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Proinflammatory Cytokine Production in Liver Regeneration Is *Myd88*-Dependent, but Independent of *Cd14*, *Tlr2*, and *Tlr4*[1]

Jean S. Campbell,[2]* Kimberly J. Riehle,[2]*† John T. Brooling,* Renay L. Bauer,* Claudia Mitchell,* and Nelson Fausto[3]*

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*, an adapter protein involved 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 production or DNA replication 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.**

Liver 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 then 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),[4] 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 the 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 nucli 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 (20, 24–26). 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

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[4] Abbreviations used in this paper: PH, partial hepatectomy; SOCS-3, suppressor of cytokine signaling-3; IRF, IFN regulatory factor; KO, knockout; Het, heterozygous; WT, wild type; LTα, lymphotoxin α.

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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 *Cd14*, *Tlr2*, and *Tlr4*, we show that the lack of these receptors does not influence liver regeneration. By contrast, there is blockage of cytokine pathways after PH in *Myd88* KO mice.

Materials and Methods

KO mice

Tlr2, *Tlr4*, and *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). *Cd14* 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 *Helicobacter* species, which can induce low-level activation of the TLR pathway (30). We then generated *Helicobacter*-free mice by neonatal rederivation (31) using foster dams housed in a *Helicobacter*-free specific pathogen-free room. DNA extracted from fecal samples was analyzed for *Helicobacter hepaticus, Helicobacter bilis*, and *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 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 μl/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: 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 (0.2 M glycine, 0.1% SDS, 1.0% Tween 20; pH 2.2), reblocked with milk and then probed with STAT-3 (Cell Signaling; no. 9132) and anti-rabbit HRP-conjugated secondary Ab. Autoradiographic film representing each phospho- and total STAT-3 blot was scanned and NIH Image analysis (version X; National Institutes of Health) was used to quantify the density of the appropriate bands. Immunoblot data are presented as the ratio of density of tyrosine 705 STAT-3 divided by the density of total STAT-3.

Electromobility shift assays

Liver tissue was snap-frozen at various time points after PH, and nuclear extracts were prepared using two different methods. For analysis of STAT-3, we used methods previously described (7, 13). Because NF-κB activation occurs predominately in nonparenchymal cells after PH (7, 8), we used a nuclear extract preparation method described by Zeini et al. (35) that lacks a sucrose gradient step and thus allows nuclear extracts to be made from all liver cell types. Protein concentration was determined by the Bradford method using BSA as a standard. Five micrograms of nuclear protein was incubated with the ^{32}P-labeled oligonucleotide probe for NF-κB or STAT-3 for 30 min at room temperature, and samples were then subjected to electrophoresis through 5% polyacrylamide Tris-glycine-EDTA gel. Gels were then dried under a vacuum and exposed to Kodak X-AR film at −80°C with intensifying screens. For supershift experiments, 1 μg of specific Abs to p50 NF-κB (sc-114X), p65 NF-κB (sc-372X), STAT-1 (C-136X), STAT-3 (C-20X) (all from Santa Cruz Biotechnology) were used as described (7, 13). A nuclear extract prepared from a WT mouse 3 h after PH was run on every STAT-3 gel as a control. We used rabbit reticulocyte lysate (1 μg; Promega) as a control for NF-κB. The dried gels were used for Phosphorimager analysis (Storm; Molecular Dynamics) and the data are presented as the density of the NF-κB p50/p65 heterodimer and STAT-3 homodimer DNA-protein complexes divided by the density of the same bands in the control sample.

Determination of IL-6 in serum, and Tnf, Socs3, Cd14, *and* Saa2 *mRNA in liver*

Serum was obtained by cardiac puncture and IL-6 levels determined using an ELISA kit (BD Pharmingen) as described (7, 13). Liver RNA was prepared using TRIzol (Ambion) according to the manufacturer's instructions and quantified by A260 absorbance. Murine *Tnf* and *Socs3* mRNA levels were determined by RT-PCR with specific primers: *Tnf* sense: 5′-ATG AGC ACA GAA AGC ATG ATC CGC GAC-3′, antisense: 5′-GAG ATA GCA AAT CGG CTG ACG-3′; SOCS-3: sense: 5′-ACC TTC AGC TCC AAA AGC GAG TA-3′, antisense: 5′-GCA GCT GGG TCA CTT TCT CAT AG-3′. Two 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 amplicon for a given primer pair. *Tnf* and *Socs3* expression levels were normalized to that of β-actin (primer to competimer ratio of 2:8) as described in the Quantum RNA kit (Ambion). *Cd14* and *Saa2* mRNA 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 amplicons 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; *, $p \leq 0.05$. Statistical analysis was performed using GraphPad Prism software.

