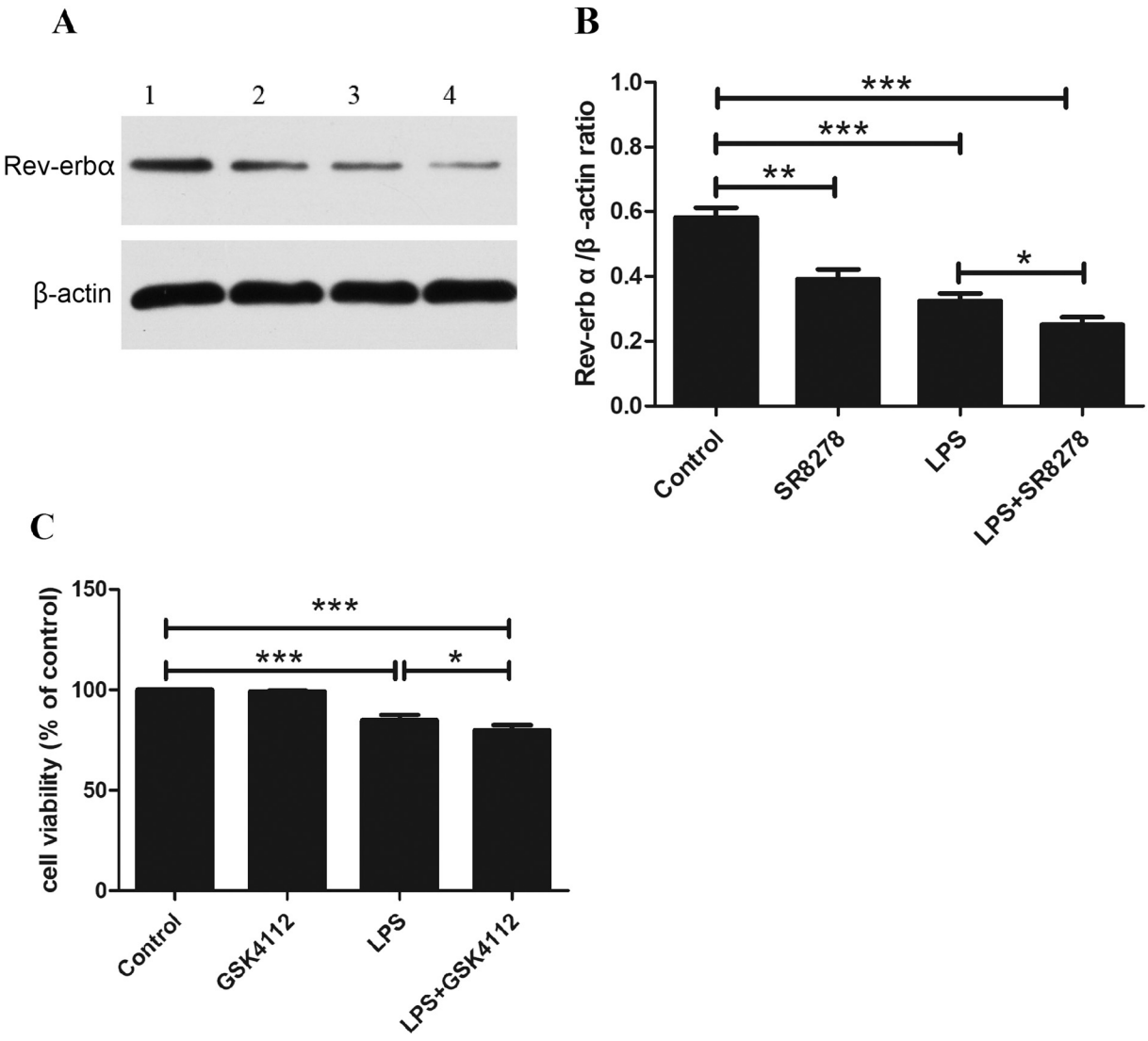

Fig. 3. Rev-erbα expression under treatment of Rev-erbα antagonist, SR8278, in lung tissues of mice, and the toxic effect of the Rev-erbα agonist GSK4112 on cell viability in RAW 246.7 cells. (A-B) Rev-erbα levels under treatment of SR8278 were determined by Western blot analyses of the lung tissues. Lanes 1–4 were control, SR8278, LPS, and LPS + SR8278 groups. (C) Cell viability of GSK4112 toxicity was measured using an MTT assay after 18 h treatment. The data are presented as the mean ± S.E.M. (n = 10); *P < 0.05, ** P < 0.01, *** P < 0.001.

