Intracellular Osteopontin Inhibits Toll-like Receptor Signaling and Impedes Liver Carcinogenesis

Xiaoyu Fan¹², Chunyan He¹, Wei Jing³, Xuyu Zhou³, Rui Chen¹², Lei Cao¹, Minhui Zhu³, Rongjie Jia¹², Hao Wang¹², Yajun Guo¹², and Jian Zhao¹²

Abstract

Osteopontin (OPN) has been implicated widely in tumor growth and metastasis, but the range of its contributions is not yet fully understood. In this study, we show that genetic ablation of Opn in mice sensitizes them to diethylnitrosamine (DEN)-induced hepatocarcinogenesis. Opn-deficient mice (Opn⁻/⁻ mice) exhibited enhanced production of proinflammatory cytokines and compensatory proliferation. Administering OPN antibody or recombinant OPN protein to wild-type or Opn⁻/⁻ mice-derived macrophages, respectively, had little effect on cytokine production. In contrast, overexpression of intracellular OPN (iOPN) in Opn⁻/⁻ deficient macrophages strongly suppressed production of proinflammatory cytokines. In addition, we found that iOPN was able to interact with the pivotal Toll-like receptor (TLR) signaling protein MyD88 in macrophages after stimulation with cellular debris, thereby disrupting TLR signaling in macrophages. Our results indicated that iOPN was capable of functioning as an endogenous negative regulator of TLR-mediated immune responses, acting to ameliorate production of proinflammatory cytokines and curtail DEN-induced hepatocarcinogenesis. Together, our results expand the important role of OPN in inflammation-associated cancers and deepen its relevance for novel treatment strategies in liver cancer.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignant carcinomas in the world and is known for its high malignancy, quick progression, and poor prognosis. It leads to more than 500,000 deaths per year (1). Major HCC risk factors include viral infection, toxicant and drug metabolic intermediates, and alcohol (2, 3). Diethylnitrosamine (DEN) is a hepatic carcinogen, which can be metabolized into an alkylating agent that induces DNA damage and mutation (4). Because DEN-induced HCC has histologic and genetic features similar to human HCC (5), it has been widely used as a hepatic carcinogen to explore human HCC.

Osteopontin (OPN) is a phosphorylated glycoprotein that is expressed in various tissues (6). In liver, OPN is related to steatohepatitis (7), liver fibrosis progression (8), and liver cancer. In patients with HCC, elevated plasma OPN level is closely related to liver function deterioration and has a positive correlation to tumor stage. Therefore, plasma OPN levels are also regarded as a potent diagnosis biomarker (9). Meanwhile, its disturbance can hamper the growth and metastasis of HCC in vitro and in vivo (10). All these results reveal that OPN aggravates growth and metastasis of HCC. Nevertheless, the role of OPN during HCC initiation is poorly understood.

Alternative splicing of OPN results in three isoforms, OPN-a, OPN-b, and OPN-c (11) and alternative translation of OPN generates two isoforms, secreted OPN (sOPN) and intracellular OPN (iOPN; ref. 12). sOPN has been recognized as an extracellular protein and participates in several physiologic and pathologic events, including immune regulation (6), in inflammation (13), tumor progression, and metastasis (14). iOPN is a shortened protein that lacks the N-terminal signal sequence of sOPN and mainly localizes to cytoplasm (12). It was first found in rat calvarial cells (15) and now has been found in other kinds of cells, such as dendritic cells (12, 16), macrophages (17), and nerve cells (18). iOPN takes part in several biologic functions. In the nucleus of 293 cells, iOPN colocalizes with polo-like kinase 1 and ERM (ezrin/radixin/moesin) complex at perimembrane regions and plays a role in cell migration (20). iOPN is also an adaptor molecule in innate immunity. In plasmacytoid dendritic cells, it interacts with myeloid differentiation primary response gene 88 (MyD88) and enhances interferon-α production through Toll-like receptor 9 (TLR9) signaling (16). In antifungal innate immunity, it participates in cluster formation of fungal receptors, as an adaptor molecule of TLR2 and dectin-1 signaling pathways, to enhance phagocytosis and clearance of fungus (21).

TLRs are pattern recognition receptors, and participate in innate immune responses against microbial pathogens (22). TLRs activate activator protein-1 (AP-1)/nuclear factor-κB (NF-κB) and...
interferon-regulatory factor (IRF) to increase proinflammatory cytokine expression through adaptor molecules MyD88 and TIR-domain−containing adapter-inducing interferon-β (TRIF; ref. 23). Here, we found that iOPN is recruited to MyD88 under stimulation of cellular debris released by necrotic hepatocytes, and negatively regulates TLR signaling in macrophages, which leads to reduced proinflammatory cytokine production and hepatocarcinogenesis in DEN-treated mice. These findings suggest that iOPN may function as an endogenous negative regulator of TLR-mediated immune responses to ameliorate inflammation-associated hepatocarcinogenesis.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Sciences, and the strain was introduced from The Jackson Laboratory in 2005. Opg−/− mice (B6.Cg-Spp1tm1bhi/J; cat. no. 004936) were purchased from The Jackson Laboratory. Mice in this study were hosted in a pathogen-free facility under standard 12-hour light-dark cycle, fed standard rodent chow, and water ad libitum. All animals were maintained in accordance with the guidelines of the Committee on Animals of the Second Military Medical University (Shanghai, China).

