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# Insensitivity of Human iPS Cells-Derived Mesenchymal Stem Cells to Interferon-γ-induced HLA Expression Potentiates Repair Efficiency of Hind Limb Ischemia in Immune Humanized NOD Scid Gamma Mice

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**Key Words.** Induced pluripotent stem cells • Mesenchymal stem cells • Human leukocyte antigen class II • Immune humanized mice

## ABSTRACT

Adult mesenchymal stem cells (MSCs) are immunoprivileged cells due to the low expression of major histocompatibility complex (MHC) II molecules. However, the expression of MHC molecules in human-induced pluripotent stem cells (iPSCs)-derived MSCs has not been investigated. Here, we examined the expression of human leukocyte antigen (HLA) in human MSCs derived from iPSCs, fetuses, and adult bone marrow (BM) after stimulation with interferon-γ (IFN-γ), compared their repair efficacy, cell retention, inflammation, and HLA II expression in immune humanized NOD Scid gamma (NSG) mice of hind limb ischemia. In the absence of IFN-γ stimulation, HLA-II was expressed only in BM-MSCs after 7 days. Two and seven days after stimulation, high levels of HLA-II were observed in BM-MSCs, intermediate levels were found in fetal-MSCs, and very low levels in iPSC-MSCs. The levels of p-STAT1, interferon regulatory factor 1, and class II transactivator exhibited similar phenomena. Moreover, p-STAT1 antagonist significantly reversed the high expression of HLA-II in BM-MSCs. Compared to adult BM-MSCs, transplanting iPSC-MSCs into hu-PBMNC NSG mice revealed markedly more survival iPSC-MSCs, less inflammatory cell accumulations, and better recovery of hind limb ischemia. The expression of HLA-II in MSCs in the ischemia limbs was detected in BM-MSCs group but not in iPSC-MSCs group at 7 and 21 days after transplantation. Our results demonstrate that, compared to adult MSCs, human iPSC-MSCs are insensitive to proinflammatory IFN-γ-induced HLA-II expression and iPSC-MSCs have a stronger immune privilege after transplantation. It may attribute to a better therapeutic efficacy in allogeneic transplantation. STEM CELLS 2015;33:3452–3467

## SIGNIFICANCE STATEMENT

Allogenic transplantation of MSCs derived from bone marrow has been used clinically in graft versus host disease, multiple sclerosis, diabetes or ulcerative colitis, Crohn's disease and the other diseases. Here we identified that human MSCs derived from human-induced pluripotent stem cells (iPSCs) exhibited poor immunogenicity after IFN-γ stimulation in vitro and exerted more cell retention, less inflammation and better efficacy in a model of limb ischemia in immune humanized mice compared to BM-MSCs. It suggests that these cells may have more potential clinical implications with a lower risk of rejection and a better therapeutic efficacy in allogenic transplantation.

## INTRODUCTION

Mesenchymal stem cells (MSCs) have attracted attention due to their potential use in transplantation for the treatment of regenerative medicine and immune diseases. MSCs have been demonstrated to be poor stimulators of an in vitro allogeneic T-cell response and fail to induce the activation of allogeneic T cells [1]. Transplanted MSCs should not trigger a strong host inflammatory response [2]. The implantation of allogeneic, major histocompatibility complex (MHC)-mismatched MSCs into baboons has been well tolerated in most animals [3, 4]. Clinical studies have shown that allogenic MSCs can be transplanted into children with imperfecta with beneficial effects

and without rejection [5]. Allogenic bone marrow (BM)-MSC infusions into patients have shown to exert benefits in clinical trials of graft-versus-host disease (GvHD) [6, 7], type 2 diabetes [8], and systemic lupus erythematosus [9]. The cotransplantation of autologous MSCs and hematopoietic stem cells (HSCs) may enhance HSC engraftment [10]. These findings suggest that even human leukocyte antigen (HLA)-mismatched MSCs can be used in many novel applications in clinical stem cell-based therapies.

The allogenic response is usually mediated by the HLA and it is expected that the transplantation of cells that do not express HLA-II antigens may minimize the alloreactivity risk. MSCs express low levels of MHC class I molecules on their surface but lack the expression of MHC class II and the costimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD40 [1, 11], which may limit immune recognition. Transplanted MSCs differentiate in vivo and are also exposed to proinflammatory cytokines during infection and inflammation. MSCs are treated with interferon-$\gamma$ (IFN-$\gamma$), a proinflammatory cytokine, to represent the "worst case scenario," which corresponds to the implantation of MSCs into sites of inflammation [12]. IFN-$\gamma$ is known to increase the cell surface expression of MHC molecules in antigen-presenting cells (APCs) and has been demonstrated to upregulate MHC-II molecules in MSCs.

Most of the above-mentioned studies focused on adult MSCs, such as MSCs derived from the BM. We recently succeeded in inducing MSCs from human-induced pluripotent stem cell (iPSCs) [13]. We found that iPSC-MSCs express well-known adult BM-MSC markers, display the potential for adipogenesis, osteogenesis, and chondrogenesis, and prevent airway allergic inflammation after xenogenic transplantation [14]. Furthermore, iPSC-MSCs display a higher capacity of both cell proliferation and repair of tissue ischemia compared with their adult BM-MSC counterparts [13]. However, the MHC expression kinetics and immunogenicity of iPSC-MSCs have not yet been investigated.

The aim of this study was to characterize the immunological properties and patterns of both HLA class I and II and the possible mechanisms activated in MSCs derived from different origins after exposure to IFN-$\gamma$. Moreover, the cell retention, relative immune responses, HLA II levels in MSCs, and repair efficiency of human iPSC-MSCs on the transplantation sites were examined in a model of hind limb ischemia in immune humanized mice. Our study demonstrated that, compared to adult MSC, human iPSC-MSCs express lower levels of HLA-II even stimulated by inflammatory cytokines (i.e., IFN-$\gamma$) and exhibit higher cell survival as well as lower immune responses after transplantation in mice, which make them attractive candidates for clinical application in allogeneic transplantation.

## MATERIALS AND METHODS

### Preparation of Human iPSC-MSCs, Fetal-MSCs, and BM-MSCs

Two independent clones of iPSCs were differentiated into MSCs according to our previous study [15]. iMR90-iPSC-MSCs 10 were generated from iPSC-iMR90-5 (WiCell Research Institute, Madison, WI, www.wicell.org) [13]. The N1-iPSC-MSC clone was prepared from iPSCs reprogrammed from human fibroblast cells [16]. The details are presented in Supporting

Information Methods. The iPSC-MSCs were morphologically highly similar to BM-MSCs, had the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts, and exhibited a similar expression profile of surface antigens.

Human MSCs derived from fetal BM (fMSCs, passage 2, catalog No. HUXMF-01001) and BM-MSCs (passage 2, catalog No. HUXMA-01001) derived from the BM of healthy adults were purchased from Cyagen Biosciences, Inc. (Suzhou, JiangSu, People's Republic of China). The cells were separately cultured with human umbilical cord-MSC growth medium with human umbilical cord MSC basal medium, 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin (Cat. No. HUXUC-90011, Cyagen Biosciences, Inc., Suzhou, JiangSu, China, www.cyagen.com.cn) or human BM-MSC growth medium with human BM-MSC basal medium, 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin (Cat. No. HUXMA-90011, Cyagen Biosciences, Inc., Suzhou, JiangSu, China, www.cyagen.com.cn).

### Stimulation of MSCs

Previous studies have shown that the expression of HLA class II is increased in BM-MSCs [12, 17–19], adipose-derived stem cells (ASCs) [20], and fMSCs after exposure to IFN-$\gamma$ [21]. In our study, two clones of iPSC-MSCs at passages 7–9, fMSCs, and BM-MSCs at passages 4–6 were exposed to recombinant human IFN-$\gamma$ (200 ng/ml, Gibco, Invitrogen Corporation, CA) and then subjected to analyses of HLA expression, costimulatory molecules, and transcription factors using flow cytometry, Western blotting, immunocytochemistry, and quantitative real-time PCR.

### Flow Cytometry Analysis of HLA, Surface Marker, and Costimulatory Molecule Expression

After 2 or 7 days of stimulation with IFN-$\gamma$, the MSCs were harvested and analyzed to determine their expression profile of immunological molecules using specific monoclonal antibodies against HLA class I (ABC), HLA class II (DR), CD80/B7-1, CD 86/B7-2, CD40, CD3, CD105, CD90, CD34, CD45, CD14, CD44, CD73, CD56 (BD Biosciences, San Diego, CA), and a BD FACSCalibur flow cytometer (BD Biosciences, NJ) in accordance with the manufacturer's instructions. Isotype-matched immunoglobulin (BD Biosciences) was used as the negative control for each assay. The results represent five independent experiments.

### Western Blotting for HLA Expression

The MSCs were cultured for 2 and 7 days with or without IFN-$\gamma$ for the examination of HLA class I and HLA class II. Peripheral blood mononuclear cells (PBMCs) from healthy human volunteers, which were obtained as described in our previous study [22], were used as the positive control. Furthermore, MSCs stimulated with IFN-$\gamma$ for 0 minute, 5 minutes, 30 minutes, 60 minutes, 6 hours, 2 days, and 7 days were subjected to Western blot analyses to detect the levels of p-STAT1 and total STAT1. The results represent four independent experiments. The following antibodies were used: human HLA-I and HLA-II (Abcam, Inc., Cambridge, MA), phospho-STAT1 and STAT1 (Cell Signaling Technology, Beverly, MA). The details are presented in Supporting Information Methods.

## Immunocytochemistry for the Analysis of HLA Expression

We then further assessed the expression of HLA-I and HLA-II via immunocytochemistry. The different types of MSCs were plated in 24-well plates with poly(L-lysine)-coated coverslips and grown in culture medium with IFN-$\gamma$. After 2 or 7 days, the cells were fixed with 4% paraformaldehyde for 20 minutes, washed, permeated in 0.25% Triton X-100/phosphate-buffered saline (PBS), and blocked with 10% goat serum. The cells were then incubated with mouse antibodies against human HLA-I (Abcam, Inc.) and human HLA-II (Abcam, Inc.) at 4°C for 16 hours. After washing, the cells were incubated with a goat anti-mouse Alexa 488-conjugated secondary antibody (Molecular Probes, CA) for 2 hours at room temperature. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, MO). The results represent four independent experiments. The sections were analyzed under a fluorescent microscope.

## RNA Extraction and Quantitative Real-Time PCR

The total RNA from the N1-iPSC-MSCs, iMR90-iPSC-MSCs, fMSCs, and BM-MSCs cultured with IFN-$\gamma$ for 0, 2, and 7 days was extracted using the TRIzol reagent (Invitrogen, CA). The cDNA was synthesized using the M-MLV Reverse Transcriptase kit (Promega, CA). The target transcripts of class II transactivator (CIITA), IFN-$\gamma$ receptor 1 (IFN-$\gamma$R1), IFN-$\gamma$ R2, interferon regulatory factor 1 (IRF-1), and regulatory factor X 5 (RFX5) were quantified by real-time PCR with SYBR Premix Ex Taq (TaKaRa, Japan) using the 18S rRNA as the internal control. The primers used in the reverse transcription and quantitative PCR assays are shown in Supporting Information Table S1.

