

Original Article

Met-CCL5 modifies monocyte subpopulations during liver fibrosis regression

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Abstract: Fibrosis or scarring of the liver parenchyma is a mainstay of chronic liver diseases and is associated with increased morbidity and mortality. Since complete scarring of the liver develops over several decades, therapeutic intervention with the aim of ameliorating fibrosis is of great clinical interest. In a recent study, we could identify the chemokine receptor antagonist Met-CCL5 as a potential compound to inhibit fibrosis progression and accelerate its regression. In the current study we characterized immune changes during fibrosis regression associated with the treatment with the CCL5 (RANTES) chemokine receptor antagonist Met-CCL5 in an established mouse model of chronic liver damage. Met-CCL5 or PBS was given after fibrosis induction (8 weeks of CCl₄) and mice were sacrificed three and seven days after peak fibrosis. Mouse livers were analyzed for immune cell infiltration and cytokine gene expression. The results show that overall monocyte recruitment was not affected by Met-CCL5, but there was a significant shift to a pro-inflammatory Gr1⁺ monocyte population in the livers of mice treated with Met-CCL5. These monocytes were mostly iNOS⁺, a phenomenon which was also evident when analyzing the overall gene expression profiles in the livers. Since a shift in monocyte subpopulations has recently been identified to contribute to fibrosis regression, our results help explaining the efficacy of CCL5 chemokine antagonism as a novel treatment option for fibrotic liver diseases.

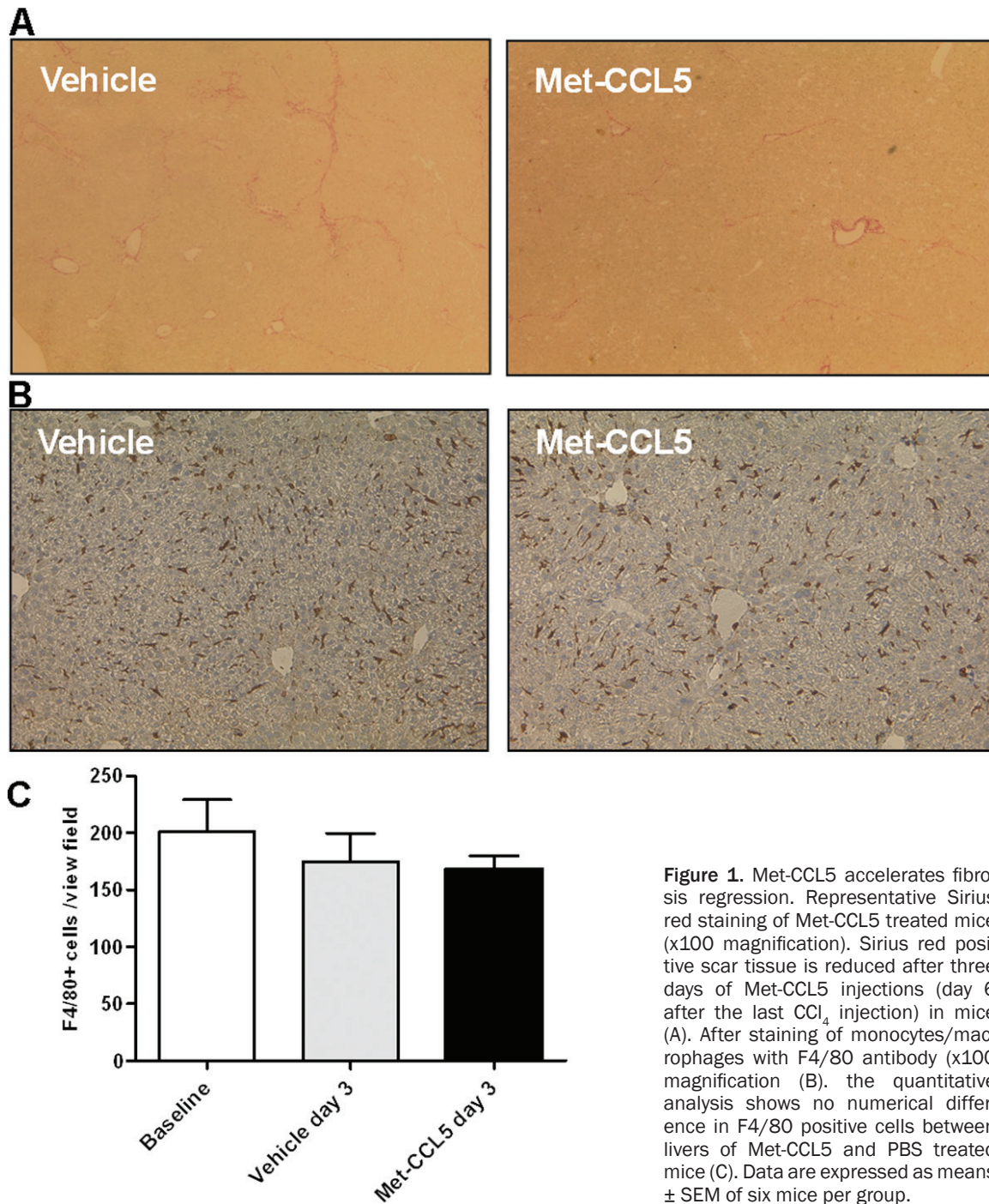
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Introduction

