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REGULAR ARTICLE

Vascularized composite allograft rejection is delayed by infusion of IFN-γ-conditioned BMSCs through upregulating PD-L1

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Abstract

Mesenchymal stromal cells (MSCs) have been applied in prevention from allograft rejection based on their immunomodulatory effects. However, conflicting results have been presented among recent studies, for which one possibility being acknowledged is that the exact effect is determined by the microenvironment when MSCs are applied in vivo. Using a hind limb composite tissue allograft model, we investigate the influence of IFN-γ-preconditioning on the immunomodulatory effects of MSCs and the subsequent allograft survival. Firstly, different doses of IFN-γ were respectively used to incubate with bone marrow–derived MSCs (BMSCs). We found that IFN-γ altered the expression of PD-L1, a major suppressor gene in the immune system during allograft rejection, in a strictly dose-dependent manner in BMSCs. Ten nanograms per milliliter IFN-γ-incubated BMSCs significantly stimulated PD-L1 expression and suppressed T cell proliferation and differentiation, while 50 ng/mL IFN-γ-incubated BMSCs sharply reduced PD-L1 expression. Moreover, we observed that, in contrast to the naive BMSC transplantation group, BMSCs pre-conditioned with 10 ng/mL IFN-γ (BMSCs-IFN-γ) significantly delayed the allograft rejection in vivo. In vitro mixed lymphocyte reaction (MLR) indicated that BMSCs-IFN-γ inhibited T lymphocyte proliferation and activation via PD-L1. Moreover, BMSCs-IFN-γ did not influence the proliferation and activation of T lymphocytes when PD-L1 protein was neutralized by the PD-L1 antibody. These data collectively reveal a role of recipient ongoing immune microenviroment in BMSC-based immunesuppressive therapy.

Keywords Allograft rejection · IFN-γ · BMSC · PD-L1 · T cell proliferation and activation

Introduction

During the past decade, human composite tissue allogeneic transplantation (CTA) has triggered pronounced interest for restoring function, appearance and dignity to patients experiencing tissue defects of various reasons. However, the concomitant side effects of life-long immunosuppressive drug administration aiming to prevent graft rejection have raised misgivings whether the benefits of CTA outweigh the risks. The increased risk for opportunistic infections and malignancies hamper the clinical application of a life-long immunosuppressive drug. Therefore, it is urgent to explore reliable means to improve graft outcomes while minimizing requirements for lifelong immunosuppression.

Mesenchymal stromal cells (MSCs), besides their multilineage-differentiation capabilities, have been suggested to be immunomodulatory via an unknown mechanism involving cell-to-cell contact or the release of soluble factors, such as nitric oxide and indoleamine-2,3-dioxygenase (IDO) (Crowder et al. 2013). Based on this, they have been used to control a series of experimental immune-mediated disorders and even some clinical cases of graft-versus-host diseases (GVHDs) (Tolar et al. 2013). Their roles in improving outcomes of allogeneic grafts including skin, solid organs and CTA were also investigated. In most of the studies, a

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prolonged allograft survival was observed after MSC delivery, potentiating the clinical application of these cells in transplantation indications (Obermajer et al. 2014). However, with the experimental body increasing, some results demonstrated that MSCs only modestly prolong graft survival or even fail to prevent graft rejection (Baron and Storb 2012; Mielcarek et al. 2011), which implicated an unconvincing therapeutic efficacy of bone marrow–derived MSCs (BMSCs) and in turn impede its widespread application. Numerous explanations have been proposed to account for the discrepancies, which include infusion approach/route, infusion timing, condition of the MSCs as well as the microenvironment that MSCs reside after in vivo application (Collins et al. 2014; Baron and Storb 2012). Therefore, the feasibility of BMSC application in tissue transplantation needs further exploration, while revealing the specific mechanism of BMSC regulation contributes to the application.

It is widely recognized that key pro-inflammatory cytokines incubated with MSCs in vitro could substantially affect MSC-mediated immunosuppression effect (Pourgholaminejad 2016). What is more, exposure to a pro-inflammatory environment is reported to underlie the therapeutic effects of MSC cells (Treacy et al. 2014; Yang et al. 2016). It was demonstrated that when incubated with interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), BMSCs are more potent immunosuppressive than unincubated BMSCs (Takeshita et al. 1998; Yang et al. 2016). By expanding the circulating population of regulatory T (Treg) cells, BMSCs possess immunosuppression properties (Tasso et al. 2012). However, the way BMSCs regulate Treg cells is unclear. Recent studies also indicated that BMSCs require a sufficient, ongoing immune response to exert their immunosuppressive function in a fully allogeneic heart transplantation model. These studies implied that the discrepancies of the immunosuppressive function observed previously and the mechanism BMSCs employed may be unraveled when we understand the local environmental influences on the immunomodulatory effect of MSCs.

Using a hind limb composite tissue allograft model, we investigated the impact of IFNγ-preconditioning on the immunomodulatory effects of BMSCs and the subsequent allograft survival. Different doses of IFN-γ were respectively used to incubate with BMSCs and an optimal concentration was selected and applied in the following allograft rejection therapy with BMSC transplantation. We found that IFN-γ treatment altered the expression of programmed death-ligand 1 (PD-L1), a major suppressor gene during allograft rejection, in a strictly dose-dependent manner. Then, we observed that, in contrast to the naive BMSC transplantation group that modestly prolonged the allograft survival, BMSCs preconditioned with 10 ng/mL IFN-γ (BMSCs-IFN-γ) significantly delayed the allograft rejection in vivo. Moreover, in vitro mixed lymphocyte reaction (MLR) was used to investigate the underlying mechanism from which BMSCs-IFN-γ regulated the allograft rejection.

Materials and methods

Animals

Brown-Norway (BN, RT1n) rats and Lewis (LEW, RT11) rats were purchased from Vital River, Inc. (Beijing, China). All rats were housed in a pathogen-free facility individually with a 12-h dark/12-h light cycle and fed with a standard laboratory diet. All rats received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. The research protocol was approved by the Experimental Animal Committee of the Fourth Military Medical University.