## The Treatment of p-STAT1 Inhibitor

In order to examine the role of p-STAT1 signaling pathway in the expression of HLA-II, 100 $\mu$m fludarabine (Sigma) (p-STAT1 inhibitor) was added into the medium of BM-MSCs for 1.5 days. Moreover, BM-MSCs were stimulated with IFN-$\gamma$ for 1.5 or 2 days. The cells were harvested for Western blotting for p-STAT1, total STAT1, and HLA-II, and quantitative PCR for CIITA and IRF-1.

## Isolation of Human Dendritic Cells and HLA Expression Assay

Dendritic cells (DCs) were isolated from adult healthy volunteers, and these cells were used as the positive controls for the expression of HLA-I and HLA-II, which was analyzed via flow cytometry analysis and immunocytochemistry. The details are presented in Supporting Information Methods.

## Immune Humanized Mice of Hind Limb Ischemia, Laser Doppler Imaging, and Masson Trichrome Staining

All procedures involving the immune humanized mice were approved by the Committee on the Use of Live Animals in Teaching and Research at The University of Hong Kong. Critical hind limb ischemia was established in NOD.Cg-Prkdc(Scid) Il2rg(tm1Wjl)/SzJ immunocompromised (NSG) mice (kind gifts from Dr. Camie Chan, The University of Hong Kong). These NSG mice lack mature T cells, B cells, or functional natural killer cells, and are deficient in cytokine signaling, leading to better engraftment of human hematopoietic cells [23].

Immune humanized mice were induced by intravenous injection of $3.0 \times 10^6$ PBMCs as previously described (abbreviated as Hu-PBMC mice) [24]. Hind limb ischemia of above mice was induced as our previous study [13]. Briefly, after anesthesia with xylazine (20 mg/kg) and ketamine (100 mg/kg) by intraperitoneal injection, the femoral artery of NSG mice was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. After arterial ligation with 8-0 suture, Hu-PBMC mice were randomized to receive following treatments: (a) PBS (PBS group, $n = 10$), (b) $3.0 \times 10^6$ BM-MSCs (BM-MSCs group, $n = 10$), or (c) $3.0 \times 10^6$ N1-iPSC-MSCs (iPSC-MSCs group, $n = 10$). In order to examine the MSCs engraftment post-transplantation, both injected BM-MSCs and iPSC-MSCs were labeled with green fluorescent protein (GFP). In each NSG mouse, a total of $3.0 \times 10^6$ cells in 200 $\mu$l PBS or the same volume medium PBS was intramuscularly injected into four sites of the gracilis muscle at the medial thigh of the ischemic hind limbs. Blood flow of ischemic limbs was detected by laser Doppler imaging analysis (Moor Instruments, Devon, U.K.) at Days 0, 7, 14, and 21 after cell administration. At Day 21, muscle tissues of the ischemic limbs were collected, formalin fixed, paraffin embedded, and sectioned. Hematoxylin and eosin (HE) staining and Masson trichrome staining were performed to assess the tissue damage and fibrosis formation of ischemic limbs, respectively.

## Immunostaining Assays of Cell Retention and Inflammation

To detect the engraftment of injected cells post-transplantation, muscle sections were stained with polyclonal rabbit anti-GFP (Santa Crus, CA). The inflammation of ischemic hind limbs was assessed by staining of CD45 (BD Pharmingen, San Diego, CA), CD4 (Santa Cruz), and HLA-II (Abcam, Inc.), respectively. The primary antibodies were incubated at 4°C overnight with a 1:100 dilution. After washed with PBS three times, the sections were incubated with the secondary antibodies for 1 hour at room temperature. The sections were incubated with biotin-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark) and visualized with diaminobenzidine. The nuclei were counterstained by hematoxylin. For fluorescent immunostaining, the sections were mounted with DAPI and the photographs were captured by a deconvoluted fluorescent microscope.

## Fluorescence-Activated Cell Sorting and Flow Cytometry Analysis for Cell Retention and Inflammation

To further study the survival rate of MSCs post-transplantation, the ischemic limbs were digested and then the associated cells were filtered. The surviving GFP$^+$-MSCs were counted by FACSAria II. For analysis of the inflammatory cell infiltration, associated cells were incubated with anti-CD45 (BD Biosciences) and then subjected to flow cytometry on a BD FACSCalibur. The data were analyzed by FlowJo software.

## Statistical Analysis

The experimental data are expressed as the means ± SEM. The data with a Gaussian distribution were statistically analyzed using one-way analysis of variance. For multiple comparisons, the data with variance homogeneity were then subjected to Tukey's test, whereas the data with variance
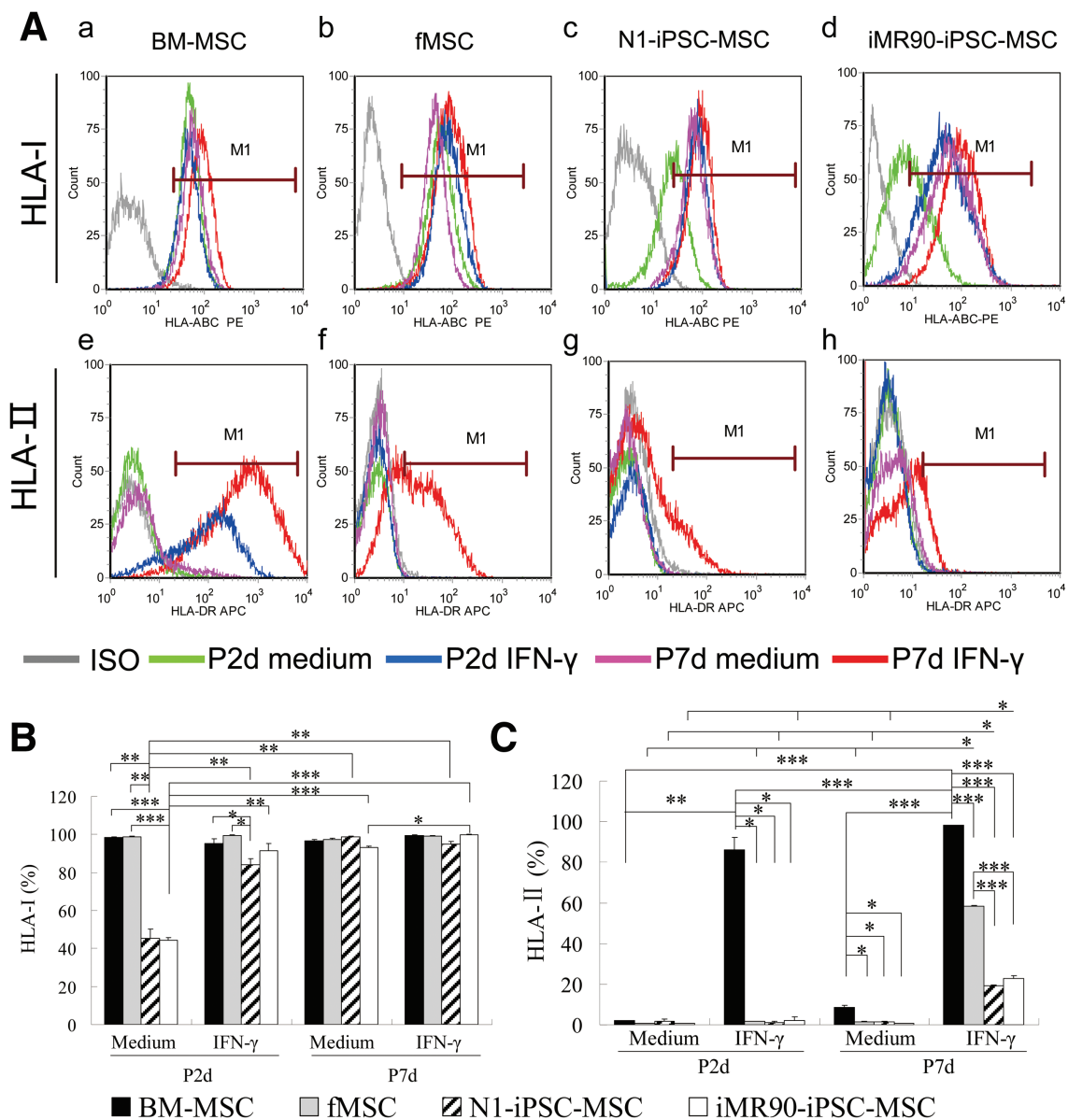
**Figure 1.** Flow cytometric detection of the expression profiles of HLA class I and II in MSCs in the absence and presence of IFN-γ. **(A):** The plots show BM-MSCs, fMSCs, N1-iPSC-MSCs, and iMR90-iPSC-MSCs with and without stimulation with IFN-γ for 2 or 7 days. The N1-iPSC-MSCs and iMR90-iPSC-MSCs exhibit low levels of HLA-I after incubation in the medium with 2 days (c, d) and low levels of HLA-II after stimulation with IFN-γ (g, h). The BM-MSCs exhibited markedly increased HLA-II expression 2 and 7 days after IFN-γ addition (e, red and blue lines). The fMSCs only exhibited a moderate increase in the HLA-II levels after 7 but not 2 days of stimulation with IFN-γ (f, red line). **(B):** The statistical analysis demonstrates lower levels of HLA-I in iPSC-MSCs compared with fMSCs and BM-MSCs. The HLA-I levels significantly increased in all the MSCs after IFN-γ addition. **(C):** The statistical analysis demonstrates the levels of HLA-II in MSCs. After 7 days of activation, the levels of HLA-II were highest in BM-MSCs, moderate in fMSCs, and lowest in N1-iPSC-MSCs and iMR90-iPSC-MSCs. Five independent experiments were performed for each group (mean ± SEM). *, $p < .05$; **, $p < .01$, and ***, $p < .001$, as determined through one-way analysis of variance with post hoc analysis for the HLA-I levels in all the MSCs and the HLA-II levels after 7 days and through the Kruskal-Wallis rank sum test followed by the Mann-Whitney $U$ test for the two-group comparisons of the other parameters. HLA-ABC, HLA-I; HLA-DR, HLA-II; ISO, isotype control; P2d, 2 days poststimulation; P7d, 7 days poststimulation. Abbreviations: BM, bone marrow; fMSC, fetal mesenchymal stem cell; HLA, human leukocyte antigen; IFN-γ, interferon-γ; iPSC, induced pluripotent stem cell.

heterogeneity were subjected to Dunnett's test. The comparisons between two groups were performed using an unpaired Student's $t$ test. The Kruskal-Wallis rank sum test and the Mann-Whitney $U$ test were performed for two-group comparisons of data with an abnormal distribution. The statistical analyses were performed using the SPSS 13.0 software (SPSS, Inc., IL), and $p < .05$ was considered statistically significant.

