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Infusion of Kupffer Cells Expanded in *Vitro* Ameliorated Liver Fibrosis in a Murine Model of Liver Injury

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Abstract

Transfer of exogenous macrophages represents an alternative technique to treat liver fibrosis. At present, bone marrow-derived monocytes and stem cells are the main sources for exogenous macrophages. Kupffer cells (KCs) are the resident macrophages in the liver and play a critical role in the liver homeostasis and diseases. It is unclear whether infusion of KCs can treat liver fibrosis. In this study, we observed that granulocyte-macrophage colony stimulating factor (GM-CSF) could improve the purity of cultured KCs and significantly up-regulate the expression of Cluster of Differentiation 11b (CD11b). The most important point is that GM-CSF could significantly promote the proliferation of KCs *in vitro*. KCs expanded *in vitro* still had the potential of M1/M2 polarization and phagocytosis. Furthermore, infusion of these KCs could ameliorate liver fibrosis induced by carbon tetrachloride (CCl_4) in mice. Together, our results suggest that KCs are likely to be another source for macrophage therapy.

Keywords

Kupffer cells, GM-CSF, cell proliferation, cell therapy, liver fibrosis

Introduction

Liver cirrhosis is a worldwide disease which seriously threatens people's health[1]. Liver transplantation, the only effective treatment for cirrhosis, is limited by the shortage of available donors. Therefore, there is an urgent requirement for the development of alternative therapies for cirrhosis. Liver fibrosis is the common path in the development of cirrhosis for most chronic liver diseases, and can be reversed[2,3]. Macrophages have appeared one of the most promising for liver fibrosis therapy[4–6]. Safety and efficacy studies of autologous macrophage therapy are underway in a Phase I/II first-in-human clinical trials for cirrhosis[6,7].

One of the main sources of transplanted macrophages is bone marrow-derived monocytes. Thomas et al.[8] have reported that infusion of bone marrow-derived macrophages (BMDMs) to mice with established carbon tetrachloride (CCl_4)-induced liver fibrosis resulted in less collagen deposition and fewer activated hepatic stellate cells (HSCs). Ma et al.[9] found that intravenous injection of M1 polarized macrophages was effective in significantly reducing fibrosis. Another source of transplanted macrophages is embryonic stem cells (ESC). Haideri et al.[10] found that intravenous delivery of murine ESC-derived macrophages could reduce fibrosis in the CCl_4-induced liver fibrosis, although a greater number of cells were required.

Kupffer cells (KCs) are the resident macrophages in the liver and constitute 80-90% of resident macrophages in the body[11]. KCs play a critical role in the phagocytosis of foreign materials, immune surveillance, and maintenance of liver homeostasis[11]. KCs originate from yolk sac-derived erythromyeloid progenitors (EMPs)[12]. In the steady state, the replenishment of KCs is independent of BM-derived progenitors but predominantly relies on self-renewal locally,

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probably due to colony stimulating factors (CSF)[13]. Kitani et al.[14] obtained enough KCs *in vitro* using a mixed primary culture of hepatocytes, which degenerated or transformed into fibroblast-like cells as supporting cells. Zeng et al.[15] separated rat liver non-parenchymal cells (NPC) from parenchymal cells by differential centrifugation and obtained KCs by using the properties of selective adhesion of macrophages. And they found that rat KCs could proliferate in normal culture, although the potential for KCs proliferation is not very strong under this condition. Liu et al.[16] further isolated mouse KCs from liver NPC by using successive gradient centrifugations, and purified by magnetic cell sorting (MACS) using surface marker F4/80.

We asked whether infusion of KCs expanded *in vitro* could treat liver fibrosis. In this study, KCs were isolated by using successive gradient centrifugations and selective adherence. We found that granulocyte-macrophage colony stimulating factor (GM-CSF) could improve the purity of cultured KCs and significantly up-regulate the expression of Cluster of Differentiation 11b (CD11b). The most important point is that GM-CSF could significantly promote the proliferation of KCs *in vitro*. KCs expanded *in vitro* had the potential of M1/M2 polarization and phagocytosis. We infused these KCs to treat CCl_4-induced liver fibrosis in mice. Our results suggested that KCs significantly ameliorated liver fibrosis and improved liver function. KCs are likely to be another source for macrophage therapy.

Materials and Methods

Cell Isolation and Culture

To isolate Kupffer cells, anesthetized mice were perfused with D-Hanks' buffer from inferior vena cava, followed by Hanks' buffer containing collagenase IV (0.2 g/L) (Sigma-Aldrich, St. Louis, MO, USA, catalog No. C5138). Single cell suspension was prepared and hepatocytes were eliminated by three centrifugations at 50 g for 3 min. KCs were purified by using successive gradient centrifugations on 8.2% and 17.6% Iodixanol (Optiprep™, Axis-Shield, Oslo, Norway, catalog No. AS1114542). The cell fraction between the interface of the 8.2% and 17.6% Optiprep was enriched with KCs and liver sinusoidal endothelial cells (LSECs). The mixed cells were counted, seeded in 24-well plates (2×10^5/well) and cultured for 2 h. Unattached cells were gently removed by washing and attached cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), murine GM-CSF (40 ng/mL, PeproTech, Rocky Hill, NJ, USA, catalog No. 315-03) for 7–14 days. Bone marrow-derived macrophages (BMDMs) were cultured as described[17].