(Fig. 5B–D). The treatment of RAW 264.7 cells with GSK4112 reduced the levels of NALP3, ASC and p20 active caspase-1 fragments caused by LPS. On the contrary, SR8278 showed opposite effect. Green cytoplasmic areas (representative of the areas containing ASCs) were observed in RAW 264.7 cells in addition to blue nuclear staining (Fig. 5E). These results indicated that LPS group increased the activation of ASCs at the single cell level, but GSK4112 decreased the activation of ASCs. Therefore, Rev-erbα can downregulate the activation of the NALP3 inflammasome. Thus, Rev-erbα is associated with the regulation of IL-1β production in macrophages.

3.7. Rev-erbα inhibits lipopolysaccharide (LPS)-induced NF-κB activation in vitro

The first step of controlling IL-1β secretion is the activation of NF-κB, which increases IL-1β transcription, as well as biologically inactive precursors (pro-IL-1β). To evaluate the effects of Rev-erbα on the NF-κB pathway, we examined the level of endogenous serine phosphorylated P65 and IkB-α, which are known markers of NF-κB activation. We found that treatment with 20 μM GSK4112 for 2 h effectively inhibited LPS-induced NF-κB activation for 24 h compared with the LPS group (Fig. 6A–C), especially inhibited the expression of phosphorylated p65 and IκBα. On the contrary, treatment with SR8278 enhanced LPS-induced NF-κB activation compared with the LPS group. We also found that GSK4112 suppressed the DNA binding activity of NF-κB by EMSA (Fig. 6D). DAPI was used to stain the nuclei. Green cytoplasmic areas (representative of the areas containing p65) were observed in RAW 264.7 cells as well as blue nuclear staining (Fig. 6E). These results indicate that the nuclear localisation of activated NF-κB p65 occurred at the single cell level. In contrast, LPS + GSK4112 -treated cells exhibited decreased levels of nuclear p65 staining compared with the LPS group, while LPS + SR8278 -treated cells increased the levels of nuclear p65 staining, in comparison to LPS –treated cells. These findings indicate that Rev-erbα is associated with the mechanism controlling IL-1β production in macrophages.

3.8. The association of the Rev-erbα and overall survival in mice

Fig. 7 shows that the survival rate was lower following LPS treatment compared with that of the control group ($P < 0.05$). Additionally, compared to LPS treatment, SR8278 (2 mg/kg) + LPS treatment was associated with a decreased survival rate ($P < 0.05$).

