Hepatic inflammatory mediators contribute to intestinal damage in necrotizing enterocolitis

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**Running Title:** Liver cytokines and NEC

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Abstract

Necrotizing enterocolitis (NEC) is a common and devastating gastrointestinal disease of premature infants. Along with pathologic effects in the ileum, severe NEC is often accompanied by multisystem organ failure, including liver failure. The aim of this study was to determine the changes in hepatic cytokines and inflammatory mediators in experimental NEC. The well-established neonatal rat model of NEC was used in this study, and changes in liver morphology, numbers of Kupffer cells (KC), gene expression and histologic localization of IL-18, TNFα and inducible nitric oxide synthase (NOS2) were evaluated. Intestinal luminal TNFα levels were also measured. Production of hepatic IL-18 and TNFα and numbers of KC were increased in rats with NEC and correlated with the progression of intestinal damage during NEC development. Further, increased levels of TNFα in the intestinal lumen of rats with NEC was significantly decreased when KC were inhibited with gadolinium chloride. These results suggest an important role of the liver and the gut/liver axis in NEC pathogenesis.

Keywords: gastrointestinal, inflammation, neonatal, gut/liver axis
Necrotizing enterocolitis (NEC) is the most common gastrointestinal disorder of premature infants (40). Despite affecting thousands of neonates in the US alone, the etiology of NEC is unknown and no effective preventative treatments exist (47). Development of NEC is likely multifactorial, with prematurity, enteral formula feeding, intestinal hypoxia/ischemia and bacterial colonization the major risk factors (11). Of the animal models developed to study this disease, the neonatal rat model incorporates the most common conditions for NEC in humans, an immature gastrointestinal tract, formula feeding and hypoxia, and thus provides an excellent paradigm for studying this disease (10, 48).

Proinflammatory cytokines, including IL-18, IL-12, TNFα and IFNγ, have been implicated in the pathophysiology of inflammatory bowel disease (6, 9, 26, 31, 33, 42). In addition, it has been suggested that the major risk factors for NEC promote an inflammatory cascade that results in the pathology associated with this disease (11, 39). We have recently shown that endogenous production of proinflammatory IL-18 and IL-12 in the distal ileum (site of injury) are increased and positively correlated with disease severity in the neonatal rat model of NEC. In contrast, production of proinflammatory TNFα and IFNγ was not elevated in the distal ileum during NEC development (20).

In addition to pathologic effects in the ileum, severe NEC injury is often followed by multisystem organ failure, including liver failure (34). While a number of studies have observed significant pathological changes in hepatic morphology and hepatobiliary function in babies with NEC (1, 36, 37), hepatic cytokine profiles during NEC development have not been examined. Transfer of ileal proinflammatory mediators into
the portal circulation may stimulate the resident hepatic macrophage, Kupffer cells (KC), to produce a variety of substances that can elicit hepatic injury. These proinflammatory compounds, including cytokines and nitric oxide, may also exacerbate intestinal injury via enterohepatic circulation connecting the liver and intestine. This type of remote organ injury to the liver and other organs has been well documented following intestinal ischemia/reperfusion (23, 57), which has also been implicated in the development of NEC (5, 51, 56).

Inducible nitric oxide synthase (NOS2) can be induced by proinflammatory cytokines, and sustained upregulation can lead to pathologic injury to the gut (28, 55). Nitric oxide (NO) can react with superoxide to produce peroxynitrite, and peroxynitrite is responsible, in part, for pathologic effects of NO (13, 14). Ford et al observed increased nitric oxide coupled with increased nitrotyrosine in intestinal enterocytes of infants with NEC (18) and we have found increased ileal NOS2 mRNA expression and protein localization in the neonatal rat model of NEC (unpublished data). However, NO has not been investigated in the liver during NEC development.

