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Proinflammatory Cytokine Production in Liver Regeneration Is Myd88-Dependent, but Independent of Cd14, Tlr2, and Tlr4

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TNF and IL-6 are considered to be important to the initiation or priming phase of liver regeneration. However, the signaling pathways that lead to the production of these cytokines after partial hepatectomy (PH) have not been identified. Enteric-derived LPS appears to be important to liver regeneration, possibly by stimulating proinflammatory cytokine production after surgery. To determine whether LPS signaling pathways are involved in the regulation of the proinflammatory cytokines TNF and IL-6 during the priming phase of liver regeneration, we performed PH on mice lacking the TLRs Tlr4 and Tlr2, the LPS coreceptor, Cd14, and Myd88, and a TNF protein involving in most TLR and IL-1R pathways. In Myd88 knockout (KO) mice after PH, both liver Tnf mRNA and circulating IL-6 levels were severely depressed compared with heterozygous or wild-type mice. Activation of STAT-3 and three STAT-3 responsive genes, Socs3, Cd14, and serum amyloid A2 were also blocked. In contrast, Tlr4, Tlr2, and Cd14 KO mice showed no deficits in the production of IL-6. Surprisingly, none of these KO mice showed any delay in hepatocyte replication. These data indicate that the LPS receptor TLR4, as well as TLR2 and CD14, do not play roles in regulating cytokine signaling pathways after PH. In contrast, Myd88-dependent pathways appear to be responsible for TNF, IL-6, and their downstream signaling pathways. The Journal of Immunology, 2006, 176: 2522–2528.

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iver regeneration following resection or injury requires a multitude of signaling and endocrine pathways that are carefully coordinated to reconstitute liver mass (reviewed in Refs. 1 and 2). The initiation of liver regeneration has been termed the priming phase, in which normally quiescent hepatocytes gain proliferative competence and become responsive to hepatic growth factors. Hepatocyte growth factor, heparin binding-epidermal growth factor-like growth factor, and TGF-α stimulate cell cycle progression, leading to DNA replication and cell proliferation (1–3). One hallmark of the priming phase is the induction of immediate early genes such as the proto-oncogenes c-myc and c-jun, and of proinflammatory cytokines (4, 5). Shortly after partial hepatectomy (PH), both serum levels of TNF and hepatic Tnf mRNA increase, leading to activation of TNFR type I and subsequent activation of the transcription factor NF-κB in nonparenchymal cells in the liver (1, 2, 6–8). Active NF-κB, in part, induces IL-6 production, which results in the activation of STAT-3. Active hepatic STAT-3 induces a number of genes involved in regulating the cell cycle, the acute phase response, and STAT-3 pathway itself, such as the suppressor of cytokine signaling 3 (Socs3) (9–11). Socs3 is part of a negative feedback loop that is rapidly induced after PH and blocks STAT-3 signaling (12, 13).

The mechanisms by which PH results in cytokine gene induction and transcription factor activation in the liver are unclear. A number of studies implicate enteric-derived LPS as the stimulating agent for proinflammatory cytokine production at the start of liver regeneration. Rats in which LPS has been depleted by antibiotic administration or other methods display impaired hepatocyte DNA synthesis following PH (14). Liver regeneration is also delayed in athymic nude mice and C3H/HeJ mice, a naturally occurring strain that is hyporesponsive to LPS (15, 16). Hepatocyte DNA synthesis is enhanced if these animals receive LPS 24 h before surgery (15, 17). In addition, Webber et al. (18) demonstrated that LPS primes hepatocytes for growth factor-induced replication in nonoperated rats.

LPS and other bacterial or viral by-products bind to a family of specific receptors, the TLRs, which regulate both innate and adaptive immunity (19–21). Studies using Tlr knockout (KO) mice and primary cell cultures from such mice have delineated the signaling pathways used by LPS (reviewed in Refs. 20–22). Circulating LPS forms complexes with LPS-binding protein and CD14, forming a ternary complex that binds to TLR4 on the surfaces of many different cell types, resulting in receptor dimerization and activation (23). Activation of TLR4 initiates both Myd88-dependent and independent signaling cascades (24, 25–26). Broadly, the TLR4 signaling pathways can be divided into those that activate NF-κB, and those that activate IFN regulatory factor (IRF). 3. LPS-induced activation of NF-κB is believed to be dependent on Myd88 interactions, while IRF3 activation is thought to be Myd88 independent (21, 26). Myd88 is also important in the intracellular signaling pathways of TLR2, a receptor that recognizes a variety of microbial by-products due to its ability to heterodimerize with TLR1 and TLR6 (21).

