Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice

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Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. Am J Physiol Gastrointest Liver Physiol 300: G433–G441, 2011. First published January 13, 2011; doi:10.1152/ajpgi.00163.2009.—Toll-like receptor 4 (TLR4) and its coreceptor, myeloid differentiation factor-2 (MD-2), are key in recognition of lipopolysaccharide (LPS) and activation of proinflammatory pathways. Here we tested the hypothesis that TLR4 and its coreceptor MD-2 play a central role in nonalcoholic steatohepatitis (NASH) and liver fibrosis in nonalcoholic fatty liver disease. Mice of control genotypes and those deficient in MD-2 or TLR4 [knockout (KO)] received methionine choline-deficient (MCD) or methionine choline-supplemented (MCS) diet. In mice of control genotypes, MCD diet resulted in NASH, liver triglycerides accumulation, and increased thiobarbituric acid reactive substances, a marker of lipid peroxidation, compared with MCS diet. These features of NASH were significantly attenuated in MD-2 KO and TLR4 KO mice. Serum alanine aminotransferase, an indicator of liver injury, was increased in MCD diet-fed genotype controls but was attenuated in MD-2 KO and TLR4 KO mice. Inflammatory activation, indicated by serum TNF-α and nictoinamide adenine dinucleotide phosphate oxidase complex mRNA expression and activation, was significantly lower in MCD diet-fed MD-2 KO and TLR4 KO compared with corresponding genotype control mice. Markers of liver fibrosis [collagen by Sirius red and α-smooth muscle actin (SMA) staining, procollagen-I, transforming growth factor-β1, α-SMA, matrix metalloproteinase-2, and tissue inhibitor of matrix metalloproteinase-1 mRNA] were attenuated in MD-2 and TLR4 KO compared with their control genotype counterparts. In conclusion, our results demonstrate a novel, critical role for LPS recognition complex, including MD-2 and TLR4, through NADPH activation in liver steatosis, and fibrosis in a NASH model in mice.

endotoxin; fatty liver; inflammation; nictoainamide adenine dinucleotide phosphate; α-smooth muscle actin

NONALCOHOLIC FATTY LIVER disease (NAFLD) is an increasingly common cause of liver disease that can progress to nonalcoholic steatohepatitis (NASH) and culminate in end-stage liver disease, featuring liver damage with fibrosis and cirrhosis (1, 10, 36). The pathogenesis of NAFLD/NASH is poorly understood. Increased hepatocyte fat deposition, apoptosis, mitochondrial dysfunction, abnormal peroxisome and microsome functions, and insulin resistance is associated with steatosis and have been observed during NAFLD (6, 32, 36, 44). More recently, a key role for inflammation was identified in NAFLD/NASH (6, 14, 28, 32, 38). Inflammation is imposed on steatosis by recruitment and activation of inflammatory cells in the liver, which contributes to steatohepatitis, a hallmark of clinically progressive NAFLD to NASH (1, 10, 14). Later, liver resident macrophages, Kupffer cells, and stellate cells induce liver remodeling with extensive fibrosis and liver cirrhosis, which dictates the final stages of the NAFLD/NASH (1, 10, 14). While the sequence of events, including fat accumulation, inflammation, and fibrosis, is not clearly delineated, inflammation seems to play a leading role in NAFLD/NASH progression (1, 10, 14, 36). Activation of inflammatory cascades can be induced by a variety of danger signals; recent studies have suggested that bacterial overgrowth and endotoxemia play a role in the pathogenesis of NAFLD/NASH (5, 11, 22, 31). The importance of increased circulating and portal blood levels of lipopolysaccharide (LPS) has been established in the pathogenesis of alcoholic liver disease (27), a disease that is historically indistinguishable from NAFLD (36).

