GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Receptor-Interacting Serine/Threonine-Protein Kinase 3 (RIPK3)—Mixed Lineage Kinase Domain-Like Protein (MLKL)—Mediated Necroptosis Contributes to Ischemia-Reperfusion Injury of Steatotic Livers

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Accepted for publication
March 21, 2019.

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Increased hepatic ischemia-reperfusion (IR) injury in steatotic livers is a major reason for rejecting the use of fatty livers for liver transplantation. Necroptosis is implicated in the pathogenesis of fatty liver diseases. Necroptosis is regulated by three key proteins: receptor-interacting serine/threonine-protein kinase (RIPK)-1, RIPK3, and mixed-lineage kinase domain-like protein (MLKL). Here, we found that marked steatosis of the liver was induced when a Western diet was given in mice; steatosis was associated with the inhibition of hepatic proteasome activities and with increased levels of key necroptosis-related proteins. Mice fed a Western diet had more severe liver injury, as demonstrated by increases in serum alanine aminotransferase and necrotic areas of liver, after IR than did mice fed a control diet. Although hepatic steatosis was not different between *Mkl* knockout mice and wild-type mice, *Mkl* knockout mice had decreased hepatic neutrophil infiltration and inflammation and were protected from hepatic IR injury, irrespective of diet. Intriguingly, *Ripk3* knockout or *Ripk3* kinase-dead knock-in mice were protected against IR injury at the late phase but not the early phase, irrespective of diet. Overall, our findings indicate that liver steatosis exacerbates hepatic IR injury via increased MLKL-mediated necroptosis. Targeting MLKL-mediated necroptosis may help to improve outcomes in steatotic liver transplantation. (*Am J Pathol* 2019, 189: 1363–1374; <https://doi.org/10.1016/j.ajpath.2019.03.010>)

Fatty liver disease, including alcoholic liver disease and nonalcoholic fatty liver disease (NAFLD), is a major health issue around the world. NAFLD is associated with obesity, diabetes, and metabolic diseases.¹ The prevalence of NAFLD is estimated to be about 20% worldwide.² NAFLD is characterized by hepatic steatosis, inflammation, and fibrosis, and can progress to cirrhosis and hepatocellular carcinoma.³

Hepatic ischemia-reperfusion (IR) injury is an important type of liver injury that occurs in many clinical conditions, including liver transplantation and liver resection surgeries.⁴ Hepatic IR injury occurs due to the depletion of oxygen and ATP, excessive inflammatory responses, and the generation of reactive oxygen species after reperfusion.⁵ Liver

transplantation remains the most effective treatment option for end-stage liver diseases. However, the practice of liver transplantation is limited by organ shortage.⁶ This issue has led to a more aggressive acceptance and usage of liver grafts from extended-criteria donors, which includes using steatotic livers.⁷ Moderate to severe liver donor steatosis (30% to 60% graft macrosteatosis) increases the risk for IR injury, as demonstrated by evidence that fatty liver has a reduced

Supported in part by NIH grants R21 AA026904 (H.-M.N.), R01 AA020518 (W.-X.D.), U01 AA024733 (W.-X.D.), P20 GM103549 (H.J.), P30 GM118247 (H.J.), and a pilot project from the Centers for Biomedical Research Excellence (COBRE; H.-M.N.).

Disclosures: None declared.

tolerance to IR injury, leading to increased mortality after transplantation.^{7,8} However, the mechanisms by which fatty liver increases IR injury are not fully understood. Without a clear understanding of these mechanisms, the potential to use fatty livers in transplantation will be limited. Furthermore, therapeutic modalities to improve postsurgical care with extensive fatty liver would also be limited.

Limited evidence indicates that necroptosis may play an important role in the pathogenesis of inflammatory liver diseases, including alcoholic liver disease, NAFLD, and hepatic IR injury.^{9–12} Necroptosis is a form of cell death also known as *programed necrosis*, and is mediated by receptor-interacting serine/threonine-protein kinase (RIPK)-1, RIPK3, and the downstream molecule mixed-lineage kinase domain–like protein (MLKL).^{13,14} Necroptosis is morphologically similar to necrosis, which is activated by a pathway in common with apoptosis, including death-receptor activation.^{15–17} In the absence of functional caspase-8, RIPK1 and RIPK3 form heterodimers, resulting in the assembly of the necrosome, which is a crucial step that leads to the phosphorylation and activation of MLKL. Activated MLKL forms oligomers or polymers that translocate to the plasma membrane, resulting in disruption of cell membrane and subsequent necrotic cell death.^{17–20} In addition, necrosome also increases inflammatory factors including IL-1 β and IL-6, which play important roles in alcoholic liver disease, NAFLD, and hepatic IR injury.⁷ There has been intensive ongoing research aiming to identify new preconditioning strategies for diminishing the adverse effects of allograft steatosis after liver transplantation. Specifically, new therapies tested in murine models have demonstrated that parenchymal cell damage and inflammation are major contributors to exacerbated hepatic IR injury in steatotic liver grafts.^{8,21,22} Necrostatin-1, a pharmacologic inhibitor of RIPK1, has been reported to protect against IR injury in heart, kidney, intestine, and liver,^{12,23–25} suggesting that targeting RIPK1-RIPK3-MLKL–mediated necroptosis may be beneficial in IR injury. However, necrostatin-1 is not a specific inhibitor of RIPK1, which has also been demonstrated to inhibit indoleamine-2,3-dioxygenase.²⁶ Therefore, the exact role of RIPK1-RIPK3-MLKL–mediated necroptosis in hepatic IR injury is still elusive.

