The Role of Bile Acids In Cholestatic Liver Injury in Mice and Man

By

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Abstract: Cholestasis is a reduction in bile flow that occurs during numerous pathologies. Cholestasis leads to significant liver toxicity, biliary hyperplasia, and liver cirrhosis. The molecular mechanisms behind the early liver injury associated with cholestasis are extensively studied, but the details, especially in man, are not well understood. The predominant hypothesis for the cause of cholestatic liver injury is that a buildup of toxic bile acids in liver and serum leads to hepatocellular apoptosis. While the direct toxicity of bile acids is supported by *in vitro* studies in primary rat hepatocytes and transfected human hepatoma lines, recent studies measuring the concentrations of individual bile acids after cholestasis *in vivo* has led us to reevaluate mechanisms of cytotoxicity during cholestasis, as bile acid levels in man may not reach the necessary concentration for onset of toxicity. *Thus, the overarching goal of this dissertation project was to determine the effect of pathophysiologically relevant concentrations of bile acids in man, with an emphasis on the human condition.* This project is focused upon understanding the mechanisms and cellular events that determine how cholestasis results in liver injury with the hope of furthering understanding the progression of the injury *in vivo* both in human patients, human hepatocyte lines and murine models. This study resulted in a number of findings that are potentially significant to the field of cholestatic liver injury. Primarily, human hepatocytes are resistant to bile acid induced apoptosis, and moreover human patients undergo relatively little apoptosis during cholestatic liver injury. This is likely due to a combination of dramatic differences between human and rodent bile acid compositions and the pathophysiology of *in vivo* models versus *in vitro* modeling in rodents. Further work is necessary to fully ascertain how, and why, human hepatocytes and human patients undergo liver injury during cholestasis.

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To my parents, Tom and Carol, and my brother Blake – Thank you for instilling in me a respect for the acquisition of knowledge and its pursuit. I can never repay how much you've done for me, and given me.

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List Of Abbreviations:

ALT - alanine aminotransferase

APAF-1 – apoptosis activating factor 1

BA - bile acid

BDL - bile duct ligation

BSEP - bile salt export pump

CA - cholic acid

CAD - caspase activated DNAse

CDCA - chenodeoxycholic acid

cK18 - caspase-cleaved cytokeratin-18 fragment

Cox-2 - cyclooxygenase-2

DAMP - damage associated molecular pattern

DCA - deoxycholic acid

Egr-1 - early growth response factor 1

ERCP - endoscopic retrograde cholangiopancreatography

FXR - farnesoid X receptor

H&E - hematoxylin and eosin

HMGB1 - high mobility group box 1

HO-1 - heme oxygenase-1

hsp86 - heat shock protein-86

Gal/End - galactosamine/endotoxin

GCA - glycocholic acid

GCDC - glycochenodeoxycholate

GDCA - glycochenodeoxycholic acid

Gpx-1 - glutathione peroxidase-1

ICAM-1 - intercellular adhesion molecule-1

IL-1ß - interleukin-1ß

IL-6 - interleukin-6

IL-10 - interleukin-10

iNOS - inducible nitric oxide synthase

LC/MS - ultra high performance liquid chromatography tandem mass spectrometry

LCA - lithocholic acid

αMCA - α-muricholic acid

ßMCA - ß-muricholic acid

miR-122 - micro RNA-122

mKC - mouse keratinocyte chemoattractant

NF-κB - nuclear factor κ B

NTCP - sodium taurocholate cotransporting polypeptide

OATP - organic anion transporting polypeptide

Mac-1 - macrophage 1 antigen

MIP-2 - macrophage inflammatory protein 2

MT-1 - metallothionein-1

RIP1 – receptor interacting protein kinase 1

RIP3 – receptor intereacting protein kinase 3

TCA - taurocholic acid

TCDC - taurochenodeoxycholic acid

TDCA - taurochenodeoxycholic acid

TLCA - taurolithocholic acid

TNF-α - tumor necrosis factor-alpha

TNFR1 - tumor necrosis factor alpha receptor

TSP-1 - thrombospondin 1

UDCA - ursodeoxycholic acid

VCAM-1 - vascular adhesion molecule-1

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Chapter 1: Introduction

1.2 A Brief History on the Theoretical Mechanisms and Modality of Cell Death during Cholestatic Liver Injury

1.1.1 Mechanisms of Cholestatic Liver Injury

Cholestasis is a reduction or stoppage in bile flow. The constituents of bile are generated in hepatocytes and cholangiocytes, and are composed of water, glutathione, proteins, lipids such as phosphatidyl choline, and bile acids. These components are excreted into the biliary tracts both through passive diffusion and through transporters present on the canalicular surface of hepatocytes and cholangiocytes. While minor to moderate cholestasis can be tolerated over a long period, complete blockage or sustained cholestasis results in significant liver injury, and can progress to fibrosis and cirrhosis if left untreated.

Cholestasis is a component of multiple different pathologies, including, but not limited to: autoimmune hepatitis-like diseases such as primary sclerosing cholangitis and primary biliary cirrhosis, numerous extrahepatic biliary obstructive diseases such as gall stones or biliary stricture, and multiple cholangiopathies such as biliary atresia. One common component of all cholestatic disorders is the accumulation of bile acids in hepatocytes that occurs due to the interruption in bile flow. This accumulation of bile acids has repeatedly been implicated in the pathogenesis of cholestatic liver injury, although the mechanisms behind how bile acids or bile salts cause toxicity are controversial (Malhi et al., 2010; Woolbright and Jaeschke, 2012). Due to their detergent-like physical nature, the idea of the inherent toxicity of bile salts or bile acids was proposed as early as the 1950s with the acknowledgement that increased systemic bile acid levels were associated

with disruption of plasma membranes via their detergent effects. Later experiments began to characterize individual bile acid's toxicity, with it becoming apparent that hydrophobicity of bile acids correlated with toxicity when bile acids were administered alone (Miyai et al., 1982). This led to the theory that bile toxicity was mediated via its hydrophobicity (or lipophilicity), and that this toxicity can be reduced by increasing the amount of hydrophilic bile acids systemically, by adding them directly, or by favoring conjugation reactions to the hydrophilic amino acid taurine (Scholmerich et al., 1984). At nearly the same time, it was noticed that lithocholic acid (LCA) was not only particularly hydrophobic and toxic (Scholmerich et al., 1984), but also led to cholestasis when given in excess (Tuchweber et al., 1983). Thus, LCA was established as the primary mediator of cholestatic liver injury and bile acid toxicity. A number of other interventions, aimed at preventing the creation of LCA from chenodeoxycholic acid (CDCA), were based on protection from LCA induced injury in squirrel monkeys that do not effectively process CDCA (Suzuki et al., 1985). This information, along with the subsequent information that hepatotoxic bile acids such as LCA and CDCA were not all that common in liver or serum of patients led to alternate theories (Takikawa et al., 1985).

Foremost was the idea that accumulation of bile acids in hepatocytes led to hepatocellular apoptosis (Spivey et al., 1993; Patel et al., 1994). Based on the fact that glycochenodeoxycholate (GCDC) was the most common bile acid found in serum, numerous groups began to administer 50-100 μM GCDC to rat hepatocytes in culture (Spivey et al., 1993; Patel et al., 1994; Webster and Anwer, 1998; Yerushelmi et al., 2001). Over the course of the next decade, a number of elaborate experiments consistently showed GCDC induced both intrinsic and extrinsic apoptosis that was

dependent on mitochondrial destabilization and Fas-induced apoptosis (Jones et al., 1998; Miyoshi et al., 1999; Faubion et al., 1999; Sodeman et al., 2000). This injury could be prevented via inhibition of caspases (Jones et al., 1998), cathepsin B (Roberts et al., 1997), or overexpression of anti-apoptotic proteins (Higuchi et al., 2001). Attempts to translate this to *in vivo* disease models appeared promising at first as the cathepsin B knockout mouse (Canbay et al., 2001) and the Fas receptor knockout mouse (Miyoshi et al., 1999) were protected from acute injury induced by bile duct ligation (BDL). At the time, this was thought to confirm signaling pathways previous established in the rat. These theories were later challenged when it was shown that inhibition of neutrophil extravasation by knockout of either intracellular adhesion molecule 1 (ICAM-1) (Gujral et al., 2004a) or cluster of differentiation protein 11b (CD11b), a component of the macrophage 1 antigen (Mac-1) complex, (Gujral et al., 2003), were protective against BDL-induced injury. Additionally, chlorotyrosine adducts, a byproduct of neutrophil-mediated cellular injury via the adduction of proteins by hypochlorous acid, were found to largely correlate with areas of focal necrosis after BDL in mice (Gujral 2004c). Further investigation into protection in the Fas receptor knockout mouse indicated that the protection was contingent not upon activation of the Fas receptor, but a blunted inflammatory response that reduced neutrophil recruitment to the area of biliary infarction (Gujral et al., 2004b). Moreover, inhibition of caspases was protective *in vivo* against established models of apoptosis (Gujral et al., 2003b), but not protective against BDL-induced injury (Gujral et al., 2004b). Further studies indicated a lack of apoptotic morphology in bile duct ligated mice, especially when compared to Jo-2 antibody induced apoptosis (Fickert et al., 2005). Finally, BH3 interacting domain death agonist

(BID) knockout mice were not protected against BDL injury, confirming the lack of bid involvement in cholestatic liver injury (Nalapareddy et al., 2009). As such, neutrophil-mediated injury was generally accepted as an alternate cause of BDL-induced injury (Copple et al., 2011). These findings were supported by data in other models of cholestasis where neutrophils were also found to play a primary role in the progression of injury (Kodali et al., 2006; Luyendyk et al., 2011).

A critical question has thus become what is the primary stimulus for inflammation in the BDL model? Interestingly, bile acids were found to induce pro-inflammatory genes when given to hepatocytes at pathophysiologically relevant concentrations (Allen et al., 2011). Taurocholic acid was found to induce expression of ICAM-1 and also increase production and secretion of pro-inflammatory cytokines such as chemokine C-X-C motif ligands 1 and 2 (abbreviated mKC and MIP-2 in this manuscript). Of critical importance to this pathophysiology is whether or not the levels of taurocholic acid (TCA) required for gene induction were attainable *in vivo* in murine models of cholestasis. To answer this question, bile acid levels were quantified at time points between 6 h and 14 days after cholestasis in multiple compartments (Zhang et al., 2012). Interestingly, levels of hydrophilic bile acids such as TCA were found to be elevated to millimolar levels in serum (Zhang et al., 2012), consistent with dramatic cytokine induction; however, levels of GCDC and other toxic bile acids were at nM concentrations. Given these data, the beginning working hypothesis of this dissertation was bile acid accumulation in hepatocytes and serum resulted in a sustained neutrophil response that recruited cytotoxic neutrophils to liver. This hypothesis was tested rigorously in primary murine, rat, and

human hepatocytes as well as in animal models of cholestasis (BDL and LCA administration) and in patients with extrahepatic cholestasis.

1.1.2. Necrosis, Apoptosis, and Necroptosis during Cholestatic Liver Injury

Multiple different cell death modalities or pathways have been identified. Primarily, these consistent of three different and distinct entities: necrosis, apoptosis and necroptosis. These pathways will be discussed individually as well as in the context of cholestatic liver injury.

The first well identified cell death pathways was oncosis, or as it is more commonly referred to in the modern literature, necrosis. Necrosis can be caused by multiple sources but is commonly seen in experimental liver injury in response to ischemia-reperfusion injury (Jaeschke and Lemasters 2003), acute drug intoxication (McGill et al., 2012) and during tumor necrosis related to hepatocellular carcinoma. Necrosis is characterized by intracellular depletion of ATP, membrane disturbances, and commonly involves mitochondrial dysfunction. As necrosis can occur in response to a varied number of stimuli there are a limited number of conserved mechanisms. Necrosis results in loss of plasma membrane potential and release of intracellular components. Overload of ions such as calcium is known to cause necrosis in a number of models, presumably due to membrane leakage and disruption. These components can be highly pro-inflammatory (Tsung et al., 2005a; Tsung et al., 2005b) and thus necrosis is a less desirable form of cell death (Jaeschke and Lemasters, 2003). For more information on release of cellular components and their inflammatory potential, see part *1.3*. Previous data from our laboratory and others has suggested cholestatic liver injury occurs through necrosis

(oncosis) (Fickert et al., 2005; Gujral et al., 2004b). Part of the dissertation will revisit this issue, as it has been challenged repeatedly in recent years.

Apoptosis is the predominant form of programmed cell death in the body. Apoptosis is necessary for both development and survival. Apoptosis is predominant during development in the body and is responsible for clefting of ridges, formation of new tissue types and more. During life, apoptosis is necessary for the removal of old, damaged cells and the replacement of these cells with new ones. Apoptotic cell death occurs through a number of different mechanisms, but the intracellular pathway is fairly conserved. Activation of apoptosis by death receptors or other stimuli results in activation or cleavage of caspases, a cysteine/aspartate protease, that eventually activate the caspase activated DNase (CAD) which translocates to the nucleus and dices DNA, killing the cell. This process is sometimes enhanced through mitochondrial involvement. In this case, Bid protein translocates to the outer leaflet of the mitochondria and forms a pore together with other Bcl-2 protein family members resulting in the release of intermembrane proteins such as cytochrome c. Cytochrome c then binds caspase-9, apoptotic protease activating factor (APAF-1) and ATP to form the "apoptosome." This complex enhances caspase activation and further drives the apoptotic process. Prevention of caspase cleavage completely inhibits apoptosis as CAD is never activated and the cells never die. Apoptosis has been proposed as a link between cell death and inflammation (Canbay et al,. 2003). During apoptosis, the cell shrinks and condenses into a morphologically distinct unit called an apoptotic body. These bodies can be phagocytized by macrophages, which activate the macrophage and causes release of pro-inflammatory mediators. Interestingly, if the macrophages are overrun with apoptotic cells, the injury

will proceed to necrosis as macrophages cannot continue to phagocytize apoptotic bodies (Gujral et al., 2004). It has been proposed previously that the apoptosis present during cholestasis, and the subsequent macrophage involvement is a mediator of inflammation during cholestatic liver injury. This hypothesis has been challenged numerous times before and may be contingent upon the model in use as macrophage depletion has been viewed both positively (Canbay et al., 2003) and negatively (Gehring et al, 2006) in the context of cholestatic liver injury. This hypothesis will be examined during this dissertation.

While previous studies assumed necrosis was not strictly controlled, a new form of necrosis called programmed necrosis or necroptosis has recently been identified (Zhang et al., 2009; Wu et al., 2013). This newly defined form of cell death occurs through interactions between caspase-8 and the receptor interacting protein kinases 1 and 3 (RIP1 and RIP3). The classical example of necroptosis comes from the tumor necrosis factor α (TNF-α) field. When TNF-α binds the TNF receptor, this activates both the NFκB pathway immediately and subsequently activates a caspase cascade that results in apoptosis. Under caspase inhibition or depletion of caspase 8 genetically, RIP3 and RIP1 instead form a complex known as the ripoptosome that mediates downstream necrosis. Depletion of RIP3 after inhibition of caspases or depletion of caspase 8 can then be protective against injury, as RIP3 seems to be the primary mediator of the switch to programmed necrosis (Zhang et al., 2009). While the downstream pathways that mediate programmed necrosis are not well characterized yet, considerable work is being undertaken in this arena in multiple models where RIP3 is presumed to play a role in the injury.

1.2 Bile Acid Metabolism, Bile Acid Transport, Bile Flow, and Enterohepatic Circulation

1.2.1 Synthesis and Conjugation

Bile acids are synthesized from cholesterol in a multistep process with two distinct pathways that produce the primary bile acids cholic acid (CA) and CDCA (Chiang, 2013). Both pathways primarily involve the oxidation of cholesterol at specific sterol ring carbons and are dependent upon which enzyme begins the process. In the classic pathway, cytochrome P450 (CYP) 7A1 oxidizes the 7 position carbon producing 7-hydroxycholesterol in the rate limiting step in the reaction. CYP8B1 and hydroxysteroid dehydrogenase 3B7 (HSD3B7) then produces cholyl-CoA in a multi-step process. The acetyl CoA group is then removed by cholyl CoA hydrolase and CA results. Alternately, the first step can be carried out by CYP27A1 resulting in chenodeoxycholic acid in what is termed the "acidic pathway" or alternate pathway. CA and CDCA can be processed by gut bacteria into deoxycholic acid (DCA) and LCA from CA and CDCA, respectively. This dehydroxylation reaction is carried out exclusively in the gut by gut bacteria and is dependent upon functional enterohepatic circulation. All of these bile acids are then commonly conjugated to either the amino acid taurine or the amino acid glycine in liver cells in a two-step process carried out by the enzymes cholyl-CoA synthetase and bile acid CoA:amino acid N-acetyl transferase (BAAT) (Figure 1). In addition to detoxifying the molecule, conjugation increases hydrophilicity which improves solubility in bile and promotes bile flow. The resulting amino acid conjugates taurocholic

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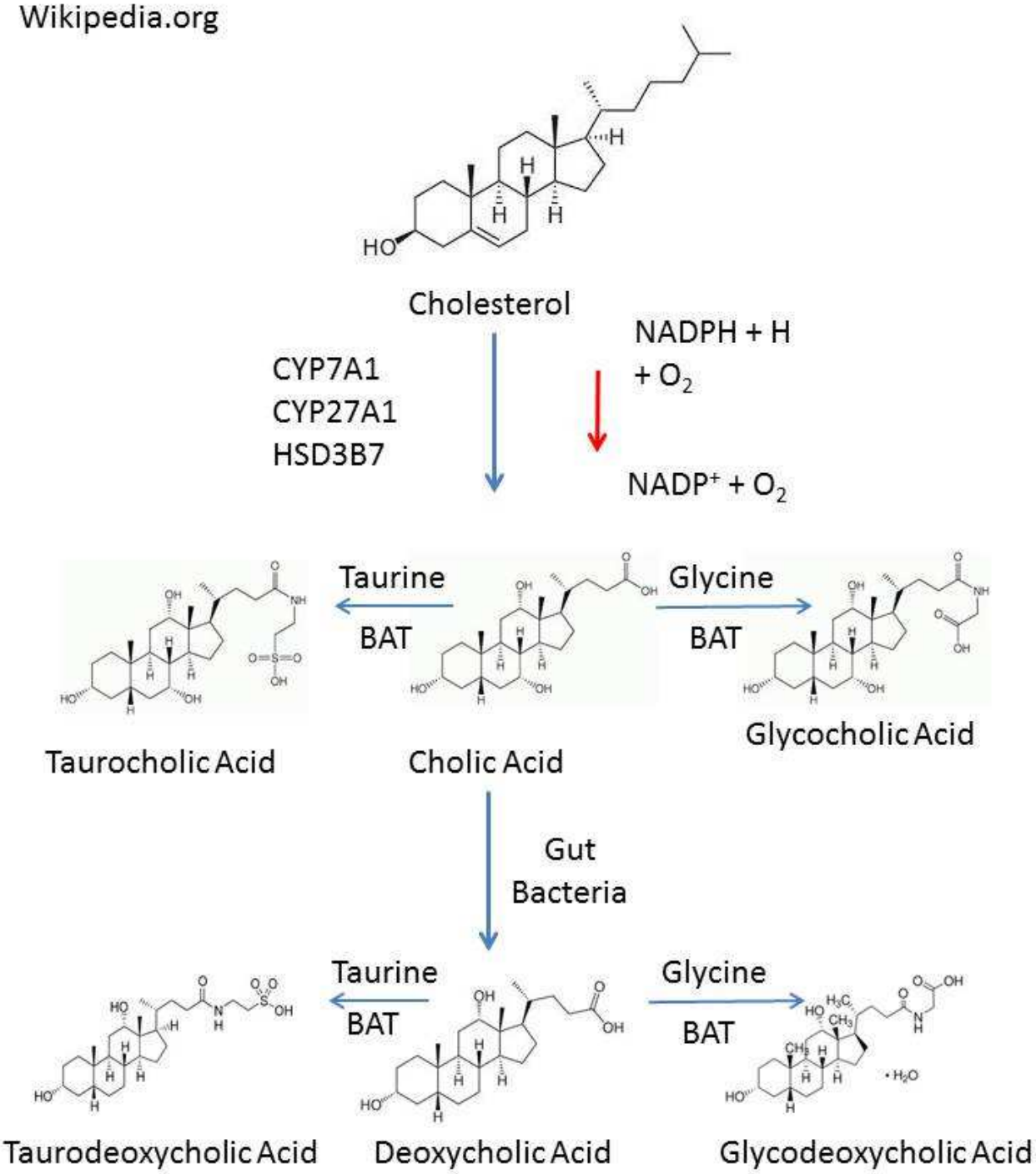

Figure 1.1: Metabolism of cholesterol into cholic acid and its derivatives.

acid (TCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDC) form the four most common species of bile acids circulating in humans (Trottier et al., 2011). In contrast, rodents produce α and ß muricholic acid from chenodeoxycholic acid through an additional hydroxylation of the 6 carbon, further reducing the hydrophobicity of the molecule. These molecules also make up a significant portion of the bile acid pool in mice (Zhang et al., 2012).

1.2.2 Bile Acid Transport and Bile Flow – Uptake and Export

Bile acids are taken up very effectively from serum, with greater than 90% of bile acids being recycled on a daily basis. This efficient uptake is mediated largely by the presence of transporters on the associated tissue that bile acids contact and includes liver, the biliary tracts, the kidney, and the intestinal tracts. While these transporters are present to some degree in the kidney and the intestines, this dissertation will focus largely on liver transporters of bile acids; these are most relevant for cholestatic liver injury. One of the primary functions of the liver is the secretion of bile acids and the maintenance of bile flow. Bile flow is contingent upon two different types of solute movement. First, the excretion of bile acids into the biliary tracts by hepatic transporters, which results in water following the organic salts and maintains biliary iso-osmolarity. This is bile acid dependent flow. Second is the active secretion of glutathione, which drives other organic solutes along with it and helps to drive biliary flow as water follows the solute gradient. This is the bile acid-independent bile flow as it is driven largely by glutathione.

A majority of bile acid export occurs through the bile salt export pump, BSEP. BSEP is a member of the ATP binding cassette class of transporters that uses ATP as an energy

source to move bile salts across the hepatic membrane against their concentrations gradient (Stieger, 2011). BSEP levels are strongly regulated by the farnesoid X receptor (FXR) (Huang et al., 2006). Intracellular increases in bile acids activate FXR which then increases BSEP expression (Zollner et al., 2003). BSEP is exclusively expressed in the liver on the canalicular side of the hepatocyte where it directly shuttles bile acids into bile. In addition to being a major determinant of bile acid-dependent bile flow, BSEP has become a major pharmacological interest as numerous recent drug and drug candidates have been shown to inhibit BSEP function (Kubitz et al., 2012). BSEP inhibition has serious clinical complications as the resulting cholestasis causes increases in liver transaminases, reduction in liver function and can lead to complications or death if left untreated and the patient remains on the drug (Fattinger et al., 2011). Currently the most recognized BSEP inhibitors are troglitazone, removed from the market for idiosyncratic liver toxicity, and bosentan, which comes with a black box warning requiring monitoring of serum transaminase levels during treatment (Padda et al., 2011). Other transporters also actively secrete bile acids include the Mdr2 protein. Deficiency of Mdr2 leads to progressive familial intrahepatic cholestasis (PFIC) in humans and a similar sclerosing cholangitis-like syndrome in mice when the corresponding gene (Mdr3) is knocked out (Trauner et al., 2013). Similarly, knockout of Mrp1 results in Dubin-Johnson syndrome, associated with an impaired ability to secrete conjugated bilirubin into bile.

In addition to canalicular export transporters, hepatocytes express a number of basolateral bile acid transporters for uptake of bile acids or excretion of bile acids into serum. The most important bile acid uptake transporter is the sodium taurocholate cotransporting polypeptide, NTCP (Hagenbuch & Meier, 1996). NTCP is a sodium dependent bile salt

uptake transporter that is a member of the solute carrier (SLC) family of proteins. Up to 80% of all bile acid uptake from serum occurs through NTCP in both mice and man (Hagenbuch & Meier, 1996; Szabo et al., 2013). NTCP function is critical to the bile acid response experimentally; NTCP protein expression is highly regulated at the genetic level. Hepatocytes left in culture will rapidly de-differentiate and downregulate NTCP (Liang et al., 1996). This results in a dramatically blunted response to bile acids. Regulation of NTCP has also been characterized during cholestasis *in vivo* after BDL. Chronic cholestasis results in a reduction in NTCP that parallels the increases seen in BSEP during cholestasis (Gartung et al., 1996; Gartung et al., 1997). This is presumably a protective response that limits the amount of bile acids in hepatocytes. Indeed injury after BDL peaks around 72 h after the onset of the ligation, and steadily declines until chronic dysfunction begins to set in (Georgiev et al., 2008). In addition to NTCP, multiple organic anion transporting polypeptides (OATPs) also participate in bile acid uptake (Hagenbuch & Gui, 2008). OATPs are present on the sinusoidal surface and can transport bile acids both into and out of the cell. While contributing significantly less total uptake, OATP function has also been shown to decrease after chronic cholestasis (Zollner et al., 2003). Bile acids are transported predominantly by OATP1B1 and OATP1B3, although NTCP retains 80% of total bile acid transport.

Given all this information, it is imperative that any cellular model for cholestatic liver injury have adequate bile acid uptake and export mechanisms to accurately recapitulate *in vivo* functions of hepatocytes. Most primary cells retain substantial expression of bile acid uptake and export transporters (Szabo et al., 2013), although this can rapidly fall if they are left in culture for a prolonged period (Liang et al., 1996). Currently, the only

non-primary line of human hepatoma cells that are known to express bile acid transporters without transfection are HepaRG cells. These cells are a bipotential line of hepatocyte-like hepatoma cells that share commonalities with primary human hepatocytes and they can be cultured indefinitely (McGill et al., 2011; Hart et al., 2011). It is not currently known how HepaRG cells respond to bile acids in regards to toxicity.

1.2.3 Enterohepatic Circulation of Bile Acids

After export into bile, conjugated bile acids travel throughout a system called enterohepatic circulation. Bile is released into the small intestine from the gall bladder post-prandial, in response to elevated levels of cholecystokinin released from the gut, where it assists in lipid digestion (Sayegh, 2013). This is also the site of synthesis of secondary bile acids such as DCA and LCA. Bile acids are then shuttled back into the liver via the portal vein after being reabsorbed into the blood steam in the ileum. Transporters on hepatocytes efficiently take up bile acids and the cycle is continued. This process is exceptionally efficient, with each bile acid being recycled numerous times. This also limits the amount of bile acids that need to be produced daily, as greater than 90% of bile acids are retained through enterohepatic circulation. Enterohepatic circulation is disrupted during cholestasis as bile is not allowed to flow into the small intestine. This causes compensatory changes in levels of transporter genes along the gut-liver axis. Principally, these changes have been discussed in previous sections.

1.3 Sterile Inflammation in the Liver

1.3.1 Components of the Innate Immune System in the Liver

The primary role of the innate immune system is host defense. The liver is a critical organ involved in innate immunity due to its responsibility in clearance of both microbial pathogens, and their components such as lipopolysaccharide (LPS). The primary cell type responsible for innate immunity in the liver is the Kupffer cell (Jaeschke, 1991). A resident macrophage, the Kupffer cell filters both pathogens and pathogenic component such as LPS from the blood. Some of these components activate Kupffer cells via pattern recognition receptors. These receptors, known as the Toll-Like Receptor (TLR) family, include TLRs 1-9 which are partially differentiated by their respective ligands (Akira et al., 2001). Activation of TLRs by endogenous ligands results in release of pro-inflammatory factors such as interleukin 1ß (IL-1ß) that stimulate further inflammation (Agostini et al., 2003). This inflammatory response, independent of infection or release of material from infectious agents, is termed sterile inflammation. In some diseases, such as hepatic ischemia reperfusion injury, prevention of this activation by blockade or depletion of TLR4 can be protective against liver injury (Tsung et al., 2005a; Tsung et al., 2005b). While Kupffer cells themselves have been implicated in the progression of some diseases, they recruit other innate immune cells to sites of injury via release of cytokines such as tumor necrosis factor-α (TNF-α), IL-1ß formed by the Nacht, LRR, and PYD domains containing protein 3 (NALP3) inflammasome, CXC chemokines and more (Szabo et al., 2013). These cytokines also serve to activate protective acute phase proteins through nuclear factor kappa B (NF-κB). This might be a protective mechanism as depletion of Kupffer cells before BDL in mice increases injury. Kupffer cells released cytokines that activated NF-κB, which resulted in protection against further insult mediated by neutrophils (Gehring et al., 2006). Whether or not this mechanism functions

protectively is probably dependent on the role of Kupffer cells in the injury as depletion of Kupffer cell activity has previously been shown to inhibit neutrophil recruitment and injury in hepatic ischemia reperfusion injury due to the loss in chemokine production (Jaeschke and Farhood, 1991). Thus, the role of Kupffer cells and traditional sterile inflammation during inflammatory liver injury is highly system dependent. In addition to Kupffer cells, the liver has a disproportionately high number of NKT cells and NK cells. These cell types are pro-inflammatory and produce numerous cytokines (Gao et al., 2009), in addition to release of cell killing proteins such as granzyme B and tumor necrosis factor related apoptosis inducing ligand (TRAIL). NKT cells may be important for the onset of cholestasis in some models, as they are implicated in the pathogenesis of biliary atresia (Shivakumar et al., 2009), as they can kill cholangiocytes during development of the post-fetal liver. Further research needs to be done to better determine the cause and progression of sterile inflammation during cholestatic liver injury *in vivo.*

Amongst these is the identification of receptors of damage associated molecular patterns (DAMPs) that result in activation of immune cells and stimulate the innate inflammatory response (Chen and Nunez, 2010). DAMP release has been proposed as both a clinical and a mechanistic biomarker that may give additional insight into clinical injury without the need for sampling the compartment (Antoine et al., 2012). Numerous DAMPs have been identified thus far, as well as nine receptors dubbed Toll-Like Receptors, (TLRs) that serve as receptors for these ligands (Chen and Nunez, 2010). Many DAMPs are released passively from necrotic cells such as high mobility group box protein-1 (HMGB1), heat shock proteins, or ATP (Brenner et al., 2013; Imaeda et al., 2009). HMGB1 is a DNA associated histone deacetylase that translocates to the cytosol during

injury (Erlandsson and Andersson, 2004; Mantell et al., 2006). Necrosis during cholestasis results in release of HMGB1 into serum as early as 6 h after injury (Woolbright et al., 2013). In addition to native HMGB-1, an acetylated form is actively secreted from macrophages that may serve as a pro-inflammatory signal (Wang et al., 1999). While there has been no direct study on the role of HMGB1 during cholestasis, this strong correlation between HMGB1 levels and inflammation after BDL merits further investigation.

TLR expression levels increase in both hepatocytes and cholangiocytes of chronically cholestatic patients (Wang et al., 2005; Benias et al., 2012). Moreover, activation of TLRs on liver immune cells stimulates local NK cells to attack and kill cholangiocytes during primary biliary cirrhosis (Shimoda et al., 2011) As blockade of TLR4 signaling via TLR4 depletion in hematopoietic cells (Tsung et al., 2005x) or the use of neutralizing antibodies to deplete HMGB1 (Tsung et al., 2004) has previously been shown to be protective against sterile inflammatory injury in the hepatic ischemia reperfusion model further studies investigating the role of TLR signaling, HMGB1 and its acetylated form, acetyl-HMGB1 are indicated. These studies are complicated by the fact that HMGB1 has additional receptors with additional functions. Amongst these is the receptor for advanced glycation end products (RAGE). The delineation of RAGE-mediated and TLR-mediated effects upon binding by HMGB1 or acetylated HMGB1are necessary to understand both in the context of cholestatic liver injury, and during general inflammation, before these molecules become therapeutic options.

