Toll-Like Receptor 4 Activity Protects Against Hepatocellular Tumorigenesis and Progression by Regulating Expression of DNA Repair Protein Ku70 in Mice

Ziyan Wang, Jun Yan, Heng Lin, Fang Hua, Xiaoxing Wang, Hanzhi Liu, Xiaoxi Lv, Jiaojiao Yu, Su Mi, Jiaping Wang, and Zhuo-Wei Hu

Hepatocellular carcinoma (HCC) is a devastating consequence of chronic inflammatory liver diseases. The goal of this study was to investigate whether Toll-like receptor 4 (TLR4) activity contributes to HCC initiation and progression in mice. A mouse model of diethylnitrosamine (DEN)-induced HCC was generated with wild-type and TLR4 mutant mice, and the development and progression of HCC and senescent responses were assessed using morphologic, immunological, and biochemical criteria. We found that genetic or pharmacologic blocking of TLR4 increased susceptibility to DEN-induced HCC carcinogenesis and progression, which was indicated by increases in number of tumor nodules, tumor volume, and animal death. The enhanced HCC was associated with a broad-spectrum reduction of immune response to DEN liver injury, as indicated by decreases in the liver-infiltrating F4/80+ macrophages, the apoptosis signal-regulating kinase 1/p38 mitogen-activated protein kinase/NF-κB and IRF3 signaling activities, and the expression of inflammatory cytokines. Suppressed immune networks resulted in a halt of cellular senescence induction in TLR4 mutant liver tissue, which promoted proliferation and suppressed programmed cell death. Moreover, TLR4 mutation resulted in a suppressed capacity of DNA repair due to a decrease in TLR4-mediated expression of DNA repair proteins Ku70/80 in liver tissue and cells. Isotopic expression of Ku70 in TLR4 mutant mice restored senescence and interrupted the positive feedback loop of DNA damage and oxidative stress, which reversed TLR4 mutation–deteriorated HCC carcinogenesis and progression. Conclusion: TLR4 plays an integrated defense role against HCC carcinogenesis by enhancing the expression and function of DNA repair protein Ku70. Our studies provide novel insight into TLR4 activity in the regulation of HCC tumorigenesis, which may be useful for the prevention of HCC development. (HEPATOLOGY 2013;57:1869-1881)
persistent oxidative/endoplasmic reticulum stress, which stimulates chronic inflammation through a unfold protein response and sustain an imbalance between programmed cell death and proliferation to confer tumorigenesis in the liver. When liver injury caused by microbes and oncotoxic agents, microbial components termed pathogen-associated molecular patterns (PAMPs) or soluble factors released from injured hepatic cells termed damage-associated molecular patterns (DAMPs) trigger inflammatory responses by interacting with pattern recognition receptors such as Toll-like receptors. Toll-like receptor 4 (TLR4) is an intensively studied member in the TLR family because of its diverse recognition ligands consisting of molecules containing PAMPs and DAMPs. However, TLR4 exhibits diverse roles in the regulation of carcinogenesis and tumor progression. For instance, a recent study indicated that the activation of TLR4 promotes cancer cell apoptosis, whereas Dapito et al. reported that inactivation of TLR4 reduces the incidence of HCC and that stimulating TLR4 with lipopolysaccharide (LPS) promotes HCC development. Blocking MyD88, a major adaptor molecule of TLR4, markedly ameliorates bacteria- or chemical-induced liver cancer. Thus, the role and mechanism of TLR4 in the pathogenesis of HCC tumorigenesis remain to be fully elucidated.

Recent studies indicate that nonhomologous end joining (NHEJ) is the major DNA double-strand break (DSB) repair pathway in human cells and that DNA repair Ku proteins are the critical NHEJ factors that regulates DNA NHEJ DSB pathway choice. Ku proteins include Ku70 and Ku80 that can form a heterodimer binding to DNA double-strand break ends to participate in the NHEJ pathway of DNA repair. In addition to its role in NHEJ, Ku proteins are involved in various genome maintenance processes such as DNA replication and repair, telomere maintenance, and chromosomal stability. Ku proteins were presumed only to recognize DSB ends and recruit other factors that process ends. However, Roberts et al. reported that Ku has a direct role in end-processing steps as well. Although the molecular mechanism of the DNA repair functions of Ku proteins is far from clear, defects in DSB repair capacity can lead to irreversible genomic instability and malignant transformation.