## RESULTS

### Detection of the Expression of HLA Proteins in Human iPSC-MSCs, fMSCs, and Adult BM-MSCs by Flow Cytometry

Many studies have demonstrated that human MSCs derived from adult tissues, such as BM-MSCs [12, 17–19], express
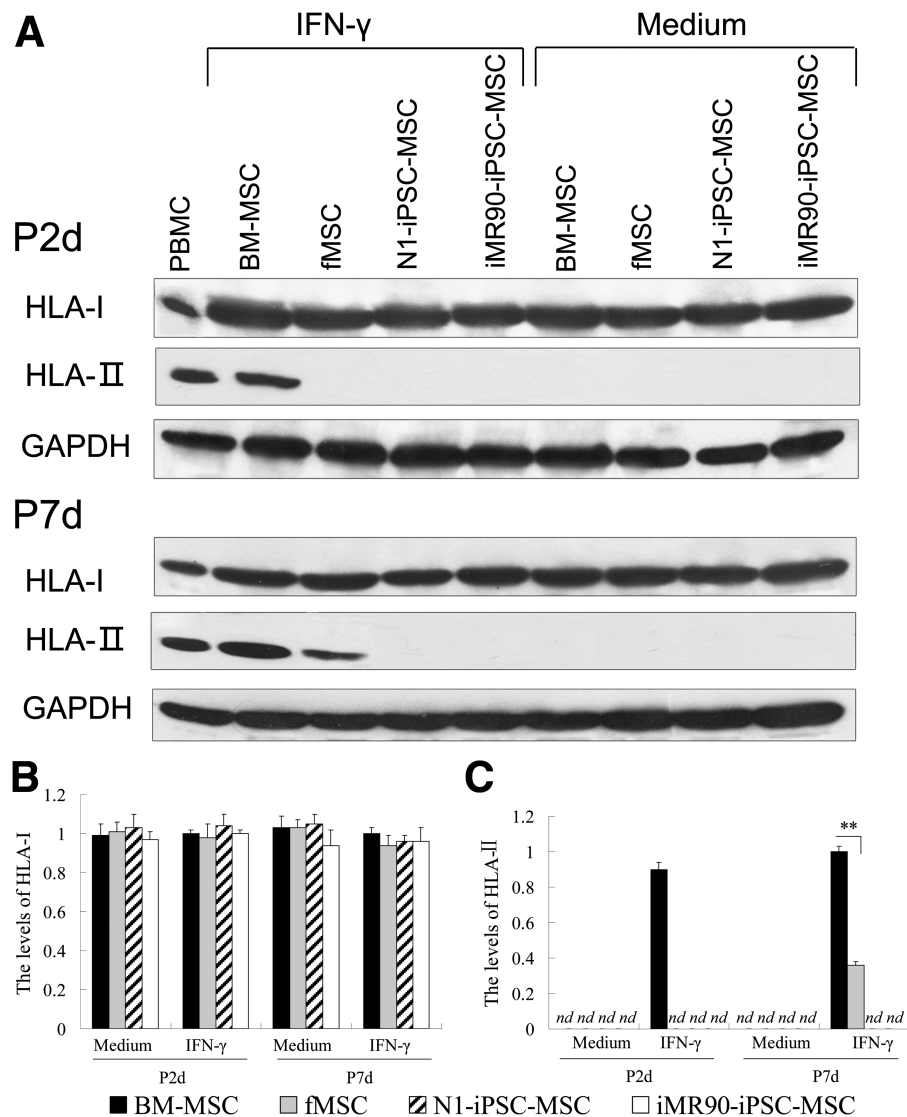
**Figure 2.** Immunoblotting of HLA class I and II proteins in MSCs after treatment with and without IFN-$\gamma$. **(A):** Western blotting of HLA-I and HLA-II proteins in different MSCs after 2 and 7 days in the presence or absence of IFN-$\gamma$. Human PBMCs were used as the positive control for the expression of HLA-I and HLA-II. HLA-I was expressed in all the MSCs, and no differences were observed between the different cells. After 2 and 7 days of IFN-$\gamma$ stimulation, HLA-II was expressed at high levels in BM-MSCs, and medium levels of HLA-II were observed in fMSCs after 7 days of stimulation, whereas HLA-II expression was not detected in iPSC-MSCs. **(B, C):** Densitometric scanning of the HLA-I and HLA-II proteins detected by Western blotting. The protein levels are expressed as relative values compared with the levels observed BM-MSCs after 7 days of stimulation with IFN-$\gamma$. The data were analyzed using an unpaired Student's *t* test for comparisons between two groups. **, $p < .01$. P2d, 2 days poststimulation; P7d, 7 days poststimulation. Abbreviations: BM, bone marrow; fMSC, fetal mesenchymal stem cell; GAPDH, glyceraldehyde phosphate dehydrogenase; HLA, human leukocyte antigen; IFN-$\gamma$, interferon-$\gamma$; iPSC, induced pluripotent stem cell; *nd*, no detection; PBMC, peripheral blood mononuclear cell.

HLA-class I but only express HLA-class II after stimulation with IFN-$\gamma$. Similarly, we found that both BM-MSCs and fMSCs express high levels (more than 90%) of HLA class I with or without treatment with IFN-$\gamma$ for 2 and even 7 days (Fig. 1A, 1B). In contrast, the levels of HLA-I observed in two clones of N1-iPSC-MSCs and iMR90-iPSC-MSCs were lower compared to those observed in fMSCs and BM-MSCs after 2 days of treatment with only medium ($p < .01$ or $p < .001$). Moreover, the N1-iPSC-MSCs and iMR90-iPSC-MSCs expressed higher levels of HLA-I after 2 and 7 days of stimulation and after 7 days without stimulation compared to the levels observed after 2 days without stimulation with IFN-$\gamma$ ($p < .01$ or $p < .001$) (Fig. 1A, 1B). In addition, the levels of HLA-I in the N1-iPSC-

MSCs were lower than those observed in fMSCs and BM-MSCs after 2 days of IFN-$\gamma$ stimulation ($p < .05$). The iMR90-iPSC-MSCs expressed lower levels of HLA-I after 7 days in the absence of stimulation compared with the levels obtained after stimulation ($p < .05$).

The HLA class II molecules were not expressed on the two clones of iPSC-MSCs, fMSCs, and BM-MSCs after 2 days in the absence of IFN-$\gamma$ stimulation (Fig. 1A, 1C). Two days after IFN-$\gamma$ activation, the expression of HLA class II molecules increased significantly to 86% in the BM-MSCs ($p < .01$), and lower increases were observed in the fMSCs, N1-iPSC-MSCs, and iMR90-iPSC-MSCs ($p < .05$). The HLA class II molecules were more highly expressed in BM-MSCs compared to the

other three types of MSCs after 7 days in the absence of stimulation with IFN-γ. Moreover, 7 days after IFN-γ treatment, the expression of HLA class II was higher in the BM-MSCs (98%) compared with the fMSCs (58%), N1-iPSC-MSCs (19%), and iMR90-iPSC-MSCs (23%) and higher in fMSCs compared to the two clones of iPSC-MSCs (all $p < .001$). In addition, a higher expression level of HLA class II was observed in the BM-MSCs 7 days after IFN-γ treatment compared with those observed after 2 days of stimulation with IFN-γ and 7 days in the absence of stimulation (all $p < .001$). We observed that the expression of HLA-II molecules in iPSC-MSCs, fMSCs, and BM-MSCs decreased successively depending on the increase in the stemness of the original source of the MSCs. We justified the results using three different passages of each MSCs, and no significant differences were observed between the cells from different passages.

Both the interaction between the peptide-MHC and the T-cell receptor and the costimulation provided by the interaction of a group of complementary molecules are required for T-cell activation. Previous studies showed that BM-MSCs [2, 11] and ASCs [20] do not express the costimulatory molecules CD80/B7-1, CD 86/B7-2, and CD40 even after stimulation with IFN-γ. In this study, we used flow cytometry to show that CD80, CD86, and CD40 are not expressed in all the tested MSCs after 2 or 7 days in the presence or absence of IFN-γ (Supporting Information Fig. S1).

We further examined the classic surface proteins of MSCs in the three different MSC types using flow cytometry. BM-MSCs, fMSCs, and two clones of iPSC-MSCs were defined as CD45−, CD14−, CD34−, CD3−, CD56−, CD44+, CD105+, CD90+, and CD73+ with and without the stimulation of IFN-γ. There was no difference for the levels of the surface proteins between three types of MSCs, and for each type of MSCs with and without the stimulation (Supporting Information Fig. S2).

### Detection of the Expression of HLA Proteins in Human iPSC-MSCs, fMSCs, and BM-MSCs Through Immunoblotting

All the MSCs derived from the adult BM, fetus, and two clones of iPSCs expressed HLA-class I molecules after 2 or 7 days in the presence or absence of IFN-γ (Fig. 2A). There was no significant difference in the HLA-I levels between the groups (Fig. 2A, 2B). As in a previous study [2, 17, 25], the BM-MSCs did not express HLA-II after 2 or 7 days in the absence of stimulation (Fig. 2A, 2C). Similarly, the fMSCs, N1-iPSC-MSCs, and iMR90-iPSC-MSCs did not express HLA-II molecules after culture in medium for 2 or 7 days. However, there was a marked increase in the HLA-II levels observed in BM-MSCs after 2 and 7 days of IFN-γ stimulation. Moreover, intermediate expression levels of HLA-II were observed in fMSCs after 7 but not 2 days of stimulation with IFN-γ, and this increased level was significantly lower than that observed in the BM-MSCs ($p < .01$). We did not observe any HLA-II expression in the two different clones of N1-iPSC-MSCs and iMR90-iPSC-MSCs after activation with IFN-γ. The HLA-II levels observed in MSCs after activation exhibited progressive reduction with an increase in the stemness of the original tissues or cells from which the MSCs were derived. The positive control, which consisted of PBMCs from healthy humans,

expressed both HLA class I and HLA class II molecules after 2 and 7 days of culture.

### Detection of the Expression of HLA Proteins in Human iPSC-MSCs, fMSCs, and BM-MSCs Through Immunofluorescence

To further confirm the expression of HLA-class I and HLA-class II molecules in MSCs, we examined their location through immunofluorescence. Few reports have described the detection of HLA expression in MSCs by immunocytochemistry. First, we investigated the expression of HLA molecules in human DCs (Supporting Information Fig. S3). As in a previous report [26], the intracellular HLA (MHC)-I staining in immature DCs exhibited a fine punctate or a silver-stained nearly reticular pattern reminiscent of the endoplasmic reticulum (ER). Some MHC I molecules also accumulated in the perinuclear region, which may correspond to cisternal Golgi elements (Supporting Information Fig. S3A). In mature DCs, most of the HLA-I was found in the plasma membrane (Supporting Information Fig. S3B). As in a previous study, the HLA (MHC)-II proteins congregated to large granules with characteristics of lysosomal structures in immature DCs and appeared at the plasma membrane in mature DCs (Supporting Information Fig. S3C, S3D).

