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IL-34 and Macrophage Colony-Stimulating Factor Are Overexpressed in Hepatitis C Virus Fibrosis and Induce Profibrotic Macrophages That Promote Collagen Synthesis by Hepatic Stellate Cells

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Chronic hepatitis C virus (HCV) infection is characterized by progressive hepatic fibrosis, a process dependent on monocyte recruitment and accumulation into the liver. The mediators expressed in chronically injured liver that control the differentiation of human monocytes into profibrotic macrophages (Mφ) remain poorly defined. We report that chronically HCV-infected patients with high fibrosis stages have higher serum levels of macrophage colony-stimulating factor (M-CSF) and interleukin (IL)−34 than HCV-infected patients with lower fibrosis stages and healthy subjects. Immunohistochemistry reveals an intense expression of IL-34 and M-CSF by hepatocytes around liver lesions. In addition, HCV infection and inflammatory cytokines enhance the *in vitro* production of IL-34 and M-CSF by hepatocytes. We next analyzed the acquisition of profibrotic properties by Mφ generated with M-CSF (M-CSF-Mφ) or IL-34 (IL-34-Mφ). M-CSF and IL-34 up-regulate the expression, by differentiating monocytes, of chemokine (C-C motif) ligand (CCL)2, CCL4, C-C chemokine receptor (CCR)1, and CCR5, which are involved in monocyte recruitment/Mφ accumulation in liver lesions. M-CSF-Mφ and IL-34-Mφ also express the hepatic stellate cell (HSC) activators, platelet-derived growth factor, transforming growth factor beta, and galectin-3. IL-34-Mφ and M-CSF-Mφ induce type I collagen synthesis by HSCs, the main collagen-producing cells in liver fibrosis. IL-13, whose expression correlates with the fibrosis stage in HCV-infected patients, decreases the expression of the collagenase, matrix metalloproteinase 1, by IL-34-Mφ and M-CSF-Mφ, thereby enhancing collagen synthesis. By inhibiting the production of interferon-gamma (IFN-γ) by activated natural killer cells, IL-34-Mφ and M-CSF-Mφ prevent the IFN-γ-induced killing of HSCs. *Conclusion:* These results identify M-CSF and IL-34 as potent profibrotic factors in HCV liver fibrosis. (HEPATOLOGY 2014;60:1879-1890)

Abbreviations: Abs, antibodies; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; CCL, chemokine (C-C motif) ligand; CCR, C-C chemokine receptor; CLDs, chronic liver diseases; Ct, threshold cycle; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony-stimulating factor; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IFN-γ, interferon-gamma; IHC, immunohistochemistry; IL, interleukin; KCs, Kuppfer cells; mAb, monoclonal Ab; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; Mφ, macrophage; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NK, natural killer; PBMCs, peripheral blood mononuclear cells; PDGF, platelet-derived growth factor; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; SEM, standard error of the mean; TGF-β, transforming growth factor beta; TIMP-1, tissue inhibitor of metalloproteinase 1; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha.

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Liver fibrosis results from the wound-healing response to repeated injury and/or inflammation and is the common scarring reaction associated with chronic liver diseases (CLDs), such as hepatitis C virus (HCV) infection, nonalcoholic steatohepatitis (NASH), or alcohol abuse. Persistent hepatocellular stress leads to chronic inflammation and, subsequently, death of parenchymal cells and progressive replacement by connective tissue and extracellular matrix (ECM).[1-3] Activation of perisinusoidal resident hepatic stellate cells (HSCs), the main collagen-producing cells, is the central event in hepatic fibrosis. Subsequent to chronic injury, activated HSCs transdifferentiate into myofibroblast-like cells that migrate at sites of tissue repair, where they secrete ECM components. HSCs are activated by transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF) produced in response to liver damage.[4,5] Etiology affects the progression and characteristics of liver fibrogenesis. During chronic HCV infection, interleukin (IL)−13 expression, rather than TGF-β, correlates with fibrotic stage, evidencing IL-13 as a profibrotic cytokine.[6] However, in 20% of advanced liver fibrosis, the existence of profibrotic mediators other than IL-13 and TGF-β is suspected.[7]

Besides the pivotal role of HSCs, progression of liver fibrosis depends on the recruitment into the liver of inflammatory monocytes, which locally differentiate into macrophages (Mφ). These Mφ, rather than Kupffer cells (KCs), contribute to activate HSCs and promote and perpetuate fibrosis.[8] Mφ exhibit remarkable plasticity and phenotypic heterogeneity, and different subsets of Mφ with distinct and sometimes opposite properties (pro- vs. anti-inflammatory, pro- vs. antifibrotic) exist. Environmental factors, including cytokines, control the polarization of Mφ.[9] IL-13/IL-4 and TGF-β induce the generation of murine profibrotic Mφ.[9,10] In contrast, the nature of the differentiation factors differs in humans and rodents, and the factors present in the liver, which promote human monocyte differentiation into profibrotic Mφ, remain unclear.[10,11]

Macrophage colony-stimulating factor (M-CSF) and IL-34 are Mφ differentiation factors that signal through the M-CSF receptor (c-fms or CD115).[12] M-CSF and IL-34 promote monocyte survival, proliferation (in mice), and induce the differentiation of human monocytes into $CD14^{high}$ $CD163^{high}$ $CD206^{low}$ Mφ, which exhibit an immunomodulatory phenotype (IL-10^{high} IL-12^{low} $ILT3^{high}$ B7-$H4^{high}$ $CD80^{low}$ $CD86^{low}$).[13,14] Accordingly, they inhibit the proliferation and the effector functions of memory $CD4^+$ T lymphocytes.[15] Although IL-34 and M-CSF control myeloid cell differentiation, their expression and role in CLDs, especially in the fibrotic process, remains largely unknown.

With approximately 170 million people chronically infected worldwide, HCV is as a major cause of CLD.[16] Over years or decades, chronic HCV infection is typically characterized by slowly progressive hepatic fibrosis, ultimately leading to cirrhosis and hepatocellular carcinoma (HCC).[17] To assess the potential role of IL-34 and M-CSF in the liver fibrotic process, we investigated their expression and function during chronic HCV infection.

Patients and Methods

Patients. M-CSF and IL-34 were quantified in serum of 148 subjects with chronic hepatitis C, naïve of antiviral treatment, and without other hepatitis virus and human immunodeficiency virus coinfection (declaration no.: DC-2011-1467; authorization no.: AC-2012-1507). Baseline characteristics of HCV-infected patients are summarized in Table 1. Hepatic histological activity (A) and liver fibrosis (F) were measured using the Metavir score (A0-3, F0-4).[18] M-CSF and IL-34 were also quantified in 30 patients suffering from nonalcoholic fatty liver disease (NAFLD). Serums from 60 healthy donors (agreement ANG-2003-02; Blood Collection Center, Angers, France) were used as controls.

