Biological effects of bone marrow mesenchymal stem cells on hepatitis B virus in vitro

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Received November 26, 2015; Accepted December 9, 2016

DOI: 10.3892/mmr.2017.6330

Abstract. The aim of the present study was to explore the effects of co-culturing bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured with hepatitis B virus (HBV)-infected lymphocytes in vitro. BM-MSCs and lymphocytes from Brown Norway rats were obtained from the bone marrow and spleen, respectively. Rats were divided into the following five experimental groups: Group 1, splenic lymphocytes (SLCs); group 2, HepG2.2.15 cells; group 3, BM-MSCs + HepG2.2.15 cells; group 4, SLCs + HepG2.2.15 cells; and group 5, SLCs + BM-MSCs + HepG2.2.15 cells. The viability of lymphocytes and HepG2.2.15 cells was assessed using the MTT assay at 24, 48 and 72 h, respectively. Levels of supernatant HBV DNA and intracellular HBV covalently closed circular DNA (cccDNA) were measured using quantitative polymerase chain reaction. Supernatant cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA). T cell subsets were quantified by flow cytometry using fluorescence-labeled antibodies. In addition, the HBV genome sequence was analyzed by direct gene sequencing. Levels of HBV DNA and cccDNA in group 5 were lower when compared with those in group 3 or group 4, with a significant difference observed at 48 h. The secretion of interferon-γ was negatively correlated with the level of HBV DNA, whereas secretion of interleukin (IL)-10 and IL-22 were positively correlated with the level of HBV DNA. Flow cytometry demonstrated that the percentage of CD3⁺CD8⁺ T cells was positively correlated with the levels of HBV DNA, and the CD3⁺CD4⁺/CD3⁺CD8⁺ ratio was negatively correlated with the level of HBV DNA. Almost no mutations in the HBV DNA sequence were detected in HepG2.2.15 cells co-cultured with BM-MSCs, SLCs, or in the two types of cells combined. BM-MSCs inhibited the expression of HBV DNA and enhanced the clearance of HBV, which may have been mediated by the regulation of the Tc1/Tc2 cell balance and the mode of cytokine secretion to modulate cytokine expression.

Introduction

In China, the yearly mortality rate for end-stage liver disease is >300,000 patients (1). Of the >30 million patients with chronic liver disease in China, ~80% are infected with the hepatitis B virus (HBV) (2). The most effective treatment for HBV-associated end-stage liver disease is liver transplantation. However, without effective prophylaxis, the risk of HBV re-infection following transplantation may reach >80% (3,4). The current treatment protocol of nucleos(t)ide analogues combined with hepatitis B immunoglobulin (HBIG) following liver transplantation, greatly reduces the hepatitis B recurrence rate (2,5,6). However, the high cost remains a heavy burden for patients (7,8), and the long-term use of nucleos(t)ide analogues may lead to HBV resistance (9,10). Application of the HBV vaccine following liver transplantation may potentially lead to the withdrawal of nucleoside analogues and HBIG therapy, however the vaccine is less effective due to the use of immunosuppressants following transplantation (11,12). Therefore, it is important to identify novel methods to prevent hepatitis B recurrence following liver transplantation.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) have demonstrated anti-inflammatory (13,14) and angiogenesis-enhancing effects (15,16) with low immunogenicity (17,18). In addition, BM-MSCs exhibit immunomodulatory capabilities in animal models of rejection following transplantation (19-21), which may represent a promising method for inducing immune tolerance. Transfusions of umbilical cord-derived MSCs for patients with HBV-associated acute-on-chronic liver failure resulted
in improved liver function and alleviated liver damage (22). However, the biological effects of BM-MSCs on HBV have not yet been reported. In the present study, the effect of BM-MSCs on HBV replication and genome mutation in vitro was investigated, as well as its associated mechanisms. The results of the current study may provide innovative strategies for the prevention of hepatitis B recurrence following liver transplantation.

