NLRC5 Mediates IL-6 and IL-1β Secretion in LX-2 Cells and Modulated by the NF-κB/Smad3 Pathway

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Abstract—Recent data have shown that nucleotide-binding domain leucine-rich repeat proteins (NLRs), a class of innate immune receptors that respond to pathogen attack or cellular stress, have gained increasing attention. NLRC5 (NLR family, CARD domain containing 5) is the largest member of the NLR family, which has recently been identified as a critical regulator of immune responses. Until recently, the function of NLRC5 has been a matter of debate. In this study, we explore the role of NLRC5 in cytokine secretion and the role of the nuclear factor-κB (NF-κB) signaling pathway in tumor necrosis factor-alpha (TNF-α)-induced NLRC5 expression in LX-2 cells. We demonstrated that overexpression of NLRC5 results in an upregulation of IL-6 and IL-1β secretion. On the other hand, knockdown of NLRC5 by transfecting siRNA decreased IL-6 and IL-1β secretion in LX-2 cells. Meanwhile, the results showed that pyrrolidine dithiocarbamate (PDTC) (a specific inhibitor of the NF-κB signaling pathway) inhibited NLRC5 expression and NLRC5 silencing could increase the expression levels of p65 in cell nucleus accompanied with upregulated phosphorylation of Smad3 protein levels in response to TNF-α. These results indicated that NLRC5 plays a significant role in TNF-α-enhanced cytokine (IL-6 and IL-1β) secretion of LX-2 cells and the NF-κB/Smad3 signal pathway is involved in its induction of expression.

KEY WORDS: NLRC5; TNF-α; IL-6; IL-1β; NF-κB signaling; PDTC.

INTRODUCTION

Hepatic fibrosis is the final common pathway for most chronic liver diseases, such as chronic hepatitis B and C, autoimmune hepatitis, and alcoholic liver disease. It is characterized by excessive deposition of extracellular matrix (ECM) proteins and exacerbated inflammatory response [1, 2]. Hepatic stellate cells (HSCs) are activated in the process of hepatic fibrosis [3]. Activation of HSCs is the central event in hepatic inflammatory injury and fibrosis [4]. Initiation of HSC activation is associated with

increases in several inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), *etc.* [5].

Notably, the NOD-like receptors (NLRs) are a family of cytoplasmic PRRs whose function is mainly to induce inflammation and cell death, facilitating quick removal of invasive pathogens, and play an important role in innate and adaptive immunity [6, 7]. Among them, NLRC5 (NLR family, CARD domain containing 5; also known as NOD4 or NOD27) is a newly identified member of the NLR family, which acts as a transcriptional regulator of the major histocompatibility complex class I [8, 9]. Human NLRC5 is located in the 16q13 locus and consists of 1866 amino acids (aa) while mouse NLRC5 is at chromosome 8 and contains 1915 aa [10]. There was a 64 % amino acid sequence identity between the human and murine proteins. Similar to other NLRs, NLRC5 contains three structural domains including the N-terminal atypical caspase activation and recruitment domain (CARD), the centrally located NACHT (named after NAIP, CIITA, HET-E, and TP-1

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proteins), and 27 leucine-rich repeats (LRRs) at the C-terminal [11]. Many studies have proved that NLRC5 was highly expressed in immune tissues or organs like the spleen, bone marrow, and thymus, suggesting that this molecule is biologically conserved in these tissues. More specifically, many recent studies have offered conflicting and alternating roles of NLRC5 in innate and adaptive immunity [12]. Previously, we demonstrated that NLRC5 plays a negative role in the regulation of IL-6 and TNF-α in lipopolysaccharide (LPS)-induced RAW264.7 cells and the JAK2/STAT3 pathway is involved in its induction of expression [13]. However, less is known about the role of NLRC5 in cytokine secretion of HSCs participating in liver inflammatory response.

It is well known that LX-2 cells are a stable and unlimited source of human HSC, which has been extensively characterized as a valuable cell-based model for studies of human hepatic fibrosis [14]. LX-2 cells retain the key features of activated HSC, such as cytokine signaling, neuronal gene expression, retinoid metabolism, and fibrogenesis [15]. Therefore, we employed the LX-2 cells to investigate the role of NLRC5 and the possible underlying mechanisms in the current study. We found that NLRC5 plays a significant role in TNF-α-enhanced cytokine secretion of LX-2 cells and the nuclear factor-κB (NF-κB)/Smad3 signal pathway is involved in its induction of expression.

MATERIALS AND METHODS

Materials and Reagents

3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO, USA). Pyrrolidine dithiocarbamate (PDTC) (a special NF-κB inhibitor) was purchased from Sigma Chemical (St. Louis, MO, USA). NLRC5, IL-6, IL-1β, and β-actin primers were produced by Sangon Biological and Technological Company (Shanghai, China). Human anti-NLRC5 polyclonal antibody and human anti-β-actin monoclonal antibody were purchased from Abcam (Cambridge, MA, USA). Human monoclonal antibodies against phospho-P65, IκBα, phospho-IκBα, ReIA/p65, Smad3, p-Smad3, β-actin, and PCNA were obtained from Boster (Wuhan, China). Secondary antibodies for goat anti-rabbit immunoglobulin (IgG) and goat anti-rabbit horseradish peroxidase (HRP) were purchased

from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nuclear and cytoplasmic protein extraction kits were obtained from Bestbio Institute of Biotechnology (China). Recombinant TNF-α was purchased from Peprotech (Peprotech, UK).

Cell Culture

LX-2 cells, immortalized human HSCs, were obtained from Dr. Scott Friedman (Mount Sinai School of Medicine) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) plus 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin–streptomycin. Cell cultures were maintained at an atmosphere of 5 % CO_2-humidified incubator at 37 °C.

Plasmid Construction

Expression plasmid for NLRC5 was generated by amplifying complementary DNA (cDNA) coding for NLRC5 from pancreas cDNA and inserting cDNA coding for NLRC5 into destination vectors by Gateway cloning (Invitrogen). The following primers were used for amplification: forward: 5′-CCGGAATTCCGGATGGCCAGGAAGCTGGA-3′; reverse: 5′-GGGATCCCGTCACCTGAGTGTCTTCCCA-3′. The N-terminal region of the NLRC5 coding region containing the predicted CARD domain was cloned into pEGFP-C2 vector by using restriction sites EcoRI and BamHI. Cell transfection was performed with the Lipofectamine™ 2000 according to the manufacturer's manuals.

