MECHANISMS OF PROINFLAMMATORY SIGNALING DURING CHOLESTASIS

BY

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ABSTRACT

Cholestasis is a condition in which bile flow from the liver to the intestines is inhibited. Loss of bile flow leads to increased concentrations of bile constituents, including bilirubin and bile acids within the liver. Cholestasis leads to liver inflammation and injury. Several studies have demonstrated that inflammation is required for injury; however, the mechanism by which cholestasis stimulates proinflammatory gene expression remains unknown.

We demonstrated previously that the transcription factor early growth response factor-1 (Egr-1) is critical for inflammation and injury during cholestasis, and that Egr-1 is upregulated in deoxycholic acid (DCA)-treated primary mouse hepatocytes. To determine the mechanism by which Egr-1 is upregulated during cholestasis we tested the hypothesis that activation of the farnesoid X receptor (FXR) or the mitogen-activated protein kinase (MAPK) pathway is required for upregulation of Egr-1 during cholestasis. The results demonstrated that loss of FXR signaling did not alter Egr-1 expression in hepatocytes treated with bile acids or during bile duct ligation (BDL), a model of cholestasis. However, loss of MAPK singling prevented upregulation of Egr-1 both *in vitro* and *in vivo*.

Next, we tested the hypothesis that bile acids upregulate proinflammatory mediators in hepatocytes by Egr-1-dependent mechanisms. The results demonstrated that the bile acids DCA, chenodeoxycholic acid (CDCA) and taurocholic acid (TCA) increased proinflammatory gene expression in primary mouse hepatocytes by Egr-1-dependent and independent mechanisms. This study also demonstrated that upregulation of proinflammatory genes by bile acids occurred independently of cell death. Further studies demonstrated that upregulation of plasminogen activator inhibitor type-1(PAI-1), vascular cell adhesion molecule-1, and Ccl7 in BDL mice required Egr-1. Lastly, Egr-1 was upregulated in the livers of patients with cholestasis and correlated with levels of IL-8, intercellular adhesion molecule-1 and PAI-1.

Next, we tested the hypothesis that interleukin-17 (IL-17) family members are required for inflammation during cholestasis. The IL-17 family of cytokines has been associated with neutrophilic inflammation and induction of proinflammatory gene expression in some cell types. Our results demonstrated that IL-17D, but no other IL-17, was upregulated in bile acid-treated hepatocytes. In addition, treatment of BDL mice with an anti-IL-17D antibody attenuated upregulation of some proinflammatory mediators, but did not affect liver injury or inflammation. Next we investigated the role of IL-17A and IL-17F in regulation of inflammation during cholestasis. IL-17A was inhibited with a neutralizing antibody, and IL-17A and IL-17F signaling were inhibited with a neutralizing antibody against IL-17 receptor A. BDL mice treated with anti-IL-17A antibody had similar liver injury and inflammation as control-treated mice. Similarly, treatment of mice with an anti-IL-17 receptor A antibody during cholestasis did not affect liver injury or inflammation.

Overall the results from our studies identified novel mechanisms of inflammation during cholestasis, and reveal potential drug targets for the treatment of cholestatic liver diseases.

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANIT	α-naphthylisothiocyanate
BCL	B lymphocyte chemoattractant
BDL	Bile duct ligation
BDECs	Bile duct epithelial cells
CA	Cholic Acid
Ccl	CC chemokine ligand
CD	Cluster of differentiation
CDCA	Chenodeoxycholic acid
CISF	Cytokine synthesis inhibitory factor
COX-2	Cyclooxygenase-2
Csf	Colony-stimulating factor
Cxcl	CXC chemokine ligand
DAMPs	Damage associated molecular patterns
DCA	Deoxycholic acid
EGFR	Epidermal growth factor receptor
EGR-1	Early growth response factor-1
Elk1/2	Ets-like transcription factor
ERK1/2	Extracellular signal-regulated kinases
FAS	Apoptosis stimulating fragment
fMLP	Formyl-methionyl-leucyl-phenylalanine
FXR	Farnesoid X receptor
GCP-2	Granulocyte chemotactic protein-2
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IL	Interleukin
IP-10	Interferon γ-inducible protein of 10 kD
I-TAC	IFN-inducible T cell alpha chemoattractant
KC	Keratinocyte-derived chemokine
LARC	Liver activation regulated chemokine
LIX	LPS-induced chemokine
LPS	Lipopolysaccharide
MAC-1	Alpha M beta 2 or CD11b/CD18
MAPK	Mitogen-activated protein kinases
MCP	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
PAI-1	Plasminogen activator inhibitor type-1
PAMPs	Pathogen associated molecular patterns
PBC	Primary biliary cirrhosis

Plaur	Plasminogen activator, urokinase receptor
PMNs	Polymorphonuclear leukocyte/ Neutrophils
PSC	Primary sclerosing cholangitis
Ptgs2	Prostaglandin-endoperoxide synthase 2
PXR	Pregnane X receptor
RANTES	Regulated upon Activation, Normal T cell Expressed and presumably Secreted
Serpine 1	Serine (or cysteine) peptidase inhibitor
SR-PSOX	Scavenger receptor for phosphatidylserine and oxidized low density lipoprotein
TDCA	Taurodeoxycholic acid
TLR4	Toll-like receptor-4
TNF-α	Tumor necrosis factor-α
UDCA	Ursodeoxycholic acid
uPAR	Urokinase plasminogen activator receptor
VCAM-1	Vascular cell adhesion molecule-1
VDR	Vitamin D receptor

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CHAPTER 1

INTRODUCTION

1.1 Causes of Cholestasis

Cholestasis is a condition in which bile flow from the liver is disrupted (Klaassen, 2001). Normally, bile, which consists mainly of bile acids, phospholipids, bilirubin conjugates and glutathione, flows from the liver into the intestines through a tree like structure called the biliary tree (Li and Crawford, 2004) (Fig 1.1). Bile formation is accomplished through hepatocyte-dependent secretion, via ATP-dependent exporters, of bile constituents into the canaliculi between adjoining hepatocytes (Esteller, 2008). Bile is a necessary vehicle for excretion of endogenous and exogenous compounds such as heme, in the form of bilirubin, some drugs and heavy metals. Bile also provides antioxidant protection to the small intestine and is required for nutrient absorption (Klaassen, 2001). Bile acids, the major constituent of bile, are amphipathic molecules that are formed from cholesterol in the liver via cytochrome p450-dependent cholesterol catabolism (Thomas et al., 2008). Bile acids aid in dietary lipid absorption and undergo enterohepatic circulation, meaning that they are secreted from the liver into the intestines where upwards of 95% of the bile acids present are reabsorbed and delivered back to the liver via the portal blood (Fig. 1.1). Levels of bile acids within the systemic circulation are generally low (approximately 5 µM in humans), however after a meal, bile acid levels within the liver, portal blood and systemic circulation increase (Thomas et al., 2008). Alterations in hepatic bile secretion into the intestines (i.e., cholestasis) can cause bile acid concentrations to increase within the liver leading to extensive pathological changes.

Causes of cholestasis can be defined as ductular or canalicular. Ductular cholestasis is divided into obstructive and non-obstructive (Paumgartner, 2010). Obstructive cholestasis results from any condition that mechanically constricts the bile duct, such as a gallstone that becomes lodged in the common bile duct or a pancreatic tumor that compresses the bile duct. Non-obstructive cholestasis results from autoimmune destruction of bile ducts, such as occurs in primary biliary cirrhosis (PBC) and biliary atresia, or from conditions that affect transport of

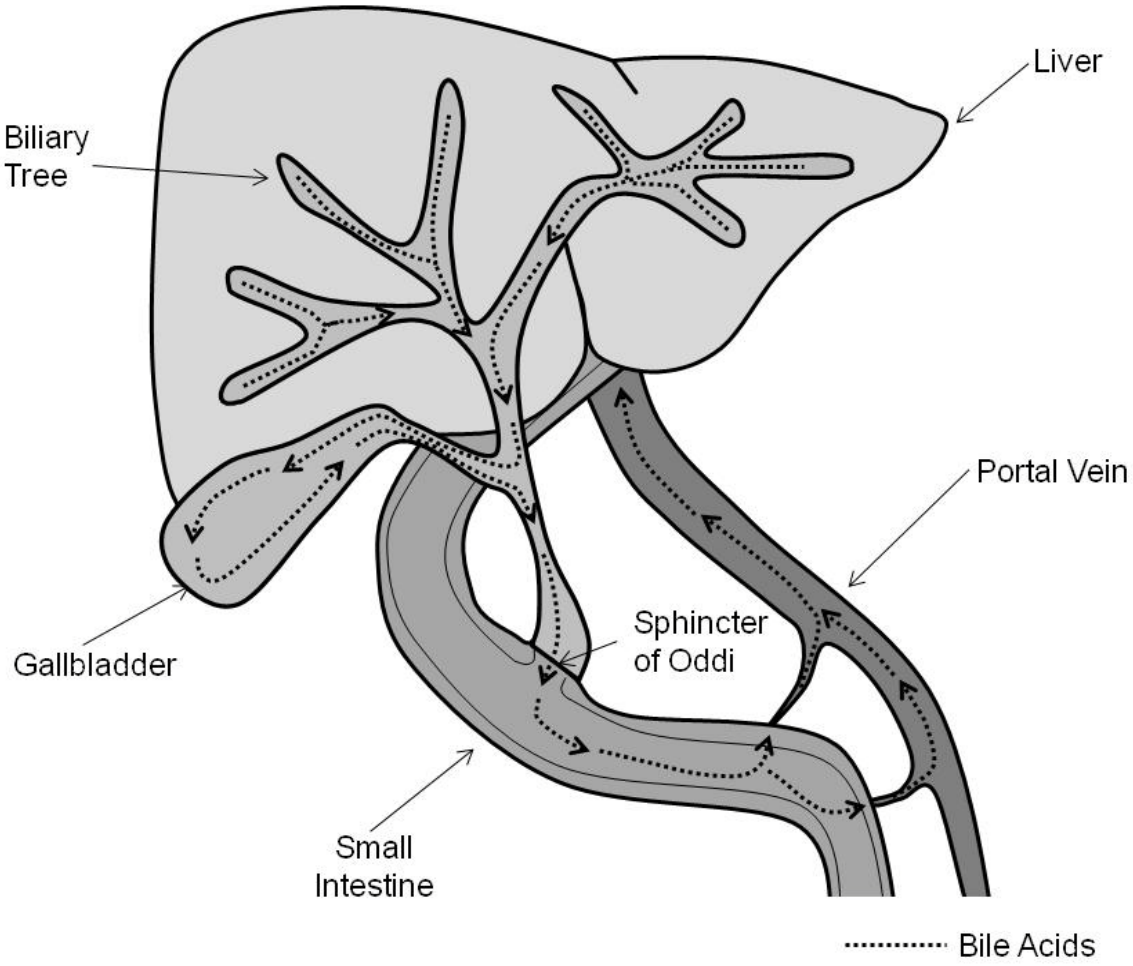

Figure 1.1 Diagram of enterohepatic circulation. Constituents of bile are transported from hepatocytes into bile canaliculi which flow into progressively larger intrahepatic ducts. The bile is then collected in the gallbladder. Upon stimulation, the gallbladder contracts releasing bile into the common bile duct. At the sphincter of Oddi, bile enters the small intestine. Nutrients within the small intestine and bile acids are absorbed into the blood by active transport. The blood is collected in the portal vein which enters the liver. The bile acids are transported into hepatocytes where they are again secreted into bile and reenter the enterohepatic circulation.

bile constituents. This is often referred to as canalicular cholestasis, which occurs when bile acid or phospholipid transport is reduced (Hirschfield and Heathcote, 2009; Paumgartner, 2010). This may result from genetic mutations in various transporters, such as those found in progressive familial intrahepatic cholestasis (Qureshi, 1999) or may result from exposure to drugs or toxicants that inhibit transporter function (Qureshi, 1999; Karlsen and Hov, 2010). Regardless of the cause, loss of transporter function alters or inhibits bile flow, leading to an increase in bile constituents within the liver.

The line between obstructive and non-obstructive is not well defined, however, and many pathologies that are considered non-obstructive may have an obstructive phenotype or component (Paumgartner, 2010). This is seen in primary sclerosing cholangitis (PSC), a cholestatic condition that is often associated with ulcerative colitis (Qureshi, 1999). During PSC strictures form within intra and extra hepatic bile ducts that obstruct normal bile flow leading to obstructive cholestasis in certain regions of the biliary tree. It has also been suggested that in patients with PBC, autoimmune destruction of bile duct epithelial cells may obstruct bile ducts thereby impeding bile flow similar to obstructive cholestasis (Paumgartner, 2010).

1.2 Treatments for Cholestasis

Currently there is only one FDA approved drug for the treatment of cholestasis regardless of the etiology. Ursodeoxycholic acid (UDCA) is given to patients therapeutically and has been shown to slow the progression of PBC in some patients. In patients with PBC, UDCA is thought to increase bile flow from the liver, and thus decrease concentrations of toxic bile constituents within the liver and serum. However, UDCA is not an effective treatment for obstructive cholestasis or PSC (Hsu et al., 1997; Sinakos et al., 2010). Consistent with this, mice given UDCA during bile duct ligation (BDL), a model of obstructive cholestasis, have greater liver injury compared to mice subjected to BDL alone (Fickert et al., 2002). Currently there is no drug therapy known to slow the progression of PSC (Aron and Bowlus, 2009). Due

to the limited efficacy of UDCA and the absence of other treatments for cholestatic conditions, liver transplantation is often the only treatment. There are considerable risks associated with liver transplantation and in the cases of PBC and PSC, the disease may reoccur (Tamura et al., 2008; Patkowski et al., 2010). Therefore, it is critical to understand the pathogenesis of cholestatic liver diseases as this may provide new insight into the treatment of these disorders.

1.3 Pathogenesis of Cholestatic Liver Disease

Although the etiologies of each specific cholestatic liver disease are distinct, the progression of these diseases can have similar effects upon serum and histological biomarkers. Serum biomarkers of cholestasis include elevated levels of alkaline phosphatase (ALP), bilirubin, bile acids and γ-glutamyl transferase (Li and Crawford, 2004). Evaluation of these biomarkers alone does not provide a definitive diagnosis of cholestasis. Histological examination of the liver is often required for definitive diagnosis and for identification of the etiology. Histological findings consistent with cholestasis include bilirubin pigment accumulation, giving hepatocytes a reddish brown color, hepatocellular degeneration, bile duct proliferation, the presence of inflammatory cells typically in periportal regions, and at later stages of the disease, peribiliary fibrosis (Li and Crawford, 2004). Histological evaluation may also show neutrophil accumulation in the liver which is most common with obstructive cholestasis, however as mentioned above, some histological features of obstructive cholestasis may be observed in non-obstructive forms of this disease (Gujral et al., 2003; Li and Crawford, 2004).

Overtime, if the underlying condition is not resolved, the liver will become fibrotic. Fibrosis is an unregulated wound healing response in which scar tissue is deposited within the liver. During liver fibrosis the composition of the extracellular matrix within the liver is altered, making the liver more rigid, leading to impaired sinusoidal perfusion and increased intrahepatic

resistance (Center, 1999). Portal biliary fibroblasts and hepatic stellate cells are known to contribute to the altered basement membrane deposition (Dranoff and Wells, 2010). Fibrosis is reversible if the underlying condition is successfully treated or resolved (Harty et al., 2010). However as fibrosis progresses, the fibrotic lesions begin to separate the liver into regenerative, abnormally functioning foci of hepatocytes. At this stage, the disease is defined as liver cirrhosis (Schuppan and Afdhal, 2008). Liver cirrhosis is typically not reversible, and the only treatment at this point is liver transplantation.

1.4 Role of Inflammation in the Pathogenesis of Obstructive Cholestasis.

Many studies have demonstrated that proinflammatory proteins such as adhesion molecules, cytokines and chemokines increase in the serum and livers of patients with cholestasis (Thomson et al., 1994; Gulubova, 1998; Tsuneyama et al., 2001; Nobili et al., 2004; Isse et al., 2007; Borchers et al., 2009). Many of these proteins have also been shown to increase in the liver and serum of animals subjected to BDL (Gujral et al., 2004a; Wang et al., 2005; Kim et al., 2006; Wintermeyer et al., 2009). With the use of knockout animals, neutralizing antibodies and small molecule inhibitors, the functions of some of these proinflammatory mediators during cholestasis have been elucidated.

Many different chemicals and treatments promote neutrophilic inflammation in the liver. The inflammatory mediators that are produced and promote inflammation and the mechanisms by which neutrophils are recruited and activated in the liver are quite similar regardless of the stimulus (Figure 1.2). Briefly, cytokines released from injured cells or activated macrophages into the general circulation cause changes in neutrophil gene expression making the neutrophil more responsive to proinflammatory signaling (Nathan, 2002). This leads to an increase in the β2-integrin, Mac-1 on neutrophils (Bajt et al., 2001; Mayadas and Cullere, 2005).

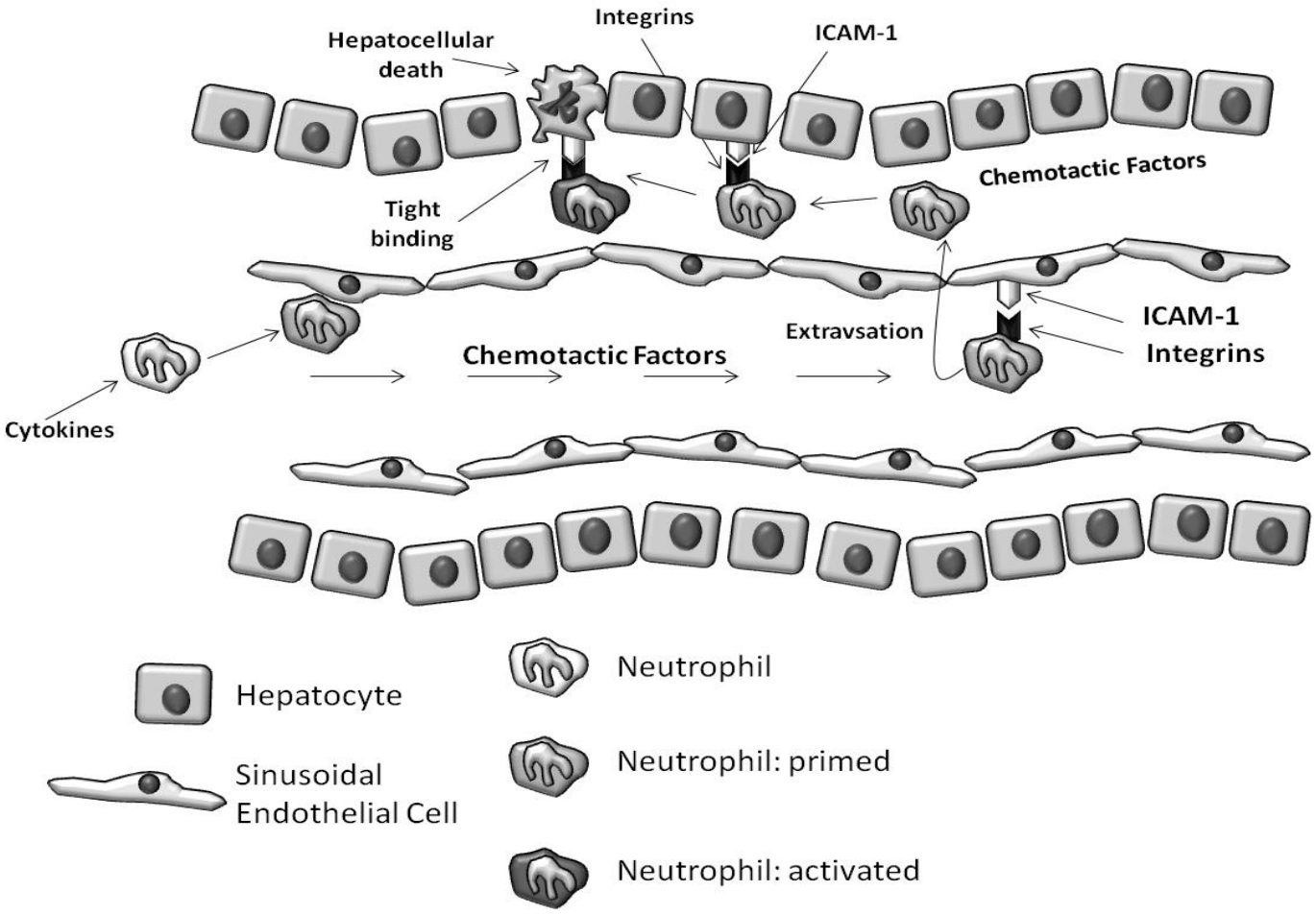

Figure 1.2 Generalized mechanism of neutrophil-dependent inflammation in the liver**.**
Cytokines, typically released from cells in the liver, prime neutrophils for activation and adhesion. The neutrophil is recruited to the sinusoids via a chemotactic gradient. Extravasation brings the neutrophil into the liver parenchyma. Tight binding of the neutrophil to the hepatocyte induces superoxide formation and the release of proteases leading to hepatocellular death.

The neutrophil is then recruited to the sinusoids of the liver via chemokines which produce a chemotactic gradient. The third step in the process is the extravasation of the neutrophil into the liver parenchyma. Extravasation requires the expression of CD18 by the neutrophil which binds to intracellular adhesion molecule-1 (ICAM-1) expressed by sinusoidal endothelial cells (Jaeschke and Smith, 1997). In addition, it is believed that a second chemotactic gradient produced by hepatocytes is also necessary for neutrophil extravasation. Once the neutrophil enters the parenchyma, a second β2-integrin- ICAM-1 interaction allows for tight binding of the neutrophil to the hepatocyte. This tight binding induces superoxide formation and the release of proteases, by mechanisms that are not completely understood, which produce necrotic hepatocellular death (Jaeschke and Smith, 1997). The function of each group of proinflammatory mediators in cholestasis and the experimental evidence that implicates them in the development of cholestatic liver injury are discussed in more detail in the following section.

1.4.1 Adhesion molecules. Soluble ICAM-1 has been shown to increase in the serum of patients with cholestatic liver disease (Thomson et al., 1994). Furthermore, serum levels of soluble ICAM-1 in patients with PBC correlates with the degree of liver injury (Thomson et al., 1994). Several animal studies have demonstrated that ICAM-1 and its integrin counterpart CD18 are critical for liver injury during cholestasis. In these studies, ICAM-1 knockout mice were subjected to BDL. ICAM-1 knockout mice had reduced numbers of neutrophils within the liver and reduced liver injury when compared to wild-type mice (Gujral et al., 2004a). In the counterpart study, inhibition of CD18 with a neutralizing antibody produced similar results (Gujral et al., 2003). These studies showed that infiltration of neutrophils into the liver and extravasation of neutrophils from the hepatic sinusoids requires ICAM-1 and CD18. Furthermore, these studies indicate that liver injury during obstructive cholestasis is largely dependent upon neutrophil infiltration.

1.4.2 Chemokines. In addition to adhesion molecules, studies have focused on the role of chemokines in the development of liver inflammation during cholestasis. Studies in which antibodies were used to neutralize the mouse neutrophil chemokines, macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC), there was a reduction in liver injury and neutrophil accumulation during BDL (Wintermeyer et al., 2009). These chemokines are the mouse orthologs of interleukin-8 (IL-8) in humans, which was shown to be elevated in the serum of patients with cholestasis. In addition neutrophils isolated from the blood of patients with cholestasis are more sensitive to the chemotatic factors formyl-methionyl-leucyl-phenylalanine (fMLP) and IL-8. The exacerbated response to these ligands is lost after biliary drainage, a procedure in which excess bile is drained from the liver (Takaoka et al., 2001). These data suggest that constituents of bile may increase the sensitivity of PMNs to chemotactic factors or may increase production of proinflammatory mediators by other cell types that prime PMNs for activation. In support of this, expression of several cytokines is increased in the serum of patients with obstructive cholestasis, with no evidence of sepsis, suggesting that bile constituents may increase production of cytokines which prime PMNs, thereby worsening liver injury (Jiang et al., 1994).

1.4.3 Potential Role of IL-17s in Cholestatic Liver Disease. The interleukin-17 family of cytokines, which consists of IL-17A through IL-17F, has been shown to increase expression of several cytokines in numerous cells types, and contribute to the development of inflammation in several animal models (Weaver et al., 2007). For example, in rats, treatment with recombinant IL-17A, via inhalation, stimulates neutrophil accumulation in the lung (Laan et al., 1999). This response was reduced when rats were pretreated with an anti-MIP-2 antibody (Laan et al., 1999). The IL-17A receptor and IL-17C receptor form a heteromeric complex which is activated by IL-17A and IL-17F. Both receptors are expressed by most cell types in the liver including, hepatocytes, bile duct epithelial cells, hepatic stellate cells and Kupffer cells (Lafdil et

al., 2010). It has also been demonstrated that treatment of cultured hepatocytes with IL-17A increases expression of several genes, including proinflammatory genes such as KC and monocyte chemotactic protein-1 (MCP-1) (Sparna et al., 2010). Harada and colleagues demonstrated that treatment of bile duct epithelial cells with IL-17A increases expression of the cytokines IL-6, IL-1β, IL-23 p19 and IL-23 p40 as well as the chemokines KC, MIP-2, MIP-2β, granulocyte chemotactic protein-2 (GCP-2) , MCP-1, and macrophage inflammatory protein-3 (MIP-3) (Harada et al., 2009). Interestingly, several of these cytokines increase expression of IL-17A by immune cells (i.e., IL-6 and IL-1β) or contribute to the maintenance of IL-17A expression by these cells (i.e., IL-23), suggesting that regulation of cytokine expression by IL-17A in BECs may contribute to a feed-forward loop that enhances inflammation (Harada et al., 2009). Collectively, these data suggest that IL-17A may contribute to inflammation in the liver during cholestasis. This is further supported by the fact that IL-17A signaling contributes to liver injury in alcoholic liver disease and concanavalin A-induced hepatitis (Lafdil et al., 2010). The role of IL-17s in the development of obstructive cholestatic liver disease; however, has not been investigated. Although, it was recently shown that neutralization of IL-17A reduces liver injury in α-naphthylisothiocyanate (ANIT)-treated mice, a chemical that damages bile duct epithelial cells thus causing cholestasis (Kobayashi et al., 2009). Therefore, in studies detailed in later chapters, the hypothesis was tested that neutralization of IL-17A in mice with obstructive cholestasis reduces inflammation and liver injury.

1.5 Potential mechanisms of Initiation of Inflammation During Cholestasis

1.5.1 Role of Toll-like receptor-4 ligands. Toll-like receptor-4 (TLR-4), a member of the toll-like receptor family, has been suggested to play an important role in the development of inflammation in the liver during cholestasis (Figure 1.3). TLR4 is activated by multiple ligands.

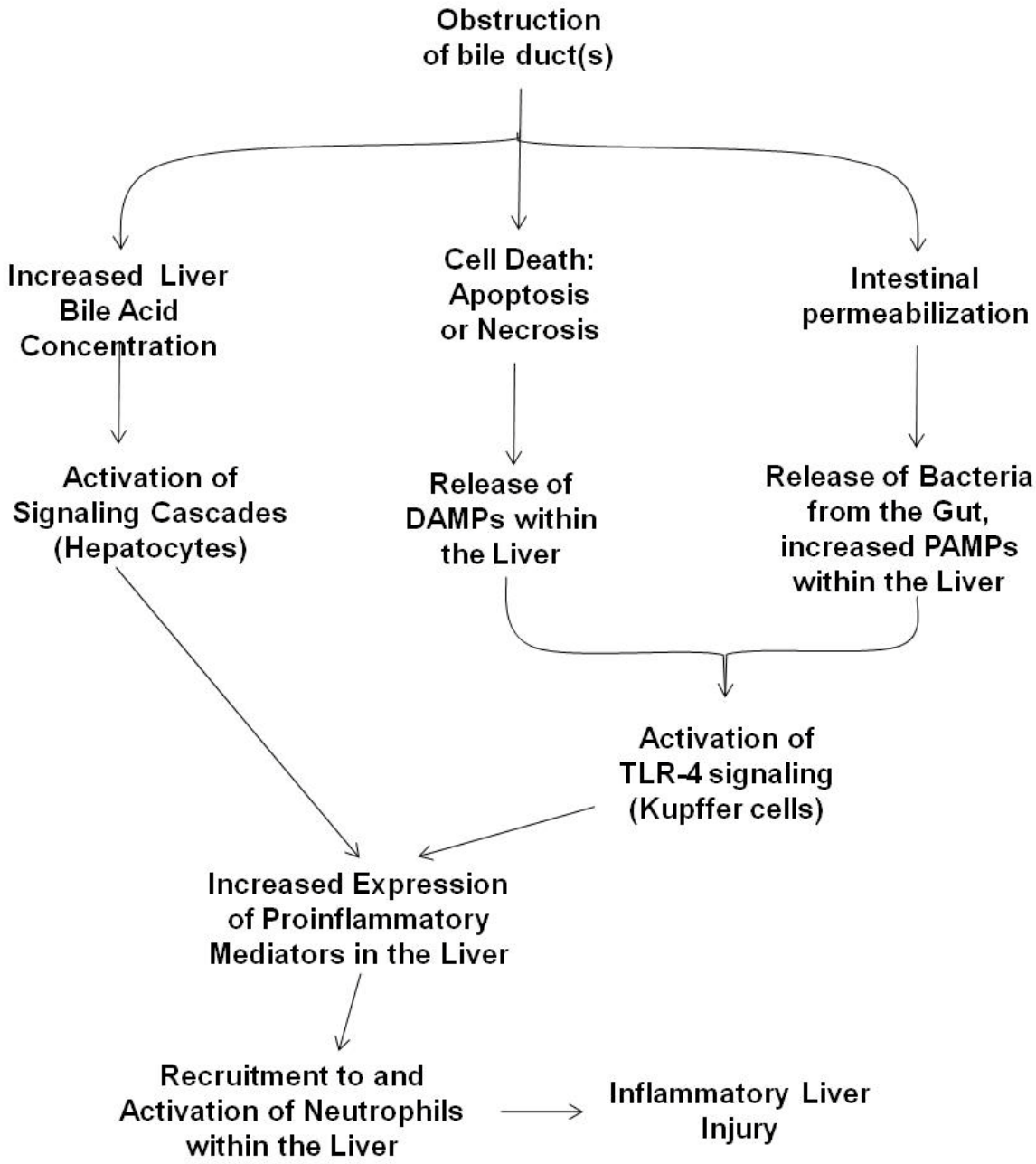

Figure 1.3 Potential Stimuli and Mechanisms of Proinflammatory Signaling during Cholestasis.

These ligands fall into two general categories; pathogen-associated molecular patterns (PAMPs), which are unique components of bacteria, virus and fungi, or damaged-associated molecular patterns (DAMPs), which are components of endogenous cells released during injury. It has been proposed that, during cholestasis, TLR4 is activated by either lipopolysaccharide (LPS), a PAMP released from bacteria which translocate from the gut, or by DAMPs released from apoptotic or damaged cells (Diamond and Rowlands, 1991; Guo and Friedman, 2010). Bacterial translocation is thought to result from a decrease in the concentration of bile acids in the intestines which allows for overgrowth of intestinal bacteria (Reynolds et al., 1996). This in combination with a reduction in immune vigilance in the liver is thought to allow for increased levels of systemic bacteria which release LPS (Abe et al., 2004). Considering that LPS is a potent inflammatory molecule and that its concentrations are increased in the serum of cholestatic mice (Reynolds et al., 1996), some have proposed that LPS may be responsible for inflammation in the liver during cholestasis (Van Bossuyt et al., 1990). Studies have shown, however, that serum levels of LPS are not significantly increased in BDL animals six to eight hours after BDL when the inflammatory response is initiated, suggesting that LPS may not be responsible for the initiation of inflammation during cholestasis (Van Bossuyt et al., 1990; Reynolds et al., 1996; Gong et al., 2002; Neuman et al., 2002; Georgiev et al., 2008; Guo and Friedman, 2010). It remains possible, however, that other TLR4 ligands, such as DAMPs, may be important for initiation of inflammation. As part of this dissertation, studies were conducted to fully evaluate the role of TLR4 ligands in regulation of inflammation during cholestasis.

1.5.2 Role of bile acids. Another potential mechanism by which cholestasis may stimulate rapid inflammation is through bile acid-dependent activation of signal transduction pathways (Figures 1.3 and 1.4). Bile acid concentrations increase rapidly within the liver and serum of BDL mice and in humans with cholestatic liver disease (Jiang et al., 1994; Tribe et al., 2009). In support of a role for bile acids in the development of liver inflammation,

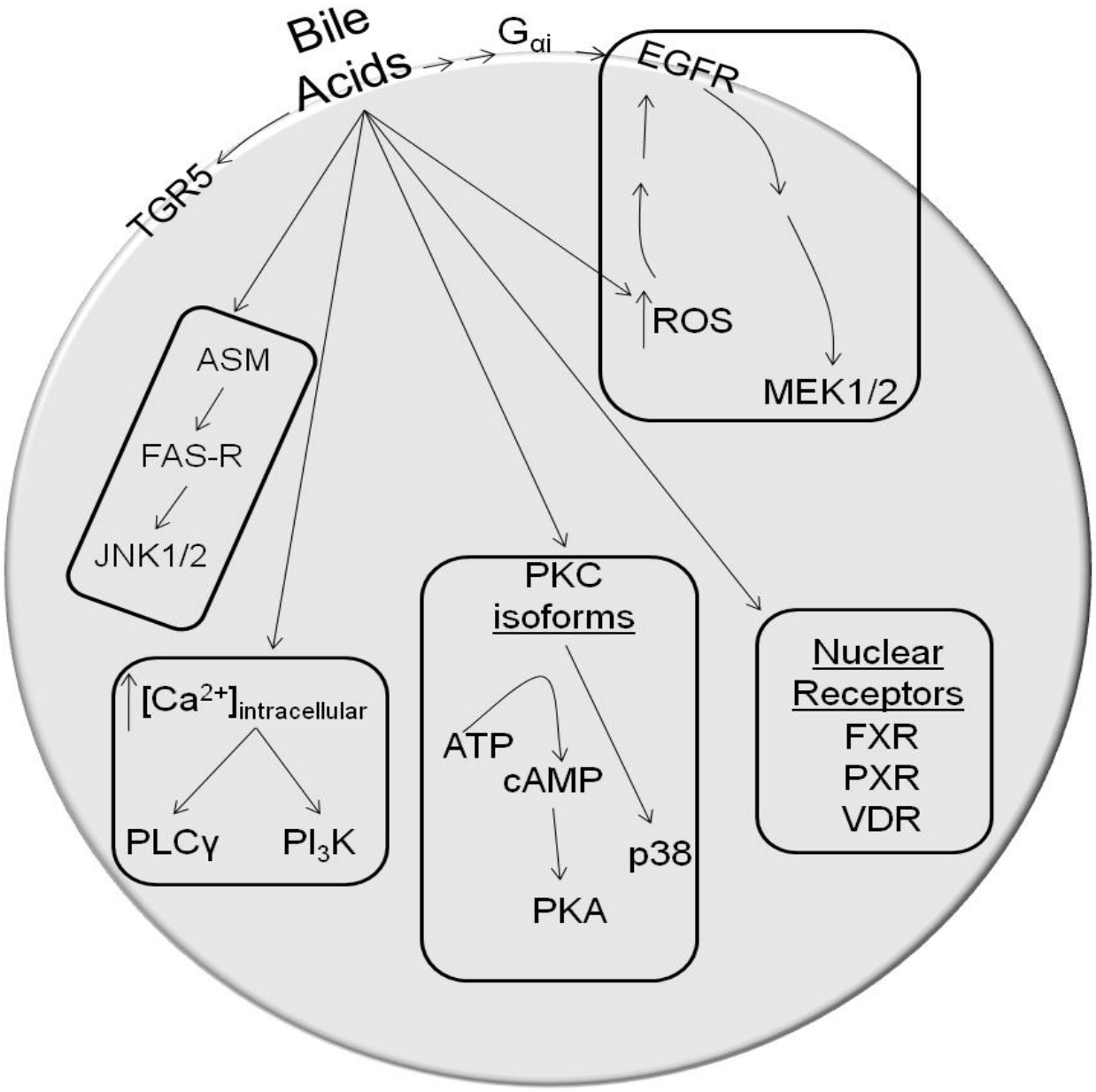

Figure 1.4 Pathways known to be Activated by Bile Acids. TGR5: G-protein coupled bile acid receptor 1; ASM: acidic sphingomyelinase; JNK: c-Jun N-terminal Kinase; PLCγ: phospholipase Cγ; PI_3K: phosphoinositide 3-kinases; PKC: protein kinase C; ATP: adenosine tri-phosphate; cAMP: cyclic adenosine mono-phosphate; PKA: protein kinase A; p38: protein of 38 kD; FXR: farnesoid X receptor; PXR; pregnane X receptor; VDR: vitamin D receptor; EGFR: epidermal growth factor receptor; ROS: reactive oxygen species; MEK: MAPK/Erk kinase.

it was shown that in patients in which the cholestasis had been decompressed (i.e., removal of excess bile from the liver), levels of inflammatory proteins within the serum and neutrophil priming were reduced. Although it is possible that some other constituent of bile contributed to hepatic inflammation in these patients, this study does provide strong evidence for bile acids in the development of liver inflammation during cholestasis, and certainly provides the impetus to evaluate the role of bile acids in initiating hepatic inflammation.

