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Co-transplantation of autologous MSCs delays islet allograft rejection and generates a local immunoprivileged site

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Abstract

Aims—Mesenchymal stem cells (MSCs) are multipotent cells with immunomodulatory properties. We tested the ability of MSCs to delay islet allograft rejection.

Methods—Mesenchymal stem cells were generated in vitro from C57BL/6 and BALB/c mice bone marrow, and their immunomodulatory properties were tested in vitro. We then tested the effect of a local or systemic administration of heterologous and autologous MSCs on graft survival in a fully allogeneic model of islet transplantation (BALB/c islets into C57BL/6 mice).

Results—In vitro, autologous, but not heterologous, MSCs abrogated immune cell proliferation in response to alloantigens and skewed the immune response toward a Th2 profile. A single dose of autologous MSCs co-transplanted under the kidney capsule with allogeneic islets delayed islet rejection, reduced graft infiltration, and induced long-term graft function in 30 % of recipients.

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Moufida Ben Nasr and Andrea Vergani have contributed equally to the paper.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed, as well as specific national laws (e.g., the current version of the German Law on the Protection of Animals) were applicable.

Informed consent This study does not involve human subjects. No informed consent needs to be obtained.

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Based on ex vivo analysis of recipient splenocytes, the use of autologous MSCs did not appear to have any systemic effect on the immune response toward graft alloantigens. The systemic injection of autologous MSCs or the local injection of heterologous MSCs failed to delay islet graft rejection.

Conclusion—Autologous, but not heterologous, MSCs showed multiple immunoregulatory properties in vitro and delayed allograft rejection in vivo when co-transplanted with islets; however, they failed to prevent rejection when injected systemically. Autologous MSCs thus appear to produce a local immunoprivileged site, which promotes graft survival.

Keywords

Islet transplantation; Mesenchymal stem cells; Immunoprivileged site; Immunoregulation

Introduction

The standard treatment for type 1 diabetes (T1D) is exogenous insulin injection, which efficiently improves glycometabolic control [1, 2]. However, insulin treatment cannot fully prevent the development of the severe chronic complications related to diabetes, such as kidney failure, coronary heart disease, retinopathy, and neuropathy [3–7]. Islet transplantation is a relatively low-invasive procedure that has been shown to normalize glycemia and effectively counteract the development of diabetic complications [8–13]. Unfortunately, the recurrence of autoimmunity and the development of alloimmune anti-islet response greatly jeopardize the long-term function of the islets, which remains poorer compared to that of other grafts (below 20 % at 5 years) [8, 14]. Various immunomodulatory strategies have been thus tested or are currently under investigation to prevent auto- [15, 16] and alloimmunity [17, 18] and thus promoting islet graft survival [8, 17, 18]. However, the potential dangerous side effects of immunosuppression are currently of concerns [19]. Since the discovery of mesenchymal stem cells (MSCs) [20], numerous clinical trials based on them have been conducted, and a large part of these studies can be attributed to the interest on their immunosuppressive properties [21–24]. MSCs, often found and isolated from the bone marrow (although not exclusive to this location), are pluripotent cells with the capability of differentiating into various cell types such as osteoblasts, chondrocytes, and adipocytes [23, 25]. Known for their plasticity, or the ability to be molded into a desired form, MSCs have been researched in many fields including diabetes, transplants, cardiology, and oncology. More recently, MSCs have also been shown to play a part in immunomodulation [26]. In vitro, MSCs inhibit T cell proliferation and promote regulatory T cell function and generation [27, 28]. In vivo MSCs have been shown to modulate autoimmune response in different murine models of diabetes [23, 29], multiple sclerosis [22], rheumatoid arthritis [30], and alloimmune response in models of islet and heart transplantation [31, 32]. In particular, MSCs have been shown to modulate anti-allogeneic islet response in an immunodeficient mouse after $CD4^+$ T cell reconstitution [32]. In our study, we evaluated the potential of bone-marrow-derived MSCs to modulate anti-islet response in immunocompetent mice; in particular, we dissected the difference in graft protection obtained by the use of autologous or heterologous MSCs and by their administration (either systemically or locally at the graft site). We believe that the data

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obtained will promote further development in the use of MSCs as immunomodulatory cells in islet transplantation.

Materials and methods

Mice

C57BL/6 and BALB/c mice were purchased from the Jackson Laboratory at Bar Harbor, Maine. All mice were housed under specific pathogen-free conditions at an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility at Boston Children's Hospital. Institutional guidelines and protocols were approved in adherence to the Institutional Animal Care and Use Committee (IACUC).

Murine islet transplantation

Islets were isolated from the pancreas of donor mice (BALB/c) [18] and transplanted under the kidney capsule of diabetic C57BL/6 mice induced with streptozotocin [(Sigma-Aldrich, St. Louis, MO) 225 mg/kg, administered i.p.]. Islet rejection is characterized by blood glucose levels >250 mg/dl for two consecutive days post-transplant [18].

MSC culture

Bone marrow mononuclear cells were isolated from the tibiae and femurs of C57BL/6 mice in order to generate MSC. $10 \times 10^6/25\ cm^2$ of MSC was seeded into flasks and was coated with M10 medium [DMEM medium (Cambrex) containing 10 % FCS (HyClone), 1 % penicillin–streptomycin, and 1 % L-glutamine (both from Cambrex)] [23].

MSC differentiation

MSC passaged at P4 was trypsinized, cultured, and induced with three varying conditions to verify pluripotentiality and the ability to differentiate into multiple cell types: osteocytes, adipocytes, and chondrocytes. Differentiation of MSCs into appropriate cell types was performed as previously described [23].

