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Essential role of suppressor of cytokine signaling 1 (SOCS1) in hepatocytes and macrophages in the regulation of liver fibrosis

Euphrasie Kawila Mafanda[a,b,1], Rajani Kandhi[a,b,1], Diwakar Bobbala[b], Md Gulam Musawwir Khan[a,b], Madhuparna Nandi[b], Alfredo Menendez[c,d], Sheela Ramanathan[b,d], Subburaj Ilangumaran[b,d,*]

[a] Immunology Graduate Program, Sherbrooke, Québec J1H 5N4, Canada
[b] Department of Anatomy and Cell Biology, Sherbrooke, Québec J1H 5N4, Canada
[c] Department of Microbiology and Infectious Diseases, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada
[d] CRCHUS, Sherbrooke, Québec J1H 5N4, Canada

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ABSTRACT

The hepatic fibrogenic response is a protective mechanism activated by hepatocyte damage and is resolved upon elimination of the cause. However, persistent injuries cause liver fibrosis (LF) to evolve into cirrhosis, which promotes the development of hepatocellular carcinoma (HCC). Development of efficient treatments for LF requires better understanding the underlying molecular pathogenic mechanisms. The loss of suppressor of cytokine signaling 1 (SOCS1) expression promotes LF and HCC in human and mice, but the underlying mechanisms remain unclear. SOCS1 is a key regulator of immune cell activation. To investigate the anti-fibrogenic functions of SOCS1 in hepatocytes and macrophages, we generated mice lacking SOCS1 in hepatocytes ($Socs1^{fl/fl}Alb^{Cre}$) or macrophages ($Socs1^{fl/fl}LysM^{Cre}$) and evaluated hepatic fibrogenic response to carbon tetrachloride (CCl_4). $Socs1^{fl/fl}Alb^{Cre}$ and $Socs1^{fl/fl}LysM^{Cre}$ mice showed severe LF characterized by increased collagen deposition, hydroxyproline content, myofibroblast accumulation along with elevated expression of *Acta2* and *Col1a1* genes. CCl_4 treatment triggered significant damage to hepatocytes in $Socs1^{fl/fl}Alb^{Cre}$ mice but not in $Socs1^{fl/fl}LysM^{Cre}$ mice. In both mice CCl_4 treatment reduced the expression of *Mmp2* and increased the expression of *Timp1*. SOCS1 deficiency in hepatocytes or macrophages did not affect *Il6*, *Tnfa* or *Tgfb*, but diminished *Infg* and augmented *Pdgfb* expression. Both $Socs1^{fl/fl}Alb^{Cre}$ and $Socs1^{fl/fl}LysM^{Cre}$ livers showed increased mononuclear cell infiltration accompanied by elevated *Ccl2* expression. Our findings show that SOCS1 exerts non-redundant functions in hepatocytes and macrophages to regulate the hepatic fibrogenic response possibly through limiting hepatocyte damage and the inflammatory response of macrophages, and support the idea of exploiting SOCS1 in LF treatment.

1. Introduction

Hepatocytes are constantly exposed to potentially toxic substances entering the liver via the portal circulation. In the process of detoxifying toxic substances, hepatocytes sustain injury that causes cellular stress, releases inflammatory mediators and initiates an inflammatory response [1,2]. Hepatitis viruses and other pathogens entering the liver via portal and systemic circulation also cause inflammation [3]. These inflammatory reactions induce hepatic fibrogenic response that aids the repair and regenerative process [4]. However, recurrent exposure to hepatotoxic substances and persistent infections cause chronic inflammation that results in extensive liver fibrosis (LF) that could progress toward cirrhosis. Whereas the normal fibrotic response is protective and resolves upon elimination of the causative agent, cirrhosis is irreversible, obliterates the liver parenchyma, compromises vital liver functions and promotes the development of hepatocellular carcinoma (HCC) [5,6]. Indeed, more than 90% of HCC arises in cirrhotic livers. Even without accompanying HCC, cirrhosis is an important cause of

Abbreviations: ALT, alanine transferase; CCl_4, carbon tetrachloride; ECM, extracellular matrix; LF, Liver fibrosis; MMP, matrix metalloproteinase; NDP, nanozoomer Digital Pathology; SOCS, suppressor of cytokine signaling; SMA, smooth muscle actin; TIMP, tissue inhibitor of MMP

* Corresponding author at: Immunology Division, Department of Pediatrics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001 North 12th Avenue, Sherbrooke QC-J1H 5N4, Canada.
E-mail address: Subburaj.Ilangumaran@Usherbrooke.ca (S. Ilangumaran).
[1] Contributed equally to this work.

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Table 1
List of qRT-PCR primers.

Gene name	Gene ID	Sense primer	Anti-sense primer (5′ to 3′)	Amplicon Size (bp)
Acta2	NM_007392.3	AGTAATGGTTGGAATGG	GTGTCGGATGCTCTTCAGG	166
Col1a1	NM_007742.4	CTCCCAGAACATCACCTATCAC	ACTGTCTTGCCCCAAGTTCCG	171
Il6	NM_031168.2	AGTCCGGAGAGGAGACTTCA	TTGCCATTGCACAACTCTTT	112
Ccl2	NM_011333.3	CAGGTCCCTGTCATGCTTCT	GTGGGGCGTTAACTGCAT	73
Ccl5	NM_013653.3	TGCAGAGGACTCTGAGACAGC	GAGTGGTGTCCGAGCCATA	149
Cx3cr1	NM_009987.4	GTTATTTGGGCGACATTGTGG	ATGTCAGTGATGCTCTTGGG	142
Mmp2	NM_008610.2	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC	152
Mmp9	NM_013599.3	GATCCCCAGAGCGTCATTC	CCACCTTGTTCACCTCATTTTG	129
Mmp14	NM_008608.4	GGATGGACACAGAGAACTTCG	AACCATCGCTCCTTGAAGAC	235
Pdgfb	NM_011057.3	CCTGCAAGTGTGAGACAGTAG	CTTTCGGTGCTTGCCTTTG	127
Rplp0 (m36B4)	NM_007475.5	TCTGGAGGGTGTCCGCAA	CTTGACCTTTTCAGTAAGTGG	154
Tgfb1	NM_011577.1	ATACGCCTGAGTGGCTGTCT	CTGATCCCGTTGATTTCCA	129
Timp1	NM_011593.2	TTGCATCTCTGGCATCTGG	TGGTCTCGTTGATTTCTGGG	136
Tnfa	NM_013693.3	CGTCGTAGCAAACCACCAAG	GAGATAGCAAATCGGCTGACG	190

