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CUTTING EDGE

Cutting Edge: TLR4 Activation Mediates Liver Ischemia/Reperfusion Inflammatory Response via IFN Regulatory Factor 3-Dependent MyD88-Independent Pathway[1]

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***The triggering molecular mechanism of ischemia-reperfusion injury (IRI), which in clinical settings results in excessive and detrimental inflammatory responses, remains unclear. This study analyzes the role of the TLR system in an established murine model of liver warm ischemia followed by reperfusion. By contrasting in parallel TLR knockout mice with their wild-type counterparts, we found that TLR4, but not TLR2, was specifically required in initiating the IRI cascade, as manifested by liver function (serum alanine aminotransferase levels), pathology, and local induction of proinflammatory cytokines/chemokines (TNF-α, IL-6, IFN-inducible protein 10). We then investigated the downstream signaling pathway of TLR4 activation. Our results show that IFN regulatory factor 3, but not MyD88, mediated IRI-induced TLR4 activation leading to liver inflammation and hepatocellular damage. This study documents the selective usage of TLR in a clinically relevant noninfectious disease model, and identifies a triggering molecular mechanism in the pathophysiology of liver IRI. The Journal of Immunology,* 2004, 173: 7115–7119.**

Ischemia-reperfusion injury (IRI)[3] is a common cause of organ dysfunction in clinical settings, such as those associated with low flow states, diverse surgical procedures, or during organ procurement for transplantation (1). In fact, IRI, an Ag-independent component of the harvesting insult, represents a serious problem affecting transplantation outcomes, because it causes early organ failure, and may lead to a higher incidence of both acute and chronic rejection.

Liver IRI activates Kupffer cells, and to a lesser degree endothelial cells (EC) and hepatocytes, leading to the formation of reactive oxygen species and secretion of proinflammatory cytokines/chemokines (2). The oxidant stress can directly damage EC and hepatocytes, whereas the soluble factors are responsible for the recruitment of neutrophils and T cells to elicit inflammatory response, the key step in the development of full-scaled IRI. Despite general consensus on the dominant role of innate immunity, the molecular details of IRI-induced innate immune cell activation remain to be elucidated. Although activation of LPS signaling has been implicated in hepatic IRI (3), specific ligand-receptor systems have not been elucidated.

By recognizing bacterial/viral-specific pathogen-associated molecular patterns, TLR represent the host sentinel system responsive to infections (3). Activation of TLRs triggers an inflammatory response that is mediated by macrophages, neutrophils, and complement. The induced chemokines/cytokines can mediate systemic responses and recruit leukocytes to sites of inflammation. In addition, APCs can be activated by TLR ligands, which may then initiate adaptive T cell responses. Relevant to the mechanism of IRI, endogenous ligands from damaged/stressed cells, including heat shock proteins (HSPs), heparan sulfate, hyaluronan, and fibronectin, have the capacity to activate TLRs (4). Indeed, endogenous TLR ligands representing the danger signal, may initiate an immune response in the absence of infection. The IRI may well fit with such a model system for testing the role of TLRs in noninfectious settings. The ATP depletion and release of oxygen-free radicals in the ischemic liver lobe may cause the initial wave of cell death, independent of inflammatory responses. Molecules released from these dead cells, including endogenous TLR ligands, may provide the initiating signals for the innate immune system, leading to the development of inflammation. This study dissects the specific function of the TLR system in a noninfectious disease model, and elucidates TLR-initiating molecular mechanisms in the pathophysiology of liver IRI.

Materials and Methods

Animals

Male wild-type (WT; C57BL/10SnJ, C57BL/6) and TLR4 knockout (KO) (C57BL/10ScCr) mice (8–12 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR2, IFN regulatory factor 3 (IRF-3), and

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[3] Abbreviations used in this paper: IRI, ischemia-reperfusion injury; EC, endothelial cell; HSP, heat shock protein; KO, knockout; IRF-3, IFN regulatory factor 3; sALT, serum alanine aminotransferase; WT, wild type; IP-10, IFN-inducible protein 10.

