Reduced Hepatic Injury in Toll-Like Receptor 4-Deficient Mice Following D-Galactosamine/Lipopolysaccharide-Induced Fulminant Hepatic Failure

Ziv Ben Ari^{1,4,*}, Orna Avlas^{4,5,*}, Orit Pappo², Veacheslav Zilbermints³, Yelena Cheporko⁴, Larissa Bachmetov⁴, Romy Zemel⁴, Asher Shainberg⁵, Eran Sharon³, Franklin Grief³ and Edith Hochhauser⁴

¹Liver Institute, ²Department of Histopathology, ³Department of Surgery A, Beilinson Hospital-Rabin Medical Center, Petah Tiqwa, ⁴Liver and Cardiac Research Laboratory, Felsenstein Medical Research Center, Petah Tiqwa, Sackler School of Medicine, Tel Aviv University, Tel Aviv ⁵Gonda (Goldschmied) Medical Diagnostic Research Center, The Mina & Everard Goodman Faculty of Life Sciences Bar-Ilan University, Ramat Gan, Israel, *Authors equally contributed to this manuscript

Key Words

GalN/LPS • cJun • I κ B • TNF- α

Abstract

Liver transplantation is the only therapy of proven benefit in fulminant hepatic failure (FHF). Lipopolysaccharide (LPS), D-galactosamine (GalN)-induced FHF is a well established model of liver injury in mice. Toll-Like Receptor 4 (TLR4) has been identified as a receptor for LPS. The aim of this study was to investigate the role of TLR4 in FHF induced by D-GalN/LPS administration in mice. Wild type (WT) and TLR4 deficient (TLR4ko) mice were studied *in vivo* in a fulminant model induced by GalN/LPS. Hepatic TLR4 expression, serum liver enzymes, hepatic and serum TNF- α and interleukin-1 β levels were determined. Apoptotic cells were identified by immunohistochemistry for caspase-3. Nuclear factor-kappa β (NF- κ B) and phosphorylated c-Jun hepatic expression were studied using Western blot analysis. All WT mice died within 24 hours after administration of GalN/LPS while all TLR4ko mice survived. Serum liver enzymes, interleukin-1 β , TNF- α level, TLR4 mRNA expression, hepatic injury and hepatocyte

apoptosis all significantly decreased in TLR4ko mice compared with WT mice. A significant decrease in hepatic c-Jun and I κ B signaling pathway was noted in TLR4ko mice compared with WT mice. In conclusion, following induction of FHF, the inflammatory response and the liver injury in TLR4ko mice was significantly attenuated through decreased hepatic c-Jun and NF- κ B expression and thus decreased TNF- α level. Down-regulation of TLR4 expression plays a pivotal role in GalN/LPS induced FHF. These findings might have important implications for the use of the anti TLR4 protein signaling as a potential target for therapeutic intervention in FHF.

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Introduction

Acute fulminant hepatic failure (FHF) is a rare clinical syndrome with multiple causes. The course of FHF is variable and the mortality rate is high. Liver transplantation is the only therapy of proven benefit, but the rapidity of progression and the variable course of FHF limit its use. Moreover, effective use of this limited

resource requires accurate prognostication and the requirement for subsequent life-long immunosuppression [1]. In the United States, spontaneous survival occurs in approximately 45%, liver transplantation in 25%, and death without transplantation in 30% of adults with FHF [1].

Sepsis and endotoxemia are frequent complications, and bacteriologically proven infection occurs in up to 80% patients with FHF [2]. Endotoxin is a gram-negative bacterial lipopolysaccharide (LPS) that releases a wide variety of inflammatory mediators, which are considered to be related to the development of FHF as well as to multiple organ failure [3-5]. D-galactosamine (GalN) together with LPS injection has been often used as an animal model of FHF [4, 5]. Mice treated with a combination of LPS + GalN selectively develop hepatic failure, which is much more severe and more rapid than FHF in humans, compared with FHF induced by a high dose of LPS alone, without sensitization [6, 7]. Upon stimulation by LPS, macrophages secrete pro-inflammatory cytokines, including IL-1, IL-6, IL-12, and TNF- α [8]. Among these factors, TNF- α is the terminal mediator of hepatic apoptosis and organ failure.

