

# Mast Cells Regulate Ductular Reaction and Intestinal Inflammation in Cholestasis Through Farnesoid X Receptor Signaling

Vik Meadows,<sup>1,2</sup> Lindsey Kennedy,<sup>2</sup> Burcin Ekser,<sup>1,2</sup> Konstantina Kyritsi,<sup>2</sup> Debjyoti Kundu,<sup>2</sup> Tianhao Zhou,<sup>2</sup> Lixian Chen,<sup>2</sup> Linh Pham,<sup>2,\*</sup> Nan Wu,<sup>2</sup> Jennifer Demieville,<sup>4</sup> Laura Hargrove,<sup>4</sup> Shannon Glaser,<sup>4</sup> Gianfranco Alpini,<sup>1,2</sup> and Heather Francis<sup>1,2</sup>

**BACKGROUND AND AIMS:** Cholestasis is characterized by increased total bile acid (TBA) levels, which are regulated by farnesoid X receptor (FXR)/FGF15. Patients with primary sclerosing cholangitis (PSC) typically present with inflammatory bowel disease (IBD). Mast cells (MCs) (i) express FXR and (ii) infiltrate the liver during cholestasis promoting liver fibrosis. In bile-duct-ligated (BDL) MC-deficient mice (B6.Cg-*Kit*<sup>W<sup>sb</sup></sup>/HNIhrJaeBsmJ [*Kit*<sup>W<sup>sb</sup></sup>]), ductular reaction (DR) and liver fibrosis decrease compared with BDL wild type, and MC injection exacerbates liver damage in normal mice.

**APPROACH AND RESULTS:** In this study, we demonstrated that MC-FXR regulates biliary FXR/FGF15, DR, and hepatic fibrosis and alters intestinal FXR/FGF15. We found increased MC number and biliary FXR expression in patients with liver injury compared with control. Histamine and FGF19 serum levels and small heterodimer partner expression increase in patients PSC and PSC-IBD compared with healthy controls. MC injection increased liver damage, DR, inflammation, biliary senescence/senescence-associated secretory phenotype (SASP), fibrosis, and histamine in *Kit*<sup>W<sup>sb</sup></sup> mice. Inhibition of MC-FXR before injection reduced these

parameters. BDL and *Kit*<sup>W<sup>sb</sup></sup> mice injected with MCs displayed increased TBA content, biliary FXR/FGF15, and intestinal inflammation, which decreased in BDL *Kit*<sup>W<sup>sb</sup></sup> and *Kit*<sup>W<sup>sb</sup></sup> mice injected with MC-FXR. MCs increased ileal FXR/FGF15 expression in *Kit*<sup>W<sup>sb</sup></sup> mice that was reduced following FXR inhibition. BDL and multidrug resistance 2/ATP-binding cassette family 2 member 4 knockout (*Mdr2*<sup>-/-</sup>) mice, models of PSC, displayed increased intestinal MC infiltration and FXR/FGF15 expression. These were reduced following MC stabilization with cromolyn sodium in *Mdr2*<sup>-/-</sup> mice. *In vitro*, MC-FXR inhibition decreased biliary proliferation/SASP/FGF and hepatic stellate cell activation.

**CONCLUSIONS:** Our studies demonstrate that MC-FXR plays a key role in liver damage and DR, including TBA regulation through alteration of intestinal and biliary FXR/FGF15 signaling. (HEPATOLOGY 2021;74:2684-2698).

Cholangiocytes are the target cells of cholestatic liver diseases, such as primary sclerosing cholangitis (PSC), which is characterized by

*Abbreviations:*  $\alpha$ -SMA, alpha smooth muscle actin; ASBT, apical Na<sup>+</sup>/bile acid transporter; ATCC, American Type Culture Collection; BA, bile acid; BDL, bile duct ligation; CCL, chemokine (C-C motif) ligand; CDCA, chenodeoxycholic acid; CK-19, cytokeratin-19; CXCL, chemokine (C-X-C motif) ligand; Cyp, cytochrome p450; DR, ductular reaction; ELA, enzyme immunoassay; FGF, fibroblast growth factor; FXR, farnesoid X receptor; Gugg, guggulsterone; HA, histamine; HR, histamine receptor; IBD, inflammatory bowel disease; IBDM, intrahepatic bile duct mass; IPA, Ingenuity Pathway Analysis; *Kit*<sup>W<sup>sb</sup></sup>, B6.Cg-*Kit*<sup>W<sup>sb</sup></sup>/HNIhrJaeBsmJ; MC, mast cell; *Mdr2*<sup>-/-</sup>, multidrug resistance 2/ATP-binding cassette family 2 member 4 knockout; OCA, obeticholic acid; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; qPCR, quantitative PCR; SASP, senescence-associated secretory phenotype; SCF, stem cell factor; SHP, small heterodimer partner; TBA, total bile acid; WT, wild type.

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\*Department of Science and Mathematics, Texas A&M University – Central Texas, Killeen, TX.

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increased hepatic fibrosis, ductular reaction (DR)<sup>(1,2)</sup> and impaired bile acid (BA) secretion.<sup>(3,4)</sup> A significant subset of patients with PSC present with inflammatory bowel disease (IBD), which increases their risk and incidence of colorectal and hepatobiliary cancer.<sup>(5)</sup> During PSC, cholangiocytes exhibit proliferative, inflammatory, fibrotic, and senescent phenotypes in response to liver injury, and several studies have demonstrated the critical contribution of the gut-liver axis during PSC.<sup>(6)</sup>

We have demonstrated that mast cells (MCs) infiltrate the liver during cholestatic liver injury and trigger biliary damage.<sup>(1)</sup> Following migration and activation, MCs induce DR<sup>(7)</sup> and senescence<sup>(1)</sup> through paracrine interactions with cholangiocytes. The PSC mouse models, multidrug resistance 2/ATP-binding cassette family 2 member 4 knockout (*Mdr2*<sup>-/-</sup>) and mice subjected to bile duct ligation (BDL), have elevated MC presence and serum histamine (HA) levels, increasing biliary expression of H1 and H2 HA receptors (HR); however, disruption of HA signaling ameliorates cholestatic liver injury.<sup>(8-10)</sup> Following BDL, MC-deficient B6.Cg-*Kit*<sup>W<sup>-sb</sup></sup>/HNIhrJaeBsmJ (*Kit*<sup>W<sup>-sb</sup></sup>) mice have reduced DR, inflammation, and hepatic fibrosis compared with BDL wild type (WT), and introduction of MCs into *Kit*<sup>W<sup>-sb</sup></sup> mice mimics cholestatic injury,<sup>(10)</sup> demonstrating the damaging role of MCs during liver injury. Moreover, MCs induce DR through TGF- $\beta$ 1<sup>(11)</sup> and VEGF<sup>(8)</sup> signaling pathways.

BA synthesis and enterohepatic circulation is tightly regulated by farnesoid X receptor (FXR) and downstream targets including FGF15/FGF19 (FGF15, mouse; FGF19, human) and cytochrome p450 (Cyp) family 7 and 27 subfamily a member 1 (Cyp7a1 and Cyp27a1).<sup>(12)</sup> FXR signaling regulates the expression

of many BA transporters such as Na<sup>+</sup>-dependent taurocholate cotransport peptide and apical Na<sup>+</sup> BA transporter (ASBT).<sup>(13)</sup> Furthermore, FXR plays a protective role in intrahepatic cholestasis by antagonizing NF- $\kappa$ B-mediated hepatic inflammation<sup>(12,14)</sup> while increasing liver injury in extrahepatic cholestasis through dysregulation of BA transporter expression.<sup>(15)</sup> *Fxr*<sup>-/-</sup> mice have decreased intrahepatic bile duct mass (IBDM) and biliary proliferation following BDL, likely because of the reduced bile flow and pressure, compared with WT mice.<sup>(16)</sup> Impairment of FGF15 signaling inhibits liver regeneration and exacerbates hepatosteatosis,<sup>(17)</sup> lending to its importance in hepatic function.

Our studies found that stabilization of MCs in *Mdr2*<sup>-/-</sup> mice reduces total BA (TBA) levels,<sup>(7)</sup> and we, among others, have shown that MCs express FXR, ASBT and FGF receptors<sup>(18,19)</sup> and migrate into tissue in response to FGF stimulation.<sup>(20)</sup> In this study, we evaluated the role of MC-FXR in regulation of BA circulation, intestinal inflammation and liver damage in models of cholestasis.

