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Mesenchymal Stem Cells Cooperate with Bone Marrow Cells in Therapy of Diabetes

VERONIKA S. URBÁN,[a] JUDIT KISS,[a] JÁNOS KOVÁCS,[b] ELEN GÓCZA,[c] VIRÁG VAS,[a] ÉVA MONOSTORI,[d] FERENC UHER[a]

[a]Stem Cell Biology, National Medical Center, Budapest, Hungary; [b]Department of Anatomy, Cell and Developmental Biology, Loránd Eötvös University, Budapest, Hungary; [c]Department of Animal Biology, Agricultural Biotechnology Center, Gödöllő, Hungary; [d]Lymphocyte Signal Transduction Laboratory, Institute of Genetics, Biological Research Center of Hungarian Academy of Sciences, Szeged, Hungary

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ABSTRACT

Several recent studies have suggested that the adult bone marrow harbors cells that can influence β-cell regeneration in diabetic animals. Other reports, however, have contradicted these findings. To address this issue, we used an animal model of type 1 diabetes in which the disease was induced with streptozotocin in mice. Freshly prepared sex-mismatched bone marrow cells (BMCs) and syngeneic or allogeneic mesenchymal stem cells (MSCs) were concomitantly administrated into sublethally irradiated diabetic mice. Blood glucose and serum insulin concentrations rapidly returned to normal levels, accompanied by efficient tissue regeneration after a single injection of a mixture of 10^6 BMCs per 10^5 MSCs. Neither BMC nor MSC transplantation was effective alone. Successful treatment of diabetic animals was not due to the reconstitution of the damaged islet cells from the transplant, since no donor-derived β-cells were found in the recovered animals, indicating a graft-initiated endogenous repair process. Moreover, MSC injection caused the disappearance of β-cell-specific T lymphocytes from diabetic pancreas. Therefore, we suggest that two aspects of this successful treatment regimen operate in parallel and synergistically in our model. First, BMCs and MSCs induce the regeneration of recipient-derived pancreatic insulin-secreting cells. Second, MSCs inhibit T-cell-mediated immune responses against newly formed β-cells, which, in turn, are able to survive in this altered immunological milieu. Thus, the application of this therapy in human patients suffering from diabetes and/or other tissue destructive autoimmune diseases may be feasible. STEM CELLS *2008;26:244–253*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Diabetes, a leading cause of morbidity and mortality in industrialized countries, is caused by absolute insulin deficiency due to autoimmune destruction of insulin secreting pancreatic β-cells (type 1 diabetes) or by relative insulin deficiency due to decreased insulin sensitivity, usually observed in overweight individuals (type 2 diabetes). In both types of the disease, an inadequate mass of functional β-cells is the major determinant for the onset of hyperglycemia and development of overt disease. Thus, intense research has been focused on possible mechanisms to promote the expansion of existing pancreatic β-cells and β-cell development from either endogenous or exogenous bone marrow-residing stem cells [1–3]. However, studies using adult bone marrow (BM) to induce β-cell expansion and/or replacement in mice resulted in a controversial outcome. In 2003, an intriguing report suggested that adult mouse BM cells developed into functionally competent pancreatic β-cells when homed to pancreatic islets after transplantation [4]. In streptozotocin (STZ)-induced diabetes, homing of transplanted bone marrow cells (BMCs) to the damaged pancreas was associated with normalization of blood sugar level and increased islet mass. Although the islet-localized BM stem cells exhibited an endothelial phenotype, their transdifferentiation into insulin-secreting β-cells was not proved. The authors speculated that β-cell regeneration from host cells was induced by unidentified growth and differentiation factors secreted by newly developed endothelial cells [3, 5, 6]. Banerjee et al. [7] successfully treated STZ-induced diabetes with multiple infusion of BM cells but did not suggest a mechanism for the recovery. Temporary islet transplantation combined with infusion of BM cells ameliorated the diabetes in NOD mice [8] when the transplantation was performed before but not after the onset of hyperglycemia [9]. Furthermore, Lee et al. [10] reported that repeated transplantation of human mesenchymal stem cells (MSCs) induced repair of pancreatic islets and renal glomeruli in NOD/*scid* mice suffering from STZ-induced diabetes. Although all of these studies strongly support that BM-derived hematopoietic and/or stromal cells can promote islet regeneration in diabetic animals, the mechanisms of the regenerative process and the appropriate conditions for using these cells for therapy are still very poorly understood.

Our work was aimed at characterizing the function of BMCs and BM-derived MSCs in healing diabetes in mice. In our model, type 1 diabetes was initiated with multiple low doses of STZ [11, 12] in female C57Bl/6 mice. Coinjection of sex-mismatched syngeneic BMCs and syngeneic or allogeneic

Correspondence: Ferenc Uher, Ph.D., National Medical Center, Stem Cell Biology, Diószegi ut 64, Budapest H-1113, Hungary. Telephone: 36-1-372-4334; Fax: 36-1-372-4352; e-mail: uher@kkk.org.hu Received April 11, 2007; accepted for publication October 2, 2007; first published online in STEM CELLS *EXPRESS* October 11, 2007. 1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2007-0267

MSCs into sublethally irradiated diabetic mice efficiently reversed the disease. The recovery was not a result of differentiation of donor BMCs into β-cells but rather a consequence of an endogenous repair process initiated by the graft and suppression of T-cell-mediated immune response against newly formed β-cells by the immunosuppressive donor MSCs.

MATERIALS AND METHODS

Induction of Experimental Diabetes and Blood Glucose Monitoring

Female C57Bl/6 mice, 8–10 weeks old (National Institute of Oncology, Hungary), were injected intraperitoneally (i.p.) with 50 mg/kg of body weight STZ (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) daily on five consecutive days. STZ was solubilized in sodium citrate buffer, pH 4.5, and injected within 15 minutes of preparation. Blood glucose was measured twice weekly with a glucometer (Accu-Chek Active blood glucose meter; F. Hoffmann-La Roche, Basel, Switzerland, http://www.roche.com). Serum insulin was quantified with a rat/mouse insulin-specific enzyme-linked immunosorbent assay kit (Linco Research, St. Charles, MO, http://www.lincoresearch.com). Those animals were considered diabetic whose blood glucose level exceeded 10 mM at days 14 and 15. All animal procedures were approved by the Animal Care and Use Committee of the National Medical Center (Budapest, Hungary).

Intraperitoneal Glucose Tolerance Test

After 4 hours fasting, mice were injected i.p. with 2 g/kg of body weight glucose. Glucose disposal was analyzed by measuring blood glucose at different time points.

