



Published in final edited form as:

Hepatology. 2021 June ; 73(6): 2397–2410. doi:10.1002/hep.31497.

Mast cells (MCs) induce ductular reaction mimicking liver injury in mice via MC-derived TGF- β 1 signaling

Konstantina Kyritsi^{2,*}, Lindsey Kennedy^{2,*}, Vik Meadows^{1,2}, Laura Hargrove⁵, Jennifer Demieville³, Linh Pham^{2,&}, Amelia Sybenga⁴, Debjyoti Kundu², Karla Cerritos², Fanyin Meng^{1,2}, Gianfranco Alpini^{1,2}, Heather Francis^{1,2}

¹Richard L. Roudebush VA Medical Center, Indiana University School of Medicine Research

²Division of Gastroenterology and Hepatology, Department of Medicine, Indiana University School of Medicine Research

³Central Texas Veterans Health Care System Texas A&M University-Central Texas

⁴UVM Health Network Texas A&M University-Central Texas

⁵Texas A&M University Health Science Center, Texas A&M University-Central Texas

Abstract

Background: Following liver injury, mast cells (MCs) migrate into the liver and are activated in cholestatic patients. Inhibition of MC mediators decreases ductular reaction (DR) and liver fibrosis. TGF- β 1 contributes to fibrosis and promotes liver disease.

Aim: To demonstrate that reintroduction of MCs induces cholestatic injury via TGF- β 1.

Methods: Wild-type, *Kit^{W-sh}* (MC-deficient), and *Mdr2^{-/-}* mice lacking l-histidine decarboxylase were injected with vehicle or PKH26-tagged murine MCs pretreated with 0.01% DMSO or the TGF- β R inhibitor, LY2109761 (TGF- β Ri, 10 μ M) three days prior to sac. Hepatic damage was assessed by H&E and serum chemistry. Injected MCs were detected in liver, spleen and lung by immunofluorescence (IF). DR was measured by CK-19 immunohistochemistry (IHC) and F4/80 staining coupled with *q*PCR for IL-1 β , IL-33 and F4/80; biliary senescence evaluated by IF or *q*PCR for p16, p18 and p21. Fibrosis was evaluated by Sirius Red/Fast Green staining and IF for SYP-9, desmin and α -SMA. TGF- β 1 secretion/expression was measured by EIA and *q*PCR. Angiogenesis was detected by IF for vonWillebrand Factor and VEGF-C *q*PCR. *In vitro*, MC TGF- β 1 expression/secretion were measured after TGF- β Ri treatment; conditioned medium was collected. Cholangiocytes and hepatic stellate cells (HSCs) were treated with MC conditioned medium and biliary proliferation/senescence was measured by MTS and *q*PCR; HSC activation evaluated for α -SMA, SYP-9 and collagen type-1a expression.

Results: MC injection recapitulates cholestatic liver injury characterized by increased DR; fibrosis/TGF- β 1 secretion; and angiogenesis. Injection of MC-TGF- β Ri reversed these parameters.

Address correspondence to: Heather Francis, Ph.D., FAASLD, Professor of Medicine, VA Research Career Scientist, Scientific Director, Indiana Center for Liver Research, Richard L. Roudebush VA Medical Center and Indiana University, Gastroenterology, Medicine, 1481 W 10th street, Indianapolis, IN 46202, heafranc@iu.edu.

*These authors contributed equally to this work

¤t address: Texas A&M University-Central Texas, Department of Science & Mathematics.

In vitro, MCs induce biliary proliferation/senescence and HSC activation that was reversed with MCs lacking TGF- β 1.

Conclusion: Our novel study demonstrates that reintroduction of MCs mimics cholestatic liver injury and MC-derived TGF- β 1 may be a target in chronic cholestatic liver disease.

Keywords

cholestasis; ductular reaction; inhibition; amelioration

Cholestatic liver injury is characterized by ductular reaction which is depicted by increased intrahepatic bile duct mass (IBDM), inflammation and biliary senescence (1). A number of rodent models are utilized to recapitulate cholestatic liver injury including bile duct ligation (BDL), carbon tetrachloride (CCl₄) treatment and genetic modifications, such as the multi-drug resistant mouse model, *Mdr2*^{-/-}, which mimics features of primary sclerosing cholangitis (PSC) (2–5). In *Mdr2*^{-/-} mice or following BDL or CCl₄ treatment, histamine (HA) levels increase and there is an upregulation of the hepatic expression of the synthesizing enzyme, 1-histidine-decarboxylase (HDC) and the H1 and H2 HA receptors (HRs) (3, 6–8).

Our previous work has demonstrated that, in various models of cholestatic liver injury, mast cells (MCs) increase in number and are found surrounding damaged bile ducts, which likely accounts for the significant upregulation of HA serum levels (7, 9, 10). In MC deficient mice (*Kit*^{W-sh}), reintroduction of cultured MCs increases ductular reaction and hepatic fibrosis thus mimicking cholestatic liver injury (10). Further, we have shown that when MCs are stabilized using cromolyn sodium treatment or depleted using a MC deficient mouse model, cholestatic liver injury is reversed implicating MCs as drivers of liver disease progression (6, 8, 10).

Along with increased MC infiltration, cholestatic liver injury is coupled with increased serum levels of TGF- β 1, which is a master regulator of hepatic fibrosis and is also a senescence-associated secretory phenotype (SASP) factor (4). The cellular source of TGF- β 1 during cholestatic liver injury is variable and a number of studies have demonstrated that hepatic stellate cells (HSCs) are the prime source for TGF- β 1 (11, 12), but studies have also shown that cholangiocytes secrete TGF- β 1 in response to damage (4). Aside from hepatic fibrosis, TGF- β 1 has been implicated in regulating biliary senescence and angiogenesis, both important features of cholestatic liver injury and ductular reaction (4, 13, 14). There is also a role for MCs in TGF- β 1 signaling and disease progression. In cardiac fibrosis it was found that MCs release TGF- β 1 upon activation and contribute to disease pathology and the differentiation of cardiac fibroblasts and macrophages, demonstrating the wide array of effects induced by MCs (15).

In our previous study, we demonstrated that injection of MCs into *Kit*^{W-sh} mice induced hepatic fibrosis and TGF- β 1 expression and secretion (10); however, we have not yet elucidated the direct effect of modulating TGF- β 1 in MCs during cholestatic liver injury. The aims of this study were to determine if (i) introduction of MCs into mice recapitulates cholestatic liver injury and (ii) MC-induced damage is regulated by MC-TGF- β 1 signaling.

Materials and Methods

Animal Models

Male wild-type C57BL/6J mice (WT) and *Kit^{W-sh}* (MC-deficient, C57BL/6J background) mice (10) were obtained from The Jackson Laboratory and colonies generated and maintained under approved current IACUC protocols at Indiana University, Indianapolis, IN and past protocols at Baylor Scott & White Healthcare, Temple, Texas. We used WT mice, which have very few to no resident MCs and *Kit^{W-sh}* mice, which are devoid of MCs and display normal morphological liver phenotypes (10). To evaluate if reintroduction of MCs induces liver damage, cultured MCs (5×10^6 cells/0.1 ml sterile 1X PBS) derived from fetal mouse liver (MC/9 (ATCC® CRL-8306™)) were tagged with the fluorescent linker, PKH26 prior to injection via tail vein as described by us (10). Male WT or *Kit^{W-sh}* mice (~10–12 weeks of age) were injected with either cultured MCs or 0.1ml sterile 1X PBS (control) three days prior to euthanasia. To demonstrate that MC-derived TGF- β 1 regulates liver damage, cultured MCs were treated with the specific TGF- β 1 receptor inhibitor, LY2109761 (TGF- β Ri, 10 μ M) (4), for up to 48 hrs prior to injection into *Kit^{W-sh}* mice. MCs were evaluated for TGF- β 1 expression and secretion following treatment with the TGF- β Ri prior to injection to confirm knockdown of TGF- β 1.

To evaluate our findings in an established cholestatic mouse model, we used male (12 wk of age) FVB/NJ (WT), *Mdr2^{-/-}* mice and a genetically modified double knockout mouse (DKO) generated by breeding *Mdr2^{-/-}* mice with l-histidine-decarboxylase knockout mice (*HDC^{-/-}*). We have recently demonstrated that DKO mice have decreased biliary damage, inflammation and hepatic fibrosis (16). In our current study, DKO mice were subjected to MC injections (control or lacking TGF- β 1, as described above) prior to euthanasia.

Liver Damage

In treated mice, liver damage was assessed by standard H&E staining (in paraffin-embedded liver sections, 4–5 μ m thick) along with serum chemistry for ALT, AST and ALP using IDEXX Catalyst One Chemistry Analyzer (IDEXX, Westbrook, ME) (17). H&E images were evaluated by a board-certified pathologist and were examined for lobular damage, necrosis, and inflammation.

Ductular Reaction, Biliary Proliferation/Damage and Inflammation

Cholestatic liver damage is characterized by increased ductular reaction and inflammation (1, 5); to demonstrate that the introduction of MCs into WT or *Kit^{W-sh}* mice (devoid of ductular reaction and inflammation (10)) recapitulates this damage, we performed CK-19 (to mark bile ducts) and F4/80 (Kupffer cell marker) immunohistochemistry in all groups of mice, and used a light microscope and VisioPharm® software to perform semi-quantification of staining (5, 7). Gene expression of inflammatory markers, interleukin (IL)-1 β , IL-33 and F4/80 were determined by *q*PCR in total liver mRNA.