## Materials and Methods

### MATERIALS

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. Z-guggulsterone (FXR antagonist)<sup>(21)</sup> was purchased from MilliporeSigma (Burlington, MA). Mouse pan Fibroblast growth factor (FGF) enzyme immunoassay (EIA) was purchased from R&D Systems (Minneapolis, MN). Mouse FGF15 EIA was

### ARTICLE INFORMATION:

From the <sup>1</sup>Richard L. Roudebush VA Medical Center, Indiana University School of Medicine, Indianapolis, IN; <sup>2</sup>Division of Gastroenterology and Hepatology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN; <sup>3</sup>Division of Transplant Surgery, Department of Surgery, Indiana University School of Medicine, Indianapolis, IN; <sup>4</sup>Department of Medical Physiology, Texas A&M University College of Medicine, Bryan, TX.

### ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Heather Francis, Ph.D., F.A.A.S.L.D.  
Indiana University, Gastroenterology, Medicine  
Richard L. Roudebush VA Medical Center  
702 Rotary Circle, Rm. 013C

Indianapolis, IN 46202-2859  
E-mail: heafranc@iu.edu  
Tel.: +1-317-274-3514

purchased from My BioSource, Inc. (San Diego, CA) and LifeSpan Biosciences, Inc. (Seattle, WA). FGF19 EIA was purchased from Abcam (Cambridge, MA). HA EIA was purchased from Cayman Chemicals (Ann Arbor, MI). Application, dilution, and vendor information for all antibodies used in this study are detailed in Supporting Table S1. RNA was reverse transcribed and amplified using Reaction Ready First Strand cDNA Synthesis kit and RT2 quantitative PCR (qPCR) Primer Assay obtained from Qiagen (Valencia, CA). All qPCR primers are listed in Supporting Table S2. TBA colorimetric kits were purchased from Cell BioLabs, Inc. (San Diego, CA). Positive immunoreactivity was quantified with Image-Pro from Media Cybernetics, Inc. (Rockville, MD).

For *in vitro* studies, we used immortalized murine intrahepatic cholangiocyte cell lines.<sup>(22)</sup> Murine hepatic MCs (MC/9, American Type Culture Collection [ATCC] CRL-8306) were purchased from ATCC (Manassas, VA). Human HSC (#5300) were obtained from ScienCell (Carlsbad, CA). All cell lines were cultured and maintained by us following vendor recommendations and as published.<sup>(8)</sup> Media and reagents were purchased from Thermo Fisher (Waltham, MA).

## IN VIVO MODELS

Commercially available homozygous *Kit*<sup>W<sup>sh</sup></sup> (MC-deficient) 10 to 12-week-old male mice were obtained from Jackson Laboratory along with sex and age-matched WT c57BL/6J mice. WT and *Kit*<sup>W<sup>sh</sup></sup> mice both display healthy liver phenotype, and WT mice have minimal MC presence.<sup>(10)</sup> To ascertain the role of MC-FXR in MC-induced damage, *Kit*<sup>W<sup>sh</sup></sup> received a single injection through tail vein of MCs (MC/9, ATCC CRL-8306,  $5 \times 10^6$  cells/0.1–0.2 mL sterile 1× PBS) treated with vehicle 0.1% DMSO (MC) or 10  $\mu$ M Z-guggulsterone (FXR inhibitor, MC-Gugg)<sup>(21,23)</sup> for 48 hours and tagged with PKH26 Red Fluorescent Cell Linker before injection. Liver (costained with cytokeratin 19 [CK-19] to mark bile ducts), lung, and spleen were evaluated for MC presence by PKH26 detection with confocal microscopy (LEICA TCS SP5 X system, Leica Microsystems, Inc., Buffalo Grove, IL). To confirm FXR inhibition, MC-FXR, FGF15, and small heterodimer partner (SHP) expression and FGF secretion were measured by qPCR and EIA, respectively. Serum, liver, isolated cholangiocytes, small intestine,

and distal ileum were collected from all animal groups (exact animal numbers are provided in the Supporting Information). Isolated cholangiocytes were collected as described.<sup>(7)</sup> Immunohistochemistry was performed on 4 to 6- $\mu$ m formalin-fixed paraffin-embedded liver or small intestine sections. Immunofluorescence was performed on 4 to 6- $\mu$ m optical cutting media-embedded liver, lung, or spleen.

To verify our findings in established models of cholestatic liver injury, male WT and *Kit*<sup>W<sup>sh</sup></sup> mice, 10 to 12-week-old, were subjected to BDL for 7 days before euthanasia along with appropriate controls as described.<sup>(10)</sup> Additionally, *Mdr2*<sup>-/-</sup> mice, an established genetic model of cholestatic liver damage, treated with saline or cromolyn sodium (MC stabilizer, 24 mg/kg BW for 1 week) were used to confirm our findings.<sup>(7)</sup> Serum, liver, small intestine, and isolated cholangiocytes and hepatocytes were collected from all mice (exact animal numbers are provided in the Supporting Information).

All animal colonies were maintained following current Institutional Animal Care and Use Committee protocols approved by the Indiana University School of Medicine Laboratory Animal Resource Center, Indianapolis, IN, and Baylor Scott and White Health Animal Facility, Temple, TX. All mice were given free access to drinking water and standard chow. Animals were kept in a temperature-controlled environment with 12:12 hr light/dark cycles.

## FXR/FGF19 SIGNALING AND MC ACTIVATION IN HUMAN PATIENTS

Human liver sections from healthy controls and patients with cholestatic liver diseases were used for immunohistochemistry for FXR costained with tryptase to determine the presence of activated MCs. Healthy nondiseased liver tissues were purchased from Sekisui XenoTech, LLC (Kansas City, KS). Healthy nondiseased, PSC and PSC with IBD comorbidity (PSC-IBD) serum were deidentified and provided by Dr. Burcin Ekser following transplantation. Liver sections (formalin-fixed, 4 to 5  $\mu$ m thick) obtained by explant from deidentified transplant patients from patients with PSC, PSC-IBD, primary biliary cholangitis (PBC), biliary atresia, and NASH were provided by Dr. Burcin Ekser. Patient demographics are detailed in Supporting Table S3. All samples were obtained under a protocol approved by Indiana University Health; the protocol



was approved by the Indiana University Institutional Review Board. Serum FGF19 (Abcam, Cambridge, MA) and HA (Cayman Chemicals, Inc., Ann Arbor, MI) levels were measured by EIA following manufacturer protocols, and levels of SHP were evaluated in total liver by qPCR from healthy nondiseased (control), PSC, and PSC-IBD samples. For all patient sample analysis, written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as described and approved by the Institutional Review Board, Indiana University. No donor organs were obtained from executed prisoners or other institutionalized persons.

Further detailed information for experiments performed *in vivo* and *in vitro* are detailed in the Supporting Methods.

## STATISTICAL ANALYSIS

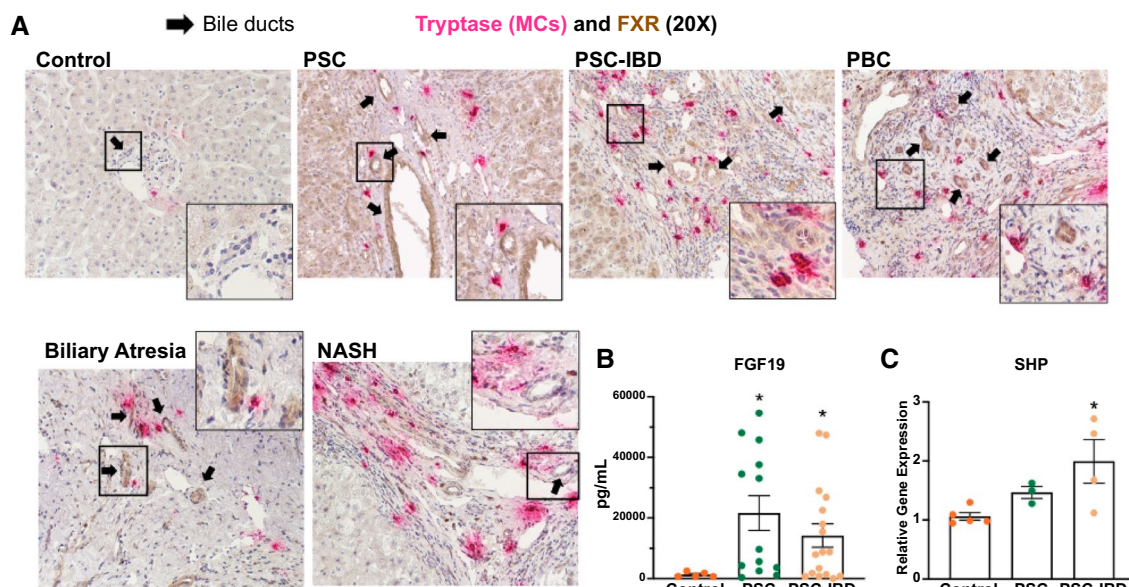
Data are presented as mean  $\pm$  SEM. Groups were analyzed by the Student's unpaired *t* test when two

groups were analyzed. Welch one-way ANOVA was used when more than two groups are analyzed, followed by appropriate post hoc test with GraphPad Prism 9 (San Diego, CA).  $P < 0.05$  was considered significant.

