From Basic Science to the Clinic

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Liver Fibrosis: From Pathogenesis to Novel Therapies

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Abstract
Chronic liver injury is accompanied by a dysbalanced scarring process, termed fibrosis. This process is mainly driven by chronic inflammation and an altered activity of a multitude of different chemokines and cytokines, resulting in the infiltration by immune cells (especially macrophages) and increase of matrix-expressing cell types. These processes might lead to cirrhosis representing the end-stage of fibrosis. Recent clinical studies comprising patients successfully treated for viral hepatitis showed that liver fibrogenesis and even cirrhosis may be reverted. The hepatic capacity to remodel scar tissue and to revert into a normal liver follows specific mechanistic principles that include the termination of chronic tissue damage, shifting the cellular bias from inflammation to resolution, initiation of myofibroblast apoptosis or senescence and, finally, fibrinolysis of excess scar tissue. The plurality of molecular and cellular triggers involved in initiation, progression and resolution of hepatic fibrogenesis offers an infinite number of therapeutic possibilities. For instance, inflammatory macrophages can be targeted via inhibition of chemokine CCL2 or its receptor CCR2 (e.g., by cenicriviroc) as well as by transfer of restorative macrophage subsets. Another target is galectin-3 that acts at various stages along the continuum from acute to chronic inflammation. Profibrogenic cytokines (e.g., transforming growth factor-β), matricellular proteins (e.g., CCN1/CYR61) or signaling pathways involved in fibrogenesis offer further possible targets. Other options are the application of therapeutic antibodies directed against components involved in biogenesis or remodeling of connective tissue such as lysyl oxidase-like-2 or synthetic bile acids like obeticholic acid that activate the farnesoid X receptor and was antifibrotic in a phase 2 study (FLINT trial). Factors affecting the gut barrier function or the intestinal microbiome further expanded the repertoire of drug targets. In this review, we discuss novel concepts in resolution of hepatic fibrosis and focus on drug targets that might be suitable to trigger resolution of fibrosis.

Introduction

Perpetuating liver damage leads to fibrosis and cirrhosis representing a common and difficult clinical challenge. Clinical hepatologists traditionally define cirrhosis as a non-reversible end stage of chronic liver injury, giving rise to a widespread distortion of normal hepatic architecture. However, recent studies have proven that the progress of ongoing liver fibrogenesis and development of cirrhosis by far is not a unidirectional process [1]. Likewise, there is evidence showing that established fibrosis is susceptible to regression and possibly even full reversion

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[2]. The most compelling confirmation that successful medical treatment of a liver disease imparts amelioration of architectural disturbances originates from chronic hepatitis B virus (HBV) infection. In HBV-infected patients with cirrhosis at baseline, 5 years of treatment with the nucleotide analogue tenofovir led to reversal of cirrhosis in almost 75% of cases [3]. However, there is still an urgent requirement to combat advanced fibrosis or established cirrhosis due to the lack of specific therapeutic tools in other disease conditions. This is a particular high unmet medical need in non-alcoholic fatty liver disease (NAFLD) encompassing non-alcoholic steatohepatitis (NASH), which can result in end-stage liver disease. Despite the fast growing prevalence of this disease worldwide, no curative therapy exists [4]. Similarly, cirrhosis occurring in the course of alcoholic liver disease cannot be reverted in most cases despite the termination of alcohol consumption [2]. Thus, novel approaches interfering with scar formation or resolution are urgently needed. Worldwide, intensive research conducted in the last decades has identified a multitude of potential novel drug targets that might be relevant to attenuate hepatic fibrogenesis, promote tissue repair and to initiate resolution. Undoubtedly, the multitude of antifibrotic treatment modalities represents an unconquered area for drug development with some of them already having a sound preclinical database or transferred into early clinical trials [5, 6]. The significant proportion of the clinical trials evaluating antifibrotic drugs is conducted in NASH and NASH-associated cirrhosis because of the exceptional epidemiological relevance. However, it is very likely that the universality of the underlying processes that control extracellular matrix (ECM) homeostasis will provide therapies with general applicability.

Inflammation as a Key Driver of Liver Fibrogenesis

The persistence of chronic inflammation is a hallmark associated with progressive hepatic fibrosis and the development of cirrhosis [7, 8]. Inflammation is a process that is initiated by tissue resident immune cells such as macrophages, mainly Kupffer cells, dendritic cells (DCs), mast cells and others [7, 8]. Over the years, the molecular understanding of inflammation and its underlying pathways has increased dramatically. The liver has a unique anatomy connected with the intestine by portal vein and bile ducts that allows delivery of products from intestinal microflora directly to the liver [9]. This architecture entails that in cases of hepatic injury or breaching of the intestinal tract mucosa, toxic substances with immunomodulatory activities such as lipopolysaccharides can penetrate into the liver and activate responsive cells. Kupffer cells, the resident hepatic macrophage population, are considered the first and most efficient responders to alterations of tissue integrity or inflammatory danger signals [10, 11]. These exogenous triggers, either pathogen-associated or danger-associated molecular patterns (PAMPs or DAMPs, respectively), initiate a sequence of events in responsive cells (Kupffer cells, possibly also stellate cells and hepatocytes) that are recognized by special pattern-recognition receptors including toll-like receptors (TLRs; fig. 1) [12, 13]. Following activation, the expression or secretion of a large variety of inflammatory cytokines can be initiated including tumor necrosis factor (TNF)-α, interleukin (IL)-1α/β, IL-6, IL-12, IL-18, granulocyte macrophage colony-stimulating factor and others. Likewise, during hepatic insult, endogenous triggers released from inherent dying cells, partly dependent on the mode of cell death (necrosis, apoptosis, necroptosis), facilitate inflammation [7, 14]. At the same time, profibrogenic cytokines such as transforming growth factor (TGF)-β1, platelet-derived growth factor, endothelial growth factor and many others are released by parenchymal and non-parenchymal liver cells. These soluble factors initiate a tightly controlled program, in which hepatic stellate cells (HSC) undergo a gradual phenotypic change from a non-proliferating, retinoid-storing cell type into a proliferating, fat- and retinoid-losing phenotype. The cellular phenotype resulting from this transdifferentiation is the myofibroblast (MFB) that increasingly expresses α-smooth-muscle actin and produces large quantities of ECM components such as collagen representing the hallmark of fibrosis. MFB not only produces almost all of the ECM components but also synthesizes a broad array of cytokines and chemokines and, furthermore, acquires contractility in response to ligands such as endothelin and nitric oxide [15]. Beside HSC/MFB, portal fibroblasts and bile duct epithelial cells might participate in fibrogenesis albeit their fractional contribution is not strictly assessed and might be of minor importance [16].