Hepatic Fibrosis and Hepatic Stellate Cell Activation

Another hallmark feature of cholestatic liver damage is exacerbated hepatic fibrosis coupled with activation of hepatic stellate cells (HSCs) (17–19); therefore, we evaluated this in all

groups of mice by Sirius Red/Fast Green staining and semi-quantified using a light microscope and ImageJ software. To evaluate HSC activation, we performed immunofluorescence (co-stained with CK-19) for desmin, synaptophysin-9 (SYP-9) or α -SMA in frozen sections from all groups of mice. The gene expression of collagen type-1a and fibronectin-1 (FN-1) was determined in total liver by *q*PCR (6, 18).

TGF- β 1 expression and serum levels are increased in models of cholestatic liver injury, including *Mdr2*^{-/-} mice and following BDL, and are indicative of hepatic fibrosis (4, 7, 10). Our work has demonstrated that MCs may influence TGF- β 1 levels (6, 16); therefore, we measured the mRNA expression of TGF- β 1 in total liver from all groups of mice, as well as serum TGF- β 1 levels by EIA. Biliary expression of TGF- β 1 and TGF- β 1R was detected in WT, *Mdr2*^{-/-}, DKO and DKO mice injected with MCs (control and lacking TGF- β 1) by immunohistochemistry.

Biliary Senescence and Angiogenesis

Recent studies have focused on the role of biliary senescence during cholestatic liver injury, and a number of groups have demonstrated that, during PSC, there is increased biliary senescence, both in animal models and human disease (13, 14, 17). Biliary senescence was evaluated in WT, *Mdr2*^{-/-}, DKO and DKO mice injected with MCs (control and lacking TGF- β 1) by immunofluorescence for p16 and p18 (co-stained with CK-19) and by *q*PCR for p18 and p21 in total liver.

The role of angiogenesis during cholestatic liver injury has not been fully evaluated and it is not clear if TGF- β 1 regulates angiogenesis; however, a number of studies have demonstrated that there is an increase in angiogenic activity during PSC and it has been shown that VEGF-A and VEGF-C expression increases following BDL and in *Mdr2*^{-/-} mice (6, 7, 10). We have demonstrated that MCs regulate angiogenesis in PSC and cholangiocarcinoma (7); therefore, we measured these parameters in WT and *Kit*^{W-sh} mice injected with MCs. Liver sections were stained for vonWillebrand Factor (vWF, co-stained with CK-19) and *q*PCR was used to measure gene expression of VEGF-C (7).

In Vitro Studies

To demonstrate a direct interaction between MC-derived TGF- β 1 and cholangiocyte proliferation and senescence, we performed *in vitro* studies using mouse MCs (described above) and cholangiocytes (SV-40 immortalized cholangiocyte line (2, 10)). In addition, human HSCs (hHSCs, LX-2, ScienCell, Carlsbad, CA) (13) were used to evaluate activation and fibrotic markers. MCs (serum starved for 24 hrs) were treated with 0.01% DMSO (vehicle) or the TGF- β Ri (10 μ M) for up to 48 hours and pellets and conditioned medium were collected. Cholangiocytes (serum starved for 24 hrs) were stimulated with pretreated MCs (vehicle and TGF- β Ri) and proliferation was determined by MTS assay and senescence by *q*PCR for p16 and p18. hHSCs (serum starved for 24 hrs) were similarly treated with MC conditioned medium and we measured α -SMA and SYP-9 by immunofluorescence in cell smears (co-stained with DAPI) and collagen type-1a and SYP-9 by *q*PCR.

Statistical Analysis

All data is expressed as mean \pm SEM. Groups were analyzed by the Student unpaired t-test when two groups are analyzed or a two-way ANOVA when more than two groups are analyzed, followed by an appropriate post hoc test. $p < 0.05$ was considered significant.

Results

Validation of MC-TGF- β 1 Levels and MC Injection/Migration

Prior to injection of MCs into WT or *Kit^{W-sh}* mice, we evaluated the level of TGF- β 1 expression and secretion in our MC line. We found that MCs express and secrete TGF- β 1, both of which are decreased when MCs are treated with the TGF- β Ri (Figure 1A – 1B). Next, we assessed MC migration after tail vein injection in both WT and *Kit^{W-sh}* mice using immunofluorescence in liver, spleen and lung sections. We found that PKH26 labeled MCs (marked by white arrows) migrate to the liver and reside near bile ducts (stained with CK-19) (Figure 1C). No tagged MCs were identified in the spleen or lung in WT or *Kit^{W-sh}* mice (Figure 1D). DKO mice injected with MCs (treated with or without the TGF- β Ri) also resulted in migration to the liver (Figure 1E), but not spleen or lung (data not shown).

MCs Induce Liver Damage that is Ameliorated when MC-TGF- β 1 is Inhibited

The introduction of MCs into WT and *Kit^{W-sh}* mice induced lobular damage, necrosis and inflammation as shown by H&E (Figure 2A). Specifically, MC injection induced chronic inflammation around the periportal area (marked by black arrows and higher magnification images) and scattered necrotic hepatocytes were visualized. When *Kit^{W-sh}* mice were injected with MCs lacking TGF- β 1 signaling, liver damage was not noted (Figure 2A). Further, ALT levels (indicative of hepatocyte damage) increased in WT mice injected with MCs compared to controls; however, similar to our previous work (10), injection of MCs into *Kit^{W-sh}* mice did not alter ALT levels; injection of MC-TGF- β 1 did not alter ALT levels in *Kit^{W-sh}* mice (Figure 2B). ALP levels (indicative of biliary damage) were increased in both WT and *Kit^{W-sh}* mice injected with MCs and when *Kit^{W-sh}* mice were injected with MCs lacking TGF- β 1, ALP levels decreased (Figure 2C). These results demonstrate that (i) injection of MCs can induce liver damage, specifically targeting cholangiocytes (but not hepatocytes), that is typical of cholestatic liver injury and (ii) MC-TGF- β 1 is a key regulator of cholangiocyte damage.

Ductular Reaction and Inflammation Increase Following MC Injection Via MC-TGF- β 1

Similar to cholestatic liver injury found in *Mdr2^{-/-}* mice or after BDL (1), we found that ductular reaction and inflammation increase in mice injected with MCs. Specifically, by CK-19 immunohistochemistry and semi-quantification, there is a significant increase in intrahepatic bile duct mass (IBDM) in both WT and *Kit^{W-sh}* mice injected with MCs compared to controls. Further, IBDM was significantly decreased in *Kit^{W-sh}* mice injected with MCs lacking TGF- β 1 (Figure 3). The number of F4/80-positive Kupffer cells increased following MC injection in both WT and *Kit^{W-sh}* mice compared to controls; *Kit^{W-sh}* mice injected with MC-TGF- β 1 had a reduced number of inflammatory Kupffer cells (Supplemental Figure 1A). The mRNA expression of IL-1 β , IL-33 and F4/80 increased in

total liver samples from WT and *Kit^{W-sh}* mice injected with MCs compared to controls, but when *Kit^{W-sh}* mice were injected with MCs lacking TGF- β 1 these markers decreased (Supplemental Figure 1B). Our data show that MCs alone can drive biliary damage and inflammation in normal mice via MC-specific TGF- β 1 signaling.

Hepatic Fibrosis and HSC Activation/TGF- β 1 Signaling Increases Following MC Injection

Mdr2^{-/-} mice and mice subjected to BDL display aggravated hepatic fibrosis, whereas WT and *Kit^{W-sh}* mice have little to no collagen deposition (6, 7). In both WT and *Kit^{W-sh}* mice injected with MCs there was a gross accumulation of collagen surround the portal area, which is indicative of cholestatic liver injury as shown by Sirius Red/Fast Green staining and semi-quantification (Figure 4A–4B). Further, the gene expression of collagen type-1a and FN-1 followed a similar trend (Figure 4C) demonstrating that MC-derived TGF- β 1 regulates hepatic fibrosis.

SYP-9 and desmin protein expression was upregulated following MC injection in both WT and *Kit^{W-sh}* mice injected with MCs compared to controls demonstrating an activation of HSCs (Figure 5A–5B). When *Kit^{W-sh}* mice were injected with MC-TGF- β 1 (to block MC TGF- β 1 signaling), both hepatic fibrosis and HSC activation were decreased (Figures 4 and 5). Finally, staining for α -SMA demonstrated a similar trend showing that MC injection increased HSC activation that was reduced when mice were injected with MCs lacking TGF- β 1 (Supplemental Figure 2A).

TGF- β 1 expression and serum levels increase in WT and *Kit^{W-sh}* mice injected with MCs as shown by *q*PCR and EIA (Figure 6A – 6B). When *Kit^{W-sh}* mice were injected with MCs lacking TGF- β 1, there was decreased TGF- β 1 expression and secretion (Figure 6A – 6B). Taken together, these data suggest that injection of MCs into normal mice induces a cholestatic liver injury phenotype, which can be regulated by modulation of MC-TGF- β 1.

