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Mesenchymal stem cells reverse high-fat diet-induced non-alcoholic fatty liver disease through suppression of CD4+ T lymphocytes in mice

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Abstract. Although the multipotency of mesenchymal stem cells (MSCs) makes them an attractive choice for clinical applications, immune modulation is an important factor affecting MSC transplantation. At present, the effect of treatment with MSCs on non-alcoholic fatty liver disease (NAFLD) has received little attention. In the present study, a compact bone-derived method was used to isolate mouse MSCs (mMSCs) and a high-fat diet was used to establish a mouse model of NAFLD. Immunophenotypic features of mMSCs were analyzed using flow cytometry. Paraffin sections were stained with hematoxylin and eosin to assess inflammation and steatosis, and with picrosirius red to assess fibrosis. Spleen leukocytes were analyzed by flow cytometry. The results demonstrated that compact bone-derived MSC transplantation decreased high-fat diet-induced weight gain, expansion of subcutaneous adipose tissue, steatosis, lobular inflammation and liver fibrogenesis. Flow cytometry analysis of spleen leukocytes demonstrated that compact bone-derived MSC transplantation suppressed the proliferation of cluster of differentiation (CD) 4+ T lymphocytes in the spleen, which had been induced by the high-fat diet. In conclusion, compact bone-derived MSCs may exhibit clinical value in the treatment of NAFLD through their capacity to suppress the activation of CD4+ T cells.

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Introduction

Due to the aging population, obesity is common and is frequently associated with non-alcoholic fatty liver disease (NAFLD), which includes non-alcoholic fatty liver and non-alcoholic steatohepatitis (NASH) (1,2). Globally, NAFLD is a widespread disorder which is now considered to be among the most common types of liver disease. Although initially benign, the disease may progress from non-alcoholic steatosis (NAS) to NASH and subsequently to hepatic fibrosis, liver cirrhosis and hepatoma (3). The prevalence of NAFLD in the general population of the western world has been reported to be 20-30% (3). In the general population of Asia, the prevalence of NAFLD has been reported to be 15-30%, and >50% in patients with diabetes and metabolic syndromes (4). In mainland China, ultrasound surveys assessing fatty liver due to any cause have been published since the mid-1990s (5,6). From these surveys, the median prevalence of ultrasonographic steatosis in the Chinese population has been observed to be 10%, with a range of between 1 and 30% (5,7).

A recent study reported that the adiponectin-derived active peptide ADP355 exerts anti-inflammatory and anti-fibrotic activity in thioacetamide-induced liver injury (8). There has been interest in the isolation and characterization of mesenchymal stem cells (MSCs) and in the potential application of these cells to the treatment of liver disease. MSCs are a heterogeneous subset of stromal stem cells, which may be isolated from various adult tissues (9). They are able to differentiate into cells of a mesodermal lineage, including adipocytes, osteocytes and chondrocytes, in addition to cells of other embryonic lineages. The multipotency of mesenchymal stem cells makes them an attractive choice for clinical applications (10-12). Immune modulation is an additional important issue in MSC transplantation. It has been demonstrated that MSCs exhibited potent anti-inflammatory and immunomodulatory activity

in vitro and *in vivo* (13). In liver disease, MSC transplantation has been observed to exert therapeutic effects in acute and chronic liver injury (14-18). In a recent study, it was demonstrated that bone-derived MSC transplantation was effective in treating experimental liver fibrosis induced by consecutive intraperitoneal injections of CCl_4, and molecules secreted by the cells ameliorated fulminant hepatic failure induced by thioacetamide (19). MSCs have been demonstrated to exert a positive effect on the immune micro-environment in animal models of fulminant hepatic failure (FHF) and chronic liver fibrosis (19).

In the present study, the potential beneficial effects of MSCs were investigated in a high fat diet (HFD)-induced NAFLD model, including further examination of whether MSCs induced immunosuppression. A mouse model of NAFLD was established through treatment with a TROPHIC (T)/HFD (20). Isolation and culture of murine MSCs from compact bone were acquired using modified previously-described procedures (19,21). The results of the present study demonstrated that NASH induced by a HFD was ameliorated by treatment with MSCs, as indicated by a decrease in obesity, the expansion of subcutaneous adipose tissue, hepatic lipid accumulation, liver inflammation and fibrosis. MSC-mediated immunomodulation resulted from a decrease in cluster of differentiation (CD)4+ T lymphocytes in the spleen.