A murine model of diet-induced hepatic steatosis was used to characterize its effects on hepatic IR injury. Hepatic steatosis was found to be associated with increased hepatic Ripk1, Ripk3, and Mkl1 proteins and with exacerbated IR injury. Furthermore, three genetically modified mouse models—*Ripk3* knockout (KO), *Mkl1* KO, and kinase-dead *Ripk3* knock-in (KDKI)—were used to examine the effects of necroptosis in hepatic IR injury. *Ripk3* KO or *Ripk3* KDKI mice were protected from IR liver injury at the late phase but not the early phase. Interestingly, *Mkl1* KO mice were protected from IR liver injury in both chow-fed and diet-induced obese conditions at both the early and the late phases. These findings may

have a significant impact on developing potential therapeutic strategies to attenuate IR injury in fatty livers by modulating RIPKs and MLKL.

Materials and Methods

Reagents

Antibodies used in this study were Mkl1 and β -actin (catalog numbers SAB1302339 and A5441, respectively; Sigma-Aldrich, St. Louis, MO); glyceraldehyde phosphate dehydrogenase (Gapdh), Ripk1, and phosphorylated (p)-Mkl1 (catalog numbers 2118, 3493, and 62233; Cell Signaling Technology, Beverly, MA); pMkl1, sodium potassium ATPase, and proteasome subunits (Psm)- α 2 and - β 5 (catalog numbers ab66596, ab76020, ab109525, and ab3330; Abcam, Cambridge, MA); Ripk3 (catalog number 2283; ProSci, Poway, CA); myeloperoxidase (Mpo; catalog number PP023AA; Biocare Medical, Concord, CA); and 26S proteasome regulatory subunit 4 (Psmc1; catalog number A303-821A; Bethyl Laboratories, Montgomery, TX). Horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Western diet (WD; catalog number TD.88137) and normal chow diet (CD; catalog number 8604) were from Envigo (Madison, WI).

Animals

Mkl1 KO mice with C57BL/6N background were generated using the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein (CRISPR/Cas)–mediated genome engineering method by Cyagen (Santa Clara, CA). Briefly, *Mkl1* exon 2 was selected as the target site. Cas9 mRNA and gRNA generated by *in vitro* transcription were then injected into fertilized eggs for KO mouse production. *Ripk3* KO (C57BL/6N) mice were generously provided by Dr. Vishva Dixit (Genentech, South San Francisco, CA) as we described previously.¹¹ *Ripk3* KDKI and *Ripk1* KDKI (C57BL/6N) mice were generously provided by Dr. Peter Gough (GlaxoSmithKline, Research Triangle Park, NC).^{27,28} Briefly, *Ripk3* KDKI mice were generated by homologous recombination using a targeting construct that mutated the catalytic lysine residue to alanine (K51A) to eliminate all kinase activity. *Ripk1* KDKI mice were generated by homologous recombination using a targeting construct that mutated the catalytic lysine residue to alanine (K45A) to eliminate all kinase activity. All mice were housed in cages (five mice per cage) and received a 12-hour light/dark cycle. All animals received humane care. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center (Kansas City, KS).

Mouse Hepatic IR Model

Four-week-old male mice were fed a WD or CD for 8 weeks, followed by hepatic IR surgery as described previously.²⁹ Briefly, hepatic ischemia was created by occluding the portal vein, hepatic artery, and bile duct just above the right branch, which provided approximately 70% of the total body's blood supply to the liver, for 45 minutes, followed by reperfusion for different periods of time (1, 6, and 24 hours). Sham control mice underwent the same procedure without vessel occlusion. Mice were euthanized and blood samples and liver tissues were collected. Livers were fixed in 10% formalin and paraffin embedded for histopathologic analysis. Liver injury was determined by measuring serum alanine aminotransferase (Alt).