Liver Angiogenesis is Exacerbated in WT and *Kit^{W-sh}* Mice Injected with MCs

By immunofluorescence and *q*PCR, when WT or *Kit^{W-sh}* mice were injected with MCs, there was an increase in vWF protein expression (Supplemental Figure 3A) and VEGF-C gene expression (Supplemental Figure 3B), markers that are indicative of increased angiogenesis. This also supports previous work demonstrating that MCs regulate angiogenesis and vascular regrowth during liver injury. Again, when *Kit^{W-sh}* mice were injected with MCs lacking TGF- β 1, angiogenic markers decreased demonstrating a link between TGF- β 1 and angiogenesis via MC interaction (Supplemental Figure 3A–3B).

Injection of MCs into DKO Mice Increased Liver Damage, IBDM, Inflammation, Biliary Senescence and Hepatic Fibrosis via MC-TGF- β 1

Similar to our recently published study in DKO mice, we found that liver damage (Supplemental Figure 4A – 4B), IBDM and inflammation (Figure 7A – 7B), and biliary senescence (Supplemental Figure 5A–5C) were reduced in DKO mice compared to *Mdr2^{-/-}* mice. Further, hepatic fibrosis and HSC activation (Figure 8A – 8C and Supplemental Figure 2B) are decreased in DKO mice compared to *Mdr2^{-/-}* mice. When DKO mice are injected with control MCs, all of these parameters increased; however, when MCs are pretreated with

the TGF- β Ri and injected into DKO mice, liver damage, IBDM and inflammation, biliary senescence and hepatic fibrosis are all ameliorated.

By immunohistochemistry, we found that biliary TGF- β 1 and TGF- β 1R expression increases in *Mdr2*^{-/-} mice compared to WT that is depleted in DKO mice (Supplemental Figure 6A – 6B). When DKO mice are injected with control MCs, biliary TGF- β 1/TGF- β 1R expression is upregulated and in DKO mice injected with MCs lacking, expression decreases (Supplemental Figure 6A – 6B). Serum levels of TGF- β 1 followed a similar trend and were upregulated in *Mdr2*^{-/-} mice and in DKO mice injected with control MCs compared to WT; however, injection with MCs lacking TGF- β 1 decreased the level of TGF- β 1 in DKO mice (Supplemental Figure 6C). This data further confirms the role of MC-TGF- β 1 during cholestatic liver injury.

Inhibition of MC-TGF- β 1 Decreases Biliary Proliferation and HSC Activation, In Vitro

There was increased biliary proliferation shown by MTS (Supplemental Figure 7A) and senescence shown by *q*PCR (Supplemental Figure 7B) following treatment with MC control conditioned medium compared to no treatment; however, when MCs were pretreated with the TGF- β Ri and placed on cholangiocytes, proliferation and senescence decreased (Supplemental Figure 7A – 7B). Similarly, HSC activation increased following treatment with MC control conditioned medium that was decreased when MCs were pretreated with the TGF- β Ri as shown by immunofluorescence (Supplemental Figure 8A) and *q*PCR assay (Supplemental Figure 8B).

Discussion

In our current study we demonstrate that reintroduction of MCs into mice that have either a normal hepatic phenotype or are devoid of MCs induces cholestatic-like liver injury characterized by ductular reaction, inflammation, biliary senescence and hepatic fibrosis. Our study further reveals that MC-derived TGF- β 1 is a potent driver of this phenotype and may be a target for cholestatic liver disease therapy. Additionally, we found that MC injection targets biliary injury, but does not promote hepatocyte damage thus demonstrating a preference for MC-cholangiocyte interaction, which is supported by our previous work that shows infiltrating MCs reside near damaged human and mouse bile ducts and contribute to increased cholestatic liver damage (2, 6, 7, 10).

There are a number of cholestatic liver injury models that are currently used to study the progression of disease including the *Mdr2*^{-/-} mouse model of PSC and BDL, an obstructive cholestasis model (16, 20, 21). Our novel findings demonstrate that a single tail vein injection of cultured MCs into mice that have a normal liver phenotype induces a recapitulation of hepatic damage similar to that of established cholestatic models. Our study also pinpoints MCs as a critical component of hepatic injury exacerbation. In a model of hepatotoxicity induced from a chemotherapy drug, cyclophosphamide, stabilization of MCs using ketotifen ameliorated liver damage by lowering serum enzymes, reducing inflammation and inhibiting oxidative stress (22), thereby supporting our studies that MCs contribute to the pathophysiology of liver damage. In human non-alcoholic steatohepatitis (NASH), MC number was found to be correlated to the degree of liver damage and fibrosis

in patients (23). The authors found an increase in MC number as the stage of fibrosis worsened, which is indicative of the detrimental role that MCs play during disease. Aside from cholestatic liver injury, during NAFLD TGF- β 1 promotes liver fibrosis via IL-13 and levels of circulating TGF- β 1 are also increased in NAFLD and NASH patients (24, 25).

To support our findings that reintroduction of MCs into mice results in alterations of tissue or organ homeostasis, a study evaluating the role of MCs during irritable bowel syndrome (IBS) found that MC-deficient (W/W^V) mice given injections of MCs derived from wild-type or Ptg2^{Y385F} mice had exacerbated visceral hypersensitivity that was absent in mice lacking MCs (26). In addition, the authors found that patients with IBS had COX2 positive MCs within their colonic mucosa (26) further supporting that MCs are implicated in tissue damage and disease phenotype. In contrast to this study, another group demonstrated that reintroduction of bone marrow-derived MCs into *Kit*^{W-sh} mice crossed with IL-10^{-/-} mice resulted in amelioration of intestinal colitis and intestinal barrier injury suggesting an alternative role for MCs during IBS (27). Our current study found that a single injection of MCs drastically exacerbates ductular reaction including increasing IBDM and biliary senescence, in WT, *Kit*^{W-sh} and DKO mice, which was reversed when MC-TGF- β 1 was inhibited.

We have looked extensively at the role of MC-derived histamine and have used a number of models and targets to demonstrate the importance of this mediator during liver disease and damage. In our study we focused on the growth factor, TGF- β 1 since this factor has repeatedly been shown to contribute to biliary damage and hepatic fibrosis (4, 7, 14, 16). MCs are known to have both preformed mediators (like histamine) and other growth factors within their granules. In addition, MCs express a wide array of receptors and ligands on their surface which, upon stimulation, can be activated to release mediators into the tissue microenvironment (28, 29). A recent study from our group demonstrated that histamine interacts with TGF- β 1 via H1HR to contribute to inflammation and liver damage (16). Our study shows that MCs express and secrete TGF- β 1 and when MC-TGF- β 1 is blocked prior to injection into mice, there is a reversal of biliary damage, inflammation and hepatic fibrosis. In support of this, it has been found that MCs treated with TGF- β 1 release an increased amount of pro-inflammatory cytokines and IL-13 (30). Another study found that inhibition of mouse MC protease-4 (MC protease implicated in cardiovascular disease) decreased TGF- β 1 expression and downstream Smad signaling (31) and it was demonstrated that MC chymase induces hypertrophic scarring of skin and fibrosis via TGF- β 1 signaling pathways (32). In this latter study, the authors found that both type I and type II collagen synthesis increased with chymase treatment and this was coupled with enhanced TGF- β 1/Smad phosphorylation. Since, during cholestatic liver damage, the level of TGF- β 1 is elevated, there is a possibility that increased TGF- β 1 (secreted from both cholangiocytes and HSCs) may activate MCs, thus promoting damage. Our hypothesis is that MCs also contribute to increased TGF- β 1 levels and blocking this growth factor ameliorates MC-induced liver damage.

While targeting MC activation has been extensively studied during allergic and asthmatic related diseases (33), the potential for targeting MCs has been evaluated in other chronic human disease models (34). Aside from our studies demonstrating that MC stabilization

decreases cholestatic liver injury (6, 8), other work has identified targets such as stem cell factor (SCF) as described in a study focused on pulmonary remodeling. Here the authors found that inhibition of SCF using anti-SCF248 significantly reduced fibrosis associated with lung remodeling and there was a reported downregulation of c-Kit positive MCs found in treated mice (35). These studies are also supported by a recent study from our group demonstrating that blocking SCF decreases infiltrated MC number, hepatic fibrosis and inflammation in *Mdr2^{-/-}* mice again showing the critical role of MCs during disease progression (17). Further, in patients with biliary atresia (BA), there is increased MC number that had elevated levels of ST-2 receptor coupled with increased IL-13 levels, which positively correlated to patients with a worse prognosis (36).

TGF- β 1 has been repeatedly found to play a role in driving liver fibrosis in a number of models and it has been demonstrated by Wu, et al. that both TGF- β 1 and TGF- β 1R are upregulated in patients with PSC (4), thus marking this growth factor as a detrimental regulator of disease progression. The level of circulating TGF- β 1 is upregulated in a number of models, including *Mdr2^{-/-}* mice and following BDL, and the prime source of TGF- β 1 during fibrosis has been assumed and demonstrated to be HSCs (37); however, more studies are also implicating cholangiocytes and other residential liver cells. The level of TGF- β 1 held inside MCs is difficult to ascertain since MCs are immature during migration and do not fully mature and activate until they have reached their destination (28). Further, many of the mediators released by MCs are regulated by the proteases contained within granules, including chymase and/or tryptase. A study found that peritoneal MCs from rats synthesize and store copious amounts of TGF- β 1 that was released when MCs were activated with a single injection of compound 48/80 (MC activator). The increase in TGF- β 1 was also associated with increased chymase 1 expression, thus implicating this protease in the regulation of TGF- β 1 (38). In our study, cultured MCs were stimulated with T-STIM prior to introduction into mice and we have also found that the addition of cultured MCs conditioned medium to either cholangiocytes or HSCs causes an upregulation of TGF- β 1 expression and secretion, thus suggesting that MCs are an additional source of TGF- β 1.

