Mesenchymal stem cells (MSC) therapy has become a promising treatment for liver fibrosis due to its predominant immunomodulatory performance in hepatic stellate cell inhibition and fibrosis resolution. However, the cellular and molecular mechanisms underlying these processes remain limited. In the present study, we provide insights into the functional role of bone marrow-derived MSCs (BM-MSCs) in alleviating liver fibrosis by targeting intrahepatic Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> macrophage subsets in a mouse model. Upon chronic injury, the Ly6C<sup>hi</sup> subset was significantly increased in the inflamed liver. Transplantation of BM-MSCs markedly promoted a phenotypic switch from pro-fibrotic Ly6C<sup>hi</sup> subset to restorative Ly6C<sup>lo</sup> subpopulation by secreting paracrine and apoptotic pathways.

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**INTRODUCTION**

Hepatic fibrosis is a dynamic process characterized by excessive accumulation of extracellular matrix (ECM), mainly fibroblastic collagens resulting from ongoing chronic liver injury and inflammation of many etiologies [1]. Activation of hepatic stellate cells (HSCs) into proliferative and fibrogenic myofibroblasts is well established as a central driver of liver fibrogenesis in experimental and human hepatic injury [2, 3]. This process is regulated by numerous extracellular signals from various resident and inflammatory cells, among which bone marrow monocyte-derived macrophages are the major modulators [4]. In mice, the circulating monocytes derived from bone marrow are composed of at least Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> subsets. These two subsets are characterized by distinct CX3CR1<sup>hi</sup>CCR2<sup>−</sup>Ly6C<sup>hi</sup> and CX3CR1<sup>lo</sup>CCR2<sup>−</sup>Ly6C<sup>lo</sup> phenotypes [5–7]. Two functional equivalents with CD14<sup>hi</sup>CD16<sup>lo</sup> and CD14<sup>−</sup>CD16<sup>hi</sup> phenotypes were preliminarily identified in humans [8–10]. In response to injury, the Ly6C<sup>hi</sup> monocytes quickly respond to inflammatory signals and migrate to the inflamed liver, wherein they differentiate into macrophages. The intrahepatic Ly6C<sup>hi</sup> macrophages are highly inflammatory and fibrogenic, whereas the Ly6C<sup>lo</sup> cells are considered alternative macrophages that can dampen inflammation and diminish liver fibrosis [11]. Ly6C<sup>hi</sup> macrophages promote HSC activation by producing a variety of cytokines and chemokines, including TGF-β, PDGF, TNF-α, IL-1β, MCP1, CCL3, and CCL5 [4]. Ly6C<sup>lo</sup> macrophages promote fibrosis resolution by secreting matrix metalloproteinases (MMPs) such as MMP12 and MMP13 and upregulating TNF-related apoptosis-inducing ligand (TRAIL) that induces HSC apoptosis [12]. The Ly6C<sup>hi</sup> monocytes/macrophages express high levels of CCR1 and CCR2, which promote the infiltration and accumulation of Ly6C<sup>hi</sup> cells in the inflamed liver [13]. Instead, Ly6C<sup>lo</sup> monocytes/macrophages display enhanced expression of CX3CR1, which inhibits inflammatory properties in macrophages by binding its ligand CX3CL1 [14].

Although mechanisms underlying hepatic fibrogenesis have been increasingly clarified, the development of effective therapies for the regression of fibrosis remains a challenge. In the past decade, transplantation of exogenous bone marrow-derived mesenchymal stem cells (BM-MSCs) has become a promising cytotherapy for treating liver fibrosis due to the predominant role of BM-MSCs in HSC inhibition and fibrosis resolution [15, 16]. Multiple immunomodulatory factors secreted from BM-MSCs were found to inhibit proliferation or induce apoptosis of HSCs, including IL-10, HGF, TGF-β, TNF-α, PGE-2, IDO, and NO [17–19]. Additionally, our previous studies found that BM-MSCs attenuate fibrosis by inhibiting Delta-like1...
expression and paracrine from hepatocytes [20]. Here, we demonstrated that BM-MSCs attenuate HSC activation and hepatic fibrosis by targeting Ly6Ch and Ly6Cl macrophages through activating the antifibrogenic-cytokine paracrine and apoptotic pathways, thereby uncovering a previously unrecognized mechanism underlying MSC-mediated therapeutic options in liver repair.

RESULTS

BM-MSCs attenuate liver fibrosis by promoting Ly6Ch/Ly6Cl macrophage conversion

A primary bone marrow-derived mesenchymal stem cell (BM-MSC) line with typical mesenchymal lineage markers and differentiation potency was transplanted in a CCL4-induced fibrotic C57BL/6 J mouse model (Fig. S1); and the transplantation remarkably attenuated liver fibrosis by decreasing serum alanine aminotransferase (ALT) level, intrahepatic collagen deposition and HSC activation (Fig. 1A–G). This outcome was accompanied by a robust reduction in profibrogenic cytokines including IL-1β, TNF-α, and PDGF derived from pro-fibrotic cells such as Ly6Ch macrophages (Fig. 1H). Quiescent and activated HSCs isolated from mouse livers were used for in vitro coculture experiments (Fig. S2). Coculture of quiescent HSCs with Ly6Ch macrophages promoted HSC activation, as examined by the upregulation of α-SMA and col1α1 mRNAs; whereas coculture of quiescent HSCs with Ly6Ch macrophages plus BM-MSCs impaired such an activation. Additionally, coculture of activated HSCs isolated from the fibrotic liver with BM-MSCs alone did not affect the expression of α-SMA and col1α1 mRNAs (Fig. 1I, J). These results suggest that BM-MSCs indirectly suppressed HSC activation through inhibiting Ly6Ch macrophages. Actually, Ly6Ch macrophages were the most prevalent and significantly increased population in the fibrotic liver (Figs. 2B, C, S3, S4). The Ly6Ch macrophages exhibited a CD45+ Ly6G−CD115−CX3CR1midCCR2low Ly6Ch phenotype, suggesting the infiltration of circulating Ly6Ch macrophocytes into the inflamed liver as previously believed. After BM-MSC transplantation, the ratio of Ly6Ch/Ly6Cl macrophages decreased by 51% compared with that of the mock PBS-administered control group (Figs. 2D, E, S6). This promotion was enhanced with the increase in the cells administered (Fig. 2F, G). Other immunocytes had no evident changes after BM-MSC infusion (Fig. S5). The Ly6Ch/Ly6Cl macrophage conversion was also evaluated by the upregulation of two regulatory genes (msig2 and mmp12) and disparate transcriptional alteration of Ly6Ch (ly6c, thiβ1, and il1β) and Ly6Cl (hgf, mmp9, mmp12, and mmp13) marker genes in CD45+Ly6G−CD115−CX3CR1midCCR2low inflammatory macrophages (named CD115−Ly6G−Ly6Ch monocytes) sorted from fibrotic liver transplanted with BM-MSCs by FACS (Fig. 2H, I, J). Additionally, the conversion was further detected by incubating CD115−Ly6G−Ly6Ch macrophages with BM-MSCs in vitro (Fig. 2K, L). These data indicated that BM-MSCs could directly promote the phenotypic switch from Ly6Ch to Ly6Cl macrophages in the fibrotic liver, which led to the conversion of macrophages from a pro-inflammatory state to pro-resolving status.

