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Original Article

Transplantation of Mesenchymal Stem Cells Attenuates Acute Liver Failure in Mice via an Interleukin-4-dependent Switch to the M2 Macrophage Anti-inflammatory Phenotype

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Abstract

Background and Aims: Transplantation of mesenchymal stem cells (MSCs) derived from bone marrow (BM) is an alternative treatment of acute liver failure (ALF) mainly because of the resulting anti-inflammatory activity. It is not known how MSCs regulate local immune responses and liver regeneration. This study explored the effects of MSCs on hepatic macrophages and the Wnt signaling pathway in ALF. ***Methods:*** MSCs were isolated from BM aspirates of C57BL/6J mice, and transplanted in mice with ALF induced by D-galactosamine (D-Gal). The proliferation of hepatocytes was assayed by immunohistochemical (IHC) staining of Ki-67 and proliferating cell nuclear antigen (PCNA). The levels of key proteins in the Wnt signaling pathway were assayed by western blotting and cytokines were determined enzyme-linked immunosorbent assays (ELISAs). A macrophage polarization assay characterized the M1/M2 ratio. The potential role of interleukin-4 (IL-4) in the biological activity of MSCs was determined by silencing of IL-4. ***Results:*** Transplantation of allogeneic MSCs significantly attenuated D-Gal-induced hepatic inflammation and promoted liver regeneration. MSC transplantation significantly promoted a phenotypic switch from proinflamatory M1 macrophages to anti-inflammatory M2 macrophages, leading to significant Wnt-3a induction and activation of the Wnt signaling pathway in mice with D-Gal-induced ALF. Of the paracrine factors secreted by MSCs (G-CSF, IL-6, IL-1 beta, IL-4, and IL-17A), IL-4 was specifically induced following transplantation in the ALF model mice. The silencing of IL-4 significantly abrogated the phenotypic switch to M2 macrophages and the protective effects of MSCs in both the ALF model mice and a co-culture model in an IL-4 dependent manner. ***Conclusions:*** *In vivo* and *in vitro* studies showed that MSCs ameliorated ALF through an IL-4-dependent macrophage switch toward the M2 anti-inflammatory phenotype. The findings may have clinical implications in that overexpression of IL-4 may enhance the therapeutic effects of allogeneic MSC transplantation in the treatment of ALF.

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Introduction

Acute liver failure (ALF) is an uncommon but life-threatening condition with high mortality.[1] Liver transplantation is an established salvage therapy for ALF, but the shortage of donors and the high cost of surgery are major considerations.[2,3] Therefore, there is an urgent need of alternative treatments for patients with ALF.

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are adult stem cells present in adipose tissue, bone marrow (BM), skeletal muscle, umbilical cord blood, and synovium.[4] MSCs are good candidates for transplantation as they are easily obtained from a variety of tissues and expanded in culture. Unlike other types of stem cells (e. g. embryonic stem cells), MSCs avoid immune rejection and ethical issues related to stem cell transplantation.[5] In addition to self-renewal and differentiation along multiple lineages, MSCs modulate innate and adaptive immune responses through secretion of mediators like transforming growth factor beta (TGF-β), indoleamine 2,3-dioxygnase, cyclooxygenase 2 (COX2), prostaglandin E2 (PGE2), interleukin (IL)-4, and TNF-alpha stimulated gene (TSG)-6.[6] Accumulating evidence indicates that MSCs

Keywords: Acute liver failure; Mesenchymal stem cells; Interleukin 4; Macrophage; Wnt signaling pathway.

Abbreviations: ALF, acute liver failure; ALT, alanine transaminase; AST, aspartate aminotransferase; BM, bone marrow; COX2, cyclooxygenase 2; D-Gal, D-galactosamine; IL-4, interleukin-4; INOS, nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; MSCs, mesenchymal stem cells; NH_3, serum ammonia; PBS, phosphate buffered saline; PGE2, prostaglandin E2; PT, prothrombin time; TBS, tris buffered saline; TGF-β, transforming growth factor β; TSG-6, TNF-α-stimulating gene 6; TUNEL, TdT-mediated dUTP nick-end labeling.

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can exert immunosuppressive effects, such as anti-inflammatory activity, which is why MSC transplantation has been used to treat inflammatory diseases.[7] For example, allo- and autotransplantation of MSCs by portal vein injection to reduce D-galactosamine (D-Gal)-induced liver failure,[8,9] which is a widely used model of immune-induced hepatic failure.[10] From the perspective of clinical application, MSCs obtained by *in vitro* expansion have advantages compared with autologous transplantation. However, the mechanisms, including those underlying MSCs-mediated attenuation of D-Gal-induced liver failure, are complex and remain to be elucidated. In this study, we performed *in vivo* and *in vitro* investigations of the mechanisms underlying the therapeutic effects of MSCs on ALF induced by D-Gal in mice.

Methods

Experimental animals

C57BL/6J mice were purchased at 4–6 weeks of age from the Experimental Animal Center of Drum Tower Hospital, Nanjing University of Medical School (Nanjing, Jiangsu, China). The mice were maintained on a 12 h light/dark cycle in a room with suitable air pressure and temperature, and given axenic water and sterile standard pellet feed. The mice were used for preparation of MSCs and *in vivo* studies and were sacrificed by cervical dislocation on completion of the study procedures. The protocols involving experimental mice were reviewed and approved by the institutional animal care and use committee of Nanjing University (Nanjing, Jiangsu, China) and conducted following the National Institutes of Health (NIH) laboratory animal care and use guidelines.

Isolation and characterization of MSCs

MSCs were isolated from fresh BM aspirates of C57BL/6J mice. Briefly, the soft tissues surrounding the femur, humerus, and tibia were cut to expose the BM cavity, followed by washing three times with phosphate buffered saline (PBS). The BM was collected and centrifuged at 1,200 rpm for 5 m. The cell pellet was resuspended in low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin and cultured in a 37°C incubator with 5% CO_2. When the cells reached approximately 80% confluence, they were passaged. MSCs were used for transplantation at passages 3 to 6. The surface markers (e.g., CD29, CD44, CD45, CD90) were assayed by flow cytometry (FACScan; Becton Dickinson, San Diego, CA, USA).

Isolation and identification of hepatic macrophages

Hepatic macrophages were isolated from the fresh liver tissues of C57BL/6J mice as previously described.[11] In brief, liver tissue was cut into small pieces and digested by incubating in 10 mL Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco, Grand Island, NY, USA) medium containing 0.1% type IV collagenase at 37°C for 30 m. The resulting tissue homogenate was filtered through 70 μm stainless steel mesh to remove undigested tissue, and the cell suspension was centrifuged at 300×*g* (Eppendorf 5810R, Germany) at 4°C for 5 m. The cell pellet was resuspended in 10 mL RPMI 1640 and centrifuged at 300×*g* for 5 m at 4°C. The pellet cells were resuspended in 10 mL RPMI 1640 and centrifuged at 50×*g* for 3 m at 4°C. The cell pellet was resuspended in 10 mL RPMI 1640, and centrifuged at 50×*g* for 3 m at 4°C. The aqueous phase (clarified cell suspension) was transferred to a 10 mL tube, and centrifuged at 300×*g* for 5 min at 4°C. The pellet was resuspended and seeded in six-well plates at a density of $1–3\times10^7$ cells/well for culture in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin in an 37°C incubator with 5% a CO_2 atmosphere for 2 h. Nonadherent cells (macrophages) were then harvested by gentle washing with PBS. The prepared hepatic macrophages were characterized by flow cytometry (FACScan; Becton Dickinson, San Diego, CA, USA) using a PE-Cyanine7-labeled anti-F4/80 monoclonal antibody (25-4801; eBioscience, USA) as previously described.

