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NLRP3 inflammasome expression is driven by NF-κB in cultured hepatocytes

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ABSTRACT

The inflammasomes are cytoplasmic multiprotein complexes that are responsible for activation of inflammatory reactions. In principle, there are four individual inflammasome branches (NLRP1, NLRP3, NLRC4/NALP4, and AIM2) that mediate the cleavage and activation of Caspase-1 and IL-1β that in turn lead to a complex network of cellular reactions initiating local and systemic inflammatory reactions. We have recently shown that NLRP3 expression is virtually absent in primary cultured hepatocytes and that *in vitro* the stimulation of hepatocytes with lipopolysaccharides results in strong activation of NLRP3 expression. We here demonstrate that this activation can be blocked by the NF-κB activation inhibitor QNZ or by infection with an adenoviral expression vector constitutively expressing a superrepressor of NF-κB. We show that QNZ blocks NF-κB-dependent expression of TNF-α, IL-1β and NLRP3. Likewise, the superrepressor of NF-κB prevents expression of NLRP3 and significantly reduces expression of inflammatory marker genes in liver cells. In a primary murine hepatoma cells, the concomitant depletion of NEMO and Caspase-8 resulted in a significant suppression of NLRP3 expression after Lipopolysaccharide challenge. Moreover, we demonstrate that a 1.3-kbp fragment located in close proximity of the most upstream transcriptional start site of the human *NLRP3* gene that harbours one putative octamer NF-κB binding site renders LPS sensitivity in reporter gene assay. We conclude that NF-κB signalling is a necessary prerequisite for proper activation of the NLRP3 inflammasome in primary hepatocytes.

1. Introduction

The term inflammasome refers to large cytosolic multiprotein complexes that sense intracellular danger signals *via* NOD-like receptors that recognize pathogen-associated molecular patterns [1]. Upon stimulation with various microbial agents such as lipopolysaccharides (LPS), these platforms induce proteolytic activation of Caspase-1 and processing of proIL-1β [1]. Inflammasome activation is presently thought to be a two-stage activation process in which the NOD-like receptors first recognizes the microbial and danger components, subsequently activating and driving inflammasome expression. This priming step is followed by a second step in which the molecular scaffold is formed that mediates the cleavage of Caspase-1 and ultimately the processing of IL-1β [2,3]. Actually, this concept is mostly accepted for NLRP3 for which it has been shown that NF-κB activators are necessary but not sufficient for NLRP3 activation and that a second stimulus such as ATP is required to activate NLRP3 and to initiate proteolytic processing of proIL-1β in murine macrophages [4]. Other studies that were also performed in macrophages have shown that reactive oxygen species are further needed for NLRP3 activation, activate Caspase-1 activation, and secretion of IL-1β [5]. However, later studies have settled reactive oxygen species upstream of NLRP3 induction but not activation [6].

In regard to inflammatory liver injury it has been shown that inflammasomes have critical roles in the initiation or progression of non-alcoholic fatty liver disease, steatosis and fibrosis [7,8]. Most recently, it was demonstrated that NLRP3 activation results in severe liver inflammation, fibrosis, and hepatocyte pyroptotic cell death [9]. In addition, it was suggested that the deregulation of NLRP3 inflammasome components is involved in the progression of hepatocellular carcinoma [10]. Another report has demonstrated the

Abbreviations: LPS, lipopolysaccharide; QNZ, N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine.

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induction and assembly of NLRP3-inflammasome complexes in human hepatoma cells that were infected with Hepatitis C virus [11].

In a previous study we have analysed inflammasome expression in various primary hepatic cell subpopulations and in experimental models of acute and chronic inflammation and ongoing hepatic fibrogenesis [12]. In summary, we found that NLRP1, NLRP3 and AIM2 expression is prominent in Kupffer cells and liver sinusoidal endothelial cells, moderate in periportal myofibroblasts and hepatic stellate cells, and virtually absent in primary cultured hepatocytes. However, the expression of NLRP3 was strongly upregulated *in vitro* when hepatocytes were stimulated with LPS suggesting an important role of NLRP3 during hepatocytic inflammation [12]. Likewise, the expression of NLRP3 was drastically increased in rat livers after ligation of the common bile duct or repeated application of carbon tetrachloride that both represent experimental models of inflammatory liver injury [12].