Neutrophils are another critical component of the innate immune response to infection. While capable of killing and phagocytosing bacteria, viruses, and other pathogens,

neutrophils are typically incapable of phagocytosing larger mammalian cells. Instead, neutrophils kill mammalian cells through the release of ROS made and released by the neutrophil (Jaeschke, 2011). Neutrophils preferentially target damaged cells through mechanisms that are not currently well understood, but likely involve the release of DAMPs or other associated injury markers from necrotic cells, and the expression of pro-inflammatory markers such as ICAM-1 on the damaged cells, allowing for firm adherence (Jaeschke, 2011). After BDL, neutrophils correlate with biliary infarcts, which define the areas of injury (Koeppel et al., 1997). These infarcts also correlate strongly with chlorotyrosine adducts, a marker of neutrophil-mediated injury (Gujral et al., 2004c). Ablation of neutrophil extravasation via knockout of CD11b, a critical component of the Mac-1 adhesion complex, or knockout of ICAM-1 protects against BDL injury (Gujral et al., 2003; Gujral et al., 2004a). Both of these models showed a significant reduction in neutrophil recruitment and extravasation in the liver which correlated with a reduction in ALT and area of necrosis. The *lpr* mouse, which has a non-functional Fas receptor, was also protected against BDL injury, a result of its impaired inflammatory phenotype (Gujral et al., 2004b). Together these data strongly implicate neutrophils as a critical component of BDL-induced injury. As of yet, the mechanisms controlling early neutrophil accumulation at sites of injury after BDL have not been well elucidated. One potential mediator is osteopontin, a multifunctional glycophosphoprotein present on bile duct epithelial cells in the liver. Induction of osteopontin is present in both models of murine cholestasis (Fickert et al., 2007; Fickert et al., 2010) and in human specimens of primary biliary cirrhosis (Huang et al., 2008). In other models of chronic liver injury, osteopontin has been shown to be a potent neutrophil chemoattractant

(Banerjee et al., 2006). In the BDL, data from our laboratory suggests osteopontin serves as a primary initial mediator of BDL-induced injury (Yang et al., 2014). $OPN^{-/-}$ mice are completely protected against BDL injury during the first day, but are not significantly different than wild-type mice at three days post injury (Yang et al., 2014). Thus, there are likely a number of redundancies present in the inflammatory process during obstructive cholestasis that prevent single mediators from being fully protective. This data is anchored by unpublished data from our laboratory indicating the chemokine receptor 2 (CXCR2) knockout mouse is also not protected at three days post injury, despite the fact that MIP-2 levels are the most increased cytokine levels we have observed thus far.

1.3.2 Mechanisms of Innate Immune-Mediated Cytotoxicity

Neutrophils execute their cell killing capacity partially through degranulation of cytotoxic molecules and release of ROS. While ROS are generated intracellularly in all cells during mitochondrial respiration, neutrophils and other innate immune cells generate ROS they are capable of releasing to cause damage to bacteria or surrounding tissue. This requires activation of the neutrophil, which is primed for the release of ROS, and then extravasation of the neutrophil, into the hepatic parenchyma, where release of these toxic particles can directly damage hepatocytes (Jaeschke and Smith, 1997). Neutrophil activation has been reported in response to both HMGB1 (Park et al., 2003) and bile acids including low concentrations of LCA (Dahm and Roth, 1990) which are elevated in serum after cholestasis (Zhang et al., 2012; Woolbright et al., 2013). Activation of neutrophils during injury results in increased expression of the Mac-1 complex (Cd11b/Cd18) on the neutrophil surface and shedding of L-selectin (Bajt et al., 2001). The Mac-1 complex is integral for neutrophil adhesion in the liver, as the liver is a low-

flow organ with limited neutrophil rolling. Without a functional Mac-1 complex on neutrophils or ICAM-1 expression on hepatocytes and sinusoidal endothelial cells, neutrophils fail to recognize the inflammatory signals, fail to extravasate and do not actively kill hepatocytes (Gujral et al., 2003; Gujral et al., 2004a). Consequently, neutrophils must anchor close to hepatocytes where ROS can diffuse into hepatocytes and directly cause damage, without first encountering secondary targets. Neutrophils produce superoxide anion through NADPH oxidase (Nox2), which then dismutates into oxygen and hydrogen peroxide (Jaeschke, 2011). The neutrophilic enzyme myeloperoxidase then converts chloride anions and hydrogen peroxide into the highly toxic hypochlorous acid. While Kupffer cells are capable of producing oxidant stress, and do so in other models of sterile inflammation such as the hepatic ischemia-reperfusion model (Jaeschke et al., 1990; Jaeschke and Farhood, 1991), the predominant oxidative stress during cholestasis likely comes from neutrophils. Kupffer cell levels stay fairly consistent during the first two weeks of cholestasis (Georgiev et al., 2008) and depletion of Kupffer cells worsens the injury due to a reduction in protective acute phase cytokine and protein production (Gehring et al., 2006). More importantly, chlorotyrosine-protein adducts are prevalent in the areas of necrosis after acute BDL injury (Gujral et al., 2004c). As hypochlorous acid is a potent oxidizer it can bind and damage various components of the cell. Hypochlorous acid has been shown to form adducts with DNA and proteins, and is particularly predisposed towards binding thiol groups on proteins (Summers et al., 2012) and towards forming toxic chloramines (Hawkins et al., 2003). These chlorotyrosine-protein adducts are indicative of an intracellular oxidant stress as they are known to bind both proteins and DNA, suggesting the hypochlorous acid must diffuse into local hepatocytes where it

initiates the toxicity. It is likely these hepatocytes exposed to neutrophilic ROS are already compromised as even low levels of toxic bile acids can impair mitochondrial function without killing the cell (Sokol et al., 2005; Woolbright and Jaeschke, 2012). Further exposure to ROS would then result in cell death due to generalized protein dysfunction as well as mitochondrial impairment. Indeed protection of the mitochondria with NIM-811, a cyclosporine analogue is protective against the early BDL injury, although it does not protect against later fibrosis (Rehman et al., 2008). Additionally, preservation or enhancement of intracellular antioxidant species is potentially protective against BDL injury as kelch-like-ECH-associated-protein-1 (Keap-1) knockdown mice with a constitutively active nuclear factor E2 related factor-2 are protected against BDL induced injury, although some portion of this may be through enhanced detoxification and hepatic export of bile acids into serum (Okada et al., 2009).

Given this information, Figure 1.2 represents a graphical representation of the presumed mechanisms behind inflammatory liver injury during cholestasis in mice.

1.4 Clinical Aspects of Cholestatic Liver Injury in Man

Cholestasis is not limited to a single pathology, and thus the study of cholestatic liver injury is not limited to a single disease. It is imperative to remember that while many of the same general mechanisms might be applicable, substantial differences are present between extrahepatic obstructive cholestasis and chronic cholangitis. While this study is focused primarily on extrahepatic obstructive cholestasis, attempts will be made when relevant to broaden mechanisms.

Cholestasis can happen in the intrahepatic bile ducts, or the extrahepatic bile ducts, in addition to inhibition of bile acid export via BSEP inhibition. The initiating point of the cholestasis has a profound effect on the resulting pathology; although in general the primary associated outcome is elevated hepatic and serum bile acid levels. Previous reports suggest extrahepatic cholestasis may result in larger increases in the serum bile acid pool as compared to intrahepatic cholestasis (Trottier et al., 2011; Trottier et al., 2012), although this might be a function of the chronicity of the cholestasis, as the kidney begins to excrete bile acids via the urine during chronic cholestasis which can lower bile acid pool size (Fickert et al., 2013). Extrahepatic cholestasis exclusively results in the formation of bile infarcts in man (Figure 1.3), which are recapitulated in the BDL model of injury (Gujral et al., 2003; Gujral et al., 2004a). Surprisingly, these infarcts in man are also characterized by the formation of bile plugs, or areas of visible stasis of bile, that are not present in murine models. It is not understood why these plugs develop in man and not mice, but may be due to substantial differences in the makeup of murine bile in comparison to human bile (Zhang et al., 2012; Dilger et al., 2012). Human bile, especially during periods of chronic cholestasis, is composed of significantly higher values of toxic bile salts such as GCDC and glycocholic acid (GCA) (Dilger et al., 2012). This is partially due to a much higher rate of glyco-conjugation as compared to taurine-conjugation in humans versus mice. Even more importantly is the fact humans lack the enzymes necessary for hydroxylation of CDCA into muricholic acid (MCA), which increases the more hydrophobic and toxic CDCA values in the total bile acid pool of humans. It is likely the murine bile acid pool, due to its largely hydrophilic nature is less toxic. This hypothesis will be tested in this dissertation. In line with this information, it

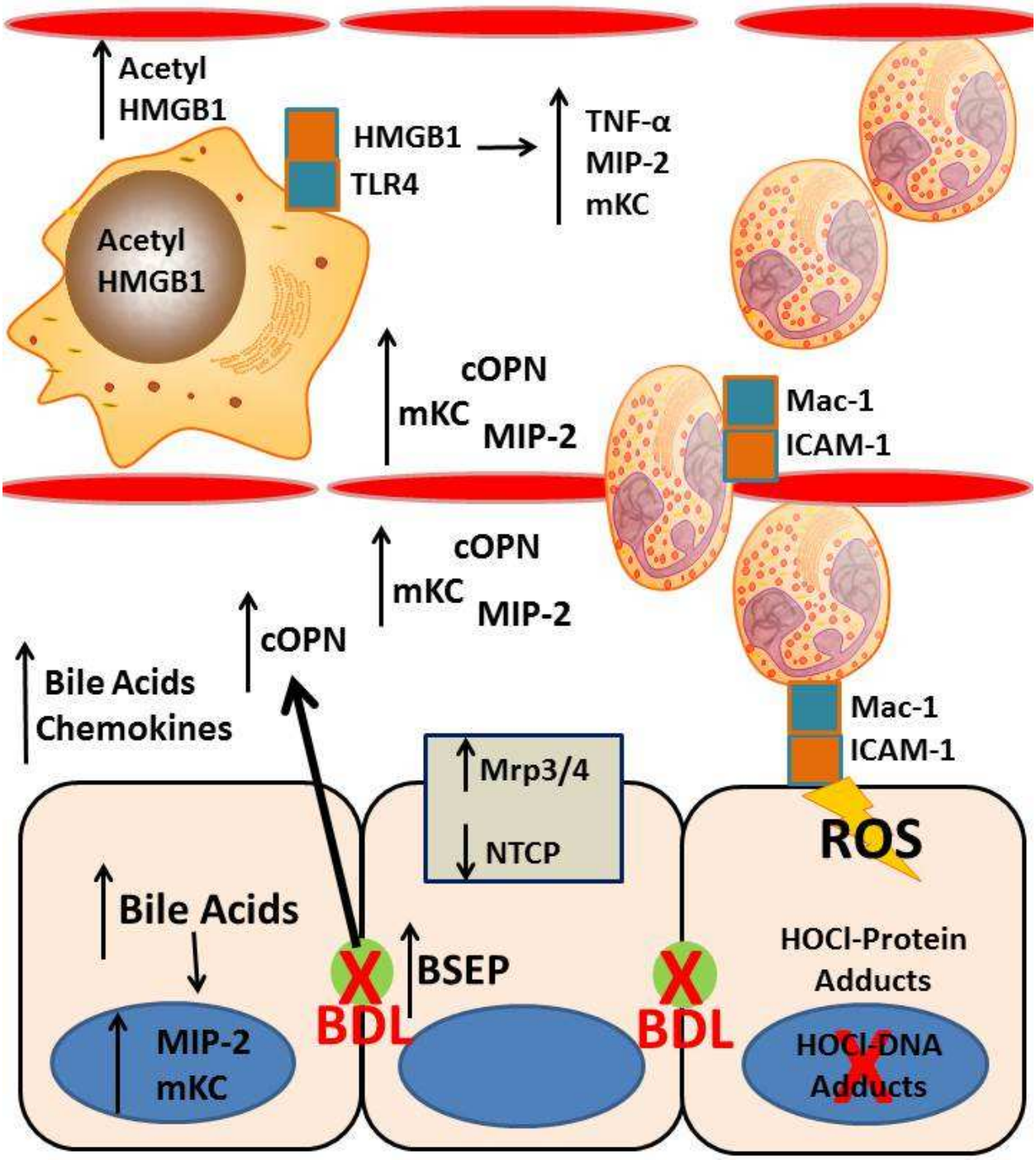

Figure 1.2: Outline of mechanisms of inflammation during cholestasis. BSEP – bile salt export protein, (c)OPN – (cleaved) Osteopontin, HMGB1 – high mobility group box 1, ICAM-1 – intracellular adhesion molecule 1, Mac-1 – macrophage antigen 1, MIP-2 – macrophage inflammatory protein 2, mKC – mouse keratinocyte factor, NTCP – sodium taurocholate cotransporting polypeptide, MRP – multidrug resistance protein, TLR – toll like receptor, TNF-α, tumor necrosis factor

may be that the more hydrophobic human bile develops plugs due to hydrophobicity, and

the bile constituents' propensity towards precipitating out of solution. Previous studies have found LCA precipitates out of the bile solution when given to excess in mice, causing rupture of the biliary tracts and cholestasis (Fickert et al, 2006). These bile plugs that develop in man may develop similarly. Extrahepatic cholestasis in man also results in spotty necrosis seen outside of areas of infarction (Figure 1.3) not typically seen in mice. These areas of spotty necrosis are flanked by morphologically apoptotic hepatocytes (Figure 1.3) which are also less apparent in murine models of cholestasis (Fickert et al., 2005).

Understanding of cholestatic liver disease in man is unfortunately less well developed than understanding of cholestatic liver disease in murine models. While the direct toxicity of bile acids towards human hepatocytes was established years ago (Galle et al., 1990), recent data suggests serum values that would mimic the concentrations necessary for toxicity are over an order of magnitude below the necessary concentration (Trottier et al., 2011; Trottier et al., 2012). Thus, it is unknown if bile acids accumulate to a degree in hepatocytes *in vivo* that results in toxicity. Numerous obstructive cholestatic liver disorders result in neutrophil accumulation. In both murine models of biliary atresia (Mohanty et al., 2010), and in human patients (Changho et al., 2010), neutrophils are seen co-localizing with injury. Neutrophil presence can also be seen in patients with extrahepatic cholestasis (Figure 1.3). Numerous disorders with prominent cholangitis have significant neutrophil infiltrate in both areas of liver necrosis and near the areas of

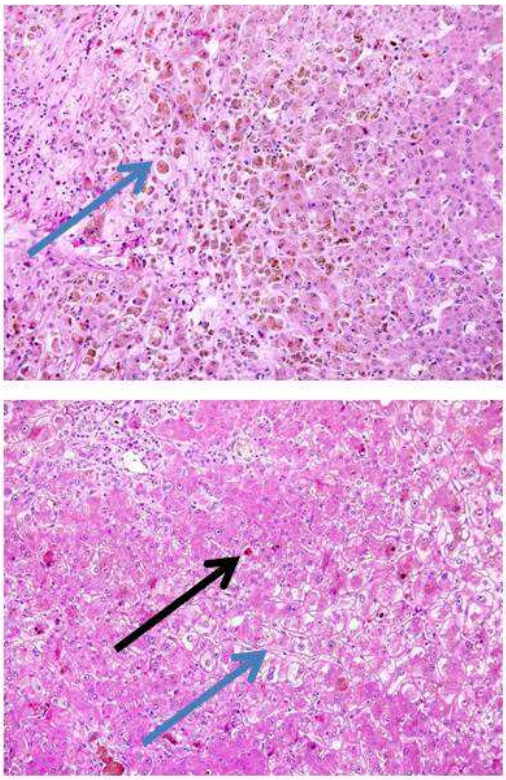

Figure 1.3: Biopsies from patients with extrahepatic cholestasis. 100x magnification. Blue arrows indicate areas of necrosis, whereas black arrows indicate individual apoptotic hepatocytes

biliary fibrosis that characterize disorders such as primary sclerosing cholangitis and primary biliary cirrhosis. While the role of neutrophils in the progression of the injury is not well defined in patients, future studies looking at neutrophil activity in cholestatic patients may be merited. As it is unethical to take punch biopsies from patients with liver injury due to bleeding complications, the use of novel, mechanistic biomarkers along with isolation of primary human hepatocytes will be necessary to further current endeavors in understanding how cholestatic liver injury occurs in man.

1.5 Conclusions

Current understanding of cholestatic liver injury is predicated upon the use of rodent models that may not accurately reflect the injury in humans (Woolbright and Jaeschke, 2012). This study seeks to answer many of the questions listed in the above introduction by shifting away from currently used models and focusing on more pathophysiologically relevant models. To this end, studies were taken up to discern the concentrations of bile acid necessary to directly cause toxicity and to additionally determine what modalities of cell death were present at these concentrations. These studies take place using clinical samples acquired from patients with well-defined cholestsais, in isolated human hepatocytes, and in well-defined *in vivo* mouse models that recapitulate human disease.

1.6 Statement of Purpose

The primary purpose of this research is a reassessment of bile acid levels in the context of cholestatic liver pathology given that new information recently has been discovered in murine models, and improvements in bile acid detection using UPLC-MS. A complete

understanding of the pathophysiology is necessary to attempt any sort of therapeutic intervention, regardless of the disease. Given our limited understanding of cholestasis progression, a complete working knowledge of bile acid concentrations in mice and man is imperative to further progression in understanding the pathophysiology. Moreover, while a working role for inflammation has been established in the BDL model, it is unknown what role this plays in man, and in other cholestatic models. We will attempt to ascertain the answer to these questions using multiple models including the BDL model, a model wherein we administer LCA via the diet, and by using both human patient samples and primary human hepatocytes. We hypothesize in this study that those models or etiologies that contain high concentrations of toxic bile acids will result in direct hepatotoxicity, whereas those models with largely non-toxic, taurine conjugated bile acids will result primarily in an inflammatory response mediated by both bile acid induced gene expression changes and via sterile inflammation and release of local DAMPs.

Chapter 2: Methods and Materials.

The following methods apply globally to the studies performed herein. Where origin of the chemical is not stated, the origin is Sigma Aldrich, St. Louis, MO, USA. All studies were done in compliance with local Institutional Animal Care and Use Committee approval. Patient studies were done after KUMC IRB approval.

Patient Selection for Cholestatic Patients and Hepatocytes Isolation Procedures: Patients admitted to the University of Kansas Hospital were enrolled in an institutional review board (IRB) approved protocol. Inclusion criteria included subjects undergoing planned endoscopic retrograde cholangiopancreatography (ERCP) for medical diagnosis and potential treatment of cholestasis. Uninjured patients were defined as patients with ALT <50U/L and ALP<110U/L. Injured patients were defined as patients with ALT≥50U/L and ALP≥110U/L and defined cholestasis as evidenced by ERCP. This study adhered to the Helsinki Declaration and all studies were done under informed consent.

Animals and experimental protocol. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals received humane care according to the criteria outlined in *Guide for the Care and Use of Laboratory Animals.* All experimental protocols were approved by the Animal Use Committees of the University of Kansas Medical Center. Thirty minutes before

surgery, the mice were injected i.m. with a cocktail of anesthetics consisting of ketamine (225 mg/kg Ketaset; Fort Dodge Animal Health, Fort Dodge, IA), xylazine (11.4 mg/kg Rompun; Bayer, Shawnee Mission, KS), and acepromazine (2.3 mg/kg acepromazine maleate; VEDCO, St. Joseph, MO). A midline laparotomy was performed and the common bile duct was ligated twice with 4-0 silk sutures and then cut between the sutures. Sham-operated animals, which were subjected to the same operation except the ligation of the bile duct, served as controls. After BDL the abdomen of each animal was closed in two layers. Blood and liver samples were collected at the indicated time of death. Pieces of the liver were snap-frozen in liquid nitrogen or fixed in phosphate-buffered formalin for immunohistochemistry and histology.

Isolation and Culture of Human Hepatocytes: Primary human hepatocytes were freshly isolated from liver resections by the Biospecimen Core in the Department of Pharmacology, Toxicology and Therapeutics at the University of Kansas Medical Center. All human tissues were obtained with informed consent from each patient, according to ethical and institutional guidelines. The study was approved by the Institutional Review Board at University of Kansas Medical Center. Cells were isolated using a multi-step collagenase procedure as in Xie et al. After an initial 3-h attachment period, cultures were washed with phosphate-buffered saline (PBS) and then culture medium, vehicle or media containing the indicated concentration of bile acid or inhibitor were added along with the appropriate vehicle.

Isolation and Culture of Rat Hepatocytes: Sprague-Dawley rats were acquired from Jackson Labs (Bar Harbor, ME). Briefly, a three-step collagenase perfusion method was used. After the

induction of anesthesia, the peritoneal cavity was opened, and a 20G catheter was inserted into the inferior vena cava. The liver was perfused *in situ* via the inferior vena cava after cutting the portal vein for 10 min with calcium and magnesium free HBSS containing 0.1mM EGTA followed by a washout step using Calcium and Magnesium free HBSS without EGTA. The final perfusion step consisted of EMEM containing 25 mM HEPES buffer and 0.025mg/ml of Liberase TM (Roche, Basel, Switzerland) and continued until the liver showed signs of digestion. The remaining portion was cut into smaller pieces with scissors to release remaining cells. The cell suspension was sequentially filtered through nylon gauze and collected in 50ml conical tubes. The cells were centrifuged for 5 min at 50 x g and 4˚C and then resuspended in fresh cold DMEM with 25 mM HEPES. This was repeated 3 times in order to isolate the hepatocyte fraction. Hepatocyte viability was using a hemocytometer and the trypan blue exclusion assay. After an initial 3-h attachment period, cultures were washed with phosphate-buffered saline (PBS) and then culture medium (controls) or media containing the indicated concentration of bile acid were added. Inhibition studies using z-VAD-fmk were carried out by pretreating for one hour with the indicated concentration of inhibitor and then adding the indicated treatment.

HepaRG and HepG2 Cell Culture

HepaRG cells were obtained from Biopredic International (Rennes, France). The cells were seeded at 1×10^5 undifferentiated cells/cm^2 in hepatocyte wash medium (Invitrogen Corporation, Carlsbad, CA) containing additives for growth (Biopredic). Cells were cultured at 37°C for 14 days before differentiation. Medium was replaced every 2-3 days. Cell differentiation was induced as described previously (McGill et al., 2011). The cells were maintained up to 4 weeks

after differentiation for use. HepG2 cells were grown to 90% confluence in DMSO-free Williams' E Medium containing penicillin/streptomycin, 2ng/mL insulin, and 10% FBS. Treatment with bile acids was performed by removing the media and then washing the cells once in PBS, before adding the indicated concentration of bile acid back onto the cells in complete Williams E media. For caspase inhibition, some cells were pretreated for 1h with 10 μM Z-VD-fmk (generous gift from Dr. S. X. Cai, Epicept Corp., San Diego, CA), then changed to medium containing 10 μM Z-VD-fmk and the indicated concentration of GCDC or 5mM galactosamine and 100ng/mL TNF (Genzyme, Cambridge, MA)

Liver injury and plasma ALT levels. ALT activities were determined in the plasma by using the Pointe Scientific ALT Kit (Pointe Scientific Canton, MI) according to the manufacturer's instructions. Sections of formalin-fixed paraffin-embedded liver samples were stained with hematoxylin and eosin (H&E) for evaluation of liver cell injury.

Hepatic neutrophil immunohistochemistry: Neutrophil accumulation in the livers was assessed by staining tissue sections using immunohistochemistry for LY-6B (AbD Serotec Raleigh, NC), a specific marker for neutrophils. Briefly, slides containing liver sections were deparaffinized, rehydrated, and then incubated with peroxidase suppressor (Sigma, St. Louis, MO) for 30 minutes. Vector Labs rat anti-mouse DAB detection system (Vector Labs Burlingame, CA) was used with the indicated primary antibody at 1:200 dilution.

Determination of circulating mechanism-based biomarkers of liver injury: Circulating biomarkers were determined as previously described for highly liver specific markers such as

microRNA-122 (miR-122) by qRT-PCR (Starkey Lewis et al., 2011), unequivocal identification and absolute quantification of full length and caspase-cleaved cytokeratin-18 was determined by mass spectrometry as previously described (Antoine et al., 2009, 2010, 2012) and total HMGB1 (Antoine et al., 2009, 2010, 2012) by immunoassay and mass spectrometry. The absolute quantification of hyper-acetylated HMGB1 was determined by mass spectrometry as previously described as a biomarker of immune cell activation (Antoine et al., 2009). For miR-122 measurement, lethal 7d (let-7d) provided biological standardization and loss of function 4 (lin-4) served as an internal standard for the assay. The investigators measuring the plasma biomarkers were blinded to the treatment groups of the animals.

Western blotting. Flash frozen liver sections were homogenized in a chaps based buffer, and then centrifuged at 14,000 rpm to remove cellular debris. The BCA assay (Pierce, Rockford, IL) was used to quantify protein levels in both liver and plasma. Briefly, equal quantities of protein were loaded into an Invitrogen Mini Blot electrophoresis system and transferred onto PVDF paper. The blot was probed with a caspase-3 antibody (Cell Signaling Danvers, MA) and visualized using an HRP conjugated secondary antibody (Santa Cruz Biotechnology Santa Cruz, CA).

Caspase activity assay: Caspase activity was determined by measuring the z-VAD-FMK-inhibitable cleavage of the fluorescent caspase-3 substrate Ac-DEVD-AMC (Sigma Aldrich St. Louis, MO) as described (Jaeschke et al., 1998).

Bile Acid Measurements: Bile acid measurements were performed as previously described (Woolbright et al., 2014). In brief, bile samples were first diluted 1:50 in water, whereas serum

samples were used as is. A methanol extraction was performed by adding 20 µL sample to 80 µL methanol to de-proteinate and centrifuging at *14,000 x g* for 10 minutes. The samples were separated using a Waters (Waters, Milford, MA) Acquity ultra-performance liquid chromatograph (UPLC) with a Waters Acquity BEH C18 column (1.7 µm, 130Å, 2.1 mm x 100 mm) and an attached Waters BEH C18 VanGuard pre-column (1.7 µm, 130Å, 2.1 mm x 5 mm). Absolute quantification was done using a standard curve of six different known concentrations ranging from 100 ng/mL to 25 µg/mL.

Cell Death Analysis: Cell death was assessed by lactate dehydrogenase (LDH) release, as described previously in detail (Bajt et al., 2004). Caspase activity was based on z-VAD-fmk inhibitable Ac-DEVD-AMC fluorescence measured as described previously using a SpectraMax spectrophotometer (Jaeschke et al., 1998).

Propidium Iodide Staining: Cells were seeded on 60 mM plastic dishes and treated with the indicated concentration of GCDC for indicated time. Propidium iodide and Hoescht dye were added for the final five minutes of the treatment, before washing the cells and adding fresh PBS. The live cells were imaged on a Zeiss Axiovert inverted fluorescence microscope through a Texas Red filter to assess PI uptake, or a 408 nM filter to visualize Hoescht staining. All fluorescence images were taken at the same exposure and later superimposed on phase contrast images or Hoescht images of the same fields using Image J software.

Real Time PCR Analysis: Gene expression measurements were performed by real-time PCR (RT-PCR) analysis as described (Gujral et al., 2004). The relative differences in expression between groups were expressed using cycle time (Ct) values generated by the ABI 7600

instrument (Applied Biosystems). Genes were normalized to GAPDH and then expressed as fold difference using the delta-delta CT formula.

Bile Acid Uptake: Hepatocytes were seeded to confluence in 12 well plates and allowed to adhere for 3 h. Uptake buffers either with or without sodium were used as in Jigorel et al., with 1μM radioactive taurocholate. Cells were lysed using 1% triton-X 100 and counted on a Micro Beta TriLux ß counter (Perkin Elmer, Waltham, MA). Values were normalized to protein level using the BCA assay.

Statistics. Statistical analysis was performed with Sigmaplot 8.0 (Systat Software, Inc., Chicago, IL). Data were assessed using one way ANOVA followed by Student-Newman-Keul's post-hoc test for comparisons between means or Dunnett's post-hoc test for comparisons to a control. For data not normally distributed, we used the Kruskal-Wallis test followed by Dunn's multiple comparisons test. $P < 0.05$ was considered significant.

Chapter 3: Plasma Biomarkers of Liver Injury and Inflammation Demonstrate a Lack of Apoptosis during Obstructive Cholestasis in Mice

This section is a reprint of the paper "Plasma Biomarkers of Liver Injury and Inflammation Demonstrate a Lack of Apoptosis during Obstructive Cholestasis in Mice" Woolbright et al., 2013 – Toxicology and Applied Pharmacology - with permission.

ABSTRACT

Cholestasis is a pathological common component of numerous liver diseases that results in hepatotoxicity, inflammation, and cirrhosis when untreated. While the predominant hypothesis in cholestatic liver injury remains hepatocyte apoptosis due to direct toxicity of hydrophobic bile acid exposure, recent work suggests the injury occurs through inflammatory necrosis. In order to resolve this controversy, we used novel plasma biomarkers to assess the mechanisms of cell death during early cholestatic liver injury. C57Bl/6 mice underwent bile duct ligation (BDL) for 6-72 h, or sham operation. Another group of mice were given D-galactosamine and endotoxin as a positive control for apoptosis and inflammatory necrosis. Plasma levels of full length cytokeratin-18 (FL-K18), microRNA-122 (miR-122) and high mobility group box-1 protein (HMGB1) increased progressively after BDL with peak levels observed after 48 h. These results indicate extensive cell necrosis after BDL, which is supported by the time course of plasma alanine aminotransferase activities and histology. In contrast, plasma caspase-3 activity, cleaved caspase-3 protein and caspase-cleaved cytokeratin-18 fragments (cK18) were not elevated at any time during BDL suggesting the absence of apoptosis. In contrast, all plasma biomarkers of necrosis and apoptosis were elevated 6 h after Gal/End treatment. In addition, acetylated HMGB1, a marker for macrophage and monocyte activation, was increased as early as 12 h but mainly at 48-72 h. However, progressive neutrophil accumulation in the area of necrosis started at 6 h after BDL. In conclusion, these data indicate that early cholestatic liver injury is an inflammatory event, and occurs through necrosis with little evidence for apoptosis.

INTRODUCTION

Cholestasis is present in multiple pathologies including gall stone obstruction of the bile duct, biliary atresia, local tumor impingement and drug toxicity (Kim et al., 2000; Poupon et al., 2000; Yang et al., 2013b). Persistent cholestasis leads to injury of hepatocytes and bile duct epithelial cells, inflammation, and progression to fibrosis, cirrhosis and death. The predominant hypothesis concerning the mechanism of cholestatic liver injury is the accumulation of hydrophobic bile salts considered to be highly toxic (Perez and Briz, 2009; Maillette de Buy Wenniger and Beuers, 2010; Higuchi and Gores, 2003), with a heavy emphasis on glycochenodeoxycholate (GCDC) or its bile acid glycochenodeoxycholic acid (GCDCA) largely due to their accumulation in human serum during cholestatic conditions (Trottier et al., 2011). Papers from multiple laboratories over the past 20 years have shown an intricate mechanism by which GCDC at 30-50 μM concentrations causes apoptosis in isolated rat hepatocytes (Spivey et al., 1993; Patel et al., 1995; Yerushalmi et al., 2001; Graf et al., 2002; Schoemaker et al., 2004; Rust et al., 2006). While GCDC-induced apoptosis is indisputable in rat hepatocytes, it is currently undetermined if this mechanism also applies to other animals or the human condition (Woolbright and Jaeschke, 2012).