Results

Deficits in circulating levels of IL-6 and activation of STAT-3 signaling pathways in MyD88 KO mice after PH

Tlr2, *Tlr4*, *Cd14* and *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 *Tlr2*, *Tlr4*, *Cd14*, and *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 *Tlr2*, *Tlr4*, or *Cd14* mice when compared with WT/Het mice. In marked contrast, only a small amount of IL-6 was detectable in *Myd88* KO mice (Fig. 1*A*). 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 *Myd88* KO mice lacked this induction (Fig. 1*B*).

IL-6 production after PH causes the activation STAT-3. In turn, this transcription factor induces a large number of genes, including those involved in hepatocyte growth and survival, and the acute

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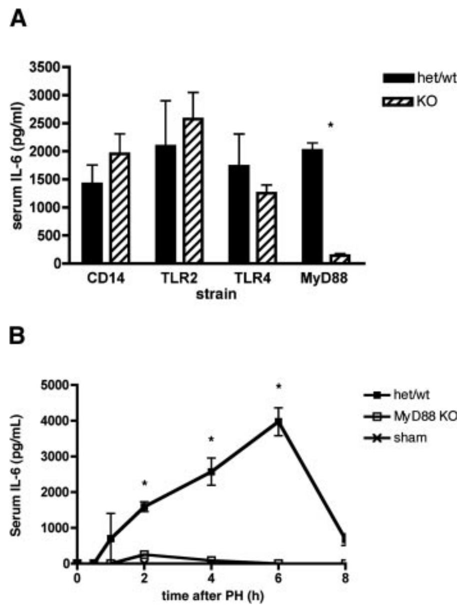

FIGURE 1. Serum IL-6 levels are decreased after PH in *Myd88* KO mice, but not in *Tlr2*, *Tlr4*, or *Cd14* KO mice. PH or sham operations were performed on *Myd88, Tlr2, Tlr4*, and *Cd14* KO mice with WT/Het littermates used as controls. Serum was collected at the indicated time points. *A*, IL-6 serum 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. *, $p \leq 0.05$ for KO vs WT/Het mice.

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. 3*A*). 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. 3, *B* and *C*). 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 *Cd14*, *Tlr2*, and *Tlr4* KO mice compared with WT/Het mice after PH.