Cytokine Quantification. Chemokine (C-C motif) ligand (CCL)2, CCL4, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-6, IL-34, interferon-gamma (IFN-γ), M-CSF, matrix metalloproteinase (MMP)−9, tumor necrosis factor alpha (TNF-α), and PDGF-A were quantified by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Lille, France). Results are expressed as a concentration or as a percentage of variation of cytokine production.

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Potential conflict of interest: Nothing to report.

Table 1. Baseline Characteristics of HCV-Infected Patients

Sexe ratio (M/F)	**2.14**
Age, years	
Median	42.6
Range	25-78
Disease duration, years	(n = 89)
Median	16
Range	2-49
HCV genotype (%)	
1	53.4 (79/148)
1a	13.5 (20/148)
1b	32.4 (48/148)
2	5.4 (8/148)
3	31.1 (46/148)
4	5.4 (8/148)
5	4.7 (7/148)
Metavir grade and stage (%)	
A0	0 (0/109)
A1	43.1 (47/109)
A2	49.5 (54/109)
A3	7.3 (8/109)
F0	5.9 (7/119)
F1	36.1 (43/119)
F2	26.1 (31/119)
F3	13.4 (16/119)
F4	18.5 (22/119)
Viral load, 10^5 Eq/mL	(n = 109)
Median	52.67
Range	1.00-856.4
AST (%)	
Normal	28.3 (39/138)
Elevated	71.7 (99/138)
ALT (%)	
Normal	12.1 (17/140)
Elevated	87.9 (123/140)

Samples were collected between February 1997 and May 2006 from untreated individuals infected with HCV followed in University Hospitals of Ajaccio, Angers, Brest, Clermont-Ferrand, Le Mans, Mulhouse, Nantes, Orléans, and Poitiers (France). Data were available in an anonymized database.

Immunohistochemistry. Paraffin-embedded liver biopsies from patients with chronic HCV infection with fibrosis and from a patient with a microvesicular steatosis (no sign of inflammation and fibrosis) were from the Department of Tissue Pathology (University Hospital, Angers, France). Liver biopsies from healthy adults were from Biochain (Newark, CA). Immunohistochemistry (IHC) was performed using rabbit anti-human IL-34 (2 μg/mL; Abcam, Paris, France) and goat anti-human M-CSF (0.5 μg/mL; Santa Cruz Biotechnology, Heidelberg, Germany) polyclonal antibodies (Abs). Bound Abs were detected with the Bond Polymer Refine Detection kit and the peroxidase detection system (Leica Microsystems, Newcastle, UK). Purified goat and rabbit immunoglobulins (Sigma-Aldrich, St Louis, MO) were used as controls.

Cell Purification and Mφ Generation. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy subjects or from chronically HCV-infected patients (naïve of treatment or not treated in the preceding 6 months) by standard density-gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). $CD14^+$ monocytes and $CD3^-$ $CD56^+$ natural killer (NK) cells were purified from PBMCs, as previously described[14,19]; purity was >99%. Macrophage subsets were generated by culturing monocytes for 7 days in RPMI 1640 culture medium (Cambrex, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS; Biowest, Nuaillé, France) and containing 50 ng/mL of M-CSF (M-CSF-Mφ) or IL-34 (IL-34-Mφ) or 20 ng/mL of GM-CSF (GM-CSF-Mφ), as previously described[14]; cytokines were from R&D systems (Abingdon, UK). In some experiments, day 7 Mφ (10^6 cells/mL) were stimulated for 2 days with 50 ng/mL of IL-13 (R&D Systems), in the presence of M-CSF, IL-34, or GM-CSF.

Human KCs and Hepatocytes. Human KCs (Life Technologies, Illkirch, France) were cultured for 4 days with 50 ng/mL of M-CSF, IL-34, or GM-CSF before phenotyping. Primary human hepatocytes (Life Technologies), cultured in collagen-coated plates, were stimulated for 24 hours with 100 ng/mL of TNF-α plus IFN-γ or IL-1β, 450 μM of etoposide (Sigma-Aldrich), or 200 ng/mL of anti-CD95 monoclonal Ab (mAb; clone CH-11; MBL, Woburn, MA); in some experiments, hepatocytes were exposed to hypoxia for 24 hours, followed by a 3-hour reoxygenation.

In Vitro *HCV Infection.* The Huh7.5 cell line, which supports HCV replication, was infected for 12 hours with 2×10^5 focus-forming units of the JFH-1 strain optimized by 10 cycles of infection in naïve Huh7.5 cells.[20] This optimized virus infects all of the cells within 3 days of infection.

Mφ-LX-2 Cell Cocultures. The human HSC line LX-2[5] was cultured in Dulbecco's modified Eagle's medium (Cambrex), supplemented with 1% FCS. LX-2 cells (2×10^5 cells/mL/well in 12-well plates) were starved for 48 hours in FCS-free culture medium. Macrophages (10^6 cells/mL/well) were then cultured with LX-2 cells in the culture medium used to generate Mφ subtypes. As controls, LX-2 cells were stimulated with 50 ng/mL of IL-13 or 5 ng/mL of TGF-β1 (R&D Systems). Cells were disrupted in TRIzol for RNA extraction or in radioimmunoprecipitation assay buffer for western blotting after 24 and 48 hours culture, respectively.

Mφ-NK Cell Cocultures. Day 6 Mφ (10^6 cells/mL/well) were stimulated with 1 μg/mL of Pam3Csk4 (Toll-like receptor [TLR]2 ligand) and 5 μg/mL of CL097 (TLR7/8 ligand; Invivogen, San Diego, CA) before the addition, after 24 hours, of autologous NK

cells (10^6 cells/mL/well). IFN-γ was quantified in the 24-hour supernatants. Results are expressed in pg/mL of IFN-γ.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis. The expression of the messenger RNA (mRNA) encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TGF-β1, PDGF-A, MMP-1, MMP-2, MMP-9, galectin-3, CCL2, CCL4, C-C chemokine receptor (CCR) 1, CCR, CCR5, lactadherin, and tissue inhibitor of metalloproteinase 1 (TIMP-1) was analyzed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) at different time points during the Mφ differentiation process after a 6-hour stimulation with 5 μg/mL of CL097. Alpha-1 type I collagen expression was analyzed on LX-2 cells exposed for 24 hours to IL-13 or TGF-β1 or cultured with Mφ. M-CSF and IL-34 mRNA were quantified in primary hepatocytes, LX-2 cells, and primary fibroblasts after 24-hour activation with proinflammatory cytokines. RNA extraction, reverse transcription, and amplification were performed as previously described.[14] Relative gene expression was calculated using the $E^{\Delta Ct}$ method, with ΔCt (threshold cycle) values calculated from the sample with the largest Ct (fewest gene copies), and then normalized against the GAPDH (determined as the most stably expressed housekeeping gene using the geNorm method).[21]

Analysis of Type I Collagen Expression by Western Blotting. Type I collagen expression in cell lysates was analyzed by western blotting. Proteins were electrophoretically separated on a 10% polyacrylamide gel in reducing conditions and then transferred to an Immobilon membrane (Millipore, Bedford, MA). Membranes were incubated with 1 μg/mL of polyclonal sheep anti-type I collagen Ab before incubation with horseradish-peroxidase–conjugated anti-sheep immunoglobulin G Ab (R&D Systems); bound Abs were detected using the ECL system (Amersham Biosciences, Foster City, CA).

