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REGENERATIVE MEDICINE

The Antidiabetic Effect of Mesenchymal Stem Cells Is Unrelated to Their Transdifferentiation Potential But to Their Capability to Restore Th1/Th2 Balance and to Modify the Pancreatic Microenvironment

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disease that results from cell-mediated autoimmune destruction of insulin-producing cells. In T1DM animal models, it has been shown that the systemic administration of multipotent mesenchymal stromal cells, also referred as to mesenchymal stem cells (MSCs), results in the regeneration of pancreatic islets. Mechanisms underlying this effect are still poorly understood. Our aims were to assess whether donor MSCs (a) differentiate into pancreatic β-cells and (b) modify systemic and pancreatic pathophysiologic markers of T1DM. After the intravenous administration of 5×10^5 syngeneic MSCs, we observed that mice with T1DM reverted their hyperglycemia and presented no donor-derived insulin-producing cells. In contrast, 7 and 65 days post-transplantation, MSCs were engrafted into secondary lymphoid organs. This correlated with a systemic and local reduction in the abundance of autoaggressive T cells together with an increase in regulatory T cells. Additionally, in the pancreas of mice with T1DM treated with MSCs, we observed a cytokine profile shift from proinflammatory to antinflammatory. MSC transplantation did not reduce pancreatic cell apoptosis but recovered local expression and increased the circulating levels of epidermal growth factor, a pancreatic trophic factor. Therefore, the antidiabetic effect of MSCs intravenously administered is unrelated to their transdifferentiation potential but to their capability to restore the balance between Th1 and Th2 immunological responses along with the modification of the pancreatic microenvironment. Our data should be taken into account when designing clinical trials aimed to evaluate MSC transplantation in patients with T1DM since the presence of endogenous precursors seems to be critical in order to restore glycemic control. STEM CELLS *2012; 30:1664–1674*

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

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disease that results from a cell-mediated autoimmune destruction of insulin-producing cells. At the time of clinical diagnosis, 60%–80% of pancreatic β-cells has been destroyed [1]. Thus, the regeneration of the endocrine pancreatic tissue has been the focus of T1DM therapeutic strategies under development [2, 3]. For this, multipotent mesenchymal stromal cells, also referred as to mesenchymal stem cells (MSCs), appear as an ideal tool since they (a) can be easily isolated from bone marrow aspirates and rapidly expanded ex vivo [4]; (b) are hypoimmunogenic, allowing allogeneic transplantation without the requirement of histocompatibility, recipient conditioning, and/or further immunosuppression [5]; (c) when systemically administered, home into injured organs and contribute to tissue regeneration [6]; and (d) have been transplanted to human patients with different diseases without major toxicity and with beneficial effects [7–10].

The antidiabetic effect of MSCs has been demonstrated in different animal models of T1DM [11–16]. Transplantation of syngeneic or allogeneic MSCs proves to be useful in preventing diabetes onset and also retarding its progression. We have shown that intravenously administered MSCs revert hyperglycemia, recover pancreatic islets, and restore the anatomical distribution of α-and β-cells in mice with T1DM [13, 14]. The relative contribution of MSC regenerative mechanisms to this therapeutic effect is still poorly understood. According to the classic view of stem cells, MSCs should acquire the phenotype of parenchymal cells and replace dead cells [17]. Nevertheless, in most of the published studies, the reported number of donor MSCs that reach and functionally integrate into damaged tissue is too low to support a physiological change (0.1%–2% of the total cells of the damaged tissue) [18]. On the other hand, MSCs might contribute to tissue

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regeneration due to their immunomodulatory potential [19]. It has been shown that they limit the expansion and cytotoxic activity of T lymphocytes and stimulate the appearance of regulatory T cells [20]. Furthermore, MSCs secrete antinflammatory cytokines and inhibit the expression of proinflammatory cytokines by immune cells [21, 22]. In animal models of autoimmune diseases (e.g., experimental encephalomyelitis and arthritis), systemically administered MSCs home into the affected organ and prevent the autoimmune destruction of both remnant and newly generated cells [23–25]. Finally, MSCs are able to produce both in vitro and in vivo antiapoptotic and mitogenic factors, among them are epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF) [18, 26]. The biological effects of these trophic factors can be direct (triggering intracellular signaling) or indirect (inducing neighboring cells to secrete other bioactive factors). Thus, MSCs might modify the organ microenvironment preventing the apoptosis of parenchymal cells and inducing the proliferation and differentiation of local progenitors [18, 26].

In order to approach to the elucidation of the mechanisms underlying the antidiabetic effect of MSCs, we assessed whether intravenously administered cells (a) differentiate into pancreatic β-cells and (b) modify systemic and pancreatic pathophysiologic markers of T1DM. For this, diabetes was induced in C57BL/6 adult male mice by the administration of five low doses of streptozotocin (STZ) [13, 14, 27]. Under this regimen, some but not all pancreatic β-cells are destroyed [28, 29]. Also, autoaggressive T cells are activated [30]. Hence, animals gradually loss their pancreatic islets and became hypoinsulinemic and hyperglycemic [13, 14]. Without insulin supplementation, they survive at least for 3 months, maintain body weight relatively constant, and slightly increase food and water intake. Twenty-five days after the first STZ dose, diabetic mice were randomly assigned into two groups: one group received a single dose of 5×10^5 bone marrow-derived MSCs or $MSCs^{GFP}$ that have been ex vivo expanded and characterized according to their plastic adherence, mesenchymal differentiation potential, and immunophenotype as previously described [31] (T1DM + MSC), and the other group received the vehicle (T1DM) (Supporting Information Fig. S1). A third group of nondiabetic animals (Normal) was also included in the study. Diabetes progression was evaluated according to the levels of glucose, insulin, and glycated hemoglobin in blood samples. Seven and 65 days post-transplantation, we determined: (a) the presence of pancreatic cells coexpressing insulin and green fluorescent protein (GFP) by immunohistofluorescence, (b) the biodistribution of $MSCs^{GFP}$ by immunohistofluorescence and flow cytometry, (c) the systemic and pancreatic abundance of autoaggressive and regulatory T cells by flow cytometry, (d) the functionality of systemic and pancreatic T lymphocytes by ex vivo culture and flow cytometry, (e) the systemic and pancreatic expression of proinflammatory and antinflammatory markers by real time reverse transcription-polymerase chain reaction (RT-PCR) and protein antibody arrays, (f) the intraislet apoptosis by immunohistofluorescence and real time RT-PCR, and (g) the systemic and local expression of trophic factors by real time RT-PCR and protein antibody arrays.