The HLA-I proteins exhibited a similar pattern with fine punctates in the cytoplasm and conglomeration outside the nucleus in BM-MSCs, fMSCs, and iPSC-MSCs (Fig. 3A-a). The HLA-I staining was weak in both N1-iPSC-MSCs and iMR90-iPSC-MSCs but strong in BM-MSCs and fMSCs cultured in the medium for 2 days. Most of the HLA-I proteins remained in intracellular locations but not at the membrane in all types of MSCs after IFN-γ administration. Interestingly, we observed that a portion of the HLA-I molecules in BM-MSCs and particularly fMSCs were recruited to the end of the polar bodies and formed a fissional pattern between adjacent cells 7 days after the addition of IFN-γ (Fig. 3A-b).

No positive staining for HLA-II was observed in any of the MSCs in the absence of IFN-γ stimulation 2 or 7 days. We observed a similar intracellular granular pattern of HLA-II staining in a small number of BM-MSCs after 2 days of IFN-γ stimulation (Fig. 3B). It was interesting that some of the HLA-II staining resembled a bead with a little eye in the center (Fig. 3B-a). No positive staining for HLA-II was found in fMSCs, N1-iPSC-MSCs, and iMR90-iPSC-MSCs after IFN-γ stimulation for 2 days. Seven days after IFN-γ addition, almost all BM-MSCs expressed positive and strong HLA-II staining. Furthermore, some of the fMSCs also exhibited HLA-II-positive staining. Only a few of the N1-iPSC-MSCs and iMR90-iPSC-MSCs expressed HLA-II proteins. More importantly, we observed HLA-II expression only at the surface of a few BM-MSCs, but no surface expression was detected in the fMSCs and iPSC-MSCs (Fig. 3B-b), which indicates their potential ability to fully present exogenous antigens. These data suggest that HLA-II molecules are actively synthesized in a time-dependent manner after IFN-γ stimulation in BM-MSCs and that some of the proteins were delivered to the cell surface. In contrast, the iPSC-MSCs synthesized few HLA-II molecules even after long-term stimulation with IFN-γ for 7 days.
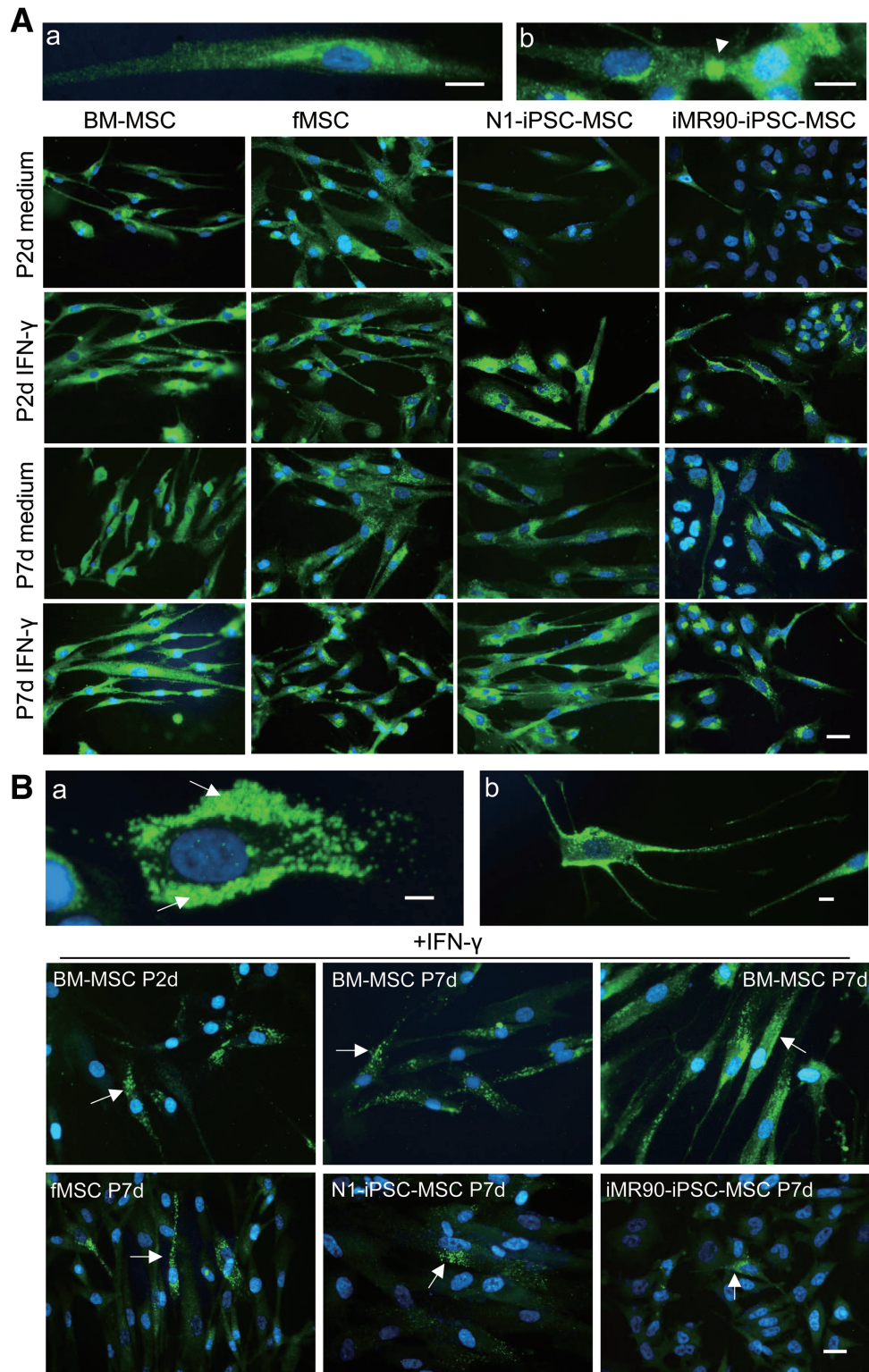
**Figure 3.** Immunofluorescence of HLA class I and II proteins in MSCs with and without IFN-$\gamma$ treatment. **(A):** HLA-I expression. The HLA-I proteins were stained as fine punctates in the cytoplasm and exhibited conglomeration outside of the nucleus (a). Some of the HLA-I proteins were recruited to the end of the polar bodies and formed a fissional pattern with the adjacent cells (b, white arrowhead). Strong HLA-I expression was detected in all the MSCs under the different conditions with the exception of N1-iPSC-MSCs and iMR90-iPSC-MSCs after 2 days of culturing in the absence of IFN-$\gamma$. **(B):** HLA-II expression. The HLA-II staining was observed as intracellular granules (white arrows) and sometimes presented as beads with little eyes in the center, following a nearly lysosomal pattern (a). A few of the BM-MSCs exhibited HLA-II-positive staining at the cell surface after 7 days of IFN-$\gamma$ stimulation (b). HLA-II-positive staining was found in BM-MSCs but not fMSCs and iPSC-MSCs 2 days after IFN-$\gamma$ stimulation. Seven days after IFN-$\gamma$ stimulation, the highest HLA-II expression was found in the BM-MSCs, whereas moderate HLA-II expression was detected in the fMSCs, and the lowest HLA-II expression was found in the iPSC-MSCs. Aa, Ba, Bb, Representative photographs of BM-MSCs 7 days after IFN-$\gamma$ addition. Ab, Representative photograph of fMSCs 7 days after IFN-$\gamma$ addition. The green staining represents the HLA molecules. The blue staining shows the cell nuclei, which were counterstained with 4′,6-diamidino-2-phenylindole. Scale bar is 10 $\mu$m in A-a,b and B-a,b, and 25 $\mu$m for the rest pictures. P2d, 2 days poststimulation; P7d, 7 days poststimulation. Abbreviations: BM, bone marrow; fMSC, fetal mesenchymal stem cell; IFN-$\gamma$, interferon-$\gamma$; iPSC, induced pluripotent stem cell.

## Phosphorylation of STAT-1 and Activation of IRF-1 and CIITA Among BM-MSCs, fMSCs, and iPSC-MSCs

Upon the binding of IFN-$\gamma$ to its cognate receptor, STAT1 phosphorylation is activated in APCs and other cell types.

Phospho-STAT1 and IRF-1 drive CIITA, which is obligatory for the transcription of MHC-II molecules. To further examine the possible mechanism underlying the differential activation of HLA-II in iPSC-MSCs, fMSCs, and BM-MSCs, we investigated
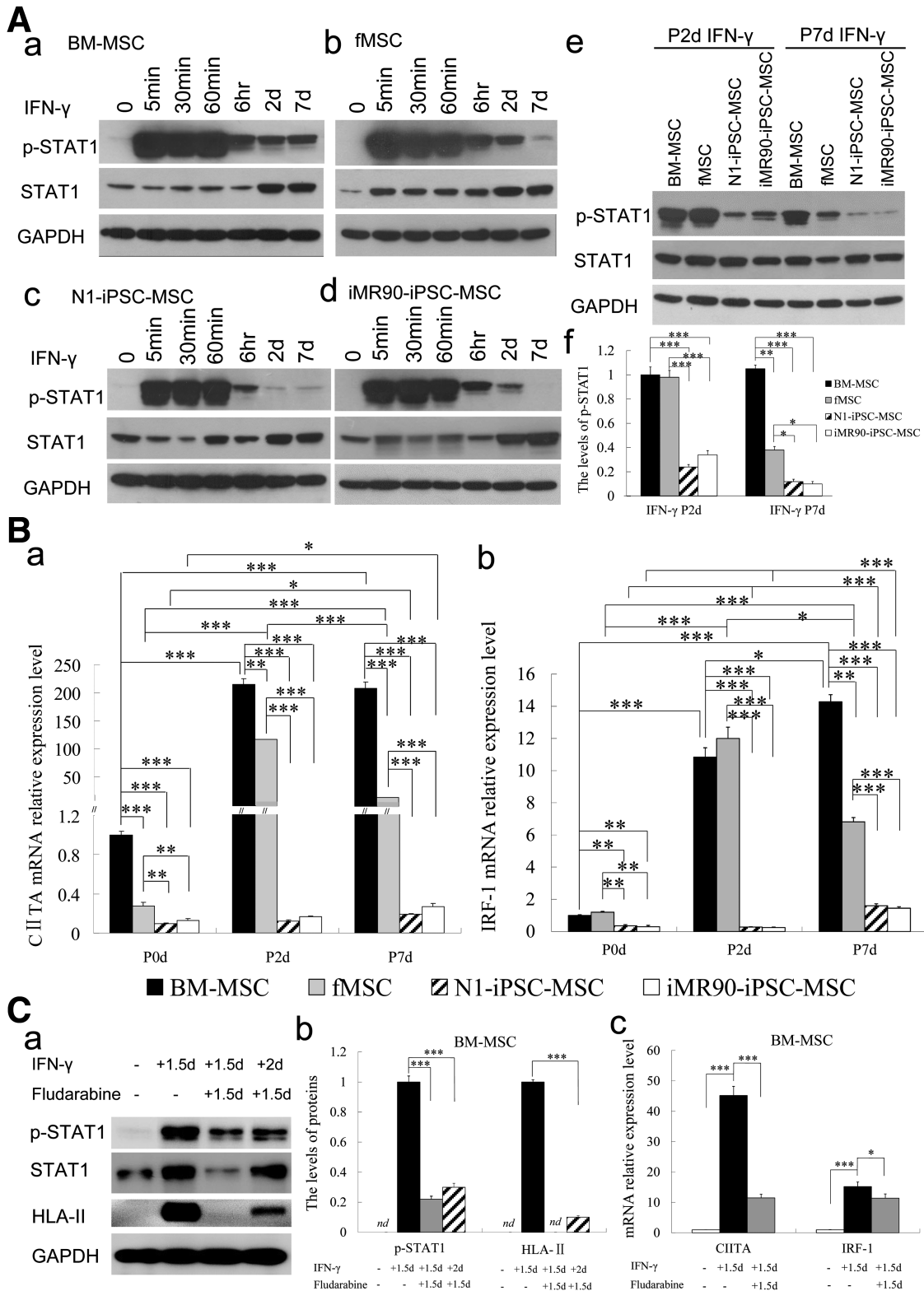