BM-MSCs hardly prevent the recruitment of Ly6Ch monocytes to injured liver

Given that bone marrow-derived Ly6Ch monocytes that highly express CCR2 can be recruited from peripheral circulation to inflamed liver and differentiated into Ly6Ch macrophages during fibrogenesis, we explored whether the decrease in Ly6Ch macrophages in the fibrotic liver after BM-MSC transplantation is caused by preventing Ly6Ch monocytes from recruiting into the liver. For this purpose, the proportion of CD11B+F4/80+ monocytes/macrophages in the fibrotic liver was examined by FCM analysis. Minimal alteration in the proportion of CD11B+F4/80+ cells was detected after BM-MSC transplantation (Fig. 3A, B). Consistently, the mRNA expression levels of cd122, cc2r, and cd11b (myeloid lineage markers) were not changed in the fibrotic livers upon BM-MSC transplantation (Fig. 3C). For further clarification, an adoptive transfer assay was performed with cognate BM-MSCs and bone marrow-derived Ly6Ch monocytes in a CCR2-deficient (CCR2−/−) mouse strain in an attempt to exclude the interference of endogenous bone marrow-derived Ly6Ch monocytes. For this procedure, BM-MSCs and FACS-sorted bone marrow-derived CD115−CD117−Ly6Ch monocytes (Fig. S7) were co-transferred into the fibrotic CCR2−/− mice through the splenic pathway. Control fibrotic CCR2−/− mice received CD115−CD117−Ly6Ch monocytes alone. CD11B+F4/80+ cells from the fibrotic liver were examined by FCM analysis (Fig. 3D). Expectedly, the proportion of CD11B+F4/80+ monocytes/macrophages was not significantly decreased in the experimental group (Fig. 3E, F). These observations suggested that BM-MSCs did not have an inhibitory effect on the recruitment of Ly6Ch monocytes into the injured liver, but the conversion of Ly6Ch to Ly6Cl macrophages in the fibrotic liver may be directly regulated by BM-MSCs.

BM-MSCs regulate Ly6Ch/Ly6Cl macrophage conversion by IL-4 and IL-10

BM-MSCs possess immunomodulatory activities by secreting immunoregulatory factors, we next explored whether the immunoregulatory factors potentially regulate Ly6Ch/Ly6Cl macrophage conversion. The expression levels of IL-4, IL-10, TGF-β, PGE2, TSG-6, IL1Rα, IL-6, and IDO in transplanted BM-MSCs under the fibrotic liver microenvironment were initially examined by qPCR. For this procedure, GFP® BM-MSCs derived from C57BL/6-Tg(CAG-EGFP)10osbJ strain were transplanted into the fibrotic liver. After infusion for 48 h, GFP® BM-MSCs were retrieved from the fibrotic liver by FACS for qPCR analysis. The results showed that IL-4 and IL-10 mRNAs in the retrieved BM-MSCs increased by 840 and 530 times compared with those in unstimulated control GFP® BM-MSCs without undergoing transplantation, however, minimal change was detected in IL1Rα and IL-6 transcripts (Fig. 4A, B). These observations suggested that IL-4 and IL-10 may play a major role in the BM-MSC-promoted conversion of Ly6Ch to Ly6Cl macrophages. For clarification, in vitro polarization assay was conducted by incubating fibrotic liver-derived CD45+Ly6G−CD11B+F4/80+Ly6Ch macrophages with different concentrations of recombinant mouse IL-4 and IL-10 proteins. As expected, both IL-4 and IL-10 remarkably promoted the Ly6Ch/Ly6Cl macrophage conversion in a dose-dependent manner, and a maximal conversion was observed in a combination of IL-4 (20 ng/mL) with IL-10 (20 ng/mL) (Fig. 4C, D). This finding suggested the existence of a synergistic effect between IL-4 and IL-10. Accordingly, a significant increase in the ratio of Ly6Ch/Ly6Cl macrophages was shown in BM-MSC-transplanted fibrotic liver treated with anti-IL-4 and anti-IL-10 neutralizing antibodies compared with that treated with isotype control IgG2b (Fig. 4E, F). Furthermore, we conducted a direct experiment functionally linking Ly6Ch/Ly6Cl macrophage plasticity and IL-4/IL-10 secretion through BM-MSCs. For this, BM-MSCs and Ly6Ch macrophages were cocultured in the presence of anti-IL-4 and anti-IL-10 antibodies (for neutralizing the activity of IL-4 and IL-10) and injured liver conditional medium (ILCM, v/v; for inducing the production of IL-4 and IL-10). The changes in macrophage plasticity were examined by FCM analysis. The results showed that the phenotypic switch from Ly6Ch to Ly6Cl macrophages was significantly inhibited in the neutralization group treated with anti-IL-4 and anti-IL-10 antibodies compared with the control group treated with the isotype IgG (Fig. 4G, H). These results suggested that BM-MSCs regulated Ly6Ch/Ly6Cl macrophage conversion largely through secreting IL-4 and IL-10 upon stimulation in the fibrotic liver microenvironment.
Fig. 1 BM-MSC transplantation attenuated CCl4-induced inflammatory levels and liver fibrosis. A ALT release level in the serum of normal control mice and fibrotic mice transplanted with BM-MSCs or PBS (control). Serum samples were collected at 2 weeks post-transplantation (n = 6). B, C Liver tissue sections were stained with Sirius Red (B), and the degree of liver fibrosis was semi-quantified by Image software (C) (n = 7). Scale bars, 200 μm. D, E Immunohistochemistry staining of α-SMA in each group (D) and α-SMA+ areas were semi-quantified by Image software (E) (n = 7). Scale bars, 200 μm. F Quantification of col1α1 mRNA expression levels related to GAPDH by quantitative real-time PCR (n = 6). G Examination of hydroxyproline contents in livers at 2 weeks post-transplantation of BM-MSCs (n = 5). H Relative expression levels of il1β, tnfα, and pdgf mRNAs in livers of normal control and fibrotic mice transplanted with BM-MSCs or PBS examined by quantitative real-time PCR (n = 6). I Relative expression levels of col1α1 and acta2 mRNAs in HSCs, HSCs cocultured with Ly6Chi macrophages, and HSCs cocultured with Ly6Chi macrophages plus BM-MSCs examined by quantitative real-time PCR (n = 4). Ly6Chi M is the abbreviation of Ly6Chi macrophages. J Relative expression levels of col1α1 and acta2 mRNAs in HSCs cocultured with BM-MSCs or without BM-MSCs (control) were examined by quantitative real-time PCR (n = 4). Bars = means ± SD. Statistical evaluation of two groups was performed using an independent Student t-test. Statistical evaluation of multiple groups was performed using one-way ANOVA with posthoc LSD test; n.s. p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 2 Evaluation of the contribution of BM-MSCs to the conversion of Ly6C\(^{hi}\) to Ly6C\(^{lo}\) macrophages. A Schematic of the work model. C57BL/6 J mice were i.v. injected with CCl\(_4\) (1 mg/kg) twice a week for 4 weeks, followed by FCM analysis to detect different immunocytes at 1, 2, 3 (fibrogenesis stage), and 7 days (resolution stage) after the final CCl\(_4\) injection. B The proportion of various immunocytes in fibrotic livers at fibrogenesis and resolution after the final CCl\(_4\) injection analyzed by FCM. C FCM analysis showing a strong response of the intrahepatic macrophage population to CCl\(_4\) injury at the early fibrogenesis stage \((n = 4)\). D, E Alteration in the ratio of Ly6C\(^{hi}/Ly6C^{lo}\) macrophages in fibrotic livers 7 days post-BM-MSC transplantation by FCM analysis \((n = 7 \text{ and } n = 9)\). F, G FCM analysis showed that the conversion of Ly6C\(^{hi}\) to Ly6C\(^{lo}\) macrophages in fibrotic livers was conducted in a dose-dependent manner in response to BM-MSC transplantation with different concentrations \((0, 1 \times 10^4, 1 \times 10^5, \text{ and } 1 \times 10^6); n = 3\). In figure (G), M is an abbreviation of experimental groups with BM-MSC transplantation. H–J Quantitative real-time PCR analysis for the transcriptional expression levels of pro-inflammation markers (H), pro-resolution markers (I), and transcription factors for Ly6Clo monocyte differentiation (J) in CD11B\(^{hi}\)F4/80\(^{int}\) macrophages sorted from fibrotic livers at 24 h post-final CCl\(_4\) injection \((n = 4 \text{ and } n = 3)\). K, L FCM analysis for the phenotypic switch from Ly6C\(^{hi}\) to Ly6C\(^{lo}\) macrophages by coculturing BM-MSCs with equivalent CD11B\(^{hi}\)F4/80\(^{int}\) macrophages isolated from the fibrotic liver for 36 h \((n = 4)\). Bars = means ± SD. Statistical evaluation of two groups was performed using an independent Student t-test. Statistical evaluation of multiple groups was performed using one-way ANOVA with posthoc LSD test; n.s. \(p > 0.05\), *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
Additionally, we explored the potential regulatory role of BM-MSCs in Ly6C<sup>hi</sup>/Ly6C<sup>lo</sup> macrophage conversion by a cell–cell contact-dependent mechanism. For this, CD11B<sup>hi</sup>F4/80<sup>int</sup> macrophages sorted from fibrotic livers were cocultured with equivalent BM-MSCs in the presence of inhibitors for three promising contact-dependent signaling pathways: Wnt, Notch, and Hedgehog. FCM analysis showed that the ratio of Ly6C<sup>hi</sup>/Ly6C<sup>lo</sup> macrophages did not significantly decline in inhibitor-administered cocultures compared with that of the control coculture that received mock DMSO (Fig. S8). These observations largely exclude the potential regulatory role of BM-MSCs in the conversion of Ly6C<sup>hi</sup> to Ly6C<sup>lo</sup> macrophages in cell–cell contact manner.