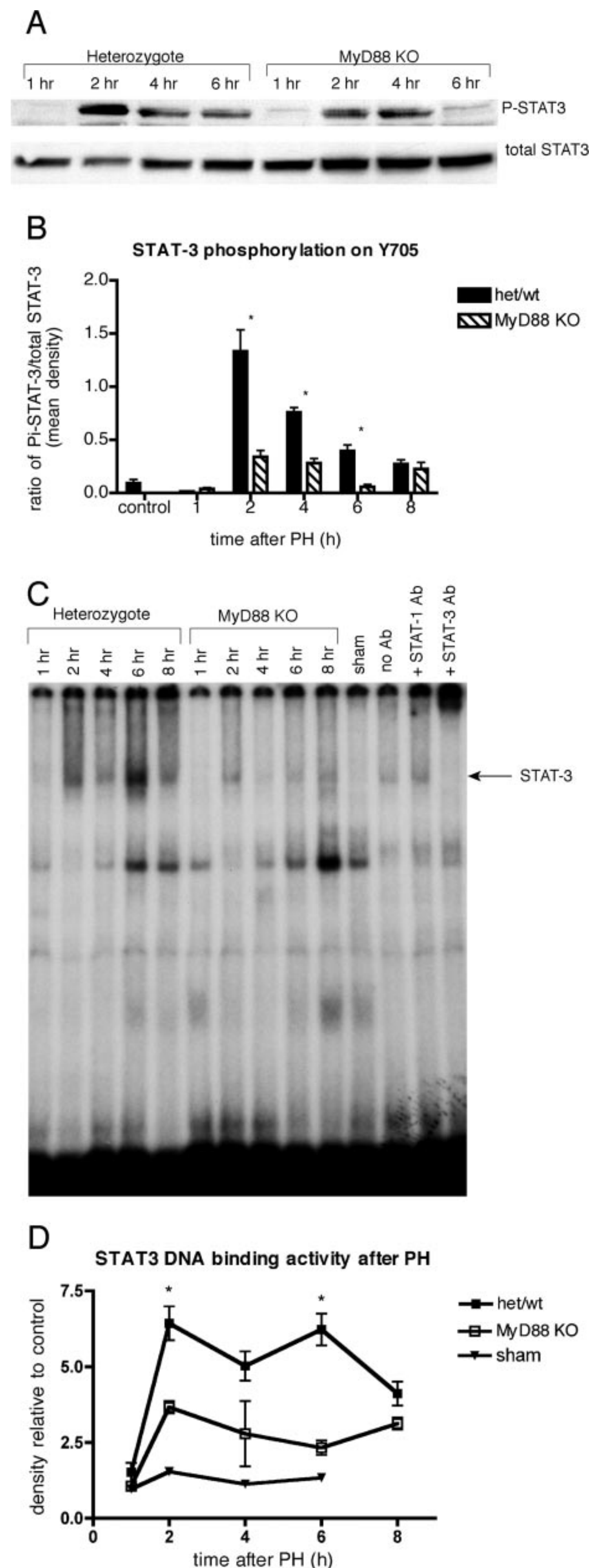

FIGURE 2. Phosphorylation and activation of STAT-3 is blocked in *Myd88* KO mice after PH. Liver tissue was collected from *Myd88* KO mice and WT/Het littermates at the indicated time points after PH. *A*, Phosphorylation of STAT-3 on tyrosine 705 (*top blot*) and total STAT-3 levels (*bottom blot*). *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; *, $p \leq 0.05$ for KO vs WT/Het mice.

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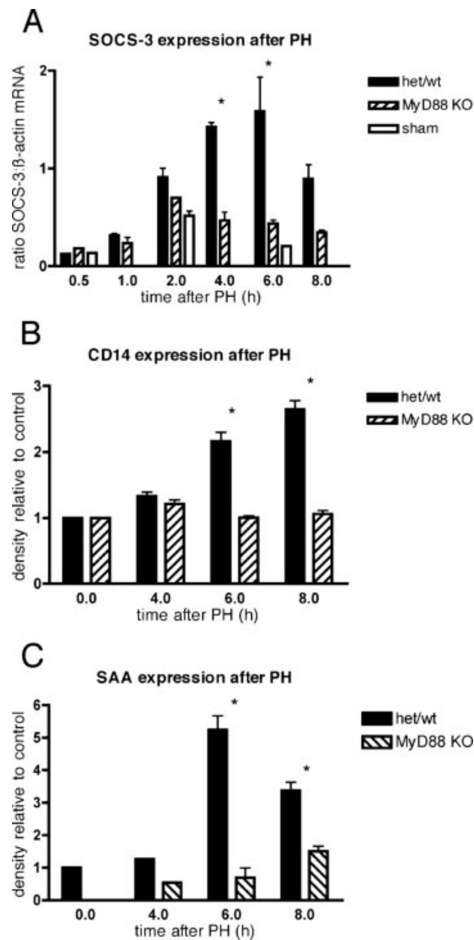

FIGURE 3. STAT-3 signaling pathways are blocked in *Myd88* KO mice after PH. Liver tissue was collected from *Myd88* KO mice and WT/Het littermates at the indicated time points after PH. *A*, *Socs3* mRNA levels as determined by RT-PCR. *B*, *Cd14* mRNA expression by Northern blot. *C*, *Saa2* mRNA expression by Northern blot. Data are presented as the average ± SEM for four to six mice per time point per strain; *, $p \leq 0.05$ 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. 4*A*). *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. 4*A*). 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.

