TLR2 Limits Development of Hepatocellular Carcinoma by Reducing IL18-Mediated Immunosuppression

Shinan Li1, Rui Sun1,2, Yongyan Chen1, Haiming Wei1,2, and Zhigang Tian1,2,3

Abstract

Immune mechanisms underlying hepatocellular carcinoma (HCC) are not well understood. Here, we show that the Toll-like receptor TLR2 inhibits production of the proinflammatory cytokine IL18 and protects mice from DEN-induced liver carcinogenesis. On this protocol, Tlr2−/− mice exhibited more aggressive HCC development associated with impaired CD8+ T-cell function. Furthermore, Ly6C(high)IL18Rα+ myeloid-derived suppressor cells (MDSC) were increased in number in the livers of Tlr2−/− mice before tumor onset. MDSC in this setting exhibited higher iNOS levels that could inhibit IFNγ production and CD8+ T-cell proliferation in vitro. Notably, Tlr2−/− hepatocytes produced more mature IL18 after DEN treatment that was sufficient to drive MDSC accumulation there. IL18 administration was sufficient to induce accumulation of MDSC, whereas hepatocyte-specific silencing of IL18 in Tlr2−/− mice decreased the proportion of MDSC, increased the proportion of functional CD8+ T cells, and alleviated HCC progression. IL18 production was mediated by caspase-8 insufficiency as the decrease in its silencing was sufficient to attenuate levels of mature IL18 in Tlr2−/− mice. Furthermore, the TLR2 agonist Pam3CSK4 inhibited both caspase-8 and IL18 expression, decreasing MDSC, increasing CD8+ T-cell function, and promoting HCC regression. Overall, our findings show how TLR2 deficiency accelerates IL18-mediated immunosuppression during liver carcinogenesis, providing new insights into immune control that may assist the design of effective immunotherapies to treat HCC.

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Introduction

Hepatocellular carcinoma (HCC) is a major threat to human health, and individuals with this disease are usually given a poor prognosis. Inflammatory factors in the HCC microenvironment are implicated in HCC progression. Indeed, various inflammatory cytokines, such as IL1β, IL18, IL6 (1, 2), and IL17 (3, 4), participate in chronic hepatic inflammation, leading to tumorigenesis. IL1β and IL18, both of which belong to the IL1 superfamily and are products of inflammasome activation, have been shown to be important in tumorigenesis. In mice, IL1β promotes metastasis (5) and tumor cell adhesion to endothelial cells (6). In patients with chronic hepatitis C virus (HCV) infection, the IL1β–31 genotype T/T and the IL1β−31 allele T contribute to increased HCC risk (7). In patients with HCV-related HCC, serum IL18 was increased in stage IV patients compared with patients with earlier stage tumors (8), and this increase was accompanied by lower survival rates. In addition, the overall survival rate was significantly lower in IL18 receptor−positive patients than in IL18 receptor−negative patients (9). Although these clinical data suggest the importance of IL18 in HCC progression, the mechanism by which IL18 promotes HCC is unclear.

As important inflammatory initiators, Toll-like receptors (TLRs) are involved in tumorigenesis. Although the role of TLRs in tumorigenesis has been extensively studied (10–14), the role of TLR2 in particular remains obscure. For example, TLR2 was reported to promote gastric tumorigenesis due to STAT3 activation (15), and blocking TLR2 attenuated pulmonary metastases of melanoma and increased both the survival rate and recovery of tumor-killing cells (16). In contrast, TLR2 activation was also shown to inhibit tumor growth by activating mast cells in lung cancer and melanoma (17). Furthermore, TLR2 deficiency led to the downregulation of macrophage-mediated inflammation, which caused a loss of normal hepatocyte senescence and an enhanced susceptibility to HCC (18). This discrepancy suggests that TLR2 can regulate tumor development via different immune mechanisms in different pathologic conditions. However, the role of TLR2 in immunosurveillance of carcinogen-induced HCC is still unknown.