**BM-MSCs undergo apoptosis in fibrotic liver after transplantation**

To determine the fate of BM-MSCs in the fibrotic liver after transplantation, Luc<sup>+</sup> BM-MSCs derived from C57BL/6J-TgN (Chicken-β-actin-LUC) ZLFlaS male mice were transplanted into syngeneic female fibrotic mice by spleen injection. Livers were harvested from recipient mice at different times after BM-MSC infusion, and the signals of Luc<sup>+</sup> BM-MSCs were detected by an in vivo imaging system. The results showed that the Luc<sup>+</sup> BM-MSC signals remarkably increased in the fibrotic liver at 12 h post-infusion followed by a drop in 72 h (Fig. 5A, B). The amount of Luc<sup>+</sup> BM-MSCs in fibrotic livers was quantified via qPCR by detecting the copy number of the Ym1/2 gene specialized on the
Y chromosome calculated by a standard curve. Consistent with the in vivo imaging result, the transplanted Luc+ BM-MSCs declined by 43% and 95% at 48 and 72 h, respectively, after infusion compared with the 12 h group (Fig. 5C). These observations suggested that BM-MSCs underwent rapid rejection through apoptotic cell death after transplantation. For clarification, an in vivo activation assay for apoptotic caspase-3 was examined in Luc+ BM-MSCs. In this case, the activation of caspase-3 was detected as luciferase activity by the administration of Z-DEVD-aminoluciferin. As expected, remarkable luminescence signals
were detected in Luc⁺ BM-MSCs in the fibrotic liver, which indicated the occurrence of apoptosis accompanied by caspase-3 activation (Fig. 5D). Additionally, significant luminescence signals were detected after culturing Luc⁺ BM-MSCs with 50% injured liver conditional medium (ILCM, v/v) in vitro (Fig. 5E). To determine the fate of apoptotic BM-MSCs, the CM-Dil-stained BM-MSCs were transplanted into fibrotic livers and harvested 48 h after transplantation. Single-cell suspensions were prepared and stained by leukocyte markers for FCM analysis. The results showed that the CM-Dil⁺ signals were mostly found in CD11B⁺ mononuclear cells, including macrophages, neutrophils, and dendritic cells. Further analysis showed that the majority of CM-Dil⁺ signals was found in CD11B⁺ F4/80⁺ macrophages, whereas dendritic cells and neutrophils were the minority of CM-Dil⁺ signals. Thus, the infiltrating macrophages, rather than dendritic cells and neutrophils, played a major role in clearing the infused apoptotic BM-MSCs. There were 84.8% ± 4.2% CM-Dil⁺ signals in Ly6G₀ macrophages and 4.0% ± 1.1% CM-Dil⁺ signals in Ly6G⁺ macrophages in the macrophage subset, which indicated that Ly6G₀ macrophages exerted a significant role in apoptotic cell clearance (Fig. 5F, G).

**Ly6G₀ macrophages upregulate MMP12 by engulfment of apoptotic bodies**

The above observations demonstrated the apoptotic fate of BM-MSCs in the fibrotic liver. For further support, the death of BM-MSCs was induced by in vitro stimulation with the ILCM. As expected, the proportions of apoptotic (Annexin V⁺ PI⁻ and Annexin V⁺ PI⁺) and necrotic cells (Annexin V⁺ PI⁺) were detected to be 67.1% and 17.0% by FCM analysis (Fig. 6A). The majority of BM-MSCs underwent apoptosis upon inducers from the fibrotic liver. Thus, the apoptotic bodies derived from BM-MSCs in fibrotic livers may facilitate immunomodulation for fibrosis. Ly6G₀ macrophages are highly restorative for fibrosis by upregulating MMPs including MMP9, MMP12, and MMP13 to promote matrix degradation; this process is associated with the phagocytosis of Ly6G₀ macrophages that highly express PtdSer-dependent receptor tyrosine kinases (MerTK) for the transduction of signals from PtdSer. In support of this viewpoint, BM-MSCs were transplanted in the fibrotic liver, and the expression of MerTK was examined by FCM analysis. The results revealed that the expression of MerTK increased from 30%–50% in Ly6G₀ macrophages after BM-MSC infusion compared with the PBS control (Fig. 6B). Given that considerable BM-MSC-derived apoptotic bodies were engulfed by Ly6G₀ macrophages (Fig. 5F), we speculated that this engulfment initiated the upregulation of MMPs. For clarification, the primary bone marrow-derived Ly6C₀ monocytes were prepared and induced to CD11B⁺ F4/80⁺ Ly6G₀ mature phenotype (BMDMs) by L929 conditioned medium. The apoptosis of BM-MSCs was induced by ILCM, and the apoptotic bodies were isolated by FACS sorting (Fig. 6C). Subsequently, apoptotic bodies were added into the CD11B⁺ F4/80⁺ Ly6G₀ macrophages, and the transcriptional mRNA levels of mmp9, mmp12, and mmp13 in cells were quantified by qPCR. The results showed that mmp12 and mmp13 were remarkably upregulated in response to apoptotic body stimulation (Fig. 6D). Furthermore, the apoptotic bodies pretreated with Annexin-V recombinant protein for the blockade of PtdSer impaired the phagocytosis. The results showed that the treatment significantly inhibited the expression of MPP12 (Fig. 6F). These outcomes suggested that MPP12 is likely a major effector in Ly6G₀ macrophage-mediated fibrosis resolution upon stimulation with BM-MSC-derived apoptotic bodies. For in vivo assay, BM-MSCs, apoptotic bodies, and BM-MSCs that were pretreated with Z-VAD-FMK were transplanted in fibrotic mice for liver fibrosis treatment. Expectedly, BM-MSC-infused livers showed low hydroxyproline levels and α-SMA staining area fractions compared with those of PBS-administered control groups. However, treatment with apoptotic bodies alone and BM-MSCs treated with Z-VAD-FMK showed no significant improvement in fibrosis compared with those of PBS-administered control groups (Fig. 6E, I, G, J, and H).