Flow Cytometry

KCs were harvested, and stained with PE-labeled anti-F4/80 (eBioscience, San Diego, CA, USA, catalog No. 124801), APC-labeled anti-CD11b (Biolegend, San Diego, CA, USA, catalog No. 101212). Dead cells were excluded by propidium iodide staining. Analysis was performed using a FACSCalibur™ flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA) and Flowjo X software (TreeStar, Ashland, OR, USA).

For cytoplasmic staining, cells (3×10^5) were fixed and permeated using the Cytofix/Cytoperm™ kit (BD Biosciences, San Jose, CA, USA, catalog No. 554714), followed by staining with PE-labeled anti-BrdU antibody (Cell Signaling Technology, Boston, MA, USA, catalog No. 50230).

For Cell cycle analysis, KCs were harvested and fixed with 70% ethanol at room temperature for 20 min. Then cells were washed and resuspended in PBS containing 50 μg/mL of propidium iodide, 0.1 mg/ml RNase A, and 0.1% Triton X-100 for 10 min. Cell cycle was analyzed using a FACSCalibur™. Data analysis was performed using the Cell Quest software (BD Biosciences, San Jose, CA, USA).

RNA Extraction and Quantitative Reverse Transcription (qRT)-PCR

Total RNA was isolated from KCs or liver tissues by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA, catalog No. 15596018) according to the manufacturer's instructions. RNA was reversely transcribed into cDNA using PrimeScript™RT Master Mix (Takara, Dalian, China, catalog No. RR036A). After reverse transcription, qRT-PCR was performed by using the TB Green™ Premix EX Taq™ II (Tli RNaseH plus) kit (Takara, Dalian, China, catalog No. RR820A) and Quantstudio 5 (Bio-rad, Hercules, CA, USA), with β-actin as an internal control. Primers used are listed in Supporting Table S1.

Macrophage Polarization and Phagocytosis

KCs (1×10^5) were seeded in 24-well plates. To polarize KCs into M0, M1 or M2 macrophages, KCs were stimulated for 24 h with PBS, lipopolysaccharide (LPS, 100 ng/ml, Sigma-Aldrich, St. Louis, MO, USA, catalog No. L4391) or Interleukin 4 (IL4) (20 ng/mL, PeproTech, Rocky Hill, NJ, USA, catalog No. 214-14), respectively.

To test phagocytosis, *Escherichia coli* (*BL21*) (1×10^6) carrying an EGFP expressing vector[18] were cocultured with KCs (1×10^5) for 2 h. Free *E. coli* were removed by washing, then KCs were collected and stained with PE-labeled anti-F4/80 antibody. Samples were analyzed by flow cytometry, and the percentage of engulfed $EGFP^+$ *E. coli* was calculated.

Liver Fibrosis Model

To induce liver fibrosis, male C57BL/6 mice were injected intraperitoneally (i.p) with 0.75 ml/kg of CCl_4 (Tianli, Tianjin, China, catalog No.202009) diluted in sterile olive oil twice a week for 8 weeks, with olive oil as a control. Three days after the last injection, mice were sacrificed for

further experiments. All animal experiments were performed following a guideline from the Animal Experiment Administration Committee of the university.

Histology

Formaldehyde-fixed liver tissues were paraffin-embedded, sectioned at 6 μm thickness, and stained with hematoxylin-eosin (H&E), Sirius red according to standard protocols. For immunohistochemistry, sections were prepared from mouse livers according to standard procedures. The primary antibodies included anti-mouse Collagen type I alpha 1 chain (Col1α1) (Servicebio, Wuhan, China, catalog No. GB11022) and anti-mouse α smooth muscle actin (αSMA) (Servicebio, Wuhan, China, catalog No. GB111364). The sections were developed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (Servicebio, Wuhan, China, catalog No. GB23303) and a DAB kit (Servicebio, Wuhan, China, catalog No. G1212-200 T). For immunofluorescence, cells were grown on cover slides until confluence, and fixed in 4% paraformaldehyde for 15 min, followed by three washes in PBS. Cells were incubated with primary antibodies including anti-mouse F4/80 (eBioscience, San Diego, CA, USA, catalog No. 144801) and Marker of Proliferation Ki-67 (MKI67, Ki67) (Thermo Fisher Scientific, Astmoor Runcorn, UK, catalog No. MA5-14520). Cells were then incubated for 1 h with the secondary antibodies, including Alexa Fluor 488-labeled donkey anti-rat IgG (Invitrogen, Carlsbad, CA, USA, catalog No. A21208) and Cy3-labeled goat anti-rabbit IgG (Boster BioTec, Wuhan, China, catalog No. BA1032). Nuclei were counter-stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA, catalog No. 94403). Photographs were taken using a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

Cell Infusion

Mice were infused with PBS or KCs (1×10^6 /mouse) after the fourth week CCl_4 injection through the tail vein, and were continually injected with CCl_4 for another four weeks. Three days after the last injection, mice were sacrificed humanely for further analyses.

