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The Immunogenicity and Immunomodulatory Function of Osteogenic Cells Differentiated from Mesenchymal Stem Cells

Hua Liu,* David Michael Kemeny,† Boon Chin Heng,* Hong Wei Ouyang,‡ Alirio J. Melendez,§ and Tong Cao1*

Multipotent mesenchymal stem cells (MSC) are reported to be immunoprivileged as well as immunosuppressive. Hence, they are ideal candidates for allogeneic transplantation to induce regeneration of diseased tissues and organs. However, it is not known whether MSC would retain their immunoprivileged and immunomodulatory properties after differentiating into the local cell types of the transplantation site. This study sought to investigate this question with a novel New Zealand White rabbit osteogenesis model. Results showed that osteogenic cells differentiated from MSC (DOC) in vitro did not express the MHC class II molecule, were incapable of inducing allogeneic lymphocyte proliferation in mixed lymphocyte culture or generating CTL, were inhibitory in ongoing lymphocyte proliferation, and secreted anti-inflammatory cytokines (IL-10 and TGF-β). There was a significantly higher secretion of IL-10 by DOC than that by MSC, while there was no significant difference between the TGF-β secretion of MSC and DOC in vitro. However, after IFN-γ treatment, TGF-β secretion by DOC significantly decreased despite the increased production by MSC. Four weeks after local DOC implantation, despite MHC class II expression, second-set allogeneic skin rejection showed similar survival to first-set allogeneic skin rejection and DOC appeared to function as osteoblasts. In conclusion, DOC retained their immunoprivileged and immunomodulatory properties in vitro, but the latter was lost following transplantation. The Journal of Immunology, 2006, 176: 2864–2871.

B one marrow-derived mesenchymal stem cells (MSC) possess extensive capacity to differentiate into multiple lineages (1–4). Several recent studies demonstrated the ability of transplanted bone-marrow derived MSC to repair bone defects in vivo, either through infusion or local implantation (5–8). It is envisioned that MSC would be routinely used for clinical therapy of bone diseases in the future.

To avoid the immunological barrier, autologous MSC are favored for bone grafting. However, there are several limitations of using an autologous cell source, such as inadequate cell numbers and donor site morbidity (9). The alternative would be to use allogeneic MSC for bone grafting. Indeed, stem cell companies and stem cell banks worldwide offer an abundant source of allogeneic MSC. Most of these commercially available MSC have been subjected to stringent biosafety testing and are usually certified to be pathogen-free. Additionally, their homogeneity and growth characteristics are likely to have been standardized and quality guaranteed. Hence, they represent a stable cell source for therapeutic applications.

However, it is well-established that allogeneic transplantation encounters the problem of immunorejection. Various strategies have been devised to overcome the immunological barrier in allogeneic transplantation including irradiation-induced immune depletion of the host, administration of immunosuppressive drugs, in utero transplantation, and MHC matching. For experimental studies, nude mice are most often used to study allogeneic engraftment biology in vivo.

Recently, there have been several exciting reports that MSC are not only capable of reconstituting the bone marrow microenvironment for hemopoiesis, but are also nontargeted by MHC-mismatched immune cells (immunoprivileged). Furthermore, they have also been demonstrated to be immunosuppressive by inhibiting the MHC-mismatched lymphocyte response. Interestingly, it appears that this immunosuppressive activity is independent of MHC expression (10–19). MSC have been shown to express MHC class I and other immune-related molecules, such as VCAM-1 and LFA-3, but, however. lack expression of B7-1, B7-2, CD40, and CD40L costimulatory molecules. Upon inducement by IFN-γ, ICAM-1 and MHC class II can be expressed on MSC, but the costimulatory molecules are not detected. In addition to the immune-related molecule deficiency theory, it was also hypothesized that MSC could migrate into the thymus to play a role in T cell-positive selection after bone marrow transplantation (20–22), as well as integration into the bone marrow microenvironment to influence early immune cell development. Besides inhibiting naive, activated and memory T cell activity, MSC can also exert a suppressive effect on NK cell activity as well as dendritic cell differentiation and maturation (19, 23). However, MSC are capable of increasing the proportion of regulatory T cells within cocultured populations of lymphocytes (24). Additionally, biologically active molecules secreted by MSC may also participate in immunoregulatory pathways involving intercellular contact (14). These cytokines could mediate a shift in proinflammatory immune response to an anti-inflammatory immune response (24). Although it is still not fully understood how transplanted MSC perform these immunomodulatory functions, their therapeutic efficacy has been demonstrated by
successful allogeneic MSC infusion in children with osteogenesis imperfecta (25). Such allogeneic MSC have been shown to safely engraft onto patients’ bone and promote osteogenesis without the need for marrow-ablative chemotherapy. Similar results have also been observed with animal models (10, 26).

