ELUCIDATION OF THE MECHANISMS OF INTERACTION BETWEEN BILE ACIDS AND THE INTERLEUKIN-23/INTERLEUKIN-17 AXIS DURING CHOLESTASIS

By

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ABSTRACT

ELUCIDATION OF THE MECHANISMS OF INTERACTION BETWEEN BILE ACIDS AND THE INTERLEUKIN-23/INTERLEUKIN-17 AXIS DURING CHOLESTASIS

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Cholestatic liver disease occurs when bile flow is disrupted, intrahepatically and/or extrahepatically, leading to neutrophil accumulation and elevated bile acid concentrations especially taurine-conjugated bile acids. The robust neutrophilic inflammatory response propagates hepatocellular injury, which is dependent upon macrophage inflammatory protein-2 (MIP-2) expression. Previously, we demonstrated that taurocholic acid (TCA) induces the expression of MIP-2 via ERK activation of early growth response factor-1 (Egr-1) in hepatocytes. However, the mechanisms of bile acid induced neutrophilic inflammation are still not well understood.

Interleukin-17A (IL-17A) is a Th17 cytokine that regulates neutrophil recruitment, defense against bacterial infection, and inflammation induced injury in autoimmune diseases. IL-17A expression is increased in patients with primary biliary cirrhosis and murine models of liver disease. Therefore, we hypothesized that IL-17A promotes inflammation in cholestatic liver disease. To test this hypothesis, we performed bile duct ligation (BDL), a murine model of obstructive cholestasis, on mice treated with an anti-IL-17A antibody. The neutralization of IL-17A reduced alanine aminotransferase (ALT) activity and areas of necrosis in mice subjected to BDL. The expression of pro-inflammatory mediators and neutrophil cell count were decreased in anti-IL-17A BDL mice. Furthermore, IL-17A synergistically enhanced MIP-2 induction by TCA in hepatocytes. IL-17F, another member of the IL-17 family, did not affect hepatocellular injury or inflammation in BDL mice. However, IL-17F had a similar synergistic interaction with TCA

in vitro. These data demonstrate that IL-17A is the predominant Th17 cytokine that contributes to the pathogenesis of cholestasis.

To determine the molecular mechanisms that underlined this synergistic interaction, we investigated known signaling pathways (i.e. C/EBPβ, p38, and JNK) that regulate IL-17A synergistic enhancement of pro-inflammatory mediators by other cytokines. The knockdown of C/EBPβ did not reduce IL-17A induction of MIP-2, but the upregulation of MIP-2 mRNA levels was attenuated in TCA treated hepatocytes. However, hepatocellular injury and inflammation was unaffected in C/EBPβ heterozygous knockout mice subjected to BDL for 3 days. Both p38 and JNK signaling pathways are activated by bile acids; whereas, treatment with IL-17A inhibited the activation of p38 and JNK. Consistently, inhibition of p38 and JNK signaling further promoted MIP-2 induction by bile acids. These results demonstrate the transcription factor, C/EBPβ, as an alternative pathway by which bile acids regulate the inflammatory response in hepatocytes. Furthermore, these data suggest that IL-17A inhibition of p38 and JNK signaling promotes the synergistic enhancement of MIP-2 induction by bile acids.

Interleukin-23 (IL-23) is critical for Th17 cellular expansion and secretion of IL-17A. Mice subjected to BDL had a biphasic induction of IL-23 mRNA levels similar to TCA serum concentrations. Therefore, we tested the hypothesis that bile acids regulate hepatic IL-23 expression. IL-23 protein expression was increased in mice fed a 0.3% cholic acid diet. Furthermore, IL-23 protein and mRNA levels were elevated in hepatocytes treated with TCA via a JNK- and AKT-dependent manner. These data suggest that pathophysiological concentrations of bile acids promote a positive feedback loop in the IL-23/IL-17A axis during cholestasis.

Overall, these studies demonstrate the molecular mechanisms underlying bile acid induction of the hepatic Th17 inflammatory response in cholestatic liver disease.

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KEY TO ABBREVIATIONS

MIP-2	Macrophage inflammatory protein-2
KC	Keratinocyte-derived chemokine
IL	Interleukin
C/EBP	CCAAT-enhancer binding protein
JNK	c-Jun N-terminal kinase
p38	p38 mitogen-activated kinase
EGFR	Epidermal Growth Factor Receptor
Egr-1	Early growth response factor-1
ERK1/2	Extracellular signal-regulated kinase1/2
FXR	Farnesoid X receptor
ICAM-1	Intercellular adhesion molecule-1
MAPK	Mitogen-activated protein kinase
PAI-1	Plasminogen activator inhibitor-1
PBC	Primary Biliary Cirrhosis
PSC	Primary Sclerosing Cholangitis
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
PMNs	Polymorphonuclear leukocytes/neutrophils
TCA	Taurocholic acid
CA	Cholic acid
CDCA	Chenodeoxycholic acid

Ccl	CC chemokine ligand
BDL	Bile duct ligation
S1PR2	Sphingosine-1-phosphate receptor 2
LPAR	Lysophosphatidic acid receptor
EP	Prostaglandin E2 receptor
CYP7a1	Cytochrome P450 7a1
BSEP	Bile salt export pump
MRP	Multidrug resistance protein
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP	Organic anion transport polypeptide
GGT	γ-glutamyl transpeptidase
SHP	Small heterodimer partner
RXR	Retinoid x receptor
LRH-1	Liver receptor homolog-1
HNF-4α	Hepatocyte nuclear factor-4 alpha
FGF15	Fibroblast growth factor 15
ABC	ATP-binding cassette
cAMP	Cyclic adenosine monophosphate
UDCA	Ursodeoxycholic acid
VCAM	Vascular cell adhesion molecule
PECAM	Platelet endothelial cell adhesion molecule
LFA1	Lymphocyte function-associated antigen-1
MAC1	Macrophage-1 antigen

CD	Cluster of differentiation
Th	T helper cell
TGF-β	Transforming growth factor-β
ROR	Retinoid-related orphan receptor
STAT	Signal transducer and activator of transcription
TRAF6	TNF receptor associated factor-6
CCl_4	Carbon tetrachloride
Col1a1	Type 1 collagen
TNF-α	Tumor necrosis factor-α
DCA	Deoxycholic acid
PTPase	Protein tyrosine phosphatase
GPCR	G-protein coupled receptors
S1PR	Sphingosine-1-phosphate receptor
PI3K/AKT	Phosphoinositide 3-kinase/AKT
FDA	Food and Drug Administration
HSC	Hepatic stellate cell
ROS	Reactive oxygen species
ASM	Acid sphingomyelinase
FASR	FAS receptor
CREB	cAMP response element-binding protein
MKP-1	Mitogen kinase phosphatase-1
MKK	Mitogen-activated protein kinase kinase
MRP	Multidrug resistance-associated protein

Chapter 1

Introduction

1.1 Anatomy and Physiology of the Liver

The liver is a multi-functional organ of the hepatobiliary tree that is located in the upper abdominal cavity beneath the diaphragm and above the stomach, right kidney, and small intestines. The liver is organized into hexagonal lobules that are further subdivided into three functional zones based on the principal function of the hepatocytes in those regions. Zone 1 is the periportal area which consists of the hepatic artery, portal vein, and bile ducts, known as the portal triad. The functions of periportal hepatocytes are β-oxidation, gluconeogenesis, cholesterol synthesis, bile formation, and amino acid metabolism. Hepatocytes around the central vein are located in the centrilobular region, also known as zone 3. Centrilobular hepatocytes have a high concentration of drug metabolizing enzymes called cytochrome P450s. Glycolysis and lipogenesis also occur in the centrilobular region. Finally, zone 2 is the midzonal region which is the region located between zone 1 and 3 (Sendensky and Dufour, 2011).

The flow of blood and bile are in opposite directions in the liver. Blood is supplied from two sources that enter the liver through the portal triad. The hepatic artery delivers cardiac arterial blood; whereas, the portal vein carries blood drained from the spleen, gastrointestinal tract, and its associated organs. Blood flows through the sinusoids toward the central vein and coalesces into the hepatic veins. The blood then returns to the heart via the vena cava. However, bile flows from the centrilobular region towards the portal triad where bile is drained into the bile ducts (Abdel-Misih and Bloomston, 2010). The liver anatomy is outlined in figure 1.1.

1.2 Causes of Cholestasis

Bile is a heterogeneous fluid containing electrolytes, bilirubin, proteins, phospholipids, cholesterol, and bile acids. Bile acids are amphipathic molecules in which their formation and movement into bile is critical for proper nutrient absorption. Cholesterol is converted to the

primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), by cytochrome P450 7A1 (CYP7a1) in the hepatic parenchymal cell, the hepatocyte. These primary bile acids are further metabolized by conjugation to amino acids such as taurine and glycine (Reshetnyak, 2013). Hepatocytes export bile salts into the bile canaliculi by the bile salt export pump (BSEP) or multidrug resistance protein, MRP2 (Dawson et al., 2009). Bile acids are then stored in the gallbladder, whereupon the release of cholecystokinin by endocrine cells of the duodenum and proximal jejunum, elicits the release of bile acids into the small intestine. Bile acids aid in the breakdown and absorption of fat and vitamins in the small intestine (Reshetnyak, 2013). Bile acids can be deconjugated in the small intestine through dehydroxylation at the carbon 7 position and/or epimerization at the carbon 3 position by bacteria. These secondary bile acids are known as lithocholic acid (LCA) which is derived from CDCA and deoxycholic acid (DCA) which is derived from CA (Reshetnyak, 2013). Approximately 95% of bile acids are reabsorbed by enterocytes and recycled back to the liver via the portal vein in a process called enterohepatic circulation (Dawson et al., 2009). Bile salts are transported into the hepatocytes via the Na^+-taurocholate cotransporting polypeptide (NTCP) or organic anion transport polypeptides (OATPs) from the blood (Dawson et al., 2009). If bile transport out of hepatocytes or bile flow through the biliary tree is disrupted, this results in a condition called cholestasis (Reshetnyak, 2013).

Cholestasis can be classified as obstructive or nonobstructive, which is further subdivided into intrahepatic or extraheptic (Li and Crawford, 2004). Nonobstructive cholestasis results from the destruction of intrahepatic and/or extrahepatic bile ducts due to an autoimmune reaction, such as occurs in primary biliary cirrhosis or primary sclerosing cholangitis. In addition, nonobstructive cholestasis can be caused by a functional defect in any of the apical or basolateral bile acid transporters on hepatocytes which occurs in patients with progressive familial genetic

disorders, xenobiotic-induced cholestasis, or intrahepatic cholestasis of pregnancy. Obstructive cholestasis results from a blockage of bile flow through the bile duct either by a tumor compressing the bile duct or a gallstone obstructing flow. Both obstructive and nonobstructive cholestasis cause severe hepatocellular and bile duct injury (Beuers, 2009; Li and Crawford, 2004; Trauner et al., 1998).

1.3 Pathogenesis of Cholestasis

The diagnosis of any form of cholestatic liver disease is dependent upon serum biomarkers and histological evaluation of a liver biopsy (Li and Crawford, 2004). Though the particular clinical biomarkers and the histological features may be different among the different cholestatic liver diseases, there are similar pathophysiological characteristics among the various cholestatic diseases. Clinically, patients present with jaundice, discolored urine, pale stools, and pruritus (i.e., itching). During cholestasis, there is an increase in serum bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), and bile acid concentrations (Heathcote, 2007). Due to the inability to excrete excess bilirubin and copper, hepatic histology shows a brown pigmentation from bilirubin and its glucuronides accumulating in perivenular regions along with copper accumulation in periportal hepatocytes (Li and Crawford, 2004).

During cholestasis, bile acid concentrations are increased in the serum and liver. Exposure of the liver to pathophysiological concentrations of bile acids during cholestasis activates a protective pathway that decreases bile acid synthesis and bile acid uptake into hepatocytes (Gupta et al., 2001; Trauner et al., 1998). This is accomplished through activation of the bile acid nuclear receptor, farnesoid X receptor (FXR) (Wang et al., 1999). Activation of FXR suppresses *de novo* bile acid synthesis via two mechanisms. First, FXR heterodimerizes with retinoid X receptor

(RXR) and increases expression of the co-repressor, small heterodimer partner (SHP) (Chiang et al., 2000). SHP represses the transcription factors, liver receptor homolog-1 (LRH-1) and hepatocyte nuclear factor-4 alpha (HNF-4α), which downregulates CYP7a1 (Goodwin et al., 2000; Gupta et al., 2001; Lee et al., 2000). Secondly, bile acids, through activation of FXR, upregulate fibroblast growth factor 15 (FGF15) in enterocytes. FGF15 is released into the portal circulation and activates the fibroblast growth factor receptor 4/β-Klotho complex on hepatocytes, which represses CYP7a1 in a JNK-dependent manner (Inagaki et al., 2005; Ito et al., 2005; Song et al., 2009). In addition to affecting bile acid synthesis, activation of FXR modifies bile acid uptake into hepatocytes. FXR dowregulates the basolateral transporter, NTCP, in a SHP-dependent manner which reduces bile acid uptake into hepatocytes (Denson et al., 2001). In addition, FXR increases bile canalicular expression of BSEP, MDR3, and ABCG5 which promote bile acid, phospholipid, and cholesterol secretion into the bile (Ananthanarayanan et al., 2001; Freeman et al., 2004; Huang et al., 2003). These adaptive changes, which include decreased bile acid synthesis and modified hepatic transport of bile acids, occur to reduce hepatic exposure to potentially toxic bile acids.

During cholestasis, bile duct epithelial cells proliferate to repair damaged bile ducts and to expand the biliary tree which promotes bile efflux from the liver (Munshi et al., 2011). During this process, gastrointestinal and neuroendocrine hormones, as well as paracrine and autocrine signaling, promote cAMP and Ca^{2+} mediated mitotic activity in these cells during severe cholestatic liver injury (Munshi et al., 2011). Furthermore, the regenerative properties of the liver are initiated at the Canal of Hering and progress throughout the liver to replace dead hepatocytes (Li and Crawford, 2004). An imbalance between repair and damage can further contribute to the pathogenesis of cholestasis.

If proper bile flow is not reestablished, the liver will become fibrotic (Hirschfield et al., 2010). Fibrosis occurs when hepatic stellate cells and peribiliary fibroblasts differentiate into myofibroblasts and begin to proliferate and deposit extracellular matrix, such as type 1 collagen (Penz-Osterreicher et al., 2011). Persistent deposition of extracellular matrix ultimately leads to hepatic dysfunction and the development of cirrhosis. Ultimately, breakdown of hepatic architecture and function in the cirrhotic liver leads to end-stage liver failure. The pathogenesis of cholestatic liver disease is outlined in figure 1.2.

1.4 Treatment of Cholestasis

Unfortunately, there is no effective therapy for patients with cholestatic liver disease. Ursodiol®, ursodeoxycholic acid (UDCA), is the only FDA approved drug for patients with cholestatic liver disease. The beneficial effects of UDCA are due to its anti-oxidant and anti-cholestatic effects. UDCA prevents cholestasis by stimulating choleresis (i.e., increased bile flow) via upregulation of transporters on hepatocytes (Jazrawi et al., 1994). UDCA has only been shown to be effective for the treatment of primary biliary cirrhosis, although the response rate is low in these patients (Grattagliano et al., 2011; Tsochatzis et al., 2013). Most other types of cholestatic liver disease do not show improvement with UDCA administration (Carey and Lindor, 2012). In these patients, the only treatment available is a liver transplant. Considering the limited number of transplantable livers, better treatment options are needed for cholestatic liver disease.

1.5 Mechanism of Hepatocellular Injury during Cholestasis

1.5.1 Direct Toxicity of Bile Acids. Due to the detergent like properties of bile acids, it was originally proposed that hepatocellular injury during cholestasis results from direct cytotoxicity by bile acids. In support of this, treatment of primary rodent hepatocytes with the secondary bile acids, LCA and DCA stimulated JNK- and FAS-dependent apoptosis, suggesting

that hepatocyte apoptosis may be the main mechanism of liver injury during cholestasis (Gupta et al., 2004; Qiao et al., 2001). However, studies in bile duct ligated mice, an animal model of obstructive cholestasis, demonstrated that hepatocytes die by necrosis and not apoptosis during cholestasis (Li and Crawford, 2004; Woolbright et al., 2013). A possible explanation for the discrepancy between *in vitro* and *in vivo* studies is that in the *in vitro* studies hepatocytes were treated with secondary conjugated bile acids which stimulate apoptosis. *In vivo*, however, taurine-conjugated and glycine-conjugated primary bile acids are the predominant bile acids that increase in concentration in cholestatic patients and in murine models of cholestasis, whereas concentrations of secondary bile acids either decrease or remain unchanged (Trottier et al., 2012; Zhang et al., 2012). *In vitro*, pathophysiological concentrations of taurine-conjugated bile acids do not induce hepatocyte apoptosis (Allen et al., 2011). These studies indicate that hepatocyte injury during cholestasis does not result from direct toxicity by bile acids. More recent studies have suggested that inflammatory cells are key mediators of liver injury during cholestasis.

1.5.2 Role of Inflammation in Hepatocellular Injury during Cholestasis. Neutrophilic inflammation is observed in the livers of patients with obstructive cholestasis and in animal models of cholestasis (Gujral et al., 2003; Zimmermann et al., 2011). Neutrophils are leukocytes that freely circulate in the blood (Galli et al., 2011; Pillay et al., 2010). These cells are critical for host defense against invading pathogens, such as bacteria (Howard et al., 1977). At sites of inflammation, a chemotactic gradient of various chemokines is formed which recruits neutrophils (Kolaczkowska and Kubes, 2013). Neutrophil infiltration begins with the process of rolling within blood vessels, a process which is facilitated by interactions between selectins on the endothelial surface and ligands on the neutrophil. Next, neutrophils firmly adhere to the endothelium and transmigrate into the parenchyma via an interaction between adhesion molecules, such as

intercellular adhesion molecule (ICAM-1/2), vascular cell adhesion molecule-1 (VCAM-1), and platelet endothelial cell adhesion molecule (PECAM) on endothelial cells, and the integrins, LFA1 (CD11a/CD18) and MAC1 (CD11b/CD18) on neutrophils (Kolaczkowska and Kubes, 2013). The membrane expression of LFA1 or MAC1 and the shedding of selectins lead to neutrophil priming (Galli et al., 2011). Once neutrophils reach a site of infection, they become activated and release reactive oxygen species and proteases that kill invading pathogens (Gujral et al., 2003; Kolaczkowska and Kubes, 2013). In conditions where inflammation becomes dysregulated, neutrophils can damage host tissue (Kolaczkowska and Kubes, 2013; Smith, 1994). Studies suggest that this occurs during cholestasis, and may be responsible for much of the hepatocellular injury that occurs.

The β_2 integrin, CD18, is a common subunit among the neutrophil integrins that regulates the interaction between neutrophils and the endothelium (Kolaczkowska and Kubes, 2013). Studies using CD18 knockout mice have shown that this integrin is essential for neutrophil-dependent liver injury in several models of liver disease (Gujral et al., 2003; Kodali et al., 2006). To investigate a role for neutrophils in liver injury during cholestasis, bile duct ligation (BDL) was performed in CD18 knockout mice. Deletion of CD18 attenuated the increase alanine aminotransferase (ALT) activity in serum, a biomarker of hepatocellular injury, and decreased areas of necrosis indicating a key role for neutrophils in liver injury during obstructive cholestasis (Gujral et al., 2003). In addition to CD18, ICAM-1 has been shown to be critical for neutrophil extravasation and hepatocyte injury in models of inflammatory liver injury. BDLs in ICAM-1 knockout mice decreased neutrophil extravasation and reduced hepatic injury (Gujral et al., 2004). Collectively, these studies demonstrated an essential role for neutrophils in the development of hepatocellular injury during cholestasis.

1.5.2.1 Role of Cytokines and Chemokines in Hepatocellular Injury during Cholestasis

Cholestasis. Levels of several cytokines and chemokines are increased in cholestatic patients and in murine models of cholestasis (Georgiev et al., 2008; Lisboa et al., 2012; Plebani et al., 1999; Zimmermann et al., 2011). Furthermore, several studies have identified cytokines and chemokines that are important for inflammatory liver injury during cholestasis. For instance, neutralizing antibodies against the chemokines, macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC), reduced neutrophil accumulation and hepatocellular injury in bile duct ligated mice; thereby, indicating a key role for these chemokines in development of inflammation during cholestasis (Wintermeyer et al., 2009). These findings are in agreement with clinical observations which show that interleukin-8 (IL-8), the human homolog of MIP-2 and KC, are at the highest concentrations in the serum of cholestatic patients with greatest neutrophil infiltration into the liver (Zimmermann et al., 2011).

1.5.2.2 Interleukin-17A. In addition to cells of the innate immune system, such as neutrophils, cells of the adaptive immune system are recruited to the liver during cholestasis. One of these cell types, $CD4^+$ Th17 cells, is increased in the liver of patients with primary biliary cirrhosis and in murine models of obstructive cholestasis (Meng et al., 2012; Rong et al., 2009). Murine Th17 differentiation from naïve $CD4^+$ T cells requires transforming growth factor-β (TGF-β) with either IL-6 or IL-21 (Korn et al., 2007; Mangan et al., 2006; Veldhoen et al., 2006). In humans, Th17 differentiation requires TGF-β in combination with IL-1β, IL-6, IL-23, and/or IL-21 (Liu et al., 2013; Tuomela et al., 2012; Wilson et al., 2007). These cytokines promote induction of the orphan nuclear receptor, RORγt in mice or RORc in humans, which promote Th17 differentiation (Ivanov et al., 2006). Furthermore, differentiation and activation of Th17 cells occurs through activation of the transcription factors, RORα and STAT3 (Figure 1.3) (Yang

et al., 2007; Yang et al., 2008c). Inhibition of Th17 cell population expansion reduced hepatic neutrophil accumulation and serum concentrations of ALP and bilirubin, biomarkers of cholestatic liver disease in CD279, a regulator of T cell proliferation, knockout mice subjected to bile duct ligation (Licata et al., 2013). These studies demonstrate that Th17 cells also contribute to inflammation and injury in cholestatic liver. Upon activation, Th17 cells secrete a variety of interleukins, including IL-17A, that regulate the inflammatory response during injury.

Interleukin-17A (IL-17A) is the best characterized cytokine belonging to the Th17 lymphocyte family (Yao et al., 1995). In addition to IL-17A, the IL-17 family consists of IL-17F, IL-17E, IL-17D, IL-17B, and IL-17C with IL-17F having the closest homology to IL-17A, and IL-17E having the most divergent homology (Aggarwal and Gurney, 2002). IL-17A is primarily produced by Th17 cells, but can also be secreted by natural killer T cells, natural killer cells, γδ T cells, and neutrophils (Eustace et al., 2011; Pandya et al., 2011). IL-17A acts as a homodimer or as a heterodimer with IL-17F (Chang and Dong, 2007). The IL-17A receptor is a heterodimer complex composed of a subunit of IL-17RA and a subunit of IL-17RC (Toy et al., 2006). Upon ligand binding, the receptor complex signals through the adaptor proteins, ACT1 and TRAF6 (Chang et al., 2006; Liu et al., 2011a; Onishi et al., 2010; Qian et al., 2007). The receptor complex has a higher affinity for IL-17A homodimer and a lower affinity for IL-17F homodimers with IL-17A/IL-17F heterodimers being in between (Korn et al., 2009). IL-17A is critical for host defense against invading pathogens, such as bacteria. It primarily mediates this effect through recruitment of neutrophils to sites of infection (Curtis and Way, 2009). Furthermore, IL-17A has been shown to contribute to inflammation and injury in various autoimmune diseases, such as rheumatoid arthritis (Gaffen, 2009a; Moran et al., 2009).

IL-17A serum concentrations and hepatic IL-17A positive Th17 cells are increased in cholestatic liver disease patients (Lan et al., 2009; Qian et al., 2013). Similarly, IL-17A expression is increased in the livers of bile duct ligated mice, and hepatocellular injury is reduced in IL-17A and IL-17RA knockout mice after bile duct ligation (Meng et al., 2012; O'Brien et al., 2013). In addition to liver injury, bile duct ligated IL-17A and IL-17RA knockout mice had reduced liver fibrosis (Hara et al., 2013; Meng et al., 2012). Collectively, these studies demonstrated an important role for IL-17A in the development of liver injury and fibrosis during cholestasis. Whether IL-17A contributes to the development of neutrophilic hepatic inflammation during cholestasis is not known. Furthermore, the mechanism by which cholestasis stimulates production of IL-17A is not known.

1.5.2.3 Interleukin-23. As stated above, IL-23 is required for maintenance of Th17 cells. IL-23 is principally secreted by immune cells and belongs to the interleukin-12 family in which it shares a p40 subunit and has a unique p19 subunit (Oppmann et al., 2000). IL-23 is not required for Th17 differentiation, but it is critical for the maintenance and stabilization of the Th17 population (Stritesky et al., 2008; Veldhoen et al., 2006). Similar to IL-17A, IL-23 has been extensively investigated during autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (Chen et al., 2006; Murphy et al., 2003). In those autoimmune diseases, IL-23 promotes inflammation and injury by stimulating persistent expression of pro-inflammatory mediators, such as IL-17A from Th17 cells (Murphy et al., 2003). Recently, the role of IL-23 in liver diseases has been investigated. Neutralization of IL-23 reduced hepatic lesions and reduced ALT activity in mice injected with adenovirus, a murine model of viral hepatitis (Hou et al., 2013). In addition, neutralization of IL-23 attenuated the hepatic inflammatory response in mice subjected to hepatic ischemia/reperfusion (Husted et al., 2006). In a model of fibrotic liver,

fibrosis was reduced in CCl_4 treated mice depleted of bone marrow IL-23 expression (Meng et al., 2012). These studies demonstrate the ability of IL-23 to propagate injury in multiple disease states. However, the effect of hepatic IL-23 expression during cholestasis is still not well understood.

1.6 Bile Acid Signaling in Hepatocytes

The mechanism by which inflammation occurs in the liver during cholestasis is not fully understood, however, our recent studies suggest that bile acids may be important for initiation of this process. In cholestatic patients, the predominant bile acids that increase in serum concentration are either taurine- or glycine- conjugated primary bile acids (Trottier et al., 2012). Similar observations have been made in an animal model of obstructive cholestasis. Zhang et al reported that taurocholic acid concentrations reach as high as 3 mM in the serum of bile duct ligated mice (Zhang et al., 2012). Because bile acids activate signaling pathways in hepatocytes, we tested the hypothesis that bile acids stimulate hepatocytes to produce pro-inflammatory cytokines, thereby initiating inflammation during cholestasis. In these studies, primary mouse hepatocytes were treated with taurocholic acid (TCA), at concentrations that occur in the serum during cholestasis (Zhang et al., 2012). Although TCA did not affect hepatocyte viability, it did upregulate various pro-inflammatory cytokines, chemokines, and adhesion molecules in primary mouse hepatocytes in an ERK-dependent and early growth response factor-1 (Egr-1)-dependent manner (Figure 1.4) (Allen et al., 2011; Allen et al., 2010). Furthermore, Egr-1 knockout mice subjected to bile duct ligation had reduced hepatocellular injury and inflammation (Kim et al., 2006). These studies demonstrated that bile acids activate ERK, which upregulates Egr-1. Egr-1, a transcription factor, then upregulated pro-inflammatory mediators that promote hepatic

inflammation during cholestasis. What remains unknown, however is the mechanism by which bile acids activate ERK and upregulate Egr-1 in hepatocytes.

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that has been shown to be activated by both primary and secondary bile acids (Dent et al., 2005). EGFR is activated by bile acids through the generation of mitochondrial reactive oxygen species (Qiao et al., 2001). Reactive oxygen species activate EGFR by inhibiting the protein tyrosine phosphatase (PTPase) that deactivates EGFR (Fang et al., 2004). Ligand independent activation of EGFR in this way stimulates downstream signaling through activation of the Raf-1/MEK/ERK pathway (Rao et al., 2002). Whether activation of this signaling pathway is required for upregulation of inflammatory mediators by bile acids is not known and a focus of my studies.

G-protein coupled receptors (GPCR) are seven transmembrane receptors that signal through a specific heterotrimeric g-protein coupled to the cytoplasmic tail of the receptor. One of those G-proteins, $G_{\alpha i}$, transactivates tyrosine kinase receptors such as EGFR (Dent et al., 2005; Melien et al., 2000; Melien et al., 1998). For example, hepatocytes treated with pertussis toxin, an inhibitor of $G_{\alpha i}$, prevented the phosphorylation of EGFR by conjugated bile acid. (Figure 1.5) (Dent et al., 2005; Fang et al., 2004). However, the GPCR that promotes activation of EGFR in hepatocytes has not been elucidated. TGR5 is a GPCR and a well characterized bile acid receptor, but is not expressed on hepatocytes (Keitel et al., 2008; Keitel et al., 2007). A closely related GPCR that is coupled to $G_{\alpha i}$ is the spingosine-1-phosphate receptor 2 (S1PR2). Recently, taurocholic acid was shown to activate ERK and AKT signaling pathways in a S1PR2 dependent-manner (Studer et al., 2012). The role of S1PR2 in bile acid induced inflammation has not been

investigated. Furthermore, whether other GPCRs are activated by bile acids and contribute to inflammatory signaling in hepatocytes has not been studied and will be a focus of my studies.

1.7 Purpose

If proper bile flow is not restored, cholestatic liver disease can result in cirrhosis and ultimately liver failure (Li and Crawford, 2004). At present, UDCA, the only FDA approved drug to treat cholestasis, is only moderately effective in a small subset of cholestatic patients (Tsochatzis et al., 2013). Therefore, it is imperative that we investigate the mechanisms that underlie the pathogenesis of cholestasis to identify better treatment options. Because of the importance of inflammation to the development of liver disease during cholestasis, we have investigated the role of various inflammatory mediators in the pathogenesis of cholestasis. Neutrophils have been shown to extravasate into the hepatic parenchyma in cholestatic patients and in animal models of cholestasis. Gujral et al demonstrated that neutrophil infiltration contributes to hepatocellular injury through the production of reactive oxygen species (Gujral et al., 2003). Furthermore, the adaptive immune response, through Th17 cellular expansion, has been shown to be activated in patients with PBC (Lan et al., 2009; Qian et al., 2013). Differentiation and activation of Th17 cells promotes induction of the IL-23/IL-17A axis (Aggarwal et al., 2003). What remains unknown is the role of the IL-23/IL-17A axis in cholestatic liver disease.

Cholestatic liver disease is characterized by an increase in serum and hepatic bile acid concentrations (Li and Crawford, 2004). We have shown previously that primary conjugated bile acids promote an inflammatory response in hepatocytes, a process that is dependent upon ERK activation and upregulation of the transcription factor, Egr-1 (Allen et al., 2011; Allen et al., 2010). However, the interaction between bile acids and the Th17 cellular response during

cholestasis has not been investigated. Collectively, the goal of the present studies is to investigate the role of IL-17 family members in the pathogenesis of cholestatic liver disease, and to determine whether there is an interaction between taurine-conjugated primary bile acids and the IL-23/IL-17A axis during cholestasis. The studies, organized by chapter, are diagramed in figure 1.6.