Although TLRs and their signaling pathways are critical for the first line of defense against bacterial and viral infection, and as
reviewed above, there is suggestive evidence that LPS may trigger cytokine production to initiate liver regeneration, there is no direct evidence linking LPS and the TLRs to the initiation of liver regeneration. Using KO mice for \(\text{Cdl}4\), \(\text{Tlr}2\), and \(\text{Tlr}4\), we show that the lack of these receptors does not influence liver regeneration. By contrast, there is blockage of cytokine pathways after PH in \text{Myd88} KO mice.

**Materials and Methods**

**KO mice**

\(\text{Tlr}2\), \(\text{Tlr}4\), and \text{Myd88} KO mice were obtained from Dr. T. Hawn (Department of Medicine, University of Washington, Seattle, WA) and originally generated by Dr. S. Akira (Osaka University, Osaka, Japan) (24, 27, 28). \text{Cdl}4 heterozygous mice (29) were purchased from The Jackson Laboratory. All mouse strains (C57BL/6) were initially housed in a specific pathogen-free facility with 12-h light/dark cycles with free access to standard food and water. After initial experiments were done, we found that all strains harbored \text{Helicobacter} species, which can induce low-level activation of the TLR pathway (30). We then generated \text{Helicobacter}-free mice by neonatal rederivation (31) using foster dams housed in a \text{Helicobacter}-free specific pathogen-free room. DNA extracted from fecal samples was analyzed for \text{Helicobacter hepaticus}, \text{Helicobacter bilis}, and \text{Helicobacter muridarum} as described (32) and confirmed by the Research Animal Diagnostic Laboratory (RADIL) at the University of Missouri (Columbia, MO). All animal studies were conducted under approved Institutional Animal Care and Use Committee protocols at the University of Washington.

**Surgeries**

PH resulting in removal of two-thirds of the liver was performed on 8- to 11-wk-old male KO, heterozygous (Het), and wild-type (WT) littermates. Surgeries were performed under inhalational isoflurane anesthesia in the morning (33) after a night of fasting, and consisted of midline laparotomy with separate ligation and removal of the left and anterior (median) lobes (7, 13, 34). Laparotomy with gentle liver manipulation was performed as a sham operation. At various times after surgery, mice were sacrificed by \(\text{CO}_2\) inhalation, and livers were harvested for analysis. BrdU (50 mg/kg) was given by i.p. injection 2 h before killing. In some cases, 3-day continuous osmotic pumps (Alzet; 1 \(\mu\)l/hour) containing BrdU (10 mg/ml) were implanted s.c. at the time of surgery and mice were killed 48 h later.

**Histology and BrdU labeling**

At the indicated time points, liver tissue was fixed in 10% neutral-buffered formalin or methacarn (60% methanol, 30% chloroform and 10% acetic acid; \(\text{v/v/v}\)) (Fisher Scientific) overnight. BrdU immunohistochemistry was performed as described (7). Data are presented as the number of nuclear BrdU-labeled hepatocytes present in 30–40× fields (1.3 mm\(^2\); \(~3000\) hepatocytes). Mitotic figures were also counted in 30–40× fields.

**Immunoblotting**

Whole liver homogenates were prepared using 1% Triton X-100 lysis buffer and quantified using Bradford reagent with BSA as a standard. Fifty micrograms of total protein was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes as described (13). After blocking with 5% milk, membranes were probed overnight with Abs that detect STAT-3 phosphorylated on tyrosine 705 (Cell Signaling; no. 9131). Protein-Ab complexes were detected using anti-rabbit HRP-conjugated secondary Ab (Amersham) and visualized with an ECL reagent (Pierce). To detect total STAT-3, membranes were first stripped with glycine stripping buffer and quantified using Bradford reagent with BSA as a standard. Fifty micrograms of RNA was reverse-transcribed, and the cDNA was then used as a template for a radioactive PCR. In all cases, PCR cycle analysis was first performed to determine the linear range of each amplon for a given primer pair. \(\text{Tnf}\) and \text{Socs3} expression levels were normalized to that of \(\beta\)-actin (primer to competitor ratio of 2:8) as described in the Quantum RNA kit (Ambion). \text{Cdl}4 and \text{Saa2} mRNAs were detected using murine cDNA probes (provided by Drs. M. Chaisson, Amgen, Seattle, WA, and O’Brien, University of Washington, Seattle, WA, respectively) using established Northern blotting procedures and normalized to cyclophilin (Ambion) (13). All radioactive amplions and detected bands in Northern blots were quantified using Phosphorimager analysis.