Materials and methods

Animals and diet. A total of 18 of male C57BL/6 mice, aged 6-8 weeks, weighing 16-18 g, were purchased from the Academy of Military Medical Science (Beijing, China) and were housed in a pathogen-free room, with a 12 h light/dark cycle at 20-25˚C. They were maintained on a normal diet or HFD obtained from TROPHIC Animal Feed High-Tech Co., Ltd. (Nantong, Jiangsu, China). The food compositions of the two dietary groups are presented in Table I. The total energy and cholesterol content in the two dietary groups are presented in Table II. The total protein, carbohydrate and total fat content within the total energy in the two dietary groups are presented in Table III. The mice were randomized into two groups: i) Normal mice; and ii) T/HFD mice. A total of 21 weeks subsequently, the T/HFD mice were randomized into two groups: i) T/HFD mice; and ii) T/HFD+MSC mice, which were intravenously injected twice with $1x10^6$ MSCs/mouse, at 21 and 23 weeks. A total of 21 weeks subsequently, the diets of the T/HFD and T/HFD+MSC mice were replaced with a normal diet. A total of 28 weeks subsequently, all of the animals were sacrificed and tissues were harvested. Mice were weighed weekly. The present study was approved by the Animal Ethics Committee of Tianjin Medical University (Tianjin, China).

Isolation and culture of bone-derived MSCs. MSCs obtained from murine compact bone were isolated and culture-expanded as described previously (19,21). Femurs and tibiae were collected from a total of 3 2-3-week-old female C57BL/6 mice (weighing 6-10 g) and were purchased from the Academy of Military Medical Science, Beijing, China. They were housed in a pathogen-free room, with a 12 h light/dark cycle at 20-25˚C. Bone marrow was flushed with PBS or α-modified minimal essential medium (α-MEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using a syringe. The compact bones were excised into chips of ~1 mm into plastic culture dishes, and washed with PBS or α-MEM until the released cells were removed. The cells were incubated in α-MEM containing 10% select fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C, in an atmosphere containing 5% CO_2. The medium was replaced every 4-5 days. Adherent cells (passage 0) were confluent following 1-2 weeks of incubation and were harvested using a cell scraper. The cells were passaged by digestion with 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.). Subsequent to 5-8 passages, the cells were used for further experiments.

Examination of immunophenotypic features of MSCs. The prepared MSCs, as described above, were harvested by digestion with trypsin, and stained for 30 min at 4˚C with fluorescein isothiocyanate-conjugated anti-mouse CD11b, CD45, CD105 and ataxin-1 (Sca-1) antibodies, or phycoerythrin-conjugated anti-mouse CD29, CD44 and CD135 antibodies (all eBioscience, Inc.; Thermo Fisher Scientific, Inc.). Cells were analyzed using a FACSCalibur instrument using a laser at a wavelength of 488 nm (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometric data were analyzed using Flow Jo software (version 7.6; Tree Star, Inc., Ashland, OR, USA).

Histological analysis of livers. Livers were perfused with PBS, removed, weighed and sliced into 0.5x0.5 cm sections. The sections were embedded in paraffin subsequent to being fixed in 4% (w/v) paraformaldehyde and were cut into 6-μm-thick sections. The paraffin sections were stained with 0.5 % w/v hematoxylin for 5 min and 1% w/v eosin for 10 sec (HE) at room temperature to assess inflammation and steatosis, and picrosirius red to assess fibrosis. Evaluation of the extent of resultant NASH (3) was performed using the following scaling scores.

Steatosis. Hepatocytes containing fat vacuoles were subjectively visualized and graded according to the following scale: 0, Normal, no hepatocytes affected; 1, minor, <5% of hepatocytes affected; 2, mild, 5-33% of hepatocytes affected; 3, moderate, 34-66% of hepatocytes affected; and 4, severe, >66% of hepatocytes affected.