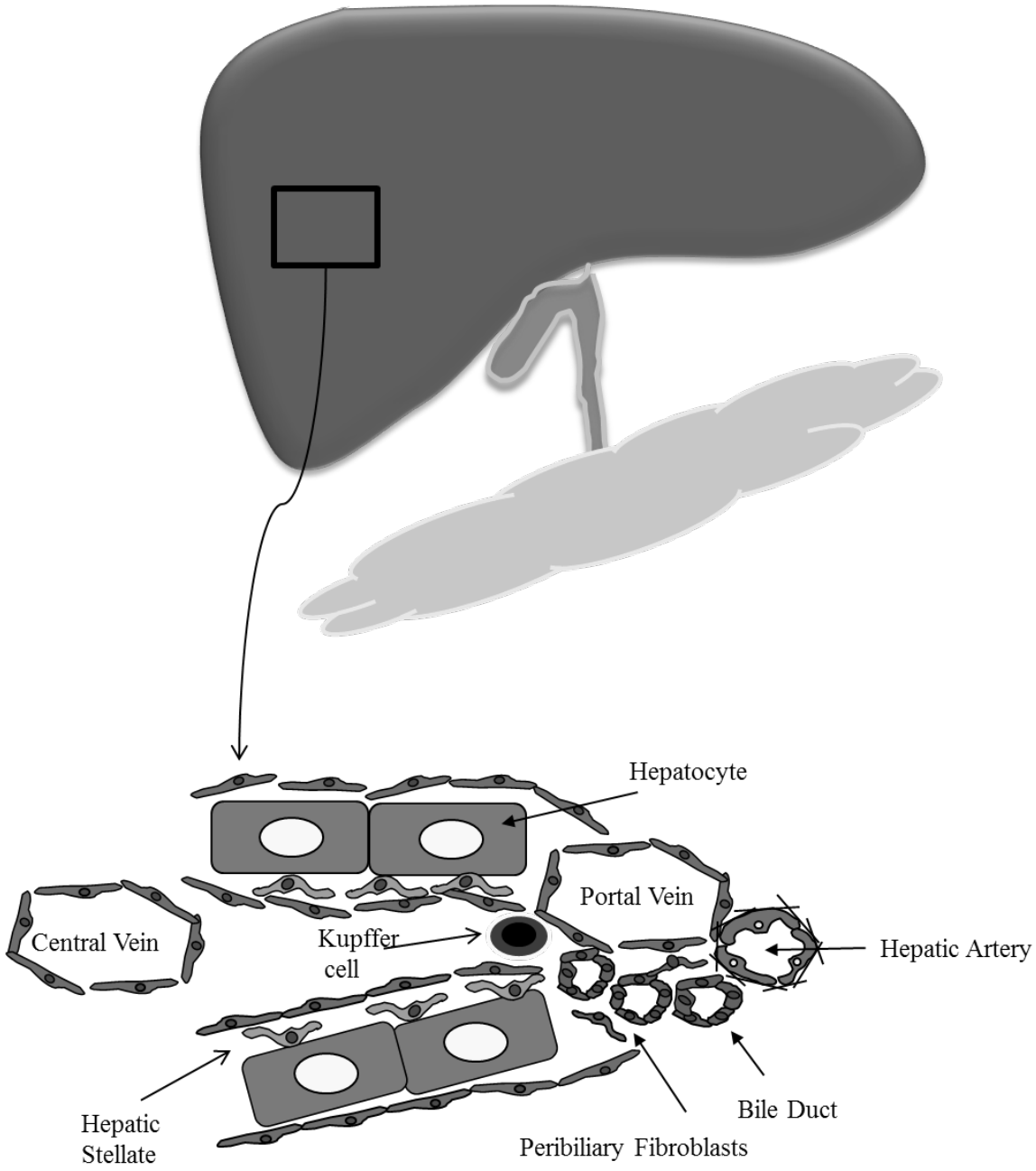


Figure 1.1 Basic Liver Anatomy. Liver is located in the upper abdominal cavity above the small intestine. Blood flows from the portal triad (hepatic artery, portal vein, and bile duct) to the central vein; whereas, bile flows from the central vein to the portal triad. The hepatic parenchymal cells are the hepatocytes which carry out the principal functions of the liver.

Figure 1.1 (cont'd)

Hepatic stellate cells are located in the space of Disse. Other cell types are the Kupffer cells, the resident macrophage, portal fibroblast, and cholangiocytes (bile duct epithelial cells). Bile acids generated by the liver are stored in the gallbladder located underneath the liver. Upon endocrine stimulus, the gallbladder releases bile acids into the small intestine via the common bile duct. Bile acids are critical for proper nutrient absorption in the small intestines.

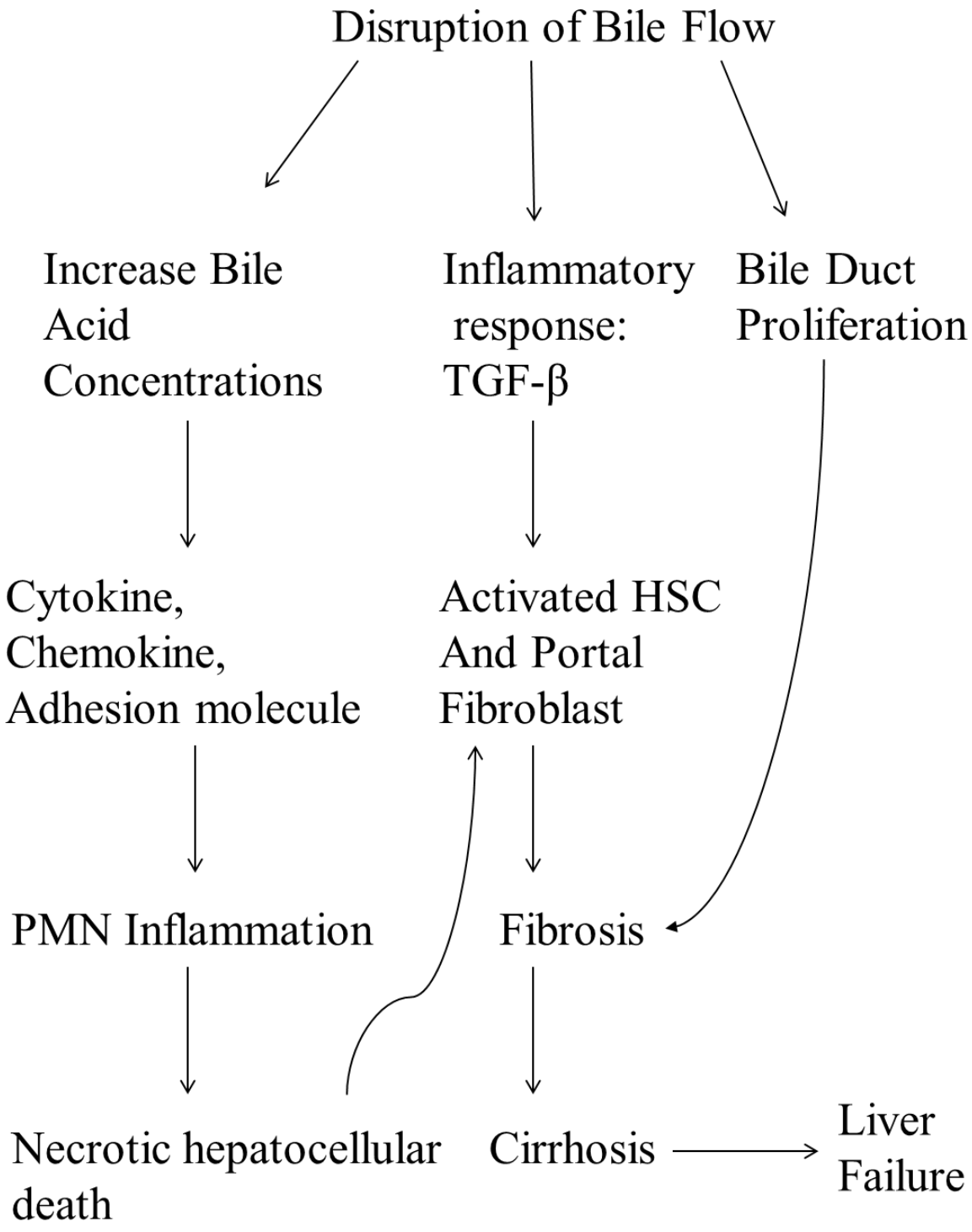


Figure 1.2 Outline of the Pathogenesis of Cholestatic Liver Disease. A disruption of bile flow causes cholestatic liver disease leading to an increase in hepatic bile acid concentrations.

Figure 1.2 (cont'd)

Pathophysiological concentrations of bile acids induce the hepatic expression of pro-inflammatory mediators, which promote neutrophilic inflammation and necrotic hepatocellular death. Furthermore, the activation of the inflammatory response can lead to the differentiation of hepatic stellate cells which will secrete extracellular matrix. An imbalance of extracellular matrices will ultimately lead to cirrhosis. In addition, the dysregulation of bile flow promotes bile duct proliferation. Proliferating cholangiocytes secrete growth factors that propagate the fibrotic response during cholestasis. Collectively, these pathways will lead to end-stage liver failure.

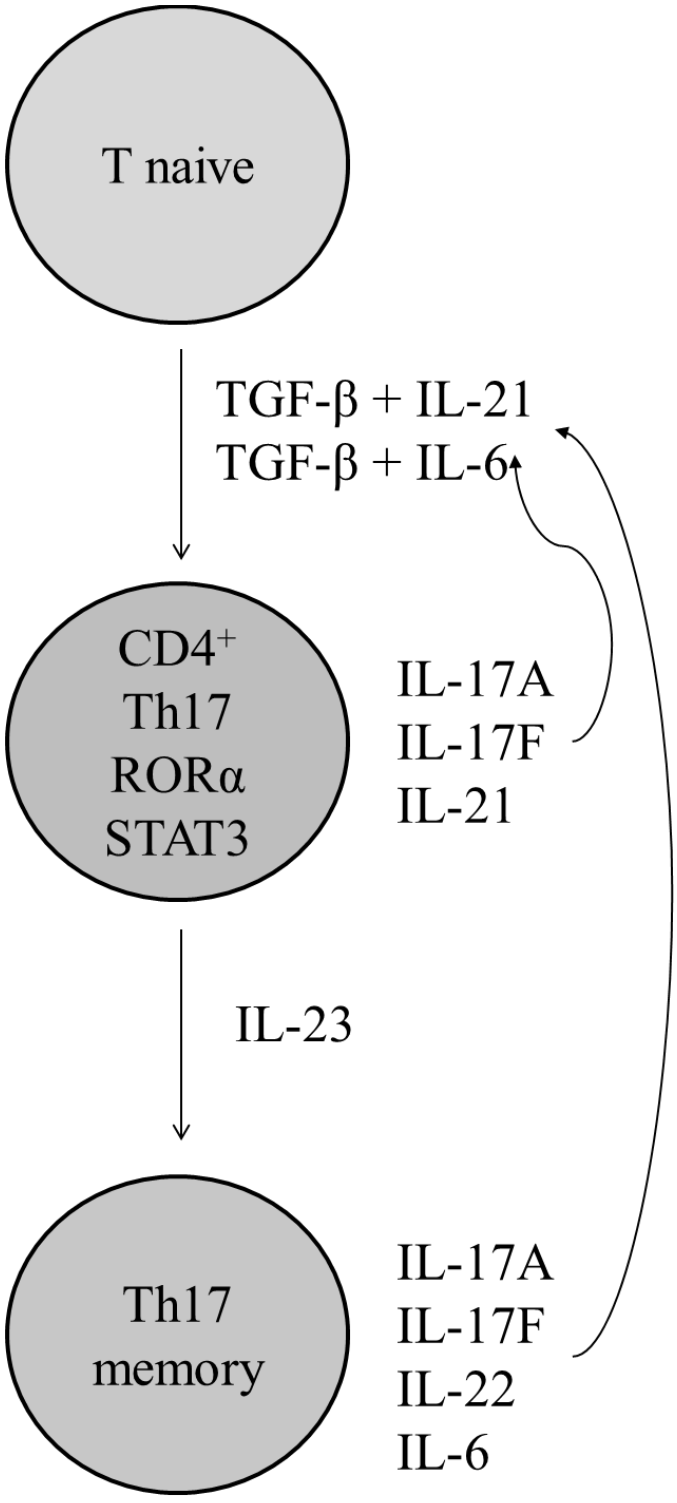


Figure 1.3 The Mechanism of Murine Th17 Cell Differentiation and Population Expansion.

Exposure to transforming growth factor-β (TGF-β) with either IL-6 or IL-21 leads to the

Figure 1.3 (cont'd)

activation of the transcription factors, RORα and STAT3, which promote murine naïve $CD4^+$ T cell differentiation to the Th17 phenotype. IL-23 maintains and stabilizes the $CD4^+$ Th17 cell into effector memory Th17 cells. Th17 cells secrete a variety of cytokines such as IL-17A, IL-17F, IL-22, and IL-21. These cytokines further propagate the differentiation and expansion of Th17 cells.

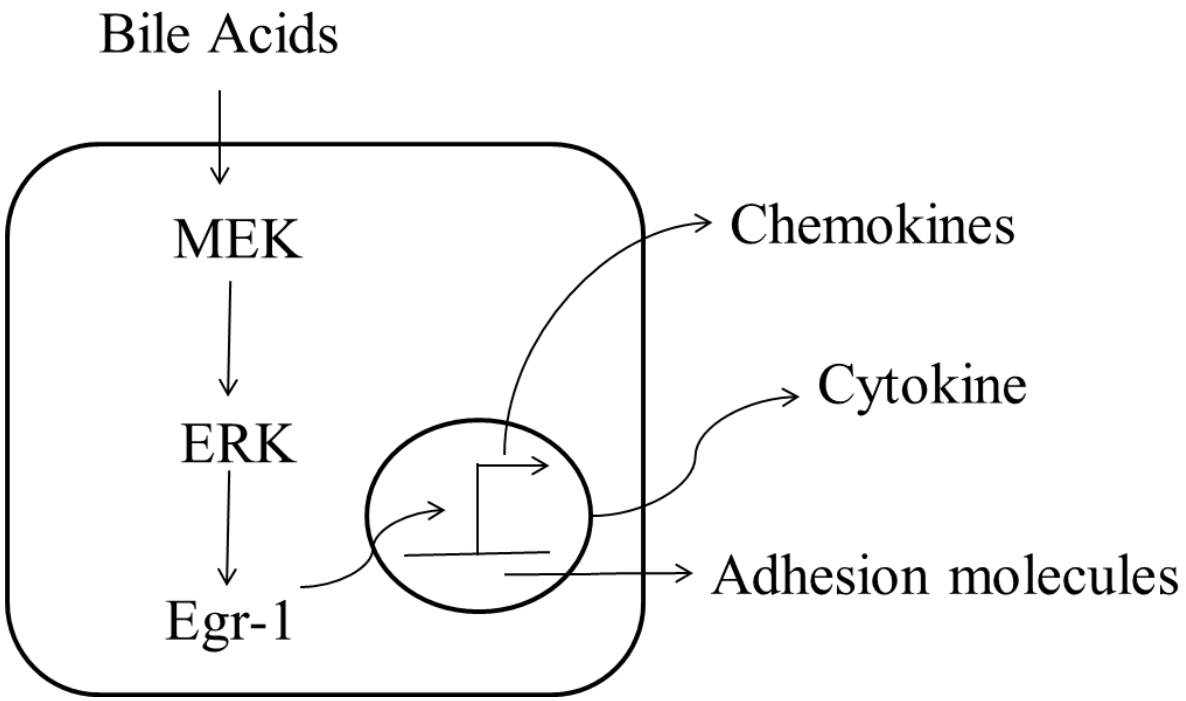


Figure 1.4 Signaling Pathway of Bile Acid Induced Inflammatory Response in Hepatocytes. Bile acids upregulate pro-inflammatory mediators such as cytokines, chemokines, and adhesion molecules via an ERK-dependent activation of the transcription factor, early growth response factor-1 (Egr-1) in hepatocytes.

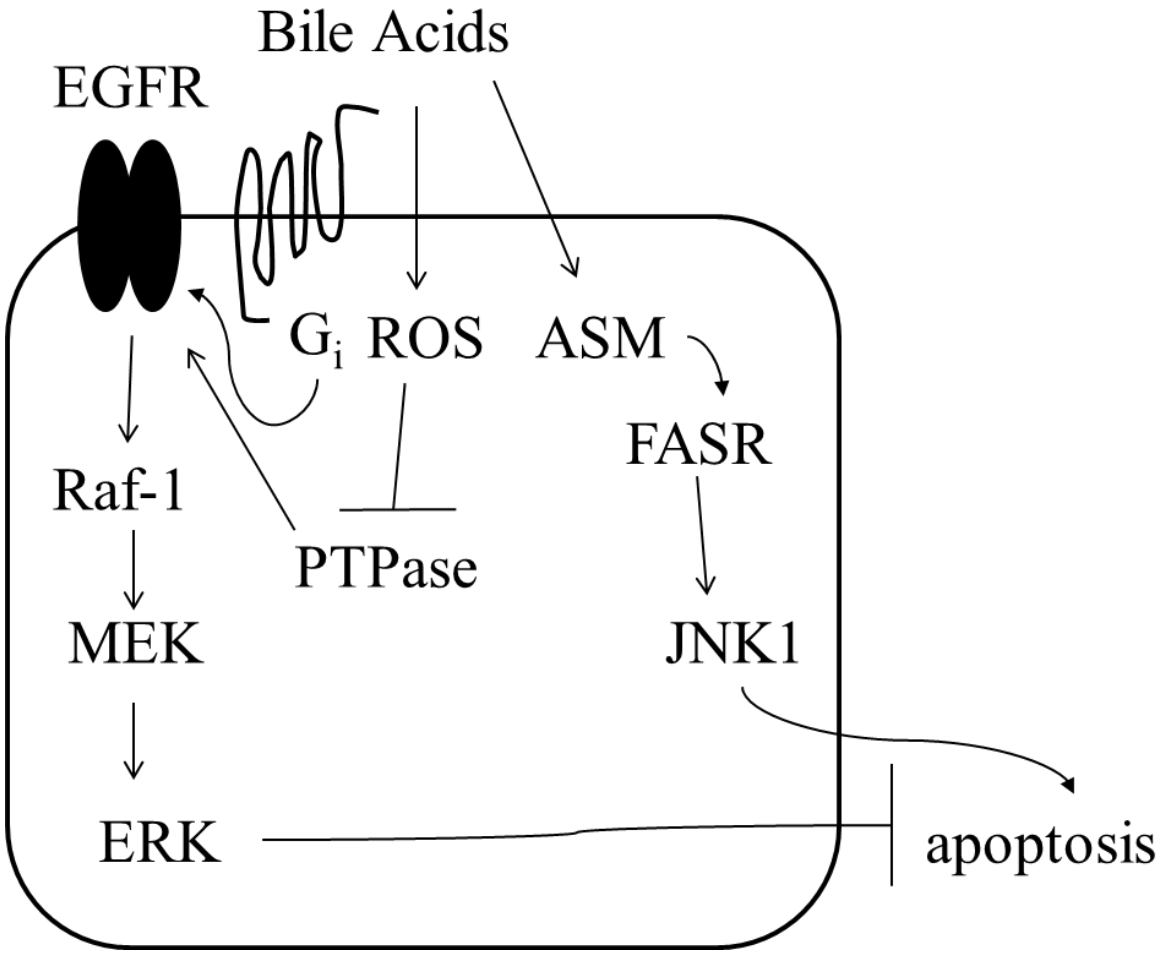


Figure 1.5 Previously Characterized Bile Acid Signaling in Primary Hepatocytes. The epidermal growth factor receptor (EGFR) and the Fas receptor are activated by conjugated bile acids, in a ligand-independent manner. Mitochondrial reactive oxygen species (ROS) inhibits the protein tyrosine phosphatase (PTPase) activity which will promote EGFR activation. In addition, bile acid activation of a $G_{\alpha i}$ coupled GPCR will lead to induction of EGFR signaling.

Hepatocellular apoptosis is inhibited by ERK activation. Whereas, the activation of JNK1 by the FAS receptor promotes apoptotic cell death.

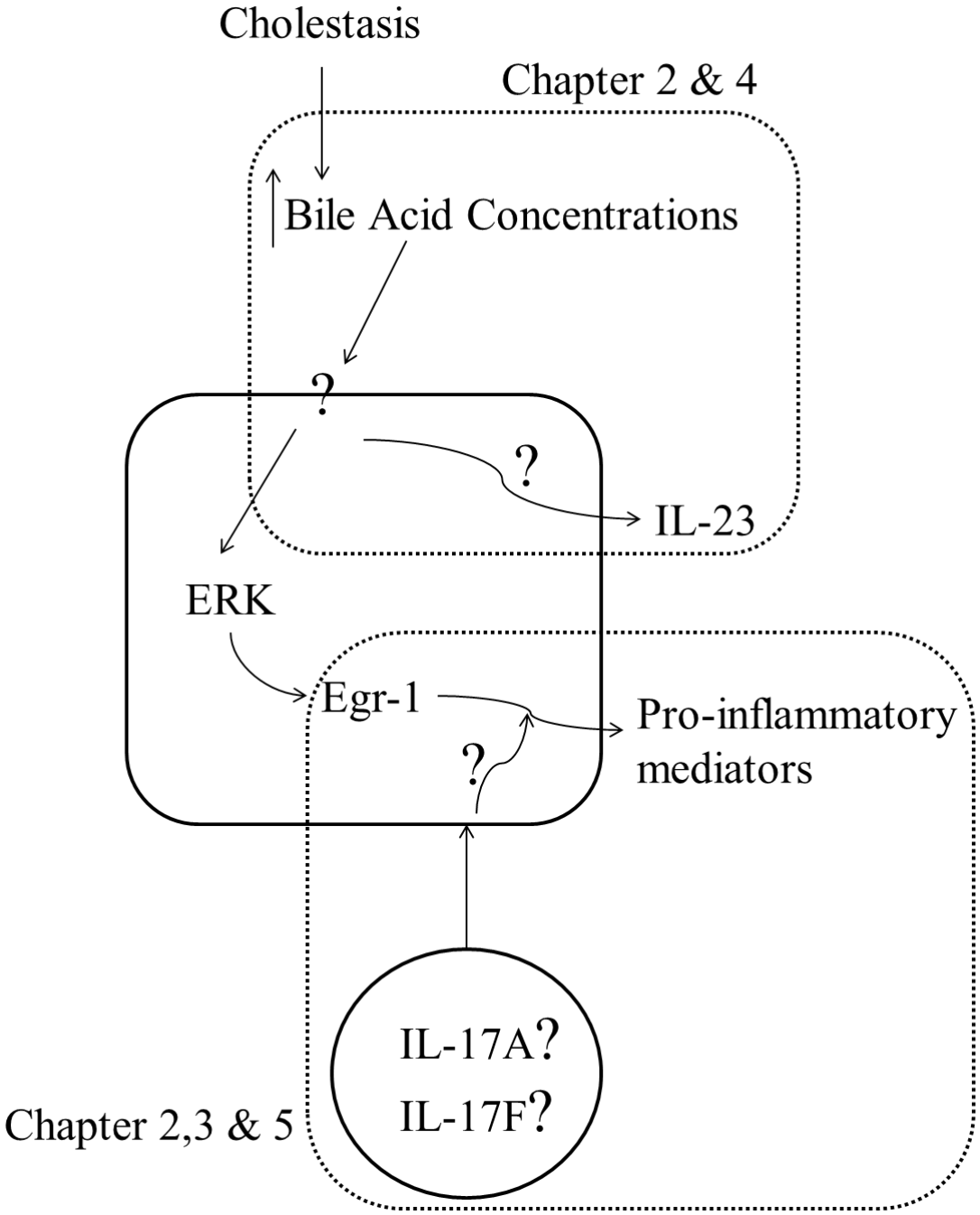


Figure 1.6 Proposed Studies Investigated in this Dissertation. In chapter 2, the role of the IL-23/IL-17A axis during obstructive cholestasis will be investigated. Chapter 2 will demonstrate that bile acids induce IL-23 mRNA levels in a JNK- and AKT-dependent manner. Chapter 2 also

Figure 1.6 (cont'd)

shows that IL-17A promotes hepatocellular injury and inflammation in a murine model of obstructive cholestasis, and that IL-17A synergistically enhances the hepatic inflammatory response by bile acids. Chapter 3 will investigate the mechanism(s) underlying the synergistic interaction between IL-17A and bile acids. Chapter 4 will elucidate the receptor activated by bile acids that regulates IL-23 mRNA levels. Lastly, the role of IL-17F during obstructive cholestasis will be studied in Chapter 5.

Chapter 2

Interleukin-17A Synergistically Enhances Bile Acid-induced Inflammation during Obstructive Cholestasis

Kate M. O'Brien, Katryn M. Allen, Cheryl E. Rockwell, Keara Towery, James P. Luyendyk,

and Bryan L. Copple American Journal of Pathology. 2013; 183(5): 1498-1507.

2.1 Abstract

During obstructive cholestasis, increased concentrations of bile acids activate Erk1/2 in hepatocytes which upregulates early growth response factor-1 (Egr-1), a key regulator of pro-inflammatory cytokines, such as macrophage inflammatory protein-2 (MIP-2). Activation of this inflammatory pathway exacerbates cholestatic liver injury. Recent studies indicated that interleukin-17A (IL-17A) contributes to hepatic inflammation during obstructive cholestasis, suggesting that bile acids and IL-17A may interact to regulate hepatic inflammatory responses. To investigate this, mice were treated with an IL-17A neutralizing antibody or control IgG, and subjected to bile duct ligation. Neutralization of IL-17A prevented upregulation of pro-inflammatory cytokines, hepatic neutrophil accumulation, and liver injury, indicating an important role for IL-17A in neutrophilic inflammation during cholestasis. Treatment of primary, mouse hepatocytes with taurocholic acid (TCA) increased expression of MIP-2. Interestingly, co-treatment with IL-17A synergistically enhanced upregulation of MIP-2 by TCA. In contrast to MIP-2, IL-17A did not affect upregulation of Egr-1 by TCA indicating that IL-17A does not affect bile acid-induced activation of signaling pathways upstream of Egr-1. In addition, bile acids increased expression of IL-23, a key regulator of IL-17A production, in hepatocytes *in vitro* and *in vivo*. Collectively, these studies identify bile acids as novel triggers of the IL-23/IL-17A axis. In addition, these studies suggest that IL-17A promotes hepatic inflammation during cholestasis by synergistically enhancing bile acid-induced production of pro-inflammatory cytokines by hepatocytes.

2.2 Introduction

Cholestatic liver diseases have many causes such as familial genetic disorders, xenobiotic exposure, autoimmune disease, and tumors that disrupt bile flow (Li and Crawford, 2004).

Common features of most types of cholestatic liver disease is an elevation in pro-inflammatory cytokines and hepatic accumulation of immune cells (Gehring et al., 2006; Georgiev et al., 2008; Gujral et al., 2003). Studies in animal models have identified several immune cell types and immunomodulatory cytokines that are essential for the pathogenesis of cholestatic liver diseases (Gehring et al., 2006; Gujral et al., 2003; Gujral et al., 2004; Wintermeyer et al., 2009). The mechanisms that regulate hepatic inflammation during cholestasis, however, are not fully understood. Elucidation of these pathways could provide important insight pertinent to the development of therapeutics for cholestatic liver disease. Our recent studies indicated an important role for bile acids in the development of inflammation during cholestasis.

In obstructive cholestasis, elevated biliary pressure disrupts the integrity of intrahepatic bile ducts resulting in the leakage of bile into the liver (Fickert et al., 2002). As a result, concentrations of bile acids, in particular taurine and glycine conjugates, increase in the liver and serum in patients and in animal models (Trottier et al., 2012; Zhang et al., 2012). During bile duct ligation (BDL) in mice, serum concentrations of bile acids exceed 3 mM with the highest fraction of bile acids being conjugated with taurine (Zhang et al., 2012). Modest increases in bile acid concentrations modulate metabolic pathways in hepatocytes that promote detoxification of bile acids (Wang et al., 1999). However, pathological concentrations of bile acids trigger a strong inflammatory response in hepatocytes characterized by induction of diverse classes of cytokines that promote infiltration of inflammatory cells, including neutrophils, macrophages, and lymphocytes (Allen et al., 2011; Allen et al., 2010). Accumulating neutrophils extravasate from sinusoids and adhere to hepatocytes, which stimulates their activation. The activated neutrophils release toxic mediators that exacerbate hepatocyte injury during cholestasis (Gujral et al., 2003).

We recently identified a bile acid-activated signaling network that elicits production of pro-inflammatory cytokines by hepatocytes. In these studies, pathological concentrations of taurocholic acid (TCA) activated ERK1/2 and upregulated the transcription factor early growth response factor-1 (Egr-1) in hepatocytes (Allen et al., 2011; Allen et al., 2010; Kim et al., 2006). Egr-1 induction by TCA promotes the production of several cytokines, chemokines, and adhesion molecules that are crucial for neutrophil accumulation and activation during cholestasis (Allen et al., 2011; Allen et al., 2010). In addition, bile acids increased expression of many pro-inflammatory mediators in an Egr-1-independent manner, suggesting that bile acids promote inflammation by multiple mechanisms (Allen et al., 2011). Collectively, these studies indicated that bile acids are central mediators of hepatic inflammation during cholestasis.

It was recently reported that the cytokine, interleukin-17A (IL-17A), contributes to the hepatic production of pro-inflammatory mediators and the progression of fibrosis in BDL mice (Meng et al., 2012). IL-17A is the best characterized member of the IL-17 cytokine family and is primarily produced and secreted by $CD4^+$ Th17 cells (Korn et al., 2009). Differentiation of murine naïve T-cells into Th17 effector cells is mediated by the cytokines, transforming growth factor-β (TGF-β) with either interleukin-6 (IL-6) or interleukin-21 (IL-21) (Gaffen, 2009b; Korn et al., 2009). In addition, interleukin-23 (IL-23) is required for maintenance and stabilization of Th17 cells (Stritesky et al., 2008). IL-17A signals via its receptor complex, IL-17RA and IL-17RC, as a homodimer or as a heterodimer with IL-17F (Gaffen, 2009b). Through adaptor proteins, IL-17A elicits production of chemokines and cytokines that promote neutrophil recruitment and thus an immune reaction to extracellular pathogens (Chang et al., 2006; Schwandner et al., 2000). It was reported that IL-17A positive cells are increased in the livers of patients with primary biliary cirrhosis (Lan et al., 2009). Similarly, it was demonstrated that IL-

17A levels are increased in mice subjected to BDL (Meng et al., 2012). In this study, liver injury and fibrosis were reduced in IL-17RA knockout mice 3 weeks after surgery. Furthermore, there were reduced levels of the cytokines, TNF-α, IL-1β, and IL-6, indicating an important role for IL-17A in the development of liver disease during cholestasis (Meng et al., 2012).

Considering the importance of both bile acids and IL-17A to hepatic inflammation during cholestasis, there might be a potential link between these inflammatory mediators in the pathogenesis of hepatocellular injury during cholestasis. Accordingly in the following studies, the hypothesis was tested that bile acids and the IL-17 signaling axis interact to promote hepatic inflammation during cholestasis.

2.3 Materials and Methods

2.3.1 Animal Care. Studies were performed on 8-10 week old male C57BL/6 mice (Jackson Laboratories; Bar Harbor, ME). All of the mice were maintained on a 12-h light/dark cycle under controlled temperature (18–21°C) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. For bile acid feeding, mice were given AIN-93M diet supplemented with 0.3% cholic acid or AIN-93M alone (Dynets Inc; Bethlehem, PA) for one week. All of the 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.

2.3.2 Bile Duct Ligation. Mice were treated with 100 μg of anti-IL-17A antibody (clone 50104, R&D systems, Minneapolis, MN; BioCell, West Lebanon, NH) or control IgG_{2A} (clone 54447, R&D systems; BioCell) by intraperitoneal injection 1 hour prior to surgery (Kobayashi et al., 2010). BDL was then performed on mice as described previously (Kim et al., 2006). The mice were given an additional 50 μg of anti-IL-17A antibody or control IgG on days 3 and 6 after surgery. Nine days after surgery, livers and blood were collected from the mice for analysis.

2.3.3 Hepatocyte Isolation. Hepatocytes were isolated from mice by collagenase perfusion as described previously (Kim et al., 2006). Hepatocytes were plated in Williams' medium E (Invitrogen, Carlsbad, CA) supplemented with10% FBS (Sigma Aldrich; St. Louis, MO) and penicillin-streptomycin (Sigma Aldrich). After 2 hours, hepatocytes were washed with 1X phosphate buffered saline (PBS) and cultured in serum-free Williams' medium E. Hepatocytes were treated with 10 ng/mL of recombinant mouse IL-17A (R&D systems) or 4mM HCl as vehicle followed by 200 μM TCA dissolved in endotoxin free water for the time indicated. For signal transduction studies, hepatocytes were treated with 100 nM wortmannin (Cayman Chemical; Ann Arbor, MI), 30 μM SP600125 (Cayman Chemical), 10 μM SB203580 (Cayman Chemical), or DMSO as vehicle in the presence or absence of 200 μM TCA (Sigma Aldrich) in endotoxin free water for 3 hours.

