CHRONIC LIVER DISEASE: NEW TARGETS AND NEW MECHANISMS

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CHRONIC LIVER DISEASE: NEW TARGETS AND NEW MECHANISMS

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EDITORIAL
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Editorial: Chronic Liver Disease: New Targets and New Mechanisms

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Editorial on the Research Topic

Chronic Liver Disease: New Targets and New Mechanisms

INTRODUCTION

Chronic liver diseases (CLD), including non-alcoholic fatty liver disease (NAFLD), chronic hepatitis B (CHB), alcoholic liver disease (ALD), affect 800 million people globally and cause 2 million deaths per year (Asrani et al., 2019; Xiao et al., 2019; Kochanek et al., 2020; Cheemerla and Balakrishnan, 2021; Kostallari et al., 2021). Without effective treatment, CLD is likely to progress to liver cirrhosis and hepatocellular carcinoma (HCC), accounting for 3.5% of worldwide deaths (Moon et al., 2020). Although significant efforts have been made to prevent CLD, the morbidity and mortality remain high. It is an unmet need to explore the mechanisms and novel therapeutic strategies to treat liver disease. This Research Topic presents the most recent advances in CLD, including novel molecular and cellular mechanisms, promising therapeutic targets, new drug delivery methods, and biomarker discovery for liver fibrosis.

NEW TARGETS IN NON-ALCOHOLIC FATTY LIVER DISEASE AND NON-ALCOHOLIC STEATOHEPATITIS

NAFLD, which affects 25% of the global population, is mainly characterized by hepatic steatosis (Younossi et al., 2016). About 10%–15% of NAFLD patients develop a severe form of NAFLD called NASH, which is characterized by increased inflammation and hepatocyte injury (Zhang et al.; Torres et al.). Although NAFLD represents the highest prevalent type of CLD (Younossi et al., 2019), its pathogenesis has not been fully understood. It was reported that inflammasomes, including the NOD-like receptor protein 3 (NLRP3), are linked to the pathophysiology of NASH (Gan et al., 2022). Torres et al. demonstrated that NLRP3 blockage by its antagonist IFM-514 decreased inflammation and fibrosis in a murine model of NASH, suggesting that NLRP3 inhibition may be an attractive therapeutic approach for NASH patients. Several other pathways were also reported as potential therapeutic targets for NAFLD. Since NAFLD is the result of the abnormal accumulation of lipids, understanding the mechanisms of excessive biogenesis of lipids is of high interest. Hydroxysteroid 17β-dehydrogenase 13 (HSD17B13), a newly identified hepatocyte-specific lipid droplet-associated protein, promotes hepatic lipogenesis (Su et al., 2014). Previous studies revealed that HSD17B13 was increased in the liver of NAFLD patients. However, HSD17B13 gene knockout failed to protect the liver from steatosis. Although murine models produced inconsistent results, human genetic surveys uncovered that loss-of-function human HSD17B13 variants are associated with decreased severity of NAFLD/NASH (Zhang et al.). Consistently, clinical

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trials showed that downregulation of HSD17B13 with RNA interference (RNAi) therapeutic approaches or the selective HSD17B13 inhibitor INI-678 reduced serum ALT and AST levels and fibrosis markers (NCT04565717, NCT04202354 and https://inipharm.com/), suggesting that this strategy presents a great therapeutic potential. Lipid homeostasis is also regulated by the endoplasmic reticulum (ER)-related degradative signaling pathway, which is necessary to eliminate misfolded proteins, limit ER stress, and maintain cell activity (Maiers et al., 2017; Liu et al., 2021). Dysfunction of ER-related degradative signaling pathway influences the metabolism of hepatocytes and biosynthesis of cholesterol in NAFLD (Duwaerts and Maiers).

The Notch signaling pathway is crucial in regulating cell differentiation, proliferation, and apoptosis. Xu and Wang summarized the role of the Notch signaling pathway in hepatic lipid accumulation, insulin resistance, oxidative stress, fibrogenesis, and autophagy progression in NAFLD. In addition to inflammation as one of the main features of NASH, biliary senescence and senescence-associated secretory phenotype (SASP) were also identified as significant contributors to the progress of NAFLD and NASH *via* the recruitment of immune cells (Meadows et al.). Interestingly, in regards to inter-organ communication, liver-eye cross-talk was found to play a possible role in NAFLD progression and diabetic retinopathy was considered a risk factor for HCC (Yuan et al.). Further studies are needed to transfer the above knowledge into clinical application.

NEW MARKER AND THERAPEUTIC TARGETS IN CHRONIC HEPATITIS B

CHB patients tend to develop liver fibrosis with subsequent cirrhosis and HCC. Thus, accurately assessing the stage of CHB is important for clinical management. Xu et al. introduced leukocyte cell-derived chemotaxin 2 (LECT2) as a novel biomarker of liver fibrosis for CHB patients. LECT2 is secreted by hepatocytes and is present in both, liver tissue and serum. LECT2 levels were significantly increased in patients with CHB or liver cirrhosis, and further enhanced with disease severity, suggesting that LECT2 may be a direct and reliable biomarker for CHB. Although the progress in staging cirrhosis is helpful for clinical decision-making, the current therapies for HBV infection are not sufficient to fully eliminate the virus and restore normal immunity. To this end, Zhong et al. discussed the role of cytokines and chemokines in HBV infection and revealed new potential candidates to be considered for immunotherapies. In summary, the novel staging marker for CHB patients and innovative immunotherapies approaches have the potential to improve the clinical management of CHB patients.

ER-RELATED DEGRADATIVE SIGNALING PATHWAYS IN ALCOHOLIC-ASSOCIATED LIVER DISEASE (ALD)

ALD is an increasing CLD worldwide with high morbidity and mortality (Sehrawat et al., 2020). The uncertainty of ALD pathogenesis makes it difficult to control the disease (Yin et al., 2022). ER contains the majority of the machinery required for xenobiotic detoxification and is important for cellular homeostasis. It is reported that ER-associated degradation (ERAD) plays a protective role in response to alcohol through promoting cytochrome p450 enzyme E1 (CYP2E1) turnover (Duwaerts and Maiers). It is worth mentioning that ERAD also contributes to remit ER stress of hepatocytes in α1-antitrypsin deficiency (Duwaerts and Maiers). Further studies are needed to better understand ERAD and develop novel protective strategies.

PORTAL FIBROBLASTS AND BILIARY SENESCENCE IN CHOLESTATIC LIVER DISEASE

Cholestatic liver disease is one of the most common liver disorders associated with inadequate bile flow concomitant with a noxious bile acid accumulation in the liver and/or systemic circulation (Gijbels et al., 2021). Myofibroblasts, including activated hepatic stellate cells (HSCs) and portal fibroblasts (PFs), are the major source of extracellular matrix in the injured liver to drive liver fibrosis (Lan et al., 2022). Unlike toxic liver injury, cholestatic liver injury activates not only HSCs but also PFs. PFs are activated and play a dominant role in the early stage of liver injury (Lan et al., 2022); however, due to technical difficulties in isolating PFs, the contributions of PFs to liver fibrosis remain only partially understood. Fuji et al. commented on the role of mesothelin (Msln), mucin 16 (Muc16), and thymocyte differentiation antigen 1 (Thy-1) in PFs activation during cholestatic liver fibrosis. They suggested that therapies targeting Msln or PFs may be promising therapeutic strategies for cholestatic liver diseases. Biliary secretory functions of cholangiocytes regulate liver inflammation and fibrosis through the secretion of various molecules. Biliary senescence and senescence-associated secretory phenotype (SASP) were reported to play a pro-inflammatory role and contribute to the progression of biliary diseases, including primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), biliary atresia, as well as ALD (Meadows et al.). Thus developing strategies to target PFs and biliary senescence would present a high therapeutic potential.

NEW MECHANISMS, TARGETS AND THERAPEUTIC APPROACHES IN LIVER CIRRHOSIS

Liver cirrhosis is the end stage of CLD, which is featured by irreversible extracellular matrix deposition and damage of liver structure (Cai et al., 2021). Cellular cross-talks, signals from the microenvironment, as well as intracellular signaling are crucial in the development of liver fibrosis and cirrhosis (Kostallari et al., 2018; Gao et al., 2020; Gao et al., 2021; Zeng et al., 2021). Two reviews in this issue stated how the major types of liver cells,

including HSCs, hepatocytes, liver sinusoidal endothelial cells, PFs, cholangiocytes and inflammatory cells, participate in the pathogenesis and development of liver cirrhosis. The relevant signaling pathways that contribute to liver fibrosis and prospective therapeutic targets were described thoroughly (Zhang et al.; Gu et al.). Additionally, Li et al. emphasized the influence of mitochondria dysfunction and hypoxia inducible factor-1α (HIF1α)-induced oxygen imbalance on metabolism and immunity in liver fibrosis.

In addition to developing new drugs, exploring the anti-fibrotic capacity of existing medicines targeting CLD is also interesting. The widely used anti-HBV infection drug tenofovir disoproxil fumarate (TDF) was found to alleviate liver fibrosis *via* its direct antiviral-independent effects; however, the mechanism involved in reducing fibrosis has not been elucidated, yet. Duan et al. applied genomics analysis to prove that TDF may ameliorate CLD by affecting the expression of genes involved in hepatic immune response and metabolic processes *via* mmu-miR-155-5p-NF-κB signaling.

Each liver cell type might respond in a different way to a given drug; thus, targeting a signaling pathway in a specific cell type would be more effective. The review from Gu et al. discussed the recent nano-delivery approaches specific-targeting HSCs, immune cells, hepatocytes, and liver sinusoidal endothelial cells for liver fibrosis. The nanoparticles (NPs), including metal NPs, lipid NPs, polymer NPs, and protein NPs, with controllable size, shape, diverse components, and modifiable surface characteristics, can reduce drug adverse effects meanwhile improve therapeutic effects. However, the efficacy, quality, safety, and cost-effectiveness of NPs need further research.

CONCLUSION AND PERSPECTIVES

The Research Topic encompasses articles spanning from new clinical and basic research findings to reviews that summarize the recent advancements in the mechanisms of CLD. The role of hepatic myofibroblasts, immune cells, sinusoidal endothelial cells, and related signaling pathways involved in inflammation, immunity, and metabolism in CLD is of great interest. Among the numerous potential therapeutic targets for CLD, treatments targeting HSD17B13 achieved encouraging preliminary results in clinical studies. Meanwhile, the present anti-fibrotic agents are neither liver nor fibrosis specific, leading to insufficient efficacy and several side effects. Therefore, seeking a more specific and effective therapeutic strategies is of great interest and presents an urgent need. Fortunately, with the development of nano-technology, NPs show advantages in targeted drug delivery, combination therapy, and theranostics. Potential anti-fibrotic targets combined with nano-technology might bring a new perspective to future therapies. Up to date, the heat shock protein 47 siRNA delivery through lipid NP to HSC is the only NP-based strategy in the clinical stage for the treatment of liver fibrosis (Gu et al.). Further research is needed to explore more appropriate and reliable nano-delivery approaches and integrate them with the novel therapeutic targets.

AUTHOR CONTRIBUTIONS

EK, JG, and HW conceived and supervised the study; YY, HW, JG, and EK wrote and revised the manuscript.

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ORIGINAL RESEARCH
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The Specific NLRP3 Antagonist IFM-514 Decreases Fibrosis and Inflammation in Experimental Murine Non-Alcoholic Steatohepatitis

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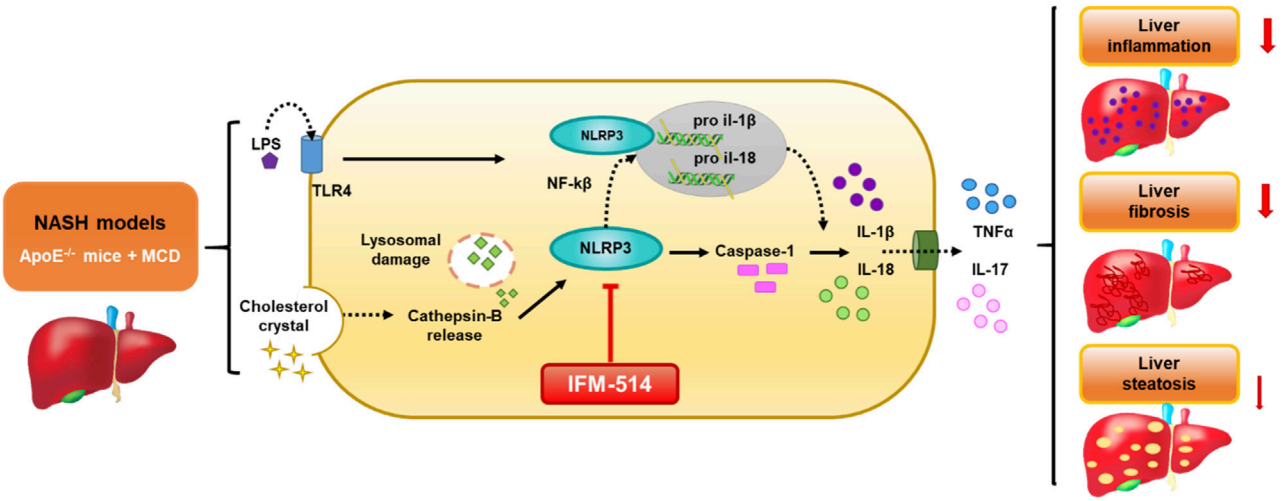
Background and Aims: Activation of the inflammasome NLRP3 (NOD-, LRR- and pyrin domain containing 3) contributes to the development of non-alcoholic fatty liver disease (NAFLD) and progression to non-alcoholic steatohepatitis (NASH). Therefore, this study explored the therapeutic effects of a novel and selective NLRP3 antagonist in a murine dietary model of NASH.

Methods: Groups of 12-week-old *ApoE*$^{-/-}$ mice were fed ad lib for 7 weeks with a methionine/choline deficient (MCD) and western diet (WD). After 3 weeks of diet-induced injury, mice were injected i. p. with the NLRP3 antagonist IFM-514 (100 mg/kg body weight) or vehicle (0.5% carmellose) every day, 5 days/week for a further 4 weeks. Several markers of inflammation, fibrosis and steatosis were evaluated. Whole transcriptome sequencing and panel RNA expression analysis (NanoString) were performed.

Results: IFM-514 inhibited IL-1β production in mice challenged with 20 mg/kg lipopolysaccharide, and in mouse and human inflammatory cells *in vitro*. IFM-514 inhibited hepatic inflammation in the *in vivo* non-alcoholic steatohepatitis model assessed by H&E staining and in the hepatic gene expression of inflammasome-related proinflammatory cytokines. This effect was associated with significant reduction in caspase-1 activation. Similarly, IFM-514 was efficacious *in vivo* in MDC-fed *ApoE*$^{-/-}$ mice, markedly reducing portal pressure, Sirius red staining and 4-hydroxyproline content compared to vehicle-treated mice. Moreover, IFM-514 significantly reduced hepatic steatosis in MCD-fed *ApoE*$^{-/-}$ mice, as evidenced by NAFLD scores, oil red O staining, hepatic triglycerides and gene expression. In WD treated animals, similar trends in inflammation and fibrosis were observed, although not sufficient IFM-514 levels were reached.

Conclusion: Overall, IFM-514 reduced liver inflammation and fibrosis, with mild effects on liver steatosis in experimental murine NASH. Blocking of NLRP3 may be an attractive therapeutic approach for NASH patients.

Keywords: inflammasome, liver fibrosis, steatosis, NASH, caspase-1



GRAPHICAL ABSTRACT | Schematic representation of the IFM-514 treatment effect in experimental murine NASH. The activation of NLRP3 inflammasome in response to NASH models is due to the gut-derived PAMPs, such as LPS, and cholesterol crystals. LPS activates nuclear factor kappa B (Nf-kB) via triggering of the TLR4 receptor and consequently promotes the expression of pro-IL-1β and pro-IL-18. Cholesterol crystals produce lysosomal damage with cathepsin-B release that also activates NLRP3, followed by caspase-1 activation that allows the release of IL-1β and IL-18 into the extracellular space and increasing TNF-α and IL-17. All these cytokines promote an increase in liver inflammation, fibrosis and steatosis. In our study, IFM-514, an NRLP3 antagonist, could protect prior to the pathogenesis produced by NASH murine model, with mild effect in liver steatosis.

HIGHLIGHTS

- IFM-514 reduced liver inflammation and fibrosis in MCD-fed *ApoE*$^{-/-}$ mice.
- IFM-514 produced a mild reduction in liver steatosis in MCD-fed *ApoE*$^{-/-}$ mice.
- Blocking NLRP3 might be an attractive therapeutic approach for NASH patients.

LAY SUMMARY

The activation of NLRP3 inflammasome in response to NASH models is due to the gut-derived PAMPs, such as LPS, and cholesterol crystals. LPS activates nuclear factor kappa B (Nf-kB) *via* triggering of the TLR4 receptor and consequently promotes the expression of pro-IL-1β and pro-IL-18. Cholesterol crystals produce lysosomal damage with cathepsin-B release that also activates NLRP3, followed by caspase-1 activation that allows the release of IL-1β and IL-18 into the extracellular space and increasing TNF-α and IL-17. All these cytokines promote an increase in liver inflammation, fibrosis and steatosis. In our study, IFM-514, an NRLP3 antagonist, could protect prior to the pathogenesis produced by NASH murine model, with mild effect in liver steatosis.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), characterized by fat accumulation in the liver (steatosis) in the absence of chronic alcohol use, is a common and emerging cause of chronic liver disease (Williams et al., 2011; Rinella, 2015). Interestingly, NAFLD is present in obese as well as lean patients. However, little is known about the differences between these two NAFLD types and thus, no tailored therapeutic approaches are available. Moreover, an estimated 10–15% of NAFLD patients develop non-alcoholic steatohepatitis (NASH), while a quarter of those will develop liver cirrhosis and potentially hepatocellular carcinoma (Macaluso et al., 2015). Therefore, NASH represents the severe and dangerous form of NAFLD characterized by hepatocyte injury, inflammation and fibrosis - the most critical outcome in NASH - , that may result from inflammasome activation (Ganz et al., 2015).

Inflammasomes are cytoplasmic multiprotein complexes that can sense danger signals from damaged cells and pathogens. They assemble to mediate caspase-1 activity, secretion of cytokines and other pro-inflammatory mediators, including IL-1β and IL-18 (Martinon et al., 2002; Schroder and Tschopp, 2010; Szabo and Petrasek, 2015), as a result of tissue damage or cellular stress. Several members of the NLR family (nucleotide-binding and oligomerization domain and leucine-rich-repeat-containing proteins), including the NOD-like receptor protein 3 (NLRP3), have been linked to the pathophysiology of NASH (Abstract diagram). This has inspired strategies to block inflammasome activation by pharmacological targeting of NLRP3 (Próchnicki et al., 2016). In fact, inhibition of NLRP3 can be achieved by limiting Toll-like receptor- and tumor necrosis factor-mediated increases in NLRP3 expression. However, since this approach lacks specificity and is likely to produce many off-target effects (Bahia et al., 2015), this study tested a novel selective NLRP3 antagonist for therapeutic effects in murine models of NASH. Since both NASH and NLRP3 have been linked to metabolic syndrome we have chosen murine models for which we have

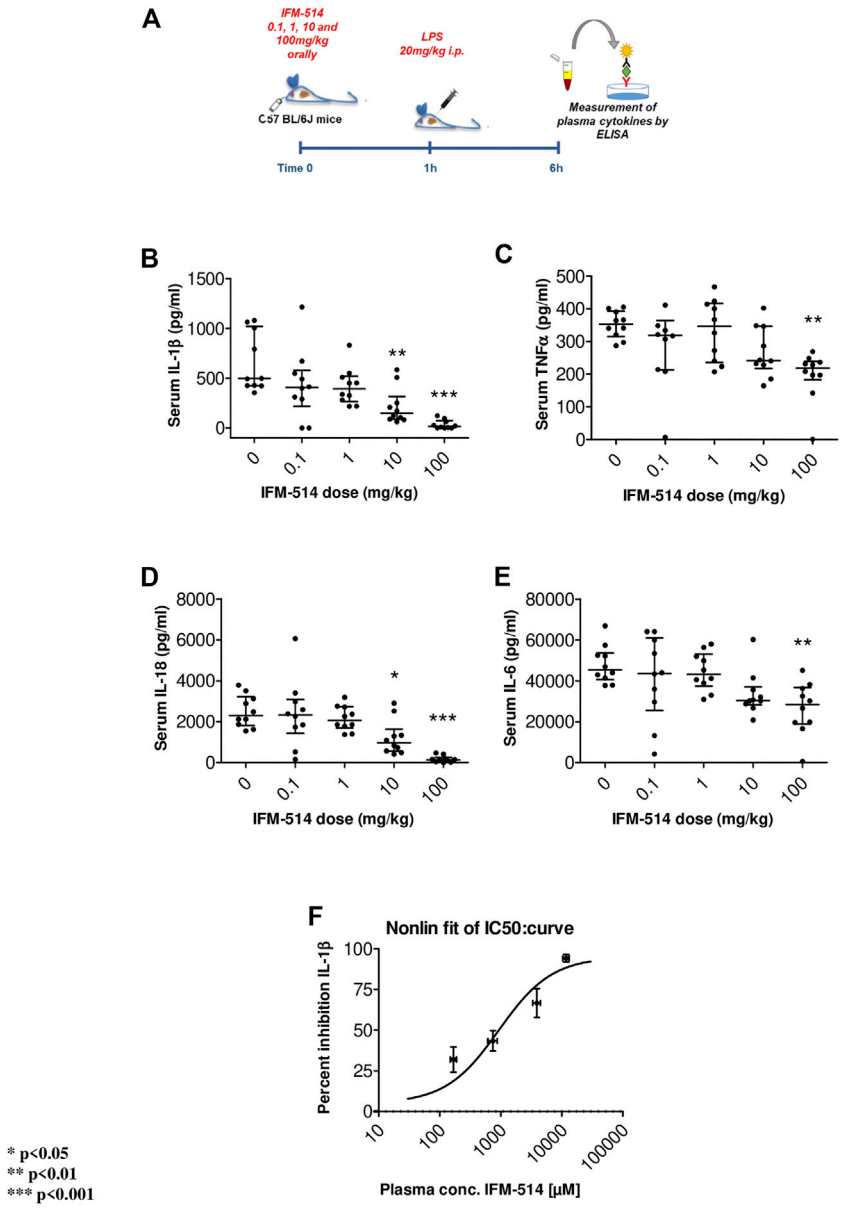


FIGURE 1 | Experimental design for mouse *in vivo* pharmacodynamics model: LPS stimulation of plasma cytokines. IFM-514 was dosed orally in WT C57BL/6 mice at 0.1, 1, 10 and 100 mg/kg 1 h before LPS (20 mg/kg i. p.) **(A)**. LPS-induced production of plasma cytokines IL-1β **(B)**, TNFα **(C),** IL-18 **(D)** and IL-6 **(E)** measured by ELISA 5 h after LPS challenge. IFM-514 significantly inhibited IL-1β production *in vivo* when dosed orally with an IC50 = 947 ng/ml; 95% CI 499.5–1,683 ng/ml **(F).** Results are expressed as mean ± standard error of the mean (SEM); n = 10/group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Abbreviations: ELISA, enzyme-linked immunosorbent assay; IC50, half maximal inhibitory concentration; IL1β, interleukin 1β; IL-16, interleukin 16; IL18, interleukin 18; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α.

previously demonstrated that it is suitable for investigation of liver of metabolic syndrome (Schierwagen et al., 2015, 2016).

RESULTS

IFM-514 Inhibits the NLRP3 Inflammasome *In Vivo* and *In Vitro*

In human cells, IFM-514 inhibited gramicidin-induced IL-1β release in PMA-primed human THP-1 cells (half maximal inhibitory concentration, IC_{50} 0.275 ± 0.115 μM, n = 9) and was a potent inhibitor of gramicidin-induced IL-1β release in LPS-primed monocyte-derived macrophages (IC_{50} 0.156 ± 0.042 μM, n = 8). Similarly, IFM-514 was a potent inhibitor of gramicidin-induced IL-1β release in LPS-primed immortalized macrophages from WT C57BL/6 mice (IC_{50} 0.146 ± 0.042 μM, n = 6), displayed similar potency in primary bone marrow-derived macrophages (BMDM) from C57BL/6 mice (data not shown) and in BMDM from Balb/c mice (IC_{50} 0.313 ± 0.013 μM, n = 6; free IC_{50} 10.9 ± 0.5 nM. To determine the recommended dose *in vivo*, WT C57BL/6 mice were

TABLE 1 | Age, body weight (week 0 and 7), liver weight and liver-to-body weight ratio of *ApoE*$^{-/-}$ mice receiving normal diet, MCD, WD and IFM-514-treated MCD-fed and WD-fed mice.

Mice group	Age (weeks)	Week 0 body weight (g)	Week 7 body weight (g)	Liver weight (g)	Liver-to-body weight ratio
Normal Diet	19 ± 0.0	25.2 ± 1.20	28.6 ± 1.18	1.4 ± 0.13	4.9 ± 0.38
MCD	21.2 ± 1.40	26.6 ± 3.75	16.7 ± 2.73[b]	0.9 ± 0.15	5.2 ± 0.59
MCD + IFM-514	16.3 ± 1.96	22.3 ± 3.88	14.3 ± 2.27[c]	0.7 ± 0.07	5.4 ± 0.40
WD	12.3 ± 2	23.3 ± 2.90	24.3 ± 3.86[b]	1.3 ± 0.25	5.5 ± 0.45
WD + IFM-514	13 ± 2.13	21.8 ± 3.83	24.0 ± 3.62[d]	1.3 ± 0.26	5.8 ± 2.25

[a]*No significant differences in body weight between any groups in week 0.*
[b]*versus control diet.*
[c]*versus MCD.*
[d]*versus WD.*

used in a pharmacodynamic (PD) model of lipopolysaccharide (LPS) mediated cytokine release. IFM-514 was dosed orally in mice at 0.1, 1, 10 and 100 mg/kg 1 h before LPS challenge (20 mg/kg i. p.) and plasma cytokines were measured 5 h later by ELISA (**Figure 1A**). IFM-514 strongly and dose-dependently abrogated the LPS-induced production of NLRP3-dependent plasma cytokines IL-1β and IL-18, and had a mild but significant effect on TNFα and IL-6 at the highest dose tested (**Figures 1B–E**). Moreover, the 100 mg/kg dose of IFM-514 inhibited IL-1β production *in vivo* by more than 90%, even when dosed orally. The plasma concentration of IFM-514 was measured at the time of cytokine measurement and plotted against the percentage inhibition of IL-1β production, resulting in an IC_{50} of 947 ng/ml (95% CI 499.5–1,683 ng/ml) (**Figure 1F**). IFM-514 was highly protein bound in plasma (99.59% bound), to an extent that, when adjusted for plasma protein binding, the free IC_{50} *in vivo* was 3.9 ng/ml or 9.2 nM, which corresponded with the free *in vitro* IC_{50} in mouse BMDM. Consistently, single-dose pharmacokinetic (PK) studies in WT C57BL/6 mice suggested that the recommended dose for *in vivo* use was 100 mg/kg free base (corresponding to 105 mg/kg Na + salt) when delivered i. p. or orally once-per-day (**Supplementary Table 1**). Using modeled PK data, this dose gave an average free liver concentration above the free *in vivo* IC_{90} in the LPS challenge model (83 nM) and the free *in vitro* IC_{90} in the BMDM assay (40.0 ± 3.5 nM) for approximately 24 h. Therefore, this dose was used for the *in vivo* experiments in NASH-induced *ApoE*$^{-/-}$ mice (**Table 1**).

Here, 12-week-old *ApoE*$^{-/-}$ mice were fed for a total of 7 weeks with MCD diet. After 3 weeks of diet-induced injury, mice received i. p. injections of IFM-514 (100 mg/kg) or vehicle for 5 days/week for a further 4 weeks (**Figure 2A**). To determine whether the liver was adequately exposed to IFM-514, the concentration of IFM-514 was measured by HPLC-MS in liver and serum 8 hours after the last dose of IFM-514 in MCD-fed *ApoE*$^{-/-}$ mice (**Figures 2B,C**). The free IFM-514 concentration in the liver was above the free plasma concentration and sufficient to inhibit 90% of the IL-1β synthesis in the LPS-PK/PD model. IFM-514-treated NASH *ApoE*$^{-/-}$ mice had similar circulating IL-1β and IL-1α as vehicle-treated NASH *ApoE*$^{-/-}$ mice (**Figures 2D,E**).

IFM-514 Reduces Hepatic Inflammation in MCD-Fed *ApoE*$^{-/-}$ Mice

Since NLRP3-mediated inflammation is linked to NASH (Mridha et al., 2017), we sought to test whether IFM-514 has an effect in MCD-fed *ApoE*$^{-/-}$ mice. Hematoxylin and eosin (H&E) staining (**Figure 3A**), inflammation score (**Figure 3B**) and NAS scores (**Figure 3C**) showed that IFM-514 reduces hepatic inflammation in MCD-fed *ApoE*$^{-/-}$ mice, being significant the NAS score. Liver samples were further processed to analyze the activation of caspase-1 and IL-1β protein expression, a pathway that is extremely important in NASH (Szabo and Petrasek, 2015). Protein analysis showed that the activation of p20 and p33-caspase-1 subunits and pro-IL-1β were significantly reduced in IFM-514-treated vs vehicle-treated MCD-fed *ApoE*$^{-/-}$ mice, but mature IL-1β and pro-caspase-1 showed a tendency to decrease with IFM-514-treatment (**Figure 3D**). To further pinpoint the mechanism of IFM-514 in hepatic inflammation, liver RNA was extracted for NanoString mouse myeloid innate immunity panel of 770 genes. MCD diet induced upregulation of genes at least 1.5-fold, and some of these genes were clearly reduced in IFM-514-treated vs vehicle-treated NASH *ApoE*$^{-/-}$ mice. This set of genes included several pro-inflammatory genes, such as chemokine ligands (Ccl) 1, 4 and 28, Il10 and Tnfα (**Figure 3E**), also shown in the heatmap (**Figure 3F**). In agreement with these data, IFM-514 had a pronounced effect on cytokine and inflammasome-related genes in the MCD-fed mice. Moreover, IFM-514 clearly reduced the hepatic expression of many genes related to the cytokine-cytokine receptor interaction (C-C) pathway, which is not limited to inflammasome function, but inflammation in general. These results suggest that IFM-514 inhibits the NLRP3 inflammasome and the subsequent activation of caspase-1 in MCD-fed *ApoE*$^{-/-}$ mice. The effect of IFM-514 decreasing liver inflammation was supported by immunohistochemistry of hepatic macrophages and activated kuffer cells (F4/80-positive cells) (**Supplementary Figure 1**), such as the reduction of *Adgre1* expression indicated in the heatmap (**Figure 3F**).

IFM-514 Reduces Hepatic Fibrosis in MCD-Fed *ApoE*$^{-/-}$ Mice

Given that MCD feeding induced hepatic fibrosis in *ApoE*$^{-/-}$ mice, we tested whether the inhibition of NLRP3 with IFM-514 had an effect on markers of fibrosis. IFM-514 treatment significantly reduced liver fibrosis as shown by quantitative Sirius red (SR) staining (**Figures 4A,B**), and the 4-hydroxyproline content (**Figure 4C**). Collagen type 1 α1 chain (Col1a1) protein expression (**Figure 4D**) had a significant reduction and hepatic mRNA expression levels (**Figure 4E**) also had a tendency towards reduction in MCD-fed *ApoE*$^{-/-}$ mice treated with IFM-514. In addition, NanoString

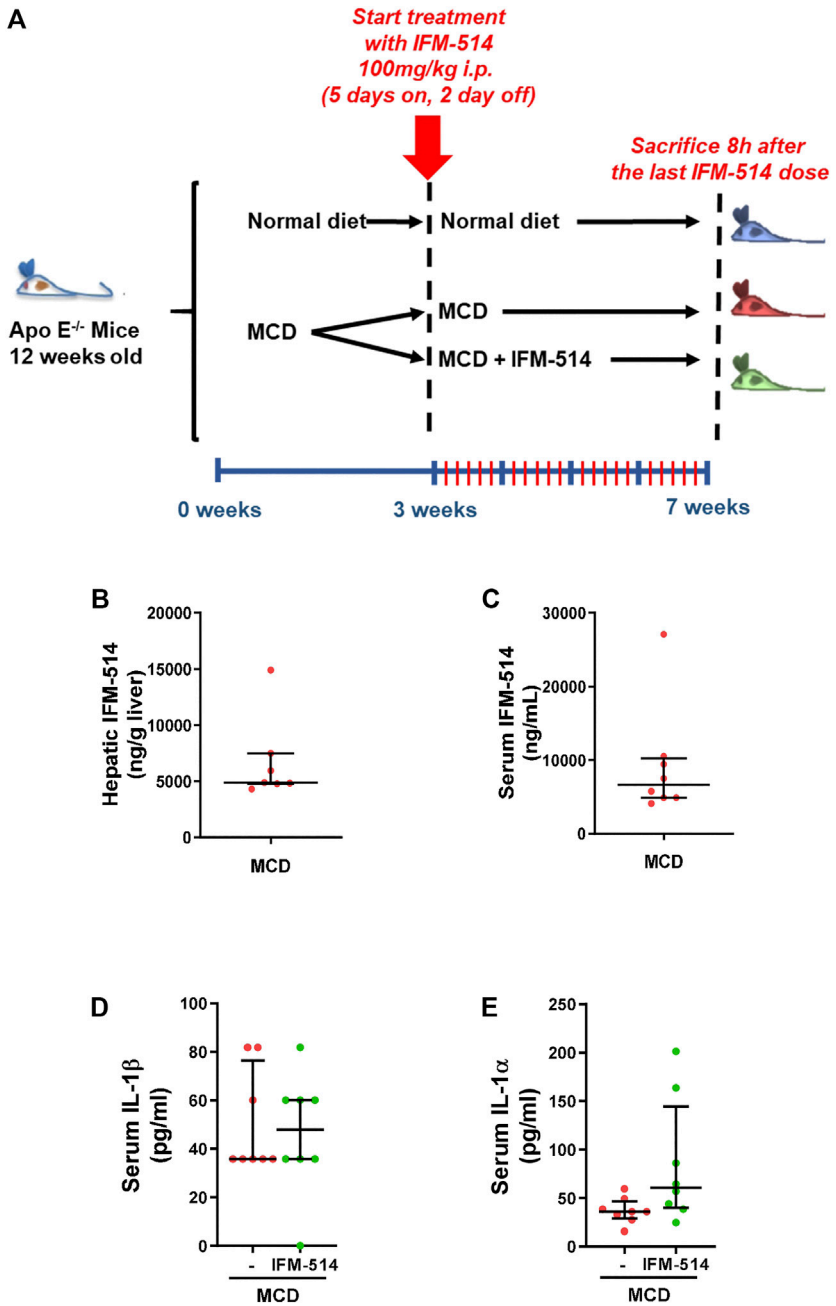


FIGURE 2 | Study design and interleukin levels. Groups (n = 10) of 12-week-old *ApoE*$^{-/-}$ mice were fed *ad lib* for 7 weeks with methionine/choline deficient (MCD) diet. After 3 weeks of diet-induced injury, mice were injected i. p. with the NLRP3 antagonist IFM-514 (100 mg/kg body weight) or vehicle (0.5% carmellose) every day, 5 days/week for a further 4 weeks **(A)**. Concentration of IFM-514 in liver (ng/g of tissue) and serum (ng/ml) following i. p. administration of IFM-514 in NASH-induced mice for 20 consecutive days. The concentration was measured by HPLC-MS **(B–C)**. Circulating IL-1β and IL-1α in IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice **(D–E)**. Results are expressed as mean ± standard error of the mean (SEM); n = 10/group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Abbreviations: IL1α, interleukin 1α.

gene expression analyses indicated that several fibrotic genes that were increased by MCD feeding were decreased by treatment with IFM-514, as shown in the heatmap (**Figure 4F**). Taken together, these data indicate that IFM-514 treatment markedly reduces key players in liver fibrosis.

Hepatic Stellate Cell Activation and Portal Hypertension in MCD-Fed *ApoE*$^{-/-}$ Mice

We next examined hepatic stellate cell activation (HSC) as an important mechanism for development of portal hypertension in addition to fibrosis (Abdulla et al., 2014), using quantitative

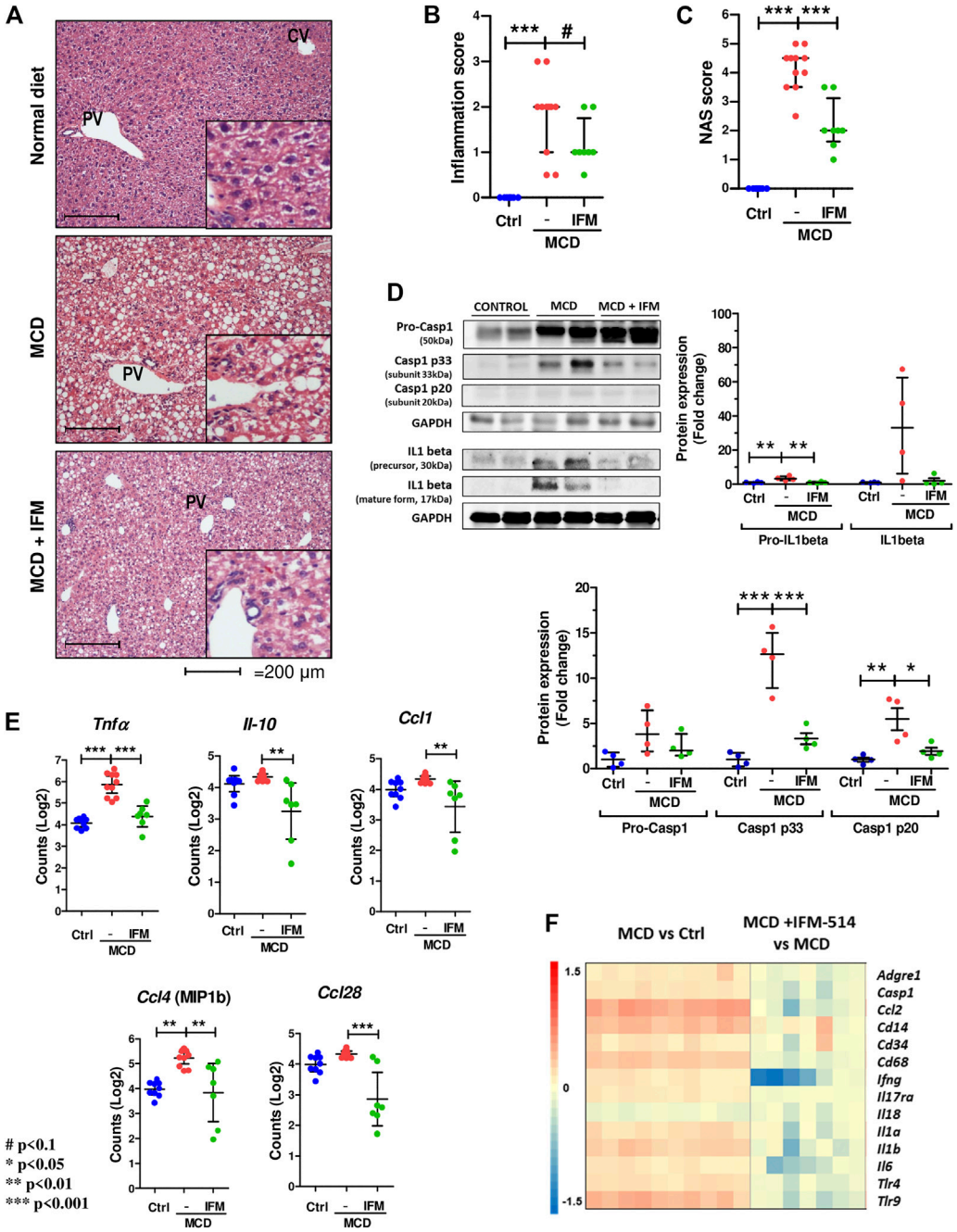


FIGURE 3 | IFM-514 reduces hepatic inflammation in MCD-fed *ApoE*$^{-/-}$ mice. H&E staining **(A)** inflammation score **(B)** and NAS score **(C)** in IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice. Caspase-1 p33, caspase-1 p20, pro-caspase-1, mature IL-1β and pro-IL-1β protein expression **(D)**. Inflammasome-related gene expression **(E)**. Heatmap for hepatic inflammation set of genes **(F)**. Scale and genes are provided on heatmap. Results are expressed as the mean ± standard error of the mean (SEM); n = 10/group, $^{\#}p <$ 0.1. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Representative photomicrographs were captured at ×100 (scale bars = 200 µm) and ×200 magnification (scale bars = 100 µm). Abbreviations: Ccl1, chemokine (C-C motif) ligand 1; Ccl4, chemokine (C-C motif) ligand 4; Ccl28, chemokine (C-C motif) ligand 28; CV, central vein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; *Il-10*, interleukin 10; PV, portal vein. See **Supplementary Table 3** for Heatmap genes.

immunostaining for α-smooth muscle actin (Acta2) (**Figures 5A,B**). Activation of HSC in MCD-fed mice was increased compared to controls, while IFM-514 treatment showed a tendency towards reduced αSMA expression. Similarly, the mRNA expression level of *Acta2* had a decreasing trend in IFM-treated MCD-fed *ApoE*$^{-/-}$ mice (**Figure 5C**). Portal pressure-measured in the spleen pulp-was significantly reduced in IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice (**Figure 5D**). Genes of key players in portal hypertension and

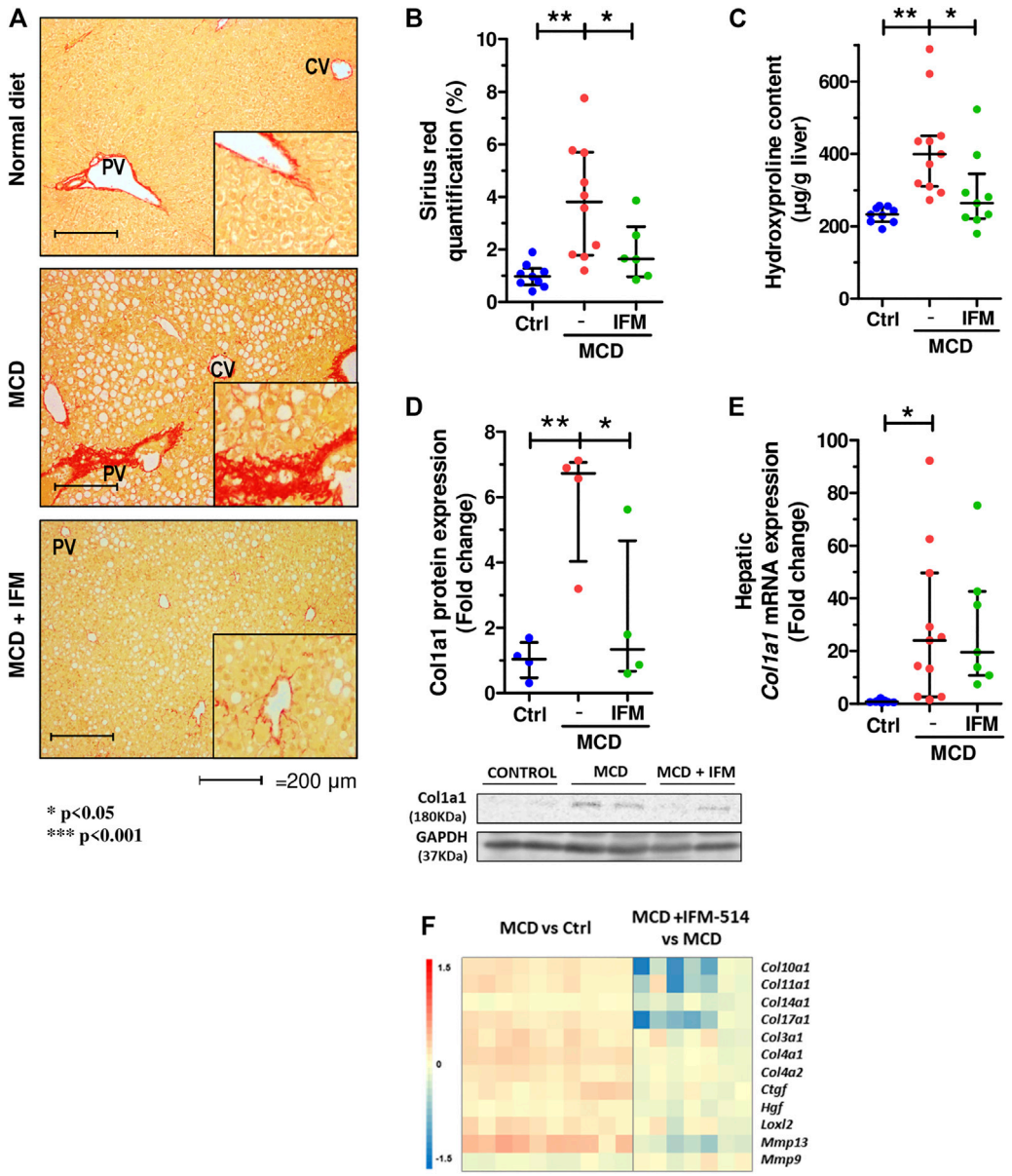


FIGURE 4 | IFM-514 reduces liver fibrosis in MCD-fed *ApoE*$^{-/-}$ mice. Sirius red (SR) staining **(A)** with SR quantification **(B)** and hepatic 4-hydroxyproline content **(C)**. Hepatic Col1a1 protein **(D)** and mRNA expression **(E)** from IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice. Heatmap for hepatic fibrosis set of genes **(F).** Results are expressed as mean ± standard error of the mean (SEM); n = 10/group, #$p < 0.1$, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. $^{\$}p < 0.05$, if outliers are excluded. Scale bars = 200 µm. Abbreviations: col1a1, collagen type 1 α1. See **Supplementary Table 3** for Heatmap genes.

activation of HSC were analyzed by NanoString gene expression (see heatmap, **Figure 5E**), showing a reduction of these genes with IFM-514 treatment.

IFM-514 Reduces Hepatic Steatosis in MCD-Fed *ApoE*$^{-/-}$ Mice

The steatosis score assessed by a liver pathologist demonstrated a significant reduction in mice treated with IFM-514 compared to vehicle treated mice (**Figure 6A**). Similarly, analysis of hepatic steatosis using quantitative hepatic triglycerides and oil red O staining revealed a trend towards a reduction in IFM-treated mice, although this was not significant, likely due to the persistence of numerous oil red O-positive small lipid droplets (**Figures 6B–D**). Similar trends were seen on protein and mRNA expression levels of central regulators of lipid homeostasis, sterol regulatory element binding factor 1 (Srebf1) and fatty acid synthase (Fasn), being significant for *Fasn* gene expression (Dinarello et al., 2012) (**Figures 6E–G**). The heatmap of the steatosis selected genes confirms the effect of IFM-514 treatment in MCD-fed mice (**Figure 6H**).

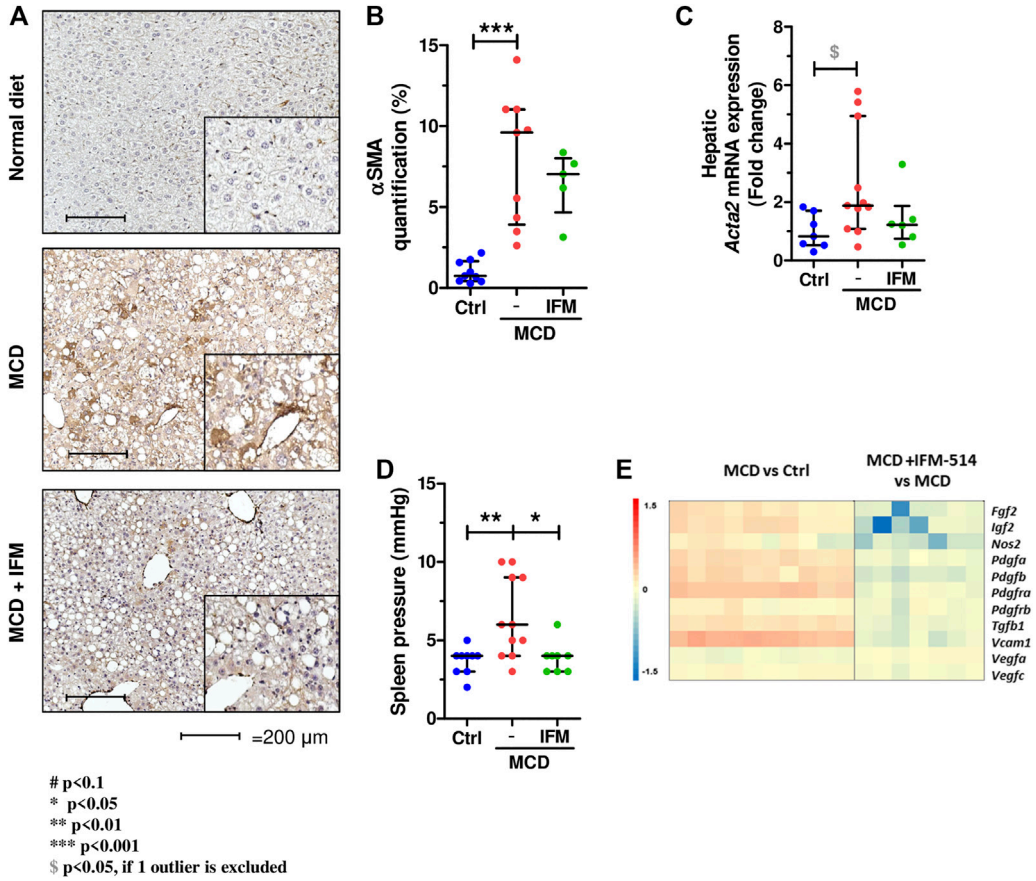


FIGURE 5 | Hepatic stellate cell activation in MCD-fed *ApoE*$^{-/-}$ mice. α-smooth muscle actin immunostaining **(A)** with quantification **(B)** from IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice. *Acta2* mRNA expression **(C)**. Spleen pressure analyses **(D).** Heatmap for portal hypertension set of genes **(E).** All mRNA data were normalized to the expression of *18s*. Results are expressed as mean ± standard error of the mean (SEM); n = 10/group, #$p < 0.1$, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. $^{\$}p < 0.05$, if outliers are excluded. Scale bars = 200 µm. Abbreviations: CV, central vein; PV, portal vein; acta2, α-smooth muscle actin gene; αSMA, α-smooth muscle actin. See **Supplementary Table 3** for Heatmap genes.

IFM-514 Reduces Hepatic Gene Expression of Inflammasome-Related and Proinflammatory Cytokines in MCD-Fed *ApoE*$^{-/-}$ Mice

To assess the extent of the genes differentially expressed following IFM-514 treatment in MCD-fed *ApoE*$^{-/-}$ mice, we analyzed the sets of genes upregulated in MCD-fed *ApoE*$^{-/-}$ mice (**Figures 7A–C**). To further characterize the upregulated pathways, we performed gene ontology (GO) analysis of all significantly upregulated genes in MCD-fed *ApoE*$^{-/-}$ mice. In biological processes, we found a one- to eight-fold enrichment in inflammation-related GO terms, such as regulation of tumor necrosis factor superfamily cytokine production and inflammatory response (**Figure 7A**). Through Venn analyses, a total of 236 genes were significantly upregulated in MCD-fed *ApoE*$^{-/-}$ mice (**Figure 7B**). Venn analyses revealed that a total of 126 genes specifically upregulated by MCD diet were found to be downregulated by IFM-514 (**Figure 7B**; **Table 2**). To examine the transcriptomic inflammatory signature in response to the IFM-514 treatment, we compared the sets of genes which were downregulated following IFM-514 treatment in MCD-fed *ApoE*$^{-/-}$ mice. GO analysis revealed enrichment for several genes related to calcium-mediated signaling, G protein-coupled receptor signaling pathway, inflammatory and immune responses in IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice (**Figure 7C**).

Thus, IFM-514 clearly reduced the expression of several genes specifically upregulated in the MCD model, not limited to inflammasome function, but related to inflammation in general. All in all, these results strongly support that the primary targets of the NLRP3 blockade are liver inflammation and fibrosis, with a mild effect on hepatic steatosis in MCD-fed *ApoE*$^{-/-}$ mice.

IFM-514 Effect on Hepatic Inflammation and Fibrosis in WD-Fed *ApoE*$^{-/-}$ Mice

Since MCD-fed mice recapitulate the histology of human NAFLD (hepatic steatosis, and fibrosis) but lack the metabolic component (obesity, high cholesterol and diabetes) to assess the impact of metabolic imbalances in NLRP3 activation in NASH, *ApoE*$^{-/-}$ mice

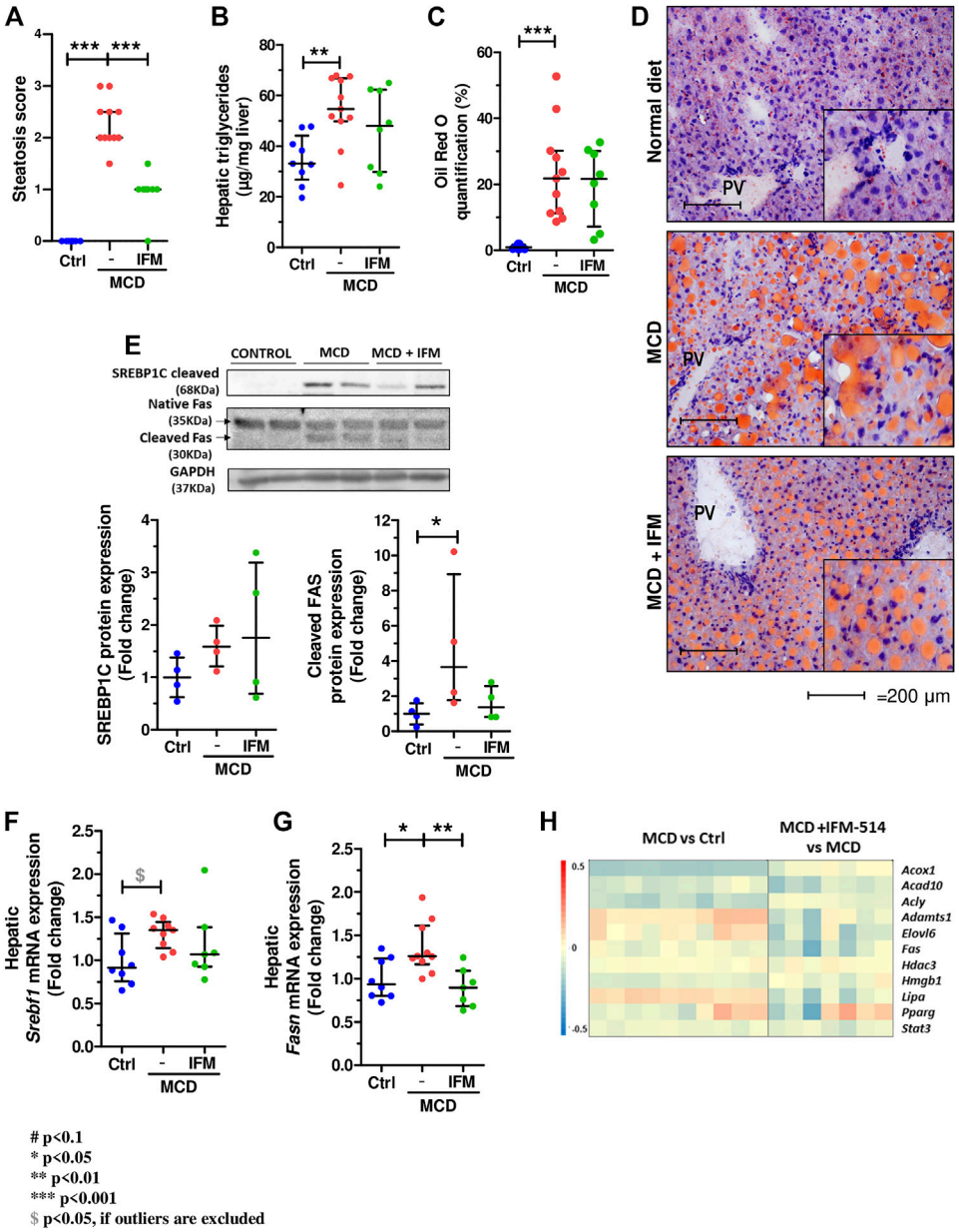


FIGURE 6 | IFM-514 reduces hepatic steatosis in MCD-fed *ApoE*$^{-/-}$ mice. Steatosis score **(A)**. Hepatic triglycerides levels **(B)**. Oil red O staining and quantification as shown as percentage of the positive area **(C–D)**. Srebp1c, native Fas and cleaved Fas protein **(E)** and mRNA expression **(F–G)** from IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice. Heatmap for hepatic steatosis set of genes **(H)**. All mRNA data were normalized to the expression of *18s*. Results are expressed as the mean ± standard error of the mean (SEM); n = 10/group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Abbreviations: srebp1c; sterol regulatory element-binding protein 1c; *srebf1*, sterol regulatory element-binding transcription factor 1; *fas*, fatty acid synthase. See **Supplementary Table 3** for Heatmap genes.

were fed with WD. 12-week-old *ApoE*$^{-/-}$ mice were fed for a total of 7 weeks with WD diet. After 3 weeks of diet-induced injury, mice received i. p. injections of IFM-514 (100 mg/kg) or vehicle for 5 days/week for a further 4 weeks (**Supplementary Figure 2A**). IFM-514 was measured by HPLC-MS in liver and serum 8 h after the last dose of IFM-514 in WD-fed *ApoE*$^{-/-}$ mice which was rather low compared to MCD fed *ApoE*$^{-/-}$ (**Supplementary Figures 2B–C**). IFM-514-treated NASH *ApoE*$^{-/-}$ mice had similar circulating IL-1β and IL-1α as vehicle-treated NASH *ApoE*$^{-/-}$ mice (**Supplementary Figures 2D–E**).

We further investigated the effect of IFM-514 treatment on inflammation and fibrosis in WD-fed *ApoE*$^{-/-}$ mice. No difference in inflammation score between *ApoE*$^{-/-}$ and WD fed *ApoE*$^{-/-}$ mice was found (**Supplementary Figure 3B**), but WD induced an increase in NAS score (**Supplementary Figure 3C**). In addition, WD significantly increased *Tnf-α* gene expression

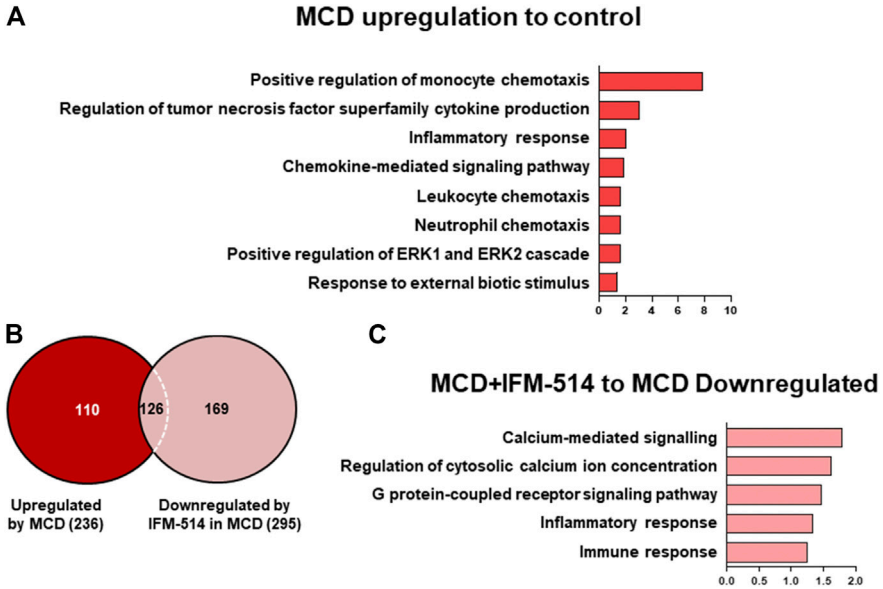


FIGURE 7 | Transcriptomic analysis of IFM-514-treated MCD-fed *ApoE*$^{-/-}$ mice. Gene ontology (GO) analysis and fold enrichment of genes of biological process upregulated in MCD-fed **(A)**. Venn diagram of downregulated genes in IFM-514-treated MCD-fed **(B)**. Enriched GO terms for genes downregulated in IFM-514-treated MCD-fed **(C)**. The signaling pathways are based on statistical significance. Genes were considered significantly down expressed ($p < 0.05$) in IFM-treated mice by a minimum of 1.5 fold above that of vehicle-treated MCD-fed *ApoE*$^{-/-}$ mice.

(**Supplementary Figure 3E**) while IFM-514 showed a tendency towards reduction in some inflammatory genes, being significant for Ccl1 and Ccl28 (**Supplementary Figures 3F–G**). Liver fibrosis was assessed by sirius red staining and hepatic hydroxyproline content, which also tend to decrease by the IFM-514 treatment (**Supplementary Figures 3A,C,E**). Activation of hepatic stellate cells assessed by α-sma staining (**Supplementary Figures 4B,D**), was significantly increased in WD fed *ApoE*$^{-/-}$ mice, and showed a tendency to decrease after the IFM-514. Similar effects showed spleen pressure (**Supplementary Figure 4F**) and the heatmaps of fibrotic and portal hypertension related genes (**Supplementary Figure 4G–H**).

DISCUSSION

Currently, therapies targeting NLRP3-dependent cytokines, i.e. canakinumab, an IL-1β-neutralizing antibody, and rilonacept, a soluble receptor that binds IL-1β and IL-1α, are applied in humans. However, they have notable immunosuppressive disadvantages compared to selective NLRP3 antagonism (Dinarello et al., 2012; Ridker et al., 2017). This is of particular relevance when considering that in a targeted anti-NLRP3 therapy, other pathogen-recognizing inflammasomes can be engaged to produce IL-1β, thus reducing the risk of immune suppression and opportunistic infections. Supporting this idea, similar concentrations of circulating IL-1β and IL-1α detected in IFM-514-treated vs untreated NASH mice might lower the risk of immunosuppressive effects of this anti-inflammasome therapy, rendering the IFM-514 treatment very suitable in NASH.

A recent study investigated the effect of the NLRP3 antagonist CP-456,773 (Jiang et al., 2017) (also called MCC950 or CRID3) on the development of NASH in dietary models (Mridha et al., 2017). Similar to our data, CRID3 caused a significant reduction in hepatic infiltration of macrophages and neutrophils and modulated fibrotic progression of steatohepatitis in MCD-fed as well as foz/foz mice fed with atherogenic diet. These effects correlated with a significant reduction of NLRP3 activation in the liver, suggesting an important role for NLRP3 in the progression of NASH. Nevertheless, further work is still required regarding the precise molecular target and safety of CRID3. Indeed, liver toxicity has been observed in humans, probably due to either the high dose of CRID3 (1,200 mg per day) or a reactive metabolite of its furan moiety or both (Mangan et al., 2018). The latter two are well-known causes of drug-induced liver toxicity. By contrast, in our study, the daily administration of 100 mg/kg of IFM-514 reached adequate concentrations in liver and serum. These concentrations are low enough not to induce liver toxicity, but still sufficient to inhibit NLRP3 *in vitro*, as shown by the observed effects of IFM-514 in the *in vivo* LPS-PD/PK studies.

NAFLD is generally considered the hepatic manifestation of the metabolic syndrome and its predominant underlying risk factors appear to be increased body weight and obesity. However, NAFLD can occur in lean subjects with a body mass index <25 kg/m^2 (Voss et al., 2011), suggesting that other predisposing factors and inherited disorders play a critical role in lean NAFLD patients, which is an important consideration in Asian populations (Wong et al., 2018). In the MCD diet, the absence of methionine leads to increased hepatic inflammation, fibrosis, liver damage and macrovesicular steatosis (Caballero et al., 2010). Therefore,

TABLE 2 | IFM-514 changes the hepatic gene expression of inflammasome-related and proinflammatory cytokines in MCD-fed *ApoE*$^{-/-}$ mice. Table shows all genes significantly downregulated in IFM-514-treated MCD-fed mice.

Hepatic-specific downregulated gene set in IFM-514-treated in MCD mice								
Gene	***p*-value**	**x-Fold**	**Gene**	***p*-value**	**x-Fold**	**Gene**	***p*-value**	**x-Fold**
2810417H13Rik	0.00667	−1.69064	Cxcr3	0.00068	−2.14185	Lpl	0.01570	−1.59407
Adam8	0.00011	−2.62183	Cxcr4	0.00319	−1.56080	Ltb	0.00006	−2.02311
Amica1	0.00011	−1.89605	Cybb	0.00019	−1.73405	Ltb4r2	0.02524	−1.71289
Anxa1	0.00870	−1.60182	Cytip	0.00017	−1.72377	Mmp12	0.00015	−4.50916
Areg	0.01426	−1.61760	Dusp2	0.00008	−2.06807	Mmp13	0.00068	−2.16102
Atf3	0.00372	−1.82585	Ear3	0.00000	−1.89868	Mpeg1	0.00011	−1.64865
Btk	0.00004	−1.58950	Fcgr1	0.00016	−1.61185	Ncf2	0.00003	−1.75356
C3ar1	0.00040	−1.65176	Fcgr3	0.00005	−1.75651	Nfatc2	0.00925	−1.57946
C5ar1	0.00300	−1.55740	Fcgr4	0.00165	−1.55248	Nlrp3	0.00041	−1.51407
Casp1	0.00018	−1.68863	Fgfr1	0.00169	−1.52777	Olr1	0.00057	−1.78952
Ccl17	0.00398	−1.67018	Flt3	0.00014	−2.02110	Pdgfb	0.00000	−1.83201
Ccl19	0.00039	−1.65110	Fpr2	0.01386	−1.54496	Plau	0.00058	−1.95671
Ccl2	0.00421	−2.19227	Fut4	0.00114	−2.06766	Psmb9	0.00493	−1.58966
Ccl22	0.00000	−2.95526	Gem	0.00001	−1.70950	Ptafr	0.00035	−1.84001
Ccl3	0.00237	−2.03073	Gpr65	0.00000	−1.95593	Ptprc	0.00083	−1.53368
Ccl4	0.00127	−2.61716	H2-Aa	0.00000	−2.89129	Retnla	0.00136	−6.02874
Ccnb2	0.00001	−2.47353	H2-Ab1	0.00000	−2.68355	Rgs1	0.02413	−1.69550
Ccr2	0.01111	−1.73233	H2-DMa	0.00001	−2.11027	Selplg	0.00063	−1.54297
Ccr7	0.00013	−1.99150	H2-DMb1	0.00014	−2.01112	Siglecf	0.00001	−2.47059
Ccr9	0.00000	−2.87416	H2-Eb1	0.00000	−2.66633	Sirpa	0.00024	−1.59139
Cd180	0.00101	−1.66737	Havcr2	0.00006	−1.90452	Syk	0.00000	−1.97188
Cd247	0.00020	−1.99446	Hdc	0.00035	−1.50346	Tgfb1	0.00001	−1.62258
Cd274	0.00005	−1.85501	Icam1	0.00005	−1.50435	Tlr13	0.00012	−1.85556
Cd40	0.00587	−1.53065	Icosl	0.00075	−1.58259	Tlr2	0.00000	−1.80107
Cd68	0.00003	−1.71272	Id3	0.00890	−1.54175	Tlr4	0.00071	−1.55125
Cd69	0.00014	−1.88949	Ikzf1	0.00020	−1.61504	Tlr6	0.00250	−1.50599
Cd74	0.00000	−2.45256	Il15	0.00373	−1.54588	Tlr7	0.00027	−1.85520
Cd80	0.00001	−1.96156	Il1b	0.02191	−1.64653	Tlr8	0.00000	−1.95168
Cd83	0.00899	−2.14652	Il1r2	0.00569	−2.68834	Tlr9	0.00017	−1.81612
Cd84	0.00009	−1.64947	Il1rn	0.00014	−1.90339	Tnf	0.00003	−2.76967
Cdc20	0.00000	−2.32831	Irf5	0.00017	−1.58824	Tnfaip3	0.00665	−1.77645
Cdh4	0.00324	−1.57430	Irf8	0.00003	−1.83892	Tnfaip8	0.00012	−1.53840
Clec5a	0.00002	−2.26914	Isg15	0.01062	−1.68263	Tnfrsf11a	0.00000	−1.76821
Clec7a	0.00003	−2.82955	Itga4	0.00001	−1.98049	Top2a	0.01684	−1.70561
Clec9a	0.01630	−1.56250	Itgal	0.00002	−1.79662	Trem2	0.00008	−2.01950
Ctsd	0.00001	−1.57586	Itgax	0.00000	−3.08656	Tspan8	0.00348	−1.84056
Ctss	0.00000	−2.23524	Itgb2	0.00002	−1.79929	Tyrobp	0.00021	−1.61424
Cx3cr1	0.00011	−2.38809	Kif20a	0.00001	−2.58010	Usp18	0.00012	−1.83401
Cxcl10	0.00001	−2.98911	Laptm5	0.00000	−1.82459	Vav1	0.00029	−1.52158
Cxcl16	0.00023	−1.58510	Lat2	0.00004	−2.22728	Vcam1	0.00017	−1.83038
Cxcl2	0.00007	−2.36479	Lgals3	0.00001	−2.09902	Vsir	0.00021	−1.62241
Cxcl9	0.00843	−2.14758	Lipa	0.00002	−1.66341	Was	0.00009	−1.76558

MCD-fed mice show severe steatohepatitis but is not caused by metabolic syndrome or insulin resistance and show weight loss and increased mortality (Larter and Yeh, 2008). For that reason, this study was conducted in *ApoE*$^{-/-}$ mice, which have a genetic predisposition to develop chronic inflammation (Harja et al., 2008), including metabolic syndrome (Schierwagen et al., 2015) as a useful tool to explore the therapeutic effects of a novel selective NLRP3 antagonist in lean NAFLD.

There is compelling evidence that NLRP3 inflammasome acts as a mediator of inflammation, lipotoxicity, and fibrosis (Duewell et al., 2010; Bracey et al., 2014; Mehta et al., 2014). Also, NLRP3 has been identified as a central insult sensor that triggers and sustains disease driven by metabolic dysfunction and fibrosis following either acute tissue injury or chronic inflammation (Christ et al., 2018). Similarly, NLRP3 activation occurs when oxidation of LDL, cholesterol, and fats are increased (Shi et al., 2006; Saberi et al., 2009). Thus, it is likely that blockage of the NLRP3 signaling pathway will decelerate the progression from NAFLD to NASH. Therefore, we tested a novel selective NLRP3 antagonist in a dietary *in vivo* study to reduce fibrosis and inflammation in NASH-induced *ApoE*$^{-/-}$ mice.

The essential findings of the present study are that an anti-NLRP3 approach in NASH can arrest established liver fibrosis and chronic inflammation in *ApoE*$^{-/-}$ mice. As for liver fibrosis, this was confirmed by robust readouts. Importantly, the ability of IFM-514 to reduce liver fibrosis is highly relevant as liver fibrosis is associated with adverse liver outcomes in NASH (Angulo et al., 2015). Concerning hepatic chronic inflammation, the NanoString panel and the analysis of the specific gene set enrichment accompanied by reduced Tnfα mRNA expression clearly

demonstrate a reduction in cytokine- and inflammasome-related genes by IFM-514. These results indicate that IFM-514 exerts both anti-fibrotic and anti-inflammatory effects in NASH *ApoE*$^{-/-}$ mice.

Several lines of evidence suggest that a specific NLRP3 antagonist could be an effective novel therapy in NASH for several reasons. First, the transition from NAFLD to NASH correlates with the accumulation of hepatic cholesterol crystals, a known NLRP3 trigger (Duewell et al., 2010; Ioannou et al., 2013). Second, genetic NLRP3 deficiency (Stienstra et al., 2011; Vandanmagsar et al., 2011; Wree et al., 2014a) as well as a specific NLRP3 antagonist (Mridha et al., 2017) halt the progression of NAFLD into NASH. Third, the gain-of-function NLRP3 knock-in mice exhibited enhanced NASH-induced fibrosis when fed with MCD (Wree et al., 2014a). Collectively, these data suggest that NLRP3 inhibitors are potential targets in obesity-induced inflammation and insulin resistance (Vandanmagsar et al., 2011), NAFLD (Henao-Mejia et al., 2012; Wree et al., 2014b) and NASH (Mridha et al., 2017). IFM-514 inhibited NLRP3 inflammasome and the subsequent caspase-1 proteolytic activation, thus preventing the development of NASH in *ApoE*$^{-/-}$ mice.

Our study has several limitations. The treatment regimen, although based on previous data using NLRP3 inhibitor (Mridha et al., 2017), may have been more efficient if the treatment would be administrated daily. In addition, the lower exposure from IP dosing in those mice may be the reason for reduced efficacy. Moreover, MCD diet in *ApoE*$^{-/-}$ mice is not the standard model of NASH, but still a model with high liver inflammation and fibrosis, and without substantial weight loss (Schierwagen et al., 2015). Finally, although NLRP3 inhibition with IFM-514 reduces lipogenic genes, it fails to decrease oil red staining in the livers of treated animals. One explanation may be that the NLRP3 inhibition decreases the inflammatory response and thereby the lipogenic stress in a second step.

Our data indicate that the beneficial effects of IFM-514 against steatohepatitis in the MCD *ApoE*$^{-/-}$ model were due to reduction of hepatic inflammation and fibrosis with marginal changes in lipid accumulation. Thus, these findings demonstrate a link between NLRP3 inflammasome activation and the progression to NASH.

CONCLUSION

IFM-514 reduced liver inflammation and fibrosis, with mild effect in liver steatosis in MCD-fed *ApoE*$^{-/-}$ mice. These data suggest that blocking NLRP3 might be an attractive therapeutic approach for lean NASH patients.

METHODS

Formulation of IFM-514

NLRP3 inhibitor, IFM-514, was kindly provided by the manufacturer (IFM Therapeutics, Boston, MA, United States) and it was supplied in the form of a sodium salt that is soluble in water at concentrations up to 2 mM. To prepare a suspension for i. p. or oral (100 mg/kg, 10 ml/kg) dosing, the appropriate amount of dry powder was weighed out and added to an appropriate volume of 0.5% carboxymethylcellulose (CMC) in PBS to obtain a 10 mg/ml suspension. The physicochemical properties of IFM-514 are shown in **Supplementary Table 1**.

In Vitro Experiments

To analyze the *in vitro* potency and selectivity of IFM-514, mouse and human inflammatory cells were used. To determine the activity of IFM-514 on gramicidin-induced release of IL-1β in cell lines, PMA-differentiated human THP-1 cells were treated with IFM-514 for 1 h and then stimulated with gramicidin (5 μM) for 2 h; an immortalized murine macrophage cell line was primed with LPS (200 ng/ml) for 2 h, treated with IFM-514 for 1 h and then stimulated with gramicidin (2 μM) for 2 h. Primary human CD14^{+} monocytes were treated with GM-CSF and IL-4 for 6 days to induce differentiation to macrophages. The cells were pre-treated with IFM-514 for 1 h, primed with LPS for 2 h and then triggered with gramicidin for 2 h. In addition, BALB/c bone marrow-derived cells were treated with M-CSF for 6 days to induce differentiation to macrophages. After an overnight rest, cells were pre-treated with compounds for 1 h, primed with LPS (10 ng/ml) for 3 h and stimulated with gramicidin (5 μM final) for 1 h. In all cases, the activity of NLRP3 was evaluated by measuring the production of IL-1β in the supernatant. In all experiments, the IL-1β production was analyzed by homogeneous time resolved fluorescence (HTRF) in cell-free supernatant.

Mice

Female C57BL/6 mice used for the acute lipopolysaccaride (LPS)-induced peritoneal inflammation model (*p*K/PD) were obtained from Jackson Laboratory. Mice were kept under specific pathogen-free conditions and provided with food and water ad libitum. The animal studies were conducted under protocols approved by the TSRL Institutional Animal Care and Use Committee (IACUC) (application number EFF-21001-02).

A total number of 30 male Apolipoprotein E-deficient mice (*ApoE*$^{-/-}$ mice) were used for all studies. *ApoE*$^{-/-}$ mice are predisposed to hypercholesterolemia, atherosclerosis, and obesity. All experiments were performed in accordance with the German animal protection law and statutory guidelines of the animal care facility (Haus für experimentelle Therapie, University Clinics Bonn, Germany), and approved by the North Rhine-Westphalia State Agency for Nature, Environment, and Consumer Protection (LANUV, file reference LANUV NRW, 84-02.04.2014.A137).

LPS-Induced Cytokine Release in C57BL/6 Mice

C57BL/6 mice (8–10 weeks of age, Jackson Laboratories) in groups of 10 were pre-dosed orally 1 h before study start with IFM-514 or vehicle (0.5% CMC in water) at various concentrations in a total volume of 10 ml/kg. The mice were then returned to their cage and allowed food and water ad libitum. After 1 h, mice were then injected IP with 20 mg/kg of LPS (0.1 ml in saline) and again returned to their cages. After an five additional hours (t = 6 h post drug), blood was collected for cytokine analysis by ELISA and quantification of IFM-514 was performed by HPLC using standard reverse-phase conditions.

Induction of NASH With Liver Fibrosis in Mice

10–12-week-old *ApoE*$^{-/-}$ male and female C57BL/6 mice were fed ad lib for 7 weeks with a methionine/choline deficient (MCD) diet (Diet#E15653-94, Ssniff Spezialdiäten GmbH, Soest, Germany) or with a high-fat cholesterol-rich Western diet (WD) (Diet#S0279-S011, 1.25% cholesterol, Ssniff Spezialdiäten GmbH, Soest, Germany). In MCD-fed mice, hepatic steatosis became histologically evident after 10 days and fibrosis after 8–10 weeks (Schierwagen et al., 2015, 2016). The MCD model shows severe steatohepatitis but is not caused by overnutrition or associated with insulin resistance (Larter and Yeh, 2008). After 3 weeks of diet-induced injury, mice were injected i. p. with 100 mg/kg/day of an NLRP3 antagonist IFM-514 (IFM Therapeutics Inc., Boston, United States) (100 mg/kg body weight) or vehicle (0.5% carmellose) every day, 5 days/week for a further 4 weeks. Serum and liver were collected for histological and molecular readouts of fibrosis, inflammation and steatosis. Portal pressure measured invasively in the spleen pulp, a cannula made from a 25-gauge needle connected to a saline-filled manometer was inserted into the spleen pulp, as previously described (Ackerman et al., 2008). The catheters were connected to a pressure transducer (Hugo Sachs Elektronik, March-Hugstetten, Germany) for blood pressure measurement. Splenic pulp pressure was measured as an index of portal venous pressure (Ppv). For assessment of liver injury, inflammation, fibrosis and steatosis, liver samples were fixed in 10% neutral-buffered formalin and paraffin embedded. Liver sections were stained with H&E and Sirius red (SR). Fibrosis severity was determined by SR morphometry and hepatic 4-hydroxyproline content as previously described (Schierwagen et al., 2015, 2016). To demonstrate steatosis, hepatic triglycerides were measured using an enzyme-linked colorimetric assay TG liquicolor mono (Human Diagnostics, Wiesbaden, Germany) and fresh-frozen hepatic sections were stained with oil red O for neutral fat.

Immunohistochemical Staining

Immunohistochemical staining for αSMA was performed in paraffin-embedded sections (5 µm). The sections were incubated with a mouse-anti-SMA antibody (Actin clone 1A4; Dako, Hamburg, Germany). Thereafter, biotinylated goat-anti-mouse (Dako, Hamburg, Germany) secondary antibody was used. Finally, sections were counterstained with hematoxylin.

Pathology

Blind analysis by a liver pathologist was carried out to determine the activity scores according to Kleiner et al. (Kleiner et al., 2005). The NAFLD Activity Score (NAS) was calculated as the sum of scores for steatosis, lobular inflammation, pigmented macrophages, and fibrosis.

Mouse Serum Chemokines

Blood was collected at the time of euthanasia, maintained at RT for 30 min to retract the clot and centrifuged at 2,000 rpm for 5 min for serum collection. Cytokines in mouse sera were measured with the BioPlex mouse cytokine assay on a Bio-Plex 200 system powered by Luminex xMAP Technology. The analysis was performed according to the manufacturer's protocol.

Gene Expression Analysis by NanoString Technology

We determined hepatic expression of specific mRNAs by reverse-transcriptase quantitative PCR (RT-qPCR), following extraction of total RNA from liver tissue. Primer sequences are shown in **Supplementary Table 2**. Expression of mRNA was relative to 18 s.

The prevalence of hepatic innate inflammation was evaluated by determining expression of a panel of mouse myeloid innate immunity (770 immune-related genes) using the NanoString technology (NanoString Technologies, Seattle, United States) according to manufacturer's instructions. Briefly, 200 ng of total RNA was hybridized, quantified, and loaded into Partek Genomics Suite (Partek Inc. St. Louis, MO, United States). The gene enrichment analysis was performed using Gene Ontology and Consensus Pathway DB. Heatmaps were generated using the gplots library in R. Genes were considered significantly down expressed ($p < 0.05$) in IFM-treated mice by a minimum of 1.5 fold above that of vehicle-treated MCD-fed *ApoE*$^{-/-}$ mice, using a hypergeometric test with FDR <0.05. Venn diagram was used to intersect the predicted target genes and the distribution of the 1.5-fold differentially expressed genes, showing the overlap in genes significantly upregulated and downregulated.

Western Blotting

Protein levels were analyzed by Western blot as described previously (Granzow et al., 2014; Brol et al., 2019). Briefly, snap-frozen livers were homogenized and diluted. Protein quantification was performed using a colorimetric BCA protein assay kit (Cat 23225, Thermo Fisher Scientific Inc., IL, United States). Forty micrograms of protein samples was subjected to SDS-PAGE under reducing conditions (10% gels), and proteins were blotted on nitrocellulose membranes. The membranes were blocked and incubated with primary antibody against collagen 1α1 (SC-12895; Santa Cruz Biotechnology, Santa Cruz, CA), srebp1c (ab28481, Abcam), fas (ab82419, Abcam), caspase-1 (sc-56036, Santa Cruz Biotechnology, Santa Cruz, CA), IL-1β (NB600-633). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an endogenous control (sc-47724; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with the corresponding secondary antibody, and blots were developed using enhanced chemiluminescence. Protein quantification was performed by ImageJ (version 1.51q, NIH, United States) and results were corrected for GAPDH levels.

Statistical Analysis

Statistical analyses among groups were performed using Prism V.5.0 (GraphPad, San Diego, CA). Comparisons between two groups were done by non-parametric Mann-Whitney U t-tests and one-way ANOVA followed by Tukey's Multiple Comparison test were used for statistical comparisons between the three groups, with $p < 0.05$ considered as significant (#< 0.1. *$p <$

0.05, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). \$ $p < 0.05$, if the point is excluded as an outlier. Data were expressed as mean ± SEM. All experiments were performed in triplicate at least four times and a representative image or blots are shown on the manuscript. For transcriptome analysis, statistical parameters were computed between groups, and results are shown as log2-fold change and visualized by heatmaps. *p*-values were calculated using paired *t*-test and corrected according to the adaptive Benjamini-Hochberg procedure. A FDR-adjusted *p*-value below 0.05 was considered statistically significant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the TSRL Institutional Animal Care and Use Committee (IACUC) (application number EFF-21001-02). All experiments were performed in accordance with the German animal protection law and statutory guidelines of the animal care facility (Haus für experimentelle Therapie, University Clinics Bonn, Germany), and approved by the North Rhine-Westphalia State Agency for Nature, Environment, and Consumer Protection (LANUV, file reference LANUV NRW, 84-02.04.2014.A137).

AUTHOR CONTRIBUTIONS

ST, FM, JT drafted the manuscript. FM, ST, MB, JSc and JSt acquired, analysed and interpreted the data. DB, AK, FU, CO, OT, BS and RS acquired and analysed the data. WR designed the compound. RSI performed pathology analysis. KB and LF provided substantial material and methods and analysed and interpreted data. EL, JT designed the original study, interpreted the data, supervised the study and obtained financial support for the study. All authors reviewed the draft for important intellectual content and approved the final article for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.715765/full#supplementary-material

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LECT2, A Novel and Direct Biomarker of Liver Fibrosis in Patients With CHB

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Chronic hepatitis B (CHB) patients with severe liver fibrosis would be more likely to progress to a poorer prognosis. Treatment is considered once the liver fibrosis reaches significant liver fibrosis (≥S2). Leukocyte cell-derived chemotaxin-2 (LECT2) has been shown to contribute to liver fibrosis progression. No research has focused on the role of LECT2 in liver fibrosis in CHB patients. This study enrolled 227 CHB patients and divided them into the training group (n = 147) and validation group (n = 80), respectively. The expression of LECT2 in serum, protein and mRNA of the human liver tissues was detected to analyze the possible associations between LECT2 and liver fibrosis. A receiver operating characteristic curve (ROC) was used to estimate the efficacy of LECT2 for predicting liver fibrosis. The data showed that there was a positive relationship between LECT2 and the progression of liver fibrosis. In the training group, LECT2 was demonstrated to have better effectiveness than APRI and FIB-4. The AUC was 0.861, 0.698, and 0.734 for significant liver fibrosis, and 0.855, 0.769, and 0.752 for advanced liver fibrosis. Besides, the efficacy of LECT2 in different statuses of patients with CHB was examined and the effectiveness of LECT2 had also been confirmed in the validation group. All the results confirmed that LECT2 could act as a perfect predictor and thus offers a novel and direct biomarker to estimate liver fibrosis more accurately.

Keywords: LECT2, direct biomarker, diagnosis, liver fibrosis, CHB

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INTRODUCTION

Patients with chronic hepatitis B (CHB) represent an escalating worldwide health concern (Polaris Observatory Collaborators, 2018; Moon et al., 2020). The World Health Organization (WHO) reported that during 2019, there were 296 million people with CHB and 1.5 million people are newly diagnosed with CHB worldwide (WHO, 2021). A total of 15–40% of CHB patients may develop complications, such as liver fibrosis, liver cirrhosis, and liver cancer (WHO, 2017; Ma et al., 2021). Every year, an estimated 1.34 million people die from CHB or chronic hepatitis C, so the WHO called for achieving a realistic goal: "In 2030, the hepatitis-related mortality and new hepatitis-infections could be reduced 65 and 90%" (WHO, 2016; Cooke et al., 2019). The worse prognosis of patients with CHB has been related to an aggravated liver fibrosis progression (Dulai et al., 2017), and the guidelines of CHB had mentioned that it is an indication for antiviral therapy once patients have been diagnosed with moderate and above fibrosis (Terrault et al., 2018). Considering the significant impact of liver fibrosis, it is very necessary for the progression of liver fibrosis to be accurately described as soon as possible (Revill et al., 2019; Berumen et al., 2021).

