

# Bone marrow mononuclear cell transplant therapy in mice with CCl<sub>4</sub>-induced acute liver failure

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**Background/aims:** Stem cell transplantation has theoretical potential for the treatment of certain liver diseases. However, the use of bone marrow mononuclear cells as a therapy for liver disease has received little attention. The present study was to examine whether bone marrow mononuclear cells might be useful in the management of acute liver failure in an animal model. **Materials and Methods:** Bone marrow mononuclear cells were harvested from BALB/c mice and then labeled with the fluorescent dye PKH26. The labeled cells were subsequently infused into the tail veins of mice in which hepatic injury had been induced by CCl<sub>4</sub> toxicity. After transplantation, the labeled cells in the liver were studied by fluorescent microscopy, and the levels of proliferating cell nuclear antigen and albumin were quantified in bone marrow mononuclear cell-treated and untreated groups. Serum aminotransferase activity was also monitored at various time points post-liver injury. **Results:** Transplanted bone marrow mononuclear cells labeled with PKH26 were found to populate the damaged liver around the portal and centrilobular regions, and they appeared to differentiate into albumin-producing hepatocyte-like cells. Animals that received bone marrow mononuclear cells also showed a trend toward improved liver enzymes as well enhanced survival rates, relative to controls. **Conclusions:** These findings suggest that systemically delivered bone marrow mononuclear cells may relocate to and be retained by the injured liver; transplantation of bone marrow mononuclear cells showed an overall beneficial effect in a murine model of acute liver failure.

**Key words:** Liver disease, bone marrow, cell transplantation

## Farelerde CCl<sub>4</sub> ile indüklenmiş akut karaciğer yetmezliğinde kemik iliği mononükleer hücre transplantasyonu terapisi

**Amaç:** Bazı karaciğer hastalıklarının tedavisinde kök hücre transplantasyonunun etkisi teorik olarak mevcuttur. Ancak, karaciğer hastalıklarının tedavisinde kemik iliği mononükleer hücrelerinin kullanımı daha az dikkat çekmiştir. Bu çalışma akut karaciğer yetmezliği hayvan modelinde kemik iliği mononükleer hücre kullanımının yararının olup olmadığının araştırılmasını amaçlamaktadır. **Gereç ve Yöntem:** Kemik iliği mononükleer hücreleri BALB/c farelerden toplanmış, floresan boya PKH26 ile işaretlenmiştir. İşaretlenmiş hücreler, CCl<sub>4</sub> ile karaciğer hasarı indüklenmiş farelerin kuyruk veninden verilmiştir. Transplantasyondan sonra, karaciğere ulaşan işaretlenmiş hücreler floresan mikroskop ile incelenmiş, bölünen hücre nükleer antijeni ve albumin düzeyleri kontrol ve kemik iliği mononükleer hücreleri almış farelerde kantifiye edilerek karşılaştırılmıştır. Karaciğer hasarından sonra serum aminotransferaz aktivitesi belirli zaman aralıkları ile ölçülmüştür. **Bulgular:** Nakledilen kemik iliği mononükleer hücrelerinin hasarlanmış karaciğerin periportal ve sentrolobuler bölgelerinde yerleştiği ve albumin üreten hepatosit benzeri hücrelere dönüştükleri tespit edilmiştir. Kemik iliği mononükleer hücreleri alan farelerin, kontrol grubu ile karşılaştırıldığında, daha iyi karaciğer enzim düzeylerine, saha iyi sağkalım oranlarına sahip oldukları görülmüştür. **Sonuç:** Bu bulgulara göre sistemik yoldan verilen kemik iliği mononükleer hücreleri hasar görmüş karaciğere yerleşebilmekte ve burada gelişebilmektedir; kemik iliği mononükleer hücre naklinin kemirgenlerde akut karaciğer hasarı modelinde yararlı olduğu düşünülmektedir.

**Anahtar kelimeler:** Karaciğer hastalığı, kemik iliği, hücre transplantasyonu

## INTRODUCTION

Acute liver failure (ALF) is a clinical condition that may occur in patients with partly compensated, acquired or hereditary liver diseases when a precipitating event leads to an acute decompensation of liver function. The current medical treatment is limited to patient support until the liver recovers spontaneously or a replacement liver becomes available (1). Although liver transplantation has become a somewhat standard treatment in these patients, a number of disadvantages of this option remain in such cases, such as organ shortage, long-term administration of immunosuppressant, acute or late complications after liver transplantation, and poor outcomes in patients not eligible for liver transplant. Accordingly, alternative approaches have been proposed (2). The prudent application of bone marrow cells (BMCs) is considered a promising cell therapy for various diseases. Donor BMCs transplanted into animal models of various diseases have been shown to differentiate into various cell types, including bone, cartilage, cardiac muscle, vascular endothelial, and neuronal cells (3,4). Recently, Schwartz et al. (5,6) reported that mesenchymal stem cells can differentiate into functional hepatocyte-like cells *in vitro*. This phenomenon has potential as treatment for acute and chronic hepatitis. Such a procedure may be particularly beneficial in the treatment of liver disease with limited regeneration potential of the liver in cirrhotic conditions and in the presence of cirrhosis (7). Hence, we hypothesized that bone marrow mononuclear cell (BMMC) therapy could be used to attenuate ALF or improve recovery in mice. To test this possibility, mononuclear cells were isolated from the bone marrow of mice in lymphocyte separation medium and then labeled with the red fluorescent dye PKH26 (8).

