ORGAN FIBROSIS: PATHOGENESIS, BIOMARKERS AND THERAPEUTIC TARGETS

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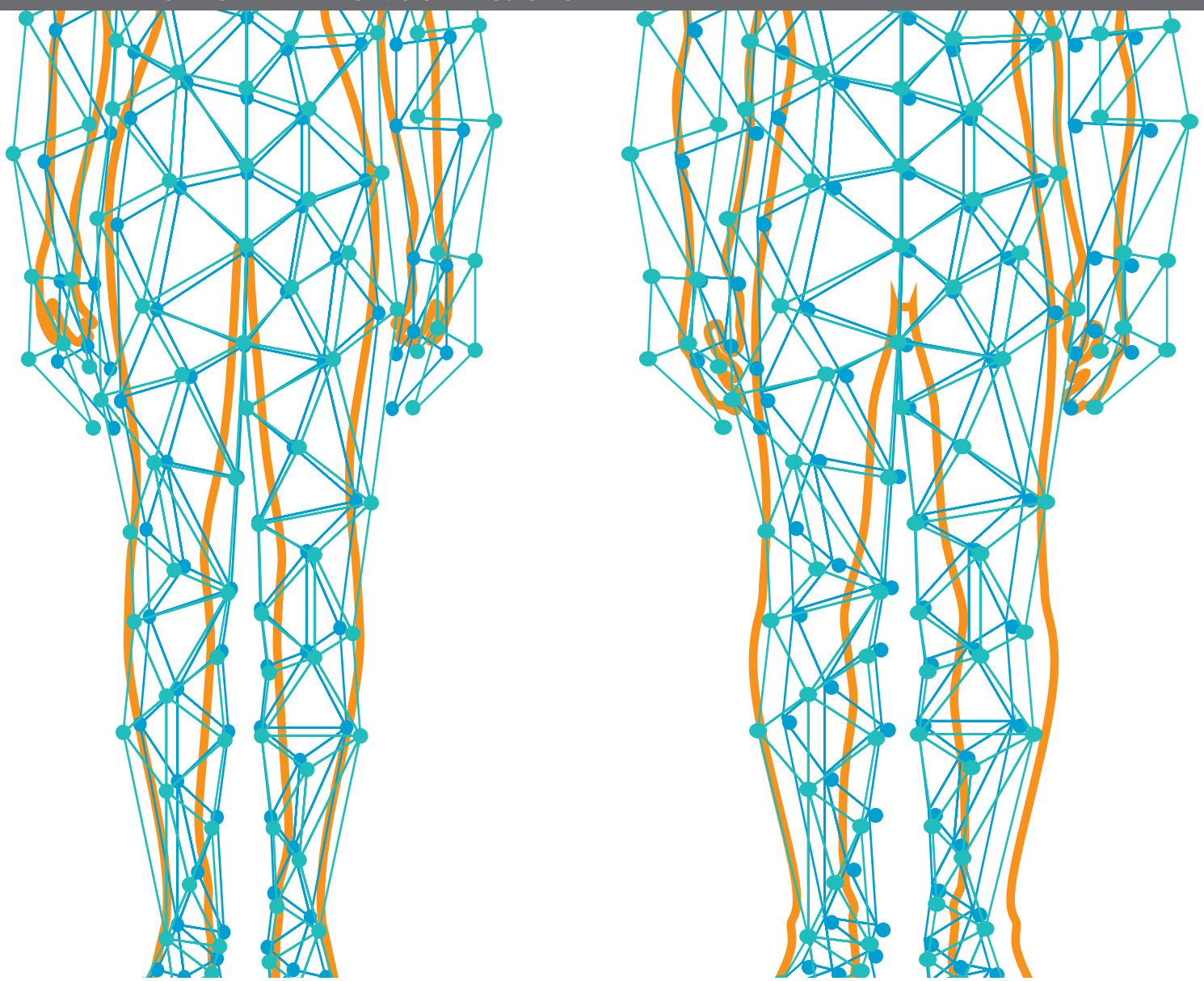

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ORGAN FIBROSIS: PATHOGENESIS, BIOMARKERS AND THERAPEUTIC TARGETS

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EDITORIAL
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Editorial: Organ Fibrosis: Pathogenesis, Biomarkers and Therapeutic Targets

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Keywords: fibrosis, chronic kidney disease, non-alcoholic fatty liver disease, cirrhosis, pulmonary fibrosis, renal fibrosis, collagen deposition

Editorial on the Research Topic

Organ Fibrosis: Pathogenesis, Biomarkers and Therapeutic Targets

The prevalence of various chronic and metabolic diseases is on the rise due to the increase in, amongst other factors, lifespan, hypertension, insulin resistance, stress, and obesity. Examples of such diseases include chronic kidney disease and non-alcoholic fatty liver disease (NAFLD), all of which are associated with high levels of morbidity and mortality, and significantly impact health-care budgets. A hallmark of most chronic conditions is the development of fibrosis in the affected organ. Fibrosis is characterized by the excessive production and deposition of extracellular matrix (ECM) proteins, including collagens, mainly by activated (myo)fibroblasts (1). This pathological process has a detrimental impact on organ architecture and function, ultimately necessitating organ transplantation. The fibrotic process is orchestrated by a wide variety of different cells and signaling pathways, which tremendously complicates the identification of relevant biomarkers and druggable therapeutic targets. Consequently, there is still no approved treatment available in clinical practice for most forms of organ fibrosis. Thus, there remains an unmet clinical need. In this Research Topic, experts from all over the world delineate the latest findings pertaining to the pathogenesis of fibrosis as well as arising biomarkers and therapeutic targets.

Interestingly, most contributions to this Research Topic focused on liver fibrosis, which does seem to reflect the recent spike in the number of publications related to this disease indexed in PubMed (**Figure 1**), whereas in the preceding years (2015–2019) the body of literature concerning liver, renal, cardiac, and lung fibrosis had a fairly similar growth rate.

As stated above, the prevalence of NAFLD is on the rise and it currently affects approximately 25% of the world population. Moreover, it is expected that there will be a 178% increase in non-alcoholic steatohepatitis (NASH)-related liver deaths by 2030 (2). Therefore, it is paramount to improve our understanding of NAFLD in order to improve clinical care. The review by Heyens et al., provides a comprehensive overview of the pathological mechanisms underlying the progression from simple steatosis to cirrhosis. In addition, they delineate the current state-of-the-art pertaining to non-invasive tools for diagnosis and monitoring of disease progression and drug responses. Lastly, they report on current and future treatment modalities. This work provides a solid foundation for future work on NAFLD and liver fibrosis. Another aspect of liver fibrosis that is gaining more and more attention from the scientific community is the role of immune cells, especially macrophages, in disease progression. Wan et al. describe in detail the regulatory role of T helper 17 cells, T helper 9 cells, T helper 22 cells, mucosa-associated invariant T cells, regulatory T cells, innate lymphoid cells, and γδ T cells in liver fibrosis. This work highlights the immunological intricacies of the fibrotic process, and reveals that although some work has been done regarding

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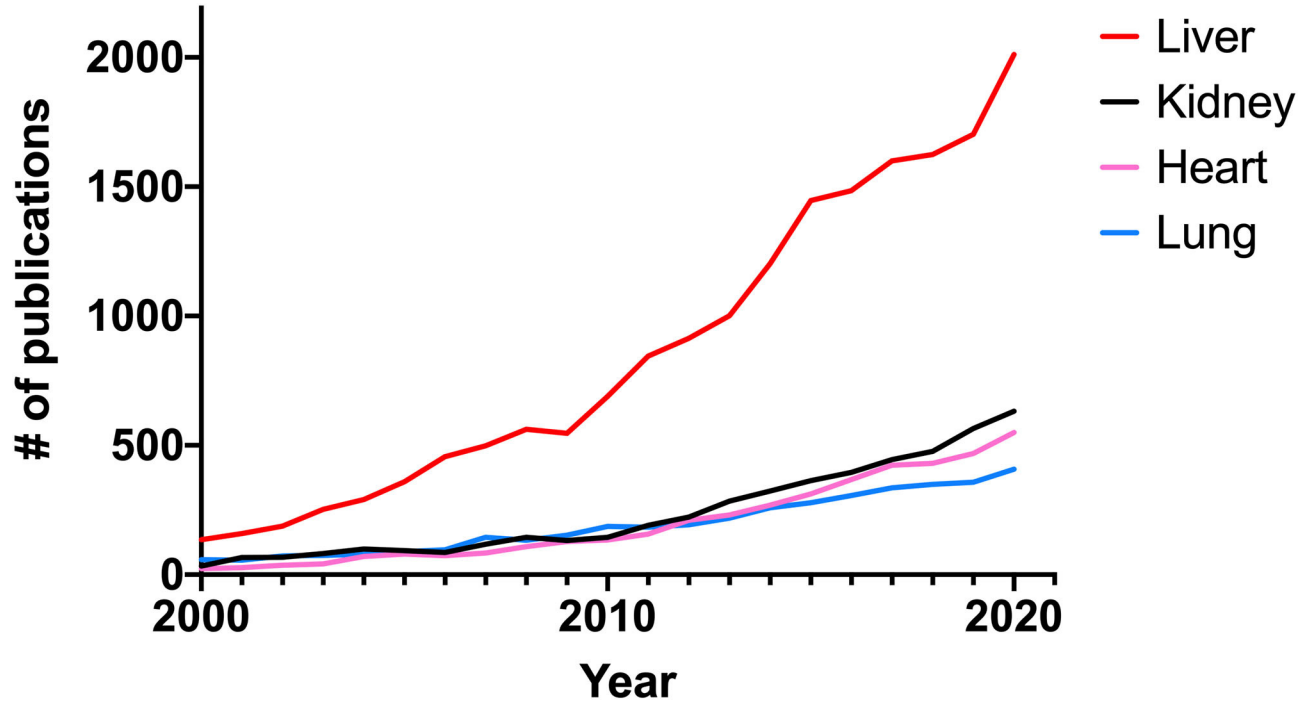

FIGURE 1 | Number of publications related to fibrosis per year indexed in PubMed from 2000 to 2020.

the therapeutic potential of modulating T helper 17 and regulatory T cell responses, more research is clearly needed before we can harness the spectrum of immune cells as therapeutic targets. The work by Gantzel et al., delineates how the scavenger receptors CD163 and mannose receptor (CD206), expressed and secreted by macrophages, can be used as biomarkers to estimate the degree of ongoing inflammation in acute and chronic liver diseases in general and in assessing fibrosis severity in patients with NAFLD as well as chronic hepatitis B and hepatitis C (HCV) infections. These findings were corroborated by the study of Xi et al., who showed that CD163 expression correlated with liver fibrosis progression. Moreover, they demonstrated that CCL2 and TGF-β stimulation increased CD163 expression *in vitro*. However, the combination of CD163 or CD206 with transient elastography (TE) did not appear to improve fibrosis prediction, as compared to TE alone, in two independent cohorts from Italy and Sweden, as shown by Kazankov et al. Thus, the search for highly predictive soluble biomarkers continues. Another marker that might be useful in the clinic is bromodomain-containing protein 4 (BRD4)—a transcriptional co-activator of several pro-fibrotic genes such as collagen 1. Wu et al., reported that BRD4 is up-regulated in liver fibrosis, regardless of etiology, and its expression levels positively correlated with disease severity. However, more research is needed to evaluate whether this marker is superior to currently used tools such as TE. Next to biomarkers, it is also important to identify patient-specific (genetic) risk factors for liver fibrosis, which can contribute to improved and more personalized clinical care. The study by Pineda-Tenor et al. revealed an association between *MTHFR* (methylenetetrahydrofolate reductase; an essential gene for the folate cycle and homocysteine metabolism) rs1801133 polymorphisms and liver fibrosis progression in HCV-infected patients. Their work demonstrated that *MTHFR* rs1801133 C allele carriers presented a diminished risk of liver fibrosis progression as compared to rs1801133 T allele carriers. Even though the study only included a small number of patients, it is a valuable and thought-provoking addition to the current literature regarding the impact of homocysteine homeostasis on NAFLD/NASH and liver fibrosis (3). The final addition to this body of work on liver fibrosis deals with biliary atresia (BA) and matrix metalloproteinase-7 (MMP-7). Nomden et al., provide an extensive overview of the molecular mechanisms of BA and the potential role of MMP-7 herein. As stated by the authors "BA and MMP-7 have an interesting relationship which is yet to be specified," and their work is an essential part of the foundation for future research into BA pathogenesis.

With regards to pulmonary fibrosis, the review by Ding et al. delineates how neutrophils can contribute to tissue destruction, thereby unveiling potential therapeutic targets related to neutrophil functions, such as the formation of neutrophil extracellular traps, for the treatment of lung fibrosis. The study by Ruigrok et al., demonstrated that silencing heat shock protein 47—a chaperone protein of collagen—did not prevent TGF-β-induced fibrosis in murine precision-cut lung slices. Directly after publication this paper received quite some attention on social media and was applauded for presenting "negative results," underscoring the fact that the scientific community needs an outlet for this type of work (4).

Models for fibrosis are constantly being improved to enhance translatability. The paper by Puerta Cavanzo et al. describes the use of macromolecular crowders to promote collagen deposition *in vitro* thereby improving the Scar-in-a-Jar system. As such, the model is a great addition to the toolbox for fibrosis research.

All authors contributing to this Research Topic have made a tremendous effort to provide an overview of the current state of the art in the field of fibrosis research. It was a pleasure to work on this Topic and we hope that the articles will provide the readers with a better understanding of fibrosis and showcase the cornucopia of unanswered questions, thereby stimulating new avenues of research.

AUTHOR CONTRIBUTIONS

HAMM, RN, and PO conceived and wrote the manuscript. All authors approved the final version of the manuscript and fully agree with its content.

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BRIEF RESEARCH REPORT
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MTHFR rs1801133 Polymorphism Is Associated With Liver Fibrosis Progression in Chronic Hepatitis C: A Retrospective Study

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Background: The *MTHFR* (methylenetetrahydrofolate reductase) rs1801133 polymorphism leads to higher circulating levels of homocysteine, which is related to several liver diseases. We aimed to evaluate the relationship between *MTHFR* rs1801133 polymorphism and liver fibrosis progression in HCV-infected patients.

Methods: We conducted a preliminary retrospective cohort study in 208 non-cirrhotic HCV-infected patients. These subjects had at least two liver stiffness measurements (LSM), which were assessed using transient elastography, and no patient had cirrhosis at baseline. We analyzed the association between *MTHFR* rs1801133 and outcome variables using Generalized Linear Models.

Results: HCV-infected patients were 47 years old, around 54% were males, a low frequency of high alcohol intake (13.5%) or prior use of intravenous drugs (10.1%). A total of 26 patients developed cirrhosis (LSM1 $\geq$ 12.5) during a median follow-up of 46.6 months. The presence of the rs1801133 C allele showed an inverse association with the LSM2/LSM1 ratio (adjusted AMR = 0.90; 95%CI = 0.83–0.98; $p = 0.020$) and the cirrhosis progression (adjusted OR = 0.43; 95%CI = 0.19–0.95; $p = 0.038$). Besides, rs1801133 CT/CC genotype had an inverse association with the LSM2/LSM1 ratio (adjusted AMR = 0.80; 95%CI = 0.68–0.95; $p = 0.009$) and the cirrhosis progression (adjusted OR= 0.21; 95%CI = 0.06–0.74; $p = 0.015$).

Conclusions: *MTHFR* rs1801133 C allele carriers presented a diminished risk of liver fibrosis progression and development of cirrhosis than rs1801133 T allele carriers. This statement supports the hypothesis that *MTHFR* rs1801133 polymorphism appears to play a crucial role in chronic hepatitis C immunopathogenesis.

Keywords: chronic hepatitis C, liver stiffness measure, hepatic fibrosis, cirrhosis, MTHFR (C677T), SNPs (single nucleotide polymorphism)

INTRODUCTION

According to the world health organization, viral hepatitis is a significant public health problem that causes 1.34 million deaths per year due to chronic liver disease (720,000 by cirrhosis) and primary liver cancer (47,000 by hepatocellular carcinoma) (1). Globally, around 71 million people suffer chronic hepatitis C (CHC) and the development of the previously described events is frequent (2, 3), even after a sustained virological response (SVR) to treatment with direct-acting antivirals (DAAs) (4, 5). The pathogenic mechanisms involved in the progression of fibrosis and cirrhosis depends, among others, on the genetic background of individuals, including several single nucleotide polymorphisms (SNPs) (6, 7).

The staging of liver fibrosis provides essential clinical information that allows the adequate management and prognosis of CHC patients (8). The liver biopsy has been used to grade the necroinflammatory activity and to stage fibrosis, together with rating scales like METAVIR, which stratify fibrosis as: (i) F0, no fibrosis; (ii) F1, mild fibrosis; (iii) F2, significant fibrosis; (iv) F3, advanced fibrosis; and (v) F4, cirrhosis (9). Nevertheless, non-invasive approaches, as the transient elastography or FibroScan, have been widely used to the liver fibrosis assessment, with excellent accuracy in advanced fibrosis and cirrhosis (10). In this context, the evaluation of liver stiffness measurement (LSM), an intrinsic physical property of liver parenchyma, provides quantitative data that correlates with fibrosis stage in CHC (11).

The methylenetetrahydrofolate reductase (*MTHFR*) gene encodes an enzyme that plays an important role in the folate metabolism, allowing the conversion from homocysteine to methionine (12). The rs1801133 SNP (also named C677T) is a non-synonymous variant A (Ala) > V (Val) (missense variant) (13). The substitution A > V in the aminoacid 222 (Ala222Val) produces a decrease of the activity of MTHFR protein and an elevation of plasma homocysteine levels (13). Hyper-homocysteinemia was related to several diseases, including hepatocellular carcinoma, steatosis, and cirrhosis (14–16), and also the development of liver fibrosis in CHC (17). There are a few articles published about *MTHFR* rs1801133 in CHC patients. *MTHFR* C677T polymorphism has been related to hepatic steatosis (18) and development of liver fibrosis (16, 17), but no association was found in other articles in patients infected with HCV (19–21). Additionally, this polymorphism could also be related to a direct profibrogenic effect, modifying the action of proteins implicated in the degradation of collagen (22).

The main objective in the current study was to investigate the association of *MTHFR* rs1801133 polymorphism with the progression of liver fibrosis and cirrhosis development, evaluated by LSM, in patients with CHC.

Abbreviations: CHC, chronic hepatitis C; HCV, hepatitis C virus; DAAs, direct-acting antivirals; SNPs, single nucleotide polymorphisms; LSM, liver stiffness measurement; MTHFR, methylenetetrahydrofolate reductase; F4; LSM1 ≥ 12.5 kPa, cirrhosis; LSM1, baseline LSM; LSM2, final LSM; ΔLSM, increase of LSM; GLM, generalized linear models; AMR, arithmetic mean ratio; OR, odds ratio; SPSS, statistical package for the social sciences; IBS, Iberian population in Spain.

MATERIALS AND METHODS

Study Population

A preliminary retrospective study was performed in 208 patients from the Hospital Virgen de la Salud (Toledo, Spain). All patients suffered from chronic hepatitis C and were enrolled between 2008 and 2016, as previously described (see **Supplementary Figure 1**) (23).

We selected the patients according to these criteria: (i) available DNA sample; (ii) detectable plasma HCV RNA at baseline and during follow-up; and (iii) baseline LSM (LSM1) and final LSM (LSM2) available with a separation of 12 months at least. Regarding the exclusion criteria, we considered: (i) liver cirrhosis at baseline (F4; LSM1 ≥ 12.5 kPa); (ii) coinfection with human immunodeficiency virus or hepatitis B virus; and (iii) autoimmune liver disease.

The study was conducted with the consent of all patients and following the 1975 Declaration of Helsinki. It was approved by the Institutional Review Board of the Instituto de Salud Carlos III ("Comité de Ética de la Investigación y Bienestar Animal" –April 4, 2013).

Clinical Data

As described previously (24), clinical and epidemiological data were collected from medical records. These data included virological, demographic, clinical, and laboratory data. Clinical guidelines available at that time (25, 26) were followed to perform the clinical management of patients during the follow-up.

At baseline, we only included non-responder patients (patients treated for HCV infection before the study), although it was possible to administrate the HCV therapy before or after being included in the study. During the follow-up, the monitoring was stopped when a patient started the HCV therapy and obtained an SVR.

DNA Genotyping

Genomic DNA obtained from 200 microliters of peripheral blood was extracted using the QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany). *MTHFR rs1801133* polymorphism was genotyped at the CeGen (Spanish National Genotyping Center; http://www.cegen.org/). Agena Bioscience's MassARRAY platform (San Diego, CA, USA) and the iPLEX® Gold assay design system were used according to the method described by Gabriel et al. (27).

Evaluation of Liver Fibrosis

The hepatic fibrosis was evaluated using the transient elastography (FibroScan, Echosens, Paris, France), by a trained hepatologist and with a single machine, as we previously described (24). LSM has a range of 2.5 to 75 kilopascals (kPa). When the interquartile-range-to-median ratio for at least 10 successful measurements was <0.30, it was considered reliable. The cut-offs of LSM proposed by Castera et al. were followed for the stratification of patients: (i) <7.1 kPa (F0–F1—absence or mild fibrosis); (ii) 7.1–9.4 kPa (F2—significant fibrosis); (iii) 9.5-12.4 kPa (F3—advanced fibrosis); and (iv) ≥12.5 kPa (F4—cirrhosis) (28).

TABLE 1 | Epidemiological and clinical characteristics of HCV-infected patients at baseline.

Characteristic	All patients	*MTHFR* rs1801133 polymorphism			
		TT	CT	CC	*P*-value
No.	208	29	112	67	
Male	112 (53.8%)	14 (48.33%)	65 (58%)	33(49.3%)	0.423
Age (years)	47.1(41.5; 57.6)	46.96 (39.5; 57.3)	46.85 (41.3; 55.4)	48.2 (42.6; 60.8)	0.988
Time of HCV infection (years)	8.2 (3.2; 13.2)	7.8 (3.3; 11.9)	8.1 (2.9; 13.3)	9 (2.7; 13.3)	0.959
High alcohol intake	28 (13.5%)	1 (3.4%)	20 (17.9%)	7 (10.4%)	0.087
Prior injection drug use	21 (10.1%)	1 (3.4%)	18 (16.1%)	2 (3%)	**0.008**
HCV genotype ($n = 204$)					
1	174 (85.3%)	26 (92.9%)	90 (82.6%)	58 (86.6%)	0.366
3	14 (6.9%)	1 (3.6%)	9 (8.3%)	4 (6%)	0.641
4	15 (7.4%)	1 (3.6%)	9 (8.3%)	5 (7.5%)	0.698
5	1 (0.5%)	0 (0.0%)	1 (0.9%)	0 (0%)	-
Prior failed IFN therapy	47 (22.6%)	6 (20.7%)	26 (23.2%)	15 (22.4%)	0.958
Baseline LSM (kPa)	6.1 (5.2; 7.7)	6.6 (5.2; 7.5)	6.1 (5.2; 7.7)	5.9 (5.1; 7.7)	0.961
F0–F1 (<7.1 kPa)	149 (71.6%)	21 (72.4%)	79 (70.5%)	49 (73.1%)	0.928
F2 (7.1–9.4 kPa)	38 (18.3%)	5 (18.2%)	22 (19.6%)	11 (16.4%)	0.873
F3 (9.5–12.4 kPa)	21 (10.1%)	3 (6.8%)	11 (9.8%)	7 (10.4%)	0.904

Statistics: Values are expressed as absolute numbers (%) or median (percentile 25; percentile 75). P-values were estimated with Chi-square test for categorical variables and non-parametric Mann-Whitney U test for continuous variables.
HCV, hepatitis C virus; IFN, interferon; kPa, kilopascal; LSM, liver stiffness measure; MTHFR, methylenetetrahydrofolate reductase.
Significant differences are shown in bold.

Outcome Variable

We analyzed how LSM values changed during the follow-up, considering: (i) the date of the first LSM (LSM1) and (ii) the date of the last LSM (LSM2), or the date when the HCV therapy started in responder patients who cleared HCV infection. For this propose, we consider two outcome variables: (1) LSM2/LSM1 ratio; (2) the cirrhosis progression (F4; LSM $\geq$ 12.5 kPa) measured as +1 [if a patient with LSM <12.5 kPa (F $\leq$ 3) changed to LSM $\geq$12.5 kPa (F4)] or 0 (if a patient with F $\leq$ 3 did not evolve to F4).

Statistical Analysis

To compare independent groups, we used the Mann-Whitney *U* test for continuous variables and the Chi-square test or Fisher's exact test for categorical variables. In the case of paired measurements, we used the Sign test for categorical variables and the Wilcoxon signed-rank test for continuous variables.

We used Generalized Linear Models (GLM) according to recessive, dominant, and additive inheritance models for the genetic association study with the aim of comparing the outcome variables according to *MTHFR* rs1801133. First, we used a GLM with a gamma distribution (log-link) to analyze continuous variables (LSM2/LSM1 ratio) and a GLM with a binomial distribution (logit-link) to analyze dichotomous variables (progression to cirrhosis). These tests provide the arithmetic mean ratio (AMR) or difference between groups, and the odds ratio (OR) or probability of occurrence of an event. The most relevant patient characteristics were used to adjust the GLM tests: gender, age, diabetes, high alcohol intake, injection drug use, time since HCV diagnosis, HCV genotype, baseline LSM, time of follow-up, HCV antiviral therapy before baseline and during the follow-up (patients who failed therapy), and other SNPs previously described in this study population (*PNPLA3* rs738409 (29), *MERTK* rs4374383 (30), *IL7RA* rs6897932 (24), and *DARC* rs12075 (23). To avoid overfitting the statistical models, we made a previous selection of covariables with the Stepwise algorithm, retaining covariables with a p-value < 0.20 at each step.

For all statistical tests, we used the Stata 15.0 (StataCorp, Texas, USA) and SPSS 24.0 (SPSS INC, Chicago, IL, USA). Statistical significance was defined as $p < 0.05$ and all p-values were two-tailed.

RESULTS

Characteristics of the Patients

The baseline characteristics of our study population are described in **Table 1**. HCV-infected patients were 47 years old, around 54% were males, a low frequency of high alcohol intake (13.5%) or prior use of intravenous drugs (10.1%). HCV genotype 1 was the predominant (85.3% of patients), 22.6% of patients previously failed the interferon therapy, and 71.6% of patients had LSM $<$ 7.1 kPa. Concerning rs1801133 genotypes, 29 patients were TT genotype, 112 were CT genotype, and 67 were CC genotype. No significant differences in baseline characteristics were found among rs1801133 genotypes, except for prior injection drug use ($p = 0.008$).

Characteristics of *MTHFR* rs1801133 Polymorphism

Table 2 describes the allelic and genotypic frequencies of the rs1801133 SNP, which showed <5% of missing values, was in

TABLE 2 | Allelic and genotypic frequencies and Hardy Weinberg Equilibrium test for *MTHFR rs1801133* polymorphism in HCV-infected patients compared to Iberian population (data from 1,000 Genomes Project Phase 3) (http://grch37.ensembl.org/index.html).

		HCV cohort	IBS group	*P*-value
No.		208	107	
Alleles	T	141 (44.1%)	95 (44.4%)	0.940
	C	179 (55.9%)	119 (55.6%)	
Genotype	TT	29 (13.9%)	18 (16.8)	0.665
	CT	112 (53.8%)	59 (55.1%)	
	CC	67 (32.2%)	30 (28.0%)	
HWE (*p*-value)		0.252	0.241	

Statistics: Values are expressed as absolute numbers (%) p-values were estimated with Chi-squared test.
HCV, hepatitis C virus; HWE, Hardy Weinberg Equilibrium; IBS, Iberian populations in Spain; MTHFR, methylenetetrahydrofolate reductase.

TABLE 3 | Clinical characteristics related to hepatic fibrosis in patients with chronic hepatitis C during the follow-up.

	All Patients		
Characteristic	**Baseline**	**End**	***P*-value**
LSM (kPa)	6.1 (5.2; 7.7)	6.8 (5.5; 9.4)	**<0.001**
F0–F1 (<7.1 kPa)	149 (71.6%)	110 (52.9%)	**<0.001**
F2 (7.1–9.4 kPa)	38 (18.3%)	47 (22.6%)	0.382
F3 (9.5–12.4 kPa)	21 (10.1%)	25 (12%)	0.571
F4 (≥12.5 kPa)	0 (0%)	26 (12.5%)	**<0.001**

Statistics: Values are expressed as absolute numbers (%) or median (percentile 25; percentile 75). P-values were calculated with Sign test for categorical variables and nonparametric Wilcoxon test for continuous variables.
HCV, hepatitis C virus; kPa, kilopascal; LSM, liver stiffness measurement; F ≥ 2, significant fibrosis; F ≥ 3, advanced fibrosis; F4, cirrhosis.
Significant differences are shown in bold.

Hardy-Weinberg equilibrium ($p = 0.252$) and had a minimum allele frequency more than 40%. We compared the genetic frequencies between patients included in this study and the Iberian population in Spain (IBS), a population of healthy subjects published by the 1,000 Genomes Project website (http://www.1000genomes.org/home). No significant differences were found for alleles ($p = 0.940$) or genotypes ($p = 0.665$).

MTHFR rs1801133 SNP and Related Liver Fibrosis Progression

The mean follow-up time (the period between the LSM1 and the LSM2) for all patients was of 46.6 months. In this context, we found a decrease in the proportion of patients with a low stage of fibrosis (F0–F1; $p < 0.001$), whereas both LSM values and the rate of patients who developed an F4 stage raised ($p < 0.001$) (**Table 3**).

We did not find significant differences among rs1801133 genotypes in the time intervals between the LSM1 and the LSM2 (48.5 months in TT genotype, 46.9 months in CT genotype, and 45.5 months in CC genotype; $p = 0.921$). We observed decreased values of the LSM2/LSM1 ratio and the rate of cirrhosis progression in rs1801133 T allele carriers (**Table 4**). Moreover, we analyzed the association between *MTHFR* rs1801133 polymorphism and liver fibrosis/cirrhosis progression through multivariate GLMs (**Table 4,** full description in **Supplementary Table 1**). The presence of the rs1801133 C allele showed an inverse association with the LSM2/LSM1 ratio (adjusted AMR = 0.90; 95%CI = 0.83–0.98; $p = 0.020$) and the cirrhosis progression (adjusted OR = 0.43; 95%CI = 0.19–0.95; $p = 0.038$). Besides, rs1801133 CT/CC genotype had an inverse association with the LSM2/LSM1 ratio (adjusted AMR = 0.80; 95%CI = 0.68–0.95; $p = 0.009$) and the cirrhosis progression (adjusted OR = 0.21; 95%CI = 0.06–0.74; $p = 0.015$) (**Table 4**).

DISCUSSION

The present study focused on the potential relationship of *MTHFR* rs1801133 polymorphism and the development of liver fibrosis and cirrhosis on HCV-infected patients, using two LSM values with an interval of at least 12 months. We observed that CT/CC genotype was related to a decreased risk of progression of liver fibrosis and the occurrence of cirrhosis.

The C677T genetic substitution at the *MTHFR* gene results in the Ala222Val replacement in the MTHFR protein, resulting in a thermolabile variant associated with lower activity, and therefore, higher circulating levels of homocysteine (13). Moreover, *MTHFR* rs1801133 SNP is in high linkage disequilibrium (LD) with other *MTHFR* SNPs, such as A1298C (rs1801131), also related to a decrease in MTHFR activity (31). The LD between the two SNPs is strong in the Spanish population (coefficient of LD = 0.98) (32), so we think that our results may be extrapolated to the A1298C variant and other *MTHFR* SNPs in LD with rs1801133.

The *MTHFR* rs1801133 SNP was previously linked to a long list of conditions and diseases, such as bone disorders, cardiovascular disease, thrombosis, neurological/neuropsychiatric conditions (33), pre-eclampsia, diabetes mellitus (34), longevity (35), and several types of neoplasia (36). Regarding liver diseases, the *MTHFR* rs1801133 polymorphism is related to altered lipid metabolism (37), which would contribute to the development of steatosis and fibrosis in HCV-infected patients (38), as well as the development of cirrhosis (14, 39–41). Furthermore, in CHC, the *MTHFR* rs1801133 variant has also been linked to liver fibrosis/cirrhosis (16, 17). Toniutto et al. described that recipients with the presence of *MTHFR* rs1801133 TT homozygote evolved with more frequency to a significant fibrosis degree during recurrent hepatitis C after liver transplantation (16). Similar to this, Adinolfi et al. described that the T allele is related to a higher prevalence of steatosis, accelerating the fibrosis development and liver disease progression (17). These analyses support that the *MTHFR* rs1801133 polymorphism and the subsequent hyperhomocysteinemia are associated with liver fibrosis among HCV-infected patients. However, there are also other articles that found no association between the *MTHFR* rs1801133 variant and liver fibrosis/cirrhosis (19–21). These articles,

TABLE 4 | Association between *MTHFR* rs1801133 polymorphism and progression of liver fibrosis in patients with chronic hepatitis C (longitudinal analysis).

	***MTHFR* rs1801133 genotypes**			**Unadjusted**		**Adjusted**	
Outcome	**TT**	**CT**	**CC**	**AMR (95%CI)**	***P*[a]**	**aAMR (95%CI)**	***P*[b]**
LSM2/LSM1							
Additive	1.19 (1.00; 1.43)	1.14 (0.95; 1.44)	1.10 (0.89; 1.34)	0.85 (0.77; 0.94)	**0.001**	0.90 (0.83; 0.98)	**0.020**
Dominant	1.19 (1.00; 1.43)	1.13 (0.95; 1.43)		0.77 (0.64; 0.93)	**0.006**	0.80 (0.68; 0.95)	**0.009**
Progression to F4	**TT**	**CT**	**CC**	**OR (95%CI)**	***P*[a]**	**aOR (95%CI)**	***P*[b]**
Additive	7 (24.10%)	13 (11.60%)	6 (9.00%)	0.55 (0.29; 1.04)	0.069	0.43 (0.19; 0.95)	**0.038**
Dominant	7 (24.10%)	19 (10.60%)		0.37 (0.14; 0.99)	**0.047**	0.21 (0.06; 0.74)	**0.015**

Statistics: Values expressed as absolute numbers (%), median (percentile 25; percentile 75), arithmetic mean ratio (AMR), odds ratio (OR), and 95% of confidence interval (95%CI). [a]*P-values were calculated by univariate regression;* [b]*P-values were calculated by multivariate regression adjusted by the most important clinical and epidemiological characteristics (see statistical analysis section). Significant differences are shown in bold.*

aAMR, adjusted arithmetic mean ratio; aOR, adjusted odds ratio; 95%CI, 95% confidence interval; p-value, level of significance; LSM, liver stiffness measure; F4, cirrhosis; MTHFR, methylenetetrahydrofolate reductase.

both those that showed an association and those that did not, had a cross-sectional design, and fibrosis was evaluated by biopsy. Our article, by contrast, had a longitudinal design that provides robustness to our data, and liver fibrosis was evaluated by transient elastography, which has excellent accuracy for cirrhosis diagnosis.

Other issues should be considered for the correct interpretation of the data. Firstly, we performed a retrospective study, which could induce ascertainment and selection biases. Secondly, the low sample size per group could limit the statistical power of the tests performed and increasing the rate of false positives. Therefore, further studies should be conducted to corroborate our preliminary findings on the potential use of *MTHFR* rs1801133 SNP as a predictive marker of liver fibrosis/cirrhosis progression in HCV-infected patients. Thirdly, the follow-up time (between LSM1 and LSM2) varied between different subjects, but 75% of the patients had more than 28 months of follow-up, and globally, all patients presented more than 12 months of follow-up. Besides, the time of follow-up in patients stratified by *MTHFR* rs1801133 genotypes was comparable. Fourthly, due to the retrospective design of our investigation, we did not take into account some important clinical variables, including obesity, abdominal ultrasound, metabolic syndrome, and pathological study of the liver (fibrosis, necroinflammation, and steatosis), among others; and biomarkers, such as HCV viral load, transaminases, platelet counts, APRI score, and FIB4 index, among others. We did not have access to these data at the time of the LSM. Moreover, we had not plasma samples available to measure concentrations of homocysteine. Finally, we included in the study more than 20% non-responders to previous interferon therapy, but this HCV therapy does not seem to protect against the progression of CHC in the long term studies (42).

CONCLUSION

In summary, in this preliminary study, our data suggest an association between *MTHFR* rs1801133 SNP and the progression of liver fibrosis and the development of cirrhosis in HCV-infected patients. Specifically, *MTHFR* rs1801133 C allele carriers presented a diminished risk of liver fibrosis progression and development of cirrhosis than rs1801133 T allele carriers. Further studies with higher numbers of patients would be needed to confirm the role of *MTHFR* in the immune-pathogenesis of CHC.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study may be made available by the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the study was conducted following the 1975 Declaration of Helsinki. The Institutional Review Board of the Instituto de Salud Carlos III (Comité de Ética de la Investigación y Bienestar Animal 04/04/2013) approved the study, and all patients gave their consent for the study. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MJ-S and SR: funding body, study concept and design. AG-M, MJ-S, JS-R, and TA-V: patients' selection and clinical data acquisition. DP-T, MJ-S, and AG-M: sample preparation, DNA isolation and genotyping. DP-T, MJ-S, AV-B, and SR: statistical analysis and interpretation of data. DP-T, MJ-S, and SR: writing of the manuscript. DP-T, AF-R, and PM: critical revision of the manuscript for relevant intellectual content. SR: supervision and visualization. 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/fmed.2020.582666/full#supplementary-material

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Macrophage Markers Do Not Add to the Prediction of Liver Fibrosis by Transient Elastography in Patients With Metabolic Associated Fatty Liver Disease

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Background and Aims: Non-invasive fibrosis staging is essential in metabolic associated fatty liver disease (MAFLD). Transient elastography (TE) is a well-established method for liver fibrosis assessment. We have previously shown that the macrophage marker sCD163 is an independent predictor for fibrosis in MAFLD. In the present study we tested whether the combination of macrophage markers and TE improves fibrosis prediction.

Methods: We measured macrophage markers soluble (s)CD163 and mannose receptor (sMR) in two independent cohorts from Italy ($n = 141$) and Sweden ($n = 70$) with biopsy-proven MAFLD and available TE.

Results: In the Italian cohort, TE and sCD163 showed similar moderate associations with liver fibrosis (rho = 0.56, $p < 0.001$ and rho = 0.42, $p < 0.001$, respectively). TE had an area under the Receiver Operating Characteristics curve (AUROC, with 95% CI) for fibrosis; F ≥ 2 = 0.79 (0.72–0.86), F ≥ 3 = 0.81 (0.73–0.89), F4 = 0.95 (0.90–1.0). sCD163 also predicted fibrosis well [F ≥ 2 = 0.71 (0.63–0.80), F ≥ 3 = 0.82 (0.74–0.90), F4 = 0.89 (0.76–1.0)]. However, combining sCD163 and TE did not improve the AUROCs significantly [F ≥ 2 = 0.79 (0.72–0.86), F ≥ 3 = 0.85 (0.78–0.92), F4 = 0.97 (0.93–1.0)]. In the Swedish cohort, TE showed a closer association with fibrosis (rho = 0.73, $p < 0.001$) than sCD163 (rho = 0.43, $p < 0.001$) and sMR (rho = 0.46, $p < 0.001$). TE predicted fibrosis well [F ≥ 2 = 0.88 (0.80–0.97), F ≥ 3 = 0.90 (0.83–0.97), F4 = 0.87 (0.78–0.96)], whereas sCD163 did not (best AUROC 0.75). sMR showed a better prediction [F ≥ 2 = 0.68 (0.56–0.81), F ≥ 3 = 0.82 (0.71–0.92), F4 = 0.79 (0.66–0.93)], but the addition of sMR did not further improve the prediction of fibrosis by TE.

Conclusion: In these cohorts of MAFLD patients, TE was superior to macrophage markers for fibrosis prediction and in contrast to our hypothesis the addition of these markers to TE did not improve its predictive capability.

Keywords: macrophages, cirrhosis, biomarkers, NAFLD, Fibroscan

INTRODUCTION

Metabolic associated fatty liver disease (MAFLD), a newly instituted and more appropriate term for non-alcoholic fatty liver disease (NAFLD) (1), is an increasingly prevalent liver condition estimated to affect a quarter of the world's population with even higher prevalence in the constantly growing group of subjects with obesity and type 2 diabetes (2). The majority of patients with MAFLD do not develop significant liver disease, however, some of them may progress to cirrhosis and liver failure ultimately warranting a liver transplant. In this respect, the stage of fibrosis has repeatedly proved to be the most important determinant of outcome in MAFLD patients (3, 4), making accurate identification of patients with advanced fibrosis crucial in the management of MAFLD. So far, liver biopsy remains the gold standard for fibrosis staging in MAFLD, however it is invasive and thus not suitable for general use given the vast numbers of subjects in need of examination. Therefore, a number of non-invasive tools for fibrosis assessment have been established (5).

Transient elastography (TE) provides the value of liver stiffness as a measure of fibrosis. It has shown good accuracy in MAFLD, particularly for advanced fibrosis and cirrhosis detection (6), and has been introduced into the practical guidelines for MAFLD diagnosis and management (7). However, TE has several limitations including imperfect prediction (8). Therefore, combinations of TE with other markers have been explored, for instance, a recent multi-center study developed and validated a score consisting of TE, controlled attenuation parameter (CAP) and aspartate aminotransferase (AST) to identify MAFLD patients with inflammatory activity, steatohepatitis and significant fibrosis (9).

Macrophages play an important role in MAFLD (10), and we and others have shown good predictive capability of the macrophage specific marker soluble (s)CD163 for fibrosis in MAFLD (11, 12). Furthermore, the addition of sCD163 to the established NAFLD fibrosis score (NFS) improved its performance (13). Another macrophage marker, the soluble mannose receptor (sMR), is associated with acute and chronic liver disease (14–16) and has not been investigated in adult MAFLD before.

We hypothesized that combining the macrophage markers sCD163 or sMR with TE would result in improvement of fibrosis prediction, and we tested this hypothesis in two independent cohorts of MAFLD patients with biopsy-proven disease.

METHODS

Study Population

This cross-sectional study was performed in two established cohorts of MAFLD patients from liver centers in Italy and Sweden. The Italian cohort consisted of patients from the Division of Gastroenterology and Hepatology, Department of Medical Sciences, University of Torino, Italy, while the Swedish cohort comprised patients included at the Karolinska University Hospital, Stockholm, Sweden. Both of these cohorts have been described in previously published reports (13, 17).

At both sites, all patients were referred for the investigation of abnormal liver tests or steatosis detected by ultrasound, and MAFLD was diagnosed by liver biopsy. In the Italian cohort, all patients had an alcohol intake of <20 g/day assessed by interviews with the patient or close family members, and in the Swedish cohort an intake of <30 g/day (males) an <20 g/day (females), assessed with phosphatidylethanol (PEth) and the alcohol-use disorders identification test (AUDIT) and the lifetime drinking history (LDH) questionnaires (18, 19).

Liver disease of other etiology was excluded. The number of patients with biopsy-proven MAFLD and available TE was 141 in the Italian cohort and 70 in the Swedish cohort. At the time of liver biopsy, demographic and clinical data were recorded, including age, gender, ethnicity, height, weight, and waist circumference. Body Mass Index (BMI) was calculated. Diabetes was defined as hemoglobin A1c $\geq$ 48 mmol/mol, fasting blood glucose $\geq$7.0 mmol/L, previous diagnosis of diabetes or use of anti-diabetic drugs.

At the time of biopsy, a fasting blood sample was obtained and routine biochemical tests were performed. Additional blood samples were drawn and frozen at -80°C for future research. All patients signed an informed consent form in accordance with the Helsinki Declaration. The acquisition, storage, and use of blood samples were approved by the Ethics Committee of the University Hospital San Giovanni Battista of Torino and by the Regional Ethics Committee of Stockholm (2011/13-31-/1 and 2018/134-32).

Biochemical Analyses

Liver and hematological parameters, fasting glucose and insulin, triglycerides and cholesterol and its components were determined using standard assays and methods. All sCD163 and sMR measurements were performed at the Department of Clinical Biochemistry, Aarhus University Hospital.

This was done in duplicate by in-house enzyme-linked immunosorbent assays (ELISAs) using a BEP-2000 ELISA-analyzer (Dade Behring) essentially as previously described (20, 21). Soluble CD163 and sMR are both resistant to repeated freezing and thawing (20–22). Control samples and serum standards were included in each run to avoid bias. We have previously established reference intervals for sCD163 (0.69–3.86 mg/L) and sMR (0.10–0.43 mg/L) in large cohorts of healthy individuals using the same assays (21, 23).

Histological Analysis

Liver biopsies were stained and examined locally by experienced pathologists as described by Kleiner et al. (24) and in accordance with the Fatty Liver Inhibition of Progression (FLIP) algorithm for the diagnosis of steatohepatitis (25). All biopsies had a minimum of 11 portal tracts, and inadequate biopsies were excluded.

Transient Elastography

Vibration controlled transient elastography (FibroScan, Echosens, Paris, France) was performed within 2 weeks prior to the liver biopsy by expert operators in accordance with the instructions by the manufacturing company, including at least 3 h of fasting. The M probe was used as standard, and the XL probe as per the automatic probe selection tool or when the M probe failed.

TE was expressed in kilopascal (kPa) and calculated as the median value of 10 successful acquisitions, defined by a success rate of >60%, and by an interquartile range <30%.

Calculation of the NAFLD Fibrosis Score and FIB-4

The NFS was calculated using the existing formula: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ body mass index (kg/m^2) $+ 1.13 \times$ impaired glucose tolerance/diabetes mellitus (yes = 1, no = 0) $+ 0.99 \times$ aspartate aminotransferase/alanine aminotransferase $- 0.013 \times$ platelets (x10^9/L) $- 0.66 \times$ albumin (g/dL) (26). The FIB-4 was calculated using the following formula: age (years) $\times$ aspartate aminotransferase(U/L)/[platelets (x10^9/L) $\times$ square root (alanine aminotransferase(U/L))] (27).

Statistical Methods

Student's *t*-test was used for the comparison of normally distributed variables between the groups. For non-normally distributed data, the Mann-Whitney test was used. The relationship between sCD163/sMR and TE was analyzed by linear regression. Spearman's rank test was used to study the relationships of TE, sCD163, and sMR with histological scores. For differences in proportions, we used the χ^2-test or Fisher's exact test.

Multiple ordered logistic regression was used to assess the relationship between the histological fibrosis score and TE, sCD163, and sMR. This analysis provides odds ratios (OR) describing the increase in the odds for a given fibrosis stage in a patient who has a specific increase in a parameter compared with another patient. We chose to present the results corresponding to a 25% increase in TE, sCD163 and sMR based on our previous experience (13, 28) and the distribution of these parameters according to fibrosis stages. We used multiple logistic regression analysis with given fibrosis stages (separate analysis for F $\geq$ 2, F $\geq$ 3, F4 stages) as the dependent variable and TE and sCD163/sMR as the explanatory to identify the best fitting models for predicting a given fibrosis stage, separately for the Italian and the Swedish cohort. The coefficients from this analysis were used as relative weights to compute the respective combined models. We then used the non-parametric Receiver Operating Characteristics (ROC) analysis to assess the performance of TE, sCD163/sMR, and their combinations in the prediction of fibrosis stages, followed by tests of equality of areas under the ROC curve (AUROCs) to compare the performance of individual markers and composite models. All data are expressed as means $\pm$ *SD* and medians with interquartile ranges (IQR) or proportions. A $p \leq 0.05$ was considered statistically significant. STATA version 14.0 ®StataCorp LP was used for data analysis.

RESULTS

Patients Characteristics

Demographic, clinical, biochemical, and histological data for the patients from both cohorts are shown in **Table 1**. The patients in the Swedish cohort were older, and a higher proportion had diabetes. Likewise, the Swedish patients had histologically more severe MAFLD, with more frequent steatohepatitis and advanced fibrosis stages. In addition, the levels of sCD163 and AST were also higher in the patients from the Swedish cohort.

Associations of Macrophage Markers and TE With Fibrosis

In the Italian cohort, sCD163 showed a moderate association with fibrosis (rho = 0.42, $p < 0.001$), as shown in **Figure 1A**. TE also correlated well with fibrosis (rho = 0.56, $p < 0.001$; **Figure 1B**). TE showed an even closer association with fibrosis in the Swedish cohort (rho = 0.73, $p < 0.001$; **Figure 1B**), which was better than for sCD163 (rho = 0.43, $p < 0.001$; **Figure 1A**), and sMR (rho = 0.46, $p < 0.001$; **Figure 1C**).

In the multiple ordered logistic regression analysis, both TE and sCD163 were significantly associated with the stage of fibrosis (OR = 1.05, $p < 0.001$ and OR = 1.13, $p = 0.013$, respectively) in the Italian cohort. In the same analysis including TE and sCD163 in patients from Sweden, TE showed a significant association (OR = 1.03, $p = 0.002$) and sCD163 showed a trend (OR = 1.05, $p = 0.088$). Similarly, the multivariate analysis with TE and sMR as the explanatory variables resulted in a significant association by TE (OR = 1.03, $p = 0.004$) and a trend by sMR (OR = 2.38, $p = 0.062$). When including both TE, sCD163, and sMR in the analysis in the Swedish cohort, TE remained significantly associated with fibrosis (OR = 1.03, $p = 0.006$), whereas sCD163 and sMR lost significance (OR = 1.03, $p = 0.32$ and OR = 1.91, $p = 0.21$, respectively).

Prediction of Fibrosis by TE, Macrophage Markers, and Combined Models

In the ROC analysis, both TE and sCD163 showed good prediction of fibrosis in the Italian cohort, especially for F $\geq$ 3 and F4 stages. Combining TE and sCD163 resulted in slightly higher AUROCs than for TE alone, however, this improvement was not statistically significant (**Table 2**).

Nevertheless, we explored whether the numerically higher AUROC of the combined model translated into an improvement in the negative and positive predictive values (NPV and PPV). We thus determined the predictive characteristics of TE and the combined model (TE + sCD163) for advanced fibrosis (F $\geq$ 3) based on its importance for long-term outcome in MAFLD (4). We used the established cut-off values for TE (<7.9 and ≥9.6

TABLE 1 | Patient characteristics.

	Italian cohort	Swedish cohort	*P*
	(*n* = 141)	(*n* = 70)	
Age (years)	43 ± 11	51 ± 14	<0.001
Sex [m/f (%)]	106 (75%)/35 (25%)	48 (69%)/22 (31%)	0.31
BMI (kg/m^2)	28 ± 4	31 ± 4	<0.001
Diabetes [*n* (%)]	34 (24%)	19 (35%)	0.02
Steatohepatitis (FLIP algorithm) [*n* (%)]	78 (55%)	49 (70%)	0.04
Fibrosis stage [*n* (%)]			
0	50 (35%)	9 (13%)	<0.001
1	29 (21%)	26 (37%)	
2	30 (21%)	14 (20%)	
3	25 (18%)	8 (11%)	
4	7 (5%)	13 (19%)	
F ≥2, NAS ≥4 and steatohepatitis [*n* (%)]	31 (22%)	25 (36%)	0.03
sCD163 (mg/L)	1.6 (1.2–2.3)	3.4 (2.3–4.8)	<0.001
sMR (mg/L)	-	0.28 (0.22–0.39)	-
ALT (IU/L)	66 (42–94)	68 (47–114)	0.23
AST (IU/L)	36 (28–48)	47 (34–68)	<0.001
Albumin (g/L)	46 ± 4	39 ± 3	<0.001
Platelets (x10^9/L)	230 ± 70	216 ± 57	0.16

Parameters are presented as means ± SD for normally distributed and medians (interquartile range) for non-normally distributed variables, and as total number (%) for categorical variables. BMI, Body Mass Index; FLIP, Fatty Liver Inhibition of Progression; sCD163, soluble CD163; sMR, soluble mannose receptor; ALT, alanine transaminase; AST, aspartate transaminase.

kPa) (29), and determined 2 cut-offs values of the combined model, a low cut-off for ruling out and a high cut-off for ruling in advanced fibrosis, based on the ROC curve (**Figure 2**). The combined model showed slightly higher NPV (92 vs. 88%) and PPV (62 vs. 50%) (**Table 3**).

In the Swedish cohort, the AUROCs of sCD163 and sMR were moderately good, whereas TE performed even better. Adding sCD163, sMR or both of these markers to TE failed to change its AUROCs (**Table 2**), for which reason we did not pursue the predictive values of the combined models.

Prediction of Combined Significant Activity and Fibrosis

Besides fibrosis, the presence of necroinflammatory activity and steatohepatitis may be determinants of progressive disease and pharmacological response, and a combination of at least significant (≥F2) fibrosis, steatohepatitis and NAFLD activity score (NAS) ≥4 has been proposed to identify patients at risk and inclusion into clinical trials (9). In the Italian cohort, 22 percent of the patients had a combination of these features, and 36 percent in the Swedish cohort (**Table 1**). We tested the ability of macrophage markers and TE to predict this composite endpoint. Neither sCD163 nor sMR performed well, with AUROCs below 0.70. TE showed better prediction, but still with AUROCs lower than 0.80 in both cohorts (**Table 4**). Combining the parameters did not improve the AUROCs (**Table 4**). AST, which was included in the composite score in the report mentioned above (9), showed AUROCs similar to those of sCD163 (highest AUROC 0.69), and the addition of AST to TE did not improve its performance (highest AUROC 0.77).

Of note, sCD163 was not significantly higher in the patients with NASH [1.69 (1.19–2.4) vs. 1.52 (1.15–2.24) mg/L, $p = 0.43$] in the Italian cohort. In the Swedish cohort, this was the case for both sCD163 [3.59 (2.35–4.94) vs. 2.97 (1.97–4.61) mg/L, $p = 0.31$] and sMR [0.29 (0.24–0.39) vs. 0.25 (0.22–0.32) mg/L, $p = 0.42$]. Furthermore, looking at the histological grades of steatosis, lobular inflammation and hepatocyte ballooning, sCD163 was only significantly associated with steatosis in the Italian cohort, whereas sMR tended to associate with ballooning in the Swedish cohort. TE showed no significant correlation with any histological measures other than fibrosis in any cohort (**Supplementary Tables 1, 2**).

Macrophage Markers in Relation to NAFLD Fibrosis Score and FIB-4

We calculated the established markers of liver fibrosis NAFLD Fibrosis Score and FIB-4 in both cohorts. In the Italian cohort, FIB-4 and NFS had higher AUROCs for F ≥ 2 compared with sCD163, whereas sCD163 had higher AUROCs for F ≥ 3 and F4 stages (**Table 5**). A combination of sCD163 and NFS resulted in an AUROC higher than both of these markers alone for F ≥ 2 fibrosis, and combinations of sCD163 with FIB-4 and NFS showed slightly higher AUROCS for F ≥ 3. The other possible combinations did not result in increasing AUROCs (**Table 5**).

In the Swedish cohort, FIB-4 had higher AUROCs than both sCD163 and sMR for all fibrosis stages. Combining FIB-4 with sMR provided higher AUROCs for F ≥ 2 and F ≥ 3 stages than both these markers, whereas combinations with sCD163 did not result in additional predictive value.

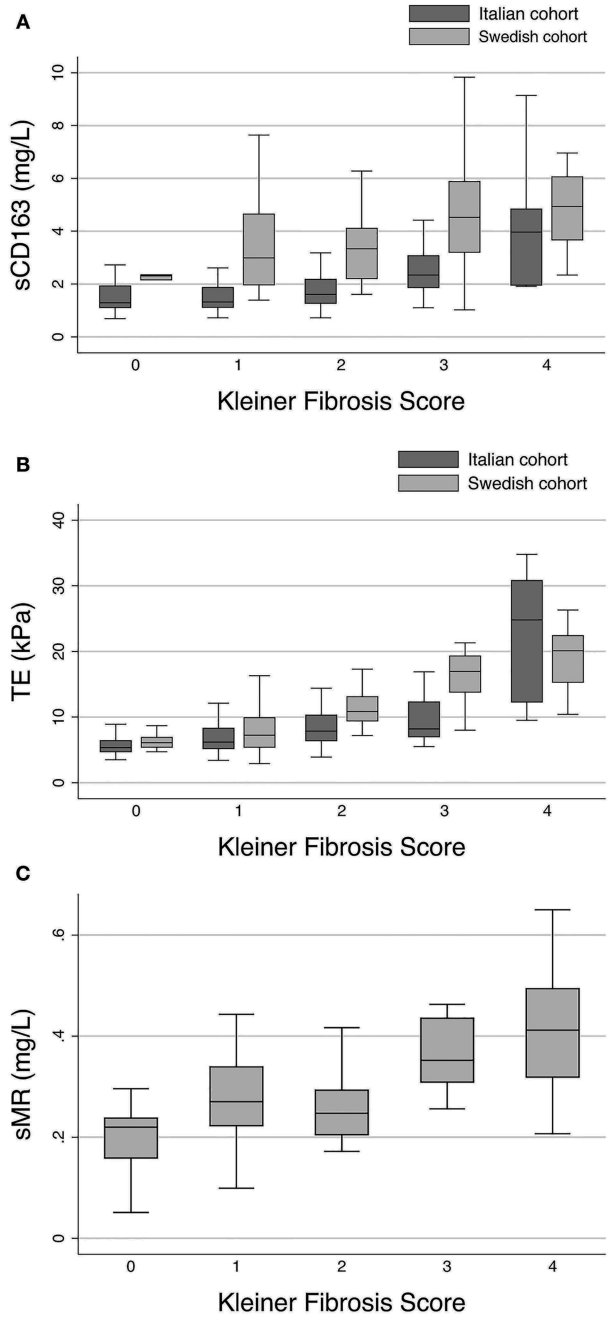

FIGURE 1 | Associations of macrophage markers and transient elastography with fibrosis in the two cohorts. **(A)** sCD163 in the Italian and Swedish cohorts. Italian cohort, rho = 0.42, $p < 0.001$; Swedish cohort, rho = 0.43, $p < 0.001$. **(B)** TE in the Italian cohort and Swedish cohorts. Italian cohort, rho = 0.56, $p < 001$; Swedish cohort, rho = 0.73, $p < 0.001$. **(C)** sMR in the Swedish cohort, rho = 0.46, $p < 0.001$. Boxes represent interquartile ranges (IQR) with medians; whiskers show adjacent values. sCD163, soluble CD163; TE, transient elastography; sMR, soluble mannose receptor.

NFS showed moderate prediction for fibrosis in the Swedish cohort, however, a combination of sCD163 and NFS had an AUROC for F $\geq$ 2 higher than both these markers alone (**Table 5**).

DISCUSSION

In this study of two independent cohorts of patients with biopsy-proven MAFLD and available TE, we measured macrophage markers sCD163 and sMR and combined them with TE hypothesizing that this would improve the prediction of fibrosis. The markers independently predicted fibrosis moderately well, but in contrast to our hypothesis, adding sCD163 and sMR to TE did not significantly improve its predictive capability.

Our study has strengths and limitations. The key strength was the well-characterized patients with histologically classified disease in two independent cohorts. Robust well-established and validated methods including the XL probe were used for the measurement of liver stiffness and for macrophage markers. The most significant limitation was the lower number of patients in the Swedish cohort, which inherently could raise the issue of inadequate power, however, as the combinations of TE with macrophage markers had generally lower AUROCs than TE alone, we do not believe that a larger number of patients in this cohort would have resulted in a better performance of the combined models. A more general drawback may be the cross-sectional design of the study and its focus on liver histology. Liver biopsy is an imperfect gold standard due to potential sampling error and interobserver variability, and the possible resulting bias is not easily predictable.

Combinations of elastography with biochemical markers and composite scores have been explored in MAFLD before. Gaia et al. showed no improvement in the fibrosis prediction of TE with the addition of ultrasonographic and biochemical measures in a smaller study (30). A larger study of MAFLD patients showed similar AUROCs for the FibroMeter^{TM}-TE combination and TE alone, however, with a markedly better PPV (31), followed by a more recent study demonstrating higher AUROCs for all fibrosis stages for the FibroMeter^{TM}-TE model (32). Similarly, another commercially available score, the Enhanced Liver Fibrosis (ELF^{TM})-test, showed improved prediction when combined with TE (33). The readily available and highly validated models NAFLD fibrosis score (NFS) and FIB-4 have been investigated in sequential algorithms with TE resulting in the increase of correctly classified MAFLD patients (34–36).

In our study, sCD163 and sMR predicted fibrosis with moderate accuracy. Furthermore, both sCD163 and sMR showed independent associations with fibrosis in the multiple ordered logistic regression analysis with TE as a covariate. These findings supported our hypothesis and held promise of an add-on value of the macrophage markers for fibrosis prediction when combined with TE. Of note, in the Italian cohort the combination of sCD163 and TE did in fact result in numerically higher AUROCs than TE alone, as well as higher NPV and PPV for advanced fibrosis. However, the increase in the AUROCs was not significant and while the NPV of the combined model was strong at 92%, TE in itself already had an adequate NPV at 88%. At the same time, the PPV of the combined model remained low at 62%, a value not fit to rule in advanced fibrosis. In the Swedish cohort, the AUROCs of the combined models were even slightly lower compared to TE. Thus, based on our data, sCD163 and sMR did not contribute to the detection of advanced fibrosis beyond TE.

TABLE 2 | Areas under the Receiver Operating Characteristics curve with 95% Confidence Intervals for fibrosis stages prediction by sCD163, sMR and transient elastography in the Italian and Swedish cohorts.

	F ≥ 2	F ≥ 3	F4
Italian cohort			
sCD163	0.71 (0.63–0.80)	0.82 (0.74–0.90)	0.89 (0.76–1.0)
TE	0.79 (0.72–0.87)	0.81 (0.73 – 0.89)	0.95 (0.90–1.0)
TE + sCD163	0.79 (0.72–0.86)	0.85 (0.78–0.92)	0.97 (0.93–1.0)
Swedish cohort			
sCD163	0.70 (0.56–0.82)	0.75 (0.62–0.88)	0.75 (0.62–0.89)
sMR	0.68 (0.56–0.81)	0.82 (0.71–0.92)	0.79 (0.66–0.93)
TE	0.88 (0.80–0.97)	0.90 (0.83–0.97)	0.87 (0.78–0.96)
TE + sCD163	0.87 (0.78–0.96)	0.90 (0.83–0.96)	0.86 (0.76–0.95)
TE + sMR	0.88 (0.79–0.96)	0.89 (0.81–0.97)	0.85 (0.74–0.95)
TE + sCD163 + sMR	0.87 (0.78–0.96)	0.89 (0.82–0.97)	0.84 (0.74–0.95)

sCD163, soluble CD163; TE, transient elastography; sMR, soluble mannose receptor.

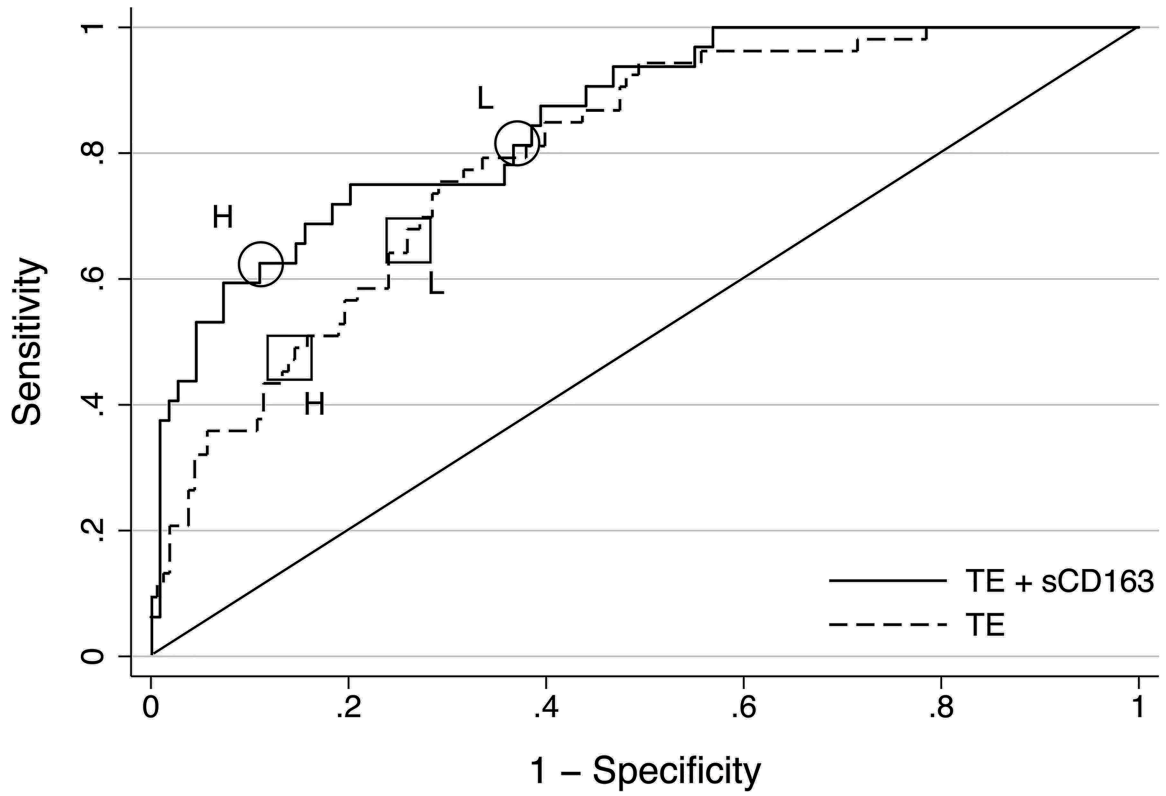

FIGURE 2 | Receiver Operating Characteristics curves for transient elastography and combined transient elastography + sCD163 for advanced fibrosis ($F \geq 3$) in the Italian cohort. Area under the ROC curve for transient elastography (TE) 0.81 (95% CI: 0.73–0.89), for the combined model 0.85 (95% CI: 0.78–0.92). The squares show the high (H) (9.6 kPa) and low (L) (7.9 kPa) cut-off values for TE, and the circles the high (3.72) and low (2.93) cut-off values for the combined model.