TNF- α -induced hepatocyte apoptosis has been identified as an early and possibly causal event during LPS/GalN-induced liver failure [3, 5, 9]. Hence, massive hepatocyte apoptosis induced by TNF- α from macrophages is the dominant mechanism of liver injury in this model [10].

Recognition of many microbial toxins occurs through the activation of Toll-like receptors (TLR) that cause the induction of an innate immune response [11-13]. TLR 2 mediates responses in a wide variety of bacterial products including lipoproteins derived from gram-negative and -positive bacteria. In contrast, TLR4 is specific for LPS derived from gram negative bacteria [11, 13]. The signaling of TLR4 in response to LPS induces production of reactive oxygen species and expression of proinflammatory cytokines such as TNF- α , which is mediated by activation of nuclear factor κ B (NF- κ B) [14]. Furthermore, studies in TLR4-deficient and TLR4 mutant (C3H/HeJ) mice showed hyporesponsiveness to LPS, demonstrating that TLR4 is a critical receptor for LPS signaling [15-17]. We have recently found that cardiomyocyte TLR4 is involved in heart dysfunction following septic shock or myocardial ischemia [18].

The aim of this study was to investigate the role of TLR4 in FHF induced by GalN/LPS administration in mice.

Materials and Methods

Animals and treatments

Male wild type mice (C57BL) were purchased from Harlan (Jerusalem, Israel), and male TLR4 deficient mice (C57BL/10ScN) were a generous gift from Dr. Stephan Jung, The Weizmann Institute, Rehovot, Israel. All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of Tel Aviv University, and according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

All male mice were maintained in a pathogen-free facility and were fed pellet food and water ad libitum, until the start of the experiment (12 weeks old). All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of the Felsenstein Medical Research Center and Tel Aviv University (Tel Aviv, Israel).

General Experimental Protocols

A total of 40 mice (12-15 weeks old) were divided into 4 groups (n = 10 each), as follows: transgenic homozygotic knockout mice (TLR4ko) and wild type (WT) controls C57Bl. Each group was further divided into two subgroups (n=10): LPS/GalN -treated and LPS/GalN -untreated subgroup (administered saline). Another 12 mice (6 WT mice and 6 TLR4ko) were treated with LPS/GalN for 24 survival curve.

Fulminant hepatic failure model-LPS/GalN

Mice were injected intra-peritoneal (IP) to induce FHF, with 10 mg/kg LPS. The LPS origin is from *Escherichia coli* (A phenol extract of serotype 011:B4; Sigma Chemical San Diego, CA, USA) in combination with GalN (300 mg/kg, i.p.). The control mice were injected IP with saline. Mice were sacrificed 4 hours following treatment when all mice were still alive. Long-term survival (24 h) was assessed in 2 additional groups of mice: C57Bl GalN/LPS treated mice (n=6) and TLR4ko GalN/LPS -treated mice (n=6).

Huh7 cells were stimulated with LPS (0.5 μ g/ml) and GalN (2.5mM) for 4 hours, and then subjected to total RNA isolation.

Liver enzyme levels

Serum liver enzymes levels were collected 4 hours post administration of LPS and were kept on ice until processed. Levels of aspartate transaminase (AST) and alanine transaminase (ALT) were determined in the perfusate in duplicate using commercial kits, according to the manufacturers' protocols.

TNF- α and IL-1 β level after LPS/GalN challenge

Serum and hepatic cytokines levels were assessed using ELISA semi-kits for mouse TNF- α and mouse IL-1 β (ELISA-Max, BioLegend, CA, USA). Cytokines in the liver were measured using 100 μ g of protein. Standard reference cytokines were provided by the manufacturer. Assays were done in duplicate with a micro titer plate reader (450-nm wavelength). Values are reported as picograms per milligram of protein [18].

