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Research Article

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Research Article

NRF1 knockdown alleviates lipopolysaccharide-induced pulmonary inflammatory injury by upregulating DKK3 and inhibiting the GSK-3β/β-catenin pathway

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Abstract

Excessive inflammatory injury is the main cause of the incidence of severe neonatal pneumonia (NP) and associated deaths. Although dickkopf-3 (DKK3) exhibits anti-inflammatory activity in numerous pathological processes, its role in NP is still unknown. In this study, human embryonic lung WI-38 and MRC-5 cells were treated with lipopolysaccharide (LPS) to induce inflammatory injury of NP *in vitro*. The expression of DKK3 was downregulated in LPS-stimulated WI-38 and MRC-5 cells. DKK3 overexpression decreased LPS-induced inhibition of cell viability, and reduced LPS-induced apoptosis of WI-38 and MRC-5 cells. DKK3 overexpression also reduced LPS-induced production of pro-inflammatory factors such as ROS, IL-6, MCP-1, and TNF-α. Nuclear respiratory factors 1 (NRF1) knockdown was found to upregulate DKK3 and inactivate the GSK-3β/β-catenin pathway in LPS-injured WI-38 and MRC-5 cells. NRF1 knockdown also suppressed LPS-induced inhibition on cell viability, repressed LPS-induced apoptosis, and inhibited the accumulation of ROS, IL-6, MCP-1, and TNF-α in LPS-injured WI-38 and MRC-5 cells. DKK3 knockdown or re-activation of the GSK-3β/β-catenin pathway reversed the inhibitory effects of NRF1 knockdown on LPS-induced inflammatory injury. In conclusion, NRF1 knockdown can alleviate LPS-triggered inflammatory injury by regulating DKK3 and the GSK-3β/β-catenin pathway.

Keywords: neonatal pneumonia, NRF1, DKK3, inflammatory injury, GSK-3β/β-catenin

Abbreviations: AP-1: activator protein-1; DKK3: dickkopf-3; IL-6: interleukin-6; LPS: lipopolysaccharide; MCP-1: chemokines monocyte chemotactic protein-1; NFκB: nuclear factor κB; NP: neonatal pneumonia; NRF1: nuclear respiratory factors 1; STR: short tandem repeat; TNF-α: tumor necrosis factor-α.

Introduction

Neonatal pneumonia (NP) is a respiratory inflammatory disease that frequently seriously threatens the health and life of infants; clinically it mainly manifests as cough and damp rales in the lungs [1]. NP has a high probability of occurrence and mortality, and hundreds of thousands to millions of babies are reported to die of pneumonia and its complications annually [2]. The onset and development of NP are related to multitudinous factors, such as the inhalation of amniotic fluid or meconium, infection with mycoplasma, bacteria, or viruses and so on [3]. However, due to the particularity of newborns, the pathogenesis of NP has not been fully elucidated, and its clinical treatment is also difficult. Thus, understanding the pivotal pathogenesis of NP and exploring more constructive treatments are of great significance to newborns.

Continuously activated multiple cascading signals and excessive inflammation have been confirmed to be closely linked with the damage to lung-related cells and the pathological process of pneumonia. Inhibition of activator protein-1 (AP-1), nuclear factor κB (NFκB), and Wnt/GSK3β/β-catenin and other signaling pathways can improve the progression of pneumonia [4, 5]. Toll-like receptors (TLRs) are a type of important innate immune receptors in neonates and have been reported to mediate inflammatory response to lipopolysaccharide (LPS) [6], which is a major pathogenic constituent of Gram-negative bacteria that cause severe inflammation in the lungs. LPS-induced activation of TLR4 reportedly inhibits the canonical Wnt/β-catenin signaling, which was considered as a molecular target to many pathogenic bacteria [7]. Dickkopf-3 (DKK3), one of the regulators of Wnt/GSK-3β/β-catenin signaling, has a variety of important biological functions; e.g. it is involved in affecting immune response, regulating cell apoptosis, proliferation, and metabolism [8–10]. TLR4 knockdown was reported to upregulate DKK3 expression in U-87 xenograft glioblastomas [11]. TNF-α, a well-known downstream effector molecule of the LPS–TLR4 signaling, was reported to downregulate Dkk3 expression in normal skin keratinocytes [12]. The expression of DKK3 was found to be downregulated in lung cancer A549 cells exposed to LPS

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[13]. DKK3 ablation can exacerbate NF-κB activation in chondrocytes [14], and aggravate apoptosis and inflammation of cardiomyocytes [15], while DKK3 overexpression can relieve neuroinflammation in mice with intracerebral hemorrhage via regulating JNK and AP-1 expression [16]. These findings suggested that DKK3 is involved in the regulation of inflammation mediated by LPS/TLR4 signaling. However, whether and how DKK3 affects LPS-induced NP remains to be fully expounded.

In this study, DKK3 was found to be downregulated in the LPS-induced pneumonia cell model, which was established by infecting human embryonic lung cells with LPS. DKK3 overexpression repressed LPS-evoked inhibition of cell viability and reduced LPS-induced apoptosis and production of pro-inflammatory factors. Nuclear respiratory factors 1 (NRF1) knockdown alleviated LPS-triggered inflammatory injury in LPS-injured WI-38 and MRC-5 cells via upregulating DKK3 and inactivating the GSK-3β/β-catenin pathway. Thus, NRF1 and DKK3 may be promising targets for NP therapy.

Materials and methods

Cell culture and LPS-induced pneumonia cell model

Human embryonic lung WI-38 and MRC-5 cells, which have been verified by short tandem repeat (STR) analysis, were purchased from the BeNa Culture Collection (Beijing, China). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Beyotime, Shanghai, China) at 37°C in a humidified 5% CO_2 ambience. All the experiments were performed using the third passage of cells after resuscitation. To establish the cell model of NP, the cells were stimulated by 5, 10, or 20 μg/ml of lipopolysaccharide (LPS) from *Klebsiella pneumoniae* (*Cat. No.* L4268, Sigma, St. Louis, MO, USA) to instigate inflammatory injury. In addition, 1 μg/ml of etanercept (MCE, NJ, USA) was added to the medium to inhibit the bioactivity of TNF-α. And, 5 mM of lithium chloride (LiCl; Sigma), a Wnt signaling agonist, was added into the medium when the GSK-3β/β-catenin pathway needed to be activated.