## MATERIALS AND METHODS

### Experimental Animals

Male and female BALB/c mice, 8-10 weeks of age and weighing 20-22 g, were purchased from the Animal Center of Jilin University (Changchun, China). All mice were housed in rooms maintained at constant temperature and humidity with 12-hour (h) light/dark cycles. Mice received normal rodent chow and water *ad libitum*. The animals were housed and experiments conducted in accordance with guidelines established by Jilin University. The animal experiments were approved by the Su-

pervisory Committee of Jilin University Animal Council.

### Study Design

Acute liver failure (ALF) was induced as described below, and subsequently the animals were randomly divided into two groups: (a) BMMC-treated (n=35):  $5 \times 10^6$  PKH26-labeled BMMCs were harvested from donor animals and infused via the tail vein after  $\text{CCl}_4$  treatment, and (b) controls (n=35): animals were infused with saline after  $\text{CCl}_4$  treatment. The hepatic tissue in the two groups was harvested and evaluated histologically. The activities of serum aminotransferases, alanine and aspartate (ALT, AST), were measured by an automatic biochemical analyzer (Sinnova D336, SINNO-WA Medical Science & Technology Co., Ltd, Nanjing, China) to assess biochemical changes in animals that received BMMCs compared with controls. These measures were performed prior to and in four successive weeks after treatment. The number of surviving mice was recorded for the two groups.

### Principal Reagents

Mouse anti-proliferating cell nuclear antigen (PCNA) antibody was obtained from Boster Co. (BM0104) Santa Cruz Biotechnology. Lymphocyte isolation medium,  $1.077 \text{ g/cm}^3$  and red fluorochrome PKH26 were purchased from the Sigma Company (MINI26, St. Louis, MO, USA). Polyclonal rabbit anti-human albumin (Code No. A0001, Dako, CA, USA), Alexa Fluor® 488 conjugated donkey anti-mouse or rabbit IgGs (Invitrogen, Carlsbad, CA), and liquid Macrogel (PEG 400, Macrogelum 400, Apoteksbolaget, Sweden) were purchased from Invitrogen Company (CA, USA) and paraformaldehyde from Sigma-Aldrich (St. Louis, MO). The following were obtained from Biolegend, San Diego, CA: FITC rat IgG  $2\alpha$   $\kappa$  Isotype ctrl. Clone: RT K 2758 Cat. No. 400505; APC rat IgG  $2\alpha$   $\kappa$  Isotype ctrl. Clone: RT K 2758 Cat. No. 400511; Percp rat IgG  $2b$   $\kappa$  Isotype ctrl. Clone: RT K 2758 Cat. No. B118419; PE rat IgG  $2b$   $\kappa$  Isotype ctrl. Clone: RT K 2758 Cat. No. B119316; APC anti-mouse CD34 Clone: MEC 14.7 Cat. No. 119309; FITC anti-mouse LY-6A/EC Sca-1 Clone: E13-16.1.7 Cat. No. 122505; PE anti-mouse/human CD44 Clone: IM 7 Cat. No. 103007; and Percp anti-mouse CD45 Clone: 30-F11 Cat. No. 103129.

### Bone Marrow Cell Harvest and Fluorescent Labeling

Femoral bones from male BALB/c mice were asep-

tically resected under ether anesthesia (8). The bone marrow in the medullary cavity was bathed with heparin, 50 U/ml, dissolved in normal saline. Then, the BMCs were suspended in lymphocyte isolation medium under sterile conditions. After dilution with 2 ml phosphate-buffered saline (PBS, 0.01 mol/L, pH=7.4) at a 1:1 ratio, the cells were slowly added to the lymphocyte isolation solution with matching relative density, 1.077 g/cm<sup>3</sup>, followed by centrifugation at 2000 rpm for 20 minutes (min). The cells were removed and washed with PBS, and centrifuged at 1200 rpm for 10 min. The top of the centrifuge tube was shaken lightly to detach the cells and DMEM/F12 medium was added (DMEM/F12, 15% fetal bovine serum (FBS), 100000 U/L penicillin, pH=7.4) to prepare the cell suspension at a cell density of more than 5x10<sup>7</sup> cells/ml. Finally, BMMCs were labeled with PKH26 according to the manufacturer's instructions. The density of labeled cell suspensions was adjusted to 3x10<sup>7</sup> cells/ml. BMMCs with viability greater than 95% as measured by trypan blue exclusion were used in subsequent experiments. In the experimental group, one million BMMCs were injected via the tail vein. The control mice were injected via tail vein with 0.1 ml of saline (0.9% NaCl with no cells) (8).