Materials and Methods

Animals

C57BL/6 and C57BL/6-Tg(ACTB-EGFP)1Osb mice (Jackson Laboratory, Bar Harbor, ME, www.jax.com) were housed at constant temperature and humidity, with a 12:12 hours light-dark cycle and unrestricted access to a standard diet and water. When required, animals were lightly or deeply anesthetized with sevofluorane (Abbott, Tokyo, Japan, www.abbott.com) or ketamine (Drag Pharma, Santiago, Chile, www.dragpharma.cl) plus xylazine (Centrovet, Santiago, Chile, www.centrovet.cl), respectively. Animal protocols were approved by the Ethic Committee of Facultad de Medicina Clinica Alemana-Universidad del Desarrollo.

Diabetes Induction

Eight-week-old male mice were lightly anesthetized. STZ (Calbiochem, La Jolla, CA, www.merckmillipore.com) was dissolved in 0.1 M citrate buffer pH 4.5 and immediately injected intraperitoneally at a dose of 40 mg/kg per day, for 5 consecutive days. Normal animals received citrate buffer only [14].

MSC and MSC^{GFP} Isolation, Ex Vivo Expansion, and Characterization

Eight- to 10-week-old male C57BL/6 or C57BL/6-Tg(ACTB-EGFP)1Osb mice were lightly anesthetized and sacrificed by cervical dislocation. Bone marrow cells were obtained by flushing femurs and tibias with sterile phosphate-buffered saline (PBS). After centrifugation, cells were suspended in α-minimum essential medium (Gibco, Auckland, New Zealand, www.invitrogen.com) supplemented with 10% selected fetal bovine serum (FBS) (Gibco) and 80 mg/ml gentamicin (Sanderson Laboratory, Santiago, Chile, www.sanderson.cl) and plated at a density of 1×10^6 nucleated cells per square centimeter. Nonadherent cells were removed after 72 hours by media change. When foci reached confluence, adherent cells were detached with 0.25% trypsin, 2.65 mM EDTA, centrifuged and subcultured at 7,000 cells per square centimeter. After two subcultures, adherent cells were characterized according to their adipogenic and osteogenic differentiation potential, as previously described [14]. Although there are currently no consensus markers for murine MSCs as there exist for human MSCs [32], immunophenotyping was performed by flow cytometry analysis after immunostaining with monoclonal antibodies against lymphocyte markers B220, CD4, and CD8-PE-Cy5 (BD Pharmingen, San Jose, CA, www.bdbioscience.com) and putative murine MSC markers SCA1-APC, CD90-FITC, and CD44-PE-Cy5 (all from eBioscience, San Diego, CA, www.ebioscience.com) [13].

MSC or MSC^{GFP} Intravenous Administration

Lightly anesthetized mice received via the tail vein 5×10^5 MSCs or $MSCs^{GFP}$ suspended in 0.2 ml of 5% mice plasma (T1DM + MSC) or 0.2 ml of 5% mice plasma (T1DM).

Blood Glucose Quantification and Diabetes Diagnostic Criterion

From nonfasted alert animals, blood samples were collected from the tail vein and glucose levels were determined with the glucometer system Accu-Chek Go (Roche Diagnostic, Mannheim, Germany, www.roche.com). Mice were considered diabetic if blood glucose levels were above 250 mg/dl, on three consecutive determinations.

Glycated Hemoglobin Quantification

From fasted alert animals, blood samples were collected from the tail vein and HbA_{1c} percentages were assessed using the DCA2000 analyzer (Bayer Corporation, Pittsburgh, PA, www.bayer.com) [33].

Plasma Insulin Quantification

From fasted alert animals, blood samples were collected from the tail vein. Plasmas were recovered by centrifugation and insulin concentrations were measured using mouse insulin ultrasensitive ELISA kit (Mercodia, Uppsala, Sweden, www.mercodia.se).

Donor MSC^{GFP} Detection in Recipient Organs

Animals were deeply anesthetized, exsanguinated, and intracardially perfused with 0.8% NaCl, 0.8% sucrose, and 0.4% glucose

for 10 minutes. Pancreas, intestine, liver, kidney, heart, blood, bone marrow, spleen, Peyer's patches, inguinal, mesenteric, and pancreatic lymph nodes were procured. For in situ detection of $MSCs^{GFP}$, organs were fixed in 4% paraformaldehyde pH 7.4. One day later, they were cryoprotected with 30% sucrose in buffered paraformaldehyde, rapidly frozen at −20°C in isopentane and sectioned. Ten-micrometer-thick sections were mounted, washed with PBS, stained with rabbit anti-GFP antibody (eBioscience) at 4°C overnight, incubated with goat anti-rabbit-FITC antibody (Vector Labs, Burlingame, CA, www.vectorlabs.com) at room temperature for 1 hour, and counterstained with 4′-6′-diamidino-2-phenylindole (DAPI) (Invitrogen, Grand Island, NY, www.invitrogen.com). To determine whether donor cells differentiated into insulin-producing cells, pancreatic sections were costained with goat anti-mouse insulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com) and donkey anti-goat-AlexaFluor555 antibody (Invitrogen). Sections were examined with the Fluoview FV10i confocal microscope (Olympus, Tokyo, Japan, www.olympus.com). Data were analyzed with the Olympus FV10-ASW2.1 software.

For flow cytometry analysis, solid organs were weighed, washed twice with ice-cold PBS, chopped and digested with 1 mg/ml collagenase type II (Gibco) at 37°C for 30 minutes. Cell suspensions were filtered through a 100 μm strainer and washed twice with ice-cold PBS. To ensure MSC^{GFP} recognition, isolated cells were fixed and permeabilized with BD Cytofix/Cytoperm kit (BD Pharmingen) and suspended in 1 ml of PBS with 2% FBS plus 0.5 μl of undiluted anti-GFP AlexaFluor647 antibody (Molecular Probes, Grand Island, NY, www.invitrogen.com). After incubation at 4°C for 12 hours, cells were washed, filtered through a 30 μm mesh, and acquired in a CyAn ADP flow cytometer (DakoCytomation, Carpenteria, CA, www.dako.com) as previously described [31]. Data were analyzed with Summit v4.3 software. Criteria used to consider an event as an MSC^{GFP} were FSC and SSC similar to ex vivo expanded MSCs and positive fluorescence both in FL1 (GFP) and FL8 (anti-GFP, AlexaFluor647) channels. Tissues from untreated diabetic mice were used as control of autofluorescence. For each organ, acquisition was performed up to detection of 100 events that fulfilled the criteria of an MSC^{GFP}. In the case of organs where donor $MSCs^{GFP}$ were not detectable, a total of 500,000 events were acquired. Results are presented as presence (detectable) or absence (under the detection limit).