RNA Interference Analysis

Small interfering RNA targeting NLRC5 was purchased from GenaPharma Corporation (Shanghai, China). NLRC5-siRNA (human) 5′-AAGAACGAGAGACUCUGCCAACUGCdTdT-3′ for the sense strand and 5′-GCAGUUGGCAGAGUC UCUCGUUCUUdTdT-3′ for the antisense strand. A negative scrambled siRNA, sense: 5′-CGUACGCGGAAUACUUCGA-3′, antisense: 5′-UCGAAGUAUUCCGCGUAC G-3′ (GenePharma, Shanghai, China), was used in parallel. LX-2 cells were cultured in 6-well plates with antibiotic-free DMEM (Opti-MEM) for 24 h before operation. Then, the siRNAs were transfected into LX-2 cells using Lipofectamine™ 2000 according to the manufacturer's protocol. Knockdown efficiency was determined by Western blot analysis. Three independent transfection experiments were performed.

Table 1. Primer Sequences for Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

| Gene	Primer pair	
NLRC5	(F)5′-ATCAACTGCCCTTCCACAAT-3′	(R)5′-CTATCTGCCCACAGCCTACC-3′
IL-6	(F)5′-GCCACTCACCTCTTCAGAACG-3′	(R)5′-CCGTCGAGGATGTACCGAATT-3′
TNF-α	(F)5′-GATCAATCGGCCCGACTATC-3′	(R)5′-TCCTCACAGGGCAATGATCC-3′
IL-1β	(F)5′-TGGCAATGAGGATGACTTGT-3′	(R)5′-GTGGTGGTCGGAGATTCGTA-3′
β-actin	(F)5′-GCCAACACAGTGCTGTCTGG-3′	(R)5′-CTCAGGAGGAGCAATGATCTTG-3′

PDTC Treatment

LX-2 cells were treated with different stimuli and divided into five groups. Two groups were treated with, or without, TNF-α (20 ng/ml). The other three groups were separately pretreated with 2, 4, 8 μM PDTC (NF-κB inhibitor, Sigma Chemical, Aldrich Ltd) for 3 h and then exposed to TNF-α (20 ng/ml) for 3 h. Normal LX-2 cells were used as control group cultured without any treatment. All cells were incubated in the CO_2 incubator before being used in assays.

Cell Proliferation Assay

Cell proliferation assay was determined by standard MTT assay. LX-2 cells were seeded into a 96-well plate at a density of 1000 cells per well and deprived of serum for 24 h before experiments. Then, the cells were transfected with NLRC5-siRNA and negative control as described above. Cell proliferation was assessed 24 h later. After culture, 5 mg/ml MTT (Sigma, USA) was added and incubated at 37 C for another 4 h; thereafter, the medium was replaced and the formazan crystals were dissolved in 150 μl DMSO. The optical density (OD) was determined with the Thermomax Microplate Reader (Bio-Tek EL, USA) at a 490-nm wavelength. All experiments were performed in triplicate and repeated at least three times. The percentage of viability was calculated according to the following formula: viability $\% = T/C \times 100$ %, where T and C refer to the absorbance of transfection group and cell control, respectively.
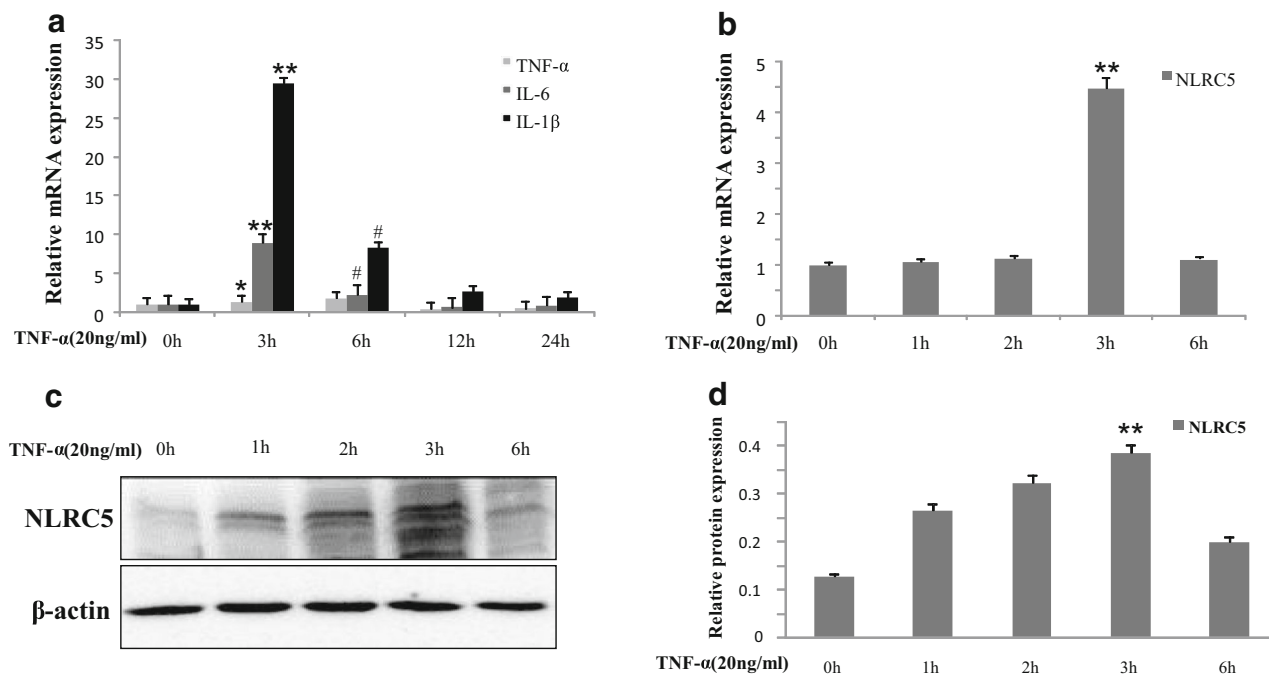
Fig. 1. TNF-α time-dependent expression of NLRC5 in LX-2 cells. **a** Using qRT-PCR, a significant upregulation of TNF-α, IL-6, and IL-1β mRNA was shown within 3 h after stimulation with TNF-α. **b** Using qRT-PCR, a significant upregulation of NLRC5 mRNA was shown within 3 h after stimulation with TNF-α. **c** Using Western blot, a significant upregulation of NLRC5 protein was shown within 3 h after stimulation with TNF-α. The results are expressed as the mean±SD of three different experiments. *$P < 0.05$ compared to the normal group. **$P < 0.01$ compared to the normal group.

Quantitative Real-Time PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was generated using a Transcriptor First Strand cDNA Synthesis Kit (TaKaRa, Japan). Relative levels of specific mRNA were determined using the Thermo PIKOREAL 96 Real-Time PCR Detection System with QIAGEN SYBR® Green supermix (Valencia, CA, USA) according to the manufacturer's instructions. The β-actin gene was used as an internal control for normalization. The primers used for PCR amplification are listed in Table 1.

