Comparison of Immunological Characteristics of Mesenchymal Stem Cells Derived from Human Embryonic Stem Cells and Bone Marrow

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Mesenchymal stem cell (MSC) has great potential for both regenerative medicine and immunotherapy due to its multipotency and immunomodulatory property. The derivation of MSCs from human tissues involves an invasive procedure and the obtained MSCs often suffer from inconsistent quality. To overcome these issues, the approaches of deriving a highly potent and replenishable population of MSCs from human embryonic stem cells (hESCs) were established. However, few studies compared the immunological characteristics of MSCs derived from hESCs with tissue-derived MSCs or demonstrated differences and the underlying mechanisms. Here, we differentiated H9 hESCs into MSC-like cells (H9-MSCs) through an embryoid body outgrowth method and compared the immunological characteristics of H9-MSCs with bone marrow-derived MSCs (BMSCs). Both sources of derived cells exhibited typical MSC morphologies and surface marker expressions, as well as multipotency to differentiate into osteogenic and adipogenic lineages. A immunological characterization study showed that H9-MSCs and BMSCs had similar immunoprivileged properties without triggering allogeneic lymphocyte proliferation as well as equivalent immunosuppressive effects on T-cell proliferation induced by either cellular or mitogenic stimuli. Flow cytometry analysis revealed a lower expression of human major histocompatibility complex class II molecule human lymphocyte antigen (HLA)-DR and a higher expression of coinhibitory molecule B7-H1 in H9-MSCs than in BMSCs. Interferon gamma (IFN- γ) is a proinflammatory cytokine that can induce the expression of HLA class II molecules in many cell types. Our results showed that pretreatment of H9-MSCs and BMSCs with IFN- γ did not change their immunogenicity and immunosuppressive abilities, but increased the difference between H9-MSCs and BMSCs for their expression of HLA-DR. Further detection of expression of molecules involved in IFN- γ signaling pathways suggested that the lower expression of HLA-DR in H9-MSCs could be partially attributed to the lower expression and the less nuclear translocation of its transcriptional factor CIITA. The present study provides evidence that the hESC-derived MSCs share similar immunogenicity and immunosuppressive abilities with BMSCs, but differ in the expression profile of immunological markers and the responsiveness to certain inflammatory cytokines, which suggests that H9-MSCs could be a safe and efficient candidate for MSC treatment in patients with inflammatory disorders.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are multipotent nonhematopoietic progenitors present in the stromal fraction of adult and fetal tissues, in particular, from the bone marrow and adipose.¹⁻⁴ Apart from their multilineage differentiation potentials, the immunoprivileged and immunomodulatory properties of MSCs have led them to be attractive

candidates for cell therapy and regenerative medicine.^{5,6} Nevertheless, loss of potency, inconsistent quality, and invasive procedure involved in cell isolation severely limit the use of MSCs in clinical applications.⁷⁻⁹ Therefore, the derivation of MSCs from other consistent sources has been considered as an alternative. The successful isolation and *in vitro* culture of human embryonic stem cells (hESCs), being capable of unlimited self-renewal and differentiation

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into all cell lineages of the body,¹⁰ opened a new avenue for MSC derivation. There are well-established approaches for the differentiation of hESCs into functional MSCs with immunomodulatory properties.^{11–15} However, the differences between hESC-derived MSCs and tissue-derived MSCs in their immunological characteristics and the underlying mechanisms have not been well defined as yet.

The immunoprivileged status of MSCs was partly due to the lack of expression of major histocompatibility complex class II (MHC-II) and costimulatory molecules.¹⁶ The MHC-II antigen is constitutively expressed on professional antigen-presenting cells (APC). It binds peptide fragments derived from pathogens and displays them on the cell surface for recognition by the appropriate T cells.^{16,17} The best-studied costimulatory molecules belong to the B7 family, which consists of seven known members: CD80 (B7.1), CD86 (B7.2), B7-H1 (programmed death-1 ligand [PD-L1]), B7-H2 (inducible costimulator ligand), B7-DC (programmed death-2 ligand [PD-L2]), B7-H3, and B7-H4. They bind to receptors on lymphocytes and regulate immune responses by directing either costimulatory or coinhibitory signals.¹⁸ Interferon gamma (IFN- γ), a major proinflammatory cytokine secreted by activated T cells and natural killer (NK) cells,¹⁹ can markedly stimulate or increase the expression of MHC-II molecules in many cell types and control the APC function of MSCs by regulating the expression of class II transactivator (CIITA), the master regulator of MHC-II.^{16,19–21} However, the influence of IFN- γ on the immunogenicity of MSCs is still surrounded by controversy.^{22–26}

The immunomodulatory properties of MSCs have been extensively studied both *in vitro* and *in vivo*.^{27–29} *In vitro*, MSCs act in a dose-dependent manner to suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli.^{30,31} Other attempts using MSC *in vivo* studies have demonstrated that MSCs could promote engraftment of CD34+ hematopoietic stem cells,³² reduce incidence of graft-versus-host diseases in patients receiving allogeneic transplants,³³ ameliorate autoimmune diseases,^{34,35} and prevent the rejection of allogeneic skin grafts.³⁶ Direct cell contact with immune cells³⁷ and soluble factors produced by MSCs, such as IL-6,³⁸ TGF- β ,^{39,40} hepatocyte growth factor,⁴¹ nitric oxide,⁴² indoleamine 2,3-dioxygenase,^{43,44} and prostaglandin E₂,^{45,46} have been implicated in the mechanisms of MSC-mediated immunosuppression.