**Statistical analysis**

Statistical analysis was done by nonparametric analysis (Mann-Whitney or an unpaired \(t\) test with Welch’s correction). Data are presented as average ± SEM with the following symbol indicating the level of significance; *\( p<0.05\), **\( p<0.01\), ***\( p<0.001\). Statistical analysis was performed using GraphPad Prism software.

**Results**

Deficits in circulating levels of IL-6 and activation of STAT-3 signaling pathways in \text{Myd88} KO mice after PH

\(\text{Tlr}2\), \(\text{Tlr}4\), \text{Cd14} and \text{Myd88} KO mice and macrophages obtained from these animals fail to produce TNF and IL-6 after LPS treatment (24, 27–29). We thus used \text{Tlr}2, \text{Tlr}4, \text{Cd14}, and \text{Myd88} KO mice to determine whether the rapid increases in IL-6 and STAT-3 that occur after PH would be blocked in animals deficient in LPS signaling. Before performing experiments with KO mice, we ascertained that Het animals lacking a single copy of each of these genes showed no alterations in cytokine induction after PH when compared with WT littermates. For these reasons, both WT and Het mice were used as controls in subsequent experiments. As determined by ELISA, circulating levels of IL-6 determined at 4 h after PH were not affected in \text{Tlr}2, \text{Tlr}4, or \text{Cd14} mice when compared with WT/Het mice. In marked contrast, only a small amount of IL-6 was detectable in \text{Myd88} KO mice (Fig. 1A). An analysis of the time course of IL-6 production after PH revealed that IL-6 amounts transiently increased by 50-fold or more in WT/Het mice, whereas \text{Myd88} KO mice lacked this induction (Fig. 1B).

IL-6 production after PH causes the activation STAT-3. In turn, this transcripion factor induces a large number of genes, including those involved in hepatocyte growth and survival, and the acute
phase response (10, 11, 36). It also activates a feedback mechanism through SOCS-3 activity, which blocks further STAT-3 activation during liver regeneration (13). We used phosphoimmunoblotting of STAT-3 and EMSA to analyze whether the deficit in circulating IL-6 seen in Myd88 KO mice would result in a lack of activation of STAT-3 after PH (Fig. 2). Tyrosine 705 phosphorylated STAT-3 was 2- to 4-fold lower in the Myd88 KO mice compared with WT/Het mice at 2, 4, and 6 h after PH (Fig. 2, A and B). EMSA analysis confirmed deficient STAT-3 DNA binding in Myd88 KO mice after PH as well (Fig. 2, C and D).

We next determined whether levels of IL-6-STAT-3 target genes were affected by the decreased levels of circulating IL-6 and blunted STAT-3 activation in Myd88 KO mice. We performed RT-PCR on mRNA extracted from whole liver to evaluate Socs3 expression after PH in Myd88 KO mice. WT/Het mice showed a 7-fold induction of Socs3 mRNA at 4 h after PH, while Myd88 KO animals showed significantly less induction of Socs3 (Fig. 3A). Interestingly, at 2 h after PH, when there is only a 3-fold induction of Socs3 mRNA in WT/Het mice, there were no differences between WT/Het, Myd88 KO, and sham-operated mice, suggesting that there may be multiple pathways capable of inducing Socs3 in the liver. We next determined whether levels of Cd14 and Saa2 mRNA, two acute phase response genes, were also blunted in Myd88 KO mice, because the expression of Cd14 and Saa2 has previously been shown to be dependent on IL-6 (10, 37, 38). The levels of Cd14 and Saa2 mRNA were elevated 2- to 5-fold in WT/Het mice at 6 and 8 h after PH, but there was little to no induction of these genes in Myd88 KO mice (Fig. 3B, C and D). Taken together, these data indicate that there is a block in IL-6 production in Myd88 KO mice after PH with a subsequent deficit in IL-6-signaling pathways. Importantly, there were no deficits in circulating levels of IL-6 in Cdl4, Tlr2, and Tlr4 KO mice compared with WT/Het mice after PH.

FIGURE 1. Serum IL-6 levels are decreased after PH in Myd88 KO mice, but not in Tlr2, Tlr4, or Cdl4 KO mice. PH or sham operations were performed on Myd88, Tlr2, Tlr4, and Cdl4 KO mice with WT/Het littermates as controls. Serum was collected at the indicated time points. A, IL-6 serum levels were measured at 4 h after PH in all strains using a specific ELISA. Data are represented as picograms per milliliter ± SEM. B, Time course of IL-6 secretion in Myd88 KO mice. *, p ≤ 0.05 for KO vs WT/Het mice.