Biochemistry

Serum albumin was analyzed with a Bio-Tek EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Statistical Analysis

Images were imported into the Image Pro Plus 6.0 software (MediaCybernetics Inc., Bethesda, MD, USA), and positive area was analyzed. Data were analyzed with Graph Pad Prism software, version 8.0. Comparisons between groups were undertaken using unpaired or paired Student's t test. Results were expressed as means ± SD. $P<0.05$ was considered as significant.

Results

GM-CSF Improved cell Purity and up-Regulated the Expression of CD11b of Kupffer Cells in Vitro

The cell fraction containing KCs was initially obtained by collagenase perfusion and density gradient centrifugation as described[16]. After incubation for 2 h in culture dishes, attached KCs were selectively harvested. However, the purity of primary KCs was not high, only about 30% of F4/80$^+$ cells (Fig. 1A, D0). Then cytokine GM-CSF was added into the culture medium. After 3 days of culture, the cell purity of F4/80$^+$ reached more than 65% (Fig. 1A, D3). Furthermore, the purity of F4/80$^+$ cells was 97% after 7 days of culture (Fig. 1A, D7 and Fig 1B).

On the other hand, GM-CSF could significantly promote the expression of CD11b on KCs surface. CD11b expression in primary KCs was low (Fig. 1A, D0). After 3 days of GM-CSF stimulation, there were two subsets of KCs, F4/80$^+$ CD11b$^-$ and F4/80$^+$ CD11b^{intermediate} (Fig. 1A, D3). Fluorescence-activated cell sorting (FACS) data showed that almost all KCs transformed into a single population of F4/80$^+$CD11b^{high} cells after 7 days of culture (Fig. 1A, D7). At this time, CD11b and F4/80 expression of KCs were similar to that of BMDMs (Fig. 1C).

GM-CSF Promoted the Proliferation of Kupffer Cells in Vitro

Interestingly, we observed that the number of KCs increased significantly in the present of GM-CSF *in vitro*. After 14 days of culture containing GM-CSF, the number of KCs was about 10 times that of the original separation (Fig. 2A). In order to detect the proliferation of KCs, three experiments were adopted. First, BrdU was added into the culture medium for 18 h, FACS data indicated that about 50% of the cells were BrdU positive (Fig. 2B). Second, cell cycle analysis showed that nearly half of the cells were in S and G2 phase (Fig. 2C). Third, immunofluorescence staining of Ki67, a marker of cell proliferation, showed that about 50% of KCs were Ki67 positive (Fig. 2D). Furthermore, we extended the culture time to 21 days and observed that KCs still had a strong capacity to proliferate. As shown in Supplemental Fig. S1, there were still lots of F4/80$^+$Ki67$^+$ cells.

Kupffer Cells Expanded in Vitro Had the Potential of M1/M2 Polarization and Phagocytosis

We asked if KCs expanded *in vitro* had similar function as BMDMs. We investigated the polarization and phagocytosis of KCs cultured for 14 days. KCs were stimulated with PBS (M0), Lipopolysaccharides (LPS) (M1), or IL4 (M2) for 24 h. The polarized phenotypes of KCs were assessed by