We here extended these studies and show that LPS-induced NLRP3 expression in primary hepatocytes is directly linked to activation of the NF-κB pathway and that inhibitors of NF-κB signalling are effective in inhibiting NLRP3 expression in cultured hepatocytes. The impact of NF-κB signalling on NLRP3 expression was also confirmed in a primary murine hepatoma cell line that was depleted for NEMO and Caspase-8. In addition, we identified a DNA stretch upstream of the transcriptional start site of the human *NLRP3* promoter that contains putative NF-κB binding site motif rendering LPS sensitivity in reporter gene assays.

2. Material and methods

2.1. Cell culture and stimulation

Primary hepatocytes were isolated from male C57BL/6 mice according to the collagenase method of Seglen [13]. Animal maintenance and experimentation was approved by the animal welfare committee of the LANUV (Recklinghausen, Germany) and performed according to the Federation for Laboratory Animal Science Associations recommendations. Isolated hepatocytes were plated in collagen-coated 6-well dishes using serum-free HepatoZYME-SFM medium (Life Technologies) and stimulation was performed on the second day after plating. One hour before stimulation, the medium was refreshed and indicated concentrations of LPS (Sigma–Aldrich) or QNZ (Calbiochem) added for indicated time intervals. Thereafter, the cells were harvested for RNA isolation or preparation of protein extracts. Each experiment was repeated three times in total. CFSC-2G cells [14,15] were cultured in medium containing 10% FCS (Perbio Science), 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Cambrex), and 1% nonessential amino acids (Lonza). For NF-κB Western blot analysis, CFSC-2G cells were seeded in 6 well plates and infected with adenoviral constructs for 16 h. The medium was renewed and the cells were incubated for an additional 12 h. Thereafter, the cells were starved overnight (0.5% FCS). On the next day, the serum was reduced to 0.2% FCS and the cells were incubated with indicated combinations and concentrations of LPS (400 ng/ml, Sigma) and QNZ (5 μM, Calbiochem) or left untreated (control). QNZ was given 1 h before LPS stimulation. After LPS/QNZ incubation for 8 h, protein extracts were prepared and subjected to Western blot analysis.

2.2. Generation of primary murine hepatoma cells with floxed NEMO and Caspase-8 genes

For the generation of NEMO and Caspase-8 floxed primary hepatoma cells (TW60) we took advantage of mice with floxed alleles of Caspase-8 and NEMO as recently published [16] and used a recently described protocol [17] with some modifications. Briefly, we injected fourteen-day old male Caspase-8f/fNEMOf/f mice in C57BL/6 background once with 25 mg diethylnitrosamine (Sigma–Aldrich)/kg body weight (i.p.). Forty weeks after treatment, these mice had developed hepatocellular carcinoma nodules. Animals were anesthetized and subjected to collagenase perfusion *via* the portal vein as described before [18] to isolate hepatocytes. In addition, solid tumours were isolated from livers, mechanically minced and incubated for 30 min in a digestion buffer containing collagenase. Hepatocytes and tumour-derived cells were filtered through a 100 μm cell strainer and cultivated for up to 4 weeks in petri dishes containing 20% (v/v) FBS, 10 mg/ml insulin (Novo Nordisk), 10 mg/ml hydrocortisone hemisuccinate, 0.25 mg/ml amphotericin B, 1 mM phenobarbital and 20 ng/ml EGF (Sigma–Aldrich). Afterwards, cells were passaged once a week and frozen at early passages. Cells were cultured in DMEM containing 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% Penicillin/Streptomycin, and 10% FCS.

