

An in Vitro and in Vivo Study of the Effect of Dexamethasone on Immunoinhibitory Function of Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells

Cell Transplantation
2018, Vol. 27(9) 1340–1351
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DOI: 10.1177/0963689718780194
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Abstract

Induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) represent a promising cell source for patient-specific cell therapy. We previously demonstrated that they display an immunomodulatory effect on allergic airway inflammation. Glucocorticoids are powerful anti-inflammatory compounds and widely used in the therapy of allergic diseases. However, the effect of glucocorticoids on the immunomodulatory function of iPSC-MSCs remains unknown. This study aimed to determine the effect of dexamethasone (Dex) on the immunomodulatory function of iPSC-MSCs in vitro and in vivo. A total of three human iPSC-MSC clones were generated from amniocyte-derived iPSCs. Anti-CD3/CD28-induced peripheral blood mononuclear cell (PBMC) proliferation was used to assess the effect of Dex on the immunoinhibitory function of iPSC-MSCs in vitro. Mouse models of contact hypersensitivity (CHS) and allergic airway inflammation were induced, and the levels of inflammation in mice were analyzed with the treatments of iPSC-MSCs and Dex, alone and combined. The results showed that Dex did not interfere with the immunoinhibitory effect of iPSC-MSCs on PBMC proliferation. In CHS mice, simultaneous treatment with Dex did not affect the effect of iPSC-MSCs on the inflammation, both in regional draining lymph nodes and in inflamed ear tissue. In addition, co-administration of iPSC-MSCs with Dex decreased the local expression of interferon (IFN)- γ and tumor necrosis factor (TNF)- α in the ears of CHS mice. In the mouse model of allergic airway inflammation, iPSC-MSC treatment combined with Dex resulted in a similar extent of reduction in pulmonary inflammation as iPSC-MSCs or Dex treatment alone. In conclusion, Dex does not significantly affect the immunomodulatory function of iPSC-MSCs both in vitro and in vivo. These findings may have implications when iPSC-MSCs and glucocorticoids are co-administered.

Keywords

Induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs), dexamethasone (Dex), immunoinhibitory, contact hypersensitivity (CHS), allergic airway inflammation

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells with immunomodulatory functions on immune cells¹, which have been being clinically explored as a new therapeutic treatment for various immune-related diseases, including graft-versus-host disease (GvHD)², systemic lupus erythematosus (SLE)³, and Crohn's disease⁴. Most preclinical and clinical studies are performed using MSCs derived from adult bone marrow (BM) and adipose tissue^{5–8}. However, several disadvantages may restrict these adult MSCs for clinical applications, including the requirement of invasive techniques for

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Submitted: February 6, 2017. Revised: October 13, 2017. Accepted: January 5, 2018.

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their isolation, a limited number of cells obtained initially from a single donor, limited capacity to proliferate when cultured *in vitro*, and varying cell qualities from different donors^{9–12}.

In the last decade, several reprogramming techniques generating human pluripotent stem cells (iPSCs) from adult somatic cells were successfully developed¹³. These iPSCs hold enormous promise for patient-personalized cell therapies¹⁴ and for research into various human diseases^{15,16}, and importantly, represent an important alternative source of functional MSCs^{17,18}. Recently, we and others have successfully induced MSCs from iPSCs (iPSC-MSCs)^{19–24}, which are similar to BM-MSCs in terms of morphology, multipotent differentiation potential, and expression of common MSC surface markers. The iPSC-MSCs display a strong immunomodulatory effect on natural killer (NK) cells²², dendritic cells and T-cells^{20,25}. The induced iPSC-MSCs are potent in the protection of limb ischemia and doxorubicin-induced cardiomyopathy^{19,26}. We have previously demonstrated that, similar to BM-MSCs, iPSC-MSCs can inhibit the Th2 phenotype both *in vitro* and *in vivo*^{20,21}. Given the advantages of iPSC-MSCs over adult MSCs, there are large expectations for the use of iPSC-MSCs for the clinical application of the treatment of allergic diseases. MSCs in combination with glucocorticoids may provide further potential benefits in the treatment of several disorders, such as ulcerative colitis, kidney transplantation, and multiple sclerosis^{27–31}. Therefore, it is foreseen that iPSC-MSCs may be used for clinical purposes in combination with glucocorticoids, which is currently the gold standard for controlling inflammation in allergic diseases. Then, the effects of glucocorticoids on the function of iPSC-MSCs should be well studied in the case of combined administration.

Few studies have investigated the interaction between BM-MSCs and glucocorticoids. Human BM-MSCs have been reported to inhibit allogeneic lymphocyte proliferation in the presence of dexamethasone (Dex) *in vitro*³². Conversely, a recent study demonstrated that Dex can eliminate the immunomodulatory effect of BM-MSCs on lymphocyte proliferation *in vitro* and in a murine model of liver fibrosis³³. In addition, a clinical trial using combined therapy with BM-MSCs and glucocorticoids in GvHD patients performed no better overall than a placebo³⁴. However, whether glucocorticoids interrupt the immunomodulatory function of iPSC-MSCs remains unknown.

In this study, we investigated the effect of Dex on the immunosuppression of iPSC-MSCs on the proliferation of anti-CD3/CD28-activated human peripheral blood mononuclear cells (PBMCs) *in vitro*, and examined the interactions between Dex and iPSC-MSCs in mouse models of contact hypersensitivity (CHS) and allergic airway inflammation. We found that Dex (at tested concentrations) had no significant effects on the immunomodulatory function of iPSC-MSCs, both *in vitro* and *in vivo*. It may be important for the potential clinical use of iPSC-MSCs in combination with glucocorticoids.