Pattern recognition toll-like receptor 4 (TLR4) is expressed on many cell types in the liver, including Kupffer cells, stellate cells, and hepatocytes (37). Initially discovered as a receptor for LPS, a cell wall component of gram-negative bacteria also referred to as endotoxin, TLR4 is implicated in recognition of heparan sulfate, fibrinogen, hyaluronan, high-mobility group box 1 (HMGB1), and potentially several other danger signals (18, 19). The diversity of TLR4 ligands, and the fact that the mediators that drive the systemic inflammatory response in the setting of sepsis or sterile tissue injury are strikingly similar (15), suggests that TLR4 and its coreceptors may play a key role in persistent inflammatory diseases for which the clear pathogen etiology has not been well established, including the NAFLD/NASH.

At least for LPS recognition, TLR4 forms a complex with its coreceptor, myeloid differentiation factor-2 (MD-2) (18, 21). MD-2 is a glycoprotein that binds to both LPS and the extracellular domain of TLR4 (2, 40, 41). Ligand engagement of the TLR4-MD-2 complex results in activation of a plethora of downstream signaling pathways that generate a variety of cellular responses. TLR4-MD-2-mediated activation of MAP kinases and NF-κB leads to activation of the proinflammatory cascade and oxidative stress through the components of the NADPH complex (25). Activation of the inflammatory cascade and induction of reactive oxygen species are particularly relevant to NAFLD/NASH, since inflammatory cytokines, such as TNF-α and oxidative stress, through NF-κB activation, contribute not only to liver inflammation but also to insulin resistance, a hallmark of NAFLD (20, 44).

Here, we investigated the hypothesis that MD-2 and TLR4 receptor complex play a role in the development of NAFLD/NASH. Using MD-2 and TLR4 KO mice and methionine choline restriction as a tool to evaluate mechanisms of inflam-
mice at generation eight, we found a mechanistic role for MD-2 and TLR4 receptor complex in several steps of the pathogenesis of NAFLD/NASH, including liver inflammation, steatosis, and fibrosis.

MATERIALS AND METHODS

Animals and experimental protocol. Three-month-old female mice were employed. MD-2-deficient or TLR4-deficient animals [knockout (KO)] (a kind gift from Dr. K. Miyake from Tokyo, Japan) were backcrossed with C57BL/6 and genotyped by PCR of tail DNA. MD2 KO mice were tested for microsatellite (99% identical with C57BL/6), and littermate controls were used (n = 6–8 mice/group). For TLR4 KO mice, all tests were initially performed using mice backcrossed (x) with C57BL/6J and later confirmed using mice at backcross generation x = 8; given the similarity of the results, the data were compiled, and overall there were n = 14–16/group. Testing shown in Figs. 1, C, 1D, and 2F was performed on TLR4 KO mice at generation eight (x = 8) after backcrossing. C57Bl/6J mice were used as controls for all TLR4 KO mice, based on their genetic proximity to the C57Bl6 strain and in agreement with recommendations for genetic background use from Jackson Laboratory (43).

This study was approved by the Institutional Animal Use and Care Committee at the University of Massachusetts Medical School. All animals were cared for in accordance with the Institutional Animal Care and Use Committee regulations at the University of Massachusetts Medical School. The mice were fed a methionine choline-deficient diet supplemented with 10% FBS, stimulated with a cocktail of PMA (50 ng/ml), ionomycin (1 μg/ml), and brefeldin A (10 μg/ml) in RPMI 1640 + 10% FBS for 4 h, and stained for surface CD68 and intracellular TNF-α using specific fluorescent-labeled antibodies and CytoFix/CytoPerm Kit (BD Bioscience, San Jose, CA). The cells were gated by size and granularity, and their fluorescence was analyzed using the LSR flow cytometer.

Cytokine measurements. Serum TNF-α levels were determined using the Pierce Multiplex Cytokine Array (Pierce, Woburn, MA).

mRNA analysis. Total RNA extraction from liver tissue and mRNA quantification using SYBR Green-based real-time quantitative polymerase chain reaction was performed as previously described (39). All specific mRNA levels were normalized against the housekeeping gene, 18S, in the same sample. The specific PCR primer sequences for target genes 18S, p22 phox, p47 phox, p67 phox, and gp91 phox have been published previously (27); additional genes studied here are listed in Table 1.