Interventional studies

5×10^5 recipient-derived MSC was administered through i.v. (systemic) or intragraft (local) during BALB/c islet into C57BL/6 kidney transplant (islet tx). After transplantation, mice were monitored daily by blood glucose measurement and the return to hyperglycemia was considered a sign of graft rejection. Blood glucose was measured using a BD Logic blood glucose meter (Becton–Dickinson, Franklin Lakes NJ).

Histology and immunohistochemistry

Kidney tissues were snap frozen embedded in OCT. Immunohistochemistry was performed as previously described [33]. Photomicrographs (400× or 200×) were taken using an Olympus BX41 microscope (Center Valley, PA). The following primary antibodies were used: anti-CD4, anti-CD8 (both from eBioscience, San Diego, CA), and anti-insulin (Dako North America, Carpinteria, CA). Immunohistochemistry was performed as well on formalin-fixed, paraffin-embedded tissue sections related to the graft. Photomicrographs

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(original magnification 40×) were taken using an Olympus BX41 microscope (Center Valley, PA). The antibodies used were Mouse anti-CD31 (PECAM-1) (Life Technologies, Carlsbad, CA, USA). Histology was evaluated by an expert pathologist.

Insulitis score

Insulitis scoring was performed as published [34] on hematoxylin- and eosin-stained graft sections (kidneys + islets). (i) *Insulin staining*: 0: no insulin staining, 1: presence of scattered insulin-positive cells, 2: presence of insulin-positive cell aggregates, 3: presence of preserved insulin-positive islets; (ii) *Islet cell infiltrate*: 0: no cell infiltrate, 1: presence of cell infiltrate around graft islets, 2: presence of cell infiltrate inside graft islets, 3: presence of cell infiltrate throughout the graft area without preserved islet structure.

Quantitative real-time PCR

RNA extracted from cultured MSCs was purified using an RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA). Transcript amplifications were read through a 7300 real-time PCR system. Primers were purchased from Applied Biosystems (Foster City, CA) and quantified by normalization of cytokines (CX3CR1, Fractalkine, CXCL19, CXCR6, SDF-1α, CXCR4, VEGF, CXCR2, IP-10, MIG, ITAC, MIP-10, IL-10, IL-12, IFN-γ, and TGF-β) to copies of GAPDH [35].

ELISPOT

To measure cytokine production by MSCs, an ELISPOT assay, following manufacturer's protocol (BD Biosciences, San Jose, CA), was used to assess IFN-γ-, IL-4-, and IL-5-producing cells. Spots were counted using an Immunospot analyzer (Cellular Technology Ltd., Cleveland, OH) [36].

Flow cytometric analysis and intracellular cytokine staining

Flow cytometry was performed to analyze surface expression markers of MSCs. Anti-mouse MHC Class I, CD28, CD90, CD13, and CD34 were purchased from BD Pharmingen and eBioscience (San Diego, CA). CD4, CD44, CD62L, CD25, CD105, interferon-γ, IL-17A, IL-10, and FoxP3 were purchased from BD Pharmingen and eBioscience (San Diego, CA). Cells recovered from spleens and peripheral bloods were subjected to FACS analysis and were run on a FACSCalibur™ (Becton–Dickinson). Data were analyzed using FlowJo software version 6.3.2 (Treestar, Ashland, OR).

Mixed lymphocyte reaction (MLR)

BALB/c splenocytes were irradiated at 3000 rads and used to stimulate C57BL/6 splenocytes at a ratio of 1:1. T cell proliferation was measured at day 3 following pulsing for 3 h with TdR (Perkin Elmer, Wellesley, MA), and cell counts were quantified using a liquid scintillation counter.

Statistical analysis

All data are expressed as mean ± standard error of mean. Survival data were analyzed through the Kaplan–Meier analysis. A two-sided unpaired Student's *t* test (for parametric

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data), or a Mann–Whitney test (for nonparametric data), was used to compare groups. A p value of less than 0.05 (by two-tailed testing) was considered to be statistically significant. All data and graphs were generated using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Results

MSCs phenotype

We first evaluated and characterized MSCs generated from the bone marrows of C57BL/6 mice. MSCs were passaged at P4 and then characterized through flow cytometry with a wide array of antibodies, including: MHC Class I, CD28, Thy-1 (also referred to as CD90), CD34, and CD13. The results obtained confirmed the MSCs phenotype of the generated cells (Fig. 1a). In order to assess the pluripotency of the generated cells, we evaluated their potential of differentiating under specific conditions (Fig. 1b). Color changes in the media were indicative of MSCs passages into appropriate cell types: chondrocytes, osteoblasts, or adipocytes. Similar to the phenotype data, differentiation assays confirmed that the generated cells were indeed MSCs [23].

MSC secretome

We then assessed the secretome profile of MSCs alone through ELISPOT assays; particularly, the number of interferon-γ (IFN-γ)-, interleukin-4 (IL-4)-, and interleukin-5 (IL-5)-producing cells was evaluated. To determine the optimal dosage of C57BL/6 MSCs to be injected, we tested four different concentrations of MSCs alone: (1) 10,000 (2) 25,000 (3) 50,000, and (4) 100,000 (Fig. 1c). Results show a concentration-dependent increase in IL-4 production (100,000 C57BL/6-MSCs vs. 10,000 C57BL/ 6MSCs; $p = 0.0002$; Fig. 1d). Partial production of IFN-γ was also observed, while only marginal levels of IL-5 were detected (Fig. 1d). We then further quantified the cytokine profile of the MSCs through RT-PCR. RNA was extracted from the MSCs and normalized against GAPDH. Data show that the MSCs were positive for SDF-1α and TGF-β (Fig. 1e, f).

