Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells

Xiao-Xia Jiang, Yi Zhang, Bing Liu, Shuang-Xi Zhang, Ying Wu, Xiao-Dan Yu, and Ning Mao

Introduction

Dendritic cells (DCs), the most potent antigen-presenting cells (APCs), are rare and ubiquitously distributed in the human body. They are derived from CD34+ bone marrow stem cells and can be generated from blood monocytes in vitro by culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). DCs play a major role in the uptake, transport, and presentation of antigens with the unique capacity to stimulate naive T lymphocytes. The ability of DCs to initiate an immune response depends on their transition from antigen-processing to antigen-presenting cells, during which they up-regulate class II major histocompatibility complex (MHC) and T-cell costimulatory molecules (CD80, CD86) in the cell surface, a process referred to as DC maturation. This transition constitutes an important checkpoint in mounting an immune response because immature DCs not only fail to prime T cells effectively but also serve to promote tolerance induction. In addition to their polarizing capacity on naive T cells, they can interact with B cells and natural killer (NK) cells. Thus, DCs are critical in the initiation of primary immune responses, becoming an important target for immunosuppression to prevent allograft rejection.

Mesenchymal stem cells (MSCs) are multipotential cells, most of which reside within the bone marrow. They can be induced to differentiate into various components of the marrow microenvironment, such as bone, adipose, and stromal tissues. Studies conducted in both human and animal models have demonstrated that MSCs are capable of long-term engraftment and multilineage differentiation in vivo. Furthermore, the intravenous administration of MSCs leads to a modest but significant prolongation of skin graft survival similar to the immunosuppressive agents currently being used clinically. These findings suggest that MSCs may harbor an immunoregulatory activity. Indeed, human MSCs share cell surface markers with thymic epithelium. They express adhesion molecules involved in T-cell interaction, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and leukocyte function-associated antigen-3 (LFA-3). Moreover, MSCs express human leukocyte antigen (HLA) MHC class I and negligible levels of both MHC class II and Fas ligand; they do not express B7-1, B7-2, CD40, or CD40L. Recently, human and baboon MSCs displayed immunosuppressive properties on T-lymphocyte proliferation in vitro. However, the induction of immune responses does not rely solely on T cells but also on essential and complicated interactions between DCs and T cells. It is highly possible that MSCs may modulate the generation and antigen-presenting capacity of DCs prior to antigen-specific lymphocyte activation and proliferation.

Based on this hypothesis, we addressed here the negative regulation of MSCs on monocyte-derived DCs regarding from initial differentiation, IL-12 secretion to ultimate stimulatory ability, hence revealing DCs as one of the targets of MSCs displaying immunosuppressive effects.

Materials and methods

Generation of human MSCs

The Research Ethics Committees of North Tai Ping Lu Hospital approved human bone marrow samples for research purposes. The health donors gave written consent for the use of bone marrow for research purposes.

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MSCs were generated from normal human bone marrow samples. The mononuclear cells were prepared by gradient centrifugation at 900g for 30 minutes on Percoll (Amersham Biosciences, Uppsala, Sweden) at a density of 1.073 g/mL. Then cells were washed, counted, and plated at 2.0 × 10⁵/cm² onto 24-well tissue culture plates (Corning Costar, Corning, NY) in low-glucose Dulbecco modified Eagle medium (LG-DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Medium was replaced, and the nonadherent cells were removed after 48 hours of initial culture and every 3 or 4 days thereafter. When 70% to 80% confluent, adherent cells were trypsinized (0.05% trypsin at 37°C for 5 minutes), harvested, and expanded in larger flasks. A homogenous cell population was obtained after 2 weeks of culture. Before further expansion and experimental use, MSCs were tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes. Adipocytic differentiation was induced by DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine, 5 μg/mL insulin, 1 μM dexamethasone, and 60 μM indomethacin, whereas 1 mM β-glycerophosphate, 0.1 μM dexamethasone, and 50 μM ascorbate were used for osteoblastic differentiation. Oil red O and von Kossa dyes were used to identify adipocytes and osteoblasts, respectively. Medium with DMEM containing 2.5% FBS, 50 ng/mL transforming growth factor-β1 (Peprotech, Rocky Hill, NJ), 50 μg/mL ascorbic acid, 1 mM sodium pyruvate, 6.25 μg/mL bovine insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenious acid, and 1.25 μg/mL bovine serum albumin was used for chondrogenic differentiation. Extracellular matrix, used to assess chondrogenic differentiation, was detected by Alcian blue staining. The identity of MSCs was also confirmed by immunophenotypic criteria based on the expression of CD73 and CD105 and the absence of hematopoietic (with anti-CD45, -CD14, -CD34 antibodies) and endothelial (with anti-CD31 antibodies) markers. All antibodies were purchased from BD Pharmingen (San Diego, CA). MSCs were maintained in culture for no more than 15 passages in vitro.