### Experimental Acute Liver Failure in Mice

Acute CCl<sub>4</sub> administration is widely used to generate an experimental model mimicking acute liver injury caused by toxic substances. On the 1<sup>st</sup> day, 20% v/v CCl<sub>4</sub> (2.0 ml/kg, dissolved in vegetable oil) was administered intraperitoneally. On the 2<sup>nd</sup> day, one million BMMCs were transplanted via the tail vein. On the 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> days, mice were sacrificed by cervical spine dislocation. Blood serum was used for biochemical analyses. The livers were dissected out and frozen in liquid nitrogen. The number of BMMCs in the hepatic tissue was examined by a laser scanning microscope (ZEISS, LSM510 META, Germany), using hematoxylin-eosin stain and by immunohistochemistry.

### Detection Index

The number of PKH26-labeled cells was quantified by immunofluorescence (FITC), and immunohistochemistry. Albumin and PCNA expressions were also detected in separate sections by immunohistochemistry. Serum aminotransferase activity was measured to evaluate liver damage. The survival rate in the two groups was compared re-

garding the potential effect of BMMC transplantation.

### Flow Cytometry Analysis

BMMCs (1x10<sup>6</sup>) were incubated in 2% FBS/PBS at 4°C for 30 min with 1 µl of monoclonal antibody specific for CD34, CD44, CD45, or sca-1 (BioLegend, San Diego, CA) or left unstained for analysis by fluorescence-activated cell sorting (FACS) Calibur with CellQuest software (Becton Dickinson, USA).

### Tissue Preparation

Following tracheal intubation, the chest was opened and animals were perfused for 2 min at 120 mmHg with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in 0.01 M PBS (pH=7.4) under an overdose of anesthesia (sodium pentobarbital 100 mg/kg, i.p.) (9). Fixed livers were cryopreserved in 30% sucrose at 4°C overnight, embedded into optimal cutting temperature (OCT) compound, and cut into 6 µm-thick sections in a cryostat (10).

### Immunohistochemistry

One out of every six serial sections per liver was collected for the examination of PKH26-labeled cells. Additional sections were processed for immunoperoxidase staining of PCNA and albumin. Thus, sections were treated 1% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min, and pre-incubated in 5% normal goat or horse serum in PBS with 0.3% Triton X-100 for 1 h. Sections were then incubated with PCNA (1:400) or albumin (1:100) antibody with blocking serum at 4°C overnight. Sections were further reacted with biotinylated goat anti-rabbit or horse anti-mouse IgGs at 1:400 (BM0104 Boster Company, Santa Cruz Biotechnology) for 2 h, and subsequently with ABC (1:400) (Vector Laboratories, Burlingame, CA) for another hour. The immunoreaction product was visualized in 0.003% H<sub>2</sub>O<sub>2</sub>, 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO). Three 10-min washes were used between incubations (11). Sections were mounted on slides, allowed to air-dry, and covered with cover slips.

To investigate whether pre-labeled PKH26 cells were still proliferating at the time of perfusion, PKH26 cells were examined for co-localization with PCNA. Immunofluorescence labeling was carried out by incubating sections in PBS containing 5% donkey serum and mouse anti-PCNA primary antibody (1:400) at 4°C overnight, followed by reaction with Alexa Fluor® 488 conjugated

donkey anti-mouse IgGs (1:200, A21206 Invitrogen, Carlsbad, CA) for 2 h. Sections were then washed and mounted with anti-fading medium before microscopic examination. Sections from BMMC-treated and control groups were processed in parallel under identical conditions to minimize experimental error.

### Statistical Analysis

One liver section from each animal was selected randomly. In each frozen section, 10 microscopic fields were examined under a microscope at 200x magnification. The total number of fluorescently labeled cells was determined for each slice. Results were expressed as mean  $\pm$  standard deviation. Statistically significant differences between the experimental and control groups were compared by Student's t-test. A value of  $p < 0.05$  was considered statistically significant. All data were processed by statistical software, the Statistical Package for the Social Sciences (SPSS) 10.0. A paired Student's t-test was used for comparison of PKH26 fluorescence intensity values and the expression of albumin quantified with point count methods.