Quantitative RT-PCR

Mice. Total RNA was purified from the livers by using TRIzol (Ambion, Cambridgeshire, UK). cDNA was synthesized from total RNA (1 µg) using the TaqMan High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR analysis by Assay-on-Demand Gene Expression Products for mouse TLR4 (Mm00445274_A1) was performed using the ABI 7000 Sequence Detection system (Applied Biosystems; Foster City, CA, USA). The results are shown as the fold change of relative quantity of the TLR4 mRNA normalized to endogenous gene TATA-box mRNA (Mm00446973_A1) values; the control cells were assigned a value of 1 and results were normalized to control [18].

Cell Culture Studies. Cells of human hepatocellular carcinoma cell line (Huh7) were maintained in complete growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin, and 1% amino acid supplement mixture). Huh7 cells were stimulated with LPS (0.5 µg/ml) and GalN (2.5 mM) for 4 hours, and then subjected to total RNA isolation. This part was conducted in order to show the changes in TLR4 and TLR2 mRNA in purified hepatocytes after LPS/GalN challenge. Assay-on-Demand Gene Expression Products (Applied Biosystems) were used for the measurement of TLR2 (Hs00152932_m1), TLR4 (Hs00152939_m1), and GAPDH (Hs99999905_m1). The results are shown as the fold change of relative quantity of TLR mRNA normalized to GAPDH mRNA endogenous gene values, the control cells (non treated cells) were assigned a value of 1 and results were normalized to control.

Pathological Evaluation

Specimens from the mice livers in all groups were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin. Pathological findings were assessed by one of the authors (OP) blind to the group allocations.

Immunohistochemistry for caspase-3

Apoptotic hepatocytes were identified using Caspase-3 immunohistochemistry. For activated caspase-3 immunostaining, 5 µm sections were dewaxed and hydrated through graded ethanols, cooked in 25 mM citrate buffer at pH 6.0 in a pressure cooker at 115°C for 3 min (decloaking chamber; Biocare Medical, Concord, CA, USA), transferred into boiling deionized water, and let to cool for 20 min. After 5 min of treatment in 3% H₂O₂, the slides were incubated with rabbit polyclonal activated caspase-3 (Cell Signaling) antibodies diluted 1:100 in CAS-Block (Zymed) for 3 h at room temperature or overnight at 4°C, washed 3 times with Optimax (HK583; Biogenex), incubated for 30 min with antirabbit Envision+ (K4007, Dako, Copenhagen, Denmark), and developed with 3,30-diaminobenzidine or 3-amino, 9-ethylcarbazole.

Western blot analysis of liver tissue

Liver tissue samples (20 mg) were homogenized in lysis buffer and quantified for protein levels using a commercial assay (Bio-Rad). Liver extracts (30 µg protein/lane) were electrophoresed and subjected to SDS-PAGE under reducing conditions

Fig 1. Kaplan-Meier survival curve: while 5/6 mice (83%) who were treated with GalN/LPS died within less than 8 hours and the sixth mouse died within the first 24 hours, all TLR4ko mice (6/6) survived following GalN/LPS. Thus, survival was improved dramatically (Kaplan-Meier, $p < 0.001$).

using 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with the appropriate antibodies [18]. Mouse monoclonal antibodies directed against the phosphorylated c-Jun NH₂-terminal kinase [c-Jun (N) (sc45)], rabbit anti-IκBα (the inhibitory protein of NF-κB) and COX-1 (used as loading control) polyclonal antibody were purchased from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit anti- c-Jun polyclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Detection was carried out by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Peroxidase-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) and peroxidase-conjugated goat anti-rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, MO). Quantification of enhanced chemiluminescence was carried out by VersaDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA).

Statistical analysis

Results are expressed as means ± standard deviation. Differences between groups were assessed by the analysis of variance (ANOVA) with Bonferroni adjustment or by Student's 2-tailed t test. The Kaplan-Meier method was used for survival, and differences were analyzed by the log-rank sum test. Statistical significance was defined as $p < 0.05$.