Materials and Methods

Generation of Human iPSC-MSCs

Amniocyte-derived iPSCs were purchased from Cell Inspire Bio (IPSN-0008, Shenzhen, China). Prior to generating the iPSC-MSCs, iPSCs were plated into a Matrigel-coated six-well plate, and cultured in mTeSR1 basal medium containing 20% mTeSR1 supplement (Stem Cell, MA, USA), 1% penicillin/streptomycin (Gibco, CA, USA). Once 60% confluence was reached, the mTeSR1 medium was replaced with induction medium containing minimum essential medium eagle- α modified (α -MEM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 200 mM l-glutamine and 10 mM non-essential amino acids (Gibco, CA, USA), sodium pyruvate, 10 mM l-ascorbate-2-phosphate (Sigma, MO, USA) and changed every other day. At 2 weeks later, cells were split with a 1:2 ratio using 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco, CA, USA), plated in a gelatin-coated six-well plate as passage one (P1). After two passages, cells were seeded in an uncoated six-well plate and cultured in iPSC-MSCs growth medium containing high glucose Dulbecco's modified eagle's medium (Hyclone, UT, USA), 10% FBS, 5 ng/ml fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (Gibco, CA, USA), 1% penicillin/streptomycin. Overall, three human iPSC-MSC clones were generated. Passage 9 to passage 17 iPSC-MSCs were used in this study.

Differentiation of Human iPSC-MSCs

Functional differentiation of iPSC-MSCs, including adipogenesis, osteogenesis and chondrogenesis, were carried out at passage 9. For adipocytic differentiation, cells were incubated in growth medium in six-well plates and changed to adipogenic differentiation medium (medium A for 3 days and medium B for 1 day; Cyagen Biosciences, CA, USA) when confluence was reached. After six cycles, cells were cultured in medium B for an additional 7 days, then stained with Oil Red O working solution (Cyagen Biosciences, CA, USA) and visualized under a light microscope. For osteogenic differentiation, cells were re-plated in growth medium at 3×10^4 cells/cm² in six-well plates pre-coated with gelatin solution. After 24 hours, the medium was changed to osteogenic differentiation medium (Cyagen Biosciences, CA, USA). After 3 weeks in culture, cells were fixed and stained with Alizarin red. For chondrogenic differentiation, 2.5×10^5 iPSC-MSCs were transferred to a 15-ml centrifugation tube to wash twice with incomplete chondrogenic medium (Cyagen Biosciences, CA, USA), then resuspended in complete chondrogenic medium to a density of 5.0×10^5 cells per ml. Cell suspension was transferred into a 15-ml polypropylene culture tube and centrifuged at $150 \times g$ for 5 minutes. The tube was incubated at 5% CO₂, 37°C, avoiding aspirating the supernatant or resuspending the pellet. After 24 hours, cell pellets were fed with fresh complete chondrogenic medium every 2–3 days.

Chondrogenic pellets were harvested after 28 days in culture, formalin fixed and paraffin embedded for Alcian blue stain.

PBMC Proliferation Assay

The buffy coats from anonymous healthy donors provided by Guangzhou Blood Center were used for human PBMC collecting as described previously²⁰. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University, China (No. 2014-C-053), and exemption of written informed consent for using human buffy coats was approved. Cells were suspended in 500 μ l of phosphate-buffered saline (PBS) containing 10% FBS and stained by 2 mM carboxyfluoresceinsuccinimidyl amino ester (CFSE; Sigma, MO, USA). After 10 minutes, cells were washed twice with 10 ml RPMI 1640 medium (Hyclone, UT, USA) with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. Cells were resuspended and dispensed in 24-well plates at a density of 2×10^5 cells/well. Then PBMCs with a stimulation of 1 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 (BD Biosciences, NJ, USA) were cultured alone or co-cultured with iPSC-MSCs in a ratio of 10:1, which was determined in our previous study²⁰, in the absence or presence of Dex at concentrations ranging from 10 ng/ml to 100 μ g/ml for 3 days.

Flow Cytometry of PBMCs and iPSC-MSCs

CFSE-stained PBMCs were harvested after 3 days of co-culture with iPSC-MSCs or Dex, and then the PBMC proliferation was assessed by flow cytometry (Beckman Gallios, IN, USA) using standard techniques. Cell surface antigens and human indoleamine 2,3-dioxygenase (IDO) expression in human iPSC-MSCs (passage 9) were also analyzed by flow cytometry. Antibodies against human antigens CD166, CD146, CD34, CD44, CD45, CD73, CD90, CD105 were purchased from BD Bioscience. Antibody against IDO (# P14902) was purchased from R&D systems (MN, USA). Data were analyzed by Kaluza Analysis Software (Beckman Coulter Life Sciences, IN, USA).

Enzyme-linked Immunosorbent Assay

Interleukin (IL)-6 and IL-10 levels in serum were determined using the ELISA Kit (KeyGEN BioTECH, Shanghai, China).

Animals

Female BALB/c mice (6–8 weeks) were purchased from Experimental Animal Center, Sun Yat-sen University (Guangzhou, China) and housed under specific pathogen-free conditions, maintained on a 12 h light/dark cycle, and provided food and water ad libitum.

All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee, Sun Yat-sen University.

Mouse Contact Hypersensitivity Model

Mice were sensitized to oxazolone (Sigma, MO, USA) by the application of 20 μ l of 1% oxazolone in an acetone/sesame seed oil vehicle (4:1 v/v) to both ears on day 1 and day 7³⁵. iPSC-MSCs (1×10^6 per mice, intravenous injection) or/with Dex (5 mg/kg, intraperitoneal injection) were injected into mice at the same time on day 6. Control mice received PBS. Ears and draining auricular lymph nodes at the base of the ear were photographed on day 8 and 9 respectively and harvested on day 9. The biggest lymph node was weighed immediately after excision. Serum samples were collected on day 9 and serum IL-6 and IL-10 levels were determined by ELISA assay.

Mouse Model of Allergic Airway Inflammation

Ovalbumin (OVA)-induced mouse model of allergic airway inflammation was established as our previous study²¹. Briefly, mice were sensitized by intraperitoneal injection of 40 μ g of OVA (grade V; Sigma, MO, USA) and 4 mg of aluminum hydroxide (Sigma, MO, USA) in 200 μ l PBS on day 1 and day 14. From day 21 to 24, mice were challenged daily with aerosolized 5% OVA in a plexiglass chamber and through an air-compressing nebulizer for 30 minutes. iPSC-MSCs (1×10^6 per mice) was injected via the tail vein on day 20. Mice were injected intraperitoneally with Dex (1.25 mg/kg/day) from day 21 to 24. The animals were sacrificed after the last challenge on day 24.

