GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Toll-Like Receptor-7 Signaling Promotes Nonalcoholic Steatohepatitis by Inhibiting Regulatory T Cells in Mice

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Toll-like receptor 7 (TLR7) signaling regulates the production of type 1 interferons (IFNs) and proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , implicated in the control of regulatory T (Treg) cell activity. However, the mechanistic interplay between TLR7 signaling and Treg cells in nonalcoholic steatohepatitis (NASH) has not been elucidated. Our aim was to clarify the role of TLR7 signaling in the pathogenesis of NASH. Steatohepatitis was induced in wild-type (WT), TLR7-deficient, IFN- α / β receptor 1-deficient, and Treg cell-depleted mice. TLR7-deficient and IFN- α / β receptor 1-deficient mice were more protective to steatohepatitis than WT mice. Of interest, both TNF- α and type 1 IFN promoted apoptosis of Treg cells involved in the prevention of NASH. Indeed, Treg cell-depleted mice had aggravated steatohepatitis compared with WT mice. Finally, treatment with immunoregulatory sequence 661, an antagonist of TLR7, efficiently ameliorated NASH *in vivo*. These results demonstrate that TLR7 signaling can induce TNF- α production in Kupffer cells and type I IFN production in dendritic cells. These cytokines subsequently induce hepatocyte death and inhibit Treg cells activities, leading to the progression of NASH. Thus, manipulating the TLR7-Treg cell axis might be used as a novel therapeutic strategy to treat NASH. (*Am J Pathol* 2018, 188: 2574–2588; <https://doi.org/10.1016/j.ajpath.2018.07.011>)

Nonalcoholic fatty liver disease (NAFLD) is a major form of chronic liver diseases in adults and children.¹ Nonalcoholic steatohepatitis (NASH) is characterized by simple steatosis, hepatitis, and progressive fibrosis. It can ultimately lead to end-stage liver disease.² NASH has been observed in a subset of patients with NAFLD. However, the exact mechanisms by which a simple steatosis leads to NASH remain poorly understood.

Recent studies have suggested that overgrowth of intestinal bacteria and increased intestinal permeability might play roles in the development of NASH.^{3–5} In chronic liver diseases, including NASH, intestinal permeability is increased because of bacterial overgrowth or altered compositions of bacterial microflora.⁶ Systemic inflammation related to NASH also injures epithelial tight junctions,³ resulting in deregulation of intestinal barrier functions. Indeed, plasma levels of lipopolysaccharides (LPSs) are

elevated in patients with chronic liver diseases, including NASH.⁷ These findings suggest that hepatic immune cells might be exposed to high levels of Toll-like receptor (TLR) ligands derived from gut bacterial products, which might trigger liver injury and progress to NASH. Several reports have detailed the importance of TLR4 and intestine-derived LPS in animal models of NASH.^{8,9} Recently, it has been revealed that TLR7, initially considered a receptor of single-stranded RNA virus, can also recognize RNA from

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bacteria.^{10,11} Furthermore, host-derived denatured RNA from apoptotic cells can also be recognized by TLR7 as an endogenous ligand.^{12,13} As a consequence, hepatic immune cells might be exposed to effective levels of TLR7 ligands after liver injury, thus playing a role in the pathogenesis of NASH. Indeed, TLR4- or TLR9-deficient mice have exhibited reduced liver injury and NASH induced by a methionine-choline-deficient (MCD) or choline-deficient amino acid-defined diet,^{9,14} indicating that TLR ligands originated from bacteria (bacterial LPS or DNA) or host (host DNA) might play a crucial role in NASH pathogenesis.^{15–17} Recently, it has been reported that TLR7 affects lipid accumulation in hepatocytes by controlling autophagy and lipid peroxidation.¹⁸ However, it is currently unclear what mechanisms regulate steatohepatitis by TLR7 signaling *in vivo*.

Therefore, the objective of this study was to clarify the role of TLR7 in the pathogenesis of NASH. We fed the MCD diet to mice to induce experimental NASH because of the reproducibility of this well-established model, which allows the assessment of inflammatory pathway.^{19–21} Our data indicate that TLR7 signaling mediated TNF- α and type 1 interferon (IFN) production in Kupffer cells (KCs) and dendritic cells (DCs), respectively, subsequently suppressing regulatory T (Treg) cells and leading to steatohepatitis.

Materials and Methods

Animals and Experimental Protocol

In the current study, TLR7 knockout (KO) mice, type 1 IFN receptor (IFNAR1) KO mice, depletion of regulatory T cells (DEREG) mice, and C57BL/6 littermate controls (eight to nine weeks of age, 25 to 30 g of weight) were used. TLR7-deficient mice were kindly provided by Dr. Shizou Akira (Osaka University, Suita, Japan). IFNAR1 KO mice were purchased from B&K Universal Limited (Hull, UK) and backcrossed with C57BL/6 mice for at least 10 generations. DEREG mice were provided by Dr. Tim Sparwasser (Technische Universität München, Munich, Germany).²² CD11c-DTR transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice received humane care according to US National Institutes of Health recommendations outlined in the *Guide for the Care and Use of Laboratory Animals*.²³ Experimental and animal management procedures were undertaken in accordance with requirements from the Animal Care and Ethics Committees of Chonbuk National University.

Mice were used to prepare two different NASH models: acute and chronic. For the acute model, TLR7 KO, IFNAR1 KO, and WT mice ($n = 6$ mice per group) were fed an MCD or methionine-choline-sufficient (MCS) diet (Dyets Inc., Bethlehem, PA) for 17 days. For the chronic model, TLR7 KO, IFNAR1 KO, DEREG, and WT mice ($n = 10$ mice per group) were fed an MCD diet for six weeks, an established

protocol to prepare NASH model.²⁴ After establishing the short-term NASH model, mice were intraperitoneally injected with a single dose of R848 (2.5 mg/kg, Invivogen, San Diego, CA) or normal saline. At 6 hours after injection, mice were sacrificed to obtain serum samples and liver tissues. Pentoxifylline (100 mg/kg, Sigma-Aldrich Co., St. Louis, MO), an inhibitor of TNF- α production, was intraperitoneally injected at 1 hour and 24 hours before R848 treatment.