Regardless of the manner in which MSC are implanted or infused, the basic functional unit in the repair of bone defects is the differentiated osteogenic cell (DOC) derived from them. A major limitation in these experiments is the relatively low level of engraftment efficiency with in vitro-cultured MSC. It has been suggested that osteogenic differentiation of MSC before implantation might be a shortcut to expedite the healing process by shortening the interval between implantation and subsequent osteogenesis in situ (5, 27, 28). However, because differentiated progenitor cells are likely to display similar immune characteristics to mature somatic cells that are capable of eliciting MHC-mismatched host immune response (immunogenicity), it is unclear whether they would be immunoprivileged upon implantation like their undifferentiated precursors. At present, the immune characteristics of DOC have not been well-studied. Hence in this study, we investigated alloimmunogenicity of New Zealand White rabbit (NZW) DOC within both in vitro and in vivo models. At the same time, to figure out the mechanism behind the immune reaction, expression of the major immunogenic molecule (MHC class II) and immunomodulatory cytokines (TGF-β and IL-10) by DOC were assessed, in comparison with undifferentiated MSC.

Materials and Methods

Animals

Thirty-two female NZW, ~4 mo of age and weighing 2.5–3.0 kg, were purchased from the Laboratory Animals Center of the National University of Singapore (Sembawang, Singapore). The animals were acclimatized under controlled temperature (25°C), lighting (lights on from 7:00 a.m. to 7:00 p.m. h), and humidity (50–70%) for at least 1 wk before use. Four rabbits from different pairs of parents were grouped as one experimental batch and were separately fed from birth. All experimental protocols involving animals were approved by the Ethics Committee for Experimental Animals of the National University of Singapore.

Media, reagents, and labware consumables

Unless otherwise stated, all media, reagents, and labware consumables were obtained from Sigma-Aldrich; all culture flasks were purchased from Falcon Products. After anesthesia, bone marrow was collected from the 6-well culture supplemented with 10 mM sodium bicarbonate, and 100 U/ml penicillin G, and 0.1 mg/ml streptomycin) at 37°C, 5% CO2, and humidity (50–70%) for at least 1 wk before use. Four rabbits from different pairs of parents were grouped as one experimental batch and were separately fed from birth. All experimental protocols involving animals were approved by the Ethics Committee for Experimental Animals of the National University of Singapore.

MSC separation and differentiation

MSC isolation and culture. After anesthesia, bone marrow was collected from healthy NZW donors. Heparinized bone marrow was cultured with MSC medium (DMEM-low glucose, 10% (v/v) of FBS (HyClone), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin) at 37°C, 5% CO2, and 95% humidity for 4 days. Adherent cells were expanded for 7 days and were detached by 0.05% (w/v) trypsin/EDTA (Life Technologies). The cells were subsequently passaged two to three times to achieve desired cell numbers. The medium was changed every 3 days during the expansion period.

Osteogenic and chondrogenic differentiation of MSC in vitro. MSC at passage 2 (P2) were induced to the osteogenic lineage with MSC medium supplemented with 10 mM sodium β-glycerophosphate, 50 μg/ml l-ascorbic acid, and 10−8 M dexamethasone (29) for 21 days. During differentiation, MSC were separately cultured in 150 flasks for immunological tests, and in 6-well culture plates (1 × 105 cells/cm2) for characterization (29) (photos not shown). Medium was collected from the 6-well culture plates on day 21 for the osteocalcin ELISA test (30) (data not shown).