Bile acids activate several signal transduction pathways in hepatocytes that may regulate production of proinflammatory mediators (Ramadori et al., 2008). For example, we demonstrated previously that the bile acid, deoxycholic acid (DCA), upregulates early growth response factor-1 (Egr-1), a transcription factor, in hepatocytes. Egr-1 has been shown to regulate many inflammatory genes in several cell types (Yan et al., 2000; Harja et al., 2004; McMullen et al., 2005; Kim et al., 2006; Pritchard et al., 2007). Consistent with a role for Egr-1 in regulating inflammatory genes in the liver during cholestasis, upregulation of ICAM-1 and MIP-2 was prevented in Egr-1 knockout mice subjected to BDL (Kim et al., 2006). These data suggest that exposure of hepatocytes to pathological concentrations of bile acids during cholestasis upregulates Egr-1 which regulates expression of inflammatory mediators that stimulate neutrophil-dependent liver injury. The mechanism(s) by which bile acids increase expression of Egr-1 and potentially other inflammatory mediators in hepatocytes during cholestasis, however, has not been elucidated. Bile acids activate several signal transduction pathways in hepatocytes, including nuclear receptors, G-protein coupled receptors, receptor tyrosine kinases, and several kinase cascades, including the mitogen-activated protein kinase pathway (MAPK) which may be responsible for upregulation of Egr-1 and other inflammatory mediators in hepatocytes (Figure 1.5) (Rao et al., 2002; Kawamata et al., 2003; Fang et al., 2004; Gupta et al., 2004; Dent et al., 2005; Nguyen and Bouscarel, 2008; Fiorucci et al., 2010a; Fiorucci et al., 2010b; Han et al., 2010).

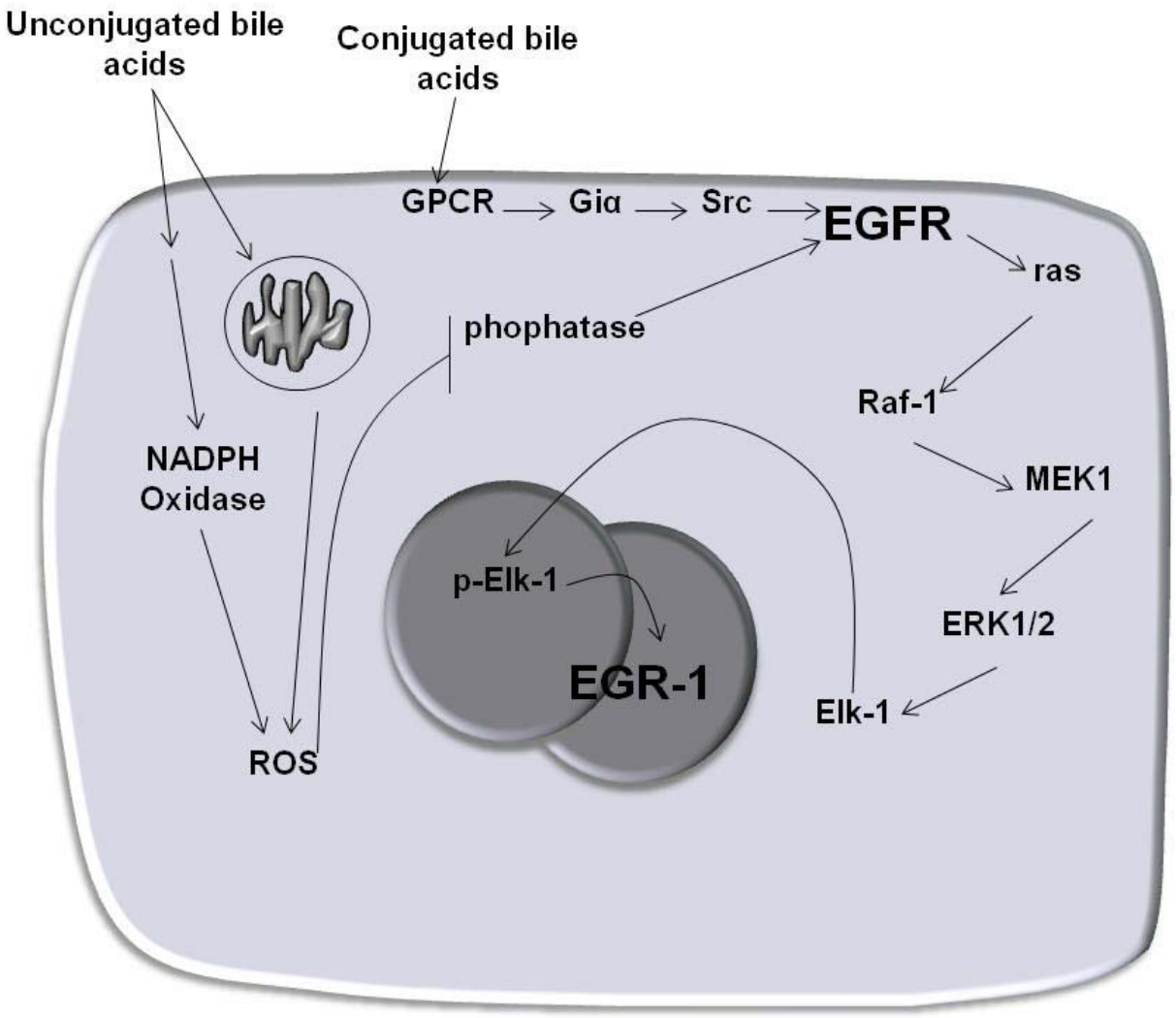

Figure 1.5 Activation of EGFR Signaling by Bile Acids in Hepatocytes. Unconjugated bile acids stimulate production of reactive oxygen species within hepatocytes which inhibit the activity of phophatases. Inhibition of phophatases prevents inactivation of EGFR. Conjugated bile acids activate EGFR through activation of a G-protein coupled receptor. Activation of EGFR leads to activation of the MAPK cascade, which activates the transcription factor Elk1/2. Elk1/2 has been shown to regulate the expression of Egr-1.

The various signaling pathways activated by bile acids and their potential contribution to regulation of inflammation during cholestasis are discussed in further detail below.

1.5.2.1 Signaling Pathways Activated by Bile Acids. The vitamin D receptor (VDR), a nuclear receptor, is activated by bile acids including taurodeoxycholic acid (TDCA), chenodeoxycholic acid (CDCA) and cholic acid (CA). Not only is the VDR a transcription factor, it also activates signal transduction pathways (Han et al., 2010). Activation of the VDR with an agonist during BDL inhibited expression of the proinflammatory genes interleukin-1β, IL-6, IL-10, tissue necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), suggesting that activation of VDR during BDL is beneficial and not detrimental. However, there was not an effect of VDR activation on liver injury suggesting that the anti-inflammatory contribution of VDR during cholestasis was minimal (Ogura et al., 2009).

The pregnane X receptor (PXR), a nuclear receptor is also activated by bile acids (Staudinger et al., 2001; Xie et al., 2001; Moore et al., 2002; Guo et al., 2003). Recent studies suggest that activation of PXR may be anti-inflammatory. For instance, activation of PXR inhibits the expression of cyclooxygenase-2 (COX-2) and TNF-α (Fiorucci et al., 2010b). Considering that mice lacking PXR had similar levels of necrosis and ALT elevation after BDL as did their wild-type counterparts, however, suggests that PXR does not modulate the inflammatory response in the liver during cholestasis (Stedman et al., 2005).

As noted previously, FXR is the endogenous nuclear receptor for bile acids. Activation of FXR with the FXR-specific agonist, GW4064, increased ICAM-1 expression in hepatocytes (Qin et al., 2005). Similarly, CDCA increased ICAM-1 expression in the same system (Qin et al., 2005). These data suggested that bile acids may increase proinflammatory gene expression in hepatocytes in an FXR-dependent manner. In contrast to this, however,

pretreatment of rats with GW4064 during BDL or after treatment with ANIT, a chemical that causes cholestasis reduced liver injury (Liu et al., 2003). This suggested that activation of FXR may be anti-inflammatory *in vivo* during cholestasis. Consistent with this, recent studies demonstrated that activation of FXR suppressed upregulation of inflammatory mediators in the liver after treatment with LPS (Wang et al., 2008). Collectively, however, it remains unclear whether activation of FXR is inflammatory or anti-inflammatory during cholestasis. Accordingly, studies were conducted in this dissertation to evaluate the role of FXR in the development of inflammation during cholestasis.

In addition to nuclear receptors, bile acids activate receptor tyrosine kinases (Nguyen and Bouscarel, 2008). The epidermal growth factor receptor (EGFR) is indirectly activated by both conjugated and unconjugated bile acids in hepatocytes (Figure 1.6) (Qiao et al., 2001; Rao et al., 2002). Activation of EGFR leads to subsequent activation of the extracellular signal-regulated kinase (Erk1/2) MAPK pathway and the transcription factor Elk-1 (Rao et al., 2002; Mayer et al., 2008). In some cell types, Elk-1 regulates Egr-1, suggesting that activation of EGFR may contribute to bile acid-dependent upregulation of proinflammatory genes. This remains to be evaluated, however.

The proapoptotic death receptor, FAS, is also activated in hepatocytes by bile acids (Bajt et al., 2000; Qiao et al., 2001; Gujral et al., 2004b; Gupta et al., 2004). Activation of the FAS receptor by certain bile acids causes downstream events that culminate in apoptosis of hepatocytes. Interestingly, FAS receptor knockout mice are resistant to liver injury during cholestasis and have reduced numbers of infiltrating neutrophils and reduced levels of proinflammatory cytokines within the liver, suggesting a potential role for activation of the FAS receptor in regulation of inflammation in the liver during cholestasis (Gujral et al., 2004b). It has

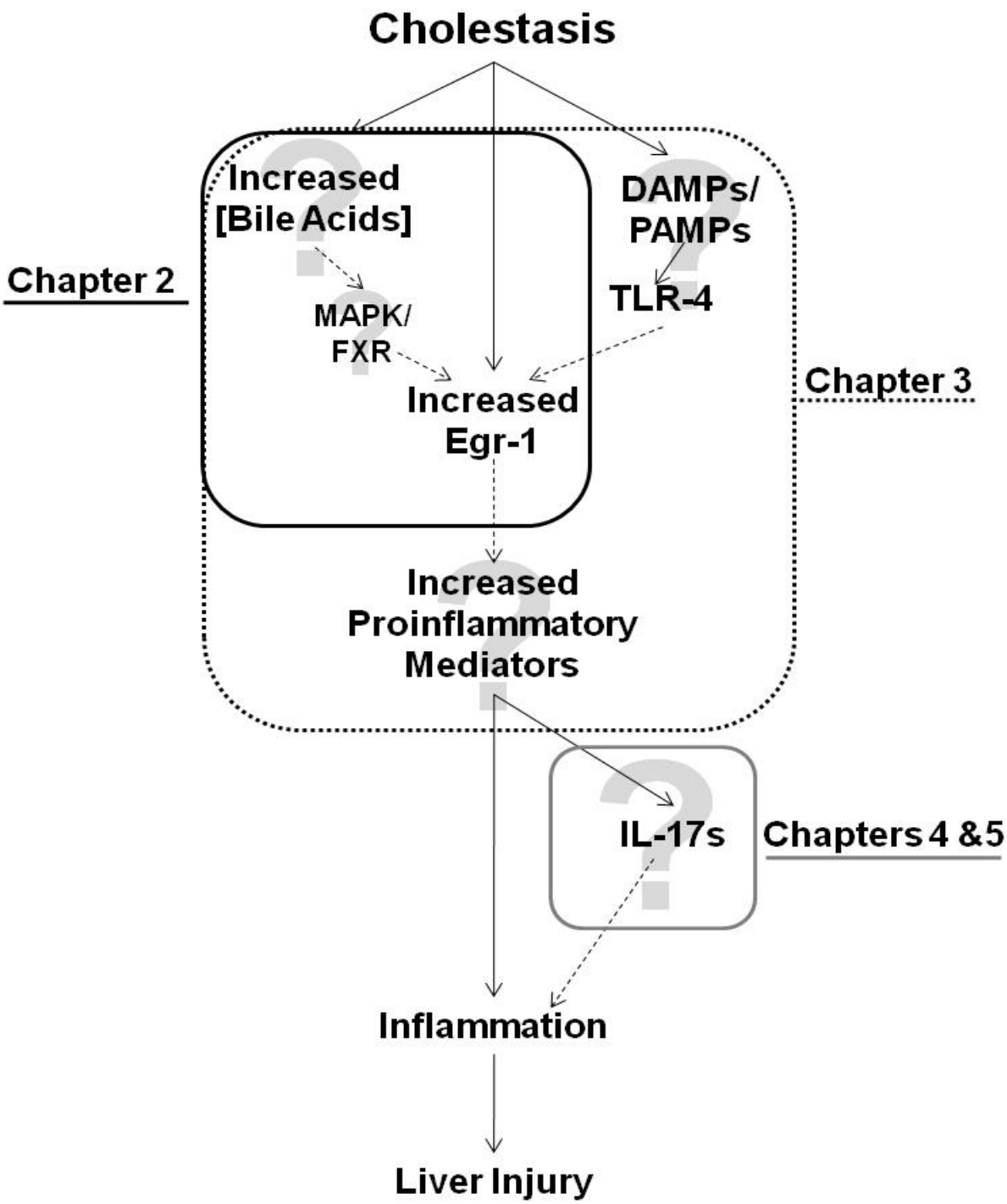

Figure 1.6 Outline of Studies Detailed in this Dissertation.

been proposed that the protection inferred due to FAS loss could be due to a reduction in natural killer T-cells, which have been shown to contribute to inflammation during cholestasis (Nalapareddy et al., 2009). Therefore, activation of FAS by bile acids may not directly regulate production of inflammatory mediators in hepatocytes, however, this has not been fully evaluated.

1.6 Purpose

Cholestatic liver diseases can cause irreversible liver injury and liver failure (Li and Crawford, 2004). Currently the only available treatment for cholestatic liver disease is UDCA which may help to attenuate liver injury and the progression of cholestasis in PBC but the efficacy of UDCA treatment in other forms of cholestasis is limited (Hsu et al., 1997; Aron and Bowlus, 2009; Sinakos et al., 2010). Inflammatory cells are known to infiltrate the liver during cholestasis, and have been shown to contribute to liver injury (Gujral et al., 2003; Gujral et al., 2004a; Li and Crawford, 2004; Kim et al., 2006). Therefore, a better understanding of the mechanism(s) that initiate inflammation during cholestasis may provide potential drug targets for the treatment of these diseases. The transcription factor Egr-1 has been shown to regulate the proinflammatory mediators ICAM-1 and MIP-2 in the liver after BDL. Furthermore, Egr-1 knockout mice subjected to BDL have reduced liver injury and reduced numbers of hepatic neutrophils. It was also demonstrated that Egr-1 was upregulated in hepatocytes after BDL and that bile acids upregulate Egr-1 in hepatocytes (Kim et al., 2006). Therefore, our overall hypothesis is that during cholestasis, pathological concentrations of bile acids upregulate Egr-1 in hepatocytes which stimulates production of inflammatory mediators that promote neutrophil accumulation and activation. What remains unknown, however, is the mechanism by which bile acids upregulate Egr-1 in hepatocytes, and which inflammatory mediators are upregulated in hepatocytes by bile acids in an Egr-1-dependent manner. In addition, the role of TLR4 and FXR in regulation of inflammation in the liver during cholestasis is not fully understood. Therefore,

studies are described in Chapter 2 that determined the mechanism by which Egr-1 is upregulated in hepatocytes after BDL using both *in vitro* and *in vivo* techniques (Figure 1.6). In Chapter 3, studies were conducted to identify those inflammatory mediators that are upregulated in bile acid-treated hepatocytes in an Egr-1-dependent manner. In addition the role of TLR4 and FXR were investigated (Figure 1.6) in this chapter. Lastly, recent studies indicate an important role for various members of the IL-17 family in regulation of neutrophilic inflammation. Since new drugs that inhibit IL-17s are in development and in clinical trials, we determined the role of IL-17s in the development of hepatic inflammation during obstructive cholestasis. These studies are described in Chapters 4 and 5 of the dissertation (Figure 1.6). Taken together these studies will give a better understanding of the steps leading to inflammation during cholestasis and the roles of proinflammatory mediators during development of this disease.

CHAPTER 2

Upregulation of Early Growth Response Factor-1 by Bile Acids Requires Mitogen-activated Protein Kinase Signaling

Katryn Allen, Nam Deuk Kim, Jeon-OK Moon, and Bryan L. Copple

Toxicology and Applied Pharmacology. 2010 Feb 15; 243 (1): 63-7

2.1 Abstract

Cholestasis results when excretion of bile acids from the liver is interrupted. Liver injury occurs during cholestasis, and recent studies showed that inflammation is required for injury. Our previous studies demonstrated that early growth response factor-1 (Egr-1) is required for development of inflammation in liver during cholestasis, and that bile acids upregulate Egr-1 in hepatocytes. What remains unclear is the mechanism by which bile acids upregulate Egr-1. Bile acids modulate gene expression in hepatocytes by activating the farnesoid X receptor (FXR) and through activation of mitogen-activated protein kinase (MAPK) signaling. Accordingly, the hypothesis was tested that bile acids upregulate Egr-1 in hepatocytes by FXR and/or MAPK-dependent mechanisms. Deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) stimulated upregulation of Egr-1 to the same extent in hepatocytes isolated from wild-type mice and FXR knockout mice. Similarly, upregulation of Egr-1 in the livers of bile duct-ligated (BDL) wild-type and FXR knockout mice was not different. Upregulation of Egr-1 in hepatocytes by DCA and CDCA was prevented by the MEK inhibitors U0126 and SL-327. Furthermore, pretreatment of mice with U0126 prevented upregulation of Egr-1 in the liver after BDL. Results from these studies demonstrate that activation of MAPK signaling is required for upregulation of Egr-1 by bile acids in hepatocytes and for upregulation of Egr-1 in the liver during cholestasis. These studies suggest that inhibition of MAPK signaling may be a novel therapy to prevent upregulation of Egr-1 in liver during cholestasis.

2.2 Introduction

Cholestasis is a condition that occurs when bile flow from the liver is disrupted (Li and Crawford, 2004). This results in increased concentrations of toxic bile acids in the liver and blood (Lindblad et al., 1977; Setchell et al., 1997). If cholestasis is left untreated, it results in hepatocellular injury, bile duct proliferation, and activation of hepatic stellate cells and portal fibroblasts, leading to fibrosis (Gujral et al., 2003; Ramadori and Saile, 2004). Over time as this

disease progresses, the liver becomes cirrhotic and the patient will be in danger of liver failure (Paumgartner, 2006).

Inflammation, consisting primarily of neutrophils, has been shown to play an integral role in the development of hepatocellular injury during cholestasis (Gujral et al., 2003; Gujral et al., 2004a). Neutrophil accumulation with in the liver parenchyma has been shown to occur in animals and humans with obstructive jaundice (Gulubova, 1998; Yamashiki et al., 1998; Neuman et al., 2002). Studies from our laboratory have shown that early growth response factor-1 (Egr-1), a transcription factor, is important for inflammation and injury during cholestasis (Kim et al., 2006). In these studies, Egr-1 was upregulated in hepatocytes of mice subjected to bile duct ligation (BDL), a model of cholestatic liver disease. Furthermore, Egr-1 knockout mice subjected to bile duct ligation had reduced liver injury, fewer neutrophils in the liver, and reduced expression of proinflammatory mediators compared to wild-type mice (Kim et al., 2006). These studies suggested that Egr-1 is a key mediator of inflammation in the liver during cholestasis. Our results demonstrated further that the bile acid, deoxycholic acid (DCA), increased Egr-1 protein levels in primary mouse hepatocytes (Kim et al., 2006). This suggested that the stimulus for upregulation of Egr-1 in liver during cholestasis may be bile acids. What remains unknown, however, is the molecular mechanism by which bile acids increase Egr-1 levels in hepatocytes. Identification of the signaling pathways that stimulate upregulation of Egr-1 could lead to the development of therapeutics that attenuate the inflammatory response in the liver during cholestasis.

Bile acids modulate gene expression in hepatocytes by several mechanisms. For example, bile acids activate the nuclear receptor, farnesoid X receptor (FXR). Upon bile acid binding, FXR heterodimerizes with RXR (Makishima et al., 1999) and binds to response elements in the promoters of genes, and modulates gene transcription by suppressing or enhancing gene expression (Claudel et al., 2005). In addition to FXR, bile acids modulate gene

expression by activating mitogen-activated protein kinase (MAPK) signaling (Rao et al., 2002). Bile acids stimulate ligand-independent activation of the epidermal growth factor receptor which stimulates phosphorylation and activation of raf kinase, MEK, and Erk1/2 (Rao et al., 2002). Whether activation of FXR and/or MAPK signaling is required for upregulation of Egr-1 by bile acids is not known. Accordingly, the hypothesis was tested that upregulation of Egr-1 in hepatocytes *in vitro* by bile acids and *in vivo* during cholestasis requires FXR and/or MAPK signaling.

2.3 Materials and Methods

2.3.1 Animals care. C57BL/6 mice (Harlan, Madison, WI) and FXR knockout mice with a congenic C57BL/6 background were used for all studies. Generation of the FXR knockout mice was described previously (Sinal et al., 2000). Mice were maintained on a 12-h light/dark cycle under controlled temperature (18-21ºC) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed ad libitum. All procedures on animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health and were approved by the institutional IACUC committee at the University of Kansas Medical Center.

2.3.2 Hepatocyte Isolation. Hepatocytes were isolated from the livers of mice by collagenase perfusion as described in detail by us previously (Kim et al., 2006). The hepatocytes were cultured in Williams' medium E containing 10% FBS and Penicillin-Streptomycin. After a 3 hour attachment period, the medium with unattached cells was removed, and fresh medium added. The cells were cultured overnight before addition of inhibitors and bile acids.

2.3.3 Bile Duct Ligation. Male, C57BL/6 and FXR knockout mice, 8-12 weeks of age, were anesthetized with isoflurane. A midline laparotomy was performed and the bile duct

ligated with 3-0 surgical silk. The abdominal incision was closed with sutures, and the mice received 0.2 mg/kg Buprenex by subcutaneous injection. For studies with U0126, mice received 100 mg/kg U0126 dissolved in DMSO or DMSO alone by intraperitoneal injection, 1 hour before surgery.

2.3.4 Real-time PCR. RNA was isolated from livers and hepatocytes using TRI reagent (Sigma Chemical Company, St. Louis, MO), and reverse transcribed into cDNA as described by us previously (Kim et al., 2006). Real-time PCR was used to quantify the mRNA levels of Egr-1 and 18S on an Applied Biosystems Prism 7300 Real-time PCR Instrument (Applied Biosystems, Foster City, CA) using the SYBR green DNA PCR kit (Applied Biosystems) as described (Kim et al., 2006). The sequences of the primers were as follows: 18S Forward: 5'-TTG ACG GAA GGG CAC CAC CAG-3'; 18S Reverse: 5'-GCA CCA CCA CCC ACG GAA TCG-3'; Egr-1 Forward: 5'-GGC AGA GGA AGA CGA TGA AG-3'; and Egr-1 Reverse: 5'-GAC GAGTTATCC CAG CCA AA-3'.

2.3.5 Western Blot. Total protein was isolated from hepatocytes and liver. In brief, hepatocytes and liver were lysed in RIPA buffer (1XPBS, 1% IGPAL, 0.5% sodium deoxycholate and 0.5% SDS) containing Complete protease inhibitor (Roche Applied Science Indianapolis, IN) and PhosSTOP phosphatase inhibitor (Roche Applied Science Indianapolis, IN). The lysates were sonicated, and total protein concentrations measured using the BCA Protein Assay Kit (Pierce Rockford, IL). Phospho-p44/42 MAPK (Erk1/2), total p44/42 MAPK, Egr-1 and α-actin levels were measured using western blotting. Equal amounts of protein were separated on a 4.5-15% gradient Criterion polyacrylamide gel (Bio Rad Hercule, CA) and transferred to a PVDF membrane (Immoblion-P Bedford, MA). The membrane was incubated with anti-phospho-p44/42 MAPK antibody, anti-p44/p42 MAPK antibody, anti-Egr-1 antibody (all from Cell Signaling Technology, Inc.) or anti-α-actin antibody (Sigma Chemical Co., St. Louis, MO) followed by incubation with the appropriate horseradish peroxidase-conjugated secondary

antibody (Santa Cruz Biotechnology Santa Cruz, CA). The bands were visualized using the ECL detection kit (Bio Rad Hercules, CA).

2.3.6 Statistical Analysis. Results are presented as the mean $\pm$ SEM. Data were analyzed by Analysis of Variance (ANOVA). ANOVAs were performed on log X-transformed data in instances in which variances were not homogenous. Comparisons among group means were made using the Student-Newman-Keuls test. The criterion for significance was $p < 0.05$ for all studies.

2.4 Results

2.4.1 Deoxycholic Acid and Chenodeoxycholic Acid Increase Egr-1 mRNA and Protein Levels in Primary Mouse Hepatocytes. Exposure of primary mouse hepatocytes to deoxycholic acid (DCA) or chenodeoxycholic acid (CDCA) caused a dose-dependent increase in Egr-1 mRNA and protein in primary mouse hepatocytes (Figure 2.1).

2.4.2 Upregulation of Egr-1 by Bile Acids does not require FXR. FXR is a nuclear receptor activated by bile acids and represents a possible signaling mechanism by which bile acids could affect Egr-1 expression (Makishima et al., 1999). To determine whether FXR is required for upregulation of Egr-1 by bile acids, hepatocytes were isolated from wild-type and FXR knockout mice and treated with either DCA or CDCA. Treatment of hepatocytes from wild-type mice with DCA or CDCA increased Egr-1 mRNA levels (Figure 2.2). Bile acids increased Egr-1 mRNA levels in hepatocytes isolated from FXR knockout mice to the same extent as hepatocytes from wild-type mice (Figure 2.2). Next it was determined whether FXR is required for upregulation of Egr-1 in the livers of BDL mice. For these studies, wild-type and FXR knockout mice were subjected to either sham operation or BDL, and Egr-1 mRNA levels were quantified in the liver. Egr-1 mRNA was increased to the same extent in the livers of wild-type and FXR knockout mice subjected to BDL (Figure 2.2).

2.4.3 Upregulation of Egr-1 by Bile Acids Requires MAPK Activation. Bile acids have been shown activate the MAPK cascade in hepatocytes (Rao et al., 2002). Accordingly, we next determined whether MAPK activation is required from upregulation of Egr-1 in hepatocytes. Treatment with either DCA or CDCA for 15 minutes stimulated phosphorylation of ERK1/2 in hepatocytes. Pretreatment with U0126, a MEK inhibitor, prevented phosphorylation of ERK1/2 after bile acid treatment (Figure 2.3 A). Treatment of hepatocytes with either DCA or CDCA increased Egr-1 mRNA levels (Figure 2.3 B and 2.3 C) and protein levels (Figure 2.4). Pretreatment with U0126 completely prevented upregulation of Egr-1 mRNA and protein by both DCA and CDCA (Figure 2.3 and 2.4). To confirm these results, hepatocytes were pretreated with SL-327, another MEK inhibitor. Pretreatment of hepatocytes with SL-327 attenuated the increase in Erk1/2 phosphorylation and the increase in Egr-1 mRNA and protein in hepatocytes treated with DCA and CDCA (Figure 2.5 and 2.6).

2.4.4 Increased Egr-1 expression is prevented with U0126 pretreatment *in vivo*. Since U0126 prevented upregulation of Egr-1 by bile acids *in vitro*, we next determined whether U0126 would prevent upregulation of Egr-1 in the livers of mice after BDL, a model of cholestasis. BDL in mice stimulated phosphorylation of ERK (Figure 2.7). Pretreatment of the mice with U0126 completely prevented phosphorylation of the ERK (Figure 2.7). BDL in mice increased Egr-1 protein levels (Figure 2.7). Pretreatment of mice with U0126 prevented the increase in Egr-1 protein after BDL (Figure 2.7).

2.5 Discussion

Inflammatory cell infiltration into the liver during cholestasis has been shown to exacerbate liver injury (Gujral et al., 2004a). Studies have shown that upregulation of inflammatory mediators is important for inflammatory injury during cholestasis (Gujral et al., 2004a; Wintermeyer et al., 2009). Our studies have identified Egr-1 as a key regulator of these proinflammatory molecules in hepatocytes *in vivo* during cholestasis (Kim et al., 2006).

Furthermore, our studies demonstrated that bile acids stimulate upregulation of Egr-1 in hepatocytes (Figure 2.1), suggesting that bile acids may be the stimulus for upregulation of Egr-1 in liver during cholestasis (Kim et al., 2006). What remained unknown from these studies, however, is the molecular mechanism by which bile acids upregulate Egr-1 in hepatocytes. The present studies demonstrated that MAPK signaling is important for upregulation of Egr-1 by bile acids *in vitro* and *in vivo*.

As discussed, bile acids activate many different signaling pathways in hepatocytes, including FXR and the MAPK cascade. FXR is the endogenous bile acid receptor, and has been shown to be activated during cholestasis and regulate expression of numerous genes (Makishima et al., 1999; Wagner et al., 2003). Our studies indicate that FXR is not required for upregulation of Egr-1 by bile acids *in vitro* or *in vivo* (Figure 2.2). Consistent with these results, previous studies demonstrated that BDL in FXR knockout mice does not reduce liver injury or increase survival in these mice when compared to wild-type mice (Wagner et al., 2003). We saw similar results in our BDL studies (data not shown). If FXR were a key regulator of Egr-1, it would be anticipated that liver injury would be reduced in FXR knockout mice, since Egr-1 is required for inflammation and injury in the liver during cholestasis.

Our studies demonstrated that MAPK activation is required for upregulation of Egr-1 *in vitro* after bile acid treatment (Figures 2.3, 2.4, 2.5 and 2.6) and *in vivo* during cholestasis (Figure 2.7). The MAPK cascade has been shown to be activated in primary rat hepatocytes treated with bile acids *in vitro* (Rao et al., 2002). Studies have shown that MAPK activation results in phosphorylation and activation of the transcription factor Elk-1 (Mayer and Thiel, 2009). Mayer et al. demonstrated that phosphorylated Elk-1 binds to the Egr-1 promoter in response to cellular Ca^{2+} changes in some cell types (Mayer et al., 2008; Mayer and Thiel, 2009). This suggests that Elk-1 may be the transcription factor responsible for upregulation of Egr-1 in bile acid-treated hepatocytes. Further studies are needed, however, to test this

possibility. Our results also demonstrated that MAPK signaling is activated in the liver during cholestasis, and that inhibition of Erk phosphorylation by a MEK inhibitor prevents upregulation of Egr-1 (Figure 2.7). Interestingly, it was recently demonstrated that the kinase inhibitor, sorafenib, prevents Erk activation in the livers of BDL rats and that this inhibitor reduces portal hypertension (Hennenberg et al., 2009). Whether sorafenib also affected inflammation in the liver was not investigated in these studies. These results in combination with ours suggest that Erk inhibition may not only be effective at reducing portal hypertension in patients with cholestasis, but may be effective at alleviating inflammatory liver injury in these patients.

Rao et. al. demonstrated previously that most conjugated and unconjugated bile acids stimulate Erk1/2 phosphorylation in hepatocytes (Rao et al., 2002). Therefore, it is likely that most bile acids will also promote upregulation of Egr-1. Activation of MAPK signaling in hepatocytes by bile acids was shown to be dependent upon ligand-independent activation of the epidermal growth factor receptor (Rao et al., 2002). Consistent with a role for this pathway in regulation of Egr-1 in hepatocytes, it was shown that epidermal growth factor upregulates Egr-1 in hepatocytes both *in vitro* and *in vivo* (Tsai et al., 2001). Therefore, therapeutic inhibition of the epidermal growth factor receptor may also be useful at preventing bile acid-induced inflammation in the liver during cholestasis by preventing upregulation of the proinflammatory transcription factor, Egr-1.

Collectively, our studies indicate that during cholestasis, elevated concentrations of bile acids activate MAPK signaling in hepatocytes. MAPK signaling then stimulates upregulation of Egr-1 which regulates expression of proinflammatory mediators that stimulate neutrophil accumulation and activation in the liver. Our studies also suggest that therapeutic targeting of the MAPK pathway in patients with cholestasis may be effective at inhibiting Egr-1 expression.

