HEPATOPROTECTIVE FUNCTIONS OF THE HEMOSTATIC SYSTEM IN EXPERIMENTAL XENOBIOTIC-INDUCED BILIARY INJURY

By

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ABSTRACT

HEPATOPROTECTIVE FUNCTIONS OF THE HEMOSTATIC SYSTEM IN EXPERIMENTAL XENOBIOTIC-INDUCED BILIARY INJURY

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Liver fibrosis is a common pathologic consequence of persistent liver injury and inflammation and is observed in patients with chronic cholestatic liver diseases such as primary sclerosing cholangitis (PSC). Liver fibrosis, documented by excess expression and accumulation of collagen proteins, disrupts liver function and ultimately may lead to liver failure. Currently, there are no specific treatments for liver fibrosis, nor are antifibrotic strategies available clinically, leaving liver transplantation as the principle treatment alternative. Consequently, a crucial therapeutic goal in patients with liver fibrosis is to limit collagen deposition and promote the resolution of fibrosis.

One potential area of research is the hemostatic system, which is activated in PSC patients and in experimental settings of chronic liver injury and fibrosis. Increased coagulation is evident in experimental settings of chronic liver injury and fibrosis. The coagulation protease, thrombin, converts soluble fibrinogen, present in blood, to fibrin monomers that are subsequently deposited in tissue as fibrin polymers. The functional contribution of fibrin deposition has been assumed to be universally pathologic due to its association with areas of cellular injury. Contrary to this untested hypothesis, complete fibrin(ogen) deficiency was found to exacerbate chronic cholestatic liver injury in mice exposed to the biliary toxicant, α-naphthylisothiocyanate (ANIT). These results contradict the assumption that fibrin uniformly supports tissue damage, and instead suggest the novel hypothesis that fibrin exerts protective effects in this model of liver fibrosis. However, the

mechanisms mediating this protective effect are not fully understood. The work described in this dissertation reveals the mechanisms mediating the protective effects of fibrin(ogen) in chronic cholestatic liver injury and fibrosis and highlights its potential as a therapeutic target for this difficult to treat disease.

A major focus of the work presented here was discovering the molecular pathways that link fibrin(ogen) deposition and its hemostatic function to liver damage during cholestatic liver disease. We found that platelet activation by thrombin-mediated protease activated receptor (PAR)-4 signaling inhibits peribiliary fibrosis in mice exposed chronically to ANIT. Moreover, hepatocellular necrosis and associated peribiliary fibrosis were significantly exacerbated in ANIT-exposed Fib$\gamma^{\Delta 5}$ mice that express a mutant form of fibrin(ogen) incapable of binding platelet integrin $\alpha_{IIB}\beta_3$. Complementing its role in hemostasis, fibrin(ogen) has a non-hemostatic function in that it can engage and activate inflammatory cells by binding the leukocyte integrin $\alpha_M\beta_2$. Another major focus of this dissertation was to determine if fibrin engages leukocytes via $\alpha_M\beta_2$ integrin to enhance inflammation and liver fibrosis in ANIT-induced chronic liver disease. The fibrin(ogen)-$\alpha_M\beta_2$ integrin interaction was found to suppress IFNγ-driven biliary hyperplasia, thereby inhibiting hepatic fibrosis in ANIT-exposed mice. Furthermore, our proof-of-principle studies with the novel small molecule leukadherin-1 suggest that this pathway can be pharmacologically targeted to reduce established liver fibrosis.

Overall these studies reveal novel pathways whereby the coagulation protein fibrinogen inhibits experimental chronic cholestatic liver injury and fibrosis. The insight provided by these findings pinpoints unique drug targets (e.g., $\alpha_M\beta_2$), and informs repurposing of available coagulation-directed therapeutics in chronic liver disease.

To my family

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introducing me to the Society of Toxicology (SOT) community. Jim fostered my leadership goals and supported my decision to serve on several committees notably as the Michigan Regional chapter of SOT student representative and liaison to the SOT GSLC, Women in Toxicology Awards Nomination Committee and my departments Graduate Student Organization (GSO) as Vice President and President. I hope I can one day mirror his enthusiasm and perseverance for toxicological research as I move forward in my career. I will also try my best to emulate his positive and exuberant attitude where he sees every perceived failure as an opportunity to find novel ways of investigating a research problem. Aside from being a fantastic mentor, Jim and his wife Liz are truly kind and thoughtful. Jim genuinely cares about the well-being of his trainees. I have lost count of the number of times he provided "ground transportation" (as he terms it), took care of administrative issues on my behalf and stood up for me. I will truly miss working with him. I do not exaggerate when I say that joining Jim's lab was one of the best decisions of my life. Jim truly is THE best mentor a student could hope for and I am proud to have worked under his mentorship.

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KEY TO ABBREVIATIONS

ABCB11	ATP-binding cassette subfamily B member 11
ADP	adenosine diphosphate
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANIT	alpha-naphthylisothiocyanate
BDEC	bile duct epithelial cell
BDL	bile duct ligation
bid	bi-daily
BSA	bovine serum albumin
CCL2	chemokine (C-C motif) ligand 2
CCL3	chemokine (C-C motif) ligand 3
CCl_4	carbon tetrachloride
CCL4	chemokine (C-C motif) ligand 4
CK19	cytokeratin19
COL1A1	collagen type 1 alpha 1
CTGF	connective tissue growth factor
CXCL1	chemokine C-X-C motif ligand-1
CYP7A1	Cytochrome P450 7A1
DMSO	dimethyl sulfoxide
EGR-1	early growth response-1
FGA	fibrinogen α chain

FGB	fibrinogen β chain
FXIII	transglutaminase coagulation factor 13
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSH	glutathione
H&E	hematoxylin and eosin
HCV	hepatitis B virus
HPC	hepatocyte
HSC	hepatic stellate cell
i.p.	intraperitoneal
ICAM-1	intracellular adhesion molecule 1
IFN-γ	interferon-γ
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide synthase
ITGβ6	integrin beta 6
JAG1	Jagged 1
KC/GRO	keratinocyte chemoattractant/growth-regulated oncogene
LA-1	leukadherin-1
MCP-1	monocyte chemoattractant protein-1
mCSF	macrophage colony stimulating factor
MDR2	multidrug resistance protein 2
MIP-2	macrophage inflammatory protein-2
MMP	matrix metalloproteinase

MRP2	multidrug resistance associated protein 2
NK	natural killer
NPC	non parenchymal cell
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP	organic anion transport polypeptide
PAI-1	plasminogen activator inhibitor-1
PAR	protease activated receptor
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PC	phosphatidyl choline
PDGF	platelet-derived growth factor
PSC	primary sclerosing cholangitis
PT	prothrombin time
PTT	partial thromboplastin time
RBC	red blood cell
TA	tranexamic acid
TAT	thrombin anti-thrombin
TF	tissue factor
TGFβ	transforming growth factor β
TIMP	tissue inhibitor of matrix metalloproteinase
TNFα	tumor necrosis factorα
tPA	tissue plasminogen activator
UDCA	ursodeoxicholic acid

uPA	urokinase plasminogen activator
vWF	von willebrand factor
WT	wild-type
$\alpha_{IIb}\beta_3$	alphaIIbbeta3 integrin
$\alpha_M\beta_2$	alphaMbeta2
α-SMA	α–smooth muscle actin
αVβ6	alphaVbeta6 integrin

CHAPTER 1

Introduction

General overview

Chronic cholestatic liver diseases, such as primary sclerosing cholangitis (PSC), result from chronic injury to bile duct epithelial cells (BDECs, cholangiocytes). Chronic BDEC injury can lead to liver fibrosis, characterized by excessive deposition of collagen proteins, which can compromise liver function and ultimately lead to liver failure [1-5]. PSC is an asymptomatic disease that is difficult to detect, and is diagnosed late in the disease pathogenesis when liver fibrosis has already provoked hepatic dysfunction [4, 6]. The grand challenge here is that currently, there are no specific treatments for PSC, nor are antifibrotic strategies available clinically, leaving liver transplantation as the principle treatment alternative [7-10]. Consequently, a key therapeutic goal in patients with biliary fibrosis is to limit the deposition of collagen and promote the resolution of fibrosis. There is a critical need to identify and therapeutically exploit pathways responsible for limiting hepatic fibrosis in chronic cholestatic liver disease. However, despite decades of fundamentally important research which has primarily focused on stellate cells, the mechanisms controlling liver fibrosis are incompletely understood and no curative therapies exist [11, 12]. A lack of detailed understanding of these mechanisms will make it impossible to develop effective therapies to inhibit fibrosis in patients with chronic biliary injury. Thus, it is time to discover other unexplored avenues for novel therapies.

One potential area of exciting research focuses on the role of the hemostatic system. The hemostatic system, comprising the blood coagulation proteases, various substrates, and platelets, is activated in humans with chronic liver disease, and this is reproduced in experimental settings of chronic liver injury and fibrosis. Specifically, increased activity of the coagulation protease thrombin and hepatic deposition of its substrate fibrin(ogen), is evident in humans with PSC and in some experimental settings resembling hepatic PSC pathology [13-16]. Although fibrin

deposition is a likely consequence of tissue injury, its impact on chronic liver disease remains poorly defined. Identifying the mechanisms whereby fibrin(ogen) inhibits chronic liver injury and fibrosis could potentially facilitate identification of novel therapeutic targets for this difficult-to-treat fibrotic disease. The purpose of this dissertation is to uncover entirely new strategies to inhibit fibrosis by targeting this understudied and misunderstood hemostatic protein.

Overview of the liver and biliary tree

The liver is the largest organ in the body and is comprised of several cell types. Hepatocytes (HPCs) are the parenchymal cells of the liver making up 60% of the total cell population. Non-parenchymal cells constituting the remaining cell population are found within the sinusoidal space and lining the bile ducts [17]. These include endothelial cells, Kupffer cells, lymphocytes, natural killer (NK) cells, stellate cells, erythrocytes, leukocytes, bile duct epithelial cells (BDECs, cholangiocytes) and other resident immune cells that perform numerous functions [18, 19]. Sinusoidal endothelial cells are fenestrated, forming a general barrier against pathogenic agents, and serve as a selective sieve for substances passing from the blood to parenchymal and fat-storing cells, and vice versa. Kupffer cells are resident macrophages involved in phagocytosis while NK cells exert their cytotoxicity by secreting cytokines when activated [20, 21]. Hepatic stellate cells (HSCs) are profibrogenic cells that store vitamin A, and produce collagens when activated [22]. BDECs that line the bile ducts represent 3% of the total cell population of the liver and form a part of the biliary tree [23]. Bile acids are a primary component of bile and are potent proinflammatory molecules [24, 25]. High concentrations of bile acids are usually restricted to bile within intrahepatic bile ducts [24, 26]. BDECs, the ciliated epithelial cells that line the bile ducts, play an essential role in bile formation and separation of concentrated bile acids from the hepatic

parenchymal cells. In addition to being active participants in maintenance of bile composition and flow, BDECs are involved in a number of processes essential for liver physiology such as angiogenesis and they are also targets and participants in cholestatic liver disease [23, 27, 28].

The biliary tree in the liver is a system of interconnecting ducts that begin as canals of Hering within the hepatic parenchyma. The ducts ("branches") of the biliary tree progressively become larger until the common bile duct ("trunk") ends on the duodenum [29]. Besides playing an important role as a major site of metabolism for endogenous and exogenous molecules, the liver has an essential role in synthesizing bile, which aid in the digestion of fats and absorption of vitamins within the intestine [26, 30]. Cytochrome P450 7A1 (CYP7A1) converts cholesterol into the primary bile acids, cholic acid and chenodeoxycholic acid in the hepatocyte. Primary bile acids are further metabolized by conjugation to amino acids such as taurine and glycine [29, 31]. Hepatocytes export bile salts into the bile canaliculi by the bile salt export pump or the multidrug resistance protein 2 (MRP2) [32]. The small and large ducts of the biliary tree guide bile out of the liver and into the gall bladder which serves as the storage vessel. After a meal, release of cholecystokinin by endocrine cells elicits the release of bile from the gall bladder into the small intestine [33]. Nearly 95% of bile acids are reabsorbed by enterocytes and recycled back to the liver via the portal blood in a process known as enterohepatic recirculation [32]. Bile salts are then transported back into the hepatocytes via the Na^+-taurocholate cotransporting polypeptide (NTCP) or organic anion transport polypeptides (OATPs) [33].

The biliary tree is nourished by its own arterial supply known as the peribiliary plexus, which supports the metabolic and functional needs of BDECs. Injury to BDECs disrupts the flow of bile, resulting in cholestasis. This exposes the liver parenchyma to abnormally high levels of bile acids, proinflammatory and toxic detergent-like molecules normally constrained to bile [33,

34]. Chronic BDEC injury occurring by multiple mechanisms, including genetic disorders, xenobiotic exposure and autoimmune disease, results in cholestasis [34, 35].Cholestasis can be classified as obstructive or non-obstructive [34]. Obstructive cholestasis can result from a blockage of bile flow through the bile duct either due to a gallstone, a tumor or bile duct strictures compressing the bile duct and obstructing bile flow. Non-obstructive cholestasis can result from damage or destruction of the intrahepatic/extrahepatic bile ducts either by xenobiotic exposure or due to an autoimmune reaction [34, 36].

There are several mechanisms by which cholestasis can occur, such as a decrease in the expression or activity of bile acid uptake transporters on HPCs [36]. Genetic conditions such as progressive familial intrahepatic cholestasis and benign recurrent intrahepatic cholestasis are associated with mutations in the ATP-binding cassette subfamily B member 11 (ABCB11) gene, which encodes the bile salt export pump protein [35, 37]. Similarly, mutations in another ABCB member gene, ABCB4, are recognized to provoke conditions such as intrahepatic cholestasis of pregnancy. Likewise, mutations in JAG1 (Jagged 1) are associated with Alagille syndrome which is characterized by chronic cholestasis [38, 39]. Furthermore, direct injury to BDECs causes an increase in bile duct permeability and subsequently causes cholestasis that originates from the portal area. Among other causes, cholestasis is thought to occur as a consequence of injury to BDECs in biliary diseases such as primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC). Although the origin of PSC is diverse and not entirely understood, a combination of risk factors including genetics, environmental chemical exposure and autoimmunity are considered to be the cause of the underlying pathologies [4, 8, 40, 41].

Chronic bile duct injury and liver fibrosis in humans

In patients with cholestatic liver disease, hepatobiliary production and/or excretion of bile are impaired. The etiology of cholestatic liver injury is varied, but a common pathology is chronic damage to BDECs. The initial hepatic response to biliary injury is compensatory, and includes expansion of the bile ducts. Proliferation of BDECs in response to biliary damage is a crucial step in the repair mechanisms aimed at compensating the functional and anatomical loss of injured bile ducts [42, 43]. The proliferating BDECs are characterized by active production of growth factors and cytokines [25]. Other compensatory responses to biliary injury include inflammation and altered expression of bile acid transporters [44-47]. Persistent cellular injury and hepatic inflammation elicits activation of portal fibroblasts that reside near intrahepatic bile ducts, and HSCs found in the vicinity of the liver sinusoids [1, 48]. Continual activation of portal fibroblasts results in exaggerated production of extracellular matrix proteins, such as collagens, which can lead to liver fibrosis. Excessive collagen deposition around the bile ducts is termed peribiliary or periductal fibrosis. If left unchecked, this can lead to extensive liver cirrhosis that compromises liver function and ultimately leads to liver failure [1, 49]. Liver fibrosis accounts for an estimated one million deaths annually owing to cirrhosis and various liver cancers [50].

Treatment of liver fibrosis is challenging and typically aimed at reducing the inciting disease process or limiting exposure to a persistent toxic insult [51]. The preponderance of liver fibrosis is caused by clinical conditions such as alcoholic liver disease, non-alcoholic steatohepatitis and viral hepatitis. However, in many of these cases, the etiology of liver fibrosis is partially understood and in some cases can be addressed as a component of clinical care. Viral hepatitis can be targeted for treatment by a combination of anti-viral drugs. For example lamivudine (brand name Epivir) therapy for hepatitis B has been shown to reduce collagen

synthesis and improve liver fibrosis and cirrhosis [52-54]. Moreover, a reduction in the progression of liver fibrosis and subsequent improvement in liver function has been observed after sustained combined interferon and ribavirin (brand name Rebetron) or combined ledipasvir and sofosbuvir (brand name Harvoni) therapies for hepatitis C [55-58]. Additionally, in alcoholic hepatitis or non-alcoholic steatohepatitis, reducing alcohol consumption or making lifestyle/dietary changes, while recognized as challenging, can reduce liver fibrosis [59-61]. Advances in the treatment of these etiologies of liver disease have the field in a position to observe whether reduction of liver disease itself will promote regression or resolution of cirrhosis, a condition largely considered irreversible. However, treating the clinical condition is not an option for patients with PSC, where the disease etiology is complex and poorly understood. Here, therapies are aimed only at relieving disease symptoms [2, 4, 8].

Development of autoimmune cholestatic liver disease is linked to environmental chemical exposure and autoimmunity, two insults not easily targeted by specific treatments. PSC is an asymptomatic, chronic cholestatic liver disease characterized by bile duct proliferation, strictures prohibiting bile flow, hepatic fibrosis, predisposition to cancer, and end-stage liver disease [4, 6, 8, 62]. Population-based studies of PSC indicate an incidence of 0.9–1.3 cases per 100,000/year and prevalence of 8.5–14.2 per 100,000/year for these populations [63, 64]. Although PSC is an uncommon disease, advanced-stage PSC remains among the most common indications for liver transplant in the U.S. and in Europe [9, 62]. Few treatments are available to slow the progression of liver fibrosis, hence, liver transplantation remains the only effective treatment for disorders of this type, and the number of patients awaiting liver transplantation far exceeds the number of available livers [8]. Even more devastating is the fact that disease recurrence in these patients after liver transplantation is frequent [10]. The naturally occurring hydrophilic bile acid

ursodeoxycholic acid (UDCA) has shown some benefit in reducing cholestasis in some patients with PSC, as it is thought to increase bile flow from the liver thereby decreasing concentrations of toxic bile components within the liver and serum. However, it's utility to reduce inflammation and fibrosis in PSC patients and patients with chronic cholestatic liver disease is extremely limited [65]. To this end, there is an immediate need to identify novel pharmacologic targets for the treatment of chronic cholestatic liver disease.

Mechanisms of liver fibrosis

Liver fibrosis is stimulated by numerous processes including cellular injury. These processes activate certain cells in the liver to synthesize and secrete proteins and mediators which in turn stimulate cells such as portal fibroblasts and HSCs to differentiate into myofibroblasts [66, 67]. Injury to epithelial or endothelial cells triggers the release of inflammatory mediators and initiates activation of the blood coagulation cascade [68]. Macrophages and neutrophils are some of the early responders that release mediators and eliminate tissue debris and dead cells. Soluble mediators, such as cytokines, chemokines and growth factors, produced in response to liver injury stimulate activation, proliferation and migration of myofibroblasts. Activated myofibroblasts then promote wound contraction and synthesis of collagen, the main component of fibrosis [69-71]. While present within the injured liver, there is currently no evidence that bone-marrow derived fibroblast-like cells contribute to fibrosis [1, 12, 72]. Myofibroblasts eventually undergo apoptosis and inactivation when the underlying causes are cleared [12]. Myofibroblasts in the liver can be derived from several different cell types such as portal fibroblasts and quiescent HSCs [11, 48, 49, 73, 74]. Once activated these fibroblasts transform into α–smooth muscle actin (α-SMA)-expressing myofibroblasts [75, 76]. After acute injury, expression of several profibrogenic genes

such as transforming growth factor β (TGFβ) and type I collagen (COL1A1) contribute to wound healing, helping stabilize the injured area by increasing collagen protein deposition in the injured area [77-80]. The breakdown and re-establishment of the extracellular matrix proteins is coordinated by matrix remodeling and collagen turnover proteins such as matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinases (TIMPs) [81-83]. However, continual injury to the liver can create an insidious cycle of tissue injury, inflammation and repair. Pathways leading to liver fibrosis are also activated after acute liver injury. Nevertheless, persistent activation of these pathways under chronic conditions results in exaggeration of the response, progressively leading to extensive liver fibrosis [49].

Several profibrogenic mediators have been identified that promote liver fibrosis. In certain types of liver injury, mediators such as platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF) and reactive oxygen species are produced, which then stimulate the differentiation of HSCs into myofibroblasts [49, 84, 85]. On activation, these cells migrate (via chemotaxis) to the site of injury, proliferate and produce collagen proteins. PDGFβ, released by activated macrophages, BDECs and activated HSCs, is one of the most potent and mitogenic stimulators of HSC activation and proliferation [84]. Alternatively, in scenarios of liver injury where the bile ducts are injured (e.g. cholestatic liver injury), in addition to PDGF, profibrotic mediators such as TGFβ produced by macrophages and BDECs, activate portal fibroblasts eventually resulting in peribiliary/periductal fibrosis [86].

While our understanding of the general mechanisms underlying liver fibrosis has increased considerably over the past few years, currently no therapies exist to prevent or reverse liver fibrosis in patients. Further studies are required to identify novel and effective therapeutic targets to treat this disease.

Modeling chronic biliary injury and periductal fibrosis in mice

Chronic liver injury and fibrosis can be modeled in rodents employing a range of approaches including chronic exposure to toxic xenobiotics, genetic and surgical interventions. With chronic exposure, hepatic fibrosis develops in rodents exposed to xenobiotics such as alpha-naphthylisothiocyanate (ANIT), carbon tetrachloride (CCl_4), thioacetamide, 3,5-diethoxycarbonyl-1,4-dihydrocollidine or dimethyl or diethyl-nitrosamine [87]. CCl_4 is a commonly used model to study liver injury and fibrosis. CCl_4 is a hepatotoxic carcinogen that exerts its toxicity on hepatocytes via its conversion to a free radical by CYP2E1. Acute single dose administration of CCl_4 causes centrilobular necrosis which is reversible after the wound healing process is initiated [88]. Chronic, repetitive exposure to CCl_4, every 3-4 days for several weeks, results in progressive development of liver fibrosis. With 2 weeks of chronic exposure to CCl_4 via the intraperitoneal (i.p.) route, deposited collagen resembles borders of the hepatic acinus, and this worsens over time [89-92]. Bile duct ligation (BDL) is a frequently used surgical model of cholestatic liver damage. This experimental model closely resembles bile duct obstruction in humans by a gall stone or tumor, which can be corrected by removal of the obstruction. In BDL, injury is mediated by an increase in biliary pressure causing a rupture of the intrahepatic bile ducts resulting in spilling of bile acids into the parenchyma of the liver [93]. Studies suggest that focal necrosis develops as a result of direct cytotoxic and proinflammatory effects of the spilled bile acids in a mechanism involving recruitment of neutrophils [46, 47, 94, 95]. Long term experimental setting of BDL in rodents for a period ranging from 9 - 28 days serves as a model of liver fibrosis [96, 97]. In the fibrosis model, elevations in transaminase levels and liver necrosis are observed along with fibrosis which is observed around the proliferating bile ducts and within areas of necrosis [97-99]. The multidrug resistance 2 (MDR2) flippase protein is a canalicular

translocator for phosphatidylcholine (PC) and is encoded by the ABCB4 gene [100]. The $Mdr2^{-/-}$ mouse has been used to model liver fibrosis resembling the pathology of PSC [101]. These mice develop biliary fibrosis owing to decreased levels of PC in the bile resulting in the leakage of bile into the portal tracts as PC is essential for maintaining bile acids in their non-toxic state [101, 102].

Among the established models of periductal fibrosis in rodents is exposure to the biliary toxicant ANIT [103-105]. Chronic exposure of mice to the xenobiotic ANIT provides a suitable experimental setting to define mechanisms of peribiliary fibrosis elicited by persistent BDEC injury [14, 106-108]. Because of its unique metabolism and transport into the bile, ANIT selectively injures BDECs [109]. Hepatocytes *in vivo* are relatively insensitive to ANIT toxicity, as these cells detoxify ANIT through conjugation to glutathione (GSH), and this conjugate is transported into the bile by the cannalicular transporter MRP2 [110-112]. Of interest, Tr(-) rats, which lack MRP2, are protected from acute ANIT hepatotoxicity, suggesting that the transport of this conjugate into the bile is essential for acute ANIT-induced liver damage [113]. Indeed, for reasons that are not entirely clear, the ANIT–GSH conjugate is unstable in bile [111]. What is known is that enterohepatic recirculation allows multiple rounds of ANIT metabolism, conjugation and biliary transport, resulting in concentration of ANIT in the bile and ultimately produces selective injury to the BDECs [110, 113]. Recognized by toxicologists for more than 50 years to produce acute cholestatic liver injury in rodents, prolonged ANIT exposure in mice is also documented to elicit a unique recapitulation of PSC-like periductal liver fibrosis in mice [108, 114, 115].

Single, large doses of ANIT produce acute cholestatic liver injury in mice and rats sharing pathological features and mechanistic similarity to the early phase of injury after BDL [15, 116, 117]. Acute studies with ANIT denote a steep dose-response curve and doses between 50-80 mg/kg

produce similar liver pathologies in mice [103, 118, 119]. This includes significant elevation in serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and bile acids and severe hepatocellular necrosis [119]. Injury progression in acute ANIT exposure involves factors such as neutrophils and platelets [120, 121]. Indeed, neutrophils accumulate within areas of hepatic necrosis after acute ANIT intoxication and contribute to ANIT-induced liver injury in a CD18-dependent manner [117, 119, 120].

Prolonged exposure (>2 weeks) of mice to ANIT via chow recapitulates many of the pathological features of PSC, particularly PSC affecting small ducts [4]. These pathological features include biliary hyperplasia, increased serum bile acids, portal lymphocytic infiltration and peribiliary fibrosis [13, 106, 122-124]. The clinical chemistry profile of chronic ANIT exposure is characterized by increase in the markers of biliary injury (ALP) with minimum to no change in markers of hepatocyte injury (ALT) [13, 14, 107, 125]. Interestingly, this clinical chemistry profile is also observed in patients with PSC [2, 4, 6, 41]. Some of the features observed with chronic ANIT exposure resemble pathological features of long-term BDL which also elicits cholestasis, inflammation and biliary fibrosis [99, 126, 127]. Additionally, fibrosis progression in both models is similar and involves peribiliary activation of latent TGFβ by the integrin $\alpha_v\beta_6$, which is expressed by BDECs. Specifically, ITGβ6 expressed on BDECs activates latent TGFβ to cause fibrosis in the ANIT model [13, 128]. Due to the selectivity of ANIT toxicity to BDECs, worsening peribiliary fibrosis is not always reflected by an increase in hepatocyte necrosis as seen in other models of hepatotoxin-induced fibrosis. For these reasons, chronic ANIT exposure in mice serves as a unique and robust experimental setting to define mechanisms of biliary fibrosis caused directly by chronic BDEC cytotoxicity.

Overview of the hemostatic system

The blood coagulation cascade is composed of a sequence of serine proteases, cellular receptors and soluble mediators and is tasked with maintaining normal hemostasis. Normally, the coagulation cascade pathway is kept in an inactive, but "ready to go" state. This ensures that in response to harmful stimuli such as vessel injury, tissue damage, or infection, the blood coagulation cascade can be promptly activated to prevent hemorrhage or initiate cellular inflammation [129]. Tissue factor (TF) is a transmembrane protein and the principal activator of the extrinsic blood coagulation cascade. TF is expressed on the extracellular membrane of various cells in a tissue-specific manner [129, 130]. High levels of TF are expressed in the brain, lung and placenta, while intermediate levels in the heart, kidney and intestine. In comparison, relatively low levels of TF are expressed in the liver and spleen [131, 132]. Tissue injury causes activation of coagulation cascade initiated by TF which complexes with coagulation factor VII/VIIa. This complex initiates the extrinsic blood coagulation serine protease cascade, activating factors X and XI by proteolytic cleavage and culminating in the generation of the serine protease thrombin. The extrinsic pathway is essential for hemostasis. The intrinsic blood coagulation pathway is physiologically activated by thrombin cleavage of factor XI. The intrinsic pathway comprising factors XIIa, XIa, IXa and VIIIa, functions in concert with the extrinsic coagulation pathway. The small amounts of thrombin produced by the extrinsic pathway activates other components in the cascade that permits amplification of the cascade through the intrinsic pathway to produce large amounts of thrombin. Recently many coagulation factors, including the intrinsic factor XII, have been shown to play a role in thrombosis (i.e. pathologic clotting) and not hemostasis, suggesting hemostasis and thrombosis can be separated. The common pathway consists of the proteases factors Xa, Va and thrombin. Anticoagulant pathways that prevent excessive clotting and ensure

that the coagulation response is arrested at the injured site balance the procoagulant response. Three major anticoagulant pathways comprising tissue factor pathway inhibitor, antithrombin and protein C regulate and balance the coagulation cascade [133-135].

Among thrombin's key substrates is fibrinogen, a 340kD plasma glycoprotein comprised of 2 pairs each of Aα, Bβ and γ chains [136, 137]. Thrombin cleaves fibrinopeptides from the N-termini of the Aα and Bβ chains of fibrinogen to form insoluble fibrin monomers. Thrombin also activates the transglutaminase enzyme factor XIII to XIIIa. Factor XIIIa then cross-links the insoluble fibrin monomers to form the polymerized and stable fibrin clot [132, 138, 139]. Formation of fibrin clots (which include interactions with platelets and/or erythrocytes) is one of the central functions of the coagulation cascade, in that it forms a physical barrier in the injured tissue and prevents hemorrhage [129, 140]. To maintain blood flow, fibrin clots are broken down and removed from the circulation by an enzymatic process termed fibrinolysis [136, 141]. Clot lysis is mediated by plasmin, an enzyme circulating in a zymogen form called plasminogen, which adopts a closed, activation-resistant conformation. Upon binding to clots, or to the cell surface, plasminogen adopts an open conformation that can be converted into active plasmin by a variety of enzymes. Plasmin then degrades the fibrin clot into fibrin degradation products. Fibrinolysis is inhibited by plasminogen activator inhibitor-1 (PAI-1) via inhibition of the urokinase and tissue plasminogen activators, uPA and tPA respectively [141-143]. Pharmacologically, the activity of plasmin can be reduced by administration of antifibrinolytic drugs such as tranexamic acid. Tranexamic acid is an FDA-approved hemostatic drug that inhibits conversion of plasminogen to plasmin [144]. Fibrinolysis is also inhibited by the physiological inhibitors α2-antiplasmin and thrombin activatable fibrinolysis inhibitor [129, 141].

Complementing its role in hemostasis, fibrin(ogen) contains unique binding sites for cellular receptors, some of which become exposed as a consequence of fibrin(ogen) cleavage and cross-linking. Through these interactions, fibrin controls a number of cellular activities, including mitogenic, chemotactic, and immune-regulatory activities through integrin (e.g., $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_M\beta_2$), as well as non-integrin (e.g., ICAM-1, cadherin) receptors [145-150]. Moreover, polymerized and cross-linked fibrin is capable of engaging and activating inflammatory cells (such as macrophages, neutrophils and NK cells) by binding the integrin $\alpha_M\beta_2$. This interaction has been recognized as being key to disease pathogenesis in a number of models of inflammatory conditions such as rheumatoid arthritis, colitis, neuromuscular disease and infection [151-154].

Platelets also play a key role in coagulation by coordinating the formation of the primary hemostatic plug and by providing an efficient catalytic surface for the assembly of the components of the blood coagulation cascade [155]. Platelets circulate in a quiescent state and are activated upon contact with a variety of stimuli including collagen, von Willebrand factor, adenosine phosphate (ADP), and the coagulation protease thrombin [156]. Thrombin is a potent activator of platelets [156-159] and can directly trigger platelet activation through activation of G-protein coupled protease activated receptors (PARs) [160, 161]. PAR-1 is essential for the activation of platelets in humans while a complex of PAR-3/4 does the same in mice, as mouse platelets do not express PAR-1 [162-164]. Thrombin can also initiate platelet activation indirectly by coordinating the formation of fibrin. Fibrin is capable of engaging and activating platelets by binding $\alpha_{IIb}\beta_3$ integrin, a platelet adhesion molecule expressed on activated platelets. This interaction promotes processes such as platelet aggregation [165, 166]. The action of thrombin on platelets produces a highly efficient catalytic surface for further generation of thrombin. Thrombin activation of platelets results in platelet degranulation and the release of additional mediators, such as ADP and

serotonin [155]. The G-protein coupled receptor $P2Y_{12}$ serves as a chemoreceptor for ADP and its interaction promotes additional platelet activation and aggregation [167]. $P2Y_{12}$ is the target of antiplatelet drugs such as clopidogrel [168].

Altogether, the blood coagulation cascade is a highly dynamic and complex pathway aimed at maintaining hemostasis by preventing hemorrhage and balancing it by preventing thrombosis via anticoagulant and fibrinolytic pathways. In addition to maintaining hemostasis, interactions between components of the coagulation cascade and cellular receptors mediate multiple functions essential for physiologic and pathologic processes including local inflammation, signaling and extracellular matrix remodeling.

Coagulation in chronic liver disease

Biomarkers of coagulation activation such as thrombin-antithrombin levels and D-dimer are elevated in patients with chronic liver disease [16, 169-172]. Experimental settings of chronic liver disease, such as ANIT-exposed mice or BDL, are also associated with activation of the coagulation cascade. Biliary injury in ANIT-exposed mice is associated with TF-dependent activation of the blood coagulation cascade, increased plasma levels of the coagulation protease thrombin and platelet activation. The increase in thrombin activity is associated with the deposition of insoluble fibrin clots in livers of mice exposed to ANIT for 4 weeks [14, 15]. Thrombin generation, fibrin deposition and platelet activation are likewise observed in mice subjected to BDL [116]. Importantly, increased plasma thrombin levels and hepatic fibrin deposition are also features of chronic cholestatic liver disease in humans and are also observed in PSC patients [16].

Several previous studies have emphasized the role of the thrombin receptor PAR-1 in liver fibrosis. ANIT and BDL-induced hepatic fibrosis was found to decrease after PAR-1 inhibition

likely owing to the expression of PAR-1 by macrophages and HSCs [121, 173]. However, the functional contribution of other thrombin targets, such as fibrin, to cholestatic liver disease is essentially unknown. Because fibrin deposition is evident in the liver after hepatic injury, the role of fibrin polymers in liver fibrosis is often assumed to be pathologic given their association with areas of cellular injury, however this connection is seldom tested. Dispelling this widely-held assumption, complete, genetically-imposed fibrin(ogen) deficiency increased liver injury and inflammation in mice exposed to ANIT for 4 weeks [14]. Moreover exaggerated fibrinolysis, indicated by elevated levels of D-dimer, has been noted in patients with liver disease [16, 172]. These findings imply that fibrin(ogen) could have a hepatoprotective function in chronic cholestatic liver disease. However, the mechanisms responsible for the hepatoprotective effects of fibrin in this experimental setting are not currently understood.

One possibility is that fibrin-dependent platelet aggregation/activation protects the liver from chronic ANIT induced injury. Platelets have been demonstrated to contribute to cholestatic liver injury after acute ANIT toxicity and acute short-term BDL [174]. Indeed, while the role of platelets in liver toxicity and disease is certainly context-dependent, this hypothesis is supported by the evidence that blood platelet count is often affected in patients with chronic liver disease [175-177]. Thrombocytopenia is not only an indicator of advanced liver disease in patients, it also exacerbates cholestatic liver injury and fibrosis in mice subjected to long-term BDL [178]. Furthermore, work by Clavien *et. al.* recently demonstrated that platelet-derived serotonin reduces the serum bile acid pool in mice after BDL thereby conferring protection from cholestatic liver injury [179]. Although the role of PAR-1 in liver fibrosis has been well studied, PAR-1 is not expressed by mouse platelets and a complex of PAR-3/PAR-4 contributes of activation of platelets in mice [163, 180]. Thus, the profibrogenic effects of PAR-1 cannot be attributed to platelet

activation. However, thrombin activation of mouse platelets via PAR-4 could be central to platelet activation in chronic liver disease. The integrin-binding functions of fibrin(ogen) could also underlie its protective functions in chronic cholestasis. Of note, in activated platelets (for e.g. activation by thrombin), the $\alpha_{IIb}\beta_3$ integrin assumes a conformation capable of engaging both soluble fibrin(ogen) and polymerized fibrin as a ligand, whereas fibrin(ogen) does not bind to quiescent platelets [165, 181-183]. Whether platelet activation driven by coagulation plays a role in cholestatic liver injury and fibrosis is not known.

Another possibility is that fibrin engagement of inflammatory cells via the integrin $\alpha_M\beta_2$ protects the liver from chronic liver injury and fibrosis [184]. Integrin $\alpha_M\beta_2$ belongs to the β_2 family of integrins that play important roles in the development of an effective inflammatory response *in vivo*. While soluble fibrin(ogen) binds to activated platelet integrin $\alpha_{IIb}\beta_3$, it has little affinity for integrin $\alpha_M\beta_2$. Polymerized fibrin on the other hand, is a strong ligand for $\alpha_M\beta_2$ integrin [165, 181-183]. Integrin $\alpha_M\beta_2$ is expressed on the surface of inflammatory cells such as NK cells, neutrophils, monocytes, macrophages and mast cells and it mediates diverse biological functions such as phagocytosis and neutrophil aggregation [184, 185]. Of importance, several of these cell types (e.g., macrophages, NK cells) have been shown to participate in liver fibrosis. Emerging evidence strongly indicates that, depending on their programming, macrophages can contribute to the progression and/or resolution of hepatic fibrosis [186]. Strong evidence supports an antifibrotic role of NK cells, mediated by upregulation of antifibrotic cytokines such as interferon-γ (IFN-γ), and via HSC-directed cytolytic activity [187, 188]. However, it is not known whether fibrin(ogen) activation of $\alpha_M\beta_2$ integrin modifies the activity of these cells in chronic liver disease. While the fibrin - $\alpha_M\beta_2$ integrin interaction has proven central to disease pathogenesis in a number of models of inflammatory conditions such as rheumatoid arthritis, colitis, neuromuscular disease, and

infection [151-154], additional studies are required to ascertain the role of this interaction in liver fibrosis.

Summary, aims and overview of dissertation

Liver fibrosis occurs as a consequence of chronic liver damage and inflammation. Fibrosis is the exaggerated accumulation of extracellular matrix proteins, mainly collagens, which can compromise liver function and ultimately lead to liver failure. Currently, no anti-fibrotic therapies are available clinically to reduce liver fibrosis [8]. In part, this may be due to the focus of the majority of work on mechanisms immediately connected to myofibroblast activation. Although not all mechanistic details are understood, I propose that by exploring the underdeveloped area of interactions between the hemostatic system and liver disease, we could identify novel mechanisms of fibrosis in chronic cholestatic liver disease. Activation of the blood coagulation cascade is associated with chronic liver injury. Indubitably, several aspects of the coagulation system control the progression of chronic cholestatic liver disease and this pathway can serve as a viable target for therapies aimed at treating liver fibrosis. Indeed, increased thrombin activity and deposition of fibrin polymers in liver are conspicuous features of both chronic liver disease in humans and experimental xenobiotic-induced liver fibrosis [14-16, 116]. Although the impact of fibrin deposition on liver disease has been inferred as damaging, complete fibrin(ogen) deficiency worsened cholestatic liver injury in the ANIT liver fibrosis model. Thus, the overall objective of this research is to identify the mechanisms whereby fibrin(ogen) inhibits chronic liver injury and fibrosis.