TABLE 1 | Demographic and laboratory data of patients with CHB in the training group and validation group.

Characteristic	Training group (*n* = 147)	Validation group (*n* = 80)	*p* value
Gender (M/F)	90/57	48/32	0.857
Age	38.00 (32.00, 48.00)	35.50 (31.00, 43.75)	0.080
He-positive, *n*	107	58	1.000
ALT	30.00 (20.00, 50.00)	32.00 (21.25, 64.75)	0.269
AST	23.00 (19.00, 32.00)	25.50 (19.00, 40.75)	0.243
GGT	18.00 (12.00, 30.25)	16.00 (12.00, 25.25)	0.296
Tbil	13.70 (10.80, 17.30)	14.45 (10.38, 17.73)	0.833
ALB	47.60 (45.20, 49.20)	47.10 (44.43, 50.30)	0.779
PLT	196.00 (154.00, 235.00)	203.00 (157.50, 235.00)	0.998
LECT2	4.97 (2.41, 7.65)	4.76 (2.84, 7.26)	0.995
APRI	0.24 (0.18, 0.39)	0.28 (0.21, 0.42)	0.105
FIB-4	0.91 (0.63, 1.34)	0.89 (0.64, 1.30)	0.943
Liver fibrosis, n			0.982[a]
S1	61	32	
S2	55	30	
S3	25	14	
S4	6	4	

Data are expressed as the median and quartiles.

[a]*Fisher's exact test.*

ALT, aspartate aminotransferase (IU/L); AST, alanine aminotransferase (IU/L); PLT, platelet count (×109/L); GGT, gamma-glutamyl transpeptidase (U/L); Tbil, total bilirubin (μmol/L); ALB, albumin (g/L); APRI, aspartate aminotransferase-to-platelet ratio index; FIB-4, fibrosis index based on the four factors; LECT2, leukocyte cell-derived chemotaxin 2 (ng/ml).

Liver histology is the classic diagnostic method to estimate the progression of liver fibrosis which relied on professional pathologists, because it is time-consuming, invasive, difficult to operate, and expensive, and poses a variety of complications, the application of liver biopsy is limited in clinic (McGill et al., 1990). Several noninvasive methods have been reported that could be used in evaluating the severity of liver fibrosis (Cao et al., 2020; Loomba and Adams, 2020). One of the most common noninvasive scoring systems is the aspartate aminotransferase-to-platelet ratio index (APRI) and the other is fibrosis index based on the four factors (FIB-4), both of them have been indicated to predict severe liver fibrosis or liver cirrhosis (Wai et al., 2003; Vallet-Pichard et al., 2007). However, these tests relied on some indirect markers of liver fibrosis, including liver biochemical profile and clinical parameters (Bertrais et al., 2017). This makes it impossible for APRI and FIB-4 to accurately evaluate the degree of liver fibrosis once these indirect makers are too high or too low. Some studies have reported the low efficacy of APRI and FIB-4 and confirmed this statement (Jia et al., 2015; Singh et al., 2017).

It has been reported that Leukocyte cell-derived chemotaxin 2 (LECT2) is involved in immune reactions (Jung et al., 2018; Lu et al., 2020), severe liver injury (Segawa et al., 2001; Okumura et al., 2017; Slowik et al., 2019), cancer (L'Hermitte et al., 2019), nonalcoholic steatohepatitis (NASH) (Takata et al., 2021), nonalcoholic fatty liver disease (NAFLD) (Yoo et al., 2017) and so on. Our previous study reported the phenomenon that LECT2 played an important role in promoting the aggravation of liver fibrosis, and liver cirrhosis patients showed more concentration of LECT2 in the serum (Xu et al., 2019). However, no one had analyzed the association between LECT2 and liver fibrosis in CHB patients. Consequently, our group aimed to detect whether the expression of LECT2 was associated with liver fibrosis in CHB patients and confirm the effectiveness of LCET2 for predicting liver fibrosis.

METHODS AND MATERIALS

Patients and Criteria

From August 2018 to August 2021, 227 patients with CHB passed the review of the inclusion and exclusion criteria and were recruited in this study. The training group enrolled 147 patients and the validation group enrolled 80 patients. The inclusion criteria were: 1) the diagnosis of CHB is consistent with the American Association for the Study of Liver Diseases (AASLD) 2018 hepatitis B guidance (Terrault et al., 2018); 2) patients were treatment-naive; 3) patients had undergone liver biopsy in the Department of Infectious Diseases, the First Affiliated Hospital of Anhui Medical University. The exclusion criteria were: 1) underlying liver diseases, such as chronic hepatitis C, chronic hepatitis D, autoimmune liver disease; 2) alcohol consumption, alcohol-related diseases; 3) obesity, NASH, NAFLD; 4) complications of systemic diseases, such as digestive system disease, cardiovascular system disease, and rheumatic immune system disease. This study was performed following the Declaration of Helsinki and was approved by the Ethical Committee of the First Affiliated Hospital of Anhui Medical University, Hefei, China.

Laboratory Tests and Noninvasive Scoring Systems

Blood samples were collected and performed on the day of the liver biopsy. Two general validated noninvasive scoring systems were calculated using the original equations (Wai et al., 2003;

TABLE 2 | Levels of APRI, FIB-4 and LECT2 in different stages of liver fibrosis in the training group and validation group.

Group		Stages of liver fibrosis				*p* value
		S0-S1	S2	S3	S4	
Training group	APRI	0.21 (0.16, 0.27)	0.25 (0.18, 0.39)	0.43 (0.23, 0.62)	0.65 (0.53, 4.48)	0.000
	FIB-4	0.69 (0.57, 1.00)	1.01 (0.63, 1.52)	1.25 (0.82, 2.24)	1.69 (0.94, 11.65)	0.000
	LECT2	2.57 (1.99, 3.54)	5.93 (3.48, 7.86)	7.74 (6.76, 10.50)	10.48 (8.76, 11.38)	0.000
Validation group	APRI	0.24 (0.16, 0.29)	0.31 (0.24, 0.46)	0.36 (0.28, 0.60)	0.73 (0.33, 1.19)	0.000
	FIB-4	0.79 (0.48, 1.02)	0.88 (0.68, 1.24)	1.38 (0.78, 1.53)	2.65 (1.57, 3.35)	0.000
	LECT2	2.70 (1.68, 3.72)	5.06 (3.99, 6.89)	8.41 (6.31, 9.41)	9.44 (6.86, 14.14)	0.000

Data are expressed as the median and quartiles, APRI: aspartate aminotransferase-to-platelet ratio index; FIB-4: fibrosis index based on the four factors; LECT2: leukocyte cell-derived chemotaxin 2 (ng/ml).

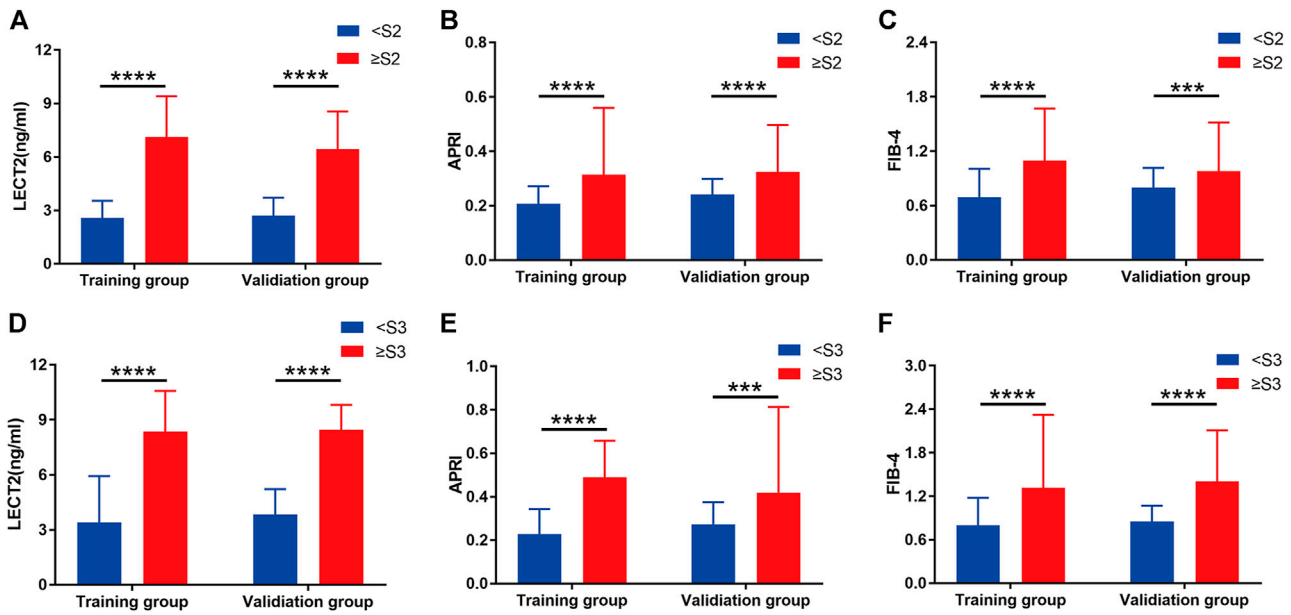


FIGURE 1 | Grouped bar chart depicting the serum liver fibrosis marker levels in patients with CHB in the training group and validation group. **(A)**, **(B)**, and **(C)** show the serum LECT2, APRI, and FIB-4 in the liver fibrosis stage <S2 and ≥S2, respectively. **(D)**, **(E)**, and **(F)** show the serum LECT2, APRI and FIB-4 in the liver fibrosis stage <S3 and ≥S3, respectively. The differences between <S2 and ≥S2 or <S3 and ≥S3 for each of the three liver fibrosis markers were significant. ***$p < 0.001$, ****$p < 0.0001$.

Sterling et al., 2006). In the calculation of APRI, the upper limit of normal (ULN) for AST level was defined as 50 IU/L.

$$APRI = \frac{\frac{AST}{ULN}}{platelets} * 100$$

$$FIB-4 = age\ years * AST / \left(platelets * \left(ALT\hat{}\ (1/2)\right)\right)$$

Test for LECT2

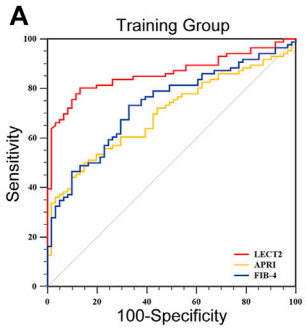
The serum level of LECT2 in patients with CHB was measured by ELISA, as previously reported (Xu et al., 2019). The assay kits were purchased from Wuhan USCN Business (Cat No: SEF541Hu). RNA *in situ* hybridization (ISH) was completed using an RNAscope® 2.5 HD Duplex Assay manual kit (ACDBio). RNA probes for LECT2 were customer-designed at ACDBio (Newark, CA, United States). LECT2 protein levels in various stages of liver fibrosis were detected by immunohistochemical (IHC) staining. A LECT2 monoclonal antibody (Cat No: Sc-398071) was purchased from Santa Cruz Biotechnology.

Liver Histology

Liver tissues were obtained by liver biopsy using ultrasound-guided needles in patients with CHB. The progression of liver fibrosis was assessed by two experienced pathologists through the Scheuer scoring system, the description from S0 to S4 represents the aggravation of liver fibrosis (Yan et al., 2020). The patients were classified as having significant liver fibrosis when the fibrosis stage was ≥S2 and advanced liver fibrosis when the stage was ≥S3.

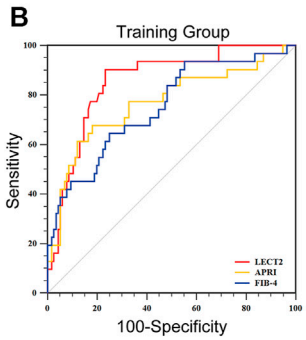
Statistical Analysis

Spss 22.0 statistical software package (SPSS Inc., Chicago, Illinois, United States) was adopted for data analysis. The categorial parameters and continuous parameters were expressed as



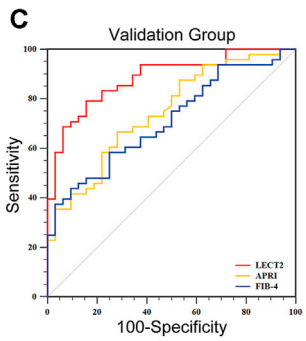
Predicting ≥S2 fibrosis

ALL patients (n=147)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.861 (0.795-0.913)	80.23	86.89	89.61	86.89	82.99
APRI	0.698 (0.617-0.771)	50.00	85.25	82.69	54.74	64.63
FIB-4	0.734 (0.654-0.803)	73.26	67.21	75.90	64.06	70.75
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		3.202			0.001	
LECT2 & FIB-4		2.326			0.020	
APRI & FIB-4		0.905			0.366	



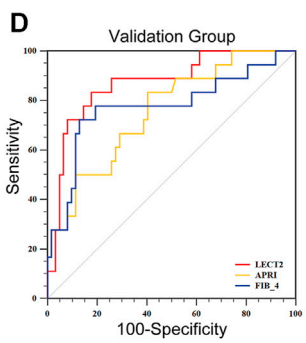
Predicting ≥S3 fibrosis

ALL patients (n=147)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.855 (0.788-0.908)	90.30	76.70	50.92	96.74	79.59
APRI	0.769 (0.692-0.834)	67.74	81.90	50.02	90.47	78.91
FIB-4	0.752 (0.674-0.819)	64.52	75.00	40.83	88.77	72.79
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		1.297			0.195	
LECT2 & FIB-4		1.690			0.091	
APRI & FIB-4		0.426			0.670	



Predicting ≥S2 fibrosis

ALL patients (n=80)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.883 (0.792-0.944)	79.17	84.37	88.37	72.98	81.25
APRI	0.745 (0.635-0.836)	66.67	71.87	78.05	58.97	68.75
FIB-4	0.711 (0.599-0.807)	43.75	90.62	87.50	51.79	62.50
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		2.040			0.041	
LECT2 & FIB-4		2.457			0.014	
APRI & FIB-4		0.721			0.471	

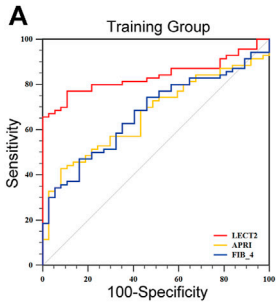


Predicting ≥S3 fibrosis

ALL patients (n=80)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.870 (0.776-0.935)	83.33	82.26	57.69	94.44	82.50
APRI	0.756 (0.647-0.845)	83.33	59.68	37.50	92.50	65.00
FIB-4	0.775 (0.668-0.861)	72.22	87.10	61.90	91.53	83.75
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		1.403			0.161	
LECT2 & FIB-4		0.993			0.321	
APRI & FIB-4		0.323			0.747	

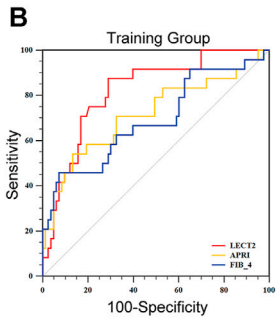
FIGURE 2 | The diagnostic efficacy of LECT2, APRI, and FIB-4 for identifying liver fibrosis in all CHB patients. **(A,B)** Identification of significant liver fibrosis (≥S2) or advanced liver fibrosis (≥S3) in the training group. **(C)** and **(D)** show the further verification of the validation group to the training group. AUC, the area under the ROC; PPV, positive predictive value; NPV, negative predictive value.

numbers or median and quartiles. Chi-square test and Fisher's exact test were used to analyze the difference in the number of different liver fibrosis stages and patients with HBeAg-negative between the training group and validation group, and the Mann-Whitney U-test was used to determine intergroup differences of continuous data. Kruskal-Wallis H-test was used to compare the expression of these predictors in various stages of liver fibrosis. Spearman correlation coefficient was used to count the relationship between liver fibrosis



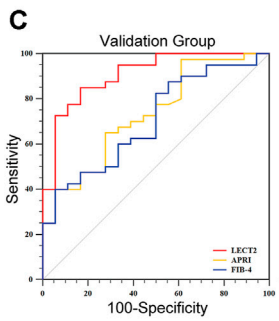
Predicting ≥S2 fibrosis

HBeAg-negative patients (n=107)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.838 (0.754-0.903)	77.14	89.19	93.10	67.36	81.31
APRI	0.669 (0.571-0.757)	42.86	91.89	90.90	45.97	59.81
FIB-4	0.672 (0.575-0.760)	47.14	83.78	47.14	45.61	59.81
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		2.605			0.008	
LECT2 & FIB-4		2.421			0.016	
APRI & FIB-4		0.080			0.936	



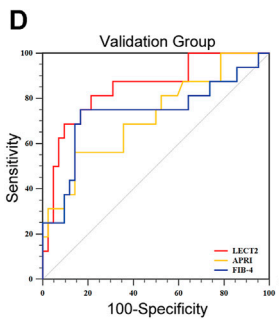
Predicting ≥S3 fibrosis

HBeAg-negative patients (n=107)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.821 (0.735-0.888)	71.08	87.50	62.14	91.29	84.11
APRI	0.718 (0.623-0.801)	54.17	86.75	54.13	86.77	79.44
FIB-4	0.681 (0.594-0.776)	45.83	92.77	64.66	85.58	82.24
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		1.206			0.228	
LECT2 & FIB-4		1.637			0.102	
APRI & FIB-4		0.555			0.556	



Predicting ≥S2 fibrosis

HBeAg-negative patients (n=58)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.907 (0.801-0.967)	85.00	83.33	91.90	71.40	84.48
APRI	0.733 (0.600-0.840)	65.00	72.22	83.89	48.11	67.24
FIB-4	0.704 (0.570-0.817)	40.00	94.44	94.13	41.42	56.88
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		2.083			0.037	
LECT2 & FIB-4		2.304			0.021	
APRI & FIB-4		0.486			0.627	



Predicting ≥S3 fibrosis

HBeAg-negative patients (n=58)	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	Accuracy
LECT2	0.847 (0.728-0.928)	81.25	78.57	59.11	91.66	79.31
APRI	0.719 (0.586-0.830)	56.25	85.71	60.02	83.71	77.58
FIB-4	0.735 (0.603-0.842)	75.00	83.33	63.17	89.74	81.03
Comparison of AUC		Z value			*P* value	
LECT2 & APRI		1.315			0.188	
LECT2 & FIB-4		0.975			0.329	
APRI & FIB-4		0.229			0.819	

FIGURE 3 | The diagnostic efficacy of LECT2, APRI, and FIB-4 for identifying liver fibrosis in CHB patients with HBeAg-negative. **(A,B)** Identification of significant liver fibrosis (≥S2) or advanced liver fibrosis (≥S3) in the training group. **(C)** and **(D)** show the further verification of the validation group to the training group. AUC, the area under the ROC; PPV, positive predictive value; NPV, negative predictive value.

and the predictors. The univariate and multivariate regression analyses were used to analyze independent influencing factors. The diagnostic efficacy of LECT2, APRI, and FIB-4 was evaluated by the receiver operating characteristic curve (ROC) and the area under the ROC (AUC). The AUC of LECT2, APRI, and FIB-4 were compared using the method of DeLong et al. (Demler et al., 2012). A two-sided *p* value of <0.05 was deemed statistically significant.

RESULTS

Patient Clinical Characteristics

The demographic and laboratory data of the 227 patients and the number of patients with each different stage of liver fibrosis are presented in **Table 1**. In the training group, stages of liver fibrosis using the modified Scheuer scoring system as the reference method

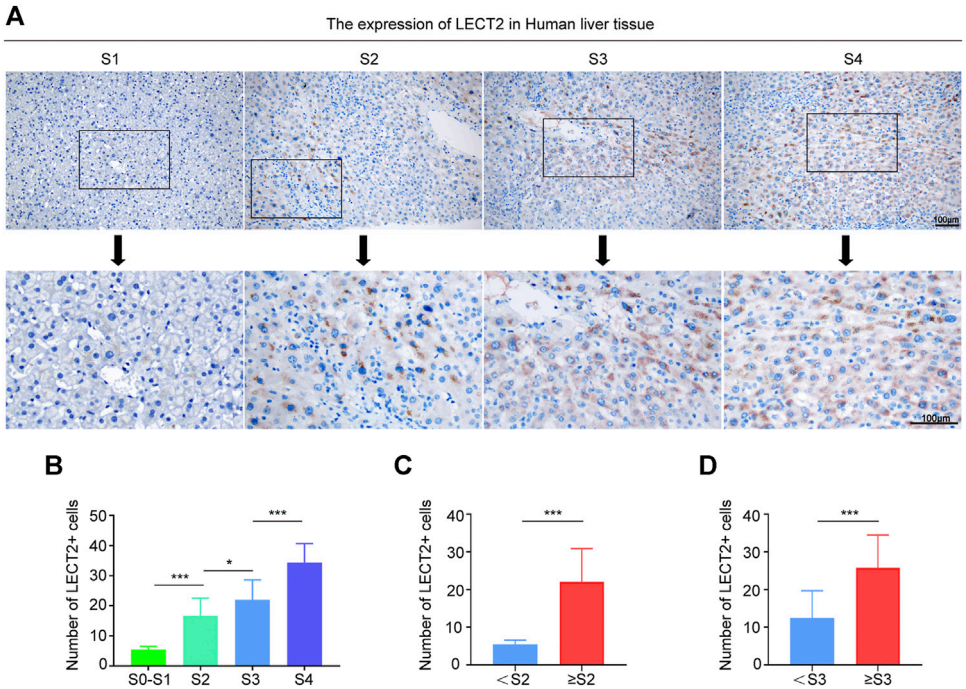


FIGURE 4 | Expression of LECT2 protein levels in the human liver tissue. **(A)** Immunohistochemical staining of LECT2 in liver tissues sections from CHB patients in different stages of liver fibrosis. **(B)**, **(C)**, and **(D)** show the quantitative analysis of LECT2+ cells in **(A)**. *$p < 0.05$, ***$p < 0.001$.

were as follows: S0–S1 in 61 individuals (41.50%), S2 in 55 (37.41%), S3 in 25 (17.01%), and S4 in 6 (4.08%). In the validation group, S0–S1 contained 32 individuals (40.00%), S2 in 30 (37.50%), S3 in 14 (17.50%), and S4 in 4 (5.00%). The concentration of LECT2, APRI, and FIB-4 were 4.97, 0.24, and 0.91 in the training group, and 4.76, 0.28, and 0.89 in the validation group, respectively. The main characteristics and the numbers in various stages of liver fibrosis showed no significant differences between the training group and the validation group.

Serum LECT2 is Positively Associated With the Stage of Liver Fibrosis

The expression of LECT2, APRI, and FIB-4 in various stages of liver fibrosis were shown in **Table 2**. It is obvious that whether in the training group or the validation group, the level of serum LECT2, APRI, and FIB-4 increased with the aggravation of liver fibrosis (all $p < 0.05$), and the patients with S4 showed the highest LECT2. In the training group, the Spearman correlation coefficient was used to demonstrate that LECT2, APRI, and FIB-4 were positively associated with the degree of liver fibrosis. And the LECT2 was shown to be more positively correlated with the stages of liver fibrosis than the others (r = 0.673, 0.423, 0.450 for LECT2, APRI, FIB-4, respectively). Furthermore, we tried to investigate the potential predictors of liver fibrosis through the univariate and multivariate regression analyses. Data showed that LECT2 was the independent predictor of significant liver fibrosis (OR = 2.311, $P = 0.000$) and advanced liver fibrosis (OR = 1.555, $P = 0.000$) (**Supplementary Tables S1, S2**). In the detailed analysis, we compared the expression of LECT2, APRI, and FIB-4 in the condition of ≥S2 or ≥ S3, respectively. The results showed that the level of these predictors was increased in patients with more serious liver fibrosis in the training group and validation group (**Figure 1**).

Diagnostic Performance of LECT2 in all CHB Patients

As mentioned above, the serum level of LECT2 was upregulated in various liver fibrosis groups, suggesting the possibility that LECT2 could discriminate the liver fibrosis stage. The efficacy of LECT2, APRI, and FIB-4 was compared by calculating the AUC of these predictors. In the training group, for predicting significant liver fibrosis (≥S2), the AUC was 0.861 of LECT2, which was shown to be significantly higher than that of APRI (0.698), and FIB-4 (0.734) (all $p < 0.05$) (**Figure 2A**). In the validation group, the AUC of LECT2 was also superior to that of APRI, and FIB-4 (0.883, 0.745, 0.711 for LECT2, APRI, FIB-4) (**Figure 2C**). The optimal cutoff value of LECT2 to predict ≥S2 liver fibrosis was 4.13 and 4.20 ng/ml in the training group and validation group. The diagnostic accuracy of LECT2 in predicting significant liver fibrosis was 82.99 and 81.25% in the training group and validation group, respectively.

We further validated the efficacy of these indicators for the prediction of advanced liver fibrosis (≥S3). In the training group, the AUC of LECT2 was 0.855, which was higher than those of APRI, and FIB-4 (0.769 and 0.752, respectively) (**Figure 2B**). In the validation group, the AUC was 0.870, 0.756, 0.775 for LECT2,

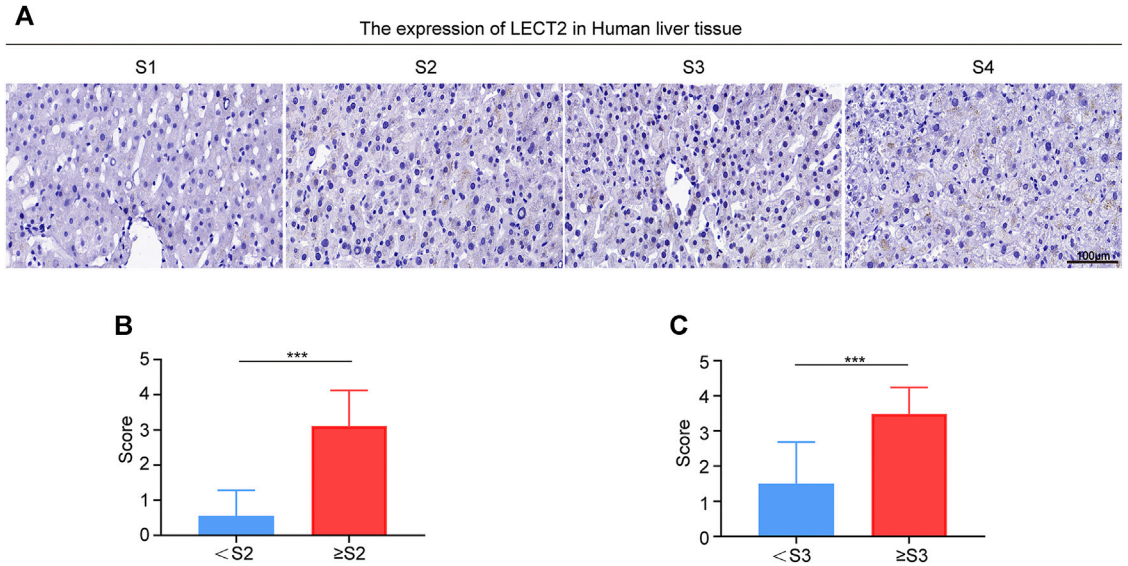


FIGURE 5 | Expression of LECT2 mRNA in the human liver tissue. **(A)** Representative photomicrographs of RNAscope *in situ* hybridization (ISH) showing LECT2 expression in liver tissues sections from CHB patients in different stages of liver fibrosis. **(B)** and **(C)** show the quantitative analysis of the results of RNAscope *in situ* hybridization (ISH) in **(A)**. ***$p < 0.001$.

APRI, FIB-4, respectively (**Figure 2D**). The optimal cutoff value of LECT2 for predicting ≥S3 liver fibrosis was 6.10 ng/ml in the training group and 5.92 ng/ml in the validation group. The accuracy of LECT2 in predicting advanced liver fibrosis was 79.59 and 82.50% in the training and validation group, respectively.

Diagnostic Performance of LECT2 in CHB Patients With HBeAg-Negative

In the training group, there were 107 CHB patients with HBeAg-negative, and in the validation group, there were 58 CHB patients with HBeAg-negative, respectively. We compared the efficacy of LECT2 in predicting significant liver fibrosis (≥S2) to APRI, FIB-4 in the training group first. As shown in **Figure 3A**, the AUC of LECT2 (0.838) was higher than that of APRI (0.669) and FIB-4 (0.672) in CHB patients with HBeAg-negative (all $p < 0.05$). Then, we validated the efficacy of LECT2 in the validation group, as shown in **Figure 3C**, the excellent efficacy of LECT2 had also been demonstrated in the validation group, the AUC was 0.907, 0.733, and 0.704 for LECT2, APRI, and FIB-4 (all $p < 0.05$). The optimal cutoff value of LECT2 to predict ≥ S2 liver fibrosis was 4.11 ng/ml in the training group and 4.20 ng/ml in the validation group. We calculated the accuracy of LECT2 in predicting significant liver fibrosis separately, and it was 81.31 and 84.48% in these two groups, respectively.

For predicting advanced liver fibrosis (≥S3), in the training group, LECT2, APRT, and FIB-4 gave an AUC of 0.821, 0.718, and 0.681, respectively (**Figure 3B**). The AUC of these predictors in the validation group was the same as the training group (0.847 for LECT2, 0.719 for APRI, and 0.735 for FIB-4) (**Figure 3D**). In the training group, the optimal cutoff of LECT2 was 6.09 ng/ml and yielded 84.11% accuracy. In the validation group, the optimal cutoff of LECT2 was 5.92 ng/ml, yielding 79.31% accuracy.

Correlation Between the Protein Levels of LECT2 in the Liver Tissues and Liver Fibrosis Stage

To verify that LECT2 is a direct predictor of liver fibrosis, we not only detected serum LECT2 but also examined the protein levels of LECT2 in the liver tissues. The expression of LECT2 was detected from liver tissues through an IHC assay. As shown in **Figure 4A**, in liver samples with a lower fibrosis stage, lower protein levels of LECT2 were observed in hepatocytes. In contrast, significantly stronger LECT2 expression was observed in the liver tissues with significant (≥S2) and advanced (≥S3) liver fibrosis. The number of LECT2+ cells in different liver fibrosis stages was calculated. It is obvious that with the aggravation of liver fibrosis, the number of LECT2+ cells increased gradually (**Figures 4B–D**).

Correlation Between the Levels of LECT2 mRNA in the Liver Tissues and Liver Fibrosis Stage

In subsequent analysis, we tested the level of LECT2 mRNA in the liver tissues. The staining results of LECT2 mRNA levels revealed that there was a higher abundance of LECT2 mRNA in the advanced fibrosis stage (**Figure 5A**). We interpreted the results according to the official scoring criteria. The expression of LECT2 mRNA was compared between the <S2 group and the ≥S2 group, data showed that it was significantly higher in the ≥S2 (significant liver fibrosis) group ($p < 0.01$) (**Figure 5B**). The concentration of

LECT2 mRNA in the ≥S3 (advanced liver fibrosis) group was more ($p < 0.01$) than that in the <S2 group (**Figure 5C**).

DISCUSSION

No study has investigated the possible relationship between LECT2 and the degree of liver fibrosis in patients with CHB until recently. This is the first report that aimed to figure out the association between the expression of LECT2 and the severity of liver fibrosis. Epidemiological studies have reported that the staging of liver fibrosis is positively linked with significant clinical complications, and an increased liver fibrosis stage is related to higher mortality than in patients without liver fibrosis (Berumen et al., 2021). Besides, the development of histopathology often precedes any overt clinical features and it is usually difficult to find in time in the clinic. The guidelines of CHB also require that treatment should be started once significant liver fibrosis is reached. For CHB patients, it is necessary that they are warned of the severity of liver fibrosis as soon as possible (EASL, 2017).

Liver biopsy is the most classic method to estimate liver fibrosis through expert histological interpretation. But it had some limitations in clinical application due to invasiveness, high price, and multiple complications. Considering the shortcomings of liver biopsy, finding some noninvasive methods to describe the degree of liver fibrosis is a very attractive option. Several noninvasive tests had been reported to predict liver fibrosis in NAFLD and HCV, and so on (Chinnaratha et al., 2014; Boursier et al., 2016). These noninvasive tests include 1) simple blood tests using common parameters, but their accuracy is low; 2) specialized blood tests that are more direct but not widely available and require specialist laboratory assessment; 3) some require costly elastography (Parola and Pinzani, 2019). Hence, there is a need for a new and direct biomarker that is suitable and economical.

Noninvasive scoring systems such as APRI and FIB-4 were used to evaluate the degree of liver fibrosis that had been reported earlier (WHO, 2015). The efficacy of LETC2 in predicting liver fibrosis was checked by comparing it with the above predictors. The results of this study displayed that the AUC of APRI in predicting significant and advanced liver fibrosis was 0.698 and 0.745 in the training group and validation group, respectively. In the study of Lu et al., the AUC of APRI for predicting significant and cirrhosis was 0.66 and 0.72 (Lu et al., 2020), which is consistent with this research. In this study, the ACU of FIB-4 was 0.734 for predicting significant liver fibrosis. Reviewing previous studies, the AUC of FIB-4 for significant liver fibrosis ranged from 0.72 to 0.76 (Xiao et al., 2015; Park et al., 2016; Tseng et al., 2018), and our results fit this range. Besides, several previous articles had reported that the AUC of APRI and FIB-4 for predicting advanced liver fibrosis (≥S3) were 0.717 and 0.760, respectively (Li et al., 2018; Gao et al., 2020). In our study, the ACU of APRI and FIB-4 was 0.769 and 0.752, which seems slightly higher than previous studies. All the above evidence reminds us that APRI and FIB-4 only showed modest predictive performance for the identification of liver fibrosis in CHB patients.

Recently, accumulating evidence has indicated that LECT2 played crucial roles in various diseases, including diabetes (Lan et al., 2014), lung cancer (Hung et al., 2018), liver cancer (Ong et al., 2011; Chen et al., 2014; L'Hermitte et al., 2019), NAFLD (Yoo et al., 2017), and so on. More importantly, our previous studies confirmed for the first time that LECT2 can bind to Tie1 as a ligand to promote the progress of liver fibrosis by affecting angiogenesis, and verified the conclusion *in vitro* and experimental animal models. In the process of studying the mechanism of LECT2 promoting liver fibrosis, we found that serum LECT2 was raised significantly in patients with liver cirrhosis and was increased as the Child-Pugh score progressed from A to D (Xu et al., 2019). However, whether LECT2 can be used as a direct biomarker to diagnose the early stage of liver fibrosis in CHB patients remains unknown.

To verify the efficacy of LECT2, firstly we compared the AUC of LECT2, APRI, FIB-4 in detecting significant (≥S2), and advanced (≥S3) liver fibrosis in CHB patients in the training group. The results showed that the AUC of LECT2 in predicting significant and advanced liver fibrosis was 0.861 and 0.855, which was higher than APRI and FIB-4. The optimal cutoff value of LECT2 is 4.13 ng/ml to predict significant liver fibrosis (≥S2), 6.10 ng/ml to predict advanced liver fibrosis (≥S3). Then, according to the guidelines of CHB (EASL, 2017; Terrault et al., 2018) which have emphasized that the degree of liver fibrosis and the various situations of CHB patients should be taken into full consideration. We discussed the efficacy of LECT2 on the premise of considering different situations of CHB patients, such as HBeAg-negative and ALT (ALT < ULN or ALT < 2ULN). We calculated and compared the diagnostic efficacy of LECT2, APRI, and FIB-4 in patients with HBeAg-negative, ALT < ULN or ALT < 2ULN (data shown in the **Supplementary Figure S1**), respectively. Beyond these different grouping conditions, the efficacy of LECT2 to predict significant and advanced liver fibrosis was still superior to APRI and FIB-4. Furthermore, we verified the efficacy of LECT2 in the validation group which was consistent with the training group, the AUC of LECT2 was 0.883 and 0.870 for significant and advanced liver fibrosis, respectively. All these results indicated that LECT2 is good at detecting liver fibrosis in CHB patients.

Our data showed that there was an obvious correlation between the high expression of serum LECT2 and the deterioration of liver fibrosis. Moreover, we detected the expression of protein and mRNA levels of LECT2 in CHB patient liver samples using IHC analysis. Our results showed that the LECT2 protein levels and mRNA levels of LECT2 in hepatic tissue were upregulated in CHB patients who were diagnosed with significant and advanced liver fibrosis by liver biopsy. It seems that LECT2 is more reliable than APRI and FIB-4 since these were obtained by calculating some liver-related laboratory indicators. More importantly, LECT2 is a protein secreted by hepatocytes and expressed in both serum and tissues. It means that LECT2 could be easily detected from serum by ELISA or flow cytometry and did not require additional histological interpretation, which is a huge advantage of LECT2 in clinical application.

In conclusion, LECT2 is a novel and direct predictor, which is suitable as a screening biomarker for significant and advanced liver fibrosis, and the diagnostic efficacy of LECT2 in different situations of patients with CHB had been confirmed. The detection of LECT2 contributes to the more accurate evaluation of liver fibrosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical committee of Anhui medical university (20190196). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YG and JBL were responsible for the study concept and design. LZ, JS, JYL, and JQ contributed to the acquisition of clinical data, and ZW, XL, and YS contributed to the detection of the expression of LECT2, XL, and YW and contributed to statistical analysis and data interpretation. XL and ZW wrote the article. HX, JK, YW, and YG verified the study. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.749648/full#supplementary-material

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Novel Therapeutic Targets in Liver Fibrosis

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Liver fibrosis is end-stage liver disease that can be rescued. If irritation continues due to viral infection, schistosomiasis and alcoholism, liver fibrosis can progress to liver cirrhosis and even cancer. The US Food and Drug Administration has not approved any drugs that act directly against liver fibrosis. The only treatments currently available are drugs that eliminate pathogenic factors, which show poor efficacy; and liver transplantation, which is expensive. This highlights the importance of clarifying the mechanism of liver fibrosis and searching for new treatments against it. This review summarizes how parenchymal, nonparenchymal cells, inflammatory cells and various processes (liver fibrosis, hepatic stellate cell activation, cell death and proliferation, deposition of extracellular matrix, cell metabolism, inflammation and epigenetics) contribute to liver fibrosis. We highlight discoveries of novel therapeutic targets, which may provide new insights into potential treatments for liver fibrosis.

Keywords: liver fibrosis, hepatic stellate cells, therapeutic targets, molecular mechanism, non-alcocholic fatty liver disease

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INTRODUCTION

Liver Fibrosis

Liver fibrosis is a repair response to chronic liver injury caused by various pathogenic factors, and it is characterized mainly by the excessive accumulation of extracellular matrix (ECM), especially collagen fibers (Reeves and Friedman, 2002). If the pathogenic factor is not removed, liver fibrosis can progress to liver cirrhosis and even hepatocellular carcinoma, which elevates risk of mortality.

Liver fibrosis has become one of the most common liver diseases worldwide, and it has also become one of the leading indications for liver transplantation. The global prevalence of nonalcoholic fatty liver disease (NAFLD) is 25.24%, and its prevalence is particularly high in the Middle East, South America and Asia. Just over half of patients (59.1%) with NAFLD, and in particular 40.76% of patients with liver fibrosis, progress to nonalcoholic steatohepatitis (NASH) (Younossi et al., 2016). Liver disease accounts for approximately 2 million deaths per year worldwide, among which 1 million deaths occur due to complications from cirrhosis, which is currently the 11th most common cause of death globally (Asrani et al., 2019).

Alcohol abuse, chronic viral hepatitis, obesity, autoimmune hepatitis, metabolic syndrome and cholestasis are the most common causes of liver fibrosis (Bataller and Brenner, 2005). If the pathogenic factors are acute or self-limiting, wound-healing responses are transient, and the liver architecture can return to normal. When the factors persist, the inflammatory phase begins and hepatic stellate cells (HSCs) activate, leading to ECM deposition and destruction of the liver architecture. The pathogenesis of liver fibrosis is complicated and involves

multiple types of liver cells and inflammatory reactions. Its main pathological features are collagen deposition and damage of liver structure. Numerous studies of anti-fibrosis targets have focused mainly on collagen deposition and various types of hepatic cells.

HEPATIC CELLS IN LIVER FIBROSIS

Liver fibrosis is a complex process of liver self-repair that involves multiple types of hepatic cells. Intercellular crosstalk within the liver microenvironment is critical for the maintenance of normal hepatic functions and cell survival (Marrone et al., 2016). The chronic presence of external pathological factors can injury hepatocytes; activate inflammatory cells such as macrophages; promote infiltration of lymphocytes; trigger proliferation of sinusoidal endothelial cells and capillarization of sinusoidal endothelial cells, blocking perfusion between blood and liver cells and causing abdominal aortic hypertension; and activate bile duct cells (Boyer-Diaz et al., 2021). These changes ultimately lead to the activation of HSCs, the most important source of myofibroblasts, to synthesize excess ECM, resulting in liver fibrosis. HSC activation is considered central in liver fibrosis. However, other types of liver cells also play important roles in fibrosis. Indeed, HSCs activation depends on the interaction with other hepatic cells, including hepatocytes, liver sinusoidal endothelial cells, inflammatory cells and biliary cells (**Figure 1**). These cells interact with each other and promote or inhibit the activation of HSC through production of cytokines and other signalling molecules. Targeting the crosstalk between HSCs and other hepatic cells might be a novel option for liver fibrosis treatment.

Hepatic Stellate Cells

Activation of HSCs, often referred to as their "*trans*-differentiation", is the major cellular source of matrix protein-secreting myofibroblasts, which are the major driver of liver fibrogenesis (Higashi et al., 2017; Tsuchida and Friedman, 2017; Cai et al., 2020). HSCs, which are also called vitamin A-storing cells, lipocytes, interstitial cells, fat-storing cells or Ito cells, exist in the space between parenchymal cells and liver sinusoidal endothelial cells of the hepatic lobule. They store 50–80% of the total vitamin A in the body; they store the vitamin A in the form of retinyl palmitate in lipid droplets in the cytoplasm (Senoo et al., 2010). HSCs in the space of Disse are also thought to contribute reversibly to portal hypertension (McConnell and Iwakiri, 2018).

Stimulating HSCs with external factors such as lipid peroxides or pro-fibrotic cytokines leads them to lose lipid droplets, proliferate, and transform into myofibroblasts. The cells then begin to produce ECM and they acquire contractile, pro-inflammatory, and fibrogenic properties (Bataller and Brenner, 2005). The disordered accumulation of ECM results in scar and liver fibrosis (Gandhi, 2017). Many current anti-fibrosis treatments aim to prevent HSCs from contributing to fibrosis, such as by blocking their activation by external factors, inhibiting their proliferation, promoting their apoptosis (Trivedi et al., 2021), and preventing their adoption of a high metabolic state (Du et al., 2018; Khomich et al., 2019).

The major signaling pathways involved in HSCs activation contains: growth factors and ligand-receptor signaling pathways (Wnt/β-catenin, Hedgehog, YAP/TAZ, FGF, cAMP-PKA-CREB), profibrogenic response pathways (TGF-β, PDGF/VEGF/CTGF, ROCKs, Axl/Gas6, Notch, renin angiotensin system), cell death signaling (autophagy, ER stress, oxidative stress), immune-related signaling (TLRs, LPS, DAMPs, interleukin), metabolic regulated pathways (Acc, Hedgehog, YAP, leptin), Nuclear receptors (FXR, LXR, PPARs, VDR) and epigenetic changes (miRNA, lncRNA, DNA methylation, histone modification) (**Figure 2**). Although some clinical drugs were found to be positive for liver fibrosis treatment in clinical trails including PPAR-γ agonist (pioglitazone), angiotensin receptor blockers (losartan, telmisartan, olmesartan and candesartan), and glucagon-like peptide-1 receptor agonists (liraglutide), the safety and effect of these drugs need to be further confirmed (Georgescu, 2008; Mantovani et al., 2021). Clinical existing drugs like angiotensin receptor blockers, PPAR-γ agonist may be accompanied by side effects due to their wide range of effects, and they may not be suitable for the treatment of simple liver fibrosis. It is necessary to find new therapeutic targets.

Myofibroblasts, which are not present in the healthy liver, are activated in response to liver injury (Friedman, 2008; Kisseleva and Brenner, 2021). They form from resident mesenchymal cells, epithelial cells (e.g. hepatocytes and cholangiocytes), endothelial cells, bone marrow stem cells, portal fibroblasts and HSCs (Wynn, 2008). The most important characteristic of liver fibrosis is the excessive deposition of ECM, in which myofibroblasts play the most important role (Karsdal et al., 2016). This makes ECM production a primary target for anti-fibrotic therapy. Inflammation often accompanied liver injury (Koyama and Brenner, 2017), which involves the production and release of such cytokines as CTGF, PDGF, and TGF-β. These cytokines activate myofibroblasts to produce abundant ECM (Wynn, 2008), inducing liver fibrosis.

Hepatocytes

Hepatocytes are the most important parenchymal cells in liver; they account for more than 80% of all liver cells. Together with cholangiocytes, hepatocytes help maintain liver homeostasis (Dai et al., 2020). Damage to hepatocytes, together with subsequent inflammatory and fibrogenic signaling cascades, is thought to trigger fibrosis (Tu et al., 2017). After damage by microenvironmental factors, hepatocytes secrete pro-inflammatory and pro-fibrotic factors, activating inflammatory cells and HSCs, in turn promoting fibrosis (Wang et al., 2016a; Zhang et al., 2019; An et al., 2020). The epithelial-mesenchymal transition (EMT) in hepatocytes promotes the progression of liver fibrosis. Fructose induces hepatocytes to upregulate fibroblast-specific protein1 and vimentin, while downregulating E-cadherin, thereby promoting EMT (Cicchini et al., 2015; Song et al., 2019).

In early-stage liver disease, if liver cell damage can be reversed, then hepatocytes can be promoted and liver fibrosis reversed.

This is the aim of hepatoprotective drugs currently used in the treatment of liver diseases. In late-stage liver disease, in contrast, it is often impossible to inhibit the death of hepatocytes. This may be because HSCs are in a highly activated state, and they can secrete pro-fibrotic factors. Drugs that fail to target liver cells selectively and that instead are taken up by HSCs can promote HSC proliferation and inhibit their apoptosis. The problem of this "two-way" action must be taken into account when designing treatments that promote apoptosis or inhibit proliferation.

Liver Endothelial Cells

LSECs act as permeable barriers and portal pressure regulators, they mediate the transcript of nutrients, they recruit lymphocytes from the blood, and they secrete cytokines and growth factors from their sinusoidal side (Asahara et al., 1999). LSECs have the highest endocytotic capacity of all human cells (Poisson et al., 2017). They also interact with HSCs and hepatocytes, and they are critical to maintain HSC quiescence and regenerate hepatocytes (Hu et al., 2014), thus inhibiting intrahepatic vasoconstriction and fibrosis. LSECs maintain HSC quiescence via a pathway that is stimulated by vascular endothelial growth factor (VEGF) and that depends on nitrous oxide (NO) (Asahara et al., 1999; Marrone et al., 2013; DeLeve, 2015). In chronic liver injury, LSECs undergo capillarization, they downregulate eNOS and NO synthesis, and they secrete profibrogenic and proinflammatory cytokines such as TGF-β1, PDGF, TNF-α and IL-6, thereby promoting liver fibrosis (Lafoz et al., 2020).

Cholangiocytes

Cholangiocytes are epithelial cells lining the intra- and extra-hepatic bile ducts; they are heterogeneous in size and function and mediate solute transport processes that determine the composition and flow of bile (Banales et al., 2019). Their dysfunction lies at the heart of cholangiopathies. During biliary disease, various pathological stimuli such as gastrointestinal hormones, bile acids, angiogenic factors, and nerve growth factor can activate cholangiocytes, leading to biliary proliferation, known as a ductular reaction. The result is an epigenetically-regulated transcriptional program involving secretion of TGF-β1, CTGF, p16, CCL2 and SA-β-gal, ultimately leading to a profibrogenic micro-environment, HSC activation, and enhanced liver fibrosis (Zhou et al., 2018; Elssner et al., 2019; Jalan-Sakrikar et al., 2019). The ductular reaction contributes to the initiation and progression of liver fibrosis (Glaser et al., 2009).

Many recent studies have explored the role of cholangiocytes in liver fibrosis. For example, non-canonical NF-κB can contribute to cholangiocyte proliferation and the ductular reaction, accelerating liver fibrosis (Elssner et al., 2019). The long non-coding RNA H19, present in exosomes from cholangiocytes, can activate HSCs and promote cholestatic liver fibrosis (Liu et al., 2019a). Knockout of the secretin receptor reduces biliary damage and liver fibrosis by slowing cholangiocyte senescence (Zhou et al., 2018). These findings suggest that targeting the activation of cholangiocytes and the ductular reaction can mitigate biliary fibrosis.

Inflammatory Cells

The acute inflammation that arises in response to liver injury is thought to help mitigate infection and promote liver repair and regeneration (Karin and Clevers, 2016). Chronic inflammation, in contrast, is detrimental and contributes to liver fibrosis through the involvement of multiple types of inflammatory cells, including Kupffer cells, recruited macrophages, neutrophils, Th17 cells and Tregs (Berumen et al., 2021; Wen et al., 2021). Macrophages play a dual role in the progress of fibrosis. M1 macrophages produce inflammatory cytokines, while M2 macrophages regulate inflammatory responses and secret matrix metalloproteases (MMPs), the main enzymes that degrade ECM, thereby reversing fibrosis (Pradere et al., 2013; Luo et al., 2019). Thus, the balance between M1 and M2 macrophages influences whether fibrosis progresses or not (Sica et al., 2014). Interestingly, recent studies have suggested that M1, but not M2 macrophages, inhibit liver fibrogenesis by recruiting endogenous macrophages and "polarizing" them into a restorative $Ly6C^{lo}$ phenotype, which secrets high levels of MMPs for collagen degradation, as well as high levels of hepatocyte growth factor for hepatocyte proliferation (Ramachandran et al., 2012; Ma et al., 2017).

Kupffer cells, the resident macrophages of the liver, play a central role in liver inflammation. They are resident macrophages that localize within the lumen of the liver sinusoids, and they account for about 30% of sinusoidal cells (Bouwens et al., 1986; Koyama and Brenner, 2017). In response to hepatocyte injury, Kupffer cells become active and secret pro-inflammatory and pro-fibrosis factors. TGF-β, which is secreted mainly by Kupffer cells and plays a key role in liver fibrosis (Xu et al., 2020), binds to a receptor on HSCs to activate them and induce production of collagen (Wang et al., 2019a). Hepatic macrophages also enhance liver fibrosis through the release of IL-1β, TNF-α, CCL2 and PDGF. During liver steatosis, neutrophils and Kupffer cells release reactive oxygen species (ROS), promoting HSC activation and liver fibrosis (Jiang et al., 2012; Dat et al., 2021). Th17 cells produce IL-17, which activates Kupffer cells and express the proinflammatory cytokines IL-6, IL-1β and TNF-α. IL-17 also directly activates HSCs and promotes collagen production via the STAT3 pathway (Meng et al., 2012). Th22 cells, for their part, produce IL-22, which drives TGF-β-dependent liver fibrosis (Fabre et al., 2018).

In this way, many types of inflammatory cells and complex molecular pathways are involved in liver fibrosis. Future studies aiming to treat liver fibrosis by targeting inflammatory cells should cautiously consider the potentially complex effects of such treatment.

NEW THERAPEUTIC TARGETS

Liver fibrosis can be reversed in early stages if the pathological insult can be removed. Here we summarize recent reports on signaling pathways that contribute to liver fibrosis and on efforts to target such pathways as a therapeutic strategy.

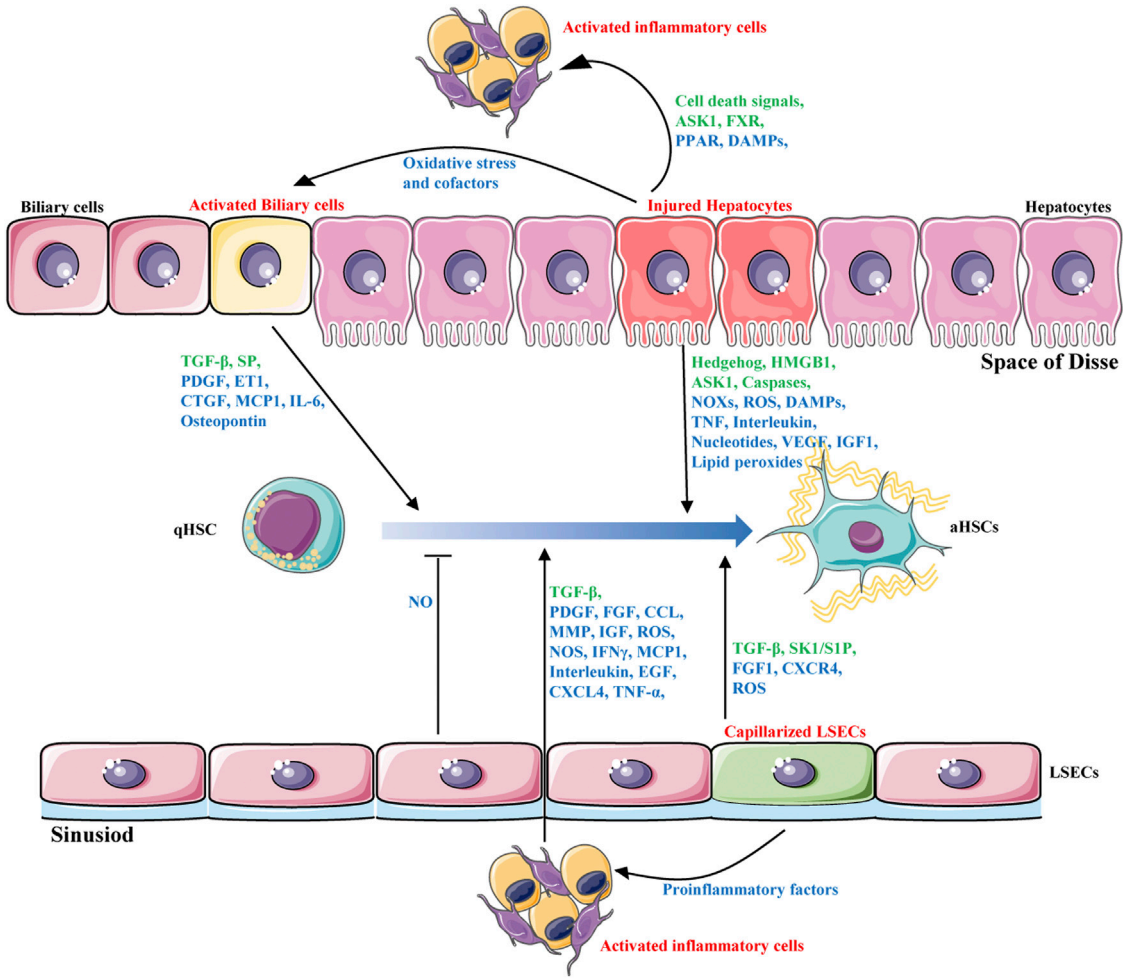

FIGURE 1 | Intercellular crosstalk during liver fibrosis. HSCs activation is the major driver of liver fibrosis that depends on the interaction with other hepatic cells, including hepatocytes, biliary cells, liver sinusoidal endothelial cells and inflammatory cells. These cells interact with each other and promote or inhibit the activation of HSCs through production of hormones, cytokines(blue) and other signalling molecules(green).

TGF-β Signaling

TGF-β signaling is a core regulator of fibrosis, and it can induce fibrosis via canonical and non-canonical (non-Smad) pathways (**Figure 3**) (Finnson et al., 2020). In both cases, myofibroblasts are activated, leading to excessive ECM production and inhibition of ECM degradation (Meng et al., 2016). TGF-β binds to its cognate receptor TGF-β type II receptor, inducing the nuclear translocation of Smad2 and Smad3, which regulate the transcription of fibrotic target genes (Zhang et al., 2021a).

In the canonical pathway, Smad2/3 is activated by phosphorylation but potentially also by lysine acetylation to promote liver fibrosis (Bugyei-Twum et al., 2018; Wang et al., 2019a; Zhong et al., 2020; Zhang et al., 2021a). The chromatin deacylase Sirtuin 6 is also an important regulator of liver fibrosis through its influence on metabolism, DNA repair, gene expression, and mitochondrial biology (Andrew et al., 2020). Sirtuin6 deficiency induces aging-dependent fibrosis in liver and other organs in mice. Sirtuin6 may deacetylate Smad3 as well as Lys-9 and Lys-56 in histone 3 to repress the expression of key TGF-β signaling genes (Maity et al., 2019). By deacetylating lysines 333 and 378 of Smad3, sirtuin6 may inhibit Smad3 activity, protecting against liver fibrosis (Zhong et al., 2020). Like Smad3, Smad2 is also a major acetylated substrate of sirtuin6. Sirtuin6 deacetylates lysine 54 on Smad2, reducing TGF-β/Smad2 signaling in HSCs and thereby alleviating liver fibrosis. By deacetylating Smad2, sirtuin6 influences its phosphorylation and nuclear translocation (Zhang et al., 2021a). These findings suggest that TGF-β signaling is a master regulator of fibrosis and warrants multilayer control, and that sirtuin6 may regulate TGF-β signaling at multiple levels.

TGF-β also regulates other signaling pathways through non-Smad signaling pathways, such as pathways involving Wnt/β-catenin, MAPK, mTOR, IKK, PI3K/Akt, and Rho GTPase, thereby contributing to liver fibrosis (Zhang, 2017; Mi et al., 2019). TGF-β-mediated upregulation of FoxO3a and the DNA demethylase TET3 in HSCs facilitates hepatic fibrogenesis (Xu

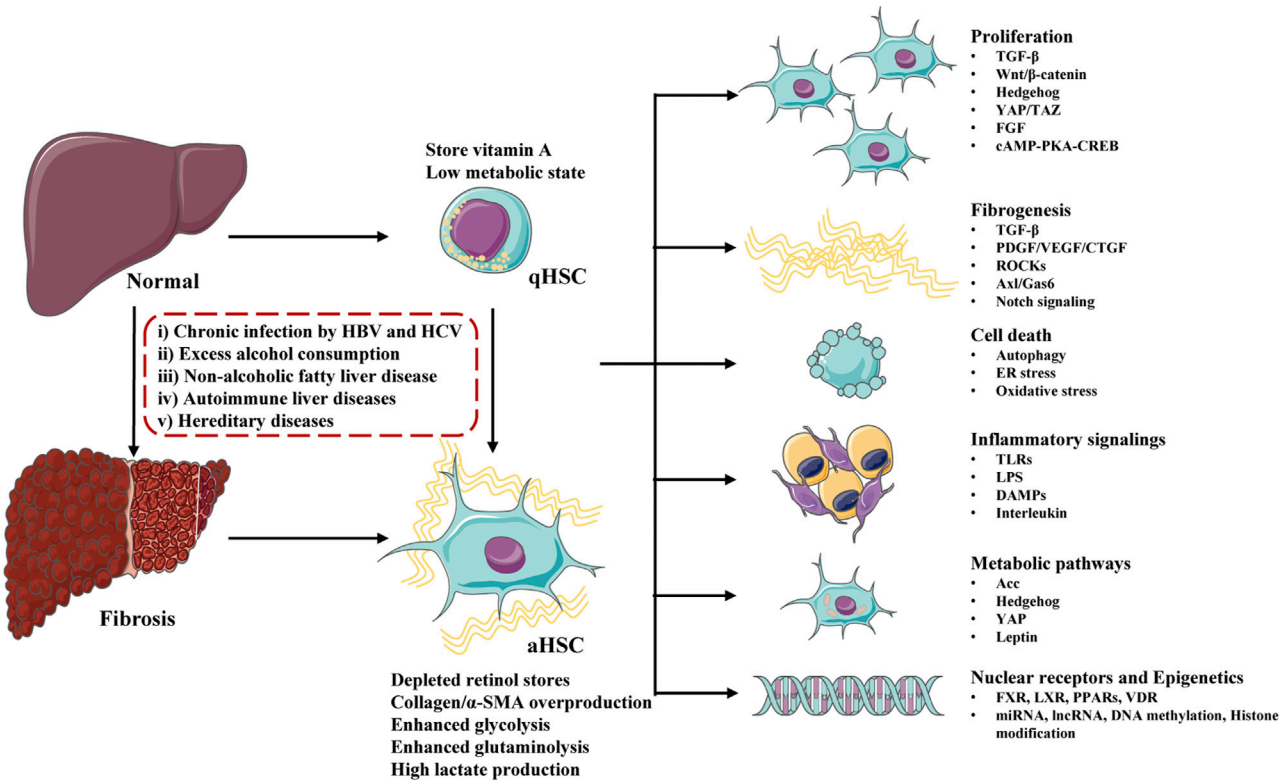

FIGURE 2 | Major molecules and signaling pathways regulating hepatic stellate cells (HSCs) activation and liver fibrosis. Undergo physiological conditions, HSCs exist as a quiescent phenotype (qHSCs), which store vitamin A and stay in a low metabolic state. After stimulating by a serious of factors (including chronic infection, excess alcohol consumption, non-alcoholic fatty liver disease, autoimmune liver diseases and hereditary diseases), qHSCs activate into activated HSCs (aHSCs) and liver fibrosis develop. Activated HSCs lose stored retinol, produce excessive collagen/α-SMA and stay at a high metabolic state. HSCs activation is regulated by a number of signaling pathways and molecules, including proliferation, fibrogenesis, cell death, inflammatory signalings, metabolic pathways, nuclear receptors and epigenetics.

et al., 2020; Kim et al., 2021). TGF-β can also regulate proteasomal degradation of EZH2 in cholangiocytes, supporting biliary fibrosis (Jalan-Sakrikar et al., 2019). TGF-β upregulates hyaluronan (HA) synthase 2, leading to increased production of HA, a major extracellular matrix glycosaminoglycan and biomarker for cirrhosis. HA promotes the fibrogenic, proliferative, and invasive properties of HSCs via pathways involving the receptors CD44, Toll-like receptor 4 (TLR4), and Notch1 (Yang et al., 2019). TGF-β1 activates the p65/MAT2A pathway to decrease levels of S-adenosylmethionine, thereby facilitating liver fibrosis (Wang et al., 2019b).

Several pathways inhibit TGF-β signaling, making them interesting as therapeutic strategies. Transcriptional intermediary factor 1γ, a negative regulator of the TGF-β pathway, interacts with Smad2/3 and binds to the promoter of the α-smooth muscle gene (α-SMA), downregulating α-SMA and activating HSCs (Lee et al., 2020). ECM1 interacts with αv integrins to keep TGF-β in an inactive form, thereby preventing HSC activation and liver fibrosis (Fan et al., 2019). Recent studies have explored epigenetic regulation of Smad- and non-Smad-mediated pathways in TGF-β signaling, highlighting the complex role of such signaling in fibrosis.

Notch Signaling

Notch signaling is a conversed intercellular signaling pathway that regulates interactions between physically adjacent cells. Accumulating evidence suggests that Notch signaling participates in liver fibrosis by mediating myofibroblasts *trans*-differentiation and the EMT (Hu and Phan, 2016). When any one of five ligands (Delta-like1/3/4, Jagged-1/2) binds to the receptor for Notch1-4, the Notch intracellular domain (NICD) is released and translocates to the nucleus, where it binds to transcription factor CBF1/Suppressor of hairless/Lag1 (CSL) and modulates gene expression (Kovall and Blacklow, 2010). Notch activity in hepatocytes correlates with disease severity and treatment response in patients with NASH, and Notch is upregulated in a mouse model of diet-induced NASH and liver fibrosis. Forced activation of Notch in hepatocytes induces fibrosis by upregulating Sox9-dependent Osteopontin (Opn) secretion from hepatocytes, which activates resident HSCs (Zhu et al., 2018). Endothelial Notch1 overexpression results in LSEC dedifferentiation and accelerates liver fibrogenesis through eNOS-sGC signaling, and it alters the angiocrine profile of LSECs to compromise hepatocyte proliferation and liver regeneration (Duan et al., 2018). DLL4, a ligand of Notch signaling, is up-regulated in the LSECs of fibrotic liver of

patients and of mice treated with CCl_4, consistent with LSEC capillarization involving endothelin-1 (Chen et al., 2019a). Notch signaling is an attractive target for treating liver fibrosis; so far, Wnt/β-catenin signaling, miR-30c, liver fibrosis-associated lncRNA1 have been found to influence such signaling (Zhang et al., 2017; So et al., 2018; Gu et al., 2021). Notch signaling can also cross-talk with other signaling pathways involving TGF-β and Hedgehog, which together can regulate liver fibrosis (Xie et al., 2013; Wang et al., 2017; Fan et al., 2020).

Some compounds attenuate liver fibrosis by targeting Notch signaling and so may be novel potential therapeutic candidates for the treatment of liver fibrosis. For example, capsaicin shows liver fibrosis progression by regulating Notch signaling to reduce secretion of inflammatory cytokine TNF-α, which attenuates myofibroblast regeneration and fibrosis mediated by HSCs (Sheng et al., 2020). The natural sesquiterpene costunolide exerts potent antifibrotic effects by disrupting the WWP2/PPM1G complex, promoting Notch3 degradation and inhibiting the Notch3/HES1 pathway (Ge et al., 2020). The Notch inhibitor niclosamide exerts hepatoprotective effects against BDL-induced liver fibrosis (Esmail et al., 2021). Dibenzazepine, a bioavailable γ-secretase inhibitor and Notch antagonist, prevents activation of Notch receptors and is already in clinical trials as an anticancer treatment (Takebe et al., 2014). A nanoparticle system has been developed to deliver dibenzazepine to the liver for treatment of liver fibrosis and obesity-induced type 2 diabetes mellitus (Richter et al., 2020).

Wnt Signaling

During liver fibrosis, canonical(β-catenin-dependent) and non-canonical (β-catenin-independent) pathways of Wnt signaling are activated and some proteins in the pathways are upregulated (Wang et al., 2018; Hu et al., 2020; Yu et al., 2020). In β-catenin-dependent pathways, Wnt ligation to cell surface receptors induces downstream phosphorylation and stabilization of β-catenin, which then translocates to the nucleus, where it acts together with p300 or CBP as a transcriptional co-activator of the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) promoter (Miao et al., 2013; Lien and Fuchs, 2014; Nusse and Clevers, 2017). Non-canonical pathways comprise the β-catenin-independent planar cell polarity pathway and the non-canonical Wnt/Ca^{2+} pathways (De, 2011). Better understanding of Wnt signaling may provide novel insights into the pathophysiology of liver fibrosis.

Wnt also interacts with other pathways to influence liver fibrosis. For example, it blocks the phosphorylation of Smad3 and ERK to inhibit TGF-β1-induced *trans*-differentiation of fibroblasts into myofibroblasts (Liu et al., 2020a). The Wnt/β-catenin pathway may interact with the Smo-independent Gli1 pathway to promote HSC contraction via TCF4-dependent transrepression of Sufu (Zhang et al., 2021b). The "protein regulator of cytokinesis 1" (PRC1), which regulates the Wnt/β-catenin signaling pathway, may induce Gli1-dependent osteopontin expression to contribute to liver fibrosis (Rao et al., 2019). Wnt signaling may play a dual role in liver repair and liver ECM deposition: it promotes liver fibrosis in the BDL mouse model of liver fibrosis, but it protects the liver in the MDR2 KO mouse model of cholestatic liver disease (Jarman and Boulter, 2020). Thus, efforts to target the Wnt signaling to alleviate liver fibrosis should consider how to reduce scarring without affecting repair.

Numerous molecules have been identified that can inhibit Wnt signaling, such as antagonists, short interfering RNA (siRNA), soluble receptors, and the transcription inhibitors DKK1, ICG-001, PRI-724, and honokiol (Miao et al., 2013; Akcora et al., 2018; Nishikawa et al., 2018; Hu et al., 2020; Lee et al., 2021). These molecules may be candidate drugs for fibrosis treatment.

YAP/TAZ Signaling

YAP/TAZ, a downstream effector of the alternative Wnt signaling pathway, is involved in liver fibrosis (Park et al., 2015). The YAP/TAZ-TEAD transcriptional complex plays an important role in the activity of the Hippo pathway (Bai et al., 2012; Crawford et al., 2018). YAP is activated in HSCs in patients with fibrotic livers, and inhibiting YAP using verteporfin impedes fibrogenesis in CCl_4 mice (Mannaerts et al., 2015). Thus, inhibition of YAP may be a novel approach for treating fibrosis. Blockade of YAP reduces HSC activation and proliferation, while promoting their apoptosis. Loss of YAP also inhibits Wnt/β-catenin activity (Yu et al., 2019). Dynein-mediated interaction between YAP and acetylated microtubules may drive nuclear localization of YAP in the soft matrix, increasing TGF-β1-induced transcriptional activity of Smad for myofibroblast differentiation (You et al., 2020). Interestingly, activation of YAP attenuates hepatic damage and fibrosis in studies of liver ischemia-reperfusion injury, which may reflect the complex role of YAP in liver repair and fibrosis through processes such as Wnt signaling (Konishi et al., 2018; Liu et al., 2019b). The expression of YAP and TAZ in HSCs as well as hepatocytes promotes parenchymal inflammation and fibrosis (Mooring et al., 2020).

Verteporfin is the most commonly used small molecule inhibitor of YAP. Many other molecules alleviate fibrosis via inhibiting YAP/TAZ signaling, including magnesium isoglycyrrhizinate, acid ceramidease, dopamine receptor D1 agonist, and liquiritigenin (Li et al., 2018; Haak et al., 2019; Lee et al., 2019; Alsamman et al., 2020). Given its complex role in liver regeneration and HSC proliferation in different stages of NAFLD, balancing the activity of YAP in hepatocytes and HSCs during different disease stages is key for efficacy.

Hedgehog Signaling

Growing evidence indicates that the hedgehog pathway is a critical regulator of adult liver repair and, hence, a potential diagnostic and/or therapeutic target in cirrhosis (**Figure 4**) (Machado and Diehl, 2018). Gli1 is the downstream transcriptional activator of hedgehog signaling, and it is also a marker of mesenchymal cells. Previous studies have confirmed perivascular $Gli1^+$ mesenchymal-like cells to be a major driver of organ fibrosis (Kramann et al., 2015). Peribiliary $Gli1^+$ mesenchymal cells are a subset of stromal cells characterized by active hedgehog signaling; these cells proliferate, acquire a myofibroblast phenotype, and surround the biliary tree in response to cholestatic injury, promoting liver fibrosis (Gupta

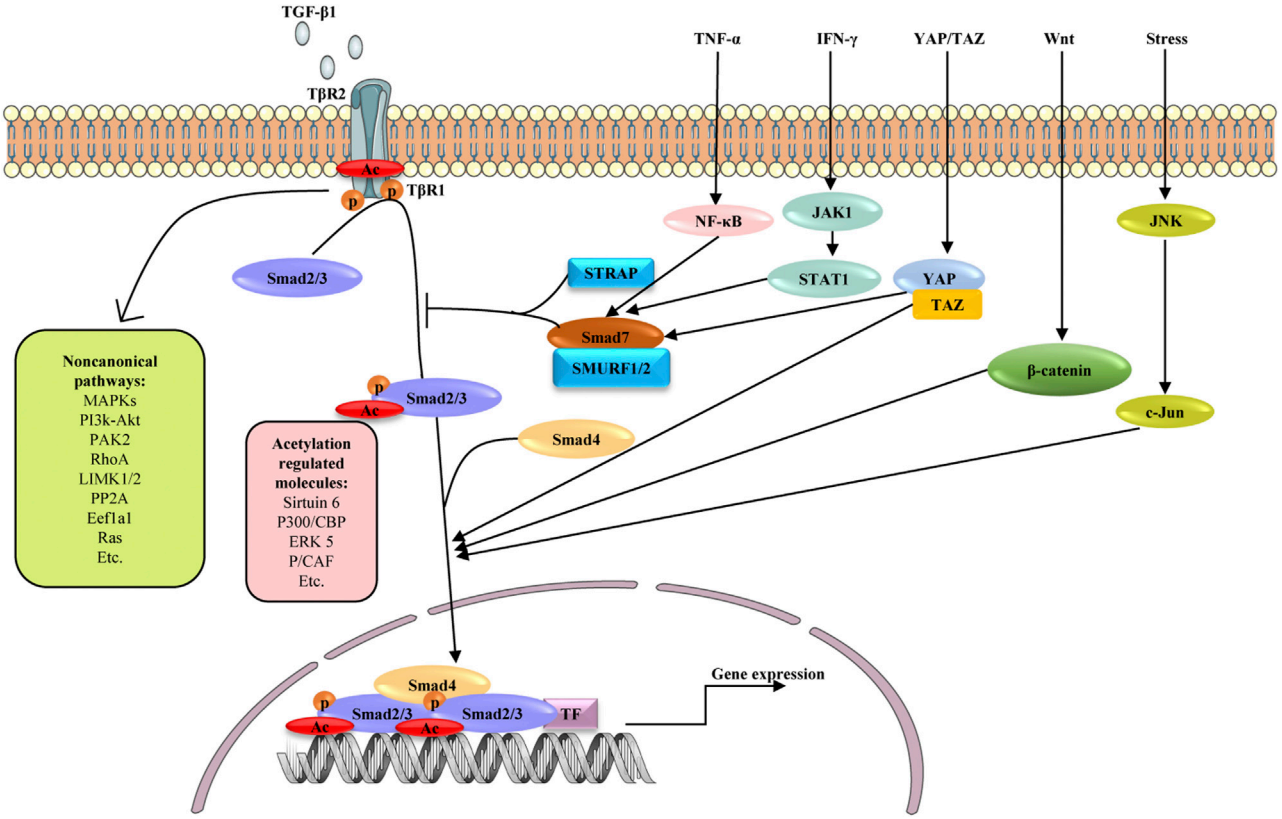

FIGURE 3 | Schematic diagram depicting possible mechanisms involved in the fibrogenesis of TGF-β signaling and its crosstalk with other signaling pathways. Signaling starts with TGF-β binding to TGFβR2 (TβR2), which activates TGFβR1 (TβR1). The active TβR1 phosphorylates and acetylates Smad2/3, which complex with Smad4, translocate into nucleus and promote profibrotic genes expression. As another active form of Smad2/3, acetylated Smad2/3 are reported to be regulated by Sirtuin6, P300/CBP, ERK5 and P/CAF. Smad7 negatively regulates TGF-β signaling through competing with Smad2/3 for interaction with TβR1 in the presence of STRAP and SMURF1/2. TGF-β can also activate non-canonical TGF-β pathways, including MAPK, PI3K/AKT, PAK2, RhoA, LIMK1/2, *p*P2A, Eefla1and Ras pathways. In addition to canonical and non-canonical pathways, TGF-β/Smad signaling shares crosstalk with other signaling pathways in liver fibrosis, including TNF-α, IFN-*γ*, YAP/TAZ, Wnt and JNK signaling. These pathways influence TGF-β signaling by affecting the activation and nuclear translocation of Smad2/3. TF: transcription factors, p: phosphate group, Ac: acetylate group.

et al., 2020). In fact, aberrant activation of hedgehog signaling in not only mesenchymal cells but also HSCs is considered crucial in liver fibrosis (Li et al., 2020a).

Some signaling factors and epigenetic modifications regulate hedgehog signaling and thereby influence HSC activation. These factors include lipopolysaccharide, palmitic acid, and the protein "predicted paired box 6" (Duan et al., 2017; Pan et al., 2017; Li et al., 2020a; Zhu et al., 2021), which may therefore be therapeutic targets in liver fibrosis. One study also suggested that miR-200a inhibits Gli3 expression and may function as a novel anti-fibrotic agent (Li et al., 2020b). Treating HSCs with the DNA methylation inhibitor 5-azadC prevents their proliferation and activation by restoring expression of Patched (PTCH1) (Yang et al., 2013). The metabolic state of HSCs affects their activation, and hedgehog signaling regulates metabolism (Chen et al., 2012; Du et al., 2018). Further work is needed to clarify exactly how hedgehog signaling regulates HSC activation and metabolism, thereby influencing liver.

Many chemical inhibitors of hedgehog inhibitors have been identified, including Gant61, GDC-0049, MD85, and vismodegib. These compounds have shown promise against liver fibrosis *in vivo* and *in vitro* (Li et al., 2019a; Kumar et al., 2019; Jiayuan et al., 2020). The naturally occurring iridoid glucoside geniposide, extracted from *Gardenia jasminoides* Ellis, inhibits hedgehog and thereby HSCs (Lin et al., 2019). These compounds are less effective against liver fibrosis in part because they cannot be delivered specifically to the liver. Two studies have achieved such delivery using nanoparticles, which improved drug efficacy. One group replaced the sulfonamide group of the hedgehog inhibitor GDC-0449 with two methylpyridine-2yl groups at the amide nitrogen, generating MDB5. This inhibitor was more potent at inhibiting hedgehog signaling and HSC proliferation *in vitro*. The research group also developed MDB5-loaded micelles, which enhanced systemic delivery of the drug and efficacy against liver fibrosis (Kumar et al., 2019). In another study, the hedgehog inhibitor vismodegib was loaded into cRGDyK-guided liposomes, which markedly inhibited the fibrogenic phenotype *in vivo*. The delivery system targeted the delivery of vismodegib to activated HSCs rather than

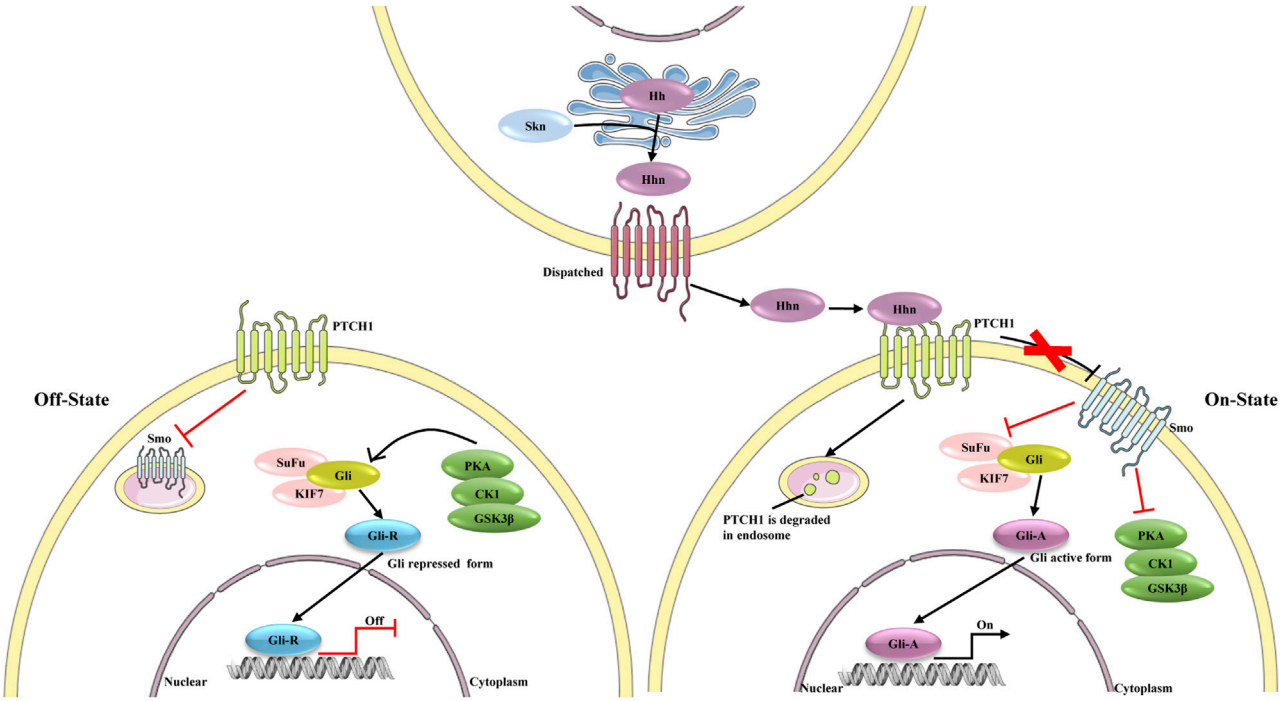

FIGURE 4 | The different state of hedgehog signaling pathway. In the absence of ligands, the signaling is on "Off state", PTCH1 inhibits the activity of Smo. Protein kinase (including PKA, CK1 and GSK3β), SuFu and KIF7 phosphorylate Gli, which leads proteolytic cleavage of Gli to Gli-repressor (Gli-R). Gli-R represses the expression of target genes. In Hhn secreting cells, the precursor of Hh is auto-cleaved and can be modified by a cholesterol at C-terminus to form Hhn on ER membrane. After this process, Hhn is secreted from the secreting cells and bind to PTCH1. PTCH1 is degraded in endosome, and consequently Smo repression is removed. Activated Smo inhibits the effect of PKA on Gli proteins, leading to the dissociation of SuFu, and Gli active form (Gli-A) is formed. Gli-A promotes the expression of target genes.

quiescent HSCs, leading to preferential accumulation in fibrotic liver. These finding illustrate the promise of delivering therapeutic agents to activated HSCs to treat liver fibrosis (Li et al., 2019a).

Fibroblast Growth Factor Signaling

Fibroblast growth factor (FGF) signaling is a prerequisite for adequate would healing, repair and homeostasis in various tissues and organs (Seitz and Hellerbrand, 2021). Since liver fibrosis is a wound healing response to liver injury, FGFs play an important role in hepatic fibrosis by acting as paracrine, and endocrine mediators of hepatocyte regeneration and HSC migration, proliferation and *trans*-differentiation (Schumacher and Guo, 2016). The paracrine FGFs (FGF1-10, FGF16-18, FGF20 and FGF22) bind strongly to heparan sulphate proteoglycans, which limits FGF diffusion through ECM and restricts their action to the site of secretion (Ornitz and Itoh, 2015; Seitz and Hellerbrand, 2021).

The three endocrine FGFs, FGF19 (mouse homolog FGF15), FGF21 and FGF23 participate in phosphate, bile acid, carbohydrate and lipid metabolism and thereby affect liver homeostasis (Itoh, 2010; Itoh et al., 2016; Kuro-o, 2019). Recent studies illustrate how FGF15/19 and FGF21 affect hepatic fibrosis and HSC activation. Hepatic accumulation of bile acid is central to the pathogenesis of cholestasis-induced liver injury, and excessive levels of cytotoxic bile acids in the liver can lead to liver fibrosis (Schaap et al., 2014). Expression of FGF15/19 is strongly induced by farnesoid X receptor (FXR) in the ileum, and the protein is secreted into the portal blood and transported to the liver, where it represses the expression of CYP7a1, a rate-limiting enzyme in bile acid synthesis, thereby mitigating liver fibrosis (Inagaki et al., 2005; Liu et al., 2020b). While enterocytes of the terminal ileum likely produce most FGF15/19, HSCs express FGFR4, while HSCs secrete FGF19. Enhanced FGF19/FGFR4 signaling blocks HSC proliferation and activation, which may help explain the anti-fibrotic effects of FGF19 observed in previous studies (Zhou et al., 2017; Hirschfield et al., 2019; Tian et al., 2021). FGF15 deficiency inhibits the development of hepatic fibrosis in animal models of NASH or liver fibrosis (Uriarte et al., 2015; Schumacher et al., 2017; Schumacher et al., 2020).

These findings suggest that FGF15/19 exert hepatoprotective effects via a pathway independent of bile acids. In contrast to FGF15/19, FGF21 is expressed predominantly in hepatocytes and is released in response to high levels of glucose and free fatty acids as well as low levels of amino acids. In this way, FGF 21 can prevent fatty liver, hepatic steatosis and hepatotoxicity (Reinehr et al., 2012; Seitz and Hellerbrand, 2021). FGF21 also inhibits HSC activation via TGF-β and NF-κB pathways, and it can induce HSC apoptosis through caspase-3, which attenuates hepatic fibrogenesis (Xu et al., 2016). FGF21 may participate in metabolism-related liver disease.

Gas6 Signaling

Growth arrest-specific gene 6 (Gas6), a ligand of the TAM receptor (Tyro3, Axl, Mer), is a vitamin K-dependent protein expressed primarily by Kupffer cells, whereas Axl is found in both macrophages and quiescent HSCs in normal liver (Bárcena et al., 2015; Shrivastava et al., 2016). Serum levels of Gas6 correlate directly with liver stiffness and are significantly higher in patients with advanced fibrosis and primary biliary cholangitis (Bellan et al., 2016; Hayashi et al., 2020). In fact, the Gas6/TAM system has recently emerged as an important player in the progression of liver fibrosis and as a novel biomarker of liver fibrosis (Bellan et al., 2019; Smirne et al., 2019). CCl_4-induced liver fibrosis activates the Gas6/Axl pathway, which in turn promotes HSC activation. Disrupting the pathway through Gas6 deficiency, Axl knockout or pharmacological inhibition attenuates hepatic fibrosis (Lafdil et al., 2006; Fourcot et al., 2011; Bárcena et al., 2015). Similarly, the rs4374383 polymorphism in the gene encoding Mer modulates HSC activation, affecting the severity of fibrosis in NAFLD (Petta et al., 2016). Gas6 also participates in both cardiac and pulmonary fibrosis by binding TAM (Espindola et al., 2018; Chen et al., 2019b; Li et al., 2019b). Targeting Gas6 signaling may be a potential treatment for liver fibrosis, so how Gas6 contributes to liver fibrosis should be further explored.