2.6 Figures

Figure 2.1

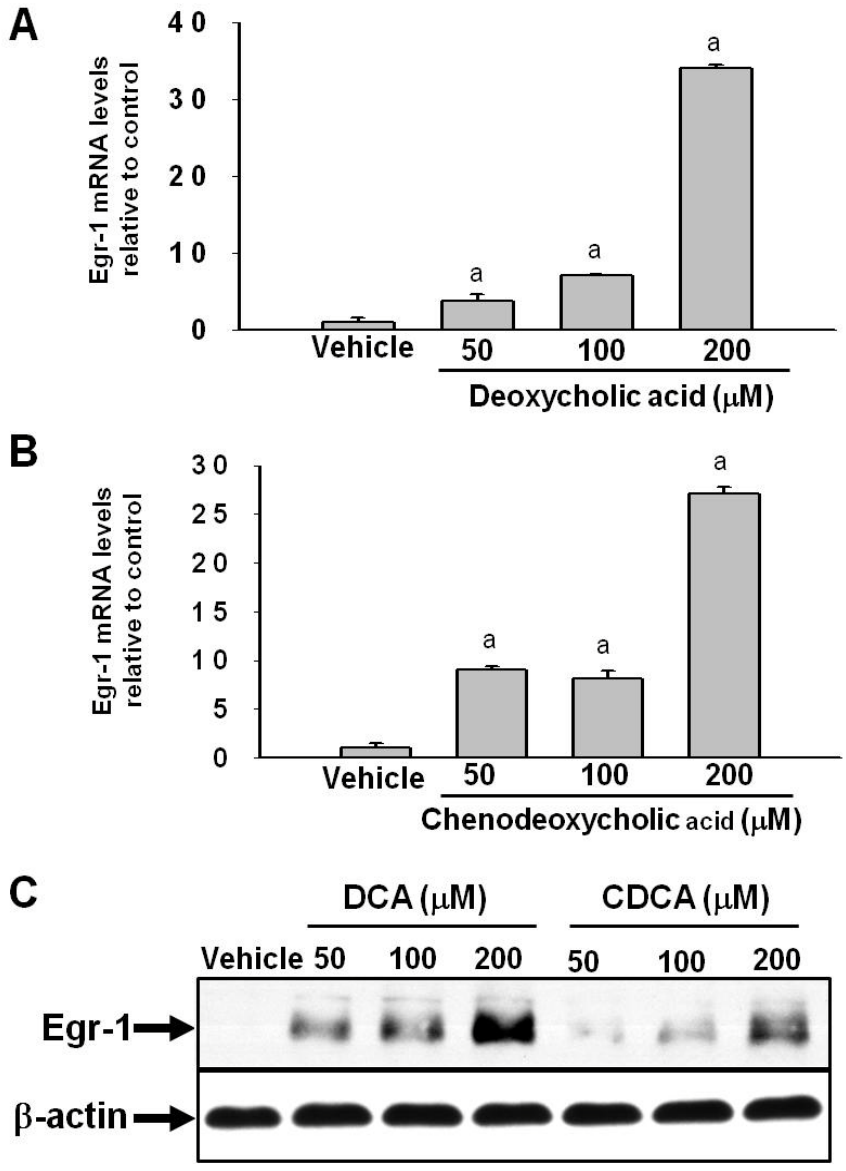

Figure 2.1 Upregulation of Egr-1 mRNA and Protein in Primary Mouse Hepatocytes Treated with Bile Acids. Primary mouse hepatocytes were isolated and treated with either deoxycholic acid (DCA), or chenodeoxycholic acid (CDCA). Two hours later, Egr-1 mRNA (A and B) was quantified. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from vehicle-treated hepatocytes. Total protein was isolated two hours after bile acid treatment and Egr-1 (C) levels were visualized by western blot the blot was then reprobed to visualize ß-actin (C).

Figure 2.2

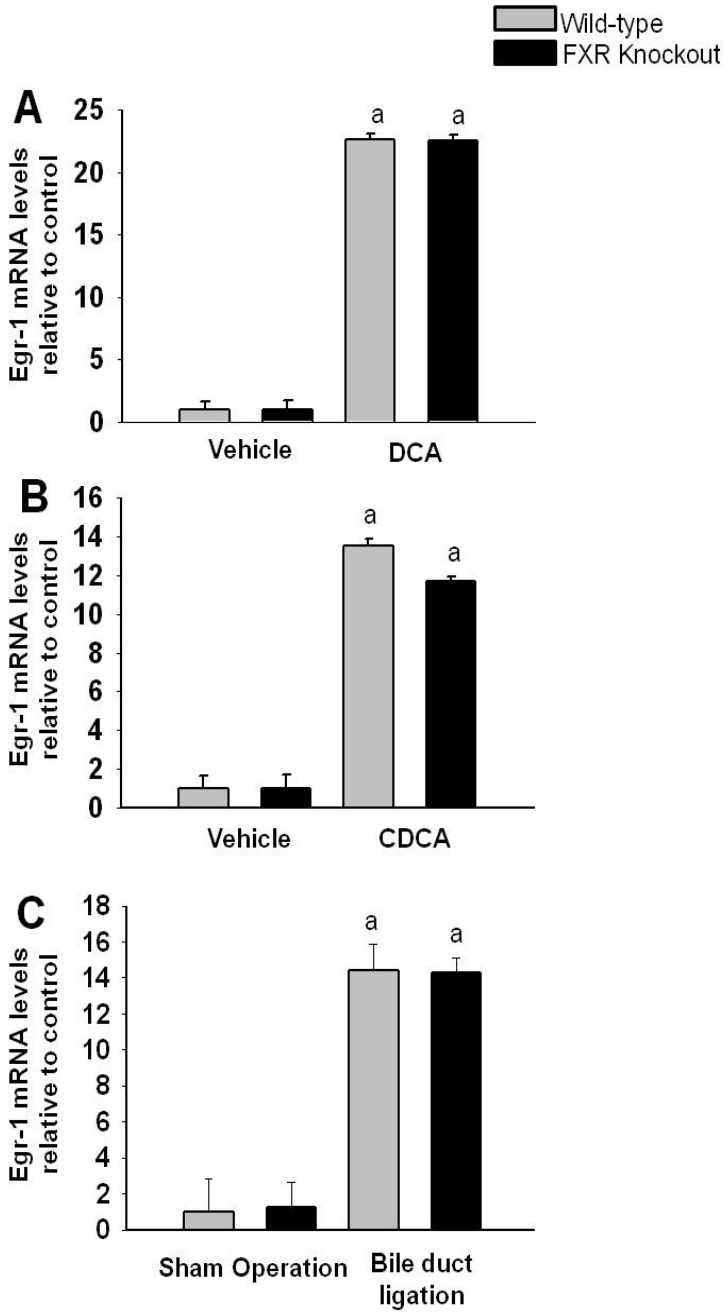

Figure 2.2 Role of FXR in Upregulation of Egr-1. Primary mouse hepatocytes were isolated from wild-type or FXR knockout mice and treated with either deoxycholic acid (DCA), or chenodeoxycholic acid (CDCA). Two hours later, Egr-1 mRNA (A and B) was quantified. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from vehicle-treated hepatocytes. Wild-type or FXR knockout mice were subjected to BDL or sham surgery (C). Three days after surgery the livers were collected and total mRNA was isolated. Egr-1 mRNA (C) was quantified by qPCR. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from sham operated control.

Figure 2.3

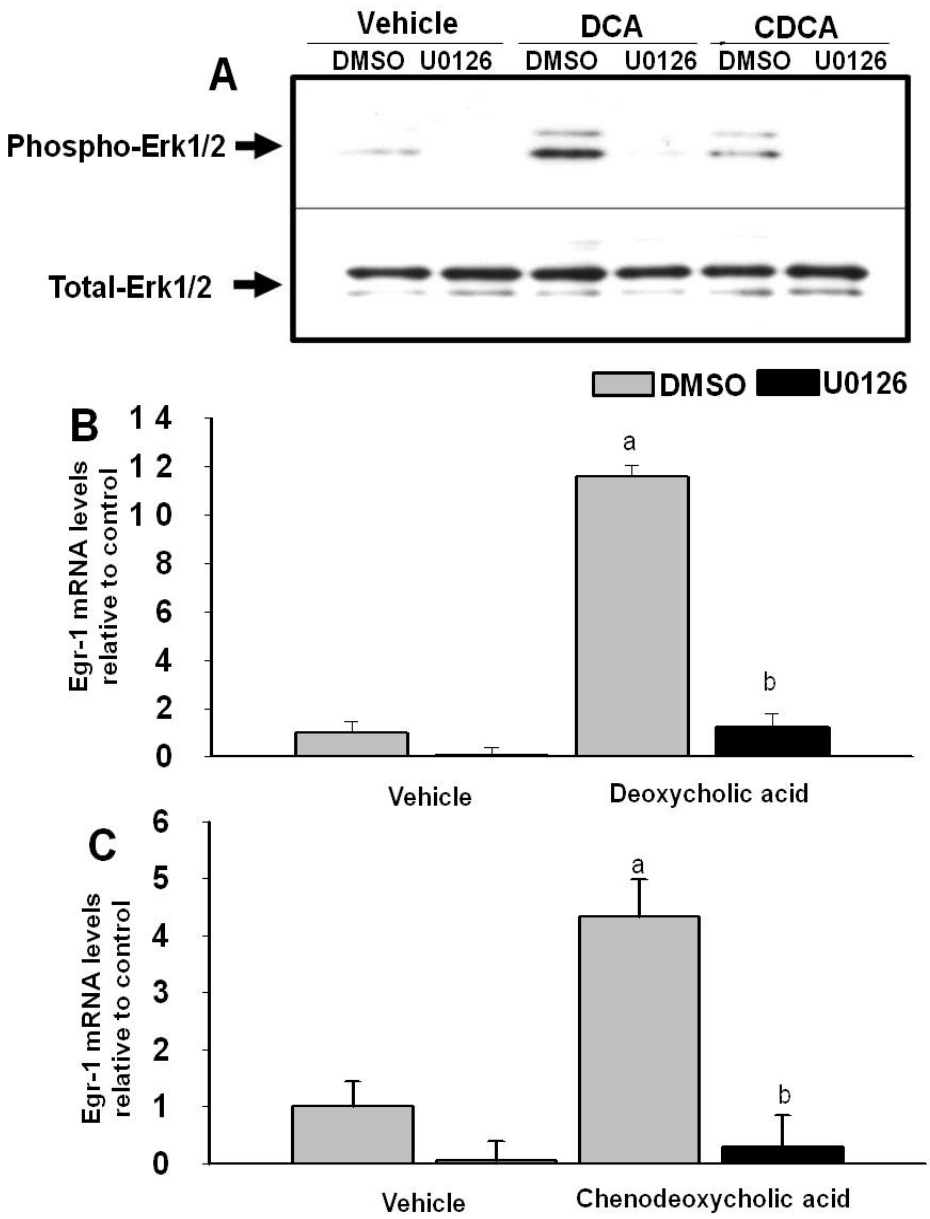

Figure 2.3 Role of MAP Kinase Signaling in Upregulation of Egr-1 mRNA by Bile Acids.
Primary mouse hepatocytes were treated with 10 µM U0126 followed by either 200 µM DCA (A) or 200 µM CDCA (B) 30 minutes later. The cells were incubated for 15 minutes or for 2 hours after which total protein or mRNA was isolated respectfully. Phospho-Erk and total-Erk protein (A) levels were visualized by western blot (A). Egr-1 mRNA (B and C) was quantified by qPCR. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from control treated hepatocytes. [b]Significantly different ($p < 0.05$) from hepatocytes treated with U0126 and bile acid.

Figure 2.4

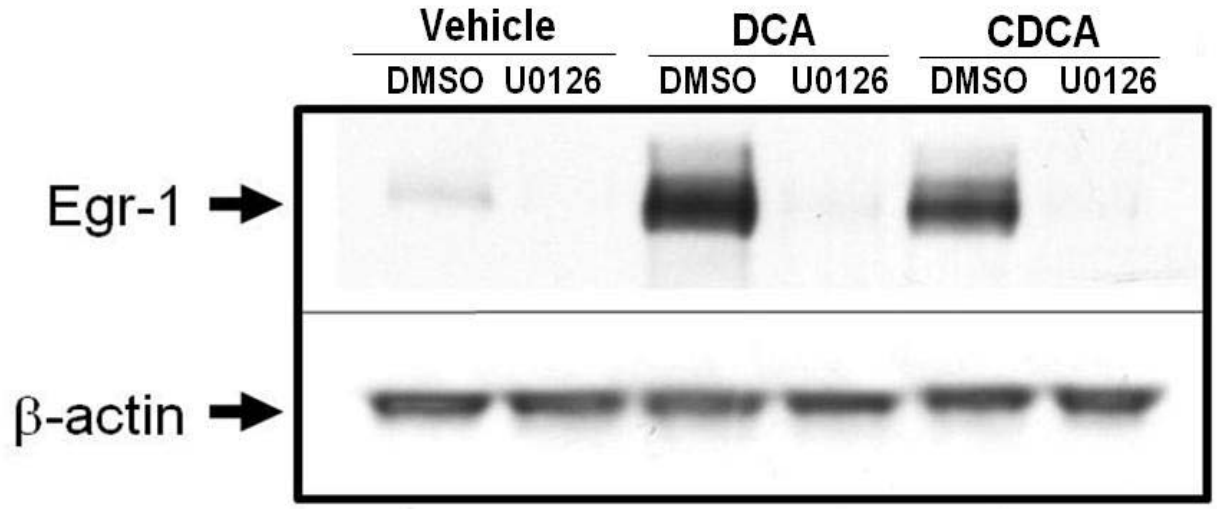

Figure 2.4 Role of MAP Kinase Signaling in Upregulation of Egr-1 Protein by Bile Acids. Primary mouse hepatocytes were treated with 10 μM U0126 followed by either DCA or CDCA 30 minutes later. The cells were incubated for 2 hours and total protein was isolated. Egr-1 protein levels were visualized by western blot. The blot was then reprobed to visualize ß-actin.

Figure 2.5

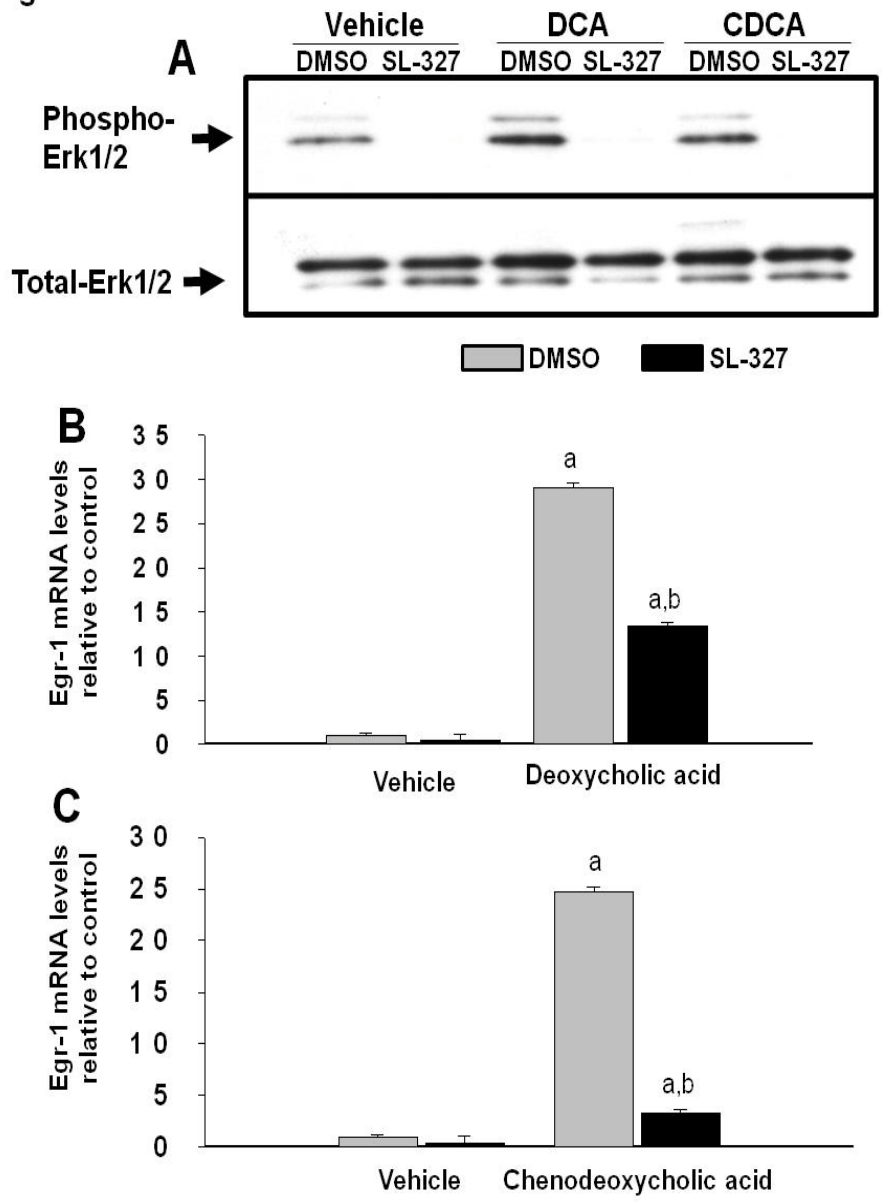

Figure 2.5 Role of MAP Kinase Signaling in Upregulation of Egr-1 mRNA by Bile Acids.
Primary mouse hepatocytes were treated with 20 µM SL-327 followed by either 200 µM DCA or 200 µM CDCA 30 minutes later. The cells were incubated for 2 hours and total mRNA or total protein was isolated. Phospho-Erk and total-Erk protein (A) levels were visualized by western blot the blot. Egr-1 mRNA (B and C) was quantified by qPCR. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from control treated hepatocytes. [b]Significantly different ($p < 0.05$) from hepatocytes treated with SL-327 and bile acid.

Figure 2.6

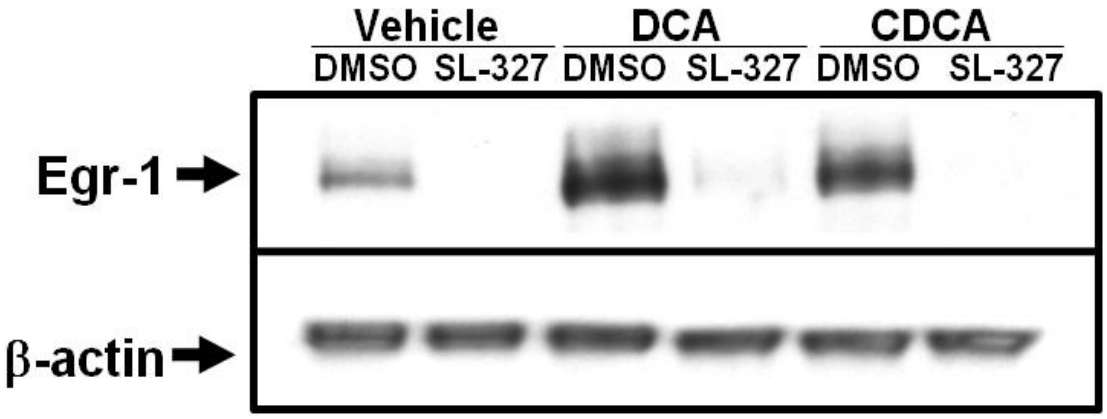

Figure 2.6 Role of MAP Kinase Signaling in Upregulation of Egr-1 Protein by Bile Acids**.** Primary mouse hepatocytes were treated with 20 µM SL-327 followed by either DCA or CDCA 30 minutes later. The cells were incubated for 2 hours and total protein was isolated. Egr-1 protein levels were visualized by western blot. The blot was then reprobed to visualize ß-actin.

Figure 2.7

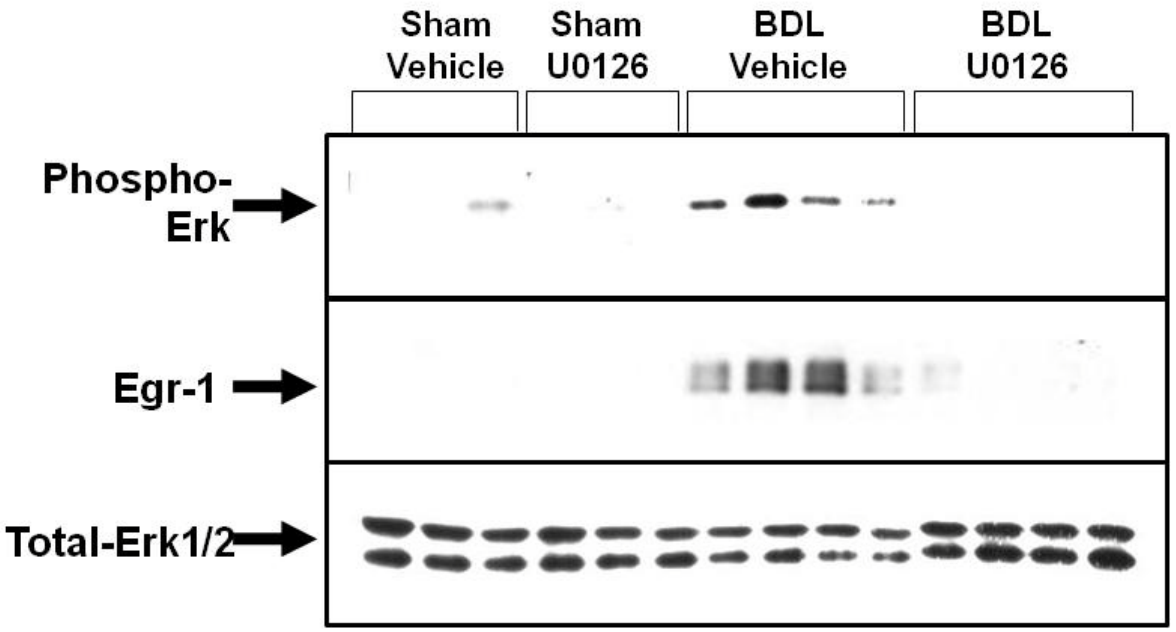

Figure 2.7 U0126 prevents upregulation of Egr-1 in the liver after BDL. Mice were treated with 100 mg/kg U0126 or vehicle. One hour later, the mice were subjected to BDL or sham operation. After 6 hours, phosphorylated Erk1/2, total Erk1/2 and Egr-1 protein were detected in the liver by western blot. n=3 for sham-operated mice and n=4 for BDL mice.

CHAPTER 3

Bile Acids Induce Inflammatory Genes in Hepatocytes: a Novel Mechanism of Inflammation during Obstructive Cholestasis

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American Journal of Pathology. 2011Jan;178(1):175-86.

3.1 Abstract

Inflammation contributes to liver injury during cholestasis. The mechanism by which cholestasis initiates an inflammatory response in the liver, however, is not known. Two hypotheses were investigated in the current studies. First, activation of toll-like receptor 4 (TLR4) by either bacterial lipopolysaccharide (LPS) or damage-associated molecular pattern molecules (DAMPs) released from dead hepatocytes triggers an inflammatory response. Second, bile acids act as inflammagens, and directly activate signaling pathways in hepatocytes that stimulate production of proinflammatory mediators. Liver inflammation was not affected in LPS-resistant C3H/HeJ mice after bile duct ligation (BDL), indicating that TLR4 is not required for initiation of inflammation. Treatment of hepatocytes with bile acids did not directly cause cell toxicity but increased expression of numerous proinflammatory mediators including cytokines, chemokines, adhesion molecules, and other proteins that influence immune cell levels and function. Upregulation of several of these genes in hepatocytes and in the liver after BDL required early growth response factor-1 (Egr-1), but not farnesoid X receptor (FXR). In addition, Egr-1 was upregulated in the livers of patients with cholestasis, and correlated with levels of inflammatory mediators. These data demonstrate that TLR4 is not required for the initiation of acute inflammation during cholestasis. In contrast, bile acids directly activate a signaling network in hepatocytes that promotes hepatic inflammation during cholestasis.

3.2 Introduction

Cholestatic liver disease develops when bile flow from the liver is interrupted (Qureshi, 1999; Li and Crawford, 2004). This can occur during autoimmune reactions, congenital disorders, pregnancy, drug hepatotoxicities and other forms of liver diseases (Li and Crawford, 2004). Cholestasis causes concentrations of bile acids to rapidly increase in liver and plasma (Lindblad et al., 1977; Setchell et al., 1997). As this disease progresses, hepatic inflammation and hepatocyte injury ultimately develop (Gujral et al., 2003). If cholestasis is not corrected and

hepatocyte injury persists, portal myofibroblasts and hepatic stellate cells are stimulated to proliferate and produce extracellular matrix, a process that ultimately results in the development of biliary fibrosis and eventually cirrhosis (Ramadori and Saile, 2004).

A major consequence of acute cholestasis, especially obstructive cholestasis, is the development of severe liver injury (Patel and Gores, 1995). It is generally assumed that exposure of hepatocytes to high concentrations of potentially toxic bile acids is mainly responsible for cholestatic liver injury (Guicciardi and Gores, 2002). In support of this, several studies have demonstrated that high concentrations of certain bile acids produce hepatocyte cell death *in vitro* through activation of proapoptotic pathways (Faubion et al., 1999; Graf et al., 2002; Reinehr et al., 2003). In these studies, deoxycholic acid (DCA), lithocholic acid (LCA), and their metabolites are frequently used (Rodrigues et al., 1998; Graf et al., 2002; Yang et al., 2009). One caveat of these studies, however, is that plasma and liver concentrations of secondary bile acids, such as LCA and DCA, do not dramatically increase in humans with cholestasis or in animal models of cholestasis (Wagner et al., 2003; Burkard et al., 2005; Marschall et al., 2006), because these bile acids are formed in the intestine from primary bile acids, and intestinal concentrations of primary bile acids decrease during cholestasis due to diminished biliary excretion (Wagner et al., 2003; Marschall et al., 2006). On the other hand, serum levels of the most abundant primary bile acids, e.g., cholic acid (CA) and muricholic acid (MCA), increase to 200-250 µM in mice subjected to BDL for 7 days (Marschall et al., 2006). In contrast to DCA and LCA, however, these bile acids are relatively nontoxic to hepatocytes in culture (Sokol et al., 1995). Accordingly, these data raise the question as to whether direct bile acid toxicity can be entirely or even partially responsible for liver injury observed during obstructive cholestasis(Copple et al., 2010).

Recent studies suggest that inflammatory cells such as neutrophils are activated and recruited into the liver during obstructive cholestasis and cause significant liver injury in a

murine model of bile duct ligation (BDL) (Gujral et al., 2003). This inflammatory response correlated with substantial formation of pro-inflammatory cytokines and expression of intercellular adhesion molecule-1 (ICAM-1) (Gujral et al., 2004a). Because liver injury in mice deficient in CD18 or ICAM-1 was almost eliminated, it was concluded that infiltrating neutrophils and not bile acids were responsible for hepatocellular injury (Gujral et al., 2003; Gujral et al., 2004a). In support of these animal studies, serum levels of the neutrophil chemokine, interleukin-8, are increased in patients with cholestatic liver disease (Yamashiki et al., 1998; Neuman et al., 2002). Furthermore, ICAM-1 is upregulated and neutrophils are present in the livers of these patients (Gulubova, 1998). Thus, both animal and human studies support the hypothesis that an inflammatory response during obstructive cholestasis plays a critical role in the injury process. What remains unclear, however, is the molecular mechanism by which cholestasis initiates this inflammatory response in the liver. Two hypotheses were investigated in the current studies to explain this mechanism. First, activation of toll-like receptor 4 (TLR4) by either bacterial lipopolysaccharide (LPS) released from the gut or damage-associated molecular pattern molecules (DAMPs) released from dead hepatocytes, exposed to pathological concentrations of bile acids, triggers an inflammatory response. Second, bile acids act as inflammagens, and directly activate signaling pathways in hepatocytes that regulate production of proinflammatory mediators that stimulate recruitment of neutrophils into the liver. A better understanding of the initiating events of the inflammatory response may uncover clinically relevant therapeutic strategies against cholestatic liver injury and even fibrosis without affecting the vital host defense functions of neutrophils.

3.3 **Materials and Methods**

3.3.1 Animals Care.. Male, C57BL/6 (Harlan, Indianapolis, IN), C57BL/6NTac (Taconic, Germantown, NY), Egr-1 knockout mice (B6.129-*Egr1*^{tm1Jmi} N12, Taconic), C3H/HeJ (Jackson Laboratories, Bar Harbor, ME), and C3Heb/FeJ (Jackson Laboratories) ranging from 8-10

weeks of age were used for these studies. FXR knockout mice have been described previously and were provided by Dr. Grace Guo, Ph.D., University of Kansas Medical Center (Sinal et al., 2000). All animals were maintained on a 12-h light/dark cycle under controlled temperature (18-21ºC) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health.

3.3.2 Hepatocyte Isolation. Hepatocytes were isolated from the livers of mice by collagenase perfusion as described in detail by us previously (Kim et al., 2006). The hepatocytes were cultured in Williams' medium E containing 10% FBS and Penicillin-Streptomycin. Hepatocytes were treated with bile acids within 16 hours of plating. For caspase inhibition studies, cells were incubated with the pan-caspase inhibitor, quinoline-val-asp-difluorophenoxymethylketone (QVD) (MP Biomedicals, Santa Ana, CA), for 30 minutes prior to bile acid treatment.

3.3.3 Bile Duct Ligation. Mice were subjected to BDL as described previously (Kim et al., 2006).

3.3.4 Human Liver Samples. Research involving human livers was reviewed by the University of Kansas Medical Center Human Research Protection Program. The specimens were provided by the KU Liver Center Tissue Bank. Diseased liver tissue utilized for these studies was collected from patients with primary biliary cirrhosis (4 Females, age 59, 56, 60, 61; 1 Male, age 47) and primary sclerosing cholangitis (3 Males, age 44, 45, 57). Liver samples without histological evidence of cholestasis, fibrosis, or severe inflammation were selected as control tissues (5 Females, age 41, 48, 56, 52, 66; 5 Males, age 57, 45, 57, 58, 48).

3.3.5 Measurement of Hepatocyte Viability. Cells were plated and treated as described above. Cell death was evaluated by measuring the release of alanine

aminotransferase (ALT) into the medium, as an indicator of necrotic cell death as described previously (Copple et al., 2009). Cleavage of AC-DEVD-AMC was measured to quantify caspase 3 activity. Briefly, cells were lysed with cell lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 0.1mM EDTA, 1 mM DTT). The lysate was centrifuged at10,000 g for 10 minutes at 4 degrees C. The supernatant was collected and incubated with 200 µM of AC-DEVD-AMC substrate (BD Biosciences San Diego, CA) in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 0.1mM EDTA, 10% glycerol, 10 mM DTT). Cleavage of the AC-DEVD-AMC substrate was measured kinetically by liberation of AMC using a Synergy 2 plate reader (BioTek, Winooski, VT) (380 nm excitation and 430-460 nm emission wavelengths).

3.3.6 Real-time Polymerase Chain Reaction (PCR). RNA was isolated using TRI reagent (Sigma Chemical Company, St. Louis, MO), and reverse transcribed into cDNA as described by us previously (Kim et al., 2006). Real-time PCR was performed on an Applied Biosystems 7900 Real-time PCR Instrument (Applied Biosystems, Foster City, CA) with the SYBR green DNA PCR kit (Applied Biosystems). Sequences of primers are shown in Table 3.1. A customized TaqMan qPCR array (Applied Biosystems Carlsbad, CA) was used to measure mRNA levels of the genes listed in Table 3.2 PCR was performed as per manufacturer's recommendations.

3.3.7 Immunohistochemistry. Sections of frozen liver were incubated with rat anti-mouse vascular cell adhesion molecule-1 (VCAM-1) antibody diluted 1:50 (Millipore, Temecula, CA). The sections were washed and incubated with goat anti-rat conjugated to Alexa 594

3.3.8 Protein Quantification. Hepatocytes were lysed in RIPA buffer containing Complete protease inhibitor cocktail (Roche Applied Science Indianapolis, IN) and PhosSTOP phosphatase inhibitor (Roche Applied Science Indianapolis, IN). Equal amounts of protein were separated on a 7.5% or 4.5-15% gradient Criterion polyacrylamide gel (Bio Rad Hercule, CA) and transferred to a PVDF membrane (Immoblion-P Bedford, MA). The membrane was

incubated with anti-ICAM-1 antibody (R&D Systems Minneapolis, MN), anti-Egr-1 antibody (Cell Signaling Technology, Inc.) or anti-α-actin antibody (Sigma Chemical Co., St. Louis, MO) followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Santa Cruz, CA). The bands were visualized using the ECL detection kit (Bio Rad Hercules, CA). Macrophage inflammatory protein-2 (MIP-2) was measured in cell culture medium using the Bio Plex assay (Bio Rad Hercules, CA) as per manufacturer's instructions.

3.3.9 Quantification of Liver Injury in BDL Mice. ALT was measured in serum using a commercially available kit as described previously (Kim et al., 2006).

3.3.10 Quantification of Neutrophil Accumulation and Extravasation. Neutrophil accumulation and extravasation were quantified as described previously (Gujral et al., 2003).

3.3.11 Statistical Analysis. Results are presented as the mean $\pm$ SEM. Data were analyzed by Analysis of Variance (ANOVA) or t test. ANOVAs were performed on log X-transformed data in instances in which variances were not homogenous. Comparisons among group means were made using the Student-Newman-Keuls test. The Spearman Correlation was performed to identify correlations between Egr-1 mRNA levels and mRNA levels of other inflammatory mediators in human livers. The criterion for significance was $p < 0.05$ for all studies.

3.4 RESULTS

3.4.1 Neutrophil Accumulation and Extravasation in the Livers of BDL Mice Occurs Independently of TLR4 Signaling. To determine whether TLR4 is required for production of inflammation in the liver during cholestasis, C3H/HeJ mice, which have an inactivating mutation in the TLR4 gene (Poltorak et al., 1998), and C3Heb/FeJ mice, TLR4 wild-type, were subjected

to BDL. Plasma ALT activity and % area of liver necrosis after BDL were unaffected in C3H/HeJ mice, consistent with previous studies (Figure 3.1) (Seki et al., 2007). Similarly, total numbers of neutrophils in the liver and numbers of extravasated neutrophils after BDL were unaffected in C3H/HeJ mice (Figure 3.1). These results suggest that LPS does not initiate inflammation through activation of TLR4 during cholestasis.

3.4.2 Bile Acids Increase Expression of ICAM-1 and MIP-2 in Hepatocytes. Bile acids activate several signaling pathways in hepatocytes (Allen et al.; Qiao et al., 2001; Dent et al., 2005). Accordingly, whether bile acids increase expression of proinflammatory mediators in hepatocytes was determined. For this study, hepatocytes were exposed to DCA, CDCA, and TCA. DCA was chosen because several studies have shown that DCA activates signal transduction pathways in hepatocytes (Allen et al.; Qiao et al., 2001; Dent et al., 2005). CDCA was chosen because serum levels of CDCA increase in BDL mice (Marschall et al., 2006). TCA was chosen because serum levels of CA increase in BDL mice (Marschall et al., 2006), and we recently determined that greater than 99% of cholic acid is conjugated with taurine in mice (Copple, Jaeschke, and Klaassen, unpublished data). Exposure of primary mouse hepatocytes to DCA, CDCA, or TCA increased mRNA levels of ICAM-1 and macrophage inflammatory protein-2 (MIP-2), a neutrophil chemokine involved in cholestatic liver injury (Figures 3.2 A and 3.2 B) (Wintermeyer et al., 2009).

3.4.3 Bile Acids Increase Expression of ICAM-1 and MIP-2 in Hepatocytes Independent of Effects on Viability. Next we investigated the mechanism by which bile acids increase expression of ICAM-1 and MIP-2 in hepatocytes. Studies have demonstrated that bile acids kill hepatocytes by apoptosis and necrosis *in vitro* (Qiao et al., 2001). Apoptotic bodies and proteins released from necrotic cells have been shown to increase expression of proinflammatory mediators in some cells types (Canbay et al., 2003). Accordingly, we next determined whether bile acids caused apoptosis or necrosis in our studies and determined

whether apoptosis contributed to the increase in ICAM-1 and MIP-2 mRNAs in bile acid-treated hepatocytes. Exposure of hepatocytes to DCA, CDCA, and TCA did not increase caspase 3 activity (Figure 3.3 A), stimulate ALT release (Figure 3.3 B), or cause morphological changes consistent with apoptosis or necrosis (Figures 3.3 C-F). Lastly, treatment of hepatocytes with QVD, a pan-caspase inhibitor, did not prevent upregulation of MIP-2 or ICAM-1 by DCA (Figure 3.3 G and 3.3H).

3.4.4 Farnesoid X Receptor (FXR) is not Required for Upregulation of ICAM-1 and MIP-2 in Bile Duct-Ligated (BDL) Mice. Previous studies demonstrated that FXR regulates ICAM-1 in HepG2 cells (Qin et al., 2005). To determine whether this occurs *in vivo* during cholestasis, wild-type and FXR knockout mice were subjected to BDL. BDL increased ICAM-1 mRNA levels to the same extent in wild-type and FXR knockout mice (3.4A). MIP-2 mRNA levels were increased to a greater extent in FXR knockout mice subjected to BDL when compared to BDL wild-type mice (Figure 3.4B). Hepatic neutrophil numbers were not significantly different between BDL wild-type and FXR knockout mice (Figure 3.4C).