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MyD88 KOs were obtained from Dr. S. Akira (Osaka University, Osaka, Japan), and bred into C57BL/c background at University of California, Los Angeles. All mice were housed under pathogen-free conditions, and received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences and published by the National Institute of Health.

Mouse warm hepatic IRI model

We have used a warm hepatic IRI model in mice, as developed by Zwacka et al. (5) and modified by us (6). Briefly, mice were injected with heparin (100 μg/kg), and an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the cephalad lobes of the liver. After 90 min of warm ischemia, the clip was removed, initiating hepatic reperfusion. Mice were sacrificed after 6 h to analyze the acute phase of liver IRI. Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured (ANTECH Diagnostics, Los Angeles, CA). Liver sections (4 μm) were stained with H&E, and then analyzed blindly. Sham WT controls underwent the same procedure, but without vascular occlusion.

RT-PCR

Five micrograms of RNA was reverse-transcribed into cDNA using oligo(dT) primers with Omniscript reverse transcriptase (Qiagen, Valencia, CA). Quantitative PCR was performed, as described (7). In a final reaction volume of 25 μl, the following were added: *Taq* polymerase, 1× *Taq* buffer (Stratagene, La Jolla, CA), 125 μM dNTP, SYBR Green I (Molecular Probes, Eugene, OR), and fluoroscein (Bio-Rad, Hercules, CA), together with cDNA and primers. Amplification conditions were as follows: 95°C (3 min), 40 cycles of 95°C (20 s), 55°C (30 s), 72°C (20 s). The primers used to amplify a specific 100- to 200-bp fragment of IFN-inducible protein 10 (IP-10), TNF-α, IL-6, and L32 have been listed (7).

Statistical analysis

All values are expressed as mean ± SD. Comparisons between the groups were done using repeated-measure ANOVA. If differences were established, we used an unpaired two-tailed Student's *t* test, with *p* values of <0.05 considered statistically significant.

Results

TLR4, but not TLR2, is required for liver IRI

To address the question of whether and which TLR system mediates the innate immune activation in liver IRI, we contrasted in parallel livers from groups of TLR4 KO and TLR2 KO mice with their WT counterparts in our 90-min warm ischemia model. The IRI was assessed at 6 h postreperfusion, when the hepatocellular injury has been shown to be T cell independent (5), by measuring sALT levels, and liver histology (6). Interestingly, livers in TLR4, but not TLR2, KO mice were protected from IRI. Thus, as shown in Fig. 1, sALT levels (international units/liter ± SD) in TLR4 KO mice were consistently markedly lower, as compared with WT controls (276 ± 50 and 2149 ± 485, respectively; $p < 0.001$), data confirmed by sALT levels in TLR4 mutants (not shown). In contrast, sALT levels in TLR2 KO mice remained comparable with those of WT controls (1979 ± 1122 and 2992 ± 833, respectively).

These functional data correlated with histological liver evaluation. As shown in Fig. 2, ischemic livers in WT mice suffered severe damage, as evidenced by coagulative necrosis involving multiple hepatic lobules (*a* and *b*). A decrease in cytoplasm staining, marked red cell congestion, and scattered apoptotic-appearing cells in sinusoids characterized the necrosis, which was panlobular and perivenular. There was no inflammatory infiltrate, and adjacent areas were normal, demonstrating mild ballooning change or mild steatosis. TLR2 KO and WT livers suffered comparable IRI (Fig. 2, *c* and *d*). In contrast, TLR4 KO mice showed near-normal liver parenchyma without zonal or panlobular necrosis, and no T and PMN infiltrate (Fig. 2, *e* and *f*). Thus, the intact TLR4, but not TLR2, system is required in the mechanism of liver IRI.

