

Regulatory mechanism of NOV/CCN3 in the inflammation and apoptosis of lung epithelial alveolar cells upon lipopolysaccharide stimulation

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Abstract. Lipopolysaccharide (LPS) induces inflammatory stress and apoptosis. Pulmonary epithelial cell apoptosis has been shown to accelerate the progression of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), and is the leading cause of mortality in patients with ALI/ARDS. Nephroblastoma overexpressed (NOV; also known as CCN3), an inflammatory modulator, is reported to be a biomarker in ALI. Using an LPS-induced ALI model, we investigated the expression of CCN3 and its possible molecular mechanism involved in lung alveolar epithelial cell inflammation and apoptosis. Our data revealed that LPS treatment greatly increased the level of CCN3 in human lung alveolar type II epithelial cells (A549 cell line). The A549 cells were also transfected with a specific CCN3 small interfering RNA (siRNA). CCN3 knockdown not only largely attenuated the expression of inflammatory cytokines, interleukin (IL)-1 β and transforming growth factor (TGF)- β 1, but also reduced the apoptotic rate of the A549 cells and altered the expression of apoptosis-associated proteins (Bcl-2 and caspase-3).

Furthermore, CCN3 knockdown greatly inhibited the activation of nuclear factor (NF)- κ B p65 in the A549 cells, and TGF- β /p-Smad and NF- κ B inhibitors significantly decreased the expression level of CCN3 in A549 cells. In conclusion, our data indicate that CCN3 knockdown affects the expression of downstream genes through the TGF- β /p-Smad or NF- κ B pathways, leading to the inhibition of cell inflammation and apoptosis in human alveolar epithelial cells.

Introduction

Acute lung injury (ALI) or its severe form, acute respiratory distress syndrome (ARDS), is characterized by an excessive and uncontrolled inflammatory response, which results in increased permeability of the alveolar-capillary barrier, alveolar flooding and acute respiratory failure (1,2). Type II alveolar epithelial cells (AEC II), the progenitor cells in the corners of alveoli, are thought to play a central role in the pathogenesis of ALI by synthesizing, secreting and reutilizing surfactant (3). In addition, the apoptosis of AEC II directly accelerates the progression of ALI, which is the leading cause of mortality in patients with ARDS (4-6).

The nephroblastoma overexpressed protein (NOV/CCN3), is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins with a variety of functions (7,8). CCN3 has been previously shown to be involved in regulating a variety of chronic inflammatory diseases, such as atherosclerosis, rheumatoid arthritis and liver disease (7,9,10). In addition, studies have also shown that CCN proteins are key signaling and regulatory molecules involved in the pathophysiology of various lung diseases, including lung cancer, chronic obstructive pulmonary disease and ventilator-induced lung injury (11-15). By integrating the proteomic profiles of inflammatory mediators with clinical informatics, our previous study (16) indicated that the plasma levels of

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CCN3 and other inflammatory mediators were significantly increased in patients with severe pneumonia-induced ARDS compared to the healthy controls. Thus, we hypothesized that CCN3 could play a role in the pathophysiology of ALI/ARDS.

Proteins involved in transcriptional regulation, such as nuclear factor (NF)- κ B and transforming growth factor (TGF)- β , have been implicated in the development and progression of ALI and ARDS (17-21). Human A549 alveolar epithelial cells have been widely used as an epithelial cell injury model to examine lipopolysaccharide (LPS)-induced acute lung inflammatory response (22-28). The objective of this study was to reveal the potential role and underlying mechanism of CCN3 in lung dysfunction from the perspective of inflammation and apoptosis using siRNA-mediated transfection approach.

Materials and methods

Reagents and chemicals. Human lung alveolar type II epithelial A549 cells were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). LPS (Sigma-Aldrich; Merck KGaA), anti-TGF- β 1 antibody (cat. no. ab64715, Abcam), ALK5 inhibitor (TP0427736; cat. no. S8700, Selleck Chemicals), pyrrolidine dithiocarbamate (PDTC; cat. no. S363302, Selleck Chemicals) and immunohistochemistry reagents and kits (Beyotime, China) were used in this study. Other reagents and chemicals were obtained from Western Biotechnology (China).

Lung alveolar epithelial cell culture. Human A549 cells were cultured in F12K medium (Gibco; Thermo Fisher Scientific, Inc.), containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, in a 37°C/5% CO₂ atmosphere. The medium was routinely changed every 3 days to remove non-adherent cells.

siRNA transfection. Cells were transfected with 100 nM negative control (NC)-siRNA or CCN3-siRNA (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions (29).

Enzyme-linked immunosorbent assay (ELISA). The levels of tumor necrosis factor (TNF)- α , IL-1 β and TGF- β 1 in the supernatant of cultured cells were analyzed using sandwich ELISA kits (cat. nos. F02810, F01220 and F02750; Bio-Tek Inc., USA), respectively, according to the manufacturer's instructions. The optical density of each well was assayed at 450 nm on a microplate reader.