Ferroptosis Signaling

Ferroptosis is a recently recognized form of regulated cell death, characterized by the presence of unusually small mitochondria with quite dense mitochondrial membrane, loss of mitochondria crista, rupture of the outer mitochondrial membrane, and accumulation of iron-based lipid reactive oxygen species (Xie et al., 2016). Ferroptosis is a defensive mechanism against cancer, neurotoxicity and ischemia/reperfusion-induced injury (Liang et al., 2019; Li et al., 2020c; Song and Long, 2020). In mice, an MCD diet induces iron accumulation, cell death and hepatic ferroptosis. Conversely, ferroptosis inhibitors alleviate MCD-diet induced inflammation, fibrogenesis and liver injury, suggesting an important role of ferroptosis in NASH (Li et al., 2020d). Ferroptosis is also an iron-dependent form of regulated cell death triggered by toxic lipid peroxidation, which is inhibited by glutathione peroxidase 4 (GPX4) in steatohepatitic liver (Qi et al., 2020).

Ferroptosis is now considered a new strategy for inhibiting HSCs to alleviate liver fibrosis (Zhang et al., 2018; Zhang et al., 2020a; Zhang et al., 2020b). How ferroptosis is regulated in HSCs remains unclear, although the RNA-binding protein ELAVL1/HuR, ZFP36/TTP, iron regulatory protein2 and the BRD7-p53-SLC25A28 complex appear to be involved. Ferroptosis can be trigged by inhibiting GPX4 (e.g., altretamine), inhibiting system Xc- (e.g., sorafenib, erastin, and sulfasalazine), depleting glutathione (e.g., BSO), or applying certain environmental conditions (e.g., high extracellular glutamate, amino acid starvation, cystine deprivation) (Alim et al., 2019). Thus, these treatments may be effective against liver fibrosis.

cAMP-PKA-cAMP-Responsive Element-Binding Signaling

Cyclic cAMP (cAMP) is well-known as an antifibrogenic second messenger (Li et al., 2019c). The downstream cAMP-responsive element-binding (CREB) protein is a nuclear protein that binds to the cAMP-responsive element (CRE) in the promoter of the gene encoding neuropeptide, and CREB has been implicated in HSC activation and liver fibrosis (Cui et al., 2021). CREB-1, following its activation by phosphorylation, inhibits HSC proliferation and collagen expression *in vitro* (Deng et al., 2011), and it is involved in TGF-β3 auto-regulation in HSCs (Houglum et al., 1997). Phosphorylation or acetylation of CREB-1 in rat HSCs inhibits the TGF-β1 pathway, downregulating collagen I (Deng et al., 2016).

In contrast to these studies suggesting that CREB-1 can inhibit fibrosis, some studies indicate that it can promote hepatic fibrosis. One study, for example, suggested that phosphorylated CREB-1 promotes fibrosis by transactivating TGF-β1 expression (Wang et al., 2016b). Acetaldehyde can activate HSC-T6 cells, while caffeine can act via the adenosine A_{2A} receptor to inhibit the cAMP/PKA pathway and thereby suppress such activation (Wang et al., 2015). Blocking the interaction between CREB and β-catenin using the selective inhibitor PRI-724 reduces liver fibrosis induced by CCl_4 or bile duct ligation (Osawa et al., 2015).

The potentially opposite effects of CREB in different cellular contexts suggest its complex involvement in liver fibrosis, which requires further investigation. Many studies have detected interaction between cAMP-PKA-CREB signaling and pathways mediated by TGF-β and Wnt. This may be a fruitful direction for future research. The molecules currently known to interact with CREB and to show therapeutic potential against liver fibrosis inhibit cAMP-PKA-CREB signaling. These molecules include ICG-001, a selective inhibitor of the CBP/β-catenin interaction (Henderson et al., 2010); PRI-724, which is phosphorylated on C-82 and is rapidly hydrolyzed *in vivo* into its active form, which shows acceptable toxicity and efficacy in preclinical studies (Lenz and Kahn, 2014; Osawa et al., 2015), and caffeine (Wang et al., 2015).

Cellular Metabolism

Under normal circumstances, HSCs are in a resting state and show low metabolism. Their main function is to store small vitamin A fat droplets, which contain more than 70% of vitamin A in the body (Puche et al., 2013). Pro-inflammatory and pro-fibrotic cytokines can activate HSCs to release their stored vitamin A, proliferate, and produce ECM, with the cells adopting a high metabolic state (Chen et al., 2012; Higashi et al., 2017). In this way, HSCs undergo dramatic metabolic changes to meet the increased bioenergetic and biosynthetic demands of mitogenesis and ECM synthesis (Xie et al., 2015; Para et al., 2019; Zhao et al., 2020; Hewitson and Smith, 2021). These metabolic changes are often accompanied by increased glycolysis and mitochondrial respiration in order to optimize glucose consumption in HSCs and redirect them to support fibrogenic *trans*-differentiation (Lian et al., 2016).

Recent studies suggest that by changing the metabolism of activated HSCs, they can be converted into the resting type, offering opportunities for liver fibrosis treatment. In diseased livers of animals and patients, the number of glycolytic stromal cells is associated with the severity of fibrosis. Glycolysis is upregulated and lactate accumulates in quiescent HSCs that have been activated to become myofibroblasts. Hedgehog signaling regulates glycolysis to control the fate of HSCs (Chen et al., 2012). Increased aerobic glycolysis alone cannot meet the high metabolic demands of active HSCs: it works together with glutaminolysis (conversion of glutamine to α-ketoglutarate) to sustain energy metabolism and permit anabolism, and this is controlled by hedgehog signaling to YAP (Du et al., 2018). *In vivo*, glutaminolysis in HSCs is a marker of active fibrogenesis, and its cell-specific antagonism represents a potential therapeutic target by depriving the cells of glutamine (Du et al., 2020). Acetyl-CoA carboxylase (ACC), a regulator of fatty acid β-oxidation and *de novo* lipogenesis, has been implicated in metabolic reprogramming during HSC activation. ACC inhibitors prevent the *de novo* lipogenesis that is necessary for induction of glycolysis and oxidative phosphorylation during HSC activation, and such inhibitors thereby mitigate fibrosis (Bates et al., 2020). In addition to elevated levels of glutamine, elevated levels of fructose can increase risk of liver fibrosis (Song et al., 2019; Roeb and Weiskirchen, 2021). More studies are needed that explore metabolic regulation of HSCs, since this is a promising therapeutic strategy against liver fibrosis (Trivedi et al., 2021).

Epigenetics

With the rise of advanced molecular methods, studies have begun to describe the epigenetic landscape of liver fibrosis, involving changes in DNA methylation, histone modifications and levels of non-coding RNAs that control chromatin structure and DNA accessibility to the transcriptional machinery. DNA methylation is carried out by three enzyme: DNA methyltransferase 1 (DNMT1), DNMT3A and DNMT3B (Jin et al., 2011). MCP2 influences methylation of the gene encoding PPARγ, leading to its silencing, which in turn promotes HSC activation (Mann et al., 2010). DNMT1 and DNMT3B methylates the genes encoding regulator of calcineurin 1 (RCAN1), prostacyclin synthase (PTGIS), Septin9 and SAD1/UNC84 domain protein-2 (SUN2), promoting HSC activation and liver fibrosis (Wu et al., 2017; Chen et al., 2018; Pan et al., 2018; Pan et al., 2019). These findings suggest that gene methylation is important for HSC activation. In fact, DNA methylation affects other epigenetic process, including the expression and activity of long non-coding RNAs, which in turn influence HSC activation and fibrosis. One example is the DNMT1-LncRNA H19 epigenetic pathway, which is involved in HSC activation and liver fibrosis (Yang et al., 2018a). Hypermethylation of the gene encoding PSTPIP2 not only activates HSCs but also polarizes macrophages in mice with CCl_4-induced hepatic fibrosis (Yang et al., 2018b).

It is not surprising, then, that the various histone modifications, which include methylation, acetylation, phosphorylation, ubiquitination, deamination, and sumoylation, are considered targets for fibrosis treatment (El Taghdouini and van Grunsven, 2016). For example, the enhancer of zeste homologue 2 (EZH2), which is responsible for the trimethylation of histone 3 at lysine 27(H3K27me3), is involved in TGF-β dependent fibrogenic pathways (Martin-Mateos et al., 2019). EZH2 and the demethylase JMJD3 regulate HSC activation and liver fibrosis (Jiang et al., 2021). Histone deacetylases 1/2 (HDAC1/2) may regulate liver fibrosis and may therefore be therapeutic targets (Liu et al., 2021; Zhu et al., 2021). Many genes are regulated through cross-talk between histone and DNA mehyltransferases such as G9a and DNMT1. CM272, a first-in-class reversible inhibitor of G9a and DNMT1, can halt fibrogenesis without causing toxic effects (Barcena-Varela et al., 2021).

Numerous non-coding RNAs such as microRNAs and long non-coding RNAs play important roles in liver fibrosis. For example, miR-199a, miR-200a/b, miR-122, miR-194/192, miR-223, miR-21, miR-155 and miR-29 are expressed or enriched in several types of hepatic cells or in the circulation specifically in the presence of liver disease, implying that they play important roles in pathogenesis (Murakami et al., 2011; Wang et al., 2021). Nanoparticle-based delivery of miR-30c to LSECs inhibits the DLL4/Notch pathway and angiogenesis, ameliorating liver fibrosis *in vivo* (Gu et al., 2021). The lncRNA-ATB is upregulated in fibrotic liver tissues and activated LX-2 cells. Knockdown of lncRNA-ATB downregulates β-catenin by upregulating the endogenous miR-200a and suppressing activation of LX-2 cells (Fu et al., 2017). HOTAIR act as an endogenous "sponge" for miR-148b to facilitate expression of DNMT1, which in turn promotes HSC proliferation and activation (Bian et al., 2017).

Several inhibitors of DNA methylation (e.g., 5-azadC, Sennoside A) and histone modifications (e.g., givinostat, DZNep, GSK-503, GSK-J4) as well as epigenetic inhibitors such as CM272 have shown promise for treating liver fibrosis (Yang et al., 2013; Martin-Mateos et al., 2019; Zhu et al., 2020; Barcena-Varela et al., 2021; Ding et al., 2021; Huang et al., 2021; Jiang et al., 2021). Epigenetic biomarkers may be useful not only as treatment targets but also for assaying in tissue and liquid biopsies in order to predict prognosis of patients with liver fibrosis. For example, the levels of H3K27ac in specific oncogenes and of TS, PPARγ-mediated DNA methylation have been suggested for this purpose (Hardy et al., 2017; Arechederra et al., 2021; Jühling et al., 2021).

CANDIDATE DRUGS DURING CLINICAL TRAILS FOR LIVER FIBROSIS

Recently, with the in-depth understanding of the pathogenesis of liver fibrosis, some new compounds with anti-fibrosis potential have emerged and are in clinical trial (Rotman and Sanyal, 2017; Lambrecht et al., 2020; Attia et al., 2021) (**Table 1**). The therapeutic targets of these compounds contain metabolism, gut-liver axis, inflammation and cell death, which share their effects among whole body. After fully confirming drug's efficacy on liver fibrosis, finding a suitable targeted delivery system for

TABLE 1 | New pharmacotherapeutics with antifibrotic effects currently in clinical trial.

Drugs	Mechanism	Therapeutic target	Current status
Saroglitazar	PPARα/γ agonist	Metabolism	Phase 2
K-877	PPARα agonist	Metabolism	Phase 2
Elafibranor	PPAR α/δ agonist	Metabolism	Phase 2
IVA337	PPARα/δ/γ agonist	Metabolism	Phase 2
BMS-986036	FGF21 analogue	Metabolism	Phase 2
LIK066	SGLT1/2 inhibitor	Metabolism	Phase 2
GS-0976	ACC1/2 inhibitor	Metabolism	Phase 2
Liraglutide	GLP-1 receptor agonist	Gut-liver	Phase 2
Cotadutide	GLP1/glucagon receptor agonist	Gut-liver	Phase 2
Semaglutide	GLP-1 receptor agonist	Gut-liver	Phase 2
Aldafermin (NGM282)	FGF19 analogue	Gut-liver	Phase 2
Balapectin	Galectin-3 inhibitor	Inflammation	Phase 2
Selonsertib (GS-4997)	ASK1 inhibitor	Cell death	Phase 3
Emricasan	Caspase inhibitor	Cell death	Phase 3
MGL-3196	THR-β agonists	Metabolism	Phase 3
Obeticholic Acid	FXR agonist	Metabolism	Phase 3
Aramchol	Scd-1 inhibitor	Metabolism	Phase 3
Cenicriviroc	CCR2/5 antagonist	Inflammation	Phase 3

drugs may help for its clinical use with better treatment efficacy and lower side effects. The future candidate drugs for liver fibrosis may develop from the novel targets or its combinatorial use.

CONCLUSION

In this review, we have outlined how major types of hepatic cells participate in liver fibrosis, and we have described several novel targets for fibrosis therapy. Our hope is to provide directions for future investigations.

Early research on the mechanism of fibrosis has focused mainly on HSC activation and collagen deposition. More recent research has focused on cellular state and processes, including metabolism, HSC proliferation and apoptosis, and epigenetic modifications. These studies have broadened our understanding of the pathogenesis of fibrosis, and have pointed out new directions for research into anti-fibrotic drugs. The many in-depth studies on the pathogenesis of liver fibrosis have identified novel signaling pathways as well as signaling crosstalk between TGF-β and Wnt/β-catenin, TGF-β and hedgehog, or YAP and hedgehog.

These studies will gradually build a complete picture of the pathogenesis of fibrosis and provide new ideas in the search for treatment targets. These studies highlight that exploiting crosstalk between signaling pathways may lead to the development of more effective drugs against liver fibrosis.

AUTHOR CONTRIBUTIONS

JZ and YL wrote the manuscript.; QL and JH contributed to the conclusion and reviewed the manuscript. JH and YL obtained funding. JZ, JH and YL are the guarantors of this work and as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Potential Molecular Targets of Tenofovir Disoproxil Fumarate for Alleviating Chronic Liver Diseases *via* a Non-Antiviral Effect in a Normal Mouse Model

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Accumulating evidence suggests that tenofovir disoproxil fumarate (TDF) can attenuate liver fibrosis directly, the mechanism of which, however, has not been fully elucidated, and there is a paucity of data concerning whether TDF can also mitigate other chronic liver diseases (CLDs). We aimed to identify the molecular targets and potential mechanism of TDF itself in ameliorating CLDs. RNA-sequencing was performed on mouse liver tissues treated with TDF or normal saline. Then the differentially expressed genes (DEGs) were screened, and enrichment analyses of the function and signaling pathways of DEGs were performed with Database for Annotation, Visualization, and Integrated Discovery (DAVID) and Metascape. Next, protein-protein interaction (PPI) networks were constructed and module analyses were utilized to identify significant genes. Subsequently, the DisGeNET platform was used to identify the potential target genes of TDF in mitigating these diseases. Finally, prediction of the transcription factors (TFs) and microRNAs (miRNAs) of the target genes was done to conjecture the underlying mechanism by which TDF relieved CLDs. As a result, a total of 854 DEGs were identified, and the DEGs were involved mainly in "immunity," "inflammation," and "metabolism" processes. In addition, 50 significant genes were obtained *via* PPI construction and module analyses. Furthermore, by means of DisGeNET, 19 genes (*Adra2a, Cxcl1, Itgam, Cxcl2, Ccr1, Ccl5, Cxcl5, Fabp5, Sell, Lilr4b, Ccr2, Tlr2, Lilrb4a, Tnf, Itgb2, Lgals3, Cxcr4, Sucnr1,* and *Mme*) were identified to be associated with nine CLDs. Finally, 34 miRNAs (especially mmu-miR-155-5p) and 12 TFs (especially Nfkb1) were predicted to be upstream of the nine target genes (*Cxcl1, Cxcl2, Ccl5, Ccr2, Sell, Tlr2, Tnf, Cxcr4,* and *Mme*) of TDF in ameliorating CLDs. In conclusion, our study suggests that TDF have the potential to ameliorate CLDs independently of its antiviral activity by affecting the expression of genes involved in hepatic immune, inflammatory, and metabolic processes *via* mmu-miR-155-5p-NF-κB signaling. These findings provided *prima facie* evidence for using TDF in CHB patients with concurrent CLDs.

Keywords: tenofovir disoproxil fumarate, chronic liver diseases, non-antiviral effect, immunity, inflammation, metabolism, miR-155-5p, NF-κB

INTRODUCTION

Tenofovir disoproxil fumarate (TDF), an orally administered ester prodrug of tenofovir, is widely used for effective treatment of hepatitis B virus (HBV) infection (Perry and Simpson, 2009). The REVEAL-HBV study group reported an increased serum level of HBV DNA at baseline to be a strong and independent risk predictor of chronic liver diseases (CLDs) development (Chen, 2006; Iloeje et al., 2006). Numerous studies have shown that TDF can achieve sustained suppression of HBV in the long-term management of chronic hepatitis B (CHB) patients regardless of hepatitis B e antigen's status and ethnicity (Heathcote et al., 2011; Gordon et al., 2013; Marcellin et al., 2019). Meanwhile, long-term studies have demonstrated sustained suppression of HBV replication with TDF to be associated with regression and a reduced risk of CLDs in CHB patients (Marcellin et al., 2013; Liu et al., 2019). Those effects were considered to be due mainly to reduced hepatic damage caused by HBV infection, but the direct non-antiviral effects of TDF might also be involved.

Two abstracts demonstrated that TDF could regress liver fibrosis directly by blocking proliferation (Signal Transduction and Cell Function, 2013) and inducing apoptosis of activated hepatic stellate cells (Abstracts, 2020). Recently, a basic study showed that TDF could attenuate liver fibrosis by upregulating expression of hepatitis C virus's non-structural protein 5A transactivated protein 9 (NS5ATP9), thereby inhibiting TGFβ1/Smad3 and NF-κB/NLRP3 signaling pathways (Zhao et al., 2020). However, the mechanism by which TDF mitigates liver fibrosis has not been elucidated fully. Furthermore, there are no data suggesting whether TDF can also alleviate other CLDs independently of its antiviral activity.

We wished to explore the potential mechanism and molecular targets of TDF in improving CLDs. Hence, we undertook RNA-sequencing (RNA-seq) on the liver tissues of wild-type mice treated with TDF and employed an integrated bioinformatic analysis.

MATERIALS AND METHODS

TDF

TDF was kindly gifted by Guangshengtang Co., Ltd (Fujian, China). The purity of TDF was 99.5%.

Animals and TDF Treatment

The study protocol was approved by the Animal Protection Organization and Ethics Committee of Chongqing Medical University (Chongqing, China). Female C57BL/6J mice (8 weeks) from the Animal Center of Chongqing Medical University were housed in a room with a 12-h light and dark cycle at 22°C with free access to mouse chow and water. After 1 week of acclimatization, mice were divided randomly into two groups. Mice in the TDF group were administered with TDF solution (455 mg of TDF powder + 0.5 g of sodium carboxymethyl cellulose +100 ml of normal saline were mixed thoroughly with a homogenizer until the solution was transparent) at 45.5 mg/kg/day by oral gavage for 4 months. Dose determination of TDF was performed *via* conversion of human equivalent doses to murine doses based on the body surface area (Reagan-Shaw et al., 2008; Ng et al., 2015). Mice in the control group received an equivalent volume of vehicle (100 ml of normal saline + 0.5 g of sodium carboxymethyl cellulose were mixed thoroughly with a homogenizer until the solution was transparent) for 4 months. Each mouse was weighed once a week. At study termination, mice were killed after 12 h of fasting. Blood samples were taken by excising the eyeballs. The liver was collected and weighed for subsequent experiments. A flowchart of this study is shown in **Figure 1**.

Biochemical Parameters

Serum was collected after centrifugation at 1,000 rpm for 15 min. Serum ALT levels were measured in the Clinical Laboratory, The Second Affiliated Hospital, Chongqing Medical University.

Hematoxylin and Eosin Staining

Liver tissues of mice were fixed in 4% paraformaldehyde, embedded in paraffin, sliced, and stained with hematoxylin and eosin (H&E).

Isolation and Sequencing of RNA

According to manufacturer instructions, TRIzol® (Invitrogen, United States) was used to extract total RNA from the liver. Then a bioanalyzer (2,200 series; Agilent Technologies, United States) was used to evaluate the concentration, purity, and quality of RNA. Then RNA was sequenced using the DNBseq platform in BGI (Shenzhen, China).

Differential Gene Expression

Low-quality reads, adaptor reads, and reads with > 10% unknown bases (poly-N) were removed from raw data to obtain high-quality "clean" reads for subsequent analyses. The Q20 (percentage of bases with a quality value ≥ 20) and Q30 content of clean data were also calculated. Clean reads were mapped to the *Mus musculus* reference genome (GRCm38.p6) using HISAT2 (http://daehwankimlab.github.io/hisat2/) (Kim et al., 2015). Expression was calculated by RSEM (Li and Dewey, 2011) and represented in fragments per kilobase per million (FPKM) reads. Differential gene expression was identified using the "DEGseq" package with R (R Institute for Statistical Computing, Vienna, Austria) (Wang et al., 2010). The absolute value of fold change (FC) ≥ 2 and adjusted *p*-value (Q-value) ≤ 0.001 were adopted as criteria for determining the significance of differential expression of a particular gene.

Functional Enrichment Analyses of DEGs *via* DAVID and Metascape

The Gene Ontology (GO) database and Kyoto Encyclopedia of Genes and Genomes (KEGG) database were employed to identical enrichment of function and signaling pathways, respectively, based on Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) (Huang et al., 2009). Following the instructions of the DAVID

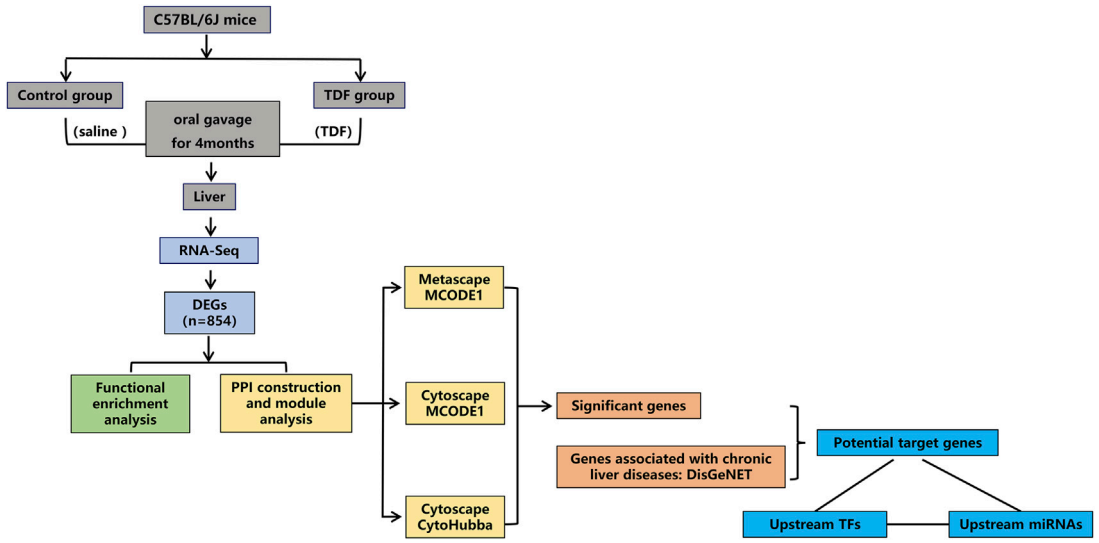

FIGURE 1 | Flowchart of this research.

manual, first, we clicked on the "Start Analysis" on the Internet website. Second, we entered the DEGs list, selected identifiers as "entrez gene ID," selected list types as "gene lists," and submitted lists. Third, we chose to limit annotations and background by *M. musculus*. Finally, the enrichment results of GO and KEGG databases were presented. $p < 0.05$ and gene count ≥ 2 were considered significant.

Furthermore, additional analyses of enrichment of function signaling pathways were done using Metascape (https://metascape.org/gp/index.html#/main/step1/) (Zhou et al., 2019). First, we pasted the gene list as "entrez gene ID." Second, we chose to input the species as *M. musculus*. Third, we clicked on "Express Analysis." Finally, analyses of enrichment of function and signaling pathways were carried out with the following ontology sources: Biological Process (BP) within the GO database, KEGG Pathway, Reactome Gene Sets, CORUM, TRRUST, PaGenBase, WikiPathways, and PANTHER Pathway. Terms with $p < 0.01$, minimum count of 3, and enrichment factor >1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) were collected and grouped into clusters based on their membership similarities.

Construction of Protein-Protein Interaction (PPI) Networks, Significant Modules, and a "Hub Gene" Network

First, Metascape was utilized to construct a PPI network and identify the significant modules. Besides, the Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org/), a user-friendly online system that provides predicted and experimental interactions of proteins (Szklarczyk et al., 2019), was used to establish a PPI network of DEGs with a confidence score ≥ 0.7 for significant differences. Then the PPI network was visualized using Cytoscape 3.6.1 (www.cytoscape.org) (Shannon, 2003). Molecular Complex Detection (MCODE) 1.5.1 (a plugin of Cytoscape) (Bader and Hogue, 2003) was used to screen and identify the most significant modules in the PPI network. MCODEs were extracted when the node score cutoff was 0.2 and K-core was 2. cytoHubba (a plugin of Cytoscape) was employed to calculate the properties of the network topology for nodes to identify hub genes with a degree ≥ 10. The "degree" indicates the number of edges connected with a specific node. Nodes with a high degree are identified as hub genes (i.e., may contribute to vital biological behaviors).

Identification and Analyses of Significant Genes

A Venn diagram was delineated to identify significant union genes among "Metascape_MCODE," "Cytoscape_MCODE," and "Cytoscape_cytoHubba" by Bioinformatics (www.bioinformatics.com.cn/), an online platform for the analyses and visualization of data. Summaries for the basic information of the significant genes were obtained *via* Mouse Genome Informatics (www.informatics.jax.org/). A hierarchical clustering heatmap of significant genes was plotted by using OriginPro 2021 based on gene expression, and classified by the biological function of genes. Correlation analyses among significant genes were achieved through Pearson's correlation test.

Identification of Potential Target Genes of TDF for Ameliorating CLDs

DisGeNET 7.0 (www.disgenet.org/) is a discovery platform containing one of the largest publicly available collections of genes and variants associated to human diseases (Piñero et al., 2019). The current version of DisGeNET contains 1,134,942 gene–disease associations (GDAs), between 21,671 genes and

30,170 diseases, disorders, traits, and clinical or abnormal human phenotypes. The relationships between the significant genes and nine common CLDs were analyzed *via* this tool. First, we entered the name of the diseases in the "Search" box. Subsequently, the summary of the GDA score and evidence for GDAs were presented, and the results were downloaded. Finally, the target genes associated with CLDs were identified from the results.

Prediction of Transcription Factors (TFs) and MicroRNAs (miRNAs) and Construction of TF-miRNA Co-regulatory Networks of Target Genes

To further explore how TDF improves CLDs, Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST 2, https://www.grnpedia.org/trrust/), a database containing 6552 TF–target interactions for 828 mouse TFs (Han et al., 2018), was employed to predict the TFs of target genes. First, we selected the species as "mouse," and submitted the list of target genes in the bottom panel of the search area titled "Find key regulators for query genes." Then the results were downloaded. Meanwhile, prediction of miRNAs of the target genes and TFs identified from TRRUST was done using DIANA-TarBase v8 (www.microrna.gr/tarbase/), a database that curates experimentally verified miRNA targets manually and contains 665,843 unique miRNA–target pairs (Karagkouni et al., 2018). We defined the species as *M. musculus*, entered the genes and TFs one-by-one, and tabulated the results. Ultimately, pairwise-related genes, TFs, and miRNAs were screened, and Cytoscape was utilized to visualize the TF–miRNA co-regulatory networks of the target genes.

RESULTS

TDF had No Significant Effect on Liver Function of Normal Mice

TDF was administered to mice by oral gavage for 4 months to simulate the long-term use of TDF in humans. Before intervention, there was no significant difference in the body weight (BW) of mice (**Supplementary Figure S1A**) between the control group and TDF group. At study termination, no mice died from TDF administration. Besides, no significant differences were found between the TDF group (n = 9) and control group (n = 11) in BW (**Supplementary Figure S1B**), liver weight (LW) (**Supplementary Figure S1C**), liver index (LW/BW) (**Supplementary Figure S1D**), ALT level (**Supplementary Figure S1E**), and liver histology (**Supplementary Figure S1F**). Taken together, these results indicated that the TDF dose we employed was non-toxic, and had no significant effect on liver function of normal mice.

RNA-Seq and Read Mapping

To explore the transcriptional changes in the liver induced by TDF administration, RNA-seq was done using the liver tissues of the mice: 639.99 million raw reads (**Supplementary Table S1**) were generated. After removing adaptors and low-quality reads, we obtained 616.74 million clean reads, with a high quality of Q30 ≥ 93.36%. Then the trimmed clean reads were mapped onto the *M. musculus* reference genome, and 76.32–81.35% of clean reads were mapped uniquely to the genome (**Supplementary Table S1B**). The uniquely mapped reads were used in all subsequent analyses.

A Total of 854 Annotated Genes Were Identified to be DEGs Induced by TDF in Mouse Livers

A total of 17,199 mRNAs were annotated (**Supplementary Table S2**). DEGseq was employed to screen for DEGs. A total of 1,341 annotated genes were identified to be differentially expressed when considering exclusively a stringent threshold of Q-value ≤ 0.001 and an absolute value of FC ≥ 2, which is presented as a volcano plot (**Figure 2A**). After removing DEGs that caused differences between groups due to abnormal expression within the group, we obtained 854 DEGs eventually (217 downregulated DEGs and 637 upregulated DEGs) (**Figure 2B** and **Supplementary Table S3**). Hence, TDF could affect gene expression in mouse livers directly. To obtain a global view of these 854 DEGs, hierarchical clustering (**Figure 2C**) was done with normalized FPKM values, and indicated that our samples were of "good" quality with gene expression of similar proportion in each group.

Functional Enrichment Analyses Indicated That the 854 DEGs Induced by TDF Were Involved Mainly in "Immunity," "Inflammation," and "Metabolism" Processes

First, enrichment analyses using the GO database and KEGG database were undertaken using DAVID. We discovered that 774 out of 854 profiled DEGs were assigned to 394 GO terms: 264 for biological process (BP), 32 for cellular component (CC), and 98 for molecular function (MF). Variations in DEGs related to BP were involved mainly in "immune system process," "inflammatory response," "lipid metabolic process," and "glucose metabolic process" (**Figure 3A**). With regard to CC, DEGs were significantly enriched in the "extracellular region," "extracellular space," "organelle membrane," and "cell surface" (**Figure 3B**). Variations in DEGs associated with MF were significantly enriched in "small molecule binding," "insulin-activated receptor activity," "iron ion binding," and "chemokine activity" (**Figure 3C**). The KEGG database indicated that 356 out of 854 profiled DEGs were assigned to 46 signaling pathways. The significant pathways relevant to DEGs were immune, inflammatory pathways ("cytokine-cytokine receptor interaction," "NOD-like receptor signaling pathway," "chemokine signaling pathway," and "TNF signaling pathway"), and metabolic pathways ("retinol metabolism," "steroid hormone biosynthesis," "arachidonic acid metabolism," and "glutathione metabolism") (**Figure 3D**). The data of GO and KEGG classifications are shown in **Supplementary Table S4**.

To gain further insight into the functions of DEGs, analyses of enrichment of signaling pathways and function were carried out

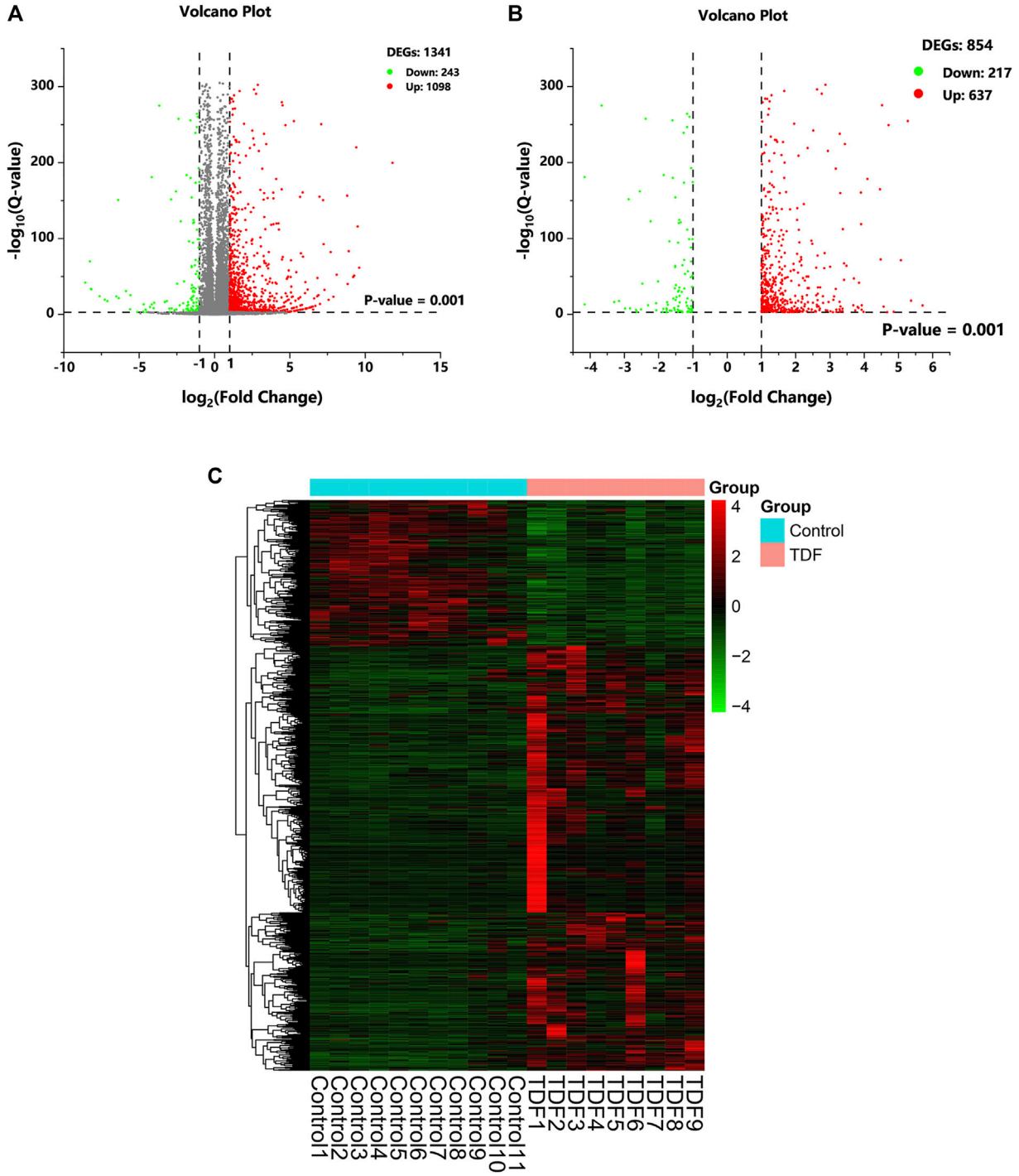

FIGURE 2 | Differentially expressed genes (DEGs) in response to TDF. **(A, B)** The volcano plot of DEGs. The cutoff values fold change ≥ 2 and Q-value ≤ 0.001 were utilized to identify DEGs. Downregulated DEGs are marked in green, upregulated DEGs are marked in red, and unchanged genes are marked in gray. **(C)** Hierarchical clustering of DEGs.

via Metascape. The results (in accordance with the results using DAVID) indicated that the DEGs induced by TDF were significantly enriched in inflammatory processes ("leukocyte migration," "neutrophil degranulation," "acute inflammatory response," "positive regulation of leukocyte migration," and "regulation of interleukin-1 production") and metabolic processes ("retinol metabolism" and "monocarboxylic acid metabolism") ($p < 0.05$, **Figures 3E–G**).

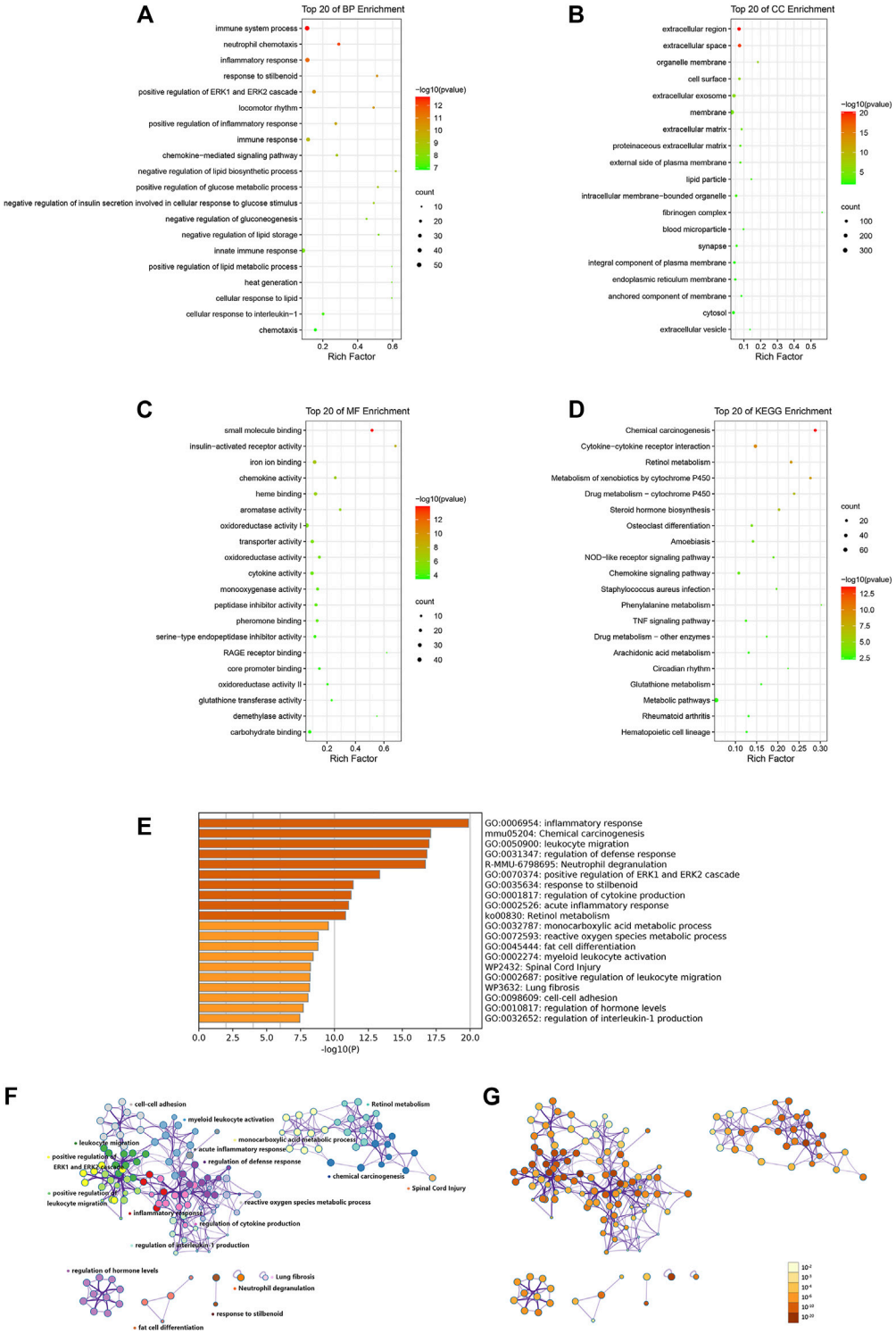

FIGURE 3 | Functional enrichment analyses of DEGs *via* DAVID **(A–D)** and Metascape **(E–G)**. Variations in DEGs associated with **(A)** biological process, **(B)** cellular component, **(C)** molecular function, and **(D)** KEGG analysis. Rich factor is the ratio of the DEG number to the total gene number in a certain pathway. The color and size of the dots represent the range of the p-value and the number of DEGs mapped to the indicated pathways, respectively. **(E)** Bar graph of enriched clusters across inputted DEGs lists, colored by p-values. Network of enriched terms: **(F)** colored by cluster ID, where nodes that share the same cluster ID are typically close to each other, **(G)** colored by p-value, where terms containing more genes tend to have a more significant p-value. The top 20 significant enriched pathways are shown.

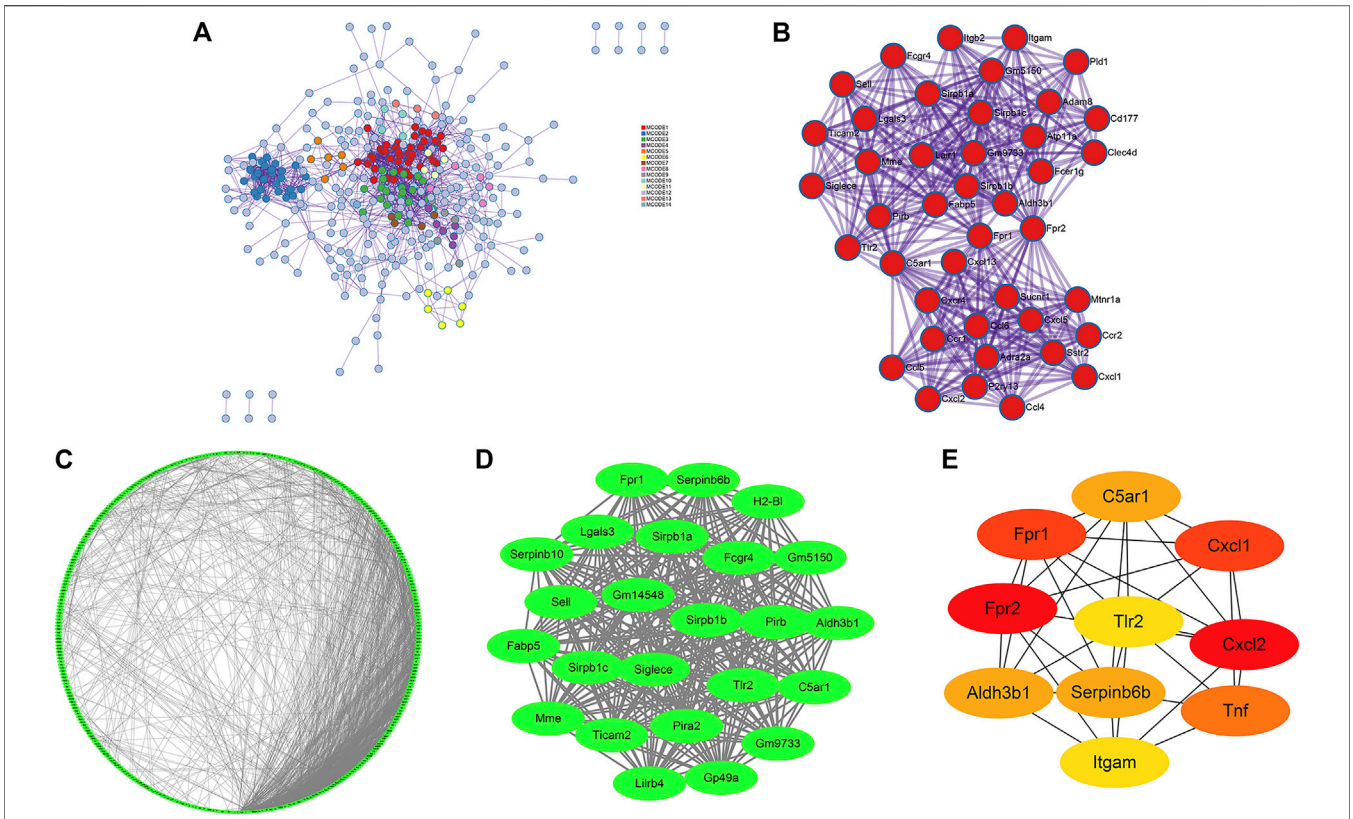

FIGURE 4 | PPI network, significant modules and hub gene networks constructed through Metascape **(A, B)**, STRING **(C)**, and Cytoscape **(D, E)**. **(A)** A PPI network of DEGs including 14 MCODE modules was established *via* Metascape. **(B)** The Metascape_MCODE1 consists of 42 genes. **(C)** A PPI network of DEGs consisting of 1937 edges and 443 nodes was established *via* STRING with a confidence score of ≥ 0.7 for significant differences. **(D)** The Cytoscape_MCODE1 consists of 24 genes. **(E)** Ten genes were identified as hub genes with Cytoscape_cytoHubba.

PPI Construction and Module Analyses Identified 50 Genes as Significant Genes, and These Genes Were Involved Mainly in "Immunity," "Inflammation," and "Metabolic" Processes

First, a PPI network of DEGs was constructed through Metascape (**Figure 4A**). Fourteen MCODE modules were identified from the PPI network. Notably, MCODE1 with the highest score consisted of 42 genes: *Sirpb1c, Gm9733, Sirpb1b, Gm5150, Sirpb1a, Fcgr4, Ticam2, Sucnr1, Siglece, P2ry13, Cd177, Aldh3b1, Cxcl13, Lair1, Atp11a, Tlr2, Sstr2, Sell, Cxcl5, Cxcl2, Ccl6, Ccl5, Ccl4, Pld1, Pirb, Mtnr1a, Clec4d, Mme, Lgals3, Fabp5, Itgb2, Itgam, Cxcl1, Fpr1, Fpr2, Fcer1g, Ccr2, Ccr1, Cxcr4, C5ar1, Adra2a,* and *Adam8* (**Figure 4B**).

Simultaneously, construction of the PPI network was also established by STRING with a confidence score of ≥ 0.7 for significant differences. There were 1937 edges and 443 nodes in the PPI network (PPI enrichment *p*-value <0.001) (**Figure 4C**). Thirty MCODE modules were identified from the PPI network by the Cytoscape_MCODE. In particular, MCODE1 with the highest score comprised 24 genes: *Serpinb6b, Sirpb1c, Pirb, Aldh3b1, Sell, Lilrb4, C5ar1, Fabp5, Pira2, Mme, Tlr2, Siglece, Lgals3, Sirpb1b, H2-Bl, Fcgr4, Sirpb1a, Gp49a, Ticam2, Gm5150, Gm9733, Fpr1, Gm14548,* and *Serpinb10* (**Figure 4D**). With degree ≥ 10 considered as the standard of judgment, 10 genes were identified as hub genes with Cytoscape_cytoHubba: *Fpr2, Cxcl2, Fpr1, Cxcl1, Tnf, C5ar1, Serpinb6b, Aldh3b1, Tlr2,* and *Itgam* (**Figure 4E**).

Finally, a VENN diagram was delineated and showed 50 significant union genes among "Metascape_MCODE1," "Cytoscape_MCODE1," and "Cytoscape_cytoHubba," including four common genes: *Aldh3b1, Tlr2, Fpr1*, and *C5ar1* (**Figure 5A**). The basic information of the 50 significant genes is summarized in **Supplementary Table S5**. Hierarchical clustering indicated that the significant genes could largely differentiate the TDF group from the control group (**Figure 5B**). Moreover, we found that these 50 genes were involved mainly in "immunity" (44%, 22/50), "inflammation" (34%, 17/50), and "metabolic" processes (10%, 5/50), and six genes (12%, 6/50) exhibited other or undefined functions (**Figure 5B**). The Pearson correlation analysis showed a positive correlation among most genes except for *Sucnr1*, *H2-Bl*, *Mme*, and *Cxcl5* (**Figure 5C**).

Nineteen Genes Were Identified to be the Potential Targets of TDF for Alleviating Nine CLDs Directly

The DisGeNET platform was employed to identify the potential targets of TDF for alleviating CLDs. The nine most common

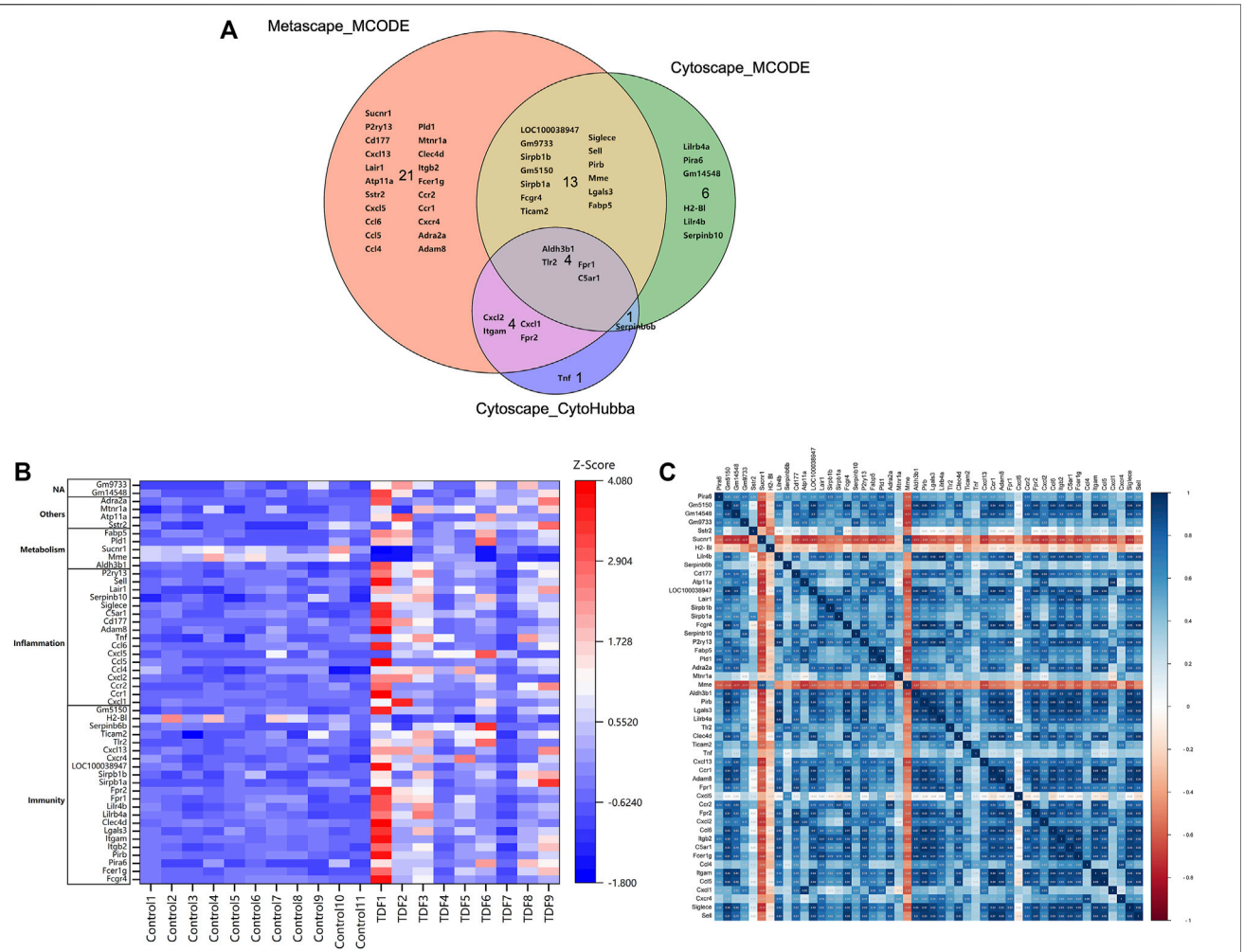

FIGURE 5 | Identification of significant genes. **(A)** A VENN diagram of the 50 significant union genes among "Metascape_MCODE1," "Cytoscape_MCODE1," and "Cytoscape_cytoHubba." **(B)** Hierarchical clustering heatmap and biological functional classification of the 50 significant genes. **(C)** Pearson correlation analysis of the 50 significant genes.

CLDs including non-alcoholic fatty liver disease (NAFLD), cholestasis, primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH), non-alcoholic steatohepatitis (NASH), liver fibrosis (LF), cirrhosis, and hepatocellular carcinoma (HCC) were analyzed. The gene–disease association score (GDAs) between the potential target genes and these CLDs are portrayed in **Figure 6A**. Nineteen genes were associated with these nine CLDs. The number of genes involved in "inflammatory," "immune," and "metabolic" processes was eight (42%), seven (37%), and three (16%), respectively. From the vertical perspective, 10 genes were related to NAFLD: *Tnf, Ccr2, Tlr2, Lgals3, Ccl5*, *Cxcl, Sell, Lilrb4a, Lilr4b,* and *Fabp5*. The four genes relevant to cholestasis were *Tnf, Ccr2, Cxcl2*, and *Mme*. Five genes (*Tnf, Tlr2, Lgals3, Ccl5*, and *Itgb2*) were connected with PBC. In addition, *Tnf* and *Ccr2* were associated with PSC. *Tnf* along with *Tlr2* were related to AIH. Moreover, seven genes were involved in NASH: *Tnf*, *Ccr2*, *Tlr2*, *Lgals3*, *Cxcl5*, *Cxcr4,* and *Adra2a*. Eight genes (*Tnf*, *Tlr2*, *Lgals3*, *Ccl5*, *Cxcl2*, *Cxcr4*, *Ccr1*, and *Sucnr1*) were relevant to LF. Besides, there were nine genes associated with cirrhosis: *Tnf, Ccr2, Tlr2, Lgals3, Ccl5, Cxcl5, Adra2a, Itgam*, and *Cxcl1*. *Tnf* together with *Ccr2* were related to HCC. In addition, according to the GDA score, the gene most associated with PBC was chemokine C-C motif ligand 5 (*Ccl5*), and the gene most closely related to the other eight liver diseases was *Tnf*. From the lateral perspective, we found that tumor necrosis factor (*Tnf*) was related to all of the nine CLDs, with cirrhosis (GDA score: 0.4) and cholestasis (GDA score: 0.34) showing the closest associations. Chemokine C-C motif receptor 2 (*Ccr2*) and toll-like receptor 2 (*Tlr2*) were associated with six CLDs, and galectin 3 (*Lgals3*) was related to five CLDs. The $\log_2$FC of genes indicated that 19 genes consisted of 17 upregulated DEGs and two downregulated DEGs, and the most significantly altered genes were adrenergic receptor alpha 2a (*Adra2a*), chemokine C-X-C motif ligand 1 (*Cxcl1*), and integrin alpha M (*Itgam*), with $\log_2$FC values of 2.84, 2.83, and 2.76, respectively (**Figure 6B**).

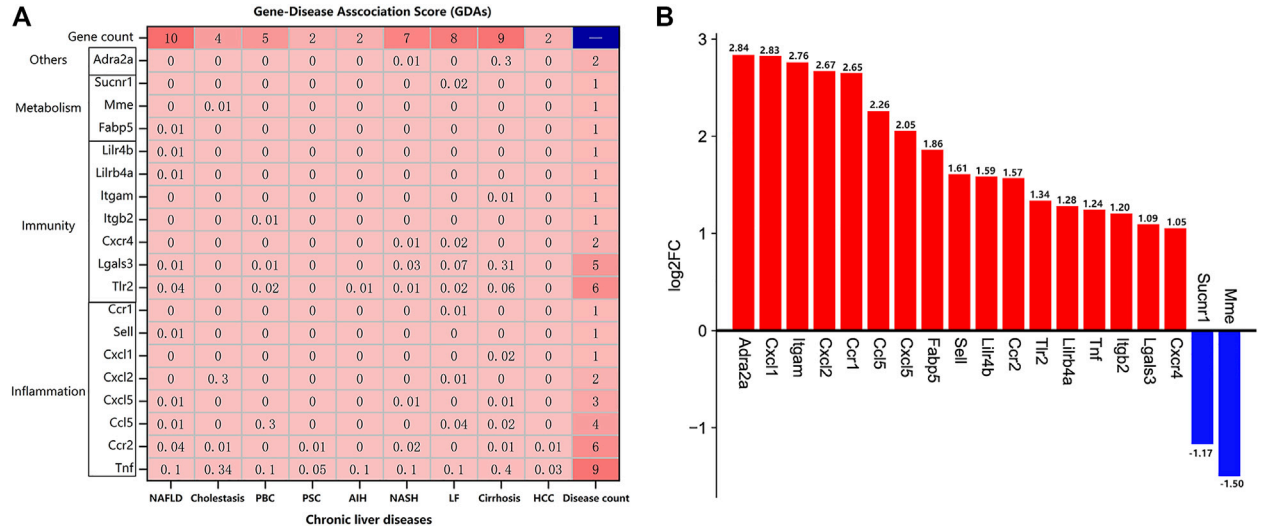

FIGURE 6 | Identification of potential target genes of TDF for direct alleviation of chronic liver diseases (CLDs). **(A)** The gene–disease association (GDAs) score between the 19 potential target genes and nine CLDs. Gene count: the number of genes associated with a certain CLD; disease count: the number of CLDs associated with the corresponding gene. **(B)** The log2FC of the 19 potential target genes using RNA-seq data.

TABLE 1 | Prediction of the transcription factors of target genes *via* TRRUST.

Key TF	Description	Overlapped genes	*p* Value	Q-value	List of overlapped genes	Log_2FC (Q-value)
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105	6	2.04E-08	2.65E-07	Ccl5, Cxcl1, Cxcl2, Itgam, Tnf, Tlr2	0.22 (<0.001)
Ikbkb	Inhibitor of kappaB kinase beta	3	1.51E-07	9.82E-07	Cxcl2, Tnf, Cxcr4	−0.14 (<0.001)
Irf1	Interferon regulatory factor 1	3	1.57E-06	6.79E-06	Ccl5, Tnf, Sell	−0.04 (0.21)
Rela	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	4	4.30E-06	1.40E-05	Ccl5, Cxcr4, Tlr2, Tnf	0.33 (<0.001)
Jun	Jun proto-oncogene	4	5.90E-06	1.53E-05	Ccl5, Cxcl1, Cxcl2, Tnf	0.83 (<0.001)
Ar	Androgen receptor	2	6.41E-05	0.000139	Tnf, Ccr2	−0.30 (<0.001)
Irf8	Interferon regulatory factor 8	2	0.000207	0.000384	Tnf, Ccl5	0.39 (<0.001)
Rel	Reticuloendotheliosis oncogene	2	0.000308	0.000477	Ccl5, Tnf	0.23 (0.29)
Twist1	Twist basic helix-loop-helix transcription factor 1	2	0.000331	0.000477	Tnf, Mme	0.39 (0.06)
Spi1	Spleen focus forming virus (SFFV) proviral integration oncogene	2	0.000631	0.00082	Ccl5, Tnf	0.85 (<0.001)
Foxo1	Forkhead box O1	2	0.000947	0.00112	Sell, Ccr2	−0.26 (<0.001)
Sp1	*trans*-acting transcription factor 1	3	0.00191	0.00198	Sell, Tlr2, Tnf	0.04 (0.22)
Egr1	Early growth response 1	2	0.00198	0.00198	Tnf, Cxcl2	1.75 (<0.001)

The basic information of these genes can be found in **Supplementary Table S5**.

A Total of 34 microRNAs (miRNAs) and 12 Transcription Factors (TFs) Were Predicted to be Upstream of the Nine Potential Target Genes

First, TF–gene analyses were undertaken with TRRUST, and 13 upstream TFs targeting 12 target genes (seven genes had no results) were identified (**Table 1**). Quite specifically, NF-κB family (*Nfkb1*, *Rela*, and *Rel*) were the most significantly enriched TFs and targeted seven genes (*Ccl5*, *Cxcl1*, *Cxcl2*, *Itgam*, *Tlr2*, *Tnf*, and *Cxcr4*). Additionally, the RNA-seq data showed that TDF could also affect expression of *Nfkb1* (log_2FC = 0.22, Q-value <0.001), *Rela* (log_2FC = 0.33, Q-value <0.001), and *Rel* (log_2FC = 0.23, Q-value = 0.29). Overall, these results indicated that NF-κB might be the most critical TF for the genes targeted by TDF to relieve CLDs.

Subsequently, DIANA-TarBase v8 was used to predict the upstream miRNAs of the 19 target genes, and 80 unique miRNAs were identified (**Supplementary Table S6A**). The most pivotal miRNAs are shown in **Table 2**. These results suggested that mmu-miR-155-5p was the most important miRNA and regulated 15 target genes.

Meanwhile, TF–miRNA interactions were predicted for the 13 identified TFs by using DIANA-TarBase, and 156 unique miRNAs were identified (Table S6B). **Table 3** shows the most

TABLE 2 | Prediction of the miRNAs of target genes *via* DIANA-TarBase.

miRNA name	Overlapped genes	List of overlapped genes
mmu-miR-155-5p	15	Cxcl1, Cxcl2, Cxcl5, Cxcr4, Ccl5, Ccr1, Ccr2, Sell, Lgals3, Tlr2, Fabp5, Tnf, Itgb2, Lilr4b, Lilrb4a
mmu-miR-1a-3p	9	Cxcl1, Cxcl5, Cxcr4, Ccl5, Ccr1, Itgb2, Tlr2, Fabp5, Adra2a
mmu-miR-21a-5p	8	Cxcl1, Cxcl2, Cxcl5, Ccr1, Sell, Tlr2, Cxcr4, Tnf
mmu-miR-122-5p	7	Cxcl1, Cxcr4, Ccl5, Ccr1, Tlr2, Sucnr1, Itgb2
mmu-miR-124-3p	6	Cxcl1, Cxcl5, Ccl5, Tlr2, Fabp5, Itgb2
mmu-miR-125b-5p	6	Ccl5, Ccr2, Sell, Adra2a, Cxcr4, Lilrb4a
mmu-miR-223-3p	5	Itgam, Fabp5, Sucnr1, Tnf, Lilr4b
mmu-miR-188-5p	4	Cxcl5, Ccl5, Tlr2, Mme
mmu-miR-196b-5p	4	Ccr2, Lgals3, Tnf, Lilr4b
mmu-let-7g-5p	3	Cxcl1, Cxcl5, Itgb2
mmu-let-7c-5p	3	Itgb2, Lgals3, Adra2a

TABLE 3 | Prediction of the miRNAs of identified transcription factors *via* DIANA-TarBase.

miRNA name	Overlapped TFs	List of overlapped TFs
mmu-miR-155-5p	8	Nfkb1, Jun, Ar, Irf8, Twist1, Spi1, Foxo1, Sp1
mmu-miR-124-3p	8	Nfkb1, Rela, Jun, Rel, Twist1, Foxo1, Sp1, Egr1
mmu-miR-106a-5p	6	Nfkb1, Irf1, Ar, Foxo1, Sp1, Egr1
mmu-miR-17-5p	6	Nfkb1, Irf1, Ar, Foxo1, Sp1, Egr1
mmu-miR-20b-5p	6	Nfkb1, Irf1, Ar, Foxo1, Sp1, Egr1
mmu-miR-1a-3p	6	Nfkb1, Jun, Rel, Twist1, Foxo1, Sp1
mmu-miR-93-5p	5	Nfkb1, Irf1, Foxo1, Sp1, Egr1
mmu-miR-22-3p	5	Nfkb1, Ikbkb, Ar, Foxo1, Sp1
mmu-miR-122-5p	5	Nfkb1, Rela, Jun, Ar, Foxo1
mmu-miR-125b-5p	5	Irf1, Jun, Irf8, Sp1, Egr1
mmu-miR-188-5p	5	Irf1, Rela, Ar, Twist1, Egr1
mmu-miR-26a-5p	5	Rela, Jun, Foxo1, Sp1, Egr1

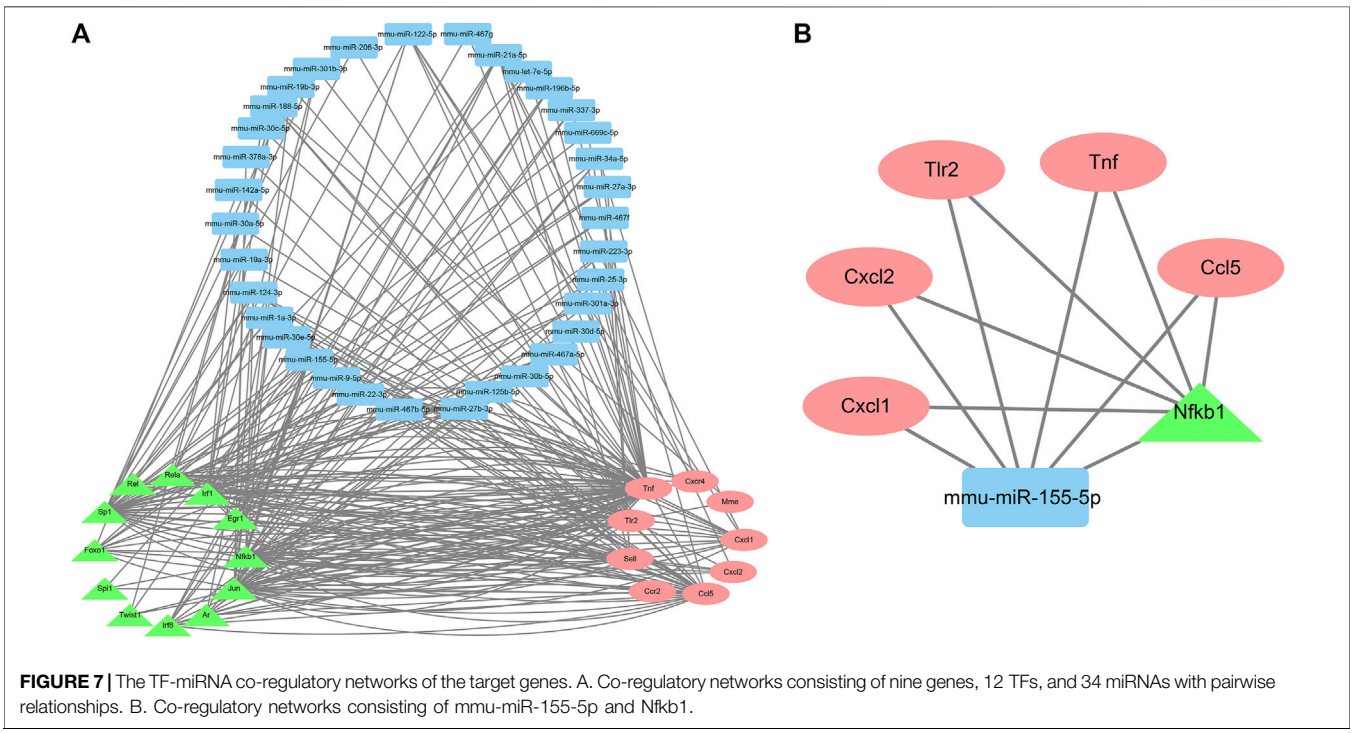

FIGURE 7 | The TF-miRNA co-regulatory networks of the target genes. A. Co-regulatory networks consisting of nine genes, 12 TFs, and 34 miRNAs with pairwise relationships. B. Co-regulatory networks consisting of mmu-miR-155-5p and Nfkb1.

pivotal miRNAs. The results indicated that mmu-miR-155-5p and mmu-miR-124-3p, interacting with eight TFs, might be the most important miRNAs.

Finally, nine genes, 12 TFs, and 34 miRNAs that were pairwise-related were screened out to construct the TF–miRNA co-regulatory networks of the target genes, which consisted of 247 edges and 55 nodes (**Figure 7A**). These results indicated that mmu-miR-155-5p (involved in 15 edges) might be the most important miRNA. The most important TF might be Nfkb1 (19 edges). Their target genes were *Tnf*, *Ccl5*, *Tlr2*, *Cxcl1,* and *Cxcl2* (**Figure 7B**).

Taken together, these results suggested that co-regulatory networks consisting of mmu-miR-155-5p and Nfkb1 might be (at least in part) the underlying mechanisms by which TDF improved CLDs.

DISCUSSION

TDF has been recommended as a first-line oral antiviral agents by the European Association for the Study of the Liver (Lampertico et al., 2017) and American Association for the Study of Liver Diseases (Terrault et al., 2018) for treatment of chronic hepatitis B (CHB) patients due to its high efficacy and genetic barrier. Evidence suggests that CHB patients predispose towards other hepatic comorbidities (Liu et al., 2018; Oh et al., 2020), in addition to viral factors, in which immune, inflammatory, and metabolic disorders also have pivotal roles (Seto et al., 2018). Meanwhile, these chronic liver diseases (CLDs) can in turn affect the disease progression (Yu et al., 2017; Choi et al., 2020; Guo et al., 2020; Bockmann et al., 2021) and the efficacy of antiviral strategies in CHB patients (Jin et al., 2012; Kim et al., 2019). Consequently, we hypothesized that TDF can inhibit the replication of HBV (a major cause of CLDs) and also attenuate CLDs directly by regulating the hepatic immune, inflammatory, and metabolic status of the host. If this is true, then TDF could be a promising antiviral drug for CHB patients with other hepatic comorbidities.

In the current study, RNA-seq of murine livers from the TDF group and control group was carried out and 854 DEGs were identified (**Figure 2**). Analyses of functional enrichment showed that the DEGs were involved mainly in "immunity," "inflammation," and "metabolism" processes (**Figure 3**). Subsequently, 50 genes were screened out as significant genes by PPI construction and module analyses, and participated mainly in "immunity" (44%, 22/50), "inflammation" (34%, 17/50), and "metabolic" processes (10%, 5/50) (**Figures 4**, **5**). Finally, 19 out of the 50 significant genes were identified to be potential target genes of TDF in alleviating nine CLDs directly, and were enriched in a greater proportion of "immunity" (37%, 7/19), "inflammation" (42%, 8/19), and "metabolic" processes (16%, 3/19) (**Figure 6**). Compelling studies have described immunity, inflammation, and metabolism to be closely related in the pathogenesis and progression of CLDs (Marra and Tacke, 2014; Heymann and Tacke, 2016; Tang et al., 2020; Ahmed et al., 2021). In addition, treatment strategies that synergistically affect hepatic immune, inflammatory, and metabolic states can regress CLDs (Hegade et al., 2016; Chen et al., 2019). Given that TDF could affect hepatic immune, inflammatory, and metabolic processes directly, one can speculate that TDF had the potential to alleviate CLDs independently of its antiviral activity.

Furthermore, it is well known that transcription factors (TFs) and microRNA (miRNAs) can jointly regulate target gene expression and contribute to multiple biological processes and different diseases. Notably, the mmu-miR-155-5p-NF-κB signaling pathway may have critical roles in CLDs by regulating expression of the genes involved in immune, inflammatory, and metabolic processes (Wang et al., 2009; Bala et al., 2011; Yuan et al., 2016; Qian et al., 2019; Ali et al., 2021). In this study, we predicted the upstream TFs and miRNAs of the 19 target genes. Then we screened nine genes, 12 TFs, and 34 miRNAs with pairwise relationships to construct the TF–miRNA co-regulatory networks of the target genes (**Figure 7**). mmu-miR-155-5p and Nfkb1 were identified to be the most important upstream moieties of these target genes. Hence, TDF might be able to attenuate CLDs by affecting hepatic immune, inflammatory, and metabolic processes by mmu-miR-155-5p-NF-κB signaling.

Our study had three main limitations. First, the enrichment analyses (using GO and KEGG databases) used in our study are based on the theory of over-representation analysis (ORA). This method considers only the DEGs list regardless of the expression and change in trends of DEGs, which may cause bias to some extent. However, considering that our study was qualitative, ORA is sufficient. Of course, the gene set enrichment analysis (GSEA) would be recommended for further studies. Second, only the direct effects of TDF on the liver were studied; other nucleoside/nucleotide analogs were not investigated. Hence, whether the direct ameliorative potential of TDF upon CLDs was unique to TDF or shared by all nucleoside/nucleotide analogs is not known. Future studies on ETV are needed to confirm this question. Third, the conclusions of our study are mainly from observations in immunocompetent mice with normal liver function, so inevitably the results will be a little overstated. Despite its descriptive nature, this study provides preliminary evidence that TDF affect the expression of genes associated with CLDs. Of course, we must verify the target genes and miRNAs by building corresponding disease models through *in vitro* and *in vivo* experiments. If we want to further translate our results to humans, chimeric mice with humanized liver is recommended to help us clarify the mechanism by which TDF improves a specific liver disease.

In conclusion, we report, for the first time, the hepatic transcriptional changes induced by TDF in healthy mice. Our findings indicate that TDF could ameliorate CLDs independently of its antiviral activity by influencing expression of the genes involved in hepatic immune, inflammatory, and metabolic processes *via* mmu-miR-155-5p-NF-κB signaling.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/ PRJNA763152.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Protection Organization and Ethics Committee of Chongqing Medical University for humanistic care.

AUTHOR CONTRIBUTIONS

YD designed the study, undertook the data processing, and drafted the original manuscript. ZC carried out the experiments. HL, WS, and YZ provided experimental guidance and technical support. MP modified the original draft of the manuscript. PH revised the manuscript and supervised the entire process. All authors contributed to this study and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.763150/full#supplementary-material

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REVIEW
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The Role of Notch Signaling Pathway in Non-Alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, and progressive NAFLD can develop into non-alcoholic steatohepatitis (NASH), liver cirrhosis, or hepatocellular carcinoma (HCC). NAFLD is a kind of metabolic disordered disease, which is commonly associated with lipid metabolism, insulin resistance, oxidative stress, inflammation, and fibrogenesis, as well as autophagy. Growing studies have shown Notch signaling pathway plays a pivotal role in the regulation of NAFLD progression. Here, we review the profile of the Notch signaling pathway, new evidence of Notch signaling involvement in NAFLD, and describe the potential of Notch as a biomarker and therapeutic target for NAFLD treatment.

Keywords: Notch signaling pathway, non-alcoholic fatty liver disease (NAFLD), steatohepatitis, lipid metabolism, insulin resistance (IR), fibrogenesis, autophagy

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), affecting over a quarter of the global population, has emerged as the highest prevalent type of chronic liver disease (Younossi et al., 2016). NAFLD encompasses a spectrum of progressive liver diseases including simple steatosis (SS), non-alcoholic fatty steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (D. Q. Huang et al., 2021; Powell et al., 2021). NAFLD is defined by the presence of steatosis in >5% of hepatocytes in histological analysis and exclusion of excessive alcohol consumption daily (≥30 g for men and ≥20 g for women) (EASL et al., 2016). Evidence suggests that NAFLD is related to liver manifestations of metabolic syndrome such as obesity, diabetes, insulin resistance (IR), and dyslipidemia (Younossi et al., 2018; Jarvis et al., 2020).

The individual clinical outcomes of patients with NAFLD are highly variable. For the majority of patients with simple steatosis, their liver disease is in non- or slow-progression. A prospective cohort study reported in a three-year period, over 20% of patients with simple steatosis developed into NASH (Wong et al., 2010), a more severe stage in which fatty liver is accompanied by necroinflammatory changes like hepatocyte ballooning and lobular inflammation (Vernon et al., 2011). In the final stages, collagen deposition and subsequent vascular remodeling result in fibrosis and cirrhosis (EASL et al., 2016). Thus far, there is no accurate non-invasive diagnostic biomarker and effective treatment toward NAFLD (Francque and Vonghia, 2019; Younossi, 2019), and current therapy is mainly focused on lifestyle changes (EASL et al., 2016; Chalasani et al., 2018).

Studies have shown that NAFLD is mainly characterized by hepatocyte inflammation and steatosis in the early stage and fibrosis and/or cirrhosis in the late stage (Wang et al., 2020). However, the pathogenesis of NAFLD has not been fully understood. In 1998, scientists first proposed the "two-hit" hypothesis to explain that steatosis (the first "hit") and other factors associated with free radicals (the second "hit") are necessary for NASH progression (Day and James, 1998). In recent years, based on animal models and descriptive clinical trials, the "multiple hits" hypothesis is widely accepted (Tilg and Moschen, 2010; Buzzetti et al., 2016; Tilg et al., 2021). The primary hit is the infiltration and pro-inflammatory state of macrophages in the visceral adipose

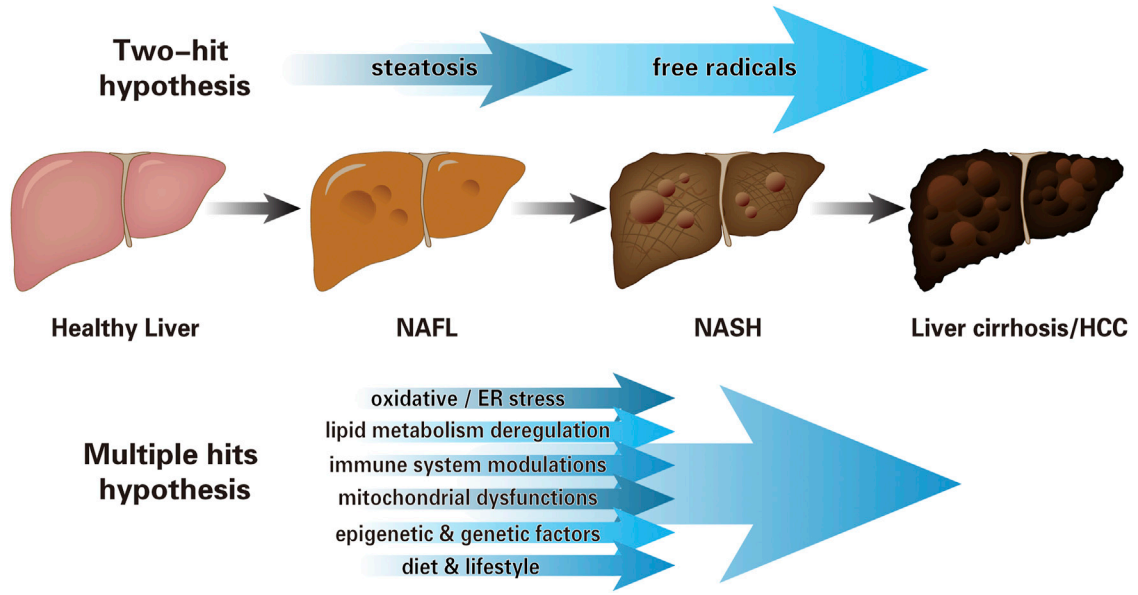

FIGURE 1 | The pathogenesis of NAFLD. Schematic representation illustrating cognition toward the progression of NAFLD: from "two-hit" hypothesis to "multiple hits" hypothesis. The early "two-hit" hypothesis, in which the first "hit" is steatosis, leads to the second "hit": oxidative stress, endotoxin, etc. The "multiple hits" hypothesis considers several parallel hits jointly affect the NAFLD pathogenesis, which includes, but is not limited to oxidative and/or ER stress, lipid metabolism deregulation, immune system modulations, mitochondrial dysfunctions, lifestyle, and epigenetic and genetic factors (NAFL, non-alcoholic fatty liver, i.e., simple steatosis without hepatocellular injury; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; ER, endoplasmic reticulum).

tissue, resulting in IR. Meanwhile, the abnormal lipolysis increases the delivery of fatty acids to the liver, and along with steatosis, aggravates the lipid metabolic burden. The imbalance results in the formation of lipotoxic lipids that generate a series of multiple hits, including oxidative and/or endoplasmic reticulum (ER) stress, inflammasome activation, and apoptotic damage, followed by inflammation, tissue regeneration, and fibrogenesis (Tilg and Moschen, 2010; Bessone et al., 2019; Sanyal, 2019). Besides, mitochondrial dysfunctions, lifestyle, and epigenetic and genetic factors also jointly affect the occurrence and progression of the NAFLD (Loomba et al., 2021) (**Figure 1**).

Notch signaling pathway plays a crucial role in cell differentiation (Amsen et al., 2009; Amsen et al., 2015), proliferation (Bartolome et al., 2019), and apoptosis (Guruharsha et al., 2012). Recently, it has also been demonstrated that Notch is involved in liver development, homeostasis, and metabolism (Bi and Kuang, 2015; Geisler and Strazzabosco, 2015; Adams and Jafar-Nejad, 2019). However, the association of the Notch signaling with NAFLD has rarely been reported. Here we review the recent advances in Notch signaling in liver pathophysiology and analyze the Notch signaling pathway as a potential target to prevent and treat NAFLD.

OVERVIEW OF NOTCH SIGNALING PATHWAY

Notch signaling is a juxtracrine signal transduction mechanism that enables cell-cell communication directly (Artavanis-Tsakonas et al., 1999). In mammals, four receptors (Notch1-4) and five ligands [Jagged (JAG) 1-2, Delta-like ligand (DLL) 1, 3, and 4] have been identified in canonical Notch signaling (D'Souza et al., 2010). In the liver of adults, four Notch receptors are expressed, while only two Notch ligands (JAG1 and DLL4) are expressed (Y. Chen et al., 2012). The ligand-receptor interaction is the initiation of Notch signaling pathway, making various cellular regulations more precise and orderly (Bray, 2016).

The core signaling pathway most commonly used to describe Notch-dependent processes is named the canonical Notch signaling pathway (Andersson et al., 2011; Guruharsha et al., 2012). The ligand presented by the Notch signal sending cell binds to the receptor on the signal-receiving cell. The endocytosis of the ligand leads to a conformational change of the Notch receptor, exposing the cleavage site of the ADAM10. Subsequent cleavage of the γ-secretase complex releases the Notch intracellular domain (NICD) (Kopan and Ilagan, 2009). NICD then migrates to the nucleus, binds to the transcription factor RBP-Jκ (also called CSL) (Kovall and Blacklow, 2010), and recruits the co-activator Mastermind-like (MAML) to initiate downstream gene transcription, including the hairy enhancer of split (*HES*) and *HES*-related (*HEY*) family genes (Nam et al., 2006; Wilson and Kovall, 2006; Bolos et al., 2007; Guruharsha et al., 2012) (**Figure 2**).

Different from other classical signal transduction processes, the canonical Notch signaling pathway is characterized by the lack of cascade amplification in the transduction process, and only NICD is generated after a Notch receptor is consumed. Therefore, its signal intensity is crucial for generating the corresponding cellular response, and any deviation in the expression level of any molecular component in the Notch

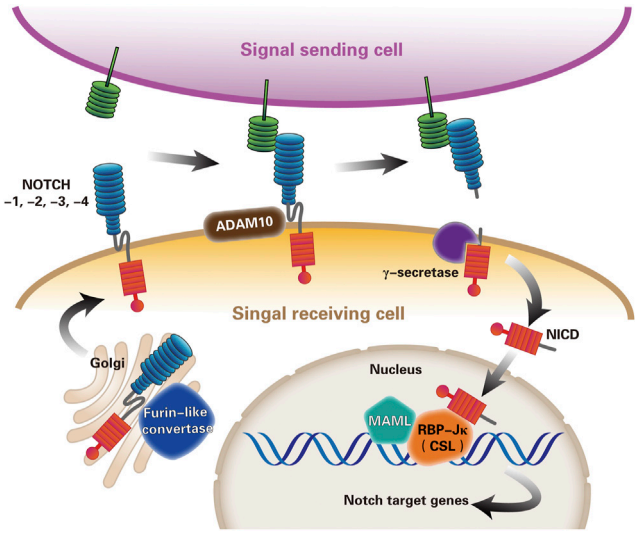

FIGURE 2 | The canonical Notch signaling pathway. Notch signaling pathway is currently thought to be activated by three steps of proteolysis. First, the mammalian Notch receptors are cleaved by a furin-like convertase in the Golgi compartment. After digestion, the extracellular subunits and transmembrane subunits formed by Ca^{2+} dependent non-covalent bonding to form heterodimers, and exocytosed to the cell membrane become mature Notch receptors. Second, Notch ligand-receptor binding enables proteolytic cleavage of the Notch extracellular domain by ADAM10 metalloprotease, and Notch receptor releases extracellular subunits. Third, γ-secretase complex cleaves the remnant receptor to allow the release and nuclear translocation of the NICD, where NICD forms a trimeric complex with transcription factor RBP-Jκ(or CSL) and the co-activator MAML, imitating the expression of Notch target genes transcription. (ADAM, a disintegrin and metalloprotease10; RBP-Jκ, recombination signal binding protein immunoglobulin kappa J; NICD, Notch intracellular domain; CSL, CBF1–suppressor of hairless–LAG1; MAML, mastermind-like).

signaling pathway may have a vital impact (Andersson et al., 2011). For example, Alagille syndrome (AGS) is caused by mutations in the gene for the Notch ligand JAG1 and NOTCH2 receptor (McDaniell et al., 2006). Currently, various Notch signaling pathway modulation approaches have been explored, including inhibition of the ligand-receptor interaction and interference with the proteolytic process of the receptor (Groth and Fortini, 2012; Shao et al., 2012; Andersson and Lendahl, 2014). Several Notch inhibitors are demonstrated effects in the NAFLD (**Table 1**).

NOTCH IN LIPID METABOLISM

As the central hub of lipid homeostasis, the liver is responsible for coordinating the whole process of lipid circulation, including the synthesis, export, redistribution, and utilization of free fatty acids (Nguyen et al., 2008). The main pathways that constitute hepatic lipid homeostasis, including uptake of circulating lipids, *de novo* lipogenesis (DNL), fatty acid oxidation (FAO), and export as very-low-density lipoprotein (VLDL) particles (Gluchowski et al., 2017; Ipsen et al., 2018). Hallmarked by hepatic steatosis, NAFLD is connected with lipid metabolism. When lipid acquisition exceeds lipid disposal in the liver, that is, the uptake of fatty acids and DNL covering oxidation and output of fatty acids, hepatic steatosis occurred (Ipsen et al., 2018). Feng et al. (2017) proposed that no significant differences between free fatty acids (FFAs) in lean or obese patients with NAFLD were observed, and the value of serum FFAs in early diagnosis of NAFLD.

The studies suggested that nutrition-induced activation of mammalian target of rapamycin (mTOR) may cause an increase in liver lipid content, which also increases the activity of basal serine/threonine kinases, leading to a self-perpetuating

TABLE 1 | Major Notch signaling inhibitors in NAFLD

Inhibitor	Target	Function	Object	References
Peroxiredoxin 6 (PRDX6)	*Notch1*	Improve lipid accumulation through induction of mitophagy	Mice	Lee et al. (2019)
Notch1 decoy	*Notch1*	Decrease hepatic glucose production	Mice	Funahashi et al. (2008) Pajvani et al. (2013)
N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)	*Notch1*	Alleviate lipid accumulation and hepatocyte injury	Mice	Zhang et al. (2021)
Delta-like1 homolog (DLK1)	*Notch1*	Reduce hepatic steatosis and improve glucose and insulin tolerance	Mice	Lee et al. (2016)
Triptolide (TP)	*Notch1*	Initiate oxidative stress in hepatocyte	Mice	Shen et al. (2019)
Hepatocyte Toll-like receptor 4 (TLR4)	*Jag1/JAG1*	Reduce NASH related liver fibrosis	Mice/ Human	Yu et al. (2021)
Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	NICD	Ameliorate hepatic lipogenesis dyslipidemia and insulin resistance	Mice	Chartoumpekis et al. (2018)
γ-secretase inhibitor (GSI)	γ-secretase	Improve glucose metabolism and ameliorate liver fibrosis	Mice	Richter et al. (2020)
Liver-specific *Rbp-jκ* knockout (L-*RBP-Jκ*)	*RBP-Jκ*	Protect from obesity-induced insulin resistance	Mice	Pajvani et al. (2011)
Silybin (SIL)	*NOTCH1*	Hepatoprotective and antitumorigenic effect in HCC cells	Human	Zhang et al. (2013)
Delta-tocotrienol (δ-T)	*NOTCH1*	Reduce biochemical markers of hepatocellular injury and steatosis	Human	Pervez et al. (2020)

lipogenic cycle (Lamming and Sabatini, 2013; Caron et al., 2015; Han and Wang, 2018). Pajvani et al. (Pajvani et al., 2013) demonstrated that inhibition of Notch signaling prevented hepatic steatosis by blocking mTOR complex 1 (mTORC1) activity, which could be reversed by rapamycin treatment. They also showed that Notch signaling augmented mTORC1 function and SREBP1c-mediated lipogenesis and that inhibition of hepatic Notch signaling protects from the fatty liver by reducing DNL.