This overall objective will be evaluated in the following manner:

Aim 1: For the first part of this dissertation, the focus is on fibrinolysis, the enzymatic process assigned with degrading fibrin clots. By inhibiting this pathway, it is hypothesized that "stabilizing" fibrin clots with the FDA-approved antifibrinolytic drug tranexamic acid reduces liver fibrosis in chronic ANIT-exposed mice.

Aim 2: Efforts were then focused on identifying mechanisms mediating these protective effects of fibrin(ogen). It was postulated that the integrin-binding functions of fibrin(ogen) could underlie its protective functions in chronic cholestasis. The role of fibrin(ogen) binding to the $\alpha_{IIb}\beta_3$ integrin, was first investigated as this process is central to platelet aggregation, clot retraction and wound healing. To examine the role of the fibrin(ogen)- $\alpha_{IIb}\beta_3$ integrin interaction in chronic cholestatic liver injury, unique Fib$\gamma^{\Delta 5}$ mice were utilized. These mice express a mutant fibrin(ogen) protein where the binding domain for $\alpha_{IIb}\beta_3$ has been mutated, thereby blocking platelet binding but preserving other clotting functions of fibrin(ogen). The impact of platelet activation via PAR-4 on chronic liver injury and fibrosis was also determined.

Aim 3: Next, the potential of fibrin to affect liver fibrosis by modulating activities of inflammatory cells via its interaction with the leukocyte integrin $\alpha_M\beta_2$ was investigated. This aspect was intriguing because the role of fibrin -$\alpha_M\beta_2$ engagement in liver fibrosis has never been examined. To dissect the role of this interaction in peribiliary fibrosis, Fib$\gamma^{390\text{-}396A}$ mice were utilized, which express a mutant fibrinogen that retains all clotting functions but cannot bind $\alpha_M\beta_2$ integrin. In proof-of-principle studies the novel small molecule called leukadherin-1 (LA-1) was utilized, which is an allosteric activator of fibrin(ogen)-bound integrin $\alpha_M\beta_2$. These studies examined the possibility that enhancing the fibrin-$\alpha_M\beta_2$ interaction could reduce liver fibrosis. Finally, the impact

of IFN-γ-mediated induction of inducible nitric oxide synthase in chronic ANIT-induced liver fibrosis was evaluated.

This dissertation is motivated by the rationale that investigation of the protective role of fibrin(ogen) in chronic liver fibrosis will facilitate identification of novel therapeutic targets to treat liver fibrosis. The outcome of the findings described here has the potential to reset the current assumption that fibrin(ogen) deposition is uniformly pathologic in chronic liver disease. These studies necessitate an important positive shift in anti-fibrotic therapies by identifying innovative drug targets (e.g., $\alpha_M\beta_2$), and informing repurposing of available coagulation-directed therapeutics in chronic liver disease.

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CHAPTER 2

The Antifibrinolytic Drug Tranexamic Acid Reduces Liver Injury And Fibrosis In A Mouse Model Of Chronic Bile Duct Injury

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Abstract

Hepatic fibrin deposition has been shown to inhibit hepatocellular injury in mice exposed to the bile duct toxicant alpha-naphthylisothiocyanate (ANIT). Degradation of fibrin clots by fibrinolysis controls the duration and extent of tissue fibrin deposition. Thus, we sought to determine the effect of treatment with the antifibrinolytic drug tranexamic acid (TA) and plasminogen activator inhibitor (PAI-1) deficiency on ANIT-induced liver injury and fibrosis in mice. Plasmin-dependent lysis of fibrin clots was impaired in plasma from mice treated with TA (1200 mg/kg, ip, bid). Prophylactic TA administration reduced hepatic inflammation and hepatocellular necrosis in mice fed a diet containing 0.025% ANIT for 2 weeks. Hepatic Type 1 collagen mRNA expression and deposition increased markedly in livers of mice fed ANIT diet for 4 weeks. To determine whether TA treatment could inhibit this progression of liver fibrosis, mice were fed ANIT diet for 4 weeks and treated with TA for the last two weeks. Interestingly, TA treatment largely prevented increased deposition of Type 1 collagen in livers of mice fed ANIT diet for 4 weeks. In contrast, biliary hyperplasia/inflammation and liver fibrosis were significantly increased in PAI-$1^{-/-}$ mice fed ANIT diet for 4 weeks. Overall, the results indicate that fibrinolytic activity contributes to ANIT diet-induced liver injury and fibrosis in mice. In addition, these proof-of-principle studies suggest the possibility that therapeutic intervention with an antifibrinolytic drug could form a novel strategy to prevent or reduce liver injury and fibrosis in patients with liver disease.

Introduction

Chronic injury to bile duct epithelial cells (BDECs) occurs in humans with cholestatic liver disease and is modeled in mice by exposure to the toxicant alpha-naphthylisothiocyanate (ANIT).

Transport of ANIT into the bile by hepatocytes injures intrahepatic BDECs [1], and exposure of mice to ANIT in the diet causes compensatory biliary hyperplasia, elevation in serum bile acids, portal lymphocytic inflammation, and mild to moderate hepatocellular injury [2-5]. Prolonged ANIT exposure in mice models progressive liver fibrosis characterized by exaggerated peribiliary collagen deposition [2-5]. BDEC injury in this model is associated with tissue factor-dependent activation of the blood coagulation cascade and increased plasma levels of the coagulation protease thrombin [2]. This increase in thrombin activity is associated with the deposition of insoluble fibrin clots in livers of mice fed ANIT diet [6]. Notably, increased plasma thrombin levels and hepatic fibrin deposition are also features of chronic cholestatic liver disease in humans [6, 7].

The formation and degradation of fibrin clots is a balanced and highly regulated process. The coagulation protease thrombin cleaves soluble fibrin(ogen) to fibrin monomers, and activates coagulation factor XIII, which cross-links fibrin monomers to form insoluble fibrin clots. Fibrin is degraded in a process termed fibrinolysis, largely by the enzyme plasmin, which is generated from plasminogen [8]. Although fibrin deposition is an inevitable consequence of tissue injury, its regulation and impact in liver disease is not completely understood [9]. Previously, we found that fibrin(ogen) deficiency increased hepatocellular necrosis in mice fed ANIT diet [6]. This suggests a protective role for hepatic fibrin deposition in this model of cholestatic liver injury, and that modulating fibrin degradation (i.e., inhibiting fibrinolysis) could prevent the progression of ANIT-induced liver injury and fibrosis.

Complete plasminogen deficiency impaired liver remodeling and regeneration in a model of carbon tetrachloride hepatotoxicity in a fibrin-independent manner [10-12]. In contrast, acetaminophen hepatotoxicity was reduced in plasminogen-null mice [13], suggesting the role of plasmin is injury/model-dependent. Plasminogen activator inhibitor-1 (PAI-1) is a primary

physiological inhibitor of fibrinolysis, via inhibition of the urokinase and tissue plasminogen activators, uPA and tPA, respectively [14]. Plasmin activity can be reduced pharmacologically by administration of tranexamic acid (TA), a drug that inhibits conversion of plasminogen to plasmin [15]. TA is an FDA-approved hemostatic agent for the treatment of traumatic bleeding and is also available over-the-counter elsewhere for indications such as heavy menstrual bleeding [16, 17]. As a hemostatic agent, TA is reported to have a favorable safety profile for existing indications [18, 19], and has been demonstrated as an inexpensive and effective treatment for traumatic bleeding, as suggested recently by the multicenter CRASH-2 trial [20]. In agreement with our findings in plasminogen-null mice, administration of TA also attenuated acetaminophen hepatotoxicity in mice, albeit to a lesser extent [13]. However, the impact of TA treatment on chronic liver injury has not been broadly investigated in humans or animal models.

We determined the impact of TA treatment on ANIT diet-induced liver injury and the progression of ANIT diet-induced liver fibrosis in mice, and compared this to the effect of PAI-1 deficiency. Based on our previous observation that fibrin(ogen) deficiency increased ANIT diet-induced liver injury, we hypothesized that TA administration would inhibit ANIT diet-induced liver injury and fibrosis in mice.

Materials and Methods

Mice: PAI-1$^{-/-}$ mice (Stock # 002507) and wild-type (WT) mice on an identical C57Bl/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained by homozygous breeding. Age-matched male mice between the ages of 8-14 weeks were used for these studies. Mice were housed at an ambient temperature of approximately 22°C with alternating 12 hour light/dark cycles and provided water and rodent chow *ad libitum* prior to study initiation.

Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at Michigan State University. All animal procedures were approved by Michigan State University Institutional Animal Care and Use Committee.

ANIT diet model and pharmacological intervention: Custom diets were prepared by Dyets, Inc. (Bethlehem, PA). The ANIT diet was an AIN-93M purified diet containing 0.025% ANIT (Sigma-Aldrich, St. Louis, MO). The control diet was the purified AIN-93M diet. Groups of mice were fed each diet for a total of 2 weeks (Expt. 1) or 4 weeks (Expt. 2), *ad libitum*. For Expt. 1, TA (1200 mg/kg, ip, bid; USP Grade) (Spectrum Chemical Company, New Brunswick, NJ) or its vehicle (sterile endotoxin-free water) was administered for the study duration. This dose was selected as approximating the range of TA doses utilized in previous mouse studies [13, 21], and was well tolerated by the mice. The dose required in mice is higher than the recommended human dose (4 grams/day), owing to the rapid elimination of TA in the kidney by glomerular filtration [22]. For Expt. 2, mice were fed control diet or ANIT diet for 4 weeks and TA or its vehicle were administered only in weeks 3-4. Wild-type and PAI-$1^{-/-}$ mice were fed ANIT diet for 4 weeks. Mice were anesthetized with isoflurane, and blood was collected from the caudal vena cava into sodium citrate (final, 0.38%) or an empty syringe for the collection of plasma and serum, respectively. The liver was removed and washed with saline. The left medial lobe of the liver was affixed to a cork with optimal cutting temperature compound (VWR Scientific, Radnor, PA) and frozen for 3 minutes in liquid nitrogen chilled isopentane. Sections of the left lateral lobe were fixed in neutral-buffered formalin for 48 h prior to routine processing. The remaining liver was cut into approximately 100 mg sections and flash-frozen in liquid nitrogen.

Histopathology and clinical chemistry: Formalin-fixed liver sections were cut at 5 microns and stained with hematoxylin and eosin (H&E) or sirius red by the Michigan State University Investigative Histopathology Laboratory for analysis of liver histopathology by light microscopy. At least 2 sections of liver from the left lateral lobe of each animal were evaluated in their entirety by a Board-certified veterinary pathologist (K.J.W). Sections were assigned a score of mild (1), moderate (2) or severe (3) for multifocal hepatocellular necrosis and bile duct hyperplasia. Total bile acids in serum were determined using a colorimetric assay (Bio-Quant, San Diego, CA). The serum activities of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined using commercially available reagents (Thermo Scientific, Waltham, MA; Pointe Scientific, Canton, MI).

Clot turbidity assay: Formation and lysis of fibrin clots was assessed by clot turbidity, as described previously [23], with slight modification. 100 μL normal pooled citrated-plasma (90% human plasma [George Kind Biomedical, Overland Park, KS]/10% pooled normal mouse plasma) was recalcified (16 mM final) and clotted with human α-thrombin (1 U/ml final) in the presence of 250 ng/ml human tissue plasminogen activator (tPA) (Molecular Innovations, Novi, MI). Clot turbidity was assessed by determining the absorbance at 405 nm over time. For select experiments, plasma from TA-treated mice and vehicle-treated mice was substituted for pooled normal mouse plasma. To approximate the TA concentration observed in TA-treated mice, the time to 50% clot lysis was determined for each sample, and compared to a standard curve generated by spiking the pooled plasma with various concentrations of TA prior to clot formation.

Immunofluorescent staining of mouse tissues and quantification: Fibrin, Type 1 collagen and cytokeratin-19 (CK19) immunostaining and quantification were performed as described previously [2]. In brief, approximately 10 low-power images (100X) for each tissue section were captured in a random and masked fashion and were analyzed using Image J (rsbweb.nih.gov/ij/). The percentage positive pixels are expressed as a fold change relative to mice fed control diet and treated with vehicle.

RNA isolation, cDNA synthesis, and real-time PCR: Total RNA was isolated from approximately 50 mg of snap-frozen liver using TRI Reagent (Molecular Research Center, Cincinnati, OH). 1 μg of total RNA was utilized for the synthesis of cDNA, accomplished using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Hepatic levels of mRNAs encoding the profibrogenic genes Type 1 collagen (COL1A1), integrin β6 (ITGB6), transforming growth factor-1 (TGFβ1) and -2 (TGFβ2) and tissue inhibitor of metalloproteinase1 (TIMP1) were determined using SYBR Green PCR, iTaq (Bio-Rad), and a CFX Connect thermal cycler (Bio-Rad). Primers were purchased from IDT (Coralville, IA). The expression of each gene was adjusted to the geometric mean Ct of two individual housekeeper genes, HPRT and 18S RNA, as described (Vandesompele et al., 2002) and the relative levels of each gene were evaluated using the ΔΔCt method. Mouse COL1A1 primer sequences were 5'-GAGCGGAGAGTACTGGATCG-3' (forward primer), 5'-GCTTCTTTTCCTTGGGGTTC-3' (reverse primer). Mouse TIMP1 primer sequences were 5'-GAGACACACCAGAGCAGATACC-3' (forward primer), 5'-CCAGGTCCGAGTTGCAGAAG-3' (reverse primer). Mouse ITGB6 primer sequences were 5'-CTCACGGGTACAGTAACGCA-3' (forward primer), 5'-AAATGAGCTCTCAGGCAGGC-3'

(reverse primer). Mouse TGFβ1 primer sequences were 5'-CTCCCGTGGCTTCTAGTGC-3' (forward primer), 5'-GCCTTAGTTTGGACAGGATCTG-3' (reverse primer). Mouse TGFβ2 primer sequences were 5'-CCCCGGAGGTGATTTCCATC-3' (forward primer), 5'-GATGGCATTTTCGGAGGGGA-3' (reverse primer).

Statistics: Comparison of two groups was performed using Student's t-test. Comparison of three or more groups was performed using one- or two-way ANOVA, as appropriate, and Student-Newman-Keul's *post hoc* test. The criterion for statistical significance was $p < 0.05$.

Results

Assessment of hepatic fibrin deposition and TA inhibition of fibrinolytic activity in mice fed ANIT diet. Compared to WT mice fed control diet for 4 weeks, marked hepatic fibrin deposition occurred in ANIT-treated WT mice (Fig. 1A). Similar hepatic fibrin deposition has been reported previously in mice fed ANIT diet for 2 weeks [6]. Interestingly, we did not observe a statistically significant increase in hepatic fibrin deposits in TA-treated mice (data not shown). The effect of TA treatment on fibrin clot lysis was analyzed *ex vivo* utilizing a fibrin clot turbidity assay. Compared to fibrin clots generated with plasma from vehicle-treated mice fed ANIT diet, fibrin

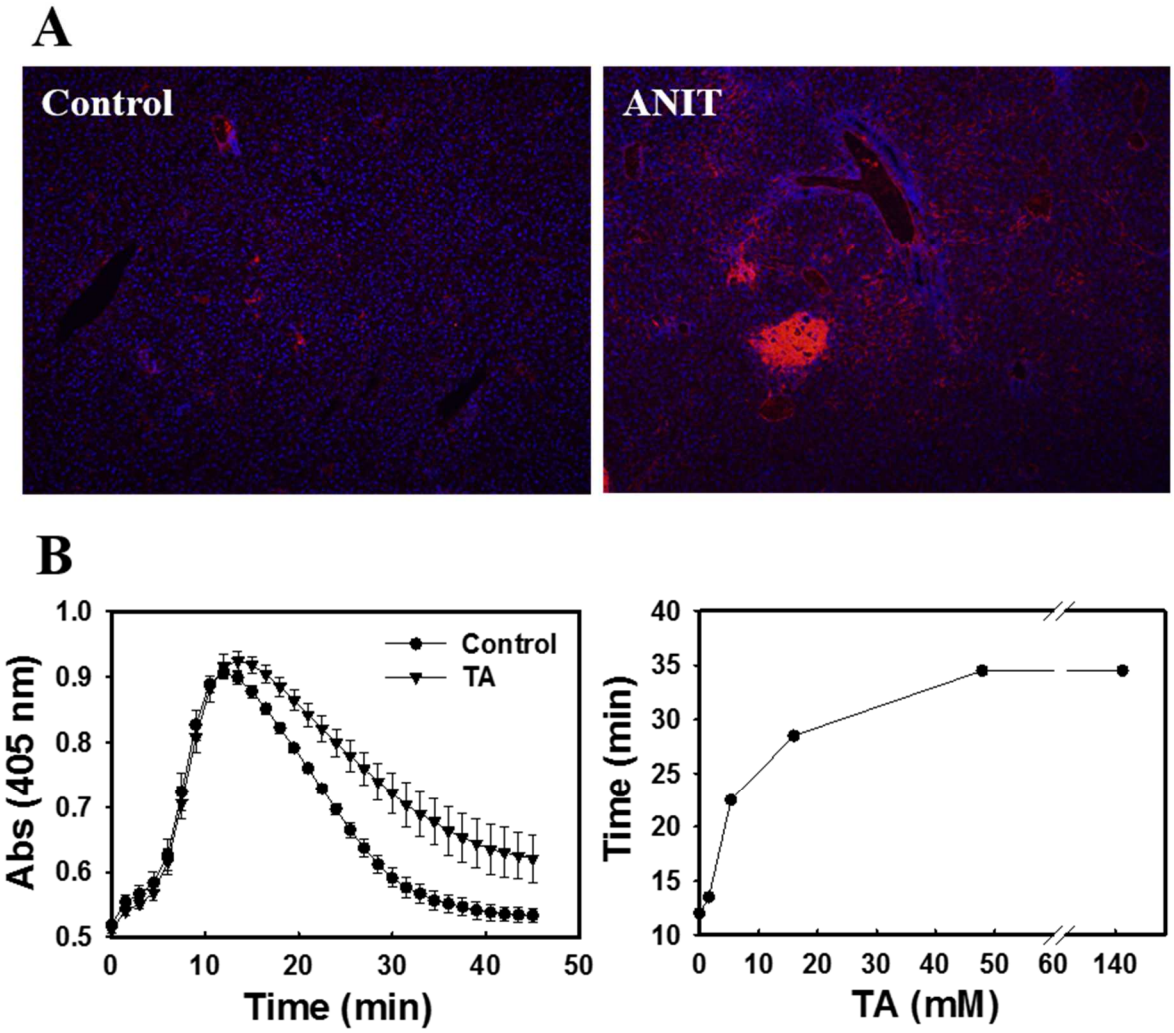

Figure 1. Hepatic fibrin deposition and antifibrinolytic activity of tranexamic acid in mice fed ANIT diet. Male, wild-type C57Bl/6J mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Representative photomicrographs (100X) show liver sections stained for fibrin (red) and counterstained with DAPI to visualize nuclei (blue). (B) Comparison of clot formation and lysis by turbidity (Absorbance [A] 450nm) in ANIT diet fed mice treated with vehicle (n=5 mice) or TA (1200 mg/kg, ip) (n=5 mice) twice daily for two weeks. Recalcified human pooled plasma was spiked with 10% mouse plasma from each group and clotting was initiated with thrombin (1 U/ml final) in the presence of 250 ng/ml human tPA. Data are expressed as mean±SEM. An increase in absorbance implies fibrin clot formation and a decrease indicates lysis.

clot lysis time was markedly prolonged when clots were generated with plasma from TA-treated mice fed ANIT diet (Fig. 1B). The time to reduce peak fibrin clot turbidity by 50% was determined and compared to a standard curve generated by spiking normal pooled plasma with TA (not shown). This analysis yielded a plasma concentration of approximately 0.2 mg/L TA, roughly 16 hours after the last dose of drug. This is similar to levels observed at this time after administration of a therapeutic dose of TA in humans [22, 24].

Effect of TA treatment on liver histopathology in mice fed ANIT diet for 2 weeks. No lesions were identified in livers of mice fed control diet for 2 weeks, irrespective of TA treatment (Fig. 2A). Vehicle-treated mice fed ANIT diet for 2 weeks developed liver injury characterized by multifocal acute hepatocellular coagulative necrosis and inflammation (6 of 7 mice), mild peribiliary fibrosis, and moderate lymphocytic inflammation/bile duct epithelial hyperplasia (7 of 7 mice) (Fig. 2A-B), in agreement with previous studies [2, 5, 8]. Treatment with TA reduced liver necrosis and inflammation in mice fed ANIT diet (Fig. 2A-B), as indicated by a reduction in the number of mice with evidence of necrosis and inflammation (3 of 6 mice), and a reduction in the average necrosis severity score from 1.8 in vehicle-treated mice to 0.5 in TA-treated mice. This corresponded to approximately a 50% reduction in necrotic area in TA-treated mice. Serum ALT activity and bile acid concentration increased in vehicle-treated mice fed ANIT diet (Fig. 2C-D). Serum ALP activity did not increase in ANIT-treated mice (not shown). Treatment with TA tended to reduce serum ALT activity, although this did not achieve statistical significance ($p=0.1$; Fig. 2C). Treatment with TA did not affect serum bile acid concentration in mice fed ANIT diet (Fig. 2D).

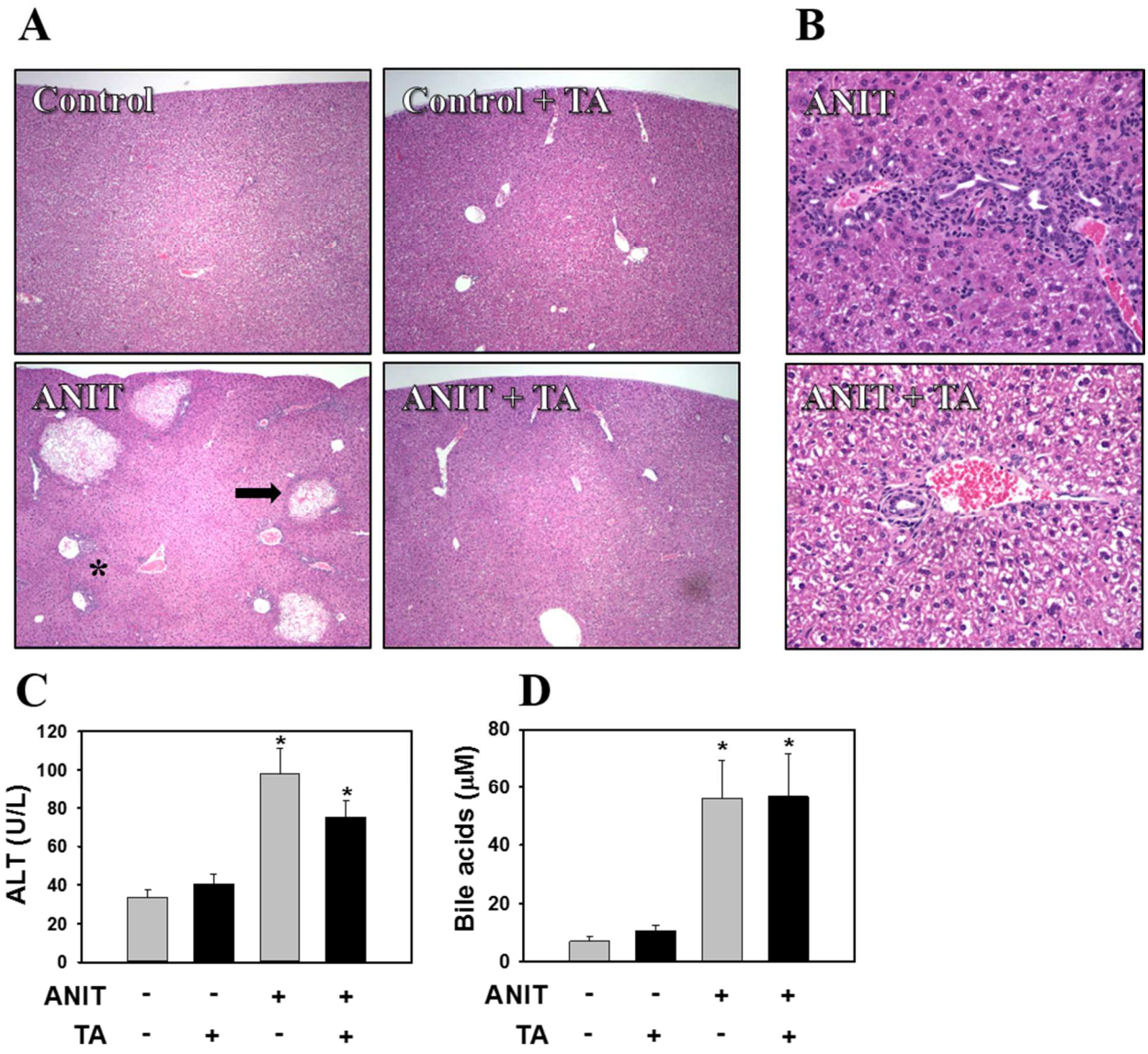

Figure 2. Effect of tranexamic acid on liver injury in mice fed ANIT diet for 2 weeks. Male, wild-type C57Bl/6J mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 2 weeks. Mice were treated with vehicle (sterile water) or tranexamic acid (TA) twice daily (1200 mg/kg, ip) for the duration of ANIT exposure. (A-B): Representative photomicrographs showing (A) low magnification (40X) and (B) high magnification (200X) hematoxylin and eosin–stained liver sections. Arrow indicates area of coagulative necrosis. Asterisk indicates area of biliary hyperplasia and portal inflammation. (C) Serum ALT activity and (D) bile acid concentration were determined as described in Materials and Methods. Data are expressed as mean±SEM; n = 3 mice per group for control diet and 6-7 mice per group for mice fed ANIT diet. *Significantly different from respective treatment fed control diet. $p < 0.05$.

Effect of TA treatment on hepatic profibrogenic gene induction and Type 1 collagen deposition in mice fed ANIT diet for 2 weeks. Peribiliary fibrosis and induction of several profibrogenic genes is evident in livers of mice fed ANIT for 2 weeks [2]. We chose to evaluate the expression of mRNAs encoding gene products that are known to participate in liver fibrosis accompanying cholestasis (eg., TIMP1, ITGB6, TGFβ), as an initial screen to identify profibrogenic pathways controlled by plasmin. Although it reduced liver injury, TA treatment did not significantly inhibit the induction of TIMP1, ITGB6, TGFβ1 or TGFβ2 mRNAs in livers of mice fed ANIT diet for 2 weeks (Fig. 3A-D). Moreover, the induction of COL1A1 mRNA (Fig. 3E) and deposition of Type 1 collagen protein (Fig. 3F) were not significantly reduced by TA treatment. This suggests that the profibrogenic and necrotic processes in mice fed ANIT diet are not interdependent at this time point.

Intervention with TA treatment reduces necrosis and biliary hyperplasia in livers of mice fed ANIT diet for 4 weeks. Next, we determined the effect of TA treatment on the progression of ANIT diet-induced liver injury and fibrosis. Mice were fed ANIT diet for 4 weeks, and given either TA or vehicle beginning in week 3. Liver histology was unremarkable in vehicle-treated mice fed control diet for 4 weeks. Tranexamic acid treatment in mice fed control diet tended to increase hepatocellular vacuolization, suggestive of glycogen or lipid droplets (Fig. 4A). Coagulative necrosis and inflammation were observed in livers of vehicle-treated mice fed ANIT diet for 4 weeks (Fig. 4A-B). However, the frequency (~50% mice with necrosis) and severity (average score 1 [mild]) were reduced compared to mice fed ANIT diet for 2 weeks. Nonetheless, intervention with TA treatment beginning in week 3 tended to reduce necrosis and peribiliary inflammation in mice

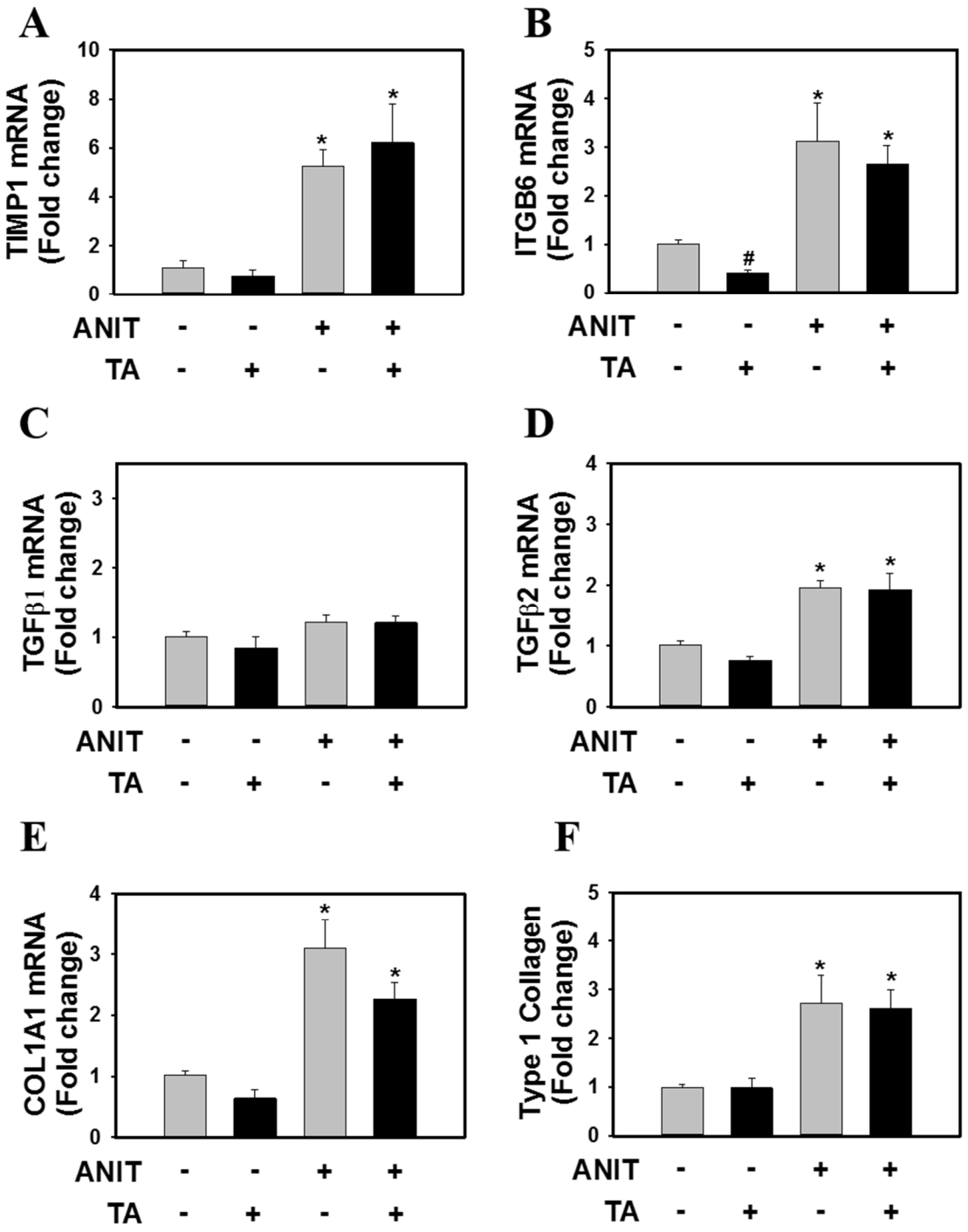

Figure 3. Effect of tranexamic acid on early hepatic profibrogenic changes in mice fed ANIT diet for 2 weeks. Male, wild-type C57Bl/6J mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 2 weeks. Mice were treated with vehicle (sterile water) or tranexamic acid (TA) twice daily (1200 mg/kg, ip) for the duration of ANIT exposure. (A-E): Hepatic levels of mRNAs encoding (A) TIMP1, (B) ITGB6, (C) TGFβ1, (D) TGFβ2 and (E) COL1A1 were

Figure 3. (cont'd). determined using real-time qPCR. (F) Deposition of Type 1 collagen protein in liver was quantified as described in Materials and Methods. Data are expressed as mean±SEM; n = 3 mice per group for control diet and 6-7 mice per group for mice fed ANIT diet. *Significantly different from respective treatment fed control diet. #Significantly different from vehicle-treated mice fed the same diet. $p < 0.05$.

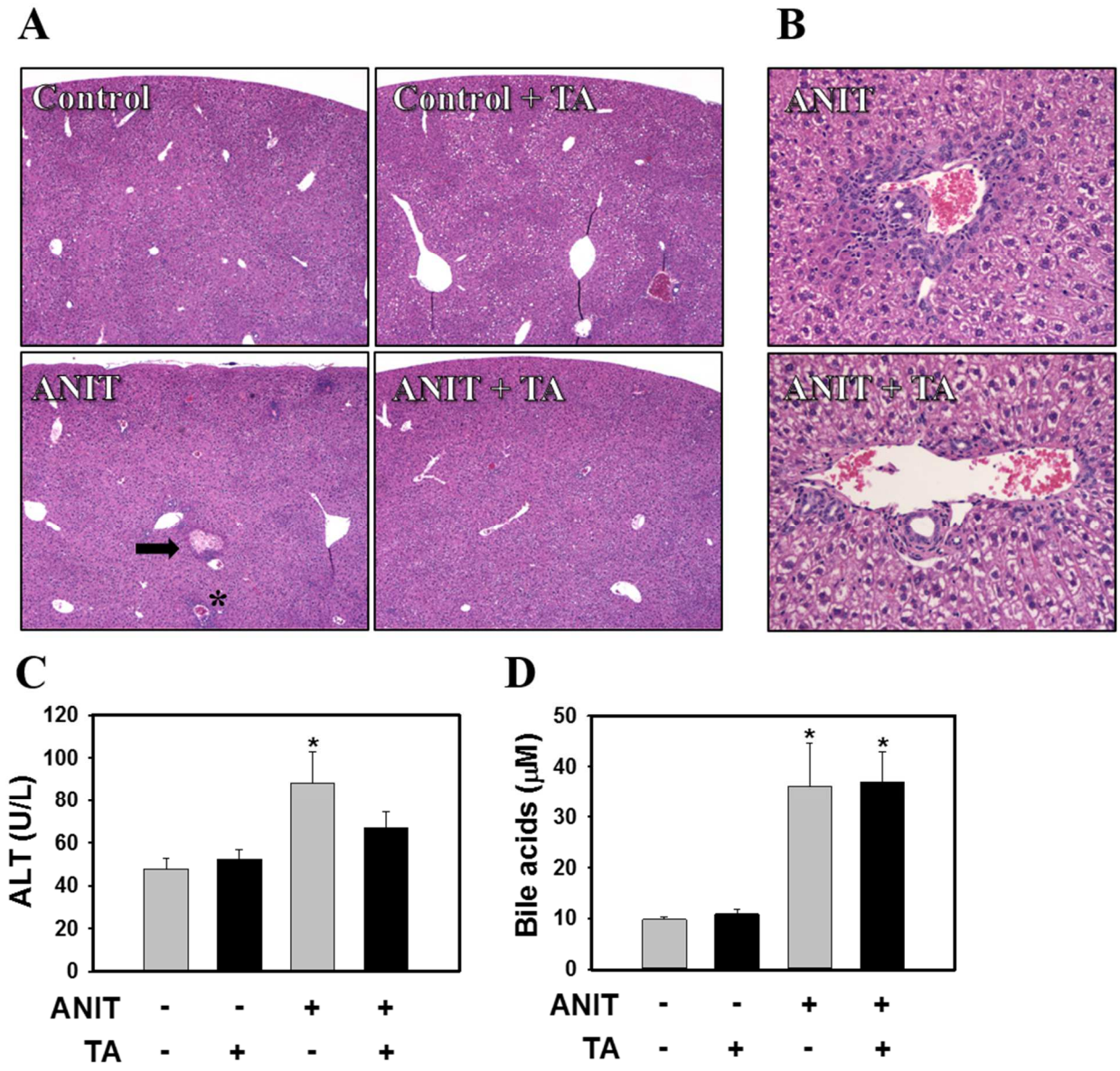

Figure 4. Effect of tranexamic acid treatment on liver injury in mice fed ANIT diet for 4 weeks. Male, wild-type C57Bl/6J mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Mice were treated with vehicle (sterile water) or tranexamic acid (TA) twice daily (1200 mg/kg, ip) beginning in week 3. (A-B): Representative photomicrographs showing (A) low magnification (40X) and (B) high magnification (200X) hematoxylin and eosin–stained liver sections. Arrow indicates area of coagulative necrosis. Asterisk indicates area of biliary hyperplasia and portal inflammation. (C) Serum ALT activity and (D) bile acid concentration were determined as described in Materials and Methods. Data are expressed as mean±SEM; n = 5 mice per group for control diet and 10 mice per group for mice fed ANIT diet. *Significantly different from respective treatment fed control diet. $p < 0.05$.

fed ANIT diet (Fig. 4A-B). Analysis of liver histopathology revealed moderate biliary hyperplasia and portal inflammation in vehicle-treated mice fed ANIT diet (average score 2), whereas these changes were noted to be mild in TA-treated mice fed ANIT diet (average score 1.1) (Fig. 4B). Serum ALT activity increased significantly in vehicle-treated mice fed ANIT diet. TA treatment tended to reduce serum ALT activity in mice fed ANIT diet (Fig. 4C), but this did not achieve statistical significance ($p=0.2$; Fig. 4C). TA treatment did not impact serum bile acid concentration in mice fed ANIT diet (Fig. 4D). BDEC hyperplasia was evaluated by immunofluorescent staining of cytokeratin 19 (CK19), a biomarker of BDECs in mouse liver (Fig. 5A-B). As determined by morphometry, the area of CK19 staining increased approximately 2.5-fold and 4.5-fold in livers of mice fed ANIT diet for 2 weeks and 4 weeks, respectively (Fig. 5C-D). Treatment with TA significantly reduced CK19 staining in livers of mice fed ANIT diet (Fig. 5C).