Systemic and Pancreatic Autoaggressive and Regulatory T Lymphocyte Determination

Animals were deeply anesthetized and exsanguinated. Spleens and pancreatic lymph nodes were procured, minced, and filtered through 100 μm strainer. Mononuclear cells were isolated using Ficoll-Hypaque and incubated with fluorescence-activated cell sorting (FACS) lysing solution at room temperature for 10 minutes. Then, an aliquot of the cells was simultaneously stained with anti-mouse CD45R-PE-TR (BD Pharmingen), anti-mouse CD19-APC-H7 (BD Pharmingen), anti-mouse CD8-AF780 (eBioscience), anti-mouse CD4-FITC (BD Pharmingen), anti-mouse CD3-PE-Cy5 (eBioscience), and anti-mouse CD40-PE antibodies (eBioscience) at 4°C for 30 minutes. Another aliquot was stained with anti-mouse CD4-FITC and anti-mouse CD25-APC (eBioscience) antibodies at 4°C for 30 minutes. Then, cells were fixed and permeabilized with BD Cytofix/Cytoperm kit and stained with anti-mouse foxp3-PE antibody (eBioscience) at 4°C for 30 minutes [34]. Isotype controls were used to distinguish nonspecific background staining from specific antibody staining. Cells were acquired in a CyAn ADP flow cytometer. Data were analyzed with Summit v4.3 software and lymphocytes were gated according to their forward scatter (FSC) and side scatter (SSC). The immunophenotypes of autoaggressive and regulatory T cells were CD45R−/CD19−/CD8−/CD4lo/CD3lo/CD40+ (Supporting Information Fig. S2) and CD4+/CD25+/foxp3+ (Supporting Information Fig. S3), respectively [35]. Results are presented as the percentage of total CD4-positive cells that displayed an immunophenotype of autoaggressive or regulatory T cells.

Systemic and Pancreatic T Lymphocyte Activation Assessment

Animals were deeply anesthetized and exsanguinated. Spleens and pancreatic lymph nodes were procured, minced, and filtered through 100 μm strainer. Mononuclear cells were purified using Ficoll-Hypaque (Sigma, St. Louis, MO, www.sigmaaldrich.com), suspended in RPMI-1640 (Gibco) supplemented with 5% FBS, 25 ng/ml phorbol-12-miristate-13-acetate (PMA) (Sigma), 1 μg/ml of Ionomycin (Sigma), and 10 μg/ml Brefeldin A (Sigma), and cultured under an atmosphere with 5% CO_2 at 37°C. Four hours later, cells were harvested and incubated with FACS lysing solution (BD Pharmingen) at room temperature for 10 minutes. Then, cells were stained with anti-mouse CD4-FITC antibody (eBioscience) at 4°C for 30 minutes. After fixing and permeabilizing with BD Cytofix/Cytoperm kit (BD Pharmingen), cells were washed once with PBS and stained with anti-mouse IL2-APC, anti-mouse IFNgamma-PE, or anti-mouse TNFalpha-APC antibodies (all from eBioscience) at 4°C for 30 minutes. Isotype controls were used to distinguish nonspecific background staining from specific antibody staining. Cells were acquired in a CyAn ADP flow cytometer. Data were analyzed with Summit v4.3 software and T lymphocytes were gated according to their FSC, SSC, and their expression of CD4 [36] (Supporting Information Fig. S4). Results are presented as the percentage of total CD4-positive cells that are positive for each cytokine after ex vivo stimulation.

Pancreatic Gene Expression Analysis

Animals were deeply anesthetized and exsanguinated. Pancreases were immediately procured. Total RNA was purified using Absolutely RNA Miniprep kit (Stratagene, Santa Clara, CA, www.stratagene.com). One microgram of total RNA was used for reverse transcription. Real time PCR reactions were performed in a final volume of 10 μl containing 50 ng cDNA, PCR LightCycler-DNA Master SYBRGreen reaction mix (Roche, Indianapolis, IN, www.roche.com), 3 mM $MgCl_2$, and 0.5 μM of each primer (Supporting Information Table S1), using a Light-Cycler 1.5 thermocycler (Roche). To ensure that amplicons were from mRNA and not from genomic DNA amplification, controls without reverse transcription were included. Amplicons were characterized according to their size evaluated by agarose gel electrophoresis and to their melting temperature determined in the LightCycler thermocycler (Supporting Information Table S1). The mRNA level of each target gene was standardized against the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), for the same sample [37]. Results are presented as fold of change versus nondiabetic mice (Normal).

Plasma Inflammation and Trophic Markers Quantification

Animals were deeply anesthetized and exsanguinated. Plasmas were recovered by centrifugation and the levels of IL1beta, TNFalpha, MCP1, ICAM1 (proinflammatory); IL4, IL10, IL13, RANTES (antinflammatory); EGF, IGF1, HGF, and bFGF (trophic) were assessed using custom mouse protein antibody arrays (RayBiotech, Norcross, GA, www.raybiotech.com). Briefly, blocked membranes were incubated with plasmas diluted 1:6 for 2 hours. After washing, biotin-conjugated streptavidin was added. One hour later, they were exposed to the chemiluminescent solution. Finally, membranes were scanned and spots were analyzed using the Custom RayBio Mouse Cytokine Antibody Array Analysis Tool (RayBiotech). To minimize assay variability, all membranes were processed and developed simultaneously. Spot intensities of positive controls were used to normalize data from different membranes. Results are presented as relative units.

Pancreatic Apoptosis Rate Determination

Animals were deeply anesthetized and exsanguinated. Pancreases were procured and immediately immersed in 4% paraformaldehyde pH 7.4. One day later, they were embedded in paraffin and sectioned. Sections of 5-μm thick were deparaffinized in xylene, rehydrated, and washed in PBS. Antigen retrieval was performed by incubation