As a result of all these intrahepatic alterations various inflammatory and profibrotic pathways are rigorously activated triggering the net accumulation of ECM in the liver. At the same time, the activity of several matrix metalloproteinases (MMPs) is altered by the enhanced expression of their inhibitor, that is, the tissue inhibitors of MMPs (TIMPs). The process of hepatic fibrogenesis is further modulated by matricellular proteins of the CCN

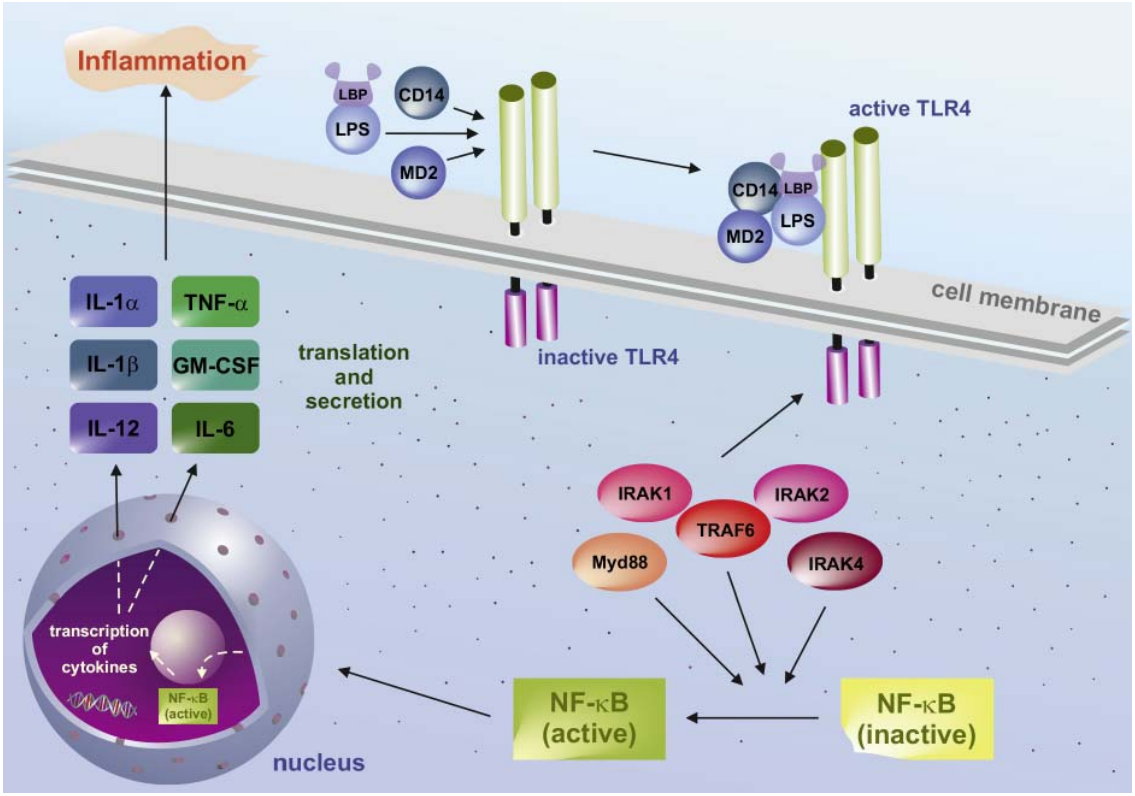

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Fig. 1. TLR signaling. LPS as a prototypical PAMP signal binds to the LBP and is recognized by the membrane-bound CD14/TLR4/MD2 receptor complex. Once activated, this receptor complex initiates a signal cascade consisting of a large variety of intracellular proteins (MyD88, IRAKs, TRAFs) resulting in the activation of the NF-κB signaling pathway and the induction of inflammatory cytokines (e.g., TNF-α, IL-1α/β, IL-6, IL-12, IL-18 and GM-CSF) driving the inflammatory response. This inflammatory signaling is prototypically found in macrophages. CD14 = Cluster of differentiation 14; GM-CSF = granulocyte macrophage colony-stimulating factor; IRAK1/2/4 = IL-1 receptor-associated kinase 1/2/4; LBP = LPS binding protein; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B-cells; TRAF6 = TNF receptor associated factor 6. For details about TLR signaling refer to [12, 13].

protein family including the cysteine-rich angiogenic inducer 61 (CYR61/CCN1) or the connective tissue growth factor (CTGF/CCN3) that are either essential factors linking inflammation and fibrosis or enhancing the effects of profibrogenic acting factors in the initiation phase [17].

Mechanisms of Liver Fibrosis Regression

The tight connection of inflammation and fibrogenesis predicts that therapies suppressing liver inflammation should also be efficient in preventing, arresting or reversing hepatic fibrosis. In general, in the regression process, a sequence of specific mechanistic principles can be distinguished. Starting from termination of chronic damage, a shift must occur that redirect the cellular bias from inflammation to resolution. Subsequently, mechanisms must guarantee a deactivation of MFB and a final degradation of excess ECM (fig. 2).

