Int J Clin Exp Pathol 2015;8(7):7694-7709
www.ijcep.com /ISSN:1936-2625/IJCEP0006861

Original Article

Culture and properties of adipose-derived mesenchymal stem cells: characteristics *in vitro* and immunosuppression *in vivo*

Fujiang Cao, Tao Liu, Yunqiang Xu, Dongdong Xu, Shiqing Feng

Department of Orthopedics of Tianjin Medical University General Hospital, Tianjin 300052, China

Received February 8, 2015; Accepted April 10, 2015; Epub July 1, 2015; Published July 15, 2015

Abstract: Objective: To compare the two sources of adipose and bone marrow derived mesenchymal stem cells (BMSCs and AMSCs) in immune regulation and to evaluate the therapeutic effects of AMSCs on Con A induced hepatitis and the possible mechanism involved in it. Methods: We isolated bone marrow and adipose derived mesenchymal stem cells respectively and compared their differences on T lymphocyte activation, proliferation and suppression. We also test the anti-apoptosis ability of AMSCs on LO2 cell line. The effects of intravenous infusion of AMSCs on liver damage were also tested and we detected donor AMSCs in liver of recipient and their effects on the activity of intrahepatic NKT cells. Results: BMSCs and AMSCs were similar in cell phenotype and the difference existed only in the expression of CD106. The results showed that the capacity of suppressing T cells proliferation and activation was weakened in AMSCs. AMSCs ameliorated liver damage and this effect was time and dose dependent. We detected donor AMSCs in liver of recipient which suggested tissue damage could be a clue for AMSCs migration. We also found AMSCs suppress the activity of intrahepatic NKT cells, but this suppress effects was not restricted in liver only, but the whole body. Conclusion: Cell origin and abundance are decisive factors in stem cells applications and with the same premise of AMSCs and BMSCs, adipose tissue is a more promising origin source of stem cells. The immunoregulatory features of MSCs might play an important role in various MSCs cellular therapies.

Keywords: Immune regulation, bone marrow derived mesenchymal stem cells (BMSCs), adipose derived mesenchymal stem cells (AMSCs), natural killer T cells (NKT), hepatitis, immunoregulatory, cellular therapy

Introduction

Mesenchymal stem cells (MSCs) are pluripotent stem cells derived from the mesoderm with self-renewal and differentiation potential [1]. A large number of studies have shown that this kind of bone marrow derived adherent cells could differentiate into a variety of cells such as osteoblasts, fat cells, nerve cells, muscle cells and endothelial cells [2-7]. What is more interesting is that MSCs have low immunogenicity [8] and immune regulation. Low immunogenicity makes them to be possible allograft cells which become an ideal seed cells for tissue engineering and immune regulation bring new hope for the treatment of autoimmune diseases. Some researchers have reported that MSCs cultured and amplified in vitro not only have immune regulatory effects on a variety of immune cells such as T, B, DC, NK [9-13], but this inhibition is not major histocompatibility complex (MHC) restricted. BMSCs from the donor, recipient or a third party have similar immunosuppressive effects, besides, MSCs cultured *in vitro* can also play a role in immune regulation *in vivo*, for example, MSCs can extend survival time of allergenic skin graft [14] and promote the proliferation of allergenic tumor cells [15]. The treatment of MSCs on graft versus-host disease (GVHD) has been proved not only in animal studies but in clinical application [16-18].

We have isolated an Flk1$^+$ mesenchymal stem cell subset from adult bone marrow and found they had not only the three germ layers of cell differentiation potential [19-22], but significant immunomodulatory capacity [23-25] which inhibited T cells proliferation to make more T cells be in G0/G1 phase and inhibit T cells acti-

Table 1. Comparison of the surface markers of BMSCs and AMSCs.

Surface markers	BMSCs	AMSCs
Flk-1	+	+
CD29	+	+
CD31	-	-
CD34	-	-
CD44	+	+
CD45	-	-
CD105	+	+
CD106	+	-
CD166	+	+
CD184	-	-
HLA-ABC	+	+
HLA-DR	-	-

+ positive rate of more than 95%; - positive rate of less than 5%.

vation. In the co-culture assay, they could change the ratio of T cells subset. Normal fat tissue is more easily got than normal bone marrow tissue and we isolated MSCs from discarded adipose tissue from liposuction and found they had morphology and phenotype similar to stem cells from bone marrow. Other laboratories got similar results [26-31], but whether AMSCs and BMSCs had a similar immune function?

Here we isolated mesenchymal stem cells from bone marrow and adipose tissue respectively and examined their effects on T cells cycle, activation, inhibition and proliferation to compare the differences of immune regulation between them.

Material and methods

Regents

L-DMEM, IMDM, DF12 (GibcoBRL); β-mercaptoethanol (Merck); basic fibroblastic growth factor (bFGF, Sigma); vascular endothelial growth factor(VEGF, Sigma); epidermal growth factor (EGF, Gibco); platelet-derived growth factor BB (PDGF-BB, Sigma); antibody: CD11a, CD29, CD31, CD34, CD44, CD45, CD73, CD105, CD106, CD166, CD184, HLA-ABC, HLA-DR (PharMingen); anti-rabbit IgG- fluorescein isothyocyanate (FITC; Sigma), anti-mice IgG-FITC (Sigma), anti-mice IgG-tetramethyl rhodamine isothiocyanate (TRITC; Santa Cruz Biotechnology), anti-rabbit IgG-TRITC (Santa Cruz Biotechnology).

Tissue specimens

Adult bone marrow and adipose samples were taken from Beijing Tiantan Hospital, Capital Medical University. All samples signed informed consent.

Cell preparations and culture of BMSCs

Isolation and culture of BMSCs were performed as described previously with some modifications [29]. Briefly, mononuclear cells were separated by a Ficoll-Paque gradient centrifugation (specific gravity 1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) and the sorted cells were plated at concentration of 1 cell/well by limiting dilution in a total of 96×10 wells coated with fibronectin (Sigma, St Louis, MO) and collagen (Sigma) for each patient. Culture medium was Dulbecco modified Eagle medium and Ham F12 medium (DF12) containing 40% MCDB-201 medium complete with trace elements (MCDB) (Sigma), 2% fetal calf serum (FCS; Gibco Life Technologies, Paisley, United Kingdom), 1× insulin transferrin selenium (Gibco Life Technologies), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 20 ng/mL interleukin-6 (Sigma), 10 ng/mL epidermal growth factor (Sigma), 10 ng/mL platelet-derived growth factor BB (Sigma), 50 ng/mL fetal liver tyrosine kinase 3 (Flt-3) ligand (Sigma), 30 ng/mL bone morphogenetic protein-4 (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco Life Technologies) at 37°C and a 5% CO_2 humidified atmosphere. Culture media were changed every 4 to 6 days.

Preparation of human AMSCs cells from the adult human fat

Human raw lipoaspirates from patients undergoing selective suction-assisted lipectomy were collected after obtaining informed consent from the patients according to procedures approved by the Ethics Committee at Beijing Tiantan Hospital, Capital Medical University. The procedure was described by Zuk et al. [30] with some modifications. The raw liposuctioned aspirate was extensively washed with D-Hanks solution to remove contaminating blood cells and local anesthetics. The extracellular matrix was digested with 0.2% collagenase II (Sigma) at 37°C for 30 min to release the cellular fractions. The cells were washed two times and plated in T-75 tissue culture asks at a density of $2×10^6$/ml. Expansion medium contained 57% DMEM/F-12 (Gibco Life Technologies, Paisley,

