

Interleukin (IL)-10 Induced by CD11b⁺ Cells and IL-10-Activated Regulatory T Cells Play a Role in Immune Modulation of Mesenchymal Stem Cells in Rat Islet Allografts

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Mesenchymal stem cells (MSCs) are suggested to be immune modulators because of their therapeutic potential in transplantation. In the present study, we evaluated the therapeutic potential of autologous MSCs for preventing graft rejection after allogeneic rat islet transplantation. We assessed the ability of MSCs to elicit an antiproliferative response in alloreactive lymphocytes and tested the immunosuppressive effect of MSCs in allogeneic islet transplantation. In islet allotransplantation, injection of autologous MSCs or a subtherapeutic dose of cyclosporine A (CsA; 5 mg/kg) alone did not prolong allograft survival. However, graft survival was attained for >100 d in 33% of autologous MSC-plus-CsA-treated recipients, indicating that graft acceptance was achieved in a subgroup of allograft recipients. Splenocytes from autologous MSC-plus-CsA-treated rats exhibited a reduced mixed lymphocyte reaction (MLR)-proliferative response to donor stimulators and increased interleukin (IL)-10 release. Interestingly, after excluding host CD11b⁺ cells, splenic T cells from autologous MSC-plus-CsA-treated rats did not produce IL-10 or did not inhibit proliferative responses under the same conditions. The use of autologous MSC-plus-CsA downregulated immune responses, inducing donor-specific T-cell hyporesponsiveness by reducing the production of proinflammatory cytokines and inducing antiinflammatory cytokine production, especially that of IL-10, during the early posttransplantation period. T-regulatory cells made a contribution at a later phase. In conclusion, the combined use of autologous MSCs and low-dose CsA exerted a synergistic immunosuppressive effect in an islet allograft model, suggesting a role for autologous MSCs as an immune modulator.

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INTRODUCTION

Type 1 diabetes is an autoimmune disease caused by destruction of insulin-producing pancreatic islet cells. Currently, islet transplantation is considered a less effective treatment modality for Type 1 diabetes than pancreas transplantation, especially from the viewpoint of long-term graft survival. There are two major impediments to the clinical application of islet transplantation: immune destruction of transplanted islets (1) and the limited supply of islet tissue (2–4). The islet rejection process is characterized by rapid infiltration of immune cells, followed by antigen-specific T-cell responses. Among the strategies used to

overcome immune rejection are the use of novel immunosuppressive agents and regimens and donor-specific induction of immune tolerance in the host. Mesenchymal stem cells (MSCs) are self-renewing, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages. It has been suggested that MSCs escape the immune system because they possess a cell surface phenotype that is poorly recognized by T cells. MSCs also mediate their immunosuppressive action through the secretion of cytokines (5). In line with their immunosuppressive capacities *in vitro*, MSCs have also been shown to display immunosuppressive capacities *in vivo*, as

evidenced by the demonstration that allogeneic MSCs prolonged allograft survival in immunocompetent mice (6). On the basis of their capacity to modulate immune responses and to promote tissue repair in experimental models, MSCs have been proposed as a treatment for autoimmune diseases, such as diabetes, rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis. The use of MSCs to prevent rejection of allogeneic grafts is still mostly limited to animal models, and the results obtained to date are conflicting. In the present study, we evaluated the therapeutic potential of autologous MSCs for prevention of graft rejection after allogeneic islet transplantation. In addition, we evaluated the expression profiles of pro- and antiinflammatory cytokine genes in the grafted site, serum cytokine levels, immune responses in splenocytes of recipients after allostimulation and the frequency of T-regulatory cells (Tregs) in secondary

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lymphoid organs. The objective of these measurements was to determine whether any of these factors played a role in modulating the immune response after MSC injection, thereby influencing islet allograft tolerance.

MATERIALS AND METHODS

Animals

Male Lewis (RT1¹) and Fisher (RT1^{1v1}) rats <8–10 wks of age were used as donors and recipients, respectively. Fisher rats were purchased from Charles River Japan (Kanagawa, Japan). Lewis rats were bred and maintained at Asan Medical Center Animal Facilities. Recipients were rendered diabetic by a single injection of streptozotocin (35 mg/kg body weight in citrate buffer, pH 4.5; Sigma, St. Louis, MO, USA) 21 d before transplantation. An animal was considered diabetic when its blood glucose level exceeded 200 mg/dL in two consecutive measurements. This experiment was approved by the Institutional Animal Care and Use Committee of Asan Institute for Life Sciences, Asan Medical Center (Seoul, South Korea; review number 2008-13-091), and conducted in accordance with the guidelines of the Asan Institute for Life Sciences for Experimental Animal Care and Use.

Islet Isolation

Lewis pancreatic islets were isolated and purified using the Ficoll purification method (7). Briefly, the pancreas of each rat was distended with a 10-mL intra-ductal injection of collagenase type XI (800 units/mL; Sigma), and pancreas digestion was performed at 37°C. Digested pancreatic tissue was mechanically disrupted by filtering through a mesh (400- μ m pore size). Islets were purified by discontinuous density gradient centrifugation using Ficoll (Sigma). Islet numbers and purity were determined by dithizone staining. Before transplantation, islets in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) were cultured overnight in a 37°C, 5% CO₂ environment.

Isolation and Culture of MSCs

Bone marrow (BM)-derived MSCs were harvested from 8-week-old (200–250 g) male Fisher rats. After removing epiphyses and gaining access to the marrow cavities, whole BM plugs were flushed out from tibial and femoral bones with low-glucose Dulbecco's modified Eagle's medium (DMEM-LG; Invitrogen, Carlsbad, CA, USA) using a 10-mL syringe with a 22-gauge needle. The cell suspension was filtered through a nylon sieve (100 μ m) and centrifuged for 5 min at 450g. Collected cells were then resuspended in serum-supplemented medium. After counting, cells in DMEM-LG supplemented with 10% (v/v) FBS and 1% (v/v) Gibco™ Antibiotic-Antimycotic (Invitrogen) were plated in 25- to 75-cm² flasks (BD Biosciences, San Jose, CA, USA) at a concentration of 5×10^6 nucleated cells/mL/cm² and incubated in 5% CO₂ at 37°C. After 24 h, the medium was changed, unattached cells were discarded, and the adherent cells were cultured until they reached 80% confluence. Cells were then detached from the bottom using a 0.25% Trypsin-EDTA (Invitrogen). The resulting suspension was then expanded by plating at 6,000 cells/cm² in 75-cm² flasks. The same conditions were used for subsequent passages. At least two independently isolated batches of MSCs were used in each experiment.

Phenotypic Analysis of MSCs

MSCs (passage 3–5) were resuspended in phosphate-buffered saline (PBS), pH 7.2, containing 1.0% (w/v) bovine serum albumin (BSA; Sigma) and 0.1% (w/v) sodium azide (Sigma). The suspensions were incubated with monoclonal antibodies (mAbs) for 30 min at 4°C. The following mAbs, purchased from Becton-Dickinson Pharmingen (San Diego, CA, USA), were used: fluorescein isothiocyanate (FITC)-conjugated antirat RT1A, CD90.1, CD 29, CD25 or CD11b; phycoerythrin (PE)-conjugated antirat RT1B, CD4 or CD54; and cychrome-conjugated antirat CD45. Each fluorescence analysis

included the appropriate FITC-, PE- or cychrome-conjugated isotype Ab controls. Cells were separated using a flow cytometry (FACSCalibur; Becton-Dickinson, San Diego, CA, USA) and not sorted.

In Vitro Differentiation

Rat BM-MSCs were also assessed for adipogenic and osteogenic differentiation *in vitro* by using the Trevigen's rat mesenchymal stem cell differentiation kit following the manufacturer's protocol (Trevigen, Gaithersburg, MD, USA).

