NKT cell modulates NAFLD potentiation of metabolic oxidative stress-induced mesangial cell activation and proximal tubular toxicity

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Over the last decade, there has been a phenomenal rise in obesity and metabolic syndrome. The obesity phenotype is often associated with fatty liver (steatosis), leading to hepatic inflammation, sinusoidal injury, and fibrosis (47). Fatty liver is also observed with liver-specific insulin and leptin resistance, leading to metabolic complications referred to as nonalcoholic fatty liver disease (NAFLD) (15, 47). Advanced and progressive NAFLD with inflammation with or without fibrosis and sinusoidal injury is termed “nonalcoholic steatohepatitis” (NASH) (5). NAFLD/NASH is linked to extrahepatic comorbidities like cardiovascular and renal complications (8, 46). For example, the cumulative evidence to date suggests that individuals with NAFLD exhibit an increased risk of developing cardiovascular disease, especially atherosclerosis and portal hypertension, type 2 diabetes, and chronic kidney disease (CKD) (2). Advanced NAFLD/NASH was associated with a higher prevalence (odd ration: 2.53, 95% confidence interval: 1.58–4.05) and incidence (hazard ratio: 2.12, 95% confidence interval: 1.42–3.17) of CKD than simple steatosis when assessed in cross-sectional and longitudinal studies with the severity of advanced NAFLD/NASH being positively associated with CKD stages (27). The strong association of advanced NAFLD/NASH with kidney disorders may lie in common factors underlying the pathogenesis of NAFLD and CKD, including insulin resistance, oxidative stress, activation of the renin-angiotensin system, and secretion of inflammatory cytokines by the stotic and inflamed liver (6, 31, 41).

We have previously shown that oxidative stress is key for the progression of NAFLD to NASH (38). Oxidative stress pathways like cytochrome P-450 (CYP)-mediated lipid peroxidation and NADPH oxidase can trigger the release of multiple proinflammatory mediators that affect distal organs like the kidney (11, 37). It is equally important that the intrinsic properties of proximal tubule cells act as immune responders and producers of a wide range of immunological, ischemic, or toxic injury that may arise from the liver (28). It is therefore not surprising that events in the proximal tubule are closely related to the pathogenesis of a vast array of kidney diseases (28). In parallel, proinflammatory mediators in the circulation are filtered through the glomerulus, and those that are secreted locally from the kidney epithelia can elicit autocrine and paracrine effects on mesangial cells (MCs), fibroblasts, vascular smooth muscle cells, and proximal tubular cells, which are crucially involved in progressive renal diseases (4, 7). The kidney immunotoxicity and inflammatory insult can be magnified by the activation of MCs, which are often found in the vicinity of Bowman’s capsule. Hyperplasia of MCs precedes or accompanies progressive glomerular scarring, as seen in chronic glomerulonephritis and diabetic glomerulosclerosis (39). MCs also generate soluble mediators that can, in a paracrine fashion, attract and activate inflammatory cells...
MATERIALS AND METHODS

Also present in advanced NAFLD/NASH-affected kidneys and oxidative stress followed by MC activation and immunotoxicity in proximal tubular cells with a second hit in the form of increased lipid peroxidation (39). MCs also resort to proliferation upon activation by proinflammatory mediator-induced oxidative stress. This is accompanied by the de novo expression of α-smooth muscle actin (α-SMA) and TGF-β secretion (13, 24, 32, 34, 48). In parallel to studies regarding the role of MCs in chronic kidney immunotoxicity, there is evidence of immunosuppressive roles of a class of T cells including invariant and CD1D-dependent natural killer (NKT) cells (17). Invariant NKT cells have been shown to inhibit the development of experimental crescentic glomerulonephritis primarily via the involvement of dendritic cells and chemokine (C-X-C motif) receptor (CXC)6 and CXC16 (33, 45). However, the cross talk between the regulatory and immunosuppressive NKT cells that have a prominent role in NAFLD has never been explored for their role in NAFLD-induced kidney immunotoxicity.

Thus, it is justifiable to assume that a preexisting condition of advanced NAFLD/NASH that has higher CYP2E1-mediated oxidative stress, release of damage-associated molecular patterns (DAMPS), higher insulin and leptin levels, and higher inflammatory mediators in the circulation can cause oxidative stress distant to the liver, especially in renal tubular cells. The presence of varied cell types within Bowman’s capsule and an activated proinflammatory phenotype of MCs raise the possibility of significant cross talk between these cells and other proximal tubular cells via autocrine and paracrine mechanisms, thus exacerbating renal injury in advanced NAFLD/NASH. In the present study, we used a diet-induced NAFLD model where hepatotoxin bromodichloromethane (BDCM) was used to generate CYP2E1-mediated oxidative stress and act as a “second hit” to allow the liver to progress into advanced NAFLD/NASH with steatohepatitis and fibrosis (38). Here, we show, using both in vivo and vitro models, that advanced NAFLD/NASH affected kidneys with a second hit in the form of increased lipid peroxidation followed by MC activation and immunotoxicity in proximal tubular cells. Interestingly, CD1D-dependent NKT cells were also present in advanced NAFLD/NASH-affected kidneys and played a crucial role in modulating the MC-induced immunotoxicity in the proximal tubule.