Recent data in a bile duct ligation (BDL) model in the mouse demonstrated that the accumulating bile acids in serum and liver consist largely of hydrophilic bile acids including taurocholate, muricholic acid and tauromuricholic acid (Zhang et al., 2012). Hepatocytes exposed to these

hydrophilic bile acids individually or in combination trigger chemokine formation and expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) but did not cause cell death (Allen et al., 2011; Zhang et al., 2012). In addition, animals deficient in CD18 or ICAM-1 are highly protected against BDL-induced liver injury suggesting that cholestatic liver injury in this mouse model is caused by neutrophils (Gujral et al., 2003a, 2004a). It has been suggested that the mode of cell death in the BDL model in rats (Shoemaker et al., 2003) or mice (Gujral et al., 2004b; Fickert et al., 2005; Nalapareddy et al., 2009; Mitchell et al., 2011) is mainly oncotic necrosis and not apoptosis. Nevertheless, this is controversial as others have concluded that apoptotic cell death is dominant (Higuchi and Gores, 2003). Part of the confusion is certainly caused by use of non-specific assays for apoptosis such as the TUNEL assay (Grasl-Kraupp et al., 1995; Jaeschke and Lemasters, 2003). However, one argument that has been used for the different interpretation is that a cell undergoing apoptosis is only identifiable for a limited time and thus many apoptotic cells may be missed when a longer time interval is used. In order to address this issue, we have used circulating biomarkers of apoptosis (caspase-cleavage fragment of cytokeratin-18 and caspase-3 enzyme activity) and necrosis (full-length cytokeratin-18, high mobility group box-1 protein (HMGB1) and miR122) in the mouse BDL model that can accumulate and provide a signature pattern of resulting cell death mechanisms. Each of these markers has been successfully used in mice (Antoine et al., 2009; Williams et al., 2011; McGill et al., 2012) and humans (Starkey Lewis et al., 2011; Antoine et al., 2012, 2013; McGill

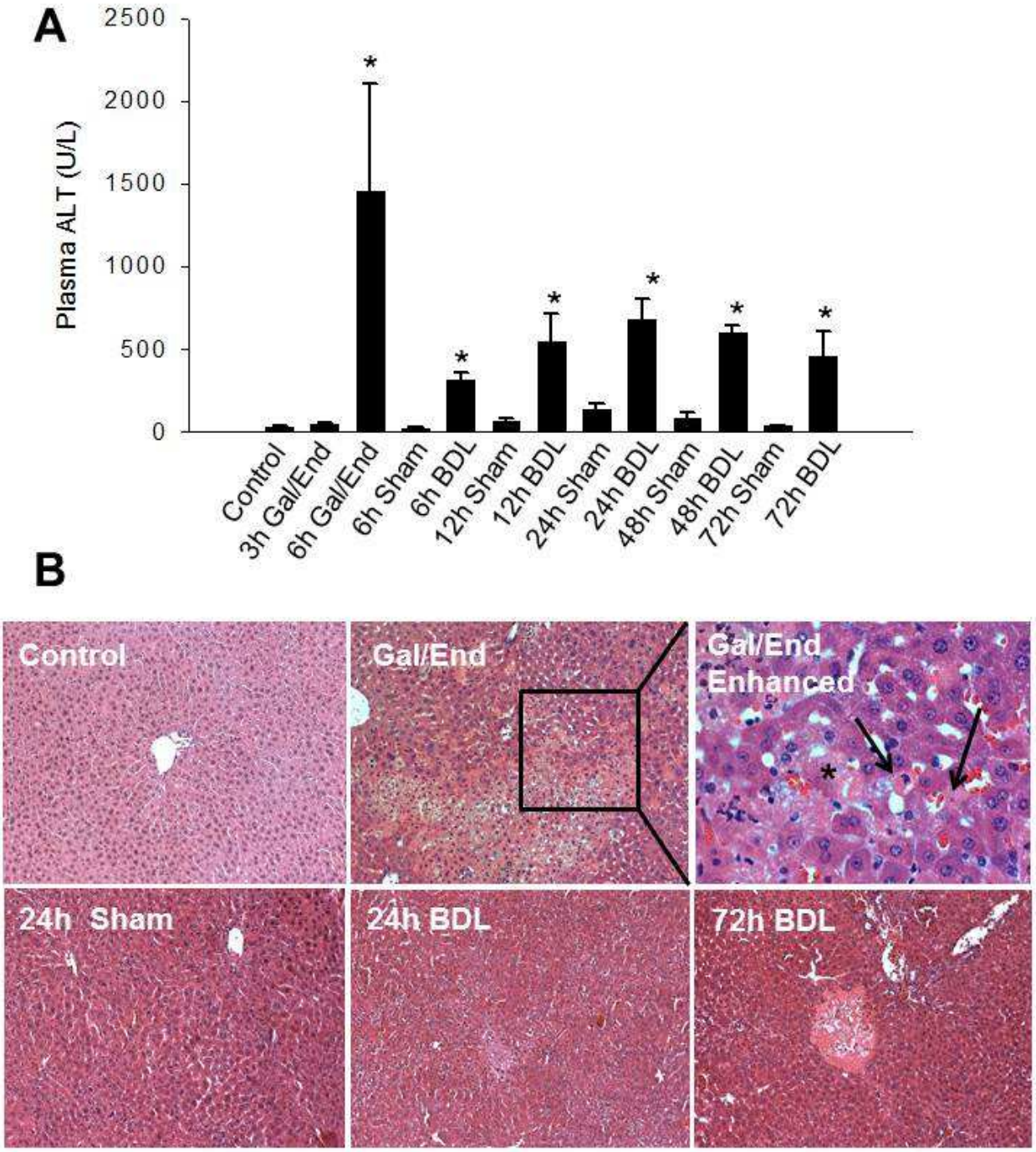

Figure 1: Liver injury in C56Bl/6 mice after treatment with galactosamine/endotoxin (Gal/End), bile duct ligation (BDL) or sham-operation. A) Plasma alanine aminotransferase (ALT) activities after Gal/End, BDL or sham-operation (6-72h). Data represent mean ± SE of n=4-6 animals per group. *P<0.05 (compared to untreated control or sham. B) Representative H&E-stained liver sections at 100x or a 400x enhanced Gal/End picture. Apoptotic cells are marked with black arrows; a necrotic cell is indicated by an asterisk.

et al., 2012; Dear et al., 2013) after acetaminophen overdose. In addition, we have compared our data in the BDL model with a traditional apoptosis/inflammation model, i.e. galactosamine/ endotoxin-induced liver injury (Leist et al., 1995; Jaeschke et al., 1998).

RESULTS

It is recognized that liver injury and bile infarcts peak between 48-72 h after BDL (Georgiev et al., 2008). Therefore, we investigated the time course of liver injury in C56Bl/6 mice between the onset of BDL and 72 h after BDL. Obstruction was confirmed by robust dilation of the gall bladder and the presence macroscopically of bile infarcts in the liver. ALT levels rose steadily through the first 24 h before subsiding slightly at 48 h and 72 h after injury (Figure 1A). H&E staining confirmed the progressive increase of liver injury and showed the characteristic bile infarcts typically seen after BDL (Figure 1B). As an apoptosis control for these experiments, mice were treated for either 3 h or 6 h with 700 mg/kg D-galactosamine and 100 μg/kg endotoxin (Gal/End), a treatment regimen that is known to trigger TNF-mediated apoptosis (Leist et al., 1995) and inflammation-induced necrosis (Jaeschke et al., 1998). While there was no ALT release at 3 h, ALT levels were increased significantly 6 h post Gal/End treatment (Figure 1A). Histology indicated the presence of both necrotic and apoptotic cells (Figure 1B).

Because it is known that apoptotic cell death is a fairly rapid process and the resulting apoptotic bodies can be removed, it has been argued that the extent of apoptosis may be underestimated when tissue sections are evaluated at a single time point (Giucciardi and Gores, 2005). In order to overcome this problem, plasma biomarkers were evaluated. We have previously shown that during apoptosis, caspase-3 activities can be measured in serum after Gal/End in rats and mice

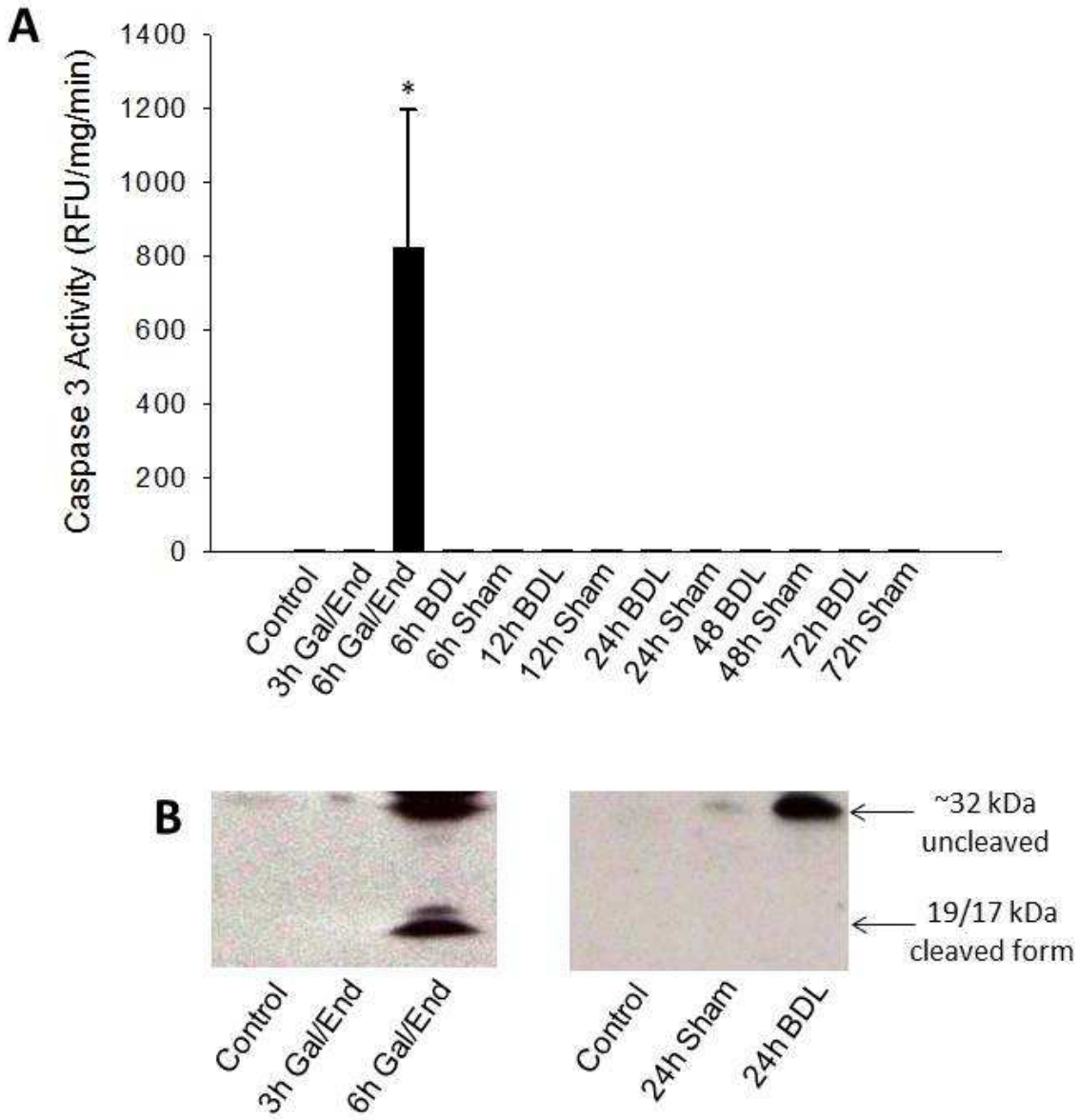

Figure 2: Assessment of apoptosis in C56Bl/6 mice after treatment with galactosamine/endotoxin (Gal/End), bile duct ligation (BDL) or sham-operation. A) Caspase-3 activity was measured in plasma after BDL or Gal/End treatment. Data represent mean ± SE of n=3 animals per group. *P<0.05 (compared to untreated control). B) Western blot of procaspase-3 (32 kD) and its active 19 and 17 kD fragments in plasma of an untreated control, 3 or 6 h after Gal/End or 24 h after BDL as a representative time point.

(Gujral et al., 2003b; McGill et al., 2012) but not after acetaminophen (APAP) overdose in mice or humans (McGill et al., 2012). No evidence of caspase-3 activity was found at any time after BDL (Figure 2A). In contrast, substantial plasma caspase-3 activities were measured 6 h after Gal/End (Figure 2A). The plasma caspase-3 activity measurements were supported by western blots showing that both procaspase-3 and an active cleavage product were detectable in the 6 h Gal/End samples (Figure 2B). However, procaspase-3 proteins were only detected in BDL samples with substantial injury (e.g. 24 h) but not in sham-operated animals (Figure 2B).

Cytokeratin 18 is a filamentous protein that can be released by damaged cells reflecting cell death. The full-length protein (FL-K18) represents cellular components released by dying, necrotic cells into the periphery whereas a caspase-specific cleavage product (cK18) provides evidence for apoptotic cell death (Biven et al., 2003). This method offers a wider analytical window enhancing the sensitivity to specifically detect apoptosis or necrosis of epithelial cells simultaneously. When FL-K18 and cK18 levels were measured in plasma of BDL and sham-operated mice, a progressive increase of FL-K18 levels was observed up to 48 h with a slight decline at 72 h after BDL (Figure 3). However, cK18 levels never exceeded sham-operated levels of <150 pmol/ml serum at any time after BDL (Figure 3). In contrast, both molecular forms of keratin-18 substantially increased during Gal/End-induced apoptosis and inflammation-induced necrosis at 6 h (Figure 3).

Serum miR-122 levels have been associated with APAP-induced necrosis in experimental animals and humans (Wang et al., 2009; Starkey Lewis et al., 2011). Similar to FL-K18, miR-122 levels increased progressively up to 48 h and moderately declined afterwards (Figure 4).

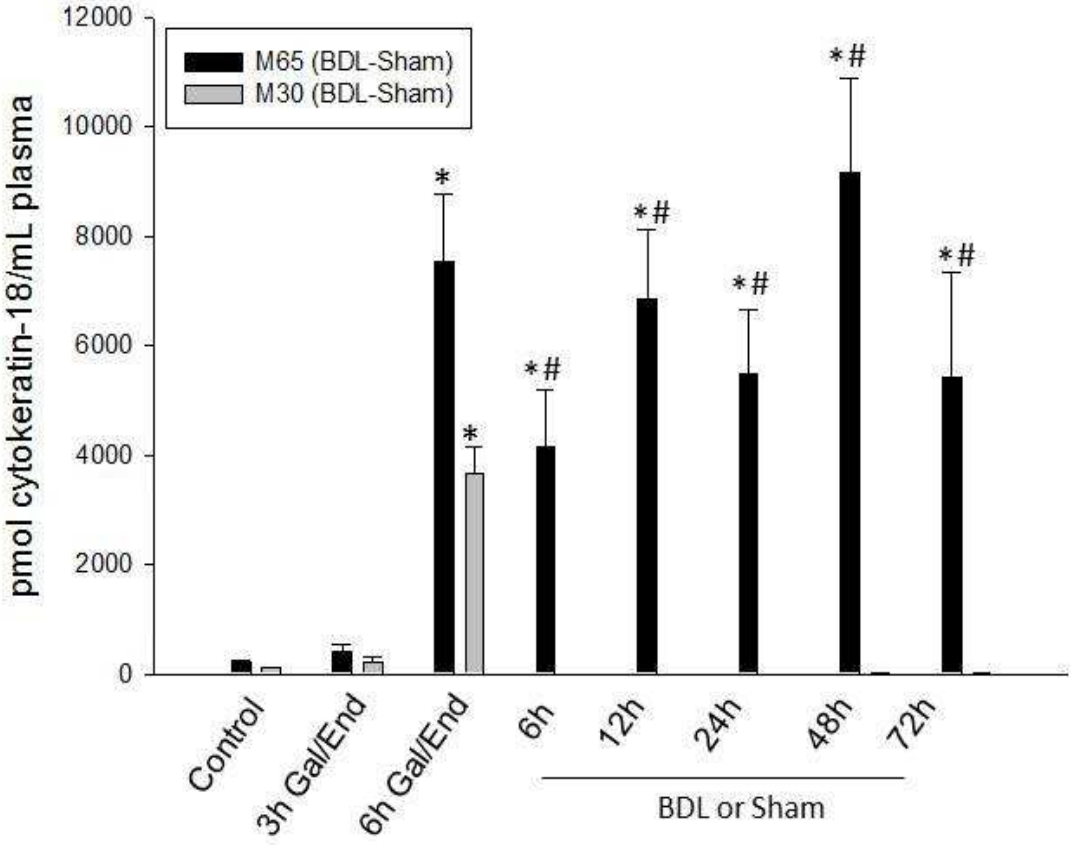

Figure 3: Full length (FL-K18) and caspase-cleaved (cK18) cytokeratin-18 levels in plasma after galactosamine/endotoxin (Gal/End) treatment, bile duct ligation (BDL) or sham-operation. The values of sham-operation (<150 pmol/ml) were subtracted from the BDL values. Data represent mean ± SE of n=4-6 animals per group. *P<0.05 (compared to untreated control); #P<0.05 (compared to cK18 values)

Only traces of miR-122 were detectable in sham-operated animals. In addition, serum miR-122 levels increased substantially at 6 h after Gal/End (Figure 4).

HMGB1 protein is a nuclear protein that can be passively released during necrotic cell death (Scaffidi et al., 2002) or actively secreted by activated inflammatory cells such as macrophages (Erlandsson Harris and Andersson, 2004). Recent work indicated that the acetylated form of HMGB1 is actively secreted mainly by inflammatory cells in response to inflammatory stimuli (Bonaldi et al., 2003). Total HMGB1 levels increased dramatically starting 6 h post BDL and further increased up to 48 h followed by a moderate decline at 72 h (Figure 5A). The increase in HMGB1 levels 6 h after Gal/End was similar to the BDL animals. In contrast to miR-122 and FL-K18, control and sham-operated animals showed low baseline levels of HMGB1with some elevation 1-3 days after BDL (Figure 5A). Analysis of the acetylated form of HMGB1 (acHMGB1) demonstrated close to undetectable levels after Gal/End and at 6 h after surgery (Figure 5B). AcHMGB1 levels were significantly increased at 12 h after BDL and peaked between 48 and 72 h (Figure 5B). However, as indicated by the percentage of acHMGB1compared to the total HMGB1, the acetylated form was only a relevant component at later time points after BDL reaching levels of 10% of the total HMGB1 compliment (Figure 5C).

Previous studies suggested that neutrophils are critical for the injury after BDL in mice (Gujral et al., 2003a). To assess how this rise in HMGB1 levels correlated with neutrophil recruitment, neutrophil levels were quantified via immunohistochemistry in liver sections. Neutrophil counts were increased significantly in all BDL samples. The time course shows a moderate infiltration of neutrophils during the first 12 h and then a more progressive increase up to 72 h (Figure 6A).

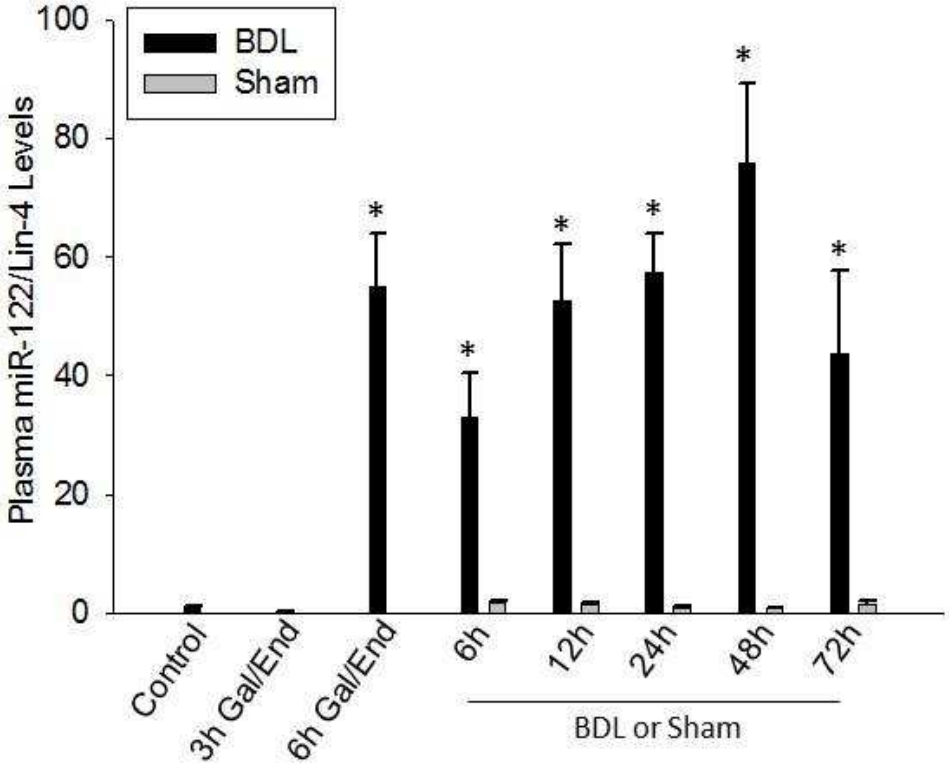

Figure 4: Plasma micro-RNA 122 (miR-122) levels were measured after galactosamine/endotoxin (Gal/End) treatment, bile duct ligation (BDL) or sham-operation. The values of miR-122 are expressed as ratio to the reference let-7d and the internal standard, Lin-4. Data represent mean ± SE of n=4-6 animals per group. *P<0.05 (compared to untreated control); #P<0.05 (compared to sham-operated control).

In contrast, high neutrophil levels were already observed 3 h after Gal/End with only a moderate further increase at 6 h (Figure 6A). Whereas neutrophils were located predominantly in sinusoids at 3 h (data not shown) and both in sinusoids and extravasated into the parenchyma at 6 h after Gal/End (Figure 6B), most neutrophils after 24-72 h after BDL are located within the area of necrosis (Figure 6B).

DISCUSSION

The objective of the current investigation was to use plasma biomarkers to assess mode of cell death and inflammation over time in a murine model of obstructive cholestasis (BDL). In addition to the mechanistic insight, the results also provide reference data for the interpretation of translational studies in humans where plasma but no tissue is available in most cases (Antoine et al., 2012; McGill et al., 2012).

Apoptosis and Necrosis During Early Cholestatic Liver Injury

A predominant hypothesis in cholestatic liver injury remains the idea that hydrophobic bile salts such as GCDC induce apoptosis in hepatocytes (Guicciardi and Gores, 2005; Maillette de Buy Wenniger and Beuers, 2010). While this mechanism applies to cell death of cultured rat hepatocytes exposed to GCDC *in vitro* (Spivey et al., 1993; Patel et al., 1995; Yerushalmi et al., 2001; Graf et al., 2002; Schoemaker et al., 2004; Rust et al., 2006), caspase levels and apoptosis after BDL in rats *in vivo* are very limited mainly due to the induction of anti-apoptotic proteins (Schoemaker et al., 2003). In mice, conclusions of apoptotic cell death after BDL were mainly based on TUNEL-positive cells and the protection in Fas receptor-deficient *lpr* mice (Miyoshi et al., 1999). However, no relevant caspase activation was detected after BDL and caspase

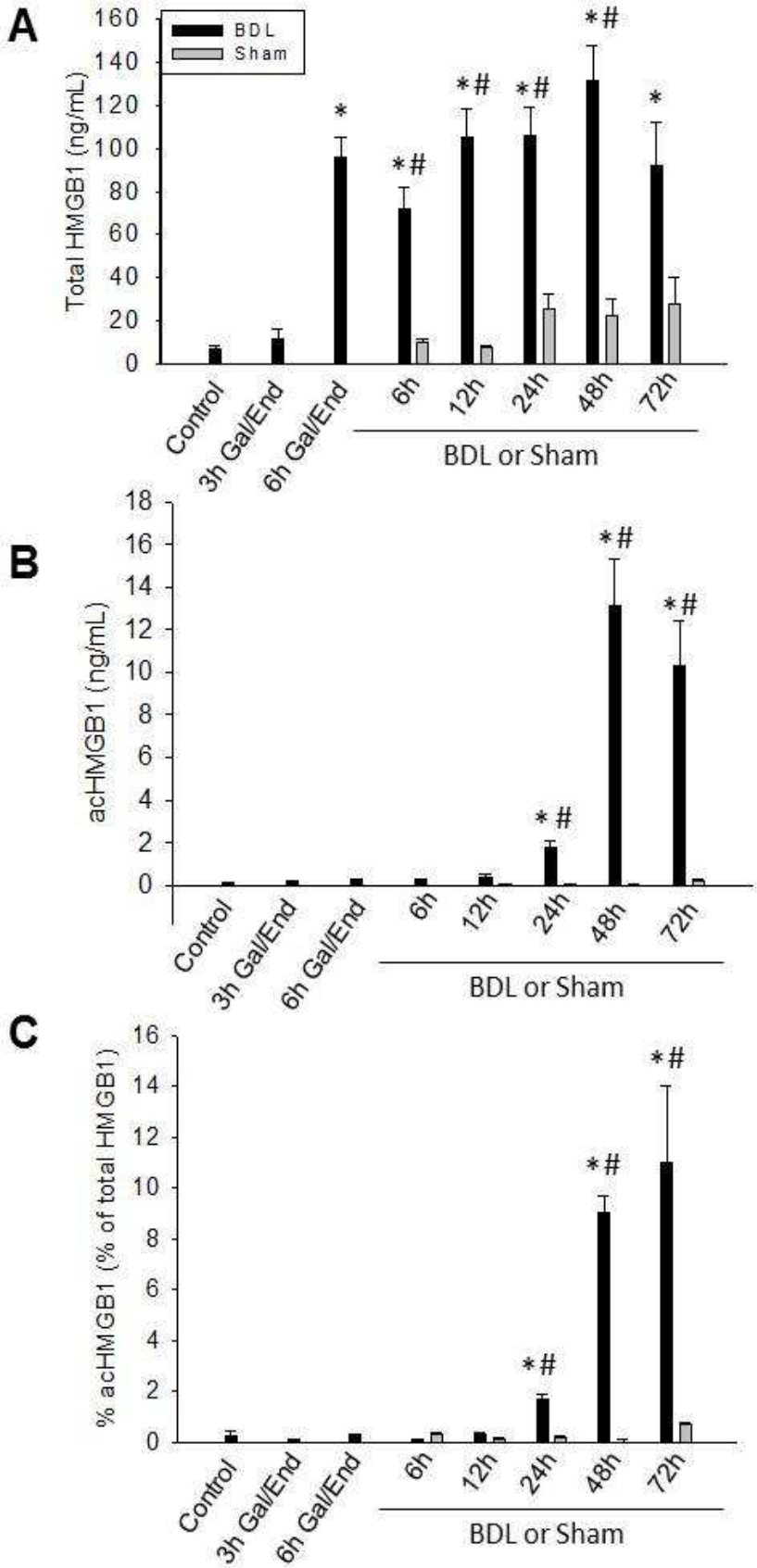

Figure 5: Total (A) and acetylated (B) forms of plasma high mobility group box 1 (HMGB1) protein were measured after galactosamine/endotoxin (Gal/End) treatment, bile duct ligation (BDL) or sham-operation. C. Percentage of acHMGB1 compared to the total HMGB1 was calculated. Data represent mean ± SE of n=4-6 animals per group. *P<0.05 (compared to untreated control); #P<0.05 (compared to sham-operated control).

inhibitors, which were highly effective in the Gal/End or Fas antibody-induced apoptosis models, did not protect against BDL-induced liver injury (Gujral et al., 2004b). The current biomarker data using full length cytokeratin-18 (FL-K18) and the caspase cleavage product (cK18), clearly indicate a lack of caspase activation from the onset of BDL to the peak of tissue damage at 2-3 days. In contrast, the dramatic increase of FL-K18 levels in plasma indicates extensive cell necrosis. Importantly, the parallel assessment of these parameters before and during liver injury after treatment with Gal/End, a model of TNF-induced parenchymal cell apoptosis and inflammation-induced necrosis (Leist et al., 1995; Jaeschke et al., 1998; Lawson et al., 1998), confirmed that substantial amounts of FL-K18 and of the caspase-cleaved fragments are released into the plasma during active necrotic and apoptotic cell death. Thus, based on the biomarker data it can be concluded that the liver injury during the first 3 days after BDL consists almost exclusively of necrosis and not apoptosis. This conclusion was further confirmed by the presence of uncleaved pro-caspase-3 in plasma after BDL and not of the active fragment. Again, the positive control for apoptosis (Gal/End) showed the presence of both pro-caspase-3 and the active fragments in plasma.

Although these plasma biomarker data are consistent and correlate with a number of tissue evaluations concluding the absence of apoptosis in the murine BDL model (Gujral et al., 2004b; Fickert et al., 2005; Nalapareddy et al., 2009; Mitchell et al., 2011), there are *in vivo* studies that claim a dominant role of apoptotic cell death (Miyoshi et al., 1999; Higuchi et al., 2001). Most of these studies rely on the TUNEL assay as an indicator of apoptosis. However, the TUNEL assay detects DNA strand breaks, which occur during apoptosis but also during necrotic cell death (Grasl-Kraupp et al., 1995; Gujral et al., 2001) suggesting that this assay is not specific for

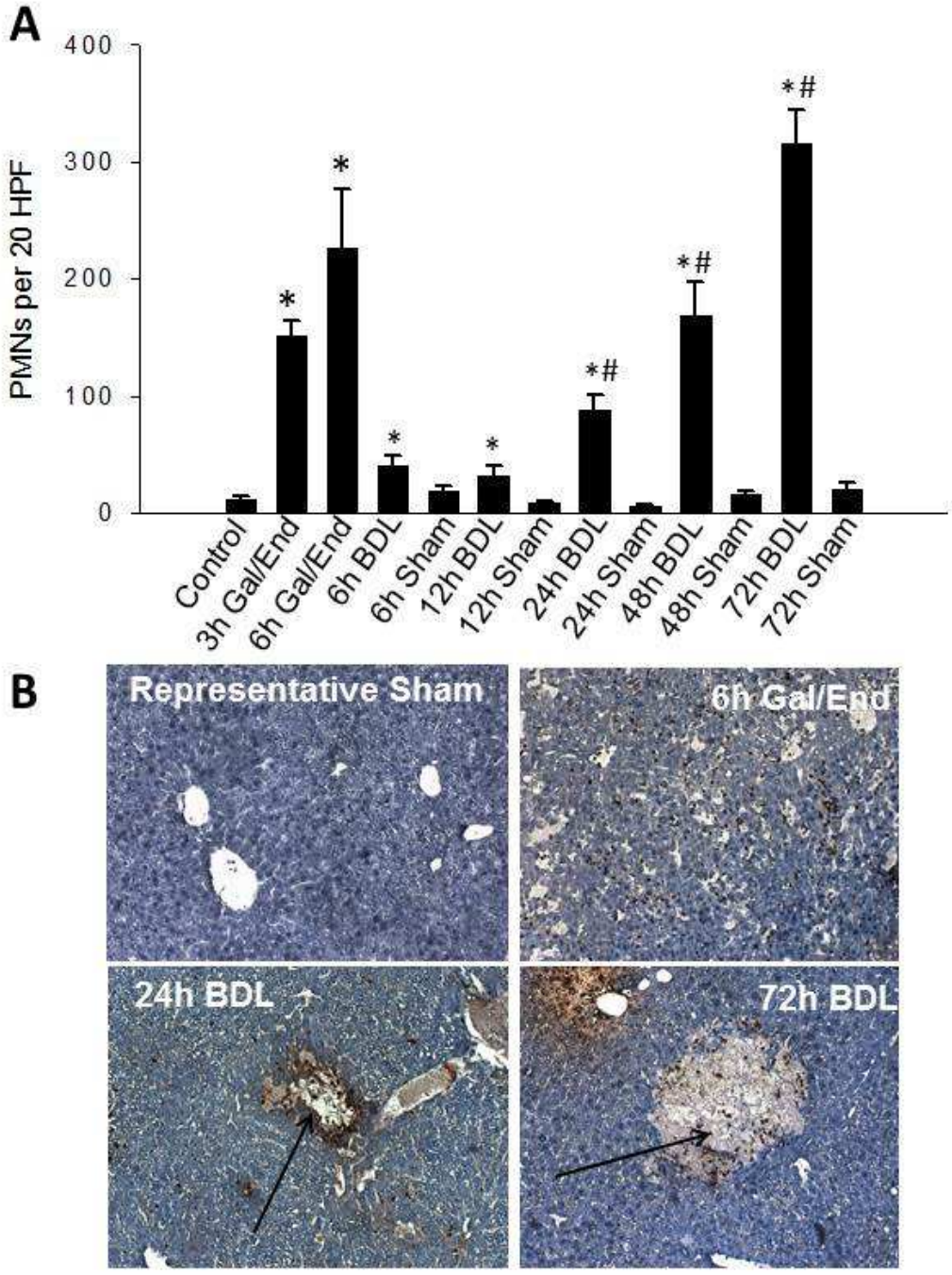

Figure 6: Neutrophil accumulation in livers after galactosamine/endotoxin (Gal/End) treatment, bile duct ligation (BDL) or sham-operation. Neutrophils were stained using an anti-neutrophil allotypic antibody against Ly6B2. A) The numbers of neutrophils were counted in 20 randomly selected high power fields (HPF) per section. Data represent mean ± SE of n=4-6 animals per group. *P<0.05 (compared to untreated control); #P<0.05 (compared to sham-operated control). B) Representative images (x100) of stained liver sections are shown. After BDL, neutrophils were largely associated with areas of infarct (arrows).

apoptosis. In addition, the argument that the Fas receptor-deficient *lpr* mouse is protected is not proof for apoptotic cell death (Miyoshi et al., 1999). *Lpr* mice show reduced inflammatory injury during BDL (Gujral et al., 2004b) and have increased Nrf2 activation, which makes them less susceptible to oxidant stress-induced injury (Williams et al., 2013). Thus, off-target effects appear to be responsible for the protection against BDL-induced injury in these mice. Likewise, the protection after anti-sense treatment knocking down the pro-apoptotic protein Bid (Higuchi et al., 2001) could not be reproduced in Bid-deficient mice (Nalapareddy et al., 2009) and therefore, off target effects need to be considered.