## Results

### PATIENTS WITH CHOLESTATIC LIVER DISEASE HAVE INCREASED MC PRESENCE AND FXR EXPRESSION

FXR agonists are currently being studied as therapeutic options for patients with cholestatic liver disease<sup>(4,12)</sup>; however, the effect of FXR on hepatic MC presence has not been defined. In patients, FXR expression (brown; found in hepatocytes and cholangiocytes) increased in cholangiocytes from late-stage PSC, PSC-IBD, late-stage PBC, biliary atresia, and NASH compared with control tissue, and the up-regulation of FXR is accompanied by elevated expression of MC



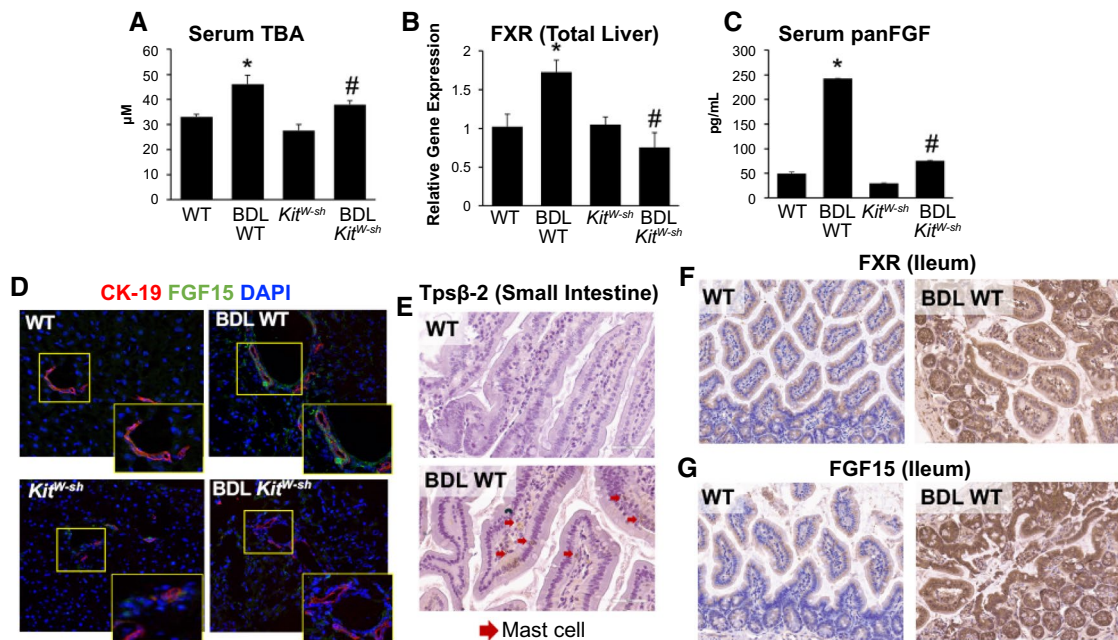
**FIG. 1.** (A) Hepatic FXR expression and MC presence increase in human liver disease explant patients. Livers from nondiseased control, PSC, PSC-IBD, PBC, biliary atresia, and NASH were stained using immunohistochemistry for FXR (brown), costained with tryptase (red) to detect activated MC presence. Enhanced biliary FXR immunoreactivity (marked by black arrows) is present in all diseased tissue explants compared with control, with a subset (PSC, PSC-IBD, and PBC) demonstrating increased hepatocyte FXR expression, indicating activated hepatic FXR function. Tryptase expression also increased in all models of disease compared with control. (B) Patients with PSC ( $n = 14$ ) and PSC-IBD ( $n = 17$ ) have increased serum FGF19 compared with healthy controls ( $n = 5$ ). (C) Patients with PSC ( $n = 3$ ) and PSC-IBD ( $n = 4$ ) also have increased hepatic SHP expression compared with controls ( $n = 5$ ). Data are mean  $\pm$  SEM of  $n = 2$  (serum) or  $n = 3$  (qPCR) experiments per patient sample. \* $P < 0.05$  vs. control. Representative images are presented as  $\times 20$  and zoom boxes are  $\times 80$ .

trypsin (red staining) found near bile ducts (Fig. 1A). Further, serum levels of FGF19 (Fig. 1B) and mRNA expression of SHP (Fig. 1C) increased in patients with PSC-IBD compared with controls. These results support the clinical implications of MC infiltration during human cholestatic liver disease and the up-regulation of FXR expression and activation of downstream signaling during the progression of chronic liver injuries, including PSC-IBD.

## TBA CONTENT AND HEPATIC FXR/FGF SIGNALING ARE DECREASED IN CHOLESTATIC MODELS LACKING MCS OR HA SIGNALING

To establish a rationale for our study, we examined changes in TBA content and FXR/FGF signaling

in established cholestatic models, including BDL and *Mdr2*<sup>-/-</sup> mice. With regard to TBA content, we found that BDL WT mice had elevated serum TBA content compared with WT mice; however, BDL *Kit*<sup>W-sh</sup> mice had reduced serum TBA content compared with BDL WT mice (Fig. 2A). BDL WT mice have significantly increased hepatic FXR mRNA expression compared with WT mice (Fig. 2B), whereas BDL *Kit*<sup>W-sh</sup> mice have lower hepatic FXR expression compared with both WT and BDL WT mice, indicating that MCs are involved in the regulation of the hepatic FXR expression. Furthermore, we found that panFGF serum secretion was minimal in WT and *Kit*<sup>W-sh</sup> mice (Fig. 2C); however, panFGF serum levels increased in BDL WT mice compared with WT mice, whereas BDL *Kit*<sup>W-sh</sup> mice displayed decreased panFGF serum secretion compared with BDL WT (Fig. 2C). Finally, we verified increased



**FIG. 2.** (A) MCs alter TBA and FXR/FGF signaling in an established model of cholestatic MC-deficient mice. Serum TBA content is increased in BDL WT compared with WT mice, but in BDL *Kit*<sup>W-sh</sup> mice, TBA content is reduced. Serum TBA was unchanged between control groups. Hepatic FXR is unchanged in WT and *Kit*<sup>W-sh</sup> mice; however, expression is elevated in BDL WT mice compared with WT and *Kit*<sup>W-sh</sup>. (B) BDL *Kit*<sup>W-sh</sup> mice exhibited reduced hepatic FXR expression compared with BDL WT mice. (C) BDL WT mice have elevated serum panFGF secretion compared with WT and *Kit*<sup>W-sh</sup>, which is reduced in BDL *Kit*<sup>W-sh</sup> mice; no significant changes were found between control groups. (D) Biliary FGF15 (green) expression was elevated in BDL WT mice shown by immunofluorescence, costained with CK-19 to mark bile ducts (red) but reduced in BDL *Kit*<sup>W-sh</sup> mice with no changes between control groups. (E) In BDL WT small intestine, there is increased MC presence detected by tryptase β-2 (Tpsβ-2) immunoreactivity and marked by red arrows. In distal ileum from BDL WT there was increased (F) FXR and (G) FGF15 immunoreactivity compared with WT mice. Data are mean ± SEM of n = 4 experiments for qPCR, panFGF EIA, and TBA from 4–6 mice per group. \**P* < 0.05 vs. WT; #*P* < 0.05 vs. BDL WT. Representative images are presented as ×20 for FXR and ×40 for FGF15 and Tpsβ-2.



FGF15 expression and immunoreactivity by immunofluorescence in bile ducts of BDL WT mice compared with WT mice, which was reduced in BDL *Kit<sup>W-sh</sup>* mice (Fig. 2D). In *Mdr2<sup>-/-</sup>* mice, we found increased FXR expression (Supporting Fig. S1A), biliary FGF15 immunoreactivity (Supporting Fig. S1B), and panFGF serum content (Supporting Fig. S1C) that were reduced in *Mdr2<sup>-/-</sup>* mice treated with cromolyn sodium.