MSCs immunomodulatory properties

We then determined in vitro the effect of generated MSCs in modulating the alloimmune response. 1×10^6 C57BL/6 responder splenocytes were stimulated with the same number of irradiated stimulator BALB/c splenocytes in the presence or absence of 1×10^5 C57BL/6 (autologous) or BALB/c (heterologous) MSCs, and in an ELISPOT assay the numbers of INF-γ-(Fig. 2a, b) and IL-4-producing cells were tested (Fig. 2c, d). While heterologous MSCs had only marginal effect on cytokines production, the use of autologous MSCs greatly modified cytokine production profile compared to cells cultured in the absence of MSCs (Fig. 2b, d). Specifically higher numbers of IFN-γ- and IL-4-producing cells were observed (IFN-γ, number of spots: no MSCs = 14, autologous MSCs = 559; $n = 6$; $p = 0.0007$; Fig. 2b; IL-4 number of spots: no MSCs = 54, autologous MSCs 1528; $n = 6$; p 0.0001; Fig. 2d) with IL-4 produced in a higher quantity (about threefold if compared to IFN-γ). The effect of the higher IL-4 production could suggest a shift toward a Th2 profile. We then ran an MLR experiment to measure in vitro alloantigen response with different dosage of autologous MSCs ($n = 3$; Fig. 2e). A dose-dependent suppression of BALB/c

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splenocytes mediated C57BL/6 splenocytes proliferation was observed, and a plateau was present with more than 1000 MSCs (Fig. 2e).

MSC co-transplantation prolongs islet allograft survival

We then tested, in vivo, the immunological properties of C57BL/6 (autologous) MSCs in promoting allogeneic islet graft survival. BALB/c islets were transplanted under the kidney capsule of streptozotocin-induced diabetic C57BL/6 mice. Untreated mice invariably rejected transplanted islets in less than 14 days [median survival time (MST) 12 days] (Fig. 3a); similarly, the systemic i.v. injection of 5×10^5 P4 autologous MSCs was not able to prolong islet graft survival (MST 15.5 days; p = ns vs. untreated). We also investigated the local immunosuppressive potential of autologous MSCs by infusing them at the site of transplantation. Generated P4 MSCs were mixed at the time of the infusion to the purified BALB/c-derived islets and then co-transplanted under the kidney capsule of diabetic C57BL/6 recipients. In this setting, delay of graft rejection was evident (MST 20 days, $n = 8$; $p = 0.01$ vs. untreated; Fig. 3b) with long-term islet graft survival achieved in about 30 % ($n = 3$) of the islet-transplanted mice (Fig. 3b). Furthermore, in order to determine whether BALB/c (heterologous) MSCs would also have a similar local effect, we infused BALB/c-derived MSCs together with BALB/c islets. The use of heterologous MSCs, however, was ineffective in promoting islet graft survival prolongation (Fig. 3c), unlike the results of the autologous MSCs.

Locally MSC-treated mice showed preserved islet allograft

Islet grafts from locally infused autologous MSC-treated ($n = 3$) and untreated recipients ($n = 3$) were harvested at day 14 after transplantation, and immunohistochemistry was performed (Fig. 4a–e, f–j). Untreated mice displayed, at the site of transplantation, lack islet structures or positive insulin staining, with $CD4^+$ and $CD8^+$ T cells evident throughout the graft area (Fig. 4c–d). On the contrary, the graft area of recipients treated with locally infused autologous MSCs showed preserved islet structure and clear positive insulin staining. $CD4^+$ and $CD8^+$ T cells are present, but mainly localized at the border of the islets with minimal infiltration of the graft (Fig. 4h–i). Furthermore, the analysis of insulin staining (Fig. 4k) and of the insulitis score or infiltrate score (Fig. 4l) confirmed that islet grafts are more preserved and less infiltrated in the MSC-treated group as compared to the untreated group. This indicates that co-transplanted MSCs may contribute to the prevention of islet infiltration and loss.

Co-transplantation of MSCs promotes revascularization of transplanted islets

To test and evaluate the impact of MSCs on the vascularization of the islets, we assessed CD31 staining, a marker of endothelial cells. A more pronounced CD31 staining was evident in locally MSC-treated mice (Fig. 4j) as compared to the untreated one (Fig. 4e), suggesting a potential role of MSCs in stimulating revascularization.

Locally MSC-treated mice showed reduced alloimmune response

Upon observing that locally or systemically infused autologous MSCs prolong islet graft survival, we decided to examine whether this was related to a local immune effect or

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whether a systemic effect was also present. Recipient splenocytes were extracted at 14 days after transplantation in both untreated and systemically or locally autologous MSC-treated mice, and proliferation in response to irradiated donor-derived splenocytes was assessed. No difference in thymidine incorporation was observed between untreated and systemically MSC-treated mice (Fig. 5a). Thymidine incorporation, as a marker of cell proliferation, was lower in the locally MSC-treated mice as compared to the systemically MSC-treated ones ($p < 0.001$), (Fig. 5a). We then tested in an ELISPOT assays the number of generated recipient IFN-γ and IL-4 producing cells in response to irradiated donor-derived splenocytes. Locally MSC-treated mice showed a significant reduction in the number of IFN-γ-producing cells as compared to systemically MSC-treated mice ($p < 0.05$), (Fig. 5b). On the contrary, we observed a significant increase in the number of IL-4 producing cells in the locally MSC-treated mice as compared to the untreated and systemically MSC-treated mice ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 5c). These data suggest that the prolongation of islet allograft survival in autologous locally MSC-treated mice may be due to the creation of a local immunoprivileged site which prevents allostimulation.