Histopathologic Analysis

For histologic review of hematoxylin and eosin-stained liver sections by light microscopy (BX-51, Olympus Corp., Tokyo, Japan), liver tissues were collected, fixed in 10% neutral buffered formalin solution for 48 hours, routinely processed, and then embedded in paraffin. Tissue sections (4 μ m in thickness) were prepared using a microtome (HM-340E, Thermo Fisher Scientific Inc., Waltham, MA) and placed on glass slides. Hematoxylin and eosin staining was performed according to standard techniques. The NAFLD score was determined by two pathologists independently (Y.S.R. and B.K.) as described previously.²⁵

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed on paraffin-embedded sections using an ApopTaq Peroxidase *in situ* apoptosis detection kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Positive reactions were visualized with 3,3'-diaminobenzidine substrate. Next, nuclear counterstaining was performed using methyl green dye. TUNEL-labeled cells were quantified by the percentage of positive area in high-power field. A total of 10 high-power fields of liver tissues were analyzed for each animal. Data are expressed as percentages of TUNEL-positive areas. Total liver section images were analyzed using a light microscope (BX-51, Olympus Corp.) and digital image software (analysis TS Auto version 5.1, Olympus Corp.).

Biochemical Measurements

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using AM101-K spectrophotometric assay kits (ASAN Pharmaceutical, Hwasung, Korea). Triglyceride and total cholesterol contents in the liver were determined using an AM202-K spectrophotometric assay kit (ASAN Pharmaceutical, Hwasung, Korea).

Real-Time Quantitative RT-PCR

Total RNA was isolated from liver tissue using Easy-Spin Total RNA extraction kit (iNtRon Biotech, Seoul, Korea). After incubation with RNase-free DNase I (Promega, Madison, WI), reverse transcription was performed using a random

primer and MultiScribe MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. cDNA was subjected to real-time PCR on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green I as a dye for binding double-strand DNA. All PCR primers were obtained from Bioneer (Daejeon, Korea). After real-time quantitative RT-PCR reaction was completed, specificity was verified by melting curve analysis. Quantification was performed by comparing Ct values of samples after normalization with glyceraldehyde-3-phosphate dehydrogenase as internal control. Sequences of primers were summarized in Table 1.

Immunoblot Analysis

Liver tissue was directly lysed with an extraction buffer (T-PER, Thermo Fisher Scientific Inc.) for 30 minutes on ice. After centrifugation at $13,000 \times g$ for 15 minutes at 4°C , protein concentration in the supernatant was measured using Bradford's reagent (Thermo Fisher Scientific Inc.). Protein (30 μg) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a gradient gel and then transferred onto polyvinylidene difluoride membranes. Blocking was performed using blocking buffer [5% nonfat dairy milk in Tris-buffered saline (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) containing 0.05% Tween-20] for 1 hour at room temperature. Primary antibodies were diluted 1:1000 in a blocking buffer and incubated at 4°C overnight. The following antibodies were used: anti-Bcl-2, anti-Bax, and β -actin (mouse or rabbit antibody, Cell Signaling Technology, Danvers, MA). To detect antigen antibody complexes, anti-rabbit or anti-mouse horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, TX) were diluted 1:3000 in blocking buffer and incubated at room temperature for 45 minutes. Immune complexes were visualized using chemiluminescent substrate (Millipore, Burlington, MA) and Kodak X-OMAT film (Eastman Kodak, Rochester, NY) according to the manufacturer's instructions.

Measurement of Thiobarbituric Acid–Reactive Substances and Plasma TNF- α

Hepatic thiobarbituric acid–reactive substances (TBARSs) as oxidative stress markers were measured using a Lab

Assay TBARS kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. Plasma concentration of mouse TNF- α was measured by enzyme-link immunosorbent assay using a Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Isolation of Hepatocytes and NPCs

To isolate hepatocytes and hepatic nonparenchymal cells (NPCs), livers of MCS or MCD-fed WT mice, TLR7 KO mice, and IFNAR1 KO mice were perfused (1 mL per minute) and digested with collagenase 1 (Worthington Biochemical Corporation, Lakewood, NJ) after cannulation of portal vein. Liver cell suspension was centrifuged at $50 \times g$ for 3 minutes. After centrifugation, the pellet representing hepatocytes was resuspended, filtered, and washed several times using Dulbecco's modified Eagle's medium (PAA Laboratories, Piscataway, NJ) supplemented with 5% fetal bovine serum (Thermo Fisher Scientific Inc.), 100 nmol/L dexamethasone (Sigma-Aldrich), 1% insulin-transferrin-selenium-X supplement (Thermo Fisher Scientific Inc.), 100 IU/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin. Viability of hepatocytes was assessed using trypan blue (Sigma-Aldrich).

After centrifuging digested liver cell suspension at $50 \times g$ for 3 minutes, the supernatant containing hepatic NPCs was collected, washed with phosphate-buffered saline, and resuspended in 40% Percoll in RPMI 1640 media. The cell suspension was gently overlaid onto 70% Percoll and centrifuged at $750 \times g$ for 20 minutes with off-brake setting. NPCs were collected from the interface, washed twice with phosphate-buffered saline, and resuspended in RPMI 1640 media.