Flow cytometric analysis. BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I (cat. no. 556547; BD Biosciences) was used to quantify apoptotic cells according to the manufacturer's instructions. After the treatments, cells were harvested, trypsinized, and rinsed twice with PBS. The suspended cells were incubated with 5 μ l Annexin V-FITC solution and 5 μ l propidium iodide (PI) solution at 4°C for 15 min. The cells were then suspended in 400 μ l binding buffer and subjected to flow cytometry (BD Biosciences), and the rates of apoptotic and necrotic cells were determined using CytExpert 2.3.0.84 (Beckman Coulter, Inc.).

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Genes	Primer sequences (5'-3')
CCN3 (153 bp)	Forward GGAGGATTCCTGGGAGGC
	Reverse ATTGACGGTTCCTATTGGTGAC
Bcl-2 (175 bp)	Forward AGGGACGGGGTGAAGTGG
	Reverse CTACCCAGCCTCCGTTATCC
TGF- β 1 (185 bp)	Forward CGTGGAGGGGAAATTGAGG
	Reverse GCCATGAGAAGCAGGAAAGG
GAPDH (146 bp)	Forward ATCCATCACCATCTTCCAGG
	Reverse GATGACCCTTTTGGCTCCC

CCN3, nephroblastoma overexpressed (also known as NOV-TGF- β 1, transforming growth factor- β 1).

Real-time quantitative PCR (qPCR). Total RNA was extracted from A549 cells using Trizol reagent (Beyotime, China), and then used for first-strand cDNA synthesis. qPCR was performed using an Applied Biosystems™ SYBR Green I Real-Time PCR Master Mix system (Funglyn Biotech, Canada). The amplification program was set to 94°C for 4 min, 35 cycles of 94°C for 20 sec and 60°C for 30 sec, and 72°C for 30 sec as a final elongation step. The gene expression level was normalized to GAPDH as the internal gene using the 2^{- $\Delta\Delta$ Cq} method (30). The primer sequences used in this study are shown in Table I.

Western blot analysis. A549 cells were seeded at a density of 1x10⁶ cells with 0.1 ml RIPA buffer. After passaging and centrifugation (12,000 x g for 15 min), the supernatants were collected and protein concentrations were assessed using the Bradford assay. An equal amount of protein (20 μ g) was loaded onto 10% SDS-PAGE gels. Proteins were resolved through SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Nonspecific sites were blocked with 5% nonfat milk at room temperature for 2 h. The blots were incubated with antibodies against Bcl-2 (dilution 1:500, cat. no. ab182858; Abcam), caspase-3 (dilution 1:500, cat. no. ab184787), TGF- β receptor (R)II (dilution 1:500, cat. no. ab113670, Abcam), p-Smad2/3 [dilution 1:500, cat. no. 8828S, Cell Signaling Technology, Inc. (CST)], CCN3 (dilution 1:500; cat. no. ab191425; Abcam) and β -actin (dilution 1:1,000; cat. no. ab8226; Abcam) at room temperature for 1.5 h or 4°C overnight. After washing with TBST three times, the blots were incubated with the secondary goat anti-rat IgG antibody (dilution 1:1,000; cat. no. AP156P; Sigma-Aldrich; Merck KGaA) at room temperature for 1.5 h, and visualized using an enhanced chemiluminescence (ECL) kit (cat. no. 34580; Thermo Fisher Scientific, Inc.) in the ImageQuant Tanon-4200 system (Tanon Science and Technology Co., Ltd., Shanghai, China). The rat anti- β -actin Ab was used as an internal control for western blotting. The densities of the detected bands were quantified in triplicate using Labworks™ Analysis Software (UVP, LLC, USA).