Several explanations for this result can be considered. In line with earlier studies, combining sCD163 and TE led to a higher PPV in the Italian cohort, which however was too low for practical use. This may partly be explained by the distribution of fibrosis in the Italian cohort with only 23% having fibrosis stage 3 or higher. The prevalence of disease affects predictive values, and in this case, favors NPV over PPV. Thus, sCD163 may have had a more pronounced impact on PPV in a cohort with more frequent advanced fibrosis. However, the distribution of fibrosis stages does not explain the similar AUROCs of TE and

TABLE 3 | Predictive value of transient elastography and combined transient elastography + sCD163 for advanced fibrosis (F ≥ 3) in the Italian cohort.

TE	**Low cut-off (<7.9 kPa)**	**Indeterminable (7.9–9.6 kPa)**	**High cut-off (≥9.6 kPa)**	**Total**
Total	92	19	30	141
F 0–2	81	13	15	109
F 3–4	11	6	15	32
Sensitivity	66%		47%	
Specificity	74%		86%	
Positive predictive value	43%		50%	
Negative predictive value	88%		85%	
TE + sCD163	**Low cut-off (≤2.93)**	**Indeterminable (2.93–3.46)**	**High cut-off (≥3.72)**	**Total**
Total	75	34	32	141
F 0–2	69	28	12	109
F 3–4	6	6	20	32
Sensitivity	82%		62%	
Specificity	63%		89%	
Positive predictive value	41%		62%	
Negative predictive value	92%		89%	

sCD163, soluble CD163; TE, transient elastography.

TABLE 4 | Areas under the Receiver Operating Characteristics curve with 95% Confidence Intervals for the prediction of combined fibrosis ≥2, NAFLD activity score ≥4 and steatohepatitis by sCD163, sMR, AST and transient elastography in the Italian and Swedish cohorts.

Italian cohort	
sCD163	0.68 (0.57–0.78)
TE	0.78 (0.68–0.87)
TE + sCD163	0.77 (0.68–0.86)
Swedish cohort	
sCD163	0.65 (0.51–0.79)
sMR	0.61 (0.47–0.75)
TE	0.76 (0.65–0.87)
TE + sCD163	0.75 (0.64–0.87)
TE + sMR	0.74 (0.62–0.85)
TE + sCD163 + sMR	0.76 (0.65–0.87)

sCD163, soluble CD163; TE, transient elastography; sMR, soluble mannose receptor.

combined models in the patients from the Swedish cohort, as they had more severe fibrosis. The lack of an improvement of TE prediction by addition of sCD163 and sMR may be rooted in the nature of these biomarkers. sCD163 and sMR were not developed with the purpose of fibrosis detection, but are functional markers of macrophage activation, and have been associated with liver fibrosis reflecting the roles of macrophages in liver disease and fibrogenesis (10). In contrast, the method of TE to determine liver stiffness was identified and refined as a dedicated test of liver fibrosis, as were composite scores such as the NFS (26), the FibroMeter™ (37), and the ELF™ (38), which may explain that these markers may have a higher usability than the macrophage markers in terms of predictive values even with similar AUROCs. In this regard, it may prove fruitful to examine combinations of TE with other specific fibrosis markers, for instance the markers of collagen turnover that also have shown promising results in MAFLD (39).

Both sCD163 and sMR failed to help detect the combination of significant fibrosis, steatohepatitis and necroinflammation suggested as the FAST algorithm to identify patients with progressive disease suitable for clinical trials (9), and the levels of the macrophage markers were not significantly elevated in patients with steatohepatitis. In some of the other cohorts of MAFLD patients investigated by our group and others, sCD163 was higher in subjects with steatohepatitis, however, the association with fibrosis was considerably stronger (11–13). We have previously explained this finding by the higher variability in the histological assessment of features such as steatosis and inflammation compared to fibrosis (24), as well as by the dynamic nature of necroinflammatory activity, whereas fibrosis remains more stable over time. It is therefore not to be expected that macrophage markers can contribute to the detection of inflammatory activity in MAFLD. However, it is important to mention that the FAST algorithm will miss patients with advanced fibrosis but no steatohepatitis, whose prognosis is very similar to those who have both advanced fibrosis and steatohepatitis (4, 40), for which reason the detection of fibrosis is more appropriate for clinical practice.

In our study, the AUROCs of sCD163 were higher compared with NFS and FIB-4 in the Italian cohort, whereas FIB-4 was superior to sCD163 and sMR in the Swedish cohort. Interestingly, in several instances the combinations of the macrophage markers with NFS/FIB-4 showed AUROC values higher than the single markers, which suggests that sCD163 and sMR may be valuable as add-ons to the biomarker-based screening of MAFLD patients

TABLE 5 | Areas under the Receiver Operating Characteristics curve with 95% Confidence Intervals for fibrosis stages prediction by sCD163, sMR, NAFLD Fibrosis Score and FIB-4 the Italian and Swedish cohorts.

	F ≥ 2	F ≥ 3	F4
Italian cohort			
sCD163	0.71 (0.63–0.80)	0.82 (0.74–0.90)*	0.89 (0.76–1.0)
NFS	0.76 (0.68–0.85)	0.70 (0.59–0.81)	0.74 (0.40–1.0)
FIB-4	0.73 (0.65–0.82)	0.68 (0.57–0.79)	0.74 (0.44–1.0)
sCD163 + NFS	**0.80 (0.72–0.88)**‡	**0.83 (0.74–0.91)**†	0.81 (0.57–1.00)
sCD163 + FIB-4	**0.76 (0.68–0.84)**	**0.83 (0.74–0.91)***	0.86 (0.69–1.00)
Swedish cohort			
sCD163	0.70 (0.56–0.82)	0.75 (0.62–0.88)	0.75 (0.62–0.89)
sMR	0.68 (0.56–0.81)	0.82 (0.71–0.92)	0.79 (0.66–0.93)
NFS	0.64 (0.49–0.79)	0.75 (0.61–0.89)	0.87 (0.76–0.94)
FIB-4	0.74 (0.61–0.86)	0.85 (0.76–0.95)	0.92 (0.85–0.99)‡
sCD163 + NFS	0.66 (0.52–0.81)	**0.81 (0.68–0.94)**	0.88 (0.78–0.98)
sCD163 + FIB-4	**0.76 (0.64–0.87)**	0.86 (0.76–0.96)‡	0.91 (0.83–0.98)‡
sMR + NFS	0.63 (0.48–0.78)	0.79 (0.67–0.91)	0.87 (0.77–0.97)
sMR+ FIB-4	**0.76 (0.64–0.87)**	**0.89 (0.82–0.97)**	0.92 (0.85–0.99)§

Combinations with possible synergy between markers in bold.
sCD163, soluble CD163; NFS, NAFLD Fibrosis Score; sMR, soluble mannose receptor.
$p < 0.05$ compared with FIB-4.
†$p < 0.05$ compared with NFS.
‡$p < 0.005$ compared with sCD163.
§$p < 0.05$ compared with sMR.

in the setting of primary care. However, this should be further tested in primary care cohorts more suitable for this purpose due to the expectedly lower prevalence of advanced fibrosis compared with our cohorts of patients referred to tertiary centers.

In conclusion, despite significant independent associations with fibrosis in two cohorts of patient with MAFLD, macrophage markers sCD163 and sMR did not improve the prediction of fibrosis by TE in combined models. This may be attributed to the nature of these markers as related to macrophage activation and not fibrosis *per se*, and other more fibrosis specific markers may be more useful and could be explored in future studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University Hospital San Giovanni Battista of Torino and Regional Ethics Committee of Stockholm. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KK: study design, conception, data analysis, interpretation, and manuscript preparation. CR, RY, AA, and HH: study subject inclusion, data acquisition, interpretation, and critical revision of the manuscript for intellectual content. HM: data analysis, interpretation, and critical revision of the manuscript for intellectual content. PS and EB: study design, conception, administrative support, data interpretation, and critical revision of the manuscript for intellectual content. HG: study design, conception, obtained funding, data interpretation, study supervision, and critical revision of the manuscript for intellectual content. All authors have approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.616212/full#supplementary-material

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REVIEW
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Current Concepts of Biliary Atresia and Matrix Metalloproteinase-7: A Review of Literature

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Biliary atresia (BA) is a rare cholangiopathy of infancy in which the bile ducts obliterate, leading to profound cholestasis and liver fibrosis. BA is hypothesized to be caused by a viral insult that leads to over-activation of the immune system. Patients with BA are surgically treated with a Kasai portoenterostomy (KPE), which aims to restore bile flow from the liver to the intestines. After KPE, progressive liver fibrosis is often observed in BA patients, even despite surgical success and clearance of their jaundice. The innate immune response is involved during the initial damage to the cholangiocytes and further differentiation of the adaptive immune response into a T-helper 1 cell (Th1) response. Multiple studies have shown that there is continuing elevation of involved cytokines that can lead to the progressive liver fibrosis. However, the mechanism by which the progressive injury occurs is not fully elucidated. Recently, matrix metalloproteinase-7 (MMP-7) has been investigated to be used as a biomarker to diagnose BA. MMPs are involved in extracellular matrix (ECM) turnover, but also have non-ECM related functions. The role of MMP-7 and other MMPs in liver fibrosis is just starting to be elucidated. Multiple studies have shown that serum MMP-7 measurements are able to accurately diagnose BA in a cohort of cholestatic patients while hepatic MMP-7 expression correlated with BA-related liver fibrosis. While the mechanism by which MMP-7 can be involved in the pathophysiology of BA is unclear, MMP-7 has been investigated in other fibrotic pathologies such as renal and idiopathic pulmonary fibrosis. MMP-7 is involved in Wnt/β-catenin signaling, reducing cell-to-cell contact by shedding of E-cadherin, amplifying inflammation and fibrosis via osteopontin (OPN) and TNF-α while it also appears to play a role in induction of angiogenesis This review aims to describe the current understandings of the pathophysiology of BA. Subsequently, we describe how MMP-7 is involved in other pathologies, such as renal and pulmonary fibrosis. Then, we propose how MMP-7 can potentially be involved in BA. By doing this, we aim to describe the putative role of MMP-7 as a prognostic biomarker in BA and to provide possible new therapeutic and research targets that can be investigated in the future.

Keywords: biliary atesia, progressive liver fibrosis, cholangiopathy, biliary fibrosis, Matrix metalloproteinase-7 (MMP)-7

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INTRODUCTION

Biliary Atresia

Biliary atresia (BA) is a rare cholangiopathy of infancy leading to obliteration of the intra- and extrahepatic bile ducts (1). The incidence of BA varies around the world from 1 case per 19,000 live births in Europe to 1 per 8,000 live births in eastern Asia (2–4). Infants that are affected present with conjugated hyperbilirubinemia, acholic stools, and dark urine (5). BA exists in an isolated or non-syndromic (IBA) form and a syndromic (SBA) form (6). The cause of both subtypes is currently unknown. It is thought, however, that SBA is caused by an error in development because other abnormalities of development are associated with this type, such as polysplenia, malrotation of the intestine and a pre-duodenal portal vein. The combination of BA and splenic malformation is a specific diagnostic subgroup, called Biliary Atresia Splenic Malformation variant (BASM) (7). Furthermore, BASM is characterized by mutations of the polycystic kidney disease 1 like 1 (*PKD1L*) gene that is associated with the rotation of the organs during embryonic development, supporting a developmental origin (8). IBA, on the other hand is thought to be caused by an (infectious) insult occurring somewhere between conception and the perinatal period (9, 10). IBA is characterized by a progressive inflammatory response resulting in injury to the bile ducts (9). The innate and adaptive immune system are therefore believed to play a prominent role in the pathophysiology of IBA. The innate immune system is the first line of defense of the immune system against pathogenic intruders. The adaptive immune system, on the other hand, is a defense system that is able to develop a very specific immune response against a pathogenic intruder (11, 12). Although there is a clear distinction, the two systems work hand in hand to rid the body of pathogens (11, 12).

Clinical diagnosis of BA is difficult; the golden standard for diagnosing BA therefore is an invasive liver biopsy or an endoscopic retrograde cholangio-pancreatography (ERCP) (1). The primary treatment of BA consists of the Kasai portoenterostomy (KPE) where bile flow is restored by removing the entire atretic extrahepatic bile duct and replacing it with a Roux-en-Y-loop of the intestine, so that bile can drain to the intestine (1, 5). KPE is deemed surgical successful when there is a potent connection between liver and intestine, allowing drainage. Therapeutic success of KPE treatment is evaluated according to the levels of bilirubin at 6 months after KPE. If clearance of hyperbilirubinemia is achieved (<20 μmol/L), KPE is deemed therapeutically successful. However, despite receiving a surgically and therapeutically successful KPE, liver fibrosis in BA patients often progresses to cirrhosis for which a liver transplantation (LTx) is required, making, BA is the most common indication for pediatric LTx (13).

Clearance of hyperbilirubinemia is associated with longer native liver survival (NLS) (14, 15). Patients that are treated at a younger age have a higher chance of achieving clearance of jaundice (3, 14). This may be related to the extent and amount of liver fibrosis that is present when KPE is performed. However, consistent proof for a relation between liver fibrosis and NLS is lacking. A relation between the aspartate transaminase-to-platelet ratio (a marker for liver fibrosis i.e.,) was not found to be correlated to NLS in BA patients, for example (16). Unfortunately, age at KPE is, as of yet, the only factor that can be used to estimate the prognosis of BA patients. Moreover, age at KPE is not a waterproof prognostic factor since most patients with a successful KPE still require an LTx eventually due to progressive liver fibrosis. This indicates that there may be other mechanisms than fibrosis due to a high level of bile acids/cholestasis involved in the progression of liver fibrosis. Immune mediated mechanisms of liver fibrosis, such as observed in related cholestatic conditions such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), show similarities but are not the same as that observed in BA (17, 18). BA-associated liver fibrosis is a unique process with unique components playing a role in the pathogenesis that can be used for diagnosis and prognosis estimation. Matrix metalloproteinase-7 (MMP-7) has recently been investigated as a possible less invasive diagnostic marker in BA as alternative to an invasive liver biopsy or ERCP and shows good sensitivity and specificity (19–21).

Matrix Metalloproteinases

MMP-7 belongs to the group of matrix metalloproteinase family (MMPs). During the initial discovery, MMPs were thought to fulfill a role in the remodeling of the extracellular matrix (ECM). As a family, MMPs are able to proteolytically cleave all the components of the ECM (e.g., collagens and fibronectin). MMPs can be produced and secreted by a variety of cells, ranging from connective tissue cells to macrophages (22). MMPs are classified in different groups according to their substrate and can act as pro- or antifibrotic proteases (23). The activity of the group of MMPs is controlled by tissue inhibitors of metalloproteinases (TIMPs) (24). In fibrotic pathologies there is an excess accumulation of connective tissue elements, such as collagen (25). Because of their involvement in remodeling of the ECM, MMPs have been investigated in fibrotic pathologies such as lung and liver fibrosis (26, 27).

Besides their function as remodelers of the ECM, numerous non-ECM related functions of multiple MMPs are being discovered, such as functions in inflammation (28, 29). MMPs are able to proteolytically cleave non-ECM proteins by a process termed "shedding." For example, MMPs are able to activate cytokines, enhancing inflammation (30). Moreover, in cancer MMPs can enhance angiogenesis, while they can also reduce cell-to-cell integrity promoting tumor growth and invasion,

Abbreviations: α-SMA, alpha-smooth muscle actin; AEC, alveolar epithelial cell; BA, biliary atresia; CC, choledochal cysts; CD, cluster of differentiation; CK7, cytokeratin 7; CTL, cytotoxic T-lymphocytes; DC, dendritic cell; ERCP, endoscopic retrograde cholangio-pancreatography; EMT, epithelial-to-mesenchymal transition; ECM, extracellular matrix; HPC, hepatic progenitor cell; HSC, hepatic stellate cell; IPF, idiopathic pulmonary fibrosis; IFN, interferon; IL, interleukin; KPE, Kasai portoenterostomy; LTx, liver transplantation; MHC, major histocompatibility complex; MF, myofibroblast; NK, natural killer; NLS, native liver survival; OPN, osteopontin; PAMP, pathogen associated molecular pattern; PRR, pathogen recognizing receptor; PF, portal fibroblasts; RDC, reactive ductular cells; Th1, T-helper 1 cell; TLR, toll-like receptor; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

respectively (31). For this reason, the role of various MMPs has been investigated in numerous pathologies, such as fibrosis and cancer in various organs (24).

MMP-7

Studies have shown that MMP-7 is present in different quantities in the livers of BA patients with different postoperative outcomes, which provides circumstantial evidence that MMP-7 may play a role in the pathophysiology of BA (32, 33). While the potential of MMP-7 to diagnose BA is clear, there is not much research performed on where exactly in the pathophysiology of BA MMP-7 can be a factor. Moreover, MMP-7 expression correlates to the extent of liver fibrosis in BA patients at time of diagnosis. However, as liver fibrosis is an abnormal process of tissue regeneration, MMP-7 might merely be a (specific) marker of this abnormal regeneration and not play an active role in the pathogenesis of BA. However, as MMP-7 is suspected to be involved in BA, its exact role and significance in the progression of BA-related liver fibrosis requires further investigation.

This review aims to describe the current concepts of the, as of yet, elusive pathophysiology of BA. Then, we aim to describe the characteristics and functions of MMP-7. Subsequently, we aim to describe where in the pathophysiology of BA MMP-7 may be involved. By doing this, we aim to further define the relation between MMP-7 and BA thereby opening up new areas that can be targeted by future research.

ETIOLOGY OF BILIARY ATRESIA

The etiology of BA is currently unknown. Much research has focused on the pathophysiology of IBA and this review will therefore discuss this subtype of BA.

One of the most popular hypotheses for the etiology of IBA is an infectious (viral) insult that leads to over activation of the immune system. The immune system subsequently targets the bile ducts and this leads to obliteration of the bile ducts (1, 9). This hypothesis has gained support due to the fact that histological evidence of inflammation in the liver and bile duct remnants at time of diagnosis in BA patients has been observed. Bill et al. (34) were the first of many studies to observe lymphocytes and other immune cells in biliary ducts of BA patients. Moreover, various viruses have been investigated in BA patients (35, 36).

Rhesus Rotavirus (RRV) has been used most often to create animal models that mimic BA and is considered the gold standard. Riepenhoff-Talty et al. (37) were the first that demonstrated the use of RRV type A in mice to mimic BA. The infected mice developed cholestasis and portal hepatitis within days after inoculation with RRV type A. A gene segment encoding a structural protein of RRV, VP4, was later identified as responsible for the specific activation of the murine immune system (38). VP4 specific inoculation of mice leads to a cholangiopathy mimicking BA, illustrating continuous improvement of the disease model (39–41).

Subsequently, studies have searched for serological and hepatic evidence of infection with Rotavirus, Reovirus or Cytomegalovirus (CMV) in BA patients (35, 36, 42). These studies showed mixed results: no conclusive evidence of a difference in infection rate of Rotavirus between BA patients and controls was observed. Studies that investigated Reovirus in infants with BA suffered from small sample sizes, lack of control groups in some studies and mixed results as well (42–44). However, recently CMV+ BA has been identified as a subgroup of BA, with a poorer prognosis than IBA patients (45). Mixed results can be due to a variety of reasons, such as an immature immune system of the BA infant resulting in a lack of proof or absence of infection or that there are multiple viruses that play a role in the activation of the immune system, for example (4, 46). Moreover, overactivation of the immune system that would eventually lead to BA via a virus can occur by mechanisms such as molecular mimicry or bystander activation. It is very hard to pinpoint to the exact virus or part of the viral genome that can cause the overactivation. It is possible that multiple viruses share a part of the genome, making it difficult to find consistent proof of infection with a single total virus in the livers of BA patients.

Other models of BA were recently described. The isoflavonoid biliatresone has been shown to selectively destruct the extrahepatic cholangiocytes in zebrafish (47). Biliatresone also mimicked BA in mice and was studied in cell cultures (48–50). These studies also investigated the mechanism of action by which the toxin could accomplish toxicity to the cholangiocytes. Biliatresone depletes glutathione (GSH), which causes disruption of microtubules in cholangiocytes (49). Microtubules are essential for lumen formation and polarity of epithelial cells and disruption of the tubules results in increased epithelial permeability and reduced cell-to-cell contact (49). RNA sequencing revealed downregulation of cell adhesion related genes (48). Therefore, Yang et al. speculate that a yet unidentified toxin may cause BA in humans by depletion of GSH (48).

The time at which the hypothesized insult leading to IBA occurs (prenatal or perinatal), remains to be determined. IBA is classically considered to originate in healthy new-borns with the insult occurring somewhere in the perinatal period with symptoms only appearing after the first weeks of life (1). Recently, evidence for BA as a disease starting *in utero*, such as hyperbilirubinemia within 24 h of life and abnormal gamma-glutamyl transferase (GGT) levels in the amniotic fluid of BA patients, is accumulating (51–53).

No conclusive evidence of what exactly causes IBA and when it is caused is available yet. There are various potential triggers that can lead to the same disease phenotype. Therefore, the cause of IBA can be multifactorial. However, regardless of the cause, the subsequent histological characteristics of immune mediated damage is similar among IBA patients. This will be described in the next section.

CELL MEDIATED IMMUNE RESPONSE IN BILIARY ATRESIA

Innate Immune Response

Cholangiocytes are the biliary epithelial cells that line the extra and intrahepatic bile ducts. Cholangiocytes are heterogeneous and reactive types of cells (17). They are able to respond to a

variety of foreign substances via pattern recognition receptors (PRRs). PRRs recognize Pathogen Associated Molecular Patterns (PAMPs) that are associated with specific types of microbes, so that a specific response against that microbe is elicited (54). Toll-like receptors (TLRs) are the most commonly described PRRs in humans. Monocytes, hepatic macrophages (i.e., Kupffer cells), dendritic cells (DCs) and cholangiocytes express various TLRs on their membrane (55). Each TLR has different specific ligands. TLR3 and TLR7 are activated by double stranded (ds) and single stranded (ss) viral RNA, respectively (56). Activation of TLRs induce the production of pro-inflammatory cytokines, chemokines, and other pro-inflammatory proteins allowing cells that express TLRs to actively participate in the immune response (12). The end result is the activation of the innate immune response that is required to clear the microbe that has infiltrated the tissue. It is likely that cholangiocytes play an active role in the pathogenesis of BA, but immune cells, such as Kupffer cells, probably play a more central role in producing and maintaining the immune response observed in BA (9, 57).

In BA, TLR activation has been investigated in liver tissue. Huang et al. (58) found increased hepatic expression of TLR7 in BA compared to choledochal cyst (CC) patients, another cholangiopathy with embryological origin (59). The increased expression was mainly located in the resident Kupffer cells and cholangiocytes in BA patients. Interestingly, they found a decreased hepatic expression of TLR7 in the late stage of BA compared to the early stage of BA, indicating that TLR7 may be more important in the initiation of the immune response rather its maintenance. Harrada et al. (60) found overexpression of TLR3 in liver tissue of BA patients compared to controls with a cholestatic liver disease. Both TLR3 and 7 induce an inflammatory cascade by inducing a type 1 interferon (IFN) response, regardless on which cell type the receptor is located as a response to viral RNA (12). Type 1 IFN uses human myxovirus resistance protein 1 (MxA) as a signaling molecule. MxA is a very specific marker of type 1 IFN signaling and was up-regulated in livers of BA patients (61). Up-regulation of TLR 3 and 7 in BA supports the finding of the involvement of RNA viruses, such as Rotavirus and Reovirus in the pathogenesis of BA (62).

Type 1 IFN signaling is part of a specific antiviral response resulting in enhanced major histocompatibility complex (MHC) expression so that infected cells can be recognized and killed easier by immune cells. Moreover, a differentiation of the immune response into a type 1 helper T-cell (Th1) specific response is promoted (12, 61). This is important for the adaptive immune response that follows the initial injury. Activation of TLR7 and type 1 IFN initiates production of interleukin-8 (IL-8) in BA by cholangiocytes and macrophages (58). IL-8 is a chemokine that attracts neutrophils mainly, but is also able to attract lymphocytes to the site of injury, such as natural killer cells (NK) which are part of the virus specific response as well (11, 63). NK cells are part of the innate immune response that can recognize and kill virus infected cells by recognition of the MHC complex (11, 63). Increased infiltration by NK cells in liver biopsies of BA patients compared to cholestatic controls has been reported (64, 65).

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and CD95 (alternatively known as Fas) can be expressed by cholangiocytes. After administration of a double stranded RNA analog, TRAIL expression was found to be increased, in contrast to Fas, that remained unchanged. Signaling via type 1 IFN can lead to increased apoptosis by TRAIL and increased expression of these apoptotic receptors therefore suggests that activation of TLR3 leads to cholangiocyte apoptosis by expression of TRAIL (60).

In summary, the innate immune response in BA initiated by the activation of TLR3 and 7, which results in apoptosis of cholangiocytes by NK cells. Moreover, a viral specific immune response via type 1 interferons and other pro-inflammatory cytokines from cholangiocytes, macrophages and dendritic cells is initiated. An overview of the initial immune response is given in **Figure 1**.

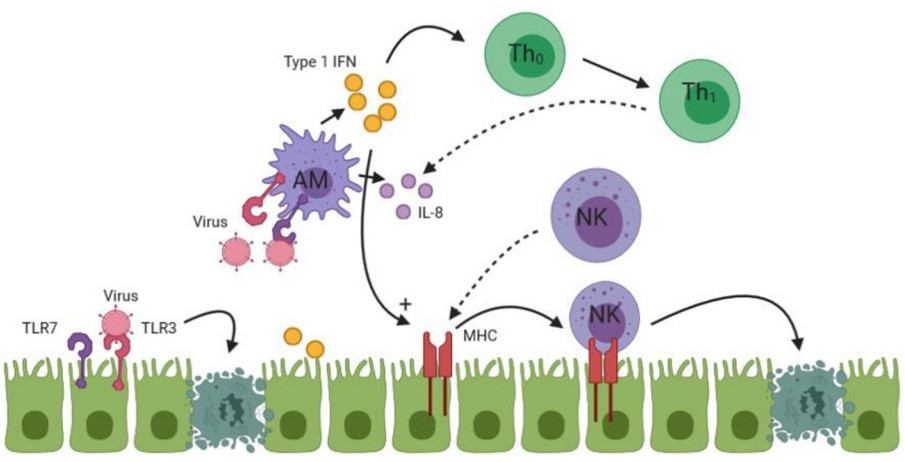

FIGURE 1 | An overview of the initial immune response in BA. Undotted arrows indicate a consequence of activation. Dotted arrows in this figure indicate homing of lymphocytes to the site of injury. TLR, Toll like receptor; Th, T-Helper cell; AM, Activated macrophage; NK, Natural killer cell. See text for more details of the process.

Adaptive Immune Response

IL-8 attracts effector immune cells to the site of injury as a result of TLR activation. When effector immune cells reach the biliary epithelium, the release of cytokines leads to proliferation and increased production of these effector immune cells and to a more tissue-specific immune response targeted at the cholangiocytes. The innate immune response stimulates and develops a more specific and potent adaptive immune response (12).

Effector cells of the adaptive immune response consist of CD4+ and CD8+ T-cells and phagocytes such as macrophages, among others (12). Macrophages play a special role in the immune system and in the pathophysiology of BA. Macrophages can recognize foreign material in the body but also have a function as effector cells of the innate and adaptive immune system. Macrophages fulfill this role by phagocytosis, a process where infected cells or cell debris is ingested by the macrophage and destroyed (11, 12). After activation, macrophages secrete tumor necrosis factor-alpha (TNF-α) and express CD68 (66). TNF-α is mainly produced by macrophages but can also be produced by other cells, such as cholangiocytes and T-cells (11, 12, 67). TNF-α is an important pro-inflammatory cytokine that can activate other immune cells but also lead to apoptosis of cells directly (12).

CD4+ T-cells are T-helper cells, which recruit phagocytes to mediate the destruction of target tissue. A subset of CD8+ T-cells are the cytotoxic T-lymphocytes (CTL). CTLs are able to kill cells that are infected with a virus. The target cell lacks the ability to eradicate the microbe that has infected the cell, so the only way to eradicate the virus is to destroy the entire cell; an action that is performed by the CD8+ CTL (or NK cell during innate immune response) (12). The effector T-cells have to be activated by a specific antigen/major histocompatibility complex (MHC) combination on the T-cell receptor (TCR). The interaction between a combination of antigen and MHC leads to the development of a very specific adaptive immune response (68). Proof of specific activation of T-lymphocytes was found by Mack et al. (68) who found expansion of CD4+ and CD8+ T-cells in BA tissue via a limited repertoire of T-cell receptors compared to controls. This indicates activation via a specific antigen.

Urushihara et al. found that in the liver specimens of patients with BA, there was an increased number and increased size of Kupffer cells at KPE and at LTx (69). This finding was replicated by Mack et al. (70). Moreover, IL-18, which is produced by these Kupffer cells, was also found to be significantly elevated in BA patients compared to controls. IL-18 is most potent when working together with IL-12 in the immune response. IL-12 is a differentiation factor that is released from macrophages to differentiate CD4+ T-helper cells into T-helper type 1 (Th1) cells (11, 12). In concert with IL-12, IL-18 induces the production of IFN-γ from Th1 cells (71). IFN-γ leads to further differentiation of the Th1 response and inhibition of the development of other T-cell lineages (12). Moreover, it activates macrophages to perform phagocytosis on infected cells and microbes (12). Mack et al. confirmed the presence of a Th1 immune response by finding extensive infiltration of CD4+ and CD8+ effector T-cells which produced IL-2, IFN-γ, and TNF-α (70). Furthermore, chemokine receptor CXCR3 is a homing factor specific for a Th1 immune response (11). CXCR3 was found to be up-regulated within portal tracts and biliary remnants in the livers of BA patients compared to controls (72). Whitington et al. investigated the presence of osteopontin (OPN) in the liver of BA patients (73). OPN is secreted by cholangiocytes and is responsible for the homing of macrophages to the bile ducts and is considered to further enhance Th1 differentiation (74). It appears that in BA, the innate immune response develops into a Th1 differentiated immune response which targets the cholangiocytes near the portal area.

During the innate immune response, NK cells are thought to kill cholangiocytes. Moreover, TLR3 activation results in their apoptosis. Macrophages can also kill cells by the production of reactive oxygen species (ROS), nitric oxide (NO) and lysosomal enzymes (11, 12). The role CD8+ CTLs play in the apoptosis of cholangiocytes remains to be determined. Ahmed et al. found a high expression of CTLs in BA patients, but not of the cytotoxic products of these CTLs (granzyme, perforin, and Fas ligand) (75). In contrast, Guo et al. found up-regulation of CD8+ CTLs and its co-stimulatory molecules in BA patients, indicating a toxic function exerted by these CTLs (65). Besides their cytotoxic function, CTLs are able to produce cytokines such as IFN-γ, thereby contributing to the Th1 immune response in BA (11, 12). In a murine model of BA, TNF-α, and IFN-γ were both required for the apoptosis of cholangiocytes (76). IL-8 is released from macrophages as a downstream product of type 1 IFN activation. IL-8 attracts neutrophils to the site of injury. Neutrophils have been studied in BA, but are thought to infiltrate the liver as a consequence of biliary tract obstruction, rather than playing a role in the initiation of bile duct damage. They may, however, play a role in cholangiocyte damage later on in the pathogenesis (77). An overview of the adaptive immune response in BA is shown in **Figure 2**.

PROGRESSIVE LIVER FIBROSIS IN BILIARY ATRESIA

Besides obliteration of cholangiocytes, progressive fibrosis of the liver is observed in the pathogenesis of BA; in particular in later stages of the disease. Continuing the hypothesis that an immune response is responsible for cholangiocyte damage and apoptosis, it seems likely that a continuation of the immune response plays a role in the progression of liver fibrosis. Most BA patients eventually require an LTx even if KPE is deemed successful (i.e., clearance of hyperbilirubinemia within 6 months after KPE), suggesting that other factors than cholestasis contribute to liver fibrosis as well in BA (3).

Characteristics of Liver Fibrosis

Liver fibrosis can result from a variety of etiologies, such as infections, alcohol, and autoimmune disorders (78). Cholangiopathies lead to a distinct type of liver fibrosis which is termed biliary fibrosis (18). Liver fibrosis develops due to the transformation of hepatic stellate cells (HSC) into myofibroblasts (MF) and the excess deposition of components

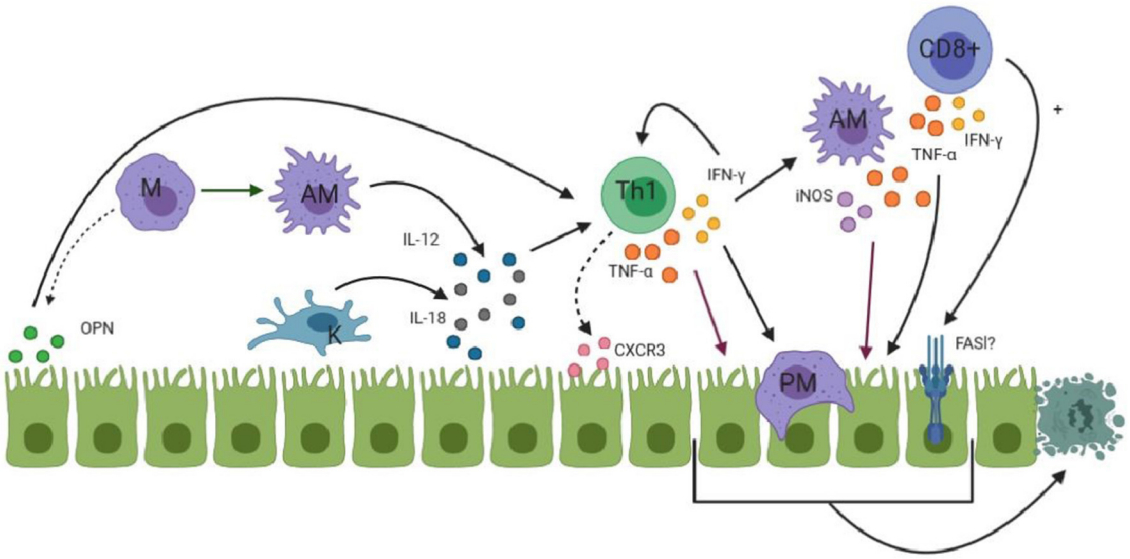

FIGURE 2 | An overview of the process where macrophages (M, purple) are attracted to cholangiocytes (green) and the Th1 (green) response starts to develop. Activated macrophages (AM) are shown as spikey purple while Kupffer cells (K) are shown in blue. PM represents phagocytosing macrophage. FASl is shown with a question mark due to the fact that its involvement in apoptosis is not proven. Dotted arrows indicates that attraction of immune cells takes place. (OPN and CXCR3). More detailed information is given in the text.

of the extracellular matrix (ECM) by MFs. Other cells can also contribute to liver fibrosis by transforming into MFs, such as portal fibroblasts (PF) (79). MFs start to produce collagen-1 and express α-smooth muscle actin (α-SMA) during liver fibrosis (79, 80). PFs and HSCs transform to their pro-fibrotic phenotype as response to pro-fibrotic growth factors such as transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF). The pro-fibrotic growth factors can be produced by a variety of cells such as macrophages in the liver but also by MFs themselves, creating a vicious cycle (12, 81, 82). Macrophages mediate survival of MFs by secreting TNF-α so that MFs can maintain a pro-fibrotic environment (18). Different etiologies lead to a different balance of cell types that are being activated and lead to fibrosis, however HSCs remain the most important producer of pro-fibrotic MFs in most models of fibrosis and in cholangiopathies (83). In the current view, PFs are the initial responders to damage to cholangiocytes and they subsequently recruit HSCs as the main contributor to biliary fibrosis.

As response to injury, the liver is able to regenerate. Regeneration of the liver during injury in a cholangiopathy is mediated by replication and proliferation of already existing cholangiocytes and by the activation of hepatic progenitor cells (HPCs) that can both differentiate toward reactive ductular cells (RDCs) (18). Proliferation of cholangiocytes and HPCs into RDCs together with the niche of inflammatory cells surrounding this proliferation is termed the ductular reaction. The ductular reaction is a hallmark of pathological repair of the damage that is being (or has been) done to the cholangiocytes or hepatocytes (18). Initiation of the ductular reaction requires (re)activation of pathways that result in the proliferation of HPCs into biliary epithelial cells. These pathways are, among others the Notch and Wnt/β-catenin signaling pathways (18). Fabris et al. investigated the ductular reaction in Alagille syndrome (AGS) and BA. AGS is characterized by defective Notch signaling (84). The authors concluded that Notch is required for the differentiation of HPCs into RDCs, as AGS patients lacked RDCs in their livers. Notch signaling increased as liver fibrosis in BA progressed (84, 85). Wnt/β-catenin signaling is involved in the determination of cell fate and proliferation (86). β-catenin acts as a signal transducer of activated Wnt. In normal circumstances, β-catenin is removed from the cytoplasm of cells, thereby preventing activation of Wnt target genes (86). When the signaling pathway is activated, β-catenin is stabilized within the cell and allows transcription of target genes in the nucleus of the cell (86). In liver fibrosis, Wnt/β-catenin signaling enhances HSC activation and survival (87). Significant up-regulation of signaling proteins of the Wnt/β-catenin in the liver of BA patients compared to controls was observed. Furthermore, protein expression of products of the Wnt/β-catenin pathway correlated to hepatic fibrosis in BA, indicating involvement of this mechanism in fibrosis progression in BA (88). Recently, a downstream product of the Wnt/β-catenin pathway, a G-protein termed RhoU, was investigated in the biliatresone induced mice model (50). It was found that RhoU was upregulated in mice treated with biliatresone, indicating activation of the Wnt/β-catenin pathway. Subsequently, they analyzed the expression in livers of BA patients and found overexpression of RhoU compared to controls, supporting their model and indicating a potential role for the Wnt/β-catenin pathway in BA (50).

Notch and Wnt/β-catenin signaling pathways have been linked to epithelial-to-mesenchymal transition (EMT). During EMT, epithelial cells lose characteristics of epithelial cells, such

as expression of E-cadherin and differentiate or transform into mesenchymal cells. Due to the loss of tight junction proteins, mesenchymal cells are able to migrate to other organs (89). EMT has been implicated in cancer metastasis and fibrosis (90). The existence and relevance of EMT remains controversial, however. Studies have investigated EMT in relation to BA and liver fibrosis, although the existence and relevance in BA remains undetermined (91). Although the Notch and Wnt β-catenin signaling pathways may not be involved by EMT in BA, they can still play a role by enhancing the proliferation of HPCs to RDCs or by activating HSCs.

Santos et al. investigated the hepatic expression of cytokeratin 7 (CK7), a marker of proliferation of cholangiocytes (i.e., ductular reaction), in patients with BA (92). They found that high CK7 expression in the liver at time of KPE predicted a poorer survival. Higher presence of ductular reactions may therefore be an indication of the presence and extent of liver fibrosis. Kerola et al. (93) found increased hepatic gene expression of α-SMA in BA patients despite receiving a successful KPE, indicating the presence of activated MFs. The hepatic gene expression of α-SMA correlated with the extent of fibrosis, expansion of the ductular reaction and was located around ductular proliferations of cholangiocytes. The cohort of Kerola et al. only contained successfully treated BA patients, suggesting other factors than cholestasis can also play a role in the progression of the ductular reaction and subsequent liver fibrosis.

Angiogenesis, the formation of new blood vessels, is a process that occurs during the growth and repair of organs. The process of angiogenesis is influenced by growth factors and hypoxia (i.e., a shortage of oxygen) (94). Angiogenesis consists of multiple sequential phases and can be induced by inflammation or hypoxia. The most important mediator during all steps of angiogenesis is members of the vascular endothelial growth factor (VEGF) family, with VEGF-A as the main component (94). Hepatic fibrosis is an inflammatory process with abnormal wound healing, which can influence angiogenesis by formation of pro-angiogenic cytokines and induction of hypoxia (95). Hypoxia can occur due to the anatomical changes during hepatic fibrosis; reduced fenestration of sinusoidal cells and accumulation of fibrotic tissue makes it more difficult for oxygen to be transported to the cells (96). During inflammation, Kupffer cells can induce the release of angiogenic factors such as VEGF by the production of oxygen radicals, such as ROS (96).

Angiogenesis in relation to BA is less well-studied and the results of the studies performed varied. In these studies, the expression of members of the VEGF family in biliary ducts and arterial walls in liver biopsies. Allam et al. found that a significant higher proportion of BA patients had positive VEGF-A (i.e., most prominent of VEGF family) staining compared to cholestatic controls (97). Moreover, they found that significantly more BA patients had positive VEGF-A expression when they received an unsuccessful KPE compared to BA patients receiving a successful KPE. Edom et al. found a characteristic staining of VEGF-A in liver biopsies of BA patients (98). They propose that there is a higher expression of VEGF-A due to hypoxia induced by portal expansion of the biliary tract. Alternatively, the increase in VEGF-A expression may be due to the fact that higher blood supply is required for the ductular reaction and proliferation of cholangiocytes. Another study found that there was increased gene expression of hypoxia-inducible factor (HIF)1a and HIF2a (99). Usually, HIF expression leads to induction of VEGF-A (100). Surprisingly, Fratta et al. found that BA patients with a high expression of HIF1a and HIF2a had a significantly lower expression of VEGF-A and its related receptor, VEGFR2, than patients with a low expression of HIF1a and HIF2a. Moreover, VEGF-A negatively correlated to expression of both HIF1a and HIF2a (99). The authors explained their findings by hepatobiliary ischemia due to an insufficient angiogenic response to the liver hypoxia. Alternatively, decreased expression of VEGF-A and VEGFR2 may be due to reduction of blood supply by blockage of the bile duct. This is contrasting with previous findings, where VEGF-A expression was correlated with cholangiocyte proliferation, a feature associated with increased liver fibrosis. Interestingly, genistein, an isoflavonoid, suppresses VEGF-A and VEGF receptor expression. Genistein can be transformed into biliatresone in the human intestine by bacteria (101).

In summary, there appears to be proliferation of cholangiocytes in patients with BA. Pathways such as the WNT/β-catenin pathway may be involved in this process. Angiogenesis is a process that is related to liver fibrosis and cirrhosis. In BA, angiogenesis has been investigated and the results have been variable. Increased or decreased VEGF-A expression may be related to biliary proliferation or an insufficient angiogenic response, respectively. More studies are required to explore the relation between cholangiocyte proliferation and induction of angiogenesis by VEGF-A in BA. Moreover, the impact biliatresone has on VEGF-A expression in the BA model could be further explored.

Immune Response and Liver Fibrosis in Biliary Atresia

Kobayashi et al. (102) found increased numbers of activated macrophages (expressing CD68) in liver biopsies of BA patients compared to controls with neonatal hepatitis. At time of KPE, patients that received an unsuccessful KPE had higher number of activated macrophages compared to BA patients that received a successful KPE. This indicates that already at KPE, the extent of macrophage proliferation and immune response can determine the outcome of BA patients. Interestingly, another study found that even despite clearance of jaundice in BA patients, serum IL-18 and the number of Kupffer cells in the liver remained elevated after KPE (69). Furthermore, Narayanaswamy et al. (103) investigated the presence of various interleukins and pro-inflammatory cytokines in the serum of BA patients at multiple time points. They observed that 6 months after KPE, BA patients had significantly higher serum values of IL-2, IFN-γ, IL-4, IL-18, and TNF-α when compared to cholestatic controls. Most cytokines appeared to increase in concentration during this 6 month period. Although there was no difference in these cytokines in the serum of BA patients who underwent a successful KPE and patients in whom the KPE was unsuccessful, the authors found significant differences when they divided the group of BA patients in those who underwent an LTx and those who still

survived with their native liver; all cytokines except IL-18 were significantly elevated at 6 months post-KPE in patients requiring early LTx.

There appears to be a continuing immune response that may be responsible for the faster liver fibrosis and subsequent LTx. Moreover, the fact that patients who are able to clear their hyperbilirubinemia and patients that are not able to clear it do not have a significant different cytokine profile over 6 months indicates that cholestasis is not the sole mechanism responsible for the progressive liver fibrosis. Macrophages may play a role in the continuation of the immune response, contributing to hepatic fibrosis by inhibiting apoptosis of MFs by secreting TNF-α, for example.

In summary, there is an initial immune response responsible for cholangiocyte damage and apoptosis. This immune response is continued into a pro-fibrotic reaction that eventually leads to liver fibrosis, despite clearance of cholestasis. Cholangiopathies can lead to liver fibrosis via cholestasis and a ductular reaction that takes place as a response to injury. The ductular reaction is defined as proliferating cholangiocytes with associated pro-inflammatory proteins (18). It is likely to think that pathways such as Wnt/β-catenin and a continuation of the inflammatory response together lead to progressive liver fibrosis in BA. The maintenance of the pro-fibrotic environment can for example be caused by the vicious cycle HSCs create by producing their own pro-fibrotic growth factors (82), but also by a yet unidentified factor such as a matrix metalloprotease (MMP).

MATRIX METALLOPROTEINASES: FUNCTIONS AND ROLE IN PATHOLOGY

Matrix metalloproteinases (MMPs) are a big family of proteases that use zinc to mediate their proteolytic activity (22, 24). Their first function to be described was the turnover and modulation of the ECM by proteolytically degrading various proteins that are present in the ECM. The ECM is a mixture of cells and non-cellular components which function as a physical scaffold to many cells. The two classes of molecules that mainly constitute the ECM are proteoglycan (PG) and fibrous proteins, such as collagen and elastin (104). PG forms a hydrophilic gel that has many differing functions such as interacting with growth factors, cell receptors and cytokines, while collagen gives the cell tensile strength (104, 105). This allows the ECM to send signals to the cell that regulate different cell functions, such as differentiation and adhesion (105). By altering the structure and components of the ECM, there is an alteration of the specific signals that are transmitted to and from the cell, affecting its behavior. In order to maintain homeostasis between the ECM and the cell, it is important that the matrix is remodeled (104, 105). Disbalance of homeostasis in the ECM leads to pathological conditions. Too little remodeling leads to accumulation of collagens and subsequent fibrosis. Too much turnover of ECM can lead to collagen loss resulting in cardiomyopathy due to decreased contractility, for example (106).

MMPs are the most important mediators of remodeling of the ECM. As a collective group, the MMPs are able to cleave all the components of the ECM (106). There are more than 20 different MMPs and they are subdivided into groups based on their substrate and their structure (gelatinases, collagenases) (22, 24). All MMPS have in common that they contain a catalytic domain with a zinc ion. When MMPs are in their inactive form (zymogen), the pro-domain containing cysteine binds to the catalytic Zn^{2+} ion in the catalytic domain of the MMP so that catalysis is prevented. This structure is the so called "cysteine switch" (22, 107). When the MMP is activated the pro-domain is cleaved off, thereby freeing the Zn^{2+} ion and allowing catalytic activity. Cleaving of the pro-domain of MMPs can be done by various mechanisms, such as cleavage by plasmin, proprotein convertases, but also by other MMPs (107).

Besides ECM turnover, MMPs play a role in inflammation, vascularization, cell migration and proliferation, among others (22). Each MMP has unique non-ECM related functions which results from the activation of bioactive proteins, a process known as 'shedding'. In inflammation, for example, MMPs can regulate the movement of leukocytes into the tissue by creating a chemokine gradient (30). For example, in a mouse model of allergic inflammation in the airway there was a reduction of the number of eosinophils in the bronchoalveolar lavage in mice with knockout MMP-9. The authors show that there was disruption of chemokine gradients due to the absence of MMP-9 (108). As mentioned briefly in the introduction, MMPs also play a role in cancer by enhancing angiogenesis. For example, MMPs promote angiogenesis by degrading ECM components so that endothelial cells can migrate to the location where new vessels are being formed (109). MMP-14 is one of the MMPs that fulfills this role. Moreover, MMP-14 is required for the production of vascular endothelial growth factor (VEGF), directly promoting angiogenesis (110).

MMPs are linked to various pathological processes and diseases. Most obvious, they play a role in fibrosis of the liver and the lung and other conditions in which alteration of the ECM leads to pathology, such as aortic aneurysms (24). They can have a pro- or anti-fibrotic role depending on where in the process of fibrosis they exert their proteolytic effect. Moreover, they can also indirectly affect fibrosis by the alteration of regulatory factors. In physiologic conditions, MMPs are secreted by a variety of cell types, such as by epithelial cells or inflammatory cells such as macrophages, when they are needed (22). Their activity is regulated by tissue inhibitors of metalloproteinases (TIMP). Pathologic conditions arise due to excess activity of MMPs or reduced inhibition of MMPs by TIMPs (22).

In hepatic fibrosis for example, MMP-9 exerts a pro-fibrotic effect by mediating leukocyte trafficking while it also correlates to the extent of hepatic inflammation but not fibrosis (111). Moreover, HSCs are thought to be activated by MMP-2 and 14 while MMP-13 leads to shedding of TGF-β (112, 113). Surprisingly, MMP-2 and 14 are also linked to fibrolysis due to their collagenolytic activity. MMP-2 and 14 activity remained elevated after termination of the administration of the toxic CCL_4 in an animal model of liver fibrosis, suggesting a role in the resolution of fibrosis. Resolution of fibrosis occurred in concert with a decrease of TIMP activity, indicating an imbalance leading to increased activity of MMP-2 and 14 (114). This

further indicates that the role of MMPs in liver fibrosis can vary depending on the substrate and the timing in the process of liver fibrosis. It requires further investigation to clarify the sources of production of MMPs and their effects at different stages of fibrosis.

MATRIX METALLOPROTEINASE-7 (MMP-7)

MMP-7 or Matrilysin-1 belongs to the group of matrilysins. MMP-7 can only degrade collagen type IV and is not able to cleave other collagens, which are the major constituents of the ECM (105). However, MMP-7 is able to degrade fibronectin, gelatin type I, III, IV and V (degraded collagen), laminin, entactin (alternatively known as nidogen) and elastin (115). In the liver, MMP-7 is thought to be produced by a variety of cells, such as glandular epithelial cells, cholangiocytes but also by macrophages (33, 116, 117).

In the ECM of the liver, collagen type IV, laminin and entactin form the main constituent of the basement membrane, which forms the outer barrier of epithelial cells (118). Fibronectin, on the other hand, is a component of the ECM that is responsible for cell to cell adhesion by binding to integrin (119). In hepatic fibrosis, the deposition of basement membrane proteins such as collagen IV actually increases (118). This could explain why MMP-7 has been described more in cancer, where disruption of the basement membrane leads to the ability of cancer cells to invade intact tissue (120). In liver fibrosis, the role of MMP-7 has not been described extensively. A recent trial, however, found that serum MMP-7 was a reliable biomarker of advanced liver fibrosis and cirrhosis (121).

As mentioned previously, VEGF is one of the most important inducers of angiogenesis (94). It is thought that VEGF is expressed in a quiescent state in normal tissue and remained in a non-active state by binding to VEGF inhibitors, such as soluble VEGF receptor-1 (sVEGFR-1). In this manner, endothelial cells are protected of angiogenesis by the inhibition of VEGF (122). Although this mechanism is not yet proven in many different cell types, a study has shown that MMP-7 is responsible for the degradation of sVEGFR-1. This will lead to liberation and activation of VEGF and subsequent initiation of angiogenesis (123). MMP-7 thus appears to exert a pro-angiogenic function by affecting the balance between VEGF and sVEGFR-1.

MMP-7 has been investigated in BA and BA-related liver fibrosis. MMP-7 has been identified as a reliable biomarker for the diagnosis of BA in cholestatic patient cohorts by different research groups (19–21, 33, 124, 125). Lertudomphonwanit et al. found that MMP-7 was released by cholangiocytes in response to injury, although it was found in an experimental model of BA in mice (125). Interestingly, using RNA sequencing techniques, it was found that there was upregulation of the MMP-7 gene in liver samples of mice with BA induced by biliatresone (48).

Bezerra et al. found that MMP-7 was one of the genes that is up-regulated in the livers of BA patients compared to cholestatic controls (126). Hsieh et al. found that there was a progressive increase of MMP-7 gene expression from KPE to LTx in BA patients (127).

Hepatic expression of MMP-7 correlated with the extent of liver fibrosis in two cholestatic patient cohorts (20, 21). In a cohort of successfully treated BA patients, hepatic expression of MMP-7 was localized in biliary epithelium where ductular proliferation took place (32). MMP-7 co-localized with CK7, which is a marker of cholangiocyte proliferation and the ductular reaction. In contrast to previous studies, hepatic expression of MMP-7 correlated to the Metavir liver fibrosis stage despite a successful KPE indicating that hepatic overexpression of MMP-7 can occur independent of cholestasis. Huang et al. found that MMP-7 was expressed by more cell types, such as Kupffer cells, as the fibrosis progressed in BA patients (33). Jiang et al. found a correlation between serum MMP-7 concentration, age and fibrosis stage in BA patients, suggesting that serum MMP-7 plays a role in fibrosis starting from a very young age in BA patients (21). Despite these correlations of MMP-7 with extent of liver fibrosis, a clear demonstration of the longitudinal effects that MMP-7 has on liver fibrosis in BA is lacking.

These results provide circumstantial evidence that MMP-7 is involved in the pathophysiology of BA. MMP-7 can be involved during the initiation of cholangiocyte damage leading to BA as well as during the progression of liver fibrosis that is observed in these patients. However, the mechanism by which MMP-7 accomplishes either or both of these processes remains to be elucidated. In liver fibrosis, the role of MMP-7 has not been accurately described. MMP-7 has been more accurately described in other pathologies, such as pulmonary and renal fibrosis, which can be used to generate hypotheses about the involvement of MMP-7 in BA.

MMP-7 IN BILLIARY ATRESIA: LESSONS LEARNED FROM OTHER PATHOLOGIES

Renal Fibrosis

Renal fibrosis is often caused by the overproduction of ECM by interstitial fibroblasts (128). The exact mechanism of renal fibrosis remains elusive. Although controversial, one of the proposed mechanisms of renal fibrosis is EMT. MMP-7 has been investigated in renal fibrosis in light of EMT and thought to be involved by a variety of mechanisms (129). In kidney fibrosis the WNT/β-catenin signaling pathway is activated when there is injury of tubular epithelial cells, leading to the transcription of target genes by β-catenin. In cancer and renal fibrosis, MMP-7 is one of the downstream molecules transcribed by β-catenin (130). MMP-7 subsequently induces apoptosis of epithelial cells by activating Fas ligand and degrading collagen IV and laminin, which destroys the basement membrane of renal tubular epithelial cells, supposedly leading to EMT and subsequent kidney fibrosis. Although EMT may not be the mechanism by which renal fibrosis occurs, MMP-7 is thought to be actively involved in the mechanism of renal fibrosis because the extent of renal fibrosis was markedly reduced in MMP-7 gene knockout mice compared with wildtype mice (130). Furthermore, pharmacological inhibition of MMP-7 reduced the extent of renal fibrosis markers (130).

In BA, EMT is also controversial. Wnt/β-catenin signaling has been shown to be activated during liver fibrosis in BA (88). As Wnt/β-catenin is involved in the regulation of cell fate, the signaling pathway is thought to enhance the ductular reaction that is seen in liver fibrosis in BA. The increased hepatic expression of MMP-7 in BA may therefore be a result of activated Wnt/β-catenin signaling, which leads to hepatic fibrosis via initiation of the ductular reaction (**Figure 3A**). Moreover, Fas is thought to be expressed on cholangiocytes in BA, leading to apoptosis. Enhanced MMP-7 expression can lead to activation of FasL resulting in apoptosis (**Figure 3B**). Although varying results concerning Fas and BA have been found, the relation between cholangiocyte apoptosis, Fas and MMP-7 should be further explored.

E-cadherin is a transmembrane protein present at cell junctions, providing cell-to-cell integrity (119). In kidney fibrosis, MMP-7 can disrupt cell-to-cell integrity by shedding of E-cadherin. E-cadherin is characteristic of epithelial cells and loss of E-cadherin expression is considered one of the first steps in EMT. In BA, the hepatic expression of E-cadherin was investigated. Hepatic expression of E-cadherin in BA patients was reduced compared to cholestatic controls (131, 132). Moreover, there was an inverse correlation between hepatic E-cadherin and apoptosis of cholangiocytes in BA patients, suggesting that reduced expression of E-cadherin can lead to apoptosis of cholangiocytes (131). Harada et al. found reduced expression of E-cadherin in damaged cholangiocytes in BA patients compared to non-damaged cholangiocytes (132). As MMP-7 is able to shed E-cadherin, it would be of great interest to investigate if MMP-7 can lead to cholangiocyte apoptosis via reduction of cell-to-cell adhesion by shedding of E-cadherin (**Figure 3C**).

β-catenin is attached to the intracellular domain of E-cadherin (133). In theory, when E-cadherin is shedded, β-catenin is freed and able to translocate to the nucleus of the cholangiocyte. Then, target genes of the Wnt/β-catenin can be transcribed, of which MMP-7 is one (130). Via this mechanism, a potential positive feedback loop between β-catenin and MMP-7 would be possible. However, no difference in the intracellular expression of β-catenin between control and BA livers and no nuclear accumulation of β-catenin were found in cholangiocytes in a study investigating this (131). Therefore, a positive feedback loop via this mechanism seems unlikely. However, as β-catenin may be involved in the pathogenesis of BA, it would still be of interest to further investigate the involvement of β-catenin and related signaling proteins in relation to shedding of E-cadherin specifically in BA (88).

Pulmonary Fibrosis

The role of MMP-7 has also been thoroughly investigated in idiopathic pulmonary fibrosis (IPF), a rapid progressing fibrotic lung disease affecting the alveolar epithelial cells (AEC) (134). MMP-7 can be used as a biomarker that predicts mortality in patients with IPF (124). Moreover, Fujishima et al. showed that proMMP-7 is produced locally by hyperplastic AECs and macrophages in patients with IPF indicating an active role of MMP-7 in the fibrotic microenvironment (116). Similarly to renal fibrosis, MMP-7 is able to shed E-cadherin in alveolar epithelial cells, leading to activation of β-catenin signaling and by the induction of Fas, leading to apoptosis of AECs (135). However, this activation is considered as a physiological process to some extent in the repair of epithelium in the airways (136).

The pulmonary expression of MMP-7 has also been investigated in concert with osteopontin (OPN). Besides a Th1 cytokine, OPN is believed to play a role in fibrosis by recruiting and differentiating MFs (137, 138). Pardo et al. found that MMP-7 and OPN co-localize on alveolar epithelial cells in patients

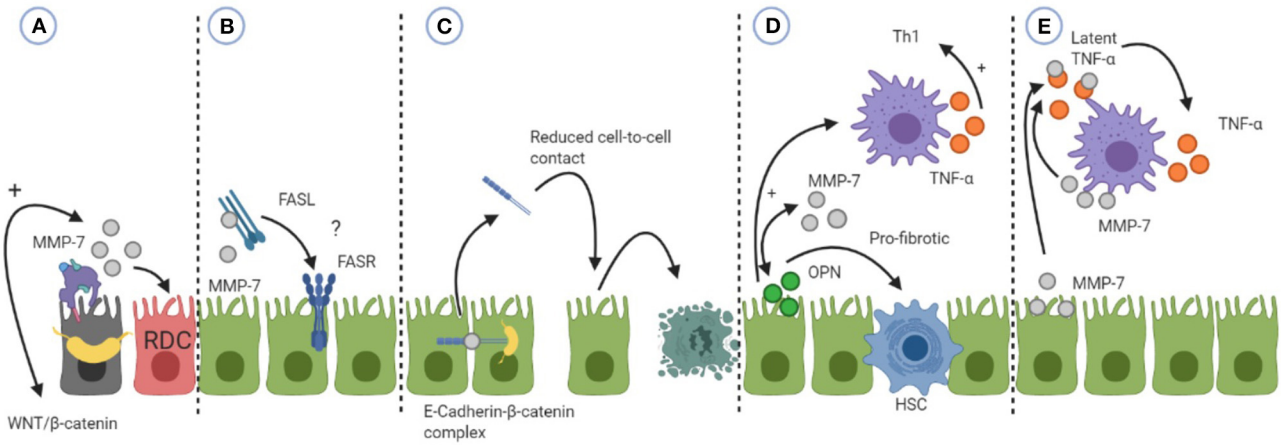

FIGURE 3 | An overview of how MMP-7 can be involved in the pathophysiology of biliary atresia (BA). **(A)** MMP-7 can be a result of activated WNT/β-catenin signaling leading to proliferation of cholangiocytes into reactive ductular cells (RDC). **(B)** MMP-7 can activate Fas-ligand (FASL) which can subsequently induce apoptosis by binding to the Fas-receptor (FASR). The relevance of this mechanism is unsure, hence the question mark. **(C)** MMP-7 can shed E-cadherin leading to reduced cell-to-cell contact and subsequent apoptosis. **(D)** MMP-7 and osteopontin (OPN) can engage in a positive feedback loop. OPN can exert its pro-fibrotic effects by activating hepatic stellate cells (HSC) or its Th1 differentiating effects by activating macrophages (M). **(E)** MMP-7 can activate tumor necrosis factor alpha (TNF-α) that is secreted by macrophages, enhancing its pro-inflammatory effects. See the text for more details of the mechanism.

with IPF (139). Since MMP-7 is able to cleave, as well as to be cleaved by OPN, the authors propose that a positive feedback loop between MMP-7 and OPN is possible. This loop leads to a sustained activation and exertion of the effects of MMP-7 and OPN, causing rapid fibrosis in the lungs of patients with IPF (139, 140). OPN exerts its pro-fibrotic effect in the lungs by increasing the secretion of collagen-1 by fibroblasts while in AECs it induced the expression of MMP-7 (139). OPN has been investigated in liver pathologies (141). In liver fibrosis, OPN mediates its pro-fibrotic effects through the activation of hepatic stellate cells (HSC) to up-regulate the deposition of collagen-1, one of the hallmarks of liver fibrosis (79). OPN supposedly accomplishes this by two pathways, either by signaling to NF-kB in HSCs or by signaling biliary epithelial cells (BEC) to up-regulate TGF-β. Moreover, in cardiac MFs, it was shown that OPN is required for TGF-β induced differentiation of MFs, indicating that OPN is important for the induction of fibrosis (138). In BA, Whitington et al. showed that OPN can be secreted by BECs and is greatly overexpressed in areas of proliferating ductal cells (73). Moreover, OPN expression in the livers of BA patients correlated very strongly to the degree of hepatic fibrosis, but also to the gene expression of TGF-β (142). It is tempting to speculate that OPN and MMP-7 may be engaged in a positive feedback loop in BA, leading to the progressive liver fibrosis that is observed in a similar manner as in IPF (**Figure 3D**). Future research should explore the mechanism by which MMP-7 and OPN interact with each other in BA. It would for example be interesting to investigate which protein induces the expression of the other and what the sources of these proteins are.

Moreover, OPN is also considered a Th1 cytokine (74). A positive feedback loop between MMP-7 and OPN can also result in a progressive inflammatory response and subsequent hepatic overexpression of both proteins in BA patients. MMP-7 can influence the inflammatory response indirectly by activating OPN. OPN is able to regulate the migration and differentiation of macrophages during the innate immune response. After OPN activated dendritic cells (DCs) and macrophages, these cells start to produce TNF-α and IL-12 so that naïve T-cells are polarized into IFN-γ producing Th1 cells (74) (**Figure 3D**).

Inflammation and Angiogenesis

MMPs can play a role in inflammation by a variety of mechanisms, such as by creating chemotactic gradients leading to influx of inflammatory cells into site of injury or by activating cytokines (30). MMP-7 activates intestinal pro α-defensins in mice, enhancing mucosal immunity (143, 144). α-Defensins are produced by Paneth cells in the intestinal crypts and have a direct antimicrobial effect and amplify the immune response by acting as a chemoattractant for immune cells. MMP-7 can also be produced by Paneth cells (143). MMP-7 has recently shown to promote an increased intestinal permeability by activating α-Defensins in Paneth cells of mice (144). Increased intestinal permeability leads to the leakage of products via the bloodstream and translocation to the liver causing inflammation. This is termed the gut-liver axis. The gut-liver axis has also been investigated in BA (145). As MMP-7 has not yet been identified in human intestinal Paneth cells, we will not discuss MMP-7 and the gut-liver axis in relation to BA here. However, this may be an interesting direction for future research. MMP-7 in relation to macrophages has been investigated in the resorption of herniated discs, a major cause of lower back pain (146). Haro et al. used a mice model to show that macrophages are responsible for the production of MMP-7 and latent TNF-α. The function of MMP-7 here is to cleave latent TNF-α to its soluble, active form. Activated TNF-α mediates macrophage invasion of the discs which is required for absorption of the disc. Importantly, the authors also show that MMP-7 is able to cleave latent TNF-α in isolated macrophages, indicating that MMP-7 ability to activate TNF-α is not restricted to the environment of herniated discs. Macrophages and its secreted TNF-α mediate damage in BA. Moreover, Kupffer cell expression of MMP-7 increases as liver fibrosis in BA progresses. MMP-7 may fulfill its role in the pathophysiology of BA by enhancing the activation of TNF-α from Kupffer cells and macrophages in the liver (**Figure 3E**).