Cell Culture

Murine normal hepatocyte cell line TIB-73 was obtained from the ATCC (Manassas, VA). Murine monocyte/macrophage cell line RAW 264.7 was obtained from the ATCC. These cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (PAA Laboratories) supplemented with 10% fetal bovine serum, 4 mmol/L L-glutamine (PAA Laboratories), 100 IU/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin.

Table 1 Sequences of Primers Used for Real-Time PCR

Gene	Forward	Reverse
<i>Tnfa</i>	5'-TCTACTCCCAGGTTCTCTTCAAGG-3'	5'-GCAAAATCGGCTGACGGTGTG-3'
<i>Foxp3</i>	5'-GGAGAGGCAGAGGACACTCAATG-3'	5'-TCAGGTTGTGGCGGATGGC-3'
<i>Ifna4</i>	5'-CCTGCTGGCTGTGAGGACATAC-3'	5'-TCTTGCCAGCAAGTTGGTTGAG-3'
<i>Lpl</i>	5'-TCTGTACGGCACAGTGG-3'	5'-CCTCTCGATGACGAAGC-3'
<i>Fabp1</i>	5'-TGGACCCAAAGTGGTCCGCA-3'	5'-AGTTCAGTCACGGACTTTAT-3'
<i>Pparg</i>	5'-CGGAAGCCCTTTGGTGACTTTATG-3'	5'-GCAGCAGGTTGTCTTGGATGTC-3'
<i>Adipoq</i>	5'-TGACGGCAGCACTGGCAAG-3'	5'-GATACTGGTCGTAGGTGAAGAGAAC-3'
<i>Gapdh</i>	5'-ACGGCAAATTCAACGGCACAG-3'	5'-AGACTCCACGACATACTCAGCAC-3'

For co-culture experiments, primary hepatocytes and NPCs isolated from WT mice, TLR7 KO mice, and IFNAR1 KO mice were co-cultured in collagen-coated 12-well Transwell plate (Sigma-Aldrich) with or without 0.4 mmol/L palmitic acid for 24 hours. Hepatocyte death was evaluated using a cytotoxicity detection kit (Sigma-Aldrich) based on the measurement of activity of lactate dehydrogenase released from the cytosol into the culture medium following the manufacturer's instruction. Absorbance of sample was measured at wavelength of 490 nm using an EMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

Quantification of Cell Death Induced by H₂O₂ and TNF- α

Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Rockville, MD) was used to detect cellular activity of dehydrogenase, a well-known hallmark of viability. A total of 2.5×10^4 primary hepatocytes per well were plated onto a 48-well collagen-coated plate in duplicates and incubated overnight. These cells were then treated with 0, 20, or 100 μ mol/L H₂O₂ for 5 minutes. After that, 20 ng/mL of TNF- α was added into each well. After incubating for 16 hours at 37°C, samples were assayed according to the manufacturer's instructions.

Phosphorothioate IRS Oligodeoxyribonucleotides Synthesis and Antagonist of TLR7

The following endotoxin-free oligodeoxyribonucleotides (ODNs, Bioneer) were used for *in vitro* or *in vivo* studies: immunoregulatory sequence (IRS) 661, 5'-TGCTTG-CAAGCTTGCAAGCA-3', and control ODNs, 5'-TCCTG-CAGGTTAAGT-3'. Various groups of mice were treated with saline, IRS 661, or control ODN at dose of 50 μ g on alternate days during the MCD diet.

Flow Cytometric Analysis

Hepatic NPCs obtained from livers were stained with fluorescein isothiocyanate anti-CD4 antibody and allophycocyanin anti-CD25 antibody (e-Biosciences, San Diego, CA) on ice for 30 minutes followed by fixation with permeabilization concentrate buffer (e-Biosciences) at 4°C for 6 hours. After fixation, cells were washed twice with permeabilization buffer and stained with phosphatidylethanolamine anti-Foxp3 antibody in permeabilization buffer at room temperature for 30 minutes. For detection of early and late apoptosis, the liver NPCs were stained with anti-CD4 antibody and anti-CD25 antibody and washed with phosphate-buffered saline. Then, cells were suspended in binding buffer and supplemented with annexin V (AV) and propidium iodide (PI) for 15 minutes at room temperature without light exposure. The cells with fluorescence AV⁺/PI⁻ were considered to be early apoptotic cells, and those with fluorescence AV⁺/PI⁺

were considered to be late apoptotic cells. Data were analyzed using CellQuest software version 3.1 (BD Biosciences, Franklin Lakes, NJ) or FlowJo software version 7.8 (FlowJo, Ashland, OR).

Statistical Analysis

All data are expressed as means $\pm$ SEM. Differences between the two groups were compared using a two-tailed *t*-test. Statistically significant difference between groups was considered at $P < 0.05$. All Western blotting, real-time quantitative RT-PCR, flow cytometry, and immunocytochemistry experiments were repeated at least twice with five to 10 different mice or samples per group. Only representative results are shown in the figures.

Results

TLR7 Deficient Mice Are Resistant to NASH

To investigate the role of TLR7 in the pathogenesis of NASH, WT and TLR7-deficient mice were fed with an MCD or MCS diet (control) for 6 weeks. After feeding with an MCD diet, livers of WT mice had marked lipid accumulation with infiltration of inflammatory cells. In contrast, livers of TLR7-deficient mice had significant reduction of steatosis and inflammation compared with those of WT mice (Figure 1A). NAFLD activity scores as determined by the degree of steatosis and inflammation²⁵ were significantly lower in TLR7-deficient mice than that in WT mice (Figure 1A). Reduced liver injury in TLR7-deficient mice was confirmed by lower serum ALT and AST levels (Figure 1B). Several lines of evidence have indicated that adiponectin and peroxisome proliferator-activated receptor gamma (PPAR γ) can negatively regulate the severity of NASH.^{26–28} To understand how TLR7 signaling might regulate lipid accumulation, expression levels of PPAR γ and PPAR γ -dependent target genes (LFABP and LPL)^{26,29} were examined in livers of WT and TLR7-deficient mice fed with an MCD diet. Expression levels of PPAR γ , LFABP, and LPL in TLR7-deficient mice fed with an MCD diet were significantly higher than those in WT mice fed with the same diet (Figure 1C). Moreover, expression level of adiponectin, an anti-inflammatory adipokine, was up-regulated in livers of TLR7-deficient mice fed with an MCD diet compared with that in WT mice fed with the same diet (Figure 1C). These results suggest that TLR7 signaling is involved in the progression of NASH.

