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Community series in hepatic immune response underlying liver cirrhosis and portal hypertension, volume II

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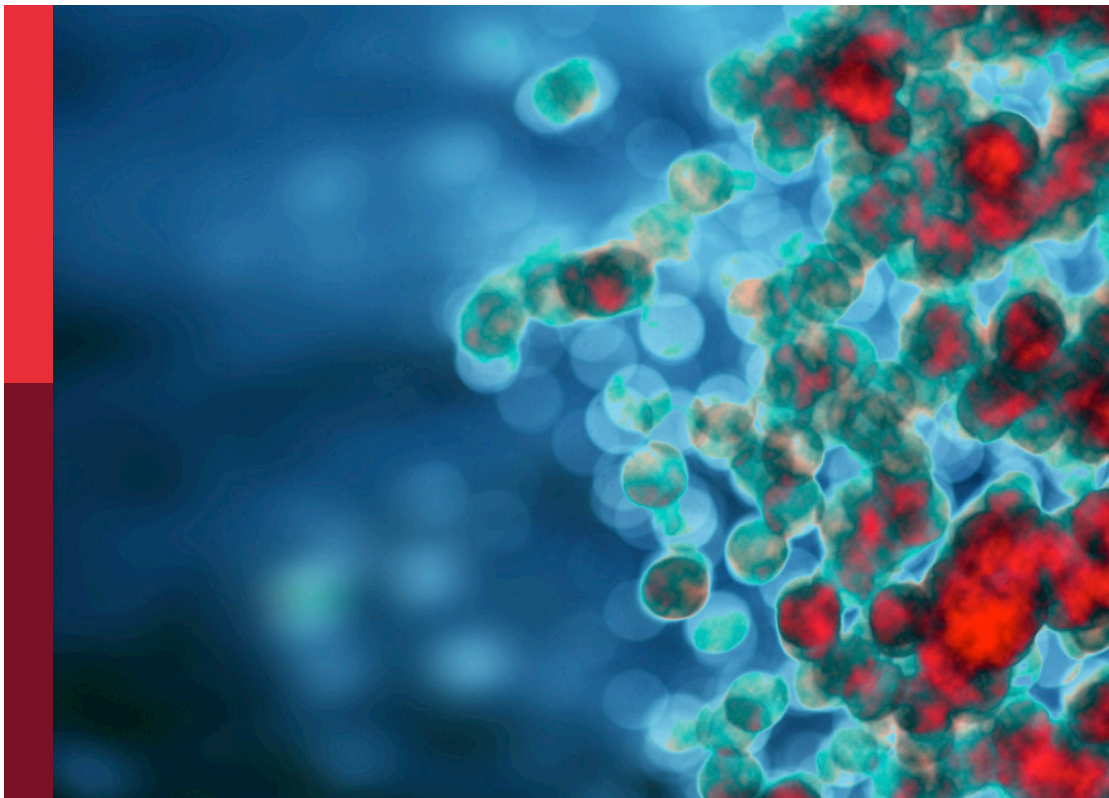

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Community series in hepatic immune response underlying liver cirrhosis and portal hypertension, volume II

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Editorial: Community series in hepatic immune response underlying liver cirrhosis and portal hypertension, volume II

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Editorial on the Research Topic

Community series in hepatic immune response underlying liver cirrhosis and portal hypertension, volume II

Introduction

The immune landscape of a healthy liver plays an important role in maintaining tissue homeostasis. However, when subjected to injury, such as metabolic disorders, alcohol consumption, hepatitis viruses infection, or autoimmune diseases, the hepatic immunologic equilibrium is impaired, leading to tissue inflammation and fibrosis and culminating with cirrhosis and liver cancer (Yi et al.; Zhang et al.) (1). Although the immune response during liver disease has received significant attention, the detailed mechanisms by which residual and infiltrating immune cells interact with the liver parenchyma and contribute to liver disease initiation and progression remain to be further explored.

This Research Topic includes 13 articles and reviews that focus on the role of immune response in different types of liver injury and summarize the current knowledge on liver immunology and pathophysiology, as well as emerging therapeutic targets.

Liver diseases and immune response

The immune response is a critical determinant in almost all kinds of liver diseases. Different types of immune cells actively participate in liver disease initiation and progression through various signaling pathways (Yi et al.; Zhang et al.) (2, 3). Several studies in this Research Topic have dissected distinct interactions between immune cells

and the liver to facilitate the understanding of the underlying mechanisms in different liver pathological settings.

Metabolic dysfunction-associated steatohepatitis

Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterized by hepatocyte steatosis, metabolic disorders, and dysregulated hepatic immune microenvironment (Zhang et al.). The growing burden of MASLD is partly attributed to the increasing prevalence of obesity and diabetes (4, 5). Sphingosine 1-phosphate (S1P) is a bioactive lipid released by stressed hepatocytes. The S1P receptor is expressed in a wide range of immune cells and, by binding S1P, can induce immune cell infiltration into the liver and contribute to liver inflammation and MASH progression (6, 7). The inhibitor of $S1P_1$, $S1P_4$, and $S1P_5$, etrasimod, showed a better effect than the single $S1P_1$ inhibitor in reducing the proportion of pro-inflammatory infiltrating immune cells and inducing the expression of anti-inflammatory markers on macrophages (Liao et al.). Liver injury and inflammation in the MASH mouse model are ameliorated after etrasimod treatment (Liao et al.). These results suggest a potential therapeutic opportunity utilizing S1P inhibitors in patients.

Chronic hepatitis B

Hepatitis B virus (HBV) infection is often chronic and difficult to eradicate because HBV can escape immune surveillance partly by impairing T-cell cytotoxic activity and cytokine production (8). $CD8^+$ T cells from chronic hepatitis B patients or $CD8^+$ T cells cocultured with hepatocytes infected with HBV have elevated expression of CD244, a regulator of immune functions (Xie et al.). Upregulation of lnc-AIFM2-1 and downregulation of miR-330-3p, two non-coding RNAs, induce the expression of CD244 on $CD8^+$ T cells, contributing to the exhaustion of $CD8^+$ T cells and subsequent HBV immune escape (Xie et al.). Non-coding RNAs have been indicated as potential contributors to liver diseases (9, 10). These findings further highlight the role of non-coding RNAs in the pathogenesis of chronic hepatitis B and propose novel therapeutic approaches by targeting HBV immune escape mechanisms.

Autoimmune liver diseases

Autoimmune liver diseases comprise autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), and primary biliary cirrhosis (PBC), which are highly associated with aberrant hepatic immune responses. In AIH, pro-inflammatory tetraspanin 1^+ B cells are enriched in the liver and correlate with the severity of AIH. In addition, the CXCR3-CXCL10 pathway might be responsible for the recruitment of this B cell subgroup to the liver (Ou et al.). PBC and PSC are two major cholangiopathies where cholangiocyte functions are impaired. However, recent studies have unveiled a more active role of cholangiocytes in the pathogenesis of liver diseases. Cholangiocytes can dramatically switch their phenotype and secretory spectrum upon injury (Cai et al.). The soluble factors secreted by activated cholangiocytes, namely cholangiokines, have multifaceted effects on the liver, including the pro-regenerative, pro-inflammatory, pro-fibrotic, and pro-tumorigenic effects (11) (Cai et al.). The immune dysregulation affecting cholangiocytes is a key mechanism in PBC (Yang et al.). Nevertheless, the potential treatment options for PBC targeting the aberrant immune response are limited (12). Recently, encouraging results have been shown by targeting immune cells (such as the anti-CD20 monoclonal antibody rituximab targeting B cells) or chemokines (such as the dual CCR2/CCR5 inhibitor cenicriviroc) in patients with PBC or 2OA-BSA-induced PBC mouse models (Yang et al.) (13, 14). More clinical investigations are needed to evaluate the effect of novel therapies on autoimmune liver diseases.

Decompensated cirrhosis

Decompensated liver cirrhosis is the advanced stage of liver cirrhosis with impaired hepatic and systemic immune responses, including immunosuppression (15). In the relatively early stage of liver cirrhosis, the replication of torque teno virus, a marker of immunosuppression, is already observed in patients (Rueschenbaum et al.). Moreover, a drop in the number of lymphocytes and a decline in T cell functions are found in patients with decompensated cirrhosis, which predict the development of acute-on-chronic liver failure (ACLF) (Rueschenbaum et al.). After ACLF is developed, while the abovementioned immunophenotype changes remain, additional changes, including neutrophilia with characterized neutrophil phenotype and increased macrophage M0-like monocytes, appear and further contribute to immunosuppression during ACLF (Weiss et al.). In decompensated cirrhotic patients with sepsis, myeloid-derived suppressor cells expand and exacerbate immunosuppression by boosting FOXP3+ T regulator cells and downregulating $CD4^+$ T cell proliferation, which can be rescued by granulocyte-macrophage colony-stimulating factor (Sehgal et al.). Further studies are needed to understand the mechanism of immunosuppression during decompensated cirrhosis and explore novel therapeutic targets to prevent the progression toward ACLF.

Emerging therapeutic approaches and targets

As the study of the immune response in liver disease has received increasing interest, potential novel anti-fibrotic or anti-cirrhotic therapies that target the hepatic immune microenvironment are being developed (Zhang et al.) (16). With the advance of regenerative medicine, mesenchymal stem cell (MSC) therapy has emerged as a promising treatment option for liver cirrhosis. MSCs are pluripotent stem cells capable of differentiating and replenishing the liver parenchyma (17). MSCs

can also inhibit hepatic stellate cell activation and accelerate the degradation of extracellular matrix (Liu et al.; Yi et al.). Furthermore, the most appealing advantage of MSCs is that they can modulate the immune cell function through direct cell contact and paracrine signaling (including secretion of extracellular vesicles) (18, 19). MSCs can block the infiltration of pro-inflammatory immune cells while recruiting anti-inflammatory cells by secreting a wide spectrum of cytokines (Liu et al.; Yi et al.) (20, 21). Clinical trials have demonstrated promising outcomes of MSC therapy in patients with liver cirrhosis, showing benefits on the long-term survival rate and liver function (22, 23).

The gut-liver axis is another important area of investigation in the field of liver diseases. Gut-derived factors such as pathogen-associated molecular patterns, bile acids, and other metabolites can influence the composition of the liver immune microenvironment and contribute to liver disease progression (24, 25). Liver cirrhosis is often accompanied by gut microbiota dysbiosis, leading to the release of microbiota-specific factors. These factors can be sensed by Toll-like receptors (TLRs), a conserved family of pattern recognition receptors, triggering hepatic immune responses and influencing the progression of liver cirrhosis (Fan et al.). Targeting altered intestinal flora or TLRs, such as fecal microbial transplantation or inhibitors of TLR signaling, has been proposed as a potential treatment option for liver cirrhosis (Fan et al.) (26, 27). Some naturally occurring metabolites, such as neuropeptide galanin, also show a beneficial effect by alleviating liver inflammation and fibrosis in mice through modulating macrophage phenotype and function (He et al.). Further investigations are needed to identify the effect of these potential treatments in humans.

Conclusion

This Research Topic highlights the role of the hepatic immune response in the progression of liver diseases. Emerging therapeutic targets and approaches for liver cirrhosis have been reviewed and discussed. Nevertheless, given the substantial heterogeneity within the cirrhotic niche (28), further research is imperative to enhance our understanding of the pathological mechanisms and discover effective treatment approaches for liver cirrhosis.

Author contributions

TL: Writing – original draft, Writing – review & editing. SL: Writing – review & editing, Writing – original draft. HY: Writing – original draft, Writing – review & editing. EK: Writing – review & editing, Conceptualization, Funding acquisition, Supervision. JG: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Dysregulated Adaptive Immunity Is an Early Event in Liver Cirrhosis Preceding Acute-on-Chronic Liver Failure

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Introduction: Acute-on-chronic liver failure (ACLF) is characterized by high levels of systemic inflammation and parallel suppression of innate immunity, whereas little is known about adaptive immune immunity in ACLF. We therefore aimed to characterize the development of the adaptive immune system during the progression of liver cirrhosis to ACLF. Patients with compensated/stable decompensated liver cirrhosis, acute decompensation of liver cirrhosis, or ACLF were recruited from a prospective cohort study. Comprehensive immunophenotyping was performed using high dimensional flow cytometry. Replication of *Torque teno* (TT) virus was quantified as a marker of immunosuppression. High frequencies of detectable TT virus were observed already in patients with compensated/stable decompensated liver cirrhosis compared to healthy controls (>50% vs. 19%), suggesting relatively early occurrence of immunosuppression in cirrhosis. In line, profoundly reduced numbers of distinct innate and adaptive immune cell populations were observed before ACLF development. These changes were accompanied by parallel upregulation of co-stimulatory (e.g. CD40L, OX40, CD69, GITR, TIM-1) and inhibitory immune checkpoints (e.g. PDPN, PROCR, 2B4, TIGIT) on CD4+ and CD8+ T cells, which again preceded the development of ACLF. On a functional basis, the capacity of CD4+ and CD8+ T cells to produce pro-inflammatory cytokines upon stimulation was strongly diminished in patients with acute decompensation of liver cirrhosis and ACLF.

Conclusion: Impaired innate and—in particular—adaptive cellular immunity occurs relatively early in the pathogenesis of liver cirrhosis and precedes ACLF. This may contribute to the development of ACLF by increasing the risk of infections in patients with liver cirrhosis.

Keywords: cellular immunity, T cells, *torque teno* virus, γδ T-cells, systemic inflammation, immune checkpoints

INTRODUCTION

Liver cirrhosis is associated with portal hypertension, impaired intestinal barrier function and intestinal dysbiosis, which result in intestinal translocation of bacteria and bacterial products (so called pathogen-associated molecular patterns, PAMPs) to the portal venous and systemic circulation (1). In addition, stress and death of parenchymal and non-parenchymal liver cells result in the release of danger-associated molecular patterns (DAMPs) such as high mobility group protein B1 (HMGB1), histones, ATP, or urate (1). The exposure of hepatic and systemic immune cells to DAMPs and PAMPs results in production of chemokines, cytokines, growth factors, reactive oxygen-species (ROS), and activation of local and further recruitment of circulating immune cells like Ly-6C+ monocytes which differentiate into macrophages (2, 3). As a consequence, already compensated liver cirrhosis is associated with low-grade chronic systemic inflammation (4). Patients with acute decompensation of liver cirrhosis show significantly higher grades of inflammation, but highest levels of systemic inflammation were consistently observed in patients with acute-on-chronic liver failure (ACLF) (5, 6). ACLF can be triggered by precipitating events such as infections, excessive alcohol exposure, or re-activation of hepatitis B (6, 7). Such precipitating events can fuel cirrhosis-associated systemic inflammation, evidenced by excessive production of inflammatory mediators such as TNF-α, IL-6, or IL-8 (5).

Importantly, liver cirrhosis is not only associated with systemic inflammation, but also with a parallel presence of profound immunosuppression (4). For example, serum concentrations of anti-inflammatory cytokines like IL-10 or IL-1RA are progressively increasing during acute decompensation of cirrhosis or development of ACLF (5). In addition, monocytes of patients with liver cirrhosis are increased in frequency and are considered to display an activated phenotype, but they do not sufficiently respond to further stimulation with LPS, a phenomenon called LPS tolerance (4). This phenomenon is partially based on high expression of the inhibitory tyrosine kinase MERTK on peripheral blood monocytes, which suppresses antibacterial monocyte functions in patients with ACLF (8).

In contrast to the well-known changes in cytokine patterns and innate immune responses during the progression of liver cirrhosis to ACLF, less is known about concomitant changes in the adaptive immune compartment and the functional evolution of immunosuppression. We therefore aimed to perform a comprehensive immunophenotyping study with a focus on cellular adaptive immunity during the progression of liver cirrhosis.

PATIENTS AND METHODS

Study Population

Since August 2013, consecutive patients hospitalized to the University Hospital Frankfurt, Germany, with acute decompensation of liver cirrhosis and/or acute-on-chronic liver failure according to the criteria of the CLIF-EASL consortium (6), were prospectively enrolled in our liver cirrhosis cohort study (9). In 2015, the cohort was extended to patients with compensated/stable decompensated liver cirrhosis, not requiring hospitalization due to decompensation.

The diagnosis of liver cirrhosis was based by combination of clinical, laboratory and imaging findings (ultrasound and transient elastography or share wave elastography) or—rarely—by liver biopsy. Acute decompensation of liver cirrhosis was defined as presence of one of the following criteria: new onset/progression of hepatic encephalopathy graded by West-Haven criteria, gastrointestinal hemorrhage, bacterial infection, or ascites grades II–III, according to the definitions used in the canonic study (6). ACLF was diagnosed according to the ACLF-criteria proposed by the CLIF-EASL consortium (6). Patients not requiring hospitalization due to hepatic decompensation and not meeting criteria of acute decompensation or ACLF were classified as compensated/stable decompensated. Patients were excluded if they were younger than 18 years, in case of pregnancy or breastfeeding, presence of hepatocellular carcinoma (HCC) beyond Milan criteria, presence of infection with human immunodeficiency virus (HIV), or therapy with immunosuppressive agents. All patients who were enrolled in this prospective cohort study until September 2017 with sufficient serum samples were included in the present analysis (TT virus cohort, details below). Comprehensive immunophenotyping analyses was performed in those patients of whom sufficient amounts of live peripheral blood mononuclear cells (PBMCs) were available (details on immunophenotyping below).

All patients provided written informed consent to the study protocol, and the study was approved by the local ethic committee of the University Hospital Frankfurt, Germany.

Detection and Quantification of TT Virus

For the detection and quantification of TT virus, DNA was isolated from plasma samples using QIAamp DNA Blood Mini Kit (Qiagen) and subjected to real-time PCR analysis using Rotor-Gene Probe PCR Kit (Qiagen, Germany) and a Rotor-Gene-Q instrument (Qiagen). TT virus specific primers and a 5′ FAM/3′TAMRA labelled probe as well as a standard plasmid containing TT virus genotype 1a DNA (AB017610.1) were used as described elsewhere (10, 11).

Immunophenotyping and Flow Cytometry

Immunophenotyping was performed in 22 patients with compensated/stable decompensated liver cirrhosis, 34 patients with acute decompensation of liver cirrhosis and 23 patients with ACLF. Due to limited numbers of PBMCs of individual patients, patients had to be distributed in two non-overlapping subgroups for immunophenotyping, i.e. a primary group to determine frequencies of innate and adaptive immune cell subpopulations (results are shown in **Figures 2** and **3**) and a second group to determine expression of co-stimulatory and inhibitory immune checkpoints on CD4+ T cells and CD8+ T cells (results shown in **Figures 4**–**7**). Of note, data of two and nine patients with compensated and decompensated liver cirrhosis, respectively, who had received a transjugular portosystemic stent shunt, were included in a previously published study (12). In addition, PBMCs of 23 healthy volunteers were analyzed as a reference.

For quantification of frequencies of innate and adaptive immune cells subpopulations, immunophenotyping was performed according to the recommendations of the Human Immunology Project Consortium (13) and according to Wistuba-Hamprecht et al. for of γδ T cells (14). Flow cytometric analysis was performed on peripheral blood mononuclear cells (PBMCs) of patients and healthy controls. PBMCs were isolated by Ficoll density gradient separation (Biocoll Separating Solution, Merck Millipore). Cells were incubated with a human FcR blocking reagent (Miltenyi Biotec) and stained with fluorochrome-coupled antibodies in Brilliant Stain Buffer (BD Horizon™). **SI Table 1** lists all antibodies for general immunophenotyping whereas antibodies listed in **SI Table 2** classify subtypes of γδ T cells. Cellular viability was estimated by 7-AAD incorporation (BioLegend). **SI Table 3** lists all antibodies for analysis of expression of co-stimulatory and inhibitory immune checkpoints on CD4+ T cells and CD8+ T cells with Zombie/Aqua (BioLegend) for liver dead cell staining. Flow cytometric measurements were performed on a BD LSRFortessa™ cytometer. For correct gating fluorescence minus one controls (FMOs), i.e. fully stained cells with the exception of one particular antibody-fluorochrome conjugate, were used to identify cells expressing a given cell surface marker, as described previously (12). Frequency of cell populations were analyzed by using FlowJo V10 software. The gating strategy to determine frequencies of innate and adaptive immune cell subpopulations including γδ T cells has been described previously (12). The gating strategy for quantification of costimulatory and inhibitory immune check points is shown in **SI Figure 1**.

Ex Vivo T Cell Function Assays

For analysis of T cell activation, PBMCs were cultured in RMPI/10%FCS/1% Pen/Strep with Golgi inhibitors 1X Monensin/1X Brefeldin A (both eBioscience) in presence or absence of 32nM PMA and 3,2μM Ionomycin (both Sigma Aldrich) for 6 h. Afterwards, cells were collected and intracellular cytokine staining for IFN-γ and TNF-α was performed using antibodies listed in **SI Table 3** with Zombie/Aqua (BioLegend) for liver dead cell staining.

Statistical Analyses

Statistical analyses were performed using BiAS, Version 11.06, and GraphPad PRISM5. Group differences were assessed by means of χ^2 contingency tables or Wilcoxon-Mann-Whitney-U-tests, as appropriate. P values < 0.05 were considered to be statistically significant. Associations of outcomes with continuous or dichotomic variables were assessed in linear and logistic regression models, respectively. After univariate analyses, multivariate analyses were performed for significant associations. Multivariate models were obtained by backward selection, using a P value >0.15 for removal from the model.

RESULTS

Baseline Characteristics of Included Patients

Overall, 131 patients with liver cirrhosis could be analyzed for the presence of TT virus, including 31 patients with compensated/stable decompensated liver cirrhosis, 46 patients with acutely decompensated liver cirrhosis, and 54 patients with ACLF. Of patients with ACLF, 23 (43%), 16 (30%), and 15 (28%) patients had ACLF grades 1, 2, and 3, respectively. Baseline characteristics of the TT virus cohort are shown in **Table 1**. Baseline characteristics of the subgroups of patients in whom immunophenotyping was performed are shown in **SI Table 4**.

TT Virus Is Frequently Detectable and Highly Replicative in Patients With Liver Cirrhosis

Torque teno virus (TT virus), is a non-enveloped virus with a circular single-stranded DNA genome, that is highly prevalent in the general population (>95%). Although TT virus replication has

TABLE 1 | Baseline characteristics of included patients.

	ACLF (n = 54)	acutely decompensated cirrhosis (n = 46)	Compensated/stable decompensated cirrhosis (n = 31)	P-value
Age (years), mean (SD)	55 (10)	55 (10)	57 (11)	n.s.
Male gender, n (%)	40 (74.1)	33 (71.7)	20 (64.5)	n.s.
BMI (kg/m^2), mean (SD)	28.0 (7.0)	26.1 (6.5)	26.0 (5.6)	n.s.
Etiology of liver disease				
Alcoholic	27 (50)	31 (67)	15 (48)	n.s.
Viral	5 (9)	7 (15)	7 (23)	n.s.
NASH	5 (9)	4 (9)	4 (13)	n.s.
Other	17 (31)	4 (9)	5 (16)	0.01
Leucocytes (/nl), mean (SD)	11.2 (6.4)	8.4 (4.8)	5.5 (2.5)	<0.0001
Hemoglobin (g/dl), mean (SD)	9.4 (1.9)	10.6 (2.6)	10.4 (2.4)	0.004
Platelets (/nl), mean (SD)	116 (78)	139 (98)	105 (59)	n.s.
CRP (mg/dl), mean (SD)	5.1 (4.3)	2.7 (2.9)	1.5 (2.1)	<0.0001
Creatinine (mg/dl), mean (SD)	2.4 (1.3)	1.1 (0.7)	0.9 (0.30)	<0.0001
Bilirubin (mg/dl), mean (SD)	12.1 (12.0)	7.0 (9.1)	4.6 (5.4)	0.004
ALT (U/l), mean (SD)	159 (240)	109 (104)	76 (11)	n.s.
INR, mean (SD)	2.0 (0.8)	1.5 (0.4)	1.4 (0.2)	<0.0001
Albumin (g/dl), mean (SD)	2.9 (0.5)	2.8 (0.4)	3.1 (0.5)	n.s.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; γGT, γ-glutamyl transferase; INR, international normalized ratio.

not been associated with any human disease, TTV DNA represents a suitable surrogate marker for immune competence (10, 15, 16). Active replication and the magnitude of replication of TT virus are considered to correlate with the extent of immunosuppression in a given patient, e.g. in the setting of medical immunosuppression in organ transplant recipients (15). We therefore performed qualitative and quantitative assessment of TT DNA viral load in our cohort. As shown in **Figure 1**, TT virus was detectable in a significantly higher proportion of patients with liver cirrhosis compared to healthy controls (>50% vs. 19%, *P*=0.0003). In addition, TT viral loads were significantly higher in patients with liver cirrhosis compared to healthy controls (**Figure 1**; *P*<0.05). Of note, frequencies of detectable TT virus were comparable between patients with compensated liver cirrhosis, acute decompensation of liver cirrhosis or ACLF (**Figure 1**). In addition, TT viral load was not significantly (though numerically) higher in patients with ACLF compared to patients with compensated/stable decompensated or acutely decompensated liver cirrhosis (**Figure 1**). Next, we performed logistic regression analyses of factors associated with detectable TT virus in patients with liver cirrhosis. Of note, only the presence of ACLF (P=0.03) and serum creatinine (P=0.02) were associated with detectable TT virus in patients with liver cirrhosis in univariate analysis, whereas in multivariate analysis only serum creatinine (P=0.02, odds ratio=1.60, 95% confidence interval=1.07–2.39) was independently associated with detectable TT virus (**SI Table 5**). Overall, these data suggest a relatively early occurrence of impaired adaptive immune responses to control TT virus in patients with liver cirrhosis, which may progress during development of ACLF.

Defective Cellular Immunity Occurs Early in Liver Cirrhosis

To better understand the evolution of changes of the innate and—in particular—adaptive immune compartments during the progression of liver disease, comprehensive immunophenotyping was performed according to the recommendations of the Human Immunology Project Consortium (13). As shown in **Figure 2A**, a progressive decrease of frequencies of all αβ T cells and of B cells was observed in patients with compensated/stable decompensated *vs.* acutely decompensated liver cirrhosis *vs.* ACLF in comparison to healthy controls. In addition, patients with acutely decompensated liver cirrhosis or ACLF had significantly lower frequencies of NK cells (**Figure 2B**), while only patients with ACLF (but not with decompensated liver cirrhosis) had significantly lower numbers of γδ T cells (**Figure 2C**). The reduced overall frequency of γδ T cells was based on significantly lower numbers of Vd2 γδ T cells, whereas the Vd1 γδ T cell compartment appeared to be expanded in patients with all stages of liver cirrhosis (**Figure 2C**).

As expected, patients with compensated/stable decompensated liver cirrhosis, acutely decompensated liver cirrhosis and ACLF had higher frequencies of myeloid cells in general compared to healthy controls (**Figure 2D**). This observation hold true for classical and non-classical monocytes, whereas reduced frequencies of myeloid dendritic cells were observed in patients with acutely decompensated cirrhosis and ACLF *versus* compensated cirrhosis and healthy controls (**Figure 2D**). Of note, changes in the T cell and myeloid cell compartments correlated strongly (**Figure 2E**).

Next, detailed subtyping of αβ T cell populations was performed, which are of primary interest in our study. Subtyping revealed reduced numbers of CD4+ T cells at all stages of liver cirrhosis and of CD8+ T cells in patients with acutely decompensated liver cirrhosis and ACLF (**Figure 3**). Changes according to the grade of ACLF, as well as comparison between patients with compensated and stable decompensated cirrhosis, are shown in **SI Figures 2** and **3**. The decrease of CD4+ T cells in acutely decompensated cirrhosis/ACLF was based on reductions of naïve and effector CD4+ T cells, whereas central memory and effector memory CD4+ T cells were not reduced or

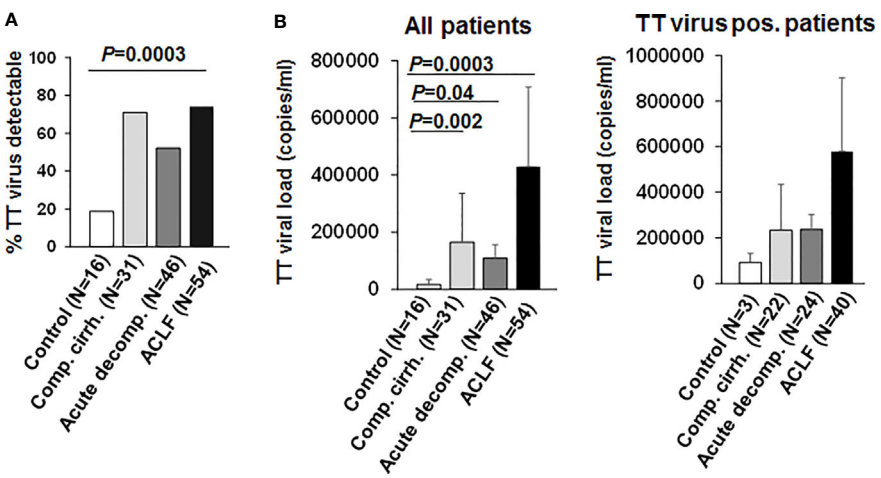

FIGURE 1 | TT virus is frequently detectable in patients with liver cirrhosis. **(A)** Frequencies of detectable TT virus DNA according to the stage of liver disease are shown in comparison to healthy controls. **(B)** Mean quantitative measurement of TT viral load according to the stage of liver disease are shown for all patients (left graph) and for the subgroup of individuals with detectable TT virus (right graph).

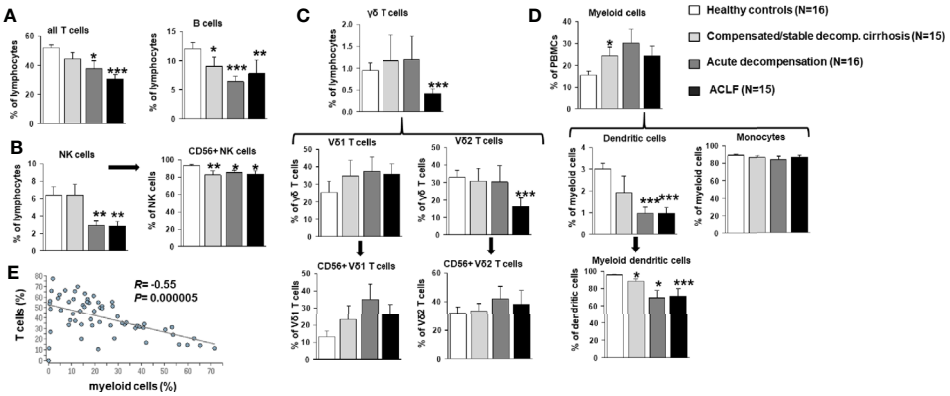

FIGURE 2 | Frequencies of T cells, B cells, NK cells, γδ T cell, and myeloid cells in patients with progressive stages of liver cirrhosis liver cirrhosis vs. healthy controls. **(A)** T and B cell frequencies are represented as frequency of all living lymphocytes. **(B)** NK cells (CD33 positive and CD16 positive) were further classified according to CD56 expression as a marker for cytotoxicity. **(C)** γδ T cells (CD3 positive and γδ TCR positive) were stained for Vδ1, Vδ2 and CD56 to identify major subpopulations. **(D)** Dendritic cells were identified as being CD14 negative and MHCII positive and classified as myeloid dendritic cells if CD11c positive. Cell frequencies are represented as frequency of all living cells for myeloid cells and as frequency of the parent population for all subtypes. **(E)** Correlation between frequencies of T cells and myeloid cells. *P<0.05, **P<0.01, ***P<0.001.

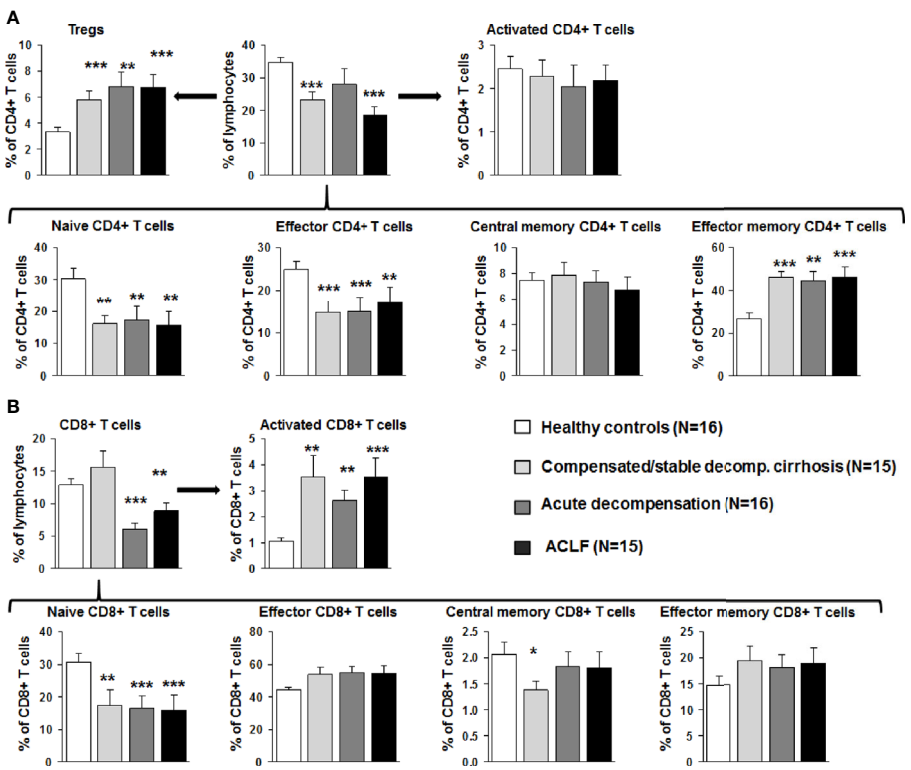

FIGURE 3 | Early changes of the αβ T cell compartment during the course of liver cirrhosis. **(A)** CD4 positive T cells were stained for CD25 and CD127 to identify Tregs (top left), for CD38 and MHCII to assess T cell activation (top right), and for CD45RA and CD197 to further characterize the status of differentiation (bottom panel). Cell frequencies are represented as frequency of all living cells for total CD4 positive T cells (top middle) and as frequency of CD4+ T cells for all subtypes. **(B)** CD8+ positive T cells were stained for CD38 and MHCII to determine activation (top right), and for CD45RA and CD197 to characterize the status of differentiation (bottom panel). Cell frequencies are represented as frequency of all living cells for total CD8 positive T cells (top left) and as frequency of CD8+ T cells for all subtypes. *P<0.05, **P<0.01, ***P<0.001.

even increased, respectively (**Figure 3A**). Of note, relative numbers of CD4+ regulatory T cells were significantly higher in patients with compensated/acutely decompensated liver cirrhosis and ACLF in comparison to healthy controls (**Figure 3A**). In the CD8+ T cell compartment, a significant reduction of naïve CD8+ T cells in patients with all stages of liver cirrhosis was observed as well, whereas relative frequencies of effector CD8+ T cells and central memory/central effector T cells were not significantly altered relative to the severity of liver cirrhosis (**Figure 3B**). Importantly, the proportion of activated CD8+ T cells was significantly higher in patients with all stages of liver cirrhosis in comparison to healthy controls (**Figure 3B**). This phenomenon was not directly observed in the CD4+ T cell compartment (**Figure 3A**). However, patients with acutely decompensated liver cirrhosis or ACLF had significantly higher frequencies of central memory Th1 cells, whereas patients with ACLF had lower frequencies of central memory Th17 cells compared to healthy controls (**SI Figure 4**). Comparable, though less pronounced differences were observed for Th1/Th17 effector memory T cell compartment (**SI Figure 4**). In contrast, Th2 cell frequencies were not altered in patients with liver cirrhosis compared to healthy controls (**SI Figure 4**).

Impaired Adaptive Immune Compartments Are Associated With Replicative TT Virus Infection

We next assessed immune cell frequencies of patients with liver cirrhosis according to the presence of detectable TT virus. As shown in **SI Figure 5**, patients with detectable (i.e. replicative) TT virus in serum revealed significantly lower frequencies of all T cells, naïve CD8+ T cells, central memory CD8+ T cells, and overall CD4+ T cells, whereas frequencies of activated CD8+ T cells were significantly higher in patients with *versus* without detectable TT virus. Of note, Vd2 γδ T cell frequencies were significantly lower in patients with detectable TT virus compared to patients without detectable TT virus as well, whereas no differences in NK cell frequencies were observed (**SI Figure 5**). No differences in myeloid cell populations were observed according to the detectability of TT virus (**SI Figure 5**). Overall, these data suggest a functional relevance of the

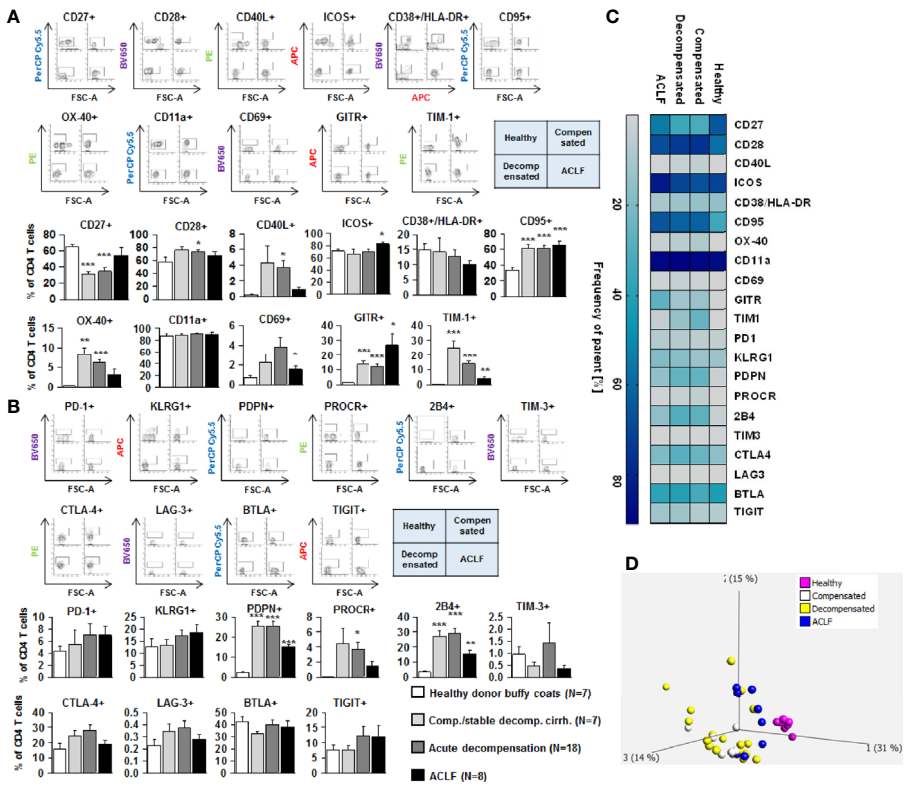

FIGURE 4 | Parallel upregulation of co-stimulatory and inhibitory immune checkpoints on CD4+ effector T cells during the course of liver cirrhosis. Expression of co-stimulatory immune checkpoints, T cells activation markers (CD38/HLA-DR, CD69) as well as of the Fas-ligand CD95 on effector CD4+ T cells are shown in **(A)**, while expression of inhibitory immune checkpoints is shown in **(B)**. Typical examples of dotplots of flow cytometric analysis of healthy controls or patients with compensated liver cirrhosis, decompensated liver cirrhosis or ACLF are shown in the upper panels whereas mean values and standard deviations are shown in graphs in lower panels. **(C)** Heatmap summarizing the mean frequencies of positive cells. **(D)** Principle component analysis of the assessed markers showed distinct clustering of patients with liver cirrhosis versus healthy controls whereas no clear clustering was observed between the stages of liver cirrhosis. Values are displayed as frequency of parent. *P<0.05, **P<0.01, ***P<0.001.

observed changes of the T cell compartments in patients with liver cirrhosis.

Profoundly Altered Expression of Co-Stimulatory and Inhibitory Immune Checkpoints on CD4 + and CD8+ T Cells in Patients With Liver Cirrhosis

We next assessed the expression of co-stimulatory and inhibitory immune checkpoints on CD4+ and CD8+ T cells. As shown in **Figures 4A** and **5A**, a number of co-stimulatory molecules, namely CD40L, OX-40, GITR, and TIM-1 were significantly upregulated on both effector (**Figure 4A**) and regulatory CD4+ T cells (**Figure 5A**) in patients with liver cirrhosis compared to healthy controls, as well as the T cell activation markers CD69 and CD38. In contrast, CD28 and CD11a were not significantly altered on CD4+ effector T cells and regulatory T cells, whereas ICOS was only upregulated on CD4+ regulatory T cells of patients with liver cirrhosis. An exceptional expression was observed for the co-stimulatory molecule CD27, which was expressed at significantly lower levels on CD4+ T cells of patients with compensated and acutely decompensated liver cirrhosis. Overall, comparable changes in the expression of co-stimulatory molecules were observed on CD8+ T cells of patients with liver cirrhosis (**Figure 6A**).

Changes of expression of co-stimulatory molecules and T cell activation markers were accompanied by a significant upregulation of the Fas-receptor CD95 on CD4+ T cells and CD8+ T cells (**Figures 4A–6A**), which promotes T cell apoptosis, as well as by an increased expression of inhibitory immune checkpoints, namely PDPN, KLGR1, PROCR, and 2B4 on CD4+ effector T cells, CD4+ regulatory T cells and CD8+ T cells, whereas upregulation of the inhibitory immune checkpoints PD-1, CTLA4, BTLA, TIM-3, LAG-3, and TIGIT in patients with liver cirrhosis was more variable (**Figures 4B–6B**). Further heatmap and principal component analyses revealed that maximal changes in the expression of co-stimulatory and inhibitory immune checkpoints relative to healthy controls were observed in patients with compensated/stable decompensated and acutely decompensated cirrhosis, while in patients with ACLF expression levels of these molecules declined to intermediate levels between healthy controls and patients with compensated/stable decompensated and acutely decompensated

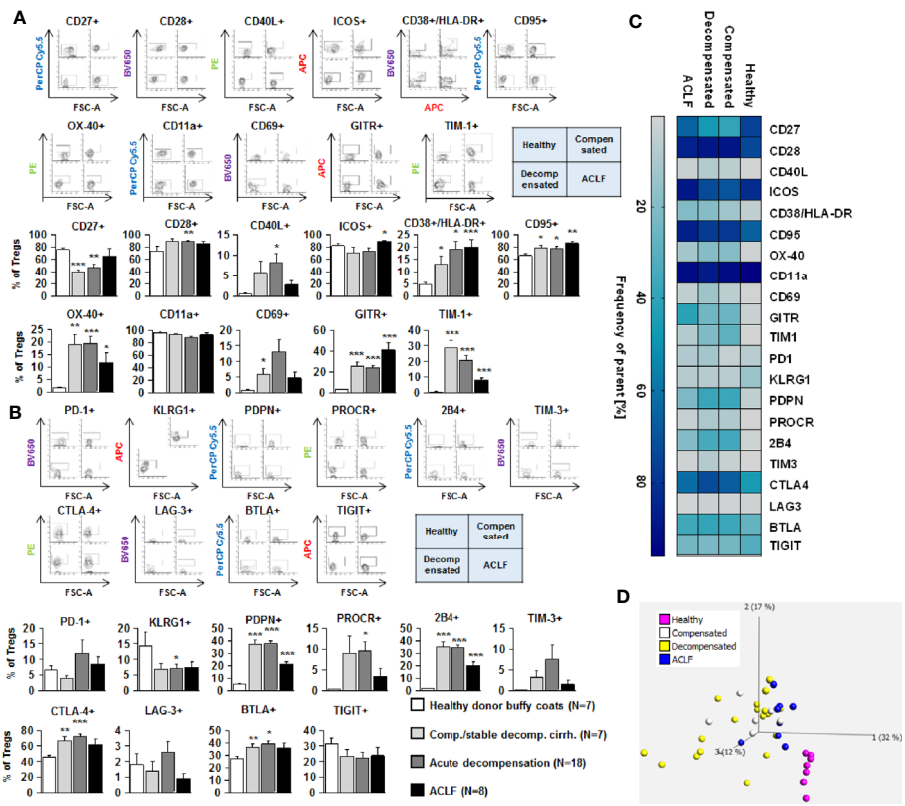

FIGURE 5 | Parallel upregulation of co-stimulatory and inhibitory immune checkpoints on CD4+ regulatory T cells during the course of liver cirrhosis. Expression of co-stimulatory immune checkpoints, T cells activation markers (CD38/HLA-DR, CD69) as well as of the Fas-ligand CD95 on regulatory (CD4+ CD25dim CD127-) T cells are shown in **(A)**, while expression of inhibitory immune checkpoints is shown in **(B)**. Typical examples of dotplots of flow cytometric analysis of healthy controls or patients with compensated liver cirrhosis, decompensated liver cirrhosis or ACLF are shown in the upper panels whereas mean values and standard deviations are shown in graphs in lower panels. **(C)** Heatmap summarizing the mean frequencies of positive cells. **(D)** Principle component analysis of the assessed markers showed distinct clustering of patients with liver cirrhosis versus healthy controls whereas no clear clustering was observed between the stages of liver cirrhosis. Values are displayed as frequency of parent. *P<0.05, **P<0.01, ***P<0.001.

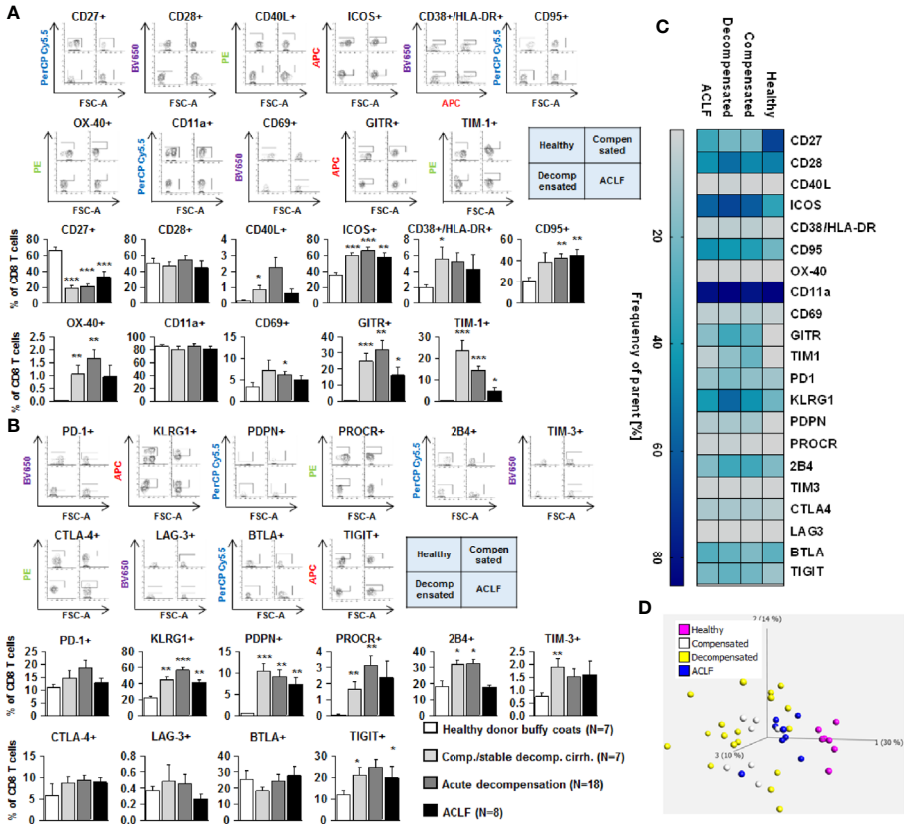

FIGURE 6 | Parallel upregulation of co-stimulatory and inhibitory immune checkpoints on CD8+ effector T cells during the course of liver cirrhosis. Expression of co-stimulatory immune checkpoints, T cells activation markers (CD38/HLA-DR, CD69) as well as of the Fas-ligand CD95 on CD8+ T cells are shown in **(A)**, while expression of inhibitory immune checkpoints is shown in **(B)**. Typical examples of dotplots of flow cytometric analysis of healthy controls or patients with compensated liver cirrhosis, decompensated liver cirrhosis or ACLF are shown in the upper panels whereas mean values and standard deviations are shown in graphs in lower panels. **(C)** Heatmap summarizing the mean frequencies of positive cells. **(D)** Principle component analysis of the assessed markers showed distinct clustering of patients with liver cirrhosis versus healthy controls whereas no clear clustering was observed between the stages of liver cirrhosis. Values are displayed as frequency of parent. *P<0.05, **P<0.01, ***P<0.001.

cirrhosis. Changes according to the grade of ACLF, as well as comparison between patients with compensated and stable decompensated cirrhosis, are shown in **SI Figures 2** and **3**.

Weak Correlation Between Systemic Inflammation and Changes in the Adaptive Immune System

Since advanced liver cirrhosis and in particular ACLF are characterized by profound systemic inflammation, we performed a correlation between markers of systemic inflammation [CRP and IL-22 (9)] and selected features of the adaptive immune system. Weak, non-significant correlations between systemic inflammation and the number of naïve CD4+ and CD8+ T cells were observed, whereas a moderate association between the extend of systemic inflammation and expression of co-stimulatory and inhibitory immune checkpoints (OX40, PRORC) on CD4+ and CD8+ T cells was observed (**Figure 7**).

Inappropriate Function of Effector T Cells of Patients With Liver Cirrhosis

Collectively, the above described findings reveal a parallel increased expression of co-stimulatory and inhibitory immune checkpoints on CD4+ and CD8+ T cells of patients with liver cirrhosis. To understand the functional consequences of these observations, live T cells of patients with liver cirrhosis or healthy controls were stimulated *ex vivo* with PMA/ionomycin. Of note, baseline expression of IFN-γ and TNF-α appeared to be higher in CD4+ and CD8+ T cells of patients with all stages of liver cirrhosis compared to healthy controls. As shown in **Figure 8**, stimulation with PMA/ionomycin of CD4+ and CD8+ T cells from healthy controls resulted in a strong and significant increase of production of IFN-γ and TNF-α, and in an attenuated but still significant increase of IFN-γ, but not of TNF-α of CD4+ and CD8+ T cells of patients with compensated/stable decompensated liver cirrhosis. In contrast, CD4+ and CD8+ T cells of patients with acutely decompensated cirrhosis or with ACLF did not respond to

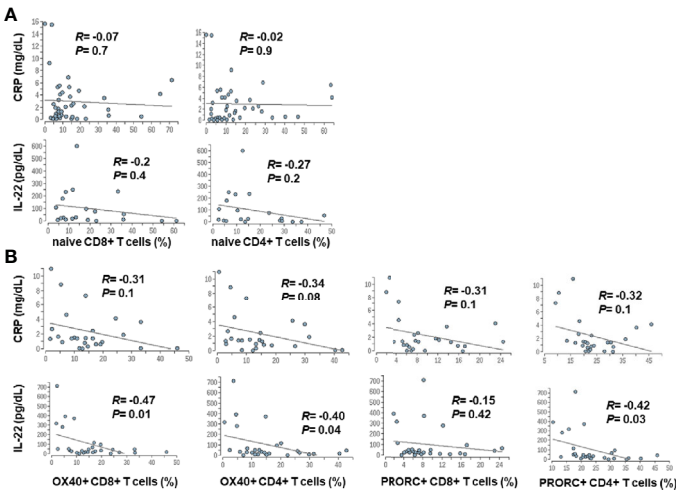

FIGURE 7 | Correlation between markers of systemic inflammation and changes in the adaptive immune system in patients with liver cirrhosis. Serum concentrations of CRP and IL-22, which are well established markers of systemic inflammation in cirrhosis (9), were correlated with frequencies of naïve CD4+ and CD8+ T cells **(A)**, was well as with the expression of selected co-stimulatory (OX40) and inhibitory (PRORC) immune checkpoints **(B)**.

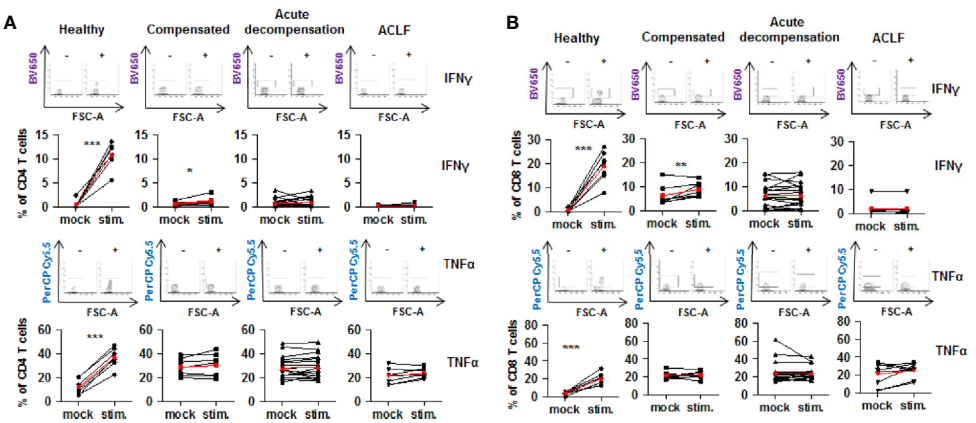

FIGURE 8 | Impaired on-demand production of pro-inflammatory cytokines in CD4+ and CD8+ T cells of patients with liver cirrhosis. CD4+ T cells **(A)** and CD8+ T cells **(B)** from healthy controls are patients with compensated/stable decompensated liver cirrhosis, acute decompensation of liver cirrhosis, or ACLF were stimulated ex vivo with PMA/Ionomycin for 6 h. Production of IFN-γ and TNF-α at baseline before stimulation and after stimulation were assessed by flow cytometry. Typical examples of dotplots of flow cytometric analysis of healthy controls or patients with compensated liver cirrhosis, decompensated liver cirrhosis or ACLF are shown in the upper panels whereas mean values and standard deviations are shown in graphs in lower panels. Red bars indicate mean increase of pro-inflammatory cytokine production from baseline to 6 h after stimulation. *P<0.05, **P<0.01, ***P<0.001.

stimulation with PMA/ionomycin with induction of IFN-γ and TNF-α.

DISCUSSION

The present comprehensive immunophenotyping study suggests a relatively early occurrence of impaired cellular immune responses during the course or liver cirrhosis, indicated by strongly reduced numbers of important cell populations of the adaptive and innate immune system like CD4+ T cells, CD8+ T cells, B cells, NK cells, and dendritic cells, which are accompanied by a parallel induction of co-stimulatory and inhibitory immune checkpoints on CD4+ and CD8+ T cells and a lost capacity to induce pro-inflammatory cytokine production. Most of the observed changes were already evident in patients with compensated/stable decompensated liver cirrhosis and were fully developed in patients with acutely

decompensated liver cirrhosis, while ACLF was associated with only few additional changes in immune cell frequencies like a restricted compartment of V2 γδ T cells and declining levels of immune checkpoint expression on αβ T cells. Hence, impaired cellular immune compartments are preceding ACLF and may contribute to the pathogenesis of ACLF. Furthermore, the high frequency of active replication of TT virus observed in our cohort of patients with liver cirrhosis supports the functional relevance of the altered cellular immune compartments.

The impaired immune cell compartments observed in our study develop in parallel to a progressive systemic inflammatory response, which is evidenced by progressively increased levels of pro-inflammatory mediators such as cytokines, chemokines, or eicosanoids in patients with compensated liver cirrhosis *versus* acutely decompensated cirrhosis *versus* ACLF (1, 5, 17). Already compensated liver cirrhosis is considered as a disorder accompanied with low-grade systemic inflammation, which promotes symptoms of liver cirrhosis such as fatigue or frailty (4). During the progression of liver cirrhosis to decompensated liver cirrhosis or ACLF, systemic inflammation augments progressively to levels which are sufficient to induce organ failures and ultimately death (1, 4, 5, 18). Importantly, systemic inflammation in patients with liver cirrhosis is paralleled by a state of immunosuppression, resulting in a high risk of development and adverse outcome of infections in liver cirrhosis and—in particular—in ACLF. This is evidenced by a kinetic of anti-inflammatory cytokines such as IL-10 or IL-1ra which completely parallels the increasing production of pro-inflammatory cytokines during the progression of liver cirrhosis to ACLF (5). The parallel increased expression of co-stimulatory and inhibitory immune checkpoints on CD4+ and CD8+ T cells observed in our study resembles the above described co-existence of upregulated pro- and anti-inflammatory cytokines and innate immune pathways which result in an ineffective host defense despite taking the hazards of inflammation-induced organ failures. Indeed, CD4+ and CD8+ T cells of patients with liver cirrhosis in our study showed high expression of pro-inflammatory cytokines at baseline which may contribute to inflammation-induced organ failures, but a lacking on-demand increase in response to further stimulation. Of note, expression of co-stimulatory and inhibitory immune checkpoints on T cells peaked in patients with compensated and acutely decompensated cirrhosis and diminished in patients with ACLF, which likely reflects extended exhaustion of adaptive immunity in patients with ACLF.

Collectively, systemic inflammation, production of inhibitory cytokines and impaired innate and adaptive cellular immune responses evolve in parallel in patients with liver cirrhosis. In this regard, liver cirrhosis could be considered as a disorder which is characterized by an impaired resolution of inflammation. The concept of resolution of inflammation includes appropriate removal of inflammatory mediators, appropriate termination and clearance of secondary anti-inflammatory cells and mediators, as well as an adequate tissue repair (19). All these features are lacking in patients with liver cirrhosis. Of note, it has been shown that an inadequate resolution of inflammation results in subsequent dysfunctional adaptive immunity characterized by T cell fate, activation of T cells, and impaired T cell function (19). The results of our study would be in line with a concept of impaired T cell immunity as a result of inappropriate resolution of inflammation, as we have indeed observed higher frequencies of activated T cells, of effector memory CD4+ T cells, as well as of induction of co-stimulatory immune checkpoints, which are however accompanied by impaired high levels of inhibitory immune checkpoints, lacking induction of cytokines upon stimulation of CD4+ and CD8+ T cells and impaired control of TT virus replication in patients with liver cirrhosis.

Of particular importance, the here observed impaired adaptive immune compartment evolves relatively early in the progress of liver cirrhosis, is almost completely established in acutely decompensated cirrhosis and appears to precede ACLF. However, it is important to note that we applied the concept of acute decompensation of liver cirrhosis in our study, which was recently introduced by the Cliff consortium and which differs from the classical concept of decompensation (6). The finding of reduced T cell numbers in patients with early stages of liver cirrhosis is in line with previous studies in patients with chronic hepatitis C and other causes of liver cirrhosis (20–22). Although our study is of associative nature and does not allow causal conclusions, one may speculate that impaired adaptive immune responses may promote the development and adverse outcome of infections, as infections are one of the most frequent causes of ACLF (6). Furthermore, infections are frequent complications of ACLF, and infection-triggered ACLF is associated with a particular poor outcome (23, 24). Collectively, these clinical data and the results of our immunophenotyping study support further research to assess whether strategies to reset the adaptive immune system to a naïve state to enable an appropriate response to pathogens (i.e. a strategy to promote resolution of inflammation), would be of benefit in the prevention and treatment of ACLF.

Our study has important limitations. First of all, we were only able to analyze immune cells in the peripheral blood of patients with liver cirrhosis, because liver biopsies in patients with advanced liver disease are rarely performed at our department. Hence, the here described immune phenotypes may differ in other relevant immunological compartment of patients with liver cirrhosis, in particular in the liver and intestines (25). Furthermore, we were not able to analyze antigen-specific T cells due to limited cell numbers available for immunophenotyping. Finally, the different stages of liver cirrhosis were analyzed in different patients and not during the progression of cirrhosis in the same individual, which—however—is not an uncommon study design.

Nevertheless, our study provides evidence of pronounced alterations of innate and—in particular—adaptive cellular immunity which precedes ACLF and may contribute to the pathogenesis of ACLF by increasing the risk of infections in patients with liver cirrhosis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the faculty of medicine, University Hospital Frankfurt. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The authors have contributed to the manuscript by planning the study (SR, CL), collecting the data (SR, SC, AQ, MW, KS, CW, AW, CL), analysis and interpretation of data (SR, SC, AQ, MW, BB, CW, HW, SZ, AW, CL), and preparation and revision of the manuscript (all authors). All authors contributed to the article 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/fimmu.2020.534731/full#supplementary-material

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Characterization of Blood Immune Cells in Patients With Decompensated Cirrhosis Including ACLF

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Background and Aims: Patients with cirrhosis and acute-on-chronic liver failure (ACLF) have immunosuppression, indicated by an increase in circulating immune-deficient monocytes. The aim of this study was to investigate simultaneously the major blood-immune cell subsets in these patients.

Material and Methods: Blood taken from 67 patients with decompensated cirrhosis (including 35 critically ill with ACLF in the intensive care unit), and 12 healthy subjects, was assigned to either measurements of clinical blood counts and microarray (genomewide) analysis of RNA expression in whole-blood; microarray (genomewide) analysis of RNA expression in blood neutrophils; or assessment of neutrophil antimicrobial functions.

Results: Several features were found in patients with ACLF and not in those without ACLF. Indeed, clinical blood count measurements showed that patients with ACLF were characterized by leukocytosis, neutrophilia, and lymphopenia. Using the CIBERSORT method to deconvolute the whole-blood RNA-expression data, revealed that the hallmark of ACLF was the association of neutrophilia with increased proportions of macrophages M0-like monocytes and decreased proportions of memory lymphocytes (of B-cell, CD4 T-cell lineages), CD8 T cells and natural killer cells. Microarray analysis of neutrophil RNA expression revealed that neutrophils from patients with ACLF had a unique phenotype

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including induction of glycolysis and granule genes, and downregulation of cell-migration and cell-cycle genes. Moreover, neutrophils from these patients had defective production of the antimicrobial superoxide anion.

Conclusions: Genomic analysis revealed that, among patients with decompensated cirrhosis, those with ACLF were characterized by dysregulation of blood immune cells, including increases in neutrophils (that had a unique phenotype) and macrophages M0-like monocytes, and depletion of several lymphocyte subsets (including memory lymphocytes). All these lymphocyte alterations, along with defective neutrophil superoxide anion production, may contribute to immunosuppression in ACLF, suggesting targets for future therapies.

Keywords: myeloid cells, innate lymphoid cells, adaptive immune cells, sepsis, organ failure, immunotherapies

INTRODUCTION

Acute-on-chronic liver failure (ACLF), which develops in patients with acutely decompensated cirrhosis, is characterized by the existence of organ failure(s) and high in-hospital mortality (1, 2). ACLF is associated with systemic inflammation as indicated by blood leukocytosis (1, 2), and high plasma levels of C-reactive protein (1, 2) and cytokines and chemokines (3, 4). White blood-cell count is a component of the chronic liver failure consortium ACLF scoring system, which accurately predicts early mortality in patients with ACLF (5). It has been suggested that, in ACLF, peripheral leukocytosis is enriched in effector immune cells that have a high potential to cause tissue damage (2). ACLF is also characterised by systemic inflammation which is known to be energetically expensive and may thus skew nutrients otherwise required for other metabolic processes towards immune cells. This would therefore deny peripheral organs the required nutrients which may result in systemic organ failure (6).

There are studies concentrating on peripheral-blood myeloid mononuclear cells involved in innate immunity (4, 7–11), showing, for example, that patients with ACLF, whether critically ill or not, have increased frequency of $CD14^+$ monocytes expressing the receptor tyrosine kinase MerTK (hereafter called MerTK) (4, 10) and $CD14^+CD15^-HLA\text{-}DR^-$ myeloid-derived suppressor cells (9). Both subsets of myeloid mononuclear cells have suppressed innate responses to bacterial pathogen-associated molecular patterns (PAMPs) (4, 9, 10). These alterations may, therefore, favor the development of bacterial infections that are frequent complications of ACLF (12, 13). Another study found decreased frequencies of other myeloid mononuclear cells (conventional dendritic cells [DCs] and plasmacytoid DCs) in patients with severe alcoholic hepatitis, including patients who had ACLF (11). Several studies have showed alterations in neutrophils (14–16) and lymphocytes (7, 17–19) in patients with decompensated cirrhosis. However, until now there has been no comprehensive description of the landscape of blood immune-cell subsets in patients with ACLF, in particular critically ill patients admitted to the intensive care units (ICUs) and who have an especially poor prognosis. A better knowledge of circulating immune-cell landscape in patients with ACLF could provide new insights into the pathophysiology of systemic inflammation and organ failures and identify targets for therapies, which are unmet medical needs (2). We therefore aimed to perform a pilot study with the objective of characterising white blood cell subsets beyond just monocytes and dendritic cells, in critically ill patients with ACLF, patients with decompensated cirrhosis but without ACLF and in healthy subjects (HS).

To address this question, we performed microarray (genomewide) analysis of RNA expression in whole-blood from study individuals. Indeed, a large number of information about a variety of circulating immune-cell subsets have been identified from genomewide analysis of RNA expression in a single blood sample obtained in each study individual (20–23).

Abbreviations: ACLF, acute-on-chronic liver failure; CIBERSORT, cell-type identification by estimating relative subsets of RNA transcripts; AD, acute decompensation; AC, advanced cirrhosis; HUVEC, human umbilical vein epithelial cell; fMLP, N-Formylmethionyl-leucyl-phenylalanine.

MATERIAL AND METHODS

Patients

The core study was a pilot aiming to investigate clinical complete blood counts and whole-blood transcriptome in patients with biopsy-proven cirrhosis admitted to the Hepatology and Liver Intensive Care Unit at Beaujon Hospital, Clichy, France (**Table S1**). The study protocol was approved by the local human research ethics committee (Supporting Methods). Written informed consent before enrollment or delayed consent was obtained from each patient, or legal surrogate. Every patient with ACLF was admitted to the ICU for organ support; blood used for whole-blood transcriptomics was taken within 24 h after admission to the ICU. We used criteria developed by the European Foundation for the Study of Chronic Liver Failure for the diagnosis of acutely decompensated cirrhosis, organ failures, and ACLF grades (**Table S2**) (1, 5, 24).

Of the 31 patients who were enrolled in the core pilot study, 24 were nonelectively admitted to the hospital [including 17 critically ill patients with ACLF (94% of them having circulatory

failure), and seven who had acutely decompensated cirrhosis without ACLF (a group hereafter called AD)], and seven were admitted for therapeutic paracentesis of refractory ascites [a group hereafter called advanced cirrhosis (AC)] (**Table 1**). Of note, patients with AC were stable; indeed, none of the patients with AC had ongoing or recently treated (less than 1 week) bacterial infection or gastrointestinal hemorrhage. During the 90 days following enrollment, the number of patients who received a liver transplant was 4, 3, and 3, in the ACLF, AD, and AC groups, respectively. The 90-day transplant-free mortality rate was 71%, 14%, and 0%, in the ACLF, AD, and AC groups, respectively.

Methods

Methods which are described in detail in the Supporting Information, are summarized as follows.

Microarray (Genomewide) Analysis of Whole-Blood RNA Expression

Blood was collected in Tempus tubes (Thermo Fisher Scientific, Waltham, MA). RNA was extracted using the 5 PRIME PerfectPure RNA blood kit (Thermo Fisher Scientific) according to manufacturer's instructions. One hundred nanograms of RNA per sample were then hybridized on Human Transcriptome Array 2.0 (HTA2.0, Affymetrix, Santa Clara, CA) at the genomic platform of the Curie Institute (Paris, France). This array was designed to measure the RNA expression of 67,528 genes. Analysis and visualization of the Human Transcriptome Array 2.0 dataset were made using EASANA® (GenoSplice technology), which was based on the GenoSplice's FAST DB® annotations (18). Expression data were log_2-transformed and used for two purposes. First, Student's *t*-test were used for identification differentially expressed genes (DEGs). Genes with a fold change >1.5-fold and P <0.05 were considered as differential expressed. Then, we performed enrichment analysis using as gene sets, the 346 blood transcription modules (hereafter referred to as BTMs) that have been developed by Li, Rouphael, Duraisingham, et al (21). and were publicly available (**Table S3**). These BTMs have been computed using transcriptomic data obtained in peripheral-blood mononuclear cells from individuals under various immunological stimuli (21). Each BTM contained a variable number of co-expressed genes and has received an identification number. A large number of BTMs were related to specific immune-cell subsets; for example, the BTMs "M.37.1: enriched in neutrophils (I)"; "M11.0: enriched in monocytes (II); "M7.0: Enriched in T cells (I)"; "M7.2: enriched in NK cells (I)"; "M47.0: enriched in B cells (I)". Other modules were related to immune signaling; for example, "M16: TLR and inflammatory signaling"; M37.0: immune activation - generic cluster" (**Table S3**). Enrichment analysis also used other publicly available data bases such as Reactome pathways (https://reactome.org) as gene sets.

Second, the CIBERSORT software package was used for deconvoluting the blood RNA microarray data (22). R values were calculated to assess correlations of complete blood counts inferred from CIBERSORT and counts measured by clinical laboratories. Inferred CIBERSORT lymphocyte counts were calculated as the sum of the counts of naive B cells, memory B cells, CD8 T cells, naive CD4 T cells, resting memory CD4 T cells, and activated memory CD4 T cells (23). The counts of monocytes, macrophage M0- and macrophage M2-like monocytes were summed to infer CIBERSORT monocyte counts (23).

Microarray (Genomewide) Analysis of RNA Expression in Blood Neutrophils

RNA was extracted from freshly isolated neutrophils. One hundred nanograms of RNA per sample were then hybridized on Clariom S Array, Human (Affymetrix) at the genomic platform of the Curie Institute (Paris, France). This array was designed to measure the RNA expression of ~20,800 genes.

RESULTS

ACLF Associates Blood Leukocytosis, Neutrophilia, and Lymphopenia

Clinical complete blood counts obtained on admission in the 31 French patients with cirrhosis showed that ACLF was characterized by significant increases in white-cell count (i.e., leukocytosis), differential and absolute neutrophil counts (i.e., neutrophilia), and significant decreases in differential lymphocyte count (i.e., lymphopenia), as compared with the two other groups (AC and AD) (**Figure 1A**, **Figure S1**). Absolute lymphocyte count, and monocyte counts (differential and absolute) did not significantly differ between ACLF and the two other groups (**Figure 1A**, **Figure S1**). No significant differences in blood counts were seen between AC and AD.

Similar findings (**Figures S2A, B**) were obtained in an independent English cohort including 91 patients with AD and 203 critically ill patients with ACLF (Supplementary Methods; **Table S4**). In both, the French cohort (**Figure 1A**) and the English cohort (**Figure S2C**), there was a significant negative correlation between differential neutrophil count and differential lymphocyte count, indicating a dichotomic regulation of circulating immune cells, opposing neutrophils to lymphocytes, and culminating in ACLF.

Validation of the CIBERSORT-Inferred Blood Counts

We found that the RNA microarray–inferred white-cell counts (calculated based on the results of the CIBERSORT method; **Table S5**) (23) were positively correlated with clinical laboratory measurements of complete blood counts (**Figure 1B**), which suggested that microarray analysis of whole-blood RNA was of sufficient quality to provide information that correlated with criterion-standard clinical measurements of blood counts.

RNA Identification of Dysregulated Blood Immune-Cell Subsets in ACLF

Genomewide analysis of whole-blood RNA expression (microarrays) was performed in the 31 patients from the French cohort and 7 HS (healthy subjects) (**Table S1**). Unsupervised hierarchical clustering analysis of the results

TABLE 1 | Characteristics of the French patients with cirrhosis enrolled for the whole-blood transcriptome analysis.

Characteristic	Advanced cirrhosis (AC, n=7)	Acute decompensation of cirrhosis (AD[a], n=7)	ACLF (n=17)	P value for the difference between all groups	P value for the difference between AC and AD	P value for the difference between ACLF and AD
Median age (IQR) – yr	59 (53–64)	57 (52–59)	58 (53–60.5)	0.81	–	–
Male sex – n (%)	7 (100)	3 (42.9)	13 (76.5)	0.06	–	–
Etiology or cirrhosis – n (%)				0.45	–	–
Alcoholic liver disease	6 (86)	3 (43)	12 (70)			
Nonalcoholic steatohepatitis	1 (14)	2 (28)	3 (18)			
Chronic hepatitis C	0 (0)	1 14)	2 (12)			
Chronic hepatitis B	0 (0)	1 (14)	0 (0)			
Acute precipitants – n (%)				–	–	0.66
Sepsis	0 (0)	2 (28.6)	8 (47.1)			
Excessive alcohol consumption	0 (0)	2 (28.6)	1 (5.9)			
Hemorrhage	0 (0)	1 (14.3)	4 (23.5)			
Other	0 (0)	1 (14.3)	2 (11.8)			
None	7 (100)	1 (14.3)	2 (11.8)			
Severity scores						
Median CLIF-C AD score (IQR)	–	55 (52–59)	–	–	–	–
Median MELD score (IQR)	10 (8–18)	21 (15–25)	35 (26–39)	<0.001	0.05	0 .003
Median CLIF-C Organ Failure score (IQR)	–	8 (7–9)	14 (12–15)	–	–	<0.001
Median CLIF-C ACLF score (IQR)	–	–	67 (59–69.5)	–	–	–
Individual organ system failures[a] – n (%)						
Kidney	0 (0)	0 (0)	11 (64.7)	<0.001	1	0.005
Lungs	0 (0)	0 (0)	10 (59.0)	0.003	1	0.02
Liver	0 (0)	1 (14.3)	4 (23.5)	0.37	–	–
Coagulation	0 (0)	1 (14.3)	11 (64.7)	0.005	0.71	0.05
Brain	0 (0)	0 (0)	3 (17.6)	0.26	–	–
Circulation	0 (0)	0 (0)	16 (94.0)	<0.001	1	<0.001
ACLF Grade[a] – n (%)				–	–	–
Grade 1	0 (0)	0 (0)	1 (5.9)			
Grade 2	0 (0)	0 (0)	2 (11.8)			
Grade 3	0 (0)	0 (0)	14 (82.4)			
Median white-cell count[b] (IQR) – per mm^3	6,000 (4,400–8,300)	5,600 (4,500–8,700)	16,100 (10,200–22,200)	0.01	0.8	0.02
Median absolute count (IQR) – per mm^3						
Neutrophils	4,250 (2595–4940)	3,680 (2,900–4,795)	12,800 (7,700–18,400)	0.022	0.9	0.013
Monocytes	700 (580–1,090)	1,000 (690–1,100)	1,100 (600–1,490)	0.28	–	–
Lymphocytes	1,300 (1,045–1,785)	1,000 (925–1,535)	1,080 (700–1,600)	0.95	–	–
Median differential count[c] (IQR) – %						
Neutrophils	66 (46–72)	60 (56–66)	81 (70–90)	<0.001	0.53	<0.001
Lymphocytes	18 (15–3)	18 (13–27)	11 (4–16)	0.008	0.9	0.016
Monocytes	12 (8–13)	13 (7–22)	9 (4–13)	.14	–	–
Median hemoglobin (IQR) – g/dl	11.6 (10.1–14.1)	7.8 (7.1–10.1)	8.8 (8.1–11.3)	0.02	0.02	0.166
Median platelet count (IQR) – per mm^3	71,000 (65,000–265,000)	80,000 (73,000–157,000)	116,000 (84,500–164,000)	0.79	–	–
Median International Normalized Ratio (IQR)	1.42 (1.20–1.86)	1.64 (1.54–2.23)	2.80 (2.10–3.10)	<0.001	0.13	0.013
Median serum sodium (IQR) – mmol/L	135 (133–138)	133 (131–135)	133 (125–137)	0.45	–	–
Median serum creatinine (IQR) – mg/dl	0.74 (0.68–0.85)	0.73 (0.56–1.51)	2.40 (1.90–3.10)	0.001	0.46	<0.001
Median C-reactive protein (IQR) – mmol/L	7 (7–10)	11 (7–24)	44 (21–94)	0.36	–	–

(Continued)

TABLE 1 | Continued

Characteristic	Advanced cirrhosis (AC, n=7)	Acute decompensation of cirrhosis (AD[a], n=7)	ACLF (n=17)	P value for the difference between all groups	P value for the difference between AC and AD	P value for the difference between ACLF and AD
Median aspartate aminotransferase (IQR) – U/L	58 (33–72)	50 (46–90)	104 (61–234)	0.05	–	–
Median alanine aminotransferase (IQR) – U/L	26 (12–33)	28 (14–37)	40 (20–83)	0.12	–	–
Median total bilirubin (IQR) – mg/dl	2.47 (0.80–4.71)	5.82 (1.81–6.23)	5.10 (3.20–11.70)	0.10	–	–
Outcome						
Death without transplantation by 90 days – n (%)	0 (0)	0 (0)	12 (71)	–	–	–
Liver transplantation – n (%)	3 (43)	3 (43)	4 (23.5)	–	–	–

P values were obtained using Kruskal–Wallis test and Mann–Whitney test and the chi-square test or Fisher's exact test, as appropriate. ACLF denotes acute-on-chronic liver failure; IQR, interquartile range; CLIF-C, Chronic Liver Failure-Consortium; and MELD, Model for End Stage Liver Disease.

[a]*Defined in* ***Table S2****.*

[b]*Normal range for blood white-cell count is 4,500–11,000 per mm*^{3}*.*

[c]*Normal ranges for differential counts are 40%–70% for neutrophils, 4%–11% for monocytes, and 22%–44% for lymphocytes.*

showed that the profiles of blood RNA expression were markedly different between patients with ACLF and all the other study persons (**Figure S3**). Microarray results were validated by using RT-qPCR in a random set of genes (**Figure S4**).

Then, the results of microarray analysis were used to establish the lists of DEGs, corresponding to 3 pairwise comparisons: AC versus HS, AD versus HS, and ACLF versus HS (hereafter referred to as AC/HS, AD/HS, and ACLF/HS, respectively; **Table S6**), that were visualized using volcano plots (**Figure 2A**). In AC/HS (**Figure 2A**, left), the 5 top upregulated genes included *SCLC38A5, CTSE, AHSP, NRG1, LINC00278* while the 5 top downregulated genes included *PPBP*, *MAP3K7CL, MS4A1, RAB27B, SDPR*. In AD/HS (**Figure 2A**, middle), the top upregulated genes included *AHSP, RSAD2, IFI27, IFI44L, THEM5* while the top downregulated genes included *MAP3K7CL, KLRC4, JCHAIN, MS4A1, LRNN3*. In ACLF/HS (**Figure 2A**, right), the top upregulated genes included *CD177, MCEMP1, ARG1, MMP9, PFKFB2* while the top downregulated genes included *CD3G, KLRC4, GNLY, FGFBP2, GPR174*. Of note, in ACLF/AC the 5 top upregulated genes (**Figure S5**; **Table S6**) were similar to the corresponding top upregulated genes in ACLF/HS (**Figure 2A**, right). In ACLF/AC, the only top downregulated gene shared with ACLF/HS was *CD3G*. In ACLF/AC, the other top downregulated genes were *FCER1A, CCR3, CLC, CX3CR1* (**Figure S5**; **Table S6**).

In addition, we used Venn diagrams to visualize the number of DEGs unique to each comparison. There were few DEGs unique to AC/HS or AD/HS (120 and 147, respectively) while there were 1497 DEGs unique to ACLF/HS, indicating changes in blood transcriptome that were specific for ACLF (**Figure 2B**).

ACLF Associates With Increased Innate Inflammatory Signatures in Blood

To gain insights into the dynamics of transcription at the single-gene level across the study groups, we first used the comparison of ACLF versus HS to rank fold-changes in gene expression, from the highest to the lowest fold-change value, and then aligned, for each gene, its fold-change values in the two other pairwise comparisons (AC/HS, AD/HS; **Table S6**). Using the fold-changes values for the 50 most upregulated genes in ACLF/HS, and the corresponding values in the two other comparisons (**Table S6**), we drew a Cleveland plot showing fold-changes for each gene across the three pairwise comparisons (**Figure 2C**, top). For each gene, the fold-change was higher in ACLF/HS than the fold-change in the two other comparisons, and, in both AC/HS and AD/HS, very few genes passed the filter defining significant upregulation (**Figure 2C**, top; **Table S6**). We also noted that 22 of the 50 most upregulated genes in ACLF/HS were related to innate immunity; for example *CD177* [that had the highest expression (**Figures 2A, C**)], *MMP8*, *MMP9, ARG1, S100A12*, were neutrophil genes. Together, these findings suggested that upregulated genes in ACLF/HS composed a distinctive gene signature of ACLF. Then, the BTM gene modules (**Table S3**) (21) and canonical pathways (in particular, Reactome pathways) were used as gene sets to perform enrichment analysis of the three complete lists of upregulated genes (ACLF/HS, AD/HS, AC/HS; **Tables S7A, C, E, G, I, K**). The 483 genes upregulated in ACLF/HS were significantly enriched in genes involved in innate immunity, including neutrophil, monocyte, Toll-like receptor and inflammatory signaling genes (**Figure 2D**, left) (**Table S7A**). In addition, analysis of Reactome pathways identified enrichment in neutrophil granule genes (**Table S7C**). Importantly, the lists of upregulated genes in the two other comparisons (AD/HS, AC/HS) had a totally different enrichment profile (**Figure 2D**, left; **Tables S7E, G, I, K**), indicating the specificity of the innate inflammatory gene signature in blood from patients with ACLF.

To capture the dynamics of upregulation of co-expressed genes across study groups, we computed gene clusters using whole-blood transcriptomic data, with the transcriptome in HS as a reference (Supplementary Methods). Each cluster was composed of significantly correlated expression values across the three patients' groups; a gene included in a given cluster was not present in another cluster. We identified nine co-expression

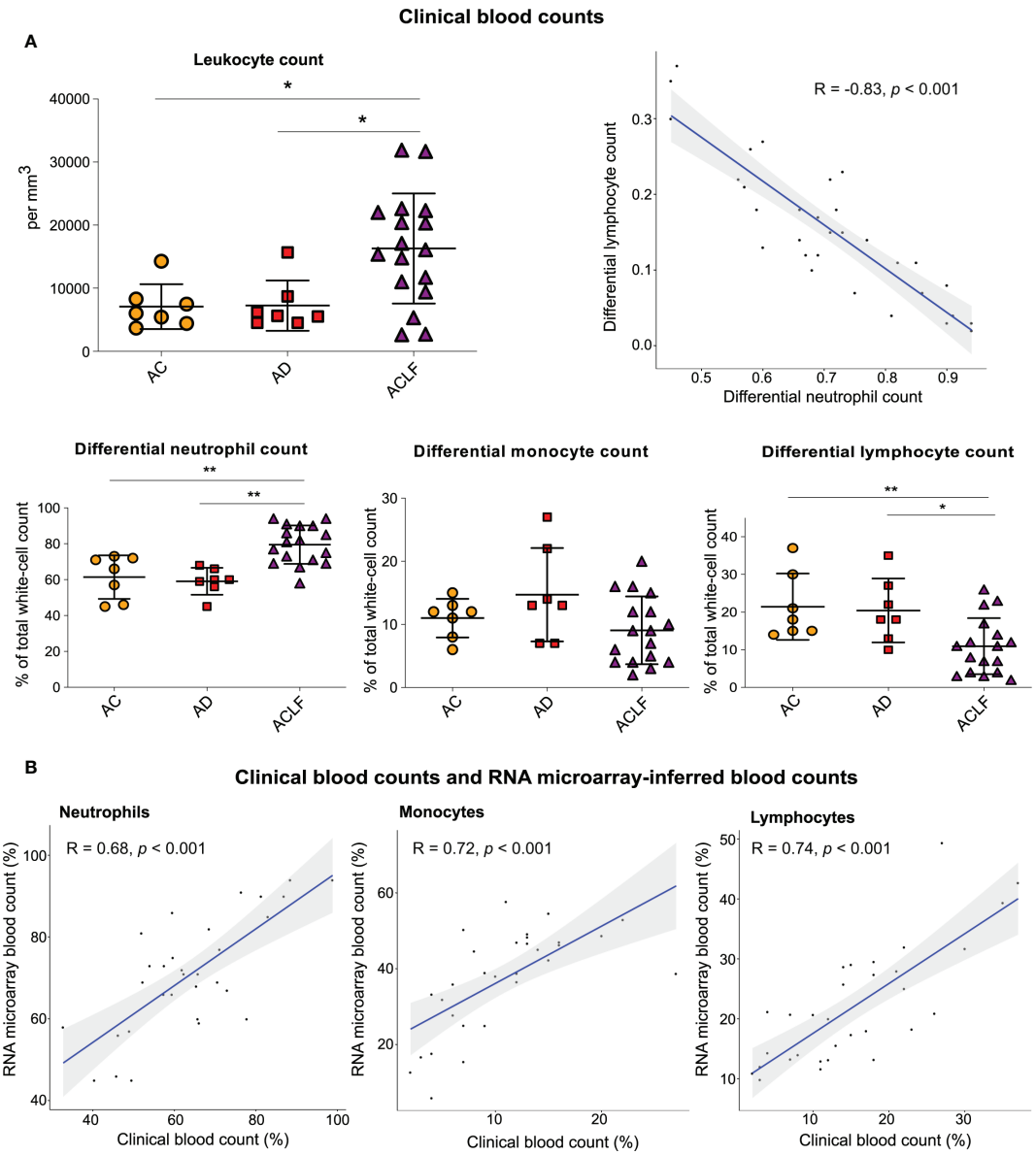

FIGURE 1 | Results of clinical blood counts and RNA microarray-inferred blood counts. **(A)** Clinical complete blood counts in three groups of patients with cirrhosis: AC (orange circles, n=7), AD (red square, n=7) and ACLF (purple triangle, n=17). P values were obtained using *Kruskal–Wallis test* and Mann–Whitney test. **P <0.01; *P <0.05. **(B)** Neutrophil, lymphocyte, and monocyte counts in paired clinical complete blood counts as compared with the CIBERSORT-inferred blood counts from RNA microarray data obtained with the use of peripheral blood (31 paired specimens). The shaded areas represent the 95% confidence intervals. AC denotes advanced cirrhosis, AD acute decompensation, and ACLF acute on chronic liver failure.

gene clusters (**Figure S6A**, **Table S8A**), which were numbered arbitrarily by the algorithm, and included a total of 2116 genes. For each cluster, we computed an eigengene (21) which is a gene summarizing the global behavior of the cluster member genes (**Figures S6A, B**). Cluster 2 was interesting because it was the second largest cluster (416 genes) and 70% of the cluster member genes were upregulated in ACLF/HS. Cluster 2 eigengene was upregulated in ACLF versus the other patients' groups and did not differ between AC and AD, indicating that cluster 2 captured gene upregulation that was specific for ACLF (**Figure S6B**). Finally, the profile of enrichment of cluster 2 (**Figure S6C**, **Table S8**) was very similar to that of upregulated genes in ACLF/HS, in particular member genes were enriched in innate inflammatory genes. Together these findings confirmed that the orchestrated activation of the blood innate inflammatory signature was a molecular hallmark of ACLF.