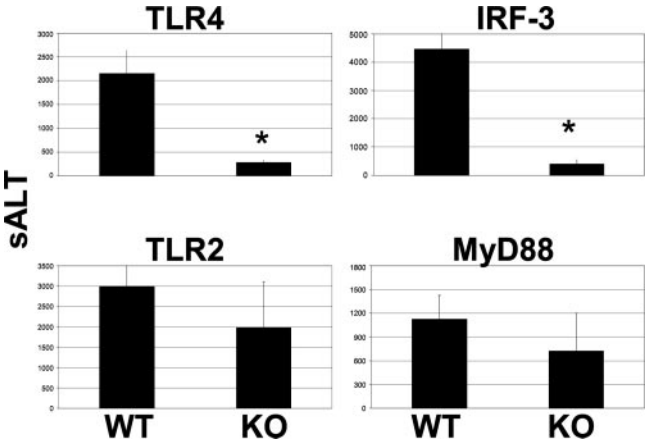

FIGURE 1. sALT (international units/liter) levels after hepatic warm ischemia/reperfusion. The sALT levels, an indicator of hepatocellular injury, were measured in parallel in blood samples from WT, and TLR4, TLR2, MyD88, and IRF3 KO mice that were subjected to 90 min of ischemia followed by 6 h of reperfusion. Mean ± SD are shown ($n = 4$–5/group). *, $p < 0.001$. Each experiment was performed at least twice at different occasions.

IRI-induced TLR4 activation is mediated by IRF3 but not MyD88

There are two major pathways downstream of TLR4 activation (8). The MyD88-dependent signaling results in the direct induction of inflammatory IL-1β, TNF-α, and IL-6, whereas MyD88-independent pathway, relying on the activation of IRF-3, results in the induction of IFN-β and IFN-inducible genes (e.g., *IP-10*). To determine the role of these two pathways in TLR4 activation leading to liver IRI, we used IRF-3 KO and MyD88 KO mice. Liver function (sALT) and histology were contrasted in parallel with those in WT counterparts. Interestingly, livers in IRF-3 KO mice remained consistently protected from IRI (254 ± 70 vs 2616 ± 283 in WT controls; $p < 0.001$; Fig. 1), similarly to those in TLR4 KO mice. However, livers in MyD88 KO mice suffered significant IRI, although somewhat less severe than WT controls (722 ± 479 and 1122 ± 306, respectively; $p = 0.35$; Fig. 1). Histological examination (Fig. 2) revealed near-normal liver parenchyma in IRF-3 KO mice (*g* and *h*), but areas of panlobular necrosis and perivenular hepatocellular change in MyD88 KO recipients (*i* and *j*). These results indicate that IRI-induced TLR4 activation leading to hepatocellular damage relies primarily on IRF-3-dependent, MyD88-independent signaling.

TLR4-mediated chemokine/cytokine profile in liver IRI

To further investigate the molecular mechanism of liver IRI, we examined intrahepatic induction of inflammatory chemokines/cytokines triggered by TLR4 activation. One of the major outcomes of IRF3-dependent signaling in TLR4 activation is the induction of IFN-β and IFN-inducible genes. We had detected a small and transient up-regulation of IFN-β in livers undergoing IRI (data not shown). The induction of IP-10, as well as TNF-α and IL-6, the proinflammatory cytokines implicated in liver IRI, was also detected by RT-PCR. Compared with sham-operated controls, livers undergoing IRI had significantly higher expression of all three genes (Fig. 3*a*). Interestingly, livers resistant to IRI in TLR4 KO, but not those that were IRI susceptible in TLR2 KO mice, revealed diminished IP-10 and TNF-α levels (Fig. 3, *b* and *c*). IL-6 induction was abolished in