global morbidity and mortality [7]. In recent years, non-alcoholic fatty liver disease arising from obesity is emerging as an important cause of fibrosis and HCC [8]. Hence, understanding the cellular and molecular mechanisms of hepatic fibrogenic response holds promise to identify new targets of therapeutic intervention to halt progression of fibrosis, hasten its resolution and prevent HCC [9].

The hepatic fibrogenic response is initiated upon activation of tissue resident macrophages that recognize danger-associated molecules released by dying hepatocytes and pathogen-derived molecules [2,10]. Cytokines and growth factors produced by activated macrophages, as well as the inflammatory molecules released by damaged hepatocytes stimulate hepatic stellate cells (HSC) to transdifferentiate into myofibroblasts, which proliferate and produce abnormal collagen fibrils and other extracellular matrix (ECM) proteins [11]. Accompanying changes in the expression of ECM modifying enzymes promote the accumulation of ECM. While fibrosis resolution restores the normal ECM, chronic liver injury deregulates this process and replaces hepatocytes with fibrous scar tissue. During the hepatic fibrogenic response, cytokines and growth factors influence hepatocyte survival and regeneration, regulate HSC activation, and modulate macrophage functions [12,13]. Regulation of these cytokines and growth factors is critical to achieve a controlled fibrogenic response and its eventual resolution, whereas their deregulated synthesis or uncontrolled activity often accompanies pathologic LF.

An important regulatory mechanism controlling cytokine and growth factor signaling in hepatocytes, macrophages, HSCs and other immune cells of the liver involves the suppressor of cytokine signaling 1 (SOCS1) protein [14]. Epigenetic repression of hepatic SOCS1 in human patients with chronic liver disease is associated with increased fibrosis [15]. A recent report has shown an association between single nucleotide polymorphism in the promoter region of the *SOCS1* gene (rs243327) and liver fibrosis [16]. Supporting a role for SOCS1 in regulating hepatic fibrogenesis, *Socs1* haplo-insufficient mice exhibit increased susceptibility to experimental fibrosis [15]. This study used $Socs1^{+/-}$ mice due to perinatal lethality of *Socs1*-null mice resulting from uncontrolled IFNγ signaling [17]. Another study used $Socs1^{-/-}Ifng^{-/-}$ mice to demonstrate IFNγ-independent anti-fibrogenic role of SOCS1 in the liver [18]. However, whether SOCS1 exerts its anti-fibrogenic functions in hepatocytes, macrophages or HSCs is not yet known. In this study, we have addressed this question using mice lacking SOCS1 in hepatocytes or macrophages.

2. Methods

2.1. Mice

$Socs1^{fl/fl}Alb^{Cre}$ and $Socs1^{fl/fl}LysM^{Cre}$ mice lacking SOCS1 expression in hepatocytes or macrophages, respectively, were generated by crossing $Socs1^{fl/fl}$ mice [19] (kind gift from Dr. Yoshimura, Tokyo, Japan) with Alb^{Cre} or $LysM^{Cre}$ mice (purchased from the Jackson labs, Bar Harbor, ME, USA), which express the Cre recombinase under the lysozyme promoter that is expressed in macrophages and other myeloid cells [20]. All experimental protocols on animals were carried out in accordance with the Université de Sherbrooke Animal Ethics Committee guidelines (Protocol number 359-14B).

2.2. Induction of liver fibrosis and measurement of serum ALT and liver hydroxyproline content

Carbon tetrachloride (CCl_4; Sigma-Aldrich, Oakville, ON) diluted in corn oil as vehicle at 1:2 ratio was used to induce LF [18]. CCl_4 was administered to 8–10-week-old male mice (0.5 µl/g body weight, i.p) every 2–3 days for five weeks. Three days after the last injection, mice were euthanized and serum and liver tissues were collected and preserved at −80 °C. For histopathology, liver tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. Serum alanine transferase (ALT) levels were measured using a kit from Pointe Scientific Inc. (Brussels, Belgium). Hydroxyproline content of the liver tissues was measured as previously described [18].

2.3. Histopathology

Sections of formalin-fixed paraffin embedded liver tissues (5 µM) were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) or Sirius red following standard methods [18]. Immunohistochemical staining of alpha smooth muscle actin (αSMA) using an antibody from Abcam (Cat #7817) as previously described [18]. Digital images of stained sections, acquired using a Nanozoomer Digital Pathology (NDP) Slide Scanner (Hamamatsu Photonics, Japan), were analyzed using the NDP software by selecting 20 random fields from 4 mice in each group. Quantification of Sirius red staining and SMA-positive areas was done using the NIH Image J software.

2.4. Gene expression analysis

RNA extraction, cDNA preparation and gene expression by RT-PCR using primers listed in Table 1 was carried out following published protocols [18]. All primers showed 90–100% efficiency and displayed a single melting curve. Expression levels of specific genes were normalized for the housekeeping gene *Rplp0* (36B4) within each experimental group and expressed as fold induction compared to $Socs1^{fl/fl}$ littermate controls treated with the vehicle.