In this scenario, macrophages are key players in the fibrotic intercellular network exerting dual functions. Depending on their phenotype, origin and functional state, they either orchestrate fibrosis progression or regression [11, 18]. In progressing fibrosis in mice, Ly6C positive monocyte-derived macrophages are massively recruited from the blood stream to the injured liver. As vigorous secretors of inflammatory cytokines including TNF-α, they drive inflammation and activate HSC thereby triggering a cascade of events leading to fibrosis [11]. In line, inflammatory macrophages foster angiogenesis, a process that is closely linked to fibrosis progression [19]. On the other hand, angiogenesis driven by myeloid-derived vascular endothelial growth factor (VEGF) is also critical for fibrosis resolution, as VEGF-stimulated sinusoidal endothelium secretes matrix degrading metallo-

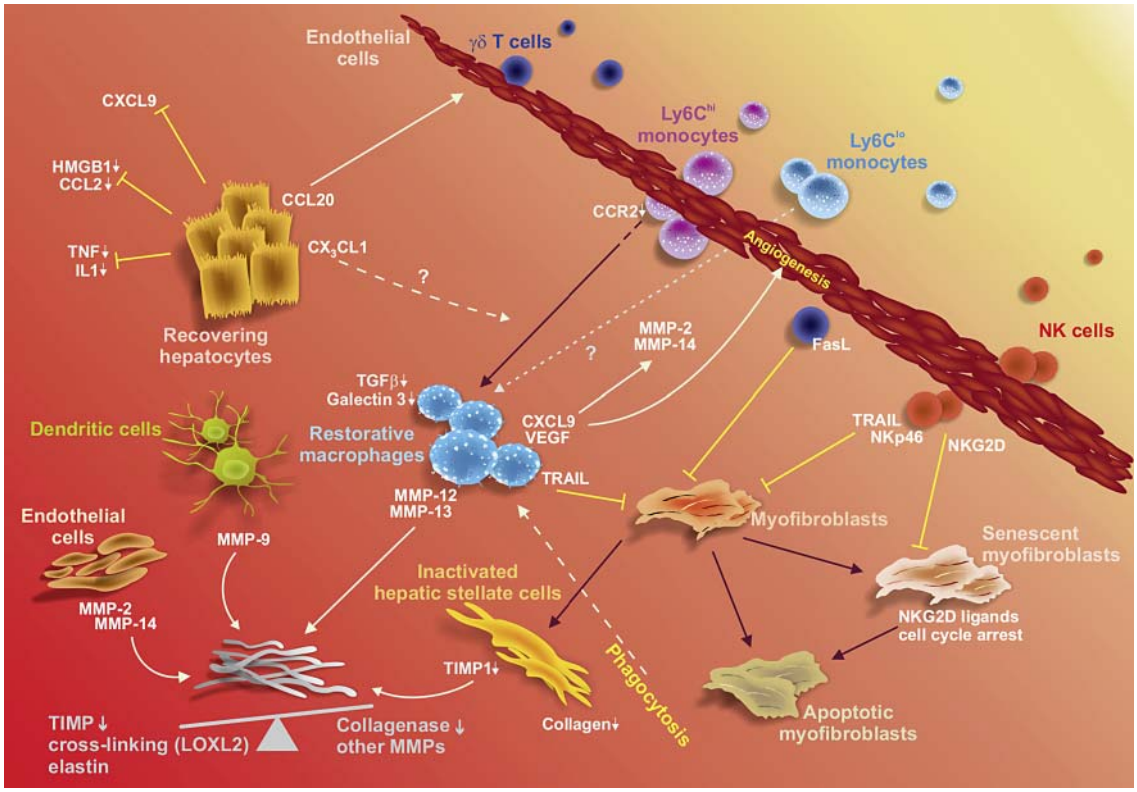

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Fig. 2. Sequential concept of liver fibrosis resolution. The figure summarizes the critical cellular and molecular events of liver fibrosis resolution that were identified in animal models. Recovering hepatocytes, restorative macrophages and deactivated MFB provide important signals for tissue regeneration, resolution of inflammation and degradation of ECM components. For details about mechanisms refer to [80]. CCL/CXCL = Chemokine; CX3CL1 = fractalkine; FasL = Fas ligand (CD95L); HMGB1 = high mobility group protein B1.

proteinases (MMP) thereby unmasking an unanticipated link between angiogenesis and the resolution of hepatic fibrosis [20].

At later stages, Ly6C^{hi} macrophages can differentiate into restorative Ly6C^{lo} macrophages that can phagocytize cellular debris and secrete MMP-9 and MMP-1/MMP-2, both of which promote scar resolution [21]. Such a therapeutic activity of macrophages was confirmed in mice by transplantation of bone marrow (BM)-derived macrophages into livers with advanced liver fibrosis induced by chronic administration of carbon tetrachloride [22]. Similarly, the transfer of autologous mixed BM-derived cells that expressed enzymatic active MMP-9 was previously shown to corroborate fibrosis resolution in a model of spontaneous regression of liver fibrosis in mice [23]. Therefore, the deliverance of immune cells to the fibrotic liver tissue is a rational therapeutic approach to modulate fibrosis regression. Importantly, the heterogeneity and the role of macrophages in human liver fibrosis are less well understood than in mouse models [24]. Nevertheless, there is preliminary clinical evidence that infusion of BM-derived cells might be beneficial in end-stage liver disease, and a large controlled multicenter clinical trial, that is, the Repeated Autologous Infusions of Stem cells in Cirrhosis (REALISTIC), in which the outcome on cirrhosis in patients will be assessed that either receive G-CSF alone or G-CSF followed by repeated infusions of hematopoietic stem cells compared with standard conservative management [25]. However, although the results of this trial need to be awaited, recovery from advanced cirrhosis to normal tissue might be somewhat limited by the occurrence of irreversible matrix crosslinking conferring a relative resistance to degradation [26].