Fibrosis or scarring of the liver is a hallmark of all chronic liver injuries. Progressive fibrosis is responsible for the largely increased morbidity and mortality in affected patients which are mainly due to decompensation of liver cirrhosis and hepatocellular carcinoma [1]. Notably, progressive liver fibrosis develops over several years or decades in most cases and many patients are nowadays identified through screening programs before complete cirrhosis is apparent. From a clinical point of view this implies the possibility for a therapeutic intervention at the stage of fibrosis in order to inhibit or even reverse its further progression [2]. However, although main molecular pathways of fibrogenesis have been identified within the last years, only a few of these investigations have yet led to a pre-clinical trial [3]. Such translational studies have been hampered by

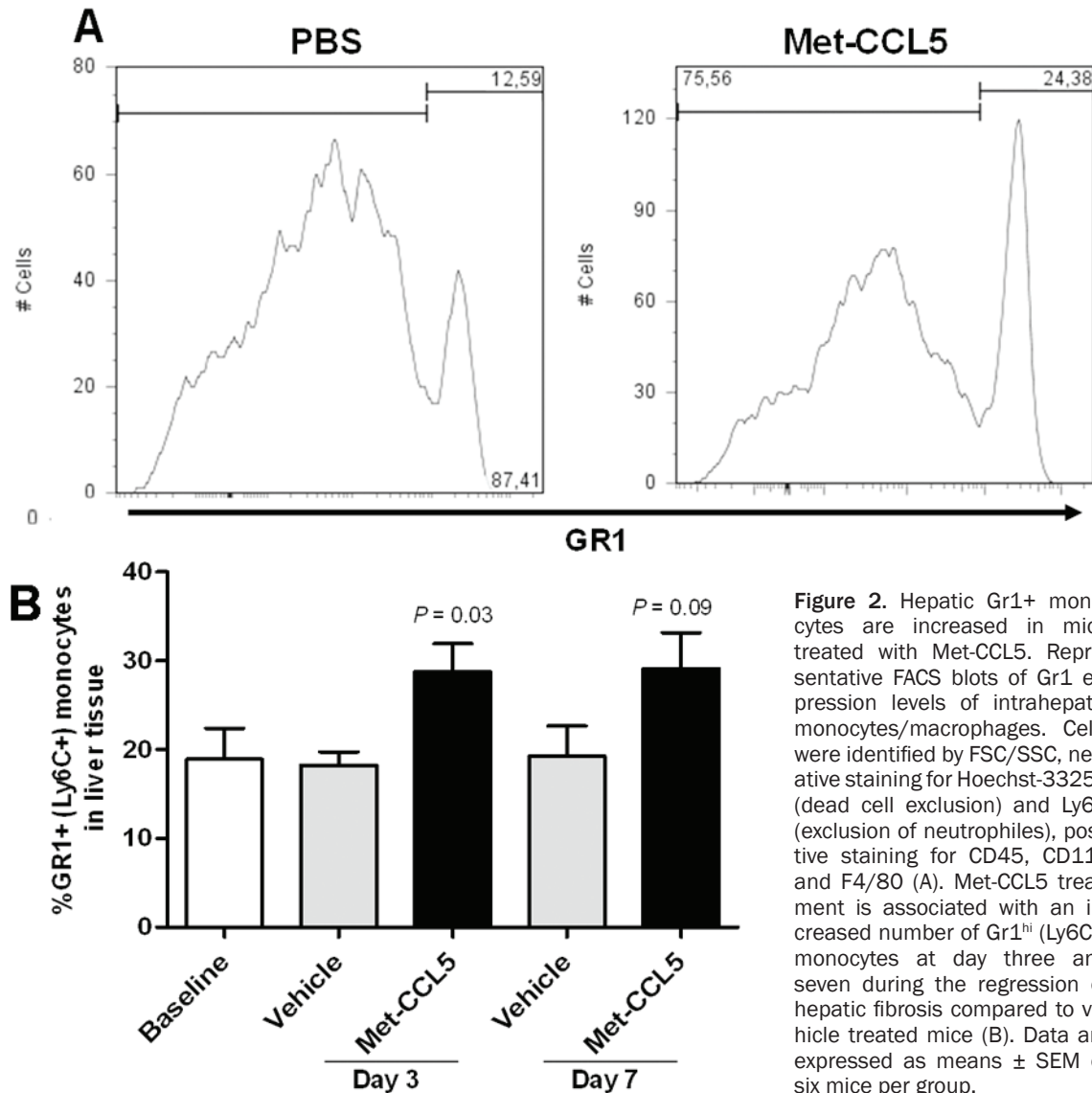
subtle differences in the pathophysiology of liver fibrosis between species (mainly rodents and humans) and the possibility of core versus peripheral fibrosis pathways which are not yet clearly identified [4, 5].

