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ORIGINAL ARTICLE

Basic Study

# Umbilical cord-derived mesenchymal stem cells alleviate liver fibrosis in rats

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Data sharing statement: The technical appendix, statistical code and dataset are available from the first author Ning-Li Chai at csxlily@163.com. All the participants gave informed consent for data sharing.



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## Abstract

**AIM:** To evaluate the efficacy of umbilical cord-derived mesenchymal stem cells (UC-MSCs) transplantation in the treatment of liver fibrosis.

**METHODS:** Cultured human UC-MSCs were isolated and transfused into rats with liver fibrosis induced by dimethylnitrosamine (DMN). The effects of UC-MSCs transfusion on liver fibrosis were then evaluated by histopathology; serum interleukin (IL)-4 and IL-10 levels were also measured. Furthermore, Kupffer cells (KCs) in fibrotic livers were isolated and cultured to analyze their phenotype. Moreover, UC-MSCs were co-cultured with KCs *in vitro* to assess the effects of UC-MSCs on KCs' phenotype, and IL-4 and IL-10 levels were measured in cell culture supernatants. Finally, UC-MSCs and KCs were cultured in the presence of IL-4 antibodies to block the effects of this cytokine, followed by phenotypical analysis of KCs.

**RESULTS:** UC-MSCs transfused into rats were recruited by the injured liver and alleviated liver fibrosis, increasing serum IL-4 and IL-10 levels. Interestingly, UC-MSCs promoted mobilization of KCs not only in fibrotic livers, but also *in vitro*. Co-culture of UC-MSCs with KCs resulted in increased production of IL-4 and IL-10. The addition of IL-4 antibodies into the co-culture system resulted in decreased KC mobilization.

**CONCLUSION:** UC-MSCs could increase IL-4 and promote mobilization of KCs both *in vitro* and *in vivo*, subsequently alleviating the liver fibrosis induced by DMN.

**Key words:** Liver fibrosis; Mesenchymal stem cells; Kupffer cells; Interleukin-4; Dimethylnitrosamine; DMN



**Core tip:** Dysregulation of the M1/M2 macrophages phenotypic balance governs the pathogenesis of liver fibrosis. Flow cytometry, immunohistochemistry and liver function tests showed that umbilical cord-derived mesenchymal stem cells (UC-MSCs) could promote the mobilization of M1 Kupffer cells (KCs) into the M2 phenotype *in vivo* and *in vitro* thereby ameliorating liver inflammation and liver fibrosis. Thus, UC-MSC transfusion yielded promising results with regard to reversal of liver injury and alleviated liver fibrosis by promoting KC mobilization and hepatocyte differentiation. The application of UC-MSCs might provide a new tool for cell therapy of liver fibrosis.

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## INTRODUCTION

Liver fibrosis is attributed to the excess deposition of collagen. It is usually caused by chronic liver injury, which triggers hepatocyte apoptosis, inflammatory cell recruitment, endothelial barrier damage, increased levels of transforming growth factor β1 (TGF-β1) and activated myofibroblast, which are responsible for scar tissue formation[1]. Inflammation might be the most critical factor in the initiation and maintenance of liver fibrogenesis[1]. When the liver is injured, the damaged epithelial and endothelial cells release inflammatory mediators, and the peripheral blood inflammatory cells are recruited to the affected liver, releasing fibrosis-related mediators such as TGF-β1 and tumor necrosis factor-α (TNF-α), inducing the activation of hepatic stellate cells and as well as deposition of collagen. Anti-smooth muscle α-actin (α-SMA) is a marker of activated hepatic stellate cells (HSCs),and HSCs play key roles in the pathogenesis of liver fibrosis. It is acknowledged that liver fibrosis can be effectively reversed[1], and the promotion of the repair process is considered a therapeutic strategy for liver fibrosis.

Currently, stem cell therapy is considered a promising treatment for various liver diseases, with most studies yielding positive results[2]. Mesenchymal stem cells (MSCs) are the most commonly used stem cells in transplantation. They are multipotent, non-hematopoietic progenitor cells that can differentiate into multiple lineages and have been applied in tissue regeneration and repair. Their hypo-immunogenicity and potential immunomodulatory capacity ensure that the MSCs have clinical value[2]. Increasing evidence suggests that MSCs contribute to the direct production of new hepatocytes[3,4]. Among MSCs, the umbilical cord-derived MSCs (UC-MSCs) possess an excellent proliferative potential, and their low immunogenicity and ease of preparation make them a good choice for use in future clinical studies[5]. Previous studies have shown that UC-MSCs are a well-tolerated therapy. They have the potential to improve the liver function and reduce ascites and mortality, especially in hepatitis B virus patients with decompensated liver cirrhosis[6] and liver failure[7]. Although the effects of UC-MSCs on liver fibrosis had been confirmed in many studies, the detailed mechanism remains unclear.

TGF-β1 is a potent fibrogenic cytokine, playing an important role in the activation of fibrogenic myofibroblasts. In fibrosis, its major source is the Kupffer cells (KCs; liver resident macrophages)[8]. Many clinical and experimental data have indicated that the activation of KCs is the key step in the initiation of liver injury[9-11]. Macrophages are divided into two major cell subpopulations: classically activated proinflammatory M1 macrophages and alternatively activated anti-inflammatory or wound repair M2 macrophages. The M1 type is induced by interferon γ (IFNγ), TLR-4 ligands and bacterial infection, while the M2 type is mostly induced by Interleukin-4 (IL-4), IL-10 or TGF-β[12]. Several studies[13-15] have demonstrated that when the liver is injured, these two functionally distinct macrophage types will be recruited to it. During the injury phase, pro-fibrogenic macrophages (M1) promote myofibroblast proliferation and apoptosis. In contrast, during the injury repair phase, the M2 macrophages predominate and mediate matrix degradation[16]. Some papers have confirmed that M2 macrophages are present during the injury repair phase when the levels of pro-fibrogenic and inflammatory mediators are decreasing[13]. Therefore, the disequilibrium between M1 and M2 macrophages appears to be the major pathogenesis that induces liver fibrosis. Strategies for restraining M1 macrophage mobilization or encouraging the M2 macrophage phenotype might prevent liver injury and thus alleviate liver fibrosis.

The goal of our study was to evaluate the efficacy of UC-MSCs transplantation to treat liver fibrosis in

rats. Furthermore, because activation of KCs is the key step in the initiation of liver injury, we were also interested in the influence of UC-MSCs transplantation on the mobilization of KCs and its mechanism.

