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Nucling, a novel protein associated with NF-κB, regulates endotoxin-induced apoptosis *in vivo*

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Sun Mi Kim[1], Takashi Sakai[1], Huy Van Dang[1], Nam Hoang Tran[1,*], Koji Ono[2], Kazunori Ishimura[2] and Kiyoshi Fukui[1,†]

[1]Division of Enzyme Pathophysiology, The Institute for Enzyme Research (KOSOKEN); and [2]Department of Anatomy and Cell Biology, Institute of Health Biosciences, The University of Tokushima, Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

*Present address: Nam Hoang Tran, Department of Maternal and Child Health, Ministry of Health, 138A Giang Vo, Hanoi, Vietnam

†Kiyoshi Fukui, Division of Enzyme Pathophysiology, The Institute for Enzyme Research (KOSOKEN), The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Tel: +81-88-633-7430, Fax: +81-88-633-7431, email: kiyo@ier.tokushima-u.ac.jp

Nucling is a proapoptotic protein that regulates the apoptosome and nuclear factor-kappa B (NF-κB) signalling pathways. Strong stimuli, such as Gram-negative bacterial lipopolysaccharide (LPS), induce the simultaneous secretion of cytokines following the activation of NF-κB. Proinflammatory cytokines can induce liver damage through several mechanisms such as increases in oxidative stress and apoptotic reactions leading to tissue necrosis. Herein, we show that Nucling-knockout (KO) mice are resistant to LPS that consistently caused mortality in wild-type (WT) counterparts. Although serum levels of cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 did not differ significantly between WT and Nucling-KO mice after the LPS challenge, hepatocytes of Nucling-KO mice were refractory to LPS- or TNF-α-induced cell death. These results were consistent with the decreased expression of proapoptotic proteins including apoptosis-inducing factor and cleaved form of poly (ADP-ribose) polymerase and terminal deoxynucleotidyl transferase dUTP nick end-labelling positive cells in the liver of Nucling-KO mice after the administration of a lethal dose of LPS. Moreover, the upregulation of NF-κB-regulated anti-apoptotic molecules including cellular inhibitor of apoptosis (cIAP) 1 and cIAP2 was observed in the liver of Nucling-KO mice after LPS treatment. These findings indicate that the Nucling deficiency leads to resistance to apoptosis in liver. We propose that Nucling is important for the induction of apoptosis in cells damaged by cytotoxic stressors through the NF-κB signalling pathway.

Keywords: apoptosis/hepatocyte/lipopolysaccharide/nucling/NF-κB.

Abbreviations: AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease-activating factor-1; cIAP, cellular inhibitor of apoptosis; COX-2, cyclooxygenase-2; DAPI, 4′, 6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IL, interleukin; iNOS, inducible nitric oxide synthase; KO, knockout; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; RT, room temperature; SDS, sodium dodecyl sulphate; TNF-α, tumour necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; WT, wild-type.

The nuclear factor-kappa B (NF-κB) signalling pathway mediates a variety of important cellular functions by regulating immune and inflammatory responses (*1*). Besides its role in the immune system, NF-κB also acts broadly to influence gene expression events that impact cell survival, differentiation, proliferation and apoptosis. As a result of such broad effects on physiology, the dysregulation of NF-κB can have severe consequences, including the development of numerous diseases (*2*). Recent studies have suggested that NF-κB activation is required to protect the adult liver from acute injury caused by an *in vivo* challenge with tumour necrosis factor (TNF)-α or lipopolysaccharide (LPS) (*3*). In hepatocytes, activation of NF-κB is induced by TNF-α and constitutes an anti-apoptotic signal, which counterbalances the proapoptotic branch of TNF-α-dependent signalling initiated by activation of caspase-8 (*4*).

Apoptosis is a cellular, self-destruction mechanism involved in a variety of biological events such as development remodelling, tissue homeostasis and removal of unwanted cells in most living tissues (*5*). Apoptosis plays a role in the pathogenesis of fulminant hepatic failure, hepatitis, organ dysfunction after liver transplantation and liver ischemia/reperfusion injury (*6*). Hepatocyte apoptosis can be initiated by exposure to toxic substances or ligation of members of the death receptor family including the TNF-α receptor and Fas (*7*). Apoptosis controlled by such death receptor pairs can cause tissue destruction. In contrast, the binding of TNF-α to the TNF receptor potentially initiates apoptosis and activates NF-κB, which suppresses apoptosis by inducing the expression of NF-κB-responsive genes, including those for TRAF1, TRAF2 and the inhibitor of apoptosis proteins (*8*). Therefore, identification of proapoptotic and anti-apoptotic pathways in death receptor–mediated hepatocyte apoptosis would help

to elucidate the pathophysiological role of apoptosis in liver diseases.