TLR7-Induced TNF- α Promotes Hepatocyte Damage

To demonstrate the mechanistic effects of the TLR7–type I IFN pathway on steatotic hepatocyte death, *ex vivo* experiments were conducted using a hepatocytes-KCs coculture system. Viabilities of WT and TLR7-deficient hepatocytes

Figure 1 Toll-like receptor7 (TLR7)-deficient mice are resistant to methionine-choline-deficient (MCD) diet-induced hepatocyte damage and inflammation. Wild-type (WT) and TLR7-deficient mice were fed a methionine-choline-sufficient (MCS) or MCD diet for 6 weeks. **A:** Liver sections stained with hematoxylin and eosin to assess steatosis and inflammation. Nonalcoholic fatty liver disease (NAFLD) activity scores were calculated based on histologic evaluation of steatosis and inflammation. **B:** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). **C:** Hepatic mRNA levels of peroxisome proliferator-activated receptor gamma (PPAR γ), LFABP, LPL, and adiponectin determined by real-time quantitative RT-PCR. They are shown as fold changes compared with those in the MCS-fed WT mice. Data are expressed as means $\pm$ SEM per group. $n = 6$ mice in the MCS group; $n = 8$ mice in the MCD group. * $P < 0.05$, ** $P < 0.01$ (two-tailed t -test). KO, knockout. Original magnification: $\times 100$ (A, top row); $\times 400$ (A, bottom row).

were comparable after treatment with palmitic acid (Supplemental Figure S1A). To mimic *in vivo* condition, hepatocytes were co-cultured with KCs. Hepatocytes co-cultured with KCs from livers of TLR7-deficient mice had significantly less cell death compared with those from livers of WT mice (Figure 2A). In contrast, these effects were dampened in the presence of TNF- α (Figure 2B). Furthermore, R848-induced cell death was significantly inhibited by pentoxifylline treatment, suggesting that TLR7-mediated hepatocyte death is dependent on KC-derived TNF- α (Figure 2C). Consistently, the expression of TNF- α was significantly decreased in TLR7-deficient mice fed with an MCD diet. The expression level of TNF- α was

markedly down-regulated in co-cultured TLR7 KO KCs compared with that in WT KCs (Figure 2D), suggesting that KCs might be the major source for inducing TNF- α -mediated hepatocyte death. R848 treatment dramatically up-regulated hepatic mRNA expression of TNF- α in MCD-fed mice compared with that in MCS-fed mice (Supplemental Figure S1, B and C). The serum ALT level was also significantly increased by R848 treatment in both diet groups (Figure 2E), which was corroborated by the results of hepatocyte apoptosis using TUNEL results (Supplemental Figure S2, A and B). These results indicate that TNF- α might be a key mediator in the pathogenesis of lipid-mediated hepatocyte death.

Figure 2 Toll-like receptor 7 (TLR7)-induced tumor necrosis factor (TNF)- α promotes hepatocyte damage. **A:** Hepatocyte death in palmitic acid (PA)-treated primary hepatocytes co-cultured with Kupffer cells (KCs) from livers of hepatic (HP) wild-type (WT) and TLR7-deficient mice. **B:** Hepatocyte death in PA-treated primary hepatocytes co-cultured with KCs from livers of HP WT and TLR7-deficient mice in the presence of phosphate-buffered saline (PBS) or TNF- α . **C:** Hepatocyte death in PA-treated primary hepatocytes co-cultured with KCs from livers of HP WT and TLR7-deficient mice in the presence of R848 or pentoxifylline (PTX). **D:** The mRNA expression of TNF- α in liver and KCs determined by real-time quantitative RT-PCR. It is shown as fold change compared with that in methionine-choline-sufficient (MCS) diet-fed WT mice or control WT non-parenchymal cells. **E:** Serum alanine aminotransferase (ALT) levels. **F:** Amounts of HP thiobarbituric acid-reactive substances (TBARSs) in MCS or methionine-choline-deficient (MCD) diet-fed WT and TLR7-deficient mice. **G:** Hepatocyte death using cell death detection assay kit after 16 hours of incubation with TNF- α . TIB-73 hepatocytes were pretreated with H₂O₂ (0, 10, 100 μ M/L) for 5 minutes before stimulation with 20 ng/mL of TNF- α . N-acetylcysteine (NAC) (10 mmol/L) was added for 30 minutes before H₂O₂ treatment. **H:** Protein expression of Bcl-2, Bax, and β -actin measured by Western blot analysis. Samples were obtained 6 hours after R848 or PTX injection in MCD diet- or MCS diet-fed WT mice (feeding for 17 days). Data are expressed as means $\pm$ SEM per group. * P < 0.05, ** P < 0.01 (two-tailed t -test). Cont, control; KO, knockout; Sal, saline.