Lobular inflammation. Grading of lobular inflammation was performed as follows: 0, None; 1, 1-2 foci/x20 field; 2, 2-4 foci/x20 field; and 3, >4 foci/x20 field.

Stages of NASH. Staging of NASH was performed as follows: 0, None; 1, extensive zone 3 perisinusoidal fibrosis; 2, zone 3 perisinusoidal, and portal or periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis.

Analysis of spleen leukocytes. Single-cell suspensions derived from spleens were prepared by mechanical disruption and filtered through a 40-μm cell strainer (BD Biosciences). The cells were placed in H_2O 30-50s, soon later added with 1/10 volume 10x PBS, and red blood cells were removed with cytolysis. The cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate, 1 μg/ml ionomycin (Enzo Life

Table I. Food composition in the two dietary groups.

Component	Mass, g	
	Normal diet	High-fat diet
Casein	193.000	262.000
Corn starch	296.500	0.000
Maltodextrin	33.000	161.000
Sucrose	332.000	89.000
Soybean oil	24.000	32.000
Lard	19.000	317.000
Cellulose	47.000	65.000
Mineral mix	43.000	58.000
Vitamin mix	9.500	13.000
L-cysteine	3.000	4.000
Choline bitartrate	3.000	3.000
TBHQ	0.008	0.069
Total	1,000.000	1,000.000

TBHQ, tertiary butylhydroquinone.

Table II. Total energy and cholesterol content in the two dietary groups.

Component	Diet	
	Normal diet	High-fat diet
Total energy, kcal/g	3.8	5.2
Total cholesterol, mg/kg	40.8	228.0

Sciences, Inc., Farmingdale, NY, USA) and 3 μg/ml brefeldin A (eBioscience, Inc.; Thermo Fisher Scientific, Inc.) for 5 h. The cells were subsequently stained for surface markers using rat anti-mouse CD4-allophycocyanin (catalog no. 17-0042-81, eBioscience, Inc., San Diego, CA, USA) for 30 min at 4˚C. The cells were analyzed using the 488 nm laser of a FACSCalibur instrument and the data generated were analyzed using FlowJo software version 7.6.1 (Tree Star Inc., Ashland, OR, USA).

Statistical analysis. GraphPad PRISM software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) was used to perform a Student's t test Results are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cells isolated from compact bone were characterized as MSCs. According to modified previously-described procedures (19), mMSCs were isolated from compact bone (from 2-3-week-old female C57BL/6 mice) and cultured. The MSCs appeared to be vortex-shaped and fibroblast-like, although not polygon-shaped (osteocytes) (Fig. 1A). In order to further identify the adherent cells, immunophenotypic features were analyzed. As presented in Fig. 1B, negative surface markers of MSCs, including CD11b, CD45 and CD135, were not expressed; however, the cells expressed surface markers characteristic of MSCs, including CD29, CD44, CD105 and Sca-1. The results of the present study indicated that these cells were MSCs.

Table III. Total Protein, carbohydrate and fat content within the total energy in the two dietary groups.

Component	Diet	
	Normal diet	High fat diet
Total protein, %	18	18
Total carbohydrate, %	72	22
Total fat, %	10	60

Treatment with MSCs reverses HFD-induced weight gain and expansion of subcutaneous adipose tissue. In order to investigate the beneficial effects of MSCs on NAFLD, a mouse model of NAFLD was established using HFD, and the mice were intravenously-injected twice with $1x10^6$ MSCs/mouse at weeks 21 and 23 (Fig. 2A). A marked difference was observed between T/HFD and T/HFD+MSC mice at 28 weeks post-treatment (Fig. 2B). Compared with normal control mice, the T/HFD-fed mice exhibited an accelerated elevation of body weight between 1 and 21 weeks (Fig. 2C). Following the two intravenous injections of MSCs, the weight of the T/HFD-fed mice decreased markedly at 21-28 weeks (Fig. 2C).

Weight gain is often accompanied by the expansion of subcutaneous adipose tissue. The results of the present study demonstrated that the mass of subcutaneous abdominal adipose tissue increased in the T/HFD-fed mice, an effect which was reversed by the MSC treatment (Fig. 3).