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 5 (version 5.02; GraphPad Software Inc., La Jolla, CA). Mann-Whitney's test was used to analyze nonparametric data. For grouped analysis, Kruskal-Wallis' test was performed, and Dunn's multiple test was used as a posttest comparison. For the comparison of mRNA expression time courses, two-way analysis of variance (ANOVA) with a posthoc Bonferroni's multiple comparison test was used. Spearman's test was used to analyze correlations of nonparametric data. Statistical significance was considered when P values were less than 0.05.

Results

M-CSF and IL-34 Serum Levels Are Elevated in HCV-Infected Patients With Advanced Liver Fibrosis. HCV-infected patients exhibited higher levels of M-CSF than healthy subjects (0.80 ± 0.03 and 0.32 ± 0.04 ng/mL, respectively; mean ± standard error of the mean [SEM]; $P < 10^{-4}$; Fig. 1A, left panel). According to the Metavir fibrosis stage, patients with cirrhosis and marked bridging fibrosis (F4 and F3 stages, respectively) had higher M-CSF levels than patients without or with portal fibrosis without septa and with portal fibrosis with few septa (F0/F1 and F2, respectively; $P = 0.0031$; Fig. 1B). IL-34 levels were low and not significantly different in patients and healthy subjects (Fig. 1A, right panel). However, patients with advanced liver fibrosis (F3-F4 stages) exhibited higher IL-34 levels than F0/1-F2 patients ($P = 0.0039$; Fig. 1C). According to the Metavir inflammation/activity grade, patients with severe inflammatory activity (A3 grade) had higher M-CSF levels than patients without or with mild (A0/A1) or moderate (A2) inflammatory activity ($P = 0.0313$; Fig. 1D). M-CSF levels were significantly higher in patients with elevated aspartate aminotransferase (AST), compared with patients with normal AST ($P = 0.006$; data not shown), and tended to be higher in patients with elevated alanine aminotransferase (ALT) than in those with normal ALT ($P = 0.07$; data not shown). No relation between IL-34 levels and inflammatory activity was observed (data not shown).

Levels of the inflammatory cytokines, GM-CSF, IL-6, IL-1β, TNFα, and CCL2, were also quantified in HCV-infected patients. IL-6 (2.5 ± 0.5 pg/mL; mean ± SEM) and CCL2 (174.2 ± 8.4 pg/mL) were increased in HCV-infected patients, compared to healthy subjects (0.8 ± 0.1 and 138.2 ± 8.5 pg/mL, respectively; Supporting Fig. 1A). Levels of IL-6 and CCL2 were correlated with M-CSF ($r = 0.4092$, $P < 0.0001$ and $r = 0.3667$, $P < 0.0001$, respectively; Spearman's rank correlation test) and IL-34 levels ($r = 0.2845$, $P < 0.001$ and $r = 0.2262$, $P < 0.01$, respectively; data not shown) and with fibrosis stage (Supporting Fig. 1A). Levels of TNF-α were elevated in 26 of 147 HCV-infected patients (587 ± 182 pg/mL; mean ± SEM) and low or undetectable in the other HCV-infected patients and in healthy subjects (data not shown). GM-CSF was elevated in 33 of 147 HCV-infected patients (126 ± 55 pg/mL; mean ± SEM; different to those with elevated TNF-α; data not shown) and low or undetectable in the other HCV-infected patients and in healthy subjects. TNF-α

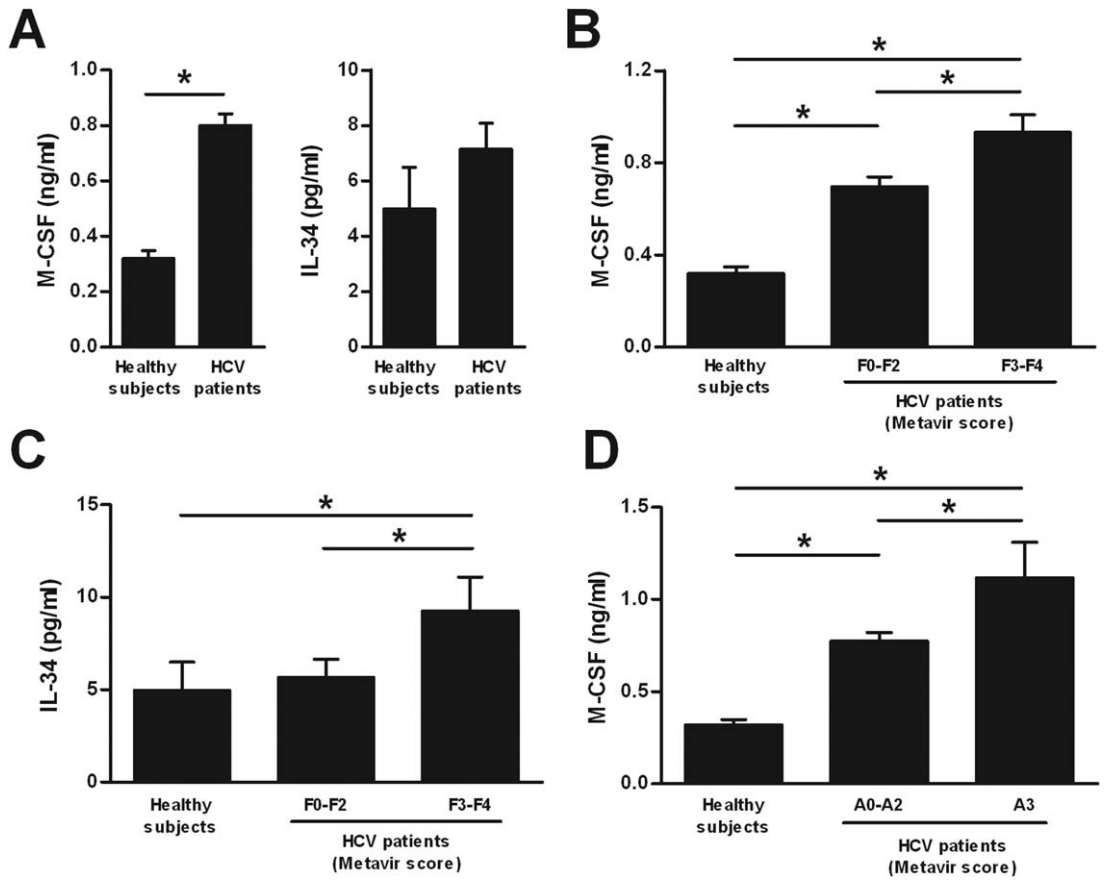

Fig. 1. HCV-infected patients with advanced fibrosis exhibit elevated serum levels of M-CSF and IL-34. (A) M-CSF and IL-34 were quantified by ELISA in the serums of 148 chronically HCV-infected patients and of 60 healthy subjects. (B, C) Serum levels of M-CSF (B) and IL-34 (C) were analyzed in 119 patients according to the Metavir fibrosis stage (F0/F1 and F2 versus F3 and F4 stage). (D) M-CSF serum levels were analyzed in patients according to the Metavir inflammatory stage (A0-A2 versus A3 stage). (A-D), results are expressed in ng/ml (mean ± SEM), $*P < 0.05$ (A, Mann-Whitney test; B-D, Kruskal-Wallis test).