In the context of inflammation, we will also discuss the role MMP-7 may play during angiogenesis in BA. As previously illustrated, findings on VEGF-A expression are variable. VEGF-A can be related to cholangiocyte proliferation because their proliferation requires a higher blood supply (97, 98). Alternatively, a lower expression of VEGF-A when BA patients experienced intense hypoxia in their liver, can be explained by an insufficient angiogenic response (99). It has been shown that MMP-7 is able to liberate VEGF-A by degrading sVEGFR-1 (123). MMP-7 localizes at the biliary epithelium, where the ductular reaction took place (32). It may be that MMP-7 is involved in the facilitation of angiogenesis by liberating VEGF-A in BA. Although the mechanism of VEGF-A liberation by MMP-7 is first to be demonstrated in liver tissue before adequate research in BA can take place, it may be a target of future research. Moreover, the exact role of VEGF-A and angiogenesis in BA should be further explored, especially in relation to progression of liver fibrosis, cholangiocyte proliferation and potentially MMP-7 as well.

CLINICAL IMPLICATIONS

Exploring and establishing the exact role that MMP-7 has in BA can provide new therapeutic targets that can be explored in order to prevent liver fibrosis or even prevent BA from occurring. Anti-inflammatory medication in the form of corticosteroids has been investigated on numerous occasions and shown to be somewhat effective in improving outcomes, such as clearance of jaundice, in BA patients (147). However, as BA is an immune mediated disease, one would expect anti-inflammatory medication to be more effective. Higher effectiveness of perioperative medication can be achieved by selective inhibition of a pathogenic factor. MMP-7 may be a key mediator linking inflammation and fibrosis that can be accurately blocked by targeted medication. In mice with renal fibrosis, MMP-7 was blocked by MMP inhibitor II. The mice had reduced extent of renal fibrosis and a reduction of renal expression of collagen-1 was observed (130). Given this potential,

the effect blocking MMP-7 has on the development of BA should be further explored.

Moreover, as serum MMP-7 correlates to the hepatic expression of MMP-7, opportunities for prognosis estimation can be explored (19–21, 33, 93). Almost all patients with BA undergo a KPE and in half of these patients, an LTx is required within the first 2 years after KPE (13). A prognostic factor that gives an estimation of the damage that is already done to the liver can help surgeons in deciding whether it is even beneficial to perform a KPE or to perform a primary LTx. Moreover, a prognostic factor after performance of a KPE can also be beneficial in BA patients. As of yet there is no factor that can accurately predict the need for an LTx. In other pathologies, MMP-7 as serum or urine marker correlates very well to clinical symptoms as well as prognosis (124, 130, 148). By having an accurate postoperative prognostic factor in BA, better triage of follow-up care for BA patients can be performed. Besides, measuring serum MMP-7 is also a lot less invasive than current diagnostic options, such as liver biopsy or ERCP (1). Before serum MMP-7 can actually be used as a prognostic biomarker, studies should investigate how hepatic MMP-7 expression progresses in the same BA patients over time; from KPE to LTx, for example. By doing this, a more accurate description of the effects of MMP-7 in BA-related fibrosis can be made. Subsequently, the predictive power of serum MMP-7 for native liver survival and occurrence of LTx in BA patients should be explored. This should be investigated as a single factor as well as in addition to other predictive markers such as postoperative bilirubin levels.

DISCUSSION

The aim of this review was to describe if and how MMP-7 can play a role in the pathophysiology of BA. An overview was given of the pathophysiology of BA, which is characterized by a Th1 immune response. Macrophages play a central role in the initial damage to cholangiocytes as well as in the progression to liver fibrosis. We described the structure and functions of MMP-7 in a physiological and pathological environment. In BA, hepatic MMP-7 expression correlates with the extent of liver fibrosis, even with minimal cholestasis after the performance of a KPE. MMP-7 co-localized with CK-7, a marker of cholangiocyte proliferation, and correlated to the extent of cholangiocyte proliferation (32). Moreover, MMP-7 localized near Kupffer cells, the residing macrophages of the liver. The co-localization of MMP-7 and important mediators of hepatic injury and fibrosis provides circumstantial evidence that MMP-7 is actively involved in the initial cholangiocyte injury in BA as well as in BA-related liver fibrosis. We described how MMP-7 is involved in other pathologies such as renal and pulmonary fibrosis and if these mechanisms could be extrapolated to the pathogenesis of BA. We hypothesized that the Wnt/β-catenin signaling pathway may be activated by MMP-7 in BA. Moreover, shedding of E-cadherin by MMP-7 leading to reduced cell-to-cell integrity and subsequent cell damage may occur in BA. In pulmonary fibrosis, OPN and MMP-7 are hypothesized to engage in a positive feedback loop leading to progressive fibrosis (139). A similar mechanism of positive feedback could well be at play in the progression of liver fibrosis in BA. Angiogenesis is a feature of liver fibrosis and cirrhosis. In BA, more research is required to clarify the role and relevance of angiogenesis and VEGF-A in the pathogenesis of BA. Due to the potential mechanisms of MMP-7 involvement in BA described, we think that the role MMP-7 can play in BA is primarily aimed at facilitating inflammation and fibrosis.

By generating hypotheses, we aimed to clarify the potential role of MMP-7 and to provide new targets for future research in BA. Moreover, determining whether MMP-7 plays an active role in the pathogenesis of BA or if MMP-7 is a marker of abnormal tissue regeneration will also provide clarity for future research. Active involvement of MMP-7 in the pathogenesis in of BA would imply possibilities for new therapeutic targets as well as new possibilities to estimate the prognosis of BA patients.

Limiting factors to this review were the fact that BA remains a very rare disease. Consequently, the studies that have been used to create this review range in their year of publication by 30 years. Moreover, BA is a heterogeneous disease with subtypes. In this review, we have tried to describe features of the isolated form of BA, but this was not specified in every study that was used. Subsequently, multiple subtypes of BA could have been included in patient cohorts, leading to different outcomes than if isolated BA only was included. We attempted to only include studies that focus on studies that investigated BA in humans so studies performed in mice with experimental BA were excluded. Future research should investigate the pathogenesis of BA in a specified, global cohort of BA patients, so that research around the globe can build further in a reliable way on results that are found.

In conclusion, BA and MMP-7 have an interesting relationship which is yet to be specified. MMP-7 can be involved in the pathogenesis of BA by multiple mechanisms that enhance inflammation, fibrosis or both. Future research can elucidate the role of MMP-7 in these processes further, so that application of MMP-7 as therapeutic target and prognostic biomarker can be explored.

AUTHOR CONTRIBUTIONS

MN: study concept and drafting of the manuscript. LB, JH, and PO: study concept and critical revision of the manuscript for important intellectual concept. HV: critical revision of the manuscript for important intellectual concept. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Macrophage Activation Markers, Soluble CD163 and Mannose Receptor, in Liver Fibrosis

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Macrophages are essential components of the human host immune system, which upon activation facilitates a broad pallet of immunomodulatory events including release of pro- or anti-inflammatory cytokines and chemokines, restoration of immune homeostasis and/or wound healing. Moreover, some macrophage phenotypes are crucially involved in fibrogenesis through stimulation of myofibroblasts, while others promote fibrolysis. During the last decades, the role of resident liver macrophages viz. Kupffer cells and recruited monocytes/macrophages in acute and chronic liver diseases has gained interest and been extensively investigated. Specifically, the scavenger receptors CD163 and mannose receptor (CD206), expressed by macrophages, are of utmost interest since activation by various stimuli induce their shedding to the circulation. Thus, quantifying concentrations of these soluble biomarkers may be of promising clinical relevance in estimating the severity of inflammation and fibrosis and to predict outcomes such as survival. Here, we review the existing literature on soluble CD163 and soluble mannose receptor in liver diseases with a particular focus on their relationship to hepatic fibrosis in metabolic associated fatty liver disease, as well as in chronic hepatitis B and C.

Keywords: liver, fibrosis, macrophages, sCD163, mannose receptor, metabolic associated fatty liver disease, hepatitis B virus, hepatitis C virus

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INTRODUCTION

The progression course from low grade hepatic inflammation and early stage fibrosis to manifest cirrhosis differs depending on disease etiology but also between subgroups within the same disease (1). With established cirrhosis follow substantial reductions in quality of life and survival, especially when decompensation develops with variceal bleeding, hepatic encephalopathy, ascites and/or hepatorenal syndrome or the development of liver cancer (2–4).

The pathomechanisms orchestrating disease progression and maintenance of inflammation and fibrosis are regulated through a complex interplay between immune cells. Resident liver macrophages viz. Kupffer cells along with recruited monocytes/macrophages are essential in the development and progression of liver diseases (5). Macrophages are key players of human innate immunity displaying an extensive array of membrane receptors, which mediate crucial immunomodulatory responses upon macrophage activation with both pro- and anti-inflammatory effects (6, 7). We review the value of two biomarkers of macrophage activation in liver diseases

and their relationship to the degree of fibrosis, ultimately concluding with the potential clinical utility of these biomarkers. We briefly comment on macrophage-related therapeutic options.

MACROPHAGES AND LIVER FIBROSIS

Kupffer cells and recruited macrophages possess the ability to exert omnipotent immunomodulation, evident by both pro-fibrotic and anti-fibrotic effects in the liver (8). Traditionally, macrophages have been divided in two subgroups with either a "pro-inflammatory" M1 or "immune-regulatory" M2 phenotype. However, this dichotomous classification is a too simplified division of a highly heterogeneous group of differently activated and functioning immune cells as recently affirmed by advanced molecular sequencing techniques (8–11).

Pathogens reaching the hepatic immune environment initiate a pro-inflammatory response with Kupffer cells as essential partakers and a major source of inflammatory signaling molecules (12). Macrophage activation by pathogen-associated molecular patterns (PAMPs) through pattern-recognizing receptors (PRRs) including Toll-like-receptors is of utmost relevance in the immunological processes during chronic inflammation. Similarly, macrophages may be activated by damage-associated molecular patterns (DAMPs) (**Figure 1**) (13). The cytokines interleukin-12 (IL-12) and interferon-γ (IFN-γ) are known to initiate a pro-inflammatory response in macrophages, resulting in the release of tumor necrosis factor (TNF), IL-1β, IL-6, IL-12 and reactive oxygen species (7–9). Furthermore, the presence of a IFNL3-IFNL4 haplotype resulting in production of IFN-γ3 is recognized as a promoter of hepatic inflammation and fibrosis progression (14). Through the release of pro-inflammatory cytokines and chemokines, macrophages interplay with other immune cells including T cells, B cells, natural killer T cells and neutrophils (8, 15). Conversely, the release of modulatory cytokines such as IL-10, transforming growth factor β (TGF-β), IL-4 and IL-13, induced by adaptive and innate signals, is of key importance in dampening inflammation and promoting wound healing (7–9, 16). In this view, TGF-β potently stimulates hepatic stellate cell differentiation into myofibroblasts (17) responsible for fibrogenesis during bouts of hepatic inflammation. Myofibroblasts undergo persistent proliferation contributing to disease progression by synthesis of extracellular matrix components and potentiation of ongoing inflammation with the release of chemokines, cytokines and fibrogenic mediators (18–20). Upon dampening of liver injury, histoarchitecture restorative events become dominating with deactivation of myofibroblasts by apoptosis or phenotypic switch and activation of protective or restorative macrophages, which promote fibrosis resolution and tissue remodeling (8, 18, 21), although the mechanisms underlying this phenotypic switch have not been fully elucidated.

MACROPHAGE ACTIVATION MARKERS

As discussed above, macrophages play a crucial role in both hepatic fibrogenesis and resolution of fibrosis, and several macrophage-released molecules are involved in these processes. Thus, plasma concentrations of such molecules may reflect the degree of fibrosis in liver diseases and help clinicians determine disease stage, prognosis, and response to intervention.

Scavenger receptors are expressed by macrophages as part of a large receptor panel accountable for regulation of endocytosis, phagocytosis, adhesion, and signaling. Some scavenger receptors are promising indicators of the inflammatory load in liver diseases and have a potential prognostic value (22). The most intensively studied proteins are cluster of differentiation 163 (CD163) and the mannose receptor (MR/CD206). CD163,

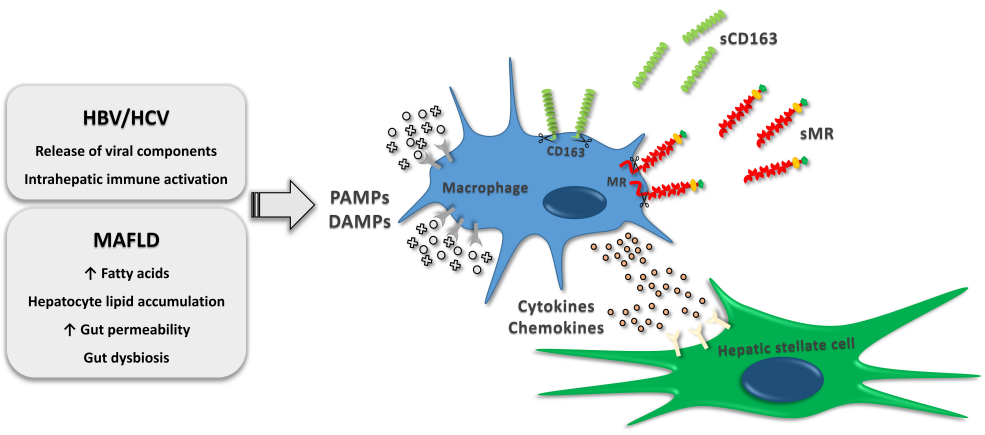

FIGURE 1 | Macrophage activation by immunomodulatory events occurring in MAFLD and chronic HBV or HCV infections. The activation increase receptor shedding, which results in release of soluble CD163 and soluble MR to the circulation. Furthermore, cytokines and chemokines released by resident and recruited liver macrophages stimulate hepatic stellate cell differentiation into myofibroblasts consequently causing synthesis of extracellular matrix components. HBV, hepatitis B virus; HCV, hepatitis C virus; MAFLD, metabolic associated fatty liver disease; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; sCD163, soluble cluster of differentiation 163; sMR, soluble mannose receptor.

a hemoglobin-haptoglobin scavenger receptor, is exclusively expressed by monocytes and macrophages (23, 24) and exerts its main biological function through elimination of hemoglobin-haptoglobin complexes during hemolysis (25). A soluble form (sCD163) is present in the plasma (26), and enzymatic receptor cleavage of CD163 by the TACE/ADAM17 system is highly upregulated in response to inflammatory stimuli, including the PAMP lipopolysaccharide (LPS) (27–30). The mannose receptor is primarily expressed by macrophages, dendritic cells and endothelial cells (31) and is involved in scavenging events including endogenous molecule clearance besides antigen presentation (32). Similar to sCD163, soluble mannose receptor (sMR) is present in plasma, and shedding by proteases is induced by PAMPs (**Figure 1**) (28, 33). Even though sCD163 and sMR share similarities regarding expression and stimuli leading to shedding, and their concentrations inter-correlate indicating concurrent shedding from activated macrophages, the biomarkers possess different immunogenic roles and may be differently regulated with fluctuations of serum concentrations in diverse immunological conditions (22). Furthermore, the very shedding intensity induced by various stimuli may differ between the two biomarkers (34).

MACROPHAGE ACTIVATION MARKERS IN LIVER DISEASES

The dynamic processes of hepatic inflammation have been extensively studied over the last decades with increasing focus on macrophage activation markers and their utility in staging the degree of ongoing inflammation and fibrosis, as well as predicting disease outcome. In this review, when discussing non-alcoholic fatty liver disease (NAFLD), we will use the recently introduced and more appropriate consensus nomenclature of metabolic associated fatty liver disease (MAFLD) (35, 36). As previously summarized and illustrated in (22, 37, 38) plasma levels of sCD163 are moderately elevated in MAFLD (39), chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infections (40), Wilson's disease (41, 42), and primary biliary cholangitis (43), and clearly reflects disease severity in MAFLD, HBV and HCV (39, 40, 44–47). In further support of a correlation between sCD163 concentrations and liver disease severity sCD163 levels show significant reductions after lifestyle interventions in MAFLD (48, 49) and after antiviral therapy in HBV and HCV (46, 50, 51). In manifest cirrhosis, sCD163 levels are even higher with a dramatic stepwise increase in parallel with Child-Pugh- and MELD-scores (52). Moreover, sCD163 values predict the degree of portal hypertension (53, 54) and are associated with variceal bleeding and prognosis (55, 56). In cirrhotic patients with hepatocellular carcinoma, sCD163 levels associate significantly with overall survival (57, 58). Hepatic expression of CD163 is significantly increased in patients with acute viral hepatitis compared with chronic viral hepatitis (59). Accordingly, the most prominent elevations of plasma sCD163 are seen in acute liver injury with intense inflammation including alcoholic hepatitis (60, 61), acute liver failure (62, 63), and acute-on-chronic liver failure (ACLF) (64), where it shows great potential as an independent predictor of short-term mortality.

Similarly, sMR has become of increasing interest as a marker of inflammation in liver disease. In children with MAFLD, sMR is elevated compared with non-overweight controls (65). sMR mimics sCD163 in chronic HBV and HCV, with increasing plasma concentrations in association with incrementing severity of hepatic inflammation (51, 66) and persistent reduction after antiviral therapies (51, 67). sMR is substantially elevated in patients with cirrhosis with significant correlation with Child-Pugh-score (56). Furthermore, it is a predictor of long-term survival and for the occurrence of cirrhosis-associated complications including decompensating events such as ascites, hepatic encephalopathy, and upper gastrointestinal hemorrhage due to portal hypertension within 1 year (56). Lastly, patients suffering from acute liver injury due to acetaminophen overdose, acute cirrhosis decompensation, ACLF, and alcoholic hepatitis have markedly increased plasma levels of sMR with 2.5- to 5-fold higher median values compared with healthy individuals (64, 68, 69). In addition, combining the CLIF-C ACLF score and sCD163 improves the prediction of 90 days mortality, while sMR in addition to the CLIF-C acute decompensation (AD) score improves the prediction of 90 and 180 days mortality (64, 70).

In summary, multiple studies have documented that plasma concentrations of the macrophage activation markers sMR and especially sCD163 are reliable indicators of ongoing hepatic inflammation, and potential useful tools to predict disease outcome including mortality.

sCD163 AND sMR IN LIVER FIBROSIS

As outlined above, a pivotal event in the hepatic inflammatory response is activation of resident and recruited macrophages responsible for further signaling events and subsequently resulting in a spectrum of scenarios ranging from resolution of inflammation to maintenance or intensification of the response, activation of hepatic stellate cells with fibrosis development, and/or induction of fibrolysis. There is a solid foundation to consider sCD163 and sMR as important and clinically useful markers of inflammation in liver disease, but they may equally reflect fibrosis severity. The latter will be reviewed below with a specific focus on MAFLD and chronic HBV and HCV where the most robust data is available.

MAFLD

The prevalence of obesity is increasing worldwide leading to a substantially increased health care burden related to diseases associated with obesity. This includes MAFLD and MAFLD with steatohepatitis with or without fibrosis (1, 35, 36). MAFLD is related to insulin resistance, obesity, type 2 diabetes mellitus (71); and suggested to be the hepatic manifestation of the metabolic syndrome (72, 73). Key immunomodulatory events comprise hepatic lipid accumulation and increased translocation of bacterial components from the gut. If the lipid load surpasses the hepatic metabolic capacity, the dysregulated

hepatic lipid metabolism will result in toxic lipid intermediates. These intermediates will be recognized as DAMPs by liver macrophages and initiate activation through PRRs. Gut derived bacterial components (e.g., LPS) potentiates this process, further increasing liver inflammation (**Figure 1**). A recent study of 40 non-diabetic and mostly non-obese patients with biopsy-proven MAFLD and varying severity of hepatic steatosis and fibrosis, indicated a link between adipose tissue insulin resistance and Kupffer cell activation since sCD163 concentrations associated with circulating free fatty acids, lipolysis rate and insulin resistance in adipose tissue (74). Persistent and excessive inflammation entails fibrogenic events including activation of myofibroblasts, which are highly dependent on release of inflammatory and pro-fibrotic cytokines such as TGF-β from activated Kupffer cells and recruited macrophages (74–76). Even though fibrosis progresses slowly in MAFLD (77) ∼20% are rapid progressors (78), and there is an urgent need for improved tools to non-invasively diagnose and evaluate liver fibrosis as well as to predict progression risks and treatment effects.

In morbidly obese adults undergoing bariatric surgery and perioperative liver biopsy, sCD163 was significantly associated with the Kleiner fibrosis score. In addition, patients with a high fibrosis score had significantly higher preoperative sCD163 levels (39), an association confirmed in two independent cohorts of patients with biopsy-proven MAFLD (**Figure 2A**). sCD163 as a sole marker performed well in detecting advanced fibrosis, and the addition of sCD163 to the established and widely applied model NAFLD Fibrosis Score (NAFLD-FS) improved the predictive capacity of the latter (44). sCD163 and NAFLD-FS had comparable sensitivities, 84 and 80%, respectively, for predicting advanced fibrosis ($F \geq 3$) using low cut-off values in an Australian cohort. However, in an Italian cohort the sensitivities of sCD163 and NAFLD-FS for predicting advanced fibrosis were much lower (∼40%), though still comparable (44). Another research group reported a similar significant association between sCD163 and hepatic fibrosis in obese individuals undergoing bariatric surgery (45). Recently, results from 40 patients with MAFLD and available liver histology and sCD163 quantifications as well as a comprehensive metabolic assessment clearly demonstrated an association between sCD163 and the stage of fibrosis (74).

In obese children undergoing lifestyle interventions, sCD163 was significantly higher at baseline compared with 1-year follow-up in children with a high level of a non-invasive surrogate measure for fibrosis [the Pediatric NAFLD Fibrosis Index (79)]. Following lifestyle intervention, sCD163 levels decreased in association with improvements in metabolic dysfunction (48). However, a recent study of sCD163 and sMR in children with MAFLD reported no significant associations between either sCD163 or sMR and fibrosis assessed from liver biopsies, which was in clear contrast with the results shown in adult cohorts and may suggest different mechanisms in terms of macrophage activation and fibrosis development in children compared with adults with MAFLD (65).

HBV and HCV

Chronic infection with HBV and HCV is maintained by continued release of viral components by infected cells (80, 81), thus interacting with the intrahepatic innate immune system including Kupffer cells and hepatic stellate cells (**Figure 1**). These are directly activated by HBV and HCV components in addition to an indirect stimulation by differentiated pro-fibrogenic liver macrophages (82, 83). Genetic studies have recently elucidated an association between sCD163 levels and expression of specific gene variants known to promote hepatic inflammation and fibrogenesis in patients with chronic HCV, thus supporting a pivotal role for Kupffer cell activation in the development and progression of hepatic fibrosis (14, 84). Several studies have measured sCD163 and sMR in chronically HBV and HCV infected individuals and related biomarker concentrations to fibrosis scores. In a small population of HCV patients assessed by the non-invasive FibroScan® sCD163 was associated with liver fibrosis (66). Data concerning liver histology and biochemistry

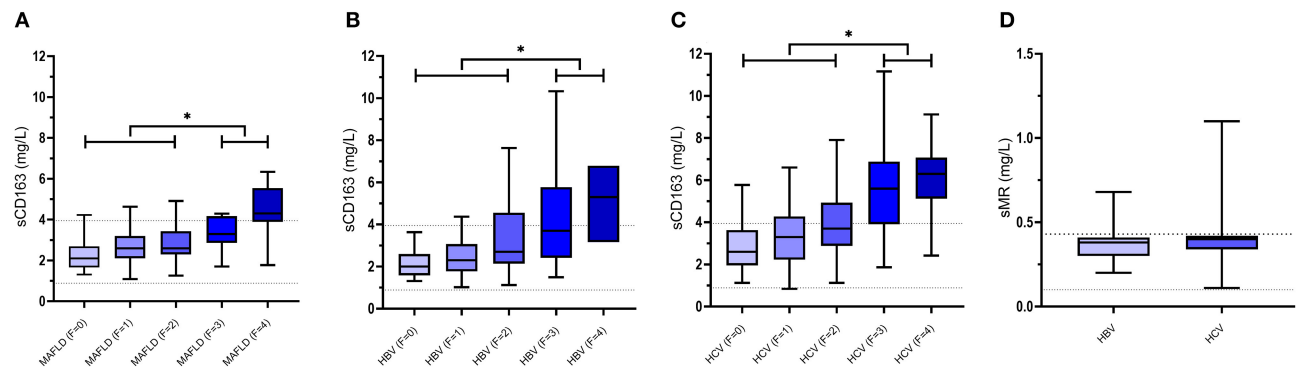

FIGURE 2 | Concentrations of sCD163 in MAFLD **(A)**, chronic HBV infections **(B)** and chronic HCV infections **(C)** as well as concentrations of sMR in chronic HBV infections and chronic HCV infections **(D)** presented by means/medians, interquartile ranges as well as minimum and maximum values. In patients with MAFLD, chronic HBV infections and chronic HCV infections plasma levels of sCD163 increase with increasing fibrosis grade (F0-F4) (40, 44, 51, 67) with significantly higher levels in patients with fibrosis grades F3-F4 compared with patients with fibrosis grades F0-F2 (*p-value <0.001). Dotted lines represent upper and lower reference values for healthy controls (26, 34). MAFLD, metabolic associated fatty liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus; sCD163, soluble cluster of differentiation 163; sMR, soluble mannose receptor.

from a large treatment-naïve cohort of 513 patients with chronic HCV and 200 patients with chronic HBV were published in 2014 (40). In general, HCV patients had higher sCD163 concentrations for the same fibrosis score as HBV patients. In both HCV and HBV, sCD163 increased in association with histological stages of fibrosis (**Figures 2B,C**). Supporting a direct association between sCD163 and fibrosis in HCV patients a CD163-HCV-fibrosis-score was superior to the common fibrosis scoring tools, APRI and FIB-4, for predicting significant fibrosis (40). The sCD163-based HCV fibrosis score has been successfully validated (50). A CD163-HBV-fibrosis-score was also presented and evaluated, but was not significantly superior to APRI and FIB-4 (40). Dultz et al. showed that chronic HBV patients with an Ishak fibrosis score $\geq$2 had significantly higher levels of plasma sCD163 than F0-F1 patients. Thus, sCD163 may be a useful biomarker to discriminate chronic HBV treatment-naïve patients with minimal fibrosis from patients with significant fibrosis (46). Recently, sCD163 and sMR were measured in a cohort of chronic HBV patients before and after nucleoside-analogue treatment (**Figure 2D**). The two macrophage activation markers showed a weak association with the Ishak fibrosis score (51). In this study, the CD163-HBV-fibrosis-score was validated and performed similarly to APRI and FIB-4 for prediction of significant fibrosis (51). A similar study of sCD163 and sMR in chronic HCV patients reported significant reductions of liver stiffness evaluated by transient elastography (FibroScan® or ARFI scan®) following antiviral treatment (**Figure 2D**). Concentrations of sCD163 and sMR correlated with liver stiffness at baseline and follow-up, though no consistent conclusion on the predictive value of the markers in relation to fibrosis severity was presented (67). Hence, the high baseline concentrations of sCD163 and sMR may not solely reflect fibrosis, since significantly higher viral load and alanine aminotransferase were present at baseline, indicating a considerable component of active liver inflammation.

In summary, there is a well-documented association between plasma concentrations of sCD163 and hepatic fibrosis in patients with chronic HBV and HCV, with a decline after antiviral treatment. The results for HCV are the most concordant.

CONCLUDING REMARKS

Plasma levels of sCD163 and sMR may be clinically useful as they directly reflect macrophage activation in liver diseases. Since macrophages play a significant immunomodulatory role in acute and chronic inflammatory liver disease, including an interplay with hepatic stellate cells, sCD163 and sMR have gained interest as potential markers of hepatic inflammation and fibrosis. Results from several studies in humans indicate usefulness of sCD163 and sMR in estimating the degree of ongoing inflammation in acute and chronic liver diseases in general and in assessing fibrosis severity in patients with MAFLD as well as chronic HBV and HCV infections. Since hepatic inflammation and fibrosis are inter-related events—both being consequences of the continuum of immunological activities in chronic liver diseases—the inflammatory burden must be considered as a possible limitation to the use of sCD163 and sMR as biomarkers of liver fibrosis. Moreover, sCD163 may be elevated in other diseases involving activation of monocytes and macrophages, including Gaucher disease (characterized by excessive macrophage proliferation), hemophagocytic syndrome, infectious diseases, chronic inflammatory diseases, and leukemia, and may be markedly elevated in septic patients (85), limiting its clinical utility. Pulmonary fibrosis may also result in elevations of circulating sCD163 (86). Hence, critical and thorough evaluation of the clinical setting is important before sCD163 and sMR are measured with the purpose of assessing liver fibrosis. However, in the urgent need for improved non-invasive disease scoring tools, especially concerning MAFLD and chronic viral hepatitis B and C, sMR and sCD163 possess great potentials toward being included in fibrosis assessments and may even reflect treatment effects. Further, from a therapeutic perspective, the membrane bound CD163 and perhaps MR may hold promise as entry molecules for liver macrophage-targeted drug delivery (87, 88). An advantage of CD163 is rapid internalization of ligands limiting systemic drug exposure (89) as well as providing a directed action of the applied drug (90). Consequently, intravenous injections of CD163-directed anti-IgG-dexamethasone conjugate in rats on a high fructose diet significantly reduced hepatic inflammation and fibrosis (88). However, future studies in humans are needed to further elucidate the therapeutic potential of macrophage receptors, thereby possibly extending the treatment options for patients with chronic liver diseases.

AUTHOR CONTRIBUTIONS

RG and HG: conceptualization. RG: writing–original draft. RG, MK, TL, KK, JG, HM, and HG: writing–review and editing. All authors approved the final version of the manuscript.

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Macromolecular Crowding as a Tool to Screen Anti-fibrotic Drugs: The Scar-in-a-Jar System Revisited

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An unsolved therapeutic problem in fibrosis is the overproduction of collagen. In order to screen the effect of anti-fibrotic drugs on collagen deposition, the Scar-in-a-Jar approach has been introduced about a decade ago. With macromolecular crowding a rapid deposition of collagen is seen, resulting in a substantial decrease in culture time, but the system has never been tested in an adequate way. We therefore have compared six different macromolecular crowders [Ficoll PM 70 (Fc70), Ficoll PM 400 (Fc400), a mixture of Ficoll 70 and 400 (Fc70/400), polyvinylpyrrolidone 40 (PVP40), polyvinylpyrrolidone 360 (PVP360), neutral dextran 670 (ND670), dextran sulfate 500 (DxS500), and carrageenan (CR)] under profibrotic conditions (addition of TGFβ1) with primary human adult dermal fibroblasts in the presence of 0.5 and 10% FBS. We found that (1) collagen deposition and myofibroblast formation was superior with 0.5% FBS, (2) DxS500 and CR results in an aberrant collagen deposition pattern, (3) ND670 does not increase collagen deposition, and (4) CR, DxS500, and Fc40/700 affected important phenotypical properties of the cells when cultured under pro-fibrotic conditions, whereas PVP40 and PVP360 did less or not. Because of viscosity problems with PVP360, we conclude that PVP40 is the most optimal crowder for the screening of anti-fibrotic drugs. Finally, the effect of various concentrations of Imatinib, Galunisertib, Omipalisib or Nintedanib on collagen deposition and myofibroblast formation was tested with PVP40 as the crowder.

Keywords: macromolecular crowding, drug testing, fibrosis, collagen, myofibroblast

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INTRODUCTION

Most cells are embedded in a tissue-specific micro-environment composed of extracellular matrix (ECM) molecules. One of the structural components of the ECM is collagen. There are several types of collagen, with the fibrillar collagens (e.g., collagen type I, II, and III) being the most common ones. The precursor procollagen needs to be processed into mature collagen to enable the formation of supramolecular structures outside the cell. Especially in culture systems, this processing is time-consuming, and extended culture times are required to obtain a sufficient amount of extracellular matrix. Already in 1986 Bateman et al. (1) showed that the addition of neutral polymers in culture medium markedly enhanced the conversion of procollagen into collagen. They showed that the rate-limiting step in culture

medium is the proteolytic cleavage of the N- and C-propeptides by propeptidases, and that adding polymers facilitated this proteolytic cleavage, which was confirmed by Hojima et al. (2). This system, which is due to macromolecular crowding (volume exclusion), has recently attracted considerable attention when it was re-invented in 2007 by Lareu et al. (3, 4).

Macromolecular crowding (MMC) is an easy and inexpensive tool to facilitate tissue engineering (5–7). It has been used to produce an extracellular micro-environment that boosts the potential of adult mesenchymal stem cells (8–13), such as enhancing adipogenic and osteogenic differentiation, and more effectively building constructs required for tissue engineering. Macromolecular crowding has further been used to improve (a) organotypic skin equivalents by promoting a functional dermal-epidermal junction in a condensed time frame (14), (b) the production of Bruch's membrane-like structures for culturing retinal pigment epithelial cells (15), and (c) bone and cartilage tissue engineering (16–18). Another interesting observation is, that the ECM produced by adult fibroblasts under MMC conditions is able to propagate human embryonic stem cells, even outperforming Matrigel (19). With respect to natural biomaterials, it was found that MMC not only enhances the polymerization rate of collagen type I, but also tunes fiber diameter and organization, a fact that can be explored for optimizing the properties of soft collagen hydrogels (20–23) or collagen films (planar constructs) (24, 25). Lastly, MMC has also been used for improving hydrogels derived from decellularized matrices (26).

A pathology with a serious burden to global health is fibrosis. At the molecular level fibrosis is, in essence, the accumulation of an excessive amount of ECM which mainly consists of collagen type I. It is stated that fibrotic phenomena play a major role in about 45% of all Western world death cases (27, 28). Despite decades of research, pharmacological treatments only modestly (if at all) attenuates the pathogenesis of fibrosis. Thus, there is a high need to develop anti-fibrotic drugs. Since the hallmark of fibrosis is the deposition of excessive amounts of collagen by activated fibroblasts (i.e., myofibroblasts) (29, 30), MMC might be a helpful tool for cellular drug screening, as it markedly shortens the culture time at which collagen deposition becomes visible. Indeed, MMC has been used for this purpose (5, 6, 31, 32), which is also known as the Scar-in-a-Jar system.

A variety of macromolecular crowders (MMCs) have been used to test the production of collagen by fibroblasts over time (3, 4, 6, 31–40). They are Ficoll PM 70 (Fc70), Ficoll PM 400 (Fc400), a mixture of Ficoll 70 and 400 (Fc70/400), polyvinylpyrrolidone 40 (PVP40), polyvinylpyrrolidone 360 (PVP360), neutral dextran 670 (ND670), dextran sulfate 10, dextran sulfate 500 (DxS500), carrageenan (CR), and polysodium-4-styrene sulfonate. Human fibroblasts used are WI-38 cells (embryonic lung fibroblasts) (3, 4, 6, 31, 38), WS-1 cells (embryonic dermal fibroblasts) (38, 39), adult dermal fibroblasts (37, 40), adult corneal fibroblasts (keratocytes) (34–36) and immortalized adult vocal fold fibroblasts (32). However, it should be stressed that it is the myofibroblast that is mainly responsible for the collagen deposition seen in fibrosis, not the fibroblast. As a consequence, testing of anti-fibrotic drugs with the Scar-in-a-Jar system should be done with myofibroblasts, or with fibroblasts that transform into myofibroblasts. The latter can be achieved by adding the strong profibrotic cytokine TGFβ1 into the culture medium (41, 42). The only reports that took this into consideration are those of Chen et al. (6, 31) and Graupp et al. (32), with DxS500 and Fc70/400 as MMCs. However, Chen et al. (6, 31) used embryonic lung fibroblasts; it is known that embryonic fibroblasts are phenotypically different from adult fibroblasts, as scarring does not occur in the embryo (43, 44). Also unfortunately, Graupp et al. (32) used immortalized adult vocal fibroblasts, not primary cells, and vocal fibroblasts are highly specialized cells. Thus, despite the advantages (short culture time in which collagen deposition as seen by optical analysis without the need for protein extraction) of the Scar-in-a-Jar principle, it seems that the system has never been tested or validated in a relevant setting.

We have compared the performance of six different MMCs (Fc70/400, PVP40, PVP360, ND670, DxS500, and CR) with primary human adult dermal fibroblasts under profibrotic conditions (presence of TGFβ1) in culture medium containing L-ascorbic acid 2-phosphate with 0.5 and 10% fetal bovine serum. These are the six MMCs that have been used in the past to accelerate collagen deposition (3, 4, 6, 31–40); the concentration used in our paper are the concentrations that have been recommended in the literature. We not only investigated collagen deposition, but also the morphology of the deposited collagen. We also tested the effect of the crowders on myofibroblast formation by means of alfa-smooth muscle actin staining. In addition, we investigated whether MMCs has an effect on the phenotypical properties of myofibroblasts. After all, if one wants to reliable test the effect of anti-fibrotic drugs, the phenotypical properties of the investigated cells should not be disturbed by the MMCs themselves. Finally, the performances of four anti-fibrotic drugs (Galunisertib, Omipalisib, Imatinib, and Nintedanib) were tested in the presence of PVP40.

Abbreviations: *ACTA2*, alfa-smooth muscle actin; BSA, bovine serum albumin; *COL*, collagen; *CTSK*, cathepsin K; DMEM, Dulbecco's modified Eagle medium; DxS500, dextran sulfate 500; ECM, extracellular matrix; FBS, fetal bovine serum; Fc70, Ficoll PM 70; Fc400, Ficoll PM 400; *FN1EDA*, fibronectin 1 containing extra domain A; Fc70/400, a mixture of Ficoll 70 and 400; MMC, macromolecular crowding; MMCs, macromolecular crowders; *MMP1*, matrix metalloproteinase 1; ND670, neutral dextran 670; *PLOD2*, lysyl hydroxylase 2; PVP40, polyvinylpyrrolidone 40; PVP360, polyvinylpyrrolidone 360; *SERPINH1*, heat-shock protein 47; α-SMA, α-smooth muscle actin; TGFβ1, transforming growth factor β1; *XBP1*, X-box binding protein 1; *YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta isoform.

MATERIALS AND METHODS

Reagents and Antibodies for Cell Culture and Immunofluorescence

Reagents and final concentrations used in culture medium were as follows: Human recombinant TGFβ1 (5 ng/ml; 100-21C, Peprotech, London, UK); ascorbic acid (0.17 mM; A8960, Sigma-Aldrich); penicillin/streptomycin (pen/strep) (50 U/L; 15140122, Thermo Fisher Scientific, Landsmeer, the Netherlands).

Macromolecular crowders were obtained from Sigma-Aldrich and used in the following concentrations in culture medium: Ficoll PM 70 (average molecular weight 70,000) (18.75 mg/ml; F2878; F2878; FVO 9%), Ficoll PM 400 (12.5 mg/ml; F4375; FVO 9%); Polyvinylpyrrolidone PVP-40 (average molecular weight 40,000) (21.5 mg/ml; PVP40; FVO 18%); Polyvinylpyrrolidone PVP-360 (average molecular weight 360,000) (11.34 mg/ml; PVP360; FVO 54%); Neutral Dextran 670 (analytical standard for GPC; average molecular weight 670,000) (100 μg/ml; 00896; FVO 5%); Dextran Sulfate 500 (sodium salt from *Leuconostoc* spp.; average molecular weight > 500,000) (100 μg/ml; D8906; FVO 5%); and Carrageenan CR (100 μg/ml; C1013; FVO 5%). The following drugs were used: Galunisertib (=LY2157299) (0.5, 1, 2.5 μM; 200-17, PeproTech); Omipalisib (=GSK2126458, GSK458, S2658) (0.01, 0.1, 1 μM; Selleck Chemicals); Imatinib (0.1, 0.5, 1 μM; I-5577, LC Laboratories, Woburn, MA); and Nintenadib (0.1, 0.5, 1 μM; Boehringer Ingelheim, Biberach, Germany). The used concentrations are in line with those of previous studies (45–49). Stock solutions of the drugs were prepared in dimethyl sulfoxide (DMSO; 100%) and stored at −20°C; the working solutions were diluted in culture medium with a final solvent concentration of ≤1%. The antibodies that were used are: mouse anti-human collagen type I (dilution 1:1,000; ab90395, Abcam, Cambridge, United Kingdom), mouse anti-human α-smooth muscle actin (α-SMA) (dilution 1:500; M0851, Dako, Glostrup, Denmark), and donkey anti-mouse IgG (H + L) Alexa Fluor 555 (dilution 1:1,000; A-31570, Thermo Fisher Scientific). The antibodies were diluted in PBS containing 2.2% bovine serum albumin (BSA) (K1106, Sanquin reagents, Amsterdam, the Netherlands).

Cell Culture

Before the onset of experiments, normal adult primary human dermal fibroblasts (CC-2511, Lonza, Basel, Switzerland) were propagated in Dulbecco's modified Eagle medium (DMEM) (12-604F, Lonza) containing 50 U/L pen/strep and 10% fetal bovine serum (FBS) (Sigma-Aldrich). Cells were negative for mycoplasm contamination. At the start of the experiment, cells were trypsinized, reseeded at a density of 10.000 cells/cm^2, and starved for 18 h in DMEM containing 50 U/L pen/strep, 0.5% FBS and 0.17 mM ascorbic acid. Experiments with the macromolecular crowders (Fc70/400, PVP40, PVP360, ND670, DxS500, or CR; see above for the used concentrations) were carried out in DMEM containing 50 U/L pen/strep, 0.17 mM ascorbic acid, 5 ng/ml TGFβ1 and 10 or 0.5% FBS. The prepared media was filtered before use through a SFCA 0.45 μm filter. For drug testing (Galunisertib, Omipalisib, Imatinib or Nintenadib; see above for the used concentrations), cells were cultured with the macromolecular crowder PVP40 dissolved in DMEM containing 50 U/L pen/strep, 0.17 mM ascorbic acid, 5 ng/ml TGFβ1 and 0.5% FBS in 24-well polystyrene plates (Costar) or 8-well Permanox chamber slides (ThermoFischer Scientific). Medium and compounds were refreshed every 24 h. It should be noted that cell density, MMC concentration and culture duration are comparable with the other studies in the field (3, 4, 6, 31–40).

Immunofluorescence

Detection of deposited (=extracellular) collagen type I was carried out on cells that were washed twice with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min. Detection of both intracellular and extracellular collagen type I was carried out on fixed cells that were permeabilized with 0.5% Triton X-100 in PBS for 10 min. For immunofluorescence of α-smooth muscle actin, cells were washed twice with PBS and fixed with ice-cold methanol/acetone (1:1) for 10 min. Methanol/acetone fixed cells were first dried and later rehydrated with PBS before use. For all immunostainings, fixed cells were subsequently incubated with PBS containing 2.2% BSA for 30 min at RT. The primary mouse anti-human collagen type I and mouse anti-human α-SMA antibodies were incubated for 1 h at RT. The secondary antibody donkey anti-Mouse IgG (H + L) Alexa Fluor 555 was incubated for 1 h at RT. Nuclei were visualized with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (1 μg/ml). All wash steps were performed in PBS. The cells were mounted with Citifluor AF1 (AF1-25, Brunschwig Chemie, Amsterdam, the Netherlands). Microphotographs were acquired in a random blind fashion with the use of a Leica DMRA microscope (Leica Microsystems, Rijswijk, the Netherlands) and a TissueFAXS microscopy system (TissueGnostics, Vienna, Austria).

Immunofluorescence Quantification by ImageJ-Fiji

For collagen type I, 36 images (20x magnification) were obtained per well from a 24-well plate (total imaged area = 13.33 mm^2). For α-SMA, 6 images (40x magnification) were acquired per well of an 8-well chamber slide (total imaged area = 1.72 mm^2). Triangle automatic thresholding was applied to discriminate between fore- and background and the resulting mean fluorescence intensity of collagen and α-SMA present in each well was used for statistical analysis.

RNA Extraction and Real-Time PCR

For gene expression analysis, total RNA was isolated with the Tissue Total RNA mini kit (Favorgen Biotech Corp., Taiwan). RNA quantity and quality were determined by UV spectrophotometry (NanoDrop Technologies, Wilmington, DE). One microgram of RNA was reverse transcribed with RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). Real-time PCR was performed with SYBR green PCR master mix (Roche, Basel, Switzerland) and VIIA7 thermal cycling system (Applied Biosystems, Carlsbad, CA). Thermal cycling conditions were 2 min at 95°C (enzyme activation), followed by 15 s at 95°C, 30 s at 60°C and 30° s at 72°C (40 cycles). Melting curve analysis was performed in order to verify the absence of primer dimers. Primers were designed and optimized to have calculated 95–105% reaction efficiency; the used sequences are shown in **Table 1**. All data were normalized against the reference gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta isoform (*YWHAZ*) (50). The following genes were measured: the extracellular matrix proteins collagen type I, III, V and fibronectin (*COL1A1, COL3A1, COL5A,* and *FN1EDA*, respectively), the collagen chaperone heat-shock

TABLE 1 | Primer sequences used for q-PCR.

Gene	Forward sequence 5′3′	Reverse sequence 5′3′
COL1A1	GCCTCAAGGTATTGCTGGAC	ACCTTGTTTGCCAGGTTCAC
COL3A1	AGGGTGCAATCGGCAGTCCA	CAATGGCAGCGGCTCCAACA
COL5A1	CCTGGATGAGGAGGTGTTTG	CGGTGGTCCGAGACAAAG
FN1EDA	AATCCAAGCGGAGAGAGTCA	GGAATCGACATCCACATCAG
SERPINH1	GCGGGCTAAGAGTAGAATCG	ATGGCCAGGAAGTGGTTTG
PLOD2	GGGAGTTCATTGCACCAGTT	GAGGACGAAGAGAACGC
MMP1	GCTAACCTTTGATGCTATAACT ACGA	TTTGTGCGCATGTAGAATCTG
CSTK	GCCAGACAACAGATTTCCATC	CAGAGCAAAGCTCACCACAG
ACTA2	CTGTTCCAGCCATCCTTCAT	TCATGATGCTGTTGTAGGTGGT
XBP1	GTGAGCTGGAGCAACAAGT	AGGCCATGAGTTTTCTCTCG
YWHAZ	GATCCCCAATGCTTCACAAG	TGCTTGTTGTGACTGATCGAC

protein 47 (*SERPINH1*), the collagen modifying enzyme lysyl hydroxylase 2 (*PLOD2*), the collagen degradation enzymes matrix metalloproteinase 1 and cathepsin K (*MMP1* and *CTSK*), the cytoskeletal protein alfa-smooth muscle actin (*ACTA2*) and the endoplasmic reticulum stress response protein X-box binding protein 1 (*XBP1*).

Statistical Analysis

All data are represented as standard error of the mean (SEM) of at least three independent experiments and were analyzed with GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA) by unpaired *t* test with Welch's correction. Values of $p < 0.05$ (95% confidence interval) were considered to be statistically significant: ns $= P > 0.05$; * $= P \leq 0.05$; ** $= P \leq 0.01$; *** $= P \leq 0.001$; **** $= P \leq 0.0001$.

RESULTS

It is well-known that TGFβ1 is a strong pro-fibrotic cytokine. To simulate fibrotic conditions, we added TGFβ1 to the primary adult human dermal fibroblasts, and checked whether the cells were responsive, both at day 2 and day 4. Indeed, as expected, a significant increase is seen in mRNA levels of genes encoding *COL1A1, COL5A1*, and *FN1EDA* (extracellular matrix proteins), *SERPINH1* and *PLOD2* (collagen-processing proteins), *ACTA2* (a marker for myofibroblasts) and *XBP1* (involved in the endoplasmic reticulum stress response), whereas a significant decrease was seen in mRNA levels of genes encoding for *MMP1* and *CTSK* (enzymes that are able to degrade collagen). We thus confirmed the fibrotic state of the cells (**Figures 1A,B**).

To test the validity of the Scar-in-a-Jar approach, we next investigated the effect of six macromolecular crowders (Fc70/400, PVP40, PVP360, ND670, DxS500, and CR) on collagen type I deposition under the same profibrotic conditions at day 4. We first tested the effect of the presence of 0.5 and 10% FBS in the culture medium, as there are contradictory data in the literature with respect to the effect of serum concentration on collagen deposition [34-36 + 39 *vs.* 38]. At 0.5% FBS, collagen deposition was seen at day 4 in all conditions (**Figure 2**). With the exception

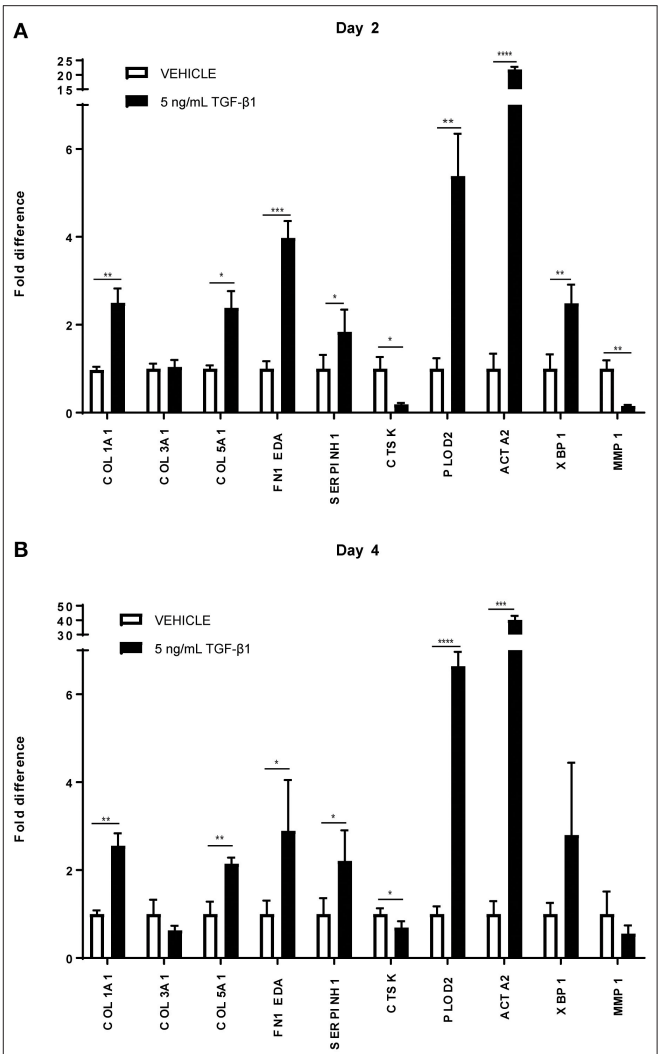

FIGURE 1 | Relative mRNA expression of fibrosis markers in human dermal fibroblasts ($n = 4$) under TGFβ1 stimulation. mRNA expression of *COL1A1, COL3A1, COL5A1, FN1 EDA, SERPINH, PLOD2, ACTA2, XBP1, MMP1,* and *CTSK* in human dermal fibroblasts with 5 ng/mL TGFβ1 stimulation and without (=vehicle). **(A)** After 48 in culture. **(B)** After 96 h in culture. * $= P \leq 0.05$; ** $= P \leq 0.01$; *** $= P \leq 0.001$; **** $= P \leq 0.0001$.

of ND670, all MMCs showed an increased collagen deposition in comparison with the control (=no added crowder). A different situation was observed with 10% FBS (**Figure 2**). Under this serum concentration, hardly any collagen deposition was seen in the control and with Fc70/400 and ND670. The crowders PVP40 and PVP360 showed a staining comparable with 0.5% FBS, whereas DxS500 and CR appeared to demonstrate increased staining in comparison with 0.5% FBS.

We also studied the transformation of fibroblasts into myofibroblasts at day 4 in the presence of 0.5 and 10% FBS under profibrotic conditions. In general, a more intense staining of α-SMA seemed to be seen with 0.5% FBS, indicating that myofibroblast formation proceeded more slowly in 10% FBS (**Figure 3**). Based on collagen deposition and α-SMA data

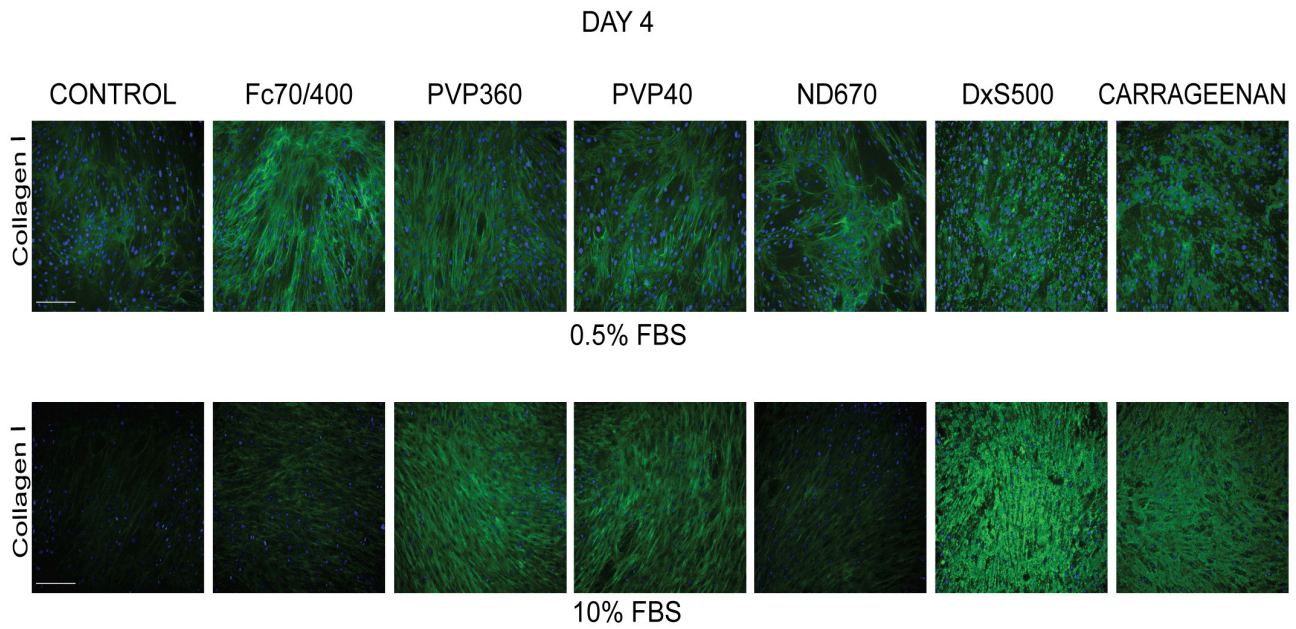

FIGURE 2 | Effect of serum concentration on collagen I deposition. Immunofluorescence staining of collagen I in human dermal fibroblasts ($n = 5$) after 96 h in the presence of TGFβ1 in DMEM containing 0.5 and 10% of FBS and macromolecular crowders Fc70/400 mixture, PVP360, PVP40, ND670, DxS500, and CR. Showing 1 FVO; DAPI, Collagen I, magnification 10x, scale bars: 200 μm.

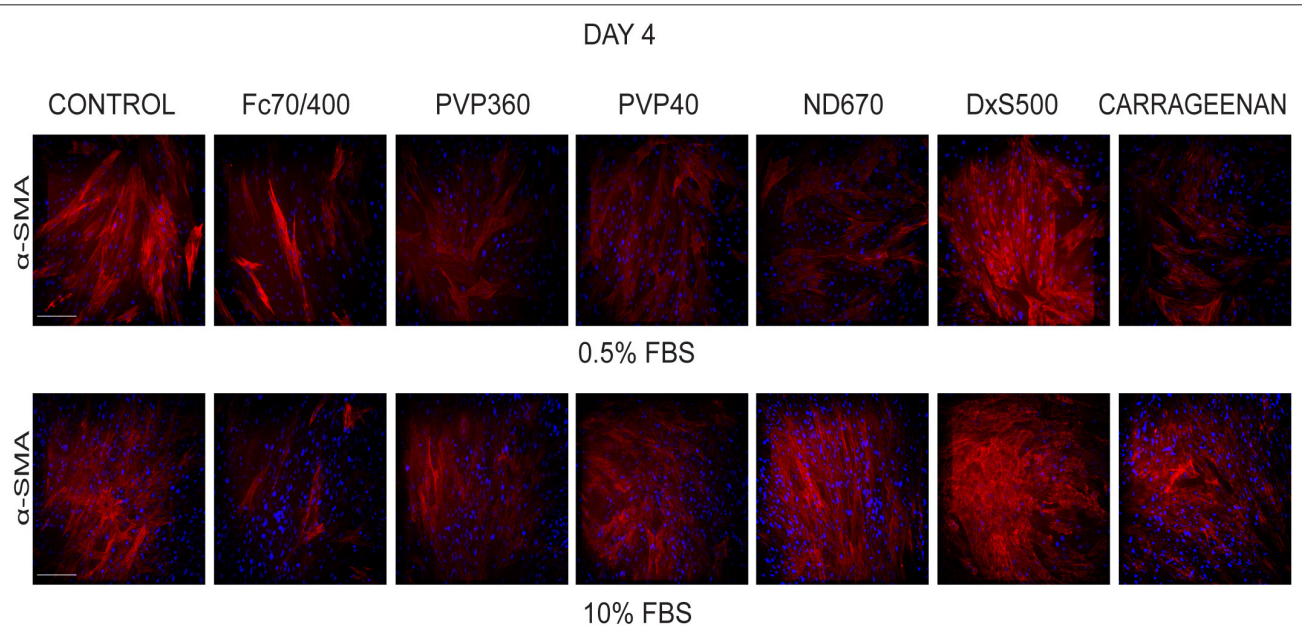

FIGURE 3 | Effect of serum concentration on myofibroblast formation. Immunofluorescence staining of α-smooth muscle actin in human dermal fibroblasts ($n = 2$) after 96 h in the presence of TGFβ1 in DMEM containing 0.5 and 10% of FBS and macromolecular crowders Fc70/400 mixture, PVP360, PVP40, ND670, DxS500, and CR. Showing 1 FVO; DAPI, α-SMA; magnification 10x, scale bars 200 μm.

we therefore decided to carry out subsequent experiments in 0.5% FBS.

Not only the amount of collagen that is deposited matters, but also the pattern of deposition. Increased magnification revealed, that with Fc70/400, PVP40, PVP360, and ND670 a reticular collagen network was formed, comparable with that of the control (**Figure 4**). In contrast, DxS500 and CR showed a highly granulated deposition. In addition, the myofibroblasts

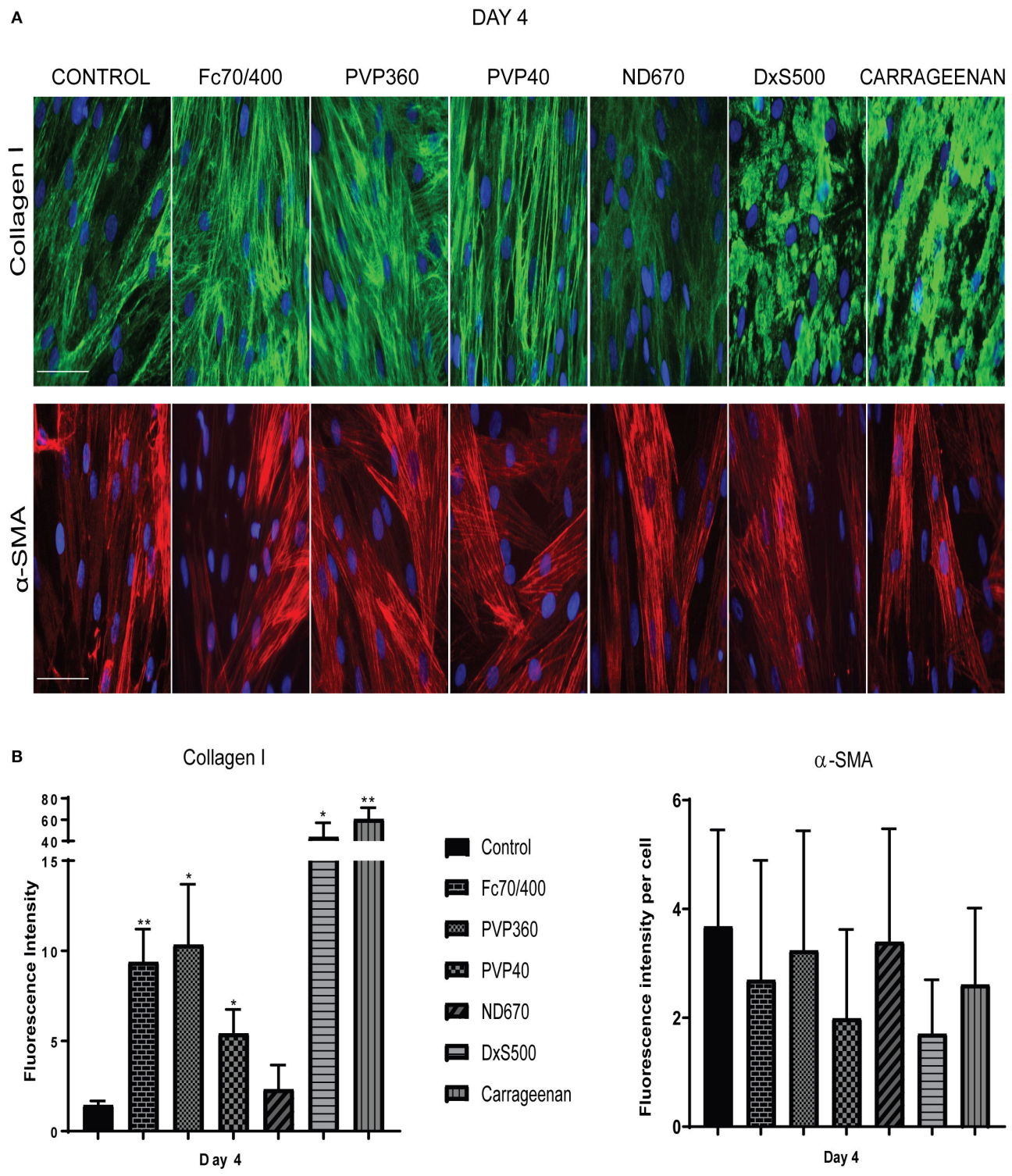

FIGURE 4 | Patterns of collagen I deposition and α-smooth muscle actin cytoskeleton at high magnification as well as its quantification. **(A)** Immunofluorescence staining of collagen I and α-smooth muscle actin in human dermal fibroblasts ($n = 3$) after 96 h in the presence of TGFβ1, cultured with macromolecular crowders Fc70/400 mixture, PVP360, PVP40, ND670, DxS500, and CR. Showing 1 FVO; DAPI, collagen I, α-SMA; magnification 40x, scale bars 100 μm **(B)** Quantification of collagen I deposition ($n = 5$) and α- smooth muscle actin ($n = 3$) vs. control. * = $P \leq 0.05$; ** = $P \leq 0.01$.

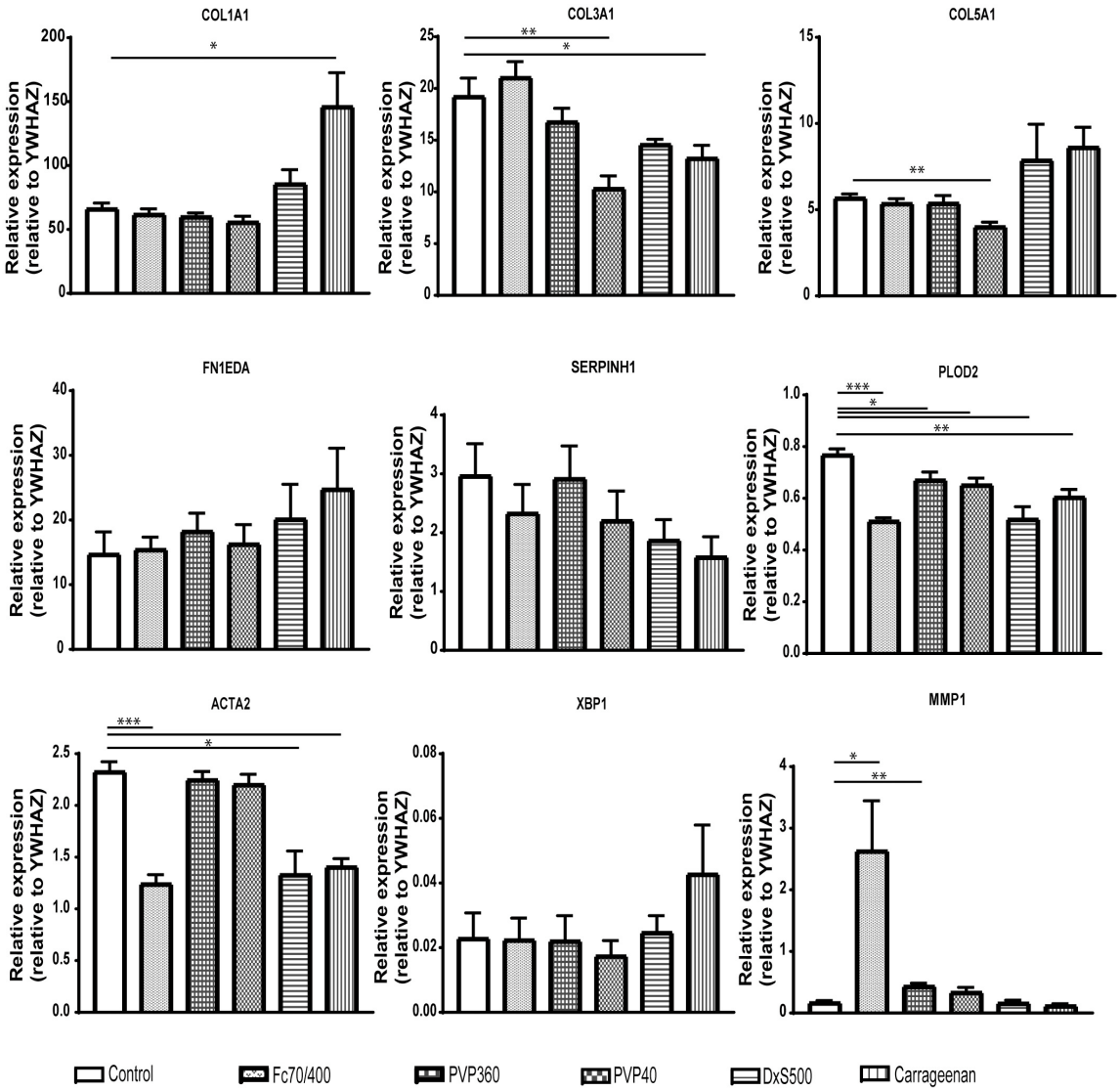

FIGURE 5 | Effects of macromolecular crowders on fibrotic markers in dermal myofibroblasts. mRNA expression of *COL1A1, COL3A1, COL5A1, FN1EDA, SERPINH1, PLOD2, ACTA2, XBP1,* and *MMP1* in human dermal myofibroblasts ($n = 4$) after 96 h in the presence of TGFβ1, cultured with macromolecular crowders Fc70/400 mixture, PVP360, PVP40, DxS500, and CR vs. control. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

cultured under DxS500 and CR showed a less prominent α-SMA cytoskeleton (thinner stress fibers; **Figure 4**). Both observations point to the direction that DxS500 and CR interferes with normal fibrotic processes, and thus seems to be less favorable as a MMC in the Scar-in-a-Jar approach for testing anti-fibrotic drugs.