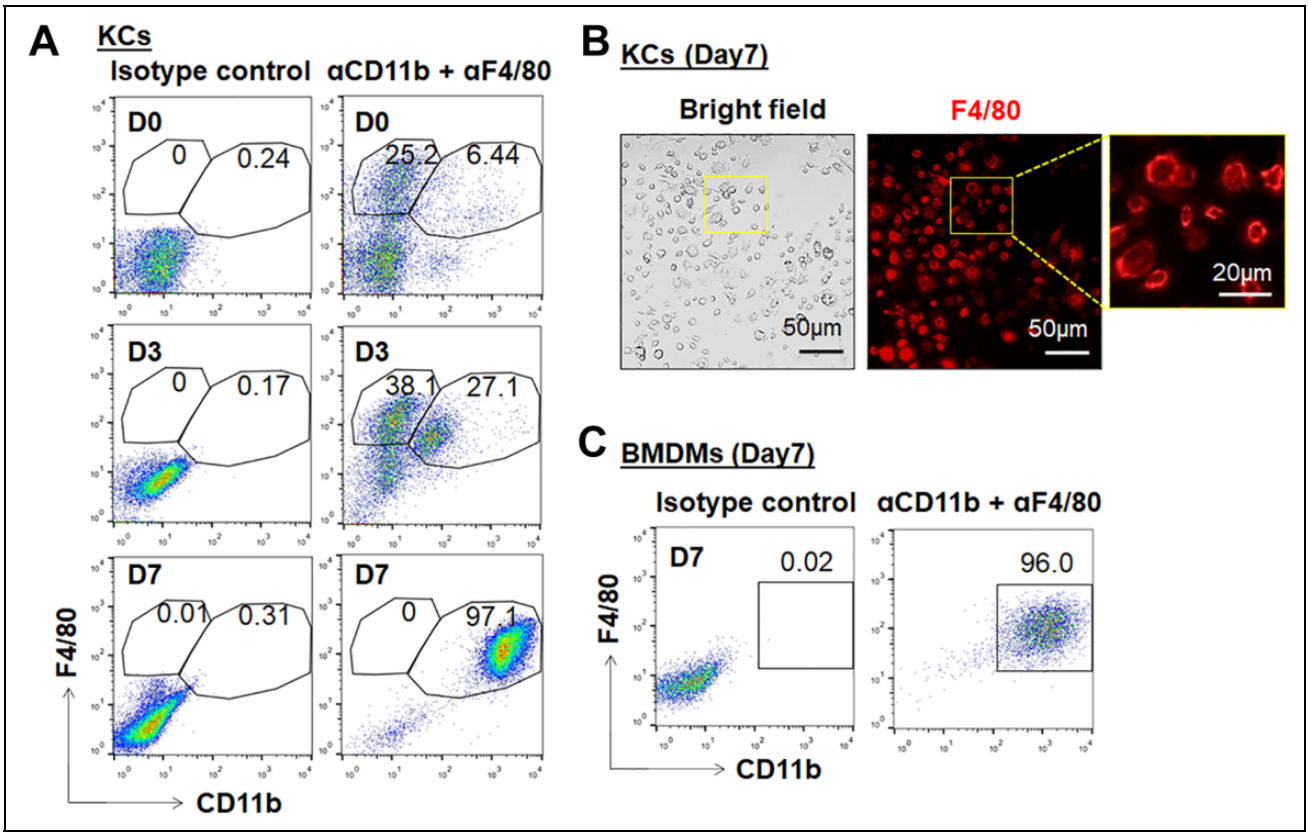

Figure 1. GM-CSF improved cell purity and up-regulated CD11b expression of Kupffer cells *in vitro*. KCs were isolated and cultured in the presence of GM-CSF for 7 days. (A) at day 0, day 3, and day 7, cells were collected and analyzed by FACS with PE-labeled anti-mouse F4/80, APC-labeled anti-mouse CD11b or isotype control. (B) Immunofluorescent staining of the cultured KCs with PE-labeled anti-mouse F4/80. The photomicrograph on the right is a larger view of the yellow frame in the middle image. (C) Mouse bone marrow cells were isolated and cultured in the presence of GM-CSF for 7 days to obtain BM-derived macrophages (BMDMs). BMDMs were also analyzed by FACS with PE-labeled anti-mouse F4/80, APC-labeled anti-mouse CD11b or isotype control. FACS: Fluorescence-activated cell sorting.

qRT-PCR. As shown in Fig. 3A, LPS up-regulated the expression of M1 markers inducible Nitric Oxide Synthase (iNOS), Tumor Necrosis Factor α (TNFα) and Interleukin 12 (IL12), while IL4 promoted the expression of M2 marker Arginase 1 (Arg1), chitinase-like 3 (Chil3, YM1/2) and Interleukin 10 (IL10). Then we examined the ability of KCs to phagocytose bacteria. KCs were incubated with $EGFP^+$ *E. coli* for 2 h. FACS analyses indicated that more than 60% $F4/80^+$ KCs phagocytosed $EGFP^+$ bacteria (Fig. 3B). In addition, KCs were incubated with Cy5-labeled Fe_3O_4 nanoparticles for 2 h. FACS data showed that nearly all of KCs incorporated the Cy5-labeled Fe_3O_4 nanoparticles (Fig. S2). These data suggested that KCs expanded *in vitro* had the potential of M1/M2 polarization and phagocytosis.

Infusion of Kupffer Cells Expanded in Vitro Ameliorated Liver Fibrosis in CCl_4-Induced Fibrotic Mice