**PtdSer–MerTK–ERK signaling axis is involved in MPP12 upregulation**

Finally, we explored whether the PtdSer-dependent MerTK signaling pathway is driven by BM-MSC-derived apoptotic bodies in the upregulation of MPP12 in Ly6G₀ macrophages. Western blot analysis showed that the MerTK was significantly upregulated in CD11B⁺ F4/80⁺ Ly6G₀ cells after incubation with apoptotic bodies (Fig. 7A, B). To clarify the activation of the MerTK signaling pathway in CD11B⁺ F4/80⁺ Ly6G₀ macrophages after being stimulated by the apoptotic bodies, the phosphorylation of MerTK was examined by immunoprecipitation and Western blot analysis. As expected, a phosphorylated 180 Kd band was clearly detected when CD11B⁺ F4/80⁺ Ly6G₀ macrophages were treated with apoptotic bodies. Furthermore, the downstream ERK1/2 was also significantly phosphorylated at Thr-202 and Tyr-204 (Fig. 7D, E). Meanwhile, the secretion of MPP12 was upregulated after stimulating CD11B⁺ F4/80⁺ Ly6G₀ macrophages by apoptotic bodies, as detected by ELISA (Fig. 7C). The correlation among MerTK, ERK1/2, and MPP12 was evaluated by utilizing UNC2541, an inhibitor for the phosphorylation of MerTK, in CD11B⁺ F4/80⁺ Ly6G₀ macrophages upon apoptotic-body stimulation. The results showed that inhibition of MerTK remarkably impaired the activation of ERK1/2 (Fig. 7F, G) and significantly decreased the expression of MPP12 (Fig. 7C). These results suggested the involvement of the PtdSer-MerTK-ERK1/2 signaling axis in apoptotic-body regulated MPP12 expression, which is crucial for matrix degradation in liver fibrosis.

**DISCUSSION**

Macrophages play pivotal roles in maintaining hepatic homeostasis and are closely associated with many liver diseases [21, 22]. Differential Ly6C expression is now used to identify the heterogeneous macrophage subsets responsible for fibrosis resolution in various tissues [22–25]. Regulation of phenotypic switch from Ly6C₀ to Ly6G₀ macrophages provides a promising therapeutic intervention for the treatment of liver fibrosis. However, it is not clear how this phenotypic conversion can be regulated. Here, we provided the first evidence that showed the dual regulatory roles of BM-MSCs in the attenuation of hepatic fibrosis by promoting Ly6G₀/Ly6G₀ macrophage switch through secreting antifibrogenic...

**Fig. 4 Examination of the phenotypic switch from Ly6Chi to Ly6Clo macrophages regulated by BM-MSCs. A Gating strategy for the isolation of GFP⁺ BM-MSCs from fibrotic livers. B Upregulation of transcriptional expression levels of immunomodulatory factors in FACS-sorted GFP⁺ BM-MSCs from fibrotic livers by quantitative real-time PCR (n = 6). C and D FCM analysis showing the stimulatory effects of IL4 and IL10 on the phenotypic switch of intrahepatic Ly6C₀/Ly6G₀ macrophages in a dose-dependent manner (n = 3). E and F FCM analysis showing the inhibitory effects of anti-IL-4 and anti-IL-10 antibodies on IL4/IL10-promoted phenotypic switch of intrahepatic Ly6C₀/Ly6G₀ macrophages. IgG2b isotype was used as a control. G and H The anti-IL-4 (0.6 μg/ml) and anti-IL-10 (1 μg/ml) antibodies and 10% ILCM (v/v) were added in BM-MScs and Ly6G₀ macrophages coculture system. FCM analysis showed the direct effect of IL4 and IL10 secreted by BM-MSCs on the phenotypic switch of Ly6C₀/Ly6G₀ macrophages. Bars = means ± SD. Statistical evaluation of two groups was performed using t-test; n.s. p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.
cytokines and actively initiating apoptotic cell death. Upon liver injury, the ratio of Ly6C^hi/Ly6C^lo macrophages was markedly decreased after BM-MSCs transplantation. This decline was not caused by preventing Ly6C^hi monocytes recruitment, as confirmed by the adoptive transfer assays in CCR2^−/− mice. Thus, BM-MSCs directly led to a decreased ratio of Ly6C^hi/Ly6C^lo macrophages in the fibrotic liver, which was evidenced by an in vitro coculture assay. qPCR analysis showed that 

\( \text{il-4} \) and \( \text{il-10} \) mRNAs were dramatically upregulated in BM-MSCs retrieved from the fibrotic liver, suggesting that these two cytokines played a major role in promoting Ly6C^hi/Ly6C^lo macrophage conversion. This suggestion was validated by in vitro polarization assays via incubating Ly6C^hi macrophages with recombinant IL-4 and IL-10 cytokines and in vivo neutralization assays via administering anti-IL-4 and anti-IL-10 blocking antibodies into the BM-MSC-transplanted fibrotic liver. The expression levels of Nr4a1 and Cebpβ transcriptional factors were upregulated in intrahepatic CD11B^hiF4/80^int cells during Ly6C^hi/Ly6C^lo macrophage conversion. This result suggested that
IL-4 and IL-10 may potentially regulate Ly6C<sup>hi</sup>/Ly6C<sup>lo</sup> macrophage conversion by upregulating the Nr4a1 and Cebp transcriptional factors, which were previously recognized as two crucial regulators for Ly6C<sup>lo</sup>/Ly6C<sup>hi</sup> macrophage polarization [26, 27]. Given that Ly6C<sup>hi</sup> macrophages are a source of fibrogenic cytokines for promoting HSC activation and hepatic fibrogenesis. The conversion of Ly6C<sup>hi</sup> to Ly6C<sup>lo</sup> macrophages significantly downregulated the expression levels of Ly6C<sup>hi</sup> macrophage-derived PDGF, TNF-α, and IL-1β in the fibrotic liver, which was accompanied with the reduced expression of HSC-derived α-SMA and Col1α1 and the reduction of liver fibrosis. These findings revealed that BM-MSCs could potentially attenuate liver fibrosis by blocking the source of fibrogenic cytokines by promoting Ly6C<sup>lo</sup>/Ly6C<sup>hi</sup> macrophage conversion at the early stage of transplantation.