In this study, we demonstrate that TLR2 inhibits production of IL18 and protects mice from DEN-induced HCC. We observed that mature IL18 levels increased after caspase-8 overactivation in Tlr2−/− mice, which then promoted T-cell dysfunction through the induction of myeloid-derived suppressor cells (MDSC). Hepatocyte-specific silencing of IL18 in Tlr2−/− mice or activation of TLR2 by injection of the TLR2 agonist Pam3CSK4 in wild-type mice...
(WT) mice decreased the proportion of MDSCs, increased CD8+ T-cell function, and alleviated HCC progression. Thus, our results reveal a novel mechanism of TLR2-mediated suppression of the tumor-induced immunosuppressive network in HCC and provide new insights into potential tumor immunotherapies.

Materials and Methods

Mice and treatments
Male WT (C57BL/6) and congenic Tlr2−/− mice were bred in specific pathogen-free (SPF) conditions. Animal experiments were approved according to the experimental animal guidelines of the University of Science and Technology of China (Hefei, Anhui, China). Fourteen-day-old WT and Tlr2−/− mice were intraperitoneally injected with 25 mg/kg DEN (cat no. N0258; Sigma-Aldrich) for long-time tumor establishment. For sorting experiments, 6-week-old male C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center at the Chinese Academy of Sciences (Shanghai, China). Mice were subjected to weekly DEN (70 mg/kg) injections for a total of eight treatments beginning at 6 weeks of age to later isolate cells for sorting MDSCs. In experiments silencing caspase-8, 6-week-old male WT and Tlr2−/− mice were intraperitoneally injected with 20 μg shRNA plasmid; 3 days later, DEN (100 mg/kg) was administered by intraperitoneal injection for 48 hours. In experiments silencing IL18, 14-day-old WT and Tlr2−/− mice were intraperitoneally injected with DEN (25 mg/kg) and hydrodynamically injected with 10 μg shIL18 or mock plasmid once every 2 weeks for 7 months beginning at 6 weeks of age. In TLR2 activation experiments, mice were subjected to weekly DEN (70 mg/kg) injections for eight times with or without Pam3CSK4 (10 μg/mouse; InvivoGen). Pam3CSK4 was administered once a week for 7 months beginning at 6 weeks of age. All mice were fed normal chow and sacrificed at the indicated time points.

Flow cytometry
The following antibodies were used for flow cytometry in this study. FITC–anti-Ly6G, TNF-α, and -CD86; PE–anti-Ly6C, and -CD69; PerCP-Cy5.5–anti-CD45 and -CD8a; APC–anti-CD11b, -CD3, and -CXC2; and PE-Cy5–anti-CD62L and -CD44 were purchased from BD Biosciences. FITC–anti-PD-1, APC–anti-perforin, and Alexa Fluor 647–anti-Ki-67 were purchased from eBioscience. Alexa Fluor 647-anti-TLR2 and -IL18R were purchased from BioLegend. Mononuclear cells (MNC) were stained with antibodies as indicated for surface antigens and intracellular cytokines staining as previously described (19, 20). Data were collected on an LSRII flow cytometer (BD Biosciences) and analyzed using the FlowJo software (TreeStar, Inc.).

Histopathologic analysis
Livers were harvested at the indicated time points. Four-micrometer-thick sections were cut from each paraffin block and used for hematoxylin and eosin, proliferating cell nuclear antigen (PCNA; ZSGB-BIO), and anti-IL18 (Santa Cruz Biotechnology) immunohistochemical staining (20).

Western blot analysis
Liver tissues and hepatocytes were collected for Western blot analysis as previously described (21). IL18, TLR2, and caspase-8 were detected after incubation with the corresponding primary antibodies: anti-IL18, anti-TLR2 (Abcam), and anti-caspase-8 (Cell Signaling Technology), respectively. The immunoreactive bands were visualized with chemiluminescence using the ECL Western Blotting Substrate (Pierce) following the manufacturer’s protocol.

ELISA
ELISA kits for IL18 (R&D Systems) and IFNγ (DAKEWE) were used to quantify cytokines.

MDSC induction in vitro
MNCs in the peripheral blood of WT and Tlr2−/− mice were obtained 5 months after DEN treatment. Cells (2 × 10^5) were cultured in flat-bottomed 96-well plates in RPMI-1640 media supplemented with 10% fetal calf serum (Gibco), 20 ng/mL GM-CSF (PeproTech), and 10 ng/mL IL4 (PeproTech). In addition, 100 ng/mL IL18 (ProSpec) was added as indicated. Cells were collected 5 days later, and the proportion of Ly6C^high cells was analyzed by flow cytometry.