Treatment with MSCs decreases HFD-induced steatosis and lobular inflammation. NFLD may progress via hepatic lipid accumulation to NAS, and via lobular inflammation to NASH (3). Hepatocytes containing fat vacuoles may be visualized as clear bubbles following liver section HE staining. As presented in Fig. 4, hepatic lipid accumulation indicated by clear bubbles occurred in T/HFD-fed mice (Fig. 4A), while it was decreased in T/HFD+MSC mice (Fig. 4B). It was additionally observed that HFD-induced lobular inflammation was present within the THFD-fed mouse livers (Fig. 4A), which was suppressed in the T/HFD+MSC mice (Fig. 4B). As indicated by scores obtained from the methods described above, increases in steatosis (Fig. 4C) and lobular inflammation (Fig. 4D) induced by HFD were significantly decreased in response to MSC treatment.

Treatment with MSCs suppresses HFD-induced liver fibrogenesis. NAFLD frequently progresses to fibrosis (3). Therefore, the stages of fibrosis in the liver samples were assessed by staining with picrosirius red. Fibrogenesis was observed within the T/HFD-fed mouse livers (Fig. 5A), and a reduction of hepatic fibrogenesis occurred in the T/HFD+MSC mice (Fig. 5B). Significant differences in fibrosis stage scores

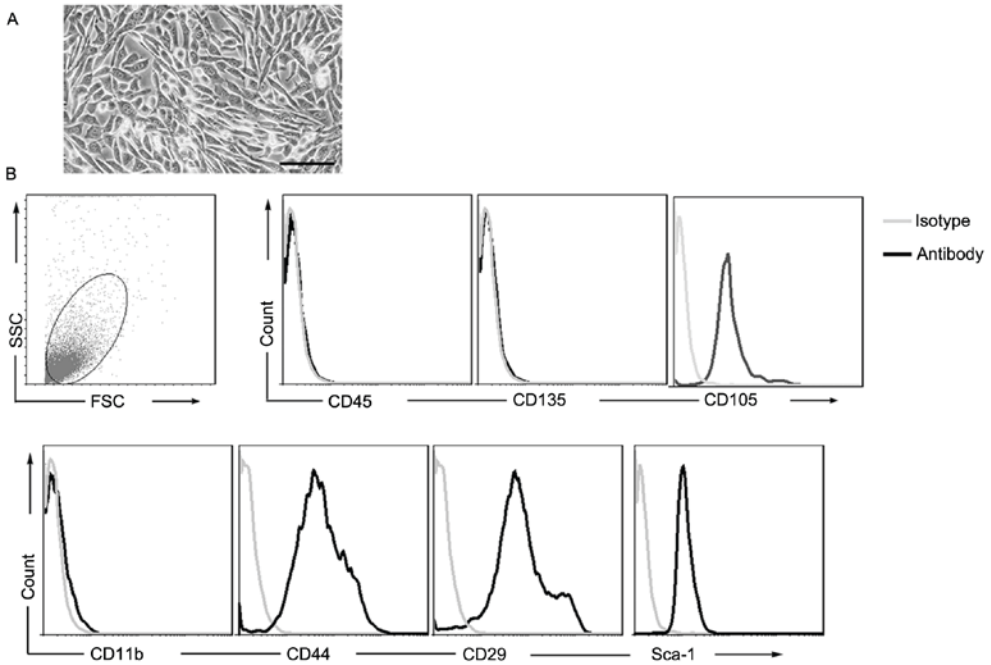

Figure 1. Identification of compact bone-derived mMSCs. (A) Morphological features. The MSCs appeared vortex-shaped and fibroblast-like. Scale bar=30 μm (B) Immunophenotypic characterization. The cells were harvested by trypsin digestion and stained with fluorescein isothiocyanate-conjugated anti-mouse CD11b, CD45, CD105 and Sca-1, or phycoerythrin-conjugated anti-mouse CD29, CD44 and CD135. The expression of each antigen is presented with the corresponding isotype control. Representative images are presented. CD, cluster of differentiation; Sca-1, ataxin-1; mMSCs, mouse mesenchymal stem cells; FSC, forward-scattered light; SSC, side-scattered light.