To strengthen this conclusion, we also measured the gene expression of *COL1A1, COL3A1, COL5A1, FN1EDA, SERPINH1, PLOD2, ACTA2, XBP1,* and *MMP1* (**Figure 5**). mRNA levels of *ACTA2* and *PLOD2* were significantly decreased in the cells cultured with DxS500 and CR, indicating important phenotypical changes of the cells. The same was the case with Fc70/400, whereas a significant change was observed with PVP40 and PVP360 of *PLOD2* only. An increase of *COL1A1* expression was seen with CR, whereas the other MMCs did not affect *COL1A1* expression. A significant decrease in mRNA levels of *COL3A1* and *COL5A1* is seen in the presence of PVP40 and CR, whereas a significant increase of MMP1 is observed with Fc70/400 and PVP360. No significant differences in *XBP1* levels were observed between the MMCs and the control, indicating a comparable endoplasmic reticulum stress response. The mRNA data reveal that DxS500 and CR indeed have an effect on the fibrotic properties of the cells, and also discloses that Fc70/400 is a less favorable MMC in the Scar-in-a-Jar approach.

Since some of the MMCs affect the phenotypical properties of fibroblasts under fibrotic conditions, we wondered whether MMCs have an effect on the phenotypical properties of non-stimulated fibroblasts. Interestingly, ***all*** MMCs significantly

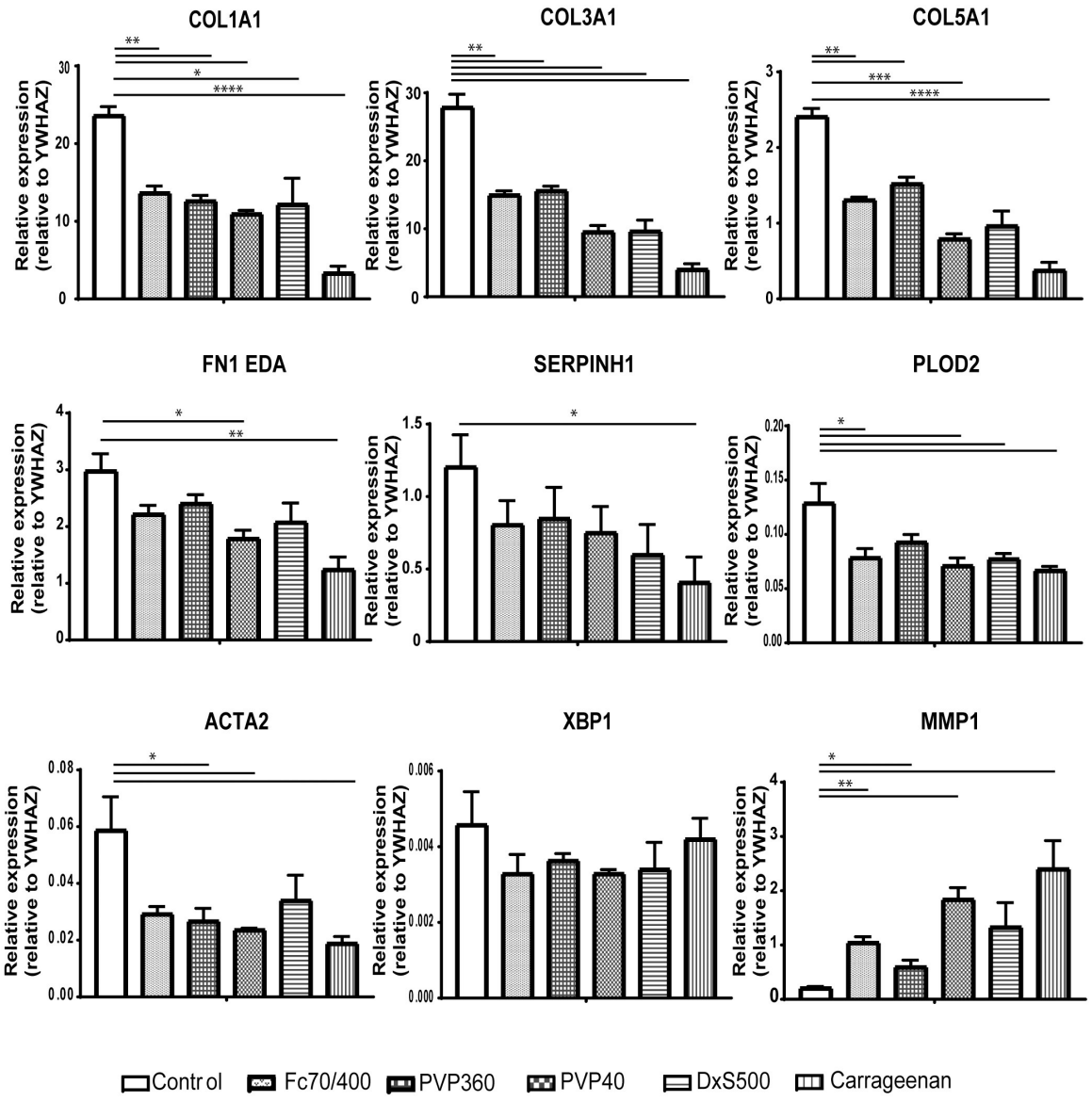

FIGURE 6 | Relative mRNA expression of fibrosis markers in dermal fibroblasts ($n = 4$) after 48 h of culture. mRNA expression of *COL1A1, COL3A1, COL5A1, FN1EDA, SERPINH1, PLOD2, ACTA2, XBP1,* and *MMP1* in human dermal fibroblasts after 48 h without stimulation with TGFβ1, cultured with macromolecular crowders Fc70/400 mixture, PVP360, PVP40, DxS500, and CR vs. control. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$.

decreased mRNA levels of *COL1A1, COL3A1, COL5A1*, and significantly increased mRNA levels of *MMP1*, both at day 2 and day 4 (**Figures 6**, **7**). This underlines the importance of the addition of TGFβ1 into the medium to mimic pro-fibrotic conditions for the screening of anti-fibrotic drugs in the presence of MMCs.

Since under pro-fibrotic conditions ND670 does not result in an increased collagen deposition, and DXS500 and CR results in an aberrant collagen deposition and important phenotypical changes, and also Fc70/400 decreases mRNA levels of *ACTA2* and *PLOD2*, it seems that PVP40 and PVP360 are the best choice for a MMC to screen anti-fibrotic drug properties. As the preparation of culture medium with PVP360 is somewhat troublesome because of an increase in viscosity, we used PVP40 in the subsequent experiment.

We visualized (**Figure 8**) collagen type I deposition and myofibroblast formation (positive staining for α-SMA) in the presence of various concentrations of Imatinib, Galunisertib, Omipalisib or Nintedanib with primary adult human dermal fibroblasts cultured for 4 days in DMEM containing 0.5% FBS, 50 U/L pen/strep, 0.17 mM ascorbic acid, 5 ng/ml TGFβ1 and 21.5 mg/ml PVP40. No decrease in collagen deposition or myofibroblast formation was seen with Imatinib, whereas a dose-dependent decrease in collagen deposition and myofibroblast formation is seen with Galunisertib and Omipalisib. On the

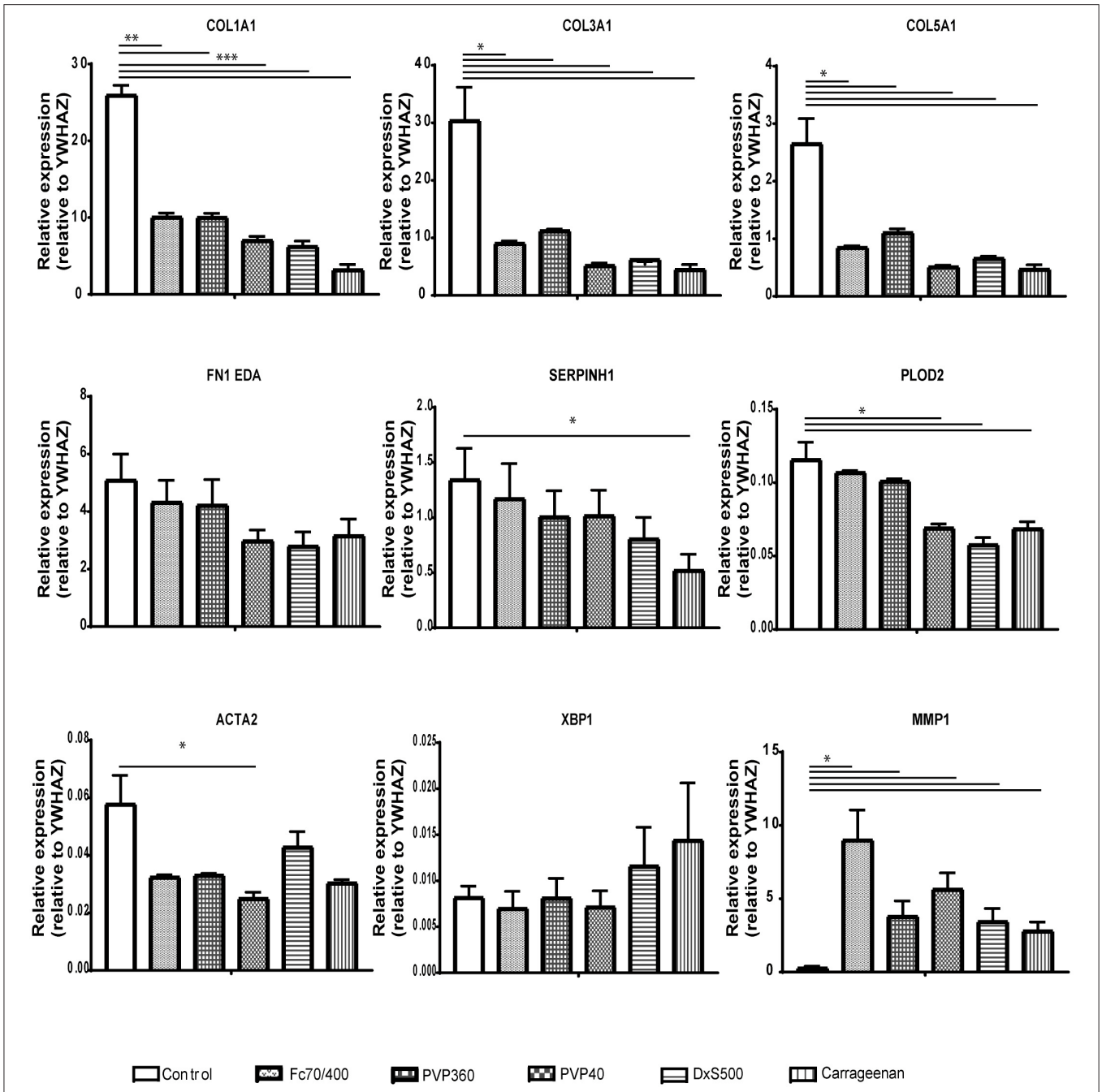

FIGURE 7 | Relative mRNA expression of fibrosis markers in dermal fibroblasts ($n = 4$) after 96 h of culture. mRNA expression of *COL1A1, COL3A1, COL5A1, FN1EDA, SERPINH1, PLOD2, ACTA2, XBP1,* and *MMP1* in human dermal fibroblasts after 96 h without stimulation with TGFβ1, cultured with macromolecular crowders Fc70/400 mixture, PVP360, PVP40, DxS500, and CR vs. control. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

other hand, Nintedanib showed a dose-dependent decrease of collagen deposition, but myofibroblast formation was unaffected. To verify whether the decrease in collagen deposition is due to a decreased synthesis of collagen, we fixed the cells with 0.5% Triton X-100. With this method mainly intracellular collagen can be visualized. Intracellular staining for collagen type I showed a marked decrease of collagen synthesis in the endoplasmic reticulum for Galunisertib, Omipalisib, and Nintedanib (**Figure 9**).

DISCUSSION

The addition of macromolecular crowders to cell cultures greatly facilitates the deposition of extracellular matrix molecules, in

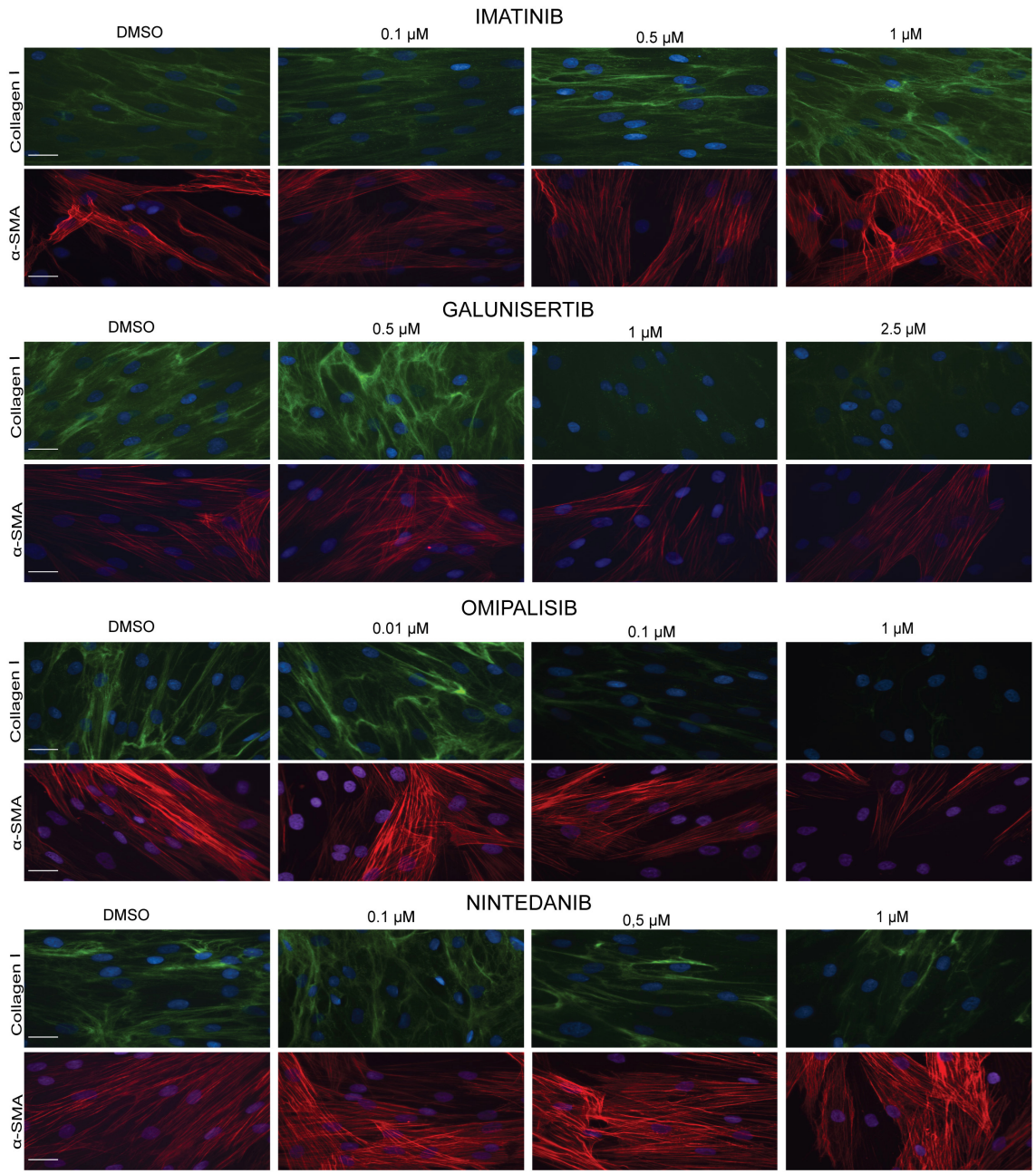

FIGURE 8 | Effect of antifibrotic compounds in dermal myofibroblasts ($n = 2$). Immunofluorescence staining of collagen I and α- smooth muscle actin in human dermal fibroblasts after 96 h in the presence of TGFβ1 cultured with macromolecular crowder PVP40 and treated with anti-fibrotic compounds Imitanib, Galunisertib, Omipalisib, and Nintedanib. Showing 1 FVO; DAPI, collagen I, α-SMA; magnification 40x; scale bars 200 μm.

particular collagens. It enhances the cleavage of the collagen propeptides, which is normally a limiting factor in collagen fibrillogenesis, resulting in a shorter culture time at which collagen deposition can be seen by optical analysis. It has been used in tissue engineering to enhance tissue production or to enhance the performance of differentiated cells and/or stem cells. It also provides the opportunity to screen in an easy way drugs for their ability to attenuate collagen deposition. This is especially of interest in the field of fibrosis, a pathology that is characterized by an excessive deposition of collagen type I; it is known as the Scar-in-a-Jar approach. When implementing this approach into our laboratory for drug screening, we observed a major hiatus in the validation of this system in the literature.

The majority of macromolecular crowding studies have been carried out with fibroblasts under non-fibrotic conditions. However, the deposition of collagen in fibrosis is mainly done

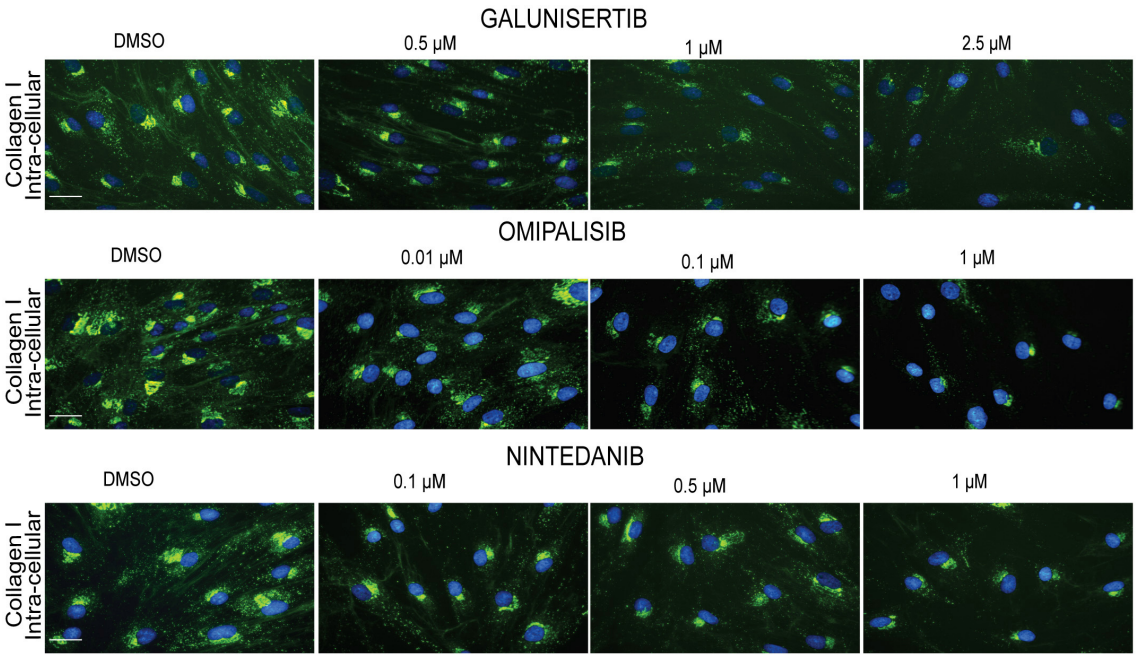

FIGURE 9 | Effect of anti-fibrotic compounds on collagen I synthesis in dermal myofibroblasts. Immunofluorescence staining of intra-extracellular collagen I in human dermal fibroblasts after 48 h in the presence of TGFβ1 cultured with macromolecular crowder PVP40 and treated with anti-fibrotic compounds Galunisertib, Omipalisib and Nintedanib. Showing 1 FVO; DAPI, collagen 1, magnification 40x, scale bars 200 μm.

by myofibroblasts, i.e., fibroblasts that are activated by pro-fibrotic cytokines such as TGFβ1. But studies that took this into consideration used embryonic fibroblasts, although it is known that wounded embryos heal without scarring. Furthermore, it has not been tested whether cells become phenotypically different in the presence of MMCs, as the focus was on extracellular matrix deposition. For our validation studies we therefore used adult human dermal primary fibroblasts cultured in the presence of TGFβ1, tested the effect of serum concentration in the medium on collagen deposition and myofibroblast formation, tested the effect of the different MMCs on the phenotypical properties of stimulated and unstimulated fibroblasts, and finally tested the system with the most favorable MMC with four anti-fibrotic drugs.

We first tested the effect of 0.5 and 10% FBS on collagen deposition and myofibroblast formation in the presence of TGFβ1. We found significant collagen deposition with 0.5% FBS with all six MMCs, whereas with 10% FBS hardly any collagen deposition was found with Fc70/400 and ND670. Furthermore, we observed a delayed myofibroblast formation in 10% FBS. It has been reported that the presence of 0.5 or 10% bovine serum has no significant effect on the amount of deposited collagen in the presence of DxS500, Fc70/400 or CR when cultured for 2–4 days under 20% oxygen tension under non-fibrotic conditions (34–36, 39), whereas another report found increased levels of collagen at 0.5% FBS with CR (38). The latter was explained by an enhanced degradation of collagen due to the presence of matrix metalloproteinases in the used serum (only CR was tested) (38). We found that collagen deposition in 10% serum is a MMC-specific phenomenon (present with PVP40, PVP360, DxS500, CR, absent with Fc70/400 and ND670); therefore, the suggested presence of matrix metalloproteinases cannot be held responsible for the presence or absence of collagen. Myofibroblast formation was not studied previously. Our data on collagen deposition and myofibroblast formation show that 0.5% FBS has a superior performance compared to 10% FBS. However, the collagen deposited by DxS500 and CR showed an aberrant pattern, whereas a normal pattern was seen with Fc40/700, PVP40, PVP360, and ND670, but the latter did not show an enhanced deposition compared to the control.

Drug screening provides the most reliable data when carried out in an environment in which the cells exhibit their normal phenotype. We found that all MMCs (we excluded ND670 because of the low collagen deposition) have serious effects on the phenotypic properties of non-stimulated fibroblasts, whereas this is much less obvious (but still present in some MMCs) when the cells are cultured under pro-fibrotic conditions. CR, DxS500, and Fc40/700 affected several major phenotypical properties of the cells when cultured under pro-fibrotic conditions, whereas PVP40 and PVP360 showed fewer perturbations. Changes in phenotypical properties by MMCs (DxS500, Fc40/700) have previously been reported for corneal fibroblasts and mesenchymal stem cells derived from bone marrow and fat tissue (10, 11, 34), but it was so far not studied in pro-fibrotic conditions. Clearly, PVP40 or PVP360 should be used as MMC in the context of drug screening.

Trichostatin, Ciclopiroxolamine and PcP56 have been tested previously as drugs with embryonic lung fibroblasts under

pro-fibrotic conditions in the Scar-in-a-Jar system (6, 31), and hepatocyte growth factor and Botox type A with adult immortalized focal fold fibroblasts (32). We have here tested Imatinib, Galunisertib, Omipalisib and Nintedanib, with PVP40 as the molecular crowder and primary adult human dermal fibroblasts as cell type. Imatinib inhibits c-abl kinase and blocks the PDGF receptor (51). Although it attenuates fibrosis in various animal models, we did not see an effect on collagen deposition by dermal fibroblasts. This is in line with previous data obtained with cardiac fibroblasts (52). Galunisertib, by being a selective inhibitor of TGFβR1, inhibits phosphorylation and activation of SMAD2/3 (53). Although it is under investigation for the treatment of different cancers, it was shown recently that it has also antifibrotic properties (47, 54). Indeed, collagen deposition was not observed in our test system. The latter was also observed with Omipalisib (GSK2126458), a potent inhibitor of phosphatidylinositol 3-kinases (PI3Ks) and mechanistic target of rapamycin (mTOR) which was also developed in the oncology setting, and is recently being tested in fibrosis (55). Both Galunisertib and Omipalisib also inhibited myofibroblast formation. Finally, Nintedanib showed reduced collagen deposits whereas myofibroblast formation was unaffected. Both observations are in line with published records (56, 57).

In conclusion, we found that the Scar-in-a-Jar approach should best be carried out under profibrotic conditions (the presence of TGFβ1) in a culture medium with 0.5% FBS and with PVP40 (or PVP360) as the macromolecular crowder. The data obtained with Imatinib, Galunisertib, Omipalisib, and Nintedanib are comparable with previously published data that were obtained without macromolecular crowder. It shows that the Scar-in-a-Jar approach as described in this paper is a reliable test system to screen antifibrotic drug properties.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NP, PO, and RB conceived the presented idea. NP, MB, and RB conceived the experimental setup. NP performed all experiments and analyzed all data. MB and EB contributed with discussion and data analysis tools. NP and RB drafted and wrote the manuscript. PO and RB revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Activated Hepatic Stellate Cells Induce Infiltration and Formation of CD163$^+$ Macrophages *via* CCL2/CCR2 Pathway

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Background: Activated hepatic stellate cells (aHSCs) regulate the function of immune cells during liver fibrosis. As major innate cells in the liver, macrophages have inducible plasticity. Nevertheless, the mechanisms through which aHSCs regulate macrophages' phenotype and function during liver fibrosis and cirrhosis remain unclear. In this study, we examined the immunoregulatory function of aHSCs during liver fibrosis and explored their role in regulating macrophage phenotype and function.

Methods: A total of 96 patients with different stages of chronic hepatitis B-related liver fibrosis were recruited in the study. Metavir score system was used to evaluate the degree of fibrosis. The expression of hepatic CCL2 and M2 phenotype macrophage marker CD163 were detected by immunohistochemistry, and the relationship among hepatic CD163, CCL2, and fibrosis scores were also explored. In the *in vitro* model, the aHSCs isolated from human liver tissues and THP-1-derived M0-type macrophages (M0MΦ) were co-cultured to observe whether and how aHSCs regulate the phenotype and function of macrophages. To explore whether CCL2/CCR2 axis has a crucial role in macrophage phenotypic changes during liver fibrosis, we treated the M0MΦ with recombinant human CCL2 or its specific receptor antagonist INCB-3284. Furthermore, we used LX2 and TGF-β-activated LX2 to mimic the different activation statuses of aHSCs to further confirm our results.

Results: In patients, the infiltration of M2 macrophages increased during the progression of liver fibrosis. Intriguingly, as a key molecule for aHSC chemotactic macrophage aggregation, CCL2 markedly up-regulated the expression of CD163 and CD206 on the macrophages, which was further confirmed by adding the CCR2 antagonist (INCB 3284) into the cell culture system. In addition, the TGF-β stimulated LX2 further confirmed that aHSCs up-regulate the expression of CD163 and CD206 on macrophages. LX2 stimulated with TGF-β could produce more CCL2 and up-regulate other M2 phenotype macrophage-specific markers, including IL-10, ARG-1, and CCR2 besides CD163 and CD206 at the gene level, indicating that the different activation status of aHSCs might affect the final phenotype and function of macrophages.

Conclusions: The expression of the M2 macrophage marker increases during liver fibrosis progression and is associated with fibrosis severity. AHSCs can recruit macrophages through the CCL2/CCR2 pathway and induce M2 phenotypic transformation.

Keywords: activated hepatic stellate cells, liver fibrosis, M2 macrophage, CCL2, hepatitis B

INTRODUCTION

Liver cirrhosis is the eleventh most common cause of mortality worldwide, causing more than 1.16 million deaths annually (1, 2). The essence of fibrosis is the wound-healing response to chronic inflammation. When this process is dysregulated, excessive scarring occurs in response to persistent injury, leading to altered tissue functions (3, 4). The immune system has a dual role in liver fibrosis pathological process by mediating the immune-inflammatory reactions and causing liver damage (4). Impaired liver immune system surveillance, which is also the main pathological feature of cirrhosis, occurs primarily due to incomplete and inappropriate activation of immune cells or impaired response of the immune system to pathogens (5–7). Some studies have shown that immune cell, including monocytes/macrophages, NK cells, and lymphocytes, show impaired functions during cirrhosis (6, 8).

Macrophages featured for strong plasticity can differentiate into pro-fibrotic and anti-fibrotic macrophages responding to different conditions (9–11). These cells exert opposite functions by producing pro-inflammation or pro-fibrotic factors in different circumstances (11–14). Typically, the classically activated macrophages (M1) secrete the pro-inflammatory cytokines that exert anti-infective effects; alternatively activated macrophages (M2) mainly regulate the inflammatory response and tissue repair (12, 15). However, persistent inflammatory response in patients with chronic hepatitis suggests that liver immune cells' tolerance might impede effective immune surveillance in the liver (6, 7). Reportedly, peripheral blood monocytes/macrophages are mainly M2 phenotypes in the late stages of hepatitis B-related cirrhosis with the decreased anti-infectivity, thereby increasing the possibility of bacterial infection (16). In clinic, high M2-specific CD163 levels indicate poor prognosis; these levels are correlated with increased tumor nodules and venous infiltration in HCC patients (17). However, the mechanisms related to M2 macrophage formation during chronic liver diseases, especially liver fibrosis/cirrhosis, are not entirely clear.

In chronic liver injury, hepatic stellate cells resting in the sinus (the Disse) lumen receive signals from damaged hepatocytes, macrophages, and other immune cells and rapidly activate to myofibroblast-like cells, also known as activated hepatic stellate cells (aHSCs) (18–21). These cells display fibrogenic properties by producing excessive extracellular matrix and collagen (22). Recently, the unexpected immunoregulatory roles of aHSCs in liver fibrosis received increasing interest (8, 21). Our previous study demonstrated that aHSCs secrete many immunomodulatory cytokines and chemokines to regulate the phenotype and function of monocytes, NK cells, and T cells (23–25). It is important to continue exploring how aHSCs regulate macrophage's phenotype and function in the long process of liver fibrosis.

CCL2, also known as monocyte chemotactic factor 1 (MCP1), regulates the migration and infiltration of monocytes/macrophages through combination with its specific receptor CCR2 (26, 27). It has been reported that CCL2/CCR2 axis has a vital role during fibrosis (28–31). CCL2-dependent infiltrating macrophages promote angiogenesis in progressive liver fibrosis. CCR2 is mainly responsible for recruiting pro-inflammatory and profibrogenic infiltrating monocytes during fibrosis progression (29). Using liquid chip screening, we previously found that CCL2 may profoundly enrich in the supernatant of aHSCs (23). Yet, so far, only a few studies have explored whether CCL2 independently affects the phenotype and function of monocytes/macrophages in the process of liver fibrosis. In the present study, we found that aHSCs aggregate the macrophages through CCL2/CCR2 pathway and induce M2 phenotypic transformation during liver fibrosis.

MATERIALS AND METHODS

Patients and Specimens

Liver tissues from different fibrosis stages were obtained from 96 patients who underwent curative liver resection of hepatocellular carcinoma at the Third Affiliated Hospital of Sun Yat-sen University in south China from 2014 to 2018. Patients with hepatitis C virus, HIV, Wilson's disease, autoimmune liver disease, genetic metabolic liver disease, and other undefined pathogenesis liver diseases were excluded from the study. The fibrotic tissues were excised at least 3 cm away from the tumor's edge, as previously described (15, 23, 32). Fresh fibrotic liver tissues from five patients were selected for the acquisition of primary aHSCs.

The experimental protocol was in accordance with the Helsinki Declaration and the local ethical guidelines. The study was approved by the Institutional Review Board of the Third Affiliated Hospital of Sun Yat-sen University.

Immunohistochemistry and Immunofluorescence Staining

The different stages of liver fibrosis were evaluated according to the Metavir score system based on the hematoxylin-eosin staining. Paraffin-embedded and formalin-fixed samples were processed for IHC. The slices were probed with primary antibody targeted against human CD68, CD163 (Zsbio, China), and

CCL2 (Sigma Aldrich, St. Louis, MO, USA), and stained with either diaminobenzidine or 3-amino-9-ethylcarbazole using the Envision System (Dako Cytomation, Glostrup, Denmark). Five representative fields were selected at 200× magnification using microscopy (Leica, Mannheim, Germany). The M2 phenotype macrophages positive with CD163 staining were calculated per field. The CCL2 protein expression was quantified based on the evaluation of staining using semi-quantitative Histoscore (H-score), which was calculated by an assessment of both the intensity of staining (graded as 0, non-staining; 1, weak; 2, median; or 3, strong) and the percentage of positive cells. The range of possible scores was from 0 to 300. The expression level of each component was categorized as low or high according to the H-score's median value. Two independent pathologists blinded to the clinical outcomes performed this analysis.

For immunofluorescence analysis, aHSCs were stained using rabbit anti-human alpha-smooth muscle actin (α-SMA, Abcam, Cambridge, MA, USA) and mouse anti-human CCL2 protein (Sigma Aldrich, St. Louis, MO, USA), followed by Alexa Fluor 555–conjugated donkey anti-mouse IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Grand Island, NY, USA). Macrophages were stained using polyclonal mouse anti-human CD163 (DakoCytomation, Glostrup, Denmark). Positive cells were detected using immunofluorescence confocal microscopy (Leica, Germany).

In vitro Co-culture System

The aHSCs were isolated as previously described (32). To minimize the clonal selection and stress during extended culture, the cells were passaged 3–8 times and then used for subsequent experiments. The LX2 cells (hepatic stellate cell line) were obtained from the ATCC cell bank. THP-1 cells (kindly provided by Dr. Songguo Zheng), a human leukemia monocytic cell line, can be differentiated into macrophages under stimulation of 100 ng/ml 12-Otetradecanoylphorbol-l3-acetate (PMA) for 48 h (33, 34). The THP1-derived M0MΦ were cultured in RPMI 1640 containing 10% FBS in 6-well flat-bottomed plates (1×10^6 cells/well) for at least 1 h before co-culture with aHSCs (including primary aHSCs or TGF-β activated LX2 at a ratio of 5:1 or 1:1).

Some reports indicated that TGF-activated LX2 tends to be in a more active status when compared with non-activated LX2. Therefore, we used LX2 and TGF-β activated LX2 as various activation status of aHSCs.

For the supernatant treatment group, we used 50% conditioned supernatant and 50% fresh complete medium to treat the macrophages for 72 h before analysis. When indicated, recombinant human CCL2 protein (rh CCL2, 2ng/ml, R&D Systems, Abingdon, UK) and INCB 3284 (100ng/ml, Tocris Bioscience, UK) were accordingly added, after which the macrophages were harvested, counted, and analyzed.

Supernatants Preparation and Testing

A total of 5×10^5 aHSCs or 1×10^6 LX2 were seeded per well into 6-well plates containing 2 ml of DMEM with 10% Fetal Bovine Serum (FBS) containing 100 ng/ml benzylpenicillin and 100 ng/ml streptomycin (all purchased from Sigma Aldrich, St. Louis, MO, USA). Once cells reached 90% confluency, the supernatants were harvested, centrifuged, and stored in aliquots at −80°C. The activated LX2 was prepared by stimulation with TGF-β (2 ng/ml, R&D Systems, Abingdon, UK) for no <24 h and then replaced with fresh medium without TGF-β for another 24 h before harvesting the supernatant. The levels of CCL2 in the conditioned supernatants were evaluated using CCL2 enzyme-linked immunosorbent assays (ELISA, R&D Systems, Abingdon, UK) according to the instructions. Next, the macrophages were treated with 50% conditioned supernatant and 50% fresh complete medium for 72 h before analysis.

Flow Cytometry and Real-Time Quantitative Polymerase Chain Reaction

Monocytes/macrophages (1×10^6) were stained with fluorochrome-conjugated monoclonal antibodies against CD14, CD163, and CD206 (all purchased from BD Biosciences) according to the manufacturer's instructions and then analyzed by flow cytometry. Data were acquired and analyzed on the Flow Cytometer (BD Biosciences). Total RNA was purified from cultured macrophages using TriPure Isolation Reagent Kit (Roche Applied Science, Germany). The cDNA was synthesized using the Transcriptor First-strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Quantitative RT-PCR was performed using SYBR Green PCR kit (Roche Diagnostics, Mannheim, Germany) on the LightCycler 480 apparatus (Roche). The relative concentrations of the target genes were calculated and normalized against that of the housekeeping gene β-actin. Primer sequences are available upon request. The following mRNAs were amplified: CCR2, CD163, ARG1, IL-10.

Chemotaxis Experiments

Briefly, the upper chamber was seeded with macrophages and separated from a lower chamber. In the lower chamber, aHSCs were seeded with or without CCR2 antagonist INCB 3284. At the specific time point, macrophages that migrated to the underside of the insert were fixed and stained for quantitation by light microscopy. Macrophages were treated with aHSCs, adding INCB plus aHSC, and Rh CCL2; blank medium was used for the control group.

Statistical Analysis

Statistical analyses were performed using SPSS 22.0. Data conforming to the normal distribution were assessed by independent sample *t*-test and paired *t*-test and expressed as mean ± standard deviation. Non-normally distributed data were compared using the Mann-Whitney *U* test and two-tailed test. *P*-value < 0.05 was considered as statistically significant.

RESULTS

Infiltration of M2 Macrophages ($CD163^+$) Increased With the Progression of Liver Fibrosis

The characteristics of the patients involved in our study are shown in **Table 1**. To examine the relationship between hepatic M2 macrophages and liver fibrosis stages, we first compared the expression of CD163 (mainly expressed on M2 macrophages) in

different stages of hepatic fibrosis among patients. The degree of liver fibrosis in different patients was assessed by hematoxylin-eosin staining according to the Metavir score system (F0, no fibrosis; F1, fibrosis around the portal vein; F2, fibrous interval around the portal vein; F3, a large number of fibrous intervals are formed between the portal vein and the central vein; F4, cirrhosis) (**Figure 1A**). F0 and F1 were defined as mild fibrosis, F2 and F3 as moderate fibrosis, and F4 as severe fibrosis. As shown in **Figures 1B,C**, the expression of CD163 in liver tissues significantly increased with fibrosis. The number of patients in mild degree was 21, in moderate degree was 34, in severe degree was 41. The CD163 IHC score in the mild degree was 34.95 ± 18.12, in moderate degree was 77.57 ± 32.48, and in severe degree was 99.62 ± 40.84; a significant difference was observed between mild and moderate fibrosis ($P < 0.001$), and between moderate and severe fibrosis ($P = 0.007$). These results indicated the correlation between M2 macrophages and fibrosis. We concluded that the fibrosis environment might affect the phenotype of macrophages.

M0MΦ Developed into M2 Phenotype When Co-cultured With aHSCs or Treated With Supernatant From aHSCs

Hepatic stellate cells are always activated during liver fibrosis and can secrete many cytokines and chemokines (23). Thus, whether aHSCs are responsible for the monocyte infiltration and M2 phenotype formation in livers is yet to be elucidated. We successfully extracted five cases of primary human aHSCs with sustainable and stable growth. To investigate the regulation of aHSCs on macrophages, the primary aHSCs were co-cultured with THP-1-derived M0MΦ (1:5 cell ratio). After 5 days of co-culture, as shown in **Figure 2,** compared with the M0 control group, the aHSCs group significantly up-regulated the expression of CD163 and CD206 on macrophages: (29.5 ± 6.1% vs. 2.7 ± 1.1%, $P < 0.001$ and 28.0 ± 4.2% vs. 2.4 ± 1.2%, $P < 0.001$). This suggested that aHSCs can regulate macrophages through certain pathways, promoting the M2 phenotype differentiation.

To further explore the mechanism of the immunomodulatory effects of aHSCs on macrophages, we detected the phenotypic changes in M0MΦ after treating with aHSCs' supernatant. As shown in **Figure 2**, the supernatant treated group independently up-regulated the expression of CD163 and CD206 on macrophages compared with the control group (26.1 ± 2.8% vs. 2.7 ± 1.1%, $P < 0.001$ and 25.8 ± 3.8% vs. 2.4 ± 1.2%, $P < 0.001$), indicating that the aHSCs might secrete specific cytokines responsible for the macrophages' phenotype transformation. Of note, macrophages in the supernatant group showed relatively lower expression of CD163 and CD206 compared with the co-culture group, indicating that cell to cell contact might also induce other ways to promote M2 phenotype transformation besides soluble molecules.

AHSCs Secrete High Levels of CCL2

In a previous study, we found that aHSCs secrete various cytokines and chemokines, including CCL2 (23). CCL2 can recruit immune cells, including monocytes. However, whether it participates in up-regulating the expression of CD163 and CD206 on macrophages is yet to be elucidated.

First, we confirmed the production of CCL2 in aHSCs as well as in those stimulated by TGF-β. To compare the results, we used the LX2 cell line as the control. **Figure 3A** showed that the primary aHSCs are typically fusiform and express the

TABLE 1 | Basic Clinical Characteristics of the Study Population ($n = 96$).

Variables	Patients, *N* (%)
Gender	
Female	15(15.63)
Male	81(84.37)
Age (years)	
≤40	15(15.63)
41–50	22(22.92)
51–60	36(37.5)
61–70	19(19.79)
>70	4(4.16)
ALT (U/L)	
≤40	58(60.42)
>40	37(30.58)
AST(U/L)	
≤40	55(57.29)
>40	41(42.71)
ALB/GLB	
>2.5	2(2.08)
1.0–2.5	91(94.79)
<1.0	3(3.13)
Total bilirubin(μmol/L)	
≤17	67(69.79)
>17	29(30.21)
HBeAg	
(+)	17(17.71)
(–)	75(78.13)
None	4(4.17)
HBV DNA	
$<10^4$	37(38.54)
10^4–10^6	29(30.21)
$\geq 10^6$	18(18.75)
None	12(12.5)
Platelets (10^9/L)	
Median (IQR)	181.5(148.75–181.5)
White blood cells (10^9/L)	
Median (IQR)	6.01(4.74–8.14)
NEUT%	59.00(50.50–68.25)
MO%	8.00(7.00–10.00)
LYM%	29.00(19.75–36.25)
Fibrosis	
F0-1(Mild)	21(21.88)
F2-3 (Moderate)	34(35.41)
F4(Severe/cirrhosis)	41(42.71)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; GLB, globulin.

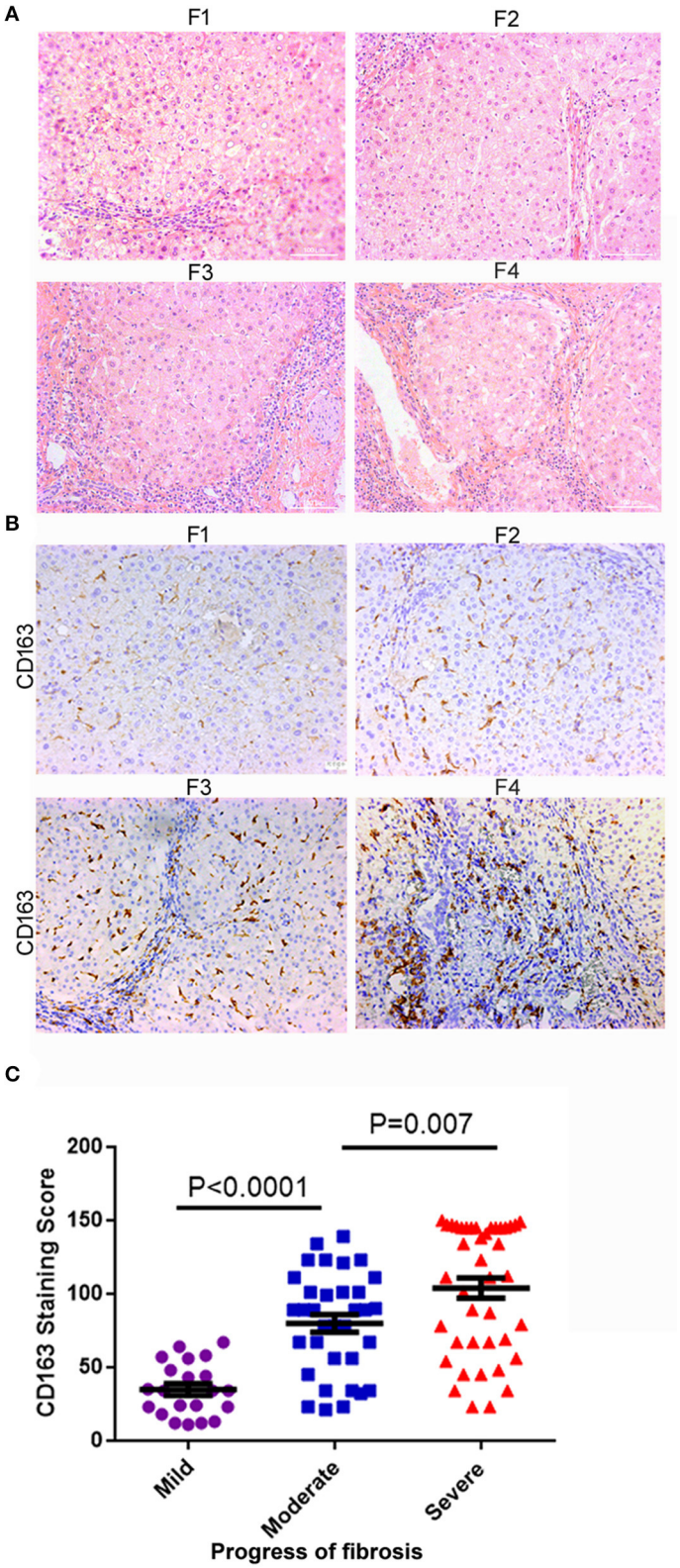

FIGURE 1 | M2 macrophages (CD163+) infiltration increased with liver fibrosis progression. **(A)** The degree of liver fibrosis in different patients was assessed by hematoxylin-eosin staining according to the Metavir score system. F1, fibrosis around the portal vein; F2, fibrous interval around the portal vein; F3, a large number of

(Continued)

FIGURE 1 | fibrous intervals are formed between the portal vein and the central vein; F4, cirrhosis. HE staining, the grade of liver fibrosis was based on Metavir score, ×200-fold. **(B)** The expression of CD163 in liver tissues significantly increased as the fibrosis aggravated. **(C)** According to the degree of F0-F4 fibrosis based on the Metavir scoring system, we defined F0-F1, F2-F3, and F4 as mild, moderate, and severe liver fibrosis, respectively. The number of patients in mild degree was 21, in moderate degree was 34, in severe degree was 41. A significant difference was observed between mild and moderate fibrosis ($P < 0.001$), and between moderate and severe fibrosis ($P = 0.007$).

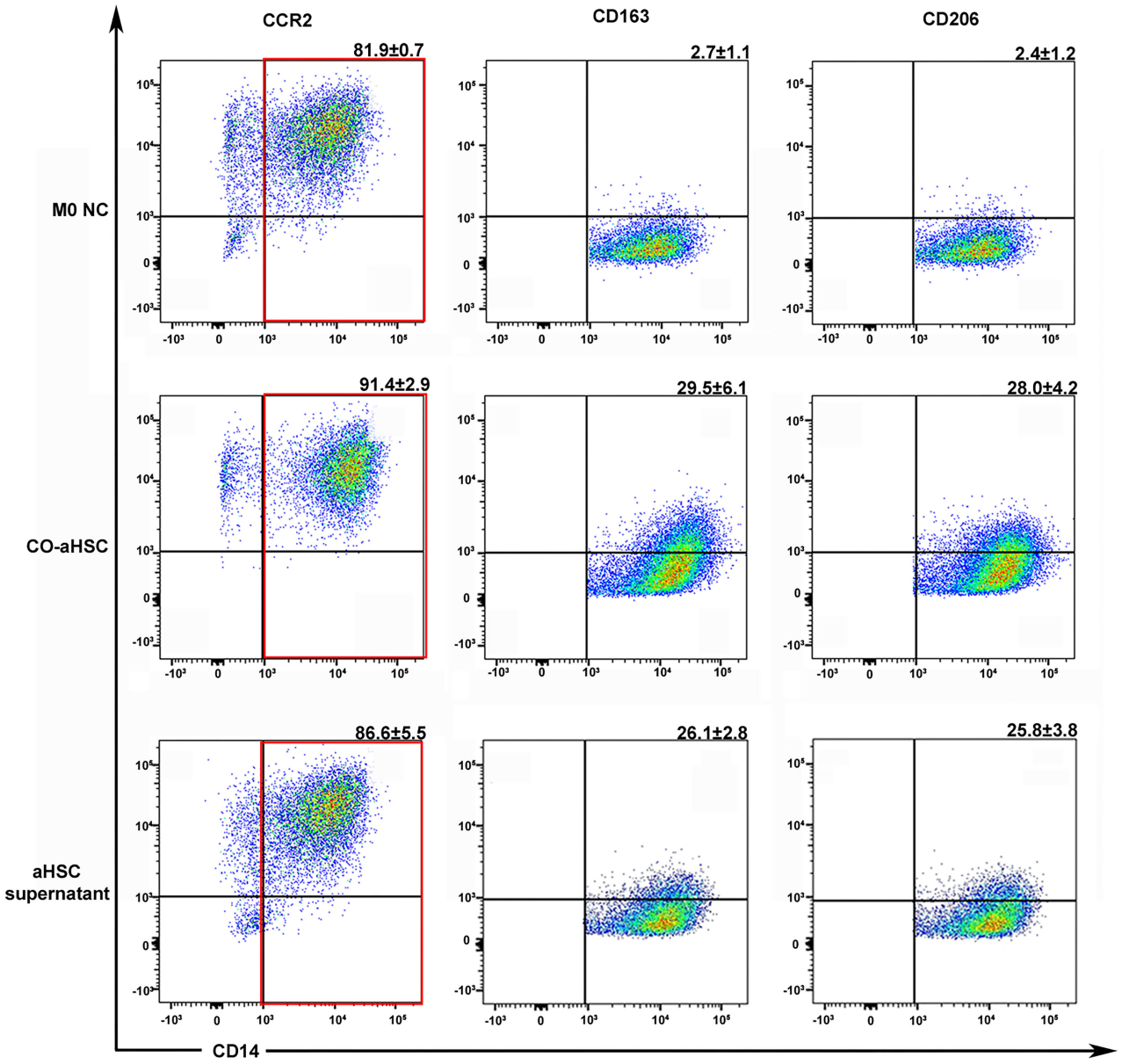

FIGURE 2 | Either the co-culture condition with aHSCs or their supernatant could independently induce the M2 macrophage differentiation. After 5 days culture, compared to the control group (M0 NC), the co-aHSC group highly expressed M2 phenotype specific proteins: CD163 (29.5 ± 6.1% vs. 2.7 ± 1.1%, $P < 0.001$) and CD206 (28.0 ± 4.2% vs. 2.4 ± 1.2%, $P < 0.001$). The aHSC supernatant group independently up-regulated the expression of CD163 and CD206 on macrophages as compared to the M0 NC group (26.1 ± 2.8% vs. 2.7 ± 1.1%, $P < 0.001$ and 25.8 ± 3.8% vs. 2.4 ± 1.2%, $P < 0.001$). These experiments were repeated at least three times.

activation marker α-SMA together with a high expression of the CCL2 protein. The expression of CCL2 in the supernatant of the primary aHSC, TGF-β (2 ng/ml)-stimulated aHSCs, LX2, and TGF-β (2 ng/ml)-stimulated LX2 was evaluated by ELISA. As shown in **Figure 3B**, aHSCs can secrete high levels of CCL2 compared to LX2. Interestingly, this ability can be further

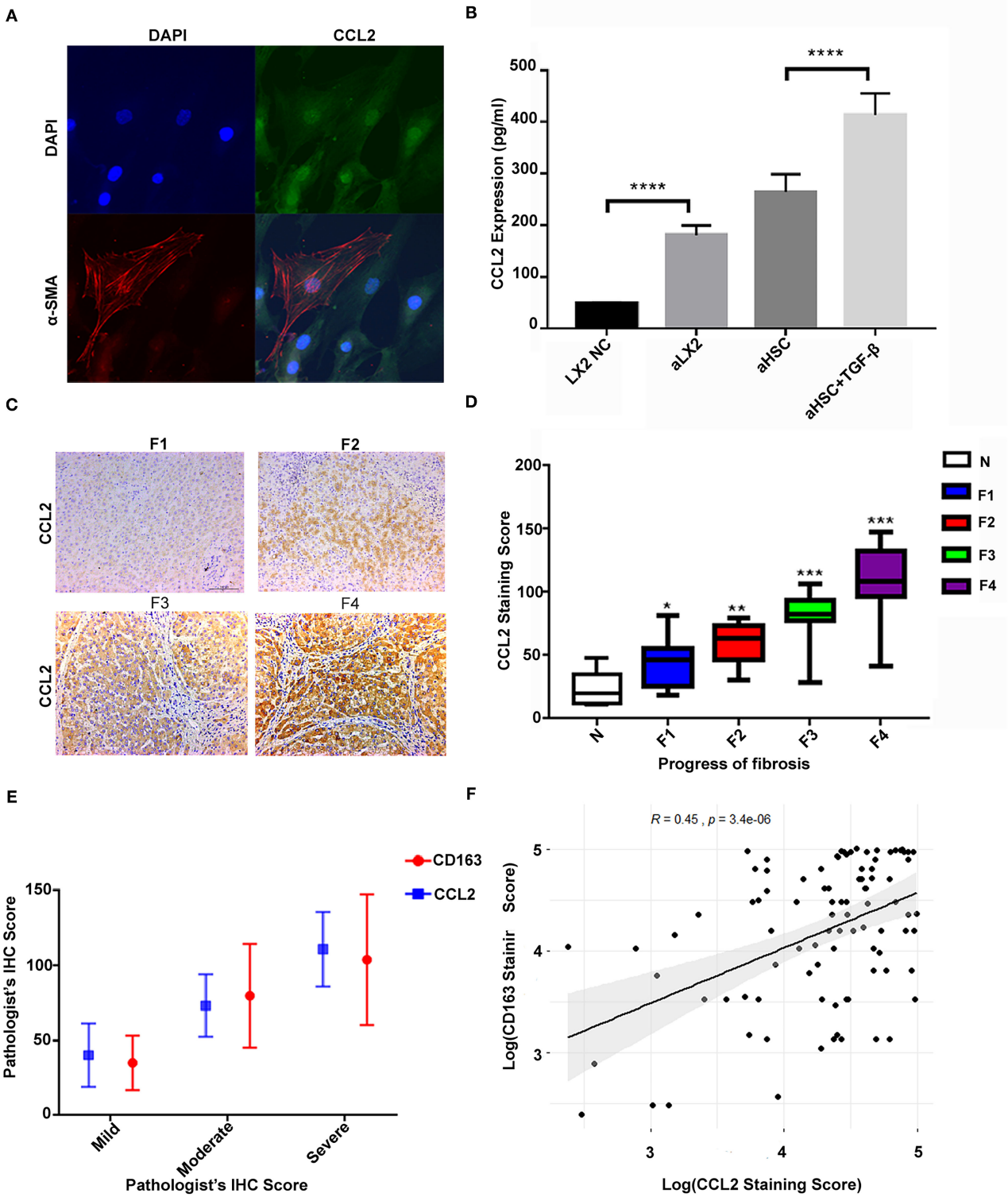

FIGURE 3 | The high level of CCL2 in aHSCs was associated with CD163+ macrophage infiltration and increased with liver fibrosis progression. **(A)** The primary aHSCs are typically fusiform and express the activation marker α-SMA, together with a high expression of CCL2 protein. **(B)** The aHSCs secrete high levels of CCL2

(Continued)

FIGURE 3 | as compared to LX2, which is further enhanced by TGF-β stimulation (LX2 vs. TGF-β-stimulated LX2, $P < 0.001$; aHSC vs. TGF-β-stimulated aHSCs, $P < 0.001$). **(C)** Representative figures for immunohistochemistry staining of liver tissues with antibody of CCL2. As the degree of liver fibrosis worsened (F1 to F4), the expression of CCL2 increased gradually. **(D)** Comparison of CCL2 staining score among different fibrotic status. The number of patients for *N* was 7, F1 was 14, F2 was 12, F3 was 14, and F4 was 41. **(E)** The correlations between CD163 and CCL2 were explored under different fibrotic degree. **(F)** A relatively strong correlation was established between M2 macrophage (CD163+) IHC staining score and CCL2 score by scatter plot ($R = 0.45$, $P < 0.05$).

TABLE 2 | Correlation analysis between clinical characteristics and CD163 and CCL2.

Demographic or Characteristics	CD163		CCL2	
	R	***P*-value**	***R***	***P*-value**
Age (years)	0.074	0.473	0.158	0.123
ALT (U/L)	0.113	0.276	0.085	0.416
AST (U/L)	0.103	0.325	0.101	0.332
Total bilirubin (μmol/L)	0.130	0.215	0.037	0.721
ALB/GLB	−0.299	0.003*	−0.445	<0.001**
White blood cells (10^9/L)	−0.019	0.859	0.027	0.795
NEUT%	−0.024	0.816	−0.029	0.784
MO%	0.060	0.565	0.107	0.303
LYM%	−0.001	0.989	0.397	<0.001**
Platelets (10^9/L)	−0.043	0.683	−0.074	0.477

**P-value ≤ 0.05; ***P-value ≤ 0.001.*
r: Pearson correlation coefficient.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; GLB, globulin.

enhanced by TGF-β stimulation (LX2 vs. TGF-β stimulated LX2, $P < 0.001$; aHSC vs. TGF-β stimulated aHSCs, $P < 0.001$).

Furthermore, in consistent to what we found in the *in vitro* study, the expression of CCL2 increased with liver fibrosis progress. As shown in **Figures 3C,D**, as the degree of liver fibrosis worsened, the expression of CCL2 gradually increased as compared to the control group (N group) (F1 vs. N, $P = 0.013$, F2 vs. N, $P < 0.01$, F3 vs. N, $P < 0.001$, F4 vs. N, $P < 0.001$). The number of patients for N was 7, for F1 was 14, F2 was 12, F3 was 14, and F4 was 41. The CCL2 staining score for N was 23.26 ± 13.85; for F1 was 48.56 ± 19.18 (F1 vs. N, $P = 0.013$); for F2 was 58.25 ± 16.24 (F2 vs. N, $P < 0.01$); F3 was 81.33 ± 18.48 (F3 vs. N, $P < 0.001$); F4 was 110.93 ± 24.75, (F4 vs. N, $P < 0.001$). Further statistical analyses were also performed for possible correlation among CD163, CCL2 as well as their correlation with other clinical variables (**Table 2**). These data indicated CCL2 was significantly related to lymphocytes' levels. Noteworthy, both CCL2 and CD163 staining scores have a statistically significant correlation with the ALB/GLB ratio.

Additionally, a significant increase in both markers (CCL2 and CD163) was detected with the advancement in fibrosis. The correlations between CD163 and CCL2 were explored under different fibrotic degree and by scatter plot (**Figures 3E,F**), which suggested a correlation between the IHC score of M2 macrophage (CD163+) and CCL2 ($R = 0.45$, $P < 0.05$). These results strengthened our hypothesis that CCL2 may regulate M2 phenotype transformation.

aHSCs Induce Macrophage Infiltration and M2 Differentiation *via* CCL2

To verify that CCL2 was responsible for macrophage infiltration and differentiation into M2 phenotype during liver fibrosis, we used rh CCL2 and its receptor antagonist INCB to confirm this pathway further. As shown in **Figures 4A,B**, macrophage infiltration increased when aHSCs were cultured in the lower chamber as compared to the control (only medium) (aHSC group vs. medium group, 237.00 ± 13.78 vs. 23.75 ± 5.32, $P < 0.01$). In contrary, the number decreased when INCB was used to block the CCL2/CCR2 pathway in the same culture system (aHSC + INCB group vs. aHSC group 83.25 ± 12.78 vs. 237.00 ± 13.78, $P < 0.01$). Rh CCL2 could also mimic aHSCs on the ability of macrophage infiltration (Rh CCL2 group vs. medium group, 143.25 ± 7.80 vs. 23.75 ± 5.32, $P < 0.01$), indicating that aHSCs promote macrophage infiltration mainly through CCL2. The experiment was repeated four times.

Furthermore, CD163 and CD206 expression was significantly up-regulated on macrophages as a result of rh CCL2 stimulation as compared to the control group. The flow cytometry result of CCL2 is shown in **Figures 4C,E** (CCL2 vs. M0, CD163: 27.6 ± 7.0% vs. 2.7 ± 1.1%, $P = 0.008$; CD206: 26.5 ± 5.1% vs. 2.4 ± 1.2 %, $P = 0.003$). The addition of INCB (100 ng/ml) inhibited the expression of CD163 and CD206 on M0MΦ (CD163: 4.5 ± 1.4%, CD206: 4.1 ± 2.6%, vs. M0 NC group, $P > 0.05$).

To avoid the clonal selection and individual differences of the primary aHSCs, we used LX2 cell lines to repeat the experiment. Treatment of LX2 cells with TGF-β induces proliferation and

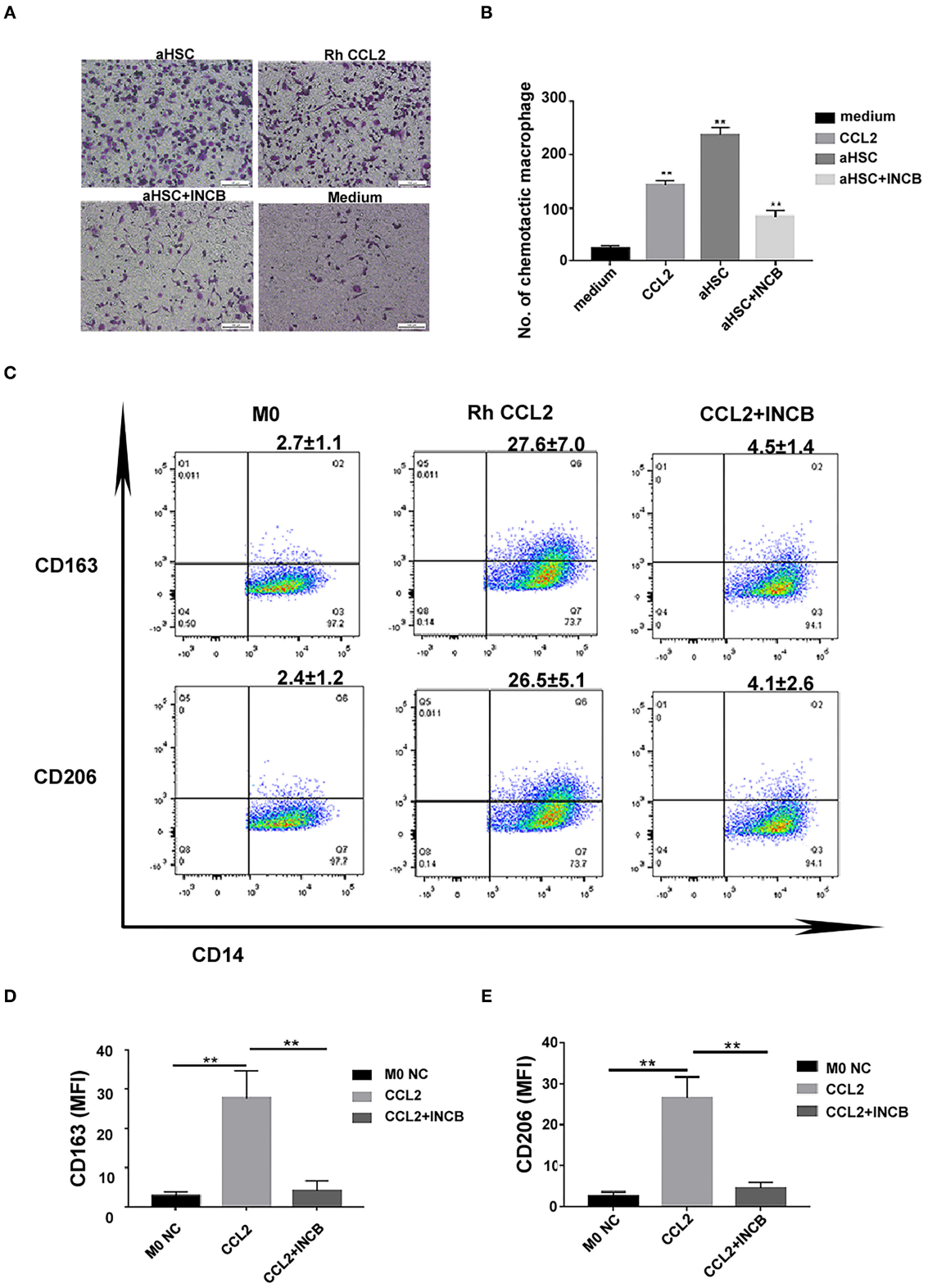

FIGURE 4 | CCL2 was responsible for macrophages infiltration and differentiation into M2 phenotype during liver fibrosis. **(A)** Representative images of macrophage infiltration under different chemotaxis treatments including aHSC, aHSC+INCB, Rh CCL2 and medium. **(B)** Statistical analysis of the number of macrophages

(Continued)

FIGURE 4 | infiltration. The aHSC group vs. medium group, $P < 0.01$, aHSC+INCB group vs. aHSC group, $P < 0.01$. Rh CCL2 vs. medium group, $P < 0.01$. **(C)** The representative flow cytometry data of macrophage phenotypic change when exposed to CCL2 treatment with or without INCB. **(D)** CCL2 significantly up-regulated CD163 expression on macrophages; this effect could be blocked by INCB (100 ng/ml). (CCL2 group vs. M0 NC group, $P = 0.008$; CCL2 group vs. CCL2 + INCB group, $P < 0.01$). **(E)** CCL2 significantly up-regulated CD206 expression on macrophages, and this effect could be blocked by INCB (100 ng/ml). (CCL2 group vs. M0 NC group, $P = 0.003$; CCL2 group vs. CCL2 + INCB group, $P < 0.01$). These experiments were repeated at least three times.

expression of ECM components. Previous studies have shown that the stimulation with TGF-β (2 ng/ml) for 24–48 h increased mRNA and protein expression of alpha-SMA, collagen type 1, PDGF, TIMP1, and TIMP2 compared to untreated LX2 cells (34, 35). Other studies have reported similar trends (36–39), thus suggesting that TGF-β activates LX2 cells to a greater extent than simple culturing of cells on a stiff surface. Compared to the LX2 group, TGF-β-stimulated LX2 (aLX2 group) up-regulated the expression of CD163 and CD206 on macrophages in both the co-culture system and when using supernatant only (**Figure 5**). Since activated LX2 also produced large amounts of CCL2 (**Figure 3B**) and CCL2-specific receptor antagonist INCB blocked the macrophages' modulation function (**Figures 4C–E**), we concluded that aHSCs induce macrophage M2 phenotype differentiation through the CCL2/CCR2 pathway.

aHSCs Upregulated the Expression of CCR2 to Form CCL2/CCR2 Positive Feedback Pathway

Interestingly, while we used qPCR to test the mRNA levels of M2MΦ-specific markers after treating THP-1-derived M0MΦ under different conditions (supernatants stimulation from aHSC, aLX2, LX2), we found that supernatants from aHSCs up-regulated the expression of macrophage CCR2, ARG-1, and IL-10 in addition to CD163 (**Supplementary Figure 1**). Furthermore, we confirmed the upregulation of CD163 on CCL2-stimulated macrophages by immunofluorescence (**Supplementary Figure 1B**). In conclusion, these results indicated that besides the secretion of high levels of CCL2, aHSCs could also up-regulate the expression of CCR2 on macrophages to activate the CCL2/CCR2 pathway. However, the mechanism needs further exploration.