NADPH oxidase assay. Whole liver tissue extract was performed as we previously described (39); protein content was quantified using Bio-Rad Protein Assay (500–0006; Bio-Rad Laboratories, Hercules, CA). NADP+/NADPH concentrations from tissue extracts with comparable protein amounts were determined using the EnzChrom NADP+/NADPH assay kit (ECNP-100) (BioAssay Systems, Hayward, CA), as recommended by the manufacturer.

Statistical analysis. Statistical significance was determined using the nonparametric Kruskal-Wallis and Mann-Whitney tests; for ALT assays, given the usage of two distinct kits for analysis in different experiments, the statistical significance of the data was confirmed using the nonparametric Wilcoxon test. Data are presented as means ± SE and were considered statistically significant at a P value <0.05.

RESULTS

MD-2 or TLR4 deficiency protects from MCD diet-induced liver fat deposition and inflammation. Inflammation is a major component of NASH (1, 10, 36). In the related condition of alcoholic steatohepatitis (ASH), endotoxin has been shown to contribute to activation of the inflammatory cascade leading to liver damage (27). MD-2 and TLR4 complex is the major receptor for endotoxin (18). Given the common pathophysiological features of ASH and NASH, we aimed to identify the role of MD-2-TLR4 complex in an experimental model of NASH using mice deficient in MD-2 or TLR4 and their endotoxin was quantified using LAL assay (detection limit 0.1 EU/ml; Cambrex, Walkersville, MD).

Isolation of liver mononuclear cells and flow cytometry analysis. Animals received anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg); the livers were perfused with Hank’s balanced saline solution (HBSS) followed by in vivo digestion with 0.33 mg/ml Liberase RI Enzyme (F. Hoffmann-La Roche, Basel, Switzerland) in HBSS. The liver mononuclear cells (LMNCs) were purified from whole liver cell suspension obtained after tissue disruption using centrifugation at slow speed (500 g) and subsequent isolation in Percoll 4070 gradient density at 800 g; LMNC were harvested from the gradient interface. The cells were further washed in saline supplemented with 2% FBS, stimulated with a cocktail of PMA (50 ng/ml), ionomycin (1 μg/ml), and brefeldin A (10 μg/ml) in RPMI 1640 + 10% FBS for 4 h, and stained for surface CD68 and intracellular TNF-α using specific fluorescent-labeled antibodies and CytoFix/CytoPerm Kit (BD Bioscience, San Jose, CA). The cells were gated by size and granularity, and their fluorescence was analyzed using the LSR flow cytometer.
genotype control counterparts. Feeding a MCS diet resulted in no signs of hepatic steatosis or inflammation in any of the mice (Fig. 1). In contrast, mice of control genotypes fed a MCD diet for 8 wk developed significant hepatic steatosis; MD-2- and TLR4-deficient mice on MCD diet showed lower liver fat accumulation, identified after OilRed O staining, compared with the mice of control genotypes (Fig. 1A). Consistent with the development of hepatic steatosis, liver triglyceride levels were significantly increased in MCD diet-fed control genotype mice but to a significantly lower extent in MD-2- or TLR4-deficient mice (Fig. 1B). These findings suggested that TLR4-MD-2 complex deficiency is partially protective against MCD-induced liver steatosis.

Feeding of MCD diet leads to accumulation of inflammatory cells into the liver in mice of control genotypes, and to a lesser extent in MD-2 or TLR4 KO mice, as indicated by the increase in content of F4/80 cells in the livers of MCD-fed animals compared with MCS diet-fed controls (Fig. 1C).

