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Hepatic Ischemia/Reperfusion Injury Involves Functional TLR4 Signaling in Nonparenchymal Cells¹

Allan Tsung, Rosemary A. Hoffman, Kunihiko Izuishi, Nathan D. Critchlow, Atsunori Nakao, Meagan H. Chan, Michael T. Lotze, David A. Geller, and Timothy R. Billiar²

Endogenous ligands from damaged cells, so-called damage-associated molecular pattern molecules, can activate innate immunity via TLR4 signaling. Hepatic warm ischemia and reperfusion (I/R) injury and inflammation is largely TLR4 dependent. We produced TLR4 chimeric mice to assess whether the TLR4-dependent injury required TLR4 expression on liver parenchymal or nonparenchymal cells. Chimeric mice were produced by adoptive transfer of donor bone marrow cells into irradiated recipient animals using reciprocal combinations of TLR4 wild-type (WT; C3H/HeOuj) and TLR4 mutant (C3H/HeJ) mouse bone marrow. Wild-type chimeric mice bearing TLR4 mutant hemopoietic cells and TLR4 mutant mice transplanted with their own bone marrow-derived cells were protected from hepatic I/R and exhibited decreased JNK and NF- κ B activation compared with WT chimeric mice transplanted with their own bone marrow. In contrast, TLR4 mutant mice transplanted with TLR4 WT bone marrow were not protected from liver I/R and demonstrated pronounced increases in JNK and NF- κ B activation when compared with autochthonous transplanted mutant mice. In addition, depletion of phagocytes taking up gadolinium chloride failed to provide any additional protection to TLR4 mutant mice, but substantially reduced damage in WT mice after hepatic I/R. Together, these results demonstrate that TLR4 engagement on actively phagocytic nonparenchymal cells such as Kupffer cells is required for warm I/R-induced injury and inflammation in the liver. *The Journal of Immunology*, 2005, 175: 7661–7668.

Ischemia and reperfusion (I/R)³ injury is a pathophysiologic process whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery to the compromised tissue. Transient episodes of hepatic ischemia occur during solid organ transplantation, trauma, hypovolemic shock, and elective liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathophysiology of liver I/R injury includes both direct cellular damage as the result of the ischemic insult as well as delayed dysfunction and damage resulting from activation of inflammatory pathways. Histopathologic changes include cellular swelling, vacuolization, endothelial cell disruption, neutrophil infiltration, and hepatocellular necrosis (1, 2).

The distal interacting elements in the cascade of inflammatory responses resulting in organ damage following hepatic I/R injury have been extensively studied. Activation of Kupffer cells with production of reactive oxygen species, up-regulation of the inducible NO synthase in hepatocytes, activation of JNK, up-regulation of proinflammatory cytokines, and neutrophil accumulation have all been identified as contributing events to the inflammation-associated damage (3–8). The extent to which the initial cellular injury contributes to propagation of the inflammatory response and leads to further tissue damage is poorly understood. Recent studies

suggest that a key link between the initial damage to cells and the activation of inflammatory signaling involves release of endogenous damage/danger associated molecular pattern (DAMP) signals from ischemic cells or disruption of the tissue matrix (9, 10).

The TLRs are one of the components by which the innate immune system senses the invasion of pathogenic microorganisms or tissue damage by recognizing specific molecular patterns that are present in microbial products (pathogen-associated molecular pattern molecules) or endogenous molecules released by damaged tissues (DAMP) (11). Perhaps more than any of the other TLR family members, TLR4 sits at the interface of microbial and sterile inflammation by responding to both bacterial endotoxin and multiple other endogenous ligands, including hyaluronic acid (12), heparin sulfate (13), fibrinogen (14), high-mobility group box 1 (HMGB1) (9, 15), and heat shock proteins (16). Both inflammation and injury responses in warm hepatic I/R are partially TLR4 dependent (9, 10). Our recent studies (9) suggest a central role for HMGB1 in the TLR4-dependent component associated with hepatocyte damage and the resultant enhanced inflammation.

The liver is well equipped to respond to these potential endogenous ligands. The liver consists of parenchymal cells (hepatocytes) and nonparenchymal cells (NPC), including Kupffer cells, sinusoidal endothelial cells, stellate cells, and hepatic dendritic cells. TLR4 is present on both hepatocytes and NPC and both cell populations possess intact TLR4 signaling pathways (17, 18). The aim of this study was to determine whether the TLR4-dependent injury requires TLR4 expression on hepatocytes or NPC. We show using TLR4 chimeric mice in a model of sterile inflammation that the NPC, not the hepatocytes, are responsible for recognizing the initial damage from ischemic cells and for activating TLR4-dependent signaling required for I/R-induced injury.

Materials and Methods

Animals

Male wild-type (WT) C57BL/6 (C3H/HeOuj) mice and TLR mutant (C3H/HeJ) mice (8–12 wk old) were purchased from The Jackson Laboratory.

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³ Abbreviations used in this paper: I/R, ischemia and reperfusion; DAMP, damage associated molecular pattern molecule; NPC, nonparenchymal cell; WT, wild type; HMGB1, high-mobility group box 1; ALT, alanine aminotransferase.

All animals were maintained in a laminar-flow specific pathogen-free atmosphere at the University of Pittsburgh (Pittsburgh, PA). Animal protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh and the experiments were performed in adherence to the National Institutes of Health Guidelines for the use of laboratory animals.