One additional mean of deconvoluting the RNA microarray data, the CIBERSORT software package (22), showed that there

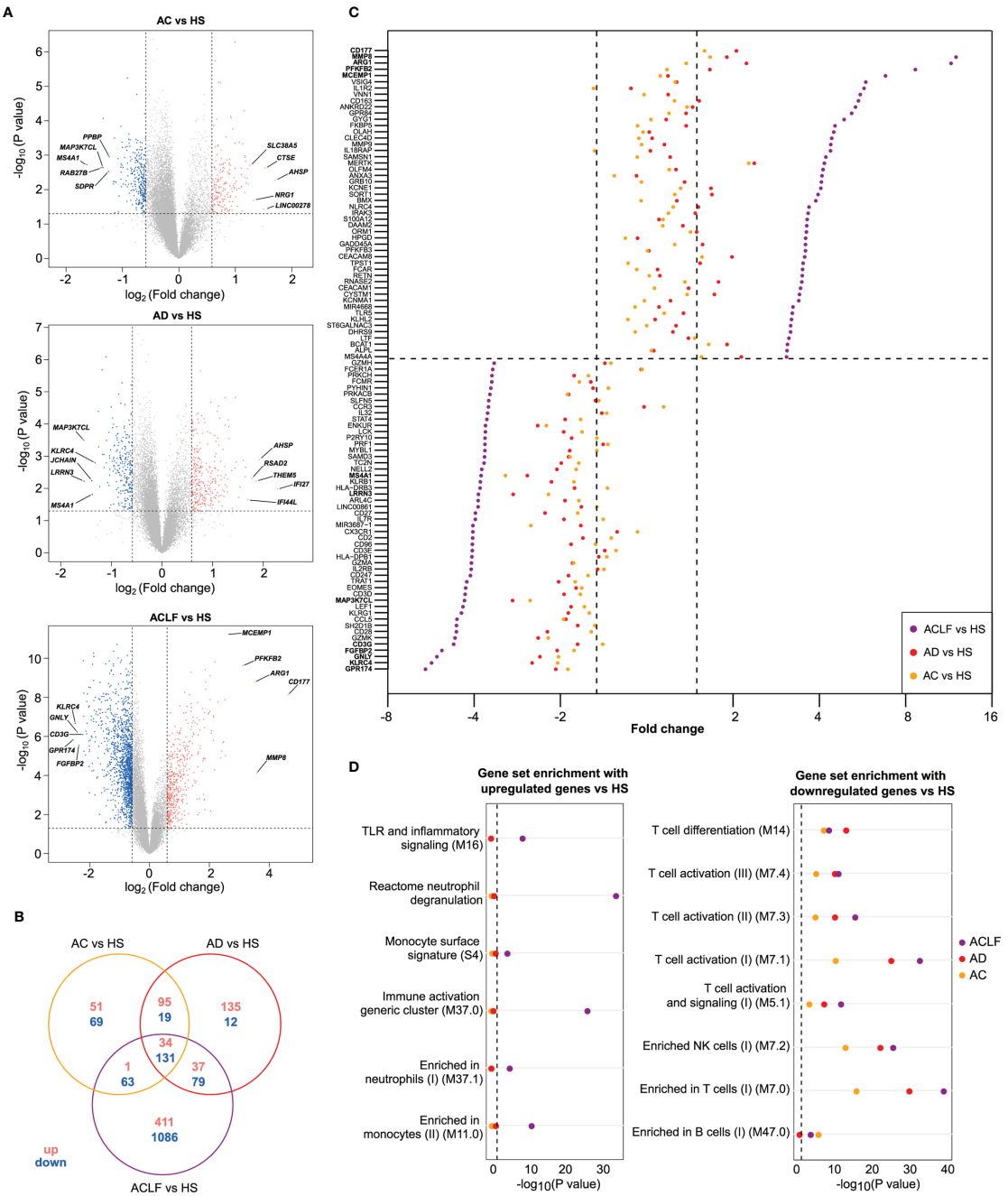

FIGURE 2 | Distinctive transcriptional characteristics of ACLF. **(A)** Volcano plots of differential gene expression between each patients' group [AC (n=7); AD (n=7); ACLF (n=17)] relative to healthy subjects [HS (n=7)], with significance ($-\log_{10}$ P value) plotted against the $\log_2$ fold-change (patients:HS ratio). Genes were considered as differentially expressed when P was <0.05 (or $-\log_{10}$ P >1.3; dashed horizontal line) and fold-change >1.5 [or $\log_2$ fold-change >0.58 for upregulation or $\log_2$ fold-change <-0.58 for downregulation (right and left dashed vertical lines, respectively)]. Gray points indicate genes with no significant difference in expression, salmon indicates genes with significantly increased expression in patients and blue indicates genes with significantly decreased expression in patients. Representative differentially expressed genes are shown. CD177 was the most upregulated gene in ACLF versus HS. Between-group comparisons were performed using Student's *t*-test. **(B)** Venn diagram showing the number of up- and downregulated genes that were unique or not to each of the three pairwise comparisons. **(C)** Cleveland plot showing the fold-changes for the 50 most upregulated genes in ACLF versus HS (top) and in the 50 most downregulated in this comparison (bottom). The dashed horizontal line separates upregulated genes from down regulated genes. For each gene, the fold-change of expression is also shown for two other pairwise comparisons: AD versus HS and AC versus HS. A dashed vertical line (right) indicates the threshold of 1.5 fold-change versus HS and the other dashed vertical line (left) indicates the threshold of -1.5 fold-change versus HS. **(D)** Results of enrichment analysis of predefined data sets (which, unless specified, were blood transcription modules (21)) with upregulated genes (left) and downregulated genes (right), both in ACLF versus HS. The enrichment of these gene sets with genes that were upregulated (left) or downregulated (right), in AC versus HS and AD versus HS genes, are also shown.

were no significant differences in the proportions of myeloid cells between AC and AD, and that these two groups had modest changes restricted to the blood monocyte/macrophage compartment relative to HS (**Figure 3**). In contrast, blood in ACLF had significant increases in the proportions of neutrophils (relative to AC) and macrophage M0-like monocytes (relative to HS and AC) (**Figure 3**). Using flux cytometry in additional individuals, we found that ACLF was associated with a significant increase in the frequency of a monocyte subset (**Figure S7**). Together these findings indicate that, in blood from patients with ACLF, the development of an innate inflammatory gene signature coincided with increases in certain myeloid cell populations (neutrophils and macrophage M0-like monocytes) that were unique to these patients.

ACLF Associates With Depletion of Several Blood Lymphocyte Subsets

Using the fold-changes values for the 50 most downregulated genes in ACLF/HS, and the corresponding values in the two other comparisons (**Table S6**), we drew a Cleveland plot showing fold-changes for each gene across the three pairwise comparisons (**Figure 2C**, bottom). For each gene, the fold-change was greater in ACLF/HS than the fold-change in the two other comparisons. However, in both AC/HS and AD/HS, several genes fulfilled the criteria defining downregulation (**Figure 2C**, bottom; **Table S6**), suggesting a progressive decrease in the expression of these genes from AC or AD to ACLF. For example, *TCF7* and *LEF1*, that are crucial for establishment of central memory CD8 T cells, were downregulated, respectively, by 1.6-, 1.6-, 2.7-fold and by 1.6-, 1.8-, 4.3-fold, in AC/HS, AD/HS, and ACLF/HS (**Table S6**).

Then, we performed BTM-based analyses and found that the 1,086 downregulated genes in ACLF/HS were significantly enriched in T-cell and NK-cell genes (**Figure 2D**, right; **Table S7B**). Moreover, Reactome pathways analysis showed that downregulated genes in this comparison were significantly enriched in genes related to RNA metabolism and translation of RNA into proteins (**Table S7D**), indicating a decrease in the constitutive apparatus required for gene induction and expression, consistent with an increased threshold for lymphocyte activation. Importantly, the lists of downregulated genes in the two other pairwise comparisons (AC/HS, AD/HS) were also significantly enriched in T-cell, and NK-cell genes (**Figure 2D**, right; **Tables S7F, J**), and genes related to RNA metabolism and translation (**Tables S7H, L**) confirming that lymphocyte gene downregulation was not unique to ACLF. Of note, our "home-made" analysis revealed that the eigengene representative of the largest gene cluster (cluster 3, 995 genes) progressively decreased from HS to ACLF; the decrease was already significant in AC relative to HS and reached its maximum with ACLF (**Figure S6B**). Ninety-two percent of cluster 3 member genes were downregulated in ACLF/HS. Moreover, the profile of enrichment of cluster 3 (**Table S8**) was very similar to that of downregulated genes in ACLF/HS, in particular member genes were enriched in genes related to T cells, B cells, and NK cells. Together these findings confirmed that decompensated cirrhosis was characterized by a progressive decline in the expression of genes related to lymphocytes, the lowest gene expression being observed in ACLF.

The use of the CIBERSORT method showed that ACLF was associated with significant decreases in several lymphocyte populations including memory B cells; resting memory CD4 T cells; CD8 T cells; and NK cells (**Figure 3**). The ACLF-associated depletion was restricted to these cells because the proportions of plasma cells; naïve CD4 T cells; activated memory CD4 T cells; and regulatory CD4 T cells (Tregs) were not affected in ACLF (**Figure 3**). Of note, there were no significant alterations in lymphocyte proportions in AC and AD (**Figure 3**). Together, these results indicated that the depletion of certain blood lymphocyte subsets was unique to ACLF, and contributed to both the decrease in clinical differential lymphocyte counts (**Figure 1A**) and the maximum reduction in lymphocyte-related genes (**Figure 2D**), observed in this syndrome. In addition, the ACLF-associated lymphocyte depletion measured with the CIBERSORT method was consistent with results of our analysis of the blood lymphocyte compartment using flux cytometry in additional individuals (**Figure S8**). In addition, flux cytometry identified a significant decrease in NK T cells (which are innate-like killer T cells; **Figure S8**).

Unique Phenotype of Circulating Neutrophils in ACLF

Blood Neutrophils Are Activated in ACLF

Because increased blood neutrophil population was a major feature of ACLF (**Figure 1A**), we performed genomewide analysis of RNA expression in freshly isolated peripheral-blood neutrophils from 15 additional individuals (five HS, five patients with AC, and five patients with ACLF; **Tables S1** and **S9**). There were only 140 DEGs (71 upregulated) in AC/HS and 832 DEGs (420 upregulated) in ACLF/HS (**Figure 4A**; **Table S10A**). Next, we focused on the list of DEGs in ACLF/HS, and pathway analysis of upregulated genes identified that 48 of these genes were neutrophil granule genes (**Figure 4B**; **Table S10B**), findings consistent with analysis of upregulated genes in whole-blood (**Figure 2D**). Granule genes encode proteins which are expressed in the lumen or membrane of different types of neutrophil granules under steady-state, and are released *via* exocytosis, following neutrophil activation (25). *CD177*, which was the second most upregulated gene in ACLF/HS (**Figure 4A**), was in the list of 48 upregulated granule genes. The CD177 protein resides in the granule membrane under basal conditions, and is mobilized to the cell surface following neutrophil activation (25, 26). Using flow cytometry, we investigated CD177 protein expression in neutrophils obtained from two additional series of individuals, one enrolled in France (seven HS, five patients with AC, and seven with ACLF, **Table S1**) and the other in India (eight patients with AC and 12 with ACLF; **Tables S1** and **S11**). The results obtained in both series showed that patients with ACLF had increases in the absolute number and frequency of $CD177^+$ neutrophils (**Figure 4C** and **Figure S9**, for French and Indian patients, respectively). Moreover, mean fluorescence intensity was significantly increased in neutrophils from French patients with ACLF (**Figure S10**), confirming increased

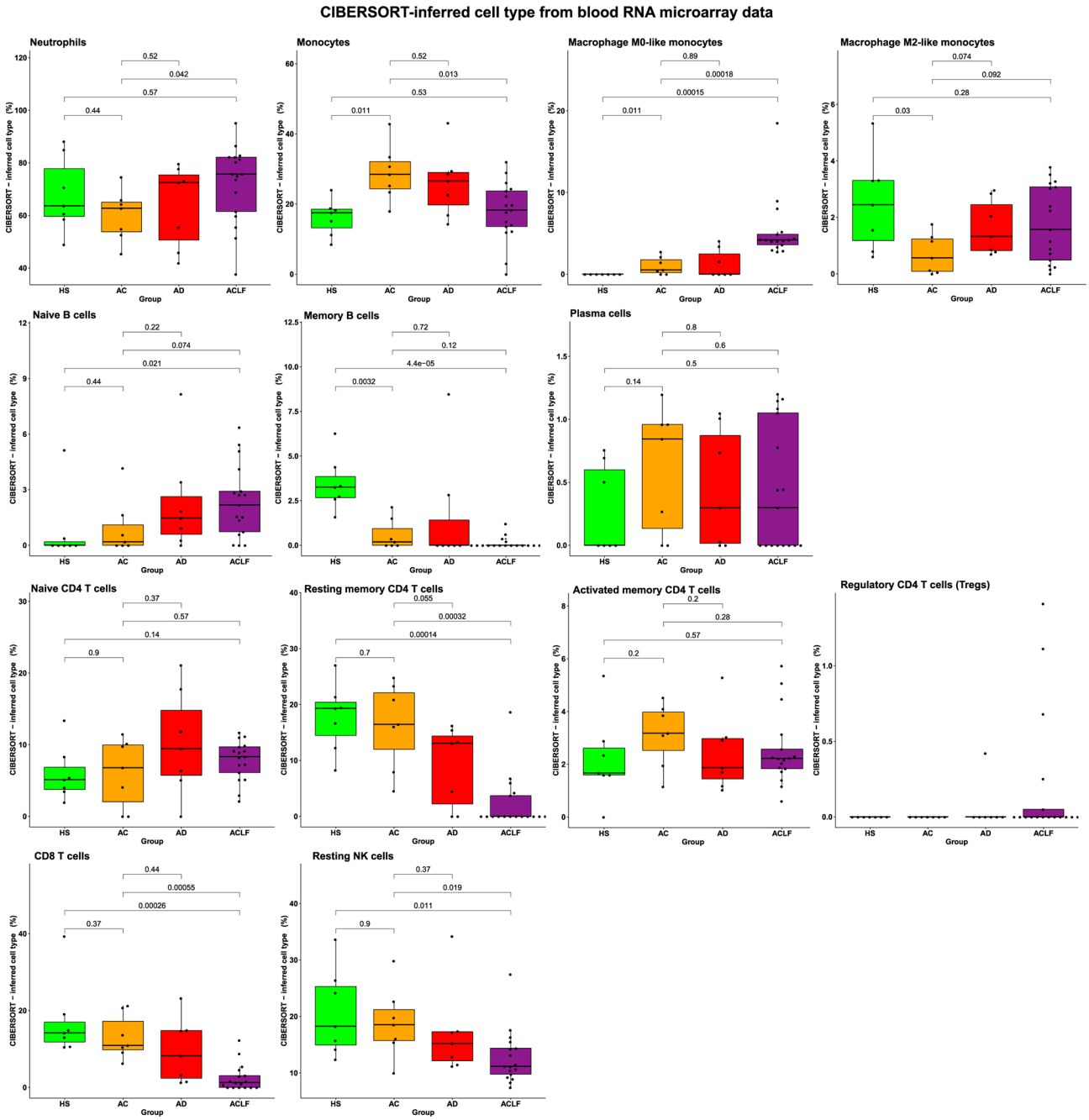

FIGURE 3 | The CIBERSORT method identifies alterations in blood immune-cell subset composition in ACLF. There were seven healthy subjects (HS), seven patients with advanced cirrhosis (AC), seven patients with acutely decompensated cirrhosis (AD), and 17 patients with ACLF. P values were obtained using one-way ANOVA and unpaired *t*-tests.

CD177 expression in these cells. Of note, upregulated genes in ACLF/HS were significantly enriched in genes related to carbohydrate metabolism (**Figure 4B**), consistent with metabolic reprogramming in activated neutrophils (27). Collectively, our analysis of upregulated genes in ACLF/HS indicated that circulating neutrophils were activated in ACLF.

In addition, pathway analysis of downregulated genes in ACLF/HS revealed that these genes were related to TP53-induced transcription (involved in cell death), activation of formins (involved in cytokinesis), cell cycle, and mitosis (both involved in proliferation) (**Figure 4B**; **Table S10C**), which are other features of unique neutrophil phenotype in ACLF (28).

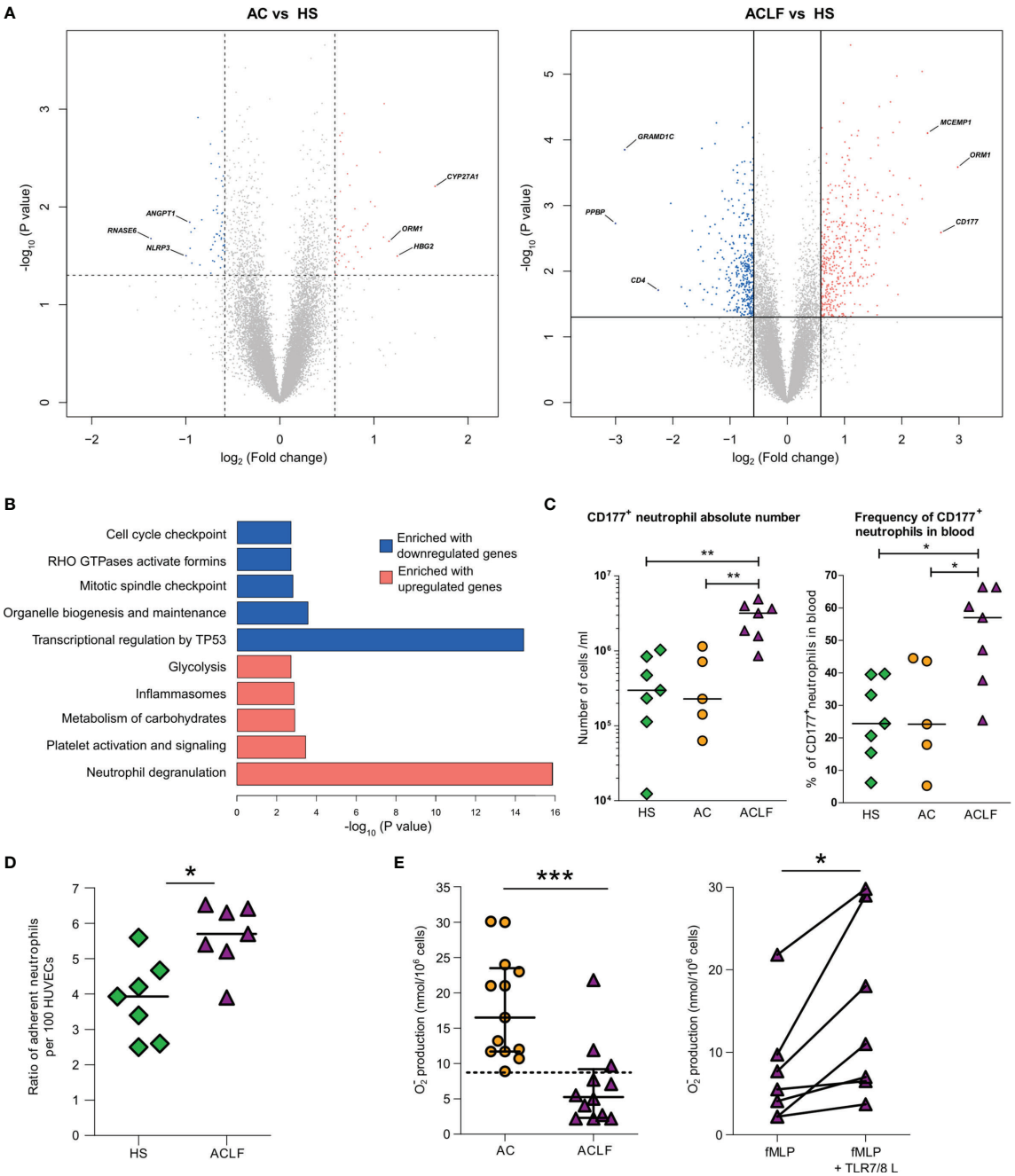

FIGURE 4 | Unique phenotype of neutrophils from patients with ACLF. **(A)** Volcano plots of differential neutrophil gene expression between AC and HS and ACLF versus HS. There were five healthy subjects (HS), five patients with advanced cirrhosis (AC), and five patients with ACLF. Between-group comparisons were performed using unpaired Student's *t*-test. *CD177* was the most upregulated gene in ACLF versus HS. **(B)** Enrichment of Reactome data sets with differentially expressed genes between ACLF and HS. **(C)** Absolute number and frequency of CD177$^+$ neutrophils in blood from HS (green diamonds, n=7), patients with AC (orange circles, n=5) and patients with ACLF (purple triangles, n=7). **(D)** Increased adhesion to HUVECs of neutrophils from ACLF patients relative to neutrophils from HS. Neutrophils from HS (n=7) and patients (n=7) were freshly isolated; their adherence to HUVECs was measured using flow cytometry as the ratio of CD66b$^+$ neutrophil per HUVEC. **(E)** Defective superoxide production in neutrophils from patients with ACLF is restored by a TLR7/8 agonist. Freshly isolated neutrophils from 13 patients with ACLF and 13 patients with AC were stimulated with fMLP (1 μM) and respiratory burst was monitored using the cytochrome c reduction assay. In seven patients, neutrophils were pre-treated with the TLR7/8 agonist CL097 (2 μg/ml) for 10 min before stimulation with fMLP. During the same period, median [IQR] superoxide production in neutrophils from healthy subjects was 19.6 [15.4–27.25]. ***P <0.001; *P <0.05. HUVEC denotes human umbilical vein endothelial cell, and fMLP N-Formylmethionyl-leucyl-phenylalanine.

Blood Neutrophils From Patients With ACLF Have Increased Adhesion to Endothelial Cells

Expression of the CD177 protein at the neutrophil plasma membrane is known to result in neutrophil arrest at the endothelium surface (26). Because of the elevated frequency of $CD177^+$ cells among neutrophils from patients with ACLF, we hypothesized that these neutrophils would exhibit enhanced adhesion to endothelial cells as compared with neutrophils from HS. We freshly isolated neutrophils from patients with ACLF and HS and compared, *in vitro*, their adhesion to human umbilical vein epithelial cells (HUVECs) (29). Patients' neutrophils had an enhanced adherence to HUVECs (**Figure 4D**). Whether this effect directly or indirectly dependent on CD177 overexpression should be further investigated.

In ACLF, Blood Neutrophils Have Defect in Superoxide Anion Production That Is Pharmacologically Reversible

Neutrophils are the first-line defense against bacterial infections (30). When stimulated by bacterial cues, such as N-Formylmethionyl-leucyl-phenylalanine (fMLP), neutrophil NADPH oxidase is activated to produce large amount of the antimicrobial superoxide anion (respiratory burst) (30). In its active form, NADPH is a multiprotein complex comprising NOX2 which catalyzes superoxide anion production (30). We (15, 16), and others (14), have shown that compared with neutrophils form HS, neutrophils from patients with AC have a defective of superoxide anion production in response to fMLP. This defect is explained, at least in part, by a degradation of the protein NOX2 involving the granule protein elastase that has been released *via* degranulation (15). Because, as mentioned earlier, there was evidence of marked neutrophil degranulation in ACLF, we wondered whether superoxide anion production was affected in neutrophils from patients with ACLF. To address this question, neutrophils from two groups of patients (ACLF and AC) were isolated to investigate the production of superoxide anion in response to the bacterial fMLP (Supplementary Methods, **Table S1**). FMLP-induced superoxide anion production by neutrophils was significantly lower in patients with ACLF than in those with AC (**Figure 4E**), suggesting that ACLF was associated with a major defect in NADPH oxidase activation.

We have previously shown that neutrophils isolated from patients with cirrhosis and exposed to CL097 or R848 (which are agonists for endosomal TLR7/8) had an increased superoxide production in response to fMLP (15). We investigated the effects of CL097 on the response to fMLP in neutrophils from patients with ACLF and found that CL097 enhanced fMLP-induced superoxide production in these cells (**Figure 4E**). These findings suggest that the mechanisms resulting in defective superoxide production in fMLP-stimulated neutrophils from patients with ACLF were reversible with endosomal TLR7/8 engagement.

DISCUSSION

This pilot study is the first to investigate the landscape of circulating immune cells in critically ill patients with ACLF seen during the first 24 h of their ICU stay. We used results of clinical complete blood count measurements and microarray (genomewide) analysis of blood RNA expression, in HS and 3 groups of patients with cirrhosis, comprising AC, AD, and ACLF. In addition to traditional bioinformatic analysis of transcriptomic data, deconvolution of these data was performed using the CIBERSORT method, enabling to enumerate the proportions of immune-cell subsets present in a given tissue, here the blood (22). The key results were that patients with ACLF had dysregulation of certain circulating immune cells, including leukocytosis fueled by increased populations of neutrophils (that had unique phenotype) and macrophages M0-like monocytes, and, as expected (31, 32), decreases in lymphocyte count related to a depletion in memory lymphocytes (of the B-cell, CD4 T-cell lineages), CD8 T cells and NK cells. Flux cytometry also identified that ACLF was associated with a decrease in circulating NK T cell population. The dysregulation of blood immune cells was unique to ACLF because it was not observed in AC and AD.

One cannot exclude that increases in blood neutrophil population associated with ACLF was partly due to the mobilization of a marginated pool of neutrophils (26). However, a more likely explanation for neutrophilia is the stimulation of a hematopoietic response program called emergency granulopoiesis (28, 33). Under acute inflammatory stresses, such as sepsis, PAMPs, cytokines (granulocyte colony-stimulating factor [G-CSF], IL-1, TNF-α), or both, stimulate large-scale *de novo* production of neutrophils from myeloid precursors in the bone marrow (33). Patients with ACLF have increased systemic levels of stimuli for emergency granulopoiesis, including PAMPs (lipopolysaccharide) (2) and cytokines, including G-CSF, IL-1, TNF-α (2–4) (**Table S12**).

In this study, microarray analysis of blood neutrophil RNA expression enabled us to show that neutrophils from patients with ACLF overexpressed genes encoding granule proteins (**Table S9**), including *CD177*, which was among the most upregulated transcript in these cells. Flow cytometry revealed that the frequency of CD177 was higher in neutrophils from patients with ACLF. CD177 degranulation at the neutrophil plasma membrane is a signal for neutrophil arrest at the endothelium surface in tissue vessels (26). Thus, using *ex vivo* experiments of neutrophil adhesion to HUVECs, we found that neutrophils from patients with ACLF were more adherent to endothelial cells than their counterpart from HS. The interaction of activated neutrophils with endothelial cells may cause endothelium injury and activate local prothrombotic mechanisms (34). Consistent with this hypothesis, patients with ACLF had elevated plasma levels of markers for endothelial dysfunction (**Table S12**). Therefore, an increased frequency of $CD177^+$ neutrophils in ACLF might play a major role in the development of organ failures in this syndrome. This could also be the case in patients without cirrhosis who have septic shock because previous studies have shown that CD177 was most upregulated gene and protein in neutrophils from these patients (35).

The ACLF-associated depletion in circulating lymphocytes can have different explanations which are not mutually exclusive.

First, like patients of the general population who have protracted sepsis, patients with ACLF may have excessive blood lymphocyte death (36). Second, one cannot exclude another that, like in other severe acute inflammatory diseases (37), ACLF was associated with an increased egress of lymphocytes, from blood to lymphoid or non-lymphoid tissues, that would contribute to circulating lymphopenia. Of note, however, in patients without cirrhosis who had protracted sepsis, blood lymphopenia was associated with extensive loss of lymphocytes in spleens, intestines, and other organs (38). Future studies are needed to elucidate the mechanisms of blood lymphopenia in blood from patients with ACLF.

Patients with ACLF have immunosuppression which is indicated by the high incidence of new or secondary bacterial of fungal infections in these patients (12, 13). Mechanisms driving immunosuppression in ACLF may include increased numbers of MerTK-expressing monocytes (4), and myeloid-derived suppressor cells (9), and exhausted T cells (39). Moreover, our study suggests additional mechanisms for immunosuppression in ACLF, related to alterations in both neutrophils and the lymphoid lineage. Indeed, although neutrophils from patients with ACLF had features of activation, these cells were defective in fMLP-induced neutrophil production of the antimicrobial superoxide anion (**Figure 4E**). This defect, therefore, was a factor of ACLF-associated systemic immunosuppression. Using the CIBERSORT method, we also found that ACLF was associated with a systemic depletion of resting memory CD4 T cells which may play a role in systemic immunosuppression in this syndrome; thus, memory CD4 T cells are essential for an immediate response against bacterial infections (40). Moreover, CD8 T cells, which are involved in elimination of infected cells (41), had a decreased proportion in ACLF. In addition, the populations of NK cells and NK T cells, which play an important role in the innate immune response against microbes (including fungi) (42) were significantly decreased in blood from patients with ACLF. Of note, the CIBERSORT method did not provide explicit information on CD8 T cells states such as exhaustion and memory. Because increased exhausted CD8 T cells and decreased in memory CD8 T cells are important contributors for immunosuppression (34, 43), we established a list of genes that are markers for each of these states (**Table S13**). In our patients with ACLF, we did not find evidence of an induction of exhaustion markers (such as negative checkpoint regulators), but identified a downregulation of the gene signature of memory CD8 T cells, suggesting that these cells may be involved in immunosuppression associated with ACLF.

A decreased number of memory CD8 T cells are involved in immunosuppression associated with cancer (43) or sepsis (36). In the context of cancer, generating more memory CD8 T cells (for example, through IL-7 or IL-15 pathways) may improve the outcomes (43). Similar approaches are considered as being of potential interest for treating immunosuppression in patients with sepsis (36). Our finding, in ACLF, of downregulation of the gene signature of memory CD8 T cells may suggests that immunotherapies with IL-7 or IL-15 might be of interest against immunosuppression associated with this syndrome. Of note, the blood expression of *IL2RG*, encoding the γ-chain receptor which is shared by IL-7 and IL-15 and is indispensable for signaling of these cytokines in target cells, was not affected in ACLF (**Table S6**). Finally, our result, that a TLR7/8 agonist stimulated production of superoxide anion in neutrophil from patients with ACLF, requires confirmation because it suggests another approach against immunosuppression associated with ACLF.

Our study has limitations, in particular because of the small number of patients investigated. However, our study enabled to validate deconvolution of RNA microarray data as a means to enumerate immune-cell subsets in the blood of patients with cirrhosis. This approach may therefore be used in large longitudinal studies assessing, for example, the impact of therapies on systemic inflammatory cells in patients with ACLF. It is also important to note that, although a co-orchestrated decrease in a large number of lymphocyte genes were significantly downregulated in AD and AC relative to HS (**Figure 2D**; **Figure S5**), the CIBERSORT-inferred proportions of lymphocyte subsets did not significantly differ in AD/HS and AC/HS (**Figure 3**). These findings, therefore, were in sharp contrast with those obtained in ACLF/HS where maximum lymphocyte gene downregulation (**Figure 2D**) coincided with decreases in certain blood lymphocyte populations (**Figure 3**). Collectively, our results suggested a model, in which blood lymphocyte gene downregulation was a characteristic of decompensated cirrhosis that preceded the decrease in the frequency of certain circulating lymphocytes; this decreased frequency being the hallmark of ACLF.

In conclusion, this pilot study showed that patients with ACLF had dysregulation in systemic immune cells including leukocytosis fueled by neutrophils and a monocyte/macrophage subset, and decreases in lymphocyte count related to a depletion in memory lymphocytes, CD8 T cells, and NK cells. All these lymphocyte alterations, along with blood enrichment in neutrophils defective in superoxide anion production, may contribute to immunosuppression in ACLF. Finally, our study suggests the existence of targets for novel therapeutic approaches for decreasing the risk of infection among patients with ACLF.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repositories and accession numbers can be found here: https://www.ncbi.nlm.nih.gov/geo/, GSE142255 (whole-blood samples); https://www.ncbi.nlm.nih.gov/geo/, GSE142254 (neutrophil samples).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de protection des personnes Ile de France III N°3194 and Comité d'Evaluation de l'Ethique des projets de Recherche Biomédicale (CEERB) Paris Nord (IRB 00006477 n° 13-043 in France F.25/5/81/ILBS/AC/2015/910 in India). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concept and design (RM, EW, PG). Acquisition of transcriptomic data (PG, AJ). Acquisition of clinical data and samples (EW, MD, LM, SS, BA, HG, MT, GM, CF, P-ER). Bioinformatic and statistical analyses (PG, JL, FA, AJ, EW, RM). Acquisition of *ex vivo* data (PH, LM, MT, JP, SS, JC). Integration of clinical and biological results and interpretation of data (PG, JL, FA, AJ, P-ER, EW, RM). Drafting of the manuscript (RM, EW, PG). Critical revision of the manuscript for important intellectual content (JC, AP, GM, RJ, CF, P-ER, SL, VA, FD). Study supervision (RM). All authors contributed to the article 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/fimmu.2020.619039/full#supplementary-material

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REVIEW
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Toll-Like Receptors Recognize Intestinal Microbes in Liver Cirrhosis

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Liver cirrhosis is one major cause of mortality in the clinic, and treatment of this disease is an arduous task. The scenario will be even getting worse with increasing alcohol consumption and obesity in the current lifestyle. To date, we have no medicines to cure cirrhosis. Although many etiologies are associated with cirrhosis, abnormal intestinal microbe flora (termed dysbiosis) is a common feature in cirrhosis regardless of the causes. Toll-like receptors (TLRs), one evolutional conserved family of pattern recognition receptors in the innate immune systems, play a central role in maintaining the homeostasis of intestinal microbiota and inducing immune responses by recognizing both commensal and pathogenic microbes. Remarkably, recent studies found that correction of intestinal flora imbalance could change the progress of liver cirrhosis. Therefore, correction of intestinal dysbiosis and targeting TLRs can provide novel and promising strategies in the treatment of liver cirrhosis. Here we summarize the recent advances in the related topics. Investigating the relationship among innate immunity TLRs, intestinal flora disorders, and liver cirrhosis and exploring the underlying regulatory mechanisms will assuredly have a bright future for both basic and clinical research.

Keywords: liver cirrhosis, dysbiosis, gut-liver axis, bacterial translocation, toll-like receptors

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INTRODUCTION

Cirrhosis is one of the leading causes of death in clinics among all digestive diseases (1). Cirrhosis refers to a process of a diffuse, progressive, fibrosing, nodular condition in liver tissue, and is a leading cause of chronic liver failure. Many different causes can induce liver cirrhosis (2–11). Cirrhosis progression can be divided into the compensatory stage and decompensated stage. As a result of the late stage, serious complications will occur, and death can hardly be avoided. There are no cure medicines to treat decompensated cirrhosis except for liver transplantation, which needs a rarely available donor liver. Although it is well known that viruses, alcohol, and non-alcoholic steatohepatitis (NASH) are closely associated with the development of cirrhosis, the exact molecular mechanism is still poorly understood. But, regardless of any causes, recent studies have shown that one common feature in cirrhosis is the alteration of gut microbiota, or dysbiosis, which is worsened with the severity of cirrhosis (12).

Intestinal microbes include bacteria, viruses, fungi, parasites, and archaea. In which bacteria are well studied and perhaps play a central role among them (13). In recent years, studies have been found that the correction of altered intestinal flora can delay or maybe revert the progress of

cirrhosis (14), and toll-like receptors recognize altered gut microbes to modulate the occurrence, development, and treatment of liver cirrhosis (15).

TLRs are one kind of critical innate immunity pattern recognized receptors (PRRs) that recognize not only pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) but also endogenous damage-associated molecular pattern molecules (DAMPs) (16, 17). Among the TLR family, some TLRs recognize different patterns in bacteria, including TLR1/2 or TLR2/6, TLR4, TLR5, and TLR9, recognizing bacterial lipoproteins, LPS, flagellin, and CpG DNA, respectively (18). TLRs recognize altered microbe in cirrhosis to activate TLR signaling pathway. TLR activation appears to be a critical molecular mechanism and is strongly associated with the progression of the diseases. Therefore, the investigation of the interaction between TLRs and the altered microbe in cirrhosis represents a promising strategy for developing an effective treatment against the deadly cirrhosis. Here we discuss the current understanding of the connection and research advances among dysbiosis, TLR recognition, and cirrhosis.

ALTERED GUT MICROBIOTA IN LIVER CIRRHOSIS

The human gastrointestinal (GI) tract, with about 200–300 m^2 mucosal surface, is continuously exposed to not only food but also microorganisms. For a healthy person, there are about trillions of microorganisms in the intestine. The gut microorganisms include not only bacteria, but also fungi and viruses, archaea, and parasites. The composition of the human intestinal flora is variable among different individuals and is influenced by many factors, including genetic variation, diet, alcohol consumption, aging, disease status, and whether antibiotics are applied (19, 20). However, the total of quantities and qualities is relatively stable in healthy people. In addition to performing their respective biological functions, the intestinal flora constantly interacts with the host; these interactions affect physiological, immunological, and pathological processes in a variety of cells and tissues (21, 22). One of the recent remarkable discoveries in cirrhosis patients, regardless of causes, is the considerable alteration of microbiota composition in the gut (called dysbiosis) (**Figure 1**) (23, 24).

Although a vast number and species of microorganisms colonize in the human intestines, the healthy gut microbiome is dominated by only some bacterial species, and the quantitative and qualitative changes in cirrhosis, including increased *Proteobacteria* and *Fusobacteria* and reduced *Bacteroidetes* (24). Meanwhile, it has been proved by the analysis of fecal bacterial content that the intestinal flora of the animal liver cirrhosis model and the human liver cirrhosis model will have a significant reduction in microbial diversity compared with the normal human intestinal flora (25, 26). Besides, a reduction in *Clostridium* resulted in significant pro-inflammatory symptoms and was inversely correlated with the Child-Pugh score (27). Furthermore, the specific correlation of mucosal-associated flora

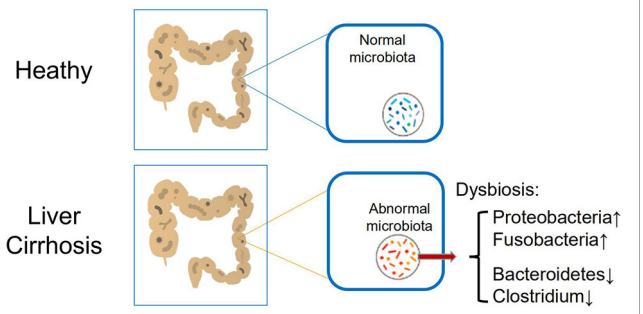

FIGURE 1 | Main alteration of intestinal microbiome in patients with cirrhosis, including increased *Proteobacteria* and *Fusobacteria*, and reduced *Bacteroidetes* and *Clostridium*.

changes has been confirmed by significant differences between patients with cirrhosis and patients with hepatic encephalopathy (see below).

How to understand the altered intestinal microbes in liver cirrhosis? Currently, it is still a topic of debate about which comes first between the changing gut microbiota and liver cirrhosis. Liver cirrhosis may induce the alteration of microbes in the intestine, but we assume that the gut microbiota may change first. The alteration of gut microbiota is then recognized by innate immunity receptors such as Toll-like receptors to induce a serial cascade of immune responses and to gradually affect liver development of cirrhosis.

TOLL-LIKE RECEPTORS RECOGNIZED INTESTINAL MICROBIOTA IN LIVER CIRRHOSIS

One of the most exciting and revolutionary discoveries in modern life science and medicine is to realize how important our innate immunity to human health and diseases. In 1989, Charles Janeway first proposed a concept of the Pattern Recognition Receptor (PRR), which recognizes pathogen-associated molecular patterns (PAMPs) to activate not only innate immunity but also adaptive immunity (28). Driven by this hypothesis, the Toll-like receptor (TLR) is the first identified PRR. TLRs are a type-I transmembrane protein capable of sensing pathogen infection. PRRs not only include Toll-like receptors (TLRs) but also include Nucleotide-binding oligomerization domain-like receptors (NLRs), C-type lectin receptors (CLRs), and Retinoic acid-inducible gene I-like receptors (RLRs). Among these PRRs, TLRs are the main receptors that recognize intestinal bacteria. It has been reported that TLRs are very important in the occurrence, development, and treatment of liver cirrhosis, but the most important role of TLRs in cirrhosis is perhaps to identify intestinal flora to trigger signaling cascades which link to the progress of liver cirrhosis.

A TLR is the key element of the innate immune system. Among 10 human TLRs, TLR2, TLR4, TLR5, and TLR9

recognize bacterial infection (**Figure 2**). TLR4 has been the most studied. Lipopolysaccharide (LPS) is a bacterial wall component from Gram-negative bacteria and is a common natural ligand of TLR4. TLR4-LPS association requires LPS binding protein, CD14, and myeloid differentiation protein 2 (MD2) to recognize LPS (29). TLR4 activated cell signal transduction has been well confirmed, mainly involving bone marrow differentiation primary response 88 (MyD88) dependent and MyD88 independent. Dependent signal transduction, which promotes mitogen-activated protein kinase and JNK aggregation to produce inflammatory activation through nuclear factor (NF-κB) (30). Cell death induced by these pathways further stimulates the recruitment of neutrophils and other inflammatory cells to the liver (31). Intestinal flora affects intestinal permeability and increases endotoxin load in portal vein circulation (32). This endotoxemia level does not produce septicemia syndrome, but it can produce a systemic pro-inflammatory and fibrotic environment, in which insulin signal transduction is damaged, resulting in increased net fat decomposition in adipose tissue and the transport of free fatty acids (FFA) from adipose tissue to the liver (33). Once excessive lipid is exposed to cellular stress in the liver, it is further expanded through intrahepatic and systemic proinflammatory environments. After that, it showed an acute reaction, hepatic fibrosis, and further progress into liver cirrhosis (34). In contrast to TLR4 recognizing Gram-negative bacteria, TLR2, heterodimer with TLR1 or TLR6, mainly recognizes Gram-positive bacteria. TLR5 and TLR9 recognize both Gram-positive and Gram-negative bacterial flagellin and CpG DNA, respectively.

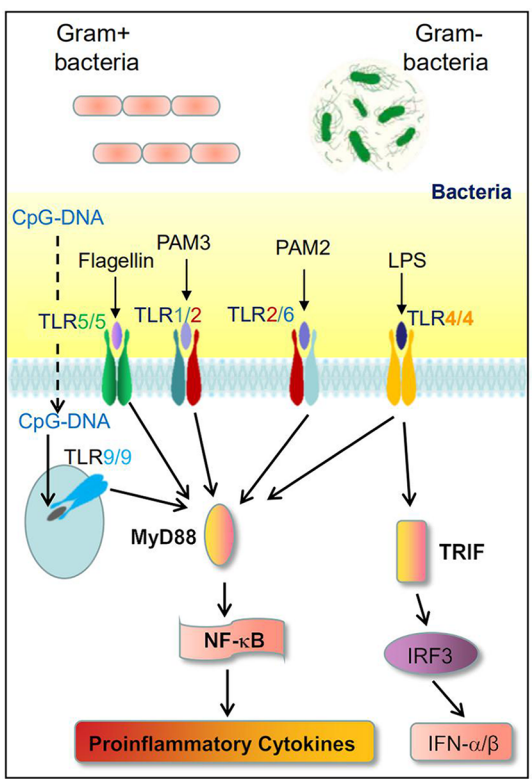

FIGURE 2 | TLRs recognize gut bacteria to induce immune response.

A lot of evidence has shown that the intestinal flora may play a major role in the pathogenesis of cirrhosis. As mentioned above, we are still not sure which one occurs first between the altered intestinal microbiome and liver cirrhosis. Next, we will discuss the potential mechanism underlying the dysbiosis in cirrhosis.

UNDERSTANDING THE LINK BETWEEN INTESTINAL MICROBIOTA AND LIVER CIRRHOSIS

As to how the intestinal flora causes cirrhosis, the following mechanisms have been proposed. The first mechanism mainly involves Toll-like receptors. As discussed above, intestinal flora in cirrhotic patients changes significantly, thus breaking the normal balance. It is still difficult to determine whether the intestinal flora changes before cirrhosis, or whether cirrhosis occurs first and then intestinal flora imbalance occurs. However, it can be determined that the imbalance of intestinal flora will cause inflammation, which can lead to and aggravate liver fibrosis. Intestinal flora imbalance triggers inflammation by activating PRR (35). The most common class of PRR is Toll-like receptors. The liver has strong innate immunity in the human body, and many TLRs are expressed and can be activated in the liver (36), which will not be discussed in detail here. Briefly, multiple TLRs play a critical role in the liver in monitoring and eliminating the invasion of pathogens under normal physiological conditions. While certain microbial-derived molecules can be tolerated for the monitoring and elimination of the liver, the liver cells may produce an immune response against these potentially harmful stimuli, thereby producing an immune response (37). Furthermore, TLRs have been found in many types of hepatocytes, including the bile duct epithelium, the dendritic cells, the endothelial cells, the stellate cells, Kupffer cells, and hepatocytes (38).

The second mechanism involves bacterial translocation. Because of abnormal immune defense, cirrhotic patients are prone to bacterial infection, especially those with advanced liver disease (Child-Pugh B and C). The Child-Pugh score consists of five clinical features and is used to assess the prognosis of liver cirrhosis, in which Child-Pugh B: 7–9 points (significant functional compromise); Child-Pugh C: 10–15 points (decompensated disease). The main causes of infection in patients with liver cirrhosis may be the decrease of phagocytic activity of bacterial translocation, reticular endothelial system from intestinal to mesenteric lymph nodes, and the decrease of antibacterial activity of ascitic fluid (39). The current pathogenic mechanism to explain the passage of bacteria or their products from the intestinal lumen through the intestinal barrier and to mesenteric lymph nodes is defined as bacterial translocation (BT). Generally proved by positive bacterial culture in mesenteric lymph nodes (40). BT also was found in patients with liver cirrhosis who

underwent laparotomy (41). Patients with advanced liver disease occur more frequently. Besides, in recent years, it has been shown that BT in patients with liver cirrhosis is related to factors other than bacterial infection, such as coagulation, renal dysfunction, and multiple organ failure (42).

The changes of BT in patients with liver cirrhosis may be due to multiple reasons including intestinal bacterial overgrowth (IBO), intestinal barrier damage, and local immune defense. It has been observed in the experimental model of liver cirrhosis and reaction of the bacterial overgrowth in patients with cirrhosis of the liver, the main growth of bacteria is aerobic Gram-negative bacteria (25), which is closely related to the development of BT and Spontaneous bacterial peritonitis (SBP). Oral antibiotics, which can inhibit Gram-negative aerobic gut bacteria, can significantly reduce the incidence of SBP in patients with cirrhosis and liver cirrhosis models of IBO, BT, and the incidence of bacterial peritonitis (43).

The most important way to prevent IBO in the small intestine is to maintain normal gastric secretion, normal intestinal movement, and ileocecal valve integrity. Inhibition of gastric acid secretion is associated with IBO, especially in the elderly (44). However, recent data show that short-term treatment of neonatal rats with H2 receptor antagonists is not conducive to the reduction of BT (45).

Decompensated cirrhotic patients often receive long-term treatment with anti-H2 receptor antagonists, which can prevent gastrointestinal bleeding caused by acute erosion of gastric mucosa or esophageal scar bleeding after endoscopic treatment of esophageal varices. Recent data show that patients with liver cirrhosis treated with gastric acid secretion inhibitors for a long time have a higher incidence of IBO, especially in patients with advanced liver disease (42). A recent retrospective study showed an increase in the incidence of SBP in patients treated with proton pump inhibitors for gastric acid inhibition (45).

MECHANISMS UNDERLYING THE DYSBIOSIS IN CIRRHOSIS

What is the underlying mechanism underlying the dysbiosis in cirrhosis? There are no clear answers yet, but there are some different pieces of evidence to support the different approaches for the alterations of microbe in the gut of cirrhosis patients. One of the resources of these bacteria mainly came from oral. Thirteen species of microbe in cirrhosis gut were closest to oral isolates. It is concluded that oral flora may invade the intestines of patients with liver cirrhosis and cause intestinal flora changes (46). Another possible mechanism is because of the bile resistance of human intestinal bacteria, the decrease of bile production in patients with liver cirrhosis makes the intestine more likely to be allowed or close to by "foreign" bacteria (47). Some experiments have also found strains such as *Campylobacter jejunum* and parainfluenza in the intestinal flora of patients. Pathogens such as *Hemophilus*, which may invade the intestines through oral pathways or contaminated food. Invasive foreign bacteria can grow not only in the colon, but also in the ileum, and contribute to the excessive growth of bacteria in the small intestine associated with liver cirrhosis, thus aggravating the development of liver cirrhosis (48).

The newly identified mechanism is Small intestinal bacterial overgrowth (SIBO). SIBO is defined as the number of bacteria in the small intestine $>10^5$ CFU/ml or the presence of colon bacteria in the upper Jejunum secretions. According to this standard, the experiment found that the incidence of SIBO in patients with liver cirrhosis was between 48 and 73% (49, 50). SIBO is particularly common in patients with severe liver cirrhosis and patients with spontaneous peritonitis or a history of hepatic brain disease. In the late stage of liver cirrhosis, SIBO is related to the occurrence and development of BT, SBP, and endotoxemia. In liver cirrhosis, SIBO is one of the main factors that promote BT, and the occurrence of BT in mesenteric lymph nodes is usually related to SIBO in experimental models (51). However, BT does not occur in as many as half of SIBO's cirrhotic models, so it seems that SIBO is permissible, but not enough in itself to cause BT to occur. Therefore, other factors, most likely, the decline in local immunity may play the most important role in inducing BT.

SIBO in liver cirrhosis is partly due to the movement of the small intestine and the decrease of the time passing through the intestine (52). The possible catalytic effect of proton pump inhibitors on SIBO and SBP has recently been questioned in many patients with liver cirrhosis. Nevertheless, it has been found that gastric acid deficiency has been observed in patients with liver cirrhosis without acid inhibitor used, resulting in an increase in pH in the small intestine, which promotes the production of SIBO (42).

It can be seen that the changes in the number and species of intestinal bacteria are related to liver cirrhosis, and maintaining the stability of intestinal bacteria plays an important role in controlling the occurrence and development of liver cirrhosis (53).

Bile acid is a new player to regulate intestinal flora. Although intestinal microbial ecological disorders and some intestinal flora may cause or aggravate liver cirrhosis, some reports in recent literature have shown that intestinal flora and its metabolites may have protective effects on the liver (54, 55). It has been recognized that both primary (hepatocytes derived) and secondary (microbial modified) bile acid (BA) act as signaling molecules in the human body. BA modulates its synthesis and regulation of key metabolic pathways and inflammatory responses by activating Farnesoid X receptor (FXR) (preferred primary BAs) and G protein-coupled receptors. Although the synthesis of BA is regulated by primary BA metabolism, the composition of intestinal flora is regulated by secondary BA. Therefore, the composition of BA, intestinal flora, and the delicate balance of living FXR activation has a profound effect on liver metabolism, anti-inflammation, and hepatoprotective process. For this reason, FXR continues to be explored as a potential treatment for liver cirrhosis in animal models and clinical trials. The above findings provide evidence for the protective effect of intestinal flora and its metabolites in the pathobiology of liver cirrhosis and disease (53, 56).

INTESTINAL FLORA ALTERATION IS ASSOCIATED WITH LIVER CIRRHOSIS COMPLICATIONS

The biggest challenge for cirrhosis is developing complications. The novel strategy to revert, or even slow down the progression is desperately needed in the clinic. With the progressive development of cirrhosis from compensated stage to decompensated stage, complications of liver cirrhosis are going to occur and are most likely also related to the more severe alteration of intestinal flora, including lower levers of Firmicutes and higher levers of *Bacteroidetes* (57). The gut flora plays a role in the development of infections and the pathogenesis of hepatic encephalopathy. Hepatic encephalopathy is characterized mainly by hyperammonemia. The increase of ammonia content in patients with hepatic encephalopathy may be due to the increase of ammonia production caused by liver dysfunction and intestinal bacterial disorder. The regulation of intestinal flora aims at reducing the number of ammonia-producing bacteria in the intestinal tract and reducing the production of ammonia, thus providing a new treatment for hepatic encephalopathy (57).

Spontaneous bacterial peritonitis is another complication of liver cirrhosis. It occurs due to the migration of intestinal bacteria to the abdominal cavity. Due to the combined effects of increased intestinal permeability and bacterial overgrowth, the risk increases with the progress of liver cirrhosis. As hepatic Encephalopathy, the standard treatment of spontaneous bacterial peritonitis also includes antibacterial therapy. When the intestinal flora is adjusted, the occurrence of spontaneous bacterial peritonitis may be reduced with decreasing in BT (58).

Bacillosis with liver cirrhosis is mainly caused by excessive growth of intestinal bacteria, immune dysfunction, and decreased bactericidal activity of phagocytes. Bacterial translocation leads to the damage of local or systemic immune defense mechanisms and plays an important role in the development of liver cirrhosis (59). Bacterial products lead to monocytes, lymphocytes activation, elevated serum TNF-α levels, inflammatory cytokines, and nitric oxide (NO) activation (60). The activation and elevated serum levels of NO lead to systemic vascular dilatation, increased cardiac output, decreased mean arterial pressure, and lead to complications of liver Cirrhosis, such as varicose veins, ascites, and hepatorenal syndrome (59, 61).

DEVELOPMENT OF NOVEL TREATMENT FOR CIRRHOSIS BY TARGETING MICROBIOTA AND TLRS AGAINST CIRRHOSIS

It is still a big challenge for the treatment of cirrhosis, and no cure medicines are available currently. The case with a clear diagnosis of cirrhosis by identifying liver pseudolobule is almost impossible to reverse the fibrosis to normal liver and the process of liver from compensating to decompensate and loss the function of liver almost cannot avoid eventually. The novel strategy/treatment is desperately needed in the clinic. Considering the gut dysbiosis is associated with the development of CLD, and then correcting the dysbiosis by modulating the gut microbiota may alter the course of the disease. Methods of modulating the gut microbiota include dietary modifications, antibiotic use, probiotics, prebiotics, and fecal microbial transplantation (FMT).

Targeting Microbiome

The progress of liver cirrhosis can be slowed down by regulating intestinal flora (56). The main focus of the prevention of bacterial infections is the use of antibiotics prophylactically. Enterobacteriaceae and Streptococcus non-enterococci are the most common pathogenic microorganisms in liver cirrhosis, and the use of antibiotics is mainly aimed at these bacteria. Intestinal bacterial overgrowth in patients with liver cirrhosis is considered to be that intestinal is the most common site of bacterial translocation (62). Rifaximin is a broad-spectrum antibiotic which eliminates intestinal microorganisms non-selectively. Rifaximin can also directly affect bacterial function by weakening the translocation ability of intestinal flora (63). The activity of rifaximin is specific in the intestinal tract, and the experiment shows that it is not absorbed into the whole-body circulation, which reduces the toxicity or side effects of rifaximin in the whole body. Rifaximin has also been shown to have a remission effect on hepatic encephalopathy in patients with liver cirrhosis and has a beneficial trend in the control of infection and bleeding rate of varicose veins (64).

Another approach to modulate microbiota is utilizing lactulose, an approved laxative. The analysis of the therapeutic effect of lactulose in the study of hepatic encephalopathy showed that the withdrawal of lactulose led to cognitive deterioration, the decrease of bacterial content in feces, and the increase of glutamic acid and glutamine in the brain (65, 66). Therefore, it seems that the regulation of intestinal flora imbalance such as by rifaximin and lactulose is a promising strategy in the treatment of cirrhosis and hepatic encephalopathy.

Lactobacillus is protective against intestinal mucosa by lowering intestinal pH. They can prevent the establishment of pathogenic species and regulate the immune response and maintain the steady-state of intestinal flora. Improve overall intestinal function (67). In the study of the liver cirrhosis model, the application of lactobacteria has been shown to effectively reduce bacterial translocation and decrease the level of serum alanine aminotransferase, thus reducing the progress of cirrhosis (68).

Fecal microbial transplantation (FMT), as a mature treatment for refractory clostridium infection (69), and as a potential solution to reverse gut dysbiosis and its downstream complications in cirrhosis. A recent study showed that FMT can reduce mouse steatohepatitis by reducing pro-inflammatory cytokines in the liver and endotoxemia (70). In theory, FMT may also influence the disease's trajectory and may alter the pathophysiology of the disease by reducing inflammation and fibrosis in the liver.

Targeting TLRs

It appears that modulating microbiome can be utilized as an effective treatment against cirrhosis, and targeting the host receptors for these microbiomes may work too. Previous studies have reported targeted TLR therapy for liver disease. In an experimental model of chronic liver fibrosis, several toll-like receptors (TLRs) are needed to make mice sensitive to liver fibrosis. It was speculated that the ligands of TLR were bacterial products from the intestinal microbiome, and TLR knockout mice had resistance to liver inflammation and fibrosis (71). The intestinal microbiota plays an important role in the pathogenesis of both nonalcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC). Moreover, TLR4 expression, alterations of bile acid metabolism, inflammatory cytokines released, may promote NAFLD-associated HCC (72). Therefore, potential targets for the prevention of HCC from NAFLD may include the composition of the microbiota, metabolites, and inhibition of TLRs. The relationship of intestinal microbiota with fibrosis development by identifying fibronectin as a TLR4 dependent mediator of the matrix and vascular changes that characterize cirrhosis (73). Intestinal sterilization confined to the late stage of HCC can reduce the occurrence of HCC, suggesting that the intestinal microbiota and TLR4 are therapeutic targets for HCC prevention in advanced liver disease (74).

However, many other approaches targeting TLRs to treat liver cirrhosis. Many TLR subtypes have been found in humans and are associated with oxidative stress (75). Oxidative stress reflects the imbalance between the production of reactive oxygen species (ROS) and the scavenging capacity of the antioxidant system. ROS leads to the progression of end-stage liver disease finally, and crosstalk between TLR and NADPH oxidase (NOX) is associated with fibrogenesis. The inhibitory effect of TLR-mediated oxidative stress in alleviating liver fibrosis has been demonstrated by a variety of natural drugs. Interestingly, the activity of natural derivatives was mainly inhibition of TLR4.

In preclinical studies, many natural drugs inhibit TLR4 or related signaling pathways to reduce oxidative stress, liver inflammation, and fibrosis (76). Besides, several compounds affecting TLR, namely curcumin, quercetin, probiotics, and have been found to have clinical effects on liver fibrosis-related diseases. Unfortunately, the link between the therapeutic efficacy of these compounds and TLR-related pathways has not been systematically tested in humans (77–79). The related inhibition of TGF- β1/Smad2 and TLR4/NF-κB p50 pathways with the prevention of liver inflammation and fibrosis emphasizes its potential as a therapeutic strategy for liver fibrosis (80).

Both probiotics and angiotensin-II type 1 receptor blocker (ARB) could inhibit hepatic fibrosis, accompanied by activation of hepatic stellate cells and inhibition of liver-specific transforming growth factor-β and TLR4 expression (81). Besides, ARB can improve hepatic fibrosis and inhibit the TLR4 signal through the angiotensin-II-mediated LPS-TLR4 signal (82).

CONCLUDING REMARKS

The homeostasis of intestinal flora plays an important role in the human body and can be involved in the regulation of various functions of the human body. The homeostasis of intestinal flora has a protective effect on the liver. The imbalance of intestinal flora will be recognized by innate immune receptors such as Toll-like receptors. A chronic inflammatory response will destroy the liver, and will gradually aggravate liver cirrhosis, and promote the occurrence and development of complications of liver cirrhosis. Existing clinical evidence shows that drug treatment of intestinal flora disorders can slow down the progress of liver cirrhosis. With the research of intestinal flora affecting the development and progress of liver cirrhosis, it provides a direction for clinical treatment. The further study of flora composition and more human research will further enrich the clinical treatment and development and provide more treatment for delaying the progress of liver cirrhosis.

AUTHOR CONTRIBUTIONS

YF and YL wrote the manuscript. YC, JL, LC, and DZ wrote and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Granulocyte-Macrophage Colony-Stimulating Factor Modulates Myeloid-Derived Suppressor Cells and Treg Activity in Decompensated Cirrhotic Patients With Sepsis

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Background: Decompensated cirrhosis patients are more prone to bacterial infections. Myeloid-derived suppressor cells (MDSCs) expand in sepsis patients and disrupt immune cell functions. Granulocyte-macrophage colony-stimulating factor (GM-CSF) therapy helps in restoring immune cell functions and resolving infections. Its role in MDSC modulation in cirrhosis with sepsis is not well understood.

Methods: A total of 164 decompensated cirrhotic—62 without (w/o), 72 with sepsis, and 30 with sepsis treated with GM-CSF—and 15 healthy were studied. High-dimensional flow cytometry was performed to analyze MDSCs, monocytes, neutrophils, CD4 T cells, and Tregs at admission and on days 3 and day 7. *Ex vivo* co-cultured MDSCs with T cells were assessed for proliferation and apoptosis of T cells and differentiation to Tregs. Plasma factors and mRNA levels were analyzed by cytokine-bead assay and qRT-PCR.

Results: Frequencies of MDSCs and Tregs were significantly increased ($p = 0.011$ and $p = 0.02$) with decreased CD4 T cells ($p = 0.01$) in sepsis than w/o sepsis and healthy controls (HCs) ($p = 0.000$, $p = 0.07$, and $p = 0.01$) at day 0 and day 7. In sepsis patients, MDSCs had increased IL-10, Arg1, and iNOS mRNA levels ($p = 0.016$, $p = 0.043$, and $p = 0.045$). *Ex vivo* co-cultured MDSCs with T cells drove T-cell apoptosis ($p = 0.03$, $p = 0.03$) with decreased T-cell proliferation and enhanced FOXP3$^+$ expression ($p = 0.044$ and $p = 0.043$) in sepsis compared to w/o sepsis at day 0. Moreover, blocking the MDSCs with inhibitors suppressed FOXP3 expression. GM-CSF treatment in sepsis patients significantly decreased MDSCs and FOXP3$^+$ Tregs but increased CD4 T-cell functionality and improved survival.

Conclusion: MDSCs have an immunosuppressive function by expanding FOXP3$^+$ Tregs and inhibiting CD4$^+$ T-cell proliferation in sepsis. GM-CSF treatment suppressed MDSCs, improved T-cell functionality, and reduced Tregs in circulation.

Keywords: myeloid-derived suppressor cells, sepsis, GM-CSF, Tregs, immune modulation, liver cirrhosis

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INTRODUCTION

Sepsis ranges from any infection to septic shock, and cirrhosis has been recognized as an independent mortality risk factor in septic shock patients (1). The development of sepsis in cirrhosis patients considerably increases both short- and long-term mortality due to immunological changes and systemic hemodynamics, while in-hospital mortality of cirrhosis patients with septic shock is higher, i.e., more than 70% (2).

Liver cirrhosis generally shows cirrhosis-associated immune dysfunction (CAID) by altering both innate and adaptive immunity (3). Impaired neutrophil and monocyte phagocytic ability and decreased HLADR expression of monocytes are the hallmarks of alcoholic liver cirrhosis patients. This disability in innate immunity leads to dysfunctional B and T cells in cirrhosis patients (4).

It is observed that defective myelopoiesis drives immature myeloid cells in the generation of myeloid-derived suppressor cells (MDSCs), instead of monocytes, dendritic cells (DCs), and neutrophils (5). MDSCs are heterogeneous in nature, and based on the expression of CD14, CD15, CD11b, CD33, and HLADR, they are distinctly characterized as of monocytic (M-MDSCs; $CD14^{+ve}CD11b^{hi}CD33^{+ve}HLADR^{lo}$) and granulocytic (G-MDSCs; $CD14^{-ve}$ $CD15^{+ve}CD11b^{hi}CD33^{+ve}HLADR^{lo}$) lineages (6). However, M-MDSCs and G-MDSCs drive their suppressive activities in two different ways. M-MDSCs secrete low ARG1 but suppress other cells by iNOS-mediated STAT1 nitration in an antigen-specific and non-specific manner (7). While G-MDSC function specifically in an antigen-specific manner with hyperactivation of ARG1, reactive oxygen species (ROS), NO, and superoxide producing peroxynitrite (PNT) (8). Furthermore, like Tregs, MDSCs are also suppressive in nature. In fact, both MDSCs and Tregs support each other: Tregs control the differentiation and function of MDSCs, while in return MDSCs help in Treg expansion (9, 10).

However, in the tumor microenvironment, secreted granulocyte-macrophage colony-stimulating factor (GM-CSF) recruits PD-L1 expressing immune-suppressive MDSCs, and blocking of GM-CSF reduced the IDO and PD-L1 expression in liver MDSCs (11). However, it was reported earlier that adding GM-CSF to the standard care reduced the infectious complications and shortened the antibiotic therapy duration in abdominal sepsis patients (12, 13). GM-CSF not only improves total leukocyte counts (TLCs) but also reversed the monocytic deactivation by increasing HLADR and TLR4 expression in sepsis patients. Furthermore, GM-CSF treatment was also correlated with increased anti-inflammatory cytokine production with less need for mechanical ventilation and longer hospital stay (12).

However, there is limited knowledge about MDSCs and their functionality in liver cirrhosis patients with and w/o sepsis. Therefore, the aim of the present study was i) to investigate the role of MDSCs in immune dysfunction and ii) modulation of MDSCs, T cells, and Tregs with GM-CSF therapy in decompensated cirrhosis (DC) patients with sepsis, which may have an impact on disease pathogenesis and patient survival.

METHODS

Study Groups and Blood Sampling

A total of 164 DC patients were enrolled: without (w/o) sepsis (n = 62), with sepsis (n = 72), with sepsis and treated with GM-CSF (n = 30), and healthy controls (HCs; n = 15) at the Institute of Liver and Biliary Sciences (ILBS), New Delhi, between 2017 and 2020 (**Supplementary Figure 1**). In an ongoing randomized controlled trial, DC patients with sepsis were given 250 µg of GM-CSF intravenously for about 6 h daily for 5 days. All the patients received standard medical treatment that included nutrition, antibiotics, and supportive care as part of standard medical treatment.

This study was approved by the Research and Institutional ethics committee with IEC No. IEC/2016/45/NA/C2, and informed consent was obtained from all the subjects enrolled in the study. In this longitudinal study, patients were closely monitored from admission and studied at baseline and on days 3 and 7. Patients with a history of any hepatitis infection [hepatitis B virus (HBV), hepatitis C virus (HCV), etc.], with HCC or any other site malignancy or any other comorbidities, and gave no consent were excluded from the study. This study was carried out in accordance with the ethical standards of the Declaration of Helsinki.

The clinical as well as biochemical assessment of all the enrolled patients with or w/o systemic inflammatory response syndrome (SIRS) or sepsis was done according to the treating physician's direction. Patients were recruited based on the following criteria: DC w/o sepsis was diagnosed when there was no evidence of SIRS or infection on any of the cultures and ultrasonography; DC with sepsis was diagnosed based on the presence of SIRS and infection, confirmed either by cultures or by imaging.

Four criteria for the diagnosis of SIRS were used (14):

a) Temperature >38.0°C or <36.0°C
b) TLC <4 or >12 × 10^9/L
c) Pulse > 90 beats/min
d) Respiratory rate (RR) >20 or <32 beats/min

Blood Sampling

Peripheral blood measuring 15–20 ml was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes from patients and healthy subjects at the time of recruitment in the study. From part of the blood, plasma was separated and stored at −80°C for assessment of cytokines, endotoxin, and other circulating analytes.

Multiparametric Whole Blood Immune Phenotyping

MDSCs, T cells, and Tregs were characterized in whole blood using specific antibodies against surface and intracellular markers labeled with different fluorochromes. MDSCs were characterized using anti-CD14 at 1:40 dilution (Clone M5E2, fluorescein isothiocyanate (FITC), 555397; BD Biosciences, San Jose, CA, USA), anti-CD11b at 1:100 dilution (Clone ICRF44,

antigen-presenting cells (APCs), 301322; BioLegend, San Diego, CA, USA), anti-CD33 at 1:40 dilution (Clone P67.6, PE-Cy7, BioLegend 366618), and anti-HLADR at 1:40 dilution (Clone L243, BV510, BioLegend 307646); naïve and memory T cells by anti-CD4 at 1:40 dilution (Clone SK3, PE-Cy7 BD Biosciences 557852), anti-CD8 at 1:40 dilution (Clone SK1,V510, BD BioLegend 344732), anti-CCR7 at 1:20 dilution (Clone G043H7, FITC, BioLegend 353216), and anti-CD45RA at 1:20 dilution (Clone HI100, V410, BioLegend 304130); and Tregs by anti-CD4 at 1:40 dilution (Clone OKT4,V410, BioLegend 317434), anti-CD25 at 1:20 dilution (Clone, PE-Cy5, BioLegend 302608), anti-FOXP3 (Clone 259D/C7, PE, BD Biosciences 560046), and anti-CD127 (Clone 259D/C7, APC, BioLegend 351316). **Supplementary Table 1** shows the markers for types of T cells, MDSCs, and Tregs.

Briefly, 100–150 µl of whole blood was incubated with appropriate concentrations of surface antibodies for 30 min at room temperature (RT) in the dark. After that, cells were fixed with 200 µl of Cytofix (BD Biosciences, #554714) for 10 min and washed twice with 1× perm wash. Furthermore, cells were incubated with intracellular antibodies for 30 more minutes at RT and washed with 1× phosphate-buffered saline (PBS). A minimum of 100,000 events were acquired using a BD FACS VERSE, and all relevant data were analyzed by using FlowJo software version 10.

Analysis of Plasma Analytes Using Cytokine Multiplex Bead Array Assay

To understand the significance of various cytokines and growth factors linked to sepsis as well as MDSC, we investigated the concentrations of forty-one plasma cytokines, chemokine and growth factors such as IL-1RA, IL-1β, IL-2, IL-12 (p40), IL-27, IL-18, IL-10, IL-17A, IFN-γ, TNF-α, IL-4, IL-6, IL-33, TGF-β1, IL-8, MIF, MIP-1α, MIP-1β, MIP-3α, ITAC, MCP-1, FRACTALKINE, ENA78, IP-10, EOTAXIN, Angiopoietin, MCSF, G-CSF/CSF3, GM-CSF, HGF, LEPTIN, TPO, VEGF-A, MMP7, MMP8, MMP1, MMP12, MMP13, P SELECTIN, E SELECTIN, and TREM1 in different patient groups by using multiplex PROCATAPLEX (Thermo Fisher, Waltham, USA), following the complete details on Luminex xponent 3.1TM Rev. 2 (Luminex Corporation, Austin, TX, USA). A standard curve was drawn using the standards provided in the kit. Values in samples were determined corresponding to the standard curve drawn. The lower detection limit of each cytokine is listed in **Supplementary Table 2**.

Preparation of Myeloid-Derived Suppressor Cells, T Cells, and Adherent Monocytes

Part of the whole blood collected from patients of different groups was used for sorting MDSCs and $CD4^+$ T cells. Cells were stained with anti-CD11b APC, anti-CD33 PE-Cy7, anti-HLADR V410, and anti-CD4 FITC antibodies and incubated for 30 min at RT in the dark, which is followed by red blood cell (RBC) lysis. Sorting of MDSC and T cells was done using BD FACS ARIA Fusion (BD Biosciences).

However, adherent monocytes were generated using 5×10^6 isolated peripheral blood mononuclear cells (PBMCs). Cells were plated in complete Roswell Park Memorial Institute (RPMI) 1640 media with 10% fetal bovine serum (FBS) for 2 h. After 2 h, supernatant and non-adherent cells were removed, and adherent monocytes were removed for further experiments.

Myeloid-Derived Suppressor Cell Functionality

T-Cell Apoptosis

To analyze the functional modulatory role of MDSCs, sorted $CD4^+$ T cells were cultured with or without fluorescence-activated cell sorting (FACS)-sorted MDSCs or adherent monocytes for 3 days. For assessment of apoptosis of T-cell cultures, cells were removed after 3 days and stained with Annexin V for 15 min followed by propidium iodide (PI). The degree of apoptosis was assessed by flow cytometry using PI and Annexin V. Apoptotic cells were defined as PI–veAnnexin V+ve cells.

T-Cell Proliferation

The proliferation of T cells was assessed using a fluorescent cell staining dye, i.e., carboxyfluorescein succinimidyl ester (CFSE). A 96-well plate was coated overnight with anti-CD3 (0.5 µg/ml). Sorted CD4 T cells measuring 0.5×10^6/ml were stained with 1 µl of CFSE (5 mM of stock solution, #21888; Sigma-Aldrich, St. Louis, MO, USA) for 15 min in the dark, cells were centrifuged at 1,200 rpm for 7 min, and the supernatant was discarded. CFSE labeling was stopped by the addition of RPMI 1640 medium (GIBCO, Thermofisher Scientific, Massachusetts, United States #22400-089) supplemented with 10% FBS (Gibco). After several washes, CFSE-labelled T cells along with soluble anti-CD28 (1 µg/ml) were plated alone, with sorted MDSCs and adherent monocytes in anti-CD3 coated wells, which were later incubated for 3–5 more days. The percentage of proliferation was assessed by flow cytometry using CFSE.

Generation of Tregs Under Th0 and Th17 Conditions

For the Th0 condition, FACS-sorted CD4 T cells were cultured alone or with MDSCs (1:1) for 3 days under the Th0 condition [anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml)]. After 3 days, Tregs ($CD4^+FOXP3^+$ cells) were determined using flow cytometry.

For the Th17 condition, FACS-sorted CD4 T cells were cultured under Th17-proliferating conditions, i.e., recombinant TGF-β (5 ng/ml, #sc-52893; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and IL-6 (20 ng/ml, #200-06; PeproTech, Cranbury, NJ, USA) along with anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml) for 3 days. After 3 days, these cells were incubated alone or with MDSC (1:1 ratio) for 3 more days in RPMI 1640 complete media. After that, cells were stained with anti-IL-17 PE-Cy7 and anti-FOXP3 PE using intracellular cytometric analysis and determined using flow cytometry.

Inhibition of Myeloid-Derived Suppressor Cells *via* Inhibitors

To unravel the mechanism of the Treg expanding effect of MDSCs, CD4 T cells were cultured with MDSCs (at a 1:1

ratio) under the Th0 condition for 72 h in the presence or absence of recombinant TGF-β (10 μg/ml, #sc-52893; Santa Cruz Biotechnology, Inc.), L-NMMA (500 μM, Sigma-Aldrich, #M7033), an iNOS inhibitor, and nor-NOHA (500 μM, Sigma-Aldrich, #399275), an Arg1 inhibitor. After that, cells were stained with anti-FOXP3 PE using intracellular cytometric analysis.

Quantitative RT-PCR Analysis

FACS-sorted MDSCs and adherent monocytes were used to isolate total RNA using a miRVANA kit (Thermo Fisher, #AM1560). cDNA was prepared using a High-Capacity cDNA Kit (ABI, Thermo Fisher Scientific, Waltham, MA, USA; #4368813). The expression of various genes like IL-10, iNOS, and Arginase1 was checked. The primer sequence genes are listed in **Supplementary Table 3**. 18S RNA served as an endogenous control for normalization. Relative expression was analyzed using SYBR Green (Thermo Fisher, # A25742) in a VIIA7 real-time PCR machine (ABI, Whitefield, India).

Statistical Analysis

Data were analyzed using the statistical software Prism (version 6; GraphPad Software, San Diego, CA, USA) and SPSS version 22 (IBM Corp, Ltd., Armonk, NY, USA). The comparison for continuous data is carried out by using the one-way ANOVA/Kruskal–Wallis test followed by probability adjustment by the Mann–Whitney test or by Bonferroni test *post-hoc* comparison as appropriate, and it is represented as mean ± SD. *p*-values <0.05 were considered statistically significant. Data with unequal distribution were used as medians. Moreover, this multinomial logistic regression was also applied along with diagnostic tests (receiver operating characteristic (ROC) curve).

RESULTS

Baseline characteristics of 164 DC patients, 62 patients w/o sepsis (age 48 ± 5 years, 87% male), 72 patients with sepsis (42 ± 9, 97% male), and 15 age-matched healthy controls were analyzed at the time of admission and enrolment in the study. Alcohol was the predominant etiology (70%) in DC patients. Sepsis patients showed a significant increase in total bilirubin, aspartate aminotransferase (AST) levels, international normalized ratio (INR), procalcitonin (PCT), lactate, MELD Na, and creatinine as compared to w/o sepsis patients in **Table 1**. The whole blood immune scan revealed lymphopenia but increased neutrophils in sepsis patients (**Supplementary Figures 2A, B**).

Increase in Myeloid-Derived Suppressor Cells in Sepsis Patients

At the Time of Admission (Day 0)

Based on the gating strategy, expression of MDSCs ($CD11b^{+}CD33^{+}HLADR^{-ve}$) was significantly increased (p = 0.011) in sepsis patients compared to w/o sepsis patients. We have further characterized subsets of MDSCs, i.e., G-MDSCs and M-MDSCs. Expression of G-MDSCs ($CD11b^{+}CD33^{+}HLADR^{-ve}CD14^{-ve}$) was found to be significantly increased in sepsis patients (p = 0.005) compared to w/o sepsis patients, while M-MDSCs ($CD11b^{+}CD33^{+}HLADR^{-ve}CD14^{+ve}$) showed no significant difference between the groups (**Figures 1A, B**). A logistic regression model positively predicted an increase in MDSCs and G-MDSCs with high sensitivity and specificity (0.732, p = 0.003 and 0.744, p = 0.002) in sepsis patients compared to w/o sepsis patients (**Figure 1C**). A comparison of gating strategies is demonstrated by (6, 15) (**Supplementary Figure 3A**).

At Follow-Up Time Points

On follow-up on day 3 and day 7, there was a significant decrease in MDSCs and G-MDSCs in sepsis patients on day 7 (p = 0.04 and p = 0.01) compared to day 0, but no difference in M-MDSCs (**Supplementary Figure 3B**). There was no change on day 3.

Decrease in $CD4^{+}$ T Cells and Its Subsets While Increase in Tregs in Sepsis Patients

At the Time of Admission (Day 0)

The presence of MDSCs modulates T-cell differentiation (10); therefore, to analyze the impact of MDSCs on CD4 T cells and T-cell differentiation, we have used CD45RA and CCR7 markers to evaluate the presence of naïve, T_{CM}, T_{EM}, and T_{EMRA} in circulation (**Figure 2A**). Sepsis patients showed a significant decrease in the total percentage of $CD4^{+}$ T cells as compared to w/o sepsis patients and HCs (p = 0.000 and p = 0.01). When $CD4^{+}$ T-cell subsets were analyzed, it was observed that although naïve T cells were not found significantly different between the groups, T_{CM} was decreased in sepsis patients compared to w/o sepsis patients (p = 0.009) (**Figure 2B**). In fact, T_{EM} and T_{EMRA} populations were also decreased in the sepsis group, but this difference was observed as compared to HCs only (p = 0.000 and p = 0.001) but not with w/o sepsis patients (**Figure 2B**). A decrease in T_{CM} in sepsis was also positively correlated with increased total bilirubin levels (p = 0.04) (**Figure 2C**).

Furthermore, Tregs were increased in sepsis patients (p = 0.02 and p = 0.01) compared to w/o sepsis patients and HCs (**Figures 2D, E**). A logistic regression model positively predicted Tregs with high sensitivity and specificity (0.769, p = 0.003) in both sepsis and w/o sepsis patients. However, Tregs were found positively correlated with MELD scores in sepsis patients (p = 0.015) (**Figure 2F**).

At Follow-Up Time Points

No difference in percentages of total CD4 T cells, T_{NAIVE} T_{CM}, T_{EMRA}, and T_{EM} was observed in follow-up between the groups. However, somehow percentage frequencies of Tregs were significantly decreased in sepsis patients on day 3 and day 7 (p = 0.002 and p = 0.008) compared to day 0, which could be the effect of normal medical treatment in sepsis patients (**Supplementary Figures 4A–D**).

TABLE 1 | Baseline clinical as well as biochemical characteristics of study groups.

Median and range	Healthy control (N = 15)	DC w/o sepsis (N = 62)	DC with sepsis (N = 72)	*p*-Value in betweenw/o and with sepsis
Age	32 (20–40)	48 (22–62)	44 (29–60)	0.07
Male:female	11:4	54:8	70:2	–
Total bilirubin (mg/dl)	1 (0.3–1.5)	4.5 (1.6–24)	14.5 (2.2–31.7)	0.00
AST (IU/ml)	20 (5–40)	59.75 (31–510)	114 (31–1037)	0.05
ALT (IU/ml)	25 (10–40)	34.5 (20–634)	41.5 (11–233)	1.00
INR (s)	1 (0.8–1.2)	1.66 (1.1–3.3)	2.58 (1.58–6.75)	0.00
PCT (ng/ml)	0.8 (0.2–2)	0.42 (0.04–3.25)	8.4 (0.07–88.4)	0.03
Lactate (mmol/L)	1.5 (1–2)	1.4 (0.6–5.2)	2.1 (0.2–13.3)	0.02
Sodium (mmol/L)	140 (136–145)	133 (124.3–142.7)	131 (113.2–148.4)	1.00
Creatinine (mg/ml)	0.6 (0.2–1)	0.86 (0.3–2.9)	1.3 (0.3–5.18)	0.04
MELD Na	8 (6–10)	23 (10–37)	32.5 (14–40)	0.00
SIRS criteria				
TLC (10^9 L)	6 (4–11)	6.3 (3.1–19.8)	12.65 (2.7–43.6)	0.00
Pulse (/min)	70 (60–100)	84 (60–110)	94 (62–132)	0.00
RR (/min)	14 (12–16)	20 (16–24)	22 (14–34)	0.02
Temperature (F)	98 (97–99)	98.2 (97–98.9)	98.4 (96–100)	1.00
Differential leukocyte count				
Neutrophils (%)	60 (40–75)	70 (59–89)	81 (36–95)	0.00
Lymphocytes (%)	30 (20–45)	16 (3.4–36)	8 (1–29)	0.00
Monocytes (%)	5 (2–10)	11 (2–18)	8 (2–30)	0.03

DC, decompensated cirrhosis; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio; PCT, procalcitonin; MELD Na, model for end-stage liver diseases-sodium; SIRS, systemic inflammatory response syndrome; TLC, total leukocyte count; RR, respiratory rate.

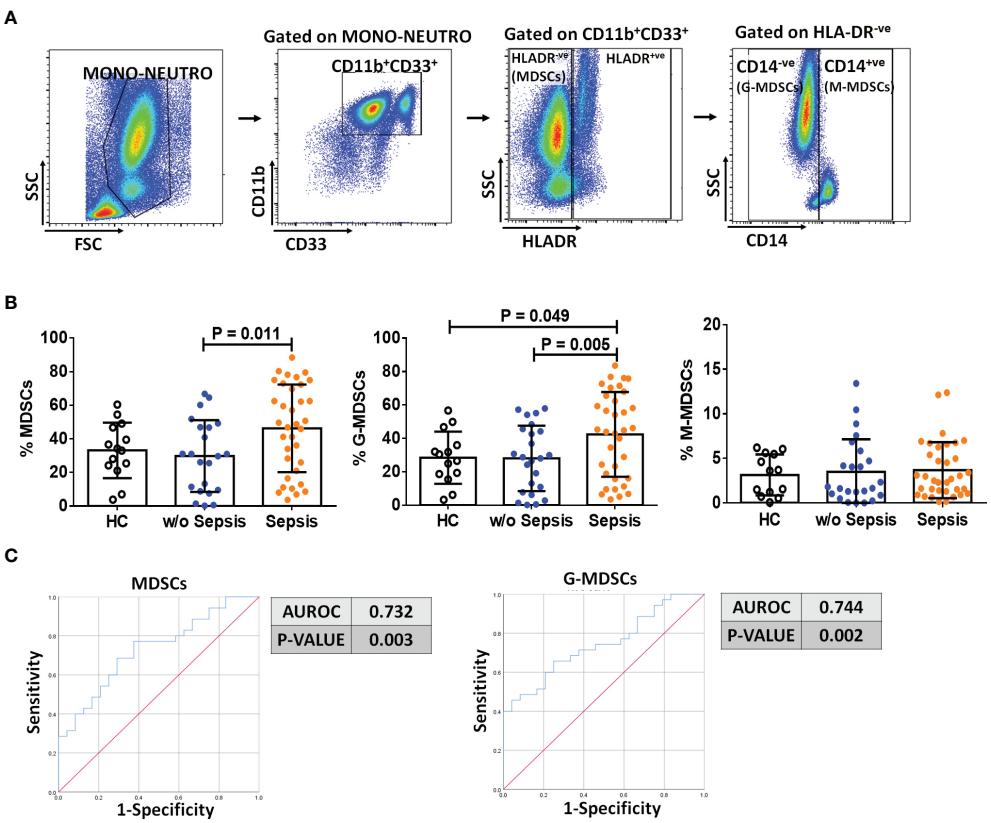

FIGURE 1 | Identification of MDSCs in patient groups. **(A)** Sequential gating strategy for identification of MDSCs and their subsets using flow cytometry. MDSCs are characterized as $CD11b^{+}CD33^{+}HLADR^{-ve}$. **(B)** Scatter dot plot shows %frequency of MDSCs, G-MDSCs, and M-MDSCs. **(C)** ROC curve shows high specificity and sensitivity of MDSCs and G-MDSCs in sepsis patients compared to w/o sepsis patients. Results are expressed as the mean ± SD. **(B)** One-way ANOVA/Kruskal–Wallis test followed by probability adjustment by the Mann–Whitney. **(C)** ROC curve *via* SPSS. MDSCs, myeloid-derived suppressor cells; ROC, receiver operating characteristic.

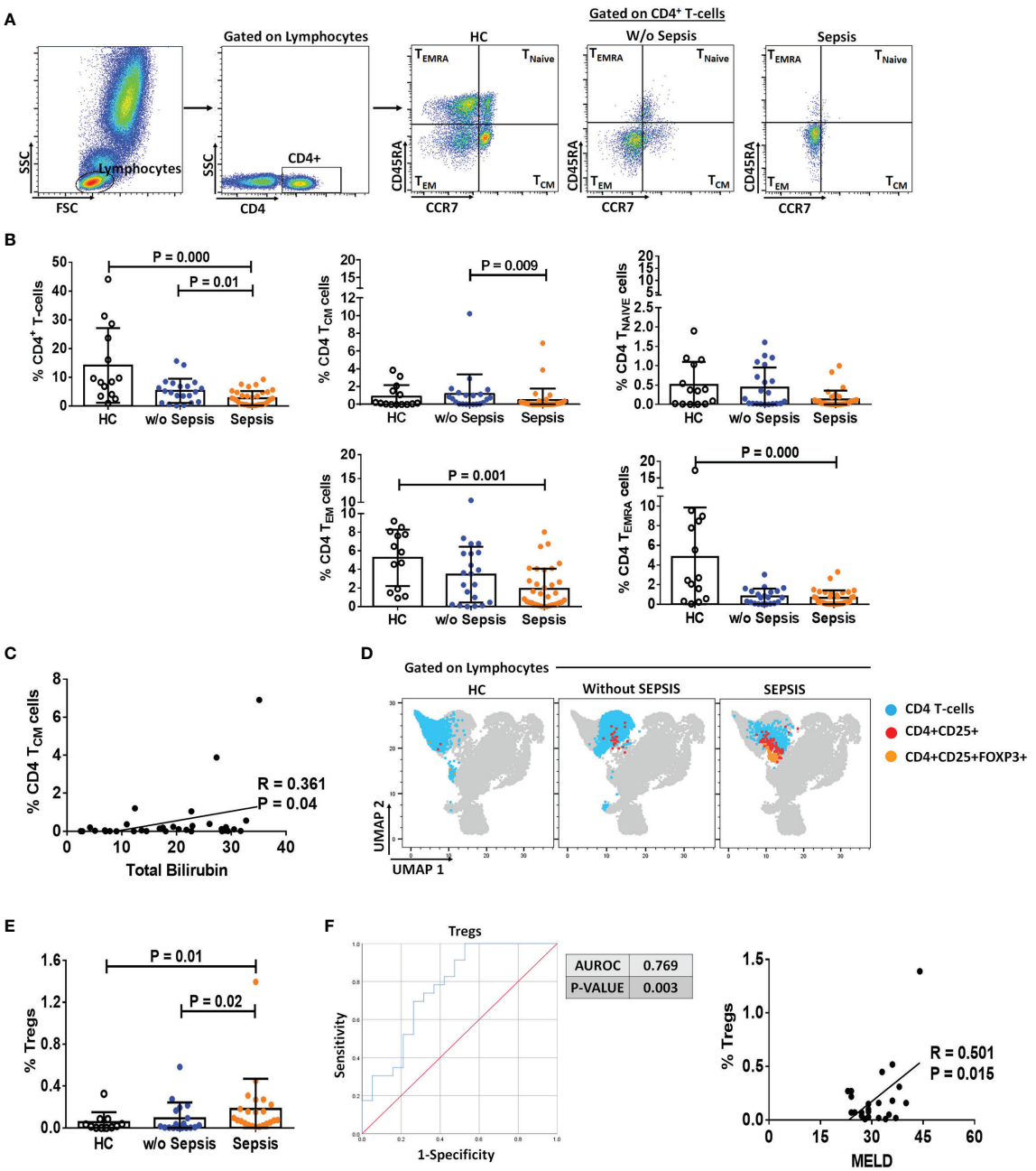

FIGURE 2 | Identification of CD4 T cells, their subsets, and Tregs in patient groups. **(A)** Sequential gating strategy for identification of CD4 and its subsets using CCR7 and CD45RA, i.e., T_{CM}, T_{NAIVE}, T_{EM}, and T_{EMRA}. Scatter dot plot shows %frequency of CD4 T cells in patient groups. **(B)** Scatter dot plot shows %frequency of T_{CM}, T_{NAIVE}, T_{EM}, and T_{EMRA}. **(C)** Correlation between %CD4 T_{CM} and total bilirubin in sepsis group. **(D)** UMAP visualization of pooled lymphocytes of HCs and patients w/o and with sepsis for characterization of Tregs. **(E)** Scatter dot plots show %frequency of Tregs. **(F)** ROC curve shows high specificity and sensitivity of Tregs in sepsis group compared to w/o sepsis group. Also, correlation of %Tregs with MELD in sepsis group. Results are expressed as the mean ± SD. **(A, B, E)** One-way ANOVA/Kruskal–Wallis test followed by probability adjustment by the Mann–Whitney. **(C–F)** Scatter diagram showing the regression line. UMAP, Uniform Manifold Approximation and Projection; HCs, healthy controls; ROC, receiver operating characteristic; MELD, model for end-stage liver diseases.

