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TLR2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation

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Abstract

Innate immune signaling associated with Toll like receptors (TLRs) is a key pathway involved in the progression of nonalcoholic steatohepatitis (NASH). Here we show that both TLR2 and palmitic acid are required for activation of the inflammasome, IL-1 α and IL-1 β , resulting in the progression of NASH. Wild type (WT) and TLR2^{-/-} mice were fed a choline deficient amino acid defined (CDAA) diet for 22 weeks to induce NASH. Bone marrow transplanted-TLR2 chimeric mice were generated after the recipient mice were lethally irradiated. Kupffer cells and hepatic stellate cells (HSCs) were isolated from WT mice and stimulated with TLR2 ligand and/or palmitic acid. WT mice on the CDAA diet developed profound steatohepatitis and liver fibrosis. In contrast, TLR2^{-/-} mice had suppressed progression of NASH. While both Kupffer cells and HSCs respond to TLR2 ligand, TLR2 bone marrow chimeric mice demonstrated that Kupffer cells were relatively more important than HSCs in TLR2-mediated progression of NASH. *In vitro*, palmitic acid alone did not increase TLR2 signaling-target genes including cytokines and inflammasome components in Kupffer cells and HSCs. The TLR2 ligand increased Nod-like receptor protein 3, an inflammasome component, in Kupffer cells, but not in HSCs. In the presence of TLR2 ligand, palmitic acid did induce caspase-1 activation and release of IL-1 α and IL-1 β in Kupffer cells, however, these effects were not observed in HSCs. *In vivo*, WT on the CDAA diet showed increased caspase-1 activation in the liver and elevated serum levels of IL-1 α and IL-1 β levels, which were suppressed in TLR2^{-/-} mice. Conclusion: TLR2 and palmitic acid cooperatively activate inflammasome in Kupffer cells/macrophages in the development of NASH.

Keywords

Kupffer cells; hepatic stellate cells; nalp3; caspase-1; interleukin-1 β

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Disclosures

No conflict of interest

Introduction

Nonalcoholic fatty liver disease (NAFLD), the hepatic consequence of the metabolic syndrome, is a significant public health issue in developed countries. A spectrum of NAFLD ranges from simple steatosis to steatosis with progressive liver inflammation and fibrosis, termed nonalcoholic steatohepatitis (NASH), eventually causing liver cirrhosis that may increase the prevalence of hepatocellular carcinoma (1, 2). However, majority of mechanisms involved in the multifactorial events in the development of NASH remain unresolved.

The hepatic innate immune system is activated in the pathogenesis of NASH (3, 4). Toll-like receptors (TLRs) are pattern recognition signal receptors for sensing bacterial and viral derived signature motifs to activate the innate immune system (5). TLR signaling functions in the host defense against invading pathogens through induction of proinflammatory cytokines in immune cells. However, the overactivation of TLR signaling or the breakdown of TLR tolerance produce a large amount of inflammatory cytokines, which ultimately leads to tissue damage (6). Among the 13 TLRs identified in mammals, TLR2, TLR4, and TLR9 are reported to be associated with steatohepatitis (4, 7, 8). TLR4 and TLR9 have been shown to promote hepatic inflammation and fibrosis in experimental NASH (8, 9). However, the role of TLR2 in the pathogenesis of NAFLD is controversial. TLR2 deficiency protected mice from hepatic steatosis induced by high fat diet (HFD) (10, 11). The NASH model induced by methionine and choline deficient (MCD) diet showed that loss of TLR2 increased susceptibility to pathogen-associated steatohepatitis (7, 12). These conflicting data imply the complexity of TLR2-mediated NAFLD.

Inflammasome activation is a pathway required for processing IL-1 β and IL-18 from their proforms to active forms through cleavage by caspase-1. Recent reports have demonstrated that loss of inflammasome components protects mice from NAFLD induced by a high fat diet (13, 14). In addition, inactivation of IL-1 receptor signaling attenuates mouse models of NASH (9, 15). These data suggest that the inflammasome contributes to the development of NAFLD. Two-signal hypothesis is accepted in inflammasome activation: the first signal produces pro-IL-1 β and inflammasome components, and the second signal assembles inflammasome components to engage in caspase-1 activation. TLRs including TLR2 activate the first signals, and the second signals are activated by danger-associated molecular patterns such as extracellular ATP and urate crystals. Emerging evidence demonstrated that free fatty acids (FFAs) activate the inflammasome (16, 17) as well as TLRs (18–20), which implicate FFAs as promoters of NASH. The elevated plasma FFA concentration in patients with the metabolic syndrome including NAFLD (21) may contribute to the development of NAFLD.

