Loss of Immunity-Supported Senescence Enhances Susceptibility to Hepatocellular Carcinogenesis and Progression in Toll-Like Receptor 2-Deficient Mice

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Hepatocellular carcinoma (HCC) is a complication at the endstage of chronic inflammatory liver diseases with dismal prognosis. Targeting of Toll-like receptor (TLR) 2 attenuates tumor metastases; we hypothesized that blocking TLR2 might also play a crucial role in reducing hepatocarcinogenesis. Surprisingly, we found that the genetic deletion of TLR2 increased susceptibility to diethylnitrosamine (DEN), a genotoxic carcinogen that can induce HCC. Indeed, TLR2-deficient mice showed a significant increase in carcinogenesis and progression of HCC as indicated by increases in tumor nodule size, tumor volume, and animal death. The enhanced susceptibility to DEN-induced HCC was associated with a broad-spectrum reduction in the immune response to DEN-induced liver injury. We found that TLR2 deficiency caused a decrease in the infiltration of macrophages and an attenuation of apoptosis signal regulating kinase 1 (ASK1) / p38 mitogen-activated protein kinase (p38 MAPK) / nuclear factor kappa B (NF-κB) signaling, which led to a decrease in the expression of interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin (IL)-1α/β, IL-6, and Cxcl-2 as well as suppression of autophagy flux and increases in oxidative stress and p62 aggregation in liver tissue. The defects in immune networks resulted in suppressed p21- and p16/pRb-dependent senescence, which caused an increase in proliferation and a decrease in apoptotic and autophagy-associated cell death in mouse livers. Restoring cellular senescence and autophagy flux by treating TLR2-deficient mice with IFN-γ, a T helper 1 (Th1) cytokine and positive modulator of senescence and autophagy, could attenuate the carcinogenesis and progression of HCC associated with TLR2-deficient animals. **Conclusion:** The loss of immune networks supporting cellular senescence and autophagy flux is attributed to enhanced susceptibility to DEN-induced hepatocellular carcinogenesis and progression in TLR2-deficient mice. These findings may be used to prevent the development of liver cancer. (HEPATOL 2013;57:171-182)

Hepatocellular carcinoma (HCC) is a complication of chronic liver disease, and it is the third leading cause of cancer deaths worldwide due to ineffective therapies. The pathogenesis of HCC is closely associated with chronic liver inflammation, which may result from microbial infection, toxic
agents, or oxidative/metabolic stress\(^2\) and can promote an imbalance between cell death and compensatory proliferation.\(^3\) Hepatic immunity is predominantly innate,\(^4\) and the liver is an organ with multiple mechanisms to defend against carcinogenesis caused by microbes and toxic agents.\(^2\) Among these, the pattern recognition receptors, especially Toll-like receptors (TLRs), play central roles in the liver defense system.\(^4\) TLRs exhibit different roles in the regulation of tumorigenesis and tumor progression.\(^5\) In certain tumor types, activation of TLRs stimulates tumor proliferation and survival, whereas in other tumor types activation of TLRs directly promotes tumor apoptosis.\(^6\) A deficiency in either TLR4\(^7\) or MyD88,\(^8\) the major adaptor molecule of TLRs, has been reported to markedly ameliorate chemically induced liver cancer.

TLR2 is a unique member of the TLR family because of its diverse ligand recognition profile, which includes a variety of pathogen- and damage-associated molecules. TLR2 can form heterodimers with other TLR subtypes or coreceptors, such as TLR1, TLR6, and CD36.\(^9\) A number of studies indicate that the TLR2 signal can not only trigger the activation of the apoptosis signal regulating kinase 1 (ASK1) / p38 mitogen-activated protein kinase (p38 MAPK) / nuclear factor kappa B (NF-kB) signaling pathway in an MyD88-dependent manner but can also stimulate the extracellular signal-regulated kinase (ERK) / Jun-amino-terminal kinase (JNK) and PI3K/Akt pathways in an MyD88-independent manner.\(^4,10\) TLR2 activity can also trigger endoplasmic reticulum (ER) stress-dependent apoptosis.\(^9\) Genetic polymorphisms of TLR2 have been reported to influence the pathogenesis of inflammatory diseases and cancer.\(^11\) The opposing roles of TLR2 activity have been observed in the regulation of tumor growth and metastasis. For instance, our recent work demonstrates that TLR2 activity promotes pulmonary tumor metastasis through the activation of signal transducer and activator of transcription 3 (Stat3),\(^12\) whereas TLR2 activity elicits tumor regression in mouse models of colitis-induced cancer\(^13\) and brain tumors.\(^14\) Thus, the function and mechanism of TLR2 activity in tumorigenesis are not fully understood.