and GM-CSF were not correlated with levels of M-CSF and IL-34 or with fibrosis stages. Finally, no IL-1β was detected in HCV-infected patients and healthy subjects (data not shown).

IL-34 and M-CSF were also elevated in NAFLD patients (12.58 ± 2.47 pg/mL and 2.86 ± 0.21 ng/mL, respectively; mean ± SEM; n = 27), compared to healthy subjects (4.98 ± 1.50 pg/mL and 0.32 ± 0.04 ng/mL, respectively; n = 68); no correlation was evidenced between levels of IL-34 and M-CSF and fibrosis stages (simple NAFLD, NASH, and NASH with cirrhosis) (Supporting Fig. 1B).

M-CSF and IL-34 Are Expressed by Hepatocytes of HCV-Infected Patients. We then investigated IL-34 and M-CSF expression in the liver. IHC revealed an intense expression of M-CSF and IL-34 in hepatocytes of HCV-infected patients, mainly in hepatocytes located around fibrotic and inflammatory lesions; no staining was observed in hepatic sinusoids and in proper hepatic arteries, hepatic portal veins, and common bile ducts of the portal tracts (Fig. 2A). A discrete and homogeneous expression of M-CSF and IL-34 was observed in a patient with microvesicular steatosis (Fig. 2A). We next analyzed the expression of M-CSF and IL-34 by *in vitro* HCV-infected hepatocytes. HCV infection up-regulated M-CSF and IL-34 mRNA expression in Huh7.5 cells, with a maximum at 7 and 11 days postinfection, respectively (Fig. 2B). M-CSF, but not IL-34, was detected in supernatants of infected Huh7.5 cells (Fig. 2C). Moreover, M-CSF and IL-34 expression is increased in primary hepatocytes in response to TNF-α plus IFN-γ and TNF-α plus IL-1β (Fig. 2D) while decreased in response to etoposide and hypoxia/reoxygenation and not modulated in response to anti-CD95 mAb and TGF-β-induced epithelial-mesenchymal transition (Fig. 2D). Expression of M-CSF and IL-34 was also increased in LX-2 cells and primary fibroblasts in response to proinflammatory cytokines (data not shown).

Finally, M-CSF and IL-34 were not expressed by immune cells infiltrating the liver of HCV-infected patients (Fig. 2A). Accordingly, M-CSF and IL-34