In conclusion, we demonstrate the novel findings that introduction of MCs into phenotypically normal mice induces biliary damage/senescence, ductular reaction, inflammation, angiogenesis and hepatic fibrosis which are all indicative of cholestatic liver injury. Our results further demonstrate that MC-derived TGF- β 1 is a critical regulator of hepatobiliary damage and blocking this growth factor ameliorated all features of cholestatic liver injury. While we recognize that our study is descriptive by nature, future studies are currently being performed to understand the signaling mechanisms that coordinate MC-derived TGF- β 1-induced ductular reaction including evaluating the role of IL-13 during liver inflammation and fibrosis since this interleukin has been implicated in other diseases; however, our findings reveal a significant role for MC TGF- β 1 during liver injury that should be fully evaluated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: The studies were supported by a SRCS Award (5I01BX000574, GA), an RCS VA Merit Award (1I01BX003031, HF) and VA Merit 1I01BX001724 (FM) from the United States Department of Veteran's Affairs, Biomedical Laboratory Research and Development Service and NIH NIDDK grants (DK108959 and DK119421, HF and DK115184 and DK076898, GA and FM). Portions of the work were also supported by the Indiana University Strategic Research Initiative (HF, GA, FM) and the Hickam Endowed Chair (GA).

Disclosures: This material is the result of work supported by resources at the Central Texas Veterans Health Care System and Richard L. Roudebush VA Medical Center. The content is the responsibility of the author(s) alone and does not necessarily reflect the views or policies of the Department of Veterans Affairs or the United States Government.

Abbreviations:

ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
α-SMA	alpha smooth muscle actin
BDL	bile duct ligation
CCl₄	carbon tetrachloride
CK-19	cytokeratin 19
c-kit	KIT proto-oncogene receptor tyrosine kinase
DKO	double knockout
EIA	enzyme immunoassay
H&E	hematoxylin and eosin
HSC	hepatic stellate cell
hHSC	human hepatic stellate cell
IBDM	intrahepatic bile duct mass
IL-1β	interleukin 1 beta
IL-33	interleukin 33
<i>Kit</i>^{W-sh}	mast cell deficient mice
MC	mast cell
Mdr2^{-/-}	multidrug resistance transporter 2/ ABC transporter B family member 2 knock out
p16	cyclin-dependent kinase inhibitor 2A
p18	Cyclin-dependent kinase 4 inhibitor C

PSC	primary sclerosing cholangitis
qPCR	quantitative PCR
SCF	stem cell factor
SYP-9	synaptophysin 9
TGF-β1	transforming growth factor beta 1
TGF-βRi	TGF- β 1 receptor inhibitor
TVI	tail vein injection
VEGF-C	vascular endothelial growth factor C
vWF	vonWillebrand Factor
WT	wild type

References

1. Sato K, Marzioni M, Meng F, Francis H, Glaser S, Alpini G. Ductular Reaction in Liver Diseases: Pathological Mechanisms and Translational Significances. *Hepatology* 2019;69:420–430. [PubMed: 30070383]
2. Graf A, Meng F, Hargrove L, Kennedy L, Han Y, Francis T, Hodges K, et al. Knockout of histidine decarboxylase decreases bile duct ligation-induced biliary hyperplasia via downregulation of the histidine decarboxylase/VEGF axis through PKA-ERK1/2 signaling. *Am J Physiol Gastrointest Liver Physiol* 2014;307:G813–823. [PubMed: 25169977]
3. Johnson C, Hargrove L, Graf A, Kennedy L, Hodges K, Harris R, Francis T, et al. Histamine restores biliary mass following carbon tetrachloride-induced damage in a cholestatic rat model. *Dig Liver Dis* 2015;47:211–217. [PubMed: 25575430]
4. Wu N, Meng F, Zhou T, Venter J, Giang TK, Kyritsi K, Wu C, et al. The Secretin/Secretin Receptor Axis Modulates Ductular Reaction and Liver Fibrosis through Changes in Transforming Growth Factor-beta1-Mediated Biliary Senescence. *Am J Pathol* 2018;188:2264–2280. [PubMed: 30036520]
5. Zhou T, Kyritsi K, Wu N, Francis H, Yang Z, Chen L, O'Brien A, et al. Knockdown of vimentin reduces mesenchymal phenotype of cholangiocytes in the *Mdr2*($-/-$) mouse model of primary sclerosing cholangitis (PSC). *EBioMedicine* 2019;48:130–142. [PubMed: 31522982]
6. Jones H, Hargrove L, Kennedy L, Meng F, Graf-Eaton A, Owens J, Alpini G, et al. Inhibition of mast cell-secreted histamine decreases biliary proliferation and fibrosis in primary sclerosing cholangitis *Mdr2*($-/-$) mice. *Hepatology* 2016;64:1202–1216. [PubMed: 27351144]
7. Kennedy L, Hargrove L, Demieville J, Karstens W, Jones H, DeMorrow S, Meng F, et al. Blocking H1/H2 histamine receptors inhibits damage/fibrosis in *Mdr2*($-/-$) mice and human cholangiocarcinoma tumorigenesis. *Hepatology* 2018;68:1042–1056. [PubMed: 29601088]
8. Kennedy LL, Hargrove LA, Graf AB, Francis TC, Hodges KM, Nguyen QP, Ueno Y, et al. Inhibition of mast cell-derived histamine secretion by cromolyn sodium treatment decreases biliary hyperplasia in cholestatic rodents. *Lab Invest* 2014;94:1406–1418. [PubMed: 25365204]
9. Hargrove L, Graf-Eaton A, Kennedy L, Demieville J, Owens J, Hodges K, Ladd B, et al. Isolation and characterization of hepatic mast cells from cholestatic rats. *Lab Invest* 2016;96:1198–1210. [PubMed: 27548803]
10. Hargrove L, Kennedy L, Demieville J, Jones H, Meng F, DeMorrow S, Karstens W, et al. Bile duct ligation-induced biliary hyperplasia, hepatic injury, and fibrosis are reduced in mast cell-deficient *Kit*(W-sh) mice. *Hepatology* 2017;65:1991–2004. [PubMed: 28120369]

11. Dewidar B, Meyer C, Dooley S, Meindl-Beinker AN. TGF-beta in Hepatic Stellate Cell Activation and Liver Fibrogenesis-Updated 2019. *Cells* 2019;8.
12. Fan W, Liu T, Chen W, Hammad S, Longerich T, Hausser I, Fu Y, et al. ECM1 Prevents Activation of Transforming Growth Factor beta, Hepatic Stellate Cells, and Fibrogenesis in Mice. *Gastroenterology* 2019;157:1352–1367 e1313.
13. Wan Y, Ceci L, Wu N, Zhou T, Chen L, Venter J, Francis H, et al. Knockout of alpha-calcitonin gene-related peptide attenuates cholestatic liver injury by differentially regulating cellular senescence of hepatic stellate cells and cholangiocytes. *Lab Invest* 2019;99:764–776. [PubMed: 30700848]
14. Zhou T, Wu N, Meng F, Venter J, Giang TK, Francis H, Kyritsi K, et al. Knockout of secretin receptor reduces biliary damage and liver fibrosis in *Mdr2(-/-)* mice by diminishing senescence of cholangiocytes. *Lab Invest* 2018;98:1449–1464. [PubMed: 29977037]
15. Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. *Cell Mol Life Sci* 2014;71:549–574. [PubMed: 23649149]
16. Kennedy L, Meadows V, Demieville J, Hargrove L, Virani S, Glaser S, Zhou T, et al. Biliary damage and liver fibrosis are ameliorated in a novel mouse model lacking l-histidine decarboxylase/histamine signaling. *Lab Invest* 2020.
17. Meadows V, Kennedy L, Hargrove L, Demieville J, Meng F, Virani S, Reinhart E, et al. Downregulation of hepatic stem cell factor by *Vivo*-Morpholino treatment inhibits mast cell migration and decreases biliary damage/senescence and liver fibrosis in *Mdr2(-/-)* mice. *Biochim Biophys Acta Mol Basis Dis* 2019;1865:165557. [PubMed: 31521820]
18. Kyritsi K, Meng F, Zhou T, Wu N, Venter J, Francis H, Kennedy L, et al. Knockdown of Hepatic Gonadotropin-Releasing Hormone by *Vivo*-Morpholino Decreases Liver Fibrosis in Multidrug Resistance Gene 2 Knockout Mice by Down-Regulation of miR-200b. *Am J Pathol* 2017;187:1551–1565. [PubMed: 28502477]
19. Meng F, Kennedy L, Hargrove L, Demieville J, Jones H, Madeka T, Karstens A, et al. Ursodeoxycholate inhibits mast cell activation and reverses biliary injury and fibrosis in *Mdr2(-/-)* mice and human primary sclerosing cholangitis. *Lab Invest* 2018;98:1465–1477. [PubMed: 30143751]
20. Mariotti V, Strazzabosco M, Fabris L, Calvisi DF. Animal models of biliary injury and altered bile acid metabolism. *Biochim Biophys Acta Mol Basis Dis* 2018;1864:1254–1261. [PubMed: 28709963]
21. Van Campenhout S, Van Vlierberghe H, Devisscher L. Common Bile Duct Ligation as Model for Secondary Biliary Cirrhosis. *Methods Mol Biol* 2019;1981:237–247. [PubMed: 31016658]
22. Abdelzاهر WY, AboBakr Ali AHS, El-Tahawy NFG. Mast cell stabilizer modulates Sirt1/Nrf2/TNF pathway and inhibits oxidative stress, inflammation, and apoptosis in rat model of cyclophosphamide hepatotoxicity. *Immunopharmacol Immunotoxicol* 2020;42:101–109. [PubMed: 32066295]
23. Lombardo J, Broadwater D, Collins R, Cebe K, Brady R, Harrison S. Hepatic mast cell concentration directly correlates to stage of fibrosis in NASH. *Hum Pathol* 2019;86:129–135. [PubMed: 30597154]
24. Hart KM, Fabre T, Scirba JC, Gieseck RL 3rd, Borthwick LA, Vannella KM, Acciani TH, et al. Type 2 immunity is protective in metabolic disease but exacerbates NAFLD collaboratively with TGF-beta. *Sci Transl Med* 2017;9.
25. Yilmaz Y, Eren F. Serum biomarkers of fibrosis and extracellular matrix remodeling in patients with nonalcoholic fatty liver disease: association with liver histology. *Eur J Gastroenterol Hepatol* 2019;31:43–46. [PubMed: 30134384]
26. Grabauskas G, Wu X, Gao J, Li JY, Turgeon DK, Owyang C. Prostaglandin E2, Produced by Mast Cells in Colon Tissues from Patients with Irritable Bowel Syndrome, Contributes to Visceral Hypersensitivity in Mice. *Gastroenterology* 2020.
27. Lennon EM, Borst LB, Edwards LL, Moeser AJ. Mast Cells Exert Anti-Inflammatory Effects in an *IL10(-/-)* Model of Spontaneous Colitis. *Mediators Inflamm* 2018;2018:7817360.
28. Halova I, Draberova L, Draber P. Mast cell chemotaxis - chemoattractants and signaling pathways. *Front Immunol* 2012;3:119. [PubMed: 22654878]