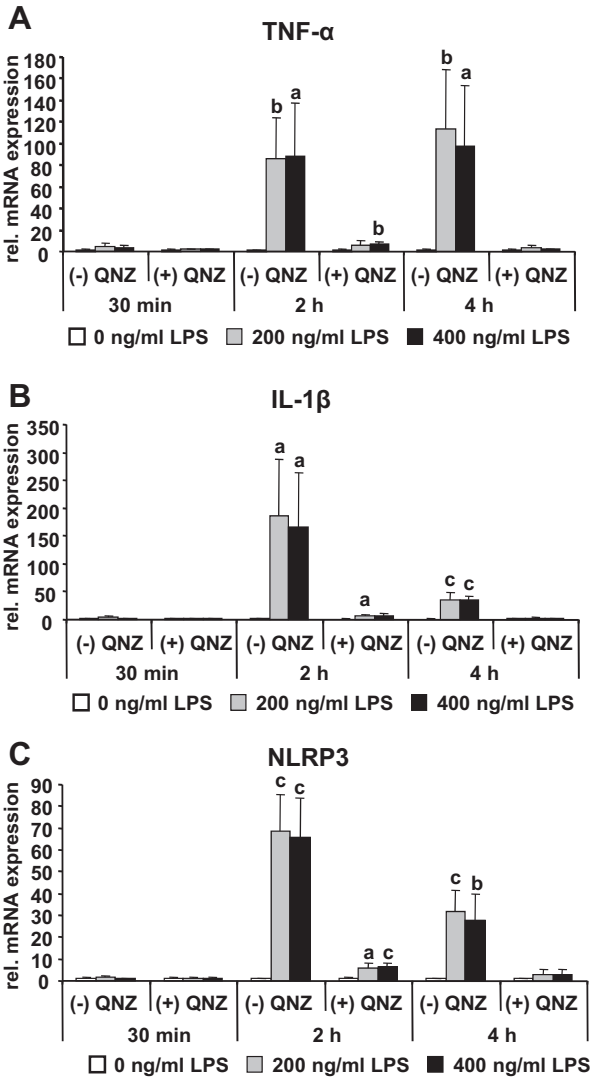

Fig. 1. Expression of the *NLRP3* gene and inflammatory associated genes in hepatocytes after stimulation with LPS. Primary murine hepatocytes were stimulated for 30 min, 2 h or 4 h with indicated concentrations of LPS in the presence (5 μM) or absence of NF-κB activation inhibitor QNZ. RNA was isolated and analysed for expression of TNF-α (A), IL-1β (B), and NLRP3 (C). p-values for significant differences in expression are marked with a ≤ 0.05, b ≤ 0.02, and c ≤ 0.01, respectively. This set of experiments is based on three independent experiments that were each done in replicate.

2.3. RNA isolation and quantitative real time PCR

Purification of total RNA, cDNA synthesis, and TaqMan PCR assays were done using primers and conditions described elsewhere [12]. Normalization of mRNA expression was done to the expression of GAPDH mRNAs.

2.4. Adenoviral infection of primary hepatocytes and murine hepatoma cells

The adenoviral expression vector Ad5-CMV-IκB(S32A/S36A) (Ad5-IκB) containing a hemagglutinin-tagged human super-repressor of NF-κB [19] was a kind gift from David A. Brenner (School of Medicine University of California, San Diego). As an adenoviral control vector, Ad5-Luc expressing the luciferase gene under regulatory control of the CMV promoter was used [20]. For depletion of NEMO and Caspase-8 in primary hepatoma cells (TW60), the serum was reduced to 4% FCS and cells were infected with adenoviral vector VQAdCMVCre/GFP (Ad5-Cre-GFP, Kerafast Inc.) for 28 h prior stimulation with LPS. As controls, cells were infected with the adenoviral vectors Ad-CMV-eGFP (Ad5-CMV-GFP, ViraQuest Inc.) or Ad5-Luc.

2.5. SDS-PAGE and Western blot analysis

Protein extracts were prepared following standard protocols [21]. Proteins were then electro-blotted on nitrocellulose membranes (Schleicher & Schuell). Successful protein transfer and equal protein loading was monitored by Ponceau S stain. Unspecific binding sites were blocked in 1 × TBST [10 mM Tris/HCl, 150 mM NaCl, 0.1% (v/v) Tween 20, (pH 7.6)] containing 5% (w/v) non-fat milk powder. The membranes were subsequently probed with antibodies given in Suppl. Table 1.