MDSC isolation and functional T-cell suppression assay
CD45^−CD11b^−Ly6G^−Ly6C^high cells were sorted from the liver and spleen of DEN-treated (3 months) mice by a FACSaria Cell Sorter (BD Biosciences). CD8^+ T cells (1 × 10^7) from normal spleen activated by anti-CD3/CD28 (10 μg/mL anti-CD3; 5 μg/mL anti-CD28) were cocultured with Ly6C^high (1 × 10^5) cells for 4 days in a round-bottomed 96-well plate at 37°C in 5% CO2. CD8^+ T cells were labeled with CFSE (0.5 μmol/L). Functional CD8^+ T-cell suppression assays were performed by evaluating both IFNγ production in the supernatant and CD8^+ T-cell proliferation.

Knockdown of IL18 and caspase-8 in hepatocytes
An effective shRNA plasmid targeting IL18 was purchased from OriGene. shRNA plasmids targeting caspase-8 were synthesized by GenePharma according to siRNA sequences confirmed to be effective (22). Additional information was provided in Supplementary Materials and Methods.

Quantitative PCR
Total RNA was isolated from liver tissue and real-time qPCR was performed. Additional information was provided in Supplementary Materials and Methods.

Statistical analysis
All statistical analyses were performed using an unpaired Student t test. The data are expressed as the mean ± SEM, and differences were considered statistically significant when P < 0.05 (*), P < 0.05; **, P < 0.01; ***, P < 0.001).

Results

The TLR2 pathway is involved in chemically induced hepatocarcinogenesis
To explore the role of TLR2 in hepatocarcinogenesis, we established a tumor model by a single injection of DEN to 14-day-old male mice. All mice developed HCC 8 months after DEN induction (Fig. 1A). Meanwhile, we observed reduced TLR2 expression on hepatocytes in the livers of DEN-treated mice (Fig. 1B). To examine the effect of TLR2 on hepatocarcinogenesis, the extensively used TLR2 agonist Pam3CSK4 was administered to DEN-induced mice. This treatment attenuated HCC development with reduced tumor number and size (Fig. 1C). Interestingly, the ratio
of liver to body weight decreased after Pam3CSK4 treatment, indicating that HCC-related hepatocyte proliferation was down-regulated (Fig. 1D). Thus, we conclude that TLR2 plays a role in the development of liver cancer.

To further investigate the role of TLR2 in HCC development, WT and Tlr2–/– mice were subjected to DEN treatment. All WT and Tlr2–/– mice developed HCC within 8 months (Fig. 2A), but tumor number and maximum tumor size were dramatically increased in Tlr2–/– mice compared with WT mice. Meanwhile, tumor incidence in Tlr2–/– mice was greater than that in WT mice at different time points (Fig. 2B). Pathologic analysis revealed that Tlr2–/– mice had larger tumor areas than WT mice (Fig. 2C). Tlr2–/– mice with HCC displayed increased PCNA levels, a marker of proliferation (Fig. 2D). Moreover, PCNA levels, a marker of proliferation (Fig. 2D). Moreover, although Tlr2–/– mice had much higher Afp levels than WT mice (Fig. 2E), no differences were observed in expression of the fibrotic markers Col1a1 and Acta2 (α-SMA) between WT and Tlr2–/– mice after DEN treatment (Fig. 2F), suggesting that the severe tumor progression in Tlr2–/– mice was independent of collagen deposition and fibrosis. Therefore, TLR2 normally plays a protective role against hepatocarcinogenesis.

**TLR2 deficiency causes CD8+ T-cell dysfunction during hepatocarcinogenesis**

It is well accepted that tumor development and progression are not only due to DNA mutations but also to the tumor microenvironment. Although T cells are reportedly involved in DEN-induced HCC in immunodeficient mice (23), the mechanism by which T cells become dysfunctional in this model remains unknown. NK cell frequency was decreased in Tlr2–/– mice, but hepatic and splenic NK cell numbers and NK cell expression of PD-1, TIM-3, and TNF-α were similar between WT and Tlr2–/– mice (Supplementary Fig. S1A–S1D). In contrast, we found that hepatic and splenic CD8+ T-cell expression of PD-1, but not TIM-3, was much higher in Tlr2–/– mice, and that TNF-α production was significantly lower (Supplementary Fig. S1C and S1D). Moreover, CD8+ T-cell perforin production was decreased in Tlr2–/– mice following DEN injection (Supplementary Fig. S1E). These data indicate that CD8+ T-cell function is impaired in Tlr2–/– mice following DEN-induced carcinogenesis, suggesting that carcinogenesis is accompanied by immunosuppression.