Arrest of Chronic Liver Damage

Most of the present knowledge of the mechanisms of liver fibrosis progression and regression originate from animal studies or from tightly observed patients, which are under effective disease-specific therapy. Although it became evident that during hepatic fibrogenesis several

restorative mechanisms are co-induced during injury, all these mechanisms are not sufficient to prevent or even arrest ongoing fibrogenesis in the presence of persisting injury. Therefore, fibrosis arrest is currently dependent on supportive treatments that might vary manifold. Of course, fibrosis arrest in patients suffering from HBV or HCV infection is best achieved by successful suppression of virus replication [2]. Similarly, increased survival and better clinical outcome during alcoholic liver disease is found after strict alcohol withdrawal [27]. However, abstinence alone is not beneficial on the prevalence of HCC development, possibly because of irreparable damages that are induced by chromosomal loss or oxidative stress after prolonged phases of alcohol abuse [28]. In mouse models, fibrosis regression is usually studied by withdrawing the causative agent (e.g., hepatotoxins or diet) [29]. As a consequence of injury cessation, the release of pro-inflammatory endogenous danger signals such as the high-mobility group protein B1 or the occurrence of free DNA that are released into the extracellular milieu during states of cellular stress or damage are halted upon termination of liver damage [14].

Shifting the Hepatic Microenvironment from Inflammation to Resolution

After arrest of chronic liver damage and withdrawal of pro-inflammatory triggers, the inflammatory milieu in the liver usually switches to a condition in which it is possible to restore the normal liver architecture. In this resolution phase, recovering hepatocytes and their neighboring non-parenchymal cells send out restorative and anti-inflammatory signals. As a consequence, the chemokine-mediated attraction of inflammatory monocytes that is majorly triggered by CCR2, the recruitment of NKT cells by CXCL16 from the circulation and the activation of intrahepatic and bypassing T cells is dramatically reduced [30]. This modified milieu allows the intrahepatic immune cells to modify their activities and adjust to a phenotype that is able to counteract the previous demolitions of the inflammatory insult. One of the most striking phenotypic immune cell switches is observed for macrophages that acquire in this phase a restorative phenotype that is characterized by low Ly6C expression in mice and high expression of MMP-9 and MMP-12, growth factors (favoring hepatocyte recovery) and phagocytosis-related genes [21]. These processes are further triggered by phagocytosis of cellular debris that is produced by apoptotic MFB and hepatocytes and activation of special signaling cascades (e.g., MAPK ERK) associated with cell proliferation [21].

At the same time, the restorative milieu within the liver is enriched with increased numbers of DCs and NK cells. This was impressively demonstrated in carbon tetrachloride-induced murine livers in which DC were conditionally depleted. These mice showed delayed fibrosis regression, while artificial DC expansion induced either by administration of the FMS-like tyrosine kinase-3 ligand or adoptive transfer of purified DCs had accelerated liver fibrosis regression [31]. This study further demonstrated that the curative effect of DCs was partially dependent on MMP-9, while NK cells induce the apoptosis of activated and senescent MFB via stress- or damage-sensitive signaling branches that require NKG2D and TNF-related apoptosis-inducing ligand (TRAIL) [32, 33]. In addition, γδ T-cell receptor expressing T-cells (γδ T-cells) restrict hepatic inflammation and fibrosis. During hepatic injury, these cells co-localize with HSC in vivo and promote apoptosis or primary HSC in a cell–cell contact-dependent manner, most likely through activation of the Fas-ligand pathway [34].

The resolution phase is further characterized by mechanisms that lead to the formation of new blood vessels from pre-existing vessels. While monocyte-promoted angiogenesis is regularly observed in progressing fibrosis [19], VEGF-stimulated sinusoidal angiogenesis might be a mandatory requirement for optimal fibrosis regression. This assumption is underpinned by the finding that both the genetic ablation of VEGF and the pharmacological inhibition of VEGF receptor 2 signaling prevent the angiogenic response and the resolution of liver fibrosis [20]. In line with these findings, VEGF neutralizing antibodies were able to prevent development of hepatic fibrosis but also disrupted fibrosis resolution in mice that were subjected to bile duct ligation [35].

MFB Deactivation and Elimination

It is obvious that the main challenge during resolution of fibrosis is the reduction or inactivation of cells that are causative for the extensive ECM production. As outlined above, the main collagen-producing cells in the liver are HSC that transdifferentiate into MFB. Therefore, the deactivation or reduction of activated HSC and transdifferentiated MFB is the key request for fibrosis regression. In general, there are a number of possibilities how the liver can facilitate the clearance or inactivation of these unwanted cells.

Genetic tracking experiments in mice using a tamoxifen-inducible CreER directed under the control of the endogenous vimentin promoter showed that the deactivation of activated and transdifferentiated MFB into a 'quiescent-like' phenotype is one option during liver fibrosis

resolution [36]. However, this reversal results in a cellular phenotype that is more prone to fibrogenic re-stimulation [36]. Similar findings were observed in experiments in which the fate of HSC/MFB was investigated during the recovery from carbon tetrachloride- or alcohol-induced liver fibrosis using Cre-loxP-based genetic labeling of MFB [37].

Another option for clearance is the initiation of MFB apoptosis. Therefore, the cells must initiate a program in which apoptotic signals trigger cell death. There is a multitude of soluble factors including growth factors (NGF, IGF-1, TGF-β) and death receptor ligands (TRAIL, FAS) that are released by neighboring inflammatory cells or hepatocytes that support the initiation of hepatic MFB apoptosis [38]. Most interestingly, MFB apoptosis is itself dependent on the presence of biologically active collagen I [39], while mutations in collagen I that confer failure in its degradation critically impairs HSC apoptosis and results in failure in liver fibrosis recovery [26]. The initiation of MFB apoptosis during fibrosis regression is further driven by the withdrawal of anti-apoptotic signals as well as by NK cells, γδ T-cells and possibly also $CD8^+$ cytotoxic T-cells [33].