ELISA Assay

The levels of IL-6 and IL-1β in the supernatants of each macrophage culture were determined using an enzyme-linked immunosorbent assay (ELISA) Kit (R&D USA) according to the manufacturer's instruction.
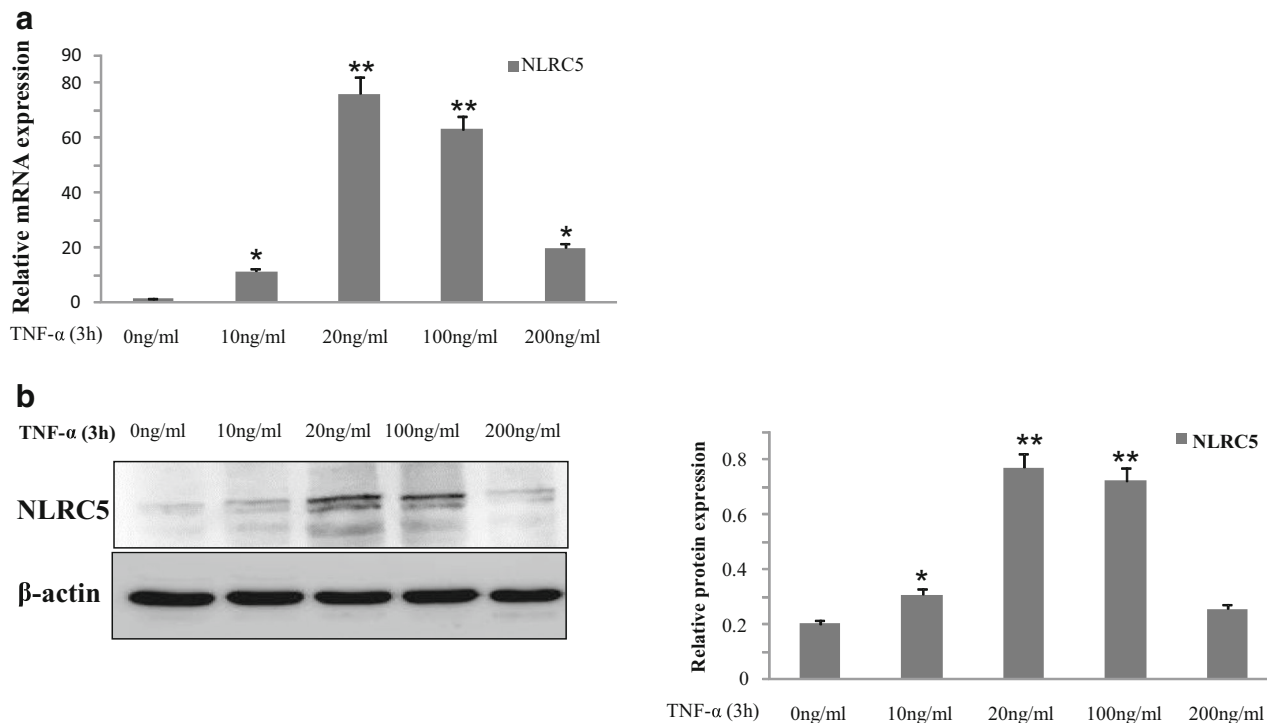
Western Blot

LX-2 cells were lysed in protein extraction solution for Western (Beyotime, China). Nuclear and cytoplasmic proteins of LX-2 cells were obtained using nuclear and cytoplasmic protein extraction kit according to the manufacturer's instructions. Protein concentration of the extract was determined by using the BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein were electrophoresed on SDS-PAGE and blotted onto PVDF membranes (Millipore Corp, Billerica, MA, USA). After blockade of nonspecific protein binding, nitrocellulose blots were incubated for 6 h with primary antibodies diluted in Primary Antibody Dilution Buffer (Beyotime, China). The primary antibody recognizing NLRC5, ReIA/P65, IκBα, p-IκBα, PCNA, and β-actin were used 1:500, 1:500, 1:500, 1:1000, 1:1000, and 1:800, respectively. The anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase were used as secondary antibodies correspondingly. After washing four times with TBST (Boster, China), the protein blots were detected using the ECL Chemiluminescent Kit (ECL-plus, Thermo Scientific).

Statistical Analysis

Data are represented as mean±SD. The data were analyzed by one-way ANOVA (LSD) using the SPSS 15.0 software to determine their significant differences. For changes in mRNA or protein levels, mRNA (relative

Fig. 2. TNF-α dose-dependent expression of NLRC5 in LX-2 cells. **a** Using real-time qPCR, a significant upregulation of TNF-α, IL-6, and IL-1β protein was shown within 3 h after stimulation with TNF-α (20 ng/ml). **b** Using Western blot, a significant upregulation of NLRC5 protein was shown within 3 h after stimulation with TNF-α (20 ng/ml). *$P<0.05$ compared to the normal group. **$P<0.01$ compared to the normal group.