Cell Separation and Transplantation of Hyperglycemic Mice

Syngeneic bone marrow cells from male C57Bl/6 mice were extracted from the femurs and tibias and transplanted by tail vein injection into lethally (900 cGy) or sublethally (450, 250, or 150 cGy) irradiated diabetic female recipients on day 15 following STZ induction of diabetes. Syngeneic or semiallogeneic MSCs from male C57Bl/6 or (C57Bl/6xDBA/2)F1 mice (National Institute of Oncology), respectively, were isolated by their adherence to surface of plastic tissue culture flasks and serially passed in vitro at least eight times before injection. Allogeneic MSCs were isolated from male CD1 mice (Charles River Laboratories, Wilmington, MA, http://www.criver.com). The enhanced green fluorescent protein (EGFP)-expressing transgenic B5/EGFP mouse line [13] was kindly provided by Andras Nagy (Mount Sinai Hospital, Toronto, ON, Canada) and maintained on CD1 background in our laboratory. Culture-expanded MSCs were coinfused with BM grafts in 0.2 ml of serum-free medium.

Isolation, Culture, and Characterization of Mouse MSCs

Isolation of MSCs was carried out according to Peister et al. [14] with some modifications. Briefly, bone marrow was collected from 8–10-week-old male mice by flushing femurs and tibias with complete medium (CM) containing Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 10% fetal bovine serum, 10% horse serum (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich), and 2 mM L-glutamine (Invitrogen) and supplemented with heparin at a final concentration of 5 U/ml. Cells were then washed twice in Hanks' balanced saline solution, plated in a 25-cm^2 flask (BD Falcon, Bedford, MA, http://www.bdeurope.com) at a density of 2–5 $\times$ 10^6 cells per cm^2 in CM, and cultured in a humidified 5% CO_2 incubator at 37°C for 72 hours. Nonadherent cells were removed by sequential changes of the medium twice a week. Confluent primary cultures were washed with phosphate-buffered saline (PBS) and lifted by incubation with trypsin/EDTA at 37°C for 5 minutes. Cells were then washed again and seeded into a 75-cm^2 flask (BD Falcon). Subsequent passages were performed similarly. Cultures were contaminated with hematopoietic cells ($CD34^+$ and/or granulocyte-macrophage colony-forming cells) until passage 6 or 7.

MSCs were retrieved by trypsin digestion, and aliquots of 5 $\times$ 10^5 cells were labeled in the dark with fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies against mouse CD3ε, CD45R/B220, CD11b, Ly-6G, TER-119, CD34, and Sca-1 (all products from BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml) for 30 minutes at room temperature. Stained cells were washed with PBS and analyzed immediately on a FACScan flow cytometer using CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com).

Osteogenic differentiation was induced by culturing confluent MSCs for 2 weeks in complete medium supplemented with dexamethasone (10^{-8} M), β-glycerophosphate (10 mM), and ascorbic acid (0.3 mM) (all from Sigma-Aldrich). To observe calcium deposition, cultures were stained with alizarin red stain (Sigma-Aldrich).

To induce adipogenic differentiation, confluent MSCs were cultured for 2 weeks in complete medium supplemented with dexamethasone (10^{-7} M) and 3-isobutyl-1-methylxanthine (0.5 mM) (Sigma-Aldrich). The cells were then fixed with 10% formalin and stained with oil red O (Sigma-Aldrich) or Giemsa and analyzed with microscopy.

Antigen-Specific T-Cell Proliferation

T-cell proliferation assays were performed as described by Horwitz et al. [15], with some modifications. Briefly, antigen-presenting cells (APCs) were enriched from spleens of untreated and STZ-treated (on day 8) female C57Bl/6 mice. Homogenized spleens were cleared up from erythrocytes by lysis with NH_4Cl. The remaining cells were resuspended in DMEM with 10% FCS at 10^7 cells per ml and incubated on Petri dishes for 4 hours at 37°C. Afterward, nonadherent cells were removed by multiple washes in cold PBS, and adherent cells were lifted by scraping. The APC-enriched population was irradiated (15 Gy) before use. T lymphocytes were prepared from the pancreas of STZ-treated mice or from the spleen of ovalbumin (OVA)-injected (10 μg of OVA plus 1 mg of $Al(OH)_3$ per animal i.p.) animals at the indicated time points. Each pancreas and spleen was removed and mechanically separated into single-cell suspension without protease digestion. Cells were washed and T cells were enriched using a Pan T-Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) following the manufacturer's instructions. APCs (5 $\times$ 10^4 cells per well) and T cells (2 $\times$ 10^5 cells per well) were incubated together on 96-well plates (BD Falcon) in the presence or absence of soluble antigens (i.e., pancreatic tissue extract or OVA) for 72 hours at 37°C. Eighteen hours before harvest, the cultures were pulsed with 1 μCi of ^{3}H-thymidine, and incorporated ^{3}H-thymidine was measured in liquid scintillation counter. Cyclosporin A (Sandimmun) was purchased from Sandoz (Novartis Hungaria Kft., Budapest, Hungary, http://www.novartis.com).

Histology and Immunohistochemistry

Pieces of pancreas, liver, and lung were fixed in 4% neutral-buffered formalin for 3 hours and embedded in paraffin wax, and the sections were stained with hematoxylin-eosin. Immunohistochemistry was performed on sections (5 μm) from formalin-fixed tissues. The sections were dewaxed, rehydrated, and incubated in hydrogen peroxide solution for 5 minutes to block endogenous peroxidases. Then, mouse monoclonal anti-insulin antibody (Sigma-Aldrich) was applied to the sections (1:1,000) for 15 minutes, followed by biotinylated rabbit anti-mouse IgG and streptavidin-peroxidase conjugate. The slides were developed with diaminobenzidine (DAB) (Peroxidase Kit; Dako, Glostrup, Denmark, http://www.dako.com) according to the manufacturer's instructions. Finally, the sections were counterstained with hematoxylin and mounted. No background staining was detected omitting the first (anti-insulin) antibody.

Combined Immunohistochemistry and Y Chromosome In Situ Hybridization

For detection of male-derived insulin-containing donor cells in the tissues of STZ-treated female recipients, we combined Y chromosome in situ hybridization with immunochemistry [16]. Sections from formalin-fixed, paraffin-embedded tissues were dewaxed, rehydrated, and blocked with Tris-buffered nonfat milk (Sigma-Aldrich) for 1 hour. After washing with TBS, mouse monoclonal anti-insulin antibody (Sigma-Aldrich) was applied (in a dilution of 1:1,000) for 1 hour, followed by rabbit anti-mouse IgG-alkaline phosphatase antibody (Sigma-Aldrich) (in a dilution of 1:100) for 1 hour. Alkaline phosphatase activity was visualized by Fast Red stain (Sigma-Aldrich). As a negative control, the first antibody was omitted.

The subsequent detection of Y chromosomes was carried out with mouse Y chromosome paint labeled with fluorescein isothiocyanate (StarFish 1189-YMF-01; Cambio, Cambridge, U.K., http://www.cambio.co.uk) following the recommendations of manufacturer. Briefly, the sections were washed in distilled water, incubated in sodium thiocyanate for 10 minutes at 80°C, and digested with 0.4% pepsin in 0.1 M HCl for 10 minutes. The reaction was quenched in glycine solution followed by washing in PBS. Then the sections were fixed in 4% paraformaldehyde, dehydrated in graded ethanol, and air-dried. A drop of prediluted StarFish Y probe was applied to the sections, and the slides were covered with glass, sealed with rubber cement, denatured at 60°C for 10 minutes, and incubated overnight at 37°C. After removing the coverslips, the sections were rinsed in formamid solution, followed by washing in either 2× standard saline citrate (SSC) or in 1× SSC at 37°C for 15 minutes, and then with 0.05% Tween 20 in SSC (37°C, 10 minutes) and PBS. Finally, the sections were counterstained with DAPI and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). The specificity of reaction was checked by comparing pancreatic slices from healthy control male and female mice.