## MATERIALS AND METHODS

### *Isolation, culture and identification of human UC-MSCs*

Research protocols that involved human participants were reviewed and approved by the Local Ethics Committee of Chinese PLA Medical Academy (Beijing, China). Written informed consent was provided by each participant in advance. Human umbilical cord samples were obtained from umbilical veins immediately after cesarean section, with the mother's consent, at the Chinese PLA Medical Academy of the PLA general hospital. We mixed the umbilical cord samples with HetaSep solution (Stemcell Technologies, Vancouver, BC, Canada) at a ratio of 5:1 and incubated the mixture at room temperature to deplete erythrocytes. We then collected the supernatant carefully and used Ficoll density-gradient centrifugation at 398 × *g* for 20 min to obtain the mononuclear cells. We washed the cells once or twice in phosphate-buffered saline (PBS) and seeded them into plates at a density of 2 × $10^5$ to 2 × $10^6$ cells/$cm^2$. We incubated these cells in a humidified atmosphere containing 5% $CO_2$ at 37 ℃. The cells were then resuspended in 10 mL MSC medium (Alpha Modified Eagles Medium, Invitrogen, Carlsbad, CA, United States), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 10% horse serum (Invitrogen), and 5 mg/mL streptomycin and 5 IU/mL penicillin (Sigma, St. Louis, MO, United States). We collected the mixture in a 15 mL Falcon tube and centrifuged it at 238 × *g* for seven minutes at 4 ℃. The cells were then counted and added to a 75 $cm^2$ flask containing 15 mL of MSC medium. The adherent cells formed colonies and grew rapidly, exhibiting a spindle-shaped morphology. When the UC- MSCs reached 85% confluence, they were passaged, and cells at the fourth passage were used for transfusion into rats. Before transfusion, UC-MSCs were subjected to quality control, including the detection of CD14, CD19, CD34, CD44, CD45, CD73 and CD105 by flow cytometry analysis, and bacteriological testing.

Standard osteogenic, adipogenic and chondrogenic assays were used to assess their differentiation potential. Osteogenic differentiation of confluent UC-MSCs monolayers obtained as described above was induced using 100 nmol/L dexamethasone, 0.05 mmol/L L-ascorbic acid-2-phosphate and 10 mmol/L β-glycerophosphate (all from Sigma). Alizarin red staining using a Sigma kit was performed to observe calcium deposition in the cultures. Adipogenic differentiation was induced in DMEM/10% FBS supplemented with 0.5 mmol/L isobutylmethylxanthine (Sigma), 60 mol/L indomethacin (ICN, Basingstoke, United Kingdom) and 0.5 mmol/L hydrocortisone (Sigma). Accumulation of lipid vacuoles was visualized using 0.5% Oil Red-O. For chondrogenic differentiation, cells (2.5 × $10^5$) were placed in serum-free medium consisting of high-glucose DMEM, 100 μg/mL sodium pyruvate, 40 μg/mL proline, 50 μg/mL L-ascorbic acid-2-phosphate, 1 mg/mL BSA, 1 × insulin-transferrin-selenium plus, 100 nmol/L dexamethasone (all from Sigma) and 10 ng/mL transforming growth factor-β3 (TGFβ3; R&D Systems, Abingdon, United Kingdom). Cell culture media were replaced every other day. Micromasses were harvested at week 3, and frozen sections (5-μm thick) were prepared.

### *Flow cytometry analysis*

Low- and high-passage MSCs were analyzed by flow cytometry. Briefly, 2 × $10^5$ to 2 × $10^6$ cells were plated into each well of a round-bottom 96-well plate. After washing with 0.1 mol/L PBS containing 1% bovine serum albumin (Sigma) and 0.1% azide (Sigma), cells were resuspended in 30 μL of primary antibodies against CD14, CD19, CD34, CD44, CD45, CD73, and CD105 (Santa Cruz Biotechnologies, Dallas, Texas, United States) for one hour at 4 ℃, respectively. The cells were then rinsed twice and incubated in secondary antibody conjugated to AlexaFluor488 (Invitrogen) for one hour at 4 ℃. Then, cells were rinsed twice, fixed using 4% paraformaldehyde for ten minutes on ice, and stored at 4 ℃ until analysis on a flow cytometer (BD Biosciences, San Diego, CA, United States).

### *Animal model of liver fibrosis and UC-MSCs transplantation*

All of the Sprague-Dawley (SD) rats chosen were male and weighed approximately 160 g. The rats were caged and raised under 12-h light-dark cycle in the specific pathogen free (SPF)-grade animal room of the experimental animal center of PLA General Hospital. Standard rat chow and water were provided for the rats. Our study was carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health strictly. The Local Ethics Committee of the Chinese PLA Medical Academy approved the protocol (Permit Number: 2012-32).

To establish the animal model of hepatic fibrosis, 48 male SD rats were randomly divided into control (*n* = 12) and dimethylnitrosamine (DMN) -treatment groups (dimethyl nitrosamine, Tianjin Chemical Reagent Research Institute, Tianjin, China) (*n* = 36). In the DMN-treatment group, all of the rats received intraperitoneal injection with DMN and saline mixture at a dose of 10 mL/kg for three consecutive days per week for three weeks. The DMN group was further subdivided into three groups (*n* = 12): Model group, DMN only; Transplantation group, 5 × $10^6$ UC-MSCs administered in 0.1 mL of normal saline by tail vein injection seven days after the first DMN treatment; Control group, DMN treatment + PBS administration

by tail vein. After 3 weeks, the rats were euthanized and serum samples collected for biochemical tests. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were detected using kits from Sigma. The right lobes of the livers were excised and soaked in 10% neutral formaldehyde for histology.

### *Evaluation of liver inflammation and fibrosis*

Hematoxylin and eosin (HE) and Masson's trichrome staining were performed to assess semi-quantitatively the pathogenesis of liver inflammation and fibrosis. An expert pathologist was arranged to assess the condition of liver inflammatory cell infiltration. This was a single-blind test and was based on the microscopic characteristics of the nucleus. The liver inflammation assessment focused on the number of polymorphonuclear leukocytes per 100 hepatocytes. Liver fibrosis was also assessed by an expert pathologist who graded it into five stages: Stage 0, normal connective tissue (no fibrosis); Stage 1, fibrous portal expansion; Stage 2, periportal fibrosis with short septa extending into lobules or rare porto-portal septa (intact architecture); Stage 3, fibrous septa reaching adjacent portal tracts and the terminal hepatic venule (architecture distortion, but no obvious cirrhosis); Stage 4, diffuse nodular formation (cirrhosis).

Micrographs were acquired using a Nikon Microphot-FXA microscope equipped with a Nikon Digital Camera DXM1200F. Digital images of HE and Masson staining were analyzed using the Image-Pro Plus software.

### *Immunohistochemical examinations*

Paraffin-embedded liver sections were deparaffinized, using xylene and alcohol. After hydration, slides were treated with a primary polyclonal antibody raised against α-SMA (1:200, Abcam, Cambridge, MA, United States), followed by biotinylated secondary antibody. Detection was carried out with streptavidin peroxidase, and the integrated optical intensity of α-SMA was semi-quantified using the Image-Pro Plus software.

