SUPPLEMENTAL MATERIALS AND METHODS

Hydroxyproline assay

The liver cirrhosis mouse model at 4 weeks after cell administration was used for measurement of hydroxyproline, which is a representative component of collagen. The liver samples (20 mg) were homogenized using the QuickZyme Hydroxyproline Assay (QuickZyme Bioscience, Zernikedreef, Netherland) according to the manufacturer's protocol, adjusted for sample, and analyzed by absorbance measurement at 570 nm. Data were expressed as the amount of hydroxyproline in 1 mg of liver, and the control, MSC100, id-BMM100, and 50/50 groups were assessed.

Co-culture

Co-culture of MSCs and id-BMMs was performed at 37°C under 21% O_2 and 5% CO_2 in transwell plates (Transwell 6-well plate; Corning) for 72 hours. The cells were harvested and mRNA expression levels in MSCs and id-BMMs were analyzed by real-time PCR.

MSC differentiation

To confirm the tri-lineage differentiation ability of MSCs into adipocytes, osteoblasts, and chondrocytes, MSCs were cultured at 37°C under 5% CO_2 in three specific media (MesenCult Adipogenic Stimulatory Supplement, Osteogenic Stimulatory Supplement, and MesenCult-ACF Chondrogenic Differentiation Medium; Stem Cell Technologies) for 10–20 days. Differentiation toward adipocytes, osteoblasts, and chondrocytes was confirmed by Oil Red O staining (Sigma-Aldrich, St. Louis, MO, USA), Alizarin Red staining (Sigma-Aldrich), and Alcian Blue staining (Sigma-Aldrich) (Supplemental Figure 1E), respectively.

Analysis of hepatobiliary enzymes

Blood samples were collected at 4 weeks after cell injection, and serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, and total bilirubin were determined by

BML Inc. (Tokyo, Japan).

Microarray analysis

Total RNA from MSCs and id-BMMs was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. Microarray analysis was performed by TaKaRa Bio Inc., (Shiga, Japan) using an Agilent expression array.

Cell culture in the presence of serum

Serum was obtained from mice that received CCl_4 i.p. for eight weeks. MSCs and id-BMMs were cultured with the serum from CCl_4-damaged mice, and compared to MSCs and id-BMMs cultured with addition of serum from normal mice. After 72 hours, cells were harvested and mRNA expression levels were analyzed by real-time PCR.

Analysis of fibrosis

To determine the extent of fibrosis in the liver, liver tissues were collected at 4 weeks after cell injection. Formalin (10%)-fixed tissue was cut into 3-μm-thick sections and stained with Sirius red. One hundred photographs (x100) were randomly captured for 4 sections of each group, and a quantitative analysis of the fibrotic area was performed by using ImageJ software (version 1.6.0 20, National Institutes of Health, Bethesda, MD, USA).

Flow cytometry (cells)

MSCs were harvested with TrypLE Express (Thermo Fisher Scientific) for 10 minutes. The resulting cells were incubated with an anti-mouse CD16/32 antibody (Becton Dickinson, Franklin Lakes, NJ, USA) for 10 minutes on ice to block non-specific binding. The cells were then incubated with the antibodies (Supplemental Table 3) for 30 minutes on ice. After washing, the cells were analyzed using a FACSCalibur system (Becton Dickinson). Flow cytometry results were obtained from at least 3

separate samples and analyzed using Flowing Software 2.5.1 (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland).

Flow cytometry (liver)

Blood cells in the whole liver three days after cell injection were harvested using a Liver Dissociation Kit (Miltenyi Biotec). The resulting cells were incubated with ACK Lysing Buffer (Thermo Fisher Scientific) for exclusion of erythrocytes. After separation, the process was as described above.

Analysis of changes in id-BMMs performing phagocytosis (co-culture of id-BMMs and hepatocyte debris)

Hepatocytes were harvested by digestion using protease (Sigma-Aldrich) and collagenase perfusion (Roche, Basel, Switzerland) from the liver of mice with CCl_4-induced liver damage. Following dissociation, an equivalent amount of PBS was added and the resulting cell suspension was strained through 70-μm cell strainers (Corning). Hepatocytes were enriched by low-speed centrifugation (50 ×g, 3 minutes), and the obtained cells were repeatedly frozen and thawed approximately 20 times to destroy mRNA (hepatocyte debris). Hepatocyte debris was added into dishes containing id-BMMs and co-cultured for 48 hours. The mRNA expression level after co-culture was analyzed by real-time PCR.