Isolation, culture and characterization of BMSCs

Two-week-old BN rat femurs and tibias were dissected out and cut into crumbs in Dulbecco's modified Eagle's medium (DMEM; Gibco, US) containing 10% FBS (Sigma Co., St. Louis, MO) and 1% penicillin/streptomycin (P/S). The bone crumbs were repeatedly pipetted to isolate cells from the crumbs. After 2-min natural sedimentation of the crumbs, the suspension was transferred to a 175-cm^2 flask without incorporating the crumbs and cultured in 15 mL of the culture medium at 37 °C in 5% CO_2. After 48 h of incubation, the nonadherent cells were removed and the remaining adherent cells were cultured with a regular exchange of the culture medium every 3 days. When 80 to 90% confluency was achieved, adherent cells were subcultured at 1:2 split ratio. Surface antigen analysis and differentiation assay were performed at passage 3. BMSCs from passage 4 to 7 were used for the subsequent experiments.

Surface antigen analysis for BMSCs was performed by using the following fluorescein isothiocyanate (FITC)-conjugated antibodies (BD Biosciences): CD29, CD90, CD44, CD73, CD105, CD166, CD45, CD14, CD31, CD34, CD45 HLA-ABC and HLA-DR. Cells (10^6) were washed in PBS containing 0.5% bovine serum albumin and labeled with above FITC-conjugated antibodies for 30 min on ice and then analyzed by using FACSCalibur (Becton Dickinson). The data were analyzed with FlowJo (TreeStar Inc. Ashland). To induce adipogenic and osteogenic differentiation, BMSCs were cultured with corresponding induction medium and characterized according to a previous protocol [12].

BMSC conditioning

BMSCs (5×10^5) were seeded into six-well plates (Greiner bio-one) and cultured with DMEM containing 10% FBS and 1% penicillin/streptomycin. On reaching 70 to 80% confluency, inflammatory cytokine IFN-γ (Sigma, St. Louie, USA) was added to the medium at final concentrations of 5, 10, 30 and 50 ng/mL for 24 h. Conditioned BMSCs were

further analyzed by AnnexinV-FITC apoptosis detection kit I. The mRNA and protein levels of VCAM-1, CTLA-4 and PD-L1 were also detected.

One-way MLR/proliferation assays

T-cells from spleens of donor and recipient rats were used as stimulator cells and responder cells, respectively. Responder T cells were labeled with CFSE according to a previous publication. In brief, spleen from Lewis rat was harvested and mashed into single cell suspension, mononuclear cells were harvested by 1.077 g/mL Ficoll density gradient solution and adjusted to 10^7/mL in PBS containing 5% FBS, followed by administration of CFSE at a final concentration of 5 μM. After incubation for 10 min at room temperature, the reaction was stopped with 10 volumes of 5% FBS-containing RPMI 1640 followed by three times of extensive washing. Stimulator T cells were isolated in the same way followed by mitomycin C inactivation at 30 μg/mL, 37 °C for 30 min. One hundred thousand CFSE-labeling responder T cells and equal numbers of inactivated stimulator T cells were then cocultured in 200 μL of RPMI 1640 medium containing 10% heat-inactivated FBS in 96-well flat-bottomed plates in a humidified 37 °C, 5% CO_2 incubation. In some experiments, 2×10^4 (T/BMSCs = 10:1) Mitomycin C inactivated BMSCs or IFN-γ incubated BMSCs were pre-plated on the 96-well in the presence or absence of anti-rat-IFN-γ mAb (Abcam, 1:100) or anti-rat-PD-L1 mAb (Abcam, 1:100) before the cocultures.

Flow cytometry

For MSCs surface phenotypic analysis, 1×10^6 cells of passage 3 were washed in PBS containing 0.5% bovine serum albumin and labeled with the above fluorescently conjugated antibodies for 30 min on ice and then analyzed by using FACSCalibur (Becton Dickinson) and data were analyzed with FlowJo V7.1.3 software (TreeStar Inc. Ashland, Ore). Washed afterward, FITC-conjugated anti-rat PD-L1 mAb was used for BMSCs immunoregulatory associated candidate molecules after IFN-γ conditioning. To obtain an insight into the ability of IFN-γ conditioned BMSCs for regulatory T cell induction (Tregs), lymphocytes from the MLR system were recovered and magnetically isolated using a $CD4^+CD25^+$ T cell isolation kit in accordance with the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated $CD4^+CD25^+$ T cells were then fixed and permeabilized with saponin 20% (Sigma-Aldrich) for 30 min at 37 °C, followed by Anti-Mouse/Rat Foxp3 PE (Cat.12577382; eBioscience) staining. Cells were quantified by flow cytometry and analyzed with Expo 32 ADC analysis software.

Detection of T cell proliferation

T cell proliferation was characterized by sequential halving of CFSE fluorescence. The percentage of CFSE divided cells was calculated by (no. of precursors that proliferated 1–6/no. of total precursors 0–6). The stimulation index (SI) was determined by assessing the ratio of the counts per minute (c.p.m.) generated in response to each stimulator to that of the culture medium alone (in the absence of stimulators). All data were analyzed with FlowJo software/Modfit Software.

Hind limb CTA and MSC delivery

A circumferential skin incision was made in the middle thigh of the recipient's hind limb, then the femoral artery, vein and nerve were dissected, clamped and transected proximal to the superficial epigastric artery, the limb was amputated at the mid-femoral level. The donor limb was prepared in the same way. Donor limb was then attached to the recipient's femoral stump with 1.5 mm Kirschner wire. After femoral vessels of the donor and recipient were anastomosed and ascertained to be patient, the incision was closed. Three milliliters of N.S. was injected intraperitoneally to compensate for perioperative fluid loss. Directly after the operation, a total of 2×10^6 BMSCs or IFN-γ incubated BMSCs in 1 mL physiology saline were injected into recipient rats through a tail vein for longer than 3 min. Control rats received an equal volume of physiology saline. After the operation, manifestations of immunological rejection were monitored. Graft rejection was defined as "the first change in the skin after erythema and edema but before progressing toward epidermolysis, desquamation, or even eschar formation."

Real-time PCR

Real-time PCR was performed in the Bio-Rad IQ^5 Real-Time System (Bio-Rad, Hercules, CA, USA) by using a SYBRH Premix Ex TaqTM II kit (TaKaRa, Dalian, China) in a 20 mL volume of the PCR reaction solution. The specific genes and their corresponding primers were designed and synthesized by GenScript Company (Nanjing, China). The expression of each gene in BMSCs cells was normalized with the housekeeping GAPDH using the $2^{-\Delta\Delta CT}$ method.