Fig. 2. Deficiency in LPS recognition complex prevents MCD diet-induced upregulation in the expression of NADPH complex and protects from lipid peroxidation. Mice of genotype control, TLR4 KO, and MD-2 KO were fed MCD or MCS diets for 8 wk. Liver thiobarbituric acid reactive substances (TBARS) levels (A) were analyzed as described in MATERIALS AND METHODS. Expression of liver p47phox (B), p67phox (C), gp91phox (D), and p22phox (E) was quantified by quantitative PCR (qPCR) using specific primers and normalization against the housekeeping gene 18S. NADPH oxidase activity was determined by measuring NADP⁺-to-NADPH ratios as described in MATERIALS AND METHODS (F). *P < 0.05 compared with the corresponding MCS group.
the proportion of TNF-α-producing CD68⁺ macrophages was increased in MCD-fed compared with MCS-fed genotype controls (Fig. 1D). More importantly, TLR4 deficiency protected from MCD diet-induced accumulation of the TNF-α-producing CD68⁺ macrophages in the liver (Fig. 1D). A significant increase in serum ALT, suggesting ongoing liver damage, was observed in the MCD diet-fed control genotype mice, and this correlated well with the steatohepatitis; however, the ALT increase was significantly attenuated in MD-2- and TLR4-deficient mice (Fig. 1E). TNF-α has been suggested as a central proinflammatory cytokine that is produced by activated inflammatory cells and mediates insulin resistance and hepatocyte apoptosis in liver disease (7, 38). Consistent with activation of the inflammatory cascade, serum TNF-α level was increased in MCD diet-fed control genotype mice compared with the MCS diet-fed controls (Fig. 1F). In contrast, MCD-induced TNF-α was significantly lower in MD-2- or TLR4-deficient MCD diet-fed mice (Fig. 1F). These data suggested that TLR4-MD-2 complex deficiency is partially protective against MCD-induced liver inflammation and damage.

**MD-2 and TLR4 deficiency attenuates oxidative stress.** Increased lipid peroxidation and oxidative stress are key in development of steatosis in NAFLD (20). We identified significantly higher levels of liver TBARS, indicative of lipid peroxidation, in MCD diet-fed compared with the MCS diet-fed genotype control mice (Fig. 2A). Consistent with our hypothesis that MD-2-TLR4 complex plays a role in NASH, we found significantly reduced induction of TBARS in the livers of MCD diet-fed MD-2- and TLR4-deficient mice (Fig. 2A).

NADPH oxidase activities play an important role in the generation of reactive oxygen radicals (25, 30). The classic NADPH complex is composed of at least six components, which include two trans-membrane flavocytochrome b components (gp91phox and p22phox) and four cytosolic components (p47phox, p67phox, p40phox, and Rac-1 protein) (30). TLR4-mediated signals are strong inducers of NADPH transcription and functional activity (25). Investigation of NADPH oxidase expression revealed a significant upregulation of the cytoplasmic components of the NADPH oxidase, including p47phox (Fig. 2B) and p67phox (Fig. 2C), in MCD diet-fed animals of control genotypes. The membrane-associated components of the NADPH complex, gp91phox (Fig. 2D) and p22phox (Fig. 2E), were also upregulated at the mRNA level in the livers of MCD diet-fed mice of control genotypes. Deficiency in MD-2 or TLR4 abrogated the MCD-induced upregulation of all of the NADPH oxidase subunits (Fig. 2, B–E), suggesting that NADPH-mediated oxidative stress is dependent on MD-2 and TLR4 expression in this model. To test for the biological significance of the mRNA increase in the NADPH subunits, we evaluated the NADPH oxidase activity. Consistent with the increased mRNA levels of NADPH oxidase complex components, NADPH oxidase activity was elevated, as suggested by the increased NADP⁺-to-NADPH ratio in livers of MCD-fed compared with MCS-fed mice of control genotypes (Fig. 2F).

More importantly, we identified that both TLR4 KO and MD-2 KO mice were protected from the MCD diet-induced activation of NADPH oxidase (Fig. 2F). Collectively, these results indicated that MD-2-TLR4 complex-induced signals contribute to liver pathology via NADPH-dependent lipid peroxidation and oxidative stress in the MCD diet-induced NASH model.