2.3.4 Serum Chemistry. Serum alanine aminotransferase (ALT) activity (Thermo Scientific; Middletown, VA), total serum bilirubin (Pointe Scientific, Inc; Canton, MI), serum alkaline phosphatase (ALP) (Pointe Scientific, Inc; Canton, MI), and total serum bile acids (Colorimetric Total Bile Acids Assay Kit; Bio-Quant, San Diego, CA) were measured using commercially available kits as per manufactures' instructions.

2.3.5 Protein Analysis. IL-23 and macrophage inflammatory protein-2 (MIP-2) proteins were quantified in culture medium by ELISA (Biolegend; San Diego, CA). Total protein was collected from hepatocytes lysed with RIPA buffer. Protein was separated on a 4-20% polyacrylamide gel (Bio-Rad, Hercules, and CA). Membranes were incubated with either anti-phospho-AKT (Thr308), anti-AKT, anti-phospho-JNK (Thr183/Tyr185), anti-JNK, anti-phospho-p38 (Thr180/Tyr182), or anti-p38 antibodies (Cell Signal Technology; Danvers, MA). Membranes were then incubated with the appropriate secondary conjugated with horseradish

peroxidase. Protein bands were detected using ECL detection kit (GE healthcare; Buckinghamshire, UK) on a LiCor Fc (Odyssey; Lincoln, NE).

2.3.6 Immunofluorescence. Liver pieces were frozen in isopentane cooled in liquid nitrogen for 8 minutes. Sections of frozen liver were cut and fixed in 4% formalin for 10 minutes. The sections were incubated with either a rabbit polyclonal anti-IL-23A antibody (Abnova; Walnut, CA), a rat anti-mouse CD68 antibody (AbD Serotec; Raleigh, NC), or a rat anti-mouse F4/80 antibody (AbD Serotec; Raleigh, NC) in 10% goat serum diluted with PBS for 3 hours. The sections were then incubated with the appropriate secondary antibody conjugated with Alexa Fluor 594 (Invitrogen). CD68 and F4/80 immunostaining were quantified and described by us previously (Copple et al., 2012).

2.3.7 Immunohistochemistry. Paraffin-embedded liver sections were incubated with rabbit anti-rat polymorphonuclear 7/4 antigen (PMN) antibody (1:100) (AbD Serotec; Raleigh, NC) for one hour or rabbit anti-α-smooth muscle actin antibody (1:100; Abcam; Cambridge, MA) overnight at 4°C. Neutrophils were quantified by counting the number of positively-stained cells in 20, 200X fields per tissue section. The analysis was performed in a blinded fashion.

2.3.8 Real-Time PCR. Trizol reagent (Sigma Aldrich) was used to isolate RNA. Contaminating DNA was removed by using the TURBO DNA-free kit (Ambion; Austin, TX). 20 ng of RNA was reverse transcribed into cDNA in a reaction containing 1X MMLV buffer, 0.1mM dNTPs, 10 mM DTT, 6.4 μg/mL random primers, 5.12 U/μL rRNasin, and 2.56 U/μL MMLV reverse transcriptase Relative mRNA levels were measured using fast SYBR green reagent (Applied Biosystems; Foster City, CA) on a 7500 Fast Real-time PCR system (Applied Biosystems). Primer sequences are shown in Table 2.1. Primers used for these studies were intron spanning.

2.3.9 Statistics. Data are expressed as mean ± SEM. When two or more groups were analyzed, a two way analysis of variance (ANOVA) was performed. Comparison between groups was done by using the Holm-Sidak method. A 95% confidence, $p<0.05$, was the criterion for significance.

2.4 RESULTS

2.4.1 Neutralization of IL-17A reduced neutrophil accumulation and liver injury after BDL. Previous studies demonstrated that neutrophils contribute to hepatic injury following BDL (Gujral et al., 2003). Given that IL-17A is instrumental in driving neutrophilic inflammation, we characterized the expression of IL-17A in the liver over time following BDL. IL-17A mRNA levels did not increase until 5 days post-BDL (Figure 2.1A). This increase in expression peaked at day 7 and remained elevated at 14 days. Next, we determined the role of IL-17A in the development of hepatocellular injury and neutrophilic inflammation after BDL. Mice were treated with either an anti-IL-17A antibody or control IgG. The mice were then subjected to BDL or sham operation for 3 or 9 days. BDL caused an increase in serum ALT activity, which was reduced in mice treated with anti-IL-17A antibody (Figure 2.1B). Consistent with this, neutralization of IL-17A decreased the area of liver necrosis in BDL mice (Figure 2.1C-E). Notably, neutralization of IL-17A did not affect hepatocellular injury 3 days post-surgery (data not shown), a time-point before upregulation of IL-17A (Figure 2.1A).

To ascertain whether blocking IL-17A specifically decreased neutrophil-mediated inflammation or instead caused a reduction in cholestasis in general, the effect of anti-IL-17A on markers of cholestasis was investigated. Alkaline phosphatase (ALP), total bilirubin, and total bile acids, were increased in the serum of mice 9 days after BDL (Table 2.2). Neutralization of IL-17A did not affect the increase in these markers of cholestasis (Table 2.2).

Next, we tested the hypothesis that neutralization of IL-17A prevents accumulation of inflammatory cells in the liver after BDL. Macrophages were detected in the liver by immunohistochemical staining for F4/80 and CD68 (Figure 2.2A-D). F4/80 and CD68 positive macrophages increased in the livers of BDL mice treated with control IgG (Figure 2.2E-F). Neutralization of IL-17A, however, did not affect accumulation of macrophages in the liver (Figure 2.2E-F). In addition, neutrophil numbers increased in the livers of BDL mice treated with control IgG (Figure 2.3). In contrast to macrophages, neutralization of IL-17A decreased neutrophil numbers after BDL (Figure 2.3).

2.4.2 Cytokine gene expression was decreased in anti-IL-17A-treated BDL mice. Because IL-17A neutralization decreased hepatic accumulation of neutrophils, we investigated the expression of cytokines and adhesion molecules upon neutralization of IL-17A in BDL mice. Hepatic mRNA levels of macrophage inflammatory protein-2 (MIP-2), Cxcl5, Ccl7, plasminogen activator inhibitor-1 (PAI-1), and intercellular adhesion molecule-1 (ICAM-1) were increased in mice treated with control IgG and subjected to BDL for 9 days (Figure 2.4A-E). Neutralization of IL-17A in BDL mice attenuated the increase in MIP-2, Cxcl5, Ccl7, and PAI-1 (Figure 2.4A-D). In contrast, ICAM-1 mRNA levels were unaffected by IL-17A neutralization (Figure 2.4E).

2.4.3 α-Smooth Muscle Actin (α-SMA) and Type I Collagen levels were unaffected by neutralization of IL-17A. Next, we determined the effect of IL-17A on the expression of profibrogenic markers 9 days post-surgery. α-SMA and type I collagen mRNA levels were increased to a similar extent in BDL mice treated with either control IgG or anti-IL-17A (Figure 2.5A-B). Similar to the mRNA levels, α-SMA protein, detected by immunohistochemistry, and collagen protein, detected by trichrome staining, were not different between control IgG treated mice and anti-IL17A treated mice subjected to BDL (Figure 2.5C-F).

2.4.4 IL-17A synergistically enhances TCA-induced upregulation of MIP-2 mRNA in primary mouse hepatocytes. We previously demonstrated that TCA promotes pro-inflammatory mediator production by hepatocytes, which is important for inflammation in the liver during cholestasis (Allen et al., 2011; Allen et al., 2010; Kim et al., 2006). Considering that IL-17A is also important for inflammation during cholestasis (Figures 2.3 and 2.4), we investigated whether IL-17A affects bile acid-induced upregulation of inflammatory mediators by hepatocytes. To determine this, hepatocytes were treated with IL-17A in the presence or absence of 200 μM TCA. Both IL-17A and TCA alone increased MIP-2 gene expression, in primary mouse hepatocytes (Figure 2.6A). Co-treatment with IL-17A and TCA synergistically enhanced induction of MIP-2 mRNA when compared to treatment with IL-17A or TCA alone (Figure 2.6A-B). MIP-2 protein concentration was increased in the medium from hepatocytes treated with either IL-17A or TCA (Figure 2.6C). Similar to MIP-2 mRNA levels, co-treatment with IL-17A and TCA substantially increased MIP-2 protein levels when compared to hepatocytes treated with either IL-17A or TCA alone (Figure 2.6C). We previously demonstrated that upregulation of MIP-2 in bile acid-treated hepatocytes is Egr-1-dependent (Allen et al., 2011). Therefore, we determined the effect of IL-17A on upregulation of Egr-1 by bile acids. As expected, TCA increased Egr-1 mRNA levels in hepatocytes (Figure 2.6D). IL-17A did not affect upregulation of Egr-1 by TCA (Figure 2.6D).

Unlike MIP-2, neutralization of IL-17A in mice did not affect upregulation of ICAM-1 in the liver after BDL (Figure 2.4E). Accordingly, we determined whether IL-17A would affect upregulation of ICAM-1 in bile acid-treated hepatocytes. Consistent with *in vivo* (Figure 2.4E), IL-17A did not enhance TCA-induced upregulation of ICAM-1 (Figure 2.6E).

2.4.5 Upregulation of IL-23 mRNA and protein in bile acid-treated primary mouse hepatocytes. Because IL-23 promotes IL-17A production, IL-23 gene expression was measured

in wild-type mice subjected to BDL over a 14 day time course. IL-23 gene expression was biphasic with an initial peak at 6 hours followed by a second peak at 5 days (Figure 2.7A). Our previous studies demonstrated that serum and liver bile acid concentrations (Zhang et al., 2012) followed a similar time course as IL-23 mRNA levels; thereby, suggesting that bile acids may contribute to upregulation of IL-23 after BDL. Therefore, we tested the hypothesis that TCA increases IL-23 production by primary mouse hepatocytes. Treatment of hepatocytes with 200 μM TCA increased IL-23 mRNA and protein levels (Figure 2.7B-C). Similar to its effects on MIP-2, IL-17A enhanced upregulation of IL-23 in hepatocytes by TCA (Figure 2.7D).

2.4.6 Signal Transduction Pathways involved in IL-23 induction by TCA. In primary mouse hepatocytes, 200 μM TCA activated AKT (Figure 2.8A), c-Jun N-terminal kinase (JNK) (Figure 2.8B), and p38 mitogen-activated kinase (p38) (Figure 2.8C). Inhibition of PI3K/AKT or JNK signaling by wortmannin or SP600125 respectively attenuated upregulation of IL-23 by TCA (Figure 2.8D-E). Inhibition of p38 by SB203580, however, enhanced IL-23 induction by TCA (Figure 2.8F).

2.4.7 Bile Acid feeding promotes hepatic IL-23 expression. To determine whether bile acids increase IL-23 expression *in vivo*, mice were fed a diet containing 0.3% cholic acid or a control diet for one week. By 1 week of feeding, this diet increased serum bile acid concentrations, including TCA (Song et al., 2011). IL-23 mRNA levels were increased in the livers of mice fed the 0.3% cholic acid diet (Figure 2.9A). Immunohistochemical staining for IL-23 in the liver demonstrated increased IL-23 protein in hepatocytes and sinusoidal cells (Figure 2.9B-C).

2.5 Discussion

During obstructive cholestasis, neutrophils accumulate in the liver, become activated, and exacerbate hepatocellular injury (Gujral et al., 2003). A recent study demonstrated that IL-17RA null mice had reduced liver injury 3 weeks after BDL, suggesting that IL-17A signaling is a prerequisite for hepatic neutrophil accumulation during obstructive cholestasis (Meng et al., 2012). Consistent with this, our current study demonstrated that neutralization of IL-17A reduced neutrophil numbers in the liver after BDL (Figure 2.3), which was associated with a reduction in ALT and area of hepatic necrosis (Figure 2.1B-E). Hepatic neutrophil accumulation depends upon upregulation of various inflammatory cytokines and adhesion molecules (Gehring et al., 2006; Gujral et al., 2004; Wintermeyer et al., 2009). In our studies, neutralization of IL-17A attenuated upregulation of several pro-inflammatory cytokines, including MIP-2, PAI-1, Ccl7, and Cxcl5 (Figure 2.4A-D), suggesting that IL-17A promotes hepatic neutrophil accumulation through upregulation of pro-inflammatory mediators which stimulate neutrophil migration to the liver during obstructive cholestasis. Unlike a previous report, however, we did not see a reduction in markers of liver fibrosis (Figure 2.5). The reason for this discrepancy may be because we investigated a much earlier time-point after BDL (i.e., 9 days in our study vs. 3 weeks in the previous study) (Meng et al., 2012).

We recently demonstrated that bile acids upregulate several cytokines, chemokines, and adhesion molecules, in hepatocytes, in an Egr-1-dependent manner (Allen et al., 2011). In addition, we demonstrated that this process is important for neutrophil-dependent liver injury during cholestasis (Allen et al., 2010; Kim et al., 2006). Our current studies demonstrate that IL-17A is also required for upregulation of inflammatory cytokines and neutrophil-dependent liver injury similar to bile acids. In many cell types, IL-17A alone is not a potent inflammatory

cytokine (Maione et al., 2009). However, IL-17A modifies the inflammatory response produced by other cytokines, such as tumor necrosis factor-α (TNF-α) and IL-6. For example, IL-17A synergistically enhances upregulation of pro-inflammatory mediators by TNF-α or IL-6 in alveolar type II cells, endothelial cells, mesangial cells, astrocytes, and fibroblasts (Griffin et al., 2012; Iyoda et al., 2010; Liu et al., 2011b; Ma et al., 2010; Ogura et al., 2008). This suggests that IL-17A may interact with other inflammatory mediators, such as bile acids, to produce inflammation during cholestasis. Our results demonstrate that IL-17A and TCA do interact to promote robust production of inflammatory cytokines by hepatocytes. IL-17A alone modestly increased MIP-2; however, MIP-2 production by hepatocytes was markedly increased in the presence of IL-17A and TCA (Figure 2.6A-C). Bile acids activate Erk1/2 in hepatocytes, which stimulate upregulation of Egr-1. Egr-1 then regulates production of MIP-2. Interestingly, IL-17A did not affect upregulation of Egr-1 (Figure 2.6D), suggesting that IL-17A does not enhance upregulation of MIP-2, by modulating bile acid signaling upstream of Egr-1.

The synergistic enhancement of MIP-2 expression by IL-17A and TCA may not only be important for obstructive cholestasis, but could be critical in other liver diseases, including primary biliary cirrhosis (PBC) and alcoholic hepatitis where IL-17A and bile acid concentrations are increased (Harada et al., 2009; Lemmers et al., 2009; Takiguchi and Koga, 1988). In these diseases, IL-17A may promote modest hepatic inflammation during early stages. When these diseases become severe and hepatic bile acid concentrations increase, however, the interaction between IL-17A and bile acids could severely exacerbate hepatic inflammation and disease progression.

Bile acids may not only modify the response of hepatocytes to IL-17A but may alter the response of other cell types, in particular Kupffer cells. Studies have shown that IL-17A

stimulates production of inflammatory mediators by Kupffer cells (Meng et al., 2012). In contrast to hepatocytes, however, bile acids inhibit production of inflammatory cytokines by Kupffer cells (Keitel et al., 2008; Sheen-Chen et al., 1998). It has been proposed that this occurs through activation of the bile acid receptor, TGR5 (Keitel et al., 2008). Accordingly, it is possible that bile acids may inhibit IL-17A-induced production of inflammatory cytokines by Kupffer cells during obstructive cholestasis, which may explain the lack of TNF-α production by hepatic macrophages during obstructive cholestasis, even though concentrations of the macrophage activators, IL-17A and lipopolysaccharide, are increased (Gehring et al., 2006). A similar effect could occur with other cell types that express TGR5, including bile duct epithelial cells and sinusoidal endothelial cells (Keitel et al., 2008; Keitel et al., 2007). Our *in vitro* data demonstrate that hepatocytes express higher levels of inflammatory mediators in the presences of bile acids and IL-17A suggesting that in liver diseases, such as cholestasis, where bile acid concentrations are increased, the production of inflammatory mediators may shift from prototypical inflammatory cells towards hepatocytes. This type of inflammation may be more resistant to commonly used anti-inflammatory drugs, such as glucocorticoids, which may explain the resistance of many types of cholestatic liver disease to anti-inflammatory therapies (Silveira and Lindor, 2008).

IL-23 is critical for the maintenance and stabilization of $CD4^+$ Th17 cells into effector T helper cells (Korn et al., 2009). After BDL, IL-23 concentrations are increased, and this increase is required for production of IL-17A (Figure 2.7A) (Meng et al., 2012). Interestingly, our studies demonstrated that the time-course of IL-23 upregulation in the liver after BDL was biphasic and identical to the time-course of bile acid concentration changes in the serum and liver (Figure 2.7A) (Zhang et al., 2012). This suggested that bile acids may be important for upregulation of IL-23 in the liver. Consistent with this, TCA increased expression of IL-23 in primary mouse

hepatocytes (Figure 2.7B-C), and IL-23 protein levels were increased in hepatocytes from mice fed a diet containing 0.3% cholic acid (Figure 2.9). This indicates that bile acids could sustain and exacerbate a Th17 response by stimulating production of IL-23, a process that was AKT- and JNK-dependent (Figure 2.8D-E). Similar to its effects on MIP-2, IL-17A enhanced TCA-induced production of IL-23 by hepatocytes (Figure 2.7D). This could initiate a positive feedback loop whereby bile acids stimulate production of IL-23 by hepatocytes which enhances IL-17A production by Th17 cells. IL-17A then enhances bile acid-induced production of IL-23 and other inflammatory mediators by hepatocytes. This process further enhances IL-17A production and ultimately IL-23 and other inflammatory cytokines that exacerbate hepatic inflammation and injury. This process is illustrated in Figure 2.10.

Our results demonstrate that IL-17A synergistically enhances bile acid-induced production of inflammatory mediators by hepatocytes. Furthermore, we demonstrated for the first time that bile acids stimulate hepatocytes to produce IL-23. Although our studies suggest that inhibition of IL-17A in patients with cholestatic liver disease may be a logical therapeutic option, systemic inhibition of IL-17A could negatively impact the ability of patients to clear bacterial infections, which may be particularly dangerous in patients with cholestasis who are at risk for developing infections. Accordingly, elucidation of the signaling mechanisms involved in the interaction between IL-17A and bile acids may provide important insight into specifically targeting the pathways involved in hepatic inflammation during cholestasis without affecting host defense against invading pathogens.

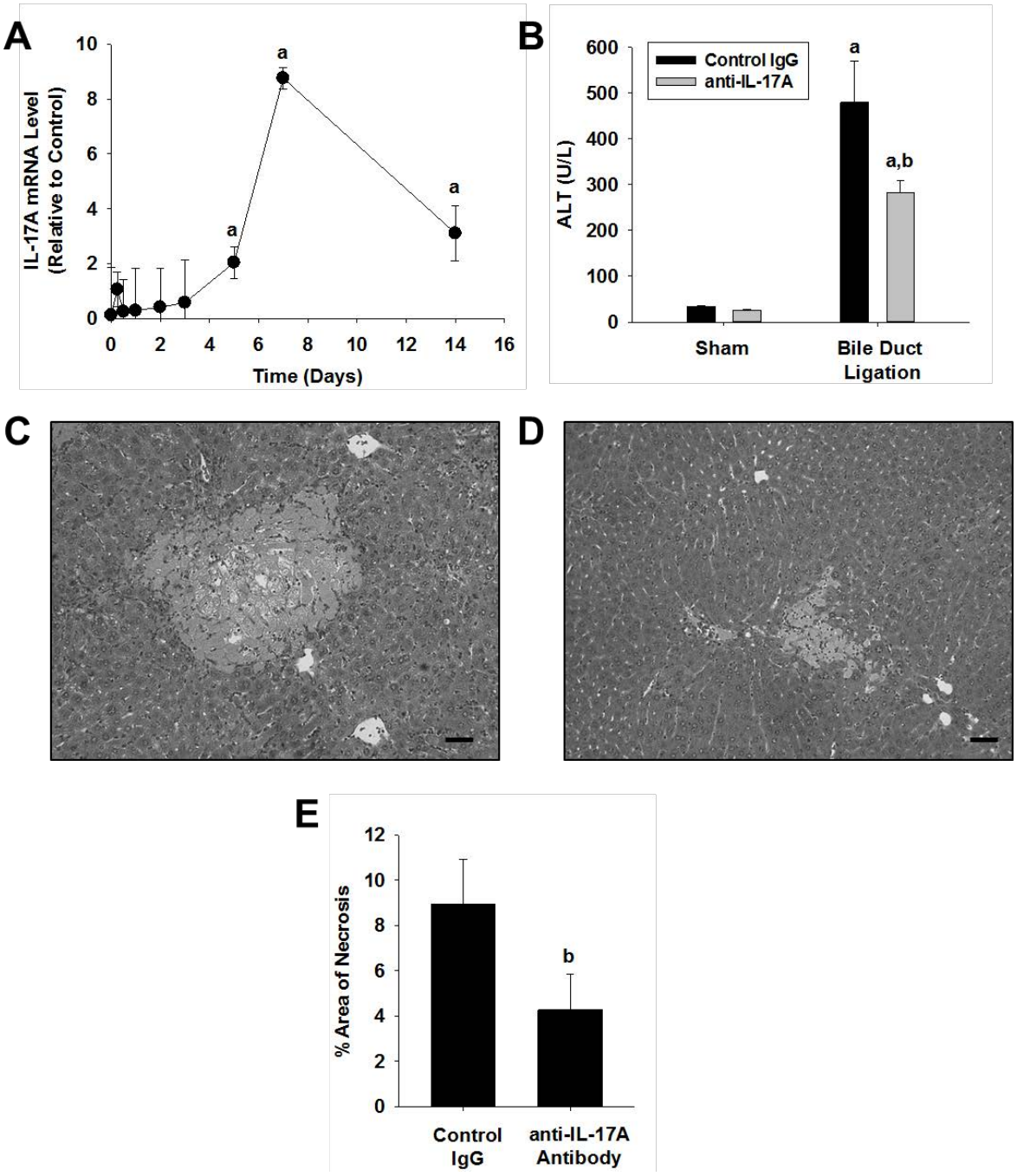


Figure 2.1 Role of IL-17A in the Development of Hepatocellular Injury after BDL. (A) IL-17A mRNA levels were quantified by real-time PCR in the livers of mice subjected to BDL for the indicated time. [a]Significantly different from day 0 ($p<0.05$). Mice were treated with either an

Figure 2.1 (cont'd)

anti- IL-17A antibody or isotype control. The mice were then subjected to BDL (n=10, n=11) or sham operation (n=3,n=3). (B) ALT activity and (C-E) percent area of liver necrosis were quantified at 9 days after surgery. The bar represents 100 μm. [a]Significantly different from sham-operated mice ($p<0.05$). [b]Significantly different from isotype control BDL mice ($p<0.05$).

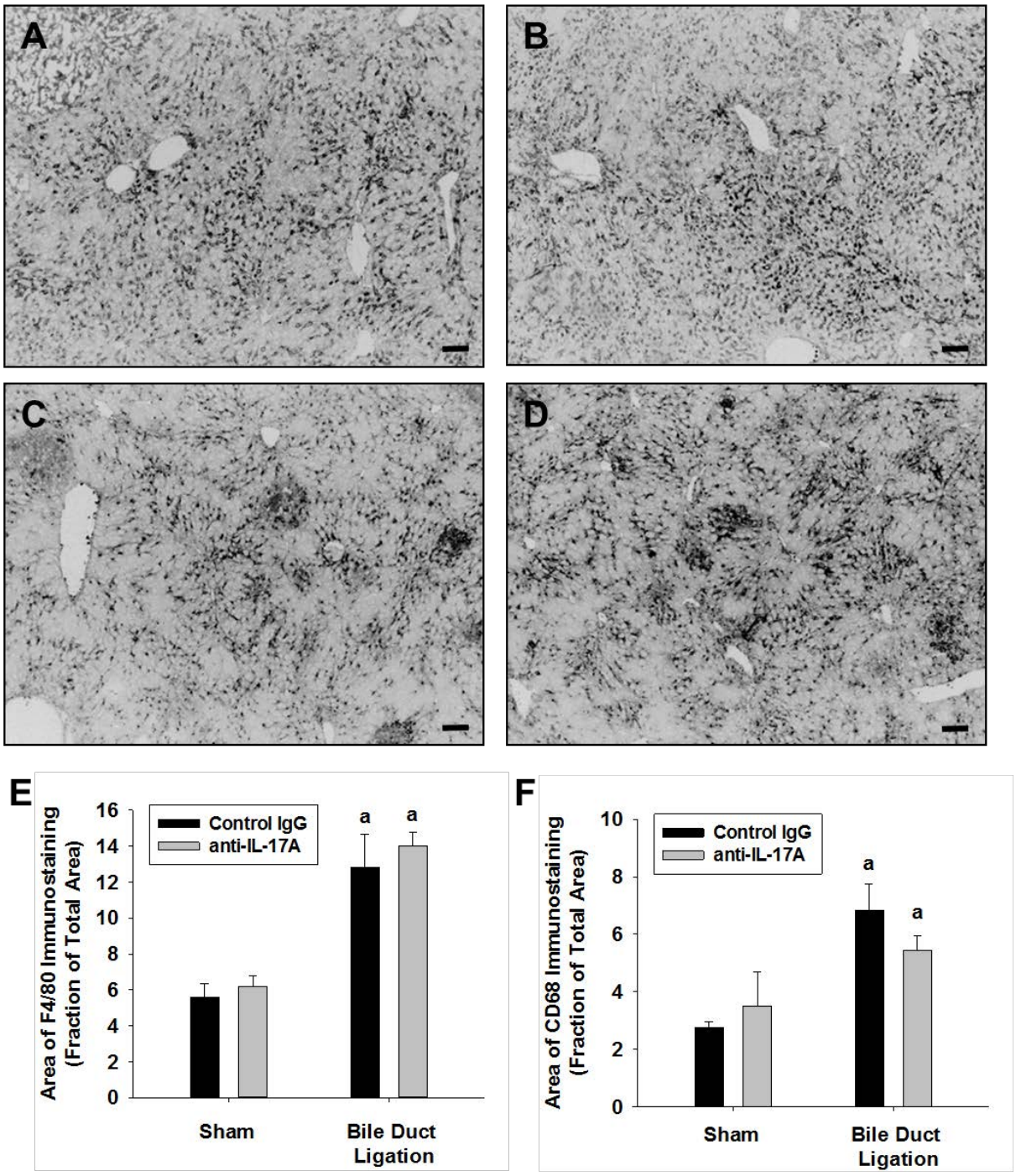


Figure 2.2 Effect of IL-17A Neutralization on Hepatic Accumulation of Macrophages in the Liver after BDL. Mice were treated with either an anti-IL-17A antibody or isotype control, and then subjected to BDL (n=4,n=5) or sham operation (n=3,n=3). At 9 days after surgery, F4/80

Figure 2.2 (cont'd)

positive macrophages were identified in the liver by immunostaining in (A) control IgG BDL mice and (B) anti-IL-17A antibody BDL mice. In addition, (C) isotype control BDL and (D) anti-IL-17A antibody BDL livers were stained for CD68 positive macrophages. The bar represents 100 μM. The area of positive staining was then quantified in sections of liver and expressed as a fraction of the total area (E- F). [a]Significantly different from sham-operated control mice ($p<0.05$).

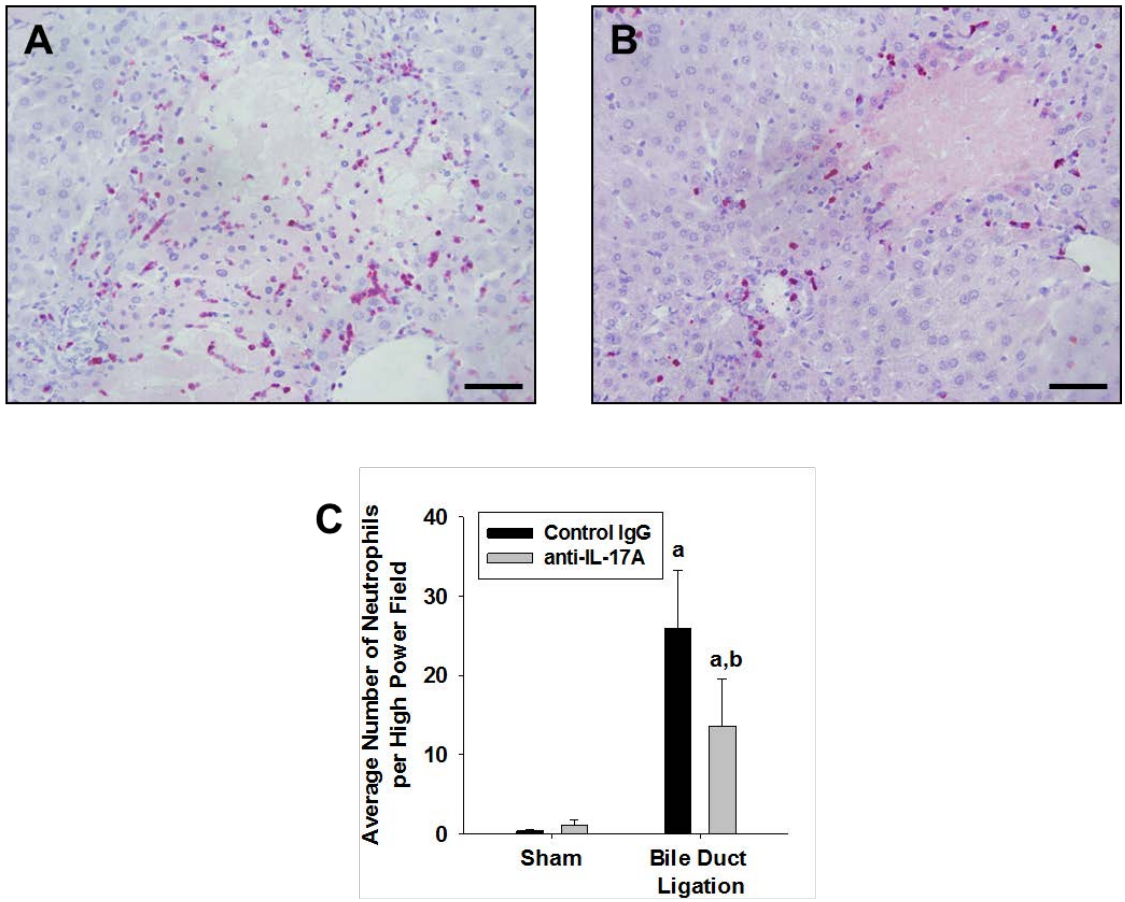


Figure 2.3 Neutralization of IL-17A decreased Hepatic Neutrophils after BDL. Neutrophils were stained in sections of liver by immunohistochemistry. (A) Section of liver from a mouse treated with isotype control and subjected to BDL. (B) Section of liver from a mouse treated with anti-IL-17A antibody and subjected to BDL. Positive cells appear red in the photomicrographs. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. The bar represents 50 μm. (C) Neutrophils were counted in sections of liver (sham: n=3,n=3) (IgG BDL n=9, anti-IL-17A BDL n=10). [a]Significantly different from sham-operated mice ($p<0.05$). [b]Significantly different from isotype control treated mice subjected to BDL ($p<0.05$).