Western blotting

Fifty micrograms of protein lysates from each sample were separated by electrophoresis on 8% SDS-PAGE gels and then transferred to nitrocellulose (NC) membranes. After being blocked with 5% non-fat milk in 1×TBS-Tween buffer, the NC membranes were incubated overnight at 4 °C with the primary antibodies for rabbit anti-rat PD-L1 and rabbit anti-rat FoxP3 in 1×TBS-Tween. Immunoreactivity was detected

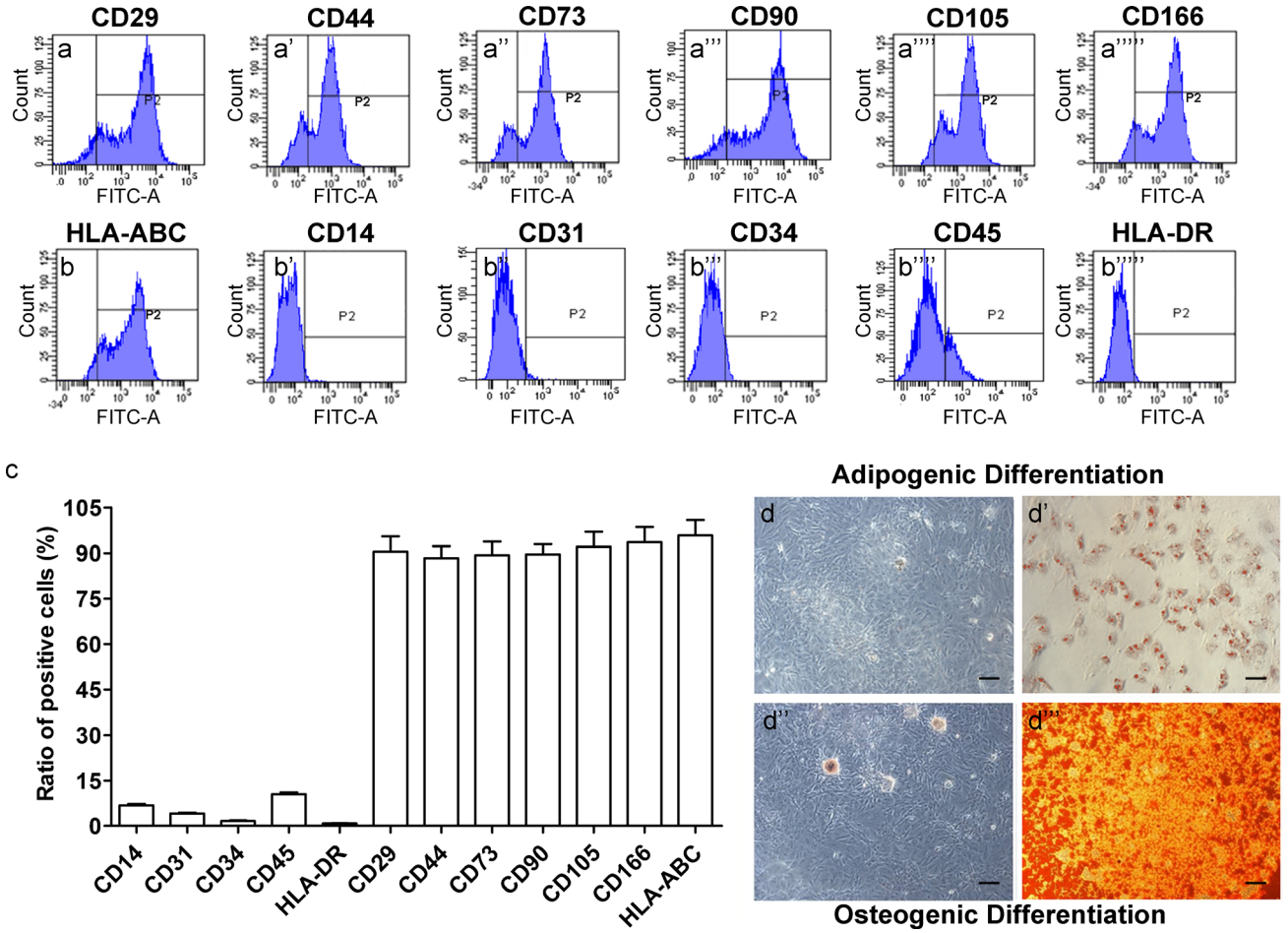

Fig. 1 Identification of rat BMSCs. (a, b) Surface antigen analysis for BMSCs using FITC-conjugated antibodies CD29 (a), CD44 (a'), CD73 (a"), CD90 (a"'), CD105 (a""), CD166 (a""'), HLA-ABC (b), CD14 (b'), CD31 (b"), CD34 (b"'), CD45 (b""), and HLA-DR (b""'). (c) Representative images of surface antigen analysis for BMSCs FITC-conjugated antibodies CD29, CD44, CD73, CD90, CD105, CD166, HLA-ABC, CD14, CD31, CD34, CD45 and HLA-DR. Adipogenic differentiation (d, d') and osteogenic differentiation (d", d"') of rat BMSCs. BMSCs were isolated from 2-week-old rat. BMSCs (10^6) were washed in PBS containing 0.5% bovine serum albumin and labeled with indicated FITC-conjugated antibodies for 30 min on ice and analyzed by using FACSCalibur. To induce adipogenic and osteogenic differentiation, BMSCs were cultured with corresponding induction medium and characterized (Oil Red O and Alizarin Red staining). Data are representative of results from four independent experiments. Scale bars 100 μm

by sequential incubation with HRP-conjugated goat anti-rabbit IgG (1:2000) and visualized by enhanced chemiluminescence system using FluorChem FC (Alpha Innotech). Quantitative analysis was performed using AlphaEase FC image process software (Alpha Innotech). In all samples, anti-rat-β-actin was used for normalization. For the detection of Foxp3 protein level, $CD4^+CD25^+$ T cells were isolated.

Statistical analysis

Data are reported as mean ± SEM. Comparisons of cell phenotype differences (FACS), anti-PD-L1 antibody levels (FACS, Western-blot), anti-FoxP3 antibody levels (Western-blot), T cell proliferations (MLR), as well as cytokine levels (ELISA) were performed using one-way ANOVA. Survival data were compared using Kaplan–Meier analysis and the log-rank test. All of the other data were analyzed by ANOVA. All statistical analyses were performed with SPSS (SPSS, Chicago, IL, USA) or GraphPad InStat (San Diego, CA, USA). $P < 0.05$ was considered significant.