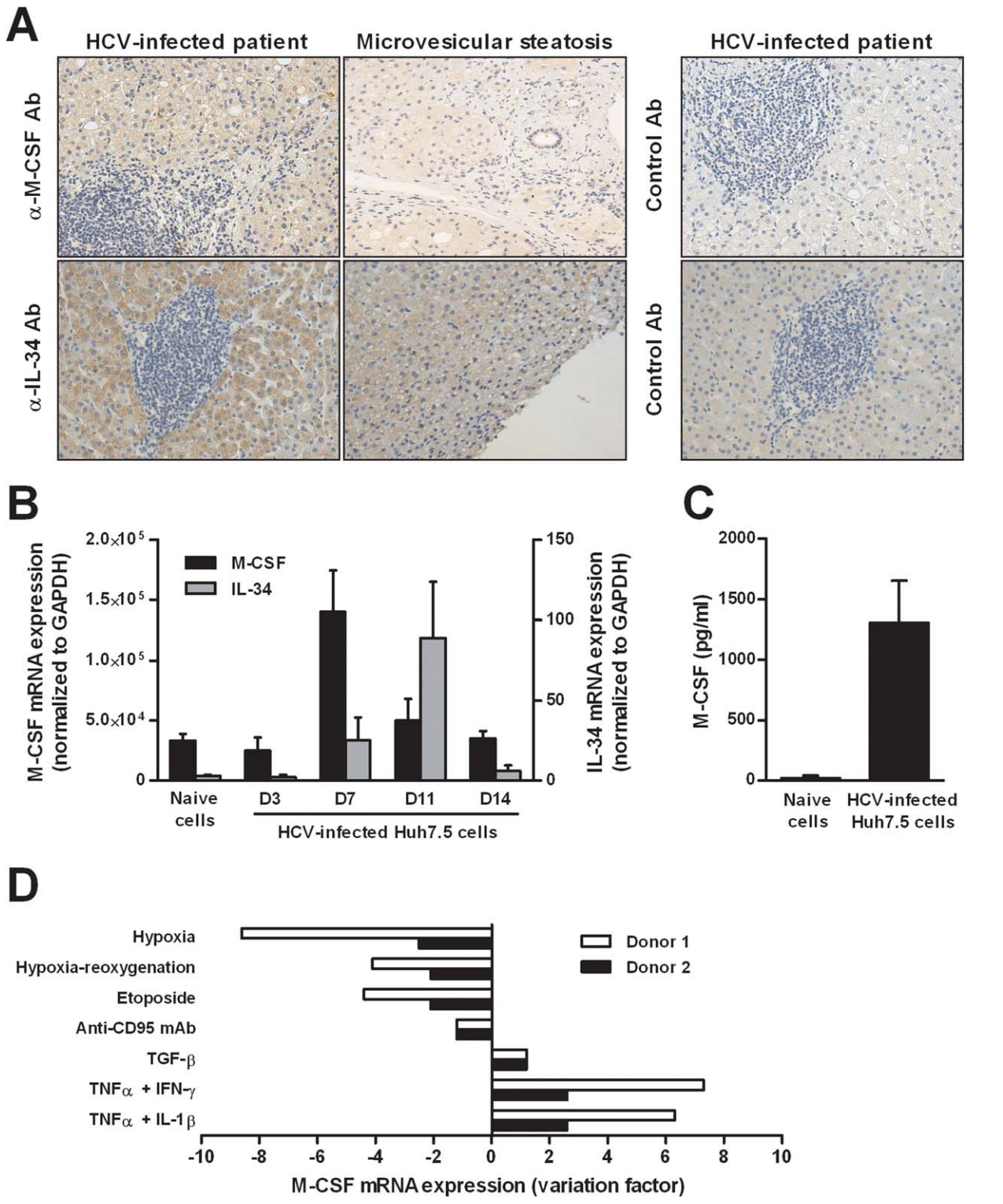

Fig. 2. Hepatocytes overexpress M-CSF and IL-34 in HCV-infected patients. (A) Liver biopsies from a HCV-infected patient and from a patient with microvesicular steatosis (without sign of fibrosis and inflammation) were labeled with anti-M-CSF, anti-IL-34 or isotype control Abs. Results are representative of one of five patients and control subjects. (B) Huh7.5 hepatoma cells were infected or not (naive cells) with HCV and M-CSF and IL-34 mRNA expression were quantified at the indicated time-points by quantitative RT-PCR. Results are expressed in mRNA expression normalized to GAPDH (mean of triplicate ± SEM) and are representative of one of two experiments. (C) M-CSF was quantified by ELISA in the 7 day supernatants of Huh7.5 cells infected or not (naive cells) with HCV. Results are expressed in ng/ml (mean of triplicate ± SEM) and are representative of one of two experiments. (D) Primary hepatocytes were cultured for 24 h with TNFα plus IL-1β or TNFα plus IFN-γ, anti-CD95 mAb and etoposide, or for 3 days with TGF-β, or subjected to hypoxia/re-oxygenation. M-CSF mRNA expression were determined by quantitative RT-PCR and normalized to GAPDH (mean of triplicate ± SEM). Results, obtained in two independent experiments, are expressed as a variation of expression compared to nonstimulated cells.

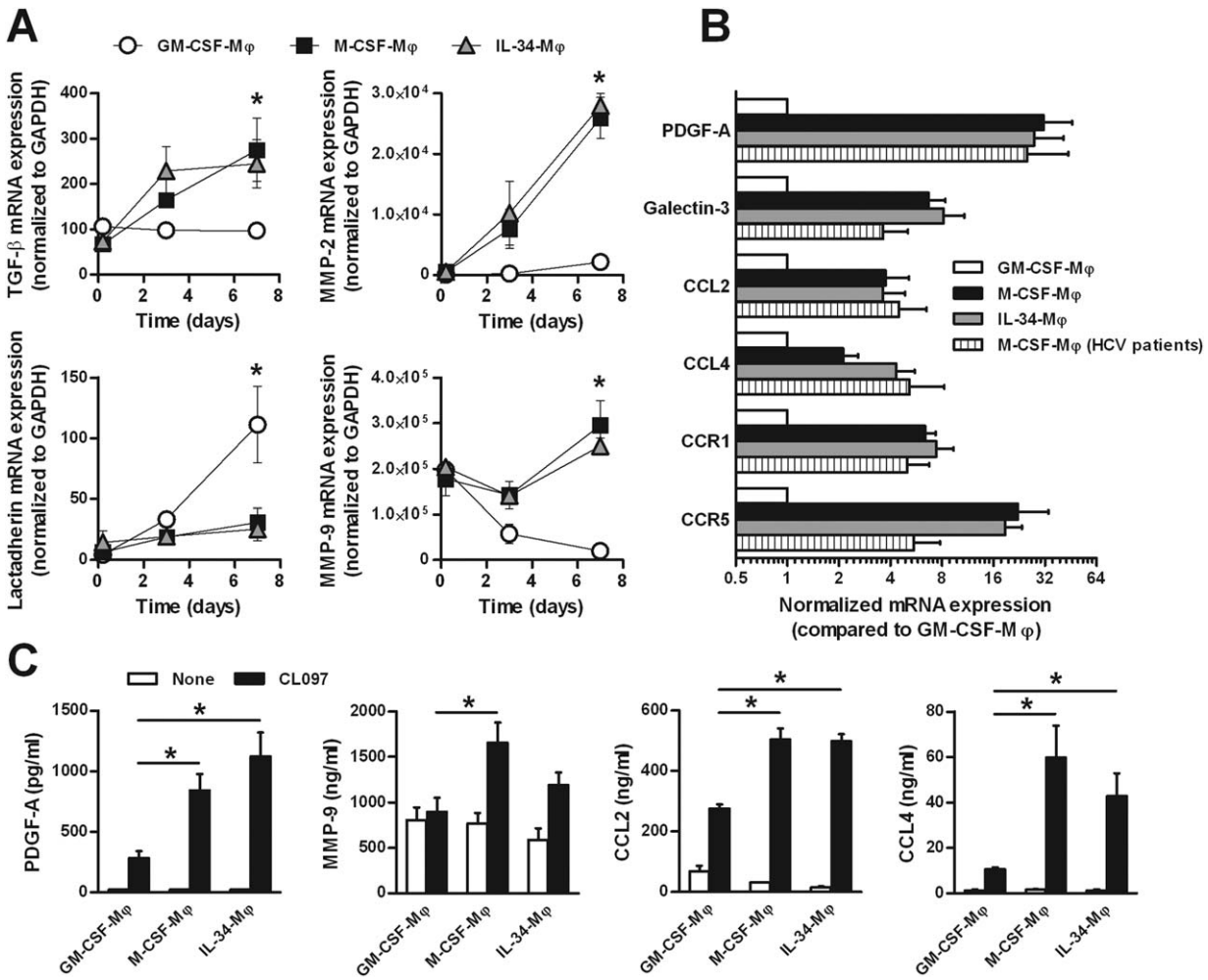

Fig. 3. M-CSF and IL-34 induce the differentiation of monocytes into profibrotic macrophages. (A) The expression of the mRNA encoding TGF-β1, MMP-2, MMP-9, and lactadherin was analyzed in monocytes cultured with GM-CSF, M-CSF, or IL-34, at the indicated time-points. Results are expressed in mRNA expression normalized to GAPDH. (B) The mRNA encoding the indicated molecules was analyzed in day 7 IL-34-Mφ, M-CSF-Mφ and GM-CSF-Mφ generated using monocytes from healthy subjects and in day 7 M-CSF-Mφ generated using monocytes from HCV-infected patients. Results are expressed in normalized ratios relative to GM-CSF-Mφ. Myeloid cells were stimulated for 6 hours with CL097 before analysis. Results are expressed as mean $\pm$ SEM, n = 4; $^*P \leq 0.05$ (two-way ANOVA with Bonferroni posttest). (C) PDGF-A, MMP-9, CCL2 and CCL4 were quantified by ELISA in the 24-hour cell culture supernatants of M-CSF-Mφ, IL-34-Mφ and GM-CSF-Mφ, stimulated or not with CL097. Results are expressed in ng/ml or pg/ml (mean $\pm$ SEM, n = 4).

mRNA expression was equivalent in PBMCs from infected patients and healthy subjects (Supporting Fig. 2).