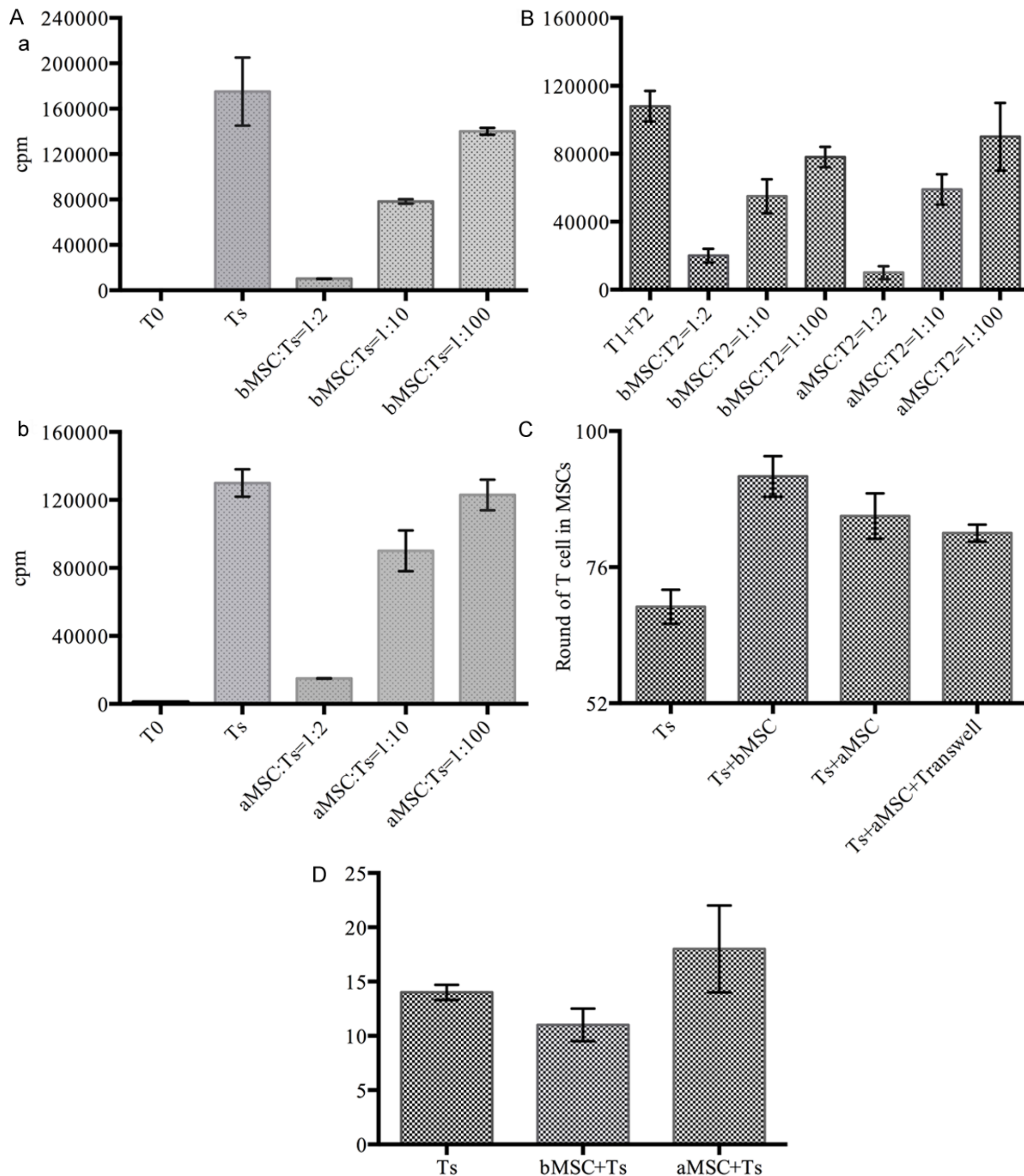

Figure 1. MSCs on T cells activity. A. MSCs on PHA stimulated T cell proliferation. T0: not adding PHA stimulated T cells; Ts: adding PHA stimulated T cells. Normal adult BMSCs or AMSCs and PHA stimulated T cells were co-cultured and the proportion of them were 1:2, 1:10 and 1:100, respectively. When MSCs and T cells were co-cultured at the ratio of 1:2, MSCs could significantly inhibit T cells proliferation and T cells could be suppressed below to 1%. When they were co-cultured at the ratio of 1:10, the ratio of suppression became 46% (BMSCs) and 63% (AMSCs) respectively in the absence of MSCs (statistically significant comparing with PHA stimulated only but in the absence of MSCs. $P < 0.05$). When they were co-cultured at the ratio of 1:100, the inhibitory effect disappeared (statistically significant comparing with PHA stimulated only but in the absence of MSCs. $P > 0.05$). B. The inhibitory effects of MSCs on MLR. T1: stimulated cells; T2: effecter cells; the ratio of T1 and T2 was 1:1. BMSCs or AMSCs were co-cultured with T1 plus T2 respectively and the ratio of MSC and T2 were 1:2, 1:10, 1:100. When MSCs co-cultured with the effecter cells at the ratio of 1:2, they could significantly be inhibited (statistically significant comparing with no co-culture, $P < 0.05$) and were inhibited to 12% and 16% respectively, and inhibited to 43% and 55% when the ratio was 1:10 (statistically significant comparing with no co-culture, $P < 0.05$), and when the ratio was 1:100, the inhibition disappeared. Both BMSCs and AMSCs was not statistical different in comparison. C. MSCs and Transwell

on T cell cycle. Ts: PHA stimulated T cells; BMSCs or AMSCs were co-culture with Ts at the ratio of 1:10. Transwell was added to part of the co-culture system of AMSCs and Ts to separate them for 3 days and the flow cytometry was used to detect the ratio of the cells in different cell cycle periods. BMSCs could inhibited T cells in the G0/G1 phase from 61.27±2.97% to 94.23±2.26% when they were co-cultured and there were statistical difference between the two ($P < 0.05$). AMSCs had a similar role and the ratio of T cells in G0/G1 phase was 85% in the co-culture assay, there existed obvious statistical difference comparing with Ts ($P < 0.05$). D. MSCs on T cell apoptosis. Ts: PHA stimulated T cells; BMSCs or AMSCs were co-culture with Ts at the ratio of 1:10 and the Annexin V detection kit was used to analyze Annexin V positive but PI-negative T-cell ratio 48 hours later. BMSCs could inhibit T cells apoptosis and the ratio of T cells apoptosis was 13.77±0.68% in the absence of BMSCs and 10.07±1.45% in the presence of BMSCs (T cells apoptosis rate was statistically different, $P < 0.05$); however, T cells apoptosis did not decrease when co-cultured with AMSCs.

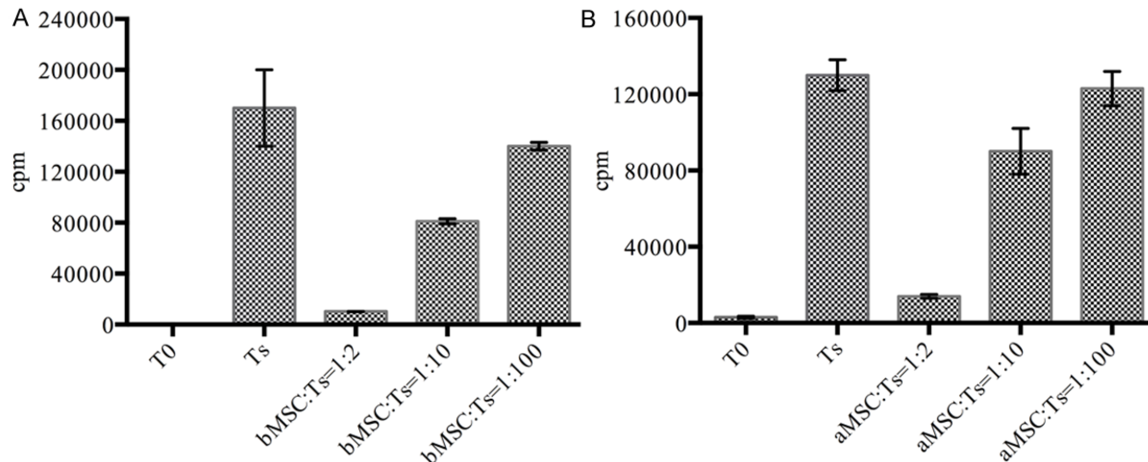

Figure 2. MSCs on the early activation of T cells. Ts: PHA stimulated T cells. BMSCs or AMSCs were co-culture with Ts at the ratio of 1:10 and CD69 was detected 12 hours later (A) and CD25 24 hours later (B). Both BMSCs and AMSCs could inhibit CD69 expression in T cells and the role of BMSCs was more significant which could inhibit CD69 from 58.76±4.83% to 11.06±3.08% (There was statistical difference, $P < 0.05$). AMSCs could only inhibit CD69 decreased to 39.26±3.74% and there was also statistically significant difference, $P < 0.05$). Both BMSCs and AMSCs could inhibit the expression of CD25 and CD25 expression could decreased from 13.5±3.77%, to 3.26±1.76% and 5.3±2.83% respectively (there were no statistically significant difference, $P > 0.05$).

UK), 40% MCDB-201 (Sigma, St. Louis, MO, USA), 2% fetal calf serum (FCS; Gibco), 1 insulin transferrin selenium (ITS; Gibco), 10^8 M dexamethasone (Sigma), 10^4 M ascorbic acid 2-phosphate (Sigma), 10 ng/ml epidermal growth factor (EGF; Sigma), 10 ng/ml platelet-derived growth factor BB (PDGF-BB; Sigma), 100 U/ml penicillin, and 100 Ig/ml streptomycin (Gibco). Once adherent cells were more than 70% confluence, they were detached with 0.125% trypsin and 0.01% EDTA, and replated at a 1:3 dilution under the same culture conditions. All the experiments were done with the 5th passage and the 20th passage, and the results present in the article are all 5th passage.

