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Chondrogenic Differentiation Alters the Immunosuppressive Property of Bone Marrow-Derived Mesenchymal Stem Cells, and the Effect Is Partially due to the Upregulated Expression of B7 Molecules

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ABSTRACT

To investigate the immunosuppressive properties of MSCs, in the present study we examined the immunogenicity of undifferentiated and trilineage-differentiated (chondrocytes, osteoblasts, and adipocytes) rat bone marrow-derived MSCs under xenogeneic conditions. After chondrogenic differentiation, rat bone marrow-derived MSCs stimulated human dendritic cells (hDCs) derived from peripheral blood monocytes, leading to eight- and fourfold higher lymphocyte proliferation and cytotoxicity than that of undifferentiated MSCs. The chondrogenic-differentiated MSCs were chemotactic to hDCs in Dunn chamber chemotaxis system and were rosetted by hDCs in rosette assays. Flow cytometry analysis revealed that chondrogenic-differentiated MSCs had promoted hDC maturation, causing higher CD83 expression in hDCs, whereas undifferentiated MSCs and osteogenic- and adipogenic-differentiated MSCs showed an inhibitory effect on hDC maturation. The costimulatory B7 molecules were upregulated only in the chondrogenic-differentiated MSCs. After blocking B7 molecules with specific monoclonal antibodies in the chondrogenic-differentiated MSCs, CD83 expression of cocultured hDCs was greatly reduced. In conclusion, chondrogenic differentiation may increase the immunogenicity of MSCs, leading to stimulation of dendritic cells. The upregulated expression of B7 molecules on the chondrogenic-differentiated MSCs may be partially responsible for this event. STEM CELLS *2007; 25:364–370*

INTRODUCTION

Unlike hematopoietic stem cells (HSCs), which keep releasing new progeny cells on a regular basis, MSCs are normally quiescent in the bone marrow stroma and serve as a stem cell reservoir. Under certain stimuli, such as trauma, MSCs are mobilized into peripheral tissues and become functionally active. In human trials and animal models, the local implantation of MSCs helped to repair bone and cartilage defects [1, 2]. The systemic infusion of MSCs into sheep and nonhuman primates migrated into a wide range of tissues and undergo site-specific differentiation [3, 4]. Indeed, MSCs are becoming a promising alternative for tissue regeneration [5].

During clinical practice, one of the primary issues concerning cell therapy is the immunocompatibility of the implanted cells. Allogeneic MSCs are immunoregulatory and do not elicit immune response in vitro [6–8] or in vivo [9–12]. It is suspected that MSCs are not immunogenic even under xenogeneic conditions. Human MSCs have engrafted and demonstrated site-specific differentiation after in utero xenotransplantation into sheep, both before and after the expected development of immune competence [13]. The xenotransplantation studies using a cardiac infarction model, however, showed conflicting outcomes with either leukocyte infiltration or functionally active chimeras reported [14, 15]. The present study aimed to investigate the xenogenicity of undifferentiated and chondrogenic-, osteogenic-, and adipogenic-differentiated rat bone marrow-derived MSCs.

MATERIALS AND METHODS

MSC Preparation and Differentiation

Green fluorescent protein (GFP)-transgenic rats (kindly provided by Prof. M. Okabe, Osaka University, Osaka, Japan) were raised and killed under the animal license from the U.K. Home Office [16]. Femur bone marrow was harvested and layered onto Lymphoprep (1.077g/ml; Nycomed, Birmingham, U.K., http://www.nycomed.com) and centrifuged at 1,840 rpm for 30 minutes. The isolated mononuclear cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 μg/ml Fungizone, and 2 mM L-glutamine (Invitrogen, Paisley, U.K., http://www.invitrogen.com) and seeded into T75 flasks (Iwaki 3110-075; Scitech Div., Chiba, Japan, http://www.isisco.ie) at a density of $1–3 \times 10^5$ cells per cm^2. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Passage 1–3 MSCs were subject to specific induction medium (all supplements were pur-

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chased from Sigma-Aldrich [Dorset, U.K., http://www.sigmaaldrich.com] unless otherwise specified). For chondrogenesis, the induction medium was serum-free DMEM supplemented with 100 nM dexamethasone, 0.2 mM ascorbic acid-2-phosphate, 1 mM sodium pyruvate, and ITS+Premix 1:100 dilution (BD Biosciences, Oxford, U.K., http://www.bdbiosciences.com). The medium was changed every other day with 10 ng/ml transforming growth factor-β1 (TGF-β1) (Peprotech EC Ltd., London, http://www.peprotech.com) freshly added each time. The expression of protein marker collagen type II was examined at day 22. The cell pellets with aliquots of 8×10^5 MSCs were also cultured in parallel with the static culture and stained with 1% Alcian Blue (pH 2.5; Sigma-Aldrich) at day 28. For osteogenesis, 10^{-8} M dexamethasone, 0.2 mM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate were added into basic medium, and the medium was changed twice weekly. Alkaline phosphatase (ALP) expression was examined at day 14, and the bone nodules were visualized by alizarin red S (Sigma-Aldrich) after 1 month. For adipogenesis, the supplements were 10^{-6} M dexamethasone, 0.50 mM isobutyl-methylxanthine, and 50 μM indomethacin. The medium was changed twice weekly. The accumulated lipid vacuoles were stained with oil red O at day 13.