M-CSF-Mφ and IL-34-Mφ Trigger Type I Collagen Secretion by Human HSCs. Based on the observations that (1) M-CSF and IL-34 are elevated in patients with advanced liver fibrosis, (2) M-CSF and IL-34 are Mφ differentiation factors,[6,14] and (3) liver fibrosis is dependent on Mφ accumulation,[8] we investigated the ability of IL-34 and M-CSF to induce profibrotic Mφ. M-CSF and IL-34, but not GM-CSF, time dependently up-regulated the expression by differentiating monocytes of the profibrotic factors, TGF-β1, MMP-2, MMP-9, PDGF-A, and galectin-3, both at the mRNA (Fig. 3A,B) and protein levels (Fig. 3C). Similar results were obtained using monocytes isolated from HCV-infected patients (Fig. 3B) and with KCs isolated from healthy subjects (Supporting Fig. 3 and data not shown). M-CSF and IL-34, but not GM-CSF, also up-regulated the expression by Mφ of CCL2, CCL4, CCR1, and CCR5 (Fig. 3B), which are involved in monocyte/Mφ recruitment and accumulation in the liver.[8,22] Moreover, M-CSF and IL-34 have additive effects on induction of mRNA encoding PDGF-A, MMP-9, CCL2, and CCL4 (Supporting Fig. 4); nevertheless, this increase in mRNA expression is directly related to the number of viable cells (data not shown). This result is in line with the fact that both cytokines act as survival and differentiation factors that signal, in myeloid cells, through the same receptor.

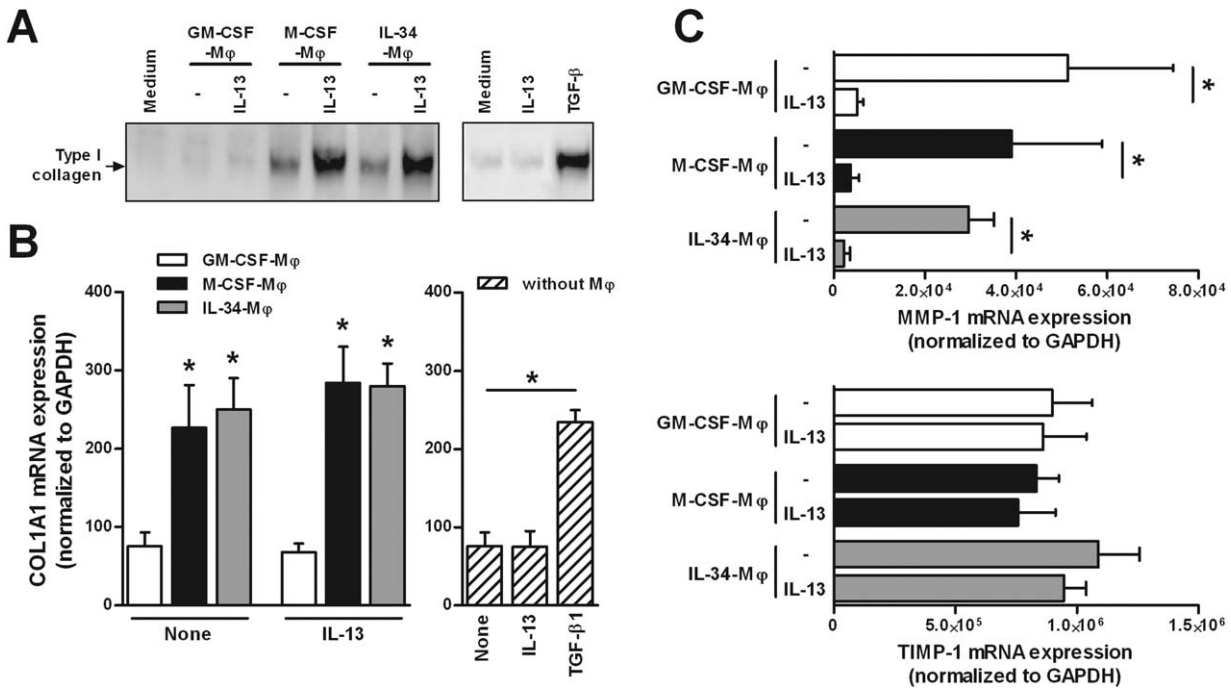

Fig. 4. M-CSF-Mφ and IL-34-Mφ trigger type I collagen expression by hepatic stellate cells. Potentiating effect of IL-13. (A&B) Day 7 GM-CSF-Mφ, M-CSF-Mφ, and IL-34-Mφ stimulated or not with IL-13, were cultured with the human hepatic stellate cell line LX-2. The expression of type I collagen was analyzed in 48-hour cell lysates by western blotting (A) and the expression of type I collagen mRNA expression was analyzed by quantitative RT-PCR after 24-hour activation (B). In some experiments, LX-2 cells were stimulated with TGF-β or IL-13 alone (A&B, right panels). Results are representative of one of three experiments. (C) MMP-1 and TIMP-1 mRNA expression was analyzed by quantitative RT-PCR in GM-CSF-Mφ, M-CSF-Mφ, or IL-34-Mφ either stimulated or not with IL-13 for 24 hours. (B&C) Results are expressed in mRNA expression normalized to GAPDH, as mean $\pm$ SEM (n = 5), $^*P < 0.05$ (Mann-Whitney test).

M-CSF-Mφ and IL-34-Mφ, but not GM-CSF-Mφ, triggered type I collagen mRNA and protein expression by LX-2 cells (Fig. 4A,B), at similar levels to TGF-β1 used as a positive control.[5] Moreover, the expression of the antifibrotic molecule, lactadherin, was up-regulated during the monocyte differentiation process with GM-CSF, but not with M-CSF and IL-34 (Fig. 3A).

IL-13 Boosts M-CSF-Mφ and IL-34-Mφ Profibrotic Property by Decreasing MMP-1. IL-13 is a profibrotic cytokine, whose expression correlates with fibrotic stages in HCV-infected patients.[6,7] Therefore, we evaluated the ability of IL-13 to modulate the profibrotic properties of M-CSF-Mφ and IL-34-Mφ. IL-13 did not modulate CCL2, CCL4, CCR2, CCR5, galectin-3, lactadherin, PDGF-A, and TGF-β expression by M-CSF-Mφ and IL-34-Mφ (data not shown). Interestingly, production of type I collagen by LX-2 cells was dramatically enhanced when cocultured with IL-13-stimulated M-CSF-Mφ and IL-34-Mφ (Fig. 4A), whereas type I collagen mRNA expression was unaffected (Fig. 4B). IL-13 alone did not modulate the expression of type I collagen by LX-2 cells (Fig. 4A,B), suggesting that IL-13 regulates expression and/or activity of collagenases in M-CSF-Mφ and IL-34-Mφ. Accordingly, IL-13 strongly down-regulated, in M-CSF-Mφ and IL-34-Mφ, expression of MMP-1, the main protease responsible for degradation of type I collagen,[23] whereas expression of TIMP-1 remained unaffected (Fig. 4C). Similar results were obtained using IL-4 (Supporting Fig. 5A and data not shown).