3.4.5 Upregulation of ICAM-1 and MIP-2 in Hepatocytes by Bile Acids Requires Early Growth Response Factor-1 (Egr-1). Next, we investigated whether upregulation of ICAM-1 and MIP-2 required the transcription factor Egr-1. Exposure of primary mouse hepatocytes to DCA, CDCA, or TCA increased Egr-1 mRNA and protein (Figures 3.5 A and 3.5 B). Exposure of hepatocytes, isolated from wild-type mice, to DCA, CDCA, or TCA increased mRNA and protein levels of ICAM-1 and MIP-2 (Figures 3.5 C-F). Upregulation of ICAM-1 and MIP-2 by bile acids was attenuated in hepatocytes isolated from Egr-1 knockout mice (Figures 3.5 C-F).

3.4.6 Bile Acids Increase Expression of Several Proinflammatory Genes in Hepatocytes by Egr-1-dependent and Independent Mechanisms. We next determined

whether inflammatory genes, other than ICAM-1 and MIP-2, are upregulated in bile acid-treated hepatocytes in an Egr-1-dependent manner. The genes that were measured are listed in Table 3.2. The results of these studies are shown in Tables 3.3-3.5. Only those genes listed in Table 3.2 that were increased by bile acid treatment are shown in Tables 3.3-3.5. Treatment of hepatocytes isolated from wild-type mice with DCA increased mRNA levels of several chemokines including, CXCL1, CXCL10, CXCL11, Ccl2, Ccl5, Ccl7, and Ccl20 (Table 3.3). In addition, DCA treatment increased mRNA levels of VCAM-1, plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator receptor (uPAR), and cyclooxygenase-2 (COX-2). Lastly, DCA treatment increased levels of the hematopoietic growth factors granulocyte macrophage colony stimulating factor (GM-CSF or Csf-2) and G-CSF (Csf-3). Of the genes upregulated by DCA, CXCL1, Ccl7, PAI-1 and GM-CSF were Egr-1-dependent (Table 3.3).

Exposure of wild-type hepatocytes to CDCA upregulated all of the same proinflammatory genes as DCA, and in addition, increased levels of CXCL16 and Ccl3 (Table 3.4). Similar to DCA, upregulation of CXCL1, Ccl7, PAI-1, and GM-CSF by CDCA were partially Egr-1 dependent (Table 3.4).

Treatment of wild-type hepatocytes with TCA increased mRNA levels of numerous proinflammatory genes. Interestingly, the profile of mRNA changes was different from the changes that occurred after treatment with the unconjugated bile acids, DCA and CDCA. Treatment of hepatocytes with TCA increased mRNA levels of several chemokines, including CXCL1, CXCL 5, CXCL10, CXCL11, CXCL13, CXCL16, Ccl2, Ccl3, Ccl4, Ccl5, Ccl7, Ccl20 and several cytokines, including IL-1β and IL-10 (Table 3.5). In addition to these genes, VCAM-1, COX-2, GM-CSF, and G-CSF were increased in hepatocytes after TCA treatment. The increase in Ccl2, Ccl7, Ccl20, IL-1β, VCAM-1, and GM-CSF mRNAs were partially Egr-1-dependent, whereas changes in CXCL1 and CXCL13 were completely Egr-1-dependent (Table 3.5).

3.4.7 Upregulation of PAI-1, VCAM-1, Ccl7, and Snail in the Livers of BDL Mice Requires Egr-1

Requires Egr-1. Next, we determined whether inflammatory genes that were upregulated in bile acid-treated hepatocytes in an Egr-1-dependent manner (Tables 3.3-3.5), were also increased in BDL mice in an Egr-1-dependent manner. BDL in Egr-1 knockout mice for 10 days resulted in decreased areas of bile infarcts and reduced ALT activity, confirming our previous studies at 14 days after BDL (data not shown) (Kim et al., 2006). BDL increased expression of PAI-1, VCAM-1, and Ccl7 mRNAs to a greater extent in the livers of wild-type mice when compared to Egr-1 knockout mice (Figure 3.6 A-C).

Levels of Snail are increased in bile acid-treated hepatocellular carcinoma cells (Fukase et al., 2008) and in the liver after BDL (Zhong et al., 2009). Studies have demonstrated that Egr-1 regulates Snail in some cell types (Grotegut et al., 2006). Accordingly, we determined whether upregulation of Snail in the liver after BDL requires Egr-1. mRNA levels of Snail increased after BDL (Figure 3.6 D). Upregulation of Snail was completely prevented in Egr-1 knockout mice (Figure 3.6D).

Minimal immunostaining for VCAM-1 was observed in sham-operated livers (Figure 3.6E). In liver sections from bile duct-ligated mice, VCAM-1 immunostaining was observed on bile duct epithelial cells and within the sinusoids primarily in periportal regions of liver (Figure 3.6 F).

3.4.8 Upregulation of Egr-1, PAI-1, ICAM-1, and IL-8 in Livers of Humans with Cholestatic Liver Disease.

Cholestatic Liver Disease. Human liver was obtained from control donors and patients with primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC). Levels of Egr-1, PAI-1, ICAM-1 and IL-8 mRNAs were increased in livers of patients with cholestasis from these diseases (Figure 3.7 A). In addition, Spearman Correlation indicated a significant correlation

between Egr-1 mRNA levels in these patients and mRNA levels of IL-8, PAI-1, and ICAM-1 (Figures 3.7 B-D).

3.5 **Discussion**

3.5.1 LPS does not promote inflammation in the liver during acute phases of cholestasis.

It has been proposed that bacterial LPS may be an important stimulus for inflammation in the liver during cholestasis (Van Bossuyt et al., 1990; Reynolds et al., 1996). Studies have demonstrated that reduced bile flow increases translocation of bacteria and LPS from the gut which may stimulate proinflammatory gene expression in the liver (Reynolds et al., 1996). In BDL animals, however, upregulation of inflammatory mediators that affect neutrophil accumulation, such as MIP-2 and ICAM-1, and hepatic neutrophil accumulation occur within the first 24 hours, whereas, plasma LPS levels are not increased at this time (Van Bossuyt et al., 1990; Reynolds et al., 1996; Gong et al., 2002; Georgiev et al., 2008; Wintermeyer et al., 2009). Furthermore, Seki and colleagues demonstrated recently that mice deficient in TLR4, the receptor for LPS, and mice deficient in TLR4 on immune cells are not protected from liver injury after BDL, suggesting that gut-derived LPS is not necessary for neutrophil-dependent injury during cholestasis (Seki et al., 2007). Consistent with this, our data demonstrate for the first time that neutrophil accumulation and extravasation in the liver 3 days after BDL is not affected in LPS-resistant C3H/HeJ mice (Figure 3.1). Collectively, these data suggest that activation of macrophages by LPS, and subsequent release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), is not important for the acute phase of inflammation that occurs during cholestasis. Consistent with this, TNF-α mRNA levels are not increased in hepatic macrophages at 24 hours after BDL and depletion of macrophages does not protect from liver injury after BDL (Zhong et al., 2003; Gehring et al., 2006). Collectively, these studies suggest that during early stages of cholestasis, activation of hepatic macrophages by LPS from the gut is not important for inflammatory liver injury.

3.5.2 Bile acid-induced hepatocyte cell death is not a stimulus for inflammation in the liver during cholestasis. Studies have demonstrated that DAMPs released from necrotic cells, such as high-mobility group protein B1, activate macrophages by a TLR4-dependent mechanism which stimulates a sterile inflammatory response (Park et al., 2006b). This mechanism is important for inflammation in the liver after hepatic ischemia-reperfusion (Tsung et al., 2005). Our results suggest, however, that this mechanism is not responsible for initiation of inflammation in the liver during cholestasis. In BDL mice, serum concentrations of CA and MCA reach approximately 200-250 µM by 7 days after BDL (Marschall et al., 2006). We recently determined that greater that 99% of CA in the serum of BDL mice is conjugated with taurine (Copple, Jaeschke, and Klaassen, unpublished data). In the present studies, exposure of hepatocytes to 200 µM TCA, a concentration which occurs in BDL mice (Marschall et al., 2006), did not produce hepatocyte cell death (Figure 3.3). Serum concentrations of CDCA and its conjugates increase to approximately 4 µM in BDL mice (Marschall et al., 2006), and in our studies, concentrations as high as 200 µM did not produce toxicity in cultured hepatocytes (Figure 3.3). Similarly, exposure of hepatocytes to 200 µM DCA did not produce toxicity in our studies (Figure 3.3). Since concentrations of DCA typically decrease during cholestasis (Wagner et al., 2003; Marschall et al., 2006), however, it is unlikely that it contributes to liver toxicity and initiation of inflammation during cholestasis. We also demonstrated that bile acid-dependent upregulation of ICAM-1 and MIP-2 was independent of caspase activation (Figure 3.3). These results are consistent with previous studies in which the pan caspase inhibitor z-Val-Ala-Asp-fluoromethylketone did not prevent inflammation or attenuate liver injury in bile duct-ligated mice (Gujral et al., 2004b). Collectively, these studies demonstrate that exposure of hepatocytes to bile acids, which increase in serum during cholestasis, do not directly cause cell death and therefore, are unlikely to initiate a sterile inflammatory response. Consistent with this, inflammation was not affected in TLR4 mutant mice (Figure 3.1), further supporting that

DAMPs released from dead cells are not responsible for initiating inflammation during cholestasis.

3.5.3 Bile acids stimulate production of inflammatory mediators by hepatocytes.

Our results indicate that LPS from the gut and DAMPs released from necrotic cells do not stimulate production of proinflammatory cytokines during early stages of cholestasis, suggesting that another mediator triggers inflammation during cholestasis. Furthermore, this stimuli elicits inflammatory mediator production from cell types, other than macrophages, since depletion of macrophages does not attenuate injury after BDL (Zhong et al., 2003; Gehring et al., 2006). Hepatocytes are exposed to high concentrations of bile acids during cholestasis (Wagner et al., 2003; Marschall et al., 2006) and bile acids activate numerous signaling pathways in hepatocytes (Allen et al.; Makishima et al., 1999; Dent et al., 2005), therefore we next investigated whether bile acids stimulate production of proinflammatory mediators by hepatocytes. Exposure of hepatocytes to bile acids increased levels of numerous mediators, including cytokines (IL-1β, IL-10), chemokines (KC, IP-10, I-TAC, MCP-1, RANTES, MCP-3, MIP-1α, MIP-1β, MIP-2, MIP-3α, LIX, SR-PSOX, MCP-3, BCL, SR-PSOX), adhesion molecules (ICAM-1, VCAM-1), enzymes in arachidonic acid metabolism (COX-2), and other proteins that influence immune cell levels and function (PAI-1, uPAR, G-CSF, GM-CSF) (Tables 3.3-3.5). The concentration of TCA that increased expression of these proinflammatory mediators was similar to concentrations that occur in the serum of BDL mice.

Several of the mediators that were upregulated by bile acids, including ICAM-1, MIP-2, as well as KC are known to be involved in neutrophil trafficking in liver and have been shown to contribute to liver injury during cholestasis (Gujral et al., 2004a; Wintermeyer et al., 2009). In addition to neutrophils, studies have shown that T cells and B cells infiltrate the liver during cholestasis (Georgiev et al., 2008). Our studies demonstrate that hepatocytes exposed to bile acids may contribute to the chemokine milieu that is responsible for lymphocyte and neutrophil

recruitment into the liver. Although systemic chemokine formation may not always contribute to inflammatory liver injury (Simonet et al., 1994; Dorman et al., 2005), a more selective chemokine formation by hepatocytes establishing a chemotactic gradient towards the parenchyma has been shown to trigger neutrophil extravasation and injury (Maher et al., 1997). Collectively, these results suggest that bile acids at pathologic concentrations are inflammatory mediators, and that hepatocytes may be an important source of cytokines and chemokines during cholestasis after exposure to high concentrations of bile acids. In further support of proinflammatory signaling in hepatocytes during cholestasis, recent studies have shown that hepatocytes stain positive for the chemokines IL-8, Gro-α, and ENA-78 by immunohistochemistry in the livers of patients with cholestasis (Dominguez et al., 2009). In addition, hepatocyte expression of chemokines has been described in animal models of cholestasis (Xu et al., 2004).

3.5.4 Bile acids increase levels of proinflammatory mediators in hepatocytes by Egr-1-dependent and independent mechanisms. Egr-1-dependent signaling has been shown to enhance inflammation in the vasculature, lungs, and liver (Yan et al., 2000; Harja et al., 2004; McMullen et al., 2005; Kim et al., 2006; Pritchard et al., 2007). We demonstrated previously that Egr-1 expression increases after BDL, and upregulation of Egr-1 was dependent upon activation of Erk1/2 signaling and occurred independently of FXR activation (Kim et al., 2006; Allen et al., 2009). We recently demonstrated that Egr-1 knockout mice subjected to BDL had reduced inflammation and liver injury, suggesting a potential role for Egr-1 in regulation of proinflammatory mediator production during cholestasis (Kim et al., 2006). These studies did not, however, identify the stimulus for upregulation of Egr-1 nor did they determine the impact of Egr-1 signaling on expression of proinflammatory mediators in hepatocytes. Accordingly, we determined whether bile acids increase cytokine and chemokine expression in hepatocytes by Egr-1-dependent mechanisms. Of the genes that increased in wild-type hepatocytes after

exposure to bile acids, ICAM-1, MIP-2, CXCL1, CXCL13, Ccl2 , Ccl7, IL-1β, PAI-1, G-CSF, GM-CSF, and VCAM-1 (Tables 3.3-3.5) were increased in an Egr-1-dependent manner. In addition, we demonstrated that Ccl7, PAI-1, VCAM-1, ICAM-1, and MIP-2 were increased in BDL mice in an Egr-1-dependent manner consistent with the *in vitro* studies (Figure 3.6) (Kim et al., 2006). VCAM-1 protein levels were primarily increased in periportal regions, where intrahepatic bile acid concentrations would expected to be highest (Figure 3.6F). This is consistent with results in humans with cholestatic liver disease where VCAM-1 levels were similarly increased in periportal regions of liver (Yasoshima et al., 1995; Medina et al., 2005).

In addition to mice, our results also demonstrated for the first time that Egr-1 expression is increased in livers of humans with cholestasis, and that levels of Egr-1 directly correlate with expression of ICAM-1, IL-8 (i.e., human homologue of MIP-2 and KC), and PAI-1 (Figure 3.7). These results suggest that Egr-1 may directly regulate expression of these genes in livers of humans with cholestasis similar to BDL mice. Furthermore, these studies suggest that bile acids may be the stimulus for upregulation of Egr-1 in the liver during cholestasis.

Recent studies indicated that the bile acid nuclear receptor, FXR, regulates ICAM-1 in HepG2 cells (Qin et al., 2005). Our results indicate, however, that FXR is not important for upregulation of ICAM-1 in the liver during cholestasis, nor is it important for hepatic neutrophil accumulation (Figure 3.4). In contrast, Egr-1 is required for upregulation of ICAM-1 in the liver during cholestasis, which is consistent with our *in vitro* studies (Figure 3.5) (Kim et al., 2006). Although these results indicate that FXR is not required for inflammation, results from FXR knockout mice need to be interpreted cautiously. FXR knockouts have higher bile acid concentrations and reduced bile duct proliferation after BDL (Sinal et al., 2000; Wagner et al., 2003). In addition, hepatocyte necrosis in FXR knockout mice after BDL is pathologically distinct from wild-type mice (i.e., less bile infarcts) (Sinal et al., 2000; Wagner et al., 2003).

Many of the genes increased in bile acid-treated hepatocytes occurred independently of Egr-1, suggesting that other signaling pathways are involved. Bile acids have been shown to activate a multitude of signaling pathways in hepatocytes. For example, MAPKs are activated in bile acid-treated hepatocytes and in livers of BDL mice (Allen et al., 2009). In addition, bile acids activate various isoforms of the protein kinase C family, p38, JNK (c-Jun NH2-terminal Kinase), and PXR (pregnane X receptor) (Rao et al., 1997; Makishima et al., 1999; Gupta et al., 2001; Kurz et al., 2001; Staudinger et al., 2001). It is possible that bile acids stimulate upregulation of the proinflammatory genes by activation of one or more of these pathways. In support of this, studies have shown that activation of JNK signaling can upregulate CXCL10 and RANTES in some cell types, and p38 signaling can upregulate MIP-1α and COX-2 in some cell types (Hiura et al., 1999; Adachi et al., 2000; Park et al., 2006a). Therefore, it seems possible that the collective upregulation of these proinflammatory genes by bile acids may require activation of several signaling pathways. However, further studies are needed to specifically identify which signaling pathways are responsible for upregulation of each mediator. Although NF-κB is an important regulator of many of these inflammatory mediators, it is unlikely that NF-κB contributes to upregulation of inflammatory mediators in hepatocytes by bile acids, because studies have shown that bile acids do not activate NF-κB in primary hepatocytes (Schoemaker et al., 2003).

In summary, our studies have identified a novel mechanism of inflammation in the liver that occurs independently of LPS and TLR4. Furthermore, these studies have identified bile acids as inflammagens that stimulate production of proinflammatory mediators by hepatocytes through Egr-1-dependent and independent mechanisms that promote neutrophil accumulation, extravasation, and activation (Figure 3.8). In addition, bile acids stimulate production of cytokines that may promote accumulation of other immune cells, such as T cells that could potentially influence liver pathology during cholestasis. Whereas this signaling network has

likely evolved to facilitate repair of the liver during acute episodes of cholestasis, during chronic cholestasis, persistent activation of this inflammatory pathway by bile acids promotes injury. Further identification of the signaling pathways that contribute to activation of this novel inflammatory signaling network in hepatocytes could be useful for the development of drugs that limit inflammation in the liver during cholestasis without affecting vital host defense function of the innate immune response.

3.7 Figures

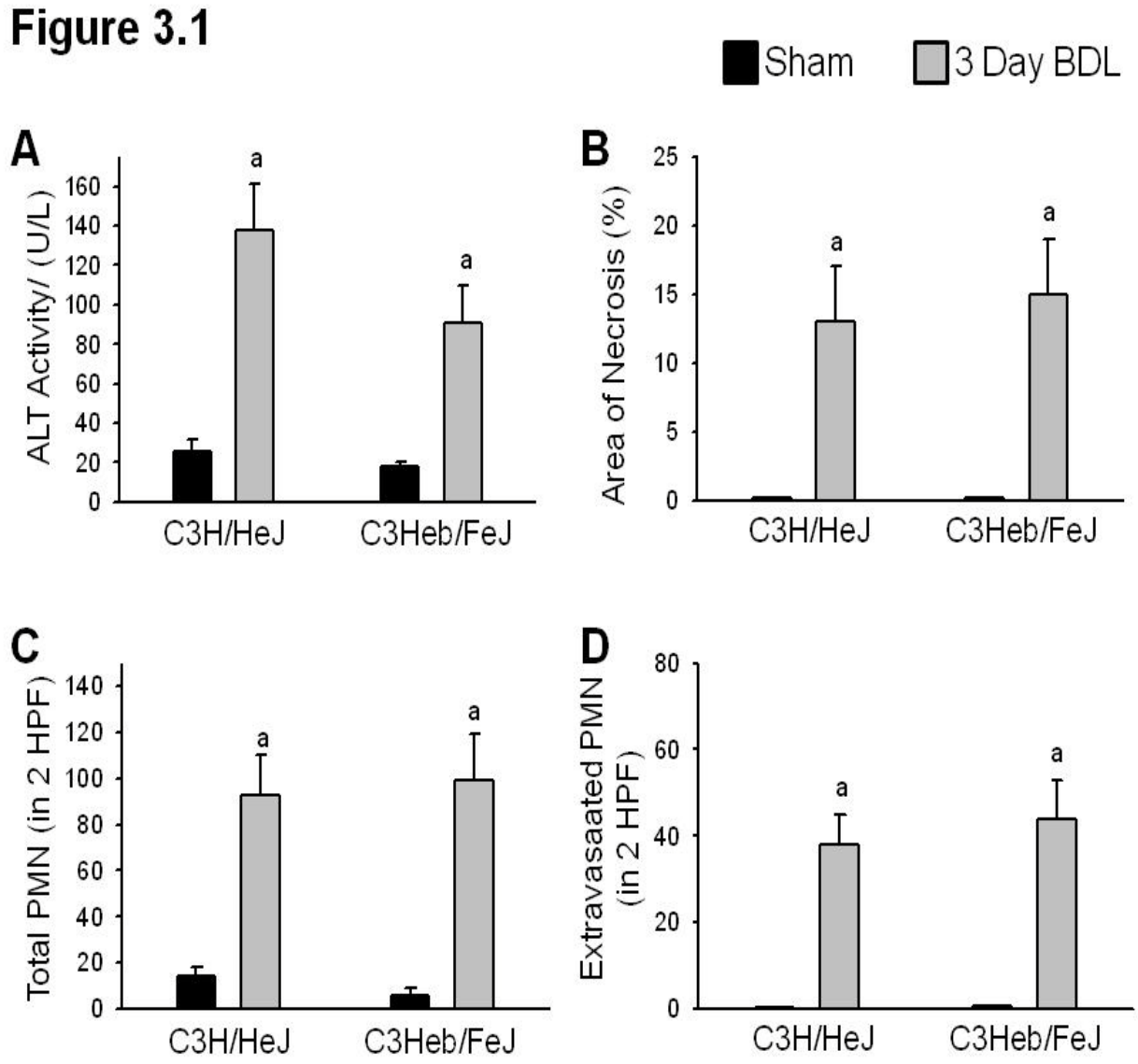

Figure 3.1 Role of TLR 4 in inflammation in the liver during cholestasis. C3H/HeJ (TLR 4 mutant) and C3Heb/FeJ (TLR 4 wild-type) mice were subjected to BDL or sham operation. Three days later, (A) plasma ALT, (B) % area of necrosis, (C) neutrophil (PMN) accumulation and (D) PMN extravasation were measured. [a]Significantly different ($p < 0.05$) from sham-operated mice.

Figure 3.2

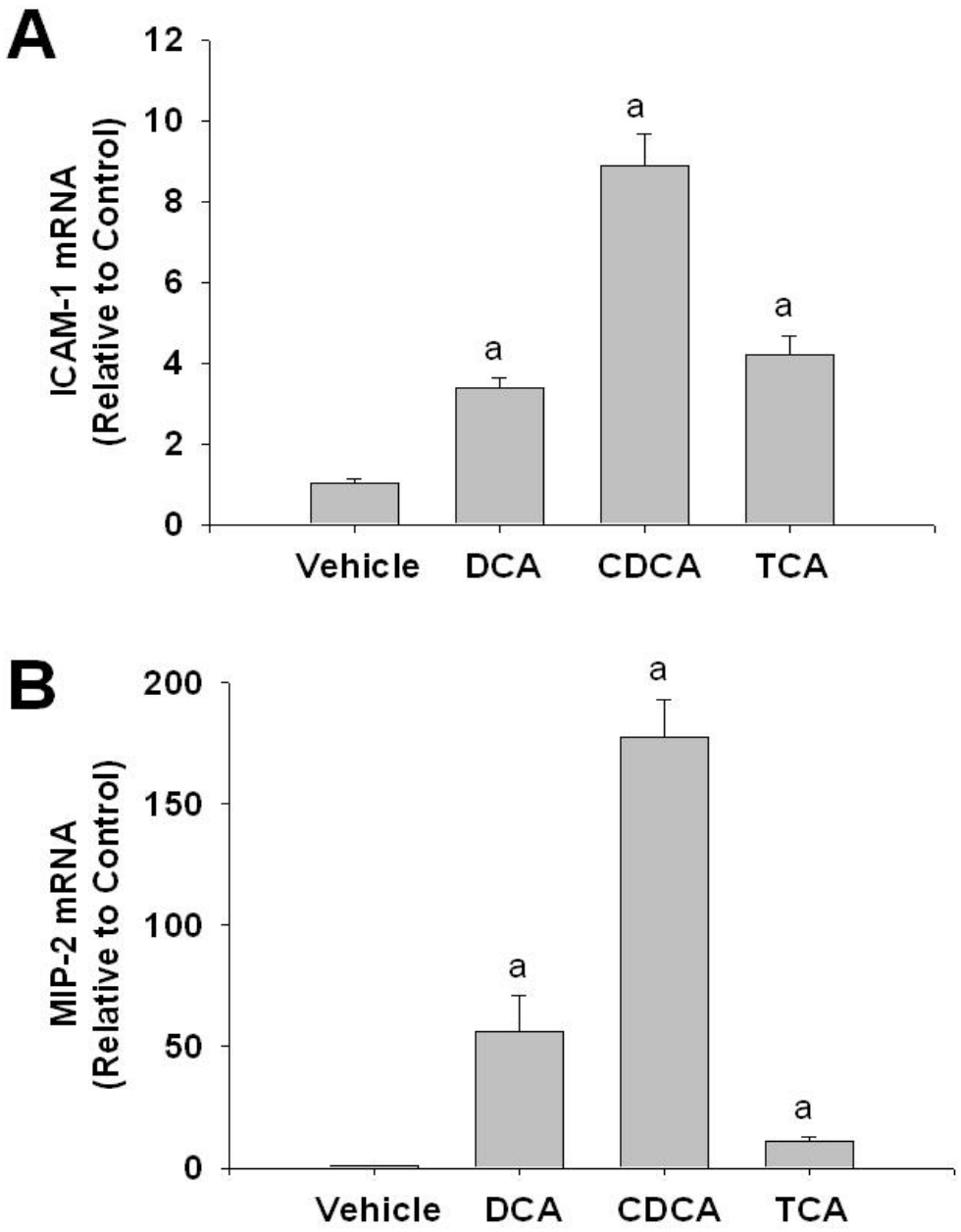

Figure 3.2 Upregulation of ICAM-1 and MIP-2 in bile acid-treated hepatocytes. Hepatocytes were isolated from mice and treated with 200 µM DCA, CDCA, or TCA. Six hours later, (A) ICAM-1 and (B) MIP-2 mRNA levels were quantified by real-time PCR. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from vehicle-treated hepatocytes.

Figure 3.3

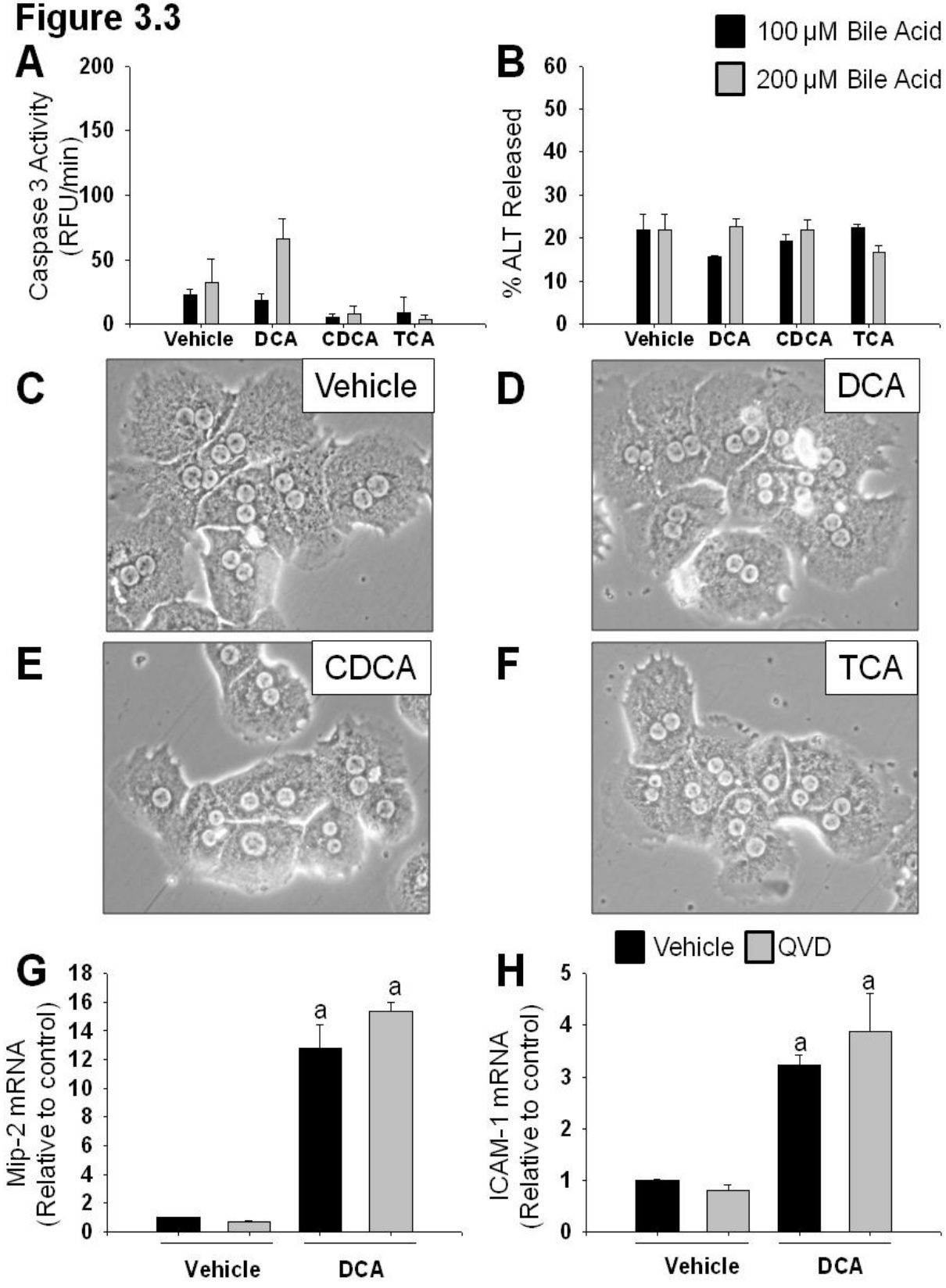
A
Caspase 3 Activity (RFU/min)
200
150
100
50
0
Vehicle
DCA
CDCA
TCA
B
100 µM Bile Acid
200 µM Bile Acid
% ALT Released
60
50
40
30
20
10
0
Vehicle
DCA
CDCA
TCA
C
Vehicle
D
DCA
E
CDCA
F
TCA
G
Mip-2 mRNA (Relative to control)
18
16
14
12
10
8
6
4
2
0
a
a
Vehicle
DCA
H
Vehicle
QVD
ICAM-1 mRNA (Relative to control)
5
4
3
2
1
0
a
a
Vehicle
DCA

Figure 3.3 Bile acids do not affect the viability of hepatocytes. Hepatocytes were isolated from mice and treated with 200 µM DCA, CDCA, or TCA. Six hours later, (A) caspase 3 and (B) ALT activities were measured. Data are expressed as mean +/- SEM; n=3. Photomicrographs of cells treated with (A) vehicle, (B) DCA, (C) CDCA, or (D) TCA were taken six hours after treatment. Primary hepatocytes were isolated and pretreated with QVD or DMSO vehicle followed by treatment with DCA. (G) ICAM-1 and (H) MIP-2 mRNA levels were measured 6 hours later. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from vehicle-treated hepatocytes.