Circulating microRNAs have been used as biomarkers for various disease processes. MiR-122 is particularly enriched in the liver (Chang et al., 2004) and was shown to be a sensitive indicator of liver injury after APAP overdose in mice (Wang et al., 2009) and humans (Starkey Lewis et al., 2011; Antoine et al., 2013; Dear et al., 2013). APAP-induced cell injury involves predominantly necrosis in both species (Gujral et al., 2002; McGill et al., 2012; Antoine et al., 2012). Our data showed a progressive increase of miR-122 levels in plasma after BDL but not after sham-operation. The time course of miR-122 release after BDL was very similar to the necrosis marker FL-K18. In addition, miR-122 levels increased 6 h after Gal/End, which involves both apoptotic and necrotic cell death at this time (Leist et al., 1995; Jaeschke et al., 1998). Thus, the release of miR-122 further supports the conclusion of extensive necrotic cell death after BDL.

A third marker of necrosis used in our study was the nuclear protein and damage associated molecular pattern (DAMP), HMGB1, which, according to current theory, is not released during

apoptotic cell death (Scaffidi et al., 2002). Again, HMGB1 levels are increased during necrotic cell death after APAP overdose in mice (Scaffidi et al., 2002; Antoine et al., 2009; Williams et al., 2011) and humans (Antoine et al., 2012, 2013). Total HMGB1 levels dramatically increased during BDL but plasma levels changed only marginally after sham-operation. The time course, progressive increase up to 48 h after BDL and then a moderate decline, was similar to FL-K18 and miR-122 levels. Together all 3 plasma biomarkers consistently indicate necrotic cell death after BDL and plasma caspase activities and cK18 levels demonstrate the lack of apoptosis. These plasma biomarkers accurately reflect what can be observed by histology with the added advantage of representing a more global picture for the entire liver.

Neutrophils and Inflammation as a Cause of Cholestatic Liver Injury

Our previous studies provided strong evidence for neutrophilic inflammation being the key mechanism of BDL-induced liver injury in the mouse (Gujral et al., 2003a, 2004a; Kim et al., 2006; Allen et al., 2011). Neutrophils cause target cell necrosis by generating reactive oxygen (Jaeschke, 2006; 2011). BDL triggers progressive recruitment of neutrophils into the liver causing extensive oxidant stress and cell death (Gujral et al., 2003a, 2004a). HMGB1 is a known damage associated molecular pattern (DAMP), which can bind to toll like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE) and induce generation of pro-inflammatory mediators, which can activate and recruit leukocytes to sites of inflammation (Yang et al., 2013a). However, the fact that TLR4-deficient mice did not show reduced hepatic neutrophil accumulation and injury after BDL did not support a role of HMGB1 or endotoxin in neutrophil recruitment (Allen et al., 2011). In addition, the release of HMGB1 from necrotic cells and the formation of pro-inflammatory cytokines and chemokines by macrophages in the

circulation are inconsistent with the focal bile infarcts caused by neutrophils. It is more likely that chemotactic factors are derived from bile as the extensive pressure building up during bile duct obstruction leads to local ruptures of cholangioles and leakage of bile into the parenchyma (Fickert et al., 2002). Preliminary data from our laboratory have suggested that chemotactic osteopontin fragments in bile can initiate the neutrophilic inflammatory response after BDL (Yang et al., 2011).

The acetylated form of HMGB1 is known to be actively secreted by monocytes/macrophages (Bonaldi et al., 2003; Erlandsson Harris and Andersson, 2004). Our data indicate that only traces of acHMGB1 are being released during the first 24 h after BDL. However, at 48 and 72 h approximately 10% of the circulating HMGB1 proteins are acetylated. This suggests increasing monocyte/macrophage activation at 2-3 days after BDL. Moreover, the delayed release of HMGB1 and detection in the circulation compared to TNFα has been shown in murine models of sepsis (Wang et al., 1999). Indeed, inflammatory mononuclear phagocytes are detectable in the liver 3 days after BDL in mice (Duwaerts et al., 2013). Our current acHMGB1 time course data are highly consistent with the presence of mononuclear phagocytes and the delayed release of HMGB1 from activated immune cells. These cells are in a more pro-inflammatory state than the resident macrophages (Kupffer cells) at this time (Duwaerts et al., 2013). This would suggest that these infiltrating monocytes are more likely the source of acHMGB1 than the resident Kupffer cells. Nevertheless, a systemically released mediator like HMGB1 cannot direct neutrophils to focal areas of necrosis and, therefore, may not be a relevant therapeutic target. Thus, the passively released, hypoacetylated form of HMGB1is a sensitive marker of cell death and the hyperacetylated form indicates monocyte/macrophage cell activation during obstructive

cholestasis. Translational studies may be warranted to assess if plasma HMGB1 levels, as demonstrated for APAP overdose (Antoine et al., 2012), may predict negative clinical outcome (acute liver failure and death).

Summary and Conclusions

Using the plasma biomarkers full length cytokeratin-18 (FL-K18), miR-122 and HMGB1, we demonstrated progressive necrotic cell death after BDL in mice. Each of these necrosis biomarkers was more responsive than plasma ALT. On the other hand, using the apoptosis biomarkers plasma caspase-3 enzyme activities, active caspase-3 fragments and the caspase-cleaved fragment of cytokeratin-18 (cK18), no evidence for apoptotic cell death was detected within the 3 days period after BDL. In contrast, in a positive control for apoptosis and necrosis, the Gal/End model, these parameters were readily detectable. The findings with the plasma biomarkers after BDL or Gal/End are in full agreement with histological assessments in the liver. Thus, the panel of plasma biomarkers indicates that liver cell death after BDL is caused exclusively by necrosis. In addition, these biomarkers may be useful to assess the mechanisms of cell death after obstructive cholestasis in humans which may ultimately lead to new therapies and stratified medicine.

Chapter 4: Bile Acid Induced Necrosis in Primary Human Hepatocytes and Cholestatic Human Patients

This section parallels many aspects of the paper "Bile Acid Induced Necrosis in Primary Human Hepatocytes and in Human Patients with Cholestasis" Woolbright et al., 2015 – Toxicology and Applied Pharmacology - with permission.

ABSTRACT

Accumulation of bile acids is a major mediator of cholestatic liver injury. Recent studies indicate bile acid composition between humans and rodents is dramatically different, as humans have a higher ratio of glycine conjugated bile acids. This increase may lead to direct toxicity that actively kills hepatocytes, and promotes inflammation under cholestatic conditions. To address this issue, this study assessed how pathophysiological concentrations of bile acids measured in cholestatic patients affected primary human hepatocytes (PHH). Individual bile acid levels were determined in serum and bile by UPLC/MS in patients with extrahepatic cholestasis with, or without, concurrent increases in serum transaminases. Bile acid levels increased in the serum of patients with liver injury, while biliary levels slightly decreased, implicating infarction of the biliary tracts. To assess bile acid induced toxicity in man, PHH were treated with relevant concentrations, derived from patient data, of the model bile acid GCDC. Treatment with GCDC resulted in substantial necrosis with no increase in apoptotic parameters. This was recapitulated by treatment with complete biliary bile acid concentrations, but not complete serum bile acid concentrations. Elevations in serum M65, HMGB1, and acetylated HMGB1 confirmed predominant inflammatory necrosis in injured patients; although, they also had mild elevations in the apoptotic M30 form of cytokeratin 18. These data suggest human hepatocytes are more resistant to bile acids than rodent hepatocytes, and die through necrosis during cholestasis. Moreover, biliary concentrations of bile acids are required for acute cell death in man, implicating biliary infarction as a driver of injury.

INTRODUCTION:

Bile acids are the primary constituent of bile and are known to be cytotoxic to hepatocytes (Malhi et al., 2010). The predominant hypothesis for the development of cholestatic liver injury is the idea that bile acids accumulate in liver and serum, which causes toxicity (Spivey et al., 1993; Jaeschke et al., 2002; Guicciardi et al., 2013). This paradigm is supported by well-characterized mechanisms of apoptosis in rat hepatocytes (Malhi et al., 2010; Guicciardi et al., 2013) and human hepatoma lines that have been transfected with the sodium taurochlate cotransporting polypeptide (NTCP) to facilitate bile acid uptake (Faubion et al., 1999; Rust et al., 2009). A majority of these studies use glycochenodeoxycholic acid (GCDC) as a model hydrophobic bile acid to induce apoptosis, as it is the major bile acid present in human serum (Spivey et al., 1993; Trottier et al., 2012). In contrast, studies in the BDL model of cholestasis suggest BA concentrations in mice may not be directly toxic (Zhang et al., 2012). Instead, bile acids act as pro-inflammatory signaling molecules that recruit neutrophils to areas of infarction (Allen et al., 2011). In support of this hypothesis, depletion of either intracellular adhesion molecule 1 (ICAM-1) or CD11b is protective against both BDL induced injury, and BDL induced neutrophil recruitment, implicating neutrophils as a major contributor to the

Table 1: Clinical Patient Data		
	UI Patients	**CLI Patients**
N	10	17
Mean Age	56 ± 3	55 ± 2
% Female	45	55
ALT (U/L)	18 ± 2	188 ± 36*
ALP (U/L)	65 ± 8	506 ± 101*
Bilirubin (mg/dL)	0.8 ± 0.1	5.6 ± 1.8*

Table 1: Patient values for bile acid analysis. Patients with presumed cholestatic liver injury were recruited to the study whereupon bile was acquired during routine ERCP, and serum acquired before intervention. Clinical chemistry and patient history were obtained by hospital staff. *$p<0.05$. ALT – alanine aminotransferase. ALP – alkaline phosphatase.

pathogenesis (Gujral et al., 2003; Gujral et al., 2004a). Thus, there appears to be species-based discrepancies in the pathogenesis of liver injury during cholestasis.

While there have been a substantial number of studies done in rodent models and transfected hepatoma lines, few studies have been performed to assess how bile acids affect primary human hepatocytes. Initial studies confirmed bile acid toxicity in human hepatocytes using GCDC, and protection by ursodeoxycholic acid (UDCA) (Galle et al., 1990). Of note, the concentrations required for even low levels of cell death were substantially higher than what is typically used in rat hepatocyte models (Galle et al., 1990). Subsequent studies examining the mechanism of injury have suggested an important role for production of ROS and caspase mediated apoptosis in bile acid induced injury in man (Gonzalez et al., 2011). However, many of the mechanisms established in primary rat hepatocytes have yet to be established in primary human hepatocytes, thus there is a need for translational studies that assess whether or not these mechanisms also occur in man. Furthermore, novel studies need to be performed that assess how hepatocytes respond to pathophysiological concentrations of relevant bile acids measured in human patients. In this study, we have determined bile acid levels in serum and bile of patients with extrahepatic cholestasis, with and without concurrent increases in liver transaminases. These measured concentrations were then applied to isolated primary human hepatocytes with the goal of assessing how cholestatic, pathophysiological, concentrations of bile acids relevant to the human

condition effect primary human hepatocytes. Herein, we demonstrate bile acid administration results in necrosis in human hepatocytes, but only when biliary levels are applied, implicating

Table 2: Bile and Serum Bile Acid Values in Patients

	Bile		***Serum***	
Bile Acid	*UI Patient (μM)*	*CLI Patient (μM)*	*UI Patient (nM)*	*CLI Patient (nM)*
LCA	<10 μM	<10 μM	<100 nM	<100 nM
UDCA	67 ± 20	71 ± 20	<100 nM	<100 nM
CDCA	<10 μM	<10 μM	854 ± 296	379 ± 141
DCA	116 ± 100	25 ± 16	1,060 ± 375	138 ± 93*#
CA	<10 μM	<10 μM	<100 nM	<100 nM
TCA	2,403 ± 223	2,460 ± 295	445 ± 76	11,757 ± 4298*
GCDC	4,325 ± 661	2,756 ± 339	2,825 ± 375	22,156 ± 5,345*
TCDC	1,067 ± 217	1,058 ± 191	480 ± 126	6,886 ± 1,643*
GCA	3,030 ± 600	1,530 ± 269	418 ± 80	7,609 ± 1,935*
GDCA	3,643 ± 742	1,152 ± 355*	782 ± 209	3,756 ± 1,337*
TDCA	712 ± 202	607 ± 320	326 ± 136	1,201 ± 289*

Table 2: Bile acid levels were measured in serum and bile of patients with and without cholestatic liver injury via UPLC/MS (as described in methods). Patient groups were defined by elevations in ALT and ALP. For GDCA and TDCA, n = 8 selected patients for both groups from within the indicated group. * $p<0.05$. #some samples not detectable within standard curve. LCA: lithocholic acid, UDCA: ursodeoxycholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, CA: cholic acid, TCA: taurocholic acid, GCDC: glycochenodeoxycholic acid, TCDC: taurochenodeoxycholic acid, GCA: glycocholic acid, GDCA: glycochenodeoxycholic acid, TDCA: taurochenodeoxycholic acid.

biliary infarction as a key mediator of injury during cholestasis in man and illustrating direct toxicity of bile acids independent of neutrophil function in man.

RESULTS

Biliary infarction as an initial mediator of cholestatic liver injury: Accumulation of bile acids in hepatocytes leads to cell death. Bile acid toxicity is dependent on the species of bile acids present though, as more hydrophobic bile acids and glycine conjugated bile acids are more toxic than the hydrophilic, taurine conjugated species (Chaterjee et al., 2013). One of the goals of the study was to better define bile acid concentrations during human cholestasis. We assessed bile acid concentrations in serum and bile of patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) for confirmation of presumed cholestasis, with an emphasis on patients with extrahepatic cholestatic liver injury. Patients were divided into those with cholestatic liver injury (cholestatic liver injury patients or CLI patients) and those without concurrent injury (uninjured patients or UI patients) as defined by clinically elevated ALT (ALT >50) and ALP (ALP>110) values. Patient values are listed in Table 1. Etiologies for patients are listed in Supplementary Table 1. Serum and biliary bile acid values were measured in both patient groups (Table 2). Glycine and taurine conjugated primary bile acids were most prevalent in serum, and increased to the highest values in serum during cholestasis. Of note, mean GCDC in serum increased to 22 μM. A large majority of bile acids in both serum and bile were conjugated in both cholestatic and non-cholestatic patients, with only a very small percentage being unconjugated. Surprisingly, while total serum bile acid values increased dramatically in CLI patients as expected (Figure 1A), biliary levels of bile acids decreased significantly in CLI patients (Figure 1B). This was largely mediated by a significant decrease in glycodeoxycholic acid (GDCA) and a trend towards a decrease in glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDC) (Table 2). Histological analysis of liver with significant

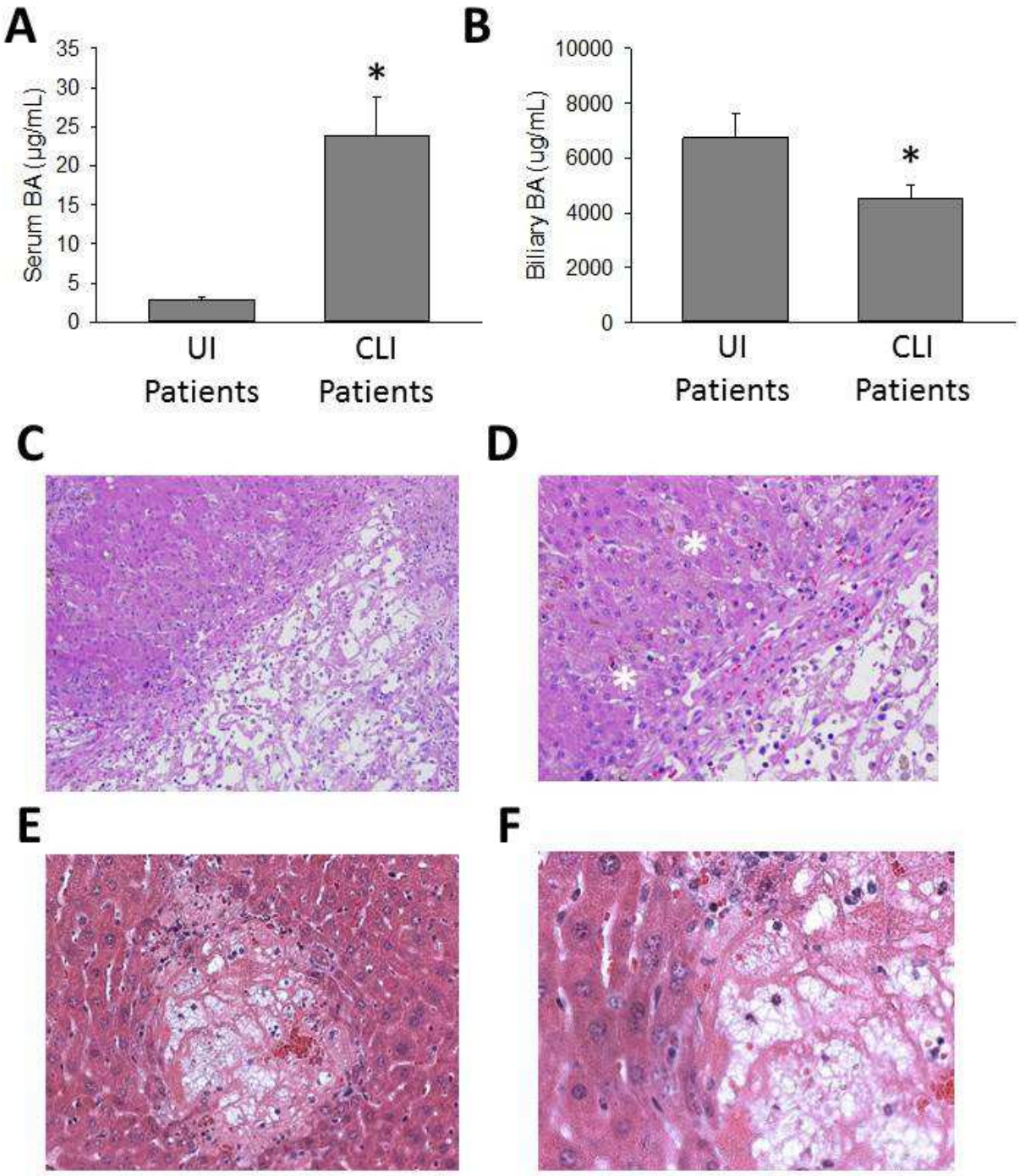

Figure 1: Infarction of the biliary tracts mediates the initial injury in cholestatic human patients. Serum (A) and bile (B) were taken from patients thought to have biliary obstruction during routine ERCP. Total and individual bile acid levels were measured via UPLC/MS. Patient data available in Table 1. H & E staining was performed on slides from human patients with extrahepatic cholestasis (C) and (D) and mice after bile duct ligation (E) and (F). Frank necrosis is seen in image (C) in an area of biliary infarction. Bile (marked by *) leaks into adjacent hepatic parenchyma in (D). Bile infarcts in mice subjected to 72 hours bile duct ligation show similar histological features.

extrahepatic cholestasis revealed bile infarcts akin to those seen in mice after BDL, with frank necrosis and significant inflammation (Figure 1C). Extra-biliary bile pools (Figure 1D, white star), were readily apparent in the hepatic parenchyma around areas of necrosis, suggesting leakage of bile and obstructive cholestasis. Areas surrounding bile displayed characteristic feathery degeneration and flocculation of cytoplasm in both mice and humans, consistent with hepatocellular necrosis. The infarcts seen in man were highly similar to infarcts seen after BDL in mice (Figure 1E and Figure 1F), which are also characterized by necrosis and inflammation (Fickert et al., 2005). Given the decrease in biliary bile acid concentrations in CLI patients, and the correlation between areas of biliary infarction and areas of frank necrosis, biliary infarction is likely an initial mediator of bile acid induced injury during extrahepatic cholestatic liver injury in man.

GCDC induced toxicity in primary human hepatocytes: Bile acids are a primary component of bile. To assess how toxic concentrations of bile acids that leaked from bile affected primary human hepatocytes, we first exposed primary human hepatocytes to increasing concentrations of the hydrophobic bile acid GCDC to assess at what point toxicity began to occur. Values used were based on maximum values from our own data as well as previously reported concentrations of these bile acid species in the serum of cholestatic patients (Trottier et al., 2011; Trottier et al., 2012). Values of GCDC consistent with serum concentrations produced no toxicity after 3 h, 6 h or 24 h (Figure 2A) exposure. Concentrations of 1 mM GCDC and above resulted in significantly increased LDH release at both 6 h and 24 h exposure (Figure 2A), although this value was not significantly higher at 24 h, suggesting the injury occurs predominantly during the initial 6 h. While these concentrations far exceed serum concentrations, they are consistent with values seen in bile of cholestatic patients (Table 2; Dilger et al., 2012). In

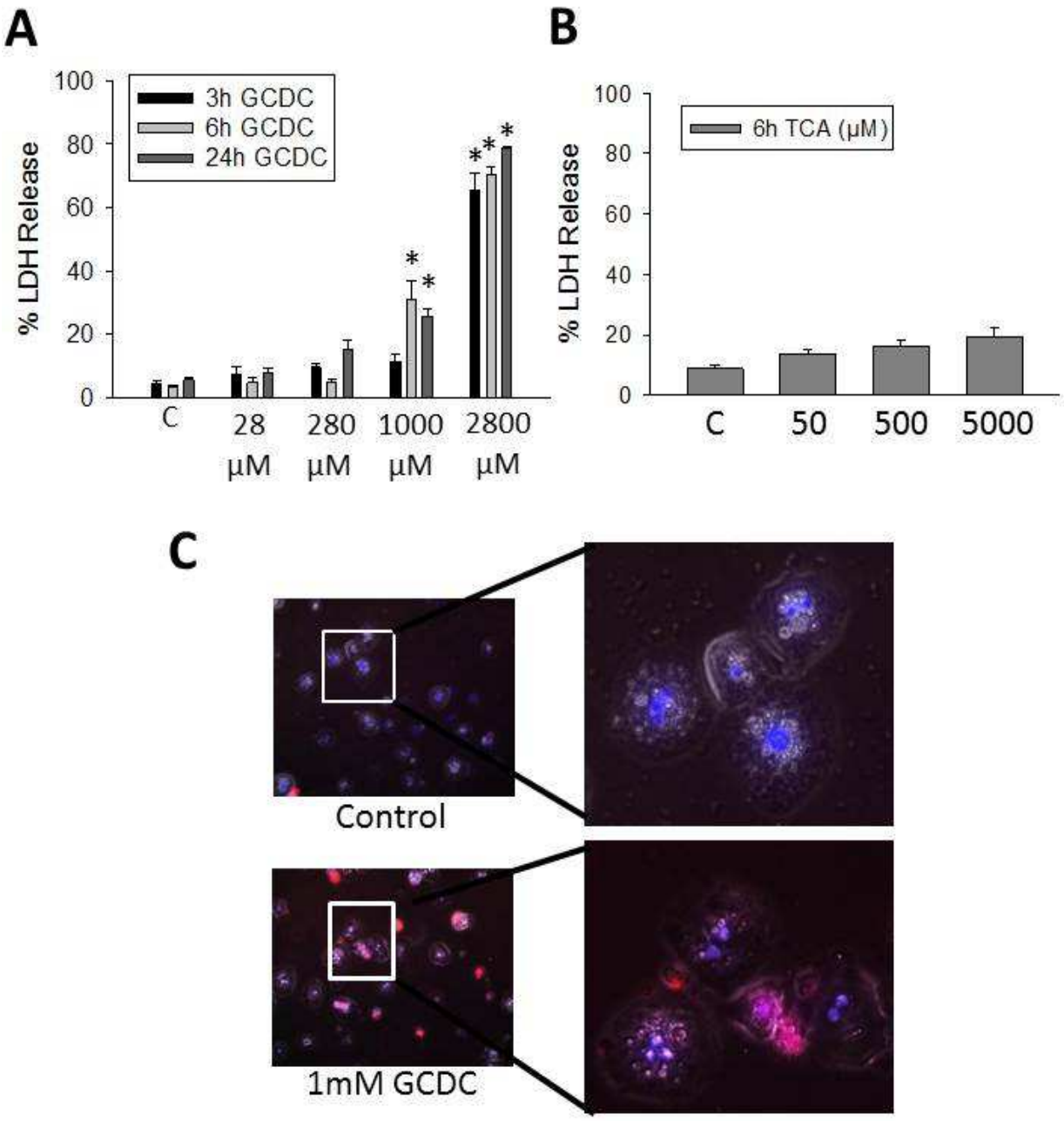

Figure 2: Bile acid exposure results in cell death in human hepatocytes. LDH was measured 3h, 6h or 24h after exposure to the indicated bile acid GCDC (A) or TCA (B). N=4, * $p < 0.05$. Propidium iodide and Hoescht stain were applied to hepatocytes after 6h treatment with 1mM GCDC (C). Images were overlaid using Image J software.

contrast, values of TCA up to 5mM showed limited toxicity after 6 h treatment (Figure 2B). To confirm the toxicity, human hepatocytes were incubated with 1 mM GCDC for 6 h and then exposed briefly to propidium iodide and Hoescht 33342 dyes. Uptake of propidium iodide was readily apparent in GCDC treated hepatocytes (Figure 2C), but not the control counterparts or TCA treated hepatocytes (data not shown). These data confirm acute toxicity after exposure to GCDC *in vitro* in human hepatocytes.

Human hepatocytes were also exposed to increasing concentrations of taurocholic acid (TCA) to see if this would induce pro-inflammatory genes, as bile acid induced inflammatory gene induction has been proposed as a mechanism for inflammation in the injury in the BDL mouse model (Allen et al., 2011; Zhang et al., 2012; Woolbright and Jaeschke, 2012). Human hepatocytes exposed to TCA showed little increase in pro-inflammatory genes, especially when compared to values seen in murine hepatocytes (Table 3). A small, but significant, increase was seen in IL-8 expression after TCA treatment. While this study does not preclude other potential inflammatory mediators being upregulated by bile acids, this facet of the murine response to bile acids *in vitro* is minimally recapitulated in the human hepatocyte.

Bile acid uptake in human hepatocytes: Bile acid uptake has previously been suggested to be a critical aspect of toxicity in mouse models of cholestatic liver injury (Gartung et al., 1996; Gartung et al., 1997). Uptake primarily occurs through the sodium-taurocholate cotransporting polypeptide (NTCP) but also occurs through organic anion transporting polypeptides (OATPs) (Hagenbuch and Gui, 2008). Bile acid uptake was measured in human hepatocytes (Figure 7A) or rat hepatocytes (Figure 7B) via uptake of radioactive taurocholate using a sodium containing or choline chloride containing media, which measure either sodium dependent (NTCP mediated) or sodium independent (OATP mediated) uptake respectively. Sodium dependent uptake was

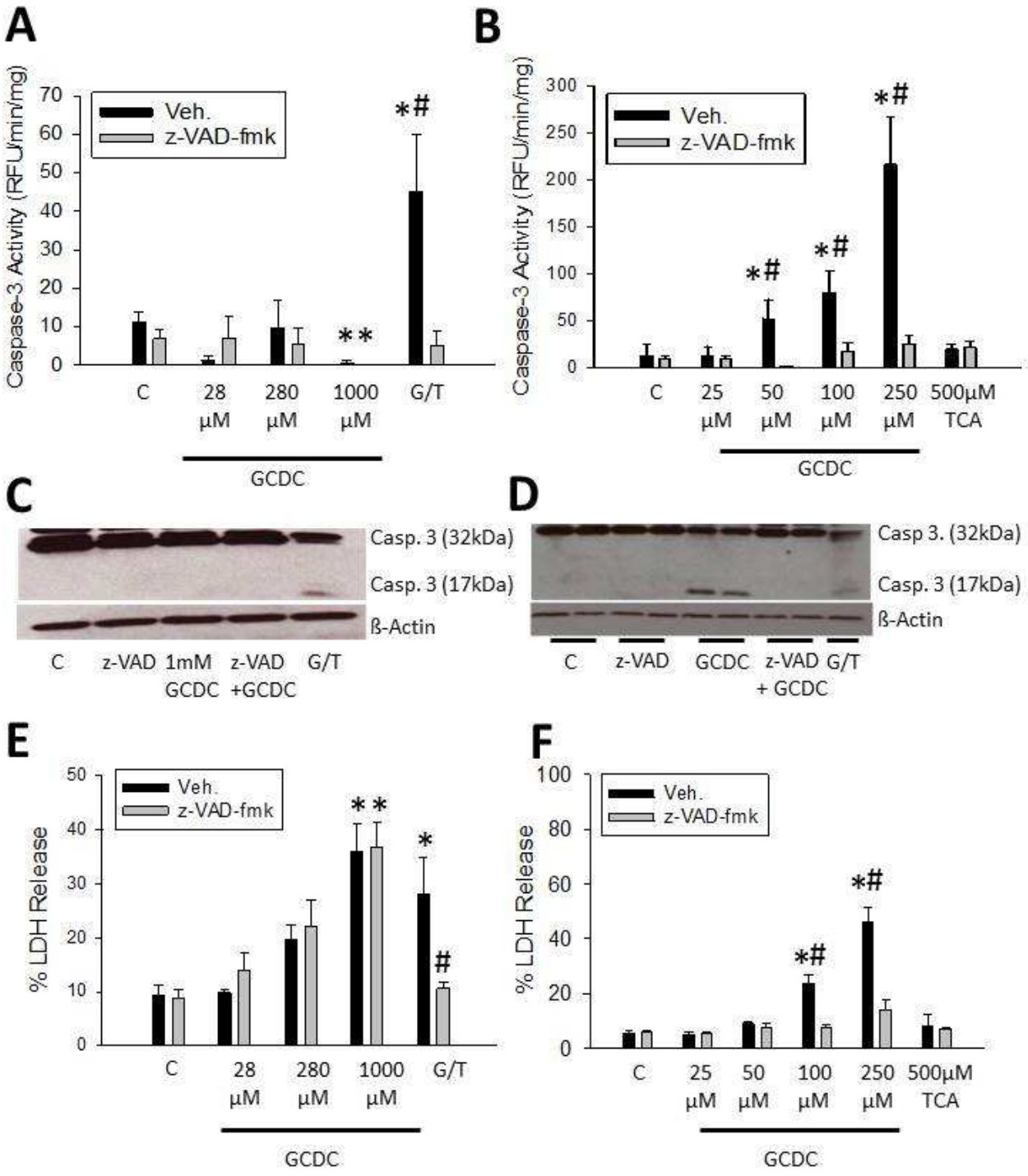

Figure 3: Bile acid exposure results in hepatic necrosis *in vitro*. Caspase-3 activity was measured using a fluorescent substrate after the indicated concentration of GCDC or TCA for 6 h or after or 12 h galactosamine/TNF treatment (G/T) in human (A) and rat (B) hepatocytes pretreated with vehicle (DMSO) or z-VAD-fmk. Caspase-3 activation was assessed via western blot in human (C) or rat (D) hepatocytes after GCDC treatment. LDH release was measured after a pretreatment with 10μM z-VAD-fmk in human hepatocytes (E) or rat hepatocytes (F). N=5 for human hepatocytes, n=3 for rats, * $p<0.05$ versus control. # $p<0.05$ versus matched pair.