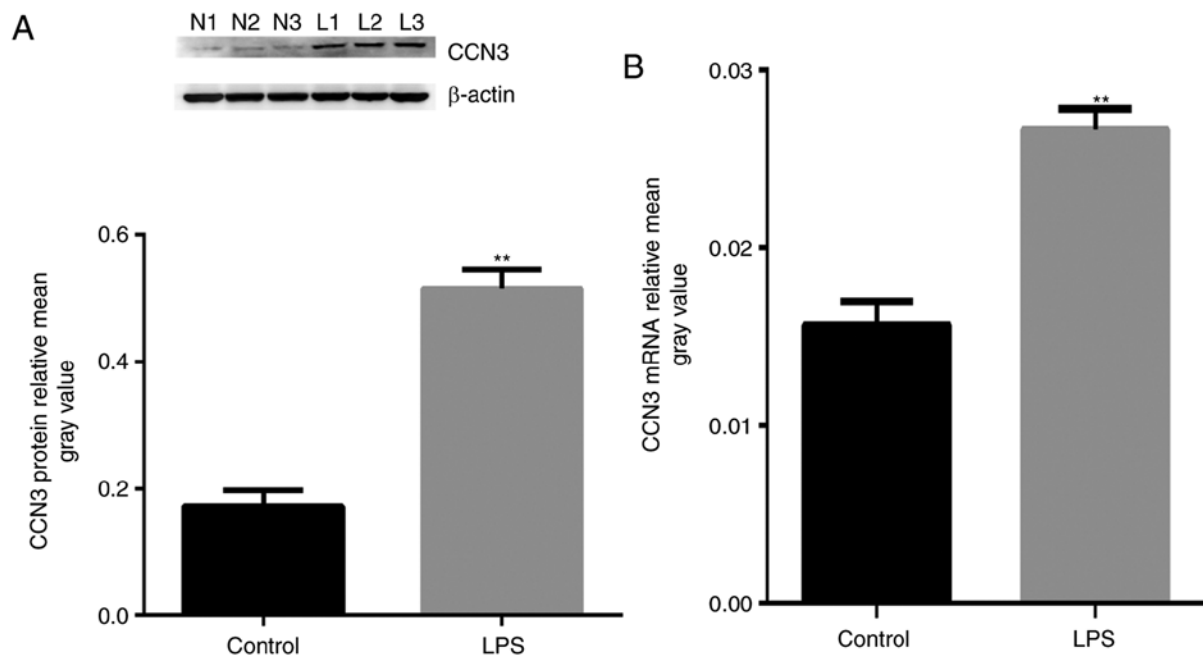


Figure 1. Effects of LPS treatment on CCN3 expression in A549 cells. (A) CCN3 protein expression in A549 cells in response to 0.1 $\mu\text{g/ml}$ LPS treatment for 12 h, as assessed by western blot analysis (N1-N3, normal; L1-L3, LPS-treated). (B) CCN3 mRNA levels in A549 cells in response to 0.1 $\mu\text{g/ml}$ LPS treatment for 12 h, as assessed with qPCR. ** $P < 0.01$ vs. the control group. LPS, lipopolysaccharide; CCN3, nephroblastoma overexpressed (also known as NOV).

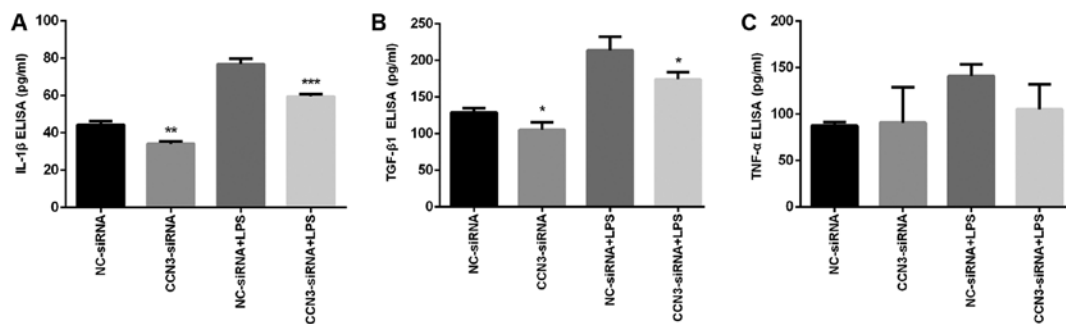


Figure 2. Effects of CCN3 siRNA treatment on the expression of inflammatory cytokines. A549 cells were treated with NC-siRNA or CCN3-siRNA for 36 h, and then incubated with or without 0.1 $\mu\text{g/ml}$ LPS for an additional 12 h. The expression levels of (A) IL-1 β , (B) TGF- β 1 and (C) TNF- α were assessed *in vitro* with ELISA. Data are presented as means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. respective siRNA-NC. NC-siRNA, siRNA-negative control group; CCN3-siRNA, CCN3 siRNA-transfected group; LPS, lipopolysaccharide; CCN3, nephroblastoma overexpressed (also known as NOV); IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

Immunofluorescence staining. Cells were grown on 12-mm glass coverslips. After transfection with CCN3-siRNA or NC-siRNA, the cells were fixed in 4% paraformaldehyde at room temperature for 15 min, permeabilized in PBS with 0.5% Triton X-100 for 15 min, and blocked with 6% goat serum for 30 min. Next, the cells were incubated with the primary NF- κ B p65 antibody (dilution 1:100, cat. no. ab32536, Abcam) solution at 4°C overnight. After washing, the cells were incubated with the secondary antibody, goat anti-rabbit IgG (Cy3; dilution 1:800, cat. no. ab6939, Abcam) at room temperature for 30 min, and DAPI was added for nuclear staining for 5 min. Finally, the cells were visualized using a confocal laser scanning microscope (Leica, Germany). The data are presented as relative fluorescence intensity.

Statistical analysis. Statistical analysis was performed with Statistical Product and Service Solutions (SPSS) v24.0 (SPSS,

Inc.) and Prism 6 (GraphPad Software). Data are presented as means \pm SEM. Statistical significance was assessed with Student's *t*-test or one-way ANOVA with Bonferroni significant analysis. Results were considered statistically significant when $P < 0.05$.