Nearly a decade ago, it was shown that senescent cells accumulate in the livers of mice that received repeated injections of carbon tetrachloride suggesting that the induction of senescence provides a barrier that limits liver fibrosis [40]. In the mentioned study it was also shown that the lack of p53 results in more fibrotic tissue and increased expression of TGF-β1 suggesting that the senescence program is p53-dependent. As a consequence, p53-deficient activated HSC can bypass the senescence response, continue to proliferate and synthesize ECM within the tissue. The list of factors that drive MFB senescence was extended by the finding that CCN1/CYR61 belonging to the family of CCN matricellular proteins acts as a trigger of cellular senescence in activated HSC and portal MFBs, most likely by engaging integrin α1β6 to induce reactive oxygen species accumulation through the RAC1-NADPH oxidase 1 enzyme complex [41]. In accordance, the adenoviral overexpression of hepatic CCN1 resulted in enhanced senescence and was capable of accelerating fibrosis regression in mice with already established fibrosis and significantly inhibited production of collagen type I at both mRNA and protein levels [41, 42]. Additionally, we demonstrated that CCN1/CYR61 attenuates TGF-β signaling by physically interacting with TGF-β thereby mitigating in vivo liver fibrogenesis in a bile duct ligation model [42]. Altogether, this suggests that the targeted transfer of this matricellular protein into ECM-producing liver cells is potentially beneficial in anti-fibrotic therapy. It should be further kept in mind that diverse factors that are secreted by senescent cells attract various innate immune cells including macrophages, neutrophils and NK cells that mediate the final clearance. NK cell-mediated immune clearance of senescent HSC for example was shown to be crucial to restrict the progression of liver fibrosis [40].

Degradation of ECM

In the last step of the restoring program, the normal liver architecture requires the degradation of superfluous ECM components composed of collagen, glycosaminoglycans, laminin, elastins and proteoglycans [7, 8]. The most important degrading effectors are MMPs representing a family of zinc- and calcium-dependent endopeptidases, which are produced by a multitude of cells and have a broad range of activity against the major constituents of ECM including fibrillar and non-fibrillar collagens and elastins [43]. There is fundamental evidence that the enzymatic activity of several MMPs in the fibrotic liver is decreased. Pioneering work has shown that one mechanism underlying this phenomenon is the elevated expression of the MMP inhibitor TIMP-1. This inhibitor is strongly upregulated during HSC activation and in experimental liver fibrosis preventing degradation of secreted collagens [44]. Likewise, in fibrotic human liver, the expression of TIMP-1 and TIMP-2 are elevated up to 5-fold compared to normal liver [45]. The importance of MMPs in regulating ECM degradation was prototypically documented in mice that lacked MMP-12. When these mice were exposed to carbon tetrachloride, they developed a similar degree of fibrosis compared to wild-type mice but increased the content of perisinusoidal elastin quantities [45]. Elastin as a characteristic feature of advanced fibrosis might also be a good target for non-invasive fibrosis detection such as molecular MRI techniques [46]. Depletion experiments further revealed that macrophages are the sole source of hepatic MMP-12 and suggest that these cells have an essential restorative function [47]. The restorative function of MMP-9 and MMP-12 was also confirmed in another study analyzing fibrosis resolution [21]. However, neutrophils and HSC also can express a repertoire of different MMPs with essential activities during fibrosis regression. It is obvious that the stimulation of MMP activity or decrease of their inhibitors should be therapeutically beneficial. This concept was proven in a study in which mice that received intraperitoneal carbon tetrachloride injections were treated with proteolytic inactive MMP-9 mutants acting as scavengers for TIMP-1 [48]. The application of these mutants led to increased apopto-

sis of activated HSC and an overall reduced hydroxyproline content in liver tissue [48]. All these studies provide the basis for novel therapeutic strategies aiming to influence the activity of the MMP/TIMP axis, particularly for preventing of matrix formation or degradation of already established scar tissue. However, some features of advanced fibrosis may confer a relative resistance to matrix degradation by formation of collagen crosslinking and deposition of elastin within the injured tissue [33].

Therapeutic Targeting of Fibrosis Resolution

Based on the different mediators and pathways that are involved in hepatic inflammation and fibrosis progression, there is a plenitude of potential drug targets that may offer the basis for new therapies in fibrosis resolution. The increasing knowledge of the cellular and molecular mechanisms of liver fibrogenesis and regression prompted extensive research on new pharmacological approaches. Several potential targets are currently investigated in preclinical and/or early clinical trials raising realistic hopes for effective antifibrotic therapies in the near future [5, 49]. The possibilities for therapeutic targeting are quite heterogeneous ranging from blocking of injury-mediated apoptosis of hepatocytes, transfer of BM-derived restorative macrophages, inhibition of inflammatory monocyte infiltration, induction of MFB apoptosis, increase of matrix degradation by inhibiting the collagen cross-linking activity, inhibiting TIMPs, activating of MMP activity and many others. In the following, we will shortly discuss some innovative examples that might translate basic findings into novel therapies and affect different drug targets.

