

The Role of Mesenchymal Stem Cells and Imatinib in the Process of Liver Fibrosis Healing Through *CCL2-CCR2* and *CX3CL1-CX3CR1* Axes

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Abstract

Background: Persistent liver damage contributes to the development of liver fibrosis, marked by an accumulation of extracellular matrix. Macrophages play a pivotal role in this process, with the *CCL2-CCR2* and *CX3CR1-CX3CL1* axes serving as key regulators of macrophage recruitment, liver infiltration, and differentiation. In this study, utilizing a rat model of carbon tetrachloride (CCL4)-induced liver fibrosis, we aimed to investigate the impact of imatinib and bone marrow-derived mesenchymal stem cells (BM-MSCs) on the expression of these axes.

Methods: Sixteen Sprague-Dawley rats were divided into four groups: healthy, liver fibrosis, imatinib-recipient, and BM-MSC-recipient. Treatment effects were evaluated using histopathology and Sirius-red staining. Quantitative real-time PCR was employed to analyze changes in the expression of the genes *CCL2*, *CCR2*, *CX3CL1*, and *CX3CR1*.

Results: Histopathological assessments revealed the efficacy of imatinib and BM-MSCs in mitigating liver fibrosis. Our findings demonstrated a significant reduction in *CCL2* and *CCR2* expression in both imatinib and BM-MSCs treatment groups compared to the liver fibrosis group. Conversely, the gene expression of *CX3CL1* and *CX3CR1* increased in both therapeutic groups compared to the liver fibrosis groups.

Conclusion: Conclusion: The notable decrease in *CCL2-CCR2* genes in both therapeutic groups suggests that BM-MSCs and imatinib may contribute to a decline in inflammatory macrophages within the liver. The lower *CCL2-CCR2* expression in imatinib-recipient rats indicates better efficacy in modulating the recruitment of inflammatory macrophages. The elevated expression of *CX3CL1* in BM-MSC-recipient rats suggests a greater impact on the polarization of LY6Chigh (inflammatory) to LY6Clow (anti-inflammatory) macrophages, warranting further investigation.

Keywords: *CCL2*, *CCR2*, *CX3CL1*, *CX3CR1*, Liver fibrosis.

Introduction

Hepatic fibrosis is a concerning health problem worldwide, observed in the patients with chronic liver diseases (CLDs) (1–3). The biological mechanism underlying the activation of hepatic stellate cells (HSCs), disruption of liver function, and accumulation of extracellular matrix proteins (ECM) and collagen type III and I, which further results in cirrhosis and hepatocellular carcinoma (HCC), as a result of the perpetual wound healing process caused by CLDs (4,5).

The inflammatory pathways and immune cells play a crucial role in the initiation and

maintenance phases of hepatic fibrosis. Kupffer cells and macrophages derived from monocytes are the two primary liver immune cells that promote liver inflammation. Kupffer cells (KCs), which are liver-dwelling macrophages, are activated upon inflammatory stimuli, and produce various pro-inflammatory cytokines and chemokines (6-8). For instance, KCs release chemokine monocyte chemoattractant protein 1 (*CCL2*) (GENE ID: 24770), known as Monocyte chemoattractant protein-1 (MCP-1), which attracts LY6Chigh monocytes through *CCR2* (GENE ID: 60463) receptors on

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LY6C^{high} monocytes surface (9-11). The well-known master regulator of hepatic fibrosis, LY6C^{high} monocytes differentiate into LY6C^{high} macrophages upon recruitment to the liver, which then cause the activation of HSCs and their trans-differentiation to myofibroblasts by secreting TGF-beta (TGF-) and platelet-derived growth factor (PDGF) (7,10,11).

CX3C chemokine ligand 1 (CX3CL1) (GENE ID: 89808), known as fractalkine, is an anti-inflammatory chemokine that serves as a protector against hepatic fibrosis by regulating macrophage infiltration, survival, and limits the differentiation of LY6C^{high} monocytes to LY6C^{high} macrophages as inflammatory macrophages in the liver (12). Additionally, CX3CL1 encourages the development of LY6C^{high} to LY6C^{low} macrophages, which help to resolve liver fibrosis by causing apoptosis in activated HSCs and degrading ECM via the release of matrix metalloproteinases 12 (MMP-12) and 13 (MMP-13). CX3CL1 receptor (CX3CR1)-deficient mice (GENE ID: 171056) showed decreased numbers of monocyte-derived macrophages and increased level of fibrosis in carbon tetrachloride (CCl4)-induced liver inflammation mice (13). Moreover, a correlation between serum CX3CL1, and quantitative serum fibrosis markers was observed in patients with liver fibrosis, indicating that serum level of CX3CL1 was increased consistent with the progression of hepatic fibrosis (12).