Osteogenic differentiation. The MSCs were plated in 24-well plates and cultured in an osteogenic medium. The osteogenic medium consisted of DMEM (Invitrogen) supplemented with 10% FBS, 50 μ g/mL ascorbic acid, 10 mmol/L β -glycerol phosphate, 10^{-7} mol/L dexamethasone and 1% penicillin/streptomycin. The cells were maintained in culture with medium changes every 3 d for 14 d. Undifferentiated MSCs were grown for 14 d in complete growth medium. After 14 d, the media were removed, and the cells were rinsed in PBS, fixed in 10% formalin and stained with alizarin red S (8). The plates were treated with the alizarin red solution and incubated for 5 min at room temperature. After 5 min, the plates were rinsed in distilled water and then examined under a light microscope and photographed. To quantify the mineralization, extraction of alizarin red staining was performed. A solution (300 μ L) containing 10% acetic acid and 20% methanol was added to each well. The plates were incubated at room temperature and shaken for 15 min. The supernatant was removed into a 1.5-mL tube, and 200 μ L were used to read at 405 nm in 96-well plates. To obtain an optical density between 0.1 and 2, all samples cultured in osteogenic medium were diluted four times and measured using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Amounts of extracted stain from wells were calculated with the alizarin red included in each sample relative to the standard curve (5–1,000 μ g/mL).

Adipogenic differentiation. For adipogenic differentiation, the MSCs were plated in 24-well plates in adipogenic medium at a cell density of 5×10^3 cells per well. The adipogenic medium was composed of DMEM with low glucose supplemented with 10% FBS, 0.1 mmol/L indomethacin, 0.5 mmol/L isobutylmethylxanthine and 10^{-6} mol/L dexamethasone (9). Undifferentiated MSCs were grown for 14 d in complete growth medium. The media were replaced every 3 d for 14 d. Adipogenic differentiation was assessed by oil red O staining at 3 wks after initial adipogenic induction. For oil red O staining, the cells were rinsed in PBS and fixed in 10% formalin followed by incubation of the cells in 2% (w/v) oil red O reagent for 5 min at room temperature. The cells were rinsed in isopropanol followed by several changes of distilled water and were then examined under a light microscope and photographed. Cells stained were dissolved in 100% isopropanol for 10 min with shaking, and 200 μ L of the sample were transferred to a 96-well plate. Extracted oil red O was measured using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices) at 500 nm and calculated the oil red O present in each sample relative to the standard curve (50–500 μ g/mL).

Mixed Lymphocyte Reaction Assay

Immunosuppressive effect of MSCs on mixed lymphocyte reaction (MLR). Fisher splenocytes (responder; 2.5×10^5 /well) and irradiated Lewis splenocytes (stimulator; 5×10^5 /well) were cocultured with or without MSCs for 3 d. Autologous or allogeneic MSCs were plated in triplicate onto round-bottom 96-well plates (BD Biosciences) in decreasing numbers (25×10^4 , 12.5×10^4 , 6.25×10^4 and 3.13×10^4 cells/well) and allowed to adhere to the plate for 1–2 h. The same procedure was followed in transwell chambers (0.2 μ m; Corning, Corning, NY, USA). MSCs (2.5×10^5 /well) were seeded in the lower chamber and allowed to adhere for 1–2 h. Equal numbers of Fisher splenocytes and irradiated Lewis splenocytes

were then cultured in the upper transwell chamber for 3 d. For secondary MLRs, Fisher splenocytes (2.5×10^5 /well), obtained at the end of the primary MLR performed with either MSCs or MSCs and cyclosporine A (CsA) (300 nmol, Sandimmune; Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA) for 7 d, were washed twice with PBS to eliminate any trace of MSCs and CsA. They were then restimulated with irradiated splenocytes (5×10^5 /well) from Lewis rats (the same allogeneic condition as for primary MLR), Wistar Furth rats (WF, RT1u, third party) or concanavalin A (ConA; 1 μ g/well) for 3 d.

T-cell proliferation in splenocytes of rats transplanted. Stimulator cells were prepared from splenocytes of Lewis rat donors. The cells were irradiated and seeded into 96-well U-bottom culture plates (NUNC, Roskilde, Denmark). Splenocytes isolated from each treatment recipient were used as responder cells and purified by centrifugation on Ficoll density gradients (Histopaque 1077; Sigma) and stored in liquid nitrogen until used. In another experiment, T cells were purified from splenocytes of transplanted rat by negative selection with anti-major histocompatibility complex (MHC) class II (OX6) microbeads (Miltenyi Biotec, Auburn, CA, USA). Thawed splenocytes (2.5×10^5 cells/well) were mixed with irradiated (25 Gy; Cs irradiator, Cisbio International, Bedford, MA, USA) allogeneic splenocytes (5×10^5 /well) and plated on 96-well U-bottom culture plates (NUNC). To investigate cytokine interactions in MLR, neutralizing Abs directed against rat IL-10 or transforming growth factor (TGF)- β (R&D Systems, Minneapolis, MN, USA) were added at a concentration of 0.5 μ g/mL on day 0 to block IL-10 or TGF- β . Normal goat IgG and IgG1 Abs (R&D Systems) at the same concentration were used as negative controls. Twenty-four hours before termination of the culture, 1 μ Ci [3 H]-thymidine (Amersham Bioscience, Arlington Heights, IL, USA) was added to each well and harvested 18 h later. Thymidine uptake was quantified in a

β -counter (TopCount NXT; PerkinElmer, Waltham, MA, USA). T-cell proliferation is expressed as mean cpm \pm standard deviation (SD) of three wells.

Islet Transplantation

Islets (4,000 islet equivalents/rat) and MSCs (2×10^6 cells/rat) were transplanted into recipient rat via the portal vein. Four groups of diabetic Fisher rats, with four to six animals per group, were transplanted with islets. CsA was diluted in PBS and administered once daily for 14 consecutive d at 5 mg/kg, intraperitoneally (i.p.). Group 1 received donor islets alone, group 2 received donor islets with MSCs, group 3 was treated with CsA (5 mg/kg/day) for 2 wks, and group 4 was treated with both MSCs and CsA (5 mg/kg/day, for 2 wks). Graft function was monitored by daily measurement of blood glucose and at least twice thereafter. Islet rejection was defined as blood glucose values >200 mg/dL on 2 consecutive days.

Immunohistochemistry

At day 5 around the date of rejection or day 100 after islet graft, recipient rats were killed in each group. Two to four sections from the median and left lateral lobe of rat liver were sampled for the pathological examination. Tissues, bearing the islet grafts, were placed in 10% (v/v) formalin before immunohistochemistry. The paraffin sections were deparaffinized in xylene, hydrated through graded ethanol and immersed in absolute methanol containing 0.3% hydrogen peroxide for 3 min to block endogenous peroxidase activity. Sections were incubated with nonimmune horse serum for 20 min to prevent nonspecific binding, then with the primary antiserum diluted in PBS for 1 h and finally with diluted biotinylated secondary antibody. Sections were incubated for 30 min with the ABC reagent (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) and then with the peroxidase substrate diaminobenzidine. The primary antibodies and their dilutions were as followed: anti-insulin (1:100; Dako,

Glostrup, Denmark), anti-CD4 (1:200; Serotec, Oxford, UK) and anti-CD8 (1:200, Serotec). Slides were counter-stained with hematoxylin and eosin (H&E).

Intracellular Staining for Foxp3 and IL-10

Peripheral blood mononuclear cells (PBMCs), graft-infiltrating lymphocytes, splenocytes and draining lymph node (LN) cells were preincubated with Fc block to prevent nonspecific binding to Fc γ receptors, and cell surface proteins were stained using FITC-labeled anti-CD25 (OX39) and PE-labeled anti-CD4 (OX38) mAbs. Intracellular Foxp3 was stained using allophycocyanin (APC)-labeled anti-Foxp3 mAb (FJK-16s; eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. For intracellular IL-10 staining, several samples were stimulated with alloantigen for 24 h; Golgi-Stop was included for the last 6 h of stimulation. The harvested cells were stained with FITC-labeled anti-CD4 and anti-CD11b mAbs and then processed for intracellular staining with PE-labeled anti-IL-10 mAb (JES5-16E3). Anti-CD4, anti-CD11b, anti-CD25 and anti-IL-10 mAbs, except those for anti-Foxp3 mAb, Fc block and isotype IgGs, were purchased from BD Biosciences. Samples were assessed on a FACSCalibur instrument using CellQuest software (BD Biosciences).