Targeting Monocytes, Macrophages and Chemokines

Based on the versatility of fibrosis formation and resolution, there are endless possibilities for therapeutic interventions. As discussed above, the infiltration of macrophage influx into the inflamed liver is critically contributing to disease progression and is therefore a good target for pharmacological intervention. This is exemplified in studies targeting the chemokine CCL2/CCR2 axis. Previous work has shown that one modality is the application of an RNA-L-aptamer-based inhibitor targeting CCL2 [26]. The respective PEGylated mirror-image L-RNA aptamer ('Spiegelmer') termed mNOX-E36 reduces the constant infiltration of $Ly6C^{hi}$ macrophage subsets in fibrotic mouse models thereby favoring the net accumulation of their restorative $Ly6C^{lo}$ counterparts and resulting in accelerated fibrosis regression [29]. Likewise, this L-aptamer proved effective in amelioration of liver steatosis [50]. Most recently, the first high-resolution crystal structure of the L-aptamer/CCL2 complex was solved at a resolution of 2.05A [51]. In this complex, the NOX-E36 L-aptamer forms a heavily distorted hairpin structure, whose exterior shell is rod-shaped that is markedly different from the secondary structure of the L-aptamer (fig. 3a). The physical interaction of the L-aptamer with monomeric CCL2 prevents CCL2 dimerization that is essential for its function in vivo [52]. Similarly, a structural model for the complement anaphylatoxin C5a with a mixed L-RNA/L-DNA aptamer was recently proposed for mouse and human C5a [53]. Since C5a is a causative gene involved in hepatic inflammation and fibrogenesis in mice and humans [54], it is supposed that this aptamer strategy will also have therapeutic potential and is in the pipeline of biopharmaceutical companies focusing on the development and commercialization of therapies to treat inflammatory liver disease.

One drug candidate that is currently evaluated in a randomized, double-blind clinical trial is cenicriviroc. This oral drug is a dual inhibitor of CCR2/CCR5 and tested in a clinical phase 2b (CENTAUR) study in adult NASH patients with fibrosis (clinical trial no. NCT02217475). Also, another chemokine-based approach that deems feasible in combating liver fibrosis is the compound Met-RANTES that counteracts the chemotactic ligand CCL5 and interferes with stellate cell activation and greatly ameliorated liver fibrosis in mice [55].

Galectin-3 is a 30 kDa protein, being widely spread among different types of cells and tissues, has a large variety of biological functions impacting cellular proliferation, differentiation, angiogenesis, adhesion, apoptosis, hypoxia homeostasis, inflammation, collagen synthesis, profibrogenic signaling and transformation [56, 57]. In animal models of steatohepatitis, galectin-3 protein expression was found to be highest in hepatic macrophages surrounding lipid-laden hepatocytes [58]. Galectin-3 is further a molecule that is acting at various stages along the continuum from acute inflammation to chronic inflammation [59], thereby also representing a promising drug target for hepatic inflammation and fibrosis. This was impressively confirmed in a rodent toxic model of cirrhosis in which fibrosis was induced by intraperitoneal injections with thioacetamide [58]. In the respective study, both the galectin-3 inhibitors, GR-myeloid differentiation factor 2 (GR-MD-02) (galactoarabino-rhamnogalaturonan) and GM-CT-01 (galactomannan), led to