Animal treatment

For hepatocarcinogenesis, mice were injected intraperitoneally (i.p.) with 25 mg/kg of DEN (Sigma) at 14 days of age and then sacrificed at the indicated times. For short-term studies of inflammation and liver injury, 6- or 8-week-old male mice were injected i.p. with 100 mg/kg of DEN and sacrificed at the indicated times. For subcutaneous injection, 6- or 8-week-old male mice were injected i.p. with 50 mg/kg of DEN 24 hours before inoculation.

Isolation and cell culture

Primary hepatocytes were isolated as described (24). Briefly, the liver was perfused in situ with liver perfusion solution (Gibco). The cell suspension was filtered through a 70 μm cell filter (BD Falcon) and the filtrate centrifuged three times at 50 × g for 1 minute. The resultant cell pellets were hepatocyte-rich fraction. Hepatocytes were identified by periodic acid-Schiff staining and the purity regularly exceeded 85%. Isolated hepatocytes were then frozen (-80°C), thawed (room temperature) three times, and filtered to prepare cellular debris released by necrotic hepatocytes. The supernatant run on an agarose gel showed no sign of DNA fragmentation, as would be seen in apoptosis. The concentration used to stimulate the macrophages was 10^6 necrotic hepatocytes/ml.

Bone marrow–derived macrophages were prepared as described (25). Both femurs and tibias were dissected and flushed. Cells were incubated with red cell lysis buffer (Beyotime) to obtain pure macrophages. After rinses, cell suspensions were cultured in DMEM (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (HyClone), 20 ng/ml Murine Macrophage Colony Stimulating Factor (Peprotech), and penicillin-streptomycin solution (1×; Bioyight) at 37°C in a humidified incubator containing 5% CO2. After overnight incubation, nonadherent cells were removed by three successive washes with PBS. Macrophages were identified by flow cytometry with PE anti-mouse F4/80 antibody and FITC anti-mouse/human CD11b antibody (BioLegend). The purity of the isolated subpopulations regularly exceeded 85%.

HEK 293T and Hepa 1-6, a mouse hepatoma cell line derived from C57BL/6 mice, were purchased from Cell Bank of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Cells were cultured at 37°C in a humidified atmosphere of 5% CO2, in DMEM supplemented with 10% FBS.

Construction of expression vectors

Murine full-length (secreted) Opg (Spp1) cDNA was generated by RT-PCR. BamHI and EcoRI sites were introduced into the PCR primers for cloning into the pcDNA3.0 vector (Invitrogen), primers: forward, CAGGGATCCATGAGATGGCGATGATTTCC; reverse, GGCGAATTCGTTGACCTGACGAAGATGAAGTC. The expression plasmid for intracellular form of Opg (iOpg) deleting the codons from 1 to 15 was generated from sOpg expression plasmid by PCR-mediated mutagenesis with the QuickChange II XL Site-directed Mutagenesis Kit (Stratagene). Murine MyD88 cDNA was generated by RT-PCR. EcoRI and XhoI sites were introduced into pcDNA3.0 vector, primers: forward, GTGAATTCTGGTGACCTGACGAAGATGAAGTC; reverse, GACTCGAGTCCAGGGCGACGACAAAAG. The preceiver-myc-IKIk1 vector was purchased from the Guangzhou Fulen Gene Company.

RNA isolation and quantitative RT-PCR

Total RNA was isolated using the Nucleospin RNA (Machery-Nagel). First-strand synthesis was performed with random primers and reverse transcription with the SuperScript III Reverse Transcriptase (Tiangen Biotech). Quantitative RT-PCR was performed using SYBR Green reagent (Roche). Reactions were performed twice in triplicate, and actin values were used to normalize gene expression. The primer sequences can be obtained from Supplementary Table S1.

Biochemical analysis and ELISA

Serum alanine transaminase (ALT) levels in liver were measured using commercial kits (Nanjing Jiancheng). Levels of IL-6 and TNFα from serum and cell supernatant were quantitated with ELISA kits (eBioscience) according to the manufacturer’s instructions. Cell supernatant of bone marrow–derived macrophages for OPN quantification was quantitated with the mouse OPN ELISA Kit (R&D Systems). Preparation of cell supernatant was as follows. 2 × 10^6 macrophages were seeded into 24-well plates; after culturing for 5 days, medium was discarded, and replaced by serum-free medium with cellular debris from Opg−/− necrotic hepatocytes. Supernatant was collected at the indicated times.