It is often debated which cell types respond to TLR ligands and are responsible for inflammasome activation. Macrophages including Kupffer cells are primary cells to activate the inflammasome and produce TLR-induced cytokines. Hepatic non-immune cells including hepatic stellate cells (HSCs) and hepatocytes also respond to TLR ligands and are the site of inflammasome activation (17, 22).

Here we show that TLR2 is a crucial molecule that promotes liver injury, inflammation and fibrosis in mice fed a choline deficient amino acid defined (CDAA) diet that develop NASH accompanied by obesity and insulin resistance (9, 23). TLR2 signaling increased the expression of proinflammatory cytokines including IL-1 β and an inflammasome component, Nod-like receptor protein (NLRP) 3. In cooperation with palmitic acid, the most abundant free fatty acid in the plasma of NAFLD (16, 24), TLR2 signaling activates inflammasome primarily in Kupffer cells, but not in HSCs or hepatocytes.

Materials and Methods

Animals and diet

Wild type (WT) C57BL/6 mice and TLR2^{-/-} mice purchased from Jackson Laboratories (Bar Harbor, ME) were bred in the UCSD vivarium. TLR2^{-/-} mice were backcrossed at least 10 generations onto the C57BL/6 background and displayed a similar hepatic phenotype as WT mice under standard laboratory chow. Male mice were divided into two groups at 8-week-old: choline-supplemented L-amino Acid Defined Diet (CSAA) (Catalog# 518754, Dyets Inc. Bethlehem, PA) and choline-deficient L-amino Acid Defined Diet (Catalog# 518753, Dyets Inc.). Each group included 7 to 10 mice. These diets were continued for 22 weeks without any interruption. The food intake on the CDAA diet was monitored for one month, and similarity in food intake was observed between WT and TLR2^{-/-} mice (Supplementary Figure 1).

To generate chimeric mice, liposomal clodronate was injected intravenously to deplete resident hepatic macrophages one day before the bone marrow transplantation. Bone marrow cells (1×10^7 cells) obtained from WT or TLR2^{-/-} mice were transplanted through tail veins after the recipient mice were lethally irradiated (10 Gy). CDAA or CSAA diet started eight weeks after bone marrow transplantation. Each group included 5 to 9 mice. The mice received humane care according to US National Institutes of Health recommendations outlined in the "Guide for the Care and Use of Laboratory Animals". All animal experiments were approved by the University of California San Diego and Akita University Institutional Animal Care and Use Committee.

Histological examination

Hematoxylin and eosin (H-E), Oil Red O, Sirius Red, immunohistochemical staining for α SMA (Dako Cytomation, Kyoto, Japan), F4/80 (eBioScience, San Diego, CA) and Ly6C (Abcam, Cambridge, MA) were performed (9). Sirius red positive area was measured on 10 low power (x40) fields/slide and quantified with the use of NIH imaging software. NAFLD activity score was determined according to the published criteria (25). F4/80- and Ly6C-positive cells were counted on 10 high power (200x) fields per slide.

Quantitative real-time PCR analysis

RNA extracted from livers and cells was converted to cDNA by reverse transcription. Then quantitative real-time PCR were performed using ABI PRISM 7000 Sequence Detector (Applied Biosystems, Foster city, CA). Genes were normalized to 18S RNA as an internal control. Sequences of primers were summarized in Supplementary Table 1.

Lipid isolation and measurement

Hepatic lipids were isolated as previously described (9). Triglyceride, total cholesterol and free fatty acid contents were measured using Triglyceride E (Wako, Osaka, Japan), Cholesterol E (Wako), and NEFA C-test (Wako) according to the manufacturer's instruction.

Western blot

Protein extracts were electrophoresed and then blotted. Blots were incubated with antibody for α SMA (Sigma, St. Louis, MO) or caspase-1 (Millipore, Temecula, CA).

Measurement for ALT, caspase-1 activity, insulin, IL-1 α , IL-1 β , and TNF α

A kit for measuring serum alanine transaminase (ALT) levels was obtained from Wako and a caspase-1 activity assay kit (catalogue #BF141000) was purchased from R&D

(Minneapolis, MN). Enzyme-linked immunoabsorbent assay (ELISA) kits were used for measuring insulin (Shibayagi, Gunma, Japan), IL-1 β , IL-1 α and TNF α (eBioscience). Insulin resistance was assessed by HOMA-IR [immunoreactive insulin (μ U/ml) $\times$ FBS (mg/dL) $\div$ 405] (26).

Cells and treatment

Kupffer cells, HSCs, and hepatocytes were isolated from mice as previously described (9). In experiments using hepatocytes, 200 μ L of liposomal clodronate was injected intravenously one day before isolation to deplete contaminated Kupffer cells. We have confirmed the intact responses of these hepatocytes to TNF α , IL-6 and insulin as assessed by activation of NF- κ B, STAT3 and Akt (data not shown). Hepatocytes and Kupffer cells were cultured in serum free media and HSCs were cultured with 1% FBS containing DMEM for 16 hours before stimulation with reagents. Pam₃CK₄ (5 μ g/ml) (EMC Microcollection, Tuebingen, Germany), CpG-ODN (5 μ g/ml) (ODN1826: 5'-tccatgacgtctctgacgtt-3') (invitrogen) and palmitic acid (200 μ M) (Sigma) were used to stimulate liver cells.