Vectors and cell transfection

The DKK3-overexpressing plasmid (pGL3–DKK3) and its control pGL3 vector, short hairpin RNA (sh-RNA) specifically against NRF1 (sh-NRF1) or DKK3 (sh-DKK3) and their negative control sh-NC, were supplied by GenePharma (Shanghai, China). Transfection of pGL3–DKK3, pGL3, sh-NRF1, sh-DKK3, and sh-NC or co-transfection of sh-NRF1 and sh-DKK3 was performed using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (qRT–PCR)

After being treated with LPS for 24 h, total RNA from WI-38 and MRC5 cells was extracted by the RNeasy Mini Kit (Qiagen, Shanghai, China), and first-strand cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Shanghai, China). RT–PCR was performed with the SYBRR FAST Universal qPCR Kit (KAPA Biosystems, Woburn, MA, USA) on the MYGOPRO PCR system (IT-IS International, Middlesbrough, UK). The housekeeping gene GAPDH served as the internal control. Each reaction was performed in triplicate, and the relative expression of each gene was calculated with the $2^{-\Delta\Delta Ct}$ method and normalized with GAPDH.

Western blot assay

After being treated with LPS for 24 h, total proteins were extracted by the RIPA lysis buffer (Beyotime). Protein samples were separated via SDS–PAGE and electroblotted onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were occluded with 5% skimmed milk and reacted with the primary antibodies against DKK3 (1:1000; Abcam, Cambridge, UK), NRF1 (1:1000; Cell Signaling, Beverly, MA, USA), Phospho-GSK-3β (p-GSK-3β; 1:1000; Cell Signaling), β-catenin (1:1000; Cell Signaling), p-JNK (1:20 000; Abcam), c-Jun/AP-1 (1:1000; Abcam), Bax (1:2000; Abcam), Bcl-2 (1:10 000; Abcam), c-Capase3 (1:5000; Abcam), and GAPDH (1:10 000; Abcam) at 4°C overnight. Subsequently, horseradish peroxidase-linked secondary antibody (1:5000, Abcam) was incubated at room temperature with these membranes for 60 min. Finally, the protein bands were visualized using a chemiluminescence system (LuminoGraph WSE-6100; ATTO, Japan) and evaluated using Image J software.

Cell counting kit-8 (CCK-8) assay

CCK-8 (Sigma) assay was performed to assess cell viability. In short, the cells were inoculated on 96-well plates and cultivated for 24, 48, and 72 h with or without 10 μg/ml of LPS in each well, followed by incubation with 10 μL of CCK-8 reagent for another 2 h. Finally, the absorbance at 450 nm was read with a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow cytometry-based apoptosis detection

According to the experimental procedures recommended by the manufacturer, flow cytometry was adopted to detect cell apoptosis by using the Annexin V-FITC/propidium iodide Apoptosis Detection Kit (Beyotime). In short, cells were exposed to LPS for 12 h and then reacted with 5 μl of propidium iodine and 5 μl of FITC-linked Annexin-V in the dark. After 15 min of incubation, apoptotic cells were detected using a flow cytometer (BD Biosciences, San Jose, CA, USA).

ELISA assay for inflammatory factors and chemokines

After being treated with or without 10 μg/ml of LPS for 12 h, the level of monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) in cell supernatants was detected with their commercially available ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China).

Measurement of reactive oxygen species (ROS)

According to the manual of Reactive Oxygen Species Assay Kit (Beyotime), the level of intracellular ROS in cells exposed to LPS for 12 h was determined with the fluorescent probe 2′,7′-dichlorfluorescein-diacetate (DCFH-DA).

Transcription factors prediction and chromatin immunoprecipitation (ChIP)

The potential transcription factors that affect the promoter activity of the DKK3 were identified using the UCSC and JASPAR database, and found that the DKK3 promoter has a

putative binding site with NRF1. ChIP assay was conducted following the instructions on the ChIP kit (Thermo Fisher Scientific, Inc., Waltham, USA). The control anti-IgG antibody and anti-NRF1 antibody were used to immunoprecipitate the cross-linked chromatin, which was then analyzed by RT–qPCR after purification.

Luciferase reporter gene assay

The promoter fragment of DKK3 was cloned into a pGL3 luciferase reporter vector (Promega, WI, USA) to construct the pGL3–DKK3 promoter vector by GenePharma. According to the manufacturer's protocols, sh-NRF1 or sh-NC was, respectively, co-transfected into cells with a pGL3 empty vector