To demonstrate their multilineage potential, MSC at P2 were also induced into the chondrogenic lineage with chondrocyte differentiation media, either as pellets or as monolayers in 6-well culture plates (29, 31, 32) for 21 days. The differentiation was confirmed by the Type II collagen ELISA test and toluidine blue staining (32) (photos and data not shown).

To serve as positive controls of differentiated lineages, primary osteogenic cells (POC) and primary chondrogenic cells were derived from NZW xiphoïd as previously described (33). These cells were cultured under the same conditions as their corresponding differentiated cells.

Alloimmunogenicity testing of DOC in vitro

MHC and other cell surface marker detection. PBMC were fractionated from heparinized NZW blood by Ficoll-Paque Plus (1.077 g/ml; Amer sham Biosciences) at 3000 g for 20 min. The interlayer cells were washed twice with HBSS and were ready for anti-rabbit mAb and other immunological tests.

Approximately 1 × 106 PBMC and MSC (P2) were incubated with mouse anti-CD34 mAb (Zymed Laboratories) (34) and mouse anti-rabbit CD45 mAb (Serotec, mouse IgG1 isotype control (BD Pharmingen)) at 4°C for 30 min. Approximately 1 × 106 DOC, MSC, INF-γ (100 U/ml) treated MSC (MSC-INF-γ), and INF-γ treated DOC (DOC-INF-γ) were incubated with mouse anti-rabbit MHC class II (Serotec) for 30 min at 4°C. After washing, cells were incubated with FITC-conjugated goat anti-mouse Ig multiple adsorption polyclonal Ab (BD Pharmingen) at 4°C for 10 min. Subsequently, the cells were fixed in 70% alcohol and detected by flow cytometry using a CyAn ADP cytometer (DakoCytomation) at 488 nm. Results were analyzed by winMDIm software.

One-way mixed lymphocyte culture (MLC). PBMC, DOC, MSC, MSC-INF-γ, DOC-INF-γ, and PBMC pretreated with INF-γ (100 IU/ml, 3 days) were exposed to 25 μg/ml mitomycin C in darkness at 37°C for 20 min, subsequently washed twice, and used as stimulators. Untreated PBMC were to be used as responders.

A total of 1 × 105 stimulating PBMC or 1 × 104 of another stimulating cell type were cocultured with 1 × 105 responding PBMC in triplicates with Biosciences’ wells of 96-well U-bottom plates, in 0.2 ml of lymphocyte culture medium (RPML 1640 supplemented with 50 μM 2-ME, 10% (v/v) FBS, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin) for 6 days. The proliferation of responding cells was assessed by the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay kit (Promega), according to the manufacturer’s instructions. Absorbance was measured with an ELx800UV ELISA plate reader at 490 nm. The following formula was used to compute the stimulation index (SI). SI = ODtargetstimulated−ODtargetspontaneous/ODtargetspontaneous−ODbackgroundspontaneous × 100.

When the allogeneic lymphocyte proliferation rate is the same as that of autologous lymphocyte proliferation, the SI is 1.

Cell-mediated lysis (CML) test. A total of 2 × 105 stimulating POC or DOC and the same number of responding PBMC was cocultured in a 24-well culture plate at 37°C for 6 days. CTL were enriched from the unattached cells and isolated by Ficoll-Paque Plus. A total of 1 × 105 cells of CTL was incubated with a serial titration of the same stimulating cells for 4 h at 37°C. The cytotoxicity was evaluated by the Cytotoxicity Assay kit (Promega) according to the manufacturer’s instructions. Absorbance was measured on an ELx800UV ELISA reader at 490 nm. The percentage of cytotoxicity was computed by the following formula: percent cytotoxicity = (ODtarget−ODbackground)×100/ODtargetspontaneous. When the allogeneic lymphocyte proliferation rate is the same as that of autologous lymphocyte proliferation, the SI is 1.

Effect of DOC on ongoing lymphocyte proliferation. A total of 5 × 105 responding PBMC and DOC were cocultured in 24-well culture plates. DOC and the same number of responding PBMC or 1 × 105 stimulating PBMC or without 8 × 105 stimulating DOC or MSC in a 96-well plate at 37°C for 3 days. The proliferation of responding cells was assessed by CellTititer 96 Aqueous Nonradioactive Cell Proliferation Assay. Absorbance was measured with an ELx800UV ELISA plate reader at 490 nm. The inhibition percentage of allogeneic proliferation was calculated as the formula: percent proliferation decrease = (1 − ODtargetstimulated/ODtargetspontaneous) × 100.