In our current study, therefore, we investigated whether the genetic inhibition of TLR2 activity could induce a similar suppressive effect on liver tumorigenesis and tumor progression in a mouse model of diethylnitrosamine (DEN)-induced HCC, a toxic chemical agent. We found that TLR2-deficient (TLR2\(^{−/−}\)) mice had increased development and progression of HCC and decreased survival compared to wildtype (WT) mice. Our studies indicate that TLR2-mediated immune networks play an integrated defense role against HCC and progression by supporting p21- and p16/pRb-dependent senescence and autophagy flux in the liver.

### Materials and Methods

All animals received care according to the Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, MD). TLR2\(^{−/−}\) mice (C57BL/6 background) were originally obtained from Jackson Laboratories (Bar Harbor, ME). Fifteen-day-old WT and TLR2\(^{−/−}\) mice were intraperitoneally injected with or without DEN (25 mg/kg) (Sigma-Aldrich, St. Louis, MO).\(^8\) The mice were fed normal chow and sacrificed at the end of the indicated months after DEN injection to observe tumor development and animal survival. To determine the role of immunity restoration and senescence, TLR2\(^{−/−}\) and WT mice were sham-treated or treated with interferon-gamma (IFN-\(γ\)) \(10^6\) U/kg every other day for 3 months at 1 day before or 3 months after DEN injection. To assess HCC, the externally visible tumors (>0.5 mm) were counted and measured using stereomicroscopy.\(^14\) The largest liver lobes were fixed in 4% formalin, paraffin-embedded, and sectioned. The sections were used for hematoxylin and eosin (H&E) staining and other analysis as described.\(^3\) Liver function was monitored by measuring serum alanine aminotransferase (ALT) and alpha-fetoprotein (AFP) staining. Western blotting of nontumor liver tissue was performed with commercial antibodies as described\(^12\) using \(β\)-actin as loading control. Detergent-soluble and -insoluble liver fractions were prepared as described.\(^16\) Immunofluorescence and immunoperoxidase activity were assayed as described.\(^12\)

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To detect reactive oxygen species (ROS), frozen liver sections or homogenates were incubated with 2',7'- dichlorofluorescein diacetate (DCFH-DA, Sigma) as described.\textsuperscript{17} TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining was performed with a kit (Roche, USA) following the manufacturer’s instructions. SA β-gal staining was detected with the senescence detection kit (Biovision, Mountain View, CA), and heterochromatin staining was performed as described.\textsuperscript{18} Student’s t test was used for statistical analysis. For the survival analysis, the log-rank test was used to assess the significance observed in the Kaplan-Meier curve. A value of $P < 0.05$ was considered statistically significant. More details are provided in Supporting Methods.

