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Regulation of Alternative Macrophage Activation in the Liver following Acetaminophen Intoxication by Stem Cell-Derived Tyrosine Kinase

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Abstract

Stem cell-derived tyrosine kinase (STK) is a transmembrane receptor reported to play a role in macrophage switching from a classically activated/proinflammatory phenotype to an alternatively activated/wound repair phenotype. In the present studies, STK^{-/-} mice were used to assess the role of STK in acetaminophen-induced hepatotoxicity as evidence suggests that the pathogenic process involves both of these macrophage subpopulations. In wild type mice, centrilobular hepatic necrosis and increases in serum transaminase levels were observed within 6 hr of acetaminophen administration (300 mg/kg, i.p.). Loss of STK resulted in a significant increase in sensitivity of mice to the hepatotoxic effects of acetaminophen and increased mortality, effects independent of its metabolism. This was associated with reduced levels of hepatic glutathione, rapid upregulation of inducible nitric oxide synthase, and prolonged induction of heme oxygenase-1, suggesting excessive oxidative stress in STK^{-/-} mice. F4/80, a marker of mature macrophages, was highly expressed on subpopulations of Kupffer cells in livers of wild type, but not STK^{-/-} mice. Whereas F4/80⁺ macrophages rapidly declined in the livers of wild type mice following acetaminophen intoxication, they increased in STK^{-/-} mice. In wild type mice hepatic expression of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-12, products of classically activated macrophages, increased after acetaminophen administration. Monocyte chemoattractant protein-1 (MCP-1) and its receptor, CCR2, as well as IL-10, mediators involved in recruiting and activating anti-inflammatory/wound repair macrophages, also increased in wild type mice after acetaminophen. Loss of STK blunted the effects of acetaminophen on expression of TNF α , IL-1 β , IL-12, MCP-1 and CCR2, while expression of IL-10 increased. Hepatic expression of CX3CL1, and its receptor, CX3CR1 also increased in STK^{-/-} mice treated with acetaminophen. These data demonstrate that STK plays a role in regulating macrophage recruitment and activation in the liver following acetaminophen administration, and in hepatotoxicity.

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Keywords

STK; liver; acetaminophen; macrophages; cytokines; inflammation

Introduction

Acetaminophen is a commonly used over the counter analgesic and the major cause of acute liver failure following accidental and intentional overdose (Bower et al., 2007). Acute intoxication results in centrilobular hepatic necrosis due to covalent binding of the reactive acetaminophen metabolite, *N*-acetyl-*p*-benzoquinone-imine (NAPQI), generated via cytochrome P450 (Cyp) mediated metabolism to critical cellular targets in these regions of the liver (Cohen and Khairallah, 1997). Evidence suggests that activated macrophages contribute to the pathogenic response to acetaminophen (reviewed in Laskin, 2009). However, the precise role of these cells in hepatotoxicity depends on the timing of their appearance in the liver and the mediators they encounter in the tissue microenvironment which dictate their phenotype and functional activities. Thus, following acetaminophen intoxication, macrophages are initially classically activated and release proinflammatory/cytotoxic mediators which promote tissue injury; subsequently, as toxicity progresses, macrophages become alternatively activated and release mediators which down regulate inflammation and initiate wound repair. The mechanisms regulating the switch of liver macrophages from a pro- to an anti-inflammatory/wound repair phenotype have not been established.

Stem cell-derived tyrosine kinase (STK) is a 180 kDa transmembrane receptor present on mouse macrophages which has been reported to negatively regulate inflammatory responses (Correll et al., 1997; Zhou et al., 2002; Morrison et al., 2004; Laskin et al., 2010a; Stuart et al., 2011). Ligand binding to STK initiates cellular signaling leading to inhibition of I κ B kinase and decreased expression of proinflammatory mediators (Chen et al., 1998; Leonis et al., 2002; Wilson et al., 2008; Ray et al., 2010; Stuart et al., 2011). Previous studies have shown that targeted disruption of the STK gene results in increased sensitivity of mice to endotoxin-induced liver injury, a process thought to be due to an inability of hepatic macrophages to become alternatively activated (Correll et al., 1997). This is supported by findings that upregulation of macrophage STK results in suppression of classical activation and induction of alternative activation in these cells (Ray et al., 2010). In the present studies, we used STK^{-/-} mice to evaluate its role in liver macrophage activation and acetaminophen-induced hepatotoxicity. Our findings that STK^{-/-} mice are hypersensitive to acetaminophen and that this correlates with aberrant alternative macrophage activation are consistent with a role of STK in regulating the phenotype and function of macrophages in the liver during the pathogenesis of hepatotoxicity.

Materials and Methods

Animals and treatments

Wild type C57BL/6 male mice (7–9 weeks, 20–25 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice with a targeted disruption of the STK gene were generated as previously described (Correll et al., 1997). All animals were maintained on food and water *ad libitum* and housed in microisolation cages. Animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Mice were fasted overnight prior to administration of acetaminophen (300 mg/kg, i.p.) or phosphate-buffered saline (PBS) control. Blood samples were collected by cardiac puncture and

analyzed for serum alanine transaminase (ALT) and aspartate transaminase (AST) using diagnostic assay kits (ThermoElectron, Pittsburgh, PA).

Histology and immunohistochemistry

Liver samples (~100 mg) were fixed overnight at 4°C in PBS containing 3% paraformaldehyde and 2% sucrose, washed 3 times with 2% sucrose/PBS, transferred to 50% ethanol, and then paraffin embedded. Six micron sections were prepared and stained with hematoxylin and eosin (Goode Histolabs, New Brunswick, NJ). For immunohistochemistry, sections were incubated overnight with rat monoclonal antibody to F4/80 or rat IgG_{2b} control (1:1000, AbD-Serotec, Raleigh, NC). Antibody binding was visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Three random liver sections from each mouse were examined.

Measurement of hepatic glutathione (GSH)

Samples of liver (25 mg) were minced in ice cold 5% metaphosphoric acid (1:10), homogenized, and then centrifuged at 3000g for 10 min at 4°C. Supernatants were filtered through a 0.2 µm syringe filter and reduced GSH quantified using a colorimetric assay kit (GSH-400, OxisResearch, Portland, OR). GSH was calculated based on the slope of a standard curve.

Acetaminophen metabolism

Blood samples (0.5 ml) were centrifuged (3000g, 4°C, 10 min), and serum frozen in liquid nitrogen and stored at -80°C until analysis. Free acetaminophen and acetaminophen-glucuronide were analyzed as previously described (Brunner and Bai, 1999). Briefly, samples were thawed and deproteinated with 6% perchloric acid containing 10 mg/ml theophylline as an internal standard. Precipitated proteins were centrifuged (13,000g, 5 min, 4°C), supernatants collected and analyzed isocratically by HPLC (Shimadzu, Columbia, MD) using a Luna 5 µm, C18 column (250 mm × 2.0 mm) (Phenomenex, Torrance, CA) with a guard column containing the same sorbent. Samples were analyzed at a constant flow rate of 0.2 ml/min with a mobile phase containing 7% acetonitrile in 0.05 mM sodium sulfate (pH 2.2) and products detected by UV absorbance at 254 nm.