Fluorescence activated cell sorting (FACS)

For immunophenotype analysis, expanded clonal cells were stained with antibodies against Flk1, CD29, CD31, CD34, CD44, CD45, CD106, (all from Becton Dickinson Immunocytometry Systems, Mountain View, CA). For intracellular antigen detection, cells were first fixed in 2% paraformaldehyde (Sigma) for 15 minutes at 4°C and permeabilized with 0.1% saponin (Sigma) for 1 hour at room temperature. Cells were washed and labeled with fluorescein isothiocyanate (FITC) conjugated secondary goat anti-mouse, goat anti-rabbit, or sheep anti-goat antibodies (Sigma), then washed and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

Mitogen proliferative assays

In mitogen proliferative assays, triplicate wells containing responder $1×10^5$ MNCs were cultured with 50 μg/ml PHA (Roche, USA) in a total volume of 0.1 ml medium at 37°C in 5% CO_2, and $Flk1^+CD31^-CD34^-$ MSCs were added on day

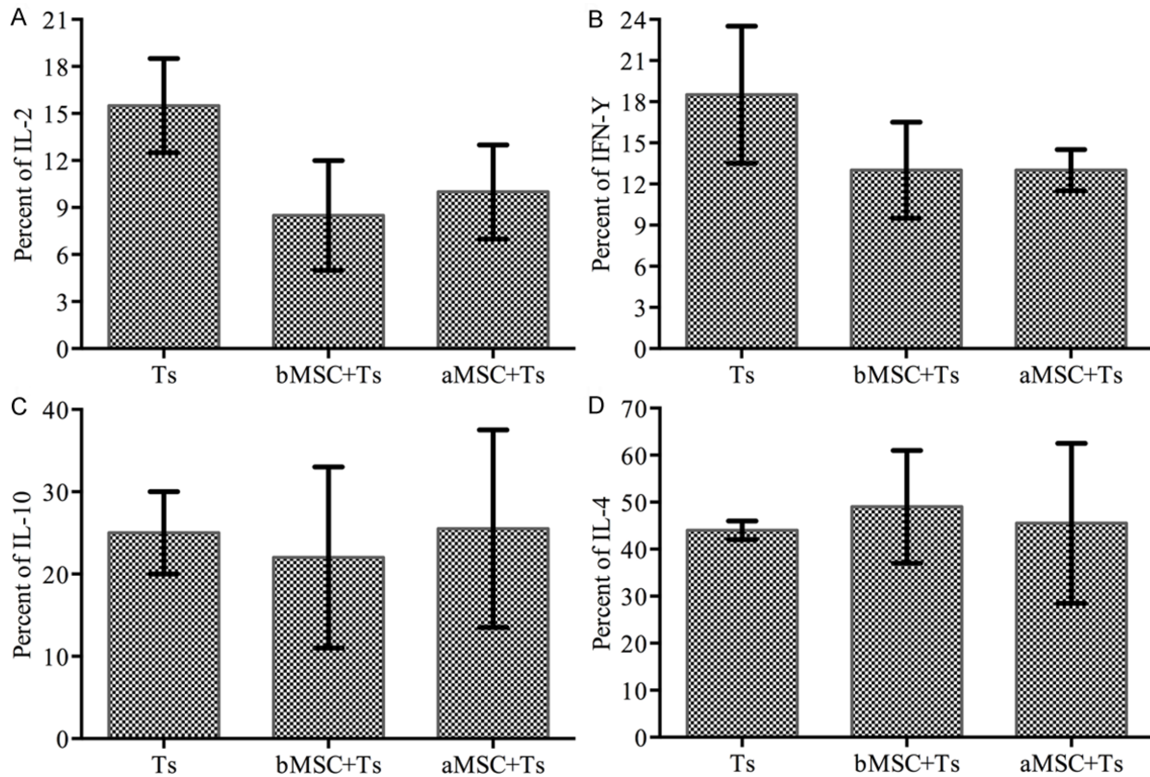

Figure 3. MSCs on T cell subgroups. Ts for the stimulation of different T-cell activator. MSC and the Ts were cocultured at the ratio of 1:10 and the ratio of IL-2 (A), IFN-γ (B), IL-10 (C) and IL-4 (D) producing cells were detected respectively. Both BMSCs and AMSCs could significantly inhibit IL-2 and IFN-γ but had no significant effects on IL-10 and IL-4.

0. Irradiated $Flk1^+CD31^-CD34^-$ MSCs (30Gy) were cocultured with the MNCs at different ratios (MSCs to MNCs = 1:2, 1:10, 1:100). Control wells contained only MNCs. Cultures were pulsed with 1 Ci/well [3H]-TdR (Shanghai Nucleus Research Institute, China) on day 2, and harvested 18 hours later with a Tomtec (Wallac Inc., Gaithersburg, MD) automated harvester. Thymidine uptake was quantified using a liquid scintillation and luminescence counter (Wallac TRILUX).

Mixed lymphocyte reaction assays (MLR)

Blood mononuclear cells (MNCs) were prepared from normal volunteers' peripheral blood by Ficoll-Paque density gradient centrifugation and suspended in RPMI 1640medium supplemented with 10% (vol/vol) FCS, 2 mM l-glutamine, 0.1 mM nonessential amino acids (Life Technologies, Grand Island, NY), 1 mM sodium pyruvate and 100 U/mL penicillin,

Effect of MSCs on T cells cycle

MSCs and MNCs were prepared as described before. T cells, stimulated with PHA (50 μg/ml, final concentration) for 3 days, were cultured alone or with MSCs or 3T3 cell line, then harvested and quantified. One million T cells were fixed with 70% cold ethanol at 4°C for 30 minutes, washed with PBS twice, and stained with 50 μg/ml PI (Sigma, USA) at room temperature for 5 minutes. Data were analyzed with Mod-FIT software.

Effect of MSCs on T cells activation

MSCs and MNCs were prepared as described before respectively. T cells were cultured alone or cocultured with prepared MSCs and stimulated with PHA (50 μg/ml final concentration). The expression of CD25 (BD, USA) and CD69 (BD, USA) was detected by flow cytometry at 24 hours, and CD44 (BD, USA) was detected at 72 hours.

Effect of MSCs on T cell apoptosis

MSCs and MNCs were prepared as described before. T cells were cultured alone or cocultured with MSCs or stimulated with PHA (50 μg/ml final concentration) for 3 days, then har-

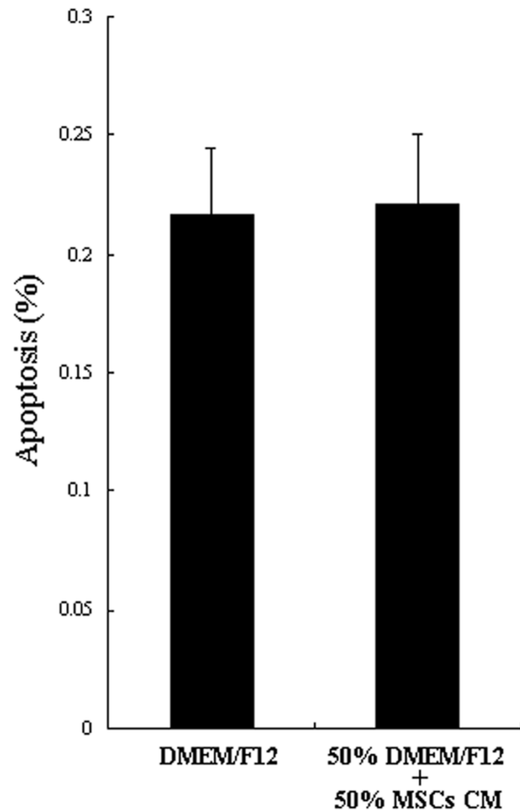

Figure 4. AMSCs condition medium didn't elevate the anti-apoptosis ability of L02 cell line. Percentage of apoptosis in both groups were 22.17±9.49% in serum-free medium DMEN/F12 group and 21.67±5.73% in 50% DMEM/F12 medium plus 50% MSCs supernatants group. There existed no difference in the number of apoptotic cells in both group (P = 0.774) (CM: condition medium).

vested and quantified, stained with Annexin-V kit (BD, USA) and analyzed by flow cytometry (FACS Vantage).

Immunofluorescence and immunocytochemistry

AMSCs were induced to differentiate into endothelium by plating in Matrigel-coated chamber slides with endothelial differentiation medium. Matrigel with cells was dispersed with dispase and the retrieved cells were allowed to attach to the gelatin-coated glass cover slip for 4 hours. Cover slip was fixed with 4% paraformaldehyde for 10 minutes at room temperature and stained for surface markers. The cover slips were placed with cells and rinsed with PBS, and then incubated with blocking buffer (1% BSA in PBS) for 30 minutes at 37°C to minimize non-specific adsorption of the antibodies to the cover slips. FITC-coupled CD31, CD34 (BD Bioscience) were used at a 1:50 dilution. Cells were examined by fluorescence microscopy. For VE-cadherin (CD144, Santa Cruz Biotechnology), a goat polyclonal antibody against VE-cadherin and streptavidin-biotin peroxidase detection system was used.

Cell culture of liver cell line L02

L02 cells were seeded in 75 cm^2 of the culture flask, with D-MEM/F-12 +10% FBS in the culture medium. When reaching 80% confluence, cells were passaged at the ratio of 1:4. In specific experiments, cells were seeded in 6-well plates or 25 cm^2 culture flasks until the confluence reaching suitable for experiments.