MATERIALS AND METHODS

BDCM and corn oil were purchased from Sigma-Aldrich (St. Louis, MO). Diallyl disulfide (DAS) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-4-hydroxynonenal (4-HNE), anti-IL-1β, anti-TNF-α, anti-interferon (IFN)-γ, anti-high mobility group box 1 (HMGB1), anti-chemokine (C-X-C motif) ligand (CXCL)16, anti-TGF-β, anti-Toll-like receptor (TLR)4, anti-CD4, anti-CD8, anti-F4/80, and anti-α-SMA primary antibodies were purchased from Abcam (Cambridge, MA). Species-specific biotinylated conjugated secondary antibody and streptavidin horseradish peroxidase were purchased from Vector Laboratories (Vectorstain Elite ABC kit, Burlingame, CA). Wild-type and gene-specific knockout (KO) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and Taconic Farms (Hudson, NY). Animal diets were purchased from Research Diets (New Brunswick, NJ). All other chemicals were of analytic grade and were purchased from Sigma-Aldrich unless otherwise specified. Paraaffinized tissue sections on slides were done by the Instrumentation Resource Facility of the University of South Carolina School of Medicine and AML Laboratories (Baltimore, MD).

Mouse models. All mice were housed with 1 mouse/cage at 23–24°C on a 12:12-h light-dark cycle with libitum access to food and water. All animals had been treated in strict accordance with the National Institutes of Health guidelines for the humane care and use of laboratory animals and local Institutional Animal Care and Use Committee standards. All experiments were approved by the institutional review board at the National Institute of Environmental Health Sciences, Duke University, and University of South Carolina (Columbia, SC). Mice were given either 60% kcal high-fat diet or Chow diet. After the animal experiment was completed, mice of all study groups were euthanized for kidney tissues and serum samples were taken for further experiments.

Mouse model of NAFLD. Male pathogen-free C57BL/6J background mice were used as wild-type mice for the NAFLD model. These mice were fed a high-fat diet (60% kcal) from 6 wk until 16 wk. All experiments were conducted in the 16-wk age group. Age-matched lean control (LC) mice were fed a diet having 10% kcal fat. Animals were housed at 1 mouse/cage before any experimental use. Mice that contained the disrupted CD1D gene (B6.129S6-Cd1d1/Cd1d2 < C57 Bl6/J), PFP/Rag2 dual gene deletion (B6.129S6- Rag2tm1Flpt/Ppfrag2tm1Crl/J), N12, Taconic Farms), and disrupted TLR4 gene (B6.129ScN-Tlr4−/−J) were fed a high-fat diet and treated identically to the NAFLD mouse model.

Administration of BDCM and induction of kidney injury. High-fat diet-fed wild-type mice (NAFLD model) and high-fat diet-fed KO mice (except for TLR4 gene KO mice) at 16 wk were administered BDCM (1 mmol/kg, diluted in corn oil) intraperitoneally for twice a week for 4 wk to assess the effects of chronic exposure to BDCM. High-fat diet-fed TLR4 gene KO mice at 16 wk were administered with the same dose of BDCM via an intraperitoneal injection for 1 wk. A set of high-fat diet-fed mice (NAFLD model) were not injected with BDCM and served as controls against high-fat diet-fed BDCM-injected wild-type mice (NAFLD + BDCM mice). A group of chow diet-fed LC mice was also treated with the same dose of BDCM for 1 mo (LC + BDCM mice). LC mice not injected with BDCM were used as a control against the LC + BDCM model.

Inhibition of CYP2E1 by DAS (CYP2E1 inhibitor). A set of high-fat diet-fed BDCM-treated mice was administered with 50 mg/kg DAS (diluted in corn oil) via an intraperitoneal injection at twice a week for 1 mo. This group was termed the NAFLD + BDCM + DAS group.

Cell culture. A kidney MC line (CRL-1927) and kidney tubular cell line (CRL-2038) were purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (Corning, Tewksbury, MA) and DMEM-F-12 (1:1, American Type Culture Collection), respectively. Media were supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO₂.

MCs were treated with 1 μM 4-HNE (Cayman Chemicals, Ann Arbor, MI) for 48 h, and equal amounts of ethanol were added to control plates. The diluent for 4-HNE and the media were collected for further experiments. Tubular cells were cultured in six-well plates. After allowing for the attachment of cells, the media were changed to obtain the following groups: the control group containing fresh DMEM, the control group with DMEM and 1 μM 4-HNE, media collected from MCs treated with alcohol only, and media collected from MCs treated with 1 μM 4-HNE for 48 h. Cells were then lysed in TRIzol (Invitrogen, Grand Island, NY) for mRNA extraction. Cells were plated on coverslips by putting the coverslips on each well of the six-well plates, maintaining the aforementioned conditions, and cells adhered on coverslips were used for immunofluorescence staining after completion of the treatment. To perform the apoptosis assay, tubular cells were seeded in 2-cm² dishes with attached coverslips.
formalin-fixed paraffin-embedded tissues were cut into 5-μm-thick sections. These sections were deparaffinized using a standard protocol (38). Epitope retrieval of the deparaffinized sections was performed using epitope retrieval solution and steamer (IHC-World, Woodstock, MD) following the manufacturer’s protocol. H2O2 (3%) was used for using epitope retrieval solution and steamer (IHC-World, Woodstock, MD) following the manufacturer’s protocol. H2O2 (3%) was used for

Quantitative real-time PCR. Gene expression (mRNA) levels in kidney tissue samples were measured by quantitative real-time RT-PCR. Total RNA was isolated from mouse kidney tissues, and cells were treated using TRIzol reagent (Life Technologies) following the manufacturer’s protocol. RNA was purified using RNAsesy mini kit columns (Qiagen, Valencia, CA). An iScript cDNA synthesis kit (Bio-Rad) was used to convert 1 μg of purified RNA to cDNA.