LPS, a strong activator of NF-κB, is one of the best studied immunostimulatory components of bacteria and can induce systemic inflammation and sepsis with a high mortality if excessive signals occur (*9*, *10*). During the course of sepsis, the production of proinflammatory cytokines such as TNF-α leads to systemic inflammatory response syndrome (*11*). The liver is the main organ that clears LPS, and the hepatocyte is a major cell type involved in the uptake of LPS (*10*). The liver is exposed to many potentially harmful agents that, under normal conditions, do not damage liver cells due to protective mechanisms and the large repair capacity of these cells. However, acute or chronic exposure to insults such as cytokines, reactive oxygen species and bile acids results in disturbed liver function (*12*). It has become increasingly evident that potent activating stimuli such as bacterial LPS induce the simultaneous secretion of cytokines, for example, TNF-α, interleukin (IL)-1α, IL-1β, IL-6 and TGF-β, frequently from the same cells, for example, macrophages in several different tissues. These proinflammatory cytokines may be detrimental to the liver in several ways including the production of oxidative stress (by TNF-α), apoptosis (by TNF-α and TGF-β) and mitochondrial dysfunction (by TNF-α), thereby leading to tissue necrosis.

Nucling is a stress-inducible protein identified in murine embryonal carcinoma cells (*13*). It is associated with the mitochondrial apoptotic pathway regulated by apoptosomes composed of cytochrome *c*, apoptotic protease-activating factor (Apaf)-1 and caspase-9 (*14*). NF-κB is spontaneously activated in tissues and cells prepared from Nucling-knockout (KO) mice (*15*). The activation of NF-κB plays a physiological role in cell proliferation and inflammation in animals. In addition, we have also reported that Nucling mediates apoptosis by inhibiting the expression of galectin-3 (*16*), an anti-apoptotic factor, through interference with NF-κB signalling pathway (*17*). Nucling is also associated with the apoptosome pathway by recruiting the Apaf-1/procaspase-9 complex following cytotoxic stress (*14*). However, Nucling-KO mice did not show any developmental abnormalities common to Apaf-1- or caspase-deficient mice because of the defect in apoptosis. Accordingly, the actual role of Nucling in the regulatory system of apoptosis in living organisms is still unclear. Conversely, Nucling-KO mice tend to suffer from inflammatory disorders including liver cancer (*15*). Cancer is one of the scenarios where too little apoptosis occurs, resulting in malignant cells that will not die (*18*). Thus, we hypothesized that a defect of apoptosis may be one of the factors in the development of liver cancer in Nucling-KO mice. To test this, we examined the effectiveness of Nucling deficiency against apoptotic stress *in vivo*. We introduced cytotoxic stress into the liver of Nucling-KO mice and their wild-type (WT) counterparts by injecting LPS.

In this report, we show that Nucling-KO mice are resistant to LPS. The objective of this study is thus to clarify the physiological roles of Nucling in LPS-induced inflammatory reactions. Specifically, we speculated that Nucling deficiency might have induced resistance of liver against cytotoxic stresses through the impairment of apoptosis regulated by apoptosome or NF-κB signalling pathway. To test this hypothesis, we performed experiments (i) to determine the time courses of proinflammatory cytokine secretion and of apoptotic features in Nucling-KO mice stimulated with LPS, (ii) to establish the proapoptotic effect of Nucling *in vivo* in the case of LPS-induced liver apoptosis and (iii) to check the expression levels of NF-κB-regulated inflammatory genes in the livers of LPS-treated mice.

Materials and Methods

Animal experiments and tissue preparation

WT and Nucling-KO mice were prepared as described (*14*, *15*). Age-matched male mice between 8 and 12 weeks old were used for all experiments. Mice were maintained at five per polycarbonate cage on hardwood chips bedding. All mice were maintained under specific pathogen-free conditions. All procedures used throughout this study were according to the Japan guidelines for animal care and approved by the Animal Care and Use Committee in the University of Tokushima (Permit number: 11093). Mice were killed with a lethal dose of anaesthetic (sodium pentobarbital), and all efforts were made to minimize suffering. Liver samples were removed and snap-frozen for protein analyses. Mice were perfused through the left ventricle with 10 ml of cold phosphate-buffered saline (PBS), followed by 25 ml of 4% paraformaldehyde in PBS. The liver was removed, weighed and fixed in 4% paraformaldehyde overnight. The next day the entire liver was embedded in paraffin in cassettes.

Application of LPS in vivo

LPS from *Escherichia coli* O127:B8 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in PBS. LPS (20 mg/kg body weight) was injected intraperitoneally. Seventeen WT and 19 Nucling-KO mice were used in each group. Mortality was monitored for 6 days after the LPS challenge.

ELISA for TNF-α

Blood samples were obtained from the retro-orbital sinus at the indicated time after the LPS injection (20 mg/kg body weight). Serum samples were collected by centrifugation (200*g* for 20 min) and used immediately. TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA) (Mouse TNF alpha ELISA Ready-SET-Go, eBioscience, San Diego, CA, USA). The assays were performed according to the manufacturer's instructions. Experiments were performed in three mice per group.