Histology and Immunohistochemistry

Paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E) for pathologic evaluation. Immunostaining for Mpo-positive neutrophils was performed.³⁰ The positive Mpo staining was quantified by counting 20 different fields under microscopy using $\times 400$ magnification. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed as described previously.¹¹ Oil red O staining was performed using liver cryo-sections as described previously.³¹

Subcellular Fractionation and Western Blot Analysis

Total liver proteins were extracted using radio-immunoprecipitation assay buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl (lauryl) sulfate in phosphate-buffered saline]. Plasma membrane fractions of liver tissue were extracted by using a Minute plasma membrane and cell fractionation kit following the manufacturer's instructions (catalog number SM-005; Invent Biotechnologies, Plymouth, MN). Protein (30 μ g) was separated on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Membranes were probed with appropriate primary and secondary antibodies and visualized with SuperSignal chemiluminescent substrate (catalog number 34577; Thermo Fisher Scientific, Waltham, MA). Densitometry analysis was performed with Un-Scan-It software version 6.1 (Silk Scientific, Orem, UT) and normalized to β -actin or Gapdh for total proteins or sodium potassium ATPase for plasma membrane proteins. All densitometry data are presented as means $\pm$ SEM.

Analysis of Proteasome Activity

Proteasome activity was analyzed using Suc-LLVY-AMC and Bz-VGR-AMC substrate (catalog numbers BML-P802 and BML-BW9375; Enzo Life Sciences, Farmingdale, NY) as described previously.¹¹ Briefly, 10 μ g of total liver lysates was added to a white 96-well flat-bottom plate. AMC

substrate (20 nmol/L) was added to each well, along with 100 μ L of assay buffer (50 mmol/L Tris pH 7.5, 25 mmol/L KCl, 10 mmol/L NaCl, and 1 mmol/L $MgCl_2$ diluted in dH_2O). Proteasome activity was determined by measuring AMC release using an excitation/emission 380/460 test filter on a plate reader (Tecan Life Sciences, Männedorf, Switzerland) after 1 hour.

Quantitative Real-Time PCR

RNA was extracted from mouse liver using Trizol (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA by RevertAid reverse transcriptase (Thermo Fisher Scientific).³² Real-time PCR was performed on a CFX384 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using SYBR Green mix (Bimake, Houston, TX). Expression levels of *Actb*, *Cd68*, *Ly6g*, *Il1b*, *Il6*, *Mip2*, and *Tnfa* were quantified using quantitative real-time PCR analysis. Primer sequences were as follows: *Actb*: forward, 5'-TGTTACCAACTGGGACGACA-3' and reverse, 5'-GGGGTGTGTAAGGTCTCAAA-3'; *Ly6g*: forward, 5'-TGCGTTGCTCTGGAGATAGA-3' and reverse, 5'-CAGAGTAGTGGGGCAGATGG-3'; *Il1b*: forward, 5'-GCCCATCCTCTGTGACTCAT-3' and reverse, 5'-AGGC-CACAGGTATTTTGTCTG-3'; *Il6*: forward, 5'-ACAAC-CACGGCCTTCCCTACTT-3' and reverse, 5'-CATTTC-CACGATTTCCCAGAGA-3'; *Mip2*: forward, 5'-CTCA-GAGGAAGACGATGAAG T-3' and reverse, 5'-CTCA-GAGGAAGACGATGAAG-3'; and *Tnfa*: forward, 5'-CGTCAGCCGATTTGCTATCT-3' and reverse, 5'-CGGACTCCGCAAAGTCTAAG-3'.

Primary Hepatocyte Culture

Murine hepatocytes were isolated by a retrograde, non-recirculating perfusion of livers with 0.05% Collagenase Type IV (Sigma-Aldrich) as described previously.³³ Cells were cultured in William's medium E supplemented with 10% fetal bovine serum for 2 hours to allow for attachment and then were switched to the same medium without fetal bovine serum. All cells were maintained in a 37°C incubator with 5% CO_2 .

In Vitro Hypoxia and Reoxygenation Model

In vitro hypoxia and reoxygenation experiments were performed as described previously.³⁴ Briefly, murine primary hepatocytes were isolated from CD- and WD-fed mice. Hepatocytes were incubated at 37°C in anaerobic jars with oxygen-absorbing packs for 3 hours, followed by reoxygenation for different periods of time. Cell death was evaluated by quantification of lactate dehydrogenase (Ldh) release into the culture medium with an assay kit following the manufacturer's instructions (Pointe Scientific, Canton, MI).

Triglyceride Analysis

Hepatic triglyceride (TG) extraction was performed as described previously.³⁵ Frozen liver tissues (20 to 50 mg) were ground into powder using a mortar and pestle followed by chloroform-methanol extraction. TG analysis was performed using GPO-Triglyceride Reagent Set (Pointe Scientific) following the manufacturer's instructions.

Caspase Activity Measurement

Caspase-3 and -8 activities were assessed as described previously.³⁶ Briefly, 15 μ g of total proteins were combined with 2 μ mol/L Ac-IETD-AFC or Ac-DEVD-AFC (Enzo Life Sciences) in a caspase assay buffer [100 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L PIPES (piperazine-N,N'-bis), 10% (w/v) sucrose, 0.1% (w/v) CHAPS (3-[(3-cholamidopropyl)dime-thylammonio]-1-propanesulfonate), 10 mmol/L dithio-threitol, pH 7.2] in a final volume of 200 μ L and were incubated for 2 hours at 37°C. The change of fluorescence was measured by a plate reader (Tecan Life Sciences).