Although the specific pathogenesis of lipid metabolism disorder in NAFLD patients is still not completely clear, studies have shown it may be associated with Notch pathway regulation (Li et al., 2019). Ding et al. (2020) investigated the dynamic role of Notch gene expression in the development of NAFLD *in vitro* and *in vivo*. They used palmitic acid (PA) and methionine-choline-deficient (MCD) models to assess notch signaling genes expression changes at different time points. Based on the characteristics of *Notch* mRNA expression levels, they evaluated that expression of *Notch3* mRNA has been dynamically changed significantly in the development of hepatic steatosis during NAFLD (Ding et al., 2020). Furthermore, Auguet et al. (2020) explored the association between the *Notch* transcriptional repressor and hepatic expression of lipid metabolism-related genes in a cohort of women with NAFLD. They found a negative relationship between hepatic *HEY2* expression and low-density lipoprotein (LDL) cholesterol (Auguet et al., 2020).

NOTCH IN INSULIN RESISTANCE

It is generally recognized that IR is pivotal in the pathogenesis and progression of NAFLD (Lomonaco et al., 2012). IR is essentially a decrease in the sensitivity of whole-body, liver, and adipose tissue to insulin, which is involved in the development of hepatic steatosis (E. Bugianesi, 2010). In NAFLD patients, increases in circulating glucose and insulin associated with IR promote hepatic DNL (Smith et al., 2020). Specifically, when IR occurs, it causes an impaired ability of insulin to inhibit adipose tissue lipolysis, resulting in increased delivery of FFAs to the liver (Bugianesi et al., 2005). Meanwhile, large lipid deposition promotes IR, which leads to fasting hyperglycemia and compensatory hyperinsulinemia, further contributing to the pathophysiology of NAFLD via exacerbating DNL (Donnelly et al., 2005).

The abnormal activation of Notch signaling pathway and IR are closely linked. It is recognized factor forkhead box protein O1 (FOXO1) has a beneficial effect on insulin-mediated glucose homeostasis (Matsumoto et al., 2007; O-Sullivan et al., 2015). Notch signal mainly affects hepatic glucose via the synergistic effect of NICD and FoxO1 transcription. Glucose-6-phosphatase catalytic subunit (G6PC) and phosphoenolpyruvate carboxykinase (PCK1) are both rate-limiting enzymes of hepatic glycogenolysis and gluconeogenesis, which would be correlated with Notch activation (Valenti et al., 2013; Dongiovanni et al., 2016). Pajvani et al. (2011) reported that combined haploinsufficiency of FoxO1 and Notch1 notably improves insulin sensitivity in diet-induced IR. Hepatic overexpression of Notch1 regulates hepatic gluconeogenesis by inducing G6PC in a FoxO1-dependent mode, in turn, aggravates insulin resistance (Pajvani et al., 2011; Bernsmeier et al., 2016). Additionally, the reduction of metabolic activity in brown adipose tissue (BAT) has been found connected with IR in human (Stanford et al., 2013; Mottillo et al., 2016). Bi et al. (2014) revealed mice in which *Notch1* or *Rbp-jκ* selectively deleted in adipocytes show upregulated expression of BAT-specific genes and improvement in glucose tolerance and insulin sensitivity.

Based on the close relation between IR and Notch, several possible pharmacological targets of NAFLD are identified. Blocking the abnormal expression of Notch at the gene level can inhibit the accumulation of liver gluconeogenesis and triglycerides (TGs), thereby reducing the risk of NAFLD. The cleavage of NICD by γ-secretase inhibitor (GSI) exhibited an improvement of glucose homeostasis and insulin sensitivity in diet-induced obese (DIO) mice (Pajvani et al., 2011). Lee et al. (2016) demonstrated that Delta-like 1 homolog (DLK1), an inhibitory regulator of Notch signaling, would reduce hepatic steatosis and hyperglycemia via exogenous administration. Chartoumpekis et al. (2018) showed nuclear factor (erythroid-derived 2)-like 2 (Nrf2) could profoundly ameliorate hepatic lipogenesis and IR by repressing NICD. Besides, researchers also found plant extracts (such as curcumin) have been shown to suppress NOTCH1, which could ameliorate fatty liver and enhance insulin sensitivity in the high-fat diet (HFD) model (Zhao et al., 2017; Saadati et al., 2019; El et al., 2021).

NOTCH IN OXIDATIVE STRESS

Oxidative stress (OS) is a concept used to describe an imbalance between pro-oxidants and antioxidants, leading to cellular damage and tissue injury (Sies, 2015). The chronic high-calorie diet causes lipid accumulation in hepatocytes and excessive generation of reactive oxygen species (ROS) (Sahini and Borlak, 2014). Meanwhile, affected by lipotoxicity from high levels of lipid metabolites, OS inhibits insulin sensitivity and facilitates DNL (Gehrke and Schattenberg, 2020).

In the pathophysiological process of NAFLD, OS is considered a pivotal mediator of the inflammatory response (Koek et al., 2011). Notch signaling has been reported to be associated with steatosis and OS. It has been proposed that ROS like H_2O_2 regulates the expression of Notch (Marinho et al., 2014). Notch1 regulates the expression of lipid oxidation genes and exhibited an obvious lipid accumulation reduction in Notch1 deficient antisense transgenic (NAS) mice (Song et al., 2016). Similarly, Notch1 inhibitor reduces ethanol-induced OS and lipid accumulation in HepG2 cells (Wang et al., 2014).

Among the multiple mechanisms that accelerate the progression of NAFLD to NASH, mitochondrial dysfunction is the prime one (Caldwell et al., 1999). Mitochondrial abnormalities disrupt the balance between pro-oxidants and antioxidants, leading to an increase of FFAs (Begriche et al., 2013). Peroxiredoxin 6 (PRDX6) is a mitochondrial antioxidant enzyme and is highly expressed in the liver (Fisher, 2011; Arriga

et al., 2019). Lee et al. (2019) demonstrated that PRDX6 induces effects of maintaining mitochondrial integrity and inhibits OS-induced Notch signaling, thereby reducing ROS production and lipid accumulation. They pointed out that PRDX6 mitophagy-mediated mechanisms offer endogenous protection against NAFLD (Lee et al., 2019).

Moreover, triptolide (TP) is the main ingredient of the medicinal herb Tripterygium wilfordii Hook f (TWHF) (Ziaei and Halaby, 2016). TP caused hepatotoxicity through initiating OS. Shen et al. (2019) investigated TP inhibited the protein expression of Notch1 and NICD, and the activation of Notch signaling has the potential to protect against TP-induced live injury. Interestingly, Huang et al. (2021) demonstrated that dose-related TP as an allosteric AMPK agonist alleviates NAFLD. Combined, the regulation of Notch signaling pathway may better enable TP to play a protective role in NAFLD.

NOTCH IN INFLAMMATION AND FIBROGENESIS

Liver fibrosis is a decisive factor of liver disease progression, particularly as it is associated with adverse prognosis and mortality in patients with NASH (Vilar-Gomez et al., 2018; Powell et al., 2021). Even in the early stage of fibrosis, it is shown a series of adverse liver-related events are gradually increasing (Angulo et al., 2015; Dulai et al., 2017; Hagstrom et al., 2017). In advanced NASH, hepatocytes are partially replaced by fibrotic scar tissue, the severe pathological change makes it difficult to treat NASH by correcting the underlying metabolic abnormality. Therefore, anti-fibrosis has become the focus of NASH therapy.

Notch activity is almost absent in healthy adult hepatocytes, mildly elevated in simple steatosis, and significantly increased in NASH (Valenti et al., 2013; Zhu et al., 2018). In various mouse models of fibrosis, over 80% of collagenous myofibroblasts are caused by hepatic stellate cell (HSC) (Mederacke et al., 2013). Notch-activated hepatocytes facilitate liver profibrogenic in NASH by both osteopontin (Opn) secretion mediated HSC activation *in vitro* and *in vivo* (Zhu et al., 2018), leading to a continuous extracellular matrix (ECM) accumulation and liver parenchyma gradually replaced by fibrous tissue (Mederacke et al., 2013). Conversely, in Notch loss-of-function mouse models, hepatocyte-specific liver inflammation and fibrosis are reduced, suggesting maladaptive hepatocytic Notch response to NASH-associated liver fibrosis (Zhu et al., 2018).

Sawitza et al. (2009) explored Jag1 as one of the cell surface ligands in Notch signaling activates HSC to stimulate α-SMA and collagen production. Yu et al. (2021) proved increasing Jag1 is responsible for fibrosis-inducing Notch reactivation. Also, other hepatic non-parenchymal cells could activate the Notch pathway to promote NASH latently through various mechanisms. Duan et al. (2018) investigated Notch activation in liver sinusoids endothelial cell (LSEC), which leads to HSC activation and the subsequent hepatic fibrosis, by downregulating eNOS-sGC signaling. Besides, researchers found that inhibitors inactivate M1 polarization of macrophage by regulating Notch signaling could reduce the secretion of inflammatory cytokine and fibrogenesis in CCl_4-induced liver injury mice (Bansal et al., 2015; Xu et al., 2015; Sheng et al., 2020). Additionally, γ-secretase inhibitor (Chen et al., 2012) and Notch3 siRNA (Y. X. Chen et al., 2012) suppressed the myofibroblastic gene expression of rat HSC line by blocking Notch signaling. Therefore, selective interruption of these Notch-related targets may provide more anti-fibrosis strategies for NAFLD (Romeo, 2019).

NOTCH IN AUTOPHAGY

Autophagy is a process in which cells degrade and metabolize their own damaged organelles or protein aggregation (Wang et al., 2019), which plays a vital role in regulating multiple liver functions and maintaining hepatic homeostasis (Ueno and Komatsu, 2017). Accumulating evidence suggests autophagy regulates liver-mediated systemic glucose and lipid metabolism (Singh et al., 2009; Galluzzi et al., 2014). Meanwhile, the liver is surrounded by exogenous substances from the portal vein circulation, including potential inflammatory mediators, in which autophagy has major cell-protective and anti-inflammatory effects (Deretic et al., 2013; Deretic and Levine, 2018; Hazari et al., 2020). All of the above suggests autophagy is associated with the occurrence and development of various liver diseases such as NAFLD.

The lipid droplets (LDs) are specialized cytosolic organelles in which some organs including the liver store neutral lipids (such as TGs) to protect from lipotoxicity (Gross and Silver, 2014). The progression of LDs degradation is regarded as a specific form of autophagy, also known as lipophagy (Garcia et al., 2018). Recent studies have revealed that disturbances in lipophagy have been linked to hepatic lipid accumulation, the process of lipophagy could be regarded as a new way of controlling NAFLD development (Grefhorst et al., 2021).

Because autophagy can remove damaged organelles, autophagy may alleviate hepatocellular injury during NASH. The protective effects of carbamazepine-induced autophagy could reduce steatosis and improve IR in the NAFLD model (Lin et al., 2013). Indeed, modulating autophagy may prevent the progression of NAFLD. Zhang et al. (2021) investigated that *Notch1* is an activated intensity of autophagy in FFA-treated HepG2 cells, and decreased *Notch1* levels may alleviate hepatocyte damage by enhancing autophagy, which could be reversed by autophagy inhibitor chloroquine. Niture et al. (2018) demonstrated that inhibition of Notch reduced the expression of autophagy biomarker and serotonin-mediated liver cell steatosis. These findings provide helpful clues for the strategy of Notch signaling pathway to regulate autophagy and thereby remit the progression of NAFLD.

NOTCH IN NAFLD-RELATED HCC

HCC is the fourth leading cause of cancer-related deaths worldwide and occurs in patients with various chronic liver diseases (Bray et al., 2018; Llovet et al., 2021). Although hepatitis B virus (HBV) infection

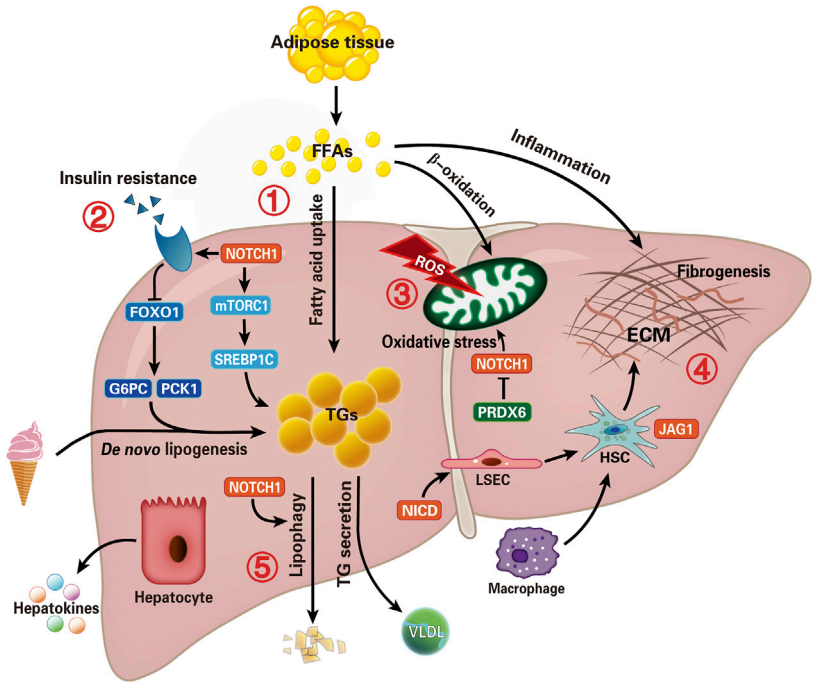

FIGURE 3 | Overview of potential therapeutic targets of Notch signal pathway in NAFLD. Summarize the recent critical advances evolving Notch signaling in the NAFLD. Intrahepatic lipid levels depend on the balance between lipid acquisition and disposal. Therefore, lipid accumulation is the result of uptake of fatty acids and *de novo* lipogenesis exceeding export as VLDL and oxidation of fatty acid, which is also a main pathophysiological change of NAFLD. The five potential pathways to intervene in NAFLD are focused on (1) hepatic lipid accumulation, (2) insulin resistance, (3) oxidative stress, (4) inflammation and fibrogenesis, and (5) autophagy (lipophagy) progression. Although several aspects of these functions remain to be fully understood, these findings offer an intriguing rationale for investigating Notch-based therapies in patients with NAFLD. Furthermore, the "hepatokines" secreted by hepatocytes may help reveal the complex molecular regulation in NAFLD. (NICD, Notch intracellular domain; FFAs, free fatty acids; mTORC1, mammalian target of rapamycin complex one; FOXO1, factor forkhead box protein O1; G6PC, glucose-6-phosphatase catalytic subunit; PCK1, phosphoenolpyruvate carboxy kinase; TGs, triglycerides; VLDL, very-low-density lipoprotein; ROS, reactive oxygen species; PRDX6, peroxiredoxin 6; ECM, extracellular matrix; HSC, hepatic stellate cell; LSEC, liver sinusoids endothelial cell).

has been the prominent risk factor of HCC, NAFLD has become the most rapidly growing driver of HCC in many countries (Younossi et al., 2019; Hester et al., 2020). The incidence in patients with NAFLD-related HCC increases with the histological stage, which is highest in patients with NAFLD-cirrhosis (Ioannou, 2021).

Thus far, the exact pathogenesis underlying NAFLD-induced HCC is only incompletely understood but mainly focuses on the effects of DNA damage response, inflammation, autophagy, and intestinal microbiota (Anstee et al., 2019; Behary et al., 2021). In addition, the chronic activation of metabolic pathways seems to play a critical role (Baffy et al., 2012). These pathways may provoke infinite hepatocyte proliferation and genomic instability, and on the other hand, provide a microenvironment conducive to malignant transformation and tumor growth.

Recent studies suggest that Notch signaling pathway is frequently associated with tumorigenesis (Nowell and Radtke, 2017). Selective blocking of Notch1 inhibits cancer cell growth and deregulates angiogenesis (Wu et al., 2010). By performing RNA sequencing of hepatocyte populations HFD-fed reporter mice, Zhu et al. (2021) illustrated that Notch-active hepatocytes showed transcriptional enrichment of ECM-related genes, which may represent a mechanism that persists in the tumorigenic process. Furthermore, they found HFD-diet mice with Notch-active mutation spontaneously formed fully developed liver tumors (Zhu et al., 2021). Therefore, it can be inferred that the continuous activation of Notch signaling pathway promotes the occurrence of NAFLD-related HCC.

CONCLUSION AND PERSPECTIVE

NAFLD is a manifestation of metabolic syndrome in the liver. With the changes in lifestyle and dietary habits, the incidence of NAFLD is rising rapidly. The previous studies have revealed the significance of the Notch signaling pathway in metabolism. The abnormal expression of Notch may lead to several metabolic disorders, thus inducing NAFLD. Although the relation between NAFLD and Notch signaling has been observed both *in vitro* and *in vivo*, most of the research findings are based on phenotypic studies and the underlying mechanisms and potential associations between different Notch molecules, and require further in-depth research.

The development of liver-specific Notch inhibitors is pivotal for the treatment of NAFLD-related hepatic lipid accumulation, IR, OS, fibrogenesis, and autophagy progression (**Figure 3**). But

until now, most intervention studies are conducted in animal models (especially mice), the potential role of Notch regulators in human NAFLD needs to be explored extensively. Recently, the rising field of "hepatokines" biology would help reveal the complex molecular regulation in NAFLD (Watt et al., 2019). If so, it would promote the development of more non-invasive diagnostic tests to improve early diagnosis rates.

There is no specific and effective pharmacotherapy toward NAFLD, however, some drugs have shown therapeutic potential by regulating a Notch signal pathway. Vitamin E (α-tocopherol) is a dietary antioxidant recommended as a treatment for NASH (Yakaryilmaz et al., 2007; Chalasani et al., 2018). Recent clinical research supports vitamin E use brought obvious histological benefits and improved prognosis in patients with NASH (Sato et al., 2015; Brunt et al., 2019; Vilar-Gomez et al., 2020). δ-tocotrienol, an isomer of vitamin E, has been explored to inhibit tumor invasion and metastasis via downregulating the NOTCH1 signaling pathway (Rajasinghe et al., 2018). Notably, Pervez et al. (2020) launched a randomized, double-blind, placebo-controlled trial of 71 patients with NAFLD. Compared with placebo, δ-tocotrienol significantly reduced biochemical markers of hepatocellular injury and steatosis in patients (Pervez et al., 2020). Silybin (SIL), a hepatoprotective drug, could be an inhibitor targeting the NICD, RBP-Jκ, and Hes1 proteins in HCC cells and exert antitumorigenic effects (Zhang et al., 2013).

The precise drug delivery without toxicity brings a wide application prospect for the treatment of NAFLD. A nanoparticle-mediated delivery system to target GSI in the liver (GSI NPs) has been developed (Richter et al., 2020), which avoids goblet cell metaplasia caused by intestinal Notch inhibition (van Es et al., 2005). Based on similar studies above would advance clinical therapy research, thereby optimizing therapies for various NAFLD subtypes to increase the cure rate while complications can be decreased. In a word, findings on Notch signaling pathway research could bring NAFLD patients a hopeful future with ever more promising targets for prevention and treatment.

AUTHOR CONTRIBUTIONS

HX designed the outline of the review and drafted the manuscript. LW contributed his scientific advice and revision of the manuscript. All authors read and approved the submitted version.

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GLOSSARY

AGS Alagille syndrome
DIO diet-induced obese
DLK1 Delta-like 1 homolog
DNL de novo lipogenesis
ECM extracellular matrix
ER endoplasmic reticulum
FAO fatty acid oxidation
FFAs free fatty acids
FOXO1 factor forkhead box protein O1
G6PC glucose-6-phosphatase catalytic subunit
GSI γ-secretase inhibitor
HCC hepatocellular carcinoma
HFD high-fat diet
HSC hepatic stellate cell
IR insulin resistanceinsulin resistance
IR insulin resistanceinsulin resistance
LDs lipid droplets
LSEC liver sinusoids endothelial cell
MCD methionine-choline-deficient
mTOR mammalian target of rapamycin
mTORC1 mTOR complex 1
NAFL non-alcoholic fatty liver
NAFLD non-alcoholic fatty liver disease
NASH non-alcoholic fatty steatohepatitis
NAS Notch1 deficient antisense transgenic
NICD Notch intracellular domain
Nrf2 nuclear factor (erythroid-derived 2)-like 2
Opn osteopontin
OS oxidative stress
PA palmitic acid
PCK1 phosphoenolpyruvate carboxy kinase
PRDX6 Peroxiredoxin 6
ROS reactive oxygen species
SIL Silybin; δ-T(Delta-tocotrienol)
SS simple steatosis
TGs triglycerides
TP triptolide
TWHF tripterygium wilfordii Hook f
VLDL very-low-density lipoprotein

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REVIEW
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Cytokines and Chemokines in HBV Infection

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Chronic hepatitis B virus (HBV) infection remains a leading cause of hepatic inflammation and damage. The pathogenesis of chronic hepatitis B (CHB) infection is predominantly mediated by persistent intrahepatic immunopathology. With the characterization of unique anatomical and immunological structure, the liver is also deemed an immunological organ, which gives rise to massive cytokines and chemokines under pathogenesis conditions, having significant implications for the progression of HBV infection. The intrahepatic innate immune system is responsible for the formidable source of cytokines and chemokines, with the latter also derived from hepatic parenchymal cells. In addition, systemic cytokines and chemokines are disturbed along with the disease course. Since HBV is a stealth virus, persistent exposure to HBV-related antigens confers to immune exhaustion, whereby regulatory cells are recruited by intrahepatic chemokines and cytokines, including interleukin-10 and transforming growth factor β, are involved in such series of causal events. Although the considerable value of two types of available approved treatment, interferons and nucleos(t)ide analogues, effectively suppress HBV replication, neither of them is sufficient for optimal restoration of the immunological attrition state to win the battle of the functional or virological cure of CHB infection. Notably, cytokines and chemokines play a crucial role in regulating the immune response. They exert effects by directly acting on HBV or indirectly manipulating target immune cells. As such, specific cytokines and chemokines, with a potential possibility to serve as novel immunological interventions, combined with those that target the virus itself, seem to be promising prospects in curative CHB infection. Here, we systematically review the recent literature that elucidates cytokine and chemokine-mediated pathogenesis and immune exhaustion of HBV infection and their dynamics triggered by current mainstream anti-HBV therapy. The predictive value of disease progression or control and the immunotherapies target of specific major cytokines and chemokines in CHB infection will also be delineated.

Keywords: hepatitis B virus, cytokine, chemokine, immune response, liver disease

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INTRODUCTION

Hepatitis B virus (HBV), a hepatotropic, non-cytopathic DNA virus, is responsible for most cases of viral hepatitis (Seeger and Mason, 2015). Although with the widespread application of two types of available approved treatment, interferons alpha (IFN-α) and nucleos(t)ide analogues (NUCs), which effectively suppress HBV replication, neither of them eliminates the virus mechanistically, leaving the risk of hepatocellular carcinoma (HCC) remains, and being a major cause of morbidity and mortality worldwide.

The clearance of HBV mainly depends on the antiviral effect of the immune system. Over 90% of people infected in adulthood will resolve the infection, presenting as the clinical manifestations of acute self-limiting infection, reflecting an optimal host immune response involving early control of viral replication (Terrault et al., 2018). Serving as the first defense line against HBV insulting, the innate immune arm reacts promptly and mightily. Subsequently, it triggers the formidable adaptive immune response by either killing virus-infected hepatocytes directly or exerting non-cytolytic mechanisms mediated by soluble cytokines (Kapoor and Kottilil, 2014). Therefore, it is safe to deduce that our fully-developed immune system is strong enough to achieve HBV containment. By contrast, unfortunately, over 90% of individuals infected in infancy will progress from acute hepatitis to lifelong chronic infection (Kwon and Lee, 2011). The causes of HBV infection chronicity remain unclear. Infectiousness at an early age is the main element accounting for persistency, which may be partially attributed to the distinct features of innate and adaptive immunity from fetal life to adulthood. Besides, viral load, genotype, route of infection, age, and genetics of the infected host jointly modify the natural course of HBV infection (Fanning et al., 2019). Chronic HBV infection causes liver injury, which is predominantly mediated by persistent intrahepatic immunopathology. The events of repetitively active viral replication, hepatic necroinflammatory, and inflammatory-induced damage repair take place iteratively, which ultimately build the pathological basis of HBV-related liver cirrhosis (LC) and HCC (Liang, 2009).

Although been controversial (Wang S. et al., 2013; Zhang Z. et al., 2020), HBV is deemed to be a stealth virus that escapes surveillance without detected by pattern recognition receptors in infected hepatocytes or acts as a suppressor of the innate defense system by multiple pathways (Yu et al., 2017). With persistent HBV-related antigen (Ag) stimulation, chronic HBV infection puts the immune system into a dilemma where expeditious and appropriate adaptive immune response cannot be evoked or be in an attrition state, leading to the anergy of anti-HBV specific immune response (Fisicaro et al., 2020). In the meantime, cytokines and chemokines, as the essential components of the immune system, undergo tremendous disturbances and participate in the exhaustion of the anti-HBV immune response. Moreover, based on its location and anatomic features, the liver harbors a specific feature of tolerance to pathogens and antigens draining from the gut, thereby avoiding severe immune-mediated damage (Robinson et al., 2016), this, however, renders the stealth virus more rampant in the liver, making it more intractable for the aggrieved adaptive response to tackle. Although the current anti-HBV therapy in clinical practice can significantly inhibit viral replication, there is still a distant way to achieve the current treatment goal of CHB patients. This functional cure is defined as undetectable HBV DNA and hepatitis B surface antigen (HBsAg) loss over a limited treatment period (Terrault et al., 2018). Meanwhile, the treatment response predictors and withdrawal criteria after long-term maintenance of NUCs therapy are still unclear. Therefore, it is urgent to seek predictive indicators of treatment response and new treatment strategies. At present, with the fabulous development of scientific-technical advances, accumulating evidence indicates that the involvement of cytokines and chemokines in the pathogenesis of HBV is more and more unambiguous, providing a proof of concept for immune-modulating therapy. Therapeutic approaches that target cytokines and chemokines associated with the severity of chronic HBV infection sound promising.

In this review, we will systematically discuss the recent literature that elucidates cytokine and chemokine-mediated pathogenesis and immune exhaustion of HBV infection and their dynamics triggered by current mainstream anti-HBV therapies. We also emphasize the potential predictive value of disease progression or control and the immunotherapies targeting specific major cytokines and chemokines in CHB infection.

CYTOKINES IN HEPATITIS B VIRUS INFECTION

Interleukin-1

The inflammatory cytokines Interleukin (IL)-1, including IL-1α and IL-1β, was first identified in the 1940s as an endogenous pyrogen (Menkin, 1943; Dinarello, 1991). IL-1α and IL-1β have similar biological activities (Netea et al., 2010), but the role of IL-1β in HBV has been attached more attention. In chronic HBV infection, HBV with its component, hepatitis B e antigen (HBeAg), have been reported to manipulate a variety of strategies to inhibit both the production and effects of IL-1β. HBeAg significantly inhibits the LPS-induced NLRP3 inflammasome activation and IL-1β production, and also attenuates its downstream pathway of NF-κB activation (Wang et al., 2019). Thereby, these may favor the establishment and maintenance of persistent infection. In contrast, hepatitis B c antigen (HBcAg) promotes LPS-induced NLRP3 inflammasome activation and IL-1β production (Wang et al., 2019). At last, the counteraction turns out to be the upregulation of IL-1β found in the serum, peripheral blood mononuclear cells (PBMCs) and *in vitro* culture primary human hepatocytes (Tian et al., 2019; Zhang Z. et al., 2020; Li and Jiang, 2021). Besides, IL-1β is also linked to IFN-α treatment response. CHB patients responded to IFN-α with clearance of HBeAg and sustained inhibition of HBV replication were accompanied by substantial rises in IL-1β in serum and spontaneous *in vitro* production from PBMC (Lei et al., 2019), suggesting the treatment response predictor and potential therapeutic roles of IL-1β. In addition, the polymorphisms of the genes encoding IL-1β are associated with disease severity in HBV infection and HBV-related hepatic complications, which might serve as a potential genetic biomarker (Tuncbilek, 2014; Dhifallah et al., 2021).

Interleukin-2

IL-2 was first identified as a T cell growth factor produced primarily by $CD4^+$ T cells (Morgan et al., 1976). IL-2 interacts with the intermediate-affinity and high-affinity IL-2 receptors functionally expressed by resting natural killer (NK) cells, $CD8^+$

T cells, and lymphocytes following their activation, respectively (Letourneau et al., 2010). IL-2 can boost the proliferation of T cells, the cytolytic activity of NK cells. On the other hand, IL-2 exerts its immunoregulatory effects by promoting the development and suppressive activity of T regulatory (Treg) cells (Liao et al., 2013). Research on the role of IL-2 in HBV infection is dominantly referred to its representative significance for the evaluation of functions in T cells, especially in the HBV-specific T cells during the natural course of HBV infection, as well as its dynamics in response to the anti-HBV treatment (Chokshi et al., 2014; Tan et al., 2018). The levels of IL-2 increase significantly in a time-dependent manner in patients with adefovir dipivoxil or telbivudine treatment, which is contrary to that with entecavir treatment (Piao et al., 2012; Li C. et al., 2013; Gao et al., 2021). Elevated levels of IL-2 on-treatment are associated with HBeAg seroconversion after treatment withdrawal (Chokshi et al., 2014), indicating that IL-2 may serve as an off-treatment predictor. During chronic HBV infection, $CD4^+$ T cell exhaustion has the absence of IL-2, which is partially restored by anti-inhibitor molecular treatment (Dong et al., 2019). Additionally, nearly a decade ago, scientists had embarked on a concerted journey to the clinical application of IL-2 but achieved highly heterogeneous results. Some studies showed that recombinant (r) IL-2 acted as an immunomodulatory agent enhancing host immune activity and might benefit CHB patients (Onji et al., 1987). In contrast, others evidenced that IL-2 therapy over short periods did not result in complete clearance of HBV, and treatment with IFN-α alone was preferable to a regimen of IFN-α/IL-2 applied (Nishioka et al., 1987; Bruch et al., 1993). Overall, although the combination of IL-2 and the conventional therapies might be a promising strategy to cure HBV, the suitable concentration, tissue-targeting, and add-on manner remain further clarified.

Interleukin-4

IL-4 is distinguished as a T helper (Th)2 cytokine that tilts the adaptive response toward humoral immunity by motivating proliferation, differentiation, and antibodies production of B cells, promoting Th2 cells differentiation from naïve $CD4^+$ T cells and inhibiting Th1 responses as well as IFN-γ production (Nelms et al., 1999).

During chronic HBV infection, IL-4 is downregulated, compared with those in healthy control (HC), and has an inverse correlation with virus load and HBsAg titers (Gu et al., 2019). Moreover, the expression levels of IFN-γ are gradually elevated, and the expression levels of IL-4 are progressively lowered from the immune tolerance phase to the inactive carrier phase (Li M. et al., 2016), indicating a shift from Th2 to Th1 responses. Interestingly, it has been reviewed that there is a consistent boost of IL-4 after NUCs treatment regardless of the specific agent (Li et al., 2017; Tavakolpour et al., 2017), whereas levels of serum IL-4 are decreased during the treatment with IFNα-2a therapy in virological responders (Park et al., 2012). As a competent cytokine, IL-4 can suppress the expression and the replication of HBV in different HCC lines (Lin et al., 2003; Yao et al., 2011). In addition, it has been suggested that the IL-4 (-590) CT genotype is a vital protective factor for the development of hepatitis among chronic HBV carriers. In contrast, the genetic variants in IL-4 -590C/T and -33C/T polymorphisms may be a risk factor for CHB in Chinese males but not for HBV-related LC or HCC (Lu et al., 2014; Saxena et al., 2014).

Interleukin-6

IL-6 a cytokine mainly produced by activated monocytes in response to inflammatory stimuli, is involved in a wide range of pleiotropic actions that affect the functions of a variety of lymphoid cells (Van Snick, 1990). IL-6 signals through membrane-bound and soluble IL-6 receptor (sIL-6R), mediating a classic signaling pathway or a trans-signaling pathway, respectively. Of note, the regenerative or anti-inflammatory activities of IL-6 are mediated by traditional signaling, whereas pro-inflammatory responses of IL-6 are mediated by trans-signaling (Rose-John, 2012).

The roles of IL-6 in HBV infection, including acute hepatitis B (AHB), CHB, and HBV-related diseases, have been widely described. However, the interpretation of the role of IL-6 in HBV is complicated because the sIL-6R can mediate trans-signaling and is implicated in a series of inflammatory diseases. During HBV infection, IL-6 is found markedly higher mediated by various pathways and closely correlated to the degree of hepatocyte damage in the HBV-related disease spectrum. Specifically, Li et al. have evidenced that HBV middle S protein and HBcAg enhance IL-6 production via p38, ERK, and NF-κB pathways (Li Y.-X. et al., 2016; Chen et al., 2017) while HBx protein not only promotes complement component 3 production by inducing IL-6 secretion from hepatocytes in mice, but stimulates the production of IL-6 in a MyD88-dependent manner, leading to HBV-mediated liver carcinogenesis (Xiang et al., 2011; Yuan et al., 2016). Furthermore, a sustained high level or dynamic elevated level of serum IL-6 indicates higher mortality in patients with HBV-acute-on-chronic liver failure (ACLF) (Zhou et al., 2020). Another research reported that HBcAg established a proinflammatory microenvironment by promoting the production of IL-6 of M2 macrophages via the TLR2 pathway (Yi et al., 2020). As for the dynamics of IL-6 upon anti-HBV treatment, two studies showed that Pegylated (Peg)-IFNα therapy induced a distinct and rapid up-regulation of IFN signaling pathway that coincided with increased detection of IL-6 (Tan et al., 2014), and IL-6 levels at 3rd and 6th months after treatment showed a predictive value of sustained virological response (Park et al., 2012). Paradoxically, another study has evidenced that, compared with baseline, the Peg-IFNα group showed a significant decrease in IL-6 during 3–6 months of treatment (Li MH. et al., 2021). In addition, serum levels of IL-6 do not reflect the inflammatory activity of hepatitis and have no predictive value of positive response to the IFN-α therapy in children with CHB (Gora-Gebka et al., 2003). In contrast to the controversy over IFN-α therapy, patients with NUCs treatment show a consistent decrease in the levels of IL-6 (Lu et al., 2008; Yu et al., 2021).

As a pleiotropic cytokine, IL-6 exerts inhibiting effects on HBV through multiple layer mechanisms. IL-6 inhibits HBV entry and transcription through sodium taurocholate cotransporting polypeptide (NTCP) down-regulation, targeting

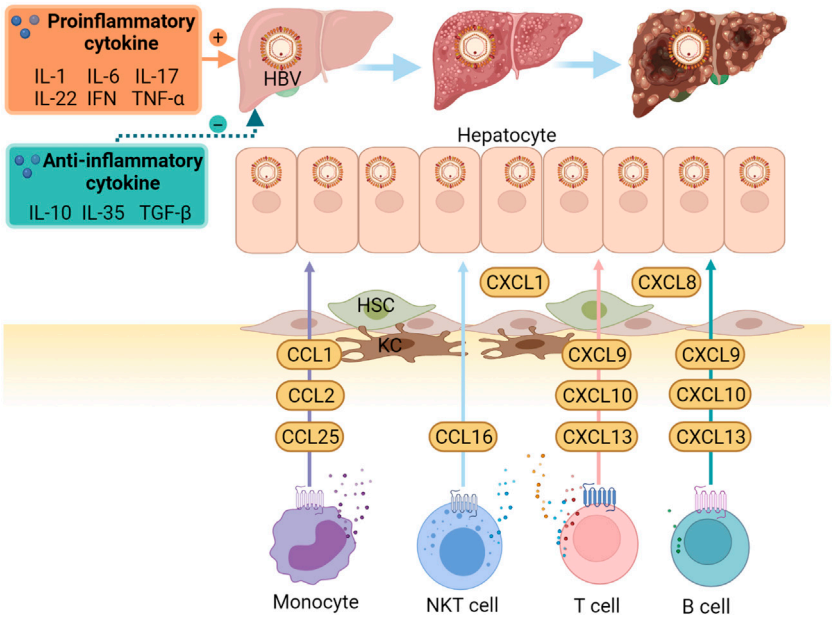

FIGURE 1 | Important cytokines and chemokines in the pathogenesis of HBV infection and HBV-related diseases. When HBV invades, local immune cells respond firstly, especially the KCs. They induce the production of chemokines and cytokines. CCL1, CCl2, and CCL25 contribute to infiltrating monocyte recruitment. CCL16 promotes the migration of NKT cells, whereas others, ligands of CXCR3 and CXCR5, including CXCL9, CXCL10, CXCL13, increase the inflow of T and B cell subsets. In addition, increased intrahepatic CXCL1 and CXCL8 mediate the recruitment of neutrophils. These, together with HSCs changes, result in the disturbance of pro- and anti-inflammatory cytokines, contributing to the development of liver cirrhosis and hepatocellular carcinoma. CCL, C-C Motif Chemokine Ligand; CXCR, C-X-C motif chemokine receptor; HBV, hepatitis B virus; HSC, hepatic stellate cell; KCs, Kupffer cells; NKT, natural killer T. (created with Biorender.com).

the epigenetic control of the nuclear covalently closed circular (ccc)DNA minichromosome, and increasing the enhancer activity of HBV enhancer 1 through signal transduction pathways (Palumbo et al., 2015). Nevertheless, the pernicious side of IL-6 on HBV infection stands out. Several studies have revealed that HBV exploited the IL-6 signal pathway to manipulate the development of LC and HCC (Chang et al., 2015; Kao et al., 2015; Zhou et al., 2018). HBV-induced mitochondrial reactive oxygen species production leads to the sustained activation of the IL-6-STAT3 pathway and ultimately contributes to HCC (Yuan et al., 2016). Overall, as there are beneficial and detrimental effects of IL-6 in HBV infection, more mechanistic research is needed to interpret this double-edged sword judiciously.

Interleukin-12

IL-12 is primarily produced by dendritic cells (DCs), monocytes, and macrophages, and to a lesser extent by B cells, whose role is predominantly associated with the differentiation of naïve T cells into Th1 cells, serving as a linkage between innate to cellular immunity (Heufler et al., 1996). It also promotes the expansion and survival of activated T cells and NK cells and modulates the cytotoxic activity of cytotoxic T lymphocytes (CTLs) and NK cells (Rossol et al., 1997). During the adaptive immune response, IL-12 primes Ag-specific T-cells for high IFN-γ production. IL-12 can also act as an adjuvant for humoral immunity by enhancing antibody (Ab) production by B cells (Metzger et al., 1997).

HBV-induced IL-12 expression involves activating the PI3K-Akt pathway by HBx, leading to the transactivation of the IL-12 p35 and p40 promoters (Wang et al., 2015). Serum levels of IL-12 are associated with alanine aminotransferase (ALT) levels, and the highest serum levels of IL-12 was accompanied by HBeAg or HBsAg seroconversion in both AHB and CHB patients (He et al., 2012; Wu et al., 2015), suggesting serum levels of IL-12 may be an available marker to evaluate cellular immunity for HBV infection. Elevated IL-12 rescues the anti-viral function of exhausted HBV-specific $CD8^+$ T cells, enhances the anti-virus properties of cytotoxicity, polyfunctionality, and multispecificity of HBV-specific T cells. Furthermore, IL-12 significantly decreases the pro-apoptotic molecule Bim, which can mediate premature attrition of HBV-specific $CD8^+$ T cells (Xiong et al., 2007; Wu et al., 2015). Co-stimulation with IL-12 is found to significantly augment the HBs/e/cAg-specific secretion of IFN-γ (Vingerhoets et al., 1998; Szkaradkiewicz et al., 2005). Besides, there is a clear consensus that the CHB patients with current available anti-HBV treatment presented higher amounts of IL-12 are associated with favorable outcomes (Ozkan et al., 2010; Yang et al., 2020).

As a promising therapeutic cytokine, IL-12 has been well explored in human studies and murine experimental models. IL-12-based vaccination therapy restores systemic HBV-specific $CD4^+$ T cell responses, elicits robust intrahepatic HBV-specific $CD8^+$ T cell responses, and facilitates the generation of HBsAg-specific humoral immunity in a mouse model of HBV carrier (Zeng et al., 2013). Moreover, combined with a plasmid expressing

TABLE 1 | Important cytokines and chemokines in HBV infection.

Mediatores	Primary effects	Roles in HBV infection	References
Cytokines			
IL-1	Proinflammatory	Predictor of treatment response to IFN-α	Lei et al., 2019
IL-2	T cell proliferation, NK cell cytolytic activity; promotes Tregs development and suppressive activity	Evaluation of HBV-specific T cell functions; immunomodulatory agent enhancing host immune activity	Chokshi et al. (2014); Tan et al. (2018) Onji et al. (1987)
IL-4	Promotes Th2 cells differentiation and humoral immunity	Suppresses the expression and replication of HBV in different HCC lines; downregulated in CHB patients	Lin et al. (2003); Yao et al. (2011); Gu et al. (2019)
IL-6	Pleiotropic actions that affect the functions of a variety of lymphoid cells	Inhibits HBV entry and transcription; manipulates the development of LC and HCC	Palumbo et al. (2015); Chang et al. (2015); Kao et al. (2015); Zhou et al. (2018)
IL-12	Promotes cellular immunity and modulates the cytotoxic activity of CLTs and NK cells	Enhances the anti-virus properties of cytotoxicity, polyfunctionality, and multispecificity of HBV-specific T cells; combination treatment with IL-12 favors HBV clearence	Xiong et al. (2007); Wu et al. (2015); Zeng et al. (2013); Carreno et al. (2000)
IL-21	B cell differentiation, germinal center responce and antibodies production	Boosts and sustains HBV-specific $CD8^+$ T cell effects by enhancing both cytolytic and noncytolytic pathways; associated with age-dependent outcome and vertical transmission of HBV infection	Li et al. (2015); Shen et al. (2021) Publicover et al. (2011); Vyas et al. (2019); Wang et al. (2021)
IL-17	Proinflammatory	Suppresses HBV replication in a noncytopathic manner; involved in the pathogenesis of liver damage, LC and HCC	Wang et al. (2013a); Bao et al. (2017)
IL-22	Tissue regeneration	Exerts dual effects in the context of inflammation	McAleer and Kolls. (2014)
IL-23	Stimulation of DC antigen presentation, generation, and maintenance of Th17 cells	Amplifies Th17 cell responses and liver inflammation; alters macrophage function and shapes a pro-cancer milieu for HCC	McKenzie et al. (2006); Wang et al. (2013b); Zang et al. (2018)
IFN	Control viral replication and dissemination	IFN-α exerts both direct antiviral and host immunomodulation effects and is the current standard treatment of HBV	Biron (2001); Sadler and Williams (2008)
		HBV specific IFN-γ producing T cells are associated with viral clearance	Wang et al. (2020)
TNF-α	Proinflammatory	Mediator of anti-HBV immunity, induces liver inflammation, leads to liver fibrosis	Wang et al. (2020)
IL-10	Regulatory cytokine, anti-inflammatory	Circulating IL-10+ Bregs and Tfr cells are associated with poor virus eradication and liver injury in CHB; IL-10-producing Breg cells suppress HBV-specific $CD4^+$ and $CD8^+$ T cell responses but enhance Treg cells	Wang et al. (2014); Das et al. (2012)
IL-35	Exerts an immunosuppressive effect on T cells	Elevates viral-specific Tregs, IL-10 production, decreases IL-17 secretion and contributes to viral persistence	Yang et al. (2019)
TGF-β	Anti-inflammatory cytokine, regulates diverse cellular processes	Boosting the activities of Treg cells; contributes to all stages of liver disease progression	Karimi-Googheri et al. (2014); Dooley and ten Dijke, (2012)
Chemokines			
CXCL9, CXCL10, CXCL11	Ligands of CXCR3, key immune chemoattractants during IFN-induced inflammatory response	Serum CXCL9, CXCL10, CXCL11 are elevated in CHB patients; CXCL10 enhances the migration of peripheral leukocytes into the liver; useful predictive indicators of disease progress and treatment response	Kakimi et al. (2001); Keating et al. (2014); Zhou et al. (2010); Sonneveld et al. (2013); Guo et al. (2016); Lee et al. (2013a)
CXCL13	Ligands of CXCR5, involed in the onset and maintenance of humoral immunity	Favors the recruitment of $CD19^+$ B cells and CXCR5+$CD8^+$ T cells into the liver; plasma CXCL13 serve as a biomarker for GC activity; increased plasma CXCL13 is distinctly observed in patients who achieve immune control of CHB infection	Li et al. (2020), (Havenar-Daughton et al., 2016), (Liu et al., 2017b)
CXCL8	Proinflammatory signaling cascade and guides neutrophils to infection site	Associated with the severity of liver inflammation/ fibrosis and resistance to IFN-α therapy	Yang et al. (2014)
CXCL12	Strong chemotaxis for lymphocytes	Involved in recruitment and retention of immune cells in CHB patients with advanced liver fibrosis	Wald et al. (2004)

HBV, hepatitis B virus; IL, interleukin; IFN, interferon; NK, natural killer; Treg, T regulatory; Th, T helper; HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; LC, liver cirrhosis; CTLs, cytotoxic T lymphocytes; DC, dendritic cells; TNF, tumor necrosis factor; Bregs, B regulatory cells; Tfr, T follicular regulatory; TGF, transforming growth factor.

IL-12, HBV DNA vaccine has a strong antigenicity in humoral and cellular immunities, enhances T cell reactivity to HBV and IFN-γ production, and showed 50% of virological response rate in CHB carriers under lamivudine treatment (Du et al., 2003; Rigopoulou et al., 2005; Yang et al., 2006). Additionally, treatment with IL-12 at suitable doses is safe and tolerable, and appears to be active against HBV in CHB patients (Carreno et al., 2000). Nevertheless, controversy still exists as other studies point out that IL-12 induces a strong immunosuppressive reaction in the liver of chronic woodchuck hepatitis virus carriers that counteracts the antiviral effect of the treatment, and the antiviral activity of rIL-12 does not appear to be advantageous in comparison to other currently available therapies in CHB patients (Zeuzem and Carreno, 2001; Otano et al., 2012). Therefore, more intensive research is needed before the application of IL-12 into clinical practice.

Interleukin-21

IL-21 is another pleiotropic cytokine, which is dominantly derived from follicular T helper (Tfh) cells, Th17 cells, and activated NKT cells (Zeng et al., 2007). Its receptor is widely expressed on various immune cells, endowing it a multifunctional role in the pathogenesis and prognosis of HBV infection.

In the course of natural HBV infection, similar to other proinflammatory cytokines, IL-21 elevates significantly at hepatic flare in AHB, CHB, and HBV-ACLF, especially in those achieving resolve or HBsAg seroconversion subsequently (Chen et al., 2014; Yoshio et al., 2018; Du et al., 2021). IL-21 has a pivotal implication in the instruction of antiviral treatment. Serum levels of IL-21 at treatment week 12 independently predicted HBeAg seroconversion in the first year of treatment in CHB patients with telbivudine treatment or salvage therapy (Ma et al., 2012; Li et al., 2021b). Additionally, serum IL-21 levels at 0, 12, 24, 52, and 104 weeks after discontinuance of entecavir are significantly higher in the durable virological remission group than in the virological relapse group (Huang et al., 2021).

Numerous studies have provided mechanistic evidence for the role of IL-21 in HBV infection. IL-21 promotes the proliferative capacity of HBV-specific $CD8^+$ T cells, down-regulates expression of the inhibitory receptors PD-1 and TIM-3, and further boosts and sustains the antiviral effects of HBV-specific $CD8^+$ T cells by enhancing both cytolytic and noncytolytic pathways (Li et al., 2015; Shen et al., 2021), thereby, contributing to viral containment and clearance. In addition, IL-21 also reduces HBV replication by inhibiting IL-10 secretion (Li et al., 2015). Our group has found that circulating $CXCR5^+CD4^+$ T cells favor HBeAg seroconversion through IL-21 in patients with CHB infection (Li Y. et al., 2013), and IL-21R deficient impairs the production of HBV-specific IFN-γ from intrahepatic $CXCR5^+CD8^+$ T cells (Li et al., 2020). Moreover, IL-21 is reported to take part in determining the age-dependent outcome of HBV infection. In an HBV mouse model, adult mice elicited a robust, diverse, long-lived HBV-specific T cell response and had increased numbers of IgG-expressing B cells in an IL-21 dependent manner, resulting in HBV clearance. Conversely, young mice and the absence of the IL-21R in adult mice resulted in Ag persistence (Publicover et al., 2011). Interestingly, IL-21 is also involved in the vertical transmission of HBV. Impaired generation of serum IL-21, Tfh cell, and plasma B cell are associated with vertical transmission of HBV to newborns, which can be improved by HBV vaccine booster-induced IL-21 production in an HBV mouse model (Vyas et al., 2019; Wang et al., 2021). Notably, the excessive or persistent inflammatory response of IL-21 leads to liver damage, HBV-related LC, and HCC (Liu B. et al., 2017; Wu et al., 2021). Taken together, a successful treatment strategy of IL-21 should be personalized tailor-made.

Th17-Associated Cytokines

Th17 cells are a subset of T helper cells that play a critical role in host defense against pathogens insult. Th17 cells arise from naïve Th0 cells initiated by IL-1, transforming growth factor-β1 (TGF-β1) and IL-6, combined with the activation of transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t, driving the development of various inflammation-related as well as autoimmune diseases (Dong, 2008). IL-17 and IL-22 are the main function executors of Th17 cells (Qu et al., 2013). Generation of the pathogenic Th17 cells requires IL-23 stimulation, and IL-23 amplifies the Th17 cell responses and causes inflammation injury (McKenzie et al., 2006). In CHB patients, Th17 cells are significantly elevated. They initiate immune-mediated pathogenesis and have a critical role in the process of HBV-related LC whose underlying mechanisms are greatly attributed to Th17-secreted cytokines.

Interleukin-17

IL-17 is an essential proinflammatory cytokine family that consists of six family members (IL-17A to IL-17F) encoded by separate genes, of which IL-17A is the representative effector cytokine secreted by Th17 (Jin and Dong, 2013). Expression of the IL-17R has been detected on almost all liver cell types (Ge and You, 2008), leading to the extensive and intricate pathogenesis process in the liver tissue during HBV infection. Similar to other proinflammatory cytokines mentioned above, peripheral and intrahepatic IL-17 was significantly increased in the setting of HBV infection, and correlated positively with the severity of liver damage and LC and HCC (Bao et al., 2017).

IL-17A suppresses HBV replication in a noncytopathic manner and over-expression of the antiviral proteins myxovirus resistance A and oligoadenylate synthetase mRNA (Wang B. et al., 2013). However, inappropriate, excessive, and non-specific Th17 effector responses are involved in the pathogenesis of liver damage, even liver failure. It has been described that the Th17-IL-17 axis is simultaneously the fuel and the flame of a sustained proinflammatory and profibrotic environment during CHB infection through a stepwise manner (Paquissi, 2017). During HBV infection, HBxAg-activated hepatic stellate cells (HSCs) recruit more Th17 cells into the liver that could, in turn, stimulate the proliferation and fibrotic marker secretion of the HSCs mediated by IL-17A and IL-22, forming a positive feedback loop that aggravates the progression of chronic liver disease with HBV infection (Tan et al., 2013; Zhang H. et al., 2020). In addition, counteraction between Th17 and Treg cell has been attached much attention by scientists (Su et al., 2013). The expression of Treg and

Th17 cells are both increased, but the ratio of Treg/Th17 is significantly decreased in patients with HBV infection (Feng et al., 2015). Reduced ratio of Treg cells and IL-10, TGF-β1 levels to Th17 cells and IL-17 levels correlate with HBV DNA suppression in CHB patients undergoing entecavir treatment, especially in the treatment response group (Zhang et al., 2010; Yu et al., 2013). In contrast, imbalance of Treg and Th17 cells might play an essential role in the occurrence, development, and outcome of CHB, although distinct pathways await elucidation (Su et al., 2013).

Interleukin-22

IL-22 is a member of the IL-10 family produced primarily by Th17 and NK cells (McAleer and Kolls, 2014). IL-22 is characterized by dual effects in the context of inflammation, and this has been attributed to its coexpression along with IL-17 (McAleer and Kolls, 2014). Evidence showed that IL-22 served as an effective adjuvant to enhance cellular immune responses during HBsAg DNA vaccination by inducing Tc17 cells to break tolerance in HBsAg transgenic mice (Wu et al., 2013). On the other hand, serum IL-22 levels and liver-infiltrating IL-22^+ cells increased stepwise from CHB to atypical hyperplasia and HCC, suggesting poor prognostic indicators for HCC (Shi et al., 2020). Additionally, IL-22 exacerbated chronic liver inflammation and fibrosis by CXCL10-and CCL20-recruited Th17 cells in HBV-infected patients and HBV Tg mice (Zhao et al., 2014). Collectively, the interpretation of the role of IL-22 in HBV infection requires prudent and comprehensive consideration for the specific disease status and distinct intrahepatic microenvironments.

Interleukin-23

IL-23, a member of the IL-12 family of cytokines with proinflammatory properties, is expressed principally by the macrophages and DCs, with the main biological functions of stimulation of DC antigen presentation, generation, and maintenance of Th17 cells (McKenzie et al., 2006). HBV induces IL-23 production in antigen-presenting cells and causes liver damage via the IL-23-IL-17 axis. HBsAg efficiently induces IL-23 secretion by myeloid DCs in a mannose receptor-dependent endocytosis manner while HBcAg-stimulated IL-23 secretion is mannose receptor- and endocytosis-independent (Wang Q. et al., 2013). Both IL-23 and IL-23R are remarkably elevated in biopsied liver tissues in HBV infection patients. Elevated IL-23 amplifies Th17 cell responses and liver inflammation. Furthermore, IL-23 upregulates IL-23R expressions on macrophages, enhancing macrophage-mediated angiogenesis (Wang Q. et al., 2013). Persistent IL-23 generation by liver inflammatory macrophages responding to damaged hepatocytes after chronic HBV infection alters macrophage function and shapes a pro-cancer milieu for HCC (Zang et al., 2018). Overall, these data provide mechanistic insights into the therapeutic potential of IL-23.

Interferon

The IFN family stands on the first line of defense activated upon viral infection and is paramount for controlling viral replication and dissemination. Although initially named after their function in interfering with viral replication, IFNs have more functional roles since being discovered for more than half a century.

IFNs bind IFN receptors on the surface of neighboring and/or immune cells, triggering a signaling cascade to induce a suite of IFN-stimulated genes (ISGs) that directly mediate the antipathogenic effects of IFNs (Schoggins et al., 2011). There are three distinct IFN families (IFN-I, -II, and -III), among which the well-characterized IFNs, IFN-α and IFN-β belong to the IFN-I family (McNab et al., 2015), and IFN-γ is the single gene product of the IFN-II family (Fenimore and Young, 2016).

Currently, IFNs are used therapeutically, with the most noteworthy example being the treatment of HBV and HCV infection, and this clinical practice has demonstrated the extraordinary value of IFNs. The fundamental mechanisms that control HBV infection with IFN-α treatment are relatively well recognized. IFN-α exerts both direct antiviral, which have been well elucidated (Sadler and Williams, 2008), and host immunomodulation effects (Biron, 2001). Specifically, IFN-α inhibits the HBV transcription and replication cycle by transcription and epigenetic modification pathways observed in human and mouse models (McNab et al., 2015). As refer to the immunoregulatory functions, numerous studies have suggested that IFN-α augments IL-27-dependent IFN-stimulated gene, induces spontaneous production of tumor necrosis factor-α (TNF-α), IL-1β (Ren et al., 2018) and improves IL-2 activity (Saxena et al., 1985), as well as triggers NK cell functionality and HBV-specific T cell responses (Bruder Costa et al., 2016). Of note, Tian et al. emphasized that effects of IFN-I on HBV replication were determined by viral load. IFN-I suppresses HBV replication when viral load is high and enhances HBV replication when viral load is low via transcriptional and post-transcriptional regulations (Tian et al., 2011). However, some patients suffer from IFN-α treatment resistance by inducing $CD24^+CD38^{hi}$ B cell and IFN-α/γ-STAT1-PD-L1 axis-mediated downregulating functions of T cells and NK cells (Fu et al., 2020; Liu et al., 2020) as well as producing anti-IFN-α Abs (Porres et al., 1989).

HBV-specific T cells execute function partially depending on IFN-γ. IFN-γ inhibits HBV replication and reduces cccDNA in hepatocytes by inducing deamination and cccDNA decay (Xia et al., 2016). HBV-specific IFN-γ producing $CD4^+$ T cells are associated with viral clearance (Wang et al., 2020). In addition, IFN-III also participates in HBV clearance. IFN-III-induced IL-10 plays a vital role in producing HBV restriction factor CBFβ (Xu et al., 2019), and nucleotide analogues show an additional pharmacological effect by inducing IFN-λ3 production, which further induces ISGs and results in a reduction of HBsAg production (Murata et al., 2018).

Tumor Necrosis Factor-Alpha

TNF-α is a potent proinflammatory cytokine mainly produced by monocytes and macrophages (Wajant et al., 2003). It elicits a particularly broad spectrum of cell proliferation, differentiation, and apoptosis in response to inflammation, infection, injury, and other environmental challenges (Baud and Karin, 2001). TNF-α

plays a dichotomous role in HBV infection, which acts as a mediator of anti-HBV immunity and induces liver inflammation, and sustained liver inflammation leads to liver fibrosis. It correlates with ongoing inflammation among chronic HBV patients with LC, which is likely attributed to the expression of biosignatures of apoptosis and activation in immune cells (Wang et al., 2020). It is reported that TNF-α producing cells are the dominant population among HBV-specific $CD4^+$ T cells and are associated with liver damage, but not viral clearance (Barathan et al., 2021). Of note, with the clinical application of anti-TNF-α therapy in inflammatory bowel and rheumatic diseases, CHB patients are faced with a challenge of reactivation of hepatitis (Lee YH. et al., 2013). Therefore, anti-HBc-positive patients undergoing anti-TNF therapy need to be carefully monitored, and prophylactic antiviral treatment is usually of great significance (Schwabe and Brenner, 2006).

ANTI-INFLAMMATORY CYTOKINES IN HEPATITIS B VIRUS INFECTION

Interleukin-10

IL-10 is a paramount regulatory cytokine that executes most, if not all, of the anti-inflammatory functions of the regulatory immune cells, namely Treg, T follicular regulatory (Tfr), Breg cells, and Myeloid-derived suppressor cells (Redford et al., 2011). It has a central role in infection by limiting the immune response to pathogens and preventing excessive immune activation and damage to the host. It also impedes pathogen clearance and leads to persistent infection. During CHB infection, serum IL-10 is elevated in the active immune group compared with immune tolerant, inactive carrier state, and HC groups. It is positively correlated with ALT and aspartate aminotransferase levels and hepatic flares (Wang et al., 2017). Moreover, increased circulating $IL\text{-}10^+$ Bregs and Tfr cells are associated with poor virus eradication and liver injury in CHB (Wang et al., 2014). IL-10-producing Breg cells suppress HBV-specific $CD4^+$ and $CD8^+$ T cell responses but enhance Treg cells in chronic HBV infection (Das et al., 2012). Nevertheless, studies also reveal the favorable characteristics of IL-10 on HBV infection. It was reported that higher serum levels of IL-10 in HBeAg-positive patients were correlated with early, spontaneous HBeAg seroconversion (Wu et al., 2010), and IL-10/HBV DNA ratio was identified as an early positive predictor for response to IFN-α treatment (Yan et al., 2015).

Interleukin-35

IL-35 is a relatively newly discovered member of the IL-12 cytokine family that has been shown to predominately exert an immunosuppressive effect on T cells (Neurath, 2008). While IL-10 and TGF-β are the most commonly studied immunosuppressive cytokines, the recently identified IL-35 is found to harbor the abilities of not only suppressing effector T cell responses directly (Tao et al., 2018) but also expanding regulatory responses by propagating infectious tolerance and generating a potent population of IL-35-expressing inducible Tregs (Olson et al., 2013). Specifically, IL-35 stimulation elevates viral-specific Tregs, accompanied by increased expression of Foxp3 mRNA and IL-10 production, and decreases IL-17 secretion and STAT3 phosphorylation in $CD4^+$ T cells, resulting in an imbalance of viral specific Treg/Th17 cells and thereby contributes to viral persistence (Yang et al., 2019). Additionally, IL-35 dampen non-specific and HBV-specific Th9 cells activity in HBV-related HCC patients (Zhang Q. et al., 2021). Accordingly, IL-35 might be pivotal for developing new therapeutic approaches for hepatitis B.

Transforming Growth Factor-Beta

TGF-β, another anti-inflammatory cytokine, plays a fundamental role in homeostasis through manipulating cell proliferation, extracellular matrix (ECM) synthesis and degradation, mesenchymal-epithelial interactions during embryogenesis, mediation of cell and tissue responses to injury, control of carcinogenesis, and modulation of immune functions (Verrecchia and Mauviel, 2002). TGF-β exerts dual regulatory functions in the immune system in response to HBV infection. TGF-β stimulates the differentiation of Th17 cells and thus, as mentioned above, favors inflammatory conditions. This contrasts sharply with its anti-inflammatory effects mediated by boosting the activities of Treg cells (Karimi-Googheri et al., 2014). The net benefits from TGF-β are context-dependent which may help to explain the conflicting studies about its role on HBV infection hitherto (Murata et al., 2009). Being recognized as a major profibrogenic cytokine, TGF-β signaling contributes to all stages of liver disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and HCC (Dooley and ten Dijke, 2012). HBV-encoded pX oncoprotein amplifies TGF-β family signaling through direct interaction with Smad4, which serves as a potential mechanism of hepatitis B virus-induced liver fibrosis (Lee et al., 2001). Indeed, functions exerted by TGF-β are not immutable, as it can shift from tumor suppression to oncogenesis accompanied by the tumor development and is adjusted by HBV (Massague, 2008; Giannelli et al., 2014).

Other Cytokines

In addition to the cytokines reviewed above, there are still many other cytokines that have directive implications in the possibility of designing immunotherapies for CHB patients. IL-7 secreted by inflamed hepatocytes, regulated TCR-mediated activation of human mucosal-associated invariant T cells enriched in the human liver, licensing them to dramatically increase Th1 cytokines and IL-17A production, which may benefit HBV eradication (Tang et al., 2013). A combination between IL-15 and IFN-α induces unprecedented efficacy of functional and specific cellular immunity in HBV transgenic mice (Di Scala et al., 2016). Furthermore, liver over-expression of IL-15 suppresses HBV replication in an IFN-β-dependent manner in mice (Yin et al., 2012). Besides, Jo et al. have evidenced that a combination between IL-12 and IL-18, secreted by monocytes, triggered activation of innate human cells in human liver, resulting the production of a high levels of IFN-γ (Jo et al., 2014). Likewise, IL-18 can inhibit HBV replication in the liver of transgenic mice (Kimura et al., 2002).

CHEMOKINES IN HEPATITIS B VIRUS INFECTION

Immune cells, as mentioned above, are the predominant source of various cytokines that continuously circulate between the lymph and blood under a homeostasis state. Once encounter pathogens, they take prompt reaction to migrate into the infection site to launch a battle. This is mainly mediated by chemokine ligand and receptor pair, which are critical to achieving precise and efficient immune response and are no exception in HBV infection. Chemokines, also known as chemotactic cytokines, are a family of small (8–12 kDa) cytokines or signaling proteins that exert their functions mainly by inducing directional movement of leukocytes and other cell types, including endothelial and epithelial cells (Raman et al., 2011). These small-molecule ligands are divided into four families based on the positioning of the conserved N-terminal cysteine residues: C, CC, CXC, and CX3C. They modulate biological processes consistently through interactions with seven-transmembrane G protein-coupled receptors (Zlotnik and Yoshie, 2000). The vast majority of known chemokines belong to the CC and CXC families. Of note, although chemokines are best known for their initially identified trafficking and guiding effects, they were later found to orchestrate a variety of additional processes, including the proliferation, differentiation, and activation of cellular responses (Atretkhany et al., 2016). Here, we will elucidate the roles of several fundamental chemokines on HBV infection.

The most critical CXC family chemokine ligand and receptor pairs related to HBV infection are CXCL9/10/11-CXCR3 and CXCL13-CXCR5. Levels of serum CXCL9, CXCL10, CXCL11, CXCL13 are elevated in patients with HBV infection compared with HCs (Kakimi et al., 2001; Keating et al., 2014). Interestingly, the increase of these chemokines happens at hepatitis with the subsequent decline of HBsAg in AHB patients and HBV-inoculated chimpanzees with HBsAg loss, indicating they might be hallmarks of functional cure of AHB or CHB patients (Yoshio et al., 2018). Numerous studies have identified the role of CXCL10, also termed IP-10, in the pathogenesis of HBV. Zhou et al. have demonstrated that HBx upregulates CXCL10 expression in a dose-dependent manner through activation of NF-κB, thereby enhancing the migration of peripheral leukocytes into the liver (Zhou et al., 2010). In an experiment exploiting an HBV transgenic mouse model, it is observed that blocking CXCL9 and CXCL10 reduces the recruitment of Ag-nonspecific lymphocytes as well as ameliorates the severity of liver diseases without affecting the effects of HBV-specific CTL (Kakimi et al., 2001; Sitia et al., 2002). On the other hand, CXCL10 is emphasized as a useful predictive indicator of disease progress and treatment response. Higher baseline serological and histological and slow reduction of CXCL10 levels indicates better prognoses in CHB patients with NUCs or Peg-IFN treatment (Sonneveld et al., 2013; Guo et al., 2016). Besides, pre-treatment CXCL9 level also has the potential to select CHB patients who can respond to Peg-IFN, especially in HBeAg-negative patients with low viral loads (Lee I.-C. et al., 2013). CXCL13, also known as B lymphocyte chemoattractant, is expressed by follicular DCs or stromal cells in lymphoid organs (Ohmatsu et al., 2007). CXCL13 is known for guiding homing of B cells, and subsets of T cells expressed CXCR5 to lymphoid follicles to form secondary lymphoid organs (Stone, 2017), and is critical for the onset and maintenance of humoral immunity. Our team previously focused on the role of CXCL13-CXCR5-mediated HBV-specific immune response. It demonstrated that increased expression of intrahepatic CXCL13 favored the recruitment of $CD19^+$ B cells and $CXCR5^+CD8^+$ T cells into the liver to shape a favorable anti-HBV immune milieu (Li et al., 2020). Besides, plasma CXCL13 can serve as a biomarker for GC activity (Havenar-Daughton et al., 2016). Increased plasma CXCL13 is distinctly observed in patients who achieve immune control of CHB infection (Liu C. et al., 2017). CXCL8 (IL-8) and CXCL12 (SDF-1) are two chemokines that have been relatively enthusiastically studied. An environment enriched in IL-7 and IL-15 licenses HBV-specific T cells to secrete CXCL8 (Gehring et al., 2011). Elevated levels of CXCL8 are associated with the severity of liver inflammation/fibrosis and resistance to IFN-α therapy (Yang et al., 2014). Moreover, HBV-induced IL8-CXCR1-TGF-β signaling cascade suppresses antitumor immunity against HCC by enhancing the accumulation of intrahepatic Treg cells and venous metastasis of hepatoma cells (Zhang C. et al., 2021). CXCL12-CXCR4 pathway is involved in recruitment and retention of immune cells in CHB patients with advanced liver fibrosis (Wald et al., 2004). HBx increases endoplasmic reticulum stress-dependent CXCL12 expression and induces HBV-induced immune cell recruitment into liver, with over 50% of liver-infiltrating lymphocytes expressing CXCR4. (Wald et al., 2004; Cho et al., 2014).Chemokines from the CC family also play a significant role in the pathogenesis of HBV infection. The CCL5 expression level in serum increases in CHB patients with aggravated liver injury and significantly decreases in cirrhosis patients with advanced liver fibrosis (Hu et al., 2019). A high expression score of CCL15 is significantly associated with the poor prognosis of HCC patients (Li et al., 2021c). CCL17 and CCL22 are induced by the contact of HBV-transfected cells with monocyte-derived DCs, which may favor the recruitment of Th17 and Tc17 cells to liver tissue in CHB (Zhang K. et al., 2020). A protective effect of CCR5Delta32 in recovery from an HBV infection is observed (Thio et al., 2007). Its ligand, CCL16 also shows an encouraging effect on inhibiting the progression of LC via inactivating HSCs (Zhuo et al., 2020). *In vitro* experiments show that CCL19 enhances the frequencies of Ag-responsive IFN-γ+$CD8^+$ T cells from patients by approximately twofold. This is further evidenced by mice overexpressing CCL19 with rapid clearance of intrahepatic HBV, likely through increasing intrahepatic $CD8^+$ T cells (Yan et al., 2021). In brief, chemokines from the CC family are critical mediators of HBV infection. Still, since the available studies are discrete and superficial, in-depth research is needed to clarify mechanisms and the possibility of therapeutic application.

DISCUSSION

Over the past half-century, tremendous progress has been made in understanding the regulation and functions of cytokines and chemokines in the liver. There is no doubt

that achieving HBV eradication relies on a well-organized immune response, which is orchestrated considerably by the spatial and temporal expression of cytokines and chemokines. With the assessment of cytokines and chemokines mentioned above, we are delighted to gain a variety of seemingly promising molecules with the predictive value of disease progression or control and immunotherapies target (**Figure 1** and **Table 1**). Actually, we yield far less than expected when transforming them into clinical applications. The measurement of serum or plasma levels of cytokines and/or chemokines is far from established to be used in daily clinical practice for CHB patients. Many chemokines have not been evaluated in-depth, and tailoring the dose of certain cytokine/chemokine administered has crucial implications in optimizing results. In addition, an important block to our understanding of HBV pathogenesis lies in dissecting the critical aspects of the cytokines and chemokines interplay in light of the conditional role these molecules play throughout infection and disease development. It is unreasonable to define the beneficial effects of a single cytokine/chemokine since a few of them play a unique and non-redundant character, and most of them share overlapping and redundant effects. In light of these considerations, defining the fundamental roles of cytokines and chemokines in HBV infection will require the basis of different species, anatomical location, and stages of liver disease development, in combined with the application of more definitive, standard tools as well as strict sample inclusion criteria, which is also meaningful for the design of clinical practice. Therefore, continued research is essential to understand better the complexity of mechanistic pathways and the pleiotropic interactions of cytokines and chemokines. Through the joined hands of scientists from different disciplines, we will eventually be able to win a future without hepatitis B.

AUTHOR CONTRIBUTIONS

SZ and LT wrote the manuscript. TZ extracted the data. LT and YL supervised the whole paper. All authors read and approved the final manuscript.

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The role of Mesothelin signaling in Portal Fibroblasts in the pathogenesis of cholestatic liver fibrosis

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Liver fibrosis develops in response to chronic toxic or cholestatic injury, and is characterized by apoptosis of damaged hepatocytes, development of inflammatory responses, and activation of Collagen Type I producing myofibroblasts that make liver fibrotic. Two major cell types, Hepatic Stellate Cells (HSCs) and Portal Fibroblasts (PFs) are the major source of hepatic myofibroblasts. Hepatotoxic liver injury activates Hepatic Stellate Cells (aHSCs) to become myofibroblasts, while cholestatic liver injury activates both aHSCs and Portal Fibroblasts (aPFs). aPFs comprise the major population of myofibroblasts at the onset of cholestatic injury, while aHSCs are increasingly activated with fibrosis progression. Here we summarize our current understanding of the role of aPFs in the pathogenesis of cholestatic fibrosis, their unique features, and outline the potential mechanism of targeting aPFs in fibrotic liver.

Keywords: cholestatic liver fibrosis, activated portal fibroblasts, mesothelin (MSLN), mucin 16 (MUC16), thymocyte differentiation antigen 1 (Thy-1)

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INTRODUCTION

Hepatic fibrosis is the outcome of chronic liver diseases, including cholestatic liver disease (primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), and secondary biliary cirrhosis (SBC)) (Lazaridis and LaRusso, 2016) and toxic liver injury (hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease and non-alcoholic steatohepatitis (NASH)) (Friedman, 2008; Dranoff and Wells, 2010). It is characterized by extensive deposition of extracellular matrix (ECM). Activated hepatic myofibroblasts, which are absent in the healthy liver, are the major source Collagen Type I which form the fibrous scar (Friedman, 2008). Hepatic stellate cells (HSCs) and portal fibroblasts (PFs) are believed to serve as the major source of the fibrous scar in the injured liver (Bataller and Brenner, 2005).

Cholestatic fibrosis is caused by chronic cholestatic injury (Lazaridis and LaRusso, 2016), hepatocyte apoptosis, ductular proliferation, inflammation, and activation of myofibroblasts. Both activated PFs (aPFs) and activated HSCs (aHSCs) (Dranoff and Wells, 2010) can produce myofibroblasts that drive cholestatic fibrosis. Despite extensive studies, the origin and contribution of hepatic myofibroblasts to cholestatic fibrosis remains controversial. Several studies in humans and

Abbreviations: Msln, mouse mesothelin (human counterpart MSLN); Muc16, mouse mucin 16 (human counterpart CA125); Thy1, mouse thymocyte differentiation antigen 1 (human counterpart THY1).

experimental models of cholestatic fibrosis implicated aPFs in the pathogenesis of cholestatic fibrosis, suggesting that aPFs might serve as the primary targets for anti-fibrotic therapy (Dranoff and Wells, 2010; Wells, 2014). In support, aPFs contribute to the fibroproliferative responses in patients with primary and secondary biliary cirrhosis (PSC and SBC), but not in patients with toxic liver fibrosis such as HBV/HCV (Koyama et al., 2017).

Under the physiological conditions, PFs comprise a small population of cells that surround the portal vein to maintain integrity of the portal tract (Dranoff and Wells, 2010). Cholestatic (but not toxic) injury (Desmoulière et al., 1997) causes their proliferation and differentiation into Collagen Type I-producing myofibroblasts((Dranoff and Wells, 2010), (Desmoulière et al., 1997), (Yata et al., 2003)), suggesting that aPFs are the "first responders" to the cholestasis-induced fibrogenic liver injury. Using the reporter Col-GFP mice (in which Collagen-1α(I) promoter drives expression of the GFP reporter gene in real time), aPFs were shown to comprise 70% of myofibroblasts at the onset of cholestatic fibrosis caused by the obstruction of the common bile duct (BDL), that mimics mechanical bile duct occlusion by liver stones or tumor mass. Similar results were obtained using another model of cholestatic injury, $Mdr2^{-/-}$ mice (Smit et al., 1993) (deficient for canalicular phospholipid flippase, Mdr2/Abcb4), which develop disruption of bile duct tight junctions and basal membranes, causing bile leakage, and periportal cholestatic fibrosis (Smit et al., 1993) that resembles PSC (Fickert et al., 2002; Fickert et al., 2004; Popov et al., 2005; Fickert et al., 2009; Baghdasaryan et al., 2010; Mair et al., 2010), and mimics MDR2 deficiency in patients (Jacquemin, 2001; Fickert et al., 2004; Popov et al., 2005). Moreover, cholestasis-activated aHSCs share more resemblance with aPFs than with CCl_4-activated aHSCs, suggesting that fibrogenic responses caused by cholestatic fibrosis differ significantly from those induced by toxic injury, and therefore the mechanism of the cholestatic fibrosis progression should be studied in further detail (Iwaisako et al., 2014).

The contribution of aPFs to liver fibrosis of different etiologies remains not well understood, mainly because of difficulties with the isolation of PFs and myofibroblasts. The most widely used method of aPF isolation is based on enzymatic digestion followed by size selection (Wen et al., 2012), as well as cell outgrowth from dissected bile and enzymatically digested liver segments(Uchio et al., 2002), (Kruglov et al., 2002). aPFs are identified by expression of Elastin, Col1a1, and other fibrogenic genes. Expression of specific markers such as Thy-1 (Knittel et al., 1999; Dudas et al., 2007; Yovchev et al., 2009; Katsumata et al., 2017), Fibulin 2 (Knittel et al., 1999), IL-6, Elastin (Goodpaster et al., 2008), the ecto-AT-Pase nucleoside triphosphate diphosphohydrolase-2 (NTPD2) (Dranoff et al., 2002), and coffilin 1 (Bosselut et al., 2010) was originally identified in aPFs, demonstrating that these cells are different from desmin, cytoglobin, GFAP, $p75^{NGFr}$, and Vitamin A expressing HSCs (Bataller and Brenner, 2005; Dranoff and Wells, 2010; Fausther and Dranoff, 2011). The development of flow cytometry-based techniques made it possible to sort purify the population of hepatic Col-GFP^{+}Thy-$1^{+}$$VitaminA^{-}$$CD45^{-}$ aPFs, which can be distinguished from Col-GFP^{+}Thy-$1^{-}$$VitaminA^{+}$ aHSCs, and identified new markers of aPFs such as Mesothelin (Msln), Muc16, CD34, Gpc3, Asporin, Bnc1 (Iwaisako et al., 2014; Nishio et al., 2021). Moreover, Msln was shown to critically regulate fibrogenic activation and proliferation of aPFs in response to cholestatic injury. This review will summarize the potential role of Msln-Thy-1 and Muc16 signaling in the activation of aPFs in experimental models of cholestatic fibrosis, and discuss the emerging strategies to target aPFs to treat cholestatic liver fibrosis.

CHOLESTATIC LIVER FIBROSIS

The etiology of cholestatic injury differs considerably from toxic liver injury. Cholestatic injury results from genetic defects or mechanical injury of the bile ducts, causing impaired hepatobiliary production and excretion of bile, accumulation of bile and liver tissue damage, apoptosis and proliferation of mature cholangiocytes and hepatocytes, inflammation, and biliary fibrosis (Fickert et al., 2009; Vavassori et al., 2009; Wagner et al., 2010). Several experimental models are routinely used to dissect the mechanism of cholestatic fibrosis, such as $Mdr2^{-/-}$ mice (Smit et al., 1993) and BDL. Despite different etiologies, these models exhibit common pathophysiological features. Reversal of the etiological cause of cholestasis may result in regression of liver fibrosis.

ACTIVATED PORTAL FIBROBLASTS PLAY A KEY ROLE IN CHOLESTATIC LIVER FIBROSIS

Activation of fibrogenic Collagen Type I producing myofibroblasts is the key event leading to the progression of cholestatic fibrosis. Myofibroblasts are characterized by a spindle or stellate shape and expression of abundant intracellular proteins (vimentin, α-smooth muscle actin (α-SMA), non-muscle myosin) (Eyden, 2008), rough endoplasmic reticulum (rER) and a Golgi apparatus producing collagen (Gabbiani et al., 1971; Majno et al., 1971; Schürch et al., 1998; Eyden, 2008).

The Origin of Myofibroblasts in Cholestatic Liver Fibrosis

The cell that secretes the fibrillary collagens leading to cholestatic fibrosis has a long and controversial history (Dranoff and Wells, 2010; Mederacke et al., 2013). Due to lineage tracing studies by our lab (Iwaisako et al., 2014; Koyama et al., 2017) and others (Asahina et al., 2009; Asahina et al., 2011), there is a clear consensus that endogenous mesenchymal cells activate to become myofibroblasts that secrete the fibrous scar proteins. Fate mapping studies have also demonstrated that epithelial mesenchymal transition (EMT) (Scholten et al., 2010; Taura et al., 2010; Chu et al., 2011), or recruited fibrocytes (Kisseleva et al., 2006; Scholten et al., 2011; Iwaisako et al., 2014) are not major contributors to the myofibroblast population. In turn, two hepatic mesenchymal cells become myofibroblasts depending on

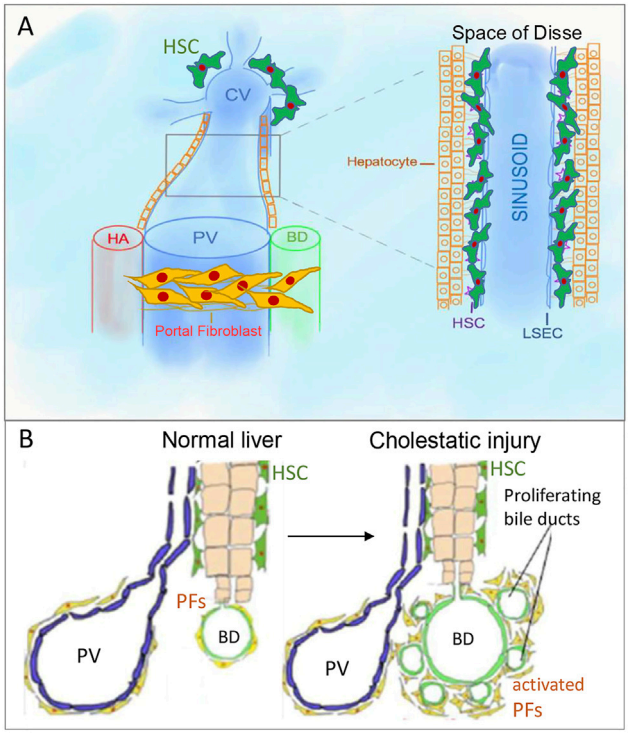

FIGURE 1 | Portal fibroblasts/myofibroblasts (aPFs/MFs) and hepatic stellate cells (HSCs). **(A)**. PFs are located around portal triads, while HSCs are located in the space of Disse, which is between sinusoidal endothelial cells and hepatocytes cluster. **(B)**. Bile ducts proliferate in response to bile duct ligation, known as "ductular reaction." PV, portal vein, CV, central vein, HA, hepatic artery, BD, bile duct.

the fibrotic stimulus (Iwaisako et al., 2014). Hepatotoxic liver injury activates HSCs to become myofibroblasts, while cholestatic liver injury activates both HSCs and aPFs (Dranoff et al., 2002; Kruglov et al., 2002; Wen et al., 2012). aPFs comprise 70% of myofibroblasts at the onset of bile duct ligation (BDL)-induced injury, while aHSCs are increasingly activated with fibrosis progression (Iwaisako et al., 2014; Karin et al., 2016) (**Figures 1A,B**).

Hepatic Stellate Cells

Under physiological conditions, quiescent HSCs express desmin, neural markers (glial fibrillar acidic protein (GFAP), synaptophysin (Bataller and Brenner, 2005), NGF receptor p75 (Sachs et al., 2007; Kendall et al., 2009)), and Vitamin A droplets((Iredale, 2007; Geerts, 2001; Senoo et al., 2007)) and reside in the space of Disse (**Figure 1A**), but in response to injury differentiate into aHSCs/myofibroblasts expressing vimentin, and collagens (Kisseleva and Brenner, 2006; Fallowfield et al., 2007).

Portal Fibroblasts

In normal liver, portal fibroblasts (PFs) comprise a small population of "periductular mesenchymal cells" that surround the portal vein and maintain integrity of the portal tract (**Figure 1B**) (Desmoulière, 2007; Dranoff and Wells, 2010; Wells, 2014). In response to cholestatic injury (but not toxic carbon tetrachloride (CCl_4)-induced injury) (Desmoulière et al., 1997), activated portal fibroblasts (aPFs) proliferate, upregulate Col1a1, TIMP1, Spp1, TGFβRI, TGFβ2, and secrete extracellular matrix (ECM) (Desmoulière et al., 1997; Yata et al., 2003; Dranoff and Wells, 2010). aPFs are identified by expression of Thy-1((Knittel et al., 1999; Dudas et al., 2007; Yovchev et al., 2009)), Fibulin 2 (Knittel et al., 1999), Elastin (Goodpaster et al., 2008), NTPD2 (Dranoff et al., 2002), coffilin 1 (Bosselut et al., 2010), Msln, Muc16, Apsorin, Bnc1, Upk1β, Calca, Gpc3 ((Koyama et al., 2017), (Iwaisako et al., 2014)). We have recently demonstrated that Msln, Muc16 (Koyama et al., 2017), and Thy-1 (Katsumata et al., 2017) play a critical role in regulation of aPF biology.

UNIQUE FEATURES OF ACTIVATED PORTAL FIBROBLASTS

Based on gene expression profiling, BDL-activated aPFs expressed genes that distinguish them from CCl_4-activated aHSCs, and were identified as "signature genes" for aPFs. In concordance with previous studies (Kawada et al., 2001; Bosselut et al., 2010), aPF signature genes included Thy-1, Elastin, Gremlin 1, Fibulin 2, and NTPD2 (Dranoff and Wells, 2010; Forbes and Parola, 2011), but also the newly identified genes, Msln, and Muc 16, Calca, Upk1β, Bnc1 and others. Human $MSLN^{+}THY1^{+}\alpha SMA^{+}$ aPFs also express aPF-specific markers (UPK1b, CD200, EMILIN2, BNC1, ASPN, GPC3, and GREM1) similar to that observed in mouse aPFs, suggesting that upregulation of these specific genes in activated PFs is preserved among species. Some of these genes Msln, Calca, Upk1β, Bnc1 were reported as signature genes of murine hepatic mesothelial (Onitsuka et al., 2010) and epicardial cells (Bochmann et al., 2010), supporting the theory that PFs originate from mesothelial cells (Asahina et al., 2009; Asahina, 2012). Expression of Msln and Muc16 is detected in $Thy\text{-}1^{+}$ aPFs but not in qHSCs, aHSCs, endothelial cells (EC), Kupffer cells (KC), or cholangiocytes. The fact that expression of Msln was detected only in isolated aPFs but not in other liver fractions suggests (Iwaisako et al., 2014) that Msln expression might be important for aPF biology.