MSCs on H_2O_2-induced apoptosis of liver cells L02

After 80% confluence of AMSCs in normal culture conditions, the medium was discarded and replaced by serum-free DMEM/F12 medium for 8 hours, followed by collection of AMSCs in conditioned medium. After L02 cells was grown to confluence, they were treated by AMSCs conditioned fluid or serum-free DMEM/F12 medium for 2 hours, and then in the absence of serum, 200 μM H_2O_2 was added to DMEM/F12 medium and cultured for 6 to 8 hours. Later, Hoechst 33342/PI double staining cells were used to test the cell apoptosis.

Con A induced liver injury model

Con A was injected intravenously into 8 weeks female C57bl/6 mice with the amount of 15 mg/kg, the experimental group mice was injected into Con A at 0 hour, 8 hour and 24 hour time points with the number of $1×10^6$ or $2×10^5$ cells and the control group received the same dose of PBS.

Isolation of intrahepatic mononuclear cells

Liver tissue was isolated after mice death and intrahepatic lymphocytes were isolated as follows: liver tissue was suspended into PBS after grinding stainless. After washing again, the cells were resuspended into 30% Percoll (containing 100 U/ml heparin) and the red blood cells were purified by erythrocyte lysis buffer (red blood cell lysis buffer composition: 0.17 mM NH4Cl, 0.01 mM EDTA, 0.1 M Tris, pH 7.3) through room temperature at 2000 rpm, and

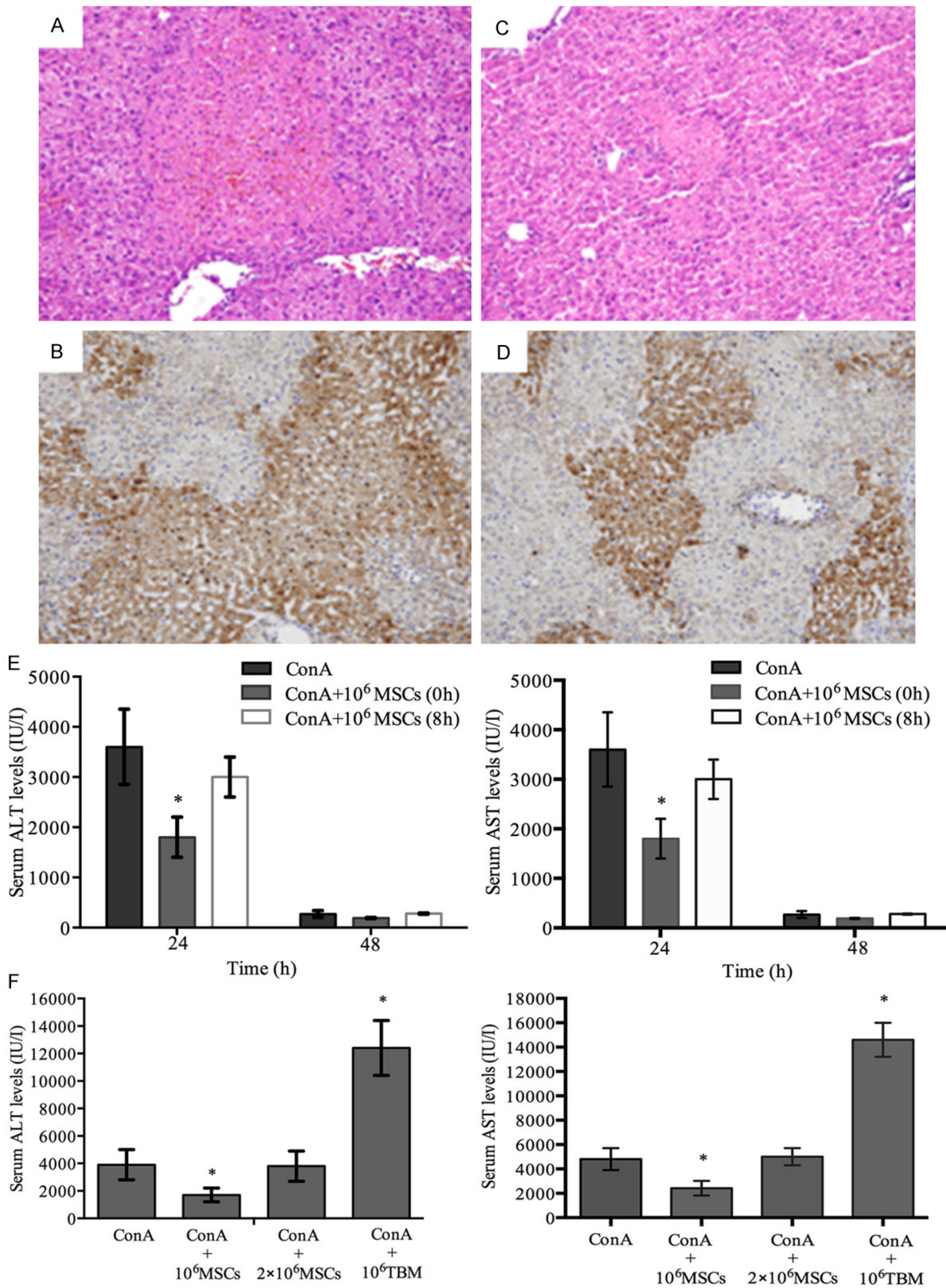

Figure 5. Reduced inflammation and liver damage in AMSCs treated mice. MSCs injection could reduce the liver injury as evidenced by accumulation of dead cells and leukocytes using TdT apoptosis test and haematoxylin and eosin (HE) stain (A-D). Besides, this protective effect was time and dose dependent as evidenced by the AST and ALT levels (E, F).

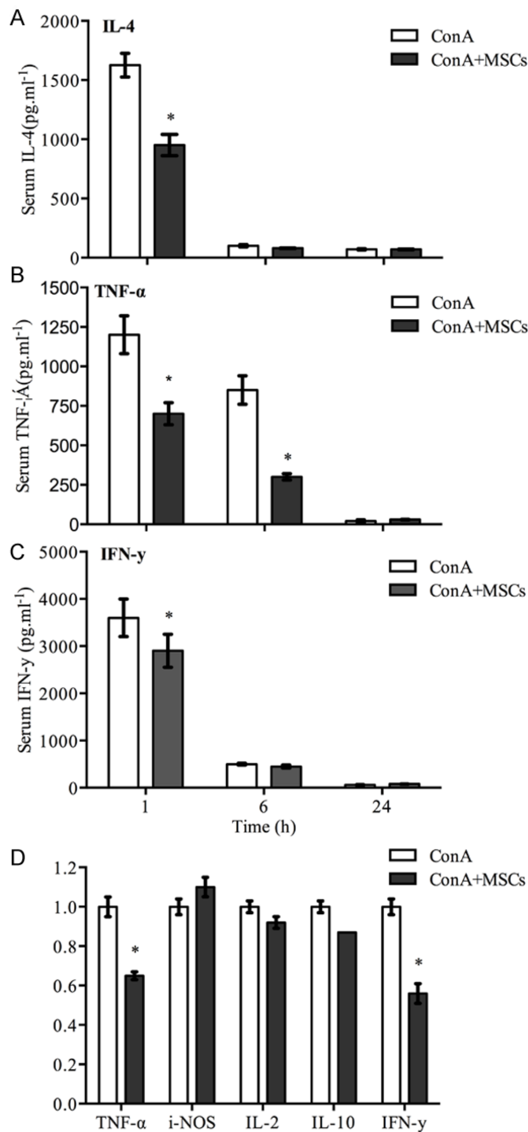

Figure 6. Expression of inflammatory cytokines and factors. Real-time PCR and Elisa were used to detect the levels of inflammatory cytokines and factors in serum and liver tissue. Infusion of MSCs reduced the TNF-α, IFN-γ and IL-4 levels in serum (A-C), but played no effects on iNOS, IL-2 and IL-10 expression (D) (*$P < 0.05$).

then they were washed by RPMI 1640 containing 10% FBS for 2 times.

Results

Comparison of BMSCs and AMSCs in cell morphology and surface markers

We derived MSCs from the bone marrow and adipose tissue and both of the two kinds of cells were spindle and fibers-like. They were all CD11a, CD31, CD34, CD45, CD184, Flk-1 and HLA-DR negative, but CD29, CD44, CD73, CD105, CD166 and HLA-ABC positive in surface markers. In all tested surface markers, only CD106 expression was different in the two kinds of cells (bone marrow mesenchymal stem cells were CD106 positive, while the adipose source were negative) (**Table 1**).