We asked whether KCs expanded *in vitro* could treat liver fibrosis like BMDMs. We established a mouse model of liver fibrosis by intraperitoneal injection of CCl_4. KCs were harvested after 14 days of culture. Mice were infused with these KCs (1×10^6 cells/mouse) via tail vein 24 h after the eighth injection of CCl_4 (Fig. 4A) as described[8]. And the mice were continually injected with CCl_4 for another 4 weeks. GFP^+ KCs could be recruited into the fibrotic liver 24 h or 1 week after infusion (Supplemental Fig. S3). H&E staining of liver sections 72 h after the last CCl_4 injection showed less inflammatory cells in the portal region of livers from KCs group mice compared with PBS group (Fig. 4C). Meanwhile, the expression of inflammatory factors C-C Motif Chemokine Ligand 2 (CCL2), TNFα, Interleukin 1β (IL1β) and Interleukin 6 (IL6) was determined at the mRNA by using qRT-PCR. The results indicated that the production of these inflammatory factors decreased significantly in the liver of mice with KCs infusion compared with the control (Fig. 4D). Moreover, serum albumin increased in KCs-recipient mice (Fig. 4B), suggested an improved liver function. Col1α1 and Sirius red staining showed that KCs significantly reduced ECM deposition compared with the PBS group (Figs. 5A, B, D, E). Hepatic stellate cells (HSCs) are the major source of the fibrous scar in liver fibrosis[2]. Therefore, we detected the activation of HSCs by staining with anti-α smooth muscle actin (SMA) antibody.

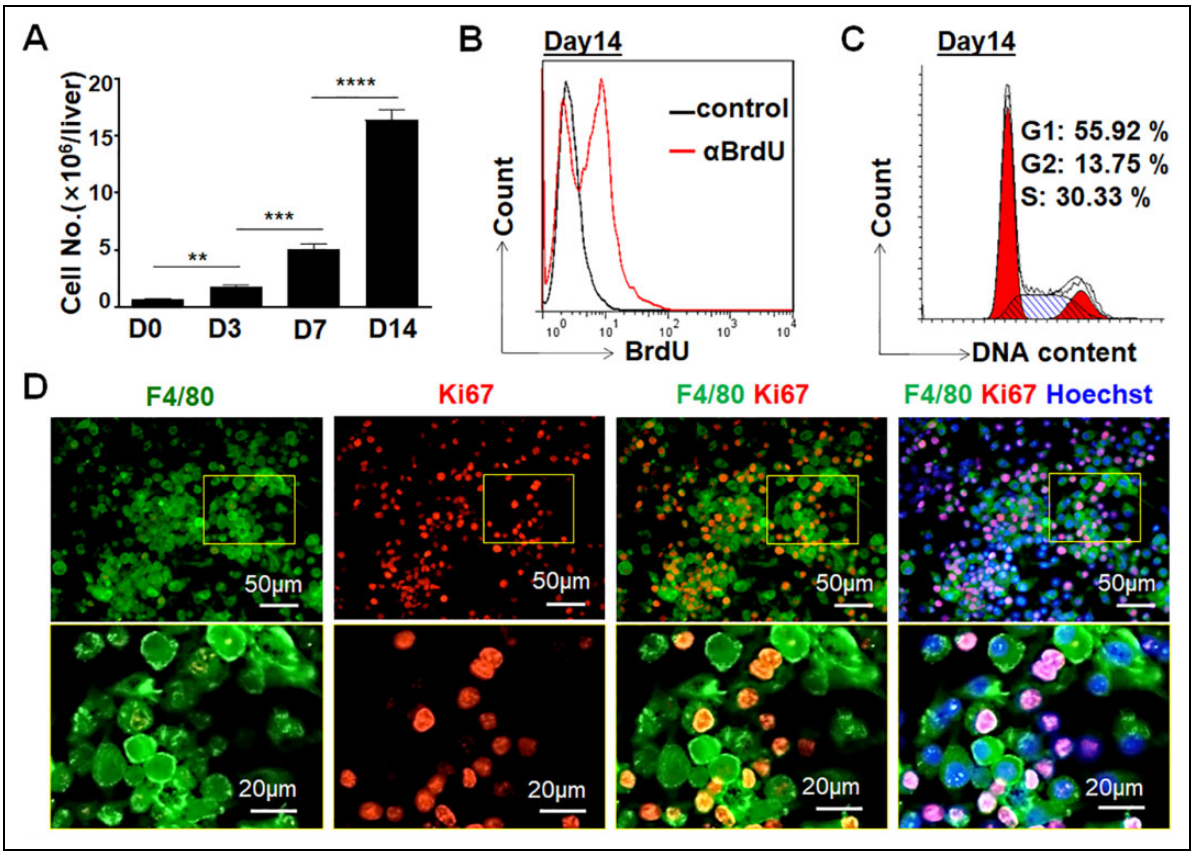

Figure 2. GM-CSF promoted the proliferation of Kupffer cells *in vitro*. KCs were cultured in the presence of GM-CSF. (A) At day 0, day 3, day 7, and day 14, cells were collected and cell numbers were counted. Cells were stained with PE-labeled anti-mouse F4/80, and analyzed by FACS, and then F4/80$^+$ cells were calculated ($n = 4$). (B) BrdU was added into the medium 18 h before the end of the experiment (Day 14). Cells were stained with anti-BrdU and analyzed by FACS. (C) Cell cycle progression of KCs (Day 14) was also determined by FACS analysis. (D) Immunofluorescent staining of KCs (Day 14) with anti-mouse F4/80 and Ki67 antibody. Nuclei were counterstained using Hoechst 33258. The lower row of photomicrographs showed a higher magnification of the yellow frames in the upper row. Data were analyzed using a paired t test. Bars = means ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. FACS: Fluorescence-activated cell sorting; GM-CSF: granulocyte-macrophage colony stimulating factor.