### *Enzyme-linked immunosorbent assays*

IL-4 and IL-10 levels in serum and cell culture supernatants were measured using ELISA kits (R&D Systems), according to the manufacturer's instructions. Samples were assessed in duplicate, and the absorbance was read at 450 nm on a Thermo Fisher microplate reader (Massachusetts, America). Cytokine concentrations were calculated using standard curves generated by the plate-reader's software.

### *Isolation, purification, culture and identification of liver macrophages*

Liver macrophages were isolated from DMN-treated rats through the collagenase perfusion method. Livers were minced and immersed in Hanks balanced salt solution at 37 ℃ for 30 min. Hanks solution consisted of 24 μg/mL Liberase (Roche Diagnostics, GmbH, Penzberg, Germany) and 1.6 U/mL DNase I (Roche Diagnostics). After that, the liver pieces were homogenized and strained through a 40 μm filter. The filtrate was then plated in RPMI containing 2% fetal calf serum on regular non-tissue medium-treated Petri dishes overnight. Non-adherent cells were washed off, and the adherent cells used for quantitation after staining with F480 monoclonal (eBioscience, San Diego, CA, United States), CD11b monoclonal (Millipore, Billerica, MA, United States) and CD206 polyclonal (eBioscience) antibodies, respectively. The purity of the macrophages was estimated by flow cytometry using F480 monoclonal antibody, and purity of > 80% was obtained. A phagocytosis test with FITC-labeled ovalbumin was used to identify liver macrophages. M2 macrophages were identified as F480+/CD206+.

To observe the effect of IL-4 on macrophage mobilization, an overdose (final concentration 250 μg/mL) of an IL-4 monoclonal antibody (Santa Cruz) was incubated with cultured macrophages to block IL-4.

### *Co-culture of UC-MSCs and macrophages*

Before carrying out co-culture of UC-MSCs and macrophages, macrophages isolated from liver tissues were activated with lipopolysaccharide (final concentration of 1 μg/mL) for 18 h. Then, $1 \times 10^5$ to $3 \times 10^5$ UC-MSCs were seeded in a 24-well plate as a supporting layer, and $1 \times 10^5$ to $3 \times 10^5$ macrophages were added to the plates.

### *Statistical analysis*

All statistical analyses were performed using the SPSS Version 16 software. Differences were considered statistically significant at $P < 0.05$. Values are presented as the mean ± SD. For semi-quantitative analysis of histological staging, non-parametric tests (Wilcoxon test) were used; other statistical analyses were performed using an unpaired Student's *t*-test.

## RESULTS

### *MSCs were isolated and purified successfully*

MSCs were long spindle-shaped cells (Figure 1A) with potent proliferation activity. MSCs derived from the umbilical cord did not express CD11b, CD19, CD34, CD45 and HLA-DR (0.61%), which was confirmed by flow cytometry. However, high numbers of cells expressing CD44 (99.99%), CD73 (99.98%), CD90 (99.99%) and CD105 (99.97%) were observed (Figure 1B). In addition, MSCs had osteogenic, adipogenic, and chondrogenic capabilities (Figure 1C). These findings suggested that these cells were MSCs.

### *Liver fibrosis is alleviated by UC-MSCs transplantation*

DMN is a potent hepatotoxin that causes centrilobular

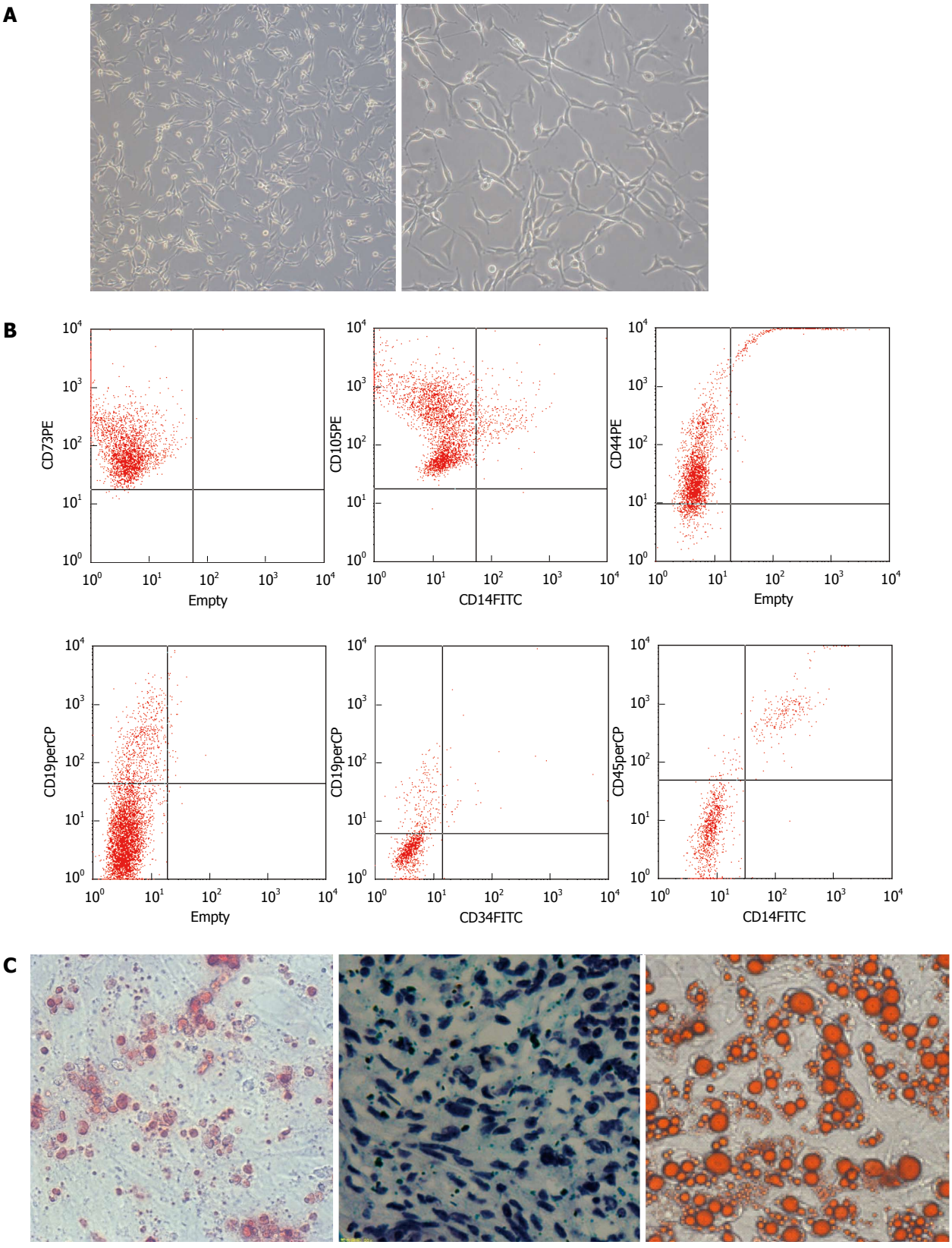


**Figure 1 Mesenchymal stem cells were isolated and purified successfully.** A: Mesenchymal stem cells (MSCs) are long spindle-shaped cells (right, magnification × 40); B: Flow cytometry showing that umbilical cord derived MSCs express high levels of CD73, CD105 and CD44, but almost no CD19, CD34, CD45 and CD14; C: The purified MSCs were capable of osteogenesis (left, magnification × 40, by Alizarin Red), chondrogenesis (middle, magnification × 40, by Toluidine Blue O) and adipogenesis (right, magnification × 40, by Oil Red-O).