Myeloid-Derived Suppressor Cells Express More IL-10, Arg1, and iNOS

Sorted MDSCs from sepsis patients showed increased IL-10, ARG1, and iNOS expression; however, a significant increase was observed in IL-10 compared to that in HCs (p = 0.016). As MDSCs have monocytic and granulocytic lineages and M-MDSCs at present, which are known to play an immunosuppressive role, i.e., suppression of T cell, Treg

expansion, and disease severity (9), we have compared monocytes (not neutrophils) with MDSCs. When IL-10, ARG1, and iNOS expression in MDSCs was compared with that in monocytes, fold-change expression of ARG1 and iNOS was found significantly increased in sepsis MDSCs ($p = 0.043$ and $p = 0.045$) compared to sepsis monocytes but has no difference in IL-10 expression (**Figure 3A**). However, plasma levels of IL-10, IL-6, and IL-8 were significantly increased in sepsis compared to HCs and w/o sepsis patients. We have also analyzed MDSC-associated plasma factors including VEGF-A, IP-10, IL-6, IL-8, IL-10, TGF-β1, MIP-3α, IL-4, IL-27, IL-1β, IL-17A, TNF-α, and GM-CSF. We observed that MIP-3α and IL-1β were decreased in sepsis, MIP-3α was negatively correlated with MDSCs/G-MDSCs, and IL-1β and IP-10 were positively correlated with M-MDSCs in sepsis patients (**Supplementary Figures 5A–C**).

Myeloid-Derived Suppressor Cells Suppressed T-Cell Functionality

To check the suppressive effect of MDSCs on T cells, FACS-sorted MDSCs and CD4 T cells were *ex vivo* co-cultured and analyzed for CD4 T-cell apoptosis and proliferation (**Supplementary Figure 6**). To know whether MDSCs and monocytes have a similar effect on T-cell apoptosis and proliferation, we have additionally co-cultured T cells with monocytes.

At the Time of Admission (Day 0)

We have observed an increase in apoptosis of T cells and a decrease in T-cell proliferation in *ex vivo* cultured MDSCs+ T cells in sepsis patients compared to w/o sepsis patients and HCs (**Figures 3B, C** and **Supplementary Figure 7A**). But monocytes did not show suppressive ability when co-cultured with T-cells, suggesting MDSCs have immunosuppressive ability in sepsis but not monocytes..

But monocytes did not show suppressive ability when co-cultured with T-cells, suggesting MDSCs have immunosuppressive ability in sepsis but not monocytes.

At Follow-Up Time Points

We found no difference in follow-up between the groups in T-cell apoptosis and proliferation (**Supplementary Figures 7B, C**).

Myeloid-Derived Suppressor Cells Induces FOXP3$^+$ Expression on T Cells

Ex vivo cultured T cells with MDSCs in the Th0 condition (without the presence of any T-cell stimulant) showed increased expression of $CD4^+FOXP3^+$ ($p = 0.044$) in sepsis patients, but no such increased expression was observed in HCs and w/o sepsis patients (**Figure 3D**).

Furthermore, it was observed that in the presence of Th17-proliferating conditions [in presence of recombinant TGF-β (5 ng/ml) and IL-6 (20 ng/ml)], MDSCs induce more $CD4^+FOXP3^+$ expression on T cells ($p = 0.043$) in sepsis patients, while IL-17 producing T cells were minimal in disease condition compared to that in HCs ($p = 0.031$) (**Figure 3E**). We concluded that MDSCs increase the Treg expression and suppress Th17 cells, causing Treg/Th17 imbalance in DC patients with sepsis.

At Follow-Up Time Points

T cells cultured with MDSCs in the Th0 condition showed no difference in expression of $CD4^+FOXP3^+$, but an expression of $CD4^+FOXP3^+$ cells in the Th17 condition significantly decreased on day 3 compared to day 0 in w/o sepsis patient (**Supplementary Figure 8A, B**).

Blocking the Myeloid-Derived Suppressor Cells Suppresses the Expression of FOXP3$^+$ Tregs

MDSCs increased FOXP3 expression on $CD4^+$ T cells in sepsis patients ($p = 0.006$). As MDSCs suppress *via* Arginase1 and iNOS, we have further explored the role of MDSC blockers: L-NMMA (iNOS inhibitor) and nor-NOHA (Arg1 inhibitor) on $CD4^+FOXP3^+$ T cells. By blocking MDSC activity with nor-NOHA and L-NMMA, there was a significant decrease in $CD4^+FOXP3^+$ T cells ($p = 0.014$ and $p = 0.045$) in sepsis patients, but no such significant difference was observed in w/o sepsis patients and HCs (**Figure 3F**).

At Follow-Up Time Points

MDSC inhibitors show a significant decrease in the expression of $CD4^+FOXP3^+$ on day 3 ($p = 0.03$ and $p = 0.03$) in sepsis patients compared to day 0, but no difference was observed in w/o sepsis patients (**Supplementary Figure 8C**).

Granulocyte-Macrophage Colony-Stimulating Factor Treatment Suppresses Myeloid-Derived Suppressor Cells and Tregs in Sepsis Patients

GM-CSF is known as a stimulant for the bone marrow to produce myeloid cells and helps in the proliferation of myeloid cells but also helps in their proliferation (11, 12). However, the effect of exogenous treatment of GM-CSF in the modulation of MDSCs and Tregs was not explored in sepsis patients. We have analyzed 30 sepsis patients who were given 250 μg of GM-CSF intravenously over 6 h for 5 days along with the standard care. Blood samples were collected post 12 h after GM-CSF administration (day 1). Baseline as well as follow-up clinical and biochemical characteristics of sepsis patients with and without GM-CSF treatment were analyzed (**Supplementary Table 4**).

After day 1 of GM-CSF therapy, neutrophils were decreased with no change in monocyte numbers but with an increase in HLADR expression on monocytes (**Supplementary Figure 9A**). Furthermore, after GM-CSF therapy on days 1 and 3, MDSCs were decreased in sepsis patients. (**Figure 4A**), although the effect was more on G-MDSCs and not on M-MDSCs (**Supplementary Figure 9B**). GM-CSF therapy also showed its impact on $CD4^+$ T cells and their subsets. We found an increase in CD4 expression on day 1 after GM-CSF therapy, while both T_{NAIVE} and T_{CM} were found to be significantly increased in the GM-CSF group ($p = 0.04$ and $p = 0.003$) compared to the group without GM-CSF (**Figures 4B, C**). Furthermore, the percentage frequency of Tregs was significantly decreased in the GM-CSF group ($p = 0.003$) compared to without GM-CSF (**Figure 4D**).

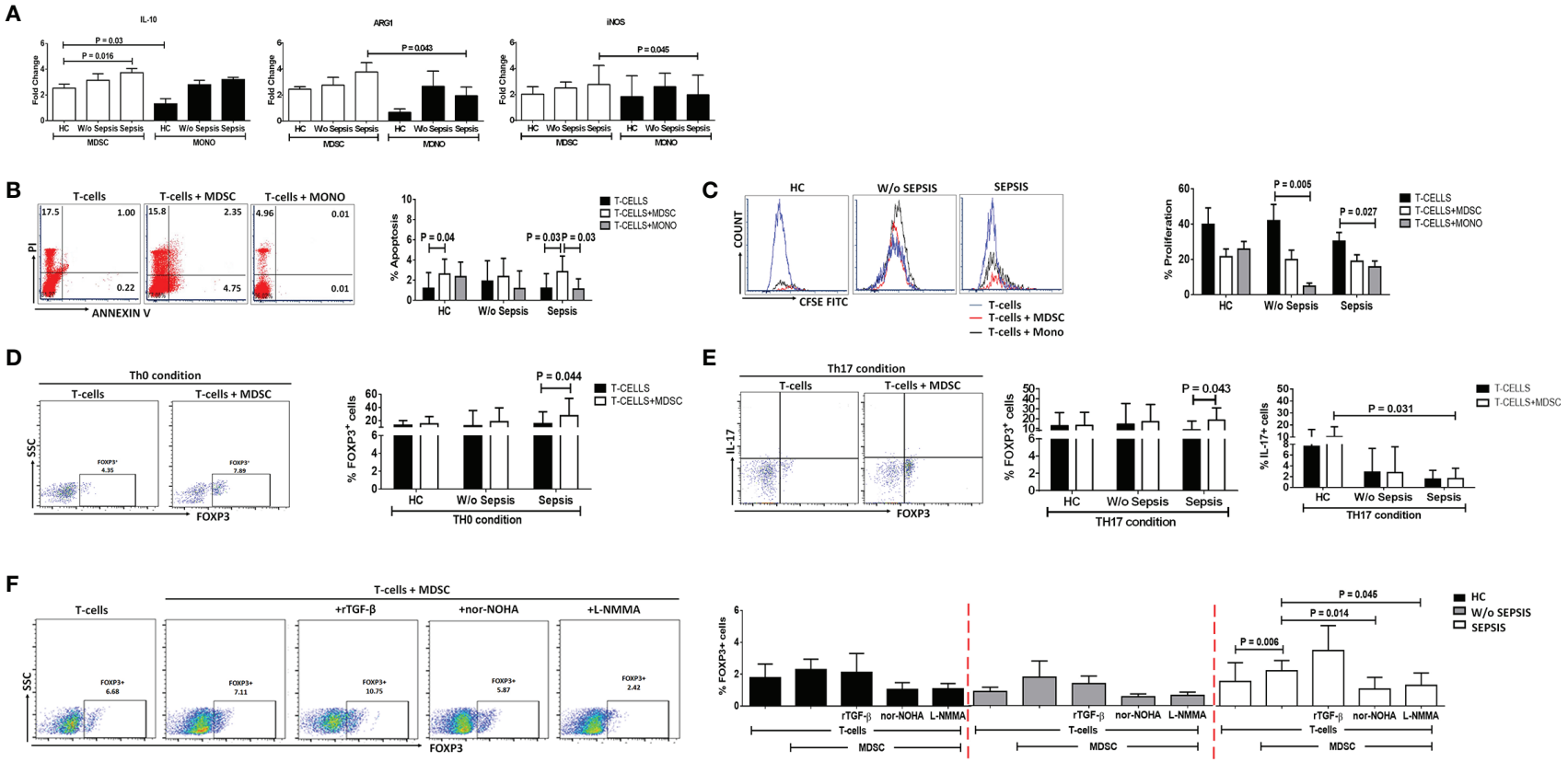

FIGURE 3 | MDSCs action on T-cell functionality and Tregs. **(A)** Fold-change expression of IL-10, Arg1, and iNOS in sorted MDSCs (white color) and monocytes (black color) in the patient groups through qRT-PCR. **(B)** %Apoptosis using PI–veAnnexin V+ve and **(C)** %Proliferation using CFSE through flow cytometry in T cells cultured alone (black color) and with MDSCs (white color) and monocytes (gray color) in the patient groups. Expression of %FOXP3$^+$ on CD4$^+$ T cells **(D)** under Th0 condition and **(E)** under Th17-proliferating condition was observed in the patient groups when T cells were cultured alone (black color) and with MDSCs (white color). **(F)** Expression of %FOXP3$^+$ on CD4$^+$ T cells in T cells cultured with MDSCs along with stimulations, i.e., rTGF-β, L-NMMA, and nor-NOHA in the patient groups. HCs (black color), without sepsis (gray color), and with sepsis (white color). Results are expressed as the mean ± SD. **(A)** One-way ANOVA/Kruskal–Wallis test followed by probability adjustment by the Mann–Whitney. **(B–E)** Mann–Whitney/t-test within the patient groups. **(F)** Kruskal–Wallis test within the group along with the multiple comparisons. MDSC, myeloid-derived suppressor cell; CFSE, carboxyfluorescein succinimidyl ester.

Granulocyte-Macrophage Colony-Stimulating Factor Treatment Reverses the Effect of Myeloid-Derived Suppressor Cells on T Cells and Tregs

After day 1 of GM-CSF therapy, *ex vivo* co-cultured MDSCs and T cells showed significantly decreased apoptosis of T cells (p = 0.005) as compared to those without GM-CSF therapy. Similarly, T-cell proliferation was significantly increased on day 1 in the GM-CSF group (p = 0.023) compared to the group without GM-CSF (**Figure 5B**). However, this effect was not observed on day 3 of GM-CSF therapy (**Figure 5A**).

Furthermore, expression of $CD4^+FOXP3^+$ on T cells (p = 0.004) in the Th0 condition was significantly decreased after GM-CSF day 1 therapy compared to without GM-CSF. At follow-up on day 3, $CD4^+FOXP3^+$-expressing T cells were constantly found to decrease in the GM-CSF group compared to the group without GM-CSF (**Figure 5C**).

Furthermore, in IL-17-proliferating conditions, MDSCs did not show an ability to induce the expression of $CD4^+FOXP3^+$ on T cells in the GM-CSF group (p = 0.000) compared to the group without GM-CSF. Similarly, till day 3, MDSCs were unable to induce $CD4^+FOXP3^+$ expression in the GM-CSF group compared to the group without GM-CSF. However, the percentage frequency of IL-17-expressing T cells was significantly increased in the GM-CSF group (p = 0.027) compared to the group without GM-CSF on day 1 and day 3 (**Figure 5D**).

Granulocyte-Macrophage Colony-Stimulating Factor Treatment Reversed Myeloid-Derived Suppressor Cell Expression on Tregs

GM-CSF therapy in sepsis patients leads to a significant decrease in $CD4^+FOXP3^+$ Tregs. In *in vitro* co-cultured assay of CD4 T cells and MDSCs with L-NMMA (iNOS inhibitor) and nor-NOHA (Arg1 inhibitor) inhibitors, a decrease in $CD4^+FOXP3^+$ Tregs was observed after GM-CSF treatment on day 1 and day 3 (**Figure 5E**).

Granulocyte-Macrophage Colony-Stimulating Factor Treatment Improves Survival of Sepsis Patients

Survival in DC patients with sepsis is mostly compromised. The Kaplan–Meier survival curves evidently proved that GM-CSF therapy along with standard care improved survival in sepsis patients compared to patients with only standard care (**Figure 5F**).

DISCUSSION

Our study observed an increase in MDSCs and Tregs in cirrhosis patients with sepsis, with a decrease in CD4 T cells. *Ex vivo* co-cultured MDSCs and T-cell experiments confirmed and supported the notion that MDSCs suppress T-cell functionality but expand $FOXP3^+$ Tregs in sepsis patients.

Our group has earlier shown that G-CSF reduces the disease severity, delays the mortality of severe alcoholic hepatitis (SAH) patients (16), and mobilizes bone marrow-derived $CD34^+$ cells in acute-on-chronic liver failure (ACLF) patients for hepatic regeneration (17). However, the focused role of GM-CSF was not addressed earlier, but in this study, usage of GM-CSF reversed the immune paralysis by suppressing the MDSCs and Tregs in sepsis patients (**Figure 6**).

Sepsis is described as organ dysfunction due to bacterial infections and induced by dysregulated host immune response resulting in a longer stay in hospitals, affecting the mortality rate (18). Immune dysfunction in liver cirrhosis patients is common, which enhances bacterial translocation from the gut to liver, resulting in endotoxemia, systemic inflammation, and septic shock (19). DC patients show leukopenia, which affects both T helper (Th) and cytotoxic T cells (Tc), monocytosis with altered function, neutrophils with impaired phagocytosis, B cells with memory B-cell dysfunction, and defective NK cells with reduced response to cytokine stimulation (4). Our study shows a decrease in both CD4 and CD8 T cells and monocytes but an increase in circulating neutrophils, MDSCs, and Tregs in DC patients with sepsis.

MDSCs are a heterogeneous population of cells, and their origin is either monocytic or granulocytic (6). Elevated levels of MDSCs have been positively correlated with severe sepsis or septic shock and longer stay of patients in the intensive care unit (ICU) (20), as MDSCs are known to have immunosuppressive activity *via* Arginase-1, iNOS, or ROS for inhibiting the functionality of immune cells, especially T cells. An increase in MDSCs acts as a potent inhibitor of T cell-mediated immunity in autoimmune hepatitis and cancer, which is attributed to the production of Arginase1, ROS, iNOS, and IL-10 (21, 22). An increase in hepatic $CD11b^+CD33^+$ MDSCs positively correlated with liver fibrosis and also linearly correlated with the tumor volume (23). Furthermore, peripheral blood MDSCs were also increased in cirrhosis and HCC patients (24). Furthermore, M-MDSCs were strongly correlated with raised ALT, AST, and decreased T-cell proliferation (25). It was earlier reported that M-MDSCs suppressed T-cell functions and antimicrobial-specific innate immune responses in ACLF patients (26). An increase in peripheral and intrahepatic G-MDSC populations was reported in ALC (Alcoholic liver cirrhosis) patients (27). In our study, we also found a significant increase in MDSCs, especially G-MDSCs, in cirrhosis patients with sepsis. *In vitro* co-cultured MDSCs with CD4 T cells suppressed the proliferation and enhanced apoptosis of CD4 T cells in DC with sepsis patients.

It is known that in the tumor microenvironment, many known circulating factors including IL-6, IFN-γ, TGF-β, VEGF, G-CSF, GM-CSF, M-CSF, and SCF induce the recruitment, accumulation, and activation of MDSCs (28) and modulate MDSCs to produce more of NO and ROS (22). Our study revealed a positive correlation of new molecules IL-1β and IP-10 with M-MDSC but a negative correlation of MIP-3α with MDSC and G-MDSC. Reduced IL-1β receptor binding ability or IL-1β levels reduces the accumulation and suppressive activity of

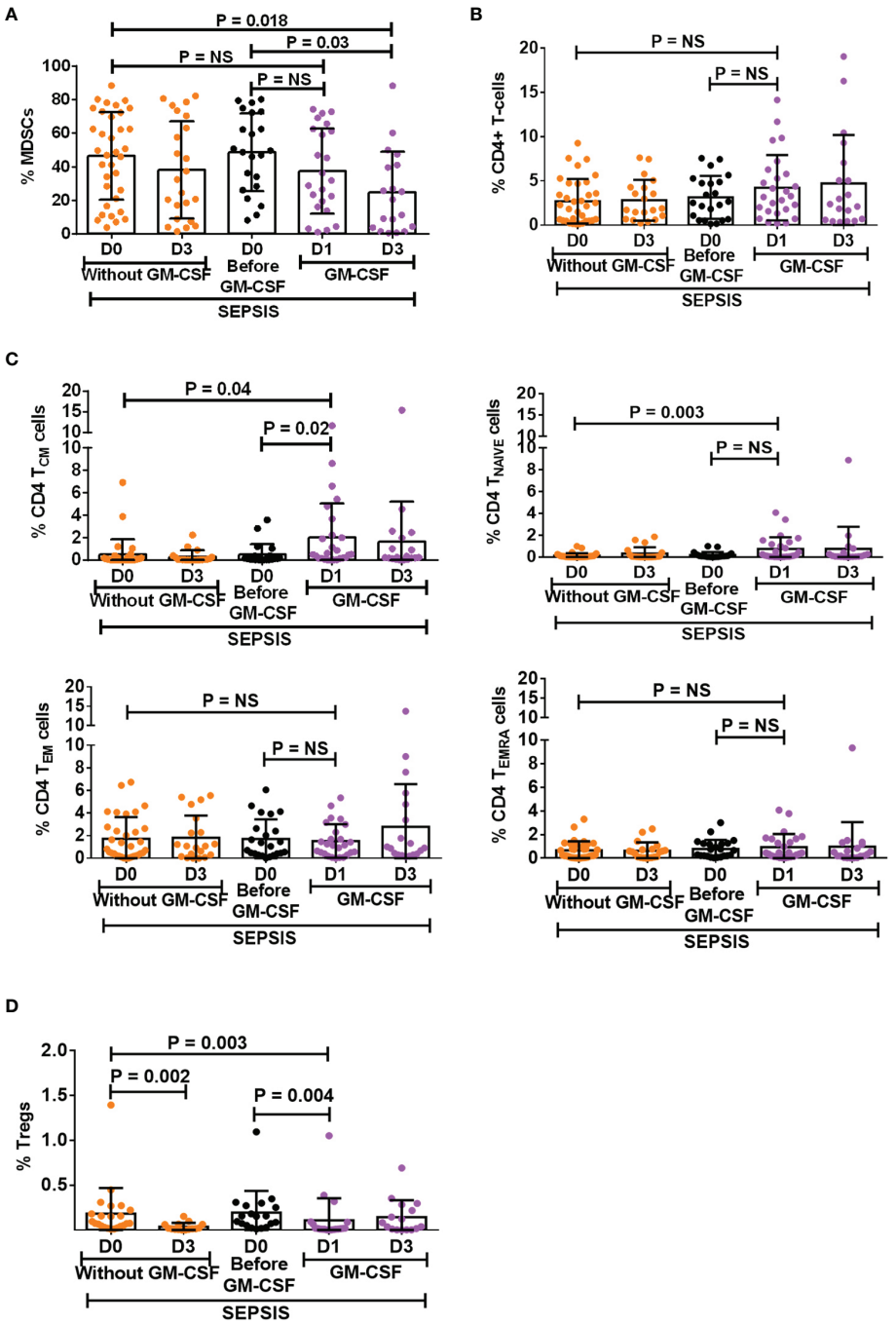

FIGURE 4 | Effect of GM-CSF treatment on the expression of MDSCs, CD4 T cells, its subsets, and Tregs. Scatter dot plot shows an expression of **(A)** %MDSCs; **(B)** %CD4 T cells; **(C)** CD4 T-cell subsets, i.e., T_{CM}, T_{NAIVE}, T_{EM}, and T_{EMRA}; and **(D)** %Tregs in sepsis patients without GM-CSF (D0 and D3), before GM-CSF (black color), and with GM-CSF (D1 and D3). Results are expressed as the mean ± SD. **(A–D)** One-way ANOVA/Kruskal–Wallis test followed by probability adjustment by the Mann–Whitney *via* SPSS. GM-CSF, granulocyte-macrophage colony-stimulating factor; MDSCs, myeloid-derived suppressor cells.

MDSC resulting in augmentation of antitumor immunity and delayed tumor growth (29). Furthermore, chemokine CXCL10/IP-10 significantly increased in a mouse model of septic shock, as they cause the activation of chemokine receptor CXCR3, an important regulator of lymphocyte trafficking and activation (30). MIP-3α (macrophage inflammatory protein-3)/CCL20 is generally expressed on several immune cells but with a stronger chemotactic effect and interaction with chemokine receptor CCR6 on lymphocytes. CCL20/CCR6 axis regulates the activation and suppression of immune cells (31).

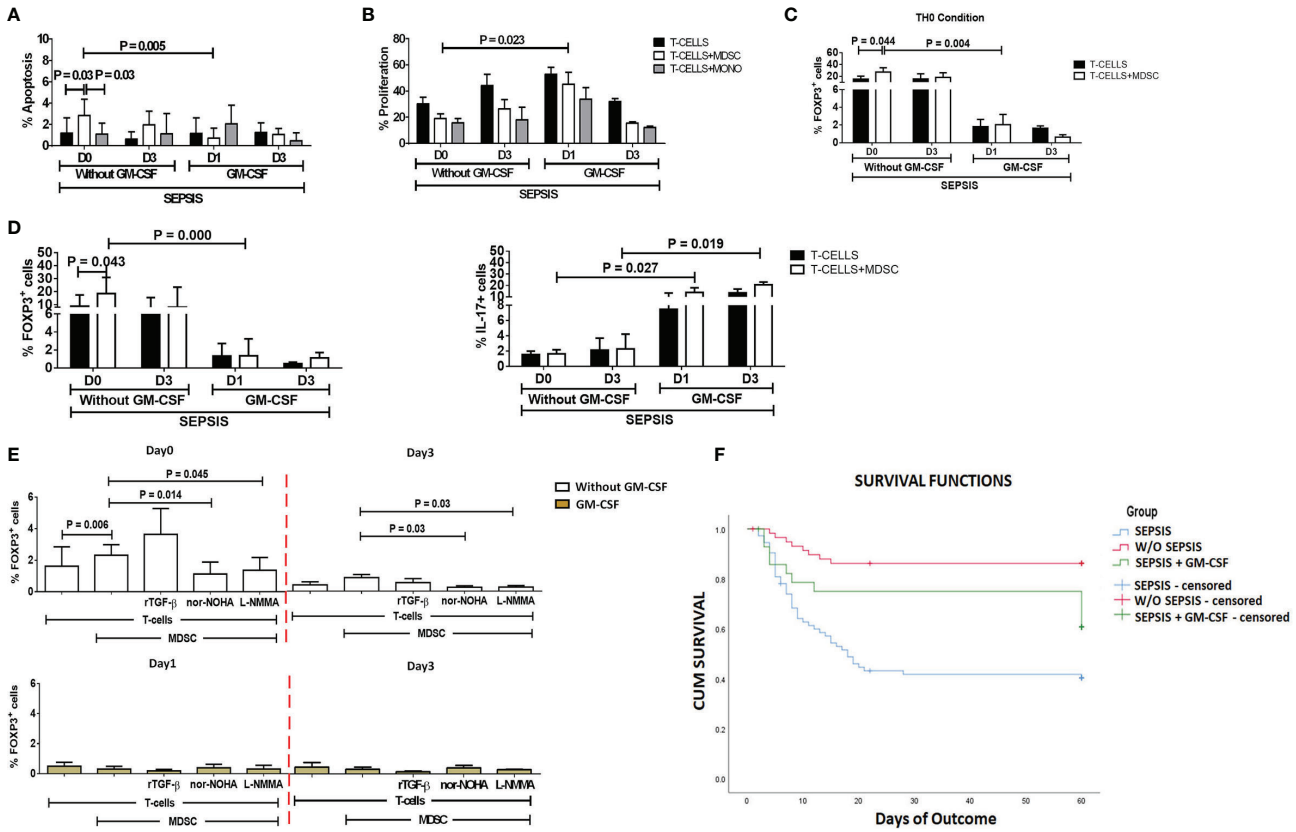

FIGURE 5 | Effect of GM-CSF treatment on T-cell functionality and Treg expression. Bar diagrams show **(A)** %Apoptosis using PI–veAnnexin V+ve and **(B)** %Proliferation using CFSE in sepsis patients with and without GM-CSF in T cells cultured alone (black color), with MDSCs (white color), and with monocytes (gray color). Bar diagrams show expression of %FOXP3$^+$ on CD4$^+$ T cells **(C)** under Th0 condition and **(D)** under Th17-proliferating condition in T cells cultured alone (black color) and with MDSCs (white color). **(E)** Expression of %FOXP3$^+$ on CD4$^+$ T cells in T cells cultured with MDSCs along with stimulations with rTGF-β, L-NMMA, and nor-NOHA; %FOXP3+ Treg expression was observed in the groups with (light brown) and without GM-CSF (white color) at different time points. Results are expressed as the mean ± SD. **(A–D)** Mann–Whitney/t-test within the patient groups along with one-way ANOVA/Kruskal–Wallis test between the groups. **(E)** Kruskal–Wallis test within the group along with the multiple comparisons. **(F)** Survival in patient groups was observed using the Kaplan–Meier survival curves *via* SPSS. GM-CSF, granulocyte-macrophage colony-stimulating factor; CFSE, carboxyfluorescein succinimidyl ester; MDSCs, myeloid-derived suppressor cells.

Both MDSC and Tregs are known suppressor cells and help each other; i.e., Tregs regulate the differentiation and function of MDSC *via* TGF-β, while MDSC helps in the expansion of Tregs in a colitis mouse model (7). In the rheumatoid arthritis mouse model, MDSC-derived IL-10 helps in the generation of Tregs but attenuates inflammation. It was found that MDSCs regulate Th17/Treg cells and control inflammation (8). Tregs/Th17 axis plays an important role in various diseases, and Treg/Th17 imbalance was known to be used in the pathogenesis of HBV-related liver cirrhosis and ACLF (32, 33). We have also shown that in DC patients with sepsis, MDSCs significantly enhanced the expression of FOXP3 on CD4$^+$ T cells and behaved as Tregs, while stimulation with L-NMMA (iNOS inhibitor) and nor-NOHA (inhibitor of Arginase1) significantly suppresses the expansion of Tregs in sepsis patients. It clearly concludes that suppression of the immunosuppressive activity of MDSCs will decrease the expansion of CD4$^+$FOXP3$^+$ cells in sepsis.

Although we had earlier observed survival benefits of G-CSF in SAH and ACLF patients (16, 17), the role of another CSF moiety, i.e., GM-CSF, was explored in sepsis patients in this study.

It was observed earlier that administration of GM-CSF in sepsis patients reversed the monocytic deactivation by increasing HLADR and TLR4-induced cytokine production, as well as decreased the time of mechanical ventilation and length of hospital (34). *In vitro* mouse model showed that the combination of GM-CSF signaling blockade and gemcitabine suppressed the MDSC phenotype and functionality (22). In another study, both GM-CSF and G-CSF prevented diabetes by reducing MDSCs and Treg cells (35). Our results clearly suggested the benefit of GM-CSF therapy in sepsis patients, as it decreases the MDSCs, FOXP3 expression on CD4$^+$ T cells, and the percentage of apoptotic T cells and increases CD4 T-cell proliferation. However, post-GM-CSF therapy on day 3, we did not observe any significant change in apoptosis and proliferation of CD4 T cells as compared to day 1. In sepsis patients, it is difficult to do a liver biopsy; therefore, our study was limited to peripheral blood. Survival rates in cirrhosis

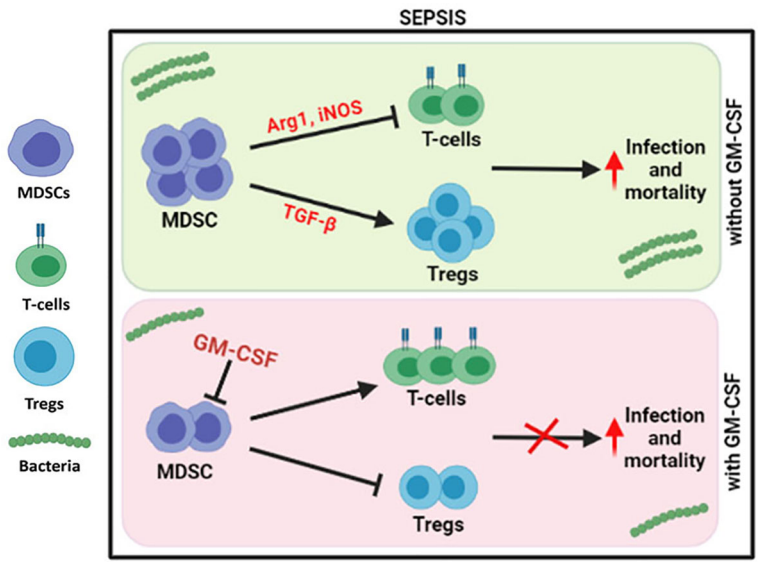

FIGURE 6 | Modulation of immune cells in sepsis with GM-CSF. In DC sepsis patients, MDSCs suppresses the T-cells using Arg1 and iNOS but expands Tregs using TGF-b. With GM-CSF treatment, immune paralysis is reversed with decline in MDSCs, and Tregs with proliferation of T-cells.

patients with sepsis are mostly compromised; however, standard care along with GM-CSF therapy has increased survival benefits in sepsis patients. This could be due to decreased MDSCs and Tregs and increased T_{CM} population. Although we have shown earlier survival benefits of G-CSF in ACLF and SAH patients, this study also showed the survival benefits after administration of GM-CSF in sepsis patients. The role of GM-CSF independent of the MDSCs can be best answered *via* mouse models, which act as a limitation to our study.

In summary, we conclude that the enhanced expression of MDSCs in DC with sepsis was found to be responsible for suppressing $CD4^+$ T cell functionality as well as expanding the $CD4^+FOXP3^+$ Treg activity. Administration of GM-CSF in sepsis patients reduced the numbers of MDSCs and Tregs and improved T-cell functionality, which are beneficial for the survival of the patients.

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 protocol was approved by the institutional review board and ethics committee (IEC No. IEC/2016/45/NA/C2). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS collected clinical samples, performed all experiments after inputs from NT, and did the initial analysis. RM, VR, and SS helped in the recruitment and characterization of patients in each group. MI helped in performing the experiments, and GK helped in the statistical analysis. An initial draft of the manuscript was written by RS. SS, RM, SB, NK, and GR have provided inputs in the manuscript. NT revised, corrected, and finalized the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.828949/full#supplementary-material

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GLOSSARY

MDSCs	myeloid-derived suppressor cells
CAID	cirrhosisassociated immune dysfunction
DC	decompensated cirrhosis
ICU	intensive care unit
IMCs	immature myeloid cells
DCs	dendritic cells
G-MDSCs	granulocytic MDSCs
M-MDSCs	monocytic MDSCs
iNOS	inducible nitric oxide synthase
ROS	reactive oxygen species
PNT	peroxynitrite
TGF-b	transforming growth factor beta
IL	interleukin
GM-CSF	granulocytemacrophage colony-stimulating factor
G-CSF	granulocyte colony-stimulating factor
M-CSF	macrophage colony-stimulating factor
VEGF	vascular endothelial growth factor
SCF	stem cell factor
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ACLF	acute-on-chronic liver failure
SAH	severe alcoholic hepatitis
FOXP3	Forkhead box P3
IDO	indoleamine 2,3-dioxygenase
PD-L1	programmed cell death ligand 1
HC	healthy control
RCT	randomized controlled trial
HCC	hepatocellular carcinoma
SIRS	systemic inflammatory response syndrome
TLC	total leukocyte count
RR	respiratory rate
INR	international normalized ratio
EDTA	ethylenediaminetetraacetic acid
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
FBS	fetal bovine serum
RPMI	Roswell Park Memorial Institute
LPS	lipopolysaccharide
RT	room temperature
RBC	red blood cells
PI	propidium iodide
CFSE	carboxyfluorescein succinimidyl ester
FACS	fluorescence-activated cell sorting
L-NMMA	Nw-methyl-L-arginine acetate salt
nor-NOHA	Nw-hydroxy-nor-Larginine diacetate salt
SPSS	Statistical Package for the Social Sciences
DLC	differential leukocyte count
PCT	procalcitonin
MELD Na	model for end-stage liver diseases-sodium
CD4+CD45RA+CCR7+	naïve T cells
TCM	CD4+CCR7+CD45RA−central memory T cells
TEM	CD4+CD45RA−CCR7−effector memory
TEMRA	CD4+CD45RA+CCR7 terminally differentiated T cells

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The immunological characteristics of TSPAN1 expressing B cells in autoimmune hepatitis

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Background and aims: Tetraspanin proteins are closely related to the functional changes of B cells, including antigen presentation, production of cytokines, and transduction. We aim to explore the potential role of Tetraspanin 1 (TSPAN1) in the biological activities of B cells in AIH.

Methods and results: Herein, this study found that numbers of cells expressing TSPAN1 were significantly increased in AIH patients compared to PBC, chronic hepatitis B, and healthy control ($P < 0.0001$). Moreover, there was a positive correlation between numbers of TSPAN1+ cells and AIH disease severity ($P < 0.0001$). Immunofluorescence staining further confirmed that TSPAN1 was primarily expressed on CD19+ B cells. Flow-cytometric analysis showed that TSPAN1+ B cells secreted more inflammatory cytokines and expressed higher level of CD86 than TSPAN1- B cells. Furthermore, compared with TSAPN1- cells, the expression of CXCR3 on TSPAN1+ cells was also higher. Meanwhile, CXCL10, the ligand of CXCR3, was significantly elevated in the liver of AIH ($P < 0.01$) and had positive correlation with the quantities of TSPAN1 ($P < 0.05$). Interestingly, the numbers of TSPAN1+ B cells were decreased in AIH patients after immunosuppressive therapy.

Conclusions: $TSPAN1^+$ B cells in the liver may promote the progression of AIH *via* secreting cytokines and presenting antigens. The chemotactic movement of $TSPAN1^+$ B cells toward the liver of AIH was possibly due to CXCR3 - CXCL10 interaction.

KEYWORDS

autoimmune hepatitis, tetraspanin 1, B cells, cytokines, antigen presentation

Abbreviations: AIH, autoimmune hepatitis; PBC, primary biliary cholangitis; CHB, chronic hepatitis B; TSPAN1, Tetraspanin 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; GGT, gamma-glutamyltranspeptidase; IgG, immunoglobulin G; PBMC, peripheral blood mononuclear cell; MFI, mean fluorescence intensity.

1 Introduction

Autoimmune hepatitis (AIH) is recognized as an immune mediated disease, characterized by hypergammaglobulinemia, female preponderance and liver-infiltrating immune cells in portal area (1, 2). The mechanisms underlying the breakdown of self-tolerance have not been fully elucidated, though there is mounting evidence that autoreactive T cell and dysfunction of regulatory T cells play a key role (3). However, anti-CD20 may be a useful treatment for patients with a poor response to conventional therapy, indicating a vital role of B cells in AIH (4, 5).

Previous studies have shown that B lymphocytes can be involved in the development of AIH. Autoreactive B cells are typically considered as the sources of autoantibodies. Seropositivity to specific autoantigens is used for distinguishing two types of AIH. Additionally, hypergammaglobulinemia with specific immunoglobulin G (IgG) is another characteristic diagnostic hallmark of AIH, which has been demonstrated to correlate with disease activity (6, 7). Moreover, B lymphocytes can drive autoimmunity as antigen presenting cells (APCs). In the absence of other APCs, activation of $CD4^+$ T cells and T follicular helper (Tfh) can be initiated by B cells. It has been reported that B cell-derived cytokines have double-edged effects on AIH (8). On one hand, pro-inflammatory cytokines like type I interferons, tumor necrosis factor-alpha (TNF-α), and interleukin (IL) 6 contribute directly or indirectly to disease progression. On the other hand, B cells can also secret IL-10 and transforming growth factor-beta (TGF-β) to induce Tfh and IL-10 producing T cells (9).

Tetraspanins are highly conserved proteins with four transmembrane domains, and known to play an important role in B and T lymphocytes (10), including cell proliferation, motility, and adhesion. For example, CD9, CD53, CD81, and CD82 all belong to the tetraspanins family, which can bind to CD19, CD21, and HLA-DR expressed on mature B cells, and are tightly associated with the functional changes of cells (11, 12). Specially, CD9, CD151, and CD81 also contribute to antigen presentation, production of cytokines, and signal transduction (13). Tetraspanin 1 (TSPAN1) as a member of tetraspanin superfamily, is mainly expressed on plasma membrane and intracellular vesicles (14). TSPAN1 has been reported to involve in a variety of biological functions, including cell proliferation, adhesion, and migration (15). It has been found that TSPAN1 might be related to poor prognosis of pancreatic cancer and ovarian cancer (16, 17). In addition, TSPAN1 silence also has effects on reducing the ratio of $CD4^+$ T cells polarizing to Th17 cells (18). However, the function of TSPAN1 on B cells remains unclear.

Herein, we reported that TSPAN1 expression was significantly elevated in the liver of AIH patients, and positively correlated with disease severity. Confocal staining further confirmed that the majority of $TSPAN1^+$ cells were CD19 positive in AIH. Interestingly, $TSPAN1^+$ B cells had stronger ability to present antigens and secret cytokines compared with $TSPAN1^-$ cells. Furthermore, CXCR3-CXCL10 interaction may be involved in the chemotaxis of $TSPAN1^+$ B cells to the liver.

2 Materials and methods

2.1 Liver samples

Liver samples were obtained from 66 patients diagnosed as AIH, 24 patients with primary biliary cirrhosis (PBC), 21 chronic hepatitis B (CHB), and 7 healthy controls (HC). All patients corresponded to diagnosis standards of AIH (19), PBC (20), and CHB (21). Additionally, 27 of the patients with AIH had a follow-up biopsy to explore whether they had histological remission after three years of standard immunosuppressive treatment. Another 28 AIH patients with secondary liver biopsy were enrolled to further investigate the relationship between histologic remission and the frequency of TSPAN1. Immunohistochemical liver tissues from patients with AIH, PBC, and CHB were derived from ultrasound-guided needle liver biopsies. Besides, the liver tissue activity was assessed by the Scheuer scoring system for inflammation and fibrosis stages (22). AIH patients were divided into histologic remission and non-remission according to the Histological Activity Index (HAI) scoring system (23). Liver samples of HC were collected from explant donors before transplantation. Peripheral blood mononuclear cells (PBMCs), from patients who met AIH diagnostic criteria, were separated and frozen in fetal bovine serum (FBS) with 10% dimethyl sulphoxide. PBMCs were used to investigate the phenotypes and functions of $TSPAN1^+$ B cells. The demographic and clinical features of these subjects were listed in Table 1 and Table 2.

2.2 Immunohistochemistry

Formalin-fixed, paraffin-embedded liver tissues were prepared for immunohistochemistry. The procedures for immunohistochemistry had been described in the previous study (24). Concisely, liver sections were blocked with goat serum for 30 minutes and then incubated with primary antibody TSPAN1 (GTX108675, GeneTex) or CXCL10 (AF-266-NA, R&D systems) overnight at 4°C. After washing in phosphate buffered saline, the incubation with a horseradish peroxidase-conjugated secondary antibody was performed at room temperature for 30 minutes. Lastly, the nuclei were stained by 3,3′-diaminobenzidine and hematoxylin. The numbers of positive sections were calculated blindly by two

TABLE 1 Clinical Features of Patients with AIH, PBC, CHB, and HC.

	AIH(diagnostic biopsy) (n = 66)	PBC(n = 24)	CHB(n = 21)	HC(n = 7)	AIH(follow-up biopsy)(n = 55)
Age (years)	46.3 ± 12.3	44.2 ± 9.3	39.6 ± 14	36 ± 11.1	48.3 ± 10.9
Gender (F/M)	57/9	19/5	9/12	3/4	49/6
ALT(U/L)	154.6 ± 136.3	50.9±32.9	69.6 ± 118.9	33.7 ± 26.3	15.1 ± 9.4
AST(U/L)	144.3 ± 160.3	40.3±20.7	42.3± 52.6	26.1 ± 6	17.7 ± 5.2
ALP(U/L)	111.8 ± 72	166.8±103.5	81 ± 21.4	85.1 ± 25.6	58.7 ± 16.6
GGT(U/L)	117.1 ± 127.9	113.8±112.7	32.4 ± 19.1	21.3± 12.6	18.7 ± 14.6
TBIL(μmol/L)	47 ± 107.4	11.7±4.2	17.7 ± 23.1	11.9 ± 3.8	10.4 ± 5.8
IgG(g/L)	17.4 ± 5.8	13.8±4	12.3 ± 3.9	\	11.9 ± 2.3

independent observers. Five fields of portal areas were selected stochastically from each liver section. Numbers of infiltrative $TSPAN1^+$ cells were acquired per high-power field. Score CXCL10 expression from zero to four points per high power field. Cases were scored at one if the expression area < 25% and two if ≥ 25% to <50%, three if ≥ 50% to < 75%, four if ≥ 75%.

2.3 Confocal staining

Procedures for confocal laser scanning microscopy had been described before (25). After formalin-fixation, paraffin embedding, the liver samples were incubated in 3% H_2O_2 for 10 minutes and goat serum for 30 minutes successively at room temperature, then washed in phosphate buffer saline. Next, the sections were incubated with two primary antibodies overnight at 4°C after undergoing antigen retrieval. The samples were incubated with AF488 and AF555 fluorochrome-conjugated secondary antibodies (1:500; Invitrogen, Carlsbad, CA) for 30 minutes. The nuclei were stained by DAPI (Southern Biotech, Birmingham, AL). The confocal scanning was performed by LSM-710 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany).

2.4 Multiplex immunofluorescence staining

Multiplex immunofluorescence staining was performed by the Opal 4-Color IHC kit (Abs50028-20T, Absin). The liver sections were processed by antigen retrieval. The primary antibody of TSPAN1 was incubated at 37°C for 1 hour. Then, the sections were washed and incubated with HRP-conjugated secondary antibody for 20 minutes at room temperature, what followed was adding TSA dye 520. The second antibody CD19 (ab134114, Abcam) was incubated overnight at 4°C, what followed was adding TSA dye 650. The last antibody CXCR3 (ab288437, Abcam) and TSA dye 570 were added sequentially. The nuclei were stained with DAPI.

2.5 *In vitro* culture

PBMCs were isolated from fresh blood through gradient centrifugation using Ficoll Hypaque Plus (GE Healthcare). $CD19^+$ B cells were further separated from PBMCs by using CD19 Microbeads (130-097-055 Miltenyi Biotec). $CD19^+$ B cells at 1×10^5 cells/mL were cultured in 96-well culture plates, where

TABLE 2 Clinical Features of Patients with AIH and HC.

	AIH (n = 14)	HC c(n = 10)
Age (years)	50.4 ± 10.8	50 ± 13.3
Gender (F/M)	9/5	8/2
ALT(U/L)	69.1 ± 45.3	20.2 ± 9.1
AST(U/L)	60.9± 54.3	20.7 ± 3.8
ALP(U/L)	110.5 ± 23	63.8 ± 9.7
GGT(U/L)	72.6 ± 85.6	15.3 ± 7.1
TBIL(μmol/L)	15.3 ± 9.9	10.3 ± 3.8
IgG(g/L)	17.5 ± 7.1	\

the complete medium consists of the Roswell Park Memorial Institute 1640 (RPMI-1640), 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 ug/mL streptomycin, and 50 mM 2-mercaptoethanol. To stimulate B cells, 2 μg/mL Cytidine-phosphate-guanosine (CPG) (Invitrogen, Carlsbad, CA), 1 μg/mL CD40 ligand (CD40L) (PeproTech, Cranbury, NJ), 50ng/mL rhIL-4 (PeproTech, Cranbury, NJ) were added in complete medium. Cells were collected after 48 hours.

2.6 Transwell assay

$CD19^+$ B cells were isolated from PBMCs of AIH patients and cultured in 96-well culture plates with CPG, CD40L, and IL-4 for 24 hours. Then, cells were harvested and placed on the upper chamber of 24-well transwell plate with 5 μm pores (Coring, Kennebunk, MA, 3421), where consist of the complete medium for 200 μL. In the lower chamber, rhCXCL10 was added with 3 μg/mL in the complete medium, while the complete medium was as the control group. Cells in the lower chamber were harvested and analyzed after 6 hours.

2.7 Flow-cytometric analysis

The frozen PBMCs were resuscitated, washed and labeled with fluorochrome-conjugated antibodies, including anti-mouse TSPAN1, CD19, CD11c, CD38, CD80, CD86, HLADR, CXCR3, CXCR4 (BD Bioscience, San Diego, CA, USA). In intracellular cytokine staining, the cells were stimulated with lipopolysaccharide for 1 hour, and then Leukocyte Activation Cocktail (BD Biosciences, San Diego, CA, USA) for 4 hours at 37°C. Specific fluorochrome-conjugated antibodies were first added to stain surface markers at 4°C for 30 minutes. Subsequently, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA, USA) at 4°C for 20 minutes. Ultimately, intracellular markers including granzyme B, interferon-γ (IFN-γ), TNF-α, and TGF-β (BD Biosciences, San Diego, CA, USA) were stained at 4°C for 1 hour. All cells were detected by LSR Fortessa X-20 analyzers (BD Biosciences). Statistics and *t*-SNE (*t*-distributed stochastic neighbor embedding) analysis were performed by FlowJo.

2.8 Statistical analysis

All statistical analysis were performed by GraphPad Prism 8.0 software. For normally distributed data, paired or unpaired Student *t*-test were used for comparisons between two groups. Statistical difference for abnormally distributed data were analyzed by Mann-Whitney U test. Wilcoxon matched-pairs test was used to analyze two paired groups. Correlation analyses were performed by Pearson's correlation test. All analyses were two-tailed, and $P < 0.05$ was considered significant.

3 Results

3.1 TSPAN1 expression was increased in AIH and correlated with disease activity

Immunohistochemical staining was performed in liver tissues of AIH (n = 66), PBC (n = 24), CHB (n = 21), and HC (n = 7), the numbers of $TSPAN1^+$ cells were significantly increased in AIH compared to PBC ($P < 0.0001$), CHB ($P < 0.0001$), and HC ($P < 0.0001$) (Figure 1A). $TSPAN1^+$ cells mainly accumulated in the portal tracts and few scatted in inter-lobular areas. To further explore the clinical significance of $TSPAN1^+$ cells in AIH, the correlation between the numbers of portal $TSPAN1^+$ cells with clinical and histological indices was analyzed. Interestingly, the frequency of $TSPAN1^+$ cells was positively correlated with hepatic inflammation degree ($r = 0.7851$, $P < 0.0001$) and fibrosis stage ($r = 0.5933$, $P < 0.0001$) (Figure 1B). Moreover, there was a positive correlation of $TSPAN1^+$ cells with serum alanine transaminase (ALT) levels ($r = 0.4451$, $P = 0.0002$), aspartate aminotransferase (AST) levels ($r = 0.2449$, $P = 0.0475$), alkaline phosphatase (ALP) levels ($r = 0.3411$, $P = 0.0051$) and gamma-glutamyltransferase (γ-GT) ($r = 0.2679$, $P = 0.0296$). However, no statistical correlation was observed between $TSPAN1^+$ cells with serum IgG and total bilirubin (TBIL) levels (Figure 1C). These results indicated that $TSPAN1^+$ cells may play an important role in the development of AIH.

3.2 TSPAN1 was mainly expressed on hepatic B cells in AIH

To investigate which category of the cells expressed TSPAN1 in AIH, we co-stained TSPAN1 with CD19, CD8, CD4 or CD68 by immunofluorescence double-staining. As shown in Figure 2A, most of TSPAN1 positive cells were colocalized with CD19. However, TSPAN1 was less expressed on CD8, CD4, and CD68 cells (Figure 2). The results indicated that infiltrating $TSPAN1^+$ cells in the liver of AIH were mainly B cells, rather than T cells or macrophagocytes. To further analyze the subpopulation of $TSPAN1^+CD19^+$ B cells in AIH, we co-stained TSPAN1 with CD27, CD138, IgG. Surprisingly, there was no co-expression between TSPAN1 and these plasma cell markers (Figure 3).

3.3 The immunological features of TSPAN1 expressing B cells in AIH

Then, we explored the frequency and immunological features of $TSPAN1^+$ B cells from PBMCs of AIH patients and HC by flow cytometry. The percentage of $TSPAN1^+$ cells in B cells was significantly higher in AIH compared with HC. *t*-SNE analysis was performed to visually present the increment of

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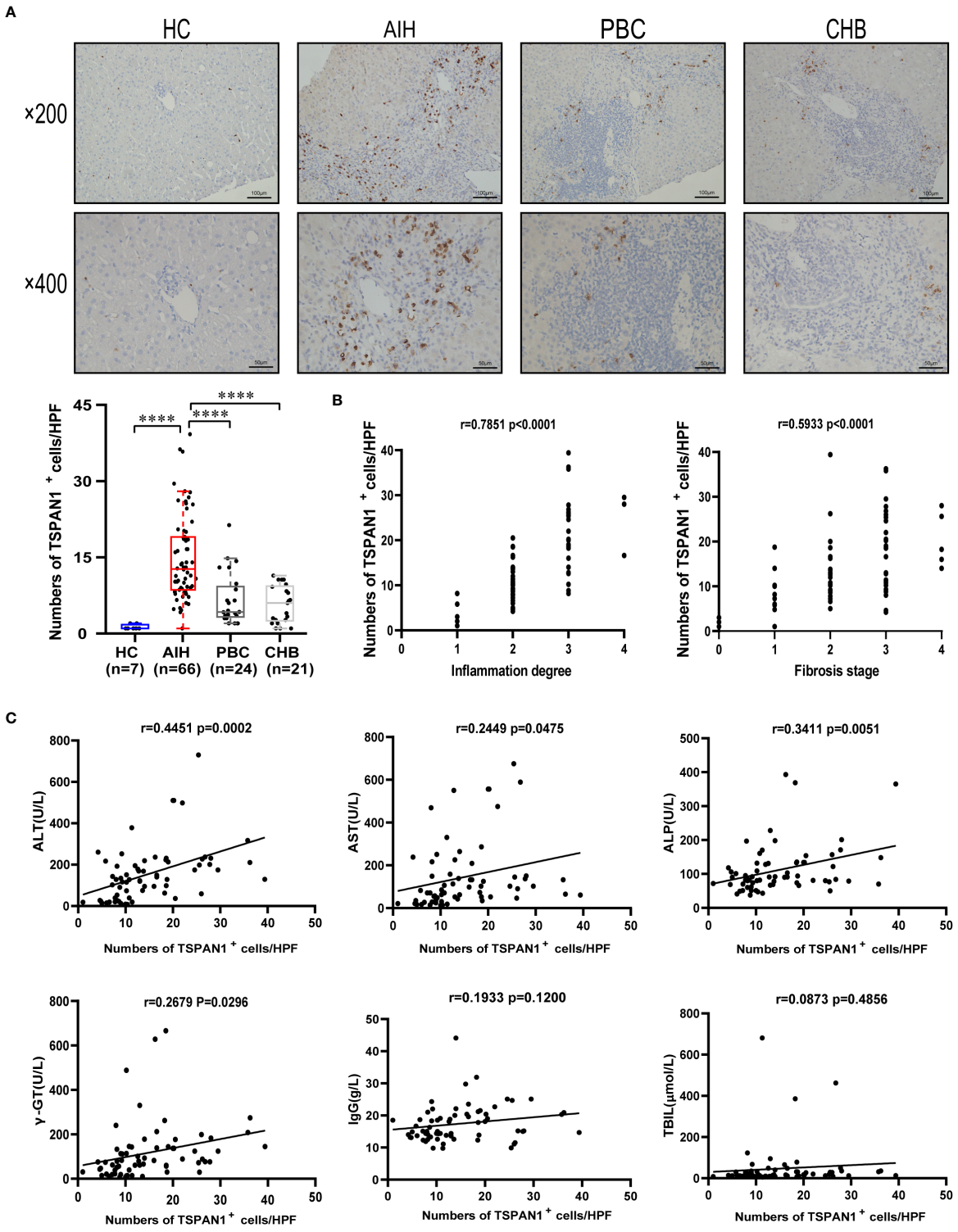

FIGURE 1
TSPAN1 expression was increased in AIH. **(A)** Representative immunohistochemical staining of TSPAN1 in AIH, PBC, CHB, and healthy controls (HCs). **(B)** The correlation analysis of the numbers of TSPAN1$^+$ cells in liver tissues of AIH with hepatic inflammation degree and fibrosis stage (×400). **(C)** The correlation analysis of the numbers of TSPAN1$^+$ cells in liver tissues of AIH with ALT, AST, ALP, γGT, IgG, and TBIL levels. ****$P < 0.0001$.

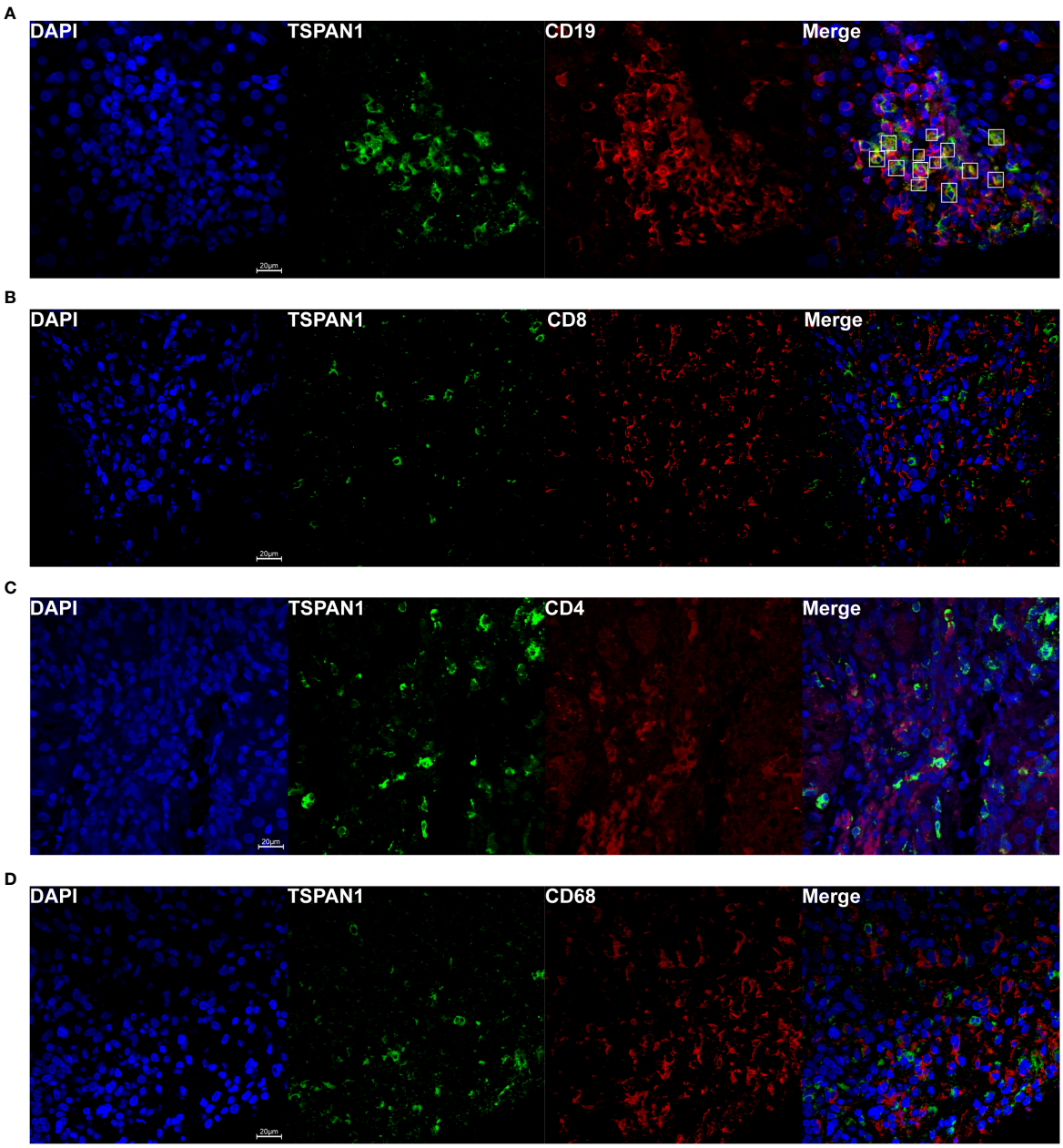

FIGURE 2
TSPAN1 was mainly expressed on hepatic B cells in AIH. Representative confocal staining of TSPAN1 with CD19 **(A)**, CD8 **(B)**, CD4 **(C)**, and CD68 **(D)** (×400) in liver tissues of AIH patients.

TSPAN1 in $CD19^+$ B cells (Figures 4A, B). We divided $CD19^+$ B cells into two groups including $TSPAN1^+$ cells and $TSPAN1^-$ cells, to further explore the characteristics of $TSPAN1^+$ B cells in AIH. Compared with $TSPAN1^-$ cells, $TSPAN1^+$ cells in AIH have higher expression of C-X-C chemokine receptor (CXCR) 3 (19.41% vs 5.73%, $P < 0.001$) and CXCR4 (66.56% vs 62.99%, $P < 0.05$), indicating enhanced chemotactic activity toward the liver. Antigen-presenting molecule CD86 (26.46% vs 13.57%, $P < 0.001$) was also highly expressed in $TSPAN1^+$ B cells (Figure 4C). Furthermore, $TSPAN1^+CD19^+$ B cells could secrete higher levels of proinflammatory cytokines, including granzyme B (55.43% vs. 33.02%, $P < 0.01$), IFN-γ (52.82% vs. 27.68%, $P < 0.01$), and TNF-α compared to $TSPAN1^-$ B cells (13.15% vs. 2.13%, $P < 0.01$). However, they also produced more TGF-β compared with $TSPAN1^-$ B cells in AIH (49.36% vs. 30.12%, $P < 0.01$) (Figure 4D).

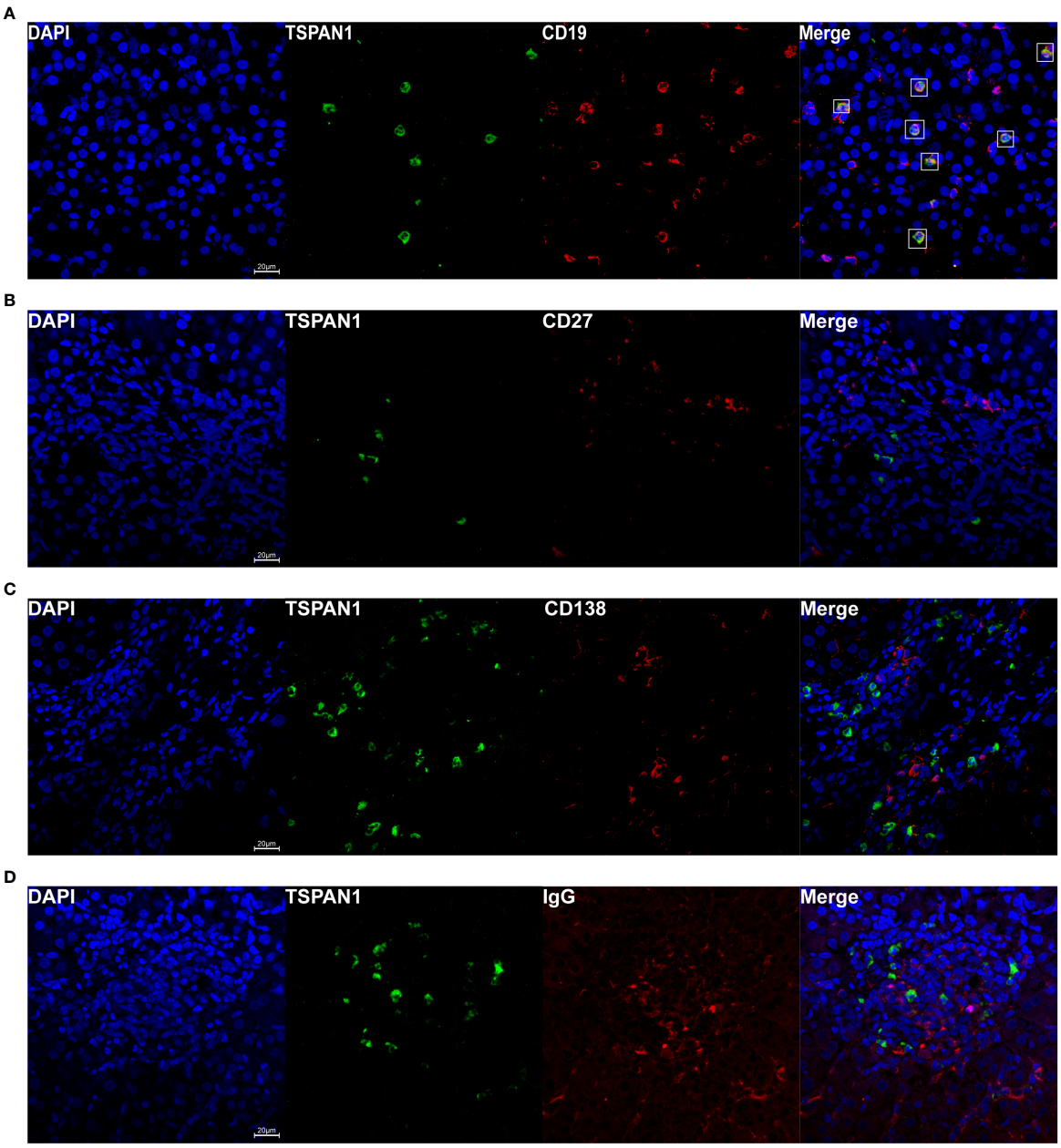

FIGURE 3
Immunofluorescence double-staining of CD19, CD27, CD138, and IgG with TSPAN1. Representative confocal staining of TSPAN1 with CD19 **(A)**, CD27 **(B)**, CD138 **(C)**, and IgG **(D)** (×400) in liver tissues of AIH patients.

3.4 TSPAN1$^+$ B cells presented a pro-inflammatory phenotype after *in vitro* stimulation

To explore whether TSPAN1$^+$ B cells have an analogous immunological function after *in vitro* culture, CPG, CD40L, and IL-4 were utilized to stimulate CD19$^+$ B cells isolated from PBMCs of healthy donors. Gating strategy was shown in Figure 5A. After stimulation, TSPAN1$^+$ B cells expressed significantly increased CD11c (5.44% vs. 3.97%, $P < 0.05$), CD38 (79.03% vs. 68.53%, $P < 0.01$), CXCR3 (24.00% vs. 11.57%, $P < 0.001$), CXCR4 (61.53% vs. 57.53%, $P < 0.05$), and

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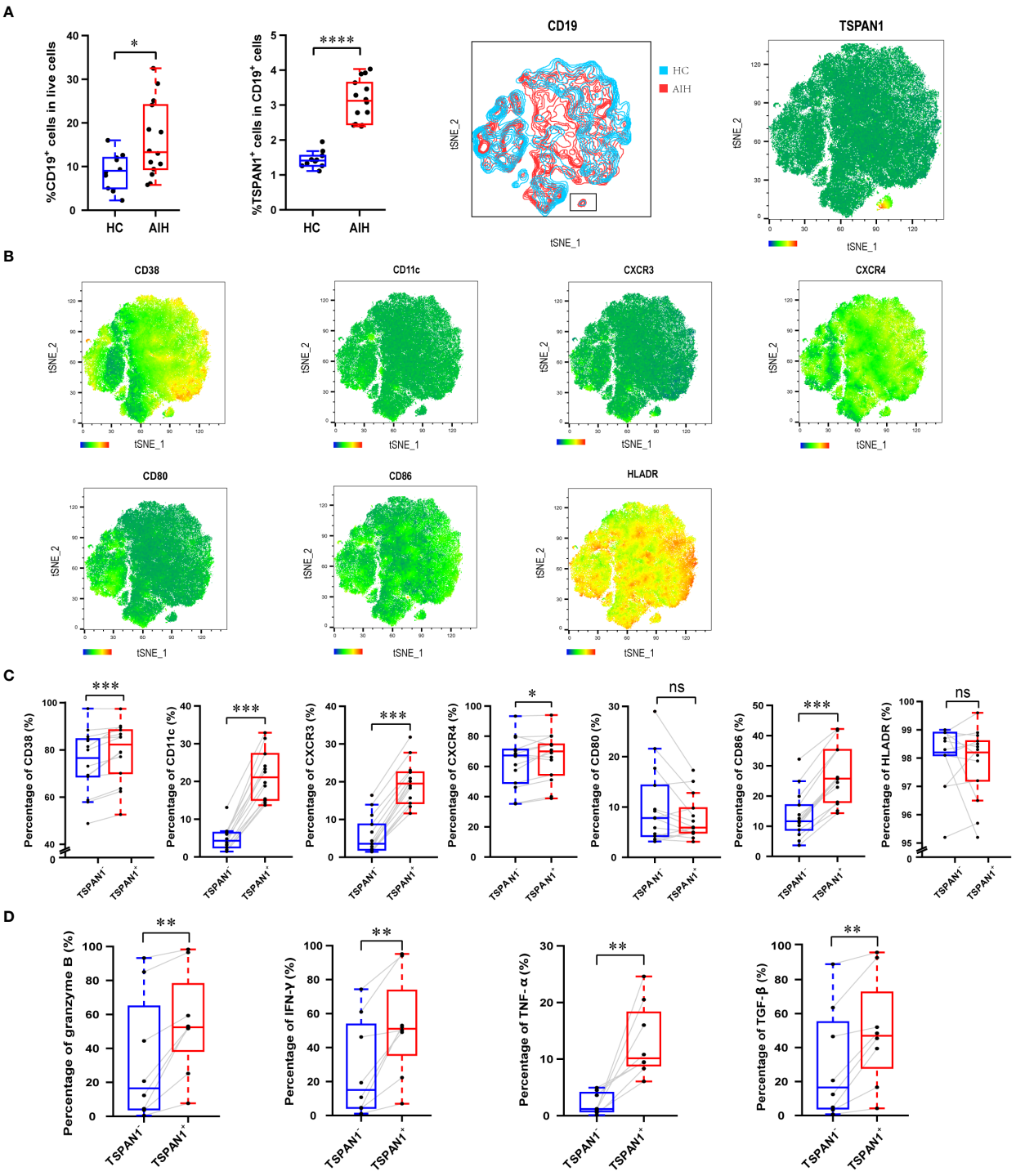

FIGURE 4
The immunological characteristics of TSPAN1$^+$ cells in AIH. **(A)** The percentage of CD19$^+$ B cells and TSPAN1$^+$ cells in peripheral blood mononuclear cells were elevated in AIH patients (n = 14) compared with HCs (n = 10). By *t*-SNE analysis of CD19$^+$ B cells, the TSPAN1$^+$ cell subset was compared between HC and AIH indicated by the black rectangle (left), and TSPAN1 expression in CD19$^+$ B cells was shown in AIH (right). **(B)** Expression of surface markers was shown by *t*-SNE analysis of CD19$^+$ B cells from AIH patients. Color is based on the expression of certain markers. **(C)** Analysis of surface markers in paired TSPAN1$^-$ cells and TSPAN1$^+$ CD19$^+$ cells from patients with AIH. Wilcoxon matched-pairs test was used. **(D)** Expression of cytokines in paired TSPAN1$^-$ cells and TSPAN1$^+$ CD19$^+$ cells from patients. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no significance.

CD86 (89.50% vs. 85.63%, $P < 0.01$) compared with TSPAN1$^-$ B cells (Figure 5B). We also observed that the expression of cytokines, including granzyme B (22.10% vs. 12.66%, $P < 0.05$), IFN-γ (8.08% vs. 6.15%, $P < 0.01$), TNF-α (10.93% vs. 1.89%, $P < 0.01$), and TGF-β (7.41% vs. 1.18%, $P < 0.05$) was higher in TSPAN1$^+$ B cells (Figure 5C). These results were consistent with the immunological feature of TSPAN1$^+$ B cells in peripheral blood from AIH.

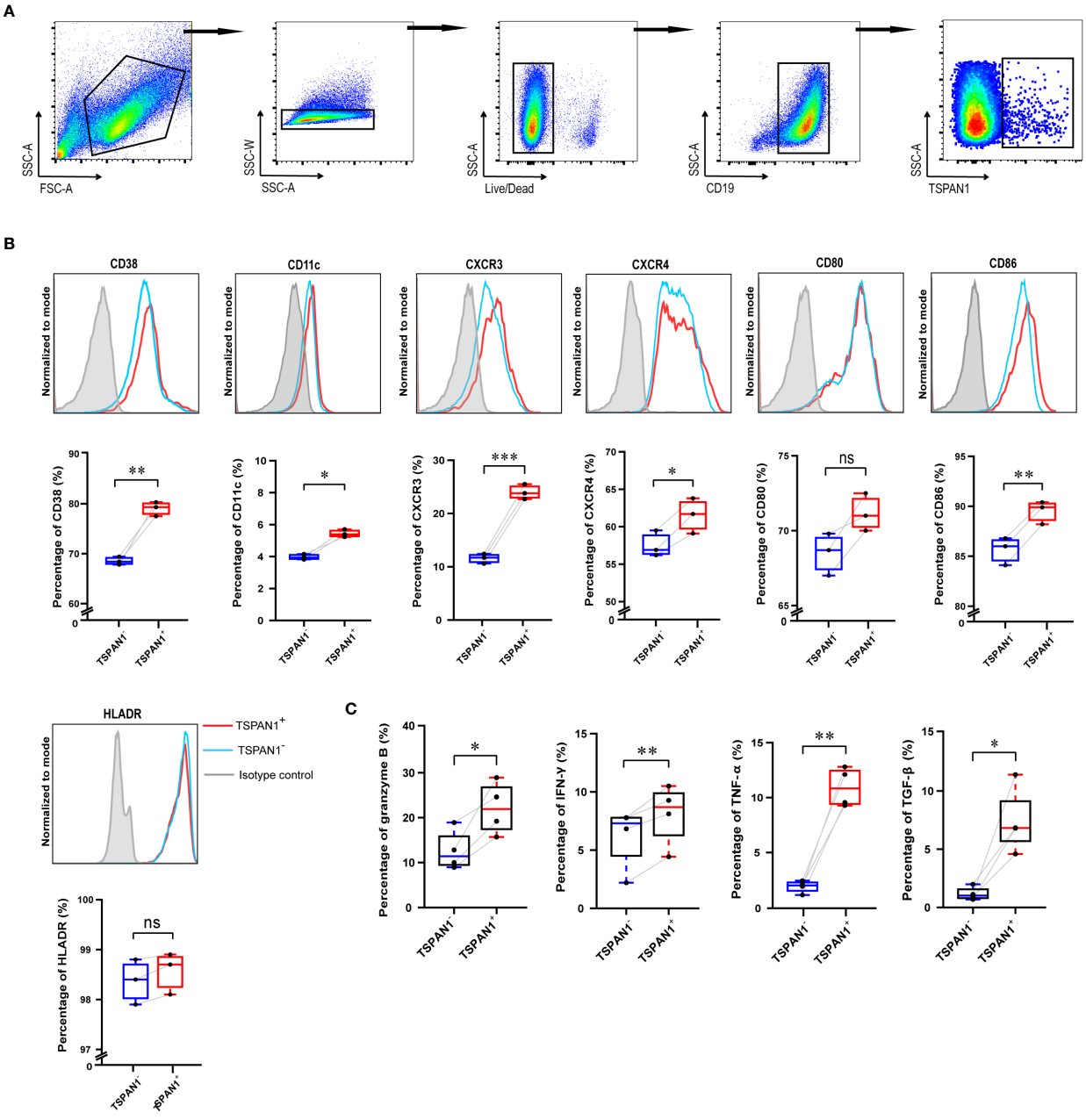

FIGURE 5
The immunological characteristics of TSPAN1$^+$ cells after *in vitro* stimulation. **(A)** The gating strategy used for flow cytometry analysis of TSPAN1$^+$ cells. **(B)** Histogram plot of surface markers of TSPAN1$^+$ B cells and paired TSPAN1$^-$ B cells (top row). Measurement of certain surface markers in paired TSPAN1$^-$ cells and TSPAN1$^+$ CD19$^+$ cells were shown (bottom row). **(C)** The expression level of cytokines in paired TSPAN1$^-$ cells and TSPAN1$^+$ CD19$^+$ cells. Paired Student *t*-test was used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: no significance.

3.5 TSPAN1$^+$ cells infiltrated in the liver through CXCR3-CXCL10 interaction

According to the results of flow-cytometric analysis, CXCR3 was highly expressed in TSPAN1$^+$ B cells. As shown in Figure 6A, multiplex immunofluorescence staining was further confirmed the co-expression of CD19, TSPAN1, and CXCR3 in liver tissues from AIH. More interestingly, TSPAN1$^+$ cells were closed to CXCL10 positive cells in the liver of AIH (Figure 6B). Since CXCL10 was the ligand for CXCR3, we detected the expression of CXCL10 in the liver tissues. The level of CXCL10 was significantly elevated in AIH compared with HC ($P < 0.01$). Besides, there is a correlation between the numbers of TSPAN1 and the expression of CXCL10 ($r = 0.4451$, $P < 0.05$) (Figure 6C). We next performed experiment to investigate the migration ability of TSPAN1$^+$ B cells *in vitro*. CD19$^+$ cells of AIH patients were isolated and stimulated for 24 hours and placed in the transwell chambers for 6 hours. As a result, a higher percentage of TSPAN1$^+$ cells in CD19$^+$ B cells were detected in lower chambers after adding rhCXCL10 compared with control group. Moreover, the percentage of TSPAN1$^+$ cells in lower chambers was higher than that in paired upper chambers after adding rhCXCL10 (Figure 6D). These results suggested that the high expression of CXCL10 in the liver of AIH may contribute to the chemotaxis of TSPAN1-positive B cells.

3.6 Histological remission was accompanied with decreased TSPAN1 expression in AIH

Patients with AIH who had biopsies before and after treatments were enrolled (n =27). The paired biopsies of the liver showed that both hepatic inflammation and fibrosis were alleviated after treatments (Figure 7A). Immunohistochemical staining for TSPAN1 was performed. The results showed that the frequency of TSPAN1 was dramatically decreased in the liver tissues of AIH patients with treatment. To expand the follow-up biopsy cohort, we enrolled another 28 patients with a follow-up biopsy. 55 patients in total were divided into histological remission and non-remission. There were 43 patients in the remission (HAI < 4) group and 12 in the non-remission (HAI > 4) group. Interestingly, the frequency of TSPAN1 positive cells in the remission group was significantly decreased compared with that in the non-remission group (Figures 7B–D). These results suggested that TSPAN1$^+$ cells may participate in the progression of AIH and were associated with histological remission.

4 Discussion

B cells have been demonstrated to be involved in the progression of AIH by producing autoantibodies, pro-inflammatory factors and functioning as APCs. In this study, we investigated the immunological feature of TSPAN1$^+$ cells in AIH. Intriguingly, the numbers of TSPAN1$^+$ cells were significantly increased in the livers of AIH and shared a close correlation with disease severity. According to confocal staining, TSPAN1$^+$ cells were mainly CD19 positive B cells. However, these cells did not express CD27 or CD138, which may be naive B cells.

B cells can secrete antibodies, proinflammatory factors, and participate in antigen presentation, which are closely related to various diseases. Herein, compared with HCs, the proportion of TSPAN1$^+$ cells in CD19$^+$ B cells increased in the PBMC of AIH patients. It is also observed that TSPAN1$^+$ cells secret more IFN-γ, TNF-α, and granzyme B, compared with TSPAN1$^-$ cells. In addition, TSPAN1$^+$ B cells expressed higher level of CD86, indicating their superior antigen-presentation ability.

Chemokine-receptor and ligand interactions have been identified as critical signals for immune cells recruitment. CXCR3 and CXCR4 were previously reported to be expressed by non-B lymphocyte immune cells and related to retention in the liver (26). Recent studies showed that CXCR3 and CXCR4 can also be expressed on B cells (27, 28). Our study demonstrated that CXCR3 was highly expressed on TSPAN1$^+$ B cells. Interestingly, the chemokine CXCL10, a ligand of CXCR3, was detected and appear to contribute to the pathogenesis of various autoimmune disease. CXCL10 can bind to CXCR3 and regulate immune responses according to activation and recruitment of leukocytes. CXCL10 was also elevated in patients of AIH (9, 29) and observed highly expressed in liver tissues of patients by immunohistochemical staining. TSPAN1$^+$ cells were adjacent to CXCL10 in the liver. Furthermore, the interaction between TSPAN1 and CXCL10 was further confirmed by transwell assay *in vitro*. CXCR3-CXCL10 interaction may contribute to the chemotaxis of TSPAN1$^+$ B cells to the liver of AIH.

In this work, to better study TSPAN1$^+$ cells *in vitro*, CPG, CD40L, and IL-4 were founded to enhance the expression of TSPAN1 in B cells. The liver is a unique immune regulatory organ characterized by complex immune activities triggered by a variety of immune cells, including B cells (30). CD40 signals together with other signals support the differentiation of B cells into plasma cells and further secrete various isotypes of antibodies. IL-4 is essential for B cell maturity and abundant in the liver (31). Combined with CD40 signaling, IL-4 promoted the proliferation of both circulating and tissue-resident B cells (32–34). Compared with peripheral blood, liver is enriched with various cytokines and lymphocytes, especially in autoimmune liver disease. Complex liver microenvironment provides a more proper condition for proliferation and aggregation of TSPAN1$^+$ B cells, which partly explained why there were more TSPAN1$^+$ B cells in the liver of AIH.

We found that the numbers of TSPAN1$^+$CD19$^+$ B cells were decreased in the liver of AIH patients after therapy. Furthermore, the frequency of TSPAN1$^+$ cells was lower in the

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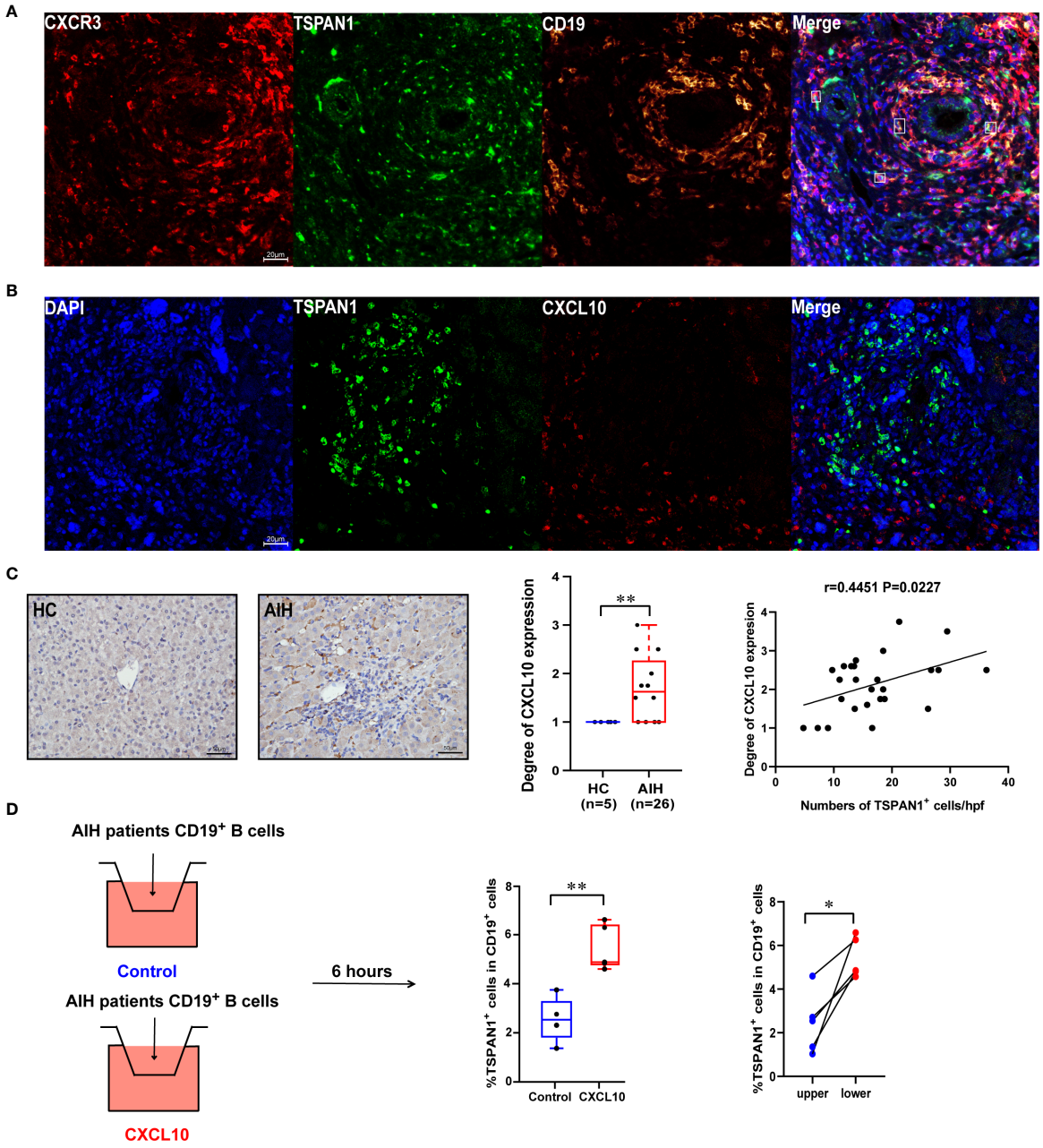

FIGURE 6
The expression of CXCR3 and CXCL10 in AIH. **(A)** Multiplex immunofluorescence staining of CXCR3 (red), CD19 (yellow), and TSPAN1 (green) in the liver of AIH patients. **(B)** Representative confocal staining of TSPAN1 and CXCL10 in the livers of AIH. **(C)** Immunohistochemical staining of CXCL10 in AIH and HC (×400). Correlation analysis of hepatic CXCL10 expression degree with the numbers of TSPAN1. **(D)** $CD19^+$ B cells were placed in the upper chambers and complete medium was added in the lower chambers with rhCXCL10 (3 μg/mL) or not for 6 hours. The percentage of $TSPAN1^+$ cells was measured by flow cytometry. Boxes represent the 25th–75th percentile of the distribution; the median is shown as a thick line in the middle of the box; whiskers extend to values with 1.5 times the difference between the 25th and 75th percentiles. $^*P < 0.05$, $^{**}P < 0.01$.

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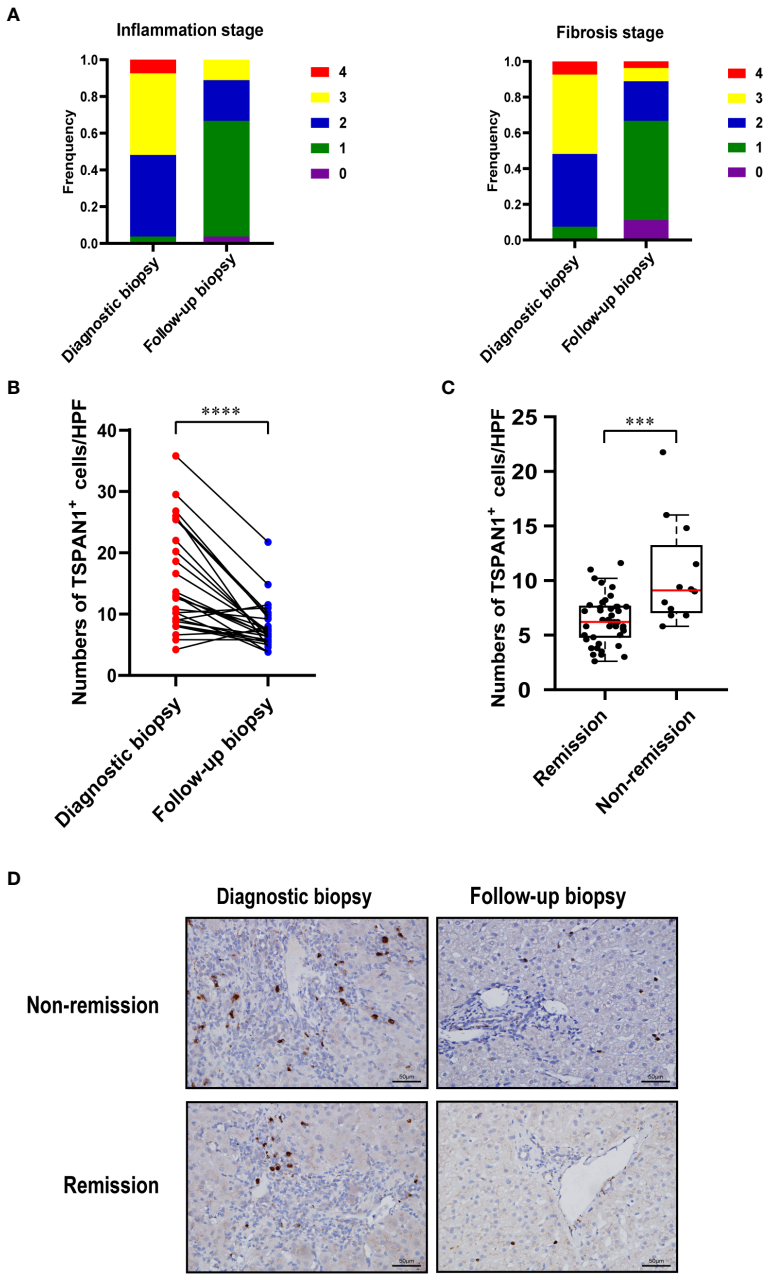

FIGURE 7
TSPAN1 expression was decreased after immunosuppressive treatment. **(A)** Hepatic inflammation and fibrosis stages were evaluated in diagnostic biopsies and follow-up biopsies in AIH patients. **(B)** Expression of TSPAN1 in paired diagnostic biopsies versus follow-up biopsies. **(C)** Expression of TSPAN1 in remission versus non-remission group. **(D)** Representative immunohistochemical staining of TSPAN1 in a diagnostic biopsy (left) and a paired follow-up biopsy (right) from non-remission (top) and remission group (bottom) (x400). ***$P < 0.001$, ****$P < 0.0001$.

remission group compared with the no-remission group. These indicated that TSPAN1$^+$ B cells may play an important role in the progression of AIH. Thus, our study provides a potential molecular target for the treatment of AIH.