## RESULTS

### Flow Cytometry Analysis of BMMCs

To assess the characteristics of BMMCs isolated from BALB/c mice, we analyzed their cell surface markers. Based on the average of four FACS analyses, 2.76% expressed CD34, a myeloid progenitor cell antigen that is also present in endothelial cells and some fibroblasts (Figure 1A); 67.10% expressed CD44, an endothelial cell marker (Figure 1B); 15.79% expressed stem cell marker Sca-1, which is present on hematopoietic (HSCs) and mesenchymal stem cells (MSCs) (Figure 1C); and 98.06% expressed CD45, a hematopoietic and leukocyte marker (Figure 1D). The Sca-1 positive fraction was approximately 15.79% of the bone marrow, representing typical HSC population as described by Okada et al. (12). Overall, the surface marker patterns of BALB/c-derived BMMCs were consistent with those used in clinical trials (13-15).

### Verification of Acute Liver Failure

As has been described previously, extensive vacuolar degeneration and swelling of hepatocytes in  $\text{CCl}_4$ -treated mice were observed, demonstrating that the model had been reproduced successfully in our experiment (Figure 2A, 2B).

### Detection of PKH26-Labeled Cells

PKH26-labeled cells appeared red when exposed to excitation light at 551 nm. These cells were detected only in sections of the liver from animals that received tail vein injection of BMMCs labeled with PKH26 (Figure 2C, 2D). Histological evaluation confirmed that BMMCs were located within the acutely injured liver. Figure 2 shows fluorescence microscopic images of a representative liver at 4 weeks following injection of BMMCs. The total number of double-positive cells labeled by PKH26 and PCNA in the BMMC-treated group was  $113 \pm 7$  cells/high power field, while in the control group, we failed to identify any PKH-26-labeled positive cells. PKH26-labeled cells (Figure 2E, E1-red color) appeared to co-localize with PCNA reactivity (Figure 2F, 2G).

### Quantitative Analysis of Proliferating Cell Nuclear Antigen

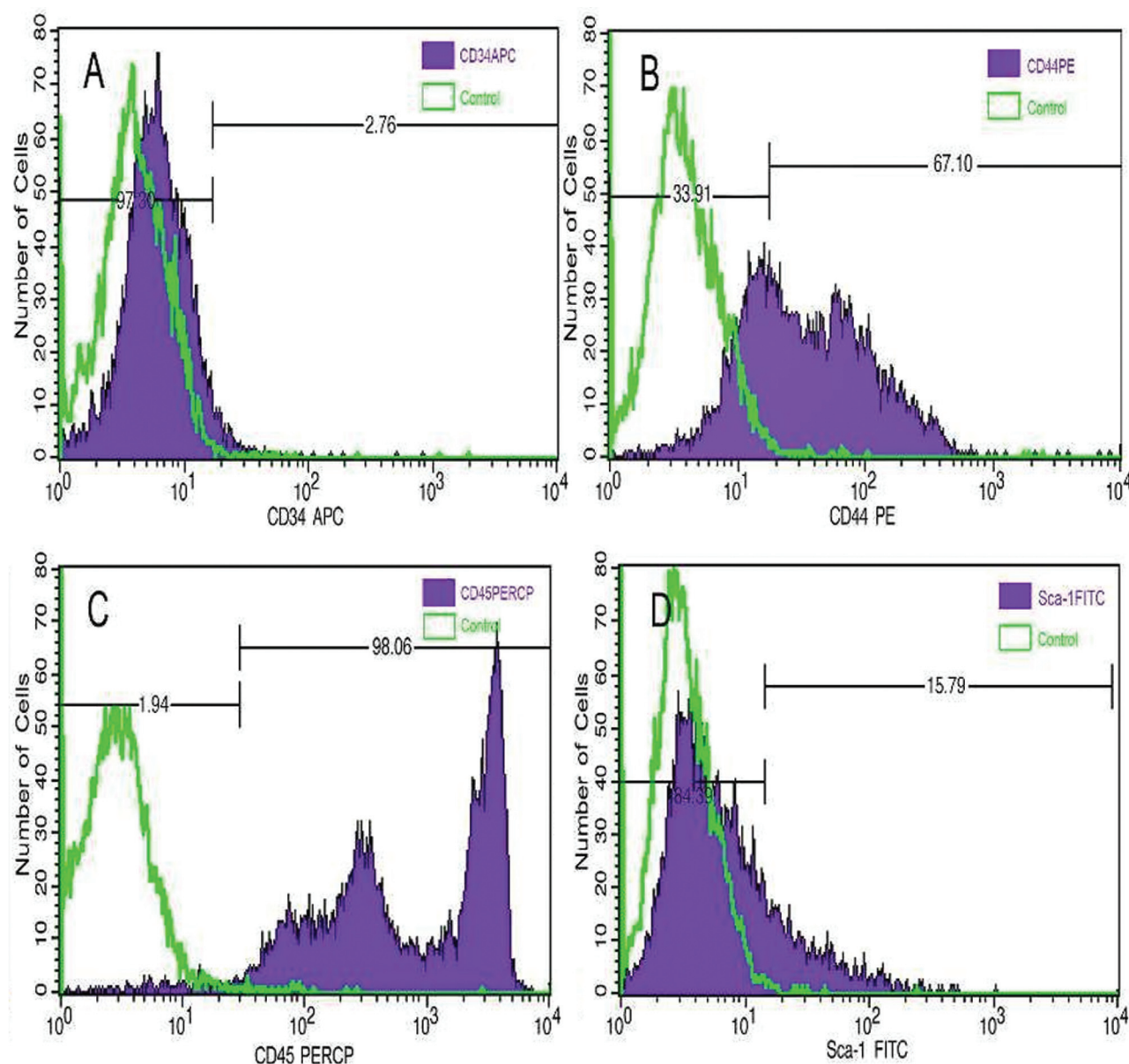
The level of PCNA was greater in the BMMC-treated versus control group. The total number of positive cells in the BMMC-treated group was  $21 \pm 2$  cells/high power field, whereas in the control group, the value was  $15 \pm 1$  cells/high power field. Statistical analysis confirmed a significant difference ( $p < 0.05$ ) between the BMMC-treated and the control groups (Figures 2H, 2I, 3).