TGF-β and IL-10 secretion of DOC and MSC. Secretion of TGF-β and IL-10 by DOC, MSC, INF-γ treated MSC, and MSC-INF-γ were tested with the medium collected from the wells of MLC plates. The assay procedure followed instructions of the BioSource Mouse IL-10 ELISA kit (BioSource International) (36) and the Quantikine TGF-β1 Immunoassay kit (R&D Systems) (37, 38). Absorbance was measured with an ELx800UV ELISA plate reader at 450 nm.

Alloimmunogenicity testing of MSC in vivo

Labeling MSC DOC. One day before implantation, DOC were stained with 25 μM CFSE, following the manufacturer’s instructions contained in the Vybrant CFDA SE Cell Tracker kit (Molecular Probes). The staining efficiency was assessed under microscope (Olympus 1×70).

Local implantation model setup. A commercial diffusion chamber model (2865) or DOC implantation of DOC without blocking nutrient exchange (39, 40). The chamber was composed of a Plexiglas ring (Millipore) bounded by two biodegradable polylactin 910 Vicryl-woven membranes (Ethicon). The chambers filled with 3.3–5 × 106 allogeneic DOC

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were either implanted close to the skull for characterizing their bone regenerative abilities or implanted i.p. for skin transplantation test.

**Fate of implanted diffusion chambers and DOC.** The skull surface-implanted DOC and diffusion chambers were harvested at days 2, 7, 14, and 28, respectively, for cryosectioning. The slides were stained with primary Abs (anti-rabbit MHC-II (1/1000), anti-osteonecrotin (ON; Developmental Studies Hybridoma Bank (DHSB); 1/50), anti-osteopontin (OPN; DHSB; 1/50), and anti-osteocalcin (OC; Takara Bio; 1/200)) for 1 h, respectively. Subsequently, they were incubated with prediluted Qdot 655 goat F(ab')2 anti-mouse IgG conjugate (H+L) (Quantum Dot; 1/200) at 37°C for 1 h. Slides were mounted and observed under confocal microscopy (Olympus FluoView 500). At the same time, the integrity of biodegradable chamber membranes was also examined.

**Skin transplantation.** Donor and recipient pairs were decided by MHC disparity through robust proliferative response in MLC and pedigree differences recorded before birth. All skin grafts were on the dorsal region of rabbits, with a spacing of at least 2 cm between separate grafts. The testing was performed at two independent times. For each time point, four rabbits were used. The skin transplantation procedure followed that of previous studies (15, 41). Each NZW received autologous, presensitized allogeneic and third-party allogeneic full-thickness skin grafts except for three controls (NZW 432, 431, and 483). NZW 432 and 431 received autologous DOC while NZW 483 did not receive any DOC. These three control rabbits received one autologous and two allogeneic skin grafts. Cephalaxin (0.1 ml/kg, i.m., bid × 3 days) and Temgesic (0.1 ml/kg, i.e., bid × 3 days) were administered into NZW postoperatively. Skin grafts were examined daily from 1 wk after operation and grafts that either failed before this time or were bitten off by rabbits were deemed a technical failure. Rejection was defined as complete eschar formation or the sloughing of epidermis. All rabbits were sacrificed 1 day after the last allogeneic skin rejection.

**Statistical analysis**

Quantitative data were analyzed by SPSS 11.5 for Windows, using either ANOVA or t tests. A value of p < 0.05 was considered significantly different.

**Results**

**Immunogenicity and immunosuppression assessment of DOC from MSC in vitro**

DOC were successfully differentiated from MSC which had been characterized by rapid plastic adherence, high proliferative potential, deficiency in the expression of hemopoietic markers (CD34 and CD45, Fig. 1), and their capacity to differentiate into osteogenic and chondrogenic cells in vitro (data and photos not shown). Previous studies reported that MSC are MHC class II-negative and are able to suppress lymphocyte proliferation in MLC, while POC are MHC class II-positive and responsible for T cell activation (42). Hence, we therefore examined MHC class II expression on DOC before (Fig. 1). However, immunogenicity does not only mean the expression of immune molecules on the cell surface; more importantly, it is the innate ability to induce an adaptive immune response by host immune cells. Thus, we investigated the activity of DOC in MLC and CML.