## MC INFILTRATION ALTERS INTESTINAL FXR/FGF15 IN CHOLESTATIC MICE

We found that BDL WT and *Mdr2<sup>-/-</sup>* mice have increased intestinal MC infiltration compared with their WT controls shown by tryptase  $\beta$ -2 (Fig. 2E and Supporting Fig. S1E). Along with increased MC presence, we found elevated expression of FXR and FGF15 in distal ileum from BDL WT mice compared with their WT controls (Fig. 2F,G). When *Mdr2<sup>-/-</sup>* mice were treated with cromolyn sodium to reduce MC activation, intestinal MC infiltration, FXR, and FGF15 expression were all reduced (Supporting Fig. S1D-F). In distal ileum from BDL WT, we also found increased mRNA expression of FGF15 and SHP, whereas ASBT decreased compared with WT mice (Supporting Fig. S2A), and, in small intestine, stem cell factor (SCF), IL-1 $\beta$ , TGF- $\beta$ 1, and chemokine (C-C motif) ligand (CCL) 3 mRNA expression are increased following BDL compared with WT mice. These parameters were decreased in BDL *Kit<sup>W-sh</sup>* mice lacking MCs (Supporting Fig. S2B)

## VALIDATION OF MC-FXR/FGF EXPRESSION AND MC INJECTION/MIGRATION AND DAMAGE OF MODEL

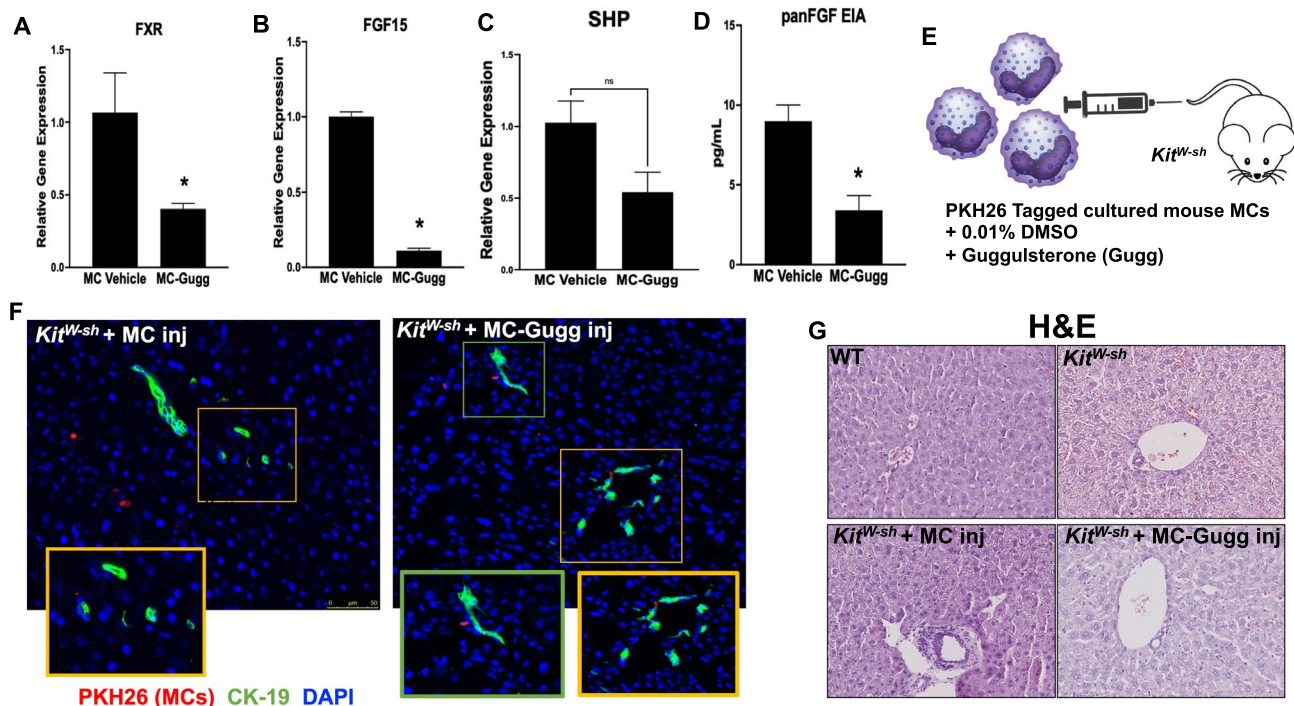
Cultured murine MCs were treated with vehicle (MC) or Z-guggulsterone (MC-Gugg), tagged with PKH26, and injected into *Kit<sup>W-sh</sup>* mice through tail vein. MC-Gugg have decreased FXR FGF15 and SHP ( $P < 0.07$ ) mRNA expression (Fig. 3A-C) and panFGF secretion (Fig. 3D) compared with vehicle-treated MCs. Similar to our previous findings,<sup>(10)</sup> we found that MCs (marked with PKH26, red) migrate to the liver and reside in close proximity to bile ducts

(stained with CK-19, green) (Fig. 3E,F). Concurrent with our previous work, we found minimal MC presence in lung and spleen from our MC-Gugg-injected mice (data not shown).<sup>(10)</sup> By hematoxylin and eosin staining, we verified that MC injection induces periportal inflammation and lobular damage that is ameliorated in *Kit<sup>W-sh</sup>* mice treated with MC-Gugg (Fig. 3G).

## MCS ALTER ENTEROHEPATIC FXR/FGF AXIS AND INTESTINAL INFLAMMATION

In *Kit<sup>W-sh</sup>* mice injected with MCs, compared with WT, there was increased (1) liver and serum TBA content (Fig. 4A,B) and (2) hepatic and biliary FXR expression (Fig. 4C,D) that was reduced in *Kit<sup>W-sh</sup>* mice injected with MC-Gugg. Downstream of FXR, hepatocyte Cyp7a1 expression also increased in *Kit<sup>W-sh</sup>* mice injected with MCs; however, hepatocyte Cyp27a1 was reduced (Fig. 4E) compared with WT, and when *Kit<sup>W-sh</sup>* mice were treated with MC-FXR, Cyp7a1 was reduced, whereas Cyp27a1 remained unchanged (Fig. 4E). Immunoreactivity of FGF15 (Fig. 4G) and liver and serum FGF15 secretion (Fig. 4H,I) all increased in *Kit<sup>W-sh</sup>* mice injected with MCs compared with WT and *Kit<sup>W-sh</sup>* mice. All of these parameters were decreased when *Kit<sup>W-sh</sup>* mice were injected with MC-Gugg (Fig. 4G-I). There were minimal changes seen between WT and *Kit<sup>W-sh</sup>* mice for TBA levels and FXR and FGF15 expression.

Interestingly, in accordance with our hepatic findings, distal ileum FXR and FGF15 expressions are minimal in WT and elevated in *Kit<sup>W-sh</sup>* mice (Fig. 5A,B). MC injection into *Kit<sup>W-sh</sup>* mice increased intestinal FXR and FGF15 expression (Fig. 5A,B), which was reduced when *Kit<sup>W-sh</sup>* mice were injected with MCs lacking FXR (Fig. 5A,B), demonstrating an impact of MC-FXR on hepatic and intestinal FXR/FGF15 signaling in MC-deficient mice. We found that small intestine mRNA levels of SCF, IL-1 $\beta$ , TGF- $\beta$ 1, and CCL3 were elevated following MC injection in *Kit<sup>W-sh</sup>* compared with WT and *Kit<sup>W-sh</sup>* mice (Fig. 5C). These inflammatory markers decreased when FXR was inhibited in MCs before injection (Fig. 5C). These findings indicate an important role for MCs in both FXR/FGF15 enterohepatic circulation and intestinal inflammation.