We further determined whether TLR2 affected hepatocyte growth itself by culturing isolated WT and Tlr2–/– hepatocytes with DEN in vitro. Pcnal and Cnd1 (cyclin D1) mRNA expression as well as PCNA protein expression were increased in Tlr2–/– mice 48 hours after DEN treatment, suggesting that hepatocyte proliferation in Tlr2–/– mice was stronger than that in WT mice (Supplementary Fig. S2A and S2B). These results indicate that the aggravated tumors in Tlr2–/– mice are not only due to enhanced hepatocyte proliferation but also to the immunosuppression present in Tlr2–/– mice.

**Hepatocyte-derived IL18 drives MDSC accumulation in the livers of TLR2-deficient mice during hepatocarcinogenesis**

MDSCs are powerful suppressors of antitumor immunity and can be divided into the following two distinct subsets: granulocytic MDSCs exhibit a CD45+ CD11b+Ly6G+Ly6Chigh phenotype, and monocytic MDSCs exhibit a CD45+ CD11b+Ly6G–Ly6Chioph phenotype. In our model, we found that Ly6Chigh MDSCs were much more prevalent in Tlr2–/– mice than in WT mice in different organs after DEN injection (Fig. 3A and Supplementary Fig. S3A and S3B). To our surprise, there were fewer Ly6G+ MDSCs in...
DEN-treated mice than in normal controls (Supplementary Fig. S3C). Evaluating the kinetics of Ly6Chigh MDSC, Ly6Chigh MDSCs were significantly increased at 5 months post-DEN injection in Tlr2−/− mice compared with WT mice (Fig. 3B and Supplementary Fig. S3D), consistent with the increased development of tumors in Tlr2−/− mice (Fig. 2B). Because Arg1 (Arginase) and Nos2 (iNOS) were increased in the liver after DEN treatment (Fig. 3C), we found that iNOS inhibition could attenuate the inhibitory effects of Ly6Chigh MDSCs on IFNγ production and CD8+ T-cell proliferation upon stimulation with anti-CD3 and anti-CD28 antibodies (Fig. 3D). Meanwhile, WT and Tlr2−/− MDSCs similarly inhibited IFNγ production and CD8+ T-cell proliferation (Fig. 3E), and we also found no differences in chemokine or chemokine receptor expression between WT and Tlr2−/− MDSCs (Supplementary Fig. S3E). Moreover, Tlr2−/− and WT MDSCs showed a similar phenotype and activation status with similar expression of CD62L, CXCR2, CD44, and CD86 (Supplementary Fig. S3F). The only observed difference between Tlr2−/− and WT MDSCs was increased Ki-67 in Tlr2−/− Ly6Chigh MDSC after DEN treatment (Supplementary Fig. S3G). These data indicate that Ly6Chigh MDSCs may promote HCC via iNOS-mediated inhibition of antitumor immunity, and the enhanced MDSC accumulation in Tlr2−/− mice may be due to their augmented proliferative ability.

To identify factors that contribute to MDSC accumulation in the liver, we examined a set of inflammatory cytokines and found that only Il18 mRNA was increased in the livers of Tlr2−/− mice (Fig. 4A). In addition, IL18 levels gradually increased in the serum and liver tissue over time (Supplementary Fig. S4A and S4B). Immunohistochemistry and immunofluorescence demonstrated that hepatocytes were the main IL18 producers (Fig. 4B and Supplementary Fig. S4C). We also isolated hepatocytes and hepatic nonparenchymal cells to analyze IL18 protein levels by Western blot analysis and found that Tlr2−/− hepatocytes produced more mature IL18 than WT hepatocytes after DEN treatment. In contrast, no differences in IL18 production by liver nonparenchymal cells were observed between Tlr2−/− and WT mice (Fig. 4C).