2.6. Immunofluorescence in primary hepatocytes

Primary murine hepatocytes were stimulated in the presence or absence of LPS (400 ng/ml) and NF-κB activation inhibitor QNZ (5 μM). Untreated cells served as control in this analysis. After stimulation, the cells were first rinsed with ice-cold phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized in 0.1% sodium citrate plus 0.1% Triton X-100 and finally blocked in 50%FCS/0.5%BSA in PBS. The cells were then subjected to immunofluorescence staining with primary antibody followed by washing with cold PBS three times for 3 min each, and incubated

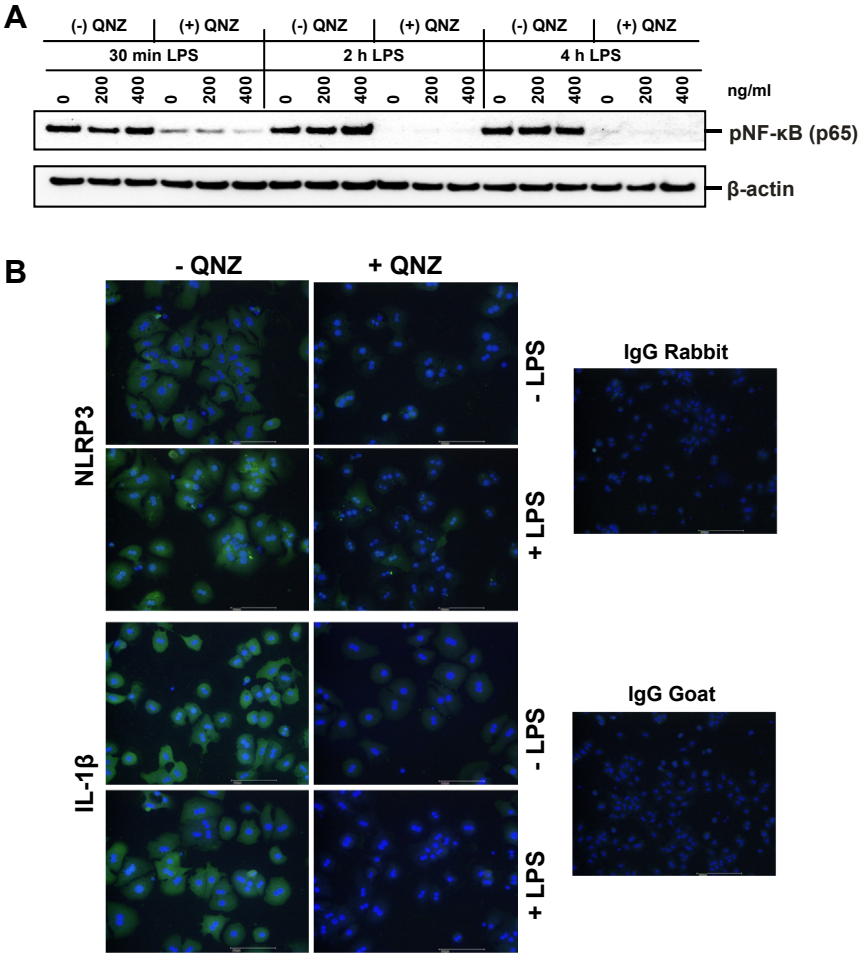

Fig. 2. Activation of NF-κB, NLRP3 and IL-1β after stimulation with LPS. (A) Cells were stimulated with indicated concentrations and combinations of LPS for 30 min, 2 h or 4 h with LPS in the presence (5 μM) or absence of NF-κB activation inhibitor QNZ. Cell protein extracts were prepared and analysed for expression of pNF-κB (p65) in Western blot. Equal protein loading was demonstrated by probing with an antibody specific for β-actin. (B) Primary murine hepatocytes were stimulated in the presence (5 μM) or absence of LPS (400 ng/ml) and NF-κB activation inhibitor QNZ. After fixation, the cells were stained with antibodies specific for NLRP3 and IL-1β. Untreated cells that were not stimulated with LPS or QNZ and stained with unspecific rabbit or goat IgGs served as controls.

with secondary antibody according to the Suppl. Table 1. Subsequently, nuclei were counterstained with DAPI and cells were fixed in Fluorescence Mounting Medium (DAKO).

2.7. Construction of the NLRP3 gene reporter

Details about the cloning of the human *NLRP3* gene reporter are given in the Supplementary Material section.