MSC co-transplantation abrogates the Th17 immune response

We next tested the effect MSCs co-transplantation on immunophenotype of recipient mice. We harvested splenocytes and peripheral blood from MSC-treated mice (systemically and locally) at day 14, and the percentages of $CD4^+$ T effectors cells ($CD4^+CD44^{high}CD62L^{low}$, or $CD4^+$ Teffs), regulatory $FoxP3^+$ T cells ($CD4^+CD25^+ FoxP3^+$), regulatory $IL\text{-}10^+$ T cells ($CD4^+IL\text{-}10^+$), $IL\text{-}17^+$ T cells ($CD4^+IL\text{-}17^+$), and IFN-γ^+ T cells ($CD4^+$IFN-γ^+) were quantified by flow cytometry analysis (Fig. 5d–m). No differences were observed in the percentage of $CD4^+$ T effector cells ($CD4^+CD44^{high}CD62L^{low}$) (Fig. 5d, i) or FoxP3+ T cells (Fig. 5h, m) in the spleen and blood of both MSC-treated mice as compared to untreated ones. The percentage of $IL\text{-}17^+$ T cells, but not of IFN-γ^+ T cells, was lower in the spleen and blood of locally MSC-treated group as compared to untreated controls and systemically treated mice (Fig. 5e–f, j–k). Furthermore, we observed a tenfold increase in the percentages of $IL\text{-}10^+$ regulatory T cells in the spleen (Fig. 5g), but not in the blood (Fig. 5l), of locally MSC-treated mice as compared to untreated controls and systemically treated mice. This suggests that co-transplantation of MSCs with islets transplantation may reshape recipients' immune system.

Locally co-transplanted MSCs remained within the islet graft

In order to track administered MSCs in mice, GFP^+MSCs were injected in islet-transplanted mice and GFP/CD105 double staining was performed and quantified (Fig. 6a–l). Flow cytometry analysis revealed that MSCs administered locally and systemically traffick to the spleen (Fig. 6d–f, $^{**}p < 0.01$) but poorly to the liver (Fig. 6j–l). While in untreated islet-transplanted mice, WT mice, and Isotype control, GFP/CD105 double-positive cells are poorly represented (Fig. 6a–c, g–i).

Discussion

T1D has been associated with a wide range of complications [37, 38], and unfortunately while immunotherapy showed some promising results in the murine model of T1D (the

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NOD mice) [39], the results in humans are poor. Of interest stem cells are now recognized as a tool for immunomodulation and regeneration [40]. Islet transplantation has been shown to normalize glycemic control and counteract the development of the chronic complications associated with T1D [41]. However, in spite of countless research and effort, long-term survival of islet grafts remains far below the survival rate of other types of grafts. In our study, we took advantage of the immunomodulatory properties of MSCs to promote allogeneic islet graft survival in a murine model of allogeneic transplantation. Specifically, (1) we generated BALB/c and C57BL/6 bone-marrow-derived MSCs under established protocols; (2) we evaluated the profile of MSCs at P4 using RT-PCR and ELISPOT showing a potential immunomodulatory profile; (3) we examined the effect of autologous (C57BL/6) MSCs on the alloimmune response in vitro, which showed higher immunomodulation obtained by responder autologous cells; (4) we tested the effect of autologous MSCs on islet graft (BALB/c → C57BL/6), with survival confirming higher effectiveness of autologous MSCs; and (5) we compared local and systemic administration of MSCs and demonstrated that locally infused MSCs promote better immunomodulation. Although the immunoregulatory properties of MSCs have been shown as beneficial in different murine models of transplantation, several issues remain to be solved in order to make MSCs a viable option for clinical practice.

The first issue is related to the high number of MSCs, often in multiple doses, that are needed to generate an in vivo relevant immunological effect [23]. In our study, we demonstrated that the use of a relatively low single-dose injection of MSCs is effective, but only if the cells are directly infused at the site of transplantation. On the contrary, the same dose of MSCs infused systemically loses its effect and is not able to promote graft survival. When we tested the immune system of mice either untreated or treated with locally injected MSCs, we did not see any difference in terms of alloantigen response. Therefore, we propose that the local injection of MSCs does not affect the priming of the immune system toward the graft antigens, but that it generates a local immunoprivileged site where islet graft is protected from the aggression of the immune system. The results of the histology are compatible with this model; in fact, $CD4^+$ and $CD8^+$ T cells appear to infiltrate the graft area, but are disposed at the border of single graft islets without the invasion typical of florid rejection. The creation of an immunoprivileged site without a systemic immunological effect could be desirable in transplantation because it may avoid the potential side effect related to systemic immunomodulation (such as reduced protection toward pathogens and reduced immunosurveillance toward dysplastic cells).

The second issue is related to the source of MSCs (autologous vs. heterologous). The source of MSCs has been debated in recent years, and autologous vs. heterologous MSCs have been challenged in different models [32, 42], with either cells resulting as desirable in different settings. In general, autologous MSCs are described to have a much better effect at prolongation; however, heterologous MSCs are often described as superior in cases such as autoimmune disease (in which autologous MSCs might be dysfunctional as well) and so would result in a better safety profile [23]. In our setting, we hypothesized that the superiority of autologous MSCs could be linked to the rejection of heterologous MSCs due to the host immunosurveillance [43–45].

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It is possible that in our study, more than an immunoprivileged local mechanism, the mild effect observed with autologous MSC could be just related to a promotion of a microenvironment that limits islet damage as indicated by other studies in syngenic models [44, 45]. Autologous MSCs may prevent islet cells loss and suppress various immune cell functions caused by pro-inflammatory cells and cytokines and facilitate islet revascularization and engraftment [44, 45]. This effect could be abrogated in the case of the heterologous MSC due to the host immunosurveillance.