expression) and protein (densitometric values) to respective housekeeping controls were compared. $P<0.05$ was considered statistically significant.
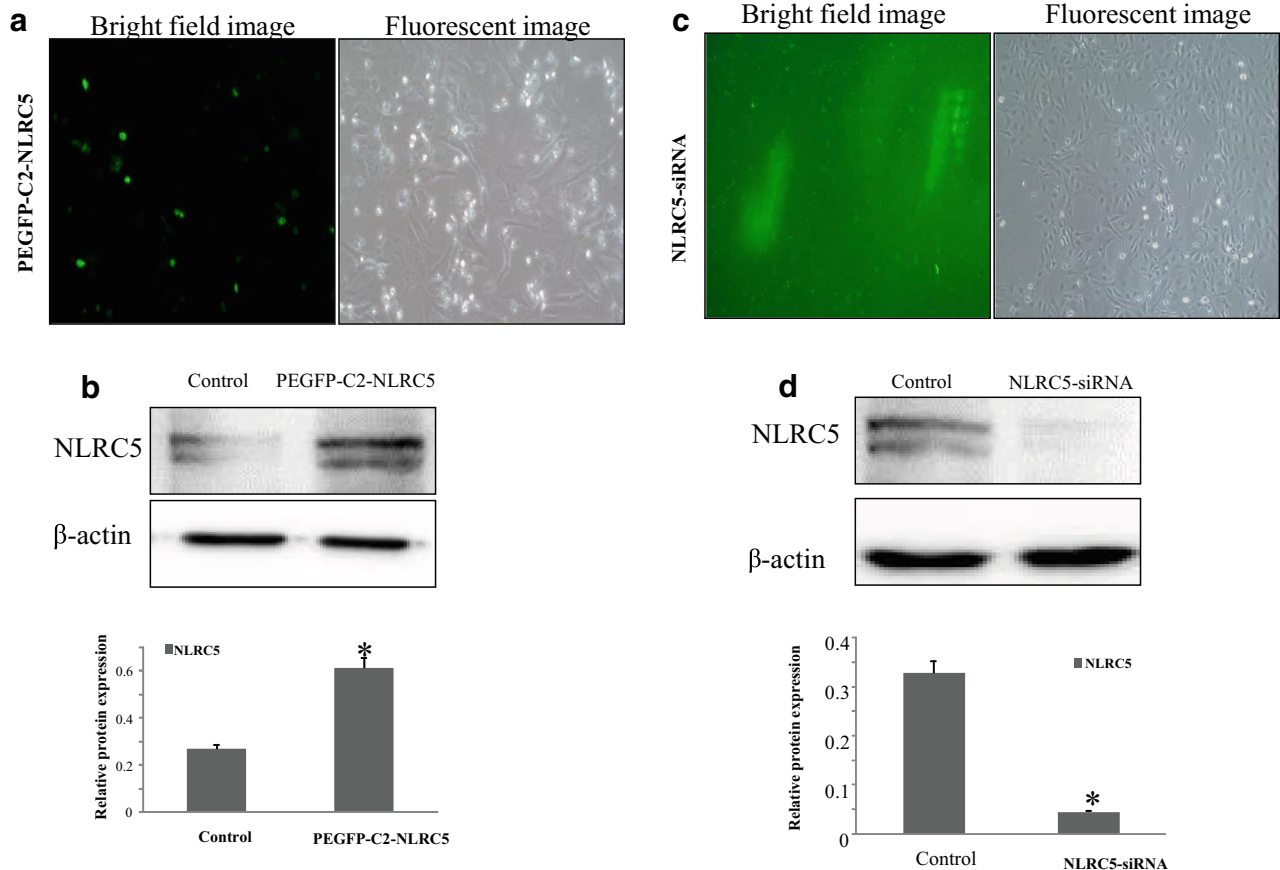
RESULTS

NLRC5 Is Induced by TNF-α in LX-2 Cells

NLRC5 has recently been identified as a critical regulator of immune responses, and it is constitutively and widely expressed [12]. Here, we observed that NLRC5 is expressed in LX-2 cells. To examine the changes of its expression in LX-2 cells during activation induced by TNF-α, which is known to activate the inflammation. We tested the kinetic profiles of NLRC5 expression. This process was time dependent; quantitative real-time PCR (qRT-PCR) analyses revealed novel

upregulation of NLRC5 at the mRNA levels, which peaked at 3 h after being induced by TNF-α treatment (Fig. 1b). Likewise, the highest mRNA levels of TNF-α, IL-6, and IL-1β were also reached after 3 h induced by TNF-α treatment (Fig. 1a). Moreover, this effect was dose dependent, the levels of NLRC5 were increased as concentrations induced by TNF-α increase, and the highest mRNA and protein levels of NLRC5 were reached at 20 ng/ml after 3 h induced by TNF-α treatment (Fig. 2). These results suggested that the induction of NLRC5 induced by TNF-α is specific.

Lipidosome-Mediated Transduction Resulted in NLRC5 Overexpression or Knockdown in LX-2 Cells

NLRC5 overexpression and knockdown were introduced into LX-2 cells by lipidosome. To corroborate the

Fig. 3. Lipidosome-mediated transduction results in NLRC5 overexpression or knockdown in LX-2 cells. **a**, **c** LX-2 cells were seeded in 6-well plates and transfected with plasmid pEGFP-C2-NLRC5 and NLRC5-siRNA. At 24 h following the transfection, the cells were photographed at ×920 magnification by fluorescence microscope. **b**, **d** 3×10^5 LX-2 cells were seeded in 6-well plates and transfected with plasmid pEGFP-C2-NLRC5 and fluorescent NLRC5-siRNA. Representative blots were from three independent experiments with densitometry. *$P<0.05$ vs control.
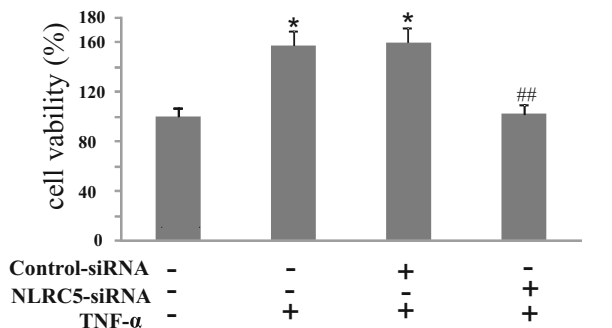
**Fig. 4.** Effect of NLRC5-siRNA on cell proliferation in TNF-α-treated LX-2 cells. Inhibition of NLRC5 significantly decreased cell proliferation in TNF-α-treated LX-2 cells. Proliferation of LX-2 cells was analyzed by MTT assay. *$p < 0.5$ and **$p < 0.01$, compared with the control condition, #$p < 0.5$ and ##$p < 0.01$, compared with control siRNA condition with TNF-α.

results are shown in Fig. 3. After detecting NLRC5 level by Western blot, we found NLRC5 protein level was elevated by pEGFP-C2-NLRC5 (Fig. 3a, b). NLRC5 siRNA induced a remarkable decrease of NLRC5 protein level in LX-2 cells (Fig. 3c, d).

Effect of NLRC5 on Proliferation in TNF-α-Treated LX-2 Cells

In order to investigate the roles of NLRC5 in regulating TNF-α-induced LX-2 cell proliferation, we tested the effect of NLRC5 silencing on the proliferation of TNF-α-treated LX-2 cells. As shown in Fig. 4, LX-2 cells transfection with NLRC5 siRNA significantly decreased mature NLRC5 expression. MTT assay showed that knockdown of NLRC5 caused a significant inhibition of cell proliferation in TNF-α-treated LX-2 cell at 24 h. The result suggested that NLRC5 regulates apoptosis in LX-2 cells positively.