PathScan Inflammation Multi-Target Sandwich ELISA kit (Cell Signaling Technology) was used to detect endogenous levels of transcription factors: NF-κB p65, phospho-NF-κB p65 (Ser536), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-STAT3 (Tyrr705), and phospho-heat shock protein (Ser32). Each phosphoprotein absorbance was corrected by the negative control and normalized by its relevant NF-κB p65 absorbance.

Western blotting

Cells were homogenized in 1× SDS lysis buffer (62.5 mmol/L Tris–HCl, 2% w/v SDS, 10% glycerol, 50 mmol/L DTT, 0.01%
w/v bromophenol blue). Frozen tissues were prepared in ice-cold RIPA lysis buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 1% w/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS) supplemented with Mammalian Protease Inhibitor Mixture (100×; BioColors) and protein concentration of the extracts was measured by the BCA Protein Assay Kit (Thermo Scientific) for Western blotting. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (0.45 μm, Millipore). After probing with individual antibodies, the antigen–antibody complex was visualized by Enhanced Chemiluminescence's Reagents Supersignal (Pierce Biotechnology). The antibodies used in this study are listed in the Supplementary Table S2.

Coimmunoprecipitation analysis

For detecting endogenous levels of interaction, with or without stimulation by cellular debris released by necrotic hepatocytes, macrophages from WT or Opn−/− mice were lysed by RIPA lysis buffer and incubated with anti-OPN antibody (Mouse monoclonal antibody 23C3, details in Supplementary Methods; 1:50), anti-MyD88 monoclonal antibody (Cell Signaling Technology; 1:50), or normal IgG (Cell Signaling Technology; 1:50) overnight at 4°C. To detect interaction in vitro, HEEK 293T cells were cotransfected with MyD88-pcDNA3.0, preceiver-myc-IRAK1, and iOpn-pcDNA3.0/pcDNA3.0 plasmids. At 48 hours posttransfection, cells were incubated with cellular debris for 4 hours and then the total cellular lysates were lysed by RIPA lysis buffer and incubated with anti-MyD88 antibody or normal IgG overnight at 4°C. Precleared protein A/G-Sepharose (Santa Cruz Biotechnology) was used to isolate antibody-bound proteins; precipitated complexes were separated by SDS-PAGE and subjected to Western blotting analysis.

Histologic, immunohistochemical assay

Formalin-fixed, paraffin-embedded liver tissues were used for hematoxylin and eosin (H&E), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), 5-bromo-2′-deoxyuridine (BrdUrd), proliferating cell nuclear antigen (PCNA), and F4/80 staining. Apoptosis was assessed by TUNEL staining with the TUNEL Detection Kit (Calbiochem) according to the manufacturer's instructions. Proliferation was assessed by BclId (Sigma) and PCNA monoclonal antibody (Cell Signaling Technology). Mice were injected i.p. with BclId (1 mg/mL) 2 hours before sacrifice for proliferation assessment and paraffin sections were stained using the BclId in situ Detection Kit (BD Biosciences) according to the manufacturer's instructions. PCNA was assessed by the PCNA antibody (1:50); more details are available in Supplementary Material and Methods.

Immunofluorescent assay

For the visualization of macrophages, immunofluorescence analysis with F4/80 specific monoclonal antibody (Santa Cruz Biotechnology) was used. The slides were dewaxed, hydrated, and washed. After microwave antigen retrieval, slides were blocked and then incubated with the antibody against F4/80 (1:50) overnight at 4°C, and Cy3-labeled anti-rat IgG (Beyotime Biotechnology; 1:500) was used as a secondary antibody. Finally, the slides were washed and mounted with DAPI (Djioi Laboratories; 1:1,000) for 5 minutes. Images were captured using a Leica DMIRB Fluorescence Microscope (OLYMPUS IX71).

Confocal microscopy

After culturing for 5 days in 24-well plates, macrophages were treated with cellular debris released by necrotic hepatocytes for indicated times, and then fixed with 4% paraformaldehyde. OPN and MyD88 were detected with mouse anti-OPN (23C3; 1:100) and rabbit anti-MyD88 polyclonal antibody (Santa Cruz Biotechnology; 1:50), respectively, followed by secondary Alexa Fluor 488–conjugated anti-mouse IgG (OPN; Life Technologies; 1:250) or Alexa Fluor 555–conjugated anti-rabbit IgG (MyD88; Life Technologies; 1:250). Nuclei were stained with DAPI. Stained cells were viewed with a confocal microscope (Zeiss LSM 510).