TA treatment inhibits induction of select profibrogenic genes and prevents the progression of collagen deposition in livers of mice fed ANIT diet for 4 weeks. Compared to mice fed control diet, expression of ITGB6, TGFβ1, TGFβ2, and TIMP1 mRNAs was increased in livers of mice fed ANIT diet for 4 weeks (Fig. 6A-D). The expression of each mRNA was notably higher than the increase observed after 2 weeks of ANIT diet (Fig. 3). TA treatment did not impact the expression of ITGB6 or TGFβ1 mRNAs in mice fed ANIT diet (Fig. 6A-B). In contrast, TA treatment reduced TGFβ2 ($P=0.08$) and TIMP1 ($P<0.05$) mRNA expression in livers of mice fed ANIT diet (Fig. 6C-D). Type 1 collagen mRNA expression increased approximately 7-fold in mice fed ANIT diet for 4 weeks compared to mice fed control

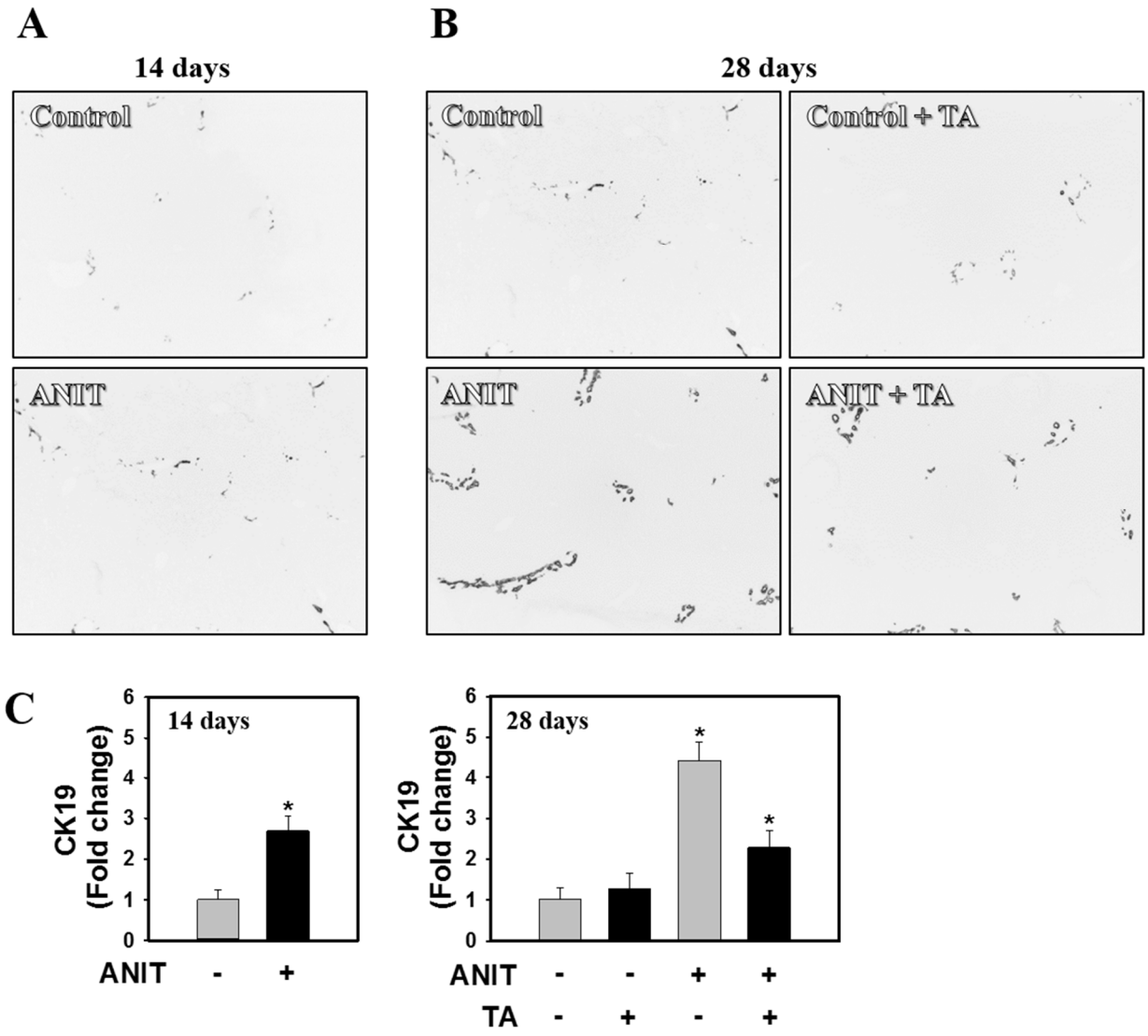

Figure 5. Effect of tranexamic acid treatment on biliary hyperplasia in mice fed ANIT diet for 4 weeks. Male, wild-type C57Bl/6J mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for up to 4 weeks. Mice were treated with vehicle (sterile water) or tranexamic acid (TA) twice daily (1200 mg/kg, ip) beginning in week 3. (A–B): Representative photomicrographs showing CK19 staining in liver sections of mice fed control and ANIT diet fed mice for (A) 2 weeks and (B) 4 weeks. Images were converted to grayscale and inverted such that CK19 staining is dark. (C) Quantification of CK19 staining area as described in Materials and Methods. Data are expressed as mean±SEM; n = 3-5 mice per group for control diet and 7-10 mice per group for mice fed ANIT diet. *Significantly different from respective treatment fed control diet. #Significantly different from vehicle-treated mice fed the same diet. $p < 0.05$.

diet (Fig. 7C). This was approximately doubled compared to mice fed ANIT diet for 2 weeks. In agreement, Type 1 collagen protein deposition increased significantly in livers of mice fed ANIT diet for 4 weeks, as indicated by Type 1 collagen immunofluorescence and sirius red staining (Fig. 7A-B). Administration of TA significantly reduced COL1A1 mRNA and protein levels in mice fed ANIT diet (Fig. 7A-C). Collagen deposition was reduced by TA treatment to a level similar to mice fed ANIT diet for 2 weeks (Fig. 7A-B).

PAI-1 deficiency increases peribiliary fibrosis in mice fed ANIT diet for 4 weeks. To complement our TA experiments, we examined the role of PAI-1 in ANIT-induced liver injury and fibrosis. PAI-$1^{-/-}$ mice are viable and do not have evidence of liver pathology [25]. Compared to ANIT-treated WT mice, histological changes including biliary hyperplasia and peribiliary inflammation were increased in ANIT-treated PAI-$1^{-/-}$ mice (Fig. 8A-B). PAI-1 deficiency did not dramatically increase liver necrosis in ANIT-treated mice, as indicated by liver histopathology (Fig. 8A-B) and serum ALT activity (Fig. 8C). Hepatic COL1A1 and α-SMA mRNA levels increased approximately 2-fold in ANIT-treated PAI-$1^{-/-}$ mice compared to ANIT-treated WT mice, but this did not achieve statistical significance (Fig. 8D). However, peribiliary Type 1 collagen protein deposition increased significantly (~3-fold) in livers of ANIT-treated PAI-$1^{-/-}$ mice compared to ANIT-treated WT mice (Fig. 8E).

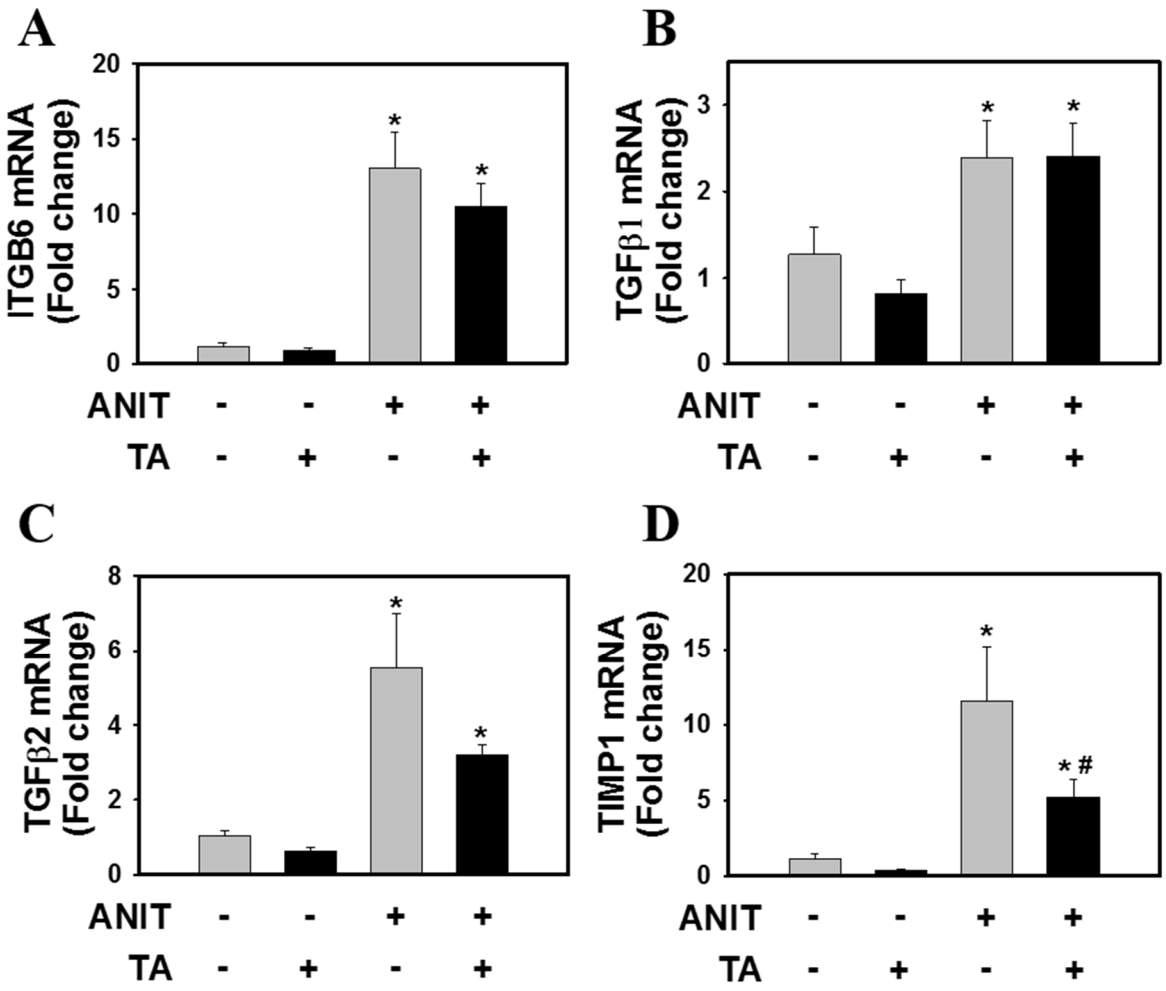

Figure 6. Effect of tranexamic acid treatment on profibrogenic gene induction in livers of mice fed ANIT diet for 4 weeks. Male, wild-type C57Bl/6J mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Mice were treated with vehicle (sterile water) or tranexamic acid (TA) twice daily (1200 mg/kg, ip) beginning in week 3. (A-D): Hepatic expression of mRNAs encoding (A) ITGB6, (B) TGFβ1, (C) TGFβ2 and (D) TIMP1 were determined using real-time qPCR. Data are expressed as mean±SEM; n=5 to 10 mice per group. *Significantly different from respective treatment fed control diet. #Significantly different from vehicle-treated mice fed the same diet. $p < 0.05$.

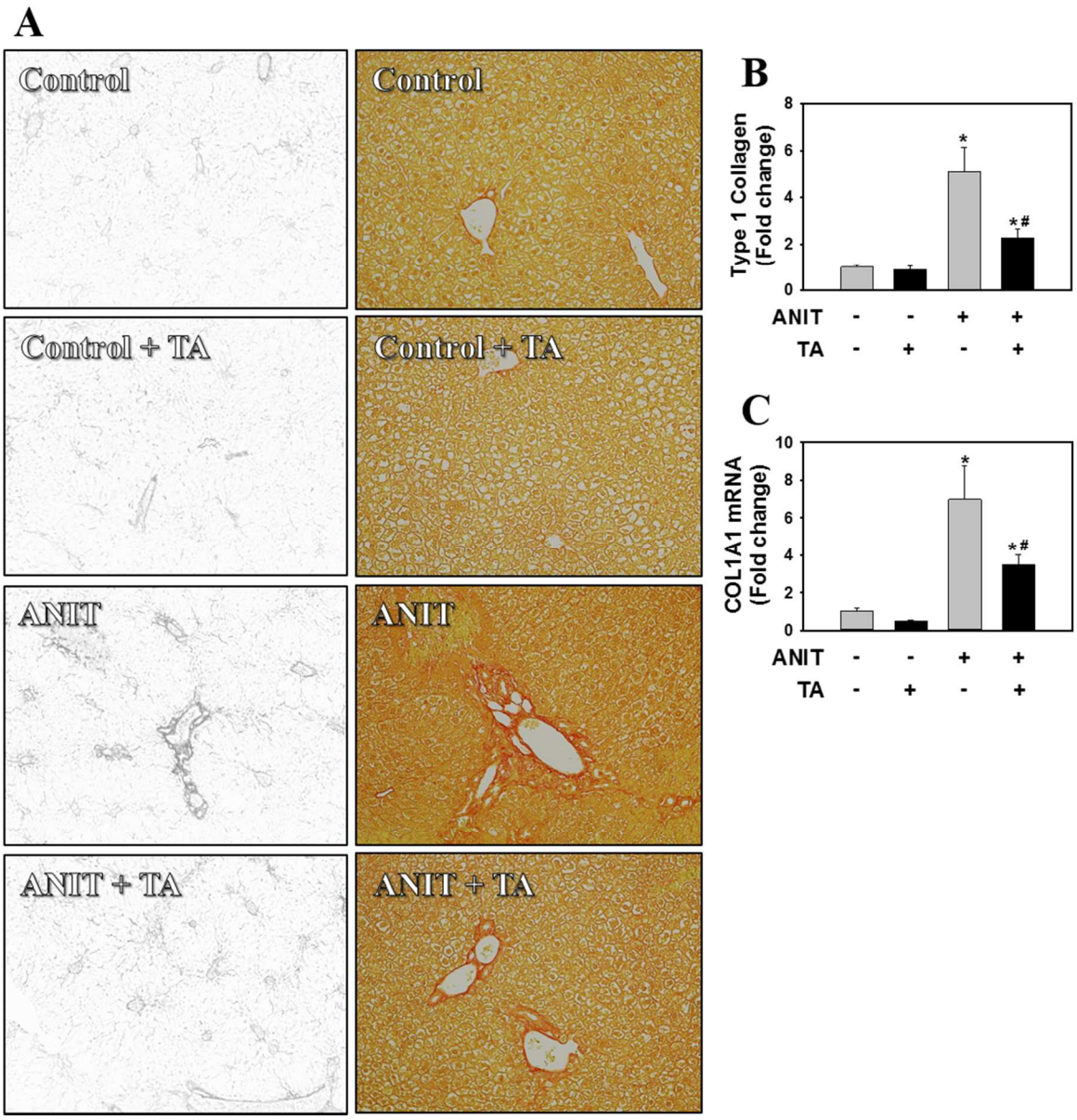

Figure 7. Effect of tranexamic acid treatment on Type 1 collagen expression and deposition in livers of mice fed ANIT diet for 4 weeks. Male, wild-type C57Bl/6J mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Mice were treated with vehicle (sterile water) or tranexamic acid (TA) twice daily (1200 mg/kg, ip) beginning in week 3. (A) Representative photomicrographs showing Type 1 collagen (100X) and Sirius red stained (200X) liver sections. Type 1 collagen images were converted to grayscale and inverted such that collagen staining is dark. (B) Collagen staining was quantified as described in Materials and Methods. (C) Hepatic mRNA expression levels of COL1A1 were determined using real-time qPCR. Data are expressed as mean±SEM; n=5 to 10 mice per group. *Significantly different from respective treatment fed control diet. #Significantly different from vehicle-treated mice fed the same diet. $p < 0.05$.

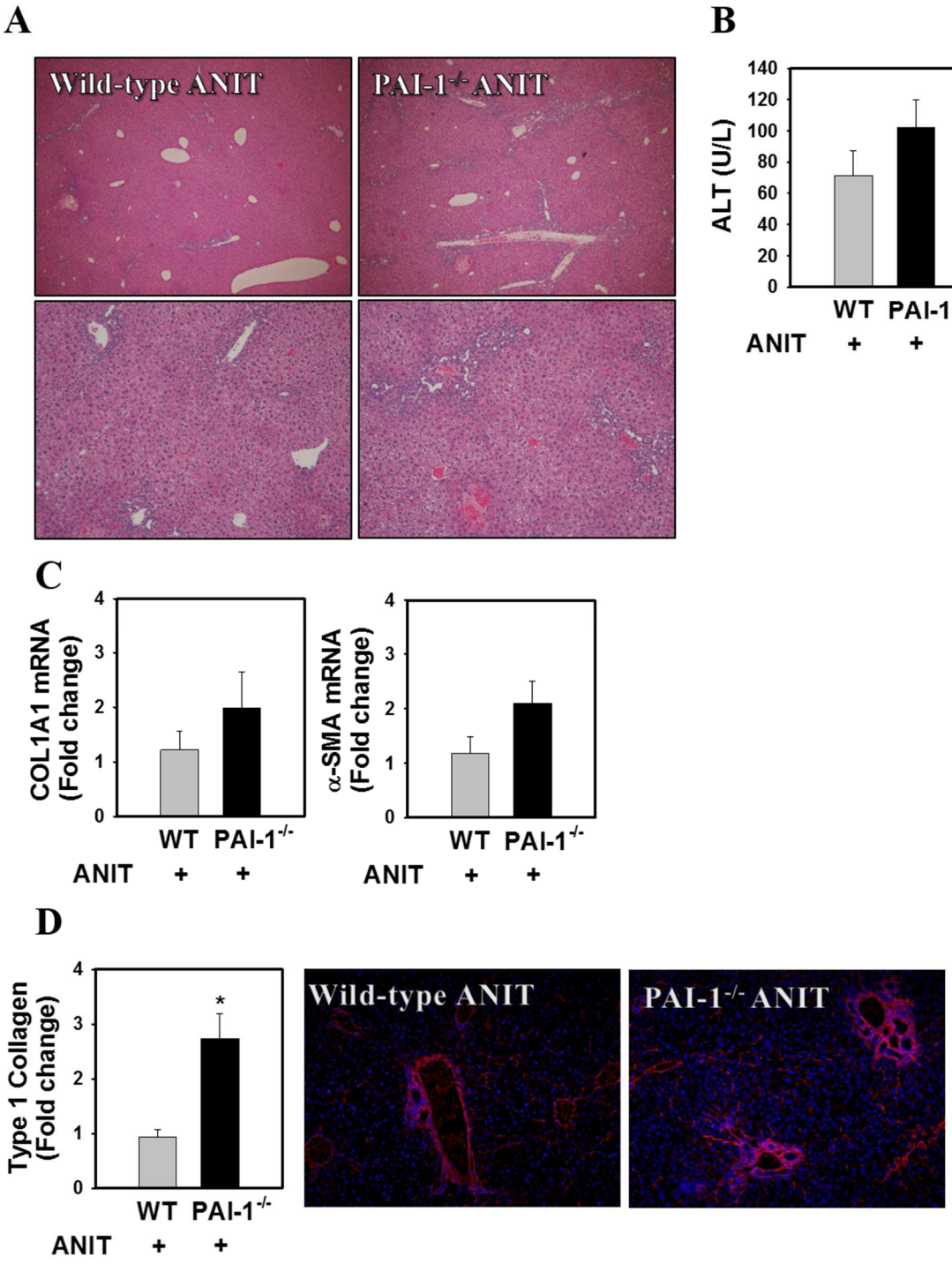

Figure 8. Effect of PAI-1 deficiency on liver injury and fibrosis in mice fed ANIT diet for 4 weeks. Male, wild-type (WT) and PAI-1$^{-/-}$ mice were fed a diet containing 0.025% ANIT for 4 weeks. Representative photomicrographs showing (A) low magnification (40X) and (B) high magnification (100X) hematoxylin and eosin–stained liver sections. Asterisks mark area of biliary hyperplasia and lymphocytic inflammation. Arrowheads mark necrotic foci (bile infarcts). (C)

Figure 8. (cont'd). Serum ALT activity was determined as described in Materials and Methods. (D) Hepatic mRNA expression levels of COL1A1 and α-SMA were determined using real-time qPCR. Data are expressed as mean±SEM; n=5 mice per group. (E) Representative photomicrographs showing Type I collagen staining (red) on DAPI-counterstained (blue) liver sections of mice fed ANIT diet for 4 weeks. Collagen protein levels were quantified as described in Materials and Methods. Data are expressed as mean±SEM; n=5 mice per group. * $p<0.05$ vs. WT mice fed the same diet.

Discussion

Hepatic fibrin deposition is evident in patients with chronic cholestatic liver disease [6] and in rodent models of chronic cholestasis [6, 26] Genetically imposed loss of fibrin(ogen) increases hepatic inflammation and injury in mice fed ANIT diet [6]. A parallel extension would suggest that uncontrolled fibrinolysis could contribute to the progression of liver disease. Clinical studies suggest exaggerated fibrinolysis in patients with liver disease, as indicated by elevated D-dimer levels, a product of fibrin degradation [27-29]. Moreover, reductions in the primary inhibitors of fibrinolysis, thrombin-activatable fibrinolysis inhibitor and alpha2-antiplasmin, have been noted in patients with liver disease [30-33]. Preserving residual hepatic fibrin deposition could form a potential strategy to reduce the progression of chronic liver disease. Our proof-of-principle study suggests that a relatively short duration of TA treatment, at plasma levels approximating those observed in humans given TA, reduces multiple aspects of cholestatic liver injury in mice. Indeed, more prolonged treatment with TA could yield more robust protection. Because TA is already FDA-approved in the U.S., and is available over-the-counter internationally for other indications, it may be a potential candidate to repurpose for the treatment of liver disease.

Our previous studies showed that fibrin(ogen) protects the liver from chronic ANIT diet-induced cholestatic liver injury. Thus, one mechanism whereby TA treatment could reduce ANIT-induced liver injury is by inhibiting plasmin-mediated fibrinolysis, thereby stabilizing hepatic fibrin deposits. Our results indicate that plasma TA levels in TA-treated mice sufficiently prolonged clot lysis *ex vivo*, consistent with inhibition of plasmin, and were similar to those observed in TA-treated humans [22, 24]. Although TA treatment likely sustained hepatic fibrin deposition in ANIT-treated mice, we were unable to detect a significant increase in hepatic fibrin deposition in ANIT-treated mice given TA. One reason is potentially because the extent of hepatic fibrin deposition represents the net balance between thrombin-driven fibrin formation and plasmin-

mediated degradation. In TA-treated mice, there was likely a reduction in injury-driven coagulation and deposition of fibrin in necrotic areas, complicating analysis of overall fibrin deposition. Nonetheless, additional studies are required to determine if fibrin is required for the protective effects of TA in chronic ANIT-induced liver injury.

It is possible that fibrin(ogen)-independent effects of plasmin contribute to liver pathology in this model. For example, plasmin has been shown to promote the activation of inflammatory cells, including neutrophils [34], which contribute to liver necrosis induced by cholestasis [35]. Of interest, at a 2-week time point where it significantly reduced liver injury, TA treatment did not impact hepatic expression of mRNAs encoding numerous chemokines including MCP-1, MIP-2 and KC (data not shown). Even so, we cannot exclude the possibility that direct activation of inflammatory cells by plasmin promotes liver injury in mice fed ANIT diet. Determining the exact mechanism whereby plasmin and fibrin(ogen) modulate ANIT-induced liver pathology is the focus of ongoing investigation in the laboratory.

TA treatment significantly reduced hepatocyte necrosis without impacting early profibrogenic changes in mice fed ANIT diet for 2 weeks. Previous studies suggest that in the ANIT diet model, the mechanisms responsible for hepatocellular injury and fibrosis are distinct at early time points (2 weeks). For example, deficiency in the thrombin receptor protease activated receptor-1 reduced fibrosis without reducing hepatocellular necrosis in mice fed ANIT diet [2]. Early growth response-1-null mice develop markedly increased ANIT diet-induced liver fibrosis without a corresponding exacerbation of hepatocyte injury [36]. Moreover, analogous to our findings with TA treatment, fibrin(ogen) deficiency increased hepatocyte injury at this time without affecting hepatic collagen expression [6]. This dichotomy likely stems from BDECs being the primary cellular target of ANIT. These cells undergo a compensatory expansion in mice

exposed to ANIT [8]. The expression of integrin $\alpha_V\beta_6$ is increased on proliferating BDECs *in vitro* and in mice fed ANIT diet [2, 37]. Previous studies have demonstrated that activation of latent-TGFβ1 by $\alpha_V\beta_6$ integrin contributes to peribiliary fibrosis in models of cholestatic liver injury [2, 37, 38]. Peribiliary collagen deposits likely represent early activation of portal fibroblasts by TGFβ1 [39, 40]. Overall, the results suggest that plasmin is not a critical mediator of the initial, peribiliary profibrogenic changes in mice fed ANIT diet.

Although TA treatment did not inhibit the early profibrogenic response to ANIT, it largely prevented the progression of liver fibrosis in mice fed ANIT diet for 4 weeks. A failure to resolve persistent liver damage in mice fed ANIT diet could result in more extensive collagen deposition. Moreover, the reduction in biliary hyperplasia by TA treatment could be the basis for reduced fibrosis. Proliferating BDECs are known to produce a number of profibrogenic mediators that coordinate matrix production by surrounding portal fibroblasts [41]. Although these resident fibroblasts likely contribute to liver fibrosis in this model, engagement of accumulating bone-marrow derived fibrocytes cannot be excluded [42]. In our initial screen of profibrogenic pathways affected by plasmin, we found that TIMP1 expression was dramatically reduced, suggesting enhanced clearance of matrix. In contrast, TA treatment did not inhibit induction of ITGB6 mRNA, a component of the $\alpha_V\beta_6$ integrin that activates latent- TGFβ1. Although TA treatment did not impact expression of TGFβ1 mRNA levels, plasmin has been shown to enzymatically activate latent-TGFβ1 [43, 44], and plasmin-catalyzed TGFβ1 activation has been implicated in other models of fibrosis [43, 45]. Collectively, additional studies investigating plasmin-driven profibrogenic protein expression and function are required, as these studies suggest multiple mechanisms whereby TA could inhibit liver fibrosis.

The role of PAI-1 in models of liver injury and disease has been extensively studied. For example, PAI-1 deficiency increases liver injury in models of acetaminophen and carbon tetrachloride hepatotoxicity [13, 46]. In agreement with our finding that inhibition of fibrinolysis with TA reduces ANIT-mediated liver injury and fibrosis, we found that PAI-1-deficiency increased ANIT diet-induced liver injury and fibrosis. In contrast, PAI-$1^{-/-}$ mice were protected from alcohol-induced liver damage [47], Moreover, PAI-1 deficiency reduced liver injury and fibrosis in mice subjected to bile duct ligation (BDL), a model of extrahepatic obstructive cholestasis [48, 49]. However, the role of fibrin(ogen) in BDL-induced liver injury and fibrosis has not been described, with the reduction in hepatic fibrin in PAI-$1^{-/-}$ mice after BDL [49] likely a reflection of reduced liver injury. Indeed, PAI-1 deficiency did not affect plasmin activity in BDL mice [50]. Moreover, PAI-1 deficiency was shown to protect BDL mice by enhancing tPA-mediated activation of hepatocyte growth factor, a process not directly impacting fibrinolysis [26]. Additional studies are required to clarify the mechanisms underlying the different functions of PAI-1 in BDL and ANIT diet models of cholestatic liver injury.

Taken together, the results indicate that administration of the antifibrinolytic TA reduces liver injury and fibrosis in a mouse model of chronic xenobiotic-induced biliary injury. The protection from liver injury after treatment with an antifibrinolytic drug is in agreement with our finding that PAI-1 deficiency increases ANIT-induced liver injury and fibrosis, and supports our previous observation that fibrin(ogen) deficiency worsens liver injury in this model. Collectively, this study provides important proof-of-principle findings on which additional studies of TA as a novel treatment for liver disease could be based.

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CHAPTER 3

Coagulation-Driven Platelet Activation Reduces Cholestatic Liver Injury And Fibrosis In Mice

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Abstract

The coagulation cascade has been shown to participate in chronic liver injury and fibrosis, but the contribution of various thrombin targets, such as protease activated receptors (PARs) and fibrin(ogen), has not been fully described. Emerging evidence suggests that in some experimental settings of chronic liver injury, platelets can promote liver repair and inhibit liver fibrosis. However, the precise mechanisms linking coagulation and platelet function to hepatic tissue changes following injury remain poorly defined. Our objectives were to determine the role of PAR-4, a key thrombin receptor on mouse platelets, and fibrin(ogen) engagement of the platelet $\alpha_{IIb}\beta_3$ integrin in a model of cholestatic liver injury and fibrosis. Biliary and hepatic injury was characterized following 4 week administration of the bile duct toxicant α-naphthylisothiocyanate (ANIT) (0.025%) in PAR-4-deficient mice (PAR-$4^{-/-}$ mice), mice expressing a mutant form of fibrin(ogen) incapable of binding integrin $\alpha_{IIb}\beta_3$ (Fib$\gamma^{\Delta 5}$), and wild-type mice. Elevated plasma thrombin-antithrombin and serotonin levels, hepatic fibrin deposition and platelet accumulation in liver accompanied hepatocellular injury and fibrosis in ANIT-treated wild-type mice. PAR-4 deficiency reduced plasma serotonin levels, increased serum bile acid concentration, and exacerbated ANIT-induced hepatocellular injury and peribiliary fibrosis. Compared to PAR-4-deficient mice, ANIT-treated Fib$\gamma^{\Delta 5}$ mice displayed more widespread hepatocellular necrosis accompanied by marked inflammation, robust fibroblast activation and extensive liver fibrosis. Collectively, the results indicate that PAR-4 and fibrin-$\alpha_{IIb}\beta_3$ integrin engagement, pathways coupling coagulation to platelet activation, each exert hepatoprotective effects during chronic cholestasis.

Introduction

Coagulation cascade activation, marked by thrombin generation, hepatic fibrin deposition, and platelet activation is a conspicuous feature of cholestatic liver disease in humans [1-3], which is recapitulated in experimental settings of chronic liver injury [2, 4]. Experimental evidence supports a role for protease activated receptors (PARs), including the thrombin receptor PAR-1, in promoting liver fibrosis [5, 6]. PAR-1 deficiency reduced hepatic collagen deposition in models of carbon tetrachloride, bile duct ligation (BDL) and alpha-naphthylisothiocyanate (ANIT)-induced liver fibrosis [2, 4, 7, 8], an observation likely connected to PAR-1 expression by macrophages and/or hepatic stellate cells [4, 8, 9]. Unlike humans, PAR-1 is not expressed by mouse platelets, and thrombin-mediated platelet activation is intact in PAR-1-deficient mice [10]. A complex of PAR-3 and PAR-4 contributes to thrombin-mediated platelet activation in mice [11, 12]. Thus, while PAR-$1^{-/-}$ mice have provided compelling evidence of profibrogenic effects of thrombin, these cannot be attributed to platelet activation.

Indeed, the mechanisms coupling thrombin activity to platelet activation in models of liver fibrosis have not been fully explored. It is conceivable that thrombin, through activation of PAR-1 (in humans) or PAR-3/4 (in mice), is central to platelet activation in liver disease. Thrombin is a very potent activator of platelets, causing degranulation and release of stored mediators, including serotonin [13]. Platelet activation by diverse mediators, including thrombin, alters the conformation of integrin $\alpha_{IIb}\beta_3$, revealing a high affinity binding site for fibrin(ogen) [14]. Fibrin(ogen) engagement of activated $\alpha_{IIb}\beta_3$ integrin can further modify platelet activation, being critical for platelet aggregation and clot retraction [14, 15]. Demonstrating the importance of this interaction, mice expressing a mutant fibrin(ogen) incapable of binding activated $\alpha_{IIb}\beta_3$ integrin have defective platelet aggregation, despite retention of other fibrin(ogen)-dependent hemostatic

functions [15]. However, the role of this functional interaction between platelets and fibrin(ogen) in chronic liver injury has not yet been defined.

The contribution of platelets in experimental settings of liver damage and fibrosis appears to be context-dependent. Studies suggest that platelets can either promote or reduce liver injury and fibrosis. The specific role of platelets depends on the etiology of the liver disease or nature of the hepatic injury [16]. Moreover, experimental variables including the degree and duration of platelet deficiency or inhibition also impacts the outcome with respect to liver injury and fibrosis [17, 18]. For example, platelets exacerbate acute cholestatic liver injury in multiple models [19-21], whereas long-term thrombocytopenia or serotonin deficiency exacerbates liver fibrosis [17, 22]. Coagulation-mediated platelet activation, through both thrombin- and fibrin(ogen)-mediated mechanisms, is central to normal hemostasis [23]. However, the impact of these platelet activation pathways on chronic cholestatic liver injury has not yet been specifically evaluated.

In the present study, we sought to identify key mechanisms that link platelet function to liver injury and fibrosis in an experimental setting of chronic bile duct injury. Utilizing PAR-4 deficient mice (PAR-4$^{-/-}$) and mice expressing a mutant form of fibrin(ogen) lacking the binding motif for integrin $\alpha_{IIb}\beta_3$ (Fib$\gamma^{\Delta 5}$) [15, 24], we determined the role of thrombin-mediated platelet activation and fibrin(ogen)-platelet interactions through the integrin $\alpha_{IIb}\beta_3$ in chronic biliary injury and fibrosis.

Materials and Methods

Mice: PAR-4$^{-/-}$ mice, Fib$\gamma^{\Delta 5}$ mice, and wild-type mice backcrossed at least 8 generations on the same C57Bl/6J background, were maintained by homozygous breeding [15, 24]. Age-matched male mice between the ages of 8-14 weeks were used for these studies. Mice were housed at an

ambient temperature of approximately 22°C with alternating 12 hour light/12 hour dark cycles and provided purified water and rodent chow *ad libitum* prior to study initiation. Mice were maintained in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities at Michigan State University or Cincinnati Children's Hospital Medical Center. All animal procedures were approved by Michigan State University or Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committees.

ANIT diet model: Custom diets were prepared by Dyets, Inc. (Bethlehem, PA). The ANIT diet was an AIN-93M diet containing 0.025% ANIT (Sigma-Aldrich, St. Louis, MO). The control diet was AIN-93M diet. Groups of mice were fed each diet for a total of 4 weeks, *ad libitum*. Mice fed ANIT diet are referred to as ANIT-treated mice. Mice were anesthetized with isoflurane, and blood was collected from the caudal vena cava into sodium citrate (final, 0.38%) or an empty syringe for the collection of plasma and serum, respectively. The liver was removed and washed with saline. The left medial lobe of the liver was affixed to cork with optimal cutting temperature compound (VWR Scientific, Radnor, PA) and frozen for 3 minutes in liquid nitrogen-chilled isopentane. Sections of the left lateral lobe were fixed in neutral-buffered formalin for 48 hours prior to routine processing. The remaining liver was cut into approximately 100 mg sections and flash-frozen in liquid nitrogen.

Histopathology and clinical chemistry: For analysis of liver histopathology by light microscopy, formalin-fixed liver sections were cut at 5 microns and stained with hematoxylin and eosin (H&E) and sirius red by the Michigan State University Investigative Histopathology Laboratory. At least 2 sections of liver from the left lateral lobe of each animal were qualitatively evaluated in their entirety by a Board-certified veterinary pathologist (K.J.W.). Quantitative measures of necrosis

(i.e., lesion frequency and size) in H&E-stained sections were performed in a masked fashion using ImageJ. For quantification of Sirius red staining (collagen deposits), images of Sirius-red stained liver sections were captured using a Virtual Slide System VS110 (Olympus, Hicksville, NY) with a 20X objective. Random images were derived from the digitized slides approximating at least 100 mm^2 tissue for each liver. The area of positive sirius red staining in each image was determined in an unbiased fashion using a batch macro and the color deconvolution tool in ImageJ. Total bile acids in serum were determined using a colorimetric assay (Bio-Quant, San Diego, CA) and serum activities of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined using commercial reagents (Thermo Scientific, Waltham, MA; Pointe Scientific, Canton, MI). Plasma thrombin-antithrombin (TAT) and serotonin levels were determined using commercial enzyme-linked immunosorbent assay kits (Siemens Healthcare Diagnostics, Deerfield, IL; Eagle Biosciences, Nashua, NH). Serum cytokine levels (IL-6, IL-4, KC/Gro, TNFα) were determined using the Meso Scale V-PLEX Proinflammatory Panel Kit and a Sector 600 Imager (Meso Scale Discovery, Rockville, MD).

Immunohistochemistry and immunofluorescence: α-smooth muscle actin (α-SMA) immunohistochemistry was performed as described previously [25], with slight modification. Briefly, sections were de-paraffinized in xylene and subjected to heat-mediated antigen retrieval in citrate buffer (10 mM, pH 6). Sections were incubated with primary rabbit-anti α-SMA antibody (1:750) (Abcam, Cambridge, MA). Fibrin(ogen) immunohistochemistry was performed on de-paraffinized formalin-fixed sections after antigen retrieval with proteinase K, using a rabbit anti-human fibrin(ogen) antibody (1:600) (Dako North America , Carpinteria, CA). Each primary antibody was detected utilizing a biotinylated goat anti-rabbit antibody (Jackson

ImmunoResearch Laboratories, West Grove, PA) and Vectastain Elite ABC kit and ImmPACT DAB substrate (Vector Laboratories, Burlingame, CA). Immunofluorescent staining of type 1 collagen, cytokeratin-19 (CK19) and integrin α_{IIb}/CD41 (platelet) were performed as described [2, 20, 26]. Prolong Gold (DAPI-containing) Antifade reagent (Life Technologies) was applied to the tissues prior to cover slipping. Fluorescent staining in liver sections was visualized using an Olympus DP70 microscope (Olympus, Lake Success, NY) and merged (as appropriate) using Olympus DP Manager software. Type 1 collagen and CK19 staining was quantified using Scion Image (Scion Corporation, Frederick, MD) as described previously [2], utilizing approximately 10 low-power images (100X) for each tissue. The percentage of pixels containing positive signal (i.e., collagen staining) was expressed as a fold change relative to wild-type mice fed control diet. Neutrophil and CD3 staining on paraffin-embedded, formalin-fixed sections was accomplished using monoclonal rat anti-mouse allotypic neutrophil marker (PMN 7/4) and rabbit polyclonal anti-CD3 (Abcam), respectively, and was performed by the Michigan State University Investigative Histopathology Laboratory. Quantification of α-SMA and CD3 positive staining was performed using digitized slides and ImageJ as described for Sirius red staining (above).

RNA isolation, cDNA synthesis, and real-time PCR: Total RNA was isolated from approximately 15 mg of snap-frozen liver using TRI Reagent (Molecular Research Center, Cincinnati, OH). 1 µg of total RNA was utilized for the synthesis of cDNA, accomplished using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Hepatic levels of mRNAs encoding the profibrogenic genes type 1 collagen (COL1A1), integrin β6 (ITGB6), transforming growth factor-1 (TGFβ1) and -2 (TGFβ2) and tissue inhibitor of metalloproteinase1 (TIMP-1) were determined using SYBR

Green PCR, iTaq (Bio-Rad), and a CFX Connect thermal cycler (Bio-Rad). Primers were purchased from IDT (Coralville, IA). The expression of each gene was adjusted to the geometric mean Ct of two individual housekeeper genes, HPRT and 18S RNA, as described [27], and the relative levels of each gene were evaluated using the ΔΔCt method. . Mouse COL1A1 primer sequences were 5'-GAGCGGAGAGTACTGGATCG-3' (forward primer), 5'-GCTTCTTTTCCTTGGGGTTC-3' (reverse primer). Mouse TIMP1 primer sequences were 5'-GAGACACACCAGAGCAGATACC-3' (forward primer), 5'-CCAGGTCCGAGTTGCAGAAG-3' (reverse primer). Mouse ITGB6 primer sequences were 5'-CTCACGGGTACAGTAACGCA-3' (forward primer), 5'-AAATGAGCTCTCAGGCAGGC-3' (reverse primer). Mouse TGFβ1 primer sequences were 5'-CTCCCGTGGCTTCTAGTGC-3' (forward primer), 5'-GCCTTAGTTTGGACAGGATCTG-3' (reverse primer). Mouse TGFβ2 primer sequences were 5'-CCCCGGAGGTGATTTCCATC-3' (forward primer), 5'-GATGGCATTTTCGGAGGGGA-3' (reverse primer).