Isolation and cultivation of primary mouse hepatocytes and Kupffer cells

Mice were fasted 16–20 h before experimentation and allowed water ad libitum. *In situ* perfusion of livers with collagenase, followed by differential centrifugation of the cellular suspension, was used to isolate hepatocytes and Kupffer cells from mice, as described previously (*15*). Hepatocytes and Kupffer cells were resuspended in complete RPMI 1640 medium (Sigma) containing 10% fetal calf serum (FCS; Hyclone, UT, USA), penicillin (100 U/ml), streptomycin sulphate (100 μg/ml), 2 mM of glutamine (Gibco, NY, USA), 28 mM of sodium bicarbonate, 8 mM of HEPES (Sigma) and 0.16 U/ml of insulin (Sigma) at 37°C in a humidified 5% CO_2 condition. The viability of hepatocyte was ~80% as determined by trypan blue exclusion. Kupffer cells were allowed to adhere to 24-well plates (BD Falcon, CA, USA) at 37°C in a humidified 5% CO_2 condition for the further purification. This procedure usually yields more than 90% Kupffer cells.

Measurement of hepatocyte damage represented by the release of lactate dehydrogenase

Isolated hepatocytes were cultured for 12 h and then resuspended with fresh RPMI 1640 medium (containing 1% FCS). Aliquots of

250 µl of hepatocytes (2×10^5 cells/ml) were seeded in 24-well, flat-bottom plates with or without Kupffer cells, followed by the incubation with 250 µl of medium containing LPS (100 ng/ml final concentration, Sigma) or TNF-α (25 ng/ml final concentration, Sigma) for 6 and 12 h.

Lactate dehydrogenase (LDH) activities in the supernatant of hepatocyte cultures were measured according to the procedures of LDH Cytotoxicity Detection Kit (TAKARA, Japan). Detection of LDH activity was performed on a spectrophotometer (infinite M200, TECAN, Austria) at 490 nm. The results of LDH activity were expressed as percentage compared with that in supernatant of Triton X-100–treated control.

Antibodies

Antibodies against cellular inhibitor of apoptosis (cIAP) 1 and NF-κB p65 were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies specific to cIAP2 (R&D systems, Minneapolis, MN, USA), β-actin (Sigma), poly (ADP-ribose) polymerase (PARP; Abcam, Japan), apoptosis-inducing factor (AIF; MBL International, Woburn, MA, USA), Apaf-1 (Chemicon international, Billerica, MA, USA) and the middle portion of Nucling (Nucl.mid) (*13*) were also used. Horseradish peroxidase–conjugated secondary antibodies were purchased from Amersham Biosciences (UK). The Alexa 488-labelled secondary antibody was from Molecular Probes (Tokyo, Japan).

Western blot analysis

Western blotting was performed according to standard procedures. All antibodies were used at recommended dilutions. Briefly, whole liver cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride) with proteinase inhibitor cocktail (Roche Diagnostics, Tokyo, Japan) and phosphatase inhibitor (Nacalai Tesque, Kyoto, Japan). The protein concentrations of the extracts were estimated using a BCA kit (Pierce, Rockford, IL, USA). Fifty micrograms of total protein was fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked for 2 h with PBS containing 5% non-fat dry milk and 0.05% Tween-20 and then incubated with appropriate antibodies in blocking buffer overnight. Bound primary antibodies were detected by incubating with horseradish peroxidase–conjugated secondary antibodies. The membrane was developed using ECL reagent (Amersham-Pharmacia). Quantification was performed by comparing densitometric scanning readings using NIH Image J 1.36b software (National Institutes of Health); numbers represent values corrected for loading with the data obtained by reprobing with β-actin. Nuclear extracts from whole liver cells of mouse were prepared as described previously (*19*), and then western blotting was carried out according to standard procedures.

TUNEL assay

Paraffin-embedded sections were de-waxed in xylene and rehydrated by passage through a graded series of ethanol solutions, finally with PBS. Slides were permeabilized with proteinase K (20 µg/ml in 10 mM Tris–HCl, pH 7.6) at 37°C for 15 min. After being washed with PBS, slides were stained using an In Situ Cell Death Detection kit, Fluorescein (Roche), according to the manufacturer's instructions. Following counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI), slides were viewed and scanned using a Nanozoomer (Hamamatsu, Japan).

Immunohistochemistry

Sections were de-waxed in xylene and hydrated through a graded series of ethanol solutions. Slides were permeabilized with proteinase K (20 µg/ml in 10 mM Tris–HCl, pH 7.6) at 37°C for 15 min, washed and incubated with 3% goat serum for 1 h at room temperature (RT) in a moist chamber for blocking. After being washed with PBS, they were incubated with anti-AIF antibody diluted 1:100 in 3% goat serum for 1 h at RT and then with Alexa 488-labelled secondary antibody diluted in 3% goat serum for 1 h at RT. Slides were washed in PBS three times and mounted with aqueous mounting medium. After being counterstained with DAPI, they were viewed and scanned using a Nanozoomer (Hamamatsu, Japan).