Antibody array

According to the manufacturer's instructions, serum of tumor-bearing mice was obtained and incubated with the mouse angiogenesis antibody array membranes (Panimics) for 2 hours at room temperature. Then serum was discarded and the biotin-labeled detection antibodies mixture was added and incubated with membranes for 2 hours at room temperature. Streptavidin–horseradish peroxidase was next added and then incubated for 1 hour at room temperature. Chemiluminescence substrated and films (Kodak) were used for detection of spots. Films were scanned and spots were quantitated using Quantity One software.

Statistical analysis

Data expressed are means ± SE. Differences were analyzed by the Student t test, and P values < 0.05 were considered as significant. For the overall survival analysis, the log-rank test was used in assessing the significance seen in the Kaplan–Meier curve.

Results

OPN ablation greatly enhances chemically induced hepatocarcinogenesis in mice

Upon DEN injection on postnatal day 14, all wild-type (WT) and Opn knockout (Opn−/−) males developed HCCs within 9 months (Fig. 1A). Strikingly, we observed a significant increase in tumor numbers in Opn−/− mice, about three times compared with that in the WT counterparts (Fig. 1B). The percentages of tumor occupied area were approximately 5% and 40% in WT and Opn−/− mice, respectively (Fig. 1C). The maximal tumor diameters were also notably larger in Opn−/− mice compared with WT controls, 17.5 ± 2.5 mm vs. 6.4 ± 2 mm (Fig. 1D). To analyze the survival difference between WT and Opn−/− mice, another group of mice was sacrificed at 16 months after DEN injection. Kaplan–Meier survival curves (Fig. 1E) clearly showed that Opn−/− mice had a significantly shorter survival time.

To further determine whether the enhanced hepatocarcinogenesis was due to the alteration of host microenvironment, Hepa1-6, a mouse hepatoma cell line derived from C57BL/6 mice, was injected subcutaneously in DEN-pretreated Opn−/− and WT mice. Hepa1-6 cells were transfected with siControl or siOpn and extracted for detecting OPN expression (Supplementary Fig. S1A). All mice were sacrificed 14 days postinoculation; gross appearance, tumor weight, and tumor volume were monitored (Supplementary Fig. S1B). Tumor tissues were lysed and then analyzed for expression of OPN and GAPDH (as internal control) protein (Supplementary Fig. S1C). Interestingly, Hepa1-6-xenografted tumors were much bigger in
mice than those in the WT mice. Moreover, when Opn was silenced in Hepa1-6 cells to eliminate endogenous tumor-derived OPN, the xenografted-tumors were still bigger in Opn−/− mice than those in WT mice. Analysis of macrophage infiltration by F4/80 staining revealed less infiltration in siOpn-transfected xenografts than that in siControl-transfected xenografts, but not significantly. And no difference was observed in macrophage infiltration between the xenografts formed from the same cells in WT and Opn−/− mice (Supplementary Fig. S1D and S1E). These findings suggest that host-derived OPN might have a negative effect on the inflammatory microenvironment in DEN-induced hepatocarcinogenesis.

Deletion of Opn exhibits enhanced cell turnover and survival signaling

Besides a high incidence of HCC, liver tumors in Opn−/− mice displayed strongly elevated apoptotic (TUNEL; Fig. 2A and C) and proliferating (BrdUrd and PCNA) tumor cells compared with those in WT mice (Fig. 2B and C), indicating enhanced cell turnover in Opn−/− tumors. Consistently, malignant liver tumors in Opn−/− mice displayed strongly increased levels of cyclin D1 and c-Myc, which are needed for cell proliferation, compared with WT controls (Fig. 2D). We next used the Inflammation Multi-Target Sandwich ELISA Kit to examine several key regulatory proteins in signaling pathways controlling the stress and inflammation response, including phospho-NF-κB p65, phospho-p38 mitogen-activated protein kinase (MAPK), phospho-signal transducer and activator of transcription 3 (STAT3), phospho-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and phospho-inhibitor-kB (IkB-α; Fig. 2E). Tumor tissues of livers were prepared. Opn−/− mice exhibited a larger degree of increase in phospho-NF-κB p65, in which activation correlates with proliferation, apoptosis, and inflammation (26). In Opn−/− tumors, there were also obvious elevations in phospho-p38 and phospho-STAT3 levels, as well as a slight increase in phospho-IκB-α. Phospho-p38 and phospho-STAT3 are activated by a variety of cellular stresses, including inflammatory cytokines, lipopolysaccharides (LPS), and growth factors (27, 28). STAT3 is activated in response to various cytokines and growth factors and mediates the expression of a variety of genes controlling cell growth and apoptosis (29). Phospho-IκB-α, an inhibitory protein of NF-κB, was too low to be detected (data not shown). Elevated activation of these proliferation and inflammation-related key regulators indicated that enhanced proliferation of hepatocytes and proinflammatory response could be induced by Opn deficiency.