Results

Knockout of TLR2 Exacerbates Hepatocarcinogenesis and Progression. To explore the role of TLR2 in liver tumorigenesis, TLR2 \textsuperscript{-/-} or WT mice were subjected to a widely used chemical carcinogenesis protocol. All male newborn WT or TLR2 \textsuperscript{-/-} mice injected with DEN (25 mg/kg) developed liver tumors within 8 months (Fig. 1B). However, only 20% of WT and TLR2 \textsuperscript{-/-} female mice developed tumors (data not shown). Pathological analysis revealed that most tumor nodules were basophilic HCC (Fig. 1A,B), and a 3-fold greater tumor area (percentage) was observed in the TLR2 \textsuperscript{-/-} mice than in the WT mice ($20.1 \pm 4.5\%$ versus $6.4 \pm 1.0\%$, $P < 0.01$) (Fig. 1A; Supporting Fig. S1A). The livers from TLR2 \textsuperscript{-/-} mice showed a lower degree of HCC differentiation than WT livers. All lesions observed in the TLR2 \textsuperscript{-/-} livers were HCC, 71.4% of the lesions from WT livers were HCC, and the remaining lesions were advanced dysplasia (Fig. S1B). Compared to WT livers, TLR2 \textsuperscript{-/-} livers exhibited a greater extent of microvascular generation (Fig. S1B) and a higher level of expression of AFP (Fig. S1C,D). Notably, the TLR2 \textsuperscript{-/-} mice developed 5-fold more visible tumor nodules than the WT mice ($29.1 \pm 2.8$ versus $5.5 \pm 0.9$, $P < 0.001$) (Fig. 1A). Additionally, an earlier onset of HCC was observed in TLR2 \textsuperscript{-/-} mice compared to WT mice, and 40% of TLR2 \textsuperscript{-/-} mice developed noticeable HCC at the end of the fourth month after DEN treatment; no WT mice presented with HCC. All TLR2 \textsuperscript{-/-} mice developed HCC at the end of the sixth month, but only 63% of WT mice developed such lesions at this time. However, 100% of WT or TLR2 \textsuperscript{-/-} mice developed liver tumors at the end of the eighth month after DEN injection (Fig. 1B). The number of HCC tumor nodules was also increased in the TLR2 \textsuperscript{-/-} mice (Fig. 1C). Although the TLR2 \textsuperscript{-/-} mice displayed no difference in a very early hepatic injury (Fig. S1E,F), they showed persistent elevated serum levels of ALT (Fig. 1E) as compared to WT mice after DEN injection. Thus, TLR2 \textsuperscript{-/-} mice with HCC had shorter mean survival times than WT mice (Fig. 1F). Collectively, the data indicate that knockout of TLR2 increases the susceptibility to the DEN-induced hepatocarcinogenesis and progression.

TLR2 Deficiency Enhances ROS Accumulation but Attenuates Cell Death. DEN is a typical chemical carcinogen and forms adducts with DNA after liver metabolism by cytochrome P450 2E1. These adducts may cause liver injury, DNA mutation, and tumorigenesis.\textsuperscript{19} No significant difference in cytochrome P450 2E1 activity was detected between TLR2 \textsuperscript{-/-} and WT mice (data not shown), indicating that the elevated HCC development in TLR2 \textsuperscript{-/-} livers did not simply result from changes in DEN metabolites. Further, the TLR2 \textsuperscript{-/-} mice exhibited enhanced accumulation of ROS in their liver tissues (Fig. 2A), which was sustained from the early to the late phase of HCC progression (Fig. 2B). Additionally, we found that TLR2 \textsuperscript{-/-} livers showed an accumulation of oxidative stress-associated products, including protein carbonyl and 8-OHdG-linked proteins (Fig. S2A) and the activation of lipid peroxides (LPO) (Fig. S2B). Generally, cellular stress and oxidative damage should induce programmed cell death in the liver through apoptosis and autophagy. However, TLR2 \textsuperscript{-/-} mice displayed a persistent decrease in cell death (Fig. 2C,D) compared to WT mice. Indeed, TLR2 \textsuperscript{-/-} mice exhibited a decrease in autophagy-associated cell death as marked by Lamp1 and TUNEL double staining ($6.0 \pm 1.7$ versus $1.5 \pm 0.3$, $P < 0.05$) (Fig. 3A,B) as well as a decrease in apoptotic cell death as evidenced by suppressed cleavage of caspase-3 (Fig. 3C,D). These results indicate that TLR2 deficiency protects liver cells from oxidative stress-induced death.

TLR2 Deficiency Suppresses Senescence in the Liver. Cellular senescence is regarded as a physiological barrier against carcinogenesis and tumor progression.\textsuperscript{20-22} Senescence can be induced by many stimuli, such as dysfunctional telomeres and other sources of DNA damage.\textsuperscript{21} As a typical biomarker of senescence, SA β-galactosidase (β-gal) staining was increased in WT livers but not in TLR2 \textsuperscript{-/-} livers after DEN treatment (Fig. 4A,B). Despite ROS accumulation (Fig. 2A) and DNA damage (Fig. S2A) in the liver tissue, γ-H2AX (phosphorylated histone H2A.X), a typical biomarker of DNA damage repair, was suppressed in
TLR2−/− livers (Fig. 4C-E), indicating a failure of the common ROS neutralizing and DNA repair mechanisms. The suppressed DNA replication is a marker of senescence, which can be detected by immunostaining for proteins such as proliferating cell nuclear antigen (PCNA).21 Compared to WT littermates, PCNA was persistently increased in the DEN-treated TLR2−/− livers (Fig. 4E,F; Fig. S2C,D). Additionally, inhibition of the p16-pRb pathway was observed in TLR2−/− liver tissue (Fig. 4E,F). This pathway has been shown to play a critical role in stress- or oncogene-induced premature cellular senescence.20,21 Interestingly, the expression of p53, an alternative trigger of cellular senescence, was induced in the sham- or DEN-treated TLR2−/− livers (Fig. 4E,F), which might be a consequence of accumulated ROS in the liver tissue.21 However, the phosphorylation level of p53 showed no difference between WT and TLR2−/− livers. The expression of p21, a downstream molecule of p53, was significantly suppressed in TLR2−/− livers (Fig. 4E,F).
These data indicate a failure induction of p16/pRb- and p21-dependent cellular senescence in the TLR2−/− liver after DEN treatment.