Unexpectedly, we found that BM-MSCs experienced severe apoptosis and produced substantial apoptotic bodies in the fibrotic liver at 12–72 h post-transplantation. This observation was quite different from the inherent cognition that MSCs can be infused into injured tissues, where they survive, proliferate, and differentiate into multiple cell lineages [28]. Here, we proved that most apoptotic bodies from BM-MSCs were engulfed by Ly6C<sup>lo</sup> macrophages in the fibrotic liver, as shown by in vivo tracking experiment using transgenic BM-MSCs from C57BL/6J-TgN mice with a chicken-β-actin-LUC reporter gene. The apoptotic bodies robustly promoted the expression of MMP12 in Ly6C<sup>lo</sup> macrophages after phagocytosis. This reaction was dramatically attenuated by the treatment of cells with Annexin-V for theophylline for theophylline stimulation with apoptotic bodies. These findings revealed that BM-MSCs could potentially attenuate liver fibrosis by blocking the source of fibrogenic cytokines by promoting Ly6C<sup>lo</sup>/Ly6C<sup>hi</sup> macrophage conversion at the early stage of transplantation.

In conclusion, we found that BM-MSCs attenuated hepatic fibrosis by converting Ly6C<sup>hi</sup> to Ly6C<sup>lo</sup> macrophages and promoting Ly6C<sup>lo</sup> macrophage restoration through activating the antifibrogenic cytokine and apoptotic pathways (Fig. 8). Thus, this work uncovered a previously unrecognized dual regulatory function of BM-MSCs in liver fibrosis. The findings largely improved the current understanding of the cellular and molecular mechanisms underlying MSC-based therapy for liver fibrosis and injury repair.

**Materials and Methods**

**Animals and experimental models**

C57BL/6J and B6/Jgpt-Ccr2<sup>αmicaoα507/Gpt</sup> were obtained from GemPharmatech. C57BL/6J-TgN (Chicken-β-actin-LUC) ZFLILAs mice were obtained from Shanghai ScienCell Digestion Biology Science&Technology. C57BL/6-Tg (CAG-EGFP) 10bx/J mice were obtained from the Jackson Laboratory. All mice were housed under standard conditions as previously described [20]. The 8- to 12-week-old and sex-matched mice were used for the experimental procedures. For liver fibrosis models, mice were intraperitoneally injected with CCL4 (1 mL/kg; 1:10 [v/v] in olive oil, Sangon Biotech) twice a week for 4 weeks. The animals were sacrificed 72 h after the final CCL4 injection, and serum and whole livers were collected for biochemical, histological, and molecular analyses as previously described [31]. For the transplantation experiment, mice were treated with CCL4 for 4 weeks with eight injections, and 1 × 10<sup>6</sup> BM-MSCs were transplanted into mice 24 h after the final injection. The mice were then continually injected with CCL4 for another week until use. All animal experiments were performed in accordance with legal regulations and approved by a local ethics committee.

**Preparation and culture of BM-MSCs and BMDMs**

Bone marrow-derived MSCs (BM-MSCs) were flushed from femurs or isolated from dissected fragments of femurs of C57BL/6J mice, EGFP transgenic mice, and luciferase transgenic mice as described previously [31, 32]. The cells were cultured in Iscove’s modified Dulbecco’s medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in 5% CO₂. The medium was refreshed once a week, and BM-MSCs were passaged using 0.25% trypsin-EDTA (Gibco) at 80–90% confluence. BM-MSCs at passages 3–6 were used in experiments and identified by their lineage differentiation potential and surface phenotypic markers through flow cytometry analysis by using antibodies against CD29, CD44, CD105, Sca-1, CD31, CD34, CD45, CD11b, CD117, and CD135 [32, 33]. Bone marrow-derived macrophages (BMDMs) were generated from C57BL/6J mice as described [34]. The whole bone marrow was cultured in DMEM (Gibco) supplemented with 10% FBS and 20% L929-conditioned medium for 2 days before removing non-adherent cells. The adherent cells were cultured for another 5 days, and this process yielded a macrophage population of >90% purity as detected by flow cytometry for CD11b and F4/80 [11].

**Isolation of hepatic nonparenchymal cells**

Hepatic nonparenchymal cells (NPCs) were isolated using a modified protocol as described previously [35]. In brief, mice were anesthetized and fixed before surgical preparation. About 20 mL of PBS (10 mM, pH 7.4)
Fig. 6 Examination of the engulfment of BM-MSC-derived apoptotic bodies by Ly6C<sup>lo</sup> macrophages. A FCM analysis for apoptotic BM-MSCs induced by 50% ILCM (v/v) for 24 h through Annexin V and PI staining. B Representative FCM gating strategy used to identify differential MerTK expression in CD11b<sup>hi</sup>F4/80<sup>int</sup>Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> macrophages in the fibrotic liver after transplanting 1 × 10<sup>6</sup> BM-MSCs or PBS control (n = 3). C Quantification of apoptotic bodies by FCM analysis. Calibration beads with diameters of 1.0 and 4.0 μm (internal reference beads) and counting beads with diameters of 7.0 μm were used to gate 1–5 μm-sized microvesicles. Apoptotic bodies were identified as AnnexinV<sup>+</sup>P62<sup>−</sup>. D Quantitative real-time PCR analysis for the transcriptional expression levels of matrix-degrading relevant mmp9, mmp12, and mmp13 genes in BMDMs (1 × 10<sup>6</sup>) with engulfment of apoptotic bodies (3 × 10<sup>7</sup>) for 12 h (n = 3 and n = 4). E Quantification of the hydroxyproline content in livers after transplantation with PBS (control), BM-MSCs, BM-MSC-derived apoptotic bodies, and BM-MSCs pretreated with Z-VAD-FMK (40 μM) (n = 3). F Quantitative real-time PCR analysis for the transcriptional expression levels of mmp9, mmp12, and mmp13 genes in BMDMs with engulfment of apoptotic bodies pretreated with Annexin V (1 μg/mL) for 12 h (n = 3 and n = 4). G, I Examination of the degree of liver fibrosis by staining tissue sections with Sirius Red (I) and semi-quantification through Image software (G) after mice were transplanted with PBS (control), BM-MSCs (1 × 10<sup>6</sup>), BM-MSC-derived apoptotic bodies (3 × 10<sup>7</sup>), and BM-MSCs (1 × 10<sup>6</sup>) pretreated with Z-VAD-FMK (40 μM) (n = 4). Scale bars, 200 μm. J Immunohistochemistry staining (J) and semi-quantification (H) of α-SMA in each group (J). The α-SMA<sup>+</sup> areas were quantified from five random non-overlapping fields of each sample (n = 4). Scale bars, 200 μm. Bars = means ± SD. Statistical evaluation of two groups was performed using the independent Student t-test. Statistical evaluation of multiple groups was performed using one-way ANOVA with posthoc LSD test; n.s. p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.
was perfused through the inferior vena cava, followed by 5 mL of collagenase-pronase perfusion. Live cells were harvested and digested in a digestive solution containing collagenase I (100 U/mL; Sigma), collagenase IV (100 U/mL; Sigma), Pronase E (25 μg/mL; Sigma), and DNase I (100 μg/mL; BioDuly) at 37 °C. After treatment for 40 min, a stopping solution containing DMEM, 10% FBS, and DNase I was added to inactivate the digestive enzymes. The livers were passed through a 40 μm cell strainer, and hepatocytes were removed by centrifugation at 5 000 g for 5 min at 4 °C. NPCs suspension was collected at 500 g for 7 min at 4 °C, followed by separation with 30 and 70% Percoll density cushion. The obtained NPC fraction was collected, counted, and prepared for flow cytometry and FACS sorting.