Co-culture of MSCs and macrophages

For co-culture of MSCs and macrophages, hepatic macrophages were isolated from normal or ALF mouse livers and plated at 4×10^5 cells/well in the lower chamber of Transwell six-well plates with 0.4 μm pore membranes (Corning Inc., Corning, NY, USA). Macrophages were seeded in the upper chambers at 2×10^5 cells/well. Macrophages cultured alone and co-cultured with MSCs with short hairpin IL-4 knockdown were controls. After incubation for 24 h, hepatic macrophages were collected for flow cytometric analysis.

Mouse model of ALF and MSC transplantation

C57BL/6J mice 4-6 weeks of age were used to establish a mouse model of ALF induced by intraperitoneal injection of D-Gal (0.6 g/kg). The C57BL/6J mice were randomly assigned to MSCs transplantation (n=20) or PBS transplantation (n=20). Twelve hours after induction of ALF, mice in the MSCs transplantation group were injected with 1×10^6 MSCs suspended in 0.5 mL PBS through the hepatic portal vein (MSCs group). Mice in the PBS-treatment group were injected with 0.5 mL PBS alone, and normal C57BL/6J mice without any transplantation were the control group.

To examine effects of MSC transplantation on survival and ALF, and to study the mechanisms, 10 mice were randomly selected from each of the MSC group and control groups. Blood and liver samples were collected at 24, 48, 72, 96, 120, 144, and 168 h after transplantation in the MSC and PBS groups. To investigate the hepatoprotection of Wnt-3a, 10 μg/kg recombinant Wnt-3a (R & D Systems, Minneapolis, MN, USA) in DMEM (n=10) and 0.1μM Wnt-C59 (SelleckChem, Houston, TX, USA) in with 0.5 mL 0.5% methylcellulose and 0.1% Tween-80 (n=10) were given by intravenous injection 24 h after injection of D-Gal.

Blood biochemistry

Blood samples were collected from the vena cava and centrifuged at 3,000×*g* for 10 m. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), ammonia (NH_3), and prothrombin time (PT) were assayed with an autoanalyzer (Fuji, Tokyo, Japan).

Cytokine assays

To evaluate the anti-inflammatory effect of MSCs transplantation, serum cytokines, including tumor necrosis factor alpha (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-10, and IL-17A, were determined by

enzyme-linked immunosorbent assay (ELISA) kits on day 3 after induction of ALF.

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using Superscript II reverse transcriptase sit (Invitrogen). qRT-PCR was performed to determine the relative mRNA expression of the genes of interest using Power SYBR Green PCR Master Mix (Takara, Tokyo, Japan). mRNA expression was calculated with the $2^{-\Delta\Delta CT}$ method and normalized to β-actin. The primer sequences are listed in Supplementary Table 1.

Labeling MSCs with DiR to track the transplanted MSCs

MSCs were incubated with 50 μmol/L DiR buffer (Fanbo Biochemicals, Beijing, China) at 37°C for 20 m. The DiR-labeled MSCs were centrifuged at 453×*g* for 5 m and resuspended in PBS, following the manufacturer's protocol. *In vivo* imaging was performed with an *in vivo* imaging system using a charged-coupled device camera (IVIS Spectrum, Caliper Life Sciences, Runcorn, Cheshire, UK) on day 3 after MSC transplantation. Living Image version 4.3.1 (Caliper Life Sciences, Hopkinton, MA, USA) was used to analyze the image data.

Western blot assay

Expression of non-phospho-β-catenin (active β-catenin), phospho-β-catenin, HGF, c-Myc, Cyclin D1, TCF1, and LEF1 was assayed by western blotting. Briefly, total protein, cytoplasmic protein, and nuclear protein were extracted from total liver tissue lysates and the cytoplasmic, and nuclear fractions following the kit manufacturer's instructions (Active Motif Company, Carlsbad, CA, USA). Protein concentration was determined with a bicinchoninic acid (BCA) assay (Sigma-Aldrich, St Louis, MO, USA). The protein extracts were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membrane were blocked with 5% (w/V) fat-free emulsion in Tris buffered saline (TBS) containing 0.05% Tween 20, and then incubated with primary antibodies overnight at 4°C. The membranes were the incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000 dil.). The antibody-bound bands were visualized and analyzed by electrochemiluminescence (ECL) and the signal intensity of each protein band was quantified with ImageJ (NIH, Bethesda, MD, USA). Primary antibodies used for western blot assays were listed in Supplementary Table 2.

Flow cytometry of M1/M2 macrophages

M1 and M2 macrophages were characterized by flow cytometry to identify their specific surface markers. The classic CD11c M1 macrophage marker and the classic M2 macrophage markers were labeled with phycoerythrin (PE)-conjugated anti-mouse CD11c (Biolegend, San Diego, CA, USA) and fluorescein isothiocyanate (FITC) anti-mouse CD206 (Biolegend, San Diego, CA). Briefly, the cells were incubated with fluorescent labeled antibody for 30 m, washed with staining buffer at 4°C, fixed in PBS containing 2% paraformaldehyde, and assayed (FACScan; Becton Dickinson) following the manufacturer's instructions.

Histological and immunohistochemical assays

Three 5 μm sections of each block of liver tissue were stained with hematoxylin and eosin (H-E) for histological evaluation. Apoptosis was assayed by TdT-mediated dUTP nick-end labeling (TUNEL) staining using a cell death detection kit (Roche, Frankfurt, Germany). The proliferation of hepatocytes was assayed by immunohistochemical (IHC) staining of Ki-67 and proliferating cell nuclear antigen (PCNA; AVIVA Systems Biology, Beijing, China). The expression of p-β-catenin, cyclin D1, and c-myc proteins was assayed by IHC staining. Semi-quantitative analysis was conducted with Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

Immunofluorescence staining of M1 and M2 macrophage phenotype biomarkers

M1/M2 macrophages were characterized by immunofluorescence staining of phenotype markers, including iNOS (an M1 marker), arginase-1 (Arg1, (an M2 marker), and F4/80 (a marker of murine macrophages). The primary antibodies were F4/80 (1:100, ab6640), arginase-1 (Arg1, 1:100, ab91279), and iNOS (1:500, ab178954 were all from Abcam and all were fluorescence labeled. Ten micrometer liver sections were blocked with a PBS blocking solution containing goat serum (Gibco) for 1 h at room temperature, and incubated with the primary antibody at 4°C overnight. The secondary antibody was added and incubated for 1h at room temperature. After washing for three times with PBS, the slides were incubated with diamidino-phenylindole (DAP for 10 m. The secondary antibodies were goat anti-rabbit IgG Alexa Fluor 488/594 (1:200, Invitrogen) and goat anti-rat IgG1 Alexa Fluor 488/594 (1:150, Invitrogen). Image Pro Plus 5.0 was used to count the number of stained macrophages and calculate the proportions of positive macrophages.

Macrophage depletion

To determine the role of liver macrophages in MSC-promoted liver regeneration, 100 μL clodronate liposomes (Cl2MBP; Vrije Universiteit, Amsterdam, The Netherlands) was injected 48 h before treatment with D-Gal.

IL-4 shRNAs and transfection

To silence IL-4 gene expression, three IL-4 specific shRNA target sequences were selected for IL-4 RNA interference short hairpin (sh)RNA1 (316–336) AAG CTG CAC CAT GAA TGA GTC; shRNA2 (181–201) AAC ACC ACA GAG AGT GAG CTC; and shRNA3 (47–67) AAT GTA CCA GGA GCC ATA TCC. The sequence of the IL-4 mismatch shRNA1 was AAG AGT AAG ATC CAC GTC. The negative control scrambled shRNA was from Ambion (Austin, TX). Lentiviral vector systems were used to deliver IL-4 shRNAs into MSCs to silence IL-4 gene expression following the manufacturer's instructions.