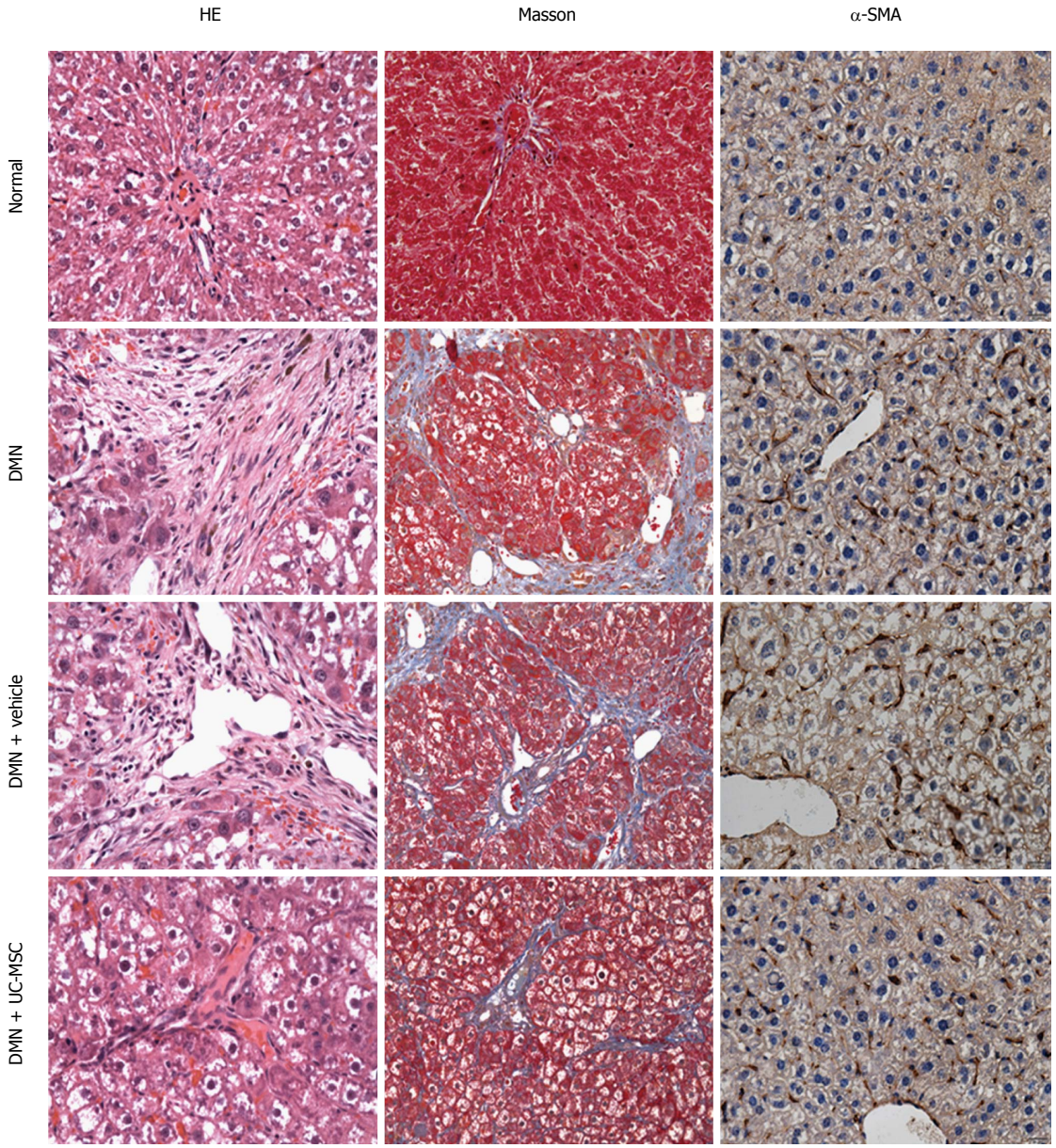


**Figure 2 Umbilical cord-derived mesenchymal stem cells transplantation alleviates liver fibrosis.** Hematoxylin and eosin (HE) staining of liver sections showing massive vacuolar degeneration of hepatocytes and intense neutrophil infiltration in DMN-treated rats and control rats (first column, HE staining). Masson's trichrome staining showed periportal fibrosis with fibrous septa extending into adjacent portal tracts and the terminal hepatic venule in DMN-treated rats and DMN + Vehicle animals (second column, Masson staining), suggesting the successful establishment of the animal model of hepatic fibrosis. Liver fibrosis and inflammation were significantly decreased by transfusion with UC-MSCs in UC-MSC-treated rats compared with the DMN + Vehicle group, which was characterized by short septa extending into lobules or portal-portal septa (first column, HE staining, and second column, Masson staining). Alpha-SMA is a marker of activated hepatic stellate cells, which plays key roles in the pathogenesis of liver fibrosis. Increased expression of α-SMA was observed in the model and DMN + Vehicle groups, and was decreased by UC-MSCs transfusion (third column, α-SMA immunohistochemical staining).

necrosis and nephrotoxic damage following peritoneal injection in rats. After treatment, rats were necropsied, and the right lobe of the liver was extracted from each animal for histology. When the liver tissue was processed with Masson's trichrome staining, periportal fibrosis was observed. The fibrous septa extended into the adjacent portal tracts and the terminal hepatic venule. In these DMN-treated rats, neutrophil infiltration and massive vacuolar degeneration were observed, which indicated the successful establishment of an animal model of hepatic fibrosis (Figure 2).