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

After transplantation, liver samples were snap-frozen in liquid nitrogen and stored at -80°C until used. Total RNA was isolated using TRIzol (Invitrogen), and cDNA was synthesized from a 1- μg RNA template using random hexamers and AccuPower[®] RT PreMix (Bioneer, Daejeon, South Korea). One-twentieth of the RT reaction mixture was added to the PCR. Rat primer sequences for IL-2 (NM_053836.1), IL-4 (NM_201270.1), interferon (IFN)- γ (NM_138880), IL-10 (X60675), TGF- β 1 (NM_021578.2), Foxp3 (NM_001108250.1)

and GAPDH (BC059110) were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-TCA TGA CCA CAG TCC ATG CCA-3', GAPDH antisense, 5'-GGG AGT TGC TGT TGA AGT CAC-3'; IL-2 sense, 5'-TTG CAC TGA CGC TTG TCC TCC TTG TCA ACA-3', IL-2 antisense, 5'-CCA TCT CCT CAG AAA TTC CAC CAC AGT TGC-3'; IL-4 sense, 5'-AGG TCA ACA CCA CGG AGA AC-3', IL-4 antisense, 5'-AGT TCA GAC CGC TGA CAC CT-3'; IFN- γ sense, 5'-TAC ACG CCG CGT CTT GGT TTT-3', IFN- γ antisense, 5'-AGC CTA AGG AAG CGG AAA AGG-3'; IL-10 sense, 5'-GGT GAC AAT AAC TGC ACC CAC-3', IL-10 antisense, 5'-GTG TCA CGT AGG CTT CTA TGC-3'; TGF- β 1 sense, 5'-GCC TCC GCA TCC CAC CTT TG-3', TGF- β 1 antisense, 5'-GCG GGT GAC TTC TTT GGC GT-3'; and Foxp3 sense, 5'-GCA CAA GTG CTT TGT GCG AGT-3', Foxp3 antisense, 5'-TGT CTG TGG TTG CAG ACG TTG-3'. PCR was performed at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 60°C for 45 s and 72°C for 1 min. Amplified products were analyzed on 1.5% agarose gels. Band densities were quantified using the Bio-Rad Quantity One program (Bio-Rad, Hercules, CA, USA). GAPDH was used as an endogenous control to normalize RNA amounts.

Statistical Analysis

Data were expressed as means \pm SD. Statistical comparisons among groups were performed by Student *t* test or analysis of variance (ANOVA) followed by Bonferroni posttests for multiple comparisons. Differences were considered statistically significant at *P* values <0.05 .

RESULTS

Characterization of MSCs from Adult Rat Bone Marrow

Adherent MSCs had a spindle-shaped fibroblastic morphology (Figure 1A, a and b). To test whether multilineage differentiation of BM-derived MSCs is possible, MSCs were induced to differentiate into osteoblast and adipocyte (Figure 1A). When MSCs were tested for their

potential to differentiate into adipocytes at passage 3, morphologic changes in the cells as well as the formation of neutral lipid vacuoles were noticeable as early as day 7 after induction. At day 14, accumulation of lipid vacuoles within and around the cells was visualized by staining with oil red O (Figure 1A, e). Osteogenic differentiation was studied by alizarin red S staining for each substrate in control and osteogenic media. Alizarin red staining is an indicator of mineralization of the extracellular matrix. Undifferentiated MSCs were used as a negative control, and staining for mineralization was negative. In contrast, MSCs cultured in the osteogenic medium were positive in alizarin staining. BM-MSCs exhibited classic mineralization nodules, and alizarin red staining was more intense for MSCs than for fibroblasts (Figure 1A, h). After adipogenic or osteogenic induction, BM-MSCs markedly increased the amount of oil red O or alizarin red stain compared with primary fibroblast (Figure 1B). These data confirm that BM-MSCs can differentiate into osteocytes and adipocytes. A phenotypic analysis of MSCs and fibroblast by flow cytometry showed that fibroblasts were positive for MHC class I molecule (RT1A), CD29 and CD54; but were negative for MHC class II molecule (RT1B), CD45, CD11b, CD25, CD90 and CD106 (Figure 1C). In contrast, MSCs were positive for MHC class I molecule (RT1A), Thy1 (CD90), CD29, CD54 and CD106; but were negative for MHC class II molecule (RT1B), CD45, CD11b and CD25 (Figure 1D).

Rat BM-Derived MSCs Inhibit the Alloantigen-Dependent Proliferative Response of T Cells

To assess whether MSCs exerted an effect on T-cell proliferation, we cocultured allogeneic lymphocytes with different numbers of allogeneic or autologous MSCs. Autologous MSCs suppressed lymphocyte proliferation (Figure 2A); the inhibitory effect of allogeneic MSCs was similar (data not shown). Fibroblasts showed no inhibition, whereas the MSCs showed a dose response in the inhibition

of T-cell proliferation (see Figure 2A). MLR assays using MSCs were also performed in transwell cultures to assess the influence of humoral substances released from MSCs and the role played by cell–cell contact between responder cells and MSCs. As shown in Figure 2B, the inhibitory effect of MSCs was partially diminished when cells were physically separated by the transwell membrane, indicating that the inhibition is mediated by both cell–cell contact and soluble factor. A comparison of the production of these cytokines during MLR showed that IL-10 secretion in cocultures with MSCs was significantly increased compared with that in cultures without MSCs ($P < 0.05$). Furthermore, IL-10 levels were higher under coculture conditions that allowed cell contact than in cultures without cell contact (Figure 2C; $P < 0.005$). CsA, added at the MLR, inhibited allogeneic lymphocyte proliferation in the dose-dependent manner of MSCs (see Figure 2C). IL-10 secretion in cocultures with MSCs was significantly increased compared with that in cultures without MSCs. When adding CsA to MLR mixed with MSCs, IL-10 secretion was significantly increased compared with other groups (see Figure 2C; $P < 0.005$). There was also a trend toward increased TGF- β secretion in MSC-containing cocultures, although this did not attain statistical significance. Next, to evaluate whether the inhibitory effect of MSCs was reversible, we cocultured recipient splenocytes with MSCs or MSC-plus-CsA for 7 d in a primary MLR and then restimulated with irradiated donor, third-party splenocytes or ConA (1 $\mu\text{g}/\text{mL}$) for 3 d. Cells cocultured with MSCs responded weakly to restimulation with donor splenocytes. Moreover, splenocytes preexposed to CsA or MSC-plus-CsA showed impaired responses to restimulation with alloantigen, compared with unexposed splenocytes. However, the preexposed cells showed normal responses to third-party antigens or nonspecific mitogens, showing that the immunosuppressive activity of MSCs was antigen specific and partially irreversible (Figure 2D).