Figure 1.
Opn ablation promotes DEN-induced hepatocarcinogenesis in mice. A, gross and histologic appearance (H&E staining) of 9-month-old DEN-treated male WT and Opn−/− mice. Scale bars, 50 μm. B, tumor numbers; C, tumor incidence; and D, maximum tumor sizes (diameters) in livers of male WT (n = 14) and Opn−/− (n = 14) mice 9 months after DEN injection. Data, mean ± SD; **, P < 0.01. E, Kaplan–Meier survival graph of 16-month-old DEN-treated male WT (n = 10) and Opn−/− (n = 10) mice. Survival of the Opn−/− mice was significantly lower than the other group. Log-rank test P < 0.001.
Oprn deficiency aggravates liver cell death and compensatory proliferation after DEN treatment

Because the deficiency of Oprn could increase the susceptibility of mice to DEN-induced hepatocarcinogenesis, which might be related to enhanced cell turnover, we therefore examined the short-term response elicited by DEN in vivo. Oprn−/− mice exhibited higher ALT level in serum, indicative of liver injury, than that in WT mice 24 and 48 hours after DEN injection (Fig. 3A). There was also an increased number of TUNEL-positive cells in Oprn−/− mice compared with WT mice (Fig. 3B and D). These results indicated more hepatocyte death in Oprn−/− mice. Liver has regenerative capacity, and cell death might lead to compensatory proliferation of surviving hepatocytes. Differences in proliferation at 24 and 48 hours after DEN injection matched the degree of injury (Fig. 3C and D). The levels of phosphorylated MAPKs, including ERK and p38, were slightly increased in liver tissues from Oprn-deficient mice, although phosphorylation of INK had no significant change (Fig. 3E), confirming stronger proliferative signaling pathways in livers from Oprn−/− mice.

To better understand how the absence of Oprn promoted tumor promotion, we further examined phosphorylation of NF-kB p65 in liver tissues, which is known to be a link of inflammation and cancer (26). Consistent with the observation in tumor tissues (Fig. 2E), absence of Oprn exhibited a great increase in phospho-NF-kB p65 relative to WT mice (Fig. 3F). Therefore, during the process of DEN-induced HCC, higher susceptibility to chemical hepatocarcinogenesis in Oprn−/− mice might be due to both enhanced cell apoptosis and compensatory proliferation of DEN-initiated hepatocytes, and enhanced NF-kB activation might be an important promoter under the circumstance of Oprn deficiency.

Oprn−/− mice exhibit elevated expression of proinflammatory cytokines

NF-kB-dependent production of proinflammatory cytokines has been demonstrated to promote DEN-induced hepatocarcinogenesis through compensatory proliferation (30). Therefore, we examined expression of NF-kB-targeted proinflammatory cytokines such as interleukin-6 (IL6), tumor necrosis factor-α (TNFα), and interleukin-1β (IL1β) after DEN administration. Significantly higher mRNA levels of IL6 and TNFα could be detected at 4 hours after DEN treatment in livers from Oprn−/− mice than those from WT mice (Fig. 4A). Tumors from WT and Oprn−/− mice were then separated. The mRNA levels of IL6 and TNFα in tumors from Oprn−/− mice were also significantly higher than those from WT mice (Fig. 4B). Next, we tested the change of tumor microenvironment through detecting proinflammatory cytokines in circulating serum of tumor-bearing mice by antibody arrays. Besides IL6 and TNFα, other NF-kB-targeted genes like IL1β, IL12, granulocyte colony stimulating factor (G-CSF), and tissue inhibitor of metalloproteinase-1 (TIMP-1) were also significantly increased in Oprn−/− tumor-bearing mice compared with WT mice (Fig. 4C and D). All the results revealed that...
**Figure 3.** Opn deficiency aggravates liver cell death and compensatory proliferation after DEN treatment. A, ALT levels in serum were determined at the indicated times after DEN injection (n = 4 mice per time point). Data, mean ± SD; *, P < 0.05. B, TUNEL assay of liver sections from WT and Opn−/− mice at 24 and 48 hours after DEN administration. Scale bars, 50 μm. C, representative images of immunohistochemistry staining for incorporating BrdUrd in the livers of DEN-treated WT and Opn−/− mice at 24 and 48 hours. Scale bars, 50 μm. D, frequencies of apoptotic cells (left) and BrdUrd-positive proliferating cells (right) after DEN injection, respectively. Data, mean ± SD; *, P < 0.05. E and F, the livers of DEN-treated WT and Opn−/− mice (n = 4 mice per time point) were removed and lysed to assess activation of phosphoproteins by Western blotting, including p-ERK, total ERK, p-p38, total p38, p-JNK, total JNK, and p-NF-κB p65; GAPDH was used as an internal control for analysis in F.

Opn−/− mice suffered from a more severe inflammatory response than WT mice, which might favor the survival and proliferation of hepatocytes.