Figure 4.

the STAT1 signaling pathway and the genes involved in the induction of MHC-II molecules. The phosphorylation of STAT1 at a tyrosine residue markedly increased starting 5 minutes and maintained the maximum level up to 60 minutes after the addition of IFN-$\gamma$ and was decreased after 6 hours of IFN-$\gamma$ stimulation in all the BM-MSCs, fMSCs, N1-iPSC-MSCs, and iMR90-iPSC-MSCs (Fig. 4A). After a decline from the maximum, BM-MSCs maintained a certain level of p-STAT1 from 6 hours up to 7 days after IFN-$\gamma$ addition (Fig. 4A-a). However, the fMSCs only maintained this level of p-STAT1 up to 2 days, then returned to almost the level of untreated controls at 7 days (Fig. 4A-b). Moreover, the N1-iPSC-MSCs and iMR90-iPSC-MSCs maintained some p-STAT1 expression up to 6 hours after IFN-$\gamma$ addition, but this level was decreased to the basal level after 2 and 7 days (Fig. 4A-c, 4A-d). The activation of p-STAT1 was significantly higher in BM-MSCs (100%) and fMSCs (98%) compared to N1-iPSC-MSCs and iMR90-iPSC-MSCs with an increase of only 24%–34% after 2 days of IFN-$\gamma$ stimulation (Fig. 4A-f, all $p < .001$). There was no difference in the p-STAT1 level between BM-MSCs and fMSCs after 2 days of stimulation. After 7 days of IFN-$\gamma$ stimulation, the BM-MSCs (105%) exhibited a higher level of p-STAT1 compared with the fMSCs (38%, $p < .001$) and the two different clones of iPSC-MSCs (10%–12%), and the fMSCs presented a higher level compared to the iPSC-MSCs ($p < .05$). Similarly to the expression of HLA-II in the MSCs derived from different origins, these data suggest a similar trend that p-STAT1 levels underwent a decline in sequence from BM-MSCs to fMSCs and to iPSC-MSCs. The different levels of p-STAT1 observed after activation may provide an explanation for the differential expression of HLA-II in these MSCs. The low level of p-STAT1 may predicate the low level of HLA-II molecules observed in iPSC-MSCs. The total STAT1 levels in BM-MSCs, fMSCs, N1-iPSC-MSCs, and iMR90-iPSC-MSCs remained at the basal levels from 0 to 6 hours and were increased after 2 and 7 days of treatment with IFN-$\gamma$.

We further investigated the mRNA expression levels of IFN-$\gamma$ R1, IFN-$\gamma$ R2, and the transcription factors CIITA, IRF-1, and RFX5, which may be involved in the activation of MHC-II molecules, using quantitative RT-PCR (Fig. 4B). Under the basal conditions, the BM-MSCs exhibited high levels of CIITA compared to the fMSCs, N1-iPSC-MSCs, and iMR90-iPSC-MSCs ($p < .001$) and presented high levels of IRF-1 compared to N1-iPSC-MSCs and iMR90-iPSC-MSCs ($p < .01$). In addition, the fMSCs had high levels of CIITA and IRF-1 compared to the N1-iPSC-MSCs and iMR90-iPSC-MSCs (all $p < .001$) (Fig. 4B-a). IFN-$\gamma$ markedly enhanced the levels of CIITA in BM-MSCs by more than 200-fold and in fMSCs by 15- and 117-fold after 2 and 7 days of stimulation, respectively (all $p < .001$). The levels of CIITA in iPSC-MSCs were increased but remained low after the addition of IFN-$\gamma$. The BM-MSCs expressed higher levels of CIITA and IRF-1 compared to the fMSCs and the two clones of iPSC-MSCs after 2 and 7 days of stimulation (all $p < .001$). Furthermore, the fMSCs expressed higher levels of CIITA and IRF-1 compared to N1-iPSC-MSCs and iMR90-iPSC-MSCs after 2 and 7 days of stimulation (all $p < .001$). Similarly to the p-STAT1 expression results, the expression of CIITA and IRF-1 exhibited a gradual decrease from BM-MSCs to fMSCs to iPSC-MSCs after activation. We did not observe any significant difference in the expression of IFN-$\gamma$ R1, IFN-$\gamma$ R2, and RFX5 between the different types of MSCs after IFN-$\gamma$ stimulation. Our data suggest that the low levels of p-STAT1 and CIITA may be responsible for the low levels of MHC-II found in iPSC-MSCs.

Furthermore, we investigated the role of p-STAT1 signaling pathway in the expression of HLA-II using fludarabine to block the phosphorylation of STAT1 and the downstreams (Fig. 4C). We observed that fludarabine had clear toxicity to MSCs after the treatment of more than 1.5 days even with low concentration of 10 $\mu$m. We finally confirmed 100 $\mu$m fludarabine of 1.5-day-treatment for our study as both more safety to the cells and the bigger effects to decrease the levels of p-STAT1. We found that fludarabine clearly but not completely decreased the phosphorylation of STAT1 to the baseline in BM-MSCs after the stimulation of IFN-$\gamma$ for 1.5 days ($p < .001$, Fig. 4C-a, 4C-b). However, even current treatment of fludarabine completely blocked the expression of HLA-II in BM-MSCs after 1.5 days-IFN-$\gamma$ stimulation, and still with little expression at 2 days-IFN-$\gamma$ stimulation. Furthermore, fludarabine dramatically reduced the high gene levels of CIITA