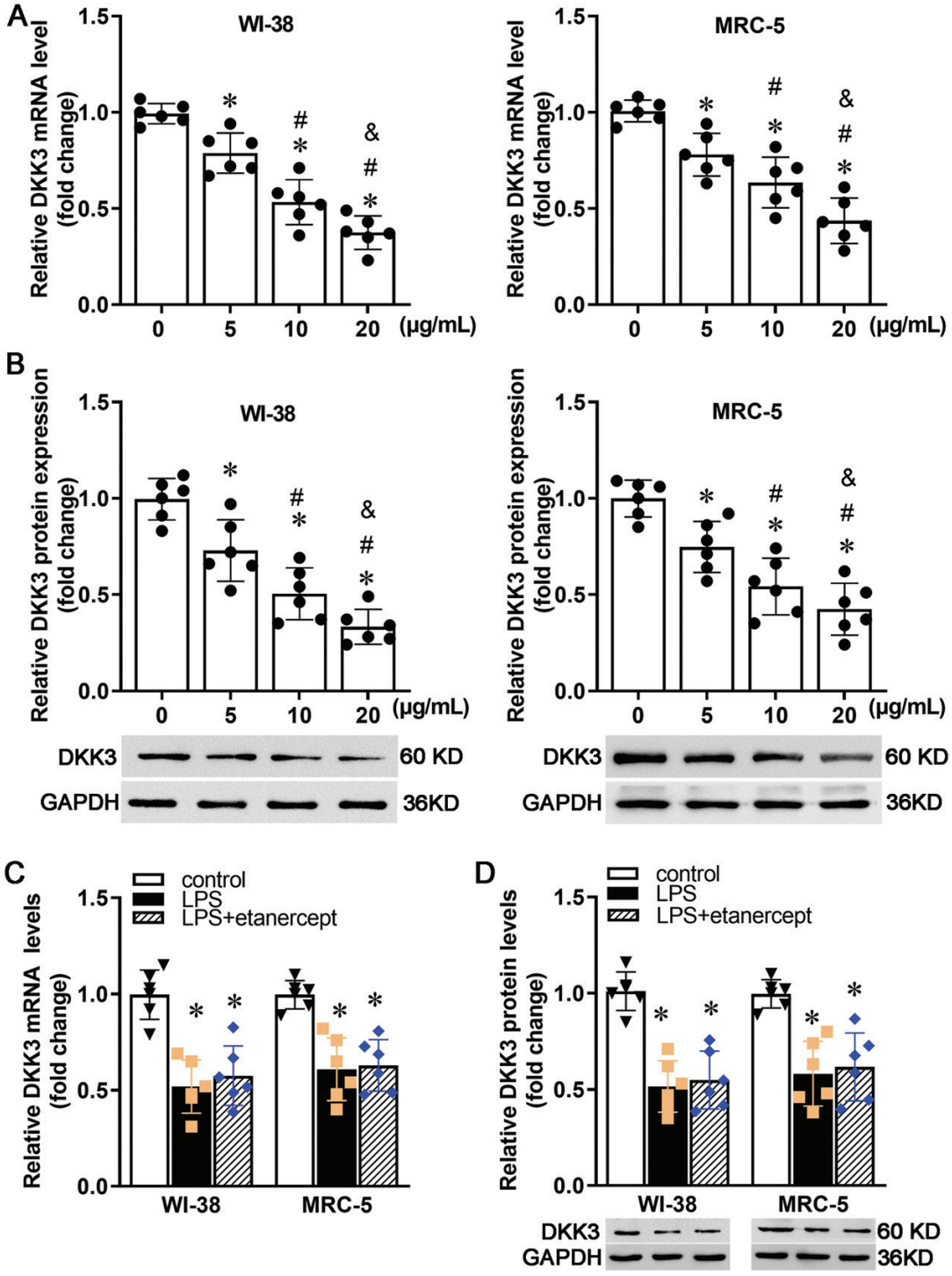

Figure 1. The expression of DKK3 in LPS-induced pneumonia cell model. WI-38 and MRC-5 cells were exposed to 0, 5, 10, or 20 μg/ml of LPS for 24 h. (A) RT–qPCR showed the DKK3 mRNA level in WI-38 and MRC-5 cells ($n = 6$). $^{*}P < 0.05$, compared with the 0 μg/ml group; $^{\#}P < 0.05$, compared with the 5 μg/ml group; $^{\&}P < 0.05$, compared with the 10 μg/ml group. (B) Representative western blotting images showed the DKK3 protein level in LPS-stimulated cells ($n = 6$). $^{*}P < 0.05$, compared with the 0 μg/ml group; $^{\#}P < 0.05$, compared with the 5 μg/ml group; $^{\&}P < 0.05$, compared with the 10 μg/ml group. (C) RT–qPCR showed the DKK3 mRNA level in WI-38 and MRC-5 cells ($n = 6$).$^{*}P < 0.05$, compared with the control group. Data were expressed as mean ± SD, and analyzed using one-way ANOVA with the Bonferroni test.

or pGL3–DKK3 promoter vector by using Lipofectamine™ 3000 (Invitrogen). Dual-Luciferase Reporter Assay System (Promega) was adopted to evaluate the luciferase activity at 48 h post-transfection.

Statistical analysis

All experiments were conducted a minimum of three times, and the results data were finally presented as mean ± SD. Statistical analysis was performed with the SPSS 22.0 software. For multiple-group comparisons, one-way analysis of variance (ANOVA) was used, followed by the Bonferroni test. Student's *t*-test was used to compare only two groups. The significant difference was identified with *P* values less than 0.05 ($P < 0.05$).

Results

DKK3 is decreased in LPS-challenged pneumonia cell model

The human embryonic lung WI-38 and MRC-5 cells were stimulated with 5, 10, or 20 μg/ml of LPS for 24 h to induce the *in vitro* cell model of NP. The mRNA and protein expression of DKK3 were both dose-dependently reduced in LPS-stimulated WI-38 and MRC-5 cells (Fig. 1A and B). Besides, compared with LPS group, LPS + etanercept group showed no significant effects on DKK3 mRNA and protein expression (Fig. 1C and D). These results suggested that LPS induces downregulation of DKK3 in WI-38 and MRC-5 cells.