In conclusion, this research found that TSPAN1$^+$ B cells were elevated and may be involved in the pathogenesis of AIH. Overexpression of CXCL10 in liver may contribute to the chemotaxis of TSPAN1$^+$ B cells. Besides, the frequency of

$TSPAN1^+$ cells was closely related to the remission of disease. TSPAN1 may be a potential target to alleviate AIH.

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 ethics committee of Renji Hospital, Shanghai Jiao Tong University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

QW, XM, and ZY designed and supervised the study. QW, RT, XM, and ZY acquired funding. YO and RC performed the experiments. YO, RC, QQ, and NC collected samples and clinical information. YO analyzed the data and drafted the manuscript. QW, XM, and ZY reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Immunomodulatory role of mesenchymal stem cell therapy in liver fibrosis

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Liver fibrosis is a fibrogenic and inflammatory process that results from hepatocyte injury and is characterized by hepatic architectural distortion and resultant loss of liver function. There is no effective treatment for advanced fibrosis other than liver transplantation, but it is limited by expensive costs, immune rejection, and postoperative complications. With the development of regenerative medicine in recent years, mesenchymal stem cell (MSCs) transplantation has become the most promising treatment for liver fibrosis. The underlying mechanisms of MSC anti-fibrotic effects include hepatocyte differentiation, paracrine, and immunomodulation, with immunomodulation playing a central role. This review discusses the immune cells involved in liver fibrosis, the immunomodulatory properties of MSCs, and the immunomodulation mechanisms of MSC-based strategies to attenuate liver fibrosis. Meanwhile, we discuss the current challenges and future directions as well.

KEYWORDS

liver fibrosis, mesenchymal stem cell, immunomodulatory effects, exosome, antifibrosis

1 Introduction

Liver fibrosis is a complex fibrogenic and inflammatory process that results from chronic liver injury. The primary pathophysiology of liver fibrosis is increased collagen deposition of types I and III in the extracellular matrix (ECM) (1, 2). Major etiological agents of liver fibrosis include alcohol, viruses, metabolic, and congenital disorders, all of which can lead to hepatocyte injury and hepatic stellate cell (HSC) activation, resulting in excessive ECM deposition and structural disorders in the liver (3, 4). Despite distinct pathogenesis, their common outcome is the development of liver cirrhosis. In recent years, the incidence and mortality of hepatic fibrosis have increased steadily and become a substantial global health burden. Other than liver transplantation, there is no curative treatment for end-stage

cirrhosis. However, expensive costs, immune rejection, and postoperative complications limit liver transplantation (5, 6).

Stem cell transplantation has emerged as the most promising treatment for liver fibrosis since the advent of regenerative medicine (7–9). Stem cells are a population of cells with self-renewal, proliferation, and pluripotent differentiation potential that can differentiate into multiple cell types under defined conditions (10, 11). Embryonic stem cells (ESCs) (12, 13), induced pluripotent stem cells (iPSCs) (14–17), and mesenchymal stem cells (MSCs) (18–22) have been investigated the most in the treatment of liver fibrosis. ESCs, the prototype of pluripotent stem cells, have nearly unlimited self-renewal capacity and differentiation potential (23). iPSCs display similar surface antigens expression, proliferation capacity, morphology, and gene expression characteristics as embryonic stem cells (24). ESCs and iPSCs achieve therapeutic effects mainly by differentiating into mature hepatocytes *in vitro* or *in vivo* (8). MSCs, on the other hand, not only have the potential for self-renewal and differentiation (25) and can regulate the immune response (7). Moreover, since abnormal immune responses are the primary cause of liver fibrosis, MSCs are the most promising stem cells for treating liver fibrosis, as they can slow or even reverse the progression of liver fibrosis (26–28).

This review focuses on the immune cells involved in liver fibrosis, the immunomodulatory properties of MSCs, and the immunomodulation mechanisms of MSC-based strategies to attenuate liver fibrosis. The challenges and future directions were also discussed.

2 Immune cells participate in the process of liver fibrosis

According to common knowledge, the liver is composed of primary hepatocytes, cholangiocytes, Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), HSCs, fibroblasts, lymphocytes, oval cells, lymphocytes, and other immune cells (29). The liver's blood supply originates from the hepatic artery and portal vein and passes through infections or toxins of systemic and intestinal origin. Hence, the liver is highly susceptible to pathogens that cause acute or chronic liver injury (30). Injury to intrahepatic parenchymal cells, such as hepatocytes or cholangiocytes, causes liver fibrosis. Varied etiologies could contribute to liver damage, including inflammation, chronic viral hepatitis, alcohol consumption, chronic cholestasis, and non-alcoholic steatohepatitis (NASH). Although the etiologies are different, the initial phase often includes hepatocyte injury, which subsequently causes the release of oxygen radicals and inflammatory molecules. These pro-inflammatory mediators stimulate KCs and hepatic sinusoidal endothelial cells, leading to the transdifferentiation of HSCs from a quiescent to an activated phenotype (1–3). In addition, other liver-specific immune cells, including natural killer (NK) cells, natural killer T cells (NKT) cells, dendritic cells (DCs), and neutrophils, react to injured hepatocytes by producing cytokines and initiating inflammatory responses. These inflammatory cytokines (such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF)) could stimulate and transdifferentiate HSCs into myofibroblasts (3, 4). HSCs are the most critical non-parenchymal cells of the liver, located in the Disse space, and play a crucial role in developing liver fibrosis. Fibroblasts in the bone marrow or circulating blood, as well as hepatocytes and cholangiocytes, can also be transdifferentiated into myofibroblasts by epithelial-mesenchymal transition (EMT). However, those derived from activated HSCs are the most common (4).

Activation of HSCs results in the generation of a massive ECM and the expression of α-smooth muscle actin (α-SMA). TGF-β is the primary cytokine involved in the activation of HSC transdifferentiation and the EMT signal. TGF-β1 activation increases ECM synthesis and inhibits ECM degradation, thereby accelerating the liver fibrosis development. In addition, myofibroblasts are known to produce tissue inhibitors of matrix metalloproteinases (TIMPs) to prevent matrix metalloproteinases (MMPs) from degrading the ECM and maintaining ECM integrity (Figure 1) (31, 32).

3 The immunomodulatory properties of mesenchymal stem cells

MSCs were initially utilized primarily for tissue repair and regeneration. Subsequently, they have been increasingly used to treat graft-versus-host disease (GVHD) (33) and autoimmune diseases like lupus (34) and Crohn's disease (35). Furthermore, the clinical potential of MSCs has been extended to treat myocardial infarction (36), stroke (37, 38), multiple sclerosis (39), liver cirrhosis (18, 40), diabetes (41), lung injuries (42, 43), and cancer (44). MSCs have been isolated and expanded from numerous adult and perinatal tissues, including bone marrow, adipose tissue, peripheral blood, fetal tissues, dental pulp, umbilical cord, and placental tissues (45).

Currently, it is believed that MSC must possess at least the following three characteristics (46): first, the cells must be able to grow on plates; second, CD90, CD73, and CD105 must be expressed, while HLA class II, CD19 or CD79a and CD14, CD34, CD45 or CD11b are negative; and third, the cells must be able to differentiate into osteoblasts, chondrocytes, and adipocytes.

MSCs are multipotent cells emerging as the most promising method of allogeneic cell treatment (45). MSCs have natural immunomodulatory properties, trophic capacities, and strong *in vitro* self-renewal capacity, and their immune-modulatory actions can be easily manipulated (44). MSCs influence most immune cell functions through direct contact and factors in the local microenvironment. Previous research has revealed that cytokines released by MSCs are primarily responsible for the

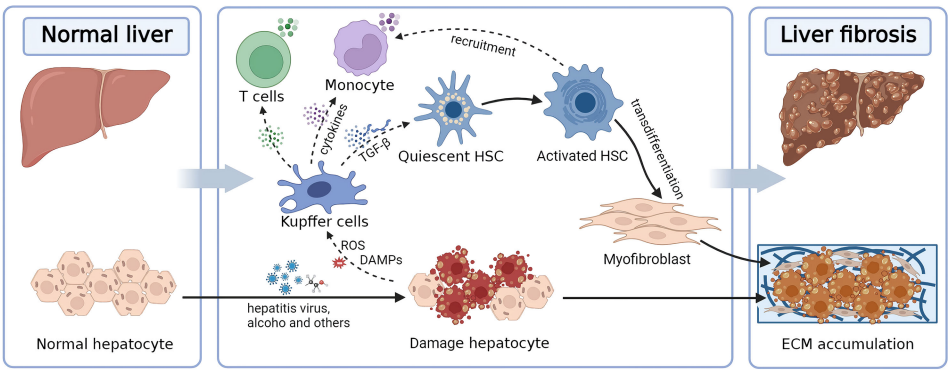

FIGURE 1
Diagram illustrating the pathological mechanisms underlying liver fibrosis. Hepatic fibrosis is induced by the imbalance between ECM production and degradation, driven by HSCs activated by hepatocyte injury. ECM, extracellular matrix; HSC, hepatic stellate cell; TGF-β, transforming growth factor-β.

immunomodulatory actions of MSCs (28). However, recent research has shown that apoptotic and metabolically inactive MSCs still possess the immunomodulatory capability, with regulatory T cells and monocytes playing a vital role (47).

MSCs regulate both innate and adaptive immunity (47), and their immunomodulatory functions are predominantly exerted through cell-to-cell contact and paracrine activity with macrophages, monocytes, neutrophils, T cells, B cells and natural killer (NK) cells. By secreting prostaglandin E2, MSCs convert pro-inflammatory M1 into anti-inflammatory M2 macrophages (PGE2) (48). MSCs can also modulate immune responses by activating the Notch 1 signaling pathway, releasing HLA-G5, PGE2, and TGF-1, and boosting the activation and proliferation of CD4+CD25+FoxP3+ regulatory T cells (Tregs) (49). MSCs decrease the proliferative potential of CD8+ T lymphocytes by producing indoleamine 2,3-dioxygenase (IDO) and heme oxygenase-1 (HO-1) and increase the rate of CD4+ T lymphocytes changing from type 1 T helper (TH1) to TH2 phenotype (50). In addition, hepatocyte growth factor (HGF) and IL-6 produced by MSCs, for instance, prevent the differentiation of monocytes into dendritic cells and reduce their propensity to cause inflammation, reduce the secretion of pro-inflammatory cytokines IL-12 and IFN-γ, and increase the production of anti-inflammatory cytokine IL-10, thereby inhibiting T-cell activation (51). MSCs reduce the generation of the pro-inflammatory cytokine TNF by inhibiting the activity of blast cells.

Successful immunomodulation and tissue regeneration depend on the interplay between MSCs and macrophages, especially juxtacrine mechanisms and cell–cell contact (52). Inflammatory factors released by M1 macrophages or activated T cells stimulate MSCs to secrete cytokines to differentiate monocytes toward the M2 type of the anti-inflammatory phenotype (53). It is known that MSCs may release anti-inflammatory or pro-inflammatory cytokines (such as IL1b, IL-6, IL-8, and IL-9) to mediate their immunomodulatory effects (54). As stated above, MSCs either inhibit or promote inflammation based on their exposure to pathological circumstances. The ultimate immunomodulatory effect may depend on the ratio of anti-inflammatory to pro-inflammatory factors in their surrounding environment (55).

4 Mesenchymal stem cell therapies for liver cirrhosis: Immune regulation plays a central role

4.1 Potential mechanisms of MSC-based treatment of liver fibrosis

Many studies have investigated the mechanisms of MSCs in the treatment of liver fibrosis from diverse perspectives, and they have been classified into three types (13, 22, 56). (1) MSCs can transdifferentiate into hepatocytes or merge with existing hepatocytes when introduced into injured liver tissue, making them a valuable resource for liver tissue regeneration and repair; (2) MSCs are capable of producing various cytokines, growth factors, and exosomes, which stimulate the regeneration of damaged liver tissue; (3) MSCs possess inhibitory effects on several other cell types, including NKs, B and T lymphocytes, allowing them to exercise immunomodulatory effects on liver diseases.

By migrating to damaged tissues, transplanted MSCs contribute to the regeneration of the damaged liver *via* hepatocyte differentiation mechanisms. Adding specific growth factors to *in vitro* culture can promote the differentiation of MSCs into hepatocyte-like cells with liver-specific morphology and functions, such as uptake of low-density lipoprotein and indocyanine green, secretion of albumin and urea, glycogen

storage, and cytochrome P450 activity (25, 57). For instance, intrasplenic-grafted MSCs transplanted into liver tissue treated with carbon tetrachloride (CCl_4) undergo hepatogenic differentiation into HLCs with typical hepatocyte morphology and form a three-dimensional structure (58). Transplantation of hepatocyte-differentiated MSCs further inhibited hepatocyte necrosis and stimulated liver regeneration, thereby enhancing the survival rate of the ALF model after transplantation into damaged liver tissue.

However, several studies have demonstrated that transplanted MSCs rarely undergo hepatic differentiation and vanish from the liver within one month (59). These studies indicate that MSCs stimulate liver regeneration *via* immune regulation and paracrine mechanisms (25, 56, 60). By regulating innate and adaptive immune cells, including T lymphocytes, regulatory T cells, helper T cells, B lymphocytes, and regulatory B cells, MSCs create a tolerant environment for maintaining immune homeostasis *in vivo* (61–64). As previously mentioned, the development of liver fibrosis is a wound-healing process involving key events, including hepatocyte injury, immune cell infiltration, HSCs activation, and excessive ECM deposition. Therefore, the immunomodulatory function of MSCs can be exploited to ameliorate liver fibrosis, and it has been an area of intense study interest (Figure 2).

4.2 MSC can inhibit immune cell infiltration

Infiltration of immune cells is a necessary stage in liver injury. MSCs create an immunological-tolerant milieu in liver tissue by reducing the infiltration of pro-inflammatory immune cells and promoting the recruitment of anti-inflammatory immune cells, preventing acute or chronic liver damage. Producing soluble factors, including TGF-β, PGE2, IDO, NO, and HGF, MSCs were able to inhibit the activation of T cells. MSCs can induce the transdifferentiation of CD4+ T cells into CD25+Foxp3+ regulatory T cells (Tregs) through the release of TGF-β (7, 49). Studies have demonstrated that MSCs dramatically decreased the number of CD4+ T cells invading the liver, the proportion of activated CD4+ T lymphocytes, and the total concentration of Th1 cells, and subsequently induced regulatory DCs and Tregs in the liver to ameliorate liver damage (65). MSCs significantly alleviated CCl_4-mediated liver fibrosis by decreasing the proportion of Th17 cells and increasing the levels of CD4+IL-10+ T cells and immunosuppressive factors (including kynurenine, IDO, and IL-10). In addition, MSCs enhanced liver function and ameliorated clinical symptoms in patients with hepatitis B virus-mediated decompensated cirrhosis by significantly downregulating the expression levels of IL-6 and TNF-α, while upregulating the expression level of IL-10 (66).

4.3 Blocking HSC is a key target for MSC to attenuate liver fibrosis

Blocking the activation of HSCs is one of the most crucial intervention targets for liver fibrosis (8). Pro-inflammatory mediators, oxidative stress molecules, and inflammatory stimulants produced by apoptosis or necrosis of liver parenchymal cells initiate the activation of HSCs (4). Activated HSCs subsequently release a variety of inflammatory chemicals that enhance the liver's inflammatory response. TGF-β is considered to be one of the most important signaling molecules for the activation of HSCs.

According to certain studies, milk fat globule-EGF factor 8 (MFGE8), one of the mediators secreted by MSC, is an anti-fibrotic protein that prevents the activation of HSCs by suppressing TGF-β type I receptor (TGFBR1) (67). Moreover, Caveolin-1, another possible target for MSC therapy, inhibited HSCs significantly (68). The expression of Wnt pathway-related proteins such as PPARγ, Wnt3a, Wnt10b, β-catenin, and WISP1 and Cyclin D1 is also known to be critical for HSC activation, and MSC has inhibitory effects on several molecules of this pathway (19). Ohara et al. (69) demonstrated that amniotic membrane-derived MSCs (AMSCs) could inhibit HSCs activation by downregulating the upstream steps of the LPS/TLR4 signaling pathway without inhibiting downstream NF-κB transcriptional activity. Qiao et al. (70) proved that hBM-MSCs significantly inhibited the proliferation of activated HSCs by inducing the apoptotic process of activated HSCs. Moreover, hBM-MSCs decreased the expression of peroxisome proliferator-activated receptor γ and α1(I) collagen and α-smooth muscle actin (α-SMA) in activated HSCs by decreasing the signalling pathway of NADPH oxidase, thereby delaying the progression of liver fibrosis.

4.4 MSC's function in ECM degradation and remodeling

As mentioned above, liver fibrosis is associated with excessive ECM deposition and decreased ECM lytic activity. ECM degradation and remodelling are considered vital targets for reversing liver fibrosis and delaying the progression of liver fibrosis. Modulating TGF-β signalling is one of the important mechanisms of MSC-based modulation of liver fibrosis (8). MSCs participate in the regulation of TGF-β downstream pathways. MSCs were able to significantly down-regulate the mRNA expression of TGF-β1 and TGFBR1 downstream molecule SMAD3 and increase the mRNA expression of SMAD7 (71). It has been demonstrated that SMAD3 upregulates the expression of the pro-fibrotic factor α-SMA or

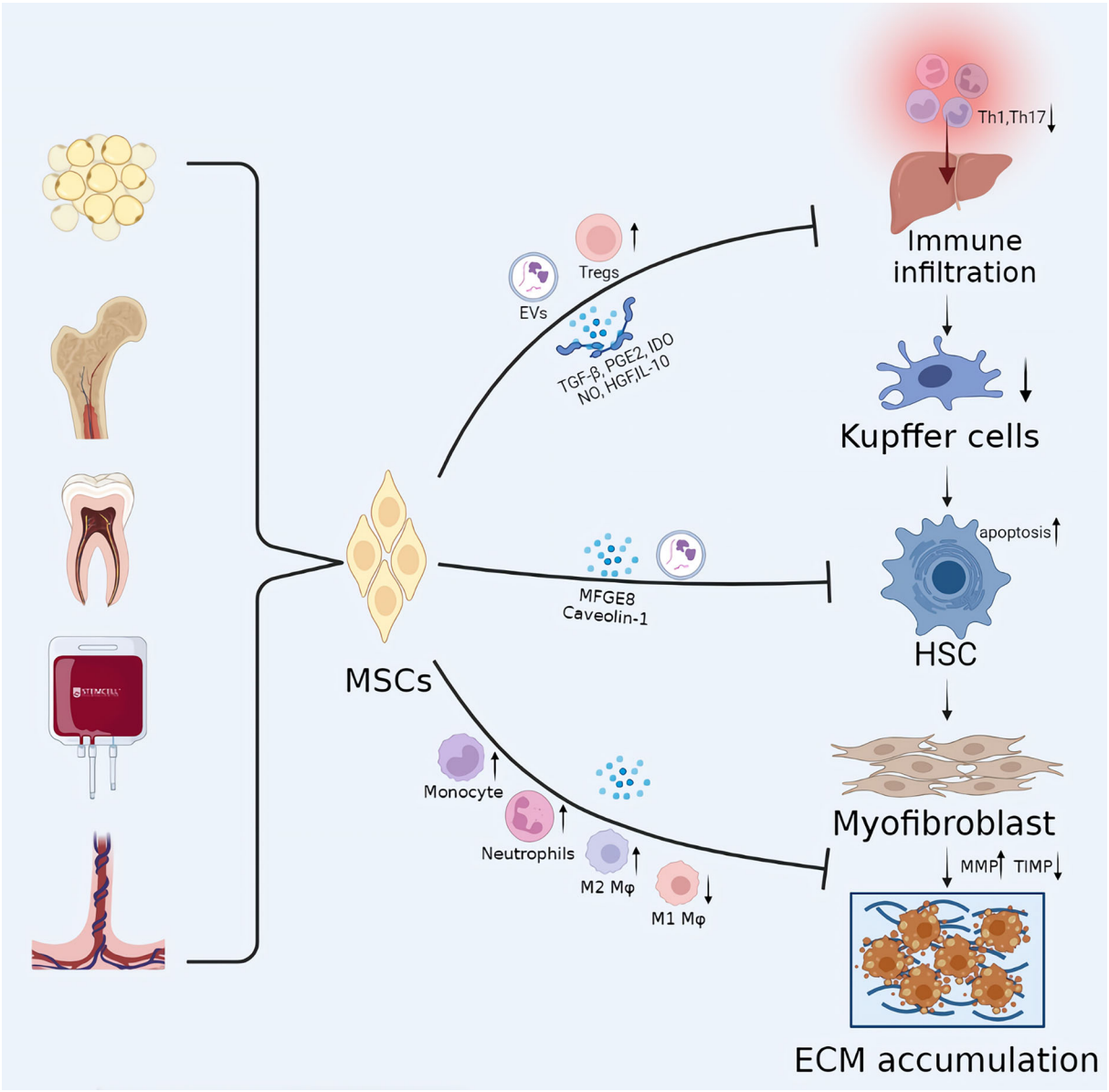

FIGURE 2
The potential mechanisms of MSCs in liver cirrhosis. The development of liver fibrosis is a wound-healing process involving key events, including hepatocyte injury, immune cell infiltration, HSC activation, and excessive ECM deposition. MSC transplantation can play a therapeutic role in every stage of liver fibrosis, with immunomodulatory effects playing a central role. EVs, extracellular vesicles; Mφ, Macrophages; IDO, indoleamine 2,3-dioxygenase.

Col1a1, whereas SMAD7 has an anti-fibrotic impact. ECM components laminin and hyaluronic acid were significantly decreased when BMSC overexpressed SMAD7.

In addition, it was demonstrated that by overexpressing SMAD7, MSC could enhance serum MMP-1 levels and reduce TIMP-1 levels. MMP-1 is a matrix metalloproteinase that degrades matrix collagen type I (Col1a1) and collagen type III (Col3a1). TIMPs, on the other hand, inhibit MMP activity by forming reversible covalent complexes with the corresponding MMPs. MSCs have been demonstrated to induce the infiltration of host monocytes and neutrophils into the liver and relieve fibrosis *via* MMP release (18). Luo et al. (72) demonstrate for the first time that BM-MSC transplantation promotes activation of MMP13-expressing M2 macrophages and suppresses M1 macrophages, which further inhibit HSCs, which play a synergistic role in attenuating liver fibrosis.

4.5 MSC can alleviate liver fibrosis *via* extracellular vehicles

In addition to direct cell-to-cell contact and paracrine cytokines, MSC can ameliorate liver fibrosis *via* extracellular vehicles (EVs). EVs produced by MSCs include exosomes (40-100 nm in diameter) and microvesicles (MVs, 0.1-1 mm in diameter). EVs contribute to the therapeutic potential of MSCs by enhancing intercellular contacts for the transport of paracrine substances during angiogenesis, tissue repair, and immunomodulation (73). Exosomes are nanoscale EVs derived from MVB, secreted into the extracellular microenvironment by the fusion of MVB with the plasma membrane. Exosomes can be taken up by target cells in the local milieu or transported to distant regions *via* biofluids. Exosomes contain numerous cytoplasmic and membrane proteins, such as nucleic acids (miRNA, mRNA, dsDNA, ssDNA, and mtDNA), ECM proteins, lipids, transcription factors, and receptors. Currently, the therapeutic mechanism of EVs is based on two main cargoes, RNA (especially miRNA) and proteins (74).

Exosomes have been shown to play an essential role in critical events in the development of liver fibrosis, including hepatocyte injury, immune cell infiltration, HSCs activation, and excessive ECM deposition (75–77). Several animal models of liver disease, including liver fibrosis and drug-induced acute liver injury, have been found to be alleviated by mesenchymal stem cell exosomes (MSCS-Ex) (78–81). For example, AMSC-Ex significantly decreased fiber accumulation, KCs number, and HSCs activation in rats with liver fibrosis. *In vitro*, AMSC-Ex significantly inhibited KC and HSC activation and suppressed the lipopolysaccharide (LPS)/toll-like receptor 4 (TLR4) signalling pathway. By decreasing collagen production and TGF-1 release, MSCs-Ex were also able to reduce the severity of liver fibrosis caused by ClC_4 (79, 82). MSC-originated exosomes circDIDO1 sponged miR143-3p in HSCs, causing cell cycle arrest, suppression, and apoptosis through promoting PTEN and repressing the ratio of p-AKT/AKT. Furthermore, umbilical cord mesenchymal stem cell exosomes (UCMSC-Ex) boosted the expression of the epithelium-associated marker E-cadherin while decreasing the expression of N-cadherin and vimentin-positive cells, suppressing EMT and preventing hepatocyte apoptosis. Jiang et al. (83) demonstrated that UCMSCs-Ex reduced CCl_4-mediated hepatocyte injury and liver fibrosis by inhibiting hepatocyte apoptosis and oxidative stress. In addition, MSCs can release immunopotent exosomes, allowing them to exert immunomodulatory effects on the differentiation, activation, and functionality of various subsets of lymphocytes. Tamura et al. (84) found that MSC-derived exosomes increased the production of anti-inflammatory cytokines and the number of T regulatory cells in mice with concanavalin A-induced liver injury, indicating immunosuppressive properties.

The ability of MSCs-Ex to execute drug transport tasks is another therapeutic use for these cells. Recent research has demonstrated that MSCs may package and distribute active medicines *via* their exosomes. These studies pave the way for researching and developing more robust and homing-capable novel medications employing MSCs. Cell-free treatment techniques avoid the potential for carcinogenesis, unneeded differentiation, embolization, cell injection, and infection dissemination associated with MSCs transplantation (21, 80). Furthermore, these therapies are safer, less costly, and more effective. Although MSCs-Ex shows considerable potential for treating liver disease, the absence of a consistent and efficient production process remains a key impediment. Thus, additional studies will be needed to address these important issues.

5 Challenges and future directions

Over the past two decades, there has been a rapid increase in cell therapy techniques for treating liver fibrosis. These technologies have accumulated knowledge on enhancing *in vitro* cell manipulation and cell transplantation processes to combat liver fibrosis and enhance liver repair. With the help of cutting-edge technologies such as bioreactors, microfluidics, and 3D bioprinting, these studies are now contributing to the development of new technologies aimed at producing *in vitro* systems that can yield liver-like tissue or whole bioengineered livers (85–87). However, it has been reported that MSCs therapy still has certain limitations for clinical application, including safety, limited cell survival, standardized production, ethical concerns, unavailability of trustworthy animal models, and lack of appropriate injection routes.

First, and perhaps most important, the safety of MSC-based therapy is still under discussion, especially in the context of long-term follow-up. A major concern is the undesired differentiation of transplanted MSCs and their propensity to impair antitumor immune responses and develop new blood vessels, which may contribute to tumor development and spread (88). In addition, neither the administration of MSCs nor their application in clinical studies has been standardized. Stem cells can readily differentiate into other cell types (except myofibroblasts), mediating communication between stem cells and HSCs, hepatocytes, or other intrahepatic cells associated with liver fibrosis, thereby triggering an immune response to promote fibrosis or prevent the reversal of liver fibrosis. Therefore, there is an urgent need for more sizeable prospective validation trials to verify the efficacy and safety of this treatment.

Second, low cell survival and poor integration with host tissue are well-recognized hurdles that confront the field of *in vivo* MSC delivery, and these may be improved by the pretreatment of stem cells (89, 90). Primary stem cells often fail to achieve the desired therapeutic effect due to poor homing ability, a low survival rate, and cellular senescence or decreased viability during *in vitro* culture. Pretreatments include transgenics, hypoxia, inflammatory factors, bioactive compounds, 3D cultures, disease-associated cells, or patient

serum. For example, the proliferation of HSCs and collagen deposition in rats with liver fibrosis were more effectively inhibited by HGF gene-transduced MSC (MSCs/HGF) (91). In a separate study, miR122-modified ADMSCs (ADMSC-122) constructed by lentivirus-mediated transfer of miR-122 had a similar effect (92). HGF is a hepatocyte growth factor that promotes hepatocyte regeneration, whereas miR-122 is crucial for inhibiting the proliferation and activation of HSCs. In conjunction with MSCs, they act on intrahepatic cells to treat liver fibrosis and overcome the lack of physiological activity of MSCs cells following transplantation.

Third, the effectiveness and safety of MSCs in treating patients with liver fibrosis have been studied in numerous clinical trials over the last decade. According to National Institutes of Health ClinicalTrials.gov, since January 1, 2012, 44 relevant clinical studies have been registered, 9 of which have been completed. Of these, China (23 cases; 52.3%), Vietnam (4 cases; 9.1%), India (3 cases; 6.8%), Japan (3 cases; 6.8%), South Korea (2 cases; 4.5%), and Belgium (2 cases; 4.5%) are the top six countries. In terms of cell origin, MSCs can be derived from human umbilical cord (14 cases; 31.82%), bone marrow (10 cases; 22.7%), adipose tissue (3 case; 6.8%), teeth (1 case; 3.2%), and unknown (16 cases, 36.4%). Simultaneously, several studies describing the outcomes of clinical trials using MSCs have been published. For instance, Yuwei et al. (93) published a meta-analysis and systematic review of randomized controlled trials (RCTs) to assess the efficacy and safety of MSCs therapy for patients with chronic liver disease. They evaluated 12 RCTs, including 846 patients who met their selection criteria. The results indicated that MSCs improved liver function compared to conventional treatment, primarily in ALB, TBIL, MELD score, and coagulation levels, but there were no significant changes in ALT and AST levels. Furthermore, MSCs treatment could not significantly improve the overall survival rate, with only a slightly positive trend. Five of these studies reported that the only side effect of MSC treatment was fever, with no other serious side effects. In terms of cell source, these clinical trials also show a tendency toward shifting from autologous to allogeneic MSCs. Allogeneic MSCs do not require the painful and complicated process of autologous cells collection (94). In addition, some MSCs can be made into off-the-shelf products due to the absence of immune rejection and the potential for massive expansion. Among these, UCMSC is a promising stem cell that is expected to be produced commercially (95). Many studies have shown that UCMSCs are an ideal source of MSCs because of young cellular age, relative ease of collection, easy batch production, and low allogeneic reactivity (96, 97).

In addition, there are various routes of MSC transplantation, including trans-portal, hepatic artery, peripheral vein transplantation, intrasplenic transplantation, peripheral vein transplantation, intrahepatic and abdominal transplantation, and the respective characteristics of these routes remain unknown. Under identical conditions, MSCs from different transplantation routes have different biodistribution *in vivo*. They may exert their effects on liver fibrosis *via* mechanisms influenced by the microenvironment, resulting in different therapeutic effects on fibrosis. Selecting a suitable stem cell transplantation route to increase the amount of stem cell colonization in liver tissue, enhance cell activity, and maintain the survival time of stem cells in liver tissue may be beneficial to improve the anti-fibrotic effect of MSCs. The indications for different transplantation routes in clinical practice must be further investigated (98, 99).

In contrast to cell-based pretreatment, cell-free therapy is a hot topic for future research. Cell-free strategies have low tumorigenic potential, low preservation costs, and low risk of exogenous infection and thrombosis. Unfortunately, no clinical trial has involved the use of stem cell-derived EVs to treat liver fibrosis and cirrhosis. This phenomenon is primarily due to the lack of standardized methods for extracting large numbers of EVs and the unknown dose and half-life of EVs.

6 Conclusion

The immune response is crucial to the genesis and progression of liver fibrosis. Due to their immunomodulatory properties, hepatic differentiation potential, and capacity to produce trophic factors, MSCs and MSC-Ex have emerged as promising agents for treating liver fibrosis. However, many issues need to be addressed before MSCs can be used clinically, including sufficient cell numbers, higher integration efficiency, consistency of *in vitro* and *in vivo* studies, and optimal timing and route of cell transplantation. Therefore, large randomized controlled clinical trials with longer follow-up periods and experimental animal studies are needed to improve the safety and efficacy of MSCs for fibrosis treatment.

Author contributions

PL: Conceptualization, methodology, investigation, funding acquisition, writing – original draft. YQ: Investigation, writing-original draft. XL: Investigation, methodology. XFZ: Investigation. YL: Conceptualization, supervision, funding acquisition. JX: Conceptualization, supervision, writing-review & editing, funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Lnc-AIFM2-1 promotes HBV immune escape by acting as a ceRNA for miR-330-3p to regulate CD244 expression

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Chronic hepatitis B (CHB) virus infection is a major risk factor for cirrhosis and hepatocellular carcinoma (HCC). Hepatitis B virus (HBV) immune escape is regulated by the exhaustion of virus-specific $CD8^+$ T cells, which is associated with abnormal expression of negative regulatory molecule CD244. However, the underlying mechanisms are unclear. To investigate the important roles of non-coding RNAs play in CD244 regulating HBV immune escape, we performed microarray analysis to determine the differential expression profiles of long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and mRNAs in patients with CHB and patients with spontaneous clearance of HBV. Competing endogenous RNA (ceRNA) was analyzed by bioinformatics methods and confirmed by the dual-luciferase reporter assay. Furthermore, gene silencing and overexpression experiments were used to further identify the roles of lncRNA and miRNA in HBV immune escape through CD244 regulation. The results showed that the expression of CD244 on the surface of $CD8^+$ T cells was significantly increased in CHB patients and in the co-culture system of T cells and HBV-infected HepAD38 cells, which was accompanied by the reduction of miR-330-3p and the elevation of lnc-AIFM2-1. The down-regulated miR-330-3p induced the apoptosis of T cells by lifting the inhibition of CD244, which was reversed by miR-330-3p mimic or CD244-siRNA. Lnc-AIFM2-1 promotes the accumulation of CD244, which is mediated by decreased miR-330-3p, and then reduced the clearance ability of $CD8^+$ T cells to HBV through regulated CD244 expression. And the injury in the ability of $CD8^+$ T cells to clear HBV can be reversed by lnc-AIFM2-1-siRNA, miR-330-3p mimic, or CD244-siRNA. Collectively, our findings indicate that lnc-AIFM2-1 on CD244 by acting as a ceRNA of miR-330-3p contributes to HBV immune escape, which may provide novel insights into the roles of interaction networks among lncRNA, miRNA, and mRNA in HBV immune escape, highlighting potential applications of lnc-AIFM2-1 and CD244 for diagnosis and treatment in CHB.

KEYWORDS

lncRNA, ceRNA, chronic hepatitis B, immune escape, CD244

1 Introduction

Chronic hepatitis B (CHB) infection continues to be a major health burden globally. Two billion people worldwide had contact with hepatitis B virus (HBV), with more than 290 million chronic HBV infections (1, 2). HBV is a noncytopathic virus, a double-stranded DNA virus, which needs to escape from the hosts' immune surveillance to survive (3, 4). Immune escape of the virus is not only related to its gene mutation, but also the host immune of T cell response to the virus (5). As the potent immune system clears the virus, the liver mainly presents as acute and self-limiting hepatitis (6). In contrast, the virus escapes the host immune response with $CD8^+$ T cell exhaustion, causing chronic hepatitis, and even progressing to cirrhosis and hepatocellular carcinoma (7). Therefore, repressing the occurrence of CHB has become a major breakthrough in treating hepatitis B and reducing the morbidity and mortality of cirrhosis and primary hepatocellular carcinoma. However, the mechanism by which antiviral $CD8^+$ T cells exhaustion plays this role in HBV immune escape is unclear.

The overexpression of signaling lymphocyte activation molecule family member 4 (SLAMF4, CD244), which is a transmembrane protein present on immune cells, enhances $CD8^+$ T cells depletion in CHB (8, 9). The ligand of CD244 is CD48, which is expressed broadly on hematopoietic cells (10). Under the stimulation of antigen-specific signals delivered through the T cell receptor (TCR), and CD244, as a co-stimulatory signal molecule, transmits the second signal and mediates the regulation of immune tolerance after being cross-linked with ligand CD48 (11). The interaction between programmed cell death receptor 1 (PD-1) and its ligand PD-L1 has been proved to play an important role in inducing hepatitis C virus (HCV) (12, 13) and HBV (14) infected T cell failure and apoptosis. Although high co-express of CD244 and PD-1 on $CD8^+$ T cells, blocking CD244 or CD48 pathway could restore normal immune function of T cells independently of PD-1 (8). Hence, CD244 may be another potential target of immunotherapy for chronic viral infection. Further exploration of the molecular regulation mechanism of CD244 in mediating the depletion of effector T cell function and viral immune escape is likely to be the focus of effective control of HBV persistence and malignant progression.

More recent studies have shown that non-coding RNAs (ncRNA), including microRNA (miRNA) and long non-coding RNA (lncRNA) may also play a significant regulatory role in HBV infection (15–17). MiRNAs participate in many vital biological processes, such as cell signal transduction and immune response, through regulating target mRNA expression (18). Long non-coding RNA (lncRNA) is another non-coding RNA molecule containing more than 200 nucleotides (19). Interactions between lncRNAs and miRNAs are predicted because lncRNAs can act as sponges or inhibitors of interacting miRNAs (20). As a class of endogenous competitive RNAs (ceRNAs), lncRNAs can mediate gene expression by acting as miRNA sponges (21). According to previous reports, infection of viruses, such as tuberculosis, induces $CD8^+$ T cells to upregulate CD244 and lncRNAs of the CD244 signaling pathway epigenetically regulate $CD8^+$ T cell immune responses (22).

Thus, we hypothesize that the evidence of interaction between ncRNA and CD244 can help elucidate functional relationship between intracellular and intercellular molecules, thereby providing insights into biological processes, pathways and interaction networks that are critical to HBV immune escape. The present study was undertaken to specifically address how CD224 of T cells is involved in the process of HBV immune escape.

2 Materials and methods

2.1 Study subjects

The present study enrolled 20 CHB patients and 23 patients with spontaneous clearance of HBV (SC HBV) at West China Hospital of Sichuan University from March 2020 to September 2020. Chinese Medical Association guideline for the diagnosis of CHB: positive for HBsAg and/or HBV-DNA more than 6 month; spontaneous patients were enrolled follow: negative for HBsAg, positive for anti-HBs and anti-HBc, alanine aminotransferase (ALT) < 50 IU/L and aspartate aminotransferase (AST) < 45 IU/L. The study was approved by the Research Ethics Committee of West China Hospital of Sichuan University. Informed consent was obtained from all patients enrolled.

2.2 RNA extraction and microarray assay

The peripheral blood mononuclear cells (PBMCs) were obtained from three patients with SC HBV and three patients with CHB above mentioned. Total RNA was isolated using miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

RNA quality assessment was performed using agilent 4200 platform. Detection of lncRNA, RNA and miRNA were performed by Gminix Informatics (Shanghai, China). Affymetrix Human Transcriptome Array 2.0 was used for differentially expressed lncRNAs and mRNAs detection, while Affymetrix miRNA 4.0 was used for miRNAs detection. The raw data for the microarray was uploaded to Gminix-Cloud Biotechnology Information (GCBI; http://www.gcbi.com.cn/gclib/html/index) for further study, and then analyzed the data using Robust Multichip Analysis algorithm. Threshold used to screen differentially expressed lncRNAs, mRNAs and miRNAs were fold change > 1.2 with a P-value < 0.05.

2.3 GO and KEGG pathway analysis

The Gene Ontology (GO; www.geneontology.org) enrichment was calculated to assess the biological process, cellular component and molecular function of the differential expression genes found (23). The differentially expressed mRNAs were mapped to terms in the GO database, and the number of genes of each term was calculated. The $P < 0.05$ denoted the significance of GO term enrichment in the deregulated expressed genes. Pathway analysis was used to investigate the differentially expressed mRNAs according to the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database (24). Fisher's exact test and χ^2 test were used to select significant GO categories and KEGG pathways, and the threshold of significance was defined by $P < 0.05$ (the FDR was used to correct the P value).

2.4 Reverse transcription and qRT-PCR

Total RNA was extracted from blood or cells, and then dissolved in Trizol reagent (Invitrogen, USA) according to the kit's instruction. The cDNA was synthesized by reverse transcribing 1 μg RNA using a Prime Script RT regent kit (TaKaRa, Japan). For the RT-qPCR, the primer sequences were designed and synthesized by Invitrogen. The amount of cDNA was amplified using a SYBR Premix Ex Taq II (TaKaRa) with primers for CD244, miR-330-3p and lnc-AIFM-2-1. GAPDH served as a loading control.

2.5 Flow cytometry

Cells were detached from the blood samples of EDTA anticoagulation or the cell culture dish with accutase (GE Healthcare), washed once with phosphate-buffered saline (PBS) and fixed in 70% ice-cold EtOH. The cell surface markers, including CD45, CD3, CD4, CD8, CD16 and CD244, were analyzed by three- or four-color flow cytometry, using fluorochrome-conjugated monoclonal antibodies (PerCP/FITC/APC/PE/BV510/BV421 anti-Human, BD). Apoptosis of T cells was examined by staining with Annexin V-FITC/PI (Beyotime Biotechnology, China). Fluorescence intensity was measured with FACSCanto Flow Cytometer and analyzed with FACSDiva Software (BD FACSCalibur, USA) and FlowJo (TreeStar Inc.) or Flowing Software (Turku Centre for Biotechnology).

2.6 Cell culture and transfection

Jurkat, HepAD38 and LO2 obtained from American Type Culture Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin under 37°C with 5% CO_2 conditions. In order to study the effect of HBV on hepatocytes mediated by T cells, Jurkat cells were co-cultured with HepAD38, a hepatocyte line infected with HBV, or LO2 cells, a normal hepatocyte line. And then the apoptosis of cells, clearance of HBV and relative gene expression were gradually detected after 24 h. In order to investigate the effect of microRNA-330-3p on the clearance of HBV mediated by T cells, antisense oligonucleotides (ASO) for miR-330-3p or miR-330-3p mimics was transfected into a co-cultured system of Jurkat and HepAD38 using Lipofectamine 2000 (Invitrogen, 11668500) according to the manufacturer's instructions. For the knockdown of CD244 or lnc-AIFM2-1, Jurkat and HepAD38 cells were performed siRNA oligonucleotide of CD244 or lnc-AIFM2-1. For the overexpression of CD244 or lnc-AIFM2-1, the plasmid of pcDNA-3.1-CD244 or pcDNA-3.1-lnc-AIFM2-1 was transfected into the co-cultured system of Jurkat and HepAD38.

2.7 Luciferase reporter assay

HEK293T cells were cultured in a 48-well cell culture dish, reaching a density of 70% confluence by the time of first transfection. ASO-miR-330-3p or miR-330-3p mimics was transfected by Lipofectamine 2000 (Invitrogen, 11668500). The medium was changed to DMEM supplement with 10% FBS after 6 h. 12 h after the first transfection, a pmirGLO plasmid (Promega) containing WT/Mut sequence of CD244 or lnc-AIFM2-1 was transfected by Lipofectamine 2000 reagent. Six hours after transfection, the medium was changed to DMEM supplement with 10% FBS, and the cells were cultured for 48 h. To test the luciferase activities, the HEK293T cells were collected and detected by a Dual-Luciferase reporter assay kit (Biyotime, RG009).

2.8 Western blotting analysis

The protein level of CD244 was determined by Western blotting. HepAD38 cells and Jurkat cells were lysed with RIPA lysis buffer for 30 min on ice, followed by differential centrifugal for fractionation. The protein concentrations were determined by a BCA protein assay kit (Thermo). Equal amounts of 20 μg proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-rad). PVDF membranes were blocked in 5% nonfat milk for 1 h at 37°C, then incubated with primary antibody (BV421 mouse anti-Human CD244, 1:1000, BD) for overnight at 4°C and appropriate secondary antibody for 1 h at 37°C. The blot was visualized using a Western Lightning™ chemiluminescence reagent (PerkinElmer, USA) and analyzed by IPP 7.0 software.

2.9 ELISA for HBV detection

The expression levels of HBV-DNA, HBsAg and IFN-γ were detected by ELISA kit (Createch Biology, Tianjin, China). 50 μl samples and control samples were added into separate wells. The wells were incubated with Ab-HRP conjugates for 1 hour at 37°C, washed 5 times with PBST. 100 μL of substrate solution was added to each well and the reaction was quenched after 15 min incubation in darkness. Absorbance at 450 nm was measured using a microplate reader (BIO-RAD, USA).

2.10 ceRNA analysis

According to the ceRNA hypothesis, lncRNAs compete for the same miRNA response elements and act as 'molecular sponges' for miRNAs, thereby regulating the derepression of all target genes of the respective miRNA family. The miRNA targets on mRNA 3′ untranslated regions (UTR) and lncRNA were calculated using the PITA algorithm (http://genie.weizmann.ac.il/pubs/mir07).

2.11 Statistical analysis

All data were analyzed using GraphPad Prism version 7 (https://graphpad.com) and shown as mean ± standard error of mean. The Student t test was performed to analyze the microarray and qRT-PCR data. ANOVA was used to compare continuous variables. The comparisons between groups were made using two-way analysis with Turkey's multiple comparisons test. The value $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Identification of differentially expressed mRNAs, miRNAs and lncRNAs in CHB and spontaneous clearance of HBV

There were 20 patients with CHB and 23 patients with SC HBV in our study. As for the liver function parameters, the average HBsAg and HBeAg levels of CHB group were obviously higher than SC HBV group (Supplementary Table 1).

The obtained RNAs expression profiles were analyzed by microarray analysis. A total of 513 lncRNAs, 256 mRNAs and 48 miRNAs were found to be differentially expressed (DE) in patients with CHB compared with patients with SC HBV (fold change > 1.2 and $P < 0.05$). Among them, 368 DE lncRNAs, 114 mRNAs and 22 miRNAs were upregulated, while 145 lncRNAs, 142 mRNAs and 26 miRNAs were downregulated in CHB patients compared with SC HBV patients. The volcano plots of these RNAs indicated that the DE RNAs can distinguish between CHB patients and SC HBV patients (Figures 1A–C).

3.2 HBV infection induced CD244 signaling expression on $CD8^+$ T Cells in CHB patients

Furthermore, the DE mRNAs and miRNAs were analyzed in heat map (Supplementary Figure 1). Then, we investigated the biological functions of DE RNAs *via* GO analysis, which supported the role of immune responses pathways evidenced in the GO term analysis including T cell receptor signaling pathway (Figures 2A–C). To determine whether CD244 signaling is involved anti-HBV immune responses, we examined CD244 expression levels in $CD4^+$ T cells, $CD8^+$ T cells, and NK cells. There is experimental strategy and cytometry plots (Supplementary Figure 2). Flow cytometric analysis showed that, compared with SC HBV patients, HBV infection induced significant increases in $CD244^+CD8^+$ T cells and $CD244^+CD4^+$ T cells, but not $CD244^+CD16^+$ NK cells (Figure 2D). In addition, percentages of $CD244^+CD8^+$ T cells were much higher than those of $CD244^+CD4^+$ T cells in PBMCs from either SC HBV patients or patients with CHB (Figures 2E, F).

3.3 HBV infection induced CD244 overexpression promoted T cells apoptosis

In order to define the roles of regulating the differential expression of CD244 in T cells of CHB and SC HBV, we established an *in vitro* model. We co-cultured Jurkat cells, a T cell line, with LO2, a normal hepatocyte line, or HepAD38 cells, a hepatocyte line infected with HBV (Figure 3A). Then, HBV DNA and HBsAg were assayed to prove the HBV infection in HepAD38 cells co-cultured with Jurkat cells (Figures 3B, C). CD244 was increased in Jurkat cells co-cultured with HepAD38 cells than co-cultured with LO2 cells (Figure 3D). The increasing of CD244 was associated with T cells apoptosis in Jurkat cells co-cultured with HepAD38 cells (Figure 3E), accompanied by higher HBV DNA and HBsAg. On the contrary, the levels of HBV DNA and HBsAg were significantly decreased after the silence of CD244 (Figures 3F–H). These data collectively suggested the importance of CD244 signaling in regulating $CD8^+$ T cell immune responses during HBV infection.

3.4 The miR-330-3p enhanced immune response to HBV infection *via* inhibiting CD244 expression

To find the cause of regulating the differential expression of CD244 in SC HBV and CHB, we analyzed the DE miRNAs. In total, we predicted 5240 target mRNAs using the miRnada and TargetScan tools (Figure 4A). GO classification and KEGG pathway analysis of these DE miRNAs showed that transcription and immune pathway significantly

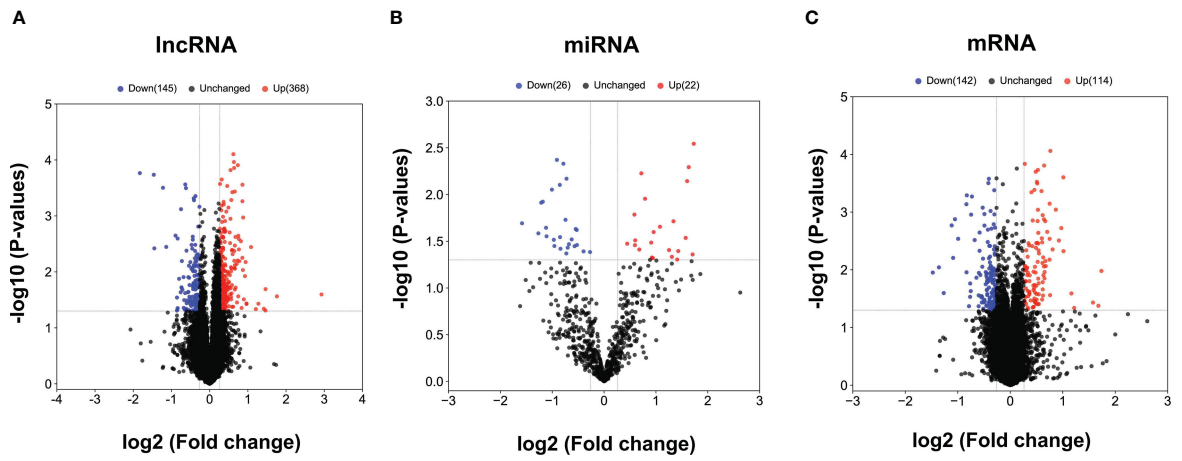

FIGURE 1
The volcano plots of differentially expressed lncRNAs, miRNAs and mRNAs between CHB patients and SC HBV controls. **(A)** Results of differentially expressed lncRNAs expression analysis between CHB (n = 3) and SC HBV (n = 3) patients. **(B)** Results of differentially expressed miRNA expression analysis between CHB (n = 3) and SC HBV (n = 3) patients. **(C)** Differentially expressed miRNA expression analysis between CHB (n = 3) and SC HBV (n = 3) patients. The abscissa is log2 (FC value) and the ordinate is -log10 (*P* value). Blue dots are downregulated genes, red dots are upregulated genes, and black dots are genes that were the same between the two groups.

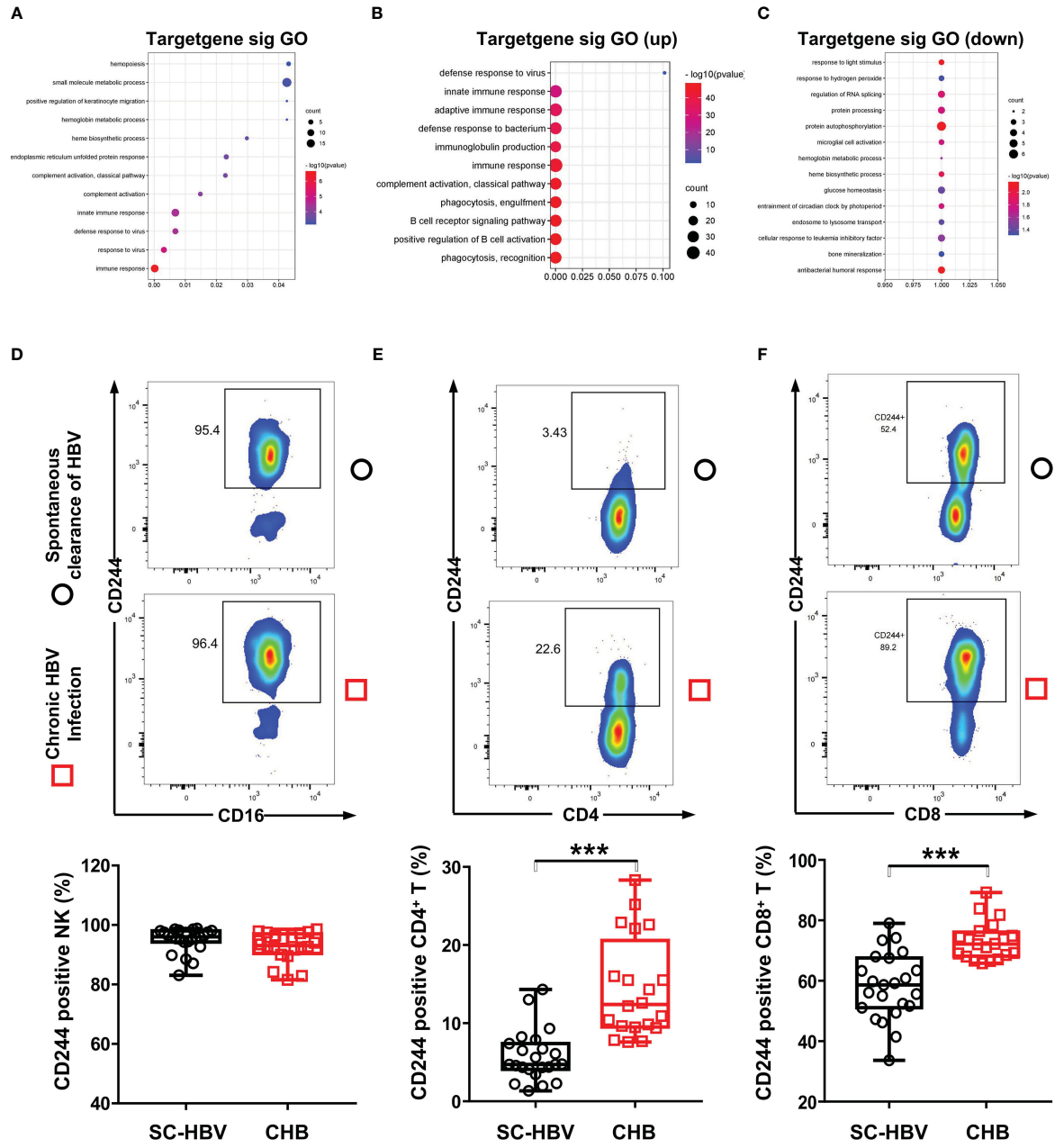

FIGURE 2
CD244 signaling expression on immune cells in CHB patients and SC HBV controls. **(A)** GO enrichment analysis of all significant genes, ($P < 0.05$). GO: Gene Ontology. **(B)** GO analysis for upregulated genes classified as signaling molecules. **(C)** GO analysis for downregulated genes classified as signaling molecules. **(D-F)** Activation and expansion of CD244 on NK cells **(D)**, $CD4^+$ T cells **(E)** and $CD8^+$ T cells **(F)** were analyzed by flow cytometry. Data (CHB, n = 20; SC HBV, n = 23) were analyzed using Student t test, *** $P < 0.001$.

increased (Figure 4B). Moreover, bioinformatics software TargetScan (http://www.targetscan.org) predicted that the target gene of DE miR-330-3p might be CD244 (Figure 4C). Then, we compared the level of miR-330-3p in CHB patients and SC HBV patients. RT-qPCR data showed that the expression of miR-330-3p was significantly decreased in CHB patients (Figure 4D). Furthermore, the co-cultured LO2 or HepAD38 cells with Jurkat cells showed that the expression of miR-330-3p was significantly decreased in HBV infection group (Figure 4E), accompanied by the higher level of CD244 (Figure 3D). On the contrary, HBV DNA and HBsAg were significantly decreased after stimulation of miR-330-3p mimics in HepAD38 cells co-cultured with Jurkat cells (Figures 4F–H).

To present the interactions between has-miR-330-3p and the mRNA of CD244, the co-cultured Jurkat cells and HepAD38 cells were transfected with mimics NC, has-miR-330-3p mimics, ASO NC, and has-miR-330- 3p ASO, respectively. After verifying that mimics and ASO of miR-330-3p work properly, we found that miR-330-3p mimics decreased the mRNA and protein level of CD244, while miR-330-3p ASO significantly increased the expression of CD244 (Figures 4I, J). In order to further explore whether miR-330-3p targeted CD244 *via* direct binding, a double luciferase test was designed. The results showed that decreased expression of CD244 induced by miR-330-3p mimics and increased expression of CD244 induced by miR-330-3p ASO in CD244-3'UTR-WT group were

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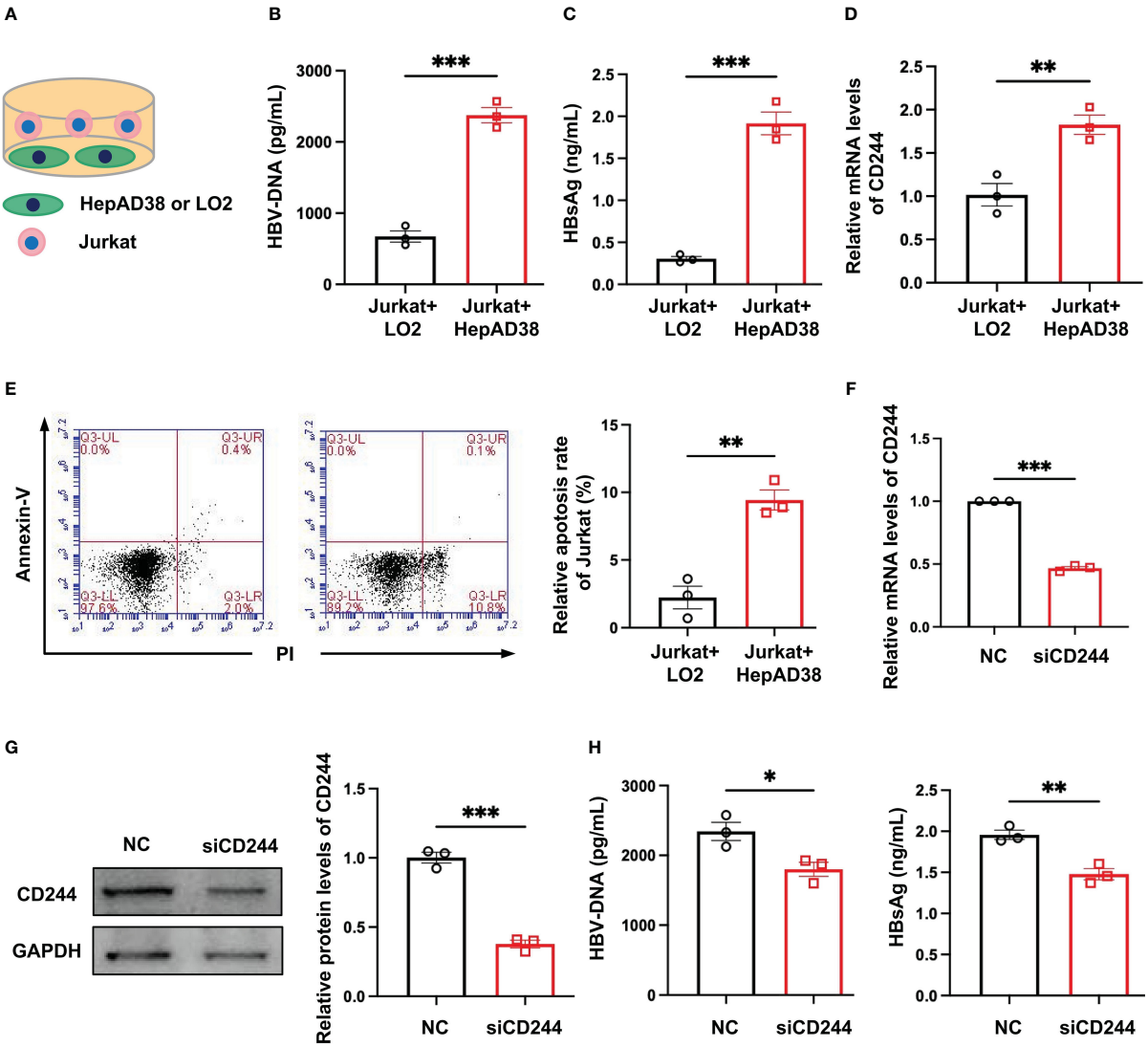

FIGURE 3
CD244 expression regulated T cells apoptosis with or without infection of HBV. **(A)** The co-culture scheme of LO2 hepatocytes or HBV infected HepAD38 cells with Jurkat cells. **(B)** HBV DNA detection in co-culture cells using ELISA. **(C)** HBsAg detection in co-culture cells using ELISA. **(D)** RT-PCR analysis of CD244 expression in Jurkat cells. **(E)** The apoptosis of Jurkat cells were identified by Annexin V/PI staining. **(F, G)** The expression of CD244 mRNA levels **(F)** and protein levels **(G)** in Jurkat cells after siCD244 transfection of co-cultured system. **(H)** Quantification of HBV DNA and HBsAg by ELISA kit. Data (n = 3 per group) were expressed as mean ± SEM and analyzed using Student t test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

eliminated by CD244-3'UTR-Mut (Figure 4K). Furthermore, the apoptosis of $CD8^+$ T cells was significantly increased in the overexpression of CD244 by CD244 plasmid transfection and the knockdown of miR-330-3p in ASO-miR-330-3p transfection (Figure 4L). Inhibition of CD244 by siRNA rescued the apoptosis of T cells, which were induced by ASO-miR-330-3p (Figure 4L). The results suggested that has-miR-330-3p targets CD244 *via* direct interaction.

3.5 Lnc-AIFM2-1 acted as a ceRNA for miR-330-3p to regulate CD244 expression

LncRNAs are emerging as important regulators in the modulation of virus infection by targeting mRNA transcription (25). Integrating the lncRNA/miRNA interactions with the miRNA/mRNA interactions, the heat maps of these RNAs indicated that the upregulated lncRNAs can distinguish between CHB patients and SC HBV patients (Figure 5A). We found that lnc-AIFM2-1 and CD244 exited similar binding sites with miR-330-3p (Figure 5B). Therefore, we compared the level of lnc-AIFM2-1 in CHB patients and SC HBV patients. RT-qPCR data showed that the expression of lnc-AIFM2-1 was significantly increased in CHB patients (Figure 5C). To present the directly interactions between has-miR-330-3p and lnc-AIFM2-1, a double luciferase test was designed. The results showed that miR-330-3p mimics significantly suppressed the expression of lnc-AIFM2-1 and miR-330-3p ASO increased the expression of lnc-AIFM2-1, which were inhibited by lnc-AIFM2-1-3'UTR-Mut (Figure 5D). Moreover, we overexpressed the lnc-AIFM2-1 by transfecting the lnc-AIFM2-1 plasmid and deleted the expression of the lnc-AIFM2-1 by

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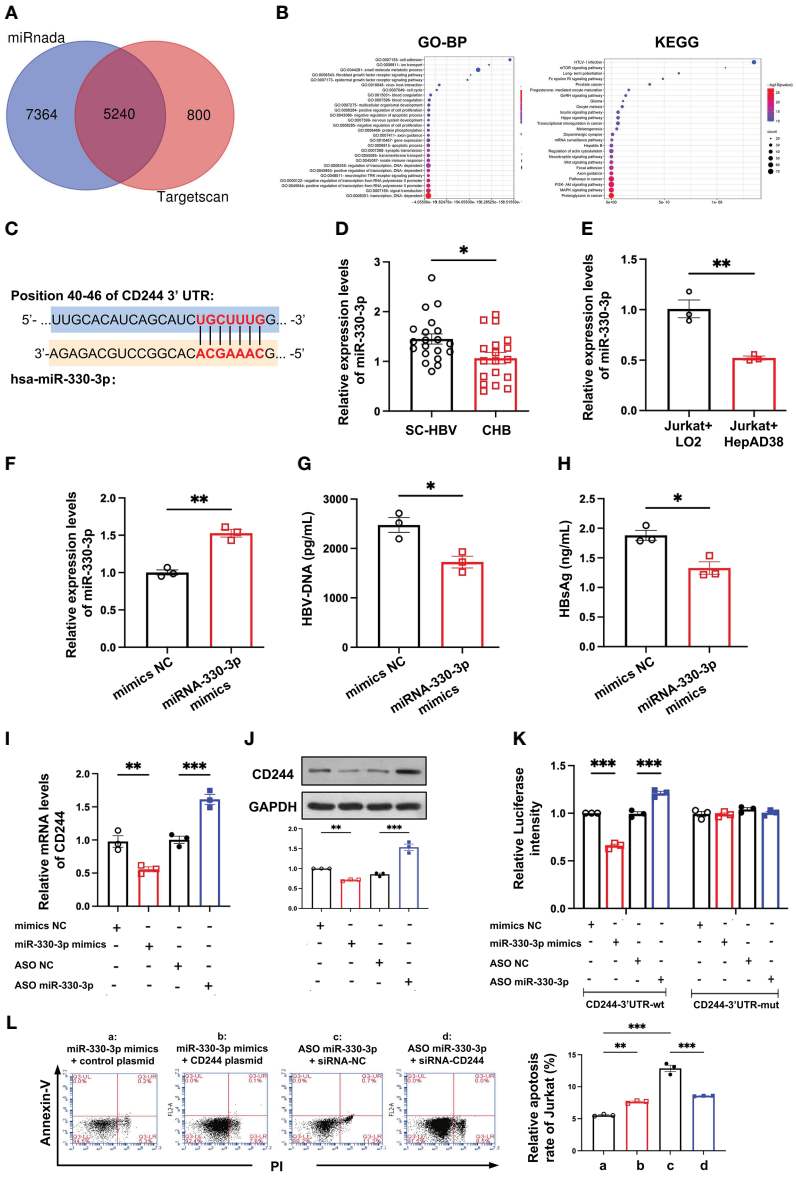

FIGURE 4
The miR-330-3p regulated immune response to HBV infection with CD244 alteration. **(A)** The target mRNAs predicted using the miRnada (www.microrna.org) and TargetScan (www.targetscan.org). **(B)** GO (biological process) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. **(C)** The prediction interaction sites of CD244 and miR-330-3p. **(D)** RT-PCR analysis of miR-330-3p expression in CHB (n = 18) and SC HBV (n = 20) patients. **(E)** The expression of miR-330-3p in co-culture system of LO2 hepatocytes or HBV infected HepAD38 cells with Jurkat cells. **(F-H)** The alteration of miR-330-3p **(F)**, HBV DNA **(G)**, and HBsAg **(H)** treated with miR-330-3p mimics or negative control miRNA in the co-culture system of HepAD38 cells with Jurkat cells. **(I, J)** The expression of CD244 mRNA levels **(I)** and protein levels **(J)** in co-culture system after miR-330-3p mimics/ASO miR-330-3p (inhibitors) or controls transfection. **(K)** The relative luciferase activity in co-culture HepAD38 cells and Jurkat cells transfected with the indicated CD244-3'UTR-wt plasmid or indicated CD244-3'UTR-mut plasmid after the intervention of miRNA mimics/ASO miR-330-3p or not. **(L)** The rescue effect of siRNA-CD244 on the inhibition of apoptosis of Jurkat cells, inducing by CD244 plasmid or ASO miR-330-3p, by flow cytometry staining with Annexin V/PI. Data (n = 3 per group) were expressed as mean ± SEM and analyzed using Student t test or two-way analysis with Turkey's multiple comparisons test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

transfecting the lnc-AIFM2-1 siRNA (Figure 5E). We found that upregulation of lnc-AIFM2-1 induced the decreased miR-330-3p and the increased CD244 (Figures 5F, G). The protein expression changes of CD244 were consistent with mRNA (Figure 5H). In addition, the downregulation of lnc-AIFM2-1 induced the increased miR-330-3p and decreased CD244 (Figures 5F, G). Accompanying by the elevated CD244, the apoptosis of $CD8^+$ T cells was significantly increased in the overexpression of lnc-AIFM2-1 group (Figure 5I). On the contrary, the apoptosis of $CD8^+$ T cells was decreased in the lnc-AIFM2-1 siRNA group, accompanying by the decreased CD244 (Figure 5I). Furthermore, the apoptosis of $CD8^+$ T cells were significantly inhibited by the treatment of miR-330-3p mimics, comparing with the group of lnc-AIFM2-1 plasmid+mimics NC (Figure 5J). The levels of HBV DNA and HBsAg were also significantly decreased in the treatment of miR-330-3p mimics than the group of lnc-AIFM2-1 plasmid+mimics NC (Figures 5K, L). These data suggested that lnc-AIFM2-1 acted as ceRNA for miR-330-3p to contribute to HBV immune escape.

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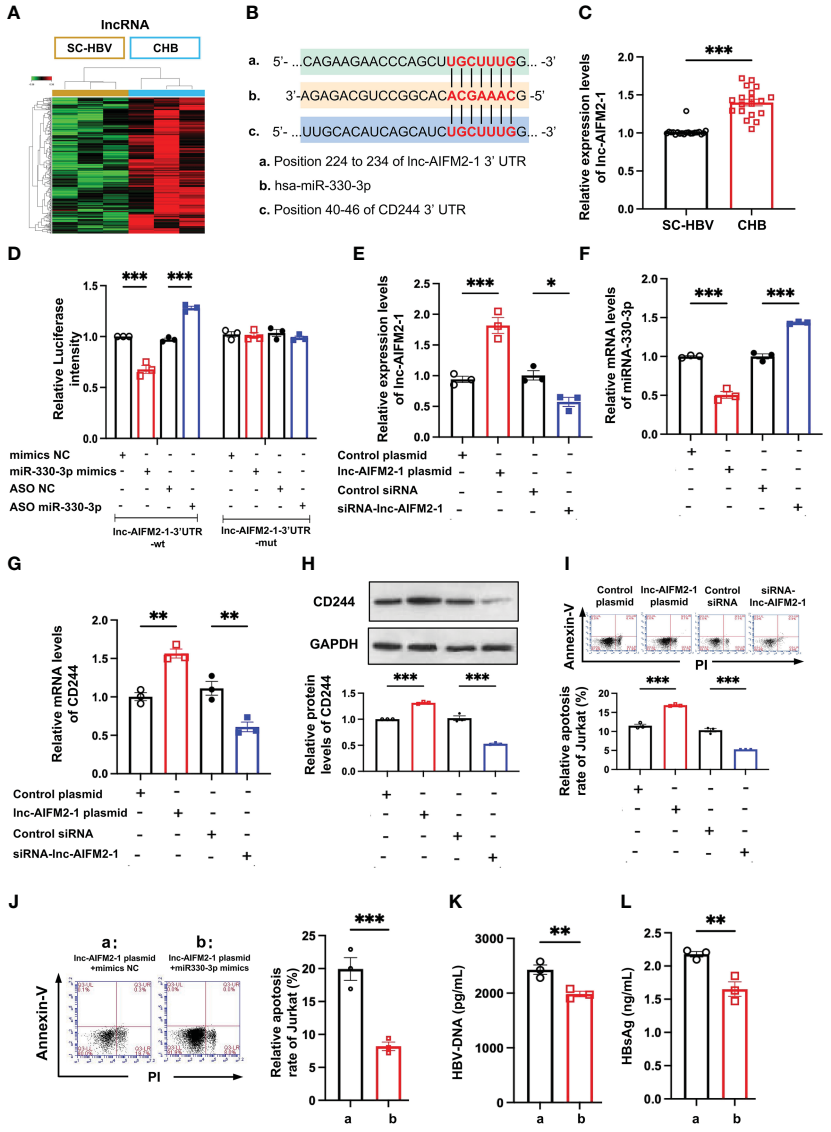

FIGURE 5
The interaction between lnc-AIFM2-1 and miR-330-3p regulated the immune response. **(A)** Heat map of up-regulated lncRNAs in CHB (n = 3) patients compared with SC HBV (n = 3) controls. Screening criteria were as follows: $P \leq 0.05$ for lncRNAs. Expression values are depicted in line with the color scale; intensity increases from green to red. **(B)** The prediction interaction sites of CD244 with miR-330-3p and lnc-AIFM2-1. **(C)** RT-PCR analysis of lnc-AIFM2-1 expression in CHB (n = 18) and SC HBV (n = 20) patients. **(D)** The relative luciferase activity in co-culture HepAD38 cells and Jurkat cells transfected with the indicated lnc-AIFM2-1-3'UTR-wt plasmid or indicated lnc-AIFM2-1-3'UTR-mut plasmid after the intervention of miRNA mimics/ASO miR-330-3p or not. **(E, F)** The expression of lnc-AIFM2-1 **(E)** and miR-330-3p **(F)** in co-culture system of LO2 hepatocytes or HBV HepAD38 cells with Jurkat cells after lnc-AIFM2-1 plasmid/siRNA-lnc-AIFM2-1 or controls transfection. **(G, H)** The expression of CD244 mRNA levels **(G)** and protein levels **(H)** in co-culture system after lnc-AIFM2-1 plasmid/siRNA-lnc-AIFM2-1 or controls transfection. **(I)** The apoptosis of Jurkat cells were identified by Annexin V/PI staining in co-culture HepAD38 cells and Jurkat cells transfected with the indicated lnc-AIFM2-1 plasmid/siRNA-lnc-AIFM2-1 or not. **(J-L)** The rescue effect of miR-330-3p mimics on the inhibition of apoptosis of Jurkat cells, inducing by lnc-AIFM2-1 plasmid, by flow cytometry staining with Annexin V/PI **(J)**, and quantification of HBV DNA **(K)** and HBsAg **(L)** detected by ELISA. Data (n = 3 per group) were expressed as mean ± SEM and analyzed using Student t test or two-way analysis with Turkey's multiple comparisons test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4 Discussion

HBV infection and CHB caused by HBV is global public health problems (26). CHB patients are at a significantly increased risk of developing liver failure, cirrhosis, and HCC (27). However, the mechanisms by which HBV evades host immunity and sustains chronic infection are not fully understood. $CD8^+$ T cells directly suppress viral replication and subsequent host dissemination by eliminating infected cells (28). In addition to TCR-mediated Ag recognition and pathogen clearance, CD244 is expressed on T cells and interact with their ligands on antigen-presenting cells upon TCR ligation, resulting in modulation of the T cell response (29, 30). CD244 is upregulated on $CD8^+$ T cells during HBV infection, and CD244 signaling reduces production of IFN-γ by $CD8^+$ T cells (8). The role of $CD8^+$ T cells in anti-HBV immunity led us to examine the expression of molecules associated with the CD244 signaling pathway in $CD8^+$ T cells during active HBV infection. In this study, samples from patients with CHB and patients with SC HBV were analyzed by

flow cytometry. The results showed that the expression of CD244 on $CD8^+$ T cells in CHB was significantly increased, which was consistent with the previous report, indicating that the abnormally high expression of CD244 was related to the chronicity of HBV.

Recently, miRNAs function in RNA silencing and post-transcriptional regulation of gene expression, and have received much attention in HBV infection (16). Previous studies demonstrated that chronic inflammation and/or viral factors can induce increased expression of miR-146a, which depresses T-cell immune function by targeting STAT1, in T cells in CHB patients (31). Here, we used microarray analysis of *miRNA* expression in CHB and SC HBV patients and further used two bioinformatics databases (miRDB and TargetScan) to speculate potential target genes for miR-330-3p, and found CD244 may be a target gene of miR-330-3p. Previous studies showed that *miR-330-3p* played an important role in the development of multiple tumors (32, 33). Moreover, miR-330-3p down-regulates the RNA level of mitogen activated protein kinase 1 (MAPK1) in liver cancer cells, thereby inhibiting the migration of liver cancer cells (34). We found that miR-330-3p was decreased in CHB patients and further determined the direct interaction between miR-330-3p and CD244, which increased $CD8^+$ T cell apoptosis and HBV immune escape. Perhaps miR330 is one of the promoting factors for chronic hepatitis B patients to develop into liver cancer, which needs further research.

Recent studies have shown that the lncRNA can extensively participate in many biological processes such as cell signal transduction and immune response through the mechanisms of epigenetic modification, transcriptional and post transcriptional regulation (35). The abnormal function of lncRNA is closely related to the occurrence and development of many diseases. The study reported that lncRNA-HULC is highly expressed in patients with CHB and hepatitis B related liver cancer, and can promote the proliferation of liver cancer cells by downregulating the tumor suppressor gene *p18 (*36, 37). In addition, Feng et al. reported that lncRNA PCNAP1, as the sponge of miR-154, regulates the proliferating cell nuclear antigen (PCNA), thereby promoting HBV replication and hepatocarcinogenesis (17). In this study, we expected to screen out the lncRNAs related to miR-330-3p, and use microarray analysis to screen out the differentially expressed lncRNAs in CHB and SC HBV patients. The bioinformatics method was used to predict the interaction targets of lnc-AIFM2-1 and miR-330-3p. Lnc-AIFM2-1 is an antisense chain located on chromosome chr10:69994626-70007836 (hg38), belonging to the intergenic lncRNA, containing three exons and a total length of 524 nt. ORF Finder and Reg RNA 2.0 software predict that it has no open reading frame and no ability to encode protein.

There are various interactions between miRNA and lncRNA to participate in the occurrence and development of diseases. As previous studies have reported that with the miRNA response elements, lncRNA could compete with mRNA to bind with miRNA, thereby freeing mRNA from the regulation of miRNA (38). There has been substantial interest in the ceRNA hypothesis in recent years, with much of the research in the area revolving around how dysregulation of ceRNA expression can affect diseases pathogenicity and progression (39). Recently, the study offers evidence that the lncRNA TUG1-miR-328-3p-SRSF9 mRNA axis function as a novel ceRNA regulatory axis, which may be associated with HCC malignancy and may be one of therapeutic targets of the anti-HCC treatment (40). According to the ceRNA hypothesis, to determine whether the lnc-AIFM2-1 acts as a ceRNA for miR-330-3p, we first examined the alterations of lnc-AIFM2-1 in CHB patients that occur CD244 upregulated and miR-330-3p downregulated. Our results demonstrated that miR-330-3p could suppress the luciferase activity of lnc-AIFM2-1, indicating the interaction between lnc-AIFM2-1 and miR-330-3p. Then, we further found that the decreasing of HBV clearance and increase of $CD8^+$ T cells apoptosis with CD244 upregulated during lnc-AIFM2-1 overexpression. Finally, miR-330-3p transfection can rescue the $CD8^+$ T cells apoptosis caused by overexpression of lnc-AIFM2-1. The results showed that lnc-AIFM2-1 and miR-330-3p play a critical role in CHB.

In summary, this study demonstrates that lnc-AIFM2-1 on CD244 by acting as a ceRNA of miR-330-3p contributes to HBV immune escape. This effect is due to the competition between lnc-AIFM2-1 and miR-330-3p to inhibit the expression of CD244 on $CD8^+$ T cells, which are key immune responses to HBV. These data provide novel insights into the roles of interaction networks among lncRNA, miRNA, and mRNA in HBV immune escape. Furthermore, these findings suggest that lnc-AIFM2-1 and CD244 may be novel targets for diagnosis and treatment in CHB.

Data availability statement

The datasets presented in this study can be found in Gene Expression Omnibus (GEO) with accession number GSE224283 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE224283).

Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Committee of West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CX: Conceptualization, Formal analysis, Data curation, Writing - original draft. SW: Conceptualization, Methodology, Investigation, Formal analysis. HZ, YZ, and PJ: Data curation, Validation. SS and YS: Validation. JC: Supervision, Project administration, Writing - review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1121795/full#supplementary-material

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Ongoing involvers and promising therapeutic targets of hepatic fibrosis: The hepatic immune microenvironment

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Hepatic fibrosis is often secondary to chronic inflammatory liver injury. During the development of hepatic fibrosis, the damaged hepatocytes and activated hepatic stellate cells (HSCs) caused by the pathogenic injury could secrete a variety of cytokines and chemokines, which will chemotactic innate and adaptive immune cells of liver tissue and peripheral circulation infiltrating into the injury site, mediating the immune response against injury and promoting tissue reparation. However, the continuous release of persistent injurious stimulus-induced inflammatory cytokines will promote HSCs-mediated fibrous tissue hyperproliferation and excessive repair, which will cause hepatic fibrosis development and progression to cirrhosis even liver cancer. And the activated HSCs can secrete various cytokines and chemokines, which directly interact with immune cells and actively participate in liver disease progression. Therefore, analyzing the changes in local immune homeostasis caused by immune response under different pathological states will greatly enrich our understanding of liver diseases' reversal, chronicity, progression, and even deterioration of liver cancer. In this review, we summarized the critical components of the hepatic immune microenvironment (HIME), different subtype immune cells, and their released cytokines, according to their effect on the development of progression of hepatic fibrosis. And we also reviewed and analyzed the specific changes and the related mechanisms of the immune microenvironment in different chronic liver diseases.Moreover, we retrospectively analyzed whether the progression of hepatic fibrosis could be alleviated by modulating the HIME.We aimed to elucidate the pathogenesis of hepatic fibrosis and provide the possibility for exploring the therapeutic targets for hepatic fibrosis.

KEYWORDS

hepatic fibrosis, hepatic immune microenvironment, chronic liver diseases, hepatic stellate cells (HSCs), immune cells

Introduction

Hepatic fibrosis is a reversible wound-healing response to liver injury, which is a major feature of any form of the chronic liver disease progressing to cirrhosis and liver failure (1). The main manifestations of hepatic fibrosis are chronic liver injury and accumulation of extracellular matrix (ECM) proteins (2). The continuous accumulation of ECM proteins could disrupt the normal function of the liver, and persistent hepatic fibrosis will progress to liver cirrhosis and even hepatocellular carcinoma (3). However, no effective drugs have been approved to target hepatic fibrosis. The therapeutic approaches to alleviate hepatic fibrosis mainly include: controlling or removing the underlying causes of hepatic fibrosis, preventing the activation and proliferation of HSCs, inhibiting the overexpression of pro-fibrotic cytokine TGF-β, and promoting fibrous tissue degradation. An emerging stem cell therapy is that the stem cells can transform into functionally active hepatocyte-like cells and participate in liver function repair and reconstruction (4).

It has been confirmed that hepatic fibrosis is driven by the activation of HSCs (5), which is the main source of ECM (6). HSCs are located in the perisinusoidal space and maintain a non-proliferative and quiescent phenotype in the normal liver tissue. The HSCs will transdifferentiate into proliferative fibrotic myofibroblasts, acquiring pro-inflammatory and pro-fibrotic properties under the chronic injury caused by many various pathogenic factors such as pathogenic microorganisms, alcohol, chemical drugs, and lipid deposition (7). The myofibroblasts transformed from HSCs account for about 82%-96% of myofibroblasts in various chronic liver diseases (8). Therefore, activated HSCs are often regarded as the main therapeutic target.

HIME is a dynamic network system composed of a variety of immune cells, immune cytokines, and related ECM (9), and it closely affects the onset and progression of hepatic fibrosis. Various immune cells are enriched in liver tissue and involved in the physiological and pathological process of hepatic metabolism and disease (10). Immune cells in the liver are diverse at a stable state and evolve further during the development of chronic liver disease, directly affecting the severity of the disease (11, 12). The recruitment and activation of immune cells in the diseased liver tissue are often initiated by the massive release and activation of cytokines and chemokines. Hepatic kuffer cells (KCs) are activated by various damage-associated molecular patterns (DAMPs, such as free DNA, ATP, high mobility group box 1) and pathogen-associated molecular patterns (such as LPS or viral DNA) that interact with Toll-like receptors (TLRs) or recombinant purinergic receptor P2X, ligand-gated ion channel 7 when hepatocytes are damaged (13, 14). This leads to the activation of the inflammasome, and the release of interleukin-1β (IL-1β), IL-18, and various other pro-inflammatory cytokines as well as chemokines. It will promote the recruitment of circulating leukocytes (monocytes and neutrophils), and activate adaptive immune T cells function. Once the infection or injury is controlled, the KCs and macrophages transform into anti-inflammatory and tissue repair phenotypes to control excessive tissue-damaging inflammatory responses (15). However, the persistent injurious stimulus in the liver will result in inflammatory cytokines continuously releasing that promote HSCs-mediated fibrous tissue hyperproliferation and excessive repair, which causes hepatic fibrosis development and progression to cirrhosis (16).

Hepatic fibrosis is an intermediate link and reversible stage in the process of chronic liver disease developing into liver cirrhosis. Intervention in this link will determine the prognostic direction of the various liver diseases. However, there is no efficient treatment strategy for hepatic fibrosis, which is mainly because profound awareness of the changes in the microenvironment of hepatic fibrosis still lacks, and no targeting and efficient intervention on the hepatic fibrosis microenvironment. Exploring the changes in local immune homeostasis and microenvironment caused by immune response under this pathological state could greatly enrich our understanding of liver disease reversal, chronicity, and even deterioration to liver sclerosis. Therefore, it is of great significance to elucidate the HIME for the study of hepatic fibrosis and the development of therapeutic strategies. In this review, we first summarized the critical components of HIME, the alteration and function of different sub-type immune cells, and their related mechanisms according to their effect on the development of progression of hepatic fibrosis. The specific changes and the related mechanisms of HIME in different chronic liver diseases were also reviewed and analyzed. Finally, we also summarized the therapeutic approaches and targets for HIME to reverse the progression of hepatic fibrosis, and we aimed to provide new research ideas and the possibility of exploring the therapy for hepatic fibrosis.

Influence of the immune cells on the development of hepatic fibrosis

The immune cells are central members of the immune microenvironment in the progression of hepatic fibrosis, and they can mediate the immune response by synthesizing and secreting different chemical inflammatory mediators. The immune cells affect the function and number of HSCs mainly through direct or indirect effects, thus influencing the progression of hepatic fibrosis. Simultaneously, they constitute a dynamically evolving microenvironment and interact as well as restrict each other, forming a complex network system, which jointly affects the process of hepatic fibrosis.