DISCUSSION

The M2 macrophage infiltration increased significantly with the progression of liver fibrosis, accompanied by the up-regulated expression of CCL2 in the fibrotic liver tissues. *In vitro* data showed that primary aHSCs isolated from fibrotic liver tissues and aLX2 could produce CCL2, promoting macrophages' conversion to the M2 phenotype, which displays its immunosuppressive function by secreting the inhibitory cytokines such as IL-10 and ARG1. This might contribute to an immunosuppressive state in the liver. It may also explain an increased risk of hepatocellular carcinoma and bacterial infection in cirrhotic patients (17, 40).

CD163 is a multifunctional receptor involved in receptor-mediated endocytosis and signal pathways upon interaction with diverse ligands. CD163-positive macrophages are usually found during the healing phase of acute inflammation and chronic inflammation in wound-healing tissues, whereas freshly infiltrated macrophages are CD163-negative (41). CD163 acts as an innate immune sensor for bacteria and inducer of local immunity, rather than as a phagocytic receptor, and has been proposed as an anti-inflammatory marker for macrophages (42). CD163 is shed from the macrophage surface into the circulation upon activation of cell surface Toll-like receptors (TLRs) and is found in the blood as soluble CD163 (sCD163). Previous studies reported increased sCD163 levels in patients with chronic viral hepatitis; these increased levels have been associated with the disease's severity and may predict fibrosis (43–45). It is also determined that the sCD163 serum level is a new independent non-invasive risk factor for death and variceal bleeding in cirrhotic patients (46). However, the link between hepatic $CD163^+$ macrophages accumulation and liver fibrosis progression remain elusive, and experimental evidence for the reasons why CD163 upregulation during liver fibrosis is still lacking. In this study, we found that the expression of CD163 in liver tissues significantly increased as the fibrosis aggravated. This may explain the increase of sCD163 levels in patients' serum as sCD163 is discarded from activated macrophages, which are mainly located in the liver.

Interestingly, we found that CCL2 improve THP1-derived M0MΦ differentiation into $CD163^+$ macrophages independently. We speculated that, besides the recruitment of immune cells, CCL2 might also play immune modulation roles. Indeed, we discovered that the supernatant from aHSCs up-regulated the expression of CD163 on macrophages more obviously than CCL2 alone, which indicated that other factors in aHSCs supernatant might also induce the expression of CD163. It has been reported that the expression of CD163 could be induced by glucocorticoids, IL-10, IL-6, and M-CSF (41). In our previous study, we found that aHSCs can secrete high levels of IL-6 and M-CSF (23), which may explain why aHSCs showed stronger up-regulation function than Rh CCL2 alone. It also indicated complicated immune modulation pathways during liver fibrosis where aHSCs dominate.

Recent studies showed that the CCL2/CCR2 axis has a vital role in the fibrotic formation in some diseases, including pulmonary fibrosis, renal fibrosis, and non-alcoholic liver fibrosis. In addition, it also regulates chemotactic macrophage infiltration (27, 47, 48). Wang et al. speculated that the CCL2/CCR2 axis is closely related to the aggregation of myeloid-derived inhibitory cells (MDSCs) and T cell function inhibition in pulmonary fibrosis and lung cancer (49). The proportions of

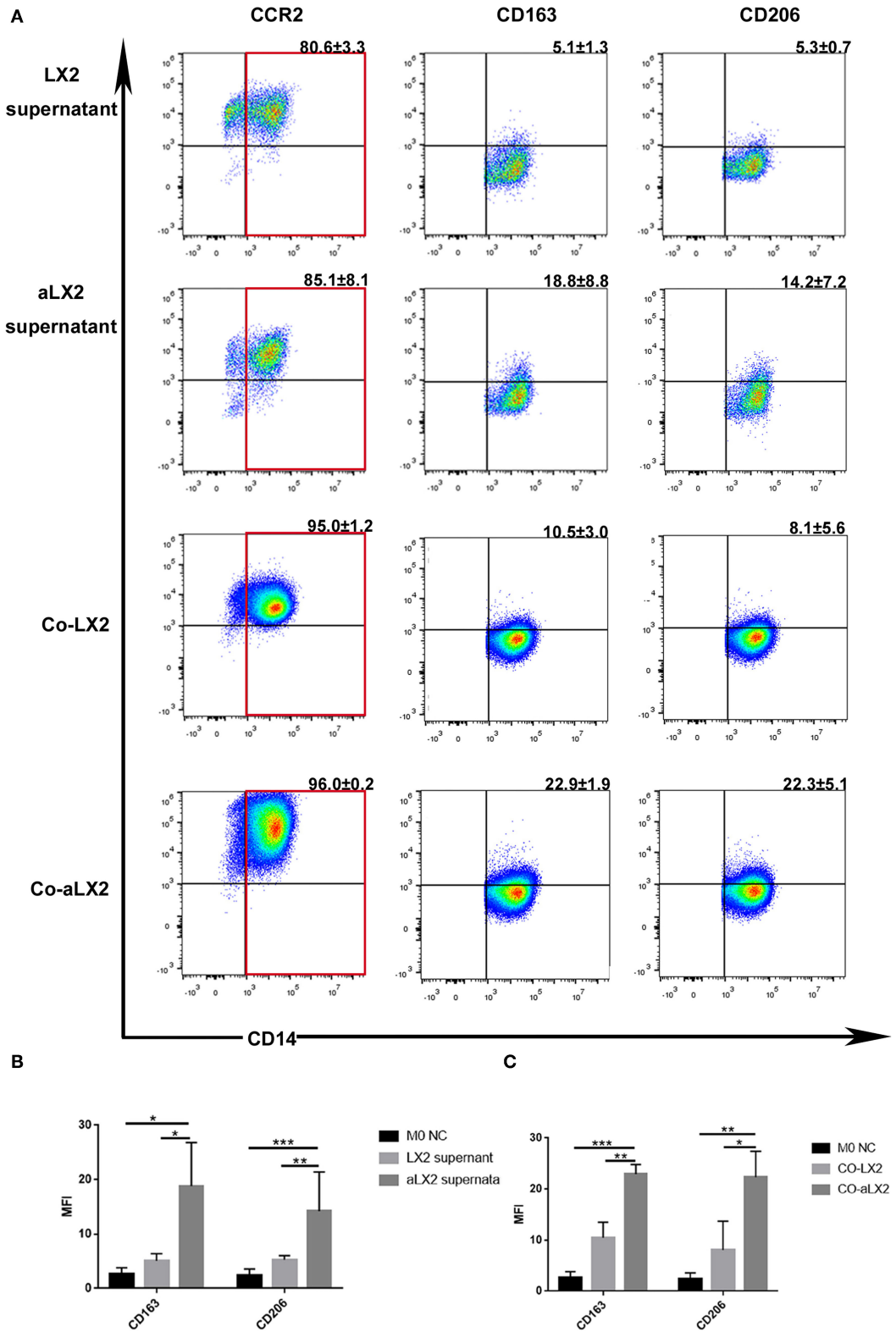

FIGURE 5 | TGF-β-stimulated LX2 up-regulated the expression of CD163 and CD206 on macrophages under co-culture or supernatant treatment condition. **(A)** The representative flow cytometry figure of macrophage phenotypic change when exposed to co-culture with LX2 or TGF-β stimulated LX2 (aLX2) or their supernatant

(Continued)

FIGURE 5 | treatment. **(B)** The aLX2 supernatant group independently up-regulated the expression of CD163 and CD206 on macrophages. (CD163: aLX2 group vs. LX2 group, $P < 0.05$), (CD163: aLX2 group vs. M0 NC group, $P < 0.05$); (CD206: aLX2 group vs. LX2 group, $P < 0.05$), (CD206: aLX2 group vs. M0 NC group, $P < 0.05$). **(C)** The co-culture with aLX2 independently up-regulated the expression of CD163 and CD206 on macrophages. CD163 (aLX2 group vs. LX2 group, $P < 0.05$; aLX2 group vs. M0 NC group, $P < 0.05$) and CD206 (aLX2 group vs. LX2 group, $P < 0.05$; aLX2 group vs. M0 NC group, $P < 0.05$). These experiments were repeated at least three times.

$CD11C^+CD206^+$ and $CCR2^+$ macrophages in adipose tissues were highly elevated in patients with NASH compared to healthy controls and patients with fatty liver, and $CCR2^+$ macrophages were also correlated with NASH severity (44). It seems that $CCR2^+$ macrophages have essential roles in inflammation, cancer, and fibrosis. Still, only a few studies examined why and how CCR2 are up-regulated on macrophages. The current study revealed the correlation between the high expression of CCL2, macrophage infiltration, and the hepatic fibrosis progress; the level of CCL2 strikingly increased with continuous activation of HSCs, which is essential in cirrhosis. Some factors from aHSCs may help to stimulate CCR2 expression. This may provide some clues and explanations to the $CCR2^+$ macrophages recruitment that have not been addressed by previous studies.

In the cell culture system, our results showed that aHSCs induce macrophages aggregation and M2 phenotype differentiation through CCL2/CCR2. On one hand, M2 macrophages might accelerate the immune surveillance disorder under cirrhotic condition by secreting immunosuppressive cytokines (5), showing a relatively weak antigen presentation, promoting vascular regeneration and tissue reconstruction (13). On the other hand, M2 macrophages secrete IL-10, TGF-β, and other cytokines, which are beneficial to the survival and sustained activation of aHSCs (40). Therefore, we propose that during the progression of liver fibrosis (especially HBV related), there might be an "amplification loop" between aHSCs and macrophages, and CCL2/CCR2 axis has essential roles in this loop. Since a study found some anti-fibrotic effects of CCL2 inhibitor in animal models of liver fibrosis (30), the pathway of CCL2/CCR2 as potential therapeutic targets should be further investigated by future studies.

The present study has some limitations. Firstly, due to the limited experiment conditions, we could not confirm our results in animal models, especially transgenic mice models. We plan to further investigate the detailed mechanisms *in vivo* in our next study. Secondly, although some experiments on CD163 and CCR2+ macrophages gave us indications that this kind of macrophages might contribute to immune suppression status in the liver and might help to develop HCC and bacterium infection (40), we do not have robust data and direct evidence to prove these hypothesis. Thirdly, a detailed mechanism such as a signaling pathway in the CCL2/CCR2 axis mediated the activation in macrophages should be investigated by future studies. Lastly, all our *in vitro* experiments were based on the THP-1 cells, a human leukemia monocytic cell line. We are not sure if this reflects the same situation in that of the primary normal human monocytes. It is warranted to repeat all the experiments with primary human monocytes if possible.

In conclusion, we found increased expression of hepatic CD163 and CCL2 in patients with HBV-related fibrosis; the expression increased dramatically with further progression of liver fibrosis. There might be an "amplification loop" between aHSCs and macrophages through CCL2/CCR2 axis; thus, more *in vitro* and *in vivo* studies in this area are needed.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Third Affiliated Hospital of Sun Yat-sen University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MZ, YC, SX: conception and design. MZ and YC: financial support. SX and XL: experiment conduction. SX and XL: provision of materials or patients. SX, XZ, YJ, ZL, and JC: collection and assembly of data. SX, XZ, YW, and JG: data analysis and interpretation. XZ: revisional author. All authors: manuscript writing. All authors: final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.627927/full#supplementary-material

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ORIGINAL RESEARCH
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Silencing Heat Shock Protein 47 (HSP47) in Fibrogenic Precision-Cut Lung Slices: A Surprising Lack of Effects on Fibrogenesis?

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Idiopathic pulmonary fibrosis (IPF) is a chronic disease that is characterized by the excessive deposition of scar tissue in the lungs. As currently available treatments are unable to restore lung function in patients, there is an urgent medical need for more effective drugs. Developing such drugs, however, is challenging because IPF has a complex pathogenesis. Emerging evidence indicates that heat shock protein 47 (HSP47), which is encoded by the gene *Serpinh1*, may be a suitable therapeutic target as it is required for collagen synthesis. Pharmacological inhibition or knockdown of HSP47 could therefore be a promising approach to treat fibrosis. The objective of this study was to assess the therapeutic potential of *Serpinh1*-targeting small interfering RNA (siRNA) in fibrogenic precision-cut lung slices prepared from murine tissue. To enhance fibrogenesis, slices were cultured for up to 144 h with transforming growth factor β1. Self-deliverable siRNA was used to knockdown mRNA and protein expression, without affecting the viability and morphology of slices. After silencing HSP47, only the secretion of fibronectin was reduced while other aspects of fibrogenesis remained unaffected (e.g., myofibroblast differentiation as well as collagen secretion and deposition). These observations are surprising as others have shown that *Serpinh1*-targeting siRNA suppressed collagen deposition in animals. Further studies are therefore warranted to elucidate downstream effects on fibrosis upon silencing HSP47.

Keywords: collagen chaperone, Gp46, gene silencing, HSP-47, lung explant culture, lung fibrosis, RNA interference

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive respiratory disease characterized by the pathological deposition of extracellular matrix (ECM) in the lungs (1). As IPF progresses, the lungs gradually lose their ability to facilitate gas exchange, leading to breathlessness and, ultimately, death. Epidemiological studies point toward an incidence of 2-30 cases per 100,000 person years and a prevalence of 10–60 cases per 100,000 people (2). In addition, patients have a poor prognosis because the median survival time after diagnosis has been estimated to be 3 to 5 years. To date, only two drugs (pirfenidone and nintedanib) have been approved for the treatment of IPF. Although pirfenidone and nintedanib do not actually cure IPF, they are often prescribed to slow its progression. These drugs, however, have also been shown to cause serious side effects, such

as gastro-intestinal bleeding, diarrhea, and liver toxicity (1). Therefore, there remains an unmet medical need for more effective and safer drugs to treat IPF.

Developing such drugs is challenging because IPF has a complex pathogenesis (3, 4). To minimize toxic effects and to maximize therapeutic effects, drug targets should remain largely confined to diseased tissue and contribute sufficiently to the pathogenesis (5). Myofibroblasts, for example, play a key role in the pathogenesis of IPF and are attractive cells to target as they produce ECM proteins (e.g., collagens and fibronectins) (6). Out of all potential drug targets in myofibroblasts, heat shock protein 47 (HSP47) is particularly interesting because it is essential for the biosynthesis of collagens, such as collagen type 1 (COL1) which is overexpressed in fibrosis (7). In the endoplasmic reticulum (ER), HSP47 facilitates the folding of procollagens into trimeric structures (triple-helices). Subsequent trafficking of these trimeric collagens from the ER to the Golgi apparatus is also mediated by HSP47. Inhibition or knockdown of HSP47 could therefore be a promising strategy to attenuate fibrosis in patients.

So far, research focused on knockdown of HSP47 rather than its pharmacological inhibition (8, 9). One of the major difficulties of developing inhibitors is that compound screening procedures are time-consuming and laborious as active human HSP47 is unstable (8, 10). As an alternative, small interfering RNA (siRNA) can be used to transiently knockdown HSP47 via RNA interference (11). Until now, several animal studies revealed that knockdown of HSP47 ameliorated fibrosis in various models (e.g., renal, peritoneal, pulmonary, hepatic, vocal fold, and skin fibrosis) (12–17). Bleomycin-induced pulmonary fibrosis, for example, was suppressed in rats after intravenous administration of vitamin A coated liposomes containing *Serpinh1*-targeting siRNA (14). Results from this study also suggest that therapeutic effects were attributable not only to reduced collagen deposition but also to apoptosis of myofibroblasts. These findings are encouraging and should be validated in other experimental models and species.

Traditional *in vitro* models (i.e., cell cultures), however, are not suitable for obtaining insights into affected molecular pathways because they lack a relevant biological context and, accordingly, offer limited insights into tissue-wide effects of siRNA-mediated HSP47 knockdown. To that end, precision-cut lung slices are interesting as they are viable explants, with a well-defined thickness and diameter, that can be cultured *ex vivo* for up to a few days (18). A key advantage of this model includes its ability to recapitulate functional and structural features of the lungs, such as the presence of different cell types and the maintenance of cell-cell and cell-matrix interactions. As a result, lung slices can be used to study airway physiology, fibrogenesis, and biotransformation (18). Furthermore, we previously demonstrated that lung slices can be successfully transfected with self-deliverable (Accell) siRNA to trigger specific mRNA and protein knockdown (19, 20). This model can therefore be used to characterize the effects of siRNA-mediated HSP47 knockdown in a biologically relevant environment.

Motivated by the need for more effective and safer drugs to treat IPF, we aimed to investigate the therapeutic potential of *Serpinh1*-targeting siRNA in lung slices prepared from murine tissue. We first confirmed whether fibrogenesis could be augmented in slices by using transforming growth factor β1 (TGFβ1)—a potent pleiotropic cytokine that plays a key role in the development of IPF (4). Various aspects of fibrogenesis were assessed, such as mRNA expression of fibrogenesis-related genes, secretion of fibronectin into culture medium, and expression of alpha smooth muscle actin (α-SMA). Furthermore, because HSP47 is involved in the biosynthesis of collagens, we monitored the secretion of COL1 and its incorporation into the ECM as well as the formation of fibrillar COL1 and collagen type 3 (COL3) networks. After characterizing the effects of TGFβ1 on fibrogenesis, we examined whether *Serpinh1*-targeting Accell siRNA triggered knockdown of *Serpinh1* mRNA and its respective protein HSP47. Finally, we set out to explore whether knockdown of HSP47 affected the development of fibrogenesis as well as the secretion and deposition of collagen.

Abbreviations: α-SMA, alpha smooth muscle actin; *Acta2*, alpha smooth muscle actin; ANOVA, analysis of variance; *Atf4*, activating transcription factor 4; ATP, adenosine triphosphate; C3M, degradation fragment of collagen type 3; COL003, 2-hydroxy-3-nitro-5-(phenylmethyl)benzaldehyde; COL1, collagen type 1; *Col1a1*, collagen type 1 alpha 1; COL3, collagen type 3; ECM, extracellular matrix; *Edem1*, ER degradation enhancer mannosidase alpha-like 1; ER, endoplasmic reticulum; *Fn*, fibronectin; FKBP65, FK506 binding protein 10; H&E, hematoxylin and eosin; HSP47, heat shock protein 47; *Hsp90b1*, heat shock protein 90 beta family member 1; *Hspa5*, heat shock protein family A member 5; IPF, idiopathic pulmonary fibrosis; LC3B, microtubule-associated protein 1 light chain 3 beta; mRNA, messenger RNA; P1NP, procollagen type 1 *N*-terminal propeptide; PVDF, polyvinylidene fluoride; qPCR, real-time quantitative polymerase chain reaction; RNAi, RNA interference; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *Serpine1*, serine (or cysteine) peptidase inhibitor clade E member 1; *Serpinh1*, serine (or cysteine) peptidase inhibitor clade H member 1; siRNA, small interfering RNA; *Syvn1*, synoviolin 1; TGFβ1, transforming growth factor β1; TMB, 3,3′,5,5′-tetramethylbenzidine; *Tnfrsf11b*, tumor necrosis factor receptor superfamily member 11b; UPR, unfolded protein response; UW, university of Wisconsin; VCL, vinculin; *Xbp1*, x-box binding protein 1; *Ywhaz*, tyrosine 3-monooxygenase activation protein, zeta polypeptide.

MATERIALS AND METHODS

Animals

Lungs were collected from male C57BL/6J mice (10–14 weeks old; 24–30 gram), which were maintained under 12 h light/dark cycles, with free access to water and food (Central Animal Facility, University Medical Center Groningen, Groningen, The Netherlands). Mice were first anesthetized with 5% isoflurane/O_2 gas (Nicolas Piramal, London, UK). Once rendered unconscious, mice were euthanized by exsanguination via the inferior vena cava followed by perforation of the diaphragm. Directly afterwards, the lungs were inflated *in situ* with 1 mL of liquefied and pre-warmed (37°C) support medium consisting of 1.5% low-gelling-temperature agarose (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 0.9% NaCl (Merck, Darmstadt, Germany). After exposing the thoracic cavity, the lungs were excised and immediately placed in ice-cold University of Wisconsin (UW) preservation solution (Dupont Critical Care, Waukegab, USA). The animal experiments were approved by the

Central Authority for Scientific Procedures on Animals (permit number: 20171290) and were conducted conform national and international legislation.

Lung Slice Preparation

After excision of the lungs, lobes were separated from each other and cylindrical tissue cores were prepared with a biopsy puncher. To preserve the viability, tissue cores were immediately transferred to ice-cold UW preservation solution. Slices with a wet weight of 4-5 mg, thickness of 250–350 μm, and diameter of 5 mm were prepared using a Krumdieck tissue slicer (Alabama Research and Development, Munford, USA), which was filled with ice-cold Krebs-Henseleit buffer supplemented with 25 mM D-glucose (Merck), 25 mM $NaHCO_3$ (Merck), and 10 mM HEPES (MP Biomedicals, Aurora, USA); saturated with carbogen gas (95% O_2 and 5% CO_2); and adjusted to a pH of 7.4 (21). Directly afterwards, slices were transferred to ice-cold UW preservation solution.

Culturing Lung Slices

After their preparation, slices were either sampled (0 h) or pre-incubated individually in 12-well plates, containing pre-warmed (37°C) culture medium (1 mL/well), at 5% CO_2 and 20% O_2 while being horizontally shaken (90 cycles/min). Culture medium was composed of DMEM/F-12 + GlutaMAX™ (Fisher Scientific, Landsmeer, The Netherlands), 100 U/mL penicillin-streptomycin (Life Technologies, Bleiswijk, The Netherlands), and 50 μg/mL gentamicin (Life Technologies). After a pre-incubation of 2 h, slices were transferred to culture plates with fresh and pre-warmed culture medium and they were incubated for either 48, 96, or 144 h. Culture medium was refreshed every 48 h. To augment fibrogenesis, slices were cultured in medium containing 5 ng/mL TGFβ1 (Roche, Basel, Switzerland). In knockdown experiments, slices were incubated without Accell siRNA (untransfected) or in medium with either 0.5 μM non-targeting (control) Accell siRNA or *Serpinh1*-targeting Accell siRNA (Dharmacon, Lafayette, USA). To determine whether pharmacological inhibition affected collagen biosynthesis, we treated slices with 2-hydroxy-3-nitro-5-(phenylmethyl)benzaldehyde (COL003), which is a selective inhibitor of HSP47 (9). Samples were obtained from three independent experiments (biological replicates), which were each carried out in triplicate (technical replicates).

ATP/Protein

Adenosine triphosphate (ATP) and protein content in slices were determined with an ATP Bioluminescence Kit (Roche Diagnostics, Mannheim, Germany) and RC DC Protein Assay (Bio-Rad, Munich, Germany), respectively (20). Briefly, slices (1/tube) were homogenized in 1 mL of ice-cold sonication solution (70% ethanol and 2 mM EDTA) using a Minibead-beater (2 cycles of 45 s). After centrifugation (16,000 x *g* at 4°C for 5 min), ATP levels in the supernatant were measured. Sample supernatants were subsequently incubated overnight at 37 °C in opened tubes to remove sonication solution through evaporation. Afterwards, upon reconstitution of the pellet, protein levels were determined. ATP values (pmol) were then normalized to the total amount of protein (μg).

TABLE 1 | Primers.

Gene	Protein	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)
Atf4	ATF4	AAGGAGGAAGACAC TCCCTCT	GTCCATGGGAAGAT GTTCTGG
Acta2	α-SMA	ACTACTGCCGAGC GTGAGAT	CCAATGAAAGATGG CTGGAA
Col1a1	COL1A1	TGACTGGAAGAGC GGAGAGT	ATCCATCGGTCATG CTCTCT
Edem1	EDEM1	GGGGCATGTTCG TCTTCGG	CGGCAGTAGATGG GGTTGAG
Fn	FN	CGGAGAGAGTGCC CCTACTA	CGATATTGGTGAA TCGCAGA
Hsp90b1	GRP94	TCGTCAGAGCTGAT GATGAAGT	GCGTTTAACCCATCCA ACTGAAT
Hspa5	BIP	GACTGCTGAGGCG TATTTGG	AGCATCTTTGGTTG CTTGTCG
Serpine1	PAI-1	GCCAGATTTATCATCAAT GACTGGG	GGAGAGGTGCACATCT TTCTCAAAG
Serpinh1	HSP47	AGGTCACCAAGG ATGTGGA	CAGCTTCTCCTT CTCGTCGT
Syvn1	HRD1	CGTGTGGACTTTAT GGAACGC	CGGGTCAGGATGCTG TGATAAG
Tnfrsf11b	OPG	ACAGTTTGCCTGGG ACCAAA	CTGTGGTGAGGTT CGAGTGG
Xbp1 (spliced)	XBP1	CTGAGTCCGAATCA GGTGCAG	GTCCATGGGAAGATG TTCTGG
Ywhaz	14-3-3ζ	TTACTTGGCCGA GGTTGCT	TGCTGTGACTGGTC CACAAT

mRNA Expression

Total RNA was isolated from slices using a Maxwell 16 LEV SimplyRNA Tissue Kit (Promega, Leiden, The Netherlands). After confirming the yield and purity with a ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, USA), isolated RNA was reverse transcribed using a Reverse Transcription System Kit (Promega) and thermal cycler (22°C for 10 min, 42°C for 15 min, and 95°C for 5 min). Real-time quantitative polymerase chain reaction (qPCR) was performed using specific primers (**Table 1**), FastStart Universal SYBR Green Master Mix (Roche, Almere, The Netherlands), and a ViiA7 qPCR machine (Applied Biosystems, Bleiswijk, The Netherlands), which was configured with 1 cycle of 10 min at 95°C and 40 consecutive cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. mRNA expression was calculated as fold induction, using *Ywhaz* as a reference gene.

Protein Secretion

Pooled culture medium samples were analyzed using a Mouse Procollagen Type 1 *N*-terminal Propeptide (P1NP) ELISA Kit (Abcam, Cambridge, USA) and Mouse Fibronectin ELISA Kit (Abcam), according to the manufacturer's instructions. Briefly, samples and standards (50 μL/well) as well as antibody cocktail

(50 μL/well) were pipetted into a pre-coated 96-well plate, which was subsequently incubated for 60 min at room temperature on a plate shaker set to 500 rpm. After washing the plate 3 times, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (100 μL/well) was pipetted in each well, and the plate was incubated for 10 min at room temperature on a plate shaker set to 500 rpm. To stop enzymatic conversion of TMB, stop solution (100 μL/well) was added. Optical densities were subsequently measured using a BioTek Synergy HT (BioTek Instruments, Vermont, USA). To account for optical imperfections in the plate, wavelength correction was applied by subtracting readings at 550 nm from readings at 450 nm. P1NP and fibronectin concentrations were interpolated from their respective standard curves. Furthermore, degradation of COL3 by matrix metalloproteinase 9 was analyzed by measuring the concentration of respective degradation fragments (C3M), using a manual competitive ELISA developed by Nordic Bioscience (Herlev, Denmark) (22).

Protein Expression

Western blotting was used to analyze α-SMA, FKBP65, LC3B, and HSP47 expression in lysate, whereas dot blotting was used to evaluate COL1 trimer content in lysate and culture medium. Lysate was prepared by isolating protein from slices with ice-cold RIPA lysis buffer (Fisher Scientific, Landsmeer, The Netherlands) and a Minibead-beater for homogenization. After centrifuging the lysate (16,000 x *g* at 4°C for 30 min), the supernatant was collected and analyzed to determine the protein concentration. To investigate protein expression through western blotting, protein (10 μg) was heated (75°C for 15 min) and then separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 10% gels, and blotted onto polyvinylidene fluoride (PVDF) membranes using a Trans-Blot Turbo Transfer System (Bio-Rad). To examine COL1 trimer content by dot blotting, undiluted culture medium samples (2 μL/dot) and diluted protein lysates (2 μg/2μL/dot) were aspirated onto nitrocellulose blotting membranes (Bio-Rad), which were air-dried for 10 min. Regardless of the blotting technique, subsequent membrane processing steps were similar. After blocking in 5% non-fat milk/TBST (Bio-Rad) for 1 h, PVDF and nitrocellulose membranes were incubated overnight (at 4°C) with primary antibodies (**Table 2**), followed by an incubation with appropriate secondary antibodies for 1 h. Clarity Western ECL blotting substrate (Bio-Rad) and a ChemiDoc Touch Imaging System (Bio-Rad) were used to visualize protein bands/dots. Vinculin (VCL) was used as a loading control for western blotting.

Tissue Stainings

Slices were fixed in formalin (4%) at 4°C for 24 h, after which they were dehydrated in graded ethanol baths, cleared in xylene, and embedded horizontally in paraffin. Before staining, sections (4 μm) were deparaffinized in xylene and rehydrated in graded ethanol baths. Tissue morphology was investigated with a routine hematoxylin and eosin (H&E) staining and fibrillar COL1 and COL3 networks were visualized using a Picro Sirius Red Stain Kit (Abcam). High-resolution digital data was then obtained by scanning stained sections with a C9600 NanoZoomer (Hamamatsu Photonics, Hamamatsu, Japan). Semi-quantitative tissue damage scores were assigned to H&E stained sections using our previously published scoring system (23). The extent of collagen deposition was estimated by visual inspection of picrosirius red-stained sections using unpolarized light.

TABLE 2 | Antibodies.

Protein	Primary antibody	Secondary antibody
α-SMA	Mouse anti-α-SMA (A2547, 1:5000, Sigma-Aldrich)	Rabbit anti-mouse HRP (P0260, 1:5000, Dako, Santa Clara, USA)
COL1	Rabbit anti-COL1 (ab34710, 1:2000, Abcam)	Goat anti-rabbit HRP (P0448, 1:2000, Dako)
FKBP65	Rabbit anti-FKBP65 (12172-1-AP, 1:500, Proteintech, Manchester, UK)	Goat anti-rabbit HRP (P0448, 1:2000, Dako)
HSP47	Rabbit anti-HSP47 (ab109117, 1:2000, Abcam)	Goat anti-rabbit HRP (P0448, 1:2000, Dako)
LC3B	Rabbit anti-LC3B (ab51520, 1:3000, Abcam)	Goat anti-rabbit HRP (P0448, 1:2000, Dako)
VCL	Mouse anti-VCL (sc-73614, 1:500, Santa Cruz, California, USA)	Rabbit anti-mouse HRP (P0260, 1:5000, Dako)

Statistics

GraphPad Prism (version 8.0) was used to analyze data with a two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

TGFβ1 Did Not Affect the Viability and Morphology of Slices

To determine whether slices remained viable upon exposure to TGFβ1, we analyzed the protein, ATP/protein, and RNA/protein content as well as the morphology (**Figure 1**). As shown, TGFβ1 did not significantly change the protein, ATP/protein, and RNA/protein content. Regardless of whether TGFβ1 was added to culture medium, the protein content remained fairly stable over time, whereas the ATP/protein content gradually increased. The RNA/protein content, however, was marked by an initial increase after 48 h of incubation, after which it gradually decreased to the same levels observed at 0 h. Furthermore, TGFβ1 did not affect the morphology of slices (tissue damage scores are shown in **Supplementary Figure 1**), and the overall morphology remained sufficiently preserved for up to 144 h because only moderate tissue damage was observed.

TGFβ1 Augmented the Development of Fibrogenesis in Slices

To establish whether TGFβ1 augmented fibrogenesis in slices, we measured the expression of fibrogenesis-related genes, secretion

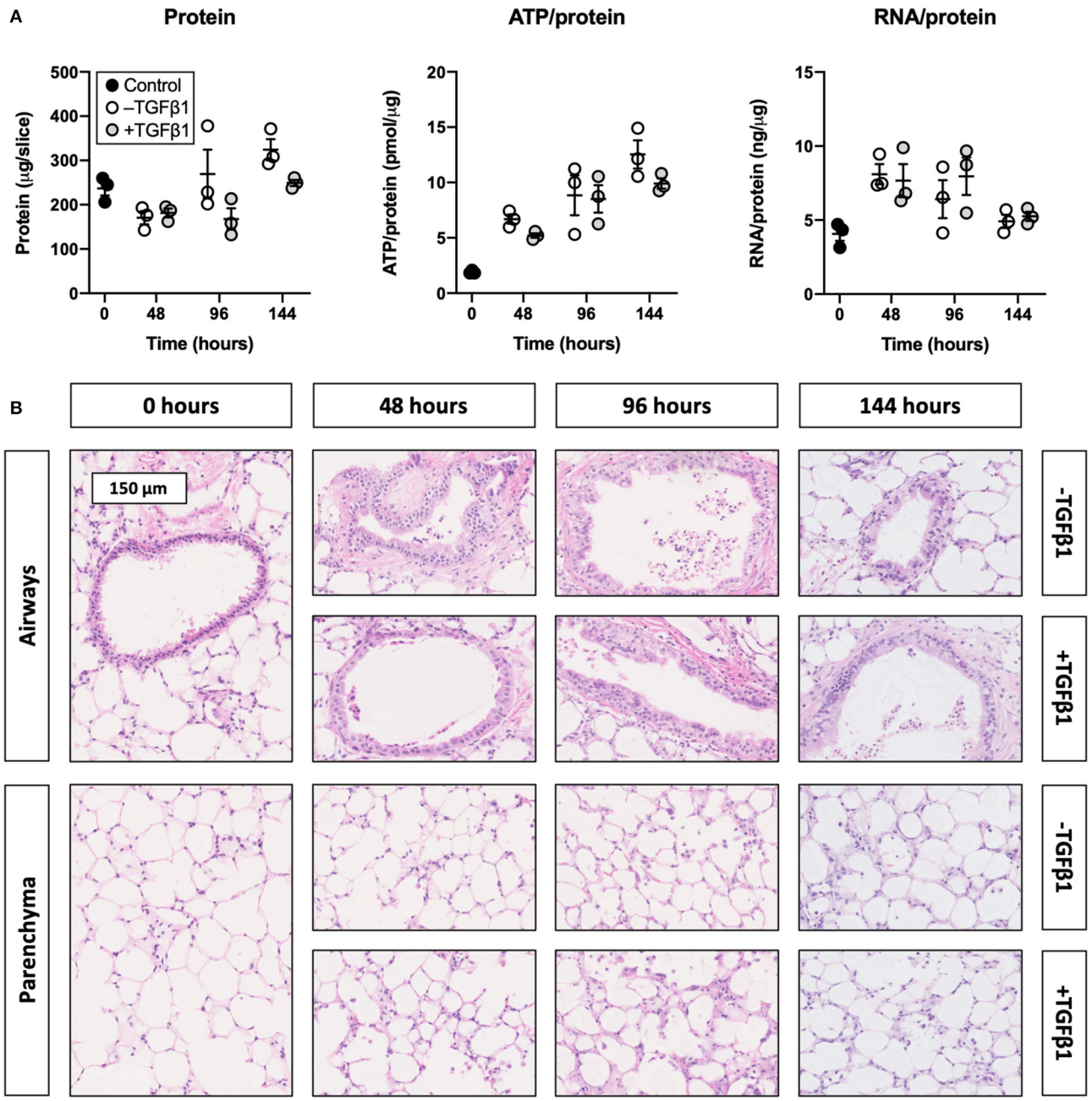

FIGURE 1 | Effect of TGFβ1 on the viability and morphology of slices. Slices were sampled after slicing (0 h) and after 48, 96, or 144 h of incubation without or with 5 ng/mL TGFβ1 ($n = 3$). Protein, ATP/protein, and RNA/protein content **(A)** were analyzed to assess the viability, and HE stainings were performed to investigate the morphology **(B)**. Values represent individual experiments performed in triplicate and are accompanied with the arithmetic mean (horizontal line) ± standard error of the mean (error bars).

of fibronectin into culture medium, and expression of α-SMA (**Figure 2**). As demonstrated, TGFβ1 significantly increased mRNA expression of all analyzed fibrogenesis-related genes. Over time, mRNA expression of *Col1a1*, *Fn*, *Serpine1*, *Serpinh1*, and *Tnfrsf11b* steadily increased, whereas mRNA expression of *Acta2* initially decreased but later increased. Treating slices with TGFβ1 also enhanced the secretion of fibronectin into culture medium. Additionally, in slices cultured without TGFβ1, α-SMA expression declined, albeit not significantly. Culturing slices with TGFβ1, however, resulted in stable α-SMA expression over time.

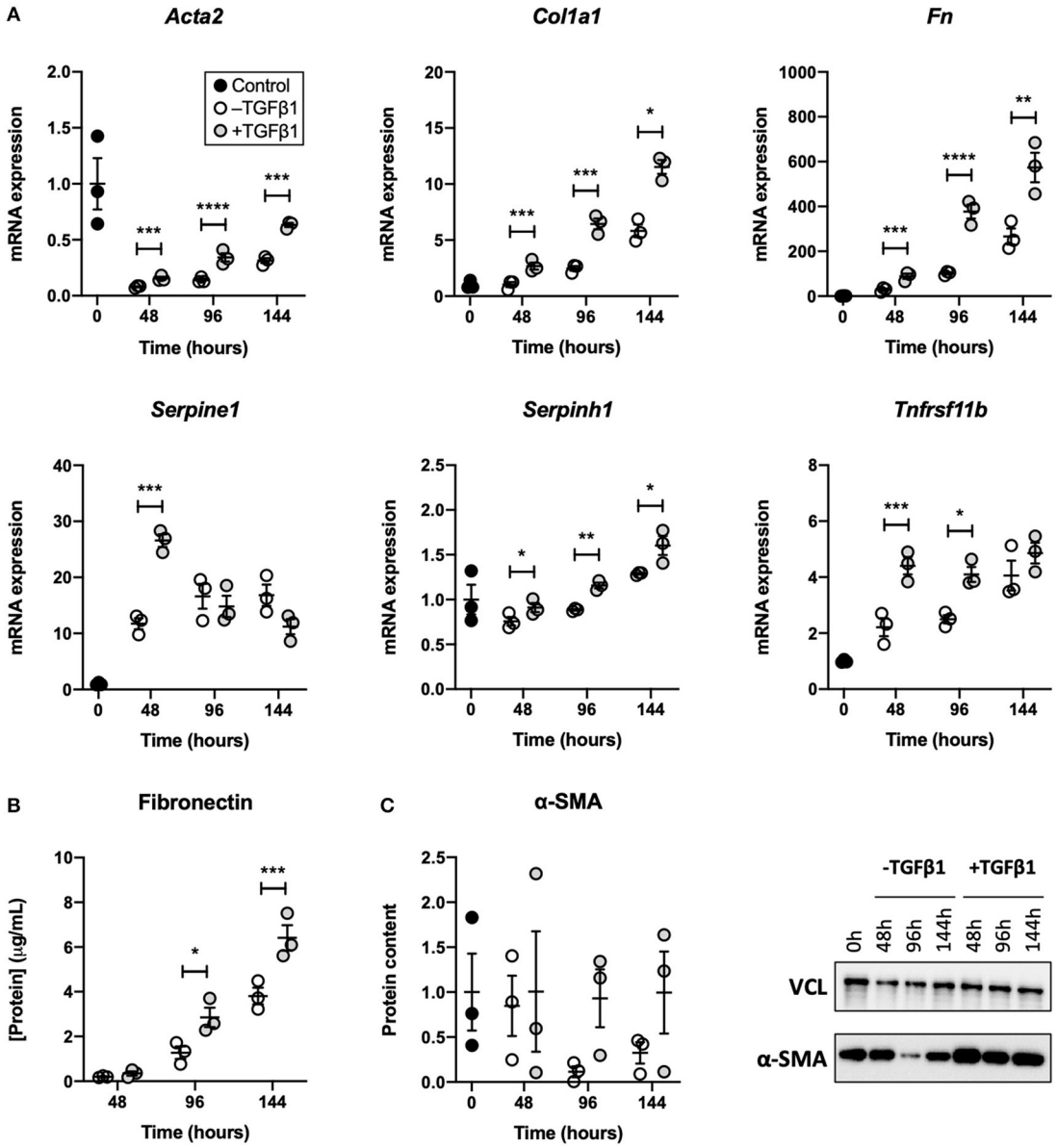

FIGURE 2 | Effect of TGFβ1 on the development of fibrogenesis in slices. Slices were collected after slicing (0 h) and after 48, 96, or 144 h of incubation without or with 5 ng/mL TGFβ1 ($n = 3$). Expression of fibrogenesis-related genes **(A)**, secretion of fibronectin into culture medium **(B)**, and expression of α-SMA **(C)** were analyzed to examine the development of fibrogenesis. Values depict individual experiments performed in triplicate and are accompanied with the arithmetic mean (horizontal line) ± standard error of the mean (error bars). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

TGFβ1 Promoted Collagen Secretion and Deposition in Slices

To identify whether TGFβ1 enhanced collagen biosynthesis in slices, we measured the concentration of P1NP and trimeric COL1 in culture medium as well as the incorporation of COL1 trimers into the ECM (**Figure 3**). As illustrated, the P1NP concentration gradually increased, and treatment with TGFβ1 further boosted this increase after 144 h of incubation. In contrast, the concentration of COL1 trimers in culture medium steadily declined and was not affected by TGFβ1. COL1 trimer content in lysate, however, increased over time and seemed to further increase, albeit not significantly, after exposing slices to TGFβ1. Similar trends were observed in sections stained with picrosirius red as networks of fibrillar COL1 and COL3 became more prominent.

Accell siRNA Did Not Affect the Viability and Morphology of Slices

To check whether Accell siRNA induced toxic effects in TGFβ1-treated slices, we assessed protein, ATP/protein, and

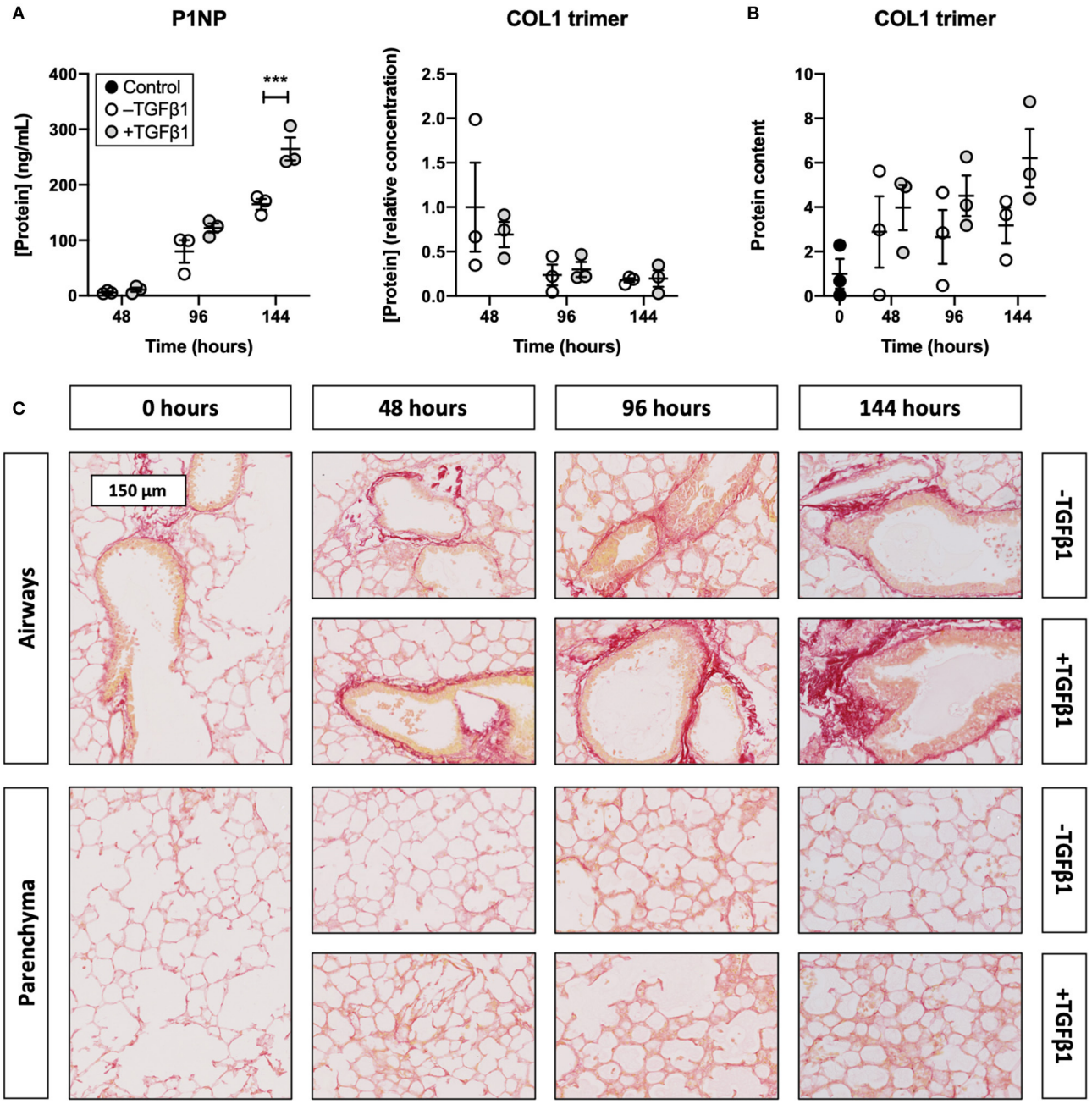

FIGURE 3 | Effect of TGFβ1 on collagen secretion and deposition in slices. Slices were sampled after slicing (0 h) and after 48, 96, or 144 h of incubation without or with 5 ng/mL TGFβ1 ($n = 3$). The concentration of P1NP and trimeric COL1 in culture medium **(A)** was measured to study collagen secretion, and COL1 trimer content in lysate **(B)** was investigated to track its incorporation into the ECM. Networks of COL1 and COL3 were visualized **(C)** with picrosirius red stainings. Values depict individual experiments performed in triplicate and are accompanied with the arithmetic mean (horizontal line) ± standard error of the mean (error bars). (***$p < 0.001$).

RNA/protein content as well as the morphology (**Figure 4**). As displayed, Accell siRNA did not significantly alter protein, ATP/protein, and RNA/protein content in slices. Similarly, no noticeable differences were observed in the morphology of untransfected and transfected slices (tissue damage scores are shown in **Supplementary Figure 2**).

Accell siRNA Induced Knockdown of mRNA and Protein in Slices

To study whether Accell siRNA induced RNA interference in slices treated with TGFβ1, we examined expression of *Serpinh1* mRNA and its respective protein HSP47 (**Figure 5**). As depicted, significant mRNA (∼65%) and protein (∼90%) knockdown was

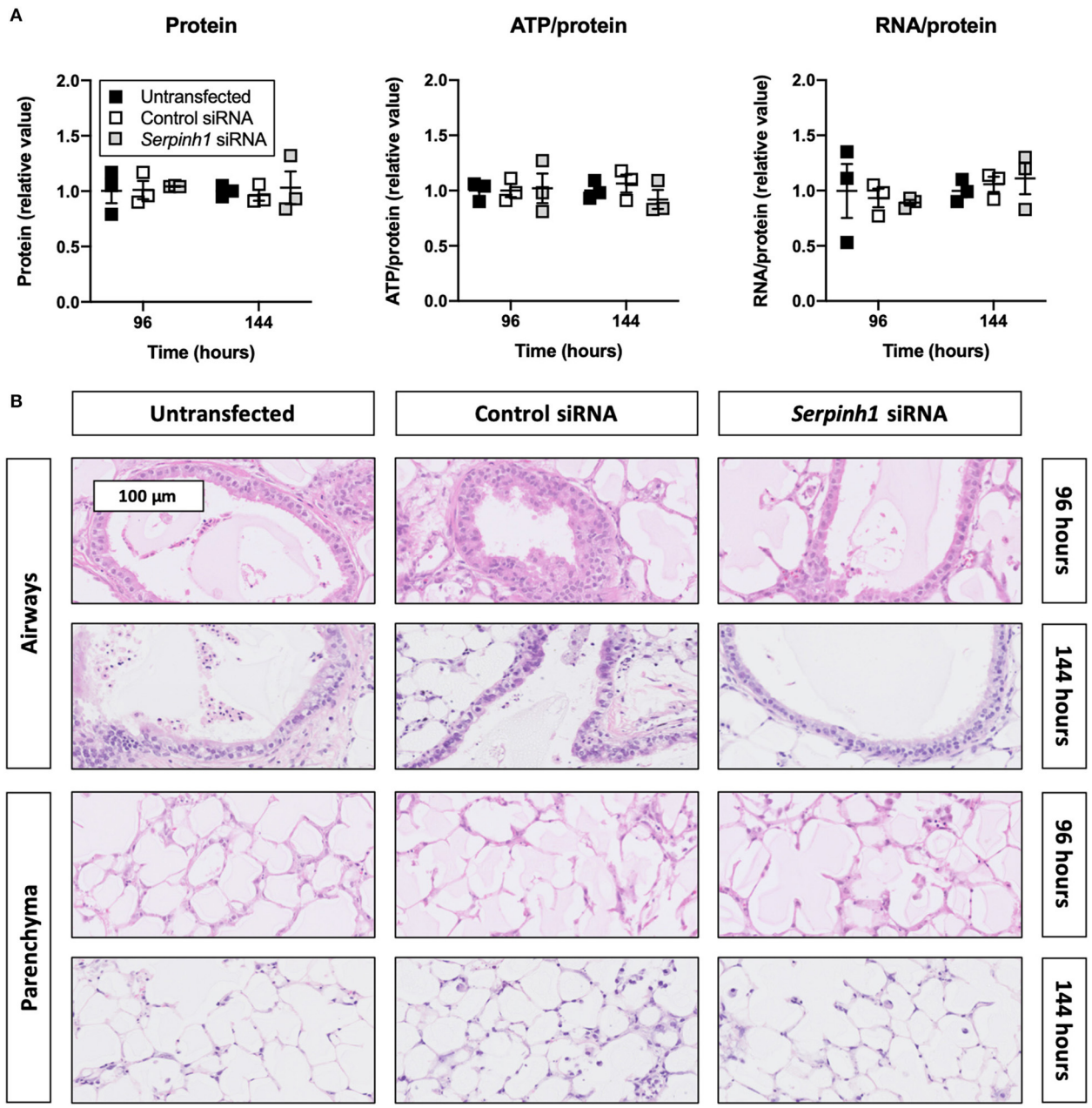

FIGURE 4 | Effect of Accell siRNA on the viability and morphology of slices. Untransfected and transfected slices were collected after 96 or 144 h of incubation with 5 ng/mL TGFβ1 ($n = 3$). Protein, ATP/protein, and RNA/protein content **(A)** were measured to study the viability, and HE stainings were conducted to assess the morphology **(B)**. Values represent individual experiments performed in triplicate and are accompanied with the arithmetic mean (horizontal line) ± standard error of the mean (error bars).

observed in slices that were treated with *Serpinh1*-targeting siRNA for 96 and 144 h. Non-targeting (control) siRNA did not cause non-specific knockdown of mRNA and protein levels.

HSP47 Knockdown Altered the Secretion of Fibronectin by Slices

To study whether knockdown of HSP47 affected fibrogenesis in TGFβ1-treated slices, we measured the expression of fibrogenesis-related genes, secretion of fibronectin into culture medium, and expression of α-SMA (**Figure 6**). As shown, no significant differences were observed between untransfected and transfected slices with respect to the expression of fibrogenesis-related genes and α-SMA. However, in comparison to untransfected slices, slices treated with *Serpinh1*-targeting siRNA displayed a significantly reduced (∼30%) secretion of fibronectin into culture medium after 144 h of incubation.

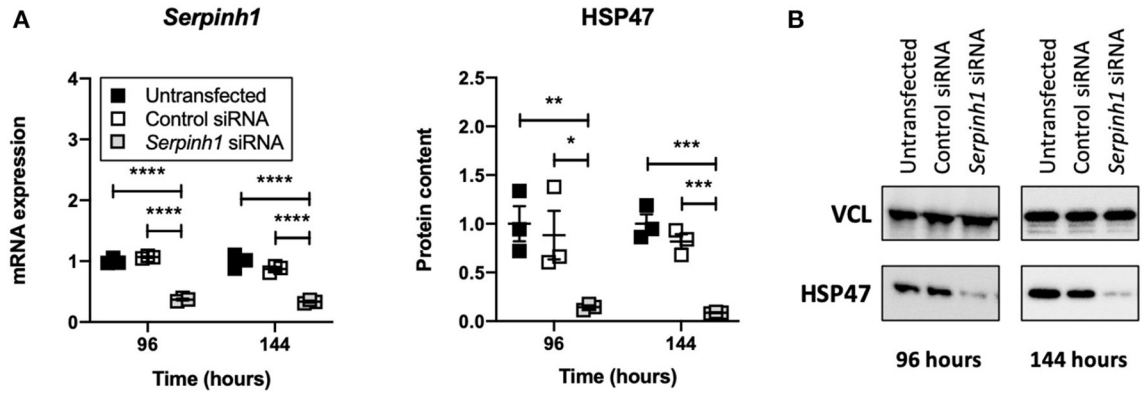

FIGURE 5 | Effect of Accell siRNA on mRNA and protein knockdown in slices. Untransfected and transfected slices were sampled after 96 or 144 h of incubation with 5 ng/mL TGFβ1 ($n = 3$). Expression of *Serpinh1* mRNA **(A)** and its respective protein HSP47 **(B)** were analyzed to verify whether Accell siRNA induced RNA interference. Values represent individual experiments performed in triplicate and are accompanied with the arithmetic mean (horizontal line) ± standard error of the mean (error bars). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

HSP47 Knockdown Did Not Diminish Collagen Secretion and Deposition in Slices

To determine whether knockdown of HSP47 affected the biosynthesis of collagen in slices, we measured the concentration of P1NP and trimeric COL1 in culture medium as well as the incorporation of COL1 trimers into the ECM (**Figure 7**). As demonstrated, no significant differences were observed between untransfected and transfected slices regarding the concentration of P1NP and trimeric COL1 in culture medium; nor was the incorporation of COL1 trimers into the ECM affected. Likewise, the formation of fibrillar COL1 and COL3 networks remained unaffected upon knockdown of HSP47.

DISCUSSION

The main objective of this study was to evaluate the therapeutic potential of *Serpinh1*-targeting siRNA in fibrogenic lung slices (**Figure 8**). Our study demonstrated that slices remained viable for up to 144 h of incubation, even when treated with TGFβ1 and Accell siRNA. In addition, TGFβ1 was shown to augment fibrogenesis as well as collagen secretion and deposition. We also observed specific and significant knockdown of HSP47 (∼90%) after culturing slices with *Serpinh1*-targeting siRNA for 96 or 144 h. Knockdown of HSP47, however, only affected the secretion of fibronectin into culture medium but no other aspects of fibrogenesis, such as mRNA expression of fibrogenesis-related genes, expression of α-SMA, and secretion of collagen as well as its incorporation into the ECM.

First of all, we analyzed protein, ATP/protein, and RNA/protein content as well as the morphology to unravel whether slices remained viable upon exposure to TGFβ1. In general, slices were viable and their morphology remained sufficiently preserved for up to 144 h in the presence of TGFβ1. Similar observations have been made for rat lung slices when they were cultured for 72 h with 10 ng/mL TGFβ1 as no differences in ATP/protein content were detected (24). Another study demonstrated human lung slices also maintained their mitochondrial activity and morphology for up to 120 h when treated with a "fibrosis cocktail," which was composed of 5 ng/mL TGFβ1, 5 μM platelet-derived growth factor AB, 10 ng/mL tumor necrosis factor alpha, and 5 μM lysophosphatidic acid (25). Though not strictly classifiable as precision-cut lung slices, cubic human lung explants (2 mm^3) were also recently shown to remain viable for up to 7 days of incubation when cultured in medium containing 10 ng/mL TGFβ1 (26). Nevertheless, due to the pleiotropic nature of TGFβ1, it cannot be ruled out that other aspects of viability were affected (e.g., cell death and/or proliferation). These findings should therefore be interpreted carefully.

After confirming that slices remained viable, we studied whether TGFβ1 augmented fibrogenesis. As demonstrated, mRNA expression of *Acta2*, *Col1a1*, *Fn*, *Serpine1*, *Serpinh1*, and *Tnfrsf11b* was clearly increased by TGFβ1, which controls a wide range of processes related to fibrosis (27). The initial decline of *Acta2* mRNA expression in slices that were not treated with TGFβ1 cannot be readily explained but we speculate it was caused by a loss of vascular smooth muscle cells because they also express this gene (28). Remarkably, in our current study, we observed a much greater induction of fibrogenesis-related genes than in our previous study (20). This can be explained by differences in incubator oxygen concentration; slices cultured at 20% O_2 (current study) are considerably more viable than slices incubated at 80% O_2 (previous study) (23). Secretion of fibronectin, which is a glycoprotein that connects ECM proteins to cells via integrins, was also increased upon exposure to TGFβ1 (29). Though we did not investigate the localization of fibronectin deposition, others have shown its deposition is generally more abundant in the outermost region of lung slices (25). Lastly, TGFβ1 appeared to only affect α-SMA expression after 96 h of incubation, albeit not significantly—perhaps, our slice incubation time was too short to reveal significant differences.

We then assessed whether TGFβ1 affected the concentration of P1NP and COL1 trimers in culture medium, the incorporation

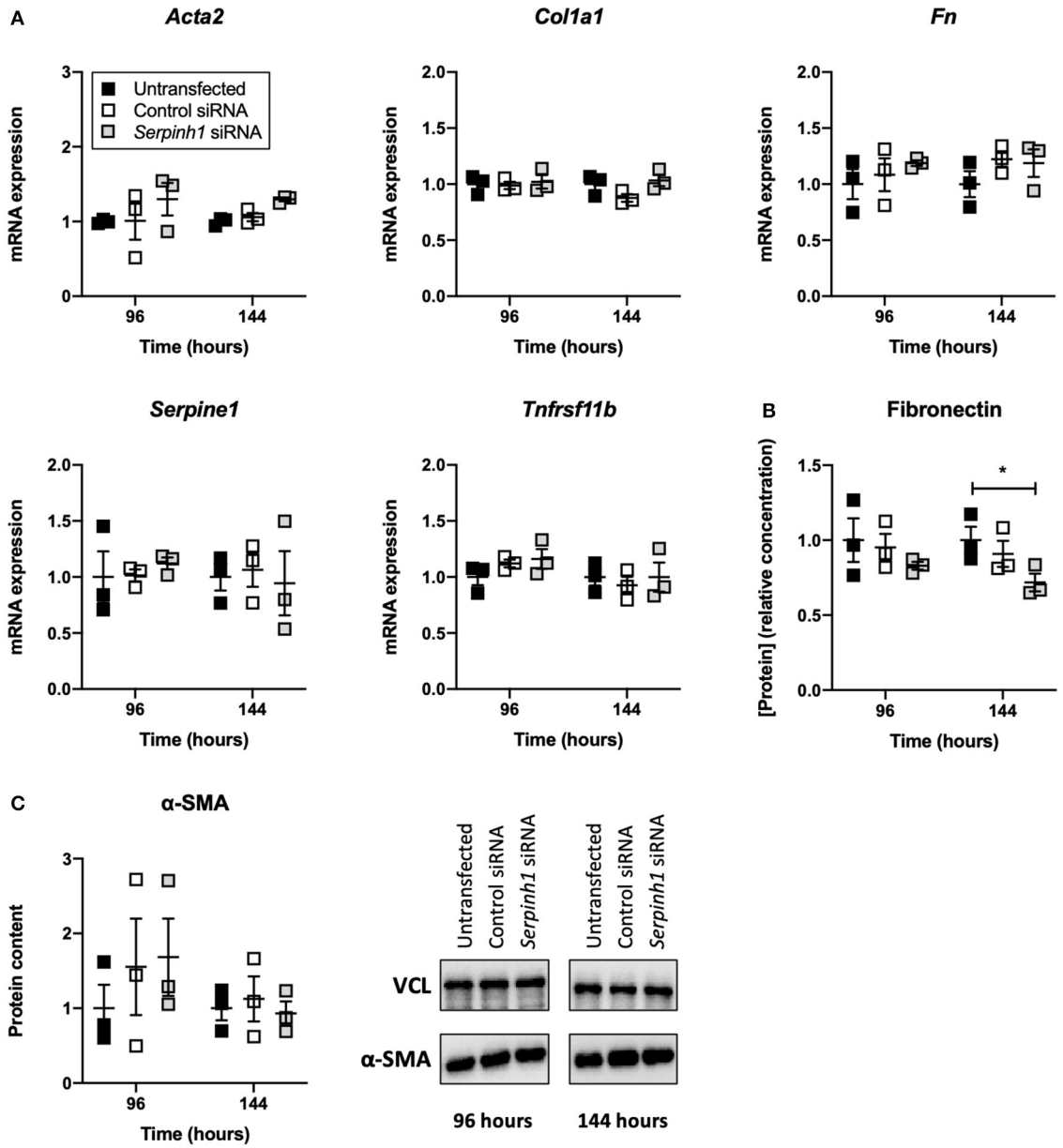

FIGURE 6 | Effect of HSP47 knockdown on the development of fibrogenesis in slices. Untransfected and transfected slices were collected after 96 or 144 h of incubation with 5 ng/mL TGFβ1 ($n = 3$). Expression of fibrogenesis-related genes **(A)**, secretion of fibronectin into culture medium **(B)**, and expression of α-SMA **(C)** were measured to identify potential downstream effects on fibrogenesis after knockdown of HSP47. Values represent individual experiments performed in triplicate and are accompanied with the arithmetic mean (horizontal line) ± standard error of the mean (error bars). (*$p < 0.05$).

of COL1 trimers into the ECM, and the formation of fibrillar COL1 and COL3 networks. In slices, the overall production of COL1 clearly increased over time and was further enhanced by TGFβ1, as demonstrated by P1NP ELISA. At first glance, this finding seems to contradict the steadily declining COL1 trimer concentration in the medium. However, this discrepancy can be explained by differences between the immunogens that were used for raising respective antibodies. The P1NP ELISA made use of antibodies raised against the *N*-terminal propeptide chains of COL1, whereas the COL1 trimer antibody was raised against trimeric COL1 with a triple-helix structure. Because the P1NP ELISA cannot differentiate between cleaved or uncleaved procollagens, these findings suggest COL1 trimers were misfolded (rendering epitopes unrecognizable) and/or incorporated into the ECM. The latter most likely occurred as COL1 trimer content in lysate was increased and fibrillar COL1 and COL3 networks became more prominent, as revealed by picrosirius red stainings.