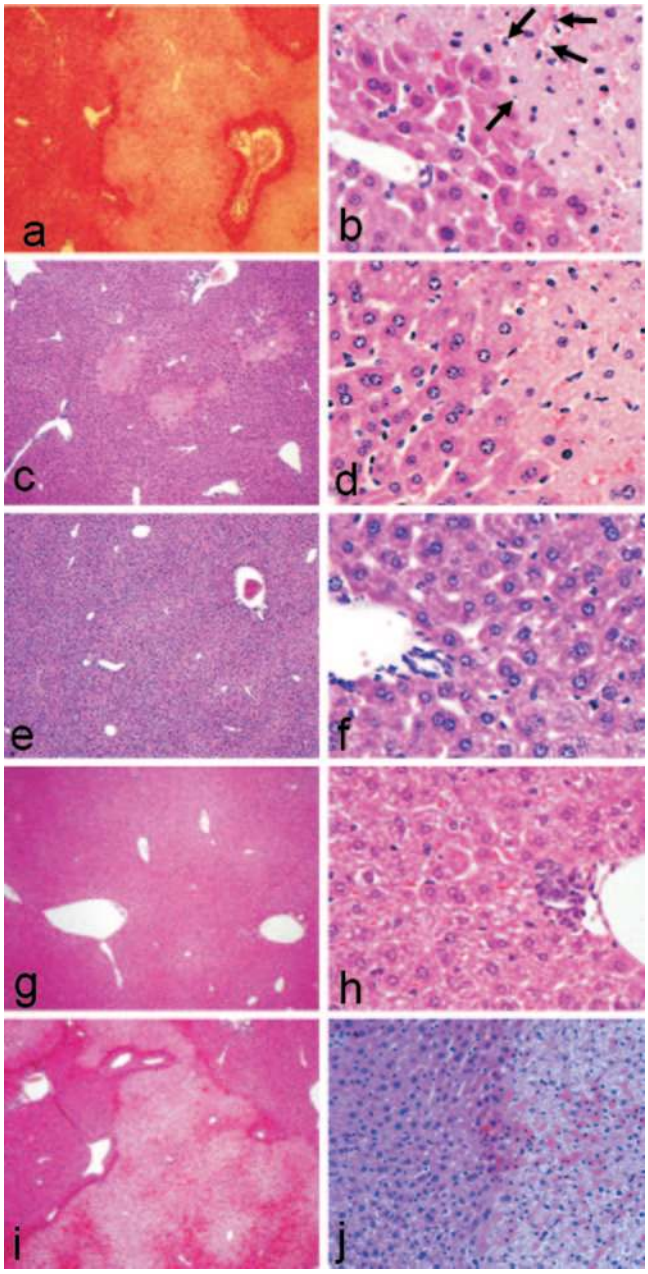

FIGURE 2. *a*, Liver IRI in WT mice with panlobular necrosis/zonal sparing (H&E, ×40). *b*, Discrete junction between necrosis and viable hepatic parenchyma; note congestion/apoptotic cells in sinusoids (arrows) of necrotic area (H&E, ×400). *c*—*j*, Low (×40) and high (×400) power micrographs of H&E-stained liver sections in IRI model: TLR2 KO with patch panlobular necrosis (*c* and *d*); TLR4 KO without histological abnormality (*e* and *f*); IRF3 KO without histological abnormality (*g* and *h*); MyD88 KO with extensive panlobular/zonal necrosis (*i* and *j*). Representative of three sections per group.

TLR4 and TLR2 KO groups. Furthermore, selective gene induction mediated by MyD88 or IRF-3-dependent signaling following IRI-induced TLR4 activation was detectable in MyD88 KO or IRF-3 KO mice. As shown in Fig. 3, *d* and *e*, IP-10 induction remained depressed in IRF-3 KO, but not in MyD88 KO mice. In contrast, IL-6 expression was diminished in MyD88 KO, but only partially decreased in IRF-3 KO livers. Interestingly, the TNF-α induction was markedly diminished in IRF-3, but not MyD88 KO livers, implying it was not the direct result of TLR4 activation, which otherwise would be an MyD88-dependent event.