HISTORICAL CHARACTERIZATION OF MSLN, CA125 AND THY-1

Mesothelin

Msln (Chang and Pastan, 1996) is Glycosylphosphatidyl inositol (GPI)-linked membrane-anchored protein (71 kDa, Msln precursor). Originally, MSLN was identified as a tumor marker. Human MSLN is strongly upregulated in several human malignancies, including mesotheliomas and ovarian cancer, and is a target for anti-cancer therapy. Anti-MSLN Abs have been generated and are being tested in clinical trials in patients with ovarian cancer.

Mucin 16

Muc16 is the murine analogue of human CA125 (McMullen et al., 2005). Studies of patients with ovarian cancer have identified the cancer antigen CA125 as a Msln ligand (Pastan and Hassan, 2014), which is widely used as a diagnostic marker (with the exception of liver and lung cirrhosis which are considered as "false positives" (Scholler and Urban, 2007). CA125 is a member of the membrane-tethered family of mucins, which contains a transmembrane domain with a short cytoplasmic domain, and highly glycosylated at N-terminus (Pastan and Hassan, 2014) and is a MSLN ligand (Gubbels et al., 2006; Kaneko et al., 2009).

Msln-Muc16 Signaling in Cancer Cells

Since its discovery in 1992 as a cancer antigen, the mechanism of human MSLN signaling remains unresolved. Until recently, CA125 (mouse Muc16) remained the only known ligand of MSLN that activates Src/Akt signaling in cancer cells. In cancer cells MSLN-Muc16 signaling increases cancer cell proliferation and metastasis. Msln-mediated secretion of MMP-7 in MUC16-expressing cancer cells occurs via a p38 MAPK-dependent pathway. Depletion of MMP-7 or inhibition of p38 activity abolishes MSLN-mediated cancer cell motility and invasion. Knockdown of Msln suppresses tumor invasiveness in xenograft models in mice (He et al., 2017). Although, $Msln^{-/-}$ and $Muc16^{-/-}$ mice have a normal phenotype until injury or stress (Bera and Pastan, 2000; McMullen et al., 2005), when subjected to experimental model of liver cancer, Msln-knockout mice developed a defect in activation of cancer associated myofibroblasts (Zhang et al., 2011).

Msln as a Mesothelial Marker

Expression of Msln is not restricted to cancer cells or cancer-associated myofibroblasts but is also induced in aPFs. Msln also serves as a mesothelial cell marker (Pastan and Hassan, 2014). Msln is highly expressed during embryonic development (Majno et al., 1971; Iwaisako et al., 2014) but minimally expressed in adulthood (Pastan and Hassan, 2014). In adult mice and humans, Msln-expressing stem-like cells reside in the mesothelial layer lining of parenchymal organs and serosal cavities (Bera and Pastan, 2000) in a dormant state, and do not proliferate until injury or stress, and have a capability to give rise to the mesenchymal and mesothelial cells, as well as fibroblasts.

Thy-1 (CD90, Cluster of Differentiation 90)

Thy-1 is a 25–37 kDa heavily N-glycosylated (GPI)-linked cell surface protein (Nosten-Bertrand et al., 1996), with a single V-like immunoglobulin domain, originally discovered as a thymocyte antigen.Thy-1 is a GPI-anchored protein (like Msln) (Nosten-Bertrand et al., 1996) expressed in fibroblasts, T cells and neurons, and considered to be a specific marker for these cell types. Thy-1 was implicated in inhibition of TGFβ1 responses in tissue fibroblasts. Studies of lung fibroblasts have demonstrated that deletion of Thy-1 in mice exacerbated bleomycin-induced lung fibrosis (Ramírez et al., 2011). Thy-1 was shown to signal via the Src-family kinase (SFK) and focal adhesion kinase (FAK) pathways (Bradley et al., 2009) to prevent TGFβ1-induced fibroblast activation (Koyama et al., 2017) and inhibition of extracellular activation of tissue-associated latent TGF-β1 via interaction with αv-β5 integrins at the cell surface (Zhou et al., 2010), suggesting that Thy-1 can function as a mechanosensor (Fiore et al., 2015). Thy-1 expression in murine lung fibroblasts is decreased with fibrosis progression (McIntosh et al., 1994; Hagood et al., 1999; Hagood et al., 2005; Sanders et al., 2007; Zhou et al., 2010; Sueblinvong et al., 2012). Thy-1 also modulates lipid raft-associated signaling promoting fibroblast adhesion and limiting migration (Bradley et al., 2009).

Thy-1 in Fibroblasts was Linked to Fibrosis

Thy-1 is silenced in lesional fibroblasts in IPF (Idiopathic Pulmonary Fibrosis), and its expression in murine lung fibroblasts is decreased with progression of experimental bleomycin induced lung fibrosis (Hagood et al., 2005; Sueblinvong et al., 2012). Thy-1 acts as a fibrosis suppressor which prevents differentiation of lung fibroblasts into myofibroblasts (including Collagen Type I expression, cytokine and growth factor expression, migration, and cell survival). Upon activation, lung myofibroblasts upregulate TGFβ1-responsive genes (Activin and PAI-1) but downregulate expression of Thy-1 (McIntosh et al., 1994; Hagood et al., 1999; Hagood et al., 2005; Sanders et al., 2007; Zhou et al., 2010). Deletion of Thy-1 exacerbates development of cholestatic fibrosis in mice (Koyama et al., 2017; Nishio et al., 2021).

MSLN SIGNALING PLAYS A CRITICAL ROLE IN ACTIVATION AND PROLIFERATION OF ACTIVATED PORTAL FIBROBLASTS

The molecular mechanisms underlying Msln signaling in experimental models of cholestatic fibrosis have been evaluated, and demonstrated that in addition to Muc16, Msln can also bind to Thy1 in aPFs and form a signaling Msln-Muc16-Thy-1 complex that regulates fibrogenic activation and proliferation of aPFs.

$Msln^{-/-}$ and $Muc16^{-/-}$ Mice are Protected From Cholestatic Liver Fibrosis

Although, $Msln^{-/-}$, $Muc16^{-/-}$, and $Thy\text{-}1^{-/-}$ mice exhibit no obvious abnormalities under physiological conditions (Bera and Pastan, 2000; McMullen et al., 2005), these molecules play a critical role in the pathogenesis of cholestatic fibrosis. Thus, cholestatic fibrosis (caused by BDL or Mdr2 deficiency) was strongly attenuated by ≈ 50% in Msln knockout mice ($Msln^{-/-}$ mice). *In vitro* analysis revealed that Msln regulates TGFβ1-inducible activation of the wild type aPFs, and facilitates their FGF-FGFRI-Act-mediated aPF proliferation (via inhibition of FGFRI turnover and re-expression). Similarly, deletion of Muc16 (the binding partner of Msln and potentially the only transmembrane signaling molecule in this complex) also attenuates development of cholestatic fibrosis, outlining the

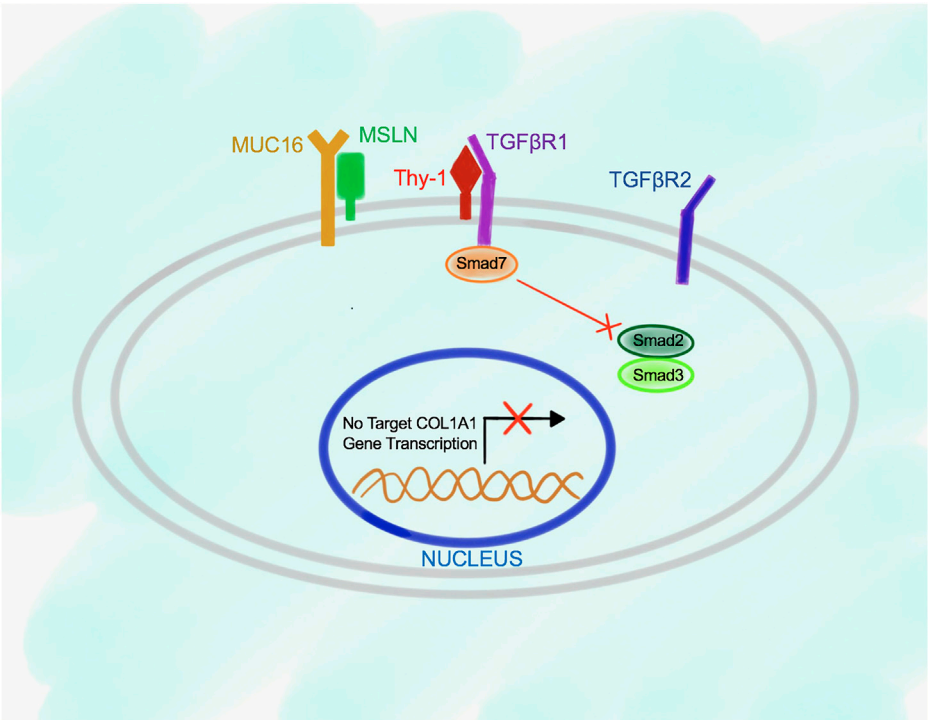

FIGURE 2 | Proposed model of Msln-Muc16 and Thy-1-TGFβRI binding in resting wild type aPFs. Msln and Muc16 form a complex in resting aPFs. Thy-1 and TGFβRI form a complex. Binding of Thy-1 to TGFβRI prevents TGFβ1 signaling, and retains Smad7 at the C-terminus of the TGFβRI.

importance of Msln-Muc16 interaction. Moreover, ductular proliferation was reduced in cholestasis-injured Msln$^{-/-}$Mdr2$^{-/-}$ mice and Muc16$^{-/-}$Mdr2$^{-/-}$ mice, suggesting that aPF activation regulates cholangiocyte proliferation.

Thy-1$^{-/-}$ mice are more susceptible to cholestatic fibrosis. Studies of the experimental models of cholestatic fibrosis in wild type, Msln$^{-/-}$ mice, Muc16$^{-/-}$ mice, and Thy-1$^{-/-}$ mice have demonstrated that Msln and Muc16 play pro-fibrogenic roles in aPF activation, while Thy-1 exhibits anti-fibrogenic properties. Consistently, cholestatic fibrosis is exacerbated in Thy-1$^{-/-}$ mice. These findings were supported by *in vitro* comparison of primary isolated mouse wild type, Msln$^{-/-}$, Muc16$^{-/-}$, and Thy-1$^{-/-}$ aPFs. In resting aPFs, Thy-1 directly binds to TGFβRI and blocks TGFβ1 binding to TGFβRI, thereby preventing TGFβ1 signaling.

MSLN, MUC16 AND THY-1 REGULATE NON-CANONICAL TGFβ1-TGFβRI SIGNALING IN CHOLESTASIS-ACTIVATED PORTAL FIBROBLASTS

Formation of Thy-1-TGFβRI in Resting aPFs Prevents TGFβ1 Signaling

The relationship between Msln, Muc16, Thy-1, and TGFβRI receptors in the wild type and Msln$^{-/-}$ aPFs was established using immunoprecipitations (IPs) with specific antibodies against each molecule. Although not quantitative, this technique allowed to determine the dynamic changes in the protein binding between Msln, Muc16 and Thy-1 in the resting wild type aPFs and in response to TGFβ1 stimulation. We have demonstrated that in resting (serum starved) aPFs Thy-1 makes an inhibitory complex with TGFβRI receptor thereby preventing TGFβ1 binding to the N-terminus of TGFβRI. Thy-1 also binds to Muc16 but has minimal interaction with Msln (**Figure 2**). Meanwhile, Msln forms a strong complex with Muc16, suggesting that Muc16 transmits intracellular signals from Msln-Muc16 complex. TGFβ1 signaling is further inhibited by Smad7 (transcription factor implicated in suppression of TGFβ1 signaling), which is bound to the C-terminus of the TGFβRI and prevents Smad2/3 docking and phosphorylation on TGFβRI.

TGFβ1 Signaling in aPFs Promotes Disruption of Thy-1-TGFβRI Complex and Formation of Msln-Muc16-Thy-1 Complex

In turn, in response to stimulation of the wild type aPFs with TGFβ1, binding of TGFβ1 to TGFβRI strongly increases the affinity of Msln to Thy-1 causing dissociation of Thy-1 from TGFβRI (**Figure 3**). Formation of Msln-Muc16-Thy-1 complex results in disruption of Thy-1-TGFβRI interaction and removal of Thy-1 from TGFβRI. TGFβI binds to TGFβRI and TGFβRII, causing dissociation of Smad7 from TGFβRI and subsequent binding of Smad2/3 to the C-terminus of TGFβRI where these transcription factors are phosphorylated and activated.

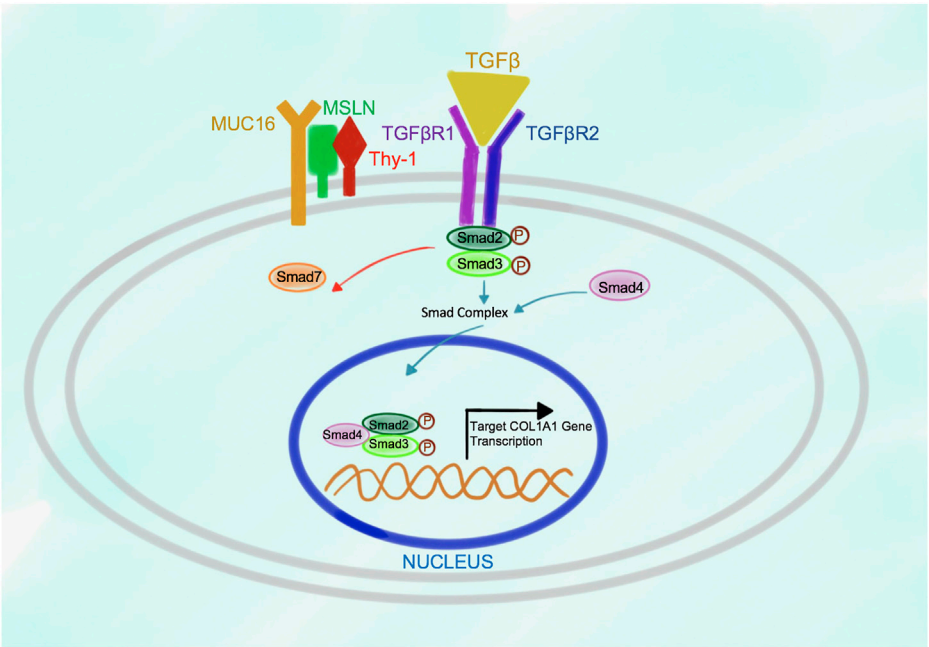

FIGURE 3 | Proposed model of Msln-Muc16-Thy-1 binding in TGFβ1-stimulated wild type aPFs. In response to TGFβ1 signaling Msln-Muc16 complex binds to Thy-1 causing dissociation of Thy-1 from TGFβRI. TGFβ1 binding to TGFβRI and TGFβR2 causes receptor crosslinking, docking of Smad2/3 to the receptors. Upon Smad2/3 phosphorylation, *p*-Smad2/3 dissociates from the receptors, forms a complex with Smad4, and translocates to the nucleus where it initiates transcription of target genes.

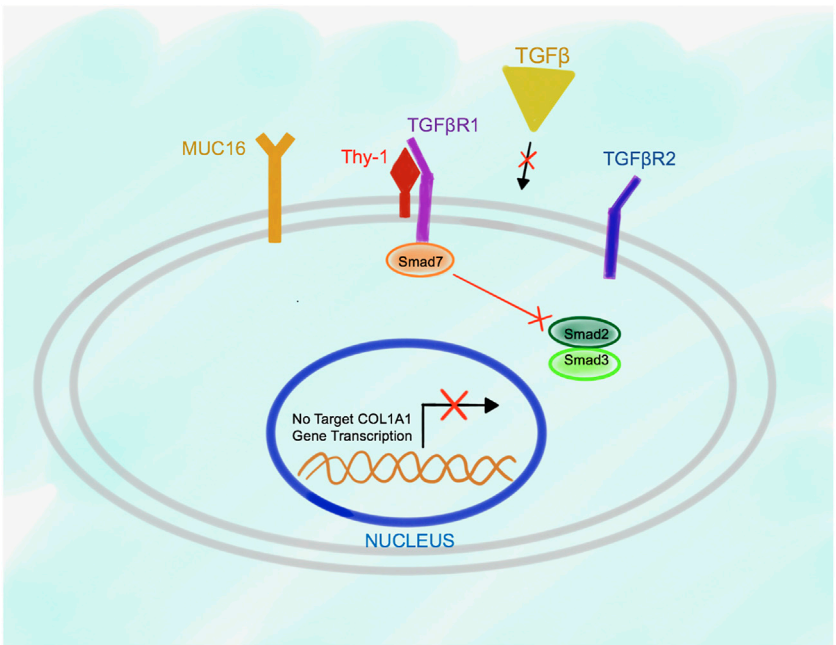

FIGURE 4 | Proposed model of Msln-Muc16-Thy-1-TGFβRI signaling in Msln$^{-/-}$ aPFs. TGFβ1 signaling is impaired in Msln$^{-/-}$ aPFs because Thy-1 forms a stable complex with TGFβRI, which hinders TGFβ1 binding to TGFβRI and TGFβR2. Smad7 is bound to the cytoplasmic tail of TGFβRI, thereby preventing docking and phosphorylation of Smad2/3.

Phosphorylated Smad2/3 are released from TGFβRI into the cytoplasm where they form a complex with Smad4. *p*-Smad2/3-Smad4 are translocated to the nucleus, where they bind to the DNA and initiate transcription of the fibrogenic genes, including Collagen Type I.

TGFβ1-TGFβRI Signaling is Suppressed in Msln-Deficient aPFs

Deletion of Msln results in suppression of TGFβ1-TGFβRI signaling in aPFs due to increased Thy-1 expression, and higher affinity of Thy-1 binding to TGFβRI (than in the wild type aPFs), indicating that Thy-1 serves as an inhibitory molecule for the TGFβ1 signaling in aPFs (**Figure 4**). Under these circumstances, Smad7 is constitutively bound to the C-terminus of TGFβRI, suggesting that lack of Msln (or increased Thy-1-TGFβ1RI binding) promotes Smad7 docking to the cytoplasmic C-terminus of the TGFβRI. As a result, activation and phosphorylation of Smad2/3 is reduced in $Msln^{-/-}$ aPFs; production of fibrogenic genes and Collagen Type I is suppressed.

TGFβ1-TGFβRI Signaling is Accelerated in Thy-1-Deficient aPFs

Moreover, deletion of Thy-1 in aPFs results in strong overexpression of Msln in $Thy\text{-}1^{-/-}$ aPFs, indicating that Thy-1 is a critical regulator of Msln. Indeed, $Thy\text{-}1^{-/-}$ aPFs produce more Col1a1 mRNA in response to TGFβ1 stimulation, and this effect is associated with increased phosphorylation of Smad2/3 and expression of TGFβRI, while binding of Smad7 to TGFβRI is decreased in $Thy\text{-}1^{-/-}$ aPFs. We speculate that genetic deletion of **Thy-1** gene results in exacerbation of Msln signaling caused by the compensatory overexpression of Msln and its target genes. It remains unknown if this effect can be solely attributed to the strong upregulation of Msln (≈7 fold over the wild type aPFs) in $Thy\text{-}1^{-/-}$ aPFs, and/or the loss of Thy-1 functions (such as binding to TGFβRI suppression of Msln expression). Since Thy-1 is a GPI-linked protein, Thy-1 might bind to another transmembrane signaling receptor (distinct from Muc16), or utilize the lipid rafts protein signaling to mediate its function.

TGFβ1-TGFβRI Signaling is Not Affected in Double Knockout $Msln^{-/-}Thy\text{-}1^{-/-}$ aPFs

Generation of double knockout $Msln^{-/-}Thy\text{-}1^{-/-}$ aPFs revealed that Thy-1 and Msln might regulate one signaling pathway, since simultaneous deletion of Msln and Thy-1 abolished both phenotypes, and double knockout $Msln^{-/-}Thy\text{-}1^{-/-}$ aPFs exhibited no obvious abnormalities. In support, simultaneous deletion of Msln and Thy-1 genes yielded a phenotype similar to that in the cholestasis-injured wild type mice, indicating that Msln and Thy-1 might regulate opposing functions within the same signaling pathway. These new findings suggest that Msln-Muc16-Thy-1 signaling plays an important role in the regulation of TGFβ1-TGFβRI signaling in cholestasis-activated aPFs.

MSLN AS A TARGET FOR ANTI-FIBROTIC THERAPY

Thy-1^{+} and $Msln^{+}$ aPFs are Expressed in Livers of Patients With Cholestatic Liver injury but not Toxic HCV Fibrosis

When the composition of myofibroblasts was analyzed in livers of patients with liver fibrosis, the expression of MSLN and THY-1 was upregulated in livers of PSC patients, patients with biliary atresia, and biliary cirrhosis (but not in livers of patients with HCV liver fibrosis). Expression of human THY-1 and MSLN correlated with the stage of cholestatic fibrosis, suggesting that $MSLN^{+}$ aPFs can be a novel target for anti-fibrotic therapy. Msln is widely expressed in embryonic mesothelium during mammalian development (Akira et al., 2006). In turn, Msln is minimally expressed in adult mice and healthy humans under physiological conditions. Upregulation of MSLN in adult humans is associated with cancer, and was recently linked to the development of cholestatic fibrosis (Pastan and Hassan, 2014).

Potential Strategies to Target aPFs

Historically high expression of MSLN was linked to increased tumor proliferation/invasion. Therefore, Msln serves as a target for anti-cancer therapy. We tested if targeting MSLN could also be beneficial for halting cholestatic fibrosis. Three classes of potential Msln inhibitors have been generated and potentially used to block MSLN-MUC16-THY-1 signaling pathway in patients: anti-human MSLN Ab-immunotoxin (that causes death of human $MSLN^{+}$ cancer cells) (Hassan et al., 2007); anti-MSLN blocking Abs can potentially suppress growth and proliferation of aPFs (Onda et al., 2005); or recombinant human soluble THY1 (hsTHY1, that neutralize reactivity to αv-β5 integrins, and bind to TGFβRI to prevent MSLN signaling) (Tan et al., 2019). These tools can potentially be used in patients with cholestatic fibrosis.

Immunotherapy to Target Cancer Cells

Immunotherapy-based strategy to target human cancer cells was developed by Dr. Pastan and colleagues, pioneers in the field of cancer research. Specifically, much progress has been made with immunotherapy-based therapeutics of human $MSLN^{+}$ malignancies. MSLN is differentially expressed between normal and cancer cells, thus making it a strong candidate for anti-cancer therapy with recombinant immunotoxins (RITs) (Liu et al., 2012). Several generations of immunotoxins, such as SS1P and LMB100, were engineered by conjugation of anti-human MSLN SS1 Ab (Hassan et al., 2007; Hassan et al., 2014) to PE38 (truncated *Pseudomo-nas* exotoxin, that causes cellular apoptosis) (Hassan et al., 2000), and successfully tested in clinical trials in patients with mesothelioma, ovarian cancer and pancreatic cancer (Liu et al., 2012; Kreitman et al., 2009; Chowdhury and Pastan, 1999; Alewine et al., 2014))(https://clinicaltrials.gov/ct2/show/NCT02810418) (Hassan et al., 2007; Hassan et al., 2014; Kreitman et al., 2009). In detail, SS1(dsFv)PE38 (SS1P) is a RIT that consists of a modified bacterial toxin

Pseudomonas exotoxin A (PE38) that is bound to the anti-MSLN Ab (SS1(dsFv)) directed against the MSLN antigen expressed on the surface of the target cells (Chowdhury and Pastan, 1999). Once bound to MSLN, the entire RIT molecule is internalized, leading to the release of PE38 into the cytosol and cellular apoptosis via inactivation of ADP-ribosylation/elongation factor 2 pathway (Hassan et al., 2000; Pastan et al., 2007).

Targeting Msln$^+$ aPFs With immunotoxins as Potential Strategy for Treatment of Cholestatic Fibrosis

The question remains if a similar strategy can be used to ablate aPFs to eliminate the source of Collagen Type I. Based on our previous findings in mice, genetic ablation of aPFs (using overexpression of Diphtheria Toxin α, DTA) causes aPF apoptosis without causing structural liver damage, and attenuates development of cholestatic fibrosis in BDL-injured mice (Koyama et al., 2017), outlining that immunotoxin-based ablation of human aPFs may become a novel strategy for treatment of PSC patients. In accord, SV40-Large SS1P and LMB100 immunotoxins (Hassan et al., 2007) can successfully kill human primary cultured aPFs *in vitro,* but also *in vivo* in the xenograft mice, generated by adoptive transplantation of human primary aPFs into the livers of adult immunodeficient Rag2$^{-/-}$ $\gamma c^{-/-}$ mice (Nishio et al., 2021). Generation of "human aPF xenograft" Rag2$^{-/-}\gamma c^{-/-}$ mice is novel, and might serve as a useful model to study *in vivo* the variability of patient-specific responses of human aPFs (fibrogenic activation/proliferation) to specific MSLN inhibitors (Nishio et al., 2021).

A potential drawback is that repeated administration of RITs (Kreitman et al., 2009) might lead to the formation of anti-drug antibodies (ADAs) and accelerated clearance of anti-MSLN-immunotoxins (Baker et al., 2010). LMB100 was engineered to reduce immunogenicity in humans compared with SS1P (Liu et al., 2012; Alewine et al., 2014). Both immunotoxins successfully showed excellent anti-tumor activity in clinical trials in patients with mesothelioma, ovarian and pancreatic cancer (Kreitman et al., 2009; Hassan et al., 2014).

Blocking of Msln Expression in aPFs May Attenuate Cholestatic Liver Fibrosis

Administration of blocking unconjugated anti-Msln As (Koyama et al., 2017) might also be beneficial in suppression of aPF proliferation and activation. Such strategy was explored in BDL-injured mice, and repetitive administration of Msln-blocking Abs (D233-3, 5ng, 10 ng, MBL Inc.; or B35 Ab, 10 ng, LSBio) was shown to inhibit aPFs and reduced cholestatic fibrosis.

Human Soluble hsTHY-1-Fc Peptide

THY-1 exhibits anti-fibrogenic properties. Human soluble THY-1 peptide shares high similarity with mouse soluble Thy-1 and crossreacts with mouse ligands. Binding of hsTHY-1 (but not hsTHY-1-RLE with mutated integrin-binding RGD-like motif) (Tan et al., 2019) to αvβ5 integrin was shown to prevent activation of latent TGFβ1 in lung fibroblasts (Zhou et al., 2010). Based on our unpublished observation, administration of hsTHY-1 peptide (1 μg/g in PBS) suppressed BDL-induced aPF activation in BDL-injured mice and attenuated development of cholestatic fibrosis (compared to mutant hsTHY-1-RLE- or vehicle-treated mice). We can speculate that administration of hsTHY-1 also prevents TBFβ1-TGFβRI signaling.

CONCLUSION

Investigation of the role of Msln, Muc16, and Thy1 in cholestatic fibrosis revealed that Msln$^{-/-}$ mice are protected from cholestatic fibrosis caused by Mdr2 deficiency, or BDL-induced obstruction of the common bile duct. There is a growing evidence that Msln is a critical activator of aPFs. Msln expression correlates with the stage of liver fibrosis in patients with PSC. Anti-MSLN Ab-immunotoxins, developed for cancer therapy, can potentially be used to target human MSLN$^+$ aPFs for treatment of cholestatic fibrosis. Overall, immunotherapy-based ablation of human aPFs might become a novel strategy for treatment of cholestatic fibrosis. It might not cure patients with cholestatic fibrosis but can decrease fibroproliferative responses to bridge PSC patients to liver transplantation, or treatment of the etiological causes.

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HF and GM wrote the manuscript. TN, YK, KL, VZ helped with the manuscript preparation. RL and DB critically revised the manuscript. TK wrote the manuscript and provided support.

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REVIEW
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Biliary Epithelial Senescence in Liver Disease: There Will Be SASP

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Cellular senescence is a pathophysiological phenomenon in which proliferative cells enter cell cycle arrest following DNA damage and other stress signals. Natural, permanent DNA damage can occur after repetitive cell division; however, acute stress or other injuries can push cells into premature senescence and eventually a senescence-associated secretory phenotype (SASP). In recent years, there has been increased evidence for the role of premature senescence in disease progression including diabetes, cardiac diseases, and end-stage liver diseases including cholestasis. Liver size and function change with aging, and presumably with increasing cellular senescence, so it is important to understand the mechanisms by which cellular senescence affects the functional nature of the liver in health and disease. As well, cells in a SASP state secrete a multitude of inflammatory and pro-fibrogenic factors that modulate the microenvironment. Cellular SASP and the associated, secreted factors have been implicated in the progression of liver diseases, such as cholestatic injury that target the biliary epithelial cells (i.e., cholangiocytes) lining the bile ducts. Indeed, cholangiocyte senescence/SASP is proposed to be a driver of disease phenotypes in a variety of liver injuries. Within this review, we will discuss the impact of cholangiocyte senescence and SASP in the pathogenesis of cholestatic disorders.

Keywords: cholestasis, fatty liver, cell cycle arrest, bile duct, aging

INTRODUCTION

Cholangiocytes, which are morphologically heterogenous, polarized cells lining the biliary epithelium (Han et al., 2013; Banales et al., 2019), have high absorptive/secretory functions and play a role in the *1*) modification of canalicular bile, *2*) paracrine communication with portal cells, and *3*) regulation of immune cell infiltration (Nathanson and Boyer, 1991; Chen et al., 2008; Banales et al., 2019). Cholangiocytes are the target of various liver diseases such as fatty liver diseases (non-alcoholic fatty liver disease [NAFLD], non-alcoholic steatohepatitis [NASH]), alcoholic liver disease (ALD) and chronic cholestatic liver diseases including primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), biliary atresia, and cholangiocarcinoma. Biliary secretory functions regulate liver inflammation and fibrosis (by both autocrine and paracrine pathways) through secretion of cytokines and other factors which may contribute to liver damage (Kennedy et al., 2021; Kyritsi et al., 2021). Cellular senescence increases in cholangiocytes of PSC patients, likely contributing to disease progression (Tabibian et al., 2014a). Moreover, many factors secreted from

cholangiocytes, such as interleukin (IL)-1β, IL-6, monocyte chemoattractant protein (MCP)-1, stem cell factor (SCF), transforming growth factor β1 (TGF-β1), and platelet derived growth factor (PDGF), are components of the senescence-associated secretory phenotype (SASP) (Rao and Jackson, 2016; Lopes-Paciencia et al., 2019; Blokland et al., 2020). Senescence and SASP secretion have gained considerable attention in studies of cholestatic liver disease progression, demonstrating a new role for senescent cholangiocytes in the pathogenesis of liver diseases (Knop et al., 1987; Wu et al., 2016).

CHOLANGIOCYTE CELL CYCLE ARREST AND PROGRESSION

The liver is composed of two main types of epithelial cells: hepatocytes and biliary epithelial cells (i.e., cholangiocytes). In normal conditions, cholangiocytes represent less than 10% of total hepatic cellular mass in humans and 2–3% in rodents (Alpini et al., 1988); however, upon stimulation, they support bile acid (BA)-independent bile flow accounting for approximately 50% of total bile secretion (Kanno et al., 2000). Small and large cholangiocytes line small (<15 μm diameter) and large (≥15 μm diameter) bile ducts, respectively (Cheung et al., 2018). Heterogeneity in size closely relates to the difference in activity and functions of cholangiocytes. Large, cyclic adenosine monophosphate (cAMP)-dependent cholangiocytes represent the mature, hormone-dependent portion of biliary epithelium, while small Ca^{2+}-dependent cholangiocytes are considered a typically quiescent population (Hall et al., 2017; Banales et al., 2019). Specifically, only small cholangiocytes proliferate when stimulated with a specific histamine receptor agonist (HRH1); however, small cholangiocytes can also acquire large cholangiocyte phenotypes after insult such as GABA treatment (Francis et al., 2008; Mancinelli et al., 2013). Secretory and proliferative activities of the cholangiocytes are finely tuned by several hormones, neuropeptides and angiogenic factors (Alvaro et al., 2007; Franchitto et al., 2013). For instance, secretin binding to the cholangiocyte-specific secretin receptor (SR, a G-protein coupled receptor) stimulates bicarbonate enriched choleresis, thereby increasing intracellular cAMP and activating the biliary cAMP/protein kinase A (PKA)-dependent cystic fibrosis transmembrane conductance regulator (CFTR) opening and subsequent anion exchange protein 2 (AE2) activation (Glaser et al., 2009; Mancinelli et al., 2013; Wu et al., 2020). Fluctuations in intracellular levels of cAMP, and its role as a second messenger, are important in the biliary epithelia not only for secretory activities, but also for proliferative processes (Baiocchi et al., 2021). Large cholangiocyte proliferation is tightly regulated by cAMP-dependent PKA/proto-oncogene tyrosine-protein kinase Src (Src)/mitogen activated protein kinase kinase (MEK)/extracellular signal-regulated protein kinases 1/2 (ERK1/2) signaling axis (Francis et al., 2004). Three proliferative modalities have been identified in experiments in normal and pathological biliary conditions (Alvaro et al., 2000). Type I (typical proliferation) is represented by an inordinate hyperplastic growth of bile ducts and is observed in the cholestatic injury model of bile duct ligation (BDL) in rodents (Alvaro et al., 2007; Glaser et al., 2009). Conversely, Type II (atypical proliferation) spreads outside the portal space, with irregular truncated ducts and is associated with chronic cholestatic diseases or severe injuries (Franchitto et al., 2013). Finally, Type III proliferation is characterized by expansion of the "oval" cells (i.e., hepatic progenitor cells, located in the Canals of Hering) and is characteristic of BDL and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet in rodent models and in human PBC (Olynyk et al., 1998; Carpino et al., 2018; Fragoulis et al., 2019). While each type of proliferative modality is separate, many cholestatic models, such as BDL, may exhibit multiple subtypes during disease progression.

On the opposite side of active eukaryotic cell division and growth stands cell cycle arrest (Toettcher et al., 2009). This molecular process promptly activates during conditions of cellular stress and DNA damage, thus preventing cell damage/repair and ceasing normal replicative activities. When cell repair cannot accommodate increased DNA damage, cell cycle arrest may evolve toward a programmed cell death route (Wiman and Zhivotovsky, 2017), such as autophagy (Glick et al., 2010), necroptosis (Khoury et al., 2020) and apoptosis (Fleisher, 1997). Homeostatic regulation of growth by apoptosis has been confirmed in biliary epithelia (Bhathal and Gall, 1985). In fact, proliferative insult to rat cholangiocytes (either BDL or α-naphthyl isothiocyanate administration) has been shown to determine an apoptosis-dependent restoration of normal bile duct mass (Lesage et al., 2001). From these and other findings, apoptosis deregulation has been suggested to play an important role in determining and maintaining ductopenic cholangiopathies, such as human PBC or PSC (Celli and Que, 1998). However, in recent years, cell cycle arrest has gained attention for the possibility to give rise to a senescent cellular phenotype. This seems associated with tissue aging, as well as acute damage from pathological conditions, including those affecting the biliary system since senescent cells are generally anti-apoptotic (Childs et al., 2014).

AGING AND SENESCENCE

Aging is a complex process. Several factors contribute to this progression including genetic, mitochondrial, peptidic and paracrine components (López-Otín et al., 2013); however, at the cellular level a specific phenotype has been consistently identified in aged tissue: the senescent cell (Campisi, 2013). This phenotype is characterized by an irreversible interruption of replicative phases possibly to prevent oncogenic proliferation after cellular injury (Sager, 1991). Two other specific features belong to a senescent cell: *1*) lack of progression toward apoptosis; and *2*) development of a SASP (Coppé et al., 2008). Following the induction of SASP, senescent cells are considered to contribute to some degenerative diseases. Experiments in transgenic mice with deletion of senescent cells, demonstrated improved outcomes of neurologic, vascular and musculoskeletal degenerative processes (Campisi et al., 2019). It is important to underscore that the

complex and heterogeneous SASP paracrine stimuli negatively or positively affect the microenvironment depending upon the pathophysiological setting. SASP may contribute to the prevention of cancer or even tissue repair (Campisi, 2013). In this perspective, the strong activity of SASP in wound healing seems in conflict with the reduced regenerative capability of aged tissue.

SENESCENCE IN CHOLANGIOPATHIES

Primary Biliary Cholangitis

PBC is an autoimmune chronic liver disease characterized by cholestasis and ductopenia of the interlobular bile ducts (Onofrio et al., 2019). PBC primarily affects middle-aged women who may present with abdominal discomfort, pruritus, fatigue or no symptoms, complicating its complex etiology (Onofrio et al., 2019). Diagnostic criteria for PBC include positive serum anti-mitochondrial antibodies (AMA) and chronic (~24 weeks) abnormal ALP levels or comparable liver histology analysis (Onofrio et al., 2019). Recent studies have advanced diagnosis and treatment options for PBC patients, but therapeutics targeting senescence have not been evaluated in the clinical settings (Onofrio et al., 2019). Due to increased bile duct senescence in PBC patients, understanding biliary senescence/SASP development remains a novel strategy to understand disease progression.

Many correlative studies have evaluated the expression of senescence and SASP in human PBC samples (Sasaki et al., 2005; Sasaki et al., 2006; Sasaki et al., 2008), but mechanistic studies are scarce. Telomere shortening has been noted in the small ducts of PBC patients and is found in conjunction with expression of p16 and p21 (Sasaki et al., 2008). Another study has shown that small ducts of PBC patients highly expressed senescent markers, and this was associated with the development of non-suppurative cholangitis and the portal infiltration of inflammatory cells (Sasaki et al., 2005). Indeed, enhanced biliary senescence is a driver of worsening disease in PBC, but the cause of senescence induction is complicated. A potential cause for this increased senescence includes enhanced oxidative stress. *In vitro* work demonstrated that biliary senescence may be driven by enhanced oxidative stress as determined by H_2O_2 and nitric oxide stimulation (Sasaki et al., 2005). This theory is supported by work that found increased endoplasmic reticulum (ER) stress, which is associated with oxidative stress, in the small ducts of patients with PBC, and the *in vitro* induction of ER stress promoted senescence in cultured cholangiocytes (Sasaki et al., 2015). Enhanced cell stress is an understandable component of biliary senescence considering the large degree of immune cell-induced damage to the cholangiocytes that occurs during PBC. Additionally, understanding the impact of enhanced biliary senescence during PBC pathogenesis is relevant considering high-risk PBC patients, identified by impaired ursodeoxycholic acid (UDCA) response, have increased p21 gene and protein expression when compared to low-risk PBC patients (Hardie et al., 2016). Recent work characterized the serum proteome of PBC patients and found that patients with attenuated response to UDCA had enhanced serum levels of chemokines associated with senescence, which the authors postulate to be released from senescent cholangiocytes (Barron-Millar et al., 2021). Specifically, the serum levels of C-X-C motif chemokine ligand 11 (CXCL11) and chemokine ligand 20 (CCL20) were significantly inversely correlated with UDCA response (Barron-Millar et al., 2021). This work underlines the importance of senescence in PBC pathogenesis, but also identifies new prognostic factors to be utilized for treatment strategies (Barron-Millar et al., 2021). Additionally, others demonstrated that inducing senescence in cholangiocytes *via* oxidative stress, DNA damage and serum deprivation *in vitro* leads to a subsequent increase in secretion of various chemokines (Sasaki et al., 2010a). These senescent cholangiocytes enhanced macrophage recruitment, which was blocked using neutralizing antibodies against CCL2 and fractalkine (i.e., chemokines) (Sasaki et al., 2010a). Of relevance, CCL2 and fractalkine expression was increased in inflamed bile ducts in human PBC samples (Sasaki et al., 2010a). Moreover, senescent small ducts in PBC also express genes associated with inflammation and immune response (Sasaki et al., 2021). This work further highlights that biliary senescence induces a pro-inflammatory secretome within damaged cholangiocytes, which promotes inflammation and the recruitment of various inflammatory cells. These mechanisms are obvious drivers of a worsening prognosis, considering the association of biliary senescence with UDCA response, and so it is imperative we define these mechanisms to understand disease progression.

Aside from oxidative stress, autophagy has been implicated as a driver of cholangiocyte senescence during PBC. Expression of autophagy markers are increased in damaged small ducts in PBC patients and are co-localized with senescence marker expression (Sasaki et al., 2010b). In cultured cholangiocytes, inhibition of autophagy blocked senescence (Sasaki et al., 2010b). Autophagy markers were expressed in the ductular reactive cells of both early- and late-stage PBC patients, whereas senescent markers were predominantly found in late-stage PBC, suggesting that autophagy precedes senescence development (Sasaki et al., 2012a). Similarly, p62 sequestrome-1 (p62, involved in autophagy) aggregates are found in inflamed small ducts in PBC and are co-expressed with obligate autophagy and senescent markers (Sasaki et al., 2012b). Knockdown of p62 reduced autophagy and senescence in cultured cholangiocytes (Sasaki et al., 2012b), lending reason to a potential link between autophagy and biliary senescence in PBC initiation and progression. These studies suggest a mechanism preceding biliary senescence in PBC, which can be used as a target to block the development of biliary senescence and subsequent liver injury. *In vitro*, treatment with hydrophobic BAs reduced AE2 expression, and enhanced oxidative stress and senescence in human cholangiocytes, showing that the hepatic microenvironment may initiate biliary oxidative stress and senescence. (Hisamoto et al., 2016). Since loss of the bicarbonate umbrella, specifically through reduced AE2 expression, is a hallmark of PBC, this data is quite compelling (Arenas et al., 2019; Banales et al., 2012). In human samples,

decreased expression of AE2 is correlated with increased bile duct senescence during PBC (Sasaki et al., 2018a), thus identifying that the bicarbonate umbrella may be an important component of PBC progression *via* increased senescence. As well, these studies link loss of the bicarbonate umbrella, a cholangiocyte-specific protective mechanism, with biliary senescence which is a novel and new finding. Interestingly, both the bicarbonate umbrella and SASP factor release can be utilized to identify unique biliary signatures in PBC, which may point to new mechanisms or markers. Upstream of AE2 activity is secretin/SR signaling, that is a determinant modulating the bicarbonate umbrella and bile flow (Jones et al., 2015). When looking at early-stage PBC, one study found enhanced secretin/SR expression in bile ducts of human samples and the dominant-negative TGF-β receptor II mouse model of PBC (Kennedy et al., 2019). In this murine model of early-stage PBC, treatment with a SR antagonist (Sec 5–27) reduced SR activation and subsequently decreased TGF-β1 expression, biliary senescence, and liver fibrosis (Kennedy et al., 2019). This study found elevated secretin/SR axis and subsequent TGF-β1 secretion and TGF-βR1 expression in human early-stage PBC samples compared to healthy control, insinuating a role for secretin/SR in PBC development and subsequent senescence (Kennedy et al., 2019). This work describes an upstream pathway regulating biliary senescence/SASP during PBC, and this mechanism may potentially be targeted for therapeutic use.

Underscoring our previous comments, direct targeting of senescence or other factors driving senescence may be therapeutic for PBC treatment, and this has been tested in experimental models. Expression of senescent markers (p16 and p21) and the anti-apoptotic marker, B cell lymphoma-extra-large (Bcl-xL), are enhanced in the small ducts of patients with PBC (Sasaki et al., 2020). Specifically, senescent cholangiocytes were found within the ductular reactions and corresponded with stage, hepatitis activity and inadequate response to UDCA (Sasaki et al., 2020). Lastly, the authors induced senescence in murine cholangiocytes *in vitro* and found that treatment with senolytics (AA-1331852, Navitoclax, Dasatinib and Dasatinib with Quercetin) induced apoptosis and effectively cleared senescent cholangiocytes (Sasaki et al., 2020). The polycomb group gene, Bmi1 downregulates p16 expression, and reduced expression of Bmi1 in the small ducts of PBC, and *in vitro* oxidative stress was able to repress Bmi1 expression in cultured cholangiocytes (Sasaki et al., 2006). Together, these findings confirm an associated theme of oxidative stress promoting senescence in PBC. Antioxidant therapies may remedy PBC-associated injury through reduced biliary senescence; however, it is imperative that we delineate the role of biliary senescence in PBC pathogenesis to define additional therapeutic options for patients. This section has highlighted the potential for antioxidant and senolytic therapies for the treatment of PBC.

Primary Sclerosing Cholangitis

PSC is an idiopathic chronic cholangiopathy characterized by increased hepatic inflammation, bridging fibrosis and progressive cholestasis (Lazaridis and LaRusso, 2015). Much like PBC, senescent cholangiocytes have been implicated in PSC progression and exacerbation of hepatic damage through paracrine secretion of inflammatory and fibrotic factors (Lazaridis and LaRusso, 2015). Therapeutic options for PSC patients are limited, with liver transplantation serving as the sole therapeutic option to improve long-term survival (Rawla and Samant, 2021). A common feature in PSC is reactive cholangiocytes, which may exhibit proliferative or senescent marker expression and increased inflammatory and fibrotic factor secretion (Lazaridis and LaRusso, 2015). The role of biliary senescence in PSC has been of rising interesting, but its exact impact on disease progression remains undefined.

Early work using primary cholangiocytes isolated from PSC patients found that these cholangiocytes highly express the senescence markers, p16 and γH2A.X, compared to normal cholangiocytes (Tabibian et al., 2014a). Similarly, another study found that cholangiocytes isolated from PSC patients and maintained in primary culture have a lower proliferative rate and high expression of SA-β-galactosidase when compared to normal cholangiocytes (Tabibian et al., 2014b). PSC cholangiocytes were also found to have increased secretion of SASP components (IL-6, IL-8, CCL2, PAI-1) compared to normal cholangiocytes, and these SASP factors induced senescence in bystander cholangiocytes as indicated by *in vitro* co-culture systems (Tabibian et al., 2014a). In this study, the induction of biliary senescence was driven by neuroblastoma RAS viral oncogene homolog (N-Ras) activation in cholangiocytes (Tabibian et al., 2014a). Others have found that cholangiocytes of PSC patients have increased expression of senescence factors as indicated by staining in liver sections (Ferreira-Gonzalez et al., 2018). Together, all of these studies suggest that biliary senescence is a key component of PSC, and similar to PBC it can confer a worsening phenotype through modulation of nearby cells. A novel murine model of inducible cholangiocyte senescence has found that upregulation of p53, through knockout of mouse double minute 2 proto-oncogene (Mdm2), leads to increased p16, p21 and γH2AX in bile ducts (Ferreira-Gonzalez et al., 2018). This work found that senescent cholangiocytes have elevated secretion of SASP factors with paracrine consequences such as increased hepatocyte senescence, exacerbated hepatic fibrosis and decreased liver regeneration (Ferreira-Gonzalez et al., 2018). Interestingly, the induction of biliary senescence in normal mice promoted portal macrophage infiltration and peribiliary fibrosis which aggravated these phenotypes when combined with DDC feeding to induce cholestasis (Ferreira-Gonzalez et al., 2018). This work demonstrates that biliary senescence alone can promote phenotypes associated with PSC, and this conclusion brings forth the question on the ability of cholangiocyte senescence to be a component of PSC etiology. In an *in vitro* model, cholangiocyte organoids (cholangoids) made from normal patient cholangiocytes that are exposed to H_2O_2 (to induce senescence, termed NHC-sen) display senescence and SASP characteristics. Further, NHC-sen cholangoids, as well as PSC cholangoids enhance macrophage recruitment compared to control (Guicciardi et al., 2018). Interestingly, isolated cholangiocytes from PSC patients have increased expression of genes associated with cell cycle arrest

and senescence, as indicated by RNA-seq, and senescent cholangiocytes formed cholangoids of a smaller size that also lacked a lumen when compared to normal cholangiocytes (Jalan-Sakrikar et al., 2021). This work is supported by other findings showing that NHC-sen cells promote the proliferation of healthy cholangiocytes and monocyte migration, which was also found with plasma EVs from multidrug resistant cassette 2 knock out (*Mdr2*$^{-/-}$) mice, a genetic murine model of PSC (Al Suraih et al., 2020). The identification of increased biliary senescence in PSC is worth intensive evaluation since it is correlated with DR, fibrosis staging and other markers of severe disease in PSC patients (Cazzagon et al., 2021). Together, the above work demonstrate that biliary senescence can induce senescence of nearby cholangiocytes and drive hepatic damage associated with cholangiopathies, such as PSC.

A feature of senescent cholangiocytes is a pro-inflammatory and pro-fibrotic secretome. One study found that biliary-derived TGF-β1 promoted paracrine cholangiocyte senescence (Ferreira-Gonzalez et al., 2018) and it has been shown that TGF-β1 blocks cell cycle progression by enhancing the transcription of senescent factors such as cyclin-dependent kinase inhibitors, p21 and p27 (Datto et al., 1995). Indeed, others have found TGF-β1 promotes cholangiocyte senescence and subsequent liver fibrosis through autocrine and paracrine signaling (Wu et al., 2016). Increased biliary TGF-β1 synthesis and secretion was found to be downstream of the secretin/SR pathway, only expressed by cholangiocytes in the liver (Wu et al., 2016) thereby demonstrating a cholangiocyte-specific mechanism regulating biliary senescence in cholangiopathies. Additionally, the inhibition of secretin/SR signaling using Sec 5–27 or SR$^{-/-}$ mice reduced biliary senescence, TGF-β1 levels and liver fibrosis in models of PSC (BDL and *Mdr2*$^{-/-}$ mice) (Wu et al., 2016; Zhou et al., 2018). It is apparent that cholangiocyte-derived TGF-β1 is an important factor mediating biliary (autocrine) and liver (paracrine) damage during PSC. Aside from cholangiocytes, mast cells secrete TGF-β1 and induce biliary senescence and other markers of cholestasis (Kyritsi et al., 2021), which is an important feature considering that mast cell migration to portal areas is a key feature and damaging component of cholestasis (Wilcox et al., 1986; Tsuneyama et al., 1999; Jones et al., 2016). Mast cell farnesoid X receptor (FXR) signaling promotes biliary senescence and associated biliary and liver damage in murine models of PSC (Meadows et al., 2021). Mast cell-derived components lend to worsening phenotypes in PSC and understanding if they can be targeted to mediate damage is a topic of research currently. Stem cell factor (SCF) is another cholangiocyte secretory component, and SASP factor, found to promote senescence in models of PSC (Meadows et al., 2019). It was demonstrated that SCF Vivo-Morpholino treatment (to reduce SCF expression) significantly decreased biliary senescence, as well as DR and liver fibrosis in *Mdr2*$^{-/-}$ mice (Meadows et al., 2019). In addition, this study demonstrated enhanced serum SCF and biliary SCF expression in human PSC compared to control samples (Meadows et al., 2019). This finding is corroborated by a correlative study demonstrating aberrant biliary SCF expression in human PSC samples (Tsuneyama et al., 1999). Interestingly, cholangiocyte SCF expression correlated with increased portal infiltration of cKit (SCF receptor) positive mast cells in human PSC (Tsuneyama et al., 1999), and SCF Vivo-Morpholino reduced hepatic mast cell presence in *Mdr2*$^{-/-}$ mice (Meadows et al., 2019), insinuating that biliary senescence mediates mast cell number and activity during PSC. This work further lends to the hypothesis that cholangiocyte secreted SASP factors can modulate the microenvironment and promote damage through the recruitment of immune cells, including mast cells.

Other signaling factors have been implicated in PSC-associated biliary senescence, such as histamine signaling, as demonstrated when *Mdr2*$^{-/-}$ mice were treated with a Vivo-Morpholino targeting the H2 histamine receptor (H2HR) inhibited biliary senescence, liver inflammation and fibrogenesis (Kennedy et al., 2020). Interestingly, this study determined that H2HR inhibition selectively targets large cholangiocyte senescence through downregulation of TGF-β1 expression (Kennedy et al., 2020), which again underlines the significance of TGF-β1 signaling in cholangiocytes during cholestatic injury. Biliary heterogeneity is an important factor influencing PSC damage. Classic PSC can affect both small and large bile ducts while small-duct PSC demonstrates fibrosis and inflammation of small ducts alone (Rawla and Samant, 2021). Understanding heterogeneity of PSC senescence is important since FoxA2, a definitive endoderm marker and transcription factor, is reduced in human PSC (McDaniel et al., 2017). Previous work has also demonstrated that this factor is predominantly expressed in small mouse cholangiocytes and liver progenitor cells *in vitro* and transplant of cultured small mouse cholangiocytes enhances FoxA2 expression, reduces biliary senescence and liver fibrosis in BDL and *Mdr2*$^{-/-}$ mice (McDaniel et al., 2017). A preferential increase in large bile duct mass has been noted in *Mdr2*$^{-/-}$ mice, demonstrating that this subpopulation may be more prone to injury and thus senescent damage (Kennedy et al., 2018a). In this regard, biliary heterogeneity may be an important aspect of biliary senescence, but more studies are warranted. It is imperative that we better define biliary heterogeneity in humans, specifically in the context of disease, to understand if these pathways identified in mouse models can hold true in the clinical setting.

Aging, in the context of disease, can work hand-in-hand with senescence to perturb liver injury (Kundu et al., 2020). Aged mice show increased expression of microRNAs (miRs) associated with aging processes (miR-1a, miR-20a, miR-30e), and increased expression of these miRs correlated with increased twinfilin-1 (twf-1) levels (Maroni et al., 2019). Interestingly, twf-1 expression increased in the DDC feeding model of cholestasis and in human PSC samples, and *Twf-1*$^{-/-}$ mice subjected to DDC had reduced biliary senescence and SASP (Maroni et al., 2019). Additionally, senescence-accelerated mice (SAMP8) had increased twf-1 expression and subsequent biliary senescence/SASP (Maroni et al., 2019). However, little work on the inappropriate induction of aging processes in cholestasis, including PSC, has not been evaluated thus far. Identifying signals inducing aging pathways cholangiocytes will be an important finding for PSC studies. Interestingly, the microbiota has been implicated in biliary senescence, as well (Tabibian et al., 2016). *Mdr2*$^{-/-}$

mice housed in a germ-free facility had exacerbated biliary senescence/SASP, associated DR and liver fibrosis (Tabibian et al., 2016). This is not surprising considering the gut-liver axis is vital for physiological function. This work also demonstrates that we can target factors outside of the liver in an effort to reduce biliary senescence during PSC, and this can potentially be applied to other cholestatic injuries as well.

Since cholangiocyte senescence and SASP are related to PSC-associated biliary and liver damage, it is of interest to evaluate if blocking biliary senescence/SASP may ameliorate damage. One study treated *Mdr2*$^{-/-}$ mice with a p16 Vivo-Morpholino and found that this treatment reduced p16 expression and senescence, TGF-β1 expression and biliary secretion of SASP components (Kyritsi et al., 2020). Furthermore, p16 Vivo-Morpholino reduced DR and portal fibrosis in *Mdr2*$^{-/-}$ mice compared to controls, which was shown to be linked to reduced miR-34a/Sirtuin-1 (SIRT1) signaling (Kyritsi et al., 2020). This is relevant considering others have found SIRT1 activity to promote cholangiocyte senescence in a model of obstructive cholestasis (Jia et al., 2021). One study generated an *Mdr2*$^{-/-}$/p16$^{-/-}$ mouse, as well an *Mdr2*$^{-/-}$ mouse line crossed with the p16Ink4a apoptosis, through targeted activation of caspase (INK-ATTAC) mouse capable of selective clearance of p16-expressing cells, and found that both of these models had reduced biliary senescence and SASP, and subsequent decreased inflammation and portal fibrosis (Alsuraih et al., 2021). Additionally, the authors found that fisetin, a flavonoid that acts as a senolytic, induced similar phenotypic changes in *Mdr2*$^{-/-}$ mice, and *in vitro* selectively targeted senescent cholangiocytes (Alsuraih et al., 2021). These studies are in parallel with those in PBC by demonstrating that blocking biliary senescence may be therapeutic for the treatment of PSC. Lastly, it is known that senescent cells are resistant to apoptotic clearance as demonstrated by enhanced Bcl-xL expression in senescent cholangiocytes, as one study displayed the protective mechanism of biliary apoptosis in a model of PSC (Moncsek et al., 2018). Specifically, the clearance of senescent cholangiocytes by Bcl-xL inhibitor treatment reduced liver fibrosis and inflammatory marker expression (Moncsek et al., 2018). These studies establish the powerful impact of targeting biliary senescence for the treatment of PSC and the gaps in knowledge to be filled in with future studies.

Biliary Atresia

Biliary atresia is a devastating pediatric cholestatic and fibrogenic liver disease with a multifactorial etiology and unknown molecular mechanism of pathogenesis (Bezerra et al., 2018; Kerola et al., 2019). Early intervention with Kasai hepatic portoenterostomy, to restore bile flow, and eventual liver transplantation increases survival of biliary atresia patients (Bezerra et al., 2018). Due to its complex pathology, a major challenge with biliary atresia treatment includes therapeutic target identification for early diagnosis. Like in adult cholestatic liver diseases, biliary and hepatic progenitor cells (HPC) senescence may exacerbate liver damage; however, research on senescence/SASP factors in biliary atresia remains severely understudied (Sasaki et al., 2018b; Wu et al., 2018; Xiao et al., 2019; Winkler et al., 2021). In the following section we highlight key studies implicating biliary senescence, and SASP factors, as contributors to biliary atresia development.

Biliary atresia patients show reduced telomere length in hepatic tissues, demonstrating telomere length negatively correlates with pediatric end-stage liver disease score (Sanada et al., 2012). The stress of biliary atresia can be measured outside of the liver, implicating far reaching effects of premature liver dysfunction (Udomsinprasert et al., 2015). Peripheral leukocytes in biliary atresia patients had reduced telomere length compared to healthy control (Udomsinprasert et al., 2015). This work found that leukocyte telomere length shortened with biliary atresia progression and had positive correlation with hepatic telomere length (Udomsinprasert et al., 2015). Interestingly, this study found that in two sets of identical twins, the twin with biliary atresia presented with reduced telomere length in hepatic tissue compared with the healthy twin (Udomsinprasert et al., 2015). These data allow us to postulate that stress from exhaustive proliferation of cholangiocytes may result in biliary senescence and diminishing immune intervention in disease progression in biliary atresia patients. Genomic instability caused by shortened telomere length initiates apoptosis and senescence and provides a therapeutic avenue for biliary atresia patients upstream of senescence (López-Otín et al., 2013). Previous work demonstrated that cholangiocytes from the adeno-associated virus (AAV) murine model of biliary atresia, expressed major histocompatibility complex (MHC) I and II, but did not serve as antigen presenting cells. Instead, these cholangiocytes modulated immune response by secretion of pro-inflammatory cytokines and chemokines (tumor necrosis factor α [TNFα] and TGF-β) (Barnes et al., 2009). This study identifies that biliary immunobiology may be an important component of biliary atresia progression through the release of inflammatory mediators. While this study does not discuss biliary immune regulation in the context of senescence/SASP, it does point to a hypothesis whereby senescent cholangiocytes may have dysregulated expression of MHC components or other immune signaling factors, or vice versa, that contribute to biliary atresia.

Senescent cholangiocyte present with enhanced p16 and p21 expression, particular in DR, in PBC and PSC; however, the role of biliary senescence in pediatric cholangiopathies like biliary atresia is limited (Sasaki et al., 2018b). Biliary and HPC p16 expression was found to be elevated in biliary atresia explant livers, indicating senescence as a component of biliary atresia pathogenesis (Sasaki et al., 2018b). Biliary atresia patients undergoing Kasai portoenterostomy have increased neural cell adhesion molecule (NCAM) positive DR cells, a stemness marker expressed by HPCs in the liver, with minimal NCAM-positive bile ducts (Sasaki et al., 2018b). However, at the time of liver transplantation, these patients had elevated NCAM expression in DR and bile duct cells, implicating differentiation during disease progression. This study also found a positive correlation between bile duct p21 expression and bile duct loss in biliary atresia patients, insinuating that the NCAM-positive DR cells (suspected to be HPCs) could be attempting to resolve bile duct loss in biliary atresia patients by differentiating into bile duct epithelial cells

(Sasaki et al., 2018b). This complex study discusses HPC proliferation and differentiation into cholangiocytes in the context of DR and cholangiocytes senescence, but the signaling mechanisms are complicated and additional work is necessary.

A murine model of inducible cholangiocyte senescence shows that senescent cholangiocytes have elevated secretion of SASP factors with paracrine consequences such as increased hepatocyte senescence, exacerbated hepatic fibrosis and decreased liver regeneration (Ferreira-Gonzalez et al., 2018). In this study, inhibition of TGF-β1 reduced biliary SASP secretion and increased hepatocyte proliferation and liver function (Ferreira-Gonzalez et al., 2018). Kasai portoenterostomy may resolve some features of cholestasis and fibrosis, but in a select group of patients' fibrosis can remain following surgery (Kerola et al., 2019). In biliary atresia, TGF-β1 and decorin expression was elevated in lobular hepatocytes and fibrotic areas and correlated with liver fibrosis (Kerola et al., 2019). At 3-year follow-up, successful Kasai portoenterostomy significantly reduced hepatic TGF-β1 and connective tissue growth factor (CTGF) expression, while TGF-β2 expression was found to be increased (Kerola et al., 2019), signifying a central role for the TGF-β superfamily in promoting continued liver fibrosis after Kasai portoenterostomy. Considering that CTGF drives senescence in fibroblasts (Capparelli et al., 2012) it is reasonable to expect cholangiocyte SASP paracrine communication as central to biliary atresia progression and successfulness of Kasai portoenterostomy. Moreover, recent work found elevated levels of long non-coding RNA (lnc) H19 in serum and hepatic lysates from biliary atresia patients compared to healthy controls (Xiao et al., 2019). These H19 levels positively correlated with fibrosis in patients, marking it as a potential biomarker in disease detection. The authors also found elevated expression of fibrogenic markers, TGF-β, α-smooth muscle actin (αSMA) and ciliary localization (Cil)-1a, along with CK-7 (bipotential marker in HPC) in biliary atresia patients (Xiao et al., 2019). The isolated serum exosome analysis from biliary atresia patients revealed elevated lncH19, high mobility group AT-Hook 2 (HMGA2) and sphingosine-1-phosphate receptor 2 (S1PR2) compared to healthy controls, further implicating that hepatic fibrosis in biliary atresia could be paracrine in nature. It is plausible that these exosomes are cholangiocyte-derived and contribute to biliary atresia-associated fibrosis *via* paracrine crosstalk. While the discussed factors are not obligate SASP components, it is known that senescent and SASP cholangiocytes have an increased secretory component and could thus be a source of these pro-fibrogenic exosomes.A previous study found that *ex vivo* ductal organoids damaged with acetaminophen had induced caspase-3, apoptosis marker, and SASP factors expression, including TGF-β1, PDGF, IL-1β, IL-6, and TNF-α (Chusilp et al., 2020). This study indicates that cholangiocyte damage drives fibrotic response during biliary atresia. While fibrosis has been well established as a characteristic of biliary atresia, the crosstalk between biliary senescence and excessive fibrosis has yet to be captured. Understanding the profibrotic and prosenescent bile ducts and HPCs in biliary atresia may serve as a therapeutic target for disease attenuation in patients waiting for liver transplantation.

SENESCENCE IN FATTY LIVER DISEASES

Non-Alcoholic Fatty Liver Disease (NAFLD)/ Non-Alcoholic Steatohepatitis (NASH)

Senescence has been the focus of recent studies with respect to various metabolic conditions including NAFLD (Zhou et al., 2021). Senescence, independent of aging, can exacerbate disease phenotypes resulting in the progression of the metabolic condition. NAFLD, or the more recent term MAFLD (metabolism-associated fatty liver disease) is the direct hepatic pathological manifestation of excess lipids and fats, primarily sourced from diet (Mantovani and Dalbeni, 2020; Younossi et al., 2021). Initiation of benign steatosis escalates into infiltration of leukocytes, mast cells and development of peribiliary fibrosis with this "second hit" (Zhu et al., 2020). This phenomenon can advance to non-alcoholic steatohepatitis (NASH) and, upon persistent insults, the liver progresses to cirrhosis and potentially hepatocellular carcinoma (HCC) which warrant liver transplantation.

The role of hepatocytes has been well studied in NAFLD (Venkatesh et al., 2017; Simon et al., 2020); however, there has been increasing interest in the contribution from cholangiocytes (Mendez-Sanchez et al., 2007; Kennedy et al., 2021; Zhou et al., 2021). A study showing evidence of DR in NAFLD was performed in rats fed with choline deficient high trans-fat diet where severe hepatic injuries were identified in areas of liver section with more CK-19 positive DR as identified by immunohistochemistry (de Lima et al., 2008). Cholangiocyte damage increases with hepatic steatosis development in NAFLD and NASH patients, further indicating cholangiocyte contribution to disease development (Natarajan et al., 2014). These early studies postulate a role from cholangiocytes in NAFLD and NASH, but true contribution is still be investigated. Cholangiocytes respond to fatty acid-induced lipo-toxicity by assuming a lipo-apoptotic phenotype *in vitro* (Natarajan et al., 2014; Natarajan et al., 2017) emphasizing a critical role for bile ducts in the phenotypic manifestation of NAFLD and describing a possible mechanism contributing to DR. It was also shown that NASH patients have increased DR and bridging fibrosis, which are two main hallmarks of cholangiocyte damage widely studied in cholangiopathies (Sorrentino et al., 2005). NAFLD and end-stage NASH patients also have increased DR and senescence (Kennedy et al., 2018b). Indeed, biliary senescence may be a significant contributor to NAFLD and NASH progression, similar to the cholangiopathies discussed above.

Similar to traditional cholangiopathies mast cells have been implicated in NAFLD/NASH progression. In histidine decarboxylase knock-out (HDC KO) mice, that have loss of endogenous histamine signaling, subjected to high-fat diet (HFD) there was reduced cholangiocyte damage and senescence, indicating a role for histamine signaling in bile duct damage during NAFLD progression (Kennedy et al., 2018b). Histamine has been previously indicated to aid in regulation of food intake and body weight *via* modulation of leptin signaling (Yoshimatsu et al., 1999; Jørgensen et al., 2007). Thus, this study crucially emphasizes the contribution of

senescent cholangiocyte-mediated histamine release in fatty liver disease progression (Kennedy et al., 2018b). These studies define a crucial role for histamine in worsening liver phenotypes in NAFLD and NASH, but few studies have described mast cells and their derived components. NASH patients have increased serum insulin growth factor-1 (IGF-1, SASP factor) compared to healthy controls and C57BL6J mice fed western diet (WD) secrete enhanced IGF-1, specifically from cholangiocytes (Kennedy et al., 2021). Importantly, *in vitro* studies demonstrated that inhibition of mast cell IGF-1 receptor *via* antagonist treatment decreased migration toward damaged cholangiocytes. Further, this work demonstrates that expression of SASP factors from senescent cholangiocytes induce mast cell migration that promotes microvesicular steatosis *via* microRNA 144-3p (miR-144-3p)/aldehyde hydrogenase 1A3 (ALDH1A3) signaling (Kennedy et al., 2021). In general, this work shows that senescent cholangiocytes may worsen NAFLD and NASH *via* the recruitment of immune cells, including mast cells. In an analysis of 1,022 NAFLD patient biopsies, microvesicular steatosis has been identified as an advanced phenotype and strongly, positively correlation with hepatic ballooning, higher NAFLD activity score (NAS) and advanced fibrosis (Tandra et al., 2011). NAFLD patients also exhibit elevated serum histamine, implicating a role for mast cell-histamine in micro-vesicular steatosis development. It can be surmised from these studies that cholangiocytes exhibit a dynamic response to the damaging stimuli in NAFLD/NASH and secrete factors that increase mast cell infiltration that perturb injury. Interestingly, HFD fed older mice (8 and 18 months) showed increased M1 macrophage (proinflammatory) infiltration in liver indicating the effect of cellular senescence in immune cell infiltration during NAFLD (Fontana et al., 2013). As well, this work describes the role of aging in cellular senescence, but more studies to fully understand the mechanism is warranted. Further characterization of cholangiocyte sub-population (small/large) in HFD/WD models is required to understand how biliary epithelia contribute to the damage caused during NAFLD/NASH and may also highlight important information on biliary heterogeneity in terms of senescence induction that may help to understand cholestatic disease processes better.

Alcoholic Liver Disease (ALD)

Liver pathophysiology associated with ALD is similar to NAFLD and includes severe steatosis, portal inflammation, and fibrosis ultimately leading to cirrhosis and liver failure (Yeh and Brunt, 2014). Although ALD can be differentiated from NAFLD based on etiology, aging is still an independent driving factor for the former. Previous work has shown that age was an influencing factor on the severity of liver injury, inflammation and fibrosis in mice fed with short- and long-term alcohol treatments (Ramirez et al., 2017). Further, the expression of SIRT1 was downregulated in aged animals and restoration of SIRT1 reversed the damage caused by binge alcohol feeding (Ramirez et al., 2017). The effects of alcohol on aging-related damage have been shown in Alzheimer's disease, where alcohol directly reduced telomere length, one of the major manifestations of senescence (Yamaki et al., 2019). Based on these data one would assume that ALD affects cellular senescence through its cellular toxicity. As well, these studies implicate aging in the promotion of cellular senescence. The main enzyme responsible for elimination of alcohol in humans is ALDH2, and when mutated, this enzyme increases the risk of developing alcohol-related HCC in Asian population (Matsumoto et al., 2016; Chang et al., 2017). Hepatocyte specific $Aldh2^{-/-}$ mice subjected to 3-h ethanol gavage showed reduced aldehyde content compared to control, suggesting a role for other hepatic cells in hepatic metabolism and clearance of alcohol (Guillot et al., 2019). Interestingly, *in vitro*, ethanol provides a dose-dependent protection against cellular senescence by activating ALDH2 in endothelial cells, thereby suggesting a pivotal role of ALDH2 in senescence (Xue et al., 2019). ALDH2 mediates anti-senescence effects during chronic ethanol challenge by activation of SIRT1/p53 pathway in human aortic endothelial cells, *in vitro* (Xue et al., 2018). siRNA mediated knockdown of ALDH2 increased senescence-associated β-galactosidase, p21 and p53 expression in human vascular endothelial cells (Nannelli et al., 2018). Small molecule activator of ALDH2, N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide (Alda-1), alleviates the reduced aldehyde clearance and reverse hepatic steatosis in male C57BL6J mice subjected to 8-week alcohol treatment (Zhong et al., 2015). These studies identify an interesting role of ALDH2 in blocking cellular senescence, and this factor may be an important target to inhibit biliary senescence in ALD and other cholestatic disorders. Further, inhibition of ALDH3A1 by administration of synthetic inhibitors, tetraethyl thiuram disulfide (disulfiram), diethylamino benzaldehyde, or 4-amino-4-methyl-pent-2-ynthioic acid, S-methyl ester (ampalthiol ester), reduced cellular proliferation (Muzio et al., 2012). The reduced proliferation could be due to the inhibition of ALDH3A1 since its downstream effectors/targets CCL20, G-protein coupled receptor-37 (GPR37), and DEAD-box helicase 3 Y-linked protein (DDX3Y) have been implicated in growth and differentiation (Ruzinova and Benezra, 2003; Moreb et al., 2008). ALDH3A1-siRNA treatment in lung cancer cell lines, H522 and A549, reduced proliferation *via* increased peroxisome proliferation activated receptor (PPAR) expression (Oraldi et al., 2011). Thus, expression of ALDHs might be inversely correlated with onset and progression of cellular senescence.

Increased cellular senescence and miR-34a expression has been reported in the NIAAA murine model of ALD (Wan et al., 2017). Inhibition of miR-34a by Vivo-Morpholino reduced ALT, fibrosis, senescence and pathology score in ethanol-treated mice (Wan et al., 2017). Moreover, miR-34a has been implicated in the regulation of cellular senescence of cholangiocytes and hepatic stellate cells in ALD mice (Annable et al., 2015; Wan et al., 2015). Due to the increasing incidence of biliary senescence in other liver diseases, there may be an important role in for cholangiocyte senescence and SASP in ALD progression. As well, defining a set of miRs associated with biliary senescence could be key for delineating a senescence-associated miR profile or identifying therapeutic, prognostic or diagnostic targets.

Taken together, these studies highlight a role for aldehyde metabolism and miRNA signaling in mediating liver cellular

TABLE 1 | Factors associated with cellular senescence in chronic liver disease.

Chronic liver disease	Cellular mechanism associated with senescence
Primary Biliary Cholangitis (PBC)	Telomere shortening, p16 and p21 expression Oxidative stress and ER stress Increased expression of CXCL11, CCL20, CCL2 and fractalkine Cholangiocyte autophagy including p62 signaling Loss of AE2 and the bicarbonate umbrella Enhanced secretin/SR signaling (early-stage PBC) Increased Bcl-xL (anti-apoptosis) expression Reduced Bmi1 (antioxidant) expression
Primary Sclerosing Cholangitis (PSC)	p16, p21, γH2A.X and SA-β-galactosidase expression Cholangiocyte secretion of SASP factors (IL-6, IL-8, CCL2, PAI-1) N-Ras activation in cholangiocytes Increased TGF-β1 signaling Upregulation of biliary secretin/SR signaling Mast cell-derived TGF-β1 and FXR signaling Cholangiocyte SCF secretion Increased biliary H2HR signaling Reduced biliary expression of FoxA2 Increased age-related miRs and Twf-1 signaling Enhanced miR-34a/SIRT1 activity Increased Bcl-xL (anti-apoptosis) expression
Biliary Atresia	Reduced telomere length in hepatic tissues and peripheral leukocytes Enhanced biliary MHC I and II expression, and secretion of TNFα and TGF-β p16, p21 and NCAM expression Enhanced TGF-β1, TGF-β2, decorin and CTGF expression Increased serum and hepatic H19 levels Biliary expression of TGF-β, αSMA and Cil-1a Serum exosome H19, HMGA2 and S1PR2 levels Increased caspase-3, TGF-β1, PDGF, IL-1β, IL-6, and TNF-α expression in *ex vivo* ductal organoids
Non-Alcoholic Fatty Liver Disease (NAFLD)/Non-Alcoholic Steatohepatitis (NASH)	Dysregulated histamine/leptin signaling in cholangiocytes Biliary IGF-1 secretion and mast cell miR-144-3p/ALDH1A3 signaling Age-related M1 macrophage infiltration
Alcoholic Liver Disease (ALD)	Reduced SIRT1 activity Inhibition of ALDH2 and ALDH3A1 Increased miR-34a

Table outlining the various signaling mechanisms and phenotypes associated with cellular senescence in chronic liver diseases, as discussed in the review. Senescence-associated factors and mechanisms are divided by disease, including PBC, PSC, NAFLD/NASH, and ALD.

senescence during ALD. The expression of ALDHs and miRNAs and their impact of biliary senescence on ALD is poorly understood. Further investigation is required to elucidate the role cholangiocytes, particularly senescent cholangiocytes, play in alcohol metabolism and damage progression in ALD.

FUTURE DIRECTIONS AND CONCLUSION

DR and biliary senescence/SASP are characteristic of cholangiopathies and are increasing in incidence during fatty liver disease progression and ALD, confirming biliary involvement, although mechanisms remain unclear. Biliary SASP factor secretion can increase the infiltration of mast cells and activate nearby cholangiocytes in cholestatic and NAFLD/NASH murine models, leading to increased inflammation and fibrosis. Mechanistic understanding of the role that biliary heterogeneity plays in SASP development has yet to be defined. Hepatocyte senescence drives hepatic steatosis in an age-dependent manner (Ogrodnik et al., 2017); however, further investigation into individual cell contribution to disease progression will provide novel therapeutic targets for patient treatment options. Future work should utilize cholangiocyte-specific mouse models to target biliary senescence and SASP factors to evaluate the role that these factors may play in the progression of cholangiopathies, NAFLD/NASH and ALD. As well, more studies are needed to define whether biliary senescence is an etiologic component of these liver disorders or a pathological consequence. Specifically in the context of NAFLD/NASH and ALD, that are not traditional cholangiopathies but nonetheless harbor cholestatic injury in a subset of patients, it is imperative to define which factors drive biliary damage in this setting. Understanding the mechanisms that promote biliary senescence in NAFLD/NASH and ALD is important since this injurious component can confer a worsening phenotype and poor prognosis. The recent onset of sophisticated experimental procedures, such as spatial transcriptomics and RNA sequencing, give researchers the capabilities to define the cellular niche during liver damage and can be utilized to describe biliary senescence in this setting. The power of RNA-sequencing

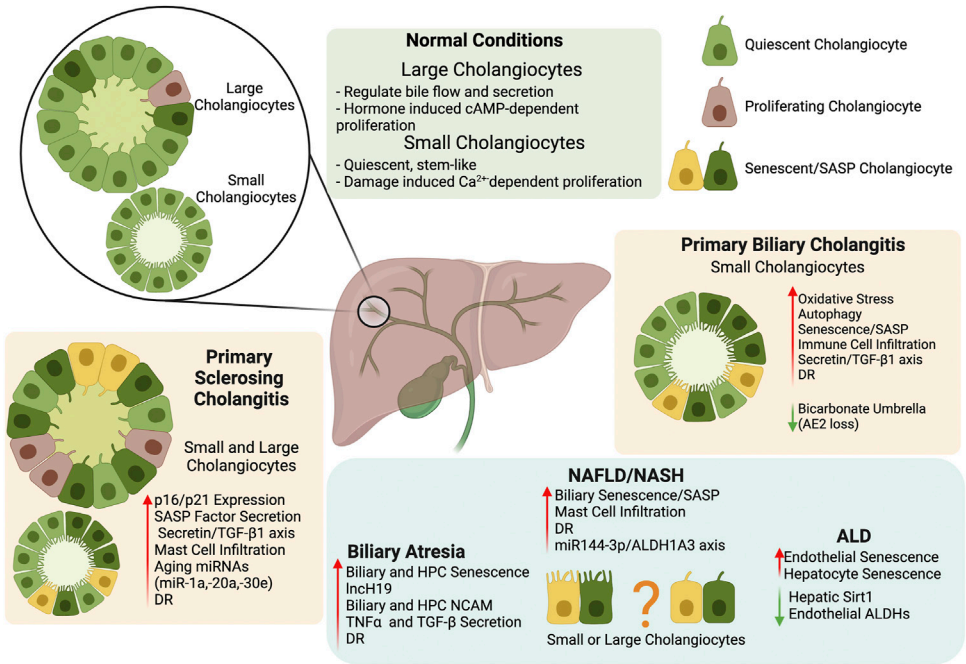

FIGURE 1 | Biliary Senescence in Liver Disease. The key findings summarized in our review indicate that biliary heterogeneity and senescence/SASP factor secretion provide a novel direction for the study and treatment of chronic liver disease. Further exploration of the mechanism behind premature cholangiocyte senescence/SASP should help clarify the role of the biliary tree in chronic liver diseases. Created with biorender.com.

can be used to evaluate the heterogeneity of biliary response to injury and induction of senescence. As well, spatial transcriptomics should be useful in defining the interaction of senescent cholangiocytes with the surrounding liver cells, and multiplex imaging assays can be used in a similar manner to define senescent cholangiocyte interaction with infiltrating immune cells. It is apparent that other experimental parameters are necessary to better define the onset of biliary senescence and the role that biliary senescence and SASP play during liver disease progression.

Biliary senescence and SASP will likely continue to gain attention in the field since cellular senescence/SASP factor secretion affects liver function and chronic liver disease progression (Meadows et al., 2019; Xiao et al., 2019; Kennedy et al., 2021). The use of senolytics may be a useful strategy to prevent senescence-associated damage in liver disease patients; however, this remains controversial. Recent work has shown that age-related NAFLD-HCC mouse models showed increased liver disease following senolytic treatment, indicating that senescence may be a consequence and not a driver of disease (Raffaele et al., 2021). Alternatively, chimeric antigen receptor (CAR) T cells can be engineered to specifically target senescent cells and were shown to ameliorate senescence-induced pathologies including hepatic fibrosis following carbon tetrachloride (CCl_4) treatment (Amor et al., 2020). As described above, some preliminary work has been performed in animal models to evaluate the use of senolytics or other factors modulating biliary senescence as therapeutic agents for liver disease, but more work is necessary. Additional work in preclinical models should be carried out to further define the usefulness of inhibiting senescence and SASP in cholangiopathies, NAFLD/NASH and ALD. While senolytics are an intuitive pharmaceutical approach for the amelioration of liver damage associated with the above disorders, there are other indirect measures by which biliary senescence/SASP can be targeted. As described above, exosomes can be implicated in liver fibrosis progression in biliary atresia, but modulation of exosomes and the utilization of this signaling mechanism as a therapeutic need to be further investigated. Additionally, targeting age-related signaling mechanisms or miRs associated with biliary senescence can be an additional route to indirectly mediate this signaling component in liver disorders. A table of the key molecules discussed in this review that regulate biliary senescence during chronic liver diseases is listed in **Table 1**.

Due to the increase of senescence in cholangiopathies and fatty liver disease, targeting senescence and SASP secretion in cholangiocytes provides a novel direction in chronic liver disease treatment and a summary of critical findings described in this review can be found in **Figure 1**. Further investigation into the mechanism for premature biliary senescence/SASP factor secretion needs to be performed before this theory can be applied to clinical practice.

AUTHOR CONTRIBUTIONS

VM = first draft, compilation, edit final draft, figure LB = first draft, edit final draft DK = first draft, edit final draft KS = first draft, edit final draft YS = literature search CW = edit final draft

SC = edit final draft SG = edit final draft GA = initial concept, funding, edit final draft LK = first draft, funding, edit final draft HF = initial concept, funding, edit final draft.

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GLOSSARY

AE2 anion exchange protein 2
ALD alcoholic liver disease
ALDH aldehyde dehydrogenase
BA bile acid
Bcl-xL B cell lymphoma-extra-large
BDL bile duct ligated/ligation
CAMK calmodulin-dependent protein kinase
cAMP cyclic adenosine monophosphate
CCL C-C like chemokine ligand
CFTR cystic fibrosis transmembrane conductance regulator
Cil1a ciliary localization 1a
CREB cAMP response element-binding protein
CTGF connective tissue growth factor
CXCL11 C-X-C motif chemokine ligand 11
DDC 3,5-diethoxycarbonyl-1,4-dihydrocollidine
DDX3Y DEAD-box helicase Y-linked protein
DNA deoxyribonucleic acid
DR ductular reaction
ER endoplasmic reticulum
ERK1/2 extracellular signal-regulated protein kinases-1/-2
FXR farnesoid X receptor
GPR g-protein coupled receptor
HFD high fat diet
HPC hepatic progenitor cells
IGF-1 insulin-like growth factor-1
IL interleukin
MCP-1 monocyte chemoattractant protein 1
***Mdr2*$^{-/-}$** multidrug resistant cassette 2 knock out
MEK mitogen activated protein kinase kinase
MHC major histocompatibility complex
miR micro RNA
NAFLD nonalcoholic fatty liver disease
NASH nonalcoholic steatohepatitis
N-Ras neuroblastoma RAS viral oncogene homolog
PBC primary biliary cholangitis
PDFG platelet derived growth factor
PKA protein kinase A
PPAR peroxisome proliferation activated receptor
pPKA phospho-PKA
PSC primary sclerosing cholangitis
SASP senescence associated secretory phenotype
SCF stem cell factor
SIRT1 sirtuin 1
αSMA α—smooth muscle actin
SR secretin receptor
Src proto-oncogene tyrosine-protein kinase Src
TGF-β transforming growth factor β
twf-1 twinfilin-1
UDCA ursodeoxycholic acid
WD western diet

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REVIEW
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Beyond the Liver: Liver-Eye Communication in Clinical and Experimental Aspects

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The communication between organs participates in the regulation of body homeostasis under physiological conditions and the progression and adaptation of diseases under pathological conditions. The communication between the liver and the eyes has been received more and more attention. In this review, we summarized some molecular mediators that can reflect the relationship between the liver and the eye, and then extended the metabolic relationship between the liver and the eye. We also summarized some typical diseases and phenotypes that have been able to reflect the liver-eye connection in the clinic, especially non-alcoholic fatty liver disease (NAFLD) and diabetic retinopathy (DR). The close connection between the liver and the eye is reflected through multiple pathways such as metabolism, oxidative stress, and inflammation. In addition, we presented the connection between the liver and the eye in traditional Chinese medicine, and introduced the fact that artificial intelligence may use the close connection between the liver and the eye to help us solve some practical clinical problems. Paying attention to liver-eye communication will help us have a deeper and more comprehensive understanding of certain communication between liver diseases and eyes, and provide new ideas for their potential therapeutic strategy.

Keywords: liver-eye communication, metabolism, non-alcoholic fatty liver disease, diabetic retinopathy, traditional Chinese medicine, communication molecule

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INTRODUCTION

In recent years, the communication between organs has received more and more attention. With the development of modern medical physiology and pathology, it has been discovered that there are some communication links between the human body's organs and organs or tissues that are established with the help of endocrine, immune and other systems, which are considered to be important for maintaining homeostasis and achieving physiological functions. The connection between the eyes and the liver has been discovered and valued in many studies (Wang et al., 2021), which is achieved through a variety of pathways including metabolism, inflammation, oxidative stress, and immunity. In addition, clinically, some possible connections have been revealed in the occurrence, development and outcome of some liver diseases and ocular diseases, such as Non-alcoholic fatty liver disease (NAFLD) and Diabetic retinopathy (DR). Therefore, if we have a clearer understanding of the communication mechanism between the liver and the eyes, it will help us better understand the development mechanism of liver and eye diseases, and provide some ideas for targeted therapy.