Oxidative stress is known to play an important role in the pathogenesis of NASH.^{9,14,30} Therefore, lipid peroxidation was assessed by quantifying levels of TBARSs, a major indicator of oxidative stress.³¹ As expected, TBARS levels were increased in the MCD diet group compared with those in the MCS diet group. However, WT mice had significant higher levels of TBARSs than TLR7-deficient mice (Figure 2F). Of note, reactive oxygen species and R848 synergistically induced TNF- α production in macrophages (Supplemental Figures S3 and S4). To access the contribution of reactive oxygen species and TNF- α to hepatocyte apoptosis, cultured hepatocytes were incubated with H₂O₂ and TNF- α . Neither H₂O₂ nor TNF- α treatment alone significantly increased apoptosis of hepatocytes, although TNF- α treatment slightly enhanced apoptosis of hepatocytes. Pretreatment with H₂O₂ synergistically enhanced TNF- α -induced hepatocyte apoptosis compared with untreated cells, whereas pretreatment with N-acetylcysteine significantly suppressed apoptosis induced by co-treatment with

H₂O₂ and TNF- α (Figure 2G). The Bcl-2 family protein is known as the key upstream signal for TNF- α -induced apoptosis.³² Therefore, expression levels of Bcl-2 and Bax were evaluated by Western blot analysis to elucidate the cell death machinery in hepatocytes induced by R848. R848 decreased the expression level of Bcl-2, an antiapoptotic protein, in whole lysate of the liver without changing the expression level of Bax, a proapoptotic protein. Of interest, pentoxifylline, an inhibitor of TNF- α production,³³ partially restored the reduction in Bcl-2 expression caused by R848 treatment (Figure 2H). Collectively, our results suggest that TLR7-induced hepatocyte apoptosis and steatohepatitis are dependent on TNF- α -mediated mechanism.

TLR7-Type I IFN Axis Is Required for Lipid-Laden Hepatocyte Death

TLR7 signaling is transduced by two cascades, the NF- κ B and interferon regulatory factor 7 pathways, that can induce

Figure 3 Toll-like receptor 7 (TLR7)—type I interferon (IFN) axis is required for lipid-laden hepatocyte death. **A:** The mRNA expression of IFN- α_4 in liver and non-parenchymal cells (NPCs) determined by real-time quantitative RT-PCR and shown as fold change compared with that in methionine-choline-sufficient (MCS)-fed wild-type (WT) mice. **B:** Hepatocyte death measured in palmitic acid (PA) (400 μ mol/L)-treated primary hepatocytes co-cultured with NPCs from WT and IFN- α/β receptor 1 (IFNAR1)-deficient liver. **C:** Serum alanine aminotransferase (ALT) levels measured in R848-treated WT and IFNAR1-deficient mice. **D:** Apoptosis determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and quantification of TUNEL-positive area. Data are expressed as means $\pm$ SEM per group. * P < 0.05, ** P < 0.01 (two-tailed t -test). Original magnification: $\times 100$ (D). Cont, control; KO, knockout; HP, hepatic; MCD, methionine-choline-deficient.

the production of proinflammatory cytokines TNF- α and type I IFN, respectively.^{34,35} Therefore, the expression levels of IFN- α_4 were examined in liver and NPCs known to be able to produce type I IFN.³⁶ As expected, expression levels of IFN- α_4 were increased in livers or NPCs of MCD-fed mice. Such increases were considerably decreased in MCD-fed mice that lacked TLR7 (Figure 3A). These results prompted us to test whether the TLR7—type I IFN pathway affected hepatocellular damage. Palmitic acid-induced hepatotoxicity was significantly down-regulated in co-cultured IFNAR1 KO NPCs compared with that in WT NPCs (Figure 3B), suggesting that NPCs might be the major source for the induction of type I IFN-mediated hepatocyte death. Consistent with results of *ex vivo* studies, liver injury was markedly decreased in MCD-fed IFNAR1 KO mice compared with that in MCD-fed WT mice based on reduced levels of serum ALT in the presence or absence of R848 administration (Figure 3C). Furthermore, hepatocyte damage induced by R848 treatment was reduced in MCD-fed IFNAR1 KO mice compared with that in MCD-fed WT mice based on TUNEL staining (Figure 3D). Taken together, our findings indicate that TLR7-dependent type I IFN is responsible for the progression of liver injury in NASH development.

Decreased Type I IFN in TLR7-Deficient Mice Contributes to Protection against NASH

To further verify the role of type I IFN in downstream pathway of TLR7 in NASH, NASH was examined in WT and IFNAR1-deficient mice. Similar to the findings in TLR7 KO mice, livers of IFNAR1 KO mice had a significant reduction of steatosis and inflammation compared with those of WT mice (Figure 4A). The NAFLD activity scores as determined by the degree of steatosis and inflammation²⁵ were also significantly lower in IFNAR1 KO mice than that in WT mice (Figure 4A). Reduced liver injury in IFNAR1 KO mice was confirmed by lower serum ALT and AST levels (Figure 4B). Consistent with these results, livers of IFNAR1 KO mice had significant reductions of inflammation and lipid peroxidation compared with those of WT mice (Figure 4, C and D). Moreover, lower lipid accumulation was observed in livers of MCD-fed IFNAR1 KO mice based on higher expression levels of PPAR γ and its target genes (Figure 4E). These findings, together with the earlier results (Figures 1, 2, and 3), suggest that ameliorated NASH in TLR7-deficient mice is mediated by the loss of detrimental TNF- α and type I IFN signaling.