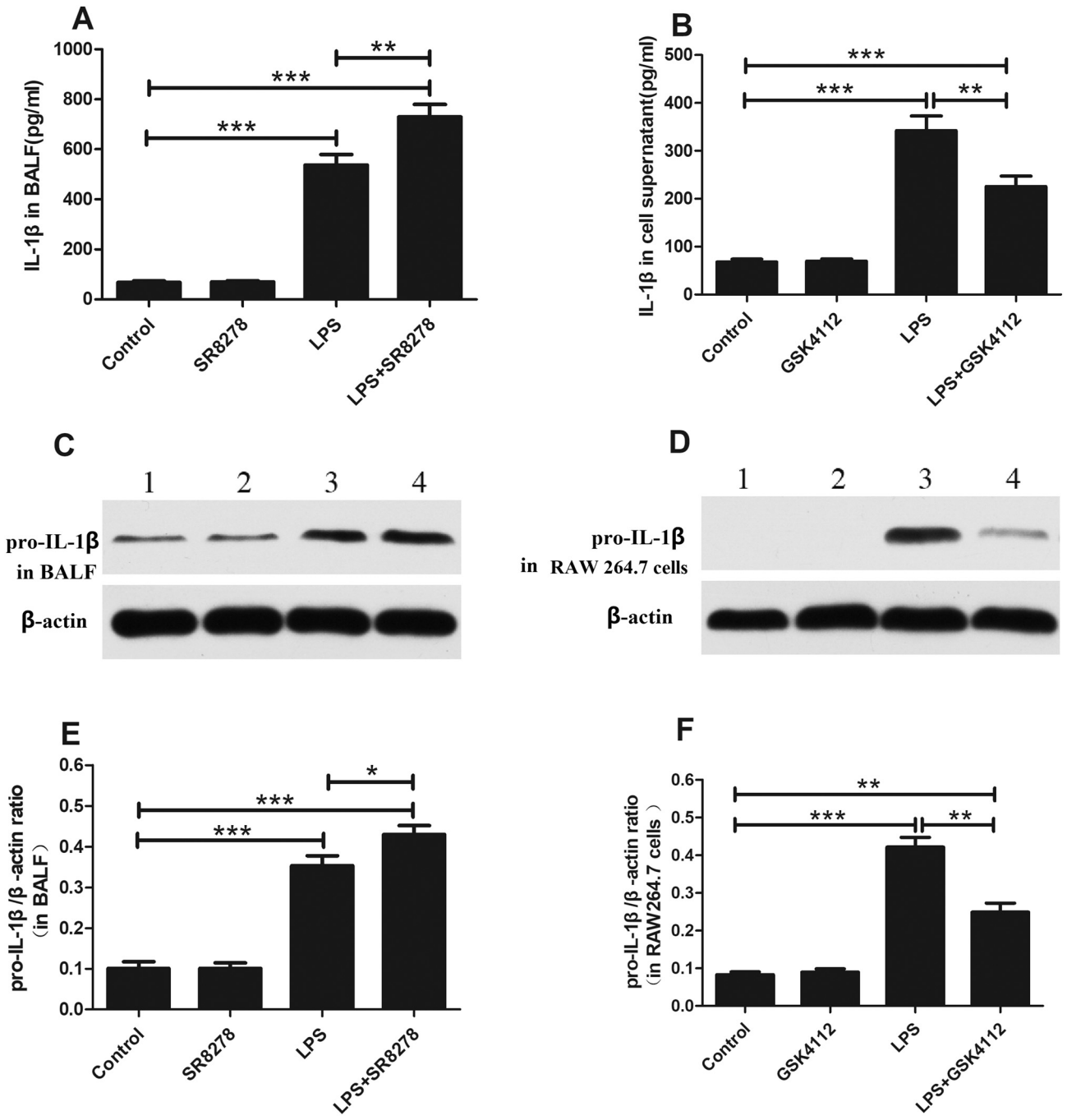

Fig. 4. Effect of GSK4112 and SR8278 on cytokine production. (A) Level of IL-1β in the BALF. (B) IL-1β in the cell supernatant. (C and E) Pro-IL-1β in the BALF. β-actin was used as the control. Groups: lane 1, control; lane 2, SR8278 only; lane 3, LPS only; lane 4, LPS + SR8278. (D and F) Pro-IL-1β in RAW 264.7 cell. The level of β-actin protein was assessed as a control. Groups: lane 1, control; lane 2, GSK4112 only; lane 3, LPS only; lane 4, LPS + GSK4112. The data are presented as the mean ± S.E.M. (n = 10); *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

However, treatment with 2 mg/kg SR8278 alone did not affect the survival rate compared the control group.

4. Discussion

The proinflammatory cytokine, IL-1β, is released by activated monocytes and macrophages, and thought to play an important role in LPS-induced lung injury [20,21]; moreover, its release induces significant tissue destruction and increases inflammation [22]. In macrophages, there are two distinct signals that induce IL-1β transcription: 1) translation and 2) activation. Initially, the activation of NF-κB induces the synthesis of pro-IL-1β precursors. Subsequently, caspase-1 of the NALP3 inflammasome cleaves the inactive pro-IL-1β precursor into mature IL-1β (Fig. 8). The NALP3 inflammasome is comprised of NLRs, ASC, and caspase-1. Activation of the NALP3 inflammasome occurs in response to pore-forming toxins, ATP and hypoxic cellular injury in conjunction with pathogen exposure or local cytokine production [23].