Picrosirius red staining. Picrosirius red staining of kidney slices was done using a Nova ultra-sirius red stain kit following manufacturer’s instructions. The primary antibodies were used as described above. After epitope retrieval, those deparaffinized sections with an Enzo immunohistochemical dual labeling kit (Farmingdale, NY) were used following the manufacturer’s protocol.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>IL-1β</td>
<td>5′-CCGCGGCACAGGAGTGTCCG-3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′-GAGGACGGCGCTGACGGGAC-3′</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>5′-GGGAGAGCGGGAACCCTCG-3′</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>5′-GGGAGAGCGGGAACCCTCG-3′</td>
</tr>
<tr>
<td>CD4</td>
<td>5′-GCACCATGTCGGCAAGGACG-3′</td>
</tr>
<tr>
<td>CD8</td>
<td>5′-GGACTTCTCTGGCAGGAAAGC-3′</td>
</tr>
<tr>
<td>VoL1</td>
<td>5′-GACTTCTCTGGCAGGAAAGC-3′</td>
</tr>
<tr>
<td>NK1.1</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
</tr>
<tr>
<td>PDDG2</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
</tr>
<tr>
<td>Fas ligand</td>
<td>5′-GGGAATCTTACCAAGGGAGC-3′</td>
</tr>
<tr>
<td>Transforming growth factor-β</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
</tr>
<tr>
<td>Chemokine (C-X-C- motif) ligand 16</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 5</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
</tr>
<tr>
<td>Toll-like receptor 4</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
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<tr>
<td>Endothelin-1</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
</tr>
<tr>
<td>Monocyte chemoattractant protein-1</td>
<td>5′-GCCAAGCTGCAACTGGGCTG-3′</td>
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Renal inflammation and fibrosis scores. A blind folded analysis of renal pathology was carried out to assess renal inflammation and fibrosis using a standard grading system. To analyze tubulointerstitial fibrosis in the renal compartments, lesions were scored using a semiquantitative scoring system (43). For inflammation, grade 0 = no foci of inflammation, grade 1 = less than one foci per two ×20 fields,
grade 2 = 1 foci per two ×20 fields to one foci per one ×20 fields, grade 3 = one to two foci per one ×20 fields, and grade 4 = more than two foci per one ×20 fields. Interstitial fibrosis was graded according to the following scale: 0 = no evidence of interstitial fibrosis, 1 = less than 25% involvement, 2 = 25–50% involvement, and 3 = less than 50% involvement.

Statistical analyses. Statistical analysis was done by ANOVA followed by Bonferroni post hoc correction for intergroup comparisons. $P$ values of <0.05 were considered statistically significant.

RESULTS

NAFLD potentiates metabolic oxidative stress via CYP2E1 and caused increased lipid peroxidation in proximal tubular cells. NAFLD shows increased CYP2E1 induction (1). We have previously shown that metabolic oxidative stress in NAFLD/NASH causes lipid peroxidation primarily dependent on CYP2E1 in the liver (38). BDCM is a substrate of CYP2E1 and is known to cause lipid peroxidation through reductive metabolism (38). The results showed that lipid peroxidation marker 4-HNE was significantly higher in the NAFLD + BDCM kidney group compared with the LC, LC + BDCM, or NAFLD alone groups ($P < 0.05$; Fig. 1, A and B). Mice treated with the CYP2E1 inhibitor DAS, a relatively specific inhibitor of CYP2E1, showed a significant decrease in lipid peroxidation, as assessed by decreased staining of 4-HNE. Peroxidation was primarily localized in proximal tubular cells and podocytes (Fig. 1A). The results suggested that a condition of NAFLD characterized by increased steatosis and metabolic oxidative stress via CYP2E1 exacerbated the lipid peroxidation in the NAFLD + BDCM kidney group and was not a result of the metabolism of the toxin alone since kidneys from the LC + BDCM group had significantly decreased 4-HNE staining compared with the NAFLD + BDCM group (Fig. 1A).

CYP2E1 metabolism in NAFLD causes increased secretion of proinflammatory cytokines in renal proximal tubules. Oxidative stress due to reductive metabolism of CYP2E1 has been shown to increase proinflammatory cytokine release (38). We studied the role of CYP2E1 reductive metabolism and its corresponding lipid peroxidation in the release of proinflammatory cytokines in NAFLD kidneys after BDCM administration. The results showed that there was a significant increase in

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Fig. 1. A: 4-hydroxynonenal (4-HNE; a marker for lipid peroxidation) immunoreactivity, as shown by immunohistochemistry in kidney slices from mice fed a chow diet [lean control (LC); i], LC mice exposed to bromodichloromethane (BDCM; ii), mice fed a high-fat diet (60% kcal fat) and used as a model of nonalcoholic fatty liver disease (NAFLD; iii), NAFLD mice exposed to BDCM (iv), and NAFLD mice coexposed to BDCM and diallylsulfide (DAS; v), a known cytochrome P-450 (CYP)2E1 inhibitor. Images were taken at ×20 magnification. B: morphometric analysis of 4-HNE immunoreactivity (mean data from 3 separate microscopic fields were plotted on the y-axis) in the LC, LC + BDCM, NAFLD, NAFLD + BDCM, and NAFLD + BDCM + DAS groups. *$P < 0.05$. 