**Differentiation of human CD14⁺ monocytes**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (1.077g/mL; Invitrogen) density gradient centrifugation. A total of 2 × 10⁶ PBMCs were cultured in 10 mL medium per 75 cm² flask (Corning). After 2 hours or overnight the cells were gently agitated and the adherent cells collected. Monocytes (more than 85% CD14⁺) were purified from adherent cells by using the MACS Monocyte Isolation Kit (Miltenyi Biotec). After 4 days, cells were pulse during the last 16 hours with 3H-thymidine (1 μCi/well [0.037 MBq/well]). Thymidine incorporation was measured by standard liquid scintillation counting. Results are expressed in counts per minute and shown as means ± SDs of triplicate values.

**KLH presentation assay**

Keyhole-limpet hemocyanin (KLH) (Sigma) was used to test antigen-specific T-lymphocyte response. The DCs were loaded with 25 μg/mL to 100 μg/mL KLH at 37°C for 60 minutes. A total of 2 × 10⁵ KLH-loaded DCs were cultured with a constant number of 1 × 10⁶ CD4⁺ T cells for 4 days. During the last 16 hours of each culture period, cells were pulsed with 3H-thymidine (1 μCi/well [0.037 MBq/well]). Results are expressed in counts per minute and shown as means ± SDs of triplicate values.

**Endocytosis**

Endocytosis was measured as the cellular uptake of fluorescein isothiocyanate (FITC)–dextran and was quantified by flow cytometry. Approximately 5 × 10⁴ cells per sample were incubated in medium containing FITC-dextran (1 mg/mL; molecular weight 40,000; Sigma, St. Louis, MO) for 60 minutes. After incubation, cells were washed twice with cold phosphate-buffered saline (PBS) to stop endocytosis and remove excess dextran and were then fixed in cold 1% formalin. The quantitative uptake of FITC-dextran by the cells was determined by fluorescence-activated cell sorting (FACS), and the results were expressed in mean fluorescence intensity (MFI) values. At least 10,000 cells per sample were analyzed.

**Mixed leukocyte reactions (MLRs)**

Monocyte-derived cells cultured with GM-CSF plus IL-4 for 7 days were irradiated (30 Gy) and cultured in triplicate at 5 × 10⁵, 1 × 10⁵, and 2 × 10⁴ cells per 200 μL per well in 96-well flat-bottomed plates with 1 × 10⁵ allogeneic CD4⁺ T cells purified from PBMCs by using the MACS CD4 isolation kit (Miltenyi Biotec). After 4 days, cells were pulse during the last 16 hours with 3H-thymidine (1 μCi/well [0.037 MBq/well]). Thymidine incorporation was measured by standard liquid scintillation counting. Results are expressed in counts per minute and shown as means ± SDs of triplicate values.

**Results**

Human MSCs strongly inhibited the initial differentiation of DCs from monocytes.