Real-time reverse-transcription polymerase chain reaction

Total RNA was extracted from the liver in Nucling-KO or WT mice using TRIZOL® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA synthesis was performed using a Superscript® III First-Strand Synthesis System for reverse-transcription polymerase chain reaction (Invitrogen), according to the manufacturer's instructions. Transcript levels of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and GAPDH were examined using a 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR® Premix Ex Taq™ II (Takara). The following primers were used: for COX-2, 5′-AAAACCGTGGGGAATGTATGAGC-3 and 5′-GATGGGTGAAG TGCTGGGCAAAG-3′; for iNOS, 5′-ACCC TCCTTGTTCAACTCAC-3′ and 5′-CTTCTGTCTCTCCAAACC CT-3′ and for GAPDH, 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′.

Results

Nucling-KO mice were resistant to the endotoxic effects of LPS

To elucidate the physiological importance of Nucling to the regulation of NF-κB signalling, we investigated the response of Nucling-KO mice to LPS, a strong activator of NF-κB. WT mice showed severe septic symptoms (*e.g.* reduced mobility, conjunctivitis, diarrhoea and fur ruffling) when administered LPS, with 59% ($n = 17$) dying within 6 days (Fig. 1). Nucling-KO mice demonstrated a significantly improved survival rate after the LPS injection compared with WT mice. One mouse died but all the others ($n = 18$) survived. This result indicated that the Nucling-KO mouse has resistance to LPS.

Nucling-KO mice showed similar pattern of cytokine release after LPS stimulation

Administration of LPS induces endotoxin shock through the release of proinflammatory cytokines including TNF-α, IL-1β and IL-6, which leads to cellular responses such as apoptosis or the activation of NF-κB. Because the number of Kupffer cells, macrophages in liver, was apoptotically decreased in

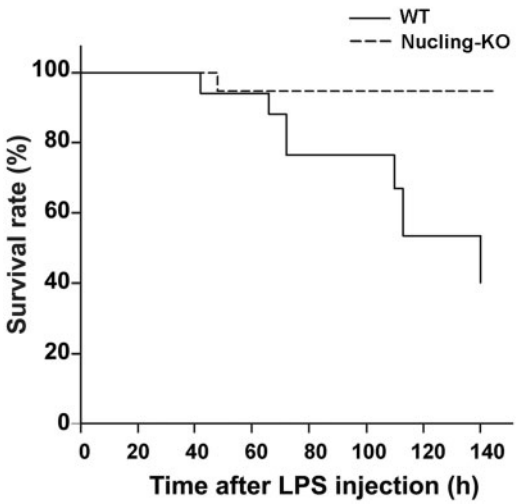

Fig. 1 Nucling-KO mice are resistant to the lethal effects of LPS. Kaplan–Meier survival curves in LPS-treated WT (solid line, $n = 17$) and Nucling-KO (dotted line, $n = 19$) mice are shown. Mice were injected intraperitoneally with LPS (20 mg/kg). Lethality was investigated for 6 days. Percent survival was determined using a Sigma Plot ($P = 0.017$ by the log-rank test).

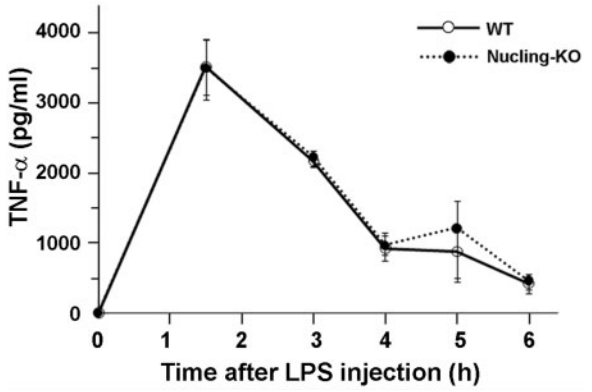

Fig. 2 LPS induces the same level of TNF-α production in WT and Nucling-KO mice. Time-dependent expression levels of TNF-α were measured in sera of WT (open circles) and Nucling-KO (closed circles) mice receiving LPS over the 6-h time period. There were no significant differences in serum TNF-α levels between the WT and Nucling-KO mice after LPS treatment. TNF-α concentrations of the control group treated with PBS were below the detection limit.

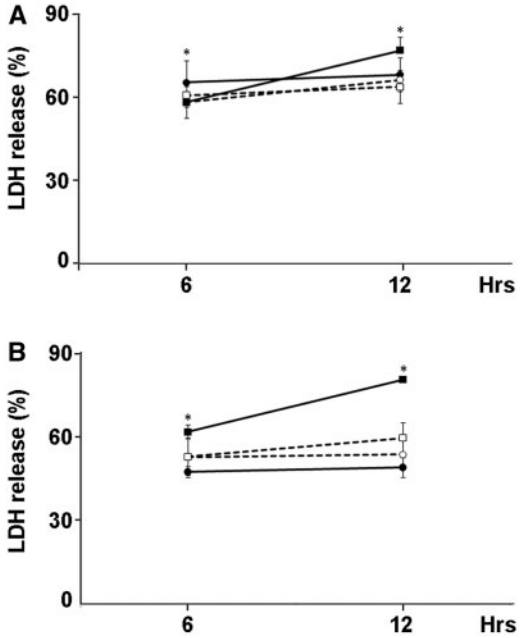

Fig. 3 Primary hepatocytes are resistant to LPS- and TNF-α induced cell death in Nucling-KO mice. LDH release from hepatocyte cultures (closed circle, WT and open circle, Nucling-KO) and Kupffer cell–hepatocyte cocultures (closed square, WT and open square, Nucling-KO) were measured after incubation with LPS (A, 100 ng/ml) or TNF-α (B, 25 ng/ml). Percentage of LDH release was compared with that in supernatant of Triton X-100–treated control. Data are means ± SD. from triplicate determinations. *Statistically significant difference in LDH release between WT and Nucling-KO induced by LPS or TNF-α ($P < 0.05$ by Student's *t*-test).