Liver fibrosis is initiated by inflammation, reflected by hepatocyte injury. Serum transaminase (ALT and AST) activities are known markers of such injury. In the DMN-treated rat model, ALT and AST levels were significantly increased compared with control rats ($P < 0.001$). Interestingly, these enzymes were significantly reduced after UC-MSCs transfusion, compared with rats in the DMN + Vehicle group ($P = 0.008$). Furthermore, liver fibrosis and cholestasis were significantly reduced by transfusion of UC-MSCs in the DMN-treated rats as compared with the DMN + Vehicle

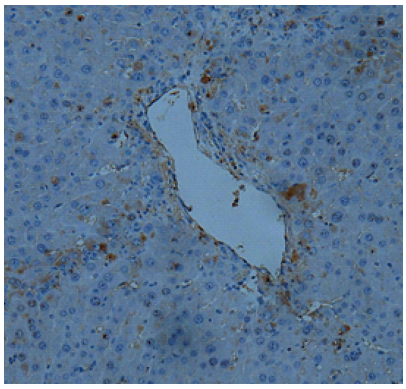

**Figure 3 Umbilical cord-derived mesenchymal stem cells transfused into rats are recruited into the injured liver.** Umbilical cord-derived mesenchymal stem cells (UC-MSCs) transfused into fibrotic liver animals were labeled with green fluorescent protein (GFP). Immunohistochemical detection with anti-GFP was performed to assess the distribution of UC-MSCs in the liver after DMN treatment. UC-MSCs were located in the injured liver together with infiltrated inflammatory cells (magnification × 40).

group, which was characterized with shorter septa extending into lobules or portal-portal septa (Figure 2). Densitometry measurements showed that the amount of collagen increased by more than 80-fold in DMN rats compared with normal rats ($P < 0.001$); after UC-MSC transfusion, this increase was reduced by about 45% compared with control rats in the DMN model ($P = 0.021$). These results indicated that UC-MSCs could ameliorate the hepatic fibrosis induced by DMN.

### *UC-MSCs transfused into rats are recruited into the injured liver*

It has been reported that US-MSCs can differentiate into hepatocytes, the main cell type in the liver. We hypothesized that UC-MSCs transfused into rats could be recruited by the injured liver, differentiate into hepatocytes, and promote recovery from liver injury. To test this hypothesis, UC-MSCs were labeled with green fluorescent protein (GFP), and immunohistochemical detection was performed to assess the distribution of UC-MSCs in the liver of DMN-treated animals. As shown in Figure 3, UC-MSCs were located in the injured liver together with infiltrated inflammatory cells, indicating that UC-MSCs transfused into rats can be recruited into the injured liver.

### *UC-MSCs transfusion promotes KC mobilization in fibrotic liver*

KCs are resident hepatic macrophages that play important roles in liver physiology by secreting inflammatory factors in response to stimulation or toxic compounds. Classical (M1) and alternative (M2) macrophages are the extreme states of macrophage phenotypes. Although M1 macrophages will trigger inflammation, such inflammation can be counterbalanced by M2 macrophages. The alternatively polarized M2 macrophages facilitate the resolution of inflammation and tissue repair.

To observe the effects of UC-MSCs on KC mobilization, UC-MSCs were transfused on day 7 into rats with liver fibrosis induced by DMN. At days 7, 14 and 21, three rats were sacrificed, respectively, and KCs were isolated, identified by positive phagocytosis test, and by CD11b and F480 expression as assessed by flow cytometry (Figure 4A-C). Furthermore, after transfusion with UC-MSCs, CD206+ KCs (M2) increased significantly in a time-dependent manner; the largest number of M2 cells appeared at day 21 (Figure 4D). Given that M2 cells are CD206+ and M1 cells are CD206$^-$, these data further confirmed that UC-MSCs transfusion could promote M1 to M2 transformation in liver fibrosis induced by DMN.

### *UC-MSCs promote KC mobilization in vitro*

We confirmed that UC-MSC transfusion promotes the mobilization of KC *in vivo*. To assess the effect of UC-MSCs on KC mobilization *in vitro*, we isolated KCs from rats treated with DMN for three consecutive weeks, and co-cultured them with UC-MSCs. KCs isolated from the same rats and cultured alone were used as the control group. Less than 4% of CD206+ M2 cells appeared in the KCs cultured alone; meanwhile, more than 80% M2 cells appeared in KCs co-cultured with UC-MSCs (Figure 5A-D). Given the anti-fibrogenic effect of M2 cells and the pro-fibrogenic effect of M1 cells, our data showed that UC-MSCs promoted KC mobilization not only *in vivo*, but also *in vitro*.

### *UC-MSCs transfusion promotes IL-4 and IL-10 production in vivo and in vitro*

After transfusion of UC-MSCs into DMN rats, serum IL-4 and IL-10 levels were assessed by ELISA. Interestingly, IL-4 and IL-10 levels declined significantly after DMN treatment, and this effect was reversed by UC-MSCs transfusion. Serum IL-4 and IL-10 levels were significantly elevated in DMN-treated rats transfused with UC-MSCs compared with animals administered with DMN alone (Figure 6A and B).

*In vitro*, we co-cultured UC-MSCs with KCs isolated from DMN rats, and cell culture supernatants were collected for ELISA: IL-4 and IL-10 levels in KCs plus UC-MSCs were, respectively, more than 3- and 4-fold higher than the values obtained for KCs cultured alone (Figure 6C).

The consistent results between *in vivo* and *in vitro* data mean that UC-MSCs promote IL-4 and IL-10 production in KCs, which subsequently resulted in KC mobilization.

### *UC-MSCs secrete IL-4 and promote KC mobilization in vitro*

We detected IL-4 production in cell culture medium of UC-MSCs by ELISA (data not shown), and confirmed that UC-MSCs promote KC mobilization, accompanied with elevated IL-4 production; therefore, we hypothesized that UC-MSCs secrete IL-4, which