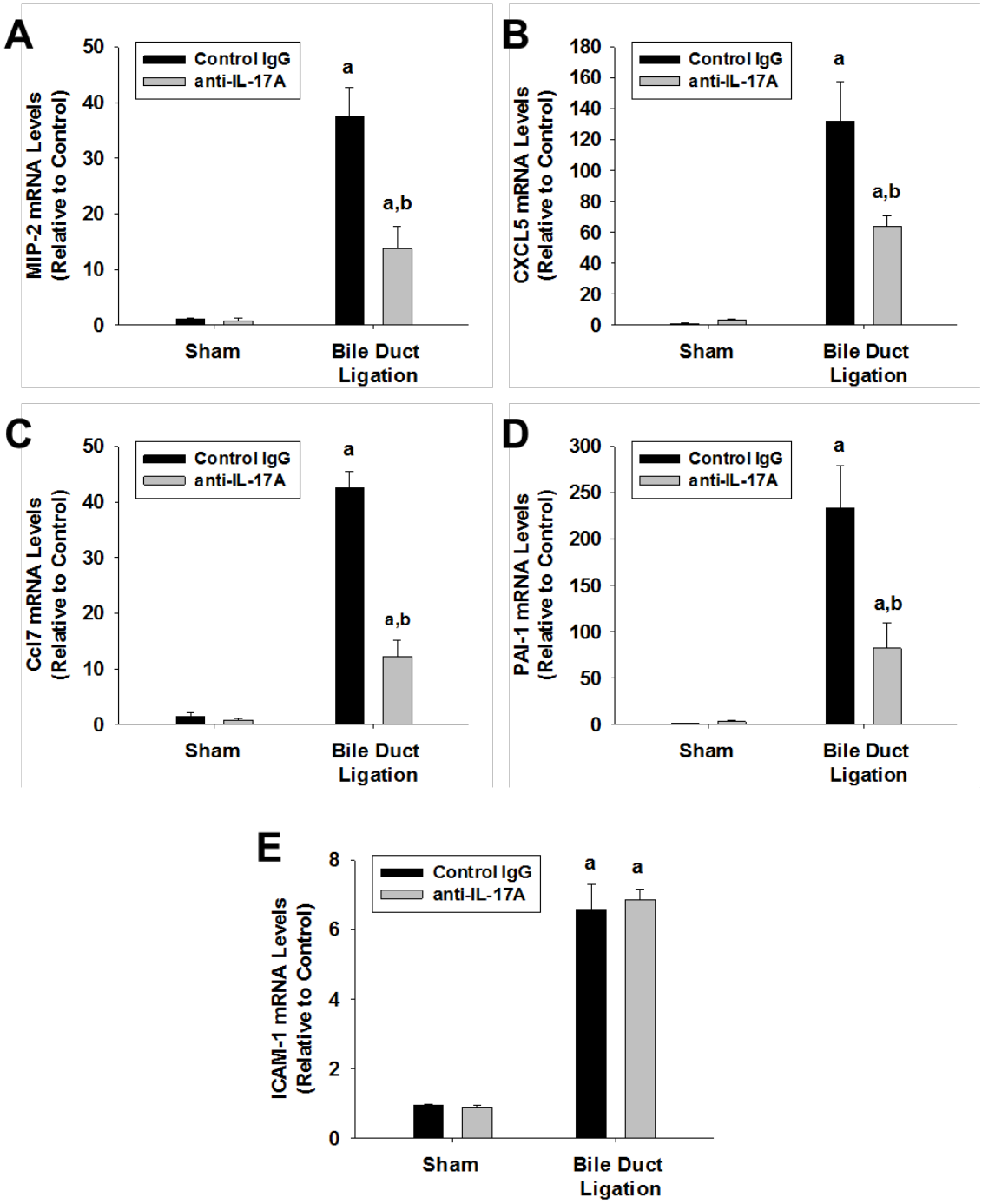


Figure 2.4 Neutralization of IL-17A decreased Expression of Pro-inflammatory Mediators after BDL. Mice were treated with anti-IL-17A antibody or control IgG and subjected to BDL

Figure 2.4 (cont'd)

(n=10, n=11) or sham surgery (n=3,n=3) as detailed in the Materials and Methods. At 9 days after surgery, mRNA levels of the indicated pro-inflammatory mediator were measured by real-time PCR. [a]Significantly different from sham operated mice ($p<0.05$). [b]Significantly different from BDL mice treated with control IgG ($p<0.05$).

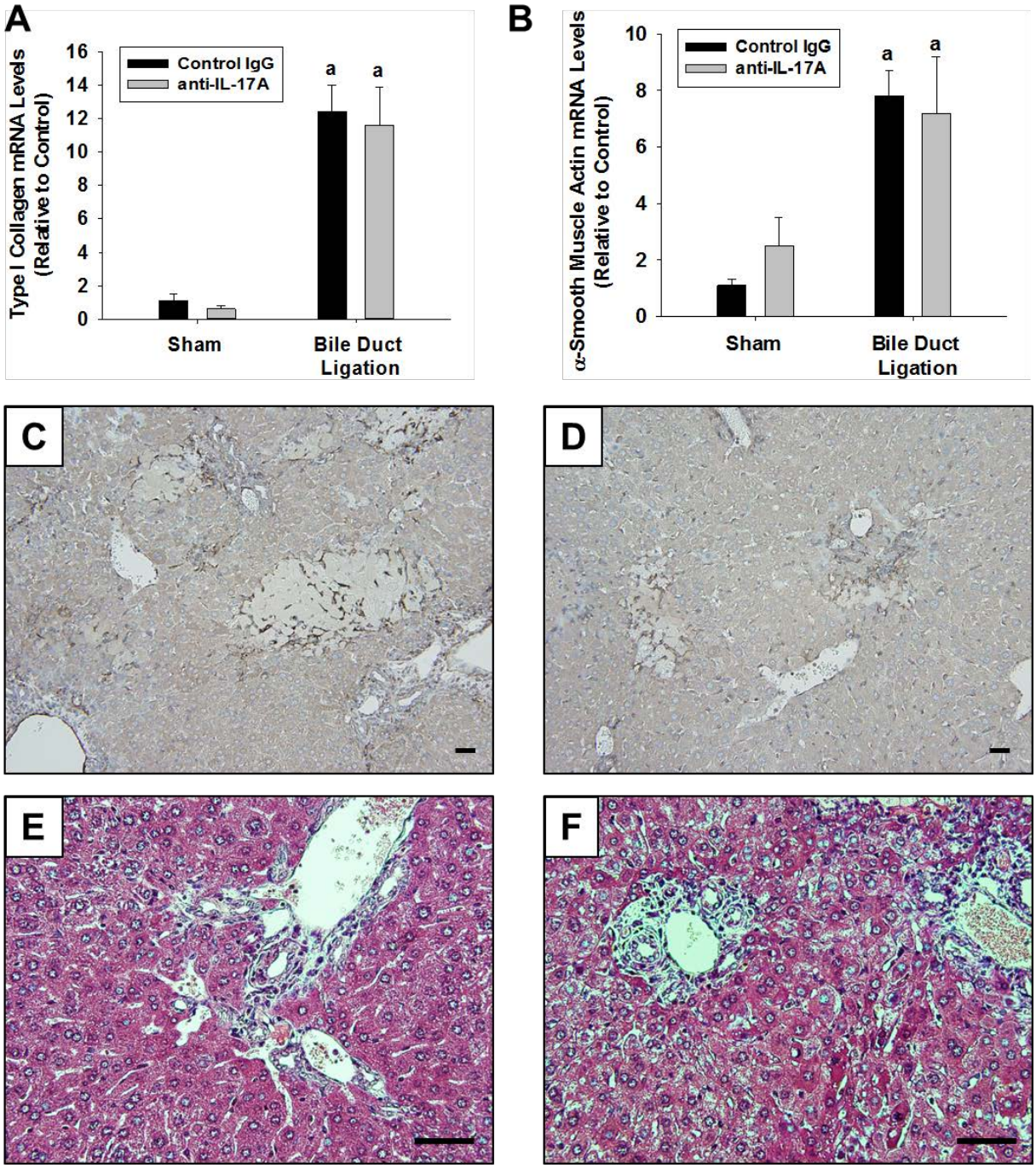


Figure 2.5 Neutralization of IL-17A did not affect Liver Fibrosis 9 days after BDL. Mice were treated with anti-IL-17A antibody or control IgG and subjected to BDL (n=10, n=11) or

Figure 2.5 (cont'd)

sham surgery (n=3, n=3) as detailed in the Materials and Methods. At 9 days after surgery, mRNA levels of (A) type I collagen and (B) α-SMA were quantified. [a]Significantly different from sham operated mice ($p<0.05$). α-SMA protein was detected in the livers of BDL mice treated with (C) control IgG or (D) anti-IL-17A by immunohistochemistry. Positive staining appears dark brown. Sections of liver from BDL mice treated with (E) control IgG or (F) anti-IL-17A were stained with Masson's trichrome. The bars represent 50 μM.

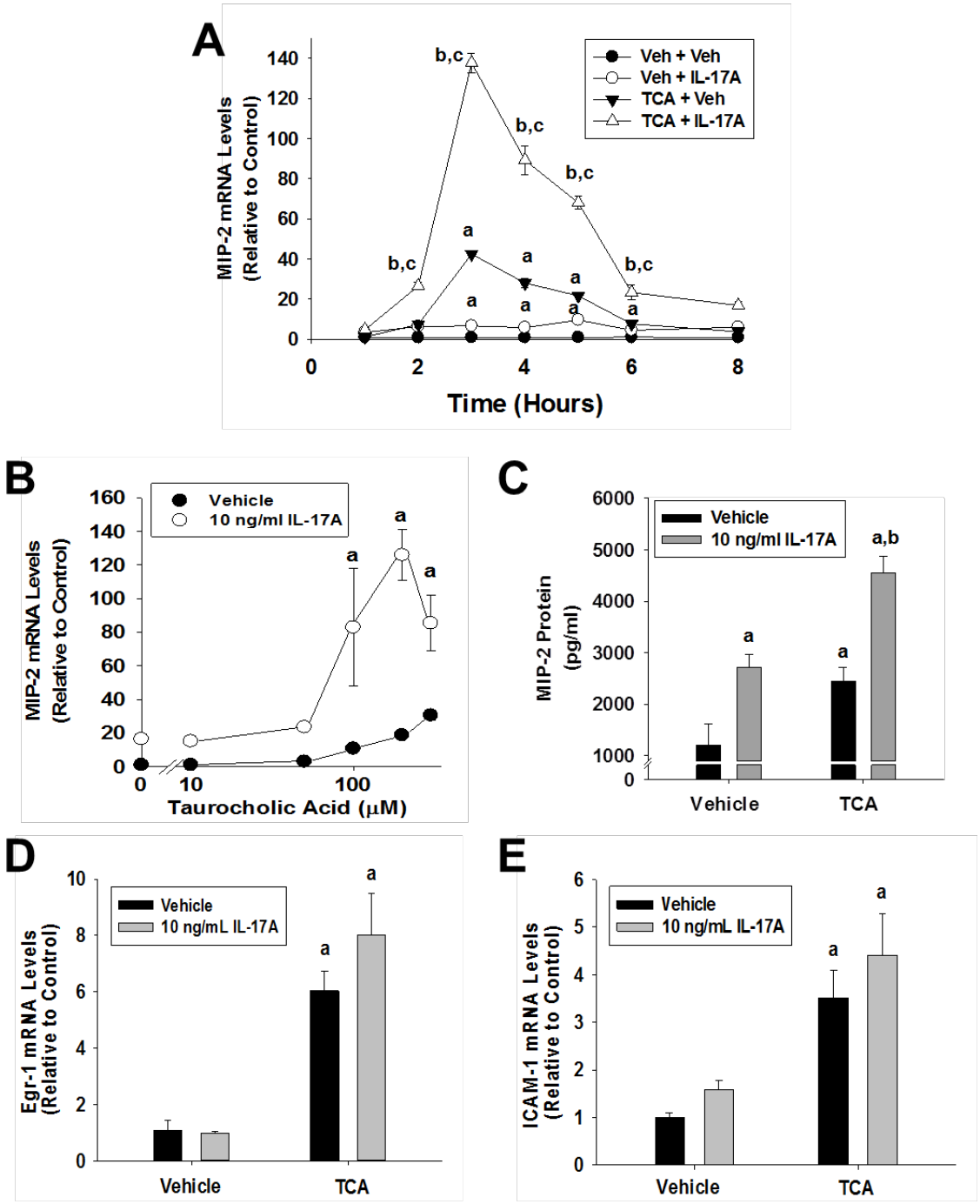


Figure 2.6 Effect of IL-17A on Upregulation of Inflammatory Mediators by TCA. (A) Primary mouse hepatocytes were treated with 10 ng/mL of IL-17A. The cells were then treated with 200 μM TCA for the indicated time. MIP-2 mRNA levels were measured by real-time PCR.

Figure 2.6 (cont'd)

[a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA-treated hepatocytes ($p<0.05$). [c]Significantly different from IL-17A-treated hepatocytes ($p<0.05$). (B) Primary mouse hepatocytes were treated with 10 ng/mL of IL-17A for 16 hours. The cells were then treated with various concentrations of TCA. MIP-2 mRNA levels were measured by real-time PCR. [a]Significantly different from hepatocytes treated with vehicle and TCA ($p<0.05$). (C) For quantification of MIP-2 protein, hepatocytes were treated with 10 ng/mL IL-17A. The cells were then treated with 200 μM TCA for 12 hours. MIP-2 protein was quantified in the medium by ELISA. [a]Significantly different from vehicle-treated hepatocytes ($p<0.05$). [b]Significantly different from hepatocytes treated with either TCA or IL-17A alone ($p<0.05$). Primary mouse hepatocytes were treated with 10 ng/mL of IL-17A followed by treatment with 200 μM TCA. (D) Egr-1 and (E) ICAM-1 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$).

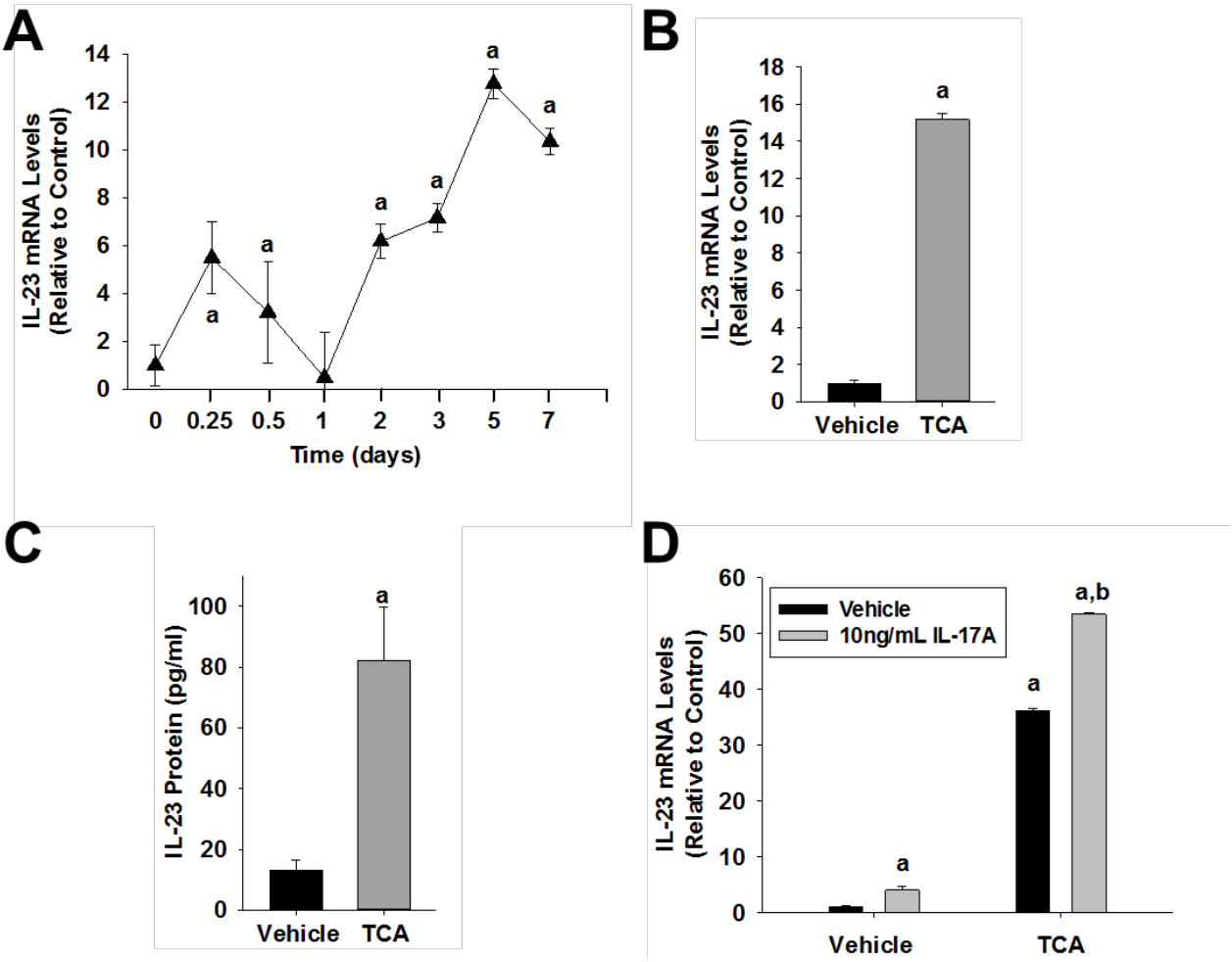


Figure 2.7 Upregulation of IL-23 in the Liver and Primary Mouse Hepatocytes. (A) Mice were subjected to BDL or sham operation. IL-23 mRNA levels were measured in in the liver. [a]Significantly different from day 0 ($p<0.05$). Primary mouse hepatocytes were treated with 200 µM TCA. IL-23 (B) mRNA and (C) protein were measured after 3 and 24 hours of TCA treatment. [a]Significantly different from vehicle- treated hepatocytes ($p<0.05$). (D) Hepatocytes were treated with 10 ng/mL of IL-17A followed by treatment with 200 µM TCA. IL-23 mRNA was measured

Figure 2.7 (cont'd)

by real-time PCR (D). [a]Significantly different from vehicle-treated hepatocytes ($p<0.05$). [b]Significantly different from hepatocytes treated with either IL-17A or TCA ($p<0.05$).

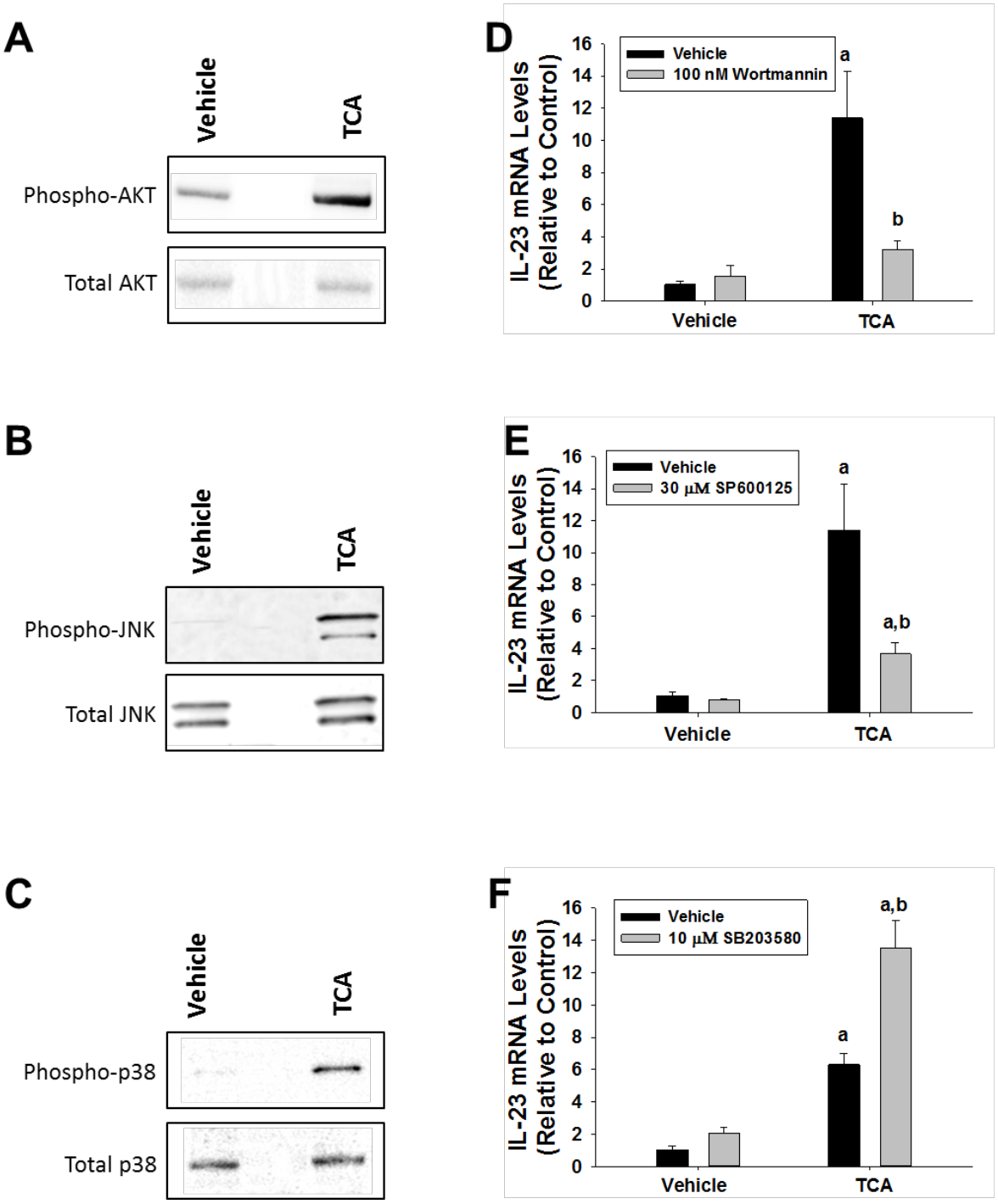


Figure 2.8 Role of Signal Transduction Pathways in Upregulation of IL-23 by TCA.

Hepatocytes were treated with 200 μM TCA for 30 minutes. Western blot was used to detect (A) phospho-and total-AKT (B) phospho-and total-JNK and (C) phospho- and total-p38. Hepatocytes

Figure 2.8 (cont'd)

were treated with (D) 100 nM wortmannin, (E) 30 μM SP600125, or (F) 10 μM SB203580 without 200 μM TCA. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from hepatocytes treated with TCA in the absence of inhibitor ($p<0.05$).

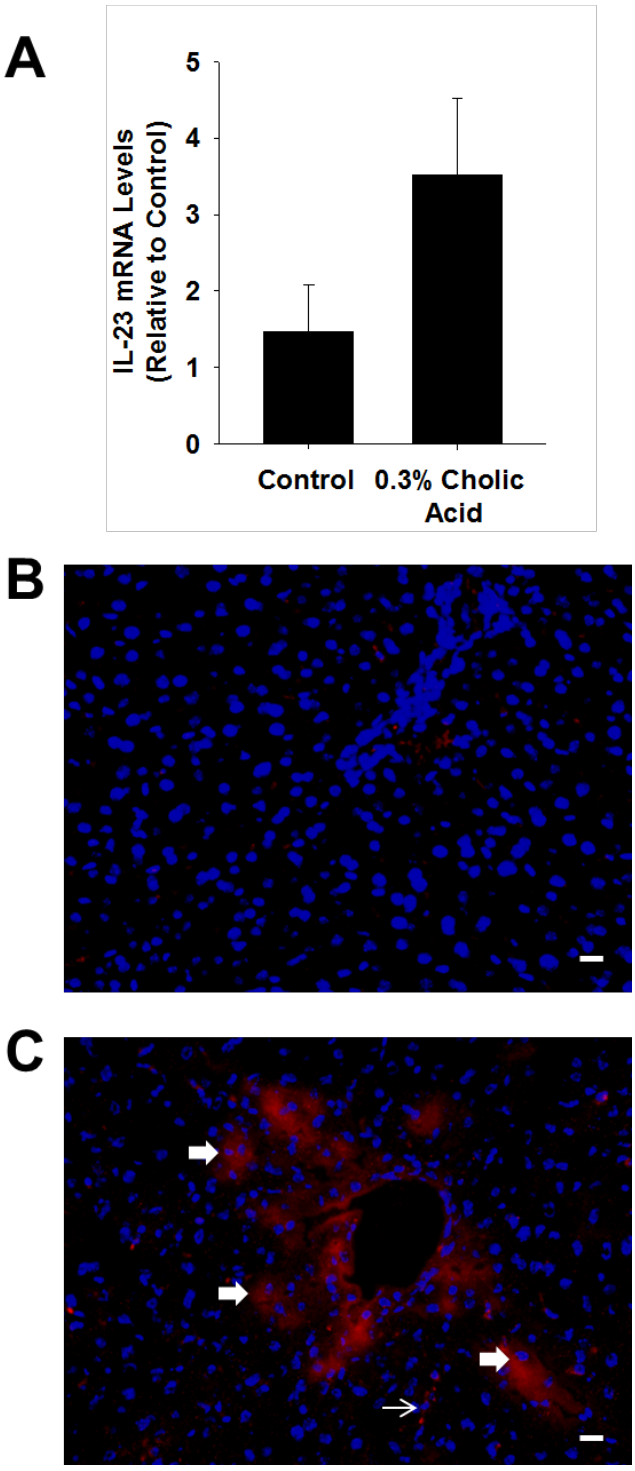


Figure 2.9 Upregulation of IL-23 in the Liver after Bile Acid Feeding. Mice were given either AIN-93M diet supplemented with 0.3% cholic (n=8) or AIN-93M diet alone (n=9). (A) IL-23

Figure 2.9 (cont'd)

mRNA was measured in the liver by real-time PCR. Immunohistochemistry was used to detect IL-23 in sections of liver from mice fed (B) AIN-93M control diet or (C) AIN-93M diet supplemented with 0.3% cholic acid. Positive staining for IL-23 appears red in the photomicrographs. The block arrows indicate IL-23 staining in hepatocytes. The thin arrow indicates area of IL-23 staining in the sinusoid. The bar represents 50 μm. DAPI, which appears blue in the photomicrographs, was used to stain nuclei.

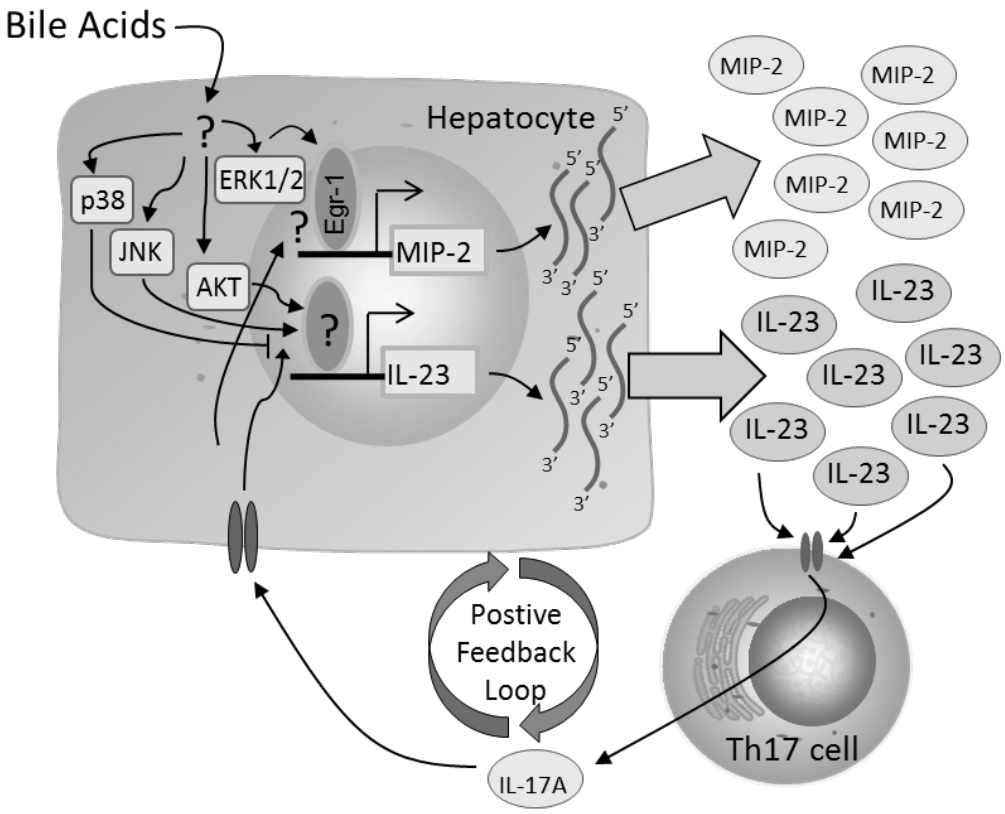


Figure 2.10 Proposed Mechanism of the Interaction between Bile Acids and the IL-23/IL-17A Axis. Bile acids upregulate MIP-2 and other cytokines in hepatocytes by an Egr-1-dependent mechanism. Bile acids also upregulate IL-23 in hepatocytes through AKT and JNK activation. IL-23 maintains Th17 cellular expansion and promotes production of IL-17A. Through an unknown signaling cascade, IL-17A synergistically enhances TCA-induced production of MIP-2 and IL-23 by hepatocytes. Enhanced production of IL-23 leads to the formation of a positive feed-back loop which further amplifies inflammation during cholestasis.

Table 2.1 Primer Pair Sequences

Gene	Forward	Reverse
MIP-2	5’-CCT CAA CGG AAG AAC CAA AGA G-3’	5’-CTC AGA CAG CGA GGC ACA TC-3’
IL-17A	5’-CCG CAA TGA AGA CCC TGA TAG A-3’	5’-TCA TGT GGT GGT CCA GCT TTC-3’
IL-23	5’-GTG ACC CAC AAG GAC TCA AGG A-3’	5’-AGC AGG CTC CCC TTT GAA GAT-3’
Cxcl5	5'-GCT GGC ATT TCT GTT GCT GTT-3'	5'-CGG TTA AGC AAA CAC AAC GCA-3'
Ccl7	5’-AAG ATC CCC AAG AGG AA TCTC A-3’	5’-CAG ACT TCC ATG CCC TTC TTT-3’
PAI-1	5’AGT CTT TCC GAC CAA GAG CA-3’	5’-ATC ACT TGC CCC ATG AAG AG-3’
RPL13a	5’-ACA AGA AAA AGC GGA TGG TG-3’	5’-TTC TCC TCC AGA GTG GCT GT-3’
ICAM-1	5’-AAC AGT TCA CCT GCA CGG AC-3’	5’- GTC ACC GTT GTG ATC CCT G-3’
Egr-1	5’-TGG GAT AAC TCG TCT CCA CC-3’	5’-GAG CGA ACA ACC CTA TGA GC-3’

Table 2.2 The Neutralization of IL-17A did not affect Markers of Cholestasis

Measurement	Control IgG Sham	Anti-IL-17A Sham	Control IgG Bile Duct Ligation	Anti-IL-17A Bile Duct Ligation
ALP (U/L)	146.7 ± 2.2	155.4 ± 14.3	973.6 ± 142.5[a]	916.6 ± 51.9[a]
Bilirubin (mg/dL)	0.1 ± 0.1	0.07 ± 0.04	16.8 ± 1.2[a]	17.4 ± 1.0[a]
Serum Bile Acid (uM)	13.6 ± 1.5	11.5 ± 0.5	274.3 ± 7.6[a]	277.0 ± 10.9[a]

The mice were treated with 100ug of anti-IL-17A antibody or control IgG. After 24 hours, the mice were subjected to bile duct ligation or sham surgery. The mice were further treated with 50ug of anti-IL-17A antibody or control IgG 3 and 6 days post-surgery. The mice were sacrificed 9 days post-surgery. ALP, bilirubin, and total bile acid were measured using commercially available kits. [a]Significantly different from sham operated mice ($p<0.05$).