**Materials and methods**

**Animals and cell lines.** A total of 12 specific pathogen-free Brown Norway (BN) male rats (age, 4-5 weeks; body weight, 200-220 g) were used for the isolation and identification of BM-MSCs. Inbred male BN rats were kept 2 rats per cage at 24°C, with 50% humidity and a 12 h light and dark cycle, with free access to water and food. An additional 6 specific pathogen-free BN male rats (age, 4-5 weeks; body weight, 200-220 g) were used for the extraction of splenic lymphocytes (SLCs), and were kept under the same conditions as described above. All animals were purchased from the Chinese Academy of Military Medical Sciences (Beijing, China). The use of animals and the animal experimental procedures employed for the purposes of this study were approved by the Ethics Committee of Tianjin First Central Hospital (Tianjin, China). The human hepatocellular carcinoma cell line HepG2.2.15 was donated by Professor Wei Lai (Hepatology Institute of Peking University Affiliated Hospital, Beijing, China), and contained the complete HBV genome, as well as expressed HBV-associated antigens and secreted whole Dane particles (23,24).

**Instruments and reagents.** The following instruments and reagents were used: Dulbecco’s modified Eagle’s medium (DMEM) and DMEM/F12 media (1:1; Hyclone, Logan, UT, USA), G418 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), fetal bovine serum (FBS; Biowest, Nuaille, France), transwell plates (Corning, Inc., Corning, NY, USA), MTT reagent (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China), dimethyl sulfoxide (DMSO; Amresco, Solon, OH, USA), lymphocyte separation medium (Beijing Dingguo Changsheng Biotechnology Co., Ltd.), TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), antibodies directed against CD29 (cat. no. RIF03; Biolegend, Inc., San Diego, CA, USA), CD34 (cat. no. sc-7324; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), CD3-APC mAb (cat. no. 11-0040-82), CD8a-PE-Cy7 (cat. no. 205208), CD45 (cat. no. 202207) and RT1B (cat. no. 205305) for the identification of BM-MSCs (Biolegend, Inc., San Diego, CA, USA), CD34-FITC (1:200), RT1A-PE (1:80) and RT1B-FITC (1:200). Cells were then washed with PBS and analyzed by flow cytometry (FACSCalibur; BD Biosciences) to determine the phenotype and purity of BM-MSCs.

**Isolation and identification of BM-MSCs.** BM-MSCs were aseptically isolated from the femur and tibia of 12 male BN rats. Red blood cells were lysed using 0.1 mol/l NH$_4$Cl, and the remaining cells were washed, resuspended and cultured in DMEM/F12 (1:1) media containing 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and 15% FBS. BM-MSCs were cultured in an incubator at 37°C and 5% CO$_2$ with saturating humidity. The medium was refreshed every 48 h. When cells at passage 3 had reached 80% confluence, cells were trypsinized, washed, centrifuged at 300 x g for 5 min at room temperature, and resuspended at 1x10$^6$ cells/ml in phosphate-buffered saline (PBS). BM-MSCs (100 µl) were incubated with the following fluorescence-labeled antibodies at 4°C for 30 min in the dark: CD29-PE (1:80), CD34-FITC (1:20), CD45-PE (1:80), CD90-FITC (1:200), RT1A-PE (1:80) and RT1B-FITC (1:200). Cells were then washed with PBS and analyzed by flow cytometry to determine the phenotype and purity of BM-MSCs.

**Harvesting of rat SLCs.** Spleens of 6 rats were extracted following sacrifice by cervical dislocation under aseptic conditions, disassociated by grinding, and then filtered through a 200-µm nylon mesh. Cell suspensions were transferred to a centrifuge tube containing Percoll lymphocyte separation medium (1.083 g/ml; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). Following centrifugation at 670 x g for 20 min at room temperature, the white middle layer was extracted and centrifuged at 330 x g for 8 min at room temperature, before the supernatant was discarded. After washing with PBS, the lymphocytes were counted and cultured in RPMI 1640 media (Gibco; Thermo Fisher Scientific, Inc.) containing 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mmol/l glutamine, and 10% FBS (5x10$^5$ cells/ml).