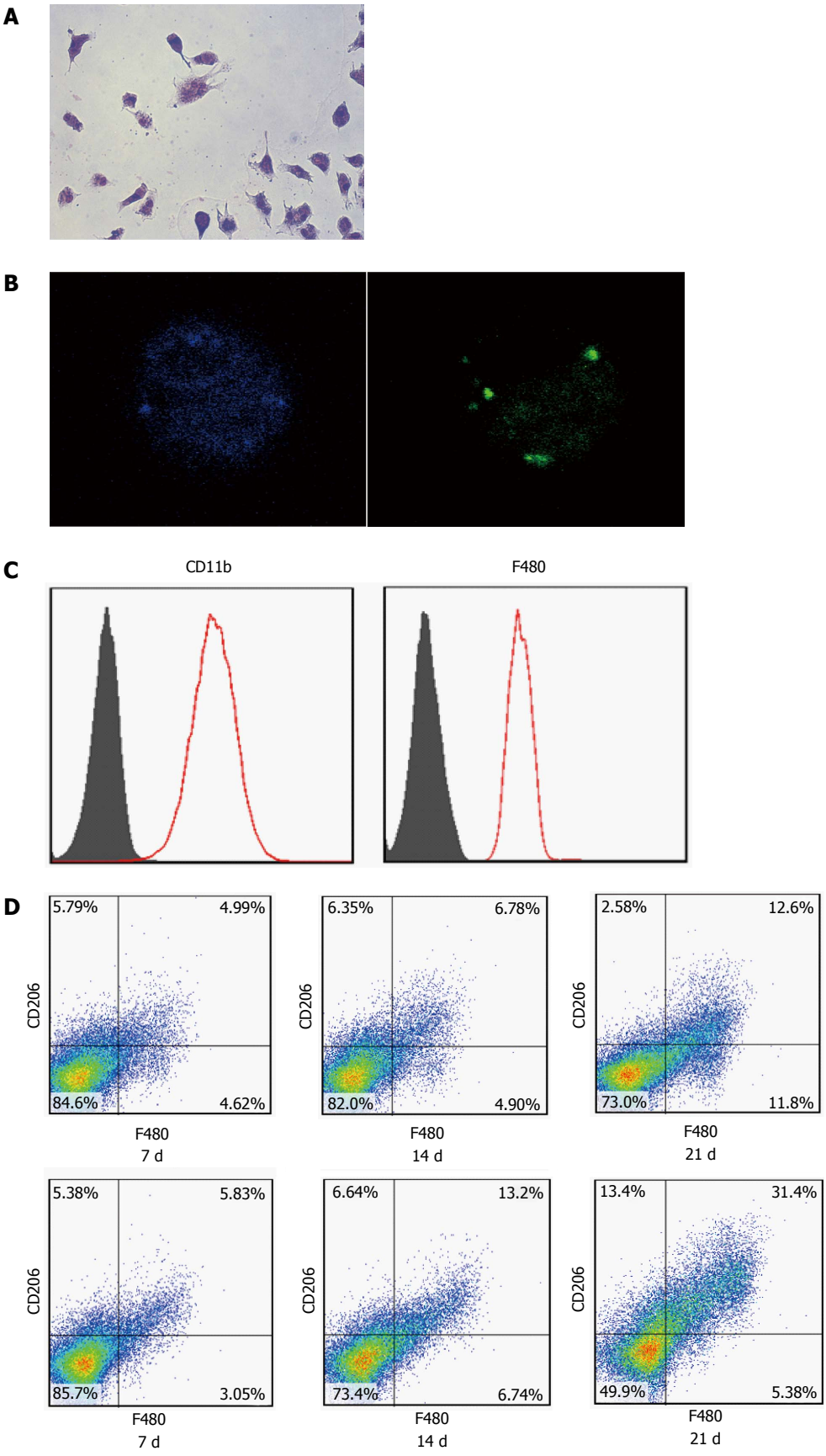


**Figure 4 Umbilical cord-derived mesenchymal stem cells transfusion promotes polarization of Kupffer cells in the fibrotic liver.** Kupffer cells (KCs) were isolated from the liver. The morphology of cells cultured with FITC-labeled ovalbumin showed green fluorescent (A, B) cells that were CD11b- and F480-positive, as assessed by flow cytometry analysis (C). After transfusion with UC-MSCs, CD206-positive KCs increased significantly in a time-dependent manner (D).

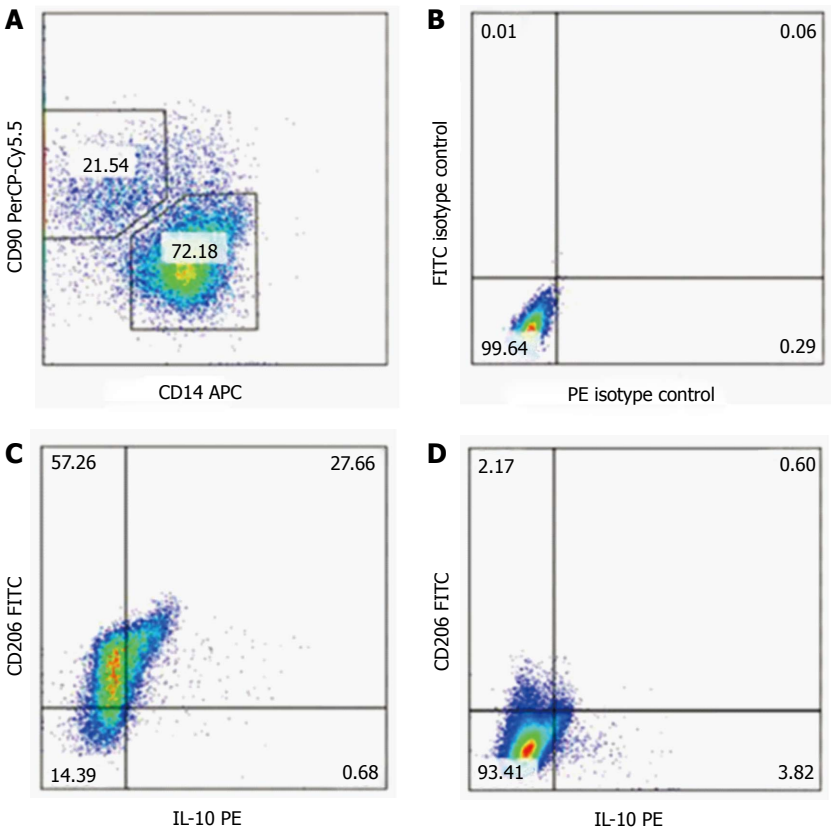


**Figure 5 Umbilical cord-derived mesenchymal stem cells promote polarization of KCs *in vitro*.** We isolated Kupffer cells (KCs) from rats treated with DMN for three consecutive weeks, and co-cultured them with umbilical cord-derived mesenchymal stem cells (UC-MSCs). KCs isolated from the same rats and cultured alone were set as the control group. Less than 4% of CD206 positive M2 cells appeared in KCs cultured alone; whereas, more than 80% M2 cells appeared in KCs co-cultured with UC-MSCs (A). Given the anti-fibrogenic effect of M2 cells, and the M1 cells' pro-fibrogenic effect, our data showed that UC-MSCs promoted KC polarization *in vitro*.

participates in KC mobilization. To test this, KCs were isolated from DMN-treated rats, and flow cytometry analysis showed that about 6% KC were CD206+ M2 cells; when UC-MSCs were added into the culture medium, the proportion of M2 cells increased to more than 70%. Interestingly, anti-IL-4 antibody treatment blocked the UC-MSCs effect and less than 30% of KCs transformed into M2 (Figure 7).

## DISCUSSION

As a heterogeneous population of cells, MSCs have the potential for multilineage differentiation. They can be isolated from various tissues, such as blood, muscle, adipose tissue, trabecular bone and even skin. Bone marrow mesenchymal stem cells (BMSCs) can differentiate into a variety of liver cells, under appropriate culture conditions[17-19]. Recent studies have demonstrated that BMSCs are useful to treat liver fibrosis and do not suffer from allograft rejection. BMSCs infusion is beneficial not only to ameliorate liver fibrosis, but also to reverse fulminant hepatic failure, which has been confirmed in rat models[20]. Many clinical studies have indicated that BMSCs are safe and effective in clinical studies. They can alleviate end-stage liver disease, and improve symptoms and liver function[21-23]. However, the invasive nature of bone marrow aspiration might limit their clinical application. Paradoxically, some studies indicated that BMSCs have the potential to promote fibrosis[24-26]. Thus, the application of BMSCs as a therapy for liver fibrosis remains controversial.