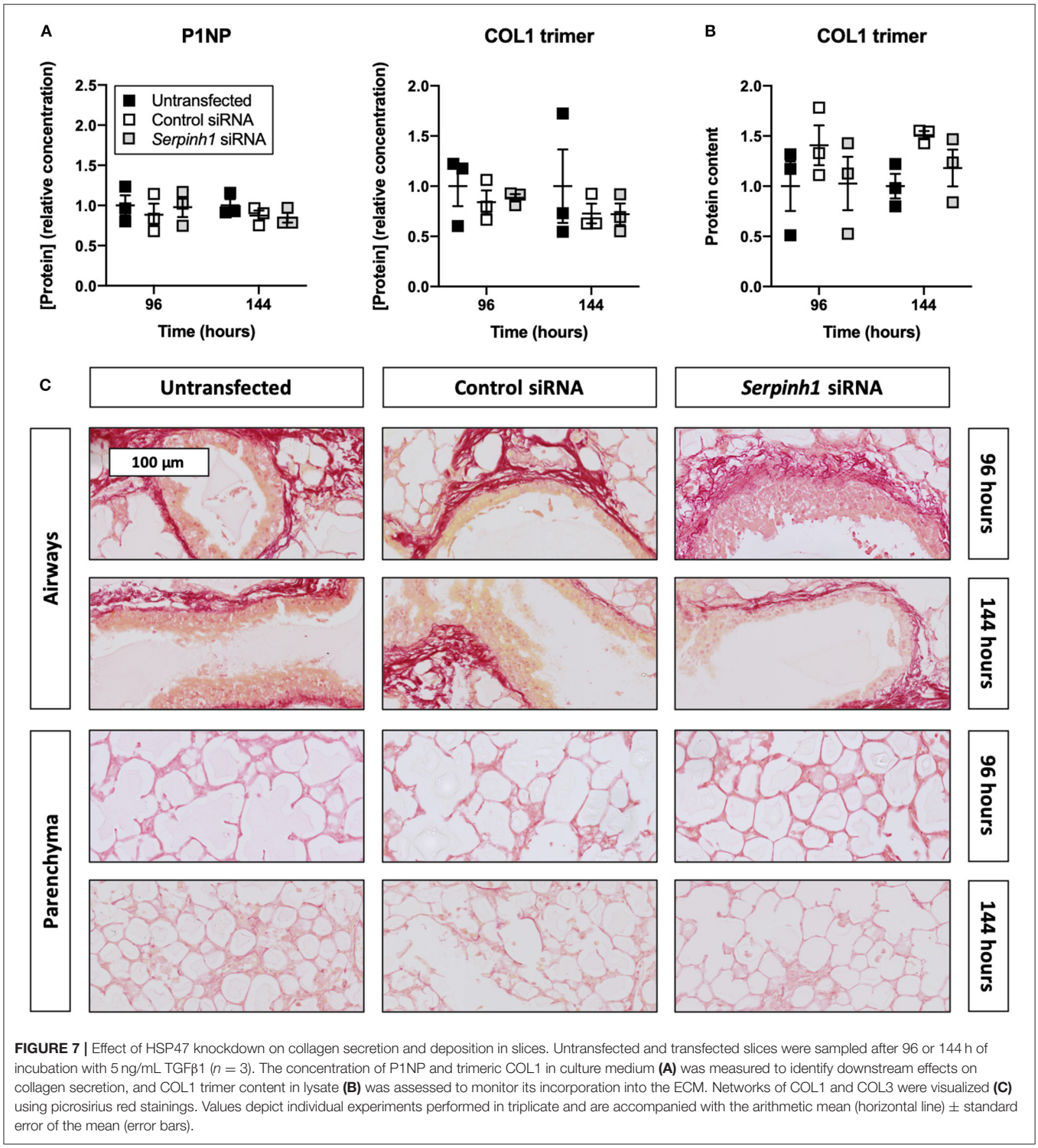

FIGURE 7 | Effect of HSP47 knockdown on collagen secretion and deposition in slices. Untransfected and transfected slices were sampled after 96 or 144 h of incubation with 5 ng/mL TGFβ1 ($n = 3$). The concentration of P1NP and trimeric COL1 in culture medium **(A)** was measured to identify downstream effects on collagen secretion, and COL1 trimer content in lysate **(B)** was assessed to monitor its incorporation into the ECM. Networks of COL1 and COL3 were visualized **(C)** using picrosirius red stainings. Values depict individual experiments performed in triplicate and are accompanied with the arithmetic mean (horizontal line) ± standard error of the mean (error bars).

Next, we validated whether our previously published transfection method could still be used to achieve mRNA and protein knockdown without affecting the viability and morphology of slices (19, 20). This was necessary because different incubation conditions were used in our current study (i.e., incubator oxygen concentration, culture medium composition, and presence of TGFβ1). As hypothesized, non-targeting and *Serpinh1*-targeting siRNA did not affect the viability or morphology of slices. Furthermore, *Serpinh1*-targeting siRNA induced significant mRNA and protein knockdown. These findings correlate well with our previous study and illustrate the usefulness of Accell siRNA to trigger

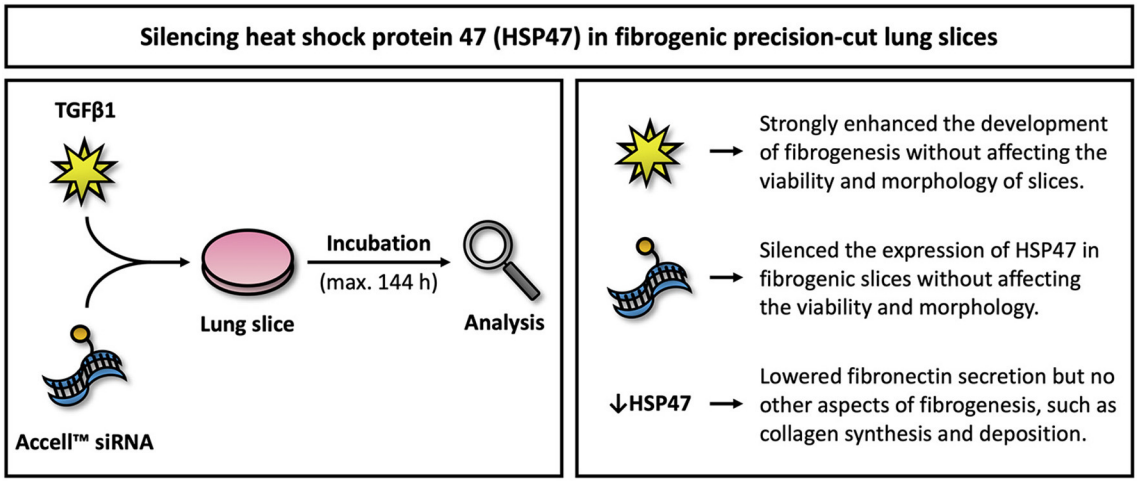

FIGURE 8 | Overview of key findings. This figure illustrates key findings of this study. Further studies are warranted to elucidate downstream effects on fibrosis upon silencing HSP47.

RNA interference in slices (20). Given that slices used in this study were more viable than those used in our previous study, we should consider our current findings as more relevant (i.e., slices represented the cellular microenvironment more accurately). Although other aspects of viability were not evaluated, sufficient evidence was collected to confirm Accell siRNA can be used to study the effects of *Serpinh1*-targeting siRNA in fibrogenic slices.

After confirming successful knockdown of HSP47, we found that neither the expression of fibrogenesis-related genes nor expression of α-SMA was affected. Aside from contradicting our previously published results (i.e., HSP47 knockdown lowered *Serpine1* and *Tnfrsf11b* mRNA expression in slices), our current findings also contradict other published studies that showed knockdown of HSP47 rapidly lowered mRNA expression of *Acta2* and *Col1a1* in mouse dermal fibroblasts, hepatic stellate cells, and embryo fibroblasts (17, 20, 30). In those studies, the quick onset of effects *in vitro* (<24 h) was achieved by using alternative transfection techniques. Cationic lipid-based transfection reagents, for instance, are typically taken up by cells very quickly (within a few hours), whereas uptake of Accell siRNA requires more time (20). Furthermore, as matrix stiffness regulates cell behavior, it cannot be ruled out that myofibroblasts cultured on plastic were phenotypically different from those in slices (31). Surprisingly, the secretion of fibronectin was lowered after knockdown of HSP47. This effect is not fully understood because, in mouse embryo fibroblasts, HSP47 has been shown not to interact directly with fibronectin or to affect its secretion (32).

Lastly, we studied whether collagen secretion and deposition were diminished in slices upon treatment with *Serpinh1*-targeting siRNA. Surprisingly, we did not identify any effects on collagen biosynthesis. Because HSP47 has been previously demonstrated to be essential for the formation of special secretory vesicles, it might have been the case that residual HSP47 (10%) was sufficient to enable the secretion of procollagens (32). This hypothesis could be tested using slices prepared from tissue of conditional HSP47-knockout mice. Furthermore, these findings contrast not only published *in vitro* studies but also *in vivo* studies; antifibrotic effects of *Serpinh1*-targeting siRNA have been previously observed in various fibrosis models, such as pulmonary, hepatic, renal, peritoneal, and dermal fibrosis (12–17, 32, 33). These studies, however, did not provide mechanistic insights into the effects over time. Effects on collagen biosynthesis were only determined after 3 or 4 weeks of (frequent) siRNA administration. The source of therapeutic effects in animals is therefore not entirely clear. Knockdown of HSP47 may have affected fibrosis through several, non-mutually exclusive mechanisms. Identifying these mechanisms will help us to better understand the therapeutic potential of *Serpinh1*-targeting siRNA. The use of advanced analytical techniques, such as single-cell sequencing, could help us to detect and characterize affected molecular mechanisms.

To gain more insight into these mechanisms, we explored whether HSP47 knockdown affected ER stress, autophagy, collagen processing, and ECM degradation (**Supplementary Figure 3**). As ER stress may have developed due to the accumulation of misfolded collagens, we measured mRNA expression of genes related to the unfolded protein response (UPR)—an evolutionary conserved stress response that becomes activated when unfolded and misfolded proteins accumulate in the ER (34). Knockdown of HSP47, however, did not significantly change mRNA expression of UPR-related genes in slices. We also studied whether autophagy was induced, but detected no significant increase in LC3B after silencing HSP47. Similarly, knockdown of HSP47 did not affect the expression of FKBP65, which interacts with HSP47 during the biosynthesis of collagens to facilitate their stabilization. Instead of solely affecting collagen biosynthesis, knockdown of HSP47 may have also caused therapeutic effects in animals by favoring the incorporation of misfolded collagens, making the ECM more vulnerable to degradation by matrix metalloproteinases. Nevertheless, we did not observe an increased degradation of COL3 in slices.

In addition to using siRNA, we also investigated whether COL003, which is a selective pharmacological inhibitor of HSP47, affected collagen biosynthesis in slices (**Supplementary Figure 4**). This compound has been previously shown to competitively inhibit interactions between HSP47 and procollagens in mouse embryo fibroblasts, thereby reducing collagen secretion (9, 35). In slices, however, the secretion of collagen was not affected by COL003 at its maximum tolerable concentration (5.0 μM); hence we did not conduct follow-up analyses regarding potential therapeutic effects or mechanisms. COL003 was also considerably more toxic in slices than in mouse embryo fibroblasts, which remained viable when cultured with 100 μM of COL003 (9). This discrepancy is probably caused by the fact that lung slices express biotransformation enzymes, which may have generated toxic metabolites (18). Safer and more effective analogs are therefore greatly desired to determine whether HSP47 is a suitable target for treating fibrosis.

CONCLUSION

The aim of this study was to evaluate the therapeutic potential of *Serpinh1*-targeting siRNA in fibrogenic lung slices. First, we demonstrated that fibrogenesis in slices could be augmented by TGFβ1 without affecting their viability and morphology. We subsequently showed that slices with a fibrogenic phenotype were successfully transfected with Accell siRNA, resulting in specific mRNA and protein knockdown. Surprisingly, upon knockdown of HSP47, the secretion of fibronectin was reduced while other aspects of fibrogenesis remained unaffected (e.g., differentiation of fibroblasts into myofibroblasts as well as collagen secretion and deposition). These observations are surprising as others have demonstrated that *Serpinh1*-targeting siRNA attenuated collagen deposition in animals when treated for 3 to 4 weeks. Although the exact source of therapeutic effects in animals remains unclear, the prospect of having a potential target for treating IPF should serve as a strong incentive for future research on HSP47.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Dutch Central Authority for Scientific Procedures on Animals.

AUTHOR CONTRIBUTIONS

MR, DL, JS, HF, WH, and PO contributed to the conception and design of this study. MR, KE, and JS carried out experiments and analyses. MR, HF, WH, and PO wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.607962/full#supplementary-material

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REVIEW
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Novel Immune Subsets and Related Cytokines: Emerging Players in the Progression of Liver Fibrosis

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Liver fibrosis is a pathological process caused by persistent chronic injury of the liver. Kupffer cells, natural killer (NK) cells, NKT cells, and dendritic cells (DCs), which are in close contact with T and B cells, serve to bridge innate and adaptive immunity in the liver. Meanwhile, an imbalanced inflammatory response constitutes a challenge in liver disease. The dichotomous roles of novel immune cells, including T helper 17 (Th17), regulatory T cells (Tregs), mucosa-associated invariant T cells (MAIT), and innate lymphoid cells (ILCs) in liver fibrosis have gradually been revealed. These cells not only induce damage during liver fibrosis but also promote tissue repair. Hence, immune cells have unique, and often opposing, roles during the various stages of fibrosis. Due to this heterogeneity, the treatment, or reversal of fibrosis through the target of immune cells have attracted much attention. Moreover, activation of hepatic stellate cells (HSCs) constitutes the core of fibrosis. This activation is regulated by various immune mediators, including Th17, Th22, and Th9, MAIT, ILCs, and γδ T cells, as well as their related cytokines. Thus, liver fibrosis results from the complex interaction of these immune mediators, thereby complicating the ability to elucidate the mechanisms of action elicited by each cell type. Future developments in biotechnology will certainly aid in this feat to inform the design of novel therapeutic targets. Therefore, the aim of this review was to summarize the role of specific immune cells in liver fibrosis, as well as biomarkers and treatment methods related to these cells.

Keywords: liver fibrosis, T helper cells, mucosa-associated invariant T cells, innate lymphoid cells, regulatory T cells, hepatic stellate cells

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INTRODUCTION

Liver fibrosis is a pathological process in which diffuse extracellular matrix (ECM) over precipitates in the liver due to abnormal hyperplasia of connective tissue caused by various pathogenic factors. The initiating event in liver fibrosis is the activation of hepatic stellate cells (HSCs), which promotes the production and accumulation of ECM (1). Liver fibrosis is a common pathological outcome of various chronic liver diseases (CLD), including chronic hepatitis B (CHB), chronic hepatitis C (CHC), non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), autoimmune hepatitis (AIH), and primary biliary cirrhosis (PBC). The treatment and prognosis of chronic liver disease depends on the degree of liver fibrosis. However, currently no treatment has demonstrated

the ability to reverse the progression of fibrosis in CLD. The aggravation of fibrosis may lead to cirrhosis, liver failure, or liver cancer, in which liver transplantation is performed as the last option (2). Therefore, early detection and inhibition of fibrosis progression is particularly important in the treatment of liver diseases.

The liver is an immune organ that plays a major role in innate and adaptive immunity. Its anatomical structure allows it to function as a filter for visceral blood, thus acting as the second line of defense for the intestinal immune system, preventing the entry of harmful substances from the intestinal tract, and their negative impact throughout the body (3). The high proportion of Kupffer cells (KCs), natural killer T (NKT) cells, γδ T cells, and dendritic cells (DCs), which are in close contact with antigen presenting cells, T cells, and B cells, serve to connect innate and adaptive immunity in the liver, while inducing immune tolerance, thereby avoiding immune responses from being mounted against foreign antigens that would otherwise cause tissue damage. These effects maintain the stability of hepatic microcirculation and tolerance to foreign antigens (4). Alternatively, inflammation generally precedes fibrosis, while immune cells are important factors in the regulation of fibrosis. Although immune cells can induce damage, they can also promote tissue repair in liver fibrosis (5). T cells and macrophages constitute the core of liver fibrosis pathogenesis with macrophage-derived transforming growth factor (TGF)-β1 known to be the strongest activator of HSCs (6). Recently, newly discovered immune cells, and their related cytokines, were shown to also participate in the process of liver fibrosis (**Table 1**). For instance, an imbalance in the ratio of regulatory T cells (Tregs)/T helper 17 cells (Th17) is characteristic of liver fibrosis progression. Indeed, some drugs function to restore the Tregs/Th17 balance, thereby alleviated liver fibrosis (7–11). Additionally, Th22, Th9, mucosa-associated invariant T (MAIT) cells, innate lymphoid cells (ILCs), γδ T cells, and their related cytokines, have been reported to regulate liver fibrosis (12–16).

The aim of this review is to provide a summary of the current understanding regarding the roles of innate immune cells in liver fibrosis, and the recent diagnostic and treatment outcomes for liver fibrosis achieved through targeting newly discovered immune cells. First, the review deals with immune cells and their associated cytokines known to promote hepatic fibrosis. Second, the roles of immune cells and their related cytokines playing in anti-hepatic fibrosis are discussed. Third, the dichotomous roles of certain immune cell types in fibrosis is discussed. Finally, conclusions and future perspectives are provided.

IMMUNE CELLS AND RELATED CYTOKINES IN PRO-HEPATIC FIBROSIS

T Helper 17 Cells (Th17)

Th17 cells are a subset of $CD4^+$ T cells characterized by RORγt expression and interleukin (IL)-17, IL-22, and IL-23 production. In acute and chronic liver injury, the amount, and proportion, of Th17 cells in the liver and peripheral blood increases. These cells have clear fibrogenic properties (17–19), with high levels of intrahepatic Th17 and IL-17 commonly observed in liver fibrosis caused by various etiologies, such as HBV (20), HCV (21), cholestatic liver injury (22), autoimmune hepatitis (23), and NAFLD (24). In fact, within a bile duct ligation (BDL) murine model, knockout of IL-17A resulted in reduced liver damage and fibrosis, accompanied by decreased levels of tumor necrosis factor (TNF)-α, TGF-β, and type I collagen in the liver compared to wild-type (WT) mice (22). Moreover, in mice with liver fibrosis induced by carbon tetrachloride (CCl4), the concentration of collagen and TGF-β in the liver of WT mice was significantly higher than in the liver of IL-17RA deficient mice. Meanwhile, *in vitro* experiments confirmed that IL-17A activates HSCs to produce collagen through the ERK1/2 and p38 signaling pathways (17). Moreover, in animal models of liver fibrosis induced by CCl4 and BDL, serum or liver IL-17 expression was positively correlated with the degree of liver fibrosis, while blocking IL-17 signaling weakened liver fibrosis. Furthermore, it has been shown that IL-17A promotes the transformation of HSCs into myofibroblasts and the production of collagen through the STAT3 signaling pathway (18).

However, Thomas et al. found that IL-17A does not directly promote HSC activation nor pro-fibrotic gene (*COL1A1, TIMP-I*, and *ACTA2*) expression, but rather requires TGF-β collaboration. Meanwhile, IL-17A upregulates and stabilizes TGF-β receptor II (TGF-βRII) expression on the surface of HSCs through the JNK signaling pathway and enhances SMAD2/3 phosphorylation to promote liver fibrosis (19). These differences may have been caused by differing experimental conditions. For instance, although both experiments conducted by Tan et al. (17) and Meng et al. (18), stimulated HSCs for 2–8 h, the latter study did not observe effects at this time point and thus, chose to further stimulate the cells for 48 h (19). Moreover, Meng et al. sought to exclude the effect of TGF-β in fetal bovine serum by performing the study under cell starvation conditions (19). Nevertheless, other studies have also confirmed that IL-17A does not induce the expression of fibrogenic genes, but rather promotes that of chemokines and pro-inflammatory factors in recruited macrophages, monocytes, and neutrophils (25, 26). Thus, IL-17 may recruit other cells to affect HSCs in complex hepatic fibrosis environments. Although advances have been made in identifying the underlying mechanism of Th17 cells and their cytokines in liver fibrosis, some challenges have arisen that require further clarification. For example, in HBV or HCV infected patients, the degree of liver fibrosis is significantly related to the virus replication rate *in vivo*. Moreover, Th17 cells and IL-17 promote viral clearance and have a certain antiviral role, similar to that of Th1 cells (27). However, both TH17 cells and IL-17 also aggravate inflammatory damage of the liver, leading to chronic HBV and HCV in patients. Due to the diverse functions of Th17 cells, determining how to exploit its anti-fibrotic effect while avoiding its pro-fibrosis potential, will serve to accelerate the clinical application of Th17 in the treatment of liver fibrosis.

T Helper 9 Cells (Th9) and IL-9

Th9 cells are a newly distinguished $CD4^+$ T cell subset characterized by the specific secretion of IL-9 and identified by PU.1 and IRF.4 (28, 29). IL-9 was originally mistaken as a type 2 cytokine until IL-4-induced differentiation of naïve $CD4^+$

TABLE 1 | The role of novel immune cells in liver fibrosis.

Cell type	Species	Molecules and signaling pathway	Effect in fibrosis	References
Th17	Mouse	IL-17	Pro-fibrosis	(17, 18)
	Human	IL-17	Pro-fibrosis	(20, 23, 24)
Th9	Mouse	IL-9	Pro-fibrosis	(13, 34)
MAIT	Mouse	TNF, TCR/MR1	Pro-fibrosis	(40)
	Human	IL-17A, TNF, TCR/MR1	Pro-fibrosis	(14, 40)
Treg	Mouse	CD39	Anti-fibrosis	(119)
	Human	TGF-β	Anti-fibrosis	(44)
		IL-8, TGF-β, CTLA-4	Pro-fibrosis	(45, 47)
Th22	Mouse	IL-22	Pro-fibrosis	(48, 62)
		IL-22	Anti-fibrosis	(12, 18, 61)
	Human	IL-22	Pro-fibrosis	(62, 63)
NK	Mouse	IFN-γ, RAE1/NKG20, NKp46, Ly49	Anti-fibrosis	(73, 77, 79, 80)
	Human	IFN-γ,TRAIL/NKG20, FasL/NKG20, NKp46	Anti-fibrosis	(75, 76, 79)
ILC2	Mouse	IL-33/IL-13	Pro-fibrosis	(85)
ILC3	Mouse	IL-17A, IL-22	Pro-fibrosis	(16)
γδ T	Mouse	IL-17A, CCR6/CCL20, FasL	Pro-fibrosis	(95, 96, 98)
		IFN-γ	Anti-fibrosis	(99)

Th, Helper T cell; IL, Interleukin; MAIT, Mucosa-associated invariant T cells; TCR, T cell receptor; MR1, Major histocompatibility complex MHC class I-related molecule; TGF-β, Transforming growth factor β; NK, Natural killer cells; IFN-γ, Interferon γ; RAE, Retinoic acid early induced transcript; NKG20, natural-killer group 2, member D; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FasL, Fas ligand; ILC, innate lymphoid cells; CCR6, CC chemokine receptor 6; CCL20, CC chemokine ligand 20.

T cells was found to generate a group of IL-9^+IL-10^+Foxp3^- T cells with no immunosuppressive capacity (30). IL-9 was further shown to be increased in the peripheral blood and liver of mice infected with *Schistosoma japonicum*, while its inhibition reduces procollagen-III (a fibrosis-related factor) expression in infected mice (31, 32). Consistently, intraperitoneal injection of anti-IL-9 antibody inhibits granulomatous inflammation in the liver and collagen deposition around the eggs of infected mice. Furthermore, direct stimulation of HSCs *in vitro* with IL-9 significantly increases the production of collagen and α-SMA (13). In addition, Th9 cells and IL-9 are increased in the blood of patients with HBV and HBV-related cirrhosis. This elevation is also present in the liver of mice with CCl4-induced liver fibrosis (33). Furthermore, Guo et al. demonstrated that CXCL10-induced IL-9 promotes liver fibrosis via the Raf/MEK/ERK signaling pathway in CCl4-induced mice (34). Hence, Th9 has clearly been shown to promote fibrosis. Consistent with studies in liver fibrotic diseases, IL-9 antibody treatment alleviates idiopathic pulmonary fibrosis and cystic fibrosis in mice (35, 36). However, Th9 cells have only been recently identified and investigated in the context of allergic reactions and parasitic infections. Therefore, the role and mechanism of Th9 cells in liver fibrosis require further analysis.

Mucosa-Associated Invariant T (MAIT) Cells

MAIT cells are a novel subset of innate-like T cells characterized by their invariant T cell receptor α-chain and their restrictive major histocompatibility complex related protein-1 (MR1), which are primarily distributed in the blood, liver, and intestinal mucosa (37). The innate functions of MAIT cells are similar to those of innate natural killer T cells (iNKT) and can be stimulated by IL-12 and IL-18 to secrete IFN-γ and granzyme (38). MAIT cells have antibacterial and immunological activities and present altered functions in chronic disease. The role of MAIT cells in liver fibrosis has been recognized due simply to their abundance in the liver, which accounts for ∼30% of all $CD3^+$ T cells present in the liver (39). In autoimmune liver disease, MAIT cells are significantly increased in the peripheral blood and liver; this increase is negatively correlated with the degree of liver fibrosis. *In vitro* studies further confirmed that IL-12 stimulates MAIT cells to produce large amounts of IL-17A. HSCs are activated by IL-17A and direct cell contact with MAIT cells, leading to HSC proliferation, pro-fibrosis, and pro-inflammatory gene expression (14). In animal models of alcoholic and non-alcoholic liver injury, MAIT cells promote the production of pro-inflammatory cytokines, such as IL-6 and IL-8 in mono-derived macrophages. Meanwhile, co-culture results demonstrate that MAIT cells promote fibroblast mitosis and pro-inflammatory properties through direct cell-cell contact (40). In addition, $MR1^{-/-}$ mice (MAIT-deficient) are resistant to liver fibrosis and have lower fibroblast density (40). Given the abovementioned results, MAIT cells play a crucial role in the process of liver fibrosis. However, the precise associated mechanism remains to be explored.

IMMUNE CELLS AND RELATED CYTOKINES IN PRO/ANTI-HEPATIC FIBROSIS

Regulatory T Cells (Tregs)

Tregs are a subset of immunosuppressive $CD4^+$ T cells characterized by transcription factor forkhead box P3 (Foxp3)

expression. The role of Tregs in liver fibrosis is complex and controversial. The number of circulating Tregs is positively correlated with the degree of liver fibrosis and serum HBV DNA load in HBV-infected patients (41). Meanwhile, Tregs inhibit HSC activation and proliferation, thereby ameliorating liver fibrosis (7, 8). However, given their immunosuppressive function, Tregs also act as a haven for hepatitis B viruses that are otherwise attacked by the immune system (41). Indeed, within HCV patients, hepatic $CD4^+Foxp3^+$ T cells are negatively correlated with liver fibrosis, whereas $CD4^+Foxp3^+$ Tregs in the blood of chronic HCV patients are less frequent than in healthy controls (42). Alternatively, Ward et al. observed no difference in the abundance of $Foxp3^+$ cells between mild and severe fibrosis in portal tract areas from HCV patients (43). Moreover, it remains unclear whether hepatic Tregs directly control HSCs and immune cells in the liver, or whether Tregs in lymph nodes or the spleen suppress the activation and migration of effector cells before infiltrating into the liver. Notably, although TGF-β is a recognized pro-fibrotic factor, that produced by Tregs in HCV negatively correlates with liver inflammation and fibrosis, suggesting that TGF-β also has anti-fibrotic properties (44). The authors suggest that this dichotomy may be due to the numerous cytokines, including IL-10, that are produced by peripheral immune cells following TGF-β stimulation, thereby effectively balancing the fibrogenic effects of TGF-β produced by other cells in the liver. Additionally, $IL\text{-}8^+CD4^+Foxp3^+$ T cells are abundant in the liver of HCV patients and are primarily distributed in the fibrosis and alpha-smooth muscle actin $(\alpha\text{-}SMA)^+$ region. Moreover, neutralization of IL-8 can block the activation of HSCs without affecting the immunosuppressive function of Tregs, suggesting that $IL\text{-}8^+$ Tregs participate in the promotion of fibrosis (45). Hence different Treg subgroups appear to have opposing effects.

The mystery of Tregs in liver fibrosis is also reflected in their regulation of other immune cells. In the BDL model, Treg depletion promotes Th17 and $CD8^+$ T cell infiltration in the fibrotic liver and increases the expression of inflammatory cytokines (IL-6, TNF-α, and IL-12p70) and chemokines (monocyte chemoattractant protein 1, macrophage inflammatory protein-1α, and regulated on activation, normal T-cell expressed and secreted chemokine), leading to the aggravation of fibrosis and suggesting that Tregs inhibit fibrosis by suppressing the formation of a pro-fibrotic niche by Th17 and $CD8^+$ T cells (46). In contrast, Tregs are enriched in liver fibrosis tissues and protect HSCs from NK cell killing in HCV patients. Tregs inhibit NK cell killing of HSCs in two ways. The first involves inhibiting NK cells by direct contact with cytotoxic T lymphocyte associated antigen-4 (CTLA-4); whereas the second, involves the production of IL-8 and TGF-β to inhibit HSCs from producing major histocompatibility complex (MHC) class I and MHC class I chain related protein A or B (MIC-A/B), which are required for NK cell activation (47). Additionally, in severe liver fibrosis, the number of Tregs in the liver is higher than that in moderate fibrosis and is positively correlated with serum ALT levels, suggesting that Tregs may be recruited to control liver cell damage (48). In addition, the immunosuppressive regulatory effect of Tregs is conducive to the formation of chronic inflammation, which maintains liver fibrosis (49). Tregs also inhibit the secretion of matrix metalloproteinas (MMPs) by KCs through TGF-β, thereby limiting liver fibrosis regression. Meanwhile, depletion of Tregs with anti-CD25 antibodies accelerates fibrosis regression in CCl4-induced liver fibrosis mice (50). Therefore, the role of Tregs is not entirely opposed to that of Th17, and may depend on the cause of liver injury, the stage of fibrosis, and the interaction between different immune cells.

Recently, Tregs were identified in visceral adipose tissue (VAT) and are now widely accepted as associated with glucose metabolism and insulin resistance (51). In obese mice induced by a high-fat diet, metabolic syndrome and non-alcoholic steatohepatitis (NASH) occur accompanied by a decrease in the proportion of Tregs in VAT (52). VAT Tregs relieve insulin resistance and glucose metabolism disorders caused by a high-fat diet in mice (51). Conversely, consumption of VAT Tregs increases the expression of inflammatory cytokines, such as TNF-α, IL-6, and C-C chemokine ligand 5 (CCL5) and promotes insulin resistance in adipose tissues (53, 54). PPARγ, a transcription factor that regulates adipocyte differentiation, is specifically expressed in VAT Tregs (55). Disabling PPARγ on Tregs results in a decrease in VAT Tregs, while Tregs in the lymphoid organs are not affected (56). In contrast, exogenous injection of a PPAR agonist (pioglitazone) in high-fat diet mice increases the number of VAT Tregs, reduces local inflammation, and improves organic metabolism. Furthermore, mice with knocked out PPARγ expression in Tregs are less responsive to pioglitazone treatment, demonstrating that VAT Tregs constitute a key factor in the regulation of insulin sensitization (55). Considering that insulin resistance promotes the development from simple fatty liver to NASH and is a common risk factor for NAFLD (57, 58), it is reasonable to assume that VAT Tregs participate in the regulation of NASH development. Revealing the mechanism whereby VAT Tregs regulate NAFLD will, therefore, contribute to elucidating the role of VAT Tregs in liver fibrosis.

T Helper 22 (Th22) Cells and IL-22

Th22 cells constitute a newly discovered subset of $CD4^+$ effector T cells that produce a high level of IL-22 rather than IL-17 or interferon (IFN)-γ. These cells are induced by IL-6 and TNF from naïve $CD4^+$ T cells. The characteristic transcription factor of Th22 cells is the aryl hydrocarbon receptor (AHR) (59). Th22 cells participate in chronic inflammation, autoimmune diseases, and cancers. Moreover, the IL-22 receptor (IL-22R) is a heterodimer composed of IL-22R1 and IL-10R2. Among them, IL-22R1 is primarily expressed on epithelial cells located in the skin and the lumen of the digestive and respiratory tracts, thereby determining the primary locations where IL-22 exerts its effects (60). However, the role of Th22 cells and IL-22 in liver fibrosis remains controversial. In CCl4-induced liver fibrosis mice, the proportion of Th22 cells in the spleen is higher than that in WT mice, and is accompanied by increased IL-22 levels in the serum and liver, suggesting that the microenvironment of liver fibrosis is conducive to the differentiation and proliferation of Th22 cells (61). Researchers who hold the view that IL-22 has

a pro-fibrotic effect have found that IL-22 relies on MAPK to promote TGF-β signaling in HSCs and induce HSCs to produce more α-SMA (48). In patients with hepatitis B cirrhosis, the infiltration of IL-22 positive cells in the liver is significantly higher than that in healthy individuals and is positively correlated with the stage of liver fibrosis. Furthermore, in HBV transgenic mice, increased IL-22 aggravates chronic liver inflammation and fibrosis by secreting CCL10 and CCL20 to recruit Th17 cells (62). Consistently, IL-22 and IL-22(+) cells are significantly increased in the peripheral blood of HCV patients, while the number of IL-22(+) cells in the liver is positively correlated with the liver fibrosis score. Further, IL-22(+) cells are primarily distributed within the fibrotic area. *In vitro* experiments have confirmed that IL-22 inhibits LX-2 (HSC line) cell apoptosis, while promoting their proliferation as well as the production of α-SMA and collagen (63).

However, several experiments have also confirmed the anti-fibrotic and protective effects of IL-22 in the liver. IL-22 resists fibrosis by inducing senescence of activated HSCs through SCOS3, p53, and STAT3 (12). Additionally, *in vivo* injection of IL-22 in BDL mice reduces collagen α1 (I) and α-SMA production to alleviate liver fibrosis (18). Moreover, administration of IL-22 inhibits HSC activation, reduces the production of pro-inflammatory factors (IL-1β, IL-6, and TNF-α), and ameliorates liver fibrosis in CCl4-induced liver fibrosis (61). In the liver of HFD-fed mice, CXCL1 is overexpressed and promotes steatosis-to-NASH progression by inducing neutrophil infiltration, oxidative stress, and stress kinase activation. However, IL-22 treatment blocks hepatic oxidative stress and its associated stress kinases via induction of metallothionein. Furthermore, although it does not target immune cells, IL-22 treatment attenuates the inflammatory functions of hepatocyte-derived, mitochondrial DNA-enriched extracellular vesicles, thereby suppressing liver inflammation in NASH (64). The functional differences of IL-22 may be related to the diversity of its sources. Indeed, various immune cells in the liver, such as Th1, Th17, Th22, γδ T, and NKT cells can produce IL-22 (65). However, none of the abovementioned studies has identified the specific cellular source of IL-22, the identification of which may provide targets for clinical therapeutic strategies. In addition, IL-22 and IL-17 are both type 3 cytokines, which can be produced simultaneously in chronic inflammatory diseases (48). In the absence of IL-17, Th22 has a protective effect against NASH. However, in the presence of IL-17, IL-22 recruits Th17 to aggravate liver fibrosis (66). Moreover, the pro-inflammatory and anti-inflammatory role of IL-22 has been shown to be regulated by IL-17 in airway inflammation (67). Thus, determining the source of IL-22 and the effect of other cytokines, such as IL-17, on IL-22 will help us better understand the role of IL-22 in liver fibrosis.

Innate Lymphoid Cells

ILCs are a group of heterogeneous lymphocytes involved in innate immunity. They do not express the antigen-specific receptors of T or B cells and are largely distributed at mucosal barrier sites where they participate in immune surveillance and regulation (68). ILCs are divided into three groups: Group 1 (ILC1 and NK cells, dependent on T-bet and producing IFN-γ), Group 2 (ILC2, dependent on GATA3 and RORα and producing type 2 cytokines, such as IL-13 and IL-5), and Group 3 (ILC3 and lymphoid tissue-inducing cells, dependent on RORγt and producing IL-17 and IL-22). The functions of ILC1, ILC2, ILC3, and NK cells correspond to Th1, Th2, Th17, and $CD8^+$ cytotoxic T cells, respectively (69). ILCs are distributed differently in different organs. The $NKP44^+$ ILC3 type predominates in the gut, where it acts as a mucosal barrier by producing IL-22. However, the $NKP44^-$ ILC3 type predominates in the liver, and have the potential to differentiate into other ILCs. $NKP44^-$ ILC3 is the only type present in fetal liver, while other ILCs can be detected with prolonged pregnancy (70).

The role of ILC1 in liver fibrosis, however, is yet to be reported. Nabekura et al. demonstrate the protective role of ILC1s in a mouse model of CCl4-mediated moderate acute liver injury. CCl4-mediated acute liver injury results in ATP and IL-12 production from DCs that activates ILC1s to produce IFN-γ. This results in upregulation of Bcl-2 and Bcl-xL by hepatocytes leading to reduced cell death and liver damage (71). Furthermore, Wang et al. found that group 1 ILCs in adipose tissues aggravate adipose fibrosis and promote the development of diabetes (72). However, since NK cells and ILC1 were not studied separately, the role of ILC1 in fibrosis could not be clearly defined.

As for NK cells in liver fibrosis, activated NK cells kill HSCs by producing IFN-γ (73–75). In addition, NK cells induce apoptosis of HSCs by direct cell contact, which involves Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and natural killer group 2, member D (NKG2D) (76). Early activated HSCs produce large amounts of retinoic acid, leading to increased expression of RAE-1 (retinoic acid early inducible 1), a ligand that activates NK cell receptor NKG2D. RAE-1 and MICA synergically trigger NK cells to kill HSCs (77, 78). Additionally, Chamutal et al. reported that primary human and mouse HSCs express unknown ligands for human NKp46 and mouse NCR1 receptors, respectively, to mediate the killing of HSCs by NK cells (79). In addition to the activation-related receptors on NK cells, inhibitory receptors Ly-49 are also reportedly involved in NK cell killing of HSCs mediated by MHC I molecules (80, 81). Notably, NK cells preferentially help to eliminate senescent HSCs and contribute to the regression of liver fibrosis (78). Besides, other immune cells can also regulate the interaction between NK cells and HSCs. In immune stimulatory conditions, such as viral liver disease or Toll-like receptor stimulation, KCs and DCs promote NK cell activation (82–84). Meanwhile, Tregs can inhibit NK cell activation to protect HSCs (47). Although the molecular mechanism underlying the NK cell anti-liver fibrosis phenomena has been extensively studied, it remains unclear whether liver resident or non-resident NK cells limit fibrosis. Moreover, the details of the interaction between NK cells and HSCs muse be further revealed before NK cells can become an immune target for anti-fibrosis strategies.

It is widely accepted that ILC2 promotes liver fibrosis. ILC2 increases at the site of hepatic fibrosis and is positively correlated with the degree of hepatic fibrosis (15). In the CCl4-induced liver fibrosis model, collagen deposition is significantly reduced following ILC2 cell depletion (85). The fibrogenic effect of ILC2

is dependent on the IL-33/IL-13 signaling pathway. Meanwhile, during chronic liver injury, increased release of IL-33 leads to the accumulation and activation of ILC2 cells in the liver through ST2 receptors on the surface of ILC2. Activated ILC2 cells then produce IL-13, which in turn activates HSCs in an IL-4Rα- and STAT6-dependent manner to aggravate liver fibrosis (85). Meanwhile, liver fibrosis is alleviated in mice lacking ST2 or IL-13, however, transfusion of ILC2 restores fibrosis (85). In addition to IL-33, thymic stromal lymphopoietin (TSLP) and IL-25 are major cytokines that drive type 2 immunity. Moreover, the activation of ILCs by the above three cytokines has been reported to lead to fibrosis in the lung and skin (86–89). Consistent with the results in other organs, simultaneously blocking IL-33, IL-25, and TSLP can improve liver and lung fibrosis and reduce IL-13 production by ILC2 in mice injected with *Schistosoma mansoni* eggs. However, other studies have found that the proportion of $CD4^{+}IL\text{-}13^{+}$ T cells increases following infection with *Schistosoma mansoni* eggs, which is accompanied by decreased ILC2 activity, suggesting that adaptive immunity may gradually replace IL-33, IL-25, and TSLP-ILC2 to maintain liver fibrosis progression (86). Although the IL-33/IL13 axis clearly promotes liver fibrosis, the specific contribution of ILC2 and type 2 immune cells remains to be investigated.

The role of ILC3 in fibrosis has only recently been discovered. In HBV patients, ILC3 is increased in peripheral blood and is positively correlated with the degree of fibrosis and inflammation. Moreover, co-culturing ILC3 with LX-2 cells demonstrated that ILC3 cells activate HSCs by producing IL-17A and IL-22. Furthermore, transferring ILC3 from normal mice to CCl4-induced *Rag-1*$^{-/-}$ mice leads to HSC activation, ECM accumulation, and aggravation of hepatic fibrosis (16). Additionally, RORγt^{+} ILCs exert a partial protective role in the hepatic immune response induced by CCl4 (90). However, the study does not distinguish between ILC3 and lymphoid tissue-inducing cells (LTi).

LTi cells are essential for peripheral lymphoid organ and tissue development (91). These cells secrete IL-17 and IL-22 in groups, both during embryo development and after birth. Activated LTi cells also produce a large number of cytokines and chemokines during induction of peripheral lymphoid organ/tissue formation, leading to lymphocyte and DC cell aggregation (92). Therefore, LTi cells have pro-inflammatory properties and are likely to participate in the inflammatory processes associated with most diseases, including liver fibrosis. However, the specific molecular mechanisms remain to be investigated.

γδ T Cells

γδ T cells make up 3–5% of total lymphocytes and 15–25% of T cells in the liver (93). These cells represent a double-edged sword in liver fibrosis. Wang et al. demonstrate that macrophages increase the number of IL-17A-producing γδT cells through the HMGB1-TLR4-IL-23 signaling pathway, recruit neutrophils to infiltrate the liver, and aggravate liver inflammation (94). Furthermore, in mice infected with *Schistosoma japonicum*, Vγ2 γδ T cells recruit neutrophils to granuloma and the liver by producing IL-17A, thereby aggravating liver fibrosis (95). However, γδ T cells do not only interact with immune cells to affect the fibrosis process but are also regulated by HSCs. In CCl4-induced acute liver injury and early stage liver fibrosis, exosomes released by hepatocytes bind to TLR3 and activate HSCs to produce IL-17A, which promotes the production of IL-17A by hepatic γδ T cells to aggravate liver fibrosis (96). However, it has also been argued that exosomes directly promote the production of IL-17 by γδ T cells (97). In addition to promoting fibrosis, γδ T cells have also been found to ameliorate liver inflammation and fibrosis. In two chronic liver injury mouse models (CCl4 and methionine-choline-deficient diet), γδ T cells are recruited to the liver through the activation of the CCR6/CCL20 signaling pathway and directly promote HSC apoptosis in a FasL-dependent manner to limit liver fibrosis (98). Liu et al. found that γδ T cells (particularly IFN-γ-producing subsets) protect the liver from fibrosis by killing activated HSCs directly or indirectly by enhancing NK cell-mediated cytotoxicity (99). Collectively, inducing the cytotoxicity of γδ T cells against HSCs can display an anti-fibrosis role, while promoting the production of IL-17 by γδ T cells aggravates fibrosis.

MICROBIOTA AND LIVER FIBROSIS

The liver is exposed to gut-derived bacterial metabolites and their products through the portal vein (100). Normally, the liver maintains a delicate balance between inflammatory and regulatory immune responses. However, when gut microbiota becomes altered, microbial stimuli affect the function of immune cells in the liver and ultimately lead to the development of liver disease (101). Liver inflammation reshapes intestinal microbiota through an unknown mechanism, leading to increased *Lactobacillus* (especially *L. johnsonii*) abundance in the gut. During the recovery stage of acute liver injury induced by concanavalin A, *Lactobacillus* was found to activate intestinal ILC3 cells to produce IL-22, which repairs the intestinal mucosal barrier and blocks further metastasis of gut microbiota to the liver. Moreover, IL-22 can induce the production of IL-10 and TGF-β by recruiting regulatory DC cells to the liver to maintain immune tolerance (102). Additionally, Hendrikx et al. report that the gut microbiota regulate ILC3 cells to reduce progression of ALD. In chronic-binge ethanol feeding mice, intestinal microbiota derived AHR ligand indole-3-acetic acid are reduced, resulting in decreased IL-22 production by ILC3s. IL-22 can also regulate the expression of intestinal REG3G, which protects mice against ethanol-induced liver disease by reducing bacterial translocation. In fact, supplementation with *Lactobacillus* to produce IL-22 effectively reduces liver damage and bacterial translocation to the liver (103). Hence, considering that systemic injections of IL-22 increase the risk of hepatocellular carcinoma in patients with CLD (104–106), altering the gut microbiota to regulate the immune cells that produce IL-22 may offer a more viable option for liver injury therapeutic interventions.

In addition to ILC3, MAIT cells are also influenced by gut microbiota in ALD. Fecal extracts from patients with ALD have reduced blood MAIT cells that are hyperactivated and exhibit defective antibacterial cytokine/cytotoxic responses (37). Moreover, in an intrahepatic cholangitis model, gut *L gasseri*

are enriched and translocate to the liver, where they amplify IL-17$^+$ γδ T cells to promote liver fibrosis and inflammation (107). Microbial-derived lipids are presented to γδ T cell receptors through CD1d on hepatocytes, which activates γδ T cells to express IL-17A, thereby aggravating NAFLD. Notably, this is unique to hepatic γδ T cells, and cannot be applied to circulating γδ T cells (108). Overall, these studies suggest that gut microbiota helps to shape the liver immune response. Although evidence does not directly suggest that an altered gut microbiota affects the role of novel immune cells in liver fibrosis, intestinal microbiota represents the core of the gut-liver axis that has been shown to drive many liver diseases to different stages. Therefore, it has important value and far-reaching significance for research in this field.

CHRONIC INFLAMMATION IN LIVER FIBROSIS

Inflammation is present in all the stages of liver fibrosis, cirrhosis, and hepatocellular carcinoma. Meanwhile, persistent activation of inflammatory responses contributes to the expansion of liver fibrosis (109). Degeneration and necrosis of hepatic parenchyma cells caused by various factors leads to the release of the inflammasome, which recruits and activates inflammatory cells. Activated inflammatory cells (particularly KCs) secrete TGF-β1, TNF-α, PDGF, and other factors, which promote the transformation of HSCs, or other fibrogenic cells, into myofibroblasts. Myofibroblasts continue to secrete and deposit extracellular matrix, and ultimately form hepatic fibrosis (110). Intrinsic cells and immune cells in the liver and HSCs together establish a complex regulatory system. These novel immune cells and their related cytokines do not only directly regulate HSCs and fibrogenesis, but also indirectly affect liver fibrosis by influencing the inflammatory microenvironment. For instance, IL-17 stimulates STAT3-mediated human endothelial cell activation and production of GRO-α, GM-CSF and IL-8, which regulate neutrophil recruitment to the liver (111). IL-17 can also induce HepG2 to produce IL-6 through activation of MAPK. Consequently, IL-6 stimulates Th17 cells and forms a positive feedback loop in AIH (23). Meanwhile, IL-17 recruits other inflammatory cells and promotes the synthesis of pro-inflammatory cytokines to exacerbate the inflammatory process, which triggers and maintains the differentiation of profibrogenic cells into myofibroblasts to amplify fibrosis. In addition to IL-17, TNF signaling controls NLRP3 inflammasome activation in myeloid derived cells to initiate liver inflammation, via recruitment of neutrophils and pro-inflammatory macrophages, leading to subsequent activation of fibrogenic pathways (112). Furthermore, the inhibitory regulation of Tregs favors the formation of chronic inflammation and contributes to the persistence of liver fibrosis. Specifically, Tregs suppress NK cells, M1 KCs, and CD8$^+$ T cells to maintain chronic liver inflammation and fibrosis (49). Hence, application of drugs capable of regulating the liver immune microenvironment while inducing the related cells to support the reversal of liver fibrosis may represent a new strategy for treating liver fibrosis.

CLINICAL RELEVANCE

The novel immune cells discussed in this review are important players in the pathogenesis of liver fibrosis. Hence, regulating their functions may represent a therapeutic strategy for the treatment of liver fibrosis. Currently, studies have shown that abrogating Th17/IL-17 signaling alleviates liver fibrosis. For instance, in *Schistosoma japonicum*-infected mice, a selective RhoA-Rho-associated kinase (ROCK) inhibitor (fasudil) limited liver fibrosis by inhibiting Th17 differentiation and IL-17 production, and upregulating Tregs (113). Moreover, abrogating inducible co-stimulator (ICOS) signaling reportedly inhibits Th17 cells, and their related cytokines, thereby reducing granulomatous inflammation and liver fibrosis around the eggs in a *Schistosoma japonicum* infection model (114). Mesenchymal stem cells have also been reported to restrict liver fibrosis by inhibiting Th17 cells (115, 116). In addition, miR-29a/miR-652, 1, 25(OH)2D3, as well as certain drugs, such as rapamycin and tofacitinib, have been shown to attenuate liver fibrosis by regulating Th17 cells (10, 11, 117, 118). Meanwhile, low dose IL-2 specifically expands and activates Treg cell populations thereby controlling autoimmune diseases and inflammation. Additionally, IL-2 and IL-2 immune complexes promote the expression of CD39 on hepatic Tregs, which inhibits the proliferation of CD8$^+$ T cells and reduces the expression of osteopontin and TNF-α to diminish biliary fibrosis in murine sclerosing cholangitis (119). Cumulatively, these results provides a theoretical basis for the treatment of fibrosing cholangiopathies with low dose IL-2. In addition to limiting liver fibrosis by targeting the novel immune cells, as described above, they may also be applied for disease prediction. In fact, γδ T cells gene signature can predict the overall and recurrence free survival of patients with HCC. Tumor microenvironments recruit γδ T cells from peripheral or peritumor regions into tumors to elicit anti-tumor effects (120). Chronic liver inflammation and fibrosis are necessary processes in the development of HCC. Therefore, if we can effectively monitor these novel immune cells, and their related molecules, as non-invasive diagnostic markers, it will be of great benefit to patients with chronic liver disease.

CONCLUSIONS

The cellular and molecular mechanisms of liver fibrosis are currently under intense investigation. Although the reversibility of liver fibrosis provides an effective early opportunity for treatment, no ideal anti-fibrotic drug is currently available for clinical practice. The activation of HSCs constitutes the core of fibrosis and is regulated by various immune mediators. Recently, novel immune cells have been discovered whose role in liver fibrosis has also been gradually recognized (**Figure 1**). Additionally, the proportion of regulatory B cells (Bregs) in peripheral blood has been positively correlated with the stage of liver fibrosis in HBV patients. Bregs inhibit effector T cells, however, enhance the function of Tregs to regulate immune tolerance in HBV-infected patients (121). In addition to regulating fibrosis by acting on HSCs, other cells also affect fibrosis controlled by the novel immune subsets and cytokines.

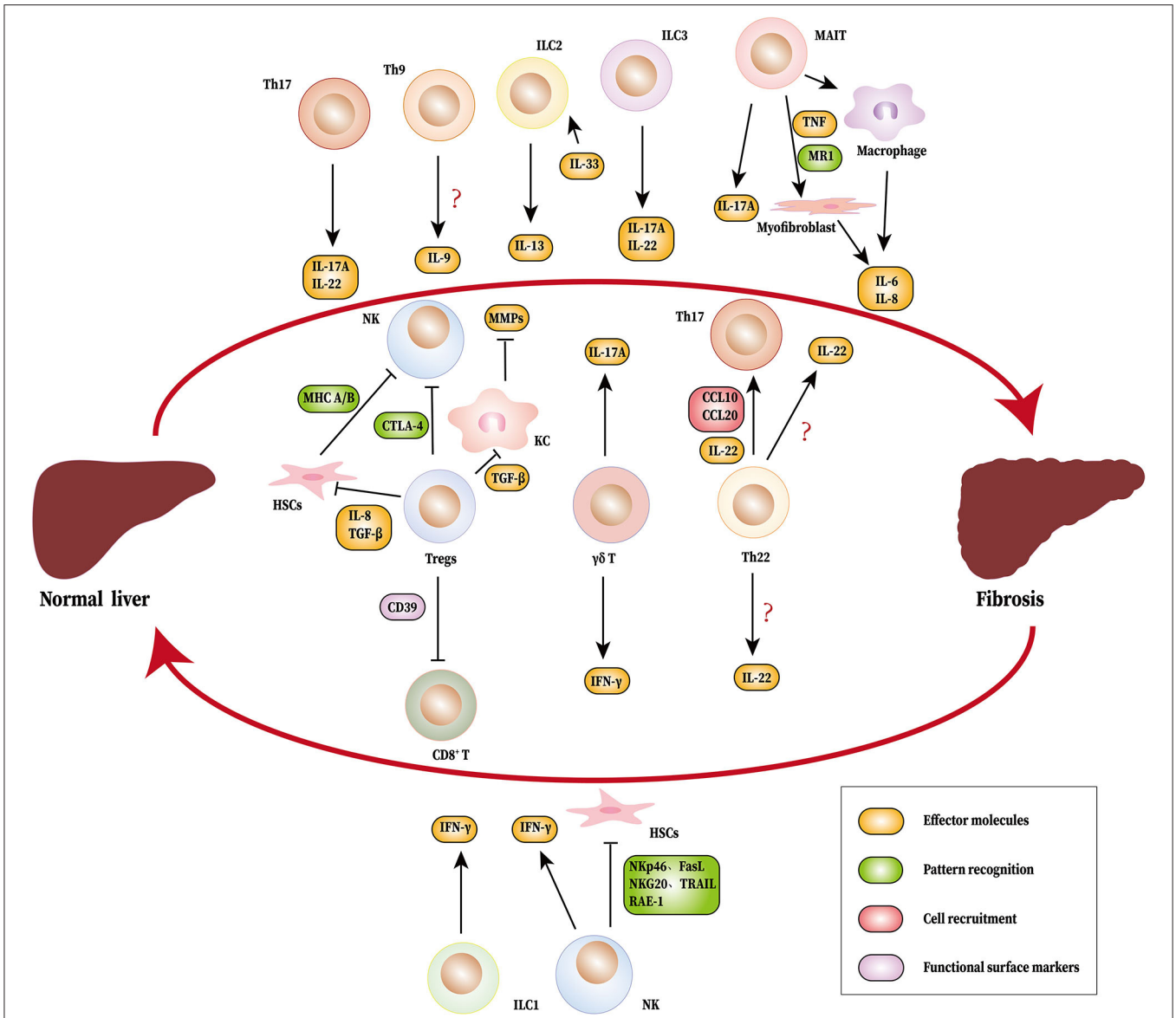

FIGURE 1 | Novel immune cells and related cytokines regulate liver fibrosis. During the process of liver fibrosis, Th17 (IL-17A and IL-22), MAIT cells (IL-17A), ILC2 (IL-13), ILC3 (IL-17A, IL-22), and γδ T cells (IL-17A) promote fibrosis. MAIT cells can induce myofibroblasts and macrophages to produce IL-6 and IL-8. Tregs inhibit NK cells directly or indirectly by HSCs. Tregs also suppress KC to produce MMPs by TGF-β. Th22 cells recruits Th17 to the liver through IL-22, CCL6, and CCL20. NK cells, ILC1 and γδ T cells produce IFN-γ, which limits fibrosis. NK cells also suppress liver fibrosis by inhibiting HSCs. The source of IL-9 and IL-22 have not been identified. HSCs, hepatic stellate cells; Th, T helper cells; MAIT cells, mucosa-associated invariant T cells; ILC, innate lymphoid cell; NK cells, natural killer cells; KC, Kupffer cells; IL, interleukin; IFN-γ, Interferon γ; TGF-β, Transforming growth factor β; MMPs, matrix metalloproteinas; RAE-1, Retinoic acid early induced transcript 1; NKG20, natural killer group 2, member D; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FasL, Fas ligand; CCL, CC chemokine ligand; TNF, tumor necrosis factor; CTLA-4, cytotoxic T lymphocyte antigen 4; MHC A/B, major histocompatibility complex A/B.

For instance, in collaboration with TNF-α, IL-17 promotes HepG2 cells to produce more periostin, which induces fibroblasts to synthesize additional type I collagen and aggravate liver fibrosis (122). IL-17A induces intrahepatic biliary epithelial cells to undergo epithelial to mesenchymal transition, during which cells obtain fibroblast-related characteristics to promote fibrosis in PBC (123). Due to the complex microenvironment of liver fibrosis, the role of one single cell type cannot be discussed while ignoring others. For example, changes in Tregs and Th17 tend to occur simultaneously and are accompanied by Th1/Th2 shifts in the initial stages of liver fibrosis. In addition, each type of immune cell produces many different cytokines, which leads to the diversity of immune cell function. Fortunately, the application of single-cell RNA sequencing technology (scRNA-seq) in liver disease enables us to identify some subsets of cells that are historically

difficult to isolate (124). In fact, scRNA-seq has identified a specific subset of macrophages ($TREM2^+CD9^+MNDA^+$ scar-associated macrophages) in human fibrotic liver that is primarily distributed in scarring regions. This subset promotes the production of collagen and proliferation of HSCs (125). Thus, these technologies allow us to capture information about key cell populations and discover new therapeutic targets. The development of biotechnology will facilitate the identification of new cell populations involved in liver fibrosis as well as to elucidate the mechanisms underlying liver fibrosis.

AUTHOR CONTRIBUTIONS

MW wrote the manuscript. JH and LD collected related literature. FH drew the figures. PG revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Liver Fibrosis in Non-alcoholic Fatty Liver Disease: From Liver Biopsy to Non-invasive Biomarkers in Diagnosis and Treatment

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An increasing percentage of people have or are at risk to develop non-alcoholic fatty liver disease (NAFLD) worldwide. NAFLD comprises different stadia going from isolated steatosis to non-alcoholic steatohepatitis (NASH). NASH is a chronic state of liver inflammation that leads to the transformation of hepatic stellate cells to myofibroblasts. These cells produce extra-cellular matrix that results in liver fibrosis. In a normal situation, fibrogenesis is a wound healing process that preserves tissue integrity. However, sustained and progressive fibrosis can become pathogenic. This process takes many years and is often asymptomatic. Therefore, patients usually present themselves with end-stage liver disease e.g., liver cirrhosis, decompensated liver disease or even hepatocellular carcinoma. Fibrosis has also been identified as the most important predictor of prognosis in patients with NAFLD. Currently, only a minority of patients with liver fibrosis are identified to be at risk and hence referred for treatment. This is not only because the disease is largely asymptomatic, but also due to the fact that currently liver biopsy is still the golden standard for accurate detection of liver fibrosis. However, performing a liver biopsy harbors some risks and requires resources and expertise, hence is not applicable in every clinical setting and is unsuitable for screening. Consequently, different non-invasive diagnostic tools, mainly based on analysis of blood or other specimens or based on imaging have been developed or are in development. In this review, we will first give an overview of the pathogenic mechanisms of the evolution from isolated steatosis to fibrosis. This serves as the basis for the subsequent discussion of the current and future diagnostic biomarkers and anti-fibrotic drugs.

Keywords: NAFLD, liver fibrosis, liver biopsy, non-invasive assessment, liver stiffness

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) refers to the presence of liver steatosis in the absence of factors that are known to induce lipid accumulation in hepatocytes, such as alcohol consumption or the use of steatogenic drugs. The diagnosis requires > 5% of the hepatocytes containing lipid droplets on histology or the amount of fat in the liver to exceed 5% of the total liver weight (1). Currently, NAFLD is the most common chronic liver disease with an estimated prevalence of 25% worldwide (2). The prevalence of NAFLD parallels the global increase in overweight and obesity which is the result of an increase of caloric intake over expenditure that leads to an increase in body mass index (BMI) (3). As a result, NAFLD will become the most common cause of liver cirrhosis, decompensated liver disease or hepatocellular carcinoma (HCC) in the short term, and thus the most important indication for liver transplantation (4).

NAFLD comprises a broad spectrum of liver lesions but also has extra-hepatic consequences. These extra-hepatic complications, including cardiovascular disease, diabetes, and non-liver malignancies, are responsible for a significant part of NAFLD-attributable morbidity and mortality (5, 6). Furthermore, there is a considerable impact on the quality of life (7, 8). With respect to the liver, the NAFLD spectrum consists of the following entities: isolated fatty liver (non-alcoholic fatty liver, NAFL); and non-alcoholic steatohepatitis (NASH), i.e., steatosis accompanied by chronic inflammation and cell damage, histologically characterized by lobular inflammation and ballooning of hepatocytes, the latter being the driving force of fibrosis that can evolve to cirrhosis and decompensated cirrhosis (**Figure 1**). HCC can also develop, even in non-cirrhotic NAFLD (9). The rate of disease progression is usually slow. About 20% of patients with NAFLD will develop NASH in three to seven years (10), which is considered the potentially progressive form of the disease (11). About 9 to 25% of individuals with NASH develop cirrhosis over a 10 to 20 year period (12).

Unfortunately, it is very hard to know which NASH patient will progress to cirrhosis due to the complex multifactorial etiology of NASH determined by genetic, epigenetic, lifestyle, and nutritional factors (13). However, the stage of liver fibrosis is the strongest predictor for liver-related mortality and development of other comorbidities (14–18), with an increase if fibrosis (F) is ≥ 2 on a scale of 0–4 as proposed by the NASH Clinical Research Network Scoring System (NASH CRN) (19). Accordingly, NASH patients with ≥F2 are considered the target population for pharmacological treatment and are most likely to benefit from antifibrotic drugs. Regression of stage F1 fibrosis is more likely with simple lifestyle changes and treatment of the metabolic comorbidities (20). Therefore, it is important to correctly diagnose the stage of liver fibrosis, preferably with non-invasive methods. Additionally, regression of advanced fibrosis should be the primary hepatic endpoint in clinical studies for antifibrotic drugs (21). Therefore, this review will tackle diagnostic methods to determine the stage of liver fibrosis and antifibrotic drugs that can reduce advanced fibrosis.

PATHOGENESIS

As understanding the pathogenesis of NAFLD and NAFLD-related fibrosis is vital in the development of biomarkers for its diagnosis and in finding targets for its treatment, we first review the most important aspects of NAFLD-related fibrogenesis.

From Liver Steatosis to NASH

Weight gain, often caused by an unhealthy lifestyle with a high-calorie diet and decreased physical activity, is one of the most important factors in the development of NAFLD. The liver plays a critical role in maintaining the metabolic balance that comes under pressure with a high caloric intake and low energy expenditure. Lipid overload, as seen in NAFLD, is a major contributor to the development of lipotoxicity (**Figure 1A**). Lipotoxicity accelerates the development of progressive inflammation, oxidative stress, and fibrosis (22). The excess energy consumed is usually stored in the form of fat in both subcutaneous and visceral depots. This capacity of the adipose tissue to store fat is genetically determined. When this capacity is exceeded, the adipose tissue experiences an overload and becomes damaged. This results in adipose tissue insulin resistance (IR) and inflammation of the tissue with imbalances in the secretion of adipokines and other inflammatory mediators (23), which causes a low-grade systemic inflammation (24). Together with ectopic fat accumulation, this leads to IR in the muscles and liver (25). The IR causes a disbalance in the homeostasis of glucose and lipid metabolism. As a result, more free fatty acids (FFA) that have to be processed by the liver, enter the circulation (26). Another consequence is that dietary carbohydrates (especially fructose) are absorbed by the liver and converted to FFA by *de novo lipogenesis*. About 40% of the fat that builds up in the liver comes from dietary carbohydrates and fat. The other 60% is derived from the dysfunctional adipose tissue (26). Thus, IR leads to an increase in FFA flux, leading to a toxic effect on the liver (27). The FFA are normally broken down in the mitochondria by beta-oxidation. Due to the FFA overload, the mitochondria are overwhelmed, and this leads to mitochondrial uncoupling. As a result, they produce reactive oxygen species (ROS) (22, 26). Combined with the dysfunctional adipose tissue and endotoxins from the gut, this leads to a pro-inflammatory and apoptotic climate in the liver, causing NASH (26–28). The Kupffer cells, the resident macrophages of the liver, as well as infiltrating immune cells, contribute to the inflammatory state of NASH. Kupffer cells absorb large amounts of FFA, which drives them toward an inflammatory phenotype. This leads to the secretion of inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor (TNF)-α and IL-10. Both IL-6 and TNF-α are associated with NASH progression (29, 30).

Taken together, NASH is the result of a complex interplay between different factors like genetic variation and obesity, which leads to a profibrotic climate in the liver (31).