Figure 4 Methionine-choline-deficient (MCD) diet-induced nonalcoholic steatohepatitis is ameliorated in interferon- α/β receptor 1 (IFNAR1)-deficient mice. Wild-type (WT) and IFNAR1-deficient mice were fed a methionine-choline-sufficient (MCS) or MCD diet for 6 weeks. **A:** Liver sections stained with hematoxylin and eosin for assessment of steatosis and inflammation. Nonalcoholic fatty liver disease (NAFLD) activity scores were calculated based on histologic evaluation of steatosis and inflammation. **B:** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). **C:** Amounts of hepatic thiobarbituric acid-reactive substances (TBARS) measured in MCS diet- or MCD diet-fed WT and IFNAR1-deficient mice. **D:** Hepatic mRNA expression levels of adipokines (TNF- α and adiponectin) determined by real-time quantitative RT-PCR shown as fold change compared with those in MCS diet-fed WT mice. **E:** Hepatic mRNA levels of peroxisome proliferator-activated receptor gamma (PPAR γ), LFABP, and LPL determined by real-time quantitative RT-PCR shown as fold change compared to those in MCS-fed WT mice. Data are expressed as means $\pm$ SEM per group. $n = 6$ mice in the MCS group; $n = 8$ mice in the MCD group. * $P < 0.05$, ** $P < 0.01$ (two-tailed t -test). Original magnification: $\times 100$ (A, top row); $\times 200$ (A, bottom row). KO, knockout.

TLR7-Induced Type I IFN and TNF- α Promotes Apoptosis of Treg Cells

The pathologic effect of TLR7 signaling against NASH implied the involvement of TNF- α or type I IFN-dependent

immune regulatory factors. Therefore, the expression level of another TLR7-dependent factor, Foxp3, a crucial transcription factor for Treg cell activation, was examined. Hepatic Foxp3⁺ Treg cell population in NASH liver was significantly increased in both TLR7- and IFNAR1-deficient

mice compared with that in WT mice (Figure 5, A and B). To identify the responsible cell type for regulating Treg cells on TLR7 activation, R848 was administered to CD11c-diphtheria toxin (DT) receptor transgenic mice after depletion of DCs by DT injection or to WT mice after depletion of KCs by clodronate liposome injection. According to a previous publication³⁶ and Supplemental Figure S5, the depletion of DCs or KCs significantly reduced the production of type I IFN and TNF- α , respectively. Of interest, TLR7 activation by R848 significantly decreased the Foxp3 expression level in the liver. Of note, such reduction was significantly recovered in DC-depleted mice and pentoxifylline-treated WT mice (Figure 5, C and D). Next, the number of hepatic Treg cells were measured after R848 treatment. Consistently, the R848-induced reduction of Foxp3⁺CD25⁺ Treg cells was recovered in DC-depleted or pentoxifylline-treated

mice (Figure 5E). Likewise, R848-induced reduction of Foxp3 expression was recovered in IFNAR1 KO mice (Figure 5F). These results prompted us to examine whether type I IFN or TNF- α affect viability of intrahepatic Treg cells on TLR7 activation. Indeed, TLR7 activation increased early (AV⁺/PI⁻) and late (AV⁺/PI⁺) apoptosis of Treg cells and this induction was reduced by DC depletion or pentoxifylline treatment (Figure 5G). Collectively, these results suggest that TLR7 signaling induces production of type I IFN and TNF- α , which in turn promotes apoptosis of Treg cells.

Foxp3⁺ Treg Cells Prevent NASH Development

On the basis of the results reported in Figure 5, whether Foxp3⁺ Treg cells might play a role in the pathogenesis of

Figure 5 Toll-like receptor 7 (TLR7)—induced type I interferon (IFN) and tumor necrosis factor (TNF)- α promotes apoptosis of T regulatory (Treg) cells. **A and B:** Hepatic Foxp3⁺CD25⁺CD4⁺ cells counted by flow cytometry. Nonparenchymal cells (NPCs) were collected from methionine-choline-sufficient (MCS) or methionine-choline-deficient (MCD)—fed wild-type (WT), TLR7-, and interferon- α/β receptor 1 (IFNAR1)—deficient mice livers after feeding for 6 weeks. **C and D:** mRNA expression levels of Foxp3 determined by real-time quantitative RT-PCR in WT mice livers at 2 hours after injection of TLR7 ligand (R848). Dendritic cells (DCs) were depleted by diphtheria toxin (DT) injections (2 ng/g of body weight) to CD11c-DT receptor (DTR) mice for 2 consecutive days. Pentoxifylline (PTX) was used to treat WT mice for a consecutive 2 days. **E:** Hepatic Foxp3⁺CD25⁺CD4⁺ cells counted by flow cytometry. NPCs were collected from DT-treated CD11c-DTR mice or PTX-treated WT mice. **F:** Foxp3 mRNA expression levels measured by real-time quantitative RT-PCR in WT and IFNAR1-deficient mice livers at 2 hours after injection of TLR7 ligand (R848). **G:** Early and late apoptosis of hepatic CD4⁺CD25⁺ cells were counted by annexin V (AV)/propidium iodide (PI) staining. Living cells (AV⁻/PI⁻); early apoptotic cells (AV⁺/PI⁻); late apoptotic cells (AV⁺/PI⁺). Data are expressed as means $\pm$ SEM per group. * P < 0.05, ** P < 0.01, and *** P < 0.001 (two-tailed t -test). Cont, control; KO, knockout.

NASH was tested. To identify the *in vivo* role of Foxp3⁺ Treg cells in NASH more specifically, bacterial artificial chromosome transgenic mice (DEREG mice) expressing a DT receptor–green fluorescent protein fusion protein under the control of Foxp3 locus were used.³⁷ Histopathologic analysis and NAFLD scoring were performed for livers of MCD-fed DEREG and WT mice. Severe steatohepatitis was observed in mice that lacked Foxp3⁺ Treg cells (Figure 6A). Consistent with the severity of NASH, a higher expression level of TNF- α and a lower expression level of adiponectin were detected in DEREG mice compared with those in WT mice (Figure 6B). Expression levels of PPAR γ and LFABP but not LPL in DEREG mice fed an MCD diet were significantly lower than those in WT mice fed an MCD diet

(Figure 6C). Taken together, these results suggest that Foxp3⁺ Treg cells have protective role against MCD-induced NASH.