29. Sibilano R, Frossi B, Pucillo CE. Mast cell activation: a complex interplay of positive and negative signaling pathways. *Eur J Immunol* 2014;44:2558–2566. [PubMed: 25066089]
30. Lyons DO, Plewes MR, Pullen NA. Soluble transforming growth factor beta-1 enhances murine mast cell release of Interleukin 6 in IgE-independent and Interleukin 13 in IgE-dependent settings in vitro. *PLoS One* 2018;13:e0207704.
31. Wang Y, Liu CL, Fang W, Zhang X, Yang C, Li J, Liu J, et al. Deficiency of mouse mast cell protease 4 mitigates cardiac dysfunctions in mice after myocardium infarction. *Biochim Biophys Acta Mol Basis Dis* 2019;1865:1170–1181. [PubMed: 30639224]
32. Chen H, Xu Y, Yang G, Zhang Q, Huang X, Yu L, Dong X. Mast cell chymase promotes hypertrophic scar fibroblast proliferation and collagen synthesis by activating TGF-beta1/Smads signaling pathway. *Exp Ther Med* 2017;14:4438–4442. [PubMed: 29104654]
33. Nakajima S, Ishimaru K, Kobayashi A, Yu G, Nakamura Y, Oh-Oka K, Suzuki-Inoue K, et al. Resveratrol inhibits IL-33-mediated mast cell activation by targeting the MK2/3-PI3K/Akt axis. *Sci Rep* 2019;9:18423. [PubMed: 31804564]
34. Lyons DO, Pullen NA. Beyond IgE: Alternative Mast Cell Activation Across Different Disease States. *Int J Mol Sci* 2020;21.
35. Rasky A, Habiel DM, Morris S, Schaller M, Moore BB, Phan S, Kunkel SL, et al. Inhibition of the stem cell factor 248 isoform attenuates the development of pulmonary remodeling disease. *Am J Physiol Lung Cell Mol Physiol* 2020;318:L200–L211. [PubMed: 31747308]
36. Liu J, Yang Y, Zheng C, Chen G, Shen Z, Zheng S, Dong R. Correlation of Interleukin-33/ST2 Receptor and Liver Fibrosis Progression in Biliary Atresia Patients. *Front Pediatr* 2019;7:403. [PubMed: 31632941]
37. Aimaiti Y, Yusufkadier M, Li W, Tuerhongjiang T, Shadike A, Meiheryayi A, Gulisitan, et al. TGF-beta1 signaling activates hepatic stellate cells through Notch pathway. *Cytotechnology* 2019;71:881–891. [PubMed: 31372876]
38. Lindstedt KA, Wang Y, Shiota N, Saarinen J, Hyytiainen M, Kokkonen JO, Keski-Oja J, et al. Activation of paracrine TGF-beta1 signaling upon stimulation and degranulation of rat serosal mast cells: a novel function for chymase. *FASEB J* 2001;15:1377–1388. [PubMed: 11387235]

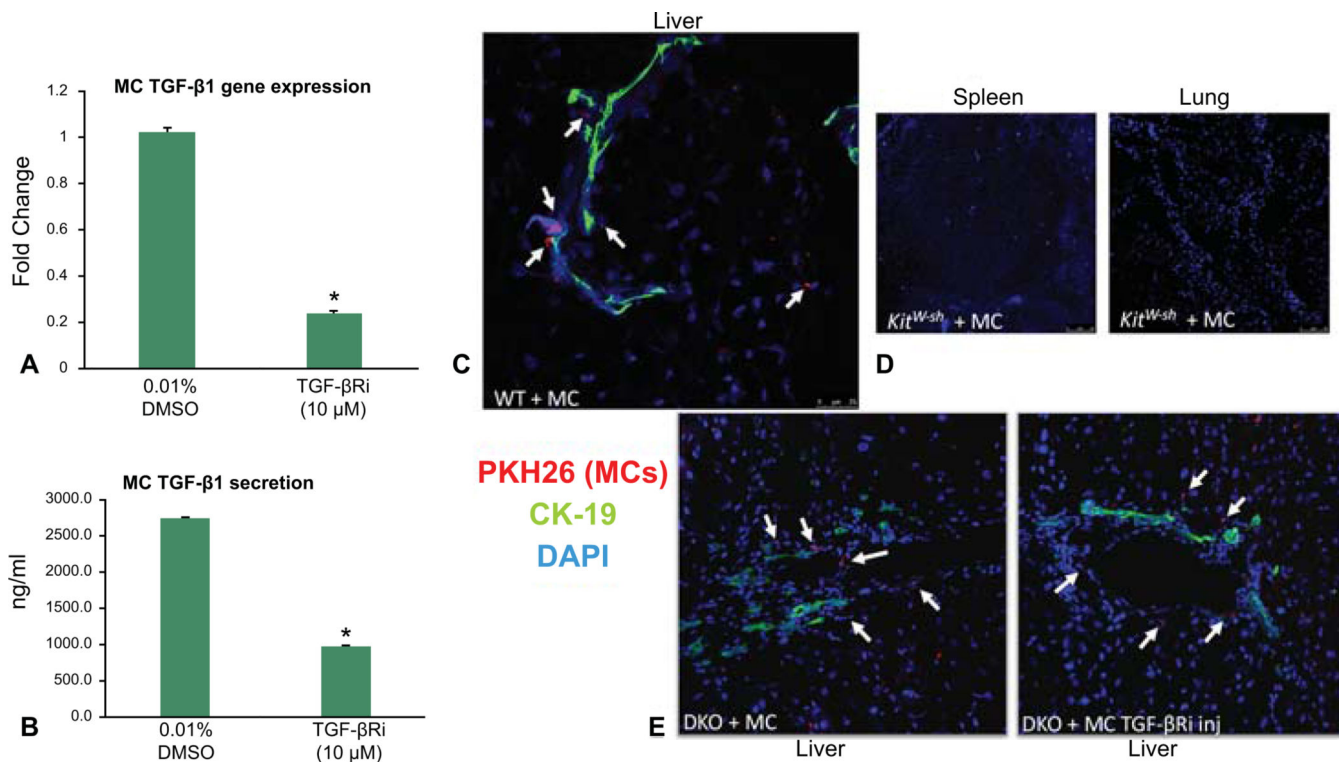


Figure 1. Model verification. Prior to injection of MCs into WT or *Kit^{W-sh}* mice, we found that MCs express and secrete TGF- β 1, both of which are decreased when MCs are treated with the TGF- β Ri as shown by *q*PCR and EIA, respectively (**A – B**). MC migration was evaluated after tail vein injection in both WT and *Kit^{W-sh}* mice using immunofluorescence in liver, spleen and lung sections. PKH26 labeled MCs (marked with white arrows) migrate to the liver and reside near bile ducts (stained with CK-19) (**C**). No tagged MCs were identified in the lung or spleen in WT or *Kit^{W-sh}* mice (**D**). DKO mice injected with MCs (treated with or without the TGF- β Ri) also resulted in migration to the liver (**E**). White arrows mark PKH26 positive MCs. Data are mean \pm SEM of 9 experiments for *q*PCR and 12 for EIA. **p*<0.05 vs. 0.01% DMSO-treated MCs. Images are 20x.