### Liver Expression of Albumin

The expression of albumin in the liver was greater in the BMMC-treated group relative to controls. The percentage of field areas occupied by albumin reactivity was calculated by point counting (16). Figures 2J and 2K show the manual count set-up consisting of an albumin image printout, and a transparent grid overlay used for point count. The total number of crossing points in the BMMC-treated group was  $159 \pm 4$  cells/high power field, and was  $50 \pm 5$  cells/high power field in the control group. Statistical analysis indicated a significant difference ( $p < 0.05$ ) between the two groups (Figure 3). Because it is well known that albumin is associated with maturity of hepatocytes (17), the markedly increased expression of albumin in the BMMC-treated group implied that the BMMCs might have differentiated into functional hepatocytes.

### Serum Aminotransferase Activity

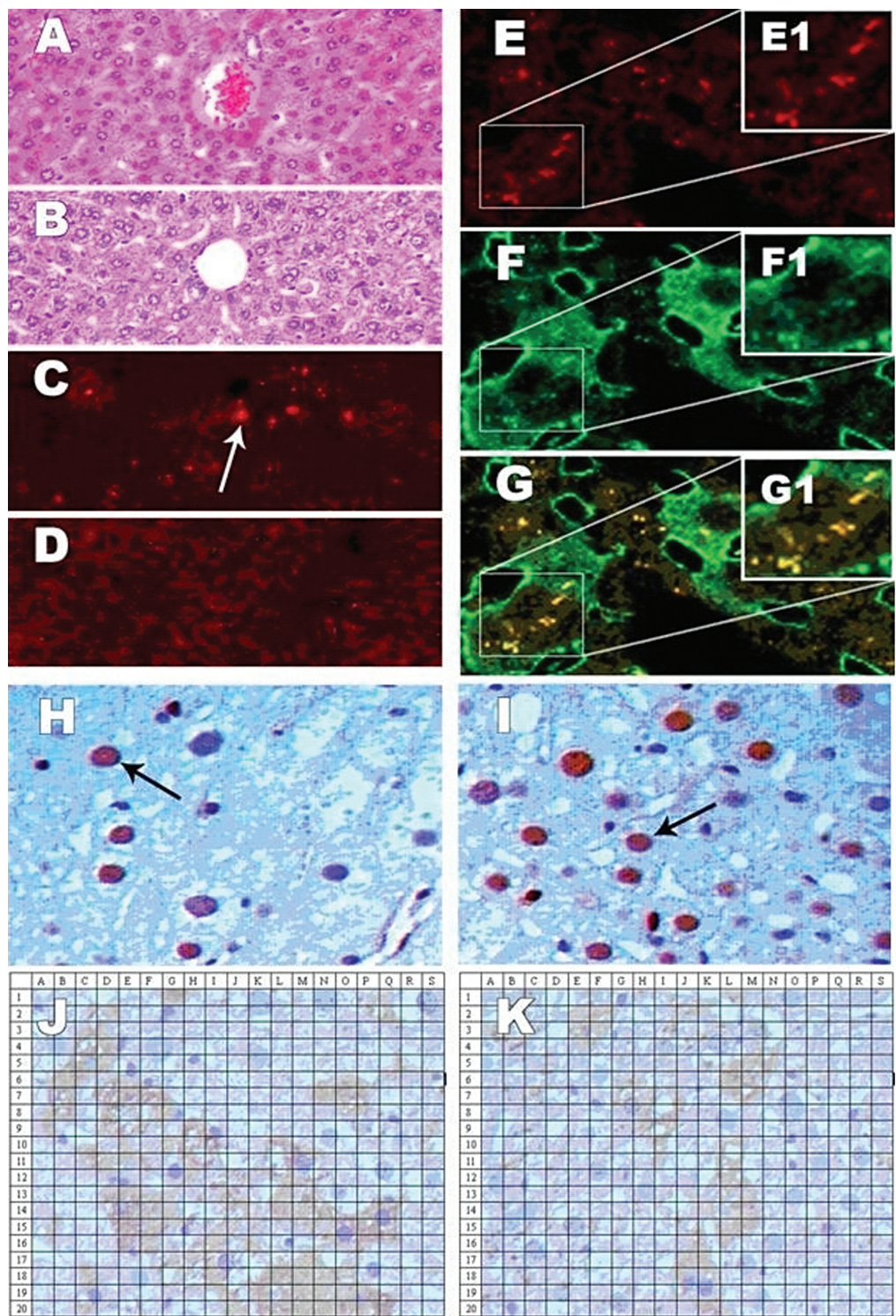
Whole blood was harvested under ether anesthesia after removing the eyes from the mice at different times following BMMC transplantation ac-