2.8. Reporter gene assay with QNZ

TW60 cells were plated in 96 well plates in DMEM containing 2.7% FCS and simultaneously transfected with/without reporter gene construct pGL-huNLRP3-Luc or pGL-NF-κB-Luc for 27 h using Lipofectamine™ 2000 (Life Technologies). Medium was changed to 2% FCS for another 20 h. Thereafter, medium was renewed, and when indicated, QNZ added to a final concentration of 20 or 50 nM. After 1 h of preincubation, the cells were finally stimulated with 200 ng/ml LPS for 3 h or left unstimulated. Luciferase activities were determined from eight wells each using commercial kits (Promega) and data were normalized to the DNA concentration in each sample. As a control, a NF-κB activity luciferase gene reporter (pGL-NF-κB-Luc) that contains a 10-bp NF-κB binding site motif (i.e., GGGGACTTTCC) rendering high susceptibility to NF-κB [22] was used as a control.

2.9. Annexin V/PI double staining of mouse hepatoma TW60 cells

Details of apoptosis and necrosis testing are given in the Supplementary Material.

3. Results and discussion

In liver cells, *NLRP3* expression is mainly restricted to Kupffer cells, liver sinusoidal endothelial cells, portal myofibroblasts, and hepatic stellate cells. However, the expression of *NLRP3* is strongly induced after LPS treatment LPS [12]. Hepatic expression is also activated during experimentally-induced inflammatory liver injury [12] and intraperitoneal injection of LPS or a mixture of D-Galactosamine and LPS [23,24]. LPS is a prototypical ligand that is recognized by the Toll-like receptor 4 playing a critical regulatory role in ongoing hepatic injury [25]. This cytokine triggers production of pro-inflammatory cytokines such as TNF-α *via* the NF-κB signalling pathway and NF-κB-controlled genes [26–28]. In the liver this receptor is expressed not only on innate immune cells such as Kupffer cells and infiltrating macrophages, but also on sinusoidal endothelial cells, hepatic stellate cells, biliary epithelial cells and hepatocytes [29,30]. In murine macrophages it has been further demonstrated that the NF-κB dependent activation of NLRP3 expression after induction with TLR agonists is mediated through binding of NF-κB to NF-κB binding sequences in the *NLRP3* promoter [31].

Therefore, we asked if our previous findings in regard to elevated expression of NLRP3 after stimulation with LPS in primary hepatocytes are the direct consequence of NF-κB activation and if the blockade of NF-κB signalling is sufficient to blunt NLRP3 expression. To do so, we first stimulated primary hepatocytes in culture with LPS in the absence or presence of QNZ which is a small synthetic aminoquinazoline component inhibiting the activation of the transcription factor NF-κB [32]. After addition of LPS at concentrations of 200 or 400 ng/ml for 2 or 4 h, we found a strong induction of TNF-α and IL-1β mRNA expression that was in both cases suppressed in the presence of QNZ (Fig. 1A and B). Similarly, the elevated expression of NLRP3 after addition of LPS was significantly blocked in the presence of QNZ (Fig. 1C). The finding that NLRP3 is highly significantly induced after the addition of LPS favours the concept that LPS first triggers an unique priming reaction of the NLRP3 inflammasome that was previously supposed in macrophages [3]. To demonstrate that the observed induction of NLRP3 expression after LPS treatment collaborates with and is caused by increased activation of the NF-κB signalling pathway in hepatocytes, we next analysed the activation status of NF-κB p65 (Ser536) by Western blot analysis. Interestingly, this analysis revealed that NF-κB p65 was already fully activated without addition of LPS and as expected inhibited by the NF-κB inhibitor QNZ (Fig. 2A). One potential explanation might be trace amount of TNF-α released by contaminating Kupffer cells in the hepatocyte culture leading to persistent activation of NF-κB. The inhibition of NF-κB phosphorylation by QNZ corroborated with a lower expression of NLRP3 and IL-1β (Fig. 2B). Previous reports have demonstrated that the phosphorylation at Ser536 is crucial for nuclear translocation of NF-κB and required for maximal transcriptional activity of NF-κB [33]. Based on these experiments we concluded that LPS-induced expression of NLRP3 in hepatocytes is mediated through NF-κB signalling but does not require enhancement of phosphorylation at serine 536 of NF-κB p65 that is essential for nuclear translocation. In this regard it is noteworthy that previous studies have suggested that the phosphorylation of Ser536 is directly dependent on TNF-α and necessitate phosphorylation of IκB proteins that is mandatory to degrade these inhibitory proteins and to fully activate NF-κB signalling [34,35]. Therefore, we speculated that the major trigger in hepatocytes for NLRP3 expression after LPS treatment is the inactivation of the IkB proteins. To prove our hypothesis, we took advantage of a superrepressor form of IκBα that contains serine-to-alanine mutations at amino acids 32 and 36 which prevent signal-induced phosphorylation at the site which is necessary for proteosomal degradation and inhibition of IκBα [19]. This superrepressor effectively blocks NF-κB signalling *in vitro* and *in vivo* and is resistant to IL-1β-induced degradation [19,36–38]. However, since this superrepressor of NF-κB signalling potentially induces increased apoptosis and cell cycle arrest that might interfere with