**MD-2 and TLR4 deficiency protects from NASH-associated liver fibrosis.** A key clinical challenge in human NASH is its progression to fibrosis and cirrhosis (1, 10, 36). In contrast to livers of MCS diet-fed control genotype animals, Sirius red (Fig. 3, A and B) and α-SMA immunohistochemistry (Fig. 3C) staining revealed that administration of MCD diet resulted in signs of fibrosis (Fig. 3, A–C). On the contrary, we found no substantial Sirius red (Fig. 3, A and B) or α-SMA (Fig. 3C) staining in either MD-2- or TLR4-deficient MCD diet-fed mice. Genes associated with fibrosis, including α-SMA (Fig. 3D), procollagen-1 (Fig. 3E), and transforming growth factor (TGF)-β (Fig. 3F), were significantly upregulated at the RNA level in MCD diet-fed control genotypes, but not or less extent in MD-2- and TLR4-deficient mice.

Liver fibrosis involves inflammation-driven tissue remodeling; matrix metalloproteinases (MMP) and their specific tissue inhibitors (TIMPs) closely regulate the metabolism of the extracellular matrix (1, 8, 14). The expression of MMP-2 (Fig. 4A) and TIMP-1 (Fig. 4B) were increased in livers of MCD-fed compared with MCS diet-fed mice of control genotypes; the induction of these genes was significantly attenuated in the absence of MD-2 or TLR4 expression.

**DISCUSSION**

Diet-induced NASH in mice mimics several features of human NASH, including steatosis, inflammation, and fibrosis (1, 10, 36). In this study, we demonstrate for the first time that deficient integrity of the danger receptor complex, including TLR4 or its coreceptor MD-2, is protective from MCD diet-induced liver steatosis and inflammation and correlates with attenuated liver injury and histological features of NASH. To this extent, our novel data also indicate that the deficiency in MD-2 or TLR4 confers protection from development of liver fibrosis in MCD diet-induced NASH.

To date, several research groups have identified that LPS, in the context of a multihit model, plays a role in development of NAFLD/NASH (10, 22, 29); the details of LPS implication per se are yet to be fully defined. Here we provide novel data indicating that danger sensing via MD-2 and TLR4 is key in the pathogenesis of NASH. Ligand recognition by the TLR4-MD-2 complex, which binds LPS to deliver intracellular signals, occurs as a result of complementary functions of MD-2 and TLR4. Neither MD-2 nor TLR4 alone can account for optimal LPS recognition (2, 40, 41). MD-2 binds LPS, however, it lacks a transmembrane domain and cannot result in intracellular signaling alone (2, 40, 41). The recently discovered crystal structure of the TLR4-MD-2 complex demonstrates the critical role of MD-2 in LPS binding and LPS-induced TLR4 activation resulting in TLR4-MD-2 complex

**Table 1. PCR primers**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>gtc gca acc atc agg gga taa</td>
<td>tgg gat tca ggg tca gga</td>
</tr>
<tr>
<td>Procollagen-I α1</td>
<td>gct ctt agg gcc cac t</td>
<td>ccc cgg ttc gtt tgg cct</td>
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<tr>
<td>MMP-2</td>
<td>att cct ggc gtc acc tgt</td>
<td>cgc cgg ttc gtt ctt ggc</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>ttt gct cgg gcc taa gga</td>
<td>ctc ctt gtg ggc tac ttc</td>
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α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; MMP-2, matrix metalloproteinase-2; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.
and conformational changes to initiate intracellular signaling through the intracellular domain of TLR4 (16). Our data suggest a major role for TLR4 and MD-2 in liver damage, as indicated by profound attenuation of features of NASH in their absence. The exact ligand(s) of TLR4/MD-2 in NASH is yet to be defined. A candidate ligand is endotoxin, most likely derived from the gut (29). This hypothesis is supported by recent reports in other models of NAFLD and is also consistent with the causal role of gut-derived endotoxin in ASH, which shares many pathological features of NASH (5, 11, 22, 27). We found a moderate but significant increase in serum endotoxin levels in MCD diet-fed mice of control genotypes (data not shown);
observation is similar to that described in the portal circulation of LPS-insensitive C3H/HeJ mice (29).