Discussion

Our results document the critical role of TLR4 and IRF-3-mediated signaling in the pathogenesis of liver IRI. This is the first study in a clinically relevant noninfectious disease model that differentiates between the two distinct TLR systems, and dissects the relevant downstream signaling pathways. Unlike the majority of TLR activation studies by endogenous ligands that were performed in vitro in cell culture systems, our study provides in vivo evidence of TLR4 activation in the absence of infection. This study addresses a key question in the pathogenesis of liver IRI, i.e., which host innate immune system becomes activated, and by which pathway to trigger the inflammatory response leading to hepatocellular damage.

TLRs are expressed on various cell types, in particular APCs, such as macrophages and DCs. TLR2 and TLR4 have been detected in parenchymal hepatocytes and Kupffer cells (9–11). IL-1, IL-6, and TNF-α enhance liver TLR2, but not TLR4 expression (10). Functionally, Kupffer cells are capable of responding to LPS stimulation (12). However, whether TLR4 expression in hepatocytes is sufficient to respond to TLR ligands remains uncertain. Thus, livers are well equipped to respond to TLR ligands, with Kupffer cells rather than hepatocytes representing the initial responder of TLR4 stimulation. Although we propose that endogenous ligands most likely activate TLR4 at the early IRI stages, increased LPS levels have been recorded in liver transplant recipients (13, 14), possibly resulting from increased endotoxin translocation from the gut due to a decrease of intestinal/hepatic blood flow. However, LPS might not be the initial TLR4 stimulant in the liver, but rather the result of IRI, possibly contributing to the secondary sustained TLR stimulation. Because the severity of transplant IRI correlates with the duration of cold preservation/warm ischemia, which were under strict sterile conditions, it is unlikely that the longer preservation would lead to the higher LPS contamination. In our murine hepatic IRI model, the liver blood flow remains largely uninterrupted. Indeed, liver IRI can develop by ex vivo blood perfusion independent of intestinal/hepatic blood flow (15). Thus, it is doubtful that gut-derived LPS initiates IRI. Increased LPS levels in circulation resulting from intestinal structural damage are most likely secondary to the initial IRI.

Which TLR4 endogenous ligands may be responsible for liver IRI? Both HSP60 and HSP70 can activate TLR2 and TLR4 in DC cultures (16, 17), whereas uric acid is the principal endogenous DC-stimulating signal (18). Heparan sulfate is shed from cell surfaces and basement membranes as a result of tissue injury. Because soluble heparan sulfate uses TLR4 to activate DC, it may well represent the mechanism by which vertebrates monitor tissue well-being. Hyaluronan, which degrades during inflammation, or catabolizes by liver EC from the blood stream (19), could also activate TLR4 (20).

Although endogenous ligands capable of activating TLRs have been identified in vitro (20–23), the function of TLRs in vivo in clinically relevant noninfectious disease models remains to be elucidated. Platt and colleagues (24, 25) have documented the role of heparan sulfate in DC activation in the infection-free environment. TLR4 activation has also been implicated in the pathogenesis of intimal lesion causing arterial obstructive disease (26) and murine myocardial IRI (27), whereas TLR2 has been involved in modulating ventricular remodeling after myocardial infarction (28). Because multiple signaling pathways may become activated by any single TLR system, determining