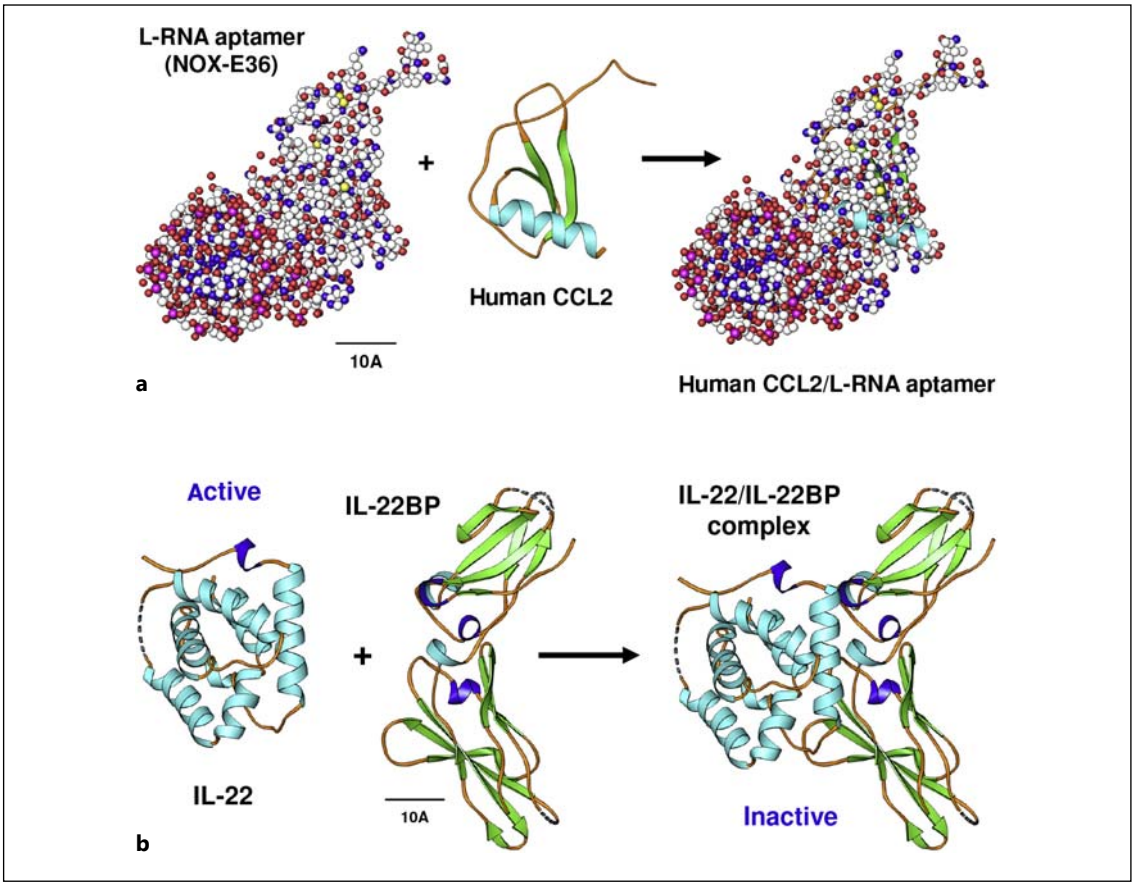

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Fig. 3. Potential drug targets for treatment of inflammatory liver injury. **a** Schematic overview of the aptamer-based inhibition of the chemokine CCL2. The aptamer NOX-E36 is a synthetic 40 mer mirror-image RNA L-oligonucleotide with the sequence 5′-GCA CGU CCC UCA CCG GUG CAA GUG AAG CCG UGG CUC UGC G-3′. This L-aptamer has capacity to bind CCL2. The individual structures were generated with the Ribbons 2.0 software using the crystal structure coordinates that are deposited in the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) under accession no. 4R8I. Please note that this cartoon does not take into account that the secondary structure of the free L-aptamer is significant different from the complex one. For more details about the L-oligonucleotide L-protein complex refer to [51]. **b** Therapeutic inactivation of IL-22 through biological sequestering by binding to IL-22BP. IL-22, physiologically protecting against fibrosis, can be biologically neutralized by the soluble IL-22 receptor IL22RA2 (also known as IL-22BP) that aggravates liver fibrosis. The modulation of the IL-22/IL-22BP axis is therefore a promising therapeutic drug target to limit ongoing fibrogenesis. The individual structures were generated with the Ribbons 2.0 software using the crystal structure coordinates that are deposited in the RCSB Protein Data Bank under accession no. 3G9V. More details about the IL-22/IL-22BP protein interactions can be found elsewhere [81].

sustained amelioration of liver scar formation and inflammation and further were effective in reversal of cirrhosis [58]. Given the putative therapeutic potential of galectin-3 in fibrosis and cirrhosis, a company (i.e., Galectin Therapeutics) was founded that pursued the therapeutic potential of galectin-3 and launched a phase-1 trial to explore the therapeutic use of GR-MD-02 in NASH patients (trial no. NCT01899859). Based on its biological relevance, this trial received fast track designation from the US Food and Drug Administration in 2013 and was completed at the beginning of 2015. The first reports of preliminary results that are discussed among experts heralded good tolerability of GR-MD-02 and the primary and secondary outcome measures further improved surrogate markers of cell death, inflammation and fibrosis.

Targeting Inflammatory Mediators

As discussed above, also the inflammatory micro-milieu of the ECM in chronic liver disease provides a substantial number of additional potential drug targets [5]. The ECM protein osteopontin, also known as bone sialoprotein 1, is composed of approximately 300 amino acids

and has wide biological activities in the liver. It is highly upregulated in fibrotic tissues and involved in wound healing and influences liver progenitor cell function in humans and mice [60]. In line with this assumption, it was demonstrated that both the application of a therapeutic aptamer as well as neutralizing antibodies were suitable to modulate liver progenitor cell response and to attenuate fibrogenesis [60]. However, future work is still necessary to proof if osteopontin is a good candidate to halt, ameliorate or reverse hepatic inflammation and fibrosis, because osteopontin is known to interact with multiple cell surface receptor that are ubiquitously expressed.

IL-22 is a member of the IL-10 family of cytokines that all act as potent mediators in cellular inflammatory response, immune system regulation, fibrosis and tissue remodeling [61]. The administration of IL-22 by either transgenic overexpression or application of exogenous IL-22 in mice reduced liver fibrosis and accelerated the resolution of liver in the carbon tetrachloride model [62]. Interestingly, the antifibrotic effect of IL-22 was associated with a significant induction of HSC senescence that expresses both the IL-22 receptors, that is, IL-10R2 and IL-22R1 [62]. Recent clinical data from patients with chronic liver infections underpin the assumption that IL-22 protects against and its competitor IL-22 binding protein (IL-22BP) aggravates liver fibrosis and cirrhosis, suggesting that the pharmacological modulation of the IL-22/IL-22BP axis may be a promising strategy to limit cirrhosis [63]. The binding of IL-22 to its decoy receptors forms a masked cytokine (fig. 3b) that is enabled to bind to its cognitive receptors thereby preventing the execution of its antifibrotic activities. Potentially, the therapeutic sequestering by components that blocks the activity or expression of IL-22BP will be attractive to modulate established fibrosis, especially in patients suffering from acute hepatic inflammation.

During the last few years, the endocannabinoid system has attracted huge interest as a modality to improve acute and chronic liver disease [64]. The endocannabinoid system with its 2 G protein-coupled receptors (CB_1 and CB_2) mainly functions in neuro- and immuno-modulation. Interestingly, the 2 endocannabinoid receptor branches display opposing functions. Whereas CB_1 downstream signaling is detrimental by promoting matrix deposition, CB_2 is hepatoprotective [64]. It has turned out that the therapeutic strategies counteracting CB_1 signaling are potentially harmful since the CB_1 antagonist rimonabant, previously licensed for treating morbid obesity, had to be withdrawn in 2009 from the market due to serious psychiatric problems and mood disorders. Albeit currently available CB_1 antagonists preclude application in man, non-blood–brain barrier penetrating second generation CB_1 antagonistic drugs might be a future option given the encouraging findings from small animal models that showed beneficial effects on glucose metabolism, fatty liver and plasma lipid profile [65]. In contrast, stimulation of the CB_2 receptor by the potent and selective CB_2 agonist JWH-133 efficiently reduced fibrosis directly through promoting HSC apoptosis/quiescence and indirectly through modulation of immune cell infiltration and various other beneficial impacts on parenchymal and non-parenchymal cells, and further by selectively reducing IL-17 production by Th_{17} lymphocytes [64, 66]. These data demonstrate that the pharmacological activation of CB_2 may be a good anti-inflammatory drug target to block ongoing hepatic fibrogenesis.