**Figure 4.** Activation of STAT1 and the expression of transcription factors involved in the synthesis of HLA-II protein in MSCs with and without IFN-$\gamma$ treatment. **(A):** The activation of STAT1 at different time points after IFN-$\gamma$ stimulation was detected through immunoblotting. (a–d) The phosphorylation of STAT1 rapidly and markedly increased starting 5 minutes and maintained the maximum level up to 60 minutes after the addition of IFN-$\gamma$, and declined 6 hours after IFN-$\gamma$ addition in all four types of MSCs. A certain level of p-STAT1 was maintained in BM-MSCs 2 and 7 days after IFN-$\gamma$ addition and in fMSCs 2 but not 7 days after IFN-$\gamma$ addition, whereas the iPSC-MSCs presented a return to the basal p-STAT1 levels within 2 days after IFN-$\gamma$ addition. (e, f) The iPSC-MSCs exhibited lower levels of p-STAT1 after 2 and 7 days of treatment compared with BM-MSCs and fMSCs. In addition, the BM-MSCs presented a higher level of p-STAT1 compared to fMSCs, and the fMSCs exhibited a higher level compared with the iPSC-MSCs. (f) Densitometric scanning of the p-STAT1 protein level detected by Western blotting. The protein levels are expressed as relative values compared with the levels observed in BM-MSCs after 2 days of stimulation with IFN-$\gamma$. **(B):** The expression levels of CIITA and IRF-1 in MSCs after treatment with IFN-$\gamma$ were detected through qRT-PCR. (a) IFN-$\gamma$ markedly enhanced the levels of CIITA in BM-MSCs and in fMSCs but not in iPSC-MSCs. The levels of CIITA in iPSC-MSCs were lower compared to those observed BM-MSCs and fMSCs 2 and 7 days after IFN-$\gamma$ addition. The fMSCs presented lower levels of CIITA compared to BM-MSCs after 2 and 7 days of stimulation. (b) IFN-$\gamma$ enhanced the levels of IRF-1 in BM-MSCs and in fMSCs but not in iPSC-MSCs. **(C):** The effects of fludarabine on BM-MSCs with the treatment of IFN-$\gamma$. (a) Western blotting for p-STAT1, STAT1, and HLA-II with the treatment of both of fludarabine and IFN-$\gamma$. (b) Densitometric scanning of the p-STAT1 and HLA-II protein levels detected by Western blotting. The protein levels are expressed as relative values compared with the levels observed in BM-MSCs after 1.5 days of stimulation with IFN-$\gamma$. (c) CIITA and IRF-1 gene levels. *, $p < .05$; **, $p < .01$; and ***, $p < .001$, as determined through one-way analysis of variance with post hoc analysis for the data with a normal distribution or through the Kruskal-Wallis rank sum test followed by the Mann-Whitney $U$ test for two-group comparisons of data with an abnormal distribution. P2d, 2 days poststimulation; P7d, 7 days poststimulation. Abbreviations: BM, bone marrow; CIITA, class II transactivator; fMSC, fetal mesenchymal stem cell; GAPDH, glyceraldehyde phosphate dehydrogenase; HLA, human leukocyte antigen; IFN-$\gamma$, interferon-$\gamma$; iPSC, induced pluripotent stem cell; IRF-1, interferon regulatory factor 1; $nd$, not detection; STAT1, the signal transducer and activator of transcription 1.
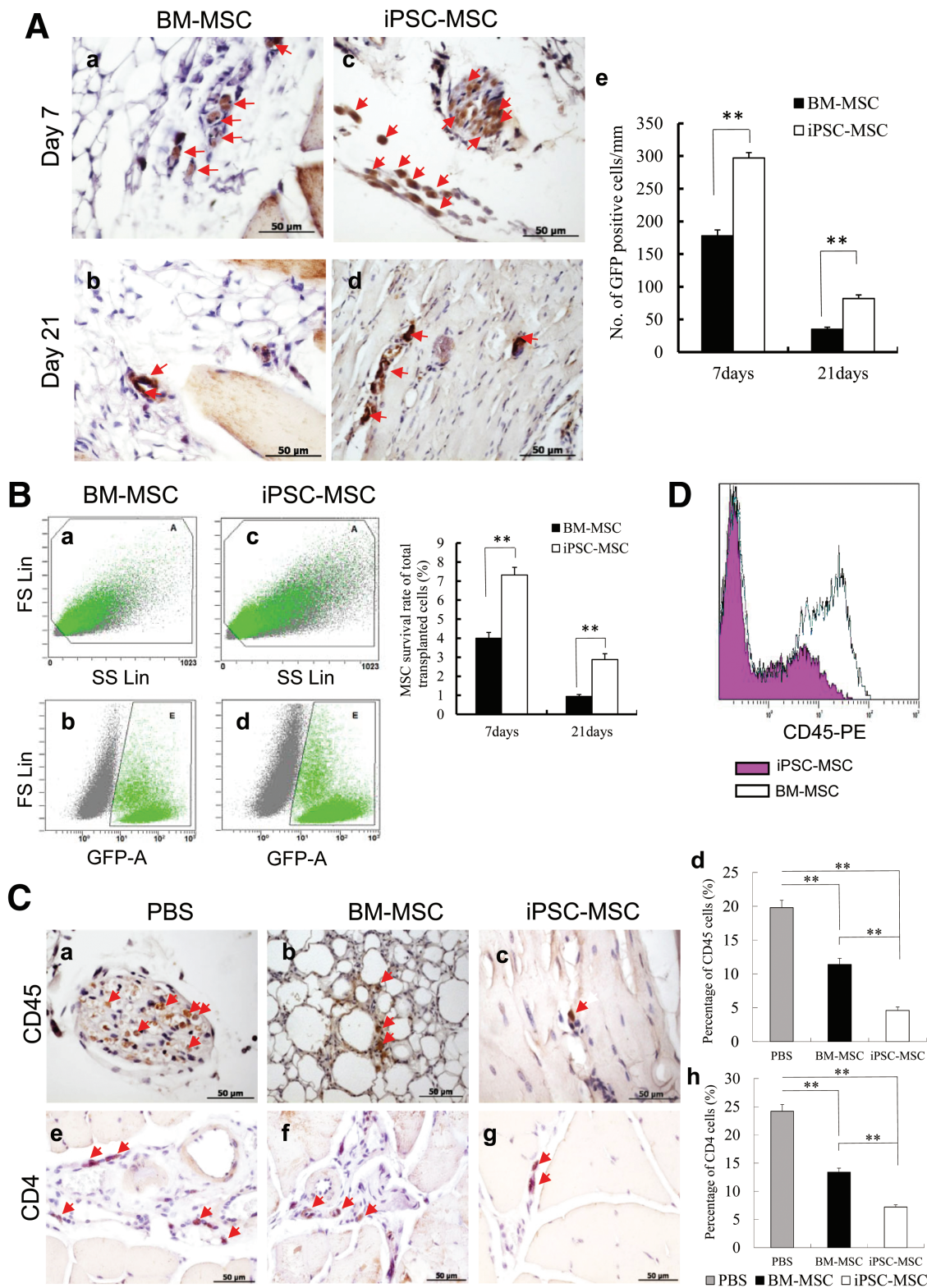
**Figure 5.** Examination of cell retention and inflammation postcell transplantation. **(A):** Cell retention was detected by staining of GFP in BM-MSCs group (a, b) and iPSC-MSCs group (c, d) in the ischemia limbs on Day 7 and Day 21, respectively. Arrows showed engrafted BM-MSCs and iPSC-MSCs in the ischemic limb with GFP positive. The engrafted cell was calculated on Day 7 and Day 21 from BM-MSCs and iPSC-MSCs group ($n = 6$, **, $p < .01$ at different time points). **(B):** Representative flow cytometric plots show surviving GFP$^+$-MSCs counted by fluorescence activated cell sorting in BM-MSCs (a, b) and iPSC-MSCs group (c, d). Gate A shows all the associated cells from the ischemia limbs. Gate E shows the GFP$^+$-MSCs out of gate A. The survival rate of MSCs at different time points were analyzed (e) ($n = 5$, **, $p < .01$ at different time points). **(C):** Representative photos of immunofluorescence staining showing CD45- (a–c) and CD4- (e–g) positive cells in the ischemia limbs at Day 21 among different groups, respectively. Arrows showed CD45- and CD4-positive cells in the ischemia limbs. The percentage of CD45- and CD4-positive cells in the ischemia limbs among different groups was analyzed at Day 21 (d, h) ($n = 6$, **, $p < .01$). Quantitative measurement of CD45 (d) and CD4 (h) was expressed as percent of positive staining versus total per myocardium area. **(D):** CD45-positive cells in the ischemia limbs at Day 21 among different groups were analyzed by flow cytometry. **(E):** The expression of MHC-II in MSCs in the ischemia limbs after transplantation at 7 days and 21 days was detected among in BM-MSCs (a–f) and iPSC-MSCs group (h–m). The percentage of MHC-II positive cells in BM-MSCs was calculated at 7 days and 21 days, respectively, ($n = 5$, **, $p < .01$ at different time points). The scale bars represent 50 $\mu$m. Abbreviations: BM, bone marrow; GFP, green fluorescent protein; iPSC, induced pluripotent stem cell; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline.
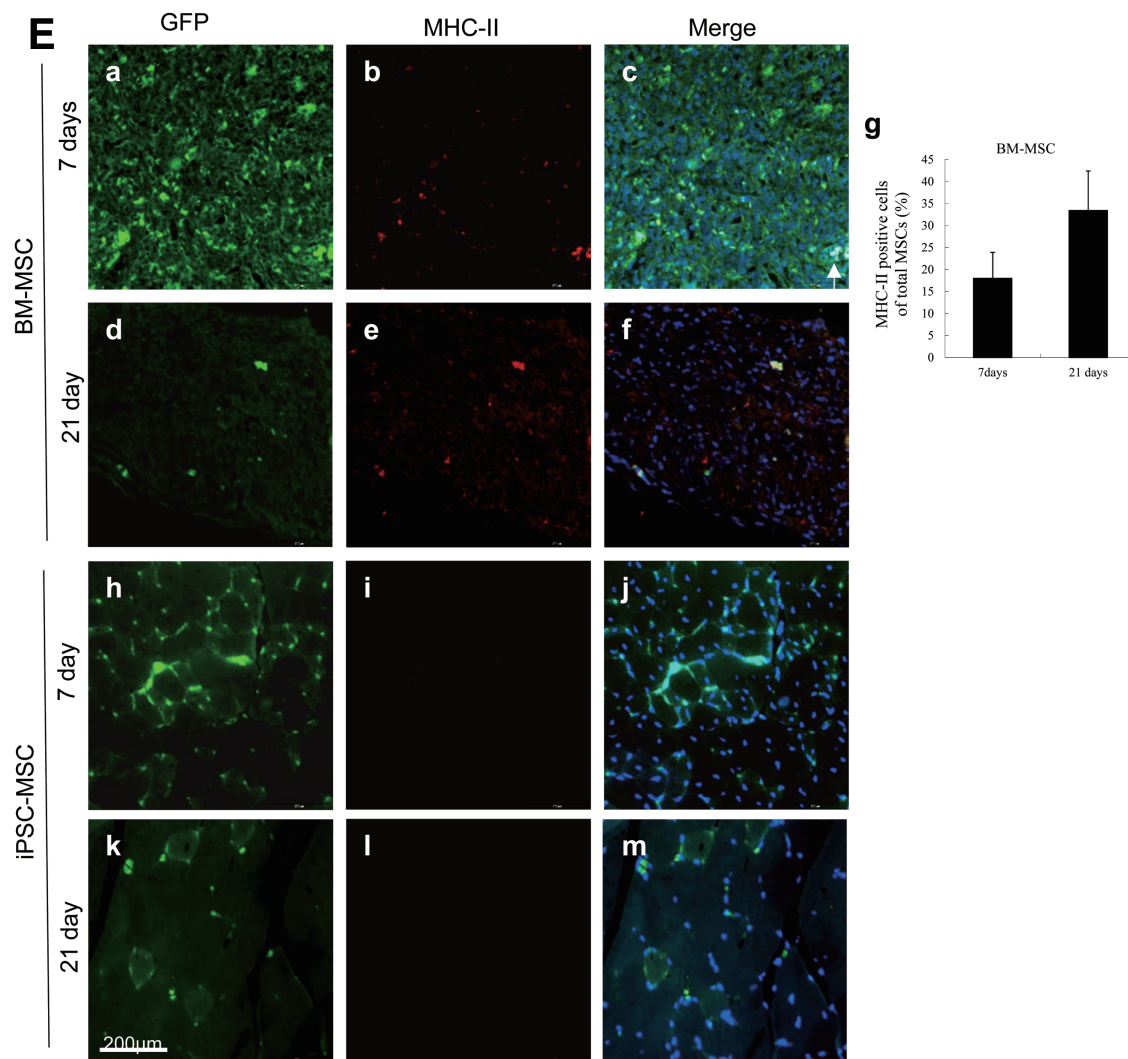
**Figure 5.** *Continued*

(*p* < .001, Fig. 4C-c) and had partly effects to decrease of IRF-1 expression (*p* < .05). The data further confirmed that p-STAT1 and CIITA genes were involved in the induction of HLA-II in MSCs after IFN-$\gamma$ stimulation.

### Examination of Cell Retention and Inflammation Post-MSC Transplantation in ischemic Limbs of Immune Humanized NOD Scid Gamma Mice

To further examine the expression of HLA-II between BM-MSCs and iPSC-MSCs and their immune privilege under the state of diseases, we transplanted them into the hind limb ischemia model of hu-PBMNC mice. The cell retention of BM-MSCs and iPSC-MSCs post-transplantation was examined at Day 7 and Day 21, respectively. At Day 7, GFP staining revealed that both BM-MSCs and iPSC-MSCs were detected. Moreover, the number of GFP-positive cells in iPSC-MSCs group was much higher than BM-MSCs group (297 $\pm$ 8.3/mm$^2$ vs. 178 $\pm$ 9/mm$^2$, Fig. 5A-a, 5A-b, 5A-e; *p* < .01). At Day 21, the number of GFP-positive cells retained in the ischemic limbs was markedly reduced in both iPSC-MSCs group and BM-MSCs group compared with Day 7. Notably,

GFP-positive cells in iPSC-MSCs group were still much higher than BM-MSCs group (82 $\pm$ 5.4/mm$^2$ vs. 35 $\pm$ 3/mm$^2$, Fig. 5A-c–5A-e, *p* < .01). To further quantify cell survival, ischemic limbs were digested at 7 days and 21 days after transplantation, respectively. As shown in Figure 5B, compared with BM-MSCs group, more GFP$^+$ cells were indentified in iPSC-MSCs group (Fig. 5B-a–5B-d). The cell survival rate was calculated by the surviving cells to the total transplanted cells. At 7 days, the cells survival rate was much higher in iPSC-MSCs group than BM-MSCs group (7.32% $\pm$ 0.4% vs. 4% $\pm$ 0.3%, Fig. 5B-e; *p* < .01). However, at 21 days, the survival GFP$^+$ cells were dramatically decreased in ischemic limbs compared with 7 days. Notably, the survival rate of iPSC-MSCs was still much higher than BM-MSCs (2.88% $\pm$ 0.3% vs. 0.94% $\pm$ 0.1%, Fig. 5B-e; *p* < .01).