Results

Effect of LPS on the expression level of CCN3 in human lung alveolar epithelial cells. To assess the effects of LPS on CCN3 expression, we analyzed mRNA expression by qPCR and protein expression by western blot analysis. The results revealed that the levels of CCN3 mRNA and CCN3 protein were significantly upregulated following treatment with 0.1 $\mu\text{g/ml}$ LPS for 12 h (Fig. 1A and B) ($P < 0.01$). Our findings suggest that CCN3 is associated with LPS-induced lung injury.

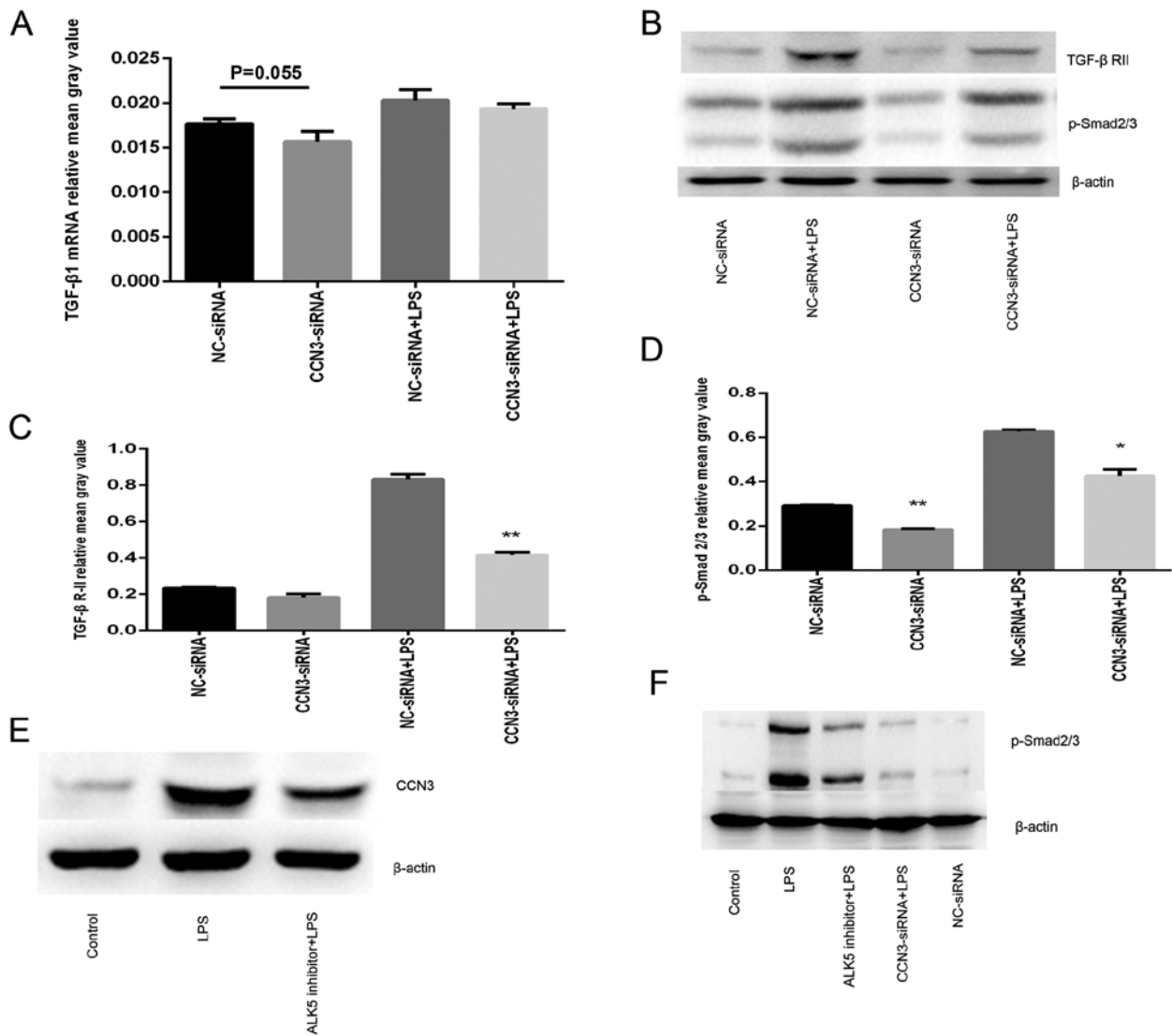


Figure 3. The anti-inflammatory activity of CCN3 gene silencing involves TGF- β /p-Smad signaling. (A) TGF- β 1 mRNA levels were determined by qPCR. (B-D) TGF- β RII and p-Smad2/3 protein levels were assessed by western blot analysis. * $P < 0.05$, ** $P < 0.01$ vs. respective NC-siRNA. (E and F) After 36 h of CCN3-siRNA transfection or not, A549 cells were pretreated with ALK5 inhibitor (10 μ M) for 30 min, followed by stimulation with LPS (0.1 μ g/ml) or 0.1% DMSO for 12 h. The protein levels of (E) CCN3 and (F) p-Smad2/3 were assessed with western blot analysis. NC-siRNA, siRNA-negative control group; CCN3-siRNA, CCN3 siRNA-transfected group; LPS, lipopolysaccharide; CCN3, nephroblastoma overexpressed (also known as NOV); TGF, transforming growth factor.