We therefore investigated whether the genetic or pharmacological inhibition of TLR4 activity induced immune suppression to limit tumorigenesis and tumor progression in liver. Using a mouse model of diethylnitrosamine (DEN)-induced HCC, we found that TLR4 mutant (TLR4mut) mice shows an increase in the initiation and progression of HCC and a decrease in the animal survival compared to wild-type (WT) littermates. Our studies indicate that TLR4-controlled immunity supporting the senescent induction and the expression of DNA repair proteins plays an integrated defense role against genotoxic carcinogenesis and tumor progression in the liver. Ectopic expression of DNA damage repairing protein Ku70 attenuates the DEN-induced HCC in TLR4mut mice, suggesting that Ku70 may act as a tumor suppressor by restoring immunity, senescent response, and autophagy flux in TLR4mut liver.

Materials and Methods

All animals received care according to the Guide for the Care and Use of Laboratory Animals. TLR4mut mice (C3H/He background) were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Fifteen-day-old WT and TLR4mut mice were injected intraperitoneally with or without DEN (25 mg/kg) (Sigma-Aldrich, St. Louis, MO). The mice were fed normal chow and sacrificed on months 1, 3, 6, and 18 after DEN injection to observe tumor development and animal survival. For adenovirus infection experiments, the mice were infected intramuscularly with or without DEN (25 mg/kg) (Sigma-Aldrich, St. Louis, MO). The mice were fed normal chow and sacrificed on months 1, 3, 6, and 18 after DEN injection to observe tumor development and animal survival. For adenovirus infection experiments, the mice were infected intramuscularly with 1 × 10⁵ viral particles (V.P.) of Ku70 adenovirus or green fluorescent protein (GFP) adenovirus per mouse on day 1, 7, and 14 after DEN injection, and were sacrificed at day 30 and month 6 after DEN injection. For assessing HCC, external visible tumors (>0.5 cm) were counted and measured by stereomicroscopy. The largest liver lobes were fixed in 4% formalin, paraffin-embedded, and sliced into sections. Sections were stained with hematoxylin and eosin and the tumor areas were measured as described. Liver function was monitored by measuring serum alanine aminotransferase (ALT).
aaminotransferase activities. Western blot assays of liver tissue were performed with commercial antibodies as described, using β-actin as loading controls. Detergent-soluble and insoluble fractions of livers were performed as described. Immunohistochemistry and immunofluorescence assays were performed as described. To detect total contents of reactive oxygen species (ROS), frozen liver sections or single cell suspensions were prepared as described and incubated with 2’,7’-dichlorofluorescein diacetate (Sigma-Aldrich, St. Louis, MO) as described. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed with detection kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Data are expressed as the mean ± SE. Groups were compared via one-way analysis of variance followed by a Tukey-Kramer or Dunnett multiple comparisons test. Comparisons between two groups were performed via an unpaired Student t test. The survival rates were analyzed using the Kaplan-Meier method. \( P < 0.05 \) was considered significant. Details are provided in the Supporting Information.

**Results**

**Loss of TLR4 Activity Confers the DEN-Induced Hepatic Tumorigenesis.** To explore the role of TLR4 in liver tumorigenesis, TLR4mut or WT mice at age 15 days were subjected to DEN-induced HCC, a well-established chemical carcinogenesis protocol. HCC developed in all of newborn WT or TLR4mut male mice injected with DEN (25 mg/kg) within 6 months (Fig. 1A). However, only 20% of female WT littersmates developed with HCC as described (data not shown). Notably, TLR4mut mice developed two-fold more visible tumor nodules than WT mice (37.76 ± 5.83 versus 18.09 ± 1.69, \( P < 0.01 \)) (Fig. 1B). Also, an earlier onset of liver tumors was observed in the TLR4mut mice than WT mice: 60% of WT male mice developed HCC at the end of 5th month after DEN treatment but all of TLR4mut male mice developed HCC (data not show). Moreover, the number of HCC tumor nodules was also found increased in TLR4mut mice than their WT littersmates (Fig. 1B). Most tumor nodules were basophilic HCC (Fig. 1A), and two-fold more tumor area (percentage) was detected in the TLR4mut mice compared with WT mice (39.35 ± 6.42 versus 16.85 ± 3.42; \( P < 0.01 \)) (Fig. 1B). Consistently, TLR4mut mice displayed a persistent hepatic injury than the WT mice as indicated by increase in the serum alanine aminotransferase (data not shown). Therefore, TLR4mut mice with HCC exhibited significantly shorter mean survival times than WT mice (Fig. 1C). Programmed cell death, including apoptosis and autophagy-associated cell death, is a crucial defense mechanism against tumorigenesis. Compared with WT mice, TLR4mut mice exhibited a remarkable decrease in apoptosis as marked by TUNEL staining (11.12 ± 0.84 versus 4.35 ± 0.45 [\( P < 0.01 \)], 9.93 ± 0.18 versus 3.84 ± 0.29 [\( P < 0.01 \)], and 10.35 ± 0.84 versus 4.30 ± 0.62 [\( P < 0.001 \)], at month 1, 3, and 6 after DEN injection, respectively) (Fig. 1D) as well as a decrease in apoptotic cell death marked by cleaved caspase-3 (Supporting Fig. 1A,B). Moreover, TLR4mut livers displayed a persistent increase in proliferation biomarker proliferating cell nuclear antigen (PCNA) as indicated by immunohistochemistry staining (Fig. 1E) and immune blotting (Supporting Fig. 1A,C). Pharmacological targeting of TLR4 with a TLR4-neuturulizing monoclonal antibody (100 µg/kg/week) for 6 months resulted in an identical phenomenon as observed in TLR4mut mice (data not shown). These data indicate that inhibition of TLR4 signaling increases susceptibility to DEN-induced carcinoogenesis and progression of HCC through enhancing proliferation and attenuating liver cell death.