Histology and Microscopic Measurement of Ear Thickness

Ear biopsy specimens were fixed with 4% paraformaldehyde and embedded in paraffin. Deparaffinized 5- μ m sections were stained with hematoxylin and eosin (H&E) and analyzed by Leica DM2500 microscope and Leica Application Suite (Leica, IL, USA) software. Ear thickness (including epidermis, dermis and subcutaneous layer) was determined by taking measurements on six individual sections.

Evaluation of the Inflammatory Cells in the Bronchoalveolar Lavage Fluids

On day 24, bronchoalveolar lavage fluids (BALFs) were collected with 1 ml of cold PBS via a 22-gauge needle inserted into the trachea. After centrifugation, cells in the BALFs were counted using a hemocytometer and then resuspended in 5 μ l of PBS. The cell suspension was streaked in a thin film over a glass slide using the second slide as a spreader and was stained with Diff-Quik kit (D030, Nanjing Jiancheng Bioengineering Institute, China, <http://www.njjcbio.com>). A total

of 250 cells per slide were evaluated for inflammatory cell counting.

Lung Histology and Inflammation Scoring

Lung sections were prepared at a thickness of 4- μ m and were stained with H&E. For the lung inflammation quantification, five sections across the main bronchus and vascular per mouse were randomly selected. The inflammation levels were scored from 0 to 3 according to the following criteria: 0 = no inflammation; 1 = occasional inflammatory cells; 2 = most bronchus or vascular were surrounded by 1–5 layers of inflammatory cells; 3 = most bronchus or vascular were surrounded by more than five layers of inflammatory cells.

Western Blotting

Western blotting analysis of the homogenized mouse ears was performed following standard procedures. Antibodies against TNF- α (Cell Signaling Technology, MA, USA) and IFN- γ (Abcam, Cambridge, UK) were used.

Statistical Analysis

All data shown are expressed as means \pm SEMs. Data were evaluated by one-way analysis of variance and further evaluated with the Bonferroni test for multiple comparisons using GraphPad Prism Software (CA, USA). A Student's *t* test was also used to determine significance when applicable. *P*-values lower than 0.05 were considered to be significant.

Results

Characterization of iPSC-MSCs

Using a modification of previously described protocols^{22,24}, three human iPSC-MSC clones were successfully generated from iPSCs reprogramed from amniocytes. The iPSC-MSCs displayed a fibroblastic morphology, which was distinct from their parent iPSCs but similar to BM-MSCs (Fig. 1(a)). Flow cytometry analysis showed that iPSC-MSCs expressed surface markers typical of adult MSCs³⁶, including CD105, CD73, CD90, CD166, CD146 and CD44, but did not express CD34 and CD45 (Fig. 1(b)). Trilineage differentiation assays demonstrated that iPSC-MSCs had the ability to differentiate along the chondrogenic, osteoblastic or adipocytic pathways when cultured in the appropriate conditions (Fig. 1(c)).

Dex had no Effects on the Immunoinhibitory of iPSC-MSCs on PBMC Proliferation

Previously, we have demonstrated that iPSC-MSCs have a potent ability to inhibit lymphocytes proliferation²⁰. However, whether Dex affects this ability remains unclear.

Therefore, we employed a co-culture system of lymphocyte proliferation induced by anti-CD3/CD28 stimulation to examine the effect of Dex on the immunoinhibitory function of iPSC-MSCs. PBMCs were separated from healthy volunteers' blood samples and stimulated with anti-CD3/CD28 in the absence or presence of iPSC-MSCs and Dex. Consistent with our previous study²⁰, iPSC-MSCs alone significantly inhibited PBMC proliferation at a ratio of 1:10 (Fig. 2(a), upper panel, and Fig. 2(b), *P* < 0.001). As expected, Dex alone showed a dose-dependent inhibition of PBMCs proliferation (Fig. 2(a), lower panel, and Fig. 2(c), *P* < 0.01 or 0.001). After iPSC-MSCs were added with Dex with a graded concentration from 10 ng/ml to 100 μ g/ml, the inhibition of PBMC proliferation was almost invariably similar to those when iPSC-MSCs were added alone (Fig. 2(a), upper and middle panel, and Fig. 2(d), *P* > 0.05), suggesting Dex does not affect the immunoinhibitory effects of iPSC-MSCs on PBMC proliferation.

Additionally, we analyzed whether IDO was involved in the inhibitory effects of iPSC-MSCs on PBMC proliferation. However, we did not observe any difference in IDO expression in iPSC-MSCs after co-culture with PBMCs in the presence or absence of Dex (Supplementary Fig. 1).

Dex had no Effects on the Immunomodulation of iPSC-MSCs on the Local Inflammation in CHS Model

We previously found that iPSC-MSCs could suppress Th2 response in a mouse model of allergic inflammation²¹. To determine whether Dex influences the immunomodulatory function of iPSC-MSCs in allergic inflammation, a mouse model of CHS, in which Th2-like reaction is involved, was developed (Fig. 3(a))³⁷. Firstly, we investigated whether Dex treatment affects the iPSC-MSC inhibition of the lymph node's weight. As expected, there was a significant enhancement of lymph node's weight in CHS models compared with control mice (Fig. 3(b)). Mice with the treatment of iPSC-MSCs or Dex alone had a marked reduction of lymph node weight (Fig. 3(b)). Interestingly, there were no differences in lymph node weight between mice with the treatment of iPSC-MSCs alone and iPSC-MSCs combined with Dex (Fig. 4(b)). These data suggested that simultaneous treatment with Dex does not affect the immunomodulation of iPSC-MSCs on the inflammation in regional draining lymph nodes in the CHS model.