In this study, we differentiated H9 hESCs into MSC-like cells (H9-MSCs) through an embryoid body (EB) outgrowth method. The immunological characteristics of H9-MSCs were characterized and compared to bone marrow-derived MSCs (BMSCs) with or without IFN- γ treatment. Moreover, the expression of genes encoding components in IFN- γ signaling pathways in H9-MSCs and BMSCs was investigated after IFN- γ treatment. Our data may provide additional insights into the responsiveness of H9-MSCs and BMSCs under inflammatory conditions after *in vivo* transplantation.

Materials and Methods

Culture and differentiation of H9 hESCs into MSCs (H9-MSCs)

The H9 hESC line was obtained from the WiCell Research Institute under a Materials Transfer Agreement (No. 11-W0039) and cultured on mitomycin C (Roche)-inactivated

mouse embryonic fibroblast, as previously described.⁴⁷ Cells were maintained in the hESC medium: DMEM/F-12 (Invitrogen), 20% knockout serum replacement (Invitrogen), 4 ng/mL fibroblast growth factor-2 (FGF-2; Invitrogen), 1 mM L-glutamine (Invitrogen), 1% nonessential amino acid (Invitrogen), and 0.1 mM β -mercaptoethanol (Sigma). Derivation of H9-MSCs was achieved following published protocol.⁴⁸ Briefly, EBs were formed by mass suspension of hESCs in a hESC culture medium without FGF-2 for 10 days. The EBs were seeded onto a gelatin-coated plate for adherent culture in the MSC growth medium: DMEM supplemented with 10% fetal bovine serum (FBS) and 1 ng/mL FGF-2. The cells at this initial passage (P0) were kept for 2 weeks followed by subsequent normal subculture. Three independent derivations of H9-MSCs were conducted.

Isolation and culture of BMSCs

Bone marrow aspirates were collected from iliac crest of five patients who receiving plastic surgery with informed consent. BMSCs were isolated by a gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare) and cultured in the MSC culture medium containing DMEM (Invitrogen) with 10% FBS (Lonza). All studies were conducted with approval from the ethics committee of Plastic Surgery Hospital.

Immunofluorescence staining

H9 hESCs were assessed by immunofluorescence staining for expression of hESC-specific markers OCT4 and SSEA-4 following protocols described previously.⁴⁷ Mouse anti-human OCT4 (611010; BD) and SSEA4 (560073; BD) were used as primary antibodies and goat anti-mouse Alexa-Fluor-594 IgG and Alexa-Fluor-488 IgG (Invitrogen) were used as secondary antibodies, respectively. The cells were mounted with a solution containing DAPI (Invitrogen) and imaged with a Nikon TE2000-S microscope.

Osteogenic and adipogenic differentiation of human MSCs

H9-MSCs P5 and BMSCs P3 were subjected to osteogenic and adipogenic differentiation following published protocols.⁴⁹ After 4 weeks in culture, H9-MSCs and BMSCs were stained by Alizarin red after osteogenic differentiation and by Oil red after adipogenic differentiation.

Flow cytometry analysis

The surface marker expressions of H9-MSCs and BMSCs were characterized using the Stemflow™ human MSC analysis kit (BD) following the manufacturer's instructions. Specific primary antibodies included CD44-PE, CD73-APC, CD90-FITC, CD105-PerCP.Cy5.5, and a negative antibody cocktail (CD31-PE, CD45-PE, CD71-PE, and CD117-PE). H9-MSCs at P5 and BMSCs at P3 were treated with IFN- γ (0 U, 10 U, 100 U, 400 U, 1000 U, or 4000 U/mL) for 72 h and then incubated with primary antibodies targeting human lymphocyte antigen (HLA) class I antigens (HLA-A,B,C-APC), HLA class II molecule (HLA-DR-APC), costimulatory molecule (CD40-PE), and B7 family members (CD80-APC, CD86-FITC, B7-H1-APC, B7-H2-PE, B7-H3-PE, B7-H4-PE, and B7-DC-APC), respectively (Biolegend). The data with

1×10^4 events per cell sample were generated by FACS Aria™ II (BD) and analyzed using FlowJo v7.6.1 software (Tree Star).