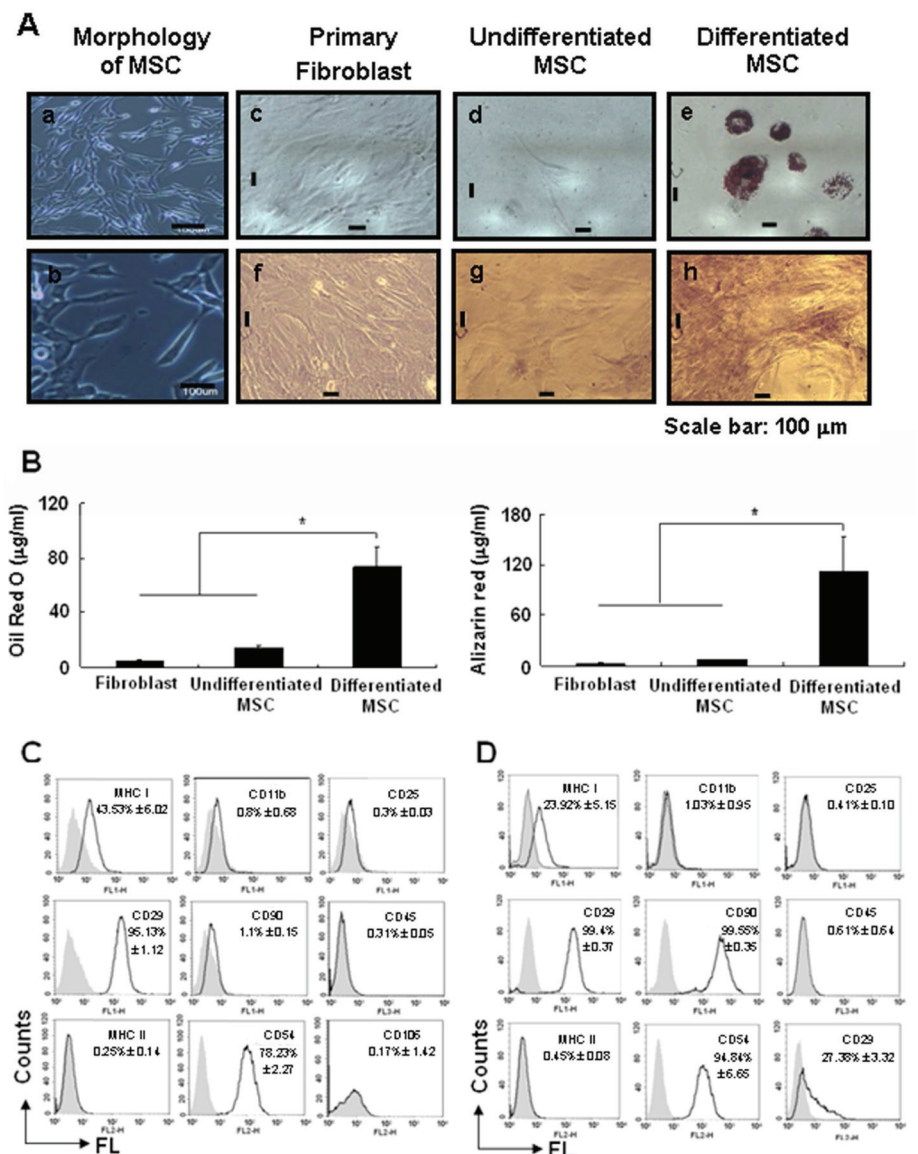


Figure 1. Expansion and characterization of MSCs from adult rat BM. (A) Morphology of MSCs isolated from rat BM and visualized under an inverted microscope (a, b). MSC and fibroblast cells grown for 14 d in adipogenic differentiation medium, fixed and stained with oil red O (c–e), and MSC and fibroblast cells grown on rat tail collagen I-coated wells for 14 d on osteogenic differentiation medium, fixed and stained with alizarin red S (f–h), are shown. The results shown are representative of three independent experiments. (B) Quantitation of oil red O staining (left) or alizarin red staining (right) of rat MSCs cultured in differentiation medium for 14 d. * $P < 0.01$ compared with fibroblast or undifferentiated MSC cells. (C) Fibroblasts and (D) MSCs were cultured for 3 to 5 passages, harvested and labeled with antibodies against MHC I, CD11b, CD25, CD29, CD90, CD45, MHC II, CD54 and CD105 and analyzed by flow cytometry. Shaded histograms indicate background signal, and open histograms show positive reactivity for the indicated antibody.

MSC-plus-CsA Administration Prolongs Islet Allograft Survival

To assess the *in vivo* function of MSCs, we investigated whether MSCs alone, or

MSCs in combination with a subtherapeutic dose of CsA (5 mg/kg) over 2 wks, had any effect on islet graft survival after islet transplantation into the

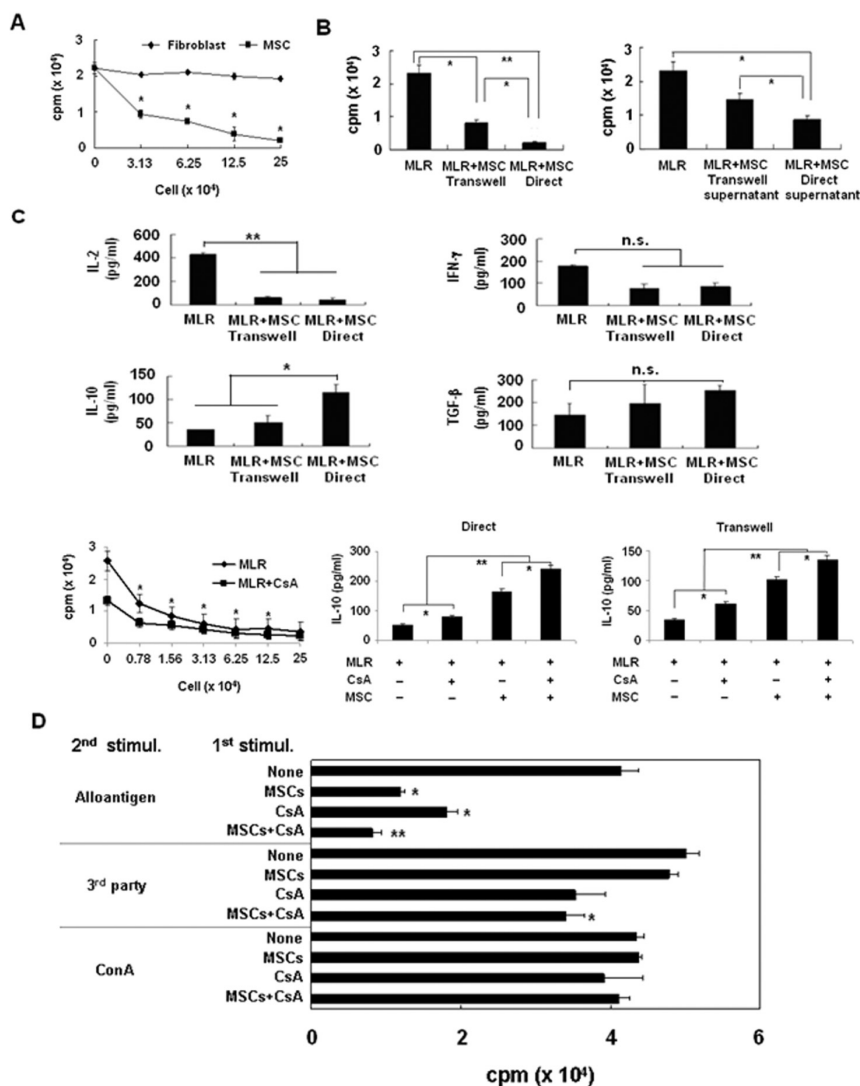


Figure 2. Rat BM-derived MSCs inhibit alloantigen-driven proliferative responses of T cells. (A) Irradiated (25 Gy) MSCs (2.5×10^5 cells/well) and allogeneic splenocytes (5×10^5 cells/well) were plated in 96-well, U-bottom plates in complete DMEM 1640 supplemented with 10% FBS 4 h before the addition of 2.5×10^5 responder cells/well; primary fibroblast cells were used as controls. MSCs or primary fibroblast cells were added in diminishing concentrations (2.5×10^5 , 1.25×10^5 , 6.25×10^4 and 3.13×10^4 cells/50 μ L/well) in an MLR assay. Cells were cocultured for 3 d with the addition of (3 H)-thymidine in the last 18 h of culture. * $P < 0.05$ compared with primary fibroblast cells. (B) Inhibitory effects of MSCs are mediated by cell contact and soluble factors. MSCs were plated into the upper chamber of transwell plates, and MLRs were set up in the lower chambers. After culturing for 3 d, cells from the lower chamber were transferred to microtiter wells in triplicate, pulsed with (3 H)-thymidine and harvested 18 h later. * $P < 0.05$, ** $P < 0.01$, compared with the MLR group. (C) MLR culture supernatants were analyzed for cytokine release by ELISA. The data are expressed as mean \pm SD of triplicate cultures. * $P < 0.05$, ** $P < 0.005$, compared with the MLR group. (D) Recipient spleen cells cocultured with MSCs or MSC-plus-CsA for 7 d in primary MLRs were subsequently restimulated with irradiated donor, third-party splenocytes or ConA (1 μ g/mL) for 3 d, pulsed with (3 H)-thymidine and harvested 18 h later. Values represent (3 H)-thymidine uptake by proliferating cells (average cpm). The results shown are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$, compared with the none group.