Figure 3. 4

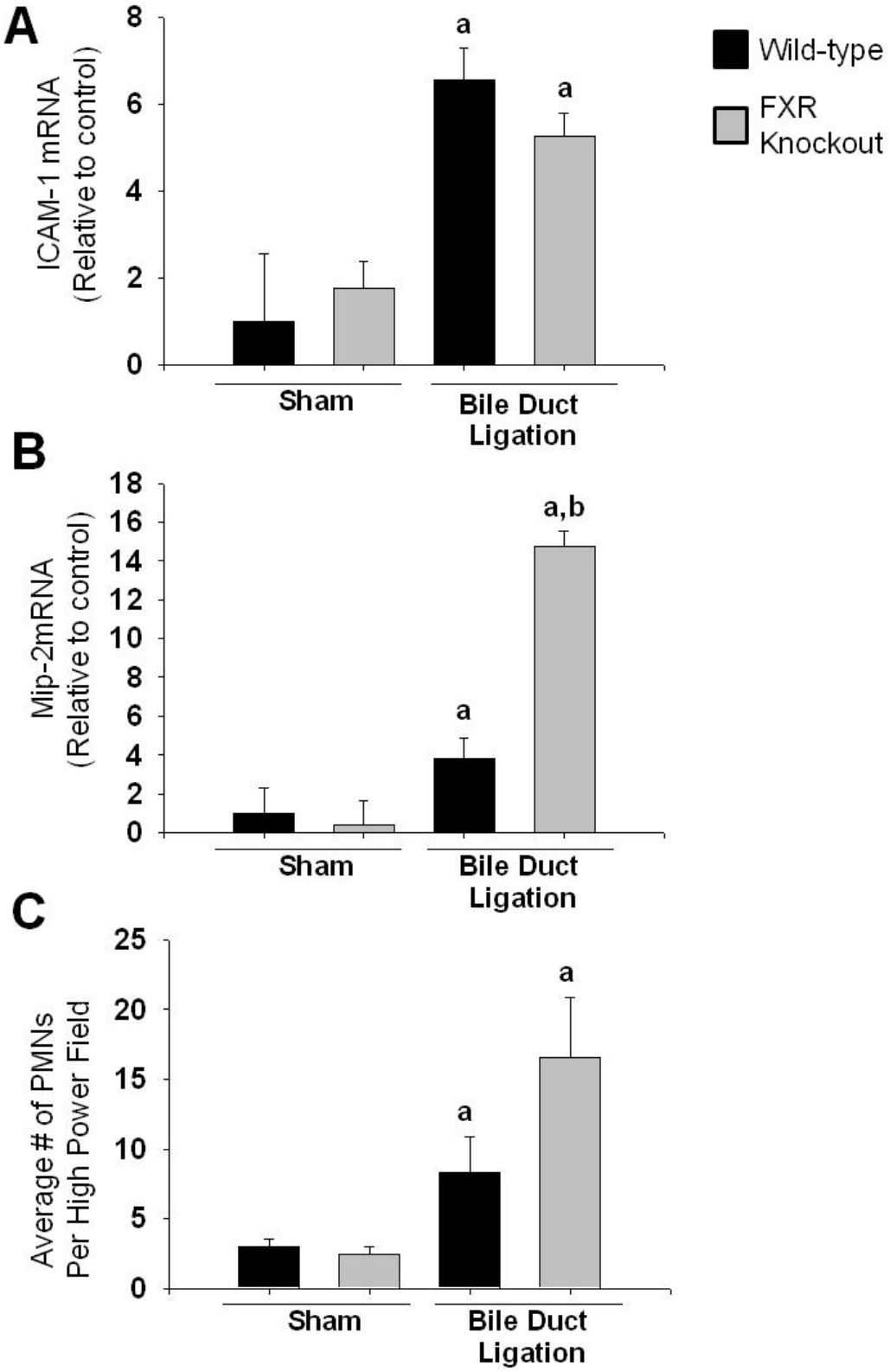
A
ICAM-1 mRNA
(Relative to control)
8
6
4
2
0
a
a
Sham
Bile Duct
Ligation
Wild-type
FXR
Knockout
B
Mip-2mRNA
(Relative to control)
18
16
14
12
10
8
6
4
2
0
a,b
a
Sham
Bile Duct
Ligation
C
Average # of PMNs
Per High Power Field
25
20
15
10
5
0
a
a
Sham
Bile Duct
Ligation

Figure 3.4 Role of FXR in inflammation in the liver during cholestasis. Wild-type and FXR knockout mice were subjected to BDL or sham operation. Three days later, mRNA levels of (A) ICAM-1 and (B) MIP-2 were quantified and (C) hepatic neutrophil accumulation was measured. [a]Significantly different ($p < 0.05$) from sham-operated mice. [b]Significantly different ($p < 0.05$) from wild-type mice subjected to BDL

Figure 3.5

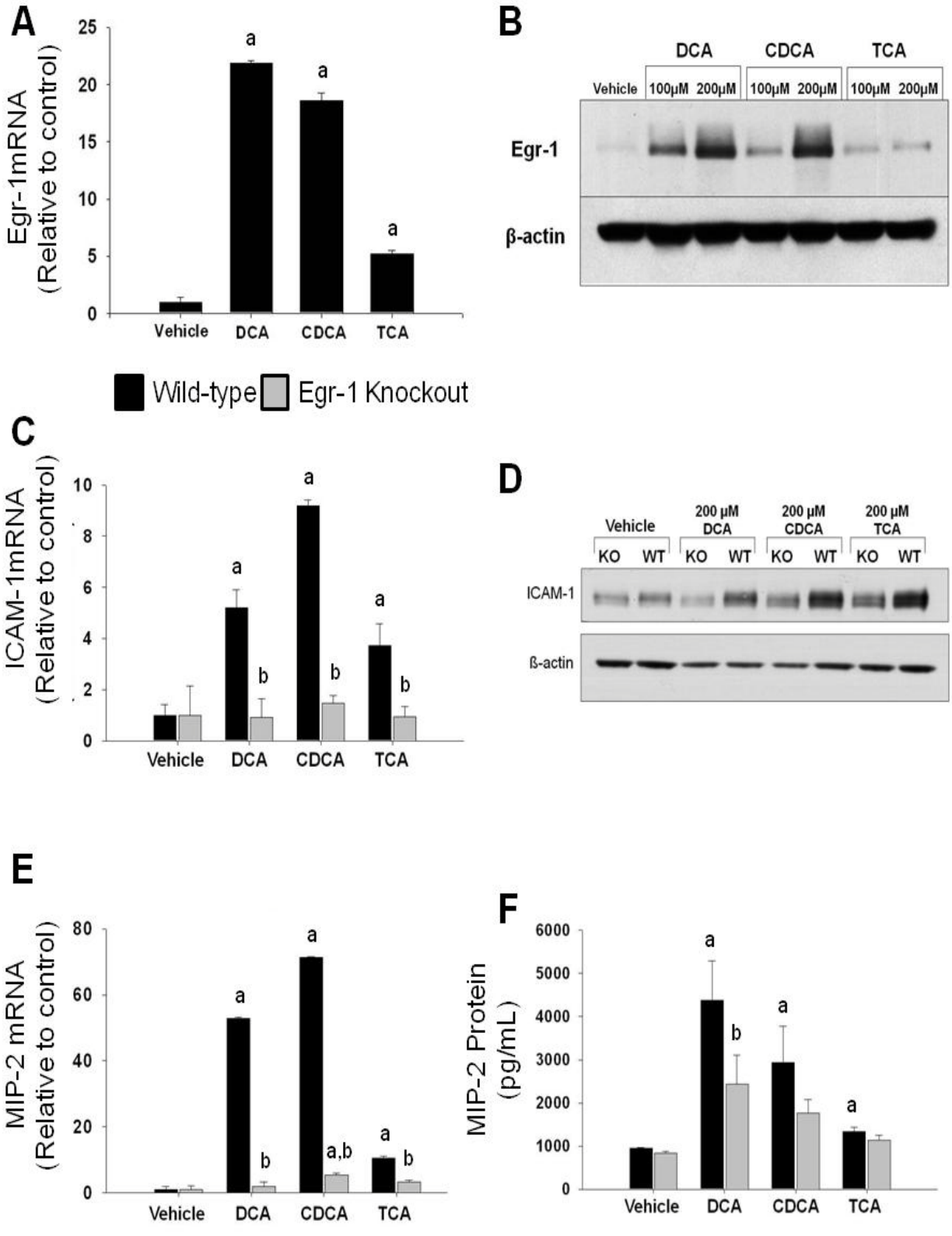
A
Egr-1mRNA (Relative to control)
25
20
15
10
5
0
a
a
a
Vehicle
DCA
CDCA
TCA
Wild-type
Egr-1 Knockout
B
DCA
CDCA
TCA
Vehicle
100μM 200μM
100μM 200μM
100μM 200μM
Egr-1
β-actin
C
ICAM-1mRNA (Relative to control)
10
8
6
4
2
0
a
b
a
b
a
b
Vehicle
DCA
CDCA
TCA
D
Vehicle
200 μM DCA
200 μM CDCA
200 μM TCA
KO WT KO WT KO WT KO WT
ICAM-1
ß-actin
E
MIP-2 mRNA (Relative to control)
80
60
40
20
0
a
b
a
a,b
a
b
Vehicle
DCA
CDCA
TCA
F
MIP-2 Protein (pg/mL)
6000
5000
4000
3000
2000
1000
0
a
b
a
a
Vehicle
DCA
CDCA
TCA

Figure 3.5 Role of Egr-1 in upregulation of ICAM-1 and MIP-2 in hepatocytes. Hepatocytes were isolated from mice and treated with 200 µM DCA, CDCA, or TCA. Two hours later Egr-1 (A) mRNA and (B) protein were measured. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from vehicle-treated hepatocytes. Hepatocytes were isolated from wild-type or Egr-1 knockout mice. Six hours later, (C) ICAM-1 and (E) MIP-2 mRNAs were measured. Eight hours later, (D) ICAM-1 and (F) MIP-2 protein levels were measured. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from vehicle-treated hepatocytes. [b]Significantly different ($p < 0.05$) from wild-type hepatocytes treated with bile acids.

Figure 3.6

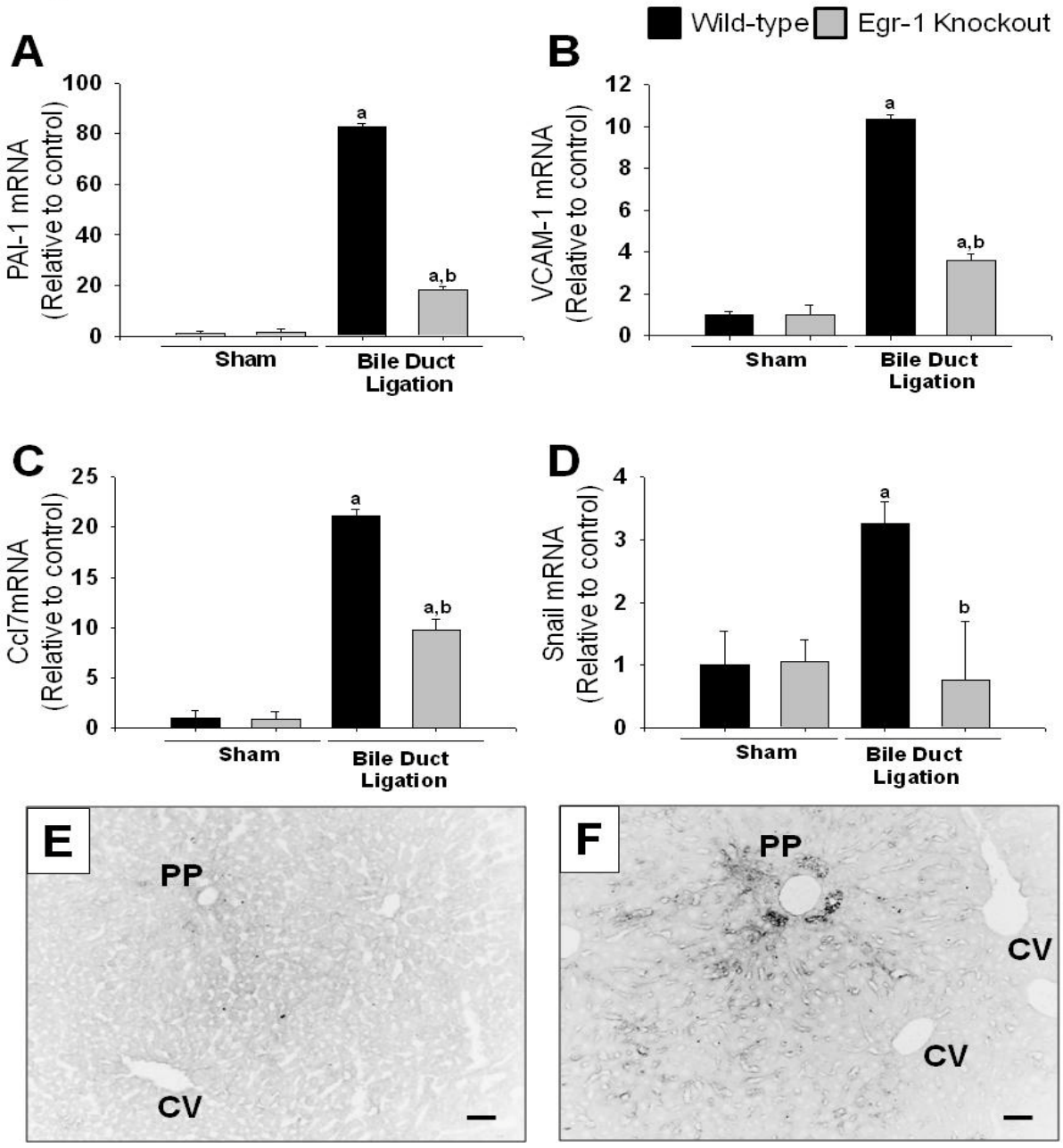

Figure 3.6 Role of Egr-1 in upregulation of PAI-1, VCAM-1, CCL7 and SNAIL in the liver during cholestasis. Wild-type and Egr-1 knockout mice were subjected to BDL or sham operation. Seven days later, mRNA levels of (A) PAI-1, (B) VCAM-1, (C) Ccl7 and (D) SNAIL were quantified by real-time PCR. [a]Significantly different ($p < 0.05$) from sham-operated mice. [b]Significantly different ($p < 0.05$) from wild-type mice subjected to BDL. VCAM-1 protein was detected in sections of liver from sham-operated (E) and bile duct-ligated mice (F). Bar indicates 50 µM. PP: periportal; CV central vein.

Figure 3.7

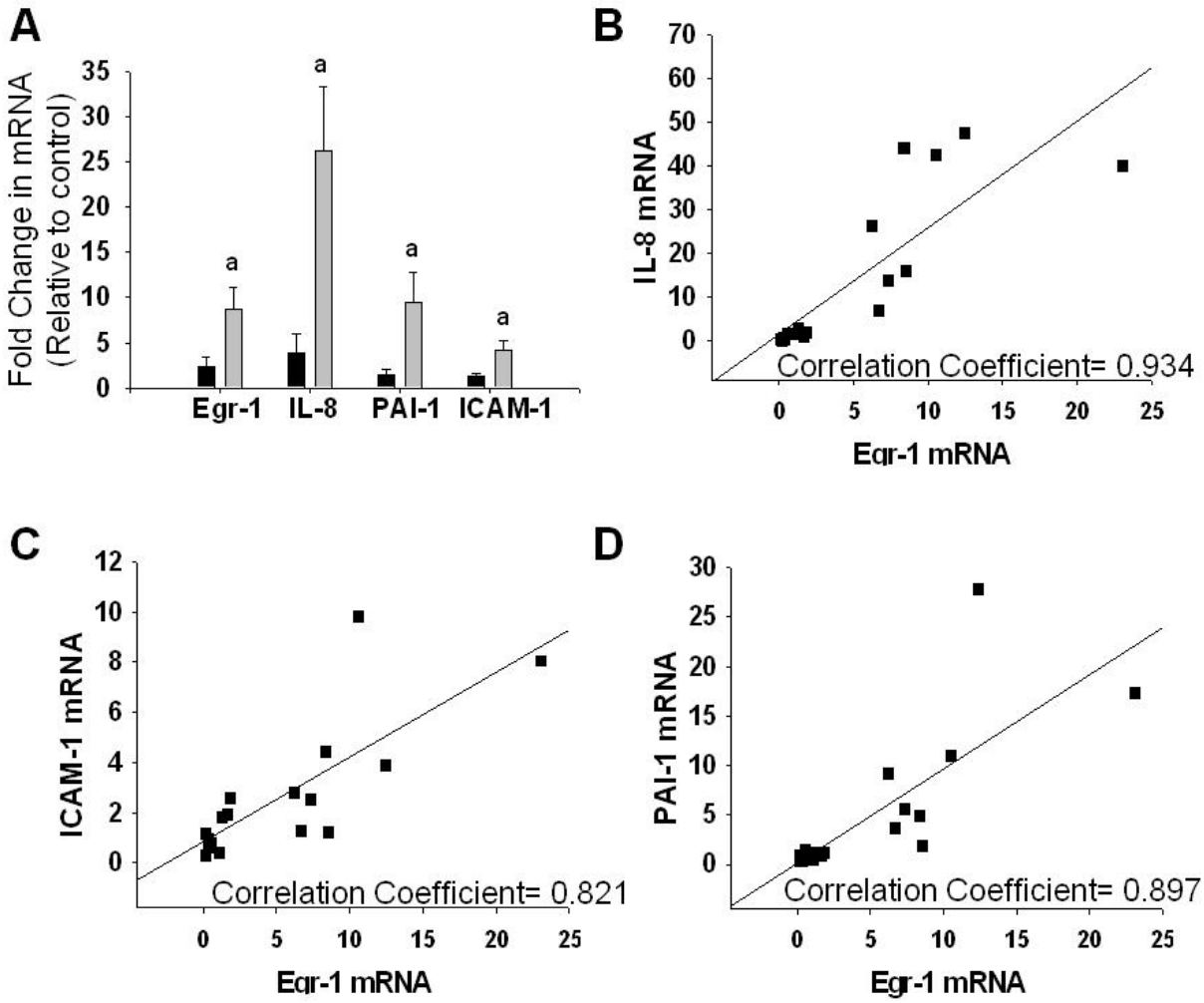

Figure 3.7 Upregulation of inflammatory mediators in the livers of humans with cholestatic liver disease. (A) Real-time PCR was used to measure mRNA levels of Egr-1, ICAM-1, IL-8 and PAI-1 in normal human livers and in cholestatic livers from patients with primary biliary cirrhosis and primary sclerosing cholangitis. [a]Significantly different ($p < 0.05$) from normal liver. The mRNA levels of (B) IL-8, (C) ICAM-1 and (D) PAI-1 were plotted against mRNA levels of Egr-1. Spearman Correlation indicated a significant correlation between Egr-1 mRNA levels and mRNA levels of ICAM-1, IL-8 and PAI-1 in normal human livers and liver from patients with cholestatic liver disease at $p<0.05$. Correlation coefficients are indicated on the graphs.

Figure 3.8

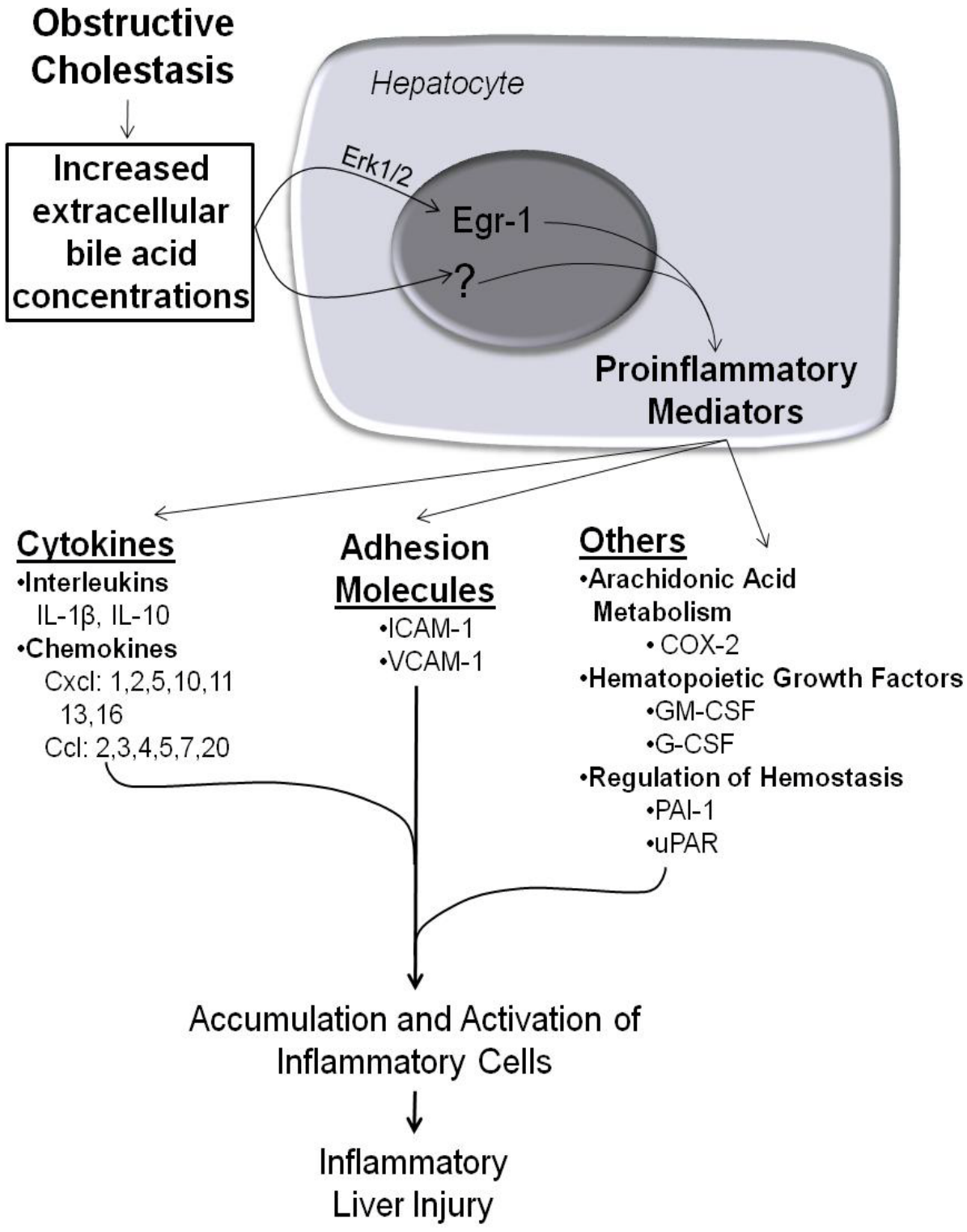

Figure 3.8 Proposed mechanism of inflammation in the liver during obstructive cholestasis.

3.7 Tables

Table 3.1 Sequences of Real-time PCR Primers

Gene	Forward Primer	Reverse Primer
Mouse Egr-1	5'-GGC AGA GGA AGA CGA TGA AG-3'	5'-GAC GAGTTATCC CAG CCA AA-3'
MIP-2	5'-CTC AGA CAG CGA GGC ACATC-3'	5'-CCT CAA CGG AAG AAC CAA AGA G-3'
Mouse ICAM-1	5'-CGA CGC CGC TCA GAA GAA-3'	5'-GTC TCG GAA GGG AGC CAA GTA-3'
Mouse 18S	5'-TTG ACG GAA GGG CAC CAC CAG-3'	5'-GCA CCA CCA CCC ACG GAA TCG-3'
Mouse PAI-1	5'-AGT CTT TCC GAC CAA GAG CA -3'	5'- ATC ACT TGC CCC ATG AAG AG-3'
VCAM-1	5'- TGG CTG TGA CTC CCC TTC TTT-3'	5'-AGA GCT CAA CAC AAG CGT GGA-3'
Ccl7	5'-AAG ATC CCC AAG AGG AAT CTC A-3'	5'-CAG ACT TCC ATG CCC TTC TTT-3'
Human 18S	5'-AAC TTT CGA TGG TCT CGC CG-3'	5'-CCT TGG ATG TGG TAG CGT TT-3'
Human Egr-1	5'-TAC TCC TCT GTT CCC CCT GCT T-3'	5'-GAA AAG GTT GCT GTC ATG TCC G-3'
Human ICAM-1	5'-CGG CTG ACG TGT GCA GTA ATA C-3'	5'-GGC TTC GTC AGA ATC ACG TTG-3'
IL-8	5'-TCC TTG TTC CAC TGT GCC TTG-3'	5'-TGC TTC CAC ATG TCC TCA CAA-3'
Human PAI-1	5'-GTT CAT TGC TGC CCC TTA TGA A-3'	5'-AGC CTG GTC ATG TTG GCC TTT C-3'
Snail	5'-TTT TGC TGA CCG CTC CAA C-3'	5'-TGC TTG TGG AGC AAG GAC AT-3'

Table 3.2: Genes Measured by RT-PCR	Symbol	Assay ID	NCBI Reference`
actin, beta	Actb	Mm00607939_s1	NM_007393.3
arachidonate 5-lipoxygenase	Alox5	Mm01182747_m1	NM_009662.2
arachidonate 8-lipoxygenase	Alox8	Mm01325281_m1	NM_009661.3
arachidonate lipoxygenase 3	Aloxe3	Mm00478628_m1	NM_011786.1
ATP-binding cassette B 11	Abcb11	Mm00445168_m1	NM_021022.3
chemokine (C motif) ligand 1	Xcl1	Mm00434772_m1	NM_008510.1
chemokine (C-C motif) ligand 1	Ccl1	Mm00441236_m1	NM_011329.2
chemokine (C-C motif) ligand 11	Ccl11	Mm00441238_m1	NM_011330.3
chemokine (C-C motif) ligand 17	Ccl17	Mm00516136_m1	NM_011332.2
chemokine (C-C motif) ligand 19	Ccl19	Mm00839967_g1	NM_011888.2
chemokine (C-C motif) ligand 2	Ccl2	Mm00441242_m1	NM_011333.3
chemokine (C-C motif) ligand 20	Ccl20	Mm00444228_m1	NM_016960.1
chemokine (C-C motif) ligand 22	Ccl22	Mm00436439_m1	NM_009137.2
chemokine (C-C motif) ligand 24	Ccl24	Mm00444701_m1	NM_019577.4
chemokine (C-C motif) ligand 25	Ccl25	Mm00436443_m1	NM_009138.2
chemokine (C-C motif) ligand 27	Ccl27	Mm00441257_g1	NM_001048179.1
chemokine (C-C motif) ligand 28	Ccl28	Mm00445039_m1	NM_020279.3
chemokine (C-C motif) ligand 3	Ccl3	Mm00441258_m1	NM_011337.2
chemokine (C-C motif) ligand 4	Ccl4	Mm00443111_m1	NM_013652.2
chemokine (C-C motif) ligand 5	Ccl5	Mm01302428_m1	NM_013653.3
chemokine (C-C motif) ligand 6	Ccl6	Mm00436446_g1	NM_009139.3
chemokine (C-C motif) ligand 7	Ccl7	Mm00443113_m1	NM_013654.2
chemokine (C-C motif) ligand 9	Ccl9	Mm00441260_m1	NM_011338.2
chemokine (C-C motif) receptor-like 1	Ccrl1	Mm02620636_s1	NM_145700.1
chemokine (C-X3-C motif) ligand 1	Cx3cl1	Mm00436454_m1	NM_009142.3
chemokine (C-X-C motif) ligand 1	Cxcl1	Mm00433859_m1	NM_008176.2
chemokine (C-X-C motif) ligand 10	Cxcl10	Mm00445235_m1	NM_021274.1
chemokine (C-X-C motif) ligand 11	Cxcl11	Mm00444662_m1	NM_019494.1
chemokine (C-X-C motif) ligand 12	Cxcl12	Mm00445552_m1	NM_001012477.1
chemokine (C-X-C motif) ligand 13	Cxcl13	Mm00444533_m1	NM_018866.2
chemokine (C-X-C motif) ligand 14	Cxcl14	Mm00444699_m1	NM_019568.2
chemokine (C-X-C motif) ligand 15	Cxcl15	Mm00441263_m1	NM_011339.2
chemokine (C-X-C motif) ligand 16	Cxcl16	Mm00469712_m1	NM_023158.6
chemokine (C-X-C motif) ligand 2	Cxcl2	Mm00436450_m1	NM_009140.2
chemokine (C-X-C motif) ligand 5	Cxcl5	Mm00436451_g1	NM_009141.2
chemokine (C-X-C motif) ligand 9	Cxcl9	Mm00434946_m1	NM_008599.4
chemokine-like factor	Cklf	Mm00459364_m1	NM_029295.2
CKLF-like MARVEL 1	Cmtm1	Mm00725383_m1	AY369078.2
colony stimulating factor 2 (granulocyte-	Csf2	Mm00438328_m1	NM_009969.4
colony stimulating factor 3 (granulocyte)	Csf3	Mm00438334_m1	NM_009971.1
complement component 2 (within H-2S)	C2	Mm00442726_m1	NM_013484.2
complement component 3	C3	Mm01232779_m1	NM_009778.2
cytochrome P450, family 7a1	Cyp7a1	Mm00484152_m1	NM_007824.2
Eukaryotic 18S rRNA	18S	Hs99999901_s1	X03205.1
G protein-coupled estrogen receptor 1	Gper	Mm02620446_s1	NM_029771.2
glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Mm99999915_g1	NM_008084.2
growth factor receptor bound protein 2	Grb2	Mm00812750_gH	NM_008163.3
intercellular adhesion molecule 1	Icam1	Mm00516024_g1	NM_010493.2
interferon gamma	Ifng	Mm00801778_m1	NM_008337.3
interferon gamma receptor 1	Ifngr1	Mm00599890_m1	NM_010511.2
interleukin 1 alpha	Il1a	Mm00439620_m1	NM_010554.4
interleukin 1 beta	Il1b	Mm00434228_m1	NM_008361.3
interleukin 10	Il10	Mm00439616_m1	NM_010548.1
interleukin 12a	Il12a	Mm00434165_m1	NM_008351.1
interleukin 13	Il13	Mm00434204_m1	NM_008355.2

Table 3.2 Cont: Genes Measured by RT-PCR	Symbol	Assay ID	NCBI Reference
interleukin 16	Il16	Mm00516039_m1	NM_010551.3
interleukin 17A	Il17a	Mm00439618_m1	NM_010552.3
interleukin 17B	Il17b	Mm00444686_m1	NM_019508.1
interleukin 17C	Il17c	Mm00521397_m1	NM_145834.3
interleukin 17D	Il17d	Mm01313472_m1	NM_145837.3
interleukin 17F	Il17f	Mm00521423_m1	NM_145856.2
interleukin 18	Il18	Mm00434225_m1	NM_008360.1
interleukin 25	Il25	Mm00499822_m1	NM_080729.2
interleukin 4	Il4	Mm00445259_m1	NM_021283.1
interleukin 5	Il5	Mm00439646_m1	NM_010558.1
interleukin 6	Il6	Mm00446190_m1	NM_031168.1
interleukin 8 receptor, beta	Il8rb	Mm99999117_s1	NM_009909.3
interleukin 9	Il9	Mm00434305_m1	NM_008373.1
leukotriene A4 hydrolase	Lta4h	Mm01246216_m1	NM_008517.2
myeloid differentiation primary response 88	Myd88	Mm00440338_m1	NM_010851.2
NF-κB gene enhancer in B-cell1	Nfkb1	Mm00476361_m1	NM_008689.2
phosphatase and tensin homolog	Pten	Mm00477210_m1	NM_008960.2
plasminogen activator, tissue	Plat	Mm00476931_m1	NM_008872.2
plasminogen activator, urokinase	Plau	Mm00447054_m1	NM_008873.2
plasminogen activator, urokinase receptor	Plaur	Mm00440911_m1	NM_011113.3
platelet factor 4	Pf4	Mm00451315_g1	NM_019932.4
platelet/endothelial cell adhesion molecule 1	Pecam1	Mm01242584_m1	NM_001032378.1
prostaglandin E receptor 1	Ptger1	Mm00443098_g1	NM_013641.2
prostaglandin E receptor 3	Ptger3	Mm00441045_m1	NM_011196.2
prostaglandin-endoperoxide synthase 1	Ptgs1	Mm00477214_m1	NM_008969.3
prostaglandin-endoperoxide synthase 2	Ptgs2	Mm00478374_m1	NM_011198.3
regulator of G-protein signaling 1	Rgs1	Mm00450170_m1	NM_015811.1
regulator of G-protein signaling 3	Rgs3	Mm00450935_m1	NM_019492.2
serine (or cysteine) peptidase inhibitor	Serpine1	Mm00435860_m1	NM_008871.2
slit homolog 2	Slit2	Mm00662153_m1	NM_178804.3
suppressor of cytokine signaling 1	Socs1	Mm00782550_s1	NM_009896.2
suppressor of cytokine signaling 3	Socs3	Mm00545913_s1	NM_007707.2
suppressor of cytokine signaling 5	Socs5	Mm00465631_s1	NM_019654.2
thymidine phosphorylase	Tymp;Sco2	Mm00460357_m1	NM_138302.1
toll-like receptor 4	Tlr4	Mm00445274_m1	NM_021297.2
transforming growth factor, beta 1	Tgfb1	Mm00441724_m1	NM_011577.1
tumor necrosis factor (ligand) 10	Tnfsf10	Mm00437174_m1	NM_009425.2
tumor necrosis factor (ligand) 14	Tnfsf14	Mm00444567_m1	NM_019418.2
tumor necrosis factor receptor 1a	Tnfrsf1a	Mm00441875_m1	NM_011609.4
tumor necrosis factor receptor 1b	Tnfrsf1b	Mm00441889_m1	NM_011610.3
vascular cell adhesion molecule 1	Vcam1	Mm00449197_m1	NM_011693.3

Table 3.3: Effect of DCA on Proinflammatory Gene Expression in Hepatocytes Isolated from Wild-type Mice and Egr-1 Knockout Mice

Gene	Other Names	Function	Wild-type Vehicle	Egr-1 Knockout Vehicle	Wild-type DCA	Egr-1 Knockout DCA
CXCL1	KC	neutrophil chemokine	1.1 +/- 0.2	1.1 +/- 0.3	3.5 +/- 0.5[a]	1.6 +/- 0.4[b]
CXCL10	IP-10	T cell chemokine	1.0 +/- 0.2	1.0 +/- 0.2	47.0 +/- 11.6[a]	33.9 +/- 14.1[a]
CXCL11	I-TAC	T cell chemokine	1.2 +/- 0.2	1.1 +/- 0.4	16.7 +/- 7.7[a]	8.1 +/- 3.6
Ccl2	MCP-1	monocyte & T cell chemokine	1.1 +/- 0.2	1.0 +/- 0.1	12.5 +/- 1.9[a]	8.6 +/- 3.7[a,]
Ccl5	RANTES	T cell, eosinophil, & mast cell chemokine	1.0 +/- 0.2	1.0 +/- 0.1	3.5 +/- 1.1[a]	4.4 +/- 1.9[a]
Ccl7	MCP-3	monocyte, T cell, neutrophil, & eosinophil chemokine	1.1 +/- 0.3	1.0 +/- 0.1	9.7 +/- 1.1[a]	2.7 +/- 0.9[b]
Ccl20	MIP-3α LARC	T cell & B cell chemokine	1.2 +/- 0.4	2.7 +/- 2.3	6.5 +/- 3.5	13.1 +/- 2.0[a]
VCAM-1	CD106	adhesion molecule	1.0 +/- 0.2	1.0 +/- 0.1	7.3 +/- 1.36[a]	4.6 +/- 1.9[a]
PAI-1	Serpine 1	plasminogen activator inhibitor	1.0 +/- 0.02	1.0 +/- 0.1	4.3 +/- 1.0[a]	1.0 +/- 0.5[b]
uPAR	Plaur	uPA receptor	1.2 +/- 0.1	1.0 +/- 0.03	2.3 +/- 0.2[a]	2.7 +/- 0.2[a]
COX-2	Ptgs2	arachidonic acid metabolism	1.0 +/- 0.2	1.0 +/- 0.1	2.9 +/- 0.5[a]	2.6 +/- 0.9[a]
GM-CSF	Csf2	hematopoietic growth factor	1.2 +/- 0.4	1.0 +/- 0.2	6.6 +/- 1.0[a]	2.4 +/- 0.3[b]
G-CSF	Csf3	hematopoietic growth factor	2.1 +/- 1.6	0.2 +/- 0.1	2.6+/- 1.0[a]	16.6 +/- 2.9[a,b]

Primary mouse hepatocytes were isolated from wild-type or Egr-1knockout mice and treated with 200 µM DCA. Six hours later, total mRNA was isolated and pro-inflammatory gene expression measured by qPCR. [a]Significantly different ($p < 0.05$) from Vehicle-treated hepatocytes. [b]Significantly different ($p < 0.05$) from WT hepatocytes treated with DCA.

Table 3.4: Effect of CDCA on Proinflammatory Gene Expression in Hepatocytes Isolated from Wild-type Mice and Egr-1 Knockout Mice

Gene	Other Names	Function	Wild-type Vehicle	Egr-1 Knockout Vehicle	Wild-type CDCA	Egr-1 Knockout CDCA
CXCL1	KC	neutrophil chemokine	1.1 +/- 0.2	1.1 +/- 0.3	7.1 +/- 1.4[a]	4.1 +/- 1.6[a]
CXCL10	IP-10	T cell chemokine	1.0 +/- 0.2	1.0 +/- 0.2	92.7 +/- 21.4[a]	102.1 +/- 33.8[a]
CXCL11	I-TAC	T cell chemokine	1.2 +/- 0.2	1.1 +/- 0.4	49.4 +/- 14.2[a]	44.7 +/- 15.0[a]
CXCL16	SR-PSOX	T cell chemokine	1.0 +/- 0.04	1.0 +/- 0.2	3.6 +/- 0.2[a]	3.7 +/- 1.6[a]
Ccl2	MCP-1	monocyte & T cell chemokine	1.1 +/- 0.2	1.0 +/- 0.1	26.5 +/- 1.3[a]	20.1 +/- 2.8[a,b]
Ccl3	MIP-1α	monocyte & T cell chemokine	1.0 +/- 0.2	1.0 +/- 0.2	2.1 +/- 0.3[a]	2.8 +/- 0.3[a]
Ccl5	RANTES	T cell, eosinophil, & mast cell chemokine	1.0 +/- 0.2	1.0 +/- 0.1	6.5 +/- 1.7[a]	8.2 +/- 3.1[a]
Ccl7	MCP-3	monocyte, T cell, neutrophil, & eosinophil chemokine	1.1 +/- 0.3	1.0 +/- 0.1	26.1 +/- 5.0[a]	10.3 +/- 4.8[a,b]
Ccl20	MIP-3α LARC	T cell & B cell chemokine	1.2 +/- 0.4	2.7 +/- 2.3	27.2 +/- 11.4[a]	34.2 +/- 8.8[a]
VCAM-1	CD106	adhesion molecule	1.0 +/- 0.2	1.0 +/- 0.1	21.9 +/- 6.3[a]	10.7 +/- 4.9[a]
PAI-1	Serpine 1	plasminogen activator inhibitor	1.0 +/- 0.02	1.0 +/- 0.1	6.6 +/- 1.6[a]	3.4 +/- 0.1[a,b]
uPAR	Plaur	uPA receptor	1.2 +/- 0.1	1.0 +/- 0.03	3.9 +/- 0.1[a]	3.87 +/- 0.4[a]
COX-2	Ptgs2	arachidonic acid metabolism	1.0 +/- 0.2	1.0 +/- 0.1	5.1 +/- 1.3[a]	3.5 +/- 1.8[a]
GM-CSF	Csf2	hematopoietic growth factor	1.2 +/- 0.4	1.0 +/- 0.2	20.2 +/- 4.1[a]	4.7 +/- 1.5[a,b]
G-CSF	Csf3	hematopoietic growth factor	2.1 +/- 1.6	0.2 +/- 0.1	3.6 +/- 1.5[a]	6.5 +/- 3.5[a]

Primary mouse hepatocytes were isolated from wild-type or Egr-1knockout mice and treated with 200 μM CDCA. Six hours later, total mRNA was isolated and pro-inflammatory gene expression measured by qPCR. [a]Significantly different ($p < 0.05$) from Vehicle-treated hepatocytes. [b]Significantly different ($p < 0.05$) from WT hepatocytes treated with CDCA.