Nucling-KO mice (*15*), we postulated that Nucling-KO mice might show milder inflammatory responses to LPS than WT. Therefore, we measured the serum levels of cytokines from mice treated with LPS and with PBS as a control. Unexpectedly, LPS treatment markedly increased serum levels of TNF-α even in Nucling-KO mice as observed in WT mice (Fig. 2). No significant differences in the levels of IL-1β and IL-6 were observed between WT and Nucling-KO mice (Supplementary Figs S1 and S2). These results indicated that the tolerance observed in Nucling-KO mice was not due to the reduced levels of cytokines released after the injection of LPS.

Nucling-KO mice were resistant to LPS- and TNF-α- induced hepatocyte damage

We next focused on the liver as a target organ to elucidate the basis for the LPS resistance. The hepatotoxicity is mainly dependent on the production and release of inflammatory mediators such as TNF-α, IL-1β and IL-6. These immunological mediators, especially TNF-α, can induce apoptotic liver injury and the infiltration of inflammatory cells (*20*). LPS (Fig. 3A) and TNF-α (Fig. 3B) induced significant elevations of LDH release in Kupffer cell–hepatocyte coculture of WT mice. Conversely, LDH release was not increased by LPS and TNF-α in Nucling-KO, indicating that Nucling-KO exhibited the resistance to LPS- and TNF-α-induced hepatocyte damage.

LPS-induced apoptosis was attenuated in the Nucling-KO mouse liver

To confirm the resistance to TNF-α-induced cytotoxicity in Nucling-KO mouse liver, we performed Terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) staining to assess the level of apoptosis. As shown in Fig. 4A, Nucling-KO mice had lower levels of TUNEL staining than WT mice. This result was consistent with AIF immunostaining of liver sections. In Nucling-KO mouse liver 24 h after the injection of LPS, staining for AIF was almost undetectable in the nucleus, whereas WT showed apoptosis with positive immunostaining for AIF in the nucleus (Fig. 4B). Although the expression level of AIF in whole liver extracts was almost the same level in both WT and Nucling-KO mice regardless of LPS injection (data not shown). These observations indicate that LPS-induced apoptosis was attenuated in the Nucling-KO mouse liver.

Expression levels of apoptosis-associated proteins, regulated by NF-κB, were altered in liver of Nucling-KO mice

To examine the involvement of apoptosis-regulating proteins in the LPS-induced apoptosis, we investigated the expression of anti-apoptotic proteins in the liver after the LPS injection. Levels of cIAP1 and cIAP2, which are required to protect against TNFα-induced apoptosis, were increased in the liver of Nucling-KO mice (Fig. 5A and B).

Next, we investigated the expression of proapoptotic proteins after the LPS challenge in the nuclear fraction of the liver. PARP is an abundant nuclear enzyme and main substrate for caspase-3. Suppression of PARP cleavage was observed in the nuclear fraction of the Nucling-KO mouse liver (Fig. 5C). In addition, we also investigated the expression of Apaf-1, which forms the molecular core of the apoptosome with caspase-9, mediating the so-called mitochondrial pathway of cell death. As shown in Fig. 5D, Nucling-KO mice showed no upregulation of Apaf-1 expression

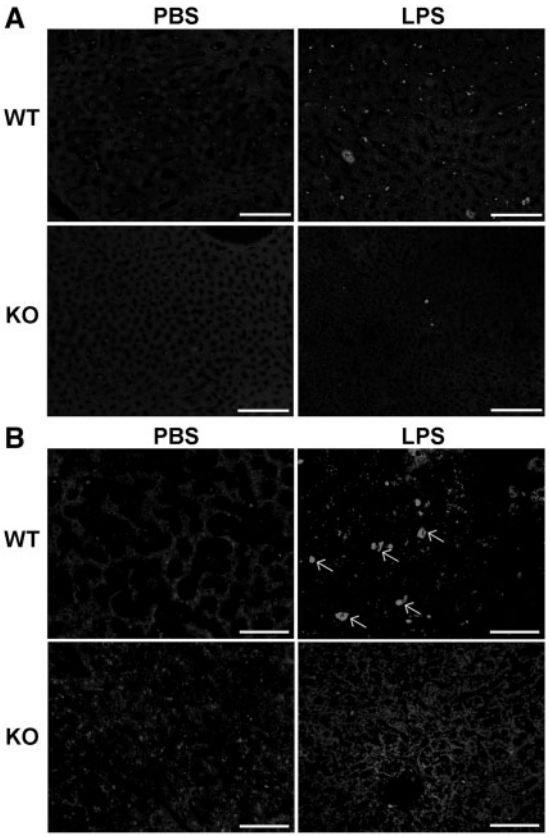

Fig. 4 LPS induces liver cell death in WT but not in Nucling-KO mice. (**A**) Representative TUNEL staining of liver sections 24 h after LPS stimulation showed apoptosis in WT but not in Nucling-KO (KO) mice. WT and Nucling-KO mice were treated with PBS as a control (bar = 100 μm). (**B**) Immunostaining for AIF in the nucleus (arrow) showed apoptosis in WT but not in Nucling-KO mice (bar = 50 μm).