Mix lymphocyte reaction and mitogen stimulation assay

Peripheral blood mononuclear cells (PBMC) were fractionated from peripheral blood of healthy volunteers by Ficoll-Paque PLUS (GE Healthcare), as reported previously.⁴⁹ PBMCs isolated from two unrelated individuals were named as PBMC1 and PBMC2. The untreated PBMCs were used as responders (PBMC^r). The inactivated PBMCs (PBMC^s), H9-MSCs, and BMSCs by 25 µg/mL of mitomycin C for 20 min at 37°C were used as stimulators. For the one-way mix lymphocyte reaction (MLR), 1×10^5 inactivated H9-MSCs or BMSCs were cocultured with 1×10^5 PBMC1^r in the RPMI-1640 + 10% FBS medium within the 96-well plates (Corning). PBMC1^r cocultured with PBMC2^s were used as the positive control. For two-way MLR, 1×10^5 PBMC1^r and 1×10^5 PBMC2^r were mixed and cocultured with inactivated H9-MSCs or BMSCs at three different ratios (1:1, 10:1, and 100:1), respectively, for 5 days. The proliferations of PBMC^r were assessed by the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay kit (Promega), and the absorbance at 490 nm was measured in the PerkinElmer EnSpire™ Multimode Plate Reader. The proliferation index (PI) for one-way MLR was calculated as follows: $PI_{one-way} = \frac{\text{Absorbance}(\text{PBMC1}^r + \text{MSC}) - \text{Absorbance}(\text{MSC})}{\text{Absorbance}(\text{PBMC1}^r + \text{PBMC2}^s) - \text{Absorbance}(\text{PBMC2}^s)}$, PI = 1 is considered as immunogenic to induce PBMC^r proliferation and PI for two-way MLR was calculated as $PI_{two-way} = \frac{\text{Absorbance}(\text{PBMC1}^r + \text{PBMC2}^r + \text{MSC}) - \text{Absorbance}(\text{MSC})}{\text{Absorbance}(\text{PBMC1}^r + \text{PBMC2}^r) - \text{Absorbance}(\text{medium})}$. For mitogen stimulation assay, inactivated H9-MSCs or BMSCs were cocultured with 1×10^5 PBMC^r at two different ratios (1:100 and 1:10) in a 96-well plate and stimulated by 10 µg/mL phytohemagglutinin (PHA; Sigma) for 72 h. PBMC^r without PHA stimulation were used as the background control. PI was calculated as follows: $PI_{PHA} = \frac{\text{Absorbance}(\text{PHA treated PBMC}^r + \text{MSC}) - \text{Absorbance}(\text{MSC})}{\text{Absorbance}(\text{PBMC}^r) - \text{Absorbance}(\text{Medium})}$. To test the effects of IFN-γ on the immunogenicity and immunomodulatory properties of H9-MSCs and BMSCs, MLR and mitogen stimulation assays were performed as above with H9-MSCs and BMSCs pretreated by IFN-γ (10 U/mL, 72 h) before the inactivation. All experiments were conducted thrice with different PBMCs and MSCs in triplicate.

Conventional and real-time polymerase chain reaction

For cDNA synthesis, 500 ng of RNA was reverse transcribed using the M-MLV reverse transcriptase (Invitrogen) following the manufacturer's instructions. The conventional polymerase chain reaction (PCR) for pluripotent markers (*OCT4*, *NANOG*) and three germ layer markers (*NEUROD1*, *BRACHYURY*, and *AFP*) was performed on Biorad DNA Engine, and the *β-actin* gene was used as a control. The real-time PCR was performed using the Fast SYBR Green Master Kit and LightCycler 480 system (Roche) according to the manufacturer's instructions, and the expression level of each gene was normalized to the expression of the housekeeping gene GAPDH. Primer sequences are summarized in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tea).

Western blotting for quantifying CIITA in cytoplasmic and nuclear protein extracts

After incubation with 0, 10, or 1000 U/mL IFN-γ for 72 h, cytosol and nuclear protein fractions of H9-MSCs and BMSCs were separated and collected individually using the Cytosol-Nuclear protein extraction kit (Applygen; BJ). Western blottings were performed using mouse anti-human CIITA (SC-376174; Santa Cruz Biotechnology) and mouse anti-human GAPDH (TA081 ZSGB-BIO) as primary antibodies and the horseradish peroxidase-conjugated donkey anti-mouse IgG (Santa Cruz) as the secondary antibody. The SuperSignal West Pico Trial Kit (Thermo Scientific) was applied for protein detection. The intensity of individual band was quantified using ImageJ densitometry software.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package. The difference was evaluated by independent Student's *t*-test and one-way ANOVA test. A *p*-value of <0.05 was considered statistically significant.