Platelet isolation and stimulation: Approximately 0.5 mL whole blood was collected from the caudal vena cava into acid citrate dextrose (ACD) and added to an equivalent volume of pipes saline glucose (PSG). Platelet-rich plasma was mixed with PSG containing 1 μM PGE_1 and 0.02U/ml apyrase and subjected to centrifugation at 500 x g for 10 min. Platelets were then subjected to one additional wash with PSG+ PGE_1/apyrase and then gently resuspended in DMEM at a density of approximately $1x10^8$ platelets/100μL. The platelets were then stimulated with thrombin (10 U/ml) or its vehicle (PBS) for 5 minutes and after centrifugation, 0.1% ascorbic acid added to the supernatant to stabilize serotonin. Supernatant serotonin levels were determined using a commercial ELISA (Eagle Biosciences).

Statistics: Comparison of two groups was performed using Student's t-test. Comparison of three or more groups was performed using one- or two-way analysis of variance (ANOVA), as appropriate, and Student-Newman-Keul's *post hoc* test. The criterion for statistical significance was $p \leq 0.05$.

Results

Increased coagulation and platelet accumulation in livers of wild-type mice. Compared to wild-type mice fed control diet, plasma TAT levels were increased in ANIT-treated mice, indicating activation of the coagulation cascade (Fig. 9A). Platelets are the primary cellular source of peripheral serotonin, a mediator shown to exert hepatoprotective effects in liver fibrosis [22, 28]. Plasma levels of serotonin were increased in ANIT-treated mice (Fig. 9B). Minimal fibrin deposition was observed in wild-type mice fed control diet (Fig. 9C). In contrast, an increase in peribiliary and sinusoidal fibrin deposits was evident in ANIT-treated mice (Fig. 9C). Fibrin was also apparent in association with focal areas of hepatocellular necrosis, although these lesions were infrequent in wild-type mice (Fig. 9C). Scattered α_{IIB} (platelet) staining was confined to sinusoids and larger vessels in mice fed control diet. Hepatic platelet accumulation was evident in livers of ANIT-treated mice (Fig. 9D). Taken together, the results indicate that ANIT toxicity in mice is

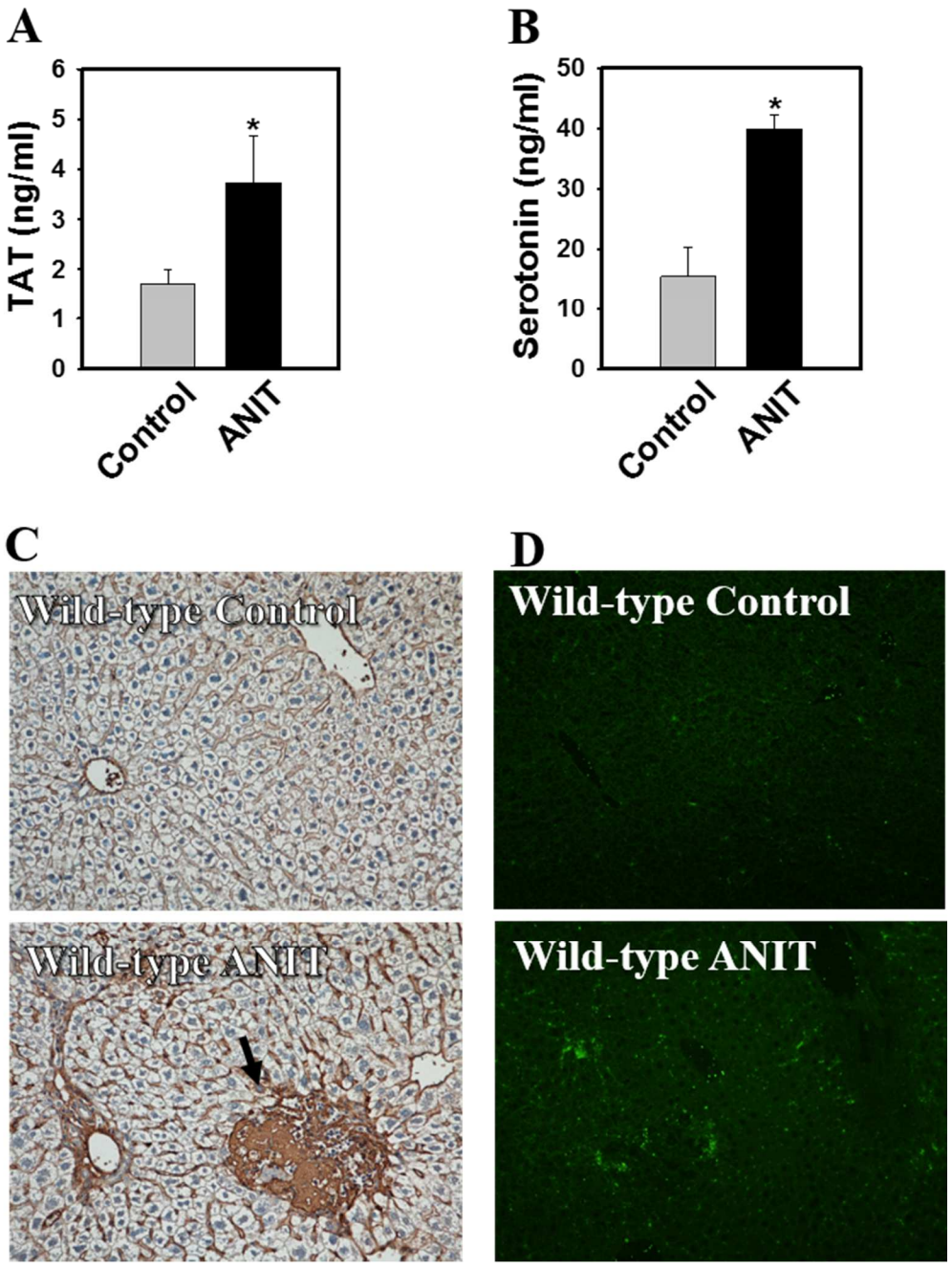

Figure 9. Coagulation and hepatic platelet accumulation in ANIT-treated wild-type mice. Wild-type mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Plasma TAT levels were determined by ELISA. (B) Plasma serotonin levels were determined by ELISA. (C) Representative photomicrographs (200X) showing liver sections stained for fibrin(ogen) (brown). Arrow indicates area of acute hepatocellular coagulative necrosis. (D) Representative photomicrographs (100X) show liver sections stained for integrin α_{IIb} (CD41, platelets). Data are expressed as mean ± SEM, n = 5 mice per group for control diet and 10 mice per group for ANIT-treated mice, *$p<0.05$ vs. control diet.

associated with activation of the coagulation cascade, hepatic fibrin deposition and platelet accumulation and activation.

Effect of PAR-4 deficiency on serotonin levels, liver injury and biliary hyperplasia in ANIT-treated mice. Plasma TAT levels were similar in ANIT-treated wild-type mice (3.8 ± 1.2 ng/ml, n=10) and ANIT-treated PAR-4$^{-/-}$ mice (3.2 ± 0.5 ng/ml, n=12). Thrombin stimulation has been shown to induce the rapid release of serotonin from human platelets [13]. Consistent with this, we found that thrombin stimulation induced serotonin release from isolated wild-type platelets, and this was significantly reduced in isolated PAR-4$^{-/-}$ platelets (Fig. 17). Plasma serotonin levels increased in ANIT-treated wild-type mice, but not in ANIT-treated PAR-4$^{-/-}$ mice (Fig. 10A). A previous study suggested that platelet-derived serotonin inhibits cholestatic liver injury, in part through regulation of the bile acid pool [22]. Consistent with this observation, serum bile acids increased significantly in ANIT-treated wild-type mice, and increased further in ANIT-treated PAR-4$^{-/-}$ mice (Fig. 10B). Serum ALT and ALP activities increased to a greater extent in ANIT-treated PAR-4$^{-/-}$ mice compared to ANIT-treated wild-type mice (Fig. 10C-D). The overall histological appearance of control diet fed WT and PAR4$^{-/-}$ mice was similar (Fig. 10F). In agreement with the increase in serum ALT, the number of necrotic lesions was significantly increased in livers of ANIT-treated PAR-4$^{-/-}$ mice compared to ANIT-treated wild-type mice, although the average size of necrotic foci was unaffected by genotype (Fig. 10E).

ANIT-treated mice developed biliary hyperplasia, which was not affected by genotype, as indicated by CK19 staining and quantification (Fig. 18A-B). Liver histopathology indicated increased portal inflammation and biliary fibrosis in ANIT-treated wild-type mice (Fig. 10F), which qualitative assessment suggested was slightly more severe in PAR-4$^{-/-}$ mice. In agreement

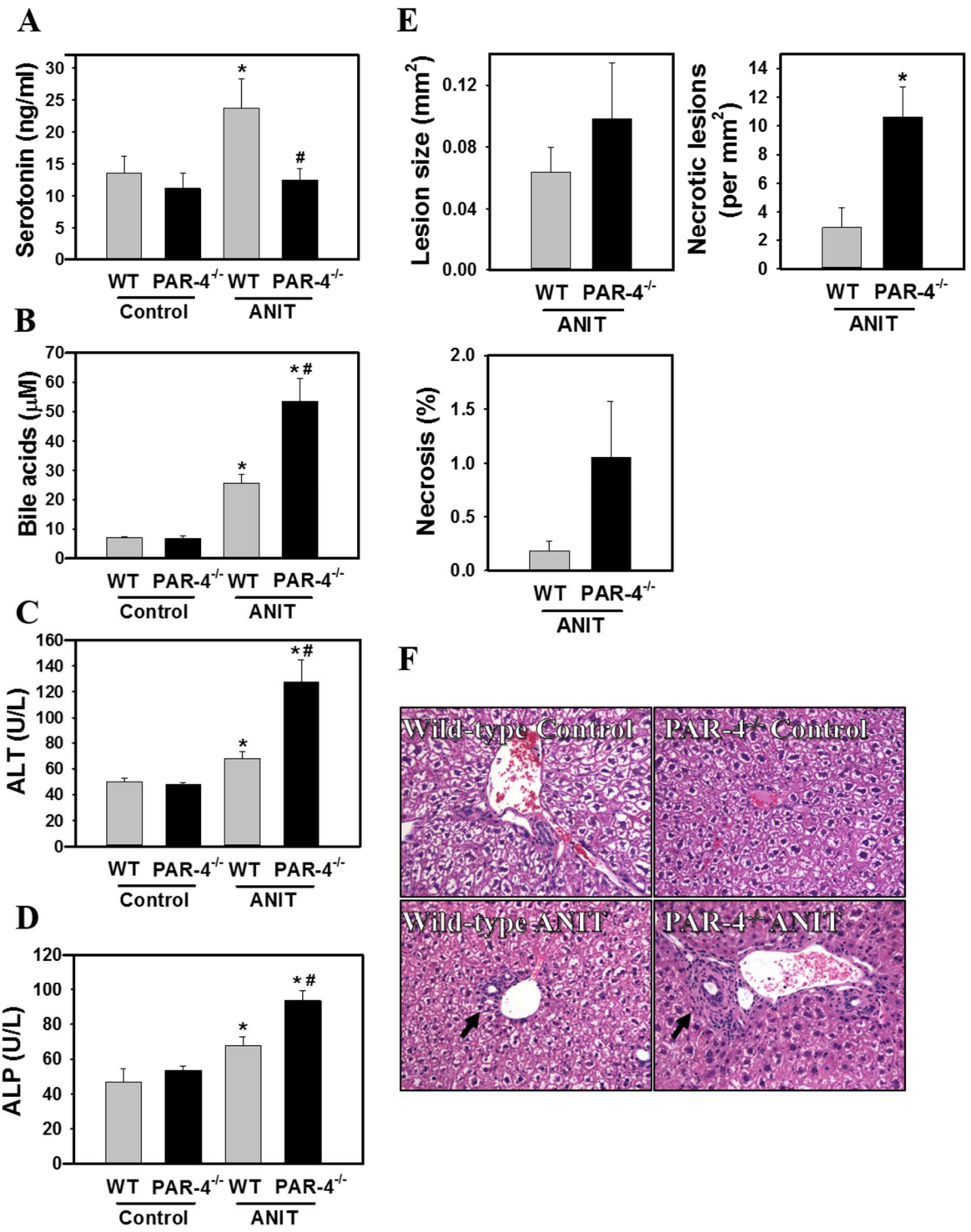

Figure 10. Effect of PAR-4 deficiency on serotonin levels and liver injury in ANIT-treated mice. Wild-type (WT) and PAR-4$^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Plasma serotonin, (B) serum bile acids, (C) serum ALT activity and (D) serum ALP activity were determined as described in Materials and Methods. (E) Necrotic

Figure 10. (cont'd). lesion size, number and area were determined as described in Materials and Methods. (F) Representative photomicrographs showing hematoxylin and eosin–stained liver sections (200X). Arrow indicates area of biliary fibrosis and portal inflammation. Data are expressed as mean ± SEM; n = 5 mice per group for control diet and 10-11 mice per group for ANIT-treated mice. *$p<0.05$ vs. control diet within genotype and #$p<0.05$ vs. WT mice fed the same diet.

with previous studies [26, 29, 30], we identified portal inflammation in ANIT-treated mice as predominantly a lymphocytic infiltrate. Indeed, accumulation of $CD3^+$ lymphocytes increased in ANIT-treated wild-type mice, and this was exacerbated in ANIT-treated PAR-$4^{-/-}$ mice (Fig. 19A-B). Consistent with enhanced inflammation, plasma IL-6 levels were significantly increased in ANIT-treated PAR-$4^{-/-}$ mice, although other cytokines examined were unaffected (Fig. 19C).

Increased liver fibrosis in ANIT-treated PAR-$4^{-/-}$ mice. Because our analysis of liver histopathology suggested more peribiliary fibrosis in PAR-$4^{-/-}$ mice, we examined the expression of profibrogenic changes and collagen deposits in ANIT-treated mice. Hepatic expression of profibrogenic COL1A1, TGFβ2, ITGB6, and TIMP-1 mRNAs was increased significantly in livers of ANIT-treated wild-type mice compared to control diet fed animals (Fig. 11A). Induction of each mRNA increased further in ANIT-treated PAR-$4^{-/-}$ mice, although increases in TGFβ1 and TGFβ2 mRNA did not achieve statistical significance (Fig. 11A). Peribiliary expression of α-SMA, a marker of activated hepatic stellate cells and portal fibroblasts, increased in livers of ANIT-treated wild-type mice (Fig. 11B), and this increase was larger in livers of ANIT-treated PAR-$4^{-/-}$ mice (Fig. 11B-C). Peribiliary collagen deposition, as indicated by sirius red staining (Fig 12A-B) and type 1 collagen immunofluorescence (Fig. 12C-D), increased in livers of ANIT-treated wild-type mice compared to control diet fed wild-type mice. In agreement with liver histology and profibrogenic gene expression, this was significantly increased in livers of ANIT-treated PAR-$4^{-/-}$ mice (Fig. 12B and 4D).

Increased hepatocellular necrosis and hepatic inflammation in ANIT-treated Fibγ$^{\Delta 5}$ mice. Fibγ$^{\Delta 5}$ mice express normal levels of a mutant fibrin(ogen) that does not bind to the platelet integrin

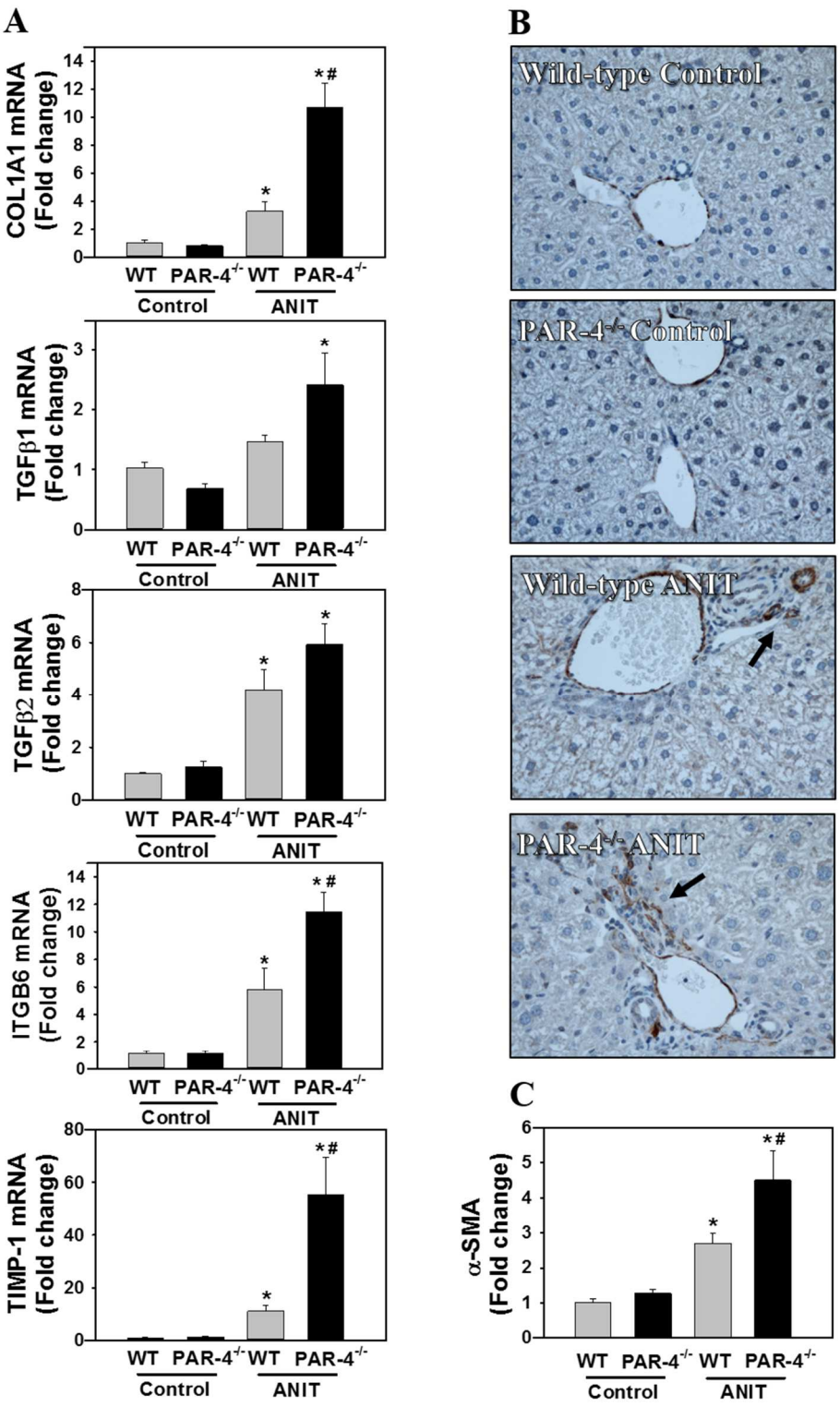

Figure 11. Increased profibrogenic gene expression in livers of ANIT-treated PAR-4$^{-/-}$ mice. Wild-type (WT) and PAR-4$^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Hepatic expression of mRNAs encoding COL1A1, TGFβ1, TGFβ2, ITGβ6 and TIMP-1 was determined by real-time qPCR.

Figure 11. (cont'd). (B) Representative photomicrographs (200X) show liver sections stained for α-smooth muscle actin (α-SMA) (brown). Arrow indicates peribiliary α-SMA staining. (C) α-SMA was quantified as described in Materials and Methods and expressed as fold change. Data are expressed as mean ± SEM; n = 5 mice per group for control diet and 10-11 mice per group for mice fed ANIT diet. *$p<0.05$ vs. control diet within genotype and #$p<0.05$ vs. wild-type mice fed the same diet.

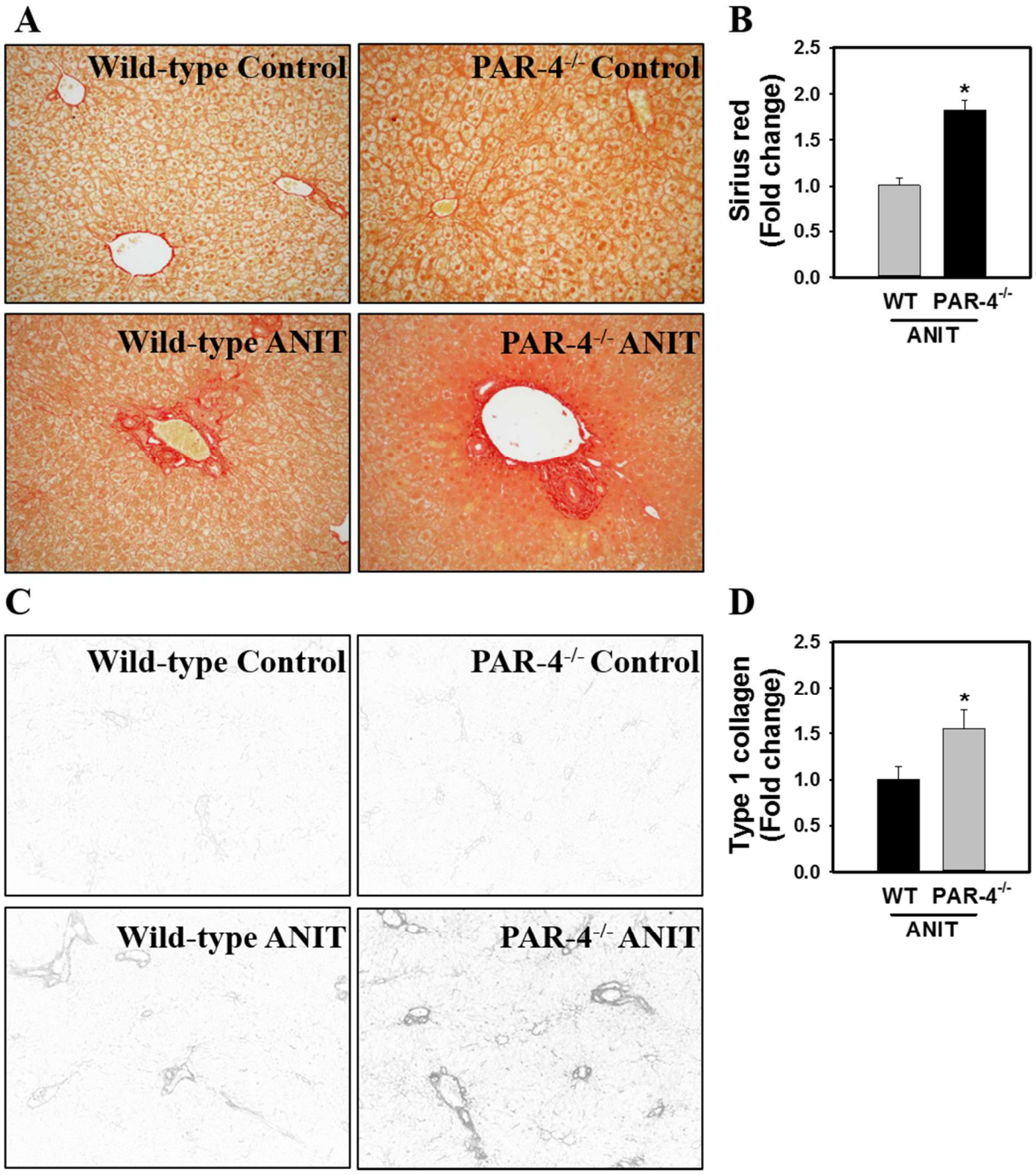

Figure 12. Increased collagen deposition in livers of ANIT-treated PAR-4$^{-/-}$ mice. Wild-type (WT) and PAR-4$^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Representative photomicrographs showing liver sections stained with (A) sirius red staining (200X) and (C) immunofluorescent type 1 collagen staining (100X), converted to grayscale and inverted such that type 1 collagen staining is dark. (B) Sirius red staining and (D) Type 1 collagen staining was quantified as described in Materials and Methods. Data are expressed as mean ± SEM; n = 5 mice per group for control diet and 10-11 mice per group for mice fed ANIT diet. *$p<0.05$ vs. ANIT-treated WT mice.

$\alpha_{IIb}\beta_3$ [15]. In contrast to PAR-4 deficiency, plasma serotonin levels were similar in ANIT-treated wild-type and Fib$\gamma^{\Delta 5}$ mice (37 ± 4 ng/ml vs. 41 ± 8 ng/ml, respectively, n=6). However, serum ALT activity and bile acids were significantly higher in ANIT-treated Fib$\gamma^{\Delta 5}$mice compared to ANIT-treated wild-type mice (Fig. 13A-B). Serum ALP activity increased in ANIT-treated mice of both genotypes (Fig. 13C). The overall histological appearance of control diet fed wild-type and Fib$\gamma^{\Delta 5}$ mice was similar (Fig. 13D). Extensive multifocal acute hepatocellular coagulative necrosis was present in ANIT-treated Fib$\gamma^{\Delta 5}$ mice, and this was minimal in ANIT-treated wild-type mice (Fig. 13D-E). Although necrotic foci were of similar size in wild-type mice and Fib$\gamma^{\Delta 5}$ mice, the number of necrotic foci and thus, total area of necrosis, were significantly increased in ANIT-treated Fib$\gamma^{\Delta 5}$mice (Fig. 13F). Potentially owing to expression of tissue factor by injured hepatocytes [31], plasma TAT levels were significantly elevated in ANIT-treated Fib$\gamma^{\Delta 5}$ mice (5.8 ± 0.9 ng/ml, n=6) compared to ANIT-treated wild-type mice (3.5 ± 0.25 ng/ml, n=6). Biliary hyperplasia was also exacerbated in ANIT-treated Fib$\gamma^{\Delta 5}$ mice, as indicated by a significant increase in CK19 staining (Fig. 18C-D).

Qualitative assessment of liver histopathology indicated markedly increased portal inflammation in livers of ANIT-treated Fib$\gamma^{\Delta 5}$mice compared to ANIT-treated wild-type mice (Fig. 13D-E). In agreement with this observation, CD3+ lymphocyte accumulation was exacerbated in ANIT-treated Fib$\gamma^{\Delta 5}$ mice compared to ANIT-treated wild-type mice (Fig. 14A-B). Neutrophils were also commonly associated with necrotic lesions in ANIT-treated Fib$\gamma^{\Delta 5}$ mice (Fig. 20). In agreement with increased cellular inflammation, plasma levels of IL-6, TNFα, IL-4 and KC/Gro were significantly increased in ANIT-treated Fib$\gamma^{\Delta 5}$ mice (Fig. 14C).

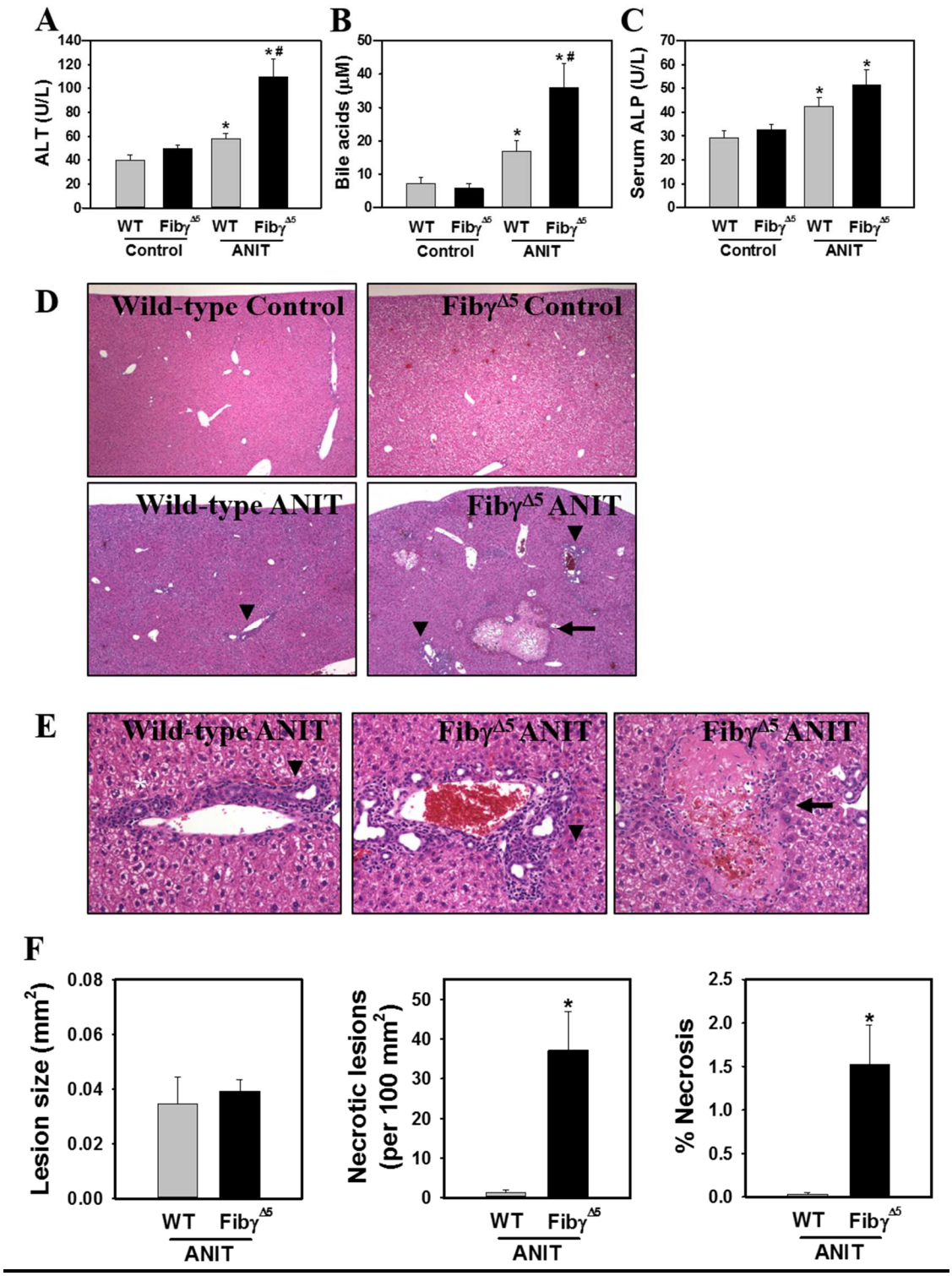

Figure 13. Increased hepatocellular necrosis in ANIT-treated Fibγ$^{\Delta 5}$ mice. Wild-type (WT) and Fibγ$^{\Delta 5}$mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Serum ALT activity, (B) serum bile acid concentration, and (C) serum ALP activity were determined as described in Materials and Methods.

Figure 13. (cont'd). Representative photomicrographs show hematoxylin and eosin–stained liver sections at (D) low magnification (40X) and (E) high magnification (200X). Arrows indicate area of hepatocellular coagulative necrosis. Arrowheads indicate area of biliary fibrosis and portal inflammation. (F) Necrotic lesion size, number and area were determined as described in Materials and Methods. Data are expressed as mean ± SEM; n = 4 mice per group for control diet and 9-10 mice per group for mice fed ANIT diet. *$p<0.05$ vs. control diet within genotype and #$p<0.05$ vs. WT mice fed the same diet.

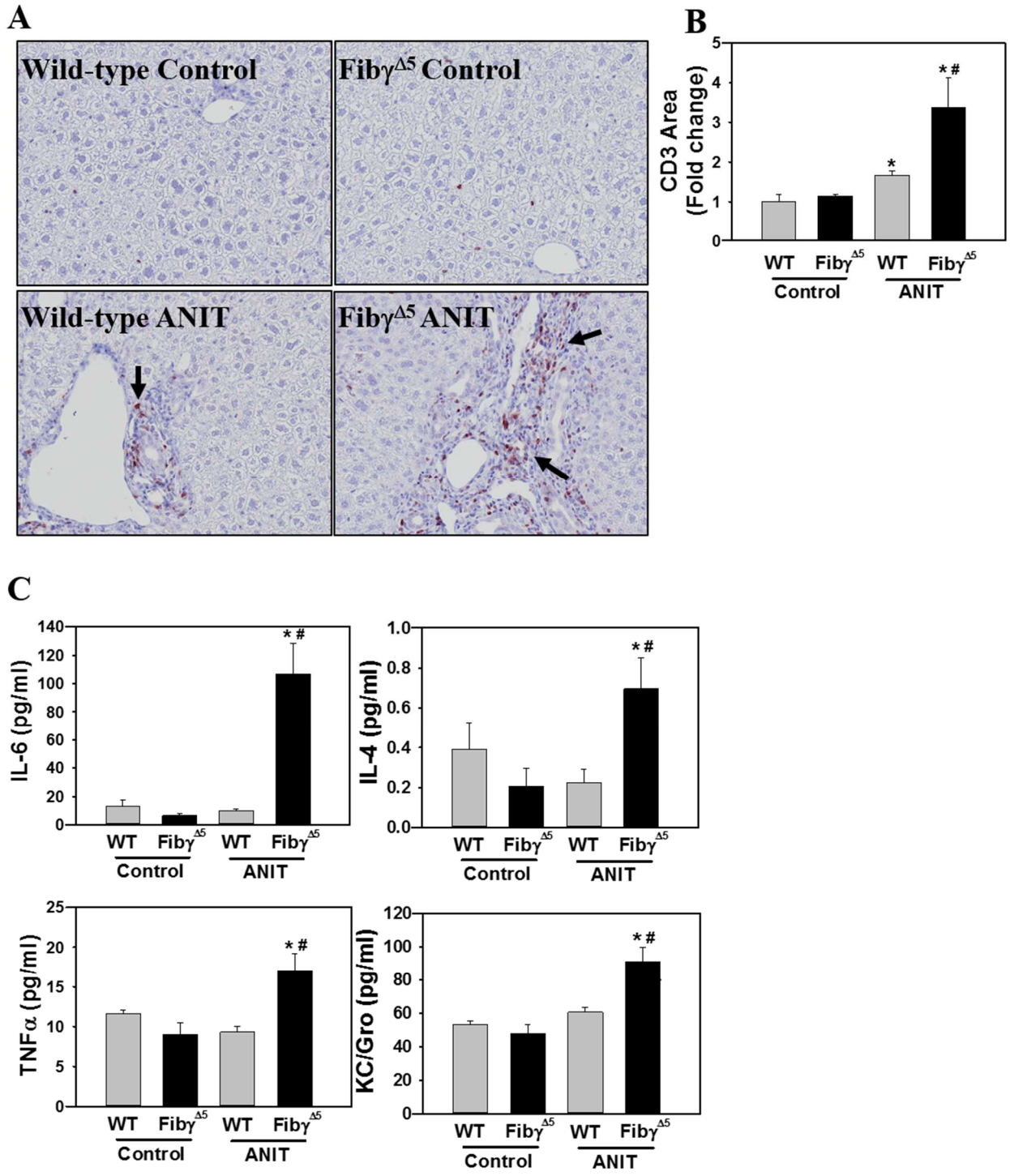

Figure 14. Increased hepatic inflammation in ANIT-treated Fibγ$^{\Delta5}$ mice. Wild-type (WT) and Fibγ$^{\Delta5}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Representative photomicrographs (200X) and (B) quantification of CD3 staining. (C) Serum levels of cytokines IL-6, IL-4, KC/Gro, and TNFα were determined as described in

Figure 14. (cont'd). Materials and Methods. Data are expressed as mean ± SEM; n = 4 mice per group for control diet and 9-10 mice per group for mice fed ANIT diet. *p<0.05 vs. control diet within genotype and #p<0.05 vs. WT mice fed the same diet.

Increased liver fibrosis in ANIT-treated Fibγ$^{\Delta 5}$ mice. Compared to wild-type mice fed control diet, hepatic expression of profibrogenic COL1A1, TGFβ2, ITGβ6, and TIMP-1 mRNAs was increased in livers of ANIT-treated wild-type mice for 4 weeks, and induction of each gene was significantly enhanced in ANIT-treated Fibγ$^{\Delta 5}$mice (Fig. 15A). Moreover, TGFβ1 mRNA levels increased in livers of ANIT-treated Fibγ$^{\Delta 5}$mice (Fig. 15A). Compared to ANIT-treated wild-type mice, α-SMA staining was dramatically increased near bile ducts in livers of ANIT-treated Fibγ$^{\Delta 5}$ mice (Fig. 15B-C). Extensive α-SMA staining was evident within and at the periphery of necrotic lesions in ANIT-treated Fibγ$^{\Delta 5}$ mice (Fig. 15B). In agreement with these indicators of a profibrogenic response, hepatic collagen deposition was dramatically increased in livers of ANIT-treated Fibγ$^{\Delta 5}$ mice compared to ANIT-treated wild-type mice, as indicated by sirius red staining (Fig. 16A-B) and type 1 collagen immunofluorescence (Fig. 16C-D).