**TLR4 Mutation Aggravates DEN-Induced DNA Damage and ROS Accumulation But Decreases the Expression of DNA Repair Protein Ku70/Ku80 in Liver Tissue.** The extent of DNA damage was analyzed by immunoperoxidase staining for 8-OHdG and the expression of γ-H2AX and H2AX in WT and TLR4 mutant liver tissue. We found that DEN insult resulted in a time-dependent increase in the expression of 8-OHdG and γ-H2AX in TLR4-deficient liver tissue (Fig. 2A-D) but a time-dependent recovery in the WT liver tissue, which was confirmed by the analysis of γ-H2AX expression in liver tissue by confocal microscopy analysis (Fig. 2E,F). Similarly, TLR4mut mice showed a time-dependent gradual increase in the accumulation of ROS in liver tissue, but WT littermates showed a time-dependent gradual decrease in ROS production (Fig. 3A,B). Notably, the expression of DNA repair protein Ku70 in the liver was significantly decreased in the DEN-treated TLR4mut mice up to 60 days after DEN injection (Fig. 3C,D and Supporting Fig. 1G). The expression of Ku80, a partner of Ku70, was also decreased in the early days after DEN injection but gradually return to the basal level after 30 days after DEN injection (Fig. 3C,D). Consistent with these observations, the expression or activity of DNA-dependent repair kinase complex ATM-DNA-PKcs was attenuated in TLR4mut liver
tissue compared with DEN-treated WT littermates (Fig. 3E,F and Supporting Fig. 1E,F). These data indicate that TLR4mut mice show a persistent increase in DNA damage response and ROS accumulation which associated with a suppressive expression of DNA repair proteins Ku70/80 and activity of DNA repair protein kinase complex in DEN-treated TLR4mut liver tissue.

**TLR4 Mutation Protects from the Activation of the Immune Networks and Cellular Senescence in the DEN-Injured Liver Tissue.** To determine the potential role of immune cells in HCC development
in TLR4mut liver, the liver-infiltrating F4/80\(^+\) macrophages were examined in sham- or DEN-treated WT and TLR4mut livers. TLR4 mutation caused a marked decrease in the liver-filtrating F4/80\(^+\) macrophages compared with WT mice (Supporting Fig. 2A,B). TLR4 mediates a diversity of cellular functions by activating the MyD88-dependent apoptosis signal-regulating kinase 1 (ASK1)/p38 mitogen-activated protein kinase (p38 MAPK)/nuclear factor kappa B (NF-\(\kappa\)B) signaling or MyD88-independent IRF3 pathway. 24 The ASK1/p38 MAPK/NF-\(\kappa\)B pathway is not only a major sensor of oxidative stress leading to programmed cell death, but it is also sufficient and necessary to induce cellular senescence against carcinogenesis after DNA damage and genomic instability. 18 Moreover, cytokine production mediated by NF-\(\kappa\)B activation plays important roles in triggering cell death and critically contributes to the cellular senescence against tumorigenesis. 25-27 We found that TLR4 deficiency resulted in a broad-spectrum decline of immune...
responses to DEN-induced liver injury. Particularly, TLR4mut liver tissue showed a striking decrease in the expression and phosphorylation of ASK1, p38 MAPK, p-IRF3, and p-NF-κB (Fig. 4A,B). The expression of inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-12, IL-17, tumor necrosis factor-α, IFN-γ,
and IFN-λ was also decreased in TLR4mut liver tissue (Fig. 4A,C). The broad-spectrum decline of immune responses caused a significant attenuation of autophagic activity as indicated by the reduced expression of LC3I/II, Beclin-1, class III phosphatidylinositol-3 kinase, and accumulation of p62 in TLR4mut liver tissue (Fig. 4A,D). Moreover, TLR4mut liver tissue showed an attenuation of p53/21- and p16/pRB-dependent cellular senescence in response to DEN-induced liver injury (Fig. 4A,E). These results indicate that TLR4 deficiency enhances susceptibility to hepatocellular carcinogenesis due to a broad-spectrum decline of immune networks, which include a decrease in liver-infiltrating macrophages, suppressed ASK1/p38 MAPK/NF-κB and IRF3/IFN signaling pathways, reduced expression of inflammatory cytokines, inactivation of autophagy, and failure of cellular senescence induction in liver tissue.