Monocytes/macrophages

Hepatic macrophages include liver-resident KCs and monocyte-derived macrophages (MDMs) (17). The MDMs, with high

Abbreviations: HSCs, hepatic stellate cells; HIME, hepatic immune microenvironment; ECM, extracellular matrix;KCs, kuffer cells; DAMP, damage-associated molecular pattern; TLR, Toll-like receptor; IL, interleukin; MDM, monocyte-derived macrophage; TNF, tumor necrosis factor; PDGF, platelet-derived growth factor; MMP, matrix metallic-proteinases; DCs, dendritic cells; NK, natural killer; lrNK, liver-resident natural killer;cNK, conventional natural killer;STAT, signal transducer-activator; SOCS, suppressor of cytokine signaling; TIMP, tissue inhibitor of metalloproteinase; NASH, non-alcoholic steatohepatitis; HBV, hepatitis B virus; MAPK, mitogen-activated protein kinase; AILD, autoimmune liver diseases; PBC, primary biliary cholangitis; ALD, alcoholic liver disease; NAFLD, non-alcoholic fatty liver disease; P2X7R, P2X7 receptor; LPS, lipopolysaccharide; JNK, Jun N-terminal kinase; TCM, traditional Chinese medicine; KD, kinsenoside.

heterogeneity and plasticity, can differentiate into functionally distinct macrophage subsets. In mice, the circulating macrophages can be divided into the Ly6C^{hi} CCR2^{hi} CX3CR1^{lo} monocytes which are considered to be potent pro-inflammatory cells, and Ly6C^{lo} CCR2^{lo} CX3CR1^{hi} monocytes, which with an anti-inflammatory patrolling function (18). Ly6C^{hi} monocytes in response to liver chronic injury are recruited to the liver and differentiate into MDM. These Ly6C^{hi} MDMs have been shown to promote liver fibrosis, and they could promote HSCs activation as well as proliferation and ECM production by expressing a large number of pro-fibrotic mediators. The MDMs are also capable of differentiating into Ly6C^{lo} restorative macrophage phenotypes, which promote ECM degradation and fibrosis resolution (19). KCs, the settled macrophages in the liver, have been identified as key regulators of hepatic inflammation. The DAMPs released from damaged hepatocytes can activate KCs and promote circulating macrophage infiltration in the liver (12). The activated macrophages have been reported to possess a bidirectional effect on the onset, progression, and reversal of hepatic fibrosis (17). Activated KCs could activate the HSCs by secreting large amounts of pro-inflammatory factors such as TGF-β1, IL-1β, and tumor necrosis factor-α (TNF-α) as well as the chemokines (20, 21). The TGF-β1 and platelet-derived growth factor (PDGF) are the most important inflammatory mediators in hepatic fibrosis. TGF-β1 regulates the activation of HSCs through the Smad pathway and inhibits the degradation of the ECM (22). PDGF activates HSCs and promotes their proliferation through the subsequent phosphatidylinositol 3 kinase (PI3K) and extracellular signal-regulated kinase pathways (23). Additionally, macrophages also mediate the reversal of fibrosis. Firstly, macrophages secrete a series of matrix metallic-proteinases (MMPs), which specifically degrade collagen and non-collagen extracellular matrix (24). Secondly, macrophages mediate HSCs apoptosis by expressing TNF-related apoptosis-inducing ligand (25), leading to the reduction of ECM and alleviation of hepatic fibrosis (26). Relaxin is an anti-fibrotic peptide hormone that can directly reverse the activation of HSCs to promote the resolution of hepatic fibrosis (27). A study showed that hepatic macrophages express primary relaxin receptors and they switch from a profibrogenic to the pro-resolution phenotype upon binding with relaxin. The latter releases exosomes that promote relaxin-mediated quiescence of activated hepatic astrocytes *via* miR-30a-5p (28). This indicates that macrophages have great potential in anti-fibrosis research and can be used as a target for anti-fibrosis therapy or reversal of fibrosis.

Dendritic cells

Dendritic cells (DCs) are the specialized antigen-presenting cells, with the function of transforming immune tolerance to immune activation, regulating the direction of the immune response in HIME of hepatic fibrosis (29). During hepatic fibrosis, DCs proliferate and undergo phenotypic changes. They stimulate adjacent T cells and natural killer(NK) cells through TNF-α, and up-regulate intercellular adhesion molecule-1 and CD40 expression of HSCs, thus, promoting the HSCs proliferation and activation (30, 31). DCs also aggravate liver inflammation by inhibiting infiltration of Treg cells and activating TLRs to produce a large number of cytokines (32). And the DCs depletion completely abrogates the elevated levels of many inflammatory mediators that are produced in the fibrotic liver (33). A similar study found knockout of DCs reduced the clearance of activated HSCs and delayed the fibrotic reversal process during the reversal phase of CCL4-induced hepatic fibrosis in mice (34).However, it also reported that although DCs can promote the activation of HSCs, the depletion of DCs does not affect the evolution of liver fibrosis in CCL4-induced hepatic fibrosis (35). Moreover, the application of FMS-liketyrosinekinase3 ligand induced the proliferation of DCs, accompanied by increased MMP-9 secretion from DCs. And the MMP-9 not only directly degrades collagen but also recruits innate immune cells such as macrophages and neutrophils, secreting MMP-8 and MMP-13 to degrade collagen, facilitating the reversal of hepatic fibrosis (34). Besides, long-term (12 weeks) alcohol intake can specifically recruit plasmacytoid dendritic cells to the liver in female mice. These plasmacytoid dendritic cells are characterized by secreting IFN-α and with anti-fibrosis function (36). These results suggest the effect of DCs in hepatic fibrosis is still controversial and need to be revealed and defined in the future research.

Natural killer cells

NK cells in liver were subdivided into CD49a^{+}DX5^{-} liver-resident natural killer (lrNK) cells and CD49a^{-} DX5^{+} conventional natural killer (cNK) cells. Both of them could kill activated HSCs in a tumor necrosis factor-related apoptosis-inducing ligand dependent manner (37). Compared to cNK cells, lrNK cells are less mature at a rest statue, but it have enhanced activity upon pathogenic stimuli and exhibit higher cytotoxicity (38). It suggested that the enrichment of lrNK cells in the liver may be to prepare for a special function against liver injury. It also was reported that lrNK cells can highly express CD107a and perforin, work together with a variety of activated receptors, release interferon-γ (INF-γ) while splitting infected hepatocytes, and inhibit the activation of HSCs in the human viral hepatitis (39). In the early stage of hepatic fibrosis, cNK cells infiltrate into the liver parenchyma and alleviate or reverse hepatic fibrosis by directly killing or inducing apoptosis of HSCs through degranulation and TNF-related apoptosis-inducing ligand expression (40). Moreover, the activated NK cells release IFN-γ through the JAK-STAT pathway to antagonize hepatic fibrosis by exerting a killing effect on the activated HSCs (41, 42). The activated NK cells can also secrete IL-10 and IL-22,which promote the senescence of activated HSCs and alleviate liver fibrosis through signal transducer-activator 3 (STAT3)/p53/p21 pathway (43, 44). And the activation of metatropic gluamate receptor 5 in NK cells can also reduce liver fibrosis by increasing their cytotoxicity and IFN-γ production (45).The NKp46 (+)NK· cells were reported to attenuate metabolism-induced hepatic fibrosis by regulating macrophage activation in mice (46). In patients with chronic HVB infection, the terminally differentiated NK cells often highly express CD57 and DNAM-1, while NKp46 and NKG2A expression at low levels. These NK cells can kill activated HCSs through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) dependent and CD44-osteopontin dependent manners (47). In contrast, during the progressive phase of hepatic fibrosis, persistent activation of HSCs will inhibit NK cell activity and weaken

its anti-fibrotic effect. It is attributed to the increased metabolism of vitamin A. Its metabolites, retinoic acid, and retinol can inhibit the activation of IFN-γ on the transcriptional STAT-1 pathway by secreting the suppressor of cytokine signaling 1 (SOCS1) (41, 48).

NKT cells, a subset of T cells, possess NK and T cell receptors and display both NK cell and T cell properties. Similar to the NK cells, NKT cells also inhibit hepatic fibrosis by directly killing the activated HSC cells or producing IFN-γ to exert the inhibiting effect on HSCs. In the CCL4-induced acute liver injury mouse model, the hepatic NKT can slow down the onset of acute liver injury and liver inflammation by inhibiting activated HSCs (49). However, the anti-fibrotic effect of NKT cells is limited to the acute liver injury period, and in persistent chronic liver injury, the anti-fibrotic effect of NKT is diminished due to functional failure of NKT and immune tolerance of the liver (50). Additionally, Park et al. found that α-galactosyl neurosphingosamine overactivated NKT cells will accelerate CCL4-induced acute liver injury, inflammation, and fibrosis (51). In the progression of the alcoholic liver, NKT cell activation can exacerbate liver injury and promote an inflammatory response (52). Another study found that NKT cells from viral-infected patients could secrete more pro-inflammatory factors IL-4 and IL-13 compared with those from non-infected patients. It suggests that NKT cells may promote fibrosis by producing pro-inflammatory factors IL-4 and IL-13 to promote the development of fibrosis in patients with chronic viral infection (53).

T cells

Different subtypes of T cells have different effects on hepatic fibrosis. Th1 and Th2 cells play the core role in this process, and they tend to exhibit mutually antagonistic effects on hepatic fibrosis. IFN-γ secreted by Th1 cells regulates the balance of MMP and tissue inhibitor of metalloproteinase (TIMP), which can inhibit HSCs activation and proliferation, induce HSCs apoptosis, and inhibit TGF-β expression in a variety of cells (e.g., hepatocytes, KCs, HSCs) to exert anti-fibrotic effects (54, 55). IL-13 secreted by Th2 induces the production of collagen I, collagen III, α-smooth muscle actin, and TIMP-1 to induce TGF-β secretion, activate HSCs, and inhibit HSCs apoptosis thus promoting the progression of hepatic fibrosis (56). Moreover, IL-4, IL-5, and IL-13 secreted by Th2 cells reduce the liver inflammatory response, but also inhibit the Th1-mediated cellular immune response and prevent the clearance of viruses and parasites, leading to the persistence of viruses and inflammation (57). In addition, Th1 and Th2 cells regulate cellular collagen synthesis by antagonistically regulating nitric oxide synthase 2/arginase activity (58). In a non-alcoholic steatohepatitis (NASH) mouse model, mice lacking the IFN-γ (typical Th1 cytokine) showed significant protection against liver damage and fibrosis (59).

$CD8^+$ T cells mainly produce cytotoxic molecules such as IFN-γ, TNF, and perforin (60). During NASH in mice, the number of $CD8^+$ T cells in the liver was increased, and lipid-conditioned $CXCR6^+$ $CD8^+$ T cells induce hepatocyte killing in a perforin-independent, FasL (CD95L)-dependent manner (61). Consequently, in a diet-induced mouse model of NASH, $CD8^+$ T cell depletion blunted liver injury (62). Thus, $CD8^+$ T cells are likely to promote hepatic injury during NASH. But the mechanism by which T cell subsets are activated and interact to promote liver inflammation is not well understood. And more research is needed to investigate the specific mechanism and how to target these pathways without compromising immune defenses (63). Additionally, hepatic tissue-resident memory T cells (TRM cells) stably occupy tissues and participate in hepatic fibrosis progression differing from T cells in the circulation. $CD8^+$T cells characterized by CD69 and CD103 are defined as $CD8^+$ TRM cells (64, 65). Koda Y et al. found that $CD8^+$ TRM cells were able to attract HSCs in a CCR5-dependent manner and mediated apoptosis of activated HSCs through the Fas- FasL pathway (66). Although little latest work had been done to verify the exist of $CD4^+$TRM fraction in human liver, its relationship with liver fibrosis has not been systematically studied and documented (67).

Gamma-delta T cells(γδ T cells), as liver tissue-resident T cells, are characterized by the gamma and delta chains of T cell receptors (68, 69). Hepatic γδ T cells account approximately for 3% - 5% of the hepatic lymphocytes and 15% - 25% of the hepatic T cells, which are identified as liver-resident cells with predominant production of IL-17as well as the IFN-γ (70). And the IFN-γ will activate macrophages to release IL-15, prompting γδ T cells to accumulate at the infectious site and participate in local anti-inflammatory and anti-fibrotic effects, whereas IL-17, a major pro-inflammatory factor, often plays a pro-fibrotic effect on the fibrosis progress (70, 71). In the CCl4-induced hepatic fibrosis murine model, γδ T cells could activate the mTOC2 signaling pathway in response to macrophages, promoted CXCR3 transcription, and drove γδ T cell accumulation in the liver (72). It also documented that hepatic γδ T cells could kill the activated HSCs in a TRAIL-or FasL-dependent manner and secrete IFN-γ to inhibit differentiation of pro-fibrotic Th 17 cells to relieve hepatic fibrosis. And it also promoted HSC lysis by enhancing the cytotoxicity of conventional NK cells and liver-resident NK cells on activated HSCs (37). However, hepatocyte-derived exosomes mediated TLR3 activation in HSCs and increased IL-17 production from γδ T cells, and the IL-17 strongly stimulated the expression of a-SMA, TGF-β, IL-6, and collagen type I-α1 in HSCs and aggravated liver fibrosis (73).

B cells

B cells are classified as B-1 cells, B-2 cells, effector B cells, and regulatory B cells (Bregs) based on their surface molecular, localization, and functional characteristics. Relatively few studies indicated that B cells can influence the development and progression of hepatic fibrosis in an antibody-independent manner (74). It has been demonstrated that B cells knockout mice given CCL4 injections had significantly lower hepatic fibrosis than normal control mice, suggesting that B cells have a pro-fibrotic effect and this effect is independent of antibodies and T cells (75). Conversely, B cells can also exacerbate hepatic fibrosis by indirectly affecting T lymphocyte function and contact with fibroblasts, phagocytes, and NK cells through the secretion of IL-1, IL-6, and IL-4 (76). Secondly, it has been found by retrospective analysis that the number of B lymphocytes was significantly lower in the cirrhotic group than in the hepatitis group and healthy individuals, which also implies that B lymphocytes are affected in the progression of cirrhosis and are involved in the development of cirrhosis (5). In addition, similarly

to T cells, regulatory B cells (Bregs) also secrete cytokines inducing immune tolerance and maintaining environmental homeostasis. Bregs from patients with chronic hepatitis B or hepatitis B virus (HBV)-associated hepatic fibrosis can inhibit the function of Th1 and Th17 through intercellular contact and secretion of IL-10. Bregs can also induce the conversion of $CD4^+CD25^-$T cells to Tregs, resulting in inflammatory response suppression and inflammatory repair (77).

In summary, the immune cells directly act on HSCs through the interaction of ligands and receptors or by secreting cytokines to regulate the activity of HSCs. Additionally, immune cells play an immunomodulatory role or indirect effect on HSCs regulating the inflammatory response through the synthesis and secretion of different cytokines and chemokines, thus promoting or inhibiting the formation and progression of hepatic fibrosis. We depicted how the main sub-types of immune cells in the HIME influence the HSCs in Figure 1.

The effect of HIME for different chronic liver diseases on the hepatic fibrosis

The HIME of viral hepatitis

Viral hepatitis is a type of infectious disease caused by the hepatitis virus and characterized by hepatocyte degeneration, necrosis, and apoptosis. Chronic hepatitis, especially chronic hepatitis B, undergoes fibrosis with the virus-induced long-term inflammation and leading to the evolutionary pathway of "hepatitis–cirrhosis–liver cancer" (78, 79).

The major mechanisms of viral hepatitis progression to cirrhosis are the direct and indirect activation of HSCs (6). The Hepatitis virus can promote HSCs activation by disturbing immune cell functions, thereby exacerbating hepatic fibrosis (80). Xie et al. (81) found that the core antigen HBeAg of HBV is the most important component of

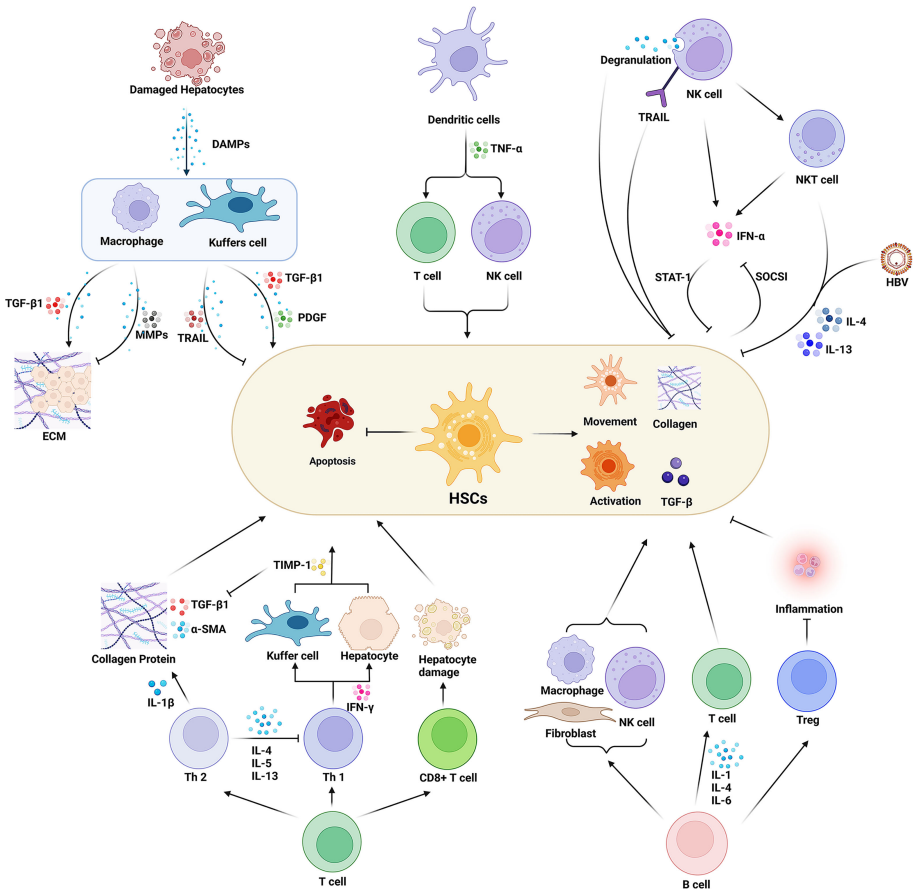

FIGURE 1
Effect of various immune cells in the hepatic immune microenvironment (HIME) on hepatic fibrosis. Various immune cells affect the process of hepatic fibrosis by promoting or inhibiting the function of HSCs. Macrophages play a dual role in this process. The peripheral macrophages and the resident liver macrophages (Kuffer cells) are activated under the damage-associated molecular pattern released from the damaged hepatocytes during injury or inflammatory conditions. Activated macrophages secrete proinflammatory factors such as TGF-β to promote ECM formation and activate HSCs. On the other hand, macrophages can also inhibit hepatic fibrosis by expressing MMPs and TRAIL. Dendritic cells secrete TNF-α to activate HSCs by enhancing the immune function of T cells and NK cells. And the activated NK cells inhibit the function of HSCs and prevent hepatic fibrosis progression. While NK cells also can directly damage HSCs through degranulation and up-regulation of TRAIL expression. Moreover, it can secrete INF-γ, and synergize NKT cells to inhibit HSCs activation through the STAT signaling pathway. However, HSCs can inhibit INF-γ secretion of NK cells *via* the SOCSI pathway. The balance of Th1/Th2 cells plays an important role in the process of hepatic fibrosis. Th1 cells secrete INF-γ to increase the expression of TIMP in macrophages and inhibit the activity of HSCs. Th2 cells can secrete a variety of cytokines to activate HSCs and inhibit the HSCs' apoptosis as well as the activity of Th1 cells. $CD8^+$ T cells secrete cytotoxic factors to mediate hepatocyte injury, thereby activating HSCs. B cells regulate macrophages, fibroblasts, and T cells and promote HSCs activation by secreting interleukins. And B cells are also able to regulate Treg cells to inhibit inflammatory responses and HSCs' activation. Created with BioRender.com.

HBV-induced macrophage activation. HBeAg binds with macrophages through the TLR-2 receptor, activates the NF-κB signaling pathway in macrophages, and releases a large number of cytokines and chemokines to inhibit viral proliferation and regulate HSCs' function, producing different pro-fibrotic effects. On the one hand, TNF-α and IL-1β can inhibit HSCs apoptosis by up-regulating the expression of TIMP-1 and down-regulating the expression of BMP and activin membrane-bound inhibitor of HSCs (82–85). Macrophages are also able to up-regulate PI3K-AKT-mTOR and p38 mitogen-activated protein kinase (p38 MAPK) pathway in HSCs to promote the HSCs movement, and also activate TGF-β/smad pathway to promote their proliferation and contraction, and promote the production of type I and type III collagen (86). Moreover, the activated HSCs further affect the function of macrophages, and they interactively promote the progression of hepatic fibrosis. Akt/PKB (protein kinase B) in HSCs promotes fibrogenic M2 polarization in macrophages (87). HBeAg directly induces up-regulate the secretion of TGF-β of HSCs, and the secreted TGF-β positively feeds back to HSCs themselves and mediates their activation and proliferation (88). The HBV and its related proteins reduce the expression of TLR in macrophages, leading to HBV immune tolerance and persistent infection. The described mechanisms of macrophage and HSCs interacted in the liver for a long time, allowing hepatic fibrosis occurrence and progress to cirrhosis or even hepatocellular carcinoma (78, 89).

Viral infection could inhibit NK cells to produce the anti-fibrotic cytokine IFN-γ, promoting the development of hepatic fibrosis. A study found that HBV infection down-regulates the expression of NK cell activating receptors NKG2D and 2B4 on NK cells *via* TGF-β1 secreted by hepatocytes, resulting in a decreased expression of their intracellular adaptor proteins DAP10 and SAP, which impair NK cell-mediated cytotoxicity ability and IFN-γ production (90). Another study found that ability to the production of IFN-γ of CD56 (dim) NK cells in patients with chronic HBV infection is impaired, leading to an increased NKG2A expression and decreased CD16 expression, thereby preventing NK cells activation and promoting hepatic fibrosis (91). Activation of the PDGF/PDGF-β receptor also plays a critical role in hepatic fibrosis. HBV core protein and HBV regulatory X protein are two proteins expressed by HBV, which can promote PDGF production by increasing the transcription of PDGF in hepatocytes. PDGF will bind to the PDGF-β receptor, a receptor located on HSCs, induce receptor phosphorylation, and initiate various intracellular signaling pathways activation such as the JAK/STAT, PI3K, PLC-γ, or MAPK pathways, leading to HSC migration, proliferation, and ECM secretion (92–94).

In the virus-infected HIEM, exosomes secreted by hepatocytes, containing miRNAs, also can regulate the function of HSCs. MiR-19, an exosome secreted from HCV-infected hepatocytes, could reduce SOCS3 production of HSC. And the SOCS3 could block JAK kinase, thus the reduction of SOCS3 will activate the JAK-STAT3 pathway, increase cyclin D1 transcription, and stimulate HSC proliferation (95). HCV-infected hepatocytes also secrete miR-192-containing exosomes and exert an effect on HSCs, increase TGF-β1 expression, and stimulate HSCs activation and differentiation into myofibroblasts phenotype (96). Figure 2 summarized the effect of viral hepatitis-induced HIME changes on the development and progression of hepatic fibrosis.

The HIME of autoimmune liver diseases

Autoimmune liver diseases (AILD) are mainly characterized by liver injury along with elevated serum immunoglobulins and the presence of multiple auto-antibodies in the blood, which is associated with autoimmunity. It can be divided into autoimmune hepatitis, primary sclerosing cholangitis, and primary biliary cholangitis (PBC), according to the cell type involved (97).

As the autoimmune tolerance of patients with AILD is impaired, a number of auto-antibodies are produced and accumulated in the HIME, including antinuclear antibodies in autoimmune hepatitis, antimyosmooth muscle antibodies, and antimitochondrial antibodies in PBC. Driven by humoral immunity, the activated $CD4^+$ T cells (including Th1 and Th2) stimulate B cells to produce antibodies against auto-antigens by directly T-B cell membranes contacting and releasing cytokines, and with chronic stimulation on hepatocytes. The damaged hepatocytes could recruit the NK cells and macrophage infiltration that exacerbate the inflammatory response within the liver (63, 98). Wu et al. (99) showed that the transcription of POU6F1 in biliary epithelial cells activates monocyte chemotactic protein-1 (MCP-1) and promotes peripheral M1 and M2 macrophage recruitment and infiltration into peribiliary gland (PBG) niche, producing chronic inflammation, resulting in dilated PBG compartments and the formation of "onionskin" fibrosis characterized by multifocal fibrosis around intrahepatic and extrahepatic bile ducts in a mouse model of primary sclerosing cholangitis.

Regulatory T cells (Tregs)/Th17 cell imbalance is critical for immune disorders in patients with AILD. Tregs are circulating auto-reactive T cells that limit autoimmune damage. A significant decrease in Tregs was found in the peripheral blood and liver of PBC patients (100, 101). In addition, Zhu et al. (102) found that knockout of AMP-activated protein kinase α1 and Tregs-specific deletion could weaken the suppressive activity of Tregs and lead to the development of alcoholic liver disease(ALD) in mice. Th17 cells were significantly increased in the bile ducts of patients with primary biliary cholangitis (103). Th17 cells are helper T cells differentiated from Th0 cells stimulated by IL-6 and IL-23, which can secrete a large number of pro-inflammatory factors such as IL-17 and IL-22, activate TNF or Fas/FasL pathway to mediate hepatocyte apoptosis, and activate HSCs to promote the development of hepatic fibrosis (104, 105). Figure 3 presented the main alterations of the HIME in the AILD.

The HIME of fatty liver disease

Fatty liver disease is mainly caused by triglyceride accumulation in the liver leading to continuous hepatocyte stimulation and arousing long-term inflammatory responses. According to whether exposure to long-term excessive alcohol consumption fatty liver disease can be divided into ALD and non-alcoholic fatty liver disease (NAFLD).

ALD As ALD patients often consume amounts of alcohol for a long time, the majority of ethanol is metabolized in the liver. Acetaldehyde, the intermediate product of ethanol, can directly damage hepatocytes and bind with the intracellular proteins as antigens to cause humoral immune responses (106, 107). This

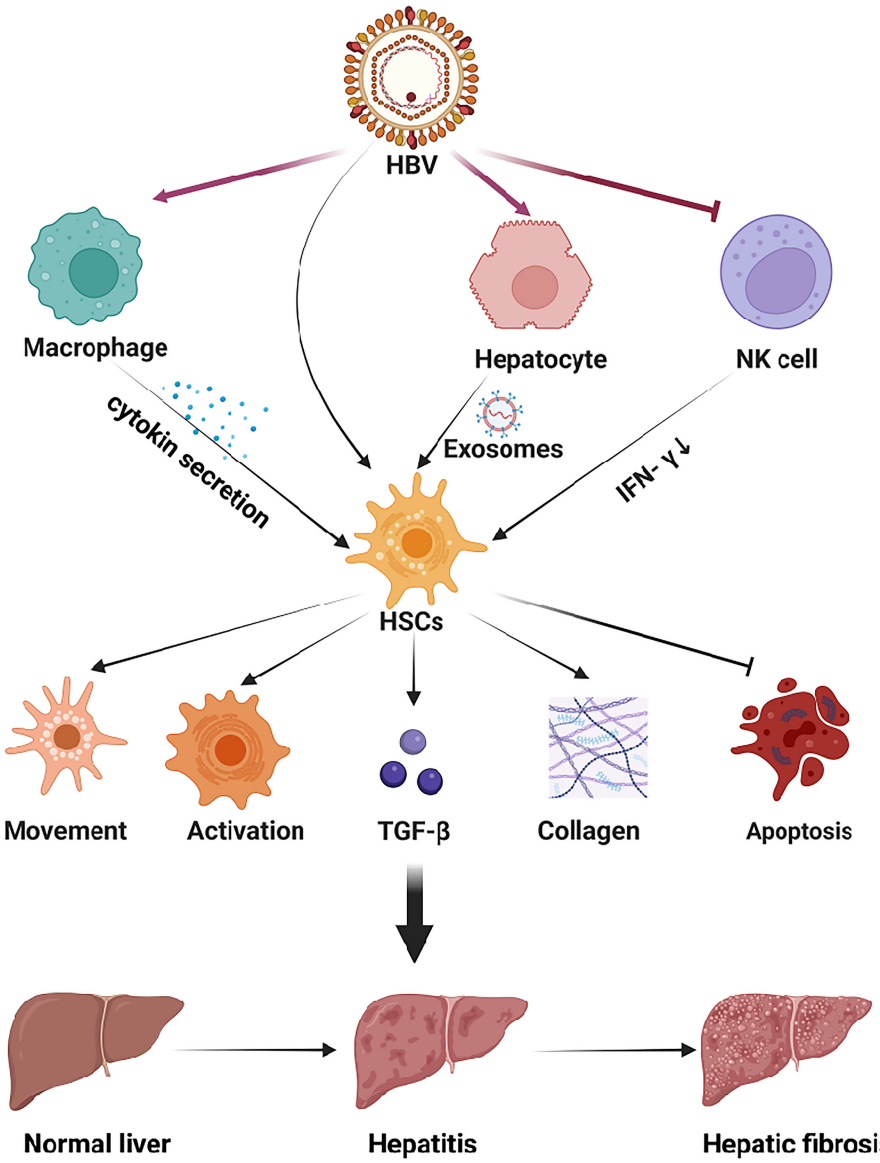

FIGURE 2
The HIME of HBV-induced viral hepatitis. The hepatitis virus replicates within hepatocytes and is secreted into the extracellular space *via* e exosomes. Viral antigens can exert multiple effects in HSCs to promote hepatic fibrosis. In addition, the virus can be recognized by macrophages and NK cells, which will up-regulate or down-regulate the secretion of cytokines to activate HSCs. Subsequently, HSCs activation aggravates hepatitis and promotes the progression of hepatic fibrosis. Created with BioRender.com.

tissue stress injury leads to an aseptic inflammation (108), which further activates the KCs, initiating the immune response and recruiting peripheral monocytes into the liver. And the KCs are capable of releasing the number of inflammatory factors and chemokines such as TGF-β, TNF-α, and IL-1β to stimulate HSCs activation. Furthermore, macrophages of the M2 type possess a powerful activating effect on HSCs (109, 110). Ethanol-induced telomerase reverses transcriptase expression in macrophages and promotes macrophage polarized into M1 type through the NF-κB signaling pathway (111). And ethanol can activate HSCs to induce collagen deposition by up-regulating P2X7 receptor (P2X7R) expression-an ATP-gated, non-selective cation channel receptor belongs to the P2X family of P2 purine receptor in human macrophages leading to NLRP3 inflammasome activation and releasing the IL-1β. Inhibition of NLRP3 inflammasome activation effectively suppresses further deterioration of alcoholic steatohepatitis and attenuates alcohol-induced liver injury (112–114). Another study found P2X7R can also mediate acetaldehyde-induced HSC activation *via* the PKC-dependent GSK3β pathway (115). Additionally, ethanol can also directly affect the expression of epigenetic regulators during HSC transdifferentiation. The histone modifying enzymes such as H3K4 and methyltransferase MLL1 are upregulated in HSCs, when exposed to ethanol and recruited to the elastin gene promoter, resulting in the up-regulation of pro-fibrotic elastin genes expression and increased ECM protein expression.

ROS also plays an important role in hepatic fibrosis resulting from alcoholic liver disease. Macrophages exposed to chronic ethanol can induce ROS release, which acts as an inducer of the TGF-β signaling pathway, thereby promoting the activation of HSCs. The dual effects of ethanol and ROS promote hepatocyte lysis and death, releasing the

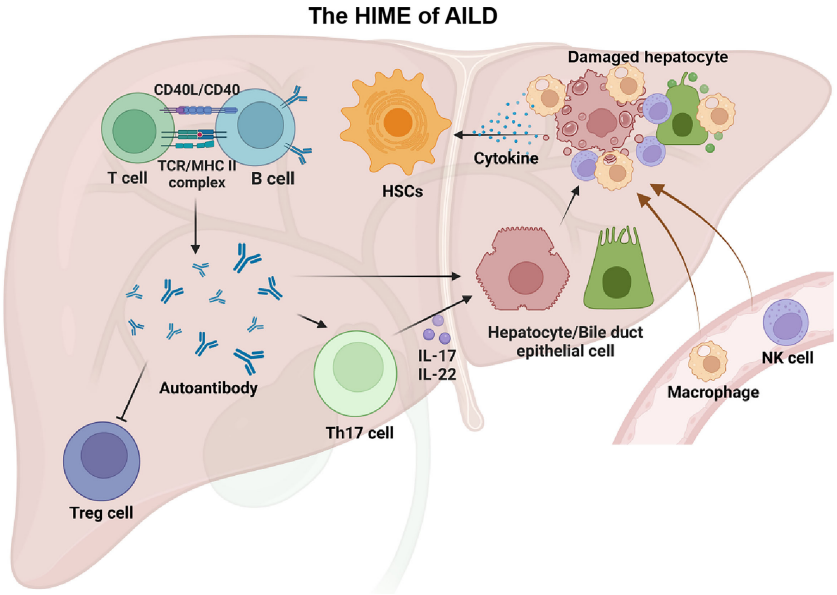

FIGURE 3
The HIME of AILD. Autoimmune liver disease happens when a large number of autoantigens are present in the liver. With the assistance of T cells, B cells can generate a large number of autoantibodies that recognize these autoantigens. These autoantibodies can impair the function of Treg cells and lead to autoimmune damage. In addition, autoantibodies can directly, or indirectly through Th17 cells, exert an effect on targeted cells (e.g., hepatocytes, biliary epithelial cells) and recruit peripheral macrophages and NK cells, causing liver injury. These injured cells release large amounts of cytokines thereby driving the HSCs activation. Created with BioRender.com.

DAMPs to the extracellular matrix, stimulating macrophage activation, and initiating liver regeneration as well as fibrosis processes (116, 117). ATP, as DAMPs released during cell lysis, acts as an endogenous danger signal to activate intracellular inflammatory response through P2X7R and exacerbate cell damage (118).

A recent study found that alcohol intake increases intestinal permeability, promotes the transfer of endotoxins such as lipopolysaccharide (LPS) from Gram-negative bacteria to the portal vein, and reaches the liver with the bloodstream. LPS activation of various TLR ligands was shown to induce miR-155 in macrophages. miR-155 plays a critical role in promoting macrophage M2 polarization. miR-155 also targets and activates SMAD2/5, Snail1, STAT3 genes that are involved in fibrosis. And the miR-155 knockout protects mice from alcohol-induced steatosis and inflammation (119–121).

NAFLD NAFLD is characterized by excessive lipids accumulation in the liver, which can affect bile acid metabolism, hepatocyte metabolism, and the function of macrophages and HSCs. And it can promote the progression of NAFLD to NASH or even cirrhosis by activating the metabolism of intrahepatic immune cells (122) (Figure 4). Innate immune activation is a key factor in the exacerbation of hepatic inflammation in NAFLD/NASH. The TLR was reported to mediate B-cell activation through activation of myeloid differentiation primary response protein 88 (MyD88) in a high-fat high-carbohydrate diet-fed mouse model of NASHA. The activated B cells secreting pro-inflammatory cytokines, modulating neighboring immune cells, and differentiating into antibody-secreting cells will result in the progression of NAFLD. A study reported that transplantation of gut microbiota from NAFLD patients into recipient mice can also increase B cell accumulation and activation in the liver (123). Studies have shown that insulin resistance is often accompanied by NAFLD-associated hepatic fibrosis, and NK cells contribute to the development of obesity-associated insulin resistance. Lipid accumulation promotes IL-6R expression in mouse NK cells, and IL-6/STAT3-dependent myeloid NK cell subsets are a critical determinant of NAFLD-associated insulin resistance *in vivo* (124). Almost all $CD4^+$ T cells are involved in the sterile inflammation associated with NASH. IL-17 secreted by Th17 cells increases c-Jun N-terminal kinase (JNK) activation in steatotic hepatocytes and exacerbates hepatocyte injury, while IL-22 secreted by Th22 cells protects hepatocytes by inhibiting PI3K/Akt-mediated JNK activation. And the balance breaking between IL-17 and IL-22 will lead to hepatocyte injury and accelerate NAFLD progression (125). Ghazarian et al. (126) reported that high-fat diet (HFD) feeding increased the expression of IFN-αR on $CD8^+$ T cells and upregulated the key transcription factors of IFN-I in the liver of the NAFLD mouse model. While the activation of IFN-I responses drives the expansion of intrahepatic pathogenic $CD8^+$ T cells, leading to the NAFLD progression. In mouse models of NAFLD, saturated free fatty acid (FFA) levels are increased in the blood, and they can bind to TLR2 and TLR4 of liver-resident Kupffer cells and peripherally infiltrating macrophages, triggering multiple mechanisms to produce the ROS, such as mitochondrial damage, endoplasmic reticulum stress, and nicotinamide adenine dinucleotide phosphate oxidase. Nicotinamide adenine dinucleotide phosphate oxidase2-derived ROS could stimulate macrophages to produce pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, which promote the development of hepatic fibrosis by up-regulating NF-κB pathway and activate JNK activator protein-1 in HSCs (127). Another study reported a high-fat diet-induced increased bile acid secretion, resulting in gut microbiota disturbances and intestinal barrier dysfunction in a mouse model of NAFLD (128). It will lead

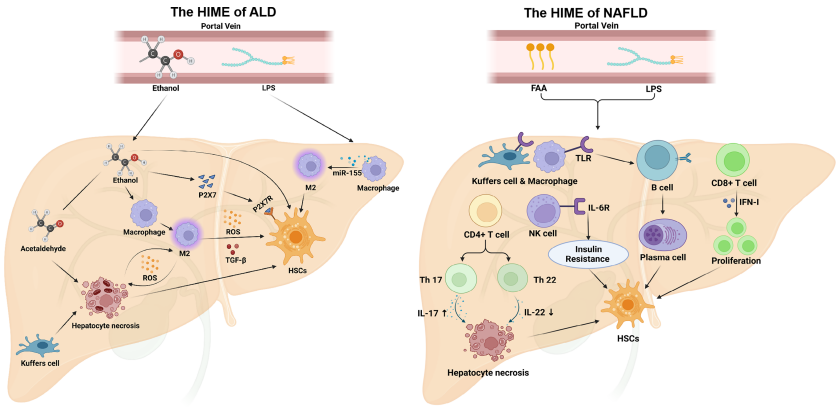

FIGURE 4
The HIME of ALD and NAFLD. In ALD, ethanol is absorbed through the intestine and entry the portal vein to the liver. Ethanol can stimulate M2 macrophage polarization that secretes reactive oxygen species and TGF-β to promote hepatic fibrosis. Ethanol also increases P2X7R(P2X7 receptor) expression on HSCs, and P2X7 can promote HSCs activation. Acetaldehyde, an intermediate product of ethanol, induces hepatocyte necrosis. And necrotic hepatocytes directly activate HSCs and release ROS to activate M1 macrophages. Additionally, ethanol can disrupt the intestinal barrier, causing LPS to enter the liver *via* the portal vein and induce M2 macrophage polarization. In NAFLD, free fatty acid and LPS from the peripheral circulation induce TLR expression in macrophages, transform B cells into plasma cells, and secrete antibodies, which activate HSCs activation. In addition, free fatty acid and LPS are also able to stimulate $CD4^+$ T cells, regulate the balance of Th17/Th22 cells, and induce hepatocyte necrosis by increasing IL-17 and reducing IL-22. Moreover, $CD8^+$ T cells and NK cells can also promote HSCs activation under this HIME. Created with BioRender.com.

to an increase of LPS and other bacteria-derived compounds in portal blood, promoting the activation of TLR and other pattern recognition receptors in the liver, and triggering local inflammatory and fibrotic responses. In addition, in a mouse model of induced NASH-fed diets rich in palmitate, cholesterol, and sucrose, the palmitate of hepatocyte specifically promotes the overexpression of the Notch pathway ligand Jag1 by activating TLR4 to upregulate NF-κB signaling. And the activation of the Notch signaling pathway in hepatocytes drives the expression of genes encoding osteopontin and secreted phosphoprotein 1 (Spp1), which promotes HSCs activation and hepatic fibrosis (129).

Effects of regulating the HIME for preventing hepatic fibrosis

The importance of immune cell populations in the development of chronic liver disease is self-evident. However, most traditional treatments for liver fibrosis are still focused on inhibiting the activation of HSCs. The role of HIME in liver fibrosis should not be underestimated. Therefore, blocking the infiltration of immune cells in the blood or altering the function of infiltrating immune cells or cytokines may be a potential target for the treatment of liver fibrosis. Table 1 summarized the details of the effects of traditional Chinese medicine(TCM) in modulating the HIME for preventing hepatic fibrosis of chronic liver disease. In four preclinical studies, researchers regulated the functions of HSCs (130, 132), macrophages or KCs (25, 131, 134–136, 138), regulatory T cells (133), DCs (30), and NK cells (25, 137) by different treatment approach in the CCl4 or bile duct ligation -induced liver fibrosis murine model. And these interventions can effectively alleviate hepatic fibrosis and tissue inflammation. Another study found inhibiting the chemokines CCR2/5 could significantly reduce circulating $Ly6C^{hi}$ monocytes and hepatic monocyte-derived macrophage infiltration in murine models of NASH, and attenuate hepatic inflammation and fibrosis (140). A chemical agent, dopamine receptor D2 antagonism, was screened to target the Yes-associated protein signaling pathway in macrophages. It antagonizes Yes-associated protein-dependent fibrotic crosstalk between selectively targeted macrophages and CTGF + VCAM1 + vascular niche, blocks fibrosis, and restores liver architecture in rodent and large animal models of NASH (139).

The occurrence and progression of liver fibrosis often involve a variety of cells and multiple signaling pathways. Clinical TCM treatments are advanced in action on multi-cellular and multi-target, which might be a new choice for alleviating liver fibrosis. Ginsenoside (KD), the active ingredient in the TCM ginseng, was reported could reduce hepatic histopathological damage, proinflammatory cytokine release, and extracellular matrix deposition in CCl4-induced hepatic fibrosis. The KD restrained the hepatic fibrosis-driven rise in CD86, MHC-II, and CCR7 levels, while upregulated PD-L1 expression on DCs, which blocked $CD8^+$ T cell activation. Additionally, KD reduced DC glycolysis, maintained DCs immature, and was accompanied by an IL-12 decrease in DCs. These effects disturbed the communication of DCs and HSCs with the expression or secretion of α-smooth muscle actin and Col-I declined in the liver (30). Lycium barbarum polysaccharides (LBPs) supplementation was reported to reduce CCl4-induced oxidative injury, inflammatory response, and TLRs/NF-kB signaling pathway expression, which alleviated CCl4-induced liver fibrosis (132). Moreover, two Chinese herbal formulas Si-Wu-Tang (134)and Yi Guan Jian (131) were reported to regulate the activation and polarization of macrophage, which significantly improved liver fibrosis and inflammatory responses. Additionally, Si-Wu-Tang also can inhibit the M1 from inhibiting the infiltration of neutrophils and promote the $CD8^+$ organization to reside memory T cells cytotoxic effect inducing the HSCs apoptosis (134). The Yi guan Jian can enhance the therapeutic effects of fetal liver stem/progenitor cells that promoted the liver regeneration differentiation of fetal liver stem/progenitor cells into hepatocytes (131). Collectively the data supports

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TABLE 1 The effects of regulating the HIME on the progression of hepatic fibrosis.

Author, publishing year [ref]	Species and disease models	Therapeutic methods	Targeted cells; Signaling pathway	The mechanisms of therapeutic effect	Effects on progression of hepatic fibrosis
Xiafei Wu, 2015 (130)	CCl4-induced hepatic fibrosis rat model	Tetramethylpyrazine (TMP)	HSCs; PDGF-bR/ NLRP3/caspase1 pathway	TMP suppressed inflammation by decreasing levels of inflammatory cytokines, including TNF-a, NLRP3, NF-kB and IL-1b, and significantly protected the liver from injury and fibrogenesis; TMP promotes the role of PDGF-bR/NLRP3/ caspase1 pathway; TMP improved histological structure of liver and decreased hepatic enzyme levels and collagen deposition in fibrotic liver.	TMP reduced hepatic inflammation and fibrosis
Pengfei Ma, 2017 (25)	CCl4 or BDL-induced hepatic fibrosis murine model	Bone-marrow-derived macrophages were polarized into M0, M1, or M2 macrophages	Macrophages and NK cells	M1 Macrophage produced matrix metalloproteinases and hepatic growth factor, which promoting collagen degradation and hepatocyte proliferation; increasing the activated NK cells in fibrotic liver and inducing HSCs apoptosis.	Bone-marrow-derived M1 macrophages alleviated hepatic fibrosis
Ying Xu, 2018 (131)	CCl4-induced hepatic cirrhosis rat model	Yiguanjian (YGJ)	Fetal liver stem/ progenitor cells (FLSPC) and macrophages; Wnt/ beta-catenin pathway	1.YGJ suppressed the macrophages activation and inhibited non-canonical Wnt and promoted canonical Wnt signaling pathway; 2. YGJ promoted the liver regeneration differentiation of FLSPCs into hepatocytes.	YGJ enhanced FLSPC-mediated repair of hepatic cirrhosis
Fang Gan, 2018 (132)	CCl4-induced hepatic fibrosis rat model	Lycium barbarum polysaccharides (LBPs)	HSCs; TLRs/NF-kB pathway	LBPs decreased α-smooth muscle actin expression and the activity of aspartate transaminase, alkaline phosphatase and alanine aminotransferase; LBPs alleviated CCl4-induced oxidative injury, inflammatory response and TLRs/NF-kB signaling pathway expression.	LBPs alleviated hepatic fibrosis
Yejin Xu, 2019 (133)	CCl4-induced hepatic fibrosis murine model	IL-10 Gene-modified bone marrow-derived dendritic cells therapy	Regulatory T Cells; TGF-β/Smad pathway	IL-10 Gene-modified bone marrow-derived dendritic cells therapy increased regulatory T cells, while ALT, AST and inflammatory cytokines were significantly reduced, and the TGF-β/smad pathway was inhibited.	The dendritic cells therapy alleviated hepatic fibrosis
Zhi Ma, 2022 (134)	BDL-induced hepatic fibrosis murine model	Si-Wu-Tang (SWT) decoction	Macrophages, neutrophils, CD8+ T cells and HSCs; Fas/FasL pathway	SWT inhibited the activation of M2 macrophages to reduce the release of profibrotic-cytokines and prevented the activation of neutrophils; SWT increased the $CD8^+$ T cells and promoted activated HSCs apoptosis through Fas/FasL pathway.	SWT improved hepatic fibrosis and inflammatory responses
Ming Xiang, 2022 (30)	CCl4 -induced hepatic fibrosis murine model	Kinsenoside (KD)	DC cells and $CD8^+$ T cells; PI3K-AKT-FoxO1 pathway	KD inhibited DCs maturation; KD promoted PD-L1 expression via PI3K-AKT-FoxO1 pathway; KD reduced IL-12 expression, blocked activation of $CD8^+$ T cells and HSCs, and reduced α-smooth muscle actin and Col-I expression.	KD alleviated hepatic fibrosis
Peng Liu, 2022 (135)	CCl4 -induced hepatic fibrosis murine model	Chitooligosaccharide (COS)	Kuffer cells and macrophages; JAK2/STAT1 pathway and JAK1/ STAT6 pathway	COS inhibited polarization of M1 and M2 macrophages; COS inhibited JAK2/STAT1 pathway on M1 macrophages and JAK1/STAT6 pathway on M2 macrophages; 3. COS rescued mice from hepatic fibrosis, marked by decreased deposition of extracellular matrix and histological lesions.	COS alleviated hepatic fibrosis
Xiang-an Zhao, 2022 (136)	CCl4 -induced hepatic fibrosis murine model	Curcumin	Kupffer cells and Ly6C(hi) MoMFs; ERK1/2 and p38 pathway	1. Curcumin decreased intrahepatic Ly6C(hi) monocyte infiltration as well as associated pro-inflammatory and profibrotic cytokines; 2. Curcumin reduced KCs activation and monocyte chemokines; 3. Curcumin prevented the M1 polarization of macrophages through ERK1/2 and p38 pathway.	Curcumin protect against hepatic fibrosis
Xixi Tao, 2022 (137)	CCl4 or BDL -induced hepatic fibrosis murine model	Sulprostone	NK cells and HSCs	Deletion of EP3 impaired the cytotoxicity of NK cells toward HSCs;	Activation of EP3 by sulprostone

(Continued)

TABLE 1 Continued

Author, publishing year [ref]	Species and disease models	Therapeutic methods	Targeted cells; Signaling pathway	The mechanisms of therapeutic effect	Effects on progression of hepatic fibrosis
				EP3 upregulated Itga4 expression in NK cells through promoting Spic nuclear translocation.	alleviated hepatic fibrosis
Jianhua Rao, 2022 (138)	CCl4 or BDL or MCD diet induced hepatic fibrosis murine model	Myeloid-specific Follistatin-like protein 1 (FSTL1)-knockout; Activation of pyruvate kinase M2	Macrophages; TLR-4/NF-κB pathway	FSTL1 deletion results in diminished hepatic macrophage and neutrophil infiltration and reduced expression of pro-inflammatory factors FSTL1 deletion inhibits M1 polarization and TLR-4/NF-κB pathway activation in macrophages; Pyruvate kinase M2 was able to counteract FSTL1-mediated M1 polarization in macrophages and inflammation	Myeloid-specific FSTL1 deficiency alleviated hepatic fibrosis
Jie Qing, 2021 (139)	CCl4-induced NASH murine and minipig models; liver biopsies from patients with cirrhosis	Dopamine receptor D2 antagonism (DRD2)	Macrophage; Hippo/YAP pathway	1. DRD2 antagonizes YAP-dependent fibrotic crosstalk promoting liver regeneration over fibrosis; 2. DRD2 antagonists block fibrosis and restore liver architecture in rodent and large animal models of NASH.	DRD2 antagonists block fibrosis and restore liver architecture in NASH
Tobias Puengel, 2022 (140)	Serum samples and liver biopsies from patients with NAFLD; CDAHFD induced NASH murine model	Chemokine receptor 2/5 (CCR2/5) inhibitor and pegylated fibroblast growth factor 21 (FGF21) agonist	Ly6C(hi) MoMFs	In NAFLD patients, serum levels of chemokines CCL2 was associated with inflammation and advanced hepatic fibrosis, FGF21 was associated with inflammation; In murine models of NASH, CCR2/5 inhibition reduced circulating Ly6C(hi) MoMFs, and the FGF21 agonist reduced body weight, hepatic triglycerides.	CCR2/5 inhibition and FGF21 agonism ameliorates steatohepatitis and hepatic fibrosis

CCl4, carbon tetrachloride 4; HSCs, hepatic stellate cells; TNF, tumor necrosis factor; NF-kB, nuclear factor kappa B; IL, interleukin; BDL, bile duct ligation; NK cells, natural killer cell; DC cells, Dendritic Cells; NASH, nonalcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; Ly6C(hi) MoMFs, Lymphocyte antigen 6C (high) monocyte-derived macrophages.
PDGF-bR/NLRP3/caspase1 pathway: platelet-derived growth factor-b receptor/NOD-like receptor thermal protein domain associated protein 3/caspase1 pathway.
TLRs/NF-kB pathway: Toll-like receptors/ nuclear factor kappa B pathway.
TGF-β/Smad pathway: transforming growth factor β/Smad pathway.
PI3K-AKT-FoxO1 pathway: phosphatidylinositol 3-kinase/protein kinase B/forkhead box O1 pathway.
JAK/STAT pathway: Janus tyrosine kinase/signal transducer and activator of transcription pathway.
ERK1/2 and p38 pathway: extracellular signal regulated kinase 1/2 and p38 signaling pathway.

the notion that multi-target therapeutics may be an effective option for t liver fibrosis treatment (Table 1).

Concluding and prospects

The immune cells and HSCs could synthesize and secrete several chemical mediators under chronic liver injury, and they constitute a dynamic HIME of an immune cells-cytokines-chemokines network system that affects the processes of hepatic fibrosis. It can be seen the HIME of hepatic fibrosis is complex not only because there are a variety of cells involved, but also because there are different sub-types of immune cells, which play different roles at different stages of liver disease progression. Moreover, in addition to local immune cells in the liver, a variety of immune cells in the circulation also can be chemotactic into the liver tissue and differentiate into different cell subtypes, exerting an effect on the initiation, progression, and regression of hepatic fibrosis. According to the summary and analysis in this review, most sub-types of immune cells have bidirectional effects on the development of hepatic fibrosis, because the immune cells interact with each other and also with other stromal cells in the microenvironment through diverse and complex cytokines networks, and eventually achieve a balance. While this balance will be broken with the persistence or elimination of the disease's causes, then these cells will form a new balance through the other signaling pathways and exert a promoting or inhibiting role in hepatic fibrosis to a different degree. Therefore, it is necessary not only to explore the function of a certain group of immune cells, but also to explore cells' diversity, and accurately analyze the cells' specific phenotype and role in different liver diseases, different stages of the same disease, and different HIME caused by different pathogenic factors stimulus. Recently, single-cell sequencing can clarify the specific role of single cells. If this detection method is applied to the investigation of the immune microenvironment of hepatic fibrosis, it may greatly enrich the existing immune network and provide comprehensive and accurate ideas for the study of hepatic fibrosis. Additionally, the specific mechanisms for the immune cells of a specific phenotype to play different roles at different stages of chronic liver disease need to be elucidated, so that a precise intervention can be performed. However, the study on specific mechanisms is currently a major vacancy for the effect of HIME on hepatic fibrosis, thus, future studies need to focus on a deeper and more specific regulatory pathway to achieve an accurate intervention target, thereby delaying or avoiding the occurrence and progression of hepatic fibrosis.

Author contributions

NZ, HY, and ZZ conceived of the subject matter and wrote the logic of this review, searched, categorized, and summarized all relevant

literature, and wrote the initial draft. ZL, XC, YZ, and RJ helped to check relevant information check the content of the initial draft. JH and HP edited the second draft and composed the manuscript. XL and YL reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Modulating sphingosine 1-phosphate receptor signaling skews intrahepatic leukocytes and attenuates murine nonalcoholic steatohepatitis

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Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid associated with nonalcoholic steatohepatitis (NASH). Immune cell-driven inflammation is a key determinant of NASH progression. Macrophages, monocytes, NK cells, T cells, NKT cells, and B cells variably express S1P receptors from a repertoire of 5 receptors termed $S1P_1$ – $S1P_5$. We have previously demonstrated that non-specific S1P receptor antagonism ameliorates NASH and attenuates hepatic macrophage accumulation. However, the effect of S1P receptor antagonism on additional immune cell populations in NASH remains unknown. We hypothesized that S1P receptor specific modulation may ameliorate NASH by altering leukocyte recruitment. A murine NASH model was established by dietary feeding of C57BL/6 male mice with a diet high in fructose, saturated fat, and cholesterol (FFC) for 24 weeks. In the last 4 weeks of dietary feeding, the mice received the $S1P_{1,4,5}$ modulator Etrasimod or the $S1P_1$ modulator Amiselimod, daily by oral gavage. Liver injury and inflammation were determined by histological and gene expression analyses. Intrahepatic leukocyte populations were analyzed by flow cytometry, immunohistochemistry, and mRNA expression. Alanine aminotransferase, a sensitive circulating marker for liver injury, was reduced in response to Etrasimod and Amiselimod treatment. Liver histology showed a reduction in inflammatory foci in Etrasimod-treated mice. Etrasimod treatment substantially altered the intrahepatic leukocyte populations through a reduction in the frequency of T cells, B cells, and NKT cells and a proportional increase in $CD11b^+$ myeloid cells, polymorphonuclear cells, and double negative T cells in FFC-fed and control standard chow diet (CD)-fed mice. In contrast, FFC-fed Amiselimod-treated mice showed no changes in the frequencies of intrahepatic leukocytes. Consistent with the improvement in liver injury and inflammation, hepatic macrophage accumulation and the gene expression of proinflammatory markers such as *Lgals3* and *Mcp-1* were

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decreased in Etrasimod-treated FFC-fed mice. Etrasimod treated mouse livers demonstrated an increase in non-inflammatory (*Marco*) and lipid associated (*Trem2*) macrophage markers. Thus, $S1P_{1,4,5}$ modulation by Etrasimod is more effective than $S1P_1$ antagonism by Amiselimod, at the dose tested, in ameliorating NASH, likely due to the alteration of leukocyte trafficking and recruitment. Etrasimod treatment results in a substantial attenuation of liver injury and inflammation in murine NASH.

KEYWORDS

lipotoxicity, fatty liver, sphingolipids, Etrasimod, Amiselimod

1 Introduction

The most common chronic liver disease in the United States, nonalcoholic fatty liver disease (NAFLD) is a heterogenous group of disorders whose spectrum ranges from isolated steatosis to nonalcoholic steatohepatitis (NASH) (1). Lipid accumulation is the hallmark of isolated steatosis, whereas NASH is a lipotoxic disorder histologically characterized by steatosis, lobular inflammation and hepatocellular ballooning, which all together lead to increased fibrosis risk (2). The histological prominence of steatosis coupled with lipidomic abnormalities noted in NASH and mechanistic studies in lipotoxic model systems have suggested a role for bioactive lipids in NASH pathogenesis, including sphingolipids (3, 4). These bioactive lipids can be generated due to activation of the hepatocellular endoplasmic reticulum stress response leading to *de novo* sphingolipid synthesis and ensuing release of sphingosine 1-phosphate (S1P) enriched proinflammatory extracellular vesicles (3). We have shown that S1P-enriched extracellular vesicles mediate the recruitment of proinflammatory monocyte-derived macrophages into the liver in NASH (5). However, several immune cell subsets are perturbed during NASH progression and the broader effects of inhibition of S1P signaling on such additional immune cells remain unknown.

S1P is formed from the hydrolysis of ceramide to generate sphingosine which is then phosphorylated by either sphingosine kinase 1 or 2 to form S1P (6). S1P binds five subtypes of G-protein couple receptors (GPCRs), namely $S1P_1$ – $S1P_5$. S1P receptors transduce intracellular signals to mediate diverse cellular responses including migration, differentiation, proliferation, lymphocyte circulation, as well as T and B cell trafficking (7–9). S1P receptors are widely expressed across various immune cell types (7). In our previous study using a dietary NASH mouse model, we found that treatment with FTY720, an $S1P_{1,\ 3,\ 4,\ 5}$ functional antagonist, ameliorates the cardinal features of NASH (10). Here we utilized Etrasimod, a fully functional antagonist of mouse and human $S1P_1$ and partial $S1P_4$ and $S1P_5$ antagonist (11), and Amiselimod, a functional $S1P_1$ antagonist (12), to examine the effects of S1P receptor pharmacological inhibition on the intrahepatic leukocyte populations associated with NASH. Additionally, we compared the effects of Etrasimod to Amiselimod to determine if $S1P_1$ receptor antagonism was sufficient to attenuate NASH (13). We report that selective modulation of S1P receptors 1, 4, and 5 through Etrasimod is more effective than $S1P_1$ modulation by Amiselimod in improving NASH in a mouse model. Etrasimod reduces the accumulation of macrophages in the liver and consequently reduces the infiltration of inflammatory cells including T cells, B cells and NKT cells, leading to attenuation of liver injury and inflammation.

2 Materials and methods

2.1 Mouse studies

Animal use was approved by the institutional care and animal use committee (IACUC) of the Mayo Clinic and conducted in accordance with the public health policy on the humane use and care of laboratory animals. C57BL/6J male mice were purchased from Jackson laboratory (Bar Harbor, Maine) at 6 weeks of age. When 12 weeks old, mice were randomized to receive either a diet high in saturated fat, fructose, and cholesterol (FFC, AIN-76A Western Diet, Test Diets 5342) or standard rodent chow diet (CD) for 24 weeks. As previously described by us, at 24 weeks of feeding, this diet recapitulates cardinal features of human NASH including histologic parameters of steatosis, inflammation, ballooning, fibrosis and metabolic parameters of obesity and insulin resistance (14). Mice had unrestricted access to food and water, and were housed in standard pathogen-free facilities, with 12-hour day-night circadian cycles. Twenty weeks into the feeding

Abbreviations: Alanine transaminase, ALT; Amiselimod, Ami; Bone Marrow-Derived Macrophages, BMDM; chow diet, CD; Dimethyl sulfoxide, DMSO; Etrasimod, Etra; extracellular vesicles, EVs; high fat, fructose and cholesterol, FFC; hepatocyte nuclear factor 4 alpha, HNF-4α; nonalcoholic fatty liver disease, NAFLD; nonalcoholic steatohepatitis, NASH; natural killer T cells, NKT cells; primary mouse hepatocytes, PMH; polymorphonuclear cells, PMNs; sphingosine 1-phosphate, S1P; sphingosine 1-phosphate receptor 1, $S1P_1$, sphingosine 1-phosphate receptor 2, $S1P_2$; sphingosine 1-phosphate receptor 3, $S1P_3$; sphingosine 1-phosphate receptor 4, $S1P_4$; sphingosine 1-phosphate receptor 5, $S1P_5$.

study mice started receiving drug or vehicle by daily oral gavage for 1 month. The drugs employed were Etrasimod (Cayman Chemical, 21661) or Amiselimod hydrochloride (Cayman Chemical, 20970). Etrasimod was dissolved at 20 mg/mL in DMSO and then diluted to 1.44 mg/mL in 0.5% sodium methylcellulose and administered to mice at a dose of 3 mg/Kg. Amiselimod was dissolved in DMSO at a concentration of 20 mg/mL and then diluted to 1 mg/mL in 0.5% sodium methylcellulose and administered to mice at a dose of 2 mg/Kg. A matched volume of DMSO, similarly diluted in 0.5% sodium methylcellulose, served as the vehicle. Each treatment group (Etrasimod or Amiselimod) had its own corresponding vehicle cohort. Upon completion of the study, liver and plasma were collected after euthanasia. Prior to liver excision, 10 mL PBS was flushed through the liver via inferior vena cava (IVC) cannulation in a retrograde fashion. Then the liver was excised, weighed, and divided for downstream analyses. Approximately 1g of liver tissue per mouse was processed for flow cytometry, described below, and the rest divided for several assays including: cryopreservation (snap frozen in liquid nitrogen) for RNA extraction and protein extraction, fixation in 10% neutral buffered formalin for paraffin embedding, and cryo-sectioning for histological analysis. Platelet poor plasma was isolated from citrated blood (described below) and stored at -20°CC till further analyses. Archived flash frozen, in liquid nitrogen, liver tissue of C57BL/6J wild type mice fed chow or FFC diet for 24 weeks were employed for RNA isolation and quantification of whole liver $S1P_{1-5}$ expression (15).

2.2 Cell culture studies

Bone marrow-derived macrophages (BMDM) were isolated from the hind legs of untreated wild type C57BL/6 mice as previously described by us (10). BMDM media was changed every 2 days and on day 7 differentiated macrophages were dissociated with Accutase (Invitrogen, 00-4555-56) for use in experiments. After dissociation, BMDMs were plated in two 100 mm petri dishes and were allowed to attach for 16 hours. Next, BMDMs were serum starved for 2 hours after which they were treated with palmitate (400 μM) complexed to 1% bovine serum albumin and volume-matched vehicle (isopropanol) for 8 hours as described by us (16). Cells were lysed in TRIzol reagent (Invitrogen, 15596026) and lysates were stored at -80°C for RNA extraction. Primary mouse hepatocytes (PMH) were isolated based on standardized techniques as described previously (17). PMHs were treated with palmitate (400 μM) and volume-matched vehicle (isopropanol) for 8 hours and collected in TRIzol reagent and stored at -80°C for RNA extraction.

2.3 Glucose tolerance testing

Intraperitoneal glucose tolerance test (GTT) was performed on mice in both CD and FFC-fed cohorts at 22 weeks on diet. Mice were fasted overnight for 16 hours and injected with 20% glucose intraperitoneally at a dose of 2 mg/Kg body mass. Blood glucose values were acquired before injection and 15, 30, 45, 60, 90, 120, and 150 minutes after injection. Tail vein blood sampling was done using AssurePlatinum blood glucose meter and test strips (Arkray, 67841996). The area under the glucose tolerance curve was calculated in Microsoft Excel and glucose disposal capacity was compared between groups.

2.4 Histology and immunohistochemistry

Five μm sections of formalin-fixed, paraffin-embedded (FFPE) liver tissues were stained with hematoxylin and eosin (H&E) using standard techniques at the Mayo Clinic Histology Core. Histology was observed and H&E slides were graded for steatosis and inflammation using the NAFLD activity score. Immunohistochemistry for Mac-2/Galectin-3 (Invitrogen, 14-5301-85) and Ki67 (Abcam, ab16667) was performed to assess macrophage accumulation and hepatocyte proliferation respectively. Five μm FFPE liver sections were dewaxed with xylene and rehydrated through graded 100%, 95%, 70%, and 50% ethanol sequentially. Slides were then immersed in 10 mM sodium citrate buffer with 0.05% Tween-20 (pH 6.0) for 20 minutes at 95°C for epitope retrieval. The slides were allowed to cool on the benchtop to room temperature. After cooling, endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 10 minutes. For Mac-2 staining, non-specific immunoglobulin binding was blocked using the Rat-IgG ABC Kit (Vector Laboratories, PK 4004) time for 30 minutes, followed by the Rodent Block M solution (Biocare Medical, RBM 961). For Ki67 blocking was performed using Rabbit-IgG ABC Kit (Vector Laboratories, PK 4001) for 30 minutes. All slides were further blocked with avidin and biotin blocking solution for 15 minutes each using the Avidin/Biotin Blocking Kit (Vector Laboratories, SP 2001). Primary antibodies for Mac-2 (1:200 dilution) and Ki67 (1:200 dilution) were applied and incubated overnight at 4°C in humidified chambers. Slides were washed and incubated for 30 minutes each in biotinylated secondary antibody (1:500 dilution) and ABC reagent from the ABC Kit per manufacturer's instructions. Signals were visualized using the ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories, SK 4105), and counterstained with Nuclear Fast Red Solution for Mac-2 (MilliporeSigma, N 8002) for 20 minutes and with Hematoxylin for Ki67 for 30 seconds. Sections were dehydrated through graded ethanol and mounted using Organo/Limonene Mount (Santa Cruz, SC-45087). The positive areas were quantified using the NIS-Elements imaging software (Nikon) on a Nikon microscope with a DS-U3 camera (Nikon, Japan) on the 20x objective lens. Images with preserved settings for light and exposure were used during image analysis and area quantification for Mac-2. Ten high power fields (20x objective lens) were manually counted for nuclear positivity for quantification of Ki67 signal and expressed as average positive nuclei per high power field. Immunohistochemistry for hepatocyte nuclear factor 4 alpha (HNF4α) (Abcam, ab201460) was performed to assess hepatic differentiation as above except for antigen retrieval, which was performed by immersing slides in Tris-EDTA Buffer with 0.05% Tween-20 (pH 9.0) for 30 minutes at 95°C. Non-specific immunoglobulin binding was blocked using the

Rabbit-IgG ABC Kit for 1 hour. Primary antibody for HNF4α (1:250 dilution) was applied and incubated overnight at 4°C in humidified chambers. Representative images were captures with the 10x objective lens.

2.5 Flow cytometry

Mouse intrahepatic leukocytes were isolated according to the published method of Blom et al. (18). Following euthanasia mouse livers were perfused with 10 mL PBS in a retrograde manner via IVC. The perfused livers were dissected out of the abdomen and the gall bladder was removed. Next, the livers were disrupted using a gentleMACS tissue dissociator in 5 mL RPMI-1640 per manufacturer's recommended preset program for 45 seconds. The dissociate was transferred to a 50 mL conical tube and RPMI-1640 was added to a volume of 40 mL. This suspension was centrifuged at 60 x g for 1 minute at room temperature and the supernatant containing hepatic immune cells was collected and centrifuged again at 480 x g for 8 minutes at room temperature. The pellet was resuspended in 10 mL of 37.5% Percoll (MilliporeSigma, P1644-1L) and centrifuged at 850 x g for 30 minutes at room temperature with no brake. The supernatant was aspirated, and the pellet was resuspended in 5 mL of red blood cell lysis buffer for 5 minutes on ice. Lysis buffer was neutralized by adding 10 mL PBS, and the remaining cells were pelleted down by centrifugation. The cells were resuspended in 200 µL of PBS and counted using the Muse® Cell Analyzer (MilliporeSigma). Approximately 1 million cells were transferred to FACS tubes and stained with 1:200 zombie aqua (Biolegend) to discriminate between viable and dead cells. Cells were washed with cell staining buffer (Biolegend) before blocking non-specific binding with TruStain FcX Plus (Biolegend). Staining for cell surface markers was performed with fluorophore-conjugated primary antibodies; CD45.2 (AF700, clone 104, Biolegend), CD3 (BUV737, clone 145-2C11, BD Bioscience), NK1.1 (APC, clone PK136, Biolegend), CD4 (BUV737, clone RM4-5, BD Bioscience), CD8a (BUV805, clone 53-6.7, BD Bioscience), B220 (BV421, clone RA3-6B2, Biolegend), CD19 (BV785, clone 6D5, Biolegend), CD11c (BV711, clone N418, Biolegend), CD11b (PE/Cy7, clone M1/70, Biolegend), and Ly6G (PE, clone 1A8, Biolegend) for 30 minutes at 4°C. Flow cytometry data was acquired on BD Fortessa X-20 and Fortessa X-30 H0081 (BD Bioscience) cytometers and analyzed using FlowJo™ Software version 10.6.1 (Becton, Dickinson & Company).

2.6 RNA purification and quantitative real time PCR

Cryopreserved mouse liver tissues of vehicle and treatment groups for both diets were recovered and homogenized in TRIzol reagent. Tissue debris was centrifuged down and the supernatant was transferred into separate Eppendorf tubes. Total RNA was extracted using the Direct-zol RNA Zymo Plus Mini Prep (Zymo Research, R 2070), then the RNA yield and quality was assessed using a NanoDrop ND1000 (ThermoScientific, Waltham, MA). 1 µg of total RNA was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, 1708891). Gene expression was analyzed using quantitative real time PCR (qPCR) with gene specific mouse primers (Table 1). qPCR was performed

TABLE 1 Mouse qPCR Primers.

Gene	Forward Primer	Reverse Primer
18S	CGCTTCCTTACCTGGTTGAT	GAGCGACCAAAGGAACCATA
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Cd68	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
Lgals3	TGGGCACAGTGAAACCCAAC	TCCTGCTTCGTGTTACACACA
Ly6c	GCAGTGCTACGAGTGCTATGG	ACTGACGGGTCTTTAGTTTCCTT
Tnfa	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Timd4	AGAATGTGCGCTTGGAGCTGAG	GGTTGGGAGAACAGATGTGGTC
Marco	ACAGAGCCGATTTTGACCAAG	CAGCAGTGCAGTACCTGCC
Trem2	CTGGAACCGTCACCATCACTC	CGAAACTCGATGACTCCTCGG
Mcp1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
S1pr1	ATGGTGTCCACTAGCATCCC	CGATGTTCAACTTGCCTGTGTAG
S1pr2	ATGGGCGGCTTATACTCAGAG	GCGCAGCACAAGATGATGAT
S1pr3	ACTCTCCGGGAACATTACGAT	CAAGACGATGAAGCTACAGGTG
S1pr4	GTCAGGGACTCGTACCTTCCA	GATGCAGCCATACACACGG
S1pr5	GCTTTGGTTTGCGCGTGAG	GGCGTCCTAAGCAGTTCCAG

using the LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics, 04707516001)), and run on LightCycler 480 (Roche). Each dataset of the target gene of interest was normalized to the geometric mean of the reference genes Hprt and 18S. All datasets from drug treated mice are expressed as fold change relative to vehicle controls in the CD group. For isolated cells, the data represent fold change compared to vehicle controls.

2.7 Biochemical assays

Platelet poor plasma was isolated by first centrifuging 500-800 µL of citrated blood for 20 minutes at 4,000 x g. The supernatant was transferred to a new Eppendorf tube and spun down at 12,000 rpm for 2 minutes. The clear supernatant was then aliquoted to new tubes for analysis or storage. Approximately 100 µL of plasma was used for analysis of alanine aminotransferase (ALT) levels using VetScan VS2 (Abaxis, Union City, CA).

2.8 Statistical and data analyses

Graphs and statistical analyses were done using GraphPad Prism version 9 for Windows (GraphPad Software, La Jolla, CA; www.graphpad.com). Data are presented as mean ± S.E.M in each graph. Each dot in the dot plot represents one mouse or biological replicate. The Grubb's test (extreme studentized deviant method) was used to identify outliers to determine if the most extreme value in a data set is a significant outlier from the rest. For statistical comparisons of multiple experimental groups against the control group, parametric data were analyzed by ANOVA and the Tukey's Multiple Comparison method was employed to determine which pairs of means are different. A p-value of < 0.05 was considered significant.

3 Results

3.1 Etrasimod and Amiselimod treatment reduce liver injury in NASH

A dietary NASH mouse model was generated by feeding mice a FFC diet for 24 weeks. Next, both CD (control) and FFC-fed groups were randomized to receive Etrasimod (3 mg/Kg) or vehicle, or Amiselimod (2 mg/Kg) or vehicle treatment based on effective doses reported in the literature (Figure 1A) (12, 13). The liver histology from FFC-fed mice showed a significant increase in steatosis and inflammation compared to those mice that received CD (Figure 1B). Steatosis and inflammatory foci were quantified using the NAFLD activity score, which showed that Etrasimod treatment did not reduce steatosis in FFC-fed mice (Figure 1C). Similarly, Amiselimod treatment did not impact steatosis (Supplementary Figures 1A, B). On the other hand, Etrasimod treatment significantly reduced inflammatory foci on histological examination of the FFC group (Figure 1D). Correspondingly, the ALT level was reduced with Etrasimod treatment in the FFC-fed group (Figure 1E), demonstrating improvement in liver inflammation and injury in FFC-fed mice. In contrast, Amiselimod treatment did not reduce inflammatory foci though lowered ALT levels (Supplementary Figures 1C, D).

3.2 Metabolic parameters are unchanged in mice treated with Etrasimod or Amiselimod

CD-fed and FFC-fed mice maintained comparable body mass, liver mass and relative liver mass (Figures 2A–C, Supplementary Figures 2A–C), regardless of Etrasimod or Amiselimod treatment, confirming that the improvement in liver injury and inflammation were not due to do a lack of weight gain. Etrasimod administration did not lower the FFC diet-induced increase of cholesterol (Figure 2D). There was a trend toward increase in fasting blood sugar in vehicle-treated FFC-fed mice; however, levels were similar to Etrasimod-treated FFC-fed mice (Figure 2E). Neither FFC-diet induced hypercholesterolemia nor elevated fasting blood sugars were lowered following Amiselimod administration (Supplementary Figures 2D, E). FFC-fed Etrasimod and Amiselimod treated mice demonstrated comparable glucose disposal to vehicle treated mice following intraperitoneal glucose challenge (Figure 2F, Supplementary Figure 2F).

3.3 Etrasimod treatment leads to changes in intrahepatic leukocyte populations

We next examined the changes in intrahepatic leukocyte populations in FFC-fed mice treated with Etrasimod or Amiselimod by flow cytometry of the intrahepatic leukocyte populations. After isolation, viable cells were selected for further analysis (Supplementary Figure 3). Our flow cytometry panel was designed to characterize the major immune cell populations within the liver (Figure 3). As expected, the FFC-diet led to an increase in the number of total $CD45^+$ cells (Figure 4A), T cells, and NKT cells (Figures 4B, C) in the liver. Etrasimod treatment led to a reduction in the frequency of T and B cells in the liver of CD- and FFC-fed mice (Figures 4B, C). $CD3^+$ $NK1.1^+$ NKT cells were also reduced in FFC-fed mice (Figures 4B, C), though they remained unchanged in CD-fed mice. Further analysis of T-cell subsets demonstrated no change in $CD4^+$ T cells in FFC-fed mice compared to CD-fed mice, though $CD4^+$ T cells were reduced by Etrasimod in both CD-fed and FFC-fed mice (Figure 4D). $CD8^+$ T cells were increased in FFC-fed mice in comparison with CD-fed mice and were decreased following Etrasimod administration. We observed an increase in double negative (DN) T cells in FFC-fed mice which were unchanged following Etrasimod administration in FFC-fed mice, though reduced by Etrasimod in CD-fed mice. Etrasimod increased the frequency and percentage of NK cells in the liver in CD-fed mice, but not in FFC-fed mice (Figures 4E, F).

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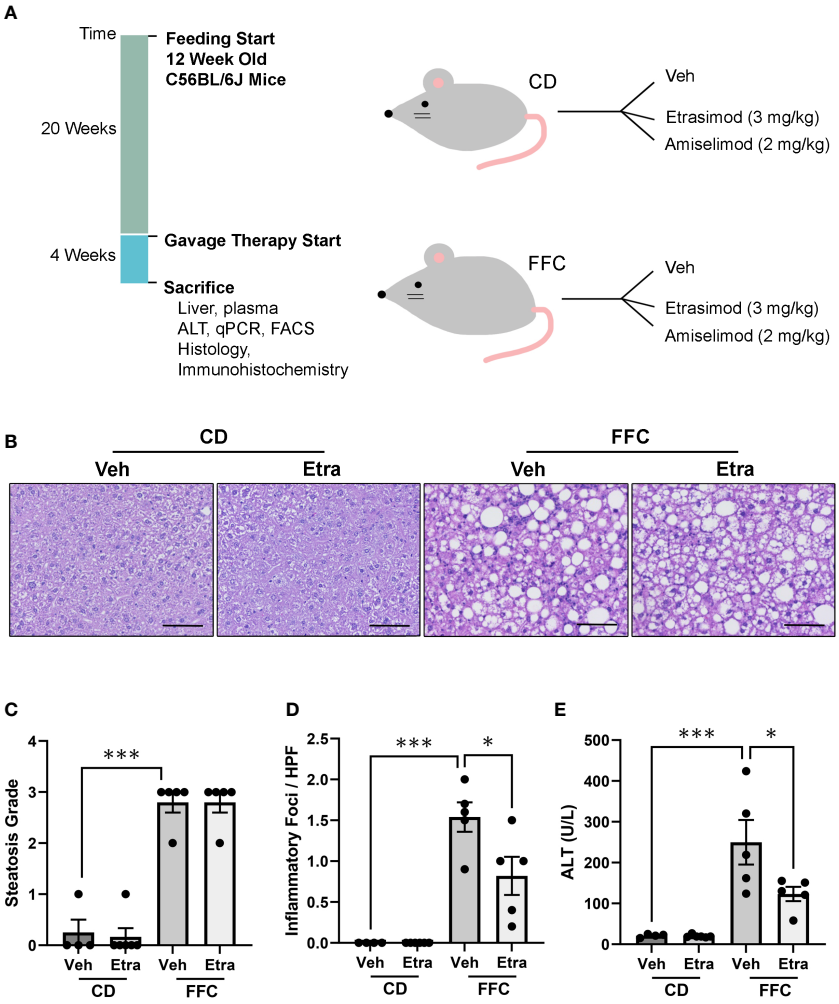

FIGURE 1
Etrasimod treatment reduces liver injury. **(A)** Timeline of mouse study and analysis after euthanasia. Dietary feeding started 6 weeks after acquisition of C57BL/6J male mice at 12 weeks of age. Mice were randomized to receive standard CD or FFC diet for 24 weeks. At 20 weeks of feeding, CD and FFC cohorts received vehicle or Etrasimod and vehicle or Amiselimod drug treatment for 4 weeks. Mice were euthanized at the end of gavage therapy and processed for analysis. **(B)** Representative liver histology shown by Hematoxylin and Eosin (H&E) staining of vehicle or Etrasimod treated mice in CD and FFC cohorts. Scale bar equals 50 µm. **(C)** Steatosis component of the NAFLD Activity Score for mouse cohorts treated with vehicle or Etrasimod. Each mouse is graded for steatosis (0–3). Less than 5% steatosis = 0, 5~33% = 1, 34~66% = 2, >66% = 3. Each dot represents one biological replicate. **(D)** The inflammatory (0-3) component score in NAS grading is shown for CD and FFC cohorts treated with vehicle or Etrasimod. If there are no inflammatory foci = 0, <2 inflammatory foci = 1, 2~4 inflammatory foci = 2, >4 inflammatory foci = 3. **(E)** Plasma ALT levels for CD and FFC cohorts treated with vehicle or Etrasimod at completion of the study. CD (n=10) and FFC (n=10). *p<0.05, ***p<0.001.

The frequency and percentage of CD11b$^+$ cells, which includes myeloid cells and polymorphonuclear cells (PMNs), were increased in both CD-fed and FFC-fed Etrasimod-treated mice. In contrast, the total number of CD45$^+$ cells in the liver were unaffected in either CD-fed or FFC-fed mice treated with Amiselimod (Supplementary Figure 4A). Intrahepatic leukocyte populations in Amiselimod treated CD-fed mice had changes similar to Etrasimod with a reduction in T cells and B cells and an increase in CD11b$^+$ myeloid cells; however, these effects were more modest in FFC-fed mice suggesting that, at the dose tested, Amiselimod was less effective than Etrasimod in modulating the distribution of immune cells under conditions of nutrient excess (Supplementary Figure 4). Though T cells were not reduced in FFC-fed mice treated with Amiselimod, the T cell subsets were altered like Etrasimod.

3.4 Macrophage accumulation and expression of proinflammatory markers are reduced with drug treatment

NASH is characterized by intrahepatic macrophage accumulation. As our flow cytometry panel did not include markers to differentiate between infiltrating monocyte-derived and tissue-resident macrophages, we assessed their accumulation and activation using immunohistochemistry and gene expression

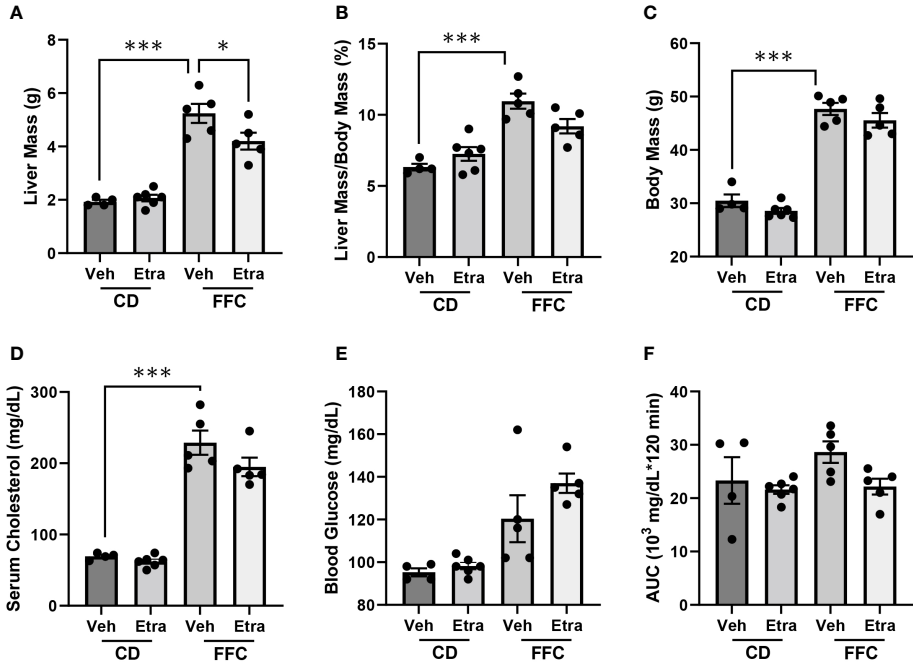

FIGURE 2
Metabolic characterization of CD and FFC-fed mice with Etrasimod treatment. **(A)** Liver mass, **(B)** liver to body mass ratio, **(C)** and body mass of groups treated with vehicle or Etrasimod. **(D)** Total cholesterol and **(E)** fasting blood glucose with vehicle or Etrasimod treatment. **(F)** Area under the curve (AUC) for the glucose tolerance test at 22 weeks on diet and 2 weeks of Etrasimod treatment. CD (n=10) and FFC (n=10). *p<0.05, ***p<0.001.

analysis. Immunohistochemistry for Mac-2/Galectin-3 in liver sections showed that activated macrophage accumulation was visibly reduced in livers of both Etrasimod and Amiselimod treated mice (Figure 5A, Supplementary Figure 5A). Quantification of *Mac-2/Galectin-3* positive areas confirmed significant decrease in macrophage accumulation in Etrasimod (Figure 5B) and Amiselimod (Supplementary Figure 5B) treated mice. There was also a significant reduction in relative mRNA expression of macrophage markers *CD68* and *Lgals3* (Figures 5C, D). Similar changes were observed in *Ly6c*, a marker of proinflammatory monocytes, though this did not achieve statistical significance (Figure 5E). By extension, we asked whether drug treatment had an effect on levels of proinflammatory chemokines and cytokines in the liver. Relative mRNA expression of monocyte chemotactic protein 1 (*Mcp1*), tumor necrosis factor alpha (*Tnfa)* and interleukin 1 beta (*Il1b)* were significantly reduced with both Etrasimod (Figures 5F–H) and Amiselimod (Supplementary Figures 5F–H) treatment in FFC mice. Thus, Etrasimod and Amiselimod treatment not only reduced inflammatory macrophage accumulation in the liver, but also reduced levels of proinflammatory cytokines that aggravate the inflammatory response. In Etrasimod treated FFC-fed livers the resident macrophage marker *Timd4* (Figure 5I) was not upregulated, unlike Amiselimod (Supplementary Figure 5I). We also observed an increase in *Marco*, a marker of non-inflammatory macrophage populations (Figure 5J, Supplementary Figure 5J) and the lipid associated macrophage marker *Trem2* (Figure 5K, Supplementary Figure 5K). These data suggest that the increase in CD11b observed by flow cytometry may be due to an increase in the abundance of resident and non-inflammatory macrophages.

3.5 Palmitate treatment induces the expression of $S1P_1$ and $S1P_2$ in macrophages

To understand whether the differential effects of Etrasimod and Amiselimod may be mediated by variations in the expression of S1P receptors, the reported expression of S1P receptors in hepatocytes, bone marrow monocytes and macrophages was compared from a publically available single cell RNA sequencing data (19) (https://www.czbiohub.org/tabula-muris/), Table 2. In this dataset $S1P_4$ mRNA was most abundant in macrophages and monocytes, and $S1P_1$ was most abundant in hepatocytes. Next, BMDM and PMH were treated with palmitate to induce lipotoxic stress (10). In BMDMs, the expression of $S1P_1$ and $S1P_2$ mRNA was induced following palmitate treatment, whereas the abundance of $S1P_4$ remained unchanged (Figure 6A). $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ were detected in hepatocytes and remained unchanged with palmitate treatment (Figure 6B). In contrast to isolated cells, analysis of the abundance of S1P receptors in whole livers demonstrated no change in $S1PR_1$ and $S1PR_3$, upregulation of $S1PR_2$, and downregulation of $S1PR_5$ in mice fed the FFC diet in comparison to CD fed controls (Figure 6C). As $S1P_1$ was upregulated in BMDM by lipotoxic stress without any induction in PMH or whole livers, this may be the primary target on macrophages for the beneficial effects of Etrasmiod and Amiselimod. Further examination of hepatocellular proliferation demonstrated low basal Ki67 positivity, consistent with the literature (20). Ki67 positivity was increased in FFC-fed vehicle treated livers, as has been reported in mouse models of NASH (21, 22) and reduced following treatment with Etrasimod

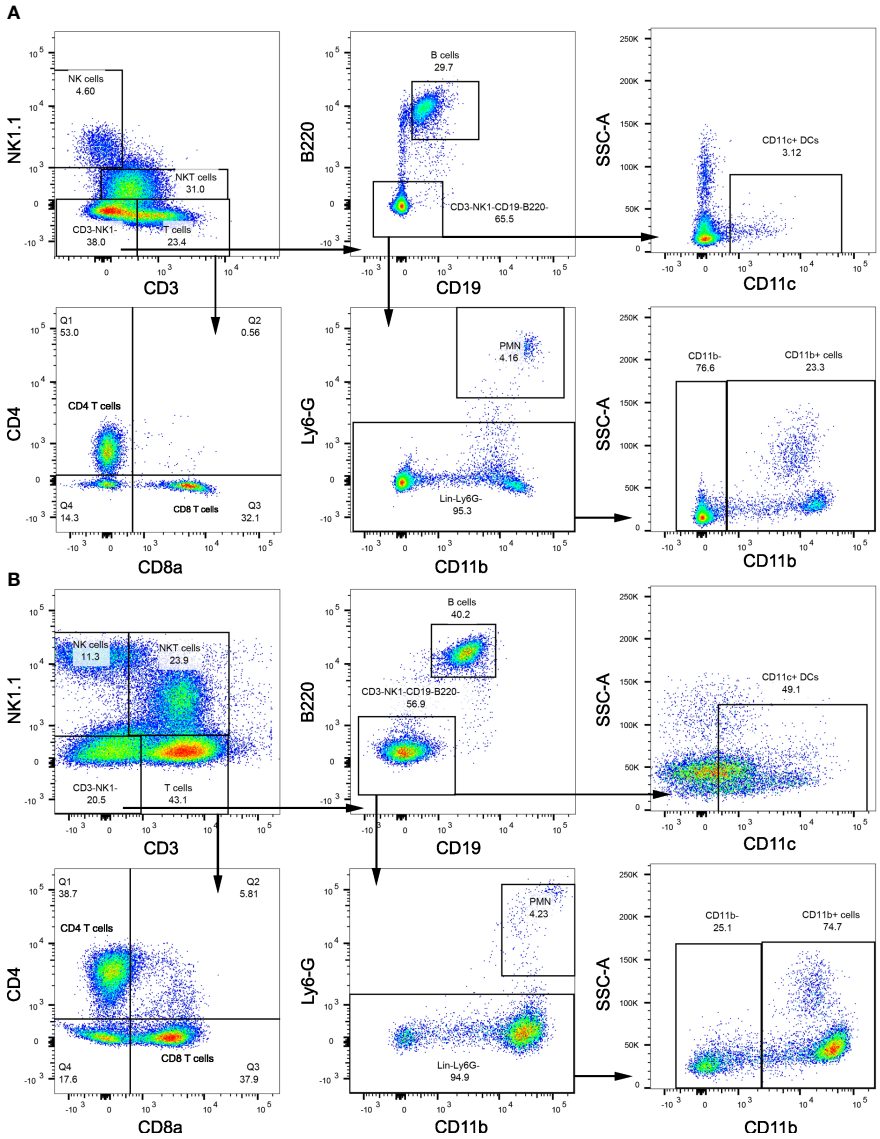

FIGURE 3
Flow cytometry gating strategy. **(A)** Flow cytometry gating strategy that is used to isolate immune populations within the liver of CD-fed mice and **(B)** FFC-fed mice. Immune populations were identified as follows: NK cells ($CD3^-NK1.1^+$), NKT cells ($CD3^+NK1.1^+$), B cells ($CD3^-NK1.1^-CD19^+B220^+$), DCs ($CD3^-NK1.1^-CD19^-B220^-CD11c^+$), PMNs ($CD11b^+Ly6G^-$), $CD11b^+$ myeloid cells ($CD11b^+$ $Ly6G^-$). T cells ($CD3^+NK1.1^-$) were selected for further analysis to identify CD4 T cells ($CD3^+NK1.1^-CD4^+CD8a^-$), CD8 T cells ($CD3^+NK1.1^-CD4^-CD8a^+$) and DNT cells ($CD3^+NK1.1^-CD4^-CD8a^-$).

and Amiselimod (Supplementary Figure 6). Hepatocellular expression of HNF4α was comparably reduced in vehicle treated and Etrasimod or Amiselimod treated FFC-fed mouse livers (Supplementary Figure 7).