Inhibition of BMSCs and AMSCs on proliferation of PHA stimulated T lymphocytes

To test and compare the normal bone marrow and adipose-derived MSCs on T lymphocyte proliferation, we co-cultured BMSCs or AMSCs with PHA stimulated T cells at different proportion and when the different sources of MSCs with T cells were co-cultured at the ratio of 1:2, MSCs could significantly inhibit T cells proliferation and T cells could be suppressed below to 1%. When MSCs and T cells were co-cultured at the ratio of 1:10, the above inhibition weakened, and the ratio of suppression became 46% (BMSCs) and 63% (AMSCs) respectively in the absence of MSCs. It was statistically significant comparing with PHA stimulated only but in the absence of MSCs ($P < 0.05$). When MSCs and T cells were co-cultured at the ratio of 1:100,the inhibitory effect disappeared (It was statistically significant comparing with PHA stimulated only but in the absence of MSCs. $P > 0.05$). These results suggested both BMSCs and AMSCs had inhibitory effect on PHA stimulated T cell proliferation and this effect was dose dependent (**Figure 1A**).

Inhibition of BMSCs and AMSCs on mixed lymphocyte reaction (MLR)

To study the inhibition of BMSCs and AMSCs on mixed lymphocyte reaction (MLR), we collected mononuclear cells from peripheral blood of two healthy volunteers, one as the stimulated cells and the other as the effecter cells, we set a different ratio of BMSCs or AMSCs to co-culture with them and we found that when BMSCs or AMSCs co-cultured with the effecter cells at the ratio of 1:2, they could significantly be inhibited (statistically significant comparing with no co-culture, $P < 0.05$) and were inhibited to 12% and 16% respectively, and they were inhibited to 43% and 55% when the ratio was 1:10 (statistically significant comparing with no co-culture, $P < 0.05$), and when the ratio was 1:100, the inhibition disappeared. Both bone marrow and adipose-derived MSCs was not statistical

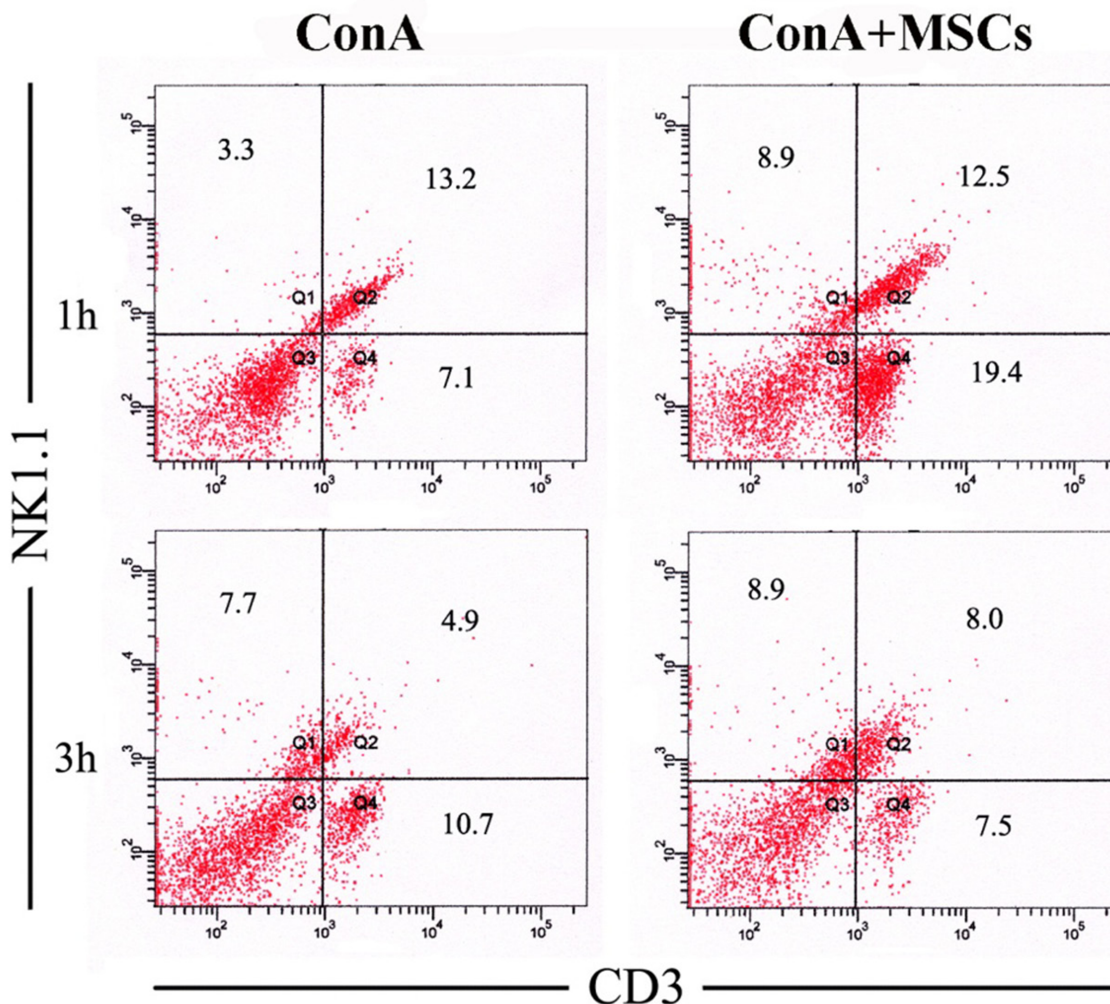

Figure 7. Intrahepatic NKT cells percents after Con A and MSCs injection. After giving Con A, NKT cell activated and secreted large amounts of inflammatory factors and cell toxic substances and we found that the factors in the liver of mice decreased after MSCs were given which indirectly reflected the reduction of NKT activation.

different in comparison. These results suggested that both BMSCs and AMSCs had inhibitory effect on MLR and this effect was dose dependent (**Figure 1B**).

Inhibition of BMSCs and AMSCs on T cells cycle and the impact on Transwell

To investigate the mechanism of inhibition of MSCs on proliferation of T cells, we further analyzed the T cell cycle correlated with proliferation. We co-cultured BMSCs or AMSCs with T cells respectively (MSC : T cell = 1:10) and tested the cells cycle 3 days later, the results showed that BMSCs could inhibited T cells in the G0/G1 phase from 61.27±2.97% to 94.23±2.26% when they were co-cultured (**Figure 1C**) and there were statistical difference between the two ($P < 0.05$). AMSCs had a similar role and the ratio of T cells in G0/G1 phase was 85% in the co-culture assay, there existed obvious statistical difference comparing with PHA stimulated T cells ($P < 0.05$). To further study whether MSCs inhibited T cell proliferation through the role of secreted factors or not, we used Transwell plate to separate AMSCs and T cells and then detected T cells cycle, the ratio of T cells in the G0/G1 phase was about 84% and there was no comparison with Transwell. These results suggested that BMSCs and AMSCs could inhibit T cells from G0/G1 phase to S phase and this role of BMSCs was more significant. Meanwhile, this role was played by factors secreted by MSCs.

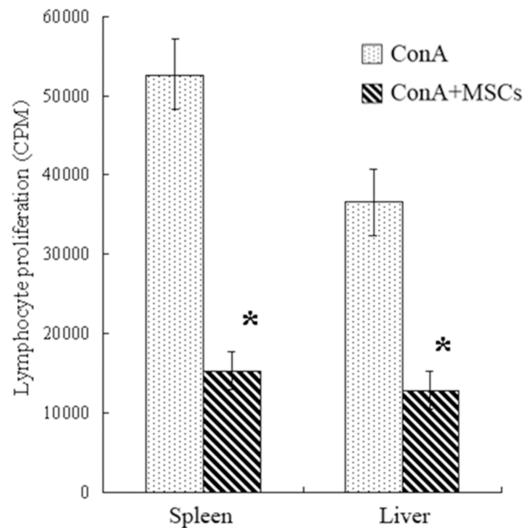

Figure 8. MSCs reduce spleen and intrahepatic lymphocytes activity *in vivo*. Lymphocytes were isolated from the liver and spleen of mice to do Con A induced lymphocyte proliferation experiments. Lymphocytes isolated from spleen and liver of mice with MSCs given corresponding lower than those with Con A injection only which indicated that immunomodulatory effects played by MSCs in vivo was likely to be in the whole body rather than just limited to the damaged liver (*$P < 0.05$).

Effects of BMSCs and AMSCs on T cells apoptosis

To study weather MSCs inhibit T cells proliferation as a result of the induction of T cells apoptosis or not, we used Annexin V kit to analysis T cells apoptosis when they were co-cultured with BMSCs or AMSCs. The results showed that BMSCs could inhibit T cells apoptosis and the ratio of T cells apoptosis (Annexin V positive, PI negative) was 13.77±0.68% in their absence of BMSCs and 10.07±1.45% in the presence of BMSCs (T cells apoptosis rate was statistically different, $P < 0.05$), however, T cells apoptosis did not decrease when co-cultured with AMSCs (**Figure 1D**). These results suggested that MSCs inhibited T cells proliferation was not due to apoptosis induced by T cells.