Statistical Analysis

All experimental data are expressed as means $\pm$ SEM and were subjected to *t*-test or one-way analysis of variance with Bonferroni *post hoc* test where appropriate. *P* < 0.05 was considered significant.

Results

Levels of Key Necroptosis Proteins Increase in Diet-Induced Steatotic Mouse Livers

To investigate the role of necroptosis in fatty liver, a mouse model of nonalcoholic fatty liver was first established. Mice fed with WD had both increased macrosteatosis (with large lipid droplets) and microsteatosis (with small lipid droplets) in hepatocytes shown by H&E and oil red O staining (Figure 1, A and B). Western blot analysis revealed increased levels of Ripk1, Ripk3, and Mkl1 in WD-fed mice compared to CD-fed mice at as early as 5 weeks and extended up to 12 weeks (Figure 1, C and D). These data indicate that diet-induced hepatic steatosis increases the levels of key necroptosis proteins in mouse livers.

Liver Steatosis Decreases Levels of Core Proteasome Subunits and Activities in Mouse Livers

Inhibition of proteasome activity by ethanol increases levels of hepatic RIPK3.¹¹ The levels of proteasome core protein subunits Psm α 2 and Psm β 5 as well as Psmc1 were decreased in the WD-fed mouse livers compared to those in the CD-fed mice (Figure 2, A and B). Moreover, hepatic trypsin-like proteasome activities were significantly decreased in the WD-fed mice compared to those in the

Figure 1 Increases of key necroptosis proteins in steatotic livers. Wild-type mice were fed with chow diet (CD) or Western diet (WD) for 5 to 12 weeks. Liver steatosis, as assessed by staining with hematoxylin and eosin (H&E; **A**) and oil red O (**B**). **C**: Western blot analysis for receptor-interacting serine/threonine-protein kinase (Ripk)-1, Ripk3, and mixed lineage kinase domain-like protein (Mkl1) after total protein extraction from liver homogenates. **D**: Densitometry analysis. Data are expressed as means $\pm$ SEM. *n* = 3 per group. **P* < 0.05 and ***P* < 0.01 versus CD.

CD-fed mice (Figure 2C). Hepatic chymotrypsin-like proteasome activities also trended to decrease in the WD-fed mice compared with the CD-fed mice (Figure 2C). These results indicate that WD decreases core hepatic proteasome subunit proteins and inhibits proteasome activities in mouse liver.

Steatosis Exacerbates IR Liver Injury that Is Blunted in *Mkl1* KO Mice

Liver tissue H&E staining revealed that IR was associated with hepatocyte necrosis mainly in the central vein areas but barely in the portal vein areas regardless of CD or WD, although the necrotic areas were much larger in the WD-fed mouse livers than in the CD-fed mouse livers (Figure 3A). Caspase-3 and -8 activities showed no changes after IR in CD- or WD-fed mouse livers (Supplemental Figure S1, D and E). TUNEL staining confirmed the nature of necrotic

Figure 2 Proteasome subunits and proteasome activities in steatotic livers. Wild-type mice were fed with chow diet (CD) or Western diet (WD) for 5 to 12 weeks. Total proteins were extracted from mouse livers. **A:** Proteasome subunit (Psm)- α 2, Psm β 5, and 26S proteasome regulatory subunit 4 (Psmc1) on Western blot analysis. **B:** Densitometry analysis. **C:** Trypsin-like and chymotrypsin-like proteasome activities as measured in liver lysates using specific substrates. Data are expressed as means $\pm$ SEM. $n = 4$ per group. * $P < 0.05$ and ** $P < 0.01$ versus CD.

cell death in the mouse livers after IR, as demonstrated by the diffuse TUNEL staining pattern, similar to acetaminophen-induced necrosis³⁷ (Figure 3B). Since the TUNEL-positive areas are similar to necrotic areas on H&E staining, necrotic areas were quantified using TUNEL staining. The TUNEL-positive areas were significantly decreased in *Mkl* KO mice after IR regardless of CD or WD (Figure 3C). The levels of serum Alt activities were also significantly lower in *Mkl* KO mice than in wild-type (WT) mice after IR in both CD- and WD-fed mice (Figure 3D), which was consistent with the TUNEL staining data. These data indicate that MLKL-mediated necrosis plays a crucial role in the pathogenesis of IR-induced liver injury. The levels of liver triglyceride were not significantly different between WD-fed WT and *Mkl* KO mice, suggesting that MLKL does not affect WD-induced steatosis (Supplemental Figure S1A).