As a nuclear receptor superfamily member, Rev-erbα recruits both the class I histone deacetylase 3 (HDAC3) and nuclear receptor corepressor-1 (NCoR) to suppress target genes in various tissues. GSK 4112 is a synthetic ligand for Rev-erbα, and is a functional receptor for porphy in heme [24]. The Rev-erbα antagonist, SR8278, is similar in

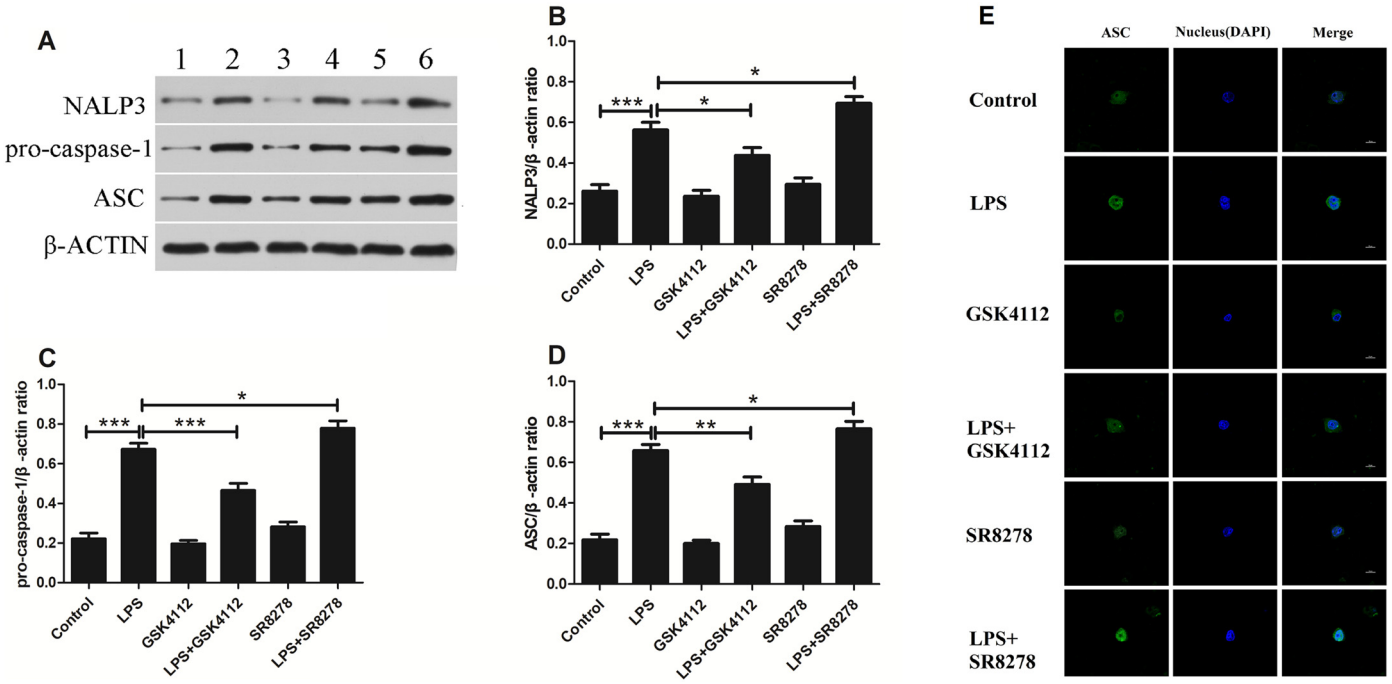

Fig. 5. The effect of GSK4112 on inflammasome expression in vitro. The expression of NALP3 protein was analysed using a Western blot and specific antibodies. The level of β-actin protein was assessed as a control. (A, B, C and D) NALP3, pro-caspase-1, and ASC in RAW 264.7 cells were assessed via Western blot. The level of β-actin protein was assessed as a control. Groups: lane 1, control; lane 2, LPS only; lane 3, GSK4112 only; lane 4, LPS + GSK4112; lane 5, SR8278; lane 6, LPS + SR8278. The data are presented as the mean ± S.E.M. (n = 10); *P < 0.05, ** P < 0.01, ***P < 0.001. (E) ASC (green color) expression in RAW 264.7 cells as evaluated by confocal microscopy. The blue staining indicates the nuclei. Magnification: ×1200. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