**The Overexpression of Ku70 Alleviates DNA Damage and Restores Cellular Senescence in TLR4 Mutant Liver Tissue.** Because we found that the expression of Ku70/Ku80 was attenuated in TLR4mut liver tissue, we examined whether the activation of TLR4 regulated the expression of Ku proteins in both
liver and liver immune cells. We found that TLR4 ligand LPS stimulated a time- and concentration-dependent Ku70 but not Ku80 expression in both liver cells and immune cells (Supporting Fig. 3A-D). We thus suspected that defeat in Ku70 expression was responsible for the enhanced susceptibility to DEN-induced HCC in TLR4mut mice. Blocking TLR4 on HepG2 cells with anti-TLR4 antibody inhibited the expression of Ku70 (Supporting Fig. 3E-G). Infection of HepG2 cells with Ku70 adenovirus could enhance the expression of Ku70 (Supporting Fig. 3H) with an identical expression level of Ku70 and GFP (Supporting Fig. 3I). Infection of TLR4mut mice with Ku70 adenovirus resulted in a significant increase in Ku70 expression in the liver tissue (Fig. 5A-C) at day 30 after DEN injection. Recent work indicates that Ku70 acts as intracellular receptor of IFNγ or IFNα. We found that overexpression of Ku70 enhanced the expression of IFNγ but not IFNα (Fig. 5A,B). Also, restoration of Ku70 expression markedly reduced DNA damage as demonstrated by a decreased γ-H2AX expression and increases in the phosphorylation of DNA-PKcs and expression of PARP-1 (Fig. 5A,B,D). Critically, overexpression of Ku70 restored both of p53/p21- and p16/pRb-dependent cellular senescence as indicated by increases in the expression or phosphorylation of p53, p21, p16, IL-1α, CXCL2 (IL-8 relevance), p-p38, and p-NF-κB expression. The data are expressed as the mean ± SE (n = 5).

Overexpression of Ku70 Reverses TLR4 Deficiency-Worsened Carcinogenesis. Six months after DEN treatment, TLR4mut mice with overexpression of Ku70 showed a significant reduction in the development of HCC, as indicated by significantly reduced numbers and volume of tumor nodules (Fig. 7A,B and Supporting Fig. 4B) and by improved liver function (Fig. 7C). Notably, 6 months after overexpression of Ku70 alleviated DNA damage and induced a senescent response in TLR4 mutant liver. (A) Expression of Ku70, Ku80, γ-H2AX, H2AX, components of ATM-DNA-PKcs complex were assessed via immune blotting. (B) Types of DNA repair and DNA damage were analyzed via confocal microscopy (red, Ku70 dots and γ-H2AX dots; blue, 4’,6-diamidino-2-phenylindole-labeled nuclei of the same fields). Scale bar = 37.5 μm. (C) Expression of p-p53, p53, p-pRb, pRb, p21, p16, IL-1α, CXCL2 (IL-8 relevance), p-p38, and p-NF-κB were assessed via immune blotting. The data are expressed as the mean ± SE (n = 5).
Ku70, the expression level of Ku70/80 was returned to the basal-below level (Fig. 7D,E); the DNA damage marker γ-H2AX, proliferation marker PCNA, and apoptosis marker activated caspase-3 were reduced to a lower level than that in the GFP-expressing TLR4mut mice (Fig. 7D,E and Supporting Fig. 4C-E). Thus, although the expression of p53 was not changed after overexpression of Ku70, the phosphorylation of p53 was significantly decreased in the Ku70-overexpressing liver tissue (Fig. 7D,E). Taken together with Figs. 5 and 6, these data show that the overexpression of DNA repair protein Ku70 can protect against HCC development and progression by restoring cellular senescent response and activation of immune networks. These effects can induce an effective autophagic degradation, clean the accumulated ROS, decrease DNA damage, attenuate proliferation, and promote the programmed cell death in TLR4mut livers (Fig. 7F).