UC-MSCs are of particular interest because of their relatively easy accessibility and abundant source, making them a good substitute for BMSCs in future clinical studies. Indeed, UC-MSCs are reported to have greater proliferative capacity, lower immunological reactivity and lower risk of graft-*vs*-host disease compared with BMSCs[5]. Rosland *et al*[27] reported that the rate of BMSCs spontaneous malignant transformation during culture is 45.8% (11 of 24), concluding that spontaneous malignant transformation might represent a biohazard in long-term *ex vivo* expansion of BMSCs. Similar properties of BMSCs from both human and murine origins have been reported in other studies[28-30]. Interestingly, Tang *et al*[31] showed that human UC-MSCs propagating in continuous culture ultimately enter senescence and are not susceptible to spontaneous malignant transformation, suggesting the biosafety of expanding human UC-MSCs *in vitro* for use in regenerative medicine.

Recently, it has been reported that transfusion of UC-MSCs could improve significantly the symptoms of primary biliary cirrhosis, with few adverse effects[32].

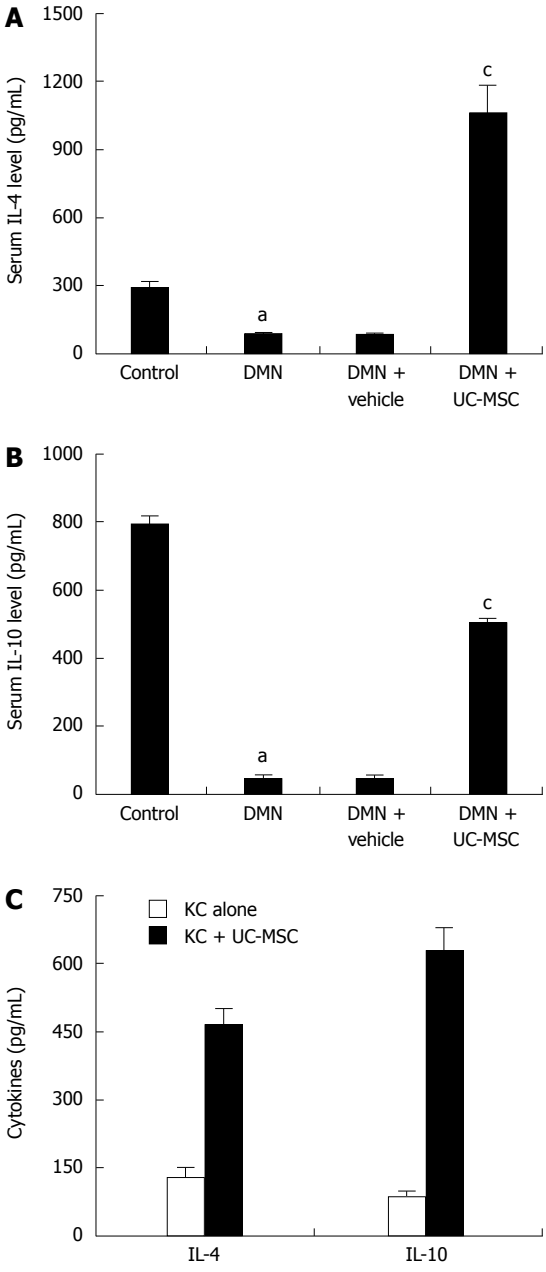


**Figure 6 Umbilical cord-derived mesenchymal stem cells transfusion promotes the production of IL-4 and IL-10 *in vivo* and *in vitro*.** Serum IL-4 and IL-10 levels were detected by ELISA. IL-4 and IL-10 levels declined significantly after DMN treatment, and this effect was blocked by umbilical cord-derived mesenchymal stem cells (UC-MSCs) transfusion. Serum IL-4 and IL-10 levels were elevated significantly in DMN rats transfused with UC-MSCs compared with rats treated with DMN alone (A and B, $^{a}P < 0.01$ compared with the control group; $^{c}P < 0.01$ compared with the DMN + vehicle group). *In vitro*, we co-cultured UC-MSCs with Kupffer cells (KCs) isolated from DMN rats, and cell culture supernatants were prepared for ELISA: IL-4 and IL-10 levels in KC plus UC-MSCs were, respectively, more than 3- and 4-fold higher than the values obtained for KC cultured alone (C). The consistent results from *in vivo* and *in vitro* experiments indicated that UC-MSCs promote IL-4 and IL-10 production in KCs, which resulted in subsequent KC polarization.

These findings suggested that UC-MSCs carry a great promise for the treatment of chronic liver disease.

MSCs have a high differentiation potential both *in vitro* and *in vivo*[17,33]. Barry *et al*[33] were the first to describe the hepatic potential of MSCs, and MSC

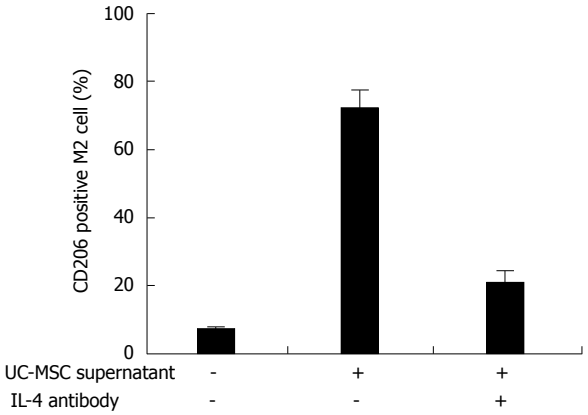


**Figure 7 Umbilical cord-derived mesenchymal stem cells secrete IL-4 and promote Kupffer cells polarization *in vitro*.** We isolated Kupffer cells (KCs) from DMN rats. Flow cytometry analysis showed that about 6% of the KCs were CD206 positive M2 cells; when umbilical cord-derived mesenchymal stem cells (UC-MSCs) were added into the culture medium, M2 cells increased to more than 70%. Interestingly, anti-IL-4 antibody treatment blocked the UC-MSC effect and less than 30% KCs became M2 cells.

transfusion could be a useful strategy for cellular therapy in liver fibrosis. Oh *et al*[34] found that two liver-specific proteins (α-feto protein and albumin) are expressed in rat bone marrow cell culture. Other studies also reported that MSCs can express albumin *in vitro*[3,4]. In the present study, green fluorescent protein (GFP) labeled UC-MSCs were transfused into rats with liver fibrosis induced by DMN. Abundant GFP positive cells were observed in DMN-induced livers 1 wk after UC-MSCs transfusion, mostly around areas with inflammatory cell infiltration. We inferred that the UC-MSCs located in the injured liver had differentiated into hepatocytes, subsequently ameliorating the DMN-induced liver injury in rats, which further supported the concept of cell therapy for the treatment of liver injury.