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F88 LIVER CYP2E1 MEDIATES KIDNEY INFLAMMATION

AJP-Renal Physiol • doi:10.1152/ajprenal.00243.2015 • www.ajprenal.org
the levels of IL-1β in the LC+BDCM group compared with the LC group (P < 0.05; Fig. 2B). Levels of the cytokine also increased in the NAFLD + BDCM group compared with the NAFLD alone group (Fig. 2, A and B), as shown by immunohistochemistry, compared with the NAFLD alone group, whereas the administration of DAS significantly decreased levels of IL-1β primarily in proximal tubular cells (Fig. 1, A and B). mRNA expression of IL1β also showed a significant increase in the NAFLD + BDCM group compared with NAFLD alone group, whereas the NAFLD + BDCM + DAS group showed a significant decrease in mRNA levels, as assessed by quantitative RT-PCR (Fig. 2C). Oxidative stress and other inflammatory stimuli of a primarily systemic nature have been shown to increase TNF-α levels and TNF-α release from proximal tubular cells, podocytes, and MCs (13, 24, 28, 34). The results showed that levels of TNF-α were significantly increased in the NAFLD alone group (Fig. 2, A and B), as shown by immunohistochemistry in kidney slices from LC (i), LC+BDCM (ii), NAFLD (iii), and NAFLD+BDCM (iv) groups of mice. *P < 0.05. C: mRNA expression of IFN-γ in kidney tissue of LC, LC+BDCM, NAFLD, NAFLD+BDCM, and NAFLD+BDCM + DAS groups of mice. D: immunoreactivity of TNF-α, as shown by immunohistochemistry in kidney slices from LC (i), LC+BDCM (ii), NAFLD (iii), NAFLD+BDCM (iv), and NAFLD+BDCM + DAS (v) groups of mice. *P < 0.05. E: morphometric analysis of TNF-α immunoreactivity in the LC, LC+BDCM, NAFLD, NAFLD+BDCM, and NAFLD+BDCM + DAS groups of mice. *P < 0.05. F: mRNA expression of IFN-γ in kidney tissue of LC, LC+BDCM, NAFLD, NAFLD+BDCM, and NAFLD+BDCM + DAS groups of mice. All immunohistochemistry images were taken at ×20 magnification, and morphometric analysis results were plotted as mean data (immunoreactivity measured in arbitrary light units) from three separate microscopic fields plotted on the y-axis. All mRNA expression was assessed by quantitative real-time PCR, and expression was normalized against the LC group. *P < 0.05.

Fig. 2. A: immunoreactivity of IL-1β, as shown by immunohistochemistry in kidney slices from LC(i), LC + BDCM (ii), NAFLD (iii), NAFLD + BDCM (iv), and NAFLD + BDCM + DAS (v) groups of mice. B: morphometric analysis of IL-1β immunoreactivity in the LC, LC + BDCM, NAFLD, NAFLD + BDCM, and NAFLD + BDCM + DAS groups. *P < 0.05. C: mRNA expression of IL-1β in kidney tissue of LC, LC + BDCM, NAFLD, NAFLD + BDCM, and NAFLD + BDCM + DAS groups of mice. D: immunoreactivity of TNF-α, as shown by immunohistochemistry in kidney slices from LC (i), LC + BDCM (ii), NAFLD (iii), NAFLD + BDCM (iv), and NAFLD + BDCM + DAS (v) groups of mice. *P < 0.05. E: morphometric analysis of TNF-α immunoreactivity in the LC, LC + BDCM, NAFLD, NAFLD + BDCM, and NAFLD + BDCM + DAS groups of mice. *P < 0.05. F: mRNA expression of IFN-γ in kidney tissue of LC, LC + BDCM, NAFLD, NAFLD + BDCM, and NAFLD + BDCM + DAS groups of mice. All immunohistochemistry images were taken at ×20 magnification, and morphometric analysis results were plotted as mean data (immunoreactivity measured in arbitrary light units) from three separate microscopic fields plotted on the y-axis. All mRNA expression was assessed by quantitative real-time PCR, and expression was normalized against the LC group. *P < 0.05.
increased primarily in tubular cells, podocytes, and MCs in the NAFLD + BDCM group compared with the NAFLD alone group (P < 0.05; Fig. 2, D and E). No significant change was observed between the LC and LC + BDCM groups. The administration of DAS significantly decreased TNF-α levels compared with the NAFLD + BDCM group (P < 0.05; Fig. 2, D and E). The increased protein levels were due to increased protein synthesis, as explained by the increased mRNA expression of the cytokine in the NAFLD + BDCM group compared with the NAFLD alone group or DAS-administered group (P < 0.05; Fig. 2F). Although the proinflammatory cytokines IL-1β and TNF-α were found to be increased in proximal tubular cells in the NAFLD + BDCM group, we investigated the role of IFN-γ, a regulatory cytokine, in the kidney. IFN-γ has been found to often be associated with a regulatory role in the kidney, especially by suppressing angiotensinogen levels via suppressor of cytokine signaling 1 (36). To study the role of CYP2E1-mediated oxidative stress in regulating the role of IFN-γ, immunohistochemistry was performed. The results showed that IFN-γ protein was primarily localized in proximal tubular cells and other cell types (Fig. 2G). Levels of IFN-γ were significantly increased in the NAFLD + BDCM group compared with the NAFLD alone group, whereas only a slight but significant change was observed between LC and LC + BDCM groups (P < 0.05; Fig. 2, G and H). The administration of DAS significantly decreased IFN-γ levels in these cells compared with the NAFLD + BDCM group (P < 0.05; Fig. 2H). mRNA expression of IFN-γ also showed a significant increase in the NAFLD + BDCM group compared with both the NAFLD alone group and DAS-administered group (P < 0.05; Fig. 2F), confirming that the increase in the levels of IFN-γ was because of increased mRNA expression and most likely by increased protein synthesis. The results assumed significance since IFN-γ can be secreted from infiltrating or resident T cells, including NKT cells, which have been shown to be protective in various forms of glomerulonephritis (45).