liver. In the untreated group, graft survival (mean \pm SD) was 5.0 ± 0.52 d. The mean graft survival times in the allogeneic and autologous MSC-treated groups were 3.7 ± 0.6 and 7.8 ± 1.26 d, respectively. Treatment with MSCs alone had no beneficial effect on graft survival. The mean graft survival time in rats receiving a subtherapeutic dose of CsA alone was 9.2 ± 4.02 d, similar to controls. In animals treated with allogeneic MSC-plus-CsA, mean graft survival time was 13.75 ± 12.3 d, not significantly different from CsA alone (Figure 3A). In contrast, treatment with autologous MSC-plus-CsA dramatically improved islet graft survival, increasing mean graft survival time to more than 89.3 ± 77.4 d ($P < 0.05$, compared with CsA alone). Collectively, these results demonstrate that allogeneic MSCs did not show an immunosuppressive effect in our rat islet transplantation model, and that treatment with autologous MSC-plus-CsA significantly enhances rat islet allograft survival compared with that after CsA alone (Figure 3B). In normoglycemic rats receiving MSCs and a short course of CsA, islets remained viable in the livers, and immunohistological analyses confirmed that insulin was generated by transplanted islets (Figure 3C). To examine cellular infiltration of the graft, we conducted a second series of islet transplants. Grafts were harvested 5 d after transplantation and analyzed immunohistochemically for insulin, CD4 and CD8 ($n = 3$ per treatment group). Control animals showed poor insulin staining at the graft site and a scattered infiltrate of CD8⁺ T cells. No differences in CD4⁺ T-cell infiltration were observed between the groups. However, the number of CD8⁺ T cells was higher in the control group than in MSC-plus-CsA groups on day 5. Grafts from MSC-plus-CsA-treated recipients showed positive staining for insulin and limited CD4 and CD8 cellular infiltration (Figure 3D). Histological analyses of pancreases from long-term surviving recipients confirmed destruction of islets.

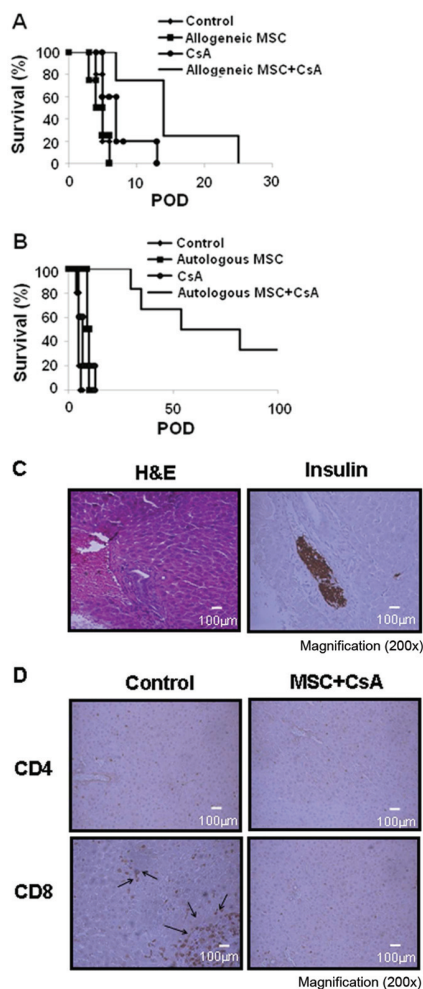


Figure 3. MSC-plus-CsA administration prolongs rat islet allograft survival. (A) Islets (4,000 islet equivalents/rat) and allogeneic MSCs (2×10^6 cells/rat) were transplanted into recipient rats. (B) Islets (4,000 islet equivalents/rat) and autologous MSCs (2×10^6 cells/rat) were transplanted into recipient rat via the portal vein. Group 1 received donor islets without MSCs, group 2 received donor islets with MSCs, group 3 was treated with CsA (5 mg/kg/day) for 2 wks, and group 4 was treated with both MSCs and CsA (5 mg/kg) for 2 wks. $*P < 0.001$, group 4 versus groups 1, 2 or 3. POD, postoperative day. (C) Immunohistochemical staining for insulin (magnification $\times 200$) and H&E (magnification $\times 200$) in liver tissue from islet-transplanted rats on day 100 after transplantation. (D) Immunohistochemical staining for CD4 (magnification $\times 200$) and CD8 (magnification $\times 200$) in liver tissue from islet-transplanted rats on day 5 after transplantation.