Results

Clinical manifestations and survival following GalN/LPS challenge

Wild type mice developed diarrhea, eye exudates and lethargy within four hours following GalN/LPS

Fig. 2. A. Real-time PCR analysis of hepatic TLR4 mRNA expression. The results are shown as the fold change of relative quantity of the TLR4 mRNA normalized to TATA-box mRNA values. In WT mice 4 hours following GalN/LPS administration, TLR4 mRNA expression significantly increased 3-fold compared with saline administration, (* $P \leq 0.005$); results are expressed as mean $\pm$ SD, $n=10$ in each group. B. Real-time PCR analysis of TLR2 (gray bars) and TLR4 (black bars) mRNA expression level in Huh7 cells following treatment with GalN (2.5 mM) and LPS (0.5 μ g/ml). The results are shown as the fold change of relative quantity of the TLR mRNA normalized to GAPDH mRNA values; the control cells were assigned a value of 1 and results are normalized to controls. Each point represents the mean $\pm$ SD of a set of data determined by at least 5 experiments. Statistical significance was calculated by 2-tailed Student's t test and is indicated as * $P \leq 0.005$.

treatment and had 100% mortality within 24 hours; 5/6 died within the first 8 hours and the sixth died within less than 24 hours. TLR4ko mice had neither morbidity nor mortality throughout the study observational period ($p < 0.0001$, Kaplan-Meier), Fig. 1.

Hepatic TLR4 gene and protein expression

All tests except for the survival curve were done on mice 4 hours after treatment when all mice were still alive. TLR4 gene expression was significantly increased (almost 3 times) in WT mice following GalN/LPS administration compared with saline administration. In TLR4ko mice, no expression of the TLR4 gene was detected in saline or in GalN/LPS administration as expected ($p < 0.05$), Fig. 2A. Since there is a controversy over the varying role of TLR4 in different organ hepatocytes, a cell line (Huh7) was incubated with GalN/LPS for gene expression changes. TLR2 was included as another TLR mRNA and served as a control. While TLR4 was elevated 3 times compared

Fig. 3. Liver enzymes. At 4 hours, serum AST and ALT levels increased significantly in WT mice administered GalN/LPS compared with mice administered saline. In TLR4ko mice administered GalN/LPS, the increase in serum liver enzymes level was significantly lower compared with WT mice, (* $P \leq 0.005$ vs WT treated with GalN/LPS). Values are presented as mean $\pm$ SD, $n=10$ in each group.

Fig. 4. Hepatic (A) and serum (B) TNF- α and IL-1 β level. Hepatic and serum TNF- α and IL-1 β levels increased significantly in WT mice administered GalN/LPS compared with mice administered saline (* $p < 0.005$). In TLR4ko mice administered GalN/LPS, hepatic TNF- α level was significantly lower than in WT mice (* $P < 0.05$) and serum TNF- α level did not rise in TLR4 compared to baseline levels or to WT treated with GalN/LPS (+ $P \leq 0.05$ vs WT treated). However, no difference in the hepatic and serum IL-1 β level was noted between WT and TLR4ko mice (Fig. 4C- D). Values are presented as mean $\pm$ SD, $n=10$ in each group.

Fig. 5. Histological findings using H&E staining. Following GalN/LPS administration in WT mice diffuse hepatic congestion and large areas of focal necrosis associated with diffused neutrophil infiltrations were noted (A). However, in TLR4ko mice following GalN/LPS administration, the inflammatory hepatic damage was much lower and liver architecture was much better preserved than in WT mice (B). Sections from WT livers following GalN/LPS administration using immunohistochemistry for caspase-3 stained very positive for the activated form of caspase-3. Many apoptotic bodies (single and in clusters) were observed. Diffused positive nuclear and cytoplasmic staining in 30%-40% of hepatocytes was noted (C). In the TLR4ko livers following GalN/LPS administration, fewer hepatocytes (<10%) stained for caspase-3 activity (D).

to PBS treated cells ($p=0.003$), TLR2 rose only 1.7 times ($p=0.002$). These results prove that TLR4 is the main

player in this response and TLR2 plays a minor role (see Fig. 2B).