Immunocytochemistry and ALP Staining

Expression of collagen type II (chondrogenesis) was examined using the avidin-biotin immunocytochemistry method described previously [17].

ALP staining was carried out using an Alkaline Phosphatase kit according to the manufacturer's instructions (Sigma-Aldrich). The osteogenic culture was incubated in a mixture of naphthol AS-MX phosphate alkaline solution with fast blue BB salt and counterstained with fast red. The resulting blue, insoluble, granular dye deposit indicated sites of alkaline phosphatase activity.

Mixed Human Peripheral Blood Lymphocyte MSC Culture

To examine the immune response of resting human peripheral blood lymphocytes (hPBLs) to xenogeneic MSCs, mixed lymphocyte culture was performed as previously described [18]. The human dendritic cell (hDC) elimination was achieved via negative selection with anti-human CD1a and CD83 antibodies using a Partec fluorescence-activated cell sorting machine (Partec GmbH, Münster, Germany, http://www.partec.de). In 96-well plates, 1×10^4 undifferentiated MSCs (stimulators) were first seeded into each well and underwent induction of trilineage differentiation for 7 days using the specific media described above. All the stimulator cells were treated with 2.5 μg/ml mitomycin C (Sigma-Aldrich) during induction culture. At the end of differentiation treatment, cells were washed twice with phosphate-buffered saline (PBS), and 1×10^5 hPBLs (responders) isolated from the same healthy donor were then added into each well. A positive control ran in parallel, where the stimulator cells were PBLs from the same GFP rat instead of MSCs. The assay was performed using a Cell Proliferation Biotrak enzyme-linked immunosorbent assay system (Amersham Biosciences, Little Chalfont, U.K., http://www.amersham.com). Absorbance was measured at 450 nm using a Multiskan RC plate reader (Alpha Technologies, Larne, U.K.) and analyzed with Genesis software (Dallas, TX, http://www.genesis-software.com).

Cytotoxicity Assay

The assay was performed using a nonradioactive cytotoxicity assay kit (Promega, Southampton, U.K., http://www.promega.com) on 96-well plates. Briefly, 5×10^4 mitomycin C-treated undifferentiated MSCs (target cells) were seeded into each well and underwent trilineage differentiation induction for 2 weeks. At the end of differentiation, the cells were washed with PBS twice. hPBLs (1×10^5) stimulated with phytohemagglutinin (Sigma-Aldrich) (effector cells) were mixed into each well in a final volume of 100 μl of RPMI 1640 with 5% human AB serum (Sigma-Aldrich) and incubated at 37°C for 4 hours in a humidified atmosphere with 5% CO_2. Target cells were lysed with 0.8% Triton X-100 and served as a positive lysis control. The colorimetric detection of lactate dehydrogenase release from cell lysis was read at 492 nm. All data presented were corrected for spontaneous release.

hDC Differentiation and Maturation

Human peripheral blood mononuclear cells were isolated by Ficoll-Paque (1.077 g/ml; Invitrogen) density gradient centrifugation and seeded into 6- and 24-well plates at a density of 1.6×10^6 cells per cm^2 in RPMI 1640 medium supplemented with 10% FBS, 25 mM/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM/l L-glutamine (Invitrogen), 1 mM sodium pyruvate plus 10 μM 2-mercaptoethanol (Sigma-Aldrich). The cells were incubated at 37°C in a humidified atmosphere for 90 minutes to allow monocytes to attach. After vigorous washing, approximately one third of plated cells remained attached, and the supernatant containing unattached cells was replaced with fresh growth medium supplemented with 50 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) and 100 U/ml interleukin 4 (IL-4) (Peprotech). The cells were incubated for 7 days at 37°C to induce hDC differentiation, and one third of the medium was changed at 3.5 days, with the full amount of induction factors added at the same time. Differentiated hDCs were then stimulated with 100 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich) for another 3 days to induce maturation.