**HepG2.2.15 cell culture.** HepG2.2.15 cells were cultured in high glucose-DMEM (Hyclone; GE Healthcare Life Sciences), which contained 10% heat-inactivated FBS, 200 mg/l G418, 6 mmol/l glutamine, 100 U/ml penicillin and 100 mg/l streptomycin, in an incubator at 37°C and 5% CO$_2$ with saturating humidity. The medium was refreshed every 48 h, and healthy cells were selected for downstream experiments.

**Co-culture of different cell types.** The following experimental groups were studied: Group 1, SLCs; group 2, HepG2.2.15
cells; group 3, BM-MSCs + HepG2.2.15 cells; group 4, SLCs + HepG2.2.15 cells; and group 5, SLCs + BM-MSCs + HepG2.2.15 cells. HepG2.2.15 cells were plated in the lower chamber of a 6-well transwell dish (pore size, 0.4 μm; Corning, Incorporated) at 1x10⁶ cells/well, and SLCs and BM-MSCs were inoculated in the upper chamber of the transwell plate at 5x10⁵ cells/well. Plates were cultured at 37°C and 5% CO₂ with saturating humidity in an incubator for 24, 48 or 72 h. Each group was plated in triplicate wells for each time point. At each time point, supernatants and cells were collected for further analysis.

**MTT cell viability assay.** Cell suspensions (200 μl) from each experimental group were added to each well of a 96-well plate (SLCs, 2x10⁴ cells/well; BM-MSCs, 2x10⁴ cells/well; HepG2.2.15 cells, 4x10⁴ cells/well), which was incubated at 37°C with 5% CO₂. Cells were cultured for 24, 48 or 72 h. MTT assay (15 μl at 5 g/l) was added to each well and incubated for 3 h. The medium was subsequently aspirated and DMSO (100 μl) was added to each well before the plates were placed on a shaker for 10 min to fully dissolve the formazan crystals. The absorbance (A) at 490 nm was measured using an automated microplate reader, and the cell survival rate was calculated using the following formula: Survival rate = (A_test well-A_blank well) / (A_control well-A_blank well) x100%.

**Detection of supernatant HBV DNA and intracellular cccDNA of HepG2.2.15 cells and BM-MSCs.** The supernatant HBV DNA levels were measured using a real-time PCR kit according to the manufacturer's instructions (Shanghai Kehua Bioengineering Co., Ltd., China) according to the manufacturer's instructions (Shanghai Kehua Bioengineering Co., Ltd., China) or BM-MSCs (5x10⁵ cells/well) using a UniversalGen DNA kit (CWBio, Co., Ltd., Beijing, China), and 2 μg HBV DNA or cccDNA was subjected to quantitative PCR analysis using an optimized quantitative PCR method described previously (25).

**HBV genomic DNA extraction and sequencing analysis.** HBV genomic DNA was extracted from the supernatants of co-cultured HepG2.2.15 cells using a Viral DNA Isolation kit (DAAN Gene, Co., Ltd., of Sun Yat-sen University, Guangzhou, China) according to the manufacturer's instructions. Briefly, cell supernatants were added to virus lysis buffer, and lysates were loaded onto a spin column. After viral DNA was bound to the membrane, each column was washed and the viral DNA was eluted.

PCR was performed using HBV genomic DNA as a template to amplify the P, S, X and C regions using the primer sequences listed in Table I. The PCR conditions were as follows: Initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were resolved by 2% agarose gel electrophoresis, and the bands were visualized under ultraviolet light following ethidium bromide staining. The DNA was recovered from the agarose gel using a MiniBEST Agarose Gel DNA Extraction kit (Takara Bio, Inc.) according to the manufacturer's protocol, and the amplified DNA was subjected to sequencing analysis by Sangon Biotech (Shanghai, China).