2.5. Statistical analysis

GraphPad Prism software (San Diego, CA) was used for data analysis

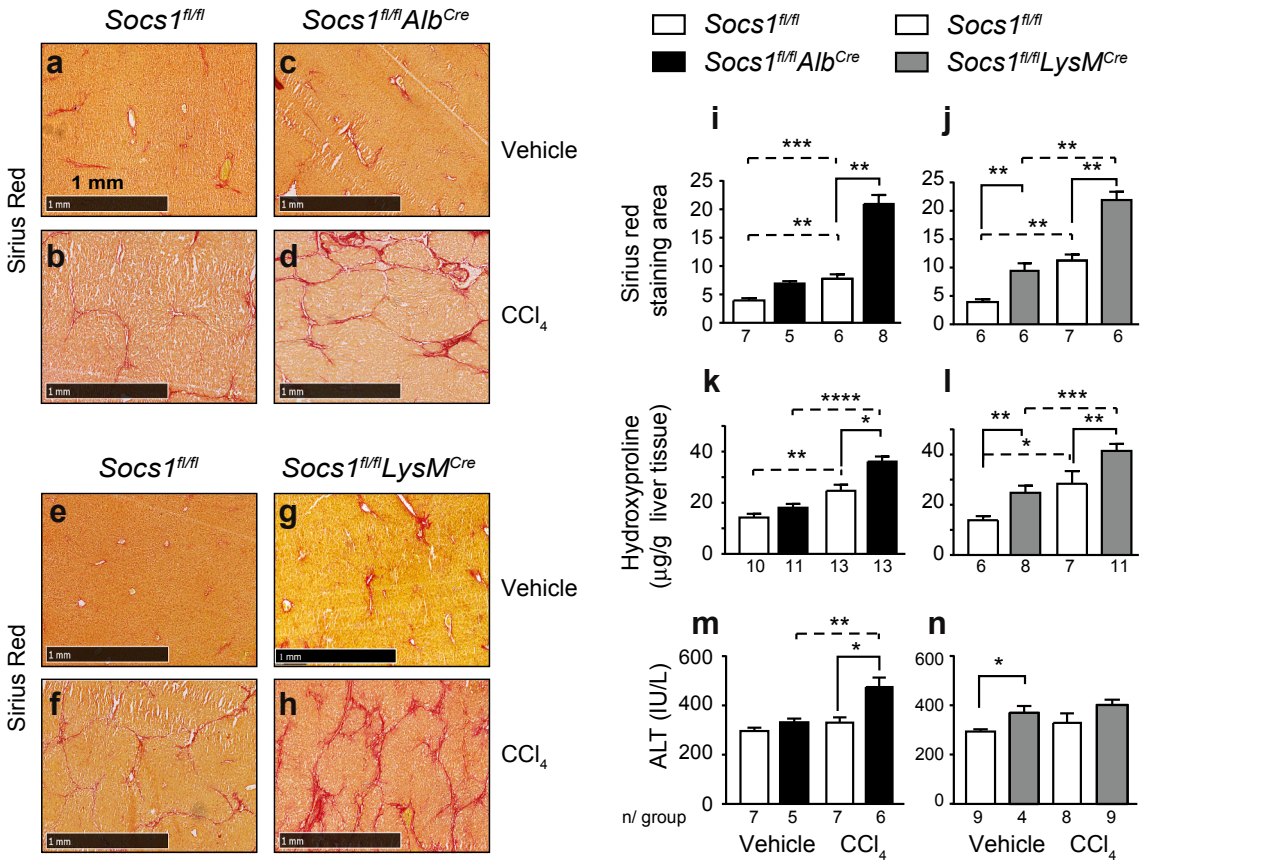

Fig. 1. Mice lacking SOCS1 in hepatocytes or in macrophages show increased liver fibrosis following chemical injury. (a–h) Representative images of Sirius red-stained sections of the livers from control (*Socs1*$^{fl/fl}$; a, b and e, f), hepatocyte-specific SOCS1-deficient (*Socs1*$^{fl/fl}$*Alb*^{Cre}; c, d) and macrophage-specific SOCS1-deficient (*Socs1*$^{fl/fl}$*LysM*^{Cre}; g, h) mice, treated with vehicle (corn oil, a, c, e, g) or CCl_4 (b, d, f, h) for five weeks. (i, j) Quantification of the Sirius red-stained areas of collagen deposition. (k, l) Liver hydroxyproline content of the indicated mice. (m, n) Serum transaminase levels in the indicated groups. Quantifications shown (i–n) are mean ± s.e.m. values from at least 4 and up to 13 mice per group from 2 or 3 independent experiments. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

and to calculate statistical significance by ANOVA or Mann-Whitney test. p values < 0.05 were considered significant.

3. Results

3.1. SOCS1 plays an essential role in both hepatocytes and macrophages to control LF

Damage to hepatocytes is often the initiating event during sterile inflammation of the liver, and macrophages play a key role in conveying this message to HSCs to mount the hepatic fibrogenic response. As the severe LF that develop in *Socs1*$^{-/-}$ mice [18] could result from the lack of SOCS1 in hepatocytes or any of the immune cells in the liver, we investigated whether SOCS1 exerts its anti-fibrogenic activity primarily in hepatocytes or macrophages using mice lacking SOCS1 in either of these cells. Following CCl_4 treatment, liver sections from *Socs1*$^{fl/fl}$*Alb*^{Cre} mice showed extensive, bridging fibrosis as revealed by Sirius red staining of collagen fibrils compared to control mice (Fig. 1a–d). *Socs1*$^{fl/fl}$*Alb*^{Cre} livers showed four times more fibrotic areas than control livers (Fig. 1i) and significantly elevated hydroxyproline content (Fig. 1k).

Socs1$^{fl/fl}$*LysM*^{Cre} mice also showed increased susceptibility to LF as revealed by increased Sirius red staining with extensive bridging pattern and high hepatic hydroxylproline content (Fig. 1e–h, j, l). Notably, *Socs1*$^{fl/fl}$*LysM*^{Cre} mice treated with the vehicle (corn oil) showed significantly higher level of LF than control mice (Fig. 1j, l, columns 1 and 2). However, serum ALT level was not significantly altered in *Socs1*$^{fl/fl}$*LysM*^{Cre} mice whereas *Socs1*$^{fl/fl}$*Alb*^{Cre} livers showed increased ALT levels (Fig. 1m, n). These findings indicated that SOCS1 plays a crucial role in hepatocytes and in macrophages to control LF induced by hepatotoxic agents, and that SOCS1 deficiency in hepatocytes increased the susceptibility to death.