Discussion

Many insults including microbial infection, genotoxic agents, and metabolic stress causing DNA damage and genomic instability can trigger so-called senescence response to defense against tumorigenesis in liver. It is evidence that immune response plays a critical role in the initiation and sustention of cellular senescence. The activation of the ASK1/p38 MAPK/NF-κB signaling as well as the expression of inflammatory cytokines IL-1α, IL-6, and IL-8 initiates and supports cellular senescence caused by a variety of stresses. Recent work further indicates that pattern recognition receptors such as TLRs can trigger cellular senescence through interacting with PAMPs and DAMPs. Our current studies demonstrate that TLR4 mutation causes a loss of immune networks supporting cellular senescent response to the DEN-induced liver injury. The suppressed immunity and senescence cannot eliminate the DEN-induced ROS accumulation and DNA damage, which stimulates hepatic proliferation, attenuates autophagy and programmed cell death, and promotes malignant transformation. We recently report that loss of TLR2 activation of the ASK1/p38 kinase/NF-κB pathway results in an enhanced susceptibility to hepatocellular carcinogenesis due to a suppressed cellular senescence and autophagic flux. The broad-spectrum decline of immune responses to DEN stress in TLR2−/− or
TLR4mut mice associated with a suppressed senescence and a defected autophagic flux, indicating a similar mechanism used by TLR2 and TLR4 to defend against HCC. However, in this study, we further find that TLR4 mutation causes a decrease in the expression of DNA repair proteins Ku70/80 in the DEN-injured livers. It is the suppressed expression of Ku70/80 leading to a persistent DNA damage and ROS/endoplasmic reticulum stress in TLR4mut liver. 36 Indeed, isotopic expression of DNA repair protein Ku70 can reverse the TLR4 mutation-enhanced susceptibility to the DEN-induced HCC through restoring the cellular senescence and activating autophagic flux in TLR4mut liver tissue. Thus, these results place TLR4 activity in the intersection of DNA damage/genome instability and senescence/autophagy/DNA repairing (Fig. 7F).

The residual hepatic cells or the liver-infiltrating immune cells have been reported to be involved in the pathogenesis of HCC development. 31,37 Indeed, microbial infection in the liver may recruit a larger number of immune cells to the liver, and the infiltrated immune cells and secreted soluble factors play a critical role in the promotion of HCC development. 10
However, if HCC is primarily caused by chemical agents or metabolic stresses, the residue liver cells undergoing premature senescence are predominant party to initiate and sustain inflammation participating in the regulation of HCC development.\textsuperscript{5} Obviously, the immunity against tumorigenesis is constituted by both liver-infiltrating immune cells and residual hepatic cells. Interestingly, in addition to its expression in immune cells, functional TLR4 is also expressed by residual hepatic cells and the TLR4-mediated responses can therefore be derived from the activated residual hepatic cells or from the liver-infiltrating immune cells. In our current work, however, a failure of cellular senescence induction in the residual hepatic cells is more likely to link to loss of TLR4-mediated immunity, enhancing susceptibility to DEN-induced hepatocellular carcinogenesis and progression. This observation is supported by the fact that the filtration of macrophages was decreased and the wide-spectrum inflammatory response was suppressed in the TLR4mut liver tissue; in addition, DNA damage, genomic instability, and malignant transformation were caused by DEN, a hepatic- but not immune-specific oncotoxic agent and a major trigger of senescent response. Thus, our study demonstrates a critical protection role of TLR4 against tumorigenesis and may help to develop new prophylactic and treatment approaches for HCC.