Since MLKL oligomer or polymer formation and plasma membrane translocation is a crucial step for triggering

necroptosis, the level of Mkl on the plasma membranes was next determined. The levels of Mkl plasma membrane translocation were increased with IR in CD-fed mice. The levels of Mkl plasma membrane translocation were already increased with WD alone, and were not further increased with IR, likely due to the nonspecific degradation of plasma membrane Mkl as a result of the massive necrosis in the WD-fed mice after IR (Figure 3E). Mkl oligomer formation was also examined in nonreducing conditions. Levels of Mkl oligomer formation were increased with IR compared with sham livers, regardless of diet. Levels of Mkl polymer were increased with IR mouse livers regardless of diet, but the levels of Mkl polymer were much higher in IR WD-fed mice than in the IR CD-fed mice (Figure 3F). Hepatocytes were next isolated from CD- and WD-fed mice, and these hepatocytes were subjected to hypoxia and reoxygenation for various periods of time. Hepatocytes isolated from WD-fed mice had significantly higher Ldh leakage compared with hepatocytes from CD-fed mice after hypoxia and reoxygenation in a time-dependent manner (Supplemental Figure S2A). The protein levels of Ripk3 and pMkl were increased with hypoxia and reoxygenation in hepatocytes isolated from both CD- and WD-fed mice (Supplemental Figure S2B).

To further confirm that the protection from IR injury in *Mkl* KO mice was not due to the protection of ischemic injury, *Mkl* KO mice and their matched WT mice were subjected to IR surgery, and liver injury was examined at 1 hour of reperfusion. There was no difference in the serum Alt levels between WT and *Mkl* KO mice at this early time point (Supplemental Figure S1B). Taken together, these data suggest that the deletion of MLKL protects against hepatic reperfusion injury but not by reducing ischemic liver injury.

Mkl KO Mice Have Decreased Hepatic Neutrophil Infiltration and Inflammation after IR

Neutrophil activation and inflammation are important causes of hepatic IR injury.³⁸ Mpo, a neutrophil marker, was stained in mouse livers. Few Mpo-positive neutrophils were present in sinusoids before ischemia in both CD- and WD-fed mice (Figure 4, A and B). An increased number of neutrophils were extravasated into the hepatic parenchyma at 6 hours, which further increased at 24 hours after reperfusion in both WT and *Mkl* KO mice. However, at 24 hours after IR, the number of neutrophils was significantly lower in the *Mkl* KO mice than in WT mice. Interestingly, the number of infiltrated neutrophils was not changed with WD in either WT or *Mkl* KO mice. Moreover, the hepatic expression of Ly6g at 6 and 24 hours was increased with IR in WT mice and was significantly inhibited in *Mkl* KO mice. Similar to the data on neutrophils, WD had no effect on the hepatic expression of Ly6g in WT or *Mkl* KO mice (Figure 4C).

The hepatic expression levels of several cytokines, including tumor necrosis factor (Tnf)- α , Il-1 β , Il-6, and the

Figure 3 Hepatic ischemia-reperfusion (IR) injury in *Mkl1* wild-type (WT) and knockout (KO) mice fed with chow diet (CD) or Western diet (WD). **A–C:** *Mkl1* KO and matched WT mice were fed with CD or WD for 8 weeks and were subjected to 45 minutes of ischemia and 6 and 24 hours of reperfusion. Liver injury as assessed by staining with hematoxylin and eosin (**A**) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; **B**), with the area of necrosis with TUNEL-positive staining measured (**C**). **D:** Serum alanine aminotransferase (ALT) levels. **E:** Expression of mixed lineage kinase domain-like protein (Mkl1) as detected by Western blot and densitometry analysis after extraction of plasma membrane fractions from livers. **F:** Monomer, oligomer, and polymer of Mkl1 on Western blot and densitometry analysis after total protein extraction from liver homogenates and gel electrophoresis under nonreducing conditions. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 per group. * $P < 0.05$ versus WT.

chemokine macrophage inflammatory protein (Mip) 2 all were increased in WT mouse livers after IR. The expression levels of Il-1 β , Il-6, and Mip2, but not Tnf- α , were further increased with WD after IR. However, the expression levels of all these proteins were decreased in *Mkl1* KO mice after IR (Figure 5, A–D). These data indicate that *Mkl1* KO mice have decreased hepatic neutrophil activation and inflammation during IR.

Deletion of Ripk3 Protects against Hepatic IR Injury at the Late Phase

Ripk3 KO mice had decreased IR injury at 24 hours but not at 6 hours, based on the H&E and TUNEL staining analyses (Figure 6, A–C). Consistent with the findings on histology, *Ripk3* KO mice had significantly lower serum Alt levels compared with WT mice at 24 but not at 6 hours after IR (Figure 6D). Caspase-3 and -8 activities also did not change after IR in *Ripk3* KO mice (Supplemental Figure S1, D and E). These observations were in contrast to those in the *Mkl1*

KO mice, which showed protection against IR injury at both the early (6 hours) and late (24 hours) phases. Similar to the finding in *Mkl1* KO mice, there was no difference in ischemic injury in *Ripk3* KO mice (Supplemental Figure S1C).