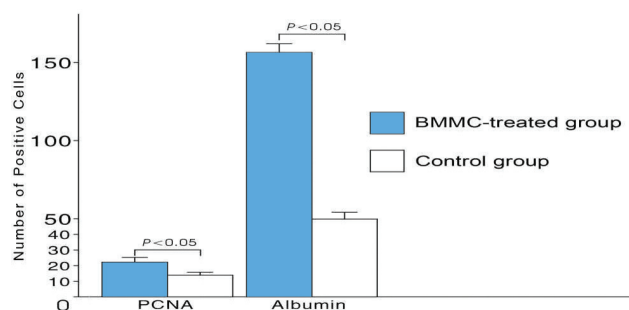
**Figure 1.** Fluorescence-activated cell sorting analysis of bone marrow mononuclear cells (BMMCs) from BALB/c mice. Purple curves with corresponding percentages represent BMMC samples labeled with CD34 (A), CD44 (B), CD45 (C) and Sca-1 (D). Green curves and percentages demonstrate negative controls (no antibody). APC: Allophycocyanin. PE: Phycoerythrin. PERCP: Peridinin. FITC: Fluorescein isothiocyanate.

cording to the protocol approved by Jilin University Animal Care Committee. Whole blood was centrifuged by hydro-extractor, and serum aminotransferase activity was measured using an automatic biochemistry analyzer (Sinnova D336, SINNOWA Medical Science & Technology Co., Ltd, Nanjing, China). No statistically significant differences in serum aminotransferase activity were found between the two groups prior to treatment (week 0). In contrast, statistically significant differences were detected at each of the different

time points during successive weeks, as shown in Figure 4A. Similar results were obtained for serum AST levels between the experimental and control groups, as shown in Figure 4B. ALT levels in the experimental and control groups were  $154.00 \pm 9.15$  vs  $149.04 \pm 1.28$ ,  $p > 0.05$ ;  $81.20 \pm 5.56$  vs  $101.04 \pm 3.65$ ,  $p < 0.05$ ;  $54.72 \pm 3.35$  vs  $82.45 \pm 5.52$ ,  $p < 0.05$ ;  $34.70 \pm 3.54$  vs  $62.50 \pm 2.35$ ,  $p < 0.05$ ; and  $33.80 \pm 4.25$  vs  $45.45 \pm 5.58$ ,  $p < 0.05$ , at 0, 1, 2, 3, and 4 weeks following cell transplantation, respectively. At the same time, the serum AST levels eva-



**Figure 2.** Histological comparisons of livers from animals treated with CCl<sub>4</sub> and normal untreated controls. **A:** Extensive vacuolar degeneration and edema of hepatocytes in acute liver failure; **B:** Normal liver tissue (hematoxylin-eosin, x200); **C, D:** Sporadic PKH26-labeled bone marrow stem cells in livers (**C:** BMMC-treated mice, **D:** control group). Positive cells labeled by PKH26 in BMMC-treated mice indicated by red fluorescence (x200); **E, F, G:** Detection of PKH26-labeled positive cells (red color, **E, E1**), PCNA-conjugated with FITC (green color, **F, F1**) and their co-localization (yellow color, **G, G1**), respectively, by laser scanning confocal microscopy (x200); **H, I:** Expression of proliferating cell nuclear antigen (PCNA) by immunohistochemistry in sporadic PKH26-labeled bone marrow stem cells in liver (**H:** BMMC-treated mice, **I:** control mice); and **J, K:** Expression of albumin by immunohistochemistry and sporadic PKH26-labeled bone marrow stem cells in liver (**J:** BMMC-treated mice, **K:** control mice). The diagram shows the manual count set-up consisting of an endothelial image printout and a transparent grid overlay used for naked-eye and point-out models. Each square of the grid measured 5.0 x 5.0 mm external area (x200).