**DOC activity in MLC.** Allogeneic PBMC of different pedigrees were cocultured with mitomycin C-treated DOC, POC, MSC, and PBMC, respectively (Fig. 2). Allogeneic DOC and MSC indicated significantly decreased lymphocyte proliferation compared with allogeneic PBMC and POC (ANOVA, p < 0.001). Although the difference between lymphocyte proliferation levels of DOC and MSC was not significant (p = 0.595), there was observed to be further suppression of DOC compared with MSC.

**DOC lysis in CML.** CTL will attack cells that have activated them in MLC, when they are subsequently exposed to each other again. To determine whether DOC were able to activate allogeneic CTL, cocultured PBMC were collected from MLC of DOC or POC, and incubated again with those derived from the same allogeneic source. As seen in Fig. 3, the degree of cytolytic activity elicited by presensitized PBMC was similar for both the allogeneic DOC and the autologous controls (ANOVA, p > 0.05). In contrast, a much higher level of cytolyis was observed with allogeneic POC (ANOVA, p < 0.001). Because DOC were not able to activate naïve lymphocytes in MLC, and also displayed obvious immunosuppressive activities, it will be interesting to investigate their suppressive effects on activated lymphocytes. Coculture of DOC with ongoing MLC demonstrated that DOC could partly inhibit ongoing allogeneic lymphocyte proliferation like their undifferentiated MSC precursors (Fig. 4). Although there is no significant difference in the inhibitory effect of DOC and MSC (t test, p > 0.05), DOC displayed a steady inhibitory effect.

**FIGURE 1.** DOC lack of MHC class II expression. MSC are negative for CD34, CD45 (A), and MHC class II Ag (C). After the treatment of IFN-γ for 3 days, expression of MHC class II increases (E). However, there is no difference in the expression of MHC class II on DOC before (D) and after IFN-γ treatment for 3 days (F), and POC displayed a steady positive signal of MHC class II (B). Solid area shows isotype-matched negative control; solid curve is fluorescence result after staining with Ag-specific Abs.

**FIGURE 2.** DOC do not elicit allogeneic lymphocyte proliferation. Approximately 1 × 10⁵ allogeneic PBMC were cocultured with mitomycin C-treated PBMC (10⁵ cells), POC (10⁴ cells), DOC (10⁴ cells), and MSC (10⁵ cells), respectively. The SI of PBMC and POC were significantly higher than those of DOC and MSC (ANOVA, p < 0.001), while there was no significant difference between SI of DOC and MSC. SI was computed as SI: ODalloproliferation/ODautoproliferation. When the allogeneic lymphocyte proliferation rate is the same as that of autologous lymphocyte proliferation, the SI is 1. Bars represent mean ± SD from 20 rabbit donors of five separate experiments performed in quadruplicate.
separate experiments conducted in triplicate. Percentages of lysed allogeneic POC were significantly higher than those of lysed allogeneic DOC and autologous controls (ANOVA, \( p < 0.001 \)), while there was no difference among the lysis of allogeneic DOC and autologous controls. The percentage of cell lysis was calculated as: percent cytotoxicity = \((\text{OD}_{\text{Exp}} - \text{OD}_{\text{Effector Spontaneous}} - \text{OD}_{\text{Target Spontaneous}})/ (\text{OD}_{\text{Target Maximum}} - \text{OD}_{\text{Target Spontaneous}})) \times 100\). Stimulating cell to CTL ratios are titrated as shown on the x-axis. Results represent mean ± SD from 12 rabbit donors of three separate experiments performed in quadruplicate. The line across the cytotoxicity curve of allogeneic POC represents its trendline.