Chemotaxis Assay

The Dunn chemotaxis chamber was set up as previously described [19]. Briefly, the inner well of an assay slide was filled with plain DMEM, and the outer well was filled with immature hDCs (chemoattractant) in RPMI 1640 medium to construct a concentration gradient. The undifferentiated and differentiated MSCs were attached to 22- × 22-mm sterile coverslips beforehand. The coverslip was then inverted onto assay slide, sealed with wax, and placed on the heated (37°C) stage of an inverted Nikon microscope equipped with a ×100 phase contrast objective linked to a charge-coupled device camera. The movement of undifferentiated and differentiated MSCs toward hDC gradient was recorded at a time-lapse interval of 10 minutes over a 10-hour period. The data were analyzed using Mathematic 3.0 and AQM 2001 software (Kinetic Imaging Ltd., Manchester, U.K., http://www.anclor.com)

Coculture of Undifferentiated and Differentiated MSCs with hDCs

After stimulation with GM-CSF and IL-4, differentiated hDCs were subject to coculture under either physical contact or separated conditions. Undifferentiated and differentiated MSCs were pretreated with mitomycin C under both conditions. For coculture, undifferentiated and differentiated MSCs were directly added into hDCs culture at a density of 1×10^4 cells per cm^2. For transwell (separated) coculture, undifferentiated and differentiated MSCs were cultured in 0.2-μm Anopore membrane inserts (Nunc, Hereford, U.K., http://www.nuncbrand.com) and loaded into culture plates. After stimulation with LPS for another 3 days, mature hDCs were harvested and ready for flow cytometric analysis. For B7 blocking, chondrogenic-differentiated MSCs were pretreated with monoclonal mouse anti-rat B7.1/CD80 (clone 3H5) and B7.2/CD86 (clone 24F) antibodies (BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen) for 12 hours at a concentration of 25 μg/ml each.

Flow Cytometry

Mature hDCs were washed twice with 5% human AB serum (Sigma-Aldrich) in cold PBS. Separate aliquots of 1×10^5 cells were suspended in the same buffer and incubated for 30 minutes at 4°C in the dark with the following antibodies against human antigens: fluorescein isothiocyanate (FITC)-conjugated anti-CD1a, phycoerythrin (PE)-conjugated anti-CD14 (DAKO, Glostrup, Denmark, http://www.dako.com), FITC-conjugated anti-HLA-DR, PE-conjugated anti-CD83, and isotype-matched FITC- and PE-conjugated negative controls (BD Pharmingen) according to the manufacturer's recommendations. After incubation, the cells were washed twice with PBS and stored at 4°C in 1% paraformaldehyde (Sigma-Aldrich). For B7 staining, the cells were incubated with unconju-

gated monoclonal mouse anti-rat B7.1/CD80 and B7.2/CD80 antibodies mentioned above, followed by a second binding with FITC rabbit anti-mouse antibody (DAKO).

Rosette Assay

After 7 days of stimulation with GM-CSF and IL-4, differentiated hDCs were labeled with red fluorescence using a PKH26 red fluorescence cell linker kit (Sigma-Aldrich) according to the instructions of the manufacturer. Similarly, undifferentiated and differentiated MSCs were treated with PKH67 green fluorescence cell linker to enhance green fluorescence. The rosette assay was performed as previously described [20]. Briefly, PKH-26-labeled hDCs were added into chamber slides (Nunc) at 1×10^5 cells per cm^2, together with 1×10^4 MSCs per cm^2. After 3 days, the cells were washed with PBS, and the dispensed cells were counted using a hemacytometer. The remaining cells were fixed in 2% paraformaldehyde and imaged using the BioRad confocal laser microscope (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com).

Reverse Transcription-Polymerase Chain Reaction and Western Blot

Total RNA was extracted from undifferentiated and differentiated MSCs using Trizol reagent (Invitrogen), and reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's recommendations. The oligonucleotide sequences for B7.1 and B7.2 molecules were from published literature [21, 22]. The polymerase chain reaction (PCR) products were electrophoresed on 1% agarose gels, visualized by ethidium bromide staining, and documented using an FX-20M electrophoresis and documentation system. Western blot was conducted as previously described [18].

Statistics

For data analysis, one-way analyses of variance were performed using statistical package SPSS 13.0 (SPSS Inc., Chicago, http://www.spss.com), followed by the post hoc test of least significant difference. For chemotaxis analysis, the Rayleigh test for unimodal clustering of directions was applied, and $p < .01$ was considered statistically significant.

RESULTS

Trilineage Differentiation of MSCs

The attached colony-forming cells in primary culture showed morphological appearance typical of MSCs (Fig. 1A). For chondrogenesis, collagen type II of static-cultured MSCs was detectable at day 22 by immunocytochemistry and Western blot (Fig. 1B). The pellets were embedded in paraffin and stained with Alcian Blue. Macromorphology showed that the outer ring of the pellet was faintly stained compared with the central core. Most of the differentiated cells in the center were encased in chondrocytic lacunae (Fig. 1C). Under osteogenic induction, the cells were positive for ALP at day 14 (Fig. 1D). One month later, the osteogenic-differentiated cells congregated and formed bone nodules, which were stained with alizarin red S (Fig. 1E). Under adipogenic induction, accumulated lipid vacuoles within cells were visualized by oil red O staining at day 13 (Fig. 1F).