From NASH to Liver Fibrosis

Immune responses in chronic liver diseases like NAFLD, not only lead to the restoration of tissue function but also to tissue injury. An overactive or exaggerated immune response can result

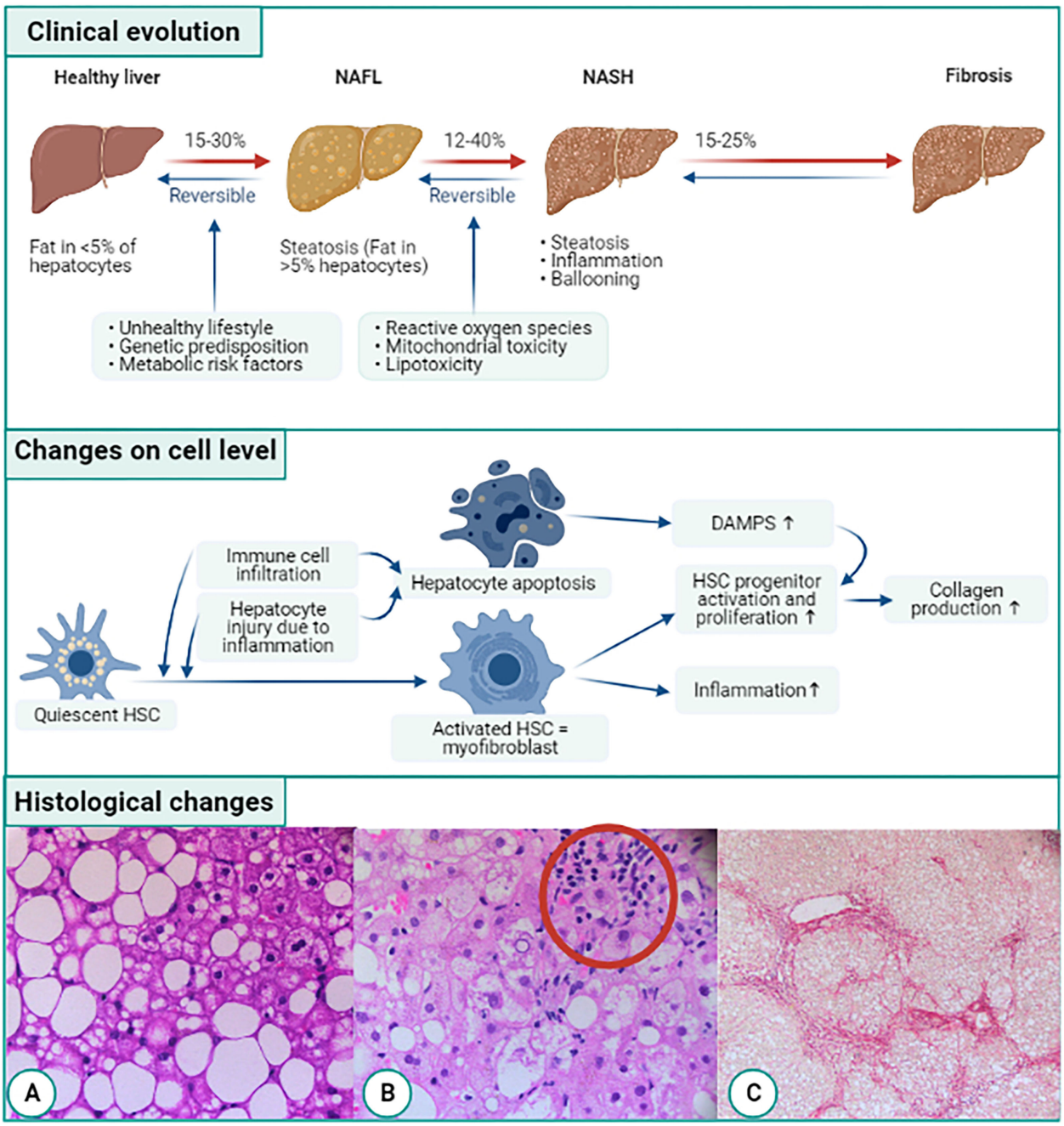

FIGURE 1 | Overview of evolution of NAFLD related fibrogenesis on clinical, cellular, and histological level. On the clinical level NAFLD starts of as simple steatosis (NAFL). The abnormal amount of liver fat triggers inflammation by infiltrating immune cells and secretion of cytokines. This is called non-alcoholic steatohepatitis or NASH which can cause liver fibrosis. On cellular level, quiescent hepatic stellate cells (HSCs) are activated by immune cell infiltration and hepatocyte injury due to inflammation. The activated HSC transdifferentiates into collagen producing myofibroblasts furthermore the myofibroblasts trigger HSC progenitor proliferation and activation. Another consequence of the immune cell infiltration and hepatocyte injury is apoptosis of hepatocytes, leading to the release of damage-associated patterns (DAMPs). DAMPs also activate hepatic progenitor cells. Both the myofibroblasts and HSCs will start producing collagen. On a histological level, first fat accumulates in the liver (A). This leads to the infiltration of immune cells (B) and ballooning and eventually liver fibrosis occurs (C). Histological pictures courtesy of Dr. P. Van Eyken, pathologist, Ziekenhuis Oost-Limburg, Genk, Belgium.

in organ dysfunction and leads to the deposition of fibrotic tissue in parallel to the cell loss (31). These immune responses comprise both innate and adaptive responses (32). For example, neutrophil infiltration is often seen in histologic samples of NASH patients (**Figure 1B**). Additionally, patients with NASH and advanced fibrosis related to NASH have a higher neutrophil/lymphocyte

ratio than patients without NASH (33, 34). Likewise, CD8+ lymphocytes have also been seen in the inflammatory infiltrate in NASH (35).

The inflammation caused by NASH causes hepatocyte death and apoptosis. The dying hepatocytes release damage-associated molecular patterns (DAMPs). The DAMPs, including nucleic acids, intracellular proteins, and adenosine triphosphate (ATP), send a danger signal to the surrounding cells (36). The danger signal activates the hepatic progenitor cells (HPCs). Apoptosis, on the other hand, produces low levels of DAMPs since most of the cell content will be retained in an apoptotic body. These apoptotic bodies will be phagocytosed by hepatic stellate cells (HSCs) and Kupffer cells. This induces a pro-fibrogenic response. Additionally, the DNA from the apoptotic hepatocytes triggers the activation of Toll-Like-Receptor (TLR)-9 on HSCs and collagen production (37).

Infiltration of the immune cells activates the trans-differentiation of HSCs into collagen-producing myofibroblasts (28). Usually, this process is involved in tissue repair upon short-term injury. When liver injury occurs, the HSCs are activated and differentiate from the quiescent phenotype to proliferative and contractile myofibroblasts (38). In their quiescent stage, HSCs store retinoids and synthesize glial fibrillary acidic protein (GFAP). When activated, a gradual loss of retinoids and GFAP coincides with their development into myofibroblasts with the synthesis of extracellular matrix (ECM) products like type I, type III, and type IV collagen but also hyaluronic acid (HA) (39). Levels of the glycosaminoglycan polymer HA increase with the amount of liver fibrosis (40). The collagen accumulation is accompanied by a rise in metalloproteinases (MMPs) such as MMP-9, which break down ECM products (41, 42). The combination of active and overexpressed MMP-9 and build-up of type III collagen leads to an abundance of cleaved type III collagen products like plasma N-terminal propeptide of type III procollagen (PIIIPN) or neo-epitope PRO-C3 (43, 44). Normally the MMPs are kept in check by tissue inhibitors of metalloproteinase (TIMPs). There are four TIMPs of which TIMP-1 is secreted by macrophages and fibroblasts (45). In murine fibrotic livers, likewise to the increase in MMPs, high concentrations of TIMP-1 were found (46). This creates a disturbance in the MMP/TIMP balance and, therefore a shift toward ECM synthesis and thus fibrogenesis (47). Alpha-2 macroglobulin (A2M) causes the balance to tip even further toward fibrogenesis by also inhibiting the MMPs (48). In addition to the ECM products, myofibroblasts also synthesize α-smooth muscle actin (α-SMA) (49). Ramzy et al. indicated that an increase in α-SMA marks the activation of HSCs (50).

During differentiation, the characteristic star-like shape of the HSCs changes to a more droplet form. The process is then balanced by the counteracting anti-fibrotic mechanisms resulting in the inactivation or apoptosis of the myofibroblasts and scar resolution. In chronic diseases, like NAFLD, there is an imbalance in these processes. The imbalance will cause persistent activation of proliferating, contractile, and migrating fibroblasts. This leads to the excessive production of ECM. The abundance of ECM will destroy the physiological architecture of the liver (51). The regulators of this balance are non-parenchymal cells (NPCs) like Kupffer cells and other immune cells, which are, as mentioned above, recruited to the site by the death and apoptosis of hepatocytes (52). NPCs will start producing pro-fibrogenic cytokines. On a molecular basis, a complex network of cytokine-induced pathways arises to coordinate the pro-fibrogenic cell interactions. The proposed signaling pathways associated with HSC activation and fibrosis progression are the transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), inflammasome (NLRP3)-caspase 1, and the WNT/β-catenin (28).

From Liver Fibrosis to Liver Cirrhosis

Progression of liver fibrosis to liver cirrhosis varies between people depending on environmental and host factors (53). Cirrhosis is a consequence of long-standing fibrogenesis that results in the encapsulation of injured liver parenchyma by a collagenous scar. Histologically, cirrhosis is characterized by fibrotic septa that connect the portal tracts with each other and with the central veins (**Figure 1C**). This leads to a disconnection of the hepatocytes from the central vein, creating islands of hepatocytes. Vascular changes also occur, including loss of sinusoidal fenestrae and appearance of a basal membrane, or so-called capillarisation of the liver sinusoids, another hallmark of cirrhosis (54). The changes in liver structure ultimately lead to an increase of intravascular resistance within the portal system and decreased hepatic perfusion (55). The consequence is a loss of liver function (56).

Molecular Signaling Pathways Involved in Liver Fibrogenesis

TGF-β Signaling

TGF-β, together with PDGF, is the most potent inducer of hepatic fibrosis (57). The TGF-β superfamily consists of 33 members, of which TGF-β1 plays an essential role in liver fibrogenesis (58). The consequences of TGF-β1 signaling are inhibition of HSC apoptosis and induction of HSCs to produce excessive amounts of ECM proteins like fibronectin and collagen types I, II, and IV (59). Additionally, the production of matrix-degrading proteins is inhibited by TGF-β1 (60). In patients with hepatic fibrosis, increased concentrations of TGF-β1 correlate with the severity of fibrosis (61, 62).

TGF-β1 mainly exerts its effects via small Mothers Against Decapentaplegic (SMAD)-dependent pathways. The SMAD family is divided into three groups based on their functions. First, there are the receptor-regulated SMADs (R-SMADs), which include SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8. Secondly, SMAD4 is the only member of the common SMAD (co-SMAD). The third group consists of the inhibitory SMADs (i-SMADs) and includes SMAD6 and SMAD7. The R-SMADs bind to membrane bound serine/threonine receptors and are activated by their kinase activity. Co-SMADs act as co-factors and attach to the R-SMADs to form a complex that will translocate to the nucleus of the cell. i-SMADs, on the other hand, counteract the effect of the R-SMADs (63). SMAD3 and SMAD4 have been found to be pro-fibrotic, whereas SMAD2 and SMAD7

are protective (64). SMAD3 induces hepatocyte death and lipid accumulation, especially in NASH (65). SMAD4 even enhances fibrogenesis by promoting SMAD3 activity. SMAD7, on the other hand, downregulates SMAD3 (66, 67). On their turn, SMADs also act as signal integrators and interact with the mitogen-activated protein kinases (MAPK) and nuclear factor kappa beta (NFκB) pathway (68).

PDGF Signaling

PDGF is a growth factor that promotes HSCs division and proliferation (28). The PDGF family consists of four members: PDGF-A, -B, -C, and -D (69). In healthy circumstances, PDGF is produced by platelets. When liver injury occurs, Kupffer cells recruited to the site of inflammation secrete PDGF (70). All PDGF members and its receptors (PDGF-R) are overexpressed in the case of liver fibrosis, and the activity increases with the degree of liver fibrosis (71–74). For example, PDGF-C activates the TGF-β/SMAD3 pathway in mice, leading to HSC proliferation, collagen production, and eventually fibrosis (72). However, of the four members, PDGF-B and -D are the most potent in activating the downstream pathways extracellular signal-regulated protein kinase/mitogen-activated protein kinase (Erk/MAPK) and protein kinase B (Akt/PKB). The activation leads to HSC proliferation (71, 75). PDGF-A expression was increased in hepatocytes from fibrotic livers compared to normal livers (76). In HSCs, on the other hand, although they express both receptors, only PDGF-BR expression was upregulated during HSC activation both *in vitro* as *in vivo* (77, 78).

The NLRP3 Inflammasome Caspase-1 Pathway

Inflammasomes are multiprotein complexes that sense danger signals like DAMPs and pathogen-associated molecular patterns (PAMPs) from damaged cells and pathogens (79). There are multiple inflammasomes implicated in liver disorders, but the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome has been studied most extensively (80–82). The NLRP3 inflammasome is activated in a two-step process. First, a bacterial signal, for example lipopolysaccharide (LPS), upregulates Nlrp3 expression via the NFκB-pathway (83). This, in turn, will enable a second signal, e.g., a DAMP, to activate the NLRP3 inflammasome. Once activated, the inflammasome binds with the adaptor molecule ASC to mediate caspase-1 cleavage, thereby activating the enzyme (79, 84). Caspase-1 activates pro-inflammatory cytokines IL-1β and IL-18 by proteolysis, though also activates the cytosolic protein gasdermin D (GSDMD) (85). GSDMD in a cleaved form will create pores in the plasma membrane of cells (86). This induces pyroptotic cell death and, consequently, the release of IL-1β and IL-18 (86–88).

In NAFLD, the NLRP3 inflammasome has been found to negatively regulate disease progression (89). In early NAFLD models, mRNA upregulation of the NLRP3 inflammasome components, like *Nlrp3*, *Asc,* and *Casp1*, was found. However, no active inflammasomes were found, indicating that not enough signals were present in a fatty liver to properly activate the inflammasome (90, 91). In NASH, on the other hand, IL-1β will stimulate the production of inflammatory cytokines, thus aggravating the already existing inflammation (92). In a mouse knock-in model of the NLRP3 inflammasome, inflammation was increased, and simultaneously a high neutrophil infiltration was found. In addition, NLRP3 also induced HSC activation and collagen deposition, thereby causing liver fibrosis (80). Blockage of NLRP3 resulted in a reduction of liver inflammation and fibrosis in an experimental mouse model of NASH (93). It is clear that NLRP3 is involved in the pathogenesis of liver fibrosis with NAFLD. Nevertheless, additional studies are necessary to provide a better insight into these mechanisms.

Wnt/β-Catenin Signaling

The Wnt signaling pathway consists of canonical and non-canonical arms and regulates a large number of cellular functions (94). The canonical pathway exerts anti-lipid formation and anti-inflammatory effects, while the non-canonical pathway promotes fat formation, lipid accumulation, and inflammation (95). An imbalance between these two pathways has been associated with NAFLD by triggering lipotoxicity and fibrogenesis (96, 97). More specifically, the Wnt signaling pathway promotes hepatic fibrosis by enhancing HSC activation and survival, and upregulation of TGF-β/SMAD pathways (49).

Other Mechanisms That Contribute to Liver Fibrogenesis

Gut Liver Axis

About 70% of the liver's blood supply comes from the intestines. This blood circulation enables the liver to interact with products derived from the intestines, like bacterial DNA, LPS, or intact bacteria due to an increased intestinal barrier permeability (98). Normally, the Kupffer cells will clear the endotoxins, maintaining the immune tolerance and homeostasis. Alteration of the gut microbiome, gut permeability, and Kupffer cell responsivity can alter this balance (31). Moreover, fructose, a compound frequently found in sugar beverages, has been shown to promote a leaky gut and liver fibrosis. Fructose induces the ethanol-inducible cytochrome P450-2E1-mediated oxidative and nitrative stress (99). In addition, the bacterial products can bind to the TLRs in the liver, thereby inducing liver inflammation. This causes the progression of liver disease due to the fact that the TLRs will activate the NFκβ and the c-Jun N-terminal kinase (JNK) pathways (100). Most of the studies are, however, performed in mice, and more research in humans is necessary (101). A study conducted by Kapil et al. in humans indicated that small intestinal bacterial overgrowth and TLR signaling are involved with liver histology in NAFLD (102). In a study by Boursier et al. it was shown that NAFLD severity was associated with gut microbiome alterations and shifts in the metabolic function of the microbiome (103). More specifically, they found that the *Ruminococcus* bacteria were independently associated with fibrosis (103). These first results in human trials concerning the microbial environment are leading to further investigations of the influence of the gut microbiome in people with metabolic disorders like NAFLD (104).

Genetic Mechanisms

In addition to environmental factors, genes play a role in NAFLD (1). Several genes have been identified through genome-wide association studies (105–107). Amongst those, Patatin-like phospholipase domain containing 3 (PNPLA3) and transmembrane 6 superfamily member 2 (TM6SF2) seem to have the biggest impact (108). The PNPLA3 gene has been most extensively studied. It is located on chromosome 22 and encodes a 481 amino acid protein that mediates triacylglycerol hydrolysis. The I148M variant of PNPLA3 (rs738409) is strongly associated with NAFLD in adults but also in obese children and adolescents (109, 110). In a mouse model of NAFLD, overexpression of the I148M variant of the PNPLA3 gene caused hepatic steatosis (111). However, the exact mechanism is not yet known (105). The TM6SF2 gene, located on chromosome 19, plays a role in the progression of NAFLD. A single nucleotide polymorphism (rs58542926) replacing a cytosine by a thymine in position 167 has been linked to an increased hepatic triglyceride content (112). This specific gene variant has also been associated with fibrosis progression (113). Both PNLPA3 and TM6SF2 thus exert an additive effect on NASH and significant fibrosis (114).

DIAGNOSIS OF LIVER FIBROSIS

As previously stated, the stage of fibrosis is the most essential determinant of liver-related progression and mortality, and a key indicator for the development of other comorbidities like type 2 diabetes (T2DM) and cardiovascular disease, indicating the need to correctly diagnose fibrosis (115).

Liver Biopsy

Liver biopsy is currently considered as the gold standard for the diagnosis and histological assessment of NAFLD (1). Unfortunately, due to its invasive nature, a biopsy is not suited for screening purposes and cannot be implemented early in the diagnostic path of potential patients (116). It is mostly reserved for patients with a high risk of advanced liver disease during long-term follow-up, to distinguish NASH from NAFL and to determine the extent of liver fibrosis (117, 118). Additionally, a biopsy is still required in more advanced stages of drug development for NASH to assess treatment efficacy (119, 120).

Histological Scoring Systems for Liver Biopsy Samples

There are different histological scoring systems for classifying liver biopsy samples (121). However, the most widely used scores are the NASH CRN including the NAFLD Activity Score (NAS) and the Steatosis-Activity-Fibrosis (SAF) score (**Table 1**) (19, 122). The NAS scoring system is initially developed for use in clinical studies, and a definition of NASH has been based on this score. The score ranges from 0 to 8 (**Table 1**) and is composed of the unweighted sum of steatosis, ballooning, and lobular inflammation. A score between 0 and 2 corresponds to no NASH, 3–4 is borderline NASH, and definite NASH has a score between 5 and 8. Yet, there are several remarks concerning this scoring system. Firstly, this definition of activity does not distinguish steatosis separately from necroinflammation. Secondly, lobular inflammation outweighs ballooning, while ballooning is an essential feature of the NASH definition. Thirdly, the grading of the ballooning is based on the number of ballooned cells, without a clear definition of how to assess ballooning. This causes a greater opportunity for interobserver variability in NASH diagnosis. The SAF score, on the other hand, assesses steatosis (S) separately from activity (A), and of course, also fibrosis (F). This scoring system was developed by the Fatty Liver: Inhibition of Progression (FLIP) consortium. The activity score is, in this case, a combination of lobular inflammation and ballooning both scored from 0 to 2, overcoming the problem of one criterium outweighing the other. Additionally, a clear definition of ballooning is given. If the size of the hepatocyte is twice as big as usual, it is considered as severe ballooning (123). Although both the NAS and SAF score have a comparable fibrosis grading system, the SAF score may potentially be more appropriate for routine diagnosis and clinical trials as it comes with an easy to use diagnostic algorithm and better-defined criteria leading to less interobserver variability (117). However, future comparative studies are needed to determine which scoring system is the most potent in scoring NAFLD related fibrosis. In contrast to differences in the concepts of activity and the scoring of the features of ballooning and lobular inflammation, the scoring of fibrosis is the same in both NASH CRN and FLIP SAF (except for the subclassification of F1 in NASH CRN). Stage one (F1) of NASH CRN system is composed of three subclasses, namely: F1a stands for mild perisinusoidal/pericellular fibrosis, F2a is moderate perisinusoidal/pericellular, and F1c is portal/periportal fibrosis. For the FLIP SAF system, the subclasses of F1 were pooled into one stage of mild perisinusoidal/pericellular fibrosis. Stage two (F2) correlates with perisinusoidal/pericellular and portal/periportal fibrosis. Next, stage three (F3) corresponds to bridging fibrosis. Lastly, stage four (F4) stands for liver cirrhosis. In a clinical situation, people speak of significant and advanced fibrosis; in this case significant fibrosis stands for $\geq$F2 and advanced for $\geq$F3. It is thus different from the MetaVir score designed for the staging of liver fibrosis caused by viral hepatitis where F1 does not have subclassifications, and for the other stage's fibrosis expansion should be located in the portal zones (124, 125). Consequently, when reviewing literature, one should pay attention to the scoring system used as a reference golden standard when studying non-invasive biomarkers.

Outside these commonly used scoring systems, there are also other more granular scoring systems though they are not used as the golden reference standard in studies with non-invasive biomarkers. For instance, the Ishak staging system ranging from 0 to 6 with 6 being cirrhosis, was previously one of the most frequently used fibrosis scoring systems in clinical trials for different etiologies of liver disease. The Ishak fibrosis stages reflect more scarring than each preceding stage. Succession from one stage to the next represents progressively more advanced liver disease (126). Another scoring system is the EPoS staging system developed by the Elucidating (E) Pathways (P) of (o) Steatohepatitis (S) consortium. This system is based on e-slides, histological glass slide images that have been turned into electronic files. It includes, similarly to the Ishak system, seven stages ranging from 0 to 6. In a first study presented

TABLE 1 | Comparison between the histologic scoring of NAFLD according to NASH CRN system and SAF system (18, 120).

	Score	NASH-CRN	SAF	Score
Steatosis	0	<5%	<5%	0
	1	5–33%	5–33%	1
	2	>33–67%	>33–67%	2
	3	>67%	>67%	3
Lobular inflammation	0	No foci	No foci	0
	1	<2 foci/20X	<2 foci/20X	1
	2	2–4 foci/20X	>2 foci/20X	2
	3	>4 foci/20X		
Ballooning	0	No ballooning	Normal hepatocytes	0
	1	Few ballooned cells	Clusters of rounded, pale hepatocytes	1
	2	Many ballooned cells	Many enlarged (2X normal size) hepatocytes	2
Fibrosis	0	No fibrosis	No fibrosis	0
	1	1a Mild, zone 3 perisinusoidal/pericellular fibrosis 1b Moderate, zone 3 perisinusoidal/pericellular fibrosis 1c Portal/periportal fibrosis	Mild fibrosis perisinusoidal/pericellular	1
	2	Perisinusoidal/pericellular and portal/periportal fibrosis	Perisinusoidal/pericellular and portal/periportal fibrosis	2
	3	Bridging fibrosis	Bridging fibrosis	3
	4	Cirrhosis	Cirrhosis	4
Composite score for activity	0–8	NAS = NAFLD Activity Score = steatosis + ballooning + lobular inflammation	A = ballooning + lobular inflammation	0-4

NAS, NAFLD Activity Score; NASH CRN, Non-Alcoholic steatohepatitis clinical research network scoring system; SAF, Steatosis-Activity-Fibrosis.

at the International Liver Congress of 2018, the EPoS scoring system showed promising results in terms of interobserver reproducibility (127).

Limitations of a Liver Biopsy

Although being the golden standard, a liver biopsy also has several limitations. The procedure comes with some discomfort and risks. As for the incidence of pain, this was reported to be 20%, though when a mildly unpleasant feeling was included in the assessment, the incidence increased to 84% (128). The incidence of severe complications and mortality was found to be between 0.3 and 0.57% and 0.01, respectively (129–131). Furthermore, the interpretation of the biopsy requires a high level of expertise and training; hence experienced physicians need to perform it. Liver biopsies are also prone to sampling error with discordance of one stage or more of 41% in a study with paired biopsies (132). This is due to the fact that a biopsy sample is only 1:50.000 of the liver mass. Fibrosis is not spread uniformly throughout the liver, which leads to this sampling error (132). Another problem in the assessment of histological liver biopsy samples is inter- and intra-observer variability (128). Evaluation of fibrosis is mostly consistent among observers. The evaluation of inflammatory activity, on the other hand, was inconsistent at a high rate in a study performed by Younossi et al. (133). Moreover, NASH can mimic other liver diseases, therefore the possibility of another etiology needs to be kept in mind (117).

Non-invasive Tests for the Detection of Liver Fibrosis

Non-invasive assessment of liver fibrosis can overcome some of the limitations of the biopsy and can be implemented and used for screening of NAFLD. There is currently an intensive search for biomarkers in NAFLD. Although stand-alone biomarkers are unlikely to provide the complex set of information that a liver biopsy offers, they can, if accurate and validated, provide an alternative to the biopsy to assess specific aspects of the disease. As outlined before, liver fibrosis is one of these crucial features, and non-invasive assessment of liver fibrosis has made significant advances in the last two decades. Currently, non-invasive assessment of liver fibrosis is composed of two different approaches: a biological approach based on the quantification of biomarkers (mostly in serum) and a physical approach based on the measurement of liver stiffness (117). A combination of the biological and physical approach results in a greater accuracy compared to the individual strategies to identify liver fibrosis, without the necessity of doing a liver biopsy (134, 135).

Methodological Aspects of Non-invasive Tests

As outlined previously, for a correct interpretation of the data, one should first of all look at the fibrosis scoring system that has been used in the design and validation of the non-invasive test. Hence, the non-invasive tests for NAFLD should be tested against

the NASH CRN or SAF grading systems and not the MetaVir. Though, one should keep in mind the differences between the NASH CRN or SAF score. Moreover, as the non-invasive tests are validated against a liver biopsy, they cannot outperform the golden standard. Second, the values of non-invasive scores mostly show substantial overlap between histological fibrosis stages. Therefore, although often proposed for that purpose, non-invasive scores are not very accurate in predicting a precise corresponding histological fibrosis stage and hence cannot be used to diagnose the histological fibrosis stage of a given patient. According to the cut-off chosen, based on a given combination of specificity and sensitivity, non-invasive scores are useful to rule-in or rule-out significant or advanced fibrosis or cirrhosis, or conversely, the absence thereof, with NPV and PPV depending on the prevalence of the condition in the population that is studied. So, the result of a non-invasive test informs you about the likelihood of finding e.g., significant fibrosis, or the absence thereof, in a given patient, but does not tell you the patient has F2.

Liver Stiffness Measurement

Vibration Controlled Transient Elastography

The physical approach to assess fibrosis consists of measuring liver stiffness, which is a physical characteristic of the liver tissue, influenced by (but not equalling) the stage of liver fibrosis. Liver stiffness can be assessed by VCTE^{TM}, as measured by the FibroScan® device, was shown to correlate with liver fibrosis in a cross-sectional analysis of patients with viral hepatitis and is now widely used as a technique to non-invasively assess liver fibrosis in various liver diseases and different circumstances, including not only screening and baseline assessment but also follow-up and assessment of treatment response (136, 137). VCTE^{TM} measures liver fibrosis via the velocity of a low-frequency (50 Hz) elastic shear wave (induced by a mechanical pulse) propagating through the liver (117). The probe uses pulse-echo ultrasound (US) to follow the propagation of the shear wave and measures its velocity. The velocity of the wave depends, amongst others, on the amount of liver fibrosis. It is a straightforward, non-invasive, and easy to use technique. The area covered by the VCTE^{TM} measurement has a volume that is 100 times bigger than an average liver biopsy sample (138). Choosing the cut-off value for the VCTE^{TM} has to be done with care and depends on the clinical situation. Low cut-off values for the VCTE^{TM}, for example 7.9 kPa, have higher negative predictive values (NPV) than positive predictive values (PPV), meaning it can more precisely rule out more severe stages of fibrosis and rule in the absence of fibrosis. In contrast, higher cut-offs have an increase in the PPV and can, therefore, be more reliably used to rule-in more severe stages of fibrosis (139). A recent meta-analysis by Hsu et al. using the following thresholds 6.2, 7.6, 8.8, and 11.8 kPa, showed a pooled area under the receiving operating curve (AUROC) of 0.82, 0.87, 0.84, and 0.83 (with 95% CI) for diagnosing ≥F1, ≥F2, ≥F3, and F4, respectively (**Table 2**) (140).

VCTE^{TM} has been found to be a cost-effective surveillance strategy to evaluate the presence of fibrosis (138). However, there are some limitations when using VCTE^{TM} measurements. Factors influencing the results of the FibroScan® measurements are ascites, elevated central venous pressure, and obesity. Gross ascites prevents an accurate measurement of liver stiffness by VCTE^{TM}. Fluid and adipose tissue attenuate the elastic wave (149–151). To overcome the latter problem, the extra-large (XL) probe was developed. It is able to assess the degree of fibrosis more accurately, though it may not be superior to the standard medium probe in obese patients (152, 153). The XL probe has a more sensitive US transducer, larger vibration amplitude, deeper focal length, and deeper signal penetration (tissue depth >35–75 mm) (154).

Liver inflammation may also reduce the accuracy of the test, as it can increase the VCTE^{TM} value by 1.3 to 3 times. This is illustrated by the rapid decline of liver stiffness after successful eradication of viral hepatitis C in a time frame that is too short to allow for substantial fibrosis regression (155). The pattern of fibrosis also differs between diseases, and the staging systems differ accordingly, as outlined previously. Accuracy and cut-offs need hence to be defined in a disease-specific way. Operator experience, sex, and metabolic syndrome can influence the FibroScan® measurements too (156, 157). A study performed by Vuppalanchi et al. demonstrated a failure rate of 5.5% because of excess skin to liver capsule distance, machine error, and invalid readings. Another study performed by the same research group indicated that fasting of at least 3 h in necessary (158). Without fasting, a significant increase ($26 \pm 25\%$, $p = 0.02$) in VCTE^{TM} was seen (159). Nonetheless, with sufficient operator experience, the failure rate and unreliability can be minimized (117, 160).

An extra feature recently added to the FibroScan® device is the possibility to measure the amount of liver fat. Since this an important characteristic of NAFLD, assessment of steatosis is therefore crucial. The fat content can be measured via the Controlled Attenuation Parameter (CAP^{TM}). The CAP^{TM} can be determined by the ultrasonic attenuation on the FibroScan® device at a frequency of 3.5 MHz on a go-and-return path (161, 162).

Other Ultrasound-Based Elastography Methods

There are several other ultrasound (US)-based methods available to determine liver elasticity (163). US elastography makes use of two different techniques, namely strain imaging or shear wave imaging. Strain imaging is used with strain elastography (SE) and acoustic radiation force impulse (ARFI) (164). Shear-wave imaging is the same technique as in the FibroScan® device (165). They have been less extensively studied in the context of NAFLD, but data on their accuracy are increasingly reported along with their use in clinical practice (166).

Point shear wave elastography (pSWE) is an ARFI-based technique that uses a short-duration, high-intensity acoustic pulse to displace tissue perpendicular to the longitudinal waves of the tissue surface (167). Next, the transducer detects the tissue displacement within a focal point along the radiation force resulting in the measurement of tissue stiffness. The big advantage of pSWE is that additional equipment is not necessary. pSWE can be incorporated in an US machine with brightness-mode. Next, direct anatomical visualization is possible, avoiding the areas with large blood vessels or parts of the biliary system (168). This implies, however, that a radiologist or sonographer is usually needed to perform the pSWE as a specific anatomical

TABLE 2 | Overview of the accuracy indices of the different non-invasive diagnostic tools for NAFLD-related liver fibrosis.

Non-invasive test	References	Meta-analysis	Fibrosis stage	Cut-off	AUROC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
VCTE™	Hsu et al. (140)	Yes	≥F1	6.2 kPa	0.82 (0.76–0.88)	66	67.	81	48
			≥F2	7.6 kPa	0.87 (0.81–0.91)	76	80	72	83
			≥F3	8.8 kPa	0.84 (0.78–0.90)	77	78.	54	91
			≥F4	11.8 kPa	0.83 (0.74–0.94)	80	81.	34	97
MRE	Liang and Li (141)	Yes	≥F1	Optimal values could not be determined	0.89 (0.86–0.92)	77	90	N.A.	
			≥F2		0.93 (0.90–0.95)	87	86		
			≥F3		0.93 (0.90–0.95)	89	84		
			≥F4		0.95 (0.93–0.97)	94	75		
pSWE	Jiang et al. (142)	Yes	≥F2	Optimal values could not be determined	0.86	70	84	N.A.	
			≥F3		0.94	89	88		
			≥F4		0.95	89	91		
APRI	Peleg et al. (143)	No	≥F3	1	0.83	78	82	N.A.	
NFS	Xiao et al. (144)	Yes	≥F2	−1.1	0.72* (0.65–0.79)	66*	83*	82*	74*
			≥F3	−1.455	0.78* (0.75–0.81)	73*	74*	50*	92*
			≥F4	−0.014	0.83* (0.76–0.89)	80	81	43	96
FIB-4	Xiao et al. (144)	Yes	≥F2	0.37–3.25	0.75* (0.70–0.79)	64*	70*	73*	61
			≥F3	1.51–2.24	0.80* (0.77–0.84)	77*	79*	66*	84*
			≥F4	1.92–2.48	0.85* (0.81–0.89)	76*	82*	39*	96*
ELF	Vali et al. (145)	Yes	≥F2	7.7	0.81 (0.66–0.89)	93	34	N.A.	
FibroMeter^{NAFLD}	Boursier et al. (146)	No	≥F2	N.A.	0.76	Not available			
			≥F3	0.311	0.76	80	62	65	83
			≥F4	N.A.	0.78	Not available			
FIBC3	Boyle et al. (147)	No	≥F3	>-0.4	0.89	83	80	74	88
NIS-4	Harrison et al. (148)	No	Exclude NAS≥4 and ≥F2	0.36	0.80 (0.77–0.84)	81	63	N.A.	78
			Include NAS≥4 and ≥F2	0.63		87	51	79	N.A.

**Mean values.*

Table based on the most recent meta-analyses if available, or otherwise on the most robust studies.

VCTE™, vibration controlled transient elastography; MRE, magnetic resonance elastography; pSWE, point shear wave elastography; NFS, NAFLD fibrosis score; FIB-4, fibrosis-4 score; ELF, enhanced liver fibrosis score; FIBC3, fibrosis C3 panel; kPa, kilopascal; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; N.A., not available.

and technical expertise is necessary to interpret the visual images (169). Furthermore, in contrast to VCTE™, pSWE has a lower failure rate of 1–2% due to the fact that it is not limited by the presence of ascites (142, 170). A meta-analysis that compared VCTE™ and pSWE showed that both provide excellent diagnostic accuracies for the diagnosis of advanced fibrosis and cirrhosis (**Table 2**) (142). However, a recent study conducted by Leong *et al.* comparing VCTE™ with pSWE for diagnosis of fibrosis stage in a biopsy-proven cohort found that VCTE™ outperformed pSWE. (171). Especially for the diagnosis of ≥F2 and ≥F3, the AUROC for VCTE™, respectively, 0.83 and 0.83, was higher than that of pSWE (0.72 and 0.69) (171).

Magnetic Resonance Elastography

Magnetic resonance elastography (MRE) is a magnetic resonance imaging-based method for quantitatively imaging tissue stiffness. These measurements can be taken rapidly during breath-hold acquisition mode. Even in the early stages, MRE can be used to detect NAFLD. The diagnostic accuracy of MRE for liver fibrosis and steatosis is higher than VCTE™ and CAP™ (172). The pooled summary receiver operating characteristics (SROC) curve of MRE in 12 studies, including 910 patients with biopsy-proven NAFLD, was 0.89 for ≥F1, 0.93 for ≥F2, 0.93 for ≥F3, and 0.95 for F4, respectively (**Table 2**) (141). Nonetheless, due to high-performance costs, MRE is usually not performed routinely to screen patients for NAFLD (172).

Non-invasive Score Calculations to Detect Liver Fibrosis

The biological approach via non-invasive score calculation is composed of routinely measured clinical and laboratory variables that can aid in predicting liver fibrosis. Different scores have been proposed to calculate the risk of fibrosis. The aspartate aminotransferase (AST)-to-platelet ratio index (APRI), developed initially for hepatitis C infection, has been suggested for predicting significant fibrosis in NASH (117, 143). The NAFLD Fibrosis Score (NFS) has been demonstrated to be useful as a prognostic marker for fibrosis. Advanced fibrosis can be excluded with an NPV of 93% when using a low cut-off value

and having a high PPV 90% (117). A meta-analysis conducted by Xiao et al. found an AUROC value of 0.78 for the exclusion of advanced fibrosis (**Table 2**) (144). The Fibrosis-4 (FIB-4) score was also designed as a parameter of fibrosis detection in patients with hepatitis C infection. This index is not influenced by BMI and is composed of routinely available laboratory data (AST, alanine aminotransferase (ALT) and platelets) (117). The FIB-4 had an AUROC of 0.80 for diagnosing advanced fibrosis, a sensitivity of 77%, a specificity of 79%, a PPV of 66%, and an NPV of 84% (**Table 2**) (144). The Enhanced Liver Fibrosis (ELF) score developed for the detection of liver fibrosis has good accuracy for the non-invasive diagnosis of advanced fibrosis in NAFLD (145). The ELF test is a panel consisting of the following markers: PIIINP, HA, and TIMP1 (173, 174). One side note, although it still has the same name, the test components and formula have been altered throughout the years. Nevertheless, not all studies reflect the accuracy of the current ELF test (145). A recent meta-analysis by Vali et al. in biopsy-proven NAFLD patients examined the accuracy of the ELF test for the diagnosis of advanced and significant liver fibrosis and NASH (145). At the recommended cut-off of 7.7, a high sensitivity (93%) was found, though specificity was limited. When the high cut-off was used (9.8), a higher specificity of 86% was reached. The cut-off value should thus be decided based on the purpose of the test in a specific clinical situation (the so-called context of use) (145).

FibroMeters, a family of blood tests specifically designed for each cause of chronic liver disease, were commercialized by Echosens (175). Although a FibroMeter^{NAFLD} is available, the FibroMeter^{V2G}, developed for hepatitis C is more accurate in NAFLD with an AUROC of respectively 0.76 and 0.8 for the detection of F≥3 (**Table 2**) (146). This is probably due to the fact that the FibroMeter^{NAFLD} only contains AST, ALT, platelets, glucose, and ferritin, whereas the FibroMeter^{V2G} uses AST, urea, platelets, prothrombin time, HA, and A2M. These last two are direct markers of liver fibrosis, while the others are indirect markers (146). In a biopsy-proven NAFLD cohort, the FibroMeter^{VCTE} was tested for accuracy. FibroMeter^{VCTE} combines the results of the VCTE^{TM} and FibroMeter^{V2G} markers in one test. In this cohort, the FibroMeter^{VCTE} was significantly more accurate than the FibroMeter or VCTE^{TM} alone (AUROC: 0.87 ± 0.012, $p \leq 0.005$) (135).

Other parameters or scores that have been proposed for the detection of NAFLD related liver fibrosis are PRO-C3 and NIS4. Measurement of type III collagen neo-epitopes (PRO-C3) as a single diagnostic marker or as part of a panel has shown to have reasonable accuracy in assessing NAFLD disease stage and activity (147). When used as a single marker, PRO-C3 performed equally to simple panels like the FIB-4 (176). This might be due to the fact that PRO-C3 is more a product of active fibrogenesis instead of static collagen accumulation. PRO-C3 might therefore be helpful to detect patients with active liver fibrogenesis (177, 178). When on the other hand, used in the FIBC3 panel in combination with age, BMI, T2DM, platelets, it was able to distinguish advanced fibrosis (≥F3) with an AUROC of 0.89, a specificity of 80%, sensitivity of 83%, PPV of 74% and an NPV of 88%, respectively, for a cut-off value of >-0.4 (147). In three independent cohorts with suspected NASH, the non-invasive blood-based diagnostic test NIS-4 was developed and validated to detect patients with NAS≥4 and ≥F2 (148). The NIS4 panel comprised of the following NASH-associated biomarkers: miR-34a-5p, A2M, YKL-40, and glycated hemoglobin (HbA1c) (179, 180). The exact functions of miR-34a-5p and YKL-40 in the development of fibrosis are not yet fully understood, though their levels are elevated in patients with liver fibrosis (181–183). In the pooled validation cohort, NAS≥4 ≥F2 patients were excluded with a cut-off of 0.36 and this with a sensitivity and specificity of 81.5 and 63%, respectively. To include NAS≥4 and ≥F2 patients, a NIS4 value of 0.63 was used. This resulted in a sensitivity of 87.1% and a specificity of 50.7%. Furthermore, the NIS4 algorithm experiences no influence of age, sex, BMI, or liver enzyme concentrations (148).

Nonetheless, scoring systems have their limits. There is no single threshold for non-invasive tests that has the perfect balance between sensitivity and specificity. Up to now, the scores are more used as a first-line risk determination, without the necessity of doing a liver biopsy. For example, the NFS works best in distinguishing advanced vs. non-advanced or any with no fibrosis (172). In 25 to 30% of the patients, however, the NFS score is intermediate (117). A recent study indicated that the NFS and FIB-4 scores were better compared to the other scores (BARD, APRI, and AST/ALT ratio) to determine fibrosis, and as good as MRE in predicting the presence of advanced fibrosis in patients with biopsy-proven NAFLD (172).

Future Biomarkers for Liver Fibrosis

Up to now, no accurate serum biomarkers are available to detect a precise stage of fibrosis. Fortunately, a lot of research is carried out on this topic. For example, Mac 2-binding protein glycan isomer (M2BPGi) is secreted by HSCs to act as a messenger for Kupffer cells during fibrosis progression (184). M2BPGi can therefore act as biomarker for detection of liver fibrosis (185). A study by Nah and colleagues indicated that M2BPGi can exclude advanced fibrosis with a sensitivity of 80% and specificity of 77.9% and a NPV of 98.9% with an AUROC of 0.85 when compared to MRE (186). Serum autotaxin (ATX) may also be a potential serum biomarker for liver fibrosis with NAFLD ADDIN EN.CITE (187, 188). ATX is responsible for the transformation of lysophosphatidylcholine to lysophosphatidate (189). The latter is involved in the process of cell migration, neurogenesis, angiogenesis, smooth muscle contraction, platelet aggregation, and wound healing (190, 191). Sinusoidal endothelial liver cells process ATX, therefore, it is thought that in the case of chronic liver injury, ATX metabolism is impaired. First results within a cohort of NAFLD patients with fibrosis show that ATX can select patients who require further evaluation. The diagnostic accuracy was, however, lower than that of MRE (187). More recently a study was published by Kimura *et al.* on the possible biomarker thrombospondin 2 (TSP2) for the detection of liver fibrosis with NAFLD. TSP2 is involved in multiple processes such as collagen/fibrin formation. TSP2 had an AUROC of 0.82 for prediction of ≥F3 (192). Lastly, the serum marker type IV collage 7s can be used to diagnose significant fibrosis with an AUROC of 0.832, a sensitivity of 91.4%, and a NPV of 87.9% with a cut-off value of ≥5.2 ng/mL (193).

A recent study by Caussy et al. demonstrated that a combination of 10 metabolites consisting of eight lipids (5α-androstan-3β monosulfate, pregnanediol-3-glucuronide, androsterone sulfate, epiandrosterone sulfate, palmitoleate, dehydroisoandrosterone sulfate, 5α-androstan-3β disulfate, and glycocholate), one amino acid (taurine), and one carbohydrate (fructose) could detect advanced fibrosis. With an AUROC of the metabolite combination of 0.94 and a sensitivity and specificity of, respectively, 90 and 79%, the metabolites performed better than the FIB-4 (0.78) and NFS (0.84) for the detection of advanced liver fibrosis (194). However, they used mass spectrometry to analyse the metabolites, which is not easily accessible and expensive and therefore not (yet) applicable in a routine clinical situation (195). However, this type of biomarkers, requiring more sophisticated techniques and all kinds of omics approaches, might show the way ahead to increase the accuracy over the currently available tools. To support biomarker development for the detection of liver fibrosis, two large projects, one in the United States of Amerika called Non-Invasive Biomarkers of Metabolic Liver Disease (NIMBLE), and one in Europe called the Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS), have been set-up (196).

The Use of Non-invasive Biomarkers in a Clinical Situation and in Trials

A non-invasive test alone has a certain accuracy depending on the context of use (147). Notwithstanding, when used in a sequential way or at the same time, the accuracy of the non-invasive tests increases significantly. The most appropriate combination is probably one with a biological test in combination with liver stiffness measurements. In a recent study by Boursier et al. different stepwise combinations were tested. The sequential combinations of the FIB-4 followed by FibroMeter^{VCTE} and the VCTE^{TM} followed by the FibroMeter^{VCTE} provided a diagnostic accuracy of 90%. A liver biopsy to confirm the results was only needed in 20% of the cases (135). Another study conducted by Srivastava et al. in a primary care cohort tested a 2-step algorithm that combined the FIB-4, followed by, if needed, the ELF test (197). Use the 2-step algorithm improved detection of advanced fibrosis and cirrhosis by 4.9-fold (197). Davyduke et al. piloted a FIB-4 first strategy, followed by a VCTE^{TM} when classified as high risk (198). When using this strategy, only 15% of the patients needed to be referred for further assessment (198). With a probabilistic decision model of a cohort of 1000 NAFLD patients, different sequential combinations of the non-invasive tests were simulated, and costs were compared. The price per case of advanced fibrosis was significantly lower when using a sequential combination (£8,932 for FIB-4/ELF, £9,083 for FIB-4/VCTE^{TM}) compared to the standard of care (£25,543) (199). Proving that the sequential combinations of non-invasive tests are cost-effective, reduce unnecessary referral and detect advanced fibrosis without the necessity of doing a liver biopsy, which can be useful for inclusion in clinical trials (199–201).

Currently, efficacy assessment in clinical trials requires histology hence biopsy in phase II trials that need to provide data to go into phase III, and for the interim analysis for conditional approval in phase III, with regression of fibrosis of 1 stage without worsening of fibrosis as one of the endpoints for regulatory approval in non-cirrhotic NASH (119, 120). Efficacy assessment in earlier phase II trials, on the other hand, can be based on non-invasive biomarkers, and trial sponsors are encouraged to collect data on biomarker response in late phase II and in phase III trials to inform future trial design. Several non-invasive markers of fibrosis have been used in several trials and mostly serve to support the data on histology. For example, in the pirfenidone (PFD) study, they evaluated the antifibrotic effects with, similarly, for the cenicriviroc (CVC) study where they used the NFS, FIB-4, APRI, and ELF scores (196, 202).

Much more data and analyses are needed to couple responses in histology to responses in biomarkers and to define criteria of response in terms of biomarkers, i.e., what magnitude of change, absolute and or relative, is clinically meaningful and correlates with a histological response or with another endpoint concerned clinically relevant and resulting in clinically significant benefit.

ANTI-FIBROTIC DRUGS

NAFLD management is centered on lifestyle modifications, weight loss, and habitual physical activity. Weight loss promotes fat reduction and NAFLD remission. A bodyweight reduction of 3 to 5% improves steatosis, and a decrease of 10% improves necroinflammation and fibrosis (1). However, dietary and lifestyle changes are hard to maintain. As a result, there is a need for appropriate drugs to treat NAFLD. The target of treatment is still a matter of debate. Fibrosis regression (mostly defined by at least one stage improvement according to NASH CRN), as a highly potential surrogate marker for clinical benefit, is one of the endpoints approved by the regulatory authorities for phase III trials in NASH (119, 120). Unfortunately, there are currently no drugs approved specifically for the treatment of liver fibrosis by the United States of America Food and Drug Administration (FDA) or European Medicines Agency (EMA) (203), despite the increased insight into the molecular and cellular mechanisms of liver fibrosis.

Current Options for NAFLD Treatment

Besides vitamin E, the only drugs that can be recommended for the treatment of NAFLD, are drugs already used for the treatment of T2DM treatment and/or obesity. Most of these drugs have a direct effect on NASH, and via this, an indirect effect on fibrosis regression (although direct antifibrotic effects might even so be present) since NASH and fibrosis are strongly intertwined.

Drugs used to treat T2DM with effects on NAFLD histology are thiazolidinediones (TZDs), glucagon-like peptide-1 receptor agonist (GLP-1 RA) and sodium-glucose cotransporter- (SGLT)-2 inhibitors (117, 203–205). TZDs like pioglitazone act on peroxisome proliferator-activated receptors (PPARs), mainly on the PPARγ isoform. If PPARγ binds to the retinoid X receptor, it has powerful insulin-sensitizing properties in adipose tissue. Although PPARγ is poorly expressed in the hepatocytes, it still exerts anti-steatogenic effects (206). In rat livers, depletion of PPARγ led to a decrease in fibrogenesis (207). However, the effect was not so strong in human trials with TZDs. TZDs cause a

reduction in liver fat, despite some overall weight gain, which is reflective of an improvement in adipose tissue function and goes along with a redistribution of fat from visceral to subcutaneous fat storage (208). PPARγ is also implicated in the activation state of HSC, so TZDs can also have direct effects on fibrogenesis. Based on the histological improvements seen with pioglitazone, several guidelines recommend the use of TZDs in patients with liver biopsy-proven NASH and T2DM (209). In the PIVENS trial, after 96 weeks of pioglitazone treatment, no improvement in fibrosis stage was seen when compared to placebo (210).

GLP-1 RAs improve glycaemic control via a decreased glucagon secretion, slowed gastric emptying, glucose-dependent insulin secretion, enhanced satiety, reduction in body weight, and BMI (204, 211–213). They also lead to the improvement of the hepatic markers ALT, AST, and gamma-glutamyl transferase (GGT). Presently, GLP-1 RA use is only recommended in case of a high BMI (>27 kg/m^2) and comorbidities like diabetes and arterial hypertension (AHT). However, NASH should be added to the list of comorbidities associated with obesity (117). In the LEAN trial, the efficacy of 48 weeks of liraglutide, a GLP-1 RA, was investigated with NASH regression without worsening of fibrosis as the primary endpoint. Despite the significant difference in the resolution of NASH between liraglutide and placebo, no significant difference in fibrosis score was detected (214). Another GLP-1 RA, semaglutide, was tested in patients at risk for NAFLD development for 104 weeks. Semaglutide significantly reduced ALT after 28 to 20 weeks of treatment (215). In a recent study by Legry et al. the effect of semaglutide in mice with induced NASH was researched. This showed a reduction in NAS though it did not reduce fibrosis (216). These results were confirmed in human trials, semaglutide was significant on the resolution of NASH, though not on the fibrosis endpoint after 72 weeks of treatment (217). Despite having an efficacy on NASH resolution, the TZD pioglitazone and GLP-1 RA semaglutide were not able to reach significance on the $\geq$1 point fibrosis reduction endpoint. Notwithstanding, a decrease in the mean fibrosis score has been reported (218, 219).

SGLT-2 is a class of oral antidiabetics that reduces hyperglycaemia by promoting urinary excretion of glucose without affecting insulin secretion (220). In rodent models of T2DM, the SGLT-2 inhibitor ipragliflozin prevented the development of NASH (221). Not only in rodent models SGLT-2 inhibitors proved their effect of NAFLD development, in patients with T2DM who received dapagliflozin or empagliflozin a decrease in hepatic steatosis was seen. However, in these studies, no effects on liver fibrosis were detected (205, 222). A recent meta-analysis by Mantovani et al. also confirmed the significant effect on hepatic steatosis, though up to now, no results on the histological response of SGLT-2 inhibitors are available, at least not from randomized placebo-controlled trials (223, 224).

Therapy in Development

As mentioned above, there are currently no approved drugs for NAFDL treatment, yet potent drugs are coming. The first drugs are already in phase III trials and are expected to be on the market by the end of 2020 (117). As mentioned, fibrosis regression of at least one point without worsening of NASH, as a likely reasonable surrogate for a clinically meaningful benefit, is one of the two regulatory endpoints for conditional approval in non-cirrhotic NASH. Obeticholic acid (OCA) is so far the only drug that demonstrated efficacy on this endpoint in phase III. OCA is a first-in-class selective farnesoid X receptor (FXR) agonist with anti-cholestatic and hepato-protective properties (225). The FXR is a bile acid nuclear receptor that plays a role in lipoprotein and glucose metabolism, hepatic regeneration, and regulation of hepatic inflammation. Activation of the FXR receptor in mice has been shown to inhibit NLRP3 inflammasome activation in hepatocytes, thereby preventing disease progression (226). OCA was FDA approved in 2016 for the treatment of primary biliary cholangitis, though it is currently in a phase III trial to test the effects and safety in NAFLD (Regenerate Study) (209). In a first interim analysis of the Regenerate study, OCA thus demonstrated statistically significant fibrosis regression of at least one point with an effect size of 11% in phase III after 72 weeks on 25 mg. OCA did, however, not meet the endpoint of NASH resolution (227).

Other drugs that are currently being tested for the treatment of fibrosis are lanifibranor, PFD, and CVC. Lanifibranor (IVA337) is a drug that activates each of the three PPAR isoforms (228). These isoforms play an essential role in the regulation of cellular differentiation, development, and tumorigenesis throughout the body. The drug has both anti-fibrotic and anti-inflammatory effects and is also beneficial for metabolic changes. Currently, lanifibranor is going into phase III, based on the significant results the drug demonstrated on both resolution of NASH and improvement of fibrosis and the combination of both (229–231). PFD is an oral antifibrotic drug approved for the treatment of idiopathic pulmonary fibrosis. In a recent study by Poo et al. the effect of prolonged-release formulation (PR-PFD) plus standard of care was tested in patients with advanced liver fibrosis (PROMETEO Study). In 35% of the patients, a significant reduction of fibrosis was seen, leading to the conclusion that PR-PFD is efficacious and safe in patients with advanced liver disease. Moreover, it showed promising antifibrotic effects (196). CVC, a drug that targets macrophages in the liver by inhibiting the C-C chemokine receptors CCR2 and CCR5 (232), met this endpoint as a key secondary endpoint in phase II after 1 year of treatment with an effect size of 9.6% ($p = 0.023$) but was not significant at 2 years and showed no efficacy on NASH resolution (202, 233). A phase III trial (AURORA) is still ongoing evaluating the effect of CVC in NASH patients with F2 or F3 (234).

Different molecules failed, despite pre-clinical studies being positive. For instance, selonsertib is an apoptosis signal-regulating kinase 1 inhibitor that has been demonstrated in patients with moderate-to-severe NASH to reduce fibrosis, steatosis, and progression to cirrhosis (235). However, in several phase III clinical trials, selonsertib did not achieve the endpoints for the reduction of fibrosis (236). This also applies to simtuzumab, a monoclonal antibody against lysyl oxidase-like 2 that is involved in fibrogenesis. In the phase IIb trials, simtuzumab was unable to reduce fibrosis (237). Likewise, for the galactin-3 inhibitor GR-MD-02, phase II trials failed to show efficacy in NASH and reduction of liver fibrosis (238, 239). Correspondingly also elafibranor, a dual PPAR agonist

that showed promising results in the reduction of NASH and liver fibrosis in a phase II trial, failed to replicate these results in a phase III trial. Therefore, the trial has been ended (240). Recent suggestions made by Ratziu et al. stated that these failed trials were caused by the rush to move compounds into clinical development without thoroughly being investigated in the pre-clinical trials (241). More attention should be paid to optimize treatment dose and regimen correctly, but most importantly, the results of small studies should be interpreted with care (241).

Future Therapeutic Targets

Although recent clinical trials have been promising in treating NAFLD-related liver fibrosis, the overall efficacy of these drugs has been modest. Only a minority of patients achieved treatment response (242). Possible new targets can be found in the gut microbiome. As stated, it plays an important role in fibrogenesis. Therefore, targeting the GLA by modulating the gut-microbiome can be a promising therapeutic approach in NAFLD (243). In mice, whole-body deletion of nucleotide-binding oligomerization domain-containing (NOD)2 caused an increase in liver steatosis and fibrosis. NOD2 can therefore potentially engage the GLA to protect against steatosis, fibrosis, and gut dysbiosis (244). Another possible treatment option is the blocking of PDGF. Blocking of PDGF signaling ameliorates experimental liver fibrogenesis. PDGF signaling can be blocked by regulation of the isoforms, regulation of the receptor binding, and finally, by inhibiting the signaling pathways (245). PDGFR kinase activity blocking is one of the most efficient ways to block the signaling pathway of PDGF. Several kinase inhibitors have been developed, though they are not entirely specific. Imatinib mesylate (Gleevec®) effectively inhibits PDGFR signaling in CCl_4-treated mice leading to improved liver regeneration *in vivo* and induced apoptosis of HSCs both *in vivo* and *in vitro* (246). In a pig serum-induced rat model of liver fibrosis, characterized by a slow progression of fibrosis, like in a human situation, imatinib had an effect in the early stages of liver fibrosis (247). Finally, Wnt3a, a canonical Wnt ligand, could be used as a future therapeutic target. In a study conducted by Wang et al. where LRP6 mutant mice were treated with Wnt3a, liver inflammation was reduced, indicating the ant-inflammatory role of Wnt3a (97).

CONCLUSION

Progressive liver fibrosis in NAFLD can lead to cirrhosis and liver-related morbidity and mortality and is also the strongest predictor of overall mortality. Halting fibrosis progression and regression of existing fibrosis are hence essential goals for treatment. Liver biopsy is still the gold standard for the staging of liver fibrosis. Accurate non-invasive diagnosis of liver fibrosis that can replace liver biopsy in most of the circumstances is obviously needed, both for initial diagnosis and monitoring of evolution over time and response to treatment. Non-invasive biological markers and liver stiffness measurement, alone or in combination, are extensively studied and further developed and validated. Currently, anti-fibrotic drugs are in development, and some have promising results that can lead to the prevention of liver fibrosis and delaying or even halting the development of cirrhosis. Moreover, based on the available data and international guidelines, a multi-disciplinary approach to treat and guide NAFLD patients is recommended due to the association with other metabolic features. These developments will potentially have an extensive impact on global health and on the healthcare costs that accompanies the rising incidence of NAFLD-related fibrosis. Furthermore, it has the possibility to lower the increasing incidence of HCC. Nonetheless, a better understanding of the complex pathology of NAFLD-related fibrogenesis is necessary to identify new targets for treatment and to find markers that will lead to new diagnostic methods that can accurately detect disease severity and fibrosis stages and its evolution over time.

AUTHOR CONTRIBUTIONS

LH collected the data and drafted the first version of the paper. All authors listed have made a substantial, direct and intellectual contribution to the review, and approved it for publication.

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REVIEW
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Neutrophils Modulate Fibrogenesis in Chronic Pulmonary Diseases

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Chronic inflammatory pulmonary diseases are characterized by recurrent and persistent inflammation of the airways, commonly associated with poor clinical outcomes. Although their etiologies vary tremendously, airway neutrophilia is a common feature of these diseases. Neutrophils, as vital regulators linking innate and adaptive immune systems, are a double-edged sword in the immune response of the lung involving mechanisms such as phagocytosis, degranulation, neutrophil extracellular trap formation, exosome secretion, release of cytokines and chemokines, and autophagy. Although neutrophils serve as strong defenders against extracellular pathogens, neutrophils and their components can trigger various cascades leading to inflammation and fibrogenesis. Here, we review current studies to elucidate the versatile roles of neutrophils in chronic pulmonary inflammatory diseases and describe the common pathogenesis of these diseases. This may provide new insights into therapeutic strategies for chronic lung diseases.

Keywords: neutrophils, asthma, chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis, fibrosis, fibrogenesis

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INTRODUCTION

Pulmonary diseases are life-threatening conditions and an important cause of death worldwide. Chronic pulmonary diseases account for about 1 in 15 deaths in the United States, and mortality is increasing (1). According to current knowledge, chronic pulmonary diseases, including chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis (CF), and idiopathic pulmonary fibrosis (IPF), are characterized by chronic inflammation, repeated lung tissue injury and repair, and eventually pulmonary dysfunction. Although therapeutic strategies have been developed, effective therapy options are currently lacking for the middle and late stages of chronic pulmonary diseases (2). Fibrogenesis is a common phenomenon in the middle-to-late stages of these diseases, but the specific mechanisms are still unclear.

Generally, chronic inflammation may result from uncontrolled acute inflammation and may lead to fibrogenesis if repeated damage-repair cycles occur. Pulmonary fibrogenesis is a process of recurrent injury to the alveolar epithelium, followed by an uncontrolled proliferation of fibroblasts and overexpression of extracellular matrix (ECM) (3). Abnormal pneumocyte apoptosis, bronchiolar proliferation, and abnormal tissue remodeling are also involved in fibrogenesis (4). Fiber deposits in the alveolar interstitial matrix eventually leads to fibrosis. Neutrophils, a prominent subpopulation of immune cells in airways, have long been regarded as effector cells in the defense against extracellular pathogens during acute inflammation. However, neutrophils also participate in chronic inflammation in various diseases, such as rheumatoid arthritis (5, 6).

Neutrophils play a modulating role in both innate and adaptive immune responses (7). On the one hand, neutrophils adhere to blood vessel walls, transmigrate to inflammatory sites following chemokine signals, and maintain cellular homeostasis by phagocytosis and degranulation or neutrophil extracellular trap (NET) formation (8). On the other hand, neutrophils and their effectors, including NETs, cytokines, exosomes, and autophagy, directly and indirectly activate fibroblasts, and promote ECM deposition. Most lung disorders, such as asthma, COPD, CF, and IPF, are inflammatory diseases that are accompanied by an increased number of neutrophils in the bronchoalveolar lavage fluid (BALF) or lung tissue (9–11). The long-term imbalance between pathogens and host defense contributes to the chronic inflammatory disease (12). High mobility group box protein 1 released by necrotic neutrophils induces inflammation and tissue remodeling by activating receptors for advanced glycation and toll-like receptor and CXC chemokine receptor (CXCR)4 signaling (13). As a result, chronic pulmonary interstitial inflammation, and fibrosis are induced and maintained.

In this review, we summarize how neutrophils restrict local inflammation and have a specific role in promoting chronic inflammation of the lung and pulmonary fibrosis. The review will reveal the various roles of neutrophils and explore common mechanisms of fibrogenesis in chronic lung diseases. This knowledge may inform treatment strategies for end-stage chronic lung diseases.

NEUTROPHILS AS DEFENDERS AND AS INDUCERS OF CHRONIC INFLAMMATION AND FIBROSIS OF THE LUNG

Neutrophils contribute to nearly 60% of all leukocytes in human body fluids (14). More neutrophils are found in the pulmonary capillaries than in the systemic circulation, which facilitates their rapid entry into the lung tissues in response to infection and inflammatory stimuli (15). Primarily, they are recruited in initial phases to exert pro-inflammatory effects. These cells are recruited within minutes after injury and involved in the removal of tissue debris. Neutrophils possess a series of effector mechanisms to play their roles in disease. Apart from phagocytosis, the recently discovered NET formation, exosomes, release of cytokines and chemokines, and autophagy all play a vital role in pulmonary diseases. These protective mechanisms favor pathogen elimination and minimization of collateral damage (16), and they have been observed in multiple respiratory diseases, such as CF and asthma (17, 18). Following phagocytosis, neutrophils activate apoptotic pathways to limit the inflammation in the lung tissue.

Although neutrophils exert powerful antibacterial and antiseptic properties, studies have found that the neutrophil/total cell ratio in lung tissue is positively correlated with the lung fibrosis degree (19). This indicates that neutrophils and/or their products may contribute to fibrogenesis (20). The earliest pathological change of pulmonary fibrosis is the injury of pulmonary endothelial cells, followed by chronic injury of lung epithelial cells and deposition of lung interstitial matrix. Neutrophils induce endothelial and epithelial cell death directly through NET formation (21) or release of invading substance, such as angiopoietin-2, which promotes vessel destabilization and facilitates neutrophil influx to lung tissue (22). Neutrophils recruited to inflammatory sites affect the function of parenchymal cells or *via* their mediators, such as exosomes or cytokines (23, 24). Neutrophils secrete toxic mediators, such as reactive oxygen species (ROS) and reactive nitrogen species, that lead directly to tissue injury, or even worse, induce another wave of chemokines that forms a positive feedback loop. Moreover, some pathogens possess properties to subvert the immune system, which causes neutrophils to respond more aggressively, leading to untoward tissue injury (25). For example, neutrophil drive the inflammation-induced tissue damage in pulmonary tuberculosis and as such, neutrophil counts are correlated with chronic inflammation in tuberculosis (26).

Phagocytosis and Secretion of Traditional Pro- and Anti-inflammatory Factors From Neutrophils in Chronic Lung Diseases

The cytoplasmic granules of neutrophils contain large amounts of lysozyme, alkaline phosphatase, antimicrobial proteins, defensin, and phagocytin. These substances eliminate foreign pathogens after their extracellular release by means of degranulation or after phagocytosis of the pathogen and fusion of the cytoplasmic granules to form the structure referred to as the phagolysosome (8). Phagolysosomes are formed from neutrophil granules (granule enzymes and ROS), lysosomes, and ingested bacteria (27). Pathogen killing is mediated by granule enzymes released from granules and ROS produced by mitochondria and NADPH oxidase (NOX) complexes. Mitochondria-generated ROS facilitate the innate immune function of neutrophils in a NOX-independent manner (28). By contrast, NOX is the only enzyme specifically used to produce ROS that participate in the regulation of infectious diseases and inflammation. However, NOX activity also leads to membrane depolarization of phagosomes to provide a proper environment for these cell organelles (29). The chemical elimination of pathogens prevents the further spread of infection and inflammation and protects the surrounding cells and tissues. Furthermore, proteinases and bactericidal proteins help in tissue repair and ECM reduction.

When phagocytosis is ineffective, degranulation occurs in response to stimuli. Neutrophils release granule contents in a degranulation manner. In this process, a series of enzymes, such as lysosomal enzymes and protease, are released. They help control bacterial challenge, but the ECM will be digested as a collateral side effect. Recurrent tissue damage and abnormal tissue repair contribute to lung tissue remodeling, degradation, and fibrogenesis. Neutrophil-derived matrix metalloproteinases (MMPs) digest the ECM, elastin, and collagen to allow for neutrophil transmigration from the bloodstream into tissues; but in some disease contexts, they weaken the host defense and contribute to tissue damage by degrading immune receptors and collectins (13). MMP2 and MMP9 are key orchestrators of emphysema (30, 31), indicating that MMPs are involved

in pulmonary structural remodeling. In asthma, neutrophil phagocytosis is decreased (32), whereas, ROS generation is increased (33), which is highly correlated with tissue inflammation. In the COPD model, a reduction in MMP9 levels is correlated with disease alleviation (34). The dysregulated release of myeloperoxidase (MPO) and neutrophil elastase contributes to tissue damage and can exacerbate CF. Increased levels of MMP8/9 and neutrophil elastase are also detected in patients with CF (35). In the BALF and lung tissues from patients with IPF, high concentrations of neutrophil-derived MMP2 and MMP9 are detected, and MMP9 activity is positively correlated with the neutrophil count in the BALF (36). Collectively, the dysfunction of phagocytosis and degranulation favors ECM destruction, which is the basis for tissue over-repair, remodeling, and fibrogenesis.