Measurement of hepatic Cyp activity

To prepare microsomes, liver samples (1 g) were homogenized at 4°C in 2 volumes (w/v) of 10 mM Tris-base (pH 7.4) containing 1.5% KCl using a Teflon-glass homogenizer. Homogenates were centrifuged at 1,000g (10 min, 4°C), supernatants collected and centrifuged at 12,000g (20 min, 4°C) to remove cellular debris, and then at 105,000g (1.5 h, 4°C). Microsomes were resuspended in homogenization buffer containing 0.5 mM phenylmethanesulfonylfluoride and centrifuged at 105,000g (90 min, 4°C). Pellets were resuspended in 0.25 M sucrose containing 10 mM Tris-base (pH 7.4) and stored at -80°C until analysis (Cooper et al., 1993). Cyp2e1 was measured by the formation of *p*-nitrocatechol (Koop, 1986). Microsomes were incubated with *p*-nitrophenol (200 µM) and NADPH (500 µM) at 37°C for 30 min, followed by trichloroacetic acid (20%, w/v) to stop the reaction. Microsomes were then centrifuged (10,000g, 5 min, 4°C), supernatants collected and mixed with 2 M NaOH. Changes in absorbance were measured spectrophotometrically at 535 nm. Concentrations of *p*-nitrocatechol in the samples were calculated based on a standard curve generated using authentic product. Cyp1a2 and Cyp3a activities were measured as previously described (McLaughlin et al., 2008) with some modifications. Briefly, liver microsomes were incubated with 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM NADPH and 5 µM 7-methoxyresorufin for Cyp1a2 or 1 µM dibenzylfluorescein for Cyp3a. Relative fluorescence

units were recorded over a 10 min interval at an excitation wavelength of 530 nm and an emission wavelength of 590 nm for methoxyresorufin, and 485 nm and 530 nm for dibenzylfluorescein on a Spectromax M5 fluorescent plate reader (Molecular Devices, Sunnyvale, CA). Rates of product formation were calculated using SoftMaxPro5 software. The concentrations of the reaction products were calculated based on a standard curve generated using authentic resorufin or fluorescein. Each determination was repeated in triplicate for all animals. To assess Cyp isoform specificity, enzyme activities were measured after the addition of 1 μ M of the Cyp1a2 inhibitor, α -naphthoflavone, or the Cyp3a inhibitor, ketoconazole (Sai et al., 2000; McLaughlin et al., 2008). Whereas, α -naphthoflavone inhibited Cyp1a2 activity by 94%, ketoconazole blocked Cyp3a activity by 99.5% (Table 1).

Western blotting

Lysates were prepared by homogenizing 30 mg of liver in buffer containing 20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM diethylene triamine pentaacetic acid, 1 mM phenylmethylsulfonylfluoride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO). Protein concentrations were measured using the bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL). Proteins were separated on 10.5–14% Tris-glycine polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes. Non-specific binding was blocked using a buffer containing 10 mM Tris-base, 200 mM NaCl, 1% polysorbate 20 and 5% non-fat milk for 1 h at room temperature. Membranes were then incubated overnight at 4°C with anti-tumor necrosis factor receptor 1 (TNFR1, 1:100, Enzo Life Sciences, Farmingdale, NY), inducible nitric oxide synthase (iNOS, 1:1000, BD Biosciences, San Jose, CA) or actin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies, followed by incubation with isotype-specific horse radish peroxidase conjugated secondary antibodies (1:5000) for 1 h at room temperature. Binding was visualized using an ECL Plus chemiluminescence kit (GE Healthcare, Piscataway, NJ). Densitometry was performed on a Bio-Rad Chem-Dox XRS using Quantity One software.

Quantitative real time PCR

Total RNA was extracted from liver samples (25 mg) using an RNeasy Miniprep kit (Qiagen Inc, Valencia, CA) and reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturers' protocol. Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Real time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT thermocycler using 96-well optical reaction plates. All PCR primer sequences were generated using Primer Express 2.0 (Applied Biosystems) and primers were synthesized by Integrated DNA Technologies (Coralville, IA). A minimum of three samples were analyzed for each experimental group, and all samples were run in duplicate. Primer sequences were: heme oxygenase-1 (HO-1), CCTCACTGGCAGGAAATCATC; interleukin (IL)-1 β , CCAAAAGATGAAGGGCTGCT; tumor necrosis factor- α (TNF α), AAATTCGAGTGACAAGCCGTA; monocyte chemotactic protein-1 (MCP-1, CCL2), GCCAGCTCTCTCTTCCTCCA; IL-10, GGTTGCCAAGCCTTATCGGA; mannose receptor, CCCAAGGGCTCTTCTAAAGCA; CCR2, TCCACGGCATACTATCAACATCTC; IL-12, CCTGGAGCACTCCCCATT; macrophage inflammatory protein-2 (MIP-2, CXCL2), AGGCTTCCCGATGAAGAG; CX3CR1, TCGGTCTGGTGGGAAATCTG; CX3CL1, GCACAGGATGCAGGGGCTTAC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TGAAGCAGGCATCTGAGGG.

Statistics

All experiments were performed using 3–9 mice/treatment group. Data were analyzed using a Student's *t* test or one-way ANOVA with Holm-Sidak post-hoc analysis.

Results

Role of STK in acetaminophen-induced hepatotoxicity

Treatment of wild type mice with acetaminophen resulted in a time-related induction of hepatotoxicity, characterized by significant elevations in ALT and AST within 6 h; this persisted for 18 h before declining (Fig. 1 and Gardner et al., 2010). Acetaminophen also induced structural alterations in the liver including hepatocellular cytoplasmic degeneration and bridging necrosis (Fig. 2 and Gardner et al., 2010). Loss of STK resulted in an increase in the sensitivity of mice to acetaminophen; thus, serum transaminases were significantly greater in STK^{-/-} mice, when compared to wild type mice, and histologic evidence of liver damage was more extensive (Figs. 1 and 2). Moreover, while administration of acetaminophen resulted in 11% mortality at 24 h in wild type mice, mortality was 43% in STK^{-/-} mice (n = 7–9 mice).

To exclude the possibility that differences in sensitivity between wild type and STK^{-/-} mice were due to alterations in acetaminophen metabolism, we measured serum levels of acetaminophen-glucuronide conjugate and hepatic Cyp2e1, Cyp3a and Cyp1a2 activities. HPLC analysis of serum samples collected from wild type and STK^{-/-} mice 30 min to 2 h after acetaminophen administration revealed the presence of unconjugated acetaminophen and acetaminophen-glucuronide (Fig. 3). No significant differences were observed between the genotypes. We also noted generally similar rates of Cyp2e1 mediated hydroxylation of *p*-nitrophenol in microsomal fractions of livers from wild type and STK^{-/-} mice (Table 1). Similarly, there were no differences noted in the activities of microsomal Cyp3a or Cyp1a2.

Effects of loss of STK on hepatic antioxidants

In further studies, we determined if increased sensitivity of mice lacking STK to acetaminophen was associated with alterations in hepatic GSH and HO-1, which are important in protecting against oxidative stress (Chiu et al., 2002; Dambach et al., 2006; Yuan and Kaplowitz, 2009). In both wild type and STK^{-/-} mice, acetaminophen caused a rapid (within 30 min) decrease in hepatic GSH, which persisted for 12 h (Table 2). Hepatic GSH levels were lower in STK^{-/-} mice, when compared to wild type mice, at all time points examined (Table 2). Following acetaminophen administration, a transient increase in hepatic HO-1 mRNA expression was observed in wild type mice which peaked at 6 h (Fig. 4 and Gardner et al., 2010). In contrast, in STK^{-/-} mice, HO-1 mRNA remained elevated for at least 12 h post-acetaminophen.