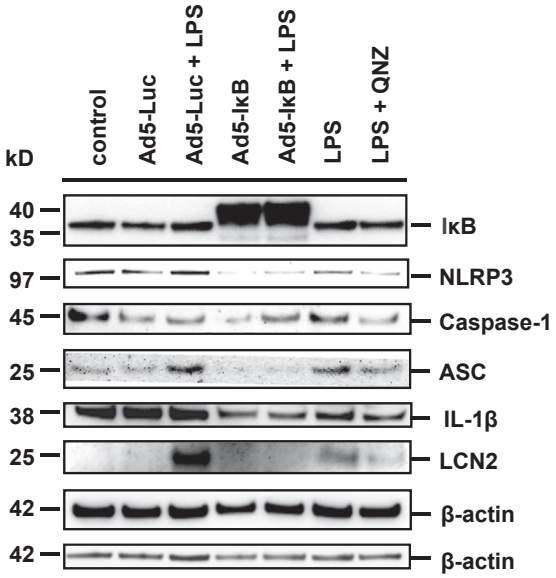

Fig. 3. Inhibitory effect of NF-κB human superrepressor on NLRP3 inflammasome expression. Immortalized hepatic stellate cells (i.e. CFSC) were incubated with indicated combinations of adenoviral expression vectors (Ad5-CMV-Luc, Ad5-CMV-IκB), LPS (400 ng/ml) and QNZ (5 μM) or left untreated (control). After the treatment (see Material and Methods for details), protein extracts were prepared and subjected to Western blot analysis using antibodies specific for IκB, NLRP3, Caspase-1, ASC, IL-1β, and LCN2. Equal protein loading was demonstrated by subsequent probing with an antibody specific for β-actin.

expression of NLRP3, we decided to use this adenoviral vector in cell line CFSC-2G that represents an immortalised rat liver fat-storing cell obtained from a CCl_4-cirrhotic rat [14,15]. This cell line was previously used by us for studies on inflammasome activation after LPS challenge [12]. When we infected the cells with this superrepressor prior to incubation with LPS, we found that the expression of NLRP3 was significantly reduced (Fig. 3). Even more, the basal expression that was observed in uninfected control cells or in cells that were infected with a control virus (Ad5-CMV-Luc) was abrogated. Likewise, the expression of NLRP3 in cells that were treated with QNZ was blocked. Similarly, the expression of basal and LPS-induced ASC expression was blunted in cells that were infected with the superrepressor. This finding is somewhat surprising since recent reports conversely demonstrated that phosphorylated IκB kinase α/β expression and NF-κB activity is modulated after ASC knockdown [39]. Since ASC is an activating adaptor that mediates NF-κB activation by direct binding to NF-κB [40] the lowering of ASC in our experiments might be potentially a direct consequence of increased ASC degradation occurring in the presence of high cellular quantities of biological inactive IκB. In line with this assumption is the finding that the treatment with QNZ was unable to affect ASC expression. The blockade of LPS-induced inflammatory signalling by the IκB superrepressor was also demonstrated by decreased expression of Lipocalin 2 (LCN2), a biomarker that is induced in liver cells by the pro-inflammatory cytokine IL-1β *via* the NF-κB pathway [20,41,42].