Statistical analysis

Differences between two groups were compared using Mann Whitney U test. Differences between multiple groups were compared using one-way ANOVA (Dr. SPSS II); $p < 0.05$ was considered significant.

Results

TLR2^{-/-} mice exhibit less inflammation in CDAA diet-induced NASH

As we reported previously (9), mice on a CDAA diet for 22 weeks resulted in severe steatosis, inflammatory cell infiltration, and hepatocyte ballooning (Figure 1A). TLR2^{-/-} mice on a CDAA diet had similar grades of steatosis (Figure 1A) and hepatic triglyceride content as WT mice (Table 1), whereas inflammatory cell infiltration and hepatocyte ballooning were markedly attenuated in TLR2^{-/-} livers (Figure 1A). Accordingly, the NAFLD activity score in TLR2^{-/-} mice was significantly lower than that in WT mice (Total score, WT versus TLR2^{-/-} mice = 6.5 versus 4.6, $p < 0.05$) (Figure 1A). WT livers on the CDAA diet increased infiltration of inflammatory cells that express F4/80 and Ly6C, while the recruitment of inflammatory cells was decreased in TLR2^{-/-} livers (Figure 1B). Serum ALT levels (Figure 1C) and the expressions of proinflammatory cytokines and chemokines (Figure 1D) were also decreased in TLR2^{-/-} mice in comparison to WT mice. The WT mice fed the CDAA diet also displayed obesity and insulin resistance (Figure 1E, 1F). While similar metabolic phenotypes were shown between WT and TLR2^{-/-} mice fed on standard chow (Table 1), the changes in weight gain and insulin resistance were blunted in TLR2^{-/-} mice on the CDAA diet (Figure 1E, 1F, Table 1). Moderate steatosis was seen in control CSAA diet fed mice, but inflammation was not evident in these animals (Figure 1 A-D).

Liver fibrosis is suppressed in TLR2^{-/-} mice after CDAA diet feeding

Liver fibrosis is a major manifestation of advanced NASH. The CDAA diet for 22 weeks resulted in perisinusoidal fibrosis in WT mice as assessed by Sirius red staining (Figure 2A, 2B). In contrast, TLR2^{-/-} mice did not show significant liver fibrosis (Figure 2A, 2B). α SMA expression was also attenuated in TLR2^{-/-} mice as examined by immunohistochemistry (Figure 2C) and immunoblotting (Figure 2D). Hepatic mRNA levels of collagen α 1(I), collagen α 1(IV), transforming growth factor β 1 (TGF- β 1, tissue inhibitor of metalloproteinase-1 (TIMP-1) and plasminogen activator inhibitor-1 (PAI-1) were decreased in TLR2^{-/-} mice compared with those in WT mice (Figure 2E). Neither WT nor

TLR2^{-/-} mice fed a control CSAA diet developed liver fibrosis or HSC activation (Figure 2 A -2E).

A TLR2 ligand activates both Kupffer cells and HSCs

Since Kupffer cells and HSCs are primary cells involved in hepatic inflammation and fibrosis, we examined whether cultured Kupffer cells and HSCs respond to a TLR2 ligand (27). Treatment with Pam₃CK₄, a synthetic TLR2 ligand, increased TNFα levels in WT-Kupffer cells at the mRNA (Figure 3A) and protein levels (Figure 3B). In both quiescent and culture-activated HSCs, Pam₃CK₄ treatment increased mRNA expression of fibrogenic genes including TIMP-1, PAI-1, and TGF-β 1 (Figure 3C), and decreased mRNA expression of Bambi, a decoy receptor for TGF-β receptor signaling (Figure 3D), demonstrating that TLR2 ligands induce a profibrogenic phenotype in HSCs.