Chimeric mice

Chimeric mice were produced by adoptive transfer of donor bone marrow cells into irradiated recipient animals using combinations of TLR4 WT (C3H/HeOuj) and TLR4 mutant (C3H/HeJ) mice in the following recipient/donor combinations: WT/WT, WT/Mutant, Mutant/Mutant, Mutant/WT. Recipient mice were exposed to an otherwise lethal 1000 cGy from a Cesium source (Nordion International) 6 h before receiving 2.5×10^6 bone marrow cells by tail vein injection. The bone marrow cells were prepared in a sterile manner from the tibia and femur bones of the donor mice. All animals were monitored two to three times weekly for the first 2 wk to ensure successful bone marrow engraftment. The chimeric mice underwent hepatic I/R after another 8–10 wk to ensure stable engraftment.

Liver ischemia

A nonlethal model of segmental (70%) hepatic warm ischemia was used. The I/R protocol was initiated with the abdominal wall being clipped of hair and cleansed with betadine. Under sodium pentobarbital (40 mg/kg, i.p.) and methoxyflurane (inhalation) anesthesia, a midline laparotomy was performed. With the use of an operating microscope, the liver hilum was dissected free of surrounding tissue. All structures in the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were occluded with a microvascular clamp (Fine Science Tools) for 60 min, and reperfusion was initiated by removal of the clamp. This method of segmental hepatic ischemia prevents mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. We have previously conducted a time course experiment to determine the optimal ischemia time period for the induction of hepatic injury (19). Less than 60 min of ischemia produced only minimal plasma transaminase elevations, whereas over 75 min of ischemia was poorly tolerated with gross evidence of poor reperfusion of the ischemic lobes. A reproducible level of hepatic damage was observed using 60 min of ischemia and was thus used for our study. After application of the clamp, the abdomen was covered with a sterile plastic wrap to minimize evaporative loss. Throughout the ischemic interval, evidence of ischemia was confirmed by visualizing the pale blanching of the ischemic lobes. The clamp was then removed and gross evidence of reperfusion based on immediate color change was assured before closing the abdomen with a continuous 4–0 diameter polypropylene suture. Either the absence of ischemic color changes or the lack of response to reperfusion was a criterion for immediate sacrifice and exclusion from further analysis. Temperature was monitored by rectal temperature probe and was maintained at 37°C by means of a warming pad and heat lamp. At the end of the observation period following reperfusion, the mice were anesthetized with inhaled methoxyflurane and were sacrificed by exsanguination. Sham animals underwent anesthesia, laparotomy, and exposure of the portal triad without hepatic ischemia. Animals were sacrificed at predetermined time points (1–6 h) after reperfusion to obtain serum and liver samples.

Gadolinium chloride treatment

Mice received either gadolinium chloride (Sigma-Aldrich) dissolved in normal saline (10 mg/kg) or normal saline alone by i.v. injection 24 h before ischemia.

NPC isolation

Live NPC were harvested using a modification of the perfusion technique of Seglen (20). The vena cava was cannulated and the liver was perfused for 3 min with $1 \times$ HBSS (Invitrogen Life Technologies) supplemented with 0.96 g of sodium bicarbonate/500 ml (Perfusate I) at a flow rate of 10 ml/min. Next, the liver was perfused with a 0.2% protease (Sigma-Aldrich) in Perfusate I (Perfusate II) for 3 min. The liver was dissected out and placed in a petri dish with Perfusate II and diced into 2- to 3-mm pieces. This slurry was then stirred in Perfusate II for 1 h at 37°C. After three washes, the cells were resuspended in culture media and counted. The NPC did not contain hepatocytes as detected by light microscopy.

Cell culture and treatment

NPC (2×10^6) were plated onto 24-well plates. The initial culture medium was Williams medium E containing 10% calf serum, 15 mM HEPES, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin. NPC were

allowed to attach to plates overnight. The cells were stimulated with LPS (0.01 μ g/ml) and the medium was collected after 6 h.

Isolation of nuclear and cytoplasmic proteins

Frozen liver tissues were suspended in buffer containing 10 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% Triton X-100 and lysed by homogenization. Nuclei were recovered by microcentrifugation at 7500 rpm for 5 min. The supernatant containing cytoplasmic and membrane protein was collected and stored at $-80^\circ C$ for Western blot analysis. Nuclear proteins were extracted at 4°C by gently resuspending the nuclei pellet in buffer containing 20 mM Tris (pH 7.5), 20% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, and 0.1% Triton X-100, followed by a 1-h incubation at 4°C with occasional vortexing. After microcentrifugation at 13,000 rpm for 15 min at 4°C, the supernatant containing nuclear protein was collected. Protein concentration was quantitated with bicinchoninic acid protein assay reagent (Pierce).

Liver damage assessment

To assess hepatic function and cellular injury following liver ischemia, serum alanine aminotransferase (ALT) levels were measured using the Opera Clinical Chemistry System (Bayer).

Histopathology

Formalin-fixed liver samples were embedded in paraffin and cut to 6- μ m thick sections. Tissues were stained with H&E, and slides were assessed for inflammation and tissue damage.