Thus, CX3CL1- CX3CR1 axes could be considered a great target for fabricating novel drug approaches as modulators of liver fibrosis.

Since their anti-fibrotic effect has already been discussed (15,16), imatinib and bone marrow-derived mesenchymal stem cells (BM-MSCs) are two prospective treatment modalities for treating liver fibrosis. Imatinib is a selective tyrosine kinase inhibitor (TKI) with the great potential of inhibiting both PDGF, and TGF- β pathways, which play a major role in fibrosis progression (14). Moreover, several studies indicated that BM-

MSCs could provide anti-fibrotic effects on the damaged liver by induction of apoptosis, and limiting the activation of HSCs, attenuating the fibrogenesis process, and elevated degradation of ECM (15,16). The goal of the present investigation was to examine the possible effects of imatinib and BM-MSCs on the CCL2-CCR2 and CX3CR1-CX3CL1 axis as prospective therapeutic strategies for liver fibrosis.

Materials and Methods

Experimental Animals

16 Male Sprague-Dawley rats, weighing 200 \pm 60 g and 8-10 weeks old, in groups of four, were placed in cages covered with woodchip. Rats were housed one week in defined standard living conditions (room temperature with access to adequate pellet food, water, and light illumination cycles of 12 h/day) before the commencement of experiments. After induction of liver fibrosis, the groups were defined as the phosphate-buffered saline (PBS)-treated group (healthy group), BM-MSCs-recipient group, imatinib-recipient group, and CCL4-treated group (fibrotic experimental group).

Liver fibrosis induction

To provoke liver damage in rats, CCL4 (1 ml/kg, 99% purified, Sigma, UK) was solved in olive oil with the ratio of (1:1). The CCL4 solution was injected intraperitoneally (IP) twice weekly for six weeks. At the same time, an equal volume of PBS was administered to the healthy group. After the 6th week, all the rats were received once-weekly administration of CCL4 or PBS as an experimental reminder. Following the last PBS or CCL4 injection, rats were euthanized after 24 h by carbon dioxide (CO₂) narcosis.

Treatment with imatinib and MSCs

BM-MSCs isolation and characterization were described completely in our previously reported articles (17,18). Rats in the BM-MSCs-recipient group received approximately 1.5 \times 10⁶ BM-MSCs via intravenous (iv) injection only once at the beginning of the

treatment. The imatinib-recipient group was administered imatinib (Osve Pharmaceutical Co, Iran) (20 mg/kg) orally by gavage daily during the 6 weeks treatment period.

Histopathological assessment

Liver tissues were immediately sectioned after euthanization and were divided into two serial sections. One part of the divided sections was stained with hematoxylin and eosin (H&E) to indicate the alternate hepatic histological features. Masson's Trichrome and Sirius-red were applied in order to demonstrate collagen deposition.

RNA extraction and complementary DNA synthesis

Total RNA was collected from liver tissue using the RNA extraction kit (Favorgen, Taiwan) according to the manufacturer's protocol. The concentration and purity of RNA were evaluated using a NanoDrop spectrophotometer (Biotek Instruments,

Winooski, VT, USA). Subsequently, Extracted RNA was reverse-transcribed using random hexamer and oligo (dT) through high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific).

Gene expression analysis

Quantitative real-time PCR was carried out on the Rotor-Gene 6000 instrument® (Qiagen AG Hilden, Germany) using an SYBR Green-based PCR Kit. Specific primers for *CCL2*, *CCR2*, *CX3CL1*, and *CX3CR1* were designed for each gene are listed in Table 1. Samples were amplified under the following conditions: 95 °C for 10 m, followed by 40 cycles of 95 °C for 20 s, 58-61 °C (*CX3CR1*: 58 °C, *CCL2*: 58 °C, *CCR2*: 60 °C, *CX3CL1*: 61 °C) for 40 s. The experiments were applied in duplicate independently. The expression level of each gene was normalized by *HPRT* as an internal control. The relative quantity of gene expressions was analyzed using REST 2009 software version 2.0.13.

Table 1. Specific primer used for expression analysis.