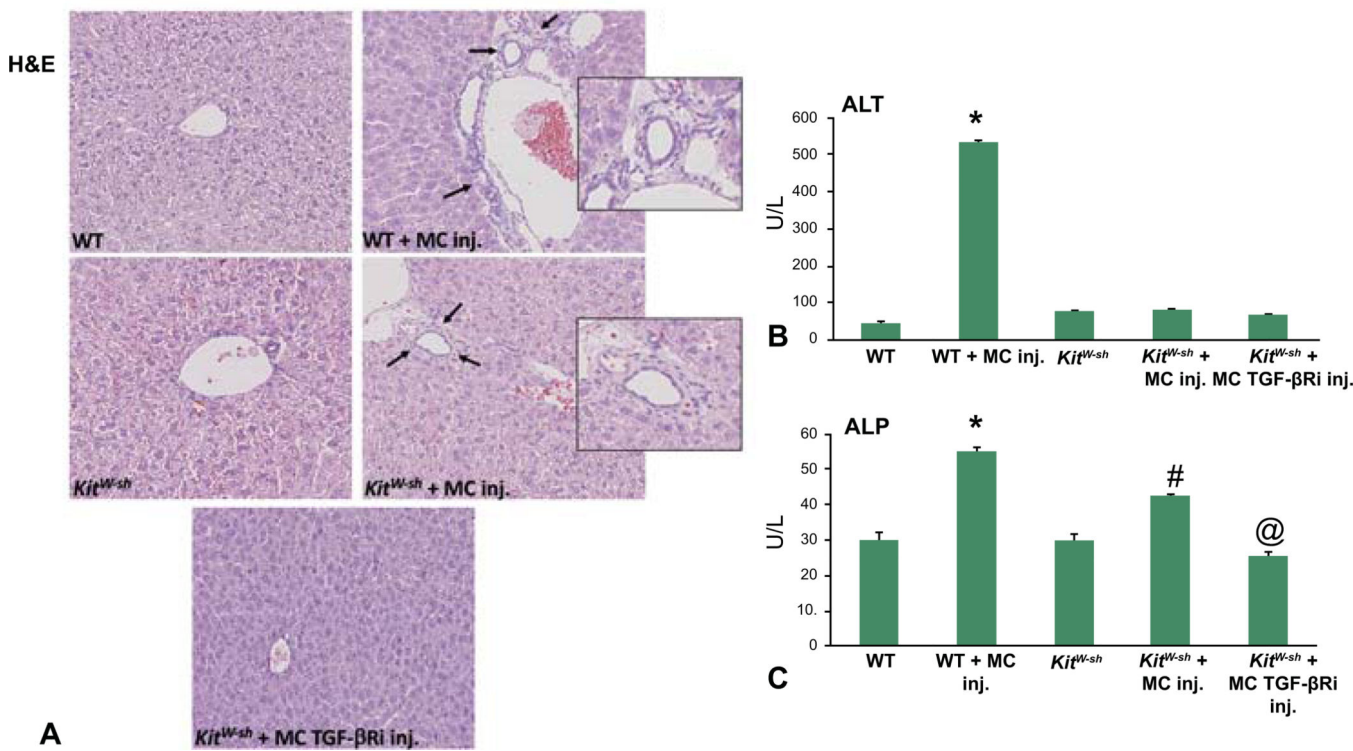


Figure 2. Hepatic damage was evaluated by H&E staining and serum chemistry. The introduction of MCs into WT and *Kit^{W-sh}* mice induced lobular damage, necrosis and inflammation as shown by H&E (marked by black arrows) and when *Kit^{W-sh}* mice were injected with MCs lacking TGF-β1 signaling, liver damage was ameliorated (A). ALT levels (indicative of hepatocyte damage) increased in WT mice injected with MCs compared to controls; however, injection of MCs into *Kit^{W-sh}* mice did not alter ALT levels and injection of MC-TGF-β1 did not alter ALT levels in *Kit^{W-sh}* mice (B). ALP levels (indicative of biliary damage) were increased in both WT and *Kit^{W-sh}* mice injected with MCs and when *Kit^{W-sh}* mice were injected with MCs lacking TGF-β1, ALP levels decreased (C). Data are mean ± SEM of 6 experiments for ALT and ALP levels. *p<0.05 vs. WT mice; #p<0.05 vs. *Kit^{W-sh}* mice; or @p<0.05 vs. *Kit^{W-sh}* mice injected with MCs. Images are 10x, inset boxes 60x.

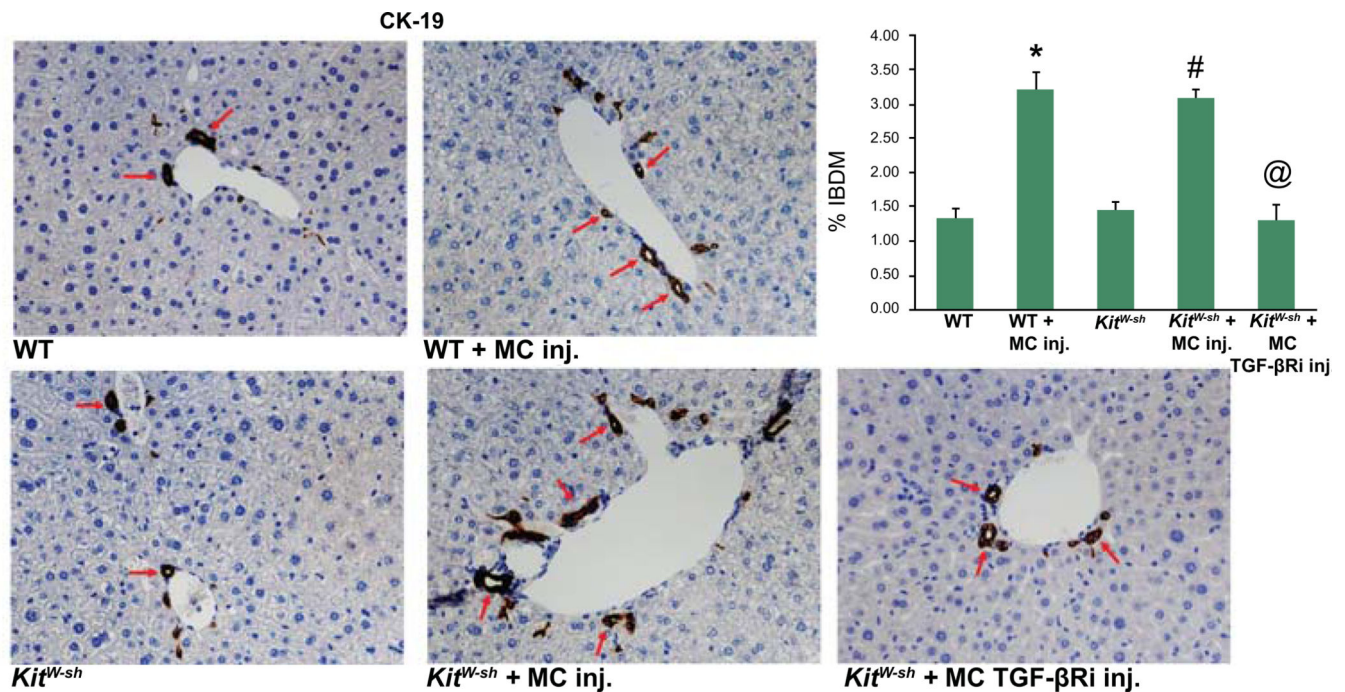


Figure 3.

Evaluation of Intrahepatic Bile Duct Mass (IBDM). Biliary mass was measured by CK-19 immunohistochemistry and semi-quantification. There was a significant increase in IBDM in both WT and *Kit*^{W-sh} mice injected with MCs compared to controls (no changes were found between control groups). IBDM was significantly decreased in *Kit*^{W-sh} mice injected with MCs lacking TGF-β1. Red arrows indicate bile ducts. Data are mean ± SEM of 10 experiments from 4–6 different mice. *p < 0.05 vs. WT mice; #p < 0.05 vs. *Kit*^{W-sh} mice; or @p < 0.05 vs. *Kit*^{W-sh} mice injected with MCs. Images are 40x.