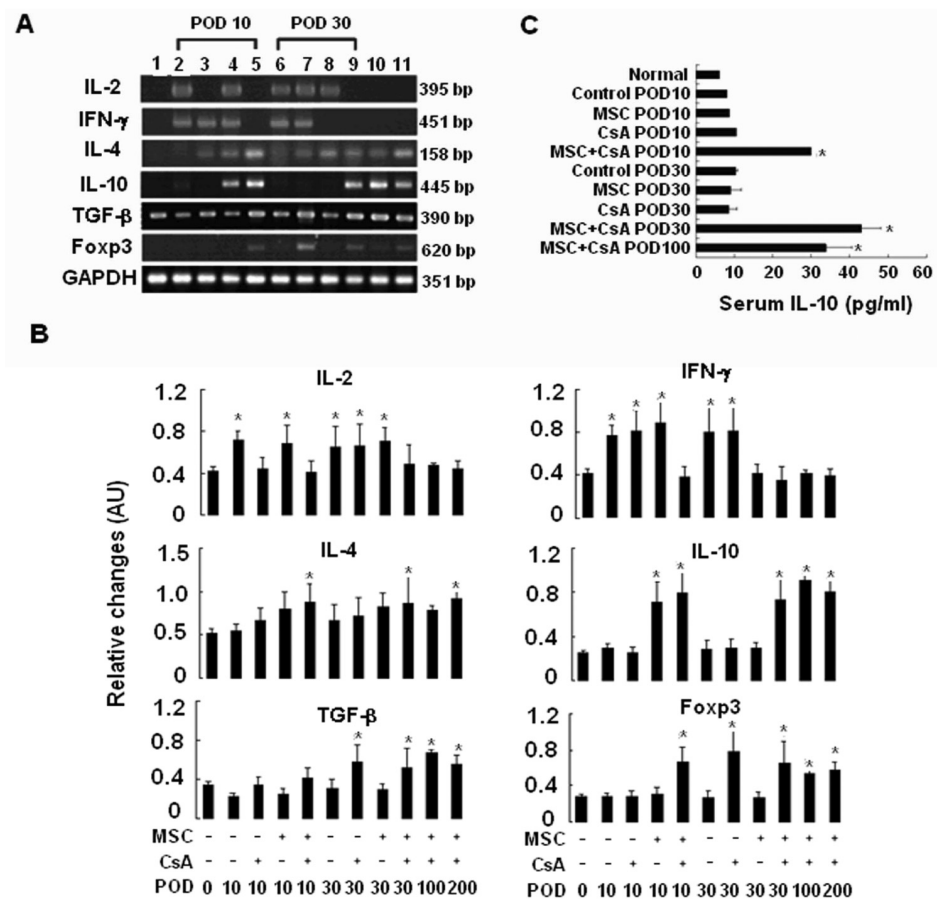


Figure 4. MSC-plus-CsA administration promotes low levels of IFN- γ production and high levels of IL-10 production. (A) Semiquantitative RT-PCR for cytokine expression was performed using RNA isolated on the indicated postoperative day (POD) from grafted livers from transplanted animals. 1: normal; 2: control POD 10; 3: CsA POD 10; 4: MSC POD 10; 5: MSC + CsA POD 10; 6: control POD 30; 7: CsA POD 30; 8: MSC POD 30; 9: MSC + CsA POD 30; 10: MSC + CsA POD 100; and 11: MSC + CsA POD 200. The results shown are representative of two separate experiments, each performed in triplicate. (B) Results are presented as the cytokine/GAPDH mRNA ratio. $*P < 0.05$ compared with normal group. (C) On day 10, 30 and 100 after transplantation, serum concentrations of IL-10 were measured by ELISA in the each group. $*P < 0.05$, MSCs + CsA versus normal, MSC or CsA-treated group. AU, arbitrary units.

MSC-plus-CsA Administration Results in Production of Low Levels of Th1-Type Cytokines and High Levels of Th2-Type Cytokines in the Grafted Liver Tissue and Serum

To elucidate potential mechanisms underlying the immunomodulatory function of MSCs on allografts, we analyzed grafted livers on posttransplantation day 10, 30 and 100 for the expression of Th1- and Th2-type cytokines, and Foxp3, by RT-PCR. In grafted livers of the MSC-

plus-CsA group, the expression of genes encoding the antiinflammatory Th2-type cytokines, IL-4 and IL-10, was significantly increased compared with controls ($P < 0.05$), whereas expression of the proinflammatory Th1-type cytokines, IL-2 and IFN- γ , was reduced compared with the MSC alone or control group (Figure 4A, B). The antiinflammatory cytokine IL-10 was upregulated continuously, beginning early after transplantation, whereas TGF- β and Foxp3 were

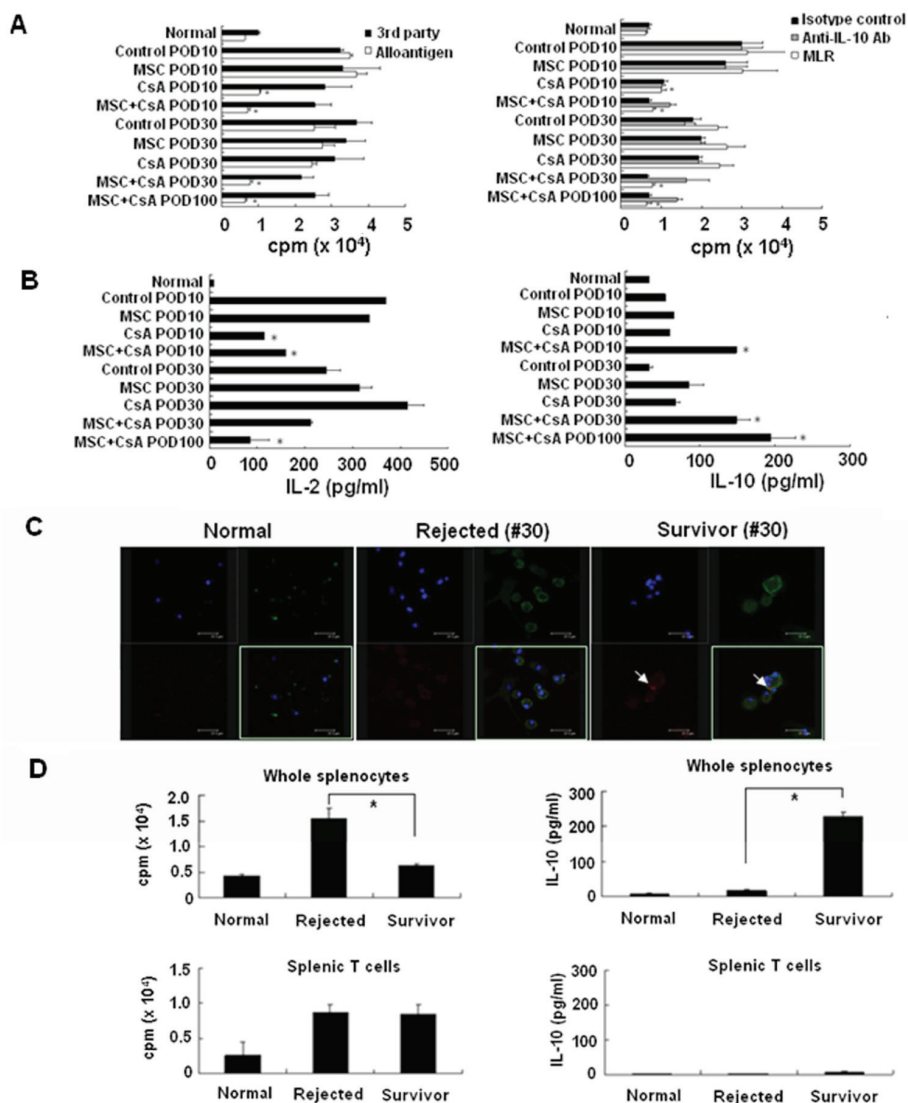


Figure 5. MSC-plus-CsA administration induces T cells hyporesponse to donor antigen *in vivo* and in IL-10-secreting CD11b⁺ cells and Tregs. (A) Isolated splenocytes (2.5×10^5) from either normal or transplanted rats were cocultured for 3 d with irradiated donor splenocytes. Results are expressed as mean cpm values \pm SD from triplicate cultures ($P < 0.05$ compared with control). (B) Isolated splenocytes from either normal or transplanted rats were cocultured for 3 d with irradiated donor splenocytes. Cytokine release in culture supernatants was determined by ELISA ($*P < 0.05$, MSCs + CsA versus control group). (C) Intracellular IL-10 in splenocytes from normal, rejected and surviving rats after alloantigen stimulation was analyzed. Splenocytes were prepared from normal, rejected and surviving rats (MSC + CsA) sacrificed on day-30 posttransplantation and were stimulated with alloantigen for 24 h. The harvested cells were stained with FITC-labeled anti-CD4 and anti-CD11b mAbs and then processed for intracellular staining with PE-labeled anti-IL-10 mAbs. Samples were examined by confocal microscopy for CD11b⁺ cells (green), IL-10 (red) and DAPI (4',6-diamidino-2-phenylindole, nucleic acid stain, blue). The results shown are representative of three independent experiments (arrow: IL-10 + cell, scale bar: 37.5 μ m). (D) Whole splenocytes or splenic T cells from splenocytes of each group were cocultured with irradiated donor splenocytes for 3 d; (³H)-thymidine was present for the last 18 h of culture. Values represent (³H)-thymidine uptake by proliferating cells (average cpm). MLR culture supernatant was collected, and IL-10 release was determined by ELISA. $*P < 0.05$, MSCs + CsA compared with rejected group.

highly expressed at a later phase. We also measured serum IL-10 levels on day 10, 30 and 100 after transplantation and found that IL-10 serum levels in the MSC-plus-CsA group were higher than those in other groups (Figure 4C). Serum IFN- γ was below the level of detection in all groups.