FIGURE 2. Phosphorylation and activation of STAT-3 is blocked in Myd88 KO mice after PH. Liver tissue was collected from Myd88 KO mice and WT/Het littermates at the indicated time points after PH. A, Phosphorylation of STAT-3 on tyrosine 705 (top blot) and total STAT-3 (bottom blot). B, Quantification of phosphorylated STAT-3 normalized to total STAT-3. C, STAT-3 EMSA. arrow indicates STAT-3 homodimer. D, Phosphorimager quantification of STAT-3 homodimer normalized to control sample. Data are presented as the average ± SEM for four to six mice per time point per strain; *, p ≤ 0.05 for KO vs WT/Het mice.
MyD88 KO mice have a delay in NF-κB activation and a failure to induce TNF after PH

Shortly after PH, there is an increase in circulating TNF levels as well as increases in hepatic mRNA levels, which coincide with NF-κB activation (1, 2). To determine whether TNF expression is altered in Myd88 KO mice after PH, we measured Tnf mRNA levels by RT-PCR. TNF expression increased at 30 min after PH in WT/Het mice, while there was no induction in Myd88 KO mice (Fig. 4A). Tnf mRNA levels did not increase in Myd88 KO mice even as late as 8 h after PH, suggesting that a compensatory rebound from this deficit does not occur (Fig. 4A). In contrast to Myd88 KO mice, induction of liver Tnf mRNA was seen in Tlr4 KO mice to a similar level observed in WT/Het littermates (data not shown). We were unable to detect TNF protein in serum or liver lysates after PH in either control or Myd88 KO mice. We next determined whether the deficit in Tnf mRNA induction in Myd88 KO mice would have an effect on NF-κB activation. In agreement with previous work (6), NF-κB was activated at 30 min after PH in WT/Het mice, but not in Myd88 KO mice (Fig. 4, B and C). However, at 1 h after PH, Myd88 KO mice showed a recovery in NF-κB activation. Thus, there is a delay rather than an overall loss of NF-κB activation after PH in Myd88 KO mice, despite the block in Tnf induction.

Normal hepatocyte proliferation in Cd14, Tlr2, Tlr4, and Myd88 KO mice after PH

To determine whether hepatocyte DNA replication after PH might be altered in Myd88 KO mice, we injected BrdU 2 h before harvesting the livers at 24–72 h after surgery. Surprisingly, there was no statistical difference in BrdU incorporation in Myd88 KO mice compared with WT/Het littermates at any of the time points examined (Fig. 5). These results suggest that the deficits in cytokine signaling present in Myd88 KO mice are not sufficient to alter hepatocyte DNA replication.

Recently, Mandell et al. (30) demonstrated that infection with Helicobacter species results in baseline activation of TLR pathways. We wondered whether the lack of effect of LPS signaling on hepatocyte DNA replication might be due to baseline activation of the innate immune response by this rodent pathogen. We performed Helicobacter genus PCR on fecal samples from mice in all four of our KO colonies. Each KO strain was infected with H. hepaticus and H. bilis (data not shown). To remove the potential confounding effect of Helicobacter infection on the role of TLR
Table I. Hepatocyte proliferation after PH in CD14, Tlr4, Tlr2, and Myd88 KO mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>BrdU Incorporation</th>
<th>Mitotic Figures</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>het/wt</td>
<td>KO</td>
</tr>
<tr>
<td>CD14</td>
<td>761 ± 73.74</td>
<td>682.5 ± 121.6</td>
</tr>
<tr>
<td>TLR4</td>
<td>761 ± 73.74</td>
<td>750 ± 159.2</td>
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<tr>
<td>TLR2</td>
<td>697 ± 121.4</td>
<td>916 ± 205.3</td>
</tr>
<tr>
<td>MyD88</td>
<td>650 ± 100.2</td>
<td>625 ± 80.4</td>
</tr>
</tbody>
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*Per 3000 cells, at 38 h for TLR4 and Myd88 KOs; 48 h for CD14 and TLR2 KOS.