The aim of this study was to investigate if the gut-liver axis plays a role in the development and progression of NEC. NEC was induced in neonatal rats via enteral formula feeding coupled with exposure to asphyxia/cold stress. We evaluated numbers of KC, gene expression and localization of proinflammatory cytokines in the liver, histologic and metabolic signs of hepatic damage, and histologic localization of NOS2 and nitrotyrosine. In addition, we examined TNFα entering the intestinal lumen.
Methods:

Animal Model and Diets: This protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801-95081). Neonatal Sprague-Dawley rats (Charles River Labs, Pontage MI) originating from 14 separate litters were utilized in 5 different experiments. Newborn rats were collected immediately after birth to prevent suckling of maternal milk. Animals were assigned to one of two experimental groups based on randomized weight: artificially fed with rat milk substitute (RMS), or dam fed (control). Briefly, pups from both experimental groups were stressed twice daily with asphyxia (breathing 100% nitrogen gas for 60 seconds) followed by cold (4°C for 10 minutes) to induce experimental NEC in the RMS group (4, 10). Using this method, 80% of the RMS-fed rats develop NEC, versus 0% of the DF rats (15, 20). RMS pups were hand-fed with 0.1 ml formula every 3-4 hours during the first 48 hours (17, 50). The hand-feeding method was replaced with mechanized artificial feeding for an additional 48 hours (17). After 96 hours, all surviving animals were terminated via decapitation.

NEC Evaluation: Pathologic changes in intestinal architecture were evaluated using our recently developed NEC scoring system (15, 20). Briefly, after termination, the gastrointestinal tract was carefully removed. A 2 cm section of distal ileum next to the ileocecal valve from each animal was fixed overnight in 70% ethanol, paraffin-embedded, microtome-sectioned at 4-6 μm, and stained with hematoxylin and eosin for histological evaluation of NEC. Histological changes in the ileum were scored by a blinded evaluator and graded as follows: 0 (normal) – no damage; +1 (mild) - slight submucosal and/or lamina propria separation; +2 (moderate) - moderate separation of
submucosa and/or lamina propria, and/or edema in submucosal and muscular layers; +3 (severe) – severe separation of submucosa and/or lamina propria, and/or severe edema in submucosa and muscular layers, region villous sloughing; +4 (necrosis) – loss of villi and necrosis (15, 20).

Liver tissue preparation and immunohistology: After termination, livers were removed and a portion was fixed overnight in 70% ethanol, paraffin-embedded, serial-sectioned at 4-6 µm, and processed as previously described (20). Briefly, after deparaffinizing, sections were blocked with 1.5% appropriate serum (Vector Laboratories, Burlingame, CA), then incubated with 2.0 ug/ml goat polyclonal anti-rat - IL-18, -NOS2, nitrotyrosine, (Santa Cruz Biotechnology, Santa Cruz, CA), TNFα (R & D Systems, Minneapolis, MN) or mouse anti-rat monoclonal ED1, ED2 (Serotec, Raleigh, NC), followed by biotinylated secondary antibody (Vector Laboratories), Vectastain Elite ABC reagent (Vector Laboratories), diaminobenzidine (DAB) and counterstained with hematoxylin. Control sections were treated with the same procedure except they were incubated with 2.0 µg/ml goat Ig (Sigma) for IL-18, NOS2 and TNFα and 2.0 µg/ml mouse IgG1 (Serotec) instead of the specific primary antibodies. No immunostaining was observed in the controls. Sections from both experimental groups were stained for a specific primary antibody at the same time so that comparisons between groups could be assessed. Stained slides were evaluated by a blinded observer and enumeration of positively stained cells was accomplished by counting ten 20X microscopic fields.

RNA Preparation: The remaining portion of liver was frozen in liquid nitrogen for mRNA extraction. Total RNA was isolated from liver tissue using the RNeasy Mini
Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer's protocol. All samples were incubated with RNase-free DNase (20 U per reaction) for 10 min. at 37°C to eliminate DNA contamination. RNA concentration was quantified by UV spectrophotometry at 260 nm and the purity was determined by the A260/A280 ratio (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA). The integrity of RNA was verified by electrophoresis on a 1.2% agarose gel containing formaldehyde (2.2 M) and ethidium bromide in 1 x MOPS buffer {40 mM MOPS (pH 7.0), 10 mM sodium acetate and 1 mM EDTA (pH 8.0)}.  