Table 3.5: Effect of TCA on Proinflammatory Gene Expression in Hepatocytes Isolated from Wild-type Mice and Egr-1 Knockout Mice

Gene	Other Names	Function	Wild-type Vehicle	Egr-1 Knockout Vehicle	Wild-type TCA	Egr-1 Knockout TCA
CXCL1	KC	neutrophil chemokine	1.1 +/- 0.2	1.1 +/- 0.3	3.1 +/- 0.2[a]	1.6 +/- 0.2[b]
CXCL 5	LIX, GCP-2	neutrophil chemokine	1.1 +/- 0.4	3.5 +/- 1.9	2.7 +/- 0.2	10.2 +/- 2.6[a,b]
CXCL10	IP-10	T cell chemokine	1.0 +/- 0.2	1.0 +/- 0.2	5.18 +/- 0.8[a]	5.9 +/- 0.7 [a]
CXCL11	I-TAC	T cell chemokine	1.2 +/- 0.2	1.1 +/- 0.4	5.9 +/- 0.9[a]	4.9 +/- 1.9[a]
CXCL13	BCL	B-Cell arrest chemokine	1.2 +/- 0.4	1.2 +/- 0.3	4.8 +/- 1.1 [a]	1.3 +/- 0.2 [a,b]
CXCL16	SR-PSOX	T cell chemokine	1.0 +/- 0.04	1.0 +/- 0.2	1.8 +/- 0.2	2.0 +/- 0.5[a]
Ccl2	MCP-1	monocyte & T cell chemokine	1.1 +/- 0.2	1.0 +/- 0.1	5.2 +/- 0.1[a]	3.6 +/- 0.5[a,b]
Ccl3	MIP-1α	monocyte & T cell chemokine	1.0 +/- 0.2	1.0 +/- 0.2	7.3 +/- 0.7[a]	9.5 +/- 1.6[a]
Ccl4	Mip-1β	T cell chemokine	1.1 +/- 0.3	1.0 +-/- 0.0	7.6 +/- 0.6 [a]	8.9 +/- 1.0 [a]
Ccl5	RANTES	T cell, eosinophil, & mast cell chemokine	1.0 +/- 0.2	1.0 +/- 0.1	1.6 +/- 0.2	2.1 +/- 0.2[a]
Ccl7	MCP-3	monocyte, T cell, neutrophil, & eosinophil chemokine	1.1 +/- 0.3	1.0 +/- 0.1	6.5 +/- 0.4[a]	2.0 +/- 0.1[a,b]
Ccl20	MIP-3α LARC	T cell & B cell chemokine	1.2 +/- 0.4	2.7 +/- 2.3	26.8 +/- 4.1[a]	13.0 +/- 0.5[a,b]
IL-1β	N/A	Lymphocyte activator, acute phase response	1.2 +/- 0.4	1.0 +/- 0.1	17.6 +/- 1.4 [a]	4.9 +/- 1.9 [a,b]
IL-10	CISF	Anti-inflammatory cytokine	1.2+/- 0.4	1.3 +/- 0.5	8.2 +/- 1.3 [a]	6.3 +/- 0.9 [a]
VCAM-1	CD106	adhesion molecule	1.0 +/- 0.2	1.0 +/- 0.1	12.0 +/- 1.2[a]	3.8 +/- 1.9[a,b]
COX-2	Ptgs2	arachidonic acid metabolism	1.0 +/- 0.2	1.0 +/- 0.1	6.9 +/- 0.3[a]	8.7 +/- 1.5[a]
GM-CSF	Csf2	hematopoietic growth factor	1.2 +/- 0.4	1.0 +/- 0.2	7.2 +/- 1.6[a]	2.0 +/- 0.7[a,b]
G-CSF	Csf3	Hematopoietic growth factor	2.1 +/- 1.6	0.2 +/- 0.1	6.0 +/- 1.8[a]	33.6 +/- 5.8[a]

Primary mouse hepatocytes were isolated from wild-type or Egr-1knockout mice and treated with 200 µM TCA. Six hours later, total mRNA was isolated and pro-inflammatory gene expression measured by qPCR. [a]Significantly different ($p < 0.05$) from Vehicle-treated hepatocytes. [b]Significantly different ($p < 0.05$) from WT hepatocytes treated with TCA.

CHAPTER 4

IL-17D Regulates Proinflammatory Gene Expression During Cholestasis

4.1 Abstract

Inflammation contributes to liver injury during cholestasis; however the mechanisms that promote inflammation are not fully elucidated. We demonstrated previously that bile acids increase expression of proinflammatory mediators in hepatocytes by Egr-1-dependent and -independent mechanisms. The interleukin-17 family of cytokines has been shown to promote inflammation in various organs. IL-17A levels are increased in patients with cholestasis caused by primary biliary cirrhosis (PBC), and in IL-2 receptor knockout mice, a genetic model of PBC. Whether bile acids stimulate hepatocytes to produce IL-17s, however, has not been investigated. Therefore, we tested the hypothesis that bile acids upregulate IL-17s in hepatocytes. Treatment of primary mouse hepatocytes with bile acids did not increase expression of IL-17A, IL-17B, IL-17C, IL-17E or IL-17F, but they did increase expression of IL-17D. IL-17D was also upregulated in bile duct ligated (BDL) mice, and in humans with cholestasis. The function of IL-17D is not known; therefore we treated mice with an anti-IL-17D antibody to elucidate the role of IL-17D during cholestasis. Compared to mice treated with control IgG, anti-IL-17D antibody treated mice had reduced levels of PAI-1 and MIP-2 after BDL. In contrast, anti-IL-17D treatment did not affect liver injury or neutrophil accumulation, suggesting the IL-17D is not a major regulator of inflammation during cholestasis. To determine whether the levels of other IL-17s increase during cholestasis, mRNA levels of IL-17A, IL-17B, IL-17E and IL-17F were measured in mice after BDL. Our results demonstrate that IL-17A, IL-17B, IL-17E and IL-17F all increase during cholestasis. These studies demonstrate that IL-17D is upregulated in bile acid-treated hepatocytes and in the livers of mice and humans with cholestasis; however the proinflammatory contribution of IL-17D is minimal in this disease. We also show that IL-17A, IL-17B, IL-17E and IL-17F increase during cholestasis, and could potentially contribute to liver inflammation.

4.2 Introduction

Cholestasis is a condition that arises when bile flow from the liver is interrupted (Klaassen, 2001). This condition causes liver injury, bile duct proliferation, biliary fibrosis, and in some cases, cancer. Studies have demonstrated that inflammation is important for liver injury during cholestasis. Proinflammatory proteins such as adhesion molecules, cytokines and chemokines increase in the serum and livers of patients with cholestasis and in the livers of BDL mice (Thomson et al., 1994; Gulubova, 1998; Tsuneyama et al., 2001; Gujral et al., 2004a; Nobili et al., 2004; Wang et al., 2005; Kim et al., 2006; Isse et al., 2007; Borchers et al., 2009; Wintermeyer et al., 2009). Consistent with a role for inflammation in liver injury during cholestasis, loss of either intracellular adhesion molecule-1 (ICAM-1) or its integrin counterpart CD18 reduces liver injury during cholestasis (Gujral et al., 2003; Gujral et al., 2004a). While the effects of several proinflammatory mediators during cholestasis have been elucidated, the mechanisms that contribute to the upregulation of these factors are not completely understood.

The interleukin-17 (IL-17) family of cytokines, comprised of several members, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F, are important regulators of immune function. IL-17A, IL-17B, IL-17C, and IL-17F have been shown to promote proinflammatory signaling in several animal models (Jovanovic et al., 1998; Laan et al., 1999; Miyamoto et al., 2003; Oda et al., 2005; Weaver et al., 2007; Yamaguchi et al., 2007; Fouser et al., 2008; Lan et al., 2009; Lemmers et al., 2009; Kobayashi et al., 2010; Zhang et al., 2010). For example, IL-17A promotes neutrophil accumulation in the lung by a mechanism that requires the chemokine, macrophage inflammatory protein-2 (MIP-2) (Laan et al., 1999). Recent studies indicate that at least one member of the IL-17 family, IL-17A, may be an important regulator of hepatic inflammation. The receptor for IL-17A, composed of a heterodimer of IL-17 receptor A and IL-17 receptor C, is expressed on several cell types in the liver including, hepatocytes, bile duct epithelial cell, hepatic stellate cells, and Kupffer cells (Toy et al., 2006; Patel et al., 2007; Ge

and You, 2008; Lemmers et al., 2009; Lafdil et al., 2010). Exposure of hepatocytes to IL-17A increases expression of several genes, including the chemokines keratinocyte-derived chemokine (KC) and monocyte chemotactic protein-1 (MCP-1) (Sparna et al., 2010). Similarly, treatment of human bile duct epithelial cells (BEC) with IL-17A increases expression of several cytokines (i.e., IL-6, IL-1β, IL-23p19, and IL-23/IL-12 p40) and chemokines (i.e., KC, MIP-2, MIP-2β, MCP-1, and MIP-3) (Harada et al., 2009). Although it is not known whether IL-17s contribute to liver inflammation during cholestasis, IL-17A levels are increased in the livers of patients with cholestasis from primary biliary cirrhosis (PBC), and levels of IL-17A are increased in IL-2Rα knockout mice, a genetic model of PBC (Harada et al., 2009; Lan et al., 2009). This suggests that IL-17s may be important mediators of inflammation in the liver during cholestasis.

We previously demonstrated that bile acids stimulate hepatocytes to produce cytokines, chemokines, adhesion molecules, and regulators of prostaglandin synthesis. This suggests that during cholestasis, exposure of hepatocytes to pathological concentrations of bile acids may initiate an inflammatory response and promote neutrophil-dependent liver injury. Whether bile acids stimulate hepatocytes to produce IL-17s, however, was not investigated in this study. Therefore, in the present studies, we tested the hypothesis that bile acids upregulate IL-17s in hepatocytes.

4.3 Materials and methods:

4.3.1 Animal Care. Male, C57BL/6 (Harlan, Indianapolis, IN), C57BL/6NTac (Taconic, Germantown, NY) or Egr-1 knockout mice (B6.129-*Egr1*^{tm1Jmi} N12, Taconic), were used for all studies. Mice were maintained on a 12-h light/dark cycle under controlled temperature (18-21ºC) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed ad libitum. All procedures on animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health and

were approved by the institutional IACUC committee at the University of Kansas Medical Center.

4.3.2 Bile Duct Ligation. Male, C57BL/6 (Harlan), C57BL/6NTac (Taconic) or Egr-1 knockout mice (B6.129-*Egr1*^{tm1Jmi} N12, Taconic) 8-12 weeks of age, were anesthetized with isoflurane. A midline laparotomy was performed and the bile duct ligated with 3-0 surgical silk. The abdominal incision was closed with sutures, and the mice received 0.2 mg/kg Buprenex by subcutaneous injection. For IL-17D neutralization studies, mice received 15 μg rat anti-mouse IL-17D (R&D Systems Minneapolis, MN) dissolved in saline or isotype control (Rat IgG2A, R&D Systems) dissolved in saline by intraperitoneal injection, 1 hour before surgery.

4.3.3 Real-time PCR. RNA was isolated from livers and hepatocytes using TRI reagent (Sigma Chemical Company, St. Louis, MO), and reverse transcribed into cDNA as described by us previously (Kim et al., 2006). Real-time PCR was used to quantify mouse mRNA levels of PAI-1, MIP-2, Egr-1, IL-17A, IL-17B, IL-17D, IL-17E and IL-17F as well as 18S on an Applied Biosystems Prism 7900 Real-time PCR Instrument (Applied Biosystems, Foster City, CA) using the SYBR green DNA PCR kit (Applied Biosystems) as described (Kim et al., 2006). The sequences of the primers were as follows: PAI-1 Forward: 5'-AGT CTT TCC GC CAA GAG CA-3'; PAI-1 Reverse: 5'-ATC ACT TGCC CCC ATG AAG AG-3'; MIP-2 Forward: 5'-CCT CAA CGG AAG AAC CAA A G-3' MIP-2 Reverse: 5'- CTC AGA CAG CGA GGC ACA TC-3'; Egr-1 Forward: 5'-GGC AGA GGA AGA CGA TGA AG-3'; Egr-1 Reverse: 5'-GAC GAGTTATCC CAG CCA AA-3'; IL-17A Forward: 5'-CCG CAA TGA AGA CCC TGA TAG A -3'; IL-17A Reverse: 5'-TCA TGT GGT GGTCCA GCT TTC-3'; IL-17B Forward: 5'-TGG AAG AGT ATG AGC GGA ACC T-3'; IL-17B Reverse: 5'-CAG GCT CCT CTT GTT GGA CAA-3'; IL-17D Forward: 5'-CAC ACA CAT CCC GTT TTC CTC-3'; IL-17D Reverse: 5'-CCA GAT CCG GAG CC TCA TTA-3'; IL-17E Forward: 5'-GGC TGT TGC ATT CTT GGC A-3'; IL-17E Reverse: 5'-CAG ATG CAG AGC TCC ACT TCA G-3'; IL-17F Forward: 5'-CGC CAT TCA GCA AGA AAT CCT-3' IL-17F

Reverse: 5'-TTG ACA CAG GTG CAG CCA ACT-3' and 18S Forward: 5'-TTG ACG GAA GGG CAC CAC CAG-3'; and 18S Reverse: 5'-GCA CCA CCA CCC ACG GAA TCG-3'. Human mRNA levels of IL-17D (Integrated DNA Technologies, Coralville, IA) and 18s (Applied Biosystems) were measured using TaqMan (Applied Biosystems) on an Applied Biosystems Prism 7900 Real-time PCR Instrument.

4.3.4 Human Liver Samples. Research involving human livers was reviewed by the University of Kansas Medical Center Human Research Protection Program. The specimens were provided by the KU Liver Center Tissue Bank. Diseased liver tissue utilized for these studies was collected from patients with primary biliary cirrhosis (4 Females, age 59, 56, 60, 61; 1 Male, age 47) and primary sclerosing cholangitis (3 Males, age 44, 45, 57). Liver samples without histological evidence of cholestasis, fibrosis, or severe inflammation were selected as control tissues (5 Females, age 41, 48, 56, 52, 66; 5 Males, age 57, 45, 57, 58, 48).

4.3.5 Quantification of Liver Injury in BDL Mice. ALT was measured in serum using a commercially available kit as described previously (Kim et al., 2006)

4.3.6 Statistical Analysis. Results are presented as the mean $\pm$ SEM. Data were analyzed by Analysis of Variance (ANOVA). ANOVAs were performed on log X-transformed data in instances in which variances were not homogenous. Comparisons among group means were made using the Student-Newman-Keuls test. The criterion for significance was $p < 0.05$ for all studies.

4.4 Results

4.4.1 IL-17D expression increases in hepatocytes treated with CDCA. Primary mouse hepatocytes were treated with 200 μM chenodeoxycholic acid (CDCA) for six hours. mRNA levels of IL-17A, IL-17B, IL-17C, IL-17E and IL-17F were unaffected by bile acid

treatment (data not shown). In contrast, mRNA levels of IL-17D increased in hepatocytes treated with CDCA (Figure 4.1). Upregulation of IL-17D by CDCA was prevented in hepatocytes isolated from Egr-1 knockout mice (Figure 4.1).

4.4.2 IL-17D expression is increased in mice and humans with cholestasis. Next whether IL-17D is upregulated in the livers of BDL mice was determined. mRNA levels of IL-17D after BDL were cyclic with an initial sharp peak at 6 hours followed by a second peak beginning at 2 days and a third broad peak beginning at 7 days after BDL (Figure 4.2 A). The highest induction of IL-17D was observed at 6 hours and 7days after BDL (Figure 4.2 A).

Next we determined whether IL-17D mRNA levels increase in the livers of patients with cholestatic liver disease caused by primary biliary cirrhosis or primary sclerosing cholangitis. The expression of IL-17D was higher in livers of patients with cholestasis when compared to normal liver samples (Figure 4.2 B).

4.4.3 Upregulation of IL-17D after BDL requires Egr-1. Our previous study indicated that upregulation of IL-17D in bile acid-treated hepatocytes was Egr-1 dependent; therefore, the expression of IL-17D in Egr-1 knockout mice after BDL was examined. IL-17D expression increased in the livers of wild-type mice after BDL (Figure 4.3). Upregulation of IL-17D was prevented in Egr-1 knockout mice (Figure 4.3).

4.4.4 Inhibition of IL-17D during cholestasis attenuates proinflammatory gene expression, but not neutrophil accumulation or liver injury. To elucidate the function of IL-17D *in vivo* mice were treated with an anti-IL-17D antibody during the course of BDL. The expression of the proinflammatory genes MIP-2 and PAI-1 increased in the livers of control IgG-treated mice after BDL (Figure 4.4 A-C). These genes were increased to a lesser extent in mice treated with anti-IL-17D (Figure 4.4 A-C). The expression of Egr-1 trended lower in the anti-IL-17D antibody treated mice. Neutrophils accumulated in the livers of control IgG-treated mice

subjected to BDL (Figure 4.5 A). Hepatic neutrophil numbers were unaffected by anti-IL-17D antibody treatment in mice (Figure 4.5 A). Similar results were observed for serum ALT levels (Figure 4.5B). H and E stained tissues were evaluated for liver injury, similar liver injury was observed between control IgG-treated and anti-IL-17D-treated mice subjected to BDL confirming the ALT results (data not shown).

4.4.5 Expression of IL-17A, IL-17B, IL-17E, and IL-17F in the livers of mice after BDL. Studies have shown that IL-17D increases expression of IL-8 (similar to MIP-2 in mouse), IL-6 and GM-CSF in human endothelial cells. These genes have also been shown to be upregulated by other IL-17s. Since there was limited effect of the anti-IL-17D antibody on liver injury and neutrophil accumulation, it is possible that other IL-17s compensate for the loss of IL-17D. Accordingly, we next determined if the expression of IL-17A, IL-17B, IL-17E and IL-17F increases after BDL. IL-17A, IL-17B, IL-17E and IL-17F mRNA levels all increased in the liver after BDL (Figure 4. 6 A-D). The expression pattern of IL-17F during the BDL time course was similar to the expression pattern of IL-17D, with peaks in expression at 6 hours, 2 days and 7 days after BDL (Figure 4.6 D). IL-17A mRNA levels were not significantly elevated until 7 days after BDL (Figure 4.6 A). IL-17B showed two peaks of expression at 2 and 7 days after BDL (Figure 4.6 B). Unlike the other IL-17 family members, IL-17E mRNA levels were increased by 2 days after BDL and continued to increase over the full time course (Figure 4.6 C).

4.5 Discussion

Neutrophil accumulation and activation within the liver parenchyma during cholestasis is known to exacerbate liver injury (Gujral et al., 2003; Gujral et al., 2004a). The mechanisms that stimulate inflammation during cholestasis, however, are not completely elucidated. Previous studies in our laboratory showed that bile acids at pathological concentrations increase expression of numerous proinflammatory genes (Allen et al., 2010). These studies did not

investigate, however, whether bile acids affect mRNA levels of IL-17 family members in hepatocytes. Therefore we determined whether bile acids increase expression of the various IL-17 family members in primary mouse hepatocytes. Our results demonstrated that mRNA levels of IL-17D increased in response to bile acids in an Egr-1-dependent manner. In contrast, the expression of IL-17A, IL-17B, IL-17E and IL-17F was not affected in hepatocytes treated with bile acids. This data suggested that IL-17D may play a unique role in cholestatic liver disease. Accordingly, we conducted studies to determine the functional role of IL-17D during cholestasis.

Our results demonstrate that IL-17D mRNA levels are increased in the livers of BDL mice and in humans with cholestasis (Figures 2 and 3). To our knowledge, this is the first time IL-17D has been measured *in vivo*, and the first time that IL-17D expression has been reported to increase in a disease state. Similar to *in vitro*, upregulation of IL-17D in the liver after BDL required Egr-1. Although it remains to be determined whether Egr-1 directly regulates IL-17D, this is the first study to identify a potential regulator of IL-17D gene expression.

To date, there is only one study that investigated the function of IL-17D. In this study, treatment of human endothelial cells increased production of IL-6, IL-8, and GM-CSF (Starnes et al., 2002). Considering that these mediators play an important role in regulation of inflammation suggested that IL-17D may modulate the inflammatory response during cholestasis. To determine if IL-17D contributed to liver injury and inflammation during cholestasis mice were treated with an anti-IL-17D antibody and subjected to BDL. Treatment with the anti-IL-17D antibody partially prevented upregulation of the proinflammatory mediators MIP-2 and PAI-1 (Figure 3). Although treatment with an IL-17D antibody limited production of some proinflammatory mediators, it did not affect neutrophil accumulation or liver injury, suggesting that either IL-17D plays a modest role in regulation of inflammatory mediators during cholestasis or that it does not affect production of other key inflammatory mediators (Figure 4).

Treatment with the IL-17D antibody only partially prevented upregulation of MIP-2 and PAI-1 after BDL. This suggested that other pathways contribute to upregulation of these genes. One possibility is other IL-17 family members. Many of the IL-17s have overlapping functions. For instance, similar to IL-17D, IL-17A and IL-17F regulate production of IL-6 and IL-8 (Laan et al., 1999; Starnes et al., 2002; Oda et al., 2005; Fouser et al., 2008). Accordingly, it may be necessary to inhibit several IL-17 family members to have a significant effect on inflammatory diseases such as cholestasis. As previously mentioned several of the IL-17 family members, including IL-17A and IL-17E, have been associated with inflammation in the liver and IL-17A levels are increased in the livers of patients with PBC (Laan et al., 1999; Pan et al., 2001; Harada et al., 2009; Lan et al., 2009; Pappu et al., 2010). No one has yet investigated whether other members of the IL-17 family are upregulated during cholestasis. Therefore we measured the expression of IL-17A, IL-17B, IL-17E and IL-17F in the livers of BDL mice (Figure 5). Interestingly, the expression of each of the IL-17 family members measured increased in the livers of BDL mice. These data suggest that one or more of the IL-17 family members could compensate for the loss of IL-17D signaling. As mentioned above, all of the genes induced by IL-17D are also known to be induced by IL-17A, suggesting that IL-17A signaling could mask the benefits of the anti-IL-17D antibody treatment.

Collectively, these studies are novel in that they show a role for IL-17D in proinflammatory signaling *in vivo*. Furthermore, these studies also provide clues as to the mechanism of regulation of IL-17D *in vivo* as our studies demonstrated that upregulation of IL-17D was prevented in Egr-1 knockout mice. These studies also identified two other inflammatory genes regulated by IL-17D, MIP-2 and PAI-1. Overall, these studies provide additional support for IL-17D as a mediator of inflammation in the liver.

4.6 Figures

Figure 4.1

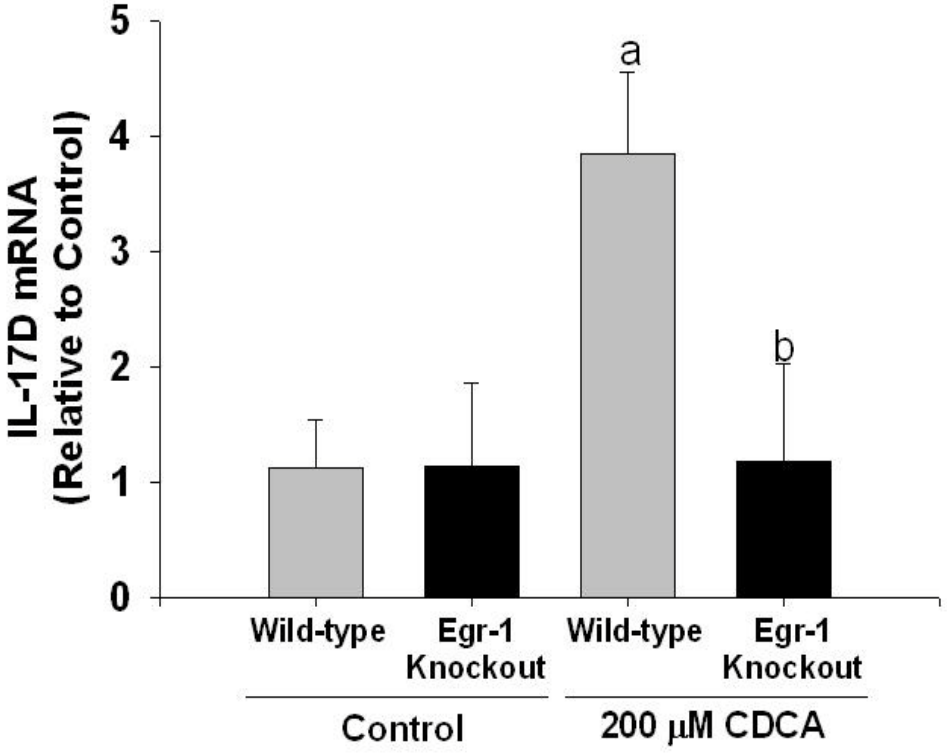

Figure 4.1 Upregulation of IL-17D in bile acid-treated hepatocytes is Egr-1 dependent. Hepatocytes were isolated from wild-type or Egr-1 knockout mice and treated with CDCA. Six hours later, IL-17D mRNA levels were quantified by real-time PCR. Data are expressed as mean +/- SEM; n=3. [a]Significantly different ($p < 0.05$) from vehicle-treated hepatocytes. [b]Significantly different ($p < 0.05$) from wild-type hepatocytes treated with CDCA.

Figure 4.2

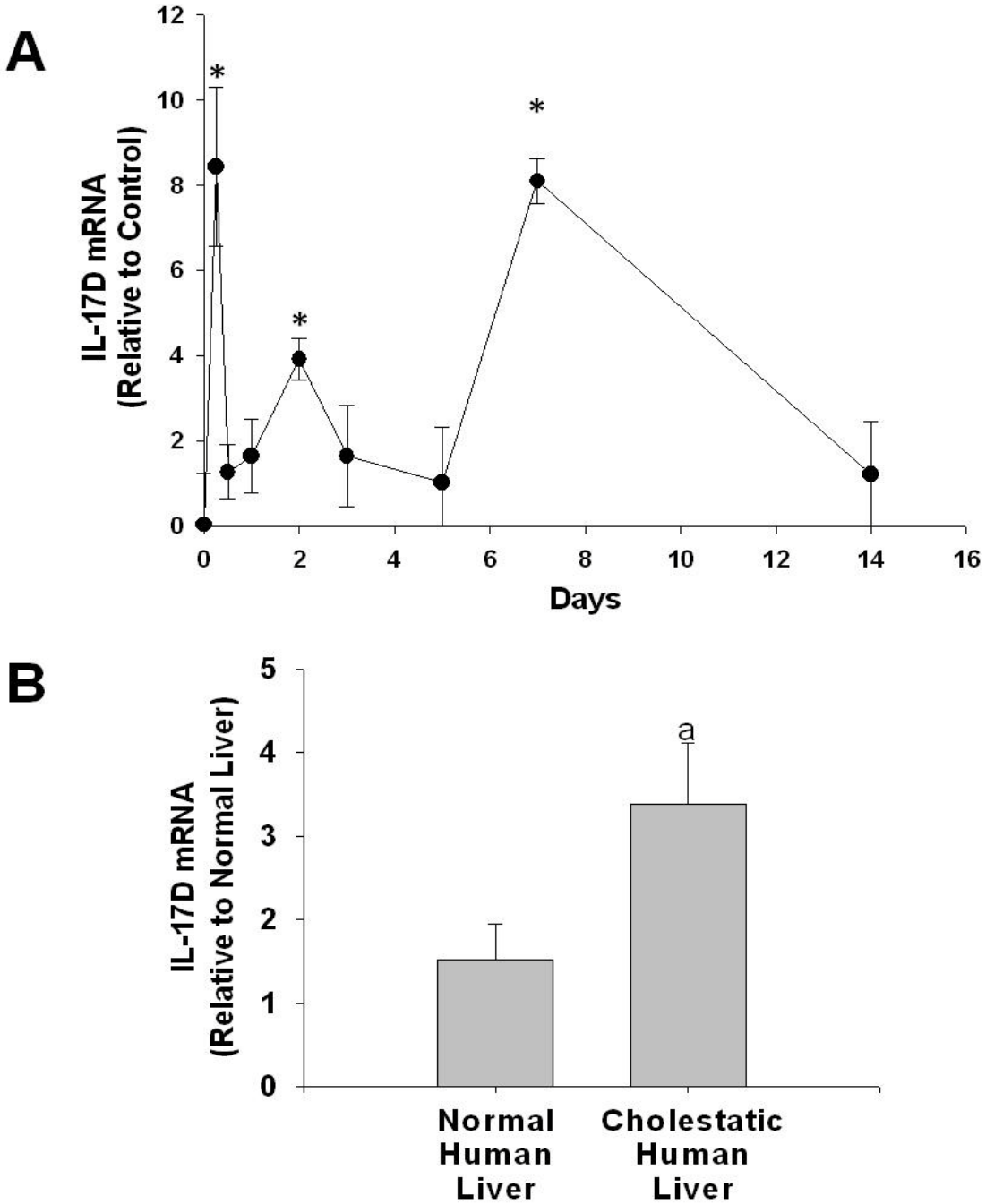

Figure 4.2 IL-17D mRNA levels in cholestatic mice and humans. (A) Mice were subjected to BDL or sham operation. 0, 6, 12, 24, 48 hours, 3, 5, 7 and 14 days later, mRNA levels of IL-17D were quantified by real-time PCR. *Significantly different ($p < 0.05$) from sham-operated mice. (B) Real-time PCR was used to measure mRNA levels of IL-17D in normal human livers and in cholestatic livers from patients with primary biliary cirrhosis and primary sclerosing cholangitis. [a]Significantly different ($p < 0.05$) from normal liver.

Figure 4.3

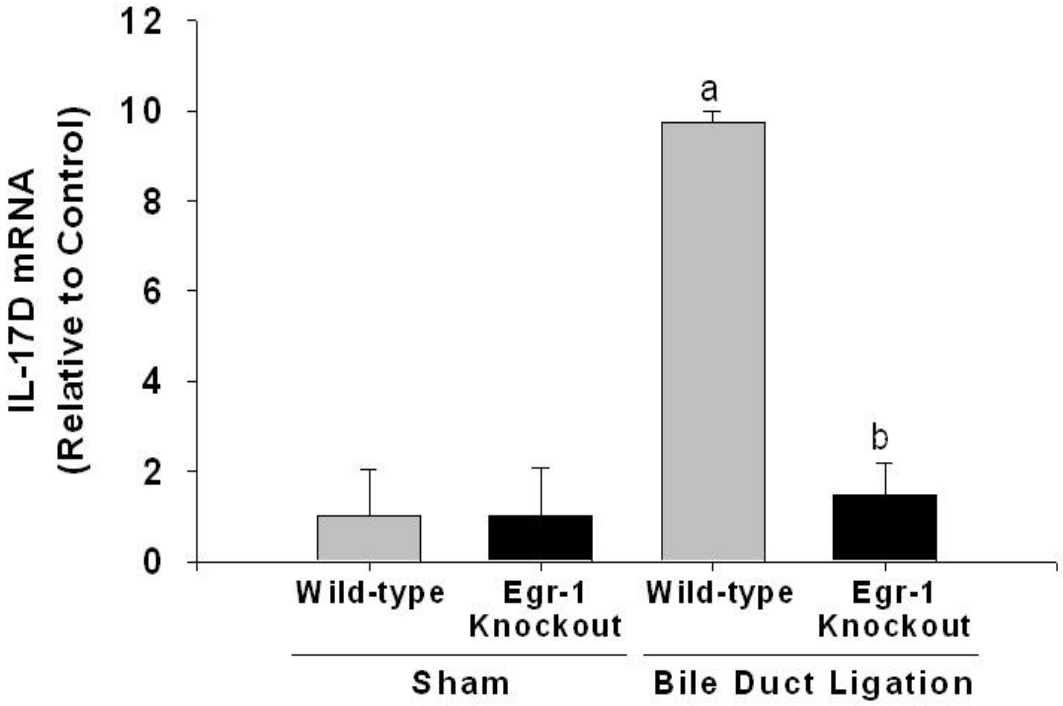

Figure 4.3 Upregulation of IL-17D in the liver after BDL requires Egr-1. Wild-type or Egr-1 knockout mice were subjected to BDL or sham operation. Seven days, later mRNA levels of IL-17D were quantified by real-time PCR. [a]Significantly different ($p < 0.05$) from sham-operated mice. [b]Significantly different ($p < 0.05$) from wild-type mice subjected to BDL

Figure 4.4

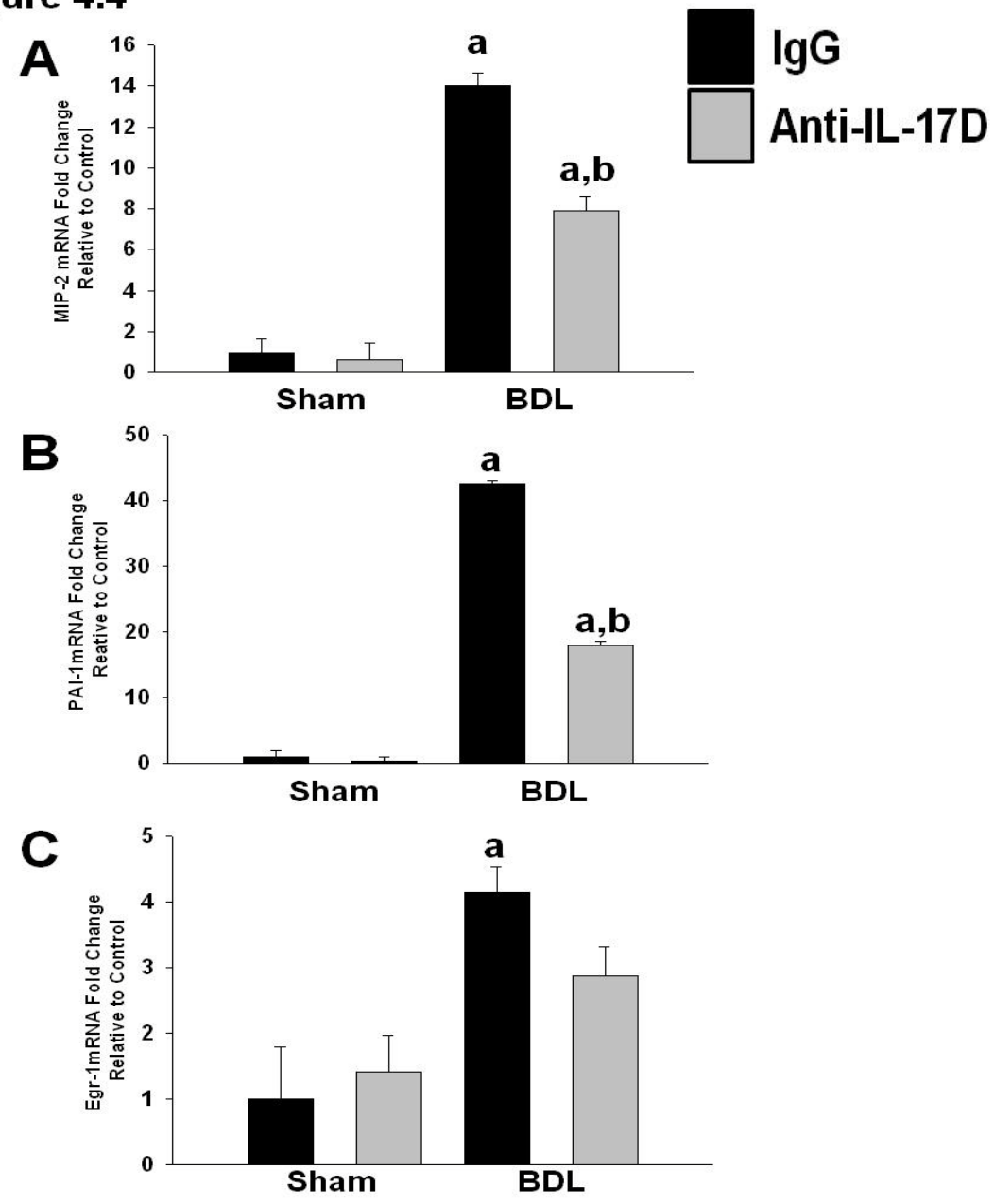

Figure 4.4 Role of IL-17D in upregulation of PAI-1, MIP-2 and Egr-1 in the liver during cholestasis. One hour before mice were subjected to BDL or sham operation, they were treated with 15 µg anti-IL-17D antibody or isotype control antibody. Forty-eight hours later, mRNA levels of (A) PAI-1, (B) MIP-2 and (C) Egr-1 quantified by real-time PCR. [a]Significantly different ($p < 0.05$) from sham-operated mice. [b]Significantly different ($p < 0.05$) from IgG-treated mice subjected to BDL.