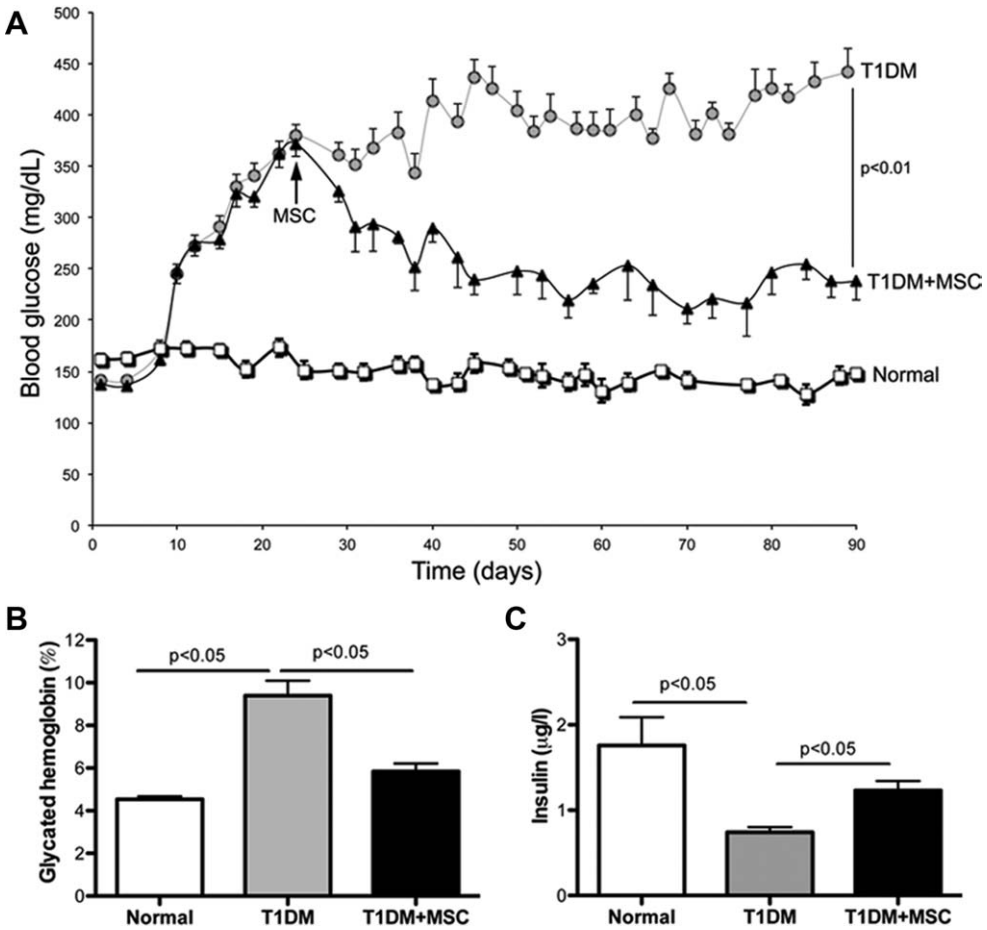

Figure 1. MSCs revert hyperglycemia, normalize glycated hemoglobin levels, and improve insulin secretion in mice with T1DM. Diabetic mice received the vehicle (T1DM) or 5×10^5 MSCs (T1DM+MSC). Blood glucose levels were assessed every 3 days **(A)**. Sixty-five days post-transplantation, glycated hemoglobin **(B)** and insulin **(C)** levels were determined ($n = 10$). Abbreviations: MSC, mesenchymal stem cell; T1DM, type 1 diabetes mellitus.

in citrate buffer 10 mM pH 6, in a boiling water bath for 30 minutes. Sections were blocked with 2% bovine serum albumin (Sigma) and incubated with goat anti-mouse insulin antibody (Santa Cruz Biotechnology) and rabbit anti-mouse active caspase-3 antibody (Cell Signaling Technology, Danvers, MA, www.cellsignal.com) at 4°C overnight. After washing, sections were incubated with donkey anti-goat-AlexaFluor555 (Invitrogen) and goat anti-rabbit-FITC antibodies (Vector Labs) at room temperature for 1 hour and counterstained with DAPI (Invitrogen). For terminal uridine nucleotide end-labeling (TUNEL) staining, sections were immunostained for insulin detection and further processed using the In Situ Cell Death kit (Roche Diagnostic), following manufacturer's instructions. Sections were examined with the Fluoview FV10i confocal microscope (Olympus). Data were analyzed with the Olympus FV10-ASW2.1 software. Results are presented as percentages of intraislet apoptotic cells.

Statistical Analysis

Data are presented as mean ± SEM. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Bonferroni post hoc test. $p < .05$ were considered statistically significant.

Results

MSCs Improve Diabetes Condition in Mice with T1DM

According to what we have already published, 7 days post-transplantation mice with T1DM that received MSCs reduced their blood glucose levels, reaching almost euglycemic values a month later (Fig. 1A) [13, 14]. This hyperglycemia reduction lasted up to the end of the study period and correlated with the normalization of the levels of glycated hemoglobin and plasmatic insulin (Fig. 1B, 1C). In contrast, mice with T1DM that did not receive MSCs remained hyperglycemic, with high levels of glycated hemoglobin and hypoinsulinemic.

MSCs Do Not Differentiate into Insulin-Producing Cells but Engraft into Secondary Lymphoid Organs of Mice with T1DM

To assess whether intravenously administered MSCs in vivo differentiate into insulin-producing cells, we transplanted $MSCs^{GFP}$ into mice with T1DM. We detected no cell co-expressing insulin and GFP in recipient pancreases (Fig. 2A).

Regarding to intravenously administered MSC biodistribution in mice with T1DM, we observed no donor cells in the pancreas, liver, kidney, intestine, and blood of these animals. In contrast, $MSCs^{GFP}$ were found in the heart, Peyer's patches, inguinal, mesenteric, and pancreatic lymph nodes procured from mice with T1DM, 7 and 65 days post-transplantation (Fig. 2B, 2C).

MSCs Restore the Systemic and Local Balance Between Autoaggressive and Regulatory T Cells in Mice with T1DM

Although several genetic factors influence diabetes, a common feature of autoimmunity is the disruption of the balance