For a next set of experiments, we generated a novel primary hepatoma cell line in which both the NF-κB essential modulator (NEMO) that has been implicated in NF-κB activation and the apical initiator Caspase-8 were floxed. Previous studies have shown that the loss of Caspase-8 protects against LPS-driven apoptotic liver injury [16] and that NEMO regulates cellular responses to inflammation [43]. Following cre-mediated deletion of Caspase-8 and NEMO using an adenoviral approach (Ad5-Cre-GFP), the challenge with LPS resulted in a highly significant decrease of NLRP3 expression compared to control cells ($p \leq 0.003$ at time point 4 h) (Fig. 4A). Similarly, the expression of IL-1β ($p \leq 0.003$ at time point 4 h) and TNF-α ($p \leq 0.000002$) were significantly blunted in cells that lacked NEMO and Caspase-8 (Fig. 4B and C) suggesting that NF-κB signalling is a key component in regulating expression of these genes in cells of hepatocytic origin. Western blot confirmed that the infection of the cells with the Cre adenovirus resulted in down-regulation of both IKKγ (NEMO) and Caspase-8 (Suppl. Figure 1). To demonstrate that the observed differences in expression of NLRP3, IL-1β, and TNF-α were not caused by toxic effects of QNZ, we determined levels of apoptosis and necrosis in these cells showing that the cells stayed viable under the chosen experimental conditions (Suppl. Figure 2). Likewise, adenoviral infection did not alter cellular apoptosis in the time interval that we have chosen in our experiments.

In line with the finding that the lack of NEMO and Caspase-8 results in transcriptional downregulation of NLRP3, the important