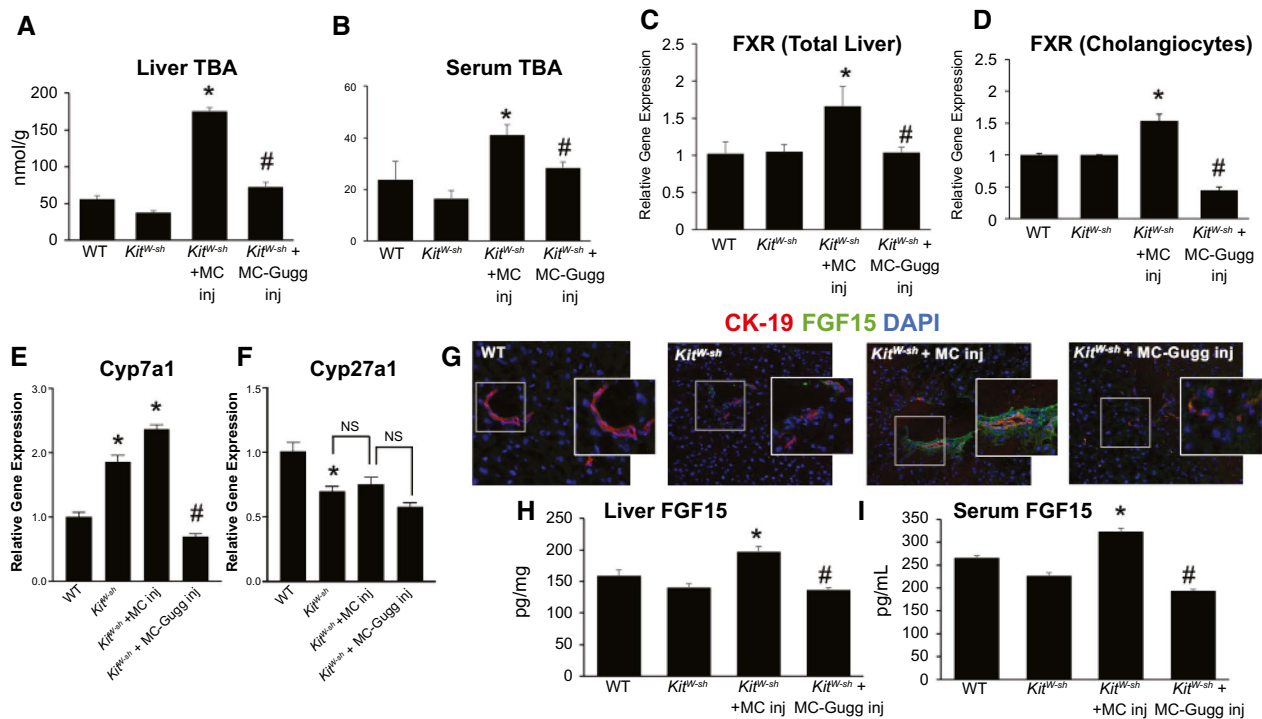
**FIG. 3.** Validation of model and hepatic damage in *Kit<sup>W-sh</sup>* mice. Cultured MCs (MC/9, ATCC CRL-8306) were stimulated with 0.1% DMSO (vehicle) or 10  $\mu$ M Z-guggulsterone for 48 hours and (A) FXR, (B) FGF15, (C) SHP mRNA expression, and (D) panFGF secretion were measured. Following treatment, MC-Gugg have reduced (A–C) FXR, FGF15, and SHP mRNA levels, measured by qPCR, and (D) panFGF secretion, measured by EIA, compared with vehicle-treated MCs. (E) Cultured MCs were treated with 0.1% DMSO or guggulsterone (Gugg) and tagged with PKH26 before injection into *Kit<sup>W-sh</sup>* mice through tail vein. MC migration to the liver was confirmed in *Kit<sup>W-sh</sup>* mice injected with both (F) vehicle-treated MCs and Z-guggulsterone-treated MCs (MC-Gugg) by immunofluorescence imaging of PKH26 (red) and CK-19 to mark bile ducts (green). (G) Hematoxylin and eosin (H&E) staining demonstrates increased hepatic damage and ductular inflammation in *Kit<sup>W-sh</sup>* + MC that is absent in WT and *Kit<sup>W-sh</sup>* mice. Hepatic damage is reduced in *Kit<sup>W-sh</sup>* + MC-Gugg mice. Data are mean  $\pm$  SEM of  $n = 4$  experiments from  $n = 2$  biological replicates for qPCR and panFGF EIA. \* $P < 0.05$  vs. MC-vehicle. Representative images are presented as  $\times 20$  for immunofluorescence, with  $\times 40$  zoom boxes, and  $\times 10$  for H&E. Abbreviation: Inj, injury.

## INHIBITION OF MC-FXR PREVENTS DR, INFLAMMATION, BILIARY SENESCENCE, SENESCENCE-ASSOCIATED SECRETORY PHENOTYPE, AND HEPATIC FIBROSIS

Introduction of MCs into *Kit<sup>W-sh</sup>* mice increased IBDM (Fig. 6A) and F4/80-positive Kupffer cells (Fig. 6B), which also confirmed by semiquantification (Fig. 6C,D), compared with WT and *Kit<sup>W-sh</sup>* mice. MC injection also enhanced hepatic inflammatory marker expression of IL-1 $\beta$ , CCL5, chemokine (C-X-C motif) ligand (CXCL) 2, and CXCL5 (Fig. 6E). Inhibition of MC-FXR reduces IBDM and hepatic inflammation compared with *Kit<sup>W-sh</sup>* +

MC mice (Fig. 6A–E). *Kit<sup>W-sh</sup>* mice injected with MCs had increased biliary proliferation shown by Ki-67 (Supporting Fig. S3A) and biliary senescence demonstrated by immunofluorescence for p16 in liver sections and mRNA expression of p18 and p21 in isolated cholangiocytes (Supporting Fig. S3B,C) compared with WT and *Kit<sup>W-sh</sup>* mice. Inhibition of MC-FXR before injection into *Kit<sup>W-sh</sup>* mice reduced these parameters (Supporting Fig. S3A–C).

*Kit<sup>W-sh</sup>* + MC mice display increased collagen deposition as shown by increased fast green-sirius red stain and semiquantification compared with *Kit<sup>W-sh</sup>* and WT mice 3 days after injection; however, inhibition of MC-FXR resulted in reduced collagen deposition and hepatic fibrosis compared with *Kit<sup>W-sh</sup>* + MC mice (Supporting Fig. S4A). Further, WT and *Kit<sup>W-sh</sup>* mice have minimal



**FIG. 4.** MC-FXR regulates hepatic and biliary FXR/FGF signaling. Introduction of MCs in *Kit<sup>W-sh</sup>* mice increased (A) hepatic and (B) circulating TBA content; however, both TBA levels were reduced with MC-FXR inhibition. (A,B) No significant changes were noted between WT and *Kit<sup>W-sh</sup>* groups. (C) Hepatic and (D) biliary FXR expression increased in *Kit<sup>W-sh</sup>* mice injected with MCs compared with WT and *Kit<sup>W-sh</sup>* mice, and inhibition of MC-FXR reduces both hepatic and biliary FXR expression in *Kit<sup>W-sh</sup>* + MC-Gugg mice. The expression of (E) hepatocyte Cyp7a1 increased in both control *Kit<sup>W-sh</sup>* mice and *Kit<sup>W-sh</sup>* mice injected with MCs compared with WT, whereas (F) hepatocyte Cyp27a1 decreased in all groups compared with WT. (E,F) In *Kit<sup>W-sh</sup>* mice injected with MC-Gugg, Cyp7a1 expression decreased and Cyp27a1 levels remained unchanged compared with control. (G) Biliary FGF15 (green) expression, costained with CK-19 to mark bile ducts (red), and (H) hepatic and (I) serum FGF15 secretion increased in *Kit<sup>W-sh</sup>* mice injected with MCs, that was subsequently reduced in mice injected with MC-Gugg (G-I). Minimal changes were noted between control groups (G-I). Data are mean  $\pm$  SEM of  $n = 8$  experiments for qPCR and  $n = 4$  experiments for FGF15 and panFGF EIA from 6–8 mice and  $n = 4$  experiments from 6–8 mice per group for serum and liver TBA. \* $P < 0.05$  vs. WT, # $P < 0.05$  vs. *Kit<sup>W-sh</sup>* + MC. Representative images are presented as  $\times 20$  with  $\times 40$  zoom boxes. Abbreviation: Inj, injury.

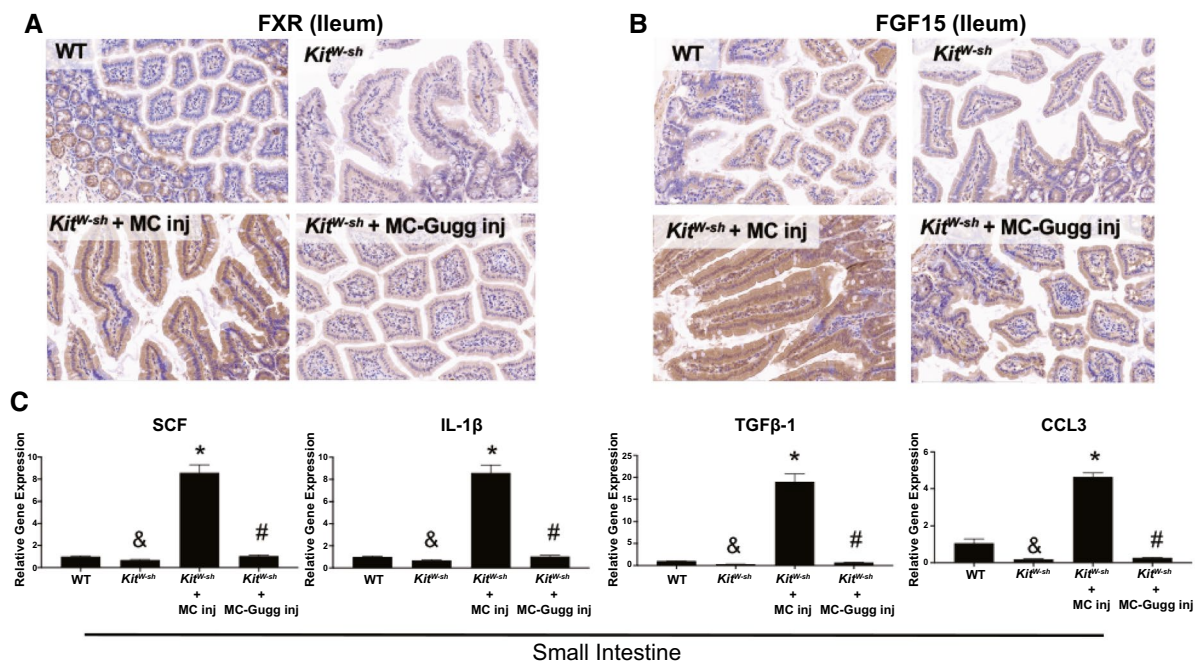
HSC presence shown by desmin that is increased in *Kit<sup>W-sh</sup>* + MC and found in the surrounding bile ducts and portal area (Supporting Fig. S4B). *Kit<sup>W-sh</sup>* + MC mice display elevated expression of alpha smooth muscle actin ( $\alpha$ -SMA) compared with WT and *Kit<sup>W-sh</sup>*, whereas in *Kit<sup>W-sh</sup>* + MC-Gugg, HSC presence and  $\alpha$ -SMA expression is reduced (Supporting Fig. S4B,C).