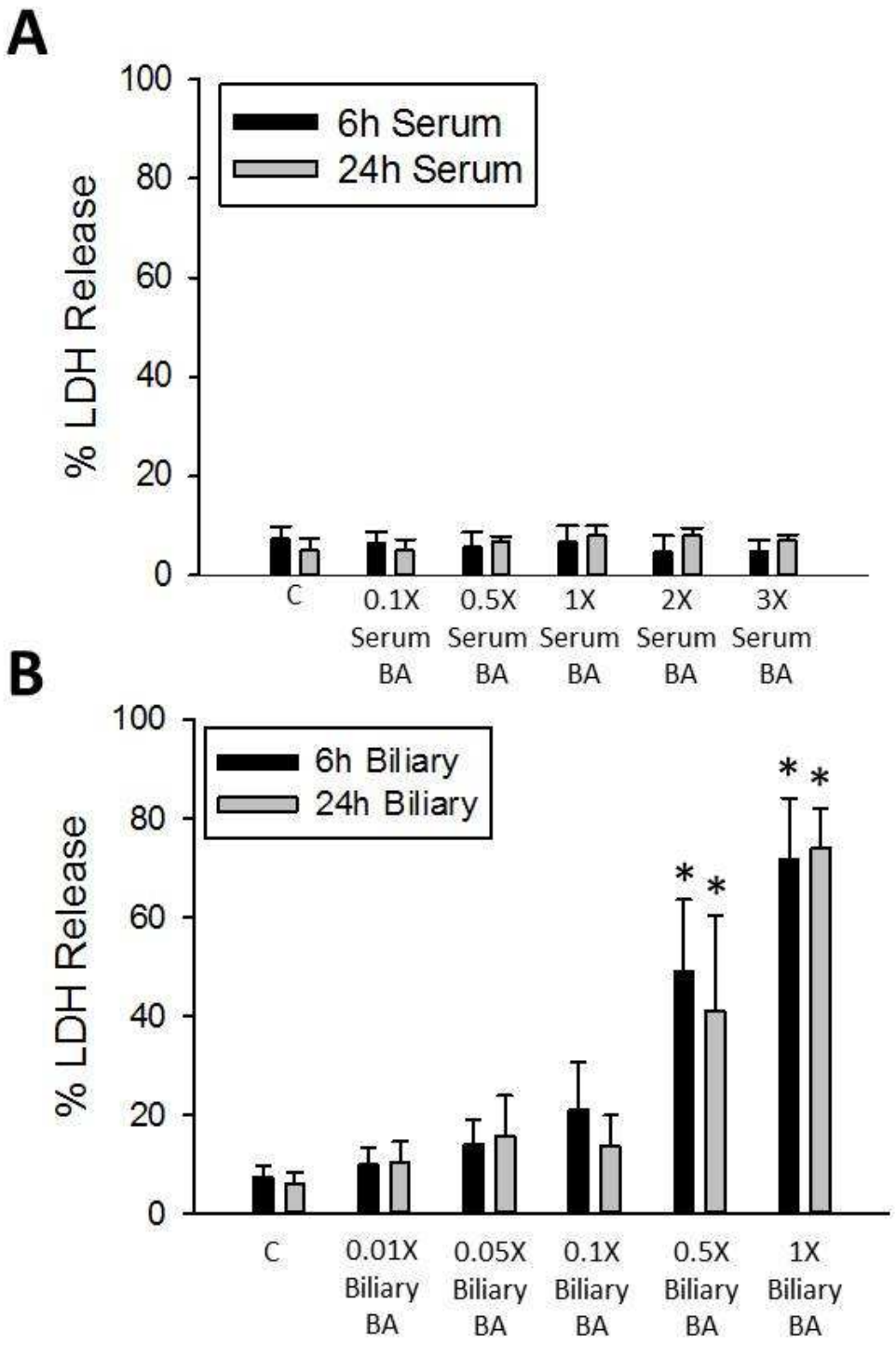

Figure 4: Biliary concentrations of bile acids and not serum concentrations are consistent with toxicity in human hepatocytes. LDH release was measured in human hepatocytes after exposure to a complete serum BA milieu or a derivation of this concentration (A) or a complete biliary bile acid milieu or a derivation of this concentration for 6h or 24h (B). N=3. * $p<0.05$ versus control. # $p<0.05$ versus matched pair.

predominant in line with previous reports (Szabo et al., 2013) in both cell lines. Human hepatocytes retained their uptake capacity 24 h after the initial plating, suggesting these cells may be usable for longer term experiments in the future. This is pertinent, as rat hepatocytes are known to decrease expression of transporters rapidly when in culture, and lose their native responses to bile acid exposure (Liang et al., 1996).

Lack of apoptosis in GCDC treated human hepatocytes: GCDC has been shown to cause apoptosis in hepatoma lines transfected with a functional sodium taurocholate transporting peptide (NTCP) and in isolated rat hepatocytes (Malhi et al., 2010). To confirm apoptosis after exposure to GCDC in human hepatocytes, caspase activity was measured in control treated, or z-VAD-fmk treated, primary human hepatocytes exposed to increasing concentrations of GCDC. While there was baseline caspase-3 activity in control primary human hepatocytes, there was no increase in caspase activity in GCDC treated human hepatocytes (Figure 3A). Caspase activity trended downwards after GCDC treatment, and was significantly different in the 1 mM GCDC treated groups (Figure 3A). These data were confirmed by Western Blot, as there was no caspase-3 activation (Figure 3C). As a positive control to insure that further induction of caspase-3 activity was achievable, human hepatocytes were also treated with 5 mM galactosamine and 250 ng/mL recombinant human TNF-α for 12 h, whereupon an increase in caspase-3 activation was observed (Figure 3A). The z-VAD-fmk treatment effectively prevented the increase in caspase-3 seen in this group. Rat hepatocytes were also treated with increasing concentrations of GCDC to confirm methodology and to serve as a control (Figure 3B). Dramatically increased caspase-3 activity was found starting at a 50 μM dose, consistent with numerous previous reports. These data were confirmed by Western Blot (Figure 3D). No increase was seen when rat hepatocytes were treated with TCA, suggesting this dose was specific

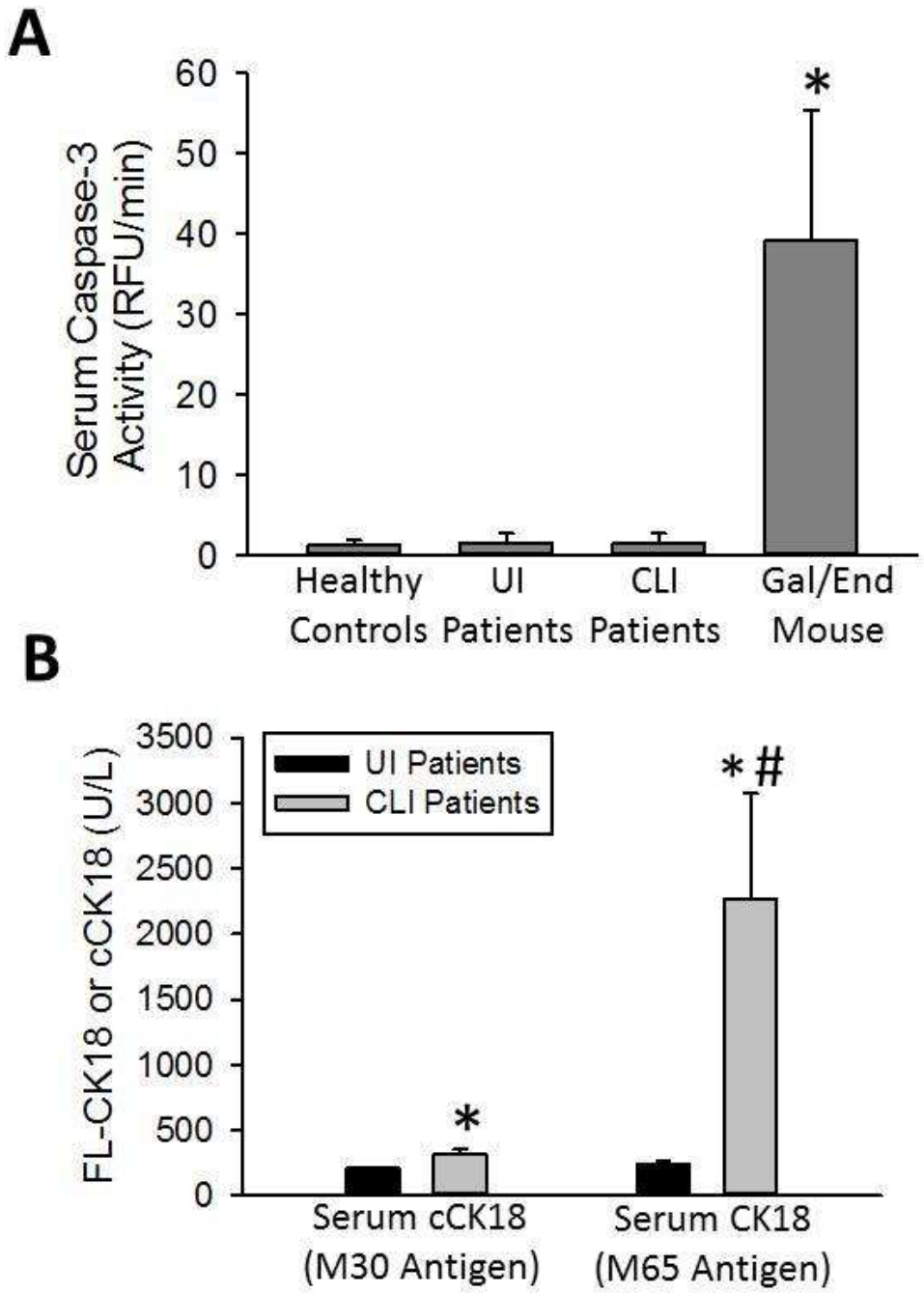

Figure 5: Necrosis predominates during cholestatic liver injury in man. Serum caspase values were measured in human patients and in galactosamine endotoxin treated mice (A). Full length cytokeratin 18 (FL-CK18) or M65 antigen values and caspase cleaved (cCK18) or M30 antigen values were measured in serum of human patients (B). Patient data available in Table 1. * $p<0.05$ versus control. # $p<0.05$ versus all other groups.

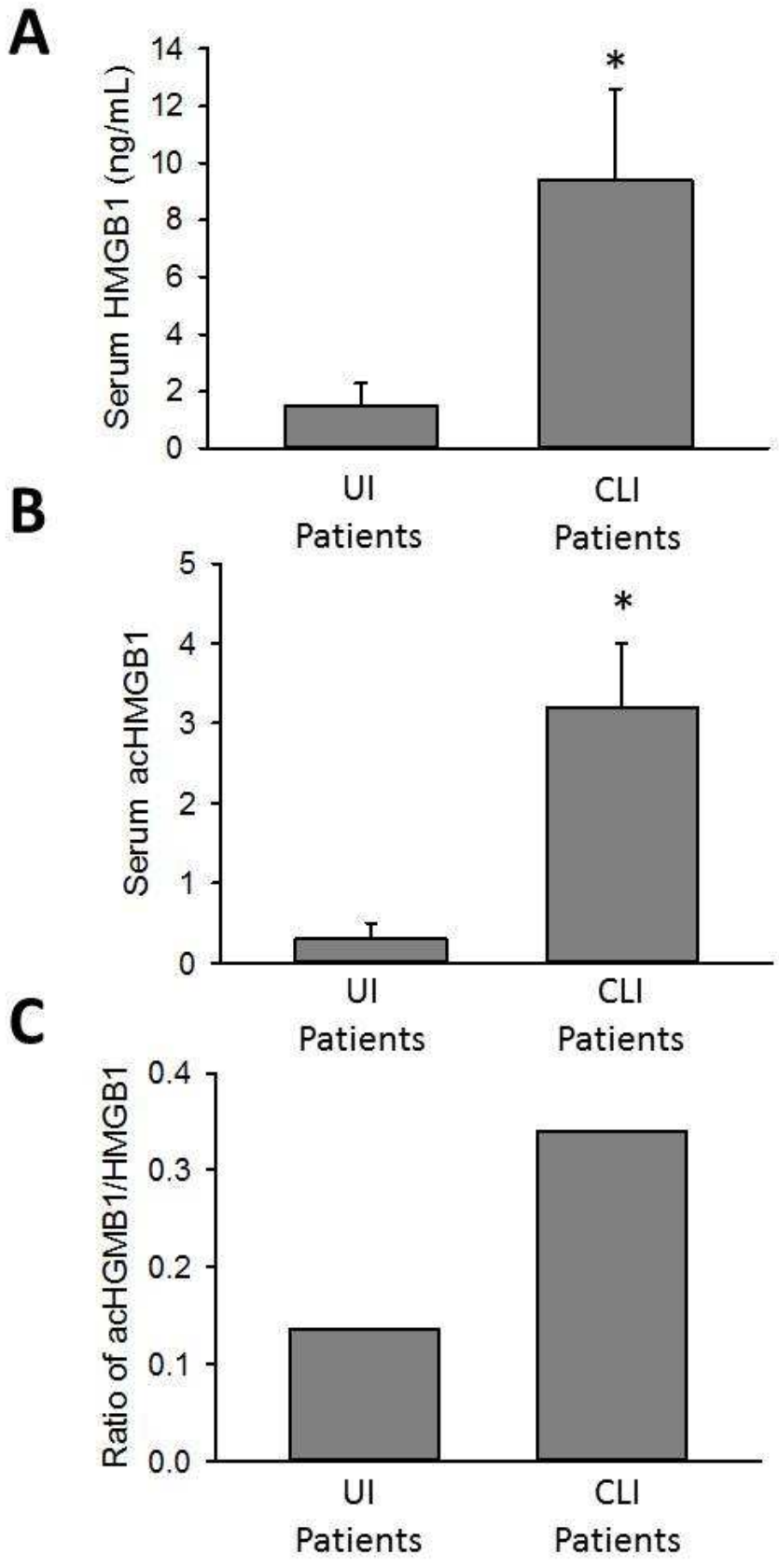

Figure 6: Elevated serum HMGB1 levels in injured patients during cholestatic liver injury in man. HMGB1 (A) and acHMGB1 levels (B) were measured in the serum of human patients. The ratio of HMGB1 to acHMGB1 was also calculated (C). Patient data available in Table 1. * $p<0.05$ versus UI group.

to the more toxic, hydrophobic bile acids such as GCDC (Figure 3B). Of note, onset of toxicity occurred at doses 10-20 fold lower in rat hepatocytes than in human hepatocytes, confirming previous observations that human hepatocytes are generally more resistant to bile acid induced cell death (Galle et al., 1990). To assess whether or not inhibition of caspases could prevent cell death, LDH release was measured in control, or z-VAD-fmk pretreated human hepatocytes, although there was no difference at any dose tested (Figure 3E). Pretreatment with z-VAD-fmk protected completely against lower doses of GCDC in rat hepatocytes, and partially against doses associated with progression to necrosis (Figure 3F). These data indicate that in contrast to *in vitro* rat hepatocytes models, bile acid induced injury occurs primarily through necrosis in human hepatocytes *in vitro*, and not through apoptosis.

Hepatocyte necrosis in response to biliary concentrations of bile acids: To test the hypothesis that biliary values of bile acids were required for cell death in human hepatocytes, human hepatocytes were exposed to a reconstituted bile acid milieu composed of a specific concentration of the total serum, or total bile derived concentrations of bile acids, measured in injured patients in this study. Exposure to the serum bile acid milieu resulted in no increases in LDH release at 6 or 24 (Figure 4A), even at exposures of up to three times the total concentration of serum bile acids (3x or three times the values of each individual measured bile acid reconstituted together into a single treatment). Exposure to the biliary bile acid milieu (Figure 4B) resulted in significant toxicity at a 0.5x dose (0.5x the value of each individual bile acid reconstituted together into a single treatment). Exposure to the complete biliary bile acid milieu resulted in near total LDH release. Direct exposure to total human bile confirmed this toxicity (data not shown). These data indicate serum bile acid concentrations are not the likely

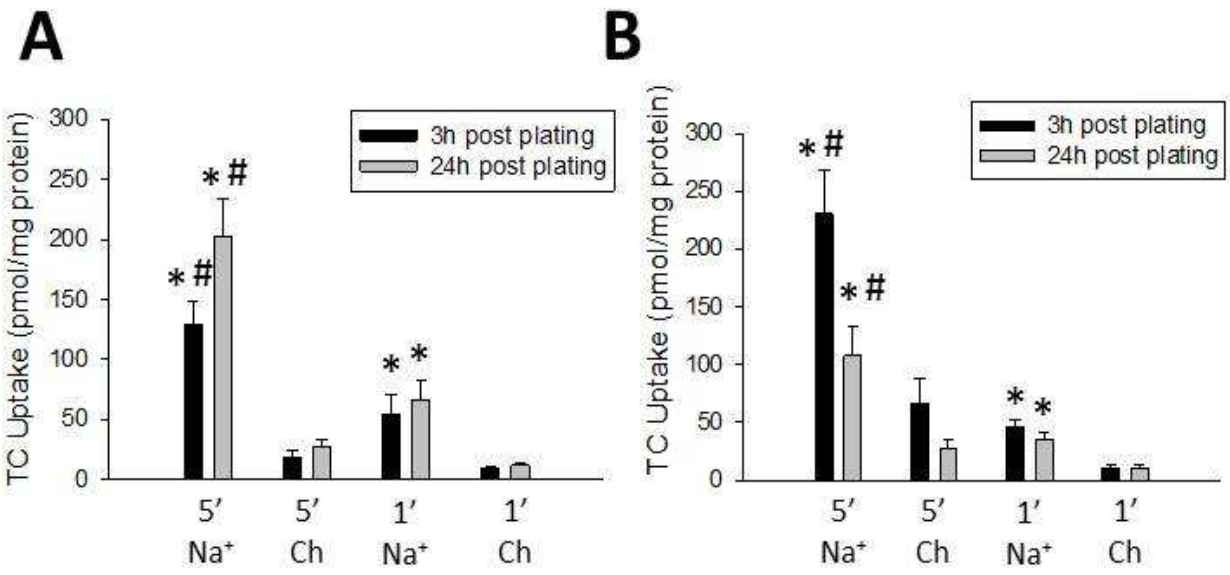

Figure 7: Uptake of bile acids is critical to bile acid induced injury *in vitro.* Taurocholate (TC) uptake (H-3) was measured in human (A) and rat (B) hepatocytes 3h and 24h post plating for 1 or 5 minutes in a sodium containing or choline chloride containing media. N=3.* $p<0.05$ versus choline media. # $p<0.05$ versus 1′ time point. (C) through (E) * $p<0.05$ versus all other treatment.

cause of bile acid toxicity during extrahepatic cholestatic liver injury, but instead biliary leakage likely accounts for much of the toxicity associated with the accumulation of bile acids.

While evidence in this study strongly favors bile acid induced necrosis, previous reports indicate significant levels of apoptosis in some populations of cholestatic patients as evidenced by elevated serum cytokeratin-18 (CK18) M30 antigen levels (Yagmur et al., 2007). To assess apoptosis *in vivo* in our study, we first assessed caspase-3 levels in the serum of our patients (Figure 5A). There was a small but insignificant increase in caspase-3 activity in the injured patient group as compared to control patients or uninjured patients; however, this paled in comparison to an established model of apoptosis, the galactosamine endotoxin treated mouse (Figure 5A). Serum M65 antigen and M30 antigen values were also calculated for the same patient set. While there was a significant increase in M30 values in the CLI group versus the UI group indicating active apoptosis in the patient population, there was a further significant increase in the M65 fragment in the injured group (Figure 5B). The M30 fragment represented approximately 13% of total circulating cytokeratin 18, indicating predominant necrosis during the injury process. To confirm this necrosis, another novel marker of necrotic injury, high mobility group box-1 (HMGB1), was measured in serum of human patients (Antoine et al., 2009; Antoine et al., 2012). HMGB1 levels were elevated in the CLI group as compared to the UI group confirming necrotic injury (Figure 6A). Additionally, elevated levels of the acetylated form of HMGB1 (acHMGB1) were also found in the CLI group (Figure 6B). CLI patients also had a higher mean ratio of acHGMB1 to HMGB1 levels in serum (Figure 6C). While the hypoacetylated form of HMGB1 is typically associated with passive necrotic release of cellular constituents (Evankovich et al., 2010), the acetylated form is actively secreted by immune cells such as macrophages (Bonaldi et al., 2003). While bile acids were not directly capable of

Table 3: Gene Expression Changes after TCA Treatment

Murine Hepatocytes		
	Control	*5000 μM TCA*
mKC	1.0 ± 0.1	25.1 ± 4.6*
MIP-2	1.0 ± 0.1	780.0 ± 122.0*
VCAM-1	1.0 ± 0.2	3.9 ± 0.2*
ICAM-1	1.0 ± 0.1	7.4 ± 0.3*
TNF-α	1.0 ± 0.2	2.2 ± 0.4*
Human Hepatocytes		
	Control	*5000 μM TCA*
CXCL-1 (mKC)	1.0 ± 0.4	1.3 ± 0.4
CXCL-2 (MIP-2)	1.0 ± 0.4	0.8 ± 0.1
ICAM-1	1.0 ± 0.1	1.5 ± 0.4
IL-8	1.0 ± 0.2	2.4 ± 0.4*

Table 3: Human or mouse hepatocytes were treated with the indicated concentration of taurocholic acid (TCA) and then RT-PCR was used to assess gene changes. No changes were found at 50 μM or 500 μM TCA treatment in human hepatocytes (data not shown). N = 3. $p<0.05$. mKC: mouse keratinocyte derived factor, MIP-2: macrophage inflammatory protein 2, VCAM-1: vascular cellular adhesion molecule-1, ICAM-1: intracellular adhesion molecule-1, TNF-α: tumor necrosis factor α, CXCL: CXC chemokine motif ligand, IL-8: interleukin-8

stimulating CXC chemokines or adhesion molecules in human hepatocytes (Table 3), passive and active release of HMGB1 during cholestatic necrosis may stimulate sterile innate immunity during the injury, which correlates with the obvious inflammatory infiltrate seen in Figures 1C and 1D. Together, these data indicate bile acids cause direct necrosis, and likely exacerbate the inflammatory response via the release of necrotic and pro-inflammatory factors.

DISCUSSION

Multiple recent papers have analyzed individual bile acids and their concentrations in human and murine serum and bile during different cholestatic disorders (Trottier et al., 2011; Dilger et al. 2012, Zhang et al., 2012, Woolbright et al., 2014a; Woolbright et al., 2014b). One hypothesis supported by these studies was the idea that humans accumulate significantly greater amounts of glycine conjugated bile acids than rodent species. While previous data from our laboratory suggests cholestatic liver injury is caused primarily via neutrophil recruitment (Gurjal et al., 2003; Gujral et al., 2004), the presence of increased concentrations of glycine conjugated bile acids in human patients suggests a potential for species based differences in initiation and promotion of the injury. Thus, the major goal of this study was to determine how pathophysiological levels of relevant bile acids affect human hepatocytes both *in vitro* and *in vivo*, as the enrichment of glycine conjugated bile acids present in man might lead to direct cytotoxicity. Paradoxically, we found human hepatocytes are highly resistant towards bile acid induced injury *in vitro*, especially when compared to rat hepatocytes (Figure 2 and Figure 3). Moreover, human hepatocytes lack the defined apoptotic response to toxic concentrations of

GCDC that rat hepatocytes exhibit (Figure 3). Using values acquired from patient bile and serum, we show hepatocytes are also resistant to the complete serum bile acid milieu, but undergo acute necrosis when exposed to biliary concentrations (Figure 4). The necrotic phenotype was confirmed using serum biomarkers of liver injury (Figures 5 and 6). These data suggest infarction of the biliary tracts and exposure of hepatocytes to biliary concentrations of bile acids results in primary necrosis, and is the initial mediator of cholestatic liver injury. This initiates an inflammatory response, potentially mediated by substantial amounts of HMGB1 and acHMGB1 present in serum of cholestatic patients (Figure 6). Histology from cholestatic liver sections confirms the data in man as the primary site of injury after extrahepatic cholestasis is characterized by frank hepatic necrosis, extra-biliary bile pools, feathery degeneration, and an inflammatory infiltrate (Figure 1).

Apoptosis and necrosis in human patients with cholestatic liver injury: Considerable discussion has occurred over the mechanisms involved in the onset and progression of cholestatic liver injury (de buy Wenninger et al., 2012; Woolbright and Jaeschke 2012; Guicciardi et al., 2013). A large portion of this debate has been over whether the primary mechanism of injury occurs through apoptosis or necrosis, with different models in different animals showing different results. Regardless, the critical question remains *what is the modality and mechanism of cell death during cholestasis in man?* Apoptosis has previously been presented as a modality of injury in bile acid treated human hepatocytes and in cholestatic human patients (Yagmur et al., 2007; Gonzalez et al., 2011). While cholestatic patients have been shown to have higher caspase-cleaved cytokeratin 18 (M30 antigen) values, M65 values were never reported in these patients, which prevents a quantitative assessment of the degree of necrosis and apoptosis (Yagmur et al., 2007). In this study, both M65 and M30 values were significantly elevated over control patients;

however, M65 values were approximately 8X higher than M30 values (Figure 5B). This suggests that while apoptosis definitely occurs during cholestasis, it makes up a smaller portion of the injury than necrosis. While both necrotic and apoptotic cells can be identified using histology in patients with obstructive cholestasis, there is some debate as to what the detection of apoptotic cells using histology means quantitatively, as actual apoptotic rates may be underestimated, since they are masked by rapid phagocytosis of apoptotic cells (Canbay et al., 2003). Given the size of the infarct in comparison to rates of detected apoptotic cells, it seems unlikely apoptosis plays a significant role in cell death unless it is a major component of the area of infarction (Figure 1). As the infarct has a clearly necrotic phenotype, it seems most likely that the initiating source, and the major modality of injury is necrosis. This is confirmed in *human* hepatocytes, which die predominantly through necrosis after toxic bile acid exposure as evidenced by propidium iodide permeable membranes, LDH release, a lack of executor caspase activity, and no protection by pretreatment with the pan-caspase inhibitor z-VAD-fmk (Figure 2 and 3) in contrast to two separate defined models of apoptosis, including administration of GCDC to rat hepatocytes (Figure 3). Bile acid uptake was similar between species (Supplementary Figure 1), thus the difference in mechanisms between man and rat is likely an intracellular event. This may be related to higher oxygen conditions experienced by hepatocyte *in vitro* (Hohenester et al., 2013). As the injury is likely due to multiple factors outside of the direct areas of biliary infarction, increases in apoptosis during cholestasis may be due to the presence altered local biliary/serum bile acid concentrations, cytotoxic neutrophils, or other inflammatory agents. Future studies examining these intracellular differences may be merited, especially given the well-founded mechanisms in place in the rat hepatocyte model (Malhi et al., 2010).

Serum and biliary concentrations of bile acids during cholestasis:

One important aspect of this study was the lack of toxicity associated with serum concentrations of bile acids (Figure 5A). Retention of bile acids in hepatocytes has long been hypothesized to be a primary source of toxicity during cholestasis. A major question is what is the primary source of bile acids that are retained by hepatocytes? Hepatocytes are known to down-regulate accumulation of the intrahepatic bile acid pool during cholestasis (Gartung et al., 1997; Gartung et al., 1998). Activation of FXR by increased intrahepatic bile acid levels decreases expression of CYP7A1 and multiple uptake transporters, and increases expression of exporters such as BSEP (Reviewed in Chiang, 2013; and Hylemon and Zhou, 2014). These measures are generally considered protective and serve to limit intrahepatic bile acid levels, partially through ablation of uptake from serum and enhanced export of bile acids into bile. In addition, the dramatic reduction in CYP7A1 leads to a major decrease in intrahepatic production of bile acids limiting intracellular toxicity. In this study, serum levels did not correlate with injury with either GCDC treatment alone (Figure 2), or exposure to a complete serum bile acid milieu (Figure 4), thus, it seems likely serum levels do not directly result in cell death during cholestasis. This meshes with histological findings, as the injury is consistent with infarction of the biliary tracts. If serum levels were integral to the injury, then periportal injury would predominate due to higher concentrations of bile acids in the periportal region, an area with highly efficient uptake of bile acids from serum, instead of the characteristic, a-zonal, focal necrosis. These data are consistent with recent hypotheses that hepatocyte injury can be prevented via protection of the cholangiocyte, as halting cholangiocyte injury and dysfunction would prevent the infarction of the biliary tracts, and halt the injury (Hohenester et al., 2012; Beuers et al., 2012). Thus, interventions designed to prevent biliary infarction via reducing biliary load or protecting

cholangiocytes against cell death would make viable pharmacological targets, in addition to reducing bile acid uptake, or preventing intracellular mechanisms of injury in hepatocytes.

One potential caveat is the acute nature of this study. Hepatocytes are exposed to serum concentrations far longer than 6 or 24 h during cholestasis in man. While there would be a much greater exposure time, this would result in FXR activation and concurrent changes in FXR dependent genes. As such, the period which hepatocytes are presumably most susceptible to bile acid induced injury would be within the first 24 h, before the compensatory changes in uptake/export and synthesis take place. Thus, while this study does not entirely preclude a role for serum bile acid concentrations in the onset of injury, the data indicate biliary levels of bile acids and biliary infarction are likely required for the onset of acute injury.

Chronic cholestasis results in rupture of the biliary tracts due to either cholangiocyte death, which is consistent with elevated ALP values, or mechanical pressure due to the dramatically increased biliary load (Fickert et al., 2002). While previous reports have suggested biliary bile acid levels mildly increase during autoimmune hepatitis (Dilger et al., 2012), our study focused on extrahepatic cholestasis. We found a decrease in biliary bile acid concentrations in patients with cholestatic liver injury (Figure 1). This decrease is likely due to rupture of the biliary tracts and spillage of bile into the hepatic parenchyma. In support of our hypothesis, mice given very high doses of UDCA during BDL have an increased biliary load and actually experience more injury, indicating that increased biliary load worsens injury by increasing the number of infarcted areas (Fickert et al., 2002). As patients are typically on lower doses of UDCA, the overall increase to the biliary load may negligible in patients given UDCA, or secondary to other positive effects UDCA has on the mitochondria (Rodrigues et al., 1998) as UDCA treatment has previously been shown to be protective against toxic bile acids *in vitro* (Galle et al., 1990).