Cytokine secretion by DOC in vitro. Without the bridge of MHC class II to APC, DOC did not stimulate allogeneic lymphocyte proliferation, nor did they display an inhibitory effect on allogeneic lymphocytes. Based on previously reported studies with MSC, it is expected that cytokines may play an important role in this. Because IL-10 and TGF-\( \beta \) are two important factors in mediating and regulating immunity, we therefore compared their secretion by DOC or MSC in vitro (Fig. 5). Results showed that there was significantly higher IL-10 secretion by DOC compared with MSC (ANOVA, \( p < 0.05 \)), while there was no significant difference in TGF-\( \beta \) secretion between them.

DOC reaction in MLC and cytokine secretion after IFN-\( \gamma \) treatment. Previous studies on treatment of MSC with IFN-\( \gamma \) demonstrated that MHC class II expression will be up-regulated without influencing their immunogenicity and immunosuppression. Interestingly, DOC did not increase MHC class II expression (Fig. 1) and they maintained nonimmune response and immunosuppression in MLC (Fig. 6). After IFN-\( \gamma \) treatment, MSC displayed a significantly increasing inhibitory effect on lymphocyte proliferation (paired-samples \( t \) test, \( p < 0.05 \)), compared with the untreated control group. Although there was observed to be an increase in the proliferation inhibition by DOC as well, there were no significant differences between treated and untreated DOC groups. In contrast to a TGF-\( \beta \) secretion increase by MSC, TGF-\( \beta \) secretion by DOC significantly decreased (ANOVA, \( p < 0.05 \)). There was a very significant difference between TGF-\( \beta \) secretion by DOC and MSC (\( p < 0.01 \)). Although there was some extent of increase in IL-10 secretion by both cell types, no significant differences were observed between the IFN-\( \gamma \)-treated and untreated groups.

FIGURE 3. DOC do not activate allogeneic CTL response. The CTL were collected from unattached cells within 6 days coulture of allogeneic PBMC and DOC (or POC, stimulating cells to responding cells ratio, 1:1), then they (10^5 cells) were cocultured with the same originated stimulating cells for 4 h. Percentages of lysed allogeneic POC represents its trendline.

FIGURE 4. DOC inhibit ongoing lymphocyte proliferation. DOC (10^4 cells) and MSC (10^5 cells) were respectively added into ongoing allogeneic lymphocyte proliferative wells at the first day of MLC. Both cell types displayed stable inhibitory effects on ongoing lymphocyte proliferation, while there was no significant difference between them (\( t \) test, \( p > 0.05 \)): percent proliferation decrease = \((1 - (\text{SI with DOC/MSC/SI without DOC/MSC}) \times 100\). Bars represent the mean ± SD of decreased percentages of allogeneic lymphocyte proliferation from six rabbit donors of two separate experiments conducted in triplicate.

FIGURE 5. DOC secrete more IL-10 than MSC while its TGF-\( \beta \) secretion significantly decrease after IFN-\( \gamma \) treatment. Both DOC and MSC were treated with IFN-\( \gamma \) (100 IU/ml) for 3 days, then were inactivated with mitomycin C. Approximately 10^5 cells of each cell type were cultured in a 96-well plate. IL-10 and TGF-\( \beta \) detection were performed after 6 days. DOC displayed significantly higher secretion of IL-10 (ANOVA, \( p < 0.05 \)) than MSC while there was no difference between IFN-\( \gamma \)-treated and untreated groups. Although there was no significant difference in TGF-\( \beta \) secretion between MSC and DOC, TGF-\( \beta \) secretion by DOC significantly decreased after IFN-\( \gamma \) treatment (ANOVA, \( p < 0.05 \)). Results represent mean ± SD from two separate experiments performed in triplicate.

FIGURE 6. DOC increase the inhibitory effect on allogeneic lymphocyte proliferation after IFN-\( \gamma \) treatment in vitro. PBMC, DOC, and MSC were treated with IFN-\( \gamma \) (100 IU/ml) for 3 days, then were inactivated and cocultured with allogeneic PBMC (cell numbers were the same as those in previous MLC). Both DOC and MSC displayed an inhibitory effect on the allogeneic lymphocyte response (ANOVA, \( p < 0.05 \)). However, MSC displayed a significant inhibition increase after IFN-\( \gamma \) treatment (ANOVA, \( p < 0.05 \)) while DOC did not. PBMC with IFN-\( \gamma \)-treatment decreased capacity to induce allogeneic proliferation but no significant difference from the IFN-\( \gamma \)-untreated group. Results represent mean ± SD from two separate experiments performed in triplicate.
Immunogenic and immunosuppressive properties of DOC as assessed in vivo within a live animal model