The immunomodulatory properties of MSC have been largely described in the literature, and several reports have described that they do exert their effects through the release of several trophic factors such as CNTF [46], already known to promote islet graft survival and other trophic factors such as hepatocyte growth factor, Von Willebrand factor, transforming growth factor-β1 [47], prostaglandin E2, or via establishing a cell-to-cell communication network [48]. Furthermore, recent published studies [48, 49] suggested that MSCs may also communicate through the release of circular membrane fragments named microvesicles (MVs) that are able to shuttle proteins and genetic information from cells of origin to target cells [49]. Favaro et al. provided a new evidence regarding the immunomodulatory mechanism governed by MSC-derived microvesicles, which were found to exert an anti-inflammatory effect by decreasing pro-inflammatory cells and cytokines, and by triggering in the meanwhile an increase in anti-inflammatory-associated cytokines, suggesting the switch to an anti-inflammatory response of T cells [48]. MVs may be released from EPCs as well and induce a protective effect on human islets via triggering neoangiogenesis, and MVs may be internalized by β-cells and islets endothelium sustaining thus insulin secretion and angiogenesis [49].

Herein, we showed and confirmed what has been reported by other investigators [44, 45] that co-transplantation of MSCs with islets from BALB/c into streptozotocin-treated C57BL/6 recipients' mice would facilitate islet revascularization, engraftment, and improved graft survival, and due to its beneficial outcomes on transplantation such procedure employing co-transplantation of MCSs with islets may be translated into the clinical settings.

Lastly, we also considered the mechanisms underlying the immunomodulatory properties of MSCs. In different models, MSCs have been shown to express a wide array of mechanisms that have been considered responsible for the immunological effect achieved [50]. These mechanisms include the inhibition of T cell activation and proliferation [51], the modulation of dentritic cell activation [52], the modulation of NK cells activity [53], and the inhibition of B cell activation [54]. In our study, we demonstrated a high IL-4 spontaneous production by MSCs, which is able to commit, in an antigen-specific assay, immune cells toward a Th2 profile. Th2 profile is well established to be associated with a reduced allospecific response and graft rejection. The production of TGF-β by MSCs might also contribute to the down-regulation of anti-islet response and the graft protection observed.

In conclusion, islet transplantation presents the unique opportunity to effectively co-transplant MSCs [55]; our data demonstrated that MSCs co-transplantation may be beneficial when co-injected with islets and may improve islet graft survival, with the

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generation of an immunoprivileged site that can halt locally anti-islet responses without any systemic effect on the recipient immune system.

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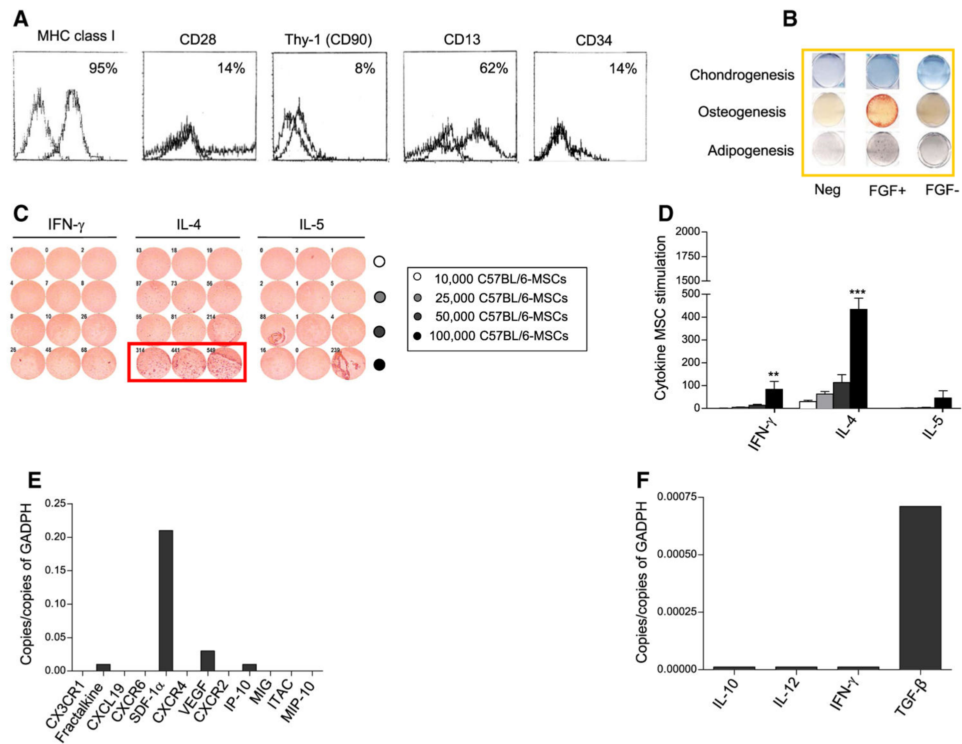

Fig. 1.

Characterization of MSCs. C57BL/6 MSCs were characterized through flow cytometry, ELISPOTs, RT-PCR, and MSCs delineation. Flow cytometry was performed to characterize the phenotype of C57BL/6 MSCs. Results show that C57BL/6 highly express MHC Class I and CD13, but express low amounts of CD28, Thy-1, and CD34 (**a**). **b** Shows the delineation of MSCs into chondrocytes, osteoblasts, and adipocytes (i.e., color = positive effect) when MSCs were cultured accordingly to protocol (**b**). **c** and **d**, MSCs secretome was assessed and C57BL/6 MSCs were plated alone, MSCs produced high amount of IL-4 cytokine at a concentration of 100,000 cells and some percentage of IFN-γ (IFN-γ $^{**}p$ <0.01, IL-4 $^{***}p$ <0.001) (**c**, **d**). Lastly, RT-PCR results show that C57BL/6 MSCs are positive for SDF-1α and TGF-β (**e**, **f**). Data are shown as mean ± SEM and are representative of $n = 3$ mice and of at least two independent experiments. $^{**}p$ <0.01; $^{***}p$ <0.001