3.2. Loss of SOCS1 in hepatocytes or macrophages promotes myofibroblast activation

Hepatic fibrogenic response is associated with trans-differentiation of HSCs to myofibroblasts, which synthesize and deposit the ECM proteins. Immunohistochemical staining of the myofibroblast marker αSMA revealed that *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} mice treated with CCl_4 showed abundant αSMA staining than vehicle-treated mice (Fig. 2a–h). The αSMA staining intensity was significantly higher SOCS1-deficient livers than in control livers following CCl_4 treatment (Fig. 2i, j; columns 3 and 4). Livers of vehicle-treated SOCS1-deficient mice also showed increased αSMA-staining area (Fig. 2i, j; columns 1 and 2). To accurately quantify myofibroblast differentiation, we evaluated the expression of *Acta2* gene, which codes for αSMA. Mice deficient for SOCS1 in hepatocytes or macrophages showed significantly elevated *Acta2* expression following CCl_4 treatment (Fig. 2k, l; columns 3 and 4). Moreover, *Acta2* expression closely correlated with that of *Col1a1,* the gene coding for collagen type 1 α1 chain (Fig. 2m, n). Unlike the αSMA-staining area, neither *Acta2* nor *Col1a1* showed increased expression in vehicle-treated *Socs1*$^{fl/fl}$*Alb*^{Cre} mice compared to controls (Fig. 2k, m). However, vehicle-treated *Socs1*$^{fl/fl}$*LysM*^{Cre} showed

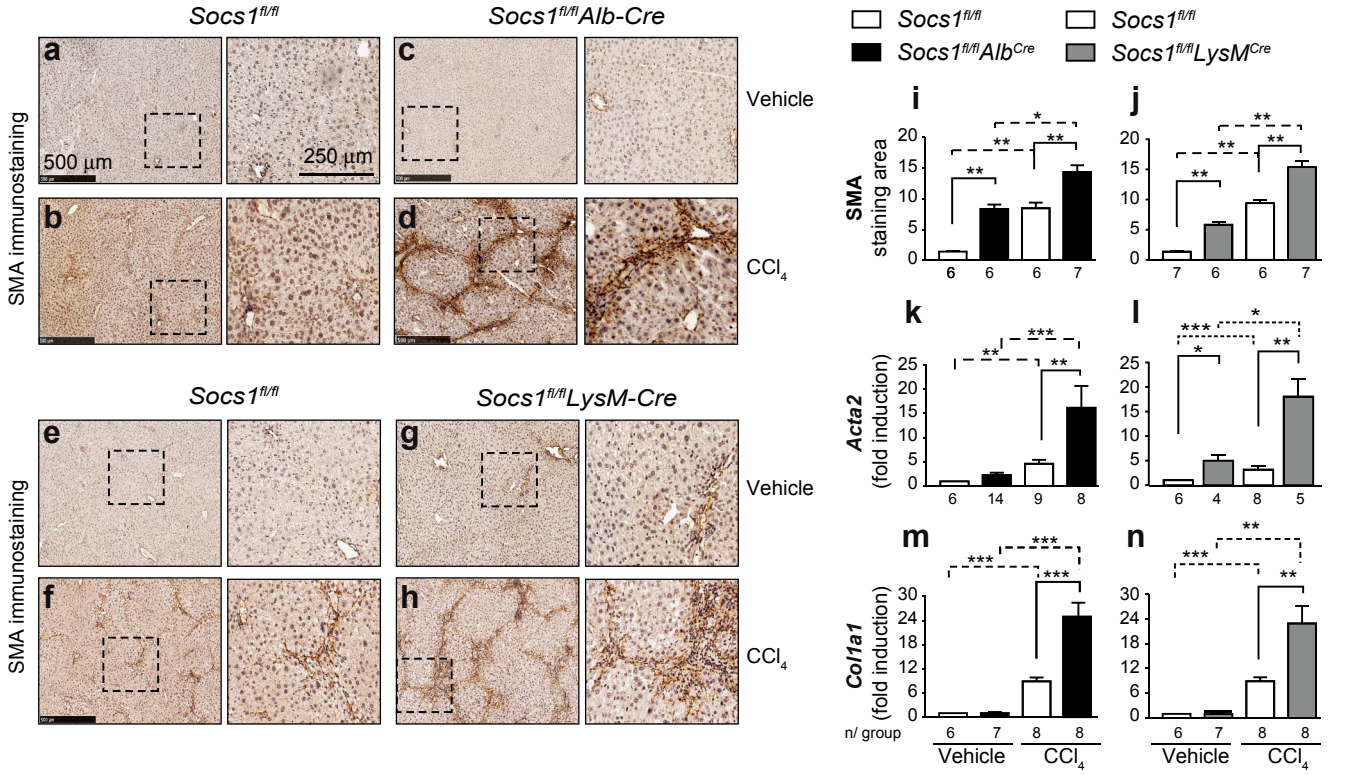

Fig. 2. SOCS1 deficiency in hepatocytes or macrophages increases liver myofibroblast content following CCl_4 treatment. (a–h) Immunohistochemical staining of α-smooth muscle actin (αSMA) in liver sections of control mice (a, b and e, f) and mice lacking SOCS1 in hepatocytes (c, d) or macrophages (g, h), treated with vehicle (corn oil, a, c, e, g) or CCl_4 (b, d, f, h). Representative images from 4 to 6 mice per group from 2 different experiments are shown. Magnified images of the indicated regions are shown below each section. (i, j) Quantification of the SMA-staining areas. (k–n) (C) Quantitative RT-PCR evaluation of *Acta2* (k, l) and *Col1a1* (m, n) genes in the livers of vehicle- or CCl_4- treated control and SOCS1-deficient mice. (i–n) Data shown are mean ± s.e.m. values from at least 4 and up to 14 mice per group from 2 or 3 independent experiments. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

a small but significant increase in *Acta2* though not *Col1a1* expression (Fig. 2l, n). These findings confirm the increased susceptibility of *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} mice to fibrogenic stimuli, and suggest that loss of SOCS1 in macrophages may render the liver susceptible to even mild fibrogenic insults.