Effects of loss of STK on acetaminophen-induced alterations in hepatic macrophages and inflammatory protein expression

F4/80 is a murine marker of mature resident tissue macrophages (Leenen et al., 1994). It is also expressed by alternatively activated anti-inflammatory/wound repair macrophages (Hume et al., 1983; Burke et al., 2010; Volarevic et al., 2011). In liver sections from wild type mice, but not STK^{-/-} mice, F4/80 positive Kupffer cells were readily identified within the sinusoids (Fig. 5). Treatment of wild type mice with acetaminophen resulted in a time-related decrease in F4/80 positive macrophages in the liver, which was evident within 6 h and persisted for 48 h (Fig. 5 and Dambach et al., 2002). In contrast, in STK^{-/-} mice, acetaminophen intoxication was associated with a time-related increase in F4/80⁺ macrophages in the liver (Fig. 5). Mannose receptor is a C type lectin reported to be upregulated on alternatively activated macrophages (Stein et al., 1992). While

acetaminophen had no effect on mannose receptor expression in livers of wild type mice, in STK^{-/-} mice an increase in expression was observed at 6 h and 12 h post treatment (Fig. 6).

We next analyzed the effects of loss of STK on acetaminophen-induced expression of pro- and anti-inflammatory mediators implicated in liver injury. Treatment of wild type mice with acetaminophen resulted in increased mRNA expression of IL-1 β , IL-12, and TNF α which was notable after 12 h (Fig. 6). mRNA expression for the chemokines MIP-2 (CXCL2) and MCP-1 (CCL2), and the chemokine receptor, CCR2, also increased in livers of mice treated with acetaminophen (Fig. 6). Whereas, MIP-2 expression reached a maximum at 6 h, MCP-1 and CCR2 peaked at 12 h post-treatment (Fig. 6). Loss of STK was associated with significant attenuation of acetaminophen-induced increases in expression of IL-1, IL-12, and TNF α , as well as MCP-1 and CCR2. In contrast, expression of MIP-2 was increased 12 h after acetaminophen administration. CX3CL1 and its receptor, CX3CR1 were also found to be upregulated in the livers of acetaminophen treated STK^{-/-} mice, but not wild type mice at 6 h and 12 h. The anti-inflammatory cytokine, IL-10 increased in livers of both wild type and STK^{-/-} mice after acetaminophen administration (Fig. 6). This response was rapid, appearing within 6 h and persisting for at least 12 h. Levels of IL-10 mRNA were significantly greater in STK^{-/-} mice, relative to wild type mice at both time points.

Expression of iNOS, the enzyme mediating nitric oxide production during inflammatory responses (Laskin et al., 2010b) was also analyzed. Western blot analysis revealed low levels of iNOS in livers of both wild type and STK^{-/-} mice (Fig. 7). While acetaminophen had no major effects on iNOS expression for up to 12 h in wild type mice, in STK^{-/-} mice, a time related increase in iNOS expression was observed beginning 6 h post treatment (Fig. 7). TNFR1 is the major receptor involved in mediating the inflammatory actions of TNF α in macrophages (Douni et al., 1995). As reported previously (Ishida et al., 2004), treatment of wild type mice with acetaminophen resulted in increased TNFR1 protein expression in the liver. This response was significantly attenuated in STK^{-/-} mice (Fig. 7).

Discussion

The outcome of acetaminophen intoxication has been linked to the phenotype and functional activity of macrophages accumulating in the liver in response to injury and the inflammatory mediators they generate (reviewed in Laskin, 2009). Whereas early classical activation of macrophages in the liver promotes acetaminophen-induced injury, subsequent alternative macrophage activation results in suppression of inflammation and initiation of tissue repair. The mechanism mediating the switch in macrophages from a classically to an alternatively activated phenotype in the liver is unknown. In the present studies, we analyzed the role of STK in acetaminophen-induced hepatotoxicity since engagement of this receptor and activation of downstream signaling pathways have been shown to play a role in polarizing macrophages towards an alternatively activated anti-inflammatory/wound repair phenotype (Ray et al., 2010). Consistent with this activity, we found that loss of STK resulted in hypersensitivity of mice to acetaminophen-induced hepatotoxicity. Thus, serum transaminase levels were significantly greater and centrilobular necrosis was more extensive, resulting in a marked increase in mortality. This did not appear to be due to altered metabolism of acetaminophen as measured by plasma levels of acetaminophen-glucuronide and hepatic Cyp2e1, Cyp3a, and Cyp1a2 activities. These findings are in accord with previous reports that macrophage-mediated repair of damaged liver is key in limiting the hepatotoxic response to acetaminophen (Hogaboam et al., 2000; Dambach et al., 2002; Ju et al., 2002; Holt et al., 2008; Si et al., 2010).

Acetaminophen-induced hepatotoxicity is known to involve oxidative stress generated as a consequence of Cyp-mediated NAPQI formation, and inflammatory cell production of

reactive oxygen and nitrogen species (Laskin and Pilaro, 1986; Michael et al., 1999; Gonzalez, 2005; Das et al., 2010). GSH plays a key role in protecting against acetaminophen-induced oxidative stress by reducing levels of NAPQI (Gonzalez, 2005). GSH is normally present in hepatocytes at relatively high concentrations (Yuan and Kaplowitz, 2009). Excessive generation of NAPQI leads to GSH depletion and hepatocyte cytotoxicity. As previously reported (Mitchell et al., 1973; Gardner et al., 2010), acetaminophen overdose was associated with a rapid decrease in liver GSH levels in wild type mice, which correlated with the development of hepatocellular necrosis. Our data showing exaggerated hepatotoxicity and mortality in STK^{-/-} mice were correlated with a greater reduction in GSH levels suggest increased oxidative stress in the livers of these animals. This is supported by our findings of a persistent increase in expression of the antioxidant, HO-1 and a rapid upregulation of iNOS in livers of STK^{-/-} mice, when compared to wild type mice. Reactive nitrogen species generated via iNOS have been shown to play a key role in acetaminophen-induced hepatotoxicity (Gardner et al., 1998; Michael et al., 1999; Gardner et al., 2002; Abdel-Zaher et al., 2007; Cigremis et al., 2009). This is thought to be due to oxidation and nitrosylation of hepatocyte lipids, proteins and DNA (Diesen and Kuo, 2011). The fact that exacerbated hepatotoxicity and increased mortality in STK^{-/-} mice in response to acetaminophen were associated with a rapid increase in hepatic iNOS suggests a potential mechanism underlying their heightened responsiveness. Similar increases in iNOS and hepatotoxicity have been reported in STK^{-/-} mice treated with endotoxin (Correll et al., 1997). These findings are consistent with a role for STK in negatively regulating macrophage iNOS activity and nitric oxide production (Correll et al., 1997; Chen et al., 1998; Liu et al., 1999). Classically activated macrophages express iNOS and are a major source of reactive nitrogen species during inflammatory reactions (Ho and Sly, 2009), suggesting that an increase in their activity following acetaminophen intoxication in STK^{-/-} mice may account for upregulation of hepatic iNOS. However, since hepatocytes have also been reported to express iNOS and to generate nitric oxide in response to inflammatory stimuli (Gardner et al., 1998; Ozaki et al., 2010), and to express STK (Stuart et al., 2011), they may also contribute to the response.