Results

Generation of H9-MSCs from H9 hESCs

To generate MSC-like cells from H9 hESCs (H9-MSCs), H9 hESCs were differentiated through an EB outgrowth method. Undifferentiated H9 hESCs showed compact colony morphology with distinct borders (Fig. 1A) and positive staining for OCT4 and SSEA4 (Fig. 1B, C). After 10 days of suspension culture, H9 hESCs formed sphere-shaped EB aggregates (Fig. 1D). The EB aggregates could adhere to the gelatin-coated plate 24 h after plating. Fibroblast-like cells migrated outward from EB aggregates within 24–48 h of attachment (Fig. 1E). The outgrowing cells, which were named as H9-MSCs, could proliferate gradually and reach confluency after 1 week of culture. After 2 passages, cells became spindle shaped with homogeneous morphology (Fig. 1F). The trends of expression changes of specified markers were analyzed using conventional and real-time PCR (Fig. 1G, H). Undifferentiated H9 hESCs expressed pluripotent markers (*OCT4*, *NANOG*), but did not express markers specific to three germ layers (*NEUROD1*, *BRACHYURY*, and *AFP*). Declining expression of *OCT4* with dramatic increase of *NEUROD1*, *BRACHYURY*, and *AFP* was observed in EBs. Expressions of *OCT4*, *NANOG*, and three germ layer markers were depleted in H9-MSCs after the second passage and exhibited a basal level in H9-MSCs at P5 (**p* < 0.05).

Surface marker expression and differentiation potentials of H9-MSCs and BMSCs

The cell surface marker expression of H9-MSCs and BMSCs was analyzed by flow cytometry analysis. During derivation, the majority of H9-MSCs gradually acquired fibroblast-like morphologies, lost SSEA-4 expression, and showed progressive acquisition of MSC-specific markers: CD90, CD105, and CD73 (Supplementary Fig. S1). Upon passage 5 (P5), more than 95% of H9-MSCs showed positive expression of CD90, CD105, CD73, and CD44, whereas uniformly negative for hematopoietic marker expression, including CD34, CD116, CD19, CD45, and HLA-DR (Fig.

2A). No significant difference in the percentage of cells expressing these markers between H9-MSCs and BMSCs was detected (Fig. 2B). Functional differentiation assays demonstrated that both H9-MSCs and BMSCs showed a mineralized nodule formation after osteogenic differentiation (detected by Alizarin red staining, Fig. 2C) and accumulation of lipid droplets in the cytoplasm after adipogenic differentiation (visualized by Oil red staining, Fig. 2D).

Effects of H9-MSCs and BMSCs in MLR and mitogen-stimulated cultures

To compare the immunogenicity and immunomodulatory abilities of H9-MSCs and BMSCs, they were subjected to MLR and mitogen stimulation assays. Results of one-way MLR showed that both H9-MSCs ($PI=0.20\pm0.12$) and BMSCs ($PI=0.29\pm0.08$) did not elicit the proliferative response of PBMC1 (Fig. 3A). In two-way MLR assay, the suppression of PBMC proliferation by two types of MSCs was presented at a cell number of 1×10^3 ($*p<0.05$) and more prominent at cell numbers of 1×10^4 and 1×10^5 ($^{#}p<0.01$) (Fig. 3B). Furthermore, both H9-MSCs and BMSCs suppressed PBMC proliferation induced by PHA stimulation in a dose-dependent fashion (Fig. 3C), in which inhibitory effects were only observed in groups coculturing 1×10^4 H9-MSCs ($PI=0.49\pm0.13$) or BMSCs ($PI=0.73\pm0.13$) ($^{#}p<0.01$), but not in groups coculturing 1×10^3 H9-MSCs or BMSCs ($p>0.05$). No significant difference between H9-MSCs and BMSCs for their suppressive effects ($p>0.05$) was observed.

Comparison of the expression of immunological markers in H9-MSCs and BMSCs

The expression profiles of immunological markers in H9-MSCs and BMSCs were analyzed using flow cytometry (Fig. 4).

Like BMSCs, the majority of H9-MSCs ($>95\%$) expressed HLA class I molecule HLA-ABC constitutively at high levels, but did not express HLA class II molecule HLA-DR ($<5\%$) (Fig. 4A, B). Neither H9-MSCs nor BMSCs expressed costimulatory molecules, including CD40 and B7 family members CD80, CD86, and B7-H2 (Fig. 4C, D). Expressions of coinhibitory B7 family members (B7-H1, B7-H3, and B7-DC), but not B7-H4, were detected in H9-MSCs and BMSCs in detail; majorities of H9-MSCs (99.9%) and BMSCs (100%) were positive for B7-H3 expression, whereas different portions of H9-MSCs and BMSCs were positive for expression of B7-H1 ($69.9\%\pm4.5\%$ and $27.6\%\pm0.4\%$) and B7-DC ($34.3\%\pm15.0\%$ and $39.3\%\pm15.7\%$), respectively (Fig. 4E, F). Results from three sets of individual experiments demonstrated that expressions of B7-H1 were significantly higher in H9-MSCs than in BMSCs, whereas the expressions of HLA-DR were significantly lower in H9-MSCs than in BMSCs ($*p<0.05$, Fig. 4G).