TLR7 Antagonism with IRS 661 Reduces NASH *in Vivo*

To test the therapeutic potential of TLR7 signaling in the pathogenesis of NASH, MCD-fed mice were treated with IRS 661, an ODN-based TLR7 antagonist. IRS 661 (50 μ g) or control ODN (vehicle) was administered to WT mice twice a week concomitantly with an MCS or MCD diet. Mice treated with IRS 661 had reduced steatosis and inflammation compared with control ODN-treated mice (Figure 7A). The NAFLD score was significantly lower in

Figure 6 Depletion of hepatic Foxp3⁺ T regulatory (Treg) cells induces more nonalcoholic steatohepatitis. Wild-type (WT) and Treg cell–depleted (DEREG) mice were fed a methionine-choline–sufficient (MCS) or methionine-choline–deficient (MCD) diet for 6 weeks. **A:** Liver sections stained with hematoxylin and eosin for assessment of steatosis and inflammation. Nonalcoholic fatty liver disease (NAFLD) activity scores were calculated based on histologic evaluation of steatosis and inflammation. **B:** Hepatic mRNA expression levels of adipokines [tumor necrosis factor (TNF)- α and adiponectin] determined by real-time quantitative RT-PCR shown as fold change compared with those in the MCS diet–fed WT mice. **C:** Hepatic mRNA levels of proliferator-activated receptor gamma (PPAR γ), LFABP, and LPL determined by real-time quantitative RT-PCR and shown as fold change compared with those in the MCS diet–fed WT mice. Data are expressed as means $\pm$ SEM per group. $n = 6$ mice in the MCS group; $n = 8$ mice in the MCD group. * $P < 0.05$ (two-tailed *t*-test). Original magnification: $\times 100$ (A, top row); $\times 200$ (A, bottom row).

Figure 7 Toll-like receptor 7 (TLR7) antagonism with immunoregulatory sequence (IRS) 661 reduces severity of nonalcoholic steatohepatitis. IRS 661- and control (Cont)- oligodeoxyribonucleotide (ODN)-treated mice were fed a methionine-choline-sufficient (MCS) or methionine-choline-deficient (MCD) diet for 6 weeks. **A:** Liver sections stained with hematoxylin and eosin for assessment of steatosis and inflammation. Nonalcoholic fatty liver disease (NAFLD) activity scores were calculated. **B:** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). **C:** Hepatic mRNA levels of adipokines [adiponectin and tumor necrosis factor (TNF)- α] measured by real-time quantitative RT-PCR shown as fold change compared with those in the Cont-ODN and MCS-treated mice. **D:** Hepatic mRNA levels of proliferator-activated receptor gamma (PPAR γ), L-FABP, and LPL determined by real-time quantitative RT-PCR shown as fold change compared with those in the Cont-ODN- and MCS-treated mice. Data are expressed as means $\pm$ SEM per group. $n = 6$ mice in the IRS 661 and Cont-ODN groups; $n = 8$ mice in the MCS and MCD groups. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (two-tailed t -test). Original magnification: $\times 100$ (A, top row); $\times 400$ (A, bottom row).

IRS 661-treated mice than that in control ODN-treated mice. Reduced liver injury in IRS 661-treated mice was confirmed by significantly lower levels of serum ALT and AST (Figure 7B). In analysis for hepatic adipokine levels,

IRS 661-treated mice had a significant decrease of TNF- α , whereas adiponectin was conversely significantly increased in IRS 661-treated mice fed with an MCD diet (Figure 7C). Reduced lipid accumulation in IRS 661-treated mice was

Figure 8 Schematic presentation showing toll-like receptor 7 (TLR7)–dependent nonalcoholic steatohepatitis (NASH) progression by inhibiting T regulatory (Treg) cells. TLR7 signaling activates Kupffer cells (KCs) and dendritic cells (DCs) to produce tumor necrosis factor (TNF)- α and type I interferon (IFN), which in turn will induce hepatocyte damages and inhibit Treg cell activity. Thus, manipulating the TLR7-Treg axis might be useful as a novel therapeutic strategy to treat NASH. IRF, interferon regulatory factor; ssRNA, single-stranded RNA.

confirmed by higher expression levels of PPAR γ and its target genes (Figure 7D). Taken together, these results support the notion that TLR7 signaling plays a key role in the pathogenesis of NASH.

Discussion

TLR7 signaling induces the production of type I IFNs and TNF- α through IRF7 and NF- κ B activation, respectively.^{34,35} Recently, it has been reported that TLR7 can regulate lipid accumulation in hepatocytes by inducing autophagy.¹⁸ Although simple steatosis has been proposed as the first hit of NASH development, steatohepatitis is a clinically important stage. However, the mechanistic role of TLR7 signaling in steatohepatitis has not been fully elucidated yet. The present study found that TLR7-deficient mice had ameliorated NASH compared with WT mice after an MCD diet. Furthermore, our data indicate that TNF- α –induced hepatocyte death and type I IFN– or TNF- α –dependent Treg cell apoptosis were pivotal in the progression of NASH based on experiments using IFNAR1-deficient, TLR7-deficient, or

DEREG mice. Consequently, we concluded that TLR7 signaling may promote NASH through regulation of Treg cell activities and hepatocyte death.