The therapeutic effects of stem cell transplantation on liver fibrosis and cirrhosis have been widely investigated in mice and humans; however, the underlying mechanisms remain obscure. Depending on the cytokine composition in the tissue environment, macrophages differentiate into distinct subclasses. Classically activated macrophages (M1) differentiate in presence of Th1 cytokines (*e.g.*, IFN-γ), or bacterial products such as lipopolysaccharide. M1 can trigger proinflammatory responses, which are needed to kill intracellular pathogens[35]. The alternatively activated macrophages (M2) are induced by Th2 cytokines, such as IL-4 and IL-13. They are associated with Th2-type immune responses, for example, in helminth parasite infections[35], and play an important role in protecting the organism against tissue damage[36] during inflammation. It has been reported that mobilization of KCs from the M1 phenotype to the M2 phenotype might promote recovery from liver injury[16]. However, we still know little about the mechanisms underlying the acquisition of the M2 phenotype.

Our data showed that UC-MSCs may promote M1 macrophage mobilization in liver fibrosis induced

by DMN not only *in vivo*, but also *in vitro*, indicating that the alleviation of liver fibrosis after UC-MSCs transfusion is partly attributed to an increase in the conversion of M1 macrophages into M2 macrophages.

A recent study by Wan *et al*[37] reported that polarized M2 macrophages promote M1 macrophage apoptosis *via* IL-4, uncovering a novel mechanism for M1/M2 balance regulation that relies on M2-induced M1 macrophage apoptosis.

The cytokines produced by different types of macrophages are very important for the development and function of both innate and adaptive immune responses. IL-10 is secreted by M2 macrophages[38], and its anti-inflammatory effect has been reported in various models of acute and chronic liver injury[39,40]. Furthermore, Suh *et al*[41] demonstrated that bone marrow cells can alleviate inflammation and fibrosis through the expression of IL-10. In addition, previous data[37] identified IL-10 as the mediator of the apoptosis of M1 KCs induced by their M2 counterparts, by showing that anti-IL-10 antibodies blunt the pro-apoptotic effects of IL-4 in conditioned media. Herein, we demonstrated increased M1 macrophage mobilization and improvement of liver fibrosis following UC-MSCs transfusion; elevated IL10 production in the plasma and liver were also found. Therefore, it is plausible that UC-MSCs transfusion improves liver fibrosis *via* the following mechanism: UC-MSCs promote M1 macrophage conversion into M2 macrophages, which secrete IL-10 and subsequently increase M1 macrophage apoptosis.

IL-4 is one of the markers of M2 macrophages. Milner *et al*[42] found that IL-4 production leads to substantial M2 macrophage accumulation in the liver. Recent evidence has suggested an association between M2 macrophage activity and restriction of fibrosis[36,43]. This likely explains the observation that IL-4 receptor-deficient mice cannot exhibit an intact alternative activation in KCs and will increase liver inflammation, fibrosis and death during acute schistosomiasis by *Schistosoma mansoni*[36]. By contrast, IL-4-activated M2 macrophages improved both steatohepatitis and fibrosis during experimental and human nonalcoholic fatty liver disease[44,45].

In this study, increased M2 macrophages in the liver treated with DMN were observed. In addition, reduced liver inflammation and liver fibrosis occurred following UC-MSCs transfusion. Finally, elevated IL-4 levels in serum and liver were also noted. These findings may help further understand how UC-MSCs mediate the repair process during liver damage, which could be associated with increased IL-4 production, accompanied by subsequent M1 macrophage mobilization into M2 counterparts.

IL-4 is also regarded as a proinflammatory cytokine, with direct cytotoxic effects on hepatocytes, as shown in a previous study by Guillot *et al*[46], in which lethal hepatitis was induced by transduction with recombinant adenoviruses coding IL-4 (AdrIL-4). The mortality of lethal hepatitis induced by AdrIL-4 transduction was dose-dependent in that study. The observed hepatotoxicity and lack of macrophage activation with AdrIL-4 transduction differ from what we observed in our study. Excessive elevation of IL-4 levels in the liver could explain the difference. Milner *et al*[42] considered that the hepatotoxicity of AdrIL-4 might be attributed to the adenovirus vector itself.

This study is the first to assess the therapeutic value of UC-MSCs in an animal model of liver fibrosis. We found that UC-MSCs transfusion by tail vein presents satisfactory results with regard to improved liver injury and alleviated liver fibrosis. Our results suggested that the therapeutic effects of UC-MSCs on liver fibrosis rely on the activation of hepatic macrophages (KCs), which provides a partial explanation of the mechanisms of UC-MSCs-mediated therapeutic benefit in liver disease. We also found that the therapeutic effects of UC-MSCs were, at least in part, caused by their upregulation of IL-10 and IL-4 in a well-known rat model of liver fibrosis. The application of UC-MSCs might provide a powerful new tool for cell therapy of liver fibrosis.

## COMMENTS

### Background

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) possess an excellent proliferative potential. Their low immunogenicity and ease of preparation provide these cells with a sound basis for their application in future clinical studies.

### Research frontiers

Currently, stem cell therapy is considered a promising treatment for various liver diseases. Increasing evidence suggests that MSCs contribute to the direct production of new hepatocytes. Among MSCs, UC-MSCs possess an excellent proliferative potential. Previous studies have shown that UC-MSCs are a well-tolerated therapy. They have the potential to improve liver function and reduce ascites and mortality, especially in hepatitis B virus patients with decompensated liver cirrhosis and liver failure.

### Innovations and breakthroughs

This study was the first to assess the therapeutic value of UC-MSCs in an animal model of liver fibrosis. UC-MSCs transfusion could improve liver injury and alleviated liver fibrosis. The therapeutic effects of UC-MSCs on liver fibrosis rely on the activation of hepatic macrophages (Kupffer cells, KCs), which probably delineate partly the mechanisms of UC-MSCs-mediated therapeutic benefit in liver diseases. The authors also found that the therapeutic effects of UC-MSCs were, at least in part, caused their upregulation of IL-10 and IL-4, in a well-known rat model of liver fibrosis.

### Applications

The application of UC-MSCs might provide a powerful new tool for cell therapy of liver fibrosis.

### Terminology

Transforming growth factor β1 (TGF-β1) is a potent fibrogenic cytokine, playing an important role in the activation of fibrogenic myofibroblasts. In fibrosis, its major source is the KCs (liver resident macrophages). Increased levels of TGF-β1 are responsible for scar tissue formation. Anti-smooth muscle α-actin is a marker of activated hepatic stellate cells, and HSCs play key roles in the

pathogenesis of liver fibrosis.

### *Peer-review*

This paper adds to the existing understanding of the use of stem cells to treat liver fibrosis.

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