**Reverse transcription (RT) and real-time reverse transcription polymerase chain reaction (real time RT-PCR):** Real-time RT-PCR Assays assays were performed to specifically quantify rat IL-18 and TNFα steady-state mRNA levels. Single-stranded complementary DNA (cDNA) was reverse-transcribed from 1 µg of total RNA in a 10 µl reaction mixture, as previously described in detail (16). The amounts of total RNA used in the RT reactions were calculated from the absorbency at 260 nm, and verified by densitometry of the 28S ribosomal RNA band separated on denaturing agarose gels (by Gel Doc 1000 Documentation System with Molecular Analyst/PC software, BIO-RAD, Hercules, CA). Real-time PCR amplification (7, 19) was performed using rat IL-18 and TNFα Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Foster City, CA) as described in the manufacturer's protocol. Samples were subjected to 40 cycles of amplification at 95° for 15 s followed by 1 min at 60° using an GeneAmp 5700 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Water controls were included to ensure specificity. Relative quantification of steady-state mRNA levels between experimental groups and all mRNA measurements were
calculated on the basis of total RNA concentration (8). Separate standard curves for IL-18 and TNFα were generated from serial dilutions of non-experimental intestinal total RNA (from 1 to 1000 ng per RT reaction). Real-time monitoring of fluorescent emission from cleavage of sequence-specific probes by the nuclease activity of Taq polymerase allowed definition of the threshold cycle during the exponential phase of amplification. Data were expressed as the fold induction of gene expression for animals with NEC (RMS fed) compared to that in the control group (dam fed) (20).

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT): Blood was obtained from each animal prior to sacrifice via cardiac puncture. Serum was stored at –70°C until utilized for diagnostic testing. AST and ALT levels were determined using a Sigma Diagnostic Transaminase kit (Sigma, St Louis, MO), procedure #505.

Gadolinium Chloride Injection: In two additional, independent studies, RMS-fed (NEC) and dam fed (control) rats were injected i.p. once daily with either 20 mg/kg gadolinium chloride (Sigma) or an equal volume of saline (personal communication with Dr. Pablo Muriel, Cinvesta, Mexico City, Mexico). This dose of gadolinium chloride decreased the number of ED2 positive KC in the liver by 87%. As previously described, all animals were subjected to asphyxia and cold stress once daily and after 96 hours, intestinal tissue was removed and flushed as described below.

Intestinal Flush: A 3 cm section of distal ileum was removed from each animal and gently flushed of intestinal content with 0.5ml sterile PBS. Flushed contents were frozen at –70°C until evaluated for TNFα levels via the Quantikine M rat TNFα Immunoassay (R & D Systems) according to the manufacturer’s protocol. All samples,
standards and controls were run in duplicate and plates were read on a SpectraMax Plus microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA).

Statistics: Statistical analyses between control and animals with NEC were performed using ANOVA followed by Fisher PLSD. Correlation analyses were performed using Spearman Rank Correlation. Analysis of NEC score was accomplished using the Mann-Whitney test for non-parametric values. All statistical analyses were accomplished using the statistical program StatView for Macintosh computers (Abacus Concepts, Berkeley, CA). All numerical data are expressed as mean ± SEM.

Results

Increased Kupffer cells (KC) in NEC liver. Newly recruited KC (ED1 positive) and resident KC (ED2 positive) were significantly increased in the livers of neonatal rats with NEC (fed RMS diet) compared to DF animals (Figure 1). Both ED1 and ED2 positive KC were distributed throughout the liver in both experimental groups (Figure 2). In addition, the number of hepatic ED2 positive cells was positively correlated with severity of intestinal damage ($r = 0.618; p \leq 0.01$).

Hepatic TNF$\alpha$ mRNA and TNF$\alpha$ and IL-18 positive cells were increased in NEC liver. TNF$\alpha$ mRNA levels in livers from animals with NEC were significantly increased compared to control pups. There was no difference in IL-18 mRNA levels between groups, however (Figure 3A). In contrast to the mRNA data, both TNF$\alpha$ and IL-18 positive cells were significantly increased in animals with NEC compared to controls (Figure 3B). TNF$\alpha$ and IL-18 positive cells were distributed throughout the liver in both groups (Figure 4). The number of TNF$\alpha$ and IL-18 positive cells were positively correlated with increasing severity of intestinal damage ($r = 0.568$ and $0.499$,
respectively; \( p \leq 0.05 \)). No differences were observed for either hepatic IL-12 mRNA or localization between groups (data not shown).