Reduction of inflammatory cytokines by CCN3-siRNA. To assess the function of CCN3 in the regulation of inflammatory cytokines, we inhibited CCN3 expression by siRNA and measured the levels of TNF- α , IL-1 β and TGF- β 1 by ELISA. As shown in Fig. 2A and B, it was demonstrated that inhibition of CCN3 by siRNA significantly downregulated the levels of inflammatory cytokines, such as IL-1 β ($P < 0.01$) and TGF- β 1 ($P < 0.05$), compared with the vehicle control (NC). Similar effects were observed after LPS treatment. Surprisingly, there were no significant changes in the levels of TNF- α after CCN-siRNA treatment compared with both the NC group and following LPS treatment (Fig. 2C).

Anti-inflammatory activity of CCN3-siRNA through modulation of TGF- β /p-Smad signaling. Activation of TGF- β through interaction with receptor-regulated Smad (Smad2/3) signaling plays a pro-inflammatory role in the resolution of lung alveolar epithelial cell injury (18,31). We next sought to explore whether

the CCN3-induced pro-inflammatory effects are mediated through the TGF- β /p-Smad signaling pathway. To investigate this, A549 cells were transfected with CCN3-siRNA for 36 h and then stimulated with or without LPS for 12 h. The mRNA level of TGF- β 1, and the protein levels of TGF- β RII and p-Smad2/3 were detected by qPCR and western blot analysis, respectively. We found that knockdown of CCN3 by siRNA largely downregulated the protein levels of TGF- β RII and p-Smad2/3 (Fig. 3B-D). However, the mRNA level of TGF- β 1 was slightly decreased, without statistical significance ($P = 0.055$) (Fig. 3A). In addition, we confirmed that pretreatment of the cells with TP0427736 (an ALK5 inhibitor), which inhibits the TGF- β /p-Smad signaling pathway, greatly prevented the overexpression of CCN3 induced by LPS treatment, while knockdown of CCN3 by siRNA effectively attenuated the LPS-induced p-Smad2/3 expression (Fig. 3E and F). To conclude, the anti-inflammatory activity of CCN3-siRNA in LPS-induced lung injury is modulated by TGF- β /p-Smad signaling.