Isolation of hepatic stellate cells
Hepatic stellate cells (HSCs) were prepared followed by a protocol as described previously [36]. Briefly, mice were anesthetized and underwent collagenase-pronase perfusion as described above. For quiescent HSC isolation, livers were harvested from healthy mice and digested in a digestive solution containing collagenase IV (100 U/mL; Sigma), Pronase E (mg/mL; Sigma), and DNase I (100 μg/mL; BioDuly) at 37 °C. For activated HSC isolation, livers were collected from fibrotic mice and digested in a digestive solution with collagenase I (100 U/mL; Sigma), collagenase IV (100 U/mL; Sigma), Pronase E (mg/mL; Sigma), and DNase I (100 μg/mL; BioDuly) at 37 °C. After treatment for 40 min, a stopping solution containing DMEM, 10% FBS, and DNase I was added to inactivate the digestive enzymes. Then, the livers were passed through a 40 μm cell strainer, and cell suspension from the digested livers was purified via 8.2% Nycodenz (Axis-shield, Oslo, Norway) gradient centrifugation at 580 g for 20 min at room temperature and filtered through a 40 μm cell strainer [31]. The obtained HSCs were cultured in plastic dishes with Iscove’s modified Dulbecco’s...
medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO₂. The purity of HSCs was detected to be >99% following the method based on HSC-specific Vitamin A fluorescence as described [36].

Histology analysis
For fibrosis examination, liver tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. Subsequently, 5 μm tissue sections were dewaxed and rehydrated in decreasing concentrations of ethanol and stained with Sirius red (Sigma). The extent of fibrosis was captured under a microscope and quantified by Image software (Carl Zeiss, Germany). For immunohistochemistry analysis, 5 μm tissue sections were boiled in sodium citrate buffer (10 mM, pH 6.0) for 30 min for antigen retrieval and treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity. The sections were treated with 5% goat serum to block nonspecific binding sites and incubated with primary anti-α-SMA antibody (Abcam) at 4 °C overnight, followed by secondary HRP-conjugated goat anti-rabbit IgG antibody (1:500, Abcam) at 37 °C for 1 h. The color was developed using a DAB mixture (Beyotime). The extent of fibrosis was captured under a microscope (Carl Zeiss Axiostar plus, Germany) and semi-quantified by detecting the average optical density of the histochemical or immunohistochemical reactant area by ImageJ software as previously described [37].

Flow cytometry and FACS analysis
For hepatic NPC analysis, cells were initially incubated with anti-rat Fc-receptor (CD16/32) for 10 min to exclude nonspecific staining. For phenotypic analysis of BM-MSCs and Ly6C<sup>hi</sup>/Ly6C<sup>lo</sup> macrophages in cultures, the BM-MSCs of passages 3−10 and primary macrophages with specified treatment were harvested by 0.25% trypsin-EDTA digestion and stained with various antibodies (Table 1). Flow cytometry (FCM) analysis and fluorescent-activated cell sorting (FACS) were performed by using BD LSR Fortessa II systems and BD FACSaria II. FACS routinely yielded cell purity levels of over 95%. FCM analysis for apoptotic bodies was performed as previously described [38].

Quantitative real-time PCR
Total RNA of liver tissues or cultured cells was prepared using Trizol reagent (Invitrogen) or iScript<sup>™</sup> RT-qPCR Sample Preparation Reagent (Bio-Rad) according to the manufacturer’s protocols. About 500 ng of RNA was reverse-transcribed into cDNA using the SuperScript III kit (Invitrogen). The PCR experiments were performed in a total volume of 10 μL by using an iQ<sup>™</sup> Universal SYBR Green Supermix (Bio-Rad). Relative expression levels were calculated using 2<sup>−ΔΔCT</sup> method with GAPDH for normalization. The transcript abundance of the target genes was analyzed through quantitative real-time PCR (qRT-PCR) on a CFX Connect Real-Time PCR Detection System (Bio-Rad) as previously described [20]. The primers for quantitative real-time PCR are listed in Supplemental Table.

Assay for IL-4/IL-10-regulated Ly6C<sup>hi</sup>/Ly6C<sup>lo</sup> macrophage conversion
The regulatory role of IL-4 and IL-10 in the phenotypic switch of Ly6C<sup>hi</sup>/Ly6C<sup>lo</sup> macrophages was evaluated by in vivo neutralization and in vitro stimulation assays. For in vivo neutralization of IL-4 and IL-10, fibrotic mice were intraperitoneally administered with rat anti-mouse IL-4 (anti-IL-4) Ab (16 mg/kg, Invitrogen) and anti-IL-10 Ab (16 mg/kg, Invitrogen) twice at 12 and 24 h after injury induction. In parallel, non-related rat isotype Ab

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**Fig. 8** Schematic of how BM-MSCs may decrease liver fibrosis by targeting Ly6C<sup>hi</sup> macrophages through activating the cytokine-paracrine and apoptotic pathways. CCL2 secreted by the fibrotic liver promoted the recruitment of Ly6C<sup>hi</sup> monocytes from bone marrow to the liver. BM-MSC transplantation promoted the conversion of Ly6C<sup>hi</sup> to Ly6C<sup>lo</sup> macrophages by secreting cytokines IL-4 and IL-10, which decreased the activation of HSCs. Meanwhile, BM-MSCs experienced severe apoptosis and produced substantial apoptotic bodies, which were engulfed by Ly6C<sup>lo</sup> macrophages, leading to MMP12 release and accelerated extracellular matrix (ECM) degradation in CCL4-induced liver fibrosis.
### Table 1. Key resources table.