The Interaction of Chondrogenic-Differentiated MSCs with Dendritic Cells

After chondrogenic differentiation, the hPBL-stimulating and cytotoxicity-inducing effects of MSCs increased approximately eight- and fourfold, respectively, compared with undifferentiated MSCs (Fig. 2). MSCs that underwent osteogenic and adipogenic differentiation showed no such effect. When dendritic cells (DCs) were removed from the hPBLs, chondrogenic-differentiated MSCs failed to stimulate hPBLs proliferation, indicating that T lymphocyte recognition of chondrogenic-differentiated MSCs required the antigen-presenting cells DCs (Fig. 2A). Similarly, the cytotoxicity assay showed that the stimulated

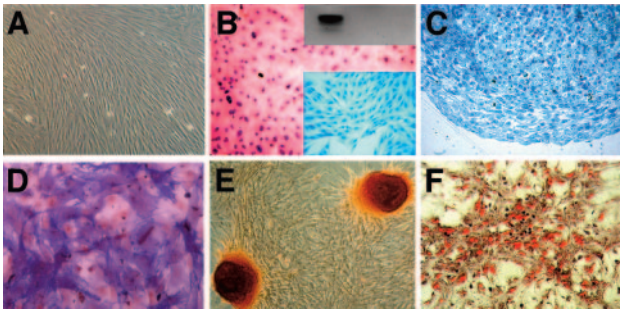

Figure 1. MSC isolation and differentiation. **(A):** Rat bone marrow-derived MSCs reached confluence (magnification, ×200). **(B):** For chondrogenic induction, collagen type II was detectable by immunostaining in two-dimensional culture cells at day 22 (magnification, ×200). Insets, negative control (bottom) and Western blot (top) of type II collagen). **(C):** After 4 weeks, the cell pellets were sectioned and stained with Alcian Blue to show typical chondrocytes (magnification, ×200). **(D):** For osteogenic induction, the cells were positive for ALP staining at day 14 (magnification, ×400). **(E):** The bone nodules were formed at day 28 as shown in alizarin red S stain (magnification, ×200). **(F):** For adipogenic induction, the accumulated lipid vacuoles were stained with oil red O at day 13 (magnification, ×200).

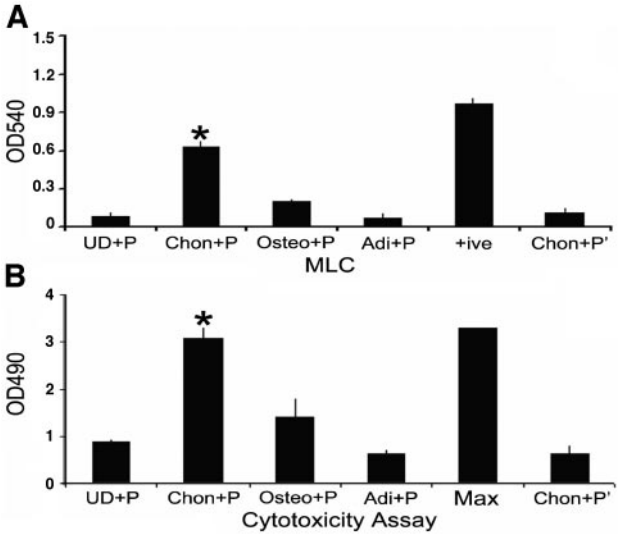

Figure 2. Chondrogenic-differentiated MSCs react with human peripheral blood lymphocytes (hPBLs) in the presence of human dendritic cells (hDCs). **(A):** Chondrogenic-differentiated MSCs stimulated hPBL proliferation in mixed hPBL MSC culture. The stimulation was diminished after the depletion of hDCs in the culture. The stimulator cells used were green fluorescent protein (GFP) rat bone marrow-derived UD, Chon, Osteo, and Adi MSCs. The responders were P or P′ from healthy donors. The peripheral blood lymphocytes from the MSCs-providing littermate GFP rat were used as stimulators in +ive. **(B):** Chon MSCs induce cytotoxic T lymphocyte-mediated cytotoxicity in cytotoxicity assay. The cytotoxic effect was not seen after the depletion of dendritic cells in the culture. The positive controls were cells lysed by 0.8% Triton X-100 (maximum). *, $p < .05$, compared with undifferentiated MSCs (UD+P) using analysis of variance. The data are pooled from three independent experiments. Abbreviations: +ive, positive control; Adi, adipogenic-differentiated; Chon, chondrogenic-differentiated; MLC, mixed lymphocyte culture; Osteo, osteogenic-differentiated; P, unpurified human peripheral blood lymphocytes; P′, human dendritic cell-depleted human peripheral blood lymphocytes; UD, undifferentiated.