MSC-plus-CsA Administration Induces T-Cell Hyporesponsiveness to Donor Antigen by IL-10-Secreting CD11b⁺ Cells

To verify that MSC-plus-CsA treatment affects the capacity of T cells from transplanted animals to mount an appropriate response upon stimulation, we evaluated the T-cell response to alloantigen from transplanted and nontransplanted control animals. In MSC-plus-CsA-treated rats, the proliferative response to alloantigen was inhibited to an extent comparable to that of control rats, whereas the proliferative response to third-party antigen was not inhibited in any of the groups (Figure 5A, left). This T-cell hyporesponsiveness was donor specific, since splenocytes from long-term surviving animals were still able to proliferate in response to third-party stimulation. To identify the soluble factors involved in suppressing MLR responses, we investigated the levels of cytokines in culture medium by enzyme-linked immunosorbent assay (ELISA) and found that IL-10 secretion by long-term surviving splenocytes was increased after incubation in the presence of donor antigen (Figure 5B). To confirm that IL-10 was involved in mediating suppression in this model, we performed MLR assays using splenocytes from long-term surviving, rejected and normal animals in the presence of mAbs that neutralize IL-10. The suppressive effect of splenocytes from MSC-plus-CsA-treated animals was clearly reversed by neutralizing anti-IL-10 Abs (Figure 5A, right). To determine the source of IL-10, which is one of the key factors prolonging survival of grafted islets in the MSC-plus-CsA group, we stained intracellular cytokine by confocal microscopy after splenocytes from trans-

planted rats were stimulated with alloantigen and found that the number of IL-10-producing cells in MSC-plus-CsA-treated animals was significantly higher than in the other groups. In addition, cells that were positive for intracellular IL-10 after alloantigen stimulation were determined to be CD11b⁺ cells and not T cells (Figure 5C). To demonstrate whether IL-10-producing cells are host-derived cells, responder T cells were negatively selected from spleen of transplanted rats and normal rats by depletion of B cells, dendritic cells and most monocytes and macrophages. These cells were stimulated with irradiated allogeneic splenocytes for 3 d. Whole-splenocyte proliferation was significantly decreased and IL-10 production was increased in long-term surviving animals. Interestingly, MLR responses and IL-10 production in the condition of CD11b⁺ cells excluded was similar in both rejected and long-term surviving animals (Figure 5D).

MSC-plus-CsA Administration Increases the Frequency of CD4⁺CD25⁺Foxp3⁺ T Cells

As CD4⁺CD25⁺Foxp3⁺ proportions had previously been reported to increase on coculturing naive T cells with MSCs *in vitro* (10), we examined whether CD4⁺CD25⁺Foxp3⁺ T cells contributed to islet allograft acceptance in MSC-plus-CsA-treated allograft recipients. Accordingly, we tested for the presence of Tregs in LN, spleen, graft-infiltrating cells and PBMCs in the MSC-plus-CsA group, by flow cytometry. We observed a significant increase in the proportion of CD4⁺CD25⁺Foxp3⁺ T cells within the total CD4⁺ population recovered from LN, graft infiltrating cells and PBMCs, but not from spleens, in the MSC-plus-CsA group compared with untreated controls on day-30 posttransplantation (Figure 6). Whereas in the frequency of CD4⁺CD25⁺Foxp3⁺ T cells in lymph node lymphocytes, splenocytes, PBMCs and graft-infiltrating cells, there was no difference between any groups on day-10 posttransplantation (data not shown).

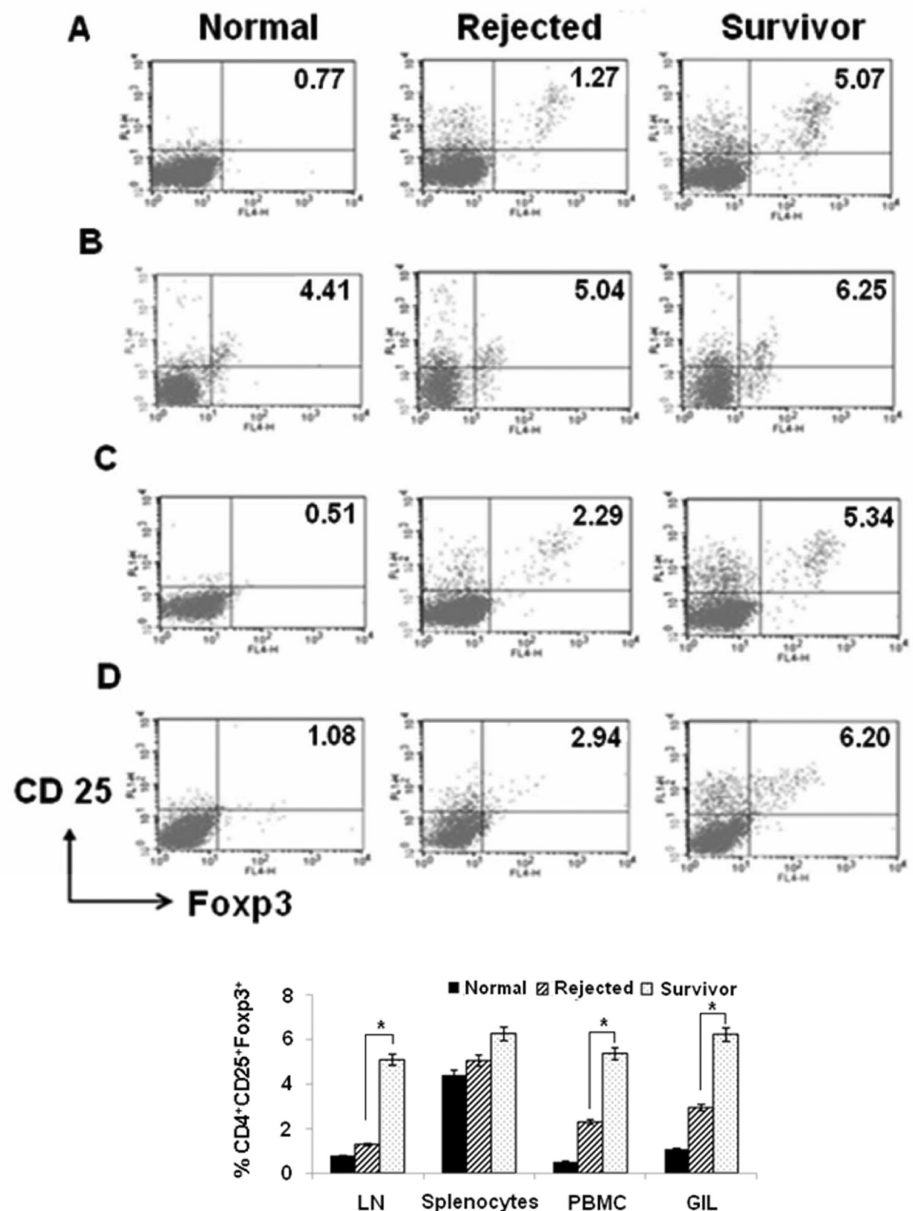


Figure 6. MSC-plus-CsA administration increases the frequency of CD4⁺CD25⁺Foxp3⁺ T cells. CD4⁺ T cells were identified in the PE-fluorescence gate on the basis of the expression of CD4. Flow cytometry diagrams showed CD25 and Foxp3 positivity in CD4⁺ T cells from lymph node lymphocytes (A), splenocytes (B), PBMCs (C) and graft-infiltrating cells (D) of normal, rejected and surviving rats at 30 d. Plots representative of three experiments are shown. **P* < 0.05, MSCs + CsA compared with rejected group. GIL, graft-infiltrating lymphocytes.