The results indicated that infusion of KCs significantly reduced the αSMA-positive signals, suggesting a reduction in the activation of HSCs (Fig. 5C, F). We also detected the expression of TGFβ, a cytokine associated with the activation HSCs[2]. The TGFβ mRNA level of the liver decreased in KCs-recipient mice (Fig. 4D). Thomas et al.[8] and Ma et al.[9] suggested that infusion of BMDMs can recruit the host effector cells improving liver fibrosis. Here, we sorted the F4/80$^+$ cells from the liver after the infusion of KCs (Supplemental Fig. S4A), and found that the mRNA level of matrix metalloproteinases (MMP) 2, MMP9, and MMP13 increased in endogenous F4/80$^+$ cells from KCs-recipient mice (Supplemental Fig. S4B). These results suggested that infusion of KCs expanded *in vitro* could attenuate CCl_4-induced liver fibrosis in mice.

Discussion

It is believed that KCs may have the capacity to self-renew through proliferation, probably due to colony stimulating factors[13,19]. This hypothesis, however, requires further investigation. In this study, we observed that cytokine GM-CSF significantly improved the purity of cultured KCs and promoted KCs proliferation *in vitro*. The expansion of KCs *in vitro* in the present of GM-CSF provided another additional evidence for KCs self-renewal.

KCs possess several important functions in the steady state, including providing barrier function against gut-derived bacteria and scavenging of damaged/aged erythrocytes[11]. What happens to KCs during liver injury? It is believed that the necrotic hepatocytes activate KCs via Toll-like receptor signaling resulting in the recruitment of circulating monocytes to the liver, where monocytes differentiate into CD11b$^+$F4/80$^+$ macrophages[5]. However, recent studies have shown in acute liver injury there is a substantial loss of CD11b^{low} F4/80$^+$ KCs at peak injury[19]. We also observed that CD11b^{low} F4/80$^+$ KCs significantly decreased, but CD11b^{high} F4/80$^+$ macrophages increased in CCl_4-induced acute liver injury (Supplemental Fig. S5). Interestingly, we observed that GM-CSF significantly upregulated the expression of CD11b in KCs *in vitro*. Therefore,

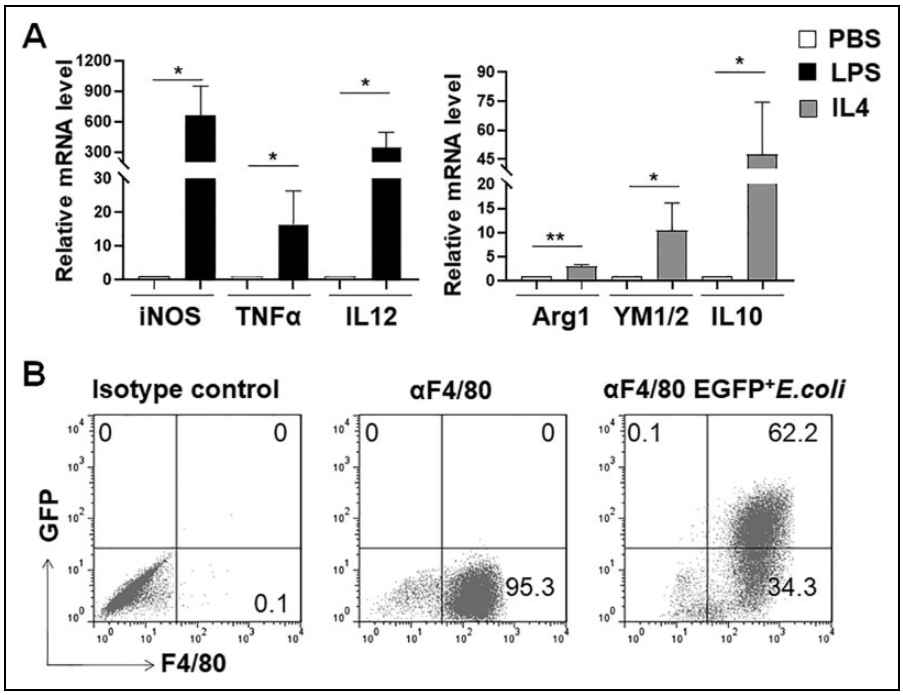

Figure 3. Kupffer cells expanded *in vitro* had the potential of M1/M2 polarization and phagocytosis. (A) KCs were stimulated with LPS (M1) or IL4 (M2), and the expression of key markers were determined by qRT-PCR, iNOS, TNFα, and IL12 as markers of M1, Arg1, YM1/2, and IL10 as markers of M2 Polarization ($n = 4$). (B) KCs (1×10^5) were cocultured with *E. coli* BL21 (1×10^6) transformed with an EGFP-expressing plasmid for 2 h. Subsequently, the cells were washed, stained with anti-mouse PE-F4/80 and analyzed by FACS. Data were analyzed using a paired *t*-test. Bars = means ± SD, $^*P < 0.05$, $^{**}P < 0.01$. FACS: Fluorescence-activated cell sorting.

