

Follistatin-like protein 1 and chronic liver disease progression: a novel pro-inflammatory and pro-fibrogenic mediator?

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Chronic liver diseases (CLD) represent a major global public health concern due to the impressive number of patients affected (more than 800 million worldwide) and the very high mortality rate reported for CLD patients, i.e., approx. 2 million deaths per year (1,2). The real clinical problem is represented by CLD progression which is a common feature in chronic liver patients, irrespective of the specific etiology. CLD progression can be induced by (I) chronic infection by hepatitis B virus (HBV) or hepatitis C virus (HCV), (II) non-alcoholic fatty liver disease or NAFLD, (III) excess alcohol consumption, (IV) autoimmune liver diseases (including primary biliary cholangitis or PBC, primary sclerosing cholangitis or PSC and autoimmune hepatitis or AIH) and hereditary diseases (mainly Wilson's disease, α 1-antitrypsin deficiency and hemochromatosis). Whatever the etiology, CLD progression is based on a longstanding history of chronic parenchymal injury, persistent activation of inflammatory response and chronic activation of wound healing reaction, with sustained fibrogenesis leading eventually to excess deposition of extracellular matrix (i.e., liver fibrosis) (3-6). This sequela of events efficiently drives CLD progression towards more advanced stages of the disease such as cirrhosis and liver failure, with cirrhotic patients being also at risk to develop HCC, the most common primary liver cancer (7).

Although any hepatic cell population has been reported to play some role in CLD progression, a prominent and critical role is attributed to liver myofibroblasts (MFs) and macrophages. As it is well known, liver MFs represent a

rather heterogeneous population of α -smooth muscle actin (α -SMA) positive cells that originate mainly from activated hepatic stellate cells (HSC), portal fibroblasts and, to a less extent, from bone marrow derived cells (mesenchymal stem cells or fibrocytes) through a process often referred to as activation/transdifferentiation (3,4,6). Liver MFs, whatever their origin, share a number of common pro-fibrogenic responses that briefly include: (I) the synthesis and remodeling of extracellular matrix components in response to reactive oxygen species (ROS) and other oxidative stress-related molecules, transforming growth factor (TGF) β and several other peptide growth factor and mediators, with an obvious major role in deposition of excess fibrillary collagen type I and III; (II) a highly proliferative attitude in response mainly to platelet-derived growth factor (PDGF), particularly PDGF-BB homodimer, or other growth factors like transforming growth factor (TGF) α , fibroblast growth factor (FGF), connective tissue growth factor (CTGF) and thrombin; the same peptide growth factors plus TGF β 1 are believed to be also responsible for the survival attitude and resistance to induction of apoptosis of MFs, at least those originated from HSC; (III) a hypoxia-dependent pro-angiogenic role mediated by the release of vascular endothelial growth factor (VEGF)-A and of other pro-angiogenic mediators (mainly PDGF-BB, Angiopoietin-1 and -2); (IV) a pro-inflammatory role which is mediated mainly by the release of chemokines like CCL2 and CCL21 as well as IL-1 β and is favored by membrane exposure of receptors for a wide range of pro-inflammatory mediators; here one should at least mention the ability of MFs to

closely interact with - and modulate the behavior of innate and adaptive immunity cells; (V) the ability to migrate in response to several chemoattractants like PDGF-BB, CCL2, VEGF-A and Angiopoietin 1 (3,4,6).

In addition to their established contribution in maintaining hepatic homeostasis under physiological conditions macrophages represent the other critical cell population in CLD progression. When talking about macrophages in liver injury (either acute or chronic) and repair processes we should first recognize the role of distinct populations including resident Kupffer cells (KCs), the first to sense injury and release cytokines and chemokines, and monocytes recruited from peripheral blood that, entering the injured liver parenchyma, sometimes defined as monocyte-derived macrophages (MoMφs) (5,8). KCs and MoMφs are then distinct subsets of macrophages that can be recognized in liver sections on the basis of the expression of surface markers. In mice KCs are CD11b^{low}, F4/80^{high} and Clec4E⁺ whereas MoMφs are CD11b⁺, F4/80^{int} (where int means intermediate level of expression), Ly6C⁺ and CSF1R⁺. In murine models of liver injury hepatic MoMφs could be further distinguished into two populations on the basis of Ly6C expression levels: pro-inflammatory Ly6C^{high} macrophages and pro-resolution Ly6C^{low} macrophages. One should note that Ly6C^{high} macrophages have a very high degree of plasticity and can shift into Ly6C^{low} cells. In human livers macrophages are similarly heterogeneous and include KCs, which are CD68⁺ and MARCO⁺, CD68⁻ and MARCO⁻ macrophages (that display a transcriptomic profile similar to that of murine pro-inflammatory Ly6C^{high} cells) and CD14⁺ monocytes. Upon chronic liver injury and whatever the specific etiology of the liver disease, KCs and MoMφs can contribute to both disease progression as well as, under certain circumstances, resolution of tissue inflammation and injury, with KCs having a role in the early stage and MoMφs being more relevant in CLD progression. In the last decade an impressive amount of either pre-clinical and clinical studies have revealed that the scenario for an ongoing CLD is even more complex and additional different subsets of hepatic macrophages have been described to be involved in disease progression, sometimes displaying different and even etiology-specific functional responses (5,8). Authoritative and up-to-date reviews and recent articles, referring also to the use of linear tracing experimental studies and single cell sequencing can offer critical and detailed information on these issues that recently have mostly focused on NAFLD and non-alcoholic steatohepatitis (NASH) (5,8-10). As