The sections were examined under an Olympus BX51 epifluorescence microscope (Olympus, Tokyo, http://www.olympus-global.com) equipped with an Fview II digital camera (Olympus Europa, Hamburg, Germany, http://www.olympus-europa.com). The pictures were taken with a ×40 0.75 numerical aperture lens and processed with AnalySIS Pro software (Olympus).

Monitoring of Bone Marrow Chimerism

Fluorescence in situ hybridization (FISH) analysis was performed on bone marrow cells prepared by standard cytogenetic techniques, including hypotonic KCl-incubation (37°C for 20 minutes) and fixation steps (acetic acid/methanol, 1:3). FISH was performed according to the manufacturer's recommendations using a rhodamine-labeled total mouse Y chromosome-specific DNA probe (Qbiogene, Irvine, CA, http://www.qbiogene.com). The best results were obtained by denaturating the probe mixture at 73°C for 5 minutes. For posthybridization wash steps, the formamide wash procedure was used. At least 50 nuclei were counted in each slide.

Statistical Analysis

Student's t test was used for p values. The Kaplan-Meier method was used to calculate the survival data, and the significance was determined by the Mann-Whitney U test.

RESULTS

Transplantation of Bone Marrow Cells Is Not Sufficient to Treat STZ-Induced Diabetes

To evaluate the potential capacity of bone marrow-derived stem/progenitor cells to restore tissue function after pancreatic damage, destructive diabetes was induced with STZ in adult female C57Bl/6 mice. Morning nonfast blood glucose level increased 4–5-fold in diabetic animals compared with that of untreated mice (5.71 ± 0.42 mM and 24.91 ± 3.93 mM, respectively) at day 35, resulting in a severely hyperglycemic condition (Fig. 1A). Elevation of blood glucose concentration starting at day 7 and continuing until day 35 correlated with gradual loss of body weight (Fig. 1B). Comparative histological study of healthy (Fig. 1C) and diabetic (Fig. 1D) pancreas sections prepared on at day 21 showed a dramatic decrease in the size of pancreatic islets containing cells with pyknotic nuclei in tissue from diabetic mice. Immunohistochemical staining of insulin-producing cells detected presence of very few insulin-positive cells in diabetic mice (Fig. 1F) compared with healthy control animals (Fig. 1E). Without administration of insulin, the sick mice died at 4–6 weeks.

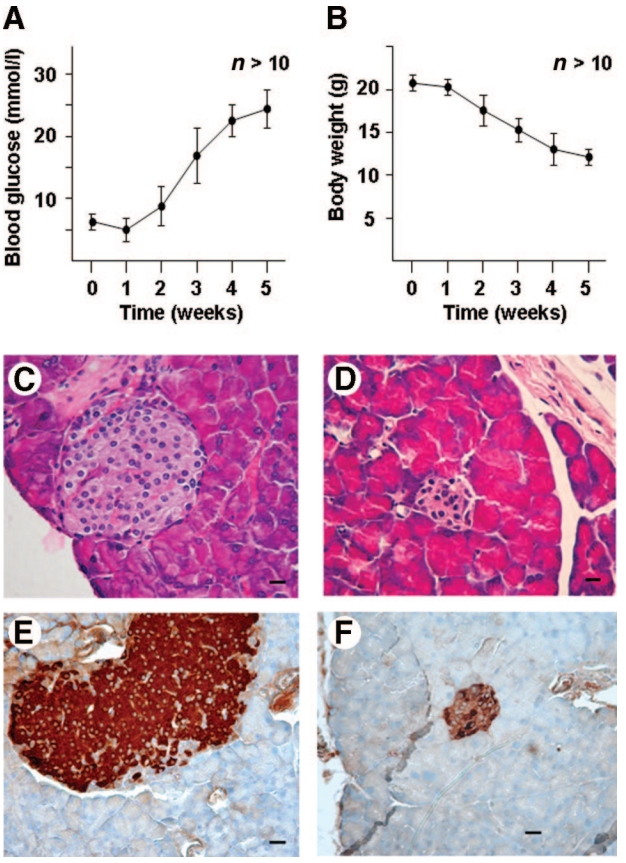

Figure 1. Induction of diabetes. Development of hyperglycemia **(A)**, weight loss **(B)**, and pancreatic damage **(C–F)** was induced with intraperitoneal injection of 50 mg/kg of body weight STZ daily for five consecutive days. Data presented in **(A)** and **(F)** are presented as mean ± SD; $n > 10$. Pancreatic islets dissected on day 21 from control **(C, E)** and STZ-treated **(D, F)** mice were stained with hematoxylin and eosin **(C, D)** or immunostained for mouse insulin **(E, F)**. Scale bars = 20 μm.

To study whether bone marrow cells were able to restore pancreas functions, 1 million nucleated BMCs, freshly isolated from adult male C57Bl/6 donors, were injected intravenously into diabetic mice after total-body irradiation (TBI) on day 15 (Fig. 2A). Different groups of animals received various doses (900, 450, 250, or 150 cGy) of TBI. The highest dose of TBI resulted in total loss of diabetic, BM-transplanted animals within a few days, whereas nondiabetic, BM-transplanted control mice survived up to 180 days (Fig. 2B). Examination of blood glucose level showed no difference in its elevation between nonirradiated, untransplanted and lowest dose-irradiated (150 cGy), transplanted diabetic animals (Fig. 2C, 2G, respectively). Elevation of blood glucose level was transiently delayed when diabetic mice were preconditioned with a medium dose (250 or 450 cGy) of irradiation (Fig. 2F, 2E) followed by BMC transplantation. However, normoglycemia was not restored, since after 2–3 weeks the blood sugar concentration rapidly