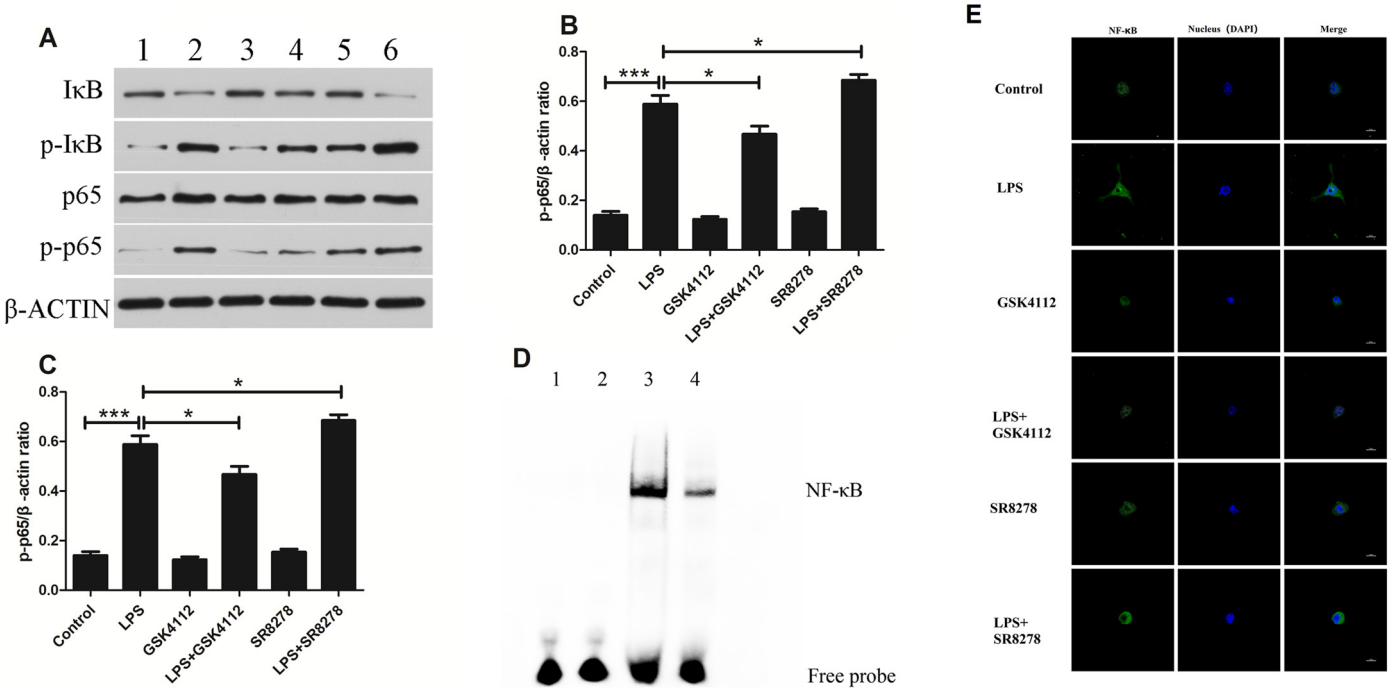

Fig. 6. The effect of GSK4112 on NF-κB expression and activation in vitro. (A, B and C) The level of IκBα and p65 protein expression in the lung tissues were detected via Western blot. The level of β-actin protein was assessed as a control. Groups: lane 1, control; lane 2, LPS only; lane 3, GSK4112 only; lane 4, LPS + GSK4112; lane 5, SR8278; lane 6, LPS + SR8278. The data are presented as the mean ± S.E.M. (n = 10); *P < 0.05, ** P < 0.01, ***P < 0.001. (D) EMSA was used to evaluate the extent of NF-κB DNA binding activity. Nuclear proteins derived from the lung tissue extracts were used to assess the level of interaction with NF-κB biotin-labelled probes. (E) NF-κB (green color) expression in the lung tissues as evaluated by confocal microscopy. The nuclei are indicated with blue staining. Magnification: ×1200; scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