Ethical approval

All animal procedures were performed with the approval

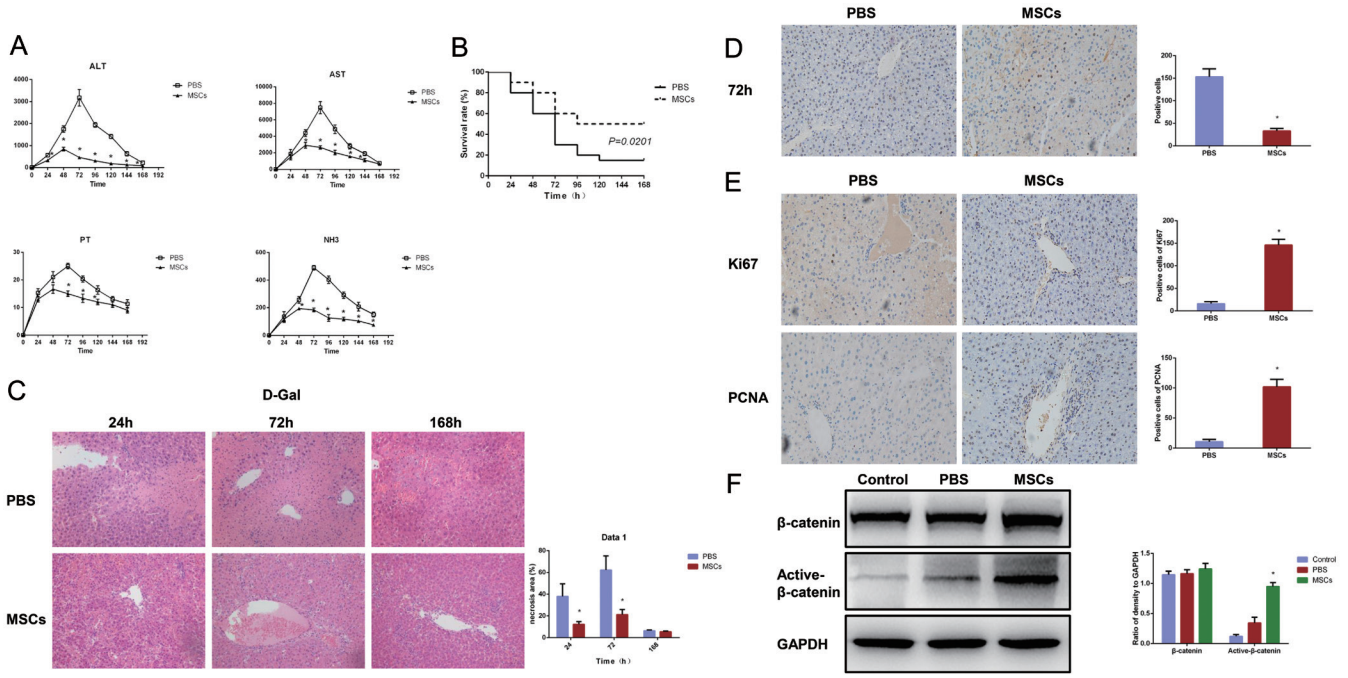

Fig. 1. Mesenchymal stem cell transplantation attenuated D-Gal-induced ALF. (A) Effects of mesenchymal stem cell (MSC) transplantation on serum levels of alanine transaminase (ALT), aspartate transaminase (AST), prothrombin (PT), and NH_3 at 24, 48, 72, 96, 120, 144, and 168 h after transplantation. Transplantation significantly decreased the levels of liver enzymes (ALT and AST), PT, and NH_3 after infusion; (B) Effects of MSC transplantation on survival included a significant reduction in mortality; (C) Hematoxylin and eosin staining of liver sections; (D) TUNEL staining assay of apoptosis; (E) Immunohistochemical staining of Ki67 and PCNA for assay of cell proliferation; (F) Western blots of β-catenin and active-β-catenin protein expression. *p<0.05 vs. phosphate buffered saline. Normal C57BL/6J mice without transplantation were controls.

of Ethics Committee for Animal Experimentation of the Affiliated Drum Tower Hospital of Nanjing University Medical School (No.20160601). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize animals suffering.

Statistical analysis

The results were expressed as means±standard derivations (SDs) from at least three independent assays. The statistical analysis was performed by Prism, version 6.0 (GraphPad Software Inc., La Jolla, California, USA). Student's *t*-test was used to evaluate between-group differences, and *p*-values <0.05 were considered statistically significant.

Results

Transplantation of MSCs attenuated D-Gal-induced ALF by promoting liver regeneration

The protective effects of MSC transplantation are shown in Figure 1A. D-Gal administration significantly elevated serum ALT, AST, PT, and NH_3, all, of which were significantly decreased in the mice with MSC transplantation compared with those given PBS (p<0.05). MSC transplantation significantly improved the survival of mice exposed to D-Gal (p<0.05, Fig. 1B). H&E staining of liver sections showed that the area of necrosis in mice with MSC transplantation was significantly smaller than that in those given PBS only (p<0.05, Fig. 1C). TUNEL analysis revealed that there significantly fewer apoptotic hepatocytes in mice with MSC transplantation than in those given PBS only (p<0.05, Fig. 1D). The proliferation assays found that there were significantly more Ki-67- and PCNA-positive hepatocytes in the MSC transplantation group than in the PBS group (p<0.01, Fig. 1E).

Western blot assays found that the expression of hepatic active-β-catenin was significantly higher in the MSCs transplantation group than in the PBS group (p<0.05, Fig. 1F). The results show that MSC transplantation attenuated D-Gal-induced liver damage in this mouse model of ALF by promoting liver regeneration, with involvement of β-catenin.

MSC transplantation promoted liver regeneration with involvement of the hepatic Wnt/β-catenin signaling pathway

Changes in the expression of proteins and mRNAs involved in the Wnt/β-catenin signaling pathway and association with liver regeneration were assayed. As shown in Figures 2 and 3, the expression of HGF, c-myc, and cyclin D1 mRNA and protein were significantly higher in the tissues from mice with MSC transplantation compared with those given PBS only (all p<0.05). The results provide additional evidence that MSC transplantation alleviated D-Gal-induced ALF by promoting liver regeneration involving the Wnt/ β-catenin signaling pathway. Wnt/β-catenin signaling modulates cytokine production. As shown in Figure 2C, cytokines levels differed significantly in mice with MSC transplantation and in those given PBS only (p<0.05). Consistent with the changes in cytokine levels, the expression of both Wnt3a mRNA and protein were significant higher in mice in the MSC transplantation in those in the PBS group (Fig. 2D, E),