**TLR2 Deficiency Results in Suppressed Autophagy Flux and p62 Aggregates in the Liver.** Autophagy serves as a barrier against tumor initiation because it acts as a crucial factor in the decision between survival and cell death when multiple stressors are present. Autophagy is an important trigger for senescence onset. In turn, senescence activates autophagy. Because suppressed senescence and autophagy-associated cell death have been observed in TLR2−/− livers, we examined the autophagy signals in the liver tissues of TLR2−/− and WT mice. The Akt activity and its downstream effector molecule, mammalian target of rapamycin (mTOR), a key inhibitor of autophagy, was markedly inhibited in the liver tissues from TLR2−/− mice compared to WT mice. The TLR2−/− liver tissues also exhibited enhanced expression of class III PI3K and beclin-1 and increased conversion of LC3B I to LC3B II, indicating the activation of autophagic signals. As a receptor and substrate of selective autophagy, the content of p62/SQSTM1 in cells is a critical indicator of autophagy flux. We found that there were no apparent differences in the expression levels of p62/SQSTM1 in cells at the indicated times after DEN injection. Eight magnified (20×) fields per liver were counted for the percentage of TUNEL-positive cells. The data are expressed as the mean ± SEM (n = 4 livers per group).
Fig. 3. TLR2 deficiency attenuates programmed cell death. (A,B) Autophagy-associated cell death was detected by Lamp-1 (red) and TUNEL (green) double staining. The cell nuclei were counterstained with DAPI (blue). Scale bar = 75 μm. The data are representative images of three assays. Eight magnified (20×) fields per liver were counted to determine the percentage of Lamp-1 and TUNEL double-positive cells. The data are expressed as the mean ± SEM (n = 4 livers per group). (C,D) The expression of cleaved caspase-3 was analyzed by western blotting. The data are representative immune blots from 4-6 assays and expressed as the mean ± SEM (n = 4). The frozen liver sections or liver extracts were prepared using the liver tissues from WT and TLR2−/− mice at the end of the 1st month after DEN or sham treatment.

Fig. 4. TLR2 deficiency suppresses p16/pRb- and p21-mediated senescence. (A,B) TLR2 deficiency suppressed SA β-gal expression. The data are representative of the SA β-gal staining (blue) of four assays and presented as the mean ± SEM (n = 4). Scale bar = 75 μm. (C,D) TLR2 deficiency suppressed the repair of damaged DNA. The data are representative images of γ-H2A.X and a quantified summary of heterochromatin staining (n = 4). Scale bar = 7.5 μm. (E,F) The expression of senescence-associated molecules was analyzed by western blotting. The data are expressed as the mean ± SEM (n = 4). The liver extracts were obtained from the livers of WT and TLR2−/− mice at the end of the 1st month after DEN treatment. Sham-treated WT and TLR2−/− mice were 1.5 months old.
TLR2<sup>−/−</sup> livers compared to the WT livers (Fig. 5C,D). Based on this finding, we further analyzed the p62 content in the detergent-soluble and -insoluble fractions of WT and TLR2<sup>−/−</sup> livers. The content of p62 in the detergent-insoluble liver fraction was significantly increased in the TLR2<sup>−/−</sup> liver tissue. Moreover, these detergent-insoluble p62 aggregates in the TLR2<sup>−/−</sup> livers were colocализed with LC-3B II and poly-ubiquitin (Fig. 5E,F). Taken together, although the autophagic signaling molecules are activated, the accumulated p62-LC-3B II- and poly-ubiquitin-positive aggregates indicate a failure of autophagy flux in the TLR2<sup>−/−</sup> livers.