Table 2.3 Additional Replicates for MIP-2 Induction by IL-17A and TCA Timecourse

Time (hr)	Vehicle	10 ng/mL IL-17	200 µM TCA	10 ng/mL IL-17A + 200 µM TCA
1	1.01±0.12, 1.00± 0.04	4.54±0.60[a], 7.73± 1.13[a]	1.29±0.08, 1.48±0.54	12.26±1.70[b,c], 10.15±0.74[b,c]
2	1.01±0.13, 1.00±0.01	3.29±0.84[a], 5.53±0.80[a]	5.62±1.09[a], 3.10±0.24[a]	31.19±3.05[b,c], 9.94±1.72[b,c]
3	1.00±0.07, 1.01±0.07	5.33±0.72[a], 5.74±1.00[a]	24.41±4.78[a], 6.55±2.25[a]	76.53±10.71[b,c], 12.43±1.60[b,c]
4	1.18±0.49, 1.02±0.16	6.88±1.74[a], 8.76±0.90[a]	20.04±5.33[a], 6.83±0.70[a]	86.36±29.56[b,c], 25.60±2.80[b,c]
6	1.12±0.38, 1.04±0.21	4.53±1.91[a], 11.65±1.47[a]	14.53±5.76[a], 2.92±0.11[a]	21.92±3.27[b,c], 22.11±4.09[b,c]

Hepatocytes were treated with 10 ng/mL of IL-17A in the presence or absence of 200 µM TCA for the time indicated. Total RNA was isolated and reverse transcribed into cDNA. MIP-2 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from IL-17A alone treated hepatocytes ($p<0.05$) [c]Significantly different TCA alone treated hepatocytes ($p<0.05$).

Table 2.4 Additional Replicates for MIP-2 Induction by IL-17A and TCA Dose Response

Treatment	Mean ± SEM
Vehicle	1.01±0.08, 1.03±0.17
10ng/mL IL-17A	7.21±0.36, 6.15±0.22
10 µM TCA	1.17±0.05, 1.21±0.03
50 µM TCA	2.55±0.14, 1.24±0.15
100 µM TCA	12.18±0.77, 1.13±0.08
200µM TCA	27.95±3.86, 1.65±0.14
300 µM TCA	18.88±0.09, 1.55±0.08
10ng/mL IL-17A + 10 µM TCA	8.34±1.12, 7.30±0.65
10ng/mL IL-17A + 50 µM TCA	17.66±1.52[a], 7.62±0.56
10ng/mL IL-17A + 100 µM TCA	41.52±5.65[a], 9.17±0.87[a]
10ng/mL IL-17A + 200 µM TCA	36.18±15.37[a], 13.83±1.44[a]
10ng/mL IL-17A + 300 µM TCA	132.86±18.20[a], 20.33±1.15[a]

Hepatocytes were treated with 10ng/mL IL-17A for 16 hours. Next, hepatocytes were treated with increasing concentrations of TCA (10-300 µM) for 3 hours. MIP-2 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle and TCA alone treated hepatocytes ($p<0.05$).

Table 2.5 Additional Replicates for ICAM-1 Induction by IL-17A and TCA

Treatment	Mean ± SEM
Vehicle	1.00±0.01, 1.00±0.06
10ng/mL IL-17A	1.34±0.11, 1.13±0.27
200 µM TCA	2.04±0.24[a], 1.92±0.11[a]
10ng/mL IL-17A + 200 µM TCA	2.33±0.03[a], 2.19±0.19[a]

Hepatocytes were pretreated with 10 ng/mL IL-17A for 16 hours and then treated with 200 µM TCA for 3 hours. ICAM-1 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$).

Table 2.6 Additional Replicates for TCA Induction of IL-23 Protein Expression

Treatment	Mean ± SEM
Vehicle	18.64±2.34, 10.18±0.70
200 µM TCA	126.09±5.82[a], 23.95±3.81[a]

Hepatocytes were treated with 200 µM TCA for 24 hour. IL-23 protein concentrations were measured in the media by ELISA. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$).

Table 2.7 Additional Replicates for IL-23 Induction by IL-17A and TCA

Treatment	Mean ± SEM
Vehicle	1.11±0.31
10ng/mL IL-17A	1.54±0.28
200 µM TCA	6.46±0.48[a]
10ng/mL IL-17A + 200 µM TCA	12.40±2.0[a,b]

Hepatocytes were pretreated with 10 ng/mL IL-17A for 16 hours, and then treated with 200 µM TCA for 3 hours. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

Table 2.8 Additional Replicates for AKT, p38, and JNK Regulation of IL-23 Induction

Treatment	Mean ± SEM
Vehicle	1.03±0.18, 1.00±0.04
200 µM TCA	21.40±3.99[a], 6.38, 0.85[a]
100nM Wortmannin	1.05±0.08, 1.14±0.22
100nM Wortmannin + 200 µM TCA	9.29±0.75[a,b], 3.06±0.93[a,b]
10 µM SB203850	7.49±0.55[a], 4.54±0.48[a]
10 µM SB203850 + 200 µM TCA	111.79±12[a,b].78, 23.57±4.07[a,b]
30 µM SP600125	4.63±0.62[a], 1.18±0.25
30 µM SP600125 + 200 µM TCA	16.23±1.82[a,b], 2.20±0.16[a,b]

Primary murine hepatocytes were pretreated with either 100 nM Wortmannin, 30 µM SP600125, or 10 µM SB203850 for 30 minutes. Next, they were treated with 200 µM TCA for 3 hours. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different for vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

Chapter 3

Role of the Mitogen Activated Protein Kinase Signaling Pathways and C/EBPβ in the Synergistic Interaction between Bile Acids and the IL-23/IL-17A Axis

3.1 Abstract

During cholestasis, there is an increase in hepatic bile acid concentrations and interleukin-17A (IL-17A) expression. Recently, we demonstrated that IL-17A synergistically enhances bile acid induction of the chemokine, macrophage inflammatory protein-2 (MIP-2). However, the mechanisms that underlie this synergistic interaction have not been elucidated.

The transcription factor, CCAAT-enhancer binding protein-β (C/EBPβ), is highly expressed in hepatocytes and has been shown to mediate IL-17A enhancement of pro-inflammatory mediators in murine hepatocytes. Therefore, we tested the hypothesis that C/EBPβ expression is required for the synergistic interaction between IL-17A and taurocholic acid (TCA). Knockdown of C/EBPβ reduced MIP-2 mRNA levels in TCA treated hepatocytes; whereas, MIP-2 expression was unaffected in IL-17A treated C/EBPβ heterozygous knockout ($C/EBP\beta^{-/+}$) hepatocytes compared to wild-type. The synergistic induction of MIP-2 was partially attenuated in the IL-17A and TCA treated $C/EBP\beta^{-/+}$ hepatocytes. Interestingly, hepatocellular injury and inflammation were unchanged in C/EBPβ heterozygous knockout mice subjected to bile duct ligation, a murine model of obstructive cholestasis. These results demonstrate that C/EBPβ is a transcriptional regulator of the bile acid induced inflammatory response in hepatocytes.

To further evaluate the mechanism underlying the synergistic interaction between bile acids and IL-17A, we investigated the p38 mitogen-activated kinase (p38) and c-Jun N-terminal kinase (JNK) signaling pathways which have been shown to mediate IL-17A synergistic interactions with various cytokines. TCA-induced activation of p38 and JNK was inhibited by IL-17A. Pharmacological inhibition of p38 and JNK activation synergistically enhanced MIP-2

induction by TCA. These data suggest that the IL-17A synergistically enhances MIP-2 induction by TCA via the inhibition of p38 and JNK activation.

3.2 Introduction

Neutrophils are required for hepatocellular injury during obstructive cholestasis (Gujral et al., 2003). The inflammatory mediators, macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), and intercellular adhesion molecule-1 (ICAM-1) are critical for neutrophil recruitment to the liver during cholestasis (Gujral et al., 2004; Wintermeyer et al., 2009). We showed recently that conjugated primary bile acids, at pathophysiological concentrations, do not affect cellular viability of hepatocytes but promote ERK1/2-dependent upregulation of the transcription factor, early growth response factor-1 (Egr-1), which upregulates MIP-2 and ICAM-1 (Allen et al., 2011; Allen et al., 2010). Interestingly, we also demonstrated that IL-17A synergistically enhances bile acid-induced inflammatory signaling in hepatocytes (O'Brien et al., 2013).

Interleukin-17A (IL-17A) is a Th17 cytokine that promotes neutrophil recruitment and host defense against bacterial infections (Curtis and Way, 2009; Kono et al., 2011; Maione et al., 2009). IL-17A positive cells are increased in patients with primary biliary cirrhosis and in the murine models of cholestasis, bile duct ligation (BDL) and α-naphthylisothiocyanate (ANIT) (Kobayashi et al., 2010; Lan et al., 2009). Inhibition of IL-17A with a neutralizing antibody in bile duct ligated and ANIT treated mice reduced liver injury, hepatic expression of inflammatory mediators, and hepatic neutrophil accumulation. These data indicate a key role for IL-17A in the development of liver inflammation, during cholestasis (Kobayashi et al., 2010; O'Brien et al., 2013). We further showed that IL-17A synergistically enhanced upregulation of inflammatory mediators in bile acid-treated hepatocytes, suggesting that IL-17A promotes inflammation during

cholestasis by interacting with bile acid inflammatory signaling in hepatocytes (O'Brien et al., 2013). What remains unknown, however, is the mechanism by which bile acids and IL-17A synergistically interact.

Others have shown that IL-17A synergizes with multiple inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), to stimulate production of inflammatory mediators by alveolar type II cells and astrocytes, which is similar to the interaction we observed between IL-17A and bile acids (Liu et al., 2011b; Ma et al., 2010). The mechanisms that underlie these synergistic interactions are varied and include modulation of mitogen activate protein kinase (MAPK) signaling by IL-17A, alterations in cytokine mRNA stability by IL-17A, and upregulation of transcription factors (Nf-kB and C/EBPβ) by IL-17A (Iyoda et al., 2010; Liu et al., 2011b; Patel et al., 2007; Sun et al., 2011). For example, inhibition of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated kinase (p38) signaling prevented the synergistic interaction between IL-6 and IL-17A in astrocytes (Ma et al., 2010). Since bile acids upregulate inflammatory mediators in hepatocytes through MAPK signaling, it is possible that IL-17A may enhance bile acid-induced inflammatory signaling by modulating MAPK signaling (Allen et al., 2010; Rao et al., 2002). Another mechanism by which this may occur is through upregulation of various transcription factors by IL-17A.

IL-17A signaling is mediated through the receptor complex, IL-17RA/IL-17RC, which activates the transcription factors, NF-kB and C/EBPβ (Maitra et al., 2007; Sonder et al., 2011). In most cell types, however, IL-17A is a weak activator of NF-kB (Granet et al., 2004; Shen and Gaffen, 2008). Nuclear translocation of C/EBPβ requires phosphorylation of regulatory domain 2 (RD2) (Williams et al., 1995). IL-17A promotes phosphorylation of C/EBPβ on two different threonine residues, Thr188 and Thr179, via ERK and glycogen synthase kinase-3β (GSK3β)

(Shen et al., 2009). It has been demonstrated that C/EBPβ expression is critical for the synergistic induction of IL-6 by TNF-α and IL-17A, in murine embryonic fibroblasts (Ruddy et al., 2004). Considering the potential of C/EBPβ to mediate synergistic interactions between IL-17A and other mediators, we also investigated its role in the interaction between bile acids and IL-17A.

3.3 Materials and Methods

3.3.1 Animal Care. All experiments were performed on either male C57BL/6 wild-type, C/EBPβ heterozygous knockouts or C/EBPβ wild-type littermates (Jackson Laboratories; Bar Harbor, ME). All of the mice were maintained on a 12-h light/dark cycle under controlled temperature (18–21°C) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. All of the 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 by collagenase H retrograde perfusion as described previously (Kim et al., 2006). Hepatocytes were treated with 10 ng/mL of mouse recombinant IL-17A reconstituted in 4 mM HCl (R&D systems; Minneapolis, MN) for either 16 hours or 30 minutes followed by treatment with 200 μM taurocholic acid (TCA) in endotoxin free water for the time indicated. For signal transduction studies, hepatocytes were treated with 10 μM SB203850, 30 μM SP600125, or DMSO as vehicle for 30 minutes followed by treatment with 200 μM TCA for 3 hours.

3.3.3 Bile Duct Ligation. C/EBPβ heterozygous knockouts or C/EBPβ wild-type littermates (Jackson Laboratories; Bar Harbor, ME) were anesthetized with isoflurane. A midline laparotomy was performed and the bile duct ligated with 4-0 surgical silk. The abdominal

incision was closed with sutures, and the mice received 0.2 mg/kg buprenex by subcutaneous injection twice daily. Mice were sacrificed 72 hours after surgery.

3.3.4 Serum Chemistry. Serum alanine aminotransferase (ALT) activity was measured by a commercially available kit (Thermo Scientific; Middletown, VA) per manufacturers' instructions.

3.3.5 Immunohistochemistry. Paraffin-embedded liver sections were incubated with a rat anti-mouse polymorphonuclear leukocytes (PMN) 7/4 antibody (1:2500) (AbD Serotec; Raleigh, NC) for one hour. Neutrophils were quantified by counting the number of positively-stained cells in 10, 200X fields per tissue section. The analysis was performed in a blinded fashion.

3.3.6 Protein Analysis. Hepatocytes were lysed in RIPA buffer containing protease and phosphatase inhibitors (Thermo Scientific; Rockford, IL). Protein was separated on a 4-20% polyacrylamide gel and transferred to PVDF membrane. The membranes were incubated with either an anti-p38, anti-phosphorylated p38 (Thr180/Tyr182), anti-JNK, anti-phosphorylated JNK (Thr183/Tyr185), anti-C/EBPβ, or anti-phosphorylated C/EBPβ (Thr188) antibodies (all from Cell Signal Technology; Danvers, MA). The membranes were then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. The bands were detected using the ECL chemiluminescence detection kit (GE Healthcare; Buckinghamshire, UK) on a LiCor Fc (Odyssey; Lincoln, NE).

3.3.7 Real-Time PCR. Total RNA was isolated by Trizol extraction, and contaminating DNA was removed using the Turbo DNA-free kit (Ambion; Austin, TX) as per manufacturer's instructions. RNA was reverse transcribed into cDNA as described in 2.3.8. mRNAs were analyzed on a 7500 Fast Real-time PCR system (Applied Biosystems; Foster City, CA) by using

the fast SYBR green reagent (Bio-rad; Hercules, CA). Primer sequences are outlined in Table 2.1. All primers used were intron spanning.

3.3.8 Statistics. Data are expressed as mean ± SEM. When two or more groups were analyzed, a two way analysis of variance (ANOVA) was performed. When the data set did not pass normality, a log transformation was utilized. Comparison between groups was done by using the Holm-Sidak method. A 95% confidence, $p<0.05$, was the criterion for significance.

3.4 Results

3.4.1 Activation of C/EBPβ by IL-17A and TCA. There are multiple downstream pathways activated by IL-17A in various cells, including C/EBPβ (Shen et al., 2009). Therefore, we evaluated whether C/EBPβ is activated in primary murine hepatocytes treated with IL-17A and TCA. Treatment of hepatocytes with either IL-17A or TCA stimulated phosphorylation of C/EBPβ, indicating activation. Surprisingly, co-treatment with IL-17A and TCA did not stimulate phosphorylation of C/EBPβ. Instead, there was a reduction of phosphorylated C/EBPβ after 10 minutes in the co-treated hepatocytes (Figure 3.1).

3.4.2 C/EBPβ is required for upregulation of MIP-2 and IL-23 in TCA and IL-17A treated hepatocytes. We next tested the hypothesis that activation of C/EBPβ is required for the synergistic interaction between IL-17A and TCA. To test this hypothesis, hepatocytes were isolated from C/EBPβ heterozygous knockout mice or wild-type littermates. For confirmation, we measured the protein expression of C/EBPβ in these livers. Hepatic C/EBPβ expression was decreased in the C/EBPβ heterozygous knockout mice, compared to their wild-type littermates (Figure 3.2A). Knockdown of C/EBPβ did not affect upregulation of MIP-2 or IL-23 by IL-17A (Figure 3.2B-C). However, MIP-2 and IL-23 mRNA levels were reduced in TCA treated C/EBPβ heterozygous knockout hepatocytes, when compared to wild-type hepatocytes.

Furthermore, the synergistic enhancement of MIP-2 and IL-23 was partially attenuated in C/EBPβ heterozygous knockout hepatocytes treated with IL-17A and TCA (Figure 3.2B-C). These results demonstrate a critical role for C/EBPβ in upregulation of MIP-2 and IL-23 in hepatocytes by TCA.

3.4.3 C/EBPβ is not required for hepatocellular injury or inflammation during cholestasis. Considering the importance of C/EBPβ for upregulation of inflammatory mediators by bile acids *in vitro,* we performed bile duct ligation in C/EBPβ heterozygous knockout mice and their wild-type littermates to determine its regulation of inflamed-induced hepatocellular injury during cholestasis. Alanine aminotransferase (ALT) activity, a clinical biomarker of hepatocellular injury, was increased in wild-type mice subjected to BDL. Partial deletion of C/EBPβ did not affect the increase in ALT activity in BDL mice (Figure 3.3A).

Next, we determined the effect of C/EBPβ on the inflammatory response during cholestasis. MIP-2 mRNA levels were increased in wild-type mice subjected to BDL. Upregulation of MIP-2 trended lower in C/EBPβ heterozygous knockout mice subjected to bile duct ligation; however, it was not significant (Figure 3.3B). IL-23 was not increased in either wild-type or C/EBPβ heterozygous knockout mice subjected to BDL (Figure 3.3C). Consistent with the cytokine mRNA levels, there was no difference in neutrophil cell count in the C/EBPβ heterozygous knockout BDL mice compared to their wild-type counterparts (Figure 3.3D).

3.4.4 IL-17A synergistically enhances MIP-2 induction by TCA through inhibition of p38 and JNK activation. Studies have shown that bile acids activate the p38 and JNK signaling pathways (Gupta et al., 2004; O'Brien et al., 2013; Qiao et al., 2003; Schoemaker et al., 2004). Therefore, we investigated whether these pathways are required for upregulation of MIP-2 by bile acids and whether their activation was affected by IL-17A. Treatment of hepatocytes

with IL-17A partially decreased phosphorylation of JNK and p38. Treatment with TCA increased phosphorylation of JNK and p38. Interestingly, IL-17A prevented phosphorylation of JNK and p38 by TCA (Figure 3.4A-B). Since IL-17A inhibited activation of JNK and p38 by TCA, we next determined the impact of blocking the signaling of these pathways with pharmacological inhibitors on upregulation of MIP-2 by TCA. Inhibition of JNK and p38 enhanced MIP-2 mRNA production by TCA; thereby, suggesting that IL-17A may induce upregulation of MIP-2 by TCA through the inhibition of JNK and p38 (Figure 3.4C-D).

3.5 Discussion

Pathophysiological concentrations of bile acids produce hepatic inflammation via the induction of MIP-2 expression in hepatocytes (Allen et al., 2011). IL-17A is a pro-inflammatory cytokine, produced by Th17 cells, that has been shown to maintain and prolong neutrophilic inflammatory responses through a synergistic interaction between cytokines in various disease states, including cholestasis (Griffin et al., 2012; Liu et al., 2011b; Maione et al., 2009). We showed previously that there is a synergistic induction of MIP-2 in hepatocytes exposed to IL-17A and TCA (O'Brien et al., 2013). However, the signaling pathway(s) that mediate the synergy between hepatocytes and Th17 cells is not understood.

Previous studies have shown that IL-17A synergistically enhances production of inflammatory mediators by TNF-α. The synergistic interaction between IL-17A and TNF-α was shown to require the activation of the transcription factor, C/EBPβ, in mouse embryonic fibroblasts (Ruddy et al., 2004). A previous study demonstrated that IL-17A promotes the phosphorylation of the full length liver activating protein (LAP) isoform of C/EBPβ (Maitra et al., 2007) (Figure 3.1). Shen et al demonstrated that IL-17A induces the phosphorylation of C/EBPβ on two threonine residues in a ERK- and GSK3β-dependent manner, which represses

gene transcription of IL-6, Ccl7, and Cxcl5, in stromal cells (Shen et al., 2009). ERK is a known regulator of the transcription factor, Egr-1 (Allen et al., 2010). Previously, we have shown that Egr-1 is critical for TCA induction of MIP-2 mRNA levels and inflammation induced injury, in a murine model of cholestasis (Allen et al., 2010; Kim et al., 2006). C/EBPβ and Egr-1 are known to have a protein-protein interaction that prolongs the transcription of target genes (Jakobsen et al., 2013; Zhang et al., 2003). Furthermore, C/EBPβ has been studied in hepatocyte proliferation, liver repair, and steatosis (fatty liver disease) due to its high expression in the liver (Birkenmeier et al., 1989; Greenbaum et al., 1998; Rahman et al., 2007). Therefore, we investigated the role of C/EBPβ in the synergistic enhancement of MIP-2 by IL-17A and TCA in murine hepatocytes. The knockdown of C/EBPβ decreased MIP-2 mRNA levels after TCA treatment and attenuated the synergistic induction of MIP-2 by TCA and IL-17A (Figure 3.2B). Interestingly, there was a complete prevention of the synergistic induction of IL-23 in C/EBPβ heterozygous knockout hepatocytes (Figure 3.2C). Our results suggest a critical role for C/EBPβ in bile acid induction of the inflammatory response by hepatocytes. However, C/EBPβ was not required for IL-17A signaling in hepatocytes (Figure 3.2B-C). Therefore, bile acid and IL-17A synergistic induction of the inflammatory response could require the interaction of both C/EBPβ and Egr-1 on the response elements of the inflammatory genes. Our *in vitro* results suggest that there are either distinct pathways that mediate the synergistic interaction, or the enhancement of MIP-2 requires the sequential activation of another transcription factor, such as Egr-1.

To further investigate C/EBPβ expression in bile acid regulation of the inflammatory response, we subjected C/EBPβ heterozygous knockout mice to bile duct ligation for 72 hours. Surprisingly, the knockdown of C/EBPβ did not affect hepatocellular injury (Figure 3.3A). In bile duct ligated mice, hepatocyte proliferation and repair begins around day 2 post-surgery

(Georgiev et al., 2008). It has been demonstrated that the deletion of C/EBPβ delays entrance into S phase of the cell cycle in mice that have undergone partial hepatectomy (Greenbaum et al., 1998; Jakobsen et al., 2013). Due to the fact that the C/EBPβ heterozygous knockout mice underwent BDL for 3 days, the impairment of liver regeneration could explain the similar serum ALT activity between the two groups. In addition, inflammation was unaffected in C/EBPβ heterozygous knockout mice compared to wild-type (Figure 3.3B-D). Although, MIP-2 mRNA levels had a lower trend in the C/EBPβ heterozygous BDL mice (Figure 3.3B). The difference between our *in vivo* and *in vitro* results could be explained by a compensatory mechanism promoted by C/EBPδ, another isoform that is also induced by IL-17A in mouse embryonic fibroblast and mediates IL-17A synergistic enhancement of IL-6 (Maitra et al., 2007; Ruddy et al., 2004; Shen et al., 2005). However, TCA may not induce C/EBPδ in primary hepatocytes, which may explain why we observed a C/EBPβ-dependent regulation of MIP-2 induction. Hepatocyte specific C/EBPβ knockout mice subjected to BDL would better indicate the role of C/EBPβ in the inflammatory response. Overall, these results further suggest that IL-17A synergistic interaction with TCA requires the activation of additional signaling cascades besides C/EBPβ.

Next, we examined known MAPK signaling pathways that are activated by either IL-17A or bile acids (Chen et al., 2011; Patel et al., 2007; Qiao et al., 2003). The synergistic interaction between IL-17A and multiple cytokines (TNF-α and IL-6) requires activation of either p38 or JNK in mesangial cells and astrocytes (Iyoda et al., 2010; Ma et al., 2010). Furthermore, bile acid activation of the JNK pathway regulates activation of the pro-apoptotic cascade in rodent hepatocytes (Qiao et al., 2003). Therefore, we determined the protein expression of phospho-JNK and phospho-p38, in hepatocytes treated with IL-17A in the presence or absence of TCA

(Figure 3.4A-B). Contrary to other cell types, IL-17A inhibited activation of JNK and p38 (Figure 3.4A-B). In addition, inhibition of JNK and p38 with pharmacological inhibitors synergistically enhanced MIP-2 mRNA levels in TCA treated hepatocytes (Figure 3.4C-D). These studies suggest that the synergistic enhancement of MIP-2 occurs through IL-17A inhibition p38 and JNK signaling. An underlying cause of this inhibition might be that IL-17A promotes activation of a protein phosphatase that would deactivate either p38 or JNK signaling. IL-17A inhibits the activity of MAPK phosphatase-1 (MKP-1) in cardiac fibroblasts (Valente et al., 2012). Due to the divergent cellular response from our results and those in other cells, IL-17A may activate MKP-1 in hepatocytes, which promotes the deactivation of the p38 or JNK signaling pathways. The role of p38 and JNK in MIP-2 synergistic induction still needs further investigation.

In conclusion, we have demonstrated that C/EBPβ mediates upregulation of pro-inflammatory chemokines in primary mouse hepatocytes by TCA *in vitro*. Furthermore, our results suggest an involvement of the MAPK signaling pathways in the synergistic interaction between IL-17A and bile acids in hepatocytes.

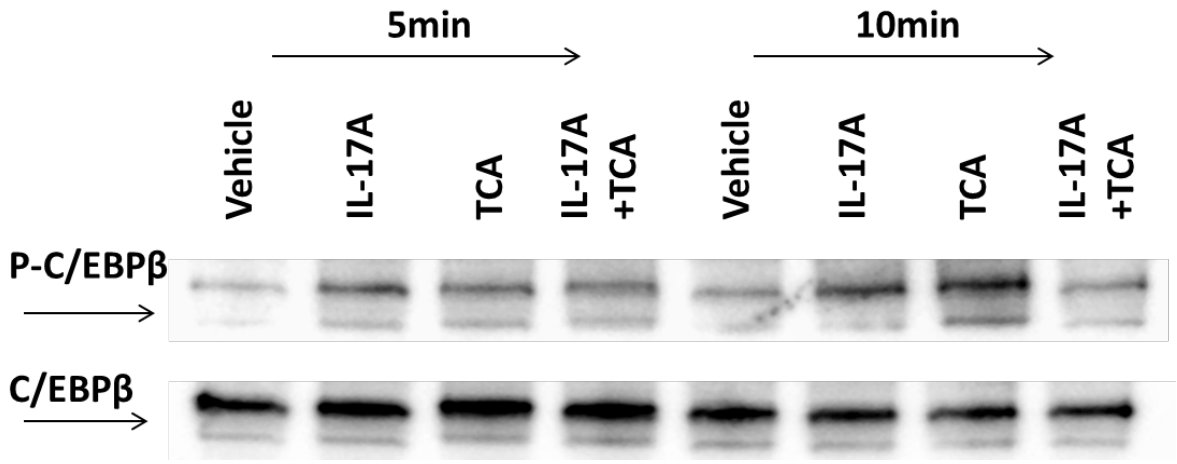


Figure 3.1 Effect of IL-17A and TCA on C/EBPβ Activation. Hepatocytes were pretreated with 10 ng/mL IL-17A for 30 minutes. Then, hepatocytes were treated with 200 μM TCA for the time indicated. Western blots were incubated with either an anti-phospho-C/EBPβ or an anti-C/EBPβ. Bands were detected using ECL chemiluminescence detection kit.

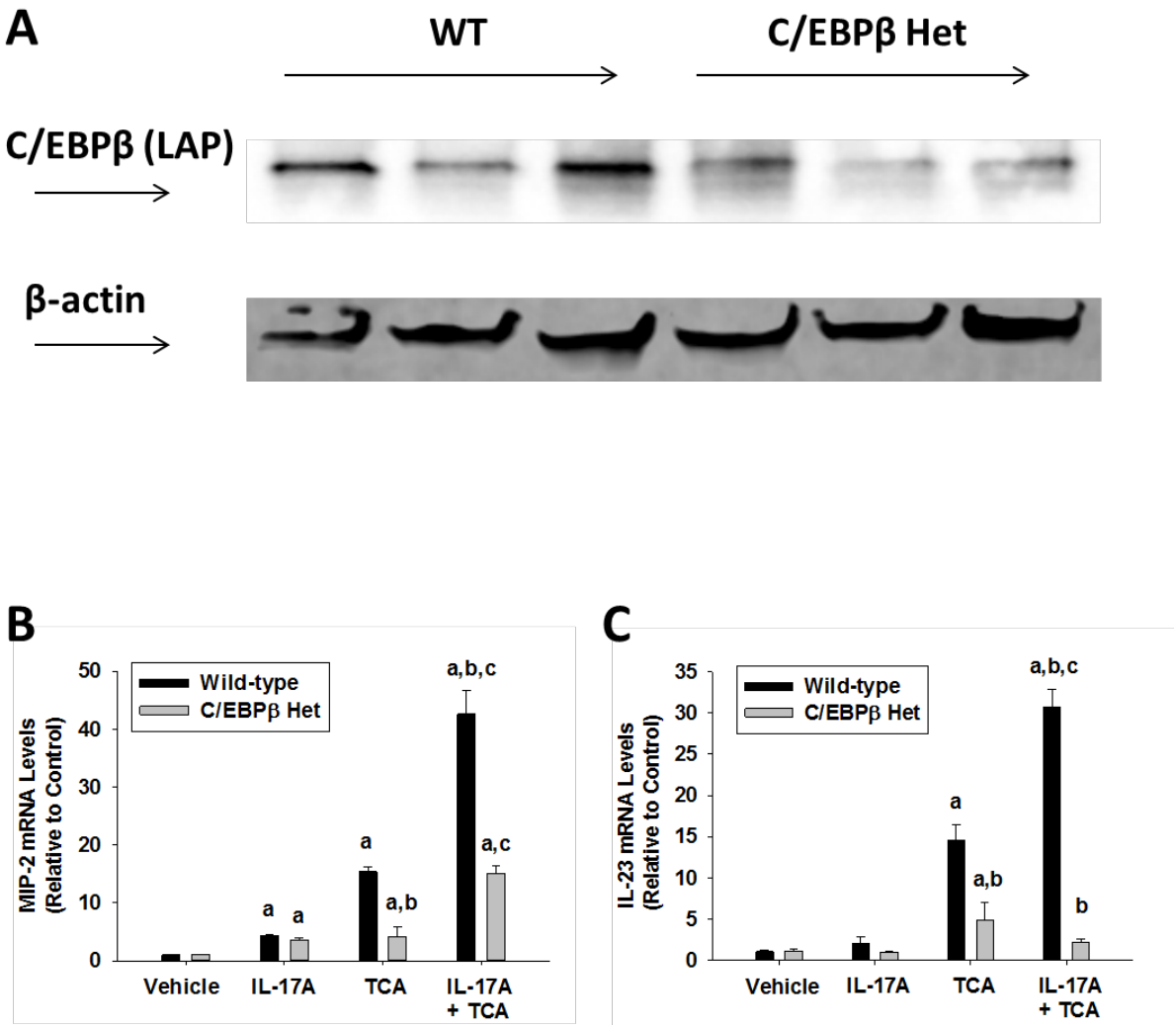


Figure 3.2 Effect of C/EBPβ on the Synergistic Interaction between IL-17A and TCA.

Whole liver from either C/EBPβ heterozygous knockout or wild-type mice were homogenized in RIPA buffer consisting of protease and phosphatase inhibitors to collect total protein. Total protein was separated on a 4-20% polyacrylamide gel and transferred to a PVDF membrane. (A) The membrane was incubated with either an anti-C/EBPβ (LAP) antibody or an anti-β-actin antibody. Bands were detected using an ECL chemiluminescence detection kit. Hepatocytes were isolated from C/EBPβ heterozygous knockout mice or their wild-type littermates.