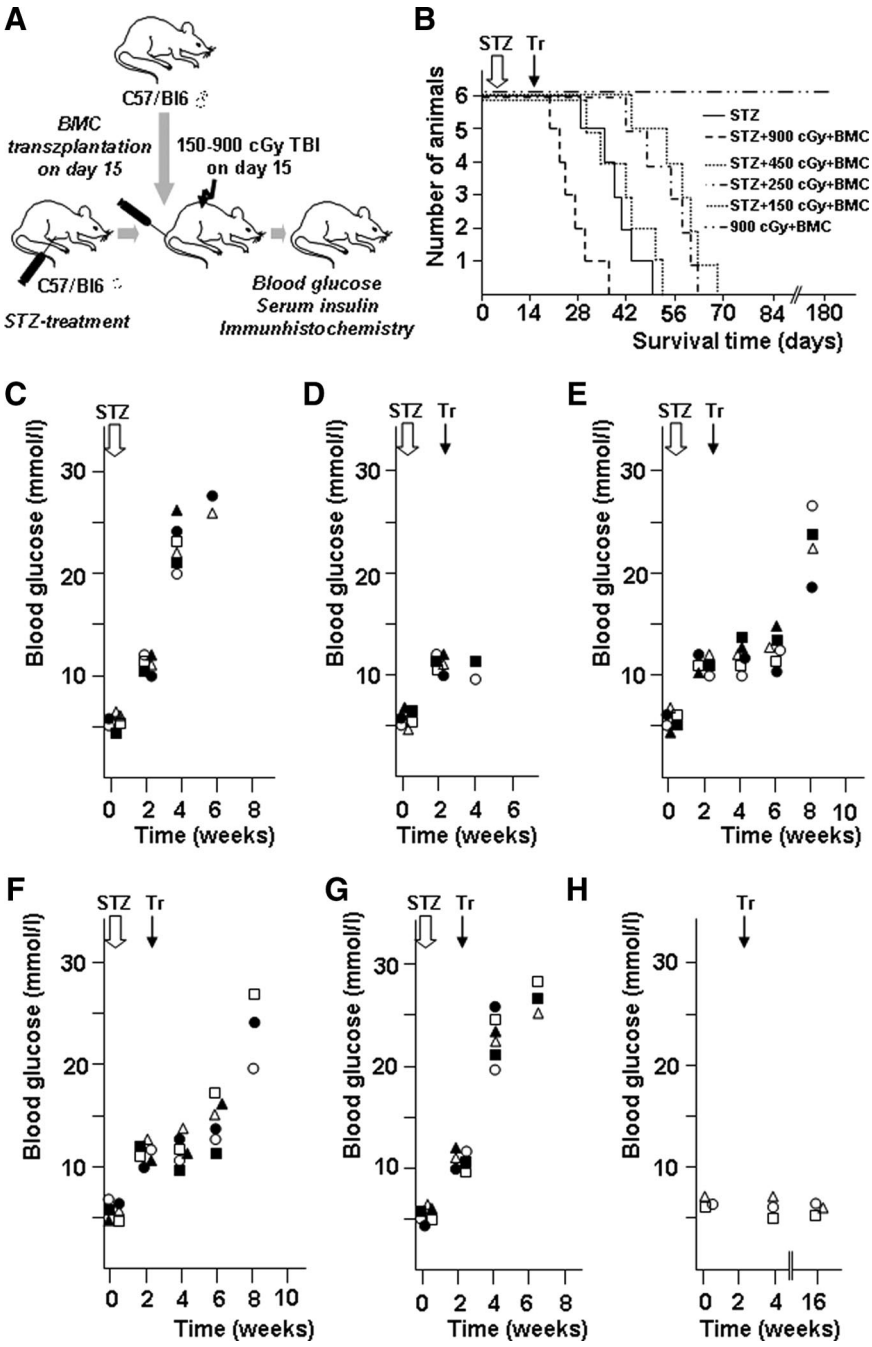

Figure 2. Transplantation of syngeneic BMCs is not sufficient to treat STZ-induced diabetes. **(A):** Experimental design for the transplantation of male BMCs into STZ-treated female mice. **(B):** Kaplan-Meier plot for survival of experimental animals ($n = 6$). **(C):** Animals were treated with STZ only ($n = 6$). **(D–G):** Diabetic (STZ-treated) mice were injected with 10^6 nucleated marrow cells on day 15 ($n = 6$) after irradiation with 900 cGy **(D)**, 450 cGy **(E)**, 250 cGy **(F)**, or 150 cGy **(G)**. **(H):** Nondiabetic control animals were conditioned with 900 cGy before BM transplantation ($n = 3$). Each symbol represents an individual animal. Disappearance of one symbol indicates the animal's death. Pooled data are presented from two independent experiments. Abbreviations: BMC, bone marrow cell; STZ, time point of streptozotocin treatment; TBI, total-body irradiation; Tr, time point of transplantation.

increased, and all mice died between 8 and 10 weeks after transplantation. Thus, transplantation of BM cells after a moderate sublethal irradiation (450 or 250 cGy) transiently reduces blood glucose levels in STZ-treated mice. Survival and blood glucose level was not affected when healthy control mice were irradiated with 900 cGy and then transplanted with syngeneic BMCs (Fig. 3H).

Mesenchymal Stem Cells Cooperate with Bone Marrow Cells in Successful Therapy of Diabetes

It has previously been shown that syngeneic or allogeneic MSCs are able to regenerate a variety of injured tissues [17–20]. To determine whether or not MSCs alone or together with unfractionated BM cells contribute to the repair of pancreatic functions, we injected syngeneic BM cells and/or different amount of semisyngeneic MSCs (isolated from male C57Bl/6xDBA/2F1 mice) intravenously into STZ-treated animals after sublethal irradiation at a dose of 250 cGy. The phenotype of culture-expanded MSCs was analyzed: these cells were strongly positive for the specific surface antigen Sca-1 and negative for differentiation markers of other cell lineages (CD34, CD3ε, CD45R/B220, CD11b, 6G, and TER-119) and were able to differentiate into the osteoblast and adipocyte lineages in vitro, verifying the MSC phenotype (supplemental online Fig. 1).

The experimental protocol is shown in Figure 3A. Blood glucose (BG) level and serum insulin (SI) concentration of diabetic animals rapidly returned to normal levels when they received 10^6 BM cells and 10^5 MSCs on day 15 (Figs. 3B and 4B, respectively), and these mice survived more than 180 days (Fig. S2). Decreasing the number of MSCs to 5×10^4 (Fig. 3C, S3C) or 2.5×10^4 (Fig. 3D, S3D) per animal greatly reduced the