In contrast, IL-13 did not confer to GM-CSF-Mφ the ability to promote collagen expression by LX-2 cells, as assessed at protein (Fig. 4A) and mRNA levels (Fig. 4B). In support, GM-CSF-Mφ, either stimulated or not with IL-13, retained similar levels of the HSC activators, PDGF, TGF-β, and galectin-3, and of lactadherin, chemokines, and chemokine receptors (data not shown). As a control,[15,24] IL-13 up-regulated expression of CCL17 and CCL22 by GM-CSF-Mφ (Supporting Fig. 5B).

M-CSF-Mφ and IL-34-Mφ Inhibit IFN-γ Production by NK Cells. The fibrotic process is restricted by activated NK cells, which produce the antifibrotic cytokine, IFN-γ, and kill activated HSCs.[25] Moreover, the ability of NK cells from HCV patients to kill HSCs inversely correlates with the stage of liver fibrosis. Therefore, we evaluated whether M-CSF-Mφ and IL-34-Mφ may reduce IFN-γ secretion by stimulated NK cells. Results showed that M-CSF-Mφ and IL-34-

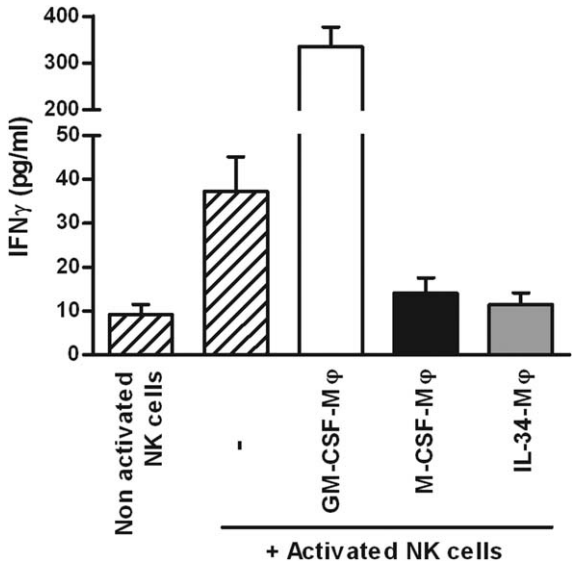

Fig. 5. M-CSF-Mφ and IL-34-Mφ inhibit the production of IFN-γ by activated NK cells. NK cells, stimulated with CL097 or Pam3CSK4, were cultured or not with autologous M-CSF-Mφ, IL-34-Mφ or GM-CSF-Mφ. IFN-γ (mean $\pm$ SEM, n = 3) was quantified in the 24-hour supernatants.

Mφ inhibited production of IFN-γ by activated NK cells, whereas GM-CSF-Mφ strongly up-regulated its expression (Fig 5). In line with their immunomodulatory properties,[15] M-CSF-Mφ and IL-34-Mφ also dramatically reduced production of IFN-γ by stimulated T cells, whereas GM-CSF-Mφ increased its production (Supporting Fig. 6).

Discussion

In this study, we identified IL-34 and M-CSF as profibrotic factors associated with chronic HCV fibrosis and are required to unveil the profibrotic activity of IL-13 on human Mφ. IL-34 and M-CSF, whose expression is enhanced in HCV-infected patients with high fibrosis stages, induce differentiation of monocytes into profibrotic Mφ that trigger type I collagen synthesis by HSCs. By decreasing MMP-1 expression by IL-34-Mφ and M-CSF-Mφ, IL-13 dramatically amplifies collagen accumulation. In line with the fact that they signal through the same receptor in myeloid cells, M-CSF and IL-34 are equivalent to confer profibrotic properties to Mφ. In contrast, the other human Mφ differentiation factor, GM-CSF, does not generate profibrotic Mφ, even in the presence of IL-13. Finally, IL-34-Mφ and M-CSF-Mφ decrease production of IFN-γ, a potent antifibrotic factor, by activated NK cells. The profibrotic properties of IL-34 and M-CSF are schematized in Fig. 6.

We report an overexpression of M-CSF and IL-34 by hepatocytes of HCV-infected patients, mainly by cells surrounding lesions. Expression of M-CSF has been reported on in human hepatoma cells,[26] with an overexpression in peritumoral liver tissues.[27] Although IL-34 mRNA is expressed in murine liver,[13] the nature of the IL-34-producing cells remained undetermined. We observed that HCV induces M-CSF and IL-34 by hepatoma cells. IL-34 mRNA was expressed 11 days postinfection, when the viral load and cytopathogenic activity are maximal, suggesting a production by dying hepatocytes. In contrast, M-CSF mRNA expression occurred earlier, suggesting a complementary role of these two cytokines in the liver. Interestingly, proinflammatory cytokines also increase expression of IL-34 and M-CSF not only by hepatocytes, but also by HSCs and fibroblasts, suggesting that their expression in the liver is mainly driven by infection and/or inflammation. The elevated levels of both cytokines in NAFLD patients support this hypothesis.

Macrophages modulate the activity of HSCs[10,28] and are crucial in the liver fibrotic process.[8] We report that M-CSF-Mφ and IL-34-Mφ promote type I collagen secretion by HSCs. M-CSF and IL-34 switch monocytes into Mφ-expressing PDGF-A and galectin-3, which promote HSC survival and proliferation, and TGF-β1, a potent HSC activator. Besides HSC activation, M-CSF-Mφ and IL-34-Mφ may favor monocyte recruitment and Mφ accumulation into liver lesions, through production of CCL2, which attract monocytes into the liver.[10,29] They also express CCR1 and CCR5 and produce CCL4, a ligand of CCR5. CCR1 and CCR5 promote hepatic fibrosis,[22] and their ligands are up-regulated in liver fibrogenesis models.[10] Finally, the resolution and/or limitation of liver fibrosis is associated with apoptosis of activated HSCs induced by activated NK cells in an IFN-γ-dependent manner.[25] We observed that IL-34-Mφ and M-CSF-Mφ inhibited IFN-γ production by NK cells, thereby evidencing another mechanism by which M-CSF and IL-34 may promote fibrosis.