Figure 3.2 (cont'd)

Hepatocytes were pretreated with 10 ng/mL IL-17A for 16 hours and then treated with 200 µM TCA for 3 hours. (B) MIP-2 and (C) IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different from wild-type vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from wild-type TCA treated hepatocytes ($p<0.05$). [c]Significantly different from wild-type IL-17A treated hepatocytes ($p<0.05$).

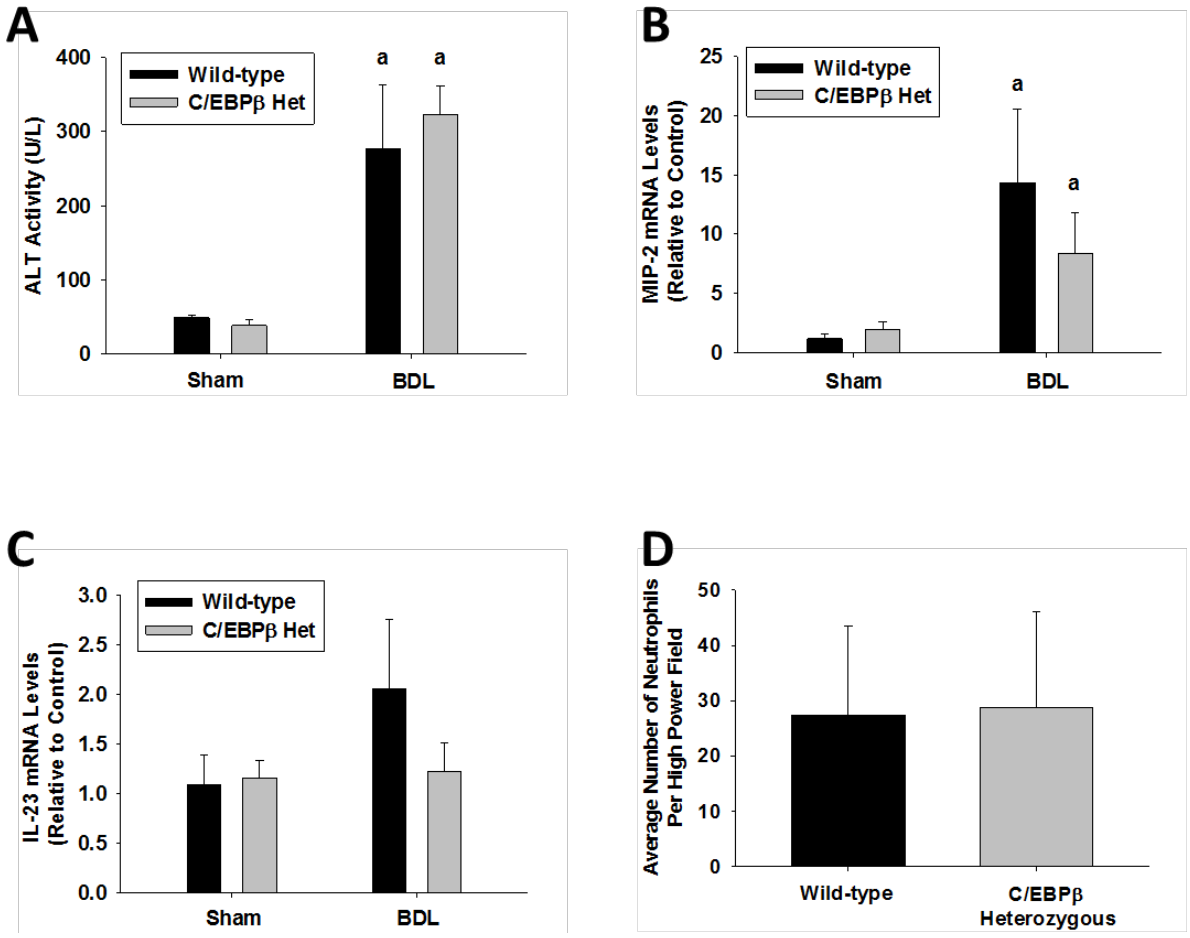


Figure 3.3 Hepatocellular Injury and Inflammation was unaffected in C/EBPβ$^{-/+}$ BDL.

C/EBPβ heterozygous (n=3, n=7) and wild-type littermates (n=3, n=6) were subjected to sham or bile duct ligation. (A) Serum ALT activity was quantified 72 hours post-surgery. (B) MIP-2 and (C) IL-23 mRNA was measured by real time PCR. [a]Significantly different from wild-type sham operated mice ($p<0.05$). Neutrophils were stained in paraffin embedded liver sections by immunohistochemistry. (D) Neutrophils were counted in sections of liver.

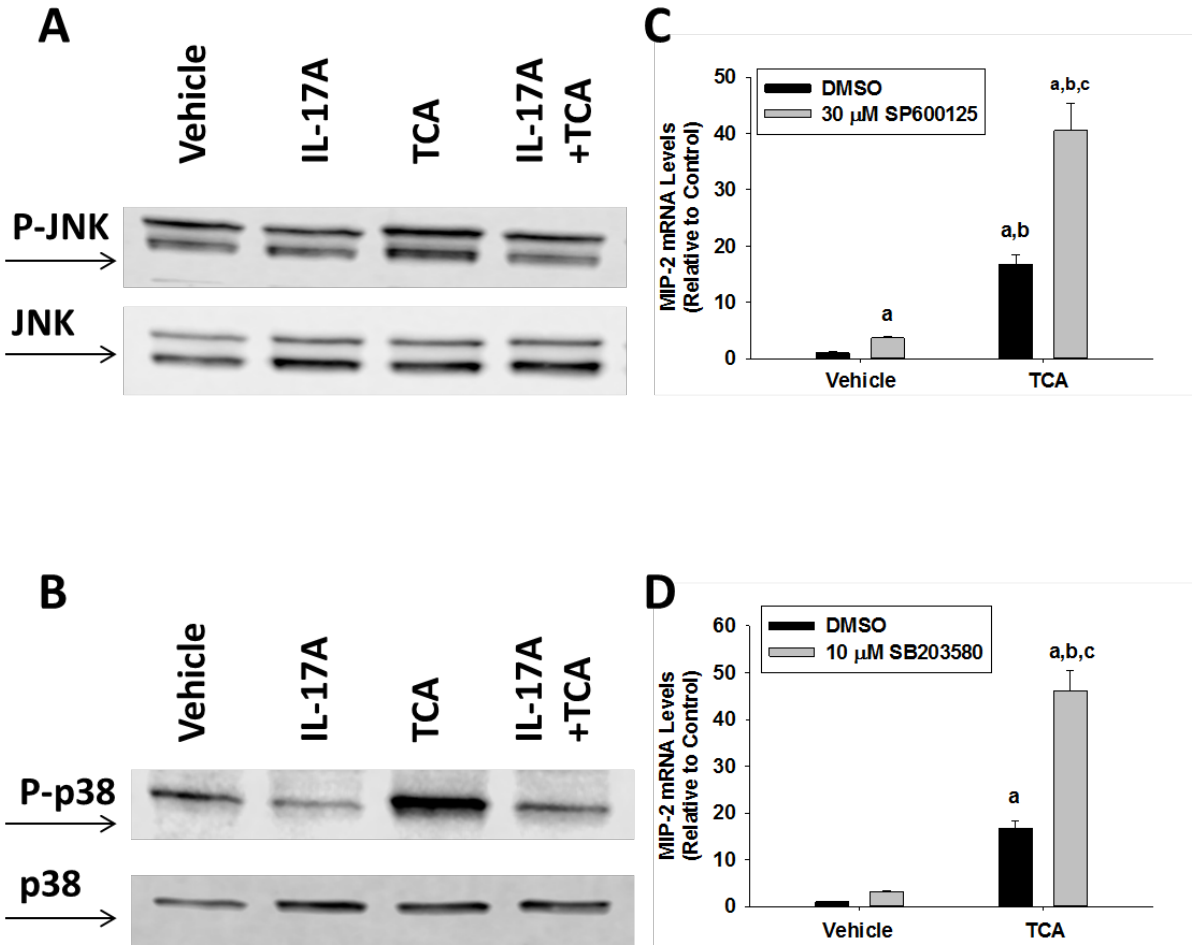


Figure 3.4 Effect of IL-17A and TCA Co-treatment on p38 and JNK Activation.

Hepatocytes were pretreated with 10 ng/mL IL-17A for 30 minutes and then treated with 200 μM TCA for 15 minutes. Western blots were incubated with either (A) anti-phospho-JNK or anti-JNK and (B) anti-phospho-p38 or anti-p38. Hepatocytes were pretreated with either (C) 30 μM SP600125 or (D) 10 μM SB203580 for 30 minutes and then treated with 200 μM TCA for 3 hours. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from inhibitor treated hepatocytes ($p<0.05$). [c]Significantly different from TCA treated hepatocytes.

Table 3.1 Additional Replicates for MIP-2 Regulation in C/EBPβ Heterozygous Knockout Hepatocytes

Treatment	Genotype	Mean ± SEM
Vehicle	Wild-type	1.01±0.11, 1.00±0.07
10ng/ml IL-17A	Wild-type	4.66±0.52[a], 4.04±0.22[a]
200 μM TCA	Wild-type	8.91±0.54[a], 8.24±0.09[a]
200 μM TCA + 10ng/mL IL-17A	Wild-type	36.55±2.89[a,b,c], 35.27±3.04[a,b,c]
Vehicle	C/EBPβ Heterozygous	1.03±0.19, 1.02±0.13
10ng/mL IL-17A	C/EBPβ Heterozygous	1.94±0.15, 4.35±0.35[a]
200 μM TCA	C/EBPβ Heterozygous	4.82±0.63[a,b], 4.33±0.32[a,b]
200 μM TCA + 10ng/mL IL-17A	C/EBPβ Heterozygous	10.62±0.99[a,c], 18.55±1.10[a,b,c]

Hepatocytes were isolated from C/EBPβ heterozygous knockout mice or their wild-type littermates. Hepatocytes were pretreated with 10 ng/mL IL-17A for 16 hours and then treated with 200 μM TCA for 3 hours. MIP-2 mRNA levels were measured by real-time PCR. [a]Significantly different from wild-type vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from wild-type TCA treated hepatocytes ($p<0.05$). [c]Significantly different from wild-type IL-17A treated hepatocytes ($p<0.05$).

Table 3.2 Additional Replicates for IL-23 Regulation in C/EBPβ Heterozygous Knockout Hepatocytes

Treatment	Genotype	Mean ± SEM
Vehicle	Wild-type	1.11±0.39, 1.04±0.20
10ng/ml IL-17A	Wild-type	3.10±1.11, 1.19±0.15
200 μM TCA	Wild-type	8.42±0.66[a], 10.99±1.02[a]
200 μM TCA + 10ng/mL IL-17A	Wild-type	15.60±1.35[a,b,c], 10.20±2.64[a]
Vehicle	C/EBPβ Heterozygous	1.00±0.06, 1.02±0.17
10ng/mL IL-17A	C/EBPβ Heterozygous	0.93±0.28, 1.14±0.11
200 μM TCA	C/EBPβ Heterozygous	1.54±0.59[b], 1.69±0.03[b]
200 μM TCA + 10ng/mL IL-17A	C/EBPβ Heterozygous	1.38±0.43[b], 1.18±0.16[b]

Hepatocytes were pretreated with 10 ng/mL IL-17A for 16 hours and then treated with 200 μM TCA for 3 hours. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different from wild-type vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from wild-type TCA treated hepatocytes ($p<0.05$). [c]Significantly different from wild-type IL-17A treated hepatocytes ($p<0.05$).

Table 3.3 Additional Replicates for MIP-2 Induction by JNK Inhibition

Treatment	Mean ± SEM
DMSO	1.00±0.02, 1.00±0.06
200 μM TCA	9.31±0.24[a,b], 15.12±1.44[a,b]
30 μM SP600125	5.01±0.11[a], 2.68±0.24[a]
200 μM TCA + 30 μM SP600125	14.55±1.11[a,b,c], 26.16±1.93[a,b,c]

Hepatocytes were pretreated with 10 ng/mL IL-17A for 30 minutes and then treated with 200 μM TCA for 15 minutes. Hepatocytes were pretreated with either 30 μM SP600125 for 30 minutes and then treated with 200 μM TCA for 3 hours. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from inhibitor treated hepatocytes ($p<0.05$). [c]Significantly different from TCA alone treated hepatocytes.

Table 3.4 Additional Replicates for MIP-2 Induction by p38 Inhibition

Treatment	Mean ± SEM
DMSO	1.00±0.06, 1.00±0.07
200 μM TCA	15.12±1.44[a,b], 5.74±0.13[a,b]
10 μM SB203580	3.55±0.12[a], 3.31±0.14[a]
200 μM TCA + 30 μM SB203580	37.96±4.08[a,b,c], 13.44±0.99[a,b,c]

Hepatocytes were pretreated with 10 ng/mL IL-17A for 30 minutes and then treated with 200 μM TCA for 15 minutes. Hepatocytes were pretreated with 10 μM SB203580 for 30 minutes and then treated with 200 μM TCA for 3 hours. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from inhibitor treated hepatocytes ($p<0.05$). [c]Significantly different from TCA alone treated hepatocytes.

Chapter 4

Identification of a Receptor that Regulates Bile Acid Induction of IL-23

4.1 Abstract

Interleukin-23 (IL-23) is critical for the maintenance and stabilization of the Th17 inflammatory response. Recently, we showed that pathophysiological concentrations of bile acids upregulate IL-23 expression in an AKT- and JNK- dependent manner. However, the mechanisms that mediate bile acid induction of IL-23 are not well known. Epidermal growth factor receptor (EGFR) and sphinogsine-1-phosphate receptor 2 (S1PR2) have been shown to be activated by taurocholic acid (TCA) in primary rodent hepatocytes. Therefore, we tested the hypothesis that EGFR or S1PR2 regulate IL-23 expression. Treatment of primary mouse hepatocytes with TCA increased expression of IL-23. Inhibition of EGFR or S1PR2, with pharmacological inhibitors, did not prevent induction of IL-23 by TCA. Next, we investigated prostaglandin E2 receptors (EP) and lysophosphatidic acid receptors (LPAR) which are related to S1PR2 and share structural homology to the bile acid receptor, TGR5. Hepatocytes treated with EP inhibitors had no effect on IL-23 expression, in the presence of TCA. However, IL-23 mRNA levels were reduced in hepatocytes pretreated with Ki16425, a LPAR1/3 competitive antagonist. Collectively, our results suggest that bile acid activation of LPARs may be important for induction of IL-23 expression in hepatocytes.

4.2 Introduction

Interleukin-23 (IL-23) is a member of the IL-12 family that is predominantly secreted by antigen presenting cells such as dendritic cells (Oppmann et al., 2000). IL-23 is required for maintenance of $CD4^+$ Th17 population expansion which stimulates them to produce Th17 cytokines, such as IL-17A and IL-17F (Stritesky et al., 2008). Patients with primary biliary cirrhosis have increased serum concentrations of IL-23 (Rong et al., 2009). Similarly, hepatic mRNA levels of IL-23 are elevated in mice subjected to bile duct ligation, a murine model of

cholestasis (O'Brien et al., 2013). Profibrogenic markers are reduced in mice that have been depleted of IL-23 in the bone marrow and subjected to bile duct ligation (Meng et al., 2012). We have demonstrated that pathophysiological concentrations of bile acids increase hepatic expression of IL-23 in a JNK- and AKT-dependent manner (O'Brien et al., 2013). However, the receptor that mediates the activation of these pathways, leading to bile acid induction of IL-23, is not known.

Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase receptor that activates ERK1/2 in hepatocytes treated with secondary conjugated bile acids in a ligand-independent manner (Rao et al., 2002). EGFR activation by secondary bile acids requires generation of mitochondrial reactive oxygen species (Fang et al., 2004; Qiao et al., 2001; Rao et al., 2002). The role of EGFR activation in the bile acid induction of IL-23 has not been investigated.

TGR5, a G protein-coupled receptor, is a well characterized bile acid receptor expressed on various cells, including macrophages and bile duct epithelial cells (Keitel et al., 2008; Keitel et al., 2010). However, studies have shown that TGR5 is not expressed by hepatocytes (Keitel et al., 2007). Recently, treatment of hepatocytes with taurine-conjugated bile acids has been shown to activate sphingosine-1-phosphate receptor 2 (S1PR2), a G protein coupled receptor with close homology to TGR5 (Hama and Aoki, 2010; Studer et al., 2012). In these studies, inhibition of S1PR2 with the selective antagonist, JTE-013, reduced bile acid activation of the ERK and AKT signaling pathways, and bile acid induction of small heterodimer partner (SHP), in primary rodent hepatocytes (Studer et al., 2012). Since AKT activation is critical for bile acid mediated upregulation of IL-23, it is possible that S1PR2 may be required for upregulation of IL-23 by TCA. Accordingly, in the following studies, the hypothesis was tested that EGFR or S1PR2 mediate the induction of hepatic IL-23 by bile acids.

4.3 Materials and Methods

4.3.1 Animal Care. All experiments were performed on 8-12 week old male C57BL/6 mice (Jackson Laboratories; Bar Harbor, ME). All of the mice were maintained on a 12-h light/dark cycle under controlled temperature (18–21°C) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. All of the 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.

4.3.2 Hepatocyte Isolation. Hepatocytes were isolated by collagenase H retrograde perfusion as described previously (Kim et al., 2006). Hepatocytes were pretreated with 5 μM AG1478, 10 μM JTE-013, 10 μM Ki16425, 10 μM tetradecyl phosphonate, 10 μM AH6809, 10 μM SC19220, or DMSO as vehicle (all from Cayman Chemicals; Ann Arbor, MI) for 30 minutes. Hepatocytes were then treated with 200 μM taurocholic acid (TCA) (Sigma Aldrich; St. Louis, MO) for 3 hours.

4.3.3 Real-Time PCR. Total RNA was isolated from isolated hepatocytes by Trizol extraction, and contaminating DNA was removed by Turbo DNA-free kit (Ambion; Austin, TX) per manufacturers' instructions. RNA was reverse transcribed into cDNA as described in 2.3.8. mRNA was analyzed on a 7500 Fast Real-time PCR system (Applied Biosystems; Foster City, CA) through the utilization of fast SYBR green reagent (Bio-rad; Hercules, CA). Primer sequences are outlined in Table 2.1. All primers used were intron spanning.

4.3.4 Statistics. Data are expressed as mean ± SEM. When two or more groups were analyzed, a two way analysis of variance (ANOVA) was performed. When the data set did not pass normality, a log transformation was utilized. Comparison between groups was done by using the Holm-Sidak method. A 95% confidence, $p<0.05$, was the criterion for significance.

4.4 Results

4.4.1 Inhibition of the Epidermal Growth Factor Receptor does not affect upregulation of IL-23 by TCA.

Both conjugated and unconjugated bile acids activate the EGFR in a ligand independent manner (Fang et al., 2004; Qiao et al., 2001). However, EGFR activation has only been investigated in the regulation of apoptotic hepatocyte death and not in the upregulation of inflammatory cytokines (Qiao et al., 2003; Rao et al., 2002). Treatment of hepatocytes with TCA increased expression of IL-23. Pretreatment with AG1478, at a concentration that prevents bile acid activation of ERK1/2, did not affect upregulation of IL-23 by TCA (Figure 4.1) (Rao et al., 2002). These data suggest that bile acid induction of IL-23 does not require EGFR activation.

4.4.2 Sphingosine-1-Phosphate Receptor 2 is not required for TCA induction of IL-23.

Activation of ERK1/2 and AKT by TCA requires S1PR2 in rodent hepatocytes (Studer et al., 2012). We recently demonstrated that bile acid induction of IL-23 is AKT-dependent. Therefore, hepatocytes were pretreated with JTE-013, a specific antagonist for S1PR2, at a concentration that prevents activation of AKT, in bile acid treated hepatocytes (Studer et al., 2012). TCA increased IL-23 mRNA levels. Inhibition of S1PR2 with JTE-013 did not prevent upregulation of IL-23 by bile acids (Figure 4.2).

4.4.3 Inhibition of Prostaglandin E2 Receptors did not affect upregulation of IL-23 by TCA.

Prostaglandin E2 receptors (EP) are GPCRs that are structurally related to TGR5, a bile acid activated GPCR (Hama and Aoki, 2010). There are four different subtypes of the prostaglandin E2 receptor (EP1-4). The antagonists, AH6809 or SC19220, which inhibit EP1 and EP2 were used to test the role of these receptors in upregulation of IL-23 by TCA (Kiriyama et al., 1997). TCA induced IL-23 gene expression in hepatocytes. This induction was unaffected in

hepatocytes pretreated with either AH6809 or SC19220 indicating no role for these receptors in upegulation of IL-23 by TCA (Figure 4.3).

4.4.4 Lysophosphatidic Acid Receptor inhibition reduces IL-23 expression.

Lysophosphatidic acid receptors (LPAR) are GPCRs that are closely related to TGR5 (Hama and Aoki, 2010). Hepatic LPAR1, LPAR2, and LPAR3 protein expression is induced in a model of chronic alcoholic liver disease (Sautin et al., 2002). Furthermore, LPAR1 and LPAR2 are expressed at low levels in freshly isolated hepatocytes and might regulate hepatocyte differentiation from oval cells (Sautin et al., 2002; Svetlov et al., 2002). To evaluate a role for LPA receptors in upregulation of IL-23 by TCA, hepatocytes were pretreated with a selective antagonist of LPAR1 and LPAR3, Ki16425 (Ohta et al., 2003). Inhibition of these receptors partially prevented upregulation of IL-23 by TCA (Figure 4.4). To determine if TCA stimulated hepatocytes to generate lysophosphatidic acid (LPA) which contribute to IL-23 induction, we utilized an autotaxin inhibitor, tetradecyl phosphonate (Durgam et al., 2005). Autotaxin, also known as lysophospholipase D, converts lysophosphatidylcholine to lysophosphatidic acid (Stracke et al., 1992; Umezu-Goto et al., 2002). Pretreatment with tetradecyl phosphonate, had no effect on upregulation of IL-23 by TCA (Figure 4.4).

4.5 Discussion

We recently showed that bile acid induction of hepatocyte interleukin-23 (IL-23) requires AKT and JNK signaling pathways (O'Brien et al., 2013). However, the receptor that regulates bile acid induction of the inflammatory response has not been elucidated. In these preliminary studies, we investigated the role of EGFR and the GPCRs: S1PR2, EP, and LPAR in the regulation of IL-23 expression in hepatocytes.

Epidermal growth factor receptor (EGFR) and sphingosine-1-phosphate receptor 2 (S1PR2) are activated by TCA treatment in primary rodent hepatocytes (Rao et al., 2002; Studer et al., 2012). Inhibition of either EGFR or S1PR2 did not prevent induction of IL-23 in hepatocytes (Figure 4.1-4.2). Similarly, prevention of EGFR and S1PR2 activation did not prevent upregulation of MIP-2 by bile acids (data not shown). Previously, we showed that TCA induction of MIP-2 is ERK-dependent (Allen et al., 2010). Therefore, the result that S1PR2 inhibition did not prevent MIP-2 induction was unexpected. However, previous studies evaluating activation of S1PR2 by TCA were performed in hepatocytes cultured in media containing dexamethasone. S1PR2 expression is not detected in hepatocytes *in vivo* under basal conditions, but mRNA levels are elevated *in vitro* in those studies (Kageyama et al., 2012). We observed that treatment with dexamethasone enhances the induction of MIP-2 by TCA. Therefore, dexamethasone may upregulate S1PR2 expression in primary hepatocytes, which will mediate TCA downstream signaling. Though TCA has been shown to activate EGFR, those studies investigated the apoptosis signaling cascade (Qiao et al., 2001; Rao et al., 2002). Our studies demonstrate that EGFR and S1PR2 do not mediate the bile acid induction of the inflammatory response (Figure 4.1-4.2). Therefore, the hepatic inflammatory response is regulated by an uncharacterized bile acid receptor.

Prostaglandin E2 receptors (EP1-4) are GPCRs that share similar homology to the known bile acid receptor, TGR5 (Hama and Aoki, 2010). There are four subtypes of prostaglandin E2 receptors, EP1-4, which are all expressed on primary murine hepatocytes (Kataoka et al., 2005). EP1 knockout mice treated with endotoxin had a reduction of hepatic injury and apoptotic hepatocyte death (Han et al., 2008). Furthermore, hepatocytes treated with an EP4 agonist promoted the upregulation of the anti-apoptotic protein, Bcl-xL, through the transactivation of

EGFR (Kataoka et al., 2005). These studies demonstrate protective and detrimental effects of prostaglandin E2 receptors in hepatic disease. Due to these studies and prostaglandin E2 receptors' relationship to TGFR, we investigated the potential role of these receptors in the regulation of the hepatic inflammatory response by bile acids. Inhibition of EP1 and EP2 had no effect on the upregulation of IL-23 in TCA treated hepatocytes (Figure 4.4). These data demonstrate that these prostaglandin E2 receptors are not involved in the upregulation of IL-23 by bile acids.

Lysophosphatidic acid receptors (LPARs) are another family of GPCRs that are closely related to the bile acid receptor, TGR5 (Hama and Aoki, 2010). There are six LPA receptors with three LPA receptors (LPAR1-3) that belong to the endothelial differentiation gene family with approximately 50% homology between them (Choi et al., 2010). LPAR1 and LPAR2 have a moderate to low expression in freshly isolated and cultured primary hepatocytes and whole liver; whereas, LPAR3 has not been shown to be expressed on hepatocytes (Choi et al., 2010; Svetlov et al., 2002). LPAR1 is induced in hepatocellular carcinoma and mediates the upregulation of matrix metalloproteinase-9 (MMP-9) via a PI3K/AKT- and p38-depedent manner in a hepatocarinoma cell line (Park et al., 2011). In this study, we used a selective inhibitor for LPAR1, LPAR3, and autotaxin to determine their involvement in bile acid induction of IL-23. Hepatocytes treated with a selective antagonist (Ki16425) for LPAR1 and LPAR3 partially prevented the induction of IL-23 by bile acids (Figure 4.4). To further investigate LPARs, we pretreated hepatocytes with tetradecyl phosphonate, an inhibitor of autotaxin enzymatic activity. Tetradecyl phosphonate treatment did not reduce IL-23 expression in hepatocytes exposed to TCA (Figure 4.4). These results suggest that bile acids do not promote LPA generation in hepatocytes, which in turn activates LPARs in autocrine and paracrine manner.

Both Ki16425 and tetradecyl phosphonate have been shown to modestly activate PPARγ with tetradecyl phosphonate having a higher affinity than Ki16425 (Durgam et al., 2005). PPARγ is a nuclear receptor that mediates an anti-inflammatory response. In addition, PPARγ downregulates IL-8, the human homolog of MIP-2 and KC, in human colon cancer cells (Nakajima et al., 2001). Considering that only Ki16425 prevented IL-23 induction, our results suggest that this off target effect is not the mechanism underlying the upregulation of IL-23 gene expression. At present, we are employing a spectrum of LPAR antagonists to further determine the effect of LPAR activation on the hepatic inflammatory response.

Autotaxin activity and LPA concentration are increased in cholestatic liver disease patients (Kremer et al., 2012). Mice injected intradermally with LPA had an increase scratch response in a dose dependent manner (Kremer et al., 2010). Furthermore, there is strong correlation between pruritus intensity and autotaxin enzymatic activity in PBC patients (Kremer et al., 2010). The potential role of LPA and autotaxin in pruritus is further demonstrated in cholestatic patients who have undergone nasobiliary drainage, a procedure that removes excess bile through the placement of catheter via the nasal cavity. After nasobiliary drainage, these patients have a drastic decrease in serum bile acid concentrations, LPA concentrations, and autotaxin activity within 24 hours. However, nasobiliary drainage is a temporary treatment for pruritus as bile acid, LPA, and autotaxin concentrations will increase in the patients over time (Kremer et al., 2010). Our results suggest that pathophysiological concentrations of bile acids do not promote pruritus via the generation of LPA by autotaxin. Therefore, the mechanisms and mediators of pruritus still need further investigation.

Overall, our results demonstrate that LPAR1 is activated by bile acids in hepatocytes and regulates the inflammatory response. As these studies are preliminary, each receptor class needs

further investigation to determine their role in bile acid promotion of the hepatic inflammatory response.

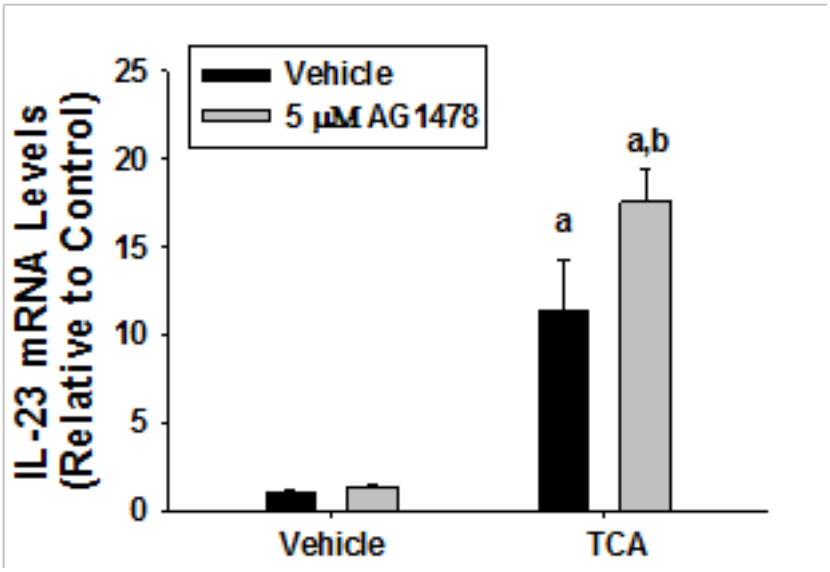


Figure 4.1 Role of EGFR on IL-23 Induction by Bile Acids. Hepatocytes were treated with 5 μM AG1478 in the presence or absence of 200 μM TCA. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

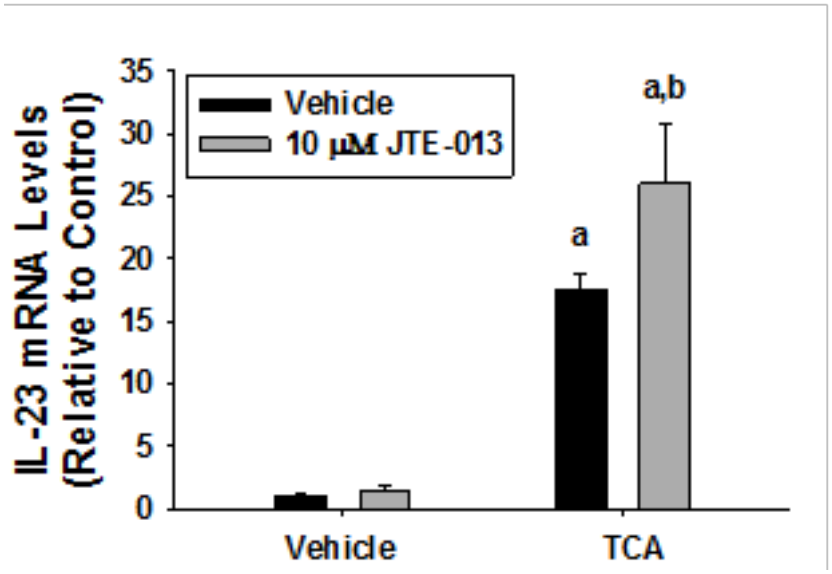


Figure 4.2 Effect of S1PR2 on IL-23 mRNA Levels in Hepatocytes. Hepatocytes were pretreated with a specific antagonist for SIPR2, JTE-013, for 30 minutes. Hepatocytes were then treated with 200 µM TCA for 3 hours. IL-23 mRNA was measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

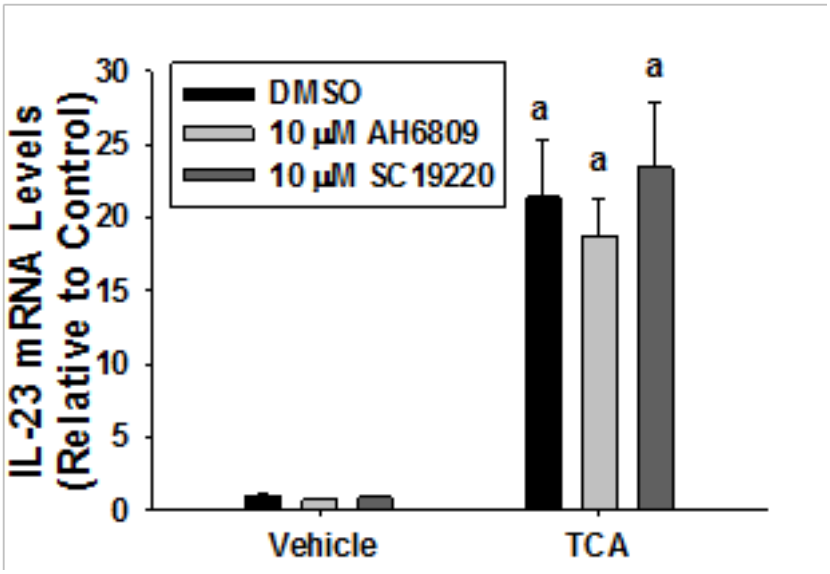


Figure 4.3 Effect of EP1 or EP2 on the Regulation of IL-23 in Primary Mouse Hepatocytes. Primary hepatocytes were treated with 10 µM AH6809 or 10 µM SC19220, selective antagonist to EP2 or EP1, in the presence or absence of 200 µM TCA. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$).