Hematopoietic cells including Kupffer cells contribute to progression of liver inflammation and fibrosis

Since both Kupffer cells and HSCs may contribute to TLR2-mediated NASH and fibrosis, we wanted to determine the responsible cells that express functional TLR2 for the development of steatohepatitis. We generated TLR2 bone marrow (BM) chimeric mice, in which hepatic macrophages were reconstituted with transplanted BM cells. As previously reported, more than 90% of Kupffer cells were replaced with transplanted BM cells three months after BM transplantation (23, 28–30). Mice transplanted with WT BM (WT BM cells transplanted to WT mice or TLR2^{-/-} mice) on the CDAA diet feeding showed severe steatosis and inflammatory cell infiltration (Figure 4A). In contrast, mice transplanted with TLR2^{-/-} BM (TLR2^{-/-} BM cells transplanted to WT mice or TLR2^{-/-} mice) had less inflammatory cell infiltration (Figure 4A). Increases in serum ALT and hepatic mRNA levels of inflammatory cytokines were suppressed in mice transplanted with TLR2^{-/-} BM cells (Figure 4B, 4C). These results indicate that hematopoietic cells including Kupffer cells are the primary cell types involved in hepatic inflammation mediated by TLR2. Consistent with the histopathology of liver inflammation, liver fibrosis was attenuated in mice reconstituted with TLR2^{-/-} BM compared to mice with WT BM as assessed by Sirius red staining (Figure 4A). Hepatic mRNA levels of fibrogenic genes were significantly suppressed in mice transplanted with TLR2^{-/-} BM (Figure 4D). Thus, hematopoietic cells including Kupffer cells, but not HSCs, are responsible for TLR2-mediated liver inflammation and fibrosis in NASH.

TLR2 ligand and palmitic acid cooperatively activate the inflammasome in Kupffer cells

We sought to elucidate the mechanism by which Kupffer cells are the primary cell types in TLR2-mediated NASH. Since palmitic acid has been reported to be a TLR2 ligand (19, 20), we tested whether palmitic acid can produce TLR2-mediated cytokines that are induced by Pam₃CK₄ (Figure 3). In contrast to previous studies (18, 19), WT Kupffer cells did not increase TNFα and CD68 in response to palmitic acid, ranging from 10 to 500 μM (Supplementary Figure 2A, 2B). Furthermore, palmitic acid did not increase mRNA expression of fibrogenic factors in HSCs (Supplementary Figure 2C). These data indicate that palmitic acid alone would not function as a TLR ligand in Kupffer cells or HSCs.

Next, we tested whether palmitic acid contributes to inflammasome activation. Pam₃CK₄ increased mRNA expression of IL-1β and IL-1α in WT Kupffer cells, but palmitic acid did not (Figure 5A, B). Neither Pam₃CK₄ nor palmitic acid increased IL-1β mRNA in HSCs (Supplementary Figure 3A). We then measured the active form of IL-1β and IL-1α in supernatant. In the presence of Pam₃CK₄, palmitic acid induced secretion of active form of IL-1β and IL-1α from Kupffer cells (Figure 5B, D). In contrast, HSCs did not secrete IL-1β by the same treatment (Supplementary Figure 3B), and palmitic acid or Pam₃CK₄ alone did

not induce IL-1 β secretion in either Kupffer cells or HSCs, suggesting that two-step stimulation with TLR2 ligand and palmitic acid is required for the inflammasome activation. To assess inflammasome activation by TLR2 ligands and palmitic acid, we measured the expression of inflammasome components including NLRP3, ASC, NLRP1 and AIM2. Among them, Pam₃CK₄ induced NLRP3 expression in Kupffer cells, but not in HSCs (Figure 5C, Supplementary Figure 3C), and NLRP3 was not induced by palmitic acid treatment alone (Figure 5C). In agreement with the findings shown above, Kupffer cells primed with Pam₃CK₄ increased caspase-1 activity in response to palmitic acid (Figure 5D, E). As expected, palmitic acid did not activate caspase-1 in HSCs primed with Pam₃CK₄ (Supplementary Figure 3D). We further examined hepatocytes to respond to Pam₃CK₄ and palmitic acid. Primary hepatocytes isolated from mice pretreated with liposomal clodronate to deplete Kupffer cells *in vivo* did not increase levels of IL-1 β mRNA and protein, NLRP3 mRNA and caspase-1 activity by Pam₃CK₄ and palmitic acid treatment (Supplementary Figure 4A-D). TNF α production was also not seen in these hepatocytes in response to Pam₃CK₄ (Supplementary Figure 4E, F). These results indicate that hepatocytes are not the responsible cell types for TLR2-mediated inflammasome activation.

Diminished inflammasome activation in TLR2^{-/-} mice

Finally, we investigated inflammasome activation *in vivo*. WT mice on the CDAA diet increased expressions of IL-1 β mRNA (Figure 1F) and the inflammasome component NLRP3 in the liver (Figure 6A). In contrast, this induction was diminished in TLR2^{-/-} mice liver (Figure 1F, 6A). Moreover, portal vein FFA levels were lower in TLR2^{-/-} mice than in WT mice (Figure 6B). In addition, WT mice on the CDAA diet exhibited increased hepatic caspase-1 activity and serum levels of IL-1 β and IL-1 α (Figure 6C-E) whereas TLR2^{-/-} mice had lower activity of caspase-1 in the liver and decreased IL-1 β and IL-1 α levels in sera (Figure 6C-E). These results demonstrate that TLR2 is required for activation of the inflammasome in mice fed the CDAA diet.