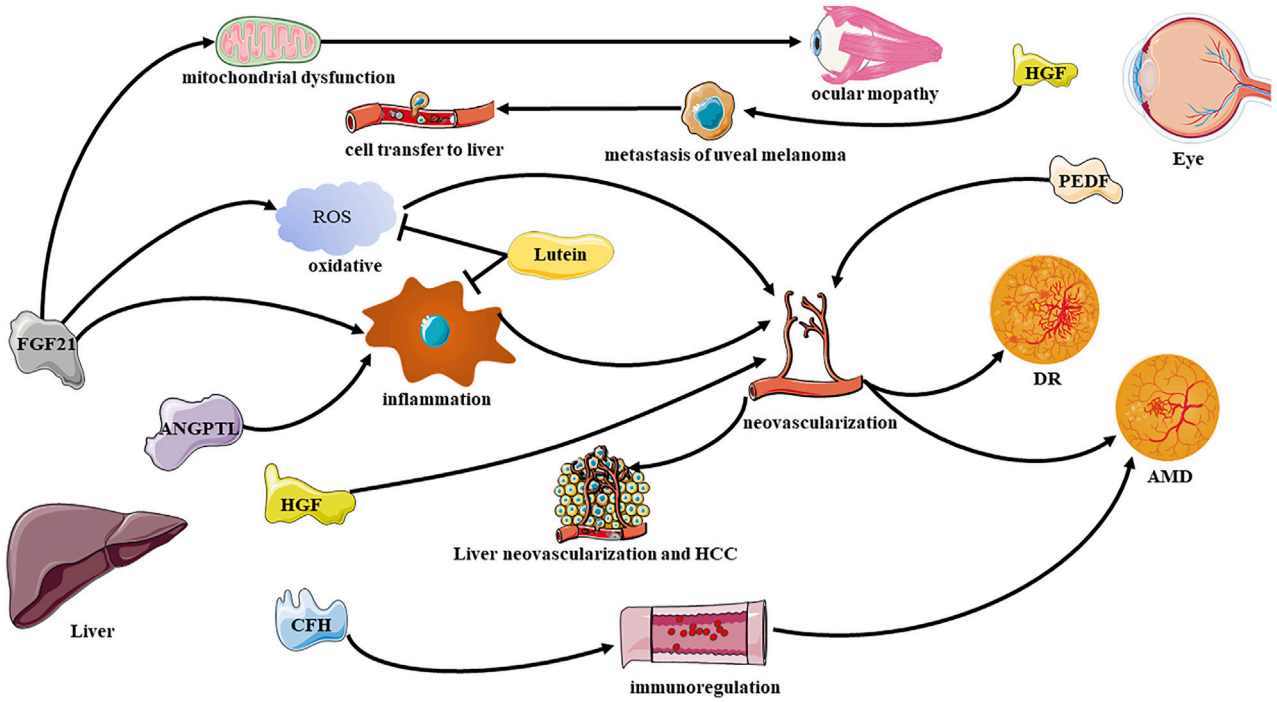

FIGURE 1 | Effective communication molecules from the liver to eye under physiological and pathological conditions. DR, diabetic retinopathy; AMD, age related macular degeneration; FGF21, Fibroblast Growth Factor 21; HGF, hepatocyte growth factor; ANGPTL, Angiopoietin-like proteins; PEDF, pigment epithelium-derived factor.

COMMUNICATING MOLECULES MEDIATE THE LIVER-EYE ASSOCIATION

The interaction between the liver and the eye is reflected in the molecular communication by their secretory factors and their associated cytokines (**Figure 1**). "Hepatokines" are certain signaling proteins that are secreted exclusively or predominantly by the liver (Wang et al., 2021),which are mostly delivered to liver or other distant organs through the human circulation system, and are involved in regulating diseases such as metabolic, inflammatory disease (Meex and Watt, 2017). Similarly, some factors secreted by the eyes also remotely affect the state of the liver. In addition, some non-organ-specific cytokines also reflect the connection between the liver and the eyes.

Fibroblast Growth Factor-21

Fibroblast growth factor-21 (FGF-21) is a hormone predominantly secreted by the liver, which could perform multiple effects on the regulation of glucose metabolism as well as insulin activity (Xu et al., 2009; Markan et al., 2014). The function of FGF-21 to regulate glucose homeostasis has been universally proven in animals (Potthoff et al., 2012) and humans (Gaich et al., 2013). In addition, FGF21 is also defined as highly predictive biomarker for mitochondrial diseases (Tsygankova et al., 2019). FGF21 has been found in clinical research to reflect the liver-eye connection in many aspects. A study has found that FGF21 is significantly related to ocular myopathy (a mitochondrial disease), especially chronic progressive external ophthalmoplegia (Morovat et al., 2017). Moreover, FGF21 can also affect autophagy, the level of FGF21 increases under fasting induction, which can dephosphorylate the transcription factor EB, in addition induce the expression of genes related to autophagy (Chen et al., 2017a). Autophagy defects, including lipofuscin accumulation, decreased mitochondrial activity, and elevated reactive oxygen levels, can affect angiogenesis. Mutations of autophagy genes and the occurrence of autophagy defects are considered to be related to the occurrence of age related macular degeneration (AMD) in animals (Zhang et al., 2017a) and humans (Golestaneh et al., 2017). FGF21 administration decreased neovascular lesions in two models of neovascular age-related macular degeneration has been observed recently (Fu et al., 2017). Oral peroxisome proliferator-activated receptors-alpha (PPARα) agonists can be used in insulin-deficient diabetic mice (Fu et al., 2018), intraperitoneal injection of streptozotocin to induce diabetes model mice (Tomita et al., 2020a), oxygen-induced retinopathy model mice (Tomita et al., 2019), and retinal ischemia model mice (Lee et al., 2021) to promote the expression of FGF21.

The production of FGF21 is induced by PPAR-α and plays a role by regulating the activities of PPAR and PGC-1α (Potthoff et al., 2009). A research has shown that FGF21 transcription cannot be induced in the liver of PPAR-α deficient mice (Lundåsen et al., 2007). The increase in FGF21 expression level boosting liver function, maintain retinal neuron activity, regulating pathological microglia proliferation, strengthening the

retinal antioxidant defense system, reducing pro-inflammatory cytokines and improving retinal function, and mediate and inhibit retinal neovascularization. Some recent basic medical studies have proved that FGF21 deficiency can lead to aggravation of retinal neovascularization. FGF21 may inhibit retinal neovascularization by inhibit the expression of tumor necrosis factor (TNF)-α and increasing the secretion of adiponectin (Lin et al., 2013; Fu et al., 2017), indicating that FGF21 may have therapeutic significance for DR. But it is worth noting that it has been reported that serum FGF21 concentration is positively correlated with the severity of DR (Lin et al., 2014), this seems to contradict the previous conclusion. The increased serum FGF21may be related to the compensation caused by FGF21 resistance, suggesting that FGF21 as a potential biomarker of DR. What's more, the analogues of long-acting FGF21 have recently been found to improve the permeability of tight junctions by increasing the level of tight junction proteins for example Claudin-1 in human vascular endothelial growth factor (VEGF)-induced human retinal microvascular endothelial cells and C57BL/6J mice, leading to the reduction of vascular leakage in retinal diseases (Tomita et al., 2020b). All in all, FGF21 has an effect on Pterygia (Yaghoobi et al., 2020), AMD, DR and many other eye diseases because of its ability to reduce ocular neovascularization. Therefore, it seems promising to use FGF21 as a treatment direction for ocular vascular diseases.

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is a cytokine mainly secreted by kupffer cells of the liver, but the expression of HGF receptors has been detected in the cornea, lens and retinal tissues of the eye, which can maintain the structure and function of corneal epithelial cells, lens epithelial cells, and retinal pigment epithelial cells (Grierson et al., 2000). Uveal melanoma is the most common primary intraocular malignant tumor, and 50% of patients eventually die of metastatic disease. The most common site of metastasis is the liver (Shields et al., 1991). HGF can play a role in promoting cell transfer. The activation of PI3K/AKT pathway induced by HGF-cMET axis participates in the down-regulation of cell adhesion molecules E-cadherin and β-catenin, which weakens cell adhesion and promotes the induction of tumor cell proliferation, movement, adhesion and invasion (Ye et al., 2008). Besides, HGF also has an effect on retinal neovascularization. HGF/NK-4 inhibits VEGF induced retinal angiogenesis by inhibiting the phosphorylation of ERK and ETS-1 in endothelial cells cultured *in vitro* and rabbits (Nakabayashi et al., 2003). Moreover, retinal pigment epithelial cells (RPE)-endothelial-mesenchymal transition is related to a variety of blinding retinal diseases. In the study of endothelial-mesenchymal transition induction on the RPE layer derived from human induced pluripotent stem cells, it was found that the HGF-MET signal showed the highest overall enrichment. In addition, they also found that HGF signaling plays a role in regulating the transcription profile of RPE (Mertz et al., 2021).Thus, it will be intriguing to study whether altered expression of HGF in liver diseases could exsert distance influence on ocular condition.

Angiopoietin-Like Proteins

Angiopoietin-like proteins 4 (ANGPTL-4) and ANGPTL-8 are a class of proteins mainly secreted by the liver, and a very small part is produced by adipose tissue and muscle (Wang et al., 2021). ANGPTL4 transcript in adipose tissue accounts for only 10% of liver in human (Romeo et al., 2009). Therefore, we have reason to believe that ANGPTL-4 in the human circulation mainly comes from the liver. The detection of protein levels in HRMEC cultured *in vitro* and rat retinal and vascular endothelial cell extracts showed that high glucose can induce the up-regulation of ANGPTL-4 expression in both models, and it may increase the expression of ANGPTL-4 by activating profilin-1 signal to generate retinal inflammation, vascular permeability, and angiogenesis (Lu et al., 2018). In the test of proliferative diabetic retinopathy (PDR) patients, it was found that the levels of ANGPTL-4 in the vitreous and serum of PDR patients were higher than those in the control group, and the expression level of VEGF was positively correlated with ANGPTL-4 (Lu et al., 2016). In addition, inflammatory factors such as interleukin (IL)-8 are also positively correlated with ANGPTL-4 levels (Wu et al., 2021). This suggests that ANGPTL-4 may promote angiogenesis in humans, and may increase retinal inflammation, thereby increasing the severity of PDR. ANGPTL-8 plays an important role in regulating lipid metabolism inside and outside EC, lipoprotein lipase activity, and inflammatory pathway NF-κB signal transduction (Abu-Farha et al., 2020). Similar to ANGPTL-4, the expression level of ANGPTL-8 was also detected in PDR patients, and it was significantly positively correlated with VEGF (Lu et al., 2017). ANGPTL reflects the tight connection between the liver and the eye in the inflammation pathway.

Complement Factor H

Complement factor H (CFH) is an essential component for the synthesis of alternative pathways of complement. It is generally believed that CFH is mainly produced in the liver in the human body (Mandal and Ayyagari, 2006), and is still controversial that whether it is expressed in the retina and RPE and choroid (Hughes et al., 2016). Importantly, the correlation between AMD and CFH has been widely concerned (Klein et al., 2005). Based on blood analysis of patients with AMD, it is found that the expression level of CFHR-4 gene, which is specifically expressed in the liver, is increased in the blood and retina. The activation of this gene will activate the complement system and exacerbate the course of the disease (Cipriani et al., 2020), implicating that the liver plays an important role in regulating the immune homeostasis of the retina. In addition, Y402H variant of CFH gene proved to be associated with increased risk of AMD (Hughes et al., 2016). CFH regulates alternative pathways of complement activation and protects host cells from inappropriate complement activation (Mandal and Ayyagari, 2006). In the C57BL/6 mouse model, the expression of CFH in RPE and choroid helps to regulate the alternative pathway of complement cascade and membrane attack complex formation, thereby preventing the occurrence of choroidal neovascularization (CNV). In addition, local inhibition of CFH also weakened the regulation of membrane attack complex

deposition, causing the disorder of membrane attack complex deposition, and aggravated the laser-induced CNV in mice (Lyzogubov et al., 2010). Further research is needed to explain how CFH mediates liver-eye contact through the immune system.

Retinal Pigment Epithelium-Derived Factor

Secretory factors produced in eyes can also act on the liver. Retinal pigment epithelium-derived factor (PEDF) was initially discovered to be secreted by retinal pigment epithelial cells (Tombran-Tink and Johnson, 1989). It is a neurotrophic factor with anti-oxidation, anti-inflammatory and anti-angiogenic effects (Elahy et al., 2014). PEDF also inhibits Wnt coreceptors and low-density lipoprotein receptor-related protein 6 (LRP6) in the eyes and liver (Protiva et al., 2015), suggesting that it may play a role in liver-eye communication. It has been widely demonstrated that PEDF and VEGF together maintain the balance of controlling angiogenesis in the eye. For example, mTORC1 signal in DR can change the proliferation and migration of endothelial cells by regulating the expression of VEGF and PEDF protein (Liu et al., 2020). Its possible mechanism of action is related to inflammation-related pathways. A study has found that PEDF can inhibit angiogenesis from endothelial cells and tumor cells by down-regulating HIF-1α in breast cancer (Mao et al., 2020). Notably, PEDF is up-regulated in the liver of cirrhotic humans and bile duct-ligated rats, and the adenovirus-mediated gene transfer in bile duct-ligated rats exogenously overexpresses PEDF, which inhibits liver angiogenesis, fibrogenesis and reduces portal pressure (Mejias et al., 2015). Interestingly, the inhibitory effect of PEDF on angiogenesis is only for pathological angiogenesis, and does not affect physiological angiogenesis, which suggests the potential of PEDF for possible therapeutic applications. In hepatocellular carcinoma (HCC), PEDF can play an anti-angiogenic effect in this typical tumor (Matsumoto et al., 2004). It can also regulate epithelial mesenchymal transition by up-regulating the expression of E-cadherin and down-regulating the expression of Slug and Vimentin, thereby reducing the migration and invasion ability of HCC cells (Chen et al., 2017b). However, it is still unveiled that if the upregulated PEDF is derived from remote delivery or from local production. There have been some researches related to knockout PEDF, but no study has specific knockout PEDF in the eye. Recent research has found that the PEDF signal of the eye in the RAD6B-deficient group changes, which leads to the occurrence of retinal degeneration (Ye et al., 2022). This may provide ideas for ocular knockout PEDF and further study the source of up-regulated PEDF expression.

Other Molecules

In addition to the oxidative stress and inflammation links between the liver and the eyes, some non-organ-specific molecules also act as mediators. Lutein is one of the carotenoids, occurs in a large green vegetables and plasma, eye of body. It has specific biological functions, especially in several ocular diseases like age-related macular degeneration (Heesterbeek et al., 2020). Mechanically, lutein can reduce light-induced oxidative damage and prevents inflammation (Li et al., 2020). Indeed, lutein supplementation improves the oxidative stress in the liver and eyes of guinea pigs on a high-cholesterol diet by reducing the binding activity of NF-κB DNA and the level of inflammatory factor TNF-α (Kim et al., 2012). Except lutein, a study showed that thioacetamide can attack the liver to secrete the pro-inflammatory cytokines IL-6 and TNF-α, which ultimately leads to brain and eye damage. More interestingly, the improvement of liver damage can improve the eyesight and cognitive ability of mice (Sun et al., 2020). It can be seen that the liver and eyes are inextricably linked with oxidative stress and inflammation.

LIVER METABOLISM AFFECTS OCULAR DISEASES

The liver is regarded as the center of metabolism in the human body, and it metabolizes carbohydrate, lipids, proteins, and many other substances. Thus, disorders of liver metabolism often influence a variety of physiological processes, and may lead to corresponding eye diseases (**Figure 2**).

Glycometabolism

The liver is an important place for the body to synthesize and store glycogen, and it plays an important role in blood sugar regulation. Abnormal liver function, such as the accumulation of fat in the liver caused by NAFLD, can induce insulin resistance and increased liver gluconeogenesis and other blood glucose regulation disorders, which induce or aggravate diabetes (Roden and Shulman, 2019; Watt et al., 2019). Diabetes can cause abnormal metabolism of vascular endothelial cells in the eye and induce DR (Li et al., 2019). It is generally recognized that there is a link between NAFLD and diabetes. For example, in an 11-year follow-up study, NAFLD was found to be a risk factor for diabetes and metabolic syndrome (Adams et al., 2009). However, the connection between NAFLD and DR is still controversial, and we will discuss it later. Regulating sugar metabolism seems to be a good target for retinal neovascular diseases. PFKFB3 is a key regulatory enzyme in the glycolysis pathway (Zhou et al., 2021). PFKFB3 inhibits endothelial cells *in vitro* and damages the sprouting of EC, and affects the growth and branching of blood vessels in the mouse retina *in vivo* (Xu et al., 2014).

Lipid Metabolism

Secondly, the liver participates in the lipid cycle by participating in the synthesis of fatty acids and lipoproteins, and plays a pivotal role in lipid metabolism (Nguyen et al., 2008). Lipid metabolism plays an important role in the pathogenesis of eye diseases, especially ocular neovascular disease. Using etomoxir to inhibit the oxidation of fatty acids in retinopathy of prematurity model mice can reduce retinal neovascularization (Schoors et al., 2015). AMD has been found to be related to a variety of lipids and lipoprotein genes, including liver lipase, cholesterol ester transferase, and apolipoprotein E, which are mainly expressed in the liver in the human body (Jun et al., 2019). Disturbance of lipid metabolism is an important pathological mechanism leading to AMD. Under oxidative stress, a type of lipid deposit called

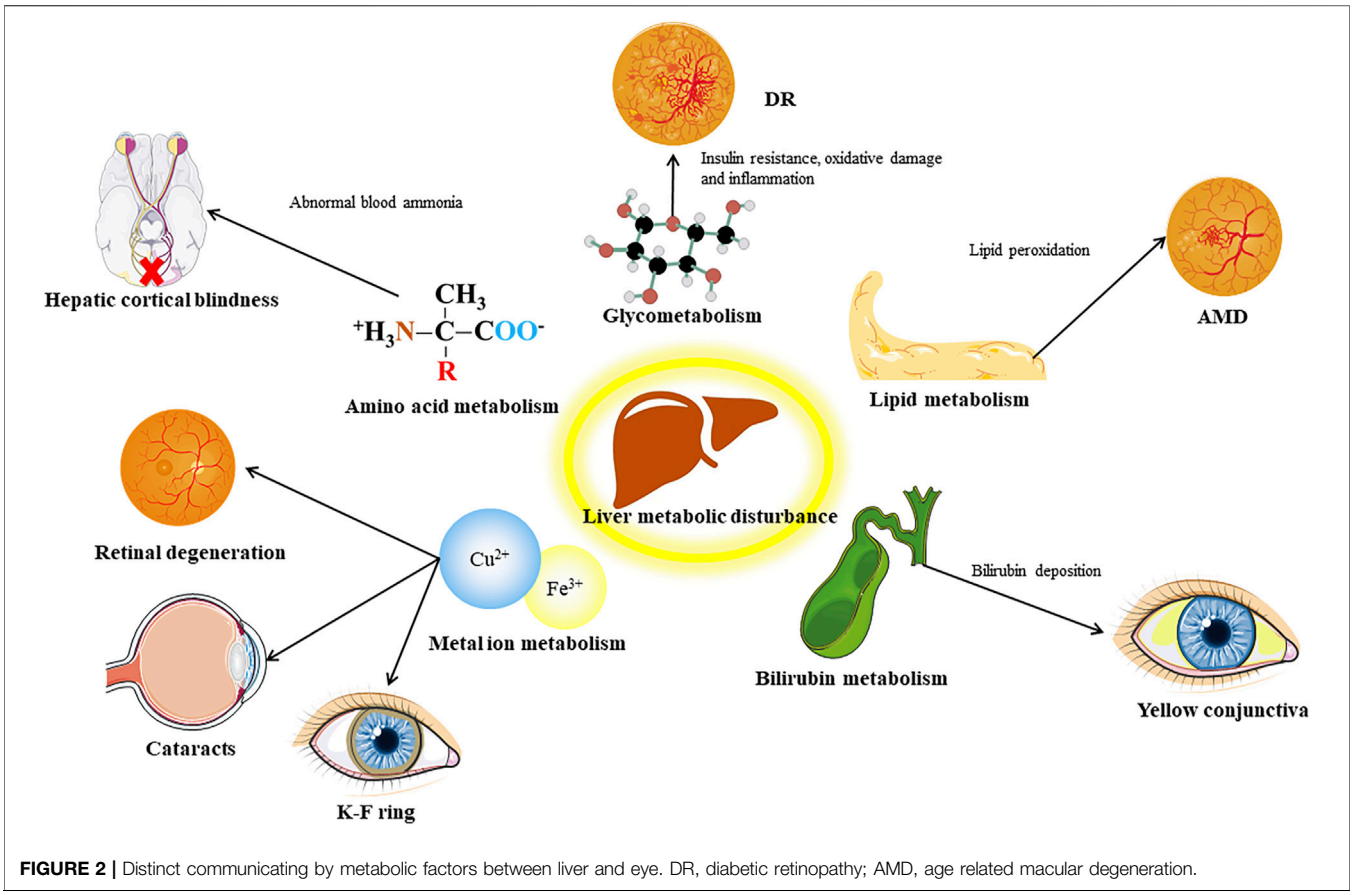

FIGURE 2 | Distinct communicating by metabolic factors between liver and eye. DR, diabetic retinopathy; AMD, age related macular degeneration.

drusen is formed in the retina, and it activates the complement system to trigger chronic inflammation (Xu et al., 2018). Studies have shown that lipid imbalance can promote the development of lesions in a variety of different animal models of AMD (Malek et al., 2005; Fujihara et al., 2009; Toomey et al., 2015). Epidemiological investigations suggest that high-fat diet is a risk factor for AMD (Clemons et al., 2005). These evidences suggest that lipid metabolism is closely related to AMD, and is related to the liver function.

Amino Acid Metabolism

Another important biological function of the liver is to deaminate and transaminate amino acids and use the ammonia produced by amino acid metabolism to synthesize urea to prevent excessive levels of ammonia in the blood (Walker, 2014). When liver disease occurs, it will affect the normal clearance of ammonia in the blood, which will have a toxic effect on the optic nerve and cause hepatic cortical blindness (Ammar et al., 2003).

Bilirubin Metabolism

The liver also plays an important role in the metabolism of bilirubin. Liver dysfunction caused by liver disease can cause liver cells to fail to normally take in unbound bilirubin in the blood, causing bilirubin metabolism disorders in the body. Elevated bilirubin can cause yellowing of the skin and conjunctiva, especially the conjunctiva. Because the conjunctiva contains more elastin, it has a higher affinity with bilirubin (Carroll et al., 2017). Yellowing of the conjunctiva in patients with jaundice is an extremely intuitive manifestation of the liver-eye connection. What's more, primary biliary cirrhosis caused by bilirubin deposition can lead to the occurrence of pigmented corneal rings (Fleming et al., 1977).

Metal Ion Metabolism

Moreover, liver also regulates the metabolism of some metal ions. The liver secretes hepcidin into the blood to reduce blood iron levels. The blood and retinal pigment epithelium iron levels of liver-specific hepcidin knock-out mice increase, and the free iron levels in the retina increase and cause RPE hypertrophy, the photoreceptors also undergo focal degeneration (Baumann et al., 2019). The pathogenic variants of the disease-causing genes of hepatolenticular degeneration led to the functional defect or loss of ATPase, which causes the biliary tract copper excretion disorder and leads to abnormal copper metabolism. This leads to copper deposits in the Descemet membrane area of the cornea, triggering a characteristic lesion called the Kayser–Fleischer ring (Richard and Friendly, 1983). In addition, copper deposits

in the eye can also cause ocular lesions called sunflower cataracts (Fahnehjelm et al., 2011).

ASSOCIATION OF NON-ALCOHOLIC FATTY LIVER DISEASE AND RETINOPATHY IMPLIES THE LIVER-EYE COMMUNICATION

NAFLD is the umbrella term for non-alcoholic simple fatty liver, non-alcoholic steatohepatitis, and hepatic cirrhosis (Farrell and Larter, 2006) and have become the leading cause of chronic liver disease in Western countries (Lazo and Clark, 2008). It has a diverse histopathological spectrum ranging from simple steatosis with mild inflammation to various stages of fibrosis, and ultimately to hepatic cirrhosis, HCC. Previous studies have found insulin resistance is a critical factor in the pathophysiology of NAFLD and can promote the accumulation of triglycerides in the liver (Zelber-Sagi et al., 2018; Abdelmoemen et al., 2019). NAFLD can cause disorders of glucose and lipid metabolism in the body, coupled with its characteristic of insulin resistance, naturally remind people of diabetes. DR is the most common chronic complication of diabetes mellitus and one of the main causes of acquired blindness in the world (Campos et al., 2017). Recognized main pathogenic mechanism of diabetic retinopathy is hyperglycemia-induced microvascular damage caused by impaired insulin action due to insulin resistance (type 2 diabetes mellitus) or insulin deficiency (type 1 diabetes mellitus) (Gardner et al., 2011), while the deep pathogenesis of DR has not yet been fully understood (Zhang et al., 2017b).

Recently, multiple clinical investigations designed to explore the effects of non-alcoholic fatty liver disease on the incidence of DR in patients with diabetes mellitus (Song et al., 2021). There also have been more and more studies have indicated that NAFLD can influence the morbidity of complications in patients of diabetes mellitus, especially microvascular complications (Hazlehurst et al., 2016; Perumpail et al., 2017), which involve chronic kidney disease and DR (Mima, 2016). Although NAFLD and DR have some similar pathogenic and molecular mechanisms (Potthoff et al., 2009; Tomita et al., 2019; Lee et al., 2021), studies on different ethnic groups have shown different results. For type 1 diabetics, a previous study showed that the prevalence of DR increased in Indian patients with nonalcoholic fatty liver disease (Vendhan et al., 2014). Our recent meta-analysis including Indian and Japanese patients with type 1 diabetes mellitus also suggested the same conclusion (unpublished). However, for type 2 diabetics, several evidence-based medicine studies from different countries reflected that NAFLD may not be a risk factor for DR and may even be beneficial. A study that mainly includes American type 2 diabetics indicated that NAFLD is not associated with retinopathy (Lin et al., 2016). Whereas several observational studies in China, Korea and Iran showed that the NAFLD group had lower retinopathy (mainly NPDR) morbidity than the non-NAFLD group in patients with type 2 diabetes mellitus (Lv et al., 2013; Kim et al., 2014; Afarideh et al., 2019; Zhang et al., 2019; Wen et al., 2021). Additionally, in an Italian research, the NAFLD was positive related with retinopathy (NPDR or PDR) (Targher et al., 2008). Some Western studies on NAFLD patients have also shown that the more severe the liver fibrosis, the higher the risk of retinopathy (Leite et al., 2021; Mikolasevic et al., 2021). These controversial opinions can be caused by different diabetic pathological characteristics of different races (Song et al., 2021). For example, the serum insulin level of Asians was lower than Caucasian (Yoon et al., 2006; Unnikrishnan et al., 2017). Regarding the connection between NAFLD and DR, the conclusions drawn by basic medical research are also controversial. As mentioned above, FGF21 plays a role in inhibiting retinal neovascularization As a regulatory secretion mainly produced by the liver (Geng et al., 2020; Keuper et al., 2020), oxidative damage and chronic inflammation of NAFLD suppressed β-klotho and FGFR expression, leading to a compensatory increase in FGF21 synthesis and secretion (Tucker et al., 2019). It has also been proved that the level of serum FGF21 in the NAFLD group is higher than control group (He et al., 2017; Keuper et al., 2020). This seems to support the conclusion that NAFLD is negatively correlated with DR, as the increased level of FGF21 may be the reason of the lower morbidity of retinopathy in patients with NAFLD after compromising with FGF21 resistance. But for retinal artery damage, in the study by Wen et al., patients with NAFLD had higher incidences of coronary artery disease and retinal artery lesion (Yang et al., 2015), the opposite conclusion was reached. However, whether FGF21 resistance also occurs in the eyes still needs to be measure. If FGF21 resistance also occurs in the eyes, then the increase in serum FGF21 does not prove that NAFLD and DR are negatively correlated. What's more, we should note that the current research on FGF21 inhibiting retinal neovascularization is through the administration of exogenous FGF21 or FGF21 receptor agonists (Tomita et al., 2019; Tomita et al., 2020a; Lee et al., 2021). At present, there is no research on whether the compensatory increase of FGF21 in serum in NAFLD is sufficient to cause the therapeutic effect of ocular neovascularization. Therefore, the compensatory elevated FGF21 level in NAFLD patients is not enough to indicate that NAFLD is negatively correlated with DR. What's more it is worth noting that in a study, the use of exogenous FGF21 inhibited the occurrence of choroidal neovascularization in mice unrelated to the occurrence of diabetes (Fu et al., 2017), which suggested that there may be more communication pathways between our liver and eyes, not just only insulin resistance.

In addition, the occurrence of retinopathy might also affect the physiological process and pathological progress of the liver. Oxidative stress in the retina induced by retinitis pigmentosa might affect soluble macromolecules in retina or damage the melanopsin system. It led to chronic circadian desynchronization, weakened the antioxidant defense of the system, and eventually led to oxidative stress in the liver (Perdices et al., 2018), which may promote NAFLD. Diabetic retinopathy was also considered as a risk factor for HCC in NAFLD patients (Azuma et al., 2019). However, the exact mechanism is still to be elucidated. Therefore, patients with

TABLE 1 | The actions and possible mechanism of TCM drugs revealing liver-eye connection.

Drug name	Pharmaceutical ingredients	The effects of drug
Qi-Ju-Di-Huang-Van	*Lycium barbarum*, *Chrysanthemum*, *Rehmannia*, *Cornus*, *Peony bark*, *Chinese yam*, *Tuckahoe*, *Alisma*	Nourishes the kidney and liver. Treatment liver and kidney yin deficiency, dizziness, tinnitus, photophobia, tears in the wind, dim vision.
Long-Dan-Xie-Gan-Tang	*Gentian*, *Gardenia*, *Scutellaria baicalensis*, *Mutong*, *Alisma*, *Plantain seed*, *Bupleurum*, *Liquorice*, *Angelica*, Radix *Rehmanniae* recen	Reduce liver and gallbladder fire, clear scorching damp heat.
Xiao-Yao-San	*Licorice*, *Angelica*, *Poria cocos*, *Paeonia alba*, *Atractylodes macrocephala*, *Bupleurum*	Soothing liver and relieving depression, nourishing blood and strengthening spleen.

NAFLD complicated with diabetic retinopathy may should be regularly screened for HCC.

In all, molecular mediators linking NAFLD with diabetic retinopathy might include an increased release of some pathogenic mediators from the liver, such as FGF21 (Fu et al., 2017), HGF (Nakabayashi et al., 2003), C-reaction protein, reactive oxygen species, IL-6 and TNF-α (Targher et al., 2008), which in turn determine the disease progression, forming eye-liver communication. However, the evidence for an association between NAFLD and diabetic retinopathy is still unclear because of the tangled association between NAFLD and hyperglycemia, insulin resistance, obesity and other traditional risk factors for diabetic retinopathy and the small study population in the published literature. More researchers are needed to elucidate the correlation and underlying molecular mechanism between NAFLD and diabetic retinopathy in diabetes.

CURRENT APPLICATION OF LIVER-EYE COMMUNICATION IN CLINICAL PRACTICE

Traditional Chinese Medicine

A long time ago, the traditional viscera theory of traditional Chinese medicine (TCM) put forward the saying that "liver resuscitation in the eyes." With the development of modern medicine, more and more evidences show that the two major organs of the liver and the eyes are complex and intimate in terms of physiology and pathology. The biological connection further supports the theory of "liver resuscitation in the eyes."

Starting from the liver to treat eye diseases, it has been widely clinical practice in TCM. According to TCM differentiation, a study have analyzed the types of patients with dry eye disease, and concluded that the liver and kidney be feeble occurs most frequently among different types, which is much higher than other types (XZ et al., 2015). Therefore, in clinical practice, some scholars treat dry eye by nourishing the liver and kidney. They choose Qi-Ju-Di-Huang-Van in treatment, which promotes the secretion of tears, prolongs the tear film rupture time, reduces dryness and recurrence rate (D and WP, 2021). Pestle therapy, which is beneficial to the liver and kidney, also promotes tear secretion, prolongs tear film rupture time, and relieves the patient's anxiety and depression (YX et al., 2020). In addition, TCM believes that the liver is the key to the treatment of inflammatory diseases. Based on this theory, people use Long-Dan-Xie-Gan-Tang, which hepatoprotective and anti-inflammatory effects, to treat uveitis (HS et al., 2015). In addition, based on the cognition of liver-eye connection, use Xiao-Yao-San, which has many benefits for the liver, to treat supraorbital neuralgia, eyeball pain, dry eye, open-angle glaucoma and achieved certain effects (J et al., 2017). We have summarized the main ingredients and effects of Chinese medicines mentioned above (**Table 1**). In addition, some of the ingredients of these Chinese medicines have been reported to have liver toxicity, including Alisma, licorice, and bupleurum (Frenzel and Teschke, 2016). These Chinese medicines are also not recommended for long-term use in clinical practice, so the safety of their long-term use should be paid attention to when using this type of medicine for treatment.

The therapeutic mechanism of TCM for eye diseases through the liver has also been extensively explained under the development of modern medicine. TCM believes that eating animal liver has the effect of improving eyesight. From the perspective of modern medicine, it is because the liver stores and transports vitamin A, and eating liver of animal supplements vitamin A. Lack of vitamin A is related to the occurrence of blindness. The photosensitive function of rod cells depends on the visual pigment composed of a molecule of 11-cis retinal and a molecule of opsin and meanwhile vitamin A is the starting material for the synthesis of 11-cis-retinal (Harrison, 2019). Because of the importance of the liver for the transportation and storage of vitamin A, damage to liver function can also lead to vitamin A deficiency in the body. For example, patients undergoing liver transplantation have a high probability of developing vitamin A deficiency (Venu et al., 2013). Therefore, night blindness and other visual disorders are more common in patients with liver disease.

More importantly, it is mostly believed that inflammatory liver disease can easily lead to some eye diseases, and Chinese medicine prescriptions for treating eye diseases have been proven to improve liver and ocular inflammation at the same time, such as lycium barbarum and chrysanthemum. Lycium barbarum polysaccharide(LBP) is the main active ingredient of lycium barbarum (Amagase et al., 2009). Studies have found that LBP is a promising neuron protective agent, which can effectively improve oxidative stress, inflammation, apoptosis and cell death (Xing et al., 2016; Zhong et al., 2020), consequently it can directly and indirectly protect the optic nerve. In addition, LBP also protect the liver. LBP significantly improve the damage induced by non-alcoholic steatohepatitis, including the increase in serum ALT and AST levels, liver oxidative stress, fibrosis, inflammation, and apoptosis (Xiao et al., 2014; Xiao et al., 2018). Chrysanthemum contains luteolin. Studies have found that

luteolin has the effect of anti-inflammatory and blocking the production of reactive oxygen species, and has anti-uveitis (Kanai et al., 2016) and anti-retinal neovascularization (Park et al., 2012) effect. In the liver, luteolin and luteolin-7-O-glucoside prevent GalN/LPS-induced hepatotoxicity in mice by regulating inflammatory mediators and antioxidant enzyme activities (Park and Song, 2019), thus play a role in protecting liver. In all, the practice of traditional Chinese medicine provides an integrative view and a novel insight into the underlying correlation between liver and eye. As liver performs a number of essential functions related to detoxication, nutrient storage, metabolism and etc. for the whole system including eye, understanding the liver-eye communicating mechanism, especially the molecular mediators are of great significance.

Artificial Intelligence Application of Liver-Eye Relationship

With the emergence of graphics processing unit, the progress of mathematical models, the availability of big data and the advent of low-cost sensors, AI has been used in many industries, including the Web of things, social media and medical fields (LeCun et al., 2015). In the medical field, especially in the image-centered departments such as radiology, dermatology, pathology and ophthalmology, AI's deep learning (DL) techniques have been widely used and made great progress due to their strong graphics processing ability (Schmidt-Erfurth et al., 2018; Ting et al., 2019a). DL approaches used complete images, and associated the entire image with a diagnostic output, thereby eliminating the use of "hand-engineered" image features (Ting et al., 2019b). In ophthalmology, DL system was mainly used in two fields. First, the DL system has been shown to accurately detect DR (Abràmoff et al., 2016; Gargeya and Leng, 2017; Ting et al., 2017), glaucoma (Ting et al., 2017), AMD (Ting et al., 2017; Burlina et al., 2018a; Burlina et al., 2018b), ROP (Brown et al., 2018) and ametropia (Poplin et al., 2018) using fundus image. Secondly, new studies have shown that several retinal conditions, such as CNV, early AMD and diabetic macular edema, can also be accurately detected by the DL algorithm used in optical coherence tomography images (Lee et al., 2017; Ting et al., 2019b). Thereby, AI's graphics processing ability and DL system make the eye a window for observing systemic diseases as well, and have the advantages of non-invasive examination and diagnosis. For example, using eye manifestations to predict neurodegenerative diseases, diabetes, etc.

Due to its non-invasiveness and convenience, analyzing ocular images via AI has a preliminary advantage in identifying liver diseases, which can also confirm the link between liver and eye from clinical perspective. In the process of liver metabolism, the direct toxicity of abnormal metabolites, excessive normal metabolites and insufficient liver energy metabolism can lead to abnormal ocular performance (Poll-The et al., 2003). Many hepatobiliary diseases, including hepatitis, cirrhosis, HCC and cholelithiasis, are often accompanied by non-specific ocular abnormalities, such as scleral jaundice caused by the accumulation of bilirubin in sclera. In addition, there are also ocular abnormalities in some rare liver diseases, including corneal Kayser-Fleischer ring in Wilson disease, cherry-red macular spots in Niemann-Pick disease, and posterior embryotoxon or optic disc drusen in Alagille syndrome. Therefore, ophthalmological examination is helpful to screen some specific hepatobiliary diseases. In view of this, the AI algorithm for screening liver diseases using ocular models has also been applied and reported for the first time (Xiao et al., 2021). In this AI algorithm, the DL system utilized patient information, including slit-lamp anterior segment photos, fundus photos and diagnostic data of hepatic diseases, to train and adjust to form a reliable DL model. By analyzing the ocular images (anterior segment photos and fundus photos), this model successfully predicted the category of hepatobiliary diseases, including HCC, hepatic cirrhosis, chronic viral hepatitis, NAFLD and cholelithiasis. Additionally, when analyzing the effect-region of the eye images, it is found that the analysis area of the DL model was mainly concentrated in the sclera, iris and the distribution area of the optic disc and inferior vascular arch (Xiao et al., 2021). This indicated that the influences of hepatic diseases on the eyes may be concentrated in these areas as well. However, at current stage, the DL model cannot describe the pathological characteristics of these areas in detail, which is need to be further investigated.

FUTURE PROSPECTIVE

In terms of communication between the liver and the eyes, there are still many worthwhile problems that still need to be resolved.

Regarding the molecular aspect, most of the current researches only describe some phenotypic links between the liver and the eye, but do not go into the specific molecular mechanisms for research. For example, how these molecules that embody liver-eye communication are produced, and the induction mechanisms of transcriptional reprogramming, protein translation, modification, and secretion of these molecules are still not well understood. We have found that Notch signaling regulates HGF and angiopoietin secreted by hepatic sinusoidal endothelium, and promotes liver regeneration and fibrosis, suggesting that Notch signaling may further affect ocular diseases by regulating hepatic sinusoidal endothelial cells (Duan et al., 2018). In addition, how these molecules are transported between organs for communication, and the transport mechanism remains to be studied. What's more, how these molecules are regulated in time and space in the pathological process, and their influence on the occurrence, development and outcome of the disease is still relatively shallow.

At present, the metabolic relationship between liver and eye is mainly reflected in the metabolism of glucose and lipids. They mainly regulate vascular endothelial cells and affect angiogenesis to reflect the liver-eye connection. In particular, the relationship between NAFLD and DR reflects the liver-eye connection. However, the current research on the deep pathogenesis of NAFLD and DR is not clear. There are also some controversies about the correlation between NAFLD and DR. Therefore, more researchers are needed to clarify the correlation between NAFLD and diabetic retinopathy. And underlying molecular mechanisms. In addition, many complications of

liver disease in the eye reflect the liver-eye connection. For example, abnormal liver copper metabolism leads to the deposition of copper ions in the eye. However, why is there such a phenomenon that abnormal metabolism of liver disease tends to deposit in the eyes?

Finally, there are some evidences of liver-eye connection in TCM, but in the past, there was no way to explain the mechanism well due to the limitation of medical level. With the development of modern medicine, some of the previously unexplainable problems have also been explained. Therefore, paying attention to the liver-eye connection embodied in TCM may provide researchers with new ideas. Moreover, artificial intelligence is now rapidly developing. Therefore, the use of artificial intelligence to examine the eyes may also provide a new idea for our non-invasive and rapid screening of liver diseases through the connection between the liver and the eye.

In the future, more research on the relationship between liver and eye will help us understand the communication mechanism between liver and eye more clearly, which will help us better understand the pathogenesis and progression of these liver diseases or eye diseases, and more contribute to clinical treatment and the development of new therapeutic targets. Moreover, the research on the communication between the liver and the eye will inspire more organs and the mechanism of communication between organs, and it helps us to understand more clearly how the human body, a sophisticated and complex system can conduct steady-state regulation, which is conducive to revealing the mysteries of the human body.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. The idea of this review was produced by LW and G-RD. The literature search and data analysis were performed by T-HY, Z-SY, G-HZ and G-RD. The work was critically revised by G-RD and LW. All authors have read and approved the final manuscript.

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HSD17B13: A Potential Therapeutic Target for NAFLD

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Nonalcoholic fatty liver disease (NAFLD), especially in its inflammatory form (steatohepatitis, NASH), is closely related to the pathogenesis of chronic liver disease. Despite substantial advances in the management of NAFLD/NASH in recent years, there are currently no efficacious therapies for its treatment. The biogenesis and expansion of lipid droplets (LDs) are critical pathophysiological processes in the development of NAFLD/NASH. In the past decade, increasing evidence has demonstrated that lipid droplet-associated proteins may represent potential therapeutic targets for the treatment of NAFLD/NASH given the critical role they play in regulating the biogenesis and metabolism of lipid droplets. Recently, HSD17B13, a newly identified liver-enriched, hepatocyte-specific, lipid droplet-associated protein, has been reported to be strongly associated with the development and progression of NAFLD/NASH in both mice and humans. Notably, human genetic studies have repeatedly reported a robust association of HSD17B13 single nucleotide polymorphisms (SNPs) with the occurrence and severity of NAFLD/NASH and other chronic liver diseases (CLDs). Here we briefly overview the discovery, tissue distribution, and subcellular localization of HSD17B13 and highlight its important role in promoting the pathogenesis of NAFLD/NASH in both experimental animal models and patients. We also discuss the potential of HSD17B13 as a promising target for the development of novel therapeutic agents for NAFLD/NASH.

Keywords: 17β-HSD13, NASH, SNPs, lipid droplet, hepatocarcinoma

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INTRODUCTION

With the development of the economy and society, the morbidity and mortality of metabolic diseases caused by overnutrition and lifestyle changes are still increasing and likely will continue to rise. As a central organ of glucose and lipid metabolism, the liver plays a critical role in developing many metabolic diseases, including nonalcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM), dyslipidemia, and obesity. Long-term nutrition overload causes both systemic and hepatic metabolic disturbance and increases the risk of developing chronic liver diseases. NAFLD is the most common chronic liver disease worldwide (Younossi et al., 2015). The typical pathology of NAFLD is characterized by hepatocellular steatosis, lobular inflammation, hepatocyte ballooning, and fibrosis. NAFLD represents a wide spectrum of liver disorders ranging from nonalcoholic fatty liver (NAFL; simple steatosis) to nonalcoholic steatohepatitis (NASH) with inflammation and hepatocyte injury, which further drives fibrosis, eventually leading to cirrhosis and hepatocellular carcinoma (HCC) (Friedman et al., 2018). Among different stages of NAFLD, NASH has been recognized as a critical stage responsible for an increasing proportion of cirrhosis, HCC, and end-stage liver disease (Peng et al., 2020).

NAFLD has a high prevalence in both developing and developed countries. It is estimated that the total number of NASH patients in the United States, Japan, and five European Union countries (Great Britain, France, Germany, Italy, and Spain) will reach 18 million in 2027. Meanwhile, the predicted number of NASH patients in the United States will reach 27 million in 2030 with an increase of 56% (Estes et al., 2018). The prevalence of NAFLD/NASH increased from 23.8 to 32.9% during 1998–2018 in China (Zhou et al., 2020). It is projected that the NASH population in China will further increase to 48.26 million in 2030 (Estes et al., 2018). As a result, from 2004 to 2016, the registration number of both male and female liver transplantation caused by NASH was increased by 80% (Noureddin et al., 2018). In Japan, NASH has become the third leading cause of death in patients with type 2 diabetes mellitus (T2DM) (Nakamura et al., 2017). It has been well-documented that although NAFL carries a very low risk of adverse outcomes, the presence of NASH significantly increases both liver and non-liver-related consequences. In addition to several severe hepatic complications, including cirrhosis, liver failure, and HCC, NASH is considered as a multi-organ disease and is associated with a markedly increased risk of developing cardiovascular disease, type 2 diabetes, hypertension, chronic kidney disease, and extrahepatic malignancy (Friedman et al., 2018). However, despite substantial advances in clarifying the underlying mechanisms of NAFLD and identifying therapeutic targets for this disease in recent years, there are currently no effective therapies available for patients.

Pathologically, NAFLD is defined as an abnormal accumulation of neutral lipids such as triglycerides and cholesterol ester stored in lipid droplets (LD), a subcellular organelle in hepatocytes (Sharma and John, 2021). LDs are complex and metabolically active organelles. Alteration of LD's biogenesis, growth, or degradation affects their sizes and numbers in liver cells. Excessive biogenesis and constant growth of LDs are the most distinctive characteristics of NAFLD and are closely associated with the progression of NAFLD towards NASH and cirrhosis (Scorletti and Carr, 2021). LDs are composed of a neutral lipid core surrounded by a phospholipid monolayer associated with various proteins. In the past decade, human genome-wide association studies (GWAS) have revealed that a group of genes encoding LD-associated proteins such as FIT2 (fat storage inducing transmembrane protein 2), adipose triglyceride lipase (ATGL; PNPLA2), and PNPLA3 (patatin-like phospholipase domain containing 3) plays an important role in the pathogenesis and progression of NAFLD. Among them, the best-characterized genetic risk factor for NFALD is a missense variant of PNPLA3 (rs738409, C > G, p.I148M). The 148M allele was associated with increased hepatic triglyceride contents and elevated serum ALT levels in a multi-ethnic population-based cohort. The 148M variant evades ubiquitylation and proteasomal degradation and accumulates on lipid droplets where it competes with ATGL to interact with ABHD5, leading to reduced ATGL activity (BasuRay et al., 2019; Gao et al., 2019; Kozlitina, 2020). These findings have provided compelling new insights into the pathogenesis of NAFLD and highlighted a novel strategy for the development of therapeutic agents by directly targeting LD-associated proteins.

By using a comparative proteomic approach, we screened differentially expressed LD-associated proteins between histologically normal and biopsy-proven steatotic human liver samples and first reported hydroxysteroid 17β-dehydrogenase 13 (HSD17B13) as a novel liver-enriched, hepatocyte-specific, LD-associated protein in the liver, where it promotes hepatic lipogenesis and the pathogenesis of NAFLD (Su et al., 2014). Subsequently, multiple human GWAS studies have been performed in different ethnic populations, in which genetic variants of HSD17B13 are reproducibly associated with the full spectrum of NAFLD and influence the risk and fate of NAFLD and severity of steatosis, inflammation, and fibrosis (Anstee et al., 2020). Here, we briefly overview the discovery, tissue distribution, and subcellular localization of HSD17B13 and highlight the important role in promoting the pathogenesis of NAFLD/NASH in both experimental animal models and patients. We also discuss the potential of HSD17B13 as a promising target for developing novel therapeutic agents for NAFLD/NASH.

CLONING, TISSUE DISTRIBUTION AND SUBCELLULAR LOCALIZATION OF HSD17B13

HSD17B13 belongs to the HSD17B family with NAD(P)H/NAD(P)$^+$-dependent oxidoreductase activity that catalyzes the interconversion between 17-ketosteroids and 17-hydroxysteroids to maintain the balance between less potent (17-keto) and more potent (17β-hydroxy) forms of estrogens and androgens (Poutanen and Penning, 2019). There are 15 members of this family identified. Most of them are related to the activation or inactivation of sex hormones (HSD17B1, HSD17B2, HSD17B3, HSD17B5, HSD17B6), and the other members are involved in fatty acid metabolism, cholesterol biosynthesis, and bile acid production (Saloniemi et al., 2012). The HSD17B13 gene was firstly cloned from the human liver cDNA library in 2007 and initially named SCDR9 (short-chain dehydrogenase/Reductase 9) (Liu et al., 2007). The human HSD17B13 (SCDR9) gene spans about 17 kb in the human genome and is located at chromosome 4q22.1 with 8 exons and 7 introns. It encodes 9 different protein isoforms through alternative splicing with the functional role of each isoform largely unknown (**Figure 1**). HSD17B13 proteins possess two conserved motifs seen in all SCDR family members. One is the TGXGXXXG motif related to NAD(P) (H) binding, and the other is the YXXXK motif critical for its catalytic activity. The protein sequence of HSD17B13 shares a high degree of homology (73.7%) with HSD17B11, another member of the HSD17B family. The HSD17B13 gene is 13 kb upstream of the HSD17B11 gene, suggesting these two genes may result from gene duplication (https://www.ncbi.nlm.nih.gov/gene/345275). Interestingly, HSD17B11 is also a lipid droplet-associated protein, although it is more broadly expressed than HSD17B13 (Horiguchi et al., 2008; Yu et al., 2018). The molecular mass of HSD17B13 proteins is between 22 and 33 kDa due to multiple isoforms. It has been reported that human HSD17B13 is most

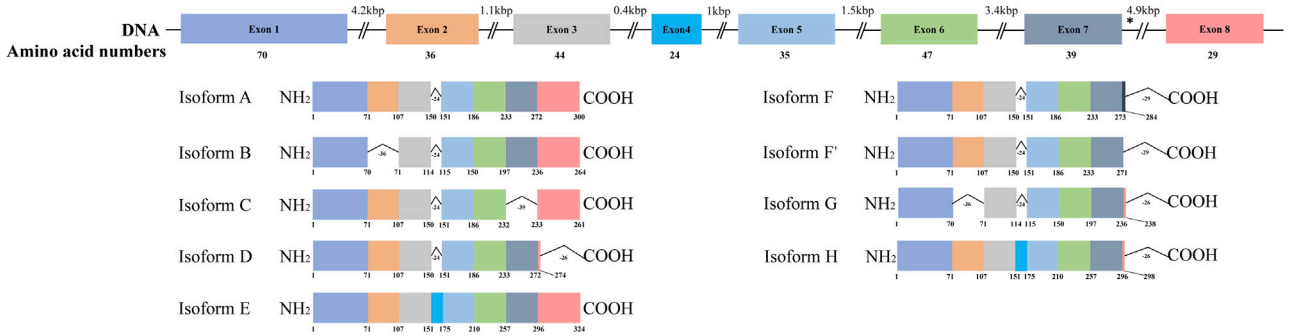

FIGURE 1 | Schematic diagram of human HSD17B13 splice variants. Human hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13) is located at chromosome 4q22.1 with 8 exons and encodes 9 different protein isoforms (HSD17B13 isoform A-H). The exons were boxed and the introns were shown as broken black lines. The newly identified exon 4 was boxed in blue. In isoform F, the C-terminal 52 amino acids (aa233-284) were encoded by the exon 7 and a part of intron 7 sequence (nt12665-13502). The asterisks indicated the position of A insertion from rs72613567 in human HSD17B13 gene. The genetic variant rs72613567 T/T gives rise to a wild type protein, while the variant rs72613567:TA results in a truncated loss-of-function of HSD17B13 protein.

abundantly expressed in the liver, with low levels in the ovary, bone marrow, kidney, brain, lung, skeletal muscle, bladder, and testis (Liu et al., 2007). Single-cell RNA-seq (scRNA-seq) analysis showed that human HSD17B13 is mainly localized in hepatocytes, with very low expression in other liver cells such as cholangiocytes, macrophages, hepatic stellate cells, liver sinusoidal endothelial cells (LSECs), T cells, and plasma cells (**Figure 2A**) (MacParland et al., 2018). Together, these findings demonstrate that human HSD17B13 is a liver-enriched, hepatocyte-specific, lipid droplet-associated protein.

Similarly, the mouse HSD17B13 gene is also mainly expressed in the liver, with much lower levels in other organs such as the ovary, kidney, brain, lung, and testis (Su et al., 2014). By searching a publically available single-cell RNA sequencing database (https://tabula-muris.ds.czbiohub.org/) which sequenced more than 100,000 cells from 20 mouse organs and tissues (Tabula Muris Consortium et al., 2018), it was found that HSD17B13 was highly expressed in mouse hepatocytes, with very low levels in Kupffer cells, LSECs, B cells and NK cells (**Figure 2B**). In addition, we analyzed a quantitative shotgun proteomics database of liver cells in different zonation of mouse liver (Berndt et al., 2021), which yielded that both mouse HSD17B13 splice variant proteins, i.e., A8Y5N4 and Q8VCR2, were selectively expressed in periportal hepatocytes at high levels (PPHs, Zone 1), with much less expression in pericentral hepatocytes (PCHs, Zone 3) (**Figure 2C**). However, it remains unclear whether a similar zonal expression pattern of HSD17B13 exists in the human liver, which requires further investigation.

As a liver-specific protein, HSD17B13 is selectively expressed in hepatocytes, where it is exclusively localized on the surface of lipid droplets. After transfecting an expression vector harboring a human HSD17B13-GFP fusion cDNA in Huh7 cells, it was found that HSD17B13 was exclusively present on the surface of LDs (Su et al., 2014). Consistently, it was also reported that in HepG2 cells, HSD17B13 was co-localized with ADRP, a LD-specific protein (Ma et al., 2019). These findings were further confirmed by immunohistochemical studies showing HSD17B13 was predominantly localized on the envelope of LDs in both human and mouse livers (Su et al., 2014). In addition, Ma Y et al. further characterized the molecular basis for LD targeting of human HSD17B13 and found that the N-terminal sequence AAs 1–28 is required for its LD localization (Ma et al., 2020). To date, little is known about the mechanisms involved in the regulation of HSD17B13 expression. A recent report showed that mouse HSD17B13 was induced by liver X receptor α (LXRα) in a SREBP1-dependent manner, suggesting it may contribute to the metabolic actions of LXRα in the liver (Su et al., 2017). In addition, in terms of its biochemical function, HSD17B13 has been shown to possess both SCDR and retinol dehydrogenase (RDH) activity (Su et al., 2014; Ma et al., 2019). Altogether, these results clearly indicate that HSD17B13 is an evolutionally conserved hepatocyte-specific LD-associated protein with similar tissue distribution and subcellular localization between species.

HSD17B13 IN NAFLD/NASH

As a liver-enriched, hepatocyte-specific, and LD-associated protein, HSD17B13 may play an important role in the regulation of liver lipid droplet biogenesis, growth, and degradation. It is anticipated that aberrant expression and dysfunction of HSD17B13 may contribute to the pathogenesis of chronic liver diseases, especially NAFLD. Two independent studies revealed that the hepatic expression of HSD17B13 was significantly induced in patients with NAFLD relative to that in healthy individuals (Su et al., 2014; Ma et al., 2019). In addition, upregulated HSD17B13 protein was mainly located in the LD subcellular fraction and on the surface of LDs (Su et al., 2014). In line with this finding, in a choline deficient diet (CD)-induced murine NASH/NAFLD model, HSD17B13 expression was found to be significantly increased (Mitsumoto et al., 2017). These findings suggested a close association of increased LD HSD17B13 levels with NAFLD development. In support, the HSD17B13 expression level was also found to be up-regulated in the livers of type 2 diabetic db/db mice and high-fat diet (HFD)-induced obese mice compared with control mice. Moreover, adenovirus-mediated hepatic overexpression

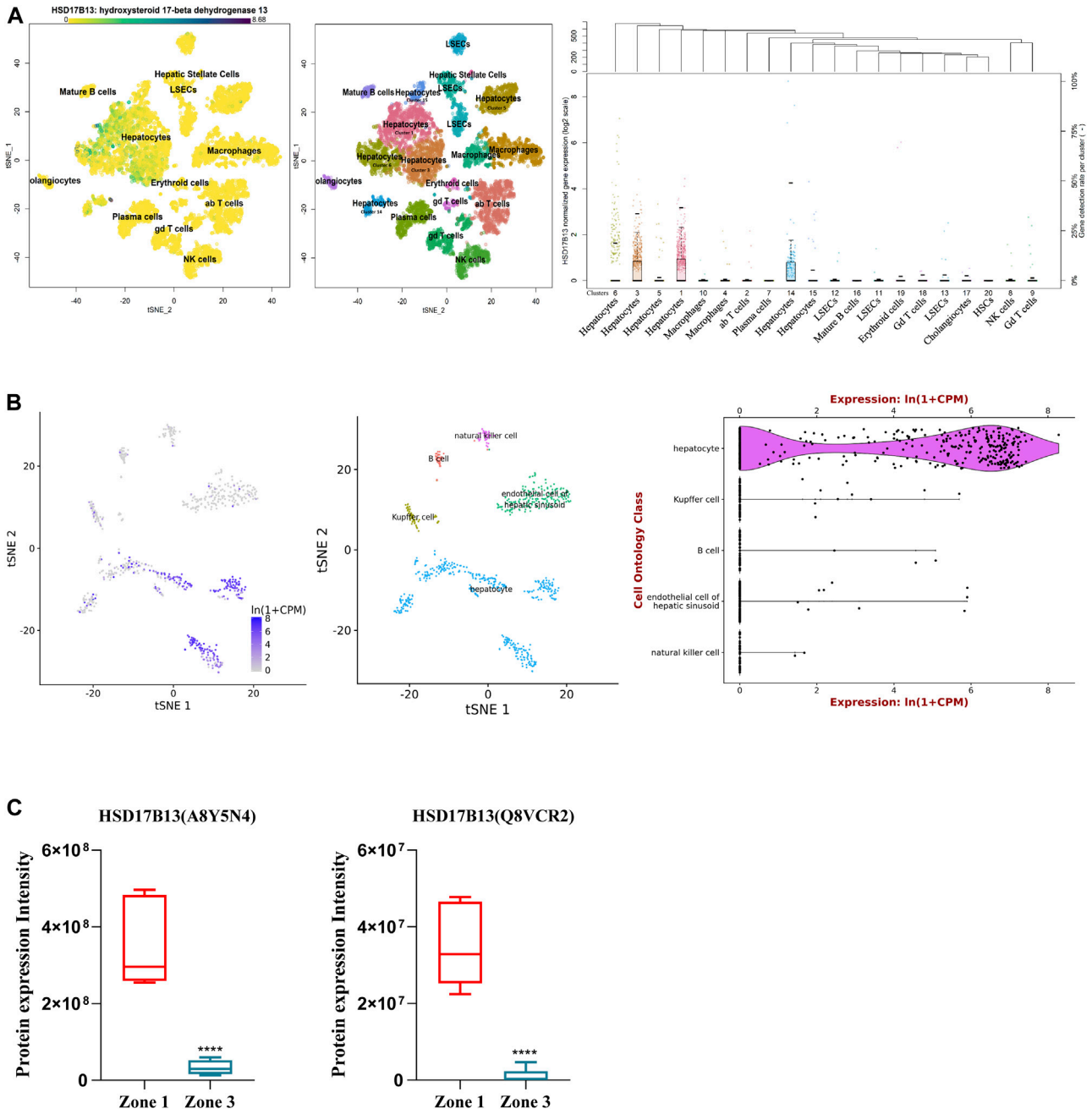

FIGURE 2 | Distribution of human and mouse HSD17B13 protein in liver cells. **(A)** Single cell RNA-sequencing (scRNA-seq) analysis showing that human HSD17B13 is mainly expressed in the hepatocytes. **(B)** Liver cell scRNA-seq analysis demonstrating that mouse HSD17B13 mRNA is highly abundant in the hepatocyte. **(C)** Two protein isoforms of mouse HSD17B13 (A8Y5N4, Q8VCR2) is abundantly expressed in the zone 1 of the liver.

of human HSD17B13 for 4 days resulted in accelerated LD biogenesis and excessive neutral lipid accumulation in the mouse liver (Su et al., 2014). In addition, it has also been reported that HSD17B13 may mediate LXRα activation-associated liver steatosis *via* a SREBP1-dependent mechanism (Su et al., 2017). These findings provide convincing evidence that increased hepatic HSD17B13 expression promotes liver LD biogenesis and NAFLD development. Surprisingly, a recent study on HSD17B13 gene knockout mice showed that HSD17B13 deficiency failed to protect the liver from high-fat diet-, Western diet- and alcohol exposure-induced steatotic damages, indicating that HSD17B13 deficiency might not have a protective role in murine NAFLD models (Ma et al., 2021). Similarly, another group reported that mice deficient of the HSD17B13 gene spontaneously developed late-onset fatty liver at the age of 9 months under normal chow (Adam et al.,

TABLE 1 | A partial list of human HSD17B13 genetic variants associated with NAFLD.

Variant Id	Location	Change	Variant type	Alleles	Transcript change	Amino Acid change	Molecular consequences
rs10433937	4p13:87308948	AAAAGCT [T/A/C/G]ATC	SNV	T > A, T > C, T > G	—		intron variant
rs10433879	4p13:87309988	GAAACCT [G/C] TCTCTA	SNV	G > C	—	—	intron variant
rs72613567	4p13: 87310241–87310242	TGTACTT [-/A] CTTCTGT	indel	dupA	—	—	splice donor variant
rs62305723	4p13:87310277	TACGATG [G/A] AACAA	SNV	G > A	c.778C > T	Pro260Ser	missense variant
rs61748262	4p13:87317092	CACTCAC [C/A/T] CAAA	SNV	C > A, C > T	c.450G > T, c.450G > A	Trp150Cys, Trp150Ter	missense variant, nonsense (stop gained)

SNV, Single nucleotide variation; indel, Insertion and Deletion; dupA, duplicate Adenine. Data from NCBI Variation Viewer (http://www.ncbi.nlm.nih.gov/variation/view).

2018). These unexpected results currently lack mechanistic explanations, which require further investigation.

Although murine models have produced inconsistent results, human GWAS studies have uncovered robust and reproducible associations between HSD17B13 gene variations and the natural history of NAFLD/NASH (Anstee et al., 2020). In a large-scale cohort study of exon-sequencing for 46,544 people of European ancestry, Abul-Husn and colleagues reported a common loss-of-function of HSD17B13 SNP (rs72613567: TA), with an insert of A allele adjacent to the splice site of exon 6 which caused the protein to be truncated prematurely. The presence of this variant reduced the risk of chronic liver diseases including NFALD, ALD and HCC. In human liver samples, the rs72613567:TA variant was associated with a reduced risk of nonalcoholic steatohepatitis, but not steatosis. They also found possible interactions between the HSD17B13-rs72613567:/A allele and PNPLA3-rs738409G-allele, which directly affected the liver PNPLA3 mRNA expression level and reduced PNPLA3 p.I148 activity (Abul-Husn et al., 2018).

The association between HSD17B13 rs72613567:TA and decreased severity of the chronic liver disease has been repeatedly confirmed in several other ethnic populations (Gellert-Kristensen et al., 2019; Ma et al., 2019; Pirola et al., 2019). In addition, Stender's group has recently reported that an unweighted genetic risk score combining three genetic variants including PNPLA3p.I148M, TM6SF2 p.E167K and HSD17B13 rs72613567 for fatty liver disease conferred up to a 12-fold higher risk of cirrhosis and up to a 29-fold higher risk of HCC in individuals from the general population in Europe (Gellert-Kristensen et al., 2020). Moreover, in patients with biopsy-proven NAFLD, PNPLA3 G/-, TM6SF2 T/- and HSD17B13 TA/- carriage were found to be associated with the severity of NAFLD, and combining PNPLA3 (rs738409, p = 0.0076), HSD17B13 (rs72613567), and TM6SF2(rs10401969, p = 0.0076), MBOAT7 (rs641738) with clinical data further increased the accuracy for predicting the severity of NASH and/or advanced fibrosis (Ioannou, 2021; Paternostro et al., 2021). Meanwhile, another study explored the impact of the HSD17B13 variant in 1,153 non-Hispanic NASH patients in the United States. The result showed that the protection of HSD17B13 rs72613567 on NASH and fibrosis might be limited to specific individuals who were aged ≥45 years, women who had class ≥2 obesity or diabetes, and those with PNPLA3 rs738409 CC genotype (Vilar-Gomez et al., 2021). These findings demonstrate that the protective effect of HSD17B13 rs72613567:TA could be influenced by the coexistence of other genetic variants such as PNPLA3 and TM6SF2 and the presence of clinical risk factors including diabetes, obesity, and alcohol consumption.

In the past years, two additional loss-of-function human HSD17B13 variants have been identified, including a protein-truncating variant rs143404524 (p.Ala192LeufsTer8) (Kozlitina et al., 2018) and a low-frequency missense variant rs62305723 G > A (p.P260S) (Ma et al., 2019). Both variants confer a partial loss of function of HSD17B13 and are associated with decreased disease severity of NAFLD. Interestingly, a few human HSD17B13 genetic variants located in various non-coding areas, including rs10433879, rs6834314, rs3923441, rs9992651, and rs72613567 were also reported to be associated with reduced severity of NAFLD (Ma et al., 2019; Namjou et al., 2019; Whitfield et al., 2019; Anstee et al., 2020; Satapathy et al., 2021) (**Table 1**). It is worth mentioning that HSD17B13 rs6834314 (A > G) was a loci initially identified by GWAS in 61,089 individuals. This variant was found to be associated with plasma concentrations of liver enzymes, including ALT, GGT, and ALP (Chambers et al., 2011) and liver fat contents (Ma et al., 2019). Collectively, both preclinical animal studies and human genetic surveys have provided compelling new insights into the pathogenesis of NAFLD/NASH and support the possibility that HSD17B13 may represent as a potential target for the treatment of NAFLD/NASH and chronic liver disease.

HSD17B13 SNPS IN OTHER CHRONIC LIVER DISEASES

In addition to NAFLD/NASH, the human HSD17B13 rs72613567: TA variant was also reported to be associated with a reduced risk of other chronic liver diseases. A whole-exon sequencing (WES) study involving 46,544 European participants found that the HSD17B13 rs72613567: TA variant was related with a markedly reduced risk of alcoholic liver disease (by 42% among heterozygotes and by 53% among homozygotes) and alcoholic cirrhosis (by 42% among heterozygotes and by 73% among homozygotes) (Abul-Husn

et al., 2018). Consistently, the genetic variant rs72613567 of the HSD17B13 gene was also found to reduce alcohol-related liver disease (ALD) risk in the Chinese Han population. In a case-control study including 769 ALD patients and 767 healthy controls, the HSD17B13 rs72613567:TA allele was associated with a decreased risk of ALD by 19% (Chen et al., 2020). Furthermore, a combination of another HSD17B13:rs68343143 variant and two other genetic variants (PNPLA3:rs738409 and TM6SF2:rs10401969) can predict the development of alcohol-related cirrhosis in heavy drinkers (Whitfield et al., 2021). Importantly, the risk of hepatocellular carcinoma was also decreased in patients heterozygous (*OR* = 0.65) and homozygous (*OR* = 0.28) for the HSD17B13 rs72613567:TA allele (Abul-Husn et al., 2018). A genetic score combining three genetic variants (PNPLA3 p.I148M, TM6SF2 p. E167K and HSD17B13 rs72613567) for fatty liver disease conferred up to a 12-fold higher risk of cirrhosis and up to a 29-fold higher risk of HCC in individuals from the general population (Gellert-Kristensen et al., 2020). Altogether, these results indicate that the genetic variants, especially the loss-of-function of SNP of HSD17B13 rs72613567, are very likely related to a reduced risk of ALD, alcohol-related cirrhosis, and hepatocarcinoma.

SUMMARY AND PERSPECTIVE

NAFLD has become the leading cause of liver cirrhosis and hepatocarcinoma in developed countries. Its prevalence in many developing countries such as China is also rapidly rising, with rates approaching those of Western countries (Wang et al., 2014). The burden of NAFLD is increasing at an alarming rate. NAFLD-related liver inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma represent a major and increasing threat to human health (Adams et al., 2017). For NAFLD, especially in its severe form NASH, there is currently no specific and effective treatment or medicine for patients. The pathogenesis of NAFLD has not been fully understood but is believed to result from a combination of environmental and genetic risk factors. In the past years, GWAS and whole exon sequencing (WES) studies have identified several genetic risk factors closely associated with the development, progression, and severity of NAFLD. PNPLA3 was the first and the best-characterized genetic risk factor, which regulates lipid droplet lipolysis in hepatocytes and is the most strongly associated genetic variant linked to NASH (Romeo et al., 2008; Friedman et al., 2018). Subsequently, a few other genetic variants, including TM6SF2, MBOAT7, and HSD17B13, have been uncovered and also considered as important genetic modifiers of NAFLD (Meroni et al., 2021).

Since HSD17B13 was first reported as a pathogenic factor for NAFLD in 2014 (Su et al., 2014), tremendous progress has been made in our understanding of its contribution to the development and progression of NAFLD. Although there are inconsistent reports in animal models, a few HSD17B13 genetic variants have been reproducibly confirmed in several large clinical and population-based studies with different ethnicities to be linked to the full spectrum of NAFLD (Kozlitina, 2020; Meroni et al., 2021) (**Table 1**). Currently, the natural substrate(s) and metabolite(s), biological roles, and underlying mechanisms of HSD17B13 in liver lipid metabolism remain incompletely understood. Nevertheless, based on the promising and convincing results from human genetic studies and some of preclinical works, HSD17B13 has been tested in patients as a therapeutic target. Alnylam Pharmaceuticals has recently launched a phase I clinical trial (https://clinicaltrials.gov/ct2/show/NCT04565717), aiming to develop an RNAi-based therapeutic approach to target HSD17B13 for the treatment of NAFLD/NASH. In addition, Arrowhead has also launched a phase I clinical trial (https://clinicaltrials.gov/ct2/show/NCT04202354) in 2019. The result showed that ARO-HSD significantly down-regulated liver HSD17B13 mRNA and protein expression and markedly reduced serum ALT and AST levels. Meanwhile, Inipharm announced that INI-678, a potent and selective small-molecule HSD17B13 inhibitor, showed reduction in key markers of liver fibrosis (α-SMA, COL-I) in a human liver cell-based 3D liver-on-a-chip-model (https://inipharm.com/). These studies are still at the early stage of clinical trials, and their effects on NAFLD/NASH require long-term observation. Moreover, the HSD17B13 genetic variant rs72613567, alone or in combination with other genetic variants and clinical risk factors, may hold great promise to predict individuals who are more susceptible to NAFLD and at high risk for NASH.

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H-bZ, HX, and WS wrote the manuscript. X-yZ and Y-fG revised and edited the manuscript.

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Nanotechnology in Drug Delivery for Liver Fibrosis

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Liver fibrosis is a reversible disease course caused by various liver injury etiologies, and it can lead to severe complications, such as liver cirrhosis, liver failure, and even liver cancer. Traditional pharmacotherapy has several limitations, such as inadequate therapeutic effect and side effects. Nanotechnology in drug delivery for liver fibrosis has exhibited great potential. Nanomedicine improves the internalization and penetration, which facilitates targeted drug delivery, combination therapy, and theranostics. Here, we focus on new targets and new mechanisms in liver fibrosis, as well as recent designs and development work of nanotechnology in delivery systems for liver fibrosis treatment.

Keywords: chronic liver diseases, liver fibrosis, nanotechnology, nanomedicine, targeted drug delivery

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INTRODUCTION

Chronic liver diseases (CLD) remain a major concern for public health around the world. About 844 million people suffer and 2 million people die per year (Byass, 2014). Liver fibrosis is a common stage of various CLD, which is an important repair and pathological process caused by different etiologies, for instance, chronic viral infections (e.g., hepatitis B virus, hepatitis C virus), metabolic disorders, alcohol abuse, autoimmune insults, or cholestatic injury (Kisseleva and Brenner, 2021). In most cases, liver fibrosis is a kind of asymptomatic disease, which is usually clinically silent and progress slowly. With persistent damage and extracellular matrix (ECM) deposition, the liver is progressively hardening and stiffening over time followed by a progressive declined function. Then liver fibrosis may develop into cirrhosis and even liver cancer, along with a suite of complications, such as portal hypertension, liver failure, and hepatic encephalopathy (HE) (Parola and Pinzani, 2019) (**Figure 1**). The only effective therapy for terminal liver disease is liver transplantation.

Fortunately, many researches have revealed that liver fibrosis can be reversed in patients when the etiological factors are removed and in experimental rodent models when hepatic stellate cells (HSCs) are deactivated (Troeger et al., 2012; Lee et al., 2015). Therefore, early diagnosis and timely treatment of fibrosis is important for clinical management. Liver biopsy has been regarded as "gold standard" for the diagnosis and staging of liver fibrosis. However, it is invasive and has some significant complications, so patient acceptance is low (Bedossa and Carrat, 2009). Lacking accurate diagnostic techniques during long-term monitoring of progressing fibrosis and responses to therapy is a major challenge for optimizing disease treatment strategies (Bansal et al., 2016). Although, many research has discovered the etiology and pathogeny of liver fibrosis, and numerous therapeutic drugs are under development. Deactivating HSCs and expediting the clearance of myofibroblasts remain effective therapeutic strategies for the regression of liver fibrosis. Currently, the main antifibrotic therapy strategies are inhibiting the activation and proliferation of HSCs, reversing the activation phenotype to the quiescent phenotype, inhibiting HSCs autophagy, inducing cell apoptosis and senescence as well as immune clearance, or promoting ECM degradation (Ezhilarasan et al., 2018)

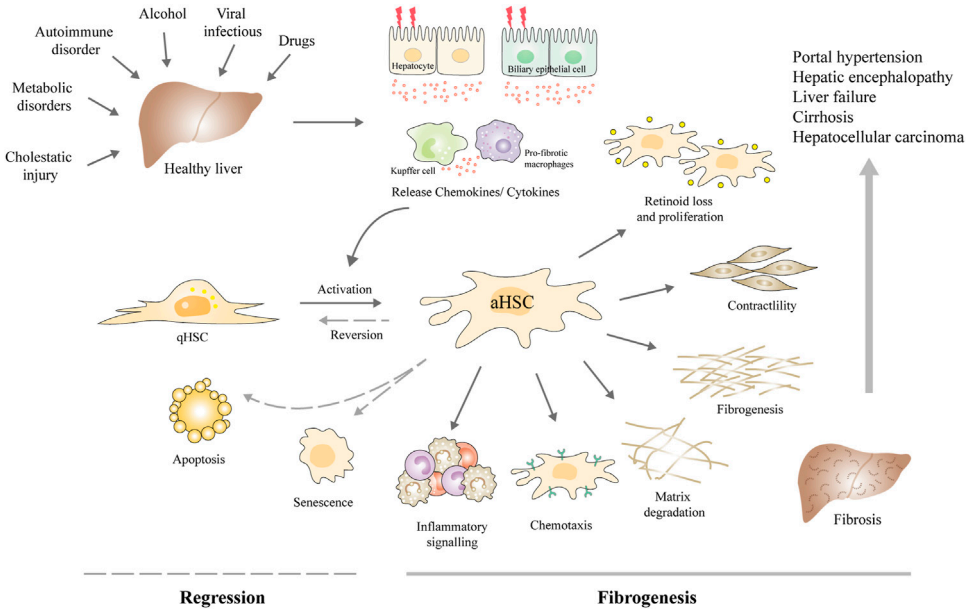

FIGURE 1 | Schematic diagram of the central role of HSCs in the fibrogenesis and regression of liver fibrosis. Upon various chronic liver injuries, the hepatocytes and biliary epithelial cells can be damaged, together with Kupffer cells and macrophages, release chemokines, and cytokines to activate the HSCs. aHSCs are prone to present characteristics including retinoid loss, proliferation, contractility, ECM production, altered matrix degradation, chemotaxis, and expressing inflammatory signals. Liver fibrosis may develop into cirrhosis and even liver cancer, along with a suite of complications, such as portal hypertension, hepatic encephalopathy, and liver failure. Currently, the main antifibrotic therapy strategies are inhibiting HSC activation and proliferation, reversing the activation phenotype to the quiescent phenotype, or inducing HSC apoptosis and senescence. Abbreviations: qHSC, quiescent hepatic stellate cell; aHSCs, activated hepatic stellate cell.

(**Figure 1**). Pharmacotherapies like Chinese herbal medicines, chemical drugs, and monoclonal antibodies have been developed against these targets (Peres et al., 2000; de Oliveira et al., 2008; Ogawa et al., 2010). However, these traditional pharmacotherapies have inadequate therapeutic efficacy and unwanted side effects. For example, sorafenib and interferon-γ (IFNγ) have displayed great antifibrotic effects *in vitro* but present poor effects *in vivo*. No licensed pharmacotherapy is currently available for liver fibrosis. The development of therapeutic approaches with enhanced efficacy and targeted capabilities is in need. Determining the new mechanisms of liver fibrosis regression is also needed for identifying new therapeutic targets to treat liver fibrosis.

In recent decades, nanotechnology has greatly contributed to the design and apply of nanomedicine in terms of diagnosis and treatment for CLD. Well-designed nanostructures have specific targeting ability as well as diagnostic capability of liver fibrosis, which can be used as therapeutic agents, contrast enhancement agents, or nanoprobes for diagnosis (Li et al., 2018b). A lot of organic or inorganic nanoparticles (NPs) have been developed for liver fibrosis, including metal nanoparticles, lipid nanoparticles, polymer nanoparticles, and protein nanoparticles. The controllable size, shape, diverse components, and modifiable surface characteristics of nanoparticles bring superior advantages, including the prolonged circulation, improved internalization and penetration, controlled drug release, high contrast, improved drug pharmacokinetics, and reduced adverse reactions (Petros and DeSimone, 2010), which facilitate targeted drug delivery, combination therapy, and theranostics. For example, a study investigated a new theranostic nanomedicine, relaxin-PEGylated superparamagnetic iron-oxide nanoparticles, which can integrate diagnosis, and therapy in one platform (Nagorniewicz et al., 2019).

In this review, we focus on new targets and mechanisms, as well as recent designs and development work of nanotechnology in delivery systems for liver fibrosis treatment (**Table 1**). Major challenges and coping strategies in nanomedicine for liver fibrosis are also discussed.

CELL TARGETS IN LIVER FIBROSIS

Various key molecular mechanisms resulting in liver fibrosis have been revealed, such as chronic hepatocyte injury, endothelial barrier damage, inflammatory cytokines release, recruitment of bone marrow-derived cells (BMDCs), secretion of transforming growth factor-β (TGFβ) by macrophages, HSC activation, excessive accumulation of ECM, as well as the production of fibrous scars (Bataller and Brenner, 2005). Fiber regression is the key to stopping fibrosis progression. Upon the removal of the above factors, the fibrosis will regress, along with the decreased pro-inflammatory or profibrogenic cytokines, disappeared aHSCs, increased collagenolytic activity, suppressed ECM production, and reduced fibrous scars (Kisseleva and Brenner, 2021). The crosstalk between hepatic myofibroblasts,

TABLE 1 | Preclinical nanomedicine for liver fibrosis.

Targeted structure	Nanoparticle formulation	Target cell, effects	Model	Reference
PDGFRβ	HMGB1-siRNA@SNALP-pPB	HSC, reduced proliferation, anti-inflammatory	TAA/CCl4-induced cirrhosis	Zhang et al. (2020)
	pPB-SSL-IFN-γ	HSC, inhibited proliferation, decreased fibrosis	TAA induced fibrosis	Li et al. (2012)
	GNR-AbPDGFRβ	HSC, decreased fibrosis, hepatic inflammation, and hepatocyte injury	CCl_4-induced fibrosis	Ribera et al. (2021)
Sigma-1 receptor	AEAA-pRLN-LPD NPs	HSC, deactivated HSC, macrophage phenotype switch	CCl_4-induced fibrosis, MCD- or CDAHFD-induced non-alcoholic steatohepatitis	Hu et al. (2021)
Integrin αvβ3	Vismodegib-cRGDyK-liposomes	HSC, inhibited hedgehog pathway signaling, reduced fibrosis	BDL/TAA induced fibrosis	Li et al. (2019)
	GMO-and miR-29b-loaded cRGD-PEG-PLGA NPs	HSC, cytotoxicity to aHSCs, inhibited production of collagen type I	CCl_4-induced fibrosis	Ji et al. (2020)
RBP	CGPVMs	HSC, inhibit collagen I accumulation	CCl_4-induced fibrosis	Qiao et al. (2018)
	siCol1α1/siTIMP-1 VLNPs	HSC, promote collagen degradation, and inhibit collagen synthesis	CCl_4-induced fibrosis	Qiao et al. (2020)
CD44	DOX-RA-CS micelles	HSC, downregulated collagen I production	CCl_4-induced fibrosis	Luo et al. (2019)
	HA-UCNP@mSiO2@RBS	HSC, HSC apoptosis, liver fibrosis relief	CCl_4-induced fibrosis	Liang et al. (2020)
Mannose receptor	TNF-α siRNA MTC NPs	Macrophage, reduced TNF-α production	LPS/d-GalN-induced hepatic injury	He et al. (2013)
PS	Cur-mNLCs	Macrophage, reduced fibrosis, increased HGF, and MMP2	CCl_4 treated rat model	Wang et al. (2018)
ASGPR	ASGPR targeting tracer	Hepatocyte, quantify, and stage liver fibrosis	CCl_4-induced fibrosis	Zhang et al. (2016)
	P-SPIONs	Hepatocyte, early diagnosis of liver fibrosis	CCl_4-induced fibrosis	Saraswathy et al. (2021)
Passive target	$S1PR_2$-siRNA GeRP	Macrophages, decreased NLRP3 inflammasome activation, attenuated hepatic inflammation, and fibrosis	BDL/MCDHF/CCl_4-induced fibrosis	Hou et al. (2020)
	TSG-6@CaP@BSA NPs	Macrophage, M2 polarization, increased MMP12 expression	CCl_4-induced fibrosis	Wang et al. (2020)
	PD-MC	Multiple cell types, reduced hepatocyte apoptosis, averted activation of macrophages, and HSCs	CCl_4-induced fibrosis	Lin et al. (2020)

*Abbreviations: PDGFRβ, platelet-derived growth factor receptor β; HMGB1, high mobility group box-1; SNALP, stable nucleic acid lipid nanoparticles; pPB, "C*SRNLIDC*" (peptide* vs. *PDGFβR); HSC, hepatic stellate cell; TAA, thioacetamide; CCl4, carbon tetrachloride; SSLs, sterically stable liposomes; IFN-γ, interferon-γ; GNR-AbPDGFRβ, PDGFRβ-antibody-conjugated gold nanorods; AEAA, aminoethyl anisamide; pRLN, plasmid DNA (pDNA) that encoded RLN; LPD, lipid–protamine–DNA; MCD, methionine-choline-deficient; CDAHFD, choline-deficient, l-amino acid-defined high-fat diet; cRGDyK, Cyclo [Arg-Gly-Asp-DTyr-Lys]; BDL, bile duct ligation; GMO, germacrone; PEG-PLGA, poly(ethylene glycol)-block-poly(lactide-co-glycolide); RBP, retinol binding protein; CGPVMs, silybin/siCol1α1 co-loaded core-shell polymer micelles; VLNPs, vitamin A-decorated and hyperbranched lipoid-based lipid nanoparticles; DOX, doxorubicin; RA, retinoic acid; CS, chondroitin sulfate; HA, hyaluronic acid; UCNP, upconversion nanoparticle; mSiO2, mesoporous silica; RBS, Roussin's black salt; MTC, Mannose-modified trimethyl chitosan-cysteine; LPS, lipopolysaccharide; d-GalN, d-galactosamine; PS, phosphatidylserine; Cur, curcumin; mNLCs, PS-modified nanostructured lipid carriers; HGF, hepatocyte growth factors; ASGPR, asialoglycoprotein receptor; P-SPIONs, pullulan stabilized iron oxide nanoparticles; S1PR2, sphingosine 1-phosphate receptor 2; GeRP, glucan–encapsulated siRNA particle; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; TSG-6, tumor necrosis factor stimulated gene 6; CaP, calcium phosphate; BSA, bovine serum albumin; MMP12, matrix metalloproteinase 12; PD-MC, polydatin-loaded micelle.*

macrophages, injured hepatocytes, and other cell types make contributions to these mechanisms (**Figure 2**).

Hepatic Stellate Cells

The activation of HSCs is regarded as the central link in liver fibrosis and therefore represents a major target for antifibrotic therapy (Higashi et al., 2017). Quiescent HSCs are located at the Disse space, an area between hepatocytes and sinusoidal endothelial cells (LSEC), with the proportion of 5–8% among the hepatocytes. Under normal physiological conditions, HSCs are in a quiescent state and responsible for storing retinoid lipid droplets (mainly vitamin A) in liver (Senoo et al., 2017). Upon liver injury, accompanied by hepatocyte inflammation, macrophages will recruit and transform the resident quiescent HSCs to a highly activated, proliferative, and contractile myofibroblast-like phenotype (Mederacke et al., 2013; Tacke and Zimmermann, 2014) (**Figure 1**). aHSCs are prone to lose lipid droplets, upregulate α-smooth muscle actin expression, and migrate to the injury site to secrete ECM to repair the damaged liver. As time goes on, the excessive accumulation of ECM and matrix metalloproteinases (MMPs) will induce remodeling of liver tissue, and the fibrous scars will eventually replace the normal tissue.