**Detection of lymphocyte surface markers CD4 and CD8 in the CD³⁺ cell by flow cytometry.** SLCs were harvested and centrifuged at 300 x g for 5 min at 4°C following culture for 24, 48 or 72 h. Then SLCs (1x10⁶ cells) were resuspended in 100 μl PBS for detection, and the fluorescence-labeled antibodies anti-CD3-APC (1:80), anti-CD4-FITC (1:200), and anti-CD8a-PE-Cy7 (1:160) were added for incubation at 4°C for 30 min in the dark, to detect the expression intensity of each cell surface marker by flow cytometry.

**Detection of supernatant cytokines.** Concentrations of IFN-γ, IL-10, and IL-22 in the cell supernatants were determined using an ELISA kit (R&D Systems, Inc.) according to the manufacturer's protocol. The absorbance at 450 nm was measured using an automated microplate reader.

**Statistical analysis.** SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Normally distributed data were presented as the mean ± standard deviation. Additional data sets were compared by analysis of variance, and Dunnett's method was used when the variance was not homogenous. Linear correlation analysis was used to test the interdependence of the variables. P<0.05 was considered to indicate a statistically significant difference. GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot data for presentation.

**Results**

**Morphology and phenotypic analysis of HepG2.2.15 cells and BM-MSCs.** HepG2.2.15 cells were confirmed to be plastic-adherent cells with a spindle-shaped morphology...
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Rat BM-MSCs were successfully established in culture and proliferated in vitro. Morphological and phenotypic examination revealed that BM-MSCs were confirmed to be plastic-adherent cells with a spindle-shaped morphology under standard culture conditions, as determined by microscopy, and some of the cells exhibited a whirlpool or chrysanthemum pattern (Fig. 1B). BM-MSCs were incubated with antibodies against CD29, CD90, RT1A, CD34, RT1B and CD45, and were analyzed by flow cytometry. Phenotypic examination of BM-MSCs at passage 3 demonstrated that 97.0% of cells expressed CD29, 96.3% of cells expressed CD90, and 96.3% of cells expressed RT1A (Fig. 1C-E). By contrast, >95% of BM-MSCs were negative for CD34, CD45 and RT1B (Fig. 1C-E), which was in accordance with the results of a previous study (26).

Detection of liver enzymes in supernatants. When co-cultured with xenogeneic SLCs or BM‑MSCs, no significant difference in liver enzyme levels in HepG2.2.15 cell supernatants was observed (Table II). This suggested that neither BM-MSCs nor SLCs induced rejection of the human hepatocellular carcinoma cell line, HepG2.2.15.

Effects of BM-MSCs on the viability of SLCs and HepG2.2.15 cells. The viability of SLCs in group 5 was significantly lower when compared with that of group 4 at each time point (24 h, P<0.05; 48 h, P<0.01; 72 h, P<0.01; Fig. 2A), which suggested that BM-MSCs may reduce the viability of SLCs.

The viability of adherent cells in group 3 was significantly lower when compared to that of groups 2 and 4 at 24 h, respectively (P<0.01 at 24 h; Fig. 2B). These results suggested that BM-MSCs exhibited stimulatory effects on HepG2.2.15 cell viability when co-cultured with SLCs.

Detection of intracellular HBV cccDNA in BM‑MSCs. No intracellular HBV cccDNA was detected in the BM-MSCs in any of the groups (data not shown), which suggested that BM-MSCs co-cultured with HepG2.2.15 cells were not infected by HBV.

HBV gene sequencing. No mutations in the C or X regions of the HBV genome were detected in HepG2.2.15 cells co-cultured with BM-MSCs, SLCs, or both types of cells (Table III). However, a T45 N mutation in the S region, and an rtR192S mutation in the P region was identified in the supernatants of BM-MSCs + HepG2.2.15 and SLCs + HepG2.2.15 groups, respectively (Table III).
Effect of BM-MSCs on lymphocyte subsets. Detection of lymphocyte surface markers by flow cytometry revealed that the percentage of CD3+CD4+ cells in group 5 was higher than that of group 4 at 24 and 72 h, but was lower at 48 h. These differences did not reach statistical significance (Fig. 4A).