In recent years an important role of chemokines, i.e. small chemotactic cytokines, has been described in different animal models of liver fibrosis. Furthermore, early data also suggests a pathophysiological role of these molecules in human liver diseases [6]. Although being considered as an overall highly redundant system, a few chemokines have been identified which appear to mediate important aspects of fibrogenesis in murine and human liver diseases [7]. One of these chemokines is the pro-inflammatory molecule CCL5 (former name "RANTES": regulated upon activation, normally T-cell expressed and secreted) [8, 9]. In extensive studies, we could characterize the role of



this particular chemokine in different murine models and provide early data for its role in human liver disease [10]. These data are congruent to a pivotal study which investigated the importance of CCL5 receptors in liver fibrosis [11]. In our study we could also show that a competitive antagonist of CCL5 receptors, Met-CCL5 [12], is sufficient to ameliorate the pro-

gression, but also accelerate the regression of murine liver fibrosis [10]. These studies set the stage for the further evaluation of this anti-chemokine strategy as a treatment option to change the natural course of liver fibrosis of different aetiologies. Nevertheless, before advancing to further investigation of this compound, the molecular mechanisms underlying



its efficacy in altering fibrogenesis are warranted. In the current study, we therefore evaluated cellular and molecular aspects of fibrosis regression induced by Met-CCL5.

Material and methods

Murine *in vivo* experiments

C57BL/6 mice were treated with CCl₄ for eight weeks to induce severe fibrosis. At the peak of fibrosis (3 days after the last CCl₄ injection), mice received either Met-CCL5 (10 μ g/mouse) i.p. or vehicle i.p. daily for the next 7 days during regression of liver fibrosis. Mice were sacrificed at day 3, 6 and 10 after the last CCl₄ injection (equivalent to day 0, 3 and 7 of fibrosis regres-

sion). In all animals, we assessed liver fibrosis histologically by quantification of the Sirius red-positive area on high-power fields per slide (magnification, 100x) using NIH ImageJ software. In addition, liver fibrosis was also quantified by the photometric measurement of the amino acid hydroxyproline, which is collagen-specific and has been described previously [10].