The inflammation in the ischemic limbs was examined by immunofluorescence staining of CD45 and CD4, respectively. As shown in Figure 5C, the percentage of CD45- and CD4-positive cells in BM-MSCs group (Fig. 5C-b, 5C-d, 5C-f, 5C-h) and iPSC-MSCs group (Fig. 5C-c, 5C-d, 5C-g, 5C-h) was significantly decreased compared with PBS group (Fig. 5C-a, 5C-d,
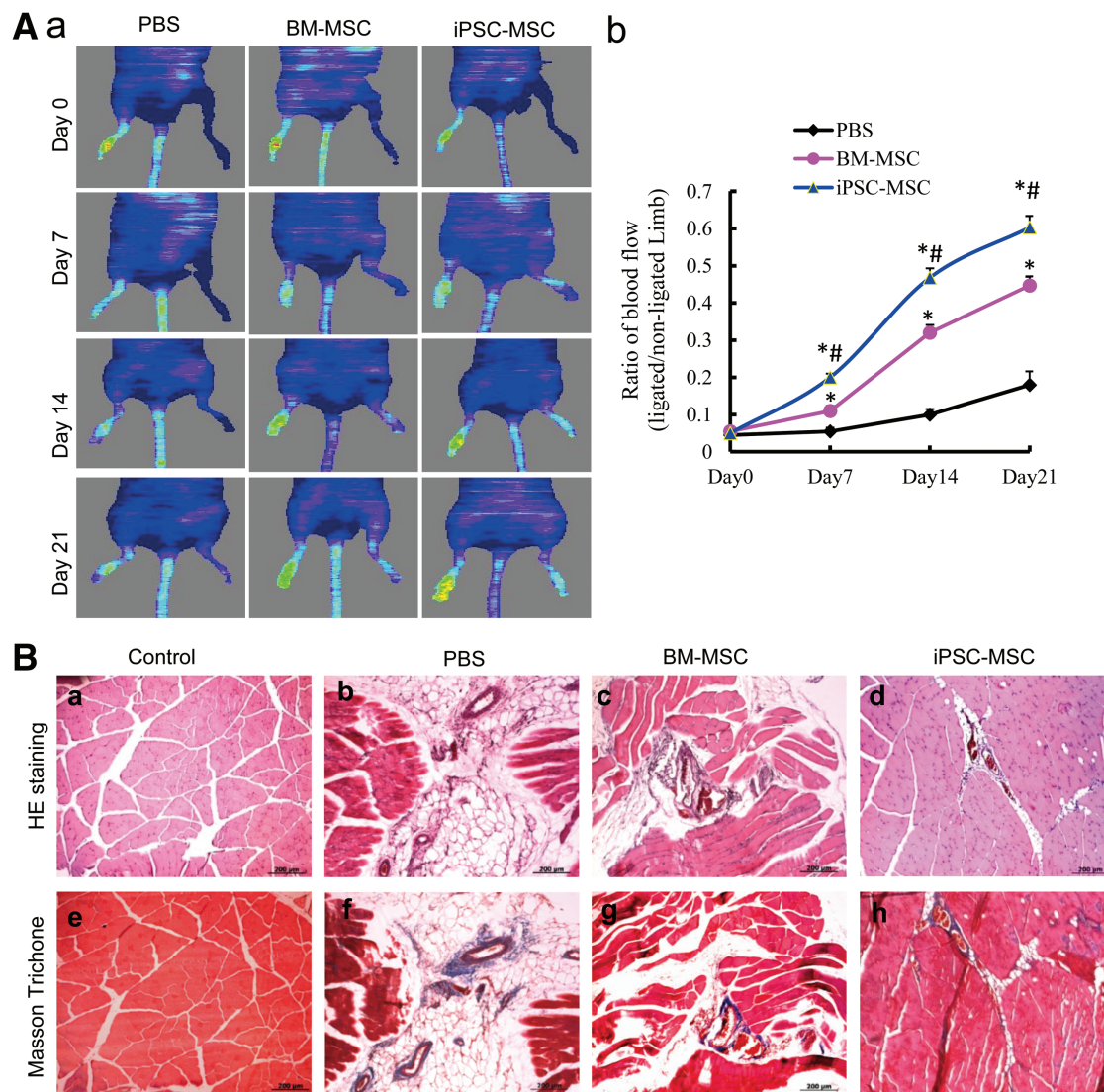
**Figure 6.** Transplantation of BM-MSCs and iPSC-MSCs in improvement of blood flow in ischemic limbs. **(A):** Representative photos showing the laser Doppler scanning at Day 0, Day 7, Day14, and Day 21 of ischemic limbs among the different groups (a). (b) The blood flow from different groups was analyzed. Compared with PBS group, there was a gradual improvement of blood flow in BM-MSCs and iPSC-MSCs group ($n = 6$, *, $p < .01$ vs. PBS group at different time points). Moreover, there was also a significant difference between BM-MSCs and iPSC-MSCs group ($n = 6$, #, $p < .01$ vs. BM-MSCs group at different time points). **(B):** Representative photos of HE staining showing the muscle degeneration among different groups. Representative photos of Masson trichrome staining showing interstitial fibrosis among different groups. HE staining demonstrated that the muscle degeneration of ischemic limbs was obviously protected in the BM-MSCs and iPSC-MSCs groups. Masson trichrome staining showed that fibrosis was remarkably attenuated in the BM-MSCs and iPSC-MSCs groups. The scale bars represent 200 $\mu$m. Abbreviations: BM, bone marrow; iPSC, induced pluripotent stem cell; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline.

5C-e, 5C-h; $p < .01$). Notably, the percentage of both CD45- and CD4-positive cells in iPSC-MSCs group was much lower than in BM-MSCs group ($p < .01$). The inflammatory cell infiltration was also examined by flow cytometry. As shown in Figure 5D, CD45 positive cells were significantly reduced in iPSC-MSCs group compared with BM-MSCs group at 21 days. Our results demonstrated that iPSC-MSCs have a stronger immune privilege than BM-MSCs.

Moreover, to further investigate the expression of HLA-II of MSCs after transplantation, GFP and HLA-II co-staining were performed. As shown in Figure 5E, the expression of HLA-II in BM-MSCs in the ischemic limbs was detected at 7 days and 21 days after injection in BM-MSCs group (Fig. 5E-a–

5E-f). Moreover, the percentage of MSCs expressing HLA-II was greatly increased at 21 days compared with 7 days (33.4% $\pm$ 9% vs. 18% $\pm$ 5.9%, Fig. 5E-g; $p < .01$). Interestingly, no HLA-II was detected in iPSC-MSCs group. These results further confirmed that iPSC-MSCs have a stronger immune privilege than BM-MSCs (Fig. 5E-h–5E-m).

### Transplantation of BM-MSCs and iPSC-MSCs Achieved Differential Improvement of Blood Flow in ischemic Limbs of Immune Humanized NOD Scid Gamma Mice

We next examined the different therapeutic effects of BM-MSCs and iPSC-MSCs on ischemic limbs. As shown in Figure 6A-a, 6A-b, compared with the nonischemic limbs, the blood

flow of the ischemic limbs was dramatically reduced at Day 0, indicating that the hind limb ischemia model was successfully established. From Day 7 on, the blood flow in BM-MSCs and iPSC-MSCs groups was gradually increased compared with PBS group (Fig. 6A-a, 6A-b; $p < .01$). At Day 21, compared with the PBS group (18% $\pm$ 3.6%), the blood flow in BM-MSCs group and iPSC-MSCs group was greatly increased to 44.7% $\pm$ 2.3% and 60.3% $\pm$ 3%, respectively (Fig. 6A-a, 6A-b; all $p < .01$). Notably, there was a significant difference between BM-MSCs group and iPSC-MSCs group (Fig. 6A-a, 6A-b; $p < .01$), suggesting iPSC-MSCs are superior to BM-MSCs in improvement of blood flow in a hind limb ischemia model of NSG mouse. HE and Masson trichrome staining demonstrated massive muscle degeneration and interstitial fibrosis formation in PBS group (Fig. 6B-b, 6B-f) compared with control group (Fig. 6B-a, 6B-e). In contrast, the muscle degeneration and interstitial fibrosis formation were remarkably reduced in BM-MSCs group (Fig. 6B-c, 6B-g) and iPSC-MSCs group (Fig. 6B-d, 6B-h). Moreover, less muscle degeneration and fibrosis were observed in iPSC-MSCs group compared with BM-MSCs group.

## DISCUSSION

In this study, we identified that human iPSC-MSCs did not express HLA-I and costimulatory molecules, and expressed very low levels of HLA-I molecules even with the treatment of IFN-$\gamma$. The levels of HLA-II molecules and the following signaling pathway of p-STAT1 and genes of IRF-1, CIITA exhibited a successive decrease in BM-MSCs, fMSCs, and iPSC-MSCs after IFN-$\gamma$ stimulation. Moreover, human iPSC-MSCs exerted more cell retention, less inflammation, no HLA-II expression, and a better therapeutic efficacy compared to BM-MSCs in a model of limb ischemia of immune humanized NSG mice. The lower levels of HLA-II molecules and the following signaling proteins and genes observed in human iPSC-MSCs but not BM-MSCs after IFN-$\gamma$ treatment indicate that these cells may have more potential clinical implications with a lower risk of rejection in allogenic transplantation.

Previous studies have shown that adult MSCs are positive for MHC class I and negative for MHC class II [12, 25]. The addition of IFN-$\gamma$ for 2 or 3 days induces most human adult BM-MSCs cells to express HLA-II [17–19, 25, 27]. Moreover, another type of adult MSCs, namely human ASCs, has been reported to express high levels of HLA-I but not HLA-II and express high levels of HLA-II after IFN-$\gamma$ stimulation [20]. Furthermore, approximately 100% HLA-I and 34% HLA-II molecules are induced by IFN-$\gamma$ treatment in epiphysis-derived MSCs [28]. In addition to adult MSCs, human fetal MSCs have been reported to express HLA-I but not HLA-II [21, 29]. Stimulation with IFN-$\gamma$ for 2 days initiates the intracellular synthesis of HLA-II, but 7 days of exposure are required for cell surface expression [21]. Our data on the expression of HLA-I and HLA-II in BM-MSCs are consistent with the findings reported by previous studies [12, 17–19]. We demonstrated that IFN-$\gamma$ induces a high expression of HLA-I molecules in human BM-MSCs, iPSC-MSCs, and fMSCs. No expression of HLA-II was observed in all the tested types of MSCs in the absence of IFN-$\gamma$. However, after IFN-$\gamma$ stimulation, HLA-II molecules were expressed at a high level in BM-MSCs, an intermediate level in fMSCs, and a minimal level in iPSC-MSCs. More impor-

tantly, we confirmed these results using three different techniques, namely flow cytometry, immunoblotting, and immunofluorescence, and two different clones of iPSC-MSCs. To the best of our knowledge, this report provides the first detailed demonstration of the expression of HLA proteins in human pluripotent stem cell-derived MSCs. It is interesting that level of HLA-II activated in MSCs derived from fetuses was between the high level observed in the adult MSCs from the BM and the low level observed in MSCs derived from iPSCs. This finding indicates that the HLA-II pattern in MSCs in response to IFN-$\gamma$ exhibits a progressive reduction depending on the enhancement of stemness of the original tissues or cells.