"FIGURE 5. Normal hepatocyte DNA replication in Myd88 KO mice. PH was performed on Myd88 KO and WT/Het mice, and liver tissue was collected and fixed as described in Materials and Methods. Nuclear BrdU labeling of hepatocytes was counted, and the data are presented as the average ± SEM for four to six mice per time point per strain; *, p ≤ 0.05 for KO vs WT/Het mice."
treatment compared with LPS-sensitive mouse strains. Although the importance of leukocytes in liver regeneration (53) is accepted, the impact of a proposed difference in composition of liver leukocytes in C3H/HeJ mice on liver regeneration is unknown. Clearly, any of these hypothetical possibilities would need in vivo experimentation to understand the potency of the mutant TLR4 allele in liver regeneration (15, 16). In any case, the data presented here strongly suggest that TLR2 and TLR4 are not involved in the initiation of liver regeneration. Our results do not exclude the possibility that other members of TLR family (e.g., TLR3, TLR5, TLR9, etc.) may be involved in liver regeneration.

Another surprising finding in our studies was that Myd88 KO mice did not have any defects in hepatocyte replication despite the lack of IL-6 production, absence of Tnf mRNA induction, and delayed NF-kB activation during the first 12 h after PH. Previous studies conducted with Il-6 KO mice indicated that IL-6 is essential for liver regeneration (9), although recent data suggests that IL-6 may be involved in hepatocyte survival rather than proliferation (54–57). We, however, did not see a difference in survival rates between Myd88 KO and WT mice at 7 days after PH (data not shown). The proliferative effects of IL-6 have undergone further scrutiny, as some groups have reported that IL-6 KO mice and mice deficient in gp130 (a necessary component of the receptor complex for the IL-6 ligand family) did not have dramatic defects in DNA replication after PH (36, 54–57). Our results with Myd88 KO mice suggest that a deficit in IL-6 production does not effect liver regeneration in these animals.

Because we did not see a block or delay in DNA replication associated with the lack of Tnf induction in Myd88 KO mice, our results suggest that Tnf may not be necessary for regeneration. Thus, a brief review of the evidence that leads to the hypothesis that Tnf plays an important role in liver regeneration is warranted. More in-depth reviews on Tnf and liver regeneration have been published (1, 59, 61–63). Other laboratories, including ours, have previously reported that Tnf protein levels transiently increase after PH (6, 64, 65). Moreover, Tnf promotes liver growth in vivo and hepatocyte proliferation in vitro (18, 59, 66–68) and injection of Tnf-neutralizing Abs blocks liver regeneration as well as multiple signaling pathways involved in this process (59, 60). We also previously demonstrated that Tnfr1 KO have defective liver regeneration after PH and carbon tetrachloride induced injury (6, 69). Based on this study and reports such as those cited above, several groups proposed that Tnf was important for liver regeneration. In opposition to this hypothesis, increases in protein levels of Tnf are not always seen immediately after PH and Tnf KO mice do regenerate their livers, suggesting that Tnf per se may not be important to regeneration (70). It is important to note that another ligand, lymphotixin α (LTA), binds to and activates Tnfr1. Recently two different groups have demonstrated that hepatocyte DNA replication is inhibited in Lta KO (71) and Lta/Tnf double KO mice (72). Thus, it appears that signaling via the Tnfr1 is important to liver regeneration irrespective of the specific Tnf superfamily ligand that binds and activates this receptor.

In contrast to our findings, Seki et al. (58) found that in addition to deficits in IL-6, Tnf and proto-oncogenes, DNA replication was also blocked in Myd88 KO mice after PH. It should be pointed out that in the work of Seki et al. (58), despite the observed deficit in hepatocyte DNA replication, Myd88 KO mice did eventually regenerate their livers as indicated by liver weight to body ratios. We currently do not know how to reconcile their findings with our own studies, in which we used identical strain of KO mice. It is possible that different surgical techniques, anesthetics used, or status of enteric pathogens contributed to a different outcome in the Seki study. Here, we tested two of these possibilities. Neither the redereivation of Myd88 KO mice to remove Helicobacter nor the use of two different surgeons, who performed the PH using slightly different surgical techniques on Myd88 KO mice, altered our results with DNA replication (data not shown).

A consistent finding, however, was that none of the single or double Tlr KO mice tested (i.e., Tlr2, Tlr4, Tlr9, or Tlr2/Tlr4) or Cd14 KO mice used in either study had any defects in either cytokine signaling or liver regeneration. Other types of liver injury appear to require TLRs. For example, TLR4 and IRF-3 but not Myd88 are important in hepatic damage and inflammation after ischemia-reperfusion injury (73). Thus, to more fully understand the role of the receptors that control innate immunity, it would be of interest to determine which TLR is responsible for Myd88-dependent activation of cytokine pathways and whether there is TLR redundancy in liver regeneration.

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Disclosures
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References