3.3. SOCS1 deficiency in hepatocytes or macrophages alters the expression of ECM modifying enzymes

Matrix metalloproteinases (MMP) and tissue inhibitors of MMPs (TIMP) are differentially expressed during hepatic fibrogenic response to favor net ECM deposition [21]. Therefore, we examined whether the increased accumulation of ECM in the livers of mice lacking SOCS1 in hepatocytes or macrophages is associated with altered expression of MMPs and TIMPs. Loss of SOCS1 in either hepatocytes or macrophages reduced the induction of *Mmp2* by CCl_4 in the liver (Fig. 3a, b; columns 3 and 4). Interestingly, loss of SOCS1 in hepatocytes or macrophages resulted in the induction of *Mmp9* and *Mmp14* by the vehicle (Fig. 3c–f; columns 1 and 2). CCl_4 treatment further augmented *Mmp9* induction in *Socs1*$^{fl/fl}$*Alb*^{Cre} mice but not in *Socs1*$^{fl/fl}$*LysM*^{Cre} mice (Fig. 3c, d; columns 2 and 4). However, CCl_4 did not modulate *Mmp14* already induced by vehicle in *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} mice (Fig. 3e, f; columns 2 and 4). Notably, CCl_4-induced expression of *Timp1* was augmented more than four-fold by the loss of SOCS1 in either hepatocytes or macrophages (Fig. 3g, h; columns 3 and 4).

3.4. Expression of inflammatory and fibrogenic cytokines in the livers lacking SOCS1 in hepatocytes or macrophages

Next, we examined the induction of pro-fibrogenic and anti-fibrogenic cytokine genes in the liver tissues of SOCS1-deficient and control mice. Induction of *Il6* by CCl_4 was comparable between SOCS1-deficient and control mice (Fig. 4a, b; columns 3 and 4). However, the SOCS1-deficient livers showed significantly elevated expression of *Il6* in response to corn oil treatment (Fig. 4a, b; columns 1 and 2) that appears to blunt the CCl_4-mediated *Il6* gene induction. Induction of *Tnfa* gene also showed similar pattern (Fig. 4c, d), although the magnitude of response was slightly less in *Socs1*$^{fl/fl}$*LysM*^{Cre} mice than *Socs1*$^{fl/fl}$*Alb*^{Cre} mice. The pro-fibrogenic cytokine gene *Tgfb* was significantly induced in CCl_4-treated control mice (Fig. 4e, f; columns 1 and 3), whereas SOCS1-deficient mice displayed discernible increase in response to vehicle (Fig. 4e, f; columns 1 and 2) that was not significantly upregulated by CCl_4 (Fig. 4e, f; columns 2 and 4). However, both *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} mice showed significant increase in the expression of *Pdgfb* following CCl_4 treatment (Fig. 4g, h).

IFNγ exerts an anti-fibrogenic role in liver fibrosis [22]. CCl_4-treated control groups showed discernible, though not significant, increase in *Ifng* gene expression, whereas SOCS1-deficient livers showed significantly reduced *Ifng* gene expression (Fig. 4i,j).

3.5. Increased leukocyte infiltration and altered chemokine expression in livers lacking SOCS1 in hepatocytes or macrophages

The livers of CCl_4-treated SOCS1-deficient mice showed increased mononuclear infiltration (Fig. 5b, d, g, h, i, j). Notably, vehicle-treated *Socs1*$^{fl/fl}$*LysM*^{Cre} mice also showed marked increase in leukocyte infiltration (Fig. 5f) that coincided with the increased fibrosis observed in these mice (Fig. 1g). As infiltration of immune cells during liver fibrosis and its resolution is mainly driven by chemokines,[23] we examined the expression of key pro-inflammatory and pro-fibrogenic chemokines *Ccl2* and *Ccl5*, and *Cx3cr1* receptor for the anti-fibrogenic chemokine CX3CL1. Notably, both *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} showed