Targeting Matrix Formation or Remodeling

Another potential approach to interfere with hepatic fibrogenesis is the direct targeting of proteins that are involved in the biogenesis of connective tissue formation. Polymerization and intermolecular crosslinking of collagen is a prerequisite during organ fibrosis that is catalyzed by the matrix enzymes lysyl oxidases. In a pioneering study, the lysyl oxidase-like-2 (LOXL2) catalyzing the first step in scaffolding and crosslinking of collagens and elastins was identified as a critical factor for scar tissue formation in liver fibrosis [67]. Targeting LOXL2 with an inhibitory monoclonal antibody (AB0023) was highly effective in targeting liver and lung fibrosis [67]. Based on these findings, a study in which a humanized monoclonal antibody against LOXL2, termed simtuzumab (formerly GS-6624), was initiated and is currently being investigated in diverse ongoing phase 2 trials involving HCV (trial no. NCT01707472), primary sclerosing cholangitis (trial no. NCT01672853) and NASH-related fibrosis (trial no. NCT01672866)/cirrhosis (trial no. NCT01672879). Since the expression of LOXL2 is also influenced by hypoxia, TGF-β and microRNAs (miR-26 and mIR-29), there are also other potential strategies for targeting LOXL2 expression and ECM crosslinking [68].

Targets for matrix remodeling are also conceivable in the CCN family of matricellular proteins. This family contains 6 individual members that are small, secreting cysteine-rich proteins with a modular architecture and implicated in the maintenance of normal liver function and the pathogenesis of liver disease [17]. Besides CCN3/CTGF, the cysteine-rich angiogenic inducer 61 (CYR61/CCN1) with all its biological activities has presently at-

tracted much attention as a potential drug target for treatment of liver inflammation and fibrosis. There are several attributes of CYR61/CCN1 that may critically impact the different steps in fibrosis progression or resolution. One study for example has shown that the expression of this matricellular protein in hepatocytes contributes to the content of macrophage infiltration in models of NAFLD in mice through the TLR4/myeloid differentiation primary response gene 88 (MyD88)/AP-1 pathway [69]. On the other hand, it was demonstrated that CYR61/CCN1 is a critical promoter of fibrosis resolution by triggering cellular senescence in activated HSC and portal MFBs through integrin-dependent mechanisms [41]. In line with this assumption, the administration of an adenoviral vector expression CYR61/CCN1 was highly effective in preventing collagen expression, induction of reactive oxygen species formation and induction of cellular senescence and apoptosis in rodent fibrotic models [42]. It will be interesting to follow-up how the increasing knowledge and encouraging findings in regard to CCN functions and in particular CYR61/CCN1 will improve disease management in hepatology.

Targeting the Gut-Liver Axis

Alterations in the intestinal microbiota or disruption of the intestinal barrier function (i.e., gut leakiness) might critically contribute to the clinical symptoms that are associated with hepatic inflammation and fibrosis. Chronic alcohol abuse for example suppresses intestinal motility, contributing to bacterial overgrowth and bacterial translocation results from increased intestinal permeability [70]. Endotoxins from the gut reaching the liver via the portal vein have been also identified as drivers of hepatic inflammation and fibrosis in animal models of toxic and metabolic liver disease [71, 72]. Comparative analysis of the gut microbiome of patients with cirrhosis and healthy control individuals revealed that bacterial species of buccal origin were enriched in the patients suggesting an invasion of the gut from the mouth in liver cirrhosis [73]. Likewise, the occurrence of obesity-associated cancer was shown to be influenced by the gut microbial metabolite [74]. In the pathogenesis of dietary or genetic obesity, induced alterations in the gut microbiota, thereby increasing the levels of the secondary bile acid deoxycholic acid that causes DNA damage provokes a senescence-associated secretory phenotype in HSC [74]. In animal models, it has already been shown that intestinal decontamination via broad-spectrum antibiotics reduces the progression of liver fibrosis, mainly via reduced TLR-dependent activation of inflammatory pathways in Kupffer cells and HSC [71]. This strategy may further help to limit hepatocarcinogenesis within inflamed livers [75]. On the other hand, commensal microbiota in comparison to germ-free gut conditions protects mice from fibrosis progression [76]. Therefore, restoration of the normal microbiome, application of probiotics or antibiotics, sequestering of deoxycholic acid (or other bile acids associated formation oxidative stress and DNA damage), as well as application of compounds that lead to elevated tightness of the intestinal barrier are promising new therapeutic strategies for the treatment of inflammatory and fibrotic liver disease.

Bile Acid Derivates as Antifibrotic Drugs

Bile acids are physiological emulsifying agents that enable digestion and absorption of lipids in the small intestine. During more recent years, it has become apparent that bile acids regulate metabolic functions via activating the intracellular nuclear receptor farnesoid X receptor (FXR) and the transmembrane G protein-coupled receptor TGR5 [77]. Activation of FXR-dependent signaling pathways improves glucose metabolism and peripheral insulin sensitivity, reduces lipogenesis and enhances fatty acid beta-oxidation and promotes anti-inflammatory actions [78]. The synthetic bile acid obeticholic acid, chemically similar to ursodeoxycholic acid, has a strong FXR agonistic activity. The antifibrotic potential of obeticholic acid was assessed in a multicenter phase II trial ('FLINT trial') on non-cirrhotic patients with NASH. This trial was stopped early upon interim analysis, because patients on obeticholic acid had improved liver histology after 72 weeks of treatment compared with placebo. The significant changes not only included reduction of steatohepatitis (45 vs. 21%), but also improvement in fibrosis (35 vs. 13%) [79]. Potentially relevant side effects of this treatment were pruritus and elevated LDL cholesterol levels. The antifibrotic potential of this bile acid derivate is currently being evaluated in a global, placebo-controlled phase 3 trial in patients with NASH and liver fibrosis ('REGENERATE', trial no. NCT02548351).

Conclusions

The thorough understanding of molecular and cellular mechanisms of liver fibrosis led to the discovery of manifold novel targets and pathways that might be suitable to block hepatic inflammation/fibrogenesis and to initiate regression of liver tissue. However, most of the present findings were generated in animal models and the findings still need to be translated to human pathogenesis.

This interesting task will need the close cooperation between basic researchers, clinical scientists and practicing clinicians. The first clinical trials with defined primary and secondary outcome measures are initiated, and the first results that are leaking from participating institutions are promising.

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