4 Discussion

S1P is a bioactive sphingolipid with pleiotropic receptor-mediated signaling responses in diverse physiological and pathophysiological contexts. S1P dysregulation and subsequent mediation of lymphocyte trafficking is implicated in chronic sterile inflammatory diseases, including NASH. Here we have explored the therapeutic efficacy of Etrasimod, a selective S1P receptor 1, 4, and 5 modulator in comparison with Amiselimod, a selective S1P receptor 1 modulator, in attenuating murine NASH and associated intrahepatic leukocyte populations via antagonism of the S1P signaling axis. We report that: 1) S1P receptor antagonism attenuates liver injury, ii) Etrasimod reduces liver inflammatory infiltrates including T cells, B cells, and NKT cells, and iii) both drugs reduce inflammatory macrophage accumulation in a dietary model of murine NASH. These data establish a mechanistic link between S1P/S1P receptor signaling and the immune inflammatory response in NASH, as selective inhibition of S1P receptors demonstrated reversal of adverse features of NASH.

S1P is a signaling sphingolipid that participates in specific receptor signaling by acting on five GPCRs linked to intracellular

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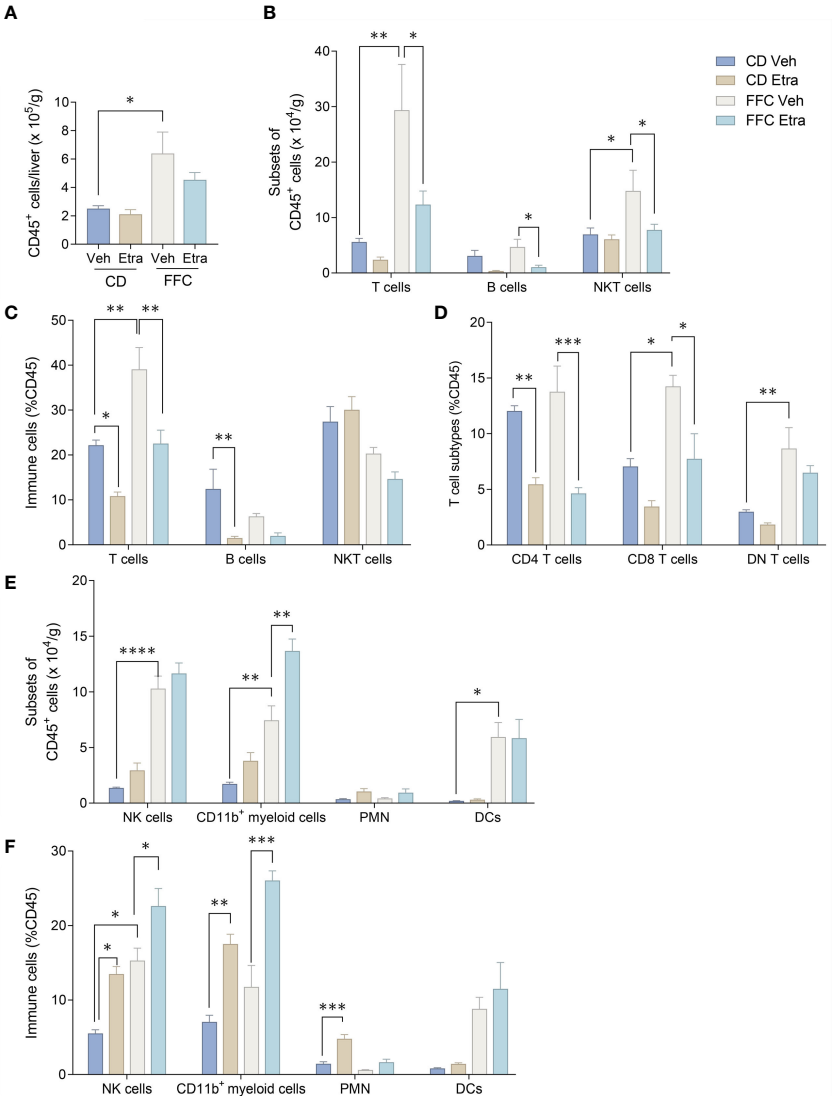

FIGURE 4
Etrasimod treatment alters intrahepatic leukocyte populations. **(A)** Number of CD45$^+$ cells per gram acquired for FACS analysis in CD and FFC cohorts. **(B)** Subsets of CD45$^+$ cells, **(C)** immune cell profiling expressed in %CD45 cells that are T cells, B cells and NKT cells by FACS analysis in livers from CD and FFC cohorts that received vehicle or Etrasimod treatment. **(D)** T-cell subtypes expressed in %CD45 cells that are CD4 positive, CD8 positive or double negative in CD and FFC cohorts. **(E)** Subsets of CD45$^+$ cells that are NK cells, CD11b$^+$ myeloid cells, PMNs and DCs. **(F)** Immune cells expressed in %CD45 that are NK cells, CD11b$^+$ myeloid cells, PMNs and DCs. CD (vehicle n=4, Etrasimod n=6) and FFC (vehicle n=5, Etrasimod n=5). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

signaling pathways (12). Dysregulation of S1P concentration is known to contribute to pathological conditions and inflammatory diseases including NASH (4). Mechanistically, toxic lipid species, such as palmitate, involved in hepatic lipotoxicity serve as precursors for downstream sphingolipids including S1P. In our previous study, we demonstrated that pharmacological treatment with Fingolimod (FTY720) effectively reversed murine NASH including reduction in ALT, inflammation, and hepatic inflammatory macrophage accumulation (10). We observed similar effects on liver injury with Etrasimod and Amiselimod. The contribution of various intrahepatic immune cell populations, including macrophages, T cells, and B cells, is being increasing appreciated in NASH (1). Having previously characterized liver injury and inflammation, our objective in this study was to examine changes in intrahepatic immune cells in NASH and how they are modified following inhibition of the S1P receptor-mediated signaling axis.

There is evidence to support that S1P receptor modulation impairs trafficking of macrophages, T cells, and B cells, all of which are implicated in NASH pathogenesis (1, 10, 23). Therefore, we examined the changes in intrahepatic leukocyte composition in mice treated with Etrasimod and Amiselimod. We found that Etrasimod treatment led to specific shifts in leukocyte populations including reduction in T cells, B cells, and NKT cells in FFC-fed mice and an increase in CD11b$^+$ myeloid cells and PMNs in both CD and FFC-fed mice. The reduction in T cell and B cell egress from lymphoid tissues is a well characterized effect of S1P antagonism, and our findings are consistent with this mechanism

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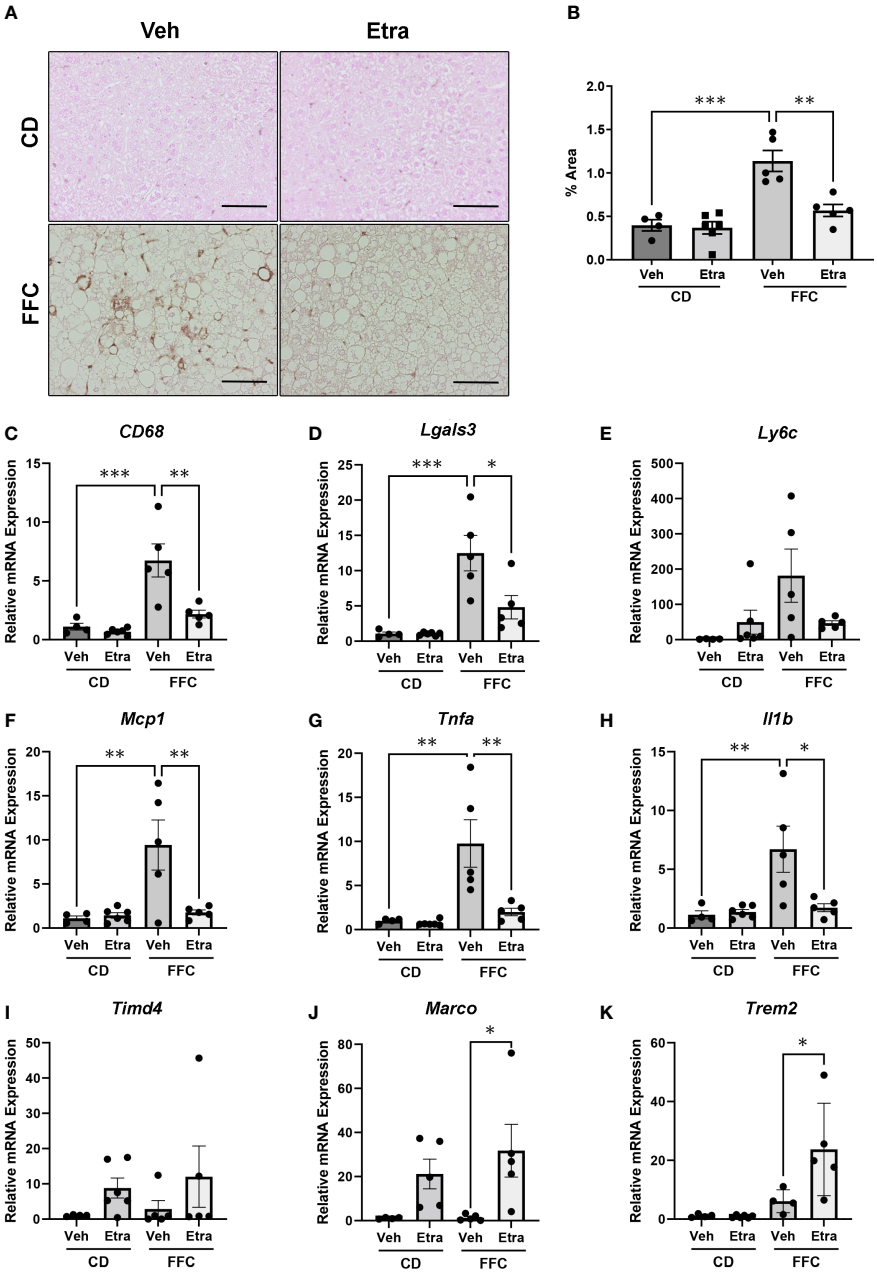

FIGURE 5
Macrophage accumulation and inflammatory markers are reduced in Etrasimod treated mice. **(A)** Representative images showing immunohistochemistry for *Mac-2* in liver tissue sections for vehicle and Etrasimod treated mice in both CD and FFC cohorts. Scale bar equals 50 µm. **(B)** Mac-2 staining was quantified as percentage of positive immunoreactive area in CD and FFC-fed mice treated with vehicle or Etrasimod. **(C–E)** Relative mRNA expression of *Cd68, Lgals3* and *Ly6c* in CD and FFC cohorts treated with vehicle or Etrasimod. **(F–H)** Relative mRNA expression of monocyte chemoattractant protein-1 *(Mcp1)*, tumor necrosis factor alpha (*Tnfa)* and interleukin-1 beta (*Il1b)* in liver tissues of CD and FFC-fed mice treated with vehicle or Etrasimod. **(I–K)** Relative mRNA expression of *Timd4, Marco* and *Trem2* in CD and FFC cohorts treated with vehicle or Etrasimod. CD (vehicle n=4, Etrasimod n=6) and FFC (vehicle n=5, Etrasimod n=5). *p<0.05, **p<0.01, ***p<0.001.

of action (24, 25). In contrast Amiselimod induced similar changes in CD-fed mice; however, this effect was prevented in mice with dietary NASH suggesting perhaps that Amiselimod was less effective under conditions of nutrient stress. These data suggest that Etrasimod, which antagonizes 1, 4, and 5, versus Amiselimod which antagonizes S1P receptor 1 alone, was more effective likely due to effects on multiple proinflammatory immune cell types, though we cannot exclude a dose-dependent effect. DNT cells are a subpopulation of regulatory T cells known to suppress immune responses in both mice and humans (26). The increase in DNT cells may contribute to the suppressive properties of Etrasimod in the immune response of NASH. The specific contributions and interrelatedness of these cell types will require further investigation aided by the incorporation of lineage specific knockout mice, for example, macrophage-specific, T cell-specific, and B cell-specific knockouts. Nonimmune cells such as

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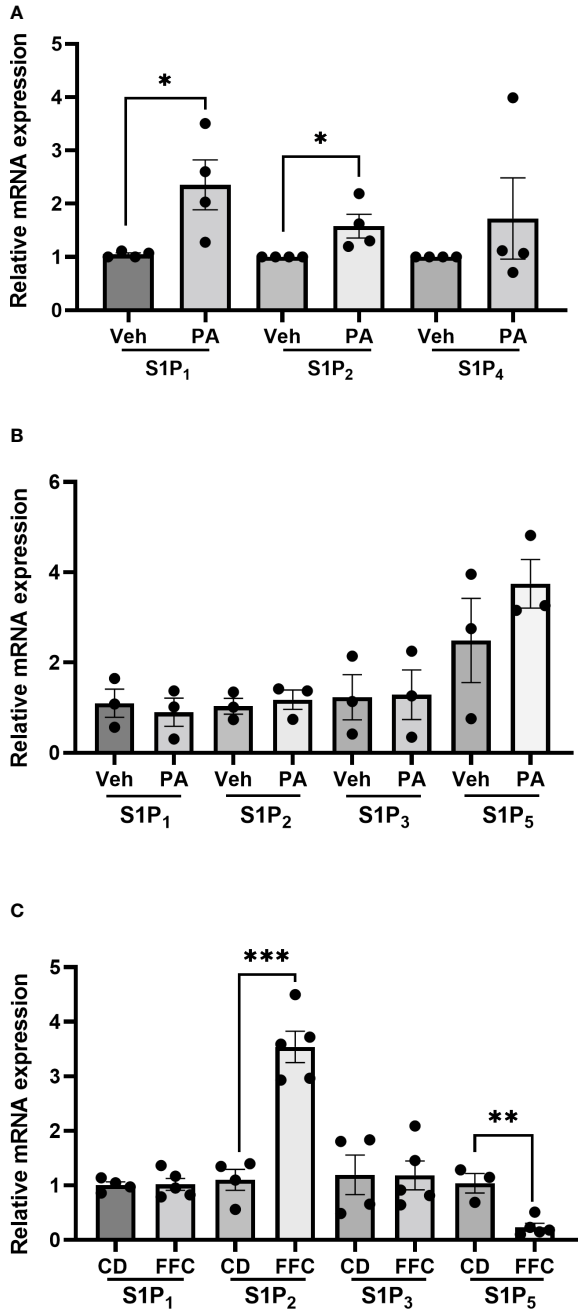

FIGURE 6
$S1P_1$ and S1P2 are upregulated in palmitate-treated BMDM. **(A)** In BMDM, expression of $S1P_1$ and $S1P_2$ mRNA was induced following palmitate treatment whereas the abundance of $S1P_4$ remained unchanged (vehicle n=4, palmitate n=4). **(B)** In PMH, $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ were detected and remained unchanged with palmitate treatment (vehicle n=3, palmitate n=3). **(C)** In whole livers, $S1PR_2$ was upregulated, $S1PR_5$ was downregulated, and no change was noted in $S1PR_1$ and $S1PR_3$ in mice fed the FFC diet in comparison to CD fed controls. CD (n=4) and FFC (n=5). *p<0.05, **p<0.01, ***p<0.001.

TABLE 2 $S1P_{1\text{-}5}$ Mean expression ln(1+CPM) in hepatocytes, macrophages, and monocytes.

S1P Receptors	Hepatocytes	Macrophage	Monocyte
$S1P_1$	2.44	0.18	0.17
$S1P_2$	0.31	0.01	0.59
$S1P_3$	0.01	0.04	0.04
$S1P_4$	0.02	2.45	1.94
$S1P_5$	0.30	0.01	0.29

(https://www.czbiohub.org/tabula-muris/).

hepatocytes and sinusoidal endothelial cells also express S1P receptors. A reduction in hepatocyte proliferation was noted in Etrasimod and Amiselimod treated livers, which could be a direct effect of the inhibitors or an indirect effect due to a reduction in inflammation. Examination of sinusoidal endothelial cells is beyond the scope of the current manuscript.

Our flow cytometry panel only included CD11b to quantify myeloid cells. Therefore, we employed a combination of immunohistochemistry and gene expression to understand the increase in $CD11b^+$ myeloid cells observed with Etrasimod, based on recent and established markers of macrophage subsets. Histologically, proinflammatory infiltrating macrophage accumulation, measured by immunohistochemistry and qPCR for galectin-3 (*Lgals3*) was increased in FFC-fed liver, consistent with our previous findings (10), and was lowered in Etrasimod treated FFC-fed mouse livers (27–29). Examination of markers of resident hepatic macrophages (*Timd4*), non-inflammatory macrophages (*Marco*), and lipid-associated macrophages (*Trem2*) suggested an increase in macrophage subsets which could explain the increase in $CD11b^+$ myeloid cells (30, 31). These data suggest a differential response of macrophage subsets to S1P receptor antagonism *in vivo*, potentially due to only minimal inhibition of $S1P_5$ by Etrasimod as $S1P_5$ is known to promote egress of monocytes from the bone marrow (32). This testable hypothesis will be approached in future studies with myeloid-specific $S1P_1$ knockout mice. Since chemokines and cytokines are secreted by multiple cell types, their reduction likely reflects overall lower immune cell infiltration in the livers of mice treated with Etrasimod and Amiselimod.

Etrasimod is an S1P receptor modulator with antagonistic functional activity against $S1P_1$ and partially against $S1P_4$ and $S1P_5$. Etrasimod has no activity towards $S1P_2$ or $S1P_3$ (33). $S1P_1$ is expressed on major immune cell types including: macrophages, neutrophils, dendritic cells, monocytes, NK cells, T cells, and B cells where they participate in recruitment and trafficking (25). $S1P_4$ is widely expressed on dendritic cells, neutrophils, macrophages, monocytes, T cells, and B cells; recent findings suggest its roles in cell differentiation, recruitment, and migration. Patrolling monocytes and NK cells both express high levels of $S1P_5$ (34). $S1P_2$ is expressed on B cell, monocytes, eosinophils, and mast cells and $S1P_3$ on T cells, B cells, macrophage, monocytes, neutrophils, eosinophils, mast cells and dendritic cells. The present study revealed that receptor-specific modulation by Etrasimod reduces accumulation of macrophages in the liver and reduces intrahepatic T cells, B cells and NKT cells in FFC-fed mice, which would be consistent with the cell-specific pattern of expression of S1P receptors and the antagonistic activity of Etrasimod. T cells are

known to egress from secondary lymphoid organs to the lymphatic and blood circulation through S1P/$S1P_1$ receptor trafficking (34, 35). The antagonistic activity of Etrasimod on $S1P_1$ receptors may have suppressed T cell egress from the thymus and secondary lymphoid organs, resulting in fewer infiltrating T cells, similar to effects in ulcerative colitis (36). In patients with relapsing multiple sclerosis, administration of low dose FTY720 and subsequent blockage of S1P-mediated T cell egress has shown beneficial disease outcomes and fewer side effects (37). On the other hand, B cells express all 5 S1P receptors, yet were reduced by Etrasimod administration suggesting a predominant role for $S1P_{1,\ 4,\ 5}$ in B cells. B cell reduction could also be a result of inhibiting S1P-mediated lymphocyte trafficking through Etrasimod, repressing lymphocyte migration to peripheral tissues. Though $S1P_1$ is more ubiquitously expressed by immune cells, at the doses we tested, Etrasimod was more effective than Amiselimod at skewing the distribution of immune cells under nutrient stress conditions, suggesting redundant roles for each receptor subtype.

In conclusion, we demonstrate that modulating $S1P_1$, $S1P_4$ and $S1P_5$ by Etrasimod treatment ameliorates cardinal features associated with progressive NASH. Additional studies are required to investigate specific S1P receptor signaling on each immune cell type and their mechanistic roles in leukocyte trafficking and subsequent biological outcomes in liver inflammation in NASH. We cannot attribute specific outcomes to any individual S1P receptor or a specific cell type; yet, antagonizing multiple receptors appears to have a greater therapeutic effect than $S1P_1$ alone. These data provide an interesting insight into the effect of S1P receptor modulation on the immune cell repertoire and its potential therapeutic benefits in NASH. Notably, FTY720 is approved for the treatment of multiple sclerosis and ozanimod is approved for ulcerative colitis. Consistent with these, Etrasimod and Amiselimod have been used in clinical trials for ulcerative colitis and multiple sclerosis, respectively (11, 38). Future studies are required to evaluate the efficacy of Etrasimod and Amiselimod in treating human NASH.

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 Institutional Animal Care and Use Committee (IACUC).

Author contributions

C-YL, NV, AM, XR, YN, TS, DD, MS, FB conducted experiments. C-YL, AM, XR analyzed and graphed data. HM designed, supervised, and analyzed experiments. C-YL and HM prepared figures and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1130184/full#supplementary-material

SUPPLEMENTARY °C
Amiselimod treatment reduces liver injury. **(A)** Liver histology shown by H&E staining of vehicle or Amiselimod treated mice in CD and FFC cohorts. Scale bar equals 50 µm. **(B)** Steatosis component of the NAFLD Activity Score (NAS) for CD and FFC mouse cohorts treated with vehicle or Amiselimod. Each mouse is graded for steatosis (0-3). Less than 5% steatosis = 0, 5~33% = 1, 34~66% = 2, >66% = 3. Each dot represents one biological replicate. **(C)** The inflammatory (0-3) component score in NAS grading is shown for CD and FFC cohorts treated with vehicle or Amiselimod. If there are no inflammatory foci = 0, <2 inflammatory foci = 1, 2~4 inflammatory foci = 2, >4 inflammatory foci = 3. **(D)** Plasma ALT levels for CD and FFC cohorts treated with vehicle or Amiselimod at completion of the study. CD (n=10) and FFC (n=10). *p<0.05, **p<0.01, ***p<0.001

SUPPLEMENTARY FIGURE 2
Metabolic characterization of CD and FFC-fed mice with Amiselimod treatment. **(A)** Liver mass, **(B)** liver to body mass ratio and **(C)** body mass of CD (n=9) and FFC (n=11) groups treated with vehicle or Amiselimod. **(D)** Total cholesterol and **(E)** fasting blood glucose with vehicle or Amiselimod treatment. CD (n=10) and FFC (n=7). **(F)** AUC for the glucose tolerance test

at 22 weeks on diet and 2 weeks of Amiselimod treatment. CD (n=10) and FFC (n=10). ***p<0.001

SUPPLEMENTARY FIGURE 3
Viability plot for flow cytometry. **(A)** Representative image shows viability of samples using live/dead cells counts by employing Zombie Aqua dye.

SUPPLEMENTARY FIGURE 4
Amiselimod treatment alters some intrahepatic leukocyte populations. **(A)** Number of $CD45^+$ cells per gram acquired for FACS analysis in CD and FFC cohorts. **(B)** Subsets of $CD45^+$ cells and **(C)** immune cells expressed in %CD45 that are T cells, B cells and NKT cells by FACS analysis in livers from CD and FFC cohorts that received vehicle or Amiselimod treatment. Quantifications are expressed as %CD45 cells or total number. **(D)** T-cell subtypes expressed in %CD45 cells that are CD4 positive, CD8 positive or double negative in CD and FFC cohorts. **(E)** Subsets of $CD45^+$ cells including NK cells, $CD11b^+$ myeloid cells, PMNs and DCs and **(F)** immune cells expressed in %CD45 that are NK cells, $CD11b^+$, PMNs and DCs. CD (vehicle n=4, Amiselimod n=5) and FFC (vehicle n=5, Amiselimod n=6). *p<0.05, **p<0.01, ***p<0.001.

SUPPLEMENTARY FIGURE 5
Macrophage accumulation and inflammatory markers are reduced in Amiselimod treated mice. **(A)** Representative images showing immunohistochemistry for *Mac-2* in liver tissue sections for vehicle and Amiselimod treated mice in both CD and FFC cohorts. Scale bar equals 50 µm. **(B)** Mac-2 staining was quantified as percentage of positive immunoreactive area in CD and FFC-fed mice treated with vehicle or Amiselimod. **(C-E)** Relative mRNA expression of *Cd68, Lgals3, Ly6c* in CD and FFC cohorts treated with vehicle or Amiselimod. **(F-H)** Relative mRNA expression of monocyte chemoattractant protein-1 *(Mcp1)*, Tumor necrosis factor alpha (*Tnfa)* and Interleukin 1 beta (*Il1b)* in liver tissues of CD and FFC-fed mice treated with vehicle or Amiselimod. **(I-K)** Relative mRNA expression of *Timd4, Marco* and *Trem2* positive macrophages in CD and FFC cohorts treated with vehicle or Amiselimod. CD (vehicle n=5, Amiselimod n=5) and FFC (vehicle n=5, Amiselimod n=6). *p<0.05, **p<0.01, ***p<0.001.

SUPPLEMENTARY FIGURE 6
FFC diet-induced increase in hepatocyte proliferation is reduced in Etrasimod and Amiselimod treated mice. **(A)** Representative images showing immunohistochemistry for Ki67 in liver tissue sections for vehicle and Etrasimod treated mice in both CD and FFC cohorts. **(B)** Positive immunoreactive staining (dark brown) was quantified as Ki67 positive nuclei per high power field in CD and FFC-fed mice treated with vehicle or Etrasimod. CD (vehicle n=4, Amiselimod n=6) and FFC (vehicle n= 5, Amiselimod n=6). **(C)** Representative images showing immunohistochemistry for Ki67 in liver tissue sections for vehicle and Amiselimod treated mice in both CD and FFC cohorts. **(D)** Positive immunoreactive staining (dark brown) was quantified as Ki67 positive nuclei per high power field in CD and FFC-fed mice treated with vehicle or Amiselimod. Scale bars represent 50 µm. CD (vehicle n=4, Amiselimod n=6) and FFC (vehicle n= 5, Amiselimod n=6). *p<0.05, **p<0.01, ***p<0.001.

SUPPLEMENTARY FIGURE 7
Hepatocyte differentiation is comparable in vehicle treated and Etrasimod or Amiselimod treated FFC-fed mouse livers. **(A)** Representative images showing immunohistochemistry for HNF4α in liver tissue sections for vehicle and Etrasimod treated mice in both CD and FFC cohorts. **(B)** Representative images showing immunohistochemistry for HNF4α in liver tissue sections for vehicle and Amiselimod treated mice in both CD and FFC cohorts. Scale bars represent 50 µm.

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Galanin ameliorates liver inflammation and fibrosis in mice by activating AMPK/ACC signaling and modifying macrophage inflammatory phenotype

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Background and aims: Galanin is a naturally occurring peptide that plays a critical role in regulating inflammation and energy metabolism, with expression in the liver. The exact involvement of galanin in non-alcoholic fatty liver disease and related fibrosis remains controversial.

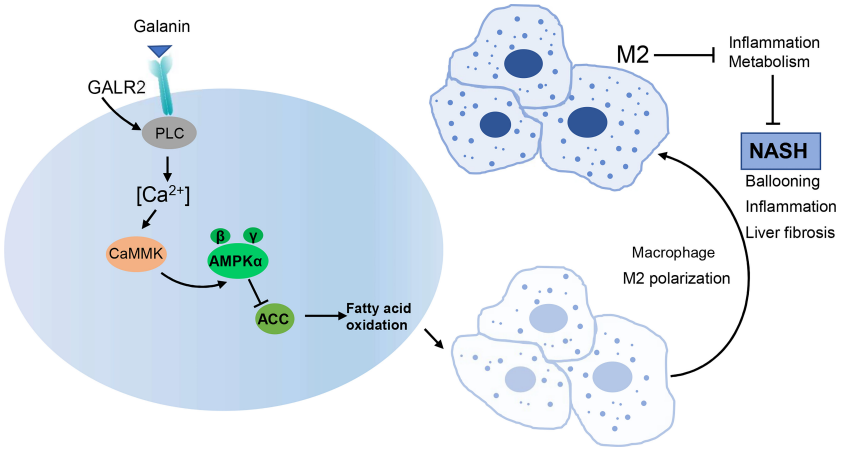
Methods: The effects of subcutaneously administered galanin were studied in mice with non-alcoholic steatohepatitis (NASH) induced by a high-fat and high-cholesterol diet for 8 weeks, and in mice with liver fibrosis induced by CCl_4 for 7 weeks. The underlying mechanism was also studied *in vitro* on murine macrophage cells (J774A.1 and RAW264.7).

Results: Galanin reduced inflammation, CD68-positive cell count, MCP-1 level, and mRNA levels of inflammation-related genes in the liver of NASH mice. It also mitigated liver injury and fibrosis caused by CCl_4. *In vitro*, galanin had anti-inflammatory effects on murine macrophages, including reduced phagocytosis and intracellular reactive oxygen species (ROS). Galanin also activated AMP-activated protein kinase (AMPK)/acetyl-CoA carboxylase (ACC) signaling.

Conclusion: Galanin ameliorates liver inflammation and fibrosis in mice, potentially by modifying macrophage inflammatory phenotype and activating AMPK/ACC signaling.

KEYWORDS

galanin, non-alcoholic steatohepatitis, liver fibrosis, macrophage, AMPK



GRAPHICAL ABSTRACT
Galanin ameliorates NASH in mice, potentially by modifying macrophage inflammatory phenotype. AMPK/ACC signaling mediates the effect of galanin on shifting inflammatory phenotype of macrophages.

Introduction

Galanin, a 29-amino-acid neuropeptide, has been shown to have a widespread distribution in central nervous system and peripheral tissues (1). The galanin receptors have three subtypes: GalR1, GalR2 and GalR3 (2), which belong to G protein-coupled receptors, and signal *via* multiple transduction pathways, including inhibition of cyclic AMP/protein kinase A (GalR1, GalR3) and stimulation of phospholipase C (GalR2) (3). This explains why one specific molecule of galanin can be responsible for different roles in different tissues. Galanin is induced in inflammation and exerts anti-inflammatory effects *via* certain galanin-receptor subtypes (4). The results of galanin on inflammation vary based on the disease and the dominant receptors on affected organ cells. This highlights the complexity of galanin's role in inflammation regulation. In addition, galanin is also associated with metabolism. It affects food intake and energy expenditure (5), as well as inhibits insulin and leptin secretion (6) (7). Galanin integrates the signals of fat stores, nutrient intake and energy expenditure to regulate energy homeostasis (8). In response to sympathetic stimulation, large amounts of galanin are produced from the liver and released into the systemic circulation (9). However, its biological effect on liver remains unclear.

Non-alcoholic fatty liver disease (NAFLD) refers to a clinicopathologic syndrome characterized by fat accumulation in the liver parenchymal cells due to insulin resistance and factors excluding alcohol (10). The disease spectrum of NAFLD includes simple fatty liver (SFL), steatohepatitis (NASH) and related liver fibrosis (11). NASH is the critical phase in NAFLD progression, while inflammatory responses are well-known as the key step from SFL to NASH (12). Therefore, investigating the inflammation in this phase in the liver and insulin resistance is particularly important.

The relationship between galanin and NAFLD pathogenesis *in vivo* remains inconclusive (8). Galanin plays a role in glucose regulation, inhibiting insulin release while promoting insulin sensitivity in skeletal muscle, heart muscle, and adipose tissue (13, 14). The role of galanin in liver fibrosis and inflammation is also complex, with some studies suggesting that it can induce scar formation in the liver, while others indicate that targeting the galanin pathway may have therapeutic potential in treating fatty liver disease (13–15). Interestingly, our previous research observed that galanin could inhibit hepatic stellate cell (HSCs) activation and suppress the profibrogenic feature of HSCs by activating GalR2 (16).

Therefore, in this study, we explored the changes in galanin levels in patients during the development of NAFLD and examined the effects of galanin in the initial stage of NASH in a mouse model induced by a high-fat and high-cholesterol diet (HFHCD). We also explored the effects of galanin on liver fibrosis by infusion of exogenous galanin into the CCl_4-treated mice. Mechanistically, galanin activates AMPK signaling and promotes M2-polarization in macrophages. Our data uncover insights into function of galanin in NAFLD and provide a molecular basis for therapeutic strategies targeting altered metabolic phenotype in NAFLD.

Materials and methods

Patient specimens

Blood samples were obtained from 62 patients with NAFLD at Xinhua Hospital following the approved IRB protocol. Sixty-two patients with NAFLD were confirmed with liver ultrasound. Three grades of steatosis have been proposed (grade 1: liver echogenicity increased; grade 2: the echogenic liver obscuring the echogenic walls of the portal venous branches; grade 3: the echogenic liver obscuring the diaphragmatic outline). Meanwhile, the blood of 38 normal adults was also collected. The age, heart rate, body mass index(kg/m^2), waist circumference (cm), systolic blood pressure (mmHg), and diastolic blood pressure (mmHg) of each patient were

recorded. The alanine aminotransferase (ALT), total cholesterol, triglycerides(mmol/L), fasting blood glucose, galanin, leptin, and insulin were detected from each blood sample.

Animal models

Male C57BL/6 mice (aged 7–8 weeks) were obtained from Slack Company (Shanghai, China). All animal handling and experimental procedures were in accordance with the ethical standards of Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiaotong University School of Medicine (Shanghai, China).

High-fat and high-cholesterol diet induced NASH models

Forty mice were randomly divided into three groups. Mice received a HFHCD (composition: 88% standard chow, 10% lard, 2% cholesterol) or a standard chow diet (Control). The effects of galanin (Bachem Co., Switzerland) were evaluated by injecting it subcutaneously (abdominal area, 8 μg/100 g body weight) daily from the beginning of the ninth week of dietary intervention for 5 weeks (17). Blood was collected before all mice were sacrificed by cervical dislocation after overnight fasting. Tissue samples were either directly snap-frozen in liquid nitrogen for molecular analysis or fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis.

CCl_4-induced liver fibrosis models

Forty mice were randomly divided into three groups. Liver fibrosis was induced by subcutaneous injection of a 2:3 solution of CCl_4 and olive oil (2.5ul/g body weight) twice per week (CCl_4 model) for 7 weeks as previously described. The effects of galanin were evaluated by injecting it subcutaneously (abdominal area, 10 μg/100 g body weight) daily. Upon sacrifice, blood was collected and serum and/or plasma were obtained. Liver tissue was either fixed in paraformaldehyde liquid, frozen in optimal cutting temperature, or snap-frozen in liquid nitrogen and stored at -80°C.

Macrophage cell lines cultures

Murine macrophage cell lines J774A.1 (ATCC TIB67) and RAW264.7 were cultured in Dulbecco's modified eagle medium (DMEM) (100U/ml of penicillin and 100μg/ml of streptomycin) containing 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere.

Histological analysis and Immunohistochemistry

Paraformaldehyde-fixed paraffin sections were stained with hematoxylin-eosin (HE) for pathological analysis. Masson's trichrome staining was performed to assess liver fibrosis, and sections were stained with Masson's trichrome stain kit (Solarbio, shanghai, China) according to the manufacturer's instructions.

After dewaxing and hydration, the sections were incubated overnight with antibodies against CD68 (ab125212, Abcam, Hong Kong). Sections were incubated with biotinylated secondary antibodies for 30 minutes. The reaction was visualized by 3,3'-Diaminobenzidine (DAB). Slides were counterstained with hematoxylin, dehydrated with sequential ethanol, hyalinized with dimethylbenzene and sealed with neutral gum. CD68-positive cells were manually counted in 10 randomly selected, non-overlapping fields.

The NAFLD activity score (NAS) was assessed based on steatosis, intralobular inflammation, and ballooning hepatocyte degeneration (18). Six sections were randomly selected from each group and observed under an electron microscope (Olympus, Japan, 400× magnification).

Serum analysis

The blood was centrifuged at 3000r/min, for 20 minutes. Serum ALT was measured using an automated analyzer. ELISAs were used to detect galanin (Bachem Co.), insulin (Alpco Immunoassays, USA), leptin and monocyte chemotactic protein-1 (MCP-1) (R&D SYSTEMS, USA) in strict accordance with the operating instructions for each product.

Quantitative real-time PCR

Total RNA was extracted using Trizol and was reverse-transcribed using a verse Transcription system (Code No.: RR036A, TAKARA, Japan). The RNA concentration was measured using a NanoDrop spectrophotometer, and 1μg of RNA was used for cDNA synthesis for each sample. Quantitative real-time PCR (qRT-PCR) of 2 ul cDNA was performed with TB Green Premix Ex Taq (Tli RNaseH Plus) (Code No.: RR420A, TAKARA, Japan). The primer sequences for amplification of each gene are listed in Supplemental Table S1. GAPDH was the internal control.

Western blot analysis

Western blot analysis was performed as previously described (19). Cells or tissues were lysed in cold RIPA buffer containing protease inhibitors. Equal amounts of total protein were separated by SDS–PAGE, transferred to PVDF membrane, and analyzed by immunoblotting. Membranes were probed with the following antibodies: anti-iNOS (ab178945, Abcam), anti-Arg1(ab233548, Abcam), anti-AMPKα (#9957, CST), anti-p-AMPKα(Thr172) (#9957, CST), anti-ACC (#9957, CST), anti-p-ACC (#9957, CST), anti-*a*-SMA(A5228, Sigma). Anti-rabbit-HRP and anti-mouse-HRP (Beyotime Biotechnology, China) were used as secondary antibodies.

Cell proliferation assay

Cell proliferation assays were performed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) following manufacturer's instructions. The cell numbers in triplicate wells were measured as the absorbance (450 nm) of reduced blank.

Phagocytic activity of cells

The phagocytotic activity of J774A.1 and RAW264.7 cells was assessed as described previously with minor modifications (20). Quiescent cells (serum-starvation 4h) were challenged by lipopolysaccharide (LPS) (0.1μg/ml) for 2 hours and then exposed to various concentrations of galanin for 24 hours. The latex beads (10%, 3μm size, Sigma) were added and incubated for an additional 4 hours. The percentage of cells containing three or more latex beads was determined by counting 200 cells.

Fluorescent measurement of intracellular reactive oxygen species

J774A.1 and RAW264.7 cells were incubated overnight in DMEM containing 10% FBS. Cells were loaded with 5μM 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) in phosphate balanced solution (PBS) for 20 minutes at 37°C. The fluorescence was monitored after 5 minutes, using excitation and emission wavelengths of 485nm and 530nm, respectively.

Transwell for migration assay

J774A.1 and RAW264.7 cells were seeded in 6-well plates and cultured for 3 days. Cell migration assay was performed in a transwell Boyden chamber (Corning). Cells suspension (3×10^5 cells/mL) was placed in the upper chamber with serum-free medium. The lower compartment contained 0.6 ml DMEM containing 10% FBS. After 24 hours of incubation at 37°C, the cells and DMEM in the upper chamber were removed. The chamber was fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet (Beyotime) for 20 minutes. The cells in 5 different fields were photographed and counted using a microscope.

Hydroxyproline content

Hydroxyproline content in liver tissue was determined using acid hydrolysis method (21). Liver tissues (0.5 g) were hydrolyzed (20 hours in 6 mol/L HCl at 100°C), diluted in ultrapure water, and centrifuged to eliminate contaminants. Samples were incubated for 10 minutes in 0.05 mol/L chloramine-T at room temperature, followed by a 15-minute incubation in Ehrlich's perchloric acid solution at 65°C. The absorbance was measured at 561 nm, and the value for each sample was computed using a hydroxyproline standard curve. Date expressed in milligrams of hydroxyproline per gram of wet liver tissue.

Statistical analysis

The results are expressed as mean ± SD. Statistical analyses were performed by using ANOVA with the statistical software GraphPad Prism 7. *Post hoc* Student–Newman–Kuels analyses were performed when >2 groups were present.

Results

Galanin is increased in the serum of patients with NAFLD

To investigate the difference in galanin content between NAFLD and normal controls, we detected serum galanin from 62 NAFLD patients and 38 normal adults. The serum galanin was upregulated in NAFLD patients in comparison with normal control group($p=0.028$). NAFLD patients showed higher body mass index ($p<0.0001$) and abdominal circumference ($p<0.0001$) (Table 1). More importantly, a high galanin content was associated with increased ALT content (Figure 1A), upregulated total cholesterol (Figure 1B), more elevated triglycerides

(Figure 1C) and leptin (Figure 1D). Serum galanin was not associated with other parameters, such as gender and heart rate.

Galanin alters metabolism in HFHCD–induced mouse NASH models

To explore the therapeutic potential of galanin in NASH, we treated HFHCD-fed mice with galanin. We first evaluated the overall metabolic status of mice. HFHCD-fed mice received additional treatment of galanin showed no significant difference in the food consumption, body weight, epididymal fat index (epididymal fat weight g/weight g) and liver index (liver weight g/weight g) compared with HFHCD-fed mice (Figures 1E–H).

In HFHCD-fed mice, galanin treatment repressed serum insulin, ALT and MCP-1 expression, suggesting metabolism changes and decreased tissue damage in these animals (Figures 1I–K). In addition, there were no significant differences in serum galanin levels among all groups (Figure 1L).

Chronic exogenous galanin infusion attenuated NASH development and fibrosis

The livers of HFHCD-fed mice exhibited varying degrees of hepatic steatosis, intralobular infiltration of inflammatory cells, loose cytoplasm and ballooning degeneration of hepatocytes. As shown in Figures 2A, C, galanin treatment decreased the mouse liver NAS in HFHCD-fed mice. Kupffer cells (KCs) are hepatic

TABLE 1 Galanin levels in patients with NAFLD.

	NAFLD(n=62)	Control(n=38)	*P* value
Sex(M/F)	46/16	15/25	-
Age(years)	43.73 ± 1.30	33.89 ± 10.37	<0.0001
Heart rate	81.04 ± 1.54	82.08 ± 2.68	NS 0.736
Body mass index (kg/m^2)	25.88 ± 0.410	20.81 ± 0.489	<0.0001
Waist circumference (cm)	82.307 ± 0.913	71.395 ± 0.937	<0.0001
Systolic blood pressure (mmHg)	131.84 ± 2.216	115.58 ± 2.340	<0.0001
Diastolic blood pressure (mmHg)	86.47 ± 1.578	77.13 ± 1.530	<0.0001
Alanine aminotransferase (U/L)	43.565 ± 6.724	19.13 ± 1.689	0.001
Total cholesterol (mmol/L)	12.476 ± 0.703	4.221 ± 0.118	<0.0001
Triglycerides(mmol/L)	1.920 ± 0.147	1.209 ± 0.094	<0.0001
Fasting blood glucose(mmol/L)	5.817 ± 0.223	5.216 ± 0.082	0.042
Galanin(ng/ml)	362.39 ± 19.558	309.11 ± 13.715	0.028
Leptin(ng/ml)	147.921 ± 10.674	81.253 ± 6.616	<0.0001
Insulin(U/ml)	23.861 ± 2.137	13.064 ± 2.137	0.001

macrophages that play a pivotal role in the key steps of fatty liver progression to fibrosis (22), with CD68 as a surface marker. CD68 immunostaining confirmed KCs infiltration in HFHCD-fed mice, which was reduced by galanin treatment (Figures 2B, D). In keeping with this observation, the mRNA levels of CD68 and those of MCP-1, a potent macrophage chemoattractant, were significantly higher in the livers of HFHCD-fed mice than in control mice($p<0.01$), and this difference disappeared following treatment with galanin ($p<0.01$). The hepatic expression of other inflammatory markers such as chemokine C-C-motif receptor 5(CCR5) and tumor necrosis factor (TNF)-α mRNA were significantly increased in HFHCD-fed mice than in control mice ($p<0.01$). This difference was abrogated or reduced following treatment with galanin($p<0.01$) (Figure 2E). Features of liver fibrosis were detected in HFHCD-induced NASH models. The results revealed that galanin reduced collagen I (COL-I) and collagen III (COL-III) mRNA in NASH models (Figure 2F).

To further explore the role of galanin in fibrosis induced by chronic liver injury and inflammation, we treated mice with carbon tetrachloride (CCl_4) or its vehicle for 7 weeks (23). HE staining of liver tissue sections showed that CCl_4-fed mice developed pericellular fibrosis, which was abrogated following galanin treatment. Masson staining also revealed that marked collagen accumulation was observed in CCl_4–treated mice, which were attenuated by galanin infusion (Figures 3A–C). Likewise, increases in hydroxyproline content observed in the livers of CCl_4–treated mice were downregulated by galanin infusion (Figure 3D). Liver mRNA levels of fibrogenesis markers (α-SMA, TGF-β1, COL-I and COL-III) were also increased in CCl_4-fed mice. They were all downregulated by galanin treatment (Figure 3E). These data conclusively demonstrated that treatment with galanin prevents the histologic features of NASH and fibrosis.

Galanin inhibits pro-inflammatory phenotype of murine macrophages induced by LPS *in vitro*

To understand how galanin regulates inflammatory role of macrophage, we assess the effect of galanin on macrophage function upon LPS challenge (1 μg/ml for 2 hours). Cells challenged by LPS showed potent phagocytic activity, which was alleviated by galanin administration (Figures 4A, B). The initially elevated mRNA level of inflammatory cytokines (TNF-α, IL-6 and IL-1β) under LPS challenge showed a significant decrease following treatment with galanin (Figure 4C). Galanin also weakened macrophage cell migration after LPS challenge (Figures 4D, E). In addition, using the peroxide-sensitive probe DCFH-DA, we found that galanin induced a significant decrease in intracellular ROS production in macrophage cells (Figure 4F). Galanin-treated macrophages underwent a marked decrease in inducible nitric oxide synthase (iNOS) and a significant increase in Arginase 1 (Arg1) compared to LPS challenge alone (Figures 5A, B). This effect was independent of cell proliferation (Figure 5C). The galanin receptors were detected in macrophages, revealing that macrophages expressed GalR2 (Supplementary Figure 1). These findings suggest galanin's novel function in blocking pro-inflammatory phenotype of macrophages expressing GalR2.

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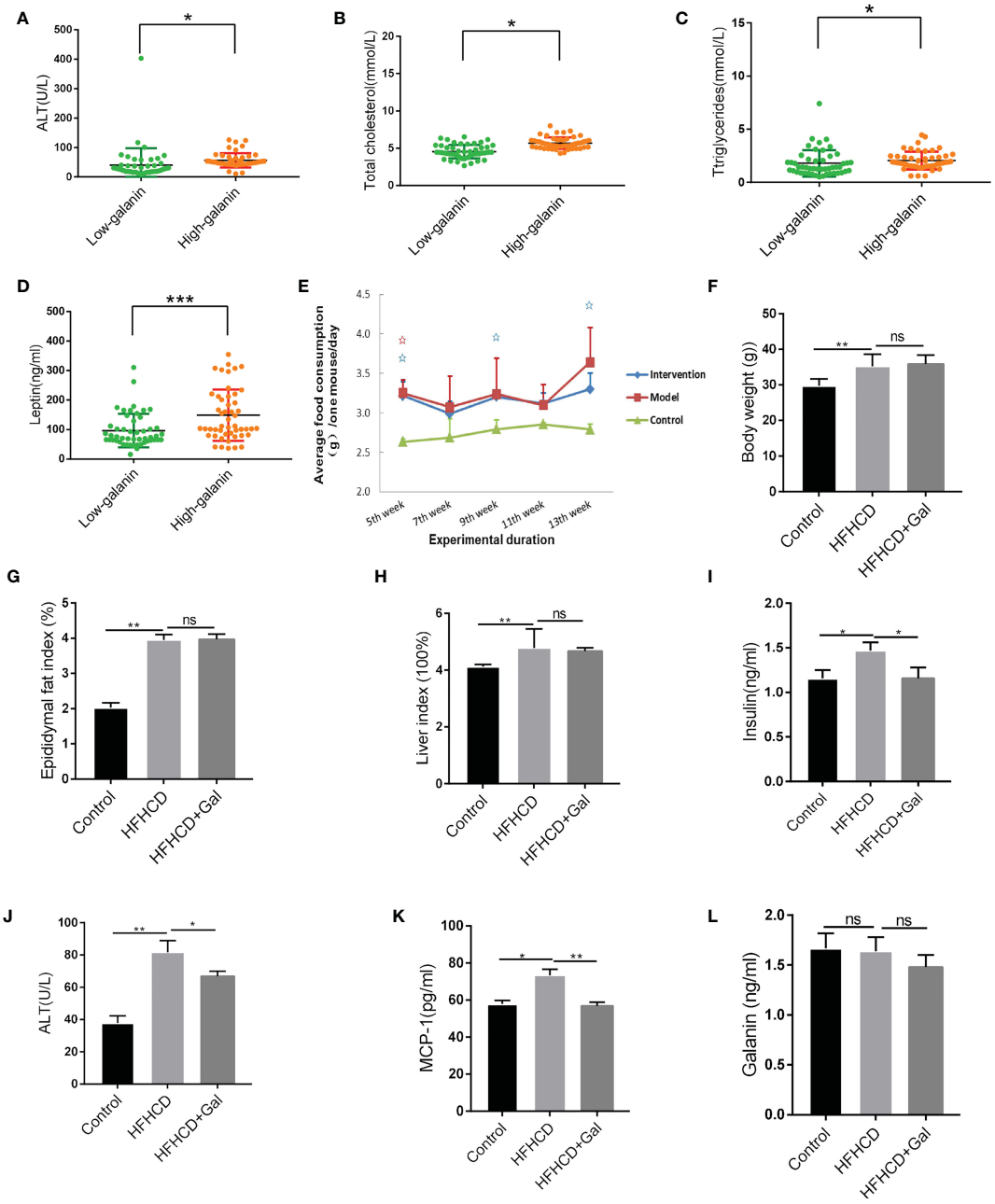

FIGURE 1
Galanin alters metabolism in HFHCD-fed mice. **(A)** Correlation analysis of ALT and galanin in human samples. *, $p < 0.05$. **(B)** Correlation analysis of total cholesterol and galanin in human samples. *, $p < 0.05$. **(C)** Correlation analysis of triglycerides and galanin in human samples. *, $p < 0.05$. **(D)** Correlation analysis of leptin and galanin in human samples. ***, $p < 0.001$. **(E)** The feed consumption of mice in each group. **(F–H)** The body weight **(F)**, epididymal fat index (epididymal fat weight g/weight g) **(G)** and liver index (liver weight g/weight g) **(H)** in HFHCD-fed mice. **, $p < 0.01$. **(I–L)** The serum insulin **(I)**, ALT **(J)**, MCP-1 **(K)** expression and serum galanin levels **(L)** in HFHCD-fed mice. *, $p < 0.05$, **, $p < 0.01$. ALT: Glutamic-pyruvic transaminase. MCP-1: Monocyte chemotactic protein -1.

AMPK/ACC signaling mediates the effect of galanin on shifting pro-inflammatory phenotype of macrophages

From our studies, galanin not only causes metabolism changes but also opposes inflammation in NASH. Insight into the role of metabolism in inflammation is supported by recent findings concerning the role of adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) as a key signaling pathway to contact inflammation and metabolism (24). To further explore the underlying mechanism of how galanin regulates pro-inflammatory phenotype of macrophages, we evaluate the effect of galanin on AMPK activation. We observed that under LPS challenge, galanin treatment induced AMPK phosphorylation on Thr172 and phosphorylates the downstream mediator acetyl-CoA carboxylase (ACC) in macrophages (Figures 6A–D). To confirm whether the

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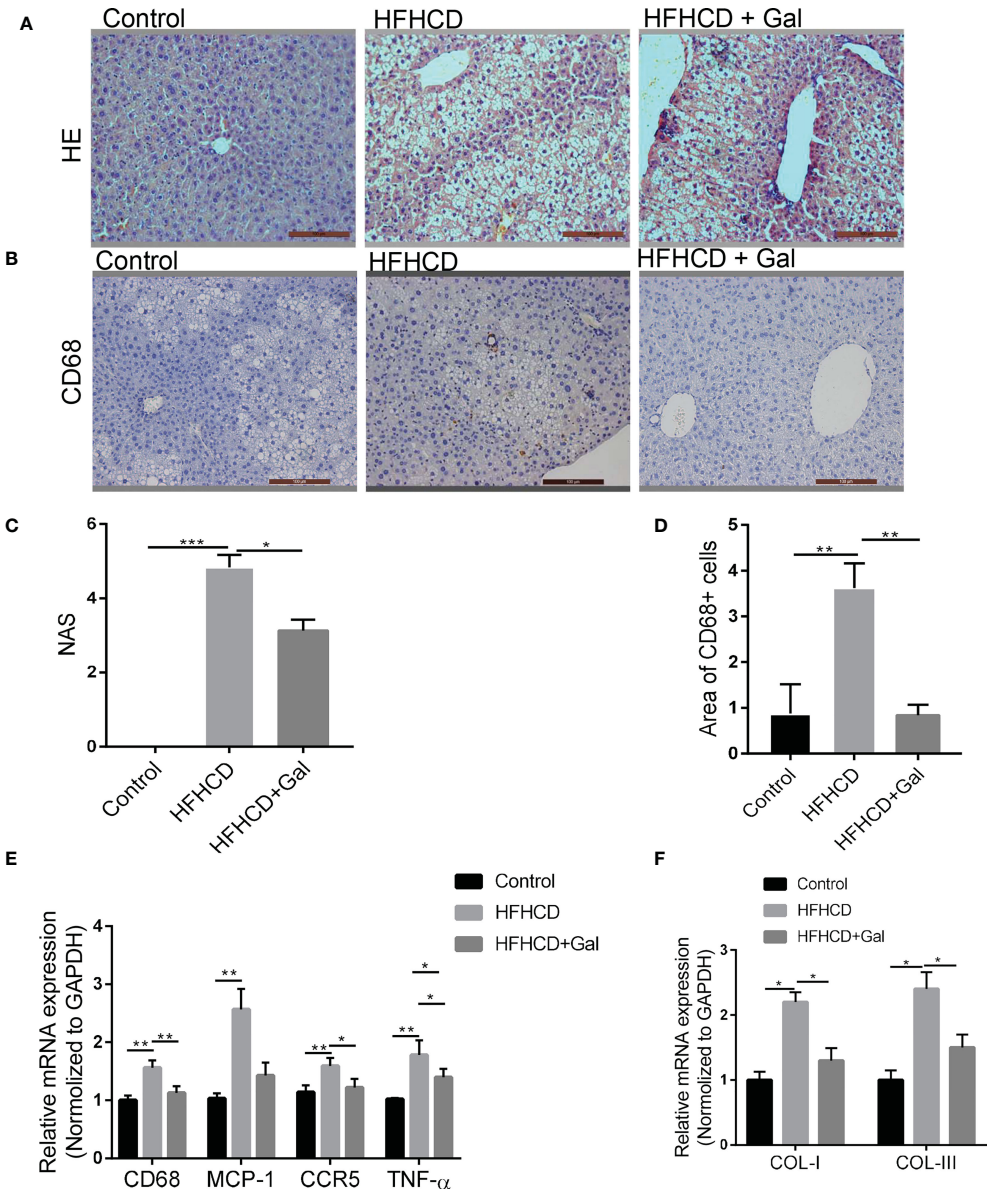

FIGURE 2
Galanin improves liver inflammation in HFHCD–fed mice. **(A)** HE staining on representative liver tissue sections from mice in each group. **(B)** Immunohistochemical analysis of CD68 on representative liver tissue sections from mice in each group. **(C)** NAS analysis using electron microscope; 6 sections per mouse were quantified. *, $p < 0.05$, ***, $p < 0.001$. **(D)** Quantitative analysis of **(B)** **, $p < 0.01$. **(E)** CD68, MCP-1, CCR5 and TNF-α mRNA levels of liver assessed by qRT-PCR. *, $p < 0.05$, **, $p < 0.01$. **(F)** Liver gene expressions of COL-I and COL-III in each group. *, $p < 0.05$. HE, Hematoxylin-eosin staining; NAS, NAFLD activity score; CCR5, C-C chemokine receptor type 5; TNF-α, Tumor necrosis factor-α; GAL, Galanin.

anti-inflammatory effects of galanin on macrophages were dependent on the activation of AMPK signaling pathway, macrophages were pretreated with Compound C, a selective AMPK inhibitor. The results showed that the M2-polarization induced by galanin on macrophages was partially blocked after treatment of Compound C (Figures 6E, F). Collectively, these findings demonstrate that galanin might promote M2 polarization of macrophages through AMPK activation.

Discussion

This study provides evidence suggesting that galanin improves histologic features of NASH including liver inflammation and fibrosis. Moreover, galanin shift inflammatory phenotype of macrophages. In addition, we show that AMPK signaling pathway mediates galanin's anti-inflammatory effects in macrophages. These data suggest that galanin could be therapeutic in NAFLD/NASH and NASH fibrosis.

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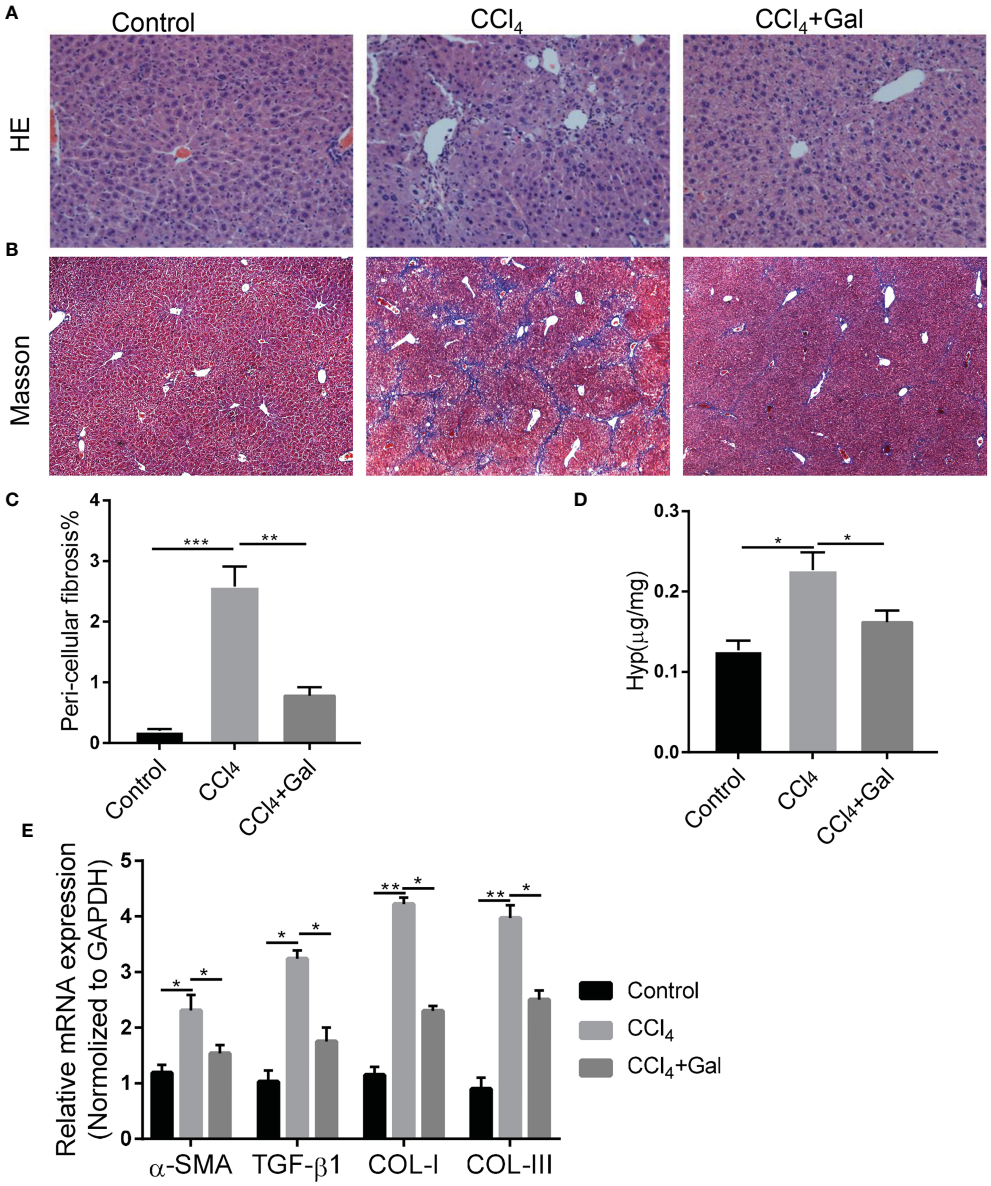

FIGURE 3
Galanin improves fibrosis induced by CCl_4. **(A, B)** Representative images of liver sections stained with HE and Masson. **(C)** Histological quantification of liver fibrosis. **, $p < 0.01$. **(D)** Hydroxyproline content in liver in each group. *, $p < 0.05$. **(E)** Liver gene expressions of α-SMA, TGF-β1, COL-I and COL-III in each group. *, $p < 0.05$, **, $p < 0.01$. α-SMA, Alpha smooth muscle actin; TGF-β1, Transforming growth factor beta1; COL-I, Type I collagen; COL-III, Type III collagen; GAL, Galanin.

The major finding in the present study was the observation that galanin alleviates NASH in mice. NASH is a potential outcome of NAFL, a condition that occurs when lipids accumulate in hepatocytes (25). Inflammation is the key rate-limiting step for NAFL to develop into NASH (26). The importance of the galanin family of peptides as inflammatory modulators is supported by data obtained from several experimental models of inflammation. The galanin family modulates inflammation in the peripheral tissues and may regulate immunity as galanin expression can be altered in inflammatory conditions (27–29). Studies indicate that galanin switches off the inflammatory response by regulating innate immunity mechanisms such as proinflammatory cytokine production (4, 30). Here we demonstrated that galanin is increased in NAFLD patients and is correlated to proinflammatory factors in NASH, including MCP-1 and leptin, suggesting a protective responder to NASH inflammation, even though early studies also suggested a deleterious effect of galanin in obesity and hepatic steatosis. Our investigation in galanin-treated mice model of NASH supported the protective effect of galanin in NASH development.

Kupffer cells are the macrophages in the liver and play a pivotal role in initiating and perpetuating the inflammatory response, with a major deleterious impact on key steps of fatty liver progression to fibrosis (31). Our results showed that galanin significantly attenuated macrophage infiltration and hepatic inflammation in HFHCD-fed mice. This aligns with previous studies that galanin modulated the expression of chemokines and cytokine levels in macrophages (32). These findings further support the concept that the body responds to

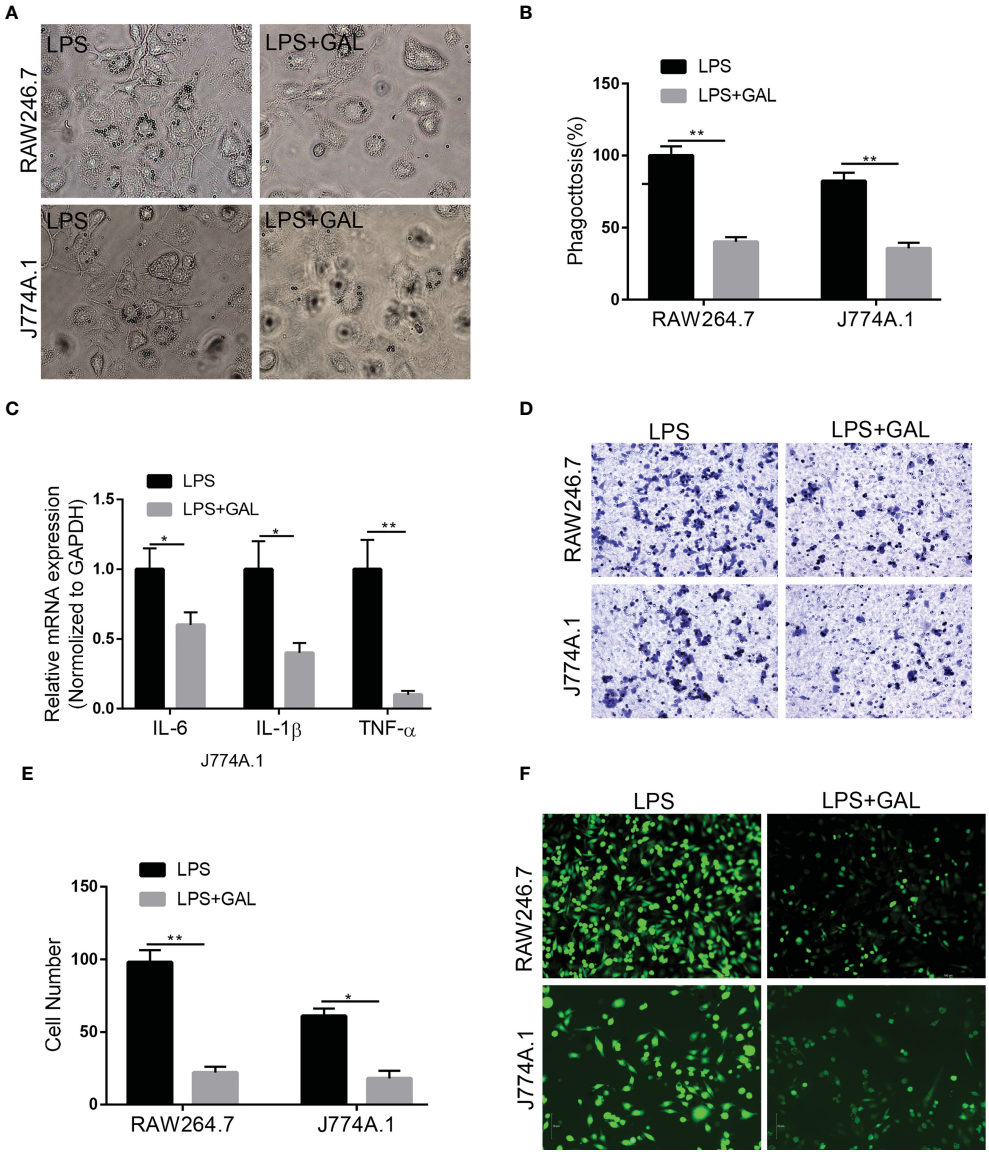

FIGURE 4
Galanin improves inflammation in murine macrophages. Murine macrophages were incubated with LPS to induce inflammation, and treated with or without galanin(1000nmol/L). **(A)** Representative images showing the internalization of latex beads by J774A.1 and RAW264.7 cells. **(B)** Quantitative analysis of **(A)** **, $p < 0.01$. **(C)** IL-6, IL-1β and TNF-α mRNA levels in J774A.1 macrophages assessed by qRT-PCR. *, $p < 0.05$, **, $p < 0.01$. **(D)** Transwell assay on J774A.1 and RAW264.7 macrophages to investigate the effect of galanin on migration. **(E)** Quantification of **(D)** *, $p < 0.05$. **, $p < 0.01$. **(F)** Representative image of detecting intracellular ROS by using the peroxide-sensitive probe DCFH-DA. IL-6, Interleukin-6; IL-1β, Interleukin-1β; DCFH-DA, 2',7'-Dichlorodihydrofluorescein diacetate; GAL, Galanin.

NASH inflammation by increasing the production of galanin peptides to switch off the inflammatory response and restore immune homeostasis, rather than exacerbating the inflammatory response (4).

Homozygous galanin transgenic mice demonstrated increased body weight and development of metabolic syndrome (33). In our study, galanin seemingly improves lipid metabolism in liver without regulating food intake and body weight, which is consistent with published studies that galanin was not associated with body weight, food intake and/or fat intake in humans (34). Nevertheless, these suggest that peripherally administered galanin is closer to the actual pathophysiology in the animal than transgenic mice. Non-selective galanin transgenic over-expression animals have long and high levels of circulating galanin, which might result in action on the central nervous system to promote food intake. However, due to the short circulating time of galanin in our study, it is unlikely to pass through the blood-brain barrier to affect the feeding center.

Interestingly, one study showed that chronic administration of oral galanin once daily in diabetic mice increased insulin sensitivity and improved several metabolic parameters such as glucose tolerance, fasting blood glucose, and insulin. The researchers concluded that oral galanin administration improves glucose homeostasis *via* the enteric nervous system, indicating its potential as a therapeutic for treating T2D. Strikingly, they did not observe a significant variation of plasma galanin in response to this oral treatment once daily, suggesting

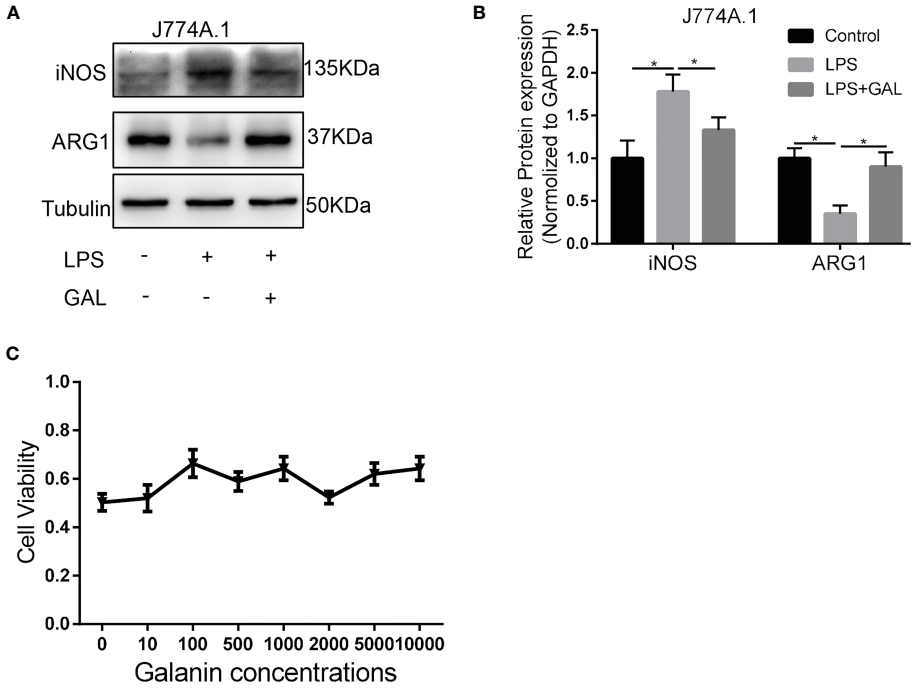

FIGURE 5
Galanin induces M2-polarization phenotype in murine macrophages. Murine macrophages were incubated with LPS to induce inflammation, or without (Control) and treated with or without galanin(1000nmol/L). **(A)** Representative image of western blot analysis of iNOS and ARG1 expression in J774A.1 macrophages. **(B)** Quantification of **(A)** *, $p < 0.05$. **(C)** CCK-8 assay of each concentration of galanin on J774A.1 macrophage proliferation. LPS, Lipopolysaccharide; iNOS, Inducible Nitric Oxide Synthase; ARG1, Arginase 1; GAL, Galanin.

that galanin's effect on glucose metabolism is short-term and related to the intestine (15). A similar phenomenon was observed in our study where an abdominal subcutaneous injection of galanin once daily did not increase circulating galanin but was protective against NAFLD progression. Whether or not a similar mechanism was involved in our study still needs further investigation.

Hepatic fibrosis is an advanced stage of NAFLD progression and HSCs are the key matrix-producing cells in liver and play a central role in hepatic fibrogenesis (35). Our previous study demonstrated that galanin inhibits HSCs activation and suppresses their profibrogenic features (16). In the present study, our research further confirmed that galanin had preventive effect on CCl_4-induced liver fibrosis *in vivo*, and improved liver function. Therefore, galanin is a promising endogenous factor involved in inhibiting NASH and fibrosis. Nonetheless, the mechanism by which transient changes in galanin are transformed into a favorable effect is still unclear and needs further investigation in future studies.

The biological activity of galanin is mediated *via* specific receptors, and the effects of galanin may differ depending on the dominant receptor(s) in different diseases and cell types (8). Studies in mice have shown that galanin treatment can increase cholangiocyte proliferation and fibrogenesis in liver fibrosis of biliary damage induced by multidrug resistance protein 2 knockout (Mdr2KO) (9). Conversely, suppressing galanin receptors has been found to reduce bile duct mass and hepatic fibrosis. In biliary hyperplasia induced by bile duct ligation in rats, galanin has been found to contribute to cholangiocyte proliferation (36). This discrepancy with our study may be due to differences in disease conditions and the galanin receptor activated.

In peripheral tissues and cells, such as adipose tissue, macrophages, and hepatocytes, the primary galanin receptor is GalR2/3. Galanin can exert its anti-inflammatory effects *via* galanin-receptor subtypes, mainly GalR2/3, while GalR1 was thought to be pro-inflammatory and pro-fibrogenic in the liver (4, 8). In these animal models of bile duct injury, the major effects are induced *via* activation of galanin receptor 1 (GalR1), which is expressed specifically on cholangiocytes, while HSCs and hepatocytes express GalR2 (9, 36) (16).

Spexin, a neuropeptide in the galanin family, has been shown to activate GALR2/GALR3 (but not GALR1) and mitigate diet-induced hepatic steatosis *in vivo* and *in vitro* by activating GALR2. Studies have demonstrated that these beneficial effects were eliminated by the GALR2 antagonist M871 in mice fed a high-fat diet and in palmitic acid-induced HepG2 cells, highlighting the critical role of the GALR2 signaling pathway in spexin's ability to improve fatty liver disease. Furthermore, spexin was found to activate GAL2 receptors and relieve skeletal muscle insulin resistance while improving metabolic parameters and adipocyte hypertrophy in obese mice by reducing M1 macrophages and subtypes and improving adipose tissue inflammation (37–40).

Macrophages are a crucial determinant for the progression of NAFLD (41). We have found that galanin regulates the inflammatory phenotype of macrophages, leading to a shift toward M2 polarization. Our results demonstrated that macrophages from LPS challenged exhibited an M1 polarized phenotype. However, galanin increased the expression of Arg1 and reduced the expression of the M1 macrophage marker iNOS in macrophages. It could be that galanin

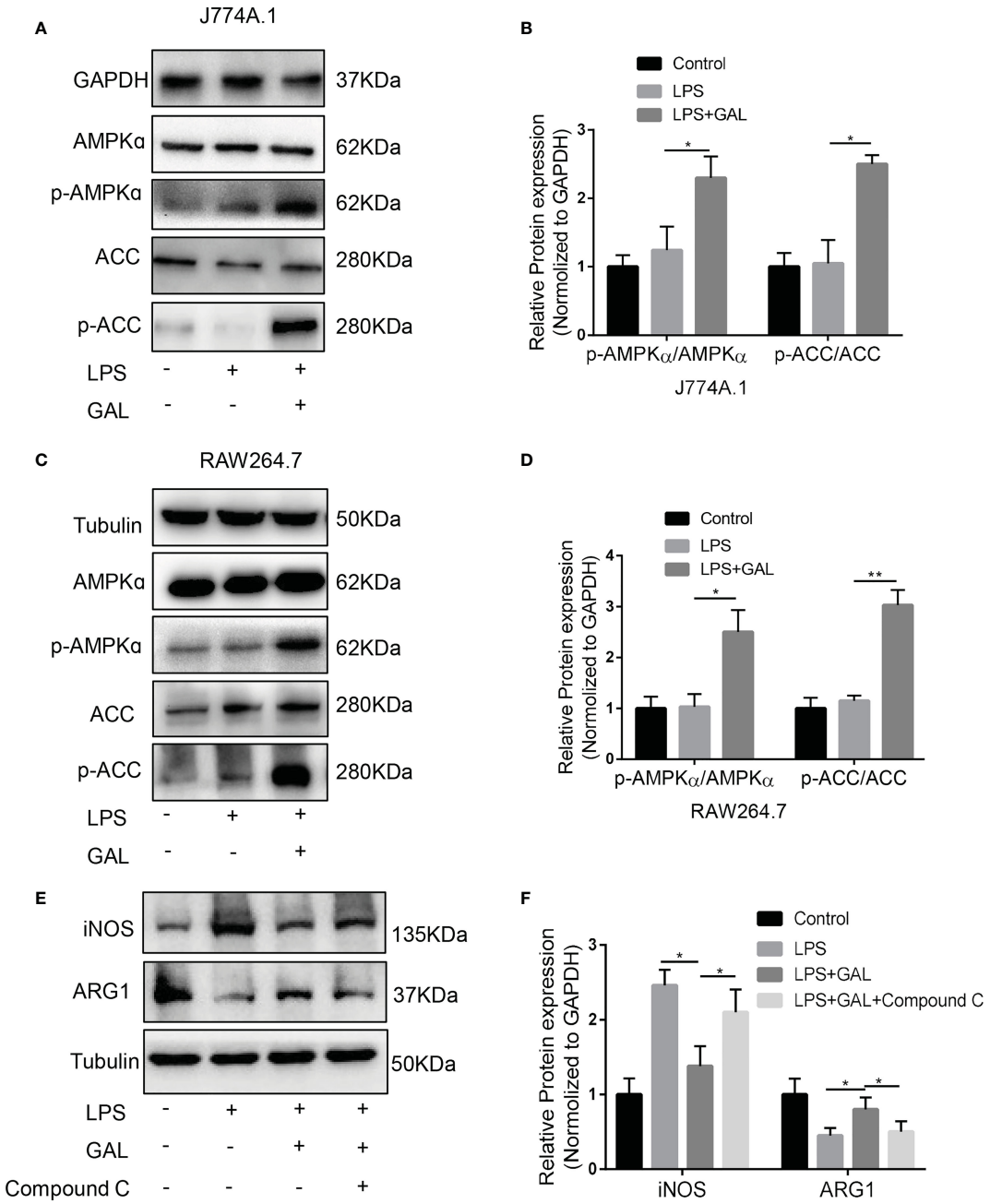

FIGURE 6
AMPK/ACC signaling mediates the effect of galanin on shifting inflammatory phenotype of macrophages. **(A)** The protein levels of AMPK, p-AMPK, ACC, p-ACC expressions in response to galanin in J774A.1 macrophages were measured by western blot. **(B)** Quantification of **(A)** *, $p < 0.05$. **(C)** The protein levels of AMPK, p-AMPK, ACC, p-ACC expressions in response to galanin in RAW264.7 macrophages were measured by western blot. **(D)** Quantification of **(C)** *, $p < 0.05$. **, $p < 0.01$. **(E)** J774A.1 macrophages were pretreated with Compound **(C)** iNOS and ARG1 expressions were detected by western blot. **(F)** Quantification of **(E)** *, $p < 0.05$. AMPK: AMP-activated protein kinase. p-AMPK, Phosphorylated AMP-activated protein kinase; ACC, Acetyl-CoA carboxylase; p-ACC, Phosphorylated acetyl-CoA carboxylase; GAL, Galanin.

induced M2 polarization and reduced M1 polarization. Arg1 production is increased in M2-polarized macrophages. This enzyme blocks iNOS activity by various mechanisms, including competing for the arginine substrate required for nitroxide production (42, 43). In adipose and liver, M2 macrophages are usually immunoregulatory and maintain insulin sensitivity, whereas M1 macrophages disrupt insulin sensitivity (44). This may explain why galanin decreased the number of CD68 positive cells in NASH in the present study.

Metabolic changes in cells that participate in inflammation, such as activated macrophages, include a shift towards enhanced glucose uptake, glycolysis and increased pentose phosphate pathway activity (45). Altered metabolism may thus participate in the signal-directed programs that promote or inhibit inflammation (46). Recent studies have shown that AMPK is a key signal in modulating inflammatory responses in immune cells and regulating lipid and glucose metabolism (47, 48). Acetyl-CoA carboxylase (ACC), a downstream target of

activated AMPK, is vital in regulating fatty acid oxidation in the liver (49). AMPK phosphorylates ACC1/2 on serine residues (Ser79/212), leading to inhibition of ACC activity (50) and decreased fatty acid synthesis (51), and increases the oxidation of fatty acid, reduced lipid storage in the liver (52). Consistent with this, we observed the activation of AMPK/ACC signaling in macrophages induced by galanin. Mechanically, activation of macrophages GalR2 is able to activate PLC activity (53), mediating the release of Ca2+ into the cytoplasm from intracellular stores and opening Ca2+-dependent channels. AMPK has been demonstrated as a multifunctional anti-inflammatory protein and can be activated *via* Ca2+/CaMKKβ pathway (54). Therefore, galanin may activate PLC activity by GalR2 in macrophages, mediating AMPK signaling *via* Ca2+/CaMKKβ pathway. It suggests that galanin activating AMPK-ACC signaling pathway might be associated with its anti-inflammatory effect. It has been revealed that metabolic reprogramming changes in hepatic macrophages play an essential role in macrophage phenotype shift (55). M2-polarized macrophages express Arg1 and convert l-arginine to urea and ornithine, competing with iNOS, which is highly expressed in M1-polarized macrophages and converts it into NO (56). The anti-inflammatory role of galanin — based on the activation of AMPK-ACC signaling pathway— seems to have been in response to metabolism changes in macrophages. As previously reported, oral administration of galanin increases total and phosphorylated AMPK protein expression, favoring glucose flux through glycolysis and activating AMPK signaling in the liver and muscles (15).

Our study has some limitations. Firstly, only male mice were used, and sex may be a crucial experimental variable in mice models. However, previous research has shown that male mice exhibit more severe liver fibrosis and inflammation with a high-fat diet (57). Future studies will include male and female mice to explore potential sex-specific differences in the effects of galanin on NASH and fibrosis. Secondly, investigating the mechanism by which transient changes in galanin through abdominal subcutaneous injection can lead to beneficial impacts even in an elevated background requires further research. Thirdly, studying the dynamic changes of galanin and its receptors during NAFLD/NASH progression may improve our understanding of our findings. Lastly, while NAFLD-related liver fibrosis models, such as long-term HFHCD or MCD, may be useful, NASH models have a major drawback, as they are unable to fully progress to severe steatohepatitis and advanced fibrosis, even after long-term feeding (11).

In conclusion, we uncovered a novel function of galanin in regulating the metabolic and pro-inflammatory phenotype of macrophage, which contributes to inhibiting NASH. This study showed that galanin caused a significant improvement in NASH features, including liver inflammation and fibrosis. All of these findings provide the enticing prospect of galanin as a promise for future practical applications in the control of NASH/NAFLD.

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 Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine. The patients/participants provided their written informed consent to participate in this study. The Ethics approval number is XHEC-NSFC-2019-262. The animal study was reviewed and approved by Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiaotong University School of Medicine. The Ethics approval number is XHEC-SHHDC-2020-063.

Author contributions

YC and ZL designed and analyzed experimental data. LH, CH, and HW performed most of the experiments. NY and JZ helped in some animal experiments. LX and TG helped with some technical guidance. LH, TG, ZL, and YC prepared figures and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Cholangiokines: undervalued modulators in the hepatic microenvironment

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The biliary epithelial cells, also known as cholangiocytes, line the intra- and extrahepatic bile ducts, forming a barrier between intra- and extra-ductal environments. Cholangiocytes are mostly known to modulate bile composition and transportation. In hepatobiliary diseases, bile duct injury leads to drastic alterations in cholangiocyte phenotypes and their release of soluble mediators, which can vary depending on the original insult and cellular states (quiescence, senescence, or proliferation). The cholangiocyte-secreted cytokines (also termed cholangiokines) drive ductular cell proliferation, portal inflammation and fibrosis, and carcinogenesis. Hence, despite the previous consensus that cholangiocytes are bystanders in liver diseases, their diverse secretome plays critical roles in modulating the intrahepatic microenvironment. This review summarizes recent insights into the cholangiokines under both physiological and pathological conditions, especially as they occur during liver injury-regeneration, inflammation, fibrosis and malignant transformation processes.

KEYWORDS

biliary epithelial cells, cholangiocyte secretome, cholangiopathies, ductular reaction, cellular senescence, inflammation, fibrosis, hepatic carcinogenesis

Introduction

Cholangiocytes, also known as biliary epithelial cells (BECs), are specialized epithelial cells forming the biliary epithelium and lining the bile ducts (1). In general, cholangiocytes are polarized with apical and basal membranes corresponding to different functions: 1) maintain bile flow *via* the cilium system and intraductal homeostasis *via* active biomolecule transport; 2) modify bile *via* secreting bicarbonate (HCO_3^-) through the plasma membrane domain; 3) maintain cross-ductal interaction in the liver, depending on their tight junctions and immunoglobulin A (IgA) secretion; 4) reabsorb different molecules, including bile salts, bile acids, glucose, amino acids and ions (2–4). Cholangiocytes represent a heterogeneous population in terms of morphological characteristics, classically described as small or large cholangiocytes (5). Accordingly, cholangiocyte

transcriptome is highly variable, so as their structural and metabolic functions. Large cholangiocytes typically line the larger branches of the biliary tree and form more complex structures than those small cholangiocytes. Simultaneously, large cholangiocytes engage in hormone-modulated bile secretion, while small cholangiocytes are able to proliferate and exhibit functional plasticity in diseases (6–8). Small cholangiocytes appear more capable of self-replication during liver injury, implying their potential in the liver regeneration and ductular reaction (DR) (9). DR is described as a complex of dynamic interactions among liver parenchymal cells, stromal cells, and immune cells, which serves a crucial machinery during liver injury-regeneration, fibrogenesis, and malignant transformation processes. Though not affirmatively being recognized as the origins of DR, cholangiocytes participate in DR as both initiators and executors (10).

To date, cholangiocyte biology has been merely studied in liver diseases, due to their relatively small population in the liver. However, a rising number of studies unveiled crucial functions of cholangiocytes in liver pathobiology. Interacting with both intra- and extrahepatic ductal environments, cholangiocytes are exposed to both hepatic molecules and gut-derived stimuli [pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) and microorganisms] (11, 12). Cholangiocytes have been identified as collateral targets of various liver diseases such as fatty liver disease [nonalcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)] and alcohol-related liver disease (ALD). BECs are also directly injured in chronic cholestatic liver diseases including primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), biliary atresia (BA) and cholangiocarcinoma (13, 14). Furthermore, stimulated cholangiocytes can adopt varying secretory phenotypes. Importantly, bile duct-derived ductular cells are considered to play active roles in liver regeneration, although contradictory results suggest the necessity of a more comprehensive analysis of their function. More interestingly, activated cholangiocytes exhibit a peculiar secretory phenotype that dramatically shapes their surrounding microenvironment by modulating immune cell recruitment and mesenchymal cell migration and activation (15). From our current understanding, the release of cholangiokines (cholangiocyte-secreted cytokines, including chemokines, growth factors, ect.) is associated with cell statuses, which are affected by tissue inflammation, infection, and metabolic dysregulations. Both acute and chronic liver disorders have been shown to alter the BEC secretory profiles (16–18). Consequently, elevating attention has been given to cholangiokines in the liver, which inspires more work on obtaining in-depth and systematic understandings.