**Intracellular OPN acts as a negative regulator for inflammatory response in macrophages**

Because OPN is expressed in a range of immune cells and reported to act as an immune modulator through its chemotactic properties (6), we first detected infiltration of macrophages by F4/80 staining in WT and Opn−/− mice. In both tumor and nontumor tissues, there were no obvious differences of macrophage infiltration between WT and Opn−/− mice (Fig. 5A and B). We therefore investigated whether Opn deficiency caused enhanced inflammation response in macrophages. Activation of NF-κB in macrophages is a critical event during progress of tumorigenesis, which links inflammation response to cancer (26). Stimulated by LPS (10 ng/mL) or cellular debris from Opn−/− necrotic hepatocytes, which could exclude the disturbance of exogenous OPN protein, macrophages from Opn−/− mice exhibited a higher expression of phospho-NF-κB p65 (Fig. 5C), as well as IL6 and
TNFαr than macrophages from WT mice did (Fig. 5D). In addition to activation of NF-κB, TLR/MyD88 signal pathway can activate members of MAPKs (23). Macrophages from WT or Opn−/− mice were treated with cellular debris (NEC) for the indicated times. Then the cells were lysed and assessed for activation of MAPKs, including ERK, p38, and JNK. The levels of phosphorylated MAPKs showed no obvious difference between macrophages from Opn−/− mice and WT mice (Supplementary Fig. S2). Thus, Opn deficiency might enhance production of proinflammation cytokines through regulating NF-κB activity in macrophages.

Alternative splicing of OPN results in three isoforms, OPN-a, OPN-b, and OPN-c (11). Our results have demonstrated that OPN deficiency causes enhanced inflammation response in macrophages, which is contradictory to the effect induced by sOPN (6). We therefore investigated whether iOPN could efficiently inhibit IL6 expression both in cell supernatant and mRNA level, whereas sOPN had no obvious effect (Fig. 6E and F). Thus, iOPN may act as a negative regulator for inflammation response in macrophages.

**iOPN interacts with MyD88 to block IRAK1 dissociation**

TLR can activate NF-κB to increase proinflammatory cytokine expression via adaptor molecule MyD88 and plays an important role in innate immune responses (23). We therefore investigated whether iOPN inhibited NF-κB activation and cytokine production by affecting the TLR/MyD88 signaling pathway. Coimmunoprecipitation of iOPN and MyD88 in macrophages demonstrated their interaction under stimulation of cellular debris (Fig. 7A). Confirmed by confocal microscopy, in WT macrophages, these two molecules showed few colocalization under no stimulation. But the circumstance changed when stimulated by cellular debris for 2 hours: colocalization was observed at perinuclear regions and when stimulated for 4 hours, they were both in the nucleus (Fig. 7B). IL1 receptor-associated kinase 1 (IRAK1) is involved in TLR signaling; phosphorylation of IRAK1 by IRAK4 can result in the activation of IRAK1 kinase activity and thus the formation of hyperphosphorylated and then phosphorylated IRAK1 is released from MyD88 and the receptor complex to activate downstream signaling (31). We detected the dissociation of IRAK1 from MyD88 complex by coimmunoprecipitation. In endogenous level, after stimulated by cellular debris for 4 hours,
Figure 5.

Opn−/− macrophages show increased sensitivity to cell death and endotoxin stimulation and produce more proinflammatory cytokines. A and B, F4/80 staining in liver sections of WT and Opn−/− HCC-bearing mice; the expression of F4/80 in mouse livers was detected by rat anti-mouse F4/80 antibody and Cy5-labeled anti-rat IgG as primary and secondary antibody, respectively. Nuclei were visualized by DAPI staining. Scale bars, 50 μm. C and D, macrophages were isolated from WT and Opn−/− mice (n = 3 mice per group). After culturing for 5 days, cells were stimulated with cellular debris released by necrotic hepatocytes (Nec) or LPS for 4 hours, and then lysed for Western blotting, including p-NF-κB p65 and GAPDH, or for RT-PCR. Data, mean ± SD; *, P < 0.05.

**Discussion**

Previous studies have suggested that OPN might play different roles in tumorigenesis when diverse carcinogens were used (32–34). In N-methyl-N-nitro-N-nitrosoguanidine–induced cutaneous squamous cell carcinoma, Opn-null mice had increased tumor growth, progression, and metastasis. Because host-derived OPN acted as a macrophage chemoattractant and at the tumor site, it could recruit or maintain macrophages, the degree of which correlated inversely with tumor growth in this model (32). In the 7,12-dimethylbenz(α)anthracene/12-O-tetradecanoylphorbol-13-acetate–induced skin papilloma model, Opn-null mice exhibited a decreased tumor/papilloma incidence by prevention of apoptosis (34). In MMTV-c-myc/MMTV-v-Ha-ras transgenic mice, OPN was not required for mammary primary tumor formation (33). In the DEN-induced HCC model, the role of OPN was largely unknown. Our previous study found that OPN was related to estrogen-mediated hepatoprotection in DEN-induced liver injury by enhancement of hepatocyte survival and inhibition of DEN biotransformation (35). Here, in accordance with liver protection at an early stage, we found OPN had a protective effect during hepatocarcinogenesis. Most chemical carcinogens act through interacting with the genetic material of the cell, especially with the DNA template (36), but there is evidence that DEN-induced HCC depends on inflammation response (24, 30). Up to 16 months of age, no Opn−/− mice exhibited spontaneous HCC. However, Opn−/− mice exhibited heavier production of proinflammatory cytokines and tumorigenesis than WT controls when DEN was administrated. These findings indicate that Opn deficiency may promote DEN-induced hepatocarcinogenesis through a robust inflammatory response.