A number of studies have demonstrated that alternatively activated macrophages play a critical role in repair of acetaminophen-induced liver damage (Hogaboam et al., 2000; Dambach et al., 2002; Ju et al., 2002; Holt et al., 2008; Si et al., 2010). A question arises as to the origin of these cells. Our data are consistent with reports that there are multiple subpopulations of phenotypically distinct alternatively activated macrophages that respond to liver injury, and that they are derived from different precursors (Si et al., 2010). F4/80 is a 160 kD membrane-associated glycoprotein and a member of the G-protein coupled receptor family present on mature macrophages and on alternatively activated macrophages participating in wound repair (Hume et al., 1983; Leenen et al., 1994; Volarevic et al., 2011; Minamino et al., 2012). We found that F4/80 was highly expressed by subpopulations of resident Kupffer cells in livers of wild type mice treated with control, which is in accord with previous studies (Dambach et al., 2002; Kinoshita et al., 2010). In contrast, F4/80 was not detectable in liver macrophages in control treated STK^{-/-} mice indicating that they are distinct from Kupffer cells in wild type mice. These findings are in agreement with reports that macrophages expressing low levels of STK also express low levels of F4/80 (Iwama et al., 1995). Following acetaminophen intoxication, the number of F4/80⁺ liver macrophages decreased in wild type mice, but increased in STK^{-/-} mice. This may reflect a compensatory attempt by the liver to generate new subpopulations of alternatively activated macrophage precursors. Previous studies have demonstrated that inhibition of macrophages with gadolinium chloride suppresses classically activated macrophages and protects against acetaminophen-induced hepatotoxicity (Blazka et al., 1995; Laskin et al., 1995; Michael et al., 1999; Abdel-Zaher et al., 2007). It has also been reported that gadolinium chloride has no effect on F4/80⁺ Kupffer cells (Devey et al., 2009). These data are in accord with the idea

that F4/80⁺ Kupffer cells represent a distinct subpopulation of resident liver macrophages that become alternatively activated following acetaminophen intoxication (Dambach et al., 2002; Devey et al., 2009). This is supported by our findings of increased expression of the anti-inflammatory cytokine IL-10, a product and inducer of alternatively activated hepatic macrophages (Thomas et al., 2011; Antoniadou et al., 2012) in the livers of acetaminophen-treated STK^{-/-} mice.

In addition to its role as an antioxidant, HO-1 possesses anti-inflammatory activity (Immenschuh et al., 2010). In the liver, HO-1 is predominantly expressed by Kupffer cells (Devey et al., 2009), and it has been reported to play a role in inducing alternative macrophage activation (Weis et al., 2009). Consistent with this activity are findings that F4/80 and HO-1 are coordinately upregulated in liver macrophages following reperfusion injury, and that this correlated with the development of an alternatively activated macrophage phenotype (Devey et al., 2009). Persistent upregulation of HO-1 in STK^{-/-} mice, but not wild type mice, following acetaminophen intoxication may similarly contribute to alternative activation of F4/80⁺ macrophages in the liver. However, it appears that an increase in this alternatively activated macrophage subpopulation alone is insufficient to initiate wound healing, as excessive hepatotoxicity and mortality are still evident in STK^{-/-} mice.

We also found that hepatic expression of mannose receptor, another marker of alternatively activated macrophages was increased in livers of STK^{-/-} mice following acetaminophen intoxication. Mannose receptors have been reported to be constitutively expressed by liver macrophages, endothelial cells and hepatocytes (Kempka and Kolb-Bachofen, 1988; Ellinger and Fuchs, 2010). However, while mannose receptor expression is upregulated in liver macrophages and endothelial cells by anti-inflammatory cytokines, this is not observed in hepatocytes (Liu et al., 2011). These data suggest that increases in mannose receptor in STK^{-/-} mice after acetaminophen are likely due to the response of nonparenchymal cells.

A number of studies have demonstrated that CCR2 plays a key role in directing monocyte precursors into the liver, which contribute to wound repair (Hogaboam et al., 2000; Dambach et al., 2002; Holt et al., 2008; Si et al., 2010). Our findings that increased hepatotoxicity in STK^{-/-} mice is correlated with reduced expression of CCR2 and its ligand MCP-1 are consistent with the idea that recruitment of these cells into the liver is essential for repair of acetaminophen injured liver. These data also provide additional support for the concept that there are multiple subpopulations of macrophages including resident F4/80⁺ Kupffer cells and CCR2⁺ infiltrating monocytes/macrophages that have the capacity to develop into alternatively activated macrophages. It remains to be determined if these subpopulations exhibit distinct functional activity.

IL-1 β , IL-12, and TNF α are proinflammatory cytokines generated by classically activated macrophages (Laskin et al., 2011). Following acetaminophen administration to wild type mice, hepatic expression of each of these cytokines increased, consistent with their potential involvement in hepatotoxicity. Surprisingly, loss of STK resulted in a significant attenuation of this response. IL-1 augments tissue damage by stimulating the production of cytokines and chemokines, and upregulating expression of cell adhesion molecules (Rosenwasser, 1998). IL-12 plays a key role in promoting the development of Th1 responses, inducing the production of interferon (IFN)- γ by inflammatory cells, and reducing IL-4 mediated suppression of IFN γ (Trinchieri, 2003). The present studies demonstrate that STK regulates expression of IL-1 β and IL-12 in the liver following acetaminophen intoxication; however, these cytokines do not appear to be involved in the exaggerated hepatotoxic response of STK^{-/-} mice. These findings are in line with reports that IL-1 β and IFN γ , a major

downstream product of IL-12, do not play significant roles in the pathogenesis of liver necrosis induced by acetaminophen (Masson et al., 2008; Williams et al., 2011).

TNF α is a macrophage-derived cytokine thought to play a dual role in acetaminophen hepatotoxicity. Thus, when released early after acetaminophen intoxication, TNF α promotes tissue injury, while at later times, it is involved in suppressing inflammation, upregulating antioxidants, and inducing hepatocyte proliferation and liver repair (Chiu et al., 2003a; Chiu et al., 2003b; Toklu et al., 2006; Campion et al., 2008; Han et al., 2010; Connolly et al., 2011). These actions of TNF α are mediated by binding to TNFR1 (Douni et al., 1995). In previous studies we demonstrated that loss of TNFR1 results in an exaggerated hepatotoxic response to acetaminophen, an effect due to blunted generation of antioxidants and reduced hepatocellular proliferation (Chiu et al., 2003a; Chiu et al., 2003b; Gardner et al., 2003). Our findings that expression of TNF α and TNFR1 are reduced in acetaminophen treated STK $^{-/-}$ mice support the idea that activation of TNFR1 signaling is hepatoprotective, and provide additional evidence that increased hepatotoxicity in these animals is due to excessive oxidative stress and impaired tissue repair.

MIP-2 (CXCL2) is a potent neutrophil chemoattractant secreted by activated macrophages (Kopydlowski et al., 1999). Previous studies have reported that MIP-2 is upregulated in the liver following acetaminophen intoxication and that this is correlated with neutrophil accumulation in the tissue (Hogaboam et al., 1999; Lawson et al., 2000). We found no differences in expression of MIP-2 mRNA between wild type and STK $^{-/-}$ mice, which is in accord with reports that neutrophils are not major contributors to the pathogenesis of acetaminophen-induced hepatotoxicity (Lawson et al., 2000).

CX3CL1/fractalkine is a CX3C chemokine that exists in both soluble and membrane-bound forms and is involved in mediating leukocyte adhesion and emigration into inflammatory sites (Imai et al., 1997). The biological activities of CX3CL1 are mediated by binding to CX3CR1, which is expressed at high levels on inflammatory monocytes/macrophages. CX3CL1/CX3CR1 interactions have been shown to contribute to the development of a variety of inflammatory disorders (D'Haese et al., 2010). Acetaminophen administration was associated with increased expression of CX3CL1 and CX3CR1 mRNA in STK $^{-/-}$ mice, but not wild type mice. Recent studies have shown that loss of CX3CL1 results in increased sensitivity of mice to toxin A-induced enteritis, which was due to impaired induction of HO-1 in F4/80 $^{+}$ repair macrophages (Inui et al., 2011). These findings, together with reports that macrophage subpopulations which express high CX3CR1 and low CCR2 exhibit an alternatively activated phenotype (Heymann et al., 2009), suggest that upregulation of CX3CL1 and CX3CR1 in STK $^{-/-}$ mice following acetaminophen intoxication may contribute to persistent HO-1 expression and increased numbers of F4/80 $^{+}$ alternatively activated macrophages.