Gene		Sequence 5'→ 3'	Product length
<i>CCR2</i>	Forward	AAAAGAGGCATAGGGCTGTGA	143 bp
	Reverse	TAAGTGCATGTCAACCACACA	
<i>CCL2</i>	Forward	AGTTAATGCCCCACTCACCT	128 bp
	Reverse	GCTTGGTGACAAATACTACAGCT	
<i>CX3CR2</i>	Forward	TGCTCAGGACCTCACCAT	102 bp
	Reverse	GCCACGATGTCAACCAAAT	
<i>CX3CL2</i>	Forward	CATCATCCTGGAGACGAGAC	145 bp
	Reverse	CACATTGTCCACACGCTTCT	
<i>HPRT</i>	Forward	GACCGTTCTGTCATGTCG	132 bp
	Reverse	ACCTGGTTCATCATCACTAATCAC	

Statistical analysis

Prism software, a statistical package, version 5 (GraphPad Software, USA) was utilized for data analyzing and visualization. Statistical analysis was performed using the student's t-test. Data were presented as the mean and standard error of the mean (S.E.M) and the significance level was considered as (P< 0.05).

Results

Histopathological assessment verified the anti-fibrotic potential of imatinib and BM-MSCs

The livers were removed at the conclusion of the therapy period for histological analysis. Through H&E staining, it was possible to see that the CCL4-treated group had liver fibrosis due to distorted lobular architecture, binuclear

hepatocytes and fatty alterations, portal inflammation, and a dilated central vein. The healthy groups (PBS recipients) exhibited the usual cellular architecture, including spherical single vesicular nuclei hepatocytes inside the central vein, absence of portal inflammation, and the usual lobular pattern. Imatinib and BM-MSc recipients showed considerable improvements in the structural pattern of the lobules compared to the CCL4-induced liver fibrosis group in terms of both portal inflammation and lobular pattern.

Furthermore, the CCL4 group displayed prolonged fibrous septa whereas Masson's trichrome staining of the negative (PBS) control group did not show any fibers surrounding the portal region. In compared to the CCL4 group, fibrous growth surrounding the portal region was less in the treatment groups. Sirius-red staining successfully confirmed the findings from Masson's Trichrome staining by detecting collagen deposition as histologic indicators of liver fibrogenesis (Fig. 1).