Results

IFN-γ treatment altered the expression of PD-L1 in a strictly dose-dependent manner in BMSCs

Mouse BMSCs were isolated and cultured, exhibiting a typical spindle-shaped morphology and achieved 80–90% confluency within 3–4 days of culture when passaged at a ratio of 1:2. To identify their characteristics, cells of the three third passages were subjected to flow cytometry for analyzing the expression of a group of cell surface molecules. As reported, these cells were found to highly express (> 90%

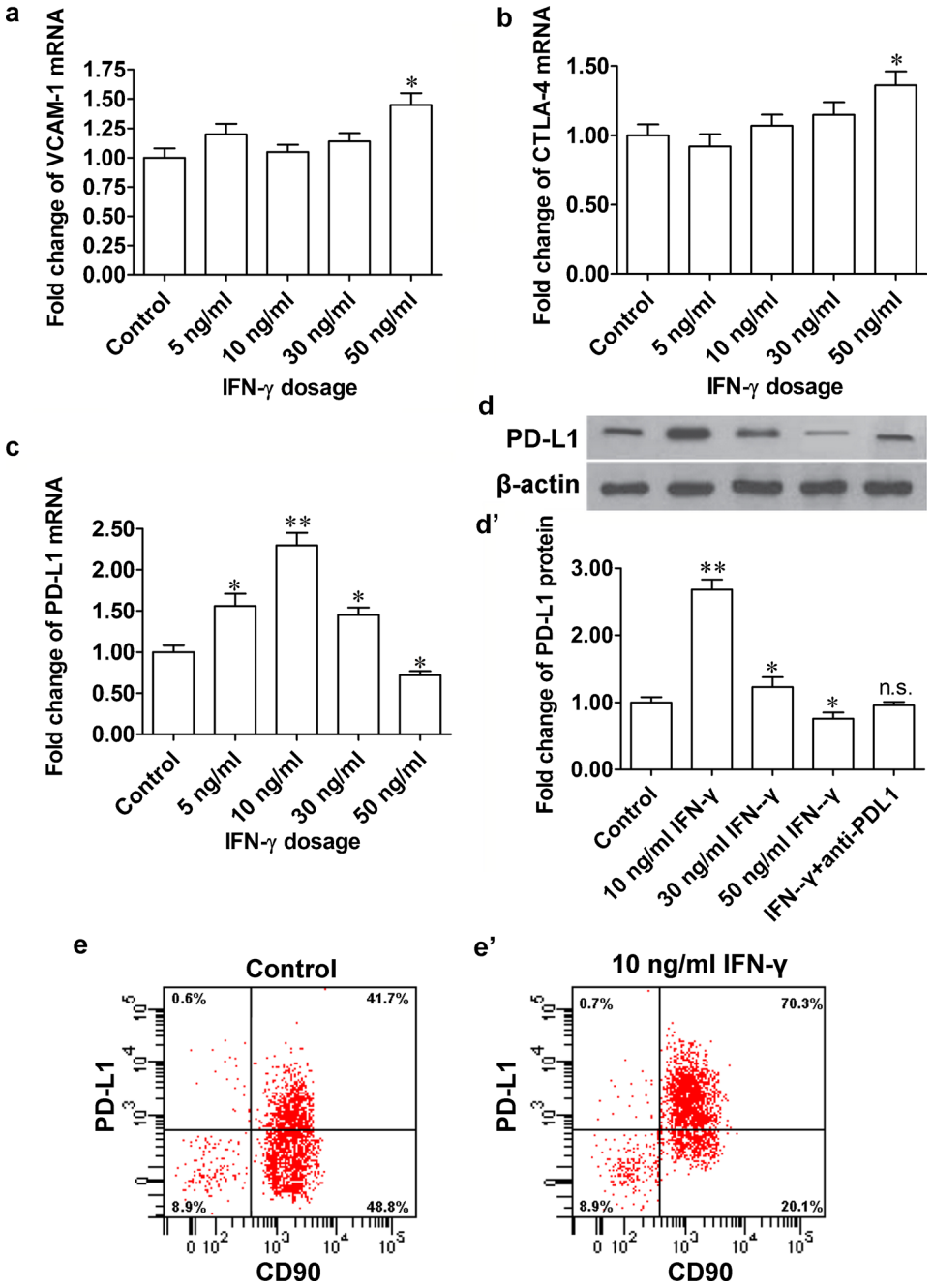

Fig. 2 IFN-γ incubation influenced the expression of PD-L1 in a dose-dependent manner in BMSCs. (a) IFN-γ incubation did not influence the expression of VCAM-1 in BMSCs unless its concentration was as high as 50 ng/mL. (b) IFN-γ incubation did not influence the expression of CTLA-4 in BMSCs unless its concentration was as high as 50 ng/mL. (c) IFN-γ incubation influenced the expression of PD-L1 mRNA in a dose-dependent manner in BMSCs. (d, d') IFN-γ incubation influenced the expression of PD-L1 protein in a dose-dependent manner in BMSCs. Five, 10, 30 and 50 ng/mL IFN-γ was used to incubate with BMSCs for 24 h. The mRNA levels of VCAM-1, CTLA-4 and PD-L1 in BMSCs were detected with qPCR and the protein level of PD-L1 was detected by analyzing with Western blotting. (e, e') The expression of PD-L1 together with BMSC markers CD90 were assessed by flow cytometry. *$P < 0.05$ compared with control (BMSCs without IFN-γ incubation). Data are representative of results from six independent experiments

positive) CD29, CD90, CD44, CD73, CD105, CD166 and HLA-ABC and weakly express (< 10% positive) CD45, CD14, CD31, CD34, CD45 and HLA-DR (Fig. 1a–c). We also utilized double staining (PE-CD73 and FITC-CD34) demonstrating that individual cells express CD73 but do not express CD34 (see supplement Fig. 1). When cultured with the adipogenic or osteogenic induction medium, almost 100% of cells underwent differentiation and displayed typical characteristics of adipocytes or osteoblasts determined by Oil Red O and Alizarin Red staining, respectively (Fig. 1d–d"').