| Reagent or resource                  | Source                  | Identifier          |
|--------------------------------------|-------------------------|---------------------|
| **Antibodies**                       |                         |                     |
| CD45, Clone 30-F11                   | eBioscience             | Cat# 53-0451-82; RRID:AB_2848416 |
| CD45, Clone 30-F11                   | eBioscience             | Cat# 56-0451-82; RRID:AB_891454   |
| CD11B, Clone M1/70                   | eBioscience             | Cat# 45-0112-82; RRID:AB_953558   |
| Ly6G, Clone RB6-8C5                  | eBioscience             | Cat# 25-5931-82; RRID:AB_469663   |
| Ly6C, Clone NK1.4                    | eBioscience             | Cat# 12-5932-82; RRID:AB_10804510 |
| Ly6C, Clone NK1.4                    | eBioscience             | Cat# 62-5932-80; RRID:AB_2735067   |
| F4/80, Clone BM8                     | eBioscience             | Cat# 11-4801-82; RRID:AB_2637191   |
| F4/80, Clone BM8                     | eBioscience             | Cat# 17-4801-82; RRID:AB_2784648   |
| CD11C, Clone N418                    | eBioscience             | Cat# 63-0114-82; RRID:AB_2722930   |
| CD11C, Clone HL3                     | BD Pharmingen™          | Cat# 550261; RRID:AB_398460       |
| CD3, Clone 17A2                      | eBioscience             | Cat# 56-0032-82; RRID:AB_529507    |
| NK1.1, Clone PK136                   | eBioscience             | Cat# 12-5941-82; RRID:AB_466050    |
| B220, Clone RA3-682                  | eBioscience             | Cat# 62-0452-82; RRID:AB_2734992   |
| CD115, Clone AF598                   | eBioscience             | Cat# 53-1152-82; RRID:AB_2016696   |
| CD117, Clone 2B8                     | eBioscience             | Cat# 62-1171-82; RRID:AB_2637141   |
| CD117, Clone 2B8                     | eBioscience             | Cat# 11-1171-81; RRID:AB_465185    |
| CD135, Clone A2F10                   | eBioscience             | Cat# 15-1351-82; RRID:AB_494219    |
| CD135, Clone A2F10                   | eBioscience             | Cat# 46-1351-80; RRID:AB_1073392   |
| CD62P, Clone Psel.KO2.3              | eBioscience             | Cat# 12-0626-82; RRID:AB_1210863   |
| CD29, Clone HMb1-1                   | eBioscience             | Cat# 11-0291-80; RRID:AB_2572448   |
| CD44, Clone IM7                      | eBioscience             | Cat# 11-0441-81; RRID:AB_465044    |
| CD105, Clone MJ7/18                  | eBioscience             | Cat# 53-1051-80; RRID:AB_2815203    |
| Sca-1, Clone D7                      | eBioscience             | Cat# 45-5981-80; RRID:AB_914370    |
| CD31, Clone 390                      | eBioscience             | Cat# 46-0311-80; RRID:AB_1834430    |
| CD34, Clone RAM34                    | eBioscience             | Cat# 11-0341-81; RRID:AB_465020    |
| MerTK, Clone D55MMER                 | eBioscience             | Cat# 63-5751-82; RRID:AB_2688139   |
| anti-rat Fc receptor (CD16/32), Clone 2.4G2 | BD Pharmingen™          | Cat# 533140; RRID:AB_394655       |
| anti-alpha smooth muscle actin Rabbit mAb | Abcam                  | Cat# ab5694; RRID:AB_2223021     |
| anti-MerTK Rabbit mAb, Clone EPR17534-139 | Abcam                  | Cat# ab184086          |
| GAPDH Monoclonal Antibody, Clone ZG003 | Invitrogen             | Cat# 39-8600; RRID:AB_2533438     |
| p44/42 MAPK (Erk1/2) Rabbit mAb      | CST                     | Cat# 4695; RRID:AB_390779        |
| Phospho-p44/42 MAPK (Erk1/2) Rabbit mAb | CST                     | Cat# 4370; RRID:AB_2315112       |
| p-Tyr mouse mAb                      | PTM Bio                 | Cat# PTM-701           |
| Goat anti Rabbit IgG H&L Polyclonal antibody, HRP conjugated | Abcam | Cat# ab6721; RRID:AB_955447 |
| Goat anti-Mouse IgG H&L Secondary Antibody | Invitrogen             | Cat# 31430; RRID:AB_228307       |
| IL4, Clone 30340                    | Invitrogen             | Cat# MA5-23722;RRID:AB_2609637   |
| IL10, Clone JES5-16E3               | Invitrogen             | Cat# 16-7101-85; RRID:AB_469225   |
| Rat IgG2b Isotype Control            | Invitrogen             | Cat# 02-9288; RRID:AB_2532966    |
| **Chemicals, Peptide, and Recombinant Proteins** |                     |                     |
| Annexin V (Ann-V) purified recombinant protein | Invitrogen             | Cat# BMS306            |
| Recombinant Mouse IL4                | Peprotech              | Cat# 214-14; RRID:AB_2609637    |
| Recombinant Mouse IL10               | R&D systems            | Cat# 417-ML            |
| Recombinant Mouse TGF-beta 1 Protein | R&D systems            | Cat# 7666-MB           |
| Recombinant Mouse Insulin R/CD220 Protein | R&D systems            | Cat# 7544-MR           |
Table 1 continued

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| Vismodegib          | Topscience | Cat# T2590 |
| iCRT3               | Topscience | Cat# T4302 |
| Semagacestat        | Topscience | Cat# T6125 |
| UNC2541             | Topscience | Cat# T7205 |
| Z-VAD-FMK           | MCE     | Cat# HY-16658B |
| I collagenase       | Sigma   | Cat# C0130 |
| II Collagenase      | Sigma   | Cat# C6885 |
| IV collagenase      | Sigma   | Cat# C5138 |
| Pronase E           | Sigma   | Cat# 1.07433 |
| DNase I             | BioDuly | Cat# E0046 |
| DAPI                | Sigma   | Cat# D5942 |
| IMDM                | Gibco   | Cat# 12200036 |
| DMEM                | Gibco   | Cat# 12800017 |
| FBS                 | Gibco   | Cat# 10091155 |
| Percoll             | GE Healthcare | Cat# 17-0891-01 |
| Nycodenz            | Axis-shield | Cat# 1002424 |
| BeyoGet™SDS-PAGE Precast Gel (4%-15%) | Beyotime Biotechnology | Cat# P0057A |
| Trizol Reagent      | Invitrogen | Cat# 15596026 |
| iScript™ RT-qPCR Sample Preparation Reagent | Bio-Rad | Cat# 170-8898 |
| D-Luciferin         | Shanghai Sciencelight Biology & Technology | Cat# luc001 |
| Z-DEVD-aminoluciferin | Promega | Cat# P1782 |
| CellTracker™ CM-Dil Dye | Beyotime Biotechnology | Cat# C7000 |
| Sirius red          | Sigma   | Cat# 365548 CAS: 2610-10-8 |
| Oil Red O           | Sigma   | Cat# 00625 CAS: 1320-06-5 |
| Toluidine blue      | Sigma   | Cat# 89640 CAS: 6586-04-5 |
| Alizarin Red S      | Sigma   | Cat# A5533 CAS: 130-22-3 |
| olive oil           | Sangon Biotech | Cat# A502795; CAS: 8001-25-0 |

**Critical Commercial Assays**

MMP12 Elisa Kit | Sangon Biotech | Cat# D721034 |
BCA protein assay kit | Beyotime Biotechnology | Cat# P04655 |
123count eBeads™ Counting Beads | Invitrogen | Cat# 01-1234-42 |
Flow Cytometry Size Calibration Kit | Invitrogen | Cat# F13838 |
Hydroxyproline assay kit | Nanjing Jiancheng Bioengineering Institute | Cat# A030-2-1 |
Alanine aminotransferase Assay Kit | Nanjing Jiancheng Bioengineering Institute | Cat# C009-2 |
Dead Cell Apoptosis Kit with Annexin V FITC and PI | Invitrogen | Cat# V13242 |
DAB Horseradish Peroxidase Color Development Kit | Beyotime | Cat# P0202 |