RNA-expression analysis of fibrosis- and macrophage-related genes

RNA from total liver tissue was isolated and reverse transcribed using RevertAid™ Premium First Strand cDNA Synthesis Kit (Fermentas). Quantitative RT-PCR was accomplished for *Timp1*, *Col1a1*, *Tgfb1*, *Mmp9*, *IL6*, *IL1b*, *IL10*,

F4/80, iNOS, Arginase1, KC and Ccl2 with SYBR® Green (Invitrogen).

Analysis of hepatic immune cells

Freshly harvested livers were cut into pieces and digested with collagenase type IV at 37°C for 30 minutes. Livers were homogenized and single cells were either stained directly for CD45 and Ly6G or underwent density gradient centrifugation for 20 min at 800g using Lympholyte-H (Cederlane Laboratories). PBMC were collected from the gradient interphase, unspecific binding was blocked with a serum cocktail and leukocytes stained with monoclonal antibodies and appropriate isotype controls for Ly6G (BD Biosciences), F4/80 (Serotec), iNOS (SantaCruz), CD45, CD3, CD4, CD8, CD45, CD206, Gr-1 or TNFa (all eBioscience). Calibrate APC beads (BD) were used as internal standard for determination of absolute cell numbers and Hoechst 33258 was added to exclude dead cells [13]. Cells were analyzed on a FACS-Cantoll (BD) and data were analyzed using FlowJo (TreeStar).

Results

Met-CCL5 accelerates fibrosis regression but does not influence overall monocyte counts within the liver

We first confirmed our prior results that Met-CCL5 is able to accelerate the resolution of scar tissue when given after peak fibrosis development, i.e. starting three days after the last CCl₄ injection. As depicted in **Figure 1A**, Sirius red positive scar tissue was reduced after three days of Met-CCL5 injections (day 6 after the last CCl₄ injection) in mice. This difference in scar tissue expression between Met-CCL5 and PBS injected animals was also true seven days after peak fibrosis, confirming our recently published data [10]. As monocytes have been identified as key cell populations during the progression and resolution of liver fibrosis [13], we next evaluated whether the difference between the Met-CCL5 and the PBS group was due to differences in the overall number of monocytes/macrophages within the liver. However, upon immunohistochemical evaluation and quantitative analyses of representative liver sections, there was not numerical difference in F4/80 positive cells between Met-CCL5 and PBS treated mice (**Figure 1B** and **1C**).

The number of pro-inflammatory Gr1+ monocytes is increased in mice treated with Met-CCL5 during fibrosis regression

Recent work has identified two main subpopulations of monocytes with different biological functions which are characterized by a high versus low expression of the lineage marker Gr1 (also known as Ly-6C/G) [13, 14]. Since the overall number of monocytes was not apparently different in our animal model, we assessed whether the distribution of Gr1+ cells is changed during Met-CCL5 treatment. Representative FACS blots are shown in **Figure 2A**. Indeed, the number of Gr1+ cells was strongly increased in animals injected with Met-CCL5 during fibrosis resolution. This difference was true for animals treated for three or seven days after peak fibrosis (**Figure 2B**).

The number of iNOS positive monocytes is increased during fibrosis resolution

A characteristic biological feature of pro-inflammatory monocytes is their increased expression of inducible nitric oxide synthetase (iNOS) [13]. In order to further characterize the role of this cell population in Met-CCL5 induced fibrosis regression, we evaluated the number of iNOS positive monocytes within the livers of our animals during fibrosis regression. Again representative blots are shown in **Figure 3A** and the overall quantification of iNOS positive cells three and seven days after peak fibrosis in **Figure 3B**. Indeed, Met-CCL5 treatment was associated with an increased number of iNOS positive intrahepatic monocytes/macrophages at both time points during the regression of liver scarring compared to PBS treated mice.