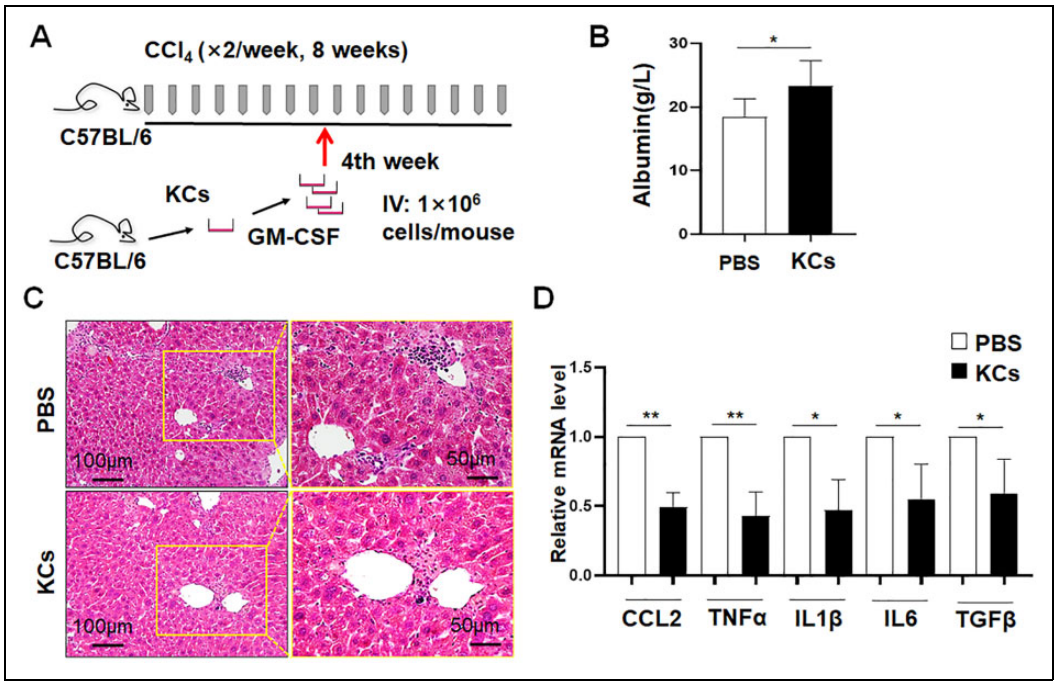

Figure 4. Infusion of Kupffer cells expanded *in vitro* attenuated liver inflammation in carbon tetrachloride (CCl_4)-induced fibrotic mice. (A) Schematic representation of the experimental procedure. Mice were injected i.p. with CCl_4 twice per week for 8 weeks, and infused with PBS or KCs expanded *in vitro* (1×10^6 cells/mouse) after the fourth week. Mice were sacrificed 72 h after the last CCl_4 injection. (B) Serum albumin levels of the mice were measured ($n = 6$). (C) Liver sections were stained by H&E staining and showed less inflammatory cells in the portal region of livers from KCs group mice compared with PBS group. (D) Fibrotic mice infused with KCs showed lower mRNA expression of CCL2, TNF-α, IL1β, IL6, and TGFβ in the liver ($n = 4$). Unpaired t test for B, and paired t test for D. Bars = means ± SD, $^*P < 0.05$, $^{**}P < 0.01$.