The present studies demonstrate that there are multiple subpopulations of macrophages in the liver, which have the capacity to develop an alternatively activated repair phenotype, and that STK is key to regulating the response of the infiltrating CCR2 $^{+}$ subpopulation. It appears that in the absence of STK, exacerbated hepatotoxicity and mortality following acetaminophen administration is due, at least in part, to reduced activity of CCR2 $^{+}$ alternatively activated repair macrophages that infiltrate into the liver in response to MCP-1. However, at present we cannot exclude the possibility that observed changes in gene and protein expression in STK $^{-/-}$ mice are a consequence of heightened sensitivity of these animals to acetaminophen, rather than the loss of STK itself.

Evidence suggests that cross talk between macrophages and hepatocytes contributes to both the development of liver pathology and to initiation of tissue repair processes. Thus,

hepatocytes injured by acetaminophen release chemokines and damage associated molecular patterns, such as HMGB1 which induce macrophage accumulation in the liver and classical activation of these cells (Laskin et al., 1986; Imaeda et al., 2009; Martin-Murphy et al., 2010; Dragomir et al., 2011). These activated macrophages in turn generate cytotoxic and proinflammatory mediators which promote hepatocyte cytotoxicity. Subsequently, as the pathogenic process progresses, surviving hepatocytes release mediators that promote alternative macrophage activation, including IL-10, TGF β , and IL-4 (Dikopoulos et al., 2004; Gressner et al., 2008). Alternatively activated macrophages then generate mediators, which down regulate inflammation and stimulate hepatocyte proliferation, extracellular matrix turnover and wound repair. It appears that the outcome of the response to acetaminophen depends on the extent to which each of these cellular response pathways is activated. Thus in the absence of STK, alternative macrophage activity is reduced, resulting in increased oxidative stress and exaggerated hepatotoxicity. These findings are intriguing as they suggest several novel approaches for developing new therapeutics to mitigate hepatotoxicity induced by acetaminophen.

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Abbreviations

STK	stem cell-derived tyrosine kinase
IL	interleukin
MCP-1	monocyte chemoattractant protein-1
NAPQI	<i>N</i> -acetyl- <i>p</i> -benzoquinone-imine
Cyp	cytochrome P450
TNFα	tumor necrosis factor- α
TNFR	tumor necrosis factor receptor
HO-1	heme oxygenase-1
GSH	glutathione
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
ALT	alanine transaminase
AST	aspartate transaminase
PBS	phosphate-buffered saline
iNOS	inducible nitric oxide synthase
MIP	macrophage inflammatory protein

References

- Abdel-Zaher AO, Abdel-Rahman MM, Hafez MM, Omran FM. Role of nitric oxide and reduced glutathione in the protective effects of aminoguanidine, gadolinium chloride and oleanolic acid against acetaminophen-induced hepatic and renal damage. *Toxicology*. 2007; 234:124–134. [PubMed: 17391827]
- Antoniades CG, Quaglia A, Taams LS, Mitry RR, Hussain M, Abeles R, Possamai LA, Bruce M, McPhail M, Starling C, Wagner B, Barnardo A, Pomplun S, Auzinger G, Bernal W, Heaton N,

- Vergani D, Thursz MR, Wendon J. Source and characterisation of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology*. 2012 (in press). 10.1002/hep.25657
- Blazka ME, Germolec DR, Simeonova P, Bruccoleri A, Pennypacker KR, Luster MI. Acetaminophen-induced hepatotoxicity is associated with early changes in NF- κ B and NF-IL6 DNA binding activity. *J Inflamm*. 1995; 47:138–150. [PubMed: 9117441]
- Bower WA, Johns M, Margolis HS, Williams IT, Bell BP. Population-based surveillance for acute liver failure. *Am J Gastroenterol*. 2007; 102:2459–2463. [PubMed: 17608778]
- Brunner LJ, Bai S. Simple and rapid assay for acetaminophen and conjugated metabolites in low-volume serum samples. *J Chromatogr B Biomed Sci Appl*. 1999; 732:323–329. [PubMed: 10517354]
- Burke ML, McManus DP, Ramm GA, Duke M, Li Y, Jones MK, Gobert GN. Coordinated gene expression in the liver and spleen during *Schistosoma japonicum* infection regulates cell migration. *PLoS Negl Trop Dis*. 2010; 4:e686. [PubMed: 20502518]
- Campion SN, Johnson R, Aleksunes LM, Goedken MJ, van Rooijen N, Scheffer GL, Cherrington NJ, Manautou JE. Hepatic Mrp4 induction following acetaminophen exposure is dependent on Kupffer cell function. *Am J Physiol Gastrointest Liver Physiol*. 2008; 295:G294–304. [PubMed: 18556419]
- Chen YQ, Fisher JH, Wang MH. Activation of the RON receptor tyrosine kinase inhibits inducible nitric oxide synthase (iNOS) expression by murine peritoneal exudate macrophages: phosphatidylinositol-3 kinase is required for RON-mediated inhibition of iNOS expression. *J Immunol*. 1998; 161:4950–4959. [PubMed: 9794431]
- Chiu H, Brittingham JA, Laskin DL. Differential induction of heme oxygenase-1 in macrophages and hepatocytes during acetaminophen-induced hepatotoxicity in the rat: effects of hemin and biliverdin. *Toxicol Appl Pharmacol*. 2002; 181:106–115. [PubMed: 12051994]
- Chiu H, Gardner CR, Dambach DM, Brittingham JA, Durham SK, Laskin JD, Laskin DL. Role of p55 tumor necrosis factor receptor 1 in acetaminophen-induced antioxidant defense. *Am J Physiol Gastrointest Liver Physiol*. 2003a; 285:G959–G966. [PubMed: 12842828]
- Chiu H, Gardner CR, Dambach DM, Durham SK, Brittingham JA, Laskin JD, Laskin DL. Role of tumor necrosis factor receptor 1 (p55) in hepatocyte proliferation during acetaminophen-induced toxicity in mice. *Toxicol Appl Pharmacol*. 2003b; 193:218–227. [PubMed: 14644624]
- Cigremis Y, Turel H, Adiguzel K, Akgoz M, Kart A, Karaman M, Ozen H. The effects of acute acetaminophen toxicity on hepatic mRNA expression of SOD, CAT, GSH-Px, and levels of peroxynitrite, nitric oxide, reduced glutathione, and malondialdehyde in rabbit. *Mol Cell Biochem*. 2009; 323:31–38. [PubMed: 19039654]
- Cohen SD, Khairallah EA. Selective protein arylation and acetaminophen-induced hepatotoxicity. *Drug Metab Rev*. 1997; 29:59–77. [PubMed: 9187511]
- Connolly MK, Ayo D, Malhotra A, Hackman M, Bedrosian AS, Ibrahim J, Cieza-Rubio NE, Nguyen AH, Henning JR, Dorvil-Castro M, Pachter HL, Miller G. Dendritic cell depletion exacerbates acetaminophen hepatotoxicity. *Hepatology*. 2011; 54:959–968. [PubMed: 21574173]
- Cooper KO, Reik LM, Jayyosi Z, Bandiera S, Kelley M, Ryan DE, Daniel R, McCluskey SA, Levin W, Thomas PE. Regulation of two members of the steroid-inducible cytochrome P450 subfamily (3A) in rats. *Arch Biochem Biophys*. 1993; 301:345–354. [PubMed: 7681660]
- Correll PH, Iwama A, Tondat S, Mayrhofer G, Suda T, Bernstein A. Dereglated inflammatory response in mice lacking the STK/RON receptor tyrosine kinase. *Genes Funct*. 1997; 1:69–83. [PubMed: 9680329]
- D'Haese JG, Demir IE, Friess H, Ceyhan GO. Fractalkine/CX3CR1: why a single chemokine-receptor duo bears a major and unique therapeutic potential. *Expert Opin Ther Targets*. 2010; 14:207–219. [PubMed: 20055718]
- Dambach DM, Durham SK, Laskin JD, Laskin DL. Distinct roles of NF- κ B p50 in the regulation of acetaminophen-induced inflammatory mediator production and hepatotoxicity. *Toxicol Appl Pharmacol*. 2006; 211:157–165. [PubMed: 16081117]
- Dambach DM, Watson LM, Gray KR, Durham SK, Laskin DL. Role of CCR2 in macrophage migration into the liver during acetaminophen-induced hepatotoxicity in the mouse. *Hepatology*. 2002; 35:1093–1103. [PubMed: 11981759]