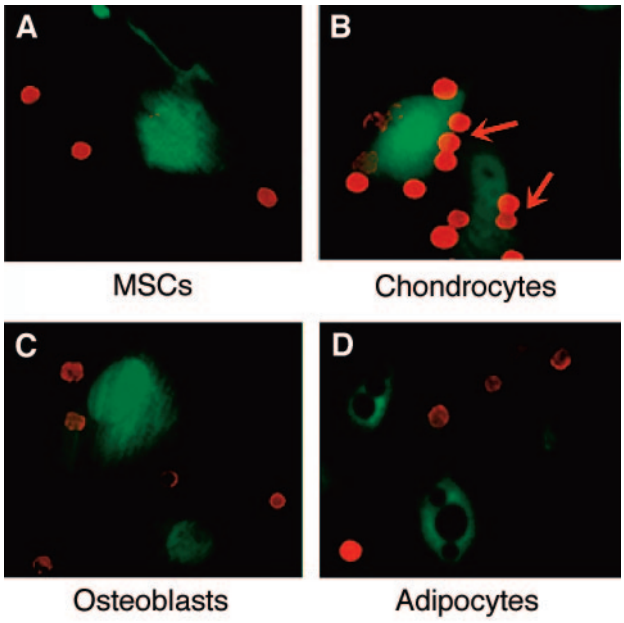

Figure 3. Chondrogenic-differentiated MSCs are capable of rosetting (binding) human dendritic cells (hDCs). PKH-26-labeled (red) hDCs (1×10^5 cells per cm^2) were cocultured with green fluorescent protein undifferentiated, chondrogenic-, osteogenic-, and adipogenic-differentiated MSCs (1×10^4 cells per cm^2) at 37°C for 3 days. After washing, the remaining cells in the culture were visualized by confocal microscopy (magnification, ×400). hDCs formed rosette rings around cocultured chondrogenic-differentiated MSCs **(B)** (red arrow), whereas only sparsely distributed hDCs were seen in other groups **(A, C, D)**. The binding rate of chondrogenic-differentiated MSCs was 45%; for the other groups, it was 4%–7%.

cytotoxic T lymphocytes were not able to recognize and destroy chondrogenic-differentiated MSCs in the absence of DCs (Fig. 2B).

Rosette binding assay and Dunn chemotaxis analysis were performed to assess the physical interaction between hDCs and MSCs. The results showed that hDCs had a high binding affinity for chondrogenic-differentiated MSCs, as seen by the typical ring-like rosettes formed around chondrogenic-differentiated MSCs by confocal microscopy (Fig. 3). No such effect was observed in any other groups wherein only sparsely distributed hDCs remained in the culture. The binding rate for chondrogenic-differentiated MSCs was 45%, significantly higher than the 4%–7% of the others ($p < .05$). For Dunn chamber chemotaxis analysis, chondrogenic-differentiated MSCs showed directional migration toward hDCs over the 10-hour observation period ($p < .01$; Fig. 4B, left), with a significant clustering of cell trajectories (Fig. 4B, right). Undifferentiated and osteogenic- and adipogenic-differentiated MSCs showed random migration, indicating the absence of chemotactic response (Fig. 4C). The migration speed was not significantly different among all groups.

The final step to demonstrate MSC-hDC interaction was to analyze the effects of undifferentiated and differentiated MSCs upon the maturation process of DCs. After coculture with chondrogenic-differentiated MSCs, 84% of hDCs were positive for CD83 expression, which was similar to normally matured hDCs (87% positive for CD83). Osteogenic- and adipogenic-differentiated MSCs did not exhibit such maturation-promoting effect upon hDCs; CD83 expressions in these two groups were similar to that of undifferentiated MSCs (14%–17%; Fig. 5). CD83 expression did not change significantly when hDCs and MSCs (undifferentiated or differentiated) were cocultured in the transwell system (data not shown), suggesting that CD83 upregulation in hDCs needs direct cell-cell interactions. The expressions of other surface markers, including HLA-DR, CD14, and CD1a, on hDCs of all groups were not significantly different after the coculture treatment.