Immunohistochemical staining

A segment of the left lobe of the liver was cryoprotected in 2% paraformaldehyde in PBS overnight and then 30% sucrose in PBS for another 24 h. The segment was then embedded and frozen in OCT compound using liquid nitrogen cooled isopentane. These samples were stored at $-80^\circ C$ until ready to be cut. To prepare the slides, the samples were cut into 6- μ m sections using a cryostat machine and placed onto slides. They were left to dry at room temperature overnight and then stored at $-20^\circ C$. Immunofluorescent staining for F4/80 was begun by first rehydrating the slides with PBS. Then, nonspecific proteins were blocked using 2% BSA for 45 min followed by rinses with 0.5% BSA. This process was followed by placing the primary Ab (F4/80; BD Biosciences) 1/100 in 0.5% BSA for 60 min. Again the slides were washed with 0.5% BSA and then the secondary Ab (goat anti-rabbit CY3; Jackson ImmunoResearch Laboratories) 1/1000 in 0.5% BSA was applied for another 60 min. A nuclear stain, Hoechst dye (bisbenzimidazole, 1 mg/100 ml), was applied for 30 s. The slides were rinsed with PBS and a coverslip with gelvatol, a water-soluble mounting medium (21 g of polyvinylalcohol, 52 ml of water, sodium azide, and 106 ml of 0.2 M Tris buffer) was placed. Slides were visualized using an Olympus BX51 epifluorescence microscope and digitized with an Olympus color video camera. In a blind manner, F4/80⁺ staining were counted in 10 high power fields ($\times 400$) per each section.

SDS-PAGE and Western blot analysis

Western blot analysis for phosphorylated and total kinase forms of p38 and JNK were performed as described (21). Primary polyclonal Ab to phosphorylated p38 and JNK (1/1000; Cell Signaling Technology) and primary polyclonal Ab to total p38 and JNK (1/1000; Santa Cruz Biotechnology) were used for Western blotting. Membranes were developed with the SuperSignal West Pico chemiluminescent kit (Pierce) and exposed to film.

Cytokine measurement

Serum concentrations of TNF, IL-6, and MCP-1 were determined using ELISA kits obtained from BioSource International.

SYBR Green real-time RT-PCR

Total RNA was extracted from the liver using the TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instruction. mRNA for TNF- α , IL-6, and inducible NO synthase and GAPDH was quantified in duplicate by SYBR Green two-step, real-time RT-PCR. After removal of potentially contaminating DNA with DNase I (Invitrogen Life Technologies), 1 μ g of total RNA from each sample was used for reverse transcription with an oligo(dT) (Invitrogen Life Technologies) and a Superscript II (Invitrogen Life Technologies) to generate first-strand cDNA. PCR mixture was prepared using SYBR Green PCR Master mix (PE Applied Biosystems) using the primers as previously described (22, 23). Thermal cycling conditions were 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE

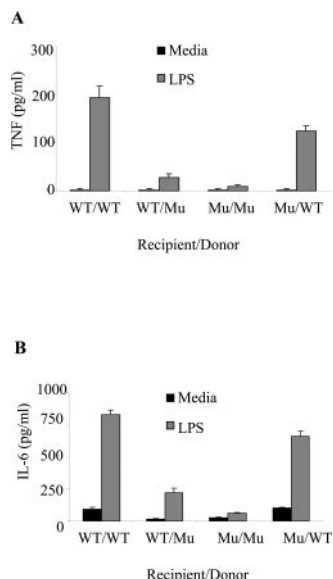


FIGURE 1. NPC from chimeric mice respond to LPS stimulation if their hemopoietic cells express functional TLR4. NPC were harvested from TLR4 chimeric mice produced by adoptive transfer of donor bone marrow cells into irradiated recipient animals using the following combinations from TLR4 WT (C3H/HeOuj) and TLR4 mutant (C3H/HeJ) mice: WT/WT, WT/Mutant (WT/Mu), Mutant/Mutant (Mu/Mu), Mutant/WT (Mu/WT). TNF (A) or IL-6 (B) levels in NPC culture supernatant were determined by ELISA. Cells were treated with 10 ng/ml LPS for 6 h. Data are representative of three independent experiments with similar results. Data shown are combined results from triplicate wells in a single experiment.

Applied Biosystems). Each gene expression was normalized with GAPDH mRNA content.

EMSA

NF- κ B DNA binding activity was measured by EMSA using nuclear extracts from liver tissues. The NF- κ B oligonucleotide (Promega) was based on the NF- κ B sequence in the Ig L chain enhancer. DNA probes were prepared by end labeling with [γ - 32 P]dATP (DuPont Merck Pharmaceuticals) and T4 polynucleotide kinase (Boehringer Mannheim) and purified in Tris-EDTA buffer containing NaCl (100 mM) using G-50 resin columns (Whatman). Typically, 5 μ l (5–10 μ g) of hepatic nuclear extract were incubated with 100,000 counts/min of 32 P-labeled oligonucleotides (0.5 ng) for 1–2 h at room temperature in a buffer containing 10 mM Tris (pH 7.6), 10% glycerol, 1 mM EDTA, 1 mg/ml BSA, and 0.2% Nonidet P-40. Additionally, 2–4 μ g of poly(dI-dC) (Boehringer Mannheim) were added to the mixture as nonspecific competitor DNA. Protein-DNA complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.4 \times running buffer containing 450 mM Tris borate and 1 μ M EDTA (pH 8.0). Gels were dried after electrophoresis and subjected to autoradiography.

Statistical analysis

Results are expressed as the mean \pm SEM. Group comparisons were performed using Student's *t* test or ANOVA. Differences were considered significant at values of *p* < 0.05.