overexpression or knockdown effect of NLRC5, we detected NLRC5 level in the conditioned medium and the
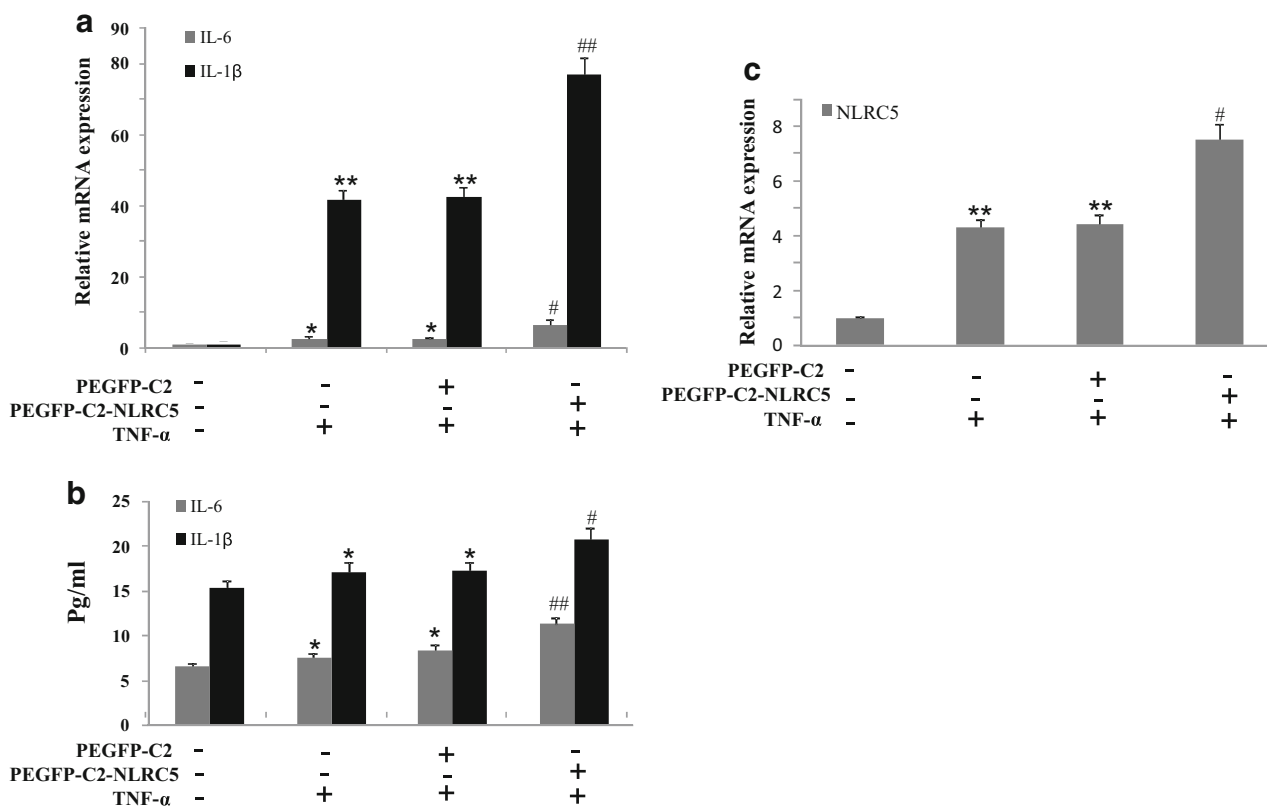
Fig. 5. The effects of NLRC5 overexpression plasmid on the secretion of IL-6 and IL-1β in TNF-α-induced LX-2 cells. LX-2 cells were transfected with pEGFP-C2-NLRC5 or pEGFP-C2, followed by TNF-α treatment. **a** The analysis of qRT-PCR showed that transfection of pEGFP-C2-NLRC5 upregulated the expression of IL-6 and IL-1β. **b** The analysis of ELISA confirmed this conclusion using cell supernatant. **c** An increase of NLRC5 was observed in cells transfected with pEGFP-C2-NLRC5 by qRT-PCR. The results are expressed as the mean ± SD of three different experiments. *$P < 0.05$ and **$P < 0.01$ compared to the normal group, #$P < 0.05$ and ##$P < 0.01$ compared to the pEGFP-C2 group.

NLRC5 Is Critical for the Cytokine Secretion of Activated LX-2 Cells

Emerging evidence suggests that IL-6 and IL-1β are participating in liver inflammation and NLRC5 may function as inflammatory cytokine mediators. In order to elucidate whether the expression of NLRC5 is involved in TNF-α-mediated cytokine secretion including IL-6 and IL-1β, we transfected LX-2 cells with plasmid pEGFP-C2-NLRC5 or negative control pEGFP-C2 then treated them with or without TNF-α. The expression levels of IL-6 and IL-1β mRNA were checked by qRT-PCR (Fig. 5a) and showed that TNF-α treatment strongly increased mRNA levels of IL-6 and IL-1β and NLRC5 upregulation potently increased IL-6 and IL-1β secretion induced by TNF-α treatment. No difference was found between pEGFP-C2 and TNF-α group. Further results of ELISA assay (Fig. 5b) also

demonstrated that pEGFP-C2-NLRC5 has a positive role on IL-6 and IL-1β secretion after the treatment of TNF-α in LX-2 cells. Thus, these effects suggest that overexpression of NLRC5 in LX-2 cells may lead to the rise of expression of IL-6 and IL-1β induced by TNF-α treatment.