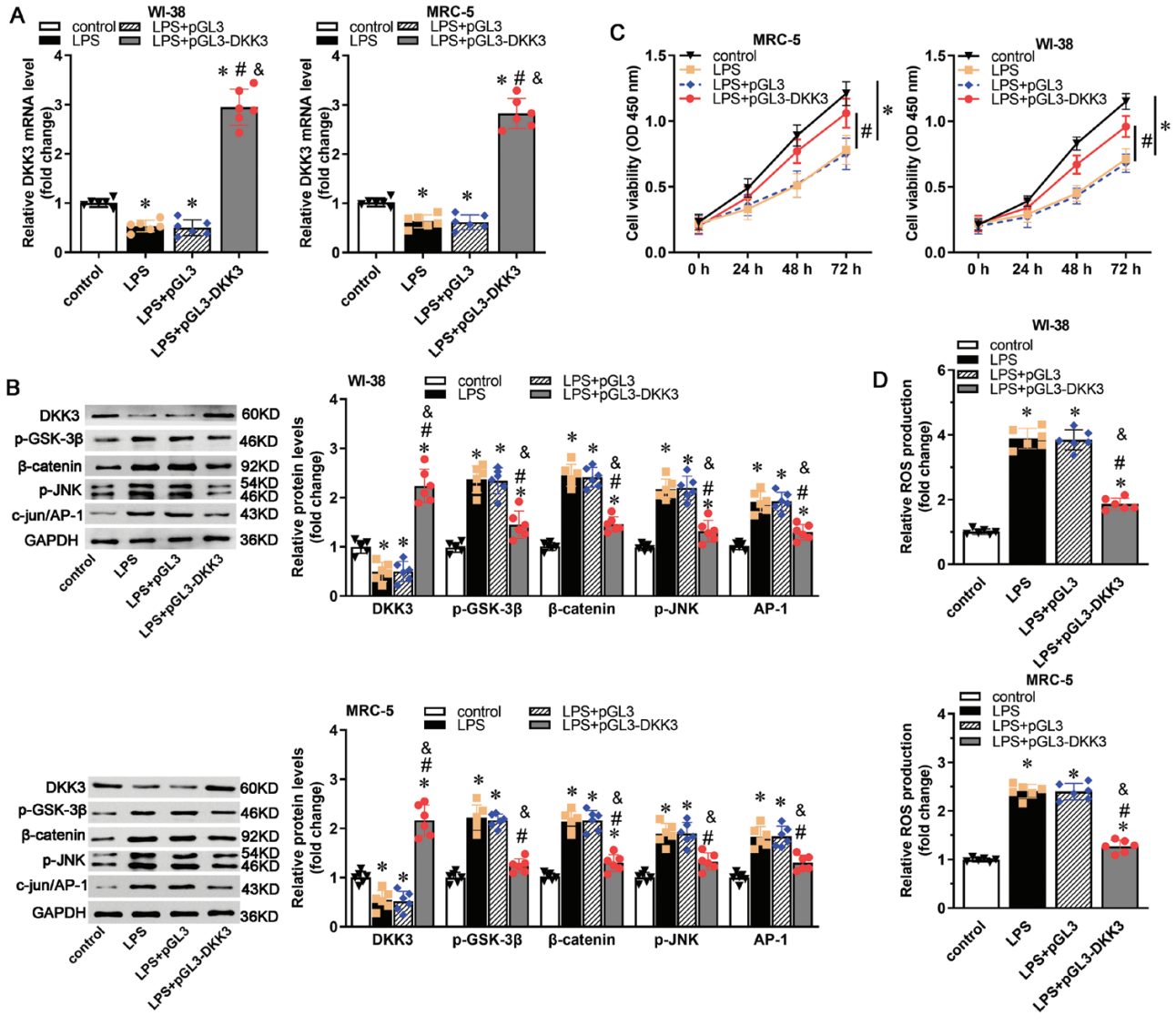

Figure 2. DKK3 overexpression increases cell viability and inactivates GSK-3β/β-catenin pathway in LPS-stimulated cells. WI-38 and MRC-5 cells transfected with pGL3–DKK3 or its control pGL3 empty vectors were exposed to 0 or 10 μg/ml of LPS. (A) RT–qPCR showed the DKK3 mRNA level in LPS-stimulated WI-38 and MRC-5 cells ($n = 6$). (B) Representative western blotting images showed showed the protein levels of DKK3, p-GSK-3β, β-catenin, p-JNK, and c-Jun/AP-1 in LPS-stimulated WI-38 and MRC-5 cells ($n = 6$). (C) CCK-8 method was used to evaluate the cell viability of WI-38 and MRC-5 cells exposed to LPS for 24 h, 48 h, or 72 h ($n = 6$). (D) Commercial kits were used to detect ROS production in cells exposed to 10 μg/ml of LPS for 12 h ($n = 6$). Data were expressed as mean ± SD, and analyzed using one-way ANOVA with the Bonferroni test. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the LPS group; $^{\&}P < 0.05$, compared with the LPS + pGL3 group.

Overexpression of DKK3 ameliorates LPS-evoked inflammatory injury in WI-38 and MRC-5 cells

Compared with the control group, the LPS group showed decreased DKK3 mRNA and protein expression, along with upregulation of p-GSK-3β, β-catenin, p-JNK and c-jun/AP-1 in WI-38 and MRC-5 cells (Fig. 2A and B , $P < 0.05$). Compared with the LPS group, the mRNA and protein expression of DKK3 were significantly increased in LPS + pGL3–DKK3 group, while the protein expression of p-GSK-3β, β-catenin, p-JNK, and c-jun/AP-1 was decreased (Fig. 2A and B, $P < 0.05$). Compared with the control group, cell viability of WI-38 and MRC-5 cells was significantly decreased in the LPS group (Fig. 2C, $P < 0.05$), accompanied by increased levels of ROS in LPS-stimulated cells (Fig. 2D, $P < 0.05$). Compared with the LPS group, cell viability was significantly increased in LPS + pGL3–DKK3 group, accompanied by decreased levels of ROS (Fig. 2D, $P < 0.05$). Compared with the control group, cell apoptosis was increased in the LPS group (Fig. 3A, $P < 0.05$), accompanied by increased expression of c-Caspase3 and Bax and decreased expression of Bcl-2 (Fig. 3B, $P < 0.05$). Compared with the LPS group, cell apoptosis was decreased in the LPS + pGL3–DKK3 group (Fig. 3A, $P < 0.05$), accompanied by decreased expression of c-Caspase3 and Bax and increased expression of Bcl-2 (Fig. 3B, $P < 0.05$). Moreover, the LPS group showed increased levels of pro-inflammatory factors IL-6, MCP-1, and TNF-α induced as compared to the control group (Fig. 3C, $P < 0.05$), while the LPS + pGL3–DKK3 group showed decreased levels of IL-6, MCP-1, and TNF-α when compared to LPS group (Fig. 3C, $P < 0.05$). These results demonstrated that LPS-induced inflammatory injury of lung cells can be reduced by DKK3 overexpression.

NRF1 contributes to the downregulation of DKK3 and activation of GSK-3β/β-catenin in LPS-challenged WI-38 cells

To explore the potential regulatory mechanism of DKK3 in NP, its candidate transcription factors were investigated by UCSC combined with the JASPAR database. As showed in