*Evaluation of damage in NEC liver.* Evaluation of the livers of animals with NEC did not reveal histologic signs of damage. Serum levels of ALT and AST were statistically, yet modestly, elevated in the NEC group (Figure 5). However, NOS2 was increased in NEC liver compared to controls (Figure 6A and 6B). Peroxynitrite, formed when NO reacts with superoxide, is too short lived to be easily detected. Fortunately, peroxynitrite forms nitrotyrosine residues that can be detected immunohistologically. Nitrotyrosine localization was also increased in rats with NEC (Figure 6C and 6D).

*Evidence of the gut/liver axis in NEC.* Our previous studies showed extremely low levels of TNF\(\alpha\) mRNA expression and protein localization in the ileum (20) and jejunum (unpublished data) of animals with or without NEC. Consequently, TNF\(\alpha\) measured in the luminal contents of the intestine should be produced primarily by the liver and enter the intestine via the biliary system. TNF\(\alpha\) from intestinal flushes of animals with NEC was statistically increased compared to controls (Figure 7). To prove that the increased TNF\(\alpha\) found in the intestinal luminal content of animals with NEC was derived from KC in the liver, RMS-fed (NEC) and dam fed rats (control) were injected with gadolinium chloride (Gad), which selectively inactivates KC *in vivo* (24). Intestinal luminal TNF\(\alpha\) was significantly decreased in the NEC + Gad group compared to the NEC group (Figure 7). Injection of neonatal rats with Gad also significantly decreased the number of both TNF\(\alpha\) and IL-18 positive cells in the liver, confirming that KC are the primary producers of both cytokines. Further, inactivation of KC significantly decreased the ileal NEC score 2-fold (Table 1) verifying that hepatic TNF\(\alpha\) plays an important role in
exacerbation of ileal damage in NEC. There were no differences in systemic blood levels of TNFα between groups, however (data not shown).

Discussion

The data presented herein show that the gut/liver axis plays an important role in NEC development. To our knowledge, this study is the first report describing dysregulation of proinflammatory cytokines and injury mediators in the liver and their association with intestinal damage during development and progression of NEC. These results suggest the importance of the liver in the pathophysiology of NEC by the release of inflammatory cytokines via the biliary system into the intestinal lumen, which can exacerbate injury in the intestine.

In this study, we demonstrate KC activation and up-regulation of both IL-18 and TNFα in the liver of animals with NEC is correlated with severity of ileal damage. Hepatic mRNA and production of the proinflammatory cytokines IL-1α, IL-1β, IL-6 and IL-12 was also examined, but there were no alterations between groups (data not shown). This is of particular interest because it appears that only certain cytokines are upregulated in the liver during the development of NEC. It has been suggested that during NEC pathogenesis, alterations in cytokine production are a result of a systemic inflammatory response syndrome (SIRS). However, in SIRS, a more generalized activation of proinflammatory cytokines occurs, including increased expression and production of IL-1 and IL-6 (12, 21, 46). Because increases in IL-1 and IL-6 are not observed, it is unlikely that a SIRS is wholly responsible for the up-regulation of hepatic IL-18 and TNFα.
In addition to ileal damage, severe NEC in infants is often accompanied by injury to the lungs, kidney and/or liver (34). A number of studies have shown significant pathologic changes in both hepatic morphology and hepatobiliary functions in infants with NEC, especially those receiving total parenteral nutrition (1, 36, 37). Moreover, hepatic injury has been documented following intestinal ischemia/reperfusion (23, 24, 57), which has been implicated in the development of NEC (5, 52, 56). However, little is known about the mechanisms initiating these changes, or proinflammatory cytokines and injury mediators during NEC development and progression. In this study, for the first time, pathologic changes in the liver during NEC were evaluated. Although minimal pathologic changes occurred in the liver, commonly utilized markers for assessing inflammatory mediated damage, nitrotyrosine, ALT and AST were increased in animals with NEC. While the increased ALT levels in animals with NEC did not approach a range generally associated with liver disease, the increase may suggest early signs of liver damage. (The more robust increase in AST is likely a reflection of damage to the intestine during NEC pathogenesis.) In the neonatal rat model of NEC, it is difficult to sustain the animals past 96 hours. It is possible that more evidence of extensive liver damage, both biochemically and histologically, would be observed if the studies could be extended. These data also suggest that in contrast to the activation of hepatic proinflammatory mediators, actual liver damage may not be a major hallmark of the pathophysiology of experimental NEC.