Figure 4.5

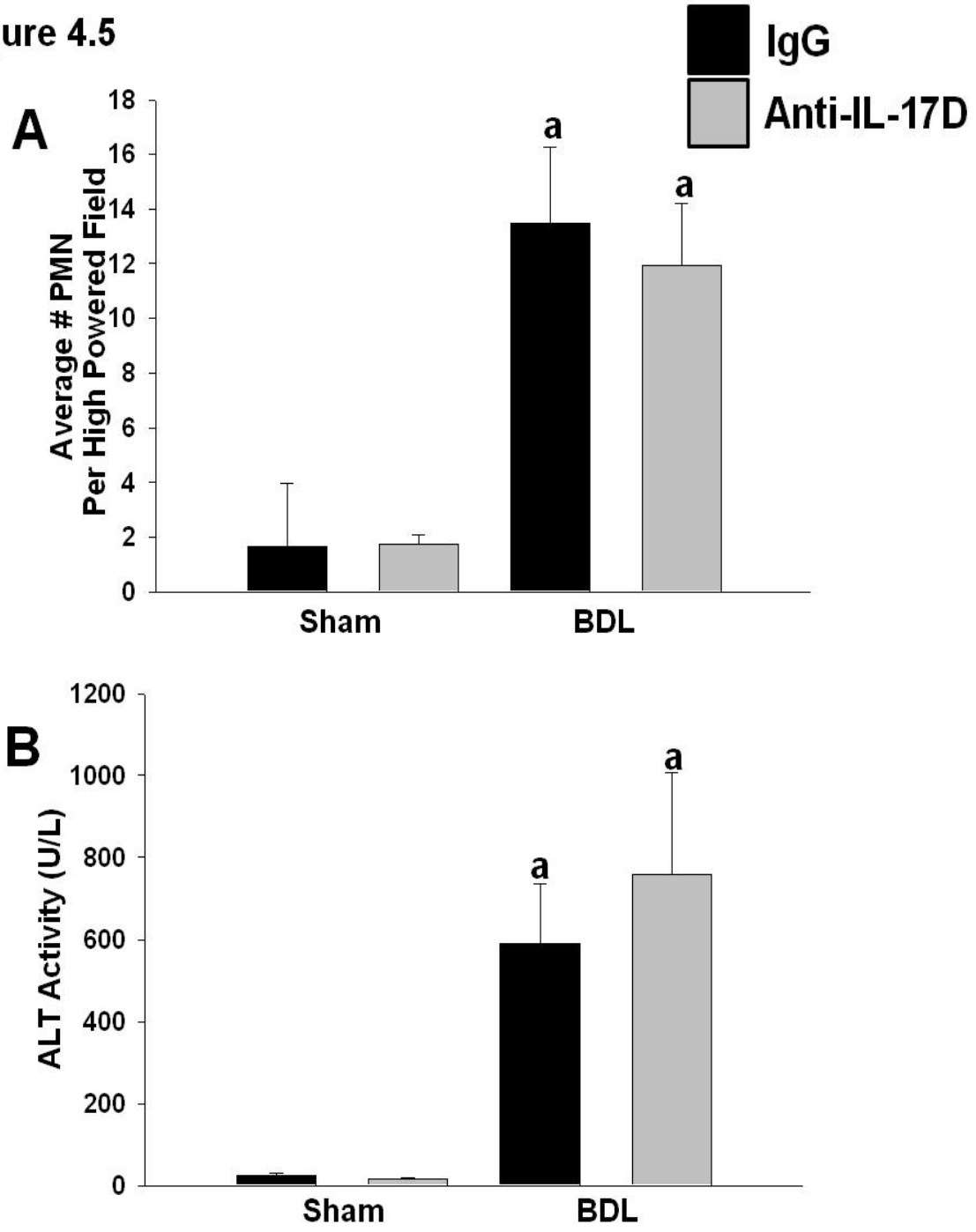

Figure 4.5 Effect of anti-IL-17D antibody on liver injury and inflammation during cholestasis. One hour before mice were subjected to BDL or sham operation, they were treated with 15 μg anti-IL-17D antibody or isotype control antibody. Forty-eight hours later, (A) hepatic neutrophil accumulation and (B) ALT levels were measured. [a]Significantly different ($p < 0.05$) from sham-operated mice.

Figure 4.6

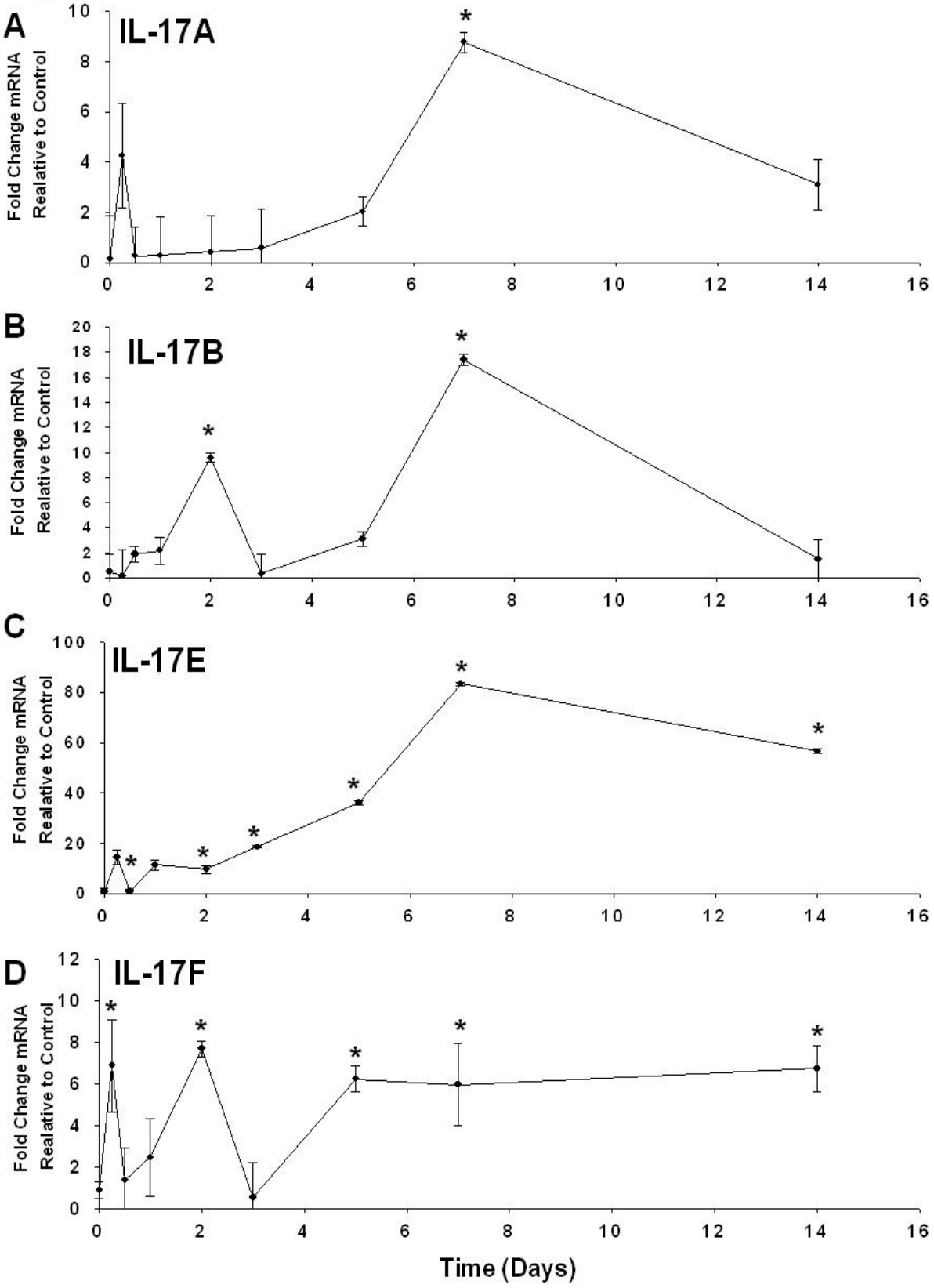
A
IL-17A
Fold Change mRNA Realative to Control
B
IL-17B
Fold Change mRNA Realative to Control
C
IL-17E
Fold Change mRNA Realative to Control
D
IL-17F
Fold Change mRNA Realative to Control
Time (Days)

Figure 4.6 Expression of IL-17A, IL-17B, IL-17E and IL-17F in the liver after BDL. Mice were subjected to BDL or sham operation. 0, 6, 12, 24, 48 hours, 3, 5, 7 and 14 days later, mRNA levels of (A) IL-17A, (B) IL-17B, (C) IL-17E and (D) IL-17F were quantified by real-time PCR. [a]Significantly different ($p < 0.05$) from sham-operated mice.

CHAPTER 5

Role of Interleukin 17A and Interleukin Receptor A in the Development of Liver Injury and Inflammation During Obstructive Cholestasis

5.1 Abstract

Inflammation contributes to liver injury during cholestasis; however the pathways that regulate inflammation are not fully understood. Interleukin-17A (IL-17A) is an important regulator of inflammation in several tissues. In a previous study, we demonstrated that IL-17A is upregulated in the liver during cholestasis. Similarly, IL-17A levels are increased in patients with primary biliary cirrhosis (PBC), and in IL-2 receptor alpha knockout mice, a murine model of PBC. The functional role of IL-17A in obstructive cholestasis, however, is unknown. To this end we tested the hypothesis that neutralization of IL-17A during bile duct ligation (BDL) would attenuate liver inflammation and injury. Our findings demonstrated that neutralization of IL-17A did not prevent upregulation of proinflammatory cytokines or chemokines after BDL. Similarly, neutralization of IL-17A did not affect liver injury or fibrosis after BDL. IL-17A and IL-17F activate the same receptor complex (i.e., IL-17RA-IL-17RC heteromeric complexes) and have similar functions. Since IL-17F is also upregulated after BDL, we determined whether neutralization of IL-17RA would affect liver injury and inflammation after BDL. Similar to IL-17A, neutralization of IL-17RA had no effect on liver injury, inflammation, or fibrosis. In conclusion, these studies indicate that IL-17A and IL-17RA are not important for liver injury and inflammation during obstructive cholestasis.

5.2 Introduction

We demonstrated previously that bile acids increase expression of many cytokines, chemokines and other proinflammatory mediators in hepatocytes, including the cytokine interleukin-17D. However, pretreatment of bile duct ligated (BDL) mice with an anti-IL-17D antibody had little effect on liver injury or inflammation. This suggested that other cytokines, such as other IL-17 family members, may compensate for the loss of IL-17D. Consistent with this, IL-17A, IL-17B, IL-17E, and IL-17F were all upregulated in the liver after BDL. Since many

of these cytokines have similar functions as IL-17D, they may also contribute to regulation of inflammation during cholestasis and thus mask the beneficial effects of IL-17D inhibition.

IL-17A is an important regulator of neutrophil–dependent inflammation in various tissues. For example, in the lung IL-17A stimulates neutrophil accumulation in a macrophage inflammatory protein-2 (MIP-2)-dependent manner (Laan et al., 1999; Weaver et al., 2007). Studies indicate that IL-17A may be an important regulator of hepatic inflammation. Exposure of hepatocytes to IL-17A increases expression of several genes, including chemokines, such as keratinocyte-derived chemokine (KC) and monocyte chemotactic protein-1 (MCP-1) (Sparna et al., 2010). Similarly, treatment of human bile duct epithelial cells (BDECs) with IL-17A increases expression of numerous cytokines (i.e., IL-6, IL-1β, IL-23p19, and IL-23/IL-12 p40) and chemokines (i.e., KC, MIP-2, MIP-2β, MCP-1, and MIP-3) (Harada et al., 2009). Studies indicate that IL-17A may contribute to inflammatory liver injury in alcoholic liver disease and in concanavalin A-induced hepatitis (Nagata et al., 2008; Lemmers et al., 2009; Zhang et al., 2010). IL-17A mediates its effects by binding to a receptor composed of a heteromeric complex of IL-17 receptor A and IL-17 receptor C. Both receptors are expressed on several cell types in the liver including, bile duct epithelial cells, hepatic stellate cells, and Kupffer cells. Furthermore, IL-17A stimulates proinflammatory signaling in hepatocytes suggesting that these cells may also express the receptor (Toy et al., 2006; Patel et al., 2007; Ge and You, 2008; Lemmers et al., 2009; Lafdil et al., 2010). Although it is not known whether IL-17A contributes to liver inflammation during obstructive cholestasis, IL-17A levels are increased in the livers of patients with primary biliary cirrhosis (PBC), a form of chronic cholestasis, and increased in IL-2Rα knockout mice, a murine model of PBC (Harada et al., 2009; Lan et al., 2009). In addition, it was recently demonstrated that neutralization of IL-17A limits liver injury in mice treated with α-naphthylisothiocyanate (ANIT), a chemical that causes cholestasis, suggesting that IL-17A may be an important regulator of inflammation during cholestasis (Kobayashi et al., 2010).

Therefore, we tested the hypothesis that IL-17A is important for inflammation and liver injury in mice with obstructive cholestasis.

5.3 Materials and methods:

5.3.1 Animal Care. C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used for all studies. Mice were maintained on a 12-h light/dark cycle under controlled temperature (18-21ºC) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed ad libitum. All procedures on animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health and were approved by the institutional IACUC committee at the University of Kansas Medical Center.

5.3.2 Bile Duct Ligation. Male, C57BL/6 8-12 weeks of age, were anesthetized with isoflurane. A midline laparotomy was performed and the bile duct ligated with 3-0 surgical silk. The abdominal incision was closed with sutures, and the mice received 0.2 mg/kg Buprenex by subcutaneous injection. For studies with anti-IL-17A, mice received 100 μg rat-anti-mouse-IL-17A (R&D systems, Minneapolis, MN) or rat IgG_{2A} (R&D systems) isotype control dissolved in saline by intraperitoneal injection, 1 hour before surgery. The mice received additional injections of 50 μg on days 3 and 6 after BDL. For studies with anti-IL-17 receptor A (IL-17RA) antibody, mice received 100 μg anti-muIL-17ra-x-muIgG (a gift from Amgen, Thousand Oaks, CA) or rat IgG control (Bio X Cell West Lebanon, NH) dissolved in saline by intraperitoneal injection, 1 hour before surgery. For the 9 day time point, the animals received follow-up injections of 50 μg on days 3 and 6.

5.3.3 Immunohistochemistry. Immunohistochemistry for type I collagen (Abcam, Cambridge, MA) and neutrophils (AbD Serotec, Raleigh, NC) was performed as described by us previously, however Alexa Fluor 594 goat anti-rat was used as a secondary antibody for

neutrophil staining and Alexa Flour 594 goat anti-rabbit (Invitrogen Carlsbad, CA) was used as a secondary antibody for type-I collagen (Kim et al., 2006).

5.3.4 Real-time Polymerase Chain Reaction. RNA was isolated from livers and hepatocytes using TRI reagent (Sigma Chemical Company, St. Louis, MO), and reverse transcribed into cDNA as described by us previously (Kim et al., 2006). Real-time PCR was used to quantify mRNA levels of IL-6, ICAM-1, Cxcl5, PAI-1, MIP-2, Egr-1, type-1 collagen, α-smooth muscle actin (α-SMA) as well as 18S and rpl13a on an Applied Biosystems Prism 7900 Real-time PCR Instrument (Applied Biosystems, Foster City, CA) using the SYBR green DNA PCR kit (Applied Biosystems) as described (Kim et al., 2006). The sequences of the primers were as follows: IL-6 Forward: 5'-ACC AGA GGA AAT TTT CAA TAG GC-3' IL-6 Reverse: 5'-TAG TGC ACT TGC AGA AAA CA-3'; ICAM-1 Forward: 5'-AAC AGT TCA CCT GCA CGG AC-3' ICAM-1 Reverse: 5'-GTC ACC GTT GTG ATC CCT G-3'; Cxcl5 Forward: 5'-GCT GGC ATT TCT GTT GCT GTT-3' Cxcl5 Reverse: 5'-CGG TTA AGC AAA CAC AAC GCA-3'; PAI-1 Forward: 5'-AGT CTT TCC GC CAA GAG CA-3' PAI-1 Reverse: 5'-ATC ACT TGCC CCC ATG AAG AG-3'; MIP-2 Forward: 5'-CCT CAA CGG AAG AAC CAA A G-3' MIP-2 Reverse: 5'- CTC AGA CAG CGA GGC ACA TC-3'; Egr-1 Forward: 5'-GGC AGA GGA AGA CGA TGA AG-3' Egr-1 Reverse:5'-GAC GAGTTATCC CAG CCA AA-3'; Type I Collagen Forward: 5'-TGT GTT CCC TAC TCA GCC GTC T-3' Type I Collagen Reverse: 5'- CAT CGG TCA TGC TCT CTC CAA-3'; α-SMA Forward: 5'- CCA CCG CAA ATG CTT CTA AGT-3' α-SMA Reverse: 5'- GGC AGG AAT GAT TTG GAA AGG-3' 18S Forward: 5'-TTG ACG GAA GGG CAC CAC CAG-3' 18S Reverse: 5'-GCA CCA CCA CCC ACG GAA TCG-3' and Rpl 13a Forward: 5'-ACA AGA AAA AGC GGA TGG TC-3' Rpl 13a Reverse: 5'- TTC TCC TCC AGA GTG GCT GT-3'.

5.3.5 Quantification of Liver Injury. ALT was measured in serum using a commercially available kit as described previously (Kim et al., 2006).

5.3.6 Statistical Analysis. Results are presented as the mean ± SEM. Data were

analyzed by Analysis of Variance (ANOVA). ANOVAs were performed on log X-transformed data in instances in which variances were not homogenous. Comparisons among group means were made using the Fisher LSD test. The criterion for significance was $p < 0.05$ for all studies.

5.4 Results

5.4.1 Inhibition of IL-17A during cholestasis has no effect on proinflammatory gene expression or markers of liver fibrosis. To elucidate the function of IL-17A during obstructive cholestasis we treated mice with 100 µg anti-IL-17A antibody by intraperitoneal injection one hour before BDL followed by 50 µg injections 3 and 6 days after BDL. Previous studies have used this dose of anti-IL-17A antibody to inhibit IL-17A function in mice (Chung et al., 2003; Miyamoto et al., 2003). The expression of IL-6, MIP-2, Cxcl5, PAI-1 and ICAM-1 were all elevated to a similar extent in IgG control-treated mice and anti-IL-17A-treated mice after BDL (Figure 5.1).

To evaluate liver fibrosis, we next measured mRNA levels of α-SMA and type I collagen. mRNA levels of α-SMA and type I collagen were elevated after BDL, however there was no difference between anti-IL-17A antibody-treated and IgG control-treated mice (Figure 5.2).

5.4.2 Neutralization of IL-17A during cholestasis does not attenuate liver injury. Serum ALT levels were elevated after BDL in both IgG control-treated mice and in mice that received the anti-IL-17A antibody. There was no difference in ALT levels between the two groups (Figure 5.3).

5.4.3 Neutralization of IL-17RA does not affect proinflammatory gene expression during obstructive cholestasis. Neutralization of IL-17A had no effect on overall injury during BDL (Figure 5.3). Because IL-17F activates the same receptor as IL-17A and is able to induce similar genes, we neutralized IL-17RA, a receptor critical for both IL-17A and IL-17F signaling.

Mice were treated with either IgG control or an anti-IL-17RA antibody during BDL. Three and 9 days after BDL, mRNA levels of proinflammatory genes were measured. mRNA levels of MIP-2, Cxcl5, and ICAM-1 were all increased after BDL. Levels of these inflammatory mediators were not affected by IL-17RA neutralization at either 3 or 9 days (Figures 5.4 and 5.5). Similar results were obtained for IL-6 at 9 days (Figure 5.5). mRNA levels of PAI-1 were increased 9 days after BDL in the IgG control group (Figure 5.4 C). Interestingly, PAI-1 mRNA levels were elevated further with anti-IL-17RA antibody treatment (Figure 5.4 C).

5.4.4 Liver injury and inflammation are not affected by IL-17RA neutralization during BDL. We next determined the effects of IL-17RA neutralization on liver injury and inflammation during cholestasis. ALT levels were measured 3 and 9 days after BDL. ALT levels were elevated to a similar extent in IgG control treated animals and anti-IL-17RA antibody treated animals after BDL (Figure 5.6 A and C). Similarly, hepatic neutrophil numbers were not different between IgG treated or anti-IL-17RA antibody treated animals subjected to BDL (Figure 5.6 B and D).

5.4.5 Liver fibrosis is unaltered by neutralization of IL-17RA during obstructive cholestasis. To examine the function of IL-17RA in liver fibrosis during BDL, mRNA levels of α-SMA and type I collagen as well as type I collagen protein were measured 9 days after BDL. mRNA levels of α-SMA and type I collagen were increased in the livers of control IgG treated mice subjected to BDL (Figure 5.7). mRNA levels of α-SMA and type I collagen trended higher in anti-IL-17RA treated mice, however, this was not significantly different from control IgG treated mice (Figure 5.7). Similar results were observed for type I collagen protein (Figure 5.7).

5.5 Discussion

Several studies have identified IL-17A as a key mediator of neutrophil-dependent injury in several tissues (Jovanovic et al., 1998; Laan et al., 1999; Miyamoto et al., 2003; Dong, 2008; Nagata et al., 2008; Harada et al., 2009; Lan et al., 2009; Lemmers et al., 2009; Kobayashi et al., 2010; Lafdil et al., 2010). We recently demonstrated that IL-17A is upregulated in the livers of mice after BDL (Figure 4.6, Chapter 4). In this model of obstructive cholestasis, neutrophils accumulate in the liver and exacerbate injury (Gujral et al., 2003; Gujral et al., 2004a; Kim et al., 2006). Accordingly, in the present studies, we tested the hypothesis that IL-17A is required for neutrophilic inflammation during cholestasis. The results demonstrated that neutralization of IL-17A had little effect on the overall pathology of cholestasis. One possible explanation for this is that IL-17A and IL-17F activate the same receptor (Wright et al., 2008), and our previous studies demonstrated that IL-17F is also upregulated in BDL mice (Figure 4.6. Chapter 4). Similar to IL-17A, IL-17F increases expression of MIP-2 and IL-6 in the lungs of mice (Oda et al., 2005). Furthermore, IL-17F stimulates neutrophil accumulation in the lung, much like IL-17A (Oda et al., 2005), and studies have shown that loss of either IL-17A or IL-17F alone is not sufficient to attenuate inflammation is some tissues (Leppkes et al., 2009). Accordingly, these studies suggested that neutralization of IL-17A alone in BDL mice may not be sufficient to prevent an inflammatory response. Therefore, we treated mice with an anti-IL-17RA antibody which abrogates signaling by both IL-17A and IL-17F.

Surprisingly, rather than reducing liver injury and inflammation during cholestasis, neutralization of IL-17RA increased PAI-1 mRNA levels and caused a trend towards an increase in liver fibrosis. These data suggest IL-17A and IL-17F do not contribute to regulation of inflammation in the liver during obstructive cholestasis. A complicating factor in this conclusion, however, is that IL-17E also requires IL-17RA for signaling. IL-17E binds to a heteromeric complex of IL-17RA and IL-17RB (Rickel et al., 2008). After activating this receptor complex, IL-17E typically stimulates a Th2 immune response (Fort et al., 2001; Hurst et al., 2002). IL-17E

has been shown to inhibit the function of Th17 cells and limit the induction of IL-17A by upregulating Th2 cytokines (Kleinschek et al., 2007). In addition, IL-17E inhibits activation of monocytes by IL-17A by upregulating suppressor of cytokine signaling-3 (SOCS-3) (Caruso et al., 2009). In our studies, we previously demonstrated that IL-17E is upregulated in the liver after BDL (Figure 4.6, Chapter 5), and it is possible that upregulation of IL-17E limits inflammation after BDL. Accordingly, it is possible that inhibiting the anti-inflammatory actions of IL-17E by treatment with anti-IL-17RA, may mask the beneficial effects of inhibiting IL-17A and IL-17F signaling. Further studies are needed, however, to test this possibility.

Overall these studies support the need for further investigation into the function of IL-17 family members during cholestasis. However, any further studies would need to be designed to elucidate the function of each specific family member in cholestasis. For example the contributions of IL-17A and IL-17F may be better defined by inhibiting IL-17RC rather than IL-17RA. IL-17RC is required for IL-17A and IL-17F function, but is not known to be necessary for signaling by any other IL-17 family members. These studies could provide detailed insight into the function of IL-17A and IL-17F in the development of liver inflammation during cholestasis.

FIGURES 5.6

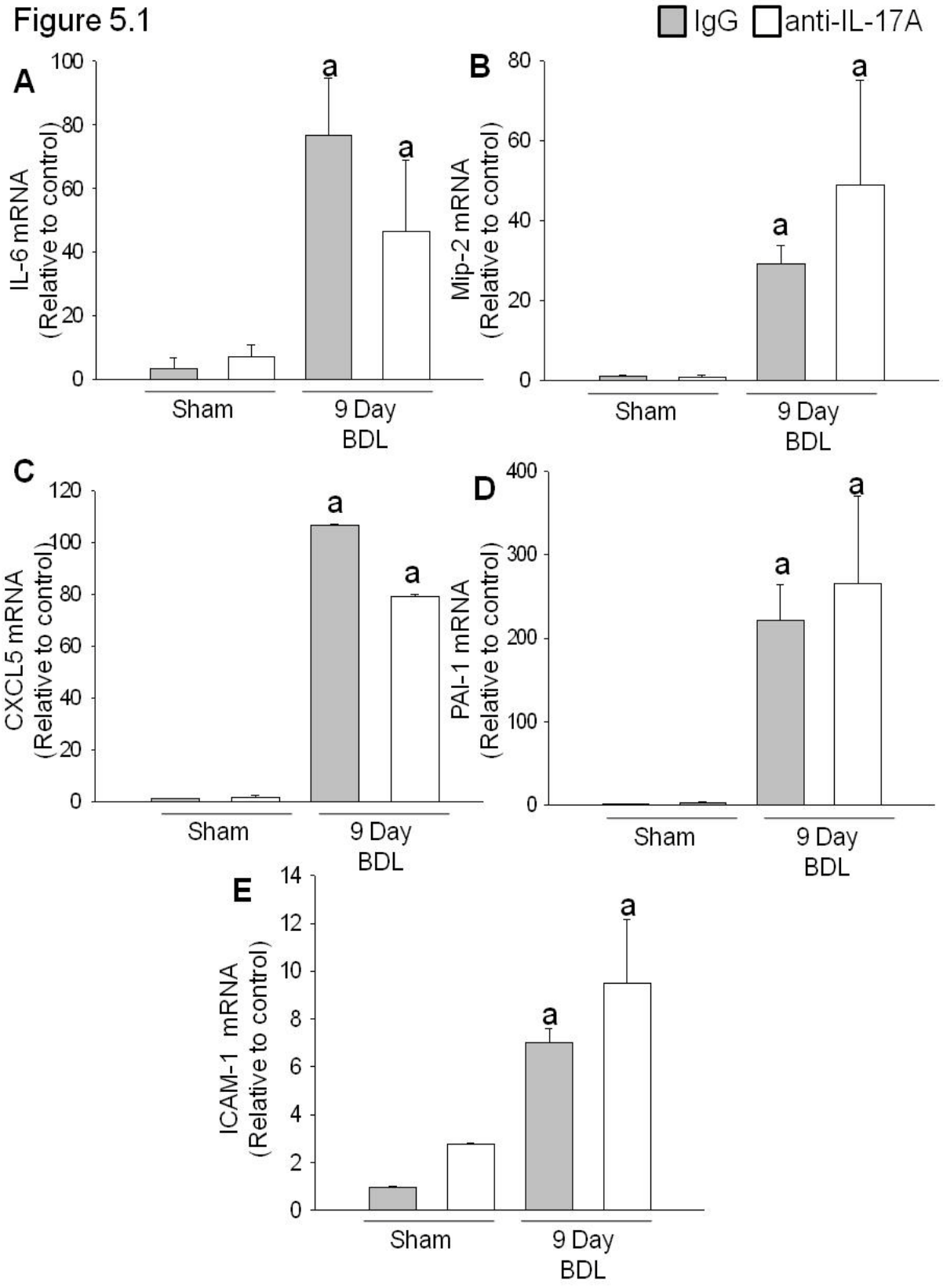
Figure 5.1
IgG
anti-IL-17A
A
IL-6 mRNA (Relative to control)
B
Mip-2 mRNA (Relative to control)
C
CXCL5 mRNA (Relative to control)
D
PAI-1 mRNA (Relative to control)
E
ICAM-1 mRNA (Relative to control)
Sham
9 Day BDL
a

Figure 5.1 Role of IL-17A in upregulation of proinflammatory mediators in the liver during cholestasis. One hour before mice were subjected to BDL or sham operation they were treated with 100 µg of anti-IL-17A antibody or IgG control with follow-up injections of 50 µg anti-IL-17A antibody or IgG control at 3 and 6 days after BDL. Nine days after BDL, mRNA levels of (A) IL-6, (B) MIP-2, (C) Cxcl5, (D) PAI-1 and (E) ICAM-1 were quantified by real-time PCR. Data are expressed as mean +/- SEM. [a]Significantly different ($p < 0.05$) from sham-operated mice.

Figure 5.2

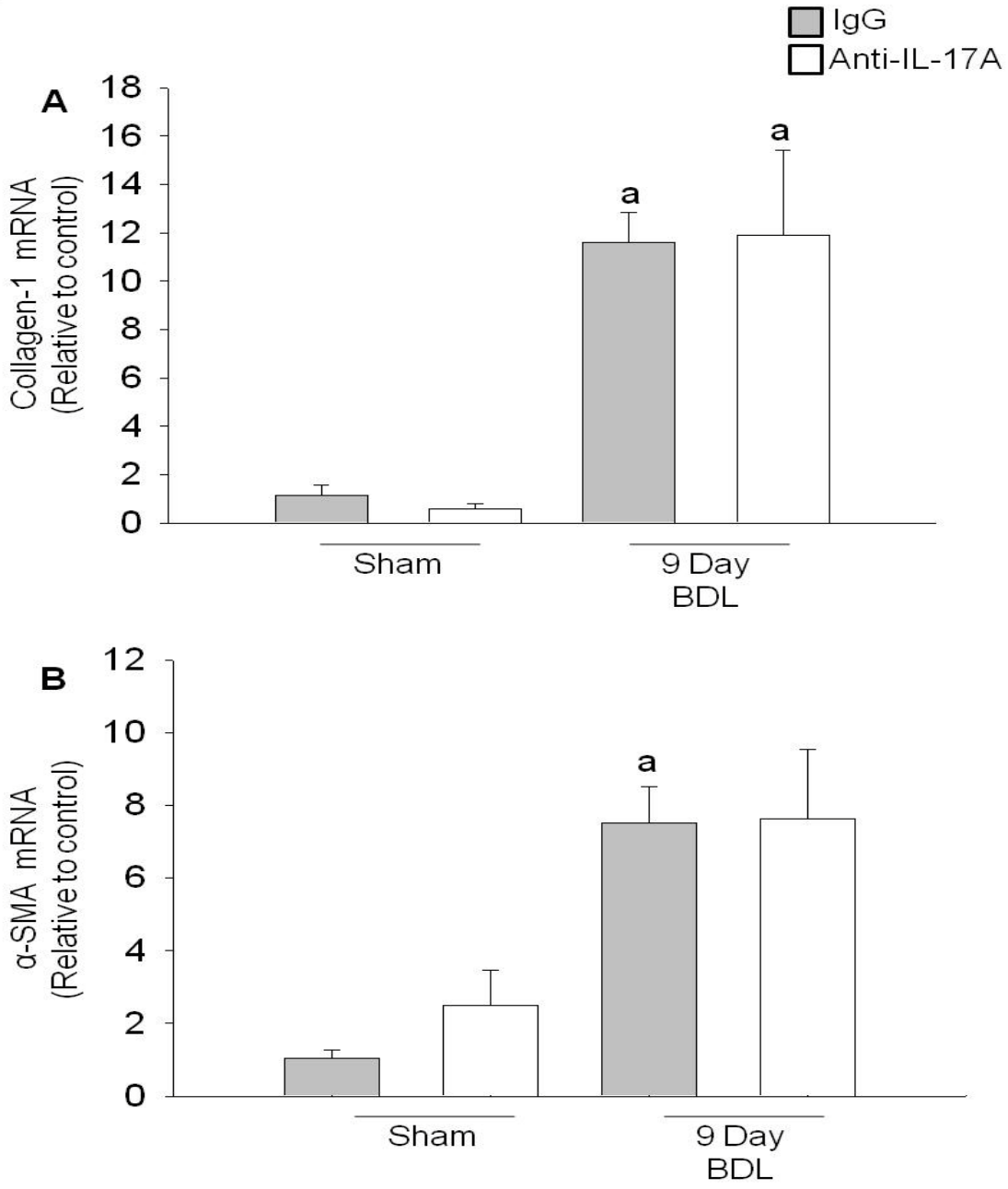

Figure 5.2 Role of IL-17A in the development of fibrosis in the liver during cholestasis. One hour before mice were subjected to BDL or sham operation, they were treated with 100 µg anti-IL-17A antibody or IgG control with follow-up injections of 50 µg anti-IL-17A antibody or IgG control at 3 and 6 days after BDL. Nine days after BDL, mRNA levels of (A) type-1 collagen and (B) α-SMA were quantified by real-time PCR. Data are expressed as mean +/- SEM..
[a]Significantly different ($p < 0.05$) from sham-operated mice.