Next, we qualitatively and quantitatively compared the effects of iPSC-MSCs with or without Dex treatment on the histopathology of the external ear. The results showed that there was a significant infiltration of inflammatory cells in the external ear of CHS mice, and the appearance of the ear was redder and more swollen than the control mice (Fig. 3(c–d)). As expected, both iPSC-MSCs and Dex treatment alone showed significant attenuation of the external ear inflammation, including swelling, inflammatory infiltration and thickness of the external ear compared with CHS mice (Fig. 3(c–d)). Interestingly, Dex treatment alone showed a stronger immunoinhibitory effect on the ear thickness of

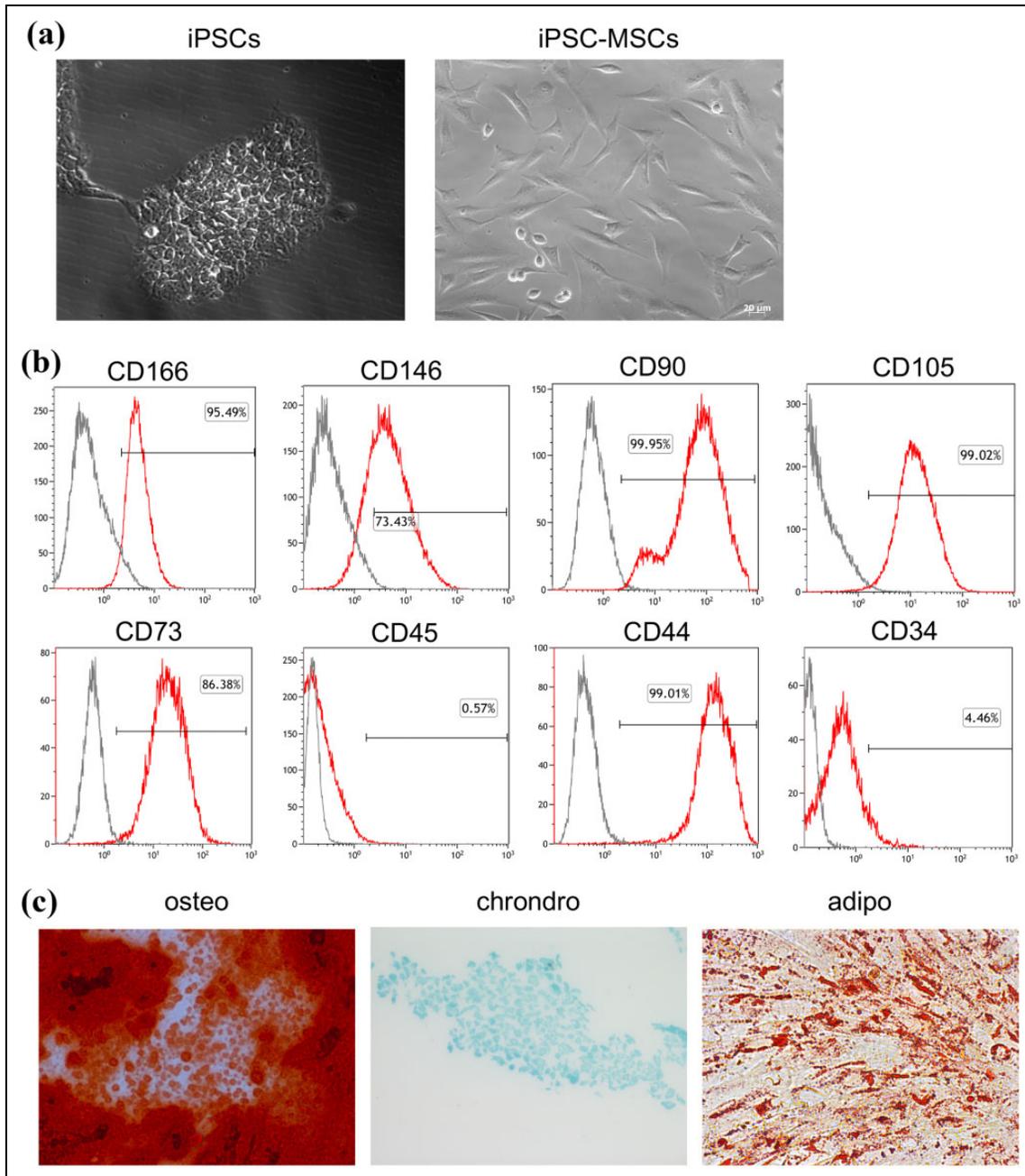


Fig. 1. Characterization of iPSC-MSCs. (a) Morphology of iPSCs and iPSC-MSCs (P4). Scale bar = 20 μ m (b) Surface antigen profiling by FACS in iPSC-MSCs (P4) cultures for CD166, CD146, CD90, CD105, CD73, CD45, CD44 and CD34. Data shown are representative of three independent experiments. Gray lines indicate isotype controls, and red lines represent the marker indicated. (c) Multipotent differentiation potential of iPSC-MSCs. Alizarin red staining for osteogenesis (left); Alcian blue staining for chondrogenesis (middle); and Oil Red O staining for adipogenesis (right). Images are representative of 3–4 independent experiments. (magnification, \times 200). FACS: fluorescent-activated cell sorting; iPSC: induced pluripotent stem cell; iPSC-MSC: induced pluripotent stem cell-derived mesenchymal stem cell.

CHS mice compared with those of iPSC-MSCs alone or combined with Dex (Fig. 3(d)). However, there was no significant difference between iPSC-MSCs alone and iPSC-MSCs combined with Dex (Fig. 3(d)), suggesting that Dex had no effects on the immunomodulation of iPSC-MSCs on the inflammation in CHS mice. Next, we measured the IL-6

and IL-10 levels in the serum of CHS mice. Unexpectedly, the results showed that there was no significant difference in IL-6 and IL-10 levels among the five groups (Supplementary Fig. 2). Taken together, these findings suggested that Dex did not affect the immunomodulation of iPSC-MSCs on the local inflammation in the CHS model.