Consistent with the data on liver injury, Mpo staining was decreased at 24 hours but not at 6 hours after IR in *Ripk3* KO mouse livers (Figure 7, A and B) compared to that in their WT littermates. The mRNA levels of Ly6G, Tnf- α , Il-1 β , Il-6, and Mip2 were also decreased at 24 hours but not at 6 hours after IR (Figure 7C and Figure 8, A–D).

The protein levels of Ripk3 and Mkl1, but not Ripk1, were increased after IR, and were further increased with WD (Figure 8E). Intriguingly, the levels of plasma membrane Mkl1 and Mkl1 oligomer or polymer were increased in both WT and *Ripk3* KO mouse livers at 6 and 24 hours after IR (Figure 8, F–G). These data suggest that MLKL plasma membrane translocation may occur independently of RIPK3, and that the late-phase protection in IR-induced liver injury in *Ripk3* KO mice is independent of Mkl1. To test whether the kinase activity of Ripk3 was required for

Figure 4 Detection of neutrophil infiltration in *Mkl1* wild-type (WT) and knockout (KO) mouse livers after ischemia-reperfusion (IR) surgery. *Mkl1* KO and matched WT mice were fed with chow diet (CD) or Western diet (WD) and subjected to 45 minutes of ischemia and 6 and 24 hours of reperfusion. **A:** Neutrophil staining with myeloperoxidase on liver sections after 6 and 24 hours of reperfusion. **B:** Mpo-positive cells were counted in 20 high-power fields (HPFs). **C:** mRNA level of a neutrophil marker Ly6G as analyzed by real-time RT-PCR. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 per group. * $P < 0.05$ versus WT.

hepatic IR injury, *Ripk3* KDKI mice were fed CD or WD, which was followed by IR. Similar to findings in the *Ripk3* KO mice, the *Ripk3* KDKI mice were protected from IR injury at 24 hours, but not at 1 and 6 hours, based on the analysis of serum Alt levels, H&E staining on liver histology, and TUNEL staining of dead cells (Supplemental Figure S3, A–C). RIPK1 is another key molecule in the necroptosis pathway. It was also tested whether the kinase activity of Ripk1 was required in IR models. *Ripk1* KDKI mice were fed CD or WD, which was followed by IR. *Ripk1* KDKI mice were not protected against IR injury at 6 hours, based on serum Alt level (Supplemental Figure S3D). Caspase-3 activity was not changed after IR in either *Ripk1* KDKI or *Ripk3* KDKI mice (Supplemental Figure S3E). Taken together, these data indicate that, in mice, Ripk3 kinase activity is required for late-phase injury against IR-induced liver injury. Moreover, MLKL may also be translocated to plasma membrane independently of Ripk3.

Discussion

Rodent studies have consistently shown that hepatic steatosis exacerbates hepatic IR injury.^{38–40} However, some studies have utilized either genetic models of obesity, such as *ob/ob* mice, or severe, but less physiologically relevant, diets, such as the methionine/choline-deficient diet. In the present study, a more physiologically relevant diet was used to induce hepatic steatosis in mice. Consistent with the findings from previous studies,^{39,40} these data show that IR injury was exacerbated in hepatic steatosis.

The type of cell death caused by hepatic IR is still controversial. One study showed that IR may induce apoptosis, as demonstrated by increased TUNEL-staining-positive hepatocytes and increased caspase activities.⁴¹ In contrast, some studies have shown necrosis without caspase activation and a lack apoptotic markers during hepatic IR injury.^{29,42} Necrotic areas

Figure 5 Detection of inflammation in ischemia-reperfusion (IR) livers. mRNA levels of cytokines tumor necrosis factor (Tnf)- α (A), IL-1 β (B), and IL-6 (C), and chemokine macrophage inflammatory protein (Mip)-2 (D) as analyzed by real-time PCR. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 per group. * $P < 0.05$ versus wild type (WT). CD, chow diet; KO, knockout; WD, Western diet.

Figure 6 Hepatic ischemia-reperfusion (IR) injury in *Ripk3* knockout (KO) mice. **A–C:** *Ripk3* KO and matched wild-type (WT) mice were fed with chow diet (CD) or Western diet (WD) for 8 weeks and were subjected to 45 minutes of ischemia and 6 and 24 hours of reperfusion. Liver injury as assessed by hematoxylin and eosin and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, with area of necrosis measured with TUNEL-positive staining. **D:** Serum alanine aminotransferase (Alt) levels. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 per group. * $P < 0.05$ versus WT.

were readily found in the mouse livers after IR by either TUNEL staining or H&E staining. More importantly, neither the cleaved caspase-3 (data not shown) nor increased caspase-3 and -8 activities were detected after IR in the mouse livers. These data clearly indicate that IR induces caspase-independent necrosis but not

apoptosis in mouse liver, which is exacerbated by liver steatosis.