The defects in DNA damage repair leading to genome instability is the hallmark of cancer, including HCC.\textsuperscript{38} Indeed, HCC is commonly secondary to cirrhosis following chronic microbe infection, genotoxic agents, and metabolic stress, which is often associated with genotoxic DNA damage and mutations of known DNA repair genes.\textsuperscript{39} For instance, the DNA repair complex and its regulatory proteins may critically influence vital cellular processes such as programmed cell death, cell proliferation, and inflammation, and thereby may play a critical role in the pathogenesis of human cancer.\textsuperscript{40-45} However, the precise mechanism of the DNA repair complex in HCC carcinogenesis remains unclear and may involve either impairing or accelerating DNA repair activity or imbalances in the activation of DNA repair proteins. Recent work demonstrates that innate immunity, especially the TLR-activated p38 kinase/NF-$\kappa$B signaling, plays a significant role in hepatic homeostasis by regulating the DNA double-strand break (DSB) repair.\textsuperscript{44} NF-$\kappa$B can be activated by DNA damage via Ataxia telangiectasia-mutated signaling.\textsuperscript{45} Volcic et al.\textsuperscript{44} report that dissected distinct DNA DSB repair mechanisms reveal a stimulatory role of NF-$\kappa$B in homologous recombination. Our present study provides the evidence to demonstrate conversely that TLR4 mutation causes a suppressed expression of DNA repair protein Ku70/80 in response to DEN insult, which may sustain DNA damage and chromosomal instability in the DEN-injured liver. TLR4 mutation-caused changes can be reversed by the ectopic expression of DNA damage repairing protein Ku70. These studies suggest that innate receptor TLR4 activity plays a key role in the regulation of DNA damage repair to protect against HCC development and progression. Additionally, Ku70 may function as an intracellular sensor activating immunity and inducing senescent response against tumorigenesis by interacting with intracellular soluble factors such as IFN$\gamma$.\textsuperscript{28} Thus, our work establishes a protective role for TLR4 activity in DEN-induced liver injury and HCC by (1) inducing programmed cell death and cleaning hepatic ROS accumulation; (2) maintaining intracellular senescent responses to avoid excessive proliferation and malignant transformation; (3) maintaining an effective autophagy flux to clear toxic p62-positive aggregates and interrupting its feedback with accumulated ROS; and (4) enhancing the expression of DNA repair proteins such as Ku70 to eliminate the risk of genome instability. By rescuing the failed programmed cell death, autophagy flux, and senescent responses, overexpression of Ku70 can reverse the deteriorated HCC in TLR4mut littermates.

The roles of TLR4 signaling are controversial in regulating hepatocarcinogenesis. Dapito et al.\textsuperscript{10} reported that TLR4 inactivation reduces the incidence of HCC and stimulating systematic TLR4 by LPS promotes HCC. The major reason for the different observations in these studies may be the different animal models used by two groups. Dapito et al. injected adult mice with 100 mg/kg of DEN plus repeated CCl$_4$, which caused DNA damage, acute and chronic liver injury, hepatocyte necrosis, and hepatic fibrosis. Additionally, chronic administration of TLR4 agonist LPS would sustain chronic liver injury and inflammation in these animals. Hence, HCC development would be reduced if TLR4 signaling was abrogated by mutation.\textsuperscript{46} However, in our work, mice were only injected with 25 mg/kg of DEN one time at the age of 15 days. DEN caused liver injury, ROS production, and DNA damage in the liver. DAMPs released from damaged liver stimulates the ASK1/p38 MAPK signaling and increases the expression of DNA repair protein Ku70/80 through activation of TLR4, which could clean ROS and DNA damage from the liver. Hence, the suppressed immune networks, including a halt of cellular senescence and autophagy due to TLR4 deficiency, fail to clean ROS and repair DNA damage.\textsuperscript{5,18}
It is the unclean ROS and unrepaird DNA damage contributing to DNA mutation, development of pre-cancerous cells, and HCC progression (Fig. 7F).

In conclusion, an intact TLR4-mediated immune network is critical for initiating and sustaining cellular senescence, autophagy flux, and expression of DNA damage repairing proteins that together build the barrier against hepatocellular carcinoma. Our studies show that Ku70 is down-regulated in TLR4mut liver tissue, which correlates significantly with enhanced initiation and progression of HCC in TLR4mut mice. Our work thus suggests an underlying mechanism in which Ku70 may act as a tumor suppressor in the liver by restoring immunity, senescence, and autophagy flux by activating p53/p21- and P16/pRb-dependent pathways. A further revelation of the molecular mechanism of the TLR4-regulated Ku70 expression and of potential strategies to induce Ku70 expression may provide a new therapeutic target for prevention and treatment of HCC.

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