A positive correlation between IL-13 expression and liver fibrosis has been reported in CLDs with different etiologies,[6] leading one to consider IL-13 as a profibrotic cytokine. We observed that IL-13 decreases MMP-1 expression by IL-34-Mφ and M-CSF-Mφ. Whereas collagenases are involved in the initiation of the fibrotic process,[30] progressive fibrosis is associated with marked increases in TIMP-1, leading to a net decrease in protease activity and therefore a more unopposed matrix accumulation. Accordingly, MMP-1 injection attenuates established liver fibrosis.[31]

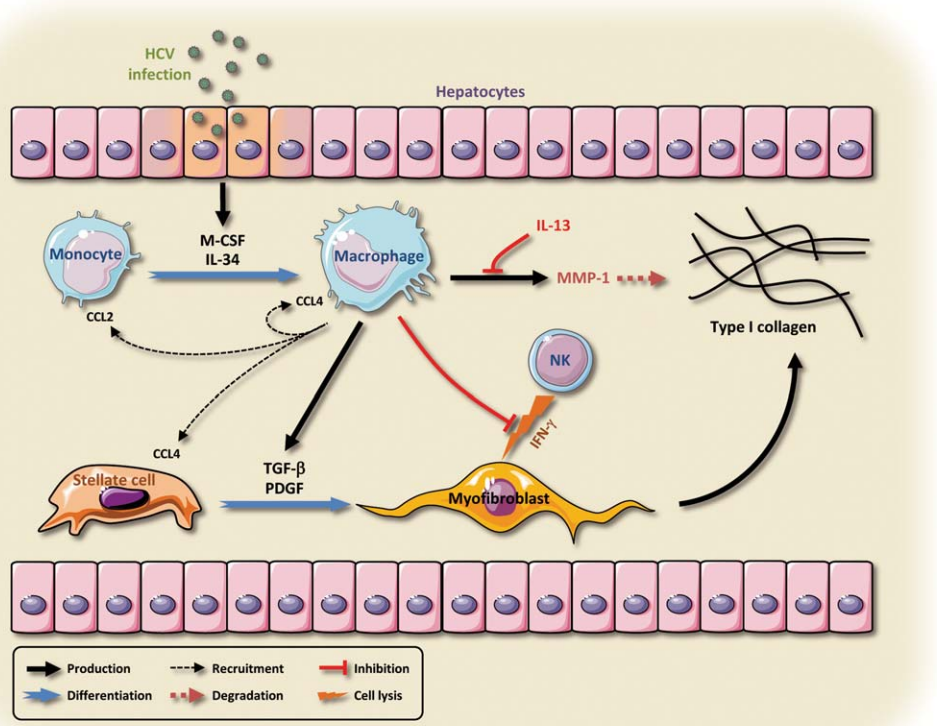

Fig. 6. Schematic representation of the potential role of IL-34 and M-CSF in the fibrosis associated to chronic HCV infection. In response to HCV infection, injured hepatocytes secrete IL-34 and M-CSF. IL-34 and M-CSF induce monocyte recruitment and differentiation into profibrogenic Mφ. More precisely, M-CSF-Mφ and IL-34-Mφ (i) promote type I collagen secretion by hepatic stellate cells, (ii) favor the survival of hepatic stellate cells by decreasing NK cell activity and, (iii) express chemokines and chemokine receptors that favor monocyte recruitment and Mφ accumulation in injured sites. Finally, the profibrotic activity of IL-13 on Mφ is dependent on IL-34 and M-CSF. IL-13 decreases MMP-1 expression by M-CSF-Mφ and IL-34-Mφ, thereby boosting their ability to promote type I collagen expression by hepatic stellate cells.

Although IL-13 induces type I collagen by the human epithelioid hemangioendothelioma stellate cell line, Li90,[32] we failed in detecting a direct effect of IL-13 on type I collagen expression by the human HSC cell line, LX-2. Thus, by decreasing MMP-1 expression in IL-34-Mφ and M-CSF-Mφ, IL-13 may favor the perpetuation of fibrosis. This hypothesis is supported by the expression of high levels of IL-13[7] and of M-CSF and IL-34 in HCV patients with advanced fibrosis.

Different studies have implicated murine Mφ polarized by IL-4 or IL-13 (also called M2a) in the pathogenesis of fibrosis.[10,11] However, their exact contribution remains controversial. As an example, they express arginase 1, successively described as an anti- and then profibrotic molecule. Notable differences also exist between human and rodent Mφ not only in terms of differentiation processes, but also of phenotypes. Moreover, the mechanisms by which IL-13 up-regulate the profibrotic properties of human and murine Mφ differ. IL-13 up-regulates TGF-β and arginase-1 expression by murine Mφ,[10] but not by human Mφ[9,10,24] (Supporting Fig. 5B). IL-13/IL-4 up-regulate CCL17 and CCL22 expression by murine Mφ[24] and by human GM-CSF-Mφ,[15] but not by human M-CSF-Mφ. Human IL-13-polarized M-CSF/IL-34-Mφ and GM-CSF-Mφ are thus distinct, each subset sharing some features of murine M2a cells, with only M-CSF/IL-34-Mφ exhibiting a profibrotic phenotype.

Accumulating data suggest that newly recruited monocytes, rather than resident Mφ and KCs, perpetuate fibrosis.[33] Blocking monocyte recruitment by targeting CCL2 inhibits liver fibrosis and HSC activation in different models of liver fibrosis.[34-36] Our results suggest that neutralizing the CD115/CD115 ligands axis represents a promising therapeutic approach in liver fibrosis. Because IL-34 and M-CSF are required to unveil the profibrogenic effect of IL-13 on Mφ, it could be more efficient to neutralize IL-34 and M-CSF than IL-13. Nevertheless, because the generation of KCs is dependent on M-CSF[37] we cannot exclude that the neutralization of the CD115/CD115 ligand axis may affect liver function.[38] Because GM-CSF (1)

does not induce profibrogenic Mφ, (2) prevents and reverses the effects of IL-34 and M-CSF on monocytes and Mφ, respectively,[14] and (3) restores the number of KCs in CSF-1-deficient mice,[39] GM-CSF administration may represent an interesting alternative to CD115/CD115 ligand neutralization. On the other hand, neutralization of the CD115/CD115 ligand axis appears of interest in HCC not only to prevent fibrosis,[27] but also to subvert the immunosuppressive properties of tumor-associated Mφ.[14]

The involvement of factors distinct from TGF-β1 and IL-13 has been suspected in the initiation and progression of human liver fibrosis.[7] Indeed, 20% of patients have a liver fibrosis that appears not dependent on TGF-β or IL-13.[7] Our results suggest that M-CSF and IL-34 may represent one of the missing factors in human fibrosis. In support, HBV-infected patients with cirrhosis have higher levels of M-CSF than patients with a chronic asymptomatic HBV carrier.[40] Moreover, M-CSF may participate in lung fibrosis. Patients with idiopathic pulmonary fibrosis have higher levels of M-CSF in bronchoalveolar lavage fluids than healthy subjects. In the model of bleomycin-induced fibrosis, $Csf1^{op}/Csf1^{op}$ mice have less lung fibrosis than wild-type mice.[41] Whether IL-34 and M-CSF also participate in liver fibrosis of other etiologies and fibrosis of other organs remains to be investigated.

In conclusion, this study highlights a central role of IL-34 and M-CSF in liver fibrosis, through the generation of profibrogenic Mφ, and also contributes to explain the profibrotic properties of IL-13. In addition to shedding new light on the puzzling factors involved in human liver fibrosis, these observations open new therapeutic approaches based on liver macrophage repolarization.

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Supporting Information

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