Effects of BMSCs and AMSCs on early activation of T cells

To investigate the inhibition of MSCs on early activation of T cells, we analyze the CD69 expression of T cells 12 hours and CD25 expression 24 hours after PHA stimulation. Both BMSCs and AMSCs could inhibit CD69 expression in T cells and the role of BMSCs was more significant which could inhibit CD69 from 58.76±4.83% to 11.06±3.08% (There was statistical difference, $P < 0.05$). AMSCs could only inhibit CD69 decreased to 39.26±3.74% and there was also statistically significant difference, $P < 0.05$). Both BMSCs and AMSCs could inhibit the expression of CD25 and CD25 expression could be decreased from 13.5±3.77% to 3.26±1.76% and 5.3±2.83% respectively (there were no statistically significant difference, $P > 0.05$). These results suggested that MSCs could inhibit early activation of T cells and the role of AMSCs was weaker than that of the BMSCs (**Figure 2**).

Effects of BMSCs and AMSCs on T cells subsets

Th0 cells has been reported to be induced into Th1 mainly through secreting IFN-γ and IL-2 and Th2 through secreting IL-10 and IL-4 stimulated by certain signals. In order to further elaborate the effects of BMSCs and AMSCs on Th0 cells to differentiate into Th1 and Th2 cells, we co-cultured mononuclear cells from peripheral blood with BMSCs or AMSCs respectively and used flow cytometry in each group to test the level of IL-10, IL-4 and IL-2, IFN-γ (**Figure 3**). Both BMSCs and AMSCs could significantly inhibit IL-2 and IFN-γ but had no significant effects on IL-10 and IL-4. These results suggested that under normal immune circumstances, BMSCs and AMSCs mainly decreased Th0 cells to differentiate into Th1 cells.

AMSCs supernatant could not improve resistance to H_2O_2 induced apoptosis in L02 cells

In many experiments, AMSCs were observed to reduce tissue injury after transplantation and the most direct reason may be the anti-apoptotic role of cytokines secreted by AMSCs. Therefore, we tested whether the ability of supernatants of BMSCs on L02 cells against H_2O_2 damage changed or not. AMSCs cultured to 80% confluence and the medium was replaced by the serum-free DMEM/F12 medium. MSCs supernatant was collected after 1 day later and 6 wells plate were inoculated with L02 cells. When the cells grew to 100% confluence, the culture medium was replaced by DMEM/F12 serum-free medium or 50% DMEM/F12 medium plus 50% MSCs supernatants, they were cultured for 1 hour, and then original medium was discarded and replaced DMEM/F12 serum-free medium adding 200 μm H_2O_2.

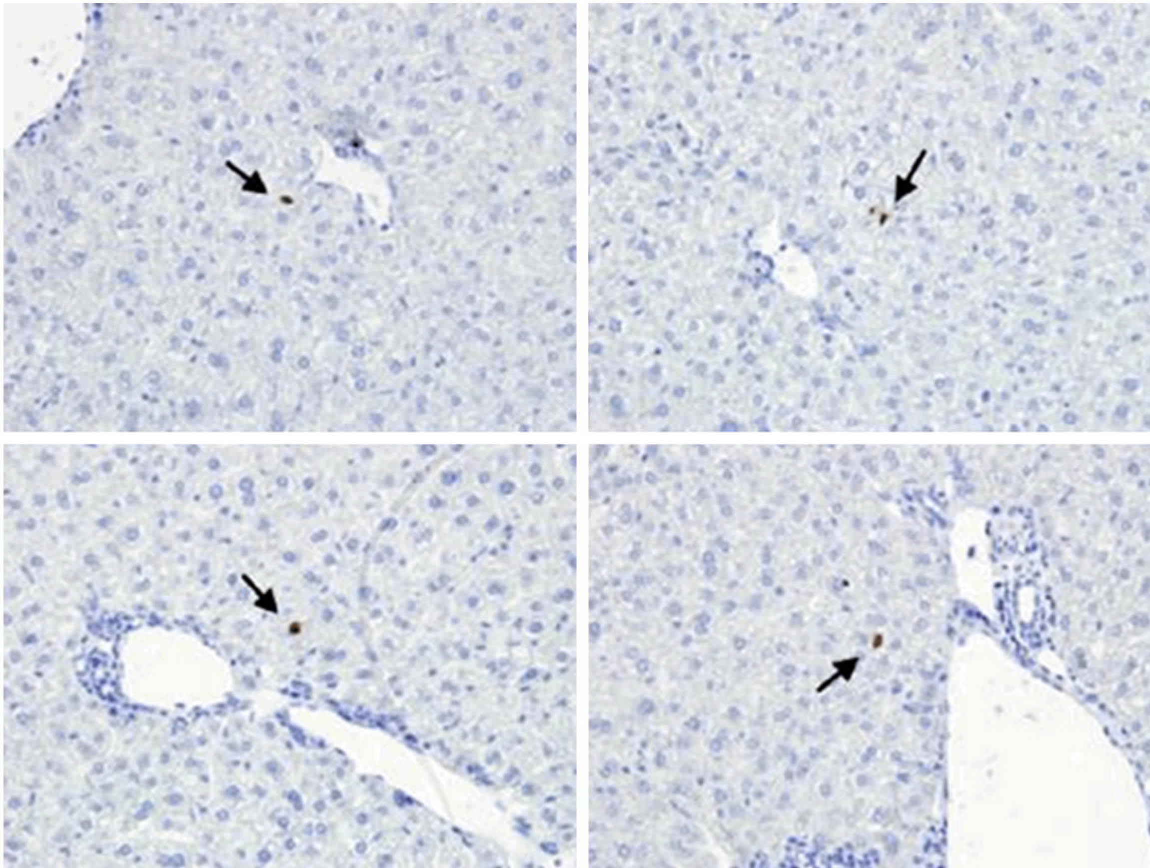

Figure 9. MSCs home to damaged liver. MSCs could be detected from liver tissue 24 hours after injection and could not be detected if they were injected into the mice without liver injury which indicated that tissue damage might be a signal of MSCs location *in vivo*. All the detected MSCs were located near the portal in the liver and all MSCs in the liver disappeared 1 month after injection and could not be detected which might be the results of self clearage or apoptosis (Arrow: eGFP positive cells).

After 8 hours, double staining with Hoechst 33342/PI was used to detect apoptosis. Percentage of apoptosis in both groups were 22.17±9.49% in serum-free medium DMEN/F12 group and 21.67±5.73% in 50% DMEM/F12 medium plus 50% MSCs supernatants group. There existed no difference in the number of apoptotic cells in both group (P = 0.774) (**Figure 4**).

AMSCs on Con A induced acute liver injury

C57bl/6 mice were injected into Con A to induce acute liver injury and the injection of AMSCs could reduce the liver injury which would be seen from HE sections and liver apoptosis biopsy detected by TUNEL (**Figure 5A-D**). After the immediate injection of Con A, AMSCs could significantly reduce liver injury. This protective effect was time and dose dependent (**Figure 5E**, **5F**). After injection of Con A, if AMSCs were immediately given, they could significantly reduced ALT and AST levels in serum, while if AMSCs were given 8 hours after Con A injection, they played no effect. Furthermore, significant effect was observed when AMSCs were given in the amount of 10^6 rather than $2×10^5$. Given that many studies using bone marrow cells or mobilized peripheral blood mononuclear cells for treatment of some diseases, we also measured the treatment effects of giving 10^7 total bone marrow mononuclear cells (TBM) after Con A injection and the results showed the injection of TBM could seriously aggravate liver damage.

Expression of inflammatory cytokines and factors

We used real-time quantitative PCR and Elisa to detect the levels of inflammatory cytokines and factors in serum and liver tissue. Infusion

of MSCs reduced the TNF-α, IFN-γ and IL-4 levels in serum (**Figure 6A-C**), but played no effects on iNOS, IL-2 and IL-10 expression (**Figure 6D**).

NKT in the liver

$CD3^+$ $NK1.1^+$ of NKT are vital cells in Con A induced liver injury [3]. After giving Con A, NKT cell activated and secreted large amounts of inflammatory factors and cell toxic substances. We found that amounts of inflammatory factors and cell toxic substances in the liver of mice decreased after AMSCs were given (**Figure 7**) which indirectly reflected the reduction of NKT activation.

Infusion of AMSCs in mice caused incompetent lymphocytes (anergy) in vivo

To verify the infusion of AMSCs induced immune suppression of lymphocytes in the liver specifically or systematically, we isolated lymphocytes from the liver and spleen of mice to do Con A induced lymphocyte proliferation experiments. Lymphocytes isolated from spleen and liver of mice with AMSCs given corresponding lower than those with Con A injection only (**Figure 8**). This showed AMSCs played immunomodulatory effects *in vivo* was likely to be in the whole body rather than just limited to the damaged liver.

AMSCs detected in the damaged liver

MSCs could be detected from liver tissue 24 hours after injection (**Figure 9**) and could not be detected if they were injected into the mice without liver injury which indicated that tissue damage might be a signal of MSCs location *in vivo*. Interestingly, all the detected MSCs were located near the portal in the liver. We did not detested whether MSCs could differentiate into liver cells, but all MSCs in the liver disappeared 1 month after injection and could not be detected which might be the results of self clearage or apoptosis. This also showed indirectly that MSCs did not differentiate into tissues consistent with their positioning tissue cells.