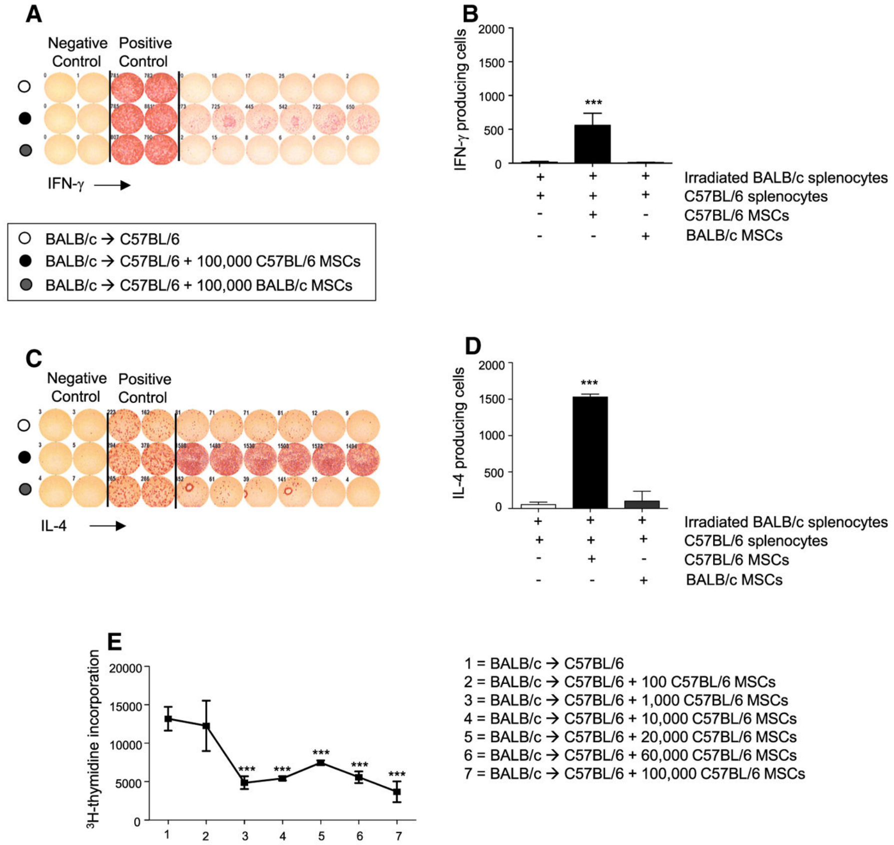

Fig. 2.

Immunomodulatory properties of MSCs. 1×10^6 C57BL/6 responder splenocytes were stimulated with irradiated stimulator BALB/c splenocytes in the presence or absence of 1×10^5 autologous-C57BL/6 or heterologous BALB/c MSCs, and in an ELISPOT assay the production of INF-γ (**a**) and IL-4 was tested (**c**). While heterologous MSCs had only marginal effect on cytokines production (**b, d**), the use of autologous MSCs greatly modified cytokine production profile compared to cells cultured in the absence of MSCs (***p <0.001; **b, d**). An MLR experiment shows a dose-dependent reduction of C57BL/6 splenocytes challenged with irradiated BALB/c splenocytes and autologous MSCs (**e**). Data are shown as mean ± SEM and are representative of at least two independent experiments. ***p <0.001

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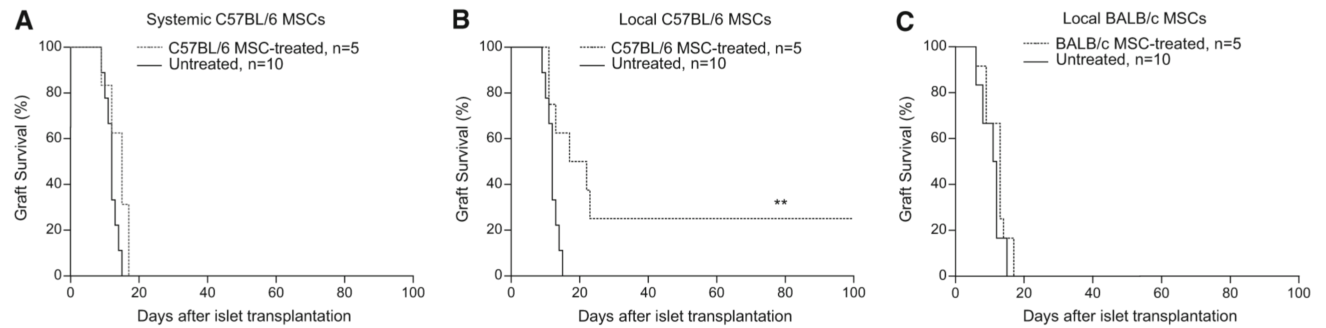

Fig. 3.