Inflammation during cholestatic liver injury

Of note, in murine models of obstructive cholestasis, inflammation plays a definitive role in the injury as both the ICAM-1 and CD11b knockout mice show a blunted neutrophil response and dramatic protection (Gujral et al., 2003; Gujral et al., 2004). It has yet to be determined what role inflammation plays in man; however, given the dramatically different bile acid concentrations in man versus mice during cholestasis (Trottier et al., 2011; Zhang et al., 2012; Dilger et al., 2012) it is likely murine bile is not as toxic, and may instead serve as an irritant that provokes the sterile inflammatory response directly without toxicity. Interestingly, previous data from our laboratory and others show the biliary bile acid pool can be toxified by feeding bile acids such as lithocholic acid, whereupon biliary toxicity overrides the inflammatory process and results in direct toxicity without input from neutrophils (Fickert et al., 2007; Woolbright et al., 2014). As human bile is also more hydrophobic than mouse bile, this supports the idea that direct exposure to toxic bile can initiate injury before the onset of inflammation. Still, inflammatory infiltrate is clearly visible in the infarcted areas of cholestatic patients in this study (Figure 1). Furthermore, while HMGB1 levels were significantly lower than levels previously measured in the mouse BDL model, values of acHMGB1 found in human patients were fairly consistent with values of acHGMB1 found in mice during the inflammatory injury phase of BDL mice (Woolbright et al., 2013; Figure 6). As we observed both elevations in HMGB1 and its acetylated form, acHGMB1, in the injured patients, it would be pertinent to further study the inflammatory response during cholestasis to better determine both if HMGB1 is a driving force for inflammation during the injury, and if this inflammation is pathogenic in nature, or occurs to clean up dead cells and assist in the regeneration process.

In conclusion, there appears to be a distinct difference in the onset and progression of cholestatic injury in humans versus rats or mice. This difference is punctuated by a very different cell death response after exposure to bile acids, and marked resistance to injury *in vitro*. Furthermore, human bile is considerably more toxic than murine bile, largely due to the increased hydrophobicity of its composition. Future studies focusing on signaling mechanisms present in human hepatocytes and cholangiocytes exposed to toxic concentrations of bile acids are warranted, as are studies defining how biliary infarction occurs and the hepatoyte's response to the pathophysiology during cholestasis.

Chapter 5: Lithocholic acid feeding results in direct hepato-toxicity independent of neutrophil function in mice.

This section is a reprint of the paper "Lithocholic acid feeding results in direct hepato-toxicity independent of neutrophil function in mice" Woolbright et al., 2014 - Toxicology Letters - with permission.

ABSTRACT

Lithocholic acid (LCA) supplementation in the diet results in intrahepatic cholestasis and bile infarcts. Previously we showed that an innate immune response is critical for cholestatic liver injury in the bile duct ligated mice. Thus, the purpose of this study was to investigate the role of neutrophils in the mechanism of liver injury caused by feeding mice a diet containing LCA. C57BL/6 mice were given control or 1% LCA containing diet for 24-96 h and then examined for parameters of hepatotoxicity. Plasma ALT levels were significantly increased by 48 h after LCA feeding, which correlated with both neutrophil recruitment to the liver and upregulation of numerous pro-inflammatory genes. The injury was confirmed by histology. Deficiency in intercellular adhesion molecule-1 (ICAM-1) expression or inhibition of neutrophil function failed to protect against the injury. Bile acid levels were quantified in serum and bile of LCA-fed mice after 96 h. Only the observed levels of LCA and its conjugate tauro-LCA in bile caused direct cytotoxicity in cultured mouse hepatocytes. These data support the conclusion that neutrophil recruitment occurs after the onset of necrosis in LCA-induced toxicity, and is not a primary mechanism of cell death when cholestasis occurs through accumulation of this hydrophobic bile acid.

INTRODUCTION

Cholestasis occurs as a result of a number of different pathologies including obstruction of the common bile duct by gall stones, biliary atresia, compression of the common bile duct from tumor growths such as cholangiocarcinoma, or during intrahepatic cholestasis of pregnancy (Zollner and Trauner, 2008; Jüngst and Lammert, 2013). During cholestasis, bile acids *accumulate in hepatocytes, which have the potential to cause cytotoxicity (Guicciardi and Gores, 2002; Perez and Briz, 2009). Studies with rat hepatocytes have shown that high concentrations of toxic bile acids such as glycochenodeoxycholic acid (GCDA) or taurolithocholic acid (TLCA) result in hepatocellular apoptosis mediated by mitochondrial and lysosomal dysfunction (Spivey et al., 1993; Botla et al., 1995; Graf et al., 2002). However, more recent data suggest these bile acids might not reach critical concentrations during obstructive cholestasis that result in hepatocellular toxicity *in vivo* (Trottier et al., 2011, 2012; Zhang et al., 2012), leading to alternate hypotheses for mechanisms of injury (Woolbright and Jaeschke, 2012).

Liver injury induced by obstructive cholestasis (bile duct ligation, BDL) in rodents is characterized by areas of focal necrosis (bile infarcts) and extensive neutrophil accumulation (Kountouras et al., 1984; Saito and Maher, 2000; Gujral et al., 2003). Animals with impaired neutrophil function developed significantly less liver injury after BDL suggesting neutrophils caused the majority of the cell damage (Gujral et al., 2003; 2004b). Prerequisite for neutrophil-induced liver injury is the activation and recruitment of neutrophils into sinusoids and a chemotactic signal for extravasation into the parenchyma (Jaeschke and Smith, 1997; Jaeschke

and Hasegawa, 2006). Recent studies demonstrated that cleaved osteopontin in bile is a critical chemotactic factor for the early neutrophil-induced injury mechanisms after BDL (Yang et al., 2014). The increased biliary pressure during obstructive cholestasis results in rupture of cholangioles causing the leakage of bile back into the parenchyma, which is responsible for the focal nature of the liver damage (Fickert et al., 2002). In addition, the exposure of hepatocytes to high levels of the typical bile acids present in mice, i.e. taurocholic acid (TCA), β-muricholic acid (βMCA) and TβMCA, does not cause directly cell death (Allen et al., 2011; Zhang et al., 2012). However, these bile acids trigger intercellular adhesion molecule-1 (ICAM-1) gene expression in hepatocytes and CXC chemokine formation that provides an additional chemotactic gradient for neutrophil recruitment (Allen et al., 2011). Thus, the acute liver injury during obstructive cholestasis in mice is a neutrophilic inflammatory injury triggered by the biliary leakage of osteopontin and the generation of CXC chemokines by hepatocytes exposed to bile acids. Given the strong evidence for a neutrophil-mediated liver injury after BDL (Gujral et al., 2003, 2004b; Kim et al., 2006; O'Brien et al., 2013; Licata et al., 2013), it remains unclear if bile acids directly cause cell death under pathophysiologically relevant conditions *in vivo.*

Recently, lithocholic acid (LCA) feeding was used as a model of liver injury (Fickert et al., 2006). LCA is a hydrophobic bile acid generated by bacterial reduction of chenodeoxycholic acid (CDCA) in the gut (Hofmann, 2004). The accumulation of high biliary concentrations of LCA and its metabolites after feeding this bile acid results in the precipitation of LCA in cholangioles and the clogging of the biliary system resembling the obstructive cholestasis caused by BDL (Fickert et al., 2006). In addition to the focal areas of necrosis, an extensive accumulation of neutrophils was also observed (Fickert et al., 2006). This raises the question whether in this

model, as observed in the BDL model, a neutrophilic inflammatory response is also the main mechanism of liver injury, or if the increased levels of the hydrophobic bile acid LCA can trigger direct cell death in hepatocytes. This issue may be clinically important as hydrophobic bile acids such as LCA and CDCA and their conjugates are more prevalent in humans (Trottier et al., 2011) than in mice (Zhang et al., 2012). Moreover, there are concerns that LCA may derive from UDCA (Sinakos et al., 2010; Hofmann, 2004), in particular when used at high doses in cholestatic conditions such as primary sclerosing cholangitis where it has been associated with adverse clinical outcomes (Lindor et al., 2009; Sinakos et al., 2010). Notably, LCA may also result in sclerosing cholangitis in some mouse strains (Fickert et al., 2006). Thus, the objective of this investigation was to evaluate the pathophysiological role of neutrophils in the LCA feeding model and, after analysis of LCA levels in bile and serum, assess the relevance of these concentrations for cytotoxicity in cultured mouse hepatocytes.

RESULTS

Lithocholic acid feeding causes extensive hepatic damage and neutrophil recruitment

C57Bl/6 mice were fed a diet containing 1% LCA or a control diet *ad libitum* for up to 96 h. In line with our previous report (Fickert et al., 2006), severe liver injury developed within 48-72 h after exposure to the LCA diet. A significant increase in plasma ALT activities as indicator of liver injury was observed as early as 48 h after the initial change in diet. The injury progressively increased up to 96 h (Figure 1A). Mean liver necrosis was found to be 55% by 96 h (Figure 1B). Plasma ALP activities were significantly increased by 96 h after the onset of LCA diet feeding, consistent with a cholestatic phenotype (Figure 1C). H&E staining of liver sections confirmed focal hepatocellular necrosis (bile infarcts) and the progressive increase in injury (Figure 1D-F).

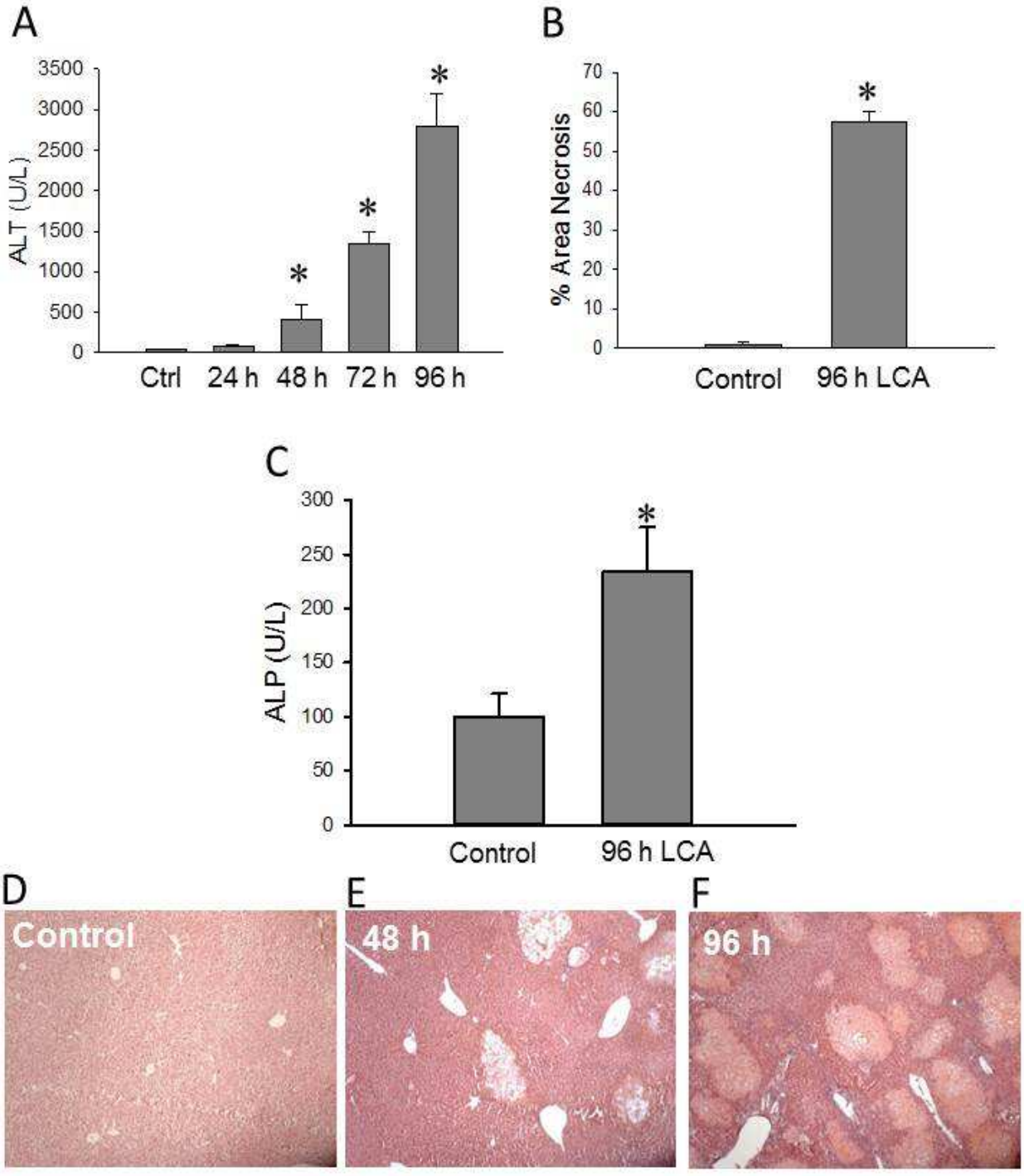

Figure 1: LCA-induced liver injury. Plasma alanine aminotransferase (ALT) activities (A) and area of necrosis (B) were determined after feeding animals a diet supplemented with 1% LCA for 0-96 hours. The area of necrosis was estimated from H&E stained liver sections. Alkaline phosphatase (ALP) activity (C) in control or mice fed 1% LCA for 96 hours. Representative images of control (D) and LCA feeding for 48 hours (E) and 96 hours (F) are shown here at 100x magnification. Data represent means ± SE of n = 4 animals per group. *P<0.05 (compared to controls, Ctrl).

To visualize the presence of neutrophils, liver sections of controls and LCA-fed mice were stained with an anti-neutrophil antibody (Ly-6b) to evaluate the inflammatory infiltrate in the liver (Figure 2A). Concurrent with the increase in ALT values, there was a dramatic increase in neutrophil recruitment to the liver by 72 h on the LCA diet (Figure 2B), although this value had declined slightly by 96 h. However, the presence of neutrophils alone does not establish a primary role for inflammation in the injury process (Jaeschke and Hasegawa, 2006; Williams et al., 2010).

LCA feeding results in hepatocellular necrosis

A large part of the current literature focuses on apoptosis as the mechanism for bile acid-induced cell death (Spivey et al., 1993; Faubion et al., 1999; Rust et al., 2009). Active caspase-3 levels, a hallmark of apoptosis, were measured via a caspase activity assay (Figure 3A) or Western blotting (Figure 3B). There was no apparent increase in caspase-3 activity in the LCA fed mice. While there was an abundant amount of procaspase-3 in the liver at every time point, there was no cleavage into the active caspase-3 fragment in any of the samples from LCA-treated animals. In contrast, high levels of caspase-3 activities (Figure 3A) correlated with the presence of active caspase-3 fragments in galactosamine/endotoxin-treated mice used as a positive control (Jaeschke et al., 2000). We also examined TUNEL-positive cells during LCA feeding. The TUNEL assay (Figure 3C-E) showed widespread nuclear and cytosolic staining indicative of DNA strand breaks in necrotic cells (Gujral et al., 2002).

lCA upregulates multiple genes involved in inflammation

Real time PCR analysis was used to analyze expression of inflammatory genes previously

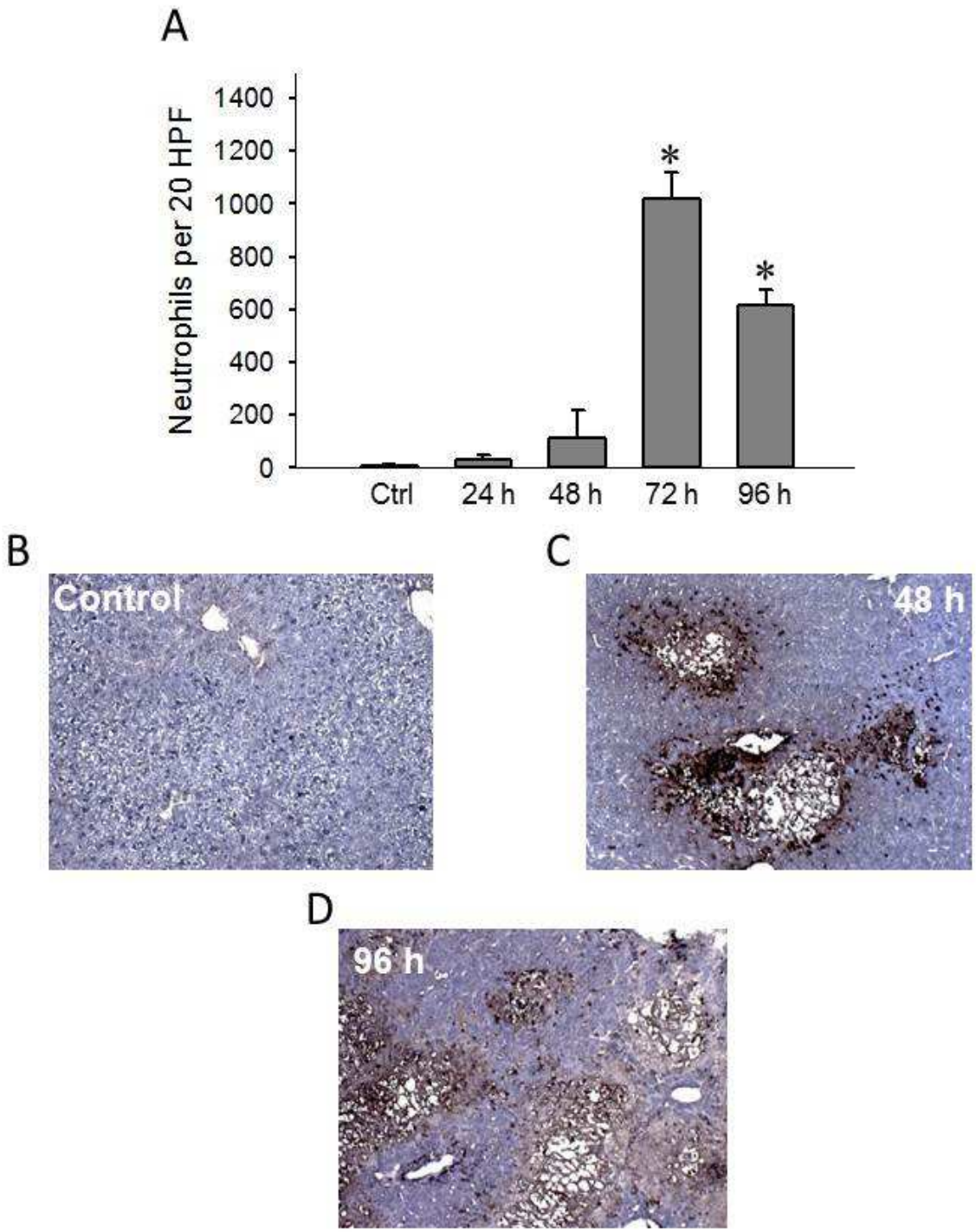

Figure 2: Hepatic neutrophil recruitment during LCA feeding. Neutrophil accumulation in the liver was quantified during up to 96 hours of feeding animals a diet supplemented with 1% LCA. Neutrophils were counted in 20 randomly selected high powered fields and totaled for each time point (A). Representative sections of controls (B) and animals fed 48 hours (C) and 96 hours (D) with the LCA diet were stained with the neutrophil marker Ly-6G. Data represent means ± SE of n = 4 animals per group. *P<0.05 (compared to controls, Ctrl).

established to be involved in cholestatic liver injury (Copple et al., 2010). Multiple inflammatory genes were strongly upregulated including the interleukins IL-1ß, IL-6, and IL-10, and the CXC chemokines MIP-2 and mKC (Table 1) and the adhesion molecule ICAM-1 (Figure 4A). In addition to the inflammatory genes, the acute phase proteins heme-oxygenase 1 (HO-1) and especially metallothionein 1 (MT-1) were induced (Table 1). These data strongly implicated inflammation in the pathology of LCA-induced liver injury. The key question remained whether these inflammatory events are a consequence of the necrotic damage caused by LCA feeding or are a critical part of the mechanism of toxicity.

No protection against LCA treatment in ICAM-1-deficient mice

ICAM-1 expression is critical for neutrophil cytotoxicity in the liver (Essani et al., 1995a). After LCA feeding, hepatic ICAM-1 mRNA (Figure 4A) and protein levels (Figure 4B, Figure 4C) were strongly induced. To test the pathophysiological role of neutrophils in LCA-induced liver injury, ICAM-1-deficient mice or WT animals were fed 1% LCA for 96 h as in previous experiments. In contrast to the extensive protection of ICAM-1-deficient mice after BDL (Gujral et al., 2004b), plasma ALT values showed no significant difference between ICAM-1-deficient and WT mice after LCA feeding (Figure 4D); similarly, the number of neutrophils in the liver of both KO and WT animals was not different (Figure 4E).

No protection against LCA treatment by inhibition of neutrophil-induced ROS formation

Neutrophils kill invading pathogens through ROS formation (Weiss, 1989; Nathan, 2006). This is largely mediated through the formation of superoxide via NAPDH oxidase and the subsequent

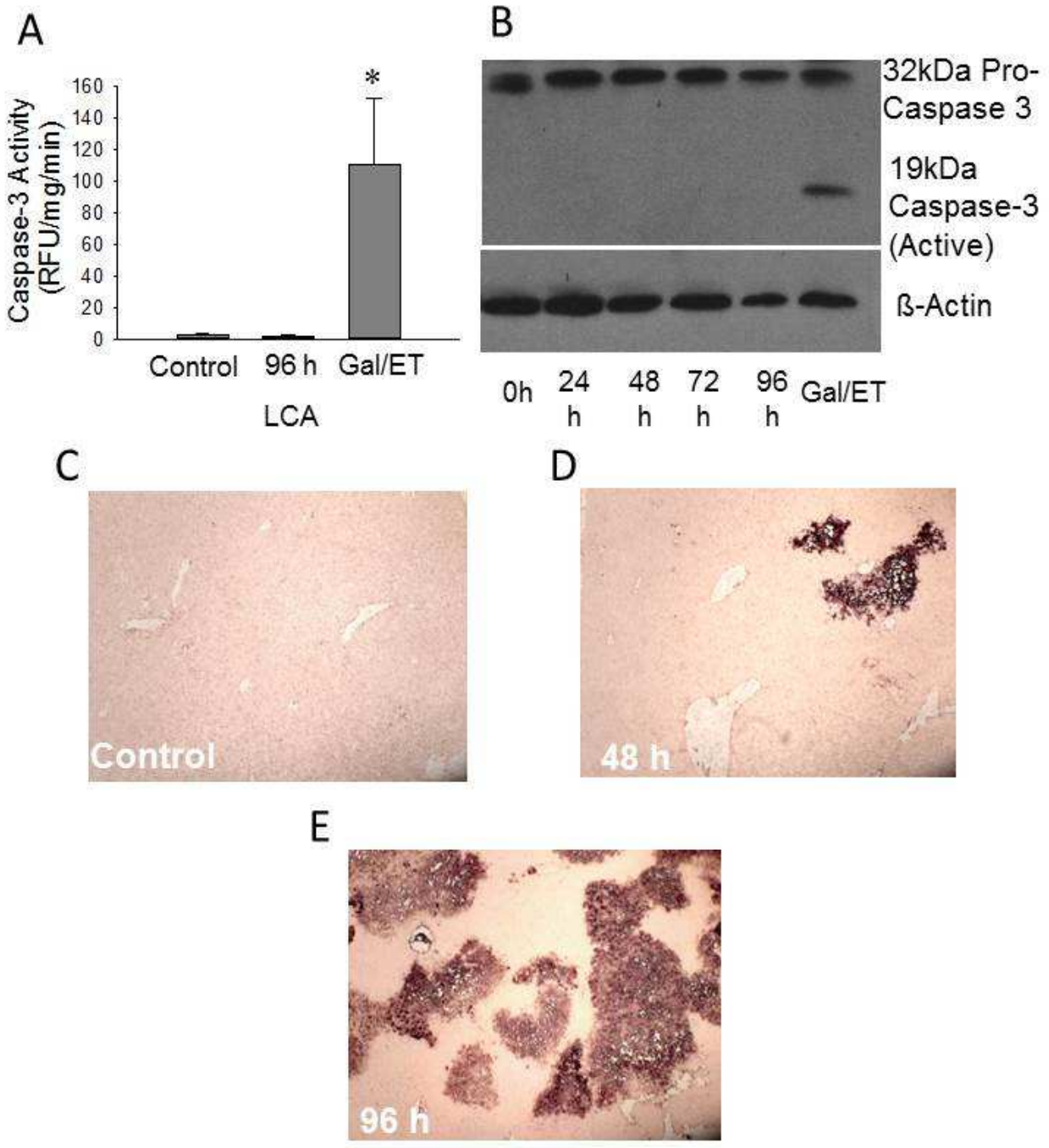

Figure 3: Necrosis versus apoptosis in LCA treated mice. Caspase activity (A) in LCA fed or galactosamine/endotoxin treated mice. Western Blot (B) for caspase activation after LCA feeding. Representative TUNEL images (x100 magnification) for control (C), 48 h (D), or 96 h (E) show widespread staining indicative of necrotic DNA damage.

generation of secondary ROS such as hypochlorous acid by neutrophil myeloperoxidase (Jaeschke, 2011). Thus, inhibition of NADPH oxidase results in a dramatically reduced potential for cytotoxicity of neutrophils. The gp91*phox*-/- knockout mice, deficient for the catalytic subunit of NADPH oxidase, were fed the 1% LCA diet for 72 h, but no difference was found in plasma ALT activities or neutrophil recruitment (Table 2). To confirm these findings, a pharmacological inhibitor of NADPH oxidase was used. LCA-fed mice were given diphenylene iodonium (DPI) chloride daily, starting at the onset of the LCA feeding. Despite the effectiveness of this treatment regimen against neutrophil-induced injury during endotoxemia (Gujral et al., 2004a), DPI had no effect on the LCA-induced liver injury (Table 2). Together, these data strongly suggest that the liver injury caused by LCA feeding is not mediated by an inflammatory mechanism involving neutrophils.

Bile acid toxicity in cultured mouse hepatocytes

To test the hypothesis that LCA or a metabolite may directly cause cell death in hepatocytes *in vivo*, we first measured bile acid levels in serum and bile of animals on the control and LCA diet at 48 and 96 h after LCA feeding. After LCA feeding, increases were seen in TCA, TLCA, and TCDCA in serum, with smaller increases seen in CDCA, DCA and CA levels (Figure 5, Supplementary Table 1). Biliary TLCA and TCDCA levels rose to concentrations of ~10mM and ~16mM respectively (Figure 5D, Figure 5F). Additionally in bile, TCA, GCA, and CA levels dropped, whereas the other bile acids largely stayed the same over 96 h (Supplementary Table 2). To test if these concentrations were consistent with toxicity, *in vitro* dose response curves were done for LCA (Figure 6A), TLCA (Figure 6B), TCA (Figure 6C) and TCDCA (Figure 6D) using primary murine hepatocytes. Measured *serum* concentrations of the bile acids did not correspond

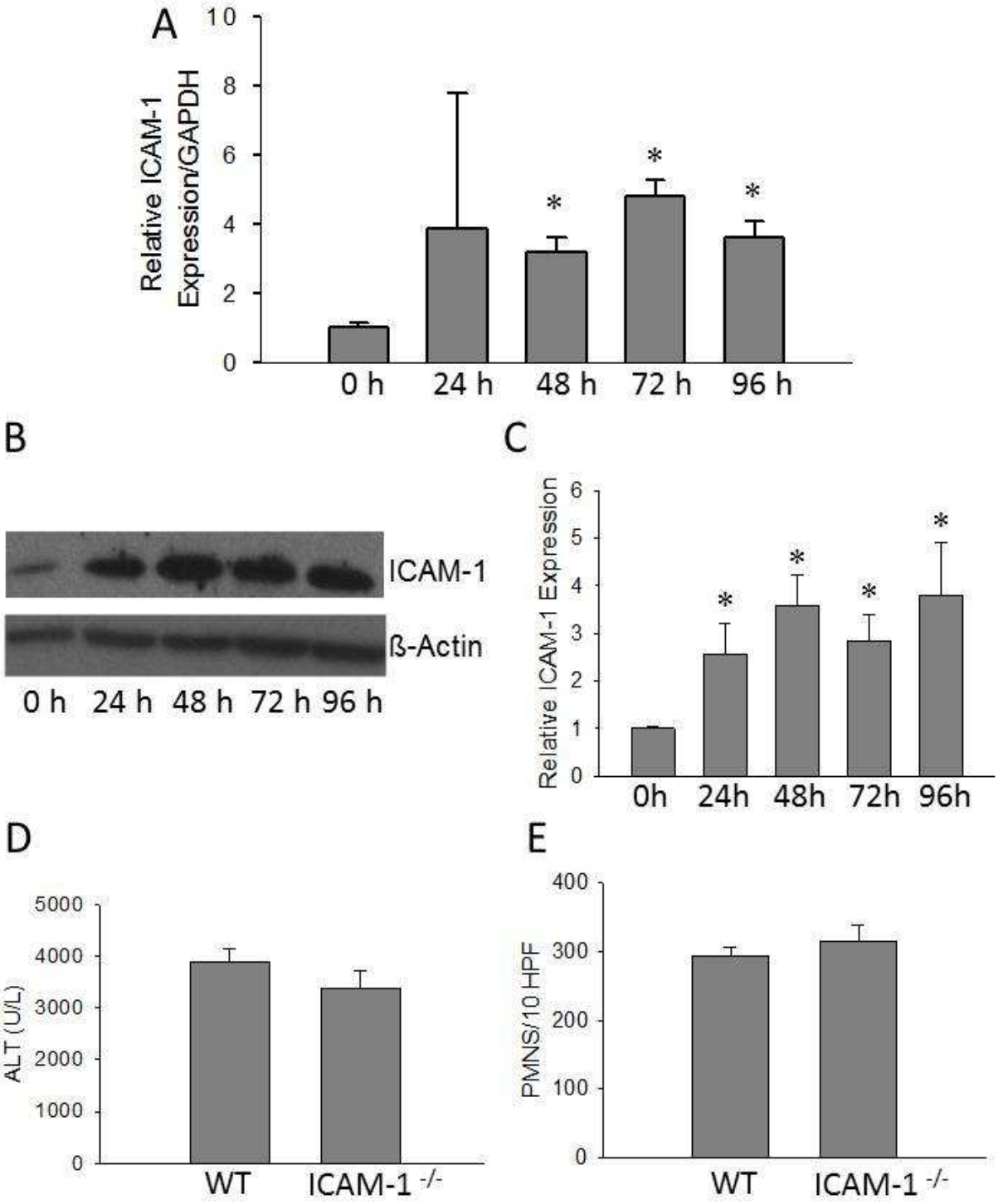

Figure 4: No protection against LCA induced liver injury in ICAM-1-deficient mice. ICAM-1 mRNA (A) and protein (B and C) levels were measured after 0-96 hours of LCA exposure. Plasma ALT activities (D) and neutrophil infiltration (E) were determined in ICAM-1-deficient and WT mice after 96 h of LCA feeding. Data represent means ± SE of n = 4 animals per group. *P<0.05 (compared to controls, Ctrl). Protein expression quantification represents means ± SE of n=3 animals per group. *P<0.05 (compared to controls, Ctrl).

with toxicity in the case of any bile acid tested (Figure 6). While LCA was toxic at doses above 500μM, this dose was never reached in the study. TLCA levels rose to the greatest degree in the study; however, issue with solubility prevented testing doses above 1mM, thus the hypothesis that TLCA levels were toxic was not able to be fully tested. While TCA levels were non-toxic at doses up to 25mM, TCDCA was highly toxic at doses of 1mM and above. As TCDCA concentrations were close to 10mM during the study, it is likely that TCDCA, if not TLCA, mediates a portion of the toxicity. To further test this, we created a biliary mixture of bile acids including 10mM TCA, 10mM TCDCA and 1mM TLCA and treated murine hepatocytes with this dose.Significant toxicity was present at this dose, confirming the hypothesis that biliary values of bile acids mediate the injury after LCA feeding (Figure 6E). These conclusions are consistent with the pattern of injury (focal necrosis).