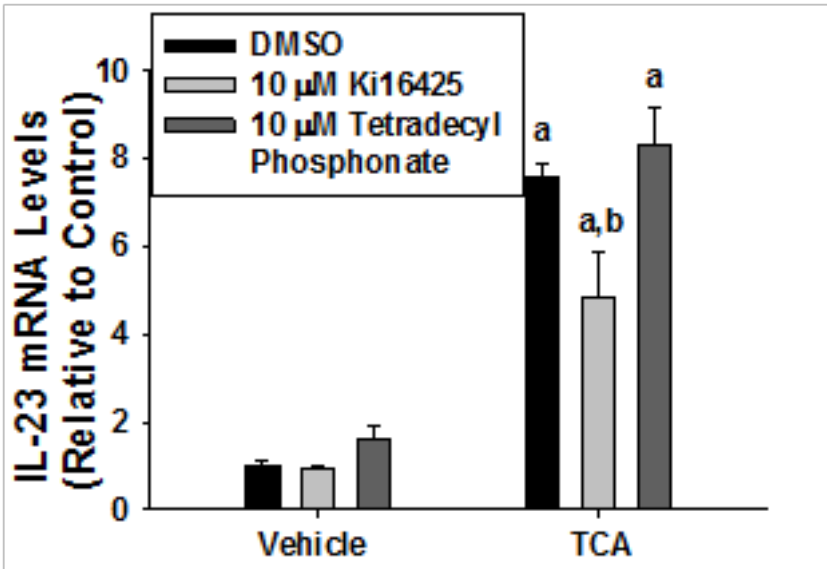


Figure 4.4 LPA Receptors mediate TCA Induction of IL-23 in Hepatocytes. Hepatocytes were treated with two different inhibitors of LPA signaling, Ki16425 and tetradecyl phosphonate for 30 minutes. Hepatocytes were then treated with 200 µM TCA for 3 hours. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

Table 4.1 Additional Replicates for the Inhibition of EGFR

Treatment	Mean ± SEM
DMSO	1.04±0.18, 1.00±0.06
200 μM TCA	6.56±1.34[a], 10.05±0.21[a]
5 μM AG1478	1.09±0.40, 1.03±0.20
200 μM TCA + 5 μM AG1478	7.27±0.21[a], 14.03±0.87[a,b]

Hepatocytes were pretreated with 5 μM AG1478 for 30 minutes and then treated with 200 μM TCA for 3 hours. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

Table 4.2 Additional Replicates for the Inhibition of S1PR2

Treatment	Mean ± SEM
DMSO	1.04±0.18, 1.00±0.06
200 μM TCA	6.56±1.34[a], 10.05±0.21[a]
10 μM JTE-013	1.52±0.50, 1.60±0.40
200 μM TCA + 10 μM JTE-013	10.54±0.80[a,b], 22.04±0.83[a,b]

Hepatocytes were treated with 10 μM JTE-013 for 30 minutes. Hepatocytes were then treated with 200 μM TCA for 3 hours. IL-23 mRNA levels were measured by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

Table 4.3 Additional Replicates for the Inhibition of Prostaglandin E2 Receptors

Treatment	Mean ± SEM
DMSO	1.04±0.22, 1.00±0.09
200 μM TCA	11.40±2.87[a], 8.84±0.22[a]
10 μM AH6809	0.80±0.12, 0.83±0.17
200 μM + 10 μM AH6809	6.13±2.13[a], 7.50±1.06[a]
10 μM SC19220	0.96±0.34, 1.11±0.18
200 μM TCA + 10 μM SC19220	6.56±1.30[a], 5.88±0.70[a]

Hepatocytes were pretreated with either 10 μM AH6809 or 10 μM SC19220 for 30 minutes; then, hepatocytes were treated with 200 μM TCA for 3 hours. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$).

Table 4.4 Additional Replicates for the Inhibition of Lysophosphatidic Acid Receptors

Treatment	Mean ± SEM
DMSO	1.02±0.16, 1.02±0.14
200 μM TCA	5.30±0.52[a], 6.08±0.70[a]
10 μM Ki16425	0.66±0.09, 0.89±0.21
200 μM TCA + 10 μM Ki16425	3.40±0.18[a,b], 2.19±0.65[a,b]
10 μM Tetradecyl Phosphonate	0.82±0.10, 0.94±0.05
200 μM TCA + 10 μM Tetradecyl Phosphonate	4.64±0.75[a], 3.32±0.12[a]

Hepatocytes were treated with either 10 μM Ki16425 or 10 μM tetradecyl phosphonate in the presence or absence of 200 μM TCA. IL-23 mRNA levels were quantified after 3 hours of TCA treated by real-time PCR. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

Chapter 5

Role of Interleukin-17F in Hepatocellular Injury during Cholestatic Liver Disease

5.1 Abstract

Activation of the inflammatory response, predominantly mediated by neutrophils, has been shown to be critical for the propagation of hepatocellular injury in cholestatic liver disease caused by a disruption of bile flow. Pathophysiological concentrations of bile acids promote the hepatic expression of macrophage inflammatory protein (MIP-2) which regulates neutrophilic inflammation. However, the critical cytokines that promote neutrophilic inflammation are not well understood. A Th17 cytokine, IL-17A, expression has been shown to be upregulated in cholestatic patients and to contribute to hepatocellular injury, inflammation, and fibrosis in a murine model of cholestasis. Interleukin-17F (IL-17F) shares 50% homology to IL-17A, but its role during cholestasis has not been elucidated. We tested the hypothesis that IL-17F promotes inflammation and hepatocellular injury during cholestatic liver disease. First, we treated hepatocytes with IL-17F in the presence or absence of taurocholic acid (TCA). There was a synergistic enhancement of MIP-2 in hepatocytes treated with IL-17F and TCA. To further evaluate the role of IL-17F during cholestasis, wild-type and IL-17F knockout mice were subjected to bile duct ligation (BDL) and markers of hepatocellular injury and inflammation were measured. 48 hours after BDL, IL-17F mRNA levels were increased in wild-type mice. There was no reduction in alanine aminotransferase (ALT) activity, a clinic biomarker of hepatocellular injury, or percent area of necrosis in IL-17F knockout mice, compared to wild-type. Furthermore, the deletion of IL-17F did not affect the expression of pro-inflammatory mediators, but there was a reduction in neutrophils in the IL-17F knockout BDL mice. Collectively, these studies demonstrate that IL-17F does not regulate the inflammatory response in the early stages of cholestasis, but IL-17F does enhance the bile acid induction of the hepatocyte inflammatory response *in vitro*.

5.2 Introduction

Cholestasis is characterized by a robust inflammatory response, hepatocellular injury, bile duct proliferation, and dysregulation of bile acid homeostasis. Persistent cholestasis stimulates differentiation of hepatic stellate cells and portal fibroblast into myofibroblasts, which produce extracellular matrix causing fibrosis (Beuers, 2009; Hirschfield et al., 2010; Li and Crawford, 2004; Trauner et al., 1998). In obstructive cholestasis, the inflammatory response is primarily neutrophilic (Gujral et al., 2003; Li and Crawford, 2004). Neutrophil accumulation during cholestasis is dependent upon the chemokines, macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC), and the adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) (Gujral et al., 2004; Wintermeyer et al., 2009). The mechanisms that regulate the inflammatory response during cholestasis are not completely understood.

Previous studies demonstrated that the cytokine, IL-17A, is increased in patients with primary biliary cirrhosis (Harada et al., 2009; Lan et al., 2009). IL-17A is a Th17 cytokine that regulates neutrophil recruitment to sites of inflammation (Maione et al., 2009). Recently, we demonstrated that the neutralization of IL-17A reduces levels of pro-inflammatory cytokines and prevents neutrophil accumulation into the livers of mice subjected to bile duct ligation. Furthermore, mice treated with IL-17A neutralizing antibody had less hepatocellular injury after bile duct ligation (O'Brien et al., 2013). We have also demonstrated that IL-17A synergistically enhances bile acid dependent upregulation of MIP-2 in hepatocytes in a p38- and JNK-dependent manner. Although these studies indicate a clear role for IL-17A in the pathogenesis of cholestatic liver disease, whether other IL-17 family members also play a part has not been investigated.

Interleukin-17F (IL-17F) is the closest family member of interleukin-17A with 50% homology (Hymowitz et al., 2001). IL-17F can be secreted by differentiated Th17 cells, lamina propria T cells, memory $CD4^+$ T cells, γδ T cells, natural killer T cells, and bronchial epithelial cells (Fujita et al., 2012; Yang et al., 2008b). Interleukin-17F and interleukin-17A activate the same heterodimer receptor complex which consists of IL-17RA and IL-17RC (Toy et al., 2006). Murine IL-17F behaves as a homodimer or as a heterodimer with IL-17A (Chang and Dong, 2007). Similar to IL-17A, IL-17F promotes inflammatory responses in various autoimmune diseases, such as asthma (Oda et al., 2005; Yang et al., 2008a). Whether it is increased in the liver during cholestasis and promotes inflammation and injury is not known. Therefore, we tested the hypothesis that IL-17F promotes inflammation and hepatocellular injury in cholestatic liver disease.

5.3 Materials and Methods

5.3.1 Animal Care. These studies were performed on 8-12 weeks old male C57BL/6 (Jackson Laboratories; Bar Harbor, ME) or IL-17F knockout mice provided by Dr. Chen Dong at M.D. Anderson Cancer Center. Generation of IL-17F knockout mice described in (Yang et al., 2008a). All of the mice were maintained on a 12-hr light/dark cycle under controlled temperature (18-21 °C) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. All of the 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.

5.3.2 Bile Duct Ligation. Mice were anesthetized with isoflurane. A midline laparotomy was performed and the bile duct ligated with 4-0 surgical silk. The abdominal incision was

closed with sutures, and the mice received 0.2 mg/kg buprenex by subcutaneous injection twice daily. Mice were sacrificed 48 hours after surgery.

5.3.3 Hepatocyte Isolation. Livers were perfused by retrograde collagenase H (Sigma Aldrich; St. Louis, MO) perfusion as described previously (Kim et al., 2006). Hepatocytes were cultured in Williams' Media E supplemented with 10% fetal bovine serum and penicillin-streptomycin. Hepatocytes were treated with 10 ng/mL recombinant mouse IL-17F (R&D systems; Minneapolis, MN) or 4 mM HCl as vehicle for 16 hours. Hepatocytes were then treated with 200 µM taurocholic acid (Sigma Aldrich) or endotoxin free water as vehicle for 3 hours.

5.3.4 Serum Chemistry. Serum alanine aminotransferase (ALT) activity was measured by a commercially available kit (Thermo Scientific; Middletown, VA) per manufacturers' instructions.

5.3.5 Immunohistochemistry. Paraffin-embedded liver sections were incubated with a rat anti-mouse polymorphonuclear leukocytes (PMN) 7/4 antibody (1:2500) (AbD Serotec; Raleigh, NC) for one hour. Neutrophils were quantified by counting the number of positively-stained cells in 10, 200X fields per tissue section. The analysis was performed in a blinded fashion.

5.3.6 Real-Time PCR. Total RNA was isolated from whole liver and isolated hepatocytes by Trizol extraction. Contaminating DNA was removed by the Turbo DNA-free kit (Ambion; Austin, TX). RNA was reverse transcribed into cDNA as described in 2.3.8. mRNA levels were evaluated using fast SYBR green reagent (Bio-rad; Hercules, CA) on a 7500 Fast Real-time PCR system (Applied Biosystems; Foster City, CA). Primer sequences are outlined in Table 5.1. All primers used were intron spanning.

5.3.7 Statistics. Data are expressed as mean ± SEM. When two or more groups were analyzed, a two way analysis of variance (ANOVA) was performed. A log transformation was performed when the data set did not pass normality. Comparison between groups was done by using the Holm-Sidak method. A 95% confidence, $p<0.05$, was the criterion for significance.

5.4 Results

5.4.1 IL-17F mRNA levels are induced during cholestasis. First, we determined the gene expression of IL-17F during cholestatic liver disease. Mice were subjected to bile duct ligation (BDL), a murine model of obstructive cholestasis, and IL-17F mRNA levels were measured by real-time PCR. IL-17F mRNA levels had an initial peak at 2 days post-surgery. Though IL-17F mRNA levels were back to basal levels at 3 days, the gene expression was increased and remained elevated after 4 days of BDL (Figure 5.1).

5.4.2 IL-17F synergistically enhances MIP-2 induction by TCA. IL-17A synergistically enhances bile acid induction of the hepatic inflammatory response via negative regulation of JNK and p38 activation. Considering the relationship between IL-17A and IL-17F, we treated hepatocytes with IL-17F in the presence or absence of TCA. Similar to IL-17A, IL-17F treatment promoted the upregulation of MIP-2 expression in hepatocytes. Co-treatment of IL-17F and TCA synergistically enhanced MIP-2 fold induction (Figure 5.2).

5.4.3 IL-17F does not affect hepatocellular injury in bile duct ligated mice. Neutralization of IL-17A reduced hepatocellular injury in a murine model of cholestasis (O'Brien et al., 2013). IL-17F is the closest family member to IL-17A, in terms of homology, and has the same physiological functions as IL-17A (Hymowitz et al., 2001). Therefore, we tested the hypothesis that injury would be reduced in IL-17F knockout mice subjected to bile duct ligation. Alanine aminotransferase (ALT) activity, a clinical biomarker for hepatocellular

injury, was increased in wild-type mice subjected to BDL. Serum ALT activity was unaffected in IL-17F knockout BDL mice (Figure 5.3A). Furthermore, there was no difference in the area of necrotic regions in the IL-17F knockout mice subjected to BDL, compared to their wild-type counterparts (Figure 5.3B-F).

5.4.4 Pro-inflammatory cytokine mRNA levels were unaffected in IL-17F knockout bile duct ligated mice. IL-17A contributes to the induction of pro-inflammatory cytokines and chemokines expression in liver diseases (O'Brien et al., 2013). Furthermore, it has been shown that both IL-17A and IL-17F are critical for neutrophil recruitment to sites of injury in autoimmune diseases (Kono et al., 2011; Liang et al., 2007). Therefore, we measured the mRNA levels of cytokines and chemokines involved in the inflammatory response during cholestasis (Bergheim et al., 2006; Gehring et al., 2006; Wintermeyer et al., 2009). MIP-2 was induced in wild-type BDL mice, but the induction was unaffected in IL-17F null BDL mice (Figure 5.4A). Plasminogen activator inhibitor-1 (PAI-1) not only regulates fibrinolysis, but PAI-1 has been shown to inhibit integrin- and vitronectin mediated cell migration (Kjoller et al., 1997). In addition, the deletion of PAI-1 reduces neutrophil infiltration and hepatocellular injury in bile duct ligated mice (Bergheim et al., 2006; Wang et al., 2005). Interestingly, mice lacking IL-17F had a higher basal level of PAI-1 gene expression than their wild-type counterparts. PAI-1 mRNA was increased in mice subjected to bile duct ligation. However, PAI-1 mRNA levels were not different in the IL-17F knockout BDL mice compared to the wild-type BDL mice (Figure 5.4B). Interleukin-6 (IL-6) is a pleiotropic cytokine that has both pro-inflammatory and anti-inflammatory functions (Scheller et al., 2011). IL-6 knockout mice subjected to BDL had exacerbating hepatocellular injury and reduced hepatocyte proliferation (Gehring et al., 2006).

IL-6 mRNA levels were increased in IL-17F null BDL mice, but it was unaffected in wild-type mice subjected to BDL (Figure 5.4C).

During cholestasis, the initiation of the inflammatory response is the recruitment of neutrophils to the hepatic parenchyma (Georgiev et al., 2008). In addition, the inhibition of neutrophil extravasation partially attenuates liver injury (Gujral et al., 2003; Gujral et al., 2004).Therefore, we counted the numbers of neutrophils in the livers of mice subjected to BDL. As expected, bile duct ligated mice had an elevation of neutrophil cell count. Bile duct ligated, IL-17F knockout mice had a reduction of neutrophil cell count compared to wild-type mice (Figure 5.5A-C).

5.4.5 IL-17F knockout mice had elevated mRNA levels of the transcription factors, C/EBPβ and early growth response factor (Egr-1). The transcription factor, CCAAT/enhancer binding protein-β (C/EBPβ), is a downstream target of the IL-17RA/IL-17RC receptor complex (Maitra et al., 2007). IL-17F knockout sham-operated mice had elevated mRNA levels of C/EBPβ, compared to wild-type mice. Subjecting mice to bile duct ligation induced C/EBPβ mRNA levels, but the deletion of IL-17F did not affect the upregulation of C/EBPβ in bile duct ligated mice (Figure 5.6A). Next, we investigated IL-17F regulation of another transcription factor, early growth response factor-1 (Egr-1). Previously, we have shown that Egr-1 is required for the induction of cytokines, chemokines, and adhesion molecule by taurocholic acid in hepatocytes (Allen et al., 2011). It has been shown that C/EBPβ activation enhances Egr-1 activity in a hepatocarinoma cell line (Zhang et al., 2003). Similar to C/EBPβ, IL-17F knockout mice had elevated levels of Egr-1 compared to wild-type mice subjected to sham-operation. Bile duct ligation increased the mRNA levels of Egr-1, but Egr-1 had the same fold induction in IL-17F knockout BDL mice compared their sham counterparts (Figure 5.6B).

5.4.6 The removal of IL-17F promotes the induction of IL-17RC, a subunit of the IL-17 receptor complex. IL-17F activates the receptor complex, IL-17RA/IL-17RC (Toy et al., 2006). It has been shown that IL-17RC has a higher affinity for IL-17F than IL-17A and is induced by IL-17F (Kuestner et al., 2007). Bile duct ligation induced the expression of IL-17RC in wild-type mice. The deletion of IL-17F promoted a higher basal level of IL-17RC mRNA levels compared to wild-type, but IL-17RC was not further elevated in IL-17F null mice subjected to BDL (Figure 5.7).

5.5 Discussion

During cholestasis, neutrophilic inflammation propagates hepatocellular injury through the release of reactive oxygen species and proteases (Gujral et al., 2003). Patients with cholestatic liver disease have elevated concentrations of conjugated primary bile acids, which promote the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules via the activation of the transcription factor, Egr-1, in hepatocytes (Allen et al., 2011; Trottier et al., 2012). We recently demonstrated that IL-17A, a Th17 neutrophil recruitment cytokine, synergistically enhances bile acid induction of the hepatic inflammatory response. Furthermore, we have shown that IL-17A contributes to inflammation and hepatocellular injury in obstructive cholestasis (O'Brien et al., 2013). IL-17F shares 50% homology to IL-17A and binds to the same heteromeric receptor complex (IL-17RA/IL-17RC) as IL-17A (Akimzhanov et al., 2007; Toy et al., 2006). Therefore, we investigated the role of IL-17F during cholestatic liver disease.

First, we determined the gene expression of IL-17F during obstructive cholestasis. Hepatic IL-17F mRNA levels are induced in bile duct ligated mice, a model of obstructive cholestasis with an initial peak at 48 hours post-surgery (Figure 5.1). Consistent with these findings, IL-17F mRNA levels are increased in mice treated with carbon tetrachloride (CCl_4), a

model of fibrotic liver (Meng et al., 2012). Through immunohistochemistry, we have observed the co-localization of portal fibroblast and IL-17F expression in the portal triad (data not shown). Therefore, portal fibroblast could be the cellular source of the initial peak of IL-17F expression at 48 hours post-surgery. To evaluate the physiological function of IL-17F in the hepatic inflammatory response, we treated hepatocytes with IL-17F with or without TCA. Similar to the IL-17A and bile acid interaction, IL-17F synergistically enhanced MIP-2 induction by TCA (Figure 5.2) but not to the same extent as IL-17A. These data suggest that IL-17F would have a similar pathophysiological function as IL-17A during cholestasis. Therefore, we subjected IL-17F knockout mice to bile duct ligation or sham-operation for 48 hours.

Hepatocellular injury and the expression of pro-inflammatory mediators were unaffected in IL-17F null mice subjected to BDL for 48 hours (Figure 5.3-5.4). However, neutrophil accumulation in the necrotic regions was drastically reduced in bile duct ligated IL-17F knockout mice (Figure 5.5). IL-17A has been shown to promote granulopoiesis via the induction of granulocyte-colony stimulating factor (G-CSF) (Schwarzenberger et al., 2000; Schwarzenberger et al., 1998). Therefore, deletion of IL-17F may suppress G-CSF dependent neutrophil granulopoiesis and the dysregulation of bone marrow neutrophil trafficking. Similarly, the neutralization of IL-17A also did not affect hepatocellular injury or inflammation in mice subjected to BDL for 3 days. Collectively, these results demonstrate the neither Th17 cytokine contributes to hepatocellular injury or inflammation at the early stages of cholestasis. Considering that T lymphocytes are the major cellular source of IL-17F, a later timepoint needs to be investigated to ensure for T cell infiltration into the parenchyma of the liver.

Histological evaluation of IL-17F knockout mice indicated steatosis (i.e., fatty liver) (Figure 5.3B-C). Though the role of IL-17F in adipose homeostasis has not been elucidated,

studies have demonstrated that the IL-23/IL-17A axis is upregulated in obese patients and contributes to the regulation of adipose tissue accumulation and adipogenesis (Sumarac-Dumanovic et al., 2009; Zuniga et al., 2010). Furthermore, IL-17RA knockout mice fed a high fat diet had increased hepatic triglycerides and white adipose tissue but reduced hepatocellular injury (Harley et al., 2013). These observations of increased fatty acid content in IL-17F knockout mice would suggest that IL-17F also contributes to adipose regulation and obesity. An induction of steatosis would account for the high basal level of the transcription factors, Egr-1 and C/EBPβ, and the pro-inflammatory mediator, PAI-1 (Figure 5.6, Figure 5.4B). Others have shown that C/EBPβ is induced in mice fed a high fat diet, and that deletion of C/EBPβ reduces injury, inflammation, and fatty acid formation during steatosis (Millward et al., 2007; Rahman et al., 2007). The interaction between C/EBPβ and Egr-1 regulates the low density lipoprotein receptor (LDLR) expression in a hepatocarinoma cell line which will regulate cholesterol homeostasis (Zhang et al., 2003). Furthermore, it has been shown that serum PAI-1 activity is closely correlated to the degree of steatosis in obese patients (Alessi et al., 2003). These results imply that antibodies against IL-17A or IL-17F should not be considered for the treatment of cholestatic liver disease due to the risk of exacerbating inflammation caused by the dysregulation of fatty acid metabolism.

Collectively, these studies demonstrate that neither IL-17F nor IL-17A contributes to the initiation of inflammation and hepatocellular injury in the early stages of cholestasis. However, IL-17F and IL-17A synergistically enhanced bile acid induction of MIP-2 in hepatocytes. The role IL-17A/IL-17F heterodimer needs to be elucidated to properly evaluate the effect of Th17 cells during cholestasis and other liver diseases.

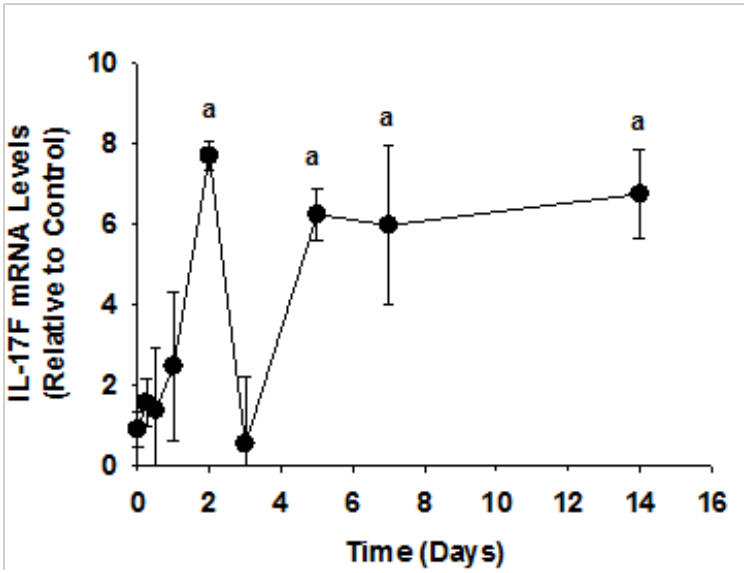


Figure 5.1 Upregulation of IL-17F mRNA Levels in Bile Duct Ligated Mice. Wild-type mice were subjected to bile duct ligation. IL-17F mRNA levels were measured in the liver at various times after bile duct ligation by real-time PCR in wild-type mice. [a]Significantly different from day 0 ($p<0.05$).

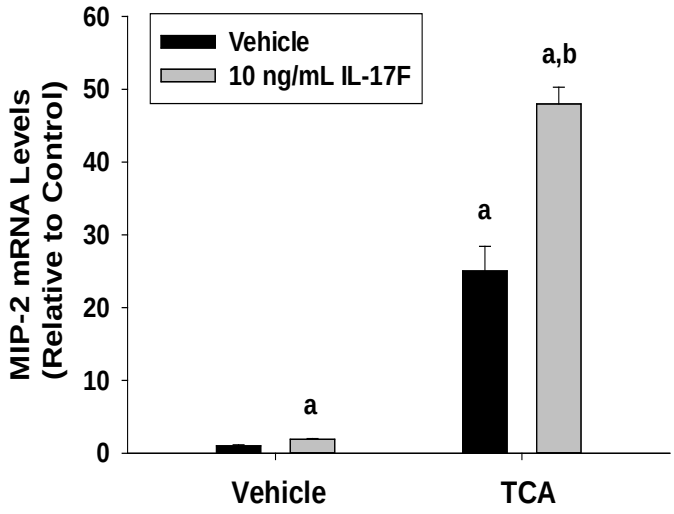


Figure 5.2 Effect of IL-17F on Bile Acid Induction of MIP-2. Primary hepatocytes were treated with 10 ng/mL of IL-17F for 16 hours. Hepatocytes were then treated with 200 µM TCA for 3 hours. MIP-2 mRNA levels were measured by real-time PCR. Graph is a representative of an n of 3. Table 2 shows MIP-2 mRNA levels of the other two sample groups. [a]Significantly different from TCA alone treated hepatocytes ($p<0.05$). [b]Significantly different from IL-17F alone treated hepatocytes ($p<0.05$).

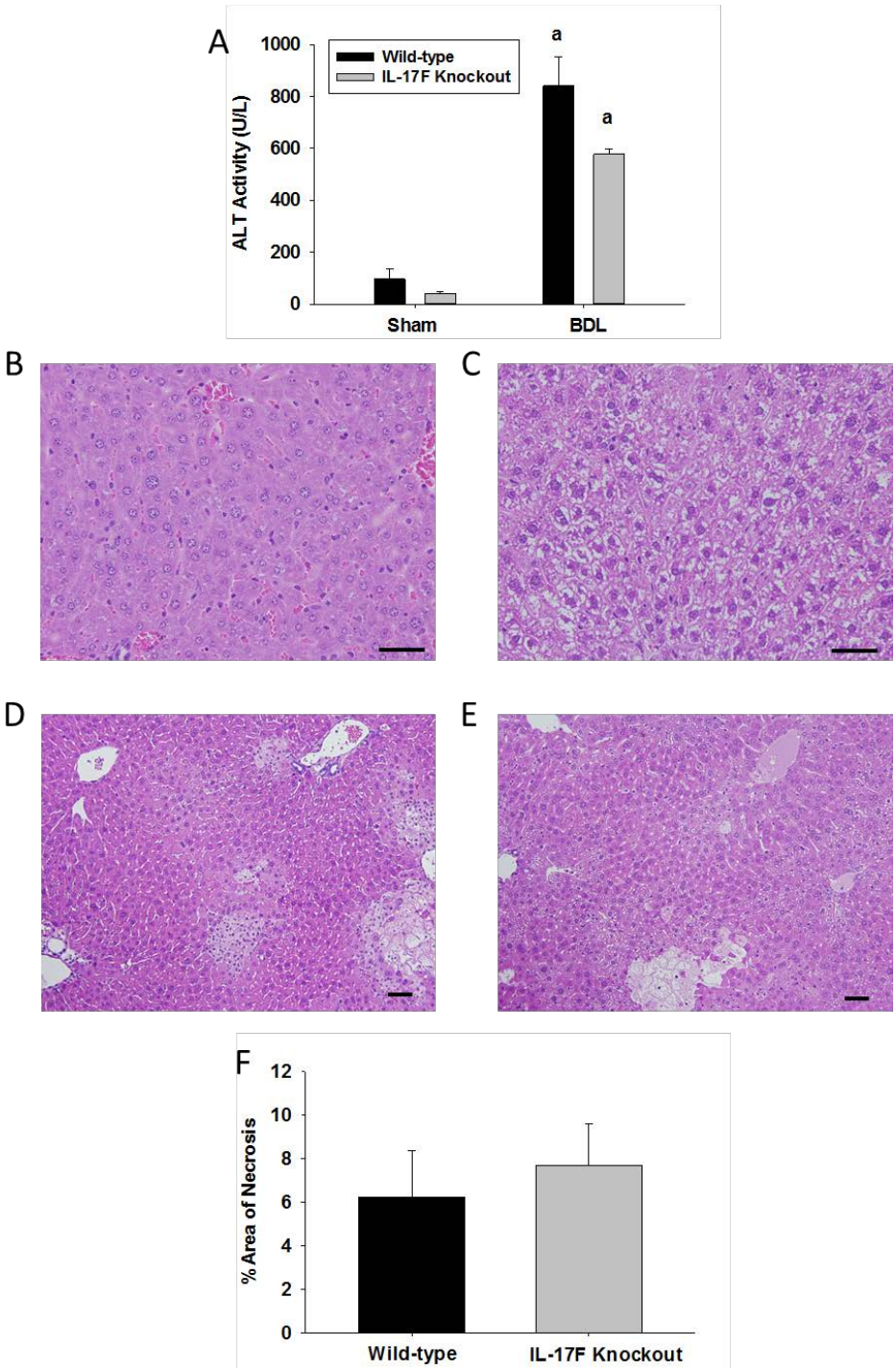
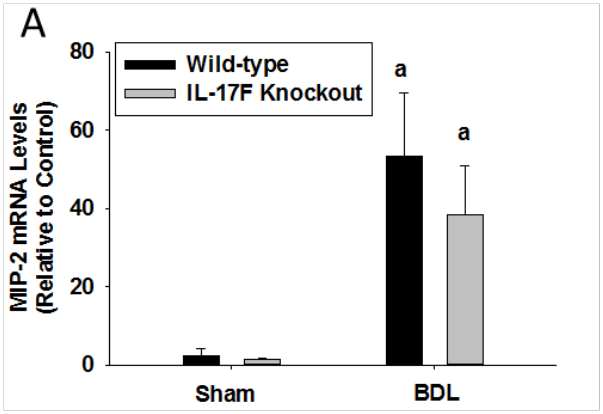


Figure 5.3 Deletion of IL-17F did not affect Hepatocellular Injury after BDL. Wild-type or IL-17F knockout mice were subjected to either sham (n=3, n=3) or bile duct ligation (n=5, n=3). (A) Alanine aminotransferase activity and (D-F) percent area of necrosis were quantified after 48

Figure 5.3 (cont'd)

hours of surgery. (B) H&E liver section from wild-type mice subjected to sham operation. (C) H&E liver section from IL-17F knockout mice subjected to sham operation. (D) H&E liver section from wild-type mice subjected to bile duct ligation. (E) H&E liver section from IL-17F knockout mice subjected to bile duct ligation. Bar represents 50 μm. [a]Significantly different from sham-operated wild-type mice ($p<0.05$).