Results

NPC from chimeric mice respond to LPS stimulation if their hemopoietic cells express functional TLR4

TLR4 chimeric mice were produced by adoptive transfer of donor bone marrow cells into irradiated recipient animals using combinations of TLR4 WT (C3H/HeOuj) and TLR4 mutant (C3H/HeJ) mice. To confirm successful engraftment of donor cells in chimeric animals, liver NPC from these mice were harvested and stimulated with LPS (10 ng/ml). As expected, NPC from WT/WT (recipient/donor) and Mutant/WT chimeric mice (animals given TLR4 WT bone marrow cells) responded to LPS stimulation with production

of TNF (Fig. 1A) and IL-6 (Fig. 1B) production. In contrast, NPC from Mutant/Mutant and WT/Mutant chimeric mice (animals given TLR4 mutant bone marrow cells) had significantly diminished responses to LPS. From previous coculture studies, the cytokines produced in our experiments derived mainly from Kupffer cells and we have shown very similar responses when purified Kupffer cells have been used instead of NPC (24). To rule out the possibility that some of the chimeric animals reconstituted fewer Kupffer cells as an explanation for the differential responses, we also performed macrophage staining with F4/80 on liver sections. WT/WT chimeric mice had 4.3 ± 0.64 positive cells per high power field ($\times 400$) compared with 4.7 ± 1.15 positive cells for WT/Mutant chimeric mice (*n* = 6 per group).

Functional TLR4 on NPC, not hepatocytes, is required for liver I/R injury

To determine the cell type in the liver important in mediating TLR4-mediated inflammation and injury after I/R, TLR4 chimeric mice were subjected to 60 min of liver ischemia and 3 h of reperfusion. In agreement with previous reports (9, 10), Mutant/Mutant chimeric mice were protected from liver I/R compared with WT/WT mice as measured by serum ALT levels (Fig. 2A). Interestingly, TLR4 WT mice that were adoptively transferred with TLR4 mutant bone marrow cells (WT/Mutant) were also protected from hepatic I/R. In contrast, serum ALT levels in TLR4 mutant mice transferred with TLR4 WT bone marrow cells (Mutant/WT) remained comparable to those of WT/WT controls. Similar results were seen using chimeric mice subjected to 60 min of liver ischemia and 6 h of reperfusion (Fig. 2B). Liver histologic examination was consistent with the serum ALT estimation of liver damage (Fig. 3). Severe sinusoidal congestion and hepatocellular necrosis were present in liver tissue from WT/WT and Mutant/WT chimeric mice, whereas minimal damage was noted in samples derived from Mutant/Mutant and WT/Mutant mice. Thus, intact TLR4 expression on bone marrow derived NPC is necessary to fully achieve the extent of liver I/R injury seen in WT mice.

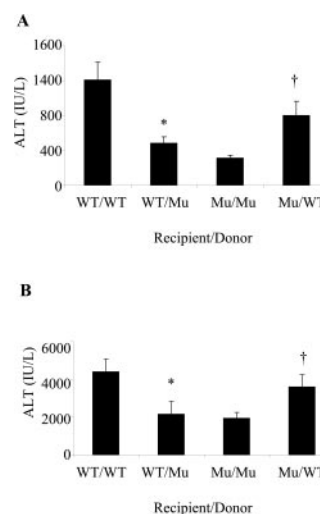
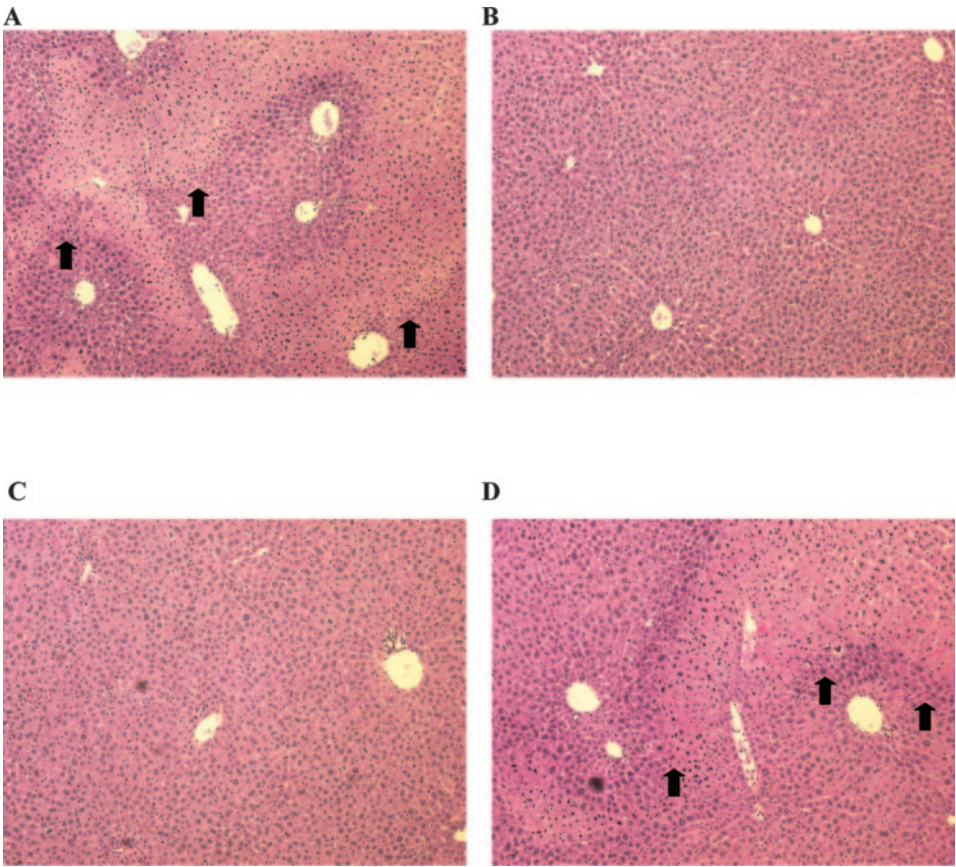