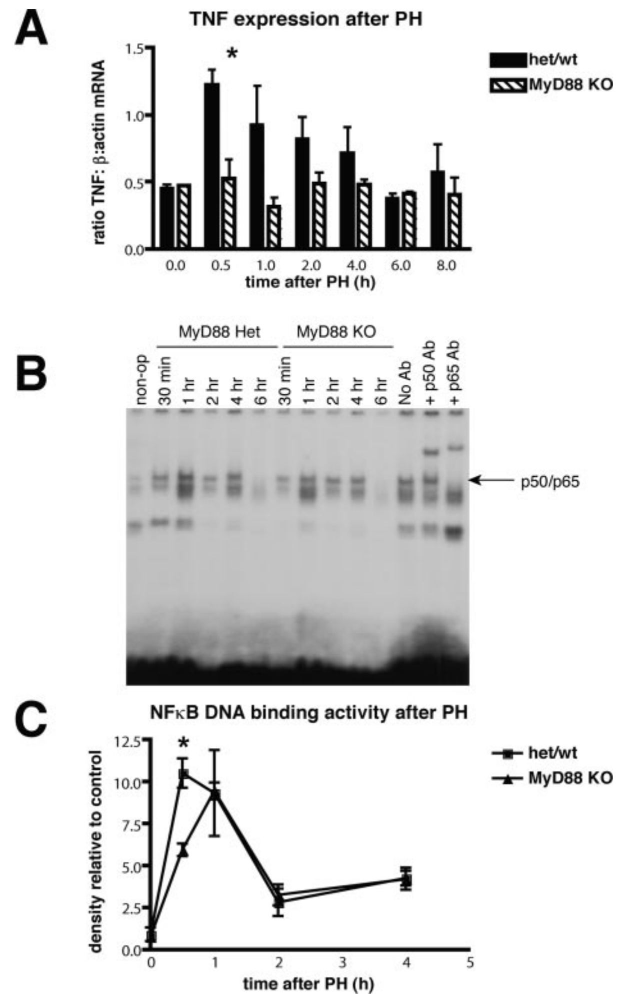

FIGURE 4. NF-κB activation and TNF expression are deficient in *Myd88* KO mice after PH. Liver tissue was collected from *Myd88* KO mice and WT/Het littermates at the indicated time points after PH. *A*, *Tnf* mRNA levels as determined by RT-PCR. *B*, NF-κB EMSA with supershift analysis for p50 and p65 proteins (*final three lanes*). The arrow indicates the p65/p50 heterodimer. *C*, Phosphorimager quantification of NF-κB p50/p65 heterodimer normalized to a control sample. Data are presented as the average ± SEM for four to six mice per time point per strain; *, $p \leq 0.05$ for KO vs WT/Het mice.

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

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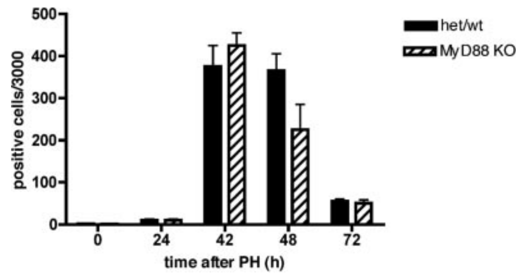

FIGURE 5. Normal hepatocyte DNA replication in *Myd88* KO mice. PH was performed on *Myd88* KO and WT/Het mice, and liver tissue was collected and fixed as described in *Materials and Methods*. Nuclear BrdU labeling of hepatocytes was counted, and the data are presented as the average ± SEM for four to six mice per time point per strain; $*$, $p \leq 0.05$ for KO vs WT/Het mice.

pathways after PH, we rederived all four strains by neonatal transfer to *Helicobacter*-free mothers. *Helicobacter*-negative status was confirmed by PCR. To study hepatocyte replication in the rederived strains by BrdU labeling, we used BrdU incorporation either by bolus injection 2 h before liver harvest or by continuous delivery through an osmotic pump implanted at the time of operation in animals killed at 38–48 h after PH (Table I). BrdU labeling of hepatocyte nuclei in the *Tlr2*, *Tlr4*, *Cd14*, or *Myd88* KO mice was not statistically different from the WT/Het littermates in each strain. Likewise, KO and WT/Het mice had similar mitotic indices as determined at 48 h after PH (Table I). The results suggest that neither the lack of LPS receptors nor the defects in cytokine signaling seen in *Myd88* KO mice affect DNA replication during liver regeneration.

Discussion

IL-6 and *Tnf* expression increases within the first 1–2 h after PH, leading to the activation of pathways that involve NF-κB and STAT-3. At 6–12 h after the operation, IL-6 production and STAT-3 activation are terminated by the induction of *Socs3*. This transient mechanism of cytokine expression allows the activation of multiple target genes to initiate liver regeneration, while preventing cytokine toxicity (1, 2). Although these pathways have been described in detail, there is great uncertainty about the mechanisms that lead to their activation. It has been hypothesized for many years that LPS is the main agent responsible for the activation of cytokine pathways at the start of liver regeneration. This hypothesis is based on several observations, which include the delay in liver regeneration associated with procedures that presumably decrease LPS release from the gut, and the delay that occurs in C3H/HeJ mice, a LPS hyporesponsive strain that carries a point mutation in the intracellular domain of *Tlr4* (14–17, 39). Moreover, the demonstration that liver regeneration is defective in mice lacking C3 and C5 is compatible with the notion that LPS release may trigger the activation of cytokine cascades by enhancing the production of complement proteins (40). Despite this compelling evidence, the potential role of LPS in the initiation of liver regeneration has not been analyzed by direct experiments in which LPS signaling is specifically blocked. In the present work, we used mice that are deficient in *Tlr2*, *Tlr4*, *Cd14*, and *Myd88* to determine the relevance of LPS signaling in the activation of cytokine pathways at the start of liver regeneration.