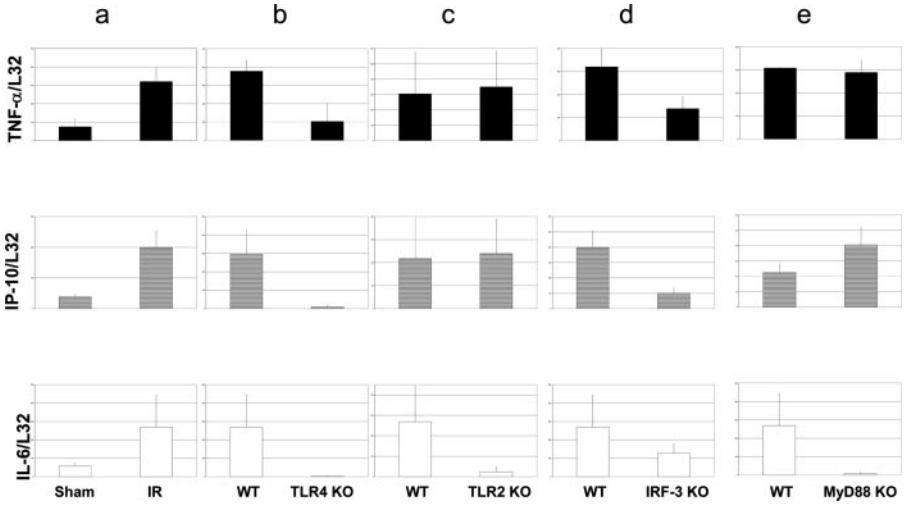

FIGURE 3. Cytokine/chemokine gene induction in livers undergoing IRI. Total RNA was extracted from ischemic (90 min) livers 6 h after reperfusion, and reverse-transcribed into cDNA. Real-time PCR was performed to measure transcript levels of TNF-α, IP-10, IL-6, and a housekeeping gene L32. The ratios of target genes/L32 were plotted with SD (average of two to four samples at the same conditions) in the following: sham-operated livers vs ischemia-reperfusion (IR)-injured WT livers (*a*); WT vs TLR4 KO livers (*b*); WT vs TLR2 KO livers (*c*); WT vs IRF-3 KO livers (*d*); and WT vs MyD88 KO livers (*e*). Means ± SD are shown (n = 4–5/group).

individual pathways downstream of TLR is vital to fully establish the role of TLRs in vivo. Our study identified specifically TLR4, and only one of TLR4 downstream signaling pathways in the development of liver IRI. These data fit well with the overall TLR transduction scheme, because MyD88 mediates both TLR4 and TLR2, whereas IRF-3 mediates selectively TLR4 activation. These results are discordant with those from transplant models, which involve both innate and adaptive immune responses. Indeed, Goldstein et al. (29) have shown the critical role of MyD88 in minor Ag-mismatched allograft rejection, although a mere TLR4 deficiency was insufficient to alter acute rejection (30), consistent with MyD88 mediating multiple TLR signaling. Thus, unlike Ag-mediated rejection, the alloantigen-independent IRI is based on the activation of TLR4 through an MyD88-independent, IRF-3 dependent mechanism.

Because IRF-3-mediated TLR4 activation results in IFN-β and IFN-inducible gene production, rather than direct induction of proinflammatory cytokines (which is mediated by MyD88), one needs to ask the question how liver inflammation is generated during IRI. As shown in this study, the induction of TNF-α, a key effector molecule in liver IRI was IRF-3 (but not MyD88) dependent. This implies that direct Kupffer cell activation via TLR4 was not responsible for TNF-α production, and multiple cell types might be involved in the pathogenesis of liver inflammation. One possible scenario of TNF-α induction is that IFN-inducible gene products, e.g., IP-10, recruit and activate intrahepatic lymphocytes to produce TNF-α. This may also provide a link between innate immune activation by liver IRI and adaptive immune responses in the absence of exogenous Ags. Indeed, IP-10 is a potent chemotactic factor for activated $CD4^+$ T cells expressing CXCR3, which by themselves can mount proinflammatory responses.

Thus, we have identified and dissected the innate immune mechanism, critical in triggering the inflammatory liver IRI response. TLR4 and one of its downstream signaling pathways mediated by IRF-3 are responsible for innate immune activation in this noninfectious, Ag-independent disease model. These results provide the rationale for designing therapeutic strategies to ameliorate IRI in the clinics.

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