To minimize the influence of postoperative aseptic inflammation, a biodegradable diffusion chamber was used. Both the fresh membrane and membrane of the diffusion chambers on day 2 postimplantation were intact and tightly woven, separating cells within the interior from the exterior leukocytes (photos not shown). Around day 14 postimplantation, the membranes had lost their integrity and became ruptured, resulting in the exposure of the contents of the biodegradable diffusion chamber to the tissue microenvironment. On day 28 postimplantation, most of the membranes were absorbed by the surrounding tissue (photos not shown). CFSE-labeled DOC were contained in the prescribed chambers, and their subsequent fate was tracked consecutively on days 7, 14, and 28.

**DOC fate in vivo.** On day 28 after rabbit head implantation, most CFSE-labeled cells migrated out of the chamber area. On the surface of chamber-adjacent skull, an obvious layer of green fluorescent cells was located (Fig. 7, C and E). The secretion of OPN and ON could be detected around implanted DOC on day 7 postimplantation by immunofluorescence staining. The level of ON secretion increased up to day 14 and then decreased afterward, but OC secretion became obvious on day 28 (Fig. 7). However, for MSC implanted in vivo, ON secretion appeared after day 14 and became obvious around day 28. A slight trace of OC could be detected on day 28 (photos not shown). After implantation, MHC class II expression was detected on DOC, regardless of whether they were derived from an autologous or allogeneic source (Fig. 8).

**Skin transplantation.** To determine whether the immunogenicity of DOC would be altered after implantation, skin transplantation after allogeneic DOC sensitization was performed. As seen in Table I, donor and recipient pairs with robust proliferative response in MLC were selected for implantation. Around 3.3–5 × 10⁶ allogeneic DOC were loaded into the diffusion chambers and implanted i.p. Twenty-eight days after implantation, the recipients received an autologous skin graft, an allogeneic DOC donor skin graft, and/or a third-party skin graft. The duration of skin graft survival are summarized in Table II. The majority (n = 10/12) of allogeneic skin grafts started to undergo necrosis before day 9 postimplantation. All rejected allogeneic skin grafts completed necrosis before day 12 postimplantation. In contrast, only two autologous skin grafts of six began necrosis before 9 days postimplantation with the necrosis of one skin graft being completed after day 12 postimplantation. Regardless of whether there was presensitization with either autologous or allogeneic DOC, there were no significant differences in the results.

**Discussion**

This study investigated the immune properties of rabbit DOC within both an in vitro and in vivo model. Our data showed that rabbit DOC in culture apparently expressed no MHC class II, like their undifferentiated MSC precursors. This implies that DOC lack the ability to present alloantigen directly to recipient CD4⁺ T cells. In MLC, APC from recipient’s PBMC provide an alternative opportunity to process and present alloantigens of DOC to recipient lymphocytes (this is termed indirect presentation). Lymphocyte proliferation would be the expected outcome after successful presentation. However, no lymphocyte proliferation was observed in MLC of DOC in this study. Thus even with the assistance of APC, DOC could not activate allogeneic CD4⁺ T cells to elicit an immune response in vitro.

To date, it is widely accepted that MHC class I is expressed on virtually all nucleated cells. As a genuine nucleated cell type, DOC have MHC class I expression (data not shown). Therefore, they could directly present alloantigens to allogeneic CD8⁺ T cells (or CTL) through MHC class I without the help of CD4⁺ T cells. Once CTL are activated, they can lyse target cells expressing the stimulating Ags. In our results, the cytolytic level of DOC by presensitized allogeneic CTL was low, similar to those induced by autologous controls. This implies that there was no CTL activation no matter the presence of alloantigens on in vitro-cultured DOC.