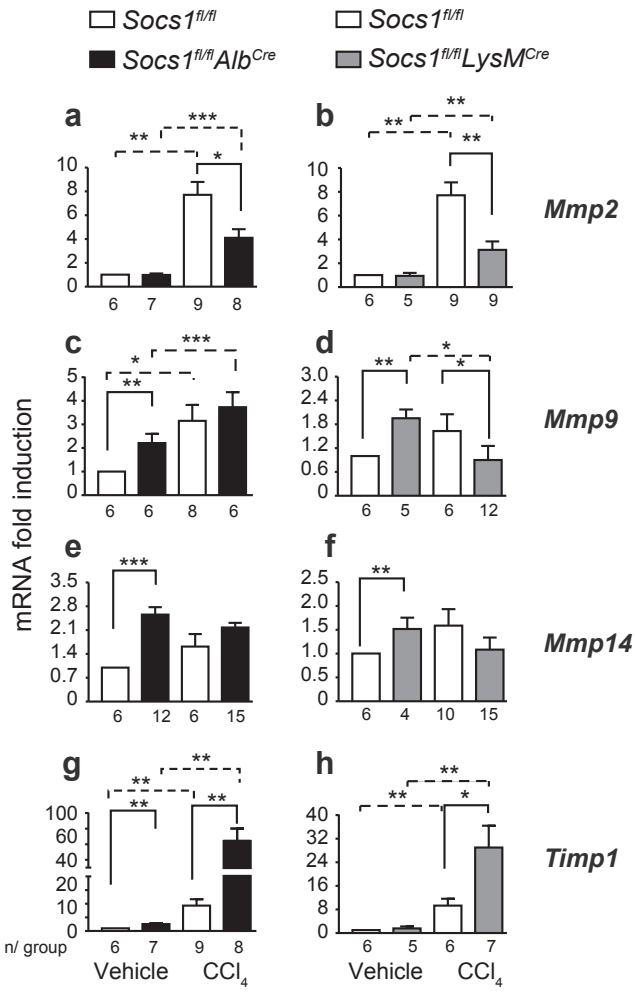

Fig. 3. Influence of SOCS1 deficiency in hepatocytes or macrophages on the expression of matrix modulating enzymes during hepatic fibrogenic response. Quantitative RT-PCR evaluation of *Mmp2* (a, b), *Mmp9* (c, d), *Mmp14* (e, f) and *Timp1* (g, h) in the liver tissues of mice lacking SOCS1 in hepatocytes (a, c, e, g) or macrophages (b, d, f, h) and the corresponding littermate controls, treated with CCl_4 or the vehicle corn oil. Data shown are mean ± s.e.m. values from at least 4 and up to 14 mice per group from 2 to 4 independent experiments. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

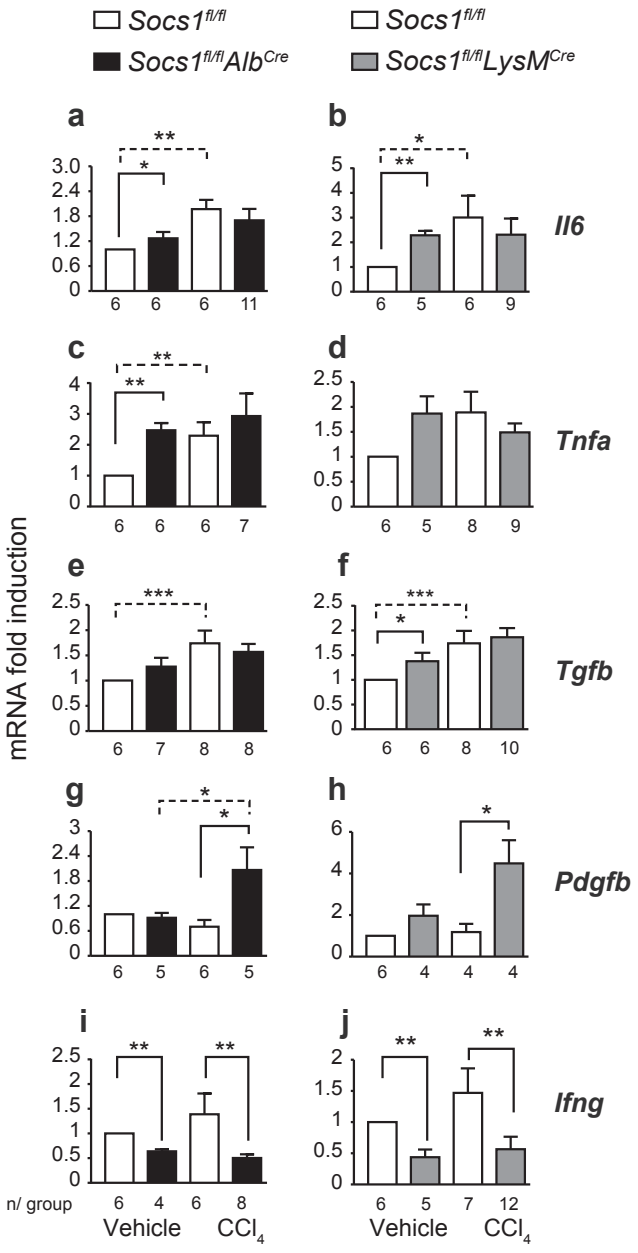

Fig. 4. Effect of hepatocyte or macrophage SOCS1 deficiency on CCl_4-induced inflammatory and fibrogenic cytokine gene expression. Induction of *Il6* (a, b), *Tnfa* (c, d), *Tgfb* (e, f), *Pdgfb* (g, h) and *Ifng* (i, j), genes in the liver tissues of mice lacking SOCS1 in hepatocytes (a, c, e, g, i) or macrophages (b, d, f, h, j) and the corresponding littermate controls, treated with CCl_4 or the vehicle corn oil. Data shown are mean ± s.e.m. values from at least 4 and up to 12 mice per group from 2 to 4 independent experiments. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

increased *Ccl2* and *Ccl5* expression following vehicle treatment (Fig. 5k–n). CCl_4 caused a moderate induction of *Ccl2* in control mice that was dramatically increased in the absence of SOCS1 in hepatocytes or macrophages (Fig. 5k, l). However, CCl_4-induced *Ccl5* expression was decreased in *Socs1*$^{fl/fl}$*Alb*^{Cre} mice but remained unchanged in *Socs1*$^{fl/fl}$*LysM*^{Cre} mice (Fig. 5m, n). Conversely, the induction of *Cx3cr1* by CCl_4 showed no significant change in *Socs1*$^{fl/fl}$*Alb*^{Cre} mice but markedly increased in *Socs1*$^{fl/fl}$*LysM*^{Cre} mice (Fig. 5o, p). These results suggest that SOCS1 deficiency either in hepatocytes or in macrophages promotes LF by modulating chemokine gene expression and immune cell infiltration.

4. Discussion

SOCS1-deficient mice are highly susceptible to LF induced by dimethylnitorsamine or CCl_4, and to experimental hepatocarcinogenesis elicited by diethylnitrosamine [18,19]. Investigations into the anti-tumor mechanisms of SOCS1 revealed that SOCS1 deficiency in hepatocytes increases their responsiveness to hepatocyte growth factor and their resistance to apoptosis [19,24,25]. However, the anti-fibrogenic mechanisms of SOCS1 remain to be elucidated. Hepatocyte damage is the initiator of hepatic fibrogenic response [1] and SOCS1 renders hepatocytes less susceptible to death following acute damage caused by diethylnitrosamine [19]. As SOCS1-deficient macrophages are highly sensitive to innate immune stimuli [26,27], we postulated that SOCS1 may exert its anti-fibrogenic functions not only in hepatocytes but also in macrophages. Our findings show that loss of SOCS1 in either hepatocytes or macrophages potentiates the hepatic inflammatory and fibrogenic responses, accompanied by very similar molecular and cellular changes with a few notable differences.