Actually, a panoply of signals drive HSCs activation. The key proliferative and fibrogenic pathways leading to fibrosis consist of TGFβ, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and connective tissue growth factor (CTGF) (Tsuchida and Friedman, 2017). It is generally accepted that TGFβ is the most potent profibrogenic cytokine in the activation process of HSCs (Hellerbrand et al., 1999). TGFβ drives the activation of HSCs mainly *via* SMAD2-SMAD3 signaling to promote transcription of type I and III collagen (Dewidar et al., 2019). Genetically overexpressing TGFβ spontaneously induce liver fibrosis, whereas genetic deletion or

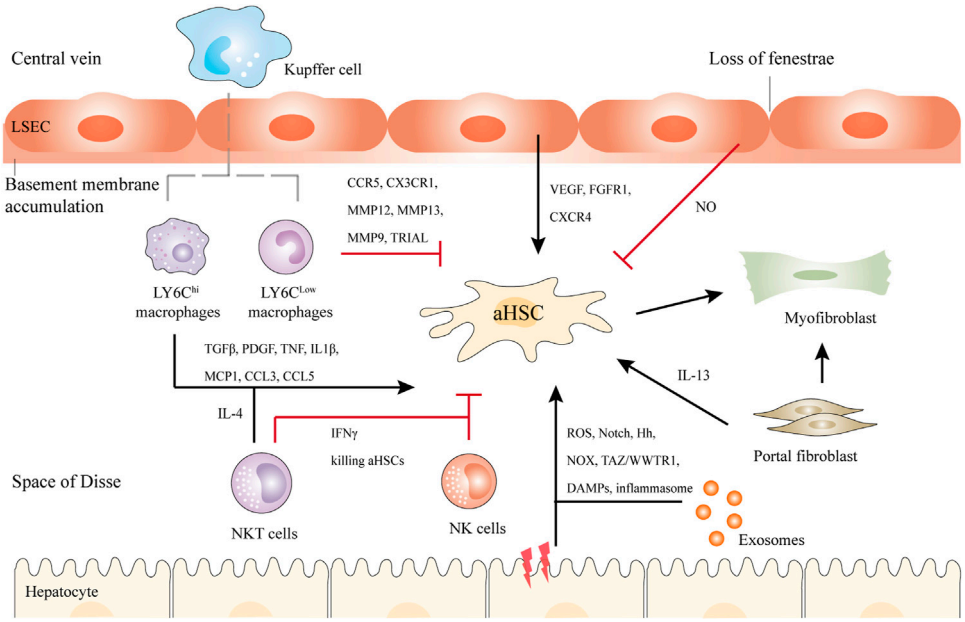

FIGURE 2 | Schematic illustration of the crosstalk between HSCs and Kupffer cells, NK cells, NKT cells, hepatocytes, bone marrow-derived macrophages, and LSECs in liver fibrosis. Briefly, upon liver injury, hepatocytes undergo damage, and inflammatory, releasing DAMPs, exosomes, etc. LY6C^{hi} macrophages activate HSCs by TGFβ, IL1β, and other cytokines, while LY6C^{low} macrophages show antifibrotic ability *via* CX3CR1, MMP12, MMP13, etc. NKT cells and NK cells secret IFNγ to kill aHSCs. NKT cells can also activate HSCs through IL-4. In addition, LSECs undergo capillarization and accumulation of basement membranes. They can promote either liver regeneration or fibrosis. Another population of myofibroblasts is the portal fibroblasts, which can also activate HSCs in cholestatic liver. Black and red arrows represent promotion or inhibition of aHSCs respectively.

pharmacological blockade of TGFβ could ameliorate fibrosis in mice (Hellerbrand et al., 1999; de Gouville et al., 2005). However, persistent depletion of TGFβ has adverse effects like poor wound healing and tumor genesis. Hedgehog signaling (Omenetti et al., 2011), innate immune signaling (especially Toll-like receptors and cytokines) (Seki et al., 2007), G protein-coupled receptors (Granzow et al., 2014), reactive oxidative stress (ROS) (Novo et al., 2011) have been implicated in HSC activation. A study using clinical liver biopsies and animal models revealed a new profibrotic function of interleukin (IL)-17A and IL-22 in a TGFβ-depend manner (Fabre et al., 2018). Blocking either IL-17 or IL-22 by antagonists resulted in reduced fibrosis. Autophagy provides HSCs with energy substrates and the process is related to upregulated endoplasmic reticulum stress (Hernández-Gea et al., 2013). Epigenetic signals regulate both activation and deactivation of HSCs. Nuclear receptors like peroxisome proliferator-activated receptor gamma (PPARγ), farnesoid X receptor (FXR), and liver X receptor (LXR) modulate the inactivation of HSC (Tsuchida and Friedman, 2017).

Portal Fibroblasts

Many cell populations, for example, HSCs, bone marrow-derived fibroblasts, portal fibroblasts, and mesenchymal cells contribute to ECM accumulation, the primary sources of the activated myofibroblasts that secrete ECM proteins are HSCs and portal fibroblasts (Kisseleva, 2017). It depends on the etiology of liver fibrosis, the major sources of myofibroblasts can differ (Iwaisako et al., 2014). aHSCs contribute >87% of myofibroblasts in hepatotoxic (carbon tetrachloride, CCl_4) liver, while activated portal fibroblasts (aPFs) are the major source in cholestatic liver (bile duct ligation, BDL), contributing >70% of myofibroblasts upon injury. Mesothelin/mucin 16 signaling might play a vital role in this biology (Koyama et al., 2017). With progressive injury by BDL, the role of aPFs lessens, HSCs gradually gain the upper hand and contribute to the myofibroblasts. Upon cholestatic injury, taurocholic acid directly stimulates COL1A1 expression in aPFs. Furthermore, IL-25 can stimulate the secretion of IL-13 in aPFs, which provides stimulus signals to HSCs (Liu et al., 2011). This understanding suggests that targeting aPFs might provide new directions for the regression of liver fibrosis.

Macrophages

The activation of macrophages also plays a key role in the fibrogenesis process. Macrophages in liver can be divided into resident Kupffer cells (KCs) and monocyte-derived macrophages. KCs have phagocytic ability and anti-inflammatory functions (Krenkel and Tacke, 2017). It is also reported that KCs have a profibrogenic role. Firstly, KCs recruit proinflammatory and profibrogenic macrophages through CCL2 secretion in the early stage of liver injury. Secondly, KCs secret TGFβ and PDGF to directly activate HSCs. Thirdly, KCs establish a profibrogenic niche by producing proinflammatory cytokines and chemokines, for instance, IL-1β, tumor necrosis factor α (TNFα), IL-6, and CCL5, which may interact with HSCs (Wen et al., 2021).

After injury, in response to inflammatory signals, BMDCs migrate to inflamed site, and differentiate into macrophages, which play a dual role in the fibrogenesis and regression process. During fibrogenesis, macrophages display a $LY6C^{hi}$ phenotype and produce cytokines and chemokines to activate HSCs, including TGFβ, PDGF, TNF, IL-1β, monocyte chemoattractant protein-1 (MCP1), CCL3, and CCL5 (Wen et al., 2021). The recruitment of this type of macrophages is CCR2-dependent in CCl_4 treated mice. CCR2-deficient mice presented an impaired recruitment of $LY6C^{hi}$ macrophages, reduction of aHSCs, and diminished fibrosis (Karlmark et al., 2009). During fibrosis regression, macrophages display a $LY6C^{low}$ phenotype expressing high levels of CCR5 and CX3CR1 and produce MMPs (MMP12 and MMP13) to promote fibrosis degradation (Krenkel and Tacke, 2017). CX3CR1 binds with its ligand to inhibit the inflammatory property of macrophages. Furthermore, $LY6C^{low}$ macrophages promote HSCs apoptosis *via* MMP9 and TNF-related apoptosis-inducing ligand (TRAIL) (Taimr et al., 2003). Moreover, the decreased tissue inhibitor of metalloproteinase (TIMP) expression contributes to the increased activity of collagenolytic enzymes. TIMPs help aHSCs survive *via* the downregulation of pro-apoptotic BAX and PUMA and upregulation of BCL2 (Parsons et al., 2004). The depletion of macrophages led to a failure of ECM degradation in CCl_4-treated mice (Duffield et al., 2005), suggesting that a more delicate and circumspect therapeutic approach is in need to effectively target different subpopulations of macrophages for treating liver fibrosis.

Recently, the critical role of c-Rel (the subunit of NF-κ-B) in regulating metabolic changes required for inflammatory and fibrogenic activities of macrophages and hepatocytes was reported by Leslie et al. (2020). In CCl_4-induced liver fibrosis, independent knockout of Rel in macrophages or hepatocytes inhibited liver fibrosis, while combined knockout in both cell types had an additive antifibrosis effect. They also identified Pfkfb3 as a key downstream mediator in this process. Targeting the c-Rel-Pfkfb3 axis might be a multiple cell targeting strategy for antifibrotic treatment.

It is worth mentioning that exosomes also play a role that cannot be ignored in the regulation by macrophages in fibrosis. Macrophages and neutrophil can release exosomes to alter liver functions. For instance, miR-233 can be delivered by macrophages *via* exosomes to many cells, including hepatocytes, controlling inflammation in liver diseases (Ismail et al., 2013; Ye et al., 2018). A recent work by Hou et al. (2021) discovered that IL-6 signaling played a critical role in controlling liver fibrosis in a macrophage-specific way. IL-6 promotes macrophages-derived miR-223-enriched exosomes to suppress the several miR-223-targeted genes expressed in hepatocytes in a nonalcoholic steatohepatitis (NASH) model.

Other Immune Cells

Besides macrophages, numerous other immune cell populations infiltrate in the fibrotic liver. Natural killer (NK) cells display an antifibrotic property generally by killing aHSCs through IFNγ and inducing HSC apoptosis in a TRAIL- and NKG2D-dependent manner (Jeong et al., 2011; Glässner et al., 2012). In murine models, enhancing NK cell activity *via* inducing the expression of IFNγ promotes fibrosis resolution (Krizhanovsky et al., 2008). By the way, NK cells also facilitate fibrosis resolution by clearing senescent myofibroblasts.

Natural killer T (NKT) cells also play a dual role in liver fibrosis. On the one hand, same as NK cells, NKT cells show antifibrotic functions by directly killing aHSCs through IFN-γ secretion (Park et al., 2009). On the other hand, in response to liver injury, $CXCR6^{+}$ NKT cells promote fibrogenesis in liver. In fibrotic livers of Cxcr6 (-/-) mice, the infiltration of macrophage and expression of inflammatory cytokines (e.g., IFN-γ, TNF-α, and IL-4) were reduced, suggesting that hepatic NKT cells maintain hepatic inflammation and fibrogenesis by providing essential cytokines (Wehr et al., 2013).

The neutrophil extracellular traps (NET) also play a certain role in CLD. Upon liver injury, neutrophils migrate into the injury site, dismantle the damaged vessels, and create channels for vessel growth. Concerning nonalcoholic fatty liver disease (NAFLD), both Treg cells and Th22 cells seem to present an overall tempering effect, while Th17 cells induce more liver damage and promote fibrosis. Th1 cells secrete IFN-γ to reduce liver fibrosis, while Th2 cells can promote fibrosis by activating HSCs *via* cytokine release (Wynn, 2004). Little is known about the role of innate T cells in liver fibrosis and further investigations are required (Bartneck, 2021).

Hepatocytes

Upon liver injury, hepatocytes generate inflammation and fibrosis. An increasing number of mediators, including ROS, Notch (Zhu et al., 2018), Hh ligands (Chung et al., 2016), NADPH oxidase (NOX) (Lan et al., 2015), and TAZ/WWTR1 (Wang et al., 2016), facilitate this process. Furthermore, exosomes containing micro RNAs secreted by injury hepatocytes might activate HSCs (Lee et al., 2017). Hepatocyte-derived exosomes promote fibrinolysis by suppressing the activation of HSCs, inhibiting macrophage activation and cytokine secretion, and inducing ECM degradation, and remodeling (Chen et al., 2019). Damage-associated molecular patterns (DAMPs) released from hepatocytes and inflammasome consisting of NLRP3, NACHT, LRR might promote HSC activation and liver fibrosis directly or indirectly (Wree et al., 2014). It is suggested that inflammation is a necessary condition for fibrosis.

Liver Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (LSECs) are fully differentiated in normal liver and have the capacity to maintain HSC quiescence via paracrine factors like nitric oxide (NO) (Deleve et al., 2008). In fibrotic livers, LSECs undergo capillarization and dedifferentiation, characterized by loss of fenestrae and basement membrane accumulation, become vasoconstrictive, proinflammatory and prothrombotic, and lose their ability of suppressing HSC activation. Additionally, fenestrae loss and basement membranes development prevent hepatocytes oxygenation, causing apoptosis and necrosis, which ultimately induce DAMP secretion to activate HSCs (Gracia-Sancho et al., 2021). Reduction of NO bioavailability and endothelial NO

synthase (eNOS) activity, together with increased ROS-mediated scavenging of NO, all lead to HSC activation, and ECM deposition in CLD (Gracia-Sancho et al., 2021). VEGF-stimulated NO can activate soluble guanylate cyclase (sGC). The activator of sGC can restore capillarization and return HSCs to the quiescent state in rats (Xie et al., 2012). Depending on the LSEC response to liver injury, LSECs can promote liver regeneration by the CXCR7-ID1 pathway, or promote liver fibrosis *via* fibroblast growth factor receptor 1 (FGFR1)-CXCR4 pathway (Ding et al., 2014).

CHALLENGES IN PHARMACOTHERAPY FOR LIVER FIBROSIS

Even though so many key targets related to liver fibrosis have been discovered, there is no available single therapeutic agent to date. Various innovative antifibrotic attempts such as IFNγ, interleukin 10, and angiotension II antagonists have shown promising results in preclinical trials, but failed in clinical trials (Bartneck et al., 2014). One major reason is likely that traditional formulations lack the specific delivery of the respective molecules. For example, IFNγ has valid antifibrotic activities, but has proinflammatory effects on macrophages (Mosser and Edwards, 2008). Since liver fibrosis is a complex process involving multiple cell types (HSCs, macrophages, LSECs, and NK cells), it is useless to target one cell type with a single pharmacological agent. This is also a reason for several antifibrotic drugs like simtuzumab who showed promising effect in preclinical experiments but failed in clinical translation. Moreover, biological differences exist between animal models and patients. So far, the targeted antifibrotic drugs neglect the pleiotropic potential of myofibroblasts (for example, activation, proliferation, releasing profibrogenic factors, and collagens) and only aim at a single mode of actions, such as, as PPARγ agonists, TGFβ inhibitors, PDGF inhibitors, etc. (Devaraj and Rajeshkumar, 2020). Many compounds derived from herbal and synthetic compounds such as silymarin and curcumin have shown promising effects for liver fibrosis. However, their poor bioavailability and water solubility, lack of specific targeting, and extra hepatic toxicity limit their application (Ezhilarasan et al., 2014).

Nanomedicines provide novel therapeutic opportunities for drug delivery in antifibrotic therapies. Nanotechnology combines the characteristics attributed to monoclonal antibodies and small molecule drug. Nanomedicine consists of the active component, for instance, small interfering RNA (siRNA), microRNA (miRNA), small molecule drug, cytokine, or protein, containing the delivery vehicle, such as liposome, protein carrier, polymeric nanoparticles, or micelles, which is guided to the specific site of the body by attaching of targeting ligands, based on ligand-receptor interactions. They may overcome many hurdles that traditional pharmacotherapy could not conquer. For instance, the drug concentration in the target cell or tissue is upregulated due to the specific targeting, while reduces adverse effects on other cell types. Additionally, nanomedicines can overcome biological barriers due to their controllable size and shape, protect the drug from being metabolized and prolong drug circulation in the bloodstream, and alter pharmacological features of the delivered drug (Bartneck et al., 2014). In preclinical models, numerous nanoformulations were explored for the treatment of liver fibrosis.

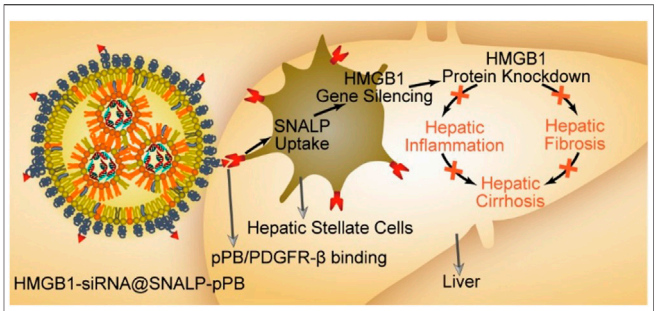

FIGURE 3 | Schematic diagram of the HMGB1-siRNA@SNALP-pPB nanoparticle targeting HSCs to silence HMGB1 to show antifibrotic and anti-inflammatory effects for hepatic cirrhosis (Zhang et al., 2020).

Current antifibrotic nanodrug therapies mainly focus on targeting HSCs, macrophages, NK cells, and LSECs, etc. Because the crosstalk between these cell populations is regarded as major contributor to liver fibrosis. The current cell therapy strategies are as follows: 1) suppressing the activation or proliferation of HSCs, promoting the apoptosis or senescence of aHSCs, switching from activated to quiescent state, inhibiting the secretion of ECM protein or promoting the expression of MMPs; 2) suppressing the production of pro-inflammatory and profibrotic factors by immune cells, or promoting the switch from fibrogenic to collagenolytic phenotype in macrophages; 3) protecting hepatocytes from injury or enhancing hepatocyte regeneration; 4) preventing the capillarization of LSECs. The following section deals with nanoparticles-based drug delivery strategies for liver fibrosis.

NANODRUG DELIVERY SYSTEM TARGETING HEPATIC STELLATE CELLS

In the last few years, numerous drug delivery approaches for specific-targeting of HSCs have been studied, for example, liposomes, protein-based delivery systems, viral vectors, and inorganic delivery systems, which can reduce drug adverse effects and further improve therapeutic effects of drugs.

Targeting Platelet-Derived Growth Factor Receptor

PDGF receptor (PDGFR) is a dimer containing two related chains connected by disulfide bonds, which is specifically overexpressed on aHSCs. In view of this, Beljaars et al. developed a receptor-recognizing cyclic peptide-modified albumin (pPB) peptide "C*SRNLIDC*" for targeting HSCs (Beljaars et al., 2003). Li et al. (2012) took use of the pPB peptide-modified sterically stable liposomes encapsulating IFNγ (pPB-SSL-IFN-γ) to

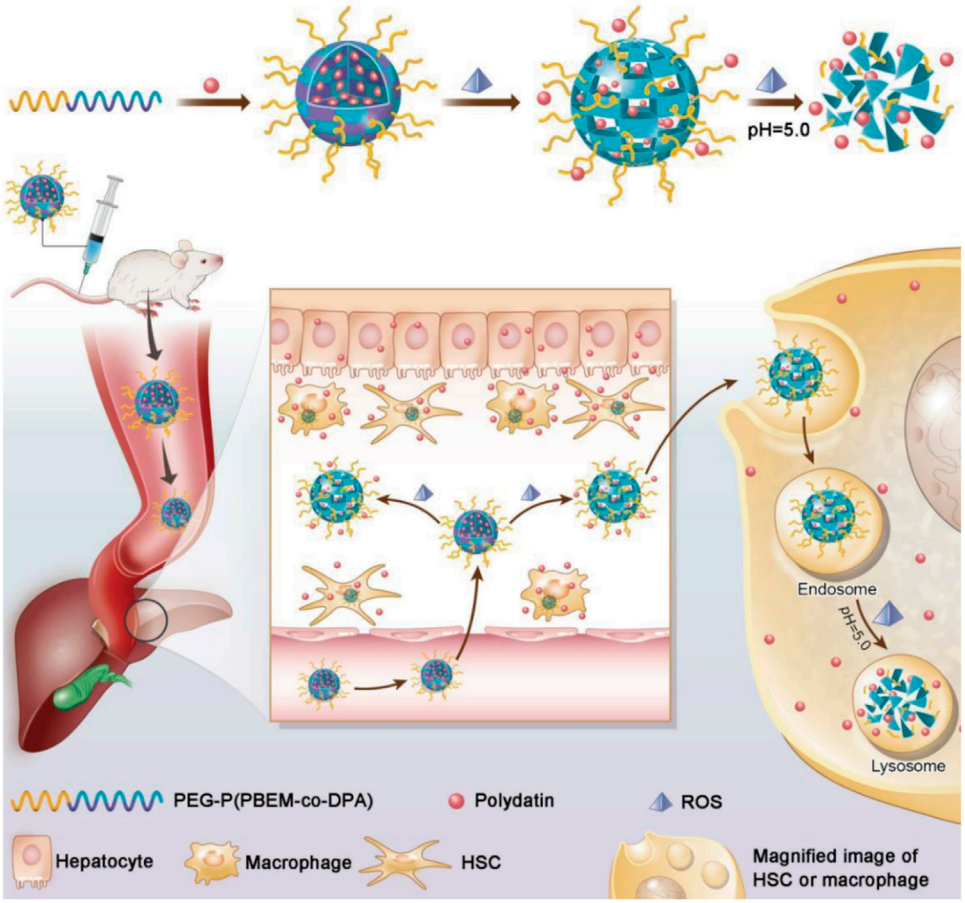

FIGURE 4 | Schematic illustration of PEG-P (PBEM-co-DPA)-Polydatin, a ROS, and pH dual-responsive nanodrug that regulates multiple cell types for the treatment of liver fibrosis (Lin et al., 2020).

specifically target HSCs. The NPs suppressed the proliferation of HSCs *in vitro* and showed decreased fibrosis in thioacetamide treated mice. The drug delievery system prolonged circulation half-life of IFNγ and decreased its side effects in fibrotic livers. Recently, Zhang et al. (2020) constructed pPB peptide-modified stable nucleic acid lipid NPs loading HMGB1 (High mobility group box-1)-siRNA (HMGB1-siRNA@SNALP-pPB) to effectively treat liver cirrhosis by their dual antifibrotic and anti-inflammatory abilities (**Figure 3**). HMGB1 protein is known as a fibroblast chemokine and pro-inflammatory factor, which promotes the proliferation of HSCs and facilitates hepatic inflammation and fibrosis. The targeted nanoparticles with both antifibrotic and anti-inflammatory effects prolonged the survival of cirrhotic mice significantly, suggesting that combination of multiple therapeutic targets would be more effective.

In addition to peptide, specific targeting mediated by antibodies has been applied for HSCs too. Inorganic delivery systems like Gold NPs can be developed into different shapes like nanocages, nanorods, and nanosatellites due to the surface characteristics and controlled particle size. Recently, Ribera et al. developed gold nanorods with surface modified anti-PDGFRβ to specifically target aHSCs (Ribera et al., 2021). Meanwhile, the gold nanorods can induce localized near infrared light (NIR)-mediated thermal ablation due to the photothermal properties, along with aHSC targeting, hepatic inflammation reduction, and fibrosis regression were observed in the CCl_4-induced fibrosis in mice. Moreover, inorganic nanomaterials themselves such as titanium dioxide (TiO_2) NPs and silicon dioxide (SiO_2) NPs demonstrate antifibrotic properties *in vitro* through acting on HSCs (Peng et al., 2018).

Targeting Sigma-1 Receptor

Relaxin (RLN) used to be considered as an antifibrotic peptide hormone which can directly reverse HSC activation for fibrosis regression. Its primary receptor, relaxin receptor family peptide-1 (RXFP1), is upregulated on aHSCs. The binding of RLN and RXFP1 can initiate the NO signalling against profibrogenic pathways (Fallowfield et al., 2014). Hu et al., 2019 engineered RLN-plasmid (pRLN)-loaded lipid-calcium-phosphate NPs (LCPs), surface modified with AEAA (aminoethyl anisamide,

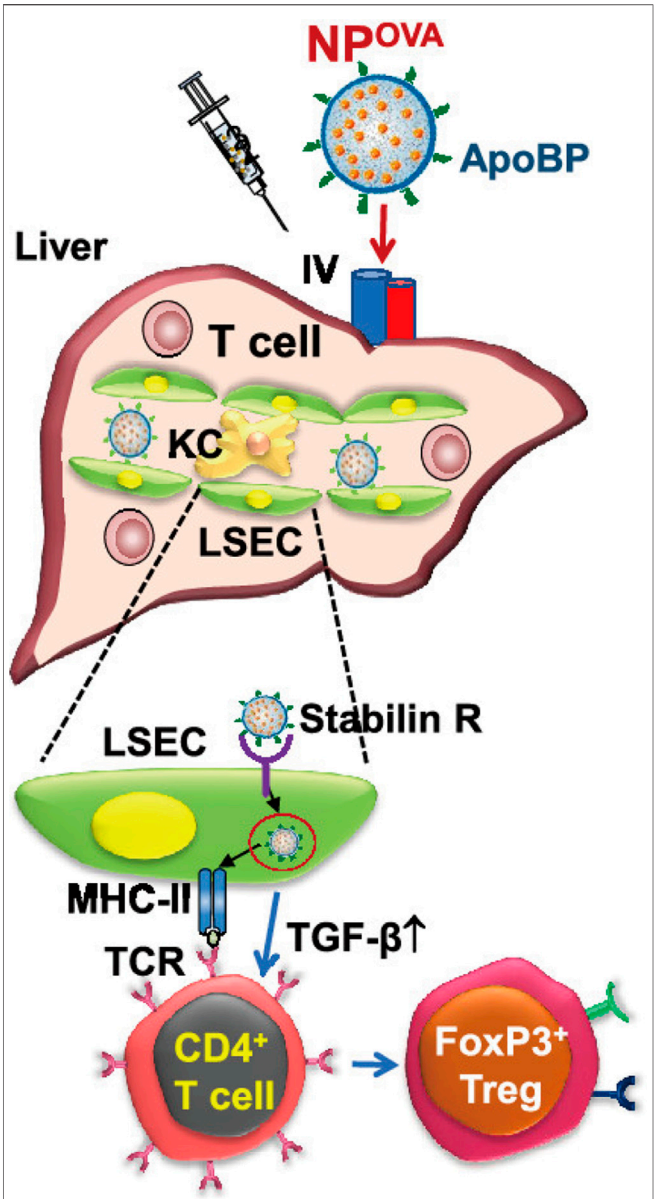

FIGURE 5 | Schematic diagram of the working model for liver-targeting tolerogenic nanoparticles. NPs attached ApoBP ligand delivering the antigens are epitopes to LSECs in the liver through endocytic uptake. Antigen processing and presentation to naive T-cells could generate Foxp3^{+} Tregs, which are recruited to the site of pathology, where they exert immunosuppressive effects (Liu et al., 2021).

ligand for the sigma-1 receptor, which is highly expressed on aHSCs), which can locally secret RLN in liver. The *in situ* enforced RLN expression by transfected aHSCs transformed themselves to quiescent phenotype and reduced ECM accumulation. In another study (Hu et al., 2021), the authors found relaxin gene therapy could reduce liver fibrosis *in vivo*, but the transmit of quiescence of aHSCs failed *in vitro* experiment. Moreover, relaxin is expressed by hepatic macrophages and on its binding, macrophages change from the pro-fibrosis to the pro-resolution phenotype via cAMP–PKA–CREB (cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cAMP-responsive element binding protein) pathway by activating Nur77 in macrophages. In view of this, they developed lipid–protamine–hyaluronic acid (LPH) nanoparticles encapsulate the relaxin gene and miR-30a-5p mimic, with surface modified AEAA, which show synergistic antifibrotic effects in rodent models. Here, miR-30a-5p derived from exosomes secreted by relaxin-educated macrophages can deactivate HSCs. The study provided a combinatory gene therapy involving macrophage phenotype switch, took into consideration the crosstalk between pro-resolution macrophages and aHSCs, synergistically and safely regressed liver fibrosis.

Targeting Integrin αvβ3

Integrins are receptors for most adhesion proteins like fibronectin and collagen type VI, take responsibility for the interaction between ECM and cells. They all contain the arginine-glycine-aspartic sequence (RGD) peptide. The RGD peptide -modified nanodelivery systems have been widely applied for HSC targeting.

Recent work by Li et al. (2019) prepared liposomes containing the cyclic peptides [cRGDyK, Cyclo (Arg-Gly-Asp-DTyr-Lys)] with high affinity to αvβ3 to specifically target aHSCs, but not quiescent HSCs, which overcomed the lack of specificity in the previous RGD-modified systems that target both quiescent and activated HSCs. Vismodegib (VIS), a Hh inhibitor, has been reported to attenuate hepatic fibrosis by inhibiting HSC activation (Philips et al., 2011). The cRGDyK modified SSLs raised therapeutic efficacy of VIS by alleviating undesirable properties, such as water insolubility, short half-life, and off-target effects. Ji et al. (2020) took use of this cyclic peptides to modify germacrone (GMO)-and-miR-29b-loaded nanoparticles, which exhibited great cytotoxicity to aHSCs and suppressed production of collagen I. In addition, cRGDyK used to be employed as an imaging modality for liver fibrosis (Li et al., 2016). It is a useful MRI tracer and can assess the extent of liver fibrosis non-invasively and quantitatively. It seems that cRGDyK-modified NPs act as an effective platform for both treatment and diagnosis of liver fibrosis.

Targeting Retinol-Binding Proteins

HSCs contain most vitamin A (VA) of the body, accounting for 80%, and it can be selectively transported into HSCs by retinol binding protein receptor (RBPR) and cell retinoic acid binding protein (CRABP) overexpressed on the surface of HSCs (Lee and Jeong, 2012). Therefore, VA-based delivery systems have been developed for liver fibrosis.

Qiao et al. (2018) developed HSC-targeted NPs grafting VA for co-delivery of chemical (silibinin) and genetic (siCol1α1) drugs, which inhibit collagen I accumulation synergistically in fibrogenesis. The team followed a tradition of multi-target therapy and prepared a lipid delivery system which carried dual siRNAs intended to both promote collagen degradation (by siTIMP-1) and inhibit collagen synthesis (by siCol1α1) (Qiao et al., 2020). They use helper lipoids (Chol-PEG-VA) for HSCs targeting and amphiphilic cationic hyperbranched lipoids (C15-PA) for siRNA complexation to generate vitamin

A-decorated and hyperbranched lipoid-based lipid nanoparticles (VLNPs). CT-VLNPs showed reduced collagen accumulation in treated mice to almost that seen in normal one. The combined therapy of co-delivering multi-target drugs achieved a substantial ideal effect, and providing a new direction for the treatment of liver fibrosis in future.

Targeting CD44

CD44 used to be regarded as a cell surface adhesion receptor highly expressed in many cancers. Later, it was discovered the high expression on aHSCs and it can interact with chondroitin sulfate (CS) and hyaluronic acid (HA) (Fujimoto et al., 2001). Therefore, CS has been suggested as a suitable candidate material to fabricate delivery systems for HSC-targeting. Recently, Luo et al. (2019) developed DOX-RA-CS (DOX, doxorubicin; RA, retinoic acid; CS, chondroitin sulfate) micelles co-delivery system for the treatment of CLD. The micelles preferentially deposited in the Golgi apparatus, destroyed the Golgi structure, and ultimately reduced collagen I production. The micelles exhibited synergistic antifibrotic effects in the CCl_4-treated rat model. In another study by Liang et al. (2020), HA was used as a candidate for HSC-specific drug delivery. Upconversion nanoparticle (UCNP) cores modified with HA and Roussin's black salt (RBS) were enveloped in mesoporous silica shells (HA-UCNP@mSiO2@RBS), which can target HSCs and locally release NO when exposing under near NIR. The release of NO can trigger HSC apoptosis and fibrosis regression.

In addition, it has been reported that CD44 is a key player in non-alcoholic steatohepatitis (NASH) (Patouraux et al., 2017). It can regulate macrophage polarization and infiltration in liver to enhance the progression of NASH. Targeting CD44 might be a potential therapeutic strategy for NASH and related fibrosis.

NANODRUG DELIVERY SYSTEM TARGETING IMMUNE CELLS

After systemic administration, NPs enter the blood stream, interact with serum proteins non-specifically, and resident macrophages of the endothelial network will remove NPs larger than 200 nm and negatively charged. Kupffer cells and LSECs in the space of Disse are inherent components of the reticuloendothelial system (RES). The diameter of the sinusoidal endothelial fenestrum is approximately 100–200 nm (Xing et al., 2021). The previous study reported that the NPs encapsulated bovine serum albumin (BSA) ranged 100–200 nm had good liver targeting ability. Nanomedicines targeting the immunologically relevant RES have been well summarized in another review by Matthias Bartneck (Bartneck, 2021).

Many nano-delivery systems have been developed for targeting macrophages, such as solid-lipid, liposomes, and polymeric NPs (Colino et al., 2020). NPs deliver drugs to macrophages by passive and active targeting. Lipoplex-based transfection has been applied for macrophage targeting, glucan encapsulating sphingosine 1-phosphate receptor 2 (S1PR2)-siRNA NPs could attenuate hepatic inflammation and fibrosis by reducing the activation of NLRP3 inflammasome (Hou et al., 2020). Transplantation of mesenchymal stem cells (MSCs) has been regarded as a promising antifibrotic strategy but facing some clinical controversies. Wang et al. (2020) found tumor necrosis factor stimulated gene 6 (TSG-6) was a major antifibrotic cytokine in MSCs. In view of the easily capture of NPs by the RES of the liver, they prepared TSG-6@CaP@BSA (CaP, calcium phosphate) NPs for antifibrotic treatment. The NPs induced M2 polarization of macrophages and increased the expression of MMP12, which could inhibit HSC activation and suppress release of pro-inflammatory cytokines.

The previous work by He et al. (2013) developed trimethyl chitosan-cysteine (MTC) conjugated anti-TNF-siRNA NPs modified by mannose, which are mostly taken by macrophages. In addition, macrophages can precisely identify phosphatidylserine (PS), an anionic phospholipid expressed by apoptotic cells. Wang et al. (2018) constructed PS-modified nanostructured lipid carriers (mNLCs) containing curcumin (Cur-mNLCs) for liver fibrosis in a CCl_4 treated rat model. The PS-modification enhanced the uptake of NPs by macrophages in diseased liver, leading to fibrosis regression and upregulation of MMP2 and hepatocyte growth factors (HGF).

Therefore, the unspecific uptake of nanoparticles by immune cells like macrophages makes these cells an interesting target cell for nanomaterials. The immune cells act as a double-edged sword for drug delivery in liver fibrosis, where macrophages are easily targeted, but there is a risk of being cleared before they reach other cell types. Hence, more powerful and more unique targeting for specific subpopulations need further investigation.

NANODRUG DELIVERY SYSTEM TARGETING HEPATOCYTES

In fibrotic liver, hepatocytes damage may have serious effect on liver function, such as, lipid, protein, sugar metabolism, and liver detoxification. Nanodrug delivery systems usually carry liver-protecting drugs to hepatocytes for maintaining the function of liver. Galactose-modified delivery systems could target hepatocytes by recognizing its ligand, asialoglycoprotein receptor (ASGPR), which is an extracellular glycoprotein receptor expressed on the surface of hepatocytes. In addition, the expression of ASGPR is fibrosis stage dependent and could be used for precise treatment in liver fibrosis (Kumar et al., 2021). A work by Zhang et al. (2016) developed a noninvasive method of imaging for SPECT (single-photon emission computed tomography) with an ASGPR targeting tracer-(99m) Tc-p (VLA-co-VNI) to quantify and stage liver fibrosis. Galactose-functionalized polyamidoamine (PAMAM) dendrimer was utilized for hepatocyte targeting by He et al. (2017). Meanwhile, the dendrimer did not show hepatic or renal toxicity, or immunotoxicity, suggesting it may be a safe and efficient hepatocyte-targeting delivery platform. Similarly, pullulan stabilized iron oxide nanoparticles (P-SPIONs) targeting ASGPR were designed for theranostic application of liver diseases (Saraswathy et al., 2021). P-SPIONs showed early

diagnosis of liver fibrosis in rodent model. Besides, lipopeptide nanoparticles (LPNP), a kind of apolipoprotein mimicking nanocarriers, have shown promising potential for hepatocyte targeting (Dong et al., 2014). The LPNP effectively and selectively delivered siRNA into hepatocytes via dynamin-dependent micropinocytosis. It is possible that bioinspired design might be a useful strategy for biomaterials as drug delivery systems for liver fibrosis.

In addition, multiple cell-targeting strategies have been applied in fibrosis targeting systems. In view of the over production of ROS in fibrotic livers, a work by Lin et al. (2020) designed PD-MC (polydatin-encapsulated micelle) with ROS and pH dual-sensitivity, realized site-specific drug release in the ROS-rich tissue and the lysosomes (**Figure 4**). Herein, nano core was synthesized and assembled into the micelle. The PDPA segment was pH-sensitive and let polydatin intracellularly release in the acidic lysosomes (pH 4.5–5.5), while the PPBEM hydrophobic core encapsulating polydatin reacted with ROS to trigger drug release and decrease ROS in liver fibrosis. PD-MC targeted multiple types of hepatic cells, effectively ameliorated liver fibrosis by inhibiting inflammatory response and ROS, prevented hepatocyte apoptosis and averted activation of HSCs and macrophages.

NANODRUG DELIVERY SYSTEM TARGETING LIVER SINUSOIDAL ENDOTHELIAL CELLS

As mentioned above, LSECs undergo the loss of endothelial permeability in CLD. Recently a study showed that TiO_2 NPs could restore sinusoidal permeability by inducing transient leakiness in primary human hepatic sinusoidal endothelial cells (HHSECs), which could improve hepatic recovery and upregulate drug uptake (Tee et al., 2018).

Moreover, in blood, over 90% of hyaluronic acid (HA) is taken and metabolized by LSECs due to the receptors on them (DeLeve and Maretti-Mira, 2017). Therefore, HA modified delivery systems can be used for LSEC targeting in liver fibrosis. Ohya et al. developed biodegradable polyanion-coated polymeric micelles conjugated with HA by polyion complex (PIC) formation, which were taken up only into LSECs (Ohya et al., 2011).

It has been reported that rather than hepatocytes, LSECs play the key role in the initial uptake of virus into the liver in a hepatitis B virus model. Fluorescent viral particles and virus protein-coated gold particles exhibited a preferential uptake of the viral substrates into LSECs (Breiner et al., 2001). Thus, utilizing viral pathways of cell targeting in liver might be a candidate for drug delivery to fibrotic liver. Abel et al. prepared mouse CD105-specific lentiviral vectors (mCD105-LV) that can specifically transduce LSECs (Abel et al., 2013). Another work took use of the endocytic uptake by LSECs to deliver antigens to the liver (Liu et al., 2021). The tolerogenic nanoparticles (TNPs) attached ApoBP ligand presented antigens to regulate the differentiation of naive T-cells into Tregs (**Figure 5**). It seems that LSECs-targeting antigen delivery might be a hopeful candidate in the immune regulation for autoimmune disorders, such as autoimmune hepatitis.

CONCLUSION

Liver fibrosis represents a common stage of CLD with a major impact on the human population, and there is an eager need for novel treatments to block and reverse the underlying pathological process. Given a vast number of drugs for liver fibrosis are under investigation, the arsenal of medicines being available is likely to prominently expand in the coming years. Up to now, most anti-fibrotic agents used clinically are neither liver nor fibrosis specific. Nanotechnology provides an opportunity to change this scenario.

However, up till now, the only nanodrug delivery system in the clinical stage for the treatment of liver fibrosis was lipid-based NPs. The targeted lipid nanoparticle delivers HSP47 (heat shock protein 47) siRNA to HSCs in Japanese subjects with moderate to extensive fibrosis (Sakamoto et al., 2018). It was in clinical phase 1b/2 and the results were safe and effective. It cannot be denied that there is still a long way to go before the clinical transformation of nanomedicine.

Firstly, although NPs have exhibited great therapeutic potential for liver fibrosis in preclinical experiments, they also show hepatotoxicity. It has been revealed that exposure to NPs could increase hepatotoxicity (Jia et al., 2017; Li et al., 2018a). The systemically evaluation is need for long-term hepatotoxicity of NPs, particularly when NPs are used in patients with CLD. Besides, it has been reported that NP structures may have strong immunomodulating activity, which can induce both immunostimulation, and immunosuppression (Di Gioacchino et al., 2011). The control of the immunological properties is also necessary for using NPs. Therefore, in preclinical and clinical stages, the safety involved in the use of NPs for liver fibrosis therapy deserve significant attention.

Secondly, many nanodrugs failed clinical development due to their failure in demonstrating a significant improvement in efficacy (Ventola, 2017). Most of the nanodrugs approved have demonstrated reduced toxicity rather than improved efficacy compared to conventional formulations. This may attribute to the difference between experimental models and human, or the lack of assurance of the quality of final products. Moreover, regulation regarding nanopharmaceuticals is still limited (Souto et al., 2020). Cost-benefit considerations should not be neglected as well. Hence, experimental animal models which can more appropriately reflect human pathophysiological processes need to be developed. To ensure the quality, efficacy, and safety of NPs for human use, clinical trials are mandatory (Souto et al., 2020). A reproducible, scalable production method must be developed and validated as well. Last but not least, to avoid investing in developing unlikely approved NPs, comparison with competitor products and a cost-benefit analysis are required.

Thirdly, it must be pointed that therapies based merely on antifibrotic effects cannot stop the driving factors of disease process such as cell stress, inflammation, and apoptosis. In addition, most research focuses on the activation process of HSCs and inflammatory pathways, but neglect the crosstalk between different cell types and different organs. It is also imperative to treat not only the etiology but also the complications of the liver disease, such as hypertension, HE, etc. Maybe no one-medication-fits-all strategy will be successful and the future focus should be on combination therapy. Relative

to traditional methods, nanomedicine might be easier to realize one-medication-fits-all strategy for liver fibrosis. Current nanotechnology particularly lipid-based RNA nanomedicines, provide new avenues through acting intracellular. Once establishing a definite mechanism route inside a cell type, lipid-based RNA nanomedicines will largely work independent of binding sites and other factors. Co-delivery of different RNAs will easily achieve the goal of multi-targeting therapy. Besides, microfluidic technology has greatly enriched the fabrication of RNA-based nanomedicines and are hoped to further do so.

In a word, nanotechnology has opened an exciting and promising field of research for the treatment of CLD. It can be envisioned that tailoring nanomedicine delivery systems will allow specific targeting to crucial cell types and achieve combination therapy, with low systemic toxicity. Nevertheless, the clinical translation of nanomedicine for antifibrotic therapies remains elusive and greater advance of our understanding on the potential targets for liver fibrosis is in need for the discovering of promising antifibrotic drug candidates. When overcoming the challenges of nanomedicine such as specific targeting, scale-up and manufacturing, regulatory and safety, it may only be a matter of time until nanomedicine alter clinical practice.

AUTHOR CONTRIBUTIONS

LG and JW designed this work of review. LG and FZ wrote the article. JW and YZ revised the article. All authors have read and agreed to the published version of the article.

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ER Disposal Pathways in Chronic Liver Disease: Protective, Pathogenic, and Potential Therapeutic Targets

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The endoplasmic reticulum is a central player in liver pathophysiology. Chronic injury to the ER through increased lipid content, alcohol metabolism, or accumulation of misfolded proteins causes ER stress, dysregulated hepatocyte function, inflammation, and worsened disease pathogenesis. A key adaptation of the ER to resolve stress is the removal of excess or misfolded proteins. Degradation of intra-luminal or ER membrane proteins occurs through distinct mechanisms that include ER-associated Degradation (ERAD) and ER-to-lysosome-associated degradation (ERLAD), which includes macro-ER-phagy, micro-ER-phagy, and Atg8/LC-3-dependent vesicular delivery. All three of these processes are critical for removing misfolded or unfolded protein aggregates, and re-establishing ER homeostasis following expansion/stress, which is critical for liver function and adaptation to injury. Despite playing a key role in resolving ER stress, the contribution of these degradative processes to liver physiology and pathophysiology is understudied. Analysis of publicly available datasets from diseased livers revealed that numerous genes involved in ER-related degradative pathways are dysregulated; however, their roles and regulation in disease progression are not well defined. Here we discuss the dynamic regulation of ER-related protein disposal pathways in chronic liver disease and cell-type specific roles, as well as potentially targetable mechanisms for treatment of chronic liver disease.

Keywords: ER associated degradation, ER-phagy, ER-lysosomal degradation, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, alcoholic liver disease, alpha-1 antitrypsin disease, fibrosis

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INTRODUCTION

The rising global prevalence of liver disease necessitates the development of effective strategies to limit disease progression. While numerous drugs and interventions have entered clinical trials, these strategies have been difficult to translate to patient care (Asrani et al., 2019). This is due in large part to the diverse etiologies and mechanisms that underly disease occurrence and progression (Asrani et al., 2019). Metabolic liver diseases, including Alcoholic Liver Disease (ALD) and Non-alcoholic fatty liver disease (NAFLD) are associated with altered proteomes, as are genetic-associated liver diseases such as Alpha-1 antitrypsin deficiency (AATD). While established as a key driver of AATD pathogenesis, a cellular process that often goes overlooked in other forms of chronic liver disease is ER-associated degradative pathways that maintain proteostasis. Also known as ER quality control pathways, these processes are critical for removing misfolded, unfolded, or modified proteins, limiting endoplasmic reticulum (ER) stress, and maintaining cell viability (Maiers and Malhi, 2019; Hetz et al., 2020). Indeed, several lines of evidence suggest that enhanced protein degradation could

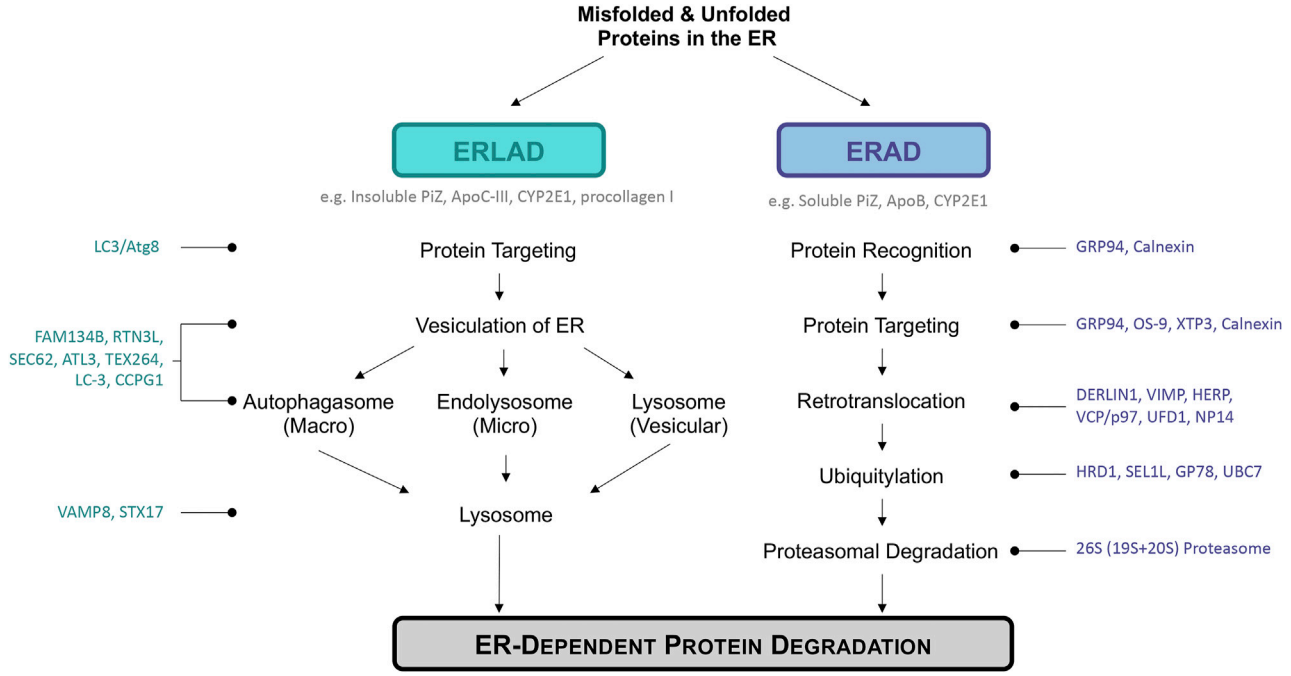

FIGURE 1 | Overview of ER disposal pathways discussed: ERLAD and ERAD. ERLAD (ER lysosomal-associated degradation) and ERAD (ER associated degradation) pathways are the main degradation pathways of misfolded or aberrant proteins during chronic metabolic liver diseases. Examples of specific proteins degraded by each pathway are listed in dark grey under pathway headers, while molecules important during each step are listed to the left or right of the figure in either teal or dark blue. ERLAD can be broken into three sub-categories based on whether the process involves the autophagosomal (macro-ER-phagy), endolysosomal (micro-ER-phagy), or lysosomal (vesicular delivery) pathways. ERAD is composed of five different steps: protein recognition, targeting, retrotranslocation, ubiquitylation, and finally protein proteasomal degradation. All pathways work to degrade distressed proteins in order to return the cell to homeostasis and avoid cell death.

limit hepatocyte damage and hepatocyte dysfunction, inflammation, and disease progression (Kim and Kim, 2020; Xia et al., 2020; Flessa et al., 2021). Furthermore, recent advances in the field of protein degradation pathways may help drive the targeting of these pathways in patients. Here we will discuss the role of protein degradation pathways associated with ER quality control: proteasomal ER-associated degradation (ERAD) and lysosomal ER-lysosomal associated degradation (ERLAD), how these pathways contribute to liver disease, and potential therapeutic strategies for targeting these pathways to limit disease progression.

The ER is Critical for Hepatic Function

The ER plays several essential roles in the liver, thus maintaining its integrity is paramount for liver health. Hepatocytes contain large amounts of both rough ER and smooth ER, which serve different functions (Baiceanu et al., 2016; Schulze et al., 2019). The rough ER is marked by ribosomes, and is the main site of protein synthesis, folding, and secretion, while the smooth ER contains the majority of the machinery required for xenobiotic detoxification, including cytochrome p450 enzymes, as well as lipid synthesis (Schwarz and Blower, 2016). Both types of ER also play roles in cellular calcium homeostasis and stress-induced RNA degradation. The ER holds an extraordinary ability to expand when physiological or pathological stimuli require (Rutkowski and Hegde, 2010; Rutkowski and Kaufman, 2004). In the liver, specifically hepatocytes, the ER is essential for maintaining cellular function and integrity. Hepatocytes produce vast amounts of secreted proteins and lipoproteins which requires ER expansion and increased expression of chaperones proteins to assist with protein folding. The ER is also a critical site of protein quality control. Misfolded proteins are prevented from entering the secretory pathway and are instead targeted for degradation by the proteosome through ERAD, or the lysosome through ERLAD (Sun and Brodsky, 2019). ER quality control is crucial for cellular homeostasis, though the distinct contribution of ERAD or ERLAD to ER physiology or pathophysiology are unclear.

ER Quality Control Pathways

ER-Associated Degradation (ERAD)

ERAD is a complex mechanism by which misfolded proteins in the ER are recognized, targeted, retrotranslocated to the cytoplasm, polyubiquitinated, and finally degraded by a proteasome (**Figure 1**). ERAD is also involved in regulating certain proteins based on metabolic signals, regardless of their configuration. In yeast, there are three types of ERAD: ERAD-L, ERAD-M, and ERAD-C, respectively targeting proteins with defects in their luminal region, membrane region, and cytoplasmic region for proteasomal degradation (Hwang and Qi, 2018). In mammals the distinction between ERAD-types is not as well established due to the complexity of ERAD

mechanisms (Kwon et al., 2020) The ERAD machinery involves numerous chaperones and factors that help in the above processes in order to maintain cellular homeostasis and avoid triggering cellular death when the ER is overloaded with misfolded proteins (Baiceanu et al., 2016; Vembar and Brodsky, 2008; Olzmann et al., 2013; Fujii et al., 2018; Kuscuoglu et al., 2018; Fregno and Molinari, 2019; Ninagawa et al., 2021). ERAD canonically progresses through five stages: recognition, targeting, retrotranslocation/extraction, ubiquitinoylation, and degradation. These steps are extensively described elsewhere, thus we will briefly outline the process of ERAD here (Fregno and Molinari, 2019; Sun and Brodsky, 2019; Molinari, 2021).

Recognition is the act of identifying an ER located protein that is misfolded and needs to undergo degradation. Recognition is based on an abnormal conformation of mannose units by proteins such as OS-9 (OS9 endoplasmic reticulum lectin), or through prolonged cycling through chaperone proteins such as calnexin or calreticulin. Protein recognition is generally done by either heat shock proteins (HSP) such as HSP70 or HSP90 or chaperons such as calnexin, calreticulin, or BiP (immunoglobulin-binding protein). Targeting is the interaction of recognized proteins with the retrotranslocation machinery. In some cases the ERAD machinery combines recognition and targeting as the same complexes are used in both. In mammals, lectins such as OS-9, XTP3-B (endoplasmic reticulum lectin 1), and mannosidases such as ERManI (ER α-1,2-mannosidase 1) and EDEM1-3 (ER degradation-enhancing α-mannosidase-like) have been implicated in protein targeting. These factors recognize the number and organization of mannose moieties on the misfolded protein and based on this information target it for degradation. Retrotranslocation/Extraction is the act of moving the targeted protein from the ER lumen or ER membrane to the cytoplasm. It is essential that the protein targeted for degradation be moved to the cytoplasm as ubiquitination ligase complexes exists only there, as does the proteasome. Proteins involved in retrotranslocation/extraction in mammals are: VIMP (VCP-interacting membrane protein), Derlin 1-3, VCP (valosin-containing protein), Sec61 complex, and HRD1. Proteins undergo Ubiquitinoylation once retrotranslocated into the cytoplasm, which marks them for proteasomal degradation. In mammals E1-E4 ubiquitin enzymes are involved, such as the conserved E3 ubiquitin ligase complex HRD1-SEL1L. Finally proteins that have been ubiquitinylated undergo proteasomal degradation by the 26S proteasome a complex formed by two 19S subunits and one central 20S unit (Baiceanu et al., 2016; Vembar and Brodsky, 2008; Olzmann et al., 2013; Kuscuoglu et al., 2018; Ninagawa et al., 2021). Together these five stages—recognition, targeting, retrotranslocation/extraction, ubiquitinoylation, and degradation—of ERAD allow for proteins to be swiftly targeted for proteasomal degradation whether located in the ER lumen or ER membrane allowing ER homeostasis to reestablish preventing liver injury.

ER-to-Lysosomal Associated Degradative (ERLAD)

ER-to-Lysosomal-Associated Degradative (ERLAD) pathways describe a subset of processes that involve targeting of proteins in the ER lumen/membrane or the ER membrane itself for lysosomal degradation (**Figure 1**). These can involve engulfment of the ER by autophagosomes followed by lysosomal degradation (macro-ER-phagy), direct targeting of ER membrane to the lysosomes (micro-ER-phagy), and LC3/Atg8-dependent vesicular deliver. These processes have been implicated in several non-physiological states, such as in the presence of mutated/misfolded proteins *in vitro*, as well as with ER turnover following ER expansion in response to stress or increased secretion. While macro-ER-phagy is the most widely studied of the ERLAD pathways, each of the ERLAD pathways have been implicated in liver disease.

Macro-ER-phagy describes a set of processes that involve targeting of ER membrane or ER-associated proteins for autophagic engulfment followed by lysosomal degradation (Fregno and Molinari, 2018; Fregno and Molinari, 2019; Sun and Brodsky, 2019). These processes have been implicated in several physiological and pathological states, such as in the presence of mutated/misfolded proteins *in vitro*, as well as with ER turnover following ER expansion in response to stress or increased secretion (Sun and Brodsky, 2019; Fregno and Molinari, 2019; Fregno and Molinari, 2018).

In macro-ER-phagy, autophagic membranes are recruited directly to the ER by membrane-localized receptors, which typically bind to LC-3/Atg8 on autophagic membranes through LC-3 interacting regions (Sun and Brodsky, 2019; Grumati et al., 2018). Several membrane-localized ER-phagy receptors have been identified, including FAM134B/RETREG1 (Family With Sequence Similarity 134, Member B/Reticulophagy Regulator 1), CCPG1 (Cell Cycle Progression 1), TEX264 (Testis Expressed 264), SEC62, ATL3 (Atlastin 3), and the long isoform of RTN3 (Reticulon 3L) (Khaminets et al., 2015; Fumagalli et al., 2016; Grumati et al., 2017; Grumati et al., 2018; Smith et al., 2018; An et al., 2019; Chen et al., 2019; Chino et al., 2019). These receptors accumulate, leading to membrane deformation. The subsequent targeting of the ER for degradation often occurs through recruitment of ATG8/LC-3 proteins located on autophagic membranes, followed by engulfment and trafficking of ER membrane to the lysosome (Sun and Brodsky, 2019; Grumati et al., 2018; Fumagalli et al., 2016; Fregno et al., 2018; Omari et al., 2018; Loi et al., 2019; Fregno et al., 2021). The mechanisms regulating engulfment/scission are unknown. Several membrane-localized ER-phagy receptors have luminal domains which may facilitate specific targeting of proteins for ER-phagy (CCPG1, FAM134B-2, TEX264. Other ER-phagy receptors do not contain a luminal domain, and instead use intermediaries to target cargo for degradation. This includes FAM134B targeting procollagen I for degradation through Calnexin (Forrester et al., 2019). In addition to cargo specificity, ER-phagy receptors show specificity based on ER morphology, with ATL3, CCPG1, and RTN3L acting at ER tubules, FAM134B facilitating ER-phagy along ER sheets, and TEX264 recruiting autophagosomes to three-way junctions of the ER (Grumati et al., 2017; Khaminets et al., 2015; Smith et al., 2018; An et al., 2019; Chen et al., 2019; Chino et al., 2019; Liang et al., 2018). This distinct localization may also have implications for specific cargo that is recruited for ER-phagic-degradation, or specific processes that require distinct ER morphology. Recently,

cytoplasmic proteins that participate in ER-phagic degradation were identified (Sequestosome 1/p62, C53, CALCOCO1 (Calcium Binding And Coiled-Coil Domain 1), and CALCOCO2) as well as several potential ER-phagic proteins identified in a recent screen; however, the distinct mechanisms for how these non ER-membrane bound proteins drive ER-phagy is unclear (Yang et al., 2016; Liang et al., 2020; Nthiga et al., 2020; Stephani et al., 2020). Due to the importance of the ER for hepatocyte function, ER-phagy is critical to maintain a healthy liver; however, little is known regarding the role and regulation of this degradative process in hepatocytes or non-parenchymal liver cells.

Micro-ER-phagy and LC3/Atg8-dependent vesicular delivery described processes where the ER is directly targeted to the lysosome without autophagic engulfment (Fregno and Molinari, 2019). Micro-ER-phagy has been observed during recovery from ER stress, and in response to aggregation of misfolded proteins in the ER that are resistant to ERAD, such as in AATD or procollagen I which will be discussed later in this review (Fregno and Molinari, 2018; Fregno et al., 2021). In mammals, the micro-ER-phagy and LC3-Atg8-dependent vesicular delivery pathways currently identified involve LC-3 lipidation, which targets the ER to interact with endolysosomal membranes followed by fusion of the membranes and degradation of the target proteins (Molinari, 2021). This process was found to degrade protein aggregates through a non-rapamycin responsive mechanism, suggesting that the autophagosome is not involved (Fregno and Molinari, 2018; Fregno et al., 2021). FAM134B was found to play a role not only in macro-ER-phagy, but also LC3/Atg8-dependent vesicular delivery. It remains unclear whether this process occurs under physiological conditions, or whether increased aggregation of misfolded proteins in the ER is required, but its potential role in liver pathology warrants further investigation.

Regulation of ER Quality Control Pathways by the Unfolded Protein Response

A major goal of ERAD and ERLAD is to remove misfolded proteins from the ER, thus it is unsurprising that these processes are regulated in part by the unfolded protein response (UPR). As the UPR is critical for liver physiology and is dysregulated in chronic liver disease, regulation of ER quality control pathways by the UPR is important to discuss. The UPR is initiated by ER stress, occurring through excess unfolded or misfolded proteins accumulating within the ER (Hetz et al., 2020; Schwarz and Blower, 2016). The UPR is propagated through three canonical ER membrane proteins: IRE1α (inositol requiring enzyme 1α), PERK (protein kinase RNA-like ER kinase), and ATF6α (activating transcription factor 6 alpha) (Schwarz and Blower, 2016; Hetz et al., 2020). Signaling through these pathways promote expression of chaperone proteins as well as proteins involved in degradative processes, while limiting non-essential protein translation. Furthermore, the UPR plays a critical role in liver disease progression and fibrosis, thus the role of the UPR in regulating protein degradation through ERAD and ERLAD is important to discuss (Maiers and Malhi, 2019). IRE1α and ATF6α are the primary regulators of ERAD in response to ER stress. Upon sensing ER stress, IRE1α splices XBP1 mRNA to induce translation of the active XBP1 transcription factor (Tirosh et al., 2006; Jurkin et al., 2014). XBP1 translocates into the nucleus and promotes transcription of several ERAD-associated genes, including ERdj3 (Endoplasmic Reticulum DnaJ Homolog 3), ERdj4, EDEM1, UBE2E1 (Ubiquitin Conjugating Enzyme E2 E1), and HERPUD1 (Homocysteine Inducible ER Protein With Ubiquitin Like Domain 1) (Oda et al., 2006; Yamamoto et al., 2007; Park et al., 2021). ATF6α is also activated in response to ER stress, promoting ATF6α trafficking to the Golgi. At the Golgi, ATF6α is cleaved and the active portion of ATF6α is released, subsequently entering the nucleus and promoting expression of XBP1 and several ERAD-associated genes (Yamamoto et al., 2007; Yoshida et al., 1998; Haze et al., 1999; Wang et al., 2000; Shen et al., 2002). Induction of the ERAD machinery is critical for identification, translocation, and degradation of misfolded proteins located within the ER lumen/membrane to relieve ER stress (Hwang and Qi, 2018). UPR regulation of ERAD likely directly contributes to hepatic steatosis and liver disease. Mice lacking hepatocyte expression of IRE1α exhibited worsened steatosis in response to ER stress (Zhang et al., 2011). Furthermore, proteosome inhibition was sufficient to promote lipid accumulation in the liver, which was exacerbated with IRE1α knockout (Zhang et al., 2011). Subsequent studies have investigated the role of IRE1α in steatosis; however, no reports have studied the link between IRE1α-regulation of ERAD to steatosis development (Maiers and Malhi, 2019).

Less is known regarding UPR regulation of ERLAD. ER stress is tightly linked to autophagy and lysosomal degradation, through UPR-mediated upregulation of LC-3B cleavage and expression of SQSTM1/p62 and Beclin (Kim and Kim, 2020; Xia et al., 2020). The ER-phagy receptor CCPG1 is upregulated by ER stress in mammalian cells potentially through XBP1, while FAM134B expression is upregulated by c/EBPβ, which is downstream of the UPR sensor ATF6α (Smith et al., 2018; Kohno et al., 2019). No other direct link between the UPR and expression of ER-phagy receptors is reported, though the UPR likely plays an important role in regulating ERLAD initiation and flux through ERLAD pathways as well.

Physiological ERAD and ERLAD in the Liver

Under physiological settings, ERAD and ERLAD serve important roles in hepatocytes (Moore and Hollien, 2012). The hepatocyte is a professional secretory cell, producing an estimated 13 million secretory proteins each minute (Schulze et al., 2019). Both the smooth and rough ER expand to accommodate induced secretion of proteins, but also need degradative pathways to remove misfolded proteins, facilitate membrane turnover, and decrease ER size upon removal of the secretory stimuli (Sun and Brodsky, 2019; Moore and Hollien, 2012). ER stress is prevalent in physiological conditions, including the post-prandial state where the increased lipid and carbohydrate presence in hepatocytes further stress the ER (Deng et al., 2013). While the transcriptional programs activated by the UPR have been,

and continue to be, extensively studied in the liver, the degradative pathways activated to remove misfolded or excess proteins are understudied in their relevance to liver physiology.

ERAD: Numerous *ERAD* components are conserved across mammals, and ubiquitously expressed across tissues. In particular, the E3 ubiquitin ligase HRD1 and its cofactor SEL1L are considered the most conserved ERAD system that facilitates retrotranslocation of misfolded proteins from the ER and subsequent proteasomal degradation (Vembar and Brodsky, 2008). In hepatocytes, HRD1/SEL1L regulates responses to fasting and feeding, regulating protein levels of the transcription factor CREBH (cyclic adenosine monophosphate (c-AMP)-responsive element binding protein H) which in turn increased transcription of FGF21 (Fibroblast growth factor 21) (Bhattacharya et al., 2018). In this manner, increased expression of HRD/SEL1L enhanced ERAD-mediated degradation of CREBH during feeding regulates metabolism in hepatocytes (Bhattacharya et al., 2018). ERAD is also associated with cholesterol biosynthesis, through targeting key enzymes involved in cholesterol synthesis, such as squalene epoxidase and 3-Hydroxy-3-Methylglutaryl-CoA Reductase for proteasomal degradation (Tan et al., 2019). Based on these physiological roles, inhibition, or suppression of ERAD could significantly influence metabolism and cholesterol synthesis, potentially driving liver disease.

ERLAD: Expression of the major ER-phagy receptors is relatively ubiquitous, with both an N-terminal truncated isoform of FAM134B (FAM134B-2) and CCPG1 showing some enrichment in the liver (Ma et al., 2018; Kohno et al., 2019). Little is known regarding the physiological or pathophysiological roles of ER-phagy receptors in liver disease, but two key studies recently provided insight into how ER-phagy regulates liver physiology. First, Kohno et al. found that FAM134B-2 increased in response to starvation in mouse livers, and this occurred through C/EBPβ-mediated transcription (Kohno et al., 2019). FAM134B is also associated with activation of the transcription factors transcription factor EB (TFEB) and Transcription Factor Binding To IGHM Enhancer 3 (TFE3) which are established regulators of autophagic genes in response to starvation (Kohno et al., 2019; Cinque et al., 2020). Supporting a physiological role for ER-phagy receptors in the liver, ER microsomes were isolated from FAM134KO or wild type mice after fasting. Proteomic analysis of proteins from the microsomes revealed 40 proteins enriched in FAM134B KO livers compared to wild type, including Apolipoprotein C-III (ApoC-III) which will be discussed later for its role in NAFLD pathogenesis (Kohno et al., 2019). Thus, FAM134B-2 may be activated under conditions of both ER stress and starvation/amino acid depletion.

ER QUALITY CONTROL PATHWAYS AND CHRONIC LIVER DISEASE

ER stress and chronic liver disease are pathologically linked, with pathogenic stimuli leading to ER stress, and ER stress driving liver disease pathology. The mechanisms through which injurious stimuli drive chronic liver disease through ER stress are described in detail elsewhere, so we will only include a brief discussion, focusing more on the role of ER quality control pathways in this review (Baiceanu et al., 2016; Maiers and Malhi, 2019; Kim and Kim, 2020; Flessa et al., 2021). ER stress occurs in response to increased triglyceride and cholesterol accumulation in hepatocytes as well as chronic alcohol consumption. ER stress and UPR signaling further potentiate liver damage through promoting hepatocyte apoptosis downstream of ATF4/CHOP signaling, inducing release of pathogenic extracellular vesicles through IRE1/XBP1, regulating steatosis, activating inflammatory pathways, and promoting fibrogenesis (Ji et al., 2005; Oyadomari et al., 2008; Cazanave et al., 2010; Pfaffenbach et al., 2010; Cazanave et al., 2011; Li et al., 2011; Malhi et al., 2013; Xiao et al., 2013; Toriguchi et al., 2014; Kakazu et al., 2016; Rahman et al., 2016; Shan et al., 2017; Dasgupta et al., 2020; Duwaerts et al., 2021). ATF6α is involved in lipid synthesis regulation, with ATF6α loss in hepatocytes limiting steatosis, but may also promote fibrogenesis in response to liver injury (Rutkowski et al., 2008; Yamamoto et al., 2010; Chen et al., 2016a; Xue et al., 2021). contributing to NAFLD development and progression. Indeed, ER stress is linked with the progression of NAFLD to NASH, as well as fibrosis progression, thus understanding the contribution of these pathways to chronic liver disease, as well as targeting these pathways to limit progression and/or promote regression of liver disease is of paramount importance. A critical and understudied UPR regulated process are the ER quality control pathways. The studies described earlier implicate ERAD and ERLAD as critical regulators of liver physiology, and potential drivers of liver pathology. We will further discuss the established and potential pathological roles for ERAD and ERLAD in genetic and metabolic liver disease, including AATD, NAFLD/NASH, ALD, and fibrosis. Finally, we will discuss these processes as potential therapeutic targets for limiting progression of chronic liver disease.

ALPHA-1 ANTITRYPSIN DEFICIENCY

Alpha-1 antitrypsin (AAT) is a serine protease inhibitor important in degrading neutrophilic elastase in the lung. Under normal conditions it is synthetized and secreted by hepatocytes, but several mutations can exist in the gene that encodes AAT, *Serpina1* (Pi), which leads to diseases of both the lung and liver. The most severe form of AATD occurs with homozygous expression of ZZ alleles (protein: PI-Z) instead of the normal MM alleles, which leads to a Glu342Lys transformation. AATD affects 1/2,000-1/5,000 people world-wide (Manne and Kowdley, 2020). This missense mutation induces an accumulation of AAT PI-Z aggregates within the hepatocyte, specifically within the ER. Hepatic accumulation of AAT PI-Z leads not only to ER stress and subsequent liver disease but also to a deficiency in circulating and pulmonary AAT, which ultimately leads to lung disease (Teckman et al., 1996; Silverman and Sandhaus, 2009; Manne and Kowdley, 2020; Narayanan and Mistry, 2020; Patel et al., 2021). Progression of AATD can range from pediatric jaundice to the development of hepatocellular carcinoma in adulthood (Narayanan and Mistry, 2020).

Hepatocytes attempt to control the accumulation of AAT aggregates *via* two main mechanisms—ERAD and macro-ER-phagy, though a direct lysosomal pathway has recently been implicated. For years ERAD was the favored mechanism through which an attempt at reestablishing protein homeostasis during AATD occurred. However, studies demonstrated that the preferred mechanism of AAT protein degradation mechanism is dependent on the state in which the PI-Z mutant proteins are found. When proteins remain soluble in monomeric or oligomeric form, they undergo ERAD rather than autophagy (Perlmutter, 2006). Most hepatic therapies for AATD have focused on increasing autophagy to reduce hepatocyte aggregates and the liver burden. The role of ERAD and ERLAD are best studied in AATD compared to other liver diseases, and may provide critical insight into how these pathways drive progression of other hepatic disorders. We will discuss these mechanisms, and lysosomal degradation, as they relate to AATD and liver disease.

ERAD in AAT Deficiency and Liver Disease

Early accounts of ERAD during AATD demonstrated that PI-Z could be degraded through proteasomal degradation involving calnexin (Qu et al., 1996). Briefly, they described a phenomenon where PI-Z bound calnexin, which in turn promoted calnexin retrotranslocation and polyubiquitination. The ubiquitinated calnexin was then recognized by cytoplasmic proteasomes for degradation. From there the involvement of ERAD in the degradation of PI-Z during AATD only became more complex. Using an array of plasmids and HEK 293 cells, Shen et al. proposed that p97/VCP (valosin containing protein) were involved in the retrotranslocation of PI-Z, which was subsequently ubiquitinated by the E3 ubiquitin ligase GP78/AMFR (autocrine motility factor receptor) and UBE2G2 (ubiquitin-conjugating enzymes E2 7) (Shen et al., 2006). Further reports added to the ERAD machinery discovery. For example, several groups demonstrated that the E3 ubiquitin ligase HRD1/SYNV1 (synoviolin1) facilitated PI-Z degradation (Christianson et al., 2008; Iida et al., 2011; Wang et al., 2011). Christianson and colleagues, through a series of *in vitro* experiments, also described upstream events in the ERAD cascade for PI-Z degradation (Christianson et al., 2008). They established that the ER resident lectins OS-9 and XTP3-B, and ER resident chaperone glycoprotein GRP94/HSP90B1 (heat shock protein 90 beta family member 1) were responsible for delivering PI-Z to the SEL1L adaptor subunit of the SEL1L/HRD1 complex for ubiquitination and degradation. Derlin-1 has also been described as a part of the ERAD machinery involved in degrading PI-Z proteins (Lilley and Ploegh, 2005; Greenblatt et al., 2011). Finally a large complex of molecules termed, Complex I, was defined in ERAD of PI-Z (Ye et al., 2004; Lilley and Ploegh, 2005; Iida et al., 2011). Complex I was composed of: OS-9, SEL1L, HRD1 (which together form Complex II), in addition to HERP (Hes related family BHLH transcription factor with YRPW motif 2), Derlin-1, VIMP (selenoprotein S), p97/VCP, NPL4 (NPL4 homolog, ubiquitin recognition factor), UFD1 (ubiquitin recognition factor in ER associated degradation 1). Molecules involved in the ERAD of mutant AAT protein PI-Z during AATD are summarized. In summary ERAD plays a key role in degrading PI-Z soluble aggregates, helping alleviate hepatocellular ER stress during AATD.

ERLAD in AAT Deficiency and Liver Disease

Accumulation of PI-Z leads to the formation of insoluble aggregates, which are resistant to ERAD (Perlmutter, 2006). Despite this insolubility, studies showed that hepatocytes shifted to promote degradation through ER-phagy (Teckman and Perlmutter, 2000). The Perlmuter group microscopically examined this degradative process in several different cell lines, PI-Z mutant mice, and liver samples from patients with AATD. They noted the formation of large insoluble aggregates of PI-Z that could be found within both the ER and autophagosomes. Around the same time, another group demonstrated that transfection of PI-Z into cells deficient for *Atg5* (autophagy related 5) led to increased PI-Z aggregate accumulation compared to WT cells (Kamimoto et al., 2006). In WT cells an increase in $LC3^{+}$ autophagosomes, in the presence of PI-Z, was noted while *Atg5* deficient cell numbers increased indicating less autophagy occurring.

While not focused directly on macro-ER-phagy, additional studies have demonstrated that the autophagic regulator TFEB is actively involved in reducing PI-Z accumulation in hepatocytes (Pastore et al., 2012; Pastore et al., 2013). Both *in vitro* and *in vivo* studies showed that when *Tfeb* was transferred into cells or PI-Z mutant mice it significantly reduced PI-Z accumulation, and reduced liver disease and fibrosis *in vivo*. Finally, Feng et al. recently described the autophagic machinery involved in PI-Z degradation in more detail (Feng et al., 2017). Using a combination of HEK 293T and HepG2 cell lines, they established that SYVN1 was involved not only in ubiquitin tagging insoluble PI-Z for proteasomal degradation but also autophagic degradation. SYVN1 promoted the interaction of PI-Z with sequestosome1 (SQSTM1), which then interacted with LC3 at autophagosomes, leading to autophagic degradation of PI-Z. Further studies are needed to fully elucidate the exact autophagic and ER-phagic pathways involved in PI-Z degradation during AATD, but the groundwork has certainly been laid.

Lysosomal Degradation Pathways in AAT Deficiency and Liver Disease

There exist very few accounts of the lysosomal pathway being involved in PI-Z degradation during AATD separate from autophagy, and of the accounts that exist, the degradation mechanisms differ. One group described an ERLAD (Fregno et al., 2018) mechanism, while the other maps out a Golgi-dependent degradation (Gelling et al., 2012) mechanism, both of which are discussed below.

ERLAD is the direct degradation of ER contained proteins, in this case polymeric PI-Z, by endolysosomes without the intervention of autophagosomes. Fregno et al. described the phenomena using a complex *in vitro* experimental system where they demonstrate that even in the absence of the

autophagosome machinery PI-Z still undergoes lysosomal degradation. The paper establishes that single-membrane ER vesicles expressing FAM134B fusion with RAB7/LAMP1 expressing endolysosomes through the formation of a complex between FAM134B and LC3-II on the endolysosomes. The process is complete when the ER vesicle fully fuses to the endolysosomes through SNARE/STX17 and SNARE/VAMP8 localized on the ER vesicle and the endolysosomes, respectively (Fregno et al., 2018).

Gelling et al., also described PI-Z removal through lysosomal degradation, but through a mechanism that involves the Golgi. In a PI-Z yeast mutation system, the group screened mutations that disrupted PI-Z degradation, and found one target of interest—*Vps10* (Sortilin in humans). Sortilin is a protein involved in transporting misfolded proteins from the Golgi to vacuoles for secretion or degradation, it is localized in the late compartment of the Golgi. When they further investigated the role of sortilin in a rat hepatoma (RH-7777) cell line, they found that in a sortilin mutant PI-Z trafficking to the lysosome was disrupted, a defect that was corrected with sortilin overexpression. The group thus concluded that the Golgi trafficked PI-Z to lysosomes for degradation in a sortilin-dependent fashion (Gelling et al., 2012).

In conclusion, AATD burdens both the liver and lungs through the aggregation of AAT and lack of AAT circulation, respectively. Numerous groups have described that both ERAD and ERLAD are highly involved in reducing the liver burden during AAT aggregate accumulation within the ER of hepatocytes. ERAD being favored when aggregates remain small and soluble while ERLAD is used when aggregates are large and insoluble. Several animal models have tested drugs to enhance autophagy/macro-ER-phagy with promising results, while carbamazepine (CZB) is currently undergoing clinical trials for the treatment of liver disease during AATD.

NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is an overarching term that incorporates both non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NAFL is the accumulation of fat, or steatosis, within the liver accompanied by little to no inflammation, while NASH is steatosis accompanied by inflammation, hepatocyte ballooning and death, as well as varying degrees of fibrosis (Kleiner et al., 2005). NASH is generally accompanied with other metabolic diseases such as obesity, diabetes mellitus, hyperlipidemia, and hypertension, and has become known as the hepatic manifestation of metabolic syndrome and insulin resistance (Kleiner et al., 2005). NAFL is generally thought of as a benign condition, while NASH is a substantial disease which can culminate into cirrhosis and hepatocellular carcinoma. As of today there are still no long-term treatment options for NASH at the exception of liver transplantation, though there is a considerable amount of research being done in the field to find treatment options. NAFLD is estimated to affect 24% of the world's population, although this is likely an underestimation given that the disease is rarely accompanied by symptoms until it reaches the late stages (Younossi et al., 2016; Younossi et al., 2018). Of the 24% with NAFLD, 59% are estimated to have NASH. Furthermore, NAFLD disproportionately affects certain races, such as people of Hispanic descent, due to the strong genetic component of this disease (Younossi et al., 2016; Younossi et al., 2018). NASH is a complex disease which affects all aspects of the hepatocyte's ability to respond to stress such as the overaccumulation of lipids. For example, in the case of excess lipids, the ER undergoes expansion. Expansion helps to accommodate for the increased physical lipid load and package and shuttle the lipid out of the hepatocyte; however, this expansion and increased load in turn triggers ER stress. ER stress plays a key role in NAFLD pathogenesis, in fact all arms of the UPR have been linked to NAFLD development, progression, and even fibrogenesis during NASH (Yamamoto et al., 2007; Zhang et al., 2011; Oyadomari et al., 2008; Rutkowski et al., 2008; Cazanave et al., 2010; Pfaffenbach et al., 2010; Yamamoto et al., 2010; Cazanave et al., 2011; Li et al., 2011; Malhi et al., 2013; Xiao et al., 2013; Toriguchi et al., 2014; Chen et al., 2016a; Kakazu et al., 2016; Rahman et al., 2016; Shan et al., 2017; Dasgupta et al., 2020; Duwaerts et al., 2021; Wang et al., 2018). In this section we will review the roles ERAD, autophagy, and lysosomal degradation pathways play in NAFLD pathogenesis, with an emphasis on lipid homeostasis.

ERAD During NAFLD

Studies investigating the role of ERAD in NAFLD pathology have primarily focused around how ERAD regulates lipid homeostasis. Some of the earlier reports on ERAD during NAFLD focused on Apolipoprotein B (ApoB) (Fisher et al., 1997; Gusarova et al., 2001; Hrizo et al., 2007; Brodsky and Fisher, 2008). ApoB is the principal apolipoprotein in VLDL (very-low-density lipoprotein) and LDL (low-density lipoprotein) and is responsible for shuttling these lipoproteins through the secretory pathway. ApoB dysfunction is strongly associated with NAFLD development, with mutations in ApoB implicated as a genetic driver of NAFLD. It is important to note that enhanced ApoB degradation leads to increased accumulation of lipids within the liver as proper lipid secretion is impaired. Fisher and colleagues demonstrated that Hsp70, an ERAD chaperone, was essential for ApoB degradation in a HepG2 overexpression system (Fisher et al., 1997). A second ERAD chaperone, HSP90, was also implicated in ApoB degradation, with transfection of HSP90 into a rat hepatoma line, RH-7777 significantly increasing ApoB degradation (Gusarova et al., 2001). Inversely, chemically disrupting the interaction between ApoB and HSP90 with Geldanamycin significantly decreased ApoB degradation. Finally the group demonstrated in HSP90 and HSP70 mutant yeast cells that ApoB was not degraded, tying in with Fisher's earlier work. Hrizo and group demonstrated that HSP110 played an essential role in stabilizing ApoB and decreased its degradation. They showed this in the cell line RH-7777 where they over expressed HSP110, which increased ApoB secretion (Hrizo et al., 2007). More recent work further tied ApoB degradation to NAFLD pathogenesis (Yamamoto et al., 2010). In an *Atf6* knock-out mouse model where ER stress was

TABLE 1 | RNAseq Analyses of patients with NAFLD/NASH.

Dataset	Comparison	ERAD: Upregulated genes	ERAD: Downregulated genes	ER-phagy: Upregulated genes	ER-phagy: Downregulated genes
GSE 17470	NASH vs. Control	UBXN1, DNAJC10	DERL2, UBE2G2, SEL11, RNF103, INSIG1	—	—
GSE 24807	NASH vs. Control	UBXN1, DNAJC10, UBE2K, TMUB2	DERL2, UBE2G2, MAN1B1, DERL3, RNF170, SEL1L, RNF103, INSIG1	—	—
GSE 48452	NASH vs. Obese	FBXO2	MAN1A2, MAN1A1	—	—
	NASH vs. Steatosis	FBXO2, DNAJB14	—	ATL3, RTN3	CDK5RAP3/C53, CALCOCO1
	Steatosis vs. Obese	—	MAN1A2, RNF170	—	SEC62
GSE 89632	NASH vs. Healthy	FBXO2, AMFR, STUB1, TMUB2, RNF170, UBXN6, TRIM25, DERL3, DNAJB12, DNAJB14, TMEM129, DERL2, EDEM2, TRIM21, MAN1A2, RNF185	FAF2, UBE2J1, UBE2G2, MANC1, INSIG1, TRIM13	CCPG1, ATL2	RTN3, RETREG1
	Steatosis vs. Healthy	FBXO2, STUB1, RNF5, TRIM13, UBXN6, TMEM129, TMUB2, RCN3, DNAJB12, RNF170, RNF139	RHBDD1, UBE2G2, MANC1, INSIG	DDRGK1, TEX264	RTN3, RETREG1
	NASH vs. Steatosis	N/A	N/A	N/A	N/A
GSE 33814	NASH vs. Healthy	WFS1, FBXO2, MAN1B1, TMUB2, NPLOC4, MAN2B1, RCN3, SORT1	TRIM13, DNAJB14, RNF170, UBE2K, XBP1, MARCHF6, MAN1C1, INSIG1	ATL3, RTN3, CCPG1, CDK5RAP3/C53	RETREG1, SEC62, CALCOCO2
	NASH vs. Steatosis	FBOX1, RCN3, MAN1B1, TMUB2, MAN2B1,UBXN1, STUB1, DNAJC18, SORT1	ERLIN1, SYVN1, UBXN4, SEL1L, RNF103, HSPA5, MAN1C1, RNF170, UBE2K, RNFT1, DNAJB14, INSIG1	—	ATL2, SEC62, CALCOCO2
	Steatosis vs. Healthy	NPLOC4, MAN1B1, TRIM25, DERL2, MAN2C1, RNF185, RHBDD1, MAN2B1, TMUB2, DNAJB12, STUB1, DNAJB14, UBE2J2	RNF139, JKAMP, UBE2K, MAN1A1, UBXN8, MAN1C1, XBP1, MARCHF6	RTN3, CCPG1, CDK5RAP3/C53	DDRGK1, RETREG1, SEC62, CALCOCO2
GSE 49541	NAFLD with Advanced Fibrosis (Stage 3/4) vs NAFLD with Mild Fibrosis (Stage 1/2)	DNAJC10	MAN1C1	—	—
GSE 159676	NASH vs. Healthy	PRKN, DNAJB14, DERL3	BAG6, UBXN8, MAN1A1, UBXN4m TMEM129, ERLIN1, UBXN6, STUB1, DNAJB11, MAN1C1, EDEM2, OS9, INSIG1, UBE2D1	RTN3, ATL3	TEX264, SEC62, RETREG1, CALCOCO1, CALCOCO2, CDK5RAP3/C53

induced with tunicamycin (1 mg/kg) for one week, an increase in steatosis and liver injury occurred. Yamamoto and colleagues went on to demonstrate that this increase in steatosis was due to increased triglyceride and cholesterol accumulation, decreased β-oxidation, and decreased VLDL secretion due to ApoB destabilization. This group speculated that the observed pathogenesis was due to the role ATF6 plays in the transcription of ERAD associated genes, with ATF6α loss limiting the levels of ERAD machinery, subsequently destabilizing ApoB and decreasing lipid export from hepatocytes (Yamamoto et al., 2007). Other studies demonstrated that ERAD is involved in increased lipogenesis *via* INSIG1 degradation and subsequent SREBP-1c activation, or through reduced TG synthesis by destabilizing DGAT2 (diacylglycerol O-acyltransferase 2) which is responsible for the last step of TG synthesis, thus leading to fatty acid accumulation (Liu et al., 2012; Choi et al., 2014). Together these studies demonstrate that targeting ApoB to increase stabilization through the potential inhibition of ERAD during NAFLD may help alleviate the ER burden and overall cellular stress helping reduce cell death and injury.

A second ERAD-associated mechanism regulating lipogenesis involves the E3 ubiquitin ligase HRD1, however the role of HRD1 in NAFLD pathogenesis remains unclear due to conflicting

reports (Wei et al., 2018; Li et al., 2021). Wei and colleagues published that liver-specific depletion of Hrd1 was beneficial to steatosis and insulin resistance (Wei et al., 2018). Liver-specific Hrd1 knock out mice fed a high fat diet displayed a significant decrease in steatosis, blood glucose levels, and expression of *de novo lipogenesis* genes compared to wild-type mice on the same diet. They proposed that Hrd1 is an important metabolic regulator, whose loss promotes AMPK and AKT hyperactivation, leading to increased lipogenesis. In contrast Li and colleagues demonstrate that in a genetic model of obesity and diabetes (db/db mice) Hrd1 is significantly decreased compared to wild-type mice (Li et al., 2021). They also establish that Hrd1 is essential for ubiquitin degradation of Acyl, which is important in *de novo lipogenesis.* In the natural absence of Hrd1, db/db mice displayed an increase in circulating Acyl which leads to increased lipogenesis. They further demonstrate their findings by overexpressing Hrd1 in db/db mice and showing a fully reversed phenotype. Very recently Yang and group established that the E3 ligase RNF5 was important in HRD1 ubiquitination and degradation (Yang et al., 2021a). Mice with liver specific *Rnf5* depletion developed NASH (increased steatosis, inflammation, and fibrosis) when fed a high fat-high cholesterol diet. Investigating human NAFLD samples, they measured significantly less RNF5 mRNA and protein in NASH than NAFL samples. This new data could point towards HRD1 having a detrimental role in NASH as indicated by Ye et al. While ERAD appeared detrimental to ApoB stabilization, its overall role in NAFLD pathogenesis may not be as clean cut. Given our current understanding of Hrd1 in NAFLD progression, it might be beneficial to increase ERAD rather than limit it as studies studying ApoB indicate. Thus targeting ERAD may not be an easy task in NASH.