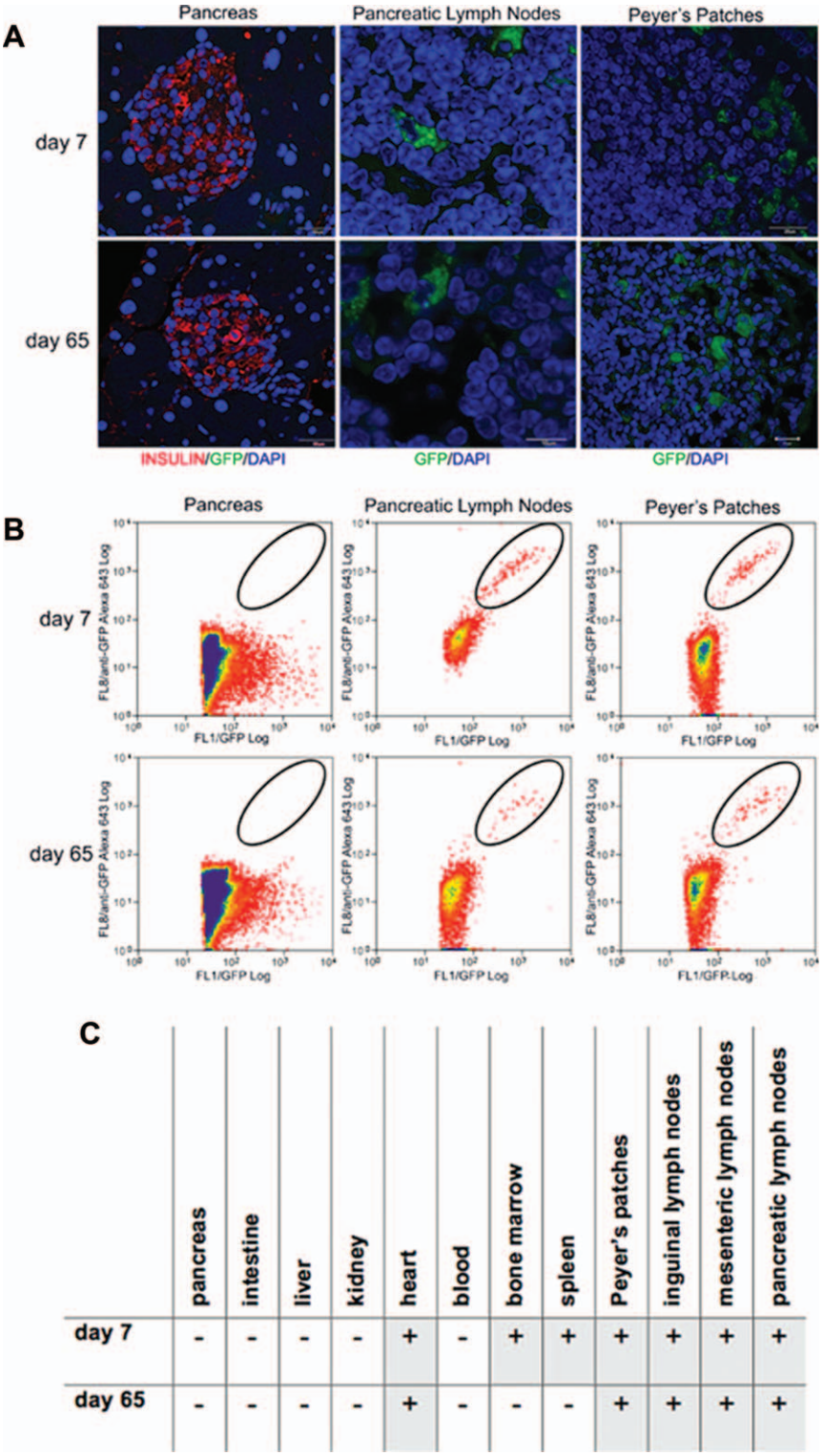

Figure 2. Mesenchymal stem cells (MSCs) do not differentiate into insulin-producing cells but engraft into secondary lymphoid organs of mice with type 1 diabetes mellitus (DMT1). Diabetic mice received 5×10^5 $MSCs^{GFP}$. Seven and 65 days later, presence of donor cells was assessed by immunohistofluorescence **(A)** and flow cytometry **(B)** in several organs **(C)**. −: under detection limit. +: detectable (representative of $n = 6$). Abbreviations: DAPI, 4′-6′-diamidino-2-phenylindole; GFP, green fluorescent protein.

between autoaggressive (destructive) and regulatory (protective) T cells [35]. Seven and 65 days post-transplantation, a significant decrease in the abundance of autoaggressive T cells was observed in both the spleen (systemic) and pancreatic lymph nodes (local) of mice with T1DM treated with MSCs, compared with untreated diabetic mice (Fig. 3A). On the other hand, the intravenous administration of MSCs resulted in an increase of regulatory T cells in the spleen (7 and 65 days) and the pancreatic lymph nodes (7 days), compared with untreated diabetic and nondiabetic mice (Fig. 3B).

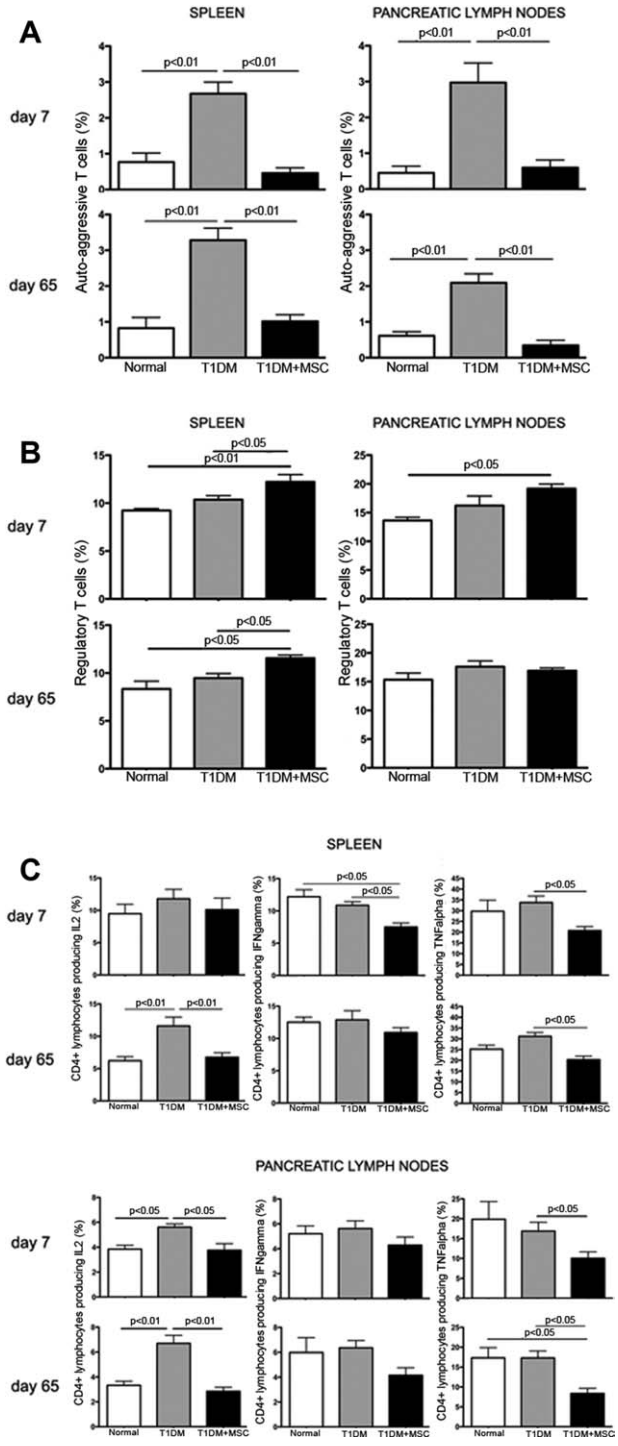

Figure 3. MSCs restore the systemic and local balance between autoaggressive and regulatory T cells in mice with T1DM. Seven and 65 days posttransplantation, mononuclear cells were isolated from the spleen and pancreatic lymph nodes. CD45R−, CD19−/CD8−/CD4lo/CD3lo/CD40+ **(A)**, CD4+/CD25+/foxp3+ **(B)**, and IL2-, INFgamma-, or TNFalpha-producing T lymphocytes **(C)** were quantified by flow cytometry ($n = 7$). Abbreviations: MSC, mesenchymal stem cell; T1DM, type 1 diabetes mellitus.

Also, we observed that T cells isolated 7 and 65 days posttransplantation from the spleen and the pancreatic lymph nodes of mice with T1DM that received MSCs were less prone to produce proinflammatory cytokines when stimulated ex vivo than the same cells isolated from untreated diabetic mice (Fig. 3C).