In evaluation of the role of TLR4-MD-2 complex in the pathogenesis of NASH, there is a need to consider that, while TLR4 recognizes exogenous danger signals, such as LPS, it also can sense multiple endogenous danger signals (19), including, but possibly not limited to, heat shock proteins (3), fibrinogen (35), fibronectin (23), and HMGB1 (26). Our results suggest protection from murine NASH when the recognition of ligands by TLR4-MD-2 complex is impaired; the role of endogenous danger signals in experimental or human NASH is yet to be evaluated.

We identified that MD-2 and TLR4 deficiency is protective in NASH due to interference with inflammation and oxidative stress. The elements of protections included prevention of inflammatory cell infiltration into the liver, diminished proinflammatory cytokine production, impaired upregulation of the liver mRNA levels of all components of the NADPH oxidase complex, and impaired function of the NADPH complex. Our observation of increased expression of the phagocyte-specific NADPH complex and increased NADPH activity in MCD-fed animals of control genotypes and lack of such effects in TLR4 or MD-2 KO animals suggests a communication between TLR4/MD-2 and NADPH oxidase activation in NASH. Several research groups have reported the key role of the proinflammatory effects of Kupffer cells (29) and TLR4 receptor (29) in NASH-associated liver inflammation; our data are in agreement with those reports. Kupffer cells are rich in TLR4-MD-2 receptor complex (37) and are a major source of NADPH in the liver. The critical role of the Kupffer cells p47phox/NADPH oxidase component has been reported in alcoholic liver disease (17).

The most important clinical challenge in NASH is the progression to liver fibrosis, which often leads to cirrhosis and liver failure (1, 10, 36). Here, we present our novel observation of increased expression of the phagocyte-specific NADPH complex and increased NADPH activity in MCD-fed animals of control genotypes and lack of such effects in TLR4 or MD-2 KO animals suggests a communication between TLR4/MD-2 and NADPH oxidase activation in NASH. Several research groups have reported the key role of the proinflammatory effects of Kupffer cells (29) and TLR4 receptor (29) in NASH-associated liver inflammation; our data are in agreement with those reports. Kupffer cells are rich in TLR4-MD-2 receptor complex (37) and are a major source of NADPH in the liver. The critical role of the Kupffer cells p47phox/NADPH oxidase component has been reported in alcoholic liver disease (17).

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some subtle differences between TLR4 KO and MD-2 KO mice upon developing MCD diet-induced NASH. For example, the extent of liver fibrosis, indicated by the Sirius red positive areas, was more pronounced in MD-2 KO compared with TLR4 KO, etc. The origin of the lack of full overlap between the TLR4- and MD-2-owed extent of protection against NASH likely lies in the final effect of these molecules on ligand recognition and/or downstream signaling events. MD-2 is an important component of LPS recognition; however, it may, or may not, be implicated in the recognition of the entire repertoire of TLR4 ligands. Alternatively, TLR4 with or without MD-2 may signal differently, or TLR4-MD-2 complex receptor may function in two separate modes; one in which full signaling occurs and one limited to MyD88-dependent signaling (13). We had previously reported a critical role of TLRs and the common TLR adaptor, MyD88, in other models of liver inflammation and injury (39); the exact signaling events downstream from TLR4-MD-2 complex in NASH are yet to be fully understood. Nevertheless, it is important to note that both TLR4 KO and MD-2 KO genotypes offered only partial protection against MCD diet-induced NASH, suggesting the possibility that TLR4-MD-2-independent events may be involved in the pathogenesis of NASH.

In conclusion, we found that danger receptor TLR4 and its coreceptor, MD-2, are critical in the development of steatosis, liver damage, inflammation, and fibrosis in the MCD diet-induced NASH in mice. The significant attenuation of steatohepatitis and the protection from fibrosis in the presence of MD-2 or TLR4 deficiency suggest that danger signals provided by MD-2-TLR4 complex play a central role in this model of hepatic lipid accumulation in mice: role of endotoxin. J Hepatol 48: 983–992, 2008.

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