Figure 5.3

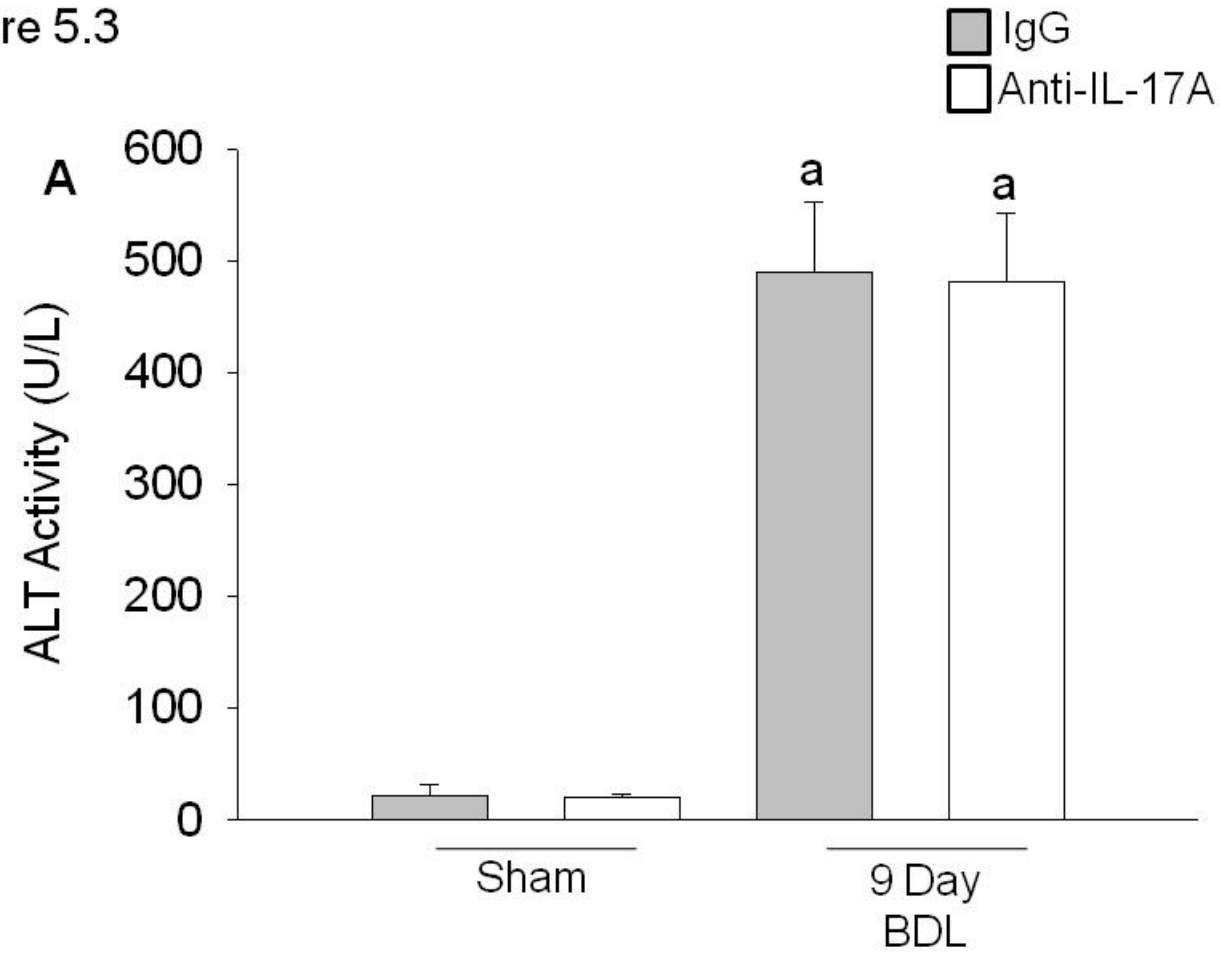

Figure 5.3 Role of IL-17A in liver injury during cholestasis. One hour before mice were subjected to BDL or sham operation, they were treated with 100 µg anti-IL-17A antibody or IgG control with follow-up injections of 50 µg anti-IL-17A antibody or IgG control at 3 and 6 days after BDL. Nine days after BDL, serum ALT activity was measured. Data are expressed as mean +/- SEM; [a]Significantly different ($p < 0.05$) from sham-operated mice.

Figure 5.4

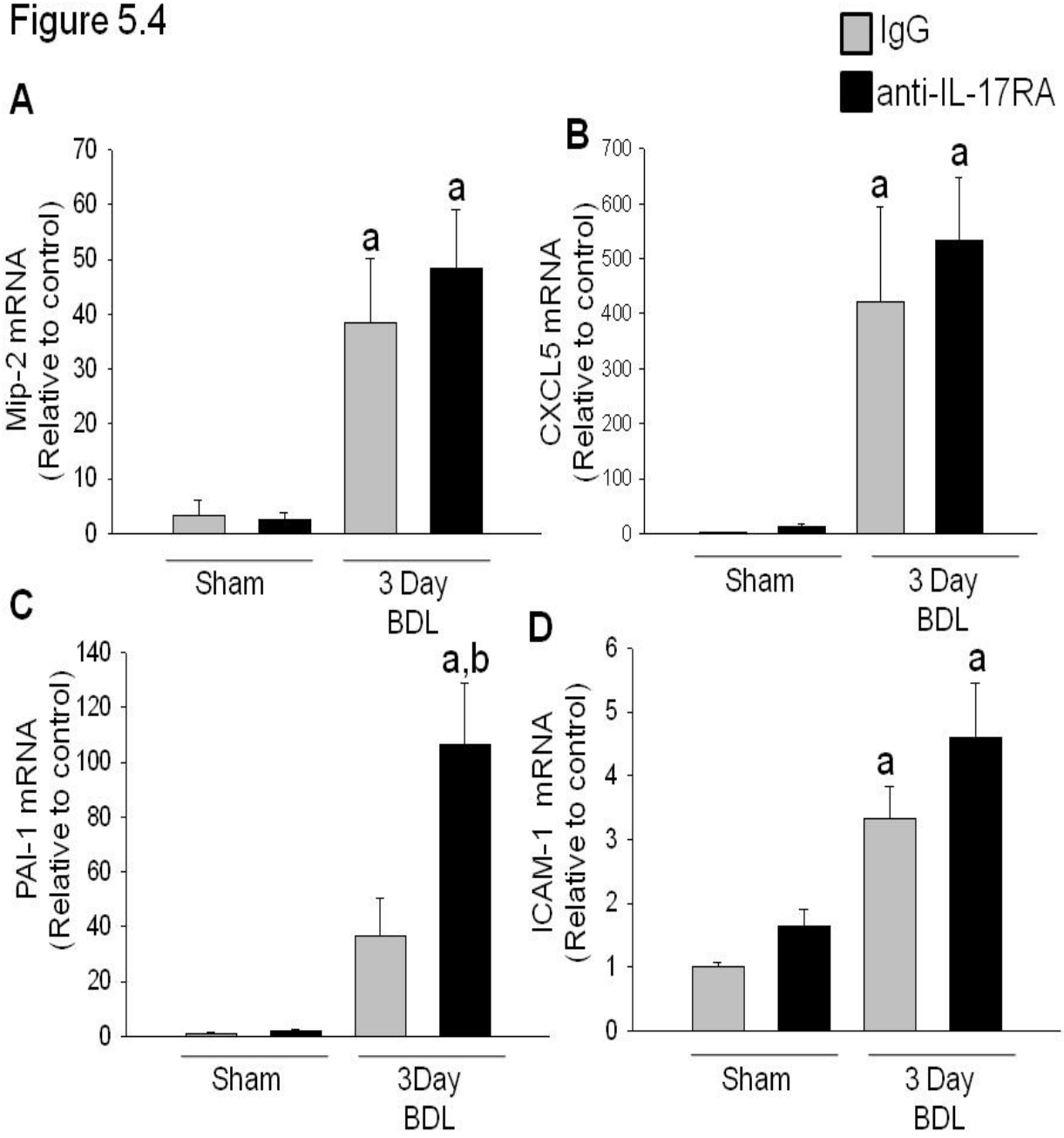

Figure 5.4 Role of IL-17RA in upregulation of proinflammatory mediators in the liver at 3 days after BDL. One hour before mice were subjected to BDL or sham operation, they were treated with 100 µg anti-IL-17RA antibody or control IgG. Three days after BDL, mRNA levels of (A) MIP-2, (B) Cxcl5, (C) PAI-1 and (D) ICAM-1 quantified by real-time PCR. Data are expressed as mean +/- SEM. [a]Significantly different ($p < 0.05$) from sham-operated mice. [b]Significantly different ($p < 0.05$) from IgG treated mice subjected to BDL.

Figure 5.5

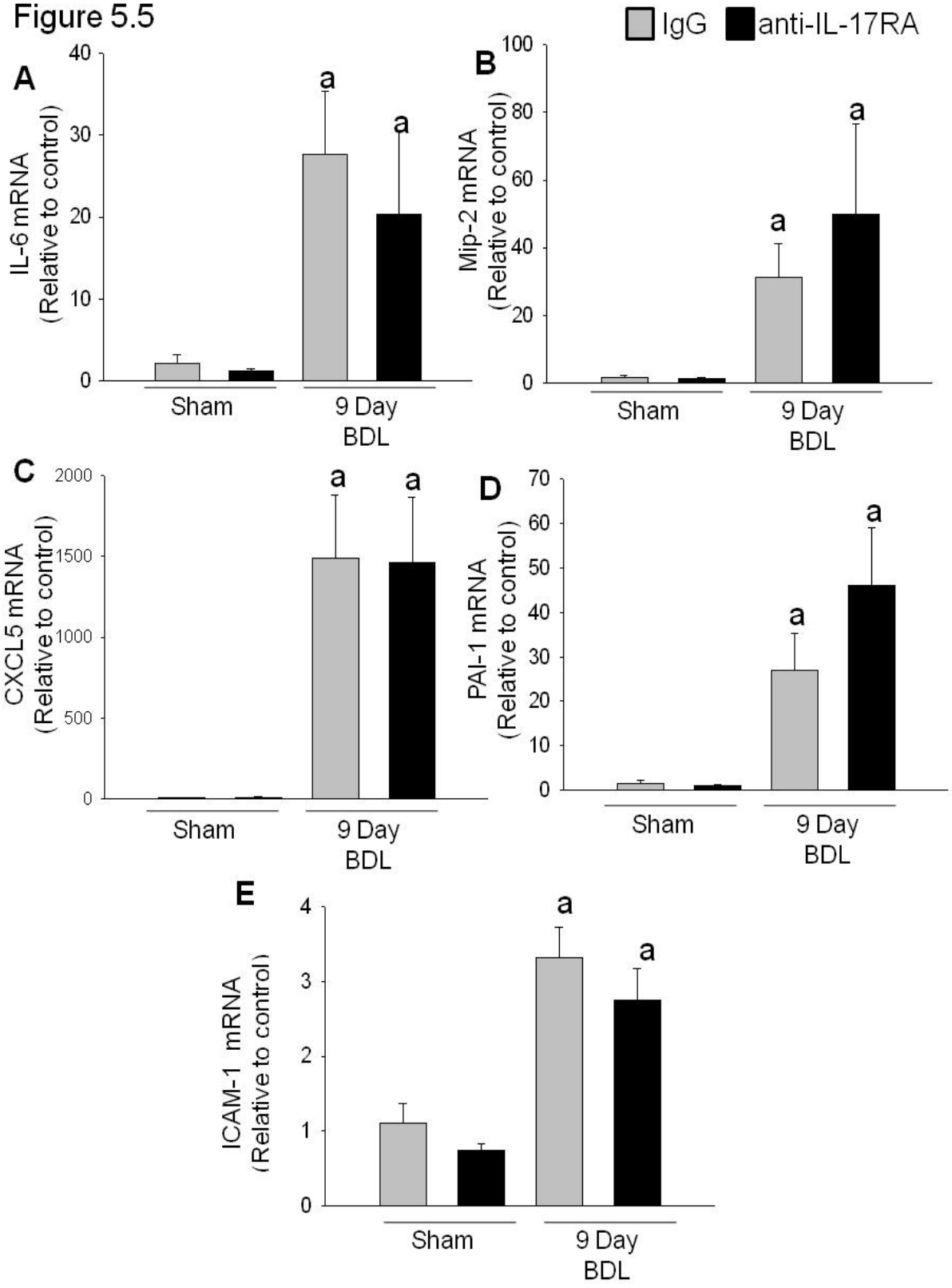
IgG
anti-IL-17RA
A
IL-6 mRNA (Relative to control)
40
30
20
10
0
a
a
Sham
9 Day BDL
B
Mip-2 mRNA (Relative to control)
100
80
60
40
20
0
a
a
Sham
9 Day BDL
C
CXCL5 mRNA (Relative to control)
2000
1500
1000
500
0
a
a
Sham
9 Day BDL
D
PAI-1 mRNA (Relative to control)
70
60
50
40
30
20
10
0
a
a
Sham
9 Day BDL
E
ICAM-1 mRNA (Relative to control)
4
3
2
1
0
a
a
Sham
9 Day BDL

Figure 5.5 Role of IL-17RA in upregulation of proinflammatory mediators in the liver at 9 days after BDL**.** One hour before mice were subjected to BDL or sham operation, they were treated with 100 µg of anti-IL-17RA antibody or IgG control with follow-up injections of 50 µg anti-IL-17A antibody or IgG control 3 and 6 days after BDL. Nine days after BDL, mRNA levels of (A) IL-6, (B) MIP-2, (C) Cxcl5, (D) PAI-1 and (E) ICAM-1 were quantified by real-time PCR. Data are expressed as mean +/- SEM. [a]Significantly different ($p < 0.05$) from sham-operated mice.

Figure 5.6

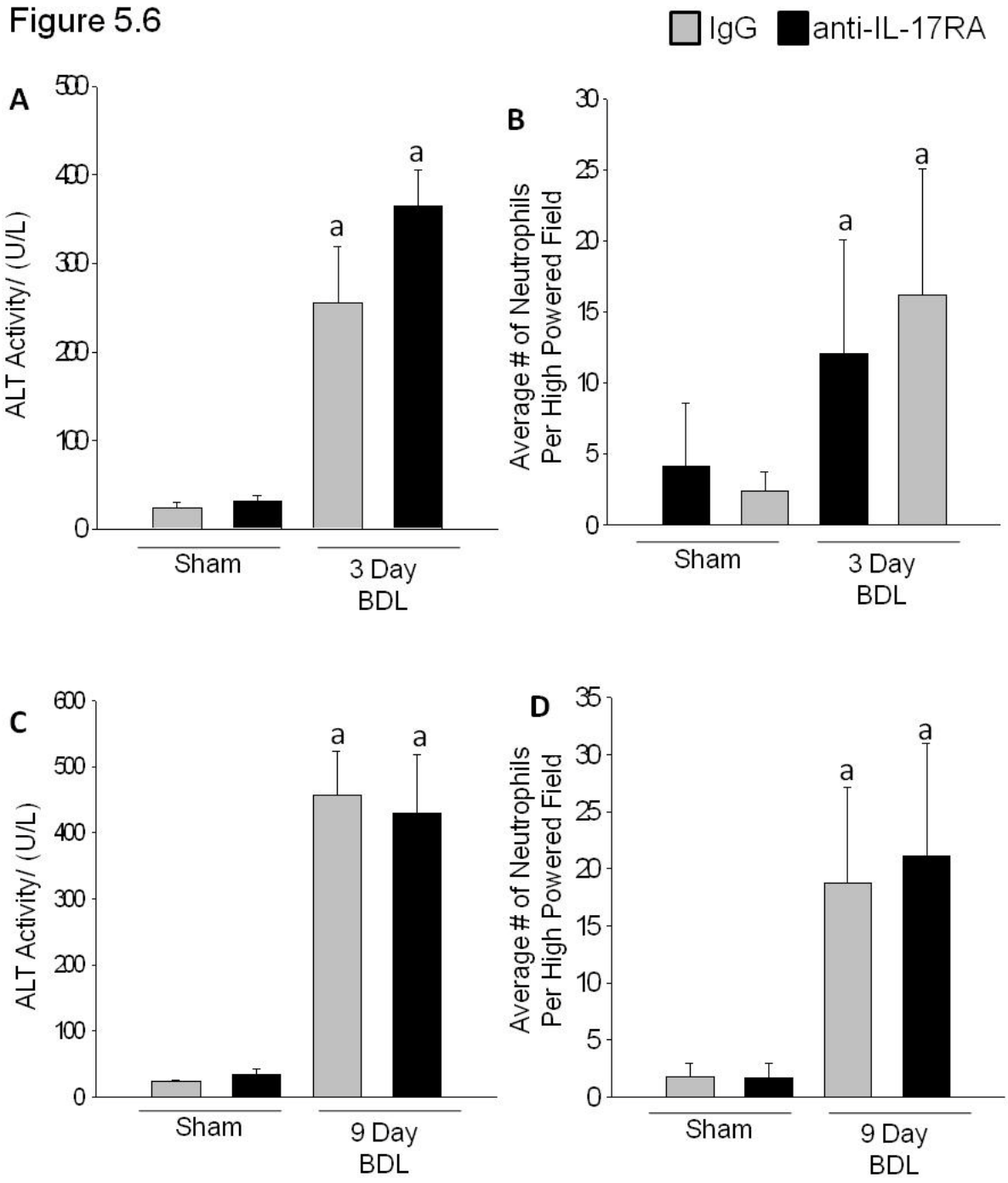

Figure 5.6 Role of IL-17RA in liver injury and inflammation during cholestasis. One hour before mice were subjected to BDL or sham operation, they were treated with 100 µg anti-IL-17RA antibody or IgG control. For the 9 day study, follow-up injections of 50 µg of anti-IL-17A antibody or IgG were given at 3 and 6 days after BDL. Three or 9 days after BDL (A and C) ALT levels were measured and neutrophils were quantified in the liver (B and D). Data are expressed as mean +/- SEM. [a]Significantly different ($p < 0.05$) from sham-operated mice.

Figure 5.7

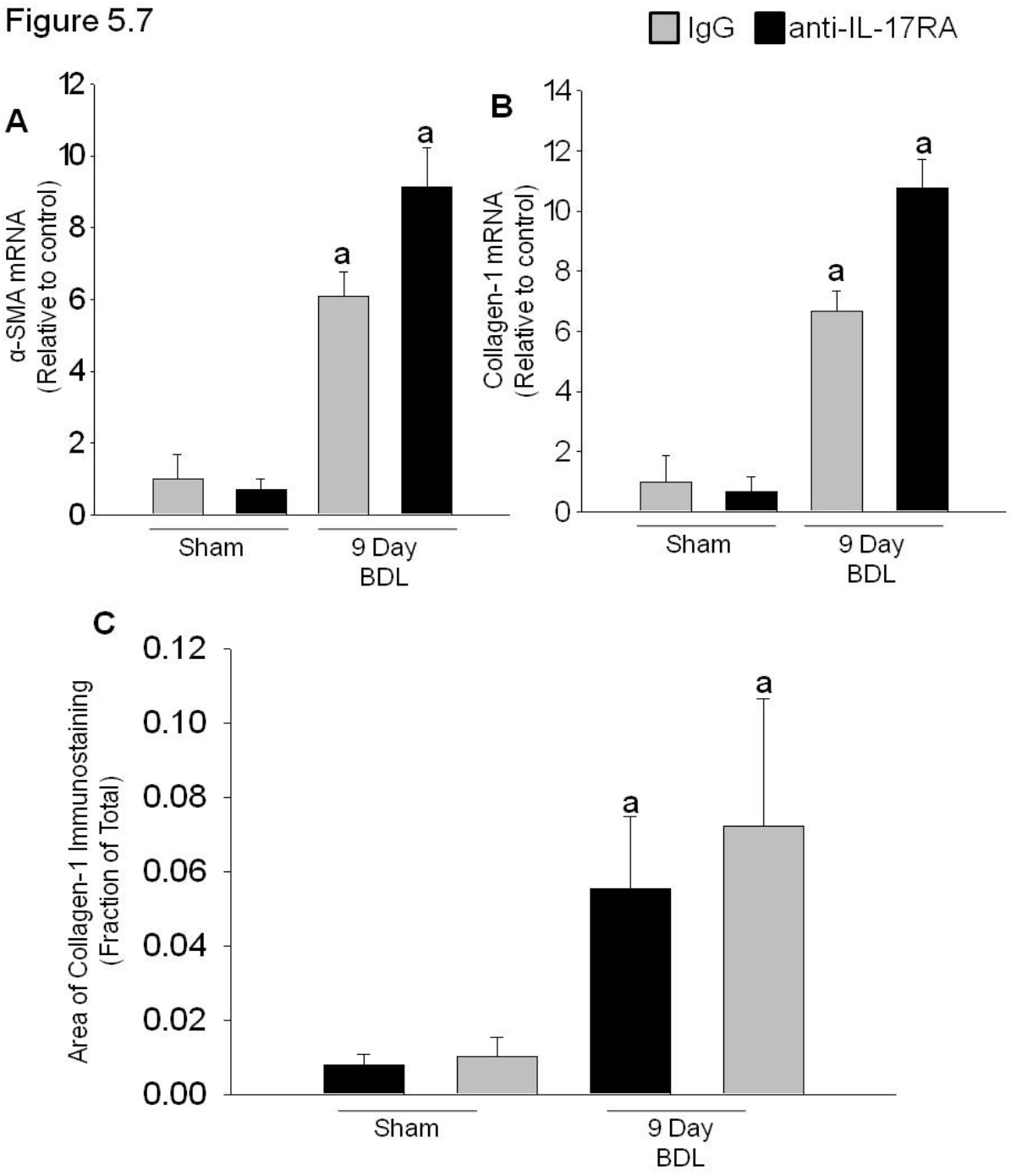

Figure 5.7 Role of IL-17RA in liver fibrosis during cholestasis. One hour before mice were subjected to BDL or sham operation, they were treated with 100 µg of anti-IL-17RA antibody or IgG control with follow-up injections of 50 µg anti-IL-17A antibody or IgG at 3 and 6 days after BDL. Nine days after BDL, mRNA levels of (A) α-SMA or (B) type I collagen were quantified by real-time PCR and (C) % area of type I collagen positive staining was quantified in sections of immunohistochemically-stained livers. [a]Significantly different ($p < 0.05$) from sham-operated mice

CHAPTER 6

DISCUSSION

6.1 Results and Summary

Cholestasis is currently a condition with limited therapies. The only FDA approved drug is the bile acid ursodeoxycholic acid which is not always effective and may be contraindicated in some forms of cholestasis (Hsu et al., 1997; Pares et al., 2006; ter Borg et al., 2006; Silveira and Lindor, 2008; Sinakos et al., 2010). If the cholestatic condition does not respond to therapy a liver transplant is the only remaining option available (ter Borg et al., 2006; Silveira and Lindor, 2008). The availability of transplantable livers is low compared to the numbers of patients requiring transplantation, and in cases of primary biliary cirrhosis and primary sclerosing cholangitis there is a possibility that the disease will reoccur (Tamura et al., 2008; Patkowski et al., 2010). Because of these factors, research is necessary to identify potential drug targets for the treatment of cholestatic disorders.

Our first study was designed to identify the mechanism by which Egr-1 is regulated in the liver during cholestasis. Previously in our laboratory it was shown that the bile acid DCA increased expression of Egr-1 in primary hepatocytes, and that Egr-1 was required for liver injury and inflammation during cholestasis. In Chapter 2 we demonstrated that expression of Egr-1 in hepatocytes was induced by the bile acids DCA and CDCA. Furthermore, we demonstrated that this induction was dependent upon MAPK signaling and not FXR. We also demonstrated that upregulation of Egr-1 within the liver of BDL mice was prevented by pretreatment with the MEK1/2 inhibitor U0126. These data demonstrated that Egr-1 induction during cholestasis was due to bile acid-dependent activation of the MAPK pathway.

In the next set of studies we determined the mechanism by which proinflammatory mediators are increased in the liver during cholestasis. These studies, detailed in Chapter 3, demonstrated that loss of TLR4 does not prevent early inflammation during cholestasis, suggesting that neither LPS nor DAMPs from dead or injured cells stimulate the initial inflammatory response. We also demonstrated that bile acid treatment of hepatocytes

increased expression of several proinflammatory mediators. Many of these proinflammatory mediators were increased in an Egr-1-dependent manner (Tables 3.3-3.5). However, the induction of several mediators was independent of Egr-1, suggesting that several pathways contribute to bile acid-dependent inflammation (Figure 1.4). Corresponding *in vivo* studies showed that several of the proinflammatory mediators, namely PAI-1, VCAM-1, and Ccl7 induced by bile acids were also increased in the livers of BDL mice, and that the induction of the these mediators was also Egr-1 dependent (Figure 3.6). We next examined the expression of PAI-1, ICAM-1 and IL-8 in human samples taken from patients with either PBC or PSC. The expression of all three increased and correlated with Egr-1 expression (Figure 3.7) suggesting that similar mechanisms of inflammation may occur in the livers of patients with PBC or PSC. This link suggests that the BDL model provides data that is constant with human cholestasis and the data we gathered from these studies was reflective of the human condition. This indicates that the findings from our current studies can be applied when designing drugs for the treatment of cholestasis.

A gene that we identified which increased in bile acid-treated hepatocytes in an Egr-1-dependent manner was IL-17D. The physiological function of IL-17D is not known, however limited evidence suggests that IL-17D may be proinflammatory (Starnes et al., 2002). Furthermore, other IL-17 family members, such as IL-17A and IL-17F, have also been shown to promote PMN-dependent inflammation via induction of proinflammatory mediators, suggesting that IL-17D may have a similar function (Laan et al., 1999; Oda et al., 2005; Weaver et al., 2007). Interestingly the expression of the other IL-17 family members was not induced in bile acid-treated hepatocytes, suggesting that IL-17D may have a unique function during cholestasis. We also demonstrated that IL-17D levels were increased during cholestasis and that induction of IL-17D was dependent upon Egr-1. To determine the function of IL-17D in cholestasis we tested the hypothesis that treatment of BDL mice with an anti-IL-17D antibody

reduces liver injury during cholestasis. These studies are detailed in Chapter 4. The data from these studies demonstrated that treatment of mice with the anti-IL-17D antibody reduced expression of MIP-2 and PAI-1 during cholestasis. However, the role for IL-17D in liver injury during cholestasis was limited. These studies are novel in that for the first time they demonstrated a functional role for IL-17D in a pathological condition and also demonstrated that IL-17D is regulated by Egr-1. Knowing that Egr-1 regulates IL-17D expression may help to elucidate the pathways by which IL-17D is regulated in other conditions. Also, knowing the genes that IL-17D regulates may help in determining the receptor(s) to which IL-17D activates to mediates its effects. However further studies will need to be conducted to fully evaluate this.

As mentioned above the expression of the other five IL-17 family members was not affected in bile acid-treated hepatocytes. However, because we saw limited effects on liver injury and inflammation in our studies with the anti-IL-17D antibody, we determined the expression of IL-17 A, IL-17B, IL-17 E and IL-17F during BDL. We found that the expression of IL-17A, IL-17B, IL-17E and IL-17F all increased during BDL, indicating that one or more of the other IL-17 family members could compensate for the loss of IL-17D during BDL.

The final studies of my dissertation determined the role of IL-17A in the development of liver inflammation and injury during cholestasis. Our rationale for investigating the role of IL-17A was that 1) the genes known to be induced by IL-17D *in vitro* are also known targets of IL-17A; 2) There were limited effects on liver injury and inflammation during BDL when mice were treated with an anti-IL-17D antibody; 3) IL-17A expression increased during cholestasis; and 4) IL-17A has been implicated in the pathology of PBC. Therefore we tested the hypothesis that neutralization of IL-17A during BDL would reduce liver inflammation and injury. Similar to the IL-17D studies, we found a modest decrease in the expression of several proinflammatory genes, including IL-6 and Cxcl5, without an effect on inflammation or liver injury. This was not surprising since both IL-17A and IL-17F activate the IL-17 receptor A /C heteromer, suggesting

that neutralization of IL-17A exclusively may not be sufficient to reduce inflammation and injury. However, there are no commercially available neutralizing antibodies for IL-17F. Therefore, we repeated the experiment using a neutralizing antibody to IL-17 receptor A. Surprisingly these studies reveal that inhibition of IL-17RA during BDL caused an increase in the expression of PAI-1 when compared to IgG control-treated mice at 3 days after BDL. Several other genes including Cxcl5, MIP-2 and ICAM-1 also trended higher in anti-IL-17RA treated mice. There was also a slight elevation in liver injury at 3 days post BDL and collagen deposition at 9 days post BDL.

Overall these studies identified a novel mechanism of bile acid-induced proinflammatory signaling in the liver during cholestasis. We also identified Egr-1 as a major regulator of inflammatory signaling during cholestasis, and defined minor roles for the IL-17 cytokine family members in this process. These studies provided insight into the unique mechanism of inflammation during cholestasis and identified potential drug targets, such as Egr-1 and Erk/MAPK signaling pathways for the treatment of cholestasis, the implications of which are discussed further below.

6.2 Significance and Future Studies

Studies from our first aim identified MEK1/2 as a regulator of Egr-1 during cholestasis and a potential target for the treatment of cholestasis. However, the outcomes of these studies were limited by the MEK1/2 inhibitors used, which have very short half-lives and are therefore, not useful for long-term *in vivo* studies. This prevented us from looking at the role of this pathway in the induction of specific proinflammatory mediators. We did attempt to use other MEK1/2 inhibitors currently available on the market such as sorafenib; however the lack of specificity and solubility issues with this compound prevented us for obtaining clear results (Adnane et al., 2006). In the future, if better inhibitors for this pathway become available, or if a

genetic model is easily obtained, these studies could be repeated to look for a direct connection between bile acid activation of MAPK signaling and the induction of proinflammatory mediators such as ICAM-1 and MIP-2 *in vivo*.

Our studies suggested that hepatocytes and not Kupffer cells produce proinflammatory mediators during cholestasis. Other studies have also demonstrated that Kupffer cells have diminished proinflammatory function during cholestasis and that depletion of Kupffer cells prior to BDL does not ameliorate liver injury (Drivas et al., 1976; Calmus et al., 1992; Zhong et al., 2003; Gehring et al., 2006). This could impact the way in which inflammation is treated during cholestasis. Because hepatocytes are not classical immune cells, traditional therapies which inhibit inflammation may not be sufficient. In support of this, ongoing studies in our laboratory have demonstrated that co-treatment of hepatocytes with bile acids and dexamethasone, a classical glucocorticoid with potent anti-inflammatory activity, actually causes a synergistic increase in the expression of MIP-2. These studies are ongoing but suggest that the inflammatory response of hepatocytes to bile acids cannot be inhibited with the use of typical anti-inflammatory drugs, and that specific targeting of pathways activated in hepatocytes by bile acids may be necessary to reduce inflammation in the liver during cholestasis. While many genes induced by bile acids were Egr-1- dependent, several proinflammatory mediators were increased in an Egr-1-independent manner. Several pathways are known to be activated by bile acids including isoforms of the protein kinase C family, p38, JNK (c-Jun NH2-terminal Kinase), and PXR (pregnane X receptor) (Figure 1.4, Chapter 1) (Rao et al., 1997; Makishima et al., 1999; Gupta et al., 2001; Kurz et al., 2001; Staudinger et al., 2001). Further studies will be needed to determine the overall contribution to inflammation of these and other pathways during cholestasis. However, collectively our *in vitro*, *in vivo* and human studies demonstrate that Egr-1 seems to play a key role in the regulation of proinflammatory gene expression during cholestasis, making Egr-1 and/or its activators the most promising potential drug targets for the

treatment of cholestasis. This study also provided new insight into the benefits of biliary drainage. As our data suggests that removal of excess bile in patients may be beneficial because it reduces the amount of bile acids with in the liver, and thus removes the inflammatory stimulus (Scott-Conner and Grogan, 1994; Hammel et al., 2001).

One mechanism by which bile acids may upregulate Egr-1 is through bile acid-dependent activation of a yet to be identified $G_{i\alpha}$ protein-coupled receptor. Studies from Dent et. al. and Fang et. al. have demonstrated that conjugated bile acids activate MAPK, AKT and glycogen synthase signaling via $G_{i\alpha}$-protein dependent mechanism in hepatocytes (Dent et al., 2005; Fang et al., 2007) . While bile acids are known to activate some G-protein coupled receptors such as TGR5 and M3 muscarinic receptors (Pols et al.), these receptors are not expressed by hepatocytes and are therefore not responsible for activation of $G_{i\alpha}$-protein in hepatocytes (Dent et al., 2005; Fang et al., 2007). The characterization of this $G_{i\alpha}$ protein-coupled receptor may allow for more specific targeting of bile acid induced inflammation in hepatocytes since this receptor is required for activation of Erk1/2 signaling in hepatocytes, which our studies demonstrate, upregulates Egr-1. While direct inhibition of Egr-1 or Erk1/2 signaling globally may have dangerous side effects due to their numerous and diverse functions, inhibition of this particular $G_{i\alpha}$ protein-coupled receptor may more selectively inhibit bile acid-induced inflammation in the liver. Further studies are necessary to identity this receptor before the contribution of this receptor to proinflammatory signaling can be determined.

Interestingly, our studies raise the question as to the possible side effects of bile acid mimetics as drugs. The bile acid backbone is being used to design some new drugs or to increase the efficacy of an old drug through altered metabolism (Enhsen et al., 1998). For example the use of a bile acid mimetic or activation of bile acid-dependent signaling has been suggested for the treatment of type 2 diabetes, inhibition of leukocyte chemotaxis, and atherosclerosis (Knop; Ferrari et al., 2006; Hageman et al., 2010). Our data would suggest the

importance of testing for inflammatory gene induction in hepatocytes by these potential drugs, because their altered structure may more robustly activate proinflammatory signaling in these cells. This could be easily tested by using primary hepatocytes and measuring proinflammatory gene expression. This simple and early screening could save lives and money in the end by eliminating drugs that could potentially induce liver inflammation and injury.

Data obtained from our studies concerning the IL-17 family of cytokines would suggest that inhibition of one family member is insufficient to prevent or attenuate liver injury. This most likely results from overlapping function of the different family members. However, our data also suggests that inhibiting the signaling of several family members at once, through receptor inhibitors, could be detrimental due to the loss of anti-inflammatory signals. In support of this, IL-17E is known to signal via a heterodimeric complex containing IL-17RA. IL-17E has been shown to inhibit the function of Th17 cells and antagonizes the function of IL-17A. We have demonstrated that IL-17E is upregulated during BDL. Taken together, loss of IL-17RA may not only limit the proinflammatory signals from IL-17A and IL-17F but also inhibit the anti-inflammatory signals from IL-17E. Further studies are needed, however, to fully define the function of IL-17E during cholestasis. One way to bypass the problem of IL-17RA neutralization could be through neutralization or inhibition of IL-17RC signaling. IL-17RC is only activated by IL-17F and IL-17A, and therefore, neutralization of this receptor would more specifically target the IL-17 F and IL-17A signaling, without effecting the signaling of IL-17E. These studies demonstrate that the role of IL-17 cytokines during cholestasis is complicated and will require further detailed studies. These studies are warranted in that IL-17A levels have been shown to increase in PBC patients.

IL-17B as well as IL-17E may contribute to anti-inflammatory signaling during cholestasis, and thus activation of IL-17B or IL-17E signaling could attenuate liver injury during cholestasis. In support of this both IL-17B and IL-17E are known to signal through the IL-

17receptor B. IL-17E activates a heterodimer of IL-17RA and IL-17RB, and IL-17B activates a homodimer of IL-17RB, suggesting that neutralization of IL-17RB will inhibit the effects of both IL-17B and IL-17E (Shi et al., 2000; Rickel et al., 2008). Preliminary studies in our laboratory indicated that neutralizing IL-17B enhances liver injury during cholestasis. This data taken together with data demonstrating that IL-17E suppresses IL-17A suggest that specific activation of IL-17RB may be beneficial during cholestasis, and that inhibition of IL-17E or IL-17B signaling may be detrimental (Kleinschek et al., 2007). However, further research is necessary.

Overall the results from our studies identified novel mechanisms of inflammation during cholestasis, and reveal potential drug targets for the treatment of cholestatic liver diseases. They also elucidated the function, all be it minor, of IL-17D, and IL-17A in cholestasis. Further studies are necessary to determine the contribution of other signaling pathways in bile acid-induced inflammation, and to determine the functional role of IL-17 cytokines in the liver during cholestasis.

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