Highly enriched CD14⁺ monocytes (more than 85% negative for CD1a) were cultured in the presence of GM-CSF (10 ng/mL) and...
IL-4 (500 μM/L) with (MSC-Mo) or without MSC coculture (Ctr-Mo). After 7 days of incubation, LPS was added for another 48 hours to promote the differentiation and maturation of DCs. As expected, Ctr-Mo acquired the size, became nonadherent and clustered, and displayed different protruding veins with abundant cytoplasm, a typical morphology of DCs (Figure 1A, upper row). In parallel, these cells displayed increased expression of CD1a in contrast with decreased or absence of CD14 expression. After exposure to LPS, they further differentiated into fully mature DCs characterized by up-regulated expression of CD83 and costimulator molecule CD80 (Figure 1B, upper row). The presence of MSCs in the initial culture did not affect cell recovery (Ctr-Mo, 52% ± 10%; MSC-Mo, 47% ± 15%). The MSC-Mo’s also acquired the size; however, they were round, scattered, lacking in veiled appearance, and developed macrophage morphology with numerous vacuoles (Figure 1A, lower row). In contrast to Ctr-Mo, MSC-Mo retained high CD14 without acquisition of CD1a and displayed no up-regulation of CD83 and CD80 even under strengthened maturation stimuli—that is, doubled concentration of GM-CSF, IL-4, and LPS to rule out the consumption of cytokines by MSCs (Figure 1B, lower row and data not shown). In some experiments, the same cell dose of MSCs was added to the Ctr-Mo group in the process of immunostaining to counteract the possible difference caused by the presence of MSCs in MSC-Mo. As a matter of fact, MSCs, before or after being cultured with monocytes and cytokines, did not express CD14, CD1a, CD80, and CD86. By using the transwell system (described in “Materials and methods”) the possible contamination of MSCs in collection of cells for phenotype analysis was further excluded. These data clearly suggested that the presence of MSCs was capable of preventing bipotential monocytes from differentiating along the DC pathway.

Fibroblasts, as previously reported by Chomarat et al., switch monocyte differentiation to macrophages rather than to DCs, and full skewing was obtained with a ratio of 1 fibroblast to 4 monocytes. While in the presence of MSCs, as shown in Figure 1C, with the MSC/monocyte ratio of 1:10, the differentiation of monocytes to DCs was completely blocked with no significant difference, and even the ratio decreased to 1:20 and 1:40 (Figure 1C and data not shown). Such inhibitory effect became minor until the ratio dropped to 1:200. Addition of TNF-α at the onset of the fibroblast/monocyte coculture is reportedly able to reverse the skewing effect of fibroblast, resulting in the generation of cells with DC phenotype. As for MSCs here, nevertheless, adding TNF-α at the initial MSC/monocyte coculture, even with doubled concentration, did not result in the generation of cells with DC phenotype. No down-regulation of CD14 and no acquisition of CD1a were found. These results further indicate, as compared with fibroblasts at least, the suppressive effect of MSCs on the initial differentiation of DCs from monocytes was strong.

**Suppression of DC generation without intercellular contact at a higher MSC/monocyte ratio**

In addition, we determined if the inhibition of MSCs on the initial differentiation of DCs required intercellular contact. For this purpose, the transwell chamber system was used to separate monocytes (upper compartment) from MSCs (lower compartment). As shown in Figure 2A, a higher MSC/monocyte ratio (1:10) could completely prevent monocyte differentiation to DCs, like the coculture system described in “Materials and methods.” As the ratio decreased to 1:20 and 1:50, monocytes were able to generate immature DCs (ie, down-regulated CD14 coupled with up-regulated CD1a expression) in the presence of GM-CSF and IL-4 and underwent maturation (significant expression of CD83, data not shown) upon LPS stimulation. The data implied that MSCs were capable of suppressing DC generation by secreting cytokines.
at a higher ratio and mainly through cell-cell interaction at a lower ratio.