- Das J, Ghosh J, Manna P, Sil PC. Acetaminophen induced acute liver failure via oxidative stress and JNK activation: protective role of taurine by the suppression of cytochrome P450 2E1. *Free Radic Res.* 2010; 44:340–355. [PubMed: 20166895]
- Devey L, Ferenbach D, Mohr E, Sangster K, Bellamy CO, Hughes J, Wigmore SJ. Tissue-resident macrophages protect the liver from ischemia reperfusion injury via a heme oxygenase-1-dependent mechanism. *Mol Ther.* 2009; 17:65–72. [PubMed: 19002167]
- Diesen DL, Kuo PC. Nitric oxide and redox regulation in the liver: part II. Redox biology in pathologic hepatocytes and implications for intervention. *J Surg Res.* 2011; 167:96–112. [PubMed: 20400112]
- Dikopoulos N, Wegenka U, Kroger A, Hauser H, Schirmbeck R, Reimann J. Recently primed CD8⁺ T cells entering the liver induce hepatocytes to interact with naive CD8⁺ T cells in the mouse. *Hepatology.* 2004; 39:1256–1266. [PubMed: 15122754]
- Douni E, Akassoglou K, Alexopoulou L, Georgopoulos S, Haralambous S, Hill S, Kassiotis G, Kontoyiannis D, Pasparakis M, Plows D, Probert L, Kollias G. Transgenic and knockout analyses of the role of TNF in immune regulation and disease pathogenesis. *J Inflamm.* 1995; 47:27–38. [PubMed: 8913927]
- Dragomir AC, Laskin JD, Laskin DL. Macrophage activation by factors released from acetaminophen-injured hepatocytes: potential role of HMGB1. *Toxicol Appl Pharmacol.* 2011; 253:170–177. [PubMed: 21513726]
- Ellinger I, Fuchs R. Receptor-mediated and fluid-phase transcytosis of horseradish peroxidase across rat hepatocytes. *J Biomed Biotechnol.* 2010; 2010:850320. [PubMed: 20168981]
- Gardner CR, Gray JP, Joseph LB, Cervelli J, Bremer N, Kim Y, Mishin V, Laskin JD, Laskin DL. Potential role of caveolin-1 in acetaminophen-induced hepatotoxicity. *Toxicol Appl Pharmacol.* 2010; 245:36–46. [PubMed: 20100502]
- Gardner CR, Heck DE, Yang CS, Thomas PE, Zhang XJ, DeGeorge GL, Laskin JD, Laskin DL. Role of nitric oxide in acetaminophen-induced hepatotoxicity in the rat. *Hepatology.* 1998; 27:748–754. [PubMed: 9500703]
- Gardner CR, Laskin JD, Dambach DM, Chiu H, Durham SK, Zhou P, Bruno M, Gerecke DR, Gordon MK, Laskin DL. Exaggerated hepatotoxicity of acetaminophen in mice lacking tumor necrosis factor receptor-1. Potential role of inflammatory mediators. *Toxicol Appl Pharmacol.* 2003; 192:119–130. [PubMed: 14550746]
- Gardner CR, Laskin JD, Dambach DM, Sacco M, Durham SK, Bruno MK, Cohen SD, Gordon MK, Gerecke DR, Zhou P, Laskin DL. Reduced hepatotoxicity of acetaminophen in mice lacking inducible nitric oxide synthase: potential role of tumor necrosis factor- α and interleukin-10. *Toxicol Appl Pharmacol.* 2002; 184:27–36. [PubMed: 12392966]
- Gonzalez FJ. Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. *Mutat Res.* 2005; 569:101–110. [PubMed: 15603755]
- Gressner OA, Rizk MS, Kovalenko E, Weiskirchen R, Gressner AM. Changing the pathogenetic roadmap of liver fibrosis? Where did it start; where will it go? *J Gastroenterol Hepatol.* 2008; 23:1024–1035. [PubMed: 18505415]
- Han D, Shinohara M, Ybanez MD, Saberi B, Kaplowitz N. Signal transduction pathways involved in drug-induced liver injury. *Handb Exp Pharmacol.* 2010; 196:267–310. [PubMed: 20020266]
- Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets.* 2009; 8:307–318. [PubMed: 19534673]
- Ho VW, Sly LM. Derivation and characterization of murine alternatively activated (M2) macrophages. *Methods Mol Biol.* 2009; 531:173–185. [PubMed: 19347318]
- Hogaboam CM, Bone-Larson CL, Steinhäuser ML, Lukacs NW, Colletti LM, Simpson KJ, Strieter RM, Kunkel SL. Novel CXCR2-dependent liver regenerative qualities of ELR-containing CXC chemokines. *FASEB J.* 1999; 13:1565–1574. [PubMed: 10463948]
- Hogaboam CM, Bone-Larson CL, Steinhäuser ML, Matsukawa A, Gosling J, Boring L, Charo IF, Simpson KJ, Lukacs NW, Kunkel SL. Exaggerated hepatic injury due to acetaminophen challenge in mice lacking C-C chemokine receptor 2. *Am J Pathol.* 2000; 156:1245–1252. [PubMed: 10751350]