Hepatocyte injury and the ensuing apoptosis or necrosis releases cellular contents that are taken up by Kupffer cells and HSCs that initiate the fibrogenic response through the release of inflammatory and fibrogenic cytokines [1]. SOCS1 deficiency in hepatocytes resulted in

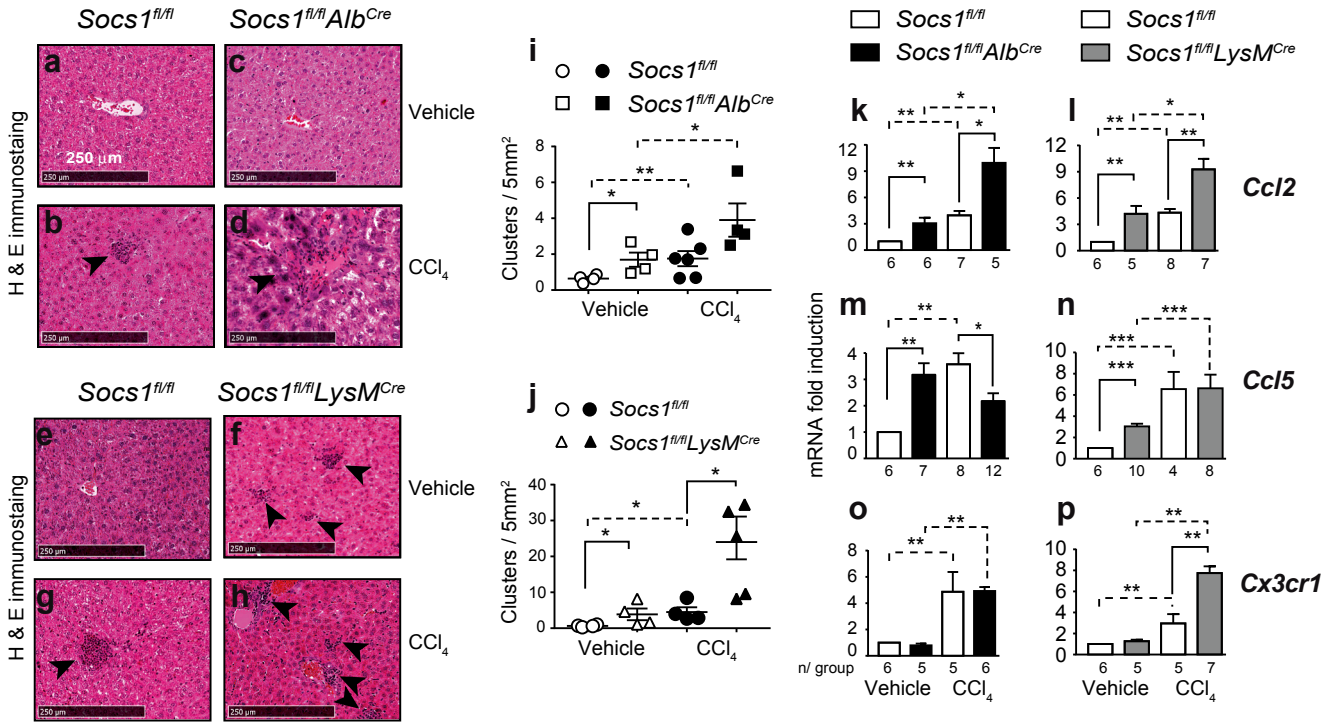

Fig. 5. SOCS1 deficiency in hepatocytes or macrophages modulates chemokine expression and promotes mononuclear cell infiltration during liver fibrosis. (a–h) Representative images of haematoxylin and eosin-stained sections of livers from control (*Socs1*$^{fl/fl}$; a, b and e, g), hepatocyte-specific SOCS1-deficient (c, d) and macrophage-specific SOCS1-deficient (f, h) mice, at the end of treatment regimen with vehicle (corn oil) or CCl_4. Clusters of mononuclear cell infiltration, indicated by arrowheads, were counted in random 5 mm^2 area of liver sections from the indicated mouse strains (i, j). Each data point represents one mouse. (k–p) Expression of *Ccl2* (k, l), *Ccl5* (m, n) and *Cx3cr1* (o, p) genes in the liver tissues of mice lacking SOCS1 in hepatocytes (k, m, o) or macrophages (l, n, p) and the corresponding littermate controls, treated with CCl_4 or the vehicle corn oil. Data shown are mean ± s.e.m. values from at least 4 and up to 10 mice per group from 2 to 3 independent experiments. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

elevated ALT levels at the end of the 5-weeks CCl_4-treatement regimen, which suggests increased hepatocyte damage and consequent augmentation of liver fibrosis. In contrast, reduced ALT levels in *Socs1*$^{fl/fl}$*Alb*^{Cre} mice following administration of diethylnitrosamine was attributed to the anti-apoptotic mechanisms of CDKN1A [19]. It is possible that the apparent difference in ALT levels following acute (diethylnitrosamine) versus chronic (CCl_4) hepatocyte injury in *Socs1*$^{fl/fl}$*Alb*^{Cre} mice could arise from the inability of CDKN1A to cope with chronic hepatocyte damage. Given the role of SOCS1 in regulating the functions of p53 and the regulation of hepatocyte survival by p53 [28,29], it is also possible that SOCS1-deficient hepatocytes may suffer severe damage and liberate abundant quantities of pro-fibrogenic mediators. Further investigations are needed to understand the how SOCS1 protects hepatocyte damage during chronic liver injury.