following LPS stimulation. In addition, NF-κB p65 (Fig. 5E) was detected in the nuclear fraction of the liver from Nucling-KO mouse with or without LPS administration. Cell survival in response to TNF-α and LPS depends on proper NF-κB activation following the translocation of NF-κB to the nucleus. We speculated that the constitutive activation of NF-κB in Nucling-KO mice was the reason for the attenuated apoptosis. To confirm the constitutive activation of NF-κB in Nucling-KO mice, we investigated the expression levels of NF-κB target genes, COX-2, and iNOS in the liver. As we expected, both transcripts were upregulated in the livers of Nucling-KO mice compared with those of WT (Fig. 6A). We also confirmed that LPS stimuli upregulated these inflammatory genes with the increase of Nucling protein in the livers of WT following LPS treatment (Fig. 6A and B).

Discussion

Our studies show that Nucling-KO mice are less sensitive to LPS than WT mice. This is consistent with the finding that Nucling-KO mice showed anti-apoptotic characteristics in the liver after the administration of LPS. We propose that the reason for the resistance of Nucling-KO mice is the constitutive upregulation of NF-κB expression.

Activation of NF-κB is a tightly regulated event. In normal cells, NF-κB becomes activated only after the appropriate stimulation and then upregulates the transcription of its target genes (*21*). NF-κB is activated in response to various stimuli, including cytokines, infectious agents, injury and other stressful conditions requiring a rapid reprogramming of gene expression (*22*).

A series of studies have demonstrated an intimate connection between the activation of NF-κB and apoptosis of hepatocytes (*23–25*). During liver injury induced by TNF-α and bile acid, activation of NF-κB led to inhibition of apoptosis in hepatocytes (*26*). In this study, we used LPS to stimulate the activation of NF-κB. Nucling-KO mice exhibited spontaneous activation of NF-κB in the liver and resistance to LPS. Nucling is thought to inhibit nuclear translocation of NF-κB by protein–protein interaction in cytoplasm of unstimulated cells (*17*). Nucling may act as an alternative and supportive molecule to IκB for the prevention of nuclear localization of NF-κB in resting cells.

However, the activation of NF-κB can cause multiorgan failure including severe hepatic damage and even death. This pathologic response is mediated by cytokines such as IL-1β, IL-6 and TNF-α, which are produced under stimulation with LPS (*27*). The early phase of endotoxin tolerance has been found to be associated with a reduced inflammatory mediator response to endotoxin (*28*). The LPS-induced TNF-α and IL-1β release is decreased in endotoxin-tolerant animals (*29*). However, injection of LPS increased the level of serum cytokines such as TNF-α (Fig. 2), IL-1β and IL-6 (Supplementary Figs S1 and S2) even in Nucling-KO mice, similar to WT mice. According to our previous studies, Kupffer cells were decreased in number by apoptosis in Nucling-KO mice (*15*). Kupffer cells are the primary source of hepatic TNF-α (*30*). We speculated that systemic levels of TNF-α in Nucling-KO mice remained high by virtue of continuous production by macrophages in other organs and in liver after the LPS challenge. We have not observed, so far, any remarkable decrease in the number of macrophages in other organs except for the liver in Nucling-KO mice (data not shown). Alternatively, the Kupffer cells in Nucling-KO contributed to the tolerance of hepatocytes to LPS. In connection with this possibility, we observed that hepatocyte of WT showed LPS resistance when co-incubated with Kupffer cells derived from Nucling-KO (data not shown).

Treatment with LPS or TNF-α leads to strong NF-κB activation and transcription of survival genes in hepatocytes. cIAP1, cIAP2 and the Bcl-2 family are known to be upregulated by NF-κB in several cell types (*31*). We found that the expression of cIAP1 and cIAP2 was increased spontaneously in the liver of Nucling-KO mice (Fig. 5A and B). On TNF-α treatment, cIAP1 and cIAP2 are required for proper TNF receptor signalling leading to NF-κB activation (*32*).