## INHIBITION OF MC-FXR REDUCES BILIARY SENESCENCE-ASSOCIATED SECRETORY PHENOTYPES AND HA/H1 SIGNALING

Ingenuity Pathway Analysis (IPA) analysis demonstrated FXR and HA signaling crosstalk through

inflammatory cytokines/senescence-associated secretory phenotypes (SASP), TGF- $\beta$ 1 and IL-1 $\beta$ , and H1HR (Fig. 7A). Biliary mRNA TGF- $\beta$ 1 and IL-1 $\beta$  expression increased in *Kit<sup>W-sh</sup>* + MC compared with WT and *Kit<sup>W-sh</sup>* mice, whereas inhibition of MC-FXR reduced these parameters (Fig. 7B). In human patients with PSC and PSC-IBD, we found increased HA secretion compared with control patients (Fig. 7C). Furthermore, WT and *Kit<sup>W-sh</sup>* mice have minimal serum HA levels that were elevated in *Kit<sup>W-sh</sup>* + MC; however, when MC-FXR was inhibited, HA levels are reduced (Fig. 7D). We have shown that HA activates cholangiocytes through paracrine interactions through H1HR signaling.<sup>(24)</sup> WT and *Kit<sup>W-sh</sup>* have minimal biliary mRNA expression of H1HR (Fig. 7E). Following MC injection, *Kit<sup>W-sh</sup>* have increased biliary H1HR mRNA expression compared





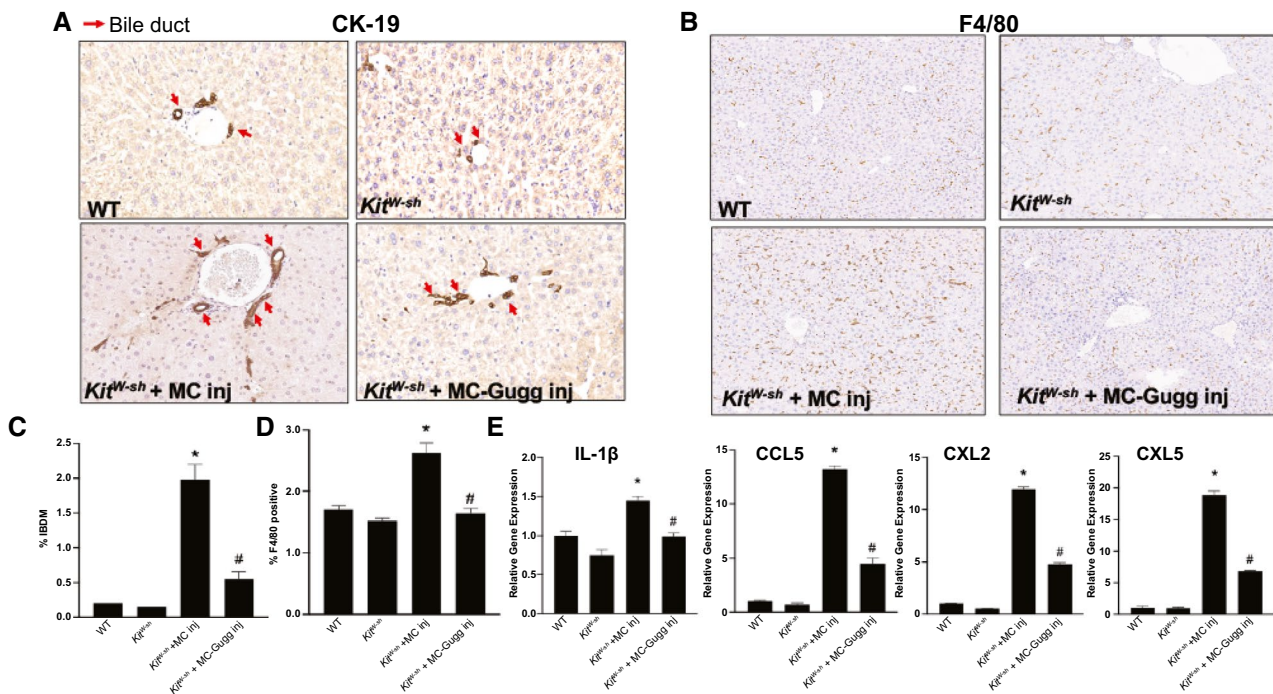
**FIG. 5.** Intestinal FXR/FGF15 and inflammation are regulated by MC-FXR. Introduction of MCs in *Kit<sup>W-sh</sup>* mice increases ileal (A) FXR and (B) FGF15 expression compared with WT and *Kit<sup>W-sh</sup>* mice and inhibition of MC-FXR decreases (A) FXR and (B) FGF15 expression, as shown in *Kit<sup>W-sh</sup>* + MC-Gugg mice. (C) MC injection increased small intestine expression of SCF, IL-1β, TGF-β1, and CCL3 in *Kit<sup>W-sh</sup>* compared with WT and *Kit<sup>W-sh</sup>* mice; however, intestinal inflammation was reduced when MC-FXR was inhibited. Representative images are presented as  $\times 20$ . Data are mean  $\pm$  SEM of  $n = 4$  experiments for qPCR from 6–8 mice (MC injection group). \* $P < 0.05$  vs. WT and *Kit<sup>W-sh</sup>*, # $P < 0.05$  vs. *Kit<sup>W-sh</sup>* + MC, & $P < 0.05$  vs. WT.

with WT and *Kit<sup>W-sh</sup>* mice, indicating activation of biliary HA signaling; however, *Kit<sup>W-sh</sup>* + MC-Gugg mice have reduced biliary H1HR expression compared with *Kit<sup>W-sh</sup>* + MC mice (Fig. 7E). Combined, these results demonstrate that MC-FXR signaling has a synergistic relationship with systemic and autocrine HA signaling through regulation of biliary HA and SASP through H1HR.

## INHIBITION OF MC-FXR BLOCKS BILIARY HA SIGNALING, *IN VITRO*

Similar to our *in vivo* findings, we found that *in vitro* murine cholangiocytes express minimal mRNA expression of IL-1β, TGF-β1, and H1HR under basal conditions; however, after stimulation with murine MC-vehicle supernatant, biliary IL-1β, TGF-β1 and H1HR increased (Supporting Fig. S5A,B). Inhibition of MC-FXR reduced biliary expression of IL-1β, TGF-β1, and H1HR (Supporting Fig. S5A,B).

Correspondingly, HA secretion increased in murine cholangiocytes treated with murine MC-vehicle supernatants, but was reduced (not significant) when murine MCs were pretreated with the FXR inhibitor (Supporting Fig. S5C). Next, we found that following murine MC-vehicle supernatant stimulation, cholangiocytes have increased mRNA expression of FGF15 compared with basal cholangiocytes (Supporting Fig. S5D). Inhibition of MC-FXR reduced biliary expression of FGF15 compared with murine cholangiocytes treated with MC-vehicle supernatants (Supporting Fig. S5D). Taken together, these results confirm our IPA findings and *in vivo* studies, wherein MC-FXR regulates systemic and hepatic MC activation through activation of IL-1β, TGF-β1, and H1HR signaling. We conclude that MCs, through activation of FXR, regulate biliary FXR/FGF15 signaling and IL-1β, TGF-β1, and H1HR expression in a synergistic manner.