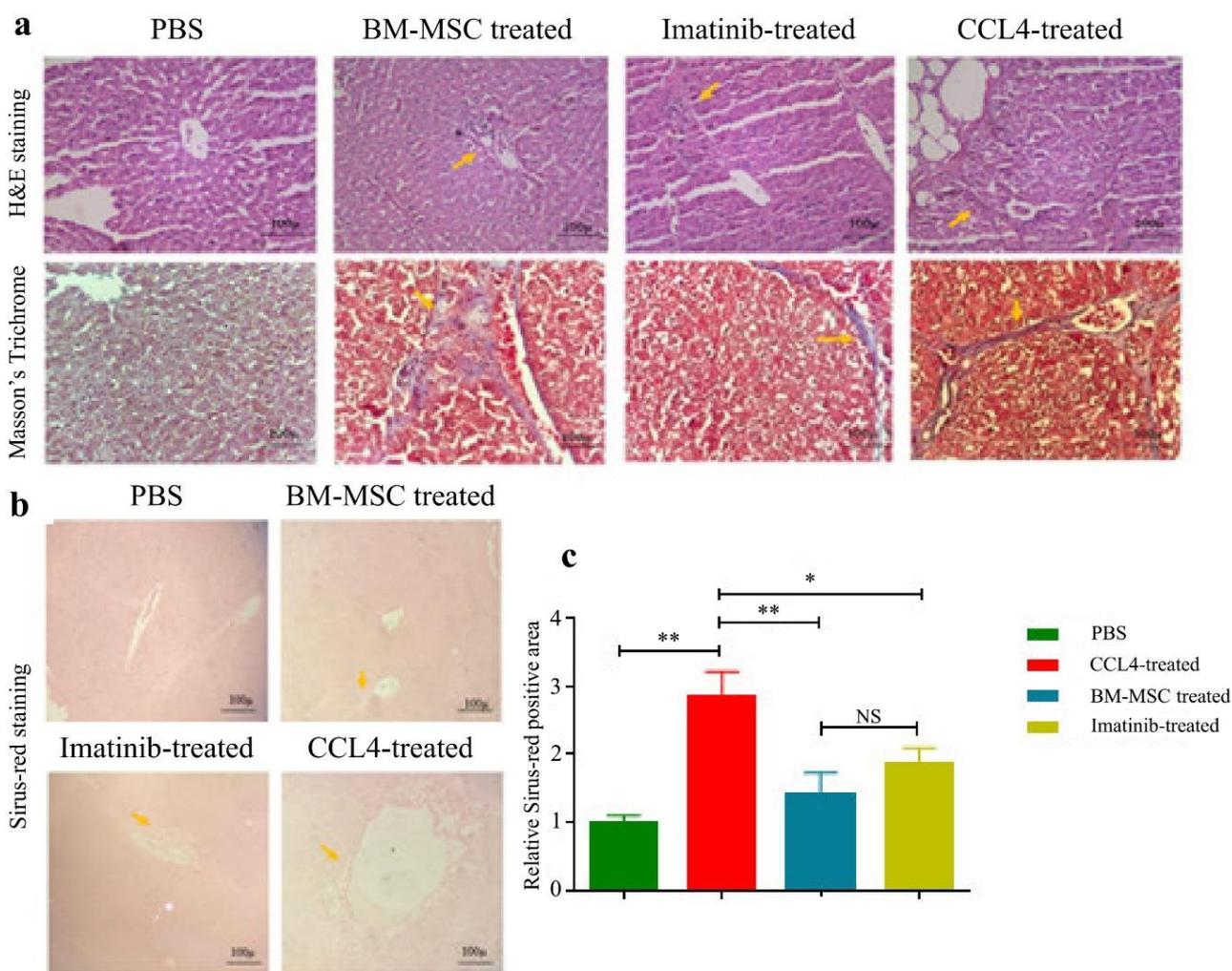


Fig. 1. Histopathological examination of fibrosis progression. **a)** Histopathological assessment of liver structure with H&E staining and Masson's trichrome (Magnification 200x). **b)** The collagen repositioning was demonstrated according to the Sirius-red staining. (Magnification 200x). Yellow arrows indicate collagen fibers. **c)** Sirius-red positive area quantified by ImageJ software compared to the PBS group. Student t-test was used for statistical analysis (*P < 0.01, **P < 0.001, and NS; not significant). (PBS, CCL4, BM-MSCs, imatinib are demonstrating of the experimental groups).

Alternate expression of CCL2-CCR2 axes in both imatinib and BM-MSCs treatment group

The expressions candidate genes (*CCL2* and *CCR2*) were evaluated in rat liver samples after treatment with imatinib and BM-MSCs. The results were compared to CCL4-induced liver fibrosis group. The expression of the *CCL2* and *CCR2* genes was elevated in CCL4-induced rats, as shown in Figures 2a and 2b. The results of qRT-PCR demonstrated that the expressions of both genes were significantly decreased in imatinib (*CCL2*: $P < 0.0001$ and *CCR2*: $P < 0.00001$), and BM-MSCs (*CCL2*: $P < 0.01$ and *CCR2*: $P < 0.0001$) treated groups than CCL4-induced liver fibrosis rats.

However, the expression levels in the imatinib-treated group were slightly lower than in BM-MSCs-treated group.

Increased expression of CX3CL1-CX3CR1 axes in both imatinib and BM-MSCs treatment group

The expression level of *CX3CL1* and *CX3CR1* genes as anti-fibrotic genes showed that treatment with BM-MSCs and imatinib results in upregulation of both genes (BM-MSC: $P < 0.0001$ and imatinib: $P < 0.01$) compared to CCL4-induced liver fibrosis rats (Fig. 3a). Imatinib-treated rats showed higher expression of *CX3CR1*, but the upregulation was not statistically significant for *CX3CL1* gene (Fig. 3b).