Next we defined what kind of factors in the supernatant contributed to the effect observed in the transwell system. Here, IL-6 and M-CSF were investigated because they are essential factors in the molecular control of antigen-presenting cell development. Actually, human MSCs did produce IL-6 (1.2 ng/mL per $2 \times 10^5$ MSCs) and M-CSF (2.6 ng/mL per $2 \times 10^5$ MSCs) as determined by ELISA. As presented in Figure 2B, the CD14$^+$ population was reduced by supplement of anti–IL-6 and anti–M-CSF neutralizing mAb respectively or combined, but increased expression of CD1a was not observed. The data suggested that MSCs might maintain the CD14 immunophenotype of monocytes/macrophages via secretion of IL-6 and M-CSF, but the cytokines counteracting DC-specific CD1a induction by GM-CSF/IL-4 deserve more investigation.

The inhibitory effect in the MSC/monocyte coculture was reversible

Monocytes are precursors of macrophages and DCs, with each cell type convertible to each other. The environment and the presence of differentiation or other stimulatory signals are the decisive factors. Then we studied whether the effect of MSCs on the initial differentiation of DCs from monocytes was persistent or reversible. MSC-Mo’s were harvested, washed intensely, and then incubated with GM-CSF, IL-4, and LPS in the absence of MSCs for another 7 days. Although prolonged culture induced cell loss, differentiation and maturation of DCs under these conditions occurred. The down-regulation of CD14 (MSC-Mo versus treated MSC-Mo: 86 versus 13), induction of CD1a (MSC-Mo versus treated MSC-Mo: 6 versus 83), and acquisition of CD83 (MSC-Mo versus treated MSC-Mo: 14 versus 63), as shown in Figure 3, indicated that the suppressive effect in the MSC/monocyte coculture was not refractory but reversible.

Moderate suppression of MSCs on mature DCs

It was important to evaluate whether MSCs, in addition to blocking initial commitment of monocytes to DCs, also affected already differentiated DCs. For this purpose, already differentiated DCs were classified into 2 categories: immature and mature DCs. The former was obtained by culture of monocytes in the presence of GM-CSF and IL-4 for 7 days and the latter under prolonged 2 days of incubation with LPS. As presented in Table 1, treatment of immature DCs with MSCs, though supplemented by GM-CSF, IL-4, and LPS, caused modest up-regulation of membrane CD14 (2.9% ± 0.8% versus 10.9% ± 3.0%) but slight reduction of maturation antigen CD83 (78% ± 12.7% versus 55% ± 15.6%). However, expression of presentation and costimulatory molecules were not altered. Surprisingly, although CD1a and CD83 expression of MSC-treated immature DCs implied increased arrestment of immature DCs, endocytic activity was significantly reduced (MFI of FITC-dextran: 63.5 ± 7.8 versus 33.5 ± 17.7), pointing to a possible inhibition of DC endocytosis by MSCs. The data suggested that some immature DCs might either convert to CD14$^+$ cells (most probably macrophages) or cease to develop into mature DCs.

On the other hand, exposure of mature DCs to MSCs (MSC-mDC), without exogenous cytokines anymore, caused a significantly decreased expression of HLA-DR, CD1a, CD80, and CD86, whereas the CD14 expression and endocytic activity almost remained unchanged. These data implied that MSC coculture could reverse mDCs to an immature state other than to CD14$^+$ monocytes/macrophages.

### Table 1. Influence of MSCs on the immunophenotype of already differentiated DCs

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<th>Immature DCs</th>
<th>Mature DCs</th>
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<tr>
<td></td>
<td>LPS</td>
<td>LPS/MSC</td>
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<td>Monocyte/macrophage antigen</td>
<td>CD14 2.9 ± 0.8</td>
<td>10.9 ± 3.0†</td>
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<td></td>
<td>CD83 78 ± 12.7</td>
<td>55 ± 15.6†</td>
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<tr>
<td>Presentation molecules</td>
<td>HLA-DR 89.5 ± 9.2</td>
<td>77.5 ± 3.5†</td>
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<td></td>
<td>CD1a 72.4 ± 13.2</td>
<td>65.6 ± 10.7</td>
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<tr>
<td>Costimulatory molecules</td>
<td>CD86 91.0 ± 8.5</td>
<td>80.5 ± 9.2</td>
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<td>CD80 52 ± 7.1</td>
<td>45 ± 1.4</td>
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<tr>
<td>Endocytosis</td>
<td>FITC-dextran* 63.5 ± 7.8</td>
<td>33.5 ± 17.7†</td>
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Monocytes cultured with GM-CSF and IL-4 for 7 days were considered as immature DCs and, after stimulation by LPS for another 2 days, as mature DCs. The immunophenotype and the uptake of dextran were analyzed by FACS. Percentage of positive cells is shown as mean ± SD from 3 separate experiments (†$P < .05$, ‡$P < .01$).