Then, different doses of IFN-γ, ranging from 5 to 50 ng/mL, were respectively used to incubate with the BMSCs. The expression of membrane immune regulatory factors VCAM-1, CTLA-4 and PD-L1 were detected with qPCR or Western blotting and cell apoptosis was detected with flow cytometry. We observed that IFN-γ, at doses of 5~30 ng/mL, increased the expression of PD-L1 (Fig. 2c–d') but had no influence on the expression of VCAM-1 and CTLA-4 (Fig. 2a, b); 50 ng/mL of IFN-γ slightly increased the expression of VCAM-1 and CTLA-4 (Fig. 2a, b) and sharply reduced PD-L1 expression (Fig. 2c–d'). IFN-γ had the most increasing effect on PD-L1 expression at the dosage of 10 ng/mL (Fig. 2c–d'). Flow cytometry showed that the expression of PD-L1 is increased after 10 ng/mL of IFN-γ treatment (Fig. 2e, e'), indicating that the IFNγ-increased expression of PD-L1 is not due to the contaminating cells. In addition, 10 ng/mL had no significant influence on the cell death of BMSCs, while 50 ng/mL led to significant cell death (Fig. 3a–b"). Thus, 10 ng/mL of IFN-γ was used to condition the BMSCs in the following experiments.

IFN-γ-conditioned BMSCs inhibited T cell proliferation and differentiation via membrane molecule PD-L1

Ten nanograms per milliliter of IFN-γ conditioned BMSCs (BMSCs-IFN-γ) was used to incubate with T cells in an in vitro MLR system at a ratio of 1:10 (BMSCs/T cells). Compared with the naive BMSC group, BMSCs-IFN-γ significantly inhibited the proliferation of T cells (Fig. 4a–a"), manifested by a relatively high proportion of parental cells (Fig. 4b) and a sharply reduced proliferation index (Fig. 4c). From the results above, we realized that PD-L1 was the only membrane immune regulatory factor significantly changed after 10 ng/mL of IFN-γ and might be involved in the regulation of BMSCs-IFN-γ induced inactivation and proliferation suppression. To verify the exact role of PD-L1 in these processes, PD-L1 antibody was used to neutralize the PD-L1 expression during incubation of T cells with BMSCs-IFN-γ. BMSCs-IFN-γ did not affect the proliferation when PD-L1 was blocked by the neutralizing antibody (Fig. 4). For T cell differentiation, BMSCs-IFN-γ had a distinct suppressive effect on the stimulation index of T cells, while the suppression could be rescued when PD-L1 expression was blocked (Fig. 5a) and this function was further confirmed by Western blotting and flow cytometry analyses: the expression of FOXP3 and ratio of $FOXP3^+$ regulatory T cells, which are required in the dynamic regulation of human immune homeostasis, were reversed to a level more or less to the naïve BMSCs group (Fig. 5b–d). These data revealed that IFN-γ- conditioned BMSCs inhibited the T cell proliferation and activation and membrane molecule PD-L1 was required in this process.

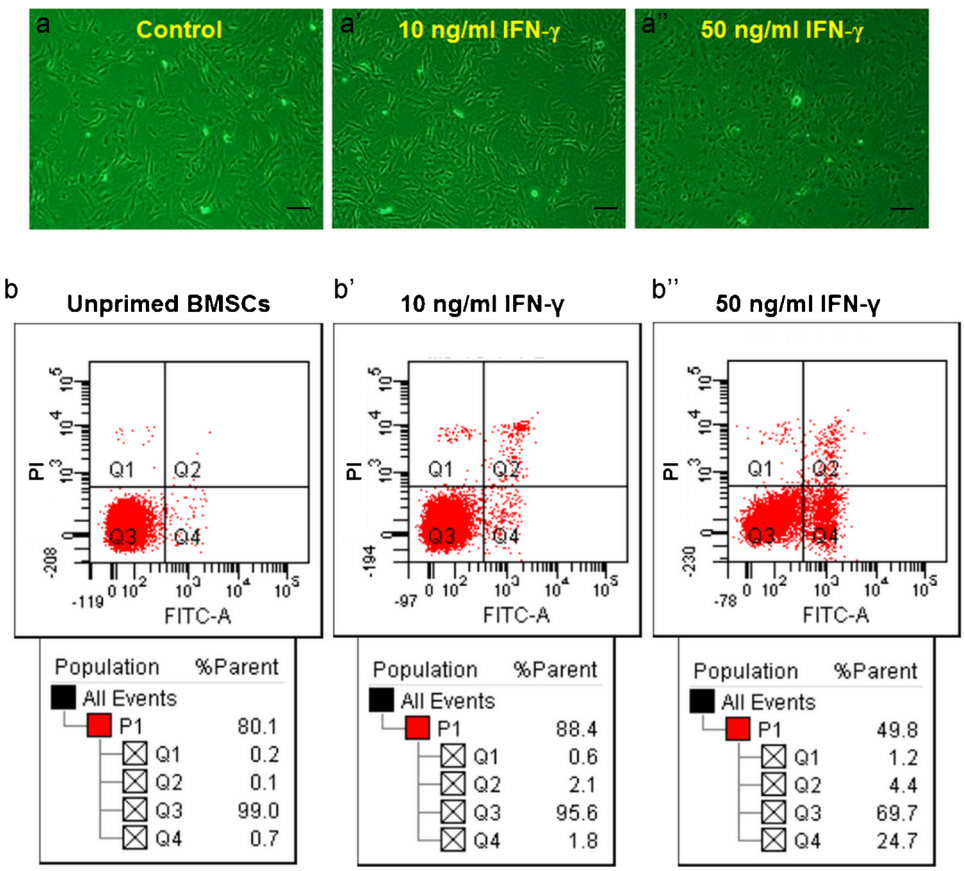

Fig. 3 High-dose IFN-γ induced cell death but low-dose IFN-γ had no influence on cell death of BMSCs. Representative images of BMSCs treated with (a', a") and without (a) IFN-γ incubation. (b–b") Statistical analysis for a–a"'. BMSCs (5×10^5) were seeded onto 6-well plates. On reaching 70 to 80% confluency, inflammatory cytokine IFN-γ was added to the medium at the final concentrations of 0, 10, and 50 ng/mL for 24 h. Images were captured and cell apoptosis was analyzed by AnnexinV-FITC apoptosis detection kit I. Data are representative of results from six independent experiments. Scale bars 100 μm