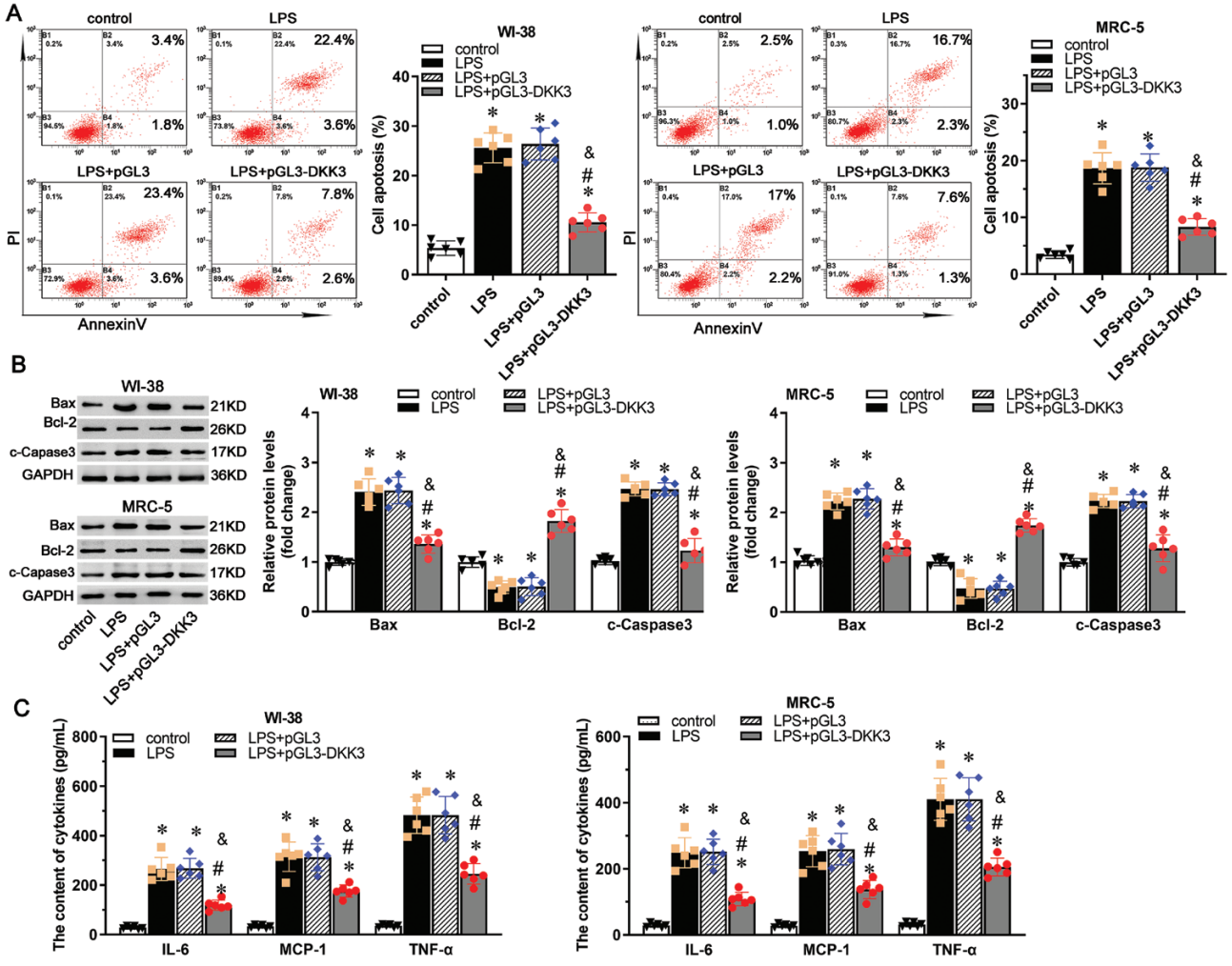

Figure 3. DKK3 overexpression ameliorates LPS-induced apoptosis and inflammatory response in WI-38 and MRC-5 cells. WI-38 and MRC-5 cells transfected with pGL3–DKK3 or its control pGL3 empty vectors were exposed to 0 or 10 μg/ml of LPS. (A) Representative flow cytometry images showed cell apoptosis in cells exposed to 10 μg/ml of LPS for 12 h ($n = 6$). (B) Representative western blotting images showed the protein level of Bax, Bcl-2, and c-Capase3 in cells exposed to 10 μg/ml of LPS for 24 h ($n = 6$). (C) ELISA assay displayed the levels of IL-6, MCP-1, and TNF-α in cells exposed to 10 μg/ml of LPS for 12 h ($n = 6$). Data were expressed as mean ± SD, and analyzed using one-way ANOVA with the Bonferroni test. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the LPS group; $^{\&}P < 0.05$, compared with the LPS + pGL3 group.

Fig. 4A, the region of the DKK3 promoter has a putative binding site with NRF1. A ChIP assay revealed a significant enrichment of the DKK3 promoter with anti-NRF1 as compared to anti-IgG (Fig. 4B, $P < 0.05$), indicating that NRF1 can bind to the DKK3 promoter. Compared with the sh-NC group, the sh-NRF1 group showed increased luciferase activity of the DKK3 promoter (Fig. 4C, $P < 0.05$). The LPS group showed increased mRNA and protein expression of NRF-1 as compared to the control group (Fig. 4D and E, $P < 0.05$), while LPS + sh-NRF1 group showed decreased mRNA and protein expression of NRF-1 when compared to the LPS group (Fig. 4D and E, $P < 0.05$). Compared with the