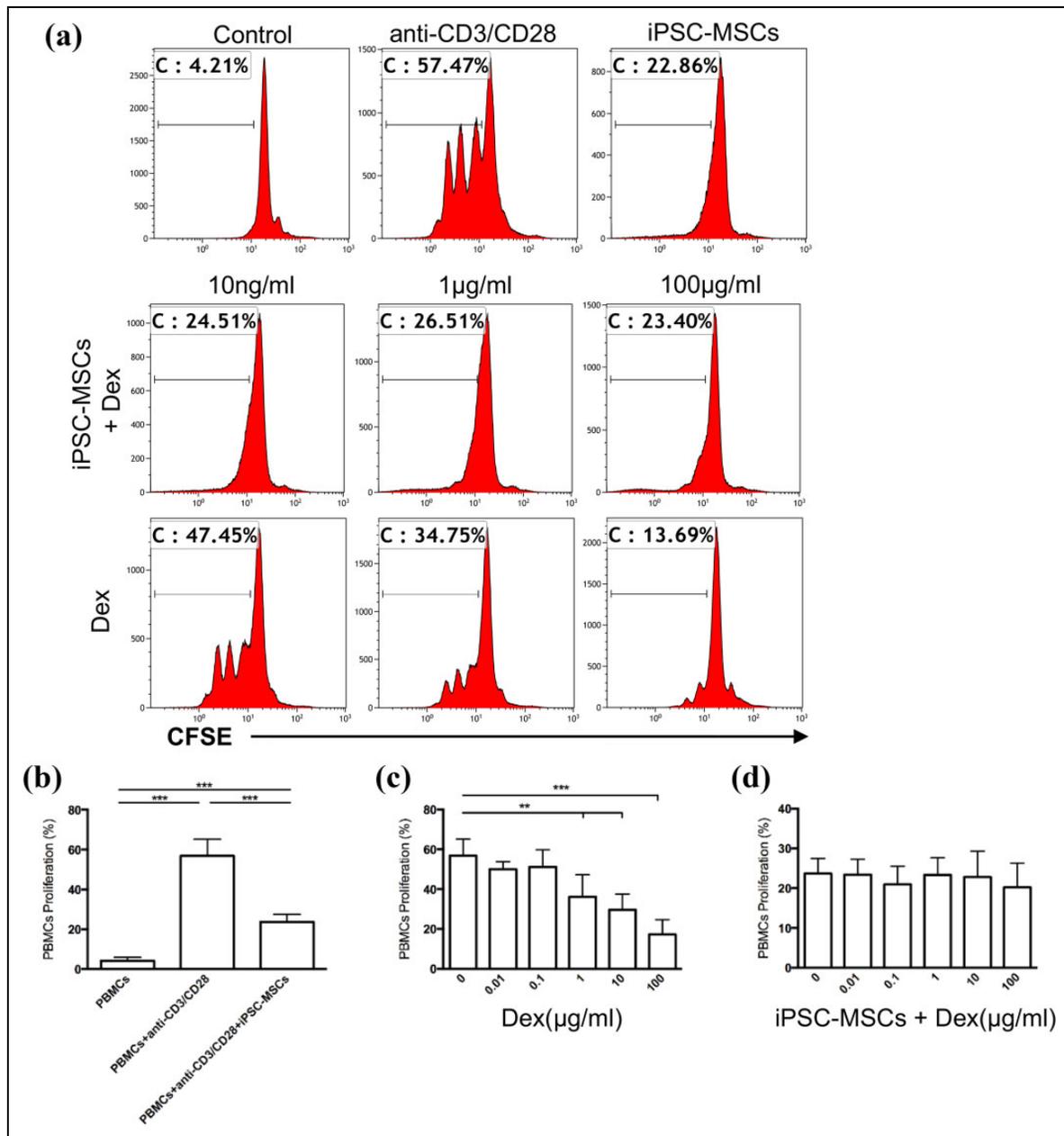


Fig. 2. Dex had no effect on the immunoinhibition of iPSC-MSCs on PBMC proliferation in vitro. PBMCs were labeled with CFSE prior to culture and stimulated by anti-CD3/CD28 with graded concentrations of Dex in the presence or absence of iPSC-MSCs. (a) The immunoinhibitory effect of iPSC-MSCs (upper panel), the combination of iPSC-MSCs and Dex (middle panel) and Dex (lower panel) on the proliferation of PBMCs. Histograms are representative of three independent experiments. The numbers in the plots indicate the percentage of CFSE-positive cells. (b–d) Quantitative analysis for the proliferation of PBMCs. Results are expressed as means \pm SEMs for $n = 3$ measured in duplicate. $*p < 0.01$, $***p < 0.001$.

CFSE: carboxyfluorescein succinimidyl ester; Dex, dexamethasone; iPSC-MSC: induced pluripotent stem cell-derived mesenchymal stem cell; PBMC: peripheral blood mononuclear cell.

Co-administration of iPSC-MSCs with Dex Decreased Local Expression of IFN- γ and TNF- α in the CHS Model

After 2 days from challenge in CHS mice, the ear tissues were harvested to determine the expressions of IFN- γ and

TNF- α by western blot analysis. CHS mice showed increased expression of IFN- γ and TNF- α compared with those of control mice (Fig. 4). Dex alone decreased the expression of IFN- γ and TNF- α in the ears of CHS mice. Treatment of iPSC-MSCs alone showed no significant effect on both IFN- γ and TNF- α expression. The levels of IFN- γ