Nucling-KO mice showed not only upregulation of anti-apoptotic proteins but also downregulation of

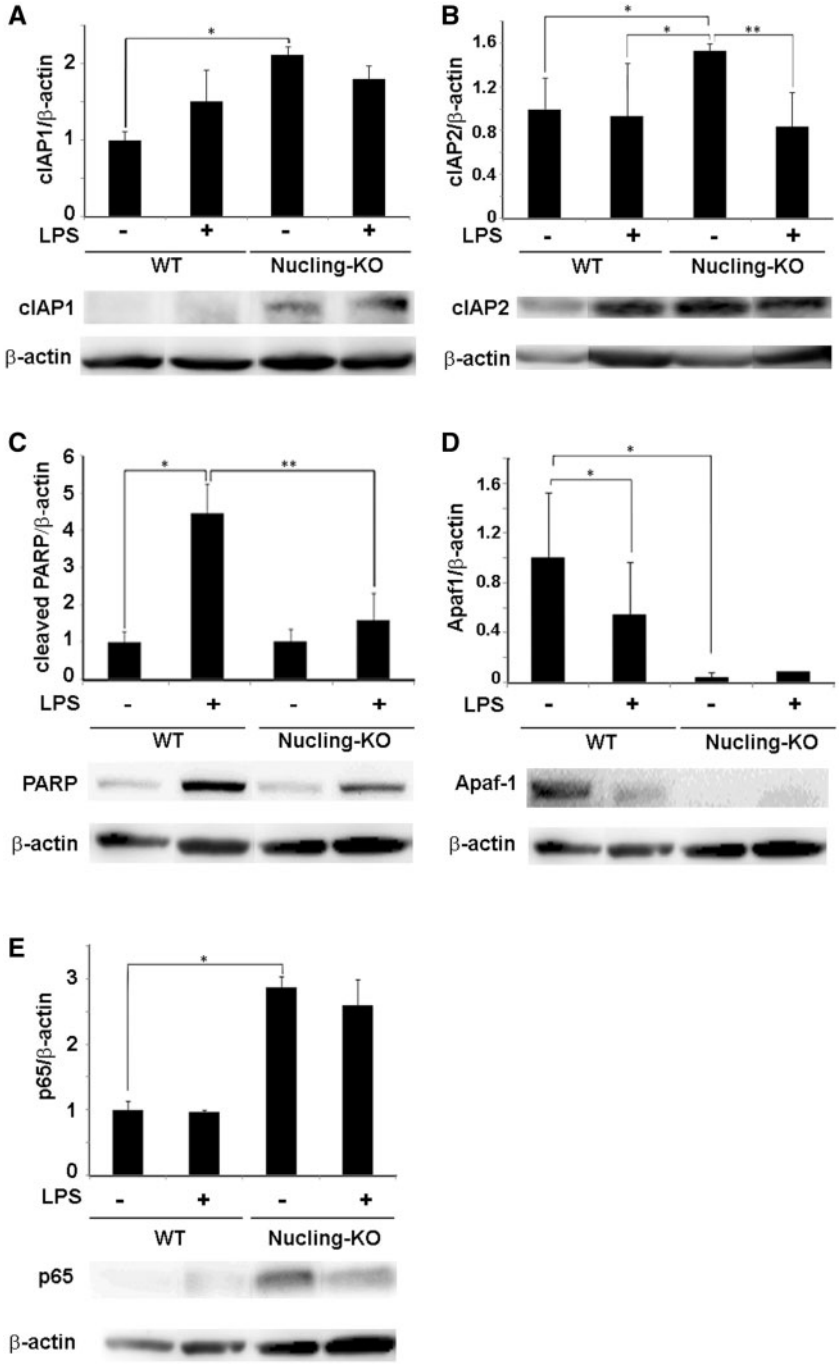

Fig. 5 Apoptosis is attenuated in Nucling-KO mice after LPS challenge through NF-κB activation. Whole liver extracts from WT and Nucling-KO mice were subjected to immunoblotting using antibodies detecting cIAP1 (**A**) and cIAP2 (**B**). Nuclear fractions of liver were prepared from WT and Nucling-KO mice sacrificed 24 h after LPS or PBS injection and subjected to immunoblotting with antibodies detecting cleaved PARP (**C**), Apaf-1 (**D**) and NF-κB p65 (**E**). The data are means ± SD for three independent experiments (*$P<0.05$, **$P<0.01$ by Student's *t*-test).

proapoptotic proteins. As shown in Fig. 5D, the expression of proapoptotic protein, Apaf-1, was decreased in the liver fraction of Nucling-KO mice. Apaf-1 forms an apoptosome with caspase-9 and regulates mitochondria-associated apoptosis with Nucling. PARP cleavage leads to cellular dysfunction and death (*33*); PARP-deficient mice were extremely resistant to LPS-induced endotoxin shock due to a defect in NF-κB-dependent transcriptional activation (*34*). Furthermore, cleavage of PARP was significantly decreased in liver on the injection of LPS in the absence of Nucling (Fig. 5C). From these points of views, we speculate that Nucling-KO mice may have a defect in NF-κB activation after LPS treatment.