Discussion

Back in the 60s, Rodbell et al. [33] had already isolated mature adipose cells from the fat tissue and fat progenitor cells that could differentiate into fat cells. In the 80s, Bukowiechi et al. [34] found that the cells in different stages of maturity existed in adipose stromal tissue, of which matrix-vascular (SV) cells was considered to be progenitor cells of fat. SV cells could be induced into mature fat cells treated with cold acclimation. In recent years, more and more people undergone liposuction surgery and many researchers began to show interests in extracting stem cells from the fat, so, adipose-derived stem cells have became the focus of attention. However, whether AMSCs and BMSCs have similar immune function or not? In a previous study in our laboratory we found that the number of colony formation of bone marrow mononuclear cells was 87±72 per 10^6 and adipose tissue 141±74 per 10^4. From the data obtained, the amount of mononuclear cells colony formation of adipose tissue was more than 150 times larger than those of the bone marrow. So if AMSCs and BMSCs have similar immune regulation, AMSCs would have a more broad application prospects in treatment for autoimmune disease, organ transplantation and graft-versus-host disease.

Puissant et al. [35] studied the immunological characteristics of AMSCs and found that AMSCs could inhibit mitogen-stimulated T cells proliferation and MLR, but they did not systematically compare the similarities and differences of AMSCs and BMSCs on T cells. In our study, we made a comprehensive comparison of the AMSCs and BMSCs on T cells proliferation, cells cycle, apoptosis, activation and the differentiation of T helper cells. We found that both AMSCs and BMSCs could inhibit T cells proliferation and the effects on mitogen stimulation and T cells proliferation were dose dependent, furthermore, the inhibitory effect was significant when MSCs and T cells were at a ratio of 1:2 and disappeared when the ratio came to 1:100. Both AMSCs and BMSCs could make more T cells to be inhibited in the G0/G1 phase and inhibited the early activation of T cells, but the role of AMSCs was weaker compared with that of BMSCs and the AMSCs did not inhibit T cells apoptosis. AMSCs and BMSCs played the same role in the differentiation of Th0 to Th1 or Th2, they mainly inhibited the differentiation of Th0 to Th1 cells (IL-2 and IFN-γ producing cells) differentiation, but had little effects on differentiation of Th0 to Th2 cells (IL-4 and IL-10 producing cells), suggesting that AMSCs and BMSCs played effects on the normal immune system mainly through affecting the cellular immune response.

We then showed use the Con A induced liver injury model to test the role of AMSCs. Con A is a mitogen that can activate T cells and Con A-induced liver injury in mice model, comparing with other models such as carbon tetrachloride, D-galactosamine models, is considered to be more suitable for the study of human viral hepatitis, the pathogenesis of autoimmune liver disease and anti-drug screening of liver injury. Con A stimulation of Th cells would activate Th cells and macrophages to produce excessive IFN-γ, TNF-α and other cytokines. TNF-α had been proven to be the terminal transmitter to mediate liver injury in the cytokine network, it acted as not only the positive triggering factor of liver cells apoptosis which directly damaged endothelial cells but the activator of neutrophil to accumulate in the liver of their chemotaxis which helped the release of proteases or oxygen free radicals to cause liver cell apoptosis or necrosis. IFN-γ and TNF-α are important inflammatory neuro-transmitters to cause acute liver injury and IFN-γ stimulated by T lymphocytes is the important activator of macrophages which helps Kupffer cells in the liver to participate in inflammation and stimulate macrophages to secret TNF-α. Studies showed that pretreatment with anti-IFN-γ or anti-TNF-α could completely block the Con A-induced liver injury in mice. The Con A induced fulminant hepatitis is mediated by TNF-α produced by macrophages and IFN-γ secreted by T cells. The Kupffer cells in the liver and NO stimulated by liver cells, are important stimulators in the production of TNF-α. We showed here that MSCs did not affect the production of iNOS in the liver, but decreased the production of TNF-α and IFN-γ which indicated that MSCc affected the production of TNF-α and IFN-γ by other means. IL-10 was involved in the inhibition of synthesis of inflammatory factors and colony stimulating factor (CSF). It also inhibited Th1 cells to produce cytokines IL-2, IL-3, IFNγ, TNFβ, GM-CSF and so on. Johann et al. [34] reported that IL-10 would be produced and progressively increased 1 hour later when C3H/HeJ mice were injected with Con A. The level of IL-10 reflected the body's natural resistance to the injury to some extent.

CD1d deficient mice (which lack NKT) did not lead to Con A-induced liver injury. Infusion of NKT and Con A into CD1d deficient mice could lead to hepatitis [35] which demonstrated NKT played an important role in hepatitis pathogenesis. We showed that infusion of MSCs in the experimental animals caused incompetence of lymphocytes and reduced the number of NKT activation. We also observed that MSCs could chemotaxis to the injured position and the suppression of lymphocytes by MSCs was systemic and not limited to the liver area. Wada et al. [29] believed this could be ascribed to the location to the lymph nodes. We did not detect whether MSCs targeted to liver could differentiate into liver cells. But the fact that we did not detected donor cells even at the 30 days indicated that MSCs had been cleared which might because this environment were not appropriate to the survival of MSCs. Many studies have showed that bone marrow cells could differentiate into liver cells [31, 33, 36], which provided a theoretical support in its treatment of liver diseases, besides, it would be more easy to use bone marrow mononuclear cells which do not need to undergo cell sorting or cell culture *in vitro*. But our research showed that bone marrow mononuclear cells transplantation in the model of acute hepatitis did not play any treatment effects, but instead aggravated the injury in the liver which suggested that non-essential cells planted into the target organ might be toxic. Hematopoietic cells could produce fibrosis and excessive pre-angiogenic factors and inflammatory factors, so, the unnecessary injection of these cells might result in edema and increase bleeding tendency of blood vessels. Therefore, we must take into account of the disease mechanism when we do stem cell therapy which therefore could not be considered merely tissue repair.

MSCs have recently been used to detect in the treatment of many diseases. In addition to autoimmune diseases, there are also ischemic disease [36-38], radiation disease [2, 17], trauma [18], chemical-induced injury [19-21] and so on. In all reports, MSCs are proved to be effective. Some of the effectiveness was due to the differentiation of MSCs by some reports [14, 20, 22] and others were attributed to some anti-apoptotic factor secreted by MSCs [19, 21, 39]. Given the fact that in all the immuno-competent animals, the injury will inevitably lead to immune responses and most of the immune responses exacerbated the injury, so, systemic immune suppression induced by MSCs infusion should played important role in the stem cells treatment.

Generally speaking, we here proved that AMSCs and BMSCs had a similar role in immune regulation. Because cell origin and abundance are decisive factors in stem cells applications and with the same premise of AMSCs and BMSCs, adipose tissue is a more promising origin source of stem cells. We also evaluate the therapeutic effect of AMSCs in Con A induced hepatitis in this study and we found intravenous infusion of AMSCs ameliorated liver damage and this effect was time and dose dependent. We also detected donor AMSCs in liver of recipient suggesting tissue damage could be a clue for AMSCs migration. Besides, AMSCs suppress the activity of intrahepatic NKT, but this suppress effect was not restricted in liver, but systematically. All these results suggested the immunoregulatory features of AMSCs might play important role in various AMSCs cellular therapies.

Acknowledgements

This work was funded by National Natural Science Foundation for Young Scholars of China(Grant No 81101362 and 81401784) and Key Project of National Natural Science Fundation of China (81330042). This work was also supported by Special Program for Sino-Russian Joint Research which was Sponsored by the Ministry of Science and Technology, China (2014DFR31210) and Key Program which was Sponsored by the Tianjin Science and Technology Committee, China (13RCGFSY19000, 14ZCZDSY00044).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shiqing Feng, Department of Orthopedics of Tianjin Medical University General Hospital, 154 Anshan Road, Heping District, Tianjin 300052, China. E-mail: fengshiqingvip2006@163.com

References

[1] Fridenshteĭn AIa. Stromal bone marrow cells and the hematopoietic microenvironment. Arkh Patol 1982; 44: 3-11.

[2] Naito H, Dohi Y, Zimmermann W, Tojo T, Takasawa S, Eschenhagen T, Taniguchi S. The effect of mesenchymal stem cell osteoblastic differentiation on the mechanical properties of engineered bone-like tissue. Tissue Eng Part A 2011; 17: 2321-9.

[3] Kasper G, Dankert N, Tuischer J, Hoeft M, Gaber T, Glaeser JD, Zander D, Tschirschmann M, Thompson M, Matziolis G, Duda GN. Mesenchymal stem cells regulate angiogenesis according to their mechanical environment. Stem Cells 2007; 25: 903-10.