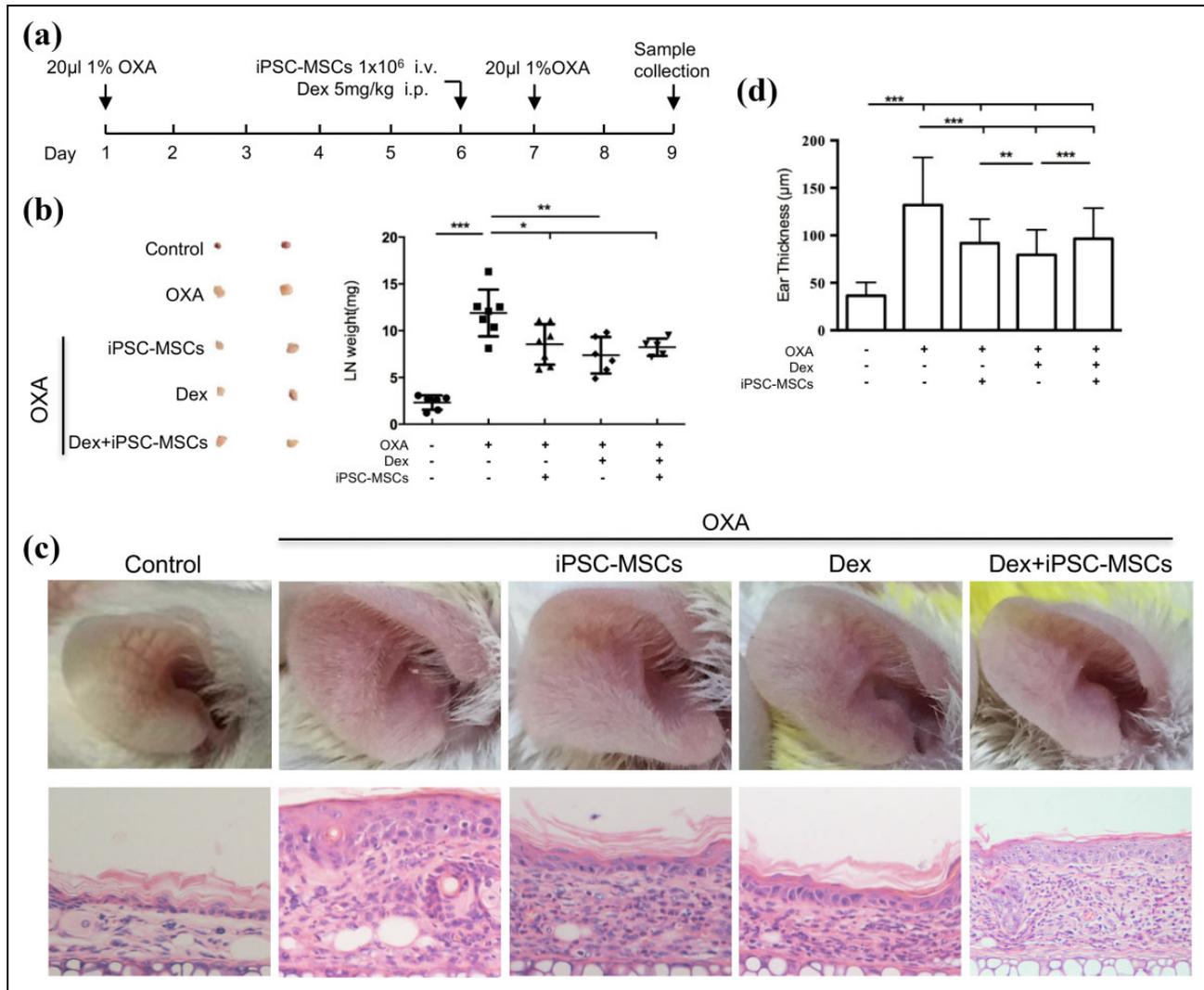


Fig. 3. Dex had no effect on the inhibition of iPSC-MSCs on the local inflammation in the CHS model. (a) The experimental protocol used to examine the effect of iPSC-MSCs and Dex. (b) The effect of iPSC-MSCs and Dex on the weight of draining lymph nodes. Representative results (left) and quantification of the size of draining lymph nodes (right). (c) Representative photomicrographs of H&E stained ear sections. (magnification, $\times 400$). (d) Statistical analysis for ear thickness. Data are expressed as means \pm SEMs. For each experiment, 5–8 animals were evaluated for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CHS: contact hypersensitivity; Dex: dexamethasone; H&E: hematoxylin and eosin; i.p.: intraperitoneal injection; i.v.: intravenous injection; iPSC-MSC: induced pluripotent stem cell-derived mesenchymal stem cell; LN: lymph node; OXA: oxazolone.

and $\text{TNF-}\alpha$ in the ears of CHS mice co-administered with iPSC-MSCs and Dex were lower than those of iPSC-MSCs administered alone, but similar to Dex alone, suggesting Dex could inhibit the production of $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$ in the CHS model independent of iPSC-MSC transplantation.

Dex did Not Affect the Immunomodulatory Effect of iPSC-MSCs on Allergic Airway Inflammation in Mice

To further confirm the effects of Dex on the immunomodulatory effect of iPSC-MSCs in vivo, we developed a mouse model of OVA-induced allergic airway inflammation. OVA-sensitized mice developed a significant pulmonary

inflammation as evidenced by higher bronchial and vascular scores in lung histology (Fig. 5(a) and (b)), and increased numbers of total inflammatory cells and eosinophils in BALF (Fig. 5(c) and (d)) compared with those in PBS-sensitized mice. In contrast, iPSC-MSC and Dex treatment, alone and combined, exhibited a significant reduction in pulmonary inflammation compared with those in OVA-sensitized mice treated with PBS (Fig. 5(a–d)). Furthermore, the mice treated with iPSC-MSCs combined with Dex showed a similar extent of reduction in pulmonary inflammation as those treated with iPSC-MSCs or Dex alone. This suggested that Dex had no effects on the immunomodulation of iPSC-MSCs on allergic airway inflammation.

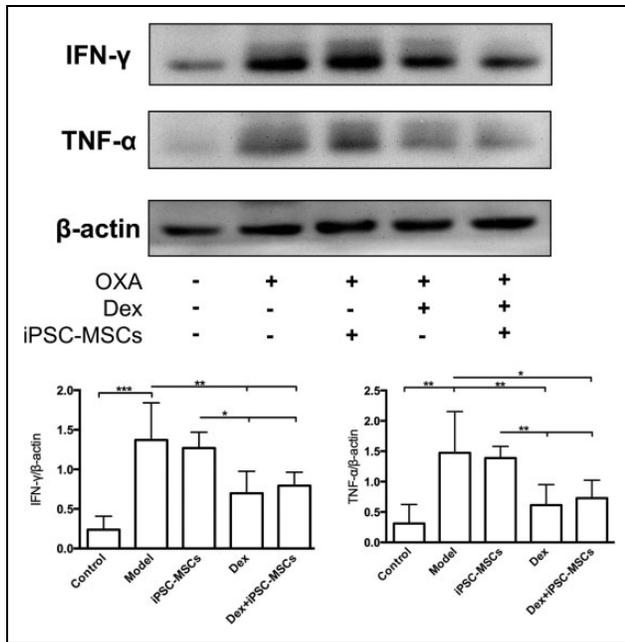


Fig. 4. Co-administration of iPSC-MSCs with Dex decreased local levels of IFN- γ and TNF- α in a CHS model. The effect of iPSC-MSCs and Dex on the levels of IFN- γ and TNF- α in the ears was determined by western blot. The results represent three independent experiments, 5–8 animals were evaluated for each group. Data are expressed as means \pm SEMs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CHS: contact hypersensitivity; Dex: dexamethasone; IFN: interferon; iPSC-MSC: induced pluripotent stem cell-derived mesenchymal stem cell; OXA: oxazolone; TNF: tumor necrosis factor.