NOS2 is known to contribute to inflammatory diseases of the colon (22, 29, 30, 44) and NO has also been shown to play a role in the ileum during NEC pathogenesis (18). Further, unpublished data from our laboratory has shown increased ileal NOS2
protein and mRNA in neonatal rats with NEC. A number of studies have shown that increased production of NO, due to up-regulation of NOS2 after LPS challenge, can cause hepatic injury (35, 58) and NO scavengers can decrease hepatocellular injury after shock (32) and endotoxin challenge (38). Here, we show increased activated NOS2 in the liver of animals with NEC. In addition, nitrotyrosine, a footprint for activation of other cytotoxic reactive nitrogen species (41, 43, 45), is markedly increased in the liver during NEC development. The presence of nitrotyrosine on its own is not conclusive evidence that NO has reacted with oxygen metabolites, and, indeed, there is no obvious structural damage in the liver during NEC. Yet significant increases in mediators of inflammation (TNFα, IL-18, activated KC) are observed in the liver during disease development. We speculate that after exposure to ileal IL-18 and IL-12 and /or hepatic TNFα and IL-18 (49, 53), NF-kB is activated and translocated to the nucleus of hepatocytes, binding promoter regions of genes encoding NOS2, and activating transcription. NO could be synthesized by NOS2, and then react with superoxide anion to form peroxynitrite and other cytotoxic reactive nitrogen species (2, 3, 27).

We have shown that TNFα in the distal ileum (20) and jejunum (unpublished data) is produced at extremely low levels in animals with or without NEC. Jackson et al, demonstrated that during endotoxemia, increased TNFα in bile contributes to intestinal injury via the luminal route (25). Therefore, intestinal luminal TNFα should be derived from bile. Although testing TNFα levels in bile from neonatal rats is technically impossible, our present data reveal that increased TNFα in the luminal contents of animals with NEC is decreased to near control levels when KC are inhibited. Further, this study shows that inhibition of KC decreases the severity of NEC, providing
additional evidence that the gut/liver axis plays an important role in NEC pathogenesis. It is likely that hepatic IL-18 also exits the liver and enters the intestine in bile, however, we did not examine IL-18 levels in luminal ileal contents because it is produced constitutively in normal neonatal rats (54), and is up-regulated in ileum during NEC (20).

Using our previous data and the currently presented results, we propose the following paradigm for cytokine dysregulation and the gut/liver inflammatory loop (Figure 8). The risk factors for NEC – prematurity, formula feeding, intestinal hypoxia/ischemia and bacterial colonization – promote the increased production of ileal IL-18 and IL-12, which contributes to tissue damage in the intestine. During the progression of NEC, ileal IL-18 and IL-12 enter the liver via the portal vein, which activates and increases the number of KC. The activated KC produce proinflammatory IL-18 and TNFα that may induce early liver injury through activation of NOS2 and induce the formation and release of additional proinflammatory mediators from hepatocytes. Hepatic-derived cytokines exit the liver and enter the gut to complete the inflammatory loop via bile and/or systemic circulation resulting in enhanced intestinal inflammation and tissue damage.

The results of this study show, for the first time, concomitant activation of KC, IL-18 and TNFα in the liver of neonatal rats with NEC and provide evidence that the gut/liver axis plays an important role in the progression of NEC. These findings suggest the importance of the liver in the pathophysiology of NEC and may provide the basis for therapeutic approaches to this disease.

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References


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NF-kappaB and enhance IFN-gamma-mediated STAT1 phosphorylation.