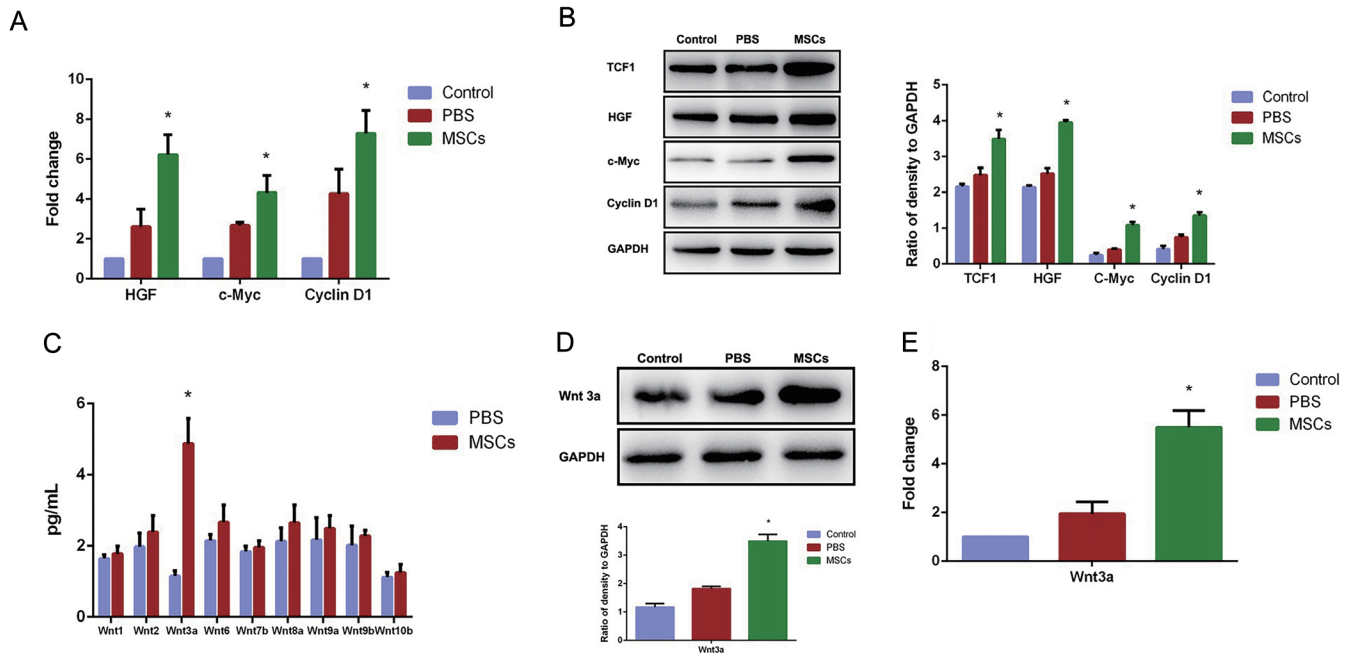

Fig. 2. Mesenchymal stem cell (MSC) transplantation increased hepatic Wnt-3a mRNA and protein expression. (A) mRNA expression of HGF, c-Myc, and Cyclin D1; (B) Western blot assays of TCF1, HGF, c-Myc, and cyclin D1 protein expression; (C) Liver cytokines associated with the Wnt signaling pathway measured by ELISA. (D) Western blots of Wnt-3a protein expression; (E) qRT-PCR assay of Wnt-3a mRNA expression. *p<0.05 vs. phosphate buffered saline. Normal C57BL/6J mice without transplantation were controls.

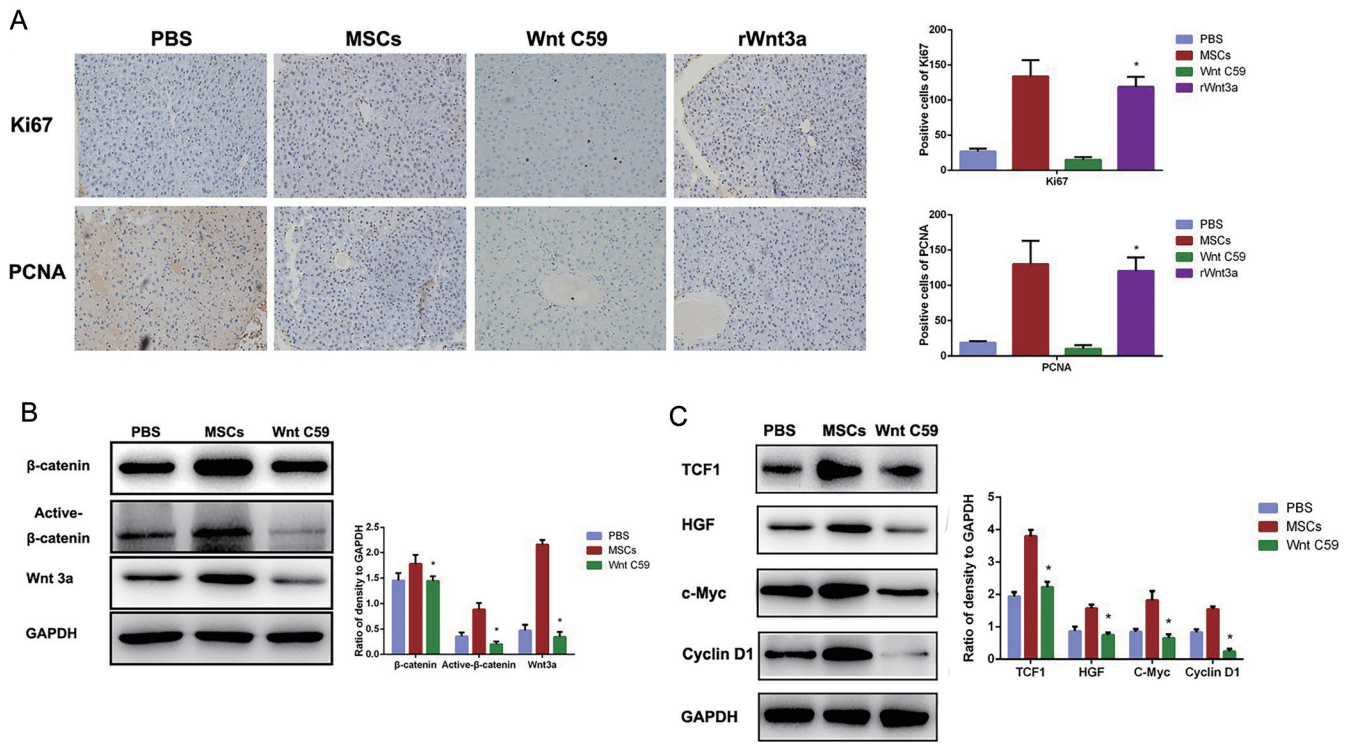

Fig. 3. Effects of Wnt-3a and the Wnt signaling inhibitor on mesenchymal stem cell (MSC) transplantation-promoted liver regeneration. (A) Immunohistochemical staining of Ki67 and PCNA (200×). Wnt-3a treatment significantly increased the number of Ki-67- and PCNA-positive hepatocytes. Wnt-C59 treatment significantly abrogated promotion of cell proliferation by MSCs. *p<0.05 vs. Wnt-C59; (B) Western blots of β-catenin, active-β-catenin, and Wnt-3a expression. Wnt-C59 significantly decreased the expression of hepatic β-catenin, active-β-catenin, and Wnt-3a proteins. *p<0.05 vs. MSCs transplantation; (C) Western blots of TCF1, HGF, c-Myc, and Cyclin D1 protein expression. Wnt-C59 significantly decreased the expression of TCF1, HGF, c-Myc, and Cyclin D1. Data are means±standard deviations. *p<0.05 vs. MSC transplantation.

which is in line with the cytokine results.

The roles of Wnt-3a and the Wnt/β-catenin signaling pathway in MSC transplantation-induced liver regeneration, mice were injected with recombinant mouse Wnt-3a or Wnt-C59, Wnt/β-catenin signaling pathway inhibitor. As shown in Figure 3, Wnt-3a significantly increased the number of Ki-67- and PCNA-positive hepatocytes ($p<0.05$), Wnt-C59 significantly abrogated the proliferation promoting effects of MSC transplantation ($p<0.05$). Wnt-C59 also significantly decreased the expression of hepatic Wnt-3a, HGF, c-myc, and cyclin D1 proteins (Fig. 3B, C). The results indicated that the Wnt/β-catenin signaling pathway was involved in the promotion of liver regeneration in mice with D-Gal-induced ALF, and that upregulation of hepatic Wnt-3a ameliorated ALF.