MSCs Restore the Pancreatic Inflammatory Balance and Increase the Systemic Antinflammatory Tone of Mice with T1DM

As expected, in the pancreas of mice with T1DM, the expression of IL1beta, IL18, TNFalpha, and MCP1 was higher than in nondiabetic animals (Fig. 4A). Interestingly, 7 days after MSC transplantation, these proinflammatory markers were downregulated to the basal levels and maintained lowup to the end of the study period. On the other hand, 7 days posttransplantation, the pancreatic mRNA levels of IL4, IL5, IL10, and IL13 were not statistically different among the experimental groups (Fig. 4B). Nevertheless, the expression of these antinflammatory molecules diminished over time in mice with T1DM but not in those that received MSCs. Regarding to circulating inflammatory markers, 7 days after MSC transplantation proinflammatory signals remained unchanged, whereas the antinflammatory markers IL13 and RANTES were increased compared with untreated diabetic and nondiabetic mice (Fig. 5).

MSCs Do Not Reduce the Pancreatic Apoptosis Rate but Increase Systemic and Local EGF Levels in Mice with T1DM

During the course of T1DM, insulin-producing cells die by apoptosis [38]. Accordingly, in the pancreatic islets of mice with T1DM, the percentages of cells positive for either active caspase-3 or TUNEL were higher than in nondiabetic mice (Figs. 6A, 6B). MSCs did not significantly modify this at the time points studied. Nevertheless, in mice with T1DM treated with MSCs, the BCL2 (antiapoptotic) versus BAX (proapoptotic) gene expression ratio was higher than in untreated diabetic mice (Fig. 6C).

MSCs are known to produce, both in vitro and in vivo, a broad range of trophic factors that have been associated with tissue regeneration [26]. Among the trophic factors assessed, only EGF increased significantly both systemically and locally in mice with T1DM treated with MSCs compared with untreated diabetic mice (Fig. 7).

Discussion

T1DM is still one of the most challenging diseases for patients, their relatives, and the health system. Its prevalence is expected to rise in the next years [39]. Hence, a therapeutic strategy aimed to recover lost insulin-producing cells and prevent the autoimmune destruction of remnant and newly generated cells is highly desirable [40]. MSCs represent a promising tool for this due to their transdifferentiation potential as well as to their immunomodulatory properties [41–44]. At preclinical level, we and other researchers have proven that systemic administration of bone marrow-derived MSCs results in a recovery of pancreatic islets, increases blood insulin secretion, and corrects hyperglycemia [11–16]. Nevertheless, the relative contribution of MSC regenerative mechanisms (transdifferentiation, immunomodulation, apoptosis prevention, and proliferation/differentiation induction) underlying this therapeutic effect is still poorly understood. Here, we show that soon after transplantation and up to the end of the follow-up period, MSC-treated diabetic mice corrected hyperglycemia and normalized glycated hemoglobin levels. Together, blood insulin levels are increased. These

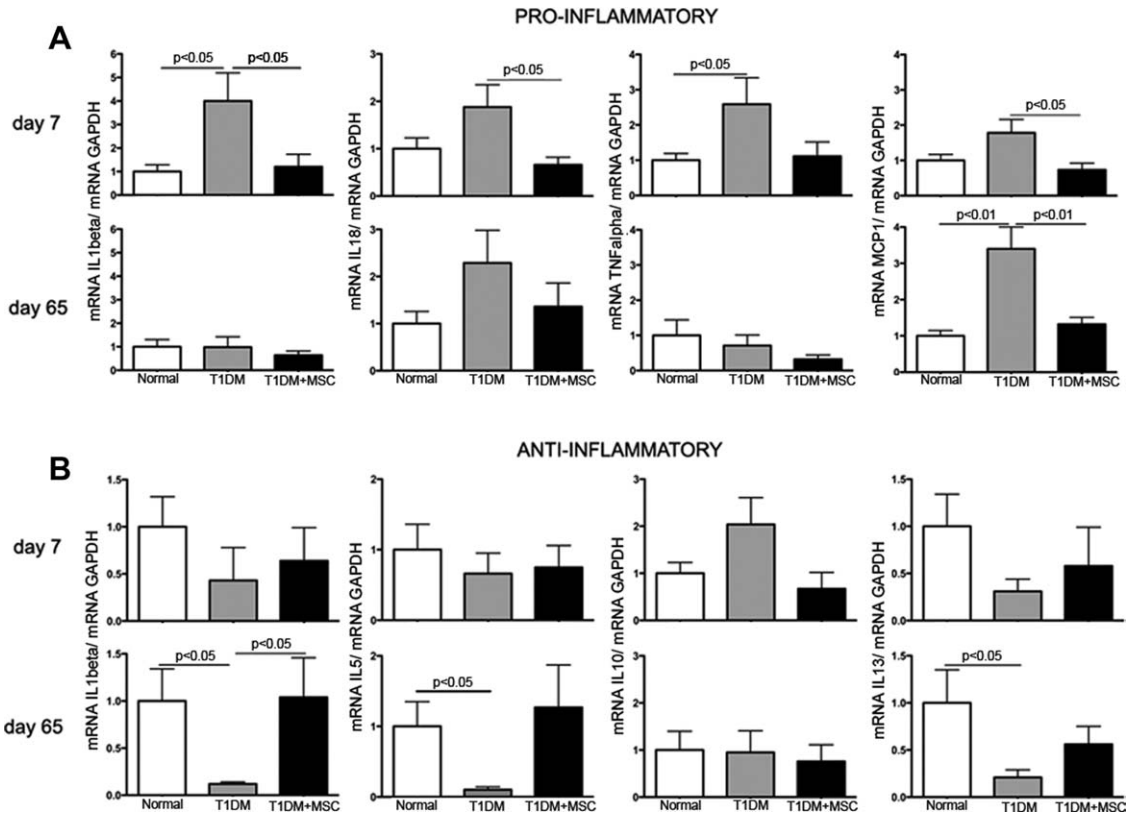

Figure 4. MSCs restore the pancreatic inflammatory balance of mice with T1DM. Seven and 65 days post-transplantation, pancreatic mRNA levels of proinflammatory **(A)** and anti-inflammatory **(B)** markers were analyzed by real time RT-PCR ($n = 7$). Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSC, mesenchymal stem cell; T1DM, type 1 diabetes mellitus.