In order to verify the effects of NLRC5 on the expression of cytokines in LX-2 cells after TNF-α induction in our system, we knocked down endogenous NLRC5 and then treated the cells with or without TNF-α for 3 h. Scrambled siRNA of NLRC5 was transfected and seen as a negative control. qRT-PCR analysis demonstrated that knockdown of NLRC5 strongly inhibited mRNA levels of IL-6 and IL-1β in LX-2 cells transfecting NLRC5-siRNA (Fig. 6a). No difference was found on IL-6 and IL-1β expression between the scrambled siRNA group and TNF-α treatment group. These results were confirmed by the ELISA assay (Fig. 6b), revealing that NLRC5-siRNA
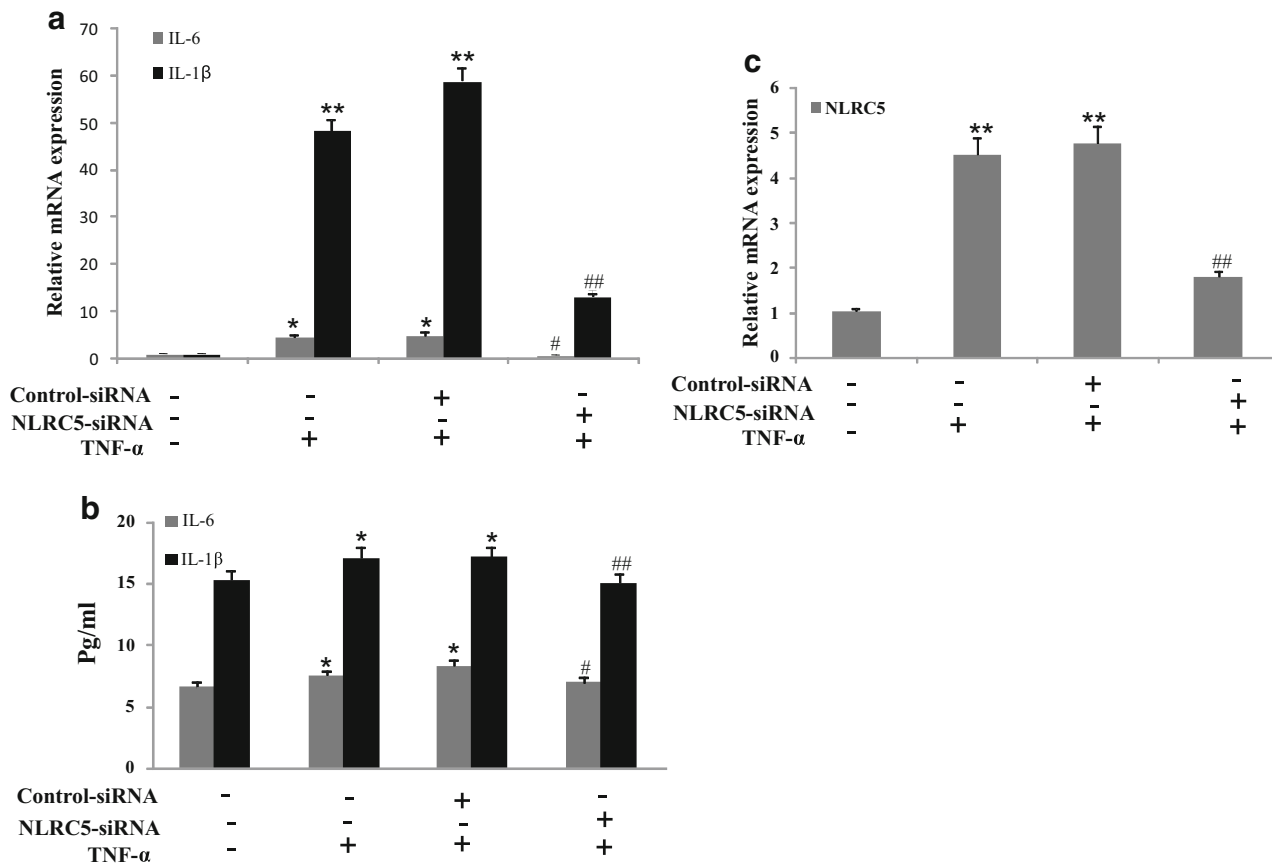
**Fig 6.** The effects of NLRC5 siRNA on the secretion of IL-6 and IL-1β in TNF-α-induced LX-2 cells. LX-2 cells were transfected with NLRC5-siRNA or scrambled siRNA, followed by TNF-α treatment. **a** The analysis of qRT-PCR showed that transfection of NLRC5 siRNA downregulated the expression of IL-6 and IL-1β. **b** The analysis of ELISA confirmed this conclusion using cell supernatant. **c** The analysis of qRT-PCR showed that a decrease of NLRC5 was observed in LX-2 cells transfected with NLRC5-siRNA. The results are expressed as the mean ± SD of three different experiments. **$P < 0.01$ and *$P < 0.05$ compared to the normal group, ##$P < 0.01$ and #$P < 0.05$ compared to the control siRNA-transfected LX-2 cells treated with TNF-α.

treatment resulted in less IL-6 and IL-1β expression in LX-2 cells. We can conclude that NLRC5 knockdown can significantly decrease IL-6 and IL-1β production stimulated by TNF-α.

Effect of NLRC5 on NF-κB/Smad3 Activity in TNF-α-Treated LX-2 Cells

Recent evidence suggests that activation of the transcription factor NF-κB plays a critical role in HSC activation [16]. We, therefore, determined whether NLRC5 regulates NF-κB leading to HSC activation. Firstly, Western blot also showed that after NLRC5-siRNA treatment, the expression of p65 was significantly increased in the

nucleus, but decreased in the cytoplasm regardless of TNF-α stimulation in LX-2 cells (Fig. 7b). Secondly, transfection with NLRC5-siRNA significantly induced the phospho-IκBα expression following TNF-α stimulation in LX-2 cells (Fig. 7a). Taking together, these data indicated that the anti-fibrotic effects of NLRC5-siRNA in LX-2 cells might be mediated by NF-κB signaling pathway. In addition, the phosphorylation of Smad3 was increased in TNF-α-treated LX-2 cells whereas the expressions of total Smad3 proteins remained unchanged (Fig. 8). In comparison with the housekeeping gene β-actin, the protein levels of p-Smad3 and Smad3 were elevated in TNF-α-activated LX-2 cells in comparison with the untreated cells. In cultured LX-2 cells, knockdown of
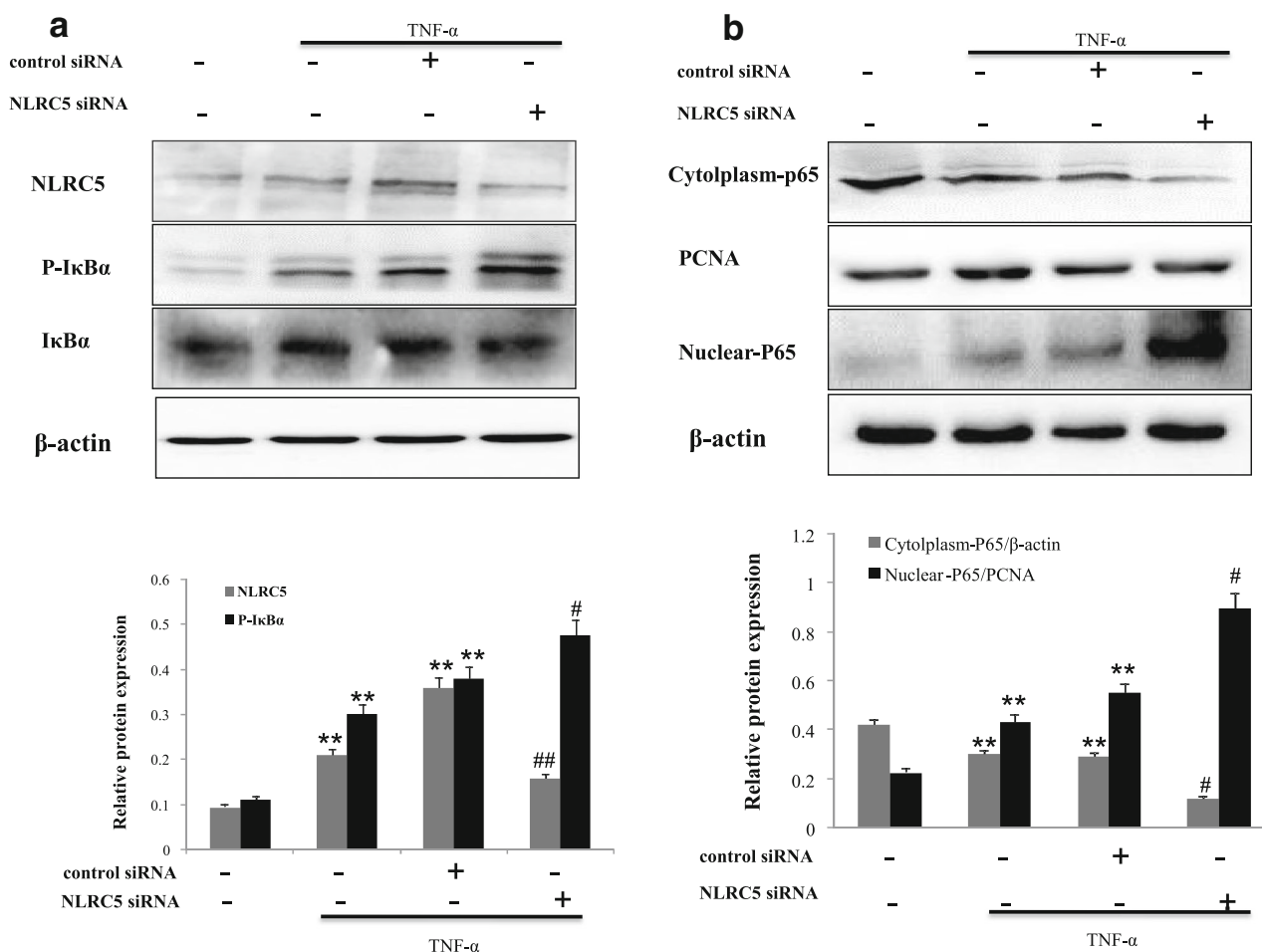