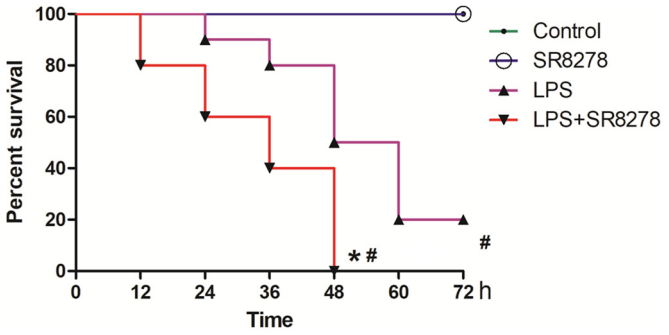

Fig. 7. The association of Rev-erbα and the overall survival in mice. Mice were injected with LPS ($n = 8$) and monitored for 72 h to determine the survival rate. The Kaplan-Meier method was used to assess the differences between each group. *P < 0.05 vs. LPS; # P < 0.05 vs. control.

structure to the agonist, but it inhibits the enhanced Rev-erbα-dependent suppression induced by GSK4112 [25]. GSK4112 (agonists), and SR8278 (antagonist) have been previously used to regulate the target genes of Rev-erbα [26].

When we used SR8278 in vitro, the expression of Rev-erbα was inhibited. Rev-erbα is a major regulator of the transcription of circulating Bmal1, and Rev-erbα expression is negatively regulated by BMAL1. Thus, Rev-erbα and BMAL1 form a feedback loop [27].

When the expression of Rev-erbα is suppressed, lung injury caused by LPS is aggravated, and the secretion of IL-1β induced by LPS increases.

It has been well-established that NALP3 promotes the recruitment of ASCs and procaspase-1 to cleave pro-IL-1β into IL-1β [28,29].

To investigate whether Rev-erbα impacts the formation of the NALP3 inflammasome, we measured the changes in NALP3 after treatment with a Rev-erbα agonist (GSK4112) in vivo. Compared with LPS treatment, treatment with GSK4112 decreased NALP3, ASC and p20 active caspase-1 fragments in RAW 264.7 cells. A substantial increase in the expression of NALP3 was observed following treatment with LPS. In contrast, this increased level of NALP3 expression was inhibited by GSK4112 treatment. As we hypothesized, GSK4112 significantly suppressed the activation NALP3 inflammasome.

NALP3 activation requires the activation of NF-κB [13]. The NF-κB signalling pathway is considered a key factor involved in the activation of inflammatory genes which induce inflammation [30,31]. Moreover, the suppression of NF-κB activation can alleviate LPS-induced inflammation [32]. It has also been reported that Rev-erbα can modulate NF-κB activation [33,34]. Thus, we hypothesized that Rev-erbα could inhibit LPS-induced NF-κB activation and reduce activation of the NALP3 inflammasome. Our findings involving treatment with the Rev-erbα agonist, GSK4112, support this hypothesis. The level of expression of NF-κB protein was determined by Western blot using phosphorylated P65 and IκB-α as well-established markers of NF-κB activation. The level of phosphorylated p65 and IκBα protein was significantly increased in the LPS group but decreased in the GSK4112 treatment groups. In addition, our results revealed that Rev-erbα decreased the DNA binding activity of NF-κB. Moreover, using confocal microscopy, we observed that Rev-erbα blocked the p65 nuclear activation induced by LPS treatment. These results strongly suggest that Rev-erbα suppresses LPS-enhanced NF-κB activity in vitro.

To investigate the anti-inflammatory effects of Rev-erbα, SR8278 was used to inhibit the expression of Rev-erbα in vivo. The results showed that Rev-erbα aggravated the degree of lung injury induced by LPS in mice, increased the infiltration of macrophages and neutrophils, elevated the production of proinflammatory mediators, and ultimately reduced the survival of LPS-treated mice.

In the present study, Rev-erbα was observed to regulate the NF-κB and NALP3 pathways to modulate LPS-induced IL-1β secretion. We found that pre-treatment with the Rev-erbα agonist, GSK 4112, significantly inhibited proinflammatory cytokine release after LPS treatment in vitro. Moreover, pre-treatment with the Rev-erbα antagonist SR8278 significantly increased proinflammatory cytokine release following treatment with LPS and aggravated lung injury in vitro. Therefore, these data indicate that Rev-erbα exhibits a general anti-inflammatory effect.

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Author contributions

L.Z., J.G. designed the study; D.Y., X.F., T.H., Y.Z., Y.G. and Y.L. collected and analysed the data; X.F. and D.Y. interpreted the data; L.Z. and D.Y. drafted the manuscript; L.Z. and H.X revised the manuscript; Y.X. conducted the experiment for revision.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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