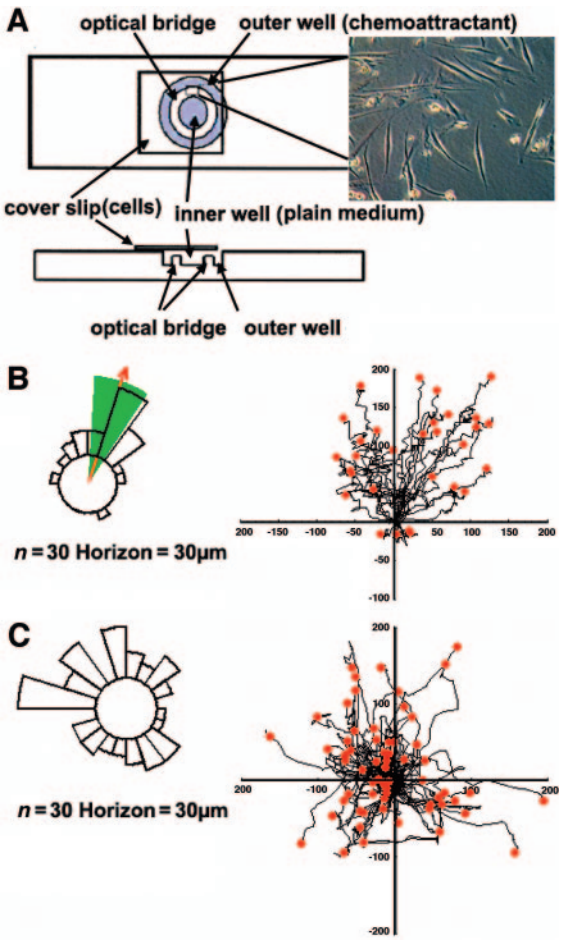

Figure 4. Dunn chemotaxis assay with human dendritic cells (hDCs) as chemoattractants. The directional movements of undifferentiated and chondrogenic-, osteogenic-, and adipogenic-differentiated MSCs toward chemoattractant hDCs were assessed by Dunn chemotaxis chamber. **(A):** The setup of Dunn chemotaxis chamber. **(B):** Chondrogenic-differentiated MSCs showed significant movement toward dendritic cells. Circular histograms represent the proportion and moving direction of chondrogenic-differentiated MSCs (left). The horizon was the distance from the starting point to a virtual horizon, which was chosen to be 30 μm for all the experiments. The cells moving beyond this distance were valid for statistical analysis. The arrows and the green sector represent the mean significant direction of migration and 99% confidence interval ($p < .01$, Rayleigh test). Scatter graphs depict trajectories and endpoints (red spots) of the cell migrations (right). **(C):** Representative chemotactic assay results from undifferentiated and osteogenic- and adipogenic-differentiated MSCs, showing random cell movement and no significant directional migration (chemotaxis). Abbreviation: N, total cell number tracked each time.

Involvement of B7 Molecules in the Regulation of Immunogenicity of Chondrogenic-Differentiated MSCs

Costimulatory B7 molecules are involved in the initiation of immune reactions, and lack of their expressions was reported in the immunosuppressive effects of MSCs [8, 23]. We therefore examined the B7 mRNA expressions of trilineage-differentiated

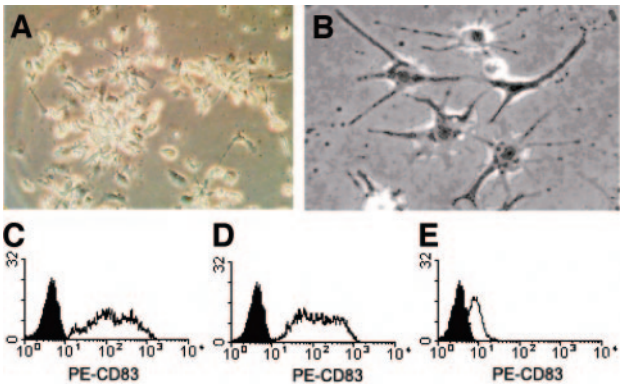

Figure 5. Phenotypic analysis of human dendritic cells (hDCs) after direct contact coculture with undifferentiated and differentiated MSCs. Human peripheral blood monocytes were incubated for 7 days in medium containing granulocyte macrophage-colony stimulating factor and interleukin 4. Differentiated but immature hDCs **(A)** (magnification, ×100) were cocultured with 1×10^4 cells per cm^2 mitomycin C-treated undifferentiated and chondrogenic-, osteogenic-, and adipogenic-differentiated MSCs or hDCs alone. After an additional 3 days of stimulation with lipopolysaccharide, mature hDCs **(B)** (magnification, ×400) were harvested and used for flow cytometric analysis using the following antibodies: fluorescein isothiocyanate (FITC) anti-CD1a, PE anti-CD14, FITC anti-HLA-DR, PE anti-CD83, and isotype-matched controls. The CD83 expression on hDCs was 84% after coculture with chondrogenic-differentiated MSCs **(C)**, similar to 87% of positive control **(D)**. In contrast, the CD83 expression on hDCs was 14%–17% after coculture with undifferentiated and osteogenic- and adipogenic-differentiated MSCs **(E)**. Shaded lines, isotype-matched controls; open lines, experimental cells. The data are typical of three independent experiments. Abbreviation: PE, phycoerythrin conjugate.