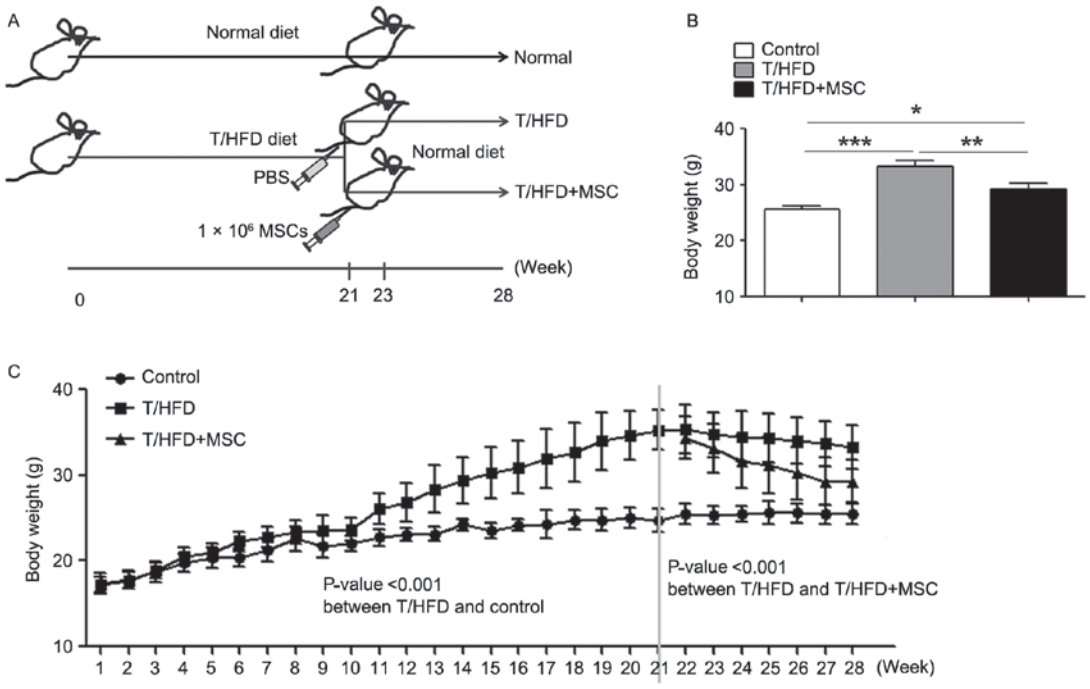

Figure 2. Treatment with MSCs reverses HFD-induced weight gain. (A) Schematic of animal experiment. Body weight data at (B) the endpoint of the experiment and (C) weeks 1-28. As presented, at 21 weeks, HFD-induced weight gain was decreased by treatment with MSCs. The data are presented as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. T/HFD, TROPHIC high-fat diet; MSC, mesenchymal stem cell.

were observed between the T/HFD and T/HFD+MSC mice (Fig. 5C).

The number of CD4^{+} T lymphocytes in the spleen is decreased by treatment with MSCs. MSCs are able to interact with innate and adaptive immune system cells, leading to the modulation of numerous effector functions (9). Analysis of splenic leukocytes was performed in order to explore the effect of MSCs on immunological responses. It was observed that, compared with T/HFD-fed mice, the number of CD4^{+} T lymphocytes

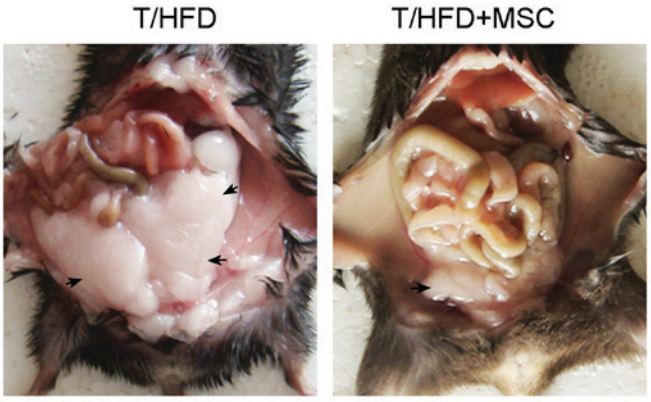

Figure 3. Treatment with MSCs reverses HFD-induced expansion of subcutaneous adipose tissue. A mass of adipose tissue (black arrows) was deposited in the peritoneal cavity of T/HFD mice, and this was decreased in the T/HFD+MSC mice. Representative images are presented. T/HFD, TROPHIC high-fat diet; MSC, mesenchymal stem cell.