FIGURE 2. Functional TLR4 on NPC, not hepatocytes, is required for hepatocellular damage after liver I/R. TLR4 chimeric mice underwent ischemia and 3 (A) to 6 (B) h of reperfusion. Serum ALT levels were analyzed as a measure of hepatocellular injury. Data represent mean \pm SE (*n* = 6 mice per group). *, *p* < 0.05 vs WT/WT chimeric mice; †, *p* < 0.05 vs Mutant/Mutant chimeric mice.

FIGURE 3. Histopathology confirms that functional TLR4 on NPC is required for injury after liver I/R. H&E stained liver sections from TLR4 chimeric mice 6 h after reperfusion (original magnification, $\times 100$). The recipient/donor combinations used for the chimeric mice are as follow: WT/WT (A), WT/Mutant (B), Mutant/Mutant (C), and Mutant/WT (D). Images are representative liver sections from six mice per group. Arrows indicate areas of hepatocellular necrosis.



JNK and NF- κ B activation involved in TLR4-mediated liver I/R injury

Among the most proximal events in I/R injury is the activation of MAPK. A role for JNK activation in liver I/R injury has been demonstrated (25, 26). To determine the differences in MAPK activation in the TLR4 chimeric mice, we assessed phosphorylation of JNK and p38. Following I/R, phosphorylation of JNK was increased in liver tissue in WT/WT and Mutant/WT mice (Fig. 4A) but less so in Mutant/Mutant and WT/Mutant mice. There was no difference in p38 phosphorylation among the chimeric mice.

NF- κ B is a transcription factor also involved in signal transduction of a variety of extracellular stress stimuli. It is activated in the setting of hepatic I/R (27) and regulates both proinflammatory and protective responses in the liver (28–30). Using EMSA, we found increases in NF- κ B DNA binding in the ischemic liver 3 h after reperfusion in WT/WT and Mutant/WT mice when compared with sham-treated animals (Fig. 4B). Mice with mutant TLR4 marrow had less activation of NF- κ B. The NF- κ B bands were specific as confirmed by cold competition in the presence of excess unlabeled NF- κ B consensus motif. We previously performed supershift studies to determine that the NF- κ B complex was a heterodimer composed of both p65 and p50 subunits (30).

Expression of functional TLR4 on NPC is important in the production of inflammatory markers

Proinflammatory cytokines, including TNF and IL-6, and expression of the adhesion molecule (ICAM-1) play key roles and can be readily assessed as markers in the pathophysiology of hepatic I/R injury (31, 32). Using real-time RT-PCR, we measured steady-state mRNA levels for each of these markers in the liver following I/R (Fig. 5). Chimeric mice lacking functional TLR4 (Mutant/Mutant) expressed lower levels of TNF, IL-6, and ICAM-1 hepatic

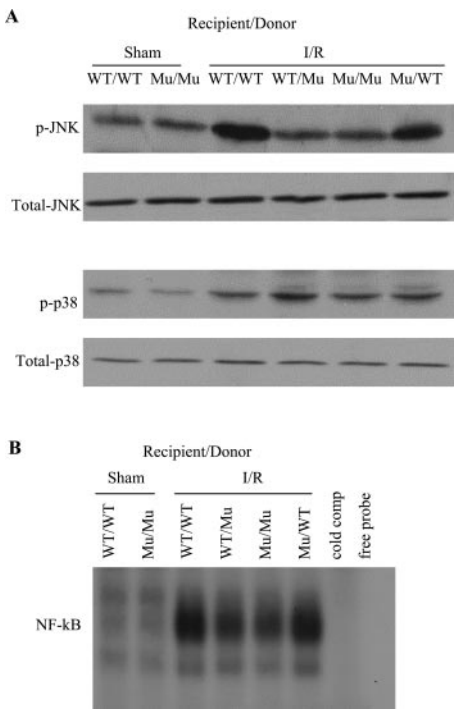


FIGURE 4. JNK and NF- κ B activation involved in TLR4-mediated liver I/R injury. A, MAPK activation was determined in TLR4 chimeric mice undergoing ischemia and 3 h of reperfusion. Western blot analysis for phosphorylated and total JNK and p38 was performed. Hepatic protein lysates from ischemic lobes were obtained. Blot shown is representative of three experiments with similar results. B, NF- κ B activation during hepatic I/R injury was assessed. TLR4 chimeric mice underwent ischemia and 3 h of reperfusion. Nuclear extracts were prepared from the ischemic livers and subjected to EMSA. Assay shown is representative of three experiments with similar results.