MSCs. Reverse transcription (RT)-PCR and flow cytometry data revealed that the expressions of both B7.1/CD80 and B7.2/CD86 were upregulated in chondrogenic-differentiated MSCs (Fig. 6), although a weak expression of B7.2/CD86 mRNA was also detected in osteogenic-differentiated MSCs using RT-PCR. Undifferentiated and adipogenic-differentiated MSCs showed no expression of either molecule.

To further examine whether B7 molecules were responsible for modulating the immunogenicity of chondrogenic-differentiated MSCs, two monoclonal antibodies specific for B7.1/CD80 and B7.2/CD86 molecules were used for blocking experiments. The expression of maturation marker CD83 on hDCs after coculture with B7-blocked chondrogenic-differentiated MSCs was reduced to 23.4%, in contrast to 80.0% of the control group without blocking antibodies (Fig. 7). If only either single molecule was blocked, there was no significant effect on CD83 expression of hDCs (data not shown).

DISCUSSION

After adopting different lineage pathways, MSCs change certain properties to suit specific functions of their terminally differentiated progenies. For chondrocytes, the anatomical location in joints somehow predisposes them to the immunological factors and contributes to the pathogenesis of various autoimmune diseases. Naturally occurring chondrocytes from many species have been recognized by immune cells in vitro [24]. After in vivo transplantation, the allogeneic chondrocytes induce both humoral and cell-mediated cytotoxicity within 2–3 weeks [25]. Yuan et al. reported that chondrocytes from many species, including human, dog, and rabbit, express B7 molecules [26]. The expression may be further upregulated under certain pathogenic conditions, such as rheumatoid arthritis. This characteristic expression of costimulatory molecules such as B7 may explain the susceptibility of cartilage tissues to the influence of immune reactions triggered by various inflammatory factors. In addition, the upregulated B7 molecules on chondrogenic-differentiated MSCs can serve as foreign antigens and directly stimulate hDCs, leading to enhanced proliferation of T lymphocytes. There are structural similarities between B7 molecules and some major histocompatibility complex (MHC)-related molecules, such as butyrophilin, myelin/oligodendrocyte glycoprotein, and the chicken MHC molecule B-G, all of which share the ability to enhance immune responses [18]. Since HLA class II is not expressed in chondrogenic-differentiated MSCs [27], the upregulated B7 molecules in these cells may serve as an alternative to HLA II and elicit immune rejection. We therefore speculate that B7 molecules serve not only as CD28 ligands to assist the immune response but also as a main immunogenic signal producer that is recognized by DCs.

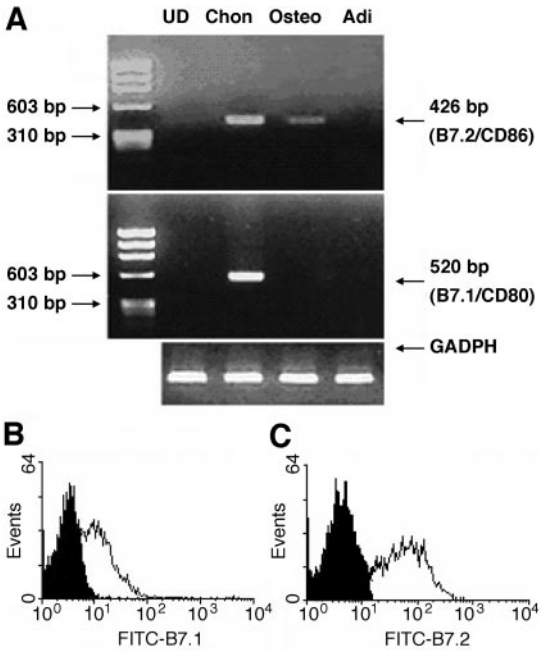

Figure 6. The expressions of costimulatory B7 molecules on undifferentiated and differentiated MSCs. Cells including UD, Chon, Osteo, and Adi MSCs were processed for analysis. **(A):** Reverse transcription-polymerase chain reaction data showed that Chon MSCs were positive for both B7 molecules, and Osteo MSCs showed faint expression of CD86/B7.2. No expression of either molecule was seen in UD or Adi MSCs. cDNA products were 520 bp for B7.1/CD80 and 426 bp for B7.2/CD86. GAPDH was the internal control. **(B, C):** Flow cytometry data showing surface expression of B7 molecules in Chon MSCs with corresponding antibodies (lined histogram) and isotype controls (shaded histogram). Abbreviations: Adi, adipogenic-differentiated; bp, base pairs; Chon, chondrogenic-differentiated; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Osteo, osteogenic-differentiated; UD, undifferentiated.