Immunological characteristics of H9-MSCs and BMSCs after IFN- γ stimulation

IFN- γ is a well-known proinflammatory cytokine produced by activated T and NK cells and functions as a stimulator to induce expression of HLA class I and II molecules in many cell types. To compare the responsiveness of H9-MSCs and BMSCs to IFN- γ , the expressions of HLA-DR and other immunomodulatory molecules in H9-MSCs and BMSCs were evaluated by flow cytometry after different dosages of IFN- γ treatment (Fig. 5). The percentages of HLA-DR-positive cells were increased in both H9-MSCs and BMSCs in a dose-dependent manner after IFN- γ stimulation, but were significantly higher in BMSCs than in H9-MSCs ($*p<0.05$) (Fig. 5A), and especially after 10 U/mL IFN- γ treatment, the percentage of HLA-DR-positive cells in BMSCs dramatically increased to $73.0\%\pm9.1\%$, which was 15-fold higher than in

FIG. 3. Effects of H9-MSCs and BMSCs in MLR and mitogen-stimulated cultures. (A) H9-MSCs had low immunogenicity as BMSCs did and could not elicit lymphocyte proliferation. PBMC1 and PBMC2 represented PBMCs isolated from two unrelated individuals. Group coculturing PBMC1⁺ and PBMC2⁺ was used as the positive control. (B) H9-MSCs could inhibit lymphocyte proliferation in two-way MLR culture in a cell number-dependent manner. Inhibitory effects of H9-MSCs and BMSCs were observed with cell number at 10^3 ($*p<0.05$). The suppressive effects of H9-MSCs and BMSCs on PBMC proliferation were more pronounced with cell number at 10^4 and 10^5 ($^{#}p<0.01$). No significant difference was observed between H9-MSCs and BMSCs for their immunosuppressive effects in two-way MLR assays. (C) H9-MSCs and BMSCs could inhibit lymphocyte proliferation by PHA stimulation. Inhibition effects were only observed in groups coculturing 1×10^4 H9-MSCs ($PI=0.49\pm0.13$) or BMSCs ($PI=0.73\pm0.13$) with 1×10^5 PHA-activated PBMCs with a cell ratio of 1:10 ($^{#}p<0.01$), but not in groups coculturing H9-MSCs or BMSCs with PHA-stimulated PBMCs with a cell ratio of 1:100. MLR, mix lymphocyte reaction; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PI, proliferation index.

FIG. 4. Analysis of the expression of immunological molecules in H9-MSCs and BMSCs by flow cytometry. Consistently, a majority of (A) H9-MSCs (98.9%) and (B) BMSCs (98.5%) were stained positively for expression of HLA class I molecules HLA-A,B,C. The percentages of HLA-DR-positive cells were negligible (<5%) in both H9-MSCs (0.12%) and BMSCs (3.74%). On the other hand, expressions of costimulatory molecules, including CD40 and B7 family members CD80, CD86, and B7-H2, were not detected in (C) H9-MSCs and (D) BMSCs. Expressions of coinhibitory B7 family members (B7-H1, B7-H3, and B7-DC), but not B7-H4 were detected in H9-MSCs (E) and BMSCs (F). Results summarized from three independent experiments (G) demonstrated that the percentages of HLA-DR-positive cells in H9-MSCs were significantly lower than in BMSCs, whereas percentages of B7-H1-positive cells in H9-MSCs were significantly higher than in BMSCs ($*p < 0.05$). No significant difference was observed in H9-MSCs and BMSCs for the percentage of cells expressing other immunological molecules. HLA, human lymphocyte antigen. Color images available online at www.liebertpub.com/tea

H9-MSCs ($4.7\% \pm 1.7\%$). The percentage of HLA-DR-positive cells went up to $91.6\% \pm 3.3\%$ in BMSCs after 1000 U/mL IFN- γ treatment, whereas gradually increased to $65.5\% \pm 6.2\%$ in H9-MSCs. Expressions of CD40 in H9-MSCs and BMSCs were elicited by IFN- γ at 1000 U/mL, and the percentage of CD40-positive cells in BMSCs was significantly higher than in H9-MSCs ($*p < 0.05$, Fig. 5B). On the other hand, the percentage of cells expressing the coinhibitory molecule B7-DC was significantly higher in H9-MSCs than in BMSCs after IFN- γ treatment at 1000 U/mL ($76.6\% \pm 11.7\%$ vs. $52.3\% \pm 2.7\%$, Fig. 5C), and B7-H1-positive cells in H9-MSCs were threefold higher than in BMSCs at 10 U/mL ($63.3\% \pm 10.9\%$ vs. $20.8\% \pm 5.9\%$, Fig. 5D). In addition, both H9-MSCs and BMSCs constitutively expressed HLA-ABC and B7-H3 and were negative for the expressions of costimulatory molecules CD80, CD86, B7-H4, and B7-H2, regardless of the IFN- γ treatment (Supplementary Fig. S2).