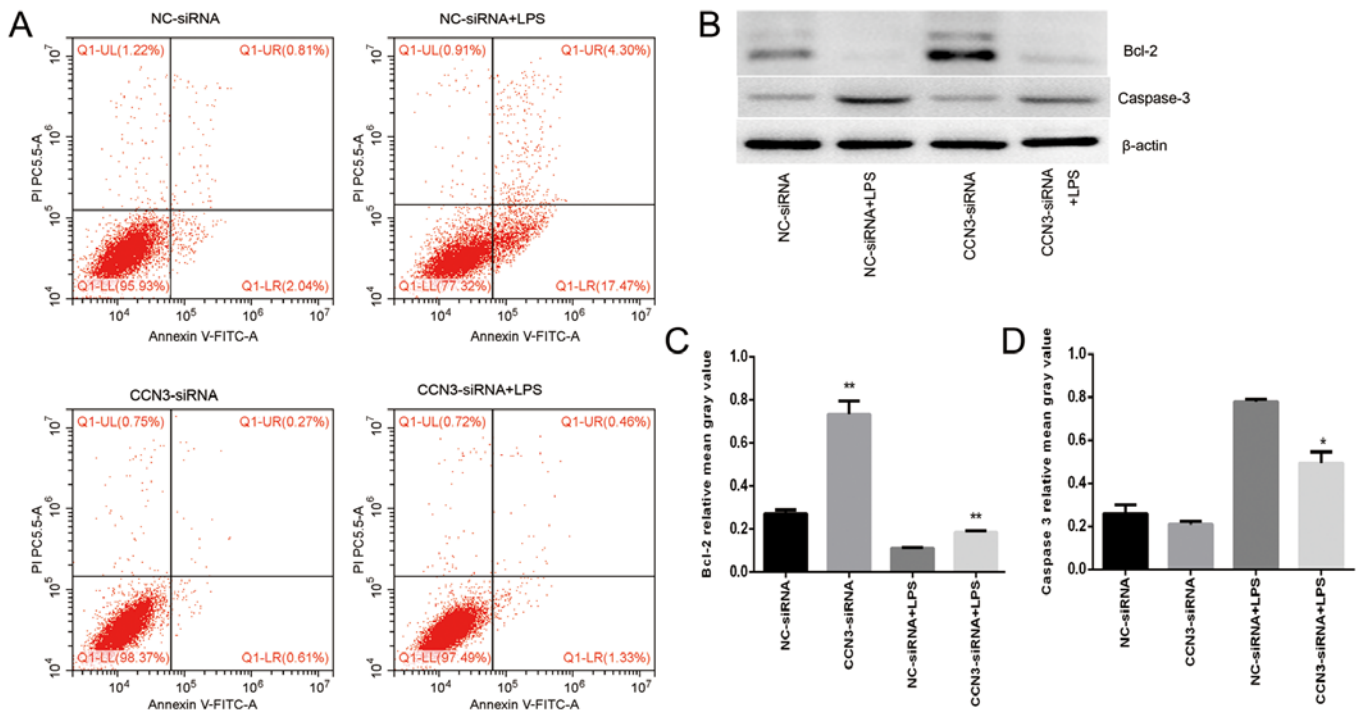


Figure 4. CCN3-siRNA mediates anti-apoptotic effects through Bcl-2/caspase-3 activation. (A) Flow cytometry was used to detect the proportion of cell apoptosis in three repeated experiments. (B-D) CCN3-siRNA-mediated anti-apoptotic effects in A549 cells through Bcl-2/caspase-3 activation. Western blot analysis was repeated at least three times. * $P < 0.05$, ** $P < 0.01$ vs. respective NC-siRNA. NC-siRNA, siRNA-negative control group; CCN3-siRNA, CCN3 siRNA-transfected group; CCN3, nephroblastoma overexpressed (also known as NOV).

Suppression of CCN3 inhibits apoptosis in A549 cells through the Bcl-2/caspase-3 pathway. Lung epithelial cell apoptosis is increased after LPS treatment (24). Bcl-2 is an important anti-apoptotic factor, and decreased expression of Bcl-2 can lead to the activation of caspase-3 and apoptosis (32). To assess the effect of CCN3 on apoptosis, we performed flow cytometry using an apoptosis detection kit. Our observations indicated that the sum of the proportions of early apoptosis and late apoptosis was significantly decreased in the CCN3 knockdown group with or without LPS treatment, but increased after LPS treatment compared with the negative group ($P < 0.001$; Fig. 4A). In addition, the western blot assays revealed that silencing of CCN3 expression upregulated Bcl-2 protein levels and downregulated the expression of caspase-3 protein, also after LPS treatment (Fig. 4B-D). Our data suggest that CCN3 is associated with the apoptosis of lung epithelial cells.

CCN3-siRNA knockdown reduces the activation of the NF- κ B signaling pathway. A previous study demonstrated that LPS could cause the activation of NF- κ B, which is involved in the process of apoptosis in ALI/ARDS (33). In order to establish whether the CCN3-induced pro-apoptotic effects are mediated through the NF- κ B signaling pathway, we used confocal microscopy to analyze the localization of NF- κ B p65 and western blot analysis of cytoplasmic and nuclear NF- κ B p65 expression levels to establish whether nuclear translocation activation occurs. The results demonstrated that activation of NF- κ B was largely reduced in the CCN3 knockdown group, whereas it was greatly stimulated after LPS exposure (Fig. 5A). As shown in Fig. 5B, pretreatment of the cells with

PDTC, which inhibits the NF- κ B signaling pathway, significantly attenuated the overexpression of CCN3 induced by LPS treatment. Meanwhile, the protein expression levels of NF- κ B p65 in the nucleus following pretreatment with PDTC or CCN3 knockdown, were significantly decreased compared to those without pretreatment, namely the LPS-treated only group ($P < 0.001$, Fig. 5C). Therefore, we suggest that CCN3 caused activation of NF- κ B in A549 cells by the promotion of nuclear translocation of NF- κ B p65.

Discussion

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a highly refractory disease, and its complex pathology in lung epithelial cells is not fully understood. In the present study, we aimed to identify a potential signaling pathway to target in order to prevent or treat the disease. This study demonstrated that nephroblastoma overexpressed (NOV; also known as CCN3) is critical for LPS-induced lung alveolar epithelial cell injury and apoptosis. Firstly, we investigated the presence of CCN3 using an *in vitro* cell culture ALI model. We found that CCN3 mRNA and protein expression were significantly increased after LPS treatment. These observations confirmed that LPS treatment strongly induced the expression of CCN3 in lung alveolar epithelial cells. This was consistent with a previous study (16), revealing elevated CCN3/NOV levels as a potential indicator for lung injury severity.

However, the precise biological role, mechanism of action and physiological function of CCN3 proteins in ARDS has remained elusive until recently. Kular *et al* (7) indicated that CCN3 expression *in vitro* is finely regulated by diverse