Fig. 6. Western blot analysis of liver tissue for c-Jun and NF-κB. In WT mice administered GalN/LPS the phosphorylated c-Jun hepatic expression was significantly higher compared with mice administered saline. In TLR4ko administered GalN/LPS p- c-Jun hepatic expression was significantly lower than in WT mice (A). In WT mice administered GalN/LPS, IκB hepatic expression was significantly lower (2-fold) than mice administered saline. In TLR4ko administered GalN/LPS, the IκB hepatic expression was significantly higher than in WT mice (B), *P ≤ 0.05 vs WT treated with saline. Values are presented as mean ±SD, n=5 in each group.

Biochemical markers of damage

Liver enzymes. Serum AST and ALT levels increased significantly in WT mice 4 hours after GalN/LPS administration compared with mice administered saline ($p < 0.05$) (Fig. 3). In TLR4ko mice administered GalN/LPS, the increase in serum liver enzymes level was significantly lower than WT mice ($p < 0.05$) (Fig. 3).

Serum hepatic TNF-α and IL-1β level after GalN/LPS challenge. Since hepatic proinflammatory cytokines are known to participate in FHF we determined whether TLR4 might play a role in the *in vivo* induction of TNF-α and IL-1β. Both serum and intrahepatic levels were assessed in WT and TLR4ko mice 4 hours post GalN/LPS administration.

In WT mice, TNF-α and IL-1β levels peaked significantly post GalN/LPS administration compared with saline administration both in serum and in the liver tissue (Fig. 4). Intrahepatic TNF-α level was 1.5 fold higher in WT compared to baseline and serum TNF-α level was

7.6 fold higher. (Fig. 4 A,B). Hepatic TNF-α of TLR4ko mice was significantly lower than WT mice, $p < 0.005$ and serum TNF-α was hardly elevated (1.1). However, intrahepatic and serum IL-1β levels were elevated in both WT and TLR4ko mice showing no differences between groups ($p = NS$) (Fig. 4 C-D).

Histologic findings

We evaluated the inflammatory hepatic damage on tissue section of treated mice with H&E staining. Histological findings following GalN/LPS administration in WT mice using H&E staining demonstrated diffuse hepatic congestion and large areas of focal necrosis associated with diffused neutrophil infiltrations (Fig. 5A). However, following GalN/LPS administration in TLR4ko, the inflammatory hepatic damage was much less compared with WT mice and liver architecture was much better preserved (Fig. 5B).

Caspase 3 was measured as an apoptotic marker

with immunohistochemistry staining. Sections from WT livers following GalN/LPS administration were highly positively stained for the activated form of caspase-3. Many apoptotic bodies (single and in clusters) were observed. Diffused positive nuclear and cytoplasmic staining in 30%-40% of hepatocytes was noted (Fig. 5C). In the TLR4ko livers following GalN/LPS administration, fewer hepatocytes (<10%) stained for caspase-3 activity (Fig. 5D).

Inflammatory signaling pathways

Effect of GalN/LPS on the hepatic expression of phosphorylated c-jun and NF- κ B. Since the inflammatory signaling pathways involve hepatic activation of phosphorylated c-jun and NF- κ B pathways, we analyzed phosphorylated c-Jun and I κ B hepatic expression in GalN/LPS treated mice. Western blot analysis of liver tissue has demonstrated that in WT mice administered GalN/LPS, phosphorylated c-Jun hepatic expression was significantly higher compared with mice administered saline 4 hours after treatment. In TLR4ko administered GalN/LPS c-Jun hepatic expression was significantly less (70%) compared with WT mice (Fig. 6A).