The increased IL18 expression and greater monocytic MDSC accumulation we observed here after DEN injection raised the possibility that hepatocyte-derived IL18 might be linked to MDSC accumulation. To test this hypothesis, we detected the IL18 receptor IL18Rα on MDSCs and found that it was upregulated on MDSCs compared with normal controls. Ly6Chigh MDSCs, but not Ly6G+ MDSCs, from Tlr2−/− mice expressed higher IL18Rα than WT mice (Fig. 4D), indicating that IL18 might drive Ly6Chigh MDSC accumulation in the livers of Tlr2−/− mice. As expected,
recombinant IL18 induced Tlr2−/−/Ly6Chigh MDSC accumulation when MNCs were cultured with IL18 in vitro, especially following DEN treatment (Fig. 4E). When IL18 was overexpressed in hepatocytes by hydrodynamically injecting an IL18-expressing plasmid in vivo (Supplementary Fig. S4D), we found a dose-dependent increase in MDSC accumulation in the liver (Fig. 4F). We also knocked down IL18 in hepatocytes by hydrodynamically injecting an shIL18 plasmid (Supplementary Figs. S4C and S5A) once every 2 weeks for 7 months (Fig. 5A). Although no difference in tumor growth was observed between WT/mock and WT/shIL18 mice after 8 months of DEN treatment, silencing IL18 in hepatocytes significantly attenuated tumor progression in Tlr2−/− mice (Fig. 5B and C), which was accompanied by decreased accumulation of Ly6Chigh MDSCs (Fig. 5D and Supplementary Fig. S5B) and recovered TNF-α production by CD8+ T cells (Fig. 5E and Supplementary Fig. S5C and S5D).

TLR2 deficiency leads to activation of the caspase-8–IL18 axis

We then examined the relationship between TLR2 and IL18. We found that Casp8 mRNA, but not Casp1 or Casp3 mRNA, was significantly increased after DEN treatment in Tlr2−/− mice, although no differences were observed in inflammasome-related Pycard or Nlph3 mRNA expression (Fig. 6A). Interestingly, Pam3CSK4 agonist-stimulated TLR2 activation inhibited caspase-8 expression (Fig. 6B and Supplementary Fig. S6A), raising the possibility that IL18 correlated with TLR2-mediated activation of caspase-8. Therefore, we designed a caspase-8 shRNA (22) to explore the effect of caspase-8 on IL18 synthesis (Supplementary Fig. S6B). Although we found no differences between WT/mock and WT/shcaspase-8 mice in terms of liver injury (ALT levels) or IL18 expression, Tlr2−/−/shcaspase-8 mice exhibited decreased serum ALT (Fig. 6C) and IL18 (Fig. 6D) levels compared with Tlr2−/−/mock mice 48 hours after DEN injection. Consistent with the higher ALT and IL18 levels in Tlr2−/− mice, we found that purified hepatocytes from Tlr2−/−/shcaspase-8 mice had lower mature IL18 than control mice (Fig. 6E), suggesting that IL18 expression might be caspase-8-dependent in Tlr2−/− mice.

TLR2 agonist attenuates DEN-induced HCC via inhibiting the IL18–MDSC–CTL interaction

In Fig. 1C, we showed that the TLR2 agonist Pam3CSK4 alleviated HCC progression. We further found that Pam3CSK4 treatment reduced both Ly6Chigh and Ly6G+ MDSCs (Fig. 7A and Supplementary Fig. S7A), and this treatment also importantly reduced serum IL18 levels (Fig. 7B). Notably, we did not observe any decreased IL18 in the serum and liver tissue of mice 8 months
after DEN and Pam3CSK4 treatment (Fig. 7B and Supplementary Fig. S7B), which might have been due to the decreased TLR2 expression on hepatocytes during the advanced stages of tumor development (Fig. 1B). Pam3CSK4 stimulation also upregulated the frequency and number of activated CD8\(^+\) T cells as assessed by CD69 expression (Fig. 7C). Moreover, in inflammatory cytokines known to play crucial roles in antitumor activity were increased after Pam3CSK4 treatment in WT mice (Fig. 7D). Together, these results demonstrate that TLR2 activation by Pam3CSK4 may inhibit HCC progression by inhibiting the IL18–MDSC–CTL interaction.