Another protein of the ERAD machinery that has been directly linked to NAFLD pathogenesis and hepatocellular carcinoma (HCC) development is the E3 ubiquitin ligase GP78 (Zhang et al., 2015). Zhang and researchers found that 1 year old *Gp78* knock-out mice naturally developed obesity. The obesity was accompanied by steatosis, liver inflammation, fibrosis, and HCC. Indeed, they found that *Gp78* levels in human HCC inversely correlated with grade of HCC. They hypothesized that loss of *Gp78* decreased ERAD which induced chronic ER stress leading to NASH and HCC. Finally when investigating RNA sequencing datasets from studies that investigated NAFL and NASH patients comparing them to healthy controls or obese controls several genes involved in ERAD emerged in most studies searched. For example the following genes were often found upregulated: *Fbxo1*, *Ubxn1*, *Tmub2*, *Man1b1*, *Stub1* while these were found to be downregulated: *Derl2*, *Man1a2*, *Ube2g2*, *and Insig* (**Table 1**) (Baker et al., 2010; Liu et al., 2011; Starmann et al., 2012; Ahrens et al., 2013; Murphy et al., 2013; Arendt et al., 2015). For example *Stub1*/Chip plays an essential role in NASH pathogenesis. When *Chip* was knocked out in mice, they developed significantly more oxidative stress, steatosis, cell death, and fibrosis than their wild-type counterparts in the absence of other stimuli (Kim et al., 2016). As for *Ube2g2*, it is involved in cholesterol synthesis, specifically it degrades the rate limiting enzymes involved in this process (Tan et al., 2019; Miao et al., 2010) Future studies into the pathological roles of these differentially regulated ERAD proteins may reveal novel mechanisms of NAFLD progression and potential therapeutic targets for future studies and therapies.

Autophagy/Macro-ER-Phagy During NAFLD

The role of autophagy in NAFLD progression has been an exciting area of research in the past decade, though the contribution of ER-phagy to NAFLD and NASH development is unclear. One of the first reports of autophagy in the liver during NAFLD was by Singh and colleagues in 2009 (Singh et al., 2009). Since then hundreds of papers have been written on the subject, of which several reviews of note (Flessa et al., 2021; Wu et al., 2018; Ramos et al., 2021). In this seminal paper, Singh et al. demonstrated in both *in vitro* and *in vivo* settings that the inhibition of autophagy led to increased lipid accumulation, reduced β-oxidation, and reduced VLDL secretion in hepatocytes coining the term "macrolipophagy". For example, culturing primary hepatocytes from *Atg5* knock out mice in the presence of oleate led to significant accumulation of triglycerides compared to wild-type hepatocytes. The group went on to demonstrate that autophagy was required for β-oxidation of lipids by adding siRNA for *Atg5* to cultured hepatocytes in the presence of MCD (methionine and choline deficient) media. They also showed microscopically that lipid droplets were associated with autophagic markers such as LC3 and LAMP1. Finally in mice fed high fat diets (HFD), autophagy decreased in the presence of HFD, leading to increased lipid droplet accumulation, which further inhibited autophagy, highlighting a vicious cycle (Singh et al., 2009). Soon after, a report from Liu and colleagues demonstrated the integral role insulin plays in hepatic autophagy (Liu et al., 2009). Autophagy was diminished in mice fed a HFD to induce insulin resistance, but when insulin was inhibited chemically with streptozotocin, autophagy was restored (Liu et al., 2009). Yang et al. further demonstrated the importance of insulin in hepatic autophagy, revealing that *in vivo* loss of *Atg7* increased both ER stress and insulin resistance (Yang et al., 2010). They also showed that all markers of autophagy were dysregulated demonstrated in an ob/ob model, resulting in overall downregulated autophagy.

Numerous other reports employing high fat or methionine-choline deficient dietary models in mice or rats have come to the same conclusions as Singh and colleagues, in the presence of a NAFLD stimulus less autophagy is observed, and consequent lipid accumulation is noted (Koga et al., 2010; Wang et al., 2010; Gonzalez-Rodriguez et al., 2014; Simon et al., 2014). Through these reports the autophagic machinery in NAFLD has been well documented and includes classic autophagic markers such as ATG5, ATG7, BECLIN-1, p62/SQSTM1, LC3-II/I, and S6K1(Singh et al., 2009; Yang et al., 2010; Gonzalez-Rodriguez et al., 2014). Finally Gonzalez-Rodriguez et al. studied liver samples from healthy control, NAFL, and NASH patients and established that patients with NASH had significantly less autophagy than both other groups as seen by their increased p62 protein levels, increased phopspho-mTOR, increased

phospho-S6K1, and decreased mRNA levels of Beclin-1 (Gonzalez-Rodriguez et al., 2014).

While not as robustly studied, macro-ER-phagy may indeed play a crucial role in NAFLD pathogenesis. As ApoB is highly regulated by ERAD, another apolipoprotein is regulated by macro-ER-phagy. ApoC-III is involved in lipid homeostasis through two distinct functions: (Asrani et al., 2019) it inhibits hepatic lipase, and (Maiers and Malhi, 2019) inhibits uptake of triglyceride-rich particles. If ApoC-III is not tightly regulated it can lead to hypertriglyceridemia and an increased risk of cardiovascular disease (Boren et al., 2020). Interestingly, a polymorphism has been found in ApoC-III lipoprotein which predisposes to NAFLD (Petersen et al., 2010). Recently published work has pointed to a role for macro-ER-phagy in the regulation of lipoprotein ApoC-III (Kohno et al., 2019). Specifically the group describes that ApoC-III is degraded in a FAM134B-2-dependent manner. This means macro-ER-phagy plays a key role in regulating lipogenesis in hepatocytes, lending it a potential central role in NAFLD pathogenesis. Changes in macro-ER-phagy during NAFLD progression could further lead to the progression of NAFLD through, for example, the regulation of ApoC-III levels in hepatocytes. A role for ERLAD in NAFLD/NASH pathogenesis is further supported by RNA sequencing data from groups that compared NASH to healthy controls. Numerous ER-phagy receptors were differentially regulated, such as ATL3, SEC62, and RTN3 (**Table 1**) (Baker et al., 2010; Liu et al., 2011; Starmann et al., 2012; Ahrens et al., 2013; Murphy et al., 2013; Arendt et al., 2015). Again, this points towards macro-ER-phagy playing an essential role during NASH and that targeting the machinery to treat NASH is a highly understudied area.

Lysosomal Degradation During NAFLD

The role of lysosomal degradation is less studied in NAFLD pathogenesis, with even less known regarding the role of ERLAD. Despite being understudied, research shows that lysosomal degradation plays a principal role in NAFLD pathogenesis. Key proteins of interest in lysosomal degradation are RABs (Ras-related protein Rab), Sortilin (VSP10), and SIMPLE (small integral membrane protein of lysosome/late endosome) (Strong et al., 2012; Zeigerer et al., 2012; Du et al., 2020; Song et al., 2021; Vos and van de Sluis, 2021). RABs are a large family of GTPase essential in early endosome, late endosomes, and lysosome formation. They are located on the surface of endosomes/lysosomes and are essential in connecting with lipid droplets and their trafficking and degradation (Liu et al., 2007). Such is the case of *Rab5*, which plays a role in the actual number of endosome/lysosomes present in hepatocytes (Zeigerer et al., 2012). In fact Zeigerer et al. found a loss of the endosomal pathway in the absence of Rab5. They also demonstrate that in the absence of *Rab5*, LDL was not able to undergo endocytosis into the primary hepatocytes used for the experiment, leading to an increased amount of circulating LDL.

Strong and colleagues investigated the role of Sortilin in lysosomal degradation of lipids (Strong et al., 2012). Sortilin is a lysosomal sorting protein found in both the Golgi and plasma membranes and is important in trafficking to the lysosome. Interestingly, this group demonstrated that increased Sortilin expression by AAV transfection into wild-type mice lead to decreased ApoB secretion and increased LDL catabolism, both were due to increased lysosomal degradation. A natural occurring mutation in Sortilin in humans significantly reduces coronary heart disease, taken together this pathway may be an interesting target for NAFLD therapeutics. Another group investigated the role SIMPLE played in NAFLD (Song et al., 2021). Song et al. investigated levels of SIMPLE in normal and NASH patient samples and found, as in their NAFLD mouse models, a significant decrease in the protein in diseased individuals. They further demonstrated that in a SIMPLE knock-out mouse model fed either an MCD, HFD, or HFHC diet they developed significant steatosis, insulin resistance, inflammation, and fibrosis. They went on to demonstrate that SIMPLE interacts with EGFR (epidermal growth factor receptor) and regulates its degradation. In the absence of SIMPLE EGFR was found hyperactivated which led to NAFLD.

When taken together it is evident that all three pathways for degradation of ER membrane/cargo are heavily involved in NAFLD pathogenesis, especially in the role they play in lipid homeostasis.

ALCOHOLIC LIVER DISEASE

ALD develops in response to chronic, excessive alcohol consumption, the prevalence of which is rising rapidly (Nagy et al., 2016). Both acute and chronic ethanol consumption lead to ER stress, and in turn, resulting UPR signaling drives ALD pathogenesis (Barak et al., 1996; Werstuck et al., 2001; Ji and Kaplowitz, 2003; Ji et al., 2011; Fernandez et al., 2013; Tsedensodnom et al., 2013; Howarth et al., 2014; Li et al., 2016; Masouminia et al., 2016; French et al., 2017; Song et al., 2020). Furthermore, ethanol-induced hepatocyte death occurs through UPR-upregulation of CHOP (Ji et al., 2005). Liver damage in ALD primarily occurs through ethanol metabolism which releases reactive oxygen species (ROS). The majority of ethanol metabolism occurs through alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) 1 and 2, but in conditions of excess ethanol, cytochrome p450 (CYP) enzymes, particularly CYP2E1, metabolize ethanol and produce ROS. Increased ROS in hepatocytes leads to mitochondrial damage, interference with protein folding, ER stress, and hepatocyte death, which in turn drives ALD progression (Wu and Cederbaum, 1996; Nagy et al., 2016; Doody et al., 2017; Lu and Cederbaum, 2018). Other ethanol-induced drivers of ER stress are protein accumulation and adduct formation, both of which impair hepatocyte function and lead to hepatocyte apoptosis, which may be aggravated by impaired ER quality control (Chen et al., 2016b; Masouminia et al., 2016; French et al., 2017).

Without dismissing other ethanol metabolizing pathways, much of our focus will be on CYP2E1 due to its relationship with ERAD and autophagy (Kwon et al., 2020; Correia et al., 2014). CYP2E1 plays a modest role in ethanol oxidation under normal conditions; however, this role increases as alcohol

TABLE 2 | RNAseq analyses from patients with Alcohol-related liver disease.

Dataset	Comparison	ERAD: Upregulated genes	ERAD: Downregulated genes	ER-phagy: Upregulated genes	ER-phagy: Downregulated genes
GSE 28619	Alcoholic hepatitis vs. Control	TRIM13, DNAJC10, TRIM25, UBE2N, UBXN8, FBXO2, JKAMP, DNAJB11, TRIM21, AMFR, UFD1, DERL2, HSPA8, RHBDD1, DNAJB14, VCP, EDEM3, SEL1L, HSPA5, NPLOC4, ERLEC1, FAF2, UBE2J1, UBE2D1	UBXN6, HERPUD1, MARCHF6, ENF5, UBE2K, MAN1A1, MAN2C1, MAN2A2, MAN1C1, INSIG1	ATL3	RETREG1, ATL2
GSE 143318	Alcoholic hepatitis vs. Control	AMFR, EDEM1, MAN1A1, TRIM25, MAN1A2, RNF5, VCP, DNAJB11, STT3B, NPLOC4, EDEM3	—	DDRGK1	—
GSE 103580	Alcoholic steatosis vs. mild alcoholic hepatitis	SEL1L, TRIM13	TMEM67, DNAJC10	CCPG1	—
	Alcoholic cirrhosis vs. alcoholic steatosis	DNAJC10, EDEM3, MAN1B1, TMEM67, DNAJC18, ERLEC1, RNFT1, UBE2J2, UBE2J1	STUB1, UBXN6, UBE2K	—	CCPG1, SEC62
	Alcoholic cirrhosis vs. mild hepatitis	INSIG1, TRIM13, DNAJC10, RNFT1, UBE2G2, SEL1L, MAN2C1, EDEM3, EDEM1, RHBDD1, DNAJC18, STT3B, ERLEC1, UBE2J1, RCN3	STUB1, DNAJB12, UBXN6, SYVN1	—	—
GSE 10356	Alcoholic cirrhosis vs. normal	MAN1A2, HSPA5, EDEM1	TRIM21	—	—
GSE 24667	Alcoholic cirrhosis vs. normal	MAN2C1, MARCHF6, PRKN, FBXO2, UBE2N, WFS1	TRIM21, TRIM13, SEL1L	—	—

exposure increases. Ethanol increases CYP2E1 production, and in turn, increased CYP2E1 levels or activity drives hepatocyte injury. Conversely, deletion or inhibition of CYP2E1 limits hepatocyte death and alcohol-induced injury (Lu and Cederbaum, 2018; Song et al., 2019). The deleterious effect of increased CYP2E1 on hepatocyte viability suggests that drugs aimed at mechanisms regulating CYP2E1 protein stability could improve the disease phenotype.

ERAD and ALD

CYP2E1 is localized at the ER membrane where it metabolizes ethanol and other xenobiotics. As an ER membrane protein, CYP2E1 undergoes turnover through ERAD under physiological conditions, but this turnover is increased with CYP2E1 damage or inactivation (Kwon et al., 2020; Correia et al., 2014). CYP2E1 metabolism of ethanol and subsequent generation of ROS damages CYP2E1, leading to degradation by ERAD (Kwon et al., 2020; Correia et al., 2014). CYP2E1 is targeted for ERAD through an initial phosphorylation event mediated by PKA and PKC, followed by ubiquitination by E2/E3 complexes (Kwon et al., 2019). These complexes include p97/Ufd1/Npl4-AAA ATPase, UbcH5a/Hsp70/CHIP, and UBC7/gp78/AMFR. Ubiquitination is followed by extraction of CYP2E1 from the ER membrane and subsequent proteasomal degradation. Loss of any of these complexes increases CYP2E1 stability, which in turn worsens drug-induced liver damage in murine models (Kwon et al., 2019; Ballinger et al., 1999; Connell et al., 2001; Jiang et al., 2001; Murata et al., 2001; Morishima et al., 2005; Kim et al., 2010). Analyses of RNAseq from patients with alcoholic hepatitis showed increased expression of numerous ERAD genes, including those involved in CYP2E1 turnover (*AMFR* and *VCP*), though this reversed in patients with alcoholic cirrhosis, who displayed reduced expression of *Stub1*/CHIP (**Table 2**) (Bourd-Boittin et al., 2011; Caillot et al., 2009a; Trepo et al., 2018; Hyun et al., 2020; Affo et al., 2013). Increased expression of ERAD genes that target CYP2E1 for degradation may reflect an adaptive mechanism of hepatocytes to remove excess CYP2E1 induced by alcohol exposure. The shift in ERAD gene expression in cirrhotic patients mirrors what we observed in other cirrhotic patients (**Table 3**), suggesting that regulation of ERAD changes with disease severity (Caillot et al., 2009a; Bourd-Boittin et al., 2011; Affo et al., 2013; Trepo et al., 2018; Hyun et al., 2020). The mechanism behind this shift is unclear but could correspond with a shift away from cells trying to adapt and resolve cellular damage, to other, more pathological signaling pathways. Other key observations from our RNAseq analyses were dysregulated expression of several mannosidases (*MAN1A1*, *MAN1A2*, *MAN2A2*, *MAN1B1*, *MAN2C1*) and ubiquitin ligases (*UBE2N*, *UBXN8*, *UBE2J1*, *UBE2J2*). Increased expression of the ERAD machinery could also be downstream of ER stress and activation of the UPR in response to hepatocyte injury. How the ERAD machinery is regulated in response to acute and chronic alcohol exposure, as well as in early and late stages of alcoholic liver disease, would provide additional insight into the role of this process in disease progression.

In keeping with a protective role of ERAD in response to alcohol, *in vitro* studies have shown that ERAD inhibition sensitizes HepG2 cells to inflammation-induced cell death. In these studies, ERAD inhibition, or deletion of SEL1L increased

TABLE 3 | RNAseq Analyses of Cirrhotic livers or Hepatic Stellate Cells.

Analysis of whole liver

Dataset	Comparison	ERAD: Upregulated genes	ERAD: Downregulated genes	ER-phagy: Upregulated genes	ER-phagy: Downregulated genes
GSE 14323	Cirrhotic vs. Healthy	RCN3, UBE2N, UBE2D1	VCP, MAN2A2, UBE2J1, EDEM2, XBP1, WFS1, UBXN4, NPLOC4, EDEM3, DNAJC10, DERL2, MAN1A2, HSPA5, EDEM1, MAN1A1, AMFR, SEL1L	RETREG1, CALCOCO1	CCPG1, RTN3
GSE 45050	Cirrhotic vs. Healthy	MAN2B1, DERL3, RCN3	RNFT1, RNF139, RNF170, MAN1A2, UBXN8, EDEM1	—	ATL2, CCPG1, RETREG1, SEC62
GSE 11536	Advanced Fibrosis vs. Mild Fibrosis	INSIG1, MAN1A2, MAN2B1, UBE2G2	MAN1B1, BAG6, EDEM1, SEL1L, UBXN8, RNF5, XBP1, MAN2A2, UBE2K, RNF103, MAN1A1, STUB1, TRIM21, UBE2D1	—	CCPG1, SEC62, RTN3, CALCOCO2

Analysis of primary or immortalized HSCs

Dataset	Comparison	ERAD: Upregulated genes	ERAD: Downregulated genes	ER-phagy: Upregulated genes	ER-phagy: Downregulated genes
GSE 68000	Primary hHSCs activated by stiffness	DNAJC10, RCN3, TRIM13, JKAMP, SEL1L, STT3B, ERLIN1, FAF2, EDEM1, ERLEC1, RHBDD1, HERPUD1, MAN1B1, UBE2K, NPLOC4, WFS1, RNF170, DNAJB14, DERL2, INSIG1, RNF185, UBE2N, UBE2D1	FBXO2, UBXN1, MAN2C1, RNF5, UBE2J1, MAN1C1, HSPA8	ATL3, RTN3	—
GSE 122710	LX-2 Cells: TGFβ vs Vehicle	DERL3, ERLEC1, DNAJC10, TRIM25, EDEM3, RNF103, RNF185, BAG6, TMEM129, SEL1L, DNAJC18, UBE2J1, JKAMP, UBXN1, AMFR	MAN1B1, VCP, UBE2N, HSPA8, WFS1, TRIM21, INSIG1, FBXO2	CCPG1, TEX264, RETREG1, ATL2, DDRGK1, CDK5RAP3/C53	—
GSE 151771	LX-2 Cells: TGFβ vs Vehicle	INSIG1, RNFT1, DNAJC18, UBE2K, RNF103, HERPUD1, UBE2D1, JKAMP, UBE2J1, DERL2, DNAJB11, UBE2G2, EDEM1, UBE2J2, STT3B, HSPA5, NPLOC4	FAF2, BAG6, UBXN1, TRIM25, UBXN6, SYVN1, TMEM129, MAN1A1, TMUB2, RNF139, RNF185, MAN1C1	CALCOCO2	ATL3, DDRGK1, TEX264, CDK5RAP3

ROS levels and disrupted mitochondrial morphology and function (Liu et al., 2020). Notably, HepG2 cells lack CYP2E1 expression, prompting the question of whether increased CYP2E1 expression in response to ERAD inhibition would protect these cells from the observed phenotypes. Indeed, E47 cells, HepG2 cells engineered to express CYP2E1, exhibited increased expression of nuclear factor-E2-related factor (Nrf2), a UPR-regulated transcription factor which increases expression of antioxidants, compared to parental HepG2 cells (Gong and Cederbaum, 2006). This may protect hepatocytes from oxidative stress caused by CYP2E1-mediated ethanol metabolism in combination with ERAD-mediated CYP2E1 degradation.

Finally, ERAD regulation of CYP2E1 may play a critical role in ALD progression outside of hepatocytes. The liver vasculature is essential for maintaining liver physiology, with changes in vascular tone or architecture leading to liver disease. Alcoholic liver disease is associated with dysregulation of liver sinusoidal endothelial cells (LSECs), and this may be related to ERAD dysfunction (Sarphie et al., 1997; Cohen and Nagy, 2011; Teschke, 2018). A recent study by Yang et al. showed that CYP2E1 expression increases in LSECs in response to ethanol, which in turn leads to acetylation of HSP90 (Yang et al., 2021b). Hsp90 is a cytoplasmic chaperone involved in protein folding but can also recruit proteins for ERAD (Giodini and Cresswell, 2008;

Donnelly et al., 2013). This study focused on the role of HSP90 acetylation, which disrupts its interaction with eNOS. HSP90 protects eNOS from ERAD, thus promoting NO production. Upon pathogenic HSP90 acetylation, NO production decreases and liver injury worsens (Yang et al., 2021b). Deacetylation of HSP90 also protected mice from alcohol-induced injury in this study. Canonically, HSP90 acetylation disrupts its affinity for binding to client proteins, thus impairing effective folding, and potentially targeting them for ERAD. Based on the study in LSECs, CYP2E1 may directly dysregulate protein folding and targeting of substrates for ERAD by promoting HSP90 acetylation, in turn driving ALD pathogenesis.

In sum, these studies indicate that therapeutics aimed at promoting CYP2E1 turnover could limit ROS production and limit ethanol-induced damage.

ERLAD in ALD

Autophagy and lysosomal degradation are tightly intertwined in ALD pathogenesis, but the contribution of ERLAD to ALD pathogenesis is unknown. Autophagy and lysosomal degradation protect hepatocytes from ethanol-mediated injury. Treatment of mice with rapamycin to promote autophagy limited injury in response to acute ethanol exposure, while overexpression of the transcription factor TFEB, which promotes lysosomal biogenesis, in mouse livers limited damage in a chronic ethanol feeding model (Ding et al., 2010; Chao et al., 2018). In turn, inhibition of autophagy or lysosomal degradation exacerbated ethanol-mediated liver injury. While little is known regarding the contribution of ERLAD to ALD progression, there is evidence that these processes are involved.

One potential role for ERLAD is through degradation of CYP2E1. Alcohol consumption, and subsequent ROS production by CYP2E1, increases autophagic flux (Kim and Kim, 2020; Chen et al., 2021). This increase is associated with removal of organelles such as mitochondria damaged by ROS, as well as in response to ER stress caused by ROS and hepatocyte dysfunction. Studies have also shown that CYP2E1 can undergo autophagic degradation in hepatocytes. This could have a critical impact on ethanol-mediated liver injury, as CYP2E1 and ethanol exposure is linked to reduced autophagy, potentially stabilizing CYP2E1 and further propagating liver damage. Our analysis of RNAseq databases revealed upregulation of ATL3 and DDRGK1 in patients with alcoholic hepatitis, as well as CCPG1 in patients with alcoholic steatosis compared to mild hepatitis (**Table 2**) (Caillot et al., 2009a; Bourd-Boittin et al., 2011; Affo et al., 2013; Trepo et al., 2018; Hyun et al., 2020). Modification of damaged CYP proteins by ubiquitination or UFMylation could target them for ERLAD by p62 or DDRGK1 respectively. p62, known for its role in autophagy, was recently reported to target ER membrane proteins for ERLAD through ubiquitination and may serve such a role in hepatocytes when ERAD is overwhelmed or inefficient at degrading CYP2E1, but this hypothesis requires further testing.

Based on the data presented above, enhanced protein degradation through ERAD or ERLAD could limit ethanol-induced hepatocyte damage and progression of ALD.

ER QUALITY CONTROL PATHWAYS IN FIBROGENESIS

A hallmark of the progression of chronic liver disease is fibrogenesis. Sustained injury to the liver promotes inflammation and activation of hepatic stellate cells (HSCs), the primary fibrogenic cell in the liver. Upon activation, HSCs produce fibrogenic proteins including procollagen isoforms I, III, VI, fibronectin, and numerous other proteins destined for secretion. Increased fibrogenic secretion requires ER expansion, increased expression of chaperone proteins, and increased ER quality control to facilitate degradation of misfolded proteins. Indeed, activated HSCs exhibit increased UPR signaling. Both IRE1α and ATF6α, upstream regulators of ERAD and autophagy, are crucial for hepatic fibrogenesis *in vivo*, though these studies have focused on transcriptional regulation of HSC activation and fibrogenesis downstream of the UPR, and not on ER quality control pathways (Xue et al., 2021; (Hernandez-Gea et al., 2013; Heindryckx et al., 2016; Liu et al., 2019). PERK, also known for a role in autophagy, promotes fibrogenesis (Koo et al., 2016; Zheng et al., 2019; B'Chir et al., 2013; Kang et al., 2017). With both IRE1α and ATF6α pathways elevated in fibrogenic HSCs, it is likely that ERAD and ERLAD also contribute to HSC activation and fibrogenesis.

The connection between ER quality control pathways and fibrogenesis are only beginning to be understood. Analysis of whole liver RNAseq from patients with advanced fibrosis or cirrhosis revealed a general downregulation of ERAD-associated genes and ERLAD-associated genes (**Table 3**) (Caillot et al., 2009b; Mas et al., 2009; Darpolor et al., 2014). As HSCs only make up a small fraction of the liver, we also analyzed RNAseq performed on primary human or immortalized human HSCs (LX-2 cells). These analyses overwhelmingly indicated upregulation of ERAD- and ERLAD-associated genes during HSC activation (Caillot et al., 2009b; Mas et al., 2009; Darpolor et al., 2014). The upregulation of ER-phagy receptors fits with recent studies in osteoblasts from zebrafish where FAM134B facilitates lysosomal degradation of misfolded or overexpressed procollagen Ia1 and 1a2 (Forrester et al., 2019). This role for FAM134B was later confirmed in mouse embryonic fibroblasts (MEFs) for a misfolded procollagen II mutant. Targeting misfolded procollagen I for degradation involved both calnexin and UGGT1 ((Fregno et al., 2021). Increased ERLAD-mediated degradation in activated HSCs could promote fibrogenesis through relieving the ER burden of misfolded procollagen I, thus limiting ER stress and promoting HSC survival. Whether this role is conserved in HSCs, and its potential for targeting *in vivo,* are unknown. Preliminary data from the Maiers lab indicates that ER-phagic flux increased in activated HSCs, and this increase is dependent on ATF6α signaling (unpublished observation). We are currently studying the roles of specific ER-phagy receptors in HSC activation and fibrogenesis, and potential targetability of these proteins to limit liver fibrosis.

THERAPEUTIC TARGETING OF PROTEIN DEGRADATION PATHWAYS

Dysregulated protein degradation is a hallmark of several diseases and has been a focus of therapeutic strategies for decades. Inhibition of proteasomal degradation successfully limits multiple myeloma progression through increasing cellular stress and driving apoptosis of cancer cells. The approach of proteasomal inhibition may be attractive if cell death is the optimal endpoint such as with removal of fibrogenic HSCs. Unfortunately, hepatocyte death is a critical driver of chronic liver disease which limits the use of a general proteasomal inhibitor. The potential for activating or inhibiting a subset of proteasomal or lysosomal degradation, such as ERAD or ERLAD could provide nuanced targeting that allows to reduced stress and injury without activating cell death pathways.

Targeting Protein Degradation in AATD

Alpha-1 antitrypsin deficiency (AATD) induces liver disease by the increased accumulation of misfolded AAT soluble and insoluble aggregates in the ER of hepatocytes. Given this, targeting ER-dependent protein degradation in AATD to alleviate the liver disease component of this illness specifically, seems a good therapeutic strategy.

The role of autophagy and macro-ER-phagy in PI-Z degradation led to numerous groups testing autophagic drugs in order to reduce hepatocyte aggregates and reduce the liver burden of AATD. Drugs that positively regulate autophagy, such as carbamazepine (CBZ), rapamycin, ezetimibe, nor-ursodeoxycholic acid (norUDCA), or glibenclamide, have been tested in AATD models and have demonstrated promising and significant results in decreasing the accumulation of PI-Z (Hidvegi et al., 2010; Kaushal et al., 2010; Yamamura et al., 2014; Tang et al., 2016; Tang et al., 2018; Wang et al., 2019). Hidvegi and colleagues demonstrated that CZB could directly increase autophagy of both insoluble and soluble PI-Z aggregates (Hidvegi et al., 2010). Furthermore mice treated with CZB for 2 weeks at a daily dose of 250 mg/kg had significantly increased autophagy of PI-Z, which directly decreased liver disease and fibrosis. Another group, giving weekly rapamycin for 12 weeks at a dose of 10 mg/kg observed a significant increase in autophagic activity as evident by decreased PI-Z aggregates within the liver (Kaushal et al., 2010). They also found reduced liver fibrosis with rapamycin treatment compared to non-treated mice. A third group tried short-term ezetimibe treatment in human primary hepatocytes, which induced significant autophagy as evidenced by increased LC3-II and decreased p62 and p-S6K (Yamamura et al., 2014). They further demonstrated that PI-Z diminished in an ezetimibe dose-dependent fashion. In recent years bile acids have gained traction as great therapeutics with little side effects, one group tried treating both AATD mice and PI-Z transfected HTOZ cells with norUDCA (Tang et al., 2016; Tang et al., 2018). The *in vitro* treatment resulted in increased autophagy of PI-Z protein aggregates in a dose-dependent manner. PI-Z mice treated with norUDCA at a daily dose of 425 mg/kg for 6 weeks developed significantly less liver disease as evidenced by diminished ALT levels, steatosis, and inflammatory foci. Finally Wang et al. screened PI-Z mutant *C. elegans* for potential therapeutics, discovering that glibenclamide increased autophagy of PI-Z aggregates (Wang et al., 2019). They initially tested the finding in a PI-Z expressing HTOZ cell line. Cells demonstrated an increase in autophagy of both soluble and insoluble PI-Z in a dose-dependent manner (1–100 μM). The group then tested analogs of glibenclamide and discovered they too had significant autophagic potential in a PI-Z mouse model and culminated in decreased fibrosis.

Excitingly, the aforementioned work informed the development of a clinical trial. David Perlmutter's group has undertaken a clinical trial with carbamazepine (CZB) in AATD patients. The results remain unknown as the study just completed, one could easily imagine the positive impact increasing autophagy in AATD could have to reduce liver injury and fibrosis in this patient population. While this is the only clinical trial currently investigating autophagy in AATD, of the 124 registered clinical trials for patients with AATD at the time this review was written, it seems further investigating ERLAD and even ERAD during AATD would be an ideal strategy to combat at least the liver disease portion of the disease, even though it would not help alleviate the lung portion.

Targeting Protein Degradation in NAFLD

There are over a thousand registered clinical trials studying NAFLD. This is unsurprising given the front stage in the therapeutic world this disease has taken in the past decade; however, it is surprising that not one trial appears to directly investigate ER-dependent protein or lipid degradation. Even autophagy, which plays a critical pathogenic role in NAFLD/NASH is not being investigated in patients. When reviewing results in mouse studies using autophagy inducing drugs (Liu et al., 2009; Lin et al., 2013), the studies were extremely promising. In a mouse model of HFD feeding for 12 weeks mice were given CBZ at 25 mg/kg dose or rapamycin at 2 mg/kg dose every other day for the last week of feeding. The treatment significantly reduced lipid droplet accumulation, hepatic and serum triglycerides, and plasma insulin. Though no difference was seen between treated and untreated controls in ALT levels (Lin et al., 2013). However, given the safety profile of CZB in the numerous neurological diseases in which it has been studied one must wonder why this drug has not been tested in the NAFLD patient population, making this truly an unmet need. NAFLD is a complex metabolic disease with multiple facets that can be targeted and given the role ER stress plays in this disease ER-dependent degradation pathways, which would alleviate ER stress, are ideal targets.

Targeting Protein Degradation in ALD

Increasing lysosomal or proteasomal degradation in ALD is a promising strategy that is understudied. Over a decade past, murine studies showed that increased autophagy, achieved through rapamycin treatment, limited the toxicity of acute ethanol exposure (Ding et al., 2010). This was attributed to enhanced degradation of lipid droplets, serving to restore lipid homeostasis in hepatocytes and limit steatosis. Subsequent studies have also suggested that autophagy protects the liver from chronic ethanol exposure (Lin et al., 2013; Lu and

Cederbaum, 2015). Currently, there are no clinical trials targeting protein degradation in patients with ALD, due in part to the unclear regulation of autophagy in response to alcohol. Recent studies aimed to understand whether activation of TFEB could limit alcohol-induced liver injury. While activation of TFEB through administration of Trehalose increased autophagic flux *in vitro*, it failed to limit alcohol-induced liver injury *in vivo* (Chao et al., 2021). Targeting mTOR to increase autophagy has also been proposed as a potential mechanism to activate autophagy in patients ALD (extensively reviewed elsewhere), but its broad impact on autophagy and lysosomal degradation could impact a wide breadth of processes (Kim and Kim, 2020; Flessa et al., 2021; Williams and Ding, 2020; Zhou et al., 2021). Specific activation of protein degradation pathways, such as ERLAD, could provide a more targeted approach for patients with ALD.

Targeting Protein Degradation in Liver Fibrosis

The secretion of procollagen I and other fibrogenic proteins is a widely pursued studied antifibrotic strategy. Several groups have sought to target degradative mechanisms in hepatic and extrahepatic disease through inhibiting the proteosome or autophagy. Bortezomib, a proteosome inhibitor used to treat Multiple Myeloma, reduces liver fibrosis in cholestatic mouse models ($MDR2^{-/-}$ Bile duct ligation), as well as renal and lung fibrosis in other mouse models (Anan et al., 2006a; Jalan-Sakrikar et al., 2019; Zhou et al., 2019; Penke et al., 2021). The mechanisms associated with the fibrosis reduction differ, with focus on EZH2, TGFβ signaling, or limiting fibroblast activation, but the anti-fibrotic results were similar. *In vitro* studies further found that proteosome inhibition leads to HSC apoptosis (Anan et al., 2006b)). These findings highlight the importance of proteasomal degradation in HSCs and fibrogenesis, but no studies have directly studied the impact of ERAD on fibrogenesis. ER stress and IRE1α signaling are elevated in activated HSCs, while disruption of IRE1α or ATF6α signaling in HSCs limits their activation and fibrosis in mice (Xue et al., 2021; Hernandez-Gea et al., 2013; Heindryckx et al., 2016; Liu et al., 2019). As IRE1α is a critical regulator of ERAD, and either proteasome inhibition or IRE1α inhibition limits HSC activation and fibrogenesis, the potential contributions of ERAD to these processes merit further study.

Regulation of autophagy has also been studied to target fibrogenesis. The autophagy inhibitor 3-MA reduced CCl_4-mediated fibrosis through promoting HSC apoptosis through the NF-kB pathway, while inhibition of autophagy using different autophagic inhibitors similarly limited HSC activation and fibrosis ((Wang et al., 2017). Other studies indicate that autophagy is protective in HSCs, with autophagy activation limiting HSC activation by TGFβ, and reducing fibrosis (Hidvegi et al., 2010; Zhu et al., 1999; Bridle et al., 2009; Xie et al., 2018). We will not expand on the dichotomous role of autophagy in HSC activation and fibrogenesis, as it is well discussed in recent reviews (Lucantoni et al., 2021; Sun et al., 2021). The major point that we hope to make is that ERLAD pathways could serve as a unique, targetable form of autophagy that directly impacts procollagen I by tipping the balance between procollagen I degradation and secretion in HSCs. Investigating the role of ERLAD in HSC activation is crucial for understanding 1) the nuances of autophagic regulation in fibrogenesis, 2) how HSCs accommodate the burden of increased procollagen I degradation, and 3) potential strategies for targeting this process.

CONCLUSION

ER Quality Control pathways are important, targetable processes which are understudied in the liver. Review of the literature and analysis of publicly available datasets clearly show that these processes are dysregulated in patients with chronic liver disease; however, their contribution to pathogenesis remains unclear. Advancements in technology such as mass spectrometry (e.g., thermal proteome profiling), drug design, high resolution microscopy, and others will allow for careful and systematic investigation of these pathways in liver physiology, different hepatic cell lineages, and under pathological conditions. These studies should provide crucial insight into an understudied area of liver physiology, and identify targetable mechanisms for limiting liver injury and disease progression.

AUTHOR CONTRIBUTIONS

CD and JM wrote, edited, designed tables for, and approved the final manuscript.

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REVIEW
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Immune and Metabolic Alterations in Liver Fibrosis: A Disruption of Oxygen Homeostasis?

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According to the WHO, "cirrhosis of the liver" was the 11th leading cause of death globally in 2019. Many kinds of liver diseases can develop into liver cirrhosis, and liver fibrosis is the main pathological presentation of different aetiologies, including toxic damage, viral infection, and metabolic and genetic diseases. It is characterized by excessive synthesis and decreased decomposition of extracellular matrix (ECM). Hepatocyte cell death, hepatic stellate cell (HSC) activation, and inflammation are crucial incidences of liver fibrosis. The process of fibrosis is also closely related to metabolic and immune disorders, which are usually induced by the destruction of oxygen homeostasis, including mitochondrial dysfunction, oxidative stress, and hypoxia pathway activation. Mitochondria are important organelles in energy generation and metabolism. Hypoxia-inducible factors (HIFs) are key factors activated when hypoxia occurs. Both are considered essential factors of liver fibrosis. In this review, the authors highlight the impact of oxygen imbalance on metabolism and immunity in liver fibrosis as well as potential novel targets for antifibrotic therapies.

Keywords: liver fibrosis, oxidative stress, hypoxia-inducible factor, immunometabolism, mitochondrial dysfunction

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BACKGROUND

Liver cirrhosis is an irreversible liver fibrosis and was the 11th leading cause of death worldwide from 2000 to 2019 according to the WHO (WHO, 2020). Liver fibrosis is a common pathological pathway for various liver diseases, including viral hepatitis, toxic hepatitis, autoimmune hepatitis, and metabolic and genetic liver diseases (Iredale and Campana, 2017). It is a response to persistent liver injury and is characterized by excessive deposition of extracellular matrix (ECM) (Alegre et al., 2017). In the end stage, most liver fibrosis progresses into liver cirrhosis, with the loss of normal function. Early liver fibrosis is reversible, but once it develops into cirrhosis, it is irreversible and leads to functional failure and many complications, such as portal hypertension. Therefore, the prevention and reversal of fibrosis is the main aim for the treatment of liver diseases (Schuppan et al., 2018).

The death of hepatocytes, activated hepatic stellate cells (HSCs), and inflammation are the main causes of the development of liver fibrosis (Alegre et al., 2017). An imbalance in oxygen supply can lead to injury to organ parenchymal cells to secrete various fibrotic and inflammatory cytokine factors and recruit inflammatory cells to the injured stroma in chronic diseases (Darby and Hewitson, 2016; Liu et al., 2017). Hypoxia is rather common in chronic liver diseases. There are several pathways regulating oxygen homeostasis, and hypoxia-inducible factor-1α (HIF-1α) is one of them. HIF-1α, activated in hypoxia, could regulate the transcription of many genes (Kietzmann,

2019). Increasing evidence shows the role of HIF-1α in the process of liver fibrosis (Strowitzki et al., 2018). On the other hand, it always shows excess oxidative stress, mitochondrial dysfunction, and excessive inflammation in chronic liver diseases. Mitochondria are the center of metabolism. With mitochondrial dysfunction in liver disease, it not only releases mitochondrial danger-associated molecular patterns (DAMPs) but also disrupts oxygen homeostasis. DAMPs are released during the condition of liver injury, activating inflammasomes. These injuries may activate HSCs, myofibroblasts, and other cells to synthesize ECM (Schuppan et al., 2018).

Here, we summarize the molecular mechanism of immune, metabolic, and oxygen homeostasis regulation in liver fibrosis to explore precise therapeutic targets and provide clinical treatment strategies.

THE MOLECULAR MECHANISM OF OXYGEN HOMEOSTASIS REGULATION

Mitochondrial Function

Mitochondria are important organelles in the generation of adenosine triphosphate (ATP) from lipid, glucose, and amino acid metabolism. The Krebs cycle in the mitochondrial matrix and oxidative phosphorylation in the mitochondrial inner membrane are important processes in energy production. Mitochondria also play a role in β-oxidation of fatty acids, reactive oxygen species (ROS) generation, cell apoptosis, autophagy, calcium homeostasis, and other biological processes (Boengler et al., 2017).

HIF Pathway

HIFs are key receptors for detecting cellular oxygen rates, consisting of an oxygen-labile α subunit (HIF1α, HIF2α or HIF3α) and a constitutively expressed β subunit (HIFβ) (Peek, 2020). HIFβ is expressed stably and continuously and does not participate in oxygen detection. HIFα subunits are regulated by oxygen at the posttranslational level. Under normal conditions, HIFα subunits are hydroxylated at specific proline residues by the prolyl hydroxylase domain (PHD) protein (Wong et al., 2013). Then, the von Hippel-Lindau (VHL)/E3 ubiquitin ligase system recognizes the hydroxylated subunits that induce protein degradation and inactivation (Günter et al., 2017). In hypoxic environments, HIFα subunits are not hydroxylated, which mediates protein stabilization. HIFα subunits heterodimerize with HIFβ subunits to form a nuclear heterodimer, which drives target gene transcription by binding to the hypoxia response element (HRE) (Baik and Jain, 2020).

THE DESTRUCTION OF OXYGEN HOMEOSTASIS IN LIVER FIBROSIS

Role of the Mitochondrial Dysfunction in Liver Fibrosis

Mitochondrial Structure and Dynamics and Liver Fibrosis

Mitochondrial dysfunction can be detected in many kinds of liver diseases and is believed to be involved in liver fibrosis (Mansouri et al., 2018). It is characterized by ultrastructural mitochondrial lesions, respiratory chain activity reduction, ATP depletion, excessive ROS levels, and mitochondrial DNA (mtDNA) damage. A recent article reported that mitochondrial dysfunction in peripheral cells along with alterations in metabolites of the urea cycle may act as biomarkers for the progression of fibrosis in nonalcoholic fatty liver disease (NAFLD) (Ajaz et al., 2021). The mitochondrial quality control system is crucial for the maintenance of mitochondria, including mitochondrial biogenesis, fusion and fission, and mitophagy.

Mitochondrial biogenesis is regulated by peroxisome proliferator-activated receptor-gamma (PPAR γ) coactivator-1alpha (PGC-1α) (Wu et al., 1999), which is a kind of transcriptional coactivator that regulates the expression of several transcription factors, such as nuclear respiratory factor (NRF)-1 and NRF-2 (Bhatti et al., 2017; Kiyama et al., 2018), and mitochondrial transcription factor A (TFAM) (Kang et al., 2007). A recent study proved that astaxanthin attenuated hepatocyte damage and mitochondrial dysfunction in NAFLD by upregulating the FGF21/PGC-1α pathway (Wu et al., 2020). PGC-1α, NRF-1, and TFAM were also elevated after melatonin treatment in carbon tetrachloride (CCl4)-treated rats. Melatonin protected against liver fibrosis by upregulation of mitochondrial biogenesis (Kang et al., 2016). Moreover, the antifibrotic effects of pomegranate seed oil (PSO), epoxyeicosatrienoic acid-agonist (EET-A), curcumin, liquiritigenin, and Solanum nigrum (AESN) may be related to the upregulation of PGC-1α (Zhai et al., 2015; Zhang et al., 2015; Tai et al., 2016; Raffaele et al., 2019; Raffaele et al., 2020).

Mitochondrial fusion is regulated by the fusion proteins mitofusin 1 (Mfn1) and Mfn2 and optic atrophy 1 (OPA1), while mitochondrial fission in mammals is regulated by dynamin-related protein 1 (Drp1) (Ni et al., 2015). The role of PGC-1α in mitochondrial dynamics has been reported. In oxidative stress-induced mitochondrial damage, the downregulation of PGC-1α is related to abnormal mitochondrial fission. Hence, PGC-1α overexpression reduced Drp1 protein levels and prevented liver fibrosis (Zhang et al., 2020). Particulate matter ≤2.5 μm (PM2.5) also contributes to mitochondrial dynamics dysfunction by increasing Drp1 and decreasing PGC-1α levels (Wang et al., 2021). The overexpression of HK domain-containing 1 (HKDC1) in the liver induced a defect in mitochondrial function by increasing Drp1 (Pusec et al., 2019). The antifibrotic effect of lipoic acid (LA) may be related to the upregulation of Drp1 (Luo and Shen, 2020).

Impaired Mitophagy and Liver Fibrosis

Mitophagy refers to the removal of dysfunctional mitochondria through fusion with lysosomes (Yoo and Jung, 2018). It can be classified into Pink1–Parkin-mediated mitophagy and Parkin-independent mitophagy. Mitophagy mediated by Drp-1 was activated by PM2.5, leading to HSC activation and fibrosis. Chronic CCl4 exposure impaired mitophagy in the liver, and melatonin may attenuate liver fibrosis by elevating PINK1 and Parkin (Kang et al., 2016). Researchers have shown that mitochondrial depolarization (mtDepo) occurs early in mice

fed a Western diet (high fat, fructose, and cholesterol) and increases mitophagic burden. Together with suppressed mitochondrial biogenesis and mitochondrial depletion, mitochondrial damage promotes the progression of liver fibrosis (Krishnasamy et al., 2019). It is worth mentioning that Parkin-independent mediators, including Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and NIX, can be regulated by HIF-1, thus removing damaged mitochondria and protecting against ROS accumulation.

Impaired mtDNA Homeostasis and Liver Fibrosis

In the case of mitochondrial dysfunction, mitochondrial DAMPs (mtDAMPs) are released to the extracellular space. This can stimulate liver inflammation and promote liver fibrosis. A recent study reported that mtDAMPs released from impaired hepatocyte mitochondria could directly activate HSCs (An et al., 2020). The role of mtDAMPs in liver fibrosis will be discussed in detail later.

Reactive Oxygen Species Generation in Mitochondria

The mitochondrial respiratory chain is considered the main source of ROS production. ROS refer to oxygen free radicals and nonradical oxidants (Zorov et al., 2014). It can be produced in both the mitochondrial matrix and the intermembrane space (Bouchez and Devin, 2019). Under normal homeostasis, mitochondria can remove physiological ROS by antioxidant mechanisms and metabolic adaptations (Lee et al., 2019) and thus maintain a balance between ROS production and removal. The antioxidant system includes superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and other antioxidants (Bhatti et al., 2017).

However, the excessive level of ROS caused by increased ROS production and decreased ROS scavenging may lead to protein, DNA, and lipid damage to mitochondria (Zorov et al., 2014; Sorrentino et al., 2018). Additionally, ROS can activate several pathways. Under hypoxia, with low-intensity ROS, the HIF-1α pathway is activated, leading to metabolic adaptation and subsequently inhibiting ROS production (Finkel, 2012). With moderate intensity of ROS in hypoxia, it can regulate inflammatory reactions, such as NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome, and mitogen-activated protein kinase (MAPK) signalling (Win et al., 2018). With a high intensity of ROS, apoptosis and autophagy may occur, preventing more damage.

Considering that changes in mitochondrial physiological processes participate in the development and progression of liver diseases, mitochondria are believed to be a promising treatment target for liver fibrosis.

Role of the HIF Pathway in Liver Fibrosis

Hypoxia and HIFs have been acknowledged as important drivers of liver fibrosis (Strowitzki et al., 2018). In fact, hypoxia is involved in a variety of liver diseases. Hypoxia levels are elevated in liver cancer and may be involved in destroying the natural immune response and creating an immunosuppressive microenvironment (Yuen and Wong, 2020). In NAFLD, hypoxia may mediate hepatic steatosis and abnormal lipid metabolism (Mesarwi et al., 2019). Hypoxia-mediated abnormal immune and metabolic microenvironments are also key factors in the development of fibrosis and liver cirrhosis.

The interaction of HIF-1α and Rho-associated coiled-coil-forming kinase 1 (ROCK1) promotes cell proliferation and collagen synthesis in rat HSCs under hypoxia (Hu et al., 2018). A recent study also showed that activated HIF-1α or HIF-2α in hepatocytes stimulates upregulation of chemokine ligand 12 (Cxcl12) by converting latent transforming growth factor β (TGF-β) to active TGF-β (Strickland et al., 2020). Cxcl12 is involved in the process of liver fibrosis through chemotaxis and activation of HSCs (Li et al., 2020). These findings indicate that HIF acts as an important regulator of liver fibrosis-targeting HSCs (Tirosh, 2018).

During liver fibrosis, hepatic sinusoidal blood flow disorder and hypoxia lead to the secretion of angiogenic factors by liver intrinsic cells. Pathological angiogenesis destroys the hepatic architecture and aggravates liver fibrosis (Poisson et al., 2017). Recent studies identified that hedgehog signalling promoted prospero homeobox protein 1 (PROX1) expression in liver fibrosis, which inhibited HIF-1α ubiquitination *via* a deubiquitinase called ubiquitin specific peptidase 19 (USP19). This hedgehog signalling-mediated hypoxia response participates in liver sinusoidal endothelial cell (LSEC) angiogenesis and the activation of HSCs (Feng et al., 2019; Yang et al., 2020). It has also been suggested that activated peroxisomal proliferator receptor γ (PPARγ) in HSCs could inhibit the expression of HIF-1α *via* an SMRT-dependent mechanism. The activation of PPARγ improved angiogenesis and vascular remodelling in carbon tetrachloride (CCl4)-induced liver fibrosis in rats. A possible negative feedback loop was raised in which HIF-1α might induce PPARγ expression in response to hypoxia or pathological stress, and then overexpressed PPARγ would inhibit HIF-1α transcription to limit amplification (Zhang et al., 2018).

THE OXYGEN IMBALANCE MEDIATED-IMMUNE AND METABOLIC ALTERATIONS IN LIVER FIBROSIS

Mitochondrial Dysfunction Mediated-Immune Regulation in Liver Fibrosis

Sterile inflammation (SI) is a common response to stress and injury in liver disease (Chen et al., 2018). This is a major process in the development of fibrosis and carcinogenesis (Kubes and Mehal, 2012). In SI, DAMPs, which are usually in the intracellular space, are released to the local microenvironment when infections, metabolic disorders, and other stimuli result in hepatocyte cell death, leading to a wide range of immune responses (Iredale and Campana, 2017). Several DAMPs have been identified, including mtDNA and nuclear DNA, high mobility group box-1 (HMGB-1), ATP and other molecules. HMGB-1 is released mostly by hepatocytes and Kupffer cells (KCs). Recent research confirmed the role of HMGB1 in liver fibrosis. It has been reported that HMGB1 could increase collagen

type I production in HSCs *via* the receptor for advanced glycation end-products (RAGE), leading to liver fibrosis. The underlying mechanism was the pMEK1/2/pERK1/2/pcJun signalling pathway (Ge et al., 2018). Furthermore, HMGB1 also links hepatocyte injury to hepatocellular carcinoma (HCC) (Hernandez et al., 2018).

MtDAMPs, including mtDNA and formyl peptides, are released to the extracellular space in the case of ROS-driven mitochondrial membrane permeability transition (Mihm, 2018). They recognize pattern recognition receptors (PRRs) on target cells to modulate the function of antigen-presenting cells (APCs), eosinophils, mast cells, and neutrophils (Krysko et al., 2011). For example, mtDNA recognizes Toll-like receptor 9 (TLR9) and NLRP3, and N-formyl peptides (NFPs) recognize TLR9 (Kubes and Mehal, 2012). Therefore, mitochondria are key stimuli of the innate immune response in liver diseases.

MtDNA is the major part of mtDAMP and is released into the cytosol when mitochondrial dysfunction and apoptosis occur. In different kinds of liver injury, the effects of mtDAMP on immune response is different. In patients with nonalcoholic steatohepatitis (NASH), mtDNA levels have been reported to increase (Garcia-Martinez et al., 2016). Furthermore, it increased in patients with active NASH (NAS 4–8 versus NAS 0–3, $p = 0.0334$) (An et al., 2020). It can activate several pathways (Zhang et al., 2019). The first is NLRP3 inflammasomes. NLRP3 can directly activate HSCs, triggering liver fibrosis (Inzaugarat et al., 2019). Furthermore, it has been reported that in the mouse NASH model, mtDNA released by KCs bound and activated the NLRP3 inflammasome to induce interleukin (IL)-1β and IL-18, triggering proinflammatory responses and resulting in liver fibrosis (Shimada et al., 2012; Pan et al., 2018; Hu et al., 2019). Likewise, KCs induced by palmitic acid (PA) induced mtDNA and activated the NLRP3 inflammasome (Pan et al., 2018). The second is TLR9. In a mouse model of NASH, the plasma level of mtDNA increased, and it could activate TLR9, leading to a proinflammatory response (Garcia-Martinez et al., 2016). Therefore, the activation of TLR9 by mtDNA is believed to play a role in the transition from steatosis to steatohepatitis (Garcia-Martinez et al., 2016). The use of the TLR7/9 antagonist IRS954 could block the ability of mtDNA, resulting in a decrease in steatosis, ballooning and inflammation, serum transaminases, and inflammatory cytokine transcript levels (Garcia-Martinez et al., 2016). This revealed the potential of TLR9 ligand therapies. In acute liver injury induced by acetaminophen (APAP), mtDNA, which binds to TLR9, can induce neutrophil activation and liver inflammation. The crucial mediator is microRNA-223 (miR-223), which acts as a negative feedback loop to limit neutrophil overactivation and liver injury (He et al., 2017). DNA-sensing pathways could induce type I interferon (IFN I) production in liver NPCs, which is related to hepatocyte necrosis (Araujo et al., 2018). The third is cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING). In a mouse model of NASH, STING deficiency attenuated steatosis, fibrosis, and inflammation in the liver; exposure to a STING agonist led to hepatic steatosis and inflammation in WT mice but not in STING-deficient mice (Yu et al., 2018). STING functions as a mtDNA sensor in KCs and increases the expression of TNF-α and IL-6 through the nuclear factor-κB (NF-κB) signalling pathway in NASH (Yu et al., 2018). The activation of STING in macrophages is also related to liver fibrosis (Luo et al., 2018). Furthermore, other mediators related to mitochondria are formyl peptides. Formyl peptides are released from dysfunctional mitochondria and bind to formyl peptide receptors (FPRs) (Krysko et al., 2011). FPRs are expressed at high levels on neutrophil granulocytes and mononuclear phagocytes (Mihm, 2018). This binding leads to the migration of immune cells to the injured tissue, activating proinflammatory responses (Raoof et al., 2010). The role of formyl peptides has been revealed in systemic inflammatory response syndrome (SIRS) and ischaemia/reperfusion injury (IRI) (Zhang et al., 2010; Hu et al., 2015). However, the direct effect of formyl peptides on liver fibrosis has not been identified.

Moreover, mitochondria are the site of ATP production, and when in disease, high concentrations of ATP may be released extracellularly. ATP binds to P2X7 receptors on neutrophils, inducing NLRP3-ASC-caspase-1 inflammasome and IL-1β secretion (Schroder and Tschopp, 2010; Karmakar et al., 2016).

These studies revealed the crucial role of mtDAMPs in modulating the immune response and liver fibrosis, which are promising biomarkers and treatment targets.

HIF Mediated-Metabolic Regulation in Liver Fibrosis

An increasing number of studies have confirmed that HIF-1 acts as a crucial regulator in metabolic reprogramming in liver fibrosis (Corcoran and O'Neill, 2016). Glucose transporters 1 (GLUT1) and pyruvate kinase isozymes M2 (PKM2) are confirmed to be the target genes of HIF-1 and the key molecules of the Warburg effect (Wan et al., 2019). Wan et al. (2019) reported that HIF-1 upregulated GLUT1 and PKM2 expression in fibrotic liver and exosomes derived from activated HSCs. Interestingly, exosomes from activated HSCs were absorbed by KCs, LSECs, and quiescent HSCs, which enhanced glycolysis of these liver nonparenchymal cells. These findings represent a novel mechanism: upon injury of parenchymal hepatic cells, HIF-1 can regulate nonparenchymal cells (NPCs) to maintain synchronization of metabolic reprogramming.

In mice fed a high-fat diet (HFD), hepatic steatosis leads to liver tissue hypoxia. The HIF1-mediated PTEN/NF-kB-p65 pathway plays a critical role in the development of NAFLD to liver fibrosis (Han et al., 2019). In an apolipoprotein E-deficient (Apoe−/−) mouse model, the circadian locomotor output cycle kaput (CLOCK) protein indirectly regulates HIF1α expression by modulating PHD protein levels. In CLOCK deficiency, HIF1α binds to the Cd36 promoter, promoting CD36 expression and uptake of fatty acids in the liver. This regulatory link among hypoxia, metabolism, and circadian locomotor promotes cirrhosis in NAFLD (Pan et al., 2020). Studies of high cholesterol diet (HCD)-induced liver fibrosis revealed that inducible nitric oxide synthase (iNOS)-mediated enhancement of fibrosis was associated with HIF1α stabilization (Anavi et al., 2015). It has been suggested that cholesterol-mediated activation of HIF-1 is ROS- and nitric oxide (NO)-dependent. Cholesterol load increased mitochondrial dysfunction and NO levels, which promoted HIF-1 stabilization and transcriptional activity (Anavi et al., 2014). Succinate, an intermediate of the tricarboxylic acid

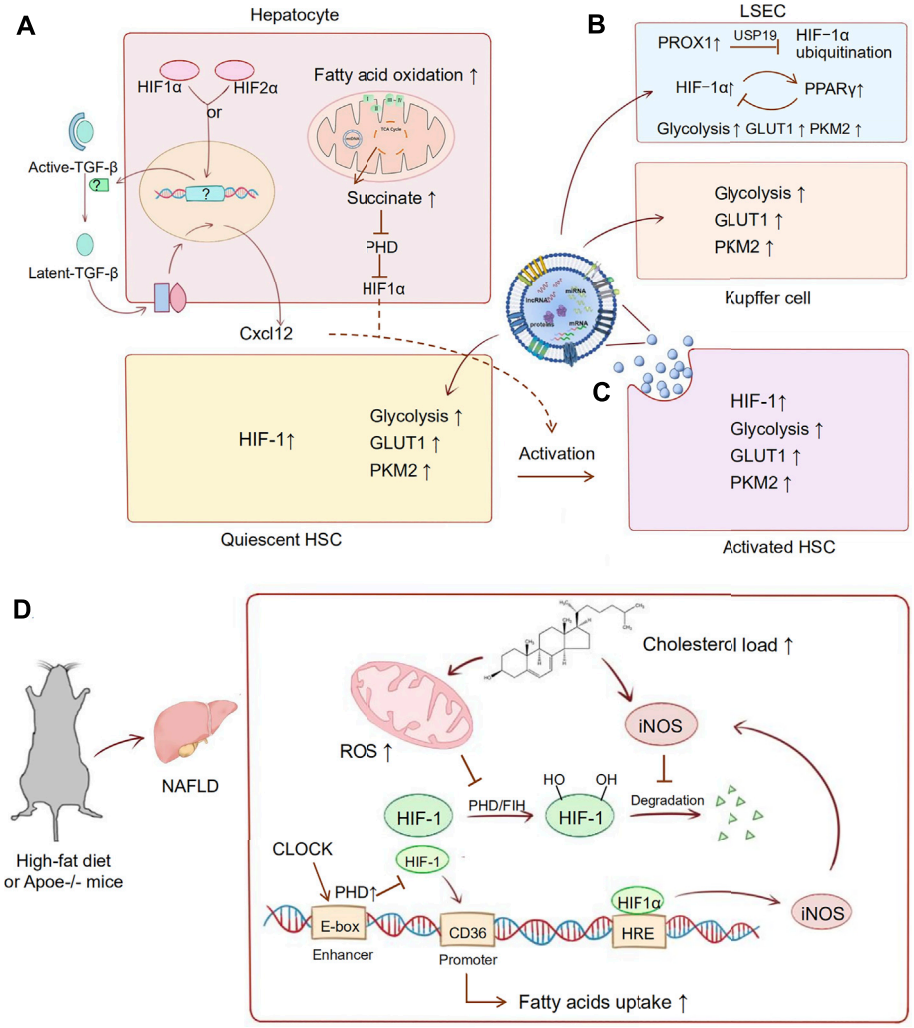

FIGURE 1 | HIF pathway applied in liver fibrosis. During the development of liver fibrosis, oxygen mediated metabolic reprogramming occurs in a variety of cells. Abnormal lipid metabolism and activation of HIF in hepatocytes mediate the fibrotic pathway and the activation of HSC cells. Besides, activated HSCs occur in intracellular metabolic reprogramming, and they also mediate the metabolic transformation of other parenchymal cells. **(A)** In hepatocytes, activated HIF-1α or HIF-2α stimulates upregulation of Cxcl12 through converting latent TGF-β to active TGF-β. Succinate accumulates in hepatocytes due to enhanced fatty acid oxidation, which stabilized and activated HIF-1α through impairing PHDs. Expression of Cxcl12 and HIF-1α is involved in activation of HSCs. **(B)** In LSECs, PROX1 inhibites HIF-1α ubiquitination *via* a deubiquitinase called USP19. A possible loop shows that HIF-1α induces PPARγ expression as a hypoxic response, then overexpressed PPARγ will inhibit HIF-1α transcription hypoxia as a negative feedback. **(C)** In activated HSCs, HIF-1 upregulates the GLUT1 and PKM2 expression in exosomes. Then these exosomes are absorbed by KCs, LSECs, and quiescent HSCs, which enhanced glycolysis of these nonparenchymal cells. **(D)** High-fat diet and Apoe knockout are common modeling methods of NAFLD. HIF-1 is also involved in metabolic disorder in the development of NAFLD to liver fibrosis. In this process, cholesterol load increased mitochondrial dysfunction and iNOS levels, which promoted HIF-1 stabilization and transcriptional activity. Then, the abnormal activation of HIF-1 promoted the production of iNOS and formed a malignant loop for fibrosis. Furthermore, HIF-1 is also involved in the circadian locomotor-related metabolic disorders in NAFLD. In CLOCK deficiency, HIF1α binds to the Cd36 promoter, promoting CD36 expression and uptake of fatty acids in the liver. High fat feeding and AP knockout mice are common modeling methods of NAFLD.

cycle, accumulates in hepatocytes due to enhanced fatty acid oxidation in fibrosis. Accumulated succinate stabilizes and activates HIF-1α by impairing PHDs, which induces inflammation and HSC activation (Cho, 2018; She et al., 2018). We summarized the details of the mechanism above in **Figure 1**.

This evidence indicates that the oxygen balance in liver fibrosis is disrupted, which mediates metabolic disorders and the pathological accumulation of metabolic substances. There is no doubt that HIF-mediated oxygen balance control is a potential target for metabolic liver disease.

HIF Mediated-Immune Regulation in Liver Fibrosis

Although HIF-α is often activated in liver diseases, the roles of HIF-1 mediated-immune regulation in different liver injuries are different. In cholestatic liver disease, nuclear HIF-1α protein was

present in hepatocytes, liver macrophages, and liver fibroblasts of patients with primary biliary cirrhosis and primary sclerosing cholangitis (Copple et al., 2012). A study of cholestatic mice indicated that chronic liver injury activated HIF-1α in macrophages. Activated HIF in macrophages may regulate the expression of platelet-derived growth factor-B (PDGF-B) to promote fibrosis, which induces HSC proliferation, chemotaxis, and collagen production.

In NASH mice, the significant upregulation of HIF-1α in hepatocytes increased proportion of M2 macrophages and promoted liver fibrosis and HCC (Ambade et al., 2016). Furthermore, HIF-1α is not only upregulated in hepatocytes, where it induces steatosis, but also in macrophages of NASH patients (Wang et al., 2019). The role of HIF-1α in macrophages in NASH was explored in the methionine-choline-deficient (MCD) diet-fed mice. Mice with stabilized HIF-1α levels in macrophages showed higher steatosis and liver inflammation. HIF-1α impaired autophagic flux in macrophages and upregulated NF-κB activation and monocyte chemoattractant protein-1 (MCP-1) production, leading to MCD diet-induced NASH (Wang et al., 2019). At the same time, digoxin was reported to be protective in inflammasome activity in macrophages and hepatic oxidative stress response in hepatocytes in NASH (Zhao et al., 2019). The protective effect was related to the downregulation of HIF-1α transactivation (Ouyang et al., 2018).

A recent article has reported the role of HIF-1α in viral hepatitis type B that HIF-1α down-regulated apolipoprotein B mRNA editing enzyme catalytic subunit 3B (APOBEC3B) and thus impaired the anti-HBV effect of A3B (Riedl et al., 2021). However, HIF-1α also induced deoxyribonucleases (DNases), which limited the duplication of hepadnaviruses (Hallez et al., 2019).

In contrast, the role of HIF-1α in chemicals-induced acute liver injury is different from its role in chronic liver diseases. Mochizuki et al. proposed a model in which liver necrotic cells could activate HIF-1α in HSCs through regional hypoxia or other mechanisms yet to be determined. HIF-1α then stimulated recruited macrophages to remove necrotic hepatocytes. In HSC-specific HIF-1α knockout, the levels of M1 macrophage activation markers and the percentage of $Gr1^{hi}$ macrophages in the liver were reduced, which impaired the clearance of necrotic cells and promoted fibrosis (Mochizuki et al., 2014). In APAP-induced liver injury, T-cell-specific Hif-1α gene knockout mice sustained severe liver damage, which was related to the aggravated inflammatory responses by enhancing aberrant innate-like γδ T-cell recruitment and excessive neutrophil infiltration (Suzuki et al., 2018).

It has also been reported that hepatocyte-specific deletion of HIF-2α improved NAFLD-associated fibrosis through downregulated histidine-rich glycoprotein (HRGP). The fraction of inflammatory $Ly6C^{high}$ hepatic macrophages, its production of IL-12, and the expression of M1 cytokines/chemokines were significantly decreased in HIF-$2\alpha^{-/-}$ mice. These findings indicated that HIF-2α/HRGP in parenchymal cells could promote proinflammatory responses of hepatic macrophages (Bartneck et al., 2016; Morello et al., 2018). These studies suggest that although liver injury is usually accompanied by the activation of HIF, different activated cells may have opposite effects on fibrosis. We summarized the oxygen imbalance mediated-immune alterations above in **Figures 2A,C**.

THERAPEUTIC SIGNIFICANCE OF OXYGEN HOMEOSTASIS IN LIVER FIBROSIS

Therapies Targeting Mitochondrial Dysfunction to Alleviate Fibrosis

Since increasing evidence has proven the crucial role of mitochondria in liver fibrosis, several efforts have been made to assess the efficacy of pharmacologic therapies targeting mitochondria.

Attenuated mitochondrial dysfunction, increased mitochondrial fission, decreased HSC migration and activation, and decreased oxidative stress are involved in the protective role of augmenting liver regeneration (ALR) in liver fibrosis (Song et al., 2011; Ai et al., 2018). Ming Song et al. first reported the therapeutic effect of ALR gene therapy (Song et al., 2011). The underlying mechanisms were attenuating mitochondrial dysfunction and oxidative stress and inhibiting the activation of HSCs. The results of Ai et al. (2018) were consistent with the former results. The inhibition of ALR expression aggravated liver fibrosis in mice that were administered CCl4 by promoting mitochondrial fusion and HSC migration. The inhibition of ALR may lead to increased mitochondrial Ca^{2+} influx in HSCs, resulting in HSC migration. ALR transfection inhibited F-actin assembly, retarded HSC migration, and promoted mitochondrial fission (Ai et al., 2018).

Poly (ADP-ribose) polymerase (PARP) activation was found in patients with hepatic cirrhosis, and the inhibition of PARP had antifibrotic effects (Mukhopadhyay et al., 2017). PARP inhibition or genetic deletion of PARP1 was reported to attenuate alcohol-induced hepatic oxidative stress and mitochondrial dysfunction by improving the activity of complexes I and IV (Mukhopadhyay et al., 2017). Xing Lin et al. reported that didymin could alleviate liver injury and fibrosis induced by CCl4 by inhibiting HSC proliferation and inducing apoptosis (Lin et al., 2016).

HSC apoptosis was partly mediated by MPTP opening. Didymin treatment led to cytochrome c release into the cytosol and decreased Bcl-2 expression, resulting in HSC apoptosis (Lin et al., 2016). Similarly, the curative effect of dihydroartemisinin (DHA) on liver fibrosis was also partly mediated by HSC apoptosis by releasing cytochrome c and activating the caspase pathway (Chen et al., 2016a).

Oxidative stress is a main stimulative factor of liver fibrosis, and it is a promising target. Melatonin may improve hepatic mitochondrial functions and thus reduce oxidative stress in some diseases (Coto-Montes et al., 2012; Jimenéz-Aranda et al., 2014; Agil et al., 2015). In CCl4-induced liver fibrosis rats, melatonin protected against liver fibrosis by attenuating mitochondrial dysfunction, which was manifested by improved mitophagy and mitochondrial biogenesis (Kang et al., 2016). Melatonin also attenuated lipid-mediated mitochondrial dysfunction and ROS generation in hepatocytes and improved mitochondrial respiratory functions, leading to decreased oxidative stress and inflammation and thus inhibition of HSC activation (Das et al., 2017). Another mitochondria-targeted antioxidant, mitoquinone,

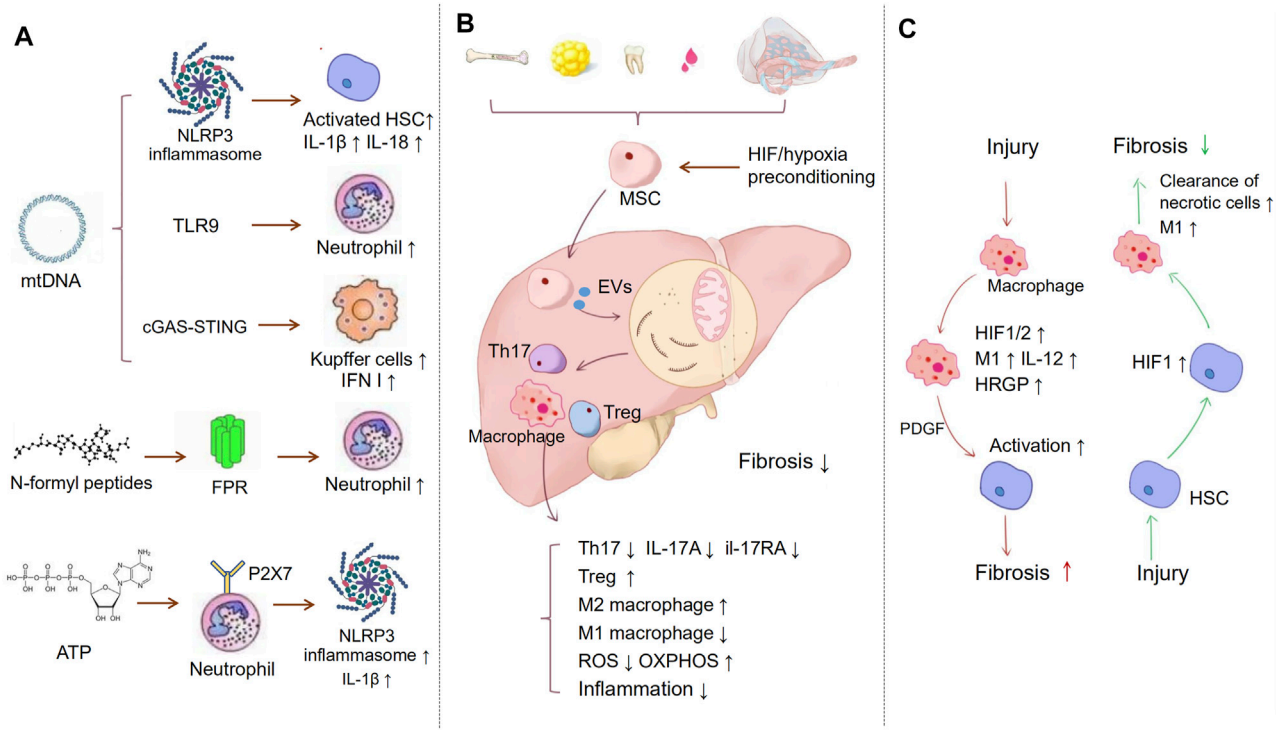

FIGURE 2 | Immune regulation in liver fibrosis. **(A)** Inflammation is a major process in the development of fibrosis. MtDAMPs, including mtDNA, N-formyl peptides, and ATP, are released to extracellular space in the case of ROS-driven mitochondrial membrane permeability transition. MtDNA can activate NLRP3 inflammasomes, TLR9, and cGAS-STING, which separately induce HSCs, neutrophil, and KCs activation. NFPs are released from dysfunctional mitochondrial, binding to FPRs, which leads to the migration of neutrophil cells to the injury tissue. Released ATP binds to P2X7 receptors on neutrophils, inducing NLRP3-ASC-caspase-1 inflammasome and IL-1β secretion. **(B)** MSCs act as a bridge to prevent inflammation and oxidative stress *via* extracellular vesicles, etc. MSCs suppress the proliferation of Th17 cells and decrease the expression of IL-17A and il-17RA. Meanwhile, MSCs promote the activation of M2 macrophages and inhibit M1 macrophages activation, hypoxia-preconditioning, and HIF-1 overexpression can improve MSC therapeutic. **(C)** HIF may play different roles in different cells. The chronic liver injury activates HIF-1α in macrophages, which may regulate the expression of PDGF-B to induce HSCs activation and fibrosis. However, liver necrotic cells can also activate HIF-1α in HSCs. HIF-1α then stimulated recruited macrophages to remove necrotic hepatocytes and alleviate fibrosis.

could attenuate liver fibrosis by reducing hepatic oxidative stress, preventing apoptosis, and promoting the removal of dysfunctional mitochondria (Turkseven et al., 2020).

It has been reported that adiponectin and its receptors enhanced fatty acid oxidation and glucose uptake and prevented the activation of HSCs induced by CCl4, thus alleviating NASH and fibrosis in mouse models (Xu et al., 2020).

Mitophagy is a selective form of autophagy that eliminates dysfunctional mitochondria (Williams et al., 2015a). It protects the liver from both acute and chronic ethanol consumption (Ma et al., 2020). Targeting mitophagy may protect the liver from acetaminophen and alcohol injury (Williams et al., 2015b). Interestingly, chronic deletion (KO) of Parkin alleviated APAP-induced liver injury, but acute knockdown of Parkin exacerbated injury (Williams et al., 2015a). This result suggested other protective pathways in the liver.

Therapies Targeting the HIF Pathway to Alleviate Fibrosis

Since hypoxia and HIFs are considered to be important drivers of liver fibrosis, targeting HIF may be an effective treatment for fibrosis (Strowitzki et al., 2018). In mice fed with HFD, curcumin can inhibit succinate-induced HSC activation by blocking the HIF-1α signalling pathway in mouse primary HSCs (She et al., 2018). In acute liver injury Tamoxifen, an agonist of the G protein-coupled oestrogen receptor (GPER), has been confirmed to inhibit the HIF1α pathway and prevent HSC activation by a mechanical mechanism (Cortes et al., 2019). In a rat model of CCl4-induced liver fibrosis, ligustrazine alleviated hepatic injury, angiogenesis, and vascular remodelling by decreasing the level of HIF-1α (Zhang et al., 2018). The combination of celecoxib and octreotide decreased thioacetamide-induced liver fibrosis in rats by inhibiting the phosphorylation of the extracellular signal-regulated kinase (p-ERK)/HIF-1α/vascular endothelial growth factor (VEGF) pathway (Gao et al., 2016). MicroRNA-122 can protect the liver from ethanol-induced injury and fibrosis by inhibiting HIF-1α expression (Satishchandran et al., 2018). In cholestatic liver fibrosis, it has also been reported that EW-7197, a TGF-β Type I receptor kinase inhibitor, can inhibit HIF1α-induced epithelial mesenchymal transition to alleviate cholestatic liver fibrosis (Kim et al., 2016). In NASH, isochlorogenic acid B was reported to have anti-fibrosis effects by inhibiting HSC activation,

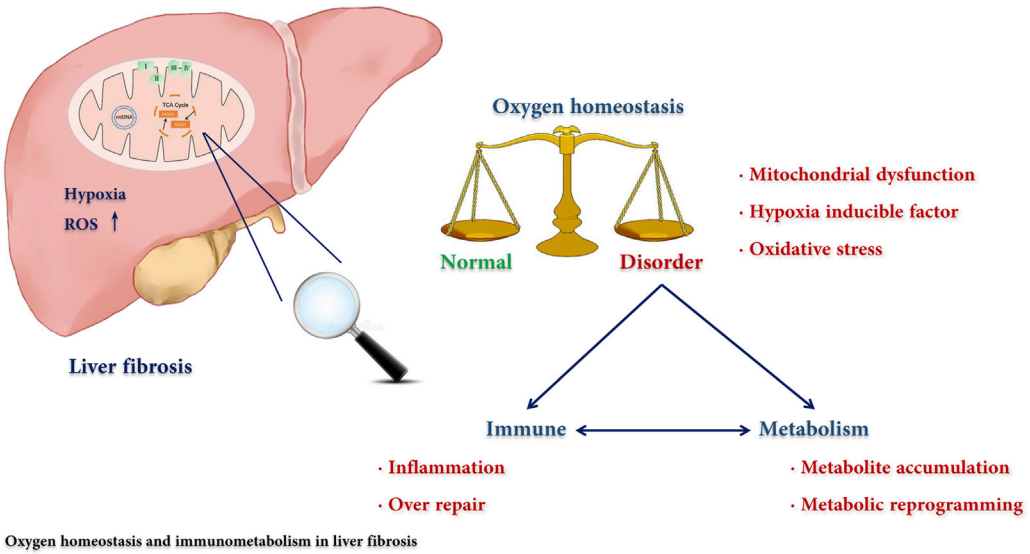

FIGURE 3 | The regulatory mechanism for immunometabolism and oxygen homeostasis in liver fibrosis. The disorders of immunity and metabolism are cross-linked in liver fibrosis and play a central role in the pathogenesis. We reviewed the effect of the destruction of oxygen homeostasis on liver fibrosis and described how oxygen participated in this process through affecting the immune-metabolism axis.

attenuating oxidative stress via Nrf2, and suppressing multiple profibrogenic factors through miR-122/HIF-1α signalling pathway (Liu et al., 2019). The protective role of digoxin in steatohepatitis was related to the inhibition of PKM2/HIF-1α signalling pathway (Ouyang et al., 2018; Zhao et al., 2019).

MSCs Act as a Bridge to Link Immunometabolism, Oxygen Homeostasis, and Fibrosis

Mesenchymal stem cells (MSCs) are pluripotent stem cells that can be induced to differentiate into several tissue cells (Chen et al., 2016b). MSC sources are diverse, such as in bone marrow, adipose tissue, placenta, amniotic tissue, cord, lung, liver, and skin (Zhuang et al., 2019). Existing studies have shown that MSC therapy is prominently effective in hepatic fibrotic diseases indirectly by regulating the immune metabolism microenvironment (El Agha et al., 2017). The secretion of IL-17A from Th17 cells can promote fibrosis by activating fibroblasts (Huang et al., 2019; Hu et al., 2020). BM-MSCs inhibited liver fibrosis by decreasing the expression of IL-17A and IL-17RA and the serum levels of IL-17 in the liver (Farouk et al., 2018). Milosavljevic et al. also confirmed that MSC-conditioned medium (MSC-CM) promoted the expansion of $CD4^{+}$-$FoxP3^{+}$-$IL\text{-}10^{+}$-T regulatory cells and suppressed the proliferation of Th17 cells, which attenuated liver fibrosis (Dong et al., 2020).

Furthermore, BM-MSC transplantation promoted the activation of M2 macrophages expressing matrix metalloproteinase 13 (MMP13) and inhibited M1 macrophage activation. Meanwhile, MSCs reduced the expression of proinflammatory cytokines and increased the expression of anti-inflammatory cytokines (van der Helm et al., 2018; Luo et al., 2019; Yu et al., 2019). Increasing mitophagy and reducing mitochondrial ROS to restrict the inflammatory activation of macrophages may be critical mechanisms by which MSCs inhibit inflammation (Li et al., 2018). In response to oxidative stress, MSCs can transport depolarized mitochondria to macrophages through extracellular vesicles (Phinney et al., 2015). Mitochondrial transfer also promotes an anti-inflammatory macrophage phenotype by enhancing oxidative phosphorylation (Morrison et al., 2017). Existing studies also confirmed that hypoxia preconditioning and HIF-1 overexpression significantly improved MSC therapy (Martinez et al., 2017). MSCs cultured under hypoxic conditions presented an enhanced therapeutic effect on liver cirrhosis, which promoted macrophage polarity to an anti-inflammatory phenotype via prostaglandin E2 (PGE2) expression (Kojima et al., 2019). In summary, MSC treatment is emerging as a connecting bridge to drive immune and metabolic regulation and oxygen balance in the fibrotic microenvironment. We summarized the details of mechanism above in **Figure 2B**.

CONCLUSION AND FUTURE PERSPECTIVES

Mitochondrial dysfunction, hypoxia, inflammation, and metabolic reprogramming are widespread in fibrotic diseases. Here, we have reviewed the regulatory mechanism for immunometabolism and oxygen homeostasis in liver fibrosis (**Figure 3**) as well as potential novel targets for antifibrotic therapies. A special metabolic immune microenvironment mediated by oxygen is described, which deeply affects the balance of tissue damage and repair. The process of fibrosis is closely related to metabolic and immune disorders, which are usually induced by the destruction of oxygen homeostasis, including mitochondrial dysfunction, oxidative stress, and hypoxia signalling pathway activation. On the one

hand, destruction of oxygen homeostasis promotes oxidative stress and releases inflammatory mediators, forming a loop with an inflammatory response and cell damage. On the other hand, cell metabolic reprogramming affects the activation of immune cells and fibroblasts, epithelial mesenchymal transformation, and angiogenesis and further promotes the development of fibrosis. Furthermore, we noticed that hypoxia-induced metabolic reprogramming of immune cells and other fibrosis-related cells is an emerging research direction, but there is still a gap to be filled in the liver fibrosis field. In summary, as immunometabolism and oxygen homeostasis are relatively new research directions, the mechanism, function, and potential clinical application in liver fibrosis need and deserve further investigation.

AUTHOR CONTRIBUTIONS

XL and QyZ: Writing the original draft. ZW: Collecting data. MZ: Conceptualization, Funding acquisition, Validation. QaZ: Conceptualization, Validation.

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GLOSSARY

ECM Extracellular matrix
HSCs hepatic stellate cells
DAMPs danger-associated molecular patterns
HIF-1α hypoxia inducible factor-1α
ATP adenosine-triphosphate
ROS reactive oxygen species
mtDNA mitochondrial DNA
NAFLD non-alcoholic fatty liver disease
PPAR γ peroxisome proliferator -activated receptor -gamma
PGC -1α peroxisome proliferator -activated receptor -gamma coactivator -1alpha
NRF nuclear respiratory factor
TFAM mitochondrial transcription factor A
CCl4 carbon tetrachloride
PSO pomegranate seed oil
EET-A epoxyeicosatrienoic acid-agonist
Mfn1 fusion proteins mitofusin 1
OPA1 optic atrophy 1
Drp1 dynamin-related protein 1
PM2.5 Particulate matter ≤ 2.5 μm
HKDC1 HK domain-containing 1
LA lipoic acid
mtDepo mitochondrial depolarization
BNIP3 Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3
mtDAMPs mitochondrial DAMPs
SOD superoxide dismutase
CAT catalase
GSH glutathione
NLRP3 NACHT, LRR and PYD domains-containing protein 3
MAPK mitogen-activated protein kinase
PHD prolyl hydroxylase domain
VHL von Hippel-Lindau
HRE hypoxia response element
ROCK1 Rho-associated coiled-coil-forming kinase 1
Cxcl12 chemokine ligand 12
TGF-β transforming growth factor β
PROX1 prospero homeobox protein 1
USP19 ubiquitin specific peptidase 19
LSECs liver sinusoidal endothelial cells
PPARγ peroxisomal proliferator receptor γ
GLUT1 glucose transporters 1
PKM2 pyruvate kinase isozymes M2
NPCs non-parenchymal cells
HFD high-fat diet
Apoe-/- apolipoprotein E-deficient
CLOCK circadian locomotor output cycles kaput
HCD high cholesterol diet
iNOS inducible nitric oxide synthase
NO nitric oxide
SI sterile inflammation
HMGB-1 high mobility group box-1
KC Kupffer cell
RAGE receptor for advanced glycation end-products
HCC hepatocellular carcinoma
PRRs pattern recognition receptors
APCs antigen-presenting cells
TLR9 Toll-like receptor 9
NFPs N-formyl peptides
NASH nonalcoholic steatohepatitis
IL interleukin
PA palmitic acid
APAP acetaminophen
miR-223 microRNA-223
cGAS cyclic GMP-AMP synthase
STING stimulator of interferon genes
NF-κB nuclear factor-κB
IFN I type I interferon
FPRs formyl peptides receptors
SIRS systemic inflammatory response syndrome
IRI ischemia / reperfusion injury
PDGF-B platelet-derived growth factor-B
MCD methionine-choline-deficient
MCP-1 monocyte chemoattractant protein-1
APOBEC3B apolipoprotein B mRNA editing enzyme catalytic subunit 3B
DNases deoxyribonucleases
HRGP histidine-rich glycoprotein
ALR augmenter of liver regeneration
DHA dihydroartemisinin
GPER G protein-coupled estrogen receptor
p-ERK phosphorylation of extracellular signal-regulated kinase
VEGF vascular endothelial growth factor
MSCs mesenchymal stem cells
MSC-CM MSC-conditioned medium
MMP13 matrix metalloproteinase 13
PGE2 prostaglandin E2

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