The percentage of CD3+CD8+ cells in group 5 was significantly lower than that of group 4 at all time points (24 h, P<0.01; 48 h, P<0.05; 72 h, P<0.05; Fig. 4B). When compared with group 4, the CD3+CD4+/CD3+CD8+ ratio in group 5 significantly increased at 24 and 48 h (P<0.01 and P<0.05, respectively; Fig. 4C), but no significant difference was observed at 72 h. The percentage of CD3+CD8+ cells was positively correlated with HBV DNA levels when co-cultured with BM-MSCs (24 h, r=0.865; 48 h, r=0.766; 72 h, r=0.912; P<0.05).

Effect of BM-MSCs on cytokine levels in co-cultured SLCs and HepG2.2.15 cell supernatants. The supernatant concentrations of INF-γ in group 5 were higher than those of group 4 at 24, 48 and 72 h (Table IV). By contrast, IL-10 and IL-22 levels in group 5 were lower than those of group 3 and group 4 at 24, 48 and 72 h (Table IV). INF-γ secretion

| Group                      | ALT (IU/l) | AST (IU/l) |
|----------------------------|------------|------------|
| HepG2.2.15                 | 1.17±0.41  | 9.53±1.63  |
| BM-MSCs+HepG2.2.15         | 1.20±0.36  | 11.30±0.40 |
| SLCs+HepG2.2.15            | 1.45±0.37  | 11.02±2.95 |
| SLCs+BM-MSCs+HepG2.2.15    | 1.17±0.40  | 11.70±3.37 |

All values are presented as the mean ± standard deviation (n=3). ALT, alanine transaminase; AST, aspartate transaminase; BM-MSC, bone marrow-derived mesenchymal stem cells; SLC, splenic lymphocytes; IU, international unit.

**Table II. Supernatant ALT and AST levels in different groups at different time points.**

| Group                      | ALT (IU/l) | AST (IU/l) |
|----------------------------|------------|------------|
| HepG2.2.15                 | 1.50±0.38  | 13.25±2.65 |
| BM-MSCs+HepG2.2.15         | 1.40±0.33  | 15.65±1.02 |
| SLCs+HepG2.2.15            | 1.72±0.20  | 17.62±3.26 |
| SLCs+BM-MSCs+HepG2.2.15    | 1.43±0.14  | 15.93±0.68 |

**Figure 2. Viability of SLCs and adherent cells as determined using the MTT assay. The viability of (A) SLCs in groups 1, 4 and 5, and (B) adherent cells in groups 2, 3, 4 and 5. *P<0.05 and **P<0.01 as indicated. SLCs, splenic lymphocytes; 1, SLCs alone; 2, HepG2.2.15 cells alone; 3, bone marrow-derived mesenchymal stem cells (BM-MSCs) + HepG2.2.15 cells; 4, SLCs + HepG2.2.15 cells; 5, SLCs + BM-MSCs + HepG2.2.15 cells.**

**Figure 3. Supernatant HBV DNA quantities and intracellular covalently cccDNA levels. The levels of (A) supernatant HBV DNA and (B) HBV cccDNA in cells from groups 2-5. *P<0.05 and **P<0.01 as indicated. HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; IU, international unit; 2, HepG2.2.15 cells; 3, bone marrow-derived mesenchymal stem cells (BM-MSCs) + HepG2.2.15 cells; 4, SLCs + HepG2.2.15 cells; 5, SLCs + BM-MSCs + HepG2.2.15 cells.**
levels were negatively correlated with HBV DNA levels (24 h, \( r = -0.900 \), 48 h, \( r = -0.982 \); 72 h, \( r = -0.968 \); \( P < 0.05 \)), whereas IL-10 and IL-22 secretion levels were positively correlated with HBV DNA levels (IL-10, 24 h, \( r = 0.860 \); 48 h, \( r = 0.972 \); \( P < 0.05 \); IL-22, 48 h, \( r = 0.858 \); 72 h, \( r = 0.742 \); \( P < 0.05 \)). In group 5, the supernatant IFN-\( \gamma \) levels at 48 h were significantly higher than those at 72 h, and the supernatant levels of IL-10 at 48 h were significantly lower than those detected at 24 and 72 h (Table IV). These findings suggested that alterations in IFN-\( \gamma \) and IL-10 levels were most evident at 48 h within the same group.