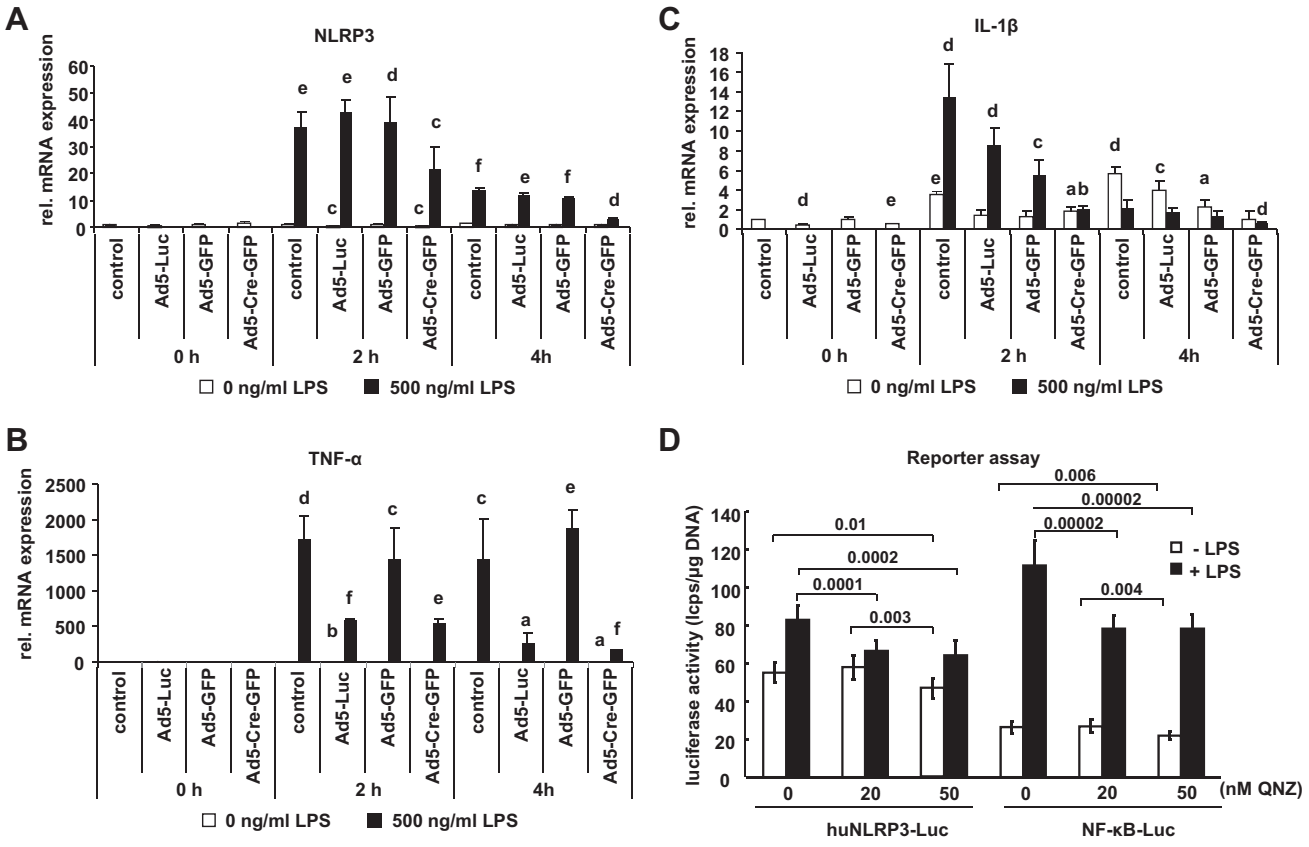

Fig. 4. Expression of TNF-α, IL-1β and NLRP3 in murine hepatoma cells lacking NEMO and Caspase-8 after LPS stimulation. Murine hepatoma cells (TW60) were depleted for NEMO and Caspase-8 using an adenoviral vector that expresses Cre recombinase. Cells were then incubated with 500 ng/mL LPS for indicated time intervals or left unchallenged. (A–C) RNA from respective cells was extracted and the expression of NLRP3 (A), TNF-α (B), and IL-1β (C) analysed by qRT-PCR. In this analysis ($n = 3$) the expression of the different genes was compared to that observed in uninfected control cells that were not challenged with LPS. All values were compared to the normal control at time point 0 h without addition of LPS. p-values are: $a \leq 0.05$, $b \leq 0.02$, $c \leq 0.01$, $d \leq 0.003$, $e \leq 0.0003$, and $f \leq 0.000002$, respectively. (D) TW60 cells were transfected with reporter huNLRP3-Luc or NF-κB-Luc gene reporters ($n = 8$). The cells were then stimulated with LPS in the presence or absence of QNZ. Cell extracts were prepared and the luciferase activity measured. Significant differences (p-values) for QNZ inhibition are indicated.

role of Caspase-8 for Toll-like-receptor-induced inflammasome priming and cytokine production *in vivo* was previously shown in mice that were challenged with LPS [44].

To understand possible transcriptional control mechanisms in NF-κB controlled expression of NLRP3, we next cloned a reporter gene construct comprising region −3968 to −2625 relative to the translational start site (ATG) of the human *NLRP3* gene (Suppl. Figure 3). In our view this region that surrounds the most upstream located transcriptional start site of human *NLRP3* [45,46] is potentially of fundamental importance for NLRP3 regulation in humans. Sequence analysis for putative transcription factor binding sites that was done with the online P-Match software (http://www.gene-regulation.com/pub/programs.html) revealed that this fragment contains a putative NF-κB binding site (−2755 to −2746).

The respective gene promoter is fully active in diverse cells of human, rat and mouse origin (not shown). We found that the activity of the respective gene reporter in the TW60 cell line is activated after stimulation with LPS ($p = 4.6 \times 10^{-7}$) with similar tendency to a reporter construct that solely contains the classical NF-κB binding motif ($p = 5.4 \times 10^{-11}$) (Fig. 4D). When we measured the LPS-induced luciferase activity after preincubation with QNZ, we found a significant reduction of the signal in a trend equally to that observed in cells transfected with the NF-κB activity reporter. This result further suggests that there is a close link of NF-κB and *NLRP3* expression.

A close link between NF-κB and NLRP3 expression was also recently suggested in diet-induced adipose tissue inflammation in which Isoliquiritigenin (i.e., a chalcone from *Glycyrrhiza uralensis*) that inhibits LPS-induced NF-κB activation was shown to significantly inhibit NLRP3 inflammasome activation [47].

In summary, our study demonstrates that NF-κB is a key regulator pathway that regulates NLRP3 expression in hepatocytes. Although we do not know if the impact of NF-κB on NLRP3 expression is specific for hepatocytes, it demonstrates that NF-κB plays an important role in mediating inflammasome activity. Therefore, the inhibition of NF-κB activity appears to be a potential new therapeutic strategy for suppression of overshooting inflammatory reactions within the liver that are a basic part of the development of hepatocellular carcinoma.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.029.

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