**TLR2 Deficiency Reduces Liver-Infiltrating Macrophage Number and Suppresses ASK1/p38 MAPK/NF-κB Signaling and Cytokine Production.** To determine the potential role of immune cells in HCC development in the livers of TLR2<sup>−/−</sup> mice, the liver-infiltrating macrophages were examined by labeling these cells with F4/80 in DEN-treated WT and TLR2-deficient livers. We found that TLR2 deficiency led to a marked decrease in the filtration of F4/80<sup>+</sup> macrophages in the liver compared to the WT condition (Fig. 6A,B). The ASK1/p38 MAPK/NF-κB signaling pathway is a major sensor of oxidative stress that promotes apoptotic cell death. Activation of this pathway leads to the production of cytokines that play important roles in triggering cell death and supporting senescence. Compared to their WT littermates, TLR2<sup>−/−</sup> liver tissue showed a striking decrease in the activity of ASK1, p38 MAPK, and NF-κB (Fig. 6C,D). However, the activity of MAPK ERK1/2 was increased in TLR2<sup>−/−</sup> liver tissue (Fig. 6C,D). These data indicate that the broad-spectrum suppression of the immune response...
Fig. 6. TLR2 deficiency suppresses the immune networks in the liver. (A,B) TLR2 deficiency decreases the number of liver-infiltrating macrophages. The macrophages were labeled with F4/80. The data are representative images of four assays and are presented as the mean ± SEM (n = 4) (B). (C,D) The expression or phosphorylation of the ASK1/p38 MAPK/NF-κB signaling molecules and ERK1/2 was analyzed by western blotting. The data are representative images of immune blots that are presented as the mean ± SEM (n = 4). (E,F) The expression of the indicated cytokines and chemokines was analyzed by western blotting. The data are representative images of immune blots and presented as the mean ± SEM (n = 4) (F).
to DEN-induced liver injury plays a critical role in the attenuated senescence and autophagy flux of TLR2−/− livers, which contributes to their enhanced susceptibility to the development of HCC.

Restoration of Senescence by IFN-γ Reverses TLR2−/−-Exacerbated HCC. Based on the preceding observations, we suspected that restoring senescence might promote the degradation of p62 aggregates and attenuate the development of HCC in TLR2−/− mice. Prophylactic treatment of TLR2−/− mice with IFN-γ, a typical TH1 cytokine that was recently identified as a positive modulator of senescence and autophagy,30-32 attenuated HCC development as indicated by a reduced number and size of tumor nodules in TLR2−/− livers (Fig. 7A,B). Indeed, IFN-γ treatment can restore senescence as indicated by an increase in the SA β-gal staining in the TLR2−/− liver (Fig. 7C,D). The data are representative images of four assays and are presented as the mean ± SEM (n = 4). Scale bar = 75 μm. The expression of senescence-associated molecules was analyzed by western blotting (E,F). The data are representative immune blots and presented as the mean ± SEM of four assays (n = 4 per group).

Fig. 7. IFN-γ alleviates TLR2−/−-exacerbated HCC by reinitiating the senescence response. (A,B) Prophylactic treatment of mice with IFN-γ decreased the numbers and sizes of HCC in the resected livers. WT and TLR2−/− mice were treated with DEN (25 mg/kg) as indicated. One day before DEN treatment the mice were treated with or without IFN-γ (10^6 U/kg every other day) for an additional 4 months and then sacrificed. The H&E staining of liver sections were prepared. Thirty magnification (×10) fields per liver were counted for the tumor numbers and the percentage of tumor area. The scale bars represent the mean ± SEM (n = 20). (C-F) IFN-γ treatment restores suppressed cellular senescence. The DEN-treated WT and TLR2−/− mice were pretreated with or without IFN-γ (10^6 U/kg every other day) for 1 month beginning 1 day before DEN injection. The prophylactic administration of IFN-γ enhanced SA β-gal staining (blue) in the DEN-treated WT and TLR2−/− liver tissues (C,D). The data are representative images of four assays and are presented as the mean ± SEM (n = 4). Scale bar = 75 μm. The expression of senescence-associated molecules was analyzed by western blotting (E,F). The data are representative immune blots and presented as the mean ± SEM of four assays (n = 4 per group).
administration of IFN-γ also attenuated HCC development (Fig. S2E,F) and decreased the appearance of p62-positive punctuate dots in TLR2−/− liver tissue (Fig. 8A). Indeed, the level of p62 in either the detergent-soluble or detergent-insoluble fraction of liver tissues was decreased by IFN-γ treatment (Fig. 8B,C), indicating a recovery of the suppressed autophagy flux in the TLR2−/− livers. Programmed cell deaths by either apoptosis (Fig. 8C) or autophagy (Fig. 8D,E) were increased in the IFN-γ-treated TLR2−/− livers. These data indicate that IFN-γ treatment reverses the TLR2 deficiency-enhanced progression of HCC by restoring the p53/p21/pRb-dependent senescence and autophagy flux in TLR2−/− liver tissue (Fig. 8F).