**FIG. 6.** Inhibition of MC-FXR reduces IBDM and inflammation. The effect of MC-FXR inhibition on IBDM and liver inflammation was measured by immunohistochemistry for (A) CK-19 and (B) EGF-like module-containing mucin-like hormone receptor-like 1 mouse homolog (F4/80), respectively, with (C,D) semiquantification. WT and *Kit<sup>W-sh</sup>* mice display minimal IBDM and F4/80 positive Kupffer cells that increased following MC injection in *Kit<sup>W-sh</sup>* + MC mice. Inhibition of MC-FXR reduced both IBDM and inflammation. (A) Red arrows indicate CK-19 positive bile ducts. (E) WT and *Kit<sup>W-sh</sup>* mice have minimal expression of hepatic inflammatory markers IL-1β, CCL5, CXCL2, and CXCL5, whereas *Kit<sup>W-sh</sup>* + MC mice have increased hepatic inflammatory marker expression that is reduced in *Kit<sup>W-sh</sup>* + MC-FXR mice. Data are mean ± SEM of n = 10–15 representative images for immunoreactivity semiquantification and of n = 4 experiments for qPCR from 6–8 mice per group. \**P* < 0.05 vs. WT, #*P* < 0.05 vs. *Kit<sup>W-sh</sup>* + MC. All representative images are presented as ×10. Abbreviation: Inj, injury.

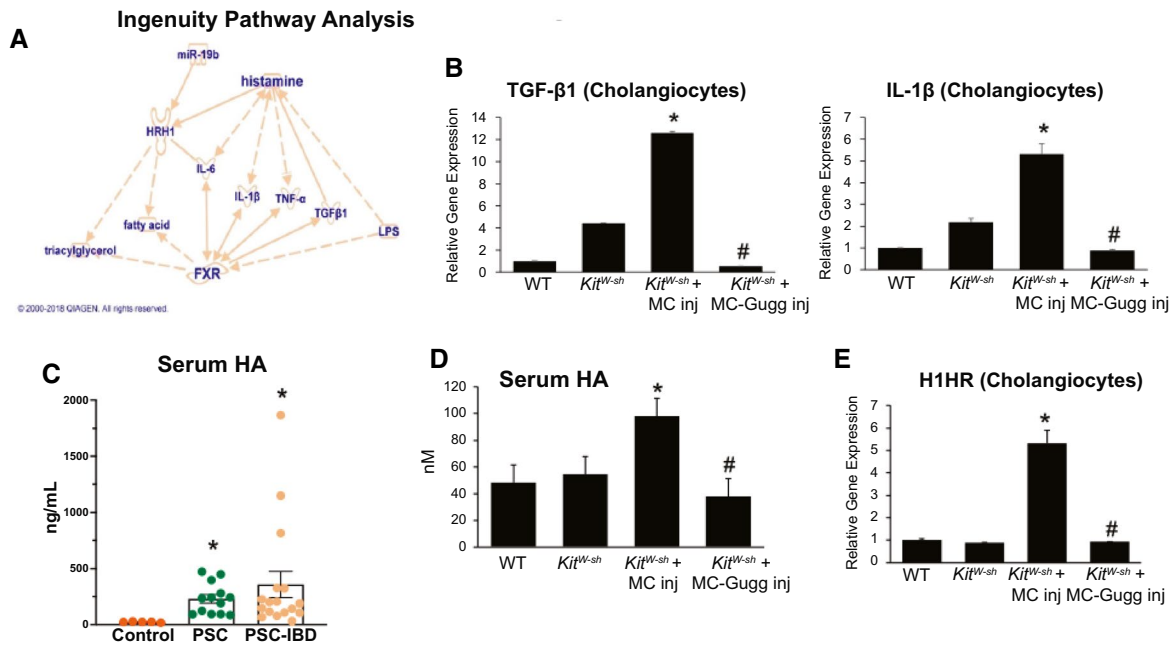
Cholangiocytes stimulated with MC-vehicle supernatant had increased proliferation compared with basal cholangiocytes as measured by bromodeoxyuridine-positive semiquantification and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Supporting Fig. S6A,B). Treatment with MC-Gugg supernatant resulted in decreased biliary proliferation (Supporting Fig. S6A,B). Taken together, we have found that MC-FXR is capable of activating an inflammatory and proliferative cholangiocyte phenotype *in vitro*, mimicking hallmarks of *in vivo* DR seen in cholestatic liver diseases.<sup>(25)</sup>

In the final sets of experiments, human HSCs stimulated with basal-treated MC supernatants had increased α-SMA and fibronectin-1 mRNA expression compared with basal treatment and inhibition of MC-FXR decreased these parameters (Supporting

Fig. S6C), demonstrating a direct interaction of MC-FXR and HSCs and further supporting our *in vivo* studies.

## Discussion

We identified a signaling pathway wherein MC-specific FXR/FGF signaling regulates DR and hepatic fibrosis, recapitulating cholestatic liver injury. We confirm previous work establishing the role for MCs in the induction and development of cholestatic liver damage characterized by DR, biliary senescence/SASP, and fibrosis in MC-deficient *Kit<sup>W-sh</sup>* mice, which are phenotypically normal. Our study reports the significant impact of MCs on TBA signaling and biliary and intestinal FXR/FGF axis during liver injury.



**FIG. 7.** FXR and HA signaling crosstalk through IL-1 $\beta$ , TGF- $\beta$ 1, and H1HR. (A) IPA demonstrated a link between HA and FXR signaling crosstalk through IL-1 $\beta$ , TGF- $\beta$ 1, and H1HR. (B) TGF- $\beta$ 1 and IL-1 $\beta$  expression increased in isolated cholangiocytes from *Kit<sup>W-sh</sup>* + MC compared with WT and *Kit<sup>W-sh</sup>* mice by qPCR and inhibition of MC-FXR reduced biliary TGF- $\beta$ 1 and IL-1 $\beta$  expression. (C) Serum HA increases in patients with PSC (n = 14) and PSC-IBD (n = 17) compared with controls. (D) WT and *Kit<sup>W-sh</sup>* have minimal HA serum secretion, which is elevated in *Kit<sup>W-sh</sup>* + MC mice. (E) Serum HA is reduced in *Kit<sup>W-sh</sup>* + MC-Gugg mice. (F) MC injection increases biliary H1HR expression in *Kit<sup>W-sh</sup>* + MC compared with WT and *Kit<sup>W-sh</sup>* mice and inhibition of MC-FXR reduces biliary H1HR expression. Data are mean  $\pm$  SEM of n = 8 experiments for qPCR, n = 4 experiments for HA EIA from 6–8 mice per group, and n = 2 experiments per patient sample for HA EIA. \*P < 0.05 vs. control group, #P < 0.05 vs. *Kit<sup>W-sh</sup>* + MC. Abbreviation: Inj, injury.

MCs are innate immune cells that, upon receptor activation, induce an inflammatory response through various mediators, including HA and FGF.<sup>(26)</sup> Our group demonstrated that MCs infiltrate the liver following injury and perpetuate cholestatic damage<sup>(8,9,24)</sup> and that stabilization of MCs in cholestatic models results in amelioration of DR, inflammation, and hepatic fibrosis.<sup>(27)</sup> Aside from traditional receptors, MCs express a number of other receptors and binding sites, including FXR $\beta$  and ASBT.<sup>(19)</sup> Our current work demonstrates that MC-derived FXR/FGF signaling regulates biliary damage and hepatic fibrosis. In support of this, during organ transplantation, MCs can produce a variety of both proinflammatory and anti-inflammatory mediators as well as release factors like FGF-2, which increases cell-to-cell interactions during regeneration.<sup>(28)</sup> In addition, FGF may induce MC recruitment, as demonstrated in a study of prostate cancer in which the authors found that

stimulation of FGF-2 induced MC recruitment and promoted angiogenesis in mice and inhibition of FGF-2 reduced tumor growth.<sup>(20)</sup>

We found that inhibition of MC-FXR signaling ameliorates biliary senescence and SASP through IL-1 $\beta$ /TGF- $\beta$ 1 and HA/H1HR signaling. Interestingly, most mediators secreted by MCs are also SASP components, and several studies have implicated TGF- $\beta$ 1 in liver disease progression.<sup>(9,11,29)</sup> We found that MCs induce IL-1 $\beta$  expression that was reduced when FXR was inhibited; however, a study by Xiong et al. found that obeticholic acid (OCA; FXR agonist), in combination with lipopolysaccharide, ameliorated liver damage and inflammation by decreasing hepatic IL-1 $\beta$ .<sup>(30)</sup>

During canonical enterohepatic circulation, intestinal FXR/FGF15 signaling becomes activated following an elevation of serum TBAs, which results in down-regulation of ileal ASBT and inhibition of