- Holt MP, Cheng L, Ju C. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J Leukoc Biol.* 2008; 84:1410–1421. [PubMed: 18713872]
- Hume DA, Robinson AP, MacPherson GG, Gordon S. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Relationship between macrophages, Langerhans cells, reticular cells, and dendritic cells in lymphoid and hematopoietic organs. *J Exp Med.* 1983; 158:1522–1536. [PubMed: 6355361]
- Imaeda AB, Watanabe A, Sohail MA, Mahmood S, Mohamadnejad M, Sutterwala FS, Flavell RA, Mehal WZ. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *J Clin Invest.* 2009; 119:305–314. [PubMed: 19164858]
- Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ, Yoshie O. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell.* 1997; 91:521–530. [PubMed: 9390561]
- Immenschuh S, Baumgart-Vogt E, Mueller S. Heme oxygenase-1 and iron in liver inflammation: a complex alliance. *Curr Drug Targets.* 2010; 11:1541–1550. [PubMed: 20704547]
- Inui M, Ishida Y, Kimura A, Kuninaka Y, Mukaida N, Kondo T. Protective roles of CX3CR1-mediated signals in toxin A-induced enteritis through the induction of heme oxygenase-1 expression. *J Immunol.* 2011; 186:423–431. [PubMed: 21131421]
- Ishida Y, Kondo T, Tsuneyama K, Lu P, Takayasu T, Mukaida N. The pathogenic roles of tumor necrosis factor receptor p55 in acetaminophen-induced liver injury in mice. *J Leukoc Biol.* 2004; 75:59–67. [PubMed: 14557383]
- Iwama A, Wang MH, Yamaguchi N, Ohno N, Okano K, Sudo T, Takeya M, Gervais F, Morissette C, Leonard EJ, Suda T. Terminal differentiation of murine resident peritoneal macrophages is characterized by expression of the STK protein tyrosine kinase, a receptor for macrophage-stimulating protein. *Blood.* 1995; 86:3394–3403. [PubMed: 7579443]
- Ju C, Reilly TP, Bourdi M, Radonovich MF, Brady JN, George JW, Pohl LR. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol.* 2002; 15:1504–1513. [PubMed: 12482232]
- Kempka G, Kolb-Bachofen V. Binding, uptake, and transcytosis of ligands for mannose-specific receptors in rat liver: an electron microscopic study. *Exp Cell Res.* 1988; 176:38–48. [PubMed: 3371424]
- Kinoshita M, Uchida T, Sato A, Nakashima M, Nakashima H, Shono S, Habu Y, Miyazaki H, Hiroi S, Seki S. Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *J Hepatol.* 2010; 53:903–910. [PubMed: 20739085]
- Koop DR. Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a. *Mol Pharmacol.* 1986; 29:399–404. [PubMed: 3702859]
- Kopydlowski KM, Salkowski CA, Cody MJ, van Rooijen N, Major J, Hamilton TA, Vogel SN. Regulation of macrophage chemokine expression by lipopolysaccharide *in vitro* and *in vivo*. *J Immunol.* 1999; 163:1537–1544. [PubMed: 10415057]
- Laskin DL. Macrophages and inflammatory mediators in chemical toxicity: a battle of forces. *Chem Res Toxicol.* 2009; 22:1376–1385. [PubMed: 19645497]
- Laskin DL, Chen L, Hankey PA, Laskin JD. Role of STK in mouse liver macrophage and endothelial cell responsiveness during acute endotoxemia. *J Leukoc Biol.* 2010a; 88:373–382. [PubMed: 20453108]
- Laskin DL, Gardner CR, Price VF, Jollow DJ. Modulation of macrophage functioning abrogates the acute hepatotoxicity of acetaminophen. *Hepatology.* 1995; 21:1045–1050. [PubMed: 7705777]
- Laskin DL, Pilaro AM. Potential role of activated macrophages in acetaminophen hepatotoxicity. I Isolation and characterization of activated macrophages from rat liver. *Toxicol Appl Pharmacol.* 1986; 86:204–215. [PubMed: 3024356]
- Laskin DL, Pilaro AM, Ji S. Potential role of activated macrophages in acetaminophen hepatotoxicity. II Mechanism of macrophage accumulation and activation. *Toxicol Appl Pharmacol.* 1986; 86:216–226. [PubMed: 3024357]
- Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: agents of defense or destruction? *Annu Rev Pharmacol Toxicol.* 2011; 51:267–288. [PubMed: 20887196]

- Laskin, JD.; Heck, DE.; Laskin, DL. Nitric oxide pathways in toxic responses. In: Ballantyne, B.; Marrs, T.; Syversen, T., editors. *General and Applied Toxicology*. Wiley-Blackwell; UK: 2010b. p. 425-438.
- Lawson JA, Farhood A, Hopper RD, Bajt ML, Jaeschke H. The hepatic inflammatory response after acetaminophen overdose: role of neutrophils. *Toxicol Sci*. 2000; 54:509–516. [PubMed: 10774834]
- Leenen PJ, de Bruijn MF, Voerman JS, Campbell PA, van Ewijk W. Markers of mouse macrophage development detected by monoclonal antibodies. *J Immunol Methods*. 1994; 174:5–19. [PubMed: 8083537]
- Leonis MA, Toney-Earley K, Degen SJ, Waltz SE. Deletion of the Ron receptor tyrosine kinase domain in mice provides protection from endotoxin-induced acute liver failure. *Hepatology*. 2002; 36:1053–1060. [PubMed: 12395314]
- Liu QP, Fruit K, Ward J, Correll PH. Negative regulation of macrophage activation in response to IFN- γ and lipopolysaccharide by the STK/RON receptor tyrosine kinase. *J Immunol*. 1999; 163:6606–6613. [PubMed: 10586055]
- Liu Y, Gardner CR, Francis M, Laskin JD, Laskin DL. Hepatic endothelial cells regulate liver cell repair following acetaminophen intoxication. *Toxicol Sci*. 2011; 120:97.
- Martin-Murphy BV, Holt MP, Ju C. The role of damage associated molecular pattern molecules in acetaminophen-induced liver injury in mice. *Toxicol Lett*. 2010; 192:387–394. [PubMed: 19931603]
- Masson MJ, Carpenter LD, Graf ML, Pohl LR. Pathogenic role of natural killer T and natural killer cells in acetaminophen-induced liver injury in mice is dependent on the presence of dimethyl sulfoxide. *Hepatology*. 2008; 48:889–897. [PubMed: 18712839]
- McLaughlin LA, Dickmann LJ, Wolf CR, Henderson CJ. Functional expression and comparative characterization of nine murine cytochromes P450 by fluorescent inhibition screening. *Drug Metab Dispos*. 2008; 36:1322–1331. [PubMed: 18420780]
- Michael SL, Pumford NR, Mayeux PR, Niesman MR, Hinson JA. Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species. *Hepatology*. 1999; 30:186–195. [PubMed: 10385655]
- Minamino T, Ito Y, Ohkubo H, Hosono K, Suzuki T, Sato T, Ae T, Shibuya A, Sakagami H, Narumiya S, Koizumi W, Majima M. Thromboxane A₂ receptor signaling promotes liver tissue repair after toxic injury through the enhancement of macrophage recruitment. *Toxicol Appl Pharmacol*. 2012; 259:104–114. [PubMed: 22206755]
- Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV Protective role of glutathione. *J Pharmacol Exp Ther*. 1973; 187:211–217. [PubMed: 4746329]
- Morrison AC, Wilson CB, Ray M, Correll PH. Macrophage-stimulating protein, the ligand for the stem cell-derived tyrosine kinase/RON receptor tyrosine kinase, inhibits IL-12 production by primary peritoneal macrophages stimulated with IFN- γ and lipopolysaccharide. *J Immunol*. 2004; 172:1825–1832. [PubMed: 14734766]
- Ozaki T, Habara K, Matsui K, Kaibori M, Kwon AH, Ito S, Nishizawa M, Okumura T. Dexamethasone inhibits the induction of iNOS gene expression through destabilization of its mRNA in proinflammatory cytokine-stimulated hepatocytes. *Shock*. 2010; 33:64–69. [PubMed: 19373131]
- Ray M, Yu S, Sharda DR, Wilson CB, Liu Q, Kaushal N, Prabhu KS, Hankey PA. Inhibition of TLR4-induced I κ B kinase activity by the RON receptor tyrosine kinase and its ligand, macrophage-stimulating protein. *J Immunol*. 2010; 185:7309–7316. [PubMed: 21078906]
- Rosenwasser LJ. Biologic activities of IL-1 and its role in human disease. *J Allergy Clin Immunol*. 1998; 102:344–350. [PubMed: 9768571]
- Sai Y, Dai R, Yang TJ, Krausz KW, Gonzalez FJ, Gelboin HV, Shou M. Assessment of specificity of eight chemical inhibitors using cDNA-expressed cytochromes P450. *Xenobiotica*. 2000; 30:327–343. [PubMed: 10821163]
- Si Y, Tsou CL, Croft K, Charo IF. CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. *J Clin Invest*. 2010; 120:1192–1203. [PubMed: 20234092]

- Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med*. 1992; 176:287–292. [PubMed: 1613462]
- Stuart WD, Kulkarni RM, Gray JK, Vasilias J, Leonis MA, Waltz SE. Ron receptor regulates Kupffer cell-dependent cytokine production and hepatocyte survival following endotoxin exposure in mice. *Hepatology*. 2011; 53:1618–1628. [PubMed: 21520175]
- Thomas JA, Pope C, Wojtacha D, Robson AJ, Gordon-Walker TT, Hartland S, Ramachandran P, Van Deemter M, Hume DA, Iredale JP, Forbes SJ. Macrophage therapy for murine liver fibrosis recruits host effector cells improving fibrosis, regeneration, and function. *Hepatology*. 2011; 53:2003–2015. [PubMed: 21433043]
- Toklu HZ, Sehrlirli AO, Velioglu-Ogunc A, Cetinel S, Sener G. Acetaminophen-induced toxicity is prevented by β -D-glucan treatment in mice. *Eur J Pharmacol*. 2006; 543:133–140. [PubMed: 16822497]
- Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol*. 2003; 3:133–146. [PubMed: 12563297]
- Volarevic V, Milovanovic M, Lujic B, Pejnovic N, Arsenijevic N, Nilsson U, Leffler H, Lukic ML. Galectin-3 deficiency prevents concanavalin A- induced hepatitis in mice. *Hepatology*. 2012 (in press). 10.1002/hep.25542
- Weis N, Weigert A, von Knethen A, Brune B. Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. *Mol Biol Cell*. 2009; 20:1280–1288. [PubMed: 19129475]
- Williams CD, Antoine DJ, Shaw PJ, Benson C, Farhood A, Williams DP, Kanneganti TD, Park BK, Jaeschke H. Role of the Nalp3 inflammasome in acetaminophen-induced sterile inflammation and liver injury. *Toxicol Appl Pharmacol*. 2011; 252:289–297. [PubMed: 21396389]
- Wilson CB, Ray M, Lutz M, Sharda D, Xu J, Hankey PA. The RON receptor tyrosine kinase regulates IFN- γ production and responses in innate immunity. *J Immunol*. 2008; 181:2303–2310. [PubMed: 18684919]
- Yuan L, Kaplowitz N. Glutathione in liver diseases and hepatotoxicity. *Mol Aspects Med*. 2009; 30:29–41. [PubMed: 18786561]
- Zhou YQ, Chen YQ, Fisher JH, Wang MH. Activation of the RON receptor tyrosine kinase by macrophage-stimulating protein inhibits inducible cyclooxygenase-2 expression in murine macrophages. *J Biol Chem*. 2002; 277:38104–38110. [PubMed: 12177064]

Article Highlights

- STK regulates alternative macrophage activation after acetaminophen intoxication.
- Loss of STK results in increased sensitivity to acetaminophen.
- Increased toxicity involves oxidative stress and decreases in repair macrophages.

Figure 1.

Effects of acetaminophen intoxication on serum transaminases. Wild type (white bars) and STK^{-/-} (black bars) mice were treated with acetaminophen or control (CTL). Serum was collected 6 h and 12 h later and analyzed for ALT and AST. Each bar represents the mean $\pm$ SE (n= 4–5 mice). ^aSignificantly different (p < 0.05) from CTL; ^bSignificantly different (p < 0.05) from wild type mice. ^cSignificantly different (p < 0.05) from 6 h post-acetaminophen.

Figure 2. Effects of loss of STK on acetaminophen-induced structural alterations in liver. Wild type and STK^{-/-} mice were treated with acetaminophen or control (CTL). Liver sections were prepared 6 h and 12 h later and stained with hematoxylin and eosin (original magnification $\times 100$). One representative section from 4–5 mice/group is shown.

Figure 3. Effects of loss of STK on acetaminophen metabolism. Serum was collected 0.5 h, 1 h and 2 h after administration of acetaminophen (APAP) or control (CTL) to wild type (white bars) and STK^{-/-} (black bars) mice, and analyzed by HPLC for the presence of APAP and APAP-glucuronide (-gluc) conjugate. Each bar represents the mean $\pm$ SE (n = 3 mice).

Figure 4.

Effects of loss of STK on acetaminophen-induced HO-1 expression. Livers were collected 6 h and 12 h after treatment of wild type (white bars) and STK^{-/-} (black bars) mice with acetaminophen (APAP) or control (CTL) and mRNA analyzed for HO-1 by real time PCR. Each bar represents the mean $\pm$ SE (n = 4–5 mice). ^aSignificantly different (p < 0.05) from CTL; ^bSignificantly different (p < 0.05) from wild type mice.

Figure 5.

Effects of loss of STK on F4/80 expression. Liver sections were prepared 6 h and 12 h after treatment of wild type and STK^{-/-} mice with acetaminophen or control (CTL). Sections were stained with antibody to F4/80. Binding was visualized using a Vectastain Elite ABC kit (original magnification X400). One representative section from 3–5 mice/group is shown.

Figure 6.

Effects of loss of STK on acetaminophen-induced alterations in expression of pro- and anti-inflammatory genes. Livers were collected 6 h and 12 h after treatment of wild type (white bars) and STK^{-/-} (black bars) mice with control (CTL) or acetaminophen (APAP) and mRNA analyzed by real time PCR. Each bar represents the mean $\pm$ SE (n = 3–5 mice). ^aSignificantly different (p < 0.05) from CTL; ^bSignificantly different (p < 0.05) from wild type mice.

Figure 7.

Effects of loss of STK on acetaminophen-induced expression of TNFR1 and iNOS. Livers were collected 6 h and 12 h after treatment of wild type mice (white bars) or STK^{-/-} (black bars) mice with acetaminophen (APAP) or control (CTL) and analyzed for TNFR1 and iNOS by Western blotting. Gels were scanned and the data presented relative to actin as the mean $\pm$ SE (n = 4–5 mice). ^aSignificantly different (p < 0.05) from CTL; ^bSignificantly different (p < 0.05) from wild type mice.

Table 1

Effects of Loss of STK on Cyp450 Activity in Mouse Liver Microsomes.

Enzymes	Wild Type	STK ^{-/-}
Cyp2e1	1.2 ± 0.05	1.3 ± 0.10
Cyp1a2	62.2 ± 15.86	44.5 ± 4.63
Cyp1a2 + α-naphthoflavone	3.9 ± 0.60 ^a	1.6 ± 0.17 ^a
Cyp3a	20.1 ± 1.43	19.3 ± 1.39
Cyp3a + ketoconazole	0.1 ± 0.01 ^a	0.1 ± 0.02 ^a

Liver microsomes were assayed for Cyp2e1, Cyp1a2 and Cyp3a activities as described in the Materials and Methods. In some experiments 1 μM α-naphthoflavone or ketoconazole was added to the assay mixtures to confirm enzyme specificity. Data are presented as pmol product/min/mg protein. Each value is the mean ± SEM of triplicate determinations from 3 mice.

^aSignificantly different (p < 0.05) from enzyme assays in the absence of inhibitor.

Table 2

Effects of Loss of STK on Hepatic GSH Content

Time after Acetaminophen (h)	Wild Type	STK ^{-/-}
CTL	100 ± 8.2 (8)	100 ± 5.7 (8)
0.5	47.6 ± 4.7 ^a (3)	38.1 ± 2.1 ^a (3)
2	35.3 ± 7.3 ^a (3)	32.8 ± 1.2 ^a (3)
6	63.6 ± 6.6 ^a (4)	30.2 ± 0.2 ^{a,b} (4)
12	69.5 ± 11.2 ^a (5)	47.4 ± 3.3 ^a (4)

Livers were collected 0.5–12 h following treatment of wild type and STK^{-/-} mice with acetaminophen or control (CTL) and analyzed for GSH content as described in the Materials and Methods. Data are presented as percentage of CTL. Each value is the mean ± SE of the number of animals indicated in parenthesis.

^aSignificantly different (p = 0.05) than CTL.

^bSignificantly different (p = 0.05) from wild type mice. Control GSH values were 3.4 ± 0.3 and 3.9 ± 0.2 μmol/g wet liver weight for wild type and STK^{-/-} mice, respectively.