DISCUSSION

The main objective of the current investigation was to evaluate the detailed mechanisms of LCA nduced hepatotoxicity in vivo. In particular, it was tested if the toxicity involved neutrophilic inflammatory mechanisms or was mainly caused by the direct cytotoxicity of LCA and its metabolites. LCA is a toxic hydrophobic bile acid, especially in humans. LCA hepatotoxicity was studied extensively over the past 50 years (Hofmann, 2004). Feeding various concentrations of LCA in the diet has been shown to cause focal necrosis in mice but the actual mechanism of cell death *in vivo* remained unclear (Fickert et al., 2006; Song et al., 2011).

The role of neutrophils in LCA-induced cholestatic liver injury

Our current study confirmed previous findings that feeding of high levels of LCA caused focal

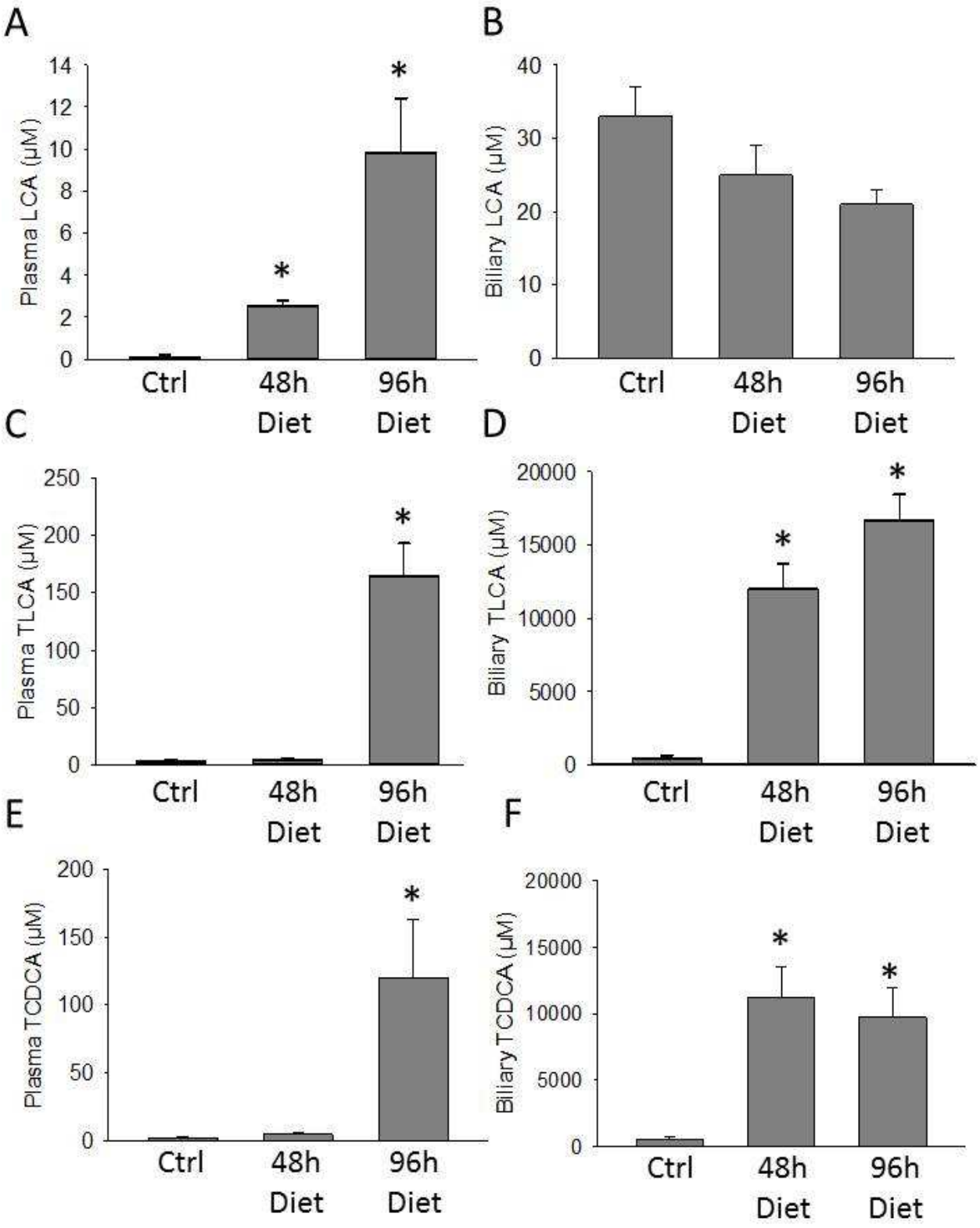

Figure 5: Major bile acid changes induced by LCA feeding. Serum and biliary concentrations of LCA (A and B), TLCA (C and D), and TCDCA (E and F) were measured in control mice or after 48h or 96 h 1% LCA feeding. Data represent means ± SE of n = 3 individual mice.* $p<0.05$ (compared to controls, Ctrl).

necrosis similar to the liver injury observed after BDL (Fickert et al., 2006). This injury pattern is caused by the precipitation of hydrophobic LCA and its metabolites in cholangioles resulting in the blockage of these biliary structures. Similar to BDL, the blocked bile ducts lead to an increased pressure build-up in the biliary system, which ultimately causes ruptures and leakage of bile back into the parenchyma (Fickert et al., 2006). Similar to BDL, a substantial number of neutrophils can be observed within and around the area of necrosis. However, in striking contrast to BDL, various interventions or gene deficiencies, which impair the cytotoxic capabilities of neutrophils, did not affect the injury. This included ICAM-1, an inducible adhesion molecule expressed on sinusoidal endothelial cells and on hepatocytes (Essani et al., 1995b). In the liver, ICAM-1 is mainly involved in the extravasation of neutrophils from sinusoids and the adhesion to hepatocytes (Essani et al., 1995a). Blocking ICAM-1 is highly effective in preventing neutrophil-induced liver injury by inhibiting neutrophil extravasation during endotoxemia (Essani et al., 1995a) or BDL (Gujral et al., 2004b). Despite the extensive protection against BDL-induced focal necrosis in ICAM-1-deficient mice (Gujral et al., 2004b), these animals experienced the same injury during LCA feeding as the wild type animals, which showed significant induction of ICAM-1 gene expression. These data support the conclusion that neutrophils are not significant contributors to the injury in LCA-fed animals.

It is well established that neutrophils kill target cells by ROS, which starts with the formation of superoxide and hydrogen peroxide by NADPH oxidase) and subsequently hypochlorous acid by myeloperoxidase (Weiss, 1989; Nathan, 2006). Consistent with these observations, neutrophil-induced liver injury depends on oxidant stress (Jaeschke, 2011; Jaeschke and Woolbright, 2012). In fact, there is direct evidence that neutrophil-derived reactive oxygen species diffuse into

hepatocytes and cause cell death in ischemia-reperfusion injury (Hasegawa et al., 2005), endotoxemia (Gujral et al., 2004a) and BDL (Gujral et al., 2003). Therefore, animals with a non-functional NADPH oxidase gp91*phox*-/- or wild type animals treated with DPI, an inhibitor of NADPH oxidase, are highly protected if the injury depends on neutrophils as was shown for ischemia (Abdelrahman et al., 2005; Lehnert et al., 2003; Harada et al., 2004) and for endotoxemia (Gujral et al., 2004a). However, neither gp91*phox*-/- mice nor animals treated with DPI showed reduced liver injury or neutrophil recruitment after LCA feeding. These data together with the lack of protection in ICAM-1 deficient mice strongly suggest that neutrophils are not relevant for the development of liver injury in this model. The lack of protection was especially interesting in light of the fact that LCA has been shown to act as a priming agent for neutrophils when exposed to low μM concentrations *in vitro* (Dahm and Roth, 1990). It seems that even though neutrophils are likely primed for oxidative burst and phagocytosis, the injury occurs through other mechanisms, indicating many of the damaged cells were necrotic or committed to cell death before the arrival of the neutrophils.

Direct cytotoxicity of bile acids

The second mechanism of cellular injury would be direct cytotoxicity of bile acids (Delzenne et al., 1992; Sokol et al., 1993). Our data indicate that serum levels of LCA are significantly elevated in LCA-fed animals compared to controls (Figure 6) or BDL animals (Zhang et al., 2012), whereas biliary levels are slightly decreased. The lack of increase in LCA in the bile is likely due to preferential export of conjugated bile acids and increased conjugation present during cholestasis. Dose-response experiments of LCA toxicity in isolated mouse hepatocytes showed an increase in cell death at concentrations between 100 and 500 μM LCA in the culture

medium (Figure 6A). LCA levels never reached this concentration though during the study. Instead, TLCA levels were dramatically elevated, consistent with the liver conjugating LCA in order to enhance excretion of the excess bile acid levels (Figure 5). Serum concentrations of TLCA were not consistent with toxicity, and TLCA was generally non-toxic at 1 mM (Figure 5, Figure 6B). Unfortunately, due to solubility problems, we were unable to test the hypothesis that biliary concentrations of TLCA were consistent with toxicity as doses above 1mM were insoluble. It is important to note though, that the tested values were 10-fold higher than previous doses tested in rats, but were not toxic, which is in strong contrast to previous data in rat hepatocytes, where TLCA was shown to be highly cytotoxic (Sokol, 1993), further exemplifying differences between rat and mouse (Woolbright and Jaeschke, 2012). Thus, *our data do not preclude the idea that biliary concentrations of TLCA are toxic,* it only suggests rats are more susceptible to mice in regards to TLCA toxicity, and that mice are resistant up to 1 mM. Instead, TCDCA levels rose dramatically and were toxic to isolated hepatocytes at concentrations reached in the bile (Figure 5, Figure 6D). While it was somewhat surprising that TCDCA rose to concentrations of 10 mM in bile, previous reports have suggested that when given to excess, LCA can be re-oxidized into CDCA and then conjugated to taurine to form TCDCA (Zhang and Klaassen, 2010). Given that TCDCA levels were approximately on par with TLCA levels, and that this aspect is not recapitulated in other models of cholestasis (Zhang et al., 2012), it seems likely that direct conversion is the source of the excess TCDCA. A mixture of bile acids present in the bile after LCA feeding was also applied to hepatocytes (Figure 6E). Interestingly, while this mixture was acutely toxic, it was slightly less toxic than TCDCA alone, lending credence to the idea that some of the less toxic, hydrophilic bile acids such as TCA might help ameliorate toxicity (Figure 6E).The selective leakage of the biliary system after LCA feeding (Fickert et al.,

2006) and the focal areas of necrosis (bile infarcts) strongly argue for the biliary bile acid concentrations as the cause of cell death. Although neutrophils were recruited into the liver and were mainly present in the area of necrosis, the ineffectiveness of multiple interventions against neutrophils suggests that direct bile acid cytotoxicity dominates under these conditions and the neutrophil response has no relevant impact.

Moderate (50-100 μM) concentrations of hydrophobic bile acids can cause apoptotic cell death in cultured rat hepatocytes (Guicciardi and Gores, 2002; Perez and Briz, 2009). However, morphological evidence and ALT release together with the lack of caspase-3 activity and caspase-3 processing clearly suggests that necrotic cell death dominates after LCA feeding *in vivo*. Although the dying cells stain positive with the TUNEL assay, which recognizes DNA strand breaks, the pattern of staining (nucleus and cytosol) is typical for necrotic cell death (Gujral et al., 2002; Jaeschke and Lemasters, 2003). In addition, the *in vitro* experiments with extensive LDH release support the conclusion that LCA causes necrotic cell death in mouse hepatocytes. The characteristics of cell death after LCA feeding are very similar to the necrotic cell death observed after BDL, which includes morphological evidence of cell swelling, vacuolation, and karyolysis. In addition, lack of caspase activation (based on enzyme activity, western blotting, immunohistochemistry, caspase-cleaved cytokeratin-18 fragments), the massive release of cell content (ALT, miR-122, full length cytokeratin-18 and high mobility group box 1 protein), and the TUNEL staining of nucleus and cytosol further supports the conclusion of necrotic cell death (Schoemaker et al., 2003; Gujral et al, 2004c; Fickert et al., 2005; Nalapareddy et al., 2009; Mitchell et al., 2011; Woolbright et al., 2013). Together these observations strongly suggest that the direct cytotoxicity of the hydrophobic bile acid LCA in

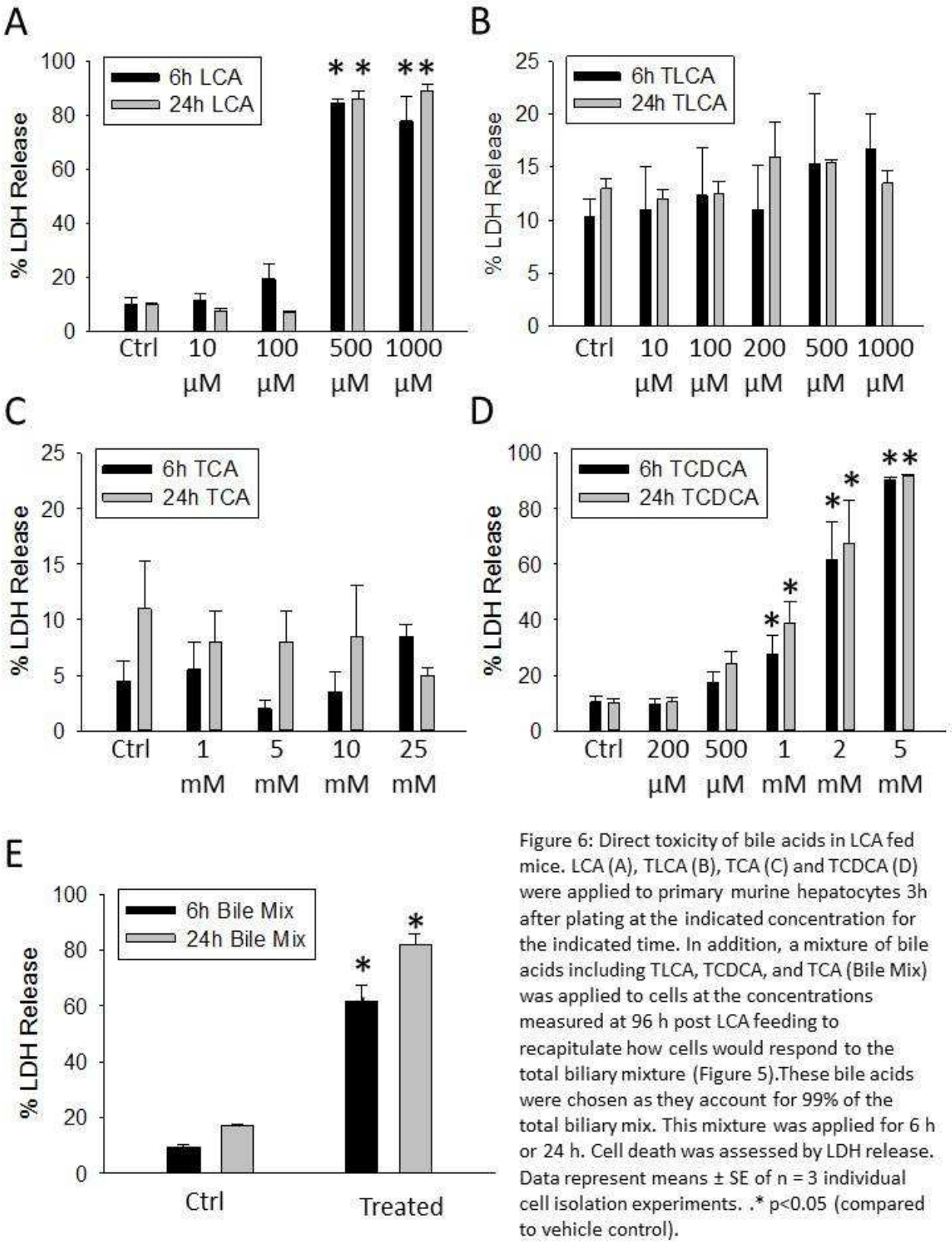

Figure 6: Direct toxicity of bile acids in LCA fed mice. LCA (A), TLCA (B), TCA (C) and TCDCA (D) were applied to primary murine hepatocytes 3h after plating at the indicated concentration for the indicated time. In addition, a mixture of bile acids including TLCA, TCDCA, and TCA (Bile Mix) was applied to cells at the concentrations measured at 96 h post LCA feeding to recapitulate how cells would respond to the total biliary mixture (Figure 5).These bile acids were chosen as they account for 99% of the total biliary mix. This mixture was applied for 6 h or 24 h. Cell death was assessed by LDH release. Data represent means ± SE of n = 3 individual cell isolation experiments. .* $p<0.05$ (compared to vehicle control).

mice *in vivo* and in cultured hepatocytes *in vitro* is caused by necrosis and not apoptosis.

Summary and Conclusions

Previous studies demonstrated the critical role of neutrophils in causing bile infarcts after obstructive cholestasis (BDL) in mice. The main reason for the limited direct toxicity of bile acids is that the dominant bile acids in mice are not cytotoxic at pathophysiological relevant concentrations. However, if the composition of the mouse bile is changed by feeding the hydrophobic bile acid LCA, the biliary concentrations of LCA, and especially its metabolites, are sufficient to directly cause necrotic cell death in mouse hepatocytes. In addition, the extensive focal necrosis *in vivo*, which was correlated with neutrophil infiltration, was not affected by various interventions against neutrophils. Together, these data indicate that the toxicity of bile leaking from ruptured cholangioles during obstructive cholestasis is dependent on the bile acid composition. Thus, if bile contains sufficient levels of toxic, hydrophobic, bile acids,, direct cytotoxicity is the main cause of hepatic necrosis with little impact of neutrophils. These findings do not reflect the mechanism of BDL injury in mice due to their high percentage of hydrophilic bile acids in bile. However, the observations may have implications for obstructive cholestasis in humans because of their higher levels of hydrophobic bile acids. This may in particular apply to patients with obstructive cholangiopathies who have higher LCA levels due to treatment with high doses of UDCA (Sinakos et al., 2010; Linor et al., 2009). Studies with human hepatocytes exposed to relevant concentrations of human bile acids are needed to address this question.

Table 1: Serum Bile Acid Values

Bile Acid	Treatments	
	Control (μM)	*96 h LCA (μM)*
TCA	0.669 ± 0.06	74 ± 14.3*
TCDCA	0.202 ± 0.02	120 ± 37.5*
GCA	0.02 ± 0.01	0.169 ± 0.05
GCDCA	0.256 ± 0.2	0.489 ± 0.12
CA	1.16 ± 0.16	18.7 ± 6.59*
CDCA	0.095 ± 0.29	9.4 ± 3.07*
DCA	0.18 ± 0.006	3.9 ± 1.00*

Table 1: Bile acids were measured in serum of control mice or after 96 h lithocholic acid (LCA) feeding. N=3. * $p<0.05$.

Table 2: Biliary Bile Acid Values

Bile Acid	Treatments	
	Control (μM)	*96 h LCA (μM)*
TCA	815 ± 239	330 ± 69*
TCDCA	15 ± 7.02	410 ± 19.59*
GCA	3.68 ± 1.59	1.14 ± 0.45
GCDCA	0.002 ± 0.001	1.46 ± .240*
CA	95 ± 64	1.33 ± 0.5*
CDCA	0.139 ± 0.04	0.3 ± 0.19
DCA	0.09 ± 0.05	0.05 ± 0.01

Table 2: Bile acids were measured in bile of control mice or after 96 h lithocholic acid (LCA) feeding. N=3. * $p<0.05$.

Chapter 6: HepaRG Cells Accurately Recapitulate the Response of Primary Hepatocytes to the Effects of Bile Acids *in vitro*

ABSTRACT

Cholestatic liver injury is a pathological component of numerous disease states. Much of the current information on cholestatic liver injury is derived from *in vitro* studies using rodent hepatocytes or human hepatoma lines transfected with bile acid (BA) uptake transporters. While these studies demonstrate BA driven apoptosis, it is debatable if these models reflect the response of human hepatocytes to BA exposure, as primary human hepatocytes respond only to higher doses of bile acids, and primarily with necrosis and not apoptosis. HepaRG cells are a bipotential, human hepatoma line that express apical and basolateral BA transporters and may more accurately reflect *in vivo* physiology. Thus, we sought to determine if HepaRG cells could replicate the response of primary human hepatocytes lines to BA exposure *in vitro*. HepG2 cells, primary murine hepatocytes (PMH) or HepaRG cells were exposed to taurocholic acid (TCA), and glycochenodeoxycholate (GCDC) and LDH release was measured to determine cell death. Cell death occurred dose responsively in HepaRG cells when exposed to GCDC; however HepG2 cells only died at very high concentrations of GCDC. Pretreatment with the caspase inhibitor z-VAD-FMK had no effect on cell death, indicating necrotic cell death. While previous data indicates PMH show a marked increase in pro-inflammatory gene expression upon exposure to TCA, there was no change in gene expression after TCA stimulation in HepaRG cells. These data provide evidence for the use of HepaRG cells as a new model for the study of the effect of BA on human hepatocytes.

INTRODUCTION

Liver injury due to cholestasis occurs in a variety of diseases, and can lead to fibrosis, cirrhosis and death (Woolbright and Jaeschke, 2012). Current understanding of cholestatic liver injury is derived partially from a number of *in vitro* studies where toxic, hydrophobic bile acids were given to either primary rodent cultured hepatocytes (Spivey et al., 1993; Patel et al., 1994) or persistent hepatoma cell lines (Roberts et al., 1997; Rust et al., 2009) that had been transfected with the necessary transporters for bile acid (BA) uptake. Many of these studies have used rats as a model, where an intricate mechanism of apoptosis in response to BA exposure, especially glycochenodeoxycholate (GCDC), has been elucidated (Spivey et al., 1993; Patel et al., 1994, Marderstein et al., 2003). While these studies have provided indisputable scientific evidence on the effects of GCDC and other pro-apoptotic bile acids on rat hepatocytes, recent studies have suggested BA exposure levels during cholestasis may be quite different in rodents and humans (Zhang et al., 2012; Trottier et al., 2011; Trottier et al., 2012). Mice show little to no accumulation of GCDC and other glycine conjugated toxic bile acids in serum and liver during cholestasis (Zhang et al., 2012), while levels of many of the less toxic hydrophilic bile acids are increased. Of note, concentrations of taurocholic acid (TCA) consistent with cholestasis have been shown to potently induce pro-inflammatory genes including adhesion factors and inflammatory cytokines (Allen et al., 2011). In contrast, human hepatocytes are exposed to higher concentrations of GCDC, respond by undergoing necrotic injury, have no response to TCA in regard to inflammation, and have prolonged bile acid uptake (Chapter 5). Thus, both murine hepatocytes and rat hepatocytes may not accurately reflect the human pathophysiology. As such, primary human hepatocytes are at a premium, although acquisition can be exceptionally

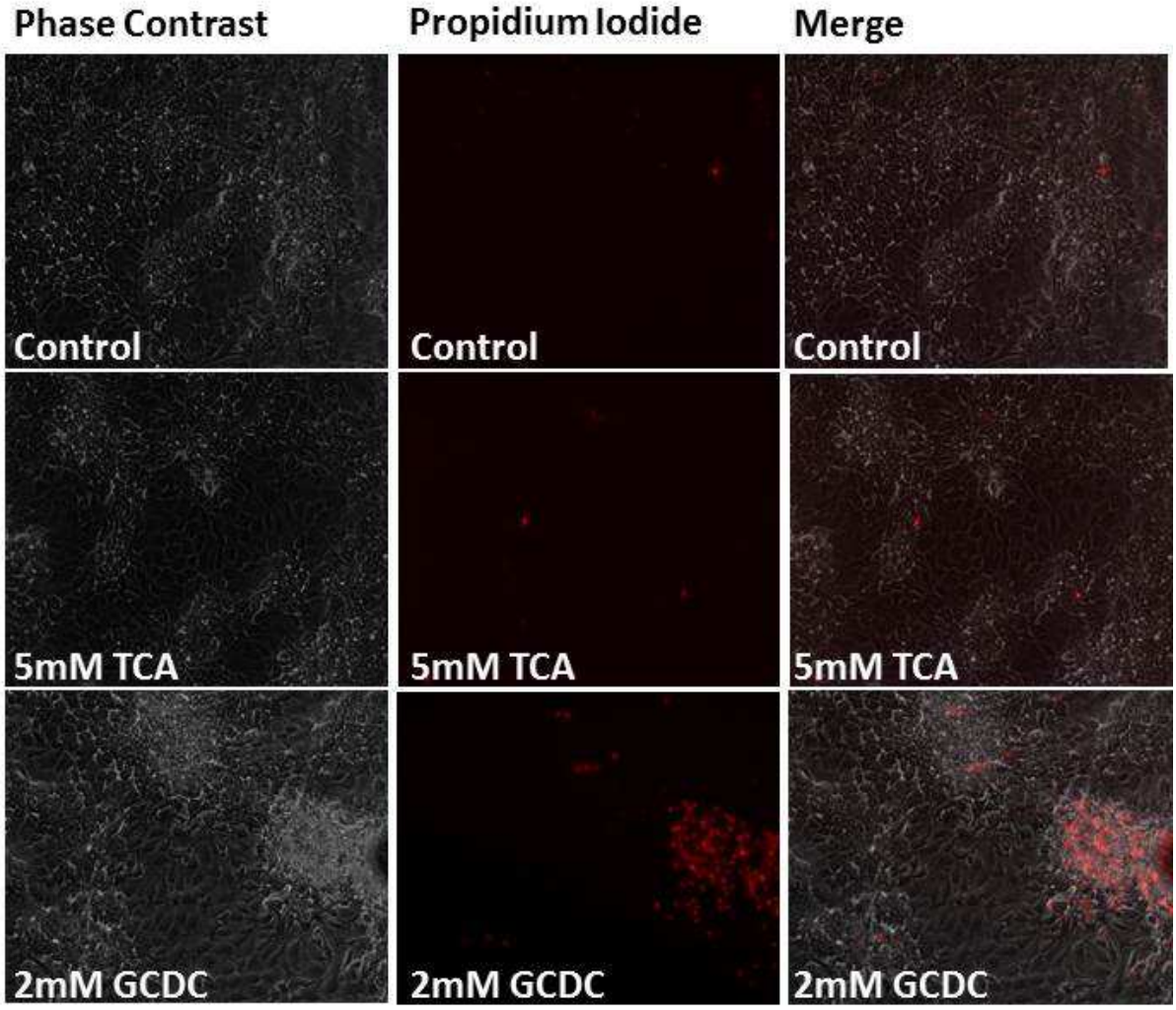

Figure 1: Propidium iodide staining after treatment of HepaRG cells for 6 hours with the indicated bile acid.

difficult, thus a primary cell line that recapitulated these processes would provide value for long term studies.

HepaRG cells are a recently isolated, bipotent, human hepatoma line that have a genetic profile that is in many ways similar to primary human hepatocytes (Parent et al., 2004; Guillozo et al., 2007). As these cells express drug metabolizing enzymes not normally expressed in hepatoma cell lines, recent studies indicate these cells may be useful for *in vitro* modeling of drug toxicity (McGill et al., 2011), and hepatitis infection (Ni et al., 2014). Currently, studies on the effects of BAs on hepatocytes require either the use of primary cells acquired directly from rodent species, or discarded human tissue as most hepatoma lines express only a fractional quantity of basolateral and apical BA transport capacity. Moreover, rodent hepatocyte lines lose transporter expression rapidly upon the initiation of cell culture (Liang et al., 1996). While primary human hepatyocytes remain the gold standard in human hepatocyte research, acquisition can be difficult and costly, and the acquired cells typically come from sub-optimal livers, which may obscure accurate results. As HepaRG cells are a persistent, human hepatocyte-like cell line that have been shown to express the necessary transporters for bile acid uptake and excretion, they may serve as a persistent human model for the effects of bile acids on human hepatocytes.

The aim of this project was to determine if the HepaRG cell line could be used to model bile acid mediated liver toxicity, and to compare that to primary cell lines previously established for BA toxicity. Herein, we demonstrate that HepaRG cells respond to bile acid exposure in a fashion similar to primary murine and human hepatocytes, recapitulating key aspects of the response to cholestatic concentrations of bile acids.

RESULTS:

As HepaRG cells are a bipotential cell line that results in the formation of hepatocyte-like and cholangiocytes-like cells, the primary question was whether or not bile acids killed both populations or only one of the populations. HepaRG cells were exposed to 2 mM GCDC or 5 mM TCA and toxicity was assessed by PI uptake. Cholangiocyte-like and hepatocyte-like populations were easily distinguishable based on morphology based on previous work (McGill et al., 2011). PI positive nuclei were exclusively seen in the hepatocyte-like population in both GCDC and TCA treated HepaRG cells (Figure 1), indicating bile acids are more toxic to the hepatocyte-like population in the HepaRG cell cultures. This confirms the idea that bile acids are toxic to HepaRG cells, and moreover, confirms the idea that hepatocytes are more susceptible to injury than cholangiocytes which are routinely exposed to biliary concentrations of bile acids.

Hepatocytes are likely exposed to biliary concentrations of bile acids in areas of hepatic infarction, as well as serum concentrations near the hepatic portal vein during cholestatic liver injury. To test what concentration of bile acid was capable of killing HepaRG cells, we exposed them to increasing concentrations of TCA as a model taurine-conjugated bile acid and GCDC as a model glycine conjugated bile acid as they are present to significant degrees in human bile (Dilger et al., 2012). HepaRG cells showed no toxicity when exposed to any concentration of TCA (Figure 1A); however, they were dose responsive to GCDC, with the onset of toxicity starting around 280 μM (Figure 1B). This was fairly consistent with previously established curves in human hepatocytes (Chapter 5). For comparison, HepG2 cells which do not express any uptake transporter were exposed to the same doses for 6 h (Figure 3). While there was no toxicity after

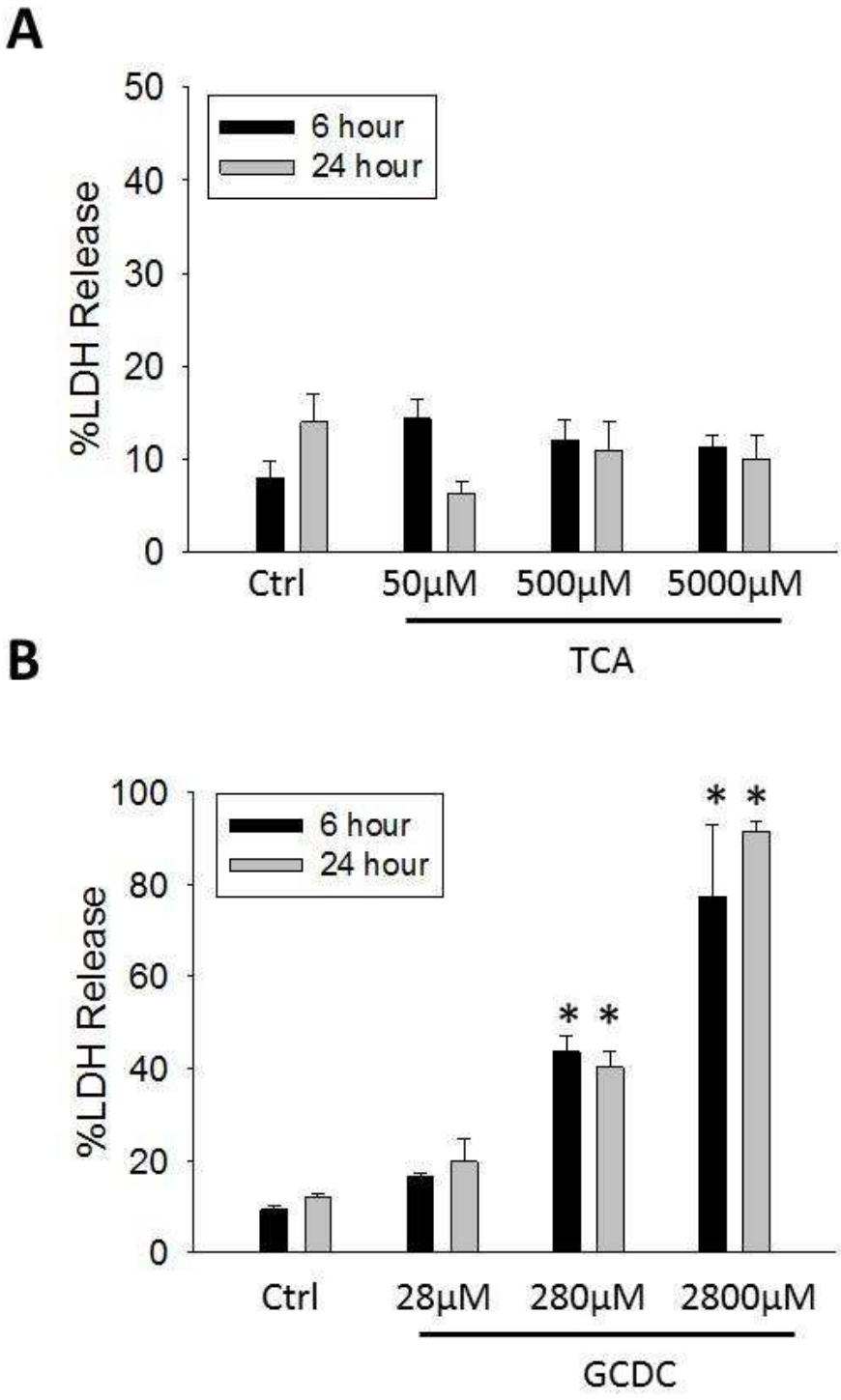

Figure 2: Cell death curves of HepaRG cells exposed to taurocholic acid (A) and glycochenodeoxycholic acid (B) for 6 hours or 24 hours. N=3. $p<0.05$ versus same time point control.