In WT mice administered GalN/LPS, I κ B hepatic expression, the inhibitory proteins of NF- κ B, was significantly lower (2-fold) compared with mice administered saline. In TLR4ko administered GalN/LPS, the I κ B hepatic expression was significantly higher compared with WT mice (Fig. 6B).

Discussion

FHF is a rare disorder with high mortality and resource cost. It can be associated with rapidly progressive multiorgan failure and devastating complications; however, outcomes have been improved by emergency liver transplantation [19]. A better understanding of the pathophysiology of the disorder, will probably lead to further improvements in survival rates. GalN/LPS-induced liver injury is an experimental model of FHF in which inflammatory cytokines play a pivotal role. This liver injury is associated with significant increases in AST, ALT, TNF- α and interleukins in serum, leading finally to extremely high lethality [10]. In the present study we demonstrated the significant contribution of hepatic TLR4 to the deleterious effects of GalN/LPS in inducing FHF. We have shown that in WT mice following GalN/ LPS administration, severe hepatic

damage was associated with a significant increase in TLR4 mRNA expression while no significant hepatic injury was demonstrated in TLR4 ko mice. In WT mice, GalN/LPS administration induced death within 8 hours. Severe hepatic damage was manifested by increased serum liver enzymes and, histologically, by diffused hepatic congestion, large areas of focal necrosis and apoptosis. GalN/LPS challenge also induced increased hepatic TNF- α level and increased hepatic expressions of NF- κ B and the phosphorylated c Jun in WT mice. Following GalN/ LPS, the absence of TLR4 receptor activation led to tolerable levels of damage in all organs, particularly the liver, thus preventing death. Our findings are compatible with other published studies in which absence of TLR4 prevented the downstream signaling that led to hepatic failure and death [14-17].

Mice are resistant to doses of D-Galactosamine (GalN) not exceeding 1g/kg [5]. In this study we administered GalN at a dose of 300 mg/kg and all animals survived (data not shown). Death occurred only when both GalN and LPS were administered. Therefore, liver damage in the GalN/LPS model of ALF is due to the activation of the inflammatory cascade by the combined action of these two agents. GalN, a hepatocyte-specific inhibitor of RNA synthesis, is known to sensitize animals to the lethal effects of both LPS and TNF- α and thus induce FHF in experimental animal models [20-22]. GalN is metabolized exclusively in hepatocytes and exerts its effect by depleting hepatic uridine nucleotides, thus leading to the impairment of the biosynthesis of several important macromolecules (e.g. proteins, glycoproteins, and glycogen) and causing hepatocyte damage and death [23]. GalN-induced uridine deficiency is thought to be responsible for the sensitization to LPS and the secretion of TNF- α [24]. GalN/LPS administration also induces the activation of NF- κ B [14]. NF- κ B activation in the resident liver macrophages causes increased expression of proinflammatory cytokines including IL-1 β , TNF- α leading to inflammation and liver failure [25]. Moreover, liver injury can be reduced in experimental fulminant hepatitis by selectively targeting NF- κ B in Kpffer cells [26] or by administering pyrrolidine dithiocarbamate, a general NF- κ B inhibitor, in GalN/LPS induced FHF [27]. Experiments with TNF- α or TNF-receptor p55 knockout mice further confirmed the critical role of TNF- α in LPS/ GalN-induced hepatotoxicity [28, 29]. LPS administration directly activates Kpffer cells to produce TNF- α and other inflammatory cytokines that cause liver injury. LPS sensitization can be prevented by the administration of UTP [5, 24, 30] while the administration of anti-TNF- α

neutralizing antibody provided complete protection against LPS/GalN-induced hepatotoxicity [31]. Gut-derived endotoxemia inducing marked elevation of serum TNF- α have been reported to be responsible for GalN-induced hepatitis [5]. These findings and our current study emphasize the pivotal role that TNF- α plays in the pathogenesis of LPS/GalN-induced FHF. In our model of LPS/GalN-induced FHF, no differences in the intrahepatic and serum IL-1 β level was noted between WT and TLR4ko mice implying that TLR4 and LPS its ligand does not play a main role in regulating this cytokine production in this model and that other downstream signaling pathways and/or other TLRs might be involved in IL-1 β secretion.