MSC transplantation induced a macrophage switch toward anti-inflammatory M2 phenotype in D-Gal-induced ALF

As shown in Figure 4A, the mRNA expression of F4/80, a cell surface marker of mouse macrophages as significantly increased in response to MSCs transplantation, but the mRNA expression of the markers of other immune cells in the liver remained unchanged. IHC staining (Fig. 4B) showed that there were significantly more F4/80-positive cells in MSC-transplanted mice than in those given PBS ($p<0.05$). Also, following transplantation, MSCs were found in the liver, but not detected in other organs, showing a great homing ability for the D-Gal-injured liver (Fig. 4C).

To determine the contributory roles of macrophages to the secretion of Wnt-3a after MSC transplantation, chloroethanol was used to promote inflammatory liver injury. After chloroethanol administration, no F4/80-positive cells were detected (Fig. 4D). Chlorohydrin treatment significantly increased serum levels of ALT/AST/PT/NH_3 and the extent of liver necrosis (Fig. 4E and F). Furthermore, chloroethanol significantly decreased the number of Ki-67- and PCNA-positive hepatocytes (Fig. 4G). The results suggested that a key protective role of liver macrophages was to increase Wnt-3a levels.

Changes in the expression of macrophage markers indicated that the transplantation of MSCs was associated with a switch from the M1 to the M2 phenotype. The mRNA expression of M1 markers, nitric oxide synthase (iNOS), TNF-α, and MCP-1, and M2 markers arginase 1 (Arg1), Mrc-2, and CD163 in mouse liver tissue is shown in Figure 5A. As shown in Figure 5B, Wnt-3a was localized to macrophages. Immunofluorescence staining that the number of iNOS-positive M1 macrophages was reduced and the number of Arg1-postive M2 macrophages was increased in MSC-transplanted mice compared the controls (Fig. 5C and D). The data indicated that MSC transplantation induced a switch in macrophage phenotypic from M1 to M2, leading to production of Wnt-3a.

MSC transplantation increased hepatic IL-4 expression in mice with D-Gal-induced ALF

To investigate the mechanisms underlying the switch to proinflammatory M1 macrophages anti-inflammatory M2 macrophages we looked for changes in the expression of paracrine factors secreted by MSCs, including G-CSF, IL-6, IL-1 beta, IL-4, and IL-17A. IL-4 mRNA and protein expression were both significantly elevated in the MSC-transplanted mice compared with the PBS controls. The differences in IL-6, IL-1β, and IL-17A (Fig. 6) were not significant. The findings indicate that IL-4 induction was specific to MSCs.

Knockdown of IL-4 abrogated MSC transplantation-mediated phenotype switch toward M2 macrophages and protective effects in D-Gal-induced ALF

To investigate the role of IL-4 in the switch from the M1 to the M2 macrophage phenotype, the IL-4 gene was silenced by shRNA-mediated interference. IL-4 expression was knocked down in mice treated with IL-4 shRNA compared with scrambled shRNA negative controls (Fig. 7A). IL-4 knockdown led to a significant increase in serum ALT/AST/PT/NH_3 (Fig. 7B) and the extent of liver necrosis (Fig. 7C). It did lead to a significant decrease in liver regeneration (Fig. 7D) and survival (Fig. 7E). Silencing IL-4 gene expression significantly affected the markers of M1 and M2 macrophages, resulting in increased hepatic iNOS mRNA expression (Fig. 7F), and decreased hepatic Arg1 mRNA expression (Fig. 7F). The results indicate that IL-4 was essential for the switch toward M2 macrophages and subsequent protective effects in D-Gal-induced ALF.

MSCs rely on IL4 to drive the phenotypic switch of mouse liver macrophages toward the M2 phenotype

The dependence of the transplantation-mediated phenotypic switch on IL-4 was investigated an *in vitro* model of co-cultured MSCs and mouse liver macrophages (Fig. 8A, B). Flow cytometry confirmed that the number of CD11c-postive M1 macrophages was reduced and the number of CD206-postive M2 macrophages was increased in the D-Gal (+) shRNA-IL-4 MSC (+), scrambled shRNA MSC (−) group versus the D-Gal (+) shRNA-IL-4 MSC (−) scrambled shRNA MSCs (−) group ($p>0.05$, Fig. 8C). The number of CD11c-postive and CD206-postive macrophages was significantly reduced in the D-Gal (+) shRNA-IL-4 MSC (−), scrambled shRNA MSCs-scramble (+) group versus the D-Gal (+) shRNA-IL-4 MSC (−), scrambled shRNA MSCs (−) group ($p<0.05$, Fig. 8C). The data indicate that MSC-induced phenotype switch was IL-4-dependent. Interference of shRNA-transfected MSCs which prevented the switch to M2 macrophage due to the knockdown of IL-4. The findings indicate that IL-4 was essential for the observed switch to M2 macrophages following MSC transplantation in this D-Gal-induced ALF model.

Discussion

Immune-mediated liver disease is often complicated by fulminant hepatitis or liver failure. MSC transplantation is an alternative treatment for ALF, but the therapeutic mechanisms need to be elucidated. The major novel findings of this study are: (1) MSC transplantation significantly ameliorated D-Gal-induced inflammation and stimulated liver regeneration (2) and induced a switch of hepatic macrophages from the M1 to the M2 phenotype. (3) Secretion of the paracrine factors IL-4 was specifically induced in following MSC transplantation compared with PBS control mice (4) Knockdown of IL-4 significantly abrogated the switch to anti-inflammatory M2 macrophages and attenuation of D-Gal-induced ALF. (5) Silencing of IL-4 in *in vitro* co-cultures of MSCs and hepatic macrophages showed that MSCs promoted the switch to M2 macrophages in an IL-4 dependent manner. The findings indicated that MSCs ameliorated ALF through IL-4-dependent macrophage switch toward M2 anti-inflammatory phenotype. These results are consistent with mediation of the protective effects of MSCs by increased levels of hepatic Wnt-3a, which in turn inhibited phosphorylation of β-catenin. The resulting increase of non-