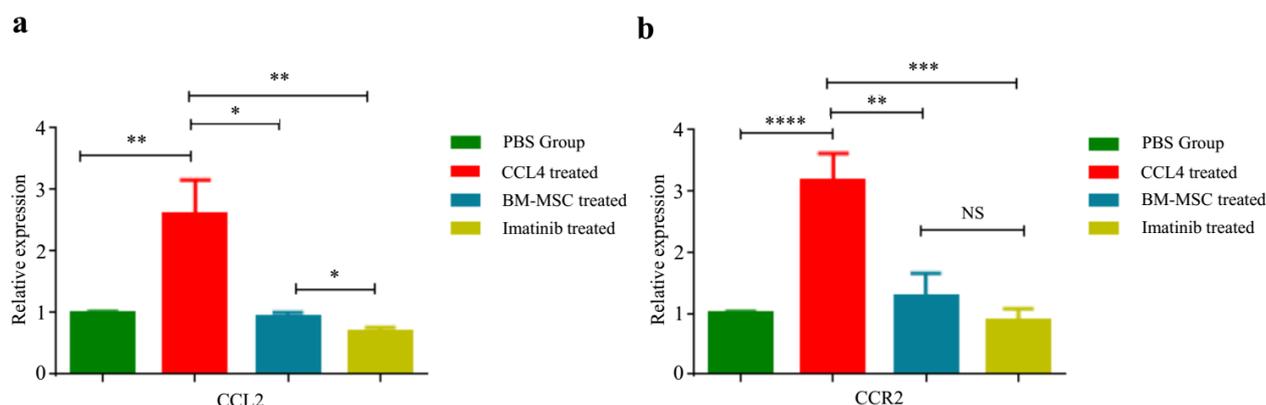


Fig. 2. Expression analysis of CCL2-CCR2 axis genes. (a) *CCL2* expression was dramatically upregulated after provoking the liver fibrosis using carbon tetrachloride. Treatment separately with BM-MSC and imatinib resulted in decreased expression of *CCL2*. (b) Similar to *CCL2*, *CCR2* showed significant decline in expression after treatment with BM-MSC and imatinib. Bars are indicating the mean + S.E.M of three independent experiments ($n = 4$ in each group). Student t-test was used for statistical analysis (* $P < 0.01$, *** $P < 0.0001$, and **** $P < 0.00001$, ns; not significant). (PBS, CCL4, BM-MSCs, imatinib are demonstrating of the experimental groups).

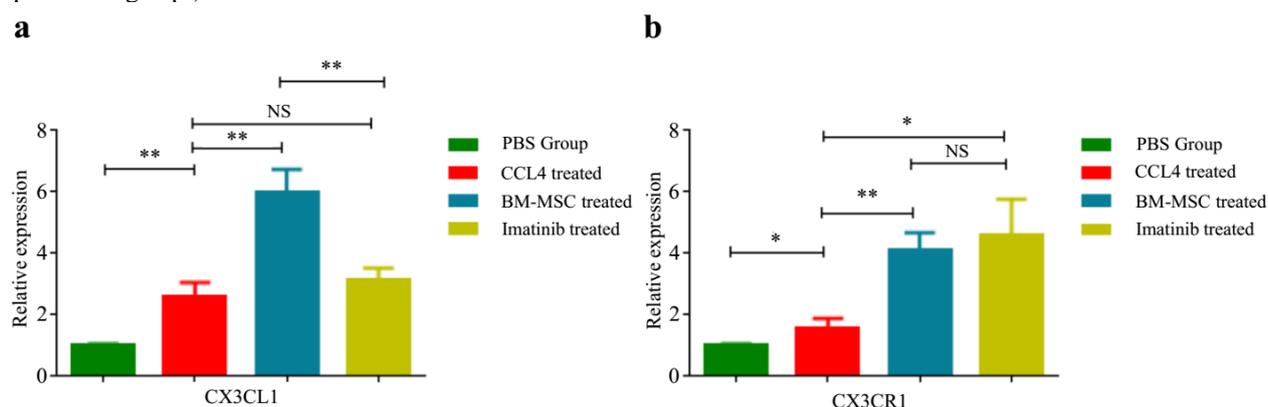


Fig. 3. Expression analysis of CX3CL1-CX3CR1 axis genes. (A) *CX3CL1* expression was notably increased upon treatment with BM-MSCs but moderately increased in imatinib treated samples. (B) *CX3CR1* expression was significantly increased in both therapeutic groups comparing to CCL4 only treated group. Bars are indicating the mean + S.E.M of three independent experiments ($n = 4$ in each group). Student t-test was used for statistical analysis (* $P < 0.01$, *** $P < 0.0001$, and **** $P < 0.00001$, ns; not significant). PBS, CCL4, BM-MSCs, imatinib are demonstrating of the experimental groups.

Discussion

Liver inflammation triggered by sustained liver damage is the main cause of liver fibrosis and ultimately cirrhosis and HCC (19). The reversible intrinsic of liver fibrogenesis trends a path towards developing therapeutic approaches leading to amelioration, and further resolution of hepatic fibrosis (20).

To date, extensive efforts were made to develop an efficient therapeutic strategy to circumvent the global health problem caused by liver fibrosis. Among these strategies, MSCs, especially BM-MSCs and imatinib have shown encouraging results (17,21–25).