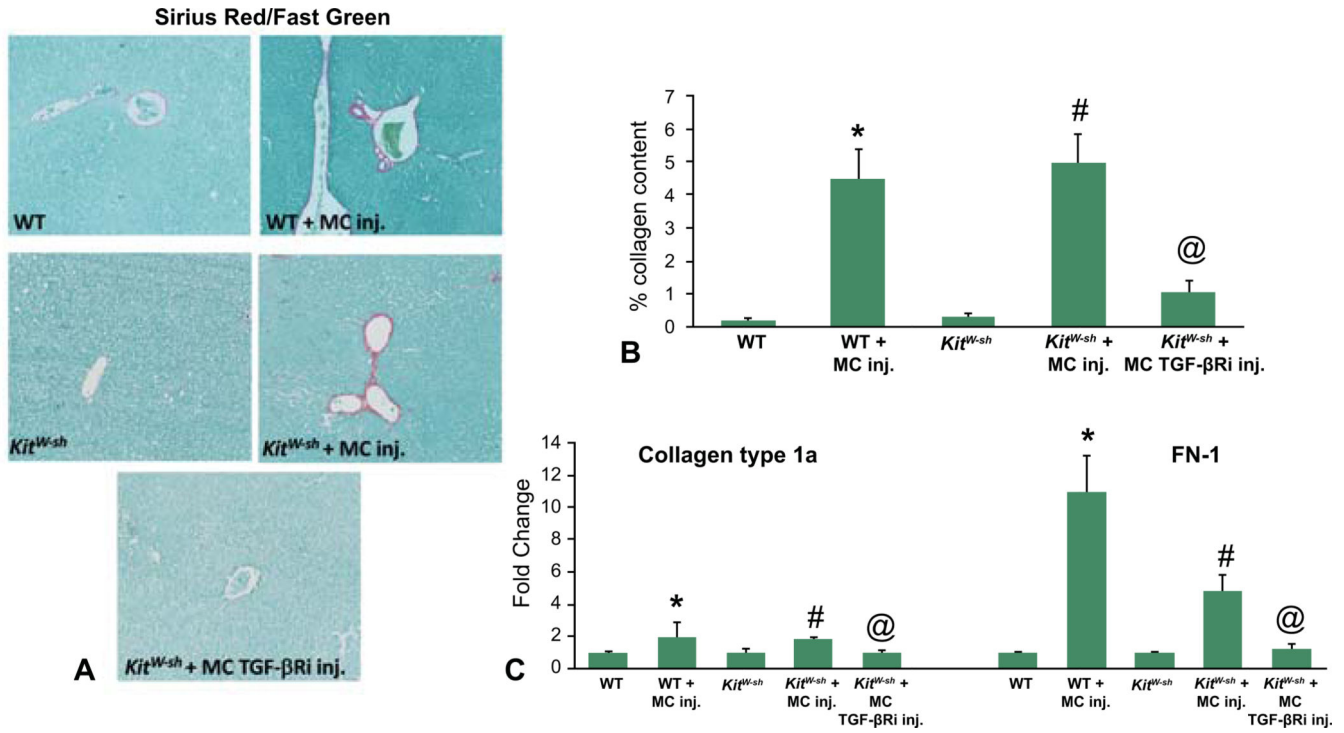


Figure 4. Hepatic Fibrosis Evaluation. In both WT and *Kit^{W-sh}* mice injected with MCs, there was a gross accumulation of collagen surround the portal area, which is indicative of cholestatic liver injury as shown by (A) Sirius Red/Fast Green staining and (B) semi-quantification. (A–B) When *Kit^{W-sh}* mice were injected with MCs lacking TGF-β1, collagen deposition was significantly reduced. By qPCR, gene expression of collagen type-1a and FN-1 increase in both WT and *Kit^{W-sh}* injected with MCs that was reduced when mice were injected with MCs lacking TGF-β1 (C). Data are mean ± SEM of 10 experiments from 4–6 different mice for staining and 12 experiments for qPCR. *p<0.05 vs. WT mice; #p<0.05 vs. *Kit^{W-sh}* mice; or @p<0.05 vs. *Kit^{W-sh}* mice injected with MCs. Images are 20x.

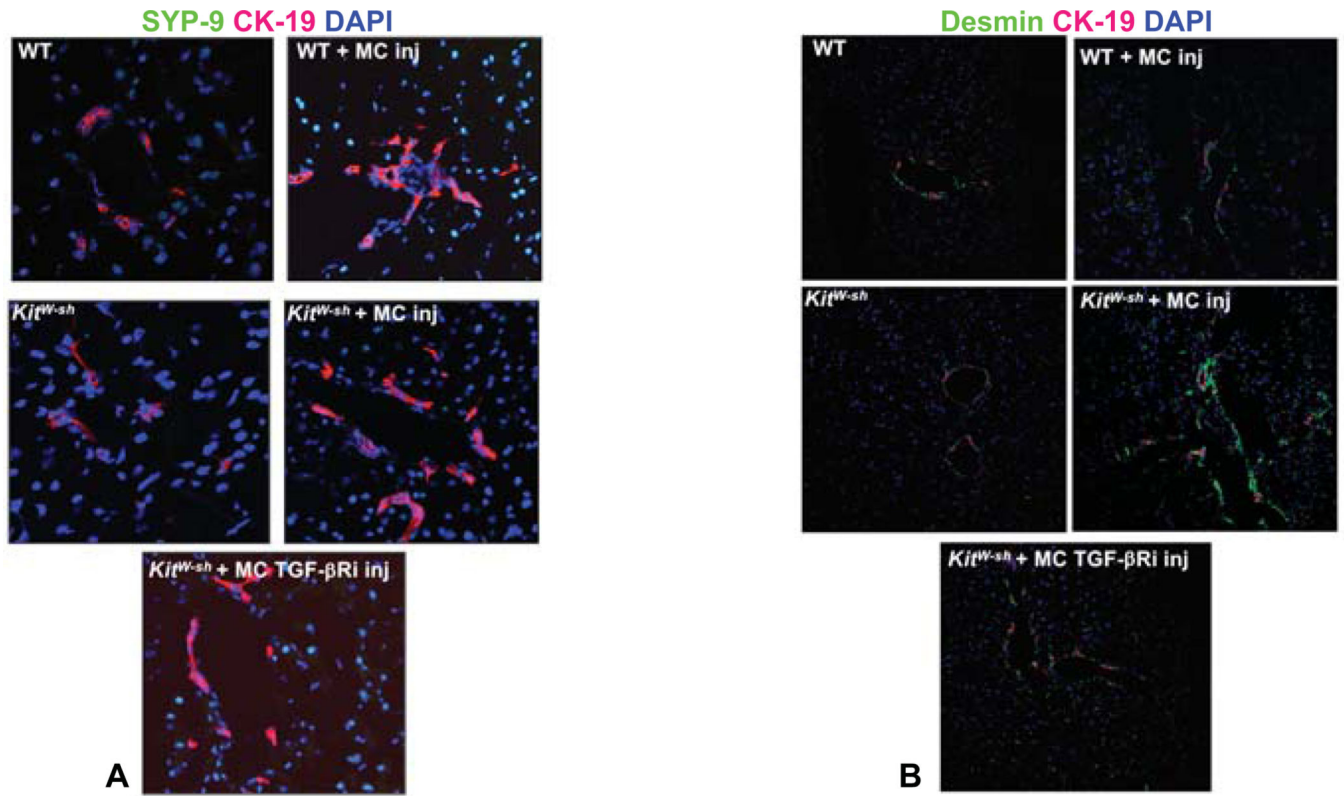


Figure 5.

HSC Activation. (A) SYP-9 and (B) desmin protein expression shown by immunofluorescence (co-stained with CK-19) was upregulated following MC injection in both WT and *Kit^{W-sh}* mice compared to controls demonstrating an activation of HSCs. When *Kit^{W-sh}* mice were injected with MC-TGF- β Ri (to block MC TGF- β 1 signaling), HSC activation decreased. Images are 40x and 20x, respectively.

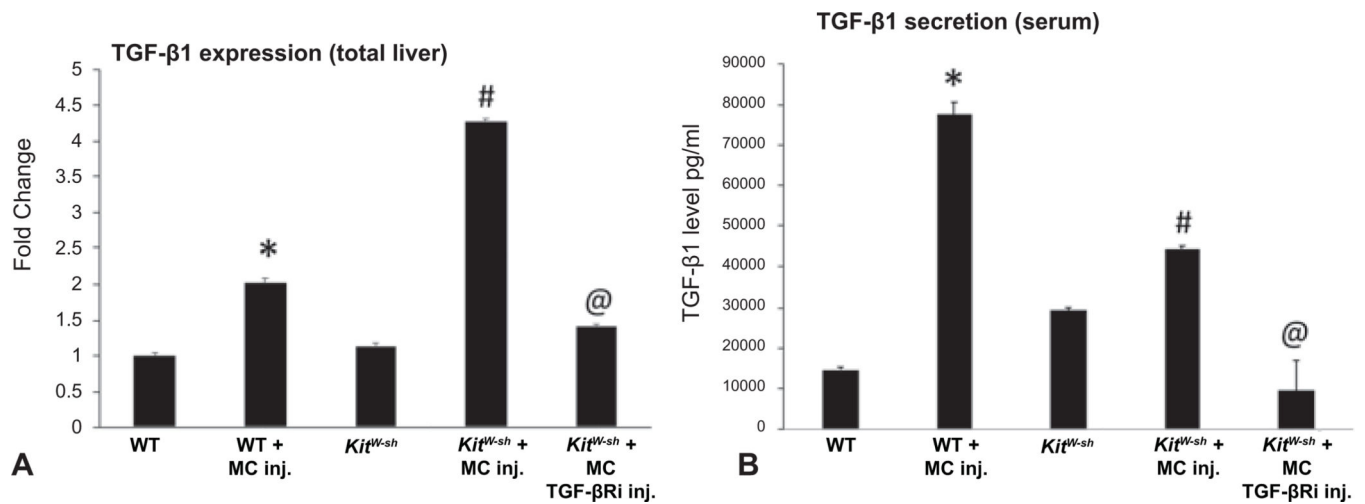


Figure 6. TGF-β1 Signaling. (A) TGF-β1 expression and (B) serum levels increase in WT and *Kit^{W-sh}* mice injected with MCs as shown by *q*PCR and EIA, respectively. (A-B) When *Kit^{W-sh}* mice injected were injected with MCs lacking TGF-β1, there was decreased TGF-β1 expression and secretion. Data are mean ± SEM of 6 experiments for *q*PCR and 12 experiments for EIA. * $p < 0.05$ vs. WT mice; # $p < 0.05$ vs. *Kit^{W-sh}* mice; or @ $p < 0.05$ vs. *Kit^{W-sh}* mice injected with MCs.