NAFLD-induced CYP2E1 metabolism leads to increased MC proliferation and TGF-β production. MCs have long been regarded as mediators of CKD (18). Hypertrophy of MCs and tubular cells is common in the progression of CKD (18). To study the involvement of MC activation, proliferation and hypertrophy in the NAFLD potentiation of CYP2E1-induced oxidative stress, and the subsequent immunotoxicity in the kidney, protein levels and localization of α-SMA were analyzed by immunohistochemistry (16). The results showed that there was a significant increase in the immunoreactivity of α-SMA in the glomerulus and proximal tubular cells in the NAFLD + BDCM group compared with the NAFLD alone group, whereas no significant change was observed between LC and LC + BDCM groups (P < 0.05; Fig. 3, A and B). DAS-administered mice had a significant decrease in immunoreactivity to α-SMA compared with the NAFLD alone group (P < 0.05; Fig. 3, A and B). mRNA expression of α-SMA was significantly higher in the NAFLD + BDCM group compared with both the NAFLD alone group or DAS-administered group, suggesting that the protein synthesis of α-SMA was intrinsic to the kidney (P < 0.05; Fig. 3C). Since MC-mediated fibroproliferative effects, especially mesangial matrix formation, are via TGF-β production, we studied the immunoreactivity of TGF-β in the glomerulus and proximal tubular cells (20, 23). The results showed that the NAFLD + BDCM group showed significantly higher protein levels of TGF-β compared with the NAFLD alone group, whereas DAS administration significantly decreased TGF-β immunoreactivity compared with the NAFLD + BDCM group (P < 0.05; Fig. 3, D and E). Interestingly, the LC + BDCM group showed a significant increase in the level of the cytokine compared with the LC group (Fig. 3E). There was increased mRNA expression of TGF-β in the NAFLD + BDCM group compared with the NAFLD alone group or NAFLD + DAS group (P < 0.05; Fig. 3F). The results suggest that MC proliferation, in part, is a significant event in NAFLD potentiation of immunotoxicity, although other cell types cannot be ruled out at this stage. MCs incubated with 4-HNE showed increased expression of IL-1β, TNF-α, PDGF2, TLR4, and α-SMA (Fig. 3, G–K). α-SMA protein expression and its myofibroblastic phenotype were also evident by immunofluorescent microscopy in the 4-HNE-treated group compared with the cells + vehicle-treated group (Fig. 3L).

NAFLD potentiation of immunotoxicity via CYP2E1-mediated oxidative stress increases T cell presence in the renal microenvironment. Differential roles of various T cell subsets have been reported in CKD (26). Our previous data in this study showed an increased IFN-γ immunoreactivity in the NAFLD + BDCM group, whereas administration of DAS had decreased protein levels of this T cell cytokine (Fig. 2, G–I). To study the presence of T cells in the NAFLD kidney after increased CYP2E1-mediated oxidative stress, quantitative real-time PCR was used to analyze the fold expression of CD4, CD8, Va14, and NK1.1 genes. The results showed that mRNA expression of CD4, CD8, Va14, and NK1.1 was significantly higher in the NAFLD + BDCM group compared with the NAFLD alone group (P < 0.05; Fig. 4, A–D), whereas the administration of DAS significantly decreased mRNA expression of T cell subset markers in the kidney. Interestingly, use of CD1D KO mice, which were deficient in CD1d-dependent NKT cells, had significantly decreased mRNA expression of Va14, CD4, and CD8, whereas the expression of NK1.1 was unchanged (P < 0.05; Fig. 4). Expression of Va14 was significantly higher in the NAFLD + DAS group, suggesting that CYP2E1 reductive metabolism might have a role in the repression of invariant NKT cells and that the administration of DAS significantly upregulated NKT cell expression of Va14 (Fig. 4C). However, protein expression of CD4 and CD8 was significantly higher in CD1d KO mice, despite the low mRNA expression of these T cell markers (Fig. 4, E and F). The observed data of higher CD4 and CD8 protein in CD1d KO mice might be due to other T cell subsets in CD1d KO mice. To show the presence of infiltrating macrophages, F4/80 immunofluorescence staining was performed. The results showed that there was no marked difference between groups. The reactivity was also diffused due to the presence of red blood cells in the kidney (Fig. 4G). These data thus suggested that CD1d-dependent NKT cells with CD4 and/or CD8 expression might be involved, although in part, in the modulation of immunotoxicity in the NAFLD kidney.

Proximal tubule immunotoxicity in NAFLD potentiation of CYP2E1-mediated renal inflammation is exacerbated in mice deficient in NKT cells. Based on the data of increased T cell presence in the NAFLD kidney and the increased expression of

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CD4, CD8, and Va14, we used mice deficient in CD1D-specific NKT cells to study the role of these T cell subsets in the NAFLD kidney. The results showed that the proinflammatory cytokines IL1-β and TNF-α were localized in proximal tubular cells and were significantly higher in CD1D KO mouse kidneys compared with the NAFLD + BDCM group (P < 0.05; Fig. 5, A, B, D, and E). The increase was three- to fourfold higher in CD1D-deficient mice. mRNA expression of the cytokines IL-1β and TNF-α was also significantly higher in CD1D KO mice (3.5-fold), suggesting an intrinsic response from the cells, most likely NKT cells dependent on CD1D (P < 0.05; Fig. 5, C and F). The results strongly suggested that the absence of CD1D significantly exacerbated the NAFLD kidney immunotoxicity. However, the increased CD4 and CD8 protein in CD1D KO mice and their strong correlation with tubular immunotoxicity in the absence of CD1D-dependent NKT cells might possibly be due to infiltrating and/or resident T cells, which are capable of increasing cytotoxicity. The study
analyzed the expression of NAFLD kidney secretion of PDGF2 and TLR4. Since there was a strong indication of the activation of MCs and the proinflammatory pathway that is downstream of TLR4 signaling, such as IL-1β and TNF-α, expression of both PDGF2 and TLR4 was performed. The results showed that mRNA expression of TLR4 was significantly higher in the NAFLD + BDCM group compared with the NAFLD alone group (P < 0.05; Fig. 5G). CD1D-deficient mice had a significant increase in the expression of TLR4 compared with the NAFLD alone group (P < 0.05; Fig. 5H). The administration of DAS significantly decreased its expression, whereas lack of CD1D had a significant increase in its expression compared with the NAFLD + BDCM group (P < 0.05; Fig. 5H). The results suggested that MC activation and the release of proinflammatory mediators were strong possibilities due to a reductive metabolism of CYP2E1 and NKT cell deficiency intrinsic to the kidney in addition to infiltrating T cell subsets (CD4+ and CD8+) might be regulating its activation and immunotoxicity.