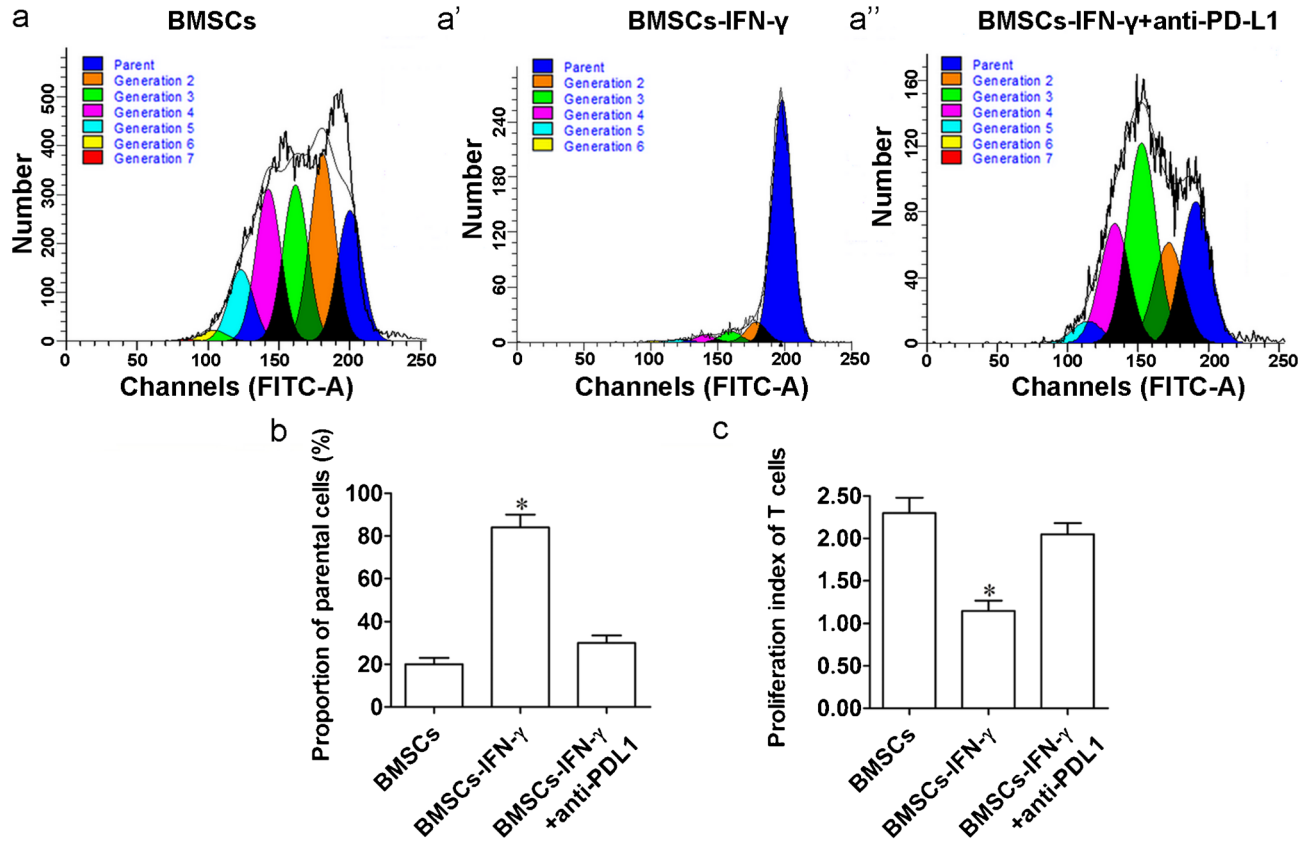

Fig. 4 Low-dose IFN-γ-conditioned BMSCs suppressed proliferation of T cells via PD-L1. (a–a") The proportion of T cells in every generation in each group sorted by flow cytometry. (b) The proportion of parental T cells in each group. (c) The proliferation index of T cells in each group. BMSCs were pre-incubated with 10 ng/mL IFN-γ or 10 ng/mL IFN-γ plus 0.5 ng/mL blocking antibody. Then, naive BMSCs or the conditioned BMSCs were used to incubate with total T cells in an in vitro MLR system. The proliferation of T cells was detected with sequential halving of CFSE fluorescence. The percentage of CFSE divided cells was calculated. Cell cycle was also detected and the proliferation index was calculated using the method: PI = (S + G2M)/(G0/1 + S + G2M). $*P < 0.05$. Data are representative of results from six independent experiments

IFN-γ-conditioned BMSCs but not naive BMSCs prolonged the survival time of the hind limb CTA

For a long time, BMSCs have been regarded to prolong allograft survival but there are also many studies demonstrating that BMSCs could accelerate rejection. A previous study demonstrated that isografts survived without signs of rejection (Wang et al. 2012). To obtain an insight into the effect of BMSCs for preventing hind limb CTA, we intraveneously infused BMSCs to the recipient directly after the transplantation. A daily monitor of rejection revealed that BMSCs hardly had an effect on the survival of hind limb CTA with the mean/average survival time similar to the control group (9 days vs 10 days), whereas, to our surprise, BMSCs incubated with 24 h of 10 ng/mL IFN-γ infusion showed delayed rejection response and prolonged survival postoperation (15 days, Fig. 6a–d). What is more, the spleen from the recipient rat of each group was collected and the percentage of Foxp3+ Tregs was analyzed by flow cytometry. As shown in Fig. 6(e, f), the percentage of Foxp3+ Tregs was 6.5% and 6.7% in the control group and the BMSC group, respectively. However, the percentage of Foxp3+ Tregs was 11.8% in the IFNγ-BMSC group, which was increased as compared to the other group.