Survival of allogeneic islet transplant with (systemically or locally) or without MSCs. The systemic injection of autologous C57BL/6 MSCs is not able to promote allogeneic islet graft survival (**a**), on the contrary the local infusion (into kidney capsule together with islet graft) of the same number of MSCs promote graft survival (**b**) (**$p<0.01$ vs. untreated). Conversely, the local infusion of heterologous BALB/c MSCs has no effect on islet graft survival (**c**). *P* values were calculated with Student's *t* test. Data are representative for at least five mice per group. **$p<0.01$

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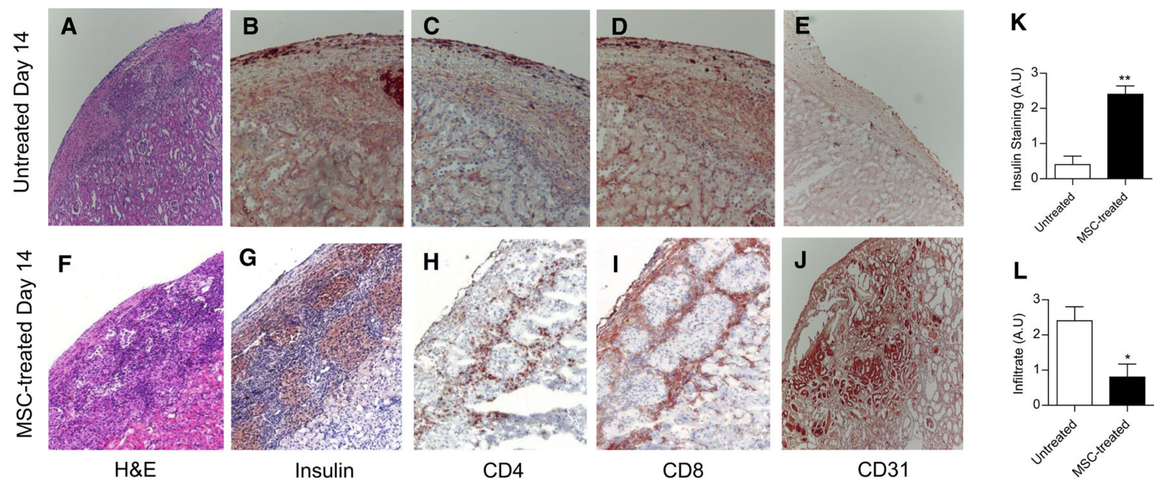

Fig. 4.

Pathology of the graft at day 14 after transplantation. The islet graft of untreated mice showed no preserved structures or insulin staining (**a, b, k**), while in locally MSC-treated mice, the islet graft showed preserved structures and positive insulin staining (**f, g, k**). $CD4^+$ and $CD8^+$ T cell infiltrate is evident (**l**) but mostly confined outside the islets (**h–i**) in locally MSC-treated mice, as compared to untreated mice, which showed overall more infiltrate (**c–d, l**). CD31 staining, a marker of endothelium and vascularization, appeared to be more evident in islet graft of mice locally MSC-treated mice (**j**) as compared to samples obtained from untreated mice (**e**). All the generated data are representative of at least $n = 3$ mice per group. *p <0.05; **p <0.01

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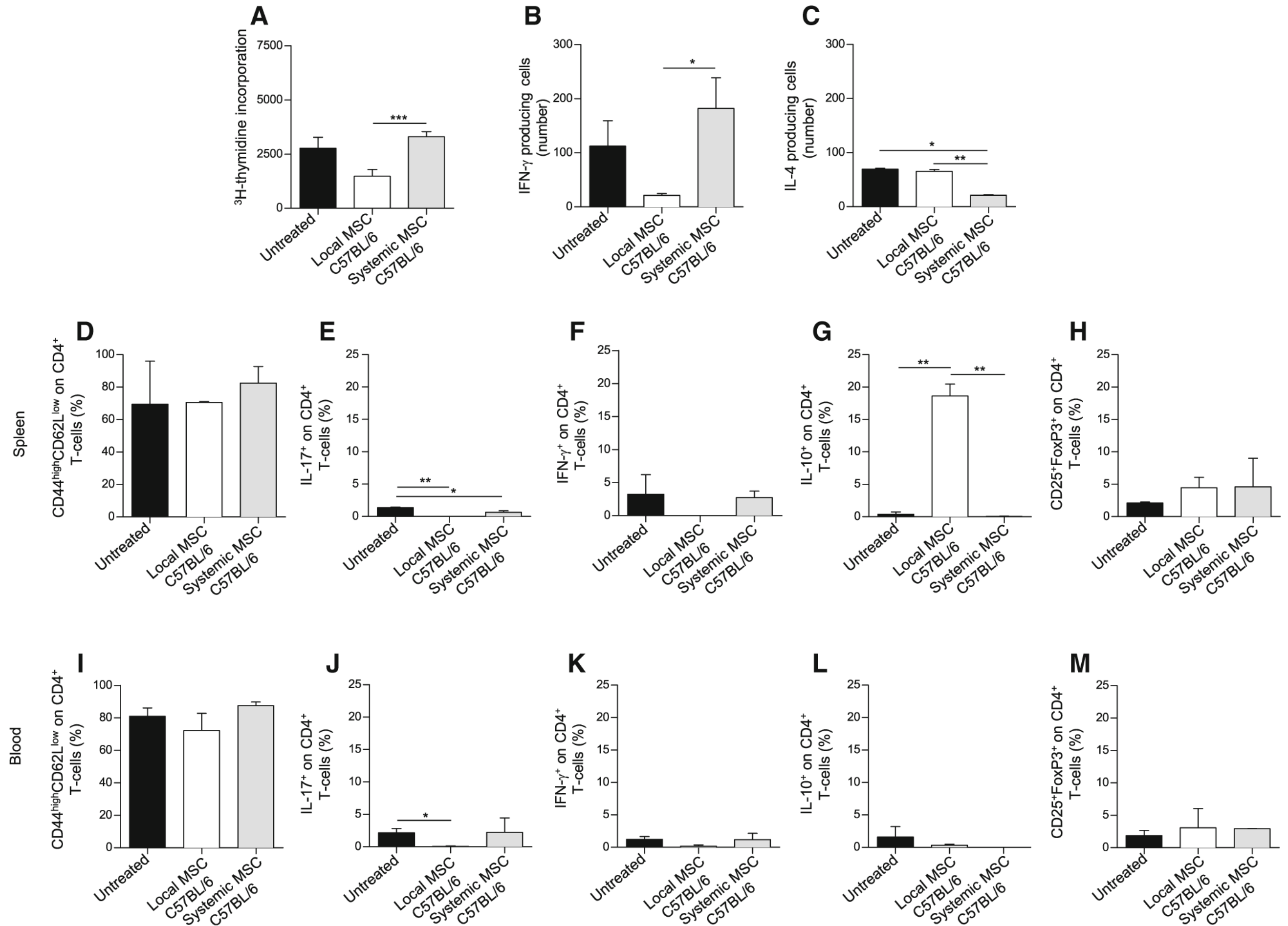

Fig. 5.