Several lines of evidence support that TLR7-dependent inhibition of Treg cells enhances NASH. First, Treg cell–mediated detrimental role of TLR7 and its downstream cytokines, TNF- α and type I IFN, have been well documented using various models.^{38–41} Second, hepatic expression of Foxp3, a key transcription factor for immunosuppressive activity of Treg cells,³⁷ is significantly higher in TLR7-deficient mice and Foxp3⁺ DEREG mice have more severe NASH than WT mice. Third, similar to TLR7 deficiency, inactivation of type I IFN signaling protected NASH and blocked Treg cell inhibition. Fourth, DCs are responsible cell type for the production of type I IFN, whereas KCs are mainly responsible for the production of TNF- α on TLR7 activation. Finally, TLR7 activation by R848 inhibits Treg cells through the production of TNF- α and type I IFN. Taken together, our results suggest that TLR7 activation–mediated TNF- α and type I IFN production are responsible for NASH development by regulating Treg cells and hepatocyte death (Figure 8).

Two possible mechanisms can activate innate immune system in NASH. First, translocated bacteria and their products can activate the innate immune network as exogenous ligands in NASH.^{5,42–44} In chronic liver diseases, including NASH, intestinal permeability is increased because of bacterial overgrowth or altered compositions of bacterial microflora.⁶ Systemic inflammation related to NASH also injures epithelial tight junctions,³ resulting in deregulation of intestinal barrier functions. Indeed, plasma levels of LPS are elevated in patients with NASH.⁷ Previous reports have found that TLR4, a receptor for LPS, can promote steatohepatitis and fibrosis.^{9,43} Increased bacterial nucleic acid (DNA and RNA) are also increased in the liver tissue of patients with NASH,⁴⁵ suggesting that translocated bacterial RNA could act as exogenous ligand in NASH. Second, accumulating evidence indicates that endogenous ligands released from damaged or stressed tissues designated as damaged tissue–associated molecular patterns can also signal through TLRs.⁴⁶ Engagement of TLRs by endogenous ligands could be a major trigger of inflammation. Recent studies have suggested that RNA released from damaged tissue or contained within endocytosed cells might serve as a ligand for TLR3.⁴⁷ Although it has been suggested that TLR3 can recognize host-derived single-stranded RNA, TLR7 is a more relevant receptor for single-stranded RNA generated by apoptotic cells than TLR3.^{12,13} Therefore, TLR7 was studied as a receptor for nucleic acid because it might play an important role in this disease.

To clarify downstream immunity of TLR7 signaling, IFNAR1-deficient mice were used and a TNF- α inhibition experiment performed. Similar to TLR7-deficient mice, IFNAR1-deficient mice had reduced steatohepatitis compared with WT mice, suggesting that the TLR7–type I

IFN axis had a detrimental role in NASH. In response to MCD diet–induced NASH, increased Foxp3⁺ Treg cells were evident in livers of TLR7-deficient and IFNAR1-deficient mice compared with those in livers of WT mice. Conversely, IFN- α_4 expression was comparable between WT and DERE mice (data not shown), suggesting that IFN- α_4 is an upstream regulator for Treg cell activities. Furthermore, TNF- α directly induced hepatocyte damage along with oxidative stress, whereas pentoxifylline treatment significantly recovered Foxp3 expression in response to R848. Therefore, it was speculated that TLR7-induced progression of NASH might be partially mediated by TNF- α and type I IFN. This notion is consistent with a previous report¹⁸ and the data indicating significantly increased hepatic Foxp3⁺ Treg cells in TLR7-deficient mice compared with those in WT mice. Moreover, treatment with the TLR7 antagonist IRS 661 ameliorated NASH development. Taken together, these findings suggest that increased Treg cells are responsible for ameliorating NASH in TLR7 or IFNAR1-deficient mice.

Although clodronate liposomes target mainly KCs, effects of KC depletion on liver diseases have been controversial according to the literature.^{48,49} In the present study, Foxp3 expression was examined after injection of clodronate liposome. Clodronate injection did not change the expression of Foxp3 compared with the R848-treated control group (data not shown). However, pentoxifylline (an inhibitor of TNF- α production) treatment significantly increased the Foxp3 expression (Figure 5D). A previous report indicated that the pretreatment of clodronate markedly decreased the proportion of the CD68⁺/F4/80⁺ KCs (high phagocytic and reactive oxygen species–producing capacity), clodronate induces proportional increase of the CD11b⁺/F4/80⁺ (high TNF-producing capacity),⁵⁰ suggesting that clodronate predominantly incorporated into CD68⁺ KCs may selectively deplete those cells. Because clodronate has off-target effects, such as depletion of monocytes⁵¹ and often altered global immune system, functional compensation could occur, thereby masking or distorting the phenotypes of these mice.⁵⁰ Therefore, pentoxifylline is a more appropriate approach than clodronate to validate the TNF- α –induced Treg cell suppression effect.

Accumulating evidence suggests that TNF- α or type I IFN induces apoptosis of Treg cells.^{40,52,53} To elucidate the mechanism of TLR7-mediated Treg cell suppression, type I IFN and TNF- α were depleted by DT injection in CD11c–DT receptor mice and pentoxifylline treatment, respectively, because DCs are the major cell type for production of type I IFN³⁶ and pentoxifylline inhibits TNF- α production. In the present study, *in vivo* activation of TLR7 by R848 injection increased apoptosis of Treg cells in the liver. Of interest, blockade of type I IFN or TNF- α significantly inhibited apoptosis of these cells. These results suggest that both TNF- α and type I IFN are cytokines responsible for regulating Treg cells by the induction of apoptosis.

In summary, TLR7 signaling activates TNF- α and type I IFN signaling, thus triggering the hepatocyte death and Treg cell suppression. This pathway plays a pivotal role in endogenous progression of NASH. Furthermore, we clarified that hepatic Foxp3⁺ Treg cells were key regulators of NASH pathogenesis. These findings indicate, for the first time, that stimulation of TLR7 promotes hepatocyte damage followed by steatohepatitis by inhibiting protective Treg cells. Understanding TLR7–Treg cell mechanisms will offer valuable insights to the prevention and treatment of NASH.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.07.011>.

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