Macrophages, which are pivotal members within the solid tumor microenvironment, play a significant role in tumor initiation when inflammation is a causal factor (37). OPN is reported to regulate function of macrophages and lead to cytokine production (13), phagocytosis and clearance of fungus (21), or bacterial infections (38). During these processes, SOPN and iOPN play different roles in adaptive and innate immunity, respectively (39). sOPN is considered as a macrophage chemoattractant and is expressed in cancer-infiltrating macrophages (9). iOPN is constitutively expressed by macrophages and participates in chemotaxis (40). Tumor-associated macrophages (TAM) provide an inflammatory microenvironment for tumor progression. Previous evidence has shown that OPN is one of the most upregulated genes in TAM, which may contribute to migration of macrophages (41). Data from exogenous OPN support that extracellular OPN is able to promote macrophage migration. However, the role of endogenous OPN in macrophage migration using Opn-deficient mice remains inconsistent. Macrophages are recruited into the peritoneum after intraperitoneal thioglycollate injection. However, the number of recruited macrophages is either fewer (42) or greater (43) in Opn-deficient mice as compared with WT mice. Moreover, in transgenic mice expressing c-myc and v-Ha-ras specifically in the mammary gland, a model of spontaneous tumor development, expression of OPN was greatly enhanced in these tumors. However, when the transgenic mice were crossed with Opn−/− mice, macrophage accumulation was found to be independent of OPN status (33). Here, we found there were no differences...
between numbers of infiltrated macrophages in intratumoral or marginal tissues of WT and Opn−/− mice in DEN-induced hepatocarcinogenesis. Our data and previous reports suggest that regulation of macrophage migration may be complex when both tumors and hosts are Opn deficient.

Here, OPN might modulate function instead of accumulation of macrophages in DEN-induced HCC. Indeed, Opn−/− macrophages exhibited more production of proinflammatory cytokines such as IL6 and TNFα. Our results further demonstrated that iOPN, rather than sOPN, acted as a negative regulator for inflammation response in macrophages. When WT macrophages were stimulated by cellular debris released by necrotic hepatocytes, protein level of iOPN was elevated, accompanied by decreased release of IL6 and TNFα by ELISA. Park and colleagues (44) have suggested that both TNFα and IL6 contribute to HCC development in mice. However, the cooperation of NF-κB, p38 MAPK, and STAT3 signaling in the regulation of tumor progression is not clear, which needs further investigation.

Kupffer cells, as resident liver macrophages, express most TLRs (46). TLR/MyD88 signaling has a strong contribution to inflammation and hepatocarcinogenesis. After incubation with either LPS or cellular debris released by necrotic hepatocytes, IL6 production in Kupffer cells from WT mice was increased, but in Kupffer cells from MyD88−/− mice, its production was elevated at a relatively low level (24). TLR4 is the major receptor recognizing endogenous ligands released from damaged or dying cells; it participates in cytokine expression mainly in myeloid cells and promotes liver tumorigenesis (47). Mice deficient in MyD88 or TLR4 had a significant reduction in tumor incidence in DEN-induced liver cancer (24, 48). These findings suggest that in DEN-induced HCC, production of cytokines by resident liver macrophages is mainly via the TLR4/MyD88 signaling.