*The data are shown as mean fluorescence intensity.
mDCs were used to stimulate allogeneic CD4 T cells. After MSC treatment, mDCs loaded with KLH were used in graded doses to stimulate CD4 T cells. Images were captured and processed as for Figure 1A. (D) Visualized using a Nikon TE2000-U microscope equipped with a Plan Fluor 10×/0.30 objective lens. (Nikon).

Because MSCs suppressed IL-12 production by DCs. Immature DCs were washed and stimulated by LPS with or without the presence of MSCs for additional 48 hours, followed by supernatant collection for quantification of IL-12. As shown in Figure 4A, IL-12 production was significantly decreased when MSCs were present (96.5 ± 0.65 versus 33.5 ± 0.56 pg per 1 × 10⁶ cells per mL, P < .01). LPS stimulation is reported to be able to induce phosphorylation of p38, an intracellular signaling pathway postulated important in the positive control of IL-12 secretion. Figure 5A shows that the phosphorylation of p38 under LPS stimulation was greatly reduced in the presence of MSCs.

Because insufficient IL-12 production of DCs has been implicated in the induction of anergy and tolerance of T cells, IFN-γ and IL-10 secretion in the MLR was quantified by ELISA to confirm the event. Notably, IL-12 secretion was also down-regulated in the MLR with MSC coculture (11.4 ± 0.4 versus 3.6 ± 0.1 pg/mL, P < .01). As shown in Figure 5B, CD4+ T cells (2 × 10⁶ per well) stimulated by MSCs/DCs (1 × 10⁵ per well) showed reduced IFN-γ secretion (1091.9 ± 73.9 versus 137.5 ± 0.7 pg/mL, P < .01) but increased IL-10 production (108.7 ± 0.2 versus 139.4 ± 5.5 pg/mL, P < .05), correlating well with impaired proliferation of CD4+ T cells (Figure 5B, MLR: 13776 ± 900 cpm versus 2459 ± 127 cpm, P < .01).

Discussion

It is well known that MSCs can inhibit T-cell proliferation induced by allogeneic cells in vitro and mediate a systemic immunosuppressive activity in vivo. In this report, we showed that human MSCs, the primitive cell type with multilineage differentiation ability, inhibited strongly but reversibly the initial differentiation of CD14+ monocytes into CD1a+ DCs via either intercellular contact or soluble factors. In the case of mature DCs, MSCs were able to down-regulate expression of costimulatory molecules CD80 and CD86 and decrease secretion of IL-12, thus suppressing T-cell

MSCs suppressed IL-12 production by DCs

Because the secretion of IL-12 is critical for the maturation and function of DCs, we next investigated whether MSCs could interfere with IL-12 production by DCs. Immature DCs were washed and stimulated by LPS with or without the presence of MSCs for additional 48 hours, followed by supernatant collection for quantification of IL-12. As shown in Figure 4A, IL-12 production was significantly decreased when MSCs were present (96.5 ± 0.65 versus 33.5 ± 0.56 pg per 1 × 10⁶ cells per mL, P < .01). LPS stimulation is reported to be able to induce phosphorylation of p38, an intracellular signaling pathway postulated important in the positive control of IL-12 secretion. Figure 5A shows that the phosphorylation of p38 under LPS stimulation was greatly reduced in the presence of MSCs.
activation and proliferation. Hence, modulation of DC differentiation and function is relevant to the immunosuppressive effect of MSCs.