Discussion

Liver-derived MSCs have been demonstrated to be crucial for the repair of damaged hepatocytes and liver regeneration (27-29). Oh et al (30) confirmed that BM-MSCs are potential sources of hepatic oval cells. When the liver is severely damaged, BM-MSCs differentiate into hepatic progenitor-like cells and mediate repair of the liver (31-33). The present study aimed to explore the effects of BM-MSCs on hepatocytes infected with HBV. Previous studies have demonstrated that human MSCs survive and exhibit protective effects on neurological and lung injuries following transplantation into rats (34-36). However, they may also stimulate an allogeneic immune response to increase lymphocyte proliferation in the host (37,38). Therefore, with the lack of stable rat cell lines transfected with HBV, and the strict ethical limits to acquire human stem cells, a xenotransplantation model was employed in the present study.

The preliminary findings demonstrated that when co-cultured with BM-MSCs, the proliferation of HepG2.2.15 cells was inhibited and HBV DNA levels were decreased. When BM-MSCs were co-cultured with SLCs, HBV DNA levels were markedly reduced. Meanwhile, BM-MSCs induced very few HBV genome sequence mutations and did not cause rejection between xenogeneic cells. To the best of our knowledge the T45N mutation in the S region, and the rtR192S mutation in the P region, are not known to be significant in the clinical treatment of hepatitis B. In addition, the preliminary results of the present study suggested that BM-MSCs may inhibit the replication of HBV cccDNA \textit{in vitro}. It is possible that BM-MSCs may suppress the proliferation of co-cultured T cells \textit{in vitro}, thereby inhibiting immune responses to induce immune tolerance (39-41). Alternatively, BM-MSCs may secrete cytokines, including fibroblast growth factor (42,43), epidermal growth factor (EGF) (44), and hepatocyte growth factor (HGF) (43,45,46) to inhibit HBV replication (47). In addition, intracellular HBV cccDNA in BM-MSCs co-cultured with HepG2.2.15 cells was not detected, which supports the conclusion that HBV is unable to replicate in BM-MSCs (48,49).

BM-MSCs are a cell type that exert immunomodulatory activities (19-21). They inhibit the proliferation and activation of T cells and exhibit immunomodulatory functions mediated by soluble factors (39,41). Prostaglandin E\( _2 \) (PGE\( _2 \)) and indole-amine dioxygenase were observed to be potentially involved in the immunomodulatory function of BM-MSCs (50). The majority of T lymphocytes can be divided into CD4\( ^+ \) T cells...
Table IV. Cytokine levels in cell culture supernatants.