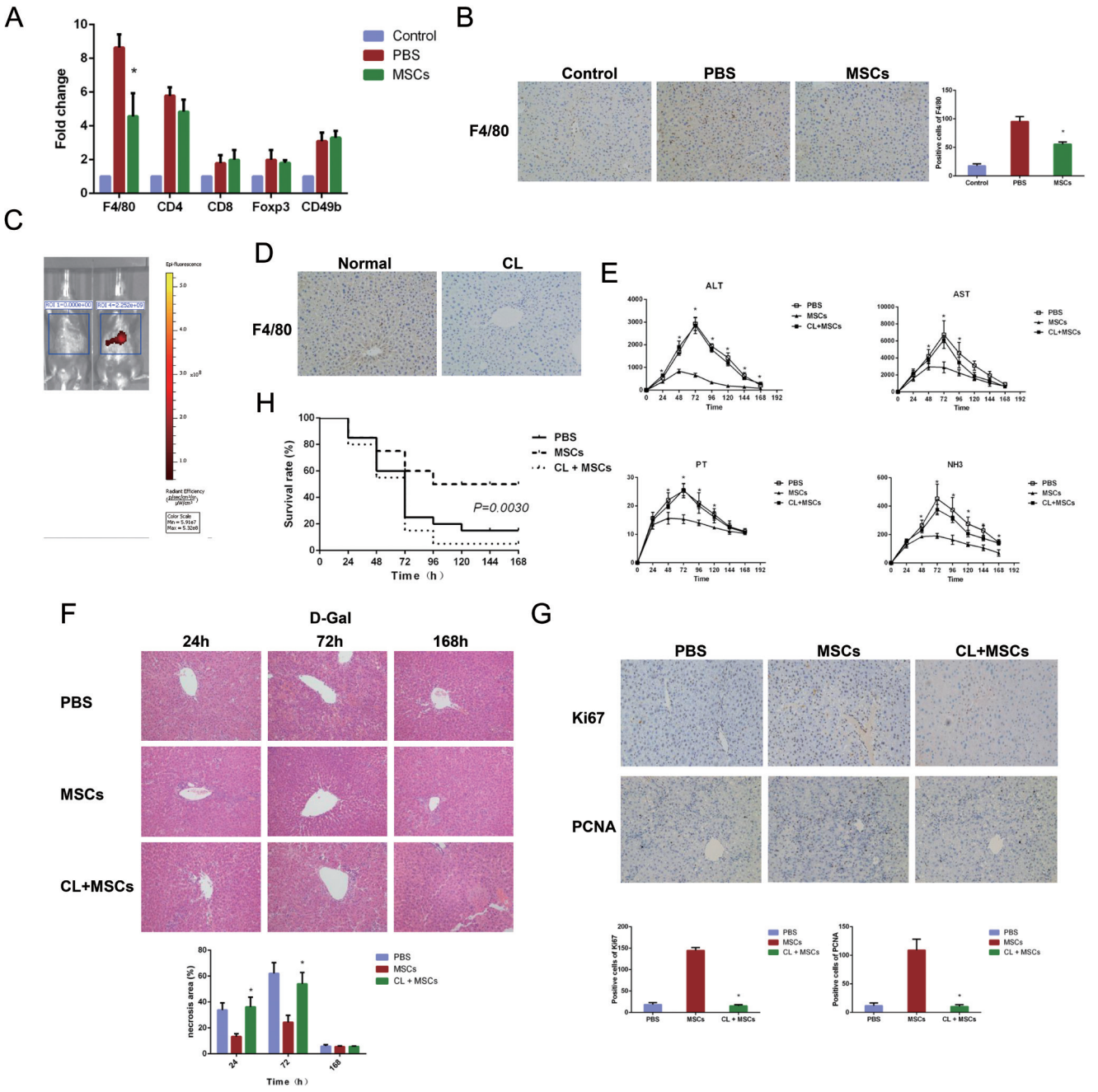

Fig. 4. Role of hepatic macrophages in mesenchymal stem cell (MSC) transplantation-mediated effects on acute liver failure (ALF). (A) F4/80, CD4, CD8, Foxp3, and CD49b mRNA expression. *p<0.05 vs. phosphate buffered saline (PBS). (B) F4/80 staining (200×). *p < 0.05 vs. PBS transplantation group. (C) IVIS was used to identify distribution of DiR-labeled mesenchymal stem cells (MSCs). (D) F4/80 staining of liver sections after chloroethanol administration (200×); (E) Serum ALT, AST, PT, and NH_3 after MSC transplantation. *p<0.05 vs. MSC transplantation. (F) Hematoxylin and eosin staining of liver tissue (200×). *p<0.05 vs. MSC transplantation. (G) Ki67 and PCNA staining (200×). *p<0.05 vs. CL+MSCs. (H) Survival analysis. *p<0.05 vs. MSCs transplantation group. Normal C57BL/6J mice without transplantation were controls.

phospho-β-catenin (active β-catenin) led to upregulation of HGF, c-myc, and cyclin D1, which promoted liver regeneration. The macrophage phenotype switch can account for the increase of Wnt-3a that was associated with MSC transplantation. Allogeneic MSC transplantation has great therapeutic potential and may be used in clinical practice in the future.

It has been reported that transplantation of autologous MSCs can prevent liver injury caused by D-Gal in animal models.[8] The transplantation of autologous MSCs to treatment patients with severe ALF is not feasible because the severity of the condition makes it challenging to obtain autologous MSCs that meet quality and quantity standards. That is the reason we used allogeneic MSCs in this study. We transplanted BM-derived MSCs into recipient mice of the same species with different genes. The results showed that allogeneic MSCs transplantation achieved protective effects

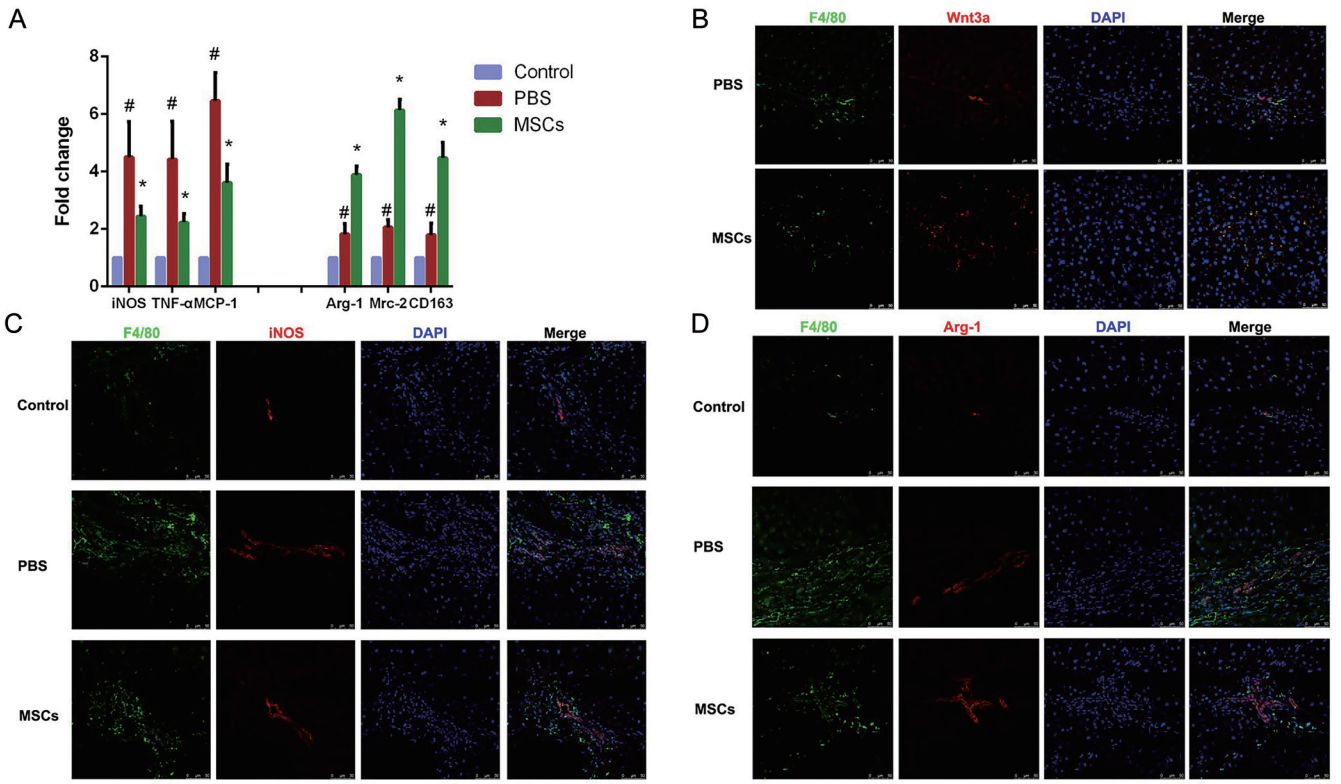

Fig. 5. Mesenchymal stem cell (MSC) transplantation induced the phenotypic switching of liver macrophages to the M2 phenotype. (A) The mRNA expression levels of nitric oxide synthase (iNOS), TNF-α, and MCP-1 markers of M1 phenotype and arginase 1 (Arg1), Mrc-2, and CD163 markers of M2 phenotype, in liver tissue. #$p<0.05$ vs. control. *$p<0.05$ vs. phosphate buffered saline (PBS). Representative images of dihydroethdium (DHE) staining of (B) Wnt-3a, (C) iNOS, and (D) Arg-1 after mesenchymal stem cell transplantation or PBS. Green and red fluorescence indicate positive staining of target proteins. Normal C57BL/6J mice without transplantation were controls.

in mice with D-Gal-induced ALF.