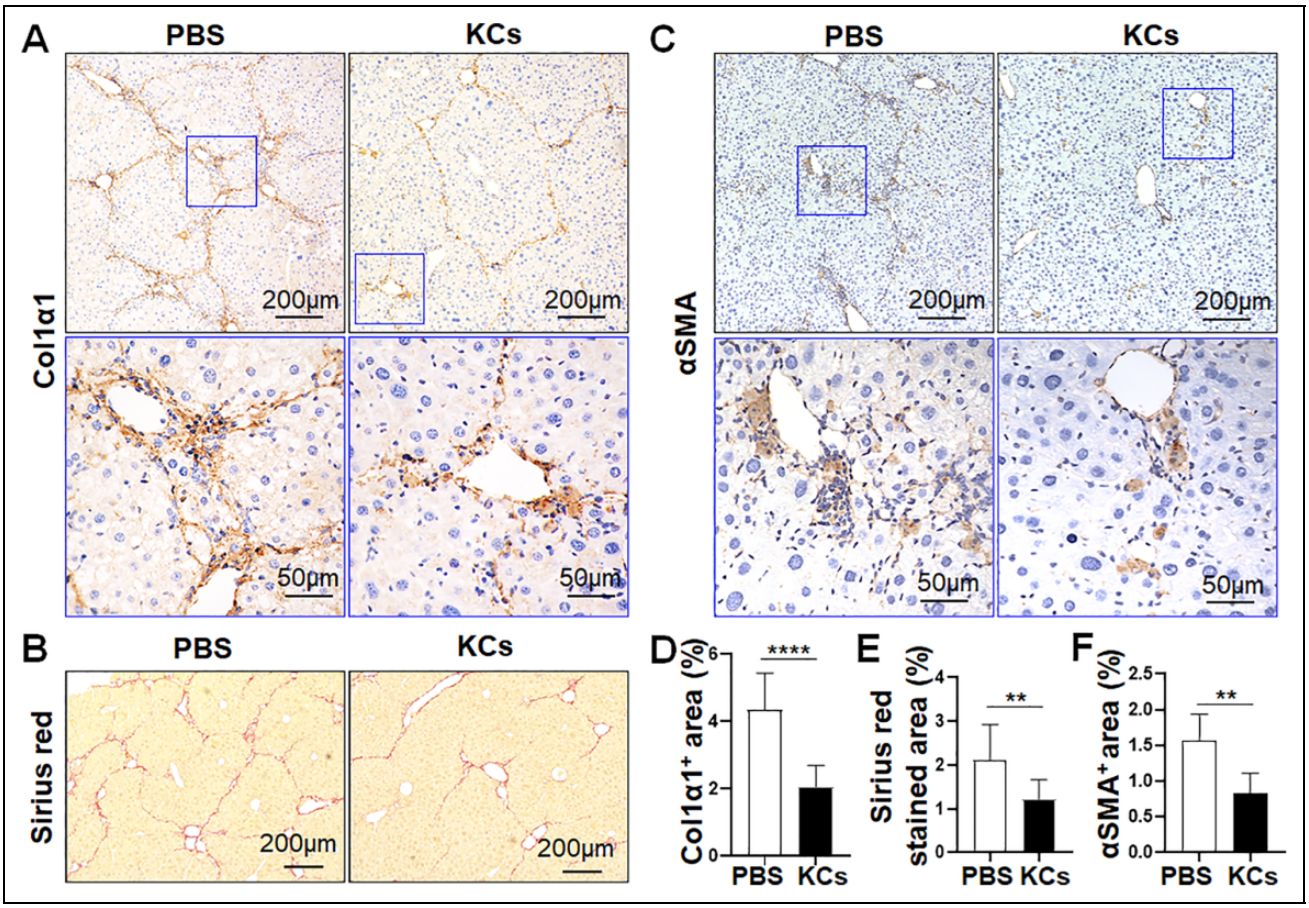

Figure 5. Infusion of Kupffer cells expanded *in vitro* ameliorated liver fibrosis induced by CCl_4 in mice. (A) Liver sections were subjected to immumohistochemical staining for Col1α1. The lower row of photomicrographs was a higher magnification of the blue frames in the upper row. (B) Liver sections were stained with sirius red staining. (C) Liver sections were stained with anti-αSMA, the marker of the activation of hepatic stellate cells (HSCs), and the lower row of photomicrographs showed a higher magnification of the blue frames of the upper row. (D) The Col1α1-positive areas in (A) were quantitatively compared ($n = 10$). (E) The positive areas of sirius red staining in (B) were quantitatively compared ($n = 12$). (F) The αSMA-positive areas in (C) were quantitatively compared ($n = 6$). Data were analyzed using unpaired t test. Bars = means ± SD, $^{**}P < 0.01$, $^{****}P < 0.0001$.

we speculate that $CD11b^{low}$ $F4/80^{+}$ KCs may change into $CD11b^{high}$ $F4/80^{+}$ macrophages *in vivo*, which could also self-renew locally in liver injury.

Thomas et al.[8] reported that BMDMs transplantation can impair liver fibrosis in mice. Considering the expansion of KCs *in vitro*, can KCs be used as a candidate for cell therapy like BMDMs? Merlin et al.[20] reported that transplanted KCs could be re-recruited into the liver and survive over the long term. In this study, we established CCl_4-induced liver fibrosis model and observed that GFP-KCs were recruited to the injured liver. Furthermore, infusion of KCs did reduce liver inflammation and impair liver fibrosis in mice. One of the mechanisms may be that transplanted KCs could promote the expression of endogenous MMPs in hepatic macrophages, which is consistent with previous reports[9]. Therefore, KCs could be one of the new cell sources for macrophage therapy. Moreover, KCs may be applied to chimeric antigen receptor (CAR) macrophage immunotherapy for tumor. Although there are still many problems to be solved, such as the acquisition of human KCs, amplification *in vitro* and allograft rejection, KCs transplantation has the potential to treat liver fibrosis and deserves further study.

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Ethical Approval

All animal procedures used in this study were approved by the Ethics Committee of Northwest University.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the approved protocols of the Ethics Committee of Northwest University.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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