Figure 6. IOPN suppresses proinflammatory cytokine production. WT macrophages were stimulated with necrotic debris at the indicated times (n = 4 mice per time point). A, the cell supernatant was collected and the expression level of sOPN was quantitated by ELISA. B, the cells were lysed to assess the expression of sOPN (60 kDa) and iOPN (55 kDa) by Western blotting, using OPN mAb (AKm2A1), with GAPDH serving as a loading control. C and D, supernatant of A was also collected for detecting the level of IL6 and TNFα by ELISA. E and F, macrophages were transfected with different plasmids for 48 hours, and then stimulated with necrotic debris for 4 hours; the expression of IL6 was quantitated by ELISA and RT-PCR. Data, mean ± SD; *, P < 0.05.
Previous studies have suggested that iOPN might perform as one of the intracellular regulators of TLR2/TLR9 signaling to balance the cytokine milieu (16, 21). In plasmacytoid dendritic cells, shortly after TLR9 engagement, iOPN interacted with MyD88 and enhanced interferon-α production (16). In antifungal innate immunity, iOPN acted as an adaptor molecule in TLR2 and dectin-1 signaling pathways and increased zymosan-induced cytokine production such as IL1β and IL10 (21). Following TLR stimulation in macrophages, iOPN was induced in vivo and in vitro and negatively regulated interferon-β production in murine macrophages (17). Here, macrophages were subjected to coimmunoprecipitation and Western blotting, using anti-IRAK1 and anti-MyD88 antibodies. D, HEK 293T cells were cotransfected with MyD88-pcDNA3.0 and pReceiver-myc-IRAK1, with or without iOpn-pcDNA3.0. At 48 hours posttransfection, cells were incubated with necrotic debris for 4 hours. The cell lysates were subjected to coimmunoprecipitation and Western blotting, using anti-IRAK1, anti-MyD88, and anti-iOPN antibodies.

Figure 7. iOPN interacts with MyD88 to block IRAK dissociation. A, macrophages from WT mice were stimulated with or without necrotic debris for 4 hours, and the endogenous association of MyD88 and OPN was coimmunoprecipitated and detected with the indicated antibodies; the expression levels of MyD88 and OPN in lysates were examined by Western blotting using anti-MyD88 and anti-OPN antibodies, respectively. B, confocal microscopy of OPN (green) and MyD88 (red) in macrophages. After treatment with necrotic debris for the indicated hours, cells were fixed. OPN and MyD88 were detected with mouse anti-OPN, rabbit anti-MyD88, followed by secondary antibodies. Scale bars, 5 μm. C, WT and Opn−/− macrophages were stimulated with or without necrotic debris for 4 hours. The cell lysates were subjected to coimmunoprecipitation and Western blotting, using anti-MyD88 antibodies. D, HEK 293T cells were cotransfected with MyD88-pcDNA3.0 and pReceiver-myc-IRAK1, with or without iOpn-pcDNA3.0. At 48 hours posttransfection, cells were incubated with necrotic debris for 4 hours. The cell lysates were subjected to coimmunoprecipitation and Western blotting, using anti-IRAK1, anti-MyD88, and anti-iOPN antibodies.

Previous studies have suggested that iOPN might perform as one of the intracellular regulators of TLR2/TLR9 signaling to balance the cytokine milieu (16, 21). In plasmacytoid dendritic cells, shortly after TLR9 engagement, iOPN interacted with MyD88 and enhanced interferon-α production (16). In antifungal innate immunity, iOPN acted as an adaptor molecule in TLR2 and dectin-1 signaling pathways and increased zymosan-induced cytokine production such as IL1β and IL10 (21). Following TLR stimulation in macrophages, iOPN was induced in vivo and in vitro and negatively regulated interferon-β production in murine macrophages (17). Here, macrophages were incubated with either LPS or cellular debris released by necrotic hepatocytes; by performing coimmunoprecipitation analysis and confocal microscopy, we demonstrated that iOPN was able to interact with MyD88 and block dissociation of IRAK1 from MyD88 complex, which led to the activation of NF-κB and enhanced production of cytokines such as IL6 and TNFα. In addition to activation of NF-κB, the TLR/MyD88 signal pathway can activate members of MAPKs (23). However, levels of phosphorylated MAPKs had no obvious difference between macrophages from Opn−/− mice and WT mice. The mechanism by which iOPN regulates TLR/MyD88-mediated NF-κB activation selectively needs further investigation. Several molecules, such as MyD88s (the short form of MyD88) and IRAKMs, have been found to be able to negatively regulate TLR-mediated immune responses (49). Our results suggest that iOPN acts as a negative regulator of TLR4/MyD88 signaling in macrophages and plays an important role in inflammation-related hepatocarcinogenesis.

iOPN has long been considered as a prosurvival factor of tumor cells via inhibition of apoptosis and inflammation regulation (50). Here, combining our observations and previous publications, we propose that in the DEN-induced hepatocarcinogenesis murine model, iOPN is able to interact with MyD88 in macrophages under stimulation of cellular debris, and thus block dissociation of IRAK1 from the MyD88 complex and subsequent NF-κB activation. Loss of OPN causes dissociation of IRAK1 from the MyD88 complex, activation of NF-κB, enhanced production of IL6 and TNFα in macrophages, which promote survival and proliferation of pre-malignant hepatocytes and leads to tumorigenesis eventually. Our findings demonstrate a new mechanism by which iOPN plays a critical role in the anti-inflammation response in macrophages and a novel insight into hepatocarcinogenesis.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Zhao
Development of methodology: X. Zhou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Fan, C. He, W. Jing
Writing, review, and/or revision of the manuscript: X. Fan, J. Zhao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Chen, L. Cao, M. Zhu, R. Jia, H. Wang
Study supervision: Y. Guo

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