**Discussion**

We observed increased ROS accumulation, cellular proliferation, and p62 aggregation as well as decreased DNA repair, programmed cell death, and autophagy flux in the TLR2−/− liver tissue in this study. All of these changes are attributable to the loss of cellular senescence as a result of TLR2 deficiency in the liver. Because ASK1/P38MAPK/NF-κB signaling or inflammatory cytokines can initiate and sustain cellular senescence,26-29 the failure of senescence induction can be attributed to the broad-spectrum reductions in the immune responses to DEN injury in the TLR2−/− livers. Indeed, senescent cells enter a unique state characterized by senescence-associated changes, including growth arrest, an arrested cell cycle, SA β-gal expression, a lack of responsiveness to cell death signals, and the senescence-associated secretory phenotype (SASP).33 SASP causes a robust increase in the expression and secretion of numerous cytokines, chemokines, growth factors, and proteases in these cells. These factors, particularly IL-1α, can activate tumor-suppressive pathways to establish and/or maintain senescent growth.
These findings are supported by observations that treatment of TLR2−/− mice with IFN-γ, a typical Th1 cytokine and positive modulator of senescence, attenuates HCC progression by restoring p53/p21-dependent senescence in the liver. Thus, our studies demonstrate a protective role for TLR2-mediated p21- and p16/pRB-dependent senescence in DEN-induced carcinogenesis.

Indeed, DEN-induced ROS production and DNA damage can trigger programmed cell death and maintain a low level of cell proliferation in WT mice because intact TLR2 activity can induce a senescence response after DEN administration. Moreover, the accumulated ROS can be cleared, and damaged DNA can be repaired by the activation of the ASK1/p38 MAPK/NF-κB signaling networks in DEN-treated mice. Together, these networks diminish the development and progression of DEN-induced HCC in WT mice. However, suppressed activation of the ASK1/p38 MAPK/NF-κB signaling pathway results in the persistent accumulation of ROS, which prevents the repair of damaged DNA, decreases programmed cell death, and increases hepatocyte proliferation; the ultimate result is the promotion of HCC development and progression in TLR2−/− mice. These observations are consistent with the findings presented in previous studies. Specifically, it has been reported that the activation of the ASK1/p38 MAPK/NF-κB pathway is critical for both neutralizing ROS/ER stress and repairing damaged DNA in stressed cells. The activation of this pathway is sufficient to activate and maintain cellular senescence. Our work, therefore, indicates that the suppression of broad-spectrum immune responses to DEN injury as a result of TLR2 deficiency plays a critical role in liver carcinogenesis and tumor progression.