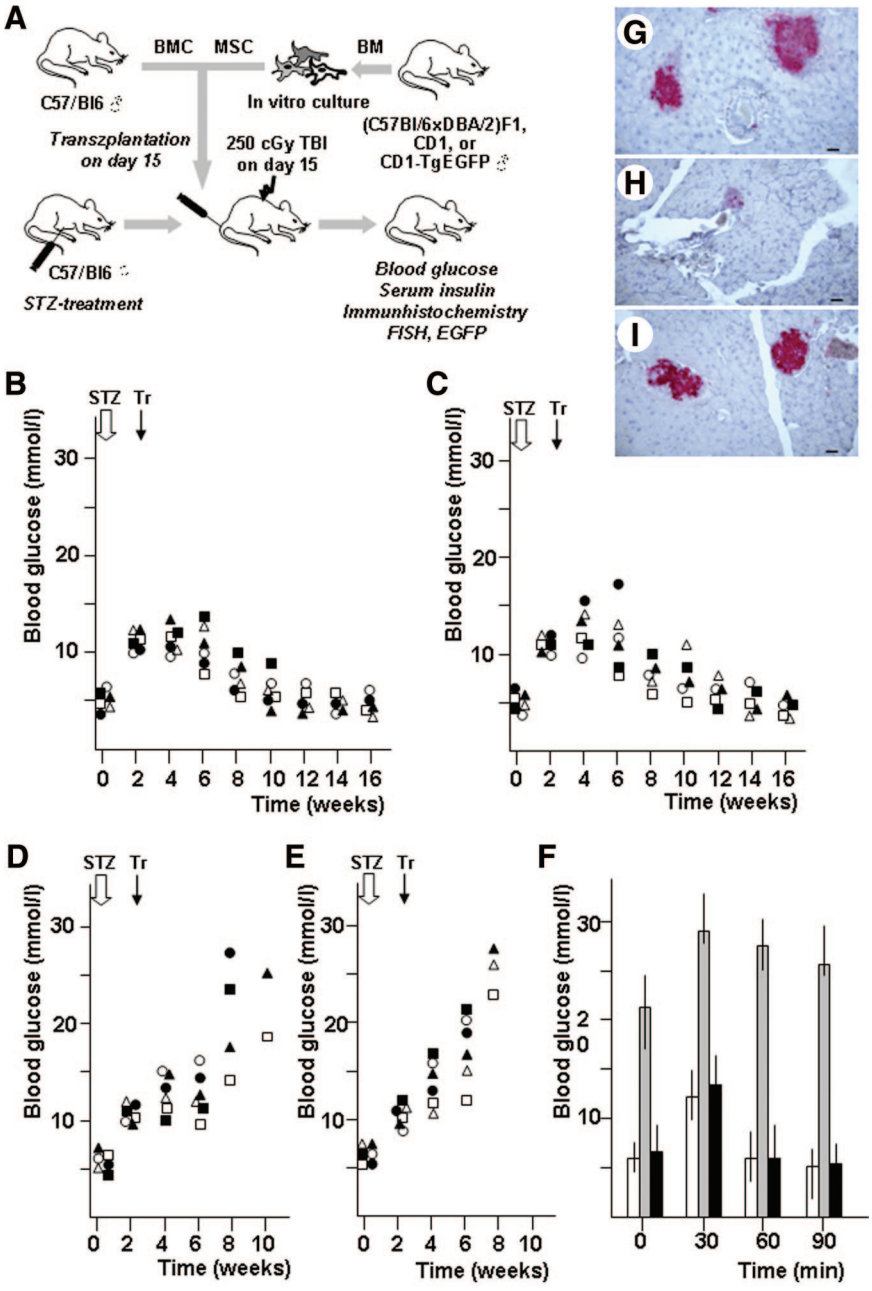

Figure 3. Mesenchymal stem cells cooperate with BMCs in successful therapy of diabetes. **(A):** Experimental design for the transplantation procedure and for the analysis of chimerism. **(B–D):** After 250 cGy TBI, STZ-treated female C57Bl/6 mice were transplanted with 10^6 nucleated BMCs isolated from syngeneic male donors and with 10^5 **(B)**, 5×10^4 **(C)**, or 2.5×10^4 **(D)** MSCs obtained from male (C57Bl/6xDBA/2)F1 animals ($n = 6$). **(E):** Diabetic mice were injected with 2×10^5 semiallogeneic MSCs only ($n = 6$). Each symbol represents an individual animal. Disappearance of one symbol indicates the animal's death. Pooled data are presented from two independent experiments. **(F):** Glucose tolerance test on week 12. All mice were fasted overnight and then injected with 2 g/kg of body weight glucose intraperitoneally. Blood sugar level was measured at the indicated time points. Open bars represent nondiabetic mice, light gray bars represent diabetic mice, and black bars represent STZ-treated, BM-plus-MSC-transplanted mice. Data are presented as mean ± SD; $n = 15$. Immunohistochemical detection of insulin (red) in pancreatic islets from healthy control **(G)**, diabetic (day 21) **(H)**, and BM-plus-MSC-transplanted diabetic (week 12) **(I)** mice. Scale bars = 20 μm. Abbreviations: BM, bone marrow; BMC, bone marrow cell; EGFP, enhanced green fluorescent protein; FISH, fluorescence in situ hybridization; STZ, time point of streptozotocin treatment; TBI, total-body irradiation; Tr, time point of transplantation.

reversing effect concerning BD and SI levels. Similarly, a minimum number of 5×10^5 nucleated marrow cells were required for successful treatment (supplemental online Fig. 3). Neither MSCs (2×10^5) nor BMCs (10^6) alone affected high blood glucose (Figs. 3E, 2F, respectively) and low serum insulin (Fig. 4D, 4C) levels and viability (supplemental online Fig. 2; Fig. 2B). Glucose tolerance test and immunohistochemical analysis of insulin-positive cells in pancreatic sections also proved the successful cooperation between MSCs and BM in the treatment of diabetes, since glucose tolerance (Fig. 3F) and mass of insulin-producing cells (Fig. 3I) became comparable to those of healthy controls (Fig. 3G) using the optimal combination of transplants. Insulin-positive cells did not appear in other organs, such as liver, spleen, lung, and bone marrow (data not shown).

Similar results were obtained when syngeneic (isolated from male C57Bl/6 mice) or allogeneic (isolated from male CD1 or EGFPTg-CD1 mice) MSCs were used for transplantation (supplemental online Table 1). Hence, MSCs and BM cells acted in concert in therapy of experimentally induced diabetes.

Timing of transplantation severely affected the outcome of the disease: six of nine animals survived longer than 20 weeks and returned to normal blood-glucose level when transplantation was carried out on day 8 after the beginning of STZ treatment, all nine animals survived and became normoglycemic with transplantation at day 15, two of nine mice survived when diabetic recipients were transplanted at day 22, and none of the animals survived if transplanted at day 29.

Recovery from Diabetes Is Not Due to Differentiation of Donor BM Cells or MSCs into Insulin-Producing β-Cells

The new pancreatic β-cells in mice that have recovered from diabetes may arise from endogenous or donor-derived sources. To determine whether donor bone marrow and/or MSCs can give rise to new insulin-producing cells, we performed a Y