Discussion

The iPSC-MSCs have advantages over BM-MSCs, including longer lifespan, various sources and easier *in vitro* expansion, which makes it an important source of MSCs^{17,38}. We have successfully derived multipotent MSCs from human iPSCs and demonstrated that human iPSC-MSCs are capable of immunomodulation, such as inhibiting lymphocyte proliferation and suppressing Th2 cytokines including IL-4, IL-5, or IL-13 in a mouse model of allergic airway inflammation^{20,21}, suggesting the potential clinical application of iPSC-MSCs for the treatment of allergic diseases. Glucocorticoids are used commonly to treat allergic diseases. Therefore, the interaction between glucocorticoids and iPSC-MSCs need to be determined before clinical application^{39,40}. In the present study, we have observed that simultaneous treatment with iPSC-MSCs and Dex showed a comparable immunoinhibitory effects on PBMCs proliferation induced by anti-CD3/CD28 *in vitro*, and local inflammation of the ear in a mouse model of CHS, as well as OVA-induced allergic airway inflammation in mice, which were similar to those treated with iPSC-MSCs alone, suggesting that Dex does not affect the immunoinhibitory effects of iPSC-MSCs.

In line with our previous studies^{20,21}, iPSC-MSCs were successfully induced from amniocyte-derived iPSCs and

were phenotypically and functionally characterized. iPSC-MSCs had the ability to differentiate into adipocytes, osteoblasts and chondrocytes. We also identified that iPSC-MSCs inhibited anti-CD3/CD28-induced lymphocyte proliferation *in vitro*, reduced local inflammation in the ears of CHS mice, as indicated by reduction of gross lymph node weight, inflammatory infiltration and the thickness of the external ear, and decreased allergic airway inflammation in mice as evidenced by lower inflammation scores in the lung and fewer inflammatory cells and eosinophils in BALF. In terms of the mechanisms underlying the action of iPSC-MSCs, we recently reported that iPSC-MSCs expressed a high level of IL-10, an important anti-inflammatory cytokine, to inhibit monocyte differentiation into dendritic cells *in vitro*⁴¹. Here we did not observe a significant difference in IL-10 levels in blood between mice treated with and without iPSC-MSCs. Additionally, no difference was observed for IDO expression in iPSC-MSCs after co-culture with PBMCs *in vitro*. These results suggested that there may be other mechanisms involved in the iPSC-MSC action in the present study.

There have been several studies investigating the effects of immunosuppressive drugs on the viability, proliferation, differentiation and function of MSCs. Mycophenolic acid, a cell cycle inhibitor, and rapamycin, an mTOR inhibitor, have been shown to suppress MSC proliferation at therapeutic doses⁴². High-dose tacrolimus, a calcineurin inhibitor, induced toxicity in adult MSCs⁴², whereas MSCs combined with low-dose tacrolimus was shown to be as effective as standard dose tacrolimus in maintaining graft survival after kidney transplantation⁴³, supporting the simultaneous application of MSCs and low-dose tacrolimus.

Only a few studies have investigated the effects of steroids on the immunomodulatory function of MSCs. One study reported that Dex did not antagonize the inhibitory effect of human BM-MSCs in a mixed lymphocyte response³². However, a recent study showed that Dex significantly eliminated the immunosuppressive effects of BM-MSCs on anti-CD3-activated T-cell proliferation *in vitro* and in a mouse model of liver fibrosis³³, in which Dex inhibited the expression of inducible nitric oxide synthase by preventing STAT1 phosphorylation, thus suggesting that concurrent application of MSCs with steroids should be avoided in clinical settings. However, our findings have shown that Dex had no effects on the immunosuppressive effects of iPSC-MSCs on lymphocyte proliferation, allergic diseases of CHS or airway inflammation. It supported the feasibility of concomitant application of iPSC-MSCs with steroids in some clinical settings. Or sometimes, concomitant application of iPSC-MSCs with a low dosage of steroid may have better results compared with a high dosage of steroid. Of course, the discrepancy underlying the interaction between steroids and adult MSCs or iPSC-MSCs should be further investigated.

We observed that the combination of iPSC-MSCs and Dex had less effect on decreasing ear thickness compared with Dex treatment alone in CHS mice. However, no