Table 1. *Effect of gadolinium chloride injection on median ileal NEC score and hepatic TNFα and IL-18 positive cells*

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<th>NEC</th>
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<tr>
<td>NEC Score</td>
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<td>TNFα positive cells</td>
<td>4.8 ± 0.3</td>
<td>0.9 ± 0.5*</td>
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<tr>
<td>IL-18 positive cells</td>
<td>31.2 ± 5.1</td>
<td>9.7 ± 1.0*</td>
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Neonatal rats with developing NEC were injected once per day with either 20 mg/kg gadolinium chloride (Gad) and an equal volume of saline. Histologic NEC score was determined by a blinded observer using our published NEC scoring system. Histologic changes in ileal structure were scored as follows: 0 = normal, undamaged; 1 = slight; 2 = moderate; 3 = severe; 4 = complete necrosis. Mean TNF and IL-18 positive cells were counted from 10 20X microscopic fields, data are expressed as mean (mean ± SEM). # *p* ≤ 0.01; *p* ≤ 0.05.
Figure Legends

Figure 1. ED1 and ED2 positive Kupffer Cells (KC) are increased in animals with NEC. ED1 and ED2 positive KC were visualized in control (n = 12) and animals with NEC (n = 12) and positive cells were counted from 10 20X microscopic fields. * $p \leq 0.05$; # $p \leq 0.01$.

Figure 2. Histologic localization of ED1 and ED2 positive KC. Representative sections from control (A and C) and rats with NEC (B and D) stained with ED1 (top row) or ED2 (bottom row). Positive cells for both markers were found throughout the liver. (Magnification 100 X).

Figure 3. TNF$\alpha$ and IL-18 mRNA expression (A) and positive cells (B) in neonatal rat liver. Mean relative mRNA was determined using real-time RT-PCR from control (n = 15) and rats with NEC (n = 17). Mean TNF$\alpha$ and IL-18 positive cells for control (n = 9) NEC (n = 12) groups were counted from 10 20X microscopic fields. * $p \leq 0.05$; # $p \leq 0.01$.

Figure 4. Histologic localization of TNF$\alpha$ and IL-18 positive cells. Representative sections from control (A and C) NEC (B and D) rats stained for TNF$\alpha$ (top row) or IL-18 (bottom row). TNF$\alpha$ and IL-18 positive cells were increased in animals with NEC (B and D). Positive cells for both cytokines were observed throughout the liver. (Magnification 100X).
Figure 5. Serum transaminase levels in animals with and without NEC. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were statistically increased in rats with NEC (n = 6) versus control rats (n = 5). * $p \leq 0.05$.

Figure 6. Histologic localization of NOS2 and nitrotyrosine. Representative sections from control (A and C) and NEC (B and D) animals stained for NOS2 (top row) or nitrotyrosine (bottom row). NOS2 and nitrotyrosine were increased in animals with NEC (B and D). (Magnification 40X)

Figure 7. Inactivation of KC decreases intestinal luminal TNF$\alpha$ in neonatal rats with NEC. Animals were injected with either 20 mg/kg gadolinium chloride (Gad) or an equal amount of saline once per day for 4 days. Intestinal luminal TNF$\alpha$ was quantified using sandwich ELISA from control (n = 7), control + Gad (n = 9), NEC (n = 10) and NEC + Gad (n = 14) groups. * $p \leq 0.001$ vs control and control + Gad. # $p \leq 0.001$ vs NEC.

Figure 8. Working model for cytokine dysregulation and the gut/liver inflammatory loop in NEC. During NEC development, the liver receives ileal proinflammatory IL-18 and IL-12 (1) via the portal vein (2). Ileal-derived IL-18 and IL-12 activate KC (3), which induces production of additional proinflammatory mediators from KC and/or hepatocytes (i.e., IL-18, TNF$\alpha$ and NOS2) (4) that can cause liver injury (5). In addition, hepatic-derived cytokines would exit the liver and enter the gut to complete the inflammatory loop via systemic circulation and/or bile (6), resulting in enhanced intestinal inflammation (7).
Figure 1.
Figure 3.

A

![Chart A](image-url)

B

![Chart B](image-url)
Figure 5.
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