**Experimental Models: Cell lines**

L929 | ATCC | Cat# CCL-1™ |

**Experimental Models: Organisms/Strains**

C57BL/6 J | GemPharmatech | Cat# N000013 |
C57BL/Jgpt-Ccr2em8Cd6657/Gpt | GemPharmatech | Cat# T006112 |
C57BL/6J-Tg (Chicken-β-actin-LUC) ZLFILAS | Scienclight Biology & Technology | N/A |
C57BL/6-Tg (CAG-EGFP)1Osb/J | The Jackson Laboratory | 3291 |

**Software and Algorithms**

GraphPad Prism 8 | Graphpad Software | [https://www.graphpad.com](https://www.graphpad.com) |
FlowJo v10.5.0 | Flowjo | [https://www.flowjo.com](https://www.flowjo.com) |
ImageJ | ImageJ software | [https://imagej.nih.gov/ij](https://imagej.nih.gov/ij) |
Image software | Carl Zeiss, Germany | N/A |
(IgG2b, 16 mg/kg) was used as control. For in vitro stimulation assay, CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>+</sup> macrophages were sorted from the fibrotic liver by FACS and cultured without (control) or with different concentrations of recombinant mouse IL-4 and recombinant mouse IL-10 (5, 10, and 20 ng/mL) for 36 h, followed by FCM analysis.

**Imaging assay**

After transplanation with Luc<sup>+</sup> BM-MSCs (1 x 10<sup>4</sup>), mice were i.p. injected with D-luciferin (150 µg/kg body weight) for 15 min before sacrificing. Liver and spleen tissues were collected and imaged using an IVIS Spectrum (PerkinElmer) imaging system for 1 min. Imaging data were analyzed and quantified with Living Image Software. The strength of the cellular signal was indicated by the spectrum. To detect apoptotic Luc<sup>+</sup> BM-MSCs in the liver, mice were injected i.p. with VivoGlo Caspase-3/-7 substrate Z-DEVD Aminoluciferine (Promega; 300 µg/kg body weight) for 1–5 min and imaged using the IVIS Spectrum imaging system. For in vitro assay, Luc<sup>+</sup> BM-MSCs were pre-treated with 50% injured liver conditional medium (ILCM; v/v) for 4 h with or without Z-DEVD-aminoLuciferin (50 µg/well) and imaged using the IVIS Spectrum (PerkinElmer) imaging system for 1–5 min.

**Preparation of injured liver conditional medium**

ILCM was prepared as described previously [28]. In brief, hepatocytes were isolated from fibrotic mice by two-step collagenase perfusion. After treatment, the perfused liver was resuspended and passed through a 40 µm cell strainer. The hepatocytes were collected by centrifugation at 50 g for 5 min and cultured in IMDM supplemented with 10% FBS at 5 x 10<sup>4</sup> cells/cm<sup>2</sup>. After 48 h, the supernatant was collected and passed through a 0.22 µm filter. The filtrate was finally defined as ILCM and stored in aliquots at -40 °C for future use.

**Preparation of apoptotic bodies**

For apoptosis induction, the cultured BM-MSCs were treated with ILCM for 24 h, and apoptotic bodies were isolated by a filter system as previously described [38]. In brief, cell debris was removed after 300 g centrifugation, and the supernatant was filtered with 5 and 1 µm filters. Apoptotic body-sized extracellular vesicles were isolated by 2000 g centrifugation at 4 °C for 20 min and stained with anti-CD62P-PE antibody and Annexin-V-APC on ice for 15 min for FCM analysis. Apoptotic bodies were characterized as positive for Annexin-V staining and negative for anti-CD62P-PE staining. The 1–5 µm apoptotic body-sized extracellular vesicles were gated by calibration beads (7 µm, Invitrogen) and counting beads (1 and 4 µm, 123 count eBeads™, Invitrogen). The number of counting beads was calculated following the manufacturer’s instructions. The number of apoptotic bodies was calculated by the number of counting beads multiplied by the ratio of apoptotic body events to counting bead events in FCM plots.

**Coculture experiments**

Coculture experiments were performed to evaluate the regulatory activity of BM-MSCs to inflammatory macrophages. CD45<sup>+</sup>Ly6G<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages were sorted from the fibrotic liver by FACS and cocultured with BM-MSCs (5 x 10<sup>4</sup>) for 36 h. Subsequently, macrophages were digested by 0.25% trypsin-EDTA for 5 min, followed by centrifugation at 300 x g for 5 min. For signaling pathway inhibition, Vismodegib (40 µM), ICRT3 (40 µM), and Semagacestat (40 µM) were added in the cocultures for 36 h, and the control coculture received mock DMSO for FCM analysis. The phenotypic changes of the macrophages were examined by FCM analysis.

**Immunoprecipitation and Western blot analysis**

For immunoprecipitation (IP), macrophages (1 x 10<sup>7</sup>) were lysed with cell lysis buffer (Beyotime Biotechnology). The lysates were centrifuged at 10,000 rpm at 4 °C for 10 min, and the supernatants were incubated with rabbit anti-MerTK mAb (Abcam) at 4 °C overnight. Protein A agarose beads (Thermo Scientific) were incubated with the mixture for 4 h. The beads were washed three times with lysis buffer, mixed with loading buffer, and incubated at 95 °C for 10 min. After centrifugation, the proteins were subjected to Western blot analysis. For this procedure, the samples were separated by BeyoGel™ SDS-PAGE Precast Gel (4–15%) and transferred to polyvinylidene difluoride membrane (EMD Millipore). After incubation with 2% BSA for 2 h at room temperature, the membranes were incubated with the primary antibody in TBST with 0.5% BSA overnight at 4 °C and then incubated with secondary antibody for 1 h at room temperature. The image was detected on a gel imaging system (Tanon 4500).

**Statistical analysis**

All data were presented as the mean ± SD of each group. Statistical analysis was performed using GraphPad Prism 8 software and SPSS. Statistical evaluation of two groups was performed using an independent Student t-test. Statistical evaluation of multiple groups was performed using one-way ANOVA with a posthoc LSD test. A value of p < 0.05 was considered statistically significant. The sample size in the studies was estimated as previously reported [39]. Grubbs criterion was used to exclude outliers from the analysis. The samples were randomly assigned to experimental groups. The investigator was blinded to the group allocation during the experiment and when assessing the outcome as far as possible.

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AUTHOR CONTRIBUTIONS

Y.H.L. and J.Z.S. conceived and designed the experiments. Y.H.L. and S.S. performed the experiments. Y.H.L., S.S., T.S., M.T.J., A.F.L., L.X.X., and J.Z.S. analyzed the data. L.X. X., D.D.F., and J.Z.S. contributed reagents/materials/analysis tools. Y.H.L., L.X.X., and J. Z.S. wrote the paper. All authors reviewed the paper and provided comments.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were performed with the approval of the Ethics Committee for Animal Experimentation of Zhejiang University.

COMPETING INTERESTS

The authors declare that they have no competing financial interests.

ADDITIONAL INFORMATION

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