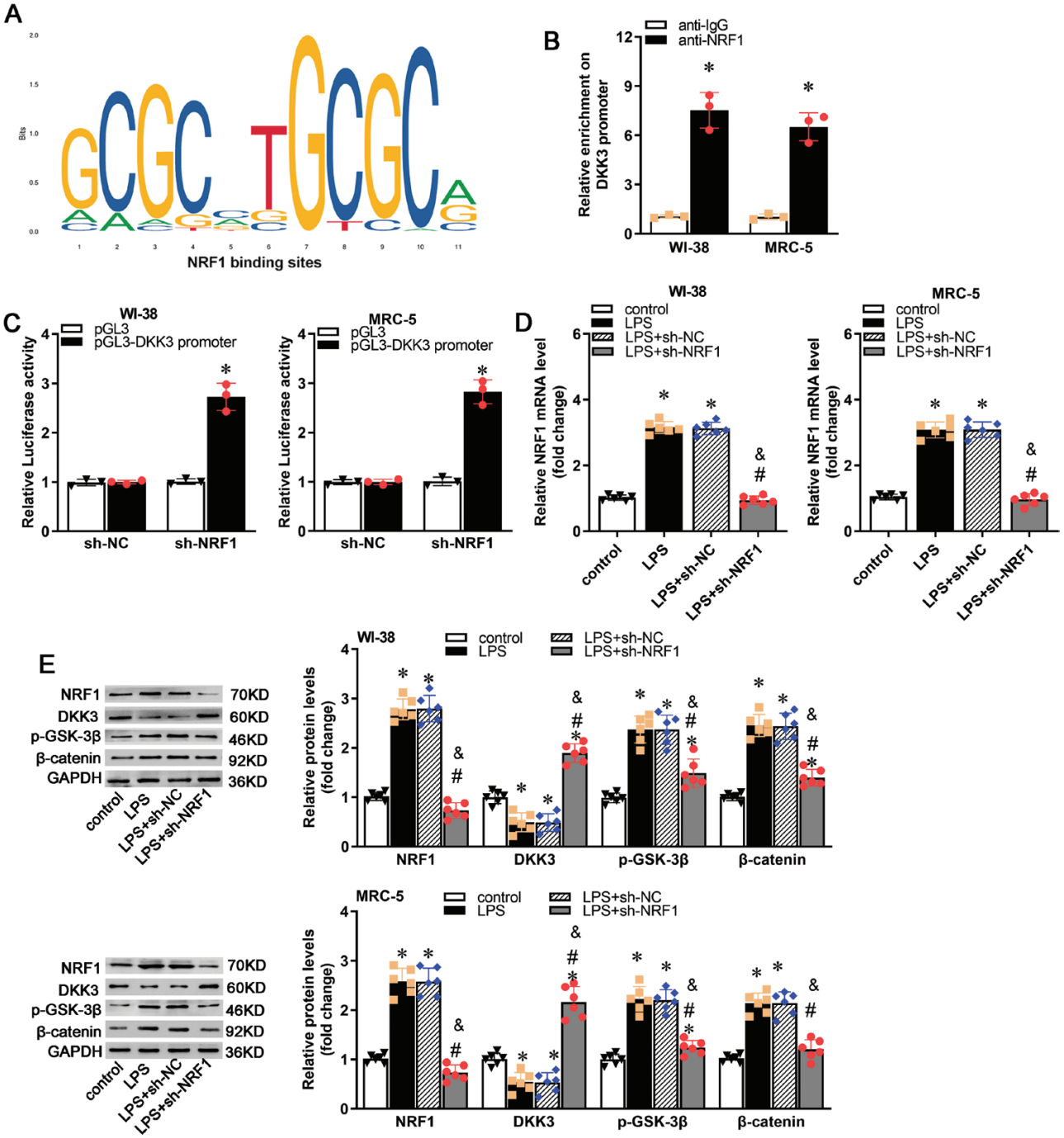

Figure 4. NRF1 downregulates DKK3 and activates the GSK-3β/β-catenin pathway in LPS-stimulated WI-38 and MRC-5 cells. (A) The putative binding sites of NRF1 on the promoter region of DKK3. (B) Enrichment of DKK3 promoter NRF1 with anti-NRF1 or anti-IgG was determined in WI-38 and MRC-5 cells by ChIP and RT–qPCR ($n = 3$). $^{*}P < 0.05$, compared with the anti-IgG group. (C) Luciferase reporter assay showed the luciferase activity in WI-38 and MRC-5 cells co-transfected with the pGL3–DKK3 promoter or pGL3 and sh-NRF1 or sh-NC ($n = 3$). $^{*}P < 0.05$, compared with the sh-NC group. (D) RT-qPCR showed the NRF1 mRNA level in sh-NC or sh-NRF1-transfected WI-38 cells treated with 0 or 10 μg/ml of LPS for 24 h ($n = 6$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the LPS group; $^{\&}P < 0.05$, compared with the LPS + sh-NC group. (E) Representative western blotting images showed the protein level of NRF1, DKK3, p-GSK-3β, and β-catenin in cells exposed to 10 μg/ml of LPS for 24 h ($n = 6$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the LPS group; $^{\&}P < 0.05$, compared with the LPS + sh-NC group showed.

LPS group, the protein expression of DKK3 was decreased while p-GSK-3β, β-catenin was increased in LPS + sh-NRF1 group (Fig. 4E, $P < 0.05$). These results demonstrated that the abnormally high expression of NRF1 induced by LPS leads to the downregulation of DKK3 and activation of the GSK-3β/β-catenin signaling pathway in WI-38 and MRC-5 cells.

NRF1 knockdown alleviates LPS-triggered inflammatory injury by upregulating DKK3 and inhibiting the GSK-3β/β-catenin pathway

Compared to the LPS group, cell viability of WI-38 and MRC-5 cells was significantly increased in the LPS + sh-NRF1 group (Fig. 5A, $P < 0.05$), accompanied by decreased levels of ROS and reduced levels of pro-inflammatory factors IL-6, MCP-1, and TNF-α (Fig. 5B and C, $P < 0.05$). However, compared with LPS + sh-NRF1 group, cell viability was significantly decreased in LPS + sh-NRF1 + sh-DKK3 group and LPS + sh-NRF1 + LiCl group (Fig. 5A, $P < 0.05$), accompanied by increased levels of ROS and increased levels of pro-inflammatory factors IL-6, MCP-1, and TNF-α (Fig. 5B and C, $P < 0.05$). Compared with the LPS group, cell apoptosis was decreased in the LPS + sh-NRF1 group (Fig. 6A, $P < 0.05$), accompanied by decreased expression of c-Caspase3 and Bax and increased expression of Bcl-2 (Fig. 6B, $P < 0.05$). However, compared with LPS + sh-NRF1 group, cell apoptosis was increased in the LPS + sh-NRF1 + sh-DKK3 group and LPS + sh-NRF1 + LiCl group (Fig. 6A, $P < 0.05$), accompanied by increased expression of c-Caspase3 and Bax and decreased expression of Bcl-2 (Fig. 6B, $P < 0.05$). These results indicated that NRF1 knockdown can attenuate LPS-triggered inflammatory damage via upregulating DKK3 and its mediated inhibition of the GSK-3β/β-catenin pathway.

Discussion

Since the excessive inflammatory response is the main pathological factor of NP, which is among the leading causes of mortality in newborns all around the world [17, 18], it is of great significance to find therapies aimed at preventing the apoptosis and inflammation of lung cells. DKK3 reportedly exhibits anti-inflammatory properties in several inflammatory diseases [14–16]. However, the roles and mechanisms of DKK3 in the progression of NP remain unclear. As a major pathogenic constituent of Gram-negative bacteria, LPS was reported to trigger a severe inflammatory response in the lungs [19, 20]. Thus, human embryonic lung WI-38 and MRC-5 cells treated with LPS were used as the cell model of NP in this study. In the present study, DKK3 was found to be reduced in LPS-stimulated WI-38 and MRC-5 cells. DKK3 overexpression showed the anti-pneumonia effects by ameliorating LPS-evoked growth inhibition and apoptosis of WI-38 and MRC-5 cells and reducing LPS-triggered inflammatory injury. NRF1 was found to inhibit the transcriptional activity of DKK3 and activate the GSK-3β/β-catenin pathway in LPS-injured WI-38 and MRC-5 cells. NRF1 knockdown alleviated inflammatory injury in both LPS-stimulated WI-38 and MRC-5 cells. The anti-pneumonia