In *Myd88* KO mice, a deficit in IL-6 production was associated with inhibition of STAT-3 activation and the loss of induction of three target genes, *Socs3*, *Cd14*, and *Saa2*. *Myd88* KO mice also had delayed activation of NF-κB and no induction of *Tnf* mRNA shortly after PH. Surprisingly, IL-6 secretion after PH was not altered by the lack of *Tlr4*, *Tlr2*, and *Cd14*. It is puzzling that loss of MyD88 created multiple defects in these cytokine pathways while the lack of TLR4 and TLR2 had no effect on cytokine activation in the regenerating liver. MyD88 functions as an adapter protein for several TLRs, and also for the IL-1 and IL-18 receptors. It is possible that MyD88 signaling in the regenerating liver depends on IL-1 and IL-18 rather than LPS. However, a number of experiments in hepatocyte cultures suggest that IL-1β inhibits hepatocyte replication (41, 42), while IL-18 expression has been associated with liver injury rather than cell proliferation (43, 44). IL-18 is also known as IFN-γ-inducing factor, and recently we and others have demonstrated that IFN-γ inhibits hepatocyte proliferation and liver regeneration (45, 46). Thus, it is unlikely that either IL-1 or IL-18 is responsible for the MyD88 signaling that results in cytokine activation at the start of liver regeneration, but experiments with IL-1 and IL-18 KO mice are needed to solidify this conclusion. Our data also suggest that complement activation after PH may be independent of LPS receptors, although no experiments were conducted to specifically examine this issue. An intriguing possibility is that signaling by some complement proteins might involve MyD88.

Our data from the TLR4 KO mice are particularly surprising in light of published data demonstrating a delay in liver regeneration in C3H/HeJ mice, an inbred strain of mice with a point mutation in *Tlr4* (i.e., *Tlr4*^{P712H}) (15, 16). Our findings, which are consistent with recent studies by Seki et al. (58), suggest that a point mutation in TLR4 has more profound effects on liver regeneration than the loss of the entire receptor. A number of possible mechanisms may exist for these seemingly contradictory results including the following: 1) other, non-TLR4, LPS receptors may exist (47, 48); 2) TLR4^{P712H} appears to act as a dominant-negative receptor (49, 50); and 3) the *TLR4* allele appears to be subject to tissue specific monoallelic expression (51). Alternatively, a possible key to this puzzle might lie in the hyporesponsive nature of C3H/HeJ mice after LPS injection. Sultzer (52) demonstrated that in C3H/HeJ mice, there was a bias toward the migration of mononuclear cells (e.g., lymphocytes and macrophages) after LPS injection, rather than neutrophils. This observation suggests that a different cytokine and chemokine milieu is present in C3H/HeJ mice after LPS

Table I. *Hepatocyte proliferation after PH in CD14, Tlr4, Tlr2, and MyD88 KO mice*

	BrdU Incorporation[a]			Mitotic Figures[b]		
Strain	het/wt	KOs	p value	het/wt	KOs	p value
CD14	761 ± 73.74	682.5 ± 121.6	0.55	22.5 ± 3.3	28.75 ± 6.63	0.73
TLR4	761 ± 73.74	750 ± 159.2	0.95	37 ± 6.1	20.8 ± 3.2	0.10
TLR2	697 ± 121.4	916 ± 205.3	0.43	35.33 ± 13.42	22 ± 6.18	0.46
MyD88	650 ± 100.2	625 ± 80.4	0.89	25.25 ± 10.13	24.0 ± 2.0	0.91

[a] Per 3000 cells, at 38 h for TLR4 and MyD88 KOs; 48 h for CD14 and TLR2 KOs.
[b] Per 3000 cells, at 48 h for all strains.

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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-κB 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.

Although we observed a lack of *Tnf* mRNA induction in *Myd88* KO mice, we were unable to see any change in TNF protein in liver tissue or in the serum immediately after PH in either *Myd88* KO or control mice. This finding is consistent with other reports of an inability to detect changes in TNF protein levels after PH despite observing induction of mRNA for this cytokine (40, 58). One possible explanation is that TNF is acting in a paracrine manner as has been previously suggested by Diehl and coworkers (59, 60). 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, lymphotoxin α (LTα), binds to and activates TNFR1. Recently two different groups have demonstrated that hepatocyte DNA replication is inhibited in *Ltα* KO (71) and *Ltα/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 rederivation 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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