Pathogenic triggers of cholangiocyte activation

More than constituents of bile ducts, cholangiocytes play a critical role in maintaining liver homeostasis, which refers to the balance of various physiological processes in the liver. One of the essential functions of cholangiocytes is to regulate biliary composition and bile flow by secreting and absorbing electrolytes, water, and other solutes. Standing to reason, cholangiocytes are vulnerable targets in cholangiopathies, which is a complex umbrella term encompassing inherited disorders, autoimmune or other poorly understood diseases (e.g., PSC, PBC and autoimmune cholangitis), exogenous stimuli-induced injury (e.g., infection and drug), ischemic injury and other undefined types of insults. Under such injury conditions, cholangiocytes assuasively secrete cholangiokines to sustain the microenvironment of the portal area. Moreover, cholangiocytes participate in the immune response and inflammation regulation through cytokine and chemokine production. Therefore, circulating immune cells are attracted and activated to promote portal inflammation (1, 19–22).

According to variable chronic liver injury mouse models, BECs actively interact with hepatocytes and liver progenitor cells (HPCs) to promote the DR, which ultimately constitutes an alternative liver regeneration process (23, 24). Synchronously, BECs have been determined to fuel DR by several cholangiokines (25, 26). Hence, this section will demonstrate intriguing secretory phenotypes that occur in cholangiocytes, triggered by a complex portal niche (Figure 1).

Cholestasis

Bile flow perturbation generally leads to an impaired bile efflux to the intestines, resulting in a pathogenic accumulation of bile acids in the intra-hepatic environment. Gradually concentrated and thickened bile exerts detrimental effects on the gut-liver axis, thus referred to as ‘toxic bile’ (27). Due to their anatomical location along the biliary tree, cholangiocytes are amongst the first cells to be affected by cholestasis. Studies have showed that higher levels of interleukin-8 (IL-8), a potent chemoattractant for neutrophils, were detected in the bile of PSC patients as compared to non-PSC patients (28, 29), which suggests that bile duct injury induces the secretion of inflammatory cytokines into the bile (30). Of note, the effects of the main bile salts, namely tauroursodeoxycholate (TUDC), taurocholate (TC), taurodeoxycholate (TDC), taurochenodeoxycholate (TCDC) and taurolithocholate (TLC), also remain to be defined. Results from Lamireau et al. indicated that TC effectively stimulated murine BECs to release monocyte chemoattractant protein-1/C-C motif chemokine ligand-2 (MCP-1/CCL-2) and IL-6 (31). In addition, oxysterols were revealed to insult cholangiocytes and induce malignancy transformation, which can be taken as a destructive part of ‘toxic bile’ (32–34).

Neuroendocrine hormones including secretin (Sct), are released by proliferating cholangiocytes (35, 36). Other than inducing biliary bicarbonate secretion by binding with its basolateral receptor (SR) (7, 37, 38), the Sct/SR axis plays a key role in the modulation of biliary proliferation and hepatic fibrosis by influencing the BEC secretome (35, 36, 39). The *SR* gene expression was shown to be elevated in the biliary obstruction animal model after bile duct ligation (BDL) (40). Furthermore, studies have shown that increased expression of vascular endothelial growth factor-A (VEGF-A) and transforming growth factor-beta 1 (TGF-β1) occurs when the Sct/SR axis is activated, leading to enhanced proliferation of ductular cells and fibrogenesis. Moreover, DR and liver fibrosis can be ameliorated when the SR expression was

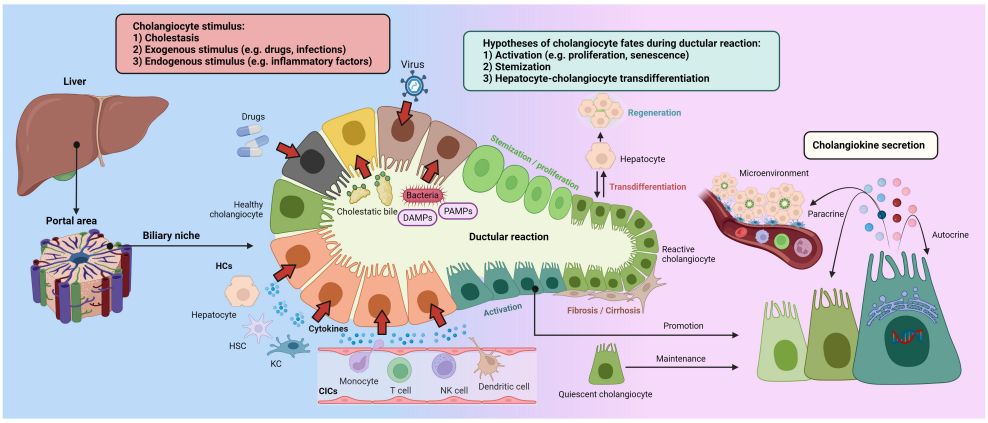

FIGURE 1
Ductular reaction and cholangiokine secretion Cholangiocytes stand as a crucial component in the hepatic portal areas, maintaining liver homeostasis. Cholangiocellular phenotypes can be altered by cholestasis, exogenous stimulus, and inflammatory factors. Besides evidence and debates on cholangiocyte stemization and hepatocyte-cholangiocyte trans-differentiation, activated cholangiocytes drive the DR by producing variable cytokines and chemokines, termed cholangiokines. Furthermore, cholangiokines are responsible for autocrine and paracrine effects in the portal microenvironment. CIC, circulating immune cell; DAMPs, damage-associated molecular patterns; HC, hepatic cell; HSC, hepatic stellate cell; KC, Kupffer cell; PAMPs, pathogen-associated molecular patterns.

genetically disrupted in BDL and *Mdr2*$^{-/-}$ (*Abcb4*$^{-/-}$) mouse models (35, 39). Additionally, increased activity of the Sct/SR/TGF-β1 axis was observed in the liver of PSC patients compared to healthy livers (39).

Furthermore, fibroblast growth factor 19 (FGF19) was found in the liver samples from patients with cholestasis (41). Exposure to FGF19 has been associated with the proliferation and IL-6 release of cholangiocytes (42). More importantly, human gallbladder cells secrete FGF19 into the bile, which is assumed to participate in cholangiopathies (43). Even though many other cell populations have been identified as sources of FGF19, it would be interesting to elucidate the functions of cholangiocyte-secreted FGF19, particularly in the hepatoportal regions (44).

It has been reported that BDL-induced bile duct obstruction in mice triggers cholangiocytes to secrete osteopontin (OPN) (45, 46). Additionally, nerve growth factor (NGF) was found to be secreted by cholangiocytes in an experimental mouse cholestasis model (47). TGR5, well-known as a G-protein-coupled bile acid receptor, is highly expressed on cholangiocytes and hepatic macrophages. It is postulated that TGR5 can participate in bile production, proliferation regulation and inflammation modulation. The beneficial secretion of bicarbonate and chloride was known attributing to the TGR5-mediated cholangiocyte activation (48). Furthermore, it is hypothesized that TGR5 might impede hepatic cell-cell communication, which either directly or indirectly affects the cholangiocyte-associated secretory characteristics. Nonetheless, the effects of TGR5 on the cholangiocyte secretome have already been discussed elsewhere (49).

Exogenous stimulus

Environmental factors (microorganisms, drugs, ischemia, etc.) serve a pivotal role in the cholangiocyte activation and pathogenesis of cholangiopathies. Notably, the microbiota has emerged as a crucial mediator of BEC functions (50). Although hepatocytes and Kupffer cells are generally responsible for the clearance of bacterial products in the liver (51, 52), cholangiocytes can also play auxiliary roles in this process, especially regarding the response to intraductal stimuli. In terms of physiology, the gut barrier stands as the first line of defense preventing external insults from entering the organism *via* the venous system, while bile duct acts as the front-line defending bile-derived insults (12). The portal channel, however, may allow certain bacteria, PAMPs, and DAMPs to enter the liver, affecting biliary inflammation or possibly inducing inflammation in the biliary tree. Paik et al. recently reported that gut-resident bacteria can inhibit Th-17 cell functions by producing bile acids (3-oxoLCA), which evidences a bacteria-induced immune turbulencein the gut-liver inflammatory modulation (53). Additionally, a growing number of studies have revealed the critical functions that gut microbiota plays in influencing the progression of liver disease, particularly in PSC and PBC (54–56). Indeed, PAMPs refluxed into the bile duct can be sensed by cholangiocytes *via* pattern recognition receptors, which can provoke a variety of inflammatory signaling pathways and cytokine secretion.

Fundamentally, cholangiocytes express the Toll-like receptor (TLR) family proteins, which are well-known as mediators in innate immune responses (57, 58). TLRs can recognize microbial and other exogenous molecules, PAMPs and DAMPs. Furthermore, the TLR activation induced by PAMPs and DAMPs triggering their conrrespondingsignaling pathways, results in the recruitment of toll/IL-1-domain containing adaptor molecules [e.g., myeloid differentiation protein 88 (MyD88)], and the activation of protein kinases [e.g., IL-1 receptor associated kinase (IRAK)]. The activation of these specific intracellular pathways leads to a nuclear factor kappa-B (NF-κB)-dependent secretion of proinflammatory cytokines/chemokines (59). Furthermore, cholangiocellular autocrine and paracrine signals are robustly enhanced by several cytokines including IL-1, IL-6, IL-8

and interferon-γ (IFN-γ) (60). Moreover, emerging studies have elucidated the mechanisms of microbiota implication on cholangiopathies. For instance, PBC patients were found to tolerate autoantibodies that can cross-react with bacterial antigens from *E. coli* and *N. aromaticivorans* (61). *E. coli* infection is known as a key factor in breaking immunological tolerance against the mitochondria, resulting in the production of PBC-specific autoantibodies (termed anti-mitochondrial autoantibodies) (62). These findings lend credence to prospective mechanisms underlying the secretome changes in association with cholangiocytes.

In the past three years, Coronavirus disease 2019 (COVID-19) has swept the world and brought new challenges to human diseases, leading to investigations and discussions on the COVID-19-interfered cholangiopathies (63, 64). A case report of COVID-19 patients discovered unique histologic features, including severe cholangiocyte injury and intrahepatic microangiopathy in their liver samples, suggesting a SARS-CoV-2-induced hepatic injury (64). Additionally, SARS-CoV-2 can infect cholangiocytes *via* the angiotensin-converting enzyme 2 (ACE2), which can be reduced by ursodeoxycholic acid while being induced by farnesoid X receptor (FXR) signaling in cholangiocytes (65).

Drug-induced cholangiopathies [also known as drug-induced vanishing bile duct syndrome (VBDS)] were first described in rare clinical cases (66). Certain medications, including carbamazepine and amoxicillin/clavulanic acid, have been shown to cause biliary damage (67). Additionally, fluorodeoxyuridines and 5-fluorouracil were revealed to selectively induce injuries in large bile ducts (68). Interestingly, cholangiocytes are implicated in drug metabolism as they were shown to express cytochrome P450 (CYP450) superfamily members (69, 70). Therefore, functional investigations linking drug metabolism or drug-induced liver damage to the secretory characteristics of cholangiocytes are highly anticipated.

Similar cholangiopathies with vanishing bile ducts, biliary strictures and protein casts also occur after ischemic insults, including ischemic-type biliary lesions (ITBL) after liver transplantation, secondary sclerosing cholangitis of critically ill patients (SC-CIP) after acute respiratory distress syndrome, COVID-19, shock and sepsis (71). Regrettably, most studies only investigated cellular injury or histological manifestations of such cholangiopathies without a detailed description of cholangiocyte-associated secretory phenotypes.

Endogenous stimulus

In comparison to injuries, endogenous stimulus, mainly inflammatory factors, play pivotal roles in modulating a variety of cholangiocyte phenotypes. In this context, cholangiocytes act as a major sensor rather than an initiator of inflammation, which possibly explains the general notion of cholangiopathies in most acute and chronic liver diseases (14).

When there is a disturbance in homeostasis, cholangiocytes are more susceptible to immunological responses, which enhances their secretion of cytokines including chemokines, and angiogenic growth factors. For instance, IL-1 and tumor necrosis factor-α (TNF-α) trigger cholangiocytes to release epithelial cell-derived neutrophil-activating protein (ENA-78) and growth-related gene products (72). Moreover, primary human cholangiocytes treated with cytokines (IL-1β, TNF-α and IL-17) or TLRs-related PAMPs [Pam3CSK4, poly(I:C) and LPS] can attract periductal Langerhans cells (Langerin$^+$ periductal cells) *via* secreting the chemokine macrophage inflammatory protein-3α (MIP-3α) to activate PAMPs-sensing TLRs, thereby regulating biliary innate immune response in PBC (73). Poly (I:C)-treated primary cholangiocytes, mimicking biliary damage in BA, also trigger a stronger release of chemokine (C-X3-C motif) ligand 1 (CX3CL1) and the subsequent attraction of malfunctional natural killer (NK) cells (74). Other cytokines (IL-1β, IL-6, and IL-23p19) and chemokines [chemokine (C-XC-C motif) ligand (CXCL)-1/2/3/6/8, CCL-2 and CCL-20) were found enriched in the interlobular bile ducts from PBC patients, which was also confirmed in the *in vitro* stimulation of primary cholangiocytes with PAMPs [Pam3CSK4, poly(I:C) and LPS] and IL-17 (75).

During persistent liver injury, cholangiocytes synthesize and release TGF-β, especially TGF-β2, which was significantly increased in reactive bile ducts of fibrotic livers. In turn, TGF-β further promotes cholangiocytes to secrete endothelin-1 and regulates, in a paracrine manner, the deposition of extracellular matrix in the adjacent mesenchymal cells (76). Cholangiocytes appear to be responsive to IFN-γ, is mainly secreted by CD8$^+$ T cells (77). IFN-γ was revealed to ameliorate fibrosis and cholestasis in carbon tetrachloride-treated mice (78). On the other hand, IFN-γ induces a shift of cytokine secretion in cholangiocytes from an acute inflammation pattern to a chronic inflammation feature, serving an important driver of persistent inflammation in cholangiopathies. Specifically, IFN-γ represses IL-8 secretion while enhancing the secretion of several cytokines including MCP-1 (79), monokine (80), interferon-inducible T cell alpha chemoattractant (ITAC) (81) and interferon-γ-inducible protein 10 (IP10) (82). Furthermore, IFN-γ and IL-6 stimulate nitric oxide (NO) production in cholangiocytes by inducing nitric oxide synthase-2 (NOS-2) expression (83). Besides, BECs exposed to IFN-γ exhibit a phenotypic flip between the acute and chronic inflammatory processes in terms of their release of inflammatory components (84).

IL-6, HGF and epidermal growth factors (EGF) can promote the proliferation of cholangiocytes *in vitro*, while the secretion of IL-6 can be further enhanced by IL-1β and phorbol myristate acetate (85). Exogenous IL-6 addition can also rescue the activin-A-induced growth inhibition of primary cholangiocytes *in vitro* (86). With the assistance of NO, IL-6 is involved in the LPS-induced sepsis-related systemic inflammatory response and is one of the most powerful mitogens for cholangiocytes (87, 88). Moreover, LPS and IFN-activated liver-derived macrophages (LDM) express high level of CD154 (also known as CD40 ligand, CD40L), which triggers the CD40-dependent changes of secreting proinflammatory cytokines with increased IL-3, IL-12p70, IL-10 and GM-CSF but reduced CXCL10, IL-6 and CCL2 in human cholangiocytes (89). Studies have showed that TNF-α and IFN-γ could disrupt the barrier function of cholangiocytes (90–92). In addition,

inflammatory macrophages secrete TNF-α in the earlier phases of liver diseases, causing an upregulation of integrin αvβ6 on the membrane of epithelial cells and leading to the binding and activation of latent TGF-β1 (93). Furthermore, BECs can produce MIP-3α/CCL-20 in response to cytokines (IL-1β, TNF-α and IL-17) and PAMPs (73). Such evidence suggests that mutual influence exists between macrophages and cholangiocytes during inflammatory hepatic processes.

Furthermore, the inflammatory milieu directly drives the alterations of the cholangiocyte secretory profile, leading to the recruitment of activated liver mesenchymal cells, thereby participating in the positive feedback loop of the inflammatory response as part of the DR. As a hallmark of epithelial–mesenchymal crosstalk, alterations in the reactive cholangiocyte secretome include the upregulation of TGF-β1, TGF-β2, IL-6, platelet-derived growth factor-B (PDGF-B) and CCL-2 (76, 94, 95). Hypothetically, endogenous stimulus derived by neighboring cells and circulating immune cells play constant roles in cholangiocyte activation and cholangiokine secretion, which eventually stimulate cholangiocytes to be a remarkable mediator in liver diseases.

Cholangiocyte-associated secretory phenotypes

Under physiological conditions, cholangiocytes stay quiescent, maintaining both local and systemic bile homeostasis (38, 96). Even though the replication rate is limited in the quiescent state, cholangiocytes are able to re-enter cell-cycle and proliferate upon various exogenous or endogenous insults (97), and even compensate for the proliferation-incapable hepatocytes to regenerate liver parenchyma (98). The secretory dynamics of cholangiocytes act in autocrine, paracrine, and endocrine manners to maintain biliary homeostasis and regulate other cell types including hepatocytes, HSCs, portal fibroblasts (PFs) and immune cells (99). Cholangiocytes detect pathogens *via* the TLRs and then secrete antimicrobial IgA into the bile, which serves a vital barrier against germs from both the duodenum and portal vein (100, 101), as well as a variety of cytokines (IL-6 and MCP-1) (102), chemokines (103) and other active anti-microbial peptides (e.g., human beta-defensin-1, hBD-1) into the portal microenvironment (104). Accordingly, these secreted substances have a variable composition depending on the cellular state of cholangiocytes, which creates a complicated secretory network that distinguishes and maintains various cholangiopathies. In general, quiescent cholangiocytes in the biliary system become activated as a result of ongoing distress (e.g., targeted BEC injury and/or inflammatory response caused by broader liver insults). Active cholangiocytes have different cell cycle fates depending on the nature and duration of the injury, primarily cell death, growth and senescence (97).

Following an acute insult, injured cholangiocytes undergo cell death, either programmed (e.g., apoptosis) or non-programmed (e.g., necrosis). The release of apoptotic bodies or DAMPs can trigger a local inflammatory response, which aids in the clearance of cell debris, leading to a time-constrained immune response. However, when a moderate injury occurs or persists, cholangiocytes may re-enter the cell cycle, or engage into an irreversible cell cycle arrest (termed cellular senescence), both of which are accompanied by unique secretory patterns. Nevertheless, several factors, including IL-1β, IL-6, MCP-1, stem cell factor (SCF), TGF-β1, and PDGF, can be secreted by both proliferative and senescent cholangiocytes (105). The similarities and differences of secreted factors from cholangiocytes in proliferative and senescent states are described in the following sections. To understand the complexity of cell status-cholangiokine association, we summarize relevant evidence in Table 1.

Quiescence-associated secretory phenotypes

The anatomic location of the biliary system makes biliary epithelium a fundamental barrier against microorganisms mainly ascending from the duodenum and partially from the portal vein, or as suggested by recent studies present in the bile (1, 124–126). Thereby, under physiological conditions, cholangiocytes establish an intricate cooperative machinery with other hepatocytes and resident immune cells through direct or paracrine factor-mediated intercellular communication. This is supported by a recent study using single-cell RNA-sequencing data of human liver samples, revealing an up-regulation of genes involved in the secretion- and inflammation-related pathway in a subset of cholangiocytes, thereby indicating a crucial role of quiescent cholangiocyte secretome in maintaining homeostatic liver-biliary microenvironment. Additionally, it shows the heterogeneity of quiescent cholangiocyte populations in the liver, suggesting that cholangiocytes may be in a dynamic physiological state as they respond to occasional microbial assaults (127).

The majority of the immunoglobulins (Igs) in human bile are secretory Igs, which significantly maintain liver homeostasis. Hepatocytes effectively secrete most of the IgA in rodents, whereas cholangiocytes represent the main source of IgA secretion in human liver (101). Biliary immunoglobulins, especially IgA, are crucial innate defenders against microorganisms in the biliary tract and upper intestine. Quiescent cholangiocytes also secrete alternative antimicrobial peptides [such as defensins (104), mucins and mucin-associated trefoil peptides (TFF) (120), lactoferrin (121) and cathelicidin (122)], contributing to the basic defense of microorganisms in the biliary tract (128).

In addition to direct immunological defense through the bile, quiescent cholangiocytes can recruit and/or maintain different immune cells by expressing immune-modulating proteins on their surface or by secreting chemokines and cytokines (2). For instance, the cholangiocytes' surface protein CD1d, which resembles the MHC class I molecule, can activate NKT cells by presenting lipid antigens (129). Cholangiocytes can also activate mucosal-associated invariant T (MAIT) cells, which are prevalent in the human liver and locate near bile ducts, by presenting bacterial antigens *via* MHC class I-related protein (130). Under normal circumstances, quiescent cholangiocytes secrete TGF-β2, which is involved in maintaining periductular connective tissues and is

TABLE 1 Cell status-associated cholangiokines.

Cell status	Cholangiokines	Conditions	Ref
Activated	MCP-1/CCL-2	Liver specimens from patients with chronic hepatitis	(106)
	CXCL-1/2/5/10/12, IL-1β and TGF-β1	CHF mouse model [Pkhd1(del4/del4]-deleted] derived primary cholangiocytes stimulated by CXCL-1 and -10	(93)
		Liver specimens from CHF patients	
	IL-8, TNF-α	PSC liver derived BECs exposed to TLR ligands (Pam3CSK4, LPS)	(107)
	IL-8	Human primary iBECs (from the non-neoplastic area of surgically resected livers of three patients with metastatic liver cancer) exposed to LPS and IL-1β and TNF-α	(108)
		Liver samples from patients with chronic viral hepatitis/liver cirrhosis/sepsis/extrahepatic biliary obstruction/fulminant hepatitis/PBC/PSC	
	Fractalkine	Human cholangiocarcinoma cell line (HuCC-T1) and human intrahepatic BEC line exposed to LPS and Th1-cytokines (IL-1β, IFN-γ and TNF-α)	(109)
Activated (proliferating)	IL-6	Human primary iBECs exposed to IL-1β and phorbol myristate acetate	(85)
	TGF-β2	Fibrotic specimens from patients with hepatitis B virus infection or alcohol abuse and rats with fibrosis secondary to bile duct ligation and scission.	(76)
	TGF-β1 and PDGF-BB	Mouse-derived iBEC organoids exposed to acetaminophen	(110)
Activated (injured)	IL-18	Mouse liver injury model (DDC diet) derived cholangiocytes exposed by LPS and ATP	(111)
		Liver samples from PSC patients	
	Fractalkine	Liver specimens from PBC patients	(109)
	CCL-2 and Integrin-β6	iBECs dissected from targeted biliary injury mouse model (*ihCD59*$^{BEC\text{-}TG}$)	(112)
Activated (senescent)	TGF-β	Liver specimens from tamoxifen-inducible K19-Mdm2$^{flox/flox}$ tdTom^{LSL} mice	(113)
	TGF-β1, MCP-1/CCL-2, IL-4, IL-5, IL-6, IL-7, IL-10 and IFN-γ	Liver specimens from PBC mouse model (dnTGF-βRII)	(114)
		Bile and liver specimens from PSC patients	
	CXCL-11, CCL-20	Serum from PBC patients	(115)
	CCL-2/3/4/5, CX3CL-1, CXCL-1, CXCL-2, CXCL-10 and CXCL-16	Mouse iBECs exposed to H_2O_2 and etoposide	(116)
	IL-6, IL-8, MCP-1/CCL-2, PAI-1	Normal human BECs exposed to LPS	(117)
		Liver specimens from PSC patients	
	MCP-1/CCL-2, CCL20, IL-3, IL-11 and IL-15	Mouse iBECs exposed to glycochenodeoxycholic acid	(118)
	TNF-α, IL-1β and MCP-1/CCL-2	Liver specimens from Mdr2$^{-/-}$ mouse model	(119)
Quiescent	hBD-1	Human normal liver tissues	(104)
	Mucins and TFF	Human normal liver tissues	(120)
	Lactoferrin and Lysozyme	Human normal liver tissues	(121)
	Cathelicidin	Human normal liver tissues	(122)
	TGF-β2	Human normal liver tissues	(76)
	CCL-2, IL-8 and IL-4	Primary iBECs from the non-cancerous liver specimens of one iCCA patient	(102)
	MCP-1/CCL-2	Human normal liver tissue	(106)
	IGF-1	Rat normal liver tissues	(123)
		Liver samples from PBC patients	

CHF, congenital hepatic fibrosis; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; iCCA, intrahepatic cholangiocarcinoma.

markedly up-regulated in the proliferating bile ducts of fibrotic livers (76). Last but not least, unstimulated primary human intrahepatic BECs secrete a panel of cytokines/chemokines *in vitro*, including IL-8, IL-4 and MCP-1 (102, 106), as well as insulin-like growth factor-1 (IGF-1) from healthy cholangiocytes (123).

Conclusively, in the healthy scenario, cholangiocytes mostly remain quiescent but retain baseline secretion of immunoglobulins, antimicrobial peptides, TGF-β2, IGF-1, etc., thereby dynamically maintaining the homeostatic hepatic microenvironment.

Proliferation-associated secretory phenotypes

When a moderate injury occurs, cholangiocytes can re-proliferate to compensate for cell loss and repair the injury, which is aided by the acute inflammatory response. Cholangiocellular proliferation can be triggered by multiple pathways and stimulus, including IL-6, hepatocyte growth factor (HGF), estrogen, acetylcholine, and bile acids, all of which function through binding to their specific receptors (8). One fundamental feature of proliferating cholangiocytes is their enhanced secretion of variable pro-inflammatory cytokines, chemokines, growth factors, defensin, and other bioactive factors (99). With the timely repair of injury, inflammation would also resolve, and this scenario represents an acute inflammatory response without inducing aberrant hyperproliferation of cholangiocytes (131). However, if the damage persists to prevails over repair processes, cholangiocytes abnormally proliferate and induce chronic inflammation through interaction with various infiltrated immune cells, causing angiogenesis and fibrotic response in the liver, termed DR (14).

In response to a variety of insults, including infections, cholestasis and ischemia, quiescent cholangiocytes can be activated (97), and acquire a hyperproliferative and neuroendocrine-like phenotype with pro-fibrotic and pro-inflammatory secretome (57). Acting in an autocrine/paracrine fashion, these released bioactive factors, including pro-inflammatory cytokines and chemokines (e.g., IL-6, IL-8, TNF-α and various growth factors), modulate cholangiocyte biology and direct the prognosis of biliary damage (108, 132–134). For example, IL-8 and TNF-α levels are substantially elevated in cholangiocytes from individuals with advanced PSC compared to those at the early disease stage (107). In the infection scenario, *Helicobacter bilis* or fluke products (Opisthorchis viverrini excretory/secretory products) can activate cholangiocytes to proliferate and massively secrete IL-6 and IL-8, thereby initiating innate mucosal immunity against microorganisms (135, 136).

Other chemokines secreted by reactive proliferating cholangiocytes including fractalkine from injured small bile ducts of PBC. Fractalkine possesses chemoattractant and cell-adhesive functions in recruiting intraepithelial monocytes/lymphocytes by binding to its receptor CX3CR1 (109). Moreover, MCP-1 expression was intensively but not exclusively up-regulated in the epithelial cells of regenerating bile ducts (106), which contributes to myofibroblastic trans-differentiation of portal fibroblasts, resulting in biliary fibrosis and cirrhosis (94).

Senescence-associated secretory phenotypes

In a chronic damage scenario or under a susceptible genetic background, injury-induced inflammation persists and causes cellular senescence in the biliary epithelium. Senescence can be induced by various factors, including repetitive replication-related telomere shortening, oncogene activation or inactivation of tumor suppressor genes, DNA-damaging interventions, and oxygen radicals (137). The first unveiled feature of senescence is the irreversible cell cycle arrest, leading to the limitation of cell division *in vitro* (138). With the deepened investigation of senescence in organisms, more hallmarks of senescence have been revealed, including intracellular accumulation of dysfunctional mitochondria, epigenetic alteration, apoptosis resistance, metabolism changes and secretion of multiple bioactive factors, so-called senescence-associated secretory phenotypes (SASP) (139). The initiation of senescence is triggered by DNA damage response (DDR), resulting in the activation of the p53 and the ERK/ETS1/2 pathways, which ultimately up-regulate the expression of $p21^{CIP1}$ (also known as *CDKN1A*) and $p16^{INK4a}$ (also known as *CDKN2A*), respectively (140, 141). As cell cycle blockers, the overexpressed $p21^{CIP1}$ and $p16^{INK4a}$ prevent cells from entering S phase from the G1 phase. Moreover, unsolvable DDR activates the retinoblastoma (Rb) and p53 pathways and promotes the formation of promyelocytic leukemia nuclear bodies, which ultimately leads to senescence-associated heterochromatin foci (SAHF) through the ASF1A and HIRA chaperones (142, 143).

Senescent cholangiocytes are accumulated in patients with PSC and alcoholic steatohepatitis and are associated with disease exacerbation (117, 144, 145). Entering senescence enhances metabolic levels, allowing cholangiocytes to resist apoptosis. Furthermore, immune cells are responsible to eliminate these apoptosis-resistant cells to prevent abnormal growth and oncogenesis (142). Nonetheless, when senescent cells persist due to an unsuccessful immunologic clearance, these senescent cells may promote aggressiveness of their neighboring malignant cells (146) or even acquire a stem cell-like phenotype themselves once being released from cell cycle withdrawal (147). The significant role of senescent cells root from not only their cell-autonomous changes but also their non-cell autonomous traits for inducing neighboring cells into senescence through their secreted TGF-β, as a bystander effect (113). The bystander effect was also unveiled in the *in vitro N-Ras*-induced senescent cholangiocytes, which promoted cell cycle arrest and SASP secretion in their surrounding cholangiocytes (117).

Senescent cholangiocytes can be found in mouse PBC samples at the early disease stage, resulting from the over-activation of the Sct/SR pathway and its induced TGF-β1 secretion, which triggered cytokine-induced senescence in an autocrine manner. SASP from these senescent cholangiocytes activates Kupffer cells and HSCs in a paracrine manner, leading to local inflammation and liver fibrosis (114). Moreover, clinical evidence also indicated an unneglected role of SASP from cholangiocytes in various cholangiopathies. For instance, C-X-C motif chemokine ligand-11 (CXCL-11) and CCL-20 from senescent cholangiocytes showed predictive value in detecting ursodeoxycholic acid (UDCA) non-responsive PBC

patients (115). Further *in vitro* study revealed that oxidative stress- and DNA damage-induced senescent BECs exhibited stronger secretion of chemokines (CCL-2/3/4/5, CX3CL-1, CXCL-1, CXCL-2, CXCL-10, and CXCL-16), thereby attracting monocyte/macrophage-like RAW264.7 cells, which suggested that the influence of senescent cholangiocytes on the pathogenesis of PBC was likely achieved by their environmental modulation (116). Furthermore, elevated secretion of pro-inflammatory factors [IL-6, IL-8, CCL-2, plasminogen activator inhibitor-1 (PAI-1)] was evident in senescent cholangiocytes in PSC (117). More evidence showed that CCL-2, CCL-20, IL-3, IL-11 and IL-15 were upregulated in senescent BECs as SASP (118).Even though senescent cholangiocytes are not well understood in BA, intrahepatic bile duct-derived organoids exhibited reduced cholangiocyte proliferation after receiving acetaminophen treatment, while enhancing the secretion of TGF-β1 and PDGF-BB, which indicated a possible role of senescence in this regard (110). The pro-inflammatory factors from SASP label senescent cholangiocytes as harmful actors involving in disease progression, which opens the door for senescence-targeted therapy, such as TGF-inhibition and senolytics, in the treatment of senescent cholangiocytes-related bile duct disorders. For example, genetic or pharmacological (Fisetin) elimination of cholangiocyte senescence reduced the release of inflammatory markers (TNF-α, IL-1 and MCP-1) and alleviated fibrosis in the progression of PSC (119).

Besides the canonical secretion of cholangiokines, cholangiocytes were reported to possibly release extracellular vesicles (EVs) containing IL-13Ra1 into the serum of PSC patients (148). Higher protein levels of Cystatin-S, IL-13Ra1, CD83, IL-1β and EMAP-2 were found in these serum EVs. However, whether and how these EVs are released by cholangiocytes are unclear due to the lack of EVs-tracing evidence. Furthermore, another study revealed that LPS-induced or PSC patient-derived senescent cholangiocytes can also release EVs, which contain multiple growth factors, including EGF, while containing low levels of cytokine/chemokine (149).

In summary, the secretory phenotypes of cholangiocytes are dynamically modified by intrinsic evolutionary factors during the life course of cholangiocytes, and by extrinsic microenvironmental factors engaging with cholangiocytes. Regarding the complexity of the cholangiocyte secretome, temporospatial regulation and cellular context must be taken into account when deciphering the role of cholangiocytes and other cell types in cholangiopathies.

Influences of cholangiokines on the hepatic environment

During liver injuries, biliary cells are susceptibly disturbed by both exogenous and endogenous stimulus, leading to cell damage. Thus, persistent damage and dysfunction in cholangiocytes trigger immune cell accumulation and inflammatory reaction, which cause variable pathological consequences, including excessive deposition of scar tissue in portal areas and biliary cirrhosis. This complex response triggered by immune cells, mesenchymal cells, and activated cholangiocytes is termed as DR (14). DR is orchestrated by a finely tuned interplay between proliferation, differentiation and trans-differentiation of cholangiocytes, hepatocytes and HPCs, ulteriorly fueling fibrogenesis and inflammation. Generally, in hepatobiliary diseases, DR refers to similar manifestations, including cholestasis, proliferation, inflammation, fibrosis, and eventually carcinogenesis (150). Nonetheless, the nature of DR remains obscure. As discussed in previous sections, cholangiocyte phenotype alterations (e.g., SASP, proliferation) during DR can drive cholangiocellular proliferation and inflammation by secreting cholangiokines, which further favors DR progression. Coinciding with current opinions, cholangiocytes are considered as not only reactors but also potential initiators in DR (14, 151, 152). In this context, cholangiokines may play crucial roles in different liver/bile duct pathological models by modulating complex cellular interactions, which will be discussed in detail in the following sections (Figure 2).

Liver regeneration

The liver has a remarkable capacity to regenerate due to the persistent occurrence of hepatocyte self-renewal. While the facultative stemization of hepatocytes has been assumed as the main origin of liver regeneration for centuries, cholangiocyte proliferation and trans-differentiation appear to be a recently recognized mechanism to enhance the liver regenerative capacity (22).

Fundamentally, HGF and ligands of epidermal growth factor receptor (EGFR), viewed as 'complete mitogens', can induce hepatocyte proliferation, even in cultures without serum supplement (153). In terms of liver regeneration, EGF, amphiregulin (AREG), TGF-α and heparin-binding EGF-like growth factor (HB-EGF) are more relevant to hepatocellular proliferation by binding to EGFR (22). Uriarte et al. discovered an increased secretion of HGF from HGF-19-treated murine cholangiocytes (154). In the $Mdr2^{-/-}$ mouse model, senescent cholangiocytes were found enriched with multiple growth factors, including EGF (149). Zhao et al. used cholangiocytes with *Cul3* (known as a tumor suppressor) gene deficiency to show that cancerous cholangiocytes are prone to secrete AREG (155). Moreover, another study indicated that cancerous cholangiocytes upregulated HSC-based HB-EGF upon TGF-β secretion (156). In addition, TNFs and IL-6 are known as 'auxiliary mitogens'. A delayed liver regeneration was recorded in mice with genetic TNF receptor 1/2 (TNFR1/2)-deficiency (157) Simultaneously, IL-6-deficient mice showed reduced activation of hepatocellular STAT3, which is a determinant in promoting proliferation (158). Interestingly, cytokines discussed above (e.g., TNF-α, IL-6) have been known as a fundamental part of cholangiocyte SASP (105).

Other than supportive functions in hepatocellular proliferation, activated cholangiocytes conduct a 'self-rescuing' program to sustain their own proliferation and survival. During this 'self-rescuing' procedure, cholangiocellular proliferation is initiated not only by genetic/epigenetic alterations but also by autocrine/paracrine cytokines. As described previously, IL-8 levels increase in PSC patients' bile. In addition, IL-8 caused cell proliferation

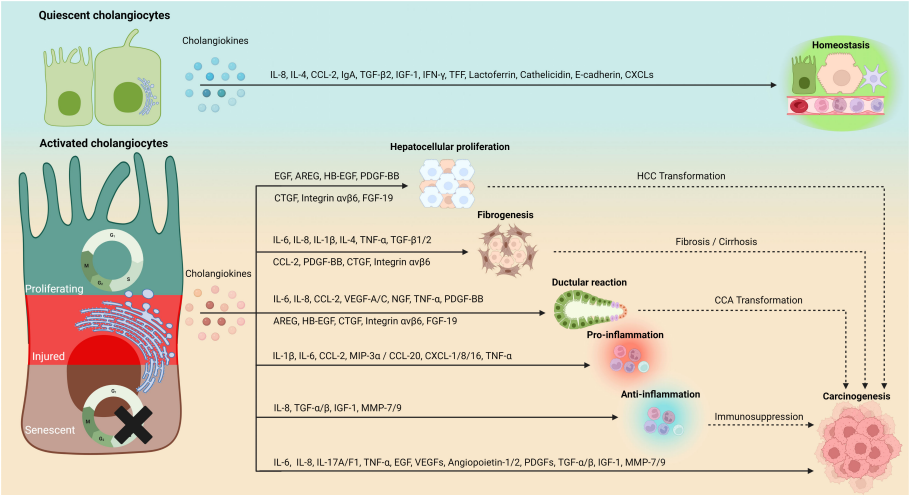

FIGURE 2
Phenotype-depending secretion and functionality of cholangiokines. Cholangiocytes convert to a major source of functional cytokines in addition to hepatocytes and immune cells. Cholangiokines exert perpetual influences on the hepatic environment. Quiescence-associated cholangiokines maintain liver homeostasis, whereas cholangiokines released by cholangiocytes at their activated statuses (e.g., proliferation, senescence and injury) mediate hepatocellular proliferation, fibrogenesis, DR and inflammation, which eventually cause hepatic carcinogenesis. IL, interleukin; CCL, chemokine (C-C motif) ligand; EGF, epidermal growth factor; IgA, immunoglobulin A; TGF-β, transforming growth factor-β; IGF-1, insulin-like growth factor 1; IFN-γ, Interferon gamma; TFF, trefoil factor; CXCL, chemokine (C-X-C motif) ligand; AREG, amphiregulin; HB-EGF, heparin-binding-EGF; FGF-19, fibroblast growth factor-19; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor, PDGF-BB, platelet-derived growth factor; CTGF, connective tissue growth factor; NGF, nerve growth factor; MIP-3α, macrophage inflammatory protein-3α; MMP, matrix metallopeptidase.

when added to primary human cholangiocyte cultures (28). The bile component TC protects cholangiocytes against injury. In mouse BDL models, TC administration can enhance VEGF-A and VEGF-C, which are key regulators of biliary proliferation during cholestasis (159, 160). Gigliozzi et al. demonstrated that cholangiocytes secrete NGF, which stimulated the proliferation of cholangiocytes *via* protein kinase B (AKT)- and ERK1/2-dependent mechanisms. *In vivo*, NGF neutralization decreased the proliferative capacity of BECs in post-BDL rats (47). More interestingly, we reported that the secretion of CCL-2 by injured cholangiocytes attracts monocytes, which in turn upregulate integrin-β6 and favor cholangiocyte proliferation. This study proposed a novel concept regarding cholangiocyte-associated cellular crosstalk during liver injury (112, 161). Taken together, bile duct repair is driven by stimulatory and inhibitory, autocrine or paracrine secretory factors originating from cholangiocytes. Promisingly, variable cells may be involved in the complex regulation of such regenerative processes.

Inflammation

Inflammation is a fundamental orchestrator of BEC response to liver injury. As discussed above, inflammatory factors effectively influence the cholangiocyte secretory programs. In turn, cholangiocytes with active secretory phenotypes regulate immune cell accumulation and polarization.

Cholangiocytes are capable of sensing exogenous stimuli, including PAMPs and DAMPs, *via* TLRs and the downstream signal pathways. Upon sensing these stimuli, signaling cascades mainly involving NF-κB, mitogen-activated protein kinase (MAPK) and inflammasome, are rapidly activated (102, 111). Consequently, a broad spectrum of proinflammatory cytokines (e.g., IL-1β, IL-8, IL-6, MCP-1, TNF-α, INF-γ and TGF-β) and chemokines (e.g., CXCL-1, -8 and -16), is released by cholangiocytes (2, 162, 163). Investigations of the liver immune landscape revealed that, the recruited leukocytes are the leading responders in the immune response towards bile duct alterations (2, 164). The first single-cell analysis of liver samples from PSC patients indicates a strong dynamism of T cells, among which naive $CD4^+$ T cells are prone to develop into T-helper (Th) 17 cells (165). Th17 cell accumulation has also been observed in the liver biopsies of PBC patients, specifically around the activated or injured intrahepatic BECs (75, 166). Reactive cholangiocytes regulate Th17 cell differentiation by IL-6 and IL-1β (167). In addition, fractalkine/CX3CL1 and CXCL1 are released by reactive cholangiocytes, which further recruit monocytes and T cells (109, 131, 168, 169). In response to biliary injury, injured or senescent cholangiocytes dramatically release TNF-α and IL-6 (105, 134). TNF-α not only activates naïve and effector T cells, but also induces apoptosis of highly activated effector T cells, further determining the scale of the pathogenic or protective conventional T-cell pool (170). Meanwhile, IL-6 is not only a key player in regulating the Th17/Treg balance, but also exerts paracrine functions to promote terminal differentiation of B cells and their subsequent secretion of immunoglobulins (171, 172).

Another important part of the liver's innate immunity is the hepatic myeloid cells, which execute crucial roles in either driving liver injury or repairing hepatic malfunction in liver diseases, such as cholangiopathies (161, 173, 174). We have revealed the cholangiocyte-monocyte crosstalk using an acute biliary cell injury mouse model. We found that the injured cholangiocytes can promote the accumulation of $CCR2^+$ monocyte-derived macrophages (MoMFs) and alter bile acid metabolism, while the

MoMFs provide important factors for cholangiocytes to proliferate and restore biliary function (112). Furthermore, we have learned from the liver samples of PSC patients and the *ex vivo* experiments that, secretion of CCL-20 and CCL-2 from human primary cholangiocytes favors monocyte infiltration (175). Additionally, Mip-3a/CCL-20 can be released by the activated cholangiocytes to induce the chemoattraction of immature dendritic cells by its binding to CC chemokine receptor 6 (CCR6) (73). To sum up, cholangiokines play a crucial role in hepatic immunomodulation. However, a more precise understanding of cholangiocyte-driven inflammation is necessary.

Fibrosis

In response to an injury, DR is driven by cholangiocyte proliferation and their secretome, participating in the complex regulation of portal inflammation and fibrogenesis (14). Inflammation generates signals that attract liver mesenchymal cells to bile ducts and portal areas. This process is considered to be the primary stage of biliary or portal fibrosis. In this context, interaction between reactive ductular cells and myofibroblast cells, so-called epithelial–mesenchymal crosstalk, is a constant key modulator in liver fibrogenesis, the process of which also involves several profibrogenic factors (e.g., IL-6, TGF-β1/2, CCL-2 and PDGF-B) (76, 94, 95).

During biliary fibrosis, proliferating BECs represent a predominant source of the profibrogenic connective tissue growth factor (CTGF) besides HSCs (176, 177). According to a recent study, reactive cholangiocytes secrete TGF-β depending on the Mothers against decapentaplegic homolog 3 (SMAD3) and lysine Acetyltransferases 2A (KAT2A). Pharmacological inhibition of Kat2a protein or cholangiocyte-selective deletion of *Kat2a* gene was protective in mouse models of biliary fibrosis (178). BECs can regulate the proliferation and myofibroblastic trans-differentiation of HSCs to provoke the portal fibrosis by the CCL-2-based paracrine (94). TGF-β1 and TGF-β2 were found upregulated in cholangiocytes during chronic liver diseases, suggesting their implication in biliary hyperplasia and fibrogenesis (76). Likewise, IL-8 secreted by the activated cholangiocytes can stimulate the production of profibrotic genes, suggesting that IL-8 may be involved in the pathogenesis of cholangiopathies (28). Grappone et al. suggested that PDGF-B chains can be produced by cholangiocytes during chronic cholestasis (179). Recently, Moncsek et al. disclosed that senescent cholangiocytes promoted the activation of quiescent mesenchymal cells in a PDGF-dependent manner (180). Another latest study has demonstrated that biliary NF-κB-inducing kinase (NIK) could trigger DR. While the ablation of NIK significantly decreased the expression of *Il-1β*, *Il-4*, *Il-6*, *iNos*, *Tnfα*, *Mcp1* and *Tgfb1*, thereby attenuating liver fibrosis (25). What's more, Liu et al. reported that cholangiocyte-derived exosomal H19 plays a critical role in the progression of cholestatic liver fibrosis by promoting the differentiation and activation of HSCs (181). Integrin αvβ6 acts as not only a crucial mediator but also a therapeutic target in liver fibrosis (182, 183). Moreover, genetic suppression of *Itgb6* (a gene encoding integrin αvβ6) in the mouse models of biliary injury is therapeutically relevant to the attenuation of DR and biliary fibrosis (184, 185). Pi et al. have revealed that CTGF and integrin αvβ6 regulate biliary cell activation and fibrosis, probably through the secretion of fibronectin and TGF-β1 (176). In conclusion, activated cholangiocytes and their cholangiokines might be promising therapeutic targets for ameliorating liver fibrosis.

Carcinogenesis

Primary liver cancers, including hepatocellular carcinoma (HCC) and CCA are a tremendous burden to global health, but their pathomechanisms are only partially understood (137, 186). From a short-term perspective, cholangiokines contribute to hyperplasia, inflammation and fibrogenesis of the hepatic portal areas. In the long run, cholangiokines may eventually fuel the malignant transformation of hepatic cells through continuous autocrine and paracrine stimulation.

IL-6 has been determined by several studies as not only a key driver but also a promising therapeutic target for liver cancers (187–189). IL-6 levels are highly presented in the serum and bile of CCA patients and culture medium of CCA cell lines (190). Recent studies concluded that HCC and intrahepatic CCA (iCCA) are significantly driven by IL-6 and its associated inflammatory processes (191, 192). IL-6 promotes the survival of transformed cholangiocytes through different pathways. In particular, the IL-6-activated p38 pathway determines cell proliferation by mediating $p21^{WAF1/CIP1}$ and p44/p42 MAPK (193). Even more intriguingly, single-cell analysis of iCCA patient specimens showed that CCA-derived exosomal miR-9-5p elicited a high secretory possibility of IL-6 in cancer-associated fibroblasts to promote tumor progression, suggesting broader roles of cholangiocyte- derived IL-6 in the tumor microenvironment (TME) (194).

EGF administration can provoke CCA progression by triggering epithelial-mesenchymal transition (EMT). In addition, the upregulation of TGF-α favors the proliferative levels of HCC cells (195). EGF and TGF-α regulate cell proliferation and differentiation by binding EGFR (196). Earlier studies also revealed a positive correlation between EGFR inhibition and HCC suppression (197, 198). Inoue et al. characterized that blocking EGFR by vandetanib in liver cancer models yielded a significantly reduced tumor vessel density and tumor growth, while enhancing tumor cell apoptosis and survival prolongation with reduced number of intrahepatic metastases (199). Moreover, it has been well elucidated that hepatic myofibroblasts promote malignancy progression in CCA patients through their HB-EGF-induced activation (156, 200), which is consistent with the fact that myofibroblasts are also prone to trigger the cholangiocyte-secreted PDGF-B (201).

TGF-β and its related signaling cascades play a central role in inflammation, fibrogenesis and immunomodulation in the TME of liver cancers (202, 203). A recent study indicated a positive feedback loop of TGF-β and LIN28B in CCA metastasis (204). TGF-β has also been found to promote the progression of CCA and HCC by interacting with non-coding RNAs (205–208). More strikingly,

TGF-β exerts immunoregulatory functions in HCC, mainly *via* suppressing T cells (202, 209). Interestingly, the blockade of TGF-β-induced activated dendritic cells enhances the lethal effects of T cells in CCA (210). Thus, TGF-β potentially disturbs immunotherapies in liver cancers, which makes it a promising target to attenuate immunotherapy resistance. Besides, TGF-β was also found to regulate monocyte/macrophages in liver cancers. Yan et al. reported that TGF-β fosters the expression of T cell immunoglobulin domain and mucin domain-3 (TIM-3/CD366) on monocytes, which augments the infiltration of tumor-associated macrophages in HCC (211). Ning et al. demonstrated that the induction of imbalanced TGF-β1/BMP-7 pathways in HCC cells could significantly reinforce the aggressiveness and stemness of HCC cells (212).

Novel observations indicate that VEGF is a master factor in lymphangiogenesis and the immune response to cholangiocarcinoma (84). The secretion of VEGFs, angiopoietin-1/2, PDGF and TGF-β from tumor cells or other cell types robustly modulate the TME, which is a critical component of tumor biology (213). The VEGF-A secretion by CCA cells can be mediated by other factors including IGF-1, its receptor IGFR as well as the estrogen receptor (ER) family (214, 215). Furthermore, estrogens induce the proliferation of CCA cells by VEGF/VEGFR2 mediation (216). VEGF-A, on the other hand, induces cholangiokines, including matrix metalloproteinase (MMP)-7 and -9, from CCA cells, which contribute to the significant remodeling of extracellular matrix (ECM) and the extensive tumor metastasis (217).

Notably, TNF-α plays contradictory roles in liver cancers. Commonly known as a participant in maintaining homeostasis of cancer immunobiology, TNF-α unveils its 'dark side' to provoke chronic inflammation, EMT and angiogenesis, which may fuel the aggressiveness of cancers (218). Interestingly, high-dose administration of TNF-α inhibits neovascularization in mice, whereas low –dosed TNF-α promotes angiogenesis by increasing the expression of VEGF, VEGFR, IL-8 and basic FGF (219). Another study underlined that TNF-α strengthened the migration behaviors of CCA cells by upregulating their EMT markers, including ZEB2, vimentin and S100A4. Moreover, TNF-α has been shown to induce *TGFB* overexpression, which eventually promotes cancer cells to migrate (220). Yuan et al. described a novel phenomenon that TNFs favor cholangiocellular proliferation, differentiation and transformation due to the induced chronic mitochondrial dysfunction and the accumulation of reactive oxygen species (ROS). This finding enriches the research directions of TNF-α meditation in CCA (221). Even though cholangiokines can hardly be concluded as a robust oncogenic secretome based on our current knowledge, various tumor-promoting cytokines secreted by cholangiocytes have been evidenced to regulate TME.

Conclusions and future perspectives

Although the quantitative contribution of cholangiocytes to the total liver mass and the hepatic secretome appears modest, cholangiocytes play essential roles in a vast array of disease-related mechanisms and shape the portal area microenvironment. Sensitized by various injuries, stimuli or immune disturbances, cholangiocytes release cholangiokines, which broadly participate in liver immunology, inflammation, fibrogenesis and malignant transformation. Particularly, cholangiokines are gaining recognition for their involvement in cholangiopathies and primary liver cancers. Of note, better characterization of the cholangiokines may provide an in-depth understanding of cholangiocyte-driven pathophysiological processes. Nonetheless, the paracrine and autocrine nature of cholangiokines poses some technical challenges, as their functions need to be interpreted in the spatiotemporal context of the hepatic microenvironment. Even though the practicability of cholangiokines as diagnostic/prognostic markers is still hidden in fog, emerging biotechnics can incarnate wind to achieve it. Recently, several novel approaches, such as multiplex immunostaining, imaging mass cytometry and spatially resolved single-cell sequencing, have emerged for *in situ* liver studies, which shed light on differential spatial heterogeneity of the hepatic parenchymal and immune cells (164, 222–224). Furthermore, by tying up the single-cell spatial or newly developed single-cell Stereo-sequencing methods (225), pathomechanisms of cholangiokines associated with time phases, zonation and functionality are anticipated to be soon and decently determined.

Author contributions

HL conceived the topic. XC and HL drafted the manuscript and prepared the figures. XC, FT, AG, and HL revised the manuscript. All authors have approved the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author FT's lab received research grants from Gilead, Allergan, Bristol-Myers Squibb and Inventiva.

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Publisher's note

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Mechanism-based target therapy in primary biliary cholangitis: opportunities before liver cirrhosis?

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Primary biliary cholangitis (PBC) is an immune-mediated liver disease characterized by cholestasis, biliary injuries, liver fibrosis, and chronic non-suppurative cholangitis. The pathogenesis of PBC is multifactorial and involves immune dysregulation, abnormal bile metabolism, and progressive fibrosis, ultimately leading to cirrhosis and liver failure. Ursodeoxycholic acid (UDCA) and obeticholic acid (OCA) are currently used as first- and second-line treatments, respectively. However, many patients do not respond adequately to UDCA, and the long-term effects of these drugs are limited. Recent research has advanced our understanding the mechanisms of pathogenesis in PBC and greatly facilitated development of novel drugs to target mechanistic checkpoints. Animal studies and clinical trials of pipeline drugs have yielded promising results in slowing disease progression. Targeting immune mediated pathogenesis and anti-inflammatory therapies are focused on the early stage, while anti-cholestatic and anti-fibrotic therapies are emphasized in the late stage of disease, which is characterized by fibrosis and cirrhosis development. Nonetheless, it is worth noting that currently, there exists a dearth of therapeutic options that can effectively impede the progression of the disease to its terminal stages. Hence, there is an urgent need for further research aimed at investigating the underlying pathophysiology mechanisms with potential therapeutic effects. This review highlights our current knowledge of the underlying immunological and cellular mechanisms of pathogenesis in PBC. Further, we also address current mechanism-based target therapies for PBC and potential therapeutic strategies to improve the efficacy of existing treatments.

KEYWORDS

primary biliary cholangitis, immune cells, bile acids, nuclear receptors, liver fibrosis

1 Introduction

Primary biliary cholangitis (PBC) is a chronic and progressive autoimmune cholestatic liver disease, which generally develop to cirrhosis and liver failure after 10–20 years without treatment. The global prevalence of PBC is estimated at 14.6 per 100 000 population, ranging from 1.91 to 40.2 (1). Both the incidence and prevalence of this condition is increasing, with the Asia-Pacific, Europe, and North America reporting annual incidences of 0.84, 1.86, and 2.75 per 100,000 population, respectively (2). The etiology and pathogenesis of PBC remain unclear, and the clinical course of the disease is insidious and heterogeneous, with variable individual responses to drug therapy. Biliary injury is a consequence of dysregulated intrahepatic and systemic immune responses, which result in cholestasis and eventual development of liver cirrhosis. The primary objective of PBC treatment is to prevent disease progression and the development of cirrhosis and liver failure. Collagen is a major extracellular matrix in fibrotic tissues (3), and its synthesis increases in PBC. The metabolic regulation of collagen biosynthesis and degradation (4) may counteract with the increased synthesis in the early stages of PBC, but cannot compensate for the extensive collagen synthesis at the late stages of PBC, resulting in gradual development of liver cirrhosis (5). Therefore, the development of new therapies for PBC requires two distinct approaches. In the early stages of the disease, the primary focus is on regulating the immune response, controlling inflammation, and improving metabolism. In the later stages, the emphasis shifts towards controlling collagen synthesis and increasing collagen degradation. Agents targeting immune-mediated pathogenesis and anti-inflammatory are probably most effective in the early stage of PBC, while anti-cholestatic and anti-fibrotic therapies are emphasized in the late stage. Although ursodeoxycholic acid (UDCA) and obeticholic acid (OCA) are approved by the Food and Drug Administration (FDA) as first and second line of therapy respectively, cirrhotic patients hardly benefit and some PBC patients are non-responsive (6, 7). This review summarizes the advances in the research of PBC pathogenesis and related treatment, with a perspective on the window of opportunity in slowing the disease progression and prevent the development of fibrosis and cirrhosis.

2 Novel advances targeting immune factors

Innate and adaptive immunity are vigorously involved at different stages of PBC. Innate immune cells include monocytes and macrophages, dendritic cells (DCs), and natural killer (NK)/natural killer T (NKT) cells are active players in the early stage of PBC (8, 9). Adaptive immune cells including antibody secreting B cells and CD3 + and CD4+ or CD8+ lymphocytes, are also critical in the early stages of the disease whereas CD8+T cells are predominant around the damaged interlobular bile ducts in early stage of PBC (10). Increasing evidence confirms the participation of different T cell subpopulations in PBC pathogenesis, including Th1, Th17, regulatory T cells (Tregs), follicular helper T (Tfh) cells, and follicular regulatory T (Tfr) cells (11). Consequently, treatment targeting immune cells and cytokines profiles have drawn much attention (Figure 1).

2.1 Targeting immune cells and related cytokines

2.1.1 Targeting B cells and related cytokines

The presence of antimitochondrial antibodies (AMA) is considered the serological hallmark of PBC. The disease specificity of AMA and high levels of serum immunoglobulin (Ig) M signifies the involvement of B cells mediated mechanisms in PBC (12, 13). Compared to healthy individuals, the frequency of $CD19^+$ B cells are highly increased in livers of PBC patients, resulting in production of higher amounts of interleukin (IL)-6, IL-10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. This can be attributed to the functional abnormality of $CD19^+CD24^{hi}CD38^{hi}$ B regulatory cells, increase in $CD3^+CD4^+CXCR5^+ICOS^+$ Tfh and $CD38^+$ plasma cells in the peripheral blood, and elevation of serum IL-21 in PBC patients in comparison to healthy controls (14). These changes positively correlated with the levels of Ig and autoantibodies in this condition (15). In patients with PBC, circulating $CD19^+$ B cells are reduced after treatment with UDCA (16).

Targeting B cell is a logical treatment strategy in PBC. Rituximab is an anti-CD20 monoclonal antibody that selectively depletes B cells. In animal studies, although anti-CD20 and anti-CD79 antibodies successfully depleted B cells and reduced the autoantibody production, it also elevated liver enzymes and aggravated PBC-like liver lesion (17). In PBC patients with incomplete response to UDCA, rituximab treatment could improve alkaline phosphatase (ALP) levels, reduce serum AMA titer, increase Treg cells numbers, and modulate cytokine production (18, 19). A phase-2 randomized controlled trial demonstrated that rituximab was safe, but did not improve symptoms of the fatigue (20). Subsequent clinical trials using a chimeric antibody against human CD20 (hCD20) showed limited efficacy. Furthermore, another humanized anti-human CD20 antibody (TKM-011) treatment, also impaired autoimmune cholangitis compared with rituximab in a mouse model of PBC (21). Hence, the efficacy of monotherapy using anti-CD20 in the treatment of PBC remains uncertain.

B-cell -activating factor (BAFF) belonging to the TNF family and a proliferation inducing ligand were thought to be involved the pathogenesis of PBC. Serum levels of BAFF are increased in PBC patients (22). BAFF inhibited IL-10 and TGF-β cytokine secretion, and induced CD4+CD25+ Tregs cell apoptosis in PBC patients (23). Bezafibrate induced BAFF activated B cells, further inhibited BAFF-induced Treg cell apoptosis (23). In an $Mdr2^{-/-}$ mice model of PBC, anti-BAFF mAb (SANDY-2) treatment reshaped hepatic B-cell receptor (BCR) repertoire and reduced the titer of the autoantibody antinuclear antibody (ANA) and the levels of its

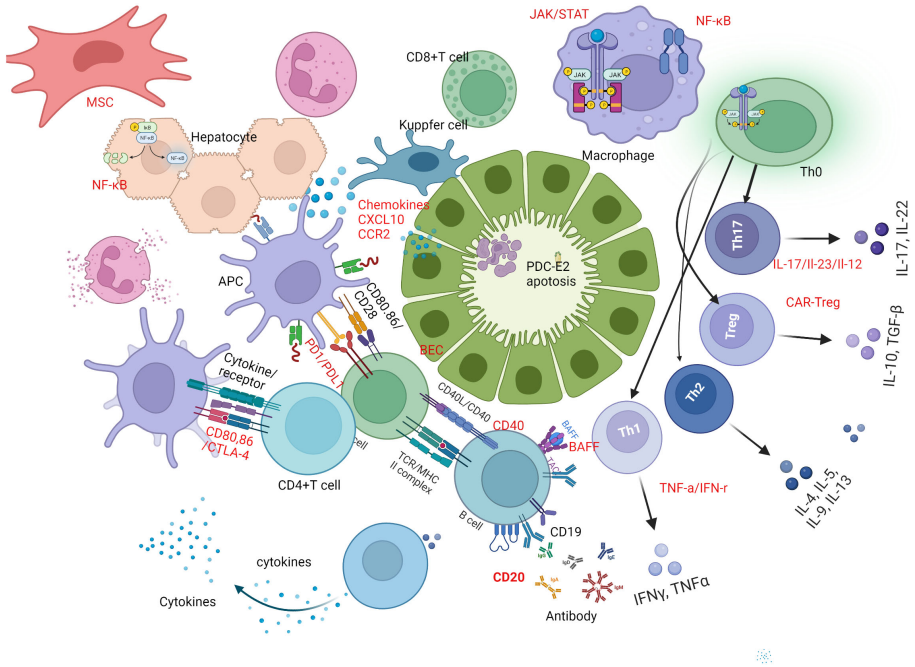

FIGURE 1
Immune mechanism based therapeutic strategies in PBC. B-cell activating factor of the tumor necrosis factor family (BAFF) and CD20-targeted therapy play a crucial role in breaking immune tolerance and stimulating immune responses in primary biliary cholangitis (PBC). Promising novel therapeutic targets for PBC treatment are highlighted in red. One potential strategy is B-cell targeted therapy, including the use of anti-CD20, anti-BAFF, or a combination of both. Another approach is T-cell-directed immunotherapy, which involves inhibiting Th1 and Th17 cell differentiation by regulating related cytokines, up-regulating Treg function and number via chimeric antigen receptor-modified Tregs (CAR-Tregs). Additionally, interfering with costimulatory signals between cells, such as targeting CTLA-4, PD-1, and CD40, has shown potential in treating PBC. Regulating related cytokines, targeting chemokines, and inhibiting signal pathways involved in PBC pathogenesis, such as monoclonal antibodies against CXCL10, JAK inhibitors, or inhibitors of the NF-κB signal pathway, represent a fourth potential approach. Finally, mesenchymal stem cells (MSCs) can be used to regulate innate and adaptive immune responses by differentiating induced pluripotent stem cells. BEC, biliary epithelial cell; PBC, primary biliary cholangitis; BAFF, B-cell -activating factor; Th1, type 1 T helper cell; Th17, type 17 T helper cell; Treg, regulatory T cell; CAR, chimeric antigen receptor; CTLA-4,cytotoxic T-lymphocyte–antigen-4; PD-1, Programmed death-1; CXCL10, chemokine (C-X-C motif) ligand 10; MSC, mesenchymal stem cells.

immune complexes. However, targeting BAFF alone could not alleviate hepatic fibrosis (24). Report from a case series found that targeting BAFF with a BAFF receptor inhibitor (belimumab) did not benefit patients with PBC; it could normalize the IgM levels but not alleviate the liver inflammation in patients with PBC (25). Binding of BAFF to its receptor on B cells is critical for the development of splenic transitional B cells to follicular cells and memory B cells. Furthermore, there is a positive correlation between serum BAFF levels, AMA titer and frequency of circulating plasmablasts (26). Hence, the use of anti-BAFF, which is most effective on transitional B cells, could down-regulate the development of B cells to memory B cells. However, BAFF receptor is not expressed on short-lived plasma cells whereas anti-CD20 therapy is effective in depleting peripheral short-lived autoreactive plasma cells. We believe strategies aiming at both peripheral short-lived autoreactive plasma cells and transitional B cells simultaneously could be effective in downregulating the B cell mediated autoimmunity in PBC. Interestingly, a recent study demonstrated that combination of anti-BAFF and anti-CD20 reduced B cells, liver portal infiltration and bile duct lesion in the ARE-Del mouse model of PBC (27). These data shed light on the potential of using a combination of biologics directed at specific immune checkpoints in B cells to treat PBC.

2.1.2 Targeting T cells and related cytokines

The relevance of IL-12/IL-23-mediated Th1/Th17 signaling pathway in the etiopathogenesis of PBC has important therapeutic implications. Livers of PBC patients are heavily infiltrated with T cells. The significance of IFN-γ secreting Th1 T cells in the immunopathology of PBC is well established (28–30). IFN-γ plays a critical role in both AMA production and autoimmune cholangitis (31–33). IFN-γ regulates key signaling pathways such as STAT, p38/MAPK, ERK, and JNK (34), blocking these IFN-γ downstream signaling pathways are likely therapeutic targets in PBC.

The predominant T cell subsets in PBC change as the disease progresses, transitioning from Th1 in the early stages to Th17 in the later stages. Specifically, Th17 activation becomes significantly dominant in the advanced and late stages of PBC. This phenomenon is well demonstrated in livers and peripheral blood of PBC patients (35, 36), as well as in animal models of PBC (37). However, studies directed to monitor and modulate the cytokine profile during disease stages are required to validate the usefulness of this strategy.

Ustekinumab is an anti-IL-12/23 monoclonal antibody used in treatment of several autoimmune conditions. IL-12/23p40 was thought to be a potential target in PBC, via the selective

suppression of IL-12 signaling (38). However, IL-12p40 also have been demonstrated to play a vital role as a negative regulator of inflammation in hepatic fibrosis of autoimmune cholangitis; in particular an animal study showed that $p40^{-/-}$IL-$2Ra^{-/-}$ mice expressed more severe portal inflammation and bile duct damage, such as portal hypertension and liver fibrosis (39). A phase 2 multicenter, open-label, proof of concept clinical trial investigating the use of ustekinumab in PBC was disappointing, as none of the patients achieved the primary endpoint. Administration of ustekinumab did not result in a decrease in alkaline phosphatase (ALP) levels of more than 40% in PBC patients who were unresponsive to UDCA treatment (40).

Th17 and mucosal-associated invariant T (MAIT) cells in the liver secreted IL-17 A, which triggered fibrosis via inducing the expression of IL-6 and other pro-fibrotic markers thus suggesting that IL-17A could be a target for anti-fibrotic treatment (41). Both IL-17A and Th17 related cytokines including IL-6 and TGF-β1 participated in the progress of liver cirrhosis. The expression of Th17 associated cytokines was also skewed in patients with PBC. The protein and mRNA levels of IL-1β, IL-6 and IL-23/p19 were up-regulated whereas (transforming growth factor) TGF-β1 and FoxP3 expression were down-regulated. Mechanistically, the synergistic activity of IL-17A and TGF-β in the production of IL-6 in dermal and lung fibroblasts depends on the convergent signaling mediated by p38 MAPK, nuclear factor-κB (NF-κB) and PI3K/Akt to some extent. Inhibiting IL-17A negatively affected TGF-β-mediated collagen-I production by SMAD signaling (42). MiR-200c is an anti-fibrotic regulator of cholestatic liver fibrosis. MiR-200c restrained the proliferative and neuroendocrine-like activation of cholangiocytes by targeting Sestrins1 and inhibiting the IL-6/AKT feedback loop to protect against cholestatic liver fibrosis (43). In this line, tocilizumab was found effective and safe in the treatment of rheumatoid arthritis (RA) in patients with PBC (44). However, there is currently only a case report available for this treatment approach, and there have been no clinical trials conducted to investigate its efficacy. Secukinumab is a human monoclonal antibody to IL-17A, which was used to treat psoriasis. Antifibrotic effect was found In 10 psoriatic patients treated with secukinumab, which could improve liver elasticity parameters (45). IL-17i treatment with secukinumab or ixekizumab improved the non-alcoholic fatty liver disease fibrosis score (46). Targeting the IL-17 axis could be a new therapeutic strategy to prevent cirrhosis of PBC. In addition, the anti-TNF-α agents, such as infliximab and adalimumab, potently suppressed IL-12/IL-23 production by inflammatory macrophage by activating FcγRs (47), but anti-TNF-α agent did not play a beneficial effect for development of cirrhosis (48).

In patients with PBC, liver infiltrating CD4+T cells and CD8+ T cells are directed against the lipoic acid binding domain of human the E2 subunits of pyruvate dehydrogenase complex (PDC-E2), and are localized at the pathological biliary epithelial cells (BECs). Elimination of these antigen specific immune responses will be critical in alleviating PBC. A Phase 2a double blind, placebo-controlled study (NCT05104853) using a nano-particle, CNP-104 harboring a PDC-E2 peptide dispersed within a negatively charged polymer matrix of poly (lactic-co-glycolic acid) (PLGA) particle is in progress to evaluate its safety, tolerability, pharmacodynamics, and efficacy in PBC patients unresponsive to UDCA. A recent study demonstrated that peptide-major histocompatibility complex class II (pMHCII)-based nanomedicines displaying PDC-E2 lipoyl domain could reprogramme cognate antigen-experienced CD4+ T-cells into disease-suppressing T-regulatory type 1 (TR1) cells in mice with characteristic pathological features of PBC. Remarkably, recruitment of TR1 cell to the liver leads to restitution of the liver microenvironment, alleviation of autoimmune cholangitis, and reversed established PBC in these mice (49).

Tregs are anti-inflammatory immune cells with a crucial role in the maintenance of peripheral tolerance. The frequency of Treg cells is lower in peripheral blood and livers of PBC patients than in healthy controls. Moreover, the number of *FoxP3*-expressing Tregs was markedly reduced in affected portal tracts in PBC livers when compared with autoimmune hepatitis (AIH) and chronic hepatitis (50). FoxP3 demethylation contributed to the reprogramming of Treg/Th17 phenotype. 5-Aza- 2- deoxycytidine (DAC) can rebuild the balance of Treg/Th17 axis via inhibiting DNA methylation of *FoxP3*, and further alleviate the progression in PBC model. Thus, DAC is also a likely future therapeutic target for reduction of inflammation in PBC (51). PDC-E2 is the major PBC autoantigen and the immunodominant epitope is well-defined at its inner lipoyl domain. The application of chimeric receptor technology to Tregs is a promising approach to induce immune tolerance in autoimmune diseases (52). The humanized mouse model *in vivo* and *in vitro* experiments showed Flagellin-specific human chimeric antigen receptor (CAR) Tregs promoted the establishment of colon-derived epithelial cell monolayers. The potential role of FliC-CAR Tregs in treating inflammatory bowel disease has been documented (53). Development of antigen/liver-specific Treg should be considered for PBC. CAR-Treg can be further gene edited to improve long-lasting outcomes in PBC (54).

AMP-activated protein kinase (AMPK) is a serine/threonine kinase known for its energy sensor function and more recently its ability to maintain *FoxP3* stability and the immunosuppressive functions of Tregs (55). AMPKα1 is a positive regulator of Tregs suppressive function. It activates AMPK to phosphorylate *FoxP3* and regulates its stability. Interestingly, Treg cell-specific AMPK α1 deletion in mice led to compromised Treg cell functions, autoantibodies production, vigorous T cell responses and autoimmune mediated liver injury (56), suggesting that AMPK activation is important for the maintenance of Treg function and the prevention of autoimmune liver disease. Moreover, the study also reported that decreased AMPK phosphorylation in Tregs and reduced in number of Tregs were also evident in PBC patients. Metformin, a pharmacological activator of AMP effectively attenuated the development of experimental autoimmune encephalomyelitis and suppressed systemic autoimmunity in C57BL/6 mice (57, 58). We contemplate that the modulation of AMPK activation treating PBC warrants to be examined.

Imbalance of Tfh cells and Tfr cells has been suggested as one of the underlying factors triggering autoimmunity (59–61). Examination of Tfh cells and Tfr cells in PBC showed that the frequency of circulating Tfh cells is increased whereas the frequency of Tfr cells are decreased in PBC livers when compared with healthy

controls. Tfr/Tfh ratio negatively correlated with serum IgM levels. A lower Tfr/Tfh ratio was more prominent in patients with cirrhosis and UDCA non-responders indicating the importance of Tfh and Tfr in the disease development of PBC to UDCA responders indicating the importance of Tfh and Tfr in the disease development of PBC (62). Moreover, cytotoxic T-lymphocyte-associated protein (CTLA)-4 expression in Tfr cells was diminished in PBC. This type of Tfr cells regulated B cell response through CTLA-4 within the germinal center (62). In addition, effector memory $CCR7^{lo}PD\text{-}1^{hi}$ Tfh cells and $CCR7^{lo}PD\text{-}1^{hi}$ Tfr cells were significantly increased in PBC patients, with their levels positively correlated with serum levels of IL-21 and ALP (62). Although UDCA therapy can alleviate such Tfr/Tfh ratio, therapeutics designed for modulating Tfh and Tfr subsets at early disease stages are desired.

Cysteine-rich angiogenic inducer 61 (Cyr61) is an immunoregulatory protein that can modulate the migration of immune cells and promote tissue repair by binding to intergins. Cheng et al. showed that administration of Cyr61 by adenovirus significantly reduced portal inflammation and biliary damage by inhibiting CD8+ T cell cytotoxicity in two mouse models of PBC (63). However, its clinical relevance remains to be determined.

The levels of serum IL-2 involved in liver inflammation and immune process; serum IL-2 levels decreased in PBC. The combination of lower serum IL-2 and higher Total BIL predicted a worse prognosis and higher tendency of liver failure in PBC patients (64). Low dose IL-2 restored immune balance of Sjögren's syndrome (SS), which was effective and well tolerated in clinical trial (65, 66). Since both pSS and PBC are autoimmune epithelitis it is tempting to speculate that low dose IL-2 could be effective for treating PBC.

2.1.3 Targeting other immune cells and related immune signals

The liver architecture is highly complex with heterogeneous functionally specific cell types such as hepatocytes, cholangiocytes, Kupffer cells, sinusoidal endothelial cells, hepatic stellate cells (HSCs), DCs and immune cells. In addition to T and B cells, immune cell populations such as DCs, NK/NKT cells, monocytes and macrophages are also involved in the pathogenesis PBC (67, 68).

Kupffer cells are sentinels of the liver-specific immune system. When activated, they can produce inflammatory cytokines and eventually damage BECs. Tyrosine-derived Clostridium metabolite p-Cresol sulfate (PCS) effectively reduced PBC related inflammation and regulated Kupffer cell polarization *in vitro* and *in vivo*. Therefore, PCS and its analogues could be effective in treating PBC (69). Mast cell (MC) infiltration are increased during liver inflammation. Activated MCs are source of pro-inflammatory mediators. MCs can indirectly manipulate Tregs functions and inhibit their suppressive and proliferative activity by influencing the intrahepatic microenvironment.

Co-stimulatory signals, cell surface molecules and mediators such as cytokines and chemokines are also vital players in the pathogenesis of PBC. Significant effort in pharmacological design is in progress focusing on those that are pertinent to liver cirrhosis. CTLA-4 gene is the first identified non-MHC susceptibility locus. There is a strong linkage between the CTLA-4 exon 1 polymorphism and PBC (70). Moreover, the number of CTLA-4 copies was found to be positively correlated with inducible co-stimulator (ICOS) and *FoxP3* expressions in PBC patients; lower number of CTLA-4 copies was associated with cirrhosis and decreased expression of CTLA-4 in late stage PBC (71). Lower levels of CTLA-4 mRNA copies were related to the immune suppression caused by cirrhosis. Decreased CTLA-4 and increased ICOS could contribute in the pathogenic process by enhancing B cell and GC response in PBC (62). Preclinical studies on CTLA-4 Ig (abatacept) in PBC murine model showed that treatment with abatacept both before and after immunization improved liver histology, reduced T cell infiltrates and biliary cell damage in the liver. CTLA-4 Ig also inhibited AMA production and autoimmune cholangitis as a preventative agent (72). However, the outcome of abatacept treatment was disappointing in clinical trial; there were no significant changes in serological levels of ALP, ALT, total BIL, albumin, Ig, or liver stiffness from baseline to week 24 after initial treatment (73). The significance of discovering therapies for established PBC cannot be overemphasized, as research studies primarily focusing on "prevention" fail to capture the true clinical conditions that exist in the real world where PBC has already manifested itself. Therefore, it is imperative to shift the focus of research efforts towards finding treatments for PBC that has already developed, to help patients manage the condition effectively.

Programmed death-1 (PD-1), a member of the CD28 superfamily of co-stimulatory molecules, is widely expressed on activated T cells and B cells. Abnormal expression of PD-1 pathway in the liver may contribute to inflammation and autoimmune injury. In the $Ae2_{a,b}^{-/-}$ mice model of PBC, PD-L1 expression in mouse BECs was induced by IFN-γ. PD-1/PD-L1 interaction resulted in intrahepatic T-cell activation and the deletion of activated intrahepatic CD8+ T cells in early stage. PD-L1 expression on biliary epithelia can be induced by IL-10 and TGF-β and plays a key role in T-cell tolerance (74). Studies have shown that abnormality in the PD-1 pathway in the liver contributes to inflammation and autoimmune injury in PBC. In patients with PBC, PD-1 was expressed abundantly on liver-infiltrating T cells around injured bile duct (BD) (75),while the mRNA levels of PD-1, PD-L1 and PD-L2 were decreased in the peripheral blood. The PD-1 ligands were regulated by IFN-γ in PBMC of PBC patients (76). Recently, Zhang et al. reported that the expression of PD-1 in peripheral CD8+T cells was decreased, while the level of PD-L1 in human intrahepatic biliary epithelial cell (HiBEC) line was also down-regulated. Hence, silencing of PD-1/PD-L1 pathway with decreased PD-1 expression in CD8+T cells, downregulation of PD-1/PD-L1 in the portal areas, increased CD8+T cell proliferation subsequently enhanced CD8+T cell-mediated cytotoxicity and induced BEC apoptosis (77). Pembrolizumab was the first anti-PD-1 antibody, which produced the therapeutic effects by inhibiting negative signaling via the PD-1/PD-L1 axis, but did not change the phenotype or function of Tregs *in vitro* (78). A case report of a melanoma patient with known PBC/AIH who was administrated with pembrolizumab suggested its safety in humans (79). Further studies including clinical trials are needed to verify the safety and efficacy of PD-1/PD-L1 pathway biologics in reducing biliary damage and liver cirrhosis in PBC.

The trans-membrane protein receptor CD40 and its ligand CD154 (CD40L) are members of the TNF receptor superfamily. CD40 is expressed by a variety of antigen-presenting cells (APCs) and CD154 is mainly expressed on activated CD4+ T-cells, they are synergistically involved in co-stimulation of immune cells. Genome-wide association studies (GWAS) and transcriptome analysis indicated that IFN-γ and CD40L were upstream regulators in both disease susceptibility and activity of PBC (80). Administration of an anti-CD40 ligand monoclonal antibody reduced peripheral T cell activation and improved cholangitis in the dnTGFβRII mice model of PBC (81). In PBC patients, the expression of CD40L mRNA increased while DNA methylation of CD40L promoter was decreased in CD4+T cells, and the level of CD40L and serum IgM were negatively correlated with the CD40L promoter methylation (82). A Phase I/II study (clinicaltrials.gov, NCT 02193360) of an anti-CD40 monoclonal antibody (FFP104; Dacetuzumab/Lucatumumab) in PBC patients was conducted to evaluate its safety, tolerability and pharmacodynamics (83).

All immune cells including T and B cells are derived from hematopoietic stem cells. Mesenchymal stem cells (MSCs) are the most common cell source for stem cell therapy. MSCs played an important role in the modulation of innate and adaptive immune responses and was considered promising therapeutic agents for PBC. MSCs therapy is a potential treatment for PBC. Experimental evidence showed that bone marrow (BM)-MSC might be effective in a PBC mouse model. PBC mouse model induced by injecting polyI:C was treated by allogeneic BM-MSC transplantation, which could regulate systemic immune response and enhance recovery in liver inflammation (84). Human umbilical cord–derived MSCs (UC-MSCs) inhibited the responses mediated by Th1 and Th17, decreased the activities of pro-inflammatory chemokines and alleviated 2-octynoic acid coupled to bovine serum albumin (2OA-BSA)-induced autoimmune cholangitis (85). There have been three MSC based clinical trials for PBC. The first one (NCT01662973) showed that umbilical cord derived MSCs therapy is safe and feasible (86); the second one (NCT01440309) showed that BM-MSC therapy could improve the quality of life and decrease the levels of liver enzymes for as long as 12 months (87), the third one is expected to enroll 140 subjects with 24 month follow up (NCT03668145). MSCs therapy is a promising treatment for PBC, technological advance in generating induced MSCs from differentiation of induced pluripotent stem cells, and application of gene editing and 3-dimensional(3D) culture can enhance the availability and potency of MSCs for therapeutic application (88). We believe that induced MSCs could represent a new breakthrough in therapy for PBC.

2.2 Targeting Immune mediators and related signaling pathway

2.2.1 Targeting chemokines of inflammation and fibrosis

Chemokines are signaling proteins that can induce directional chemotaxis in neighboring cells. Hepatocytes, stromal cells and biliary epithelial cells can secrete chemokines mitigating cell migration and tissue infiltration. In PBC, chemokines mediate leukocyte recruitment and subsequent immune mediated damage of intrahepatic BECs. The role of chemokines in pathogenesis of PBC, especially abnormality of C-X-C motif chemokine receptor (CXCR)3 axis, has been reported (89, 90). The levels of chemokines such as IFN-γ-inducible protein-10 (IP-10)/chemokine (C-X-C motif) ligand 10 (CXCL10), monokine induced by IFN-γ (MIG/CXCL9) and CXCR3 were found to be increased in PBC patients and their first-degree relatives, with the expression of IP-10 and MIG in the portal areas. In addition, the frequency of CXCR3-expressing cells in peripheral blood was significantly higher in PBC. CXCR3-positive cells were prominent in the portal areas of diseased livers, primarily on CD4+ T cells (89). The serum levels of MIG and IP-10, CXCR3 expression of peripheral blood mononuclear cells significantly decreased after UDCA administration in PBC patients (90). Serum concentrations of most chemokines primarily responsible for Th1 or Th17 cell chemotaxis, such as IP-10/CXCL10, CXCL11 and fractalkine (FKN)/CX3CL1 were increased throughout the PBC disease course. On the other hand, chemokines predominant for Th2 cell recruitment, for example CCL17, CCL22 and CXCL5, were decreased in PBC patients (91).

The serum level of CX3CL1 was the only chemokine that positively correlates with PBC stage, which increases only in advanced PBC (91). NI-0801 is a fully human monoclonal antibody against CXCL10, which inhibited the combination of CXCL10 with its receptor CXCR3. An open-label, single-arm, phase 2a, proof-of-concept, multicenter study (NCT01430429) was conducted in 29 PBC patients with inadequate response to UDCA. Unfortunately, administration of NI-0801 at a dose of 10 mg/kg did not attain the therapeutic benefit, with headache being the commonly reported adverse event (92).

The level of CXCL13 was higher in serum and liver of treatment-naïve PBC patients. The serum CXCL13 level decreased with oral UDCA, while intrahepatic CXCL13 increased the recruitment of CXCR5+ lymphocytes to liver, eventually resulted in abnormal production of autoantibodies by B cells (93). Studies targeting at intrahepatic CXCL13 should be explored.

In the 2OA-BSA induced PBC model, CCR2-deficient mice manifested milder disease. CCR2 recruited infiltrating Ly6C^{hi} monocytes into the portal zone of livers. Administration of cenicriviroc, a dual CCR2/CCR5 inhibitor, improved liver fibrosis in this PBC animal model (94). Cenicriviroc attenuated disease severity in by decreasing serum bile acids and improving histological severity scores (94). The therapeutic effects of cenicriviroc need to be further investigated in clinical trials.

Substantial data suggested that the FKN–CX3CR1 axis is involved in the pathogenesis of PBC (95, 96), CCL2 and CX3CL1 produced by senescent BECs was up-regulated. These chemokines promoted infiltration of CCR2 and CX3CR1 positive cells and further aggravate inflammation in bile duct lesion in PBC. Anti-FKN mAb E6011 inhibited recruitment immune cells by blocking the FKN–CX3CR1 axis, which was expected to be useful for Crohn's disease (CD), RA, and PBC (97). A phase II, double-blind, placebo-controlled study showed the clinical benefit of RA patients with inadequate response to methotrexate, although the primary endpoint was not achieved (98). Phase 1 study of E6011 in

patients with CD showed it was well-tolerated and might be effective (99). Unfortunately, no clinical trials for PBC have been conducted so far.

2.2.2 Targeting immune signal pathways

Genome-wide studies have identified several candidate genes responsible for antigen presentation and lymphocyte signaling, for example IL-12-JAK/STAT signaling and the NF-κB and TNF signaling pathways (100). To date, studies on signaling pathways in PBC are mainly conducted in animals, with a few clinical trials.

The role of JAK/STAT signaling pathways in many autoimmune diseases has been demonstrated and related drugs were used widely, such as RA (101). Recently the role of JAK/STAT signaling pathway in autoimmune cholangitis was reported. In animal experiments, when ARE-Del+/− mice were treated with the JAK1/2 inhibitor ruxolitinib (102), the level of splenic Tregs increased, and that of splenic CD4+ T, CD8+ T, Tfh cells and germinal center (GC) B cells decreased. The hepatic CD4+ T cells and CD8+ T cells were also suppressed. Ruxolitinib inhibited the expression of IFN-γ gene by the JAK-STAT pathway. A clinical trial for baricitinib (LY3009104) in PBC patients who did not respond to or could not take UDCA (Clinical Trials.gov Identifier: NCT03742973) was conducted to evaluate the efficacy and safety of baricitinib. The study was terminated early because of low enrollment. Two patients were enrolled and completed the trial, one was randomized to receive baricitinib 2 mg/day, and the other received placebo (103). Over the treatment period, a single non-serious treatment-emergent adverse event of moderate sinusitis was reported by the baricitinib treated patient at day 47. This patient demonstrated a rapid and significant decline in ALP, markers of inflammation, pruritus and self-reported depression during a 12-week treatment period, but ALP rebounded to pre-treatment levels during a 4-week post-treatment follow-up. The placebo-treated patient did not show improvement in such biomarkers (103).

With a growing body of evidence identified the important role of the TNF super-family and downstream inflammatory signaling pathways, including NF-κB signaling pathway, in the pathogenesis of PBC, drugs directed at this mechanism is thus of pharmacological interest. The Sirt1 signaling pathway plays a principal role via NF-κB subunit in the development of PBC (104) and therefore a future target for the treatment of PBC. Mammalian Sirtuin-1 (Sirt1), a yeast silent information regulator 2 (Sirt2) homologs, is able to regulate hepatic BAs homeostasis and central metabolic functions through deacetylation. The level of Sirt1 mRNA level was increased in liver tissue of PBC patients, while SIRT1 protein level was up-regulated in the liver during human and murine cholestasis. Over-expression of Sirt1attenuated FXR-mediated inhibition of bile acid synthesis and contributed to the accumulation of bile acids, further induced liver cells apoptosis and aggravated liver inflammation and injury (105). Resveratrol, a Sirt1 activator, suppressed inflammatory responses of PBC by p65 subunit of NF-κB in animal model. Thus far, clinical trials on such related targeted drugs in PBC have not been conducted.

The TLR4/MyD88/NF-κB signaling pathway was activated, and the TLR4 and NF-κB mRNA levels increased in liver tissues of PBC mice. This pathway resulted in liver damage and cell apoptosis by inducing the release of inflammatory factors and producing apoptotic proteins in Poly I:C model mice (106). NF-κB regulated numerous cytokines, and PPARα can interfere with NF-κB signaling. Fenobrate, a peroxisome proliferator activated receptor α-agonist, mediated PPARα activation, regulated inflammatory pathways and inhibited the production of pro-inflammatory cytokines *in vivo* as well as *in vitro* in animal studies. Recently, it was reported that fenofibrate decreased the levels of many pro-inflammatory cytokines by inhibiting nuclear NF-κB p50 and p65 protein expression on the NF-κB signaling pathway, which likely contributed to its anti-inflammatory effects in PBC (107). A CCR2 small interfering RNA silencing (siCcr2)-based therapy by loading multivalent siCcr2 with tetrahedron framework DNA nanostructure (tFNA) vehicle (tFNA-siCcr2) reduced inflammatory mediator production by blocking the NF-κB signaling pathway and attenuated liver fibrosis by regulating the immune cell function in animal experiment (108).