**Discussion**

TLR2 is an important component of innate immunity. Here, we explored the role of TLR2 in HCC progression. To our knowledge, our data are the first to show that TLR2 activation may control IL18 expression by inhibiting caspase-8 activation in the DEN-induced HCC model, leading to decreased MDSC induction and maintenance of antitumor CTL function (Fig. 4A). In Tlr2\(^{-/-}\) mice, we observed increased HCC progression, upregulation of IL18, and decreased MDSC accumulation in the liver, and dysfunctional CTL cells, which could all be reversed by hepatocyte-specific silencing of IL18 or administration of the TLR2 agonist Pam3CSK4. As important inflammatory initiators, TLRs are involved in tumorigenesis and tumor immunosurveillance. Recently, TLR activation was explored as a novel immunotherapeutic approach. For example, TLR3 activation can drive tumor infiltration of T and NK cells, leading to enhanced cancer cell death (10). A combination of agonists targeting TLR7, 8, and 9 increases the number and tumoricidal activity of tumor-infiltrating CTL and NK cells, establishing long-term protective immunity (14). In this study, we demonstrate that TLR2 may be another potential candidate target for HCC immunotherapy, similar to TLR3, 7, 8, and 9 in other tumors.

Many studies have demonstrated a correlation between TLR2 and HCC in the clinical setting. Reduced TLR2 expression on hepatocytes was noted in the livers of patients with HBeAg-positive chronic hepatitis B, and this was associated with a functional decrease in cytokine production in peripheral CD14-positive monocytes (24). This result is consistent with the conclusions drawn from our findings in DEN-induced HCC mice. Upon analysis of liver samples from patients at different stages of liver disease, Soares and colleagues found that Tlr2 and Tlr4 mRNA were decreased in patients with hepatocarcinoma (25). In addition, several studies have demonstrated that single-nucleotide polymorphisms (SNP) in Tlr2 are associated with HCC susceptibility (26, 27). The frequency of the Tlr2-196-174 del allele was significantly higher in patients with HCV-associated HCC. This phenomenon was noted not only in HCC but also in gastric cancer (28), colon cancer (29), and prostate cancer (30), further indicating the importance of TLR2 in tumor immunosurveillance.

In our study, we used an experimental HCC model to explore the role of TLR2 in hepatocarcinogenesis. Consistent with the...
work of Lin and colleagues (18), we also observed that TLR2 protected mice from DEN-induced tumors. However, we explored an entirely different mechanism for HCC progression in Tlr2<sup>-/-</sup> mice. Although Lin and colleagues focused on the TLR2-mediated loss of immune-supported (mostly via cytokines) hepatocyte senescence (e.g., loss of apoptotic and autophagic death) that made Tlr2<sup>-/-</sup> mice more susceptible to HCC, we focused instead on another aspect of the immune network (e.g., the TLR2–caspase-8–IL18–MDSC–CTL axis) and found that the proinflammatory cytokine IL18 was critical for the progression of liver cancer in Tlr2<sup>-/-</sup> mice. Both studies have revealed two different but critical mechanisms of HCC development (e.g., intrinsic and extrinsic pathways) in Tlr2<sup>-/-</sup> mice: while our work emphasizes the role of immune cells, the work by Lin and colleagues emphasizes hepatocyte senescence in inflammatory conditions.

IL18 is a well-known proinflammatory cytokine, but the precise role of IL18 in tumor immunity remains controversial. IL18 can activate immune cells against malignant cells. IL18 can convert NK cells into “helper” cells, which attract immature DCs via CCL3 and CCL4. CXCR3 and CCR5 ligands produced by DCs facilitate the recruitment of type I effector CD8<sup>+</sup> T cells against tumors (33). Conversely, studies by the Termé and colleagues show that IL18 suppresses the NK cell arm of tumor immunosurveillance in a PD-1–dependent manner by inducing Kit<sup>+</sup>CD11b<sup>+</sup> NK cells overexpressing B7-H1/PD-L1 (34, 35). Although MDSCs have been reported to play an important role in tumor progression (36, 37) and the inflammasome component NLRP3 has been confirmed to contribute to MDSC accumulation (38), the exact role of IL18 in MDSC generation is still unknown. In our study, we demonstrate for the first time that IL18 can induce MDSC accumulation in vitro and in vivo (Figs. 4 and 5). When we silenced IL18 by shRNA in vitro, we detected a decrease in LysC<sup>high</sup> MDSCs and subsequent tumor reduction in Tlr2<sup>-/-</sup> mice (Fig. 5B). This result indicates that tumor development is a complex process, and IL18 may not be the dominant mechanism regulating tumor development after DEN treatment in WT mice;
this idea is supported by our data showing that the number of Ly6C<sup>high</sup> MDSCs and CD8<sup>+</sup> T cells were mildly, but not significantly, changed after shIL18 treatment in WT mice (Supplementary Fig. S5B–S5D). In Tlr2<sup>-/-</sup> mice, loss of TLR2 likely permits potent IL18 expression, allowing the TLR2–caspase-8–IL18–MDSC pathway to become prominent. These results suggest that potential tumor therapy targeting IL18 in patients with HCC should take into account the TLR2 expression status of the individual patient.