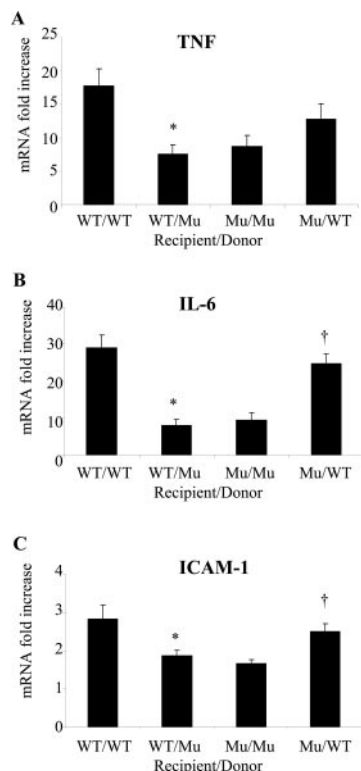


FIGURE 5. Functional TLR4 on NPC are important in the production of hepatic inflammatory mediators. Hepatic TNF- α (A), IL-6 (B), and ICAM-1 (C) mRNA expression were measured following ischemia and 3 h of reperfusion in TLR4 chimeric mice. Results were obtained using real time RT-PCR and expressed as relative increase of mRNA expression compared with sham animals. Data represent mean \pm SE ($n = 6$ mice per group). *, $p < 0.05$ vs WT/WT chimeric mice; †, $p < 0.05$ vs Mutant/Mutant chimeric mice.

mRNA compared with mice with functional TLR4 (WT/WT) after I/R. WT animals adoptively transferred with TLR4 mutant bone marrow cells (WT/Mutant) expressed lower hepatic levels of these inflammatory mediators when compared with WT/WT chimeric mice. In contrast, TLR4 mutant mice given WT bone marrow cells (Mutant/WT) expressed higher levels of IL-6 and ICAM-1 mRNA when compared with Mutant/Mutant mice. Because we observed decreases in intrahepatic production of inflammatory mediators among the TLR4 chimeric mice, we next examined systemic levels of TNF, IL-6, and MCP-1 in these mice (Fig. 6). Chimeric mice adoptively transferred with TLR4 WT bone marrow cells (WT/WT and Mutant/WT) had higher serum levels of these mediators when compared with mice receiving TLR4 mutant cells (WT/Mutant and Mutant/Mutant). These results indicate that in our chimeric mice, the bone marrow-derived NPC with functional TLR4 are important in the production of inflammatory mediators observed following liver I/R injury.

Functional TLR4 on phagocytic cells enhances liver I/R injury

Our findings suggest that liver NPC play a major role in TLR4-mediated inflammation and injury after I/R. We next sought to identify the cell types responsible for recognizing DAMP-TLR4 ligands as a result of I/R. We initially focused on phagocytic cells including Kupffer cells, which are the resident macrophages in the liver, as they produce multiple proinflammatory cytokines in response to liver I/R. We used gadolinium chloride to deplete liver Kupffer cells and other avidly phagocytic cells (i.e., dendritic cells) in both TLR4 WT and mutant animals immediately before induc-

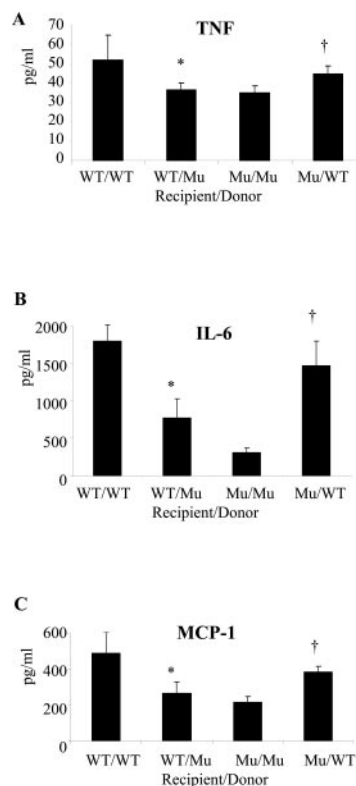


FIGURE 6. Circulating inflammatory mediator production dependent on functional TLR4 on NPC. Serum TNF- α (A), IL-6 (B), and MCP-1 (C) levels were measured following ischemia and 6 h of reperfusion in TLR4 chimeric mice. Data represent mean \pm SE ($n = 6$ mice per group). *, $p < 0.05$ vs WT/WT chimeric mice; †, $p < 0.05$ vs Mutant/Mutant chimeric mice.

ing transient hepatic I/R. In agreement with previous findings demonstrating the role of Kupffer cell activity in hepatic I/R injury (33), gadolinium chloride-treated WT mice were protected from 60 min of ischemia and 6 h of reperfusion (Fig. 7). Protection was not observed in TLR4 mutant mice undergoing hepatic I/R by gadolinium chloride pretreatment. Liver histologic findings were consistent with the serum ALT estimation of liver damage (Fig. 8). Severe sinusoidal congestion and hepatocellular necrosis was present in liver tissue from TLR4 WT mice treated with normal saline, whereas minimal damage was noted in samples from WT mice treated with gadolinium chloride. There was also little evidence of hepatocellular injury in TLR4 mutant mice after I/R after treatment with either saline or gadolinium chloride after I/R. Depletion of Kupffer cells using gadolinium chloride was confirmed