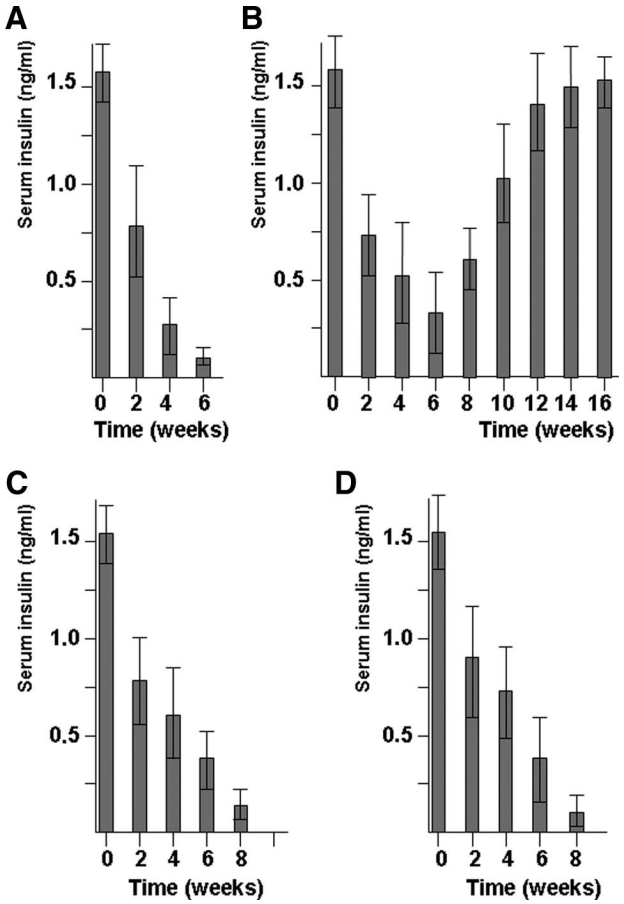

Figure 4. Serum insulin level is restored in diabetic mice after transplantation with bone marrow cells (BMCs) and MSCs. Animals were treated with streptozotocin (STZ) only (diabetic control) **(A)** or transplanted with 10^6 BMCs and 10^5 MSCs **(B)**, 10^6 BMCs alone **(C)**, or 10^5 MSCs alone **(D)** after STZ treatment. Data are presented as mean ± SD; $n = 9$. Experimental design is shown in Figure 3A.

chromosome-specific in situ FISH analysis combined with immunostaining for insulin on pancreatic sections (Fig. 5). The assay was first validated using pancreatic sections from healthy control male mice. Punctate staining for Y chromosome was evident in the nuclei of both acinar and islet cells (Fig. 5A). Labeled nuclei were not observed in tissue sections from control female animals (not shown). FISH analysis, carried out at day 2 (Fig. 5B) or day 84 (Fig. 5C) following transplantation of BM and MSCs from matched male donors, failed to give a positive signal in the nuclei of acinar and islet cells of female diabetic mice. Accordingly, using mismatched EGFPTg-CD1 transgenic mice as an MSC source, green fluorescence cells were absent in the recipient pancreas (not shown). These results clearly showed that the new β-cells did not arise from donor-derived sources.

Nevertheless, Y chromosome-carrying cells localized within the BM, indicating that the engraftment of donor-derived BM cells and/or MSCs homed into the recipient's marrow. The level of BM chimerism peaked at 10 days after transplantation (6%–12% donor-derived cells in the recipient's bone marrow) and then rapidly declined (Fig. 5D); meanwhile, the total number of nucleated cells in the bone marrow gradually increased (Fig. 5E). Green fluorescence protein-expressing MSCs did not appear in peripheral blood, bone marrow, liver, lung, or spleen of the transplanted animals (data not shown). These data indicated that the reappearance of pancreatic insulin-secreting cells and normalization of blood sugar and serum insulin level were results of an endogenous regenerative process activated in the host. We thoroughly examined the liver, spleen, stomach, intestine, lung, and bone marrow of mice transplanted with BM cells and syngeneic or allogeneic MSCs and did not observe signs of tumorogenic transformation in any of those organs (data not shown).

MSCs Suppress β-Cell-Specific T-Cell Response

Previous studies reported that direct damage of β-cells in STZ elicited diabetes was followed by activation of autoreactive T cells and, as a consequence, destruction of the islets [21, 22]. On the other hand, immunosuppressive effect of mesenchymal stem cells by inhibiting T-cell response has been also well-documented [23–25]. Therefore, we speculated that MSCs might inhibit β-cell-specific immune response, contributing to the restoration of endogenous pancreatic islet integrity. To test this hypothesis (experimental design is shown in Fig. 6A), we isolated T cells from pancreas of diabetic mice transplanted with BMCs or MSCs alone (Fig. 6D, 6E, respectively), transplanted with BM and MSCs in combination (Fig. 6C), or left untransplanted (Fig. 6B). Splenic antigen-presenting cells—isolated from diabetic mice—induced intensive proliferation of autoreactive T lymphocytes prepared from animals left untransplanted (Fig. 6B) or transplanted with BM cells alone (Fig. 6D). In contrast, MSC transplantation fully diminished T-cell proliferation when it was injected either alone (Fig. 6E) or in combination with BMCs (Fig. 6C). These findings strongly suggested that MSCs were able to suppress the β-cell-specific T-lymphocyte response.

To control the specificity of the autoimmune (e.g., β-cell antigen-triggered) T-cell proliferation and exclude an autonomous mitogenic effect or antigen-independent false response, the following experiment was carried out: T cells from diabetic animals were mixed with APCs from diabetic, control (isolated from untreated mice), or OVA-immunized spleen cells. Autoimmune T cells proliferated only when supported by diabetic or in vitro β-cell extract loaded control APC, but not in the presence of APCs from OVA-injected mice (supplemental online Table 2). These results prove that spleen APCs present β-cell-specific antigen isolated from either STZ-treated (in vivo-loaded) animals or normal APCs loaded in vitro with β-cell extract.

It has to be noted, however, that administration of Cyclosporin A (CsA), a strong immunosuppressive drug (10 mg/kg s.c., on days 15, 17, and 19), did not cause normalization of blood sugar level, and all animals died between 10 and 12 weeks (data not shown), indicating that immunosuppression was necessary but not sufficient for recovery from diabetes.

DISCUSSION

This work demonstrates that cotransplantation of syngeneic unfractionated BM cells and syngeneic or allogeneic culture-expanded MSCs can reverse STZ-induced diabetes in mice. This treatment of mice with hyperglycemia and islet destruction results in the reappearance of functional pancreatic insulin-secreting cells and normoglycemia. Transplantation with neither BM cells nor MSCs alone is sufficient for treatment of diabetes, indicating that these cells act in concert during islet regeneration. These findings are in conflict with earlier reports, since STZ-induced diabetes has been reverted with unfractionated BM cells [7], and human MSCs alone have been effective to treat diabetes in NOD/*scid* mice injected with multiple low doses of STZ [10]. Controversy between the above and our results can be explained by the various experimental design used