**MC proliferation and TGF-β production were increased in mice deficient in NKT cells.** To show that CD1D-dependent NKT cells regulated the MC proliferation, we studied the protein levels of α-SMA and TGF-β in the NAFLD kidney. The results showed that immunoreactivity of α-SMA and TGF-β were significantly increased in the NAFLD + BDCM...
group compared with the NAFLD alone group (P < 0.05; α-SMA: Fig. 6, A and C, and TGF-β: Fig. 6, B and D), whereas mice deficient in CD1D-dependent NKT cells showed a significant increase in both α-SMA and TGF-β immunoreactivity compared with the NAFLD + BDCM group. Dual labeling of IL1β/α-SMA and TGF-β/α-SMA showed a significant increase in immunoreactivity in CD1D KO mice. Interestingly, mice lacking T, B, and NK cells showed a significant decrease in α-SMA levels. The results suggested that MC activation (α-SMA +ve cells), at least in part, is dependent on the presence of NKT cells and that lack of NKT cells significantly enhanced immunotoxicity. In the absence of T, B, or NK cells, there was no significant increase in MC activation (Fig. 6, E, v and F, v).

NAFLD potentiation of renal inflammation induced by MCs causes increased levels of proximal tubular HMGB1 levels and apoptosis in part via the TLR4 pathway with exacerbation after lack of CD1D-dependent NKT cells. Our results showed that MCs were activated after CYP2E1-induced oxidative stress. Reductive metabolism of CYP2E1 has been shown to cause cell death and the release of DAMPs in the circulation (9). HMGB1 is known to be an established DAMP and a ligand for TLR4 activation (10, 19). Our previously described results in this study have shown TLR4 activation in the NAFLD kidney that was dependent on CYP2E1 reductive metabolism and the presence of CD1D. To show the presence of HMGB1 in the NAFLD kidney, we studied the immunoreactivity of HMGB1 in kidney tissues. The results showed that HMGB1 immuno-

Fig. 5. A: immunoreactivity of IL-1β, as shown by immunohistochemistry in kidney slices from NAFLD (i), NAFLD + BDCM (ii), and CD1d KO + BDCM (iii) groups. B: morphometric analysis of IL-1β immunoreactivity in NAFLD, NAFLD + BDCM, and CD1d KO + BDCM groups of mice. Data were normalized against the NAFLD group. *P < 0.05. C: mRNA expression of IL-1β in kidney tissue of NAFLD, NAFLD + BDCM, and CD1d KO + BDCM groups of mice. D: immunoreactivity of TNF-α, as shown by immunohistochemistry in kidney slices from NAFLD (i), NAFLD + BDCM (ii), and CD1d KO + BDCM (iii) groups. E: morphometric analysis of TNF-α immunoreactivity in NAFLD, NAFLD + BDCM, and CD1d KO + BDCM groups of mice. Data were normalized against the NAFLD group. *P < 0.05. F: mRNA expression of TNF-α in kidney tissue of NAFLD, NAFLD + BDCM, and CD1d KO + BDCM groups of mice. G and H: mRNA expression of TLR4 and PDGF2 in kidney tissue of LC, LC + BDCM, NAFLD, NAFLD + BDCM, NAFLD + BDCM + DAS, and CD1d KO + BDCM groups of mice. All immunohistochemistry images were taken at ×20 magnification, and morphometric analysis was plotted as mean data (immunoreactivity measured in arbitrary light units) from three separate microscopic fields plotted on the y-axis. All mRNA expression was assessed by quantitative real-time PCR, and expression was normalized against the LC group. *P < 0.05.
reactivity was significantly higher in the NAFLD + BDCM group compared with the NAFLD alone group (P < 0.05; Fig. 7, A and B), whereas deficiency of CD1D significantly increased HMGB1 protein in this group compared with the NAFLD + BDCM group (P < 0.05; Fig. 7, A and B). Further apoptotic cell death, as indicated by TUNEL-positive nuclei, was significantly increased in the NAFLD + BDCM group compared with the NAFLD alone group (P < 0.05; Fig. 7, C and D). Deficiency of CD1D further increased apoptosis compared with the NAFLD + BDCM group (Fig. 7, C and D). These results suggested that increased HMGB1 was partly due to the repression of NKT cell function in NAFLD and may be responsible for the proinflammatory surge in the kidneys. Interestingly, apoptosis indicated by TUNEL assay also followed a similar pattern, being controlled in part by NKT cells intrinsic to the kidneys. The apoptosis might be mediated by CD95L (Fas ligand) since mRNA expression was significantly higher in the NAFLD + BDCM group compared with the NAFLD alone group, whereas CD1D-deficient mice had a further increase in the expression of Fas ligand (P < 0.05; Fig. 7E). To show the role of MCs in proximal tubular immunotoxicity, conditioned medium from MCs was used to incubate proximal tubular cells. The results showed that proximal tubular cells had a significant increase in chemokine (C-C motif) ligand 5 (regulated on activation, normal T cell expressed and secreted) and endothelin-1 expression (P < 0.05; Fig. 7F and G). Tubular cells also had a significant increase in the expression of IL-1β and monocyte chemoattractant protein-1 (Fig. 7, H and I). These cells also exhibited more apoptotic cell death, as indicated by increased TUNEL-positive nuclei (shown by
Fig. 7. A: Immunoreactivity of high mobility group box (HMGB)1, as shown by immunohistochemistry in kidney slices from LC (i), LC + BDCM (ii), NAFLD (iii), NAFLD + BDCM (iv), and CD1d KO + BDCM (v) groups of mice. B: Morphometric analysis of HMGB1 immunoreactivity in LC, LC + BDCM, NAFLD, NAFLD + BDCM, and CD1d KO + BDCM groups of mice. Mean data (immunoreactivity measured in arbitrary light units) from three separate microscopic fields were plotted on the y-axis. *P < 0.05. C: Number of apoptotic nuclei as shown by TUNEL immunofluorescence staining in kidney slices from LC (i), LC + BDCM (ii), NAFLD (iii), NAFLD + BDCM (iv), and CD1d KO + BDCM (v) groups of mice. The number of TUNEL-positive cells identified by their green-stained nuclei against the nuclear fast red-stained nuclei corresponds to the number of apoptotic events. Images were taken at ×20 magnification. D: Percentage of TUNEL-positive cells (obtained by morphometric analysis done on images from three separate microscopic fields) in LC, LC + BDCM, NAFLD, NAFLD + BDCM, and CD1d KO + BDCM groups. *P < 0.05. E: mRNA expression of Fas ligand (FasL) in kidney tissue of LC, LC + BDCM, NAFLD, NAFLD + BDCM, and CD1d KO + BDCM groups of mice. *P < 0.05. F–I: A kidney MC line (CRL-1927) and kidney tubular cell line (CRL-2038) were used for these experiments. CRL-2038 cells were incubated with conditioned medium (CM) from MC + 4-HNE. mRNA expression was then assessed by quantitative real-time PCR using primers for chemokine (C-C motif) ligand 5, endothelin-1, IL-1β, and monocyte chemoattractant protein (MCP)-1 (*P < 0.05). J: Cell death (apoptotic) as shown by TUNEL (green)-stained nuclei (red). Nuclei positive for TUNEL show a yellow appearance and were markedly higher in cells CM + 4-HNE compared with the other groups.
yellow; Fig. 7J). The data showed that activated MCs caused tubular cell toxicity.