Biopotent monocytes can give rise to either antigen-presenting DCs or scavenging macrophages when crossing endothelium in vivo, and similar plasticity has been recapitulated in vitro by feeding them with GM-CSF/IL-4 or M-CSF. Like fibroblast and epithelial cell lines identified before,30 MSC-treated monocytes under GM-CSF/IL-4 stimuli showed a larger cell size with numerous vacuoles and displayed low expression of CD1a and persistently high expression of CD14, indicating a skewing from DCs to macrophages. Nevertheless, human MSCs investigated here revealed several unique properties. First, the inhibition was more powerful. The MSC/monocyte ratio that was required for complete suppression of DC commitment was far lower (1:20 and even 1:40) than that of normal skin fibroblast (1:4). Addition of TNF-α,31 a facilitator of DC induction by overriding the IL-6/M-CSF pathway, failed to alter the switch by MSCs. Second, in contrast to fibroblast, MSCs, particularly at a higher ratio (1:10), could reproduce full skewing without cell-cell contact in the transwell system. By comparative analysis of the typical coculture and transwell system, the relative contribution of intercellular contact or supernatant factors could be estimated. Whatever culture methods, a higher MSC/monocyte ratio (1:10) could completely inhibit monocyte differentiation to DCs, thus at least implying the suppression in the coculture system was saturated. As the ratio decreased to 1:20 and 1:40 (data not shown), the inhibitory effect within the transwell system dropped dramatically, but coculture could still fully reserve a typical monocyte/macrophage immunophenotype. Hence, at a lower ratio, the effect of intercellular contact prevailed. Among cytokines proven to exercise a negative control of DC generation from monocytes, IL-6 and M-CSF, rather than IL-1034 and IFN-γ,35 were indeed produced by human MSCs. Neutralizing their bioactivity, however, failed to generate CD1a+ cells with a unique veiled appearance. As expected, CD14 expression was reduced upon MSC treatment regardless of whether the starting population was either immature or mature DCs. Such alteration implied that the pool of immature DCs was enlarged at the expense of mature DCs. Nevertheless, the change contradicted with decreased or unchanged endocytosis capacity of DCs. The mechanism is most probably related to direct suppression of DC endocytosis by MSCs.

Whether naive T cells diverge into Th1 or Th2 effectors depends on the cytokine environment (IL-12) and the type and the activation state of DCs. Accordingly, insufficient IL-12 production and decreased expression of MHC class II plus costimulatory molecules of DCs upon MSC treatment might modulate the balance between Th1 and Th2 cells in favor of the latter, which showed lower IFN-γ and higher IL-10 production. Although the direct effect of MSCs on T cells may be included in the MLR, the changed morphology of DCs and the decreased secretion of IL-12 suggested a direct effect of MSCs on DCs, which subsequently regulated T-cell expansion.

Abundant studies have been carried out to show that MSCs perform a direct, strong, and reversible suppression on T-cell proliferation in vitro. Such effect occurs regardless of the species of T cells or MSCs and is independent of APCs or CD4+/CD25+ regulatory T cells. In vivo, administration of MSCs leads to prolonged skin graft survival. Lazarus et al33 have reported a phase 1 bone marrow stromal cell (BMSC) study in patients with advanced hematologic malignancies without significant toxicities. In addition, most patients have experienced a reduction of acute and chronic graft versus host disease (GVHD). Whether MSCs or their offspring participate in the regulation of T-cell proliferation via DCs in vivo calls for further investigation. Fortunately, we have successfully isolated and expanded MSCs from murine bone marrow using a novel and reliable method, which paves the way for studying the effect of MSCs on DCs in vivo.34

In summary, our data suggested that MSCs might modulate the immune system, not only acting directly on T cells but also at the very first step of the immune response through the inhibition of DC
differenciation and maturation. This study will be clinically beneficial. Because DCs serve as gatekeepers to the body’s immune system, it seems likely that by somehow suppressing DC function, it be through the limitation of antigen uptake, processing, or presentation, the likelihood of graft rejection could be significantly reduced. More valuable, the effects of MSCs on DCs are reversible, which will circumvent the flaws of long-lasting hypoinnune competence following transplantation.

References

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