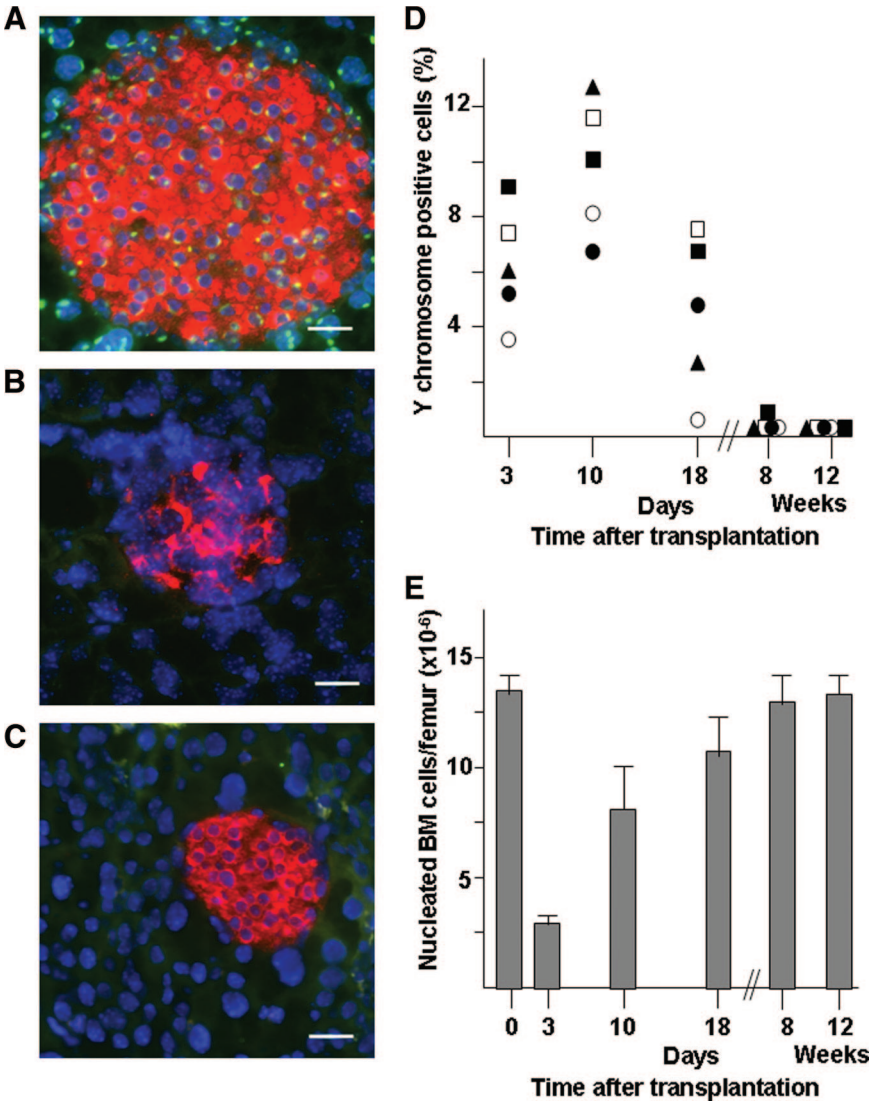

Figure 5. Male donor cells transiently home to the female recipient's bone marrow but do not appear in the regenerating pancreas. Shown are insulin (red), Y chromosome-specific fluorescence in situ hybridization (FISH) (green), and 4,6-diamidino-2-phenylindole (blue) staining of representative pancreatic sections from a healthy control male C57Bl/6 mouse (positive control) **(A)** and from diabetic, bone marrow cell (BMC)/MSC-transplanted female mice (experimental design is shown in Fig. 3A) **(B, C)**. Female animals were sacrificed at 2 **(B)** or 70 **(C)** days after grafting. Scale bar = 100 μm. Chimerism in the bone marrow of streptozotocin-treated and BMC/MSC-injected female recipients was determined by counting the Y chromosome-positive cell number on FISH-stained samples on day 10 after transplantation. Each symbol represents one individual animal ($n = 5$) **(D)**. The number of nucleated BM cells per femur in recipient mice was counted. Data are presented as mean ± SD; $n = 5$ **(E)**. Abbreviation: BM, bone marrow.

in these studies: (a) Banerjee et al. [7] reported that more than three consecutive injections of BM cells were required for successful treatment, whereas a single transplantation was enough using BM cells and MSCs in our experiments; and (b) Lee et al. [10] used a high number (2.5×10^6) of MSCs, injecting them into the left cardiac ventricle of NOD/*scid* mice through the chest wall to avoid a risk of pulmonary emboli caused by MSC aggregation. We used 10^5 MSCs for single injection through tail vein; hence, the incidence of cell aggregation was negligible.

The mechanism by which bone marrow-derived cells induce pancreatic tissue repair in our model is not yet clear. New pancreatic islets may arise from the transplanted bone marrow and/or mesenchymal stem cells. This possibility is not supported by our results, since neither Y chromosome-carrying nor $EGFP^+$ donor-derived β-cells are detected in the regenerated pancreas. Nevertheless, donor BM cells are present in the recipient's bone marrow, resulting in transient chimerism; hence, homing of donor BM cells can be followed by Y chromosome-specific FISH analysis. These results are well consistent with several other reports [5, 6, 26] showing that the differentiation capacity of adult marrow-derived cells is limited and that pancreatic β-cells are not substantially derived from the bone marrow in vivo.

The other source of the islet tissue can be endogenous. Regeneration of endocrine pancreas function has been documented after partial pancreatectomy, duct ligation, and cellophane wrapping, as well as in STZ-treated animal models [27]. Recently, Dor [28] presented evidence that existing β cells are able to regulate their own homeostasis in mouse. Evidence for β-cell self-duplication has also been reported during neonatal development, in which expression of cyclin D2 in the endocrine pancreas has been accompanied by β-cell proliferation [29]. Moreover, β-cell neogenesis can occur even in adult pancreas in animal models, since several groups have been able to isolate putative precursor cells from adult murine and human pancreas [30–33]; these cells were capable of differentiating into any endocrine components of the gland. However, these studies do not give a clear answer as to whether the newly formed insulin-secreting cells derive from replication of the few β-cells surviving the STZ treatment, transdifferentiation of nonendocrine epithelial cells [34], or proliferation and differentiation of tissue-specific stem cells. Neither we have addressed this issue in this paper; therefore, the endogenous source of the new β-cells in the cured animals remains to be elucidated.

It is clearly shown here that neither BM nor MSCs alone are sufficient to support the treatment. The BM graft itself contains a few mesenchymal cells, approximately 1–10 MSCs in 10^6