NETs – Double-Faced Newcomers in Chronic Lung Diseases

NETs are a mechanism of activated neutrophils to control infection and inflammation by "trapping" pathogens extracellularly in a suicidal or vital manner (37). The progress of active NET release is known as NETosis. NETs can be produced in response to infectious or non-infectious stimuli. Following activation of NET cell death programs, suicidal NETs are released by dying neutrophils 2–4 h after their activation (38) and are composed of decondensed chromatin (including cathelicidin LL-37, α-defensin, and neutrophil elastase), dissolved nuclear membranes, and chromatin. NETosis can be accelerated by the presence of bacteria as a way to wall off the infection and to limit the pro-inflammatory cytokine secretion, which limits the inflammatory response (39). Various agents, including bacteria, fungi, protozoa, viruses, platelets, interferon (IFN)-α, autoantibody, nitric oxide donors, can induce NET formation (40, 41). Moreover, granular enzymes and ROS positively regulate NET formation (41). *In vitro*, lipopolysaccharides can also induce the generation of NETs (42). These results indicate that NETs play a critical role in pathogen clearance and tissue homeostasis.

Connective tissue growth factor (CTGF, CCN2) is a matricellular protein implicated in fibrosis and an important downstream mediator of TGF-β induced fibrosis (43). The expression of CTGF is elevated in IPF, and confined to proliferating type II alveolar cell and myofibroblasts (44). CTGF may play a role in promoting fibroblast proliferation and extracellular matrix production in pulmonary fibrosis (44). Studies show a closely relationship between NET and CTGF. Myofibroblast CTGF expression is enhanced by NETs inducer (45), which also promote lung fibroblasts activation and myofibroblast differentiation (45, 46). Fibrotic interstitial lung biopsies demonstrate a close proximity between NETs and alpha-smooth muscle actin (α-SMA)-expressing fibroblasts (45).

NETs are reported to participate in the pathogenesis of multiple lung diseases, including pneumonia, acute lung injury, asthma, COPD, and CF. Although NETs play an important role in pathogen elimination and host defense, other evidence reveals substantial damage to the lung epithelium and endothelium, either directly or indirectly (21). Much of the literature on NETs in pneumonia, acute lung injury, COPD, asthma, and CF has already been reviewed in detail, so we will summarize major findings here (47). Recent data suggest that NETs also play an important role in autoimmune and autoinflammatory pathologies (48, 49). NETs induce the activation of lung fibroblasts, stimulate their differentiation into the myofibroblast phenotype, and promote fibrotic activity *via* the expression of interleukin (IL)-17, a primary initiator of inflammation and fibrosis (45). *In vitro*, isolated NETs significantly increase the levels of cytokines and stimulate macrophages to secrete IL-1β, which promotes neutrophil and macrophage infiltration in airways and contributes to lung fibrosis (50). As a result, NETs amplify tissue inflammation along the NET-cytokine-cell axis. Peptidylarginine deiminase 4 (PAD4), an essential enzyme for NET formation (51), is elevated in the lungs of COPD patients (52). In addition, the concentrations of NETs and their components, including extracellular DNA, LL-37, and CXCL8/IL-8, are increased in patients with neutrophilic asthma and COPD, and these elevated levels are negatively correlated with lung function (53). In COPD patients, NET levels are also elevated in the sputum, and they are similarly negatively correlated with lung function. In mice, reduced NET levels following erythromycin administration can alleviate emphysema and decrease the numbers of Th1, Th17, and myeloid dendritic cells (54). Furthermore, NET components stimulate histamine and leukotriene release, which may lead to further inflammatory changes in asthma and COPD. Neutrophils of CF patients exhibit a prolonged lifespan, which promotes NET production and increased necrosis (55). Furthermore, the neutrophil count is increased in patients with CF, and the levels of NET components (extracellular DNA, neutrophil elastase, and MPO) are correlated with disease severity (56). In addition, increased levels of autoantibodies against NET components are correlated with decreased lung function in patients with CF (56, 57). Despite no currently proven direct connection between NETs and IPF, the relationship between NET and pulmonary fibrosis formation suggests a likely relationship between IPF and NETs. A recent study showed that pulmonary surfactant protein-D can inhibit NETosis in lipopolysaccharide-stimulated human neutrophils, providing the potential to eliminate negative NET effects (58).

Exosomes – Tiny Multi-Agent Messengers in Chronic Lung Diseases

Exosomes are extracellular vesicles <150 nm in size that are released into the plasma by budding and carry proteins, lipids, and nucleic acids. Exosomes are secreted by a wide range of cells in response to different stimuli and play a role in information transmission (59). As an intercellular communicator, exosomes can reach distant cells quickly and affect their behavior *via* interaction with surface markers. Exosomes acquiring the CD63/CD66b phenotype are from neutrophils, with which they can be traced extracellularly. Studies have shown that exosomes are released into the lungs, and they are related to the pathophysiology of lung diseases (60). Functionally, exosomes eliminate pro-inflammatory chromosomal DNA fragments to

maintain cellular homeostasis (61). Functional inhibition of exosomes triggers the innate immune response to generate more ROS by accumulating nuclear DNA.

Exosomes from activated neutrophils express higher neutrophil enzyme than quiescent exosomes, which indicate that exosomes show higher destructive effect in lung (62). Neutrophil-derived exosomes can be immediately internalized by airway smooth muscle cells, thereby altering their proliferative properties and contributing to airway remodeling (17). This is associated with excessive neutrophil-driven inflammation and local elevation in neutrophil elastase levels. Exosomes from neutrophils enhance the proliferation of airway smooth muscle cells to promote airway remodeling in patients with asthma, and they play an important role in the progression of asthma (17). In COPD, exosomes containing neutrophil elastase, which is resistant to alpha1-antitrypsin, destroy the tissue architecture *via* integrin Mac-1 and neutrophil elastase (62). Although studies concerning neutrophil-derived exosomes and lung fibrotic disease have not been identified, such exosomes potentially contribute to the pulmonary architectural distortion in chronic lung diseases. In turn, exosomes from lung fibroblasts activate DNA damage response and epithelial cell senescence (63). In systemic sclerosis, a systemic fibrotic disease, neutrophil-derived exosomes inhibit the proliferation, and migration of human dermal microvascular endothelial cells *via* S100A8/A9 in neutrophils, this result indicates that exosomes from neutrophils prevent lung remodeling (64). In mice with bleomycin-induced pulmonary fibrosis, exosomes containing microRNA-22 can ameliorate pulmonary fibrosis by suppressing transforming growth factor (TGF)-β1-induced expression of α-smooth muscle actin (65). This contrary effect of exosomes in fibric diseases is mainly caused by the different functional components contained in the released exosomes.

Autophagy, an Unusual Cell Death in Chronic Lung Diseases

Autophagy is the lysosome-dependent process of cell organelle self-degradation that is mediated under various cellular stress conditions to maintain cellular homeostasis of cellular protein synthesis, degradation, and recycling (66). Autophagy is crucial for cellular function and can be seen in both physiological and pathological processes in most cells, including neutrophils. In recent studies, autophagy has shown a complicated and important moderator function in pulmonary diseases (67). In chronic lung diseases, autophagy helps to maintain the cell cycle of lung fibroblasts and reduce the production of ECM.

In patients with asthma, autophagy levels of peripheral blood cells in the sputum and peripheral blood eosinophils are increased (68). Similarly, autophagy levels in airway tissues are also increased in a murine asthma model. Inhibiting autophagy reduces airway responsiveness, eosinophilia, and inflammation in this murine asthma model (69). Smoke-exposed neutrophils present increased autophagy and lose the ability to ingest the respiratory pathogen *Staphylococcus aureus* (70). Moreover, cigarette smoke exposure induces autophagic cell death in neutrophils, leading to the development of emphysema (71). These results indicate that the upregulation of autophagy in neutrophils contributes to persistent inflammation and the development of COPD.

However, studies concerning the autophagy of neutrophils in CF and IPF are lacking. The level of autophagic Beclin-1 is decreased in fibroblasts in fibrotic autoimmune diseases, suggesting an inhibited autophagy status (72). Autophagy stimulated by rapamycin (an mTOR inhibitor promoting autophagy) causes the reduced expression of fibronectin and α-smooth muscle actin in fibroblasts *in vitro* and exerts an anti-fibrotic effect in the bleomycin model *in vivo* (73). By contrast, inhibiting autophagy leads to an increase in extracellular collagen production and fibroblast-myofibroblast differentiation in pulmonary fibrosis (74, 75). In IPF, insufficient autophagy promotes fibrogenesis by stimulating fibroblast proliferation (76) and accelerates cellular senescence and myofibroblast differentiation of lung fibroblasts, suggesting the pro-fibrotic functions of insufficient autophagy in IPF (77, 78). Macroautophagy and mitophagy are decreased in lung epithelial cells and lung fibroblasts of IPF patients (54). Mitophagy, a crucial process in cellular energy homeostasis, modulates macrophage apoptosis and stabilizes macrophages to release TGF-β1, thereby stimulating local fibroblast activation. Aging mitochondria and impaired autophagy/mitophagy are involved in the pathogenesis of IPF (75, 79). This result caters to the fact that aging is a major risk factor for IPF (80). Blocking mitophagy in alveolar macrophages protects against bleomycin-induced fibrosis in mice (81). These results indicate an intimate connection between autophagy and persistent inflammation, as well as fibrosis, but more studies are needed to verify the specific function of neutrophil autophagy in these diseases.

Some drugs show to be highly associated with autophagy. For example, hydroxychloroquine blocks autophagy by impairing the autophagosome-lysosome fusion and the degradation of the autophagosome contents (82). Metformin, an insulin-sensitizing drug, stimulates AMP-activated protein kinase, which enhances autophagy by ULK1 (a key regulating protein of autophagy) activation (83). Moreover, autophagy positively regulates NET formation (84, 85), proposing that inhibition of autophagy using pharmacological inhibitors may hinder the release of NETs. Consequently, modulation of autophagy emerges as a promising therapeutic option for the treatment of NET-driven disorders.

Cytokines and Chemokines as Positive and Negative Regulators in Chronic Lung Diseases

Neutrophils secrete a vast number of cytokines and chemokines, such as pro-inflammatory cytokines (IL-1, IL-6, IL-17, and IL-18), anti-inflammatory cytokines (IL-10, TGF-β1, and TGF-β2), chemokines (CXCL1–11, CCL2–4, and CCL17–22), and immunoregulatory cytokines IFN-γ, IL-12, and IL-23), all of which act directly or indirectly on other immune cells, contributing to tissue damage or repair (8, 29, 86). As a key connector of the innate and adaptive immune system, neutrophils interact with other immune cells, including T cells, B cells, natural killer cells, macrophages, dendritic cells, and

mesenchymal stem cells (7). Particularly, IFN-γ, TGF-β, IL-6, IL-17A, and IL-17F are functionally important in lung diseases.

IFN-γ shows anti-fibrotic effects by inhibiting the collagen formation in fibroblasts *in vitro*. It also decreases the gene expression of pro-fibrogenic factors, including TGF-β1 and CTGF (87). Clinically, INF-γ is used to treat lung fibrosis in IPF, despite its effectiveness being quite controversial (88).

TGF-β is a strong pro-fibrotic factor in the lung (89). Three major mammalian isoforms of TGF-β have been identified: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 and TGF-β2 are secreted by neutrophils. TGF-β induces the proliferation of fibroblasts and macrophages *via* platelet-derived growth factor expression and stimulates fibroblasts to generate superabundant ECM. Besides, activated macrophages also express a vast number of pro-inflammatory and fibrogenic cytokines, such as tumor necrosis factor-α, IL-1β, and IL-13, triggering persistent inflammation downstream and chronic lung fibrosis (89). Pulmonary TGF-β1 levels are increased in a model of experimental lung fibrosis, and TGF-β1 overexpression induces persistent pulmonary fibrosis *via* the SMAD3 signaling pathway (90). *Smad3* gene knockout protects mice from TGF-β1- and bleomycin-induced pulmonary fibrosis (91).

In patients with IPF or acute exacerbation of IPF, IL-6 levels in the BALF and serum are significantly higher compared to healthy controls (92–94), indicating a close connection between this cytokine and fibrosis pathogenesis. In IPF, IL-6 enhances the

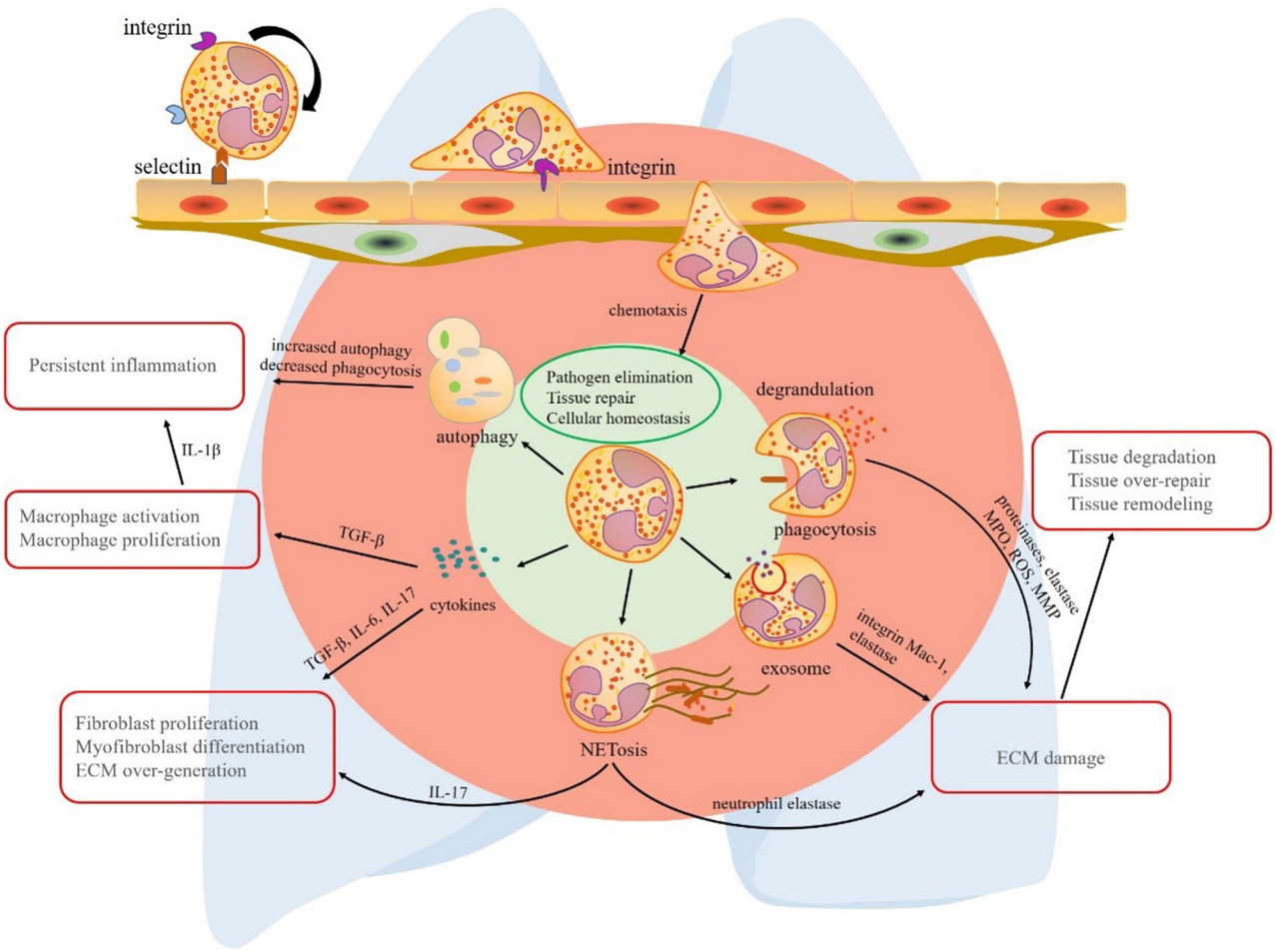

FIGURE 1 | The multiple effects of neutrophils in lung fibrosis. Circulating neutrophils roll, adhere, crawl, and transmigrate to chemokines to lung tissue. In the lung tissue, neutrophils play their roles *via* phagocytosis, degranulation, neutrophil extracellular trap (NET) formation, exosome secretion, release of cytokines, and autophagy redox balance. The primary common features of these mechanisms are pathogen elimination, tissue repair, and cellular homeostasis, respectively. However, the mechanisms that promote the progression of chronic inflammatory lung disease are quite different. Phagocytosis, degrandulation, NET formation, and exosomes contribute to ECM injury and tissue damage-repair-remodeling. In addition to ECM damage, NETs play a role in activating immune responses and release pro-fibrotic factor IL-17. Although neutrophil autophagy effect is limited, increased proportion of autophagy in neutrophil deduces the ability of eliminating inflammation. A vast of pro-fibrotic cytokines released by neutrophils contribute to lung fibrosis formation, respectively. Transforming growth factor (TGF)-β promotes persistent inflammation by macrophage activation and proliferation. interleukin (IL)-17 derived from neutrophils or NETs induces fibroblast proliferation and myofibroblast differentiation. As a result, extracellular matrix (ECM) is over-expressed in interstitial tissue of lung. TGF-β, transforming growth factor-β; IL-17, interleukin-17; NET, neutrophil extracellular trap; ECM, extracellular matrix; MPO, myeloperoxidase; ROS, reactive oxygen species; MMP, matrix metalloproteinase.

proliferation of lung fibroblasts *via* SHP-2/ERK/MAPK signaling (95). In addition, IL-6 promotes in IPF the resistance of lung fibroblasts to Fas-induced apoptosis by overexpression of the anti-apoptotic protein BCL-2 (96).

In human lung fibroblasts, IL-17A stimulates their proliferation, generation of ECM, and differentiation into the myofibroblast phenotype *via* the NF-κB pathway. IL-17 also promotes fibrosis *via* NETosis (97). IL-17 expresses in NETs and promotes the fibrotic activity of lung fibroblasts (45). Study shows that IL-17-producing cells and NETosis are synchronous increased in psoriatic lesions. The expression of IL-17 is increased in presence of NETs *in vitro* (97). Neutralization of IL-17A can ameliorate bleomycin-induced lung fibrosis in mice (98). These results indicate a pro-fibrotic role for IL-17A in human lung tissue remodeling through direct effects on lung fibroblasts (99). Superabundant neutrophils also lead to tissue damage, and pulmonary fibrosis is significantly alleviated when neutrophils are depleted (100). Collectively, neutrophils, as a major source of inflammatory factors, play vital pro- or anti-fibrotic roles in the lung. This dual effect is mainly caused by differences in environmental conditions of the distinct pulmonary diseases. Changing disease-related factors may reverse the disease progression and promote recovery.

SUMMARY

Chronic inflammatory lung diseases are a group of neutrophil-related disorders with poor prognosis in middle-to-late stages. To sum up the common features of these diseases, first, neutrophilia can be detected in the lung tissue or BALF. Second, protective factors and pro-inflammatory factors coexist, but the balance is disturbed under disease conditions. Third, specific neutrophil functions are altered, such as enhanced ROS production, aberrant NET formation, increased autophagy, and abnormal secretion of cytokines. In contrast to the traditional view on these short-lived cells, research corroborates the hypothesis that neutrophils and their products contribute to inflammation removal, but also chronic inflammation and fibrosis of lung tissue (**Figure 1**). The shift in the balance toward tissue destruction may result in persistent inflammation and fibrogenesis. These mechanisms also explain the acute exacerbation of some chronic lung diseases after experiencing infection. Avoiding infection is an important preventive measure to control pulmonary fibrosis.

Given the role of neutrophils in fibrosis, strategies focusing on neutrophil components may be effective, such as reducing neutrophil numbers in the airway, decreasing protease and ROS generation, decorating NETs, regulating autophagy, reducing the expression or activity of TGF-β protein, and providing exogenously exosomes containing microRNA. DNase and NET-associated elastase that affect the formation of NET may be helpful. Compound, such as hydroxychloroquine, which can reduce NETs *via* blocking autophagy is also considerable. Moreover, based on the tightly correlation of NETs and lung function, NET is expected to be a biomarker for evaluating criteria of lung function and fibrosis. However, the effectiveness of these strategies is limited by their off-target effects. More studies are needed to explore the precise role of neutrophils in lung fibrotic diseases, which will provide better evidence for the treatment of these diseases.

AUTHOR CONTRIBUTIONS

LD wrote this review. JY and CZ consulted relevant literature. XZ drew the figures. PG revised the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Hepatic BRD4 Is Upregulated in Liver Fibrosis of Various Etiologies and Positively Correlated to Fibrotic Severity

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Bromodomain-containing protein 4 (BRD4) has been implicated to play a regulatory role in fibrogenic gene expression in animal models of liver fibrosis. The potential role of BRD4 in liver fibrosis in humans remains unclear. We sought to investigate the expression and cellular localization of BRD4 in fibrotic liver tissues. Human liver tissues were collected from healthy individuals and patients with liver fibrosis of various etiologies. RNA-seq showed that hepatic BRD4 mRNA was elevated in patients with liver fibrosis compared with that in healthy controls. Subsequent multiple manipulations such as western blotting, real-time quantitative polymerase chain reaction, and dual immunofluorescence analysis confirmed the abnormal elevation of the BRD4 expression in liver fibrosis of various etiologies compared to healthy controls. BRD4 expression was positively correlated with the severity of liver fibrosis, and also correlated with the serum levels of aspartate aminotransferase and total bilirubin. Moreover, the expression of C-X-C motif chemokine ligand 6 (CXCL6), a factor interplayed with BRD4, was increased in hepatic tissues of the patients with liver fibrosis. Its expression level was positively correlated with BRD4 level. BRD4 is up-regulated in liver fibrosis, regardless of etiology, and its increased expression is positively correlated with higher degrees of liver fibrosis. Our data indicate that BRD4 play a critical role in the progress of liver fibrosis, and it holds promise as a potential target for intervention of liver fibrosis.

Keywords: liver fibrosis, BRD4, CXCL6, HBV, human samples

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INTRODUCTION

Hepatic fibrosis is a pathological process involving persistent injury to the liver and subsequent wound-healing responses that induce the production and deposition of extracellular matrix (ECM) proteins. Chronic inflammation in response to liver injury and excessive accumulation of ECM proteins can result in the progressive substitution of liver parenchyma by scar tissue. If left untreated, liver fibrosis can progress into cirrhosis, an end stage of liver fibrosis that affects 1–2% of the patient population and causes more than one million deaths yearly across the world (1–3). A variety of etiological factors have been identified, including viral hepatitis infections [e.g., hepatitis

B virus (HBV) infection, hepatitis C virus (HCV) infection], excessive alcohol consumption, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis (AIH), and cholestasis. Unfortunately, to date, there is no approved anti-fibrotic medication except for liver transplantation as the last resort of curative therapy for patients with liver cirrhosis. The exact mechanisms underlying the development and progression of hepatic fibrosis remain elusive. It has been documented that the activation of hepatic stellate cells (HSCs) through various inflammatory and fibrogenic pathways plays a pivotal role in liver fibrosis (4, 5). HSCs are considered the primary source of activated myofibroblasts and the cells can produce ECM proteins in the liver. Although rapid progress has been made in the basic research on experimental liver fibrosis, accurate non-invasive biomarkers and effective anti-fibrotic drugs are not available in clinical practice. Better understanding of the molecular mechanisms underlying liver fibrosis may provide new therapeutic and diagnostic targets, and in turn may accelerate the development of treatment and diagnosis for liver fibrosis.

The bromodomain and extra-terminal domain (BET) family is a class of proteins with two bromodomains and one extra-terminal domain, which can recognize acetylated lysine in histones (6, 7). There are four types of BET family members in humans: BRD2, BRD3, BRD4, and BRDT. Among them, BRD4 has recently received great interest because it seems to play a critical role in fibrosis. BRD4 is a transcriptional enhancer with two bromodomains. By binding to acetylated chromatin in interphase and mitosis, it acts as a co-activator in the transcription of various genes to modulate the cell cycle. Previous studies in cell culture systems and animal models have indicated that BRD4 promotes the gene expression of collagen type I alpha 1 chain (Col1A1) in response to transforming growth factor (TGF)-β, thereby resulting in fibrosis in the lung (8–10) and liver organs (11, 12). It has been reported that knockout of BRD4 through its specific small interfering RNA (siRNA) or inhibition using the BRD4 inhibitor JQ1 markedly reduces profibrotic mRNA expression, suppresses platelet derived growth factor (PDGF)-mediated proliferation of HSCs, and blocks the activation of HSCs in the carbon tetrachloride (CCl4)-induced mouse model of liver fibrosis (12–14).

Although BRD4 has been implicated in the development and progression of experimental liver fibrosis in cell culture systems and animal models (11, 15), its expression and function in liver fibrosis in humans has not been elucidated. Intrigued by the previous findings, we performed the first study to characterize the expression of BRD4 in patients with liver fibrosis. Specifically, we examined the BRD4 expression pattern in human liver tissues from patients with liver fibrosis of various etiologies, elucidated the specific cell types of BRD4 expression in human liver fibrosis specimens, and then analyzed the correlation of the BRD4 expression level with the fibrotic degree and clinical features in liver fibrosis caused by chronic HBV infection. A number of studies have shown that BRD4 could be a co-activator for transcription of nuclear factor-κB (NF-κB)-regulated cytokines (8, 16–18). More recently, C-X-C Motif Chemokine Ligand 6 (CXCL6) was reported to be involved in liver fibrosis (19). We therefore examined CXCL6 expression in fibrotic liver tissue and analyzed the correlation between BRD4 and CXCL6 levels. The results showed that BRD4 was highly expressed in the fibrotic liver tissue of patients and was principally localized in hepatocytes, HSCs, macrophages, and biliary tract cells. The BRD4 mRNA level was positively correlated with the CXCL6 mRNA level in the hepatic tissues of patients with liver fibrosis. Moreover, in HBV liver fibrosis, BRD4 protein expression was correlated with the severity of liver fibrosis. Our findings through conducting this study indicate that BRD4 plays important roles in the development and progression of liver fibrosis in humans.

Abbreviations: AIH, autoimmune hepatitis; AST, aspartate aminotransferase; BCA, bicinchoninic acid; BET, bromodomain and extra-terminal domain; BRD4, Bromodomain-containing protein 4; BSA, bovine serum albumin; CCl4, carbon tetrachloride; CD, cluster of differentiation; CK, cytokeratin; Col1A1, collagen type I alpha 1 chain; CXCL6, C-X-C Motif Chemokine Ligand 6; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; HBV, hepatitis B virus; HCV, hepatitis C virus; AIH, autoimmune hepatitis; NASH, non-alcoholic steatohepatitis; PBC, primary sclerosing cholangitis; HNF, hepatocyte nuclear factor; HSCs, hepatic stellate cells; IF, immunofluorescence; IHC, immunocytochemistry; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor-κB; PDGF, platelet derived growth factor; qRT-PCR, real-time quantitative polymerase chain reaction; RIPA, radioimmunoprecipitation assay; RNA-seq, RNA sequencing; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA, small interfering RNA; αSMA, α-smooth muscle actin; TBIL, total bilirubin; TBST, Tris-buffered saline/Tween 20; TGF-β, transforming growth factor β; WB, Western blot.

MATERIALS AND METHODS

Human Liver Tissues

A total of 32 fresh liver tissues, comprising 19 samples of liver fibrosis and 13 normal liver tissues, and 96 liver sections were obtained from Xiangya Hospital (Changsha, Hunan, China). Fresh hepatic tissues were collected from the study participants during either surgery or needle biopsy. The normal liver tissues and sections were obtained from transplant donors or individuals with hepatic hemangioma, while fibrotic tissues were obtained from patients with different degrees of liver fibrosis. Immediately following needle biopsy or surgery, fresh liver tissues were collected and snap-frozen in liquid nitrogen or fixed in 4% acetic paraformaldehyde. The liver sections were obtained from patients at Xiangya Hospital spanning the period between January 2015 and January 2020. The 96 liver sections evaluated consisted of 10 samples in the normal control group and 86 samples in the liver fibrosis/cirrhosis group. Liver sections in the normal control group were obtained from benign lesions such as hepatic hemangioma. The liver fibrosis/cirrhosis group contained 40 patients with HBV-associated fibrosis/cirrhosis, three with HCV-associated fibrosis/cirrhosis, one with NASH-associated fibrosis, 13 with cholestasis, five with primary sclerosing cholangitis (PBC), 12 with AIH, and 12 with overlap syndromes (**Supplementary Tables 1, 2**). The HBV liver fibrosis/cirrhosis samples were classified as F1-F4 according to the Metavir score: F1, portal fibrosis; F2, fibrosis with few septa; F3, numerous septa; and F4, cirrhosis. The clinical characteristics of the enrolled patients are listed in **Supplementary Table 3**.

Collections of tissues were performed with the approval of the Ethics Review Board of Xiangya Hospital Central

South University [IRB(S) No.201912533]. Informed consent was obtained from all patients.

RNA-Sequencing

Ten snap-frozen liver tissues were used for RNA sequencing (RNA-seq) and were obtained from eight patients who underwent surgical resection for the treatment of HBV-associated liver cancer in the liver fibrosis group ($n = 4$) or hemangioma in the control group ($n = 4$) (GEO: GSE171294). RNA-seq analysis was performed to explore potential genes related to liver fibrosis (Novogene Bioinformatics Institute, Beijing, China). Briefly, the total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Germantown, USA) following the manufacturer's manual. The NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) was used to generate sequencing libraries according to the manufacturer's instructions. Index codes were added to attribute sequences to each sample, and the index-coded samples were clustered using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) on a cBot Cluster Generation System in accordance with the manufacturer's manual. Upon the completion of cluster generation, RNA-seq was performed and 150 bp paired-end reads were generated with an Illumina Novaseq platform.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using the TRIzol reagent (Invitrogen, Grand Island, NY) following the manufacturer's instructions. RNA samples (2 μg) were reverse transcribed in cDNA with a reverse transcriptase (Promega, Madison, WI). To determine the mRNA expression levels of target genes, quantitative real-time PCR was performed using the 2×Taq PCR Master Mix (Qiagen, Beijing, China) and primers (**Supplementary Table 4**) on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Distilled water was used in place of cDNA as a negative control. The relative mRNA expression of the gene of interest was determined using the comparative Ct (threshold cycle) method as reported previously (20). Specifically, the ΔCt in each group was generated by subtracting the Ct of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from that of the target gene, yielding the $^{\Delta}$Ct in each group. The $^{\Delta\Delta}$Ct was then obtained by subtracting the $^{\Delta}$Ct of the experimental group from that of the control group. The relative expression levels of the gene of interest were normalized to those of GAPDH.

Western Blot Analysis

Total proteins were extracted from each liver tissue sample (50 mg) using a radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail tablet (Roche, Indianapolis, IN), and a bicinchoninic acid (BCA) assay was conducted to determine the protein concentrations. For Western blot analysis, 30 μg of each protein sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After completion, all proteins were transferred to nitrocellulose membranes and blocked by incubation with 5% skim milk powder dissolved in 0.1% Tris-buffered saline/Tween 20 (TBST). The resulting membranes were sequentially incubated with primary antibodies, including anti-BRD4 antibody (1:1000, ab128874; Abcam) and anti-GAPDH antibody (1:1,000; ab181602; Abcam) at 4°C overnight. The membranes were subsequently incubated with a secondary horseradish peroxidase-conjugated antibody (Proteintech Inc, Wu Han, China) at room temperature for 1 h. Immunoreactive proteins were visualized using the Enhanced Chemiluminescence Western Blotting Detection System (Santa Cruz) and the intensity of the chemiluminescent signal was analyzed using image J software. To control for sample loading error, the expression of BRD4 was normalized to GAPDH.

Histological Examinations and Laboratory Tests

Paraffin-embedded sections at a thickness of 2 μm were stained with Masson trichrome for histological examinations of the liver tissues. The pathological changes in the liver tissues were assessed by pathologists at Xiangya Hospital Central South University, and the degrees of liver fibrosis were evaluated according to the Metavir score. Blood routine tests and biochemical examinations for liver function and coagulation function were performed at the clinical lab in Xiangya Hospital Central South University.

Immunohistochemistry

Immunocytochemistry (IHC) was performed as described previously (21). In brief, paraffin sections (2 μm in thickness) were prepared from paraffin embedded liver tissue blocks, deparaffinized, and subsequently hydrated. Endogenous peroxides were then quenched with 0.3% H2O2. The sections were sequentially incubated with primary antibodies, including anti-BRD4 antibody (ab128874; Abcam, Cambridge, UK) at a dilution of 1:200 and anti-CXCL6 antibody (SC-377026, Santa Cruz) at a dilution of 1:100 at 4°C overnight. Sections with the primary antibody substituted with an equal concentration of rabbit or mouse IgG (ab172730, Abcam) were used as negative controls The bound antibodies were detected with diaminobenzidine (Sigma-Aldrich, St. Louis, MO) using the biotin–streptavidin–peroxidase system (UltraSensitive-SP-kit, MaiXin Biotechnology, Fuzhou, China). A nuclear counterstain with hematoxylin was performed in IHC.

During data analysis, both the intensity and extent of staining were taken into consideration. The intensity of staining was determined using the following criteria: 0 for negative; 1 for weak staining; 2 for moderate staining; and 3 for strong staining. The extent of staining was expressed as the area percentage of positive staining, ranging from 0 to 100%. Then, 10 areas from each section were randomly selected to examine the area percentage of positive staining and to calculate the mean extent of staining. The final score for each section was obtained by multiplying these two scores (intensity score × extent score) as reported previously (21).

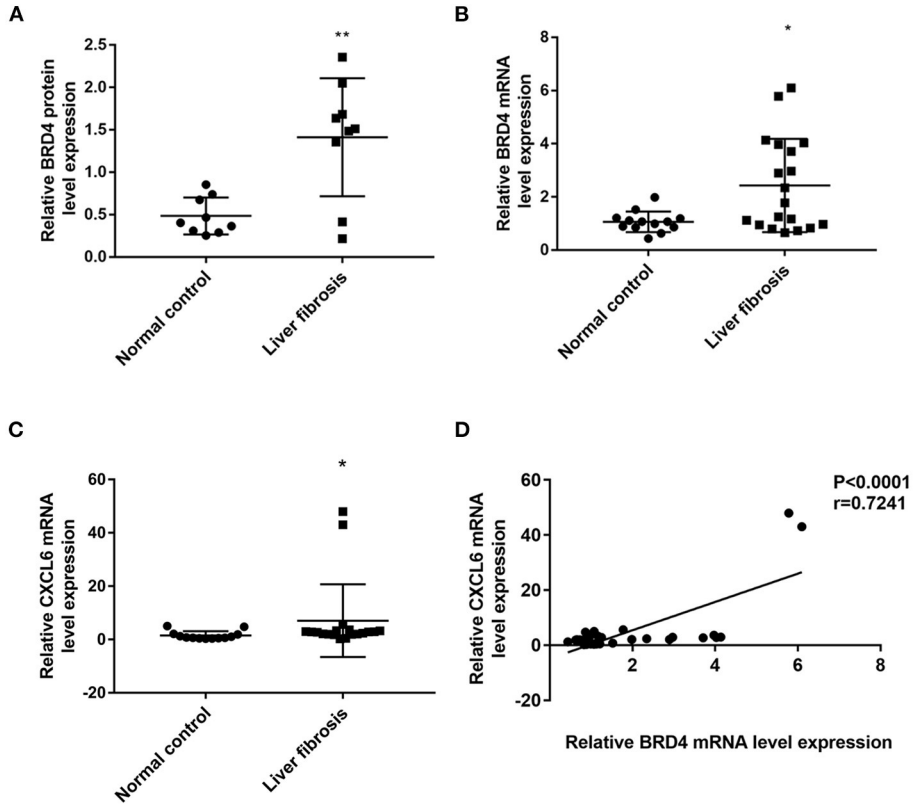

FIGURE 1 | BRD4 and CXCL6 expression in liver tissues of control and fibrosis group. The hepatic tissues were obtained as described in Materials and Methods. **(A)** BRD4 protein expression in patients with liver fibrosis ($n = 9$) and normal controls ($n = 9$). Data were expressed as mean ± SEM, Differences between groups were evaluated using two independent samples *t*-test. **(B)** mRNA expression level of BRD4 in liver tissues from patients with liver fibrosis ($n = 19$) and normal controls ($n = 13$). Data were expressed as median M (P25, P75), Differences between groups were evaluated using Mann-Whitney U rank-sum test. **(C)** CXCL6 mRNA expression in liver fibrosis group ($n = 19$) and control group ($n = 13$). Data were expressed as median M (P25, P75). Differences between groups were assessed using Mann-Whitney U rank-sum test. **(D)** Spearman correlation analysis of correlation between CXCL6 mRNA and BRD4 mRNA expression. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. BRD4, Bromodomain-containing protein 4; CXCL6, C-X-C motif chemokine ligand 6.

Immunofluorescence

Paraffin sections at a thickness of 2 μm were rehydrated, after which the slides were incubated with sodium citrate buffer (pH 6.0) for 10 min to retrieve the antigens. The slides were incubated for 1 h with 10% (v/v) bovine serum albumin (BSA), a widely used blocking solution, to minimize the background and non-specific binding. The resulting slides were sequentially incubated with primary antibodies at 4°C overnight, specifically anti-BRD4 antibody (ab128874, Abcam), anti-hepatocyte nuclear factor (HNF)4α antibody (ab41898; Abcam), anti-α-smooth muscle actin (SMA) antibody (ab7817; Abcam), anti-cluster of differentiation (CD)68 antibody (ab201340; Abcam), and anti-cytokeratin (CK)19 antibody (ab194399; Abcam). The resulting slides were washed three times with PBS, followed by incubation with secondary antibodies labeled with either Coralite Fluor 488 or Coralite Fluor 594. These procedures were performed in accordance with the manufacturer's instructions. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The slides were then mounted with cover glass, visualized, and analyzed under a Zeiss LSM 780 confocal microscope (Carl Zeiss Inc., Jena, Germany).

Statistical Analysis

Statistical analysis was conducted using the SPSS version 20 software package. Measurement data were analyzed with the Shapiro-Wilk test for normality, and data with a normal distribution were expressed as mean ± SEM. Differences between groups were evaluated using two independent samples *t*-test. Data with a non-normal distribution were presented as the median M (P25, P75) and differences between groups were assessed with the Mann-Whitney U rank-sum test. Count data were presented as the number of cases (percentage) and differences between groups were analyzed with the $\chi 2$ test. Spearman's correlation and Pearson correlation were used for the correlation analysis. *P*-values <0.05 were considered to indicate a significant difference between groups.

RESULTS

Hepatic BRD4 mRNA and Protein Expression Was Up-Regulated in Liver Fibrosis and Correlated to CXCL6 Level

RNA-seq showed that 1,246 genes were aberrantly expressed with more than log2-fold change in liver fibrosis tissues than in normal control tissues ($P < 0.05$). Among these, 687 genes were significantly up-regulated and 559 genes were down-regulated (**Supplementary Figure 1**). Notably, the BRD4 gene was increased in the liver fibrosis group vs. the normal control group (**Supplementary Figure 2**). In a relatively large scale of liver biopsy samples, qRT-PCR showed that BRD4 mRNA expression was significantly up-regulated in the fibrosis group compared with the expression in the control group (**Figure 1B**). Western blot analysis also showed that the BRD4 protein level was significantly increased in the fibrosis group compared with the level in the control group (**Figure 1A** and **Supplementary Figure 3**).

It was recently reported that hepatic CXCL6 expression is up-regulated in liver fibrosis and can promote the interaction of BRD4 with other transcriptional factors (19). RNA-seq showed that CXCL6 mRNA expression was increased in fibrotic liver tissues (**Supplementary Figure 2**). Confirmatory qRT-PCR analysis showed that the CXCL6 mRNA level was significantly increased in liver tissues from the liver fibrosis group compared with those from the control group (**Figure 1C**). Interestingly, the CXCL6 mRNA level was positively correlated to the BRD4 mRNA level in the liver fibrosis group (**Figure 1D**). Immunohistochemistry showed that CXCL6 positive staining was mainly localized in hepatocytes in liver tissues (**Supplementary Figure 4**).

Hepatic BRD4 Expression Was Increased in Liver Fibrosis/Cirrhosis With Various Etiological Factors

Considering that fresh liver samples were limited to liver fibrosis/cirrhosis samples in the vicinity of liver cancer, we examined the hepatic BRD4 expression pattern using pathologic specimens from patients with liver fibrosis/cirrhosis with a variety of etiologies, including HBV, HCV, AIH, PBC, NASH, cholestasis, and overlap syndrome. The analysis showed that BRD4 was mainly expressed in hepatocytes in the liver (**Figures 2A,B**). A low amount of positive staining for BRD4 was found in fibrotic scars in the liver. BRD4 protein expression was increased in the samples from all enrolled patients with liver fibrosis/cirrhosis compared with the expression in the control samples. As shown in **Figures 2A,B**, BRD4 protein levels were significantly increased in HBV-, AIH-, PBC-, cholestasis-, and overlap syndrome-associated liver fibrosis/cirrhosis tissues.

BRD4 Expression Was Localized in Hepatocytes, HSCs, Hepatic Macrophages, and Biliary Tract Cells

We further analyzed the cellular localization of BRD4 expression in the cell types of the liver. HNF4α, α-SMA, CD68, and CK19 are cell markers of hepatocytes, activated HSCs, macrophages, and biliary tract cells, respectively. BRD4-positive staining was detected in hepatocytes, activated HSCs, macrophages, and biliary tract cells (**Figure 3**). It was found that ∼90% of the total hepatocytes, 56% of the total macrophages and 67% of the total biliary tract cells were BRD4 positive.

BRD4 Expression Was Positively Correlated With the Severity of Fibrosis and Circulatory Aspartate Aminotransferase and Total Bilirubin Levels in HBV-Induced Liver Fibrosis

Given that HBV-induced liver fibrosis is the most common type of liver fibrosis in China due to the high prevalence of HBV infection, we further analyzed the association of the BRD4 expression level with fibrosis severity and other parameters. The male to female ratio was 1/1 in the normal control group, 1/1 in the F1 group, 4/1 in the F2 group, 9/1 in the F3 group, and 4/1 in the F4 group. No statistically significant difference in age was found among the five subgroups. The age was 51.4 ± 10.6 in the normal control group, 45.1 ± 13.4 in the F1 group, 54.8 ± 12.1 in the F2 group, 48.3 ± 12.9 in the F3 group, and 49.4 ± 14.0 in the F4 group. No statistically significant differences in gender and age were found among the five subgroups (**Supplementary Table 3**). The clinicopathological liver samples with HBV-associated fibrosis/cirrhosis were graded according to the Metavir score (F1–F4) (**Supplementary Table 3**). As shown in **Supplementary Table 3**, the alanine aminotransferase (ALT) levels of the normal control group [11.6 (9.8, 29.9) U/L] were significantly different from those of the F2 group [43.8 (27.0, 58.6) U/L], F3 group [53.3 (23.5, 76.9) U/L], and F4 group [33.5 (24.6, 49.3) U/L] ($P < 0.05$). The AST levels of the normal control group [17.8 (15.1, 28.6) U/L] were also significantly different from those of the F2 group [40.4 (31.5, 60.4) U/L], F3 group [52.7 (27.9, 74.6) U/L], and F4 group [40.4 (29.3, 45.8) U/L] ($P<0.05$). The TBIL levels of the normal control group (8.5 ± 2.2 umol/L) were significantly different from those of the F3 group (17.2 ± 6.7 umol/L), F4 group [14.3 (12.1, 17.8) umol/L] ($P < 0.05$). The white blood cell (WBC) level for the control group was significantly different from that of the F3 group ($P < 0.05$). Significant differences were observed between the control group and F4 group in ALT, AST, TBIL, prothrombin time (PT), plasma thromboplastin antecedent (PTA), international normalized ratio (INR), and platelet count (PLT) ($P < 0.05$).

As shown in **Figures 4A,B**, the BRD4 positive staining was increased with the severity of fibrosis. The positive correlation was found between BRD4 level and fibrotic severity ($r = 0.737$, $P < 0.0001$, $n = 50$) (**Figure 4C**). The BRD4 protein expression was also positively correlated with AST ($r = 0.368$, $P = 0.009$, $n = 50$), TBIL ($r = 0.401$, $P = 0.004$, $n = 50$) (**Figures 4D,E**). No significant correlation was found between the expression level of BRD4 and the ALT, PT, PTA, INR, or other clinical parameters (**Supplementary Figure 5**).

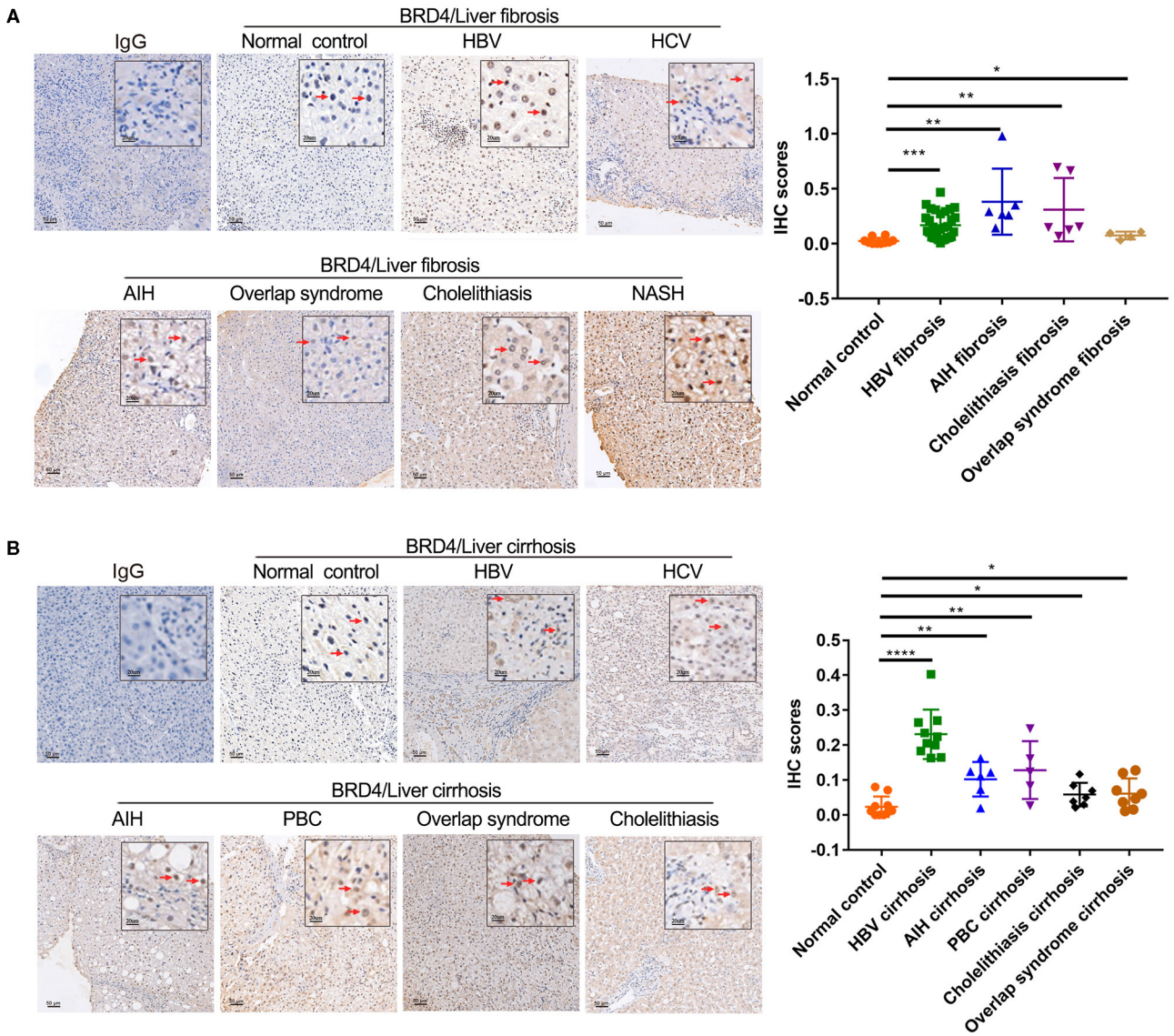

FIGURE 2 | Immunohistochemistry analysis of hepatic BRD4 protein expression in liver fibrosis/cirrhosis. **(A)** Immunohistochemistry analysis was performed to examine hepatic BRD4 protein expression patterns in normal liver tissues and liver fibrosis of various etiologies. The control sections were incubated with IgG. Representative images of normal hepatic tissues, HBV, HCV, AIH, NASH, cholelithiasis, and overlap syndrome. Scale bar: 50 μm. The expression level of BRD4 was analysis by Image J software. Data were expressed as mean ± SEM or median M (P25, P75). Differences between groups were evaluated using two independent samples *t*-test or Mann-Whitney U rank-sum test. **(B)** Immunohistochemistry analysis was performed to examine hepatic BRD4 protein expression patterns in normal liver tissues and liver cirrhosis of various etiologies. The control sections were incubated with IgG. Representative images of normal hepatic tissues, HBV, HCV, AIH, PBC, cholelithiasis, and overlap syndrome. Scale bar: 50 μm. The expression level of BRD4 was analysis by Image J software. Data were expressed as mean ± SEM or median M (P25, P75). Differences between groups were evaluated using two independent samples *t*-test or Mann-Whitney U rank-sum test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$; $^{****}P < 0.00001$ vs. normal control. BRD4, bromodomain-containing protein 4; HBV, hepatitis B virus; HCV, hepatitis C virus; AIH, autoimmune hepatitis; NASH, non-alcoholic steatohepatitis; PBC, primary sclerosing cholangitis.

DISCUSSION

The acetyl-histone binding protein BRD4 is not only involved in cell transcription, proliferation, and apoptosis (22–24), but is also essential for the transcription of aurora B kinase, an important kinase in the modulation of chromosome separation and cytokinesis during mitosis (25). More recently, it has been shown that the inhibition of BRD4 activity by its selective inhibitors displays therapeutic potential for liver cancer, gastric cancer, pituitary adenoma, aeroallergen-induced inflammation, and airway remodeling (26–28). Emerging evidence suggests that BRD4 is involved in the development and progression of fibrosis in different organs, including cardiac (29–31), pulmonary (8), and hepatic fibroses (12).

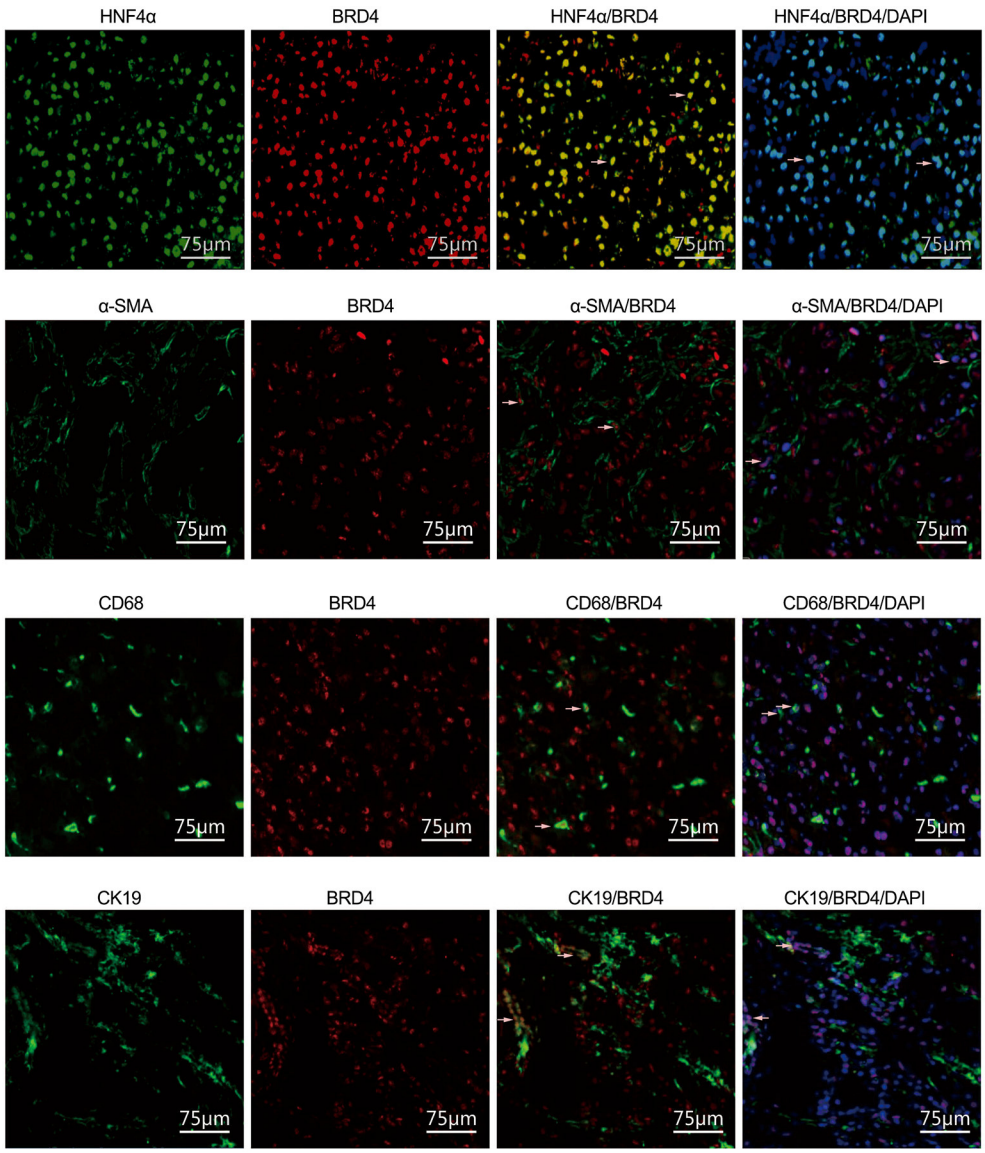

FIGURE 3 | Immunofluorescence analysis of cellular localization of BRD4 in the liver tissues. Immunofluorescence (IF) analysis was conducted to visualize the cellular localization of BRD4 in the liver tissues as described in Materials and Methods. The representative double immunofluorescence images showed expression of HNF4α (green), α-SMA (green), CD68 (green), CK19 (green), and BRD4 (red) in normal and HBV fibrotic liver tissue. Scale bar: 75 μm. BRD4, bromodomain-containing protein 4; HNF4α, hepatocyte nuclear factor 4 alpha; α-SMA, α-smooth muscle actin; CD68, cluster of differentiation 68; CK19, cytokeratin 19.

The BRD4 selective inhibitor JQ1 has been demonstrated to reverse the fibrosis response in the mouse model of liver fibrosis caused by CCl4 (12). In this study, we obtained the following major novel findings: (1) Hepatic BRD4 expression was up-regulated in patients with liver fibrosis of various etiologies, including HBV, HCV, AIH, PBC, cholestasis, and overlap syndrome; (2) Hepatic BRD4 expression was positively correlated with the severity of liver fibrosis; (3) Increased expression levels of hepatic BRD4 were positively correlated with the circulatory parameters associated with hepatic functions in HBV-induced liver fibrosis. Thus, our data suggest that the up-regulation of BRD4 may be associated with a common pathway for liver fibrosis, rather than the cause of liver fibrosis.

Previous studies have mainly focused on the effects of BRD4 on HSCs and have shown that the up-regulation of BRD4 in HSCs promotes the production of collagen I and subsequently leads to the activation of HSCs (32). However, the cell types that specifically express BRD4 have not been characterized in human liver tissues. In the present study, we demonstrated that BRD4 is expressed in various cell populations in the liver, including not only HSCs, but also hepatocytes, macrophages, and biliary tract cells. However, the functions of BRD4 in these cells in the liver remain largely unknown. It is well-known that

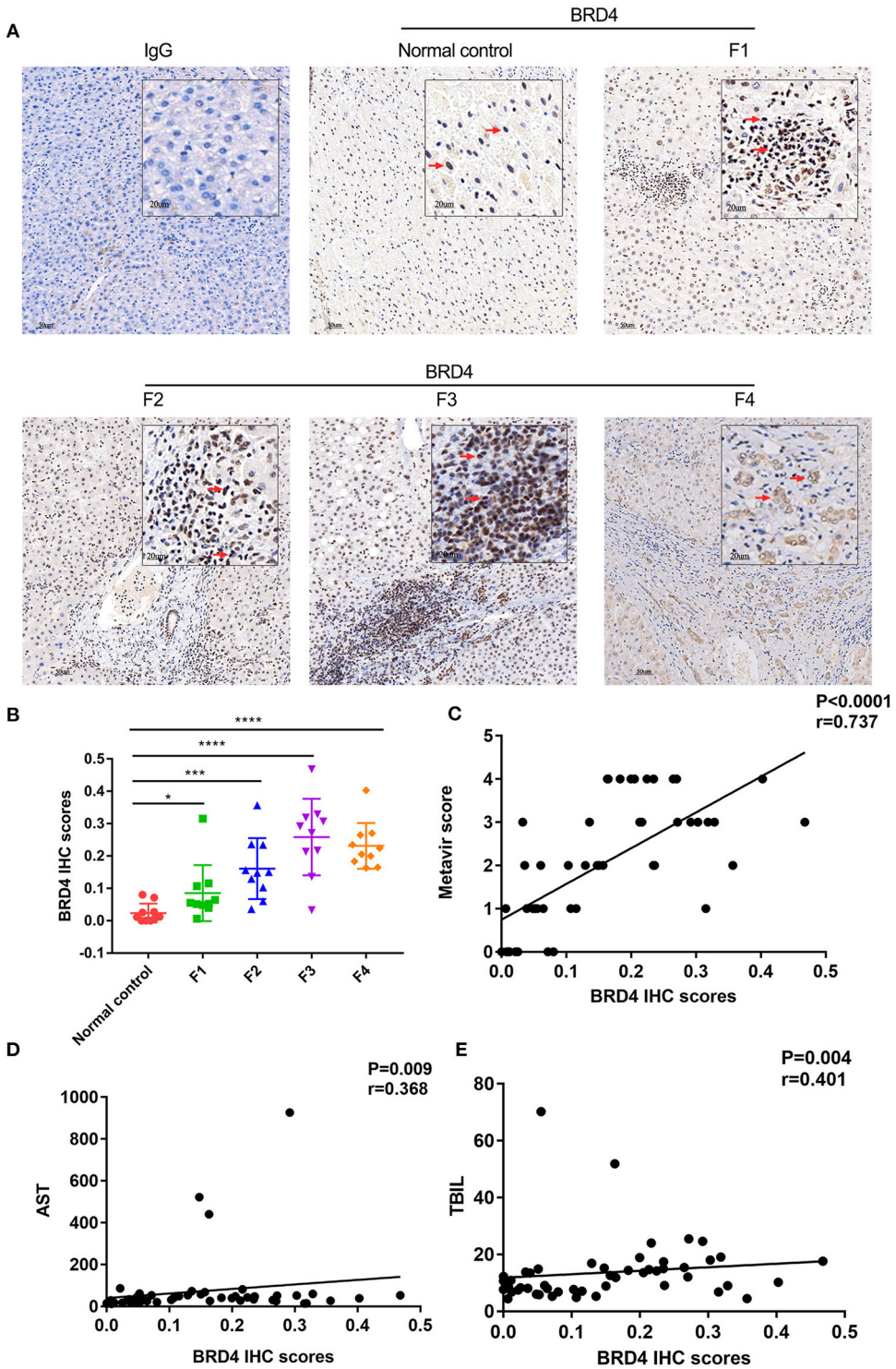

FIGURE 4 | BRD4 protein expression level in hepatic tissues of HBV liver fibrosis with different degrees. Immunohistochemistry was performed to examine hepatic BRD4 protein expression in HBV liver fibrosis with F1–F4 according to the Metavir score. The expression level of BRD4 was analysis by Image J software. **(A)** representative images of BRD4 expression in F1–F4 liver fibrosis. Scale bar: 50 μm. **(B)** cumulative data of BRD4 expression in F1–F4 liver tissues. Data were expressed as mean ± SEM or median M (P25, P75). Differences between groups were evaluated using two independent samples *t*-test or Mann-Whitney U rank-sum test. **(C)** Spearman correlation analysis of the correlation between severity of liver fibrosis and BRD4 expression. **(D)** Spearman correlation analysis was conducted to assess the correlation between BRD4 expression and AST of patients with liver fibrosis. **(E)** Spearman correlation analysis was conducted to assess the correlation between BRD4 expression and TBIL of patients with liver fibrosis. BRD4, bromodomain-containing protein 4; HBV, hepatitis B virus; AST, aspartate aminotransferase; TBIL, total bilirubin. *$P <$ 0.05; ***$P <$ 0.0001; ****$P <$ 0.00001.

the destruction and regeneration of hepatocytes occurs during the development of liver fibrosis caused by HBV. Our results showed that BRD4 protein expression was positively correlated with AST and TBIL in HBV-induced liver fibrosis. In view of the role of BRD4 in cell proliferation and apoptosis, BRD4 might be involved in the regeneration of hepatocytes in HBV-induced liver fibrosis. Some studies have demonstrated that BRD4 plays important roles in macrophages. For instance, a number of recent studies have shown that BRD4 can affect tumor-associated macrophages (TAM) in solid tumors (18, 33, 34). Ren et al. (13) reported that BRD4 participates in the production of pro-inflammatory cytokines induced by titanium particles via promoting the activation of NF-κB signaling in macrophages in mice. Macrophages have recently been shown to play a critical role in liver fibrosis in NASH (35), suggesting that the up-regulation of BRD4 in macrophages might be involved in the progression of liver fibrosis. Nevertheless, the roles of BRD4 in hepatocytes, macrophages, and biliary tract cells during liver fibrosis remain to be further studied.

It is currently recognized that inflammatory factors promote the activation of HSCs and play an important role in the development of liver fibrosis. In various tissues, BRD4 is found to be involved in inflammatory responses. It has been shown that BRD4 plays an important role in inflammation of vascular smooth muscle cells (36). BRD4 inhibitors block airway inflammation caused by the virus (37). BRD4 blockers reduce pathological cardiac hypertrophy by reducing ROS production and inhibiting fibrosis and inflammation (38). BRD4 inhibitors also have a protective effect against vincristine induced peripheral neuropathy by reducing inflammation (39). It has been demonstrated that involvement of BRD4 in inflammation is associated with NF-κB activation. BRD4 plays an central role in respiratory syncytial virus-induced inflammation and infant pneumonia through NF-κB (17, 40). BRD4 inhibitors can effectively inhibit NF-κB mediated inflammatory responses in endothelial cells (41).

Given that BRD4 is a co-activator of NF-κB and promotes the transcription of chemokines and cytokines in various cells besides macrophages, there is a possibility that BRD4 may promote the initiation, development, and progression of liver fibrosis through certain inflammatory mediators. It has been shown that chemokines, a group of small (8–14 kDa) proteins, exert their roles in the host immune responses and other physiological and pathological processes through interactions with their cell-surface receptors (42–45). Chemokines are produced by a wide range of cell types, including leukocytes, endothelial cells, fibroblasts, epithelial cells, and tumor cells (46–51). Notably, a number of previous studies have shown that the CXCL6 level is increased in the sera of patients with liver fibrosis and its expression is increased in the liver tissues of patients with liver fibrosis (19, 52, 53). It has been reported that CXCL6 is a prognostic biomarker for liver fibrosis. Consistent with these previous studies, we also found that CXCL6 expression is elevated in HBV-induced liver fibrosis. As for the potential role of CXCL6 in liver fibrosis, it has been demonstrated that CXCL6 is involved in the recruitment of monocytes into the liver following liver injury. It stimulates the release of TGF-β by Kupffer cells and subsequently promotes the activation of HSCs, thereby leading to liver fibrosis (54). Interestingly, Cai et al. (19) also showed that CXCL6 could promote interactions between BRD4 and other transcription factors (e.g., SMAD2) in HSCs. These findings are in support of our speculation that BRD4 could promote CXCL6 production through its function as a co-activator of NF-κB, and that CXCL6, in turn, promotes BRD4 function by enhancing interactions between BRD4 and other factors. Such interactions between CXCL6 and BRD4 might promote the development and progression of liver fibrosis.

The present study has some potential limitations. For instance, we did not verify whether CXCL6 is the downstream gene of BRD4 and did not assess the potential genes regulated by BRD4 in human liver tissues. Further in-depth investigations are underway in our laboratory to better understand the role of BRD4 in patients with liver fibrosis.

In summary, this study has demonstrated, for the first time to our knowledge, that hepatic BRD4 expression is markedly increased in patients with liver fibrosis with various etiological factors, and that elevated levels of BRD4 are associated with higher degrees of liver fibrosis. The results have also indicated that BRD4 is mainly expressed in hepatocytes, HSCs, hepatic macrophages, and biliary tract cells in the liver. These findings suggest that BRD4 plays an important role in the development and progression of liver fibrosis in humans and that BRD4 has potential as a target for intervention of liver fibrosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO), https://www.ncbi.nlm.nih.gov/geo/, GSE171294.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Board of The Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XN designed the experiments, wrote the manuscript, and supervised the study. SP and LF supervised the study and instructed the clinical characteristics. CW performed the experiments and wrote the manuscript. DC assisted the data analysis and experiments. YP and YL assisted experiments. CF and YW instructed the histological analysis of the section. 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/fmed.2021.683506/full#supplementary-material

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