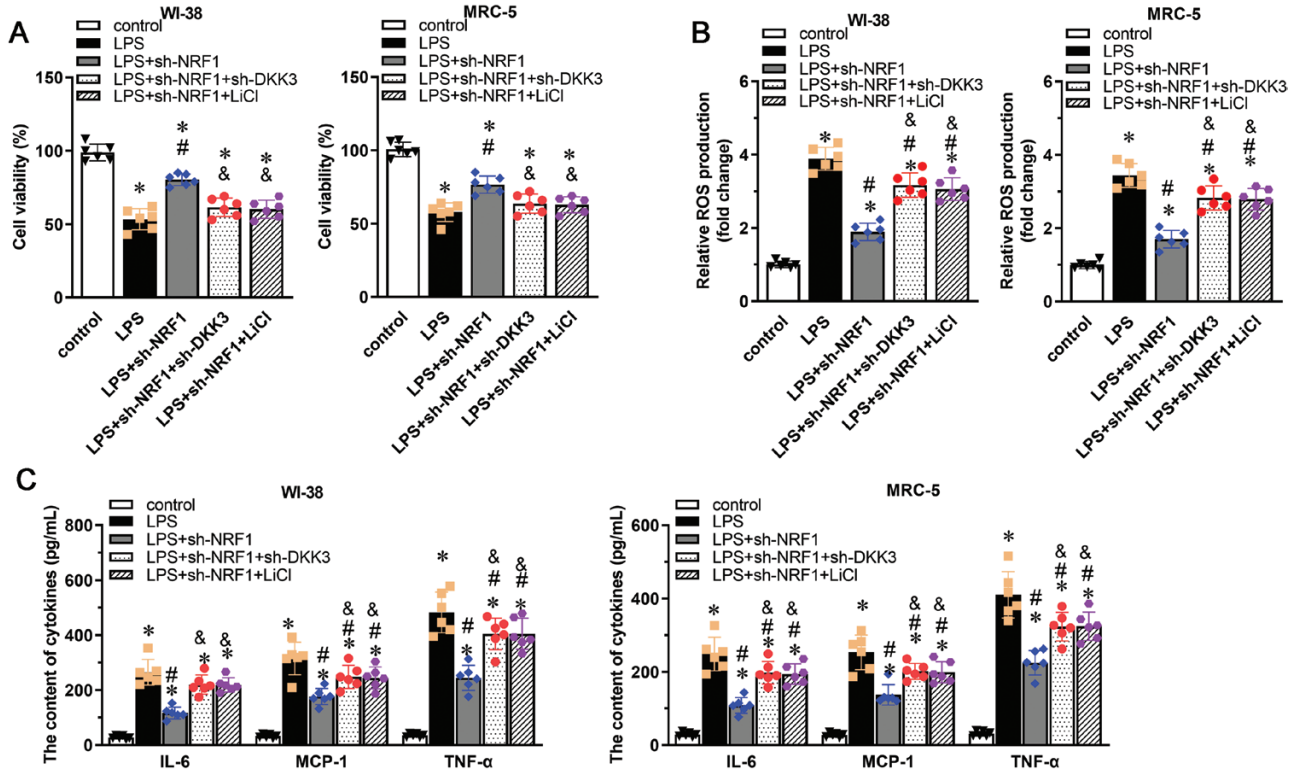

Figure 5. NRF1 knockdown alleviates LPS-triggered ROS production and inflammatory response via regulating DKK3 and the GSK-3β/β-catenin pathway. WI-38 and MRC-5 cells were transfected with sh-NRF1, or co-transfected with sh-NRF1 and sh-DKK3, and treated with or without 10 μg/ml of LPS and 5 mM of LiCl. (A) CCK-8 method evaluated the cell viability ($n = 6$). (B) Commercial kits revealed the ROS production treated 10 μg/ml of LPS for 12 h ($n = 6$). (C) ELISA assay displayed the levels of IL-6, MCP-1, and TNF-α treated at 10 μg/ml of LPS for 12 h ($n = 6$). Data were expressed as mean ± SD, and analyzed using one-way ANOVA with the Bonferroni test. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the LPS group; $^{\&}P < 0.05$, compared with the LPS + sh-NRF1 group.

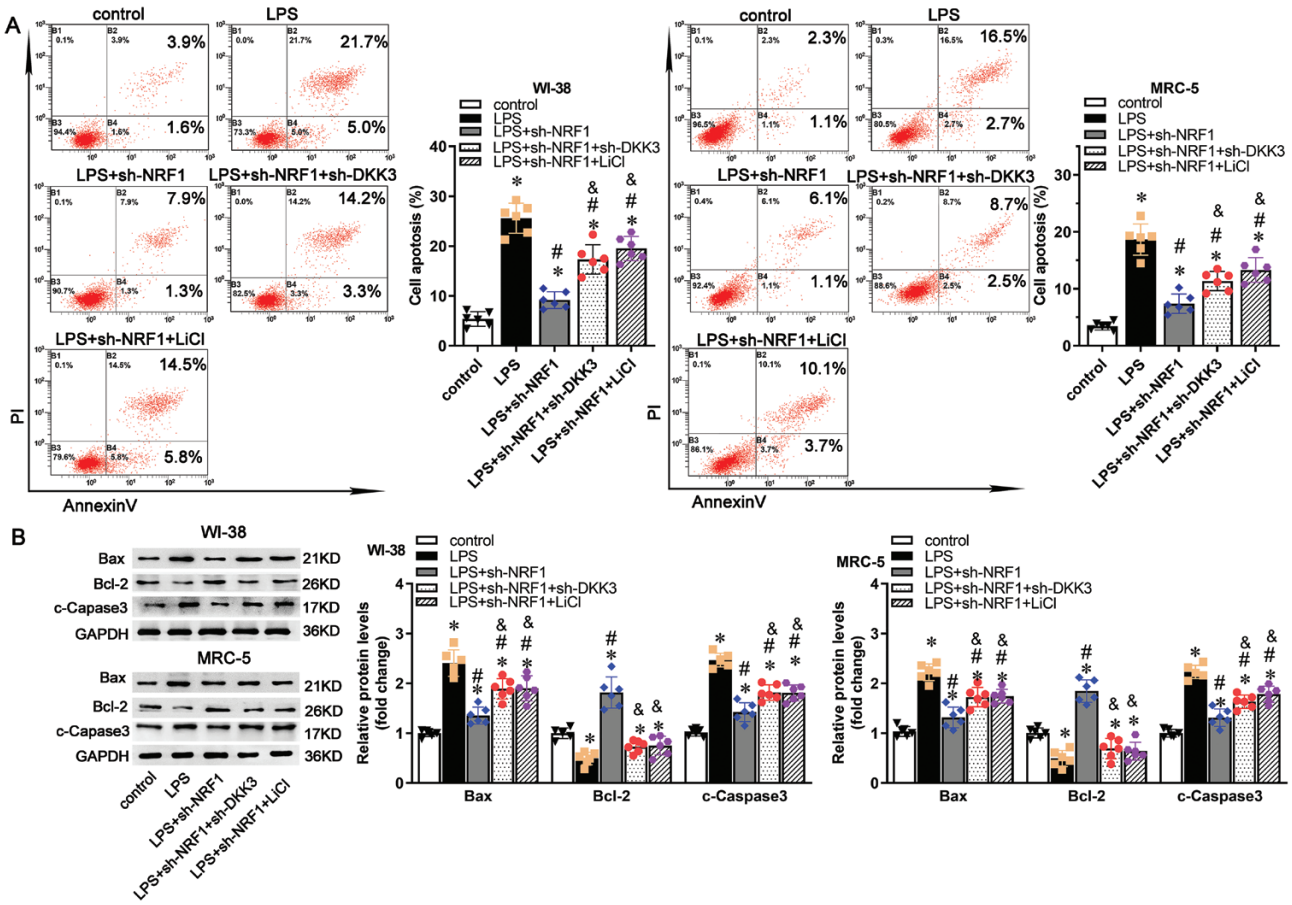

Figure 6. NRF1 knockdown alleviates LPS-induced apoptosis via regulating DKK3 and the GSK-3β/β-catenin pathway. WI-38 and MRC-5 cells were transfected with sh-NRF1, or co-transfected with sh-NRF1 and sh-DKK3, and treated with or without 10 μg/ml of LPS and 5 mM of LiCl. (A) Representative flow cytometry images showed cell apoptosis in cells exposed to 10 μg/ml of LPS for 12 h ($n = 6$). (B) Representative western blotting images showed the protein level of Bax, Bcl-2, and c-Capase3 in cells exposed to 10 μg/ml of LPS for 24 h ($n = 6$). Data were expressed as mean ± SD, and analyzed using one-way ANOVA with the Bonferroni test. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the LPS group; $^{\&}P < 0.05$, compared with the LPS + sh-NRF1 group.

effect of NRF1 knockdown may be related to its induced DKK3 upregulation and inactivation of the Wnt/β-catenin signaling pathway.