Phagocytosis assay

After culture, 1×10^5 id-BMMs were plated into a 96-well plate (Microplate 96-well with lid; Iwaki), pHrodo Green Zymosan A Bioparticles Conjugate for phagocytosis (Thermo Fisher Scientific) was added into each well, and the cells were cultured at 37°C under 5% CO_2 for 1 hour. Photographs of phagocytosis were captured from each section randomly, using an HS all-in-one fluorescence microscope (BZ-9000) (Keyence, Osaka, Japan). The number of phagocytizing id-BMMs per 100 id-BMMs was analyzed.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Characterization of mouse bone marrow-derived mesenchymal stem cells (MSCs) and induced bone marrow-derived macrophages (id-BMMs). (A) Hematoxylin staining of id-BMMs. (Scale bars: 100 μm.) (B) Flow cytometric analysis for the cell surface markers of id-BMMs. (C) Microscopic analysis of MSCs. (Scale bars: 100 μm.) (D) Flow cytometric analysis for the cell surface markers of MSCs. (E) Tri-lineage differentiation potential of MSCs. Chondrogenic (left panel), adipogenic (middle panel), and osteogenic (right panel) differentiation was confirmed by Alcian blue staining, Oil red O staining, and Alizarin red staining, respectively. (Scale bars: 100 μm.)

Supplemental Figure 2. Gene expression in cultured MSCs and id-BMMs analyzed by microarray. Heatmaps of 5 major categories are shown. mRNA expression of genes related to fibrosis, chemoattractants, pro-regeneration, inflammation/anti-inflammation, and macrophage phenotypes is shown.

Supplemental Figure 3. Phagocytic ability of id-BMMs determined by zymosan phagocytosis assay. Data are presented as the means ± SD, n = 6 in each experiment, $p = 0.038$ (co-culture compared to simple culture), $p = 0.041$ (addition of serum compared to simple culture), $p = 0.008$ (co-culture and addition of serum compared to simple culture), $p = 0.036$ (co-culture and addition of serum compared to co-culture), $p = 0.032$ (co-culture and addition of serum compared to addition of serum). (Scale bar: 50 μm.)

Supplemental Figure 4. Schematic representation of the experimental design for live imaging to show the detailed behavior of administered MSCs (DsRed; red) and id-BMMs (GFP; green). The id-BMMs derived from GFP knock-in mice and MSCs derived from DsRed knock-in mice were administered to the mice with CCl4-induced liver damage via the tail vein. (Scale bar: 100 μm.)

Supplemental Figure 5. Localization of administered MSCs and id-BMMs at 1, 3, and 7 days after cell injection in intravital imaging analysis. (A) Intravital imaging using two-photon excitation microscopy of the lung (upper panels), and spleen (lower panels) 3 days after cell administration in the MSC100 (left panels), id-BMM100 (middle panels), and 50/50 (right panels) groups. Green cells represent administered id-BMMs, red cells are administered MSCs. Nuclei are stained with DAPI (blue), the dense blue area composed of blue fibers represents fibrosis, and white spots represent debris of hepatocytes. (Scale bar: 100 μm.) (B) Comparison of localization of administered id-BMMs between the 50/50 and id-BMM100 groups at 1, 3, and 7 days, n = 12 mice in each group.

Supplemental Figure 6. *Cxcl1 and Cxcl2* mRNA levels in the id-BMM100 and 50/50 groups were markedly upregulated at 3 days after cell administration. Data are presented as the means ± SD, n = 12 in each experiment. Representatively, in the 50/50 group, mRNA levels of CXCL1 ($p < 0.001$; day 3, compared to control, $p < 0.001$; day 3, compared to MSC 100, $p < 0.001$; day 3, compared to id-BMM100) and CXCL2 ($p < 0.001$; day 3, compared to control, $p < 0.001$; day 3, compared to MSC 100, $p = 0.086$; day 3, compared to id-BMM100) are upregulated.

Supplemental Figure 7. Flow cytometric analysis of CD206-positive M2 polarized macrophages. The values represent the frequency of F4/80+/CD11b+/CD206+ cells (M2 macrophages) among all macrophages. Data are presented as the means ± SD, n = 12 mice in each group.

Supplemental Table 1. List of primers used for real-time PCR. The names of primers, catalog numbers, species origin, and company are provided.

Supplemental Table 2. List of antibodies used for immunostaining. The names of antibodies, clones, species origin, company names, dilution, antigen retrieval, and heating time are provided.

Supplemental Table 3. List of antibodies used for flow cytometry. The names of antibodies, clones, species origin, and company names are provided.

Supplemental Video 1. Intravital two-photon imaging of id-BMMs phagocytizing debris in the liver. Green cells are the administered id-BMMs, nuclei are stained with DAPI (blue), the dense blue area composed of blue fibers represents fibrosis, and white spots represent hepatocyte debris. Three minutes after starting the video, id-BMMs approached debris. After 9–16 minutes, id-BMMs surrounded and phagocytized the debris, and digested it (Phagocytosis activity). After 21–30 minutes, id-BMMs re-approached and phagocytized residual debris. Scale bar, 50 μm. Playback speed = 100×.