Immunophenotype of treated mice. Proliferation toward donor antigens was reduced in the locally autologous MSC-treated mice compared to systemically MSC-treated mice (**a**). We found significant difference in terms of cytokine production toward graft antigens between untreated, locally and systemically MSC-treated mice, with a reduction in the number of IFN-γ-producing, and an increase in IL-4-producing, cells in mice treated with MSCs locally (**b–c**). The percentage of CD4$^+$ effector T cells in the spleen (**d**) and in the blood (**i**) is unaffected by both treatments with MSCs, and MSC co-transplantation reduces the percentage of IL-17$^+$ (**e, J**) and IFN-γ$^+$ (**f, k**) cells. While the percentage of FoxP3$^+$ regulatory CD4$^+$ T cells is unaltered (**h, m**), a tenfold increase in the percentage of IL-10$^+$ CD4$^+$ T cells was evident in the spleen (**g**), but not in the blood (**l**), of mice locally treated with MSCs. Scale bars show mean ± SEM for at least $n = 3$ mice, and data are representative of two independent experiments. *p <0.05; **p <0.01; ***p <0.001

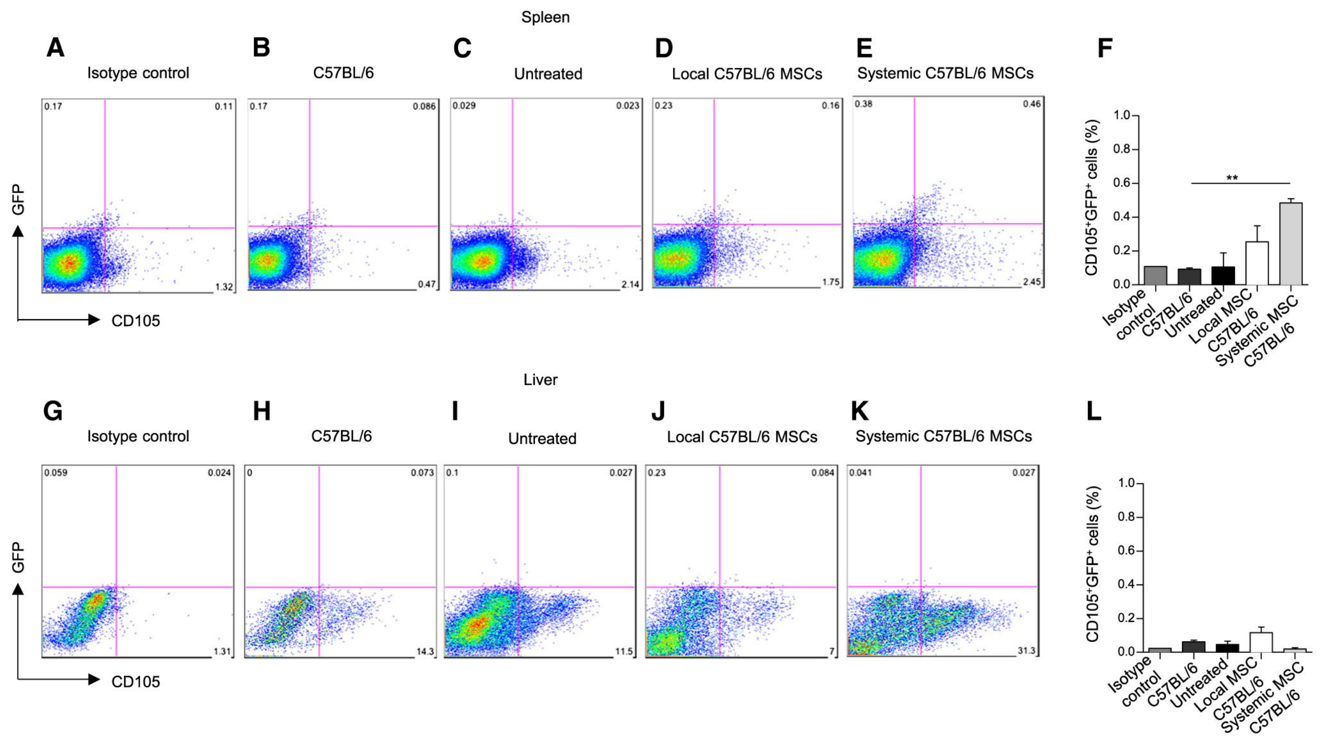

Fig. 6.

MSCs tracking. In order to track administered MSCs in mice, GFP+MSCs were injected in islet-transplanted mice and GFP/CD105 double staining was performed and quantified (**a–l**). Flow cytometry analysis revealed that MSCs administered locally and systemically traffick to the spleen (**d–f**) but poorly to the liver (**j–l**). In untreated islet transplanted mice, WT mice, and Isotype control, GFP/CD105 double-positive cells are poorly represented (**a–c, g–i**). Data are shown as mean ± SEM and are representative of $n = 3$ mice and of at least two independent experiments. ** $p < 0.01$

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