To clarify whether different expressions of immunological markers in H9-MSCs and BMSCs in response to IFN- γ would differentially affect the functional capacity of H9-MSCs and BMSCs to inhibit T-cell proliferation, H9-MSCs and BMSCs were pretreated with 10 U/mL IFN- γ and T-cell proliferation was assessed after cellular or mitogen stimuli. Results of one-way MLR showed that both pretreated H9-MSCs and BMSCs failed to generate the proliferative response of T cells (Fig. 6A). Two-way MLR assay showed that the comparable suppressive capacity of H9-MSCs and BMSCs inhibiting T-cell proliferation was presented at a cell number of 1×10^3 ($*p < 0.05$) and more pronounced at 1×10^4 and 1×10^5 ($*p < 0.01$) (Fig. 6B). Also, both pretreated H9-MSCs and BMSCs could suppress the PHA-induced proliferation of lymphocytes at a cell number of 1×10^3 and 1×10^4 ($*p < 0.05$, Fig. 6C). There was no significant difference between H9-MSCs and BMSCs for their suppressive effects.

FIG. 5. Expression of immunological markers of H9-MSCs and BMSCs in response to IFN- γ stimuli. (A) IFN- γ could enhance the expression of HLA-DR on H9-MSCs and BMSCs with concentrations from 10 to 1000 U/mL in a dose-dependent manner ($^{\#}p < 0.01$; $^{*}p < 0.05$). Results summarized from three independent experiments demonstrated that percentages of HLA-DR-positive cells were significantly higher in BMSCs than in H9-MSCs in response to IFN- γ stimulation with concentrations ranging from 10 to 4000 U/mL ($^{*}p < 0.05$). (B) Expressions of CD40 on H9-MSCs and BMSCs were not elicited by low dose of IFN- γ (10 U/mL). BMSCs showed a rapid increase in the CD40 expression after treatment with 1000 U/mL IFN- γ . A mild increase was observed in H9-MSCs treated with 1000 U/mL IFN- γ . Results summarized from three independent experiments demonstrated that percentage of CD40-positive cells was significantly higher in BMSCs than in H9-MSCs in response to 1000 U/mL IFN- γ stimulation ($^{*}p < 0.05$). (C) IFN- γ at 1000 U/mL could significantly elevate the percentage of B7-DC-positive cells in H9-MSCs, but not in BMSCs. The percentage of B7-DC-positive cells in H9-MSCs was significantly increased and was significantly higher than in BMSCs after 1000 U/mL IFN- γ treatment ($^{*}p < 0.05$). (D) The percentages of B7-H1-positive cells in H9-MSCs were significantly higher ($^{*}p < 0.05$) than in BMSCs. IFN- γ at 1000 U/mL significantly increased the percentages of B7-H1-positive cells in H9-MSCs and BMSCs and no significant differences were observed between H9-MSCs and BMSCs. IFN- γ , interferon gamma.

Expression of genes encoding components of the IFN- γ signaling pathways in H9-MSCs and BMSCs

To explore the mechanism underlying the different responsiveness of H9-MSCs and BMSCs to IFN- γ -induced HLA-DR expression, we analyzed mRNA expressions of molecules that were involved in IFN- γ signaling pathways by quantitative real-time PCR (Fig. 7A). The expressions of *HLA-DR* and *CIITA* (the master regulator that determines the transcription level of MHC-II) were upregulated in both H9-MSCs and BMSCs in an IFN- γ dose-dependent manner

($^{*}p < 0.05$ for H9-MSCs treated with 0 U and 10 U/mL IFN- γ vs. H9-MSCs treated with 100 U and 1000 U/mL IFN- γ ; $^{\Delta}p < 0.05$ between BMSCs without IFN- γ treatment vs. BMSCs with IFN- γ treatment; $^{\S}p < 0.05$ between BMSCs treated with 10 U/mL IFN- γ vs. BMSCs treated with 100 U and 1000 U/mL IFN- γ) and were significantly higher in BMSCs than in H9-MSCs ($^{*}p < 0.05$). Expression of *IFNGR1*, *IFNGR2*, *IRF1*, and *IRF2*, which are *CIITA* upstream signaling molecules involved in Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, was evaluated as well. Expressions of *IRF1* and

FIG. 6. Effects of H9-MSCs and BMSCs pretreated with IFN- γ on MLR and mitogen-stimulated cultures. (A) Pretreatment of H9-MSCs and BMSCs with 10 U/mL IFN- γ failed to elicit lymphocyte proliferation. PBMC1 and PBMC2 represented PBMCs isolated from two unrelated individuals. Group coculturing PBMC1^T and PBMC2^S was used as the positive control. (B) H9-MSCs and BMSCs could inhibit lymphocyte proliferation in a two-way MLR culture in a cell number-dependent manner. Inhibitory effects of H9-MSCs and BMSCs were observed with cell number at 10^3 ($^{*}p < 0.05$). The suppressive effects were more pronounced by H9-MSCs and BMSCs with cell number at 10^4 and 10^5 ($^{*}p < 0.01$). No significant difference for the immunosuppressive effects of H9-MSCs and BMSCs was observed. (C) Analysis of the immunosuppressive effects of H9-MSCs and BMSCs on PBMC proliferation induced by PHA. Inhibition effects were observed in groups coculturing 1×10^3 or 1×10^4 H9-MSCs and BMSCs with 1×10^5 PHA-activated PBMCs ($^{*}p < 0.05$).