The histopathological investigations, as the golden test of prognosis hepatic fibrosis showed a decrease in the progression of liver fibrosis in imatinib and BM-MSCs-recipient groups. These results were in line with previous studies, that showed the effects of imatinib (14) and BM-MSCs on the treatment of liver fibrosis. The great anti-fibrotic potential of imatinib is via the inhibition of platelet-derived growth factor (PDGF) receptors (26). BM-MSCs demonstrated anti-fibrotic effects via modulating HSC activation and suppressing collagen deposition in the damaged liver (27).

Understanding the comprehensive biological consequences of desired therapies on the reduction of liver fibrosis requires further studies. This understanding not only provides insights into the pathways, which are affected by BM-MSCs and imatinib treatment, but also lays the foundation for designing a better strategy for improving anti-fibrotic effects of therapeutical approaches.

By releasing different chemokines, liver immune cells, in particular macrophages, play a crucial role in the formation and progression of liver fibrosis. The *CCL2-CCR2* and *CX3CL1-CX3CR1* axes are among the most efficient regulators of liver fibrosis, according to an increasing body of research (7,10,12). Several attempts were made to pharmacologically inhibit *CCL2-CCR2* axis indicating that its inhibition decreases the level of liver inflammation and fibrosis (28,29). We

examined the expression level of the *CCL2* and *CCR2* genes in both imatinib, and BM-MSCs-recipient groups to see if the desired therapies affect *CCL2-CCR2* axes.

Our results were the indicative of suitable regression of *CCL2* and *CCR2* expression, upregulated after *CCL4* provocation of liver fibrosis, down to approximately their normal levels following treatment by imatinib and BM-MSCs. This data is representing that imatinib and BM-MSCs may possess the capacity to decrease the level of liver inflammation and decrease the monocyte infiltration to the liver. The imatinib had a better performance in significantly decreasing the expression level of *CCL2* and *CCR2* genes compared to BM-MSCs.

The *CX3CL1-CX3CR1* axis is a notable regulator of liver fibrosis as well. This axis plays a crucial function in preventing both the influx of monocytes into the liver and the polarization of LY6C^{high} macrophages into restorative LY6C^{low} macrophages (14), acting as a negative regulator of liver fibrosis. After receiving either imatinib or BM-MSCs, *CX3CL1* and *CX3CR1* expression was found to be upregulated, suggesting that the fibrotic phenotype has been improved. A recent study showed that there is a positive correlation between increased *CX3CL1* plasma level of newly diagnosed chronic myelogenous leukemia (CML) patients after 12 months of treatment with imatinib (30). These data suggest that imatinib actively has a great potential in activating immune responses.

Moreover, BM-MSCs increased the expression of *CX3CL1* statistically much higher than imatinib which could imply the fact that BM-MSCs trigger the activity and polarization of LY6C^{low} macrophages more than imatinib. Moreover, it brings about the idea that the effect of BM-MSCs on this axis should be more studied in the future to unravel its mechanism of action for further use in the clinical section.

Similar to our findings, a recent study showed that the administration of the Fuzheng

Huayu recipe, a locally developed medicine, improved liver fibrosis by implementing the biological function of *CX3CL1* and *CCL2* chemotaxis (31). Thus, developing therapeutic options, which can efficiently and selectively target these axes, should be considered as a great approach for tackling the treatment of liver fibrosis. Our results clearly indicated that imatinib and BM-MSCs have a notable effect on modulating inflammatory pathways of hepatic fibrosis via suppression of *CCL2-CCR2* axis and activation of the *CX3CL1-CX3CR1* axis.

In conclusion, we showed that oral imatinib treatment and intravenous delivery of BM-MSCs result in decreased expression levels of *CCL2* and *CCR2*, which are critical regulators of the migration of inflammatory macrophages to the liver in rats with CCL4-induced liver fibrosis. In parallel, the expression of *CX3CL1* and *CX3CR1* notably increased indicating that the therapeutic approaches effect on upregulation of this axis to promote the polarization of LY6C^{low} leading to the resolution of hepatic fibrosis. In general, although both mesenchymal stem cells, and imatinib appear to be effective in ameliorating fibrosis, the TKI-receiving group

has been better able to activate non-inflammatory pathways.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Ethics approval

Ethics Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran) approved all experimental procedures. (Code of Ethics: IR.SBMU.MSP.REC.1396.269).

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