The suppressed immune responses mediated by liver-infiltrating immune cells may also play a key role. In this study, decreased infiltration of F4/80+ macrophages was observed in DEN-treated TLR2-mutant livers. This finding is consistent with the suppression of immune signaling pathways and cytokine production in the TLR2-mutant livers. However, these results do not agree with a recent report in which TLR2 deficiency was shown to enhance tumor development in a mouse model of colitis-induced cancer by increasing the number of colon-infiltrating inflammatory cells and the production of inflammatory cytokines in local tissues. Obviously, the difference between the two studies can be attributed to the use of different animal models. In the DEN-induced HCC model, sterile inflammation can be induced by the unfolding protein response to oxidative/ER stress or by PRRs, such as TLR2, interacting with DAMPs released from the damaged liver cells. In the colitis-induced cancer model, the microbial infection recruits a large number of inflammatory cells to the tissue; the tissue-infiltrating inflammatory cells produce inflammatory cytokines, such as IL-6 and IL-17, to promote cancer development. Interestingly, TLR2 mutations promote tumorigenesis in two different cancer models. These studies indicate the complexity of the role of TLR2 activity in the regulation of tumor development. In future studies, it will be worthwhile to determine how TLR2 mutations can affect communication between immune and liver parenchymal cells, particularly as it relates to dissecting the significance of TLR2 in the regulation of HCC development for individual hepatic cell populations using bone marrow chimeras and other molecular approaches.

Our study demonstrates a critical protective role of TLR2-mediated p62-dependent activation of autophagy in DEN-induced tumorigenesis through the clearing of intracellularly accumulated ROS and p62 aggregates. Recent studies indicate that the accumulated ROS and p62 aggregates can form a positive feedback loop, and each of these factors is toxic to the liver and acts as a trigger for HCC development. By clearing p62 aggregates from the cell, autophagy protects the liver from ROS-related ER stress, DNA damage, and carcinogenesis. Moreover, a link has been recently established between autophagy and cellular senescence: autophagy is a consequence of cellular senescence, and it can also trigger cellular senescence. Indeed, TLR2 deficiency attenuated senescence and suppressed autophagy flux can be reversed by the administration of IFN-γ, a positive modulator of senescence and autophagy. Immune networks supporting senescence can attenuate proliferation, programmed cell death, and malignant transformation by promoting effective autophagy flux to eliminate toxic p62 aggregates and interrupt the cycle of p62 aggregation and ROS production.

In summary, although the mechanism by which TLR2 signaling participates in the regulation of cellular senescence to maintain growth arrest and promote programmed cell death remains inconclusive, our studies suggest that the loss of immune networks may play a role in the failure of initiating and maintaining cellular senescence and autophagy flux in the TLR2-mutant liver tissue. These changes account for the enhanced susceptibility of TLR2-deficient mice to DEN-induced HCC.