Discussion

PD-L1 is a 40-kDa type 1 transmembrane protein that regulates the development, maintenance and function of induced regulatory T cells (Francisco et al. 2009). It plays a major role in suppressing the immune system during a few special events such as pregnancy, autoimmune disease, some infectious diseases, as well as tissue allografts (Jacobs et al. 2009). It was reported that PD-L1 binding to PD-1 usually transmits an inhibitory signal and suppresses the proliferation and activation of T cells (Li et al. 2013; Rodig et al. 2003). Simultaneously, the PD-L1/PD-1 complex was able to restrict the accumulation of foreign antigen specific T cells via Bcl-2-mediated apoptosis (Singh et al. 2013). In this study, we showed that the upregulation of PD-L1 in the BMSCs after IFN-γ stimulus, markedly reduced the proliferation of T cells and the ratio of regulatory T cells. In addition, when PD-L1 was blocked by the neutralizing

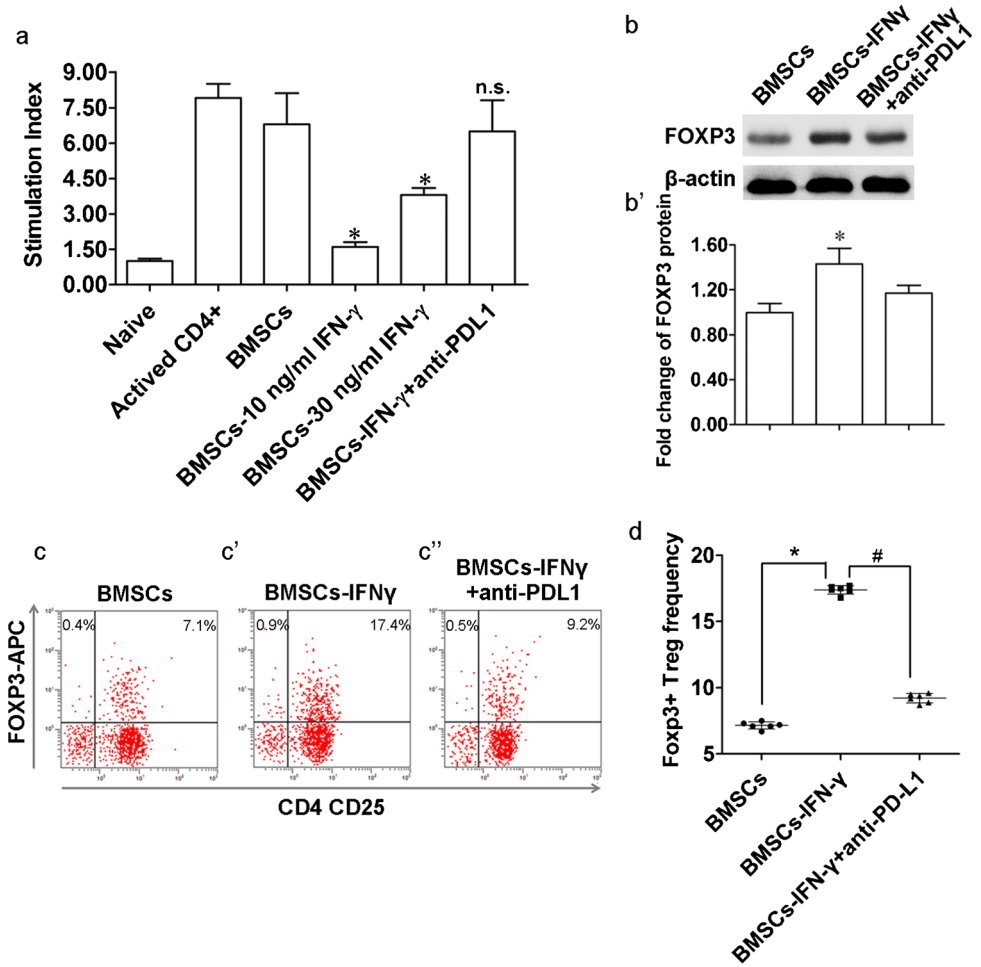

Fig. 5 Low-dose IFN-γ-conditioned BMSCs suppressed differentiation of T cells via PD-L1. BMSCs were pre-incubated with 10 ng/mL IFN-γ or 30 ng/mL IFN-γ or 10 ng/mL IFN-γ plus 0.5 ng/mL blocking antibody. Then, 100 μL of stimulator cells and 100 μL of responder cells labeled with CFSE were added. (a) The stimulation index was detected with flow cytometry. IFN-γ-conditioned BMSCs affected the simulation index of T cells in a dose-dependent manner. (b, b') The expression of FOXP3 was detected by western blot. (c–c") IFN-γ-conditioned BMSCs improved FOXP3 expression in $CD4^+CD25^+$ T cells. The $CD4^+CD25^+$ T cells were collected and cells were cultured with anti-FOXP3 mAb. The proportion of $FOXP3^+$ T cells was detected by flow cytometry. (d) Representative images of $FOXP3^+$ regulatory T cell frequency. $^*P < 0.05$. Data are representative of results from six independent experiments

antibody, the proliferation of T cells and the ratio of regulatory T cells were reversed. Therefore, PD-L1 played a key role in the regulation of proliferation and activation of the T cells during its incubation with BMSCs-IFN-γ.

There have been some studies on the regulation of PD-L1 expression by inflammatory cytokines. For example, C. Blank and his colleagues incubated tumor-specific T cells with LPS, TNFα and IFN-γ and found that IFN-γ but not LPS and TNFα, could stimulate the expression of PD-L1 and decrease T cell receptor-mediated proliferation and cytokine production (Blank and Mackensen 2007). Treatment with IFN-α, IFN-β and IFN-γ but not LPS, was observed to induce PD-L1 mRNA expression and PD-L1 protein expression on the membrane of microvascular endothelial cells. In this study, our data showed that proinflammatory cytokine IFN-γ altered the expression of PD-L1, a major suppressor gene in the immune system during allograft rejection, in a strictly dose-dependent manner in BMSCs: 10 ng/mL IFN-γ significantly promoted PD-L1 expression, 30 ng/mL IFN-γ modestly improved PD-L1 expression, while 50 ng/mL IFN-γ sharply reduced PD-L1 expression. Consistently, Hettich et al. used 50 ng/mL IFN-γ to deactivate PD-L1 signaling (Hettich et al. 2016).