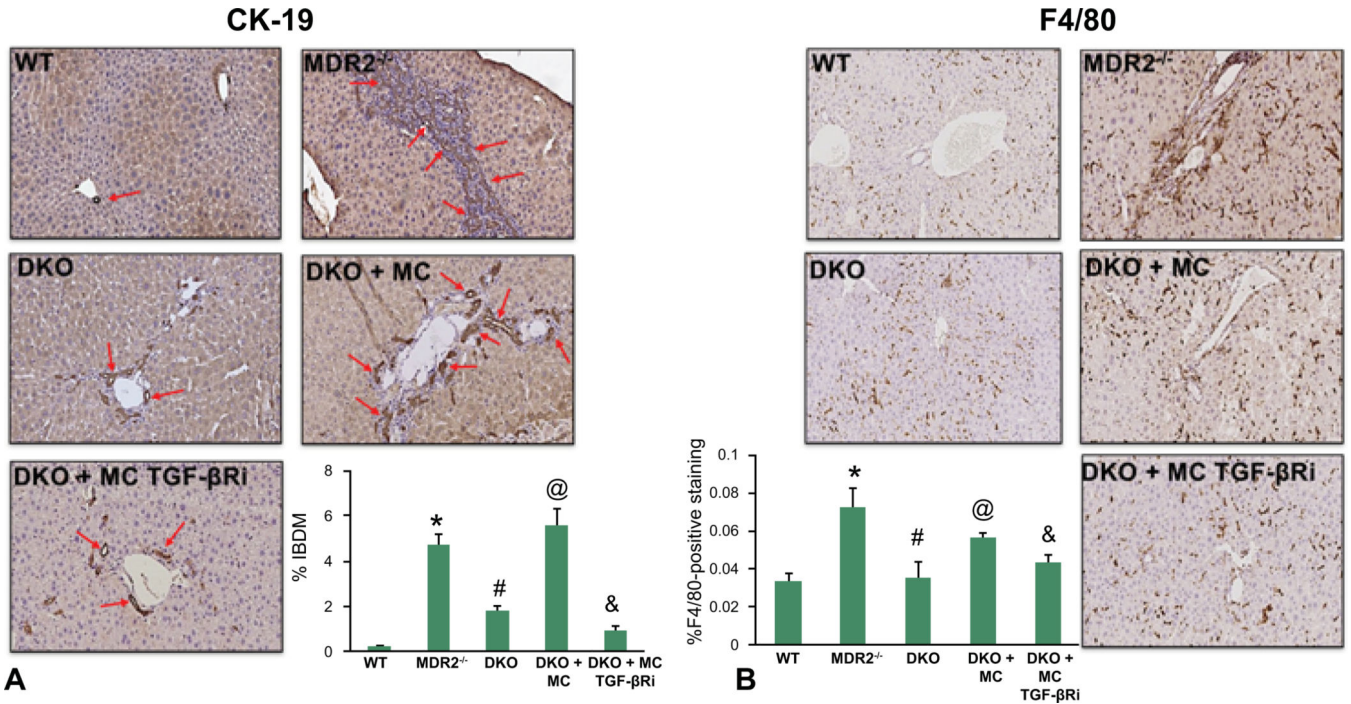


Figure 7. Evaluation of IBDM and Inflammation in DKO Mice. Biliary mass was measured by CK-19 immunohistochemistry and semi-quantification (A). In *Mdr2*^{-/-} mice, IBDM significantly increased that was downregulated in DKO mice. Further, IBDM increased in DKO mice injected with MCs compared to controls. IBDM was significantly decreased in DKO mice injected with MCs lacking TGF-β1. Red arrows indicate bile ducts. By F4/80 staining, the number of Kupffer cells increase in *Mdr2*^{-/-} mice compared to WT that are significantly downregulated in DKO mice. When DKO mice are injected with MCs, Kupffer cell number increases, which is decreased when DKO mice are injected with MCs lacking TGF-β1 (B). Data are mean ± SEM of 10 experiments from 6–8 different mice. *p<0.05 vs. WT mice; #p<0.05 vs. *Mdr2*^{-/-} mice; @p<0.05 vs. DKO mice and &p<0.05 vs. DKO mice injected with MCs. Images are 40x.

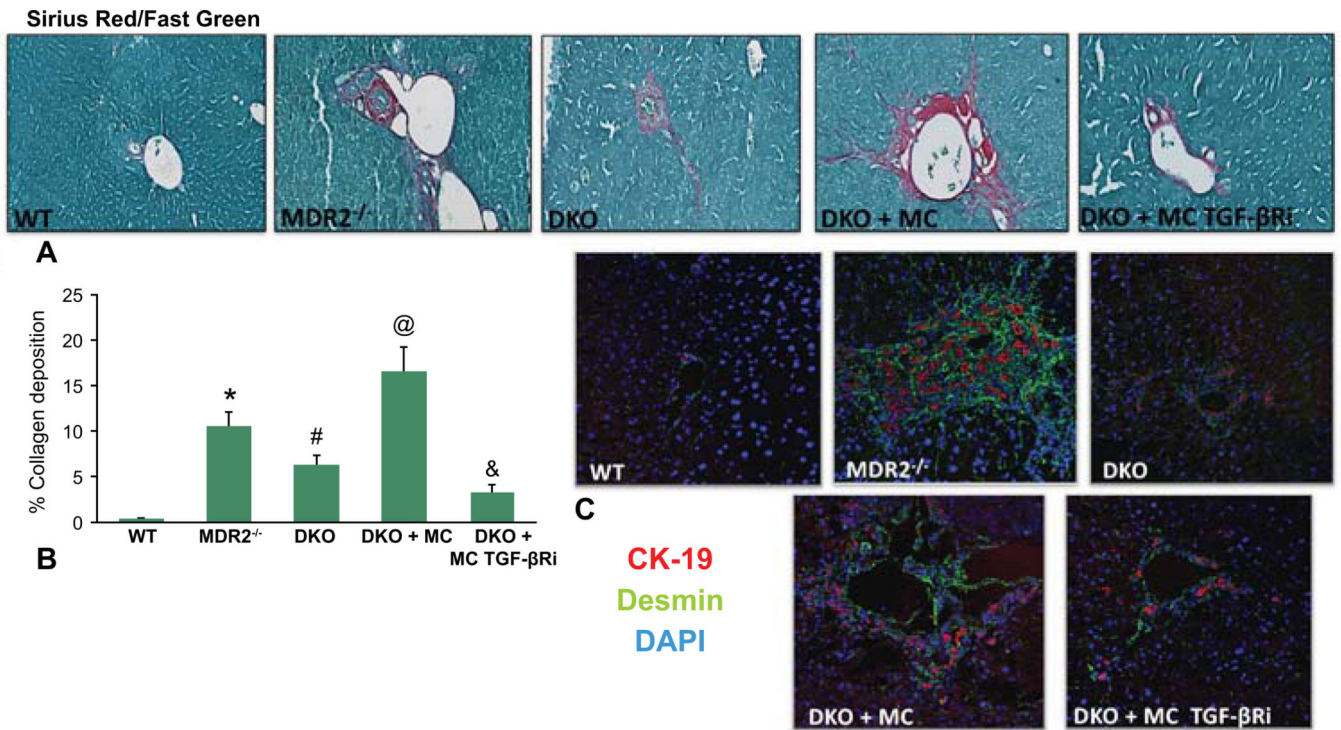


Figure 8.

Hepatic Fibrosis and HSC Activation in DKO Mice. In DKO mice, collagen deposition is decreased compared to *Mdr2*^{-/-} mice shown by (A) Sirius Red/Fast Green and (B) semi-quantification (A–B). When DKO mice are injected with MCs, there was an accumulation of collagen surrounding the portal area that is significantly reduced in DKO mice injected with MCs lacking TGF-β1. Similarly, by immunofluorescent staining, there is a downregulation of desmin positive HSCs in DKO mice compared to *Mdr2*^{-/-} mice and when DKO mice are injected with MCs, desmin staining is intensified (C). DKO mice injected with MCs lacking TGF-β1 have lower levels of desmin positive staining. Data are mean ± SEM of 10 experiments from 4–6 different mice. **p*<0.05 vs. WT mice; #*p*<0.05 vs. *Mdr2*^{-/-} mice; @*p*<0.05 vs. DKO mice and &*p*<0.05 vs. DKO mice injected with MCs. Images are 40x.