**Figure 3.** A comparison of the numbers of PCNA- and albumin-positive cells in BMMC-treated and control groups. The identity of the expression of PCNA-positive, proliferating cells was evaluated by double immunofluorescence staining of PKH26-labeled cells. To investigate whether pre-labeled PKH26 cells were still proliferating at the time of perfusion, PKH26 cells were stained with PCNA. Immunofluorescence labeling was carried out by incubating sections in PBS containing donkey serum and mouse anti-PCNA primary antibody overnight, followed by Alexa Fluor® 488 conjugated donkey anti-mouse IgGs. The percentage of field area occupied by albumin expression was calculated by point counting, as described in Materials and Methods.

lated in the two groups at the different time points were  $124.52 \pm 2.26$  vs  $121.56 \pm 2.25$ ,  $p > 0.05$ ;  $58.16 \pm 4.55$  vs  $99.30 \pm 6.25$ ,  $p < 0.05$ ;  $41.49 \pm 4.40$  vs  $78.46 \pm 6.55$ ,  $p < 0.05$ ;  $31.90 \pm 2.55$  vs  $58.64 \pm 3.22$ ,  $p < 0.05$ ; and  $31.00 \pm 5.77$  vs  $42.30 \pm 1.29$ ,  $p < 0.05$ , at 0, 1, 2, 3, and 4 weeks following cell transplantation, respectively.

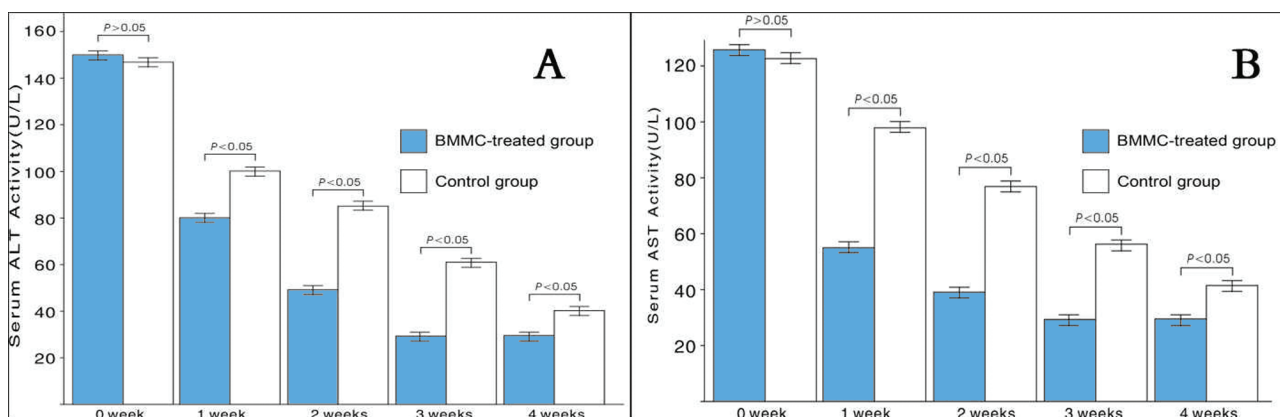
### Comparison of Survival Rates

The numbers of surviving mice as a function of time are shown in Figure 5. Data are presented as numbers of surviving animals up to 28 days after treatment. The survival rates were calculated in the experimental and control groups at different time points for days 4, 8, 12, 16, 20, 14, and 28, respectively, and were significantly different between

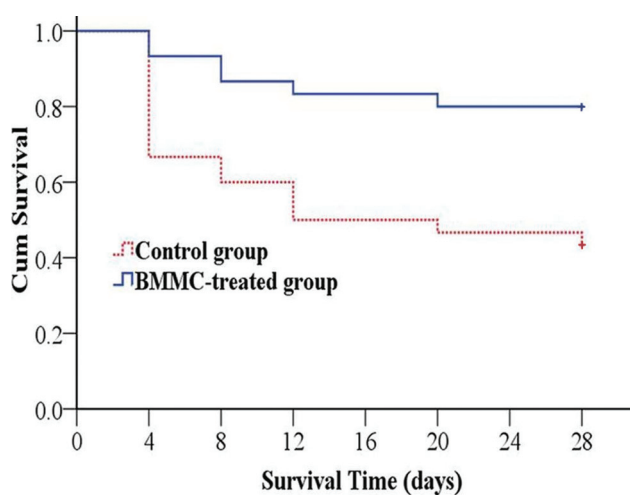
the experimental and control groups at corresponding time points (100%, 95.3%, 90.2%, 85.7%, 80.5%, 80.5%, 80.5% vs 100%, 65.7%, 60.4%, 50.7%, 48.3%, 48.3%, 48.3%, 48.3%;  $p < 0.05$ ). At each time point, the number of mice surviving the induction of ALF was greater in the BMMC-treated versus control group.