FIG. 7. Real-time PCR and western blot analysis for the expressions of molecules involved in the IFN- γ -JAK/STAT pathway in response to IFN- γ stimulation. **(A)** Expressions of *HLA-DR*, *CIITA*, *IRF1*, and *IRF2* were significantly increased in both H9-MSCs and BMSCs by IFN- γ in a dose-dependent manner ($^{\#}p < 0.05$ between H9-MSCs treated with 0 U or 10 U/mL IFN- γ vs. H9-MSCs treated with 100 U or 1000 U/mL IFN- γ ; $^{\Delta}p < 0.05$ between BMSCs without IFN- γ treatment vs. BMSCs with IFN- γ treatment; $^{\S}p < 0.05$ between BMSCs treated with 10 U/mL IFN- γ vs. BMSCs treated with 100 U and 1000 U/mL IFN- γ). Expressions of *HLA-DR* and *CIITA* were significantly higher in BMSCs than in H9-MSCs in response to different doses of IFN- γ stimuli ($^*p < 0.05$), whereas expression of *IRF2* was significantly higher in BMSCs than in H9-MSCs in response to 0 and 10 U/mL of IFN- γ stimuli ($^*p < 0.05$). **(B)** Western blot analysis for semiquantification of CIITA proteins in the cytoplasm and nuclear extracts in H9-MSC and BMSC in response to IFN- γ stimulation (0 U, 10 U, 1000 U/mL). CIITA (C) represents CIITA in the cytoplasm fraction, whereas CIITA (N) represents CIITA in the nuclear fraction. For semiquantification, western blot analysis was carried out for four times and the levels of CIITA proteins were normalized to GAPDH. The amount of CIITA (C) was significantly lower in H9-MSCs than in BMSCs after exposure to IFN- γ , whereas CIITA (N) was significantly lower in H9-MSCs than in BMSCs with or without IFN- γ treatment ($^*p < 0.05$). CIITA, class II transactivator.

IRF2 were significantly enhanced in both H9-MSCs and BMSCs after IFN- γ treatment, but *IRF2* expression was significantly lower in H9-MSCs than in BMSCs with 0 and 10 U/mL IFN- γ treatment ($^*p < 0.05$). Besides, no significant difference was observed for the expression of *IFNGR1*, *IFNGR2*, and CIITA binding proteins, including *CREB1*, RFX factors (*RFX5*, *RFXAP*, *RFXANK*), and NF-Y factors (*NFYA*, *NFYB*, and *NFYC*) in H9-MSCs and BMSCs after IFN- γ stimulations (Supplementary Fig. S3).

As nuclear translocation of CIITA plays an important role for its function, western blot analysis was used to semiquantify the amount of CIITA proteins in the cytoplasm and nuclear extracts of H9-MSCs and BMSCs (Fig. 7B). CIITA (C) represents CIITA in the cytoplasm fraction, whereas CIITA (N) represents CIITA in the nuclear fraction. Exposure to low-dose (10 U/mL) IFN- γ significantly enhanced the amount of CIITA proteins in both the nuclear and cytoplasm fractions of BMSCs ($p < 0.05$). However, CIITA proteins showed cytoplasm retention in BMSCs after exposure to high-dose (1000 U/mL) IFN- γ . In contrast, IFN- γ at two different concentrations failed to promote CIITA protein synthesis and nuclear translocation in H9-MSCs. Moreover, the amount of cytoplasmic CIITA was significantly lower in H9-MSCs than in BMSCs after exposure to IFN- γ , whereas nuclear CIITA was significantly lower in

H9-MSCs than in BMSCs with or without IFN- γ stimulation ($^*p < 0.05$).

Discussion

The hESCs have been considered as an alternative source for MSCs, which have promising therapeutic potentials for regenerative medicine and immunotherapy. To compare the immunological characteristics of MSCs derived from hESCs with tissue-derived MSCs is crucial for the applications. In the present study, H9-MSCs were successfully derived from H9 hESCs through an EB outgrowth method and their immunological characteristics were compared with BMSCs. Our results demonstrated that H9-MSCs and BMSCs with or without IFN- γ pretreatment had similar immunogenicity and inhibitory effects on lymphocyte proliferation induced by cellular and mitogenic stimuli. However, expression profiles of immunological markers in H9-MSCs and BMSCs showed significantly higher expression of B7-H1, but lower expression of HLA-DR in H9-MSCs than in BMSCs. IFN- γ stimulation assay suggested that H9-MSCs were less susceptible to IFN- γ -induced HLA-DR expression than BMSCs. Further detection of expression of molecules involved in IFN- γ signaling pathways suggested that the lower expression of HLA-DR in H9-MSCs than in BMSCs could be partially attributed

to the lower expression levels of *CIITA* and *IRF2*, and the less nuclear translocation of *CIITA*.