DISCUSSION

Most studies suggest that MSCs inhibit alloreactive/autoreactive T-cell responses and promote therapeutic effects in experimental models (5,11–13). Controversy surrounds the optimal source of MSCs for use in immune modulation. Although the immunoregulatory aspects of MSCs

are well established, other studies have indicated that these cells might not be able to completely evade the immune system. This result was initially indicated by Djouad *et al.* (11), showing that although allogeneic murine MSCs could engraft and form bone in immunocompetent mice, lymphocytic infiltrates were

seen at the periphery of the newly formed tissue. Subsequently, several studies questioned the efficacy of allogeneic MSCs *in vivo* (14–20). In particular, Nauta *et al.* demonstrated that infusion of donor MSCs, in conjunction with allogeneic bone marrow transplantation, is associated with enhanced rejection of the latter cells, indicating that allogeneic MSCs might induce a memory T-cell response (14,17). Indeed, our data show that injection of allogeneic MSCs alone or with CsA showed islet graft survival similar to the control group, although the strong suppressive reactivity was exhibited *in vitro*. Furthermore, allogeneic MSCs combined with low-dose CsA failed to prolong islet allograft survival. Allogeneic MSCs are likely recognized by host T cells and might not be able to evade immune surveillance in allogeneic recipients. In a recent report, Fiorina *et al.* (21) found that allogeneic MSCs are capable of protecting islet mass and of delaying the onset of disease when injected into prediabetic NOD mice, but monotherapy often fails to provide a durable favorable outcome. In addition, the Fiorina *et al.* study was conducted in an NOD setting, where alloimmune and autoimmune responses are both involved, whereas our study was performed in streptozotocin (STZ)-induced diabetic rats. STZ has been widely used to induce hyperglycemia by specifically destroying the insulin-producing β cells of the islets of Langerhans in experimental models of Type I diabetes. Against this backdrop, we sought to determine whether autologous MSCs downregulate the immune response and prolong graft survival. Importantly, autologous MSCs coadministered with a subtherapeutic dose of CsA suppressed the early activation of T cells and synergistically prolonged allograft survival (Figure 3). Induction of transplantation tolerance in experimental systems using CsA (22,23) typically requires treatment for at least 7–14 days. Two previous studies reported that autologous MSC-plus-CsA exerted a synergistic effect *in vitro*, an observation that could have clinical implications in transplant

recipients (24,25). Although a number of studies have documented the immunosuppressive activities of MSCs, the mechanisms underlying this effect have not been fully explained. Generally, contact-dependent mechanisms and soluble factors are thought to collaborate to induce MSC-mediated immunosuppression. Recently, Kathryn Wood's group suggested that one of the principal explanations for the immunosuppressive capacity of MSCs is the cleavage of CD25 from the T-cell surface by MSC-secreted matrix metalloproteinases *in vitro* and *in vivo*. Moreover, several soluble immunosuppressive factors, either produced constitutively by MSCs or released after cross-talk with target cells, were reported to be involved in MSC-mediated immunoregulation (5,10,13,26–29). Previously, we reported that transfection of the *vIL-10* gene into islets effectively elevated IL-10 levels within the grafts of recipients and proved beneficial in rodent allogeneic islet transplantation (30). We therefore tested whether this cytokine plays a role in MSC-mediated inhibition and further assessed the involvement of cell–cell contact in this process. We found that the conditioned medium from MSC–T-cell cocultures modulated T-cell alloreactivity, suggesting a role for soluble factors. We further found that addition of MSCs to MLRs inhibited IL-2 and IFN- γ production while increasing the levels of IL-10, suggesting that IL-10 is important in mediating the suppressive capacity of MSCs (Figure 2C). We found that in grafted livers from the allo–MSC-plus-CsA group, the expression of IL-2, IFN- γ , IL-4 and IL-10 was comparable to other groups at 20 days after transplantation. However, autologous MSC-plus-CsA treatment decreased IL-2 and IFN- γ mRNA levels but increased IL-4 and IL-10 mRNA levels in grafted livers (Figure 4). Interestingly, IL-10 was upregulated continuously from the early post-transplantation period, whereas TGF- β and Foxp3 were upregulated later. These changes in the cytokine environment were restricted to the autologous MSC-plus-CsA-treated group and were closely

related to suppression of rejection and long-term allograft survival. The proliferation of splenocytes from rats transplanted with autologous MSC-plus-CsA was suppressed upon restimulation with allogeneic splenocytes *in vitro*. These splenocytes also produced a low level of IL-2 and a high level of IL-10 compared with other groups. The functional contribution of IL-10 to the immunomodulatory function of MSCs in autologous MSC-plus-CsA-treated rats was confirmed by addition of a neutralizing anti-IL-10 antibody in MLR, which resulted in recovery of the MLR response in long-term surviving splenocytes (Figure 5A). TGF- β was readily detected in all groups, but the inhibitory activity of splenocytes from MSC-plus-CsA-treated animals was slightly abolished by addition of a neutralizing anti-TGF- β Ab (data not shown). These data support the idea that TGF- β does not play a major role in the long-term survival of grafted islets in animals treated with the combination of MSCs and low-dose CsA. Several reports demonstrated that TGF- β 1 can either enhance (31,32) or inhibit (33,34) effector T-cell proliferation. These results suggest that immunoregulatory effects of TGF- β 1 depend on strength of stimulation of effector T cells and could change tolerogenic property of TGF- β 1 *in vivo*. In our study, TGF- β and Foxp3 mRNA were upregulated late in the post-transplantation period in grafted liver. TGF- β was recognized as a critical regulator in immune responses and greatly dampens T-cell responses in particular (35). TGF- β was shown to convert peripheral CD4⁺CD25⁻ T cells into Tregs and thus could promote peripheral tolerance (36). We suggest that TGF- β may affect the generation of Tregs in later stages after transplantation and thus contribute to improved graft survival.

A recent study demonstrated that allogeneic islets transplanted with autologous MSCs induced insulin secretion and promoted long-term islet allograft survival when combined with anti-lymphocyte serum (ALS) and CsA (37). In this study, combined autologous MSC +

ALS + CsA treatment, but not autologous MSC + CsA treatment, was associated with the generation of IL-10-secreting CD4⁺ T cells. This result suggests that ALS treatment may increase IL-10 expression (38) and contribute to long-term islet allograft survival. In our present study, autologous MSCs plus low-dose CsA prolonged islet allograft survival and induced production of IL-10 from host-derived CD11b⁺ cells after transplantation (Figure 5D). In the recent reports, IL-10 produced by recipient cells other than T lymphocytes was required for Treg function and maintenance of Foxp3 expression, despite the ability of the donor Tregs to secrete IL-10. Tregs transferred into *Il10^{-/-}Rag1^{-/-}* recipient mice expanded in number *in vivo* and homed to various tissues, but these cells failed to maintain Foxp3 expression and suppressive activity in the absence of IL-10 (39). In our study, the production of IL-10 itself by host-derived CD11b⁺ cells is certainly one mechanism by which autologous MSCs may exert their suppressive activity in the early phase. Then we determined the frequency of Tregs (CD4⁺CD25⁺Foxp3⁺ cells) harvested from islet transplanted rats. Interestingly, relative to other T-cell subsets, the number of Tregs in long-term surviving rats was increased in LNs, PBMCs and graft at a later stage (around 30 days), but was relatively unchanged in splenocytes (see Figure 6). Thus, increased Tregs presumably will maintain the suppressive effect by host cell-derived IL-10 and then support long-term graft function.

In conclusion, we have demonstrated that MSCs administered with low-dose CsA prolong graft survival compared with MSCs or CsA alone, and combined treatment with MSCs and CsA prevents rejection of islet allografts by suppression of local proinflammatory cytokine production. MSCs are known to produce high levels of IL-10. Our study shows that MSCs induce monocytes to produce IL-10 in *in vivo* as well *in vitro* experiments. Furthermore, MSCs indirectly affected the Treg via IL-10 secreted from CD11b⁺ cells and TGF- β at a later phase.

Thus, upregulation of IL-10 synthesis by combined autologous MSC-plus-CsA treatment causes donor antigen-specific T-cell hyporesponsiveness during the early posttransplantation phase and maintains suppressive activity by Tregs later. The immunomodulatory effect of combined autologous MSC-plus-CsA treatment in islet transplantation, reported here, suggests a new strategy for preventing and treating rejection after transplantation and provides a preliminary experimental basis for the application of MSCs in clinical islet transplantation as well as other solid organ transplantation.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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