a relevant example of this macrophage heterogeneity, in NASH patients and murine models of experimental NASH but also in other pre-clinical and clinical conditions of CLD, a further subpopulation of macrophages has been characterized which is expressing CD9 and the scavenger receptor Triggering Receptor Expressed on Myeloid Cells 2 (TREM-2) (11). These TREM-2 positive macrophages may represent heterogeneous subsets of innate immunity cells with functions that may vary depending on the context of liver injury (8).

In the very complex scenario of ongoing CLD just summarized one should additionally stress that no validated anti-fibrotic therapies are available at present. If the goal to target the specific etiological cause or agent remains the most reliable and efficient one to counteract CLD progression, as shown by the tremendous efficacy of direct antiviral agents (DAA) in targeting and eliminating HCV, at present the most promising pre-clinical studies and related clinical trials are designed to specifically inhibit macrophage recruitment, macrophage activation or even to induce phenotype switching of macrophages [reviewed in ref. (8)].

Along these lines, a very recent and elegant study by Rao and coworkers (12) has added a novel piece of knowledge of potential interest by proposing Follistatin-like protein 1 (FSTL1) as a potentially targetable mediator able to contribute to CLD fibrogenic progression mainly by reprogramming macrophages to a pro-inflammatory (i.e., M1) polarization. FSTL1 is a multi-domain glycoprotein displaying several regulatory functions that belongs to the secreted protein acidic and rich in cysteine (SPARC) family of proteins. In its structure FSTL1 contains a secretion signal, a Follistatin- and a Kazal-like domain, two EF-hand domains, and a von Willebrand factor type C domain (13). Although the short secretion signal (involving approx. 18-20 aminoacids in mice and humans) can vary significantly between investigated species, the remaining major part of primary structure (272 aminoacids) is much more conserved and shows a very high degree of similarity between species (>94%), particularly between mice and humans. FSTL1 has as critical role during embryo development and, following gastrulation, is broadly expressed in murine embryos whereas at the end of gestation its expression is restricted to mesenchymal cells of most tissue (13). Even more relevant, FSTL1 has been described to undergo significant expression changes during various human diseases. This includes cardiovascular and lung diseases, cancer progression and systemic autoimmune diseases, with a possible proposed role for FSTL1 as a pro-inflammatory

mediator (13-15). This issue is perhaps still debated since some studies have also reported an anti-inflammatory action for this secreted glycoprotein [discussed in ref. (13)]. Moreover, available literature data have outlined a possible pro-fibrogenic role for this glycoprotein; in particular FSTL1 is up-regulated by TGF β 1 in lung fibroblasts but can also promote fibrogenesis by facilitating TGF- β signaling; in addition, haplo-depletion of FSTL1 or the use of FSTL1 neutralizing antibodies in mice significantly reduced experimental lung fibrosis induced by bleomycin (16,17). Whether liver fibrosis is concerned, FSTL1 was reported to be up-regulated in human fibrotic livers and, particularly, in activated hepatic stellate cells (HSC) (18). Moreover, experimental studies have provided evidence that silencing or targeting FSTL1 expression resulted in a significant reduction of liver fibrosis (19,20). However, differently from what reported for chronic diseases in other organs or tissues (13-15), the role of FSTL1 in relation to hepatic inflammatory response was not previously investigated. In their study Rao and coworkers (12) have investigated for the first time the possible pro-inflammatory role of FSTL1 in relation to either human and experimental CLD. Authors, by analyzing human normal and fibrotic liver tissues not only confirmed the up-regulation of FSTL1 expression in human fibrotic livers *vs.* levels in control/non fibrotic patients, as already reported by others (18), but revealed that FSTL1 transcript and protein hepatic levels were progressively increasing from patients with mild/early fibrosis to those with advanced fibrosis. Most relevant, this study is the first to report that FSTL1 expression is apparently up-regulated mainly in macrophages, as detected in human fibrotic specimens obtained by patients with mixed etiology. In this study FSTL1 expression was apparently low in HSC and not detectable in parenchymal cells. Almost homologous data were obtained by analyzing liver specimens obtained from three different murine protocols of CLD, as induced by either chronic carbon tetrachloride (CCl₄) treatment, bile duct ligation (BDL) or by feeding mice on a methionine- and choline-deficient (MCD) diet. In order to confirm the relevant issue of a prominent expression of FSTL1 in macrophages, Authors developed a murine model by using the Cre-LoxP system in order to create myeloid-specific FSTL1-deficient (FSTL1^{M-KO}) mice. According to the hypothesis, when experimental chronic liver injury was induced using the previously mentioned protocols (CCl₄ treatment, BDL or MCD diet), liver fibrosis and transcript levels of most common markers of fibrogenesis were markedly