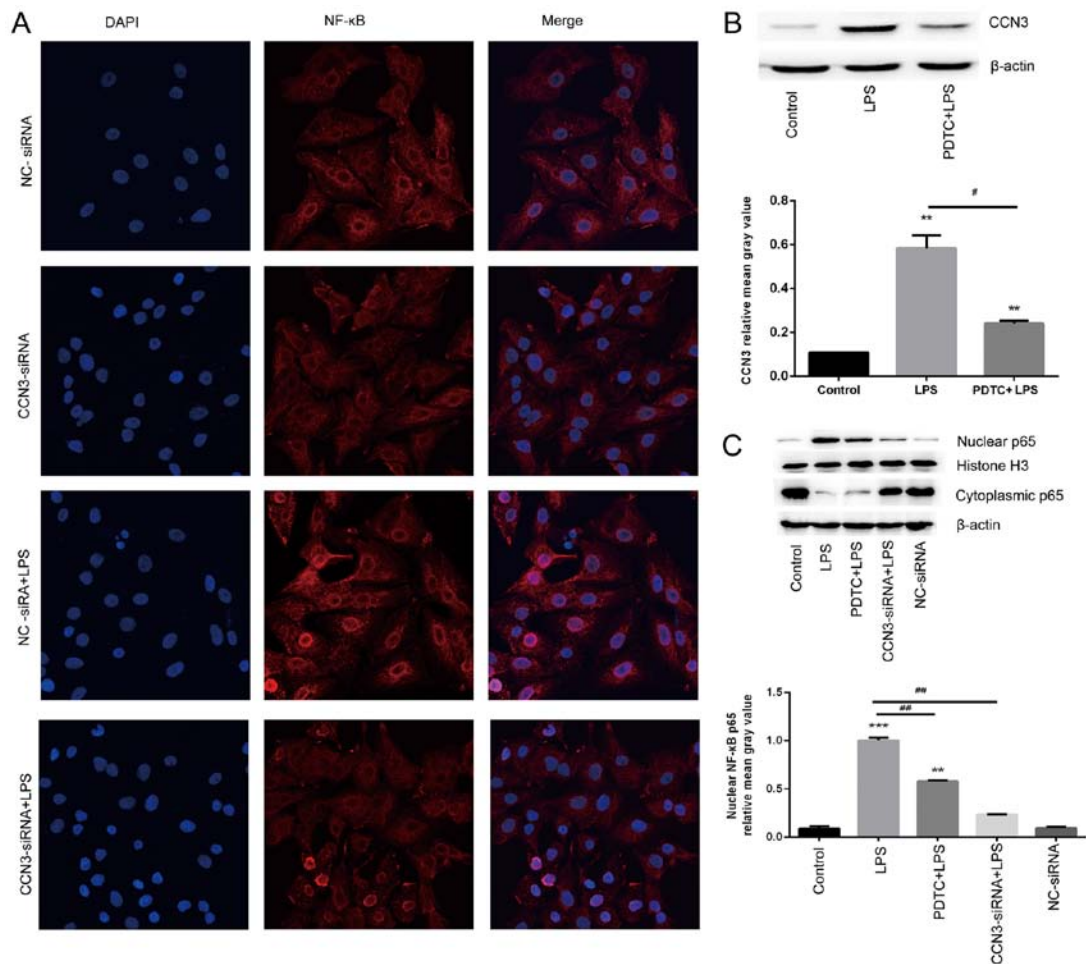


Figure 5. CCN3-siRNA knockdown inhibits the LPS-induced NF- κ B signaling pathway. (A) Confocal microscopy of NF- κ B nuclear translocation in cultured A549 cells. Magnification x600. DAPI (blue), NF- κ B p65 (red), in A549 cells challenged with LPS. (B and C) After 36 h of CCN3-siRNA transfection or not, cells were pretreated with PDTC (10 μ M) for 30 min, followed by stimulation with LPS (0.1 μ g/ml) for 12 h. The protein levels of (B) CCN3 and (C) NF- κ B p65 in the cytoplasm and nucleus were assessed by western blot analysis. ** $P < 0.01$, *** $P < 0.001$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. LPS group. NC-siRNA, siRNA-negative control group; CCN3-siRNA, CCN3 siRNA-transfected group; CCN3, nephroblastoma overexpressed (also known as NOV); LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B.

inflammatory mediators and cytokines, such as TNF- α , IL-1 β and TGF- β . In this study, an *in vitro* siRNA approach showed that inhibition of CCN3 significantly attenuated the expression levels of pro-inflammatory cytokines (IL-1 β and TGF- β 1), which have been linked to the initiation and amplification of the inflammatory response in ALI/ARDS (2,34). Surprisingly, we found no significant changes in the expression level of TNF- α after transfection with CCN3-siRNA. This could be explained by the insensitivity of TNF- α production by the alveolar epithelial cells in our study. TNF- α was possibly not involved in this process. In addition, recent studies have revealed that CCNs and TNF- α are co-expressed at sites of inflammation (35). It is therefore tempting to speculate that CCNs may help to counterbalance the inflammatory effects of TNF- α . A previous study has shown that A549 cells release IL-1 β and TNF- α after LPS stimulation through autocrine modes (36). This exerts a strong and synergic induction signal for IL-6 (37), since a high increase of IL-6 in ALI/ARDS accelerates the development of the local inflammatory microenvironment (38). Moreover, high levels of IL-1 β , IL-6 and TNF- α have been considered as the most promising biomarkers for predicting morbidity and mortality in patients with ALI/ARDS (39). Taken together, we

demonstrated that CCN3 plays an important pro-inflammatory role in lung epithelial cell injury by promoting the activities of specific cytokines, such as IL-1 β and TGF- β 1.