We further identified the possible mechanisms underlying the low expression of HLA-II in iPSC-MSCs. The MHC-II antigen is constitutively expressed on professional APCs and can be induced by IFN-$\gamma$ in other cell types, such as fibroblasts, epithelial cells, and even BM-MSCs [18, 30, 31]. Upon the binding of IFN-$\gamma$ to its cognate receptor, IFN-$\gamma$R1 and IFN-$\gamma$R2, STAT1 is activated to yield p-STAT1 [32]. Transcription factors, including p-STAT1 and IRF-1, bind to their own enhancer sequences in the IFN-$\gamma$ activation sequence element and drive the transcription of CIITA, which is obligatory for the transcription of the MHC-II gene [18, 33, 34]. In this study, similarly to the trend of HLA-II expression described in the different types of MSCs, the expression of p-STAT1, CIITA, and IRF-1 progressively decreased from BM-MSCs to fMSCs to iPSC-MSCs after IFN-$\gamma$ treatment. More importantly, very low levels of p-STAT1, CIITA, and IRF-1 gene expression were observed in iPSC-MSCs, which may result in the low induction of the MHC-II molecule observed in these cells. Moreover, p-STAT1 antagonist almost completely blocked the production of HLA-II protein and CIITA gene levels in BM-MSCs with the stimulation of IFN-$\gamma$ even still with some phosphorylation of STAT1. p-STAT1 antagonist only partly affected the IRF-1 gene levels, which may be because IRF-1 is also involved in the production of MHC-I protein or the other proteins [18]. Unlike a previous report [18], we observed no difference in the expression levels of the IFN-$\gamma$R1 and IFN-$\gamma$R2 genes even in BM-MSCs after IFN-$\gamma$ stimulation. This discrepancy may be due to the different cell origins or different culture conditions. The possible pathways involved in HLA-II expression in iPSC-MSCs are summarized in Figure 7. In human iPSC-MSCs, IFN-$\gamma$R1 and IFN-$\gamma$R2 are maintained at low levels after stimulation with IFN-$\gamma$. The phosphorylation of STAT1 remains low and thus results in low levels of p-STAT1 homodimerization. Together with low IRF-1, the few p-STAT1 dimers bind to the promoter regions of CIITA to result in low levels of transcription. The resulting low levels of CIITA protein are not sufficient to induce the expression of HLA-II molecules. In addition, low levels of p-STAT1 may result in low expression levels of the IRF-1 and CIITA genes.

Consistent with previous reports about the negative expression of costimulatory molecules in BM-MSCs [12], ASCs [20], and MSCs derived from the umbilical cord [29], we demonstrated that iPSC-MSCs, fMSCs, and BM-MSCs do not express costimulatory molecules regardless of the presence of IFN-$\gamma$. In general, cells that express MHC molecules can either stimulate T cells directly if they possess the appropriate costimulatory molecules [35] or activate T cells through an indirect pathway through cross presentation of their MHC antigens by
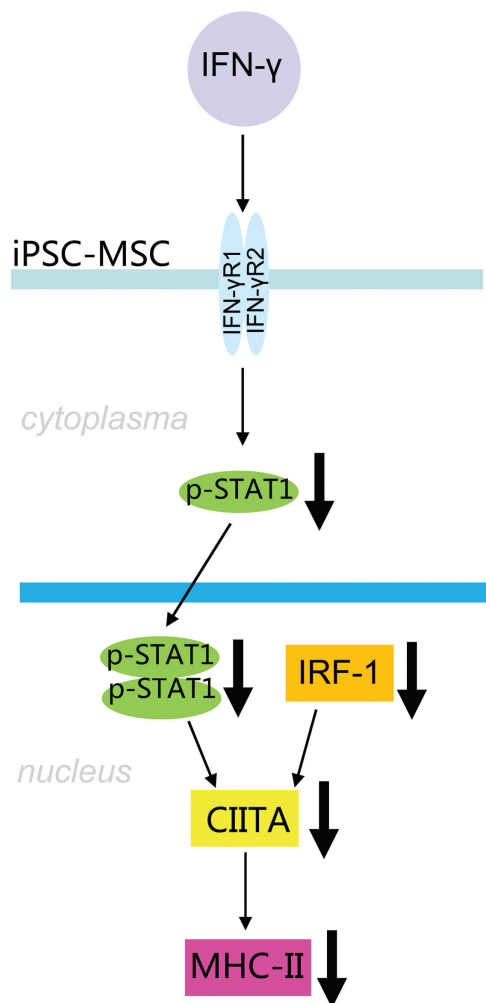
**Figure 7.** Diagram of the signaling pathways involved in the induction of HLA-II molecules in human iPSC-MSCs. In human iPSC-MSCs, IFN-γR1 and IFN-γR2 were maintained at low levels even after stimulation with IFN-γ. A low amount of STAT1 is phosphorylated, and thus few dimerized p-STAT1 molecules are available. In addition, these cells exhibit low IRF-1 and CIITA expression levels. Low levels of CIITA are transcribed due to the low level of IRF-1 and the low number of p-STAT1 dimers. Consequently, there is an insufficient amount of CIITA proteins to induce the expression of HLA-II molecules. Human iPSC-MSCs express low levels of HLA-II molecules in response to IFN-γ stimulation. Abbreviations: CIITA, class II transactivator; IFN-γ, interferon-γ; iPSC, induced pluripotent stem cell; IRF-1, interferon regulatory factor 1; MSC, mesenchymal stem cell; STAT1, the signal transducer and activator of transcription 1.

professional APCs. The cells will be rejected by the immune system unless suppressive or tolerogenic mechanisms are used. The low immunogenicity and immunosuppressive nature of MSCs are of clinical relevance to achieve low transplantation rejection in allogenic transplantation. Human or rat MSCs induce a weak immune response in the rat striatum after allo- or xeno-transplantation, and these animals express very few DCs and Tαβ- cells and no Tγδ-lymphocytes [36]. The transplantation of allogeneic MSCs into baboons has been found to be well tolerated [3, 4].

We further used the immune humanized NOD Scid gamma mice to examine the effects of iPSC-MSCs after transplantation. We induced a hind limb ischemia model and found

that more iPSC-MSCs remained in the injected muscle compared to BM-MSCs after the transplantation. There was much lower inflammation for iPSC-MSCs group compared with PBS group and BM-MSCs group. Moreover, the expression of HLA-II in MSCs was detected in BM-MSCs group but not in iPSC-MSCs group at 7 and 21 days after transplantation. iPSC-MSCs significantly improved the blood flow in above mice and superior to BM-MSCs. These data suggest that allogenic transplantation of iPSC-MSCs to humanized mice had the potential to be tolerated and exhibited good effects for the ischemia. There is some evidence that BM-MSCs are immunogenic and can induce T-cell responses through their MHC-I and MHC-II molecules or even CD80 in the presence of IFN-γ stimulation [37–39]. In our study, we found the upregulation of both MHC-II expression and the involved pathways, that is, p-STAT1, IRF-1, and CIITA, in BM-MSCs but not in iPSC-MSCs after IFN-γ stimulation. It suggests that human iPSC-MSCs have greater advantages to escape immune rejection in allogenic transplantation. Even that allogenic BM-MSCs are currently used for the patients in clinical trials such as GvHD [6, 7], the lower immunogenicity of iPSC-MSCs indicates a greater application in allogenic transplantation for patients. More importantly, it also provide a promising projection that iPSC-MSCs have the possibility to be produced as common biologicals and are easier to use with a low level of transplantation rejection in allogenic transplantation.

There are some controversial findings regarding the role of MHC molecules in MSCs. Some papers reported that ASCs, umbilical cord blood-derived MSCs, and BM-MSCs do not induce allogeneic PBMC activation and proliferation in vitro even if they express MHC-II [1, 20, 40, 41]. BM-MSCs stimulate mixed lymphocyte cultures and mitogenic responses independently of MHC expression [41]. fMSCs that upregulate HLA-II in response to IFN-γ stimulation fail to induce an immune response [21]. Therefore, the potential significance of the lack or low expression of HLA-II in human iPSC-MSCs in response to IFN-γ stimulation should be carefully further investigated. However, our study provides strong evidence that there is no expression of HLA-II molecules in human iPSC-MSCs under simulated inflammation conditions, which are represented by IFN-γ stimulation.

Moreover, adult MSCs, such as BM-MSCs, exhibit limited proliferative capacity and a rapid loss of differentiation potential [42–44]. iPSCs are unspecialized cells that are capable of renewing themselves infinitely in a culture dish, which allows them to serve as an unlimited and noninvasive resource of MSCs [13]. iPSC-MSCs have the potential to resolve the bottleneck problem that BM-MSCs are not sufficiently able to expand to the numbers needed for clinical use due to the long and complicated procedure and their limited proliferative activity. Our data suggest that iPSC-MSCs exhibited some difference or advantages compared to BM-MSCs even they had almost similar morphology, surface markers, and therapeutic effects. We previously found that there was a nearly 10-fold higher level of telomerase activity in iPSC-MSCs than in BM-MSCs [13]. This may explain our finding that the greater capacity of cell proliferation in iPSC-MSCs than BM-MSCs [13]. Although protein levels of pluripotency-associated genes were undetected in iPSC-MSCs and they did not induce any tumor formation after 4 months after subcutaneously transplanting into severe combined immunodeficiency mice [13], the safety

concern associated with the genomic instability of iPSCs still should be carefully evaluated before the clinical translation of iPSC-MSCs [45, 46].

Human iPSC-MSCs are immunosuppressive and poorly immunogenic, and these features make them attractive candidates as therapeutic agents in transplantation approaches for the treatment of diseases characterized by an abnormal activation of the immune system, such as GvHD. The combination of the proliferative capacity of human iPSC-MSCs and the immunological inertness of these cells indicates that they may be a source for more easily derived and highly acceptable MSCs that can be obtained at a higher yield.

## CONCLUSION

In summary, we demonstrated that human iPSC-MSCs are insensitive to proinflammatory IFN-$\gamma$-induced HLA-II expression and have a stronger immune privilege after transplantation. This may contribute to a better therapeutic efficacy in allogeneic transplantation.

## AUTHOR CONTRIBUTIONS

X.Li, M.X.D., Y.Y., and J.S.: collected and assembled the data; Y.Q.S. and W.X.G.: collected and assembled the data and prepared the dendritic cells; S.M.C., X.Liang, F.G., C.W.C., and H.F.T.: prepared iPSC-MSCs; Q.L.: designed experiments to derive and provide iPSC-MSCs, data interpretation, and revised the manuscript; Q.L.F.: provided the concept and design, performed the data analysis, and wrote and revised the manuscript; Y.Z.: collected and assembled the data and wrote and revised the manuscript. Y.Q.S., Y.Z., and X.Li contributed equally to this manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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