We found that most transplanted MSCs homed to D-Gal-injured liver tissue. Twenty-four hours after intracavitary injection, MSCs were detected in the liver, but not in any other organs. Amiri F. *et al*.[12] found that MSCs migrated to the liver within 24 h after blood transfusion, and were able to reducing liver failure. However, several other studies have reported that MSCs were mainly found in the lungs, after administration, and not in the liver.[13] Lee KC *et al*.[14] also found that most MSCs were retained in the lungs, but reduced hepatocyte apoptosis. The inconsistency may be related to different injection methods. MSCs infused through the portal vein flow into the liver first, while MSCs given by tail vein infusion flow into the lungs first. Most of the injected MSCs remain in the liver or lungs in the form of emboli. Although no MSCs were detected in the liver after intravenous injection, hepatitis was still remarkably attenuated in animal models. In this study, BM-derived MSCs had a beneficial effect on ALF. MSCs transplanted into the liver reduced the inflammatory reaction and the liver injury

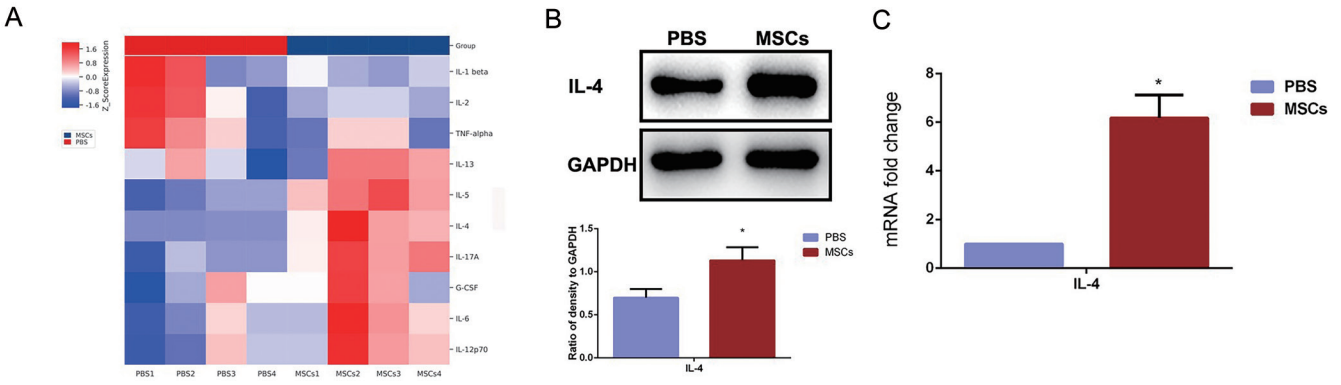

Fig. 6. Mesenchymal stem cell (MSC) transplantation increased hepatic IL-4 expression in D-Gal-induced acute liver failure (ALF). (A) G-CSF, IL-6, IL-1 beta, IL-4, and IL-17A paracrine factors secreted by MSCs determined by enzyme-linked immunosorbent assay. (B) Western blots of hepatic IL-4 protein after MSC transplantation or phosphate buffered saline; (C) qRT-PCR assay of IL-4 mRNA expression in the MSCs transplantation and control groups. *$p<0.05$ vs. PBS.

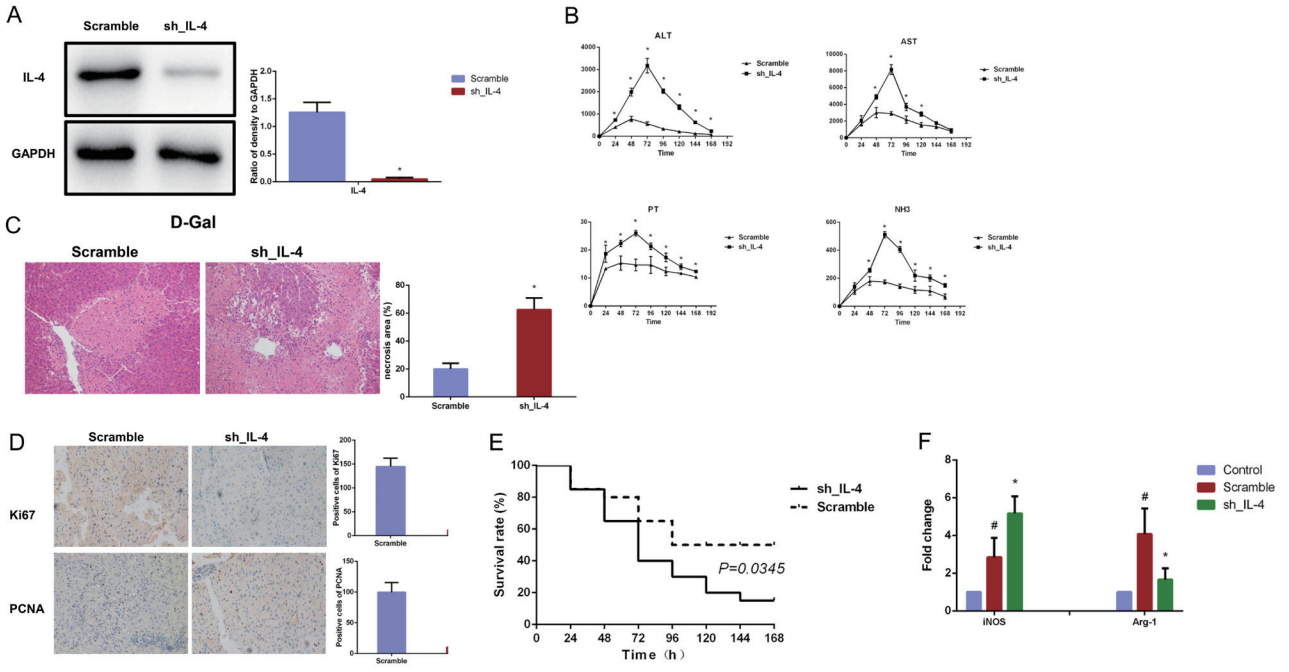

Fig. 7. Silencing of IL-4 abrogated mesenchymal stem cell (MSC) transplantation promoted a switch to the macrophage M2 phenotype. (A) Western blots of hepatic IL-4 protein in the MSCs transplantation and scrambled short hairpin RNA groups. (B) Serum alanine transaminase (ALT), aspartate transaminase (AST), prothrombin (PT), and NH_3 in the MSCs transplantation and scrambled short hairpin RNA groups. (C) Hematoxylin and eosin staining of liver tissue (200×). (D) Ki67 staining and PCNA staining (200×). (E) Survival analysis. (F) Expression of iNOS and Arg-1 mRNA. *p<0.05 vs. Scrambled short hairpin RNA negative control. #p<0.05 vs. scramble. Normal C57BL/6J mice without transplantation were controls.