Discussion

The literature is somewhat perplexing as to the role of platelets in liver disease, and it is challenging to conclude that platelets have a unified role in all forms of liver injury and fibrosis. Differences in the experimental basis of liver damage, subtle changes in the timing, duration and/or extent of thrombocytopenia, and the potency/efficacy of antiplatelet interventions (genetic or pharmacologic) may each impact interpretation of the role of platelets. For example, whereas platelet-derived serotonin is reported to promote liver repair/regeneration and inhibit liver fibrosis in some systems [22, 28, 32, 33], its role is reversed in settings of viral hepatitis and non-alcoholic steatohepatitis [34, 35]. Platelets contribute to the acute phase of cholestatic liver injury induced by BDL or a single, large dose of ANIT [19-21]. Likewise, short term (48 hour) antibody-mediated thrombocytopenia reduced hepatic α-SMA levels in 8-week-old Mdr2$^{-/-}$ mice [18]. In contrast,

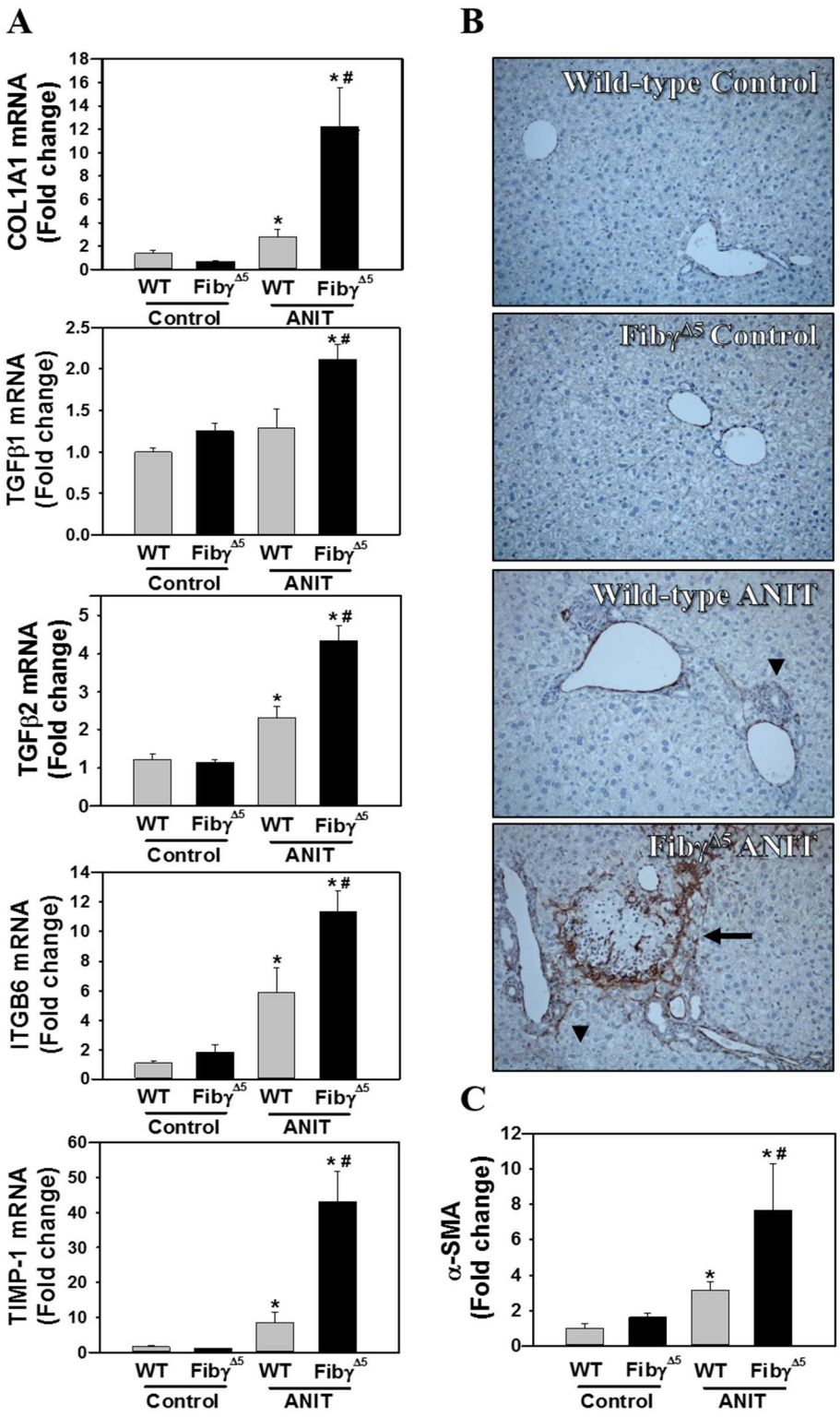

Figure 15. Increased profibrogenic gene induction in livers of ANIT-treated Fibγ$^{\Delta 5}$mice. Wild-type (WT) and Fibγ$^{\Delta 5}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks.

Figure 15. (cont'd). (A) Hepatic expression of mRNAs encoding the profibrogenic genes COL1A1, TGFβ1, TGFβ2, ITGβ6 and TIMP-1 was determined by real-time qPCR. (B) Representative photomicrographs (200X) show liver sections stained for α-smooth muscle actin (α-SMA) (brown). Arrow heads indicates area of periportal α-SMA staining. Arrow indicates area of α-SMA staining within an area of hepatocellular coagulative necrosis. (C) α-SMA was quantified as described in Materials and Methods and expressed as fold-change. Data are expressed as mean ± SEM; n = 4 mice per group for control diet and 9-10 mice per group for mice fed ANIT diet. *$p<0.05$ vs. control diet within genotype and #$p<0.05$ vs. WT mice fed the same diet.

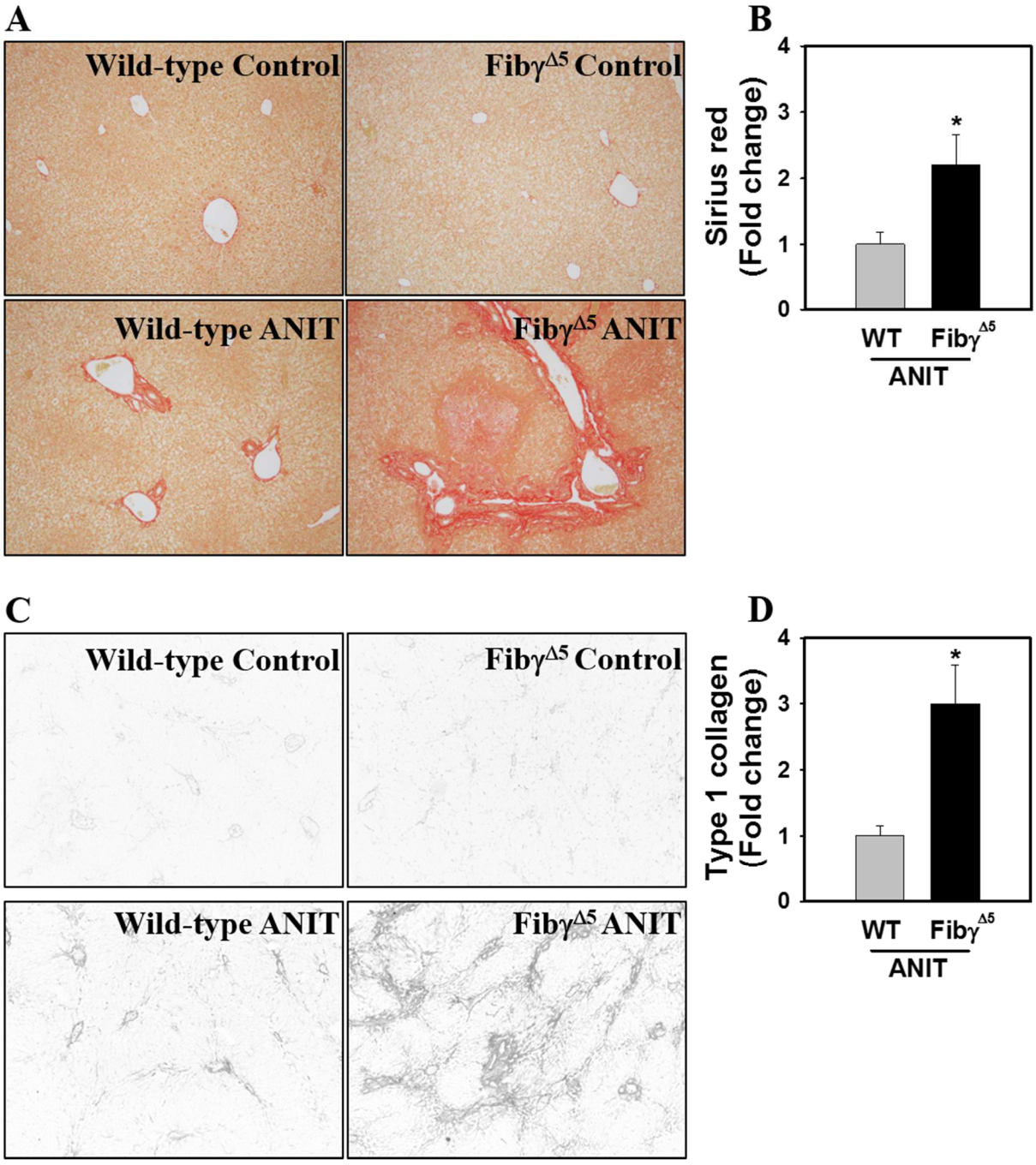

Figure 16. Increased collagen deposition in livers of ANIT-treated Fibγ^{Δ5} mice. Wild-type (WT) and Fibγ^{Δ5}mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Representative photomicrographs showing liver sections stained for (A) Sirius red (200X) and (B) immunofluorescent type 1 collagen (100X). Fluorescent images were converted to grayscale and inverted such that type 1 collagen staining is dark. (C) Sirius red and (D) Type 1 collagen stains were quantified as described in Materials and Methods. Data are expressed as mean ± SEM; n = 4 mice per group for control diet and 9-10 mice per group for mice fed ANIT diet. *p<0.05 vs. ANIT-treated WT mice.

prolonged thrombocytopenia exacerbates long-term BDL-induced liver fibrosis, as does a lack of peripheral serotonin [17, 22]. Notably, a similar dichotomy exists for the role of fibrin(ogen). For example, where fibrin(ogen) is deleterious in acute ANIT hepatotoxicity [36], our findings here in Fib$\gamma^{\Delta 5}$ mice and previously in fibrin(ogen)-null mice [26] suggest that protective properties of fibrin(ogen) emerge as the liver insult becomes chronic. Collectively, these studies highlight the diverse functions of platelets in liver disease, and emphasize a need to obtain a deeper understanding of the context-dependent role of platelets in liver disease.

PAR-1 and $\alpha_{IIB}\beta_3$ antagonists represent targets for antiplatelet therapy in humans [37, 38] and similar effects on platelet activation are observed in PAR-4-deficient mice [12], in $\alpha_{IIB}\beta_3$-deficient mice [39, 40], and in mice expressing $\gamma^{\Delta 5}$ fibrin(ogen) [15], which does not support platelet aggregation. Here, we found that PAR-4 deficiency and disruption of fibrin(ogen) engagement of $\alpha_{IIB}\beta_3$ increased fibrosis caused by chronic biliary injury. In contrast, a recent study found that aspirin significantly reduced fibrosis in Mdr2$^{-/-}$ mice, another model of peribiliary fibrosis [18]. By way of comparison, aspirin prolonged bleeding time by 1.4-fold [18], whereas bleeding times are prolonged by >5-fold in PAR-4$^{-/-}$ mice and Fib$\gamma^{\Delta 5}$ mice [12, 15]. This suggests that severe defects in platelet function (e.g., lack of thrombin signaling or fibrin(ogen) engagement) can result in increased liver fibrosis, analogous to long-term thrombocytopenia. In contrast, less potent inhibition of platelet activation (e.g., aspirin) may inhibit liver fibrosis, yet preserve protective platelet functions.

Several studies have implicated platelet-derived serotonin as a mediator capable of suppressing cholestatic liver injury and biliary fibrosis [22, 33, 41]. However, the mechanism whereby platelets are stimulated to release serotonin during cholestasis has not been fully characterized. We found that PAR-4 deficiency significantly reduced thrombin-mediated serotonin

release in isolated mouse platelets, and plasma serotonin did not increase in ANIT-treated PAR-4$^{-/-}$ mice. This strongly suggests that thrombin-mediated platelet activation is central to serotonin release in cholestasis, although additional studies will be required to elucidate whether changes in serotonin contribute to increased fibrosis in PAR-4$^{-/-}$ mice. Our results are at least consistent with those in the BDL model, where defective platelet serotonin release is associated with alterations in the bile acid pool, a proposed mechanism whereby liver fibrosis is exacerbated [22].

Disruption of thrombin signaling and fibrin-$\alpha_{IIB}\beta_3$ integrin engagement, in PAR-4$^{-/-}$ mice and Fib$\gamma^{\Delta 5}$mice, respectively, increased hepatocyte injury in mice fed ANIT diet, as indicated by serum ALT activity and liver necrosis. However, the severity of hepatocellular necrosis was more dramatic in ANIT-treated Fib$\gamma^{\Delta 5}$mice, and profibrogenic changes such as fibroblast activation and collagen deposition within necrotic areas suggest incomplete repair of necrosis. Whereas changes in plasma serotonin may account for liver pathology in PAR-4$^{-/-}$ mice, plasma serotonin levels in ANIT-treated mice were unaffected by fibrin(ogen) mutation. This suggests that the $\alpha_{IIb}\beta_3$ integrin-fibrin interaction does not augment platelet serotonin release in this context. In other settings, fibrin(ogen) engagement of integrin $\alpha_{IIB}\beta_3$ can facilitate wound repair by promoting platelet aggregation and clot retraction [42, 43]. It is conceivable that in the context of chronic cholestatic liver injury, $\gamma^{\Delta 5}$ fibrin(ogen) fails to support appropriate platelet aggregation and localized release of repair mediators. Additional studies are required to determine whether defective liver repair causes increased liver necrosis in ANIT-treated Fib$\gamma^{\Delta 5}$ mice.

Strong experimental evidence in BDL, carbon tetrachloride and ANIT models indicates that the thrombin receptor PAR-1 contributes to fibrosis in multiple tissues, including the liver [2, 4, 7, 8]. Use of PAR-1$^{-/-}$ mice does not directly address the role of thrombin-mediated platelet activation in liver fibrosis, because platelets in PAR-1 null mice are fully responsive to thrombin

[10]. Likewise, PAR-1 activation of stellate cells and portal fibroblasts would be retained in PAR-4 null mice, despite a lack of thrombin signaling in platelets. The observation that PAR-4 deficiency increased liver fibrosis in a model where PAR-1 deficiency reduces fibrosis suggests dichotomous roles of thrombin in this experimental setting. It would be interesting to observe the combined effect of platelet and non-platelet PAR signaling on liver fibrosis. Possible approaches include use of PAR-1/PAR-4 double deficient mice, although the phenotype of these mice has not been described. Alternatively, PAR-$4^{-/-}$ mice administered a PAR-1 antagonist could closely resemble the anticipated effect of a PAR-1 antagonist in patients. Similarly, it would be interesting to evaluate the impact of PAR-4 deficiency in mice expressing $\gamma^{\Delta 5}$ fibrin(ogen), a scenario representing major defects in platelet activation and hemostatic function.

Recent clinical evidence suggests that low-molecular weight heparin delays hepatic decompensation in patients with advanced liver cirrhosis, a majority of which had viral hepatitis [44]. It will be exciting to see whether novel FDA-approved oral anticoagulants (e.g., rivaroxaban, apixaban, dabigatran) that limit thrombin proteolytic activity, are similarly applied as coagulation-directed therapies for liver pathologies. If liver disease (either developing or end-stage) does emerge as an indication for these drugs, it is of importance to determine how coagulation-mediated platelet activation (ie., through fibrin(ogen) or PARs) participates in other models of liver fibrosis. This is particularly important as elements of hemostasis gain traction as biomarkers and potential therapeutic targets in liver disease.

APPENDIX

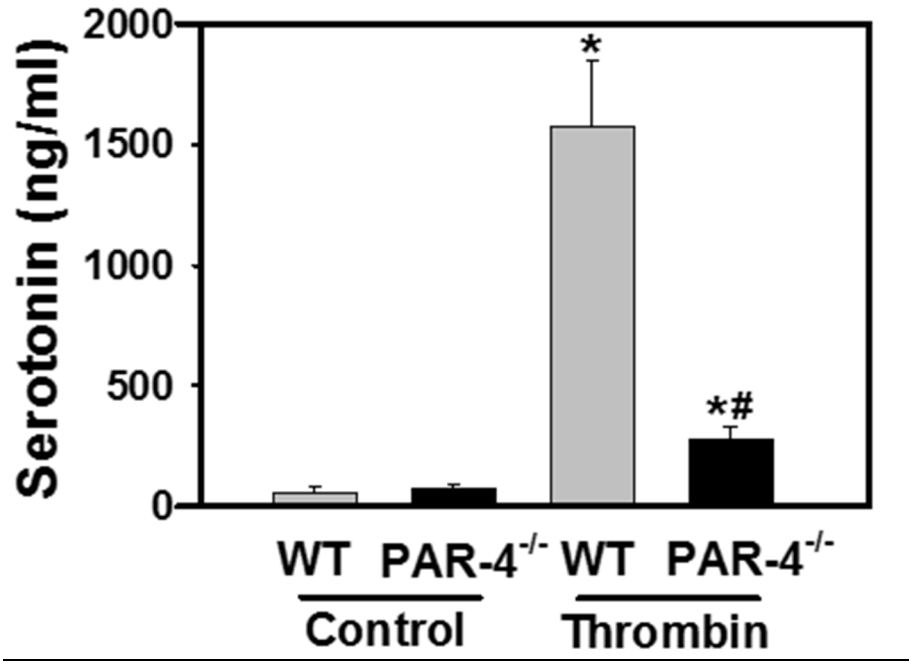

Figure 17. Thrombin-mediated serotonin release is PAR-4-dependent in mouse platelets. Approximately 1 x 10^8 platelets from wild-type (WT) mice and PAR-$4^{-/-}$ mice were stimulated with thrombin (10 U/ml) or vehicle (PBS) for 15 minutes. Serotonin levels were analyzed in supernatants by ELISA. Platelets from 3 independent mice were utilized for each treatment group. Data are expressed as mean ± SEM. *$p<0.05$ vs. control treatment within genotype and #$p<0.05$ vs. thrombin-treated WT platelets.

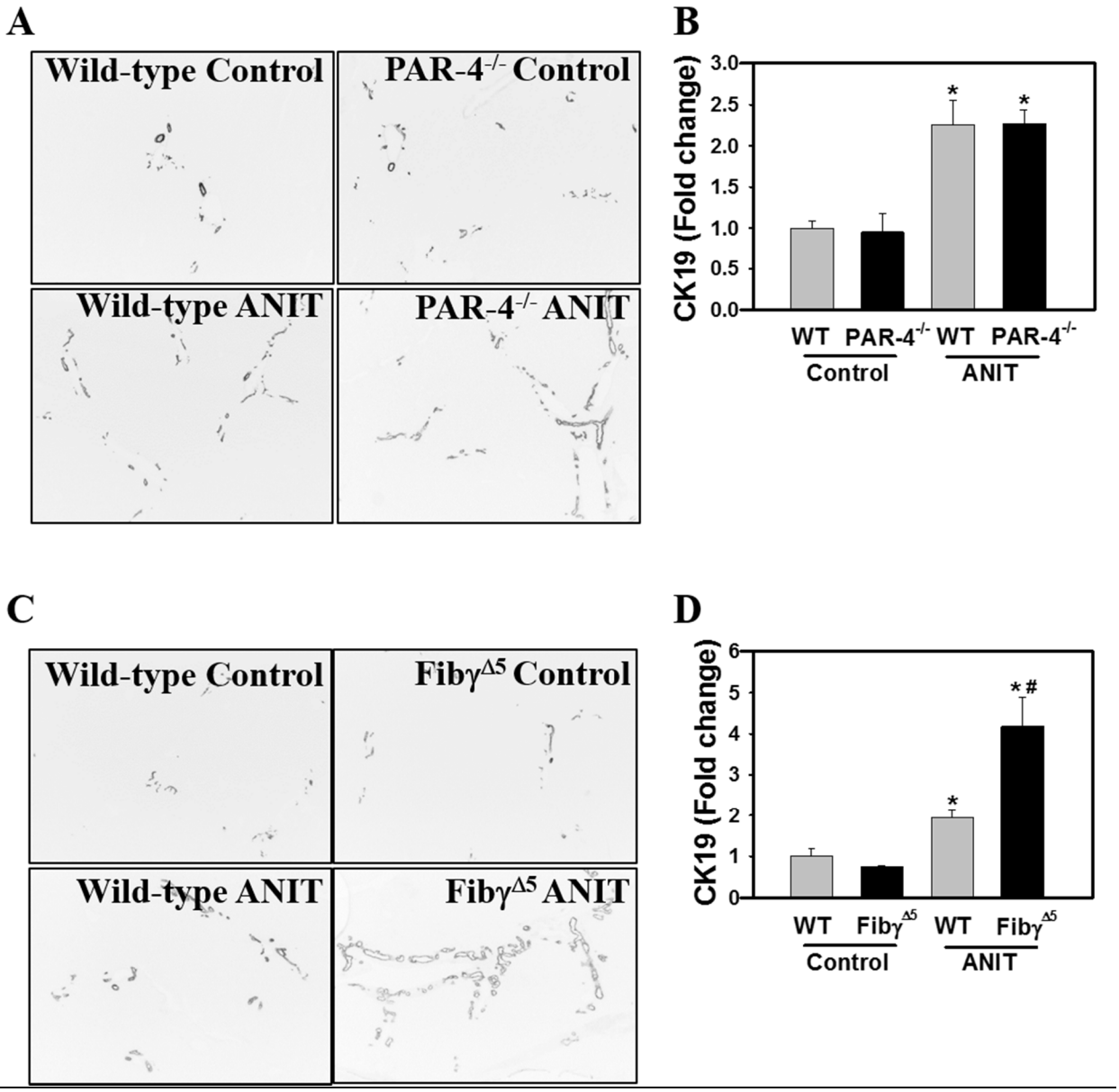

Figure 18. Biliary hyperplasia in ANIT-treated PAR-4$^{-/-}$ mice and Fib$\gamma^{\Delta 5}$ mice. Wild-type (WT), PAR-4$^{-/-}$ and Fib$\gamma^{\Delta 5}$mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A, C) Representative photomicrographs (100X) show liver sections stained for cytokeratin-19 (CK19, bile ducts). Images were converted to grayscale and inverted such that CK19 staining is dark. (B, D) The area of positive staining was quantified as described in Materials and Methods. Data are expressed as mean ± SEM; n = 4-5 mice per group for control diet and 9-11 mice per group for ANIT-treated mice. *p<0.05 vs. control diet within genotype and #p<0.05 vs. WT mice fed the same diet.

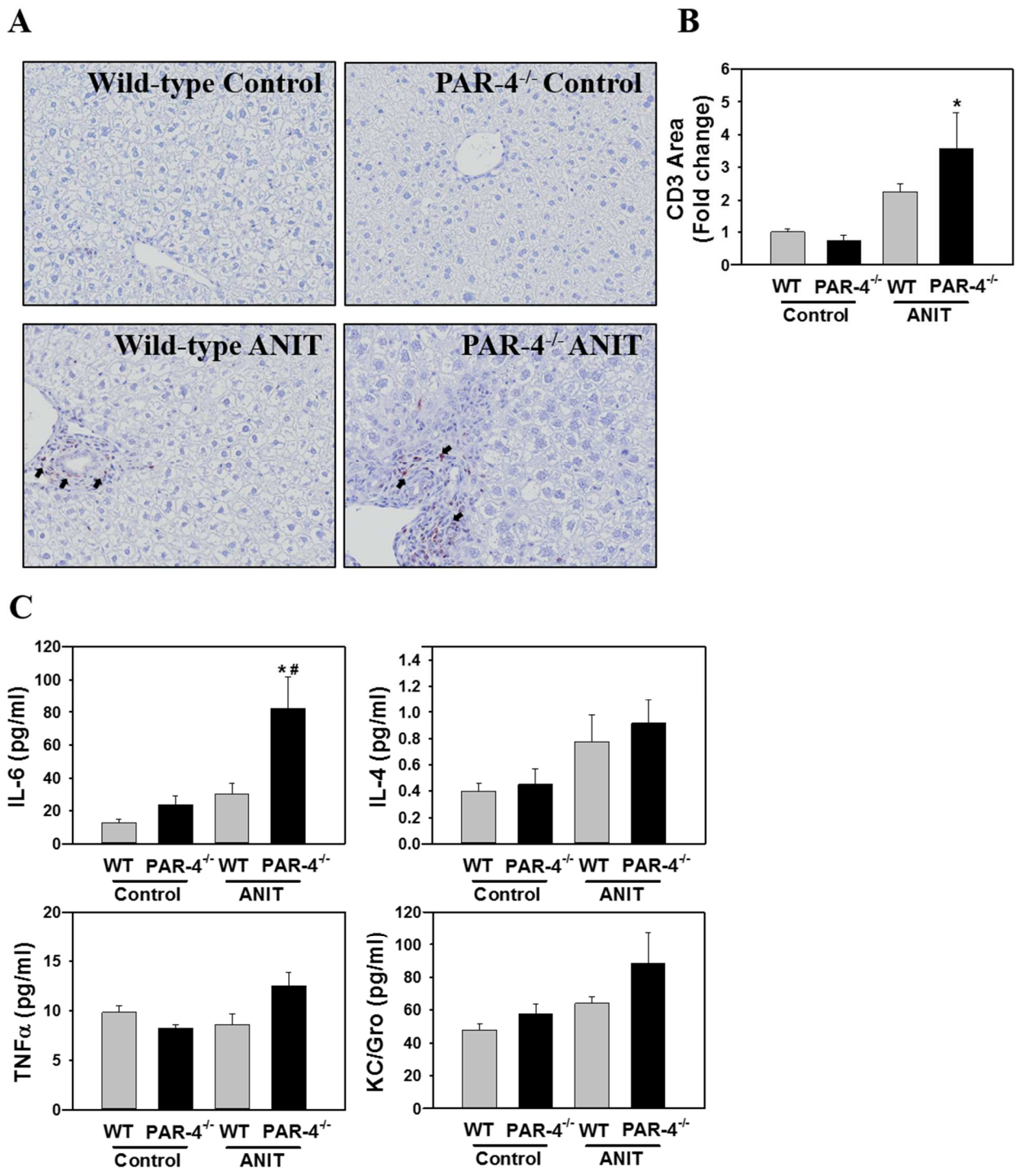

Figure 19. Hepatic inflammation in ANIT-treated PAR-4$^{-/-}$ mice. Wild-type (WT) and PAR-4$^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Representative photomicrographs (200X) and (B) quantification of CD3 staining. (C) Serum levels of cytokines IL-6, IL-4, TNFα, and KC/Gro were determined as described in Materials and

Figure 19. (cont'd). Methods. Arrows denote positive CD3 staining in A-B. Data are expressed as mean ± SEM; n = 5 mice per group for control diet and 10-11 mice per group for mice fed ANIT diet. *$p<0.05$ vs. control diet within genotype and #$p<0.05$ vs. WT mice fed the same diet.

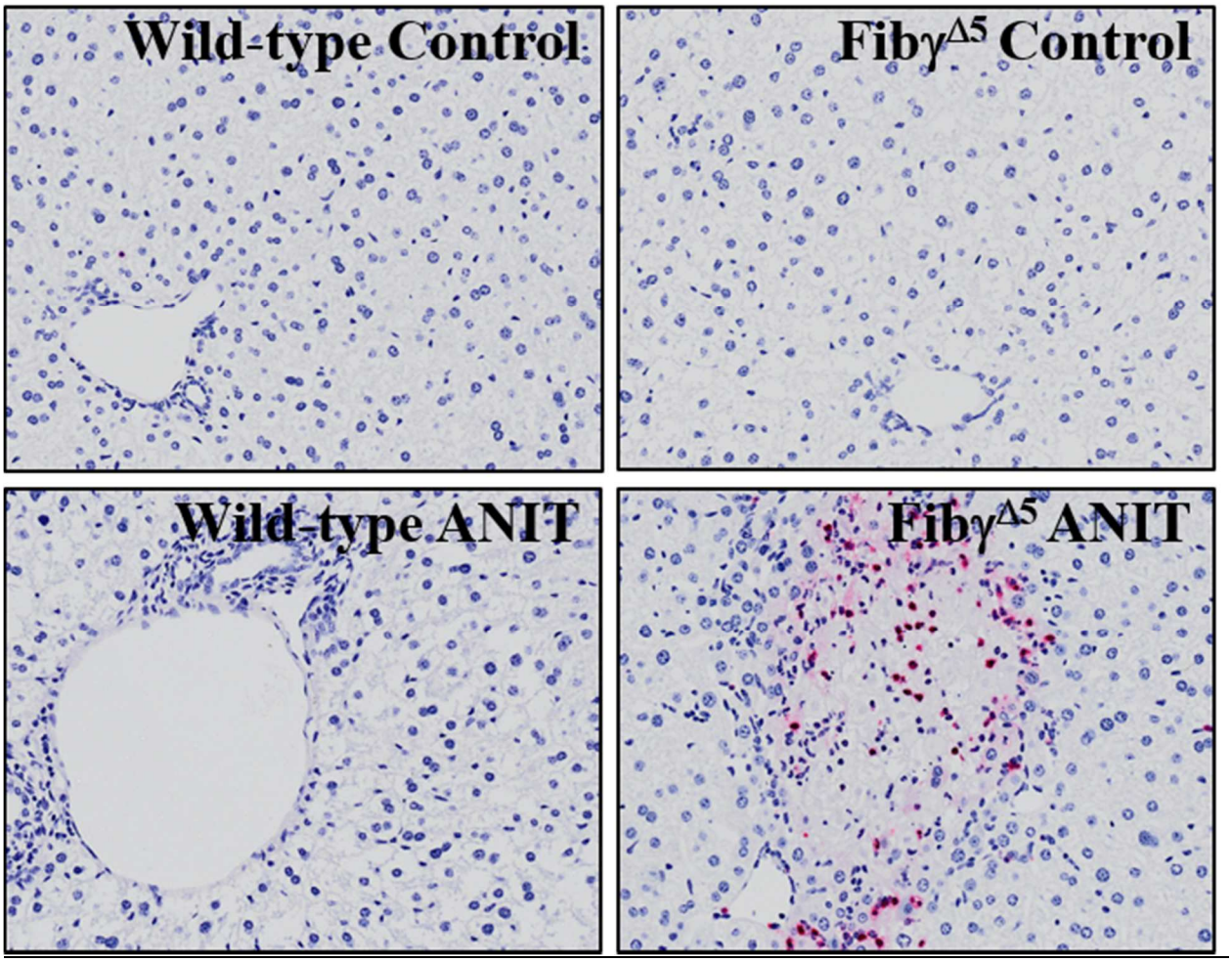

Figure 20.. Wild-type (WT) and Fibγ$^{\Delta 5}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Representative photomicrographs (200X) show neutrophil staining, particularly within areas of necrosis in livers of ANIT-treated Fibγ$^{\Delta 5}$ mice.

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CHAPTER 4

Fibrin Deposition Following Bile Duct Injury Limits Fibrosis Through An $\alpha_M\beta_2$-Dependent Mechanism

Nikita Joshi, Anna K. Kopec, Jessica L. Ray, Holly Cline-Fedewa, Atta Nawabi, Timothy Schmitt, Rance Nault, Timothy R. Zacharewski, Cheryl E. Rockwell, Matthew J. Flick and James P. Luyendyk. *Blood*. 2016, 127(22): 2751-2762.

Abstract

Coagulation cascade activation and fibrin deposits have been implicated or observed in diverse forms of liver damage. Given that fibrin amplifies pathologic inflammation in several diseases through the integrin receptor $\alpha_M\beta_2$, we tested the hypothesis that disruption of the fibrin(ogen)-$\alpha_M\beta_2$ interaction in Fib$\gamma^{390\text{-}396A}$ mice would reduce hepatic inflammation and fibrosis in an experimental setting of chemical liver injury. Contrary to our hypothesis, alpha-naphthylisothiocyanate (ANIT)-induced liver fibrosis increased in Fib$\gamma^{390\text{-}396A}$ mice, whereas inflammatory cytokine expression and hepatic necrosis were similar to ANIT-challenged wild-type mice. Increased fibrosis in Fib$\gamma^{390\text{-}396A}$ mice appeared to be independent of coagulation transglutaminase FXIII, as ANIT-challenge in FXIII-deficient mice resulted in a distinct pathological phenotype characterized by increased hepatic necrosis. Rather, bile duct proliferation underpinned the increased fibrosis in ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. The mechanism of fibrin-mediated fibrosis was linked to interferonγ (IFNγ) induction of inducible nitric oxide synthase (iNOS), a gene linked to bile duct hyperplasia and liver fibrosis. Expression of iNOS mRNA was significantly increased in livers of ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. Fibrin(ogen)-$\alpha_M\beta_2$ interaction inhibited iNOS induction in macrophages stimulated with IFNγ in vitro and ANIT-challenged IFN□-deficient mice had reduced iNOS induction, bile duct hyperplasia, and liver fibrosis. Further, ANIT-induced iNOS expression, liver fibrosis and bile duct hyperplasia were significantly reduced in wild-type mice administered leukadherin-1 (LA-1), a small molecule that allosterically enhances $\alpha_M\beta_2$-dependent cell adhesion to fibrin. These studies characterize a novel mechanism whereby the fibrin(ogen)-integrin $\alpha_M\beta_2$ interaction reduces biliary fibrosis and suggests a novel putative therapeutic target for this difficult-to-treat fibrotic disease.

Introduction

Increased coagulation activity and hepatic fibrin(ogen) deposition have been observed in models of cholestatic liver disease, wherein bile ducts are injured and often occlude [1-3]. Reflecting this experimental observation, increased deposition of thrombin-cleaved fibrin was evident clinically in livers from patients with cholestatic liver disease compared to non-diseased livers [4]. However, beyond limited clinical studies indicating sustained fibrin(ogen) expression and hypercoagulability in patients with liver fibrosis caused by cholestatic liver disease [5, 6], the exact role of fibrin(ogen) in the progression of this pathology has not been completely defined. Cholestatic liver disease is typically coupled to bile duct epithelial cell (BDEC) injury in the liver, as is the case with primary sclerosing cholangitis (PSC). Although the etiology of PSC is clearly associated with BDEC injury, the central trigger(s) and mechanism(s) driving the pathology are not fully understood. PSC is typified by proliferation of the bile ducts, strictures prohibiting bile flow, and excess collagen deposition around the bile ducts (i.e., peribiliary fibrosis), which if sustained, can lead to liver failure and predisposition to cancer [7-9]. Of importance, PSC accounts for approximately 8% of all liver transplants in the United States 10 and there is currently no established curative therapy for PSC [9].

The xenobiotic alpha-naphthylisothiocyanate (ANIT) is a chemical that selectively injures BDECs [10-13]. Long term exposure of rodents to ANIT recapitulates many clinical and histopathological features of sclerosing cholangitis [4, 7, 14, 15], including coagulation cascade activation, robust fibrin(ogen) deposition, bile duct hyperplasia and peribiliary fibrosis [2, 5, 6, 16]. Complete fibrin(ogen) deficiency in ANIT-exposed mice provoked an atypical increase in focal liver necrosis, a unique lesion that was recapitulated in mice expressing mutant fibrin(ogen) unable to engage platelet integrin $\alpha_{IIb}\beta_3$ [2, 4]. This suggests that a hemostatic function of fibrin(ogen)

inhibits necrosis in this setting; however, the presence of necrosis in these mutant mice prevented us from mechanistically isolating the specific role of fibrin(ogen)-driven inflammation in peribiliary fibrosis. Here, the longstanding, yet untested hypothesis in the field, is that fibrin matrices formed as a consequence of injury/disease-triggered coagulation, enhance inflammatory cell (i.e., leukocyte) activity leading to liver fibrosis [17]. This concept is anchored in the non-hemostatic functions of fibrin polymers, including engagement of the leukocyte integrin $\alpha_M\beta_2$, which amplifies inflammation and tissue injury in many other disease settings [18-22]. Notably, however, the precise function of the fibrin(ogen)-leukocyte integrin $\alpha_M\beta_2$ interaction has never been evaluated in experimental hepatic injury.

Anchored in previous studies indicating that fibrin(ogen)-driven inflammation exacerbates inflammatory disease, we tested the hypothesis that the fibrin(ogen)-integrin $\alpha_M\beta_2$ interaction promotes experimental liver inflammation and fibrosis. To test this hypothesis, we utilized a combination of in vivo and in vitro studies involving mice expressing a mutant form of fibrinogen lacking the binding motif for integrin $\alpha_M\beta_2$ (Fib$\gamma^{390\text{-}396A}$ mice), FXIII$^{-/-}$ mice, and leukadherin-1 (LA-1), a novel small molecule integrin agonist that allosterically increases $\alpha_M\beta_2$-dependent cell adhesion to fibrin polymers.

Materials and Methods

Mice: Fib$\gamma^{390\text{-}396A}$ mice [21], mice lacking the FXIII catalytic A subunit (FXIII$^{-/-}$ mice) [23] and wild-type mice on an identical C57BL/6 background were maintained by homozygous breeding. IFN$\gamma^{-/-}$ mice [24] and wild-type mice on an identical congenic C57Bl/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME). Age-matched male mice between the ages of 8-14 weeks were used for these studies. Wild-type C57Bl/6J mice utilized for bone marrow isolation

were purchased from The Jackson Laboratory. Mice were housed at an ambient temperature of approximately 22°C with alternating 12 hour light/12 hour dark cycles and provided water and rodent chow ad libitum prior to study initiation. Mice were maintained in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities and approved by the IACUC at Michigan State University or Cincinnati Children's Hospital Research Foundation.

ANIT and carbon tetrachloride (CCl4)-induced liver fibrosis and pharmacological interventions: Custom diets were prepared by Dyets, Inc. (Bethlehem, PA). The ANIT diet was an AIN-93M purified diet containing 0.025% ANIT (Sigma-Aldrich, St. Louis, MO). The control diet was the purified AIN-93M diet. Groups of mice were fed each diet for a total of 4 weeks or 8 weeks, *ad libitum*. For studies with leukadherin-1 (LA-1), mice were fed control diet or ANIT diet for 6 weeks and LA-1 (0.4 mg/kg/day, i.p., bid) (Tocris Bioscience, Bristol, UK) or its vehicle (1.5 μL DMSO in 100 μL sterile PBS) were administered only during weeks 5 and 6. This dosing paradigm was based on other published studies with LA-1 in mice [25] and was well tolerated by the mice. Mice fed ANIT diet are referred to as ANIT-exposed mice. For studies with carbon tetrachloride (CCl_4), CCl_4 (10% at 10 μL/g, i.p.; Sigma) or its vehicle (corn oil; Sigma) was administered to groups of wild-type or $Fib\gamma^{390\text{-}396A}$ mice twice a week for a total of 4 weeks or 8 weeks. Mice were anesthetized with isoflurane, and blood was collected from the caudal vena cava into sodium citrate (final, 0.38%) or an empty syringe for the collection of plasma and serum, respectively. The liver was removed and washed with saline. Sections of the left lateral lobe were fixed in 10% neutral buffered formalin for 48 hours prior to routine processing. The remaining liver was cut into approximately 100 mg sections and flash-frozen in liquid nitrogen.

Histopathology and clinical chemistry: For analysis of liver histopathology by light microscopy, formalin-fixed liver sections were cut at 5 microns and stained with hematoxylin and eosin (H&E), sirius red, fibrin(ogen) and cytokeratin-19 (CK-19) antigen by the Michigan State University Investigative Histopathology Laboratory. At least 2 sections of liver from the left lateral lobe of each animal were qualitatively and quantitatively evaluated in their entirety to calculate area of necrosis. Quantitative assessment of necrotic area in H&E-stained sections were performed in a masked fashion using ImageJ. For quantification of sirius red (collagen deposits) and CK-19 staining (BDECs), images of stained liver sections were captured using a Virtual Slide System VS110 (Olympus, Hicksville, NY) with a 20X objective. Random images were derived from the digitized slides approximating at least 100 mm2 tissue for each liver. The area of positive sirius red and CK-19 staining in each image was determined in an unbiased fashion using a batch macro and the color de-convolution tool in ImageJ. Immunofluorescent staining of type I collagen was performed as described [3, 4]. Prolong Gold (DAPI-containing) Antifade reagent (Life Technologies) was applied to the tissues prior to cover slipping. Fluorescent staining of liver sections was visualized using an Olympus DP70 microscope (Olympus, Lake Success, NY). Type I collagen staining was quantified using Scion Image (Scion Corporation, Frederick, MD) as described previously, [2] utilizing approximately 10 low-power images (100X) for each tissue. The percentage of pixels containing positive signal (i.e., collagen staining) was expressed as a fold change relative to wild-type mice fed control diet. Serum activities of ALT and alkaline phosphatase (ALP) were determined using commercial reagents (Thermo Scientific, Waltham, MA; Pointe Scientific, Canton, MI). Plasma fibrinogen levels were determined using commercial

reagents (Siemens Healthcare Diagnostics, Deerfield, IL) and a STart4 Coagulation Analyzer (Diagnostica-Stago, Parsippany, NJ).