Increased production of TNF- α in IR injury of steatotic livers has been well-documented.^{39,43,44} TNF- α is a pleiotropic cytokine that regulates cell death, cell survival, and inflammation. Depending on the cellular context, TNF- α

Figure 7 Detection of neutrophil infiltration in the livers of *Ripk3* knockout (KO) mice after ischemia-reperfusion (IR) surgery. *Ripk3* KO and matched wild-type (WT) mice were fed with chow diet (CD) or Western diet (WD) and were subjected to 45 minutes of ischemia and 6 and 24 hours of reperfusion. **A:** Neutrophil staining with myeloperoxidase (Mpo) on liver sections after 6 and 24 hours of reperfusion. **B:** Mpo-positive cells were counted in 20 high-power fields (HPFs). **C:** mRNA levels of a neutrophil marker Ly6G as analyzed by real-time RT-PCR. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 per group. * $P < 0.05$ versus WT.

Figure 8 Detection of inflammation in ischemia-reperfusion (IR) livers of *Ripk3* knockout (KO) mice. mRNA levels of cytokines tumor necrosis factor (Tnf)- α (A), IL-1 β (B), and IL-6 (C), and chemokine macrophage inflammatory protein (Mip)-2 (D) as analyzed by real-time PCR. Total protein and plasma membrane fractions were extracted from livers. Equal amounts of proteins from the samples of the same groups were combined. E–G: Expression levels of mixed lineage kinase domain-like protein (MLKL), receptor-interacting serine/threonine-protein kinase (Ripk)-1, and Ripk3 in total lysate (E); plasma membrane MLKL (F); and oligomer and polymer of MLKL (G) as detected by Western blot analysis. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 per group. * $P < 0.05$ versus wild type (WT). #Nonspecific band. CD, chow diet; Gapdh, glyceraldehyde phosphate dehydrogenase; WD, Western diet.

can induce either caspase-dependent apoptosis or RIPK1/3-MLKL-mediated necroptosis. There are two TNF receptors, and the various effects of TNF- α mainly depend on TNFR1. Once TNF- α binds to TNFR1, a multiprotein complex including TNFR1-associated death domain protein (TRADD), TNFR-associated factor 2, cellular inhibitor of apoptosis protein (cIAP)-1 and -2, linear ubiquitin chain assembly complex (LUBAC), and RIPK1 is formed, resulting in the ubiquitination of RIPK1. Ubiquitination of RIPK1 by cIAPs and LUBAC recruits transforming growth factor β -activated kinase (TAK)-1-binding proteins 2/3 to promote the activation of TAK1. Activation of TAK1 results in I κ B kinase complex activation and further NF- κ B activation, which increases the expression of genes for inflammation and cell survival. When RIPK1 is deubiquitinated, TNF- α then triggers either apoptosis or necroptosis. Deubiquitinated RIPK1 forms a complex with TRADD, Fas-associated death domain protein (FADD), FADD-like IL-1 β -converting enzyme inhibitory protein, and caspase-8 to activate caspase-8 and subsequently trigger apoptosis. Necroptosis is generally blocked by apoptosis through caspase-8-mediated cleavage and inactivation of RIPK1 and RIPK3. When caspase-8 is inhibited, RIPK1 and RIPK3 bind with each other via their RIP homotypic interaction motif domains, which further recruit MLKL to form the necrosome to induce necroptosis.^{13,45}

Here, the protein levels of Ripk3 and Mlkl were increased in mouse livers and in primary cultured mouse hepatocytes after WD feeding. Ripk3 protein increases in mouse livers after long-term alcohol administration, as well as in mouse and human hepatocytes of alcoholic liver disease, due to impaired proteasomal activities.¹¹ Notably, the protein levels of Psm α 2 and Psm β 5 and hepatic proteasome activity

were decreased in WD-fed mice. Therefore it is likely that decreased proteasomal degradation of RIPK3 and MLKL may lead to the accumulation of hepatic RIPK3 and MLKL after WD. The basal protein levels of RIPK1 and RIPK3 (in particular RIPK3) are much higher in spleen and thymus than in the liver, suggesting that RIPK1 and RIPK3 may play crucial roles in immune cells in normal physiologic conditions.^{37,46} However, the levels of hepatic RIPK3 protein may be increased due to the impaired proteasomal degradation of RIPK3 in response to stresses such as alcohol and high-fat diet, which then triggers RIPK3-MLKL-mediated necroptosis.