Wnt/β-catenin signaling is critical for various aspects of biliary physiology and pathology, including bile acid secretion, regeneration, and homeostasis. Wnt/β-catenin signaling takes part in hepatocyte–BEC trans differentiation and hepatobiliary repair (109). A crosstalk between TGF-β/Smad3 and Wnt/β-catenin pathway promotes abnormal extracellular matrix production, which is involved in the progression of fibrosis. β-catenin binds to the cofactor CREB binding protein (CBP) or a homolog of CBP P300 and induces target gene transcription. Inhibition of WNT/β-catenin signaling can attenuate fibrosis. Wnt/β-catenin signaling also regulate T cell development and function (110). OP-724 is the specific CBP–β- catenin antagonist. Studies in animal models have verified that OP-724 decreased the Bile acids (BAs) by Egr-1 signaling and exerted anti-fibrotic effects by inhibiting the infiltration of inflammatory cells (111). An open-label phase 1 trial showed in patients with advanced PBC, intravenous OP-724 infusion was well tolerated. Although it did not significantly improve liver function, its anti-fibrotic effects were indicated by decreased in collagen in livers of PBC patients with advanced fibrosis (112).

The Notch signaling pathway was abnormally activated in fibrotic patients (113). This pathway takes part in cholangiocytes proliferation cycle. Inhibition of Notch signaling pathway can prevent biliary liver fibrosis and the abnormal proliferation of cholangiocytes (114). Niclosamide is an FDA approved oral anthelmintic drug. It was found that niclosamide inhibited several intracellular signaling pathways including the Notch pathway during other disease therapy. Niclosamide is a promising antifibrotic agent, which significantly reduced liver enzymes and reduced inflammation by decreasing TNF-α, IL-6, NF-kB and p-STAT3 in PBC animal model (115).

3 Targeting bile acid metabolism

The metabolism of BAs, especially enterohepatic circulation, plays a vital role in cirrhosis and portal hypertension (116). BAs can activate different receptors, including nuclear receptors (NRs) and membrane receptors and subsequently affect downstream immunological

responses. A schematic representation of BAs metabolism in liver and intestine and the BA targets that are of relevance in treating PBC are shown in the figure (Figure 2). Autoimmunity and cholangitis have the potential to be improved via regulation of the immune system. BECs survival may be extended by fortifying the bicarbonate umbrella or improving cell membrane integrity (117). Drugs that antagonize BAs toxicity, such as UDCA and nor-UDCA, might be effective at all disease stages. UDCA obtained the cumulative experience over the past decades, but the study aiming at this classical and traditional drug still continue.

3.1 The advance and mechanism of classical medicine

UDCA is recommended as the standard first line treatment for PBC. The recommendation highlights a dose–response relationship and the importance of the 13–15mg/kg dose (118). UDCA is a endogenous bile acid, which plays its therapeutic role by multi-aspect mechanisms, including accelerating bile acid enterohepatic circulation, stabilizing the biliary $HCO3^-$ umbrella, anti-apoptosis, and anti-inflammatory (119). A large multicenter study indicated that UDCA therapy improved liver transplant (LT)-free survival in all patients with PBC, regardless of the disease stage and the observed biochemical response (118, 120). Unfortunately, although UDCA monotherapy improved overall LT-free survival, approximately 30-40% patients do not respond favorably to UDCA. A recent study showed that PBC patients benefited more from add-on therapies in which UDCA is combined with glucocorticoids or immunosuppressants (121). Bezafibrate combination with UDCA resulted in better biochemical response and lower predicted mortality or LT need than those treated with UDCA alone (122).

3.2 Targeting bile acid receptor

BAs consist of a group of multitudes endogenous signaling molecules, that each activates specific receptors such as farnesoid X

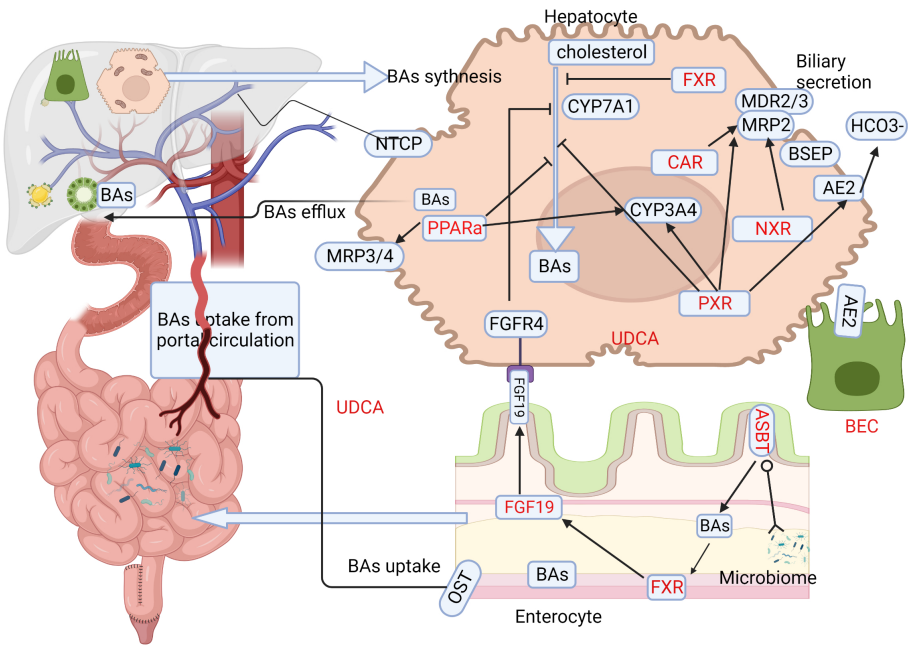

FIGURE 2
BAs metabolism in liver and intestine and associated therapeutic targets in PBC. The major process of BAs metabolism during synthesis in the liver and their uptake in the enterohepatic circulation provide various windows for developing effective treatments in PBC. Target locations for therapy are highlighted in red. Ursodeoxycholic acid (UDCA) is the classical treatment, and its basic mechanism is to adjust the metabolism of BAs. The first part of PBC treatment involves targeting BAs synthesis, and medication mainly targets nuclear receptors (NRs) such as farnesoid X receptor (FXR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPARα), and constitutive androstane receptor (CAR). Primary BAs are synthesized primarily through the classic pathway, with CYP7A1 being the limiting enzyme. FXR receptors are expressed widely in hepatocytes as well as enterocytes, and BAs inhibit CYP7A1 via the induction of small heterodimer partner in hepatocytes, while in enterocytes, they induce the production of FGF-19, which acts via FGFR4 to inhibit CYP7A1 and BAs synthesis. PXR also plays a vital role in inhibiting CYP7A1. PPARα promotes BAs efflux via MDR3 and MRP3/4, detoxifying BAs and counteracting intrinsic bile toxicity by CYP3A4. The second aspect focuses on the gut-liver axis and gut microbes, including gut microbiota and apical sodium-dependent bile acid transporter (ASBT) inhibitors. Secreted BAs are actively absorbed via luminal ASBT in the distal small bowel, from where they are transported to the portal circulation via organic solute transporter (OST). The reabsorbed BAs are taken up by hepatocyte sinusoidal membrane protein NTCP and re-secreted. The third aspect targets biliary epithelial cells (BECs), as apoptosis of BECs plays an important role in PBC pathogenesis. BECs secrete inflammatory cytokines/chemokines and other antimicrobial molecules, serving as a bridge between bile acid metabolism and the immune response. BAs,Bile acids; PBC, primary biliary cholangitis; UDCA, ursodeoxycholic acid; NRs,nuclear receptors; FXR, farnesoid X receptor; PXR, pregnane X receptor; PPARa, peroxisome proliferator-activated receptor alpha; CAR, constitutive androstane receptor; CYP7A1,cytochrome P450 family 7 subfamily A member 1; FGF-19,fibroblast growth factor 19; FGFR4,FGF receptor 4; MDR3,multidrug resistant protein 3; MRP3/4,multidrug resistance-related protein 3/4; CYP3A4, cytochrome P450 family 3 subfamily A member 4;ASBT,apical sodium–dependent bile acid transporter; OST, organic solute transporter; NTCP, sodium taurocholate cotransporting polypeptide; BECs,biliary epithelial cells.

receptor (FXR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPARα), constitutive androstane receptor (CAR), and vitamin D3 receptor (VDR), as well as the membrane G protein-coupled receptors Takeda G protein receptor 5 (TGR5) and sphingosine-1-phosphate receptor 2 (S1PR2) in the gastrointestinal tract. Physiologically, BAs receptors function as a guard in maintaining gut barrier function and portal pressure. BAs act on a myriad of NRs including FXR and PPAR to regulate cholestasis, inflammation, and fibrosis. It is not surprising that BA receptors are enthusiastically pursued as potential therapeutic targets in PBC.

3.2.1 Farnesoid X receptor

FXR-agonists target both the gut and the liver (Figure 2). A well-known agonist of FXR is OCA, which plays anti-inflammatory and anti-fibrotic role by targeting the activation of both liver sinusoidal endothelial cells and Kupffer cells. OCA was recommended as a second-line treatment for UDCA non responders. OCA was shown to be effective and safe in a 3-year clinical trial and follow-up study (123). Its efficacy was also evident in about 43% of UDCA non-responders in real-world (124). The recommended dose of OCA is 5-10mg, with incidence of pruritus increased with dose (125). The FDA issued a new warning in 2021that OCA use in PBC patients with advanced cirrhosis should be restricted due to risk of serious liver injury (126). A combination of UDCA and OCA provided satisfactory clinical outcomes for patients inadequately responded to UDCA monotherapy (127), while add-on therapy with OCA and bezafibrate improved the prognostic markers of difficult-to-treat PBC (128). The triple combination therapy of UDCA, OCA and fibrates improved or normalized the biochemical and clinical features of PBC, such as pruritus, but the safety and side effects needed to be evaluated with longer and larger studies (129). New therapies for PBC targeting NRs including FXR and PXR have generated encouraging results. The combination of FXR agonists and PXR agonists might be a potential approach in avoiding cirrhosis, such as combination therapy of OCA and budesonide for PBC (130). Long-term OCA therapy appears to optimize the prognosis of PBC. OCA is a steroidal FXR agonist, which has poor bioavailability and aqueous solubility. Further studies on pharmacological and toxicological features of OCA and its derivatives may help to enhance its efficacy. Another steroidal FXR agonist, EDP-305, suppressed liver injury and fibrosis without promoting ductal proliferation reaction in two murine models with pre-established biliary fibrosis (131). However, a phase II randomized, double-blind, placebo-controlled study (NCT03394924) on EDP-305 in patients with PBC did not achieve the primary end point (132).

Nonsteroidal synthetic FXR agonists are also therapeutically effective in treating cholestasis diseases. For example, Tropifexor (TXR) binds to the FXR ligand-binding domain and regulated FXR target genes in the liver and intestine. TXR increased FGF19 secretion by activating FXR in the ileum and suppressed bile acids synthesis in the liver. TXR inhibited cholestatic liver injury and fibrosis by modulating the gut-liver axis (133). Clinical trials have shown that TXR was generally safe and well tolerated at daily doses of 30–90 ug, which improved cholestatic markers and the hepatocellular injury marker (134). TXR improved primary bile acid diarrhea by prolonging the ascending colonic transit half-time (135), but the similar side effect of pruritis as OCA still existed because of TGR5 activation.

A Phase 2 clinical trial (NCT02943447) of another nonsteroidal FXR agonist, Cilofexor (GS-9674) yield promising results, with 9% of PBC patients reached the target endpoint of ALP less than 1.67 ULN in the 30-mg group and 14% in 100-mg group. However, 7% of patients in the 100-mg group discontinued treatment due to pruritus. Experimental study demonstrated that a non-bile acid FXR agonist PX20606 greatly improved portal hypertension in a partial portal vein ligation induced non-cirrhotic hypertension. PX20606 also reduced liver fibrosis and sinusoidal dysfunction in a carbon tetrachloride induced cirrhosis rodent model (136). The effects of PX20606 in cholestasis disease such as PBC remains to be determined.

3.2.2 Peroxisome proliferator-activated receptor agonists

Both PPAR and FXR belong to the nuclear receptor family. PPAR regulated bile formation, inflammation and fibrosis as transcriptional modifiers. The PPAR nuclear receptors have 3 isoforms, PPARα, PPARδ and PPARγ. Bezafibrate and Fenofibrate are two major types of PPARα agonist used in treating PBC. Bezafibrate is considered as the third treatment option for PBC, after UDCA and OCA. A clinical trial of add-on therapy with bezafibrate and UDCA for 24 months reported a higher rate of complete biochemical response and an improvement in liver fibrosis in the combined therapy group than the UDCA monotherapy group (137). Fenofibrate is a more selective PPARα agonist, which significantly improved liver biochemical parameters and alleviated pruritus in PBC (138). Fenofibrate in combination with UDCA therapy improved LT-free survival and histological features, including fibrosis and ductular injury, in advanced PBC or liver cirrhotic patients (139, 140). Pemafibrate, a new selective PPARα modulator, was recommended for treating PBC with dyslipidemia, or for patients with poor response to UDCA monotherapy or bezafibrate plus UDCA combination treatment (141). It is also noted that switching from Fenofibrate or bezafibrate to Pemafibrate reduced adverse effects for patients with incomplete response or some renal disorder (142, 143).

Seladelpar (MBX-8025) is a selective PPARδ agonist. A phase II trial reported that patients received Seladelpar could regain normalized ALP levels after 12 weeks of treatment, but the study was terminated early due to increased aminotransferases in the high dose group (144). A phase III trial (ENHANCE) for Seladelpar found that ALP levels were significantly reduced with mild to moderate adverse events in nearly 45% of patients treated with 10-mg dose (145). With its effectiveness in improving liver biochemistry and symptoms, Seladelpar is likely a future second-line agent for PBC (146).

Elafibranor, a dual PPARα/δ agonist, significantly reduced PBC disease activity markers over 12 weeks in a phase II clinical trial

(NCT03124108) (147). A double-blind phase III trial (ELATIVE; NCT04526665), aiming at validating effectiveness and safety of elafibranor (80 mg/day) on cholestasis in PBC, is currently ongoing. Saroglitazar is a novel dual PPAR (a/γ) agonist. Clinical trial of saroglitazar showed promising rapid and sustained improvement of ALP in treated PBC patients (148, 149).

3.2.3 Pregnane X receptor

PXR is involved in regulating the biosynthesis, transport, and metabolism of BAs. It regulates BA synthesis by down-regulating cholesterol 7a-hydroxylase (CYP7A1). PXR also has anti-fibrotic and anti-inflammatory properties. Budesonide is a dual agonist of nuclear glucocorticoid receptor and PXR, and has anti-inflammatory as well as immunosuppressive capabilities. Budesonide is also involved in BAs synthesis, metabolism and transport. Unfortunately, budesonide add-on therapy in non-UDCA responsive PBC patients UDCA was not able to reduce liver pathology (150). Moreover, budesonide is not recommended or cirrhotic patients due to risk of increased portal vein thrombosis (132).

3.2.4 Other anti–cholestatic agents beyond NRs

FGF19 is an endocrine hormone, which have antifibrotic effects through reduction of bile acid synthesis and activation of the oxidative stress response. Aldafermin (NGM282), a non-tumorigenic FGF19 analogue, improved cholestatic liver enzymes levels compared with placebo in a clinical trial (NCT02026401) and well tolerated in PBC patients (151). Future studies should focus on decreasing hepatic decompensation or cirrhosis.

3.3 Targeting gut-liver axis and gut microbes

The intricate relationship between the gut–liver axis, liver cirrhosis and portal hypertension have positionally centered targeting the gut–liver immune axis as a prospective treatment strategy in PBC. The gut–liver axis highlights the close anatomical and functional relationship between gut and liver. Gut dysbiosis impaired the intestinal barrier and altered human immunity status, enabling bacterial metabolites to reach to the liver through the portal vein (116). The pattern recognition of microbial molecules by cell surface receptors lead to activation of immune system subsequent proinflammatory responses in the liver. Gut microbial dysbiosis was evident in treatment-naïve PBC and could be partially ameliorated by UDCA (152). Gut microbiota and bacterial translocation play an important role in the pathogenesis of PBC, cirrhosis and its complications of portal hypertension (152, 153). The gut microbiota plays a key role in regulating bile acid metabolism, influence intestinal permeability and portal hypertension through the FXR. At the same time, cirrhosis and portal hypertension can have an effect on the microbiome and increase translocation.

3.3.1 The microbiome-based therapies

The three biomes of the microbiome including the immunobiome, endobiome and xenobiome, interact with the host play important roles in the pathogenesis of cholestatic liver disease (154). Molecular mimicry between bacterial proteins could generate humoral and cellular immune response to break tolerance to PDC-E2 revealed the complex orchestration of microbiome and immunobiome in the pathogenesis of PBC (155). Recent studies reported that in patients with liver fibrosis, both the microbiome composition and bile acid composition are altered, suggesting that the gut-microbiota- bile acid axis is a potential target for in treating liver fibrosis (156). The current advances of gut microbiome-based therapies include antibiotics, probiotics, fecal microbiota transplantation (FMT) and precision microbiome-centered therapies. Although these strategies have been successfully used in treating cholestatic liver and intestinal disorders (157), they have not been examined in PBC. Regulating BAs homeostasis by targeting FXR are still the main treatment strategy aiming at gut microbiome in PBC. Both liver biochemistry values and circulating levels of BAs were improved after administrating of cholestyramine PBC patients, their gut microbiota and the composition of BAs were also altered. The effect of cholestyramine on compositional and functional alterations in gut commensal was also evident (158). Bile Acid -microbiota interaction should be explored in treating PBC.

3.3.2 Apical sodium–dependent bile acid transporter inhibitors

The liver has enormous capacity to regulate cholestasis by reducing uptake systems and BAs synthesis. ASBT inhibitors can increase intestinal bile salts absorption and decrease the BA load, and logically should be considered for treating PBC. Several trials have been conducted on small-molecule ASBT inhibitors in PBC. Most of these trials focused on pruritus symptom of PBC. Linerixibat (GSK2330672), a selective inhibitor of ASBT, may treat cholestatic pruritus in this disease setting. Three trials (NCT05448170) (GLIMMER) have documented that Linerixibat effectively reduced pruritus and total serum BA concentrations compared with placebo and also well tolerated (159–161). However, data on preventing cirrhosis are not available. A phase III trial (NCT04950127) named GLISTEN (Global Linerixibat Itch study of efficacy and safety) is ongoing. This study aims to evaluate the efficacy and safety of Linerixibat in 230 participants with PBC and cholestatic pruritus.

Maralixibat (Lopixibat/LUM001/SHP625) is a selective ASBT inhibitor. In a phase II RTC (NCT01904058) study, there were no significant differences in pruritus reduction, cholestasis and hepatocellular injury markers between maralixibat and placebo groups due to a strong placebo effect.

The secretin (Sct)/secretin receptor (SR) signaling pathway regulates the bicarbonate umbrella and stimulates biliary bicarbonate via cyclic cAMP-mediated opening of the cystic fibrosis transmembrane conductance regulator (CFTR) and activates the anion exchanger protein 2 (AE2), which played a key role in maintaining biliary homeostasis. The serological expression of Sct and SR in hepatobiliary and Sct levels were increased in early-stage PBC patients. SR antagonist (Sec 5–27) reduced bile duct damage and liver fibrosis by inhibiting Sct/SR axis in early-stage PBC (162). Sct regulated biliary proliferation and

bicarbonate secretion in cholangiocytes via SR in mouse models and human samples of late-stage PBC. Reduced Sct/SR/CFTR/AE2 axis and anterior grade protein 2 (Agr2)/MUC1 levels were detected in isolated late-stage human PBC cholangiocytes, and they were restored after one week of *in vitro* treatment with Sct. Such reduction in biliary Sct/SR/CFTR/AE2 expression and bile bicarbonate levels lead to liver inflammation and fibrosis in late-stage disease in a PBC mice model. Importantly, ductular reaction and biliary senescence were ameliorated by supplying Sct (163). Both short- and long-term Sct treatment promoted bicarbonate and mucin secretion and hepatic bile acid efflux, thus reducing cholestatic and toxic BAs-associated injury in late-stage PBC mouse models (163). This indicated the expression of Sct/SR signaling can be vary with PBC disease stages. Further understanding on mechanism of differential Sct/SR expression in hepatobiliary cells PBC is necessary for designing new diagnostic and therapeutic approaches for the management of PBC.

3.4 Targeting biliary epithelial cells

BECs is the major type of hepatic epithelial cells lining both the intracellular and extracellular bile ducts, forming a biliary tree. BECs expresses MHC class I and class II and are active participants in immune-mediated liver diseases. Immunologically, BECs secrete inflammatory cytokines/chemokines and other antimicrobial molecules after TLR stimulation as innate immune cells, present antigens as APCs, as well as secrete IgA and various antimicrobial peptides into the bile (164). BECs mostly expressed CD58 (lymphocyte function-associated antigen 3), CD80 (B7), and CD95 (Fas) (165). Injured and senescent BECs can also regulate the microenvironment around bile ducts by producing associated chemokines and cytokines, which contribute to the bile duct lesions. In 2-OA-OVA-induced mouse model of autoimmune cholangitis, BEC apoptosis was evident in early stage of autoimmune cholangitis and also associated with altered gut microbiota. The apoptosis of BECs was induced bacterial mediated TLR2 signaling (65). Apoptosis of BEC is considered an initial step in the loss of tolerance in PBC, followed by infiltration of CD4+ and CD8+ T cells and liver injury. A recent study showed that emperipolesis is frequently observed in PBC liver sections; such phenomenon is more prominent in early stage than late stage PBC, was mediated by CD8+ T cells with BEC as the host cells (166). Cysteine-rich angiogenic inducer 61 (Cyr61) is a new type of dual immunomodulatory molecule that can regulate both the innate immunity and adaptive immunity. *In vitro* studies showed that Cyr61 regulated intrahepatic immunity by inhibiting the CD8 T cells cytotoxic effects on BECs and inflammation. Overexpression of Cyr61 *in vivo* could alleviate liver inflammation and BECs injury in a mouse model of PBC (63). Cyr61 can be a potential therapeutic candidate for PBC.

3.5 Targeting liver fibrosis

Setanaxib (GKT137831) is a selective inhibitor for nicotinamide adenine dinucleotide phosphate oxidase (NOX) isoform 1 and 4. This inhibitor may slow or reverse cholestatic fibrosis (132). It attenuated liver fibrosis and reactive oxygen species production in the MDR2 knockout mice (167, 168). A large phase 2 trial (NCT05014672) on setanaxib was completed, with a significant decrease in liver stiffness and substantial decreases in cholestasis marker after 24 weeks (132).

Lysyl oxidase-like protein 2 (LOXL2) is a key enzyme in the development of organ fibrosis. LOXL2 was associated with BECs injury. It is over-expressed in liver fibrosis and promoted fibrosis progression. Anti-LOXL2 therapeutic antibody inhibited LOXL2, hence attenuated both parenchymal and biliary fibrosis as well as promoted fibrosis reversal in animal experiment (169). LOXL2 is a promising therapeutic target to treat biliary and non-biliary fibrosis. Results from ongoing clinical trials of LOXL2 mAb Simtuzumab on patients with liver fibrotic disease may open the window for new anti-fibrogenic therapy in PBC.

Setanaxib (GKT137831) is a dual Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) 1/4 inhibitor, which exerts anti-inflammatory and antifibrotic effects. GKT137831 attenuated liver fibrosis, decreased hepatocyte apoptosis and reactive species of oxygen production in animal models (170). GKT137831 improved markers of cholestasis and inflammation in PBC. A multicenter phase 2 Study (NCT03226067) was designed to evaluate safety and efficacy of GKT137831 in PBC patients with incomplete response to UDCA. A six-week ad-interim analysis showed there was rapid reduction of GGT and ALP levels dose-dependent way and without side effects (171).

4 Emerging strategies

Recent advances in molecular and tissue culture technologies have greatly expanded the scope and potential of developing approaches in the treatment of autoimmune diseases (172–175). Here, we discuss some of these unprecedented opportunities and their potential applications in the development of novel PBC therapies in PBC.

4.1 Genetics and environmental factors

Genetics has long been recognized to play an important role in autoimmune disease susceptibility. Geo-epidemiological studies in PBC have provided evidence of familial risk, case control studies and genome wide association studies have identified various HLA and non-HLA alleles that are associated with PBC. However, these alleles are non-PBC specific and most of the identified non-HLA loci were also found to be susceptible genes in other autoimmune diseases and different between study populations (176). Extensive studies have addressed the association of HLA class II alleles with the development of PBC. In particular, the *DRB1*08* allele family, with *DRB1*0801*, *DRB1*0803*, *DRB1*14*, and *DPB1*0301* as susceptible and *DRB1*11*, *DRB1*13* as protective alleles (177–180). A recent study from Japan identified HLA-DQ alleles, *DQB1*06:04* and *DQB1*03:01*, as disease protective alleles (181). A high prevalence of *HLA DRB1**0301–*DQB1**0201 haplotype among PBC patients in Sardinia was also reported (182).

GWAS analyses from European countries, North America, Japan, and China have identified HLA alleles that possess strong link with susceptibility to PBC and revealed more than 40 non-HLA alleles contributing to PBC susceptibility (183–193) but they can differ among studies and populations. These alleles primarily belong to genes and pathways involved in antigen presentation and production of IL12 (*IRF5, SOCS1, TNFAIP3, NF-κB*, and *IL-12A*), activation of T cells and IFN- production (*TNFSF15, IL12R, TYK2, STAT4, SOCS1, NF-κB*, and *TNFAIP3*), as well as activation of B cells and production of immunoglobulins (*POU2AF1, SPIB, PRKCB, IKZF3*, and *ARID3A*). The association of these immune pathways with the pathogenesis of PBC provide opportunities for strategic therapeutic designs in personalized medicine. Epidemiological studies on PBC showed that frequent exposure to environmental chemicals such as nail polish, chemicals in tobacco smoke, and hormone replacement therapies are significantly associated with an increased risk of PBC (194). Bacterial infection and xenobiotics have been proposed as candidate environmental factors that may explain tolerance breakdown and production of PBC-specific AMAs (195). Large-scale case–control studies have consistently detected an association of PBC with urinary tract infections caused by *Escherichia coli*, as *E. coli* PDC-E2 is molecularly similar to human PDC-E2, the immunodominant target of AMAs (155). Detailed analysis of AMA activity to the human and *E. coli* PDC-E2 indicated that exposure to *E. coli* could elicit specific antibody to *E. coli* PDC-E2 resulting in determinant spreading and the loss of tolerance to the human autoantigen (13). Another bacterium of interest is *Novosphingobium aromaticivorans*, a ubiquitous xenobiotic-metabolizing bacterium that produces lipoylated proteins, which are highly reactive with sera from PBC patients (155). The complexity of interactions between genetics and environmental factors (196, 197) together with the changing geoepidemiology and mortality in PBC further highlight the need of novel approaches in order to understand the immunopathogenic basis of PBC to further advance therapeutic approaches towards personalized medicine (198–200).

4.2 Epigenetics

Epigenetics is the study of DNA and related factors modifications that are inheritable and do not involve changes in the DNA sequence (201). Epigenetic information controls cellular heterogeneity and identity since the genomic sequence is identical in all cells of the body (201).There are four types of epigenetic information, namely DNA methylation, post-translational changes of histones, non-coding RNAs, and chromatin organization (lack of data on PBC) (202). DNA methylation involves the addition of a methyl group preferentially involving the nucleotide cytosine in CpG sites, which typically results in gene silencing. Post-translational modifications of histones change the DNA accessibility to transcription factors or enhancers and influence transcription and activate or silence genes. These modifications include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation (201). On the other hand, non-coding RNAs (ncRNAs) are RNAs that do not code for proteins and include two main classes: small non-coding RNAs (miRNAs) and long non-coding RNAs (lncRNAs) (203, 204). These epigenetic modifications could be therapeutic targets in PBC.

Studies have shown the involvement of epigenetic dysregulation in PBC. One study found a significantly reduced methylation level of the CD40L promoter in CD4+ T cells of PBC patients, which led to higher levels of *CD40L* mRNA expression (82). Additionally, the study found that Immunoglobulin M serum levels were negatively correlated with promoter methylation patterns. Studies on methylation patterns in monozygotic twins discordant for PBC and found regions with different methylation patterns on ChrX, with hypermethylation being the common finding in PBC probands (205, 206). In addition, it was shown that imbalance on Treg/Th17 axis in PBC was likely to be affected by the *FoxP3* hypermethylation. In the same study, it was demonstrated that DAC-mediated *FoxP3* demethylation on PBC mice rebuilt the Treg/Th17 balance, resulting in the alleviation of liver lesions and inflammation (51).

PBC affects women more frequently than men, which hampers the drawing of conclusions about potential sex-dependent epigenetic abnormalities. The post-translational modifications of histones have also been implicated in PBC. For example, T lymphocytes from patients with PBC have higher expression levels of *β-Arrestin 1 (βarr1)* than controls, which is involved in T cell activation and has a pathological role in autoimmunity (207). Recently, valproic acid, a histone deacetylase inhibitor, was shown to have antifibrotic effects in the liver and kidney in the experimental adriamycin-induced nephropathy model (208, 209). Although not tested in PBC model, it warrants further study in this condition.

Dysregulation of specific miRNAs has been observed in PBC, and one study found that miR-506 is upregulated in PBC and can target AE2 mRNA, which may contribute to the breakdown of PBC tolerance (210–212). Intriguingly, *miR-506* is located on the X-chromosome. Conversely, few lncRNAs have been implicated in PBC, but *H19* has been identified as a key player in bile duct ligation-induced cholestatic liver injury and is upregulated in PBC and other cholestatic disorders (213). *H19* has multiple functions, including participation in different signaling pathways and functioning as a miRNA sponge (213). There are not reported *in vivo* models or clinical trials implementing RNA interference (RNAi) or small interfering RNAs (siRNA) therapy for treatment of PBC. Further studies targeting pathogenic RNA-associated molecules are warranted. In summary, knowledge on the epigenetic mechanisms and epigenetic contributors will help to understanding the disease process and outcome in patients with PBC so as to develop targeted directed therapies at different stages of disease.

4.3 Single-cell RNA sequencing and spatial transcriptomics

Single-cell RNA sequencing (scRNA-seq) technology and spatial transcriptomic (ST) can not only discover new cell types,

but also reveal unique changes in each cell, greatly promoting genomics research. GWAS have reported that the association of multiple genetic loci with PBC susceptibility in various populations (214, 215) but without defining any candidate genes. Single cell sequencing analysis revealed that *ORMDL3*+ cholangiocytes had higher metabolism activity and are also play important immune-regulatory roles via the VEGF signaling pathway in the pathogenesis of PBC (216). Recently, Li et al. reported the identification of $DUOX2^+$ $ACE2^+$ small cholangiocytes in human and mouse livers by ST. $DUOX2^+$ $ACE2^+$ cholangiocytes interacted with immune cells in the liver portal areas where $CD27^+$ memory B and plasma cells accumulated. Interestingly, it was also noted that: a) the number of $DUOX2^+$ $ACE2^+$ cholangiocytes decreased with the development and progression of PBC; b) the polymeric immunoglobulin receptor (pIgR) was highly expressed in $DUOX2^+$ $ACE2^+$ cholangiocytes; c) the expression of serum anti-pIgR autoantibodies was highly increased in both positive and negative AMA-M2 of PBC patients (217). Taken together, $DUOX2^+$ $ACE2^+$ small cholangiocytes and anti-pIgR autoantibody levels can be further evaluated as potential biomarkers in monitoring therapeutic regimens in patients with PBC. Targeting anti-pIgR autoantibodies is likely a potential therapeutic approach in PBC.

4.4 Organoids

Organoid technology has evolved with the use of MSCs, liver organoids can mimic different liver disease and increase the translatability of drugs for pre-clinical therapies. Organoids are three-dimensional structures that mimic the structure and function of organs *in vivo*. They are derived from stem cells and can be used to study diseases and test potential treatments (218). Biliary-like cells can indeed be isolated from human bile and cultured long term as biliary organoids (219). Organoids can be used to generate a model of PBC which could be further used to study the disease and its underlying mechanisms (220–222). Researchers can further use organoids to test potential drugs or therapies for PBC, which can then be translated to clinical trials. Some works have developed successful organoid models for primary sclerosing cholangitis which were able to recapitulate the disease inflammatory immune profile (223).

The opportunity to isolate patient biliary stem cells will allow researchers to screen pipe line drugs, develop personalized treatments and therapies that are tailored to the individual patient. This approach can help identify new drugs or repurpose existing drugs for the treatment of PBC (219). On the other hand, organoids could be used to generate new liver tissue to replace damaged or diseased tissue. This approach could potentially be used to treat end-stage liver disease caused by PBC. Using a cell engraftment in human livers undergoing *ex vivo* normothermic perfusion, Sampaziotis et al. (223) demonstrated that extrahepatic organoids were able to successfully repair human intrahepatic ducts after transplantation. It is intriguing that activation of receptor interacting protein kinase (RIPK)3-dependent necroptosis is a core event in PBC (224) and human cholangiocyte organoids can recapitulate cholangiopathy associated RIPK3-dependent necroptosis signaling pathways *in vitro* (218). The potential application of organoids for the development of new treatments for PBC and other liver diseases is promising.

5 Current research gaps and potential future developments

The pathogenesis of PBC involves many factors including immunological abnormality, BAs metabolism, gut macrobiotics, BECs injury, gut-liver axis and fibrotic formation. Although with extensive preclinical studies and clinical trials, there does not seem to be a single drug or a single mechanism that is effective in completely halting disease progression and cirrhosis. With the ultimate objective in stopping disease development early enough to avoid cirrhosis and its complications, combinatorial approaches targeting multiple mechanisms and their relevant players are necessary. Multiple immune factors or BAs metabolism play different roles in different stage of PBC disease. Stem cell therapy and anti-fibrotic therapies are potentially useful for preventing progression of PBC. Liver transplantation is currently still the most effective treatment for PBC patients with end-stage liver disease. Long-term studies are needed to evaluate the effectiveness of current treatments and to identify predictors of disease progression and adverse outcomes. Joint effort between clinicians and wet bench scientist work closely together to take advantage of recent research advances such as epigenetics, transcriptomics and the use of organoids technologies to develop unexplored territories in the therapy of PBC. Last but not least, the impact of PBC on patients' quality of life and well-being is significant, yet there is limited research on patient-reported outcomes in this condition. Future studies should focus on identifying patient-centered endpoints that reflect the impact of PBC in daily life. Clinically, PBC is heterogeneously presented with stages and clinical manifestations; we do not anticipate that there is one "magic bullet" for all PBC patients. Continuous effort in closing the gaps in deciphering mechanisms underlying the disease progress, identifying novel risk loci and vigorous research in candidate drugs will improve the diagnosis, clinical management and outcomes in patients with PBC.

Author contributions

YY, LG, MR and PL wrote the main manuscript text and prepared all figures. XH and MR, PL revised and edited the manuscript and figures. LG and PL jointly originated and supervised this work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

PBC	Primary biliary cirrhosis
UDCA	Ursodeoxycholic acid
OCA	Obeticholic acid
FDA	Food and Drug Administration
DCs	Dendritic cells
NK	Natural killer
NKT	Natural killer T
AMA	Antimitochondrial antibodies
Ig	Immunoglobulin
IL-6	Interleukin-6
IL-10	Interleukin-10
IFN-γ	Interferon-γ
TNF-α	Tumor necrosis factor-α
ALP	Alkaline phosphatase
BAFF	B-cell -activating factor
BCR	B-cell receptor
ANA	Antinuclear antibody
MAIT	Mucosal-associated invariant T
TGF-β1	Transforming growth factor-β1
NF-κB	Nuclear factor-κB
RA	Rheumatoid arthritis
BECs	Biliary epithelial cells
PLGA	Poly lactic-co-glycolic acid
PMHCII	Peptide-major histocompatibility complex class II
TR1	Regulatory type 1
PDC-E2	E2 subunit of pyruvate dehydrogenase complex
AIH	Autoimmune hepatitis
CAR	Chimeric antigen receptor
AMPK	AMP-activated protein kinase
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
Cyr61	Cysteine-rich angiogenic inducer 61
Pss	Primary Sjögren syndrome, HSCs, Hepatic stellate cells
MC	Mast cell
ICOS	Inducible co-stimulator
PD-1	Programmed death-1
BD	Bile duct
BAs	Bile acids
HiBEC	Human intrahepatic biliary epithelial cell

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APCs	Antigen-presenting cells
MSCs	Mesenchymal stem cells
BM-MSC	Bone marrow-MSC
UC-MSCs	Umbilical cord–derived MSCs
2OA-BSA	2-Octynoic acid coupled to bovine serum albumin
3D	3-dimensional
CXCR3	C-X-C motif chemokine receptor 3
IP-10	Inducible protein-10
FKN	Fractalkine
GC	Germinal center
Sirt1	Sirtuin-1
NRs	Nuclear receptors
LT	Liver transplant
FXR	Farnesoid X receptor
PXR	Pregnane X receptor
PPARα	Peroxisome proliferator- activated receptor alpha
CAR	Constitutive androstane receptor
VDR	Vitamin D3 receptor;TGR5, Takeda G protein receptor 5
S1PR2	Sphingosine-1-phosphate receptor 2
TXR	Tropifexor
FMT	Fecal microbiota transplantation
CFTR	Cystic fibrosis transmembrane conductance regulator
SR	Secretin receptor
AE2	Anion exchanger protein 2
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
LOXL2	Lysyl oxidase-like protein 2
NADPH	Nicotinamide adenine dinucleotide phosphate. ncRNAs, Non-coding RNAs
miRNAs	Small non-coding RNAs
siRNA	Small interfering RNAs
scRNA-seq	Single-cell RNA sequencing
ST	Spatial transcriptomic
RIPK	Receptor interacting protein kinase

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Immune microenvironment changes of liver cirrhosis: emerging role of mesenchymal stromal cells

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Cirrhosis is a progressive and diffuse liver disease characterized by liver tissue fibrosis and impaired liver function. This condition is brought about by several factors, including chronic hepatitis, hepatic steatosis, alcohol abuse, and other immunological injuries. The pathogenesis of liver cirrhosis is a complex process that involves the interaction of various immune cells and cytokines, which work together to create the hepatic homeostasis imbalance in the liver. Some studies have indicated that alterations in the immune microenvironment of liver cirrhosis are closely linked to the development and prognosis of the disease. The noteworthy function of mesenchymal stem cells and their paracrine secretion lies in their ability to promote the production of cytokines, which in turn enhance the self-repairing capabilities of tissues. The objective of this review is to provide a summary of the alterations in liver homeostasis and to discuss intercellular communication within the organ. Recent research on MSCs is yielding a blueprint for cell typing and biomarker immunoregulation. Hopefully, as MSCs researches continue to progress, novel therapeutic approaches will emerge to address cirrhosis.

KEYWORDS

liver cirrhosis, liver immune microenvironment, mesenchymal stromal cells, nonalcoholic fatty lives disease, autoimmune liver disease, chronic liver disease, therapy

1 Introduction

According to epidemiological data (1), approximately 1.5 billion people worldwide suffer from chronic liver disease (CLD), with about 20,000 deaths occurring annually, of which 10,000 are caused by liver cirrhosis. The global mortality for liver cirrhosis has risen by 47.15% in recent years (2, 3). Viral hepatitis, alcoholic liver disease, and non-alcoholic steatohepatitis are the leading causes of liver cirrhosis (4). Moreover, a wide range of other

factors also can lead to cirrhosis, including genetic factors, autoimmune diseases, cholestatic diseases, iron or copper overload (5). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are responsible for more than 60% of cirrhotic cases worldwide (6). The number of hospitalized patients with HCV-related cirrhosis is anticipated to decrease significantly by 2025 (7). Only a small number of patients infected with the hepatitis D virus (HDV) will develop liver cirrhosis (8). In pregnant women, low immune function often plays a role as a prerequisite for liver cirrhosis when infected with the hepatitis E virus (HEV), with up to 30% of pregnant patients dying from HEV infection (9). Alcohol-related cirrhosis (AC) has been shown to be a significant cause of hospitalization in the United States, with the number of hospitalized patients increasing rapidly (10). A survey of middle-aged women in the UK found that the higher the amount of alcohol consumed, the greater the incidence of liver cirrhosis (11). Due to the development of hepatitis virus vaccines and effective antiviral therapy, the incidence and prevalence of end-stage liver cirrhosis in non-alcoholic fatty liver disease (NAFLD) change to has risen sharply (12). The prevalence rate of NAFLD-related end-stage liver cirrhosis in China is growing at an alarming rate with the accelerating urbanization process. It is estimated that the number of NAFLD patients in China will reach 314.58 million by 2030 (13). We concluded the epidemiology and risk factors for liver cirrhosis (Figure 1).

Cirrhosis is an end-stage pathological process caused by a variety of chronic liver diseases that will result in persistent chronic liver injury (14). Cirrhosis, characterized by chronic inflammatory necrosis and dynamic fibrosis, is considered to be a diffuse pathological state with a transformation from normal liver tissue structure to abnormal nodular hyperplasia, which in turn progresses from compensated cirrhosis (asymptomatic stage) to decompensated cirrhosis (symptomatic stage) (5), eventually leading to hepatocellular carcinoma (15). However, studies have found that this process can be prevented, bringing about reversible liver fibrosis and the reversal of cirrhosis (15, 16). Regardless of the complexity and prevalence of the etiology of cirrhosis, liver fibrosis

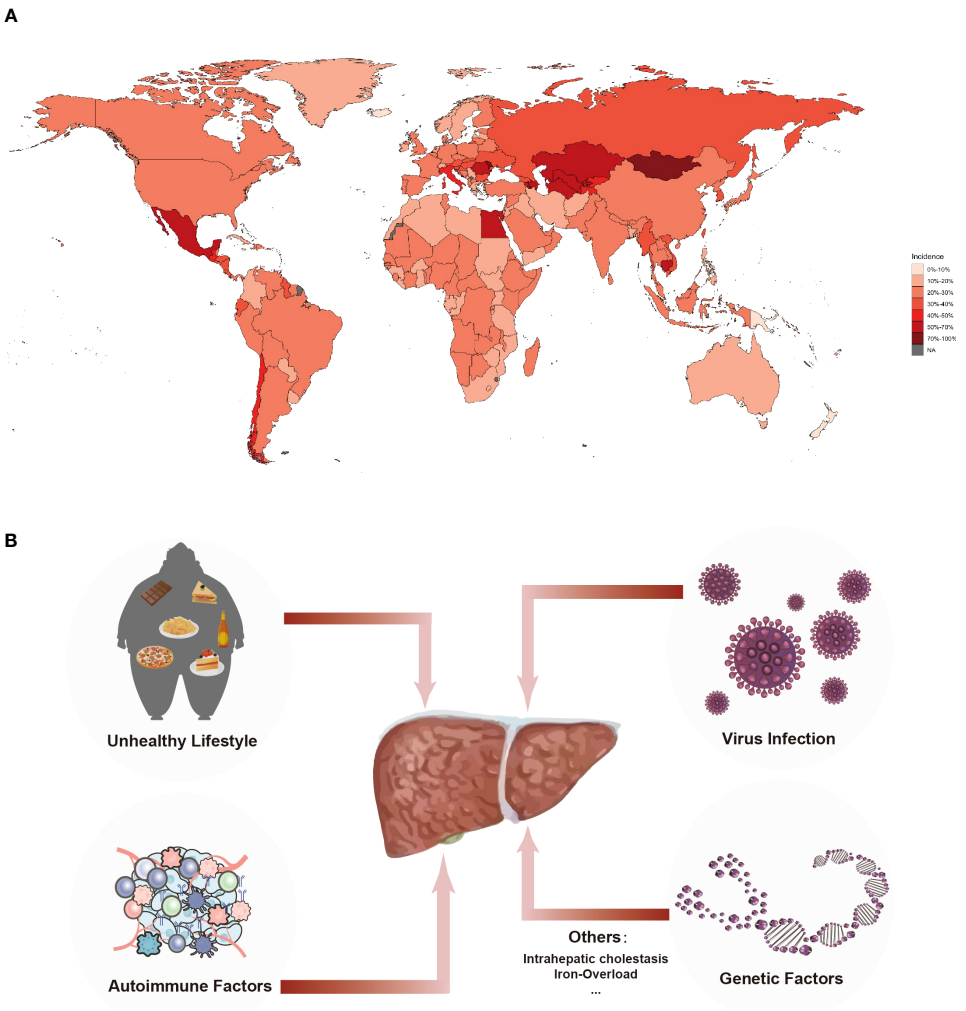

FIGURE 1
Incidence and Etiology of Liver Cirrhosis. **(A)** Incidence of liver cirrhosis in chronic liver diseases worldwide. Around 1.5 billion people worldwide suffer from chronic liver diseases that eventually progresses into fibrosis and cirrhosis, result in 10 thousand deaths globally. The data were extracted from the GHDx database (https://ghdx.healthdata.org). **(B)** The etiology of cirrhosis. Leading etiologies of cirrhosis were viral, alcoholic, unhealthy lifestyle and genetic factors.

is a mandatory part of cirrhosis. Chronic inflammatory liver injury and liver fibrosis continue to increase, leading to dysregulated crosstalk between immune cells in the liver microenvironment, which drives the progression of cirrhosis (17–19).

2 Liver cirrhosis immune microenvironment

In addition to its role in metabolism, nutrient storage, and detoxification, the liver is the body's most functionally complex immune organ. It has a profound impact on immune function (20). The liver is rich in blood circulation and the circulation system collects blood from the portal vein and the hepatic artery, which contains a large number of microbial-associated molecular patterns (MAMP), pathogen-associated molecular patterns (PAMP), damage-associated molecular patterns (DAMP), and various toxin and antigen molecules from the intestine (19, 21). Herein, the liver must simultaneously recognize antigenic components from the systemic circulation and the gastrointestinal tract. These antigens stimulate the liver through a series of pattern recognition receptors (PRR), such as Toll-like receptors (TLR) and nucleotide-binding oligomeric domain-like receptors (NOD-like receptors or NLR), which trigger unique immune responses to induce immune activation and immunomodulatory cytokine production. TLR is expressed on various hepatic cells, like Kupffer cells (KCs), dendritic cells, hepatic stellate cells, endothelial cells and hepatocytes (22). The hepatic immune microenvironment contains a variety of immune cells and molecules performing unique roles based on the association with non-immune cells, thus developing a complex and dynamic network system. Although the irreplaceable metabolic functions of the liver often obscure the perception of its role as an immune organ, hepatic metabolic functions create a microenvironment in which parenchymal and non-parenchymal cells communicate; in other words, the metabolic environment can alter the immune response in the liver (23).

The liver microenvironment consists of multiple components, including KCs, hepatic sinusoidal endothelial cells (HSECs), HSCs, immune cells, extracellular matrix (ECM), cytokines, and various growth factors (24, 25). Along with the liver's inherent immune dysfunction, viral infections, alcohol abuse, metabolic disorders, and autoimmune abnormalities can indirectly inflict liver injury, inflammation, fibrosis, and cirrhosis. Changes to the immune microenvironment in liver cirrhosis involve a decrease in $CD8^+$ T cells and natural killer (NK) cells and an increase in $CD4^+$ memory T cell infiltration (26) (Figure 2).

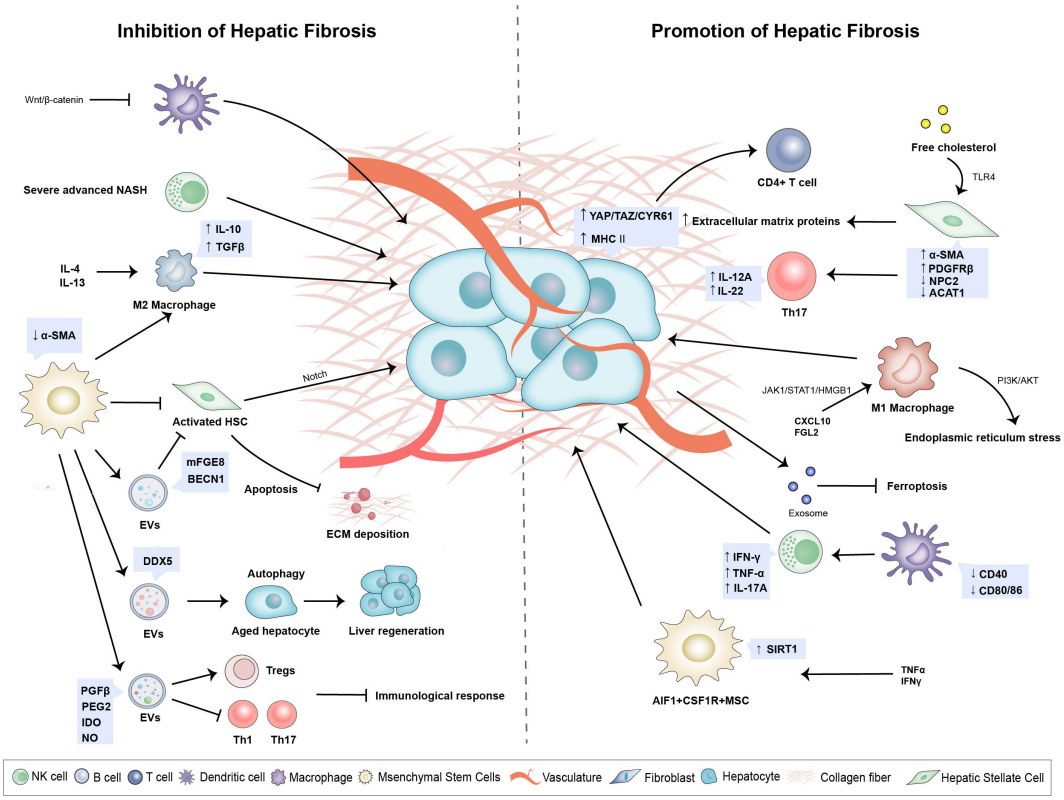

FIGURE 2
Changes of hepatic immune microenvironment play a pivotal role in hepatic fibrogenesis. A variety of immune cells and non-immune cells constitute a complex and dynamic network system. Upregulation of YAP/TAZ/CYR61 in activated Hepatocytes activating monocyte differentiation into pro-inflammatory macrophages. Activated HSCs promote lipid droplet loss and α-smooth muscle actin increase as well as secretion of extracellular matrix proteins and accelerate the development of liver fibrosis. Hepatic Macrophages promote autophagy and activation of HSC by secreting prostaglandin E2 and binding to receptor EP4, which leads to the development of liver fibrosis and cirrhosis. MSCs can inhibit the inflammation and immune response, inhibit the excessive ECM deposition, and promote the hepatocyte regeneration during liver fibrosis.

2.1 Hepatocytes

Hepatocytes are involved in the innate immune response by undergoing organelle damage and releasing stress signals in response to injury and inflammatory stimulation, promoting the development of liver cirrhosis and cancer. This process occurs with complicated crosstalk between hepatocytes and immune cells in the liver microenvironment (27, 28). In mice, activated hepatocytes can induce monocytes into pro-inflammatory macrophages with increased YAP/TAZ/CYR61, stimulating liver inflammation and fibrosis (28, 29). YAP/TAZ is the vital effector in the Hippo pathway, which regulates TGF-β2–mediated fibrogenesis (30). MHC-II is highly expressed in hepatocytes of alcoholic hepatitis, and it can activate CD4-positive lymphocytes and trigger a pro-inflammatory response (31). Lipid deposition can increase the susceptibility of hepatocytes to apoptosis in patients with nonalcoholic steatohepatitis (NASH), which had demonstrated in high-fat diet (HFD) mice. Notably, lacking AMP-activated protein kinase (AMPK) can accelerate fibrosis in NASH (32). Virus-infected and hepatocyte-derived exocrine miR-222 promoted fibrosis by inhibiting TFRC and TFRC-induced ferroptosis (33).

The overexpression of transcription factor FoxM1 was dependent on Kupffer cells, and it triggered hepatocyte death and contributed to liver inflammation and injury (34). Hepatocyte autophagy is a steady-state process that protects against hepatocyte death (27, 35). In CCl4-induced mouse models and cirrhotic patients, hepatocyte autophagy was significantly inhibited by the miR-125a/VDR axis-dependent autophagy, which finally promoted liver fibrosis (36). Autophagy disorders were also observed in alcoholic liver disease (ALD) and NAFLD (37). While the situation was different in viral hepatitis. Hepatocyte autophagy could enhance HBV DNA replication (38), while autophagy disorders could inhibit HCV replication by enhancing intracellular immunity (39). Telomere shortening and the absence of telomerase in hepatocytes could lead to cell senescence, promoting virus replication and liver cirrhosis (40, 41).

2.2 Hepatic stellate cells

HSCs reside in the Disse space between hepatic sinusoidal endothelial cells (LSECs) and hepatocytes. In their resting state, HSCs contain many retinol (vitamin A) lipid droplets (42). However, when the liver was subjected to inflammatory stimulation or hepatocyte death, HSCs received signals secreted by immune and non-immune cells in the liver microenvironment and underwent transdifferentiation into proliferative fibroblast myofibroblasts (MFs) (43). Activated HSCs lost lipid droplets and upregulated the expression of α-smooth muscle actin (α-SMA) (44), which led to the secretion of extracellular matrix proteins and the eventual development of liver fibrosis (17). The percentage of α-SMA positive hepatic stellate cells was significantly increased in patients with virus-associated cirrhosis (45). HSC activation was driven by the increased level of platelet-derived growth factor (PDGF) receptor β (46). Kupffer cells secreted PDGF, which could stimulate the production and deposition of collagen. Additionally, activation of the acid-sensing ion channel 1a (ASIC1a) via the PI3K/AKT pathway induced endoplasmic reticulum stress (ERS), thereby promoting the progression of liver fibrosis (47, 48). Apart from retinoids, cholesterol, triglycerides, phospholipids, and free fatty acids are also present in HSC lipid drops (49). The NPC2 protein expressed in resting HSC, as well as the ACAT1 isoenzyme, together bound directly to free cholesterol and played a critical role in cholesterol metabolic homeostasis. The accumulation of free cholesterol stimulated HSC through increasing TLR4 signaling and sensitizing HSC to transforming growth factor β (TGF-β) (50, 51). In a high-cholesterol diet mouse model of NASH, NPC2 and ACAT1 deficiency significantly boosted liver fibrosis progression (52, 53).

T helper cells (Th17s) collaborated with HSCs in a pro-inflammatory circumstance. Activated HSCs recruited more Th17 cells and provoked the secretion of IL-12A and IL-22 that contributed to cirrhosis in chronic hepatitis B (CHB) (54). Regulatory T cells (Tregs) possess anti-inflammatory properties. IL-8 produced by $Foxp3^{+}CD4^{+}$ Tregs activated HSCs and promoted liver fibrogenesis in chronic hepatitis C (43). 22-carbon hexanoic acid (DHA) plays a critical role in anti-fibrotic activity depending on peroxisome proliferator-activated receptor γ (PPARγ), while it is absent in liver cirrhosis patients, low level of DHA promotes NF-κB and TGF-β pathways in HSC and consecutively activates HSC (55, 56). Additionally, it was found that membrane-bound glycoprotein CD73 promoted activation and autophagy of HSCs by promoting AMPK/AKT/mTOR signaling pathway, which was conducive to alcohol-related liver fibrosis (57).

Cell-derived extracellular vesicles (EVs) have emerged as essential agents in the progression of liver injury and fibrosis (58). Delivering diverse cargo via EVs is a critical component of cell-to-cell communication (59). In the liver, EVs from injured hepatocytes and LSECs activate and migrate of HSCs (1, 58). Recent research shows that SHP2 in HSCs exerts its pro-fibrotic role by enhancing the release of fibrogenic EVs through inhibiting autophagy, REDD1, and activating the mTOR pathway (60).

2.3 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are multipotent fibroblast-like cells that have the ability to differentiate into hepatocyte-like cells (HLCs) and immunomodulatory properties have received much attention in a wide range of medical and health fields (61).MSC was reported to express a specific set of surface markers, such as CD73, CD90 and CD105 (62).

Single-cell RNA sequencing analysis unveiled that different subsets of MSCs were functionally distinct, and even though CMKLR1+ MSCs had lower proliferative capacity than CMKLR1- MSCs, the former had superior immunomodulatory functions (63). In addition, Zong et al. also identified another isoform by using the high-throughput sequencing technology, AIF1+CSF1R+MSCs, with high expression of SIRT1 and induced by TNF-α, exerting pro-inflammatory and pro-tumorigenic effects (64). Similar to HSCs, MSCs are one of the sources of MFs in the liver and are highly differentiated (65). Nevertheless, MSCs could suppress HSC

activation and protect hepatocytes from damage by inhibiting the Notch pathway, thus alleviating the progression of liver fibrosis to cirrhosis (66). It has been shown that MSCs only become immunosuppressive when exposed to sufficiently high levels of pro-inflammatory cytokines (67–69). Despite their higher pro-inflammatory potential, MSCs can exhibit pro-inflammatory phenotypes when exposed to low levels of IFN-γ and TNF-α. Through the production of chemokines, they enhance T cell response by bringing lymphocytes to areas of inflammation (67).

MSCs have been shown to exert significant therapeutic effects utilizing their soluble products, such as extracellular vesicles, cytokines, trophic factors, and chemokines. Research shows that the EVs generated by MSCs, such as exosomes, could make great contributions to the therapeutic potential in tissue repair, angiogenesis and immunomodulation by facilitating cell–cell interactions, and delivering paracrine factors (70). MicroRNA-618, the exosome of MSCs, acted as an instrumental player targeting Smad4 to reverse the progression of fibrosis to cirrhosis (71). Exosome-derived Bone marrow stromal cell-derived exosomes (BMSC-Exos) attenuated collagen deposition and liver impairment, enhanced hepatocyte proliferation and ultimately alleviated liver fibrosis in a rabbit cirrhosis model (72). BMSC-Exos suppressed hepatocyte pyroptosis by downregulating pyroptosis-related proteins which included NLRP3, caspase-1, and IL-1β, thereby remitting liver cirrhosis (73).

Moreover, the exosomes secreted by MSC have similar physiological functions as MSCs and play a major role in cellular communication (74, 75). They can also induce anti-inflammatory M2 polarization and facilitate the production of anti-inflammatory mediators such as IL-10 and TGF-β (76). HSC ferroptosis can also be mediated by MSC-exosome (MSC-Exo) to mitigate liver fibrosis (77).

It has been shown that MSCs regulate the innate and adaptive immune response through intercellular contacts or paracrine mechanisms (67). As an illustration, MSCs can produce HGF and IL-6, which inhibit monocyte differentiation into dendritic cells, lowering inflammation, decreasing the secretion of IL-12 and IFN-γ, and increasing the production of IL-10, continually weakening the activation of T cells (78). MSCs inhibit the Kupffer cell activity, reducing the production of the pro-inflammatory cytokine TNF. Furthermore, MSCs secrete PGE2 to transform pro-inflammatory M1 macrophages into anti-inflammatory M2 macrophages (79). MSCs suppress $CD8^+$ T lymphocyte proliferation and enhance $CD4^+$ T lymphocyte conversion from T-helper 1 to T-helper 2 phenotype by producing IDO and heme oxygenase 1 (80).

Furthermore, recent research suggests that autophagy and senescence are mechanisms through which MSCs acquire their antifibrotic properties. As a vital cellular process, autophagy prevents nutritional, metabolic, and infection-mediated stress while maintaining homeostasis (81). The efficacy of MSCs as a therapeutic intervention is contingent upon the maintenance of optimal levels of autophagy, which in turn can ameliorate the fibrotic cascade. Despite this, aging-related autophagic damage is associated with a decline in MSC number and function, which are crucial to liver fibrosis (82).

2.4 Liver sinusoidal endothelial cells

LSECs are non-substantial hepatic endothelial cells lacking basement membranes and rich in open window pores. These LSECs window pores serve as a parclose to protect hepatocytes from various damages and facilitate substance exchange by producing nitric oxide (NO) for stimulating vascular endothelial growth factor (VEGF) and reversing activated HSC to a resting state (83–85). The alcohol-metabolizing enzyme Cytochrome P4502E1 (CYP2E1) was expressed in alcohol-induced LSEC, leading to increased acetylation of mitochondrial heat shock protein 90 (Hsp90). This acetylation reduced the interaction between Hsp90 and nitric oxide synthase (eNOS), resulting in decreased NO production and increased alcohol-induced liver injury (86). Similarly, Notch signaling was activated in LSECs of NASH mice and exacerbated NASH progression in an eNOS-dependent mechanism (87). LSECs possessed endocytic and clearance abilities and vital immune functions, impacting the homeostasis of the liver microenvironment (88, 89). During the early stage of liver cirrhosis, LSECs exhibited anti-inflammatory effects (90). Upon microbial infection, LSECs triggered local activation of effector CD8 T cells that exerted the immune surveillance capacity of the liver (88). Immunoproteasome LMP7 levels in LSECs were elevated in cirrhosis patients and liver fibrosis mice models, and LSECs presented MCH-II antigen to CD4 T cells after liver injury stimulation (91).

Hepatocyte death can lead to LSECs capillarization, immune cell interactions, and HSCs activation (92). Hepatic sinusoidal capillarization is the underlying pathological change of liver cirrhosis (93). This transformation was deleterious in NASH to form a basement membrane on LESCs' surface, which inhibited the release of very low-density lipoprotein (VLDL) from hepatocytes into the Disse cavity and finally promoted hepatic steatosis. Capillarization also invoked hedgehog (Hh) signaling and exacerbated liver cirrhosis development (94, 95). Adipocyte fatty acid binding protein (A-FABP) regulated lipid metabolism, and elevated expression of A-FABP was observed in cirrhosis-associated NAFLD. Meanwhile, A-FABP stimulated Hh signaling and promoted LSECs vascularization, which led to HSC activation to enhance TGF-β1 activity, resulting in more severe liver fibrosis (96). In the progression of liver fibrosis, CXCR4 and CXCR7 exerted opposite effects on LSECs. With the increase of HIF-1α, CXCR4 upregulated to promote the isoform PDGF-BB secretion by LSEC and binding to its receptors, forming an intercellular crosstalk that activated HSCs and aggravated fibrosis, promoting the development of cirrhosis. While CXCR7 downregulation facilitated the capillarization of LSEC to promote hepatic cirrhosis (97).

2.5 Dendritic cells

DCs, as the most important antigen-presenting cells (APC), serve as a bridge between innate and adaptive immunity. DCs recognized and ingested pathogenic antigens through phagocytosis

with other immune cells and presenting MHC peptides to CD4 T cells and CD8 T cells to initiate the immune response for exogenous antigens (98). DCs include plasmacytoid DC (pDC) derived from common dendritic progenitor (CDP) cells and the conventional dendritic cell subtype generated by the entry of circulating cDC precursors into the peripheral environment (99).

Comprehensive single-cell RNA sequencing analysis revealed that cDCs were associated with NASH pathology. Elevated Xcr1cDC1 was observed in the NASH model to increase pro-inflammatory CD8T cells and exacerbated NASH to cirrhosis (100). The deficiency of Cbl-b and c-Cbl in DCs led to the excessive accumulation of cDC1 in the liver and promoted liver cirrhosis and premature death in mice (101). The Wnt/β-catenin pathway is crucial in liver homeostasis (102). Lack of Wnt/β-catenin signal should be triggered autoimmune hepatitis (AIH) and abnormal activation of hepatic dendritic cells (HDCs), promoting cholestatic liver injury and fibrosis (103). DCs induced NK cells to proliferate and produced IFN-γ, and DC-NK crosstalk severely impaired the ability of antiviral immune response in CHB patients (104). DCs were rapidly recruited to the liver of NASH mice model with elevated TNF-α, IL-6, and MCP-1 expression. DCs depleting delayed intrahepatic inflammation and fibrosis regression, thereby promoting NASH. Chronic alcohol consumption decreased the production of cytokines such as TNF-α, IFN-γ and IL-12 in DCs, and the number of peripheral blood DCs. It also decreased the expression of CD40, CD80, or CD86, which reduced the stimulatory function of DCs on T cells and led to immune deficiency in mice (105). CCL20, produced primarily by HSCs, is a chemoattractant for immature dendritic cells with inflammatory molecules mediating fibrosis. Interaction between immune cells resulted in the increased expression of CCL20 in NAFLD fibrosis patients (106). Indoleamine 2,3-dioxygenase 1 (IDO1), an immunomodulatory enzyme, was highly expressed in choledochotomy (BDL)-induced mice, inhibiting DCs maturation and T cell proliferation from recruiting immune cells and promoting hepatic fibrosis (107).

2.6 Natural killer cells and natural killer T cells

NK cells have a powerful killing function, whose amount and activity are always affected by the liver immune microenvironment. They serve as surveillance to monitor external infections, tumors, inflammatory stimuli, and autoimmunity and secrete cytokines and chemokines (108). NK cells are divided into two subgroups: $CD56^{dim}$ (> 90%) group and $CD56^{bright}$ group. The former has a more substantial cytotoxic effect and plays a role as an immunomodulator (109). NK cells are the first line of defense against viral hepatitis, exerting an antiviral immune response by directly clearing virally infected cells or activating antigen-specific T cells via the production of IFN-γ and TNF-α (110). The decrease of $CD56^{dim}$ NK cells, total NK cells, and their activated receptor NKG2D in peripheral blood monocytes (PBMC) of NAFLD patients can lead to NK cell dysfunction (111, 112). In particular, NK cell function was defective and inactivated in patients with CHB, and monocytes suppressed HBV-specific T cell immune responses, leading to chronic persistent HBV infection (113). The increase of $CD56^{bright}$ NK cells could be detected in patients with autoimmune-mediated liver disease, and elevated serum IFN-γ levels induced hepatocyte death by enhancing the cytotoxicity of NK cells, ultimately resulting in macrophage activation and the development of fibrosis (114).

NKT cells possess NK cell-like characteristics and express T cell receptors, which recognize lipid antigens from major MHC-1-associated protein CD1d (115, 116). Invariant natural killer cells (iNKTs) are the primary subtype of NKT cells (109). Patients with HBV-associated liver cirrhosis (HBV-LC) showed highly activated peripheral iNKT cells, which may lead to overhealing caused by extracellular matrix deposition and the progression from fibrosis to cirrhosis (117). Liver injury induced by concanavalin A (ConA) intravenous administration was considered as an experimental model of T cell-mediated AIH in mice, in which iNKTs were specifically activated to kill hepatocytes and accumulated in the mouse liver, increasing activated immune cells cytokine through upregulation of Fas/FasL in the liver, resulting in more severe immune damage (118). With the prevalence of obesity, excessive cholesterol uptake directly destroys the function of NKT cells through lipid oxidation during the progression of NAFLD disease to liver cirrhosis. Interestingly, NKT cell depletion occurred in the early stage of mild NASH. For severe advanced NASH, NKT cells were protective against disease progression and played an anti-fibrotic role (119, 120). Compared with healthy people, primary biliary cholangitis patients had more iNKT cells, which produced high levels of IL-17A and promoted the progression of PBC-related fibrosis (121).

2.7 Macrophage

For the complexity of the liver microenvironment and immune function, macrophages show great plasticity and heterogeneity (122, 123). Macrophages can polarize into M1 cells and M2 cells. The classical M1 subtype is activated by TLR ligand and IFN-γ and secretes pro-inflammatory cytokines. On the contrary, the alternative M2 subtype secretes anti-inflammatory cytokines, which are stimulated by IL-4 or IL-13 (124). In the progression of NAFLD, it was found that hepatic macrophages polarized toward M2 and promoted HSC autophagy and activation by secreting prostaglandin E2 (PGE2) and then binding with EP4, which in turn favored the development of liver fibrosis and cirrhosis (125). It was reported that fibrinogen-like 2 (Fgl2) mediated mitochondrial damage, disrupted mitochondrial HSP90-Akt interactions. Moreover, Fgl2 induced M1 Polarization to secrete pro-inflammatory factors in hepatitis B (126). CXCL10 promoted M1 polarization, resulting in the activation of the JAK/STAT1 pathway (127). The connection between macrophages and HSCs can facilitate liver fibrogenesis. Subtype M2C-like polarized macrophages activated tyrosine kinase receptors (MerTK) on their surface influencing the profibrogenic HSCs (128).

M1 Polarization of macrophages is critical in the liver. Xu et al. found that osteopontin promoted M1 Polarization in NAFLD by

activating JAK1/STAT1/HMGB1 signaling, which aggravated liver injury and cirrhosis (129). Studies in a humanized mouse model of HBV infection revealed that HBV could induce the differentiation of human monocytes/macrophages into M2 macrophages, which then expressed IL-10 and other inhibitory cytokines (113, 130). In addition, soluble CD206 (sCD206) expressed by macrophages could promote T cell activity and inhibit the antiviral effect of CD8T cells. High expression of sCD206 accelerated the progression of cirrhosis in patients with hepatitis B virus-related decompensated cirrhosis (HBV-DeCi) (131).

3 Therapies

Treatment of the etiology is the cornerstone of cirrhosis treatment. The means of treating the primary cause include alcohol abstinence for alcoholic cirrhosis, antiviral drugs for HBV and HCV, immunosuppressants for autoimmune hepatitis, and ursodeoxycholic acid for primary biliary cholangitis. Several studies have shown that etiologic treatment can effectively restrain the progression of cirrhosis and even reverse patients with decompensated cirrhosis to compensated cirrhosis (i.e., recompensated cirrhosis), thus reducing the rate of death and improving the quality of life. Therefore, better studies of the altered cirrhosis immune microenvironment would help to develop more effective targeted therapeutic regimens.

3.1 Viral hepatitis

Currently approved antiviral therapies for HBV include pegylated interferon alpha (PEG-IFN-α) with immunomodulatory activity and nucleoside (acid) analogs (NAs) that inhibit HBV polymerase. Still, neither achieves the functional cure of HBV (i.e., scavenging HBsAg) (132). The conditions for the usage of the two drugs are different. NAs can prevent severe viral hepatitis relapse, especially in patients with liver cirrhosis. While PEG-IFN-α is contraindicated in cirrhosis patients, for it can cause more severe liver damage (133). In a randomized open phase II trial, treatment with elbasvir/grazoprevir (EBR/GZR) + sofosbuvir (SOF) for 12 weeks was highly effective for HCV patients treated either with or without peginterferon (PEG-IFN-α-2a) or for cirrhosis patients (134).

The most advanced approach in clinical development to date is the competitive inhibitor myrcludex-B (MyrB) based on the PreS1 peptide now called the hepatocyte entry inhibitor bulevirtide (BLV), which has successfully blocked HBV and HDV entry (135). In HDV-associated cirrhosis patients, for whom interferon is contraindicated, treatment with BLV alone results in a sustained virologic response (136), but the optimal duration remains determined (137). The combination of BLV and tenofovir disoproxil fumarate (TDF) has a favorable safety and efficacy profile for treating HDV-related compensated cirrhosis (138). Lonafarnib (LNF) and ritonavir (RTV) are promising therapies for treating HDV, and the combination of PEG-IFN-α may increase the efficacy. A phase III clinical trial of LNF is currently underway (139).

The risk of HCV progression to cirrhosis and HCC continues to increase after treatment with direct-acting antiviral agents (DAAs). Dysfunction of $CD4^+$ and $CD8^+$ T cells has been identified in patients with hepatitis C, making liver immunotherapy urgent (140). Compared with an oral agonist of TLR 7 (GS-9620), the agonist of TLR 8 (GS-9688) stimulated the expression of IFN-γ and TNF-α in NK cells while all increased the antiviral capacity of $CD8^+$ T cells (141). The immunotherapy by GS-9688 achieved sustained efficacy in murine models of HBV (142). The safety and tolerability of oral selgantolimod (TLR 8 agonist) was evaluated in CHB patients in one phase Ib study, and the recent phase II study further supported the development of this immunomodulator (143). NASVAC, a vaccine formulation containing both hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg), targeted a lower proportion of patients who developed cirrhosis in phase III clinical trials compared with PEG-IFN (144). HCV vaccine in phase I-II clinical trials found that 78% of HCV-infected patients showed a specific response to T cells, reducing the peak of HCV RNA level, providing a basis for future immunotherapy (145). In a proof-of-concept clinical trial, combination therapy of entecavir (NA) plus PEG-IFN-α-2a followed by HBV vaccination developed a "blueprint" for serum clearance of HBsAg, suggesting that the combination of drugs and immunotherapy provides therapeutic interventions for functional cure of viral infections (146).

3.2 Alcoholic hepatitis

Campaigns for vaccination, screening, and antiviral treatment of hepatitis B and C have reduced the burden of chronic disease. However, concurrent increases in drug injection, alcohol abuse, and metabolic syndrome threaten these trends (1). A large randomized clinical trial discovered that long-term administration of albumin could improve survival in patients with decompensated cirrhosis (147). Whereas, in an open-label multicenter trial ATTIRE, increasing albumin infusion in patients with decompensated cirrhosis showed no more benefit due to most of the patients suffering alcohol-related liver disease (148). These results show that breaking down the etiology of cirrhosis is crucial for subsequent treatment.

Corticosteroids are currently recommended for the treatment of severe alcoholic hepatitis (SAH), but about 25% of SAH did not respond to prednisone treatment (149). Granulocyte colony-stimulating factor (G-CSF) can prolong the survival of alcoholic hepatitis (AH) patients, and the combination of N-acetylcysteine (NAC) with standard drug therapy (pentoxifylline) may also reduce AH liver injury and prolong survival (150). The immunomodulatory effect of G-CSF in the AH mouse model had shown to increase the number of immune cells entering the liver and promoting the polarization of macrophages toward M2, which facilitated liver repair (151). Macrophage and neutrophil infiltration diminished in AH mice treated with intraperitoneal MSC infusion, and skeletal muscle satellite cell-derived MSC counteracted ethanol-induced inflammation by secreting PEG2 and HGF, thus making MSC promising as an effective therapy for patients with alcoholic

hepatitis (152, 153). In SAH patients, the fecal microbiome transplantation (FMT) treatment for 90 days could cause a reduction in the ratio of mucosa-associated invariant T and Th17 cells and a decrease in IL-17 and IFN-γ production. Besides, FMT could attenuate the hepatic inflammatory response and finally improve survival in SAH patients, which suggests that FMT may be an alternative to prednisone treatment (154). In the Defeat Alcoholic Steatohepatitis trial (DASH), the combination of IL-1β-receptor antagonist (anakinra) with pentoxifylline plus zinc supplementation offered the opportunity for prolonged survival in patients with AH (155). Significantly elevated markers of immune cell activation stimulated by DAMP and PAMP, including macrophages and neutrophils were found in AH subjects studied in four clinical centers in the United States and correlated with the severity of AH (156). Silybin, a kind of herbal plant, normalized alcohol-induced immune regulation of the liver and induced activation of T cells and downregulation of cytokines such as TNF (157). These immune cells play an irreplaceable role in the intricate liver immune microenvironment, which guides the direction for AH to discover immune targets and provides future treatment strategies to prevent disease progression, but further prospective clinical studies are needed to confirm this good desire.

3.3 Nonalcoholic fatty liver disease

Anti-inflammatory and anti-fibrotic immunotherapy strategies play a therapeutic role for NAFLD, with no standard treatments change to currently approved (158). FXR is expressed in immune cells. Cilofexor, an FXR agonist, improved hepatic steatosis in a 24-week phase II study in NASH patients. However, Liver Fibrosis scores and liver stiffness were not observed when were used only in noncirrhotic NASH patients (159). Meanwhile, a modified and optimized FXR agonist (MET409) had the same effect in another 12-week study in patients with NASH, despite the side effect of headache. MET409 is intended to be used as first-line monotherapy for NASH, while combinations of MET409 with other agents are in development (160). More recently, results of a phase II trial showed that the FXR agonist tropifexor led to sustained reductions in ALT and liver fat, but the side effect of pruritus was unavoidable, and further investigation for antifibrotic effects in combination with other agents is needed (161). NAFLD is associated with hypertriglyceridemia. In patients receiving the FXR agonist cilofexor (CILO) and the acetyl-coenzyme A carboxylase (ACAC) inhibitor firsocostat, inhibition of triglycerides by fenofibrate was strengthened (162), providing strong evidence for the combinations of multiple drugs. Saroglitazar is a PPAR-α/γ agonist that is no less effective than fenofibrate (163). Whether it can replace fenofibrate in combination therapy for NAFLD remains to be proven.

The immunotherapy based on chimeric antigen receptor (CAR) T cells targeting and destroying myofibroblasts can be used to reduce extracellular matrix deposition in NASH mice (164). But more evidence is needed to confirm the efficacy. Cenicriviroc (CVC), as a dual CCR2 and CCR5 antagonist, had confirmed in a phase IIb (CENTAUR) study in anti-fibrosis effect in NAFLD patients (165). Unfortunately, the CVC clinical trial was prematurely interrupted in the anticipated phase III (AURORA) study (166). Oral OKT3 (anti-CD3 mouse monoclonal antibody) was administered to NASH patients with diabetes to induce Tregs activation for ameliorating insulin resistance and liver injury. Although the number of study subjects was small, the related parameters showed promising results (167). Fibroblast growth factor 19 (FGF19) analog aldafermin (also known as NGM282 or M70) reduced liver fat content and fibrosis levels by 7.7% and 1%, respectively, compared with the placebo group (168). Pegbelfermin is a pegylated FGF21 analog. FGF21 improves the condition of NAFLD and NASH by directly regulating lipid metabolism and reducing fat accumulation in an insulin-independent manner (169). Some clinical trials have shown that BMS-986036 has a good effect on improving the liver fat content, inflammation, and fibrosis of NAFLD and NASH, and is well tolerated (170, 171). As new immunotherapeutic drugs, the clinical research of FGF19 and FGF21 is in the third stage, and more studies are needed to determine their biological characteristics and therapeutic effects (172).

Given that none of the new drugs currently under evaluation shows improvements in major clinical endpoints, it is expected that a reasonable combination will be more effective in controlling or preventing further deterioration of NASH. Several studies have now shown that MSC-secreted exosomes supply natural drug delivery vectors and offer prospective strategies for the treatment of NAFLD. MSC-Exo miR-24-3p, miR-223-3p, and miR-627-5p attenuated lipid deposition and liver fibrosis in NAFLD mice, but more studies are needed to provide meaningful evidence for clinical treatment (173–176). MSC-Exo extracted from human umbilical cords stimulated M2 polarization, exhibited downregulation of pro-inflammatory factors such as TNF-α, IL-6 and IL-1β, and detected high expression of PPARα in liver tissue, thereby alleviating methionine-cholesterol deficient (MCD) diet-induced progression to NASH in NAFLD mice (177). Exosomes originated from human adipose mesenchymal stem cells (hADMSCs-Exo) were shown to inhibit HSCs activation and rectify choline metabolism disorders via PI3K/Akt/mTOR pathway to ameliorate liver fibrosis, especially caused by NAFLD (178). Deeper exploration of immune-related mechanisms and further clinical trials may be needed to cure NAFLD patients.

3.4 Autoimmune liver disease

3.4.1 Primary biliary cholangitis

Primary biliary cholangitis (formerly called primary biliary cirrhosis) is characterized by cholestasis and biliary fibrosis, with autoimmune destruction leading to immune-mediated damage (179). Ursodeoxycholic acid (UDCA) is used as first-line therapy for PBC or as second-line therapy (obectocholic acid and benzofibrate) if the patient does not respond to UDCA (180). In a phase III clinical trial, patients who did not respond to UDCA could also be treated with budesonide, which improved liver function markers in serum but had little effect on liver histology (181).

It is well recognized that PPARs are nuclear receptors that regulate a variety of immune cell functions and play an important

role in regulating innate and adaptive immunity (182). During the 52-week study, seladelpar, a selective PPARγ-δ agonist, was safe and well tolerated in patients with PBC, which overcame the side effects of skin itching and caused no serious adverse events or deaths (183). Fatigue is also one of the symptoms of PBC. RITPBC is believed to be the first randomized, controlled phase II clinical trial to investigate fatigue in PBC. Rituximab is a monoclonal antibody against CD20 on the surface of B cells to improve serum alkaline phosphatase (ALP) of refractory UDCA in PBC and decimate B cells (184). Rituximab is also considered a potential treatment for PBC fatigue (185). In addition, the anti-IL12/23 monoclonal antibody ustekinumab led to a slight decrease in PBC in a proof-of-concept study, but the efficacy and safety of its immunomodulatory effect remain to be verified (186).

3.4.2 Primary sclerosing cholangitis

Primary sclerosing cholangitis is a chronic cholestatic liver disease characterized by progressive inflammation and fibrosis of the intrahepatic and extrahepatic bile ducts, leading to multifocal biliary stricture and progressive liver disease (e.g., cirrhosis) (187). Immunotherapy for PSC is complicated. Although a variety of immunomodulators have been tested for the treatment of PSC, the general treatment regimen has not been proven to benefit patients (188). 24-Norursodoxycholic acid (norUDCA), a homolog of UDCA, is a novel bile acid that reduces ALP in PSC patients in a dose-dependent way and significantly improves cholestasis (189). According to a meta-analysis, immunosuppressants (mycophenolate mofetil, methotrexate, and tacrolimus) significantly reduced ALP and AST and improved liver function, which seemed to be the most effective treatment with severe side effects (188).

Liver transplantation (LT) is the only life-prolonging and curable treatment for PSC. However, an international observational study found that morbidity and mortality of recurrent primary sclerosing cholangitis (rPSC) increased after liver transplantation in children. It was thought that rPSC elicited a more robust immune response than PSC (190). In addition, a 36-year-old woman who had both PSC and ulcerative colitis was diagnosed with autoimmune hepatitis after treatment with the mRNA vaccine COVID-19 (191). We could speculate whether PSC or a specific immune response after vaccination led to the conversion of immune disease, but the specific mechanism was unclear. Proinflammatory cytokines, such as TNF-α and IL-1β, were highly expressed in patients with PSC and AIH, while the function of T lymphocytes and NK cells in the liver were impaired (192), so anti-TNF therapy was also one treatment option. Exploring the immunological changes in the liver microenvironment may provide a solid basis to clinical immunotherapy.

3.4.3 Autoimmune hepatitis

Autoimmune hepatitis is characterized by elevated serum aminotransferase, immunoglobulin G (IgG) levels, and positive autoantibodies (193). The International Autoimmune Hepatitis (IAIHG) defined "complete biochemical response" (CBR) as the normalization of serum transaminases and IgG (194). To achieve this goal, corticosteroids and/or azathioprine (AZA) are the standard treatment for AIH, but some patients still respond poorly to standard treatment. The CBR rate of patients treated with mycophenolate mofetil (MMF) was significantly higher than that of patients treated with AZA, so MMF became an alternative therapy for initial treatment (195–197). For children with AIH, MMF was a "life-saving drug" for children (198). In most patients with conventionally treated refractory AIH, one in three patients treated with the immunosuppressant calcium phosphatase inhibitor tacrolimus developed CBR and had good renal function after the withdrawal of the drug (199). At the same disease stage, selective depletion of B cells by rituximab, an anti-CD20 monoclonal antibody, also lowered transaminase and IgG levels (200, 201). In a multicenter study, liver stiffness was reduced compared with that before tacrolimus treatment, but no statistical significance was found, possibly due to a small sample size. It is worth noting that only one patient discontinued treatment due to serious adverse events (202). Future studies need to enlarge the number of patients and investigate the effects of immunomodulation on disease.

Depletion of Tregs was one of the methods to establish the AIH mouse model. In conjunction with the reduction of Tregs by steroid treatment, enhanced intrahepatic Tregs immunotherapy would be the preferred option for AIH patients (203, 204). The low dose of IL-2 improves the selectivity and number of Tregs after treatment, thereby re-regulating the hepatic immune microenvironment for improve AIH (204, 205).

3.5 Inherited disease

Wilson disease (WD) is an inherited disorder of copper metabolism caused by mutations in ATP7B (hepatomegaly protein) (194). It causes liver damage and neurological symptoms due to abnormal copper ion metabolism in the body, leading to copper accumulation in the liver, brain, and other tissues, which can lead to cirrhosis in the long term (195, 196). Although liver transplantation can cure WD, can also cause serious immunosuppression (197). In most WD patients, oral chelating agents such as D-penicillamine and trientine were effective (206). Trientine tetrahydrochloride (TETA4) was found to be superior to penicillamine in phase III clinical trials (199). Besides, these drugs can decrease the number of whole blood cells in patients, weakening the immune system and increasing the risk of infection (200). Therefore, targeted liver immunotherapy would provide a better cure for WD.

3.6 New strategies for MSC based therapy

The present study shows that a number of factors are upregulated by MSCs, such as MMPs and VEGF, to promote liver fibrosis regression (207). Besides, the study indicates A combination of MSCs and macrophages was more effective in reducing fibrotic gene expression and procollagen synthesis than either of them individually. In line with this, it has been observed that localized MSCs improve liver fibrosis by reducing the activation of fibroblasts and the production of collagen (208).

Evidence is mounting that MSC-mediated immune regulation can alleviate liver fibrosis through programmed death mechanisms, such as apoptosis, autophagy, ferroptosis, and pyroptosis (209). Maintaining the characteristics of MSCs is dependent on basal autophagy levels. Aged MSCs can benefit from activated or increased autophagy to slow metabolism and strengthen their functions to resist aging. Conversely, Aged MSCs are more susceptible to toxic substance accumulation and mitochondrial damage, exacerbating inflammatory responses and cell damage, and ultimately this accelerates the aging process. Increasing evidence suggests that fibrotic microenvironment induced MSC autophagy by upregulating Becn1, while Becn1 knockdown inhibited T lymphocyte infiltration, HSC proliferation and suppressed the production of cytokines by increasing PTGS2/PGE2 secretion, thereby further enhancing MSC antifibrotic activity (210). Therefore, the augmentation of the antifibrotic potential of MSCs through the manipulation of their autophagic processes presents a viable approach towards the management of liver fibrosis.

Recently, Zhang et al have proposed the therapeutic potential of extracellular vesicles derived from mesenchymal stromal cells (MSC-Evs) in facilitating liver regeneration (211). MSC-EVs have been observed to stimulate the rejuvenation of aged hepatocytes and augment their proliferation by upregulating mitophagy. Subsequent to a more in-depth inquiry into the mechanistic intricacies of this process, it was discovered that DDX5, which is abundant in MSC-EVs, can be transferred to aged hepatocytes to stimulate EF1 nuclear translocation and consequent upregulation of Atg4B expression, ultimately leading to the induction of mitophagy. The validity of these findings was confirmed through in vivo and in vitro experiments involving DDX5 knockdown in MSC-EVs. Therefore, MSC-EVs present a promising therapeutic modality for liver fibrosis patients by reversing senescence and promoting the regeneration of senescent hepatocytes (211).

4 Summary

The disruption of hepatic homeostasis may lead to a persistent inflammatory response and a reduction in immune function, thereby emphasizing the critical role of the immune microenvironment in the development of liver cirrhosis. Remarkably, recent clinical trials have demonstrated that transplantation of mesenchymal stromal cells derived from human umbilical cord blood can potentially improve the long-term survival of patients with decompensated cirrhosis (202). Moreover, the paracrine intercellular communication of MSCs may confer the benefit of effectively eliciting physiological effects through minute concentrations of EV. The diversity of MSCs derived from distinct tissue sources and the distinctive attributes of MSC-EVs indicate a significant therapeutic potential of MSCs for their antifibrotic properties. Nevertheless, the capacity of MSCs to proliferate indefinitely is limited, and they may exhibit senescence after cell division, which can disrupt homeostasis in vivo through senescent cells and cell-cell interactions. Hence, investigating the mechanisms of cellular communication in the aging microenvironment is a pressing necessity and a promising therapeutic strategy.

Author contributions

QY searched the literature and drafted the manuscript, Ywa and QC collected and sorted out the literature. JY made the figures, QY and JY designed the article structure, and Ywu revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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