Fig. 7. NLRC5 knockdown markedly induces NF-κB activity in LX-2 cells after TNF-α treatment. LX-2 cells were transfected with NLRC5-siRNA or scrambled siRNA, followed by TNF-α treatment. **a** NLRC5, p-IκBα, and IκBα levels were determined by Western blot. β-actin was used as an invariant control for equal loading. Representative blots were from three independent experiments with densitometry. **b** Nuclear and cytoplasmic p65 proteins were isolated and determined by Western blot; PCNA was used as normalized control in nuclear protein. Data were presented as mean±SD of three different experiments. *$P<0.05$ and **$P<0.01$ compared with control, [#]$P<0.05$ and [##]$P<0.01$ compared with control siRNA-transfected LX-2 cells treated with TNF-α.

NLRC5 with siRNA raised phosphorylation of Smad3 protein levels in response to TNF-α (Fig. 8). These data suggested that NLRC5 exerts its promoting role in HSC activation and may regulate the NF-κB/Smad3 pathway negatively.

NF-κB Signaling Pathway Is Involved in the Expression of NLRC5 Induced by TNF-α

To determine whether NF-κB activation is involved in TNF-α-induced expression of NLRC5, LX-2 cells were treated with PDTC (an NF-κB inhibitor) (2, 4, and 8 μM) for 2 h in the presence or absence of 20 ng/ml TNF-α for 3 h. Western blot analysis showed that LX-2 cells
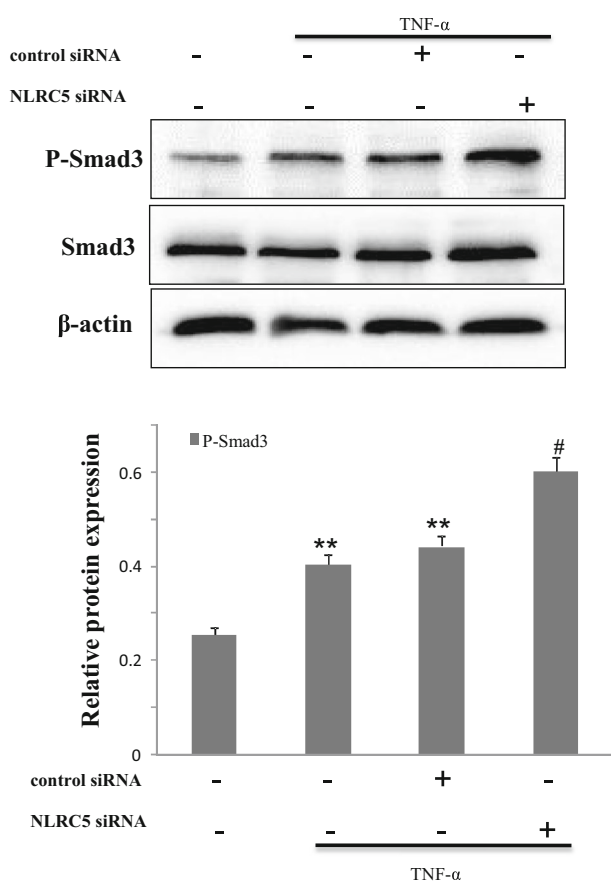

Fig. 8. Effect of NLRC5 on the activation of Smad3 pathway in LX-2 cells. LX-2 cells transfected with NLRC5-siRNA or scrambled siRNA were harvested 24 h after transfection, and whole-cell protein extracts were made. Western blot analyses of p-Smad3 and Smad3 proteins were performed. *All images on the above* were representative of at least three independent experiments. *The graph on the subjacent* showed the quantification of the band intensity. *$P<0.05$ and **$P<0.01$ compared with control, #$P<0.05$ and ##$P<0.01$ compared with control siRNA-transfected LX-2 cells treated with TNF-α.

pretreated with PDTC (2, 4, and 8 μM) inhibited TNF-α-induced expression of NLRC5, indicating that the expression of NLRC5 itself is controlled by the NF-κB signaling pathway (Fig. 9).

DISCUSSION

More and more evidence indicated that increased and sustained inflammation cause liver injury/disease and perpetuated activation of the stellate cell ultimately leading to increased hepatic injury and hepatic fibrosis. HSCs are the central cell in hepatic inflammatory injury and fibrosis [17] [20]. It is well known that hepatic fibrosis is a reversible wound-healing response and the activated HSCs decrease due to apoptosis in the recovery process. The novel studies on hepatic fibrosis will emphasize the promotion of apoptosis, inhibition of proliferation, and activation of HSCs [18, 19]. Several recent studies identified that the transcriptional factor NF-κB has been shown to be involved in the regulation of cytokine signaling and cellular apoptosis, which contributed to the activation of HSCs [16]. RNA interference (RNAi)-mediated knockdown studies suggested that NLRC5 negatively regulates the NF-κB signal pathway. Despite our increased understanding of NLRC5 function and interactions, many aspects related to mechanisms of sensing, downstream signaling, and *in vivo* functions remain elusive. However, the relationship between NLRC5 and NF-κB signal pathway is unclear and remains to be further explored in HSCs, especially in liver inflammation responses. Thus, an understanding of the molecular mechanisms by which NLRC5 can regulate inflammatory cytokines appears critical for developing novel and more effective treatments for inflammation-mediated liver diseases [20].