In addition to NAFLD, RIPK1-RIPK3-mediated necroptosis also occurs in various pathophysiologically relevant conditions including IR injury in different organs. For instance, RIPK1-RIPK3-mediated necroptosis has been implicated in ischemic brain injury,⁴⁷ IR-induced myocardial injury,⁴⁸ kidney injury,⁴⁹ as well as hepatic IR injury.^{12,39,50,51} In some studies, necrostatin-1, a pharmacologic inhibitor of RIPK1, has been used and reported to protect against IR injury, suggesting that targeting RIPK1-RIPK3-MLKL-mediated necroptosis may be beneficial in preventing I/R injury. However, necrostatin-1 is not a specific inhibitor of RIPK1, and has also been demonstrated to inhibit indoleamine-2,3-dioxygenase.²⁶ A recent study³⁹ demonstrated that increased pMlkl levels were found in hepatic steatosis and IR mouse livers. Genetic deletion of *Ripk3* and *Mlkl* and *Ripk3* or *Ripk1* KDKI mouse models were used to conduct the present study. Mlkl oligomer or polymer formation and plasma membrane translocation were increased in IR livers and were further increased in WD-fed IR livers. These findings indicate that Mlkl-dependent disruption of cellular integrity contributes to

hepatic IR injury in this model. Importantly, although a strong attenuation of IR injury was observed in *Mkl* KO mice, deletion of *Ripk3* or expression of *Ripk3* KD were associated with protection from IR injury only during the late phase. *Ripk1* KDKI mice were not protected against IR injury at 6 hours. These data suggest that *Mkl* activation and plasma membrane translocation occur independent of *Ripk3* or *Ripk1* in this model. Although *RIPK3* expression is much lower at the basal level in hepatocytes compared to immune cells, mainly Kupffer cells, *MLKL* protein level is relatively higher in liver (data not shown).⁵² In addition, the elevated levels of *MLKL* in hepatocytes are associated with hepatic injury in a mouse model of hepatitis and in autoimmune hepatitis in humans, which is also independent of *RIPK3*.⁵² The deletion of *Ripk3* does not protect against IR injury in mice.⁵⁰ However, this study determined IR injury only after 4 hours of reperfusion, which is consistent with the data that the deletion of *Ripk3* protected against IR injury after 24 hours but not after 6 hours of reperfusion.

Hepatic IR injury is caused by an excessive inflammatory response and by microcirculatory dysfunction.^{53–56} Hepatic neutrophil infiltration is important in hepatic IR models. Neutrophil infiltration starts at 4 to 8 hours of reperfusion, and these neutrophils are activated and generate reactive oxygen species.^{38,56} Our data, which relied on the identification of neutrophils in tissue sections based on a specific staining of Mpo and mRNA levels of Ly6G in tissues, demonstrated that neutrophils were recruited to the liver during the reperfusion period. Furthermore, neutrophil extravasation into the parenchyma mainly inside or surrounding necrotic areas began at around 6 hours and continued during the remaining reperfusion period. The mRNA level of Ly6G started to increase at 6 hours. Despite the very clear role of neutrophils in causing additional injury during hepatic IR, neutrophils do not always aggravate the existing injury in all acute injury models. Neutrophils do not aggravate the injury after an overdose of acetaminophen but contribute to the repair process.^{57,58} Here, the amount of neutrophils was slightly increased in WD-fed compared to CD-fed mouse livers, whereas serum Alt was increased significantly in WD-fed IR livers. The mRNA level of Ly6G did not further increase in WD-fed mouse livers. Neutrophil filtration and activation may partially contribute to IR injury, and other mechanisms, such as increased microcirculatory disturbances, in steatotic livers may contribute to the injury.³⁸ Recent evidence indicates that activation of the *RIPK1-RIPK3-MLKL* necroptosis pathway promotes the neutrophil extracellular traps in cultured neutrophils and bone-marrow-derived cells.^{59–61} Increased neutrophil extracellular traps promote IR injury in liver and heart.^{62,63} However, whether *RIPK1-RIPK3-MLKL*-mediated neutrophil extracellular traps may promote hepatic IR injury remains unclear.

In summary, hepatic proteasomal functions were inhibited, and key necroptosis proteins, *Ripk3* and *Mkl*, were increased with WD in mice. Liver steatosis exacerbated

hepatic IR injury partially with increased neutrophil infiltration and inflammation. With WD, *Mkl* plasma membrane translocations were increased to induce necroptosis, which was further increased with IR. Knockout of *Mkl* protected against reperfusion injury in both CD- and WD-fed mice. Knockout of *Ripk3* and *Ripk3* KDKI protected against reperfusion injury at later time points, which indicates that the protection of *Mkl* KO mice was partially independent of *Ripk3*. These findings may have a significant impact on the development of novel therapeutic strategies against IR injury in steatotic livers by modulating *RIPK* and *MLKL*.

Acknowledgments

We thank Dr. Vishva M. Dixit (Genentech, San Francisco, CA) for providing the *Ripk3* KO mice and Dr. Peter Gough (GlaxoSmithKline, Collegeville, PA) for the *Ripk3* and *Ripk1* KDKI mice.

H.-M.N. designed and performed experiments, analyzed data, and wrote the manuscript; X.C., J.K., F.D., and S.W. performed experiments; Y.-H.S., T.L., W.-X.D., and H.J. designed experiments and wrote the manuscript; all authors read and approved the final manuscript.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2019.03.010>.

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