Several lines of evidence indicate that the chronic inflammatory lesions in organs of Nucling-KO mice were due to the constant activation of NF-κB. Enhanced NF-κB activity has been linked to decreased apoptosis via the expression of anti-apoptotic genes (*35*). In general, NF-κB activation is an inducible

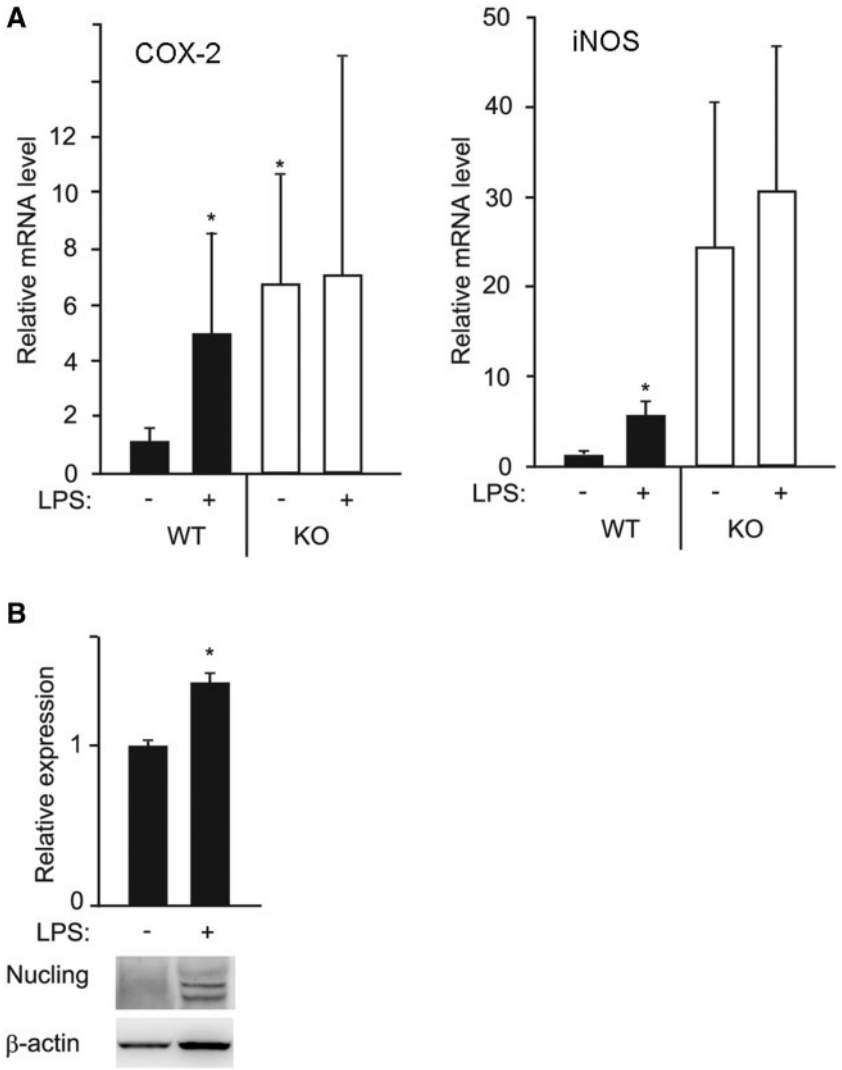

Fig. 6 Inflammation-related genes regulated by NF-κB were spontaneously activated in the livers of Nucling-KO mice. (**A**) Total RNAs prepared from the livers of WT and Nucling-KO mice were subjected to quantitative reverse-transcription polymerase chain reaction using specific primers detecting COX-2 and iNOS. The livers were prepared from WT and Nucling-KO mice sacrificed 24 h after LPS or PBS injection. (**B**) Immunoblotting was performed to check the expression of Nucling in the livers. The data are means ± SD for three independent experiments (*$P < 0.05$ by Student's *t*-test).

but transient event in normal cells. In tumour cells, different molecular changes may result in impaired regulation of NF-κB activation and NF-κB becoming spontaneously activated (*21*). Uncontrolled NF-κB expression negatively regulates apoptosis in tumour cells by affecting several anti-apoptotic genes including those for cIAP1, cIAP2, XIAP, cFLIP, Bcl family members, A1 and survivin (*36*). Therefore, our study suggests that a deficiency of Nucling accounts for liver carcinogenesis through a defect in the apoptotic signalling pathway. These observations and the data using several mammalian cells (unpublished) suggested that Nucling deficiency induced not only spontaneous activation of NF-κB in resting cells but also the impairment of NF-κB activation in stimulated cells. Previously, we reported that Nucling itself is upregulated by several cytotoxic stresses (*37*), and we think that the upregulation of Nucling is critical as the first step for the activation of apoptosis pathway. This hypothesis is consistent with our observations showing significant upregulation of Nucling in the livers of LPS-treated mice (Fig. 6B). Thus, the upregulation of Nucling is thought to be important for the LPS-induced apoptosis including PARP cleavage and NF-κB activation. The reason why cIAP2, iNOS and COX-2 were not upregulated by LPS in Nucling-KO mice (Figs 5B and 6A) may come from the defect in NF-κB activation system following LPS stimuli.

In conclusion, we observed that Nucling-KO mice had resistance to LPS. Apoptosis was attenuated in the liver of Nucling-KO mice with spontaneous NF-κB activation. Taken together, we propose that Nucling plays an important role in the regulation of apoptosis in the liver through the modulation of NF-κB signalling.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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