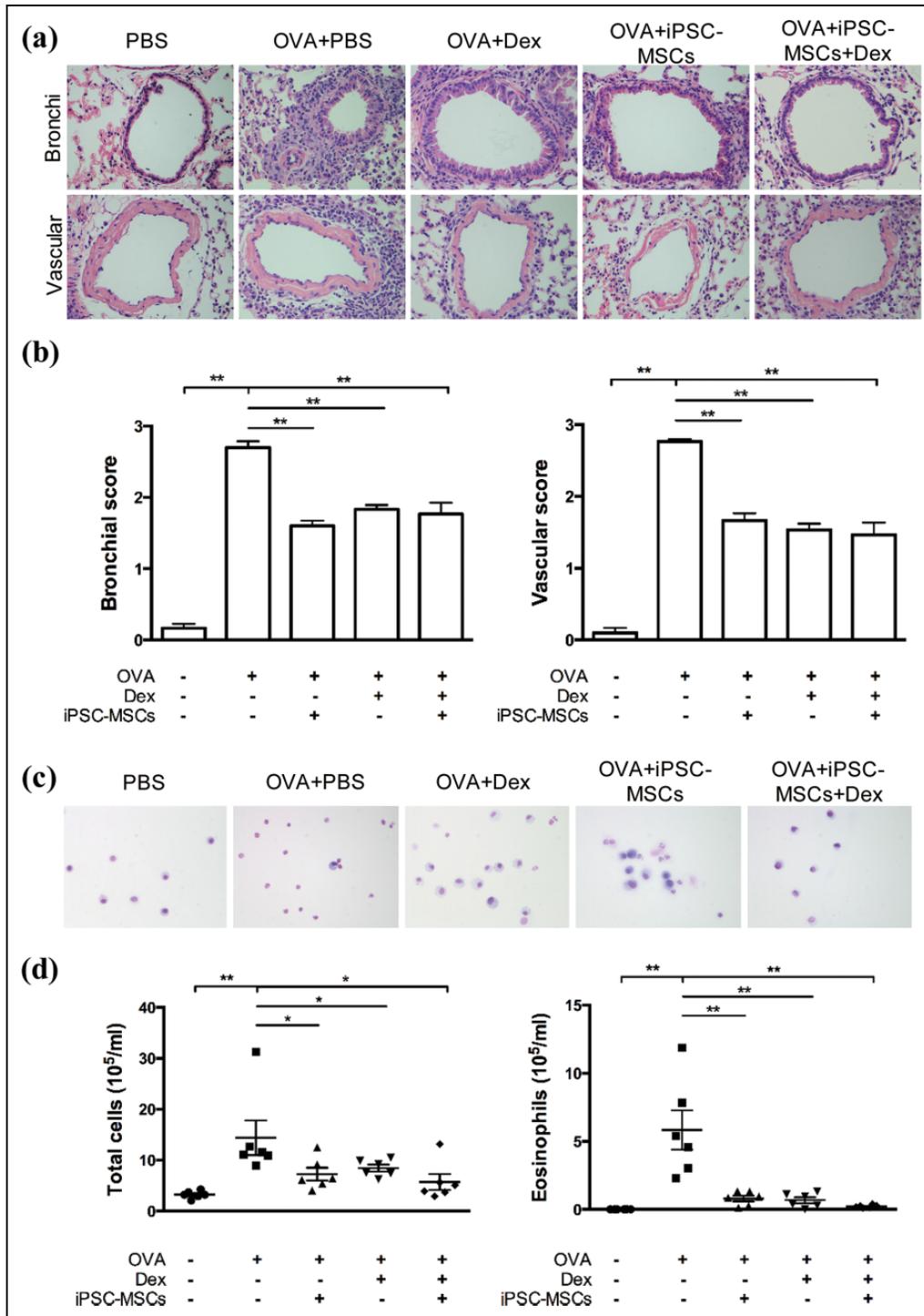


Fig. 5. Dex did not affect the immunomodulatory effect of iPSC-MSCs on allergic airway inflammation in mice. (a) Representative photomicrographs of H&E stained lung sections from each group (magnification, $\times 400$). (b) Statistical analysis for inflammation score in the lungs. (c) Representative diff-quick staining of the inflammatory cells present in the BALF (magnification, $\times 400$). (d) Total cells and eosinophils counts in the BALF. The results represent three independent experiments, 4–6 animals were evaluated for each group. Data are expressed as means \pm SEMs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

BALF: bronchoalveolar lavage fluid, Dex: dexamethasone; H&E: hematoxylin and eosin; iPSC-MSC: induced pluripotent stem cell-derived mesenchymal stem cell; OVA: ovalbumin; PBS: phosphate-buffered saline.

significant difference was found for the IFN- γ and TNF- α levels in the ears of CHS mice, or the inflammation in the lungs in allergic mice between the combination of iPSC-MSCs and Dex and Dex alone. Anyway, we are unable to exclude a possibility that iPSC-MSCs might have a disruptive effect on the anti-inflammatory effects of Dex in some settings. Their interaction should be carefully addressed in specific settings. Fortunately, we found that although Dex was able to inhibit PBMC proliferation in a concentration-dependent manner (from 0.01 to 100 μ g/ml), it showed a consistent suppressive effect on PBMC proliferation when co-cultured with iPSC-MSCs, which was equivalent to that of iPSC-MSCs in the absence of Dex. It suggested good application of iPSC-MSCs combined with an even low dosage of steroid.

We acknowledge some limitations in our current study. Although we determined the effect of iPSC-MSCs combined with Dex on local inflammation in CHS mice, we did not evaluate the differential subsets of inflammatory cells, which should be further assessed by flow cytometry analysis. Secondly, we acknowledge the possibility that iPSC-MSCs might interrupt the inhibitory effect of Dex on lymphocyte proliferation and in CHS mice in our study, which should be further studied in glucocorticoid receptor knockout mice in the future.

In conclusion, to our knowledge, we provide the first preliminary evidence that Dex does not significantly affect the immunomodulatory function of iPSC-MSCs both in vitro and in vivo. It supports the feasibility of concomitant application of iPSC-MSCs with steroids in clinical settings.

Acknowledgments

Dan Wang and Yue-Qi Sun contributed equally for this article. Dan Wang and Yue-Qi Sun performed the cell culture, collection and assembly of data, data analysis, and prepared the manuscript; Wen-Xiang Gao and Dan Wang performed flow cytometry, western blot, and preparation of stem cells; Xing-Liang Fan edited the manuscript. Jian-Bo Shi and Qing-Ling Fu contributed to the conception and design of the study. Qing-Ling Fu wrote and revised the manuscript. All authors reviewed and approved the final manuscript.

Ethical Approval

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University (No. 2014-C-053 for human) and Institutional Animal Care and Use Committee Sun Yat-sen University, China (No. DB-16-0404 for animals).

Statement of Human and Animal Rights

All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee, Sun Yat-sen University.

Statement of Informed Consent

Human blood buffy coats from 'anonymous donors' were obtained from Guangzhou Blood and an exemption of the written informed consent was approved.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The study was supported by grants from the National Natural Science Foundation of China (No. 81500768, 81322012, 81373174, 81273272, 81470069, 81670902, 81770984) and the Science and Technology Foundation of Guangdong Province of China (No. 2015B020225001, 2016A020215049, 2016A030308017).

Supplemental Material

Supplemental material for this article is available online.

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