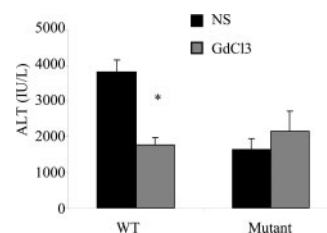
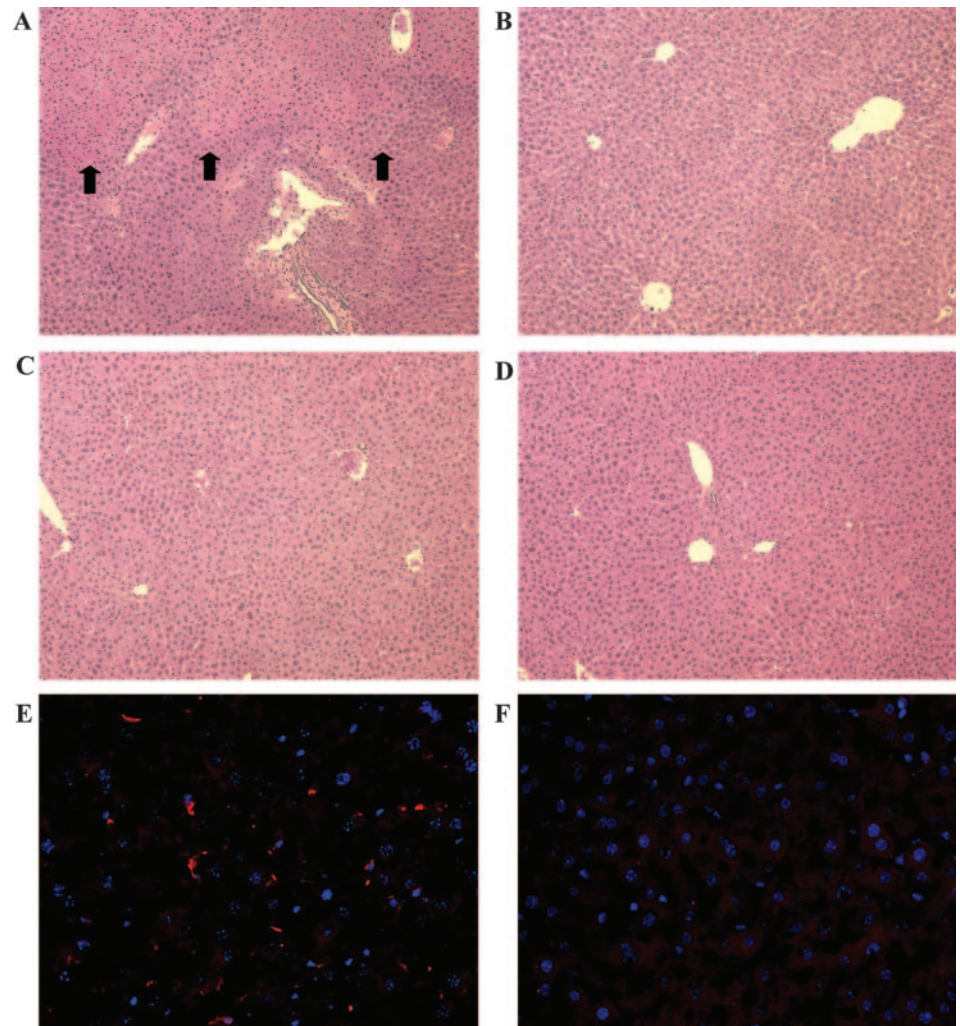


FIGURE 7. Functional TLR4 on phagocytic cells are important for liver I/R injury. TLR4 WT (C3H/HeOuj) and TLR4 mutant (C3H/HeJ) mice underwent ischemia and 6 h of reperfusion after Kupffer cell depletion with gadolinium chloride 24 h before ischemia. Serum ALT levels were analyzed as a measure of hepatocellular injury. Data represent mean \pm SE ($n = 6$ mice per group). *, $p < 0.05$ vs WT mice.

FIGURE 8. Histopathology and immunohistochemistry of livers after I/R in phagocytic cell-depleted TLR4 mice. TLR4 WT (C3H/HeOuj) and TLR4 mutant (C3H/HeJ) mice underwent ischemia and 6 h of reperfusion after Kupffer cell depletion with gadolinium chloride 24 h before ischemia. H&E stained liver sections from TLR4 WT mice (A), TLR4 WT mice treated with gadolinium chloride (B), TLR4 mutant mice (C), and TLR4 mutant mice treated with gadolinium chloride (D) (original magnification, $\times 100$). To confirm the depletion of Kupffer cells using gadolinium chloride, liver sections were stained for F4/80 Ag, a glycoprotein expressed by murine macrophages. E, Control saline treatment. F, Gadolinium chloride treatment. Original magnification was $\times 400$. F4/80 (red) and nuclei (blue) are as shown (E and F). Images are representative liver sections from six mice per group.



by staining liver sections for F4/80 Ag, a glycoprotein expressed by murine macrophages (Fig. 8).

Discussion

TLR signaling is one of the major mechanisms by which the innate immune system senses the invasion of pathogenic microorganisms by recognizing pathogen associated molecular pattern molecules that are present in microbial products (11). Increasing evidence suggests that TLRs may also be involved in the activation of the innate immune system in noninfectious settings by recognizing DAMPs (9, 10, 34, 35). Although TLR4 plays a critical role in the pathogenesis of liver I/R injury (9, 10), the initiating events by which damaged liver cells are capable of activating TLR4 remains to be elucidated. Importantly, the cell type responsible for recognizing the endogenous ligands released from damaged liver cells is unknown. The purpose of this study was to determine the cell type in the liver responsible for TLR4-dependent inflammation following ischemia and reperfusion. We found that: 1) TLR4-mediated liver I/R injury requires functional TLR4 on liver NPC, not hepatocytes; 2) chimeric mice adoptively transferred with TLR4 mutant bone marrow cells are protected from hepatic I/R injury; 3) the protection is associated with a decrease in JNK and NF- κ B activation and a decrease in local hepatic and circulating proinflammatory cytokine expression; and finally 4) evidence for Kupffer cells as a key cell type involved in TLR4-mediated damage in hepatic I/R.