[4] Chan J, Waddington SN, O'Donoghue K, Kurata H, Guillot PV, Gotherstrom C, Themis M, Morgan JE, Fisk NM. Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic mdx mouse. Stem Cells 2007; 25: 875-84.

[5] Li F, Bronson S, Niyibizi C. Derivation of murine induced pluripotent stem cells (iPS) and assessment of their differentiation toward osteogenic lineage. J Cell Biochem 2010; 109: 643-52.

[6] Krause U, Harter C, Seckinger A, Wolf D, Reinhard A, Bea F, Dengler T, Hardt S, Ho A, Katus HA, Kuecherer H, Hansen A. Intravenous delivery of autologous mesenchymal stem cells limits infarct size and improves left ventricular function in the infarcted porcine heart. Stem Cells Dev 2007; 16: 31-7.

[7] Rider DA, Dombrowski C, Sawyer AA, Ng GH, Leong D, Hutmacher DW, Nurcombe V, Cool SM. Autocrine fibroblast growth factor 2 increases the multipotentiality of human adipose-derived mesenchymal stem cells. Stem Cells 2008; 26: 1598-608.

[8] Semenov OV, Koestenbauer S, Riegel M, Zech N, Zimmermann R, Zisch AH, Malek A. Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation. Am J Obstet Gynecol 2010; 202: 193, e1-e13.

[9] Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005; 105: 1815-22.

[10] Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 2005; 105: 2821-7.

[11] Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V, Uccelli A. Human mesenchymal stem cells modulate B-cell functions. Blood 2006; 107: 367-72.

[12] Moayedi Y, Basch ML, Pacheco NL, Gao SS, Wang R, Harrison W, Xiao N, Oghalai JS, Overbeek PA, Mardon G, Groves AK. The candidate splicing factor Sfswap regulates growth and patterning of inner ear sensory organs. PLoS Genet 2014; 10: e1004055.

[13] Qu SY, Ou-Yang HF, He YL, Li ZK, Shi JR, Song LQ, Wu CG. Der p2 recombinant bacille Calmette-Guerin priming of bone marrow-derived dendritic cells suppresses Der p2-induced T helper17 function in a mouse model of asthma. Respirology 2014; 19: 122-31.

[14] Kong D, Heath E, Chen W, Cher M, Powell I, Heilbrun L, Li Y, Ali S, Sethi S, Hassan O, Hwang C, Gupta N, Chitale D, Sakr W, Menon M, Sarkar F. Erratum: Epigenetic silencing of miR-34a in human prostate cancer cells and tumor tissue specimens can be reversed by BR-DIM treatment. Am J Transl Res 2013; 6: 102-103.

[15] Reuven ML, Neely ST, Kopun JG, Rasetshwane DM, Allen JB, Tan H, Gorga MP. Effect of calibration method on distortion-product otoacoustic emission measurements at and around 4 kHz. Ear Hear 2013; 34: 779-88.

[16] Pan W, Jin Y, Chen J, Rottier RJ, Steel KP, Kiernan AE. Ectopic expression of activated notch or SOX2 reveals similar and unique roles in the development of the sensory cell progenitors in the mammalian inner ear. J Neurosci 2013; 33: 16146-57.

[17] Korrapati S, Roux I, Glowatzki E, Doetzlhofer A. Notch signaling limits supporting cell plasticity in the hair cell-damaged early postnatal murine cochlea. PLoS One 2013; 8: e73276.

[18] Slowik AD, Bermingham-McDonogh O. Hair cell generation by notch inhibition in the adult mammalian cristae. J Assoc Res Otolaryngol 2013; 14: 813-28.

[19] Maruyama K, Uematsu S, Kondo T, Takeuchi O, Martino MM, Kawasaki T, Akira S. Strawberry notch homologue 2 regulates osteoclast fusion by enhancing the expression of DC-STAMP. J Exp Med 2013; 210: 1947-60.

[20] Colpitts SL, Stonier SW, Stoklasek TA, Root SH, Aguila HL, Schluns KS, Lefrançois L. Transcriptional regulation of IL-15 expression during hematopoiesis. J Immunol 2013; 191: 3017-24.

[21] Volkenstein S, Oshima K, Sinkkonen ST, Corrales CE, Most SP, Chai R, Jan TA, van Amerongen R, Cheng AG, Heller S. Transient, afferent input-dependent, postnatal niche for neural progenitor cells in the cochlear nucleus. Proc Natl Acad Sci U S A 2013; 110: 14456-61.

[22] Shen Y, Richards VM. Bayesian adaptive estimation of the auditory filter. J Acoust Soc Am 2013; 134: 1134-45.

[23] Martin GK, Stagner BB, Lonsbury-Martin BL. Time-domain demonstration of distributed distortion-product otoacoustic emission components. J Acoust Soc Am 2013; 134: 342-55.

[24] Ory V, Tassi E, Cavalli LR, Sharif GM, Saenz F, Baker T, Schmidt MO, Mueller SC, Furth PA, Wellstein A, Riegel AT. The nuclear coactivator amplified in breast cancer 1 maintains tumor-initiating cells during development of ductal carcinoma in situ. Oncogene 2014; 33: 3033-42.

[25] Liu H, Zhou J, Cheng P, Ramachandran I, Nefedova Y, Gabrilovich DI. Regulation of dendritic cell differentiation in bone marrow during emergency myelopoiesis.J Immunol 2013; 191: 1916-26.

[26] Joo WJ, Sweeney LB, Liang L, Luo L. Linking cell fate, trajectory choice, and target selection: genetic analysis of Sema-2b in olfactory axon targeting. Neuron 2013; 78: 673-86.

[27] Viemeister NF, Byrne AJ, Stellmack MA. Spectral and level effects in auditory signal enhancement. Adv Exp Med Biol 2013; 787: 167-74.

[28] Gopisetty A, Bhattacharya P, Haddad C, Bruno JC Jr, Vasu C, Miele L, Prabhakar BS. OX40L/Jagged1 cosignaling by GM-CSF-induced bone marrow-derived dendritic cells is required for the expansion of functional regulatory T cells. J Immunol 2013; 190: 5516-25.

[29] Kiernan AE. Notch signaling during cell fate determination in the inner ear. Semin Cell Dev Biol 2013; 24: 470-9.

[30] Munnamalai V, Fekete DM. Wnt signaling during cochlear development. Semin Cell Dev Biol 2013; 24: 480-9.

[31] Rehman A, Hemmert KC, Ochi A, Jamal M, Henning JR, Barilla R, Quesada JP, Zambirinis CP, Tang K, Ego-Osuala M, Rao RS, Greco S, Deutsch M, Narayan S, Pachter HL, Graffeo CS, Acehan D, Miller G. Role of fatty-acid synthesis in dendritic cell generation and function. J Immunol 2013; 190: 4640-9.

[32] Beijer MR, Molenaar R, Goverse G, Mebius RE, Kraal G, den Haan JM. A crucial role for retinoic acid in the development of Notch-dependent murine splenic CD8- CD4- and CD4+ dendritic cells. Eur J Immunol 2013; 43: 1608-16.

[33] Mochizuki K, Xie F, He S, Tong Q, Liu Y, Mochizuki I, Guo Y, Kato K, Yagita H, Mineishi S, Zhang Y. Delta-like ligand 4 identifies a previously uncharacterized population of inflammatory dendritic cells that plays important roles in eliciting allogeneic T cell responses in mice. J Immunol 2013; 190: 3772-82.

[34] Jung JY, Avenarius MR, Adamsky S, Alpert E, Feinstein E, Raphael Y. siRNA targeting Hes5 augments hair cell regeneration in aminoglycoside-damaged mouse utricle. Mol Ther 2013; 21: 834-41.

[35] Lina IA, Lauer AM. Rapid measurement of auditory filter shape in mice using the auditory brainstem response and notched noise. Hear Res 2013; 298: 73-9.

[36] Hwang NS, Im SG, Wu PB, Bichara DA, Zhao X, Randolph MA, Langer R, Anderson DG. Chondrogenic priming adipose-mesenchymal stem cells for cartilage tissue regeneration. Pharm Res 2011; 28: 1395-405.

[37] Markel TA, Wang Y, Herrmann JL, Crisostomo PR, Wang M, Novotny NM, Herring CM, Tan J,Lahm T, Meldrum DR. VEGF is critical for stem cell-mediated cardioprotection and a crucial paracrine factor for defining the age threshold in adult and neonatal stem cell function. Am J Physiol Heart Circ Physiol 2008; 295: H2308-14.

[38] Steigman SA, Armant M, Bayer-Zwirello L, Kao GS, Silberstein L, Ritz J, Fauza DO. Preclinical regulatory validation of a 3-stage amniotic mesenchymal stem cell manufacturing protocol. J Pediatr Surg 2008; 43: 1164-9.

[39] Markovic M, Crichton K, Read JW, Lam P, Slater HK. Effectiveness of ultrasound-guided corticosteroid injection in the treatment of Morton's neuroma. Foot Ankle Int 2008; 29: 483-7.