It is suspected that TGF-β1 contributes to the B7 upregulation during chondrogenesis induction and subsequent immune reactions. However, we did not observe any direct correlation between TGF-β1 supplementation and B7 expression on primitive MSCs in preliminary experiment. In fact, the anti-inflammatory TGF-β1 has been reported in the immunosuppression of MSCs. MSCs and osteogenic-differentiated MSCs all secrete TGF-β1 under physiological condition [6, 28]. In transwell coculture, the increased expression of both transforming growth factor (TGF)-β1 and TGF-β1R provided an autocrine loop for

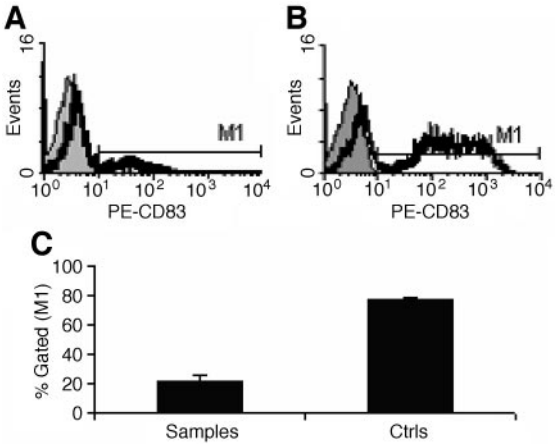

Figure 7. Maturation marker CD83 was expressed in human dendritic cells (hDCs) after coculture with B7-blocked chondrogenic-differentiated MSCs. Differentiated but immature hDCs were cocultured with B7-blocked chondrogenic-differentiated MSCs in the presence of lipopolysaccharide for 3 days. Flow cytometry examination showed that CD83 expression on hDCs was significantly (lower) inhibited after blocking B7 molecules **(A)**, whereas the corresponding control group without blocking antibodies showed normal (higher) expression of CD83 **(B)**. There were significantly more CD83 positive cells (mean × SD of gated positive cells of M1) in the three samples measured **(C)**. Shaded lines, isotype-matched Ctrls; open lines, experimental cells. Abbreviation: Ctrls, controls.

the propagation and education of immunosuppressive MSCs upon B cells [6]. Among others, TGF-β1 is involved in the suppression of T lymphocyte proliferation [23]. TGF-β1 may also involve in inhibiting of activation and maturation of DCs when cocultured with MSCs [8]. Taken together, TGF-β1 is not a decisive factor for the altered immunogenicity of chondrocytes. Even if traces of it remains in the culture, the effect should be immunosuppressive, not stimulatory, as we have seen.

Current results showed that undifferentiated MSCs prevented hDC maturation as demonstrated by the low expression of CD83. However, such effects were not observed in the transwell coculture system, suggesting that direct cell-cell contact between MSCs and DCs is required for the effect. Jiang et al. recently reported that physical contact is necessary for the inhibitory effect on CD83 expression in low-ratio MSC/DC coculture (1:20 and 1:50) [29]. On the contrary, at a higher ratio of MSCs/DCs (1:10), transwell culture produced inhibitory results similar to those of direct contact culture. The ratio of MSCs/hDCs used in the present was approximately 1:50, within the range of low-ratio culture reported in the previous study. Similarly, Krampera et al. also suggested that cell-cell contact is essential for the observed inhibitory effect of MSCs upon naïve and memory antigen-specific T-cells [30]. The suppressive signals of MSCs may be produced in the forms of soluble factors (at certain concentrations), and when the cell-cell ratio is low, direct contact is required to provide a continuous supply of these soluble factors in the coculture system to maintain the inhibitory effects.

We also observed that osteogenic and adipogenic differentiation did not change the immunological properties of MSCs. Osteogenic-differentiated MSCs from rabbit and human are low-immunogenic, but the reason remains unknown [18, 28]. In adipocyte, the most abundant protein, adiponectin, is immunosuppressive, as it induces the production of the anti-inflammatory mediators and impairs the production of the proinflammatory cytokines [31]. The adipose tissue has immunosuppressive function per se to certain extent. As the obesity develops, the immune function becomes less and less sufficient, and more and more anti-inflammatory immune cells are therefore recruited [32]. Our results reckon that adipose tissue, or adipose tissue-derived product, may be the cause for the impairment of immunological process in clinical adiposity condition [32]. However, only induced adipocytes were examined in the current study; whether naturally occurred adipocytes behave in a manner similar to the induced adipocytes needs further investigation.

In conclusion, chondrogenic differentiation may increase the immunogenicity of MSCs, leading to stimulation of DCs. The upregulated expression of B7 molecules on the chondrogenic-differentiated MSCs may be partially responsible for this event.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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