In this study, we presented the expression analysis of NLRC5 in LX-2 cells with the purpose of revealing the relationship between NLRC5 and NF-κB/Smad3 signal pathway. Furthermore, we investigated the proinflammatory cytokine expression of LX-2 cells induced by the expression change of NLRC5. First, we observed the upregulation of NLRC5 at both the mRNA and protein levels after treatment with TNF-α compared with normal LX-2 cells. These results showed that NLRC5 expression was sharply induced by TNF-α and could be used as a useful indicator for evaluating inflammation. Moreover, TNF-α is one of the most potent innate immune-activating stimuli. TNF-α exerts its toxic effects by potently activating HSC and inducing the expression of inflammatory cytokines such as IL-1β and IL-6 [21, 22]. Our study also confirmed
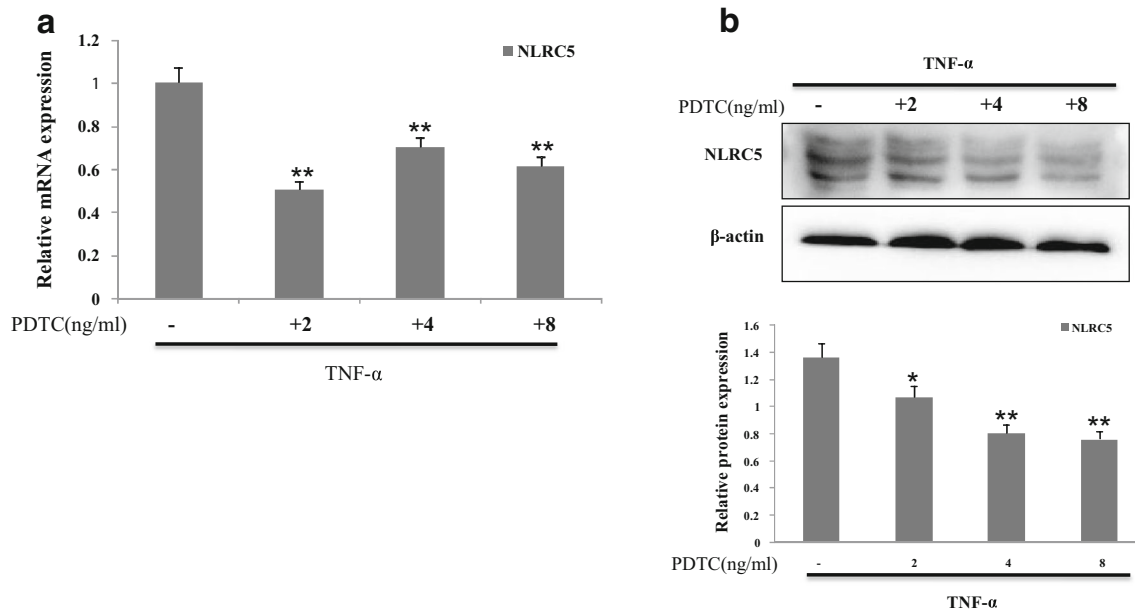
Fig. 9. The effects of PDTC on the level of NLRC5 in TNF-α-stimulated LX-2 cells. Pretreatment with the NF-κB inhibitor PDTC (2, 4, 8 μM) before infection of TNF-α in LX-2 cells. **a** Decreased expression of NLRC5 in mRNA level was detected by real-time qPCR. **b** Western blot was used for detecting NLRC5 and β-actin, and a decreased expression of NLRC5 was detected. The results are expressed as the mean±SD of three different experiments. *$P < 0.05$ and **$P < 0.01$ compared to the normal group, #$P < 0.05$ and ##$P < 0.01$ compared to the TNF-α group.

an upregulated expression of IL-1β, IL-6, and TNF-α in LX-2 cells induced by TNF-α. More recent studies suggested that NLRC5 may have a close relationship with inflammation-associated diseases; thus, we speculate that NLRC5 may participate in the secretion of cytokines IL-6 and IL-1β. According to this study, NLRC5 overexpression induced elevation of IL-6 and IL-1β expression in terms of mRNA and protein level. Furthermore, TNF-α treatment of pEGFP-C2-transfected cells was not able to rescue the expression of these cytokines. In contrast, we found that NLRC5 siRNA treatment decreased elevation of IL-6 and IL-1β expression induced by TNF-α. Based on the experimental data discussed above, we can conclude that NLRC5 is critical for the expression of cytokine genes at baseline and can positively regulate proinflammatory cytokine secretion in LX-2 cells.

Activation of NF-κB is linked to phosphorylation and proteolytic degradation of IκBα [16]. TNF-α is known as one of the strongest stimuli of NF-κB activity [23]. Indeed, we found that TNF-α induced phosphorylation of IκBα level in LX-2 cells compared with the normal group and the phosphorylation of IκBα was further induced in LX-2 cells pretreated with NLRC5-siRNA, whereas NLRC5-siRNA notably reduce cell survival. By analogy, NLRC5-siRNA treatment increased the expression of p65 significantly in the nucleus but decreased in the cytoplasm

regardless of TNF-α stimulation in LX-2 cells (Fig. 7c). In addition, NLRC5-siRNA accentuated phosphorylation of Smad3 protein levels in response to TNF-α (Fig. 9). These data suggested that NLRC5 exerts its promoting role in HSC activation through blocking of the NF-κB/Smad3 pathway. In addition, the inhibition of NF-κB signaling could also reduce NLRC5 induced by TNF-α. Indeed, PDTC effectively inhibited NLRC5 expression. Our finding of the relationship between NF-κB signal pathway and NLRC5 is novel and unexpected. Although NLRC5 was activated by TNF-α in LX-2 cells, NLRC5 may also signal through other pathways including the MAPK kinase, NF-κB signal pathways, and IFN pathways. These alternative signaling pathways may be primarily responsible for the negative regulation of NLRC5 induction in LX-2 cells, whereas NF-κB/Smad3 signaling may function to positively regulate NLRC5 expression.

In summary, this study shows that NLRC5 is critical for the cytokine secretion and modulated by NF-κB/Smad3 in LX-2 cells. Our further work would focus on primary HSCs isolated from the normal animals or the animals with hepatic inflammatory injury and fibrosis to analyze the expression and function of NLRC5 in hepatic inflammatory injury and fibrosis. Understanding the mechanisms of the negative regulators NLRC5 is an amazing task, but one that must be pursued as manipulating

negative regulators may lead to novel and useful therapeutic approaches to a range of infectious and inflammatory liver diseases of great clinical importance.

Conflict of Interest. We declare that we have no conflict of interest.

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