Overall cytokine expression is also altered by Met-CCL5 treatment

After having observed differences in monocyte subset infiltration during fibrosis regression, we next evaluated whether there are also overall differences in cytokine gene expression induced by Met-CCL5 treatment. The results are shown in **Figure 4**. Indeed, *iNos* gene expression in whole liver was significantly increased at day three in mice treated with Met-CCL5 compared to animals injected with PBS ($P = 0.04$), confirming the monocyte data described above. In contrast, mRNA expression levels of markers for anti-inflammatory cell sub-

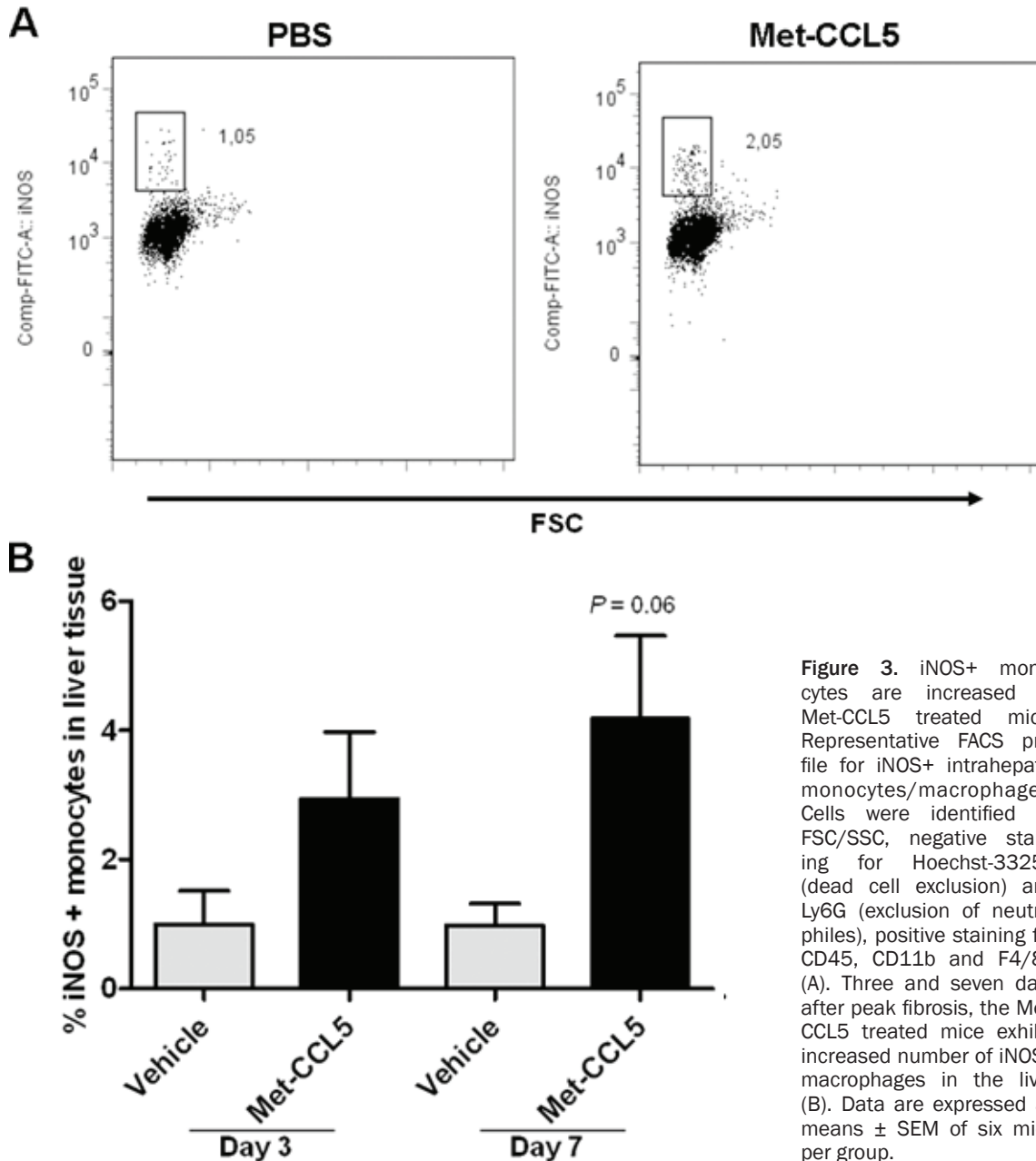


Figure 3. iNOS⁺ monocytes are increased in Met-CCL5 treated mice. Representative FACS profile for iNOS⁺ intrahepatic monocytes/macrophages. Cells were identified by FSC/SSC, negative staining for Hoechst-33258 (dead cell exclusion) and Ly6G (exclusion of neutrophils), positive staining for CD45, CD11b and F4/80 (A). Three and seven days after peak fibrosis, the Met-CCL5 treated mice exhibit increased number of iNOS⁺ macrophages in the liver (B). Data are expressed as means $\pm$ SEM of six mice per group.