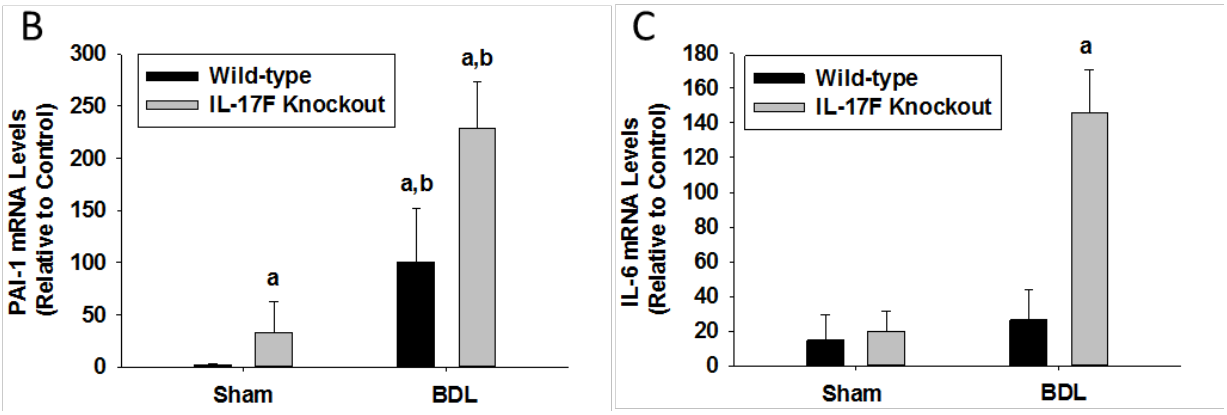


Figure 5.4 IL-17F Regulation of Inflammatory Mediators 2 days after BDL. Sham (n=3, n=3) or bile duct ligation (n=5, n=3) was performed on either wild-type or IL-17F knockout mice for 48 hours. (A) MIP-2, (B) PAI-1, and (C) IL-6 mRNA levels were measured by real-time PCR. [a]Significantly different from wild-type sham-operated mice ($p<0.05$). [b]Significantly different from IL-17F knockout sham-operated mice ($p<0.05$).

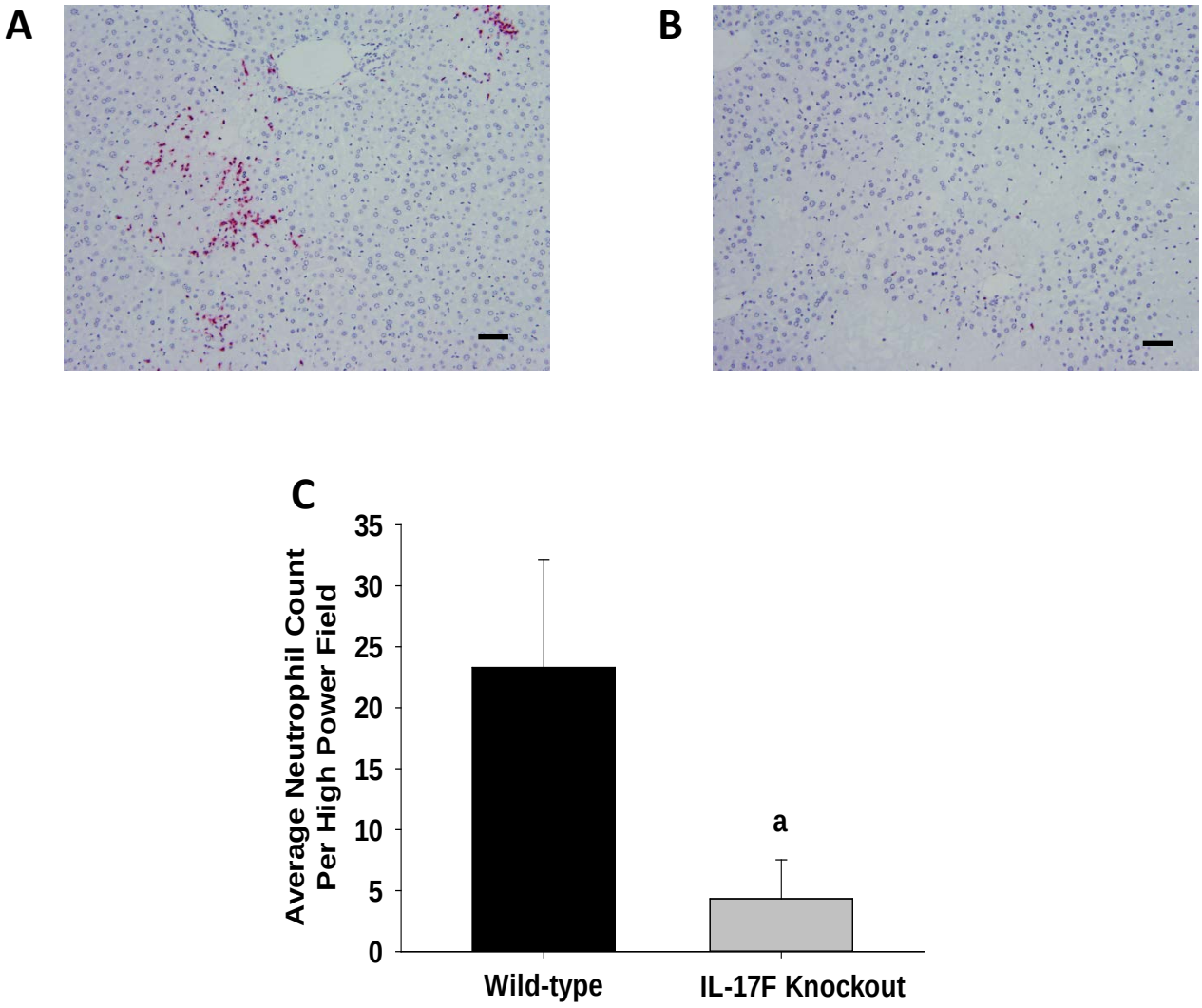


Figure 5.5 Effect of IL-17F on Neutrophil Accumulation in Bile Duct Ligated Mice. Wild-type (n=5) or IL-17F knockout mice (n=3) were subjected to bile duct ligation for 48 hours. Neutrophils were stained in paraffin embedded liver sections by immunohistochemistry. (A) Section of liver from wild-type mice subjected to BDL. (B) Section of liver from IL-17F knockout mice subjected to BDL. The bar represents 50 µm. Positive cells appear red in the photomicrograph. (C) Neutrophils were counted in sections of liver. [a]Significantly different from wild-type subjected to BDL ($p<0.05$).

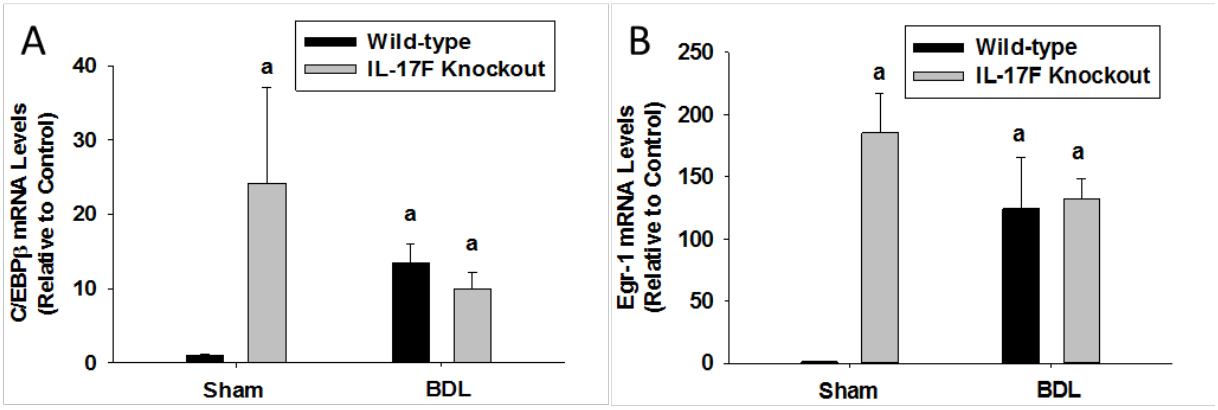


Figure 5.6 Regulation of Hepatic Expression of C/EBPβ and Egr-1 by IL-17F. Wild-type or IL-17F knockout mice were subjected to either sham-operation (n=3, n=3) or BDL (n=5, n=3) for 48 hours. (A) C/EBPβ or (B) Egr-1 mRNA levels were measured by real-time PCR. [a]Significantly different from wild-type sham-operated mice ($p<0.05$).

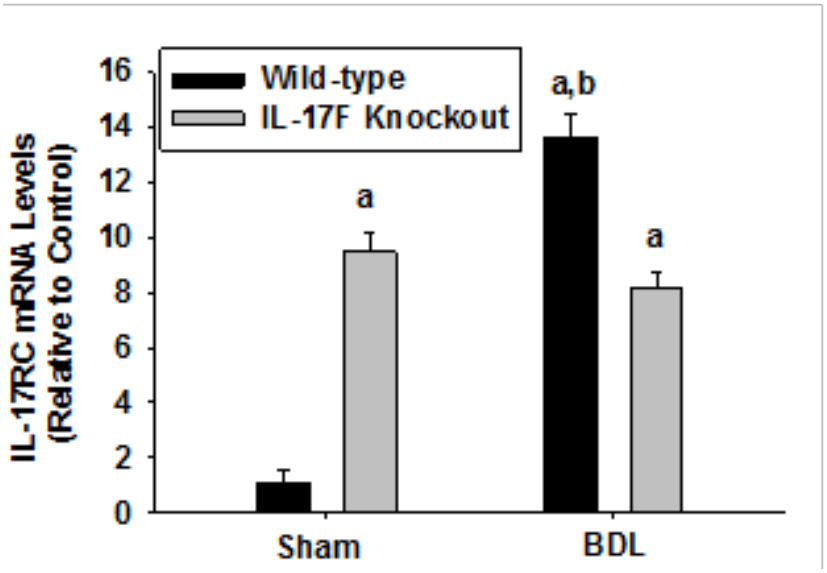


Figure 5.7 Hepatic Expression of the IL-17 Receptor Complex Subunit, IL-17RC, after BDL. Sham (n=3, n=3) or bile duct ligation (n=5, n=3) was performed on either wild-type or IL-17F knockout mice for 48 hours. IL-17RC mRNA levels were measured by real-time PCR. [a]Significantly different from wild-type sham-operated mice ($p<0.05$). [b]Significantly different from IL-17F knockout sham-operated mice ($p<0.05$).

Table 5.1: Primer Pair Sequences

Gene	*Forward*	*Reverse*
MIP-2	5’-CCT CAA CGG AAG AAC CAA AGA G-3’	5’-CTC AGA CAG CGA GGC ACA TC-3’
IL-6	5’ACC AGA GGA AAT TTT CAA TAG GC-3’	5’-TGA TGC ACT TGC AGA AAA CA-3’
RPL13a	5’ACA AGA AAA AGC GGA TGG TG-3’	5’-TTC TCC TCC AGA GTG GCT GT-3’
PAI-1	5’-AGT CTT TCC GAC CAA GAG CA-3’	5’-ATC ACT TGC CCC ATG AAG AG-3’
IL-17RC	5’-CCT GCT CCT CAG AGA CAT CC-3’	5’-ATC TGG TCC TAC ACG AAG CC-3’
C/EBPβ	5’-CCC CGC AGG AAC ATC TTT A-3’	5’-GTT TCG GGA CTT GAT GCA AT-3’
Egr-1	5’-TGG GAT AA TCG TCT CCA CC-3’	5’-GAG CGA ACA ACC CTA TGA GC-3’
IL-17F	5’-TTG ATG CAG CCT GAG TGT CT-5’	5’-AAT TCC AGA ACC GCT CCA GT-3’

Table 5.2: Additional Replicates of the *In Vitro* Studies

Treatment	Mean ± SEM
Vehicle	1.01±0.11, 1.00±0.09
10 ng/mL IL-17F	2.98±0.44, 2.67±0.34
200 µM TCA	6.15±0.21[a], 21.80±3.9[a]
10 ng/mL IL-17F + 200 µM TCA	9.91±1.6[a,b], 69.04±6.7[a,b]

Hepatocytes were pretreated with 10 ng/mL of IL-17F for 16 hours. Hepatocytes were then treated with 200 µM TCA for 3 hours. [a]Significantly different from vehicle treated hepatocytes ($p<0.05$). [b]Significantly different from TCA treated hepatocytes ($p<0.05$).

Chapter 6

Discussion

6.1 Summary and Significance

There is only one FDA approved drug, ursodeoxycholic acid (UDCA), for the treatment of cholestatic liver disease (Beuers, 2009; Calmus and Poupon, 1991). However, only a few cholestatic patients show any improvement under an UDCA treatment regimen (Heathcote, 2007). The study of the mechanisms underlying the inflammatory response during cholestasis could provide a therapeutic target for cholestatic liver disease. Therefore, we have begun to focus on the mechanisms regulating the interaction between the innate and adaptive immune response during cholestasis. In these studies, we investigated the contribution of the Th17 cellular response by elucidating the role of the IL-23/IL-17 axis during cholestasis.

There is some clinical evidence that pathophysiological concentrations of bile acids promote hepatic injury. Cholestatic patients that have undergone biliary drainage have a reduction in portal inflammation and hepatocellular injury markers (Hammel et al., 2001). Furthermore, decompressed (e.g. excess bile acid removed) cholestatic patients showed an overall improvement and an increase chance of remission (Stapelbroek et al., 2006). Consistently, we have recently shown that bile acids promote the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules that are critical for the recruitment of neutrophils into the liver parenchyma (Allen et al., 2011; Wintermeyer et al., 2009). Furthermore, we have demonstrated that taurine-conjugated bile acids induce the hepatic expression of IL-23, which is required to maintain the expansion of the Th17 cells. $CD4^+$ Th17 cells are the principal cellular source of IL-17A during cholestatic liver disease (Stritesky et al., Meng et al 2012) (Figure 2.7B-D). IL-17A expression is increased in cholestatic patients and murine models of cholestasis (Ichikawa et al., 2010; O'Brien et al., 2013). These results suggest a cross-talk between bile acids and Th17 cells that propagate the neutrophilic inflammatory response in cholestatic liver disease.

In addition, our results suggest bile acids as novel regulators of both the innate and adaptive immune response.

To investigate the relationship between bile acids and Th17 cellular response, we injected a neutralizing antibody against IL-17A and subjected mice to bile duct ligation, a murine model of obstructive cholestasis. The neutralization of IL-17A decreased ALT activity and areas of necrosis in BDL mice, after 9 days of surgery (Figure 2.1B-E). In addition, pro-inflammatory cytokine mRNA levels and total neutrophil cell count is reduced in anti-IL-17A BDL mice compared to wild-type BDL (Figure 2.3-2.4). To further elucidate the role of IL-17A, we treated primary hepatocytes in the presence or absence of TCA. IL-17A enhanced MIP-2 and IL-23 induction by TCA (Figure 2.6A, 2.7D). First, these data suggest the importance of Th17 cellular expression of IL-17A to sustain bile acid promotion of neutrophil recruitment and infiltration into the parenchyma. Second, the results demonstrate that bile acids promote a positive feedback loop between hepatocytes and the Th17 cells during cholestasis.

At present, secukinumab, a monoclonal antibody against IL-17A, has completed a phase III clinical trial for the treatment of psoriasis and rheumatoid arthritis (Genovese et al., 2013; Rich et al., 2013). Patients treated with secukinumab have shown a better improvement in their condition than those treated with entanercept, an antibody against TNF-α (Hueber et al., 2012). However, Crohn's disease patients treated with secukinumab had severe adverse effects. Most prominent of these adverse effects were neutropenia, leucopenia, infections, and candidiasis (Hueber et al., 2012). These clinical studies suggest that secukinumab would not be a valuable treatment for cholestatic patients due to their high risk of infections. Therefore, the elucidation of the mechanism underlying the synergistic interaction between bile acids and IL-17A could provide a treatment for cholestatic patients without the severe adverse effects.

To determine the mechanism of bile acids and IL-17A synergistic interaction, we studied the transcription factor (C/EBPβ) that has been shown to regulate IL-17A signaling via the IL-17RA/IL-17RC adaptor proteins, ACT1 and TRAF6 (Chang et al., 2006; Liu et al., 2009). First, hepatocytes isolated from C/EBPβ heterozygous knockout mice had a reduction in MIP-2 and IL-23 fold change after TCA treatment (Figure 3.2B-C). MIP-2 synergistic induction is partially attenuated in IL-17A and TCA co-treated C/EBPβ heterozygous knockout hepatocytes (Figure 3.2B). In addition, the knockdown of C/EBPβ returns IL-23 mRNA levels to basal levels, in co-treated hepatocytes (Figure 3.2C). Our *in vitro* results shown that C/EBPβ expression is critical for the promotion of the positive feedback loop of the IL-23/IL-17A axis during cholestasis. Furthermore, our results demonstrate that hepatic C/EBPβ activity is not required for the upregulation of MIP-2 by IL-17A.

Next, we subjected either C/EBPβ heterozygous knockout or wild-type mice to bile duct ligation for 3 days. Hepatocellular injury was unaffected in C/EBPβ heterozygous knockout BDL mice (Figure 3.3A). Pro-inflammatory mediator mRNA levels and total neutrophils were not significantly different in the C/EBPβ heterozygous BDL mice compared to wild-type (Figure 3.3B-D). However, MIP-2 mRNA levels had lower trend in C/EBPβ heterozygous BDL mice (Figure 3.3B). Though these results demonstrate that C/EBPβ does not contribute to bile acid induced neutrophilic inflammation, C/EBPβ activation is required for hepatocytes proliferation and repair as well as adipose homeostasis (Greenbaum et al., 1998; Tanaka et al., 1997). The knockdown of whole body C/EBPβ could inhibit the initiation of hepatocyte proliferation by mitogens secreted from other cellular sources. A hepatocyte specific knockout of C/EBPβ would better demonstrate C/EBPβ regulation of the synergistic interaction between bile acids and IL-17A during obstructive cholestasis.

To further evaluate the mechanism underlying the synergy between IL-17A and TCA, we studied the known MAPK pathways that are activated by bile acid signaling (Gupta et al., 2004; Schoemaker et al., 2004). IL-17A treatment prevented the phosphorylation of p38 or JNK in hepatocytes (Figure 3.4A-B). This inhibition of p38 and JNK was also observed in hepatocytes co-treated with IL-17A and TCA (Figure 3.4A-B). Similarly, hepatocytes treated with either p38 or JNK inhibitors had a further enhancement of MIP-2 induction (Figure 3.4C-D). These data suggest that IL-17A prevents the activation of p38 and/or JNK which promotes the synergistic enhancement of MIP-2 by bile acids. However, hepatocytes were pretreated with IL-17A for 30 minutes before TCA treatment. Our results might also suggest an autoregulation of p38 and JNK activation by which a phosphatase deactivates these pathways rapidly after IL-17A activation. The role of IL-17A in the activation of p38 and JNK needs further investigation.

As stated above, we have shown that bile acids promote the hepatic expression of IL-23 which requires the activation of AKT and JNK (Figure 2.7B-C, 2.8A-E, 2.9). Whereas, p38 downregulates IL-23 fold induction in TCA-treated hepatocytes (Figure 2.8F). Our data suggest that MIP-2 and IL-23 induction by bile acids are regulated by two independent pathways. However, it is possible that MIP-2 and IL-23 upregulation are receptor-specific as are the MAPK pathways that regulate those cytokines. The receptor that regulates bile acid promotion of the inflammatory response is not known. Therefore, we investigated a known bile acid activated receptor, EGFR (Qiao et al., 2001). The inhibition of EGFR did not prevent the upregulation of IL-23 by TCA (Figure 4.1). Next, we determined the role of GPCRs (S1PR2, LPAR, and EP) that are closely related to the bile acid receptor, TGR5, which is not expressed on hepatocytes (Hama and Aoki, 2010; Keitel et al., 2007). Neither S1PR2 nor EP inhibition affected IL-23 expression in hepatocytes (Figure 4.2-4.3). LPAR1 and LPAR2 have 50% homology between

them and are expressed on primary mouse hepatocytes. Consistently, human hepatocytes express low levels of LPAR1 but do not express LPAR2 (Svetlov et al., 2002). The inhibition of LPAR1 and LPAR3 by the selective inhibitor, Ki16425, attenuated IL-23 mRNA levels (Figure 4.4). Interestingly, it has been demonstrated that the inhibition of LPAR1 reduces fibrotic markers, alanine aminotransferase activity, and hepatocyte proliferation in murine models of hepatic injury (Choi et al., 2010; Murch et al., 2007; Sautin et al., 2002). Our results suggest that LPAR1 also mediates the Th17 inflammatory response by upregulating IL-23 expression in hepatocytes. Interestingly, Th17 cellular differentiation was suppressed in LPAR1 deficient naïve $CD4^+$ T cells (Miyabe et al., 2013). Collectively, these data suggest that LPAR1 could be an excellent therapeutic target for the treatment of cholestatic liver disease.

Lastly, we studied the inflammatory role of another Th17 cytokine family member, IL-17F. IL-17F is the closest family member to IL-17A (Hymowitz et al., 2001). IL-17F mRNA levels have an initial peak at 2 days post-BDL, but it returns to basal level within 3 days. However, its induction is reestablished and remains elevated after 4 days of obstruction (Figure 5.1). To determine if IL-17F would have the same pathophysiological function as IL-17A, we treated hepatocytes with IL-17F in the presence or absence of TCA. Similar to IL-17A, IL-17F synergistically enhanced MIP-2 induction by TCA (Figure 5.2). Next, we performed either sham-operation or bile duct ligation on IL-17 knockout mice for 48 hours. The deletion of IL-17F did not affect hepatocellular injury or pro-inflammatory mediator mRNA levels in mice subjected to BDL, but there was attenuation of neutrophil cell count in the areas of necrosis (Figure 5.3-5.5). These results demonstrate a critical role of IL-17F to mediate neutrophil accumulation in cholestatic liver disease. Interestingly, IL-17F knockout mice had a higher basal level of PAI-1, Egr-1, and C/EBPβ than their wild-type counterparts (Figure 5.4B, 5.6). This is

likely due to the increase in fatty acid content in the liver of IL-17F knockout mice. Overall, these results demonstrate the potential of IL-17F to regulate adipose homeostasis and granulopoiesis. These results also further suggest the impracticality of the administration of an antibody against the Th17 cytokines, IL-17A and IL-17F, due to the risk of uncontrollable weight gain and the inability to actively regulate neutrophil production in the bone marrow.

Overall, we have identified bile acids as novel inducers of the IL-23/IL-17A axis during cholestasis. The molecular mechanisms that regulate this interaction between bile acids and the IL-23/IL-17A axis are outlined in Figure 6.1. Furthermore, these studies could be extrapolated to other liver diseases that have abnormally high concentrations of bile acids such as alcoholic liver disease (Trinchet et al., 1994). Though our studies demonstrate that antibody against IL-17A or IL-17F could cause more detrimental effects than beneficial, the discovery of receptor regulating the upstream mediator of the Th17 cellular response could be a plausible option.

6.2 Future Studies

We have shown that the MAPK pathways, p38 and JNK, activation negatively regulates MIP-2 induction by TCA. In addition, IL-17A treatment reduced the protein expression of phospho-p38 and phospho-JNK indicating activation. However, it still needs to be demonstrated that IL-17A inhibition of p38 and/or JNK is the underlying cause of the synergistic enhancement of MIP-2. First, we need to investigate mitogen kinase phosphatase-1 (MKP-1) activity in IL-17A treated hepatocytes. IL-17A has been shown to inhibit the activation of MKP-1, which will promote the activation of p38 and JNK in cardiac fibroblast (Valente et al., 2012). Therefore, it is possible that there is a cellular divergent, and IL-17A is activating MKP-1 in hepatocytes. Furthermore, IL-17A has been shown to activate the protein kinase, MAPK kinase (MKK3/6), that regulates the phosphorylation of p38 in synovial fibroblast (Faour et al., 2003).

Measurement of protein expression of MKP-1 or MKK3/6 would suggest a pathway by which IL-17A prevents the activation of p38 and JNK. MKP-1 or MKK3/6 deficient hepatocytes treated with IL-17A in the presence or absence of TCA would illustrate a direct effect by IL-17A. These studies would provide further evidence to support the hypothesis that the synergistic interaction between IL-17A and bile acid requires the inhibition of p38 and JNK activity.

We have shown that either LPAR1 and/or LPAR3 mediate bile acid induction of IL-23 expression in hepatocytes (Figure 4.4). However, LPAR3 has not been shown to be expressed on hepatocytes. Another family member that shares 50% homology to LPAR1 is LPAR2 that has a low expression on primary hepatocytes (Svetlov et al., 2002). At present, we are employing a spectrum of inhibitors for the LPARs. As our results are preliminary, we need to further evaluate these receptors in the cell culture before we elucidate their role *in vivo*.

The LPA receptors are GPCR that have the ability to couple to $G_{\alpha i}$ (Fukushima et al., 1998). Bile acids activation of the ERK pathway has been shown to be pertussis toxin sensitive; thereby, suggesting that a G(αi)PCR is the underlying receptor (Dent et al., 2005). Therefore, treatment of hepatocytes with pertussis toxin would demonstrate LPAR coupled with $G_{\alpha i}$ promotes the regulation of the hepatic inflammatory response. Our results suggest that LPA generation does not activate these receptors in an autocrine or paracrine loop. However, tetradecyl phosphonate has been shown to have off target effects (Durgam et al., 2005). Therefore, we need to further evaluate the role of these receptors in the regulation of hepatic IL-23 expression. We could utilize mice deficient in LPAR1 or autotaxin. LPAR1 deficient hepatocytes should have a reduction in IL-23 mRNA levels when treated with TCA. LPAR1 deficient mice subjected to BDL would demonstrate the beneficial effects of treating cholestatic patients with an LPAR antagonist.

An interesting observation in this dissertation is the appearance of steatosis in IL-17F knockout mice (Figure 5.3B-C). Recent publications have shown that IL-17A is increased in obese patients and regulates adipose differentiation, but the mechanisms underlying this effect are not well understood (Sumarac-Dumanovic et al., 2009; Zuniga et al., 2010). Furthermore, IL-17F has not been shown to mediate adipose formation and accumulation. Therefore, the role of IL-17F during obesity needs to be determined.

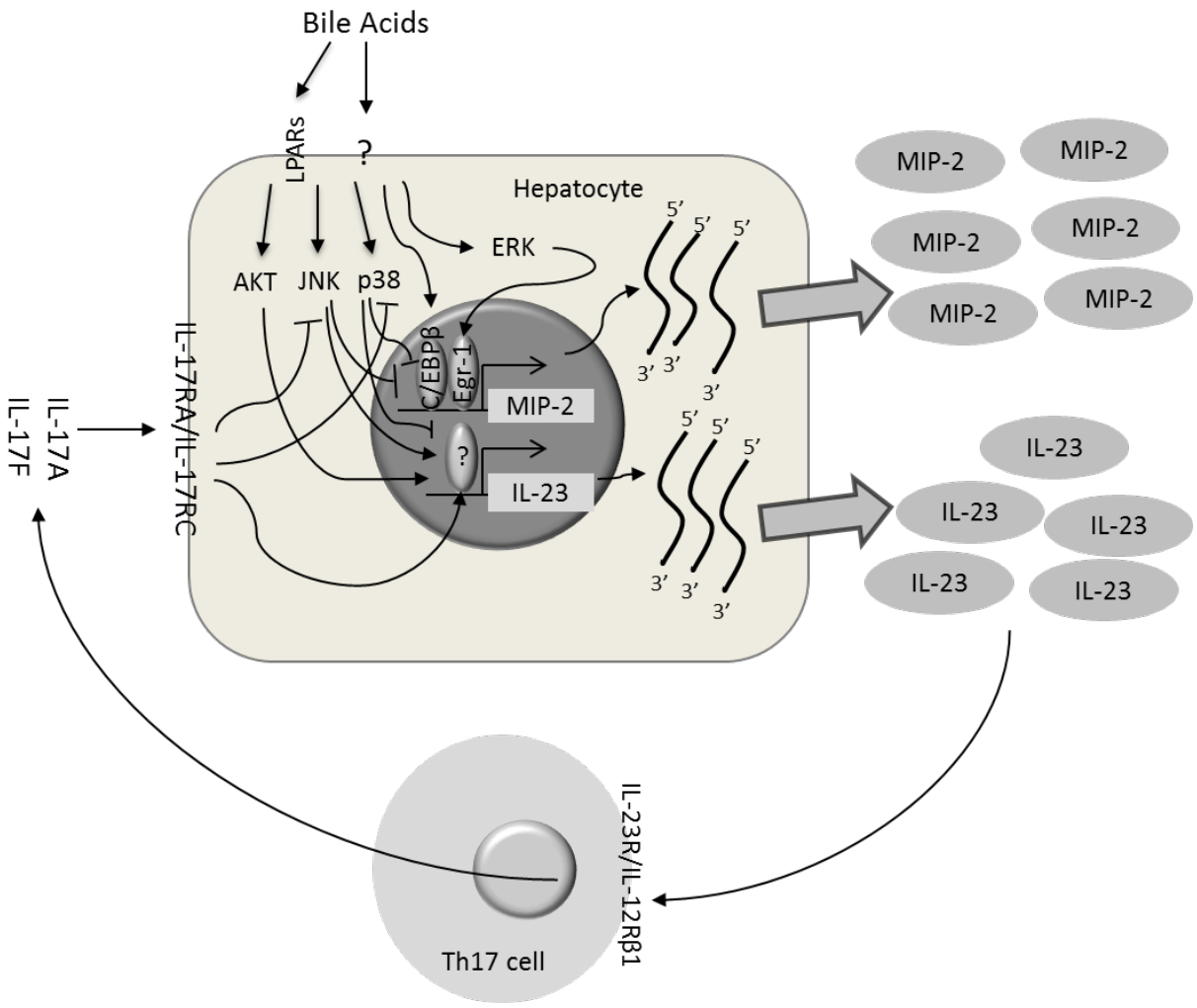


Figure 6.1 Mechanisms of the Interaction between Bile Acids and the IL-23/IL-17A Axis Outlined in this Dissertation. Bile acids promote the induction of IL-23 mRNA levels via lysophosphatidic acid receptors (LPARs). AKT and JNK signaling pathways are required for the induction of IL-23; whereas, p38 inhibits the upregulation of IL-23 mRNA levels in hepatocytes. IL-23 expression maintains the population expansion of Th17 cells, which promotes the secretion of the cytokines, IL-17A and IL-17F. IL-17A inhibits the activation of p38 and JNK

Figure 6.1 (cont'd)

signaling; thereby, leading to the synergistic enhancement of MIP-2 induction by bile acids. Furthermore, C/EBPβ is a novel pathway that mediates bile acid induction of MIP-2 in hepatocytes.

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