The collaborative effect of TLR2 and TLR9 ligands on cytokine production in Kupffer cells

Since TLR9 signaling promotes NASH progression through IL-1 β (9), we investigated whether palmitic acids participate in TLR9-mediated IL-1 production. In combination with CpG-ODN, a synthetic TLR9 ligand, palmitic acids functioned to secrete IL-1 α and IL-1 β in Kupffer cells (Figure 7A, B). Then, we examined the collaborative effect of TLR2 and TLR9 ligands in Kupffer cells. TLR2 and TLR9 ligands synergistically upregulated mRNA levels of proinflammatory cytokines including IL-1 β , IL-1 α , TNF α and MCP-1 (Figure 7C-F). The synergistic effects of TLR2 and TLR9 ligands were also observed in the secretion of IL-1 α and TNF α proteins (Figure 7A, B, E). These results suggest that TLR2 and TLR9 signaling collaboratively induce cytokine production in Kupffer cells that promote NASH.

Discussion

We have previously demonstrated that activation of TLR4 signaling is crucial for the development of liver fibrosis through amplification of TGF β signaling in HSCs (28). In murine NASH models, TLR4 ligand LPS and TLR9 ligand bacterial DNA were elevated in the plasma (9, 23), and the signaling of TLR4 and TLR9 promoted NASH development. MyD88, an adaptor molecule for TLR2, TLR4 and TLR9, is crucial for NASH progression (9). The present study demonstrates that TLR2 signaling acts as a promoter of liver inflammation and fibrosis in CDAA diet-induced NASH (Figure 1 and 2). Interestingly, TLR2^{-/-} mice displayed less liver inflammation and fibrosis but similar levels of hepatic steatosis as WT mice, indicating that TLR2 signaling contributes to liver inflammation and fibrosis, but that hepatic lipid accumulation is independent of TLR2. Although a TLR2 ligand can activate both Kupffer cells and HSCs (Figure 3), our *in vivo* study using TLR2

BM chimeric mice demonstrated that Kupffer cells are responsible for TLR2-mediated liver inflammation and fibrosis (Figure 4). The activation of the inflammasomes, and the production of IL-1 α and IL-1 β in WT mice on the CDAA diet were suppressed in TLR2-deficient mice (Figure 6). In Kupffer cells, the cooperative actions mediated by TLR2 ligand and palmitic acids are required for activation of inflammasomes and production of IL-1 α and IL-1 β (Figure 5). We also found that TLR2 and TLR9 signaling synergistically induce production of inflammatory cytokines that can promote NASH (Figure 7).

A previous study reported that TLR2^{-/-} deficiency aggravates NASH developed on an MCD diet (12). When mice fed a MCD diet, TLR2^{-/-} mice are more susceptible to LPS-induced inflammatory and fibrogenic responses than WT mice (7, 12). The present study used the CDAA diet that produces weight gain, insulin resistance and liver fibrosis, in contrast to MCD diet that causes weight loss, improvement of insulin signaling, and only very mild liver fibrosis. Our data showed that the portal vein FFA levels were elevated in mice fed the CDAA diet, but not the MCD diet (Supplementary Figure 5). Because the majority of NASH patients develop obesity, insulin resistance and liver fibrosis with elevation of FFA levels (21), we propose that the CDAA diet model is more relevant NASH model to human NASH than the MCD diet model. The murine HFD model induces obesity, insulin resistance and hepatic steatosis, but liver inflammation is very mild and fibrosis does not develop. TLR2^{-/-} mice and MyD88^{-/-} mice had less steatosis than WT mice on a HFD (10, 11). Similarly, TLR2^{-/-} mice exhibited less hepatic inflammation and fibrosis on the CDAA diet, which influences obesity and systemic insulin resistance. Notably, TLR2^{-/-} mice on the CDAA diet developed similar degree of hepatic steatosis, but insulin resistance was suppressed. Alternatively, CSAA diet caused much less hepatic steatosis than CDAA diet, but induced similar degree of insulin resistance caused by CDAA diet (Figure 1) (9, 31). These findings suggest that systemic insulin resistance, simple steatosis and NASH substantially affect one another, but could be caused by different etiologies, and steatosis is not a preliminary hepatic condition for NASH (32).