sets, i.e. *Il10* and *Tgfb1*, were markedly reduced in Met-CCL5 treated mice ($P = 0.03$ and $P = 0.08$, respectively), confirming that this CCL5 chemokine receptor antagonist affected the immune phenotype during fibrosis resolution.

Discussion

The current study was designed to further characterize molecular mechanisms underlying our observation that the chemokine receptor antagonist Met-CCL5 is able to accelerate the

regression of liver fibrosis in a well established animal model. From a clinical point of view, novel treatment options for inhibition or even resolution of liver fibrosis are of great interest as chronic liver diseases are an increasing cause of morbidity and mortality worldwide. For this reason, the compound tested in the current study might be of great interest as it antagonizes murine as well as human chemokine receptors CCR1 and CCR5 [12, 15]. Both receptors are cognate receptors for the chemokine CCL5 and have been involved in the progres-

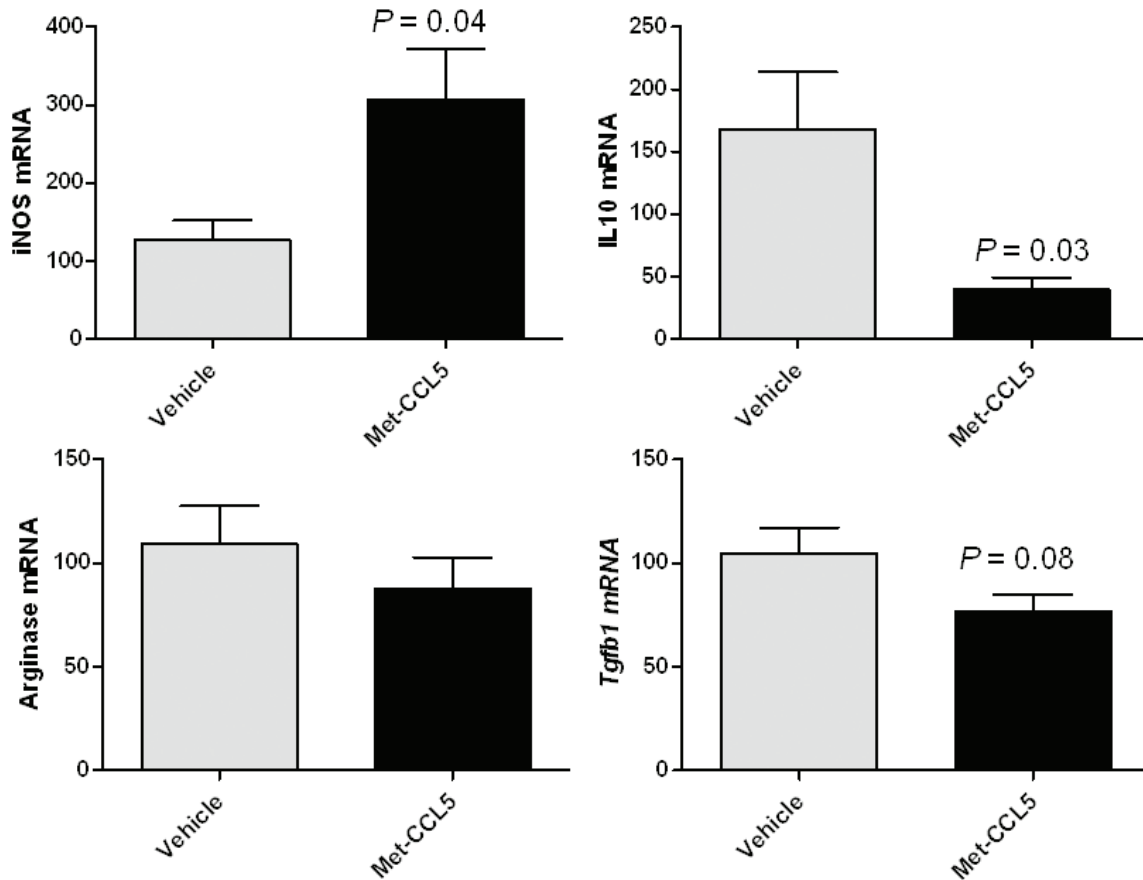


Figure 4. Cytokine expression is altered by Met-CCL5 treatment. Hepatic gene expression of iNOS was significantly increased at day three in mice treated with Met-CCL5 compared to animals injected with PBS. In contrast, mRNA expressions of markers for anti-inflammatory cell subsets such as IL10 and Tgfb1 were markedly reduced in Met-CCL5 treated mice. Expression of Arginase, as typical marker of alternatively activated macrophages, did not vary. Data are expressed as means $\pm$ SEM of six mice per group.

sion of liver scarring [11]. Thus, the results of the current study might also be helpful for translational studies designed to inhibit fibrogenesis in early clinical trials [4].

The main finding of our study was that Met-CCL5, when given during the resolution phase of liver fibrosis, did alter the phenotype of the immune response within the liver. Specifically, we observed a shift in monocyte subsets towards a predominance of Gr1+, iNOS+ monocytes in Met-CCL5 treated mice compared to control mice treated with PBS. Interestingly, there was only a qualitative change in monocyte subsets since the overall number of monocytes/macrophages did not significantly change by immunohistochemical analysis.

In earlier studies, Gr1+ (Ly6C+) monocytes have been identified as the main fibrogenic cell