The liver is a complex organ with important functions in immune surveillance and clearance of bacteria and their products. Liver cells express pattern recognition molecules, including members of the TLR system, which recognize molecular patterns of invading pathogens. TLR4, present on both hepatocytes and NPC, is involved in the response to LPS. Recent evidence suggests that the innate immune system may use the TLR system for the recognition of endogenous danger signals in addition to microbial products. In particular, TLR4, in addition to playing a critical role in LPS signaling, has also been shown to participate in the recognition of several endogenous ligands such as hyaluronic acid, heparin sulfate, fibrinogen, HMGB1, and perhaps heat shock proteins (9, 12–14, 16). We have recently reported that administration of a neutralizing Ab to HMGB1 provides a level of protection from injury and inflammation in hepatic warm I/R similar to that observed in TLR4 mutant mice (9). Furthermore, exogenous HMGB1 increased hepatic I/R-induced injury only in TLR4 WT mice. HMGB1 was up-regulated in hepatocytes in the warm I/R model and in hepatocytes made ischemic in vitro, suggesting that stressed or damaged hepatocytes provide the danger signal in hepatic I/R to the neighboring immune cells in the liver. HMGB1 has also been shown to act as a late mediator in rodent sepsis models (36, 37). In this study the source of HMGB1 is activated macrophage; however, the activation and release of HMGB1 by macrophages requires several hours. Our current study does not identify

the sources or further characterize the signals that activate the TLR4-mediated signaling.

It is not surprising that Kupffer cells may play such a role in TLR4-mediated inflammation. Kupffer cells represent the largest fixed population of macrophages in the body and ~40–65% of liver NPC (24). Upon activation, Kupffer cells can secrete potent inflammatory mediators such as cytokines, reactive oxygen species, prostanooids, and NO (38). Because the distinct types of liver cells are arrayed in close proximity to each other allowing for cell-cell interactions, the Kupffer cells are intimately involved in liver response to stress. We hypothesize that during liver I/R, endogenous ligands released from injured or necrotic hepatocytes are recognized by Kupffer cells through TLR4, thus initiating the TLR4 signaling cascade resulting in inflammation and organ damage. In our TLR4 chimeric mice, we waited 8–10 wk after irradiation and bone marrow transfer before performing liver I/R to ensure that the majority of Kupffer cells in the liver would be from the donor. Previous studies have shown that Kupffer cells repopulate the liver 14 days after their elimination by the administration of liposome-entrapped clodronate in mice (39). In addition, Kupffer cells of donor origin repopulate the recipient livers within 14–21 days after mouse bone marrow transplantation (40). Thus, the Kupffer cells at the time of liver ischemia were mainly of the donor type. However, our results do not exclude the possibility that other TLR4 expressing immune cells are activated during I/R. Other NPC, such as dendritic cells, may also play an important role in TLR4-mediated liver injury. Gadolinium chloride pretreatment is toxic to phagocytic cells in the liver, thereby eradicating Kupffer cells and dendritic cells. Indeed, much of the emphasis on DAMP-induced activation of innate immunity via TLR4 has centered on dendritic cells. DAMP not only leads to dendritic cell maturation but also stimulates the immediate production of inflammatory mediators (41–43). Gadolinium chloride would likely be toxic to these cells given their extremely active phagocytosis and pinocytosis.

To elucidate the molecular basis of protection in our chimeric mice adoptively transferred with bone marrow cells lacking functional TLR4, we investigated the MAPK and NF- κ B signaling pathways. The MAPK family represents a group of proteins involved in signal transduction of a variety of cellular stimuli. The JNK subgroup of MAPKs, also known as stress-activated protein kinase, is activated in response to environmental stresses (44). JNK activation has been found in the liver after I/R (9, 25, 26, 45, 46). NF- κ B is a transcription factor also involved in signal transduction of a variety of extracellular stress stimuli. Phosphorylation and proteolytic degradation of I κ B allows the release and nuclear translocation of NF- κ B, followed by transcription of many inflammatory genes. Further, inhibition of NF- κ B activation has been shown to ameliorate liver I/R injury (30). The reduction in JNK and NF- κ B activation in chimeric mice lacking functional TLR4 on bone marrow-derived cells suggests that the activation of TLR4 on these cells in part accounts for the proinflammatory downstream signals in the liver following I/R.

In summary, this study documents that functional TLR4 on NPC is critical to the pathogenesis of hepatic I/R injury. Our results also suggest that the Kupffer cell may be one of the NPC types mediating inflammation and organ damage after I/R. Kupffer cells may be one of the initial responders to the release of endogenous ligands from damaged or necrotic cells through activation of the TLR4 system. Interventions that inhibit TLR4 activity on Kupffer cells may be effective in settings of ischemic liver injury to minimize organ damage and may be useful in other clinical settings associated with inflammation and cellular necrosis.

Disclosures

The authors have no financial conflict of interest.

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