The inflammasome is a multi-protein platform containing NLRP1, NLRP3, NLRC4 and AIM2 that activates caspase-1 through the adaptor protein ASC. Activated caspase-1 proteolytically cleaves pro-IL-1 β and pro-IL-18 to generate active forms of IL-1 β and IL-18 (33). A recent report demonstrated that IL-1 α secretion also requires inflammasome activation (34). Inflammasome activation has been reported to be important for the development of obesity, hepatic steatosis and insulin resistance (13, 14, 16). In these reports, NLRP3^{-/-}, ASC^{-/-} and caspase-1^{-/-} mice on a HFD had less hepatic steatosis and obesity and improved insulin sensitivity (13, 14, 16). The function of the inflammasome in a NASH model induced by MCD diet is controversial. Two reports showed that activation of inflammasome components including caspase-1 promotes liver inflammation and fibrogenesis in NASH induced by MCD diet (17, 35). However, a recent study demonstrated that mice deficient in caspase-1, ASC or NLRP3 developed more steatosis by MCD diet (36). The results from our present and previous studies are in agreement with concept that inflammasome activation promotes NASH and insulin resistance. In particular, TLR2^{-/-} mice fed a CDAA diet exhibited decreased expression of inflammasome components including NLRP3, caspase-1, IL-1 α and IL-1 β , and TLR2^{-/-} mice and IL-1R^{-/-} mice showed reduced body weight gain and suppression of insulin resistance, liver injury, inflammation and fibrosis compared with WT mice (9). TLR ligands alone are sufficient for induction of NLRP3 through NF- κ B, but are insufficient to activate the inflammasomes (37, 38). Additional factors such as particular bacterial toxins, extracellular ATP or certain danger signals are required for assembly of inflammasome components and activation (33, 39, 40). Dietary factors, such as uric acids, cholesterol crystal and palmitic acid were also reported as a trigger to assemble the inflammasome components in macrophages (16, 41, 42). Wen et al demonstrated that LPS activates inflammasome in cooperation with palmitic

acid, one of the most abundant saturated fatty acids in plasma of NAFLD, in BM-derived macrophages (16, 42). Similarly, our data demonstrated that the TLR2 or TLR9 ligand alone failed to activate inflammasomes, while palmitic acid in concert with a TLR2 or TLR9 ligand induces inflammasome activation in Kupffer cells (Figure 5, 7) (9). Importantly, in hepatic macrophages/ Kupffer cells, LPS alone can activate inflammasomes to produce IL-1 β , IL-1 α and IL-18 through the MyD88-independent TRIF-dependent pathway (Figure 7A, B) (43, 44). Although palmitic acid has been characterized as a TLR2 and TLR4 ligand to induce inflammatory cytokines (18–20), data from recent research and ours did not support this effect of palmitic acid in both Kupffer cells and HSCs (45).

HMGB-1 released from damaged hepatocytes may activate TLR2 in addition to TLR4 as an endogenous ligand in NASH. Intestinal microflora can also be the source of TLR2 ligand because of contradictory phenotypes in metabolic disease in TLR2^{-/-} mice between SPF and non-SPF conditions that affect the composition of gut flora (10, 11, 46). Oral bacteria contribute to the progression of metabolic disease including atherosclerosis (47, 48). Thus, endogenous ligands, such as HMGB-1, and the components derived from intestinal microflora or oral bacteria can be a ligand for TLR2 in NASH.

The present study demonstrates that TLR2^{-/-} mice exhibited reduced NASH compared to WT mice, but seemed more NASH pathology than in TLR9^{-/-} mice (9). Our data suggest that TLR2^{-/-} Kupffer cells are more sensitive to produce inflammatory cytokines than WT Kupffer cells in response to TLR9 ligand whereas TLR9^{-/-} Kupffer cells have similar response to TLR2 ligand in comparison to WT Kupffer cells (Figure 7). It is conceivable that TLR9 signaling may compensate the expression of inflammatory cytokines in TLR2^{-/-} Kupffer cells, which may explain the mechanisms underlying suppressed NASH pathology in TLR9^{-/-} mice compared to TLR2^{-/-} mice. However, liver inflammation in TLR2^{-/-} mice was still less than WT mice because NASH progression requires the synergy of TLR2 and TLR9 signaling. This synergy induces the production of inflammatory cytokines in Kupffer cells that promote NASH. In particular, TLR2 and TLR9-mediated IL-1 induces fibrogenic responses including the upregulation of TIMP-1 and PAI-1, and the downregulation of Bambi, an endogenous inhibitor of the TGF β ; thus the combination of these responses promotes liver fibrosis in HSCs (9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard abbreviations used

ALT	alanine transaminase
BM	bone marrow

CCR	CC chemokine receptor
CDAA	choline-deficient amino-acid defined
CSAA	choline-supplemented amino acid defined
ELISA	enzyme-linked immunoabsorbent assay
HE	hematoxylin and eosin
HFD	high fat diet
HOMA-IR	homeostasis model assessment of insulin resistance
HSC	hepatic stellate cell
IL-1β	interleukin-1 β
MCD	methionine and choline deficient
MCP	monocyte chemoattractant protein
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NLRP	Nod-like receptor protein
PAI-1	plasminogen activator inhibitor-1
TGF-β1	transforming growth factor β 1
TIMP-1	tissue inhibitor of metalloproteinase-1
TLR	Toll-like receptor
TNF α	tumor necrosis factor α
WT	wild type