**Exacerbation of immunotoxicity in NAFLD kidneys after reductive metabolism of CYP2E1 is mediated by TLR4 activation.** A significant role of CYP2E1 in inflammation has been documented (3). The mechanisms, although unclear, might proceed through an oxidative stress-DAMP-TLR4 signaling pathway. To study whether the strong correlation of the events that we observed in the NAFLD kidney might be via TLR4 signaling, we used TLR4 KO mice kidneys treated identically. The results showed that the immunotoxicity was significantly decreased in TLR4 KO mouse kidneys compared with the NAFLD + BDCM group for IL-1β, TNF-α, and Fas ligand, whereas the expression of IL-6 was unchanged (P < 0.05; data not shown and Fig. 8A). LC mice treated with BDCM had a marked increase in the expression of Fas ligand compared with the LC group and NAFLD + BDCM group (Fig. 8A). HMGB1 immunoreactivity was unchanged in TLR4 KO mice, indicating that HMGB1 was upstream of TLR4 signaling, as may be perceived since HMGB1 is a known inducer of TLR4 signaling (Fig. 8, B and C). The results suggested a strong involvement of TLR4 signaling in the immunotoxicity of the kidney of mice that had NAFLD and CYP2E1 activation.

**Exacerbation of immunotoxicity in NAFLD kidneys by reductive metabolism of CYP2E1 increases the levels of CXCL16.** CXCL16 has been shown to be a proinflammatory mediator mostly released by dendritic cells and is bound by NKT cells through their surface moiety CXCR6 (33). To show whether CYP2E1 reductive metabolism in NAFLD causes an increase in levels of CXCL16 in the kidneys, we performed immunohistochemistry and mRNA expression profiles of this important cytokine. The results showed that there was a significant increase in the immunoreactivity of CXCL16 in the NAFLD + BDCM group compared with the NAFLD alone group (P < 0.05; Fig. 9, A and B). The administration of DAS significantly decreased the immunoreactivity of CXCL6, whereas CD1D-deficient mouse kidneys did not show any significant difference (P < 0.05). mRNA expression of CXCL16 also showed a similar trend while indicating that the expression was intrinsic to the kidneys (P < 0.05; Fig. 9C). These results suggested that there might be a strong correlation with increased CXCL16 in the kidneys and increased NKT cell activation, as shown in this study, because NKT cells are attracted due to the higher presence of cognate ligand and chemokine (CXCL16) in the renal microenvironment (33).