The increased hepatic fibrogenic response observed in *Socs1*$^{fl/fl}$*LysM*^{Cre} mice is consistent with the critical role of SOCS1 in regulating macrophage activation by innate immune stimuli and with the key pathogenic role of macrophages in liver fibrosis [26,27]. Importantly, our findings show that loss of SOCS1 in macrophages increases the susceptibility to liver fibrosis induction by corn oil. As the *LysM* promoter is expressed also in other myeloid cells and is constitutively active in polymorphonuclear neutrophils (PMN) [20], it is possible that SOCS1-deficient neutrophils could also contribute to the heightened fibrogenic response in *Socs1*$^{fl/fl}$*LysM*^{Cre} mice. Neutrophils are rapidly recruited to the liver during sterile inflammation as well as during infections, and contribute to hepatocyte injury through release of reactive oxygen species and proinflammatory cytokines [30]. Gao and colleagues have recently shown that neutrophils play a key role on high-fat diet- and ethanol- induced steatohepatitis and liver fibrosis [31]. In this model, GM-CSF and IL-15 secreted from activated HSCs promote neutrophil survival and their inflammatory response. It is noteworthy that SOCS1 is a regulator of IL-15 signaling in lymphocytes and IL-15 promotes neutrophil survival by upregulating the anti-apoptotic protein MCL1 [32,33]. Earlier studies have used *Socs1*$^{fl/fl}$*LysM*^{Cre} mice in the context of inflammation and infection, and some have showed increased neutrophil accumulation [34–36]. Even though increased neutrophil accumulation could occur in the inflamed tissues of *Socs1*$^{fl/fl}$*LysM*^{Cre} mice, it remains to be tested whether SOCS1 deficiency in neutrophils also increases their survival and activation in response to IL-15 and GM-CSF, contributing to the heightened inflammatory response and tissue fibrosis.

Inflammatory cytokines promote LF by inducing the key fibrogenic cytokine TGFβ and PDGF, which promotes HSC proliferation [37]. CCl_4 treatment increased the expression of *Il6*, *Tnfa* and *Tgfb* genes in control mice, whereas *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} mice showed increased expression of these genes even with vehicle treatment, suggesting the critical requirement of SOCS1 to maintain tissue homeostasis. Even though SOCS1 deficiency did not upregulate these cytokines further than what was observed in CCl_4-treated control mice, both *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} mice showed marked increase in CCl_4-induced *Pdgfb* expression. PDGFβ promotes liver fibrosis by inducing proliferation and activation of HSCs [38,39]. As PDGFβ expression predominantly occurs in inflammatory macrophages of fibrotic livers [39], it is likely that the increased inflammatory response in *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} liver augments *Pdgfb* expression in macrophages. Moreover, HSCs in these mice may also escape other regulatory mechanisms. First, TIMP1, which promotes liver fibrosis by inhibiting MMPs and by promoting HSC survival [40,41], is highly induced in the livers of CCl_4-treated *Socs1*$^{fl/fl}$*Alb*^{Cre} and *Socs1*$^{fl/fl}$*LysM*^{Cre} mice. Second, these livers showed downmodulation of *Ifng*, which was discernibly increased in control mice. IFNγ attenuates liver fibrosis by inhibiting proliferation of HSCs and their collagen production, and by augmenting NK-mediated killing of activated HSCs [22,42–45]. Hence, reduced *Ifng* induction in SOCS1-deficient livers may allow HSCs escape

NK cell-mediated killing, although the reasons for impaired *Ifng* induction in SOCS1-deficient livers remains to be addressed. Collectively, several mechanisms could contribute to the increased HSC accumulation and their transdifferentiation in $Socs1^{fl/fl}Alb^{Cre}$ and $Socs1^{fl/fl}LysM^{Cre}$ mice.

Altered chemokine and chemokine receptor expression play a key role in the pathogenesis and resolution of liver fibrosis [23]. Notable pro-fibrogenic chemokines are CCL2 and CCL5. CCL2, produced mainly by hepatocytes, Kupffer cells and HSCs, induces infiltration of monocytes and promotes migration and activation of HSCs during fibrogenesis [46,47]. Even though HSCs are the main source of CCL5, it is also produced by hepatocytes, leukocytes and platelets. CCL5 acts on HSCs in autocrine and paracrine manner to facilitate their migration, proliferation and activation [48,49]. The increased *Ccl2* and *Ccl5* expression in vehicle-treated $Socs1^{fl/fl}Alb^{Cre}$ mice could arise from SOCS1-deficient hepatocytes. However, similar increase of *Ccl2* and *Ccl5* expression in vehicle-treated $Socs1^{fl/fl}LysM^{Cre}$ mice indicate that activated HSCs at the effector phase of the fibrogenic cascade could be a major source of pro-fibrogenic chemokines. Notably, the livers of CCl_4-treated $Socs1^{fl/fl}LysM^{Cre}$ mice showed increased expression of *Cx3cr1*, which is predominantly expressed in monocytes and monocyte-derived macrophages and exerts anti-fibrogenic functions [23,50]. Whether SOCS1 deficiency simply tilts the balance towards profibrogenic inflammation, or also overcomes the antifibrotic functions of CX3CR1, remains to be addressed.

In conclusion, SOCS1 plays a non-redundant role in hepatocytes and macrophages to regulate the hepatic fibrogenic response. Whereas SOCS1 expression in hepatocytes may serve to limit cellular damage that initiate the fibrogenic response during persistent liver injury, SOCS1 in macrophages may serve to attenuate the perpetuation and amplification of the inflammatory response. SOCS1 controls ECM deposition by regulating the expression of ECM modulating enzymes MMP2 and TIMP1, and the pro-fibrogenic growth factor PDGFβ and chemokines CCL2 and CCL5. Further studies are needed to decipher the mechanisms by which SOCS1 regulates the various molecular players of liver fibrosis in different cellular compartments.

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