References
1. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557-2576.
2. Stauffer JK, Scarzello AJ, Jiang Q, Wiltrout RH. Chronic inflammation, immune escape and oncogenesis in the liver: a unique
neighboring for novel intersections. Hepatology 2012 [Epub ahead of print].
3. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell 2005;121:977-990.
4. Szabo G, Dolganius A, Mandrekar P. Pattern recognition receptors: a contemporary view on liver diseases. Hepatology 2006;44:287-298.
5. Huang B, Zhao J, Unkless JC, Feng ZH, Xiong H. TLR signaling by tumor and immune cells: a double-edged sword. Oncogene 2008;27:218-224.
6. Matijevic T, Pavelic J. Toll-like receptors: cost or benefit for cancer? Curr Pharm Des 2010;16:1081-1090.
7. Yu LX, Yan HY, Li Q, Yang W, Wu HP, Dong W, et al. Endotoxin accumulation prevents carcinogen-induced apoptosis and promotes liver tumorigenesis in rodents. Hepatology 2010;52:1322-1333.
8. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007;317:121-124.
9. Seimon TA, Nadolski MJ, Liao X, Magallon J, Nguyen M, Ficic NT, et al. Atherogenic lipids and lipoproteins trigger CD36-TLR2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress. Cell Metab 2010;12:467-482.
10. Seki E, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. Hepatology 2008;48:322-335.
11. Boraska Jelavicz T, Barisic M, Drmic Hofman I, Boraska V, Vrdoljak E, Peruzovic M, et al. Microsatellite GT polymorphism in the toll-like receptor 2 is associated with colorectal cancer. Clin Genet 2006;70:156-160.
12. Yang HZ, Cui B, Liu HZ, Mi S, Yan J, Yan HM, et al. Blocking TLR2 activity attenuates pulmonary metastases of tumor. PLoS One 2009;4:e6520.
13. Lowe EL, Brothter T, Rabizadeh S, Hu B, Wang H, Chen S, et al. Toll-like receptor 2 signaling protects mice from tumor development in a mouse model of colitis-induced cancer. PLoS One 2010;5:e13027.
14. Curtin JF, Liu N, Candolfi M, Xiong W, Assi H, Yagiz K, et al. HMGB1 mediates endogenous TLR2 activation and brain tumor regression. PLoS Med 2009;6:e10.
15. Sakurai T, Maeda S, Chang L, Karin M. Loss of hepatic NF-kappa B activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation. Proc Natl Acad Sci U S A 2006;103:10544-10551.
16. Komatsu M, Kurokawa H, Wargur S, Taguchi S, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat Cell Biol 2010;12:213-223.
17. Yang HZ, Wang JP, Mi S, Liu HZ, Cui B, Yan HM, et al. TLR4 activity is required in the resolution of pulmonary inflammation and fibrosis after acute and chronic lung injury. Am J Pathol 2012;180:275-292.
18. Kennedy AL, Morton JP, Manoharan I, Nelson DM, Jamieson NB, Pawlikowski JS, et al. Activation of the PI3KCA/AKT pathway suppresses senescence induced by an activated Ras oncogene to promote tumorigenesis. Mol Cell 2011;42:36-49.
19. Kang JS, Wanibuchi H, Morimitsu K, Gonzalez FJ, Fukushima S. Role of CYP2E1 in diethylnitrosamine-induced hepatocarcinogenesis in vivo. Cancer Res 2007;67:1141-1146.
20. Kang TW, Yeva T, Woller N, Hoenicke L, Wuestfeld T, Dauch D, et al. Senescence surveillance of premalignant hepatocytes limits liver cancer development. Nature 2011;479:547-51.
21. Nardella C, Clohessy J, Alimonti A, Pantolli PP. Pro-senescent therapy for cancer treatment. Nat Rev Cancer 2011;11:503-511.
22. Campisi J, d’Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 2007;8:729-740.
23. Mathew R, Karp CM, Beaudoin B, Visong N, Chen G, Chen HY, et al. Autophagy suppresses tumorgenesis through elimination of p62. Cell 2009;137:1062-1075.
24. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. N Engl J Med 2009;361:1570-1583.
25. Minzushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010;140:313-326.
26. Dolado I, Swat A, Ajenjo N, De Vita G, Cuadrado A, Nebreda AR. p38alpha MAP kinase as a sensor of reactive oxygen species in tumorigenesis. Cancer Cell 2007;11:191-205.
27. Nakagawa H, Hirata Y, Takeda K, Hayakawa Y, Sato T, Kinosita H, et al. Apoptosis signal-regulating kinase 1 inhibits hepatocarcinogenesis by controlling the tumor-suppressing function of stress-activated mitogen-activated protein kinase. Hepatology 2011;54:185-195.
28. Freund A, Patil CK, Campisi J. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. EMBO J 2011;30:1536-1548.
29. Kennedy NJ, Cellurale C, Davis RJ. A radical role for p38 MAPK in tumour initiation. Cancer Cell 2007;11:101-3.
30. Kim KS, Kang K, Seo YB, Baek SH, Kim JR. Interferon-gamma induces cellular senescence through p53-dependent DNA damage signaling in human endothelial cells. Mech Ageing Dev 2009;130:179-188.
31. Budhu A, Wang XW. The role of cytokines in hepatocellular carcinoma. J Hepatol 2006;48:1197-1213.
32. Yan J, Wang ZY, Yang HZ, Liu HZ, Mi S, Lv XX, et al. Timing is critical for an effective anti-metastatic immunotherapy: the decisive role of IFNalpha/STAT1-mediated activation of autophagy. PLoS One 2011;6:e24705.
33. Freund A, Orjalo AV, Desprez PY. Cancer Cell 2009;13:1062-1075.
34. Park EJ, Lee JH, Yu GY, Ali SR, Holzer RG, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. Cell 2010;140:238-246.
35. Fukata M, Hernandez Y, Conduah D, Cohen J, Chen A, Breglio K, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nat Med 2010;16:238-246.
36. Nasdala C, Ciofally J, Alinomi A, Pantolli PP. Pro-senescent therapy for cancer treatment. Nat Rev Cancer 2011;11:503-511.
37.诶est S, Quante M, Bhagat G, Takashi S, Cui G, Yang XD, et al. Interferon-gamma inhibits gastric carcinogenesis by inducing epithelial cell autophagy and T cell apoptosis. Cancer Res 2011;71:4247-4259.