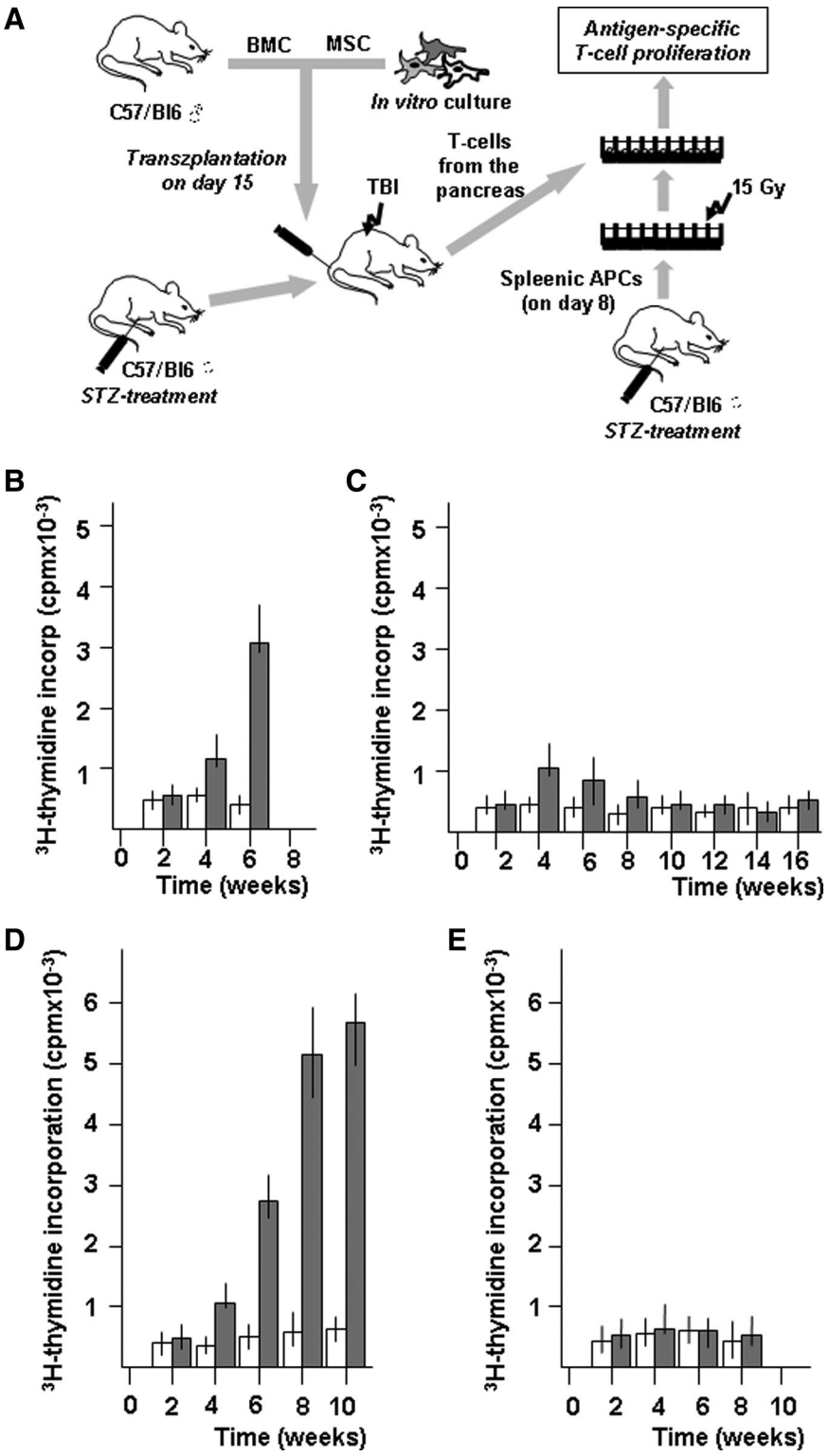

Figure 6. MSCs suppress β-cell specific T-cell response. **(A):** Experimental design for detection of antigen-reactive T cells in the pancreas of STZ-treated mice. **(B–E):** T cells (2×10^5 cells per well) from the pancreas of untransplanted animals **(B)** or animals transplanted with BMCs/MSCs (**C**), BMCs alone **(D)**, or MSCs alone **(E)** were isolated and mixed with APCs (5×10^4 cells per well) obtained from spleens of either untreated control animals or from another group of STZ-treated animals. Cell cultures were incubated on 96-well tissue culture plates for 72 hours at 37°C. Eighteen hours before harvest, cultures were pulsed with 1 μCi of ^{3}H-thymidine. Incorp ^{3}H-thymidine was measured using a liquid scintillation counter. Open columns represent cultures containing APCs derived from untreated control mice, and gray columns represent cultures containing APCs derived from STZ-treated animals. Data are presented as mean ± SD; $n = 7$. Abbreviations: APC, antigen-presenting cell; BMC, bone marrow cell; incorp, incorporated; STZ, streptozotocin; TBI, total-body irradiation.

nucleated BM cells [24], which is not a sufficient amount for successful treatment. Therefore, we have grafted diabetic animals with 10,000 times more MSCs in our setting. The important question still remains how BM cells and MSCs in combination contribute to β-cell regeneration. Transplanted BM cells and MSCs may provide essential factors inducing pancreatic tissue regeneration. Although the nature of these factors is not yet known, it has reported that hepatocyte growth factor produced by transplanted marrow cells can promote pancreatic β-cell regeneration in STZ-treated rats [35]. Others have suggested that donor marrow-derived endothelial precursor cells play an obligatory role during reversal of STZ-induced murine diabetes [3, 5], based on the fact that endothelial cell contact and signaling is necessary and sufficient for endocrine pancreatic development [36]. Mesenchymal stem cells may play a dual role: they may help in tissue repair by endogenous factors and

inhibit β-cell-specific T-cell response. The possibility of contribution to tissue regeneration emerges from the concept that MSCs have trophic effects inhibiting fibrosis and enhancing angiogenesis by secreting a variety of cytokines [37]. This hypothesis has been supported by a number of translational and clinical studies in the areas of cardiac infarct [38, 39], synovial joint regeneration [17], and stroke regeneration [40].

Other functions of MSCs in reversing degenerative β-cell disease may arise from their recently identified immunosuppressive property. Previous studies have reported inhibition of alloantigen- or mitogen-induced T-lymphocyte proliferation [41–44]. Another study has shown that MSCs influence the differentiation and maturation of dendritic cells from monocytes and interfere with their ability to activate T cells [45]. Moreover, MSCs have been used in successful management of graft-versus-host disease, another T-cell-mediated pathology [25, 46]. In agreement with these findings, we demonstrate that the β-cell-specific autoimmune response is strongly reduced in the presence of mesenchymal stem cells.

The importance of MSC immunosuppressive function in diabetes therapy is easily acceptable conception. Although STZ elicits diabetes by direct damage of β-cells, this degenerative course is followed by activation of an autoreactive T-cell response [11, 12]. The emerged autoimmune response does not allow further autoregenerative processes, similarly to human type I diabetes. Therefore, the interruption of β-cell-specific immune response may promote both the rescue of surviving β-cells in islets and the production of new β-cells, which are now able to survive in the altered immunological milieu. Previous studies have shown the feasibility of immunosuppression by anti-inflammatory drugs preventing or delaying the onset of type 1 diabetes in NOD mice [47–51]. Accordingly, treatment with monoclonal anti-CD3 antibody resulted in preserving β-cell function for at least 18 months in the majority of patients with recent onset of type 1 diabetes [52, 53]. Mesenchymal stem cells cannot be replaced with CsA in our treatment protocol, likely for the reasons that (a) in addition to their anti-inflammatory function, MSCs are involved in the tissue (pancreas) repair process, and (b) CsA has severe side effects, such as nephro- and hepatotoxicity, counteracting with the tissue regeneration [54, 55].

In conclusion, our work offers a novel potential therapeutic protocol for type 1 diabetes. Cotransplantation of syngeneic bone marrow cells with syngeneic or even allogeneic mesenchymal stem cells clearly supports pancreas tissue repair, stabilization of normal blood glucose and serum insulin level, and hence recovering and surviving of mice suffered from STZ-induced diabetes. The protocol we offer to treat diabetes is superior to others published so far: nonlethal, low-dose irradiation results in minimal tissue damage; one-step transplantation event can be carried out with a single intravenous injection of the graft without the risk of embolia caused by cell aggregation; transplanted MSCs do not require genetic identity; and no additional immunosuppression has to be applied. Important questions remain to be answered regarding the identification of bone marrow cell population and signals from BM cells and MSCs, which induce β-cell regeneration.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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