| Cytokine | Group                        | 24 h (pg/µl) | 48 h (pg/µl) | 72 h (pg/µl) |
|----------|------------------------------|--------------|--------------|--------------|
| IFN-γ    | SLCs                         | 848.557±11.409<sup>a</sup> | 468.347±20.523<sup>a</sup> | 528.111±15.640<sup>a</sup> |
|          | BM-MSCs+HepG2.2.15          | 636.650±47.047<sup>a</sup> | 460.953±38.345<sup>a</sup> | 603.735±26.848<sup>a</sup> |
|          | SLCs+HepG2.2.15             | 675.637±19.046 | 621.237±24.709 | 517.170±31.331<sup>a</sup> |
|          | SLCs+BM-MSCs+HepG2.2.15     | 735.030±18.646 | 780.463±19.879 | 676.317±34.414<sup>a</sup> |
| IL-10    | SLCs                         | 803.930±55.897<sup>a</sup> | 297.040±32.246<sup>a</sup> | 183.367±46.742<sup>a</sup> |
|          | BM-MSCs+HepG2.2.15          | 240.747±28.605 | 206.609±13.669<sup>a</sup> | 259.580±30.070<sup>a</sup> |
|          | SLCs+HepG2.2.15             | 511.553±37.490 | 413.360±14.133<sup>a</sup> | 553.133±54.416<sup>a</sup> |
|          | SLCs+BM-MSCs+HepG2.2.15     | 420.227±23.235<sup>b</sup> | 153.087±26.016 | 447.230±31.192<sup>b</sup> |
| IL-22    | SLCs                         | 344.423±36.904<sup>a</sup> | 180.337±4.672<sup>a</sup> | 164.537±35.654 |
|          | BM-MSCs+HepG2.2.15          | 183.135±18.123<sup>a</sup> | 166.264±23.206<sup>a</sup> | 164.722±12.389 |
|          | SLCs+HepG2.2.15             | 166.337±18.651 | 258.923±24.262<sup>a</sup> | 305.053±14.766<sup>a</sup> |
|          | SLCs+BM-MSCs+HepG2.2.15     | 146.007±20.407<sup>b</sup> | 208.537±6.499 | 210.857±22.527 |

Data are presented as the mean ± standard deviation (n=3). <sup>a</sup>P<0.05, and <sup>b</sup>P<0.01, vs. SLCs + BM-MSCs + HepG2.2.15. <sup>c</sup>P<0.05, and <sup>d</sup>P<0.01, vs. the same group at 48 h. IFN-γ; interferon-γ; IL, interleukin; SLCs, splenic lymphocytes; BM-MSCs, bone marrow-derived mesenchymal stem cells.

and CD8+ T cells, and the majority of CD8+ T cells are cytotoxic T lymphocytes (CTL). T cell function is exhausted during chronic HBV infection, and CTLs cannot effectively eliminate the virus. As a result, the virus persists and the proportion of T cell subsets in the peripheral blood is subsequently altered (51-53). The findings of the present study suggested that the percentage of CD8+ cells was positively correlated with HBV DNA levels, which is consistent with a previous study demonstrating that an imbalance of T cell subsets was closely associated with HBV DNA levels (54,55). The CD4+/CD8+ ratio increased at 24 and 48 h, and then decreased at 72 h. Furthermore, the reduction in the levels of intracellular HBV cccDNA was the most significant at 48 h, which suggested that the increased CD4+/CD8+ ratio was correlated with inhibitory effects on HBV cccDNA replication. To further confirm these results, the levels of cytokines were measured.

MSCs clearly inhibit the proliferation of allogeneic lymphocytes (CTL). T cell function is exhausted during chronic HBV infection, and CTLs cannot effectively eliminate the virus. Therefore, a number of preventative treatments against HBV re-infection. Although HBV does not affect the phenotype or differentiation ability of BM-MSCs, it has been demonstrated to inhibit the proliferation of BM-MSCs in vitro (64). Therefore, a number of issues require further investigation before BM-MSCs may be used as a clinical treatment option, and will be a focus of future research.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81270528, 81170444, 81572857, and 81370465). "SLCs+BM-MSCs+HepG2.2.15 146.007±20.407 258.923±23.426", "BM-MSCs+HepG2.2.15 183.367±46.742", "SLCs+BM-MSCs+HepG2.2.15 420.227±23.235", "IL-10 SLCs 803.930±55.897", "IL-22 SLCs 344.423±36.904", "SLCs+BM-MSCs+HepG2.2.15 164.722±12.389", "SLCs+BM-MSCs+HepG2.2.15 183.135±18.123", and "SLCs+BM-MSCs+HepG2.2.15 146.007±20.407".
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