## DISCUSSION

In this study, we present data showing the effectiveness of transplanting BMMCs as an approach to treatment of ALF in an experimental animal model. FACS analysis of BMMCs showed that most cells were CD44 (67.10%), Sca-1 (15.79%), CD34 (2.76%), and CD45 (98.06%) (18). Therefore, the contribution of HSCs to the therapeutic effect appears to be substantial. Hepatic oval cells involved in some forms of liver regeneration expressing many markers are also found on HSCs (19). In addition, several independent studies (20,21) have reported that BMCs can give rise to several hepatic epithelial cell types, including oval cells, hepatocytes, and duct epithelium. From these observations, a hypothesis was proposed that bone marrow resident stem cells, specifically HSCs, are an important source for liver epithelial cell replacement, particularly during ALF. The current interest in the role of bone marrow stem cells in liver regeneration began with the discovery that rat oval cells induced by a classic carcinogen regimen expressed genes typically associated with HSCs (22). Hematopoietic cells per se may also be essential for transdifferentiation. Macrophages and inflammatory cells may secrete cytokines and other factors essential for proper tissue restoration. Kupffer cells, the tissue macrophages in the liver, are replaced after BMMC transplantation, and are



**Figure 4.** Serum aminotransferase levels in whole blood measured by automatic biochemistry analyzer following treatment.



**Figure 5.** Survival curves of  $\text{CCl}_4$ -induced acute liver failure in the two groups. The number of surviving mice every fourth day after treatment was calculated in order to assess the survival function for the two groups.

involved in phagocytosis of dead cells during liver remodeling. Therefore, it is conceivable that intra-hepatic transplantation of the HSCs themselves or their differentiated offspring could have a therapeutic benefit in some types of liver damage (19). In the present experiment, we found that the transplantation of BMMCs significantly reduced the serum aminotransferase levels in  $\text{CCl}_4$ -induced ALF. Due to the genetic identity of the inbred animals, the utilization of BALB/c mice in the present project allowed us to avoid immunologic rejection. Thus, the transplantation procedure represented a syngraft. The use of PKH26 fluorescent labeling enabled us to observe whether and to what extent the BMMCs localized in the livers of the recipient group, by counting the numbers of fluorescently-labeled cells. The findings that BMMCs can result in a significant increase in albumin expression suggest that these cells could differentiate into hepatic cells, and the increased expression of PCNA suggests that the cells may have subsequently undergone cell division. It is well known that PCNA, which exists and is synthesized in the cell nucleus, is a nuclear antigen related with the cell life cycle. PCNA is expressed in the G1 and S phases, and performs the essential function of providing replicative DNA polymerases in eukaryotic cells (23). The quantity of PCNA is low in resting cells, but is substantially increased in multiplying and transformed cells. The expression of both albumin and PCNA were significantly greater in the BMMC-treated versus

the control group based on quantitative analysis. As shown by double fluorescence, our data also indicate that transplanted BMMCs seem to become hepatic-like cells and can secrete albumin. This observation is in agreement with a recent report, although the number was very small in our experimental model (24). Differentiation of transplanted BMMCs into albumin-producing hepatocytes, as indicated by the tremendous increase in albumin expression, in hepatic tissue may presumably lead to improved liver function and result in the enhanced survival of mice receiving BMMC transplantation (25). In this way, transplanted BMMCs differentiate into functional hepatocytes that can compensate for ALF in mice. The serum ALT and AST levels also markedly changed in response to transplantation of BMMCs, confirming that the liver deterioration can be ameliorated by transplanted BMMCs. The results indicate that BMMCs can locate to acutely injured livers and transform into hepatic-like cells and act like normal hepatic cells in mice.

Recently, cell fusion has been reported as an important mechanism for liver cell gene expression in the transplantation of BMMCs and tissue stem cells (26,27). Several authors have reported the existence of hepatic stem/progenitor cells in bone marrow (28,29). Exactly why the BMMCs in our model were able to differentiate into functional hepatocytes remains an intriguing question. One key difference between the methods described by Jiang et al. (30) and our protocol was that freshly isolated BMMCs were directly transplanted in our study, without the use of a culture period. Although it is not known exactly which cells trans-differentiate into hepatocytes, we believe that the liver damage induced by  $\text{CCl}_4$  might represent a key factor, facilitating a special environment that enables trans-differentiation of BMMCs into functional hepatocytes (31). In support of this speculation are data showing that special stress-induced signaling pathways are to play crucial roles in hepatogenesis. Mice in which various inflammation signal molecules have been knocked out were found to undergo massive liver degeneration (32,33). Based on these results, much remains to be explained about the differentiation of transplanted BMMCs into hepatocytes in our model. Obviously, it is also clear that in our mouse model, the condition of the recipient of BMMC transplantation represents a critical variable in the differentiation process. The protocol presented in this

report should facilitate the development of cell transplantation for ALF in future studies.

In summary, we have demonstrated the feasibility and benefit of BMMC transplantation as an alter-

native therapeutic approach to ameliorate ALF. BMMC transplantation may be useful for the treatment of ALF as well as the restoration of liver function in human clinical situations in the future.

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