Although the above data implied that DOC were nonimmunogenic in vitro, it is possible that they might be induced to express MHC class II in vivo, which could turn on the immune response. To mimic an in vivo environment, IFN-γ, a proinflammatory cytokine which is secreted by various immune cells and can upregulate expression of MHC molecules on various cells, was added.
After IFN-\(\gamma\)-evidence implied the retention of the immunoprivileged status of immune response to DOC occurred in vivo within our observation to produce memory T cells in vivo after initial sensitization. No strong seems MHC class II expression does not activate any CTL or pro-

riods as third-party skin grafts (first-set allograft rejection). It donors (second-set allograft rejection) showed similar survival pe-

servation). Additionally, allogeneic skin grafts from the DOC genesis up to day 28 postimplantation (ending time point of ob-

servation) displayed that most of the allogeneic skin grafts (\(n\) = 10/12) began to necrose before day 9 postimplantation and completed necrosis before day 12 (\(n\) = 11/12). However, a few autologous skin grafts (\(n\) = 16/6) started necrosis before day 9 and most of them (\(n\) = 56) could survive beyond 12 days postimplantation. Auto, autologous; Allo, allogeneic; the number on the right of the slash represents the total testing numbers.

### Table I. SI of skin graft donors and recipients

<table>
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<tr>
<th>Rabbit Recipient</th>
<th>Implanted DOC Numbers ((\times 10^6))</th>
<th>Rabbit Donor of DOC</th>
<th>Third-Party Rabbit</th>
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*The SI, a ratio of allogeneic to autologous lymphocyte proliferation, was used to compare recipient PBMC proliferative activity to DOC donor and third-party rabbit PBMC for skin transplantation matching. An index of 1.3 or greater was deemed to be a significant reaction.

### Table II. Preimplantation of DOC did not delay allogeneic skin graft rejection

<table>
<thead>
<tr>
<th>Skin Graft Type</th>
<th>DOC Graft Type</th>
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<th>Day Necrosis Completed</th>
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<td></td>
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<td>(\geq) Day 9</td>
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<td>Auto-DOC</td>
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<tr>
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<td>Allo-DOC</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td>Allogeneic</td>
<td>Without</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Auto-DOC</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Allo-DOC</td>
<td>4/6</td>
<td>2/6</td>
</tr>
</tbody>
</table>

*MHC class II-mismatched rabbits (SI >1.3) were selected for skin transplantation and rejection testing. Skin rejection observation displayed that most of the allogeneic skin grafts (\(n\) = 10/12) began to necrose before day 9 postimplantation and completed necrosis before day 12 (\(n\) = 11/12). However, a few autologous skin grafts (\(n\) = 16/6) started necrosis before day 9 and most of them (\(n\) = 56) could survive beyond 12 days postimplantation. Auto, autologous; Allo, allogeneic; the number on the right of the slash represents the total testing numbers.
secrete anti-inflammatory cytokines, such as IL-10 and TGF-β, which are closely related to their immunomodulatory function. After implantation, they do not provoke an immune response at the early stage.

Although the immunosuppressive function of DOC is promising, it cannot be ignored that DOC gradually expressed MHC class II and lost their suppressive activity in vivo. It is reported that POC expressed all immune surface markers which can elicit an immune response, and they are able to act as APCs (42–46). Hence, it is predicted that DOC in vivo are going the way their progeny normally does. Even though this tendency is not optimistic for allogeneic cell therapy, these day 21-differentiated DOC derived from in vitro culture displayed significantly different immune properties from POC, and implanted DOC performed obvious osteogenic function at a very early stage, which facilitates the treatment of bone lesions.

In conclusion, DOC do not express MHC class II under in vitro culture conditions. However, MHC class II expression is inducible upon implantation in vivo. These cells are nonimmunogenic and are able to suppress immune response, but their immunosuppressive function may gradually diminish upon differentiation in vivo. Hence, the question on how to maintain the immunomodulatory function and block the way to immunogenicity of osteogenic cells has attracted particular attention. Because DOC displayed osteogenic function much earlier compared with undifferentiated MSC upon transplantation in situ, this would favor their use in bone tissue engineering. Hence, the cryopreservation and storage of DOC could make a valuable contribution to speedier treatment of bone diseases. Because MSC-derived osteogenic, chondrogenic and adipogenic cells have all been shown to be nonimmunogenic (22), this could suggest that more immunoprivileged cell types can possibly be derived from MSC in the future. If such is the case, then this would bring huge benefits to the field of cell-transplantation therapy.

Acknowledgments


References