TGF- β 1 is a secretory cytokine that binds to the Type II and Type I TGF β receptor (TGF- β RII and TGF- β RI, respectively), which initiates TGF- β signaling via Smad phosphorylation and nuclear translocation (40,41). Many studies have demonstrated that the TGF- β 1 pathway plays a critical role in the development of ALI (18,40-42). One study has demonstrated that early activation of TGF- β 1/Smad2 signaling might contribute to acute pancreatitis-associated ALI, through regulation of lung permeability, epithelial ion transport, fibrinolysis and the extracellular matrix (31). In the present study, we observed a reduction in the levels of TGF- β R II and p-Smad2/3 following CCN3 silencing. Notably, overexpression of CCN3 in A549 cells might contribute to the activation of the TGF- β signaling pathway, leading to the destruction of epithelial integrity and aggregation of lung injury. Therefore, to further explore the direct roles of the TGF- β and p-Smad2/3 signaling pathways in the effect of CCN3, TP0427736, which inhibits the phosphorylation of Smad2/3 (43), was chosen to treat the A549 cells. We observed a significant decrease in CCN3 expression induced by LPS after TP0427736 treatment in

the A549 cells, suggesting that CCN3 may promote the release of inflammatory mediators by the TGF- β /p-Smad2/3 signaling pathway in A549 cells. This reveals an alternative pathway that could aid in future studies of the pathogenesis of ALI.

Apoptosis of AEC II cells plays an essential role in the pathogenesis of ARDS (44). Our study showed that 0.1 μ g/ml LPS could promote apoptosis in A549 cells. CCN3 knockdown greatly reduced the apoptotic rate of AEC II cells, while overexpression of CCN3 promoted AEC II apoptosis. LPS-triggered alveolar epithelial type II cell apoptosis is thought to primarily depend on the mitochondrial apoptosis signaling pathway (45). Bcl-2 regulates the mitochondrial apoptotic pathway by preventing the release of cytochrome *c* from mitochondria to cytosol, and activating caspase-3 (46). Doghman *et al* (47) showed that CCN3 induced human adrenocortical cell apoptosis through the activation of caspase 3. Consistent with these results, the changes in Bcl-2 and caspase-3 levels highlighted that the inhibition of CCN3 expression prevented AEC II apoptosis at the cellular level. Therefore, the activation of CCN3 induced by LPS may be involved in promoting AEC II cell apoptosis through the Bcl-2/caspase-3 pathway.

Moreover, this study showed that CCN3 activated NF- κ B in A549 cells by promoting nuclear translocation of NF- κ B p65. As a central mediator of the human immune response, NF- κ B is a critical transcription factor in the pathogenesis of ALI (48). It directly activates downstream signaling pathways by interacting with MyD88, promoting the degradation of I κ B α and the phosphorylation of NF- κ B (49), leading to epithelial cell apoptosis by the Fas/Fas ligand (Fas L) signaling pathway (50-52). In addition, previous studies have demonstrated that the NF- κ B pathway promoted lung inflammation and injury in response to local and systemic stresses in airway epithelial cells, by triggering the transcription of inflammatory cytokines and chemokines (TNF- α , IL-1 β and IL-6) (53-55). Because this study did not illuminate the relationship between IL-1 β and NF- κ B, it is not clear whether the same response to stimuli could be observed in A549 cells. Additionally, overexpression of CCN3 has been observed to have anti-inflammatory effects on endothelial cells by inhibiting the activation of NF- κ B (9), which is in contrast with our results. These differences may be due to differences in the cell types analyzed. Our results demonstrated that pretreatment with PDTC (a specific NF- κ B inhibitor) significantly suppressed LPS-induced CCN3 and nuclear NF- κ B p65 expression, indicating that CCN3 plays a role in human lung alveolar epithelial cells through the NF- κ B signaling pathway. It has been shown that excessive epithelial cell apoptosis could lead to the damage of the pulmonary alveolar-capillary barrier and aggravate the inflammatory responses in lung diseases (52,56), suggesting a close association between inflammation and apoptosis. In summary, our study revealed that CCN3 may have potential clinical value in the occurrence and development of ALI via the TGF- β /p-Smad or NF- κ B signaling pathways. Further research is needed to validate our findings using *in vivo* models, yet our data suggest a novel potential target for future clinical studies.

In conclusion, the present study demonstrated that CCN3 expression in alveolar epithelial cells was significantly increased under inflammatory conditions and/or in response to stimuli such as LPS. The overexpression of CCN3 can be perturbed by a TGF- β /p-Smad or NF- κ B inhibitor, which explains how CCN3-siRNA led to the inhibition of the release

of inflammatory cytokines and apoptosis in human alveolar epithelial cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HPZ, CSC and YGC conceived and designed the experiments, analyzed the data and wrote the manuscript. HYH, DMW, CLC, LD and ND carried out the experiments, prepared and analyzed the figures and tables. LD, CSC, CLC and YGC obtained the study materials and reagents in preparation for the experiments. All authors reviewed drafts of the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The protocol of the present study was approved by the Use Committee of Wenzhou Medical University (Wenzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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