**NAFLD-induced CYP2E1 activity and metabolic oxidative stress cause kidney injury and affect glomerular function.** To show whether CYP2E1-mediated oxidative stress affected glomerular function, mouse blood was analyzed for BUN concentrations as an indicator of glomerular function. The results showed that BUN increased significantly in the LC + BDCM group compared with the LC alone group (P < 0.05; Fig. 10A). The administration of the CYP2E1 inhibitor showed a significant decrease in BUN values. However, an underlying condition of NAFLD and metabolic oxidative stress significantly increased the concentration of BUN (>3-fold) compared with the NAFLD alone or LC groups (Fig. 10A). Picrosirius red staining, an indicator of collagen deposition, showed a marked increase in the NAFLD + BDCM group compared with the NAFLD alone group, whereas administration of the CYP2E1 inhibitor attenuated the collagen deposition (Fig. 10B). Histological scores showed that the NAFLD + BDCM group had higher inflammation fibrosis than the NAFLD alone group, whereas the CD1D KO group showed higher scores for these two indexes compared with the NAFLD + BDCM group. TLR4 KO mice had lower inflammation and fibrosis (Table 2).
Fig. 8. A: mRNA expression of IL-1β, IL-6, TNF-α, and FasL in kidney tissue of LC, LC+BDCM, NAFLD, NAFLD+BDCM groups as well as TLR4 gene-deficient (TLR4 KO) mice fed a high-fat diet and exposed to BDCM. All mRNA expression was assessed by quantitative real-time PCR, and expression was normalized against the NAFLD group. *P < 0.05. B: Immunoreactivity of HMGB1, as shown by immunohistochemistry in kidney slices from LC (i), LC+BDCM (ii), NAFLD (iii), NAFLD+BDCM (iv), and TLR4 KO + BDCM (v) groups. All images were taken at ×20 magnification. All mRNA expression was assessed by quantitative real-time PCR, and expression was normalized against the LC group. *P < 0.05. C: Immunoreactivity of HMGB1. *P < 0.05.
often associated with proximal tubular inflammation, glomerulonephritis, and glomerulosclerosis. Our study investigated the proinflammatory events in the glomerulus and proximal tubules after progressive NAFLD (NAFLD with inflammation and fibrosis in the liver) in mice. Furthermore, we aimed at identifying the cell types that might be primarily responsible for the heightened proinflammatory phenotype by targeting MCs, since it has been shown that MCs have a strong role to play in the development of CKD (20). The results showed that there was a significant increase in the levels of lipid peroxidation, IL-1β, and TNF-α in proximal tubular cells and podocytes. There was a significant increase in the levels of α-SMA, a proliferative marker of MCs, along with levels of TGF-β. Interestingly, levels of IFN-γ and the T cell markers CD4, CD8, and Va14 (an invariant NKT cell marker) were also increased in proximal tubules. The strong inflammatory phenotype and increased levels of α-SMA and TGF-β levels showed a strong bias toward the role of MCs in proinflammatory processes in the tubule, but a role of endothelial cells also could not be ruled out (39). The rationale that MC activation caused proximal tubular immunotoxicity was further strengthened by results where conditioned medium from MCs enhanced tubular cell expression of proinflammatory cytokines and cell death (Fig. 7, F–J). It was highly unlikely that the

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**Figure 9**: A: immunoreactivity of chemokine (C-X-C motif) ligand (CXCL)16, as shown by immunohistochemistry in kidney slices from LC (i), LC + BDCM (ii), NAFLD (iii), NAFLD + BDCM (iv), CD1d KO + BDCM (v), and NAFLD + BDCM + DAS (vi) groups of mice. All images were taken at ×20 magnification. B: morphometric analysis of CXCL16 immunoreactivity in LC, LC + BDCM, NAFLD, NAFLD + BDCM, CD1d KO + BDCM, and NAFLD + BDCM + DAS groups of mice. Mean data (immunoreactivity measured in arbitrary light units) from three separate microscopic fields were plotted on the y-axis and normalized against the NAFLD group. *P < 0.05. C: mRNA expression of CXCL16 in kidney tissue of LC, LC + BDCM, NAFLD, NAFLD + BDCM, CD1d KO + BDCM, and NAFLD + BDCM + DAS groups of mice by quantitative real-time PCR. Expression was normalized against the LC group. *P < 0.05.
infiltrating macrophages were responsible since there was equal F4/80 staining in kidney sections, including the CD1D KO group (Fig. 4G).

The data showed a prominent inflammatory pattern in tubules, but the presence of T cells also increased the possibility of a regulatory role of these cells in the kidney after NAFLD progression. The argument in favor of the regulatory role of T cells could not be ignored since glomerulonephritis has been shown to be attenuated by the presence of NKT cells primarily in the kidney (45). Moreover, the significant increase in the mRNA expression of Va14, a marker of invariant CD1D-dependent NKT cells, also indicated the involvement of these cell types (45). We used CD1D KO mice to probe the involvement of NKT cells since use of mice that were deficient for T, B, and NK cells did not alter the immunotoxic phenotype in the kidneys, especially regarding the activation of MCs (Fig. 6, E and F). The use of CD1D KO mice significantly exacerbated the proinflammatory phenotype in the kidneys by three- to fourfold compared with the NAFLD + BDCM group (which harbors the kidney disease phenotype).

Ectopic manifestation of the effects of progressive NAFLD, especially in conditions of NASH, has been shown (8).
DAMPS can be released in the circulation after liver injury (9). HMGB protein, adipokine leptin, or TNF-α in the circulation has been shown to cause lipid peroxidation in distant organs (40). Furthermore, HMGB1 can act as an endogenous ligand for TLR4 receptors and increase immunotoxicity (12). Our data showed increased levels of HMGB1 protein in proximal tubular cells, whereas lack of NKT cells showed a further increase in the levels of HMGB1, suggesting a modulatory role of NKT cells in preventing DAMP-induced kidney injury. However, the exact mechanism by which NKT cells can modulate the release of DAMPs is not understood in this study. To show whether HMGB1 mediated the proximal tubular inflammation through the TLR pathway, we used TLR4 KO mice. The results indicated that the mRNA expression of IL-1β and TNF-α was significantly decreased in TLR4 KO mouse kidneys, suggesting a strong involvement of TLR4 pathway activation, probably through endogenous ligands. However, the data are clearly of an indicative nature since endotoxin leaching via the gut could not be ruled out, as has been predicted in alcoholic liver disease (42).

Taken together, we report a possible mechanistic investigation of ectopic immunotoxicity in kidney proximal tubular cells after heightened CYP2E1 metabolism in progressive NAFLD. The increased oxidative stress caused MC activation and pro-inflammatory cytokine release, thus accentuating extracellular matrix formation via TGF-β production. The resultant immunotoxicity possibly mediated the altered glomerular function, as indicated by increased BUN values. The proinflammatory events were likely mediated by the TLR4 pathway, and the presence of NKT cells was found to be protective in the NAFLD potentiation of proximal tubular immunotoxicity. On the other hand, NKT cells, which might be activated in parallel by oxidative stress, played a major protective role via binding to CXCL16, which also has been shown in this study to be increased in the kidneys after reductive metabolism of CYP2E1. Future studies will need to focus on both DAMP-induced TLR4 pathway activation and a concomitant regulatory mechanism by CD1D-dependent NKT cells or other regulatory T cells.

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DISCLOSURES

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