by promoting a phenotypic switch to anti-inflammatory M2 macrophages, and the involvement of Wnt-3a, was supported by the finding that Wnt-c59, a Wnt-3a inhibitor, blocked the hepatoprotective effect of MSCs. The results indicate that Wnt-3a was involve in the liver protection of MSCs, especially in the regulation of macrophage polarization. In this study, it was found that the increase in Wnt-3a caused by MSCs promoted the recovery of liver injury by activating the Wnt signaling pathway, leading to upregulation of non-phospho-β-catenin expression.[15] It is well known that activation of the β-catenin pathway is indispensable for the repair of liver damage, and that β-catenin is a key protein in the regulation of the expression of cyclins A, D and E. Cyclins regulate the progression of cells from the G1 to the S phase of the cell cycle.[16,17] It has been found that within 24 h of D-Gal induced liver injury, the expression of nuclear and cytoplasmic β-catenin significantly increased in some hepatocytes. Elevated β-catenin could be the basis of liver lobule repair and promotion of liver regeneration. In this study, the increased expression of β-catenin was accompanied by increased expression of the HGF, c-myc and cyclin-D1. Consistent with previous studies, we found that the induction of β-catenin expression occurred before that of its target genes.[15]

Wnt-3a regulates the activation of various immune cells, such as macrophage phenotype transformation, and activation of regulatory T cell subsets and helper T cells. We found that the expression of Foxp3 was not changed, and that the number of CD4 T cells was significantly reduced. Therefore, the activation of Wnt-3a induced by MSC transplantation was closely related to macrophages. Moreover, Wnt-3a from macrophages rather than T cells has been shown to inhibit acute inflammation, which further supports our findings.[18] Ylostalo *et al*.[19] found that MSCs induced macrophage M2 polarization mainly through the COX2-dependent prostaglandin E2 pathway. In contrast to Ylostalo *et al*.,[19] we observed a significant increase in the expression of IL4 but not COX2 following MSC transplantation. In addition, the therapeutic effect of BM-derived MSCs was abrogated by IL-4 gene knockout, indicating that the IL4 gene was essential for the immunomodulatory activity of MSCs.

It merits attention that in our study, BM-derived MSC promoted a switch in macrophage phenotype from M1 to M2 by increasing Wnt-3a. The underlying mechanism involved production and secretion of IL-4 by BM-derived MSCs in the host inflammatory state, stimulated the reprogramming of the host liver macrophages, and increased the expression of Wnt-3a, which inhibited the liver inflammatory response and reduced liver injury.

In conclusion, the findings demonstrate that transplantation of allogeneic MSCs ameliorated ALF induced by D-Gal through an IL-4-dependent macrophage switch toward the M2 anti-inflammatory phenotype. Our findings may have clinical implications in that upregulation of IL-4 may enhance the therapeutic effects of allogeneic MSC transplantation in the treatment of ALF.

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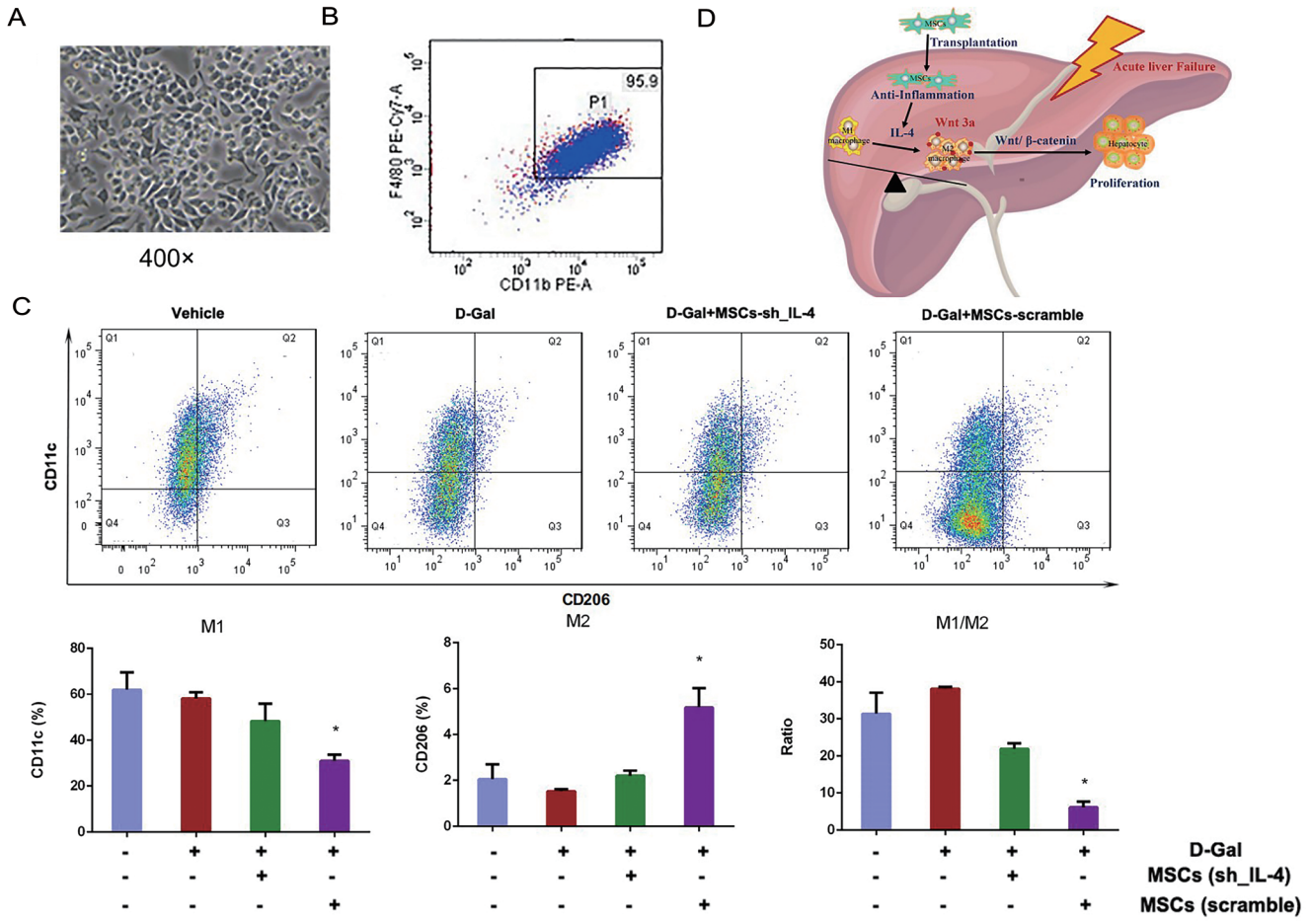

Fig. 8. Hepatic macrophage switch toward the M2 phenotype was dependent on IL-4. (A) Liver macrophages (400×). (B) Flow cytometry of liver macrophage purity. (C) Flow cytometry of the macrophage phenotype switch. (D) Graphical abstract. *p<0.05 vs. D-Gal(+) short hairpin RNA-IL-4 MSC(−), scrambled short-MSC(−) group.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived and designed the study (JW, HD, JZ, SX, XS, HR), collected the data (JW, HD, JZ, SX), assembled the data (JW, HD, JZ), performed data analysis and interpretation (JW, HD, JZ, XS, HR), wrote the manuscript (JW, HD, JZ, SX, XS, HR), provided financial support and study materials, and gave final approval of the manuscript (XS, HR). All authors read and approved the manuscript.

Data sharing statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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