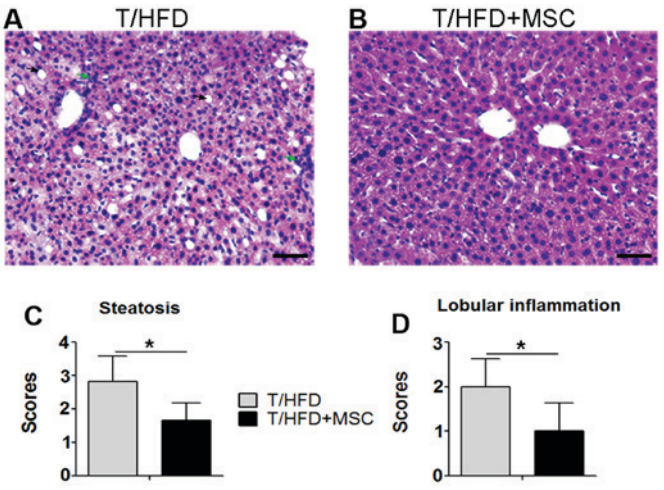

Figure 4. Treatment with MSCs decreases HFD-induced steatosis and lobular inflammation in the liver. Hematoxylin and eosin staining of liver sections from (A) T/HFD and (B) T/HFD+MSC mice. Fat vacuoles are indicated by black arrows and inflammation foci are indicated by green arrows. Scale bar=100 μm. (C) Steatosis and (D) lobular inflammation were scored. Representative images are presented. The data are presented as the mean ± standard deviation. *P<0.05. T/HFD, TROPHIC high fat diet; MSC, mesenchymal stem cell.

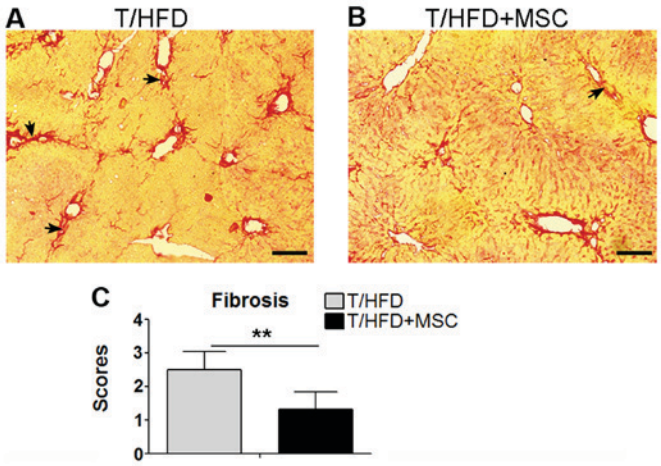

Figure 5. Treatment with MSCs suppresses liver fibrogenesis induced by HFD. Picrosirius red staining of liver section from (A) T/HFD and (B) T/HFD+MSC mice. Red-stained collagen (fibrogenesis) is indicated by black arrows. Scale bar=100 μm. (C) Liver fibrosis was scored. Representative images are presented. The data are presented as the mean ± standard deviation. **P<0.01. T/HFD, TROPHIC high-fat diet; MSC, mesenchymal stem cell.