TCA exposure similar to HepaRG cells (Figure 3A), there was also a lack of dose response in the HepG2 cells. Instead, little cell death occurred until the 2800 μM GCDC dose, which coincided with near complete cell death (Figure 3B). This may be due to inherent detergent capacity of the bile acids, suggesting a limited intracellular response. These data indicated that concentrations of bile acids consistent with biliary values were toxic to HepaRG cells, much like primary human hepatocytes.

Previously we have shown that primary human hepatocytes do not undergo the characteristic apoptosis associated with GCDC that rat hepatocytes undergo (Chapter 5); instead, these cells undergo necrosis. HepaRG cells were treated with 2mM GCDC for 6 h or 5 mM galactosamine plus 250 ng/mL TNFα for 16 h and LDH release and capsase-3 activity were measured. Caspase inhibition was almost completely protective against TNF-induced apoptosis and subsequent LDH release; however, there was no protection against GCDC (Figure 4A). Concurrently, HepaRG cells did not have any increase in caspase-3 activity, nor did z-VAD-fmk treatment protect (Figure 4B). As such, HepaRG cells again faithfully model the response of primary human hepatocytes to GCDC.

While murine hepatocytes are known to induce numerous cytokines and pro-inflammatory genes after exposure to TCA, primary human hepatocytes do not recapitulate this process in the same way (Chapter 5). To determine if HepaRG cells mimicked the murine phenotype or the human phenotype, HepaRG cells were exposed to 500 μM TCA and relevant human genes or their murine orthologues were measured by RT-PCR. While primary murine hepatocytes had increases in multiple cytokines and adhesion factors, no such increase was seen in HepaRG cells, nor was there a significant increase in the primary neutrophil chemoattractant IL-8 (Table 1).

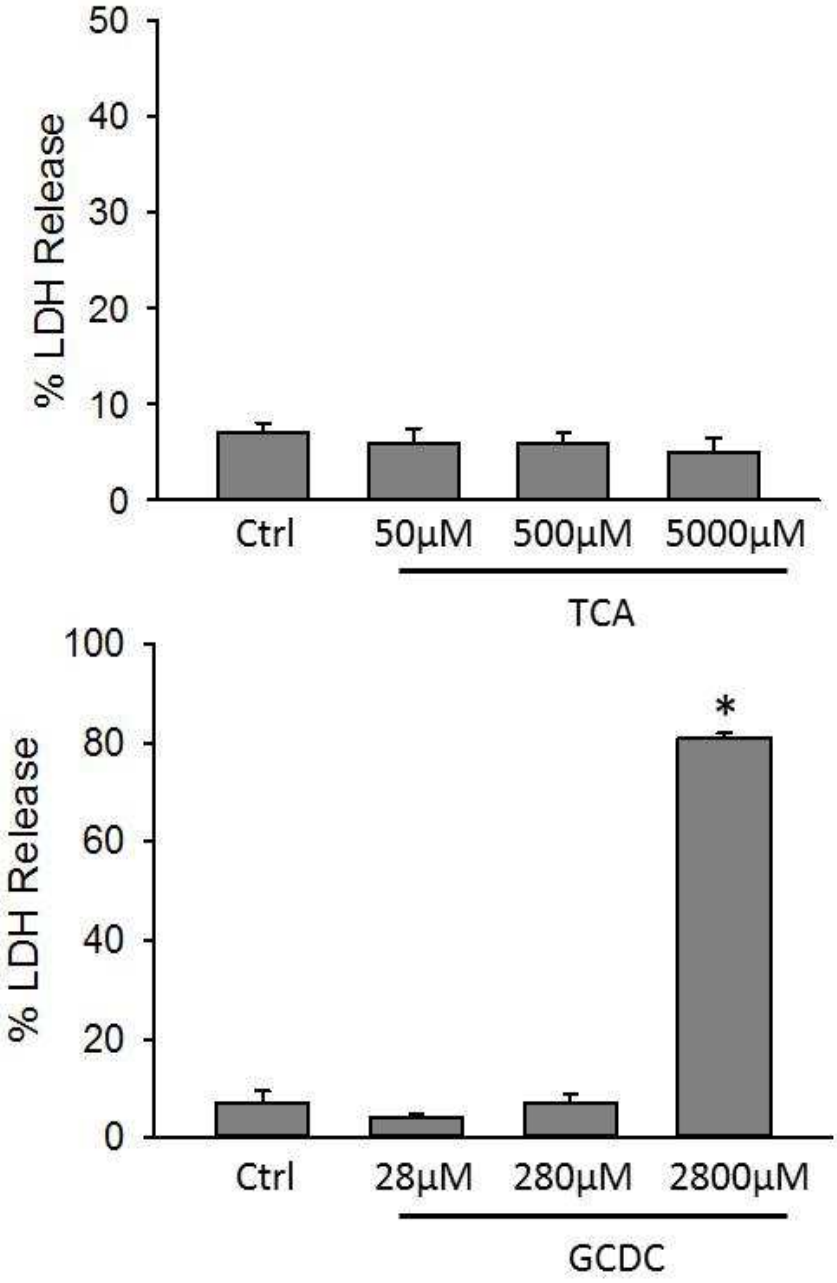

Figure 3: Cell death curves of HepG2 cells exposed to taurocholic acid (A) and glycochenodeoxycholic acid (B) for 6 hours. N=3. $p<0.05$ versus same time point control.

These data indicate HepaRG cells are more similar to primary human hepatocytes than primary murine hepatocytes in regards to their pro-inflammatory phenotype after bile acid exposure.

DISCUSSION:

HepaRG cells as a model for bile acid toxicity in vitro

Primary human hepatocytes are currently the gold standard for toxicity studies; however, they are difficult to acquire, expensive, and can have substantial variability in response to drugs and chemicals (Xie et al., 2014). Additionally, they are not self-sustaining, and thus prolonged laboratory experiments require fresh batches of hepatocytes on a regular basis. It would be beneficial to have a cell line that recapitulated the human hepatocytes response for exploratory studies. The primary goal of this study was to determine if HepaRG cells, a hepatoma cell line, could recapitulate aspects of bile acid toxicity typically seen only in primary hepatocytes or hepatoma lines transfected with bile acid transporters. Our results indicate HepaRG cells are susceptible to bile acid toxicity at concentrations similar to primary human hepatocytes (Figure 1), mimicking mechanisms of cell death found in human hepatocytes (Figure 4), and share the same inflammatory response as human hepatocytes (Table 1). These data confirm the idea that HepaRG cells may be more appropriate for bile acid culture than HepG2 cells as they express bile acid transporters (Szabo et al., 2013), and undergo dose responsive toxicity after bile acid exposure. The HepaRG cell line is bipotential and readily differentiates into two separate, morphologically distinct cell lines upon differentiation (Parent et al., 2004). Thus, a critical aspect of study in the cell line is determining which population is susceptible to injury. At doses below 3 mM GCDC, there was little to no cell death in the areas morphologically distinct from the hepatocyte islands (Figure 2). As these cells are generally thought of as cholangiocyte-like cells, this observation was not surprising, as

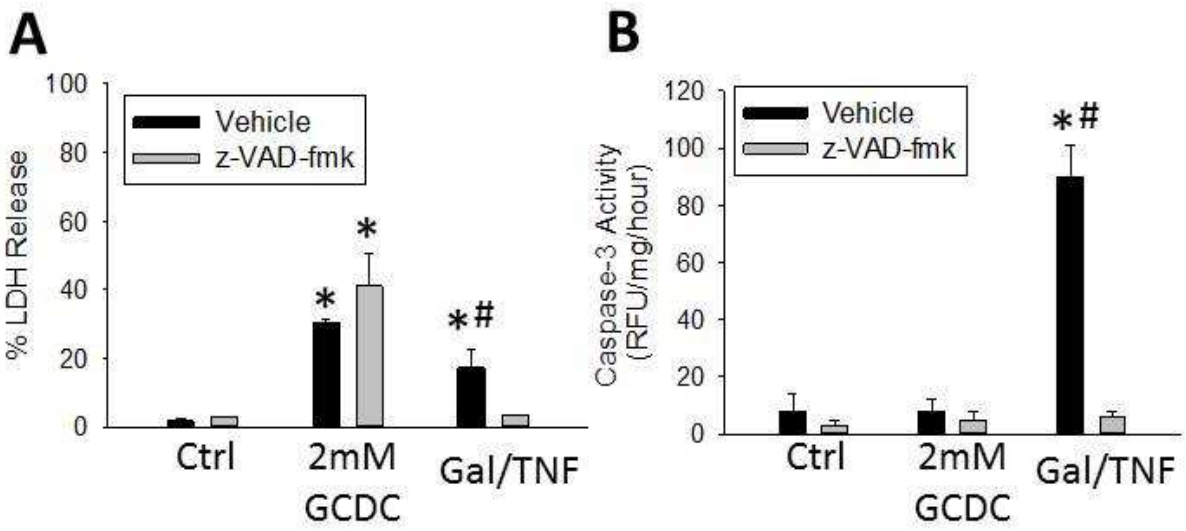

Figure 4: LDH release (A) and Caspase-3 activity (B) for HepaRG cells in response to glycochenodeoxycholic acid or galactosamine + TNF-α treatment. n=3. $p<0.05$ versus same time point control.

cholangiocytes are less susceptible to the effects of bile acids, potentially due to a local bicarbonate umbrella previously demonstrated in human cholangiocytes (Beuers et al., 2012).

While specific detection of cell death was achieved in the study using propidium iodide, one potential flaw with the cell line remains the presence of the additional cholangiocytes in the population. As the cholangiocyte population can represent up to 50% of the population, a major portion of the population's genome is contributed by these cells. As of yet, it is not possible to dissect out the hepatocyte population, although ongoing studies are attempting to force a greater degree of the cells towards the hepatocyte or cholangiocyte lineage (Dianat et al., 2014). Therefore, studies on gene expression or protein expression changes must be analyzed carefully as effects may be blunted by the presence of unresponsive cells. In HepaRG cells there was little to no induction of inflammatory cytokines, especially when compared to the rapid and dramatic induction of multiple cytokines seen in the murine hepatocyte model. Although the problem of the cholangiocyte cell line potentially blunting the effect of TCA and other conjugated bile acids was possible, this result did model what was previously observed in human hepatocytes (Chapter 5). While inflammation is a key component of the pathology in both murine models such as BDL (Gujral et al., 2003; Gujral et al., 2004), and clinically in models of autoimmune cholestatic hepatitis (Gossard et al., 2012), the mechanism through which inflammatory cells are recruited may function differently in mice and man (see Chapter 5). Further investigations are necessary in this area.

Bile Acid Exposure in vivo*: What Is Realistic in Human Patients and How Important Is It?*

A significant amount of the current understanding of cholestatic liver injury and the subsequent progression to fibrosis and cirrhosis is based on the idea that exposure to micromolar

HepaRG Cells		
	Control	*500μM TCA*
ICAM-1	1.0 ± 0.1	0.5 ± 0.1
CXCL-1	1.0 ± 0.1	0.8 ± 0.1
CXCL-2	1.0 ± 0.2	0.7 ± 0.1
IL-8	1.0 ± 0.2	1.3 ± 0.2
Primary Murine Hepatocytes		
	Control	*500μM TCA*
mKC (CXCL-1)	1.0 ± 0.1	24.6 ± 4.1*
MIP-2 (CXCL-2)	1.0 ± 0.1	310.0 ± 107.0*
VCAM-1	1.1 ± 0.2	3.5 ± 0.7*
ICAM-1	1.0 ± 0.1	6.3 ± 0.5*
TNF-α	1.0 ± 0.1	1.4 ± 0.5

Table 1: Gene expression before and after treatment with 500μM TCA in primary murine hepatocytes and HepaRG cells. ICAM-1 – intracellular adhesion molecule 1. CXCL – CXC motif chemokine ligand, IL – interleukin, VCAM-1 – vascular cellular adhesion molecule 1, TNF-α – tumor necrosis factor α

concentrations in the range of 50-100μM of hydrophobic bile acids like GCDC results in apoptosis (Malhi et al., 2006, Canbay et al., 2003). This led to the hypothesis that i*n vivo*, apoptosis leads to release of apoptotic bodies that are phagocytosed by macrophages resulting in increases in inflammatory cell recruitment and extravasation, and activation of hepatic stellate cells responsible for fibrosis (Guicciardi et al., 2010). Increasing doses based upon published human serum concentrations of bile acids during multiple cholestatic conditions were used in this paper to mimic conditions potentially seen during cholestasis *in vivo* (Trottier et al., 2011; Trottier et al., 2012). While there was no increase in cell death when hepatocytes were exposed to serum concentrations of BAs, extensive injury was seen at higher doses in every model (Figures 1-4). Therefore, the critical question becomes: *What is the real concentration of bile acids hepatocytes are exposed to* in vivo, as levels inconsistent with cell death or apoptosis would strongly refute the proposed connection between apoptosis and fibrosis or disease progression. Our data indicate hepatocytes are resistant to bile acids, or at the least, resistant towards the level of bile acids found in serum during cholestasis (Figure 1). Leakage of bile into the hepatic parenchyma has been noted in multiple animal models of cholestasis, including BDL (Fickert et al., 2002) and lithocholic acid feeding (Woolbright et al., 2014). However, injury may depend on bile acid constituency (Woolbright and Jaeschke, 2012). Liver and serum levels of bile acids in mice after BDL were found to be largely composed of non-toxic, hydrophilic bile acids such as cholic acid and its tauro-conjugate TCA (Zhang et al., 2012), suggesting injury may progress through other means in the BDL model (Gujral et al., 2004), as in this study and others TCA was found to be largely non-toxic (Allen et al., 2011). In contrast, direct feeding of LCA resulted in direct toxicity to exposure to very high concentrations of LCA-derived conjugates after biliary disruption (Woolbright et al., 2014). Thus, whether or not bile, or bile

acids, act as a direct source of injury is likely determined by concentrations of specific bile acids, in addition to the overall concentration to which hepatocytes are exposed. One important question to answer will be the concentration of bile acids in hepatocytes, even though it will be difficult to define BA levels in hepatocytes. As the liver is composed of multiple cell types and includes cholangiole tracts filled with bile, whole liver is a poor surrogate for hepatocyte BA levels. Moreoever, uptake of bile acids is regulated by the hepatocyte bile acid sensor FXR (Zollner et al., 2006). Upregulation of transport proteins such as the Mrp family and Mdr family increase bile acid efflux, while downregulation of NTCP and OATPs reduces bile acid influx during cholestasis (Gartung et al., 1996; Gartung et al., 1997). As such, it is difficult to completely define bile acid levels in hepatocytes during cholestasis, as there will be considerable flux. However, these levels may be critical to defining the cholestatic pathologies though, as hepatocytes are likely resistant to bile acid mediated cell death when exposed to serum concentrations.

In conclusion, we have provided data that suggests HepaRG cells may be a novel cell line for use in studying bile acid induced toxicity *in vitro.* HepaRG cells model numerous aspects of primary human hepatocyte cultures including dose response to model bile acids, a lack of an inflammatory response seen as in murine cultures, and predominant necrosis along with general resistance to bile acid induced cell death as compared to rat hepatocytes cultures. This cell line may have benefit studying hepatocyte specific mechanisms of cell death, and due to the co-culture with cholangiocyte-like cells, may have additional benefits.

Chapter 7: Conclusions

Cholestatic Liver Injury: Bridging the past and the present:

Scientific hypotheses are constantly evolving to better fit the current data. Bile acids have long been postulated to be the primary cause of injury during cholestasis. While initial hypotheses from *in vivo* models were that LCA mediated toxicity via its strong cholestatic, hydrophobic, and toxic properties (Yousef et al., 1983; Tuchweber et al., 1983), these hypotheses were disproven when it was shown that LCA did not accumulate to toxic levels in most mammals. This led to novel hypotheses springing up that showed apoptosis in response to concentrations of GCDC present in human serum (Malhi et al., 2010). This had led to an expansion of the "apoptosis hypothesis" of cholestasis and its prominent role in the current literature. While this hypothesis was challenged in this dissertation and in reviews associated with this material (Woolbright and Jaeschke, 2012), the toxicity of bile acids is not in question.

Whether bile acid toxicity is due to inherent hydrophobicity and detergency, or due to induction of apoptosis, a majority of data still points towards bile acids being intrinsically involved in the injury. One of the major hypotheses of this dissertation was that individual human bile acid concentrations would dictate cause and course of injury in humans. This hypothesis was tested vigorously and has led to multiple conclusions. Primarily, it was determined neither mouse, nor rat, and no current model of cholestasis faithfully models the human pathology. Of note, we found that human hepatocytes were significantly more resistant to bile acid induced injury, but also had significantly higher

concentrations of toxic bile acids, both in bile, and serum, as compared to mice. Interestingly, this led to humans having a mixed bag of responses previously noted in rodent species: 1) while there is a lack of apoptosis in models of obstructive cholestasis in mice, humans undergo minor, but statistically significant and histologically provable levels of apoptosis during *in vivo* cholestasis; 2) *however,* this is not recapitulated by *in vitro* treatment of human hepatocytes with bile acids as in rat models; 3) nor is the characteristic mouse response of increased expression of CXC chemokines and adhesion molecules in response to high concentrations of TCA; 4) although histologically, humans undergo a number of the same phenotypic changes seen in rodent *in vivo* models of cholestasis, such as inflammation and biliary infarction; 5) biliary infarction results in the exposure of human hepatocytes to very high concentrations of bile acids that likely mediate the onset of necrosis, *as humans have considerably higher concentrations of GCDC and other glycine conjugated, toxic bile acids in their bile;* 6) finally, in support of this hypothesis, bile acid levels in bile decrease during cholestatic liver injury in man. Thus, it appears that no model of rodent obstructive cholestasis currently in use will accurately reproduce what happens in human patients, largely due to very different bile acid pools, and an as of yet uncharacterized resistance to bile acid induced injury in human hepatocytes.

These data beg the following question- *What is the most appropriate rodent model to recapitulate <u>human</u> obstructive cholestatic liver injury?* Unfortunately, culture of primary hepatocytes from rodents seems to be of little use for assessing mechanisms of bile acid toxicity that are pathophysiologically relevant to humans, thus, *in vivo* models will likely continue to be the preferred model. Two different *in vivo* models were used in this

Table 7.1: Comparison of Models for Obstructive Cholestasis				
Human Phenotype	**Bile Duct Ligation**	**LCA Administration**	**DDC**	**ANIT**
Bile Acid Induced Toxicity	Negative	Positive	Unknown	Negative
Inflammation	Positive	Positive	Positive	Positive
Recapitulates Human Bile Acid Levels	Negative	Negative - Artificially Possible	Unknown	Unknown
Mimics Pathology	Some Aspects	Some Aspects	Negative	Negative
Rapid Induction	Positive	Positive	Negative	Negative
Biliary Infarction	Positive	Positive	Negative	Positive
High Level of Reproducibility	Positive	Positive	Positive	Positive
Caveats	Ultra-rapid onset unlikely in patients	Irrelevant Bile Acid	Not physiological	Not physiological

Table 7.1: Comparison of models for obstructive cholestatic liver injury. LCA, lithocholic acid. DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine. ANIT, alpha-napthylthioisocyanate.

dissertation (LCA administration and BDL), and interestingly, the two of them recapitulate different components of the human injury. Other models may also be relevant to the pathology and Table 7.1 lists the representative pros and cons: Given the information in the above table, a new model has a potential to better serve the community as a way to investigate the pathology. Given the relative differences in the BDL model and the LCA model, some combination of the two might fully recapitulate the human pathology, as you would need extrahepatic obstruction along with increased toxic bile acid levels. Previous attempts at "humanizing" the bile acid pool have focused on giving unconjugated CA, which our study does not support (Kunne et al., 2014). A new model could be used where the nearly undetectable LCA or CA is replaced with the relatively highly found GCDC. By directly giving mice GCDC you bypass their propensity for taurine conjugation, and increase the toxicity of the bile acid pool, effectively "humanizing" the pool. Low levels of GCDC could be used that would be subtoxic, but alter bile acid kinetics and concentration. BDL could then be done either using the selective BDL model or complete obstruction (Fickert et al., 2013) to recapitulate the response in humans. Other potential models would be the lithogenic diet model where gall stones are induced via feeding of cholic acid and cholesterol (Xie et al., 2013). It is possible that if you could palpate a gall stone from a sedated mouse into the biliary ducts, mimicking a loose stone, which would then induce cholestasis. This idea is currently untested; however, as gall stones represent a major source of obstructive cholestasis there is potential behind the idea. Regardless, work in this dissertation necessitates a re-evaluation of much of the recent literature, and promises to refocus future works on

mechanisms of oncotic necrosis that occur in humans and the subsequent inflammation that develops from bile acid induced toxicity.

Cholestatic Liver Injury: What holds for the future?

The major intervention for prevention of cholestatic liver injury is currently endoscopic retrograde cholangiopancreatography (ERCP) and due to its efficacy, this is unlikely to change. Thus, one might ask: Why continue to study obstructive cholestasis at all? ERCP has relatively good outcomes, is highly efficacious, and is available at numerous hospitals around the world. Primarily, current models, current knowledge, and the wealth of patients with obstructive cholestasis make it an excellent starting point to begin tackling more complex diseases. Autoimmune disorders such as primary biliary cirrhosis and primary sclerosing cholangitis present with a lower chance for successful treatment, and not surprisingly, the etiologies of these diseases are much less well understood. What is known is that, like in obstructive cholestasis, there is damage to the biliary tracts, loss of hepatocytes, a reduction in bile flow, a pronounced inflammatory infiltrate, and differential gene regulation that accompanies the lesion. This causes much of the same dysfunction such as seen in models of obstructive cholestasis, and while obstructive cholestasis can simply be fixed with surgical "replumbing" of the biliary tracts via ERCP, this technique is not available in these more complex, partially intrahepatic disorders. In order to combat these more dangerous diseases, pharmacology will be necessary. As these diseases have complex etiologies, current models are underwhelming in their recapitulation of the pathophysiology. Thus, valuable information can be applied broadly to form hypotheses that might hold across the cholestatic spectrum. Data from this dissertation supports a number of different potential pharmacological targets.

A majority of the focus on cholestatic liver injury has been on protecting hepatocytes against the toxic bile acid concentrations. While this is a potential therapeutic avenue, another potential route is the protection of cholangiocytes against biliary infarction, and cholestatic biliary injury (Beuers et al., 2012). Cholangiocytes are constantly exposed to high concentrations of bile acids in the bile. Interestingly, our data showed that patients with obstructive cholestasis do not have higher biliary bile acid values but instead, have lower values (Chapter 4). This is complicit with biliary infarction and release of bile into the hepatic parenchyma to reduce biliary pressure. Thus, one therapeutic option would be the protection against this biliary infarction. It has been hypothesized that cholangiocytes are protected against normal bile concentrations via both a protective layer of bicarbonate that blocks excessive uptake of bile acids from bile, and a siaylated glycocalyx that acts as a physical buffer, and source of bicarbonate (Hohenester et al., 2012). Desiaylation of the glycocalyx, or removal of the anion exchanger 2 protein (AE2) responsible for bicarbonate secretion increased injury in human cholangiocytes exposed to toxic concentrations of bile acids *in vitro* (Hohenester et al., 2012). As biliary bile acid levels do not change dramatically during obstructive cholestasis, the rupture of the biliary tracts likely is partially derived from a breakdown of the protective layer of bicarbonate and apical glycocalyx, and subsequent cholangiocyte cell death. As biliary bicarbonate secretion is responsible for a significant portion of bile flow, enhancing bicarbonate secretion would likely only serve to worsen the cholestatic syndrome by increasing biliary load in cases of major obstruction. Instead, therapeutics should be designed that can effectively prevent glycocalyx breakdown, buffer biliary pH concentrations without enhancing biliary flow, and reduce bile acid uptake into cholangiocytes. Therapeutics

with this sort of profile would potentially be beneficial in other disorders with significant cholangitis, such as primary sclerosing cholangitis and primary biliary cirrhosis. As biliary cell loss is thought to be a prominent cause of cholestasis in these injuries, decreasing further stress on the biliary tracts and enhancing biliary flow may alleviate some of the inflammatory signals that perpetuate the biliary injury.

Currently, UDCA is used pharmacologically in some cholestatic disorders. The presumed mechanism of action is an enhancement of choleresis and cytoprotective properties of multiple origins (Carey and Lindor, 2012). Other bile acid analogues such as obeticholic acid have been proposed as therapeutics with similar outcomes, although different predicted mechanisms (Lindor and Silveira, 2014). While both of these bile acids alter the composition of the biliary pool and enhance choleresis, neither of them have been shown to alter *the toxicity* of the biliary pool. Promoting conjugation to taurine instead of glycine will reduce the toxicity of the biliary pool, according to data from this dissertation, as taurine conjugated bile acids were generally less toxic to hepatocytes than their glycine conjugated counterpart. Moreover, taurine conjugated bile acids have much higher pKa values than glycine conjugated bile acids and are not effectively taken up by cholangiocytes, further reducing the potential damage to cholangiocytes (Hohenester et al., 2012). On the same note, excess supplementation with taurine may shift the conjugation reactions away from their normal state (approximately 50:50) and more towards a pro-taurine-conjugated state. Currently, it is not well established why humans have such a higher rate of glycine-conjugation than rodents. It could potentially be due to endogenous taurine and glycine concentrations, as changes in taurine and glycine can affect conjugation rates (Hardison, 1978). Humans, unlike mice, have limited capacity for

taurine synthesis and receive most through the diet (Huxtable and Lippincott, 1982). Supplementation of taurine in cholestatic patients has a potential to reduce the toxicity of the bile acid pool in patients benefitting both cholangiocytes and hepatocytes that are exposed to biliary bile acid levels.

Moving forward, there needs to be a better understanding of the intracellular signaling mechanisms that control bile acid induced toxicity in man. Toxic bile acid levels induce multiple stress kinase pathways (Woolbright, unpublished) including ERK, JNK, and P38. The relative contribution of each of these pathways to the injury is not currently known; however, early studies indicate JNK is not involved in the injury (Woolbright, unpublished). Activation of these pathways may be involved in multiple other intracellular effects that directly, or indirectly, affect cell survival. This can yield novel information on the mechanism of necrosis that dying cells undergo. Unfortunately, this mechanism was not well discerned in this study, despite efforts to the contrary. Given the extreme flocculation of cytoplasm seen in hepatocytes during cholestasis in man though, high concentrations of bile acids likely dysregulate ion channels that exacerbates whatever detergent effect the bile acids have, leading to necrosis. A study to gain better understanding of calcium signaling, intracellular calcium levels, and how bile acids dysregulate these channels is currently underway. These studies will take place both in primary human hepatocytes and additionally in HepaRG cells. The advent of protective interventions that functioned both in primary human hepatocytes and in HepaRG cells would solidify the HepaRG model as a way to assess interventions against injury that might also protect in human hepatocytes and *in vivo* in patients.

Finally, the role of inflammation in bile acid induced necrosis needs to be better understood. In the LCA model of bile acid feeding, significant inflammation was found, yet shown to be irrelevant to the injury process as knockout mouse models protected against BDL induced inflammation and injury were not protected against LCA administration. This is likely due to the inherent toxicity of LCA and accumulation of high levels of LCA metabolites that directly resulted in hepatic toxicity (Chapter 5). As humans have biliary bile acid levels that result in direct toxicity, this same situation likely plays out in man. On the other hand, both the mouse BDL model and human patients have significant accumulation of acetylated HMGB1 in serum and a pronounced neutrophil infiltrate histologically (Chapter 4). Additionally, human patients with liver injury during cholestasis had a notably higher absolute neutrophil counts than their counterparts without liver injury. Thus, two separate hypotheses are possible: 1) bile acids kill hepatocytes after biliary rupture which results in release of HMGB1 and other DAMPs and recruitment of cytotoxic inflammatory species, including neutrophils and macrophages that also release acHGMB1, that aggravates the injury as in the BDL model or 2) bile acids kill hepatocytes and the circulating HMGB1, acHGMB1, and inflammatory infiltrate are there to clean up surrounding dead tissue and resolve the injury. This hypothesis will be tested in the future by examining neutrophil activation, neutrophil ROS production, and neutrophil degranulation in cholestatic patients. If inflammation does play a role in the injury, common anti-inflammatory drugs such as glucocorticoids, which also protect against BDL injury (Hsieh et al., 2006), may be a viable short-term treatment option before stenting of the bile duct via ERCP.

Final Thoughts:

The ultimate goal of scientific research is to benefit the community, specifically, patients. Thus, models of cholestatic liver injury that recapitulate human pathology are at a premium. Primary human hepatocytes are currently the gold standard for toxicity studies as they are closest in nature to human hepatocytes response *in vivo.* HepaRG cells used in this dissertation may be able to help bridge the difficult gap between starting up human hepatocyte culture collaborations and working in less relevant models such as murine or rat hepatocytes. While work in this dissertation repeats studies done in other species, this work shows for the first time species differences that are pertinent for the future study of cholestatic liver injury. Moreover, new mechanisms of injury and new biomarkers associated with cholestasis were presented for the first time in this dissertation. New mouse and human cell line models need to be developed that can faithfully model what happens in man, especially in regards to more severe cholestatic liver disorders. Furthermore, *in vitro* research should be broadly re-evaluated in the context of applying bile acids to cells, both in the context of cellular injury and in the context of assessment of the role of unconjugated and conjugated bile acids in metabolism. As this research identifies actual bile acid concentrations present during control and diseased conditions, these concentrations should be focused upon, as they best represent the *in vivo* physiology. Future *in vitro* work will hopefully focus on the administration of conjugated bile acids, and evaluate results in terms of actual concentrations. These findings are broadly translatable and will hopefully push forward work not only on cholestatic liver injury, but also in the fields of biomarkers, drug induced hepatotoxicity, and metabolism.

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