reduced in FSTL1^{M-KO} mice versus respective control mice (i.e., FSTL1^{FL/FL}). Experimental data showed that FSTL1 deletion in myeloid cells also significantly attenuated inflammatory response and, more specifically, reduced recruitment of macrophage and neutrophils as well as suppressed M1 polarization and NF- κ B pathway activation in fibrotic livers. Overall these data were in agreement with the hypothesis that during chronic liver injury FSTL1 may operate as a pro-inflammatory mediator able to reprogram macrophages. In the last part of their study Authors performed experiments designed to investigate the mechanism (or mechanisms) that may explain the FSTL1-mediated reprogramming of macrophages into a pro-inflammatory phenotype. By employing various approaches, including the use of an agonist of pyruvate kinase M2 (PKM2), they were able to show that FSTL1 can operate by directly binding to PKM2, resulting in inhibition of its ubiquitin-mediated degradation, enhanced stability of cytoplasmic PKM2 and promotion of PKM2 phosphorylation and nuclear translocation in macrophages. On these basis Authors concluded that in ongoing CLD FSTL1 released by activated macrophages can promote and sustain chronic inflammatory response by inducing macrophage M1 polarization through selective targeting and activation of intracellular PKM2 (12).

The overall study is of additional interest not only for having delineated the potential role of FSTL1 in modulating macrophage function during liver fibrosis but also because Authors also preliminary tested the efficacy of DASA-58, a small molecule able to activate PKM2 (21,22). The use of this agonist drug efficiently blocked or significantly reduced FSTL1-mediated parameters of inflammation, glycolysis and TLR4/NF κ B pathway when tested particularly in bone marrow-derived macrophages overexpressing FSTL1 (12). This approach, although studied only *in vitro* on cultured cells, can open the way for a future specific therapy that may selectively target either FSTL1 or, alternatively, PKM2 to prevent or at least slow down fibrosis progression in a CLD. Of course we are waiting for properly designed pre-clinical *in vivo* studies and, in case, related clinical trials, to eventually validate such an interventional therapeutic strategy.

By concluding this editorial commentary dedicated to the study of Rao *et al.* (12), a number of issues remain unresolved that should be further investigated in the next future. A first issue is related to the limited translational potential of data obtained in the murine protocols of experimental CLD used in the study of Rao *et al.* (12),

which essentially do not reproduce or poorly reproduce human clinical conditions. One should note, for example, that MCD dietary protocol is not adequate to reproduce human progressive NAFLD (these mice significantly lose weight and do not develop hyperglycemia and/or insulin resistance). Similarly, the BDL model does not reproduce the most common human cholangiopathies (i.e., PBC, PSC) and the CCl₄ chronic model, although still largely used, has a limited significance versus human CLD conditions. The use of more appropriated murine models able to better reproduce human CLD should be undertaken. A second issue that should be further investigated is whether FSTL1 may have any role in either modulating or inducing the development of macrophage subsets that have emerged in recent years including, for example, the CD9 and TREM2 positive macrophages described in NASH but also in other CLD of different etiology (11). A third issue, potentially related to the latter, is suggested by the fact that the absolute majority of human patients analyzed by Authors were from HBV or HCV chronically infected individuals, with very few patients having a diagnosis of NASH. According to the present worldwide increasing relevance of NAFLD/NASH further studies dedicated to investigate FSTL1 in selected cohorts of NAFLD/NASH patients should be performed. Finally, one has to note that a discrepancy exists between data from Rao *et al.* (12) study, indicating macrophages as the major source of FSTL1 in CLDs, and previous reports suggesting HSC (18) or MFs (23) as a privileged source of FSTL1. In order to unequivocally unravel this point a properly designed trace lineage and diriment experiment should be designed and performed, using appropriate protocols of experimental chronic liver injury; for this purpose the use of Fstl1-CreERT2 mice may help to understand the real contribution of fibroblasts/MFs to FSTL1 expression and release (23).

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