Isolation and culture of bone marrow-derived macrophages: Bone marrow macrophages were derived as described previously [26] with slight modification. Briefly, under sterile conditions, the femurs and tibias from wild-type mice were removed and excess tissue excised. Marrow was flushed from bones using RPMI containing 10% FBS and 2% penicillin/streptomycin (BMDM medium) loaded into a 5 ml syringe fitted with a 25 gauge needle. Bone marrow aspirates were transferred into a 50 ml conical tube and collected by centrifugation at 500g for 10 minutes. Following red cell lysis, the cells were washed twice and collected by centrifugation as before. The cells were then cultured in BMDM media containing 10 ng/mL recombinant mouse macrophage colony stimulating factor (mCSF; Biolegend, San Diego, CA) at a density of 0.5 x 106 cells/mL in T75 flasks. After 4 days, cells were detached using a non-enzymatic buffer, adjusted to a density of 1 x 106 cells/mL in mCSF containing BMDM medium, and plated on 6-well culture plates (2 x 106 cells/well) coated with 10 μg/mL endotoxin-free bovine serum albumin (BSA), wild-type murine fibrinogen (Enzyme Research, South Bend IN, USA, 10 μg/mL) or 10μg/mL murine γ390-396A fibrinogen. The cells were then treated with either recombinant murine IFNγ (10 ng/mL; Biolegend, San Diego, CA), IL4 (10 ng/mL; Biolegend, San Diego, CA) or vehicle (PBS) for 24 hours prior to collection of RNA.

RNA isolation, cDNA synthesis, and real-time PCR: Total RNA was isolated from approximately 15 mg of snap-frozen liver or adherent macrophages using TRI Reagent (Molecular Research Center, Cincinnati, OH). 1 μg of total RNA was utilized for the synthesis of cDNA, accomplished

using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Hepatic levels of mRNAs were determined using iTAQ University SYBR Green (Bio-Rad), and a CFX Connect thermal cycler (Bio-Rad). Primers were purchased from IDT (Coralville, IA). The expression of each gene was normalized to the geometric mean Ct of two individual housekeeper genes, HPRT and 18S RNA, as described [27] , and the relative levels of each gene were evaluated using the ΔΔCt method. Primer sequences are listed in Table 1.

Analysis of liver nonparenchymal cells by flow cytometry: Hepatic nonparenchymal cells (NPCs) were isolated from mouse liver by collagenase perfusion and digestion as described previously [28]. Isolated NPCs from liver were washed and re-suspended in FACS buffer (PBS, 1% FCS). The cells were then labeled with antibodies targeting F4/80 and CD68 (Biolegend, San Diego, CA) conjugated to FITC. After labeling, the cells were washed and resuspended in FACS buffer. The fluorescence was then detected and quantified with a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using CFlow software (BD Accuri, San Jose, CA). The F4/80+ CD68+ population was identified within the live cell population, which was determined by forward and side scatter. Doublets were excluded by FSC-H vs FSC-A and SSC-H and SSC-A analysis.

Human liver samples: De-identified liver samples and associated clinical data from 16 human patients with primary biliary cirrhosis (PBC)- or PSC-associated cirrhosis and 27 human patients with hepatitis C (HCV)-associated cirrhosis were kindly provided by The University of Kansas

Medical Center Liver Center Tissue Bank. The project "Association of hemostatic factor expression with profibrogenic genes" is supported by the Kansas University Liver Center.

Statistical analyses: Comparison of two groups was performed using Student's t-test. Comparison of three or more groups was performed using one- or two-way analysis of variance (ANOVA), as appropriate, and Student-Newman-Keul's post hoc test. Associations between variables for human data were assessed using Pearson's r correlation. Comparisons of clinical parameters were made using the Mann-Whitney U Test. The criterion for statistical significance was $p \leq 0.05$.

Results

Fibrin(ogen)-$\alpha_M\beta_2$ binding suppresses hepatic fibrosis following chronic bile duct injury. Periportal (zone 1) fibrin(ogen) deposition increased in livers of ANIT-exposed wild-type mice (4 weeks), particularly surrounding the bile ducts and in neighboring sinusoids (Fig. 21A, arrowheads). No change in plasma fibrinogen was detectable after 4 weeks of ANIT exposure, reflecting minimal change in hepatic fibrinogen gene expression (Fig. 28A-C, G). Fib$\gamma^{390\text{-}396A}$ mice express a mutant fibrinogen that cannot bind leukocyte integrin $\alpha_M\beta_2$ [21].

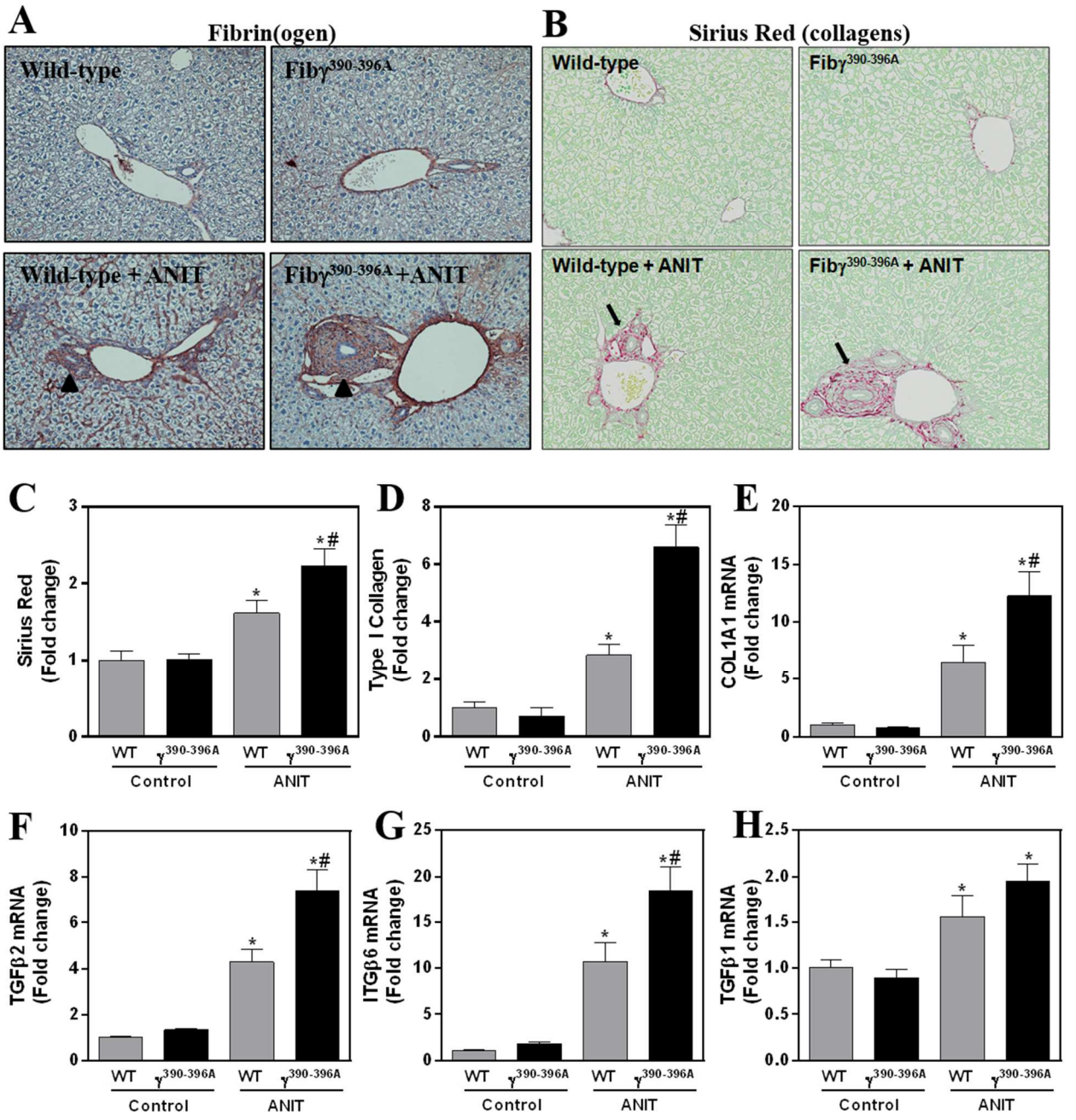


Figure 21. Increased fibrosis in livers of ANIT-exposed Fibγ$^{390\text{-}396A}$ mice. Wild-type (WT) and Fibγ$^{390\text{-}396A}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Representative photomicrographs show liver sections stained for fibrin(ogen) (brown) (200X). Arrowheads indicate peribiliary fibrin(ogen) deposits (B) Representative photomicrographs show liver sections stained for collagens with sirius red (200X). Arrows indicate peribiliary collagen deposition. (C) Sirius red and (D) type I collagen stains were quantified as described in Materials and Methods.

Figure 21. (cont'd). Hepatic expression of mRNAs encoding the profibrogenic genes (E) COL1A1, (F) TGFβ2, (G) ITGβ6 and (H) TGFβ1 was determined by real-time qPCR. Data are expressed as mean + SEM; n = 4 mice per group for control diet and 10-16 mice per group for mice fed ANIT diet. *$p<0.05$ vs. control diet within genotype and #$p<0.05$ vs. WT mice fed the same diet.

Documented evidence indicates normal hemostatic function in Fibγ$^{390-396A}$ mice, including fibrin polymer formation, PT/PTT and thrombin times, and tail-bleeding analyses identical to wild-type mice [21]. In agreement, the localization and intensity of peribiliary fibrin(ogen) staining was similar in ANIT-exposed wild-type and Fibγ$^{390-396A}$ mice, with an apparent increase in fibrin(ogen) deposition in Fibγ$^{390-396A}$mice particularly evident near areas of bile duct proliferation (Fig. 21A, arrowheads). Compared to wild-type mice, peribiliary fibrosis after 4 weeks of ANIT exposure was significantly increased in livers of Fibγ$^{390-396A}$ mice, marked by excessive total and type I collagen deposition in ANIT-exposed Fibγ$^{390-396A}$ mice (Fig. 21B-D). This increase in fibrosis was coupled to an exaggerated induction of the profibrogenic genes COL1A1, TGFβ2 and ITGβ6 in ANIT-exposed Fibγ$^{390-396A}$ mice (Fig. 21E-G). In contrast, ANIT induction of TGFβ1 was not affected by genotype (Fig. 21H). Interestingly, the exaggerated liver fibrosis in Fibγ$^{390-396A}$ mice occurred without a corresponding increase in serum enzyme biomarkers of liver injury (i.e., ALT, ALP; Fig. 29A-B). Moreover, induction of several proinflammatory mediators linked to fibrosis was unaffected by genotype in ANIT-exposed mice (Fig. 29C-H). Peribiliary fibrosis (Fig. 30, arrows) was also increased in ANIT-exposed Fibγ$^{390-396A}$ mice after 8 weeks of ANIT exposure (Fig. 30A-E), and this similarly was observed without an increase in serum ALT activity (Fig. 30F). Collectively, the results indicate that experimental bile duct fibrosis is increased in Fibγ$^{390-396A}$ mice compared to wild-type mice.

FXIII-deficient mice develop a pathologic phenotype distinct from Fibγ$^{390-396A}$ mice after ANIT exposure. Fibγ$^{390-396A}$ mice were shown to develop venous thrombi of reduced size due to RBC exclusion from venous clots [29]. This occurred secondary to a reduced rate of Factor XIII (FXIII)-mediated crosslinking [29]. To address the possibility that a reduced rate of FXIII-

mediated crosslinking could form the basis for exaggerated liver fibrosis in Fibγ$^{390-396A}$ mice, we compared hepatic fibrin(ogen) deposition in Fibγ$^{390-396A}$ mice with that of FXIII$^{-/-}$ mice exposed to ANIT, and determined the effect of FXIII deficiency on ANIT-induced liver fibrosis. Chronic exposure of mice to ANIT produces two distinct pathologies in the liver, bile duct hyperplasia and fibrosis (i.e., surrounding bile ducts in zone 1), and acute focal hepatocellular necrosis (i.e., often termed bile infarcts). Bile duct hyperplasia was evident in ANIT-exposed wild-type mice and this seemed to increase in Fibγ$^{390-396A}$ mice (Fig. 23A, arrows). In agreement with serum enzyme levels (Fig. 29A-B), focal hepatocellular necrosis was very rare in both ANIT-exposed wild-type mice and ANIT-exposed Fibγ$^{390-396A}$ mice (Fig. 22B). Fibrin(ogen) deposits observed in these infrequent necrotic lesions were similar in wild-type and Fibγ$^{390-396A}$ mice, appearing as a dense network spanning the lesion, which seemed to outline the border of previously intact hepatocytes (Fig. 22A, arrowheads).

In complete contrast to ANIT-exposed Fibγ$^{390-396A}$ mice, focal hepatocellular necrosis evident as bile infarcts (Fig. 22C, asterisks), significantly increased in ANIT-exposed FXIII$^{-/-}$ mice compared to ANIT-exposed wild-type mice (Fig. 22D). As compared to wild-type mice, fibrin(ogen) deposits within focal necrosis in ANIT-exposed FXIII$^{-/-}$ mice appeared less intense, and lacked a defined pattern within the lesion (Fig. 22C, arrowhead). The markedly elevated necrosis in FXIII$^{-/-}$ mice appeared to be secondary to significantly increased peribiliary fibrosis in ANIT-exposed FXIII$^{-/-}$ mice compared to ANIT-exposed wild-type mice (Fig. 22E-F). No obvious distinction in the peribiliary fibrin(ogen) deposits were observed in wild-type and FXIII$^{-/-}$ mice exposed to ANIT for 4 weeks (not shown). Thus, ANIT-exposed FXIII$^{-/-}$ mice developed

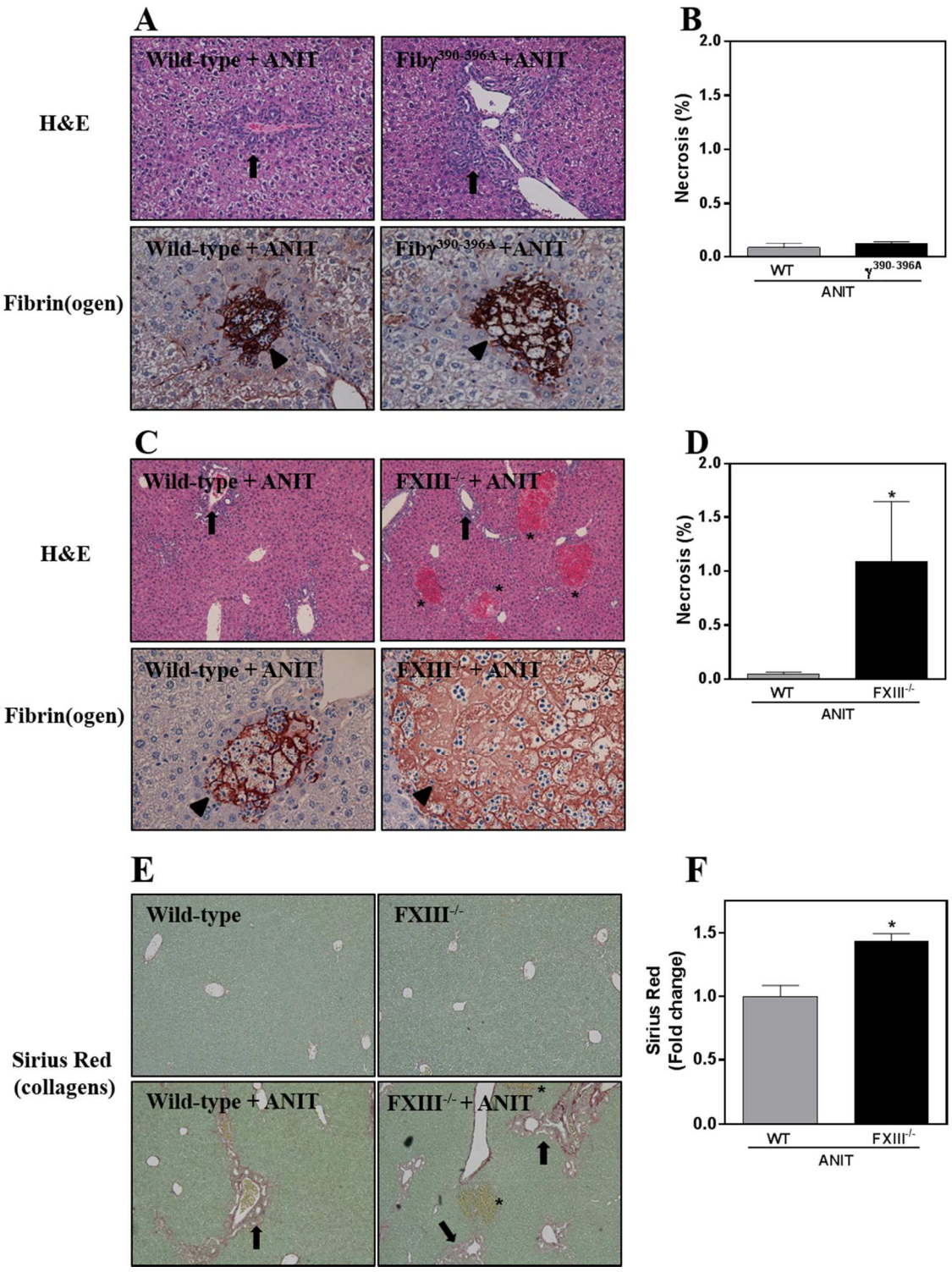


Figure 22. Hepatocellular necrosis and fibrin(ogen) deposition in livers of ANIT-exposed Fibγ$^{390-396A}$ and FXIII$^{-/-}$ mice. Wild-type (WT), Fibγ$^{390-396A}$ and FXIII$^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Representative photomicrographs show liver sections stained for hematoxylin and eosin (H&E) or fibrin(ogen)

Figure 22. (cont'd). (brown) from (A) Fib$\gamma^{390\text{-}396A}$ mice, (C) FXIII$^{-/-}$ mice, and corresponding wild-type mice exposed to ANIT (A and C). Arrows indicate areas of bile duct hyperplasia. Arrowheads indicate fibrin(ogen) deposits within areas of hepatocellular necrosis. Asterisks indicate focal hepatocellular necrosis or bile infarcts. Images are 200X except H&E-stained sections in C, which are 100X. (B, D) Area of necrosis was determined as described in Materials and Methods. (E) Representative photomicrographs show liver sections stained for sirius red (200X). Arrows indicate peribiliary collagen deposition. Asterisks indicate focal hepatocellular necrosis (i.e., bile infarcts). (F) Sirius red staining was quantified as described in Materials and Methods. Data are expressed as mean + SEM; n= 4-16 mice per group.*$p<0.05$ vs. ANIT-exposed WT mice.

increased focal hepatocellular necrosis with altered hepatic fibrin(ogen) deposition, a pathology distinct from ANIT-exposed Fib$\gamma^{390-396A}$ mice.

Fibrin(ogen)-$\alpha_M\beta_2$ binding suppresses ANIT-induced bile duct hyperplasia. BDECs express a number of profibrogenic genes that drive local periductal fibrosis by activating adjacent portal fibroblasts [30]. These include TGFB2 and ITGB6 [3, 30-33], genes expressed to a greater extent in ANIT-exposed Fib$\gamma^{390-396A}$ mice (Fig. 21F-G). Thus, we explored the possibility that increased fibrosis in ANIT-exposed Fib$\gamma^{390-396A}$ mice could be anchored to an expansion of intrahepatic BDECs. Histological analyses indicated that ANIT-exposed mice developed classic biliary hyperplasia, as indicated by cytokeratin-19 (CK-19) staining (Fig. 23A, arrows), one marker of biliary epithelium in mice. Notably, bile duct hyperplasia was significantly exacerbated in ANIT-exposed Fib$\gamma^{390-396A}$ mice compared to ANIT-exposed wild-type mice (Fig. 23A-B). Although biliary hyperplasia tended to increase in ANIT-exposed FXIII$^{-/-}$ mice, this increase was not statistically significant (Fig. 23C-D). This implies that excessive bile duct hyperplasia and related profibrogenic gene induction could be one mechanism underlying the exaggerated liver fibrosis in ANIT-exposed Fib$\gamma^{390-396A}$ mice.

Fibrin(ogen)-$\alpha_M\beta_2$ binding does not control experimental liver fibrosis in the absence of classical bile duct proliferation. If bile duct hyperplasia were central to the mechanism whereby fibrin(ogen)-$\alpha_M\beta_2$ binding controlled hepatic fibrosis, we hypothesized that expression of $\gamma^{390-396A}$ fibrinogen would not impact fibrosis in a model lacking classic bile duct hyperplasia. Thus, for comparison we selected carbon tetrachloride (CCl_4)-induced liver fibrosis, wherein chronic administration of toxicant produces liver fibrosis distinct in pattern from ANIT, and

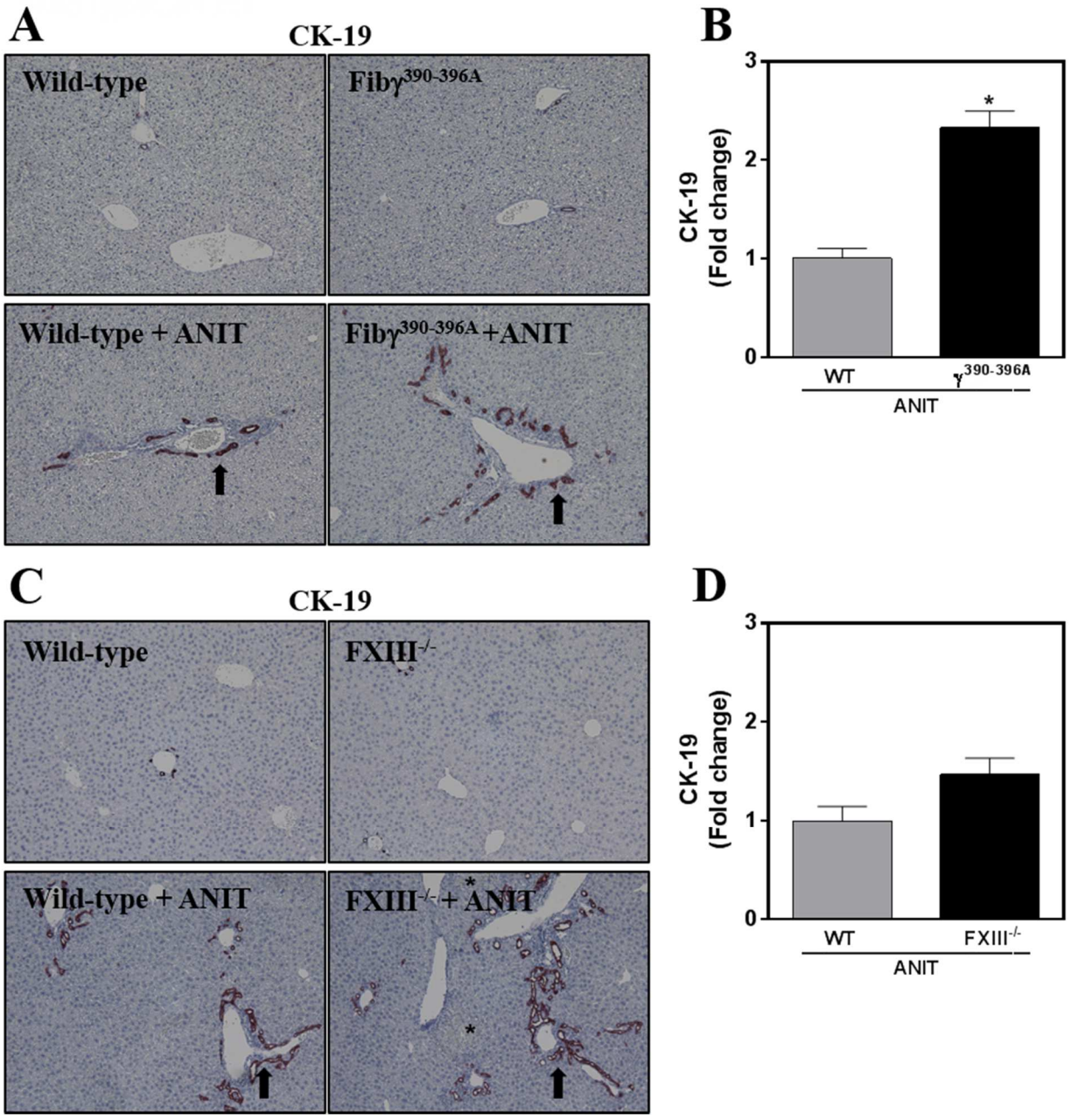


Figure 23. Biliary hyperplasia in ANIT-exposed mice. Wild-type (WT), Fibγ$^{390-396A}$ and FXIII$^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Representative photomicrographs (200X) show liver sections stained for cytokeratin-19 (CK-19, brown) in ANIT-exposed (A) Fibγ$^{390-396A}$ mice, (C) FXIII$^{-/-}$ mice and corresponding WT mice (A and C). Arrows indicate areas of biliary hyperplasia. Asterisks indicate focal hepatocellular necrosis (i.e., bile infarcts). (B,D) CK-19 staining was quantified as described in Materials and Methods. Data are expressed as mean + SEM; n= 4-16 mice per group. *p<0.05 vs. ANIT-exposed WT mice.

without classic bile duct hyperplasia [34]. Similar to a previous study [35], hepatic fibrin(ogen) deposits were also observed in wild-type mice challenged with CCl_4 (8 weeks) in a pattern resembling more the borders of the hepatic acinus (Fig. 24A, arrowhead). Fibrin(ogen) deposition was similar in CCl_4-challenged Fib$\gamma^{390\text{-}396A}$ mice (not shown). Hepatocellular injury, indicated by elevation of serum ALT activity, was similar in CCl_4-challenged wild-type and Fib$\gamma^{390\text{-}396A}$ mice after 4 and 8 weeks of exposure (Fig. 24B). The CK-19 positive cell population in CCl_4-challenged mice lacked characteristics of classic biliary hyperplasia (Fig. 31). Liver fibrosis was similar in wild-type and Fib$\gamma^{390\text{-}396A}$ mice challenged with chronic CCl_4 exposure for 4 and 8 weeks, marked by hepatic collagen deposition (Fig. 24G-H, arrows) and induction of profibrogenic genes in liver (Fig. 24C-F).

Suppression of type 1 cytokine-driven macrophage activation by fibrin(ogen)-$\alpha_M\beta_2$ binding: a potential mechanism controlling pathologic bile duct hyperplasia and fibrosis. Despite marked induction of numerous inflammatory chemokines in livers of ANIT-exposed mice (Fig. 29C-H), we did not observe an effect of ANIT exposure or genotype on the number of hepatic macrophages (Fig. 25A). Of importance, we and others have shown lymphocyte accumulation in livers of ANIT-exposed mice [2, 36]. Increased expression of type 1 cytokines, including interferonγ (IFNγ), is observed in ANIT-exposed rodents [36]. IFNγ promotes proinflammatory (i.e, "classical") macrophage activation [37]. We found that hepatic IFNγ mRNA increased similarly in ANIT-exposed wild-type and Fib$\gamma^{390\text{-}396A}$ mice (Fig. 25B). However, hepatic induction of inducible nitric oxide synthase (iNOS/NOS2) mRNA, a readout of proinflammatory macrophage activation[37] increased in livers of patients with PSC [38],

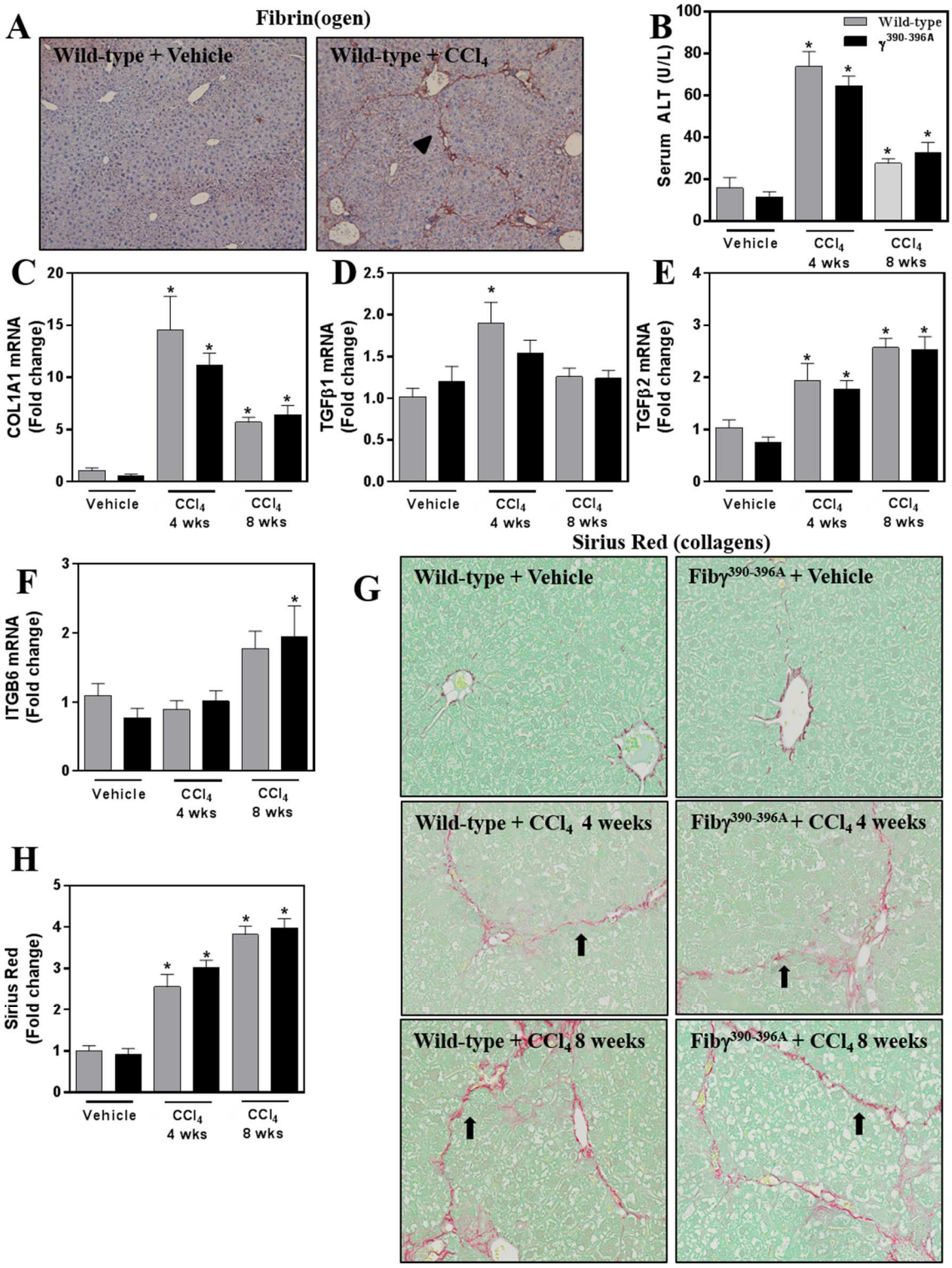


Figure 24. Effect of CCl_4 challenge on liver fibrosis in Fib$\gamma^{390-396A}$ mice. Wild-type (WT) and Fib$\gamma^{390-396A}$ mice were exposed to vehicle (Veh; corn oil) or CCl_4 (10% at 10 µL/g, i.p.) for 4 and 8 weeks (wks) as described in Materials and Methods. (A) Representative photomicrographs (200X) show liver sections stained for fibrin(ogen) (brown) in CCl_4–treated WT mice after 8

Figure 24. (cont'd). weeks. Arrowhead indicates fibrin(ogen) deposition resembling borders of hepatic acinus. (B) Serum ALT activity was determined as described in Materials and Methods. Hepatic expression of mRNAs encoding the profibrogenic genes (C) COL1A1, (D) TGFβ1 (E) TGFβ2 and (F) ITGβ6 was determined by real-time qPCR. (G) Representative photomicrographs show liver sections stained for sirius red (200X). Arrows indicate collagen deposition resembling borders of hepatic acinus. (H) Sirius red staining was quantified as described in Materials and Methods. Gray bars indicate wild-type and black bars indicate $Fib\gamma^{390\text{-}396A}$ mice. n = 4-5 mice per group treated with vehicle and n = 9-10 mice per group treated with CCl_4. *$p<0.05$ vs. vehicle within genotype.

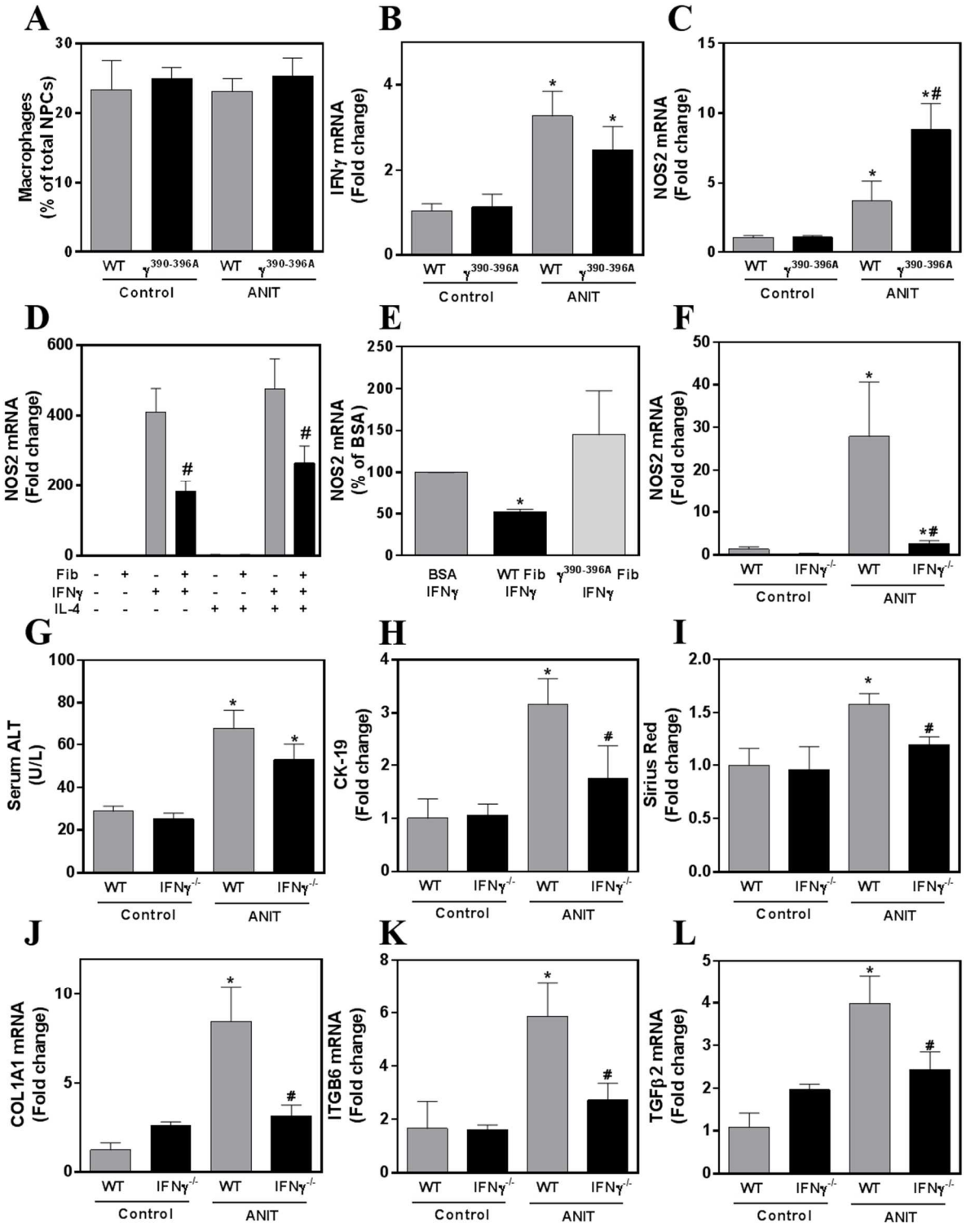


Figure 25. Suppression of type 1 cytokine-driven macrophage activation by fibrin(ogen)-$\alpha_M\beta_2$ binding. Wild-type (WT), Fib$\gamma^{390\text{-}396A}$ and IFN$\gamma^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Hepatic macrophage number was determined by flow cytometry as described in Materials and Methods.

Figure 25. (cont'd). Hepatic expression of mRNAs encoding the genes (B) IFNγ and (C, F) NOS2 was determined by real-time qPCR. (D) Expression of NOS2 mRNA in bone marrow macrophages plated on either BSA or surface adhered WT fibrinogen (10 μg/mL) and stimulated with recombinant mouse IFNγ and/or recombinant mouse IL-4 (10 ng/mL) for 24 hours. (E) Expression of NOS2 mRNA in IFNγ-stimulated bone marrow macrophages plated on BSA, WT fibrinogen or $\gamma^{390\text{-}396A}$ fibrinogen (10 μg/mL) (G) Serum ALT activity was determined as described in Materials and Methods. (H) Cytokeratin-19 (CK-19) and (I) Sirius red staining were quantified as described in Materials and Methods. Hepatic expression of mRNAs encoding the profibrogenic genes (J) COL1A1, (K) ITGβ6 and (L) TGFβ2 was determined by real-time qPCR. Data are expressed as mean + SEM; n= 4-16 mice per group for *in vivo* studies; for *in vitro* studies, results represent macrophages from 9 mice. *$p<0.05$ vs. control diet within genotype and #$p<0.05$ vs. ANIT-exposed WT mice or respective treatment on BSA.

was dramatically increased in livers of ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice compared to ANIT-exposed wild-type mice (Fig. 25C).

IFNγ and integrin signaling pathways are known to interact [39]. Thus, we tested the hypothesis that the fibrin(ogen)-$\alpha_M\beta_2$ binding could suppress IFNγ-mediated NOS2 induction. Fibrinogen adhered to a surface, such as cell culture plastic, engages $\alpha_M\beta_2$, allowing us to test this hypothesis in vitro. Compared to a BSA-coated surface, IFNγ-mediated NOS2 mRNA induction was significantly attenuated in macrophages cultured on a fibrinogen-coated surface (Fig. 25D). Similar results were obtained when the cells were treated with IFNγ and IL-4, a cytokine capable of promoting alternative macrophage activation (Fig. 25D). Whereas wild-type fibrinogen significantly inhibited IFNγ-mediated NOS2 mRNA induction, this effect was lost when the cells were cultured on $\gamma^{390\text{-}396A}$ fibrinogen (Fig. 25E). This suggests that the fibrin(ogen)-$\alpha_M\beta_2$ binding could suppress IFNγ induction of NOS2 mRNA, forming a mechanistic basis for enhanced NOS2 mRNA expression in ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. Indeed, we found that NOS2 mRNA induction in ANIT-exposed mice was dramatically reduced in IFN$\gamma^{-/-}$ mice (Fig. 25F). IFNγ deficiency had no impact on serum ALT in ANIT-exposed mice (Fig. 25G). However, bile duct hyperplasia (Fig. 25H, Fig. 32B) and hepatic fibrosis (Fig. 25I, Fig. 32A), along with hepatic profibrogenic gene induction (Fig. 25J-L), were significantly reduced in ANIT-exposed IFN$\gamma^{-/-}$ mice compared to ANIT-exposed wild-type mice. Collectively, these studies suggest a mechanism whereby fibrin(ogen)-$\alpha_M\beta_2$ binding could inhibit bile duct proliferation and liver fibrosis and are the first to define the precise role of IFNγ in ANIT-induced bile duct fibrosis.