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Figure 1. TLR2^{-/-} mice exhibit less inflammation in CDAA-induced NASH

WT and TLR2^{-/-} mice were fed CSAA diet (CS) or CDAA diet (CD) for 22 weeks. Closed bars indicate WT mice and open bars represent TLR2^{-/-} mice. (A, left) Hematoxylin and eosin (HE) and oil red O staining. Liver sections on CDAA diet are presented. The grades of steatosis were similar levels in WT and TLR2^{-/-} mice whereas inflammatory cell infiltration (arrows) and hepatocyte ballooning (arrow head) were blunted in TLR2^{-/-} mice. Original magnification, $\times 400$ for HE and oil red O staining. Bar 100 μ m. (A, right) NAFLD activity score. (B) Immunohistochemical staining for F4/80 and Ly6C. Liver sections on CDAA diet are presented. Infiltration of F4/80- and Ly6C-positive cells was suppressed in TLR2^{-/-} mice. Original magnifications, $\times 400$ for F4/80, $\times 600$ for Ly6C. Bar 100 μ m. (B, right) The

numbers of F4/80-positive and Ly6C-positive cells. (C) Serum ALT levels. (D) mRNA expression of TNF α , IL-1 β , and MCP-1. Genes were normalized to 18S RNA as an internal control. (E, F) Data on mice fed standard chow (ST) were included. (E) Body weight. (F) HOMA-IR. ND; not detected. Data represent mean $\pm$ SD, * p <0.05. n.s.; not significant.

Figure 2. TLR2^{-/-} mice develop less liver fibrosis

WT and TLR2^{-/-} mice were fed CSAA diet (CS) or CDAA diet (CD) for 22 weeks. Closed bars represent WT mice and open bars indicate TLR2^{-/-} mice. (A) Sirius red staining. Original magnification, ×400. Bar 100 μm. (B) Sirius red positive area. Liver fibrosis was attenuated in TLR2^{-/-} mice. (C) Immunohistochemical staining for αSMA. Original magnification, ×400. Bar 100 μm. (D) Western blotting for αSMA. (E) mRNA expression of fibrogenic genes. Genes were normalized to 18S RNA as an internal control. Data represent mean ±SD, *p<0.05. n.s.; not significant.

Figure 3. A synthetic TLR2 ligand activates both Kupffer cells and HSCs

WT Kupffer cells and HSCs were isolated and cultured in the presence of 5 μ g/ml Pam₃CK₄.

(A) mRNA expression of TNF α in Kupffer cells. (B) TNF α concentrations in the supernatant from WT Kupffer cell-culture. (C) mRNA expression of fibrogenic genes in quiescent HSCs and culture-activated HSCs. (D) BamBI mRNA expression in HSCs. Genes were normalized to 18S RNA as an internal control. Data represent mean $\pm$ SD, *p<0.05.

**p<0.01. n.s.; not significant.

Figure 4. Hematopoietic cells including Kupffer cells are crucial for the development of liver inflammation and fibrosis in NASH

TLR2 bone marrow (BM) chimeric mice, in which hepatic macrophages were reconstituted with transplanted BM cells, were generated. Mice on CDAA diet are presented. (A) HE staining (upper) and Sirius red staining (lower). WT BM-transplanted mice show inflammatory cell infiltration (arrows), which are blunted in TLR2^{-/-} BM transplanted mice. Liver fibrosis is attenuated in TLR2^{-/-} BM transplanted mice. Original magnification, $\times 400$. Bar 100 μ m. (B) Serum ALT levels. (C) mRNA expression of TNF α and IL-1 β . (D) mRNA expression of fibrogenic genes. Genes were normalized to 18S RNA as an internal control. Data represent mean $\pm$ SD, * $p < 0.05$.

Figure 5. Palmitic acid activates inflammasome in cooperation with TLR2 ligand in Kupffer cells
Kupffer cells were isolated from WT mice and cultured in the presence of 5 μ g/ml Pam₃CK₄ and/or 200 μ M palmitic acid. (A) mRNA expression of IL-1 β and active IL-1 β in the supernatant. (B) mRNA expression of IL-1 α and active IL-1 α in the supernatant. (C) mRNA expression in NLRP3. Genes were normalized to 18S RNA as an internal control. (D) Caspase-1 activity. (E) Immunoblotting for the active form of caspase-1 is shown. N.D.; not detected. Data represent mean $\pm$ SD, * p <0.05.