The evidence for the activation of TLR4 in acute liver failure has been scarce and there is a controversy upon TLR4 stimulation in different cells following LPS [32, 33]. Our results are similar to those of Kitazawa et al. [34] who have demonstrated increased TNF- α mRNA, TLR4 mRNA and CD14 mRNA hepatic expression in rats following GalN-induced acute severe liver failure compared with control rats. Wang et al. [35] also provided data regarding increase hepatic expression of CD14 and TLR4 mRNA in mice, 1-6 h after LPS/GalN administration. Furthermore, a recently published study has demonstrated that a TLR4 antagonist, E5564, reduced GalN/LPS-induced acute liver injury in rats and improved the overall survival rate [36]. However, TLR4 mRNA has been shown to decrease after LPS treatment in macrophages in one study [33] and no changes of TLR4 mRNA expression was detected in isolated hepatocytes in another study [32]. In order to emphasize the importance of TLR4 activation in this model of fulminant hepatitis we have used in our study purified hepatocytes (Huh7 cells) and noted that following the GalN/LPS challenge, TLR4 mRNA expression was increased 3-fold compared to PBS treated cells. Moreover, we have also analyzed TLR2 mRNA expression which is also known to be activated by LPS [11]. TLR2 mRNA expression increased by only 1.7 fold compared to TLR4 mRNA expression, suggesting that TLR4 is the main player in this model of fulminant hepatitis and TLR2 which is weakly activated, has a minor role. In our TLR4 deficient mice following GalN/LPS administration we demonstrated reduced hepatic injury and an improved overall survival rate. It seems likely that hepatic up-regulation of TLR4 following GalN/LPS-induced FHF causes hypersensitivity to endotoxemia and leads to significant hepatic dysfunction and mortality.

Several studies, including studies based on the gene-

knockout approach, have also convincingly demonstrated the role of c-Jun as a critical apoptotic mediator in GalN/LPS-induced FHF [37, 38]. Administration of the JNK inhibitor protected mice from GalN/LPS-induced FHF [38]. The JNK signaling pathway is one of the most important apoptosis-signaling pathways, and is activated by various forms of liver injury [39]. The most commonly measured parameter of JNK activity is c-Jun phosphorylation, but other important substrates may exist. TNF- α is known to trigger apoptosis by activating the JNK pathway [40], and the TNF- α gene itself, which contains a c-Jun binding site on its promoter [41]. After 3h of GalN/LPS injection, nuclear phosphorylated c-Jun (p-c-Jun) level was significantly activated together with increased NF- κ B nuclear translocation and TNF- α secretion, resulting in apoptotic activities. In our study the NF- κ B pathway in TLR4ko mice was not activated, in TLR4ko administered GalN/LPS compared with WT mice as demonstrated by the absence of a change in the level of the I κ B α , the endogenous inhibitor of NF- κ B. We also found that JNK pathway activity was attenuated, as seen by the decrease in the hepatic level of phosphorylated c-Jun in TLR4ko administered GalN/LPS compared with WT mice. Our findings are in agreement with other published studies. Up-regulated TLR4 activation in WT animals was found to be highly correlated with NF- κ B activation and enhancement of NF- κ B activation was strongly associated with a significant increase in the hepatic expression of TNF- α [42].

In conclusion, following the induction of FHF in TLR4-deficient mice, the inflammatory response and liver injury was significantly attenuated through decreased hepatic JNK and NF- κ B expression and thus TNF- α activity. Down-regulation of TLR4 expression and function plays a pivotal role in GalN/LPS induced fulminant hepatic injury. These findings might have important implications for the use of anti TLR4 protein signaling as a potential target for therapeutic intervention in FHF leading to further improvements in survival rates and reducing the need for urgent liver transplantation.

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