There are several mechanisms that can lead to IL1β and IL18 production. Inflammasome activation is a well-known pathway for IL1 and IL18 maturation. Inflammasomes are multiprotein complexes that include NLRs/AIM2, ACS, and pro-caspase-1. When they assemble and operate as structural platforms, pro-caspase-1 is cleaved into caspase-1, allowing the release of mature IL1β or IL18 (39). In addition to this canonical inflammasome activation pathway, noncanonical pathways, including caspase-8 (40) and caspase-11 (41), are also involved in generating mature...
Although inflamasome activation was shown to be important for tumor development, we did not observe IL1β or caspase-1 upregulation in Th2 / T cell- compared with WT mice in our tumor model (Figs. 4A and Fig. 6A). Only IL18 and caspase-8 were elevated after DENV treatment, suggesting that caspase-8 was responsible for IL18 maturation via a noncanonical pathway. Interestingly, a previous study also found that caspase-8 was elevated in the absence of TLR2 signaling (42) along with a decrease in c-FLIP (43)—a reported caspase-8 inhibitor (44)—leading to activation of the caspase cascade. Although our data indicate a role for caspase-8 in processing mature IL18 (Fig. 6D), the precise signaling pathway involved in the DEN model still requires further investigation.

CTL exhaustion is recognized as a key mechanism underlying the loss of immunosurveillance. During tumor progression, CD8 T-cell function is impaired, with lower cytokine secretion, cytotoxicity, and expression of exhaustion markers, such as TIM-3, LAG-3, PD-1, and CTLA-4. Suppressive cytokine, such as IL10, and immune-suppressor cells, including MDSCs and Treg cells, also contribute to CD8 T-cell dysfunction. MDSCs inhibit CD8 T-cell function by sequestering cytokine that T cells require for activation (45) and by inducing Treg cells, which occurs independently of nitric oxide (NO) production (46). Reactive oxygen species (ROS) and NO are two factors through which MDSCs induce T-cell exhaustion, where ROS leads to T-cell unresponsiveness by posttranslational modification of T-cell receptors, and NO induces apoptosis (37). Furthermore, the number of MDSCs, and PD-1–exhausted T cells were increased in patients with HCC (47), a phenomenon that we also observed in this study, illustrating that our animal model could mimic clinical observations. MDSCs, especially the monocytic subset, played an immunosuppressive role in our DEN model by directly targeting CD8 T cells.

In summary, we reveal that TLR2 plays a critical role in inhibiting hepatocarcinogenesis using the DEN-induced HCC animal model. Our data demonstrate that IL18, a cytokine dependent on caspase-8 processing, can induce MDSC accumulation in the livers of Th2 / T mice. These MDSCs can then subsequently suppress antitumor immunity. Silencing IL18 using a hepatocyte-specific shRNA in Th2 / mice or systemic administration of the TLR2 agonist Pam3CSK4 in WT mice can abolish the caspase-8/IL18-MDSC-CTL interaction and inhibit tumor growth. Thus, this study reveals for the first time that TLR2 plays a preventative role in tumor development via inhibiting the caspase-8-IL18-MDSC axis, and these findings may provide new insights into HCC immunotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Li, Z. Tian
Development of methodology: S. Li, R. Sun, H. Wei, Z. Tian
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Li, Y. Chen, Z. Tian
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Li, H. Wei, Z. Tian
Writing, review, and/or revision of the manuscript: S. Li, H. Wei, Z. Tian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Li, Y. Chen, H. Wei, Z. Tian
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