A previous study revealed that the expression of DKK3, a regulator of Wnt/GSK-3β/β-catenin signaling [21, 22], was downregulated in LPS-stimulated lung cancer A549 cells [13]. This study found that the expression of DKK3 was also downregulated in LPS-treated WI-38 cells. This is similar to previously reported findings that TLR4 knockdown upregulates DKK3 expression in U-87 xenograft glioblastomas [11]. TNF-α has been reported to reduce Dkk3 expression in keratinocytes [12], which may be one of the reasons for the LPS–TLR4-mediated downregulation of DKK3. However, the role of TNF-α between LPS–TLR4 signaling and DKK3 expression has not been explored in this study, which is one of the limitations of this study and needs to be further explored in future studies.

Previous studies have showed that DKK3 can inhibit the neuroinflammation of mice with intracerebral hemorrhage via regulating JNK and AP-1 [16], and increased cardiomyocyte apoptosis and inflammatory infiltration were found in the hearts of DKK3 knockout mice [15]. Similarly, the current study found that DKK3 overexpression, accompanied by inactivation of GSK-3β/β-catenin signaling and downregulation of p-JNK and AP-1, reduced overproduction of ROS and pro-inflammatory factors IL-6, MCP-1, and TNF-α induced by LPS. LPS-induced growth inhibition and apoptosis of WI-38 and MRC-5 cells were also inhibited by DKK3 overexpression. These findings indicated that the abnormal dysregulation of DKK3 may be implicated in the progression of NP, and LPS-induced inflammatory injury of lung cells can be reduced by DKK3 overexpression.

Previous studies have reported that many transcription factors, such as GATA6 and PAX6 [23, 24], can regulate the expression of DKK3. According to the results of the bioinformatics predictions and ChIP assay in this study, transcription factor NRF1 was found to have the putative binding site in the promoter region of DKK3. Moreover, NRF1 silencing elevated DKK3 expression and increased the luciferase activity of the DKK3 promoter, suggesting that NRF1 can upregulate DKK3 at the transcriptional level. Increased NRF1 expression has been found in LPS-activated macrophages [25] and in the LPS-induced cell model and a rat model of chronic obstructive pulmonary disease [26], suggesting its involvement in inflammatory diseases. It was reported that after LPS stimulation, the expression of NRF1 was increased in RAW264.7 macrophages and in the lung tissues of Sprague–Dawley rats with

acute lung injury [27]. Similarly, the expression of NRF1 was increased in LPS-stimulated WI-38 and MRC-5 cells in this study. According to preliminary studies, NRF1 was reported to participate in the inflammatory process of many diseases, such as atherosclerosis [28], adipocyte inflammation, and chronic obstructive pulmonary disease [26]. Overexpression of NRF1 reportedly promoted inflammation reactions in high-glucose-induced human mesangial cells by increasing the levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 [29]. The present study found that NRF1 knockdown can counteract LPS-evoked growth inhibition and apoptosis elevation of WI-38 and MRC-5 cells, along with the reduction of ROS, IL-6, MCP-1, and TNF-α. In addition, inhibition of DKK3 can neutralize the pro-proliferative, anti-apoptotic, and anti-inflammatory effects of NRF1 knockdown. These data demonstrated that the knockdown of NRF1 can attenuate LPS-triggered pulmonary inflammatory damage via regulating DKK3. In addition, NRF2/GABPA, another nuclear respiratory factor, has also been reported to be associated with lung injury and inflammatory response [30]. However, the present study mainly focused on the effect of the NRF1/DKK3 axis on LPS-induced inflammatory injury, thus the role of Nrf2 in DKK3 and NP needs to be further explored in future studies. The Wnt/GSK-3β/β-catenin pathway is known to play essential roles in cell survival and apoptosis, immune homeostasis, and inflammatory response in cells treated with pathogenic factors [7, 31, 32]. Since DKK3 is a regulator of the Wnt/GSK-3β/β-catenin pathway and NRF1 can affect DKK3 expression, NRF1 was found to activate the GSK-3β/β-catenin pathway in LPS-stimulated WI-38 and MRC-5 cells in this study. Previous studies have reported that the knockdown of NRF1 leads to the activation of β-catenin in liver HepG2 cells [33], and circNSUN2 regulated by NRF1 can activate the Wnt/β-catenin pathway in lymphocytes [34]. In the current study, the expression of p-GSK-3β and nuclear β-catenin in LPS-stimulated WI-38 and MRC-5 cells were reduced by NRF1 knockdown, suggesting that inhibition of NRF1 leads to the inactivation of the GSK-3β/β-catenin pathway in LPS-stimulated WI-38 and MRC-5 cells. Moreover, re-activation of the GSK-3β/β-catenin pathway by LiCl can reverse the anti-pneumonia effects of NRF1 knockdown, indicating that inhibition of GSK-3β/β-catenin is one of the anti-pneumonia mechanisms of NRF1. However, these findings need to be further validated in animal models of NP, which is one of the limitations of this study.

Conclusion

In summary, NRF1 knockdown can alleviate LPS-triggered pulmonary inflammatory injury by regulating DKK3 and the GSK-3β/β-catenin pathway. NRF1 and DKK3 may be novel therapeutic targets for NP.

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Ethical Approval

Not applicable.

Conflict of Interests

The authors declare no conflicts of interest.

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Data Availability

The data supporting the findings of this study are available from the corresponding author upon request.

Author Contributions

L.K. and X.H.W. designed research; L.K., X.H.W., J.F.W., J.G., W.Z., and R.R.L. performed research, L.K., X.H.W., J.F.W. and J.G. analyzed data and interpreted experimental results. L.K., X.H.W, and F.W. wrote the original draft. All authors read, edited and approved the final manuscript.

Permission to Reproduce

Not applicable.

Clinical Trial Registration

Not applicable.

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