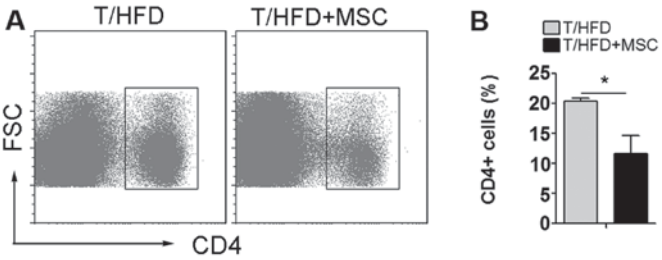

Figure 6. Splenic CD4^{+} T lymphocyte number was decreased by treatment with MSCs. Representative (A) dot plot and (B) quantification of flow cytometry analysis of CD4^{+} T lymphocytes in the spleen. The data are presented as the mean ± standard deviation. *P<0.05. CD, cluster of differentiation; MSC, mesenchymal stem cell; T/HFD, TROPHIC high fat diet; FSC, forward-scattered light.

in the spleen was decreased by treatment with MSCs in the T/HFD+MSC mice (Fig. 6).

Discussion

MSCs were originally isolated from bone marrow by Friedenstein *et al* (22) in 1976, and have subsequently been observed to exist in other organs and tissues (21). However, the bone marrow-derived method based on plastic adherence has proved unsuccessful for mMSCs, due to the low frequency of mMSCs and the contamination of hematopoietic cells in culture (23). In the present study, the compact bone-derived method established originally by Zhu *et al* (21) was used. MSCs that appeared vortex-shaped and fibroblast-like were successfully isolated, and were demonstrated to express putative surface markers of MSCs, including CD29, CD44, CD105 and Sca-1, which was consistent with a previous report (21).

MSCs exhibit potential clinical value in the treatment of liver disease. In a previous study, it was demonstrated that compact bone-derived MSCs improved gross and microscopic liver histopathology and prolonged the survival of mice with thioacetamide-induced FHF, in addition to suppressing CCl_4-induced chronic liver fibrosis (19). In addition, it was demonstrated that treatment with MSCs partially ameliorated FHF, and markedly improved chronic liver fibrosis (19). In the present study, a model of NAFLD was established using T/HFD (20), in order to explore whether MSCs exhibit potential clinical value in NAFLD. It was observed that HFD-fed mice with MSC intervention exhibited a decrease in weight gain. Obesity is often accompanied by the expansion of subcutaneous adipose tissue (4), which was demonstrated to be reversed by treatment with MSCs in the present study. In addition, the steatosis and liver fibrosis in the present model of NAFLD were ameliorated by treatment with MSCs. The results of the present study suggested that MSCs may exhibit clinical value in NAFLD.

The immunomodulatory and immunosuppressive functions of MSCs are potentially involved in the beneficial effect of MSC transplantation, in chronic and acute liver disease (24). Recently, it was demonstrated that MSC therapy suppressed liver fibrosis by downregulating immune cell infiltration (19). In the present study, MSC intervention suppressed lobular inflammatory cell infiltration, indicated by HE staining, in the livers of T/HFD mice. The results of the present study demonstrated that the immunomodulatory and immunosuppressive functions of MSCs may serve a role in the beneficial effect of MSC transplantation observed in the present model of NAFLD.

The immunosuppressive effect of treatment with MSCs, inducing an anti-inflammatory state, has been demonstrated to be associated with an altered distribution of $CD4^+$ T lymphocytes (19). Autologous and allogeneic bone marrow-derived MSCs have been demonstrated to dose-dependently and contact-independently reduce $CD4^+$ T cell proliferation, induced by cellular or nonspecific mitogenic stimuli (25). The suppressive capacities of MSCs were further confirmed in preclinical studies, demonstrating that treatment with MSC is able to modulate pathogenic T cell responses (26-28). In the present study, it was demonstrated that transplantation of compact bone-derived MSCs led to a suppression of $CD4^+$ T cell proliferation in the spleens of T/HFD mice. It is hypothesized that the suppression of $CD4^+$ T cells is one mechanism by which MSCs exert immunomodulatory and immunosuppressive functions.

In summary, compact bone-derived MSCs exhibit potential clinical value in a mouse model of NAFLD. MSC transplantation decreased weight gain and the expansion of subcutaneous adipose tissue, decreased HFD-induced steatosis and lobular inflammation, and suppressed liver fibrogenesis. MSCs exert beneficial effects in the mouse model of NAFLD via immunomodulation and immunosuppression, including the suppression of $CD4^+$ T cells.

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