Figure 6. Inflammasome activation is abolished in TLR2^{-/-} mice

WT and TLR2^{-/-} mice were fed control CSAA (CS) diet, and CDAA (CD) diet for 22 weeks. Closed bars represent WT mice and open bars indicate TLR2^{-/-} mice. (A) Hepatic mRNA expression of NLRP3. Genes were normalized to 18S RNA as an internal control. (B) FFA concentrations in the portal vein. (C) Caspase-1 activation in the liver. (D) Serum IL-1β concentrations. (E) Serum IL-1α concentrations. N.D.; not detected. Data represent mean ± SD, *p < 0.05. n.s.; not significant.

Figure 7. TLR2 and TLR9 ligands synergistically produce inflammatory cytokines in Kupffer cells

Kupffer cells were isolated from WT, TLR2^{-/-} and TLR9^{-/-} mice, and cultured in the presence of 5 μ g/ml Pam₃CK₄, 5 μ g/ml CpG-ODN and/or 200 μ M palmitic acid. (A,B,E) IL-1 β (A), IL-1 α (B) and TNF α (E, right) proteins in the supernatant. (C-F) mRNA expression of IL-1 β (C), IL-1 α (D), TNF α (E, left) and MCP-1 (F). Genes were normalized to 18S RNA as an internal control. N.D.; not detected. Data represent mean $\pm$ SD, *, significant differences to WT KCs treated with Pam₃CK₄ or CpG-ODN, #, significant differences to WT KCs treated with CpG-DNA, §, no significant difference with WT KCs treated with Pam₃CK₄.

Table 1

Body/Liver/Fat weight and Lipid Level at 22 weeks after the CSAA and CDAA diet feeding.

	Standard chow			CSAA		CDAA	
	WT mice (n = 7)	TLR2 ^{-/-} mice (n = 7)	WT mice (n = 10)	TLR2 ^{-/-} mice (n = 7)	WT mice (n = 10)	TLR2 ^{-/-} mice (n = 9)	
Body weight (g, start, wk=0)	22.5 ± 0.95	21.4 ± 0.65	21.5 ± 1.13	22.9 ± 0.93	23.0 ± 1.74	21.0 ± 0.82	
Body weight (g, end, wk=22)	30.9 ± 1.22	29.5 ± 0.73	42.0 ± 2.52 ^a	33.8 ± 1.75	42.8 ± 2.02 ^a	33.5 ± 1.94 ^c	
Liver weight (g)	1.22 ± 0.14	1.15 ± 0.22	1.88 ± 0.21 ^a	1.37 ± 0.53	2.54 ± 0.38 ^{a,b}	1.54 ± 0.28 ^c	
Liver weight (%)	3.71 ± 0.75	3.80 ± 0.66	4.44 ± 0.73 ^a	4.05 ± 0.68	5.50 ± 0.64 ^{a,b}	5.11 ± 0.58 ^c	
Epididymal fat (g)	0.96 ± 0.22	0.87 ± 0.36	2.24 ± 0.76 ^a	1.25 ± 0.51	2.39 ± 0.52 ^a	1.01 ± 0.34 ^c	
Epididymal fat (%)	2.85 ± 0.34	2.95 ± 0.54	4.82 ± 0.91 ^a	4.00 ± 0.77	4.70 ± 0.75 ^a	3.53 ± 0.67 ^c	
Plasma							
Triglyceride (mg/dl)	36.5 ± 6.25	33.8 ± 4.82	55.3 ± 7.55	48.8 ± 6.12	70.5 ± 10.6 ^a	51.1 ± 8.76	
Total cholesterol (mg/dl)	70.3 ± 6.32	66.9 ± 7.12	143 ± 12.8 ^a	99.1 ± 15.8	99.6 ± 8.69	82.8 ± 7.93	
Free fatty acid (mEq/L)	0.40 ± 0.11	0.35 ± 0.08	0.47 ± 0.19	0.41 ± 0.13	0.50 ± 0.21	0.36 ± 0.12	
Liver							
Triglyceride (mg/g liver)	33.5 ± 3.91	30.7 ± 3.67	80.4 ± 8.81 ^a	54.3 ± 6.92	155 ± 19.5 ^{a,b}	150 ± 20.6	
Total cholesterol (mg/g liver)	11.6 ± 2.56	10.6 ± 1.68	17.5 ± 2.56	12.6 ± 1.05	23.7 ± 3.09 ^a	15.1 ± 2.61	
Free fatty acid (mEq/g liver)	0.60 ± 0.16	0.57 ± 0.17	2.79 ± 0.85 ^a	2.55 ± 0.51	3.08 ± 0.88 ^a	2.48 ± 0.62	

Note: Values are mean ± SD.

^aSignificantly different from WT on Standard chow, p<0.05.

^bSignificantly different from WT on CSAA diet, p<0.05.

^cSignificantly different from WT on CDAA, p<0.05.