The novel allosteric $\alpha_M\beta_2$ integrin activator leukadherin-1 (LA-1) reduces collagen deposition in wild-type mice with established periductal fibrosis. The novel small molecule

LA-1 has previously been shown to act selectively on $\alpha_M\beta_2$ to enhance binding of the integrin to fibrin through an allosteric mechanism [25, 40]. We postulated that LA-1 treatment would suppress ANIT-induced biliary fibrosis. To evaluate the effect of LA-1 treatment on mice with established liver disease, wild-type mice were exposed to ANIT for 6 weeks and given LA-1 (0.4 mg/kg/day, i.p,) or vehicle (1.5 μL DMSO in 100 μL sterile PBS) twice daily during weeks 5 and 6. LA-1 treatment significantly attenuated bile duct hyperplasia (Fig. 26A) and hepatic fibrosis (Fig. 26B) in ANIT-exposed mice, as indicated by CK-19 and sirius red staining, respectively, without affecting serum ALT activity (Fig. 26C). Interestingly, LA-1 treatment also attenuated the induction of NOS2, TGFβ2, COL1A1, and ITGβ6 mRNAs in ANIT-exposed mice (Fig. 26D-G). Finally, we assessed whether the impact of LA-1 treatment on fibrosis required fibrin(ogen)-$\alpha_M\beta_2$ binding. Of importance, LA-1 failed to affect liver fibrosis, profibrogenic gene expression or serum ALT activity in ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice (Fig. 27A-F). This suggests that the therapeutic effects of LA-1 in this experimental setting require fibrin(ogen)-$\alpha_M\beta_2$ integrin binding.

Discussion

Fibrin deposits are often assigned a pathologic role in studies of acute and chronic liver damage. In part, this is driven by evidence that anticoagulants attenuate fibrin deposition concurrent with a reduction in experimental fibrosis [41, 42]. However, the reduction of fibrosis by anticoagulants cannot be definitively ascribed to changes in fibrin(ogen), due to the fact that thrombin has multiple downstream targets that may influence fibrotic changes. Previous studies

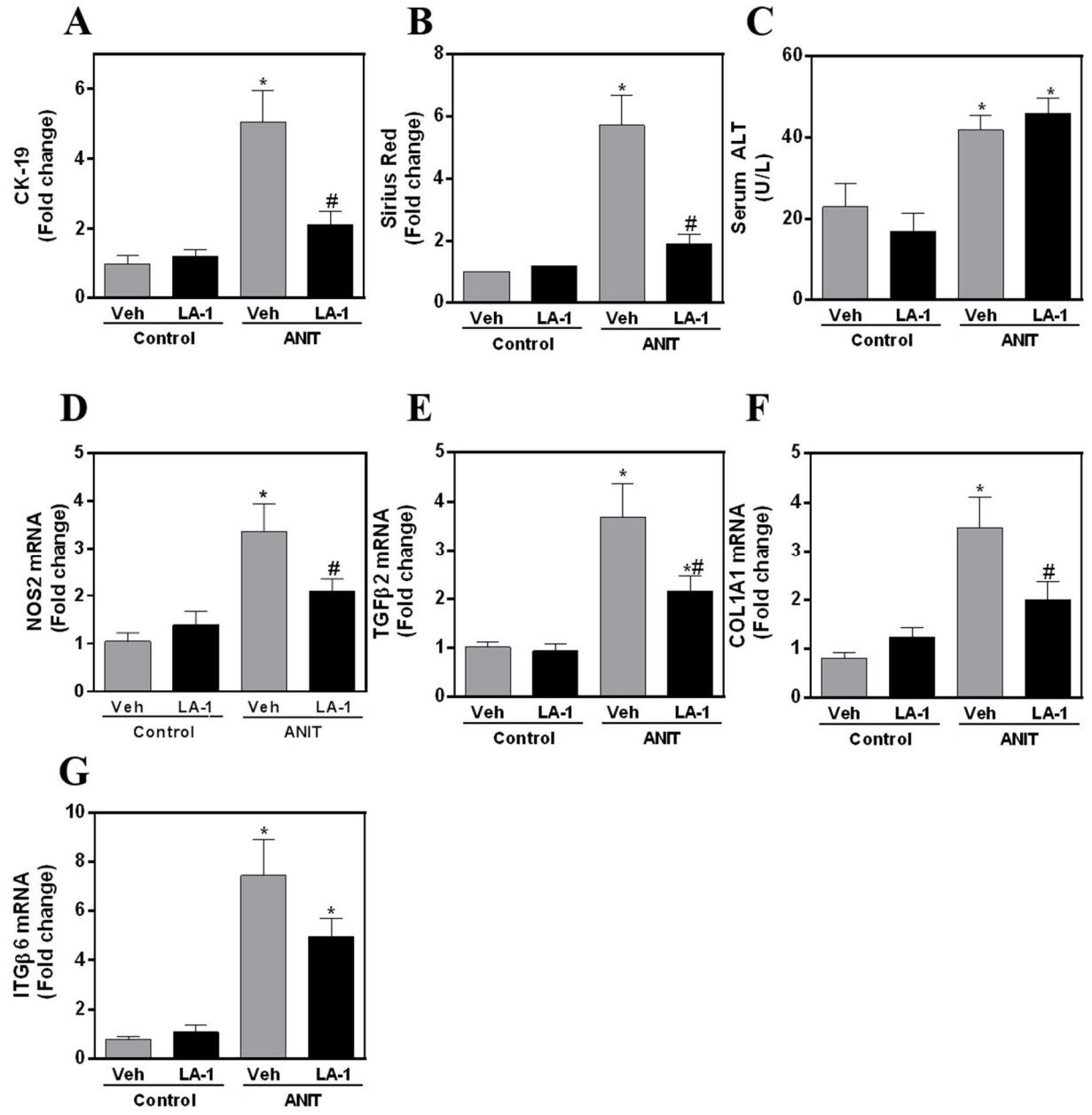


Figure 26. Treatment with the novel allosteric $\alpha_M\beta_2$ integrin activator leukadherin-1 (LA-1) reduces fibrosis in livers of wild-type mice with established periductal fibrosis. Wild-type mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 6 weeks. Mice were treated with vehicle (Veh; 1.5 µL DMSO in 100 µL sterile PBS) or LA-1 twice daily (0.4 mg/kg/day, i.p.) in weeks 5-6. (A) Cytokeratin -19 (CK-19) staining and (B) Sirius red staining were quantified as described in Materials and Methods. (C) Serum ALT activity determined as described in Materials and Methods. Hepatic expression of mRNAs encoding the genes

Figure 26. (cont'd). (D) NOS2, (E) TGFβ2 (F) COL1A1 and (G) ITGβ6 was determined by real-time qPCR. Data are expressed as mean + SEM; n = 5 mice per group for control diet and 12-13 mice per group for mice fed ANIT diet. *$p < 0.05$ vs. respective treatment on control diet. $^{\#}p < 0.05$ vs. vehicle-treated ANIT-exposed mice.

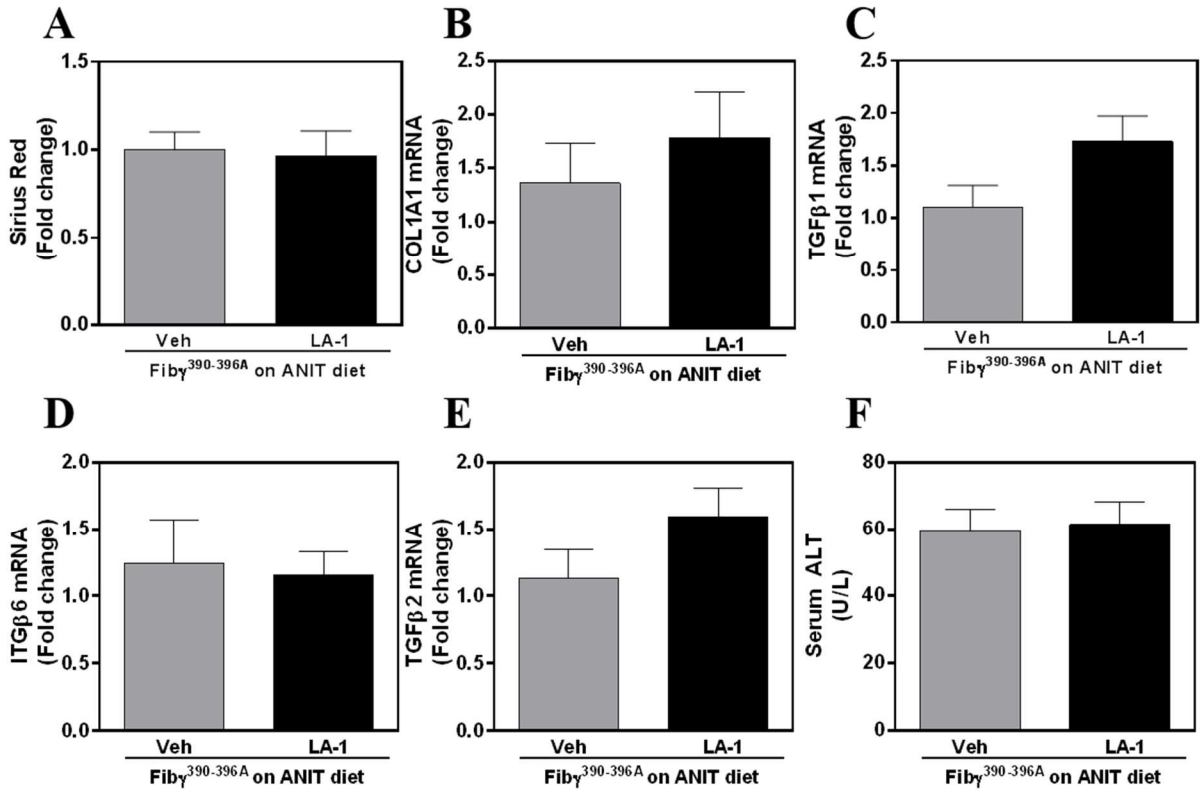


Figure 27. Leukadherin-1 (LA-1) fails to reduce fibrosis in livers of ANIT-exposed Fibγ$^{390\text{-}396A}$ mice. Fibγ$^{390\text{-}396A}$ mice were fed diet containing 0.025% ANIT for 6 weeks. Mice were treated with vehicle (Veh; 1.5 µL DMSO in 100 µL sterile PBS) or LA-1 twice daily (0.4 mg/kg/day, i.p.) in weeks 5-6. (A) Sirius red staining was quantified as described in Materials and Methods. Hepatic expression of mRNAs encoding the profibrogenic genes (B) COL1A1, (C) TGFβ1 (D) ITGβ6 and (E) TGFβ2 was determined by real-time qPCR. (F) Serum ALT activity was determined as described in Materials and Methods. Data are expressed as mean + SEM; n = 7 mice per group.

have indicated a seminal role for the thrombin receptor protease activated receptor-1 (PAR-1) in fibroblast activation in vitro and liver fibrosis in vivo in several experimental mouse models [3, 42-44]. To date, evidence supporting a proinflammatory and profibrogenic function of fibrin in experimental hepatic fibrosis has largely been "guilt by association," because fibrin deposits frequently co-localize with damaged liver tissue and/or collagen. Contradicting this assumption, we found that fibrin(ogen) engagement of leukocyte integrin $\alpha_M\beta_2$ had no impact on chronic CCl_4-induced liver fibrosis, a result consistent with previous findings of Bezerra et al [45] in mice completely lacking fibrinogen. We further describe a novel mechanism wherein fibrin(ogen) inhibits experimental bile duct hyperplasia and fibrosis, mediated by the leukocyte integrin $\alpha_M\beta_2$ binding motif located in the c-terminal domain of the fibrinogen γ-chain. Previous studies implicated this domain in driving inflammation in the context of multiple disease states [18-22]. Now, we can extend the mechanistic contributions of fibrin(ogen)-$\alpha_M\beta_2$ engagement to include modulation of leukocyte activation and anti-fibrotic function in bile duct injury.

Fib$\gamma^{390\text{-}396A}$ mice are documented to have normal hemostasis, and arterial thrombosis similar to wild-type mice [21]. However, RBCs are excluded from venous thrombi in Fib$\gamma^{390\text{-}396A}$ mice, owing to a reduced rate of FXIII-mediated $\gamma^{390\text{-}396A}$ fibrinogen crosslinking [29]. Whether intrahepatic fibrin(ogen) deposits resemble one of these forms of thrombosis, or something entirely unique, is not known. Thus, it is difficult to completely exclude a role for this process in the observed phenotype of ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. However, we observed a dramatic increase in hepatic necrosis in ANIT-exposed FXIII$^{-/-}$ mice, a pathologic feature completely distinct from ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. Thus, it is not likely that reduced FXIII fibrin crosslinking function in and of itself is the primary mechanism of increased fibrosis in ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. Interestingly, fibrin(ogen) had an altered, diffuse appearance in necrotic

lesions in livers of FXIII$^{-/-}$ mice. It is plausible that FXIII-mediated crosslinking of fibrin(ogen) within foci of necrosis is an essential precursor to protective hemostatic effect of fibrin(ogen) mediated by binding the platelet integrin $\alpha_{IIb}\beta_3$ (Fig. 34) [2]. Consistent with this, FXIII deficiency was associated with delayed liver repair after acute CCl4-induced liver injury [46]. To our knowledge, these are the first studies to examine the role of FXIII in experimental liver fibrosis, leading to unexpected discovery of a protective function of FXIII in limiting hepatocellular necrosis in ANIT-exposed mice.

Although liver pathology was quite distinct in ANIT-exposed FXIII$^{-/-}$ mice and Fib$\gamma^{390\text{-}396A}$ mice, we cannot exclude the possibility that delayed FXIII-mediated crosslinking contributes to increased fibrosis in ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. Indeed, FXIII deficiency also increased fibrosis after ANIT exposure. One hypothesis is that FXIII-mediated crosslinking increases the affinity of fibrin for $\alpha_M\beta_2$ (i.e., FXIII cross-linked fibrin is a better ligand of $\alpha_M\beta_2$ than non-cross-linked fibrin), although to our knowledge this has not yet been examined. Notably, biliary fibrosis increased in FXIII$^{-/-}$ mice without a robust increase in bile duct hyperplasia, distinguishing this response from ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice. Because hepatic necrosis was uniquely increased in FXIII$^{-/-}$ mice, the increase in fibrosis may also occur by mechanisms secondary to the necrosis. Overall, given the complex liver pathology observed in FXIII$^{-/-}$ mice exposed to ANIT, it seems premature to conclude definitively as to the precise role of FXIII in liver fibrosis. Indeed, one human study found that the FXIII Val34Leu polymorphism, which increases the rate of thrombin-mediated FXIII activation, was associated with faster fibrosis progression in patients with viral hepatitis B and C [47]. Collectively, there is a need for additional studies defining the role of FXIII, and specifically FXIII-mediated fibrin cross-linking, in experimental and clinical liver fibrosis.

The role of fibrin(ogen) in experimental fibrosis appears to be highly tissue- or even context-specific. For example, fibrin(ogen) actually promotes tissue fibrosis in models of Duchenne muscular dystrophy [18, 19] and kidney injury [48]. However, complete fibrin(ogen) deficiency had no impact on chemical-induced lung fibrosis [49]. Our results indicate that even within a single organ system, fibrin(ogen) engagement of integrin $\alpha_M\beta_2$ can have disparate roles depending on the experimental context/pathology. In ANIT-exposed mice, disruption of fibrin(ogen)-$\alpha_M\beta_2$ binding resulted in excessive bile duct hyperplasia. Proliferating BDECs express higher levels of profibrogenic mediators, [3, 32, 50] including the $\alpha_V\beta_6$ integrin, which directs local generation of active TGFβ1 protein and portal fibroblast activation [3, 32, 33, 50, 51]. Indeed, hepatic expression of the β6 subunit increased dramatically in ANIT-exposed Fib$\gamma^{390\text{-}396s}$ mice. $\alpha_V\beta_6$ expression is selective to BDECs and $\alpha_V\beta_6$ contributes to biliary fibrosis in multiple models of bile duct injury [3, 32, 33, 52]. In contrast, we found that neither classic bile duct hyperplasia or ITGβ6 mRNA expression increased in CCl_4-exposed mice, and $\alpha_V\beta_6$ does not participate in CCl_4-induced liver fibrosis [52]. Collectively, these observations suggest that suppression of liver fibrosis by fibrin(ogen) engagement of integrin $\alpha_M\beta_2$ occurs secondary to a reduction in bile duct hyperplasia.

There may be multiple mechanisms whereby fibrin(ogen)-$\alpha_M\beta_2$ binding inhibits bile duct fibrosis, but it seems plausible that liver macrophages play a central role. Depending on their gene expression profile, macrophages can promote liver fibrosis, and cytokines driving macrophages towards this profibrogenic phenotype are increased in livers of ANIT-exposed rodents (e.g., IFNγ). We found that IFNγ drives bile duct hyperplasia and fibrosis in ANIT-exposed mice, the first mechanistic validation of the suggestion that IFNγ drives this pathology [36]. In ANIT-exposed mice, IFNγ induced hepatic expression of iNOS, one proinflammatory macrophage biomarker

expressed in patients with PSC and linked to biliary hyperplasia and fibrosis [38]. Here we show for the first time that IFNγ is responsible for induction of iNOS in ANIT-exposed mice. Despite having a similar induction of IFNγ, hepatic iNOS expression was markedly enhanced in ANIT-exposed $Fib\gamma^{390\text{-}396A}$ mice. Anchoring this observation to an in vitro mechanism, we found that fibrin(ogen)-$\alpha_M\beta_2$ binding suppressed IFNγ-mediated iNOS induction in macrophages. Interestingly, there is precedent for this pathway, as other studies have suggested that ITAM-coupled integrins can inhibit IFN signaling in macrophages [39]. Collectively, the results suggest a mechanism wherein fibrin(ogen)-$\alpha_M\beta_2$ binding inhibits bile duct hyperplasia and the ensuing fibrosis in ANIT-exposed mice by inhibiting IFNγ-mediated macrophage activation (Fig. 34). Although additional experimentation is required, this hypothesis is consistent with the exciting concept that fibrin(ogen) deposition is not just reactive to tissue injury, but instead can carefully tailor local leukocyte activity to modify disease progression.

Human liver diseases vary with their etiology and thus may have differing mechanistic paths driving fibrosis. Particularly for cholestatic liver diseases, where treatment options are limited, capturing key differences in mechanism or clinical pathology may reveal unique targets for treatment. Previous studies have suggested that patients with cholestatic liver disease have preserved levels of plasma fibrinogen, [5, 6, 53, 54] whereas fibrinogen levels tend to decrease in patients with liver disease of non-cholestatic origin. This has not been observed in all cases, and may relate to differential regulation of hepatic fibrinogen. Interestingly, plasma fibrinogen levels at 4 weeks were normal in ANIT-exposed mice, but decreased after CCl_4 treatment (Fig. 28G). This difference, and the differential inhibition of fibrosis by fibrin(ogen)-$\alpha_M\beta_2$ binding in these experimental settings prompted us to explore whether there were any interesting associations between hepatic fibrin(ogen) and profibrogenic gene expression in diseased human livers.

Interestingly, we found that expression of the rate-limiting FGB chain inversely associated with COL1A1 mRNA expression in cirrhotic livers from patients with PSC/PBC, but not in cirrhotic livers from patients with a traditionally non-cholestatic liver disease (i.e., hepatitis C/HCV) (Fig. 33). The exact basis for this association in PSC patients, and lack thereof in HCV patients, is not clear, and interpretation should be cautious. However, viewed in the context of results from the two fibrosis models, this could suggest a unique association between fibrinogen expression and/or function in cholestatic liver disease. The results support a need for additional clinical analyses of fibrin(ogen) expression and regulation in patients with different types of liver disease.

Identification of the fibrin(ogen)-$\alpha_M\beta_2$ interaction as a putative mechanism to inhibit biliary fibrosis suggests a novel therapeutic target. To explore this concept, we employed the novel small molecule LA-1, an $\alpha_M\beta_2$ integrin agonist that allosterically enhances $\alpha_M\beta_2$-dependent cell adhesion to fibrin polymers [25]. Using an interventional treatment paradigm, we found that LA-1 reduced bile duct hyperplasia and fibrosis in ANIT-exposed wild-type mice, even when administered after the mice had developed disease. The precise cellular mechanism whereby LA-1 reduces bile duct hyperplasia and fibrosis requires additional investigation. However, our observation that LA-1 treatment did not reduce fibrosis in ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice implies that the mechanism whereby LA-1 reduces fibrosis in this experimental context requires the $\alpha_M\beta_2$ integrin binding domain of the fibrin(ogen) molecule. The appeal of this therapeutic strategy is increased by the fact that targeting the fibrin $\alpha_M\beta_2$-binding domain could be accomplished without necessarily compromising fibrin polymerization or the role of fibrin in hemostasis.

In the present studies, we found that the integrin $\alpha_M\beta_2$ binding domain of fibrin(ogen) inhibits liver fibrosis specifically coupled to bile duct proliferation. The mechanism appeared distinct from FXIII-mediated crosslinking, as we show for the first time that FXIII-deficient mice

develop focal hepatic necrosis as a unique phenotype accompanying biliary fibrosis. Rather, a combination of in depth in vivo and in vitro studies suggested a novel mechanism where fibrin(ogen)-$\alpha_M\beta_2$ binding inhibits IFNγ-driven leukocyte activation thereby reducing bile duct hyperplasia and fibrosis in mice. Finally, we provide proof-of-principle experimental evidence that this pathway can be pharmacologically targeted to inhibit bile duct hyperplasia and fibrosis in mice (Fig. 34). Overall, these carefully paired and well-controlled studies significantly advance the field by illuminating an entirely novel and druggable pathway wherein hemostatic factors control the progression of experimental liver disease.

APPENDIX

Gene Name	Gene Symbol	Species	Gene ID	Forward primer	Reverse primer
18S ribosomal RNA	RN18s	mouse	19791	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Chemokine (C-C motif) ligand 2	CCL2	mouse	20296	GTCCCTGTCATGCTTCTGG	GCTCTCCAGCCTACTCATTG
Chemokine (C-C motif) ligand 3	CCL3	mouse	20302	TACAGCCGGAAGATTCCACG	GTCAGGAAAATGACACCTGGC
Chemokine (C-C motif) ligand 4	CCL4	mouse	20303	TCTGTGCAAACCTAACCCCG	GAGGGTCAGAGCCCATTGGT
Collagen, type I, alpha 1	COL1A1	mouse	12842	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
Fibrinogen alpha chain	FGA	mouse	14161	GCCCAACGAGAGACTGTGAT	TGCTGGATCAAAAGCCATCCT
Fibrinogen beta chain	FGB	mouse	110135	GACTACGATGAACCGACGGA	TAGCCCCCTCCACTGATAGG
Fibrinogen gamma chain	FGG	mouse	99571	GACGACGGCATTATTTGGGC	AACGTCTCCAGCCTGTTTGG
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	mouse	14433	GTGGACCTCATGGCCTACAT	TGTGAGGGAGATGCTCAGTG
Hypoxanthine guanine phosphoribosyl transferase	HPRT1	mouse	15452	AAGCCTAAGATGAGCGCAAG	TTACTAGGCAGATGGCCACA
Interferon gamma	IFNγ	mouse	15978	CTTTGGACCCTCTGACTTGAG	TCAATGACTGTGCCGTGG
Integrin, beta6	ITGβ6	mouse	16420	CTCACGGGTACAGTAACGCA	AAATGAGCTCTCAGGCAGGC
Intercellular adhesion molecule 1	ICAM1	mouse	3383	TTTGAGCTGAGCGAGATCGG	CTCCGGAAACGAATACACGG
Interleukin 1 beta	IL1β	mouse	16176	AGCTTCCTTGTGCAAGTGTCT	GACAGCCCAGGTCAAAGGTT
Interleukin 6	IL6	mouse	16193	TCCTCTCTGCAAGAGACTTCC	TTGTGAAGTAGGGAAGGCCG
Nitric oxide synthase 2, inducible	NOS2	mouse	18126	TTCTGTGCTGTCCCAGTGAG	TGAAGAAAACCCCTTGTGCT
Transforming growth factor, beta1	TGFβ1	mouse	21803	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
Transforming growth factor, beta2	TGFβ2	mouse	21808	CCCCGGAGGTGATTTCCATC	GATGGCATTTTCGGAGGGGA
Collagen, type I, alpha 1	COL1A1	human	1277	ACATGTTCAGCTTTGTGGACC	GTGATTGGTGGGATGTCTTCG
Fibrinogen beta chain	FGB	human	2244	CAACGACAATGAGGAGGGTT	CGATAGCCACCTCCACTGAT
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	human	2597	CTCCTGTTCGACAGTCAGCC	ACCAAATCCGTTGACTCCGAC
Hypoxanthine phosphoribosyltransferase 1	HPRT1	human	3251	CCCTGGCGTCGTGATTAGTG	CGAGCAAGACGTTCAGTCCT

Table 1. Gene names and primer sequences (5'-> 3') for transcripts verified by qPCR.

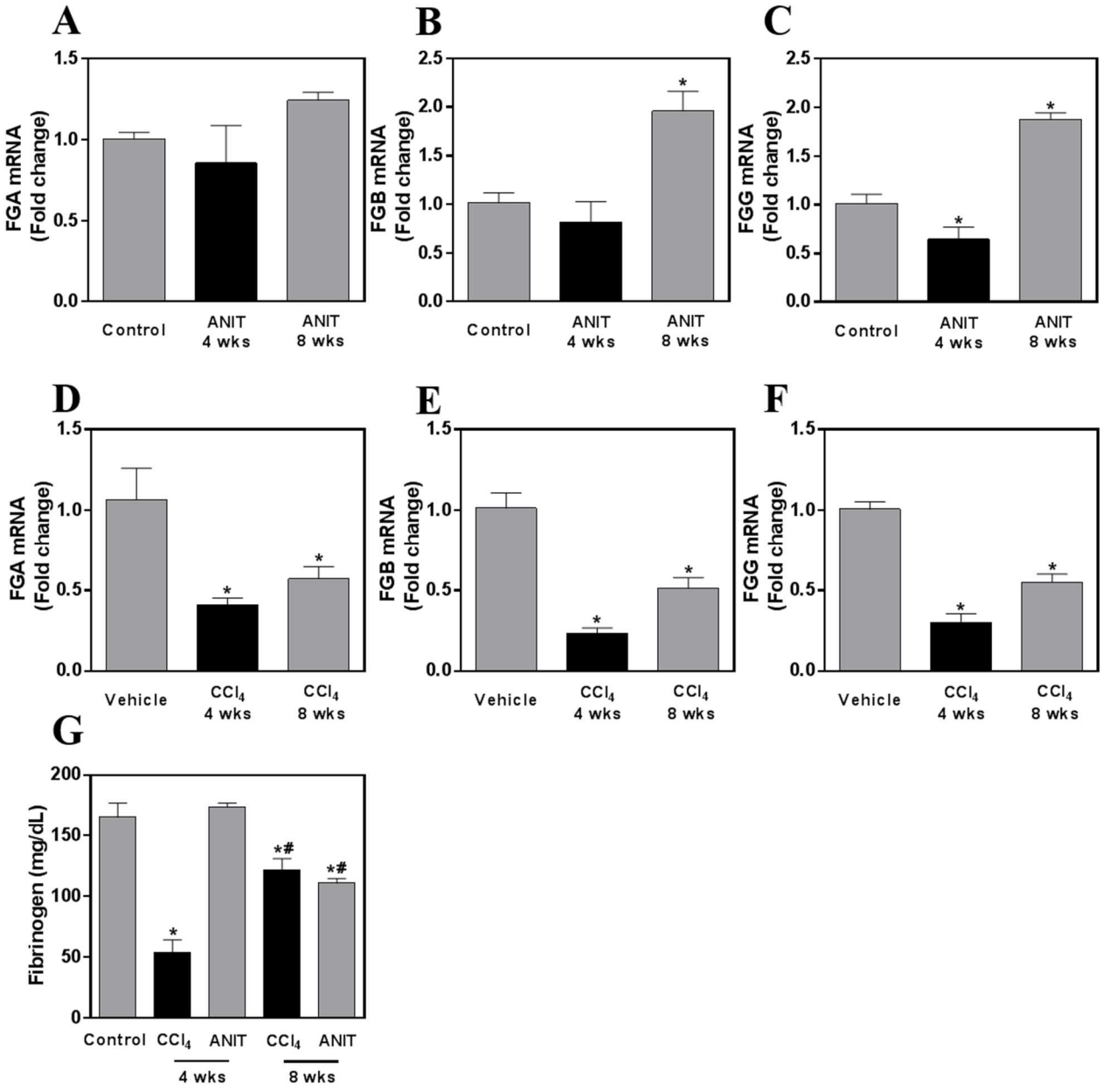


Figure 28. Fibrinogen gene expression and plasma levels in ANIT- and CCl_4-exposed Fib$\gamma^{390\text{-}396A}$ mice. Wild-type (WT) and Fib$\gamma^{390\text{-}396A}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 and 8 weeks. Separate cohorts of WT and Fib$\gamma^{390\text{-}396A}$ mice were exposed to vehicle (Veh; corn oil) or CCl_4 (10% at 10 µL/g, i.p.) for 4 and 8 weeks, as described in Materials and Methods. Hepatic expression of mRNAs encoding (A, D) FGA, (B, E) FGB and (C, F) FGG was determined by real-time qPCR. (G) Plasma fibrinogen levels were determined as described in Materials and Methods. Data are expressed as mean + SEM; n = 4-16 mice per group. *p<0.05 vs. control diet or vehicle exposed mice. #p<0.05 vs. treatment-matched group at 4 weeks.

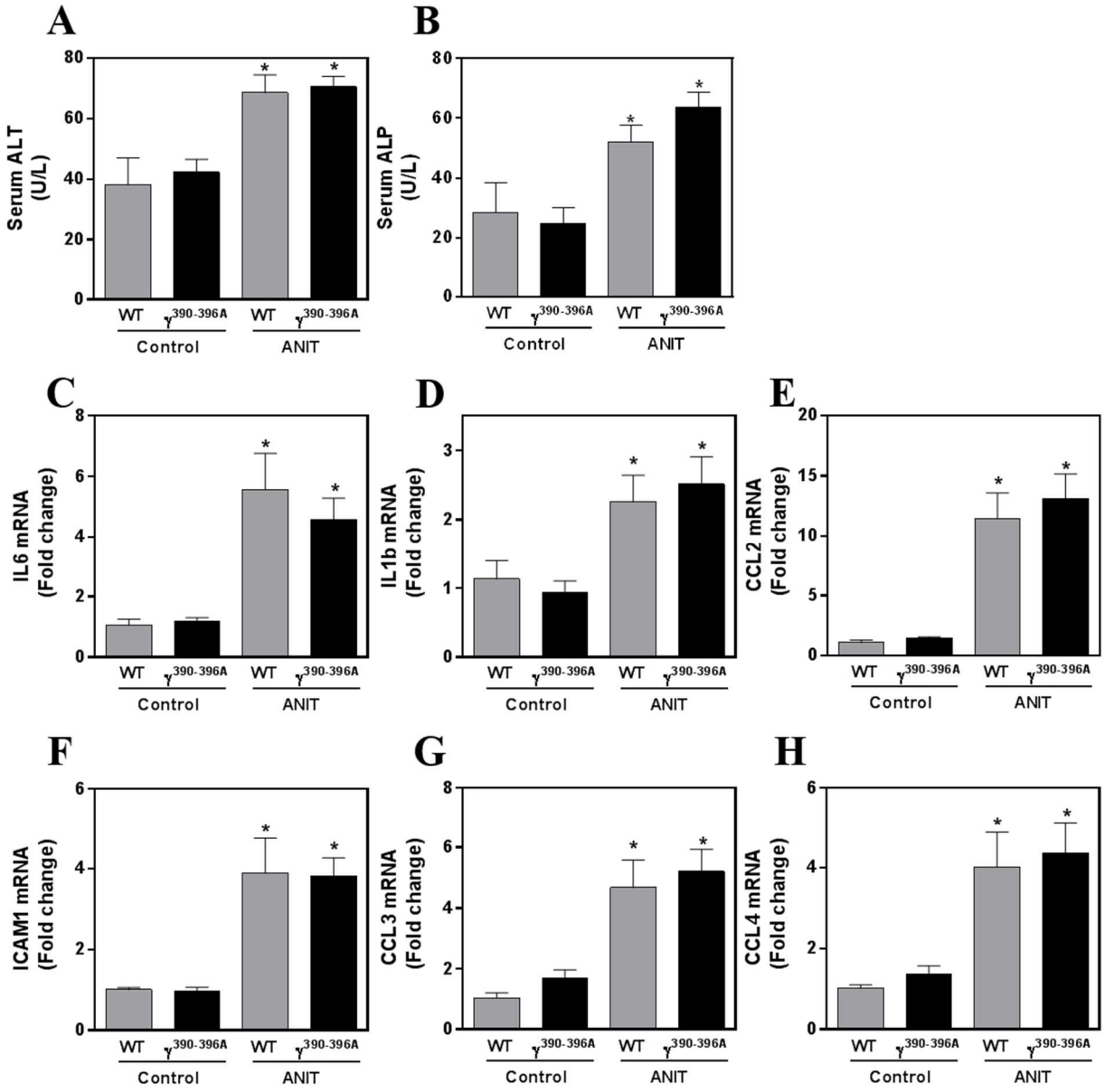


Figure 29. Hepatic injury and inflammatory gene induction in ANIT-exposed Fibγ$^{390\text{-}396A}$ mice. Wild-type (WT) and Fibγ$^{390\text{-}396A}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Serum ALT and (B) serum ALP activities were determined as described in Materials and Methods. Hepatic expression of mRNAs encoding (C) IL-6, (D) IL1β, (E) CCL2, (F) ICAM1, (G) CCL3 and (H) CCL4 were determined by real-time qPCR. Data are expressed as mean + SEM; n = 4 mice per group for control diet and 10-16 mice per group for mice fed ANIT diet. *$p<0.05$ vs. control diet within genotype.

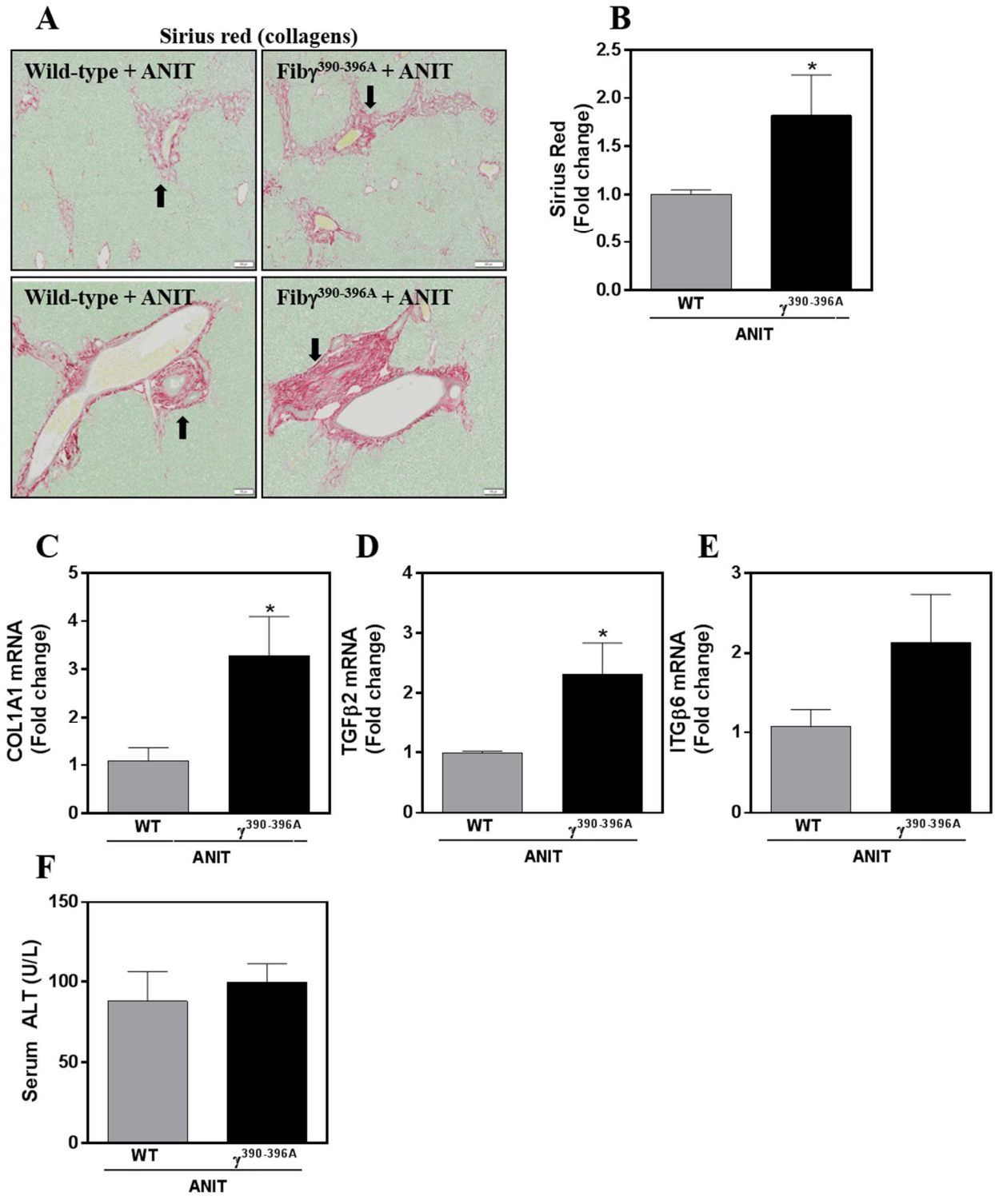


Figure 30. Increased fibrosis in livers of Fibγ$^{390\text{-}396A}$ mice exposed to ANIT for 8 weeks. Wild-type (WT) and Fibγ$^{390\text{-}396A}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 8 weeks. (A) Representative photomicrographs show liver sections stained for collagens with sirius red at low magnification (100X, top) and high magnification

Figure 30. (cont'd). (200X, bottom). Arrows indicate marked large duct collagen deposition. (B) Sirius red staining was quantified as described in Materials and Methods. Hepatic expression of mRNAs encoding the profibrogenic genes (C) COL1A1 (D) TGFβ2 and (E) ITGβ6 was determined by real-time qPCR. (F) Serum ALT activity was determined as described in Materials and Methods. Data are expressed as mean + SEM; n = 4 mice per group. *$p<0.05$ vs. ANIT-exposed WT mice.