The reason why MSCs mediated immunosuppression has not been fully elucidated. One possibility is that the inflammatory milieu/microenvironment may, in part, determine the immune function of MSCs. An increasing number of studies demonstrated that the immunosuppressive function of MSC requires MSC priming in a pro-inflammatory microenvironment that is built by pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1α and IL-1β (Broekman et al. 2016; Cortez et al. 2013). In this study, we showed that 10 ng/mL IFN-γ-incubated BMSCs dramatically suppressed T cell

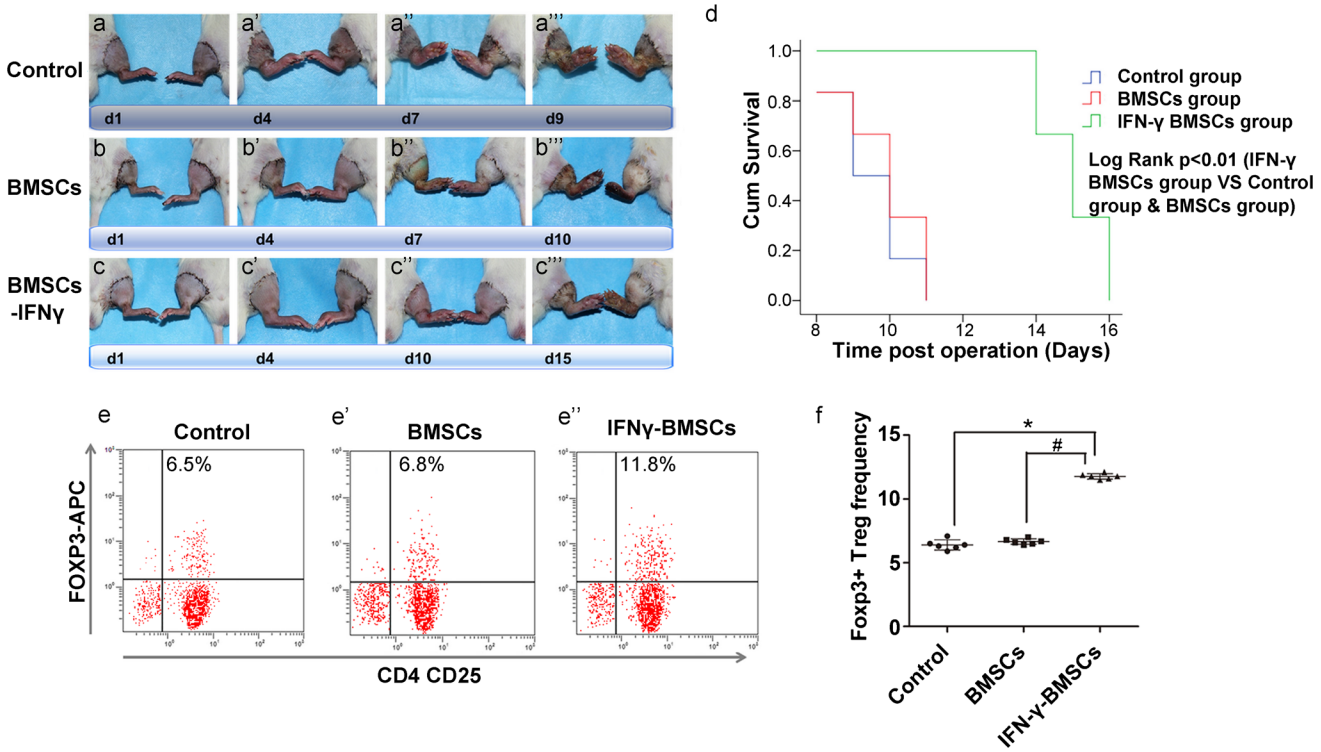

Fig. 6 Transplantation of IFN-γ-conditioned BMSCs delayed the vascularized composite allograft rejection and prolonged the survival time of rats with hind limb composite tissue allogeneic transplantation. (a–c''') The limb was amputated at the mid-femoral level. The recipients' and donors' limbs were prepared. Donor limbs were attached to the recipient's femoral stump with 1.5 mm Kirschner wire. After the operation, a total of 2×10^6 BMSCs (b–b''') or IFN-γ incubated BMSCs (c–c''') in 1 mL physiology saline were injected to recipient rats through tail vein. Control rats received an equal volume of normal saline (a–a'''). Manifestations of immunological rejection were monitored at days 1, 4, 7, 9 (10 or 15). (d) IFN-γ-conditioned BMSCs prolonged the survival time of rats with hind limb composite tissue allogeneic transplantation. Data are representative of results from six independent experiments. (e–e'') The spleen from the recipient rat of each group was collected and the percentage of Foxp3+ regulatory T cell was analyzed by flow cytometry. (f) Representative images of $FOXP3^+$ regulatory T cell frequency

proliferation and activation, which is associated with the delay of allograft rejection after BMSC transplantation in vivo.

The manner that MSCs influence the immune cells and exert their immunosuppressive effects is still undetermined. Both contact-dependent and contact-independent manners have been approved. Krampera and colleagues showed that the suppressive activity of MSC was abrogated when cells were physically separated by a transwell membrane (Krampera et al. 2006). Other studies have shown that MSC may interfere directly with T cell, or antigen-presenting cell phenotype, causing these cells to adopt regulatory functions (Calkoen et al. 2014; Fidler et al. 1996; Liao and Zhao 2015). MSC may interfere with ligand–receptor interactions required for T cell activation and proliferation (De et al. 2002). In contrast, other studies have proposed a role for soluble factors in MSC mediated immunosuppression using transwell cultures or MSC supernatant. In our current study, IFN-γ mediated the interactions between BMSCs and ligand–receptor and regulated T cell proliferation and differentiation.

Although most authors attribute these findings that contradict the tolerizing effect of BMSCs to differences in BMSC cultivation, experimental design or contamination with hematopoietic stem cells. Our results illustrate that MSC can either be inhibitory or stimulating depending on the degree of T cell preactivation. As described earlier, we also confirm that IFN-γ can direct BMSCs to function as inhibitors. This ability is not solely restricted to IFN-γ. TNF-α and IL-2 can also boost the inhibitory potential of MSC in our hands, which is in keeping with another report indicating that MSCs need to be "licensed" by IFN-γ plus either TNF-α, IL-1, or IL-1. In summary, we suggest to distinguish an "immunosuppressive MSC" (isMSC) from a "immunogeneic MSC" (igMSC) because we have outlined that MSCs are able to switch from one functional cell type to the other independently of the number of cells employed or the culture conditions used. To pave the way for MSC to be considered in first clinical trials in solid organ transplantation, this controversy has to be further investigated to ensure patient safety and to facilitate a therapeutic benefit.

In conclusion, vascularized composite allograft rejection is delayed by infusion of IFN-γ-conditioned BMSCs and this delay is associated with upregulation of PD-L1. Our data reveal a role of recipient ongoing immune microenviroment in BMSC-based immunesuppressive therapy.

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