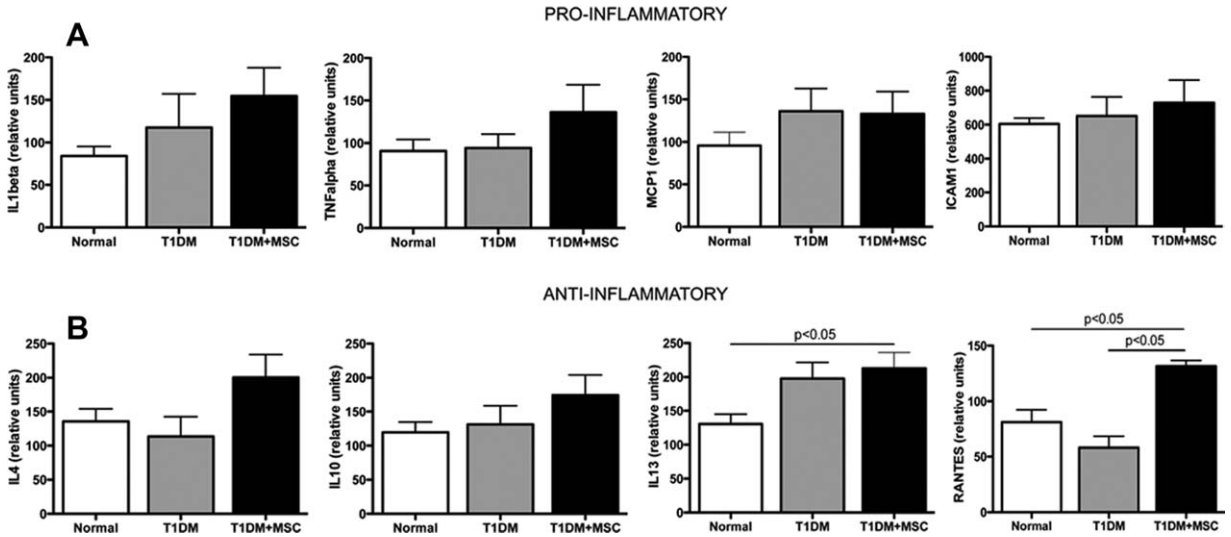

Figure 5. MSCs increase the systemic anti-inflammatory tone in mice with T1DM. Seven days post-transplantation, plasmatic levels of proinflammatory **(A)** and anti-inflammatory **(B)** markers were analyzed by custom mouse protein antibody arrays ($n = 6$). Abbreviations: MSC, mesenchymal stem cell; T1DM, type 1 diabetes mellitus.

changes were not related to donor cell differentiation into pancreatic β-cells since no cell expressing both insulin and GFP were detected in the pancreas of mice with T1DM that received $MSCs^{GFP}$. This result is consistent with data obtained in other animal models of diabetes, where it has been shown that the in vivo transdifferentiation of MSCs to pancreatic β-cells is very limited or inexistent [14, 45, 46]. Interestingly, while no donor cells were detected in pancreas, we found them in secondary lymphoid organs (spleen; Payer's patches; inguinal, mesenteric, and pancreatic lymph nodes). This is not without precedent since when administered to mice with experimental autoimmune encephalomyelitis, MSCs home into lymphoid organs where they cluster around T lymphocytes [24, 25].

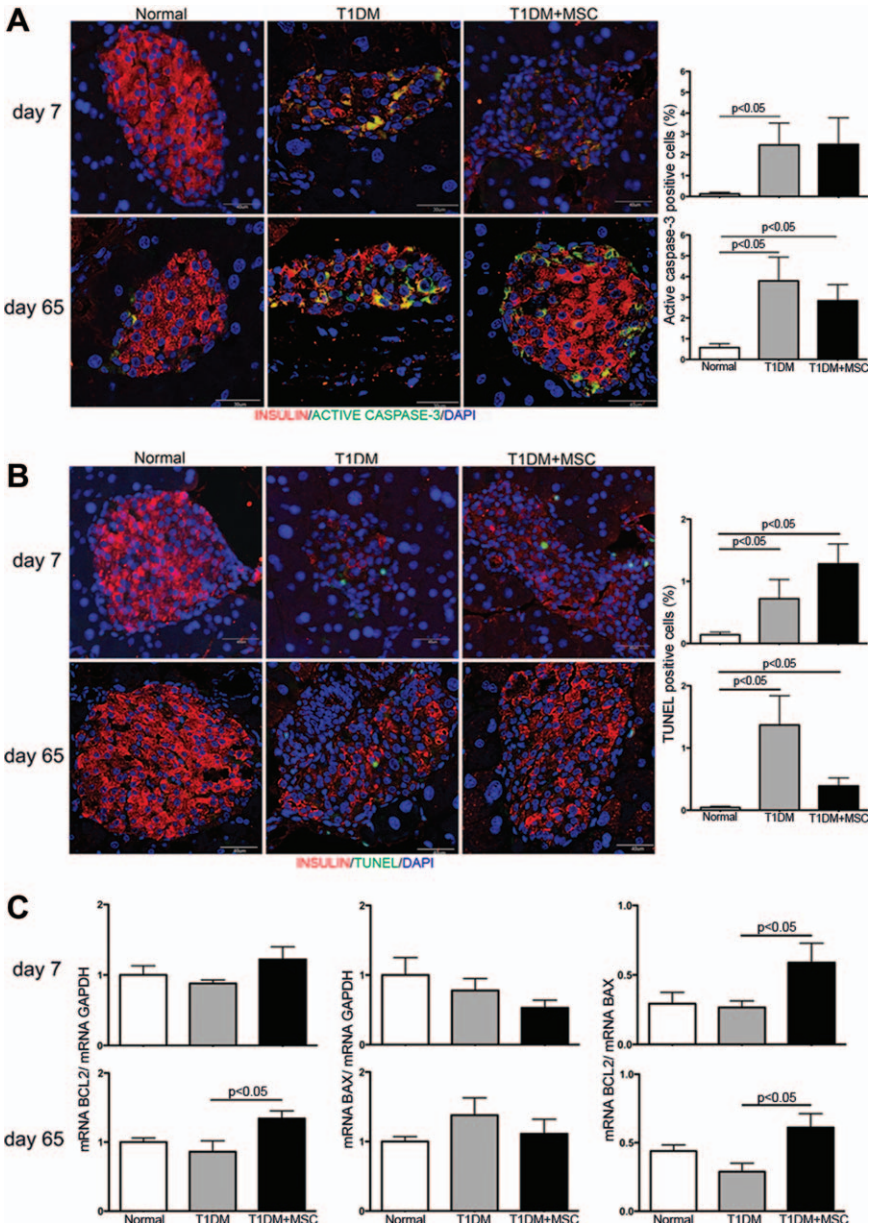

Figure 6. MSCs do not reduce pancreatic apoptosis in mice with T1DM. Seven and 65 days post-transplantation, apoptotic rate in the pancreatic islets were determined by the quantification of active caspase-3 **(A)** and TUNEL-positive cells **(B)**. Pancreatic mRNA levels of antiapoptotic and proapoptotic markers were analyzed by real time RT-PCR **(C)** ($n = 7$). Abbreviations: GAPDH, glyceraldehide-3-phosphate dehydrogenase; MSC, mesenchymal stem cell; T1DM, type 1 diabetes mellitus; TUNEL, terminal uridine nucleotide end-labeling.