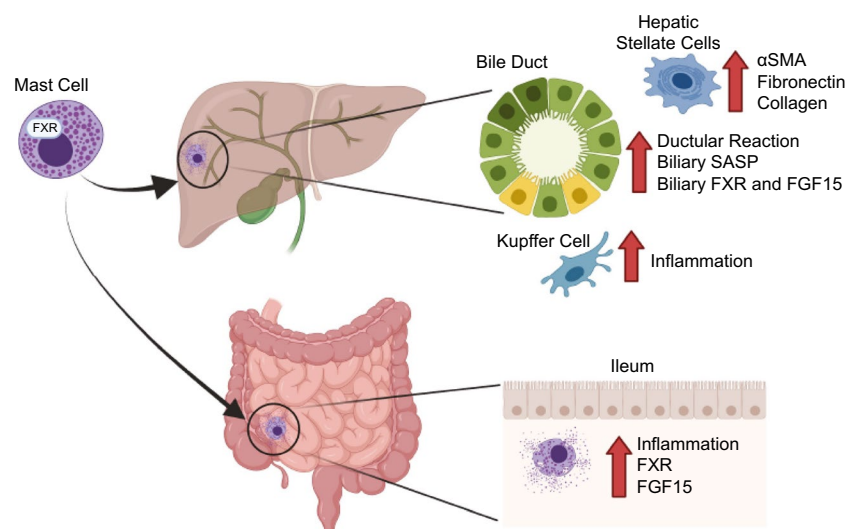
hepatic BA synthesis enzymes.<sup>(4)</sup> *Fxr*<sup>-/-</sup> mice have reduced intrahepatic BA pressure following BDL surgery, resulting in lower IBDM compared with BDL WT.<sup>(31)</sup> We found that MC injection increased TBA content and hepatic FXR/FGF15 signaling in MC-deficient mice, and these features were blunted after inhibition of MC-FXR, pinpointing MCs as critical regulators of TBA and hepatic FXR/FGF15 signaling. Interestingly, we found that intestinal FXR/FGF15 expression was increased following MC injection in MC-deficient mice and reduced in MC-FXR injected and untreated MC-deficient mice. This could be due to *Kit*<sup>W-sh</sup> phenotype, which lack melanocytes and interstitial cells, but warrants further investigation. In contrast to our findings, Verbeke et al. found that vehicle-treated BDL rats had low expression of SHP, a downstream target of FXR activation, in ileum and jejunum that was rescued in ileum only following oral gavage of OCA.<sup>(32)</sup> Nonetheless, in our current study, BDL WT and *Mdr2*<sup>-/-</sup> mice displayed elevated intestinal FXR and FGF15 expression compared with WT controls, due to the complexity of the intestinal FXR/FGF15 axis this also requires further investigation.

Previous studies found that BAs regulate the release of specific mediators from MCs, including the release of HA<sup>(33,34)</sup>; however, our studies show that MCs influence hepatic and intestinal BA signaling. Our findings are not surprising considering that MCs express BA transporters and ligands,<sup>(19)</sup> which may interact with surrounding cells to induce proliferation, senescence, or inflammation or alter BA synthesis. MC HA and intestinal H1HR activation contribute to chenodeoxycholic acid (CDCA)-induced colonic chloride secretion in rats, whereas MC-deficient rats had decreased CDCA-induced chloride secretion,<sup>(33)</sup> supporting the role for MCs in intestinal BA signaling. Interestingly, BA activation of FXR in rat colon induced mucosal MC release of nerve growth factor, which was reduced following FXR inhibition and silencing,<sup>(35)</sup> supporting our proposed crosstalk between BA signaling, FXR, and MC mediators. Furthermore, human colonic transcriptomic analysis showed up-regulation of BA signaling pathways in patients with PSC-IBD compared with those with IBD and healthy controls<sup>(36)</sup>; however, MC presence and activation must be assessed in intestinal tissue from patients with PSC-IBD in order to confirm MC and FXR intestinal crosstalk. It is unlikely that MCs

synthesize BAs, and thus, regulation of MC mediators like FGF15 or HA may act as a secondary mechanism to control BA circulation and signaling.

We demonstrate that inhibition of MC-FXR reduces biliary FXR and FGF15 *in vivo* and *in vitro*, which may result in reduced cholehepatic shunting of BAs; however, further analysis is needed to confirm this. Our data show that inhibition of MC-FXR results in a reduction of TBA levels that are otherwise elevated following cholestatic liver injury.<sup>(37)</sup> Quist et al. found that CDCA induces HA release from MCs *in vitro*, but cholic acid impeded HA release, thus supporting the concept that BAs and MCs may interact to influence HA signaling.<sup>(34)</sup>

It has been shown that there is dysregulation of FXR and FGF signaling in various liver diseases including NAFLD, PBC and PSC.<sup>(16,38,39)</sup> OCA is currently being used in clinical trials and shows potential for improved liver histology and lipid absorption in patients with PSC and NASH, respectively, and provides clinical benefits to patients with BA diarrhea<sup>(40-42)</sup>; however, the overall benefits of FXR agonism are controversial because of adverse events seen in patients with decompensated cirrhotic PSC and PBC.<sup>(3)</sup> This may be due to the increased expression of biliary FXR in patients with liver injury, as shown in our study. If FXR is already enhanced in cholangiocytes, administering OCA may be counterproductive by inducing biliary senescence or increasing damage during cholestatic injury; however, further studies should be performed to investigate this possibility. Furthermore, gut microbiota influences BA signaling and intestinal inflammation and the subsequent positive or negative effects of FXR during diseases like NAFLD or NASH.<sup>(43)</sup> In support of our study, in intestinal *Fxr*<sup>-/-</sup> knockout mice fed high-fat diet (HFD) and treated with antibiotics, HFD-associated pathologies were reduced,<sup>(44)</sup> further implicating the complex role of FXR signaling during liver disease. Interestingly, OCA but not fexaramine (FXR agonist) ameliorated altered gut-barrier vascularization in experimental cirrhotic mice (carbon tetrachloride or BDL), but both, OCA and fexaramine, were able to reduce bacterial translocation from small intestine to the liver.<sup>(45)</sup> It has been recently shown that MC presence and BA receptor increase in the duodenum of patients with irritable bowel syndrome, which, along with our study, indicates a role for MC regulation of BA receptor expression throughout the intestinal tract.<sup>(46)</sup> The role of MCs in the regulation of epithelial barrier, inflammation, and



**FIG. 8.** Graphical abstract demonstrating the role of MC-FXR during cholestatic liver damage. Patients with cholestatic liver diseases, such as PSC, often present with comorbidities like IBD. We found that MCs, which express FXR and secrete FGFs and HA, infiltrate the liver and contribute to increased DR, biliary SASP, and FXR/FGF15 during cholestatic liver injury. Furthermore, MCs can activate HSCs to increase  $\alpha$ -SMA, fibronectin-1, and collagen, exacerbating liver damage. In this study, we found that MCs infiltrate the intestine of cholestatic liver injury mouse models, increasing intestinal inflammation and altering the FXR/FGF15 axis, leading to dysregulated BA signaling. MCs may serve as an ideal target for therapeutic interventions for patients presenting with cholestatic liver injury and intestinal inflammation. Created with BioRender.com.

gut-liver axis during cholestatic liver injury is a complicated future direction for this study.

In conclusion, we have generated a model of DR and hepatic fibrosis by injecting MCs into phenotypically normal MC-deficient mice and confirmed our findings using the established BDL and PSC mouse model. Our data demonstrate that MCs regulate biliary injury and DR through alteration of TBA levels and FXR/FGF signaling through biliary SASP and HA/H1HR pathways (Fig. 8). In human samples, we found that both FXR in bile ducts and MC presence are up-regulated in cholestatic liver damage, demonstrating the close interaction between MCs and biliary FXR during liver disease. Although controversial, our results demonstrate that specific inhibition of MC-FXR ameliorates cholestatic liver injury by reducing TBA levels, biliary FXR/FGF15, and subsequent HA/H1HR signaling. Our study also confirms a role for MC-FXR in the regulation of intestinal MC infiltration, inflammation, and FXR/FGF15 in cholestatic mice (Fig. 8). Further investigation into the contribution of MC mediators is needed to fully identify clinical therapies; however, our data suggest that MCs may be a targetable link for patients with PSC alone or PSC coupled with IBD.

**Author Contributions:** V.M. was responsible for conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, and writing (original draft, review, and editing). L.K. was responsible for data curation, formal analysis, investigation, methodology, validation, and writing (review and editing). B.E. was responsible for resources and writing (editing). K.K. was responsible for data curation and writing (editing). D.K. was responsible for data curation and writing (editing). T.Z. was responsible for software and data curation. L.C. was responsible for data curation and writing (editing). L.P. was responsible for data curation and writing (editing). N.W. was responsible for data curation and editing. J.D. was responsible for data curation, formal analysis, investigation, methodology, and validation. L.H. was responsible for data curation, formal analysis, investigation, methodology, and validation. S.G. was responsible for funding acquisition and resources. G.A. was responsible for funding acquisition, resources, and writing (review and editing). H.F. was responsible for conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, visualization, and writing (original draft, review, and editing).

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## Supporting Information

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep.32028/supinfo](https://onlinelibrary.wiley.com/doi/10.1002/hep.32028/supinfo).