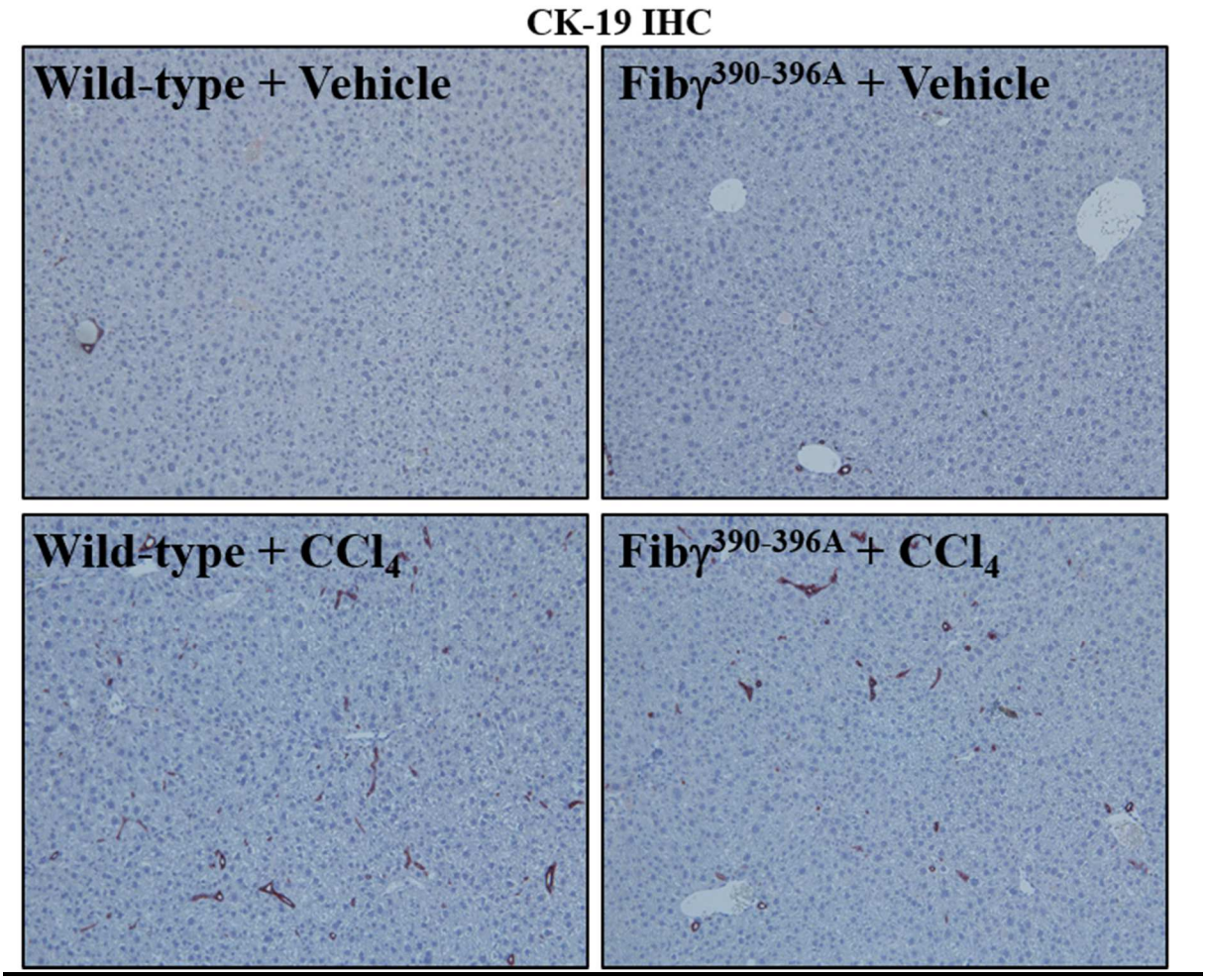


Figure 31. Lack of classic biliary hyperplasia in CCl_4- exposed mice. Wild-type (WT) and Fib$\gamma^{390-396A}$ mice were exposed to vehicle (Veh; corn oil) or CCl_4 (10% at 10 μL/g, i.p.) for 8 weeks as described in Materials and Methods. Representative photomicrographs (200X) show liver sections stained for cytokeratin-19 (CK-19, brown).

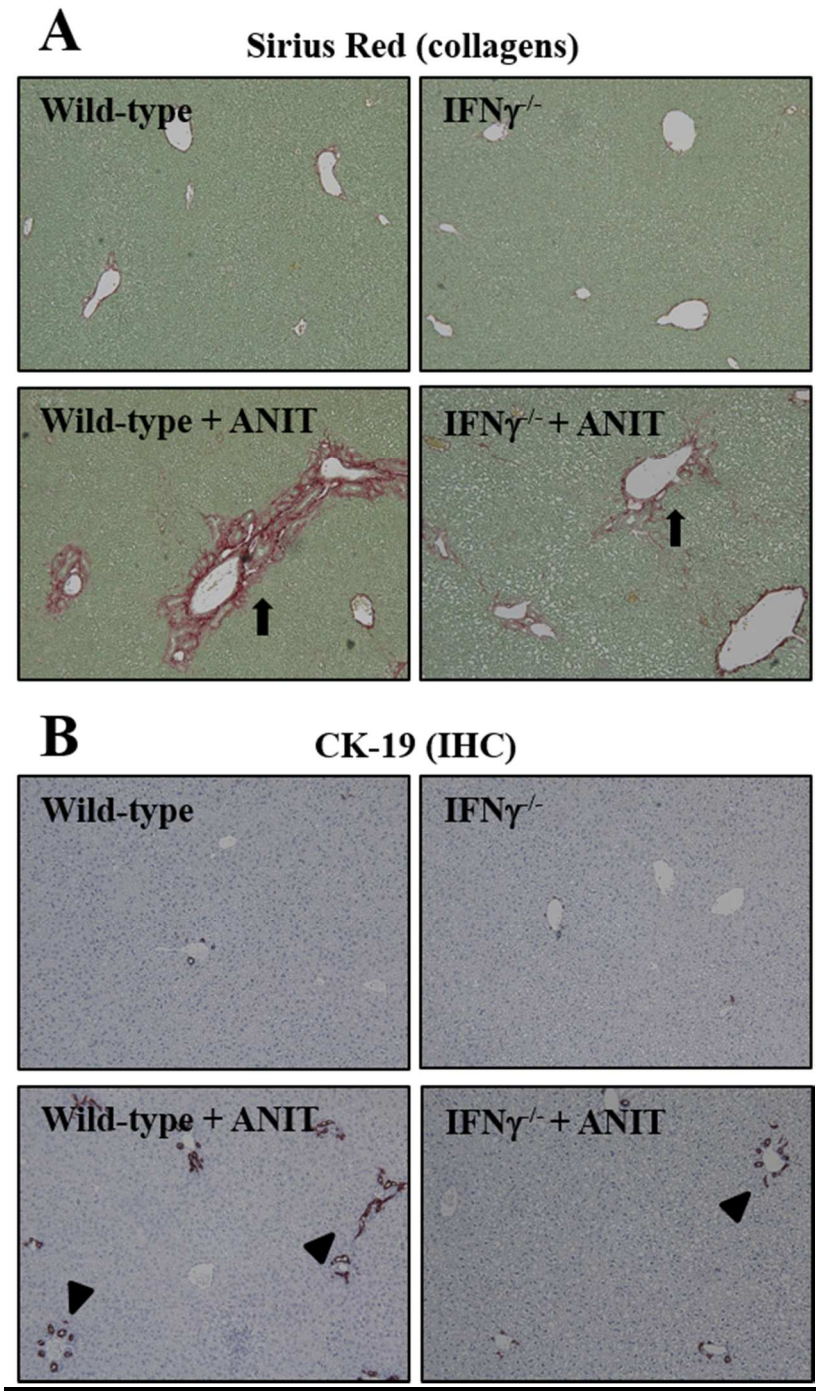
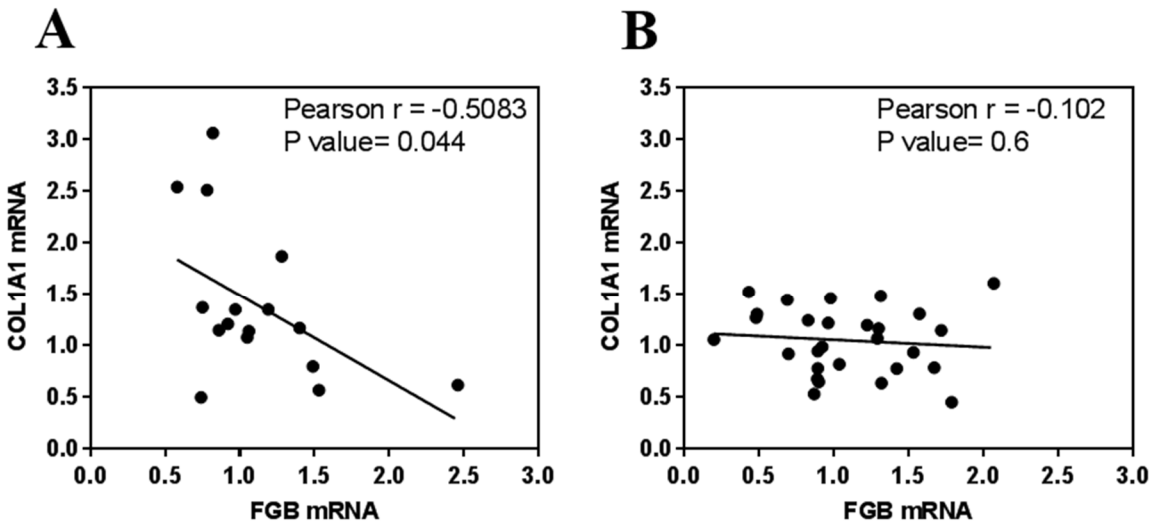


Figure 32. Reduced fibrosis and biliary hyperplasia in livers of IFNγ$^{-/-}$ mice exposed to ANIT for 4 weeks. Wild-type (WT) and IFNγ$^{-/-}$ mice were fed control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Representative photomicrographs show sirius red-stained liver sections (100X). Arrows indicate peribiliary collagen deposition. (B) Representative photomicrographs (100X) show liver sections stained for cytokeratin-19 (CK-19, brown). Arrowheads indicate areas of biliary hyperplasia.



C

	PSC/PBC	HCV
ALT (U/L)	66.5 (24-126)	164 (15-868)
Albumin	2.6 (1.8-3.8)	2.3 (1.4-3.8)
INR	1.5 (1.0-2.4)	1.8 (0.9-3.3)
Bilirubin	7.2 (1.5-20.8)	5.0 (0.8-14.5)
Number of patients	16	27

Figure 33. Correlation of hepatic FGB mRNA with COL1A1 mRNA and biomarkers of hepatic function in patients with liver fibrosis. De-identified liver samples from 16 human patients with primary biliary cirrhosis (PBC)- or primary sclerosing cholangitis (PSC)-associated liver fibrosis, and 27 human patients with hepatitis C (HCV)- associated liver fibrosis were kindly provided by The University of Kansas Medical Center Liver Center Tissue Bank. Associations between hepatic levels of COL1A1 vs. FGB mRNAs in samples from patients with (A) PBC/PSC and (B) HCV were assessed using Pearson's r correlation. (C) Corresponding clinical data are shown as mean and range. The criterion for statistical significance was $p \leq 0.05$.

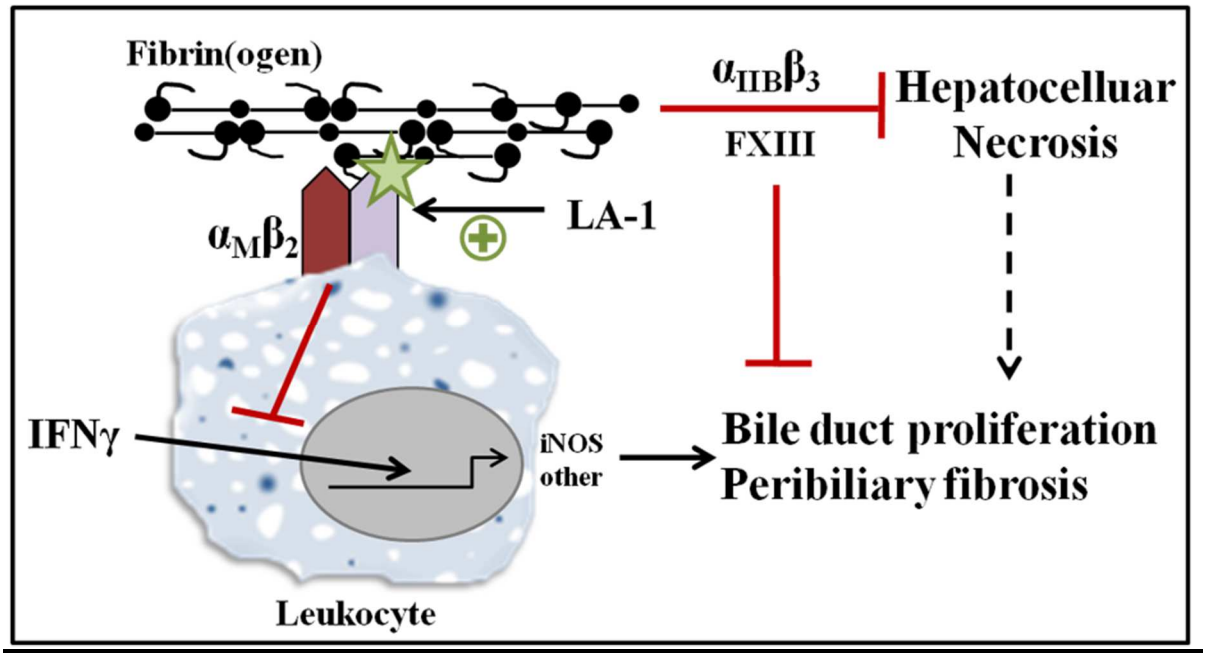


Figure 34. Proposed mechanism whereby fibrin(ogen)-integrin $\alpha_M\beta_2$ binding inhibits bile duct hyperplasia and fibrosis in experimental cholestatic liver disease. The mechanism whereby the integrin $\alpha_M\beta_2$ binding domain of fibrin(ogen) inhibits liver fibrosis relates to suppression of bile duct proliferation after ANIT challenge. Bile duct proliferation and fibrosis were driven by IFNγ *in vivo,* and fibrin(ogen) - integrin $\alpha_M\beta_2$ binding suppressed IFNγ-mediated macrophage activation *in vitro*, suggesting a possible mechanism whereby fibrin(ogen) suppresses both bile duct hyperplasia and liver fibrosis by modifying proinflammatory macrophage activation. Proof-of-principle studies with LA-1 suggest that this pathway can be pharmacologically targeted to reduce established liver fibrosis. This mechanism appears to be distinct from FXIII-mediated fibrin(ogen) crosslinking activity, which plays a role in inhibiting hepatocellular necrosis, potentially in tandem with a pathway we described previously related to fibrin(ogen) engagement of platelet integrin $\alpha_{IIb}\beta_3$ (Joshi *et al.*, *JTH* 2014). Collectively, these results and our previously published studies suggest a multifaceted mechanism by which fibrin(ogen) inhibits cholestatic liver disease pathology.

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CHAPTER 5

Significance, Implications And Future Directions

Significance of dissertation

In the context of liver fibrosis, the role of the coagulation protein fibrin(ogen) has been generally assumed as damaging and as such, its potential as a therapeutic target has been largely underestimated. This dissertation highlights the protective role of fibrin(ogen) in an experimental setting of chronic cholestatic liver injury and fibrosis. The studies described in this dissertation provide substantial insight and elucidate key concepts regarding the molecular mechanisms mediating the protective effects of fibrin(ogen) via its integrin binding functions. An innovative and potentially translational aspect of these studies is use of the investigational drug leukadherin -1 (LA-1), a small molecule allosteric activator of $\alpha_M\beta_2$ that increases affinity for fibrin(ogen) binding. This finding is extremely important because it suggests that the fibrin(ogen)-integrin $\alpha_M\beta_2$ interaction is a novel therapeutic target to treat liver fibrosis. Moreover, this knowledge paves the way for deeper mechanistic studies and identification of additional and perhaps better compounds. Significantly, drugs targeting the non-hemostatic function of fibrin could potentially be translated to the clinic without simultaneously inducing complications associated with thrombosis or hemorrhage. These studies are also significant because they have the potential to inform repurposing of existing coagulation-directed therapeutics, such as tranexamic acid, for the treatment of liver fibrosis.

Summary of findings

The impact of inhibiting fibrinolysis on ANIT-induced liver injury and fibrosis was initially assessed. These studies were based on a prior observation that complete genetic fibrin(ogen) deficiency increased cholestatic liver injury and inflammation in ANIT-exposed mice [1]. The hypothesis tested was that the FDA approved anti-fibrinolytic drug tranexamic acid (TA) inhibits ANIT-induced liver injury and fibrosis (Chapter 2). These studies revealed that administration of TA reduced markers of cholestatic liver injury in mice exposed to ANIT. Progression of liver fibrosis and biliary hyperplasia was largely prevented with TA treatment in mice exposed to ANIT diet for 4 weeks. In agreement with these findings, genetic deficiency in PAI-1 was found to exacerbate peribiliary fibrosis and biliary hyperplasia in ANIT-exposed mice.

We then set our sights on identifying mechanisms mediating the protective effects of fibrin(ogen). Our initial attention was on identifying mechanisms linking coagulation and platelet function to chronic cholestatic liver disease (Chapter 3). It was hypothesized that thrombin activation of platelets via protease activator receptor-4 (PAR-4) and fibrin(ogen) engagement of platelets via $\alpha_{IIb}\beta_3$ integrin inhibits chronic cholestatic liver disease in ANIT-exposed mice. Hepatocellular necrosis increased significantly in ANIT-exposed PAR-4 deficient mice compared to the wild-type mice. Hepatocellular necrosis and biliary hyperplasia were more severe in ANIT-exposed Fib$\gamma^{\Delta 5}$ mice, which express a mutant fibrin(ogen) protein incapable of binding integrin $\alpha_{IIb}\beta_3$. In summary, we found that both thrombin- and fibrin-mediated mechanisms inhibit hepatocellular injury and fibrosis in this experimental setting.

The findings with platelet integrin $\alpha_{IIb}\beta_3$ indicate that the hemostatic function of fibrin(ogen) inhibits chronic cholestatic liver injury. However, no study to date has addressed the long held assumption that the fibrin matrix (through its non-hemostatic function) actively enhances

inflammatory cell activity and liver fibrosis. Thus, we sought to determine if fibrin engages leukocytes via $\alpha_M\beta_2$ integrin to enhance inflammation and liver fibrosis in ANIT-induced chronic liver disease (Chapter 4). By utilizing unique Fib$\gamma^{390\text{-}396A}$ mice, expressing a mutant form of fibrinogen wherein the hemostatic function is retained but fibrinogen lacks the binding motif for integrin $\alpha_M\beta_2$, the fibrin(ogen)-integrin $\alpha_M\beta_2$ interaction was found to suppress ANIT-induced hepatic fibrosis and biliary hyperplasia. Surprisingly, the increase in liver fibrosis and BDEC hyperplasia in Fib$\gamma^{390\text{-}396A}$ mice occurred without a corresponding increase in liver injury. In contrast, hepatocellular necrosis was significantly increased in mice deficient in transglutaminase factor XIII (FXIII$^{-/-}$), that crosslinks and stabilizes the fibrin clot. Interestingly, hepatic fibrosis was similarly elevated in WT and Fib$\gamma^{390\text{-}396A}$ mice after carbon tetrachloride (CCl_4) challenge, which is a model of hepatotoxicity that lacks classic biliary hyperplasia. iNOS mRNA expression was significantly increased in ANIT-exposed Fib$\gamma^{390\text{-}396A}$ mice and ANIT-challenged IFN$\gamma^{-/-}$ mice had reduced iNOS expression, biliary hyperplasia and liver fibrosis. Finally in proof-of-principle studies the novel small molecule leukadherin-1 (LA-1), that allosterically enhances fibrin(ogen)-integrin $\alpha_M\beta_2$ interaction, was found to reduce biliary fibrosis. Overall, these studies provide important proof-of-principle findings that significantly advance the field by revealing a novel and druggable pathway to reduce liver fibrosis.

Implications of findings

Protective role of the anti-fibrinolytic drug tranexamic acid in liver fibrosis**:** Results from the powerful proof-of-principle studies with TA (Chapter 2) indicate that an anti-fibrinolytic drug inhibits disease pathology in the ANIT model of chronic cholestatic liver disease. Collectively, along with the studies in fibrin(ogen) deficient mice, this suggests that a potential translational

strategy to reduce the progression of chronic cholestatic liver disease could involve preserving residual hepatic fibrin deposition. TA is an FDA-approved anti-fibrinolytic drug that has been demonstrated to be inexpensive, safe and effective in treating traumatic bleeding [2-5]. Studies outlined in this dissertation are the first to describe the protective effect of TA in chronic cholestatic liver disease and suggest it could be utilized as an anti-fibrotic. Viewed in the context of previous studies using fibrinogen-null mice PAI-1-null mice, it seems likely that the protective effects of TA are mediated, at least in part, via inhibition of plasmin-driven fibrinolytic activity. Defining whether fibrin(ogen) is required for the protective effects of TA could potentially enable repurposing of TA and other anti-fibrinolytic drugs for patients with PSC. It would also be interesting and useful to investigate the effects of inhibiting upstream fibrinolytic proteins proteins like tissue- and urokinase-type plasminogen activators (tPA and uPA respectively) in the context of chronic cholestatic liver disease. Patients with end stage liver disease initiated by chronic cholestasis have reduced capacity to produce fibrinogen and have increased fibrinolytic capacity [6, 7]. Interestingly, fibrinolytic proteins present in human bile can accelerate lysis of plasma clots *in vitro* [8]. Clinical evidence of determinations of coagulation and fibrinolytic activity in bile from PSC patients could potentially also be coupled to these findings.

Hepatoprotective action of the hemostatic system in cholestatic liver disease via platelet activation: Platelet activation through mechanisms involving thrombin and fibrin(ogen) is essential for normal hemostasis. While the key activators of platelets are known, the pathways that are essential to activate platelets to be protective in chronic cholestatic liver injury and fibrosis are not known. The results from our studies highlight a novel hepatoprotective action of the coagulation cascade in chronic cholestatic liver disease involving platelet activation by thrombin via PAR-4 and

interaction of fibrin(ogen) with platelets via integrin $\alpha_{IIb}\beta_3$ (Chapter 3). Furthermore, these studies imply that PAR-4 is critical for platelet activation during cholestatic liver injury. Further defining the mechanisms whereby platelets protect in this context of chronic liver disease would be extremely illuminating.

The collective findings presented here are especially illuminating when considering how controversial and dichotomous the role of platelets is in liver disease. The specific role of platelets seems to depend on the etiology of the liver disease or nature of the hepatic injury. For instance, in experimental settings of viral hepatitis and non-alcoholic steatohepatitis, platelets contribute to hepatic injury and fibrosis [9, 10]. However the role of platelets is reversed in settings of cholestatic liver disease where platelets have been demonstrated to promote liver regeneration and inhibit liver fibrosis. In humans, antagonists of PAR-1 and integrin $\alpha_{IIb}\beta_3$ are FDA-approved anti-platelet strategies [11, 12]. Although several clinical studies have demonstrated a benefit for anticoagulants and antiplatelet therapies in patients with liver disease, the effect of these compounds in indications with a cholestatic origin have never been examined. Our studies suggest that that in the context of chronic cholestatic liver disease, platelet activation in conjunction with certain aspects of the coagulation cascade, plays a protective role.

Identification of the fibrin-$\alpha_M\beta_2$ interaction as a putative therapeutic target for the reduction of peribiliary fibrosis: Hepatic fibrin deposits are ubiquitous in clinical and experimental liver damage, and are often assigned a pathologic role, based on the notion that non-hemostatic and proinflammatory effects of the so-called fibrin "provisional matrix" exacerbate liver fibrosis. However, a direct link between the non-hemostatic functions of fibrinogen and liver injury/fibrosis has never been examined. The studies documented in this dissertation directly address this critical

gap in knowledge, and in fact, upend this widely held assumption (Chapter 4). Our studies describe for the first time a novel mechanism whereby fibrin(ogen) inhibits peribiliary fibrosis mediated by its interaction with the leukocyte integrin $\alpha_M\beta_2$. Importantly, the potential pathway through which this suppression in fibrosis occurs appears to be via a reduction in biliary hyperplasia. This is highlighted by experiments with CCl_4, wherein classic BDEC hyperplasia does not occur, and liver fibrosis is similar in CCl_4–exposed WT and Fib$\gamma^{390\text{-}396A}$ mice. The disparate results from the studies with Fib$\gamma^{390\text{-}396A}$ mice challenged with ANIT or CCl_4 indicate that the role of fibrin(ogen)-integrin $\alpha_M\beta_2$ interaction is distinct and contingent upon the experimental model and ensuing pathology. These studies are also the first to examine the role of FXIII-mediated fibrin crosslinking in chronic ANIT-induced liver fibrosis. FXIII deficient mice displayed pathology distinctively different from Fib$\gamma^{390\text{-}396A}$ mice with worsened hepatocellular necrosis that occurred without any change in BDEC proliferation. Bearing in mind the protective function of platelet integrin $\alpha_{IIb}\beta_3$ in ANIT-induced liver necrosis, this highlights the likelihood that FXIII crosslinking might be working to inhibit hepatocellular injury through a pathway distinct from the fibrin(ogen)-integrin $\alpha_{IIb}\beta_3$ interaction. Emerging evidence indicates that macrophages, known to express $\alpha_M\beta_2$ integrin, play a role in fibrosis progression. Moreover, cytokines such as IFNγ, that can push macrophages towards a profibrotic phenotype, are increased in ANIT-exposed rodents. Anchored in strong *in vitro* studies with bone marrow derived macrophages and *in vivo* studies with IFN$\gamma^{-/-}$ mice, our findings reveal that IFNγ drives bile duct proliferation and liver fibrosis in ANIT-exposed mice in an iNOS dependent manner.

The outcome from studies with Fib$\gamma^{390\text{-}396A}$ mice that retain their hemostatic function, are extremely exciting because blocking the fibrin-$\alpha_M\beta_2$ interaction revealed a novel mechanism by which this interaction inhibits biliary hyperplasia and liver fibrosis. The clinical challenge with

targeting the coagulation cascade has been the risk for excessive bleeding or thrombosis/clotting. Therein lies the advantage of discovering this downstream pathway because in theory, this pathway could be targeted to elicit the protective effects without compromising hemostasis. As a proof of principle for this, we utilized the novel small molecule LA-1 to demonstrate that enhancing fibrin-$\alpha_M\beta_2$ engagement inhibits ANIT-induced liver fibrosis.

Taken together, these studies have profound paradigm-shifting implications for multiple reasons. First and foremost, it overturns the widely held assumption that the fibrin matrix uniformly drives hepatic inflammation and fibrosis. Furthermore, it does so by suggesting a novel mechanism whereby fibrin-$\alpha_M\beta_2$ engagement inhibits bile duct hyperplasia and liver fibrosis. Finally, these studies identify the fibrin-$\alpha_M\beta_2$ interaction as a putative therapeutic target for the reduction of biliary fibrosis without impacting hemostatic status. These findings necessitate an important positive shift in anti-fibrotic therapies, pinpoint novel drug targets (e.g., $\alpha_M\beta_2$), and inform repurposing of available coagulation-directed therapeutics in liver disease.

Future directions

The results from the TA studies indicate that clot lysis *ex vivo* was prolonged by TA in plasma of TA-treated mice, suggesting the possibility that TA reduces liver injury in this model by inhibiting plasmin-mediated fibrinolysis. Indeed, this is consistent with plasmin inhibition and similar to what is observed in humans treated with TA [13, 14]. Whether TA mediates its protective effects in chronic ANIT-induced injury in a fibrin dependent manner is not known. We did not examine whether TA treatment increased hepatic levels of stabilized fibrin polymer. Even with the right tools, interpretation of data showing alterations in fibrin deposition would be challenging. Ideally, we would like to measure fibrin deposition and anticipate that it would track with the

intervention (i.e. treatment with anti-fibrinolytic would increase fibrin deposition, whereas an anti-coagulant would decrease fibrin deposition). However if the anti-fibrinolytic drug reduces injury, as is the case with TA, then it would reduce fibrin deposition by blocking injury-driven coagulation. Nevertheless, additional studies are required to determine if fibrin is necessary for these protective effects of TA in this model. Possible approaches include administering TA to fibrin(ogen) deficient mice or mice expressing mutant fibrinogen^{AEK} (Fib^{AEK} mice). Fib^{AEK} mice carry a mutation in the thrombin cleavage site of the fibrinogen Aα-chain (Fib^{AEK} mice). These mice express normal levels of circulating fibrinogen, but the mutant fibrinogen^{AEK} is fully resistant to thrombin proteolytic cleavage and therefore cannot form polymers [15]. The results also do not completely delineate the mechanism whereby TA reduces liver pathology and hepatic collagen deposition in ANIT-exposed mice and this area of study requires further exploration.

Plasmin might be capable of mediating its effects in a fibrin(ogen) independent manner by promoting inflammatory cell activation [16] which contributes to liver necrosis in the bile duct ligation (BDL) model of cholestatic liver disease [17]. It is not known whether plasmin directly activates inflammatory cells in the chronic ANIT-experimental setting and further studies are necessary to investigate the mechanism by which plasmin modulates ANIT-induced liver injury. Plasmin is indeed known to cleave human PAR-1 [18] and higher plasmin concentrations can activate PAR-1 on human monocytes [19] and PAR-4 on platelets [20, 21]. Thus, the possibility that plasmin exerts its effects via PARs could be of interest and further studied.

The results with PAI-1 deficiency provide an important addition to a growing body of literature indicating dual roles for PAI-1 in different models of liver injury. Several studies have shown that liver fibrosis is reduced in PAI-1-deficient mice subjected to common bile duct ligation. However this reduction in liver fibrosis could be attributed to the prevention of hepatocellular

injury in PAI-1-deficient mice after BDL [22-24]. The mechanism whereby PAI-1 exacerbates injury after BDL was determined in elegant studies using genetic and pharmacologic approaches [24], to relate to tissue plasminogen activator (tPA)-driven hepatocyte growth factor activation, and seemingly independent from plasmin to be plasmin-independent. Additional studies are required to define the mechanisms underlying these divergent roles of PAI-1 in the different models of cholestatic liver injury.

Consistent with the *in vivo* studies highlighting the pivotal role of PAR-4, thrombin-mediated release of serotonin was suppressed with PAR-4 deficiency *ex vivo*. While these findings strongly link thrombin-mediated platelet activation to serotonin release in cholestatic liver disease, further studies are required to definitively elucidate if these changes in serotonin contribute to the increase in fibrosis observed in PAR-4$^{-/-}$ mice. It would also be interesting to explore the impact of hematopoietic cell PAR-4 deficiency on cholestatic liver disease. Indeed, hematopoietic cell PAR-4 deficiency did not affect acute acetaminophen toxicity [25]. Bone marrow transplant studies similar to Miyakawa *et al* could further inform this line of investigation.

The findings with PAR-4$^{-/-}$ and Fib$\gamma^{\Delta 5}$ mice provide the impetus for additional investigation into defining the intricacies whereby different platelet activators/mediators impact disease outcome after chronic cholestatic liver damage. In addition to serotonin, platelet activation by thrombin results in the release of other mediators such as adenosine diphosphate (ADP) which signals from the $P2Y_{12}$ chemoreceptor. This receptor is the target of anti-platelet drugs such as clopidogrel. In light of the dual role of platelet activation in liver injury, it would be important to examine the role of $P2Y_{12}$ receptor activation in cholestatic live injury. Whether combined deficiencies in multiple platelet activation pathways worsen chronic cholestatic liver disease is another line of investigation that is essential and could potentially aid in informing clinical studies

and further experimental studies. Unpublished studies from our lab suggest that clopidogrel administration exacerbates ANIT-induced liver fibrosis in wild-type but not PAR-4 deficient mice. It would be fascinating and informative to extend these studies and determine the impact of clopidogrel administration in ANIT-exposed Fib$\gamma^{\Delta 5}$ mice.

Patients with liver cirrhosis have been shown to exhibit altered levels of von Willebrand factor (vWF) [26]. vWF is an essential player in adhesion of platelets to the injured endothelium. Moreover, binding of vWF to glycoprotein Ib initiates signal transduction events leading to the activation of platelet integrin $\alpha_{IIb}\beta_3$. This enables the integrin to become competent to bind vWF or fibrin(ogen) and thus mediate platelet aggregation [27]. Seeing as to how important the actions of vWF are in platelet adhesion and activation, examining the role of vWF and its cleaving protein ADAMTS 13 in chronic cholestatic liver injury and fibrosis is an obvious extension of delving further into this and are the focus of ongoing studies in our lab.

ANIT-exposed Fib$\gamma^{\Delta 5}$ mice exhibited dramatic liver histopathology compared to PAR-4$^{-/-}$ mice with severe hepatocellular necrosis and deposition of collagen within the areas of necrosis. Considering the role of the fibrin(ogen)-integrin $\alpha_{IIb}\beta_3$ interaction in platelet aggregation, clot retraction and wound healing, further studies are required to investigate whether defective repair processes resulted in the exacerbated liver necrosis in ANIT-exposed Fib$\gamma^{\Delta 5}$ mice. In the same vein, *ex vivo* studies can be conducted utilizing platelet rich plasma from ANIT-exposed PAR4$^{-/-}$ and/or Fib$\gamma^{\Delta 5}$ mice and treating with clopidogrel or other platelet inhibitors.

ANIT-exposed FXIII-deficient mice displayed exacerbated hepatocellular necrosis. Fibrin(ogen) deposits are usually observed in the livers ANIT exposed WT mice in the peribiliary areas and within infrequent areas of necrosis. In contrast, fibrin(ogen) deposition within the frequent areas of necrosis in FXIII$^{-/-}$ mice appeared to be less intense, more diffuse and lacking a

defined pattern. Given that FXIII deficiency was associated with a delay in liver repair following CCl_4 challenge [28], it is likely that FXIII-mediated crosslinking is a prerequisite for the protective effect of the fibrin(ogen)-platelet $\alpha_{IIb}\beta_3$ integrin interaction. However, further studies are required to definitively establish this connection. Despite the dissimilar necrosis, like Fib$\gamma^{390\text{-}396A}$ mice, liver fibrosis increased in FXII$^{-/-}$ mice after ANIT exposure. Additional research is required to examine whether the fibrosis after FXIII deficiency occurs as a consequence of the increased liver necrosis. Whether FXIII crosslinking makes fibrin a better ligand for $\alpha_M\beta_2$ and what the precise role of FXIII is in liver fibrosis are other areas that require additional investigation. For these studies, it would be extremely useful to utilize the viable FXIII$^{-/-}$ mice that express $\gamma^{390\text{-}396A}$ fibrinogen available with Dr. Matthew Flick at Cincinnati Children's Hospital.

Collectively our studies highlight protective properties of fibrin(ogen) and its integrin-binding functions in experimental liver fibrosis. However, the molecular form of fibrin(ogen) required to inhibit ANIT-induced liver injury is not yet known. While soluble fibrin(ogen) and polymerized fibrin clots can both bind platelet integrin $\alpha_{IIb}\beta_3$, polymerized fibrin can bind leukocyte integrin $\alpha_M\beta_2$ and further examination of the role of fibrin polymer formation in peribiliary fibrosis is required. To achieve this, studies are currently underway in our lab that utilize Fib^{AEK} mice where fibrinogen is "locked" in the soluble form that has little affinity for $\alpha_M\beta_2$.

Hemostatic and inflammatory pathways are highly integrated with considerable regulatory cross talk. Macrophages have been identified as critical regulators of fibrosis with dichotomous roles. Distinct macrophage populations can exert unique functional activities that either initiate and promote liver fibrosis or resolve and regress it [29]. Moreover, macrophages are known to express $\alpha_M\beta_2$ integrin. Our *in vitro* and *in vivo* findings with bone marrow derived macrophages and IFN$\gamma^{-/-}$ mice indicate that a potential mechanism via which fibrin(ogen)- $\alpha_M\beta_2$ binding inhibits

fibrosis is by inhibiting IFNγ-mediated macrophage activation. However, additional studies are necessary to test this possibility, and other mechanisms cannot be excluded. For instance, NK cells are enriched in the liver, and have been demonstrated to play a crucial role in liver fibrosis. In animal models, NK cells have been shown to attenuate liver fibrosis [30]. However, it is not known whether fibrin(ogen) activation of $\alpha_M\beta_2$ integrin modifies the activity of NK cells in chronic liver disease. Considering that NK cells are known to express $\alpha_M\beta_2$ and that they are an important cellular source of IFNγ, it would be interesting to explore their role in ANIT-induced chronic cholestatic liver injury and peribiliary fibrosis. Further, in addition to its direct effects on NK cell activity, fibrinogen could modify NK cell function indirectly by modifying the activity of other $\alpha_M\beta_2$ expressing cells such as macrophages. Indeed, NK cell-stimulating cytokines are derived from other cell types, and IL-12 in particular has been shown to be sourced from macrophages [31]. It is plausible that fibrin stimulates macrophages via $\alpha_M\beta_2$ integrin to produce a factor (IL-12) that impacts NK cell activation. Further investigation into this line of studies could yield additional clues into the pathways by which fibrin-integrin $\alpha_M\beta_2$ binding affects liver fibrosis. Mast cells express integrin $\alpha_M\beta_2$ and increased number of mast cells have been shown to accumulate in the portal tracts of fibrotic livers from patients and rats exposed to CCl_4 [32, 33]. Mast cells are also known to secrete profibrotic mediators and components of the extracellular matrix. Furthermore, TGFβ has been shown to be synthesized by and be chemotactic for mast cells [34]. In conflicting studies by Terada *et al* that utilized mast cell deficient rats, these cells did not contribute to the progression of CCl_4-induced liver fibrosis [33]. Even so, considering how pathologies and mechanisms differ across different experimental settings, it is conceivable that mast cells might play a role in ANIT-induced liver chronic disease via fibrin-integrin $\alpha_M\beta_2$ binding.

An examination of the role of mast cells in chronic ANIT-induced liver fibrosis could lend more insight into the pathways that link hemostasis and inflammation.

The proof of principle studies with LA-1 highlight the protective role of fibrin- $\alpha_M\beta_2$ integrin binding in liver fibrosis. Furthermore, that LA-1 completely failed to reduce liver fibrosis in the Fib$\gamma^{390\text{-}396A}$ mice indicates that the anti-fibrotic function of LA-1 requires fibrin-$\alpha_M\beta_2$ binding. However, the exact mechanism by which LA-1 exerts its protective effects in chronic cholestatic liver disease are not known and necessitate investigation. Studies indicate that while plasma fibrinogen levels are reduced in patients with liver diseases of non-cholestatic origin, fibrinogen levels are preserved in patients with cholestatic liver disease and in our studies with ANIT exposure in mice. Indeed, mechanisms controlling the development of hepatic fibrosis depend on factors like cellular basis and etiology of liver injury. Thus, it is likely that the hemostatic changes accompanying liver diseases of various etiologies are also not uniform and fibrinogen levels differ based on disease etiology. This suggests the possibility of a unique role for fibrin(ogen) in the setting of cholestatic liver disease. In agreement, our studies indicate that expression of the rate-limiting FGB chain associated inversely with COL1A1 mRNA expression in livers from patients with PSC/PBC, but not in livers from patients with non-cholestatic liver disease. Of course, interpretation of this data must be made cautiously. Nevertheless, the basis of this difference is not known and additional studies are required to define the exact role of fibrin(ogen) in patients with cholestatic liver disease. While this would understandably be challenging, our results support a need for additional clinical analyses of fibrin(ogen) in patients with liver disease.

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