A common feature of autoimmune diseases is the occurrence and persistence of autoaggressive T cells concomitant with a breakdown in the mechanisms maintaining peripheral tolerance [47]. In T1DM, autoaggressive T cells promote inflammation, stimulate B cells to produce autoantibodies, and directly attack pancreatic β-cells, leading to their death [48]. It has been shown that these cells are necessary and sufficient to transfer diabetes from the nonobese diabetic mice to immunodeficient mice [35]. Both in patients and in animal models of T1DM, autoaggressive T cells have been characterized according to the expression of CD4 and CD40 surface markers, and they are found greatly expanded when compared with nondiabetic individuals [48, 49]. Antigen-specific activation and expansion of autoaggressive T cells in secondary lymphoid organs may be suppressed by regulatory T cells [49, 50]. While a regulatory T cell functional deficiency results in T1DM, reconstitution of the regulatory T-cell pool prevents its development [51]. Therefore, the fine balance between autoaggressive and regulatory T cells must be restored in order to revert T1DM [49, 50]. We found that, 7 and 65 days post-transplantation, the abundance of autoaggressive T cells is reduced and that of regulatory T cells is increased, both systemically (spleen) and locally (pancreatic lymph nodes), in mice with T1DM that received MSCs. Furthermore, when we assessed the functionality of the T lymphocytes isolated from MSC-treated diabetic mice, we observed that they were less prone to produce proinflammatory cytokines (IL2, IFNgamma, and TNFalpha) than the same cells isolated from untreated diabetic mice. MSC administration also resulted in a shift from proinflammatory to antinflammatory cytokine expression and secretion.

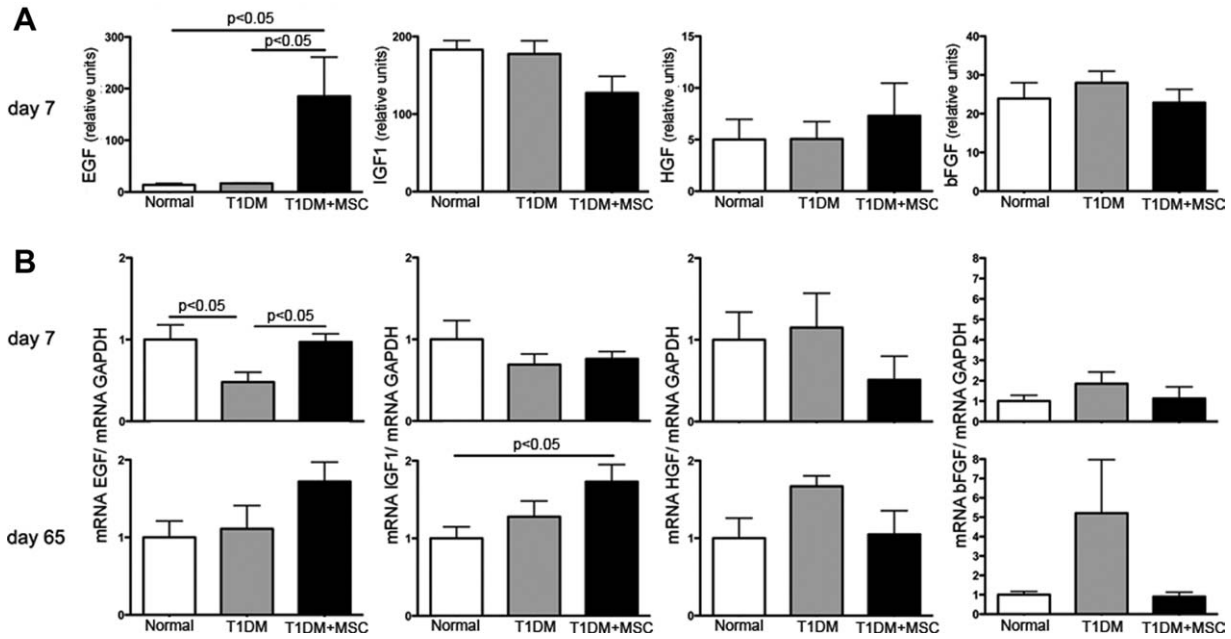

Figure 7. MSCs increase systemic and local EGF levels in mice with T1DM. Seven and 65 days post-transplantation, expression of trophic factors was assessed in plasma samples by custom mouse protein antibody arrays **(A)** and in pancreas by real time RT-PCR **(B)** ($n = 6$). Abbreviations: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GAPDH, glyceraldehide-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; MSC, mesenchymal stem cell; T1DM, type 1 diabetes mellitus.

Most robust changes of proinflammatory markers occur 7 days post-transplantation, whereas antinflammatory markers vary significantly 65 days post-transplantation.

Thus, our data generated using a correlation approach and an animal model that shares the causes, signs, main complication, and evolution of patients with T1DM, but that does not develop advanced lesions due to its short-life span [14, 38], suggest that once in secondary lymphoid organs donor MSCs inhibit the expansion of autoaggressive T cells either inducing regulatory T cells or by modifying the cytokine profile. The former is in concordance with previous reports for other autoimmune diseases, and for T1DM in other animal models [15, 23, 52]. The latter agrees with the fact that, both in vitro and in vivo, MSCs might secrete antinflammatory and immunoregulatory factors that suppress Th1 activation [15, 53, 54]. Whether the observed changes are selective for pancreatic immunogens or compromise the whole immune response is currently unknown. Nevertheless, we found no complications indicative of systemic immunosuppression (infections and tumor formation) in MSC-treated diabetic mice.

Finally, it is well known that MSCs secrete trophic factors including those that inhibit the apoptosis of mature cell and stimulate the proliferation and differentiation of endogenous progenitor of endocrine pancreas [55–58]. We observed no statistically significant reduction of intraislet apoptosis rate in mice with T1DM that received MSCs. On the other hand, in these animals both plasmatic and pancreatic levels of EGF increased prompt after MSC administration. It has been reported that the transgenic expression of EGF in the pancreas enhances β-cell proliferation and differentiation [59]. Also, combined EGF and gastrin treatment proved to increase insulin-producing cell mass in diabetic rodents due to neogenesis from pancreatic duct cells [58, 60].

Hence, we proved that MSC transplantation results in overcoming of the pathophysiological mechanisms of T1DM, mainly the imbalances between autoaggressive (destructive) and regulatory (protective) T cells and between Th1 (proinflammatory) and Th2 (antinflammatory) immune responses. Also, in the restoration of the ability of the pancreatic microenvironment to support regeneration, due to the constraint of the expression of cytokines that directly impair pancreatic β-cell function/proliferation/differentiation [61], and the recovery of the expression and secretion of trophic factors that are critical for those processes [58]. Together, lost cells seem to be replaced by either self-duplication of pancreatic β-cells or differentiation of endogenous progenitors [35, 51, 62, 63].

Conclusions

The antidiabetic effect of MSCs systemically administered is unrelated to their transdifferentiation potential but to their capability to restore the ratio between autoaggressive/regulatory T cells and Th1/Th2 cytokines together with the modification of the pancreatic microenvironment. Hence, the transplantation of MSCs restores the immune balance and allows the regeneration of pancreatic islets from endogenous cells with the concomitant reduction in blood glucose levels.

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Disclosure Of Potential Conflicts Of Interest

The authors indicate no potential conflicts of interest.

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