type within the liver by their ability to directly stimulate hepatic stellate cells, the main extracellular matrix-producing cell type within the liver [13, 16]. However, the role of this specific cell type in fibrosis resolution has not yet been systematically analysed [17]. Nevertheless, there is some indirect evidence that this monocyte subpopulation might be important in scar removal as well. The main chemokine receptor for recruitment of these cells is CCR2. Notably, while animals deficient in this particular chemokine receptor are less prone to progressive liver fibrosis, the absence of this receptor was associated with a persistence of hepatic injury [18] in the same animal model as in the current study. In accordance with our data, it might thus be hypothesized that the inability of CCR2 knockout mice to recruit Gr1+ monocytes into the liver during fibrosis resolution might be the main factor for the persistence of liver scarring.

In line, a very recent study, by using adoptive transfer and in situ labelling experiments, also identified restorative macrophages which derive from recruited Ly-6C(hi) (Gr1+) monocytes [19]. Thus, there is clear evidence that Gr1-high monocytes are a central cell population during the regression phase of liver fibrosis.

However, the question remains unanswered how application of Met-CCL5 to mice during fibrosis resolution might shift the monocyte population to a Gr1+ predominant phenotype. Met-CCL5 is not known to directly antagonize CCR2. However, many immune cells, including monocytes, do express a variety of chemokine receptors depending on the milieu and the activation status of the particular cell [20]. Thus, antagonism of CCR5 and CCR1 might have led to the up-regulation of other chemokine receptors, including CCR2, what might then trigger the preferential recruitment of certain monocyte subsets. Importantly, Gr1^{lo} (Ly6C^{lo}) monocytes have been described to express the chemokine receptor CCR5, and pharmacological antagonism of CCR5 affected the recruitment of the subpopulation in murine models of atherosclerosis [21]. Thus, the relative increase of Gr1^{hi} monocytes upon Met-CCL5 in fibrosis regression might be related to a selective inhibition of the Gr1^{lo} subtype. However, whether this is only true for resolution of fibrosis but not its progression remains unanswered at the current stage and would be beyond the scope of the current report.

In summary, we herein describe plausible cellular mechanism by which the chemokine receptor antagonist CCL5 accelerates the regression of established liver fibrosis. These results might further guide the early clinical evaluation of chemokine antagonistic strategies in subjects with chronic liver diseases.

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Conflict of interest

The authors declare no conflict of interest.

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