It is well-known that T-lymphocyte activation required two signals. The first signal is provided by an interaction of the T-cell receptor with antigenic peptide presented by MHC-II molecules, and the second costimulatory signal can only be delivered by costimulatory molecules.¹⁸ Indeed, a recent study reported that a significant increase in the proliferation of antigen-challenged lymphocytes from allergic rhinitis subjects is correlated with increased expressions of MHC-II and CD86 on MSCs in response to increased production of inflammatory cytokines from T cells.⁵⁰ IFN- γ is a proinflammatory cytokine produced by activated immune cells in both innate and adaptive immune responses.¹⁹ It has been reported that IFN- γ can markedly stimulate or increase the expression of MHC-II molecules in many cell types, including MSCs.^{16,19,20} Our finding that H9-MSCs and BMSCs pretreated with IFN- γ had a dramatic increasing of HLA-DR and failed to generate T-cell proliferation *in vitro* could be explained by the lack of expression of costimulatory markers. Interestingly, the prompt emergences of T-cell costimulatory molecule CD40 on BMSCs and H9-MSCs after incubating with 1000 U/mL IFN- γ were observed in our study. Together with the reports that administration of allogeneic MSC after IFN- γ stimulation is in fact immunogenic *in vivo*,^{51,52} our results suggested that MSCs might not be totally immunoprivileged in inflammatory conditions. Moreover, we first reported that H9-MSCs expressed a significantly higher level of B7-H1 than BMSCs did and more sensitive to IFN- γ -induced expression of B7-DC after exposure to 1000 U/mL IFN- γ . B7-H1 and B7-DC were found to serve as coinhibitory molecules by binding to their receptor CD279 (PD-1) and suppressing the lymphocyte proliferation.^{53,54} Hence, it was suggested that the immunosuppressive ability of H9-MSCs might be activated earlier and easier compared with BMSCs.

IFN- γ signaling is mediated by the JAK/STAT signaling pathway.⁵⁵ Binding of IFN- γ to its cognition receptors IFN- γ R1 and IFN- γ R2 results in receptor dimerization and subsequent activation of receptor-associated JAK1 and JAK2, leading to phosphorylation and nuclear translocation of STAT-1. STAT-1 cooperates with its downstream transcription factor interferon regulatory factor 1 (IRF1), ubiquitously expressed factors USF-1 and IRF2 to activate the transcription of *CIITA*. *CIITA* in turn forms transcriptosome with the heterotrimeric NF-Y transcription factor, CREB, and the trimeric RFX complex to regulate transcription of the MHC-II genes.⁵⁶ Our findings first showed that the expressions of signaling molecules involved in the IFN- γ signaling pathway were comparable in H9-MSCs and in BMSCs with or without IFN- γ treatment, except for *IRF2* and *CIITA*. *CIITA* is the master regulator that determines the transcription level of MHC-II,⁵⁷ and its nuclear translocation is crucial to its function. In our study, low concentration (10 U/mL) of IFN- γ treatment enhanced nuclear translocation of *CIITA* protein, whereas high concentration (1000 U/mL) of IFN- γ treatment resulted in cytoplasmic retention of *CIITA* in BMSCs, consistent with a previous report.²¹ However, we did not observe obvious nuclear translocation of *CIITA* in H9-MSCs exposed to either low or high concentrations of IFN- γ . *CIITA* is composed of four functional domains: acidic transactivation domain, proline/

serine/threonine (PST)-rich domain, GTP binding domain (GBD), and Leucine-rich repeats (LRR), and three nuclear localization regions.⁵⁸ Previous studies demonstrated that LRR of *CIITA* controlled its rate of nuclear accumulation.⁵⁹ Mutations in GBD and LRR, as well as hypophosphorylation in the PST domain could impair the nuclear translocation of *CIITA*.^{60,61} Thus, it could be speculated that the structures or epigenetic modification of the functional domains of *CIITA* in hESC-derived MSCs might be distinct from BMSCs and responsible for the decreased nuclear translocation of *CIITA*. Further investigations are needed to determine the underlying mechanism.

In conclusion, we demonstrated that hESC-derived MSCs shared similar immunoprivileged status and immunosuppressive abilities as BMSCs. Also, the evidences that H9-MSCs differ from BMSCs in the expression profile of immunological markers and their responsiveness to the proinflammatory cytokine IFN- γ , suggested that MSCs derived from hESCs could be a safe and efficient candidate for MSC treatment in patients with inflammatory disorders. Moreover, we first reported that H9-MSCs were less susceptible to IFN- γ -induced HLA-DR expression than BMSCs by expressing lower mRNA levels of *IRF2* and *CIITA*, and less nuclear translocation of *CIITA* proteins. These data provide a new insight into the molecular mechanism that determines the immunological characteristics of MSCs from different developmental origins.

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Disclosure Statement

No competing financial interests exist.

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