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Pretransplant Infusion of Mesenchymal Stem Cells Prolongs the Survival of a Semiallogeneic Heart Transplant through the Generation of Regulatory T Cells

Federica Casiraghi,* Nadia Azzollini,* Paola Cassis,** Barbara Imberti,† Marina Morigi,‡ Daniela Cugini,** Regiane Aparecida Cavinato,* Marta Todeschini,** Samantha Solini,** Aurelio Sonzogni,† Norberto Perico,** Giuseppe Remuzzi,**† and Marina Noris**

In this study, we investigated whether mesenchymal stem cells (MSC) had immunomodulatory properties in solid organ allograft transplantation, using a semiallogeneic heart transplant mouse model, and studied the mechanism(s) underlying MSC tolerogenic effects. Either single (portal vein, day −7) or double (portal vein, day −7 and tail vein, day −1) pretransplant infusions of donor-derived B6C3 MSC in B6 recipients induced a profound T cell hyporesponsiveness and prolonged B6C3 cardiac allograft survival. The protolerogenic effect was abrogated when donor-derived MSC were injected together with B6C3 hematopoietic stem cells (HSC), suggesting that HSC negatively impact MSC immunomodulatory properties. Both the induction (pretransplant) and the maintenance phase (>100 days posttransplant) of donor-derived MSC-induced tolerance were associated with CD4+CD25+Foxp3+ Treg expansion and impaired anti-donor Th1 activity. MSC-induced regulatory T cells (Treg) were donordirectional since adoptive transfer of splenocytes from tolerant mice prevented the rejection of fully MHC-mismatched donor-specific secondary allografts but not of third-party grafts. In addition, infusion of recipient-derived B6 MSC tolerized a semiallogeneic B6C3 cardiac allograft, but not a fully MHC-mismatched BALB/c graft, and expanded Treg. A double i.v. pretransplant infusion of recipient-derived MSC had the same tolerogenic effect as the combined intraportal/i.v. MSC infusions, which makes the tolerogenic protocol applicable in a clinical setting. In contrast, single MSC infusions given either peritransplant or 1 day after transplantation were less effective. Altogether these findings indicate that MSC immunomodulatory properties require HSC removal, partial sharing of MHC Ags between the donor and the recipient and pretransplant infusion, and are associated with expansion of donor-specific Treg.


Transplantation is regarded as the only therapeutic choice for the end-stage failure of several organs; however, the prolonged acceptance of transplanted organs requires long-term use of combinations of immunosuppressive drugs. This treatment risks infection and a range of side effects, which, along with the inexorable chronic allograft injury, limit the life of the transplanted organs and the patients (1). The most appealing solution to these problems is the induction of transplantation tolerance, defined as lifelong, donor-specific unresponsiveness without the need of chronic immunosuppression (2). In recent years, several clinically relevant tolerance-induction regimens have been reported in experimental models. Many of these approaches incorporate infusion with either mature cells or stem cells (3–7).

Mesenchymal stem cells (MSC) produce the stromal matrix, which constitutes the bone narrow microenvironment, and support the growth of hematopoietic progenitor cells (8, 9). Potential interest to transplant medicine derives from the observation that MSC are immunoprivileged and display immunosuppressive capacities (10, 11). Due to low expression of MHC class II (MHCII) in unstimulated conditions (12–14) and absence of costimulatory molecules such as B71, B72, CD40, and CD40L on the cell surface (13, 15, 16), MSC escape the immune system and therefore could be infused into an allogeneic host without being rejected, eventually avoiding the need of conditioning regimen (12, 17). In vitro studies with human (13, 14, 16, 18–20), baboon (21), rat (22), and murine (23–26) MSC have shown that they prevent T cell responses to cellular and to nonspecific mitogenic stimuli, targeting both naive and memory CD4+ and CD8+ T cells (13, 25). Moreover, in vitro MSC-induced T cell suppression occurs independently of MHC matching with either stimulatory cells or responder lymphocytes in a MLR (21, 23) in a dose-dependent manner (13).

Notwithstanding the considerable amounts of in vitro data supporting the nonimmunogenicity and immunomodulatory effects of MSC, scanty and conflicting data are available on the immunomodulatory capacities of these cells in vivo. Allogeneic MSC infusion slightly prolonged skin allograft survival in immunocompetent baboons (21), prevented the rejection of allogeneic B16

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3 Abbreviations used in this paper: MSC, mesenchymal stem cell; HSC, hematopoietic stem cell; BM, bone marrow; Foxp3, forkhead box p3; Treg, regulatory T cell; MHCII, MHC class II; MST, median survival time; A1, arbitrary unit.

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melanoma cells in immunocompetent C3H mice (23), and attenuated graft-versus-host disease in mice (27) and in humans (28, 29). In mice, administration of autologous MSC also prevented the development of autoimmune encephalomyelitis (30). On the contrary, MSC infusion failed to prevent graft rejection (22) or did result in a very modest prolongation of graft survival (31, 32) in fully MHC-mismatched vascularized heart transplant models in rodents.

The present study was designed 1) to investigate the effectiveness of MSC in promoting immunosuppression/tolerance in the context of vascularized solid organ transplantation in a mouse model of semiallogeneic heart transplantation; 2) to dissect the mechanism(s) underlying the potential MSC tolerogenic effects in vivo with a main focus on the role of regulatory T cells (Treg); and 3) to find out the best tolerogenic MSC infusion protocol.

Materials and Methods

Mice

Male and female inbred C57BL/6 (B6, H-2b), (C57BL/6 × C3H)F1, (B6C3, H-2k), C3H (C, H-2), and BALB/c (H-2d) mice were purchased from Charles River Laboratories. Animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national (DL n.116, GU suppl 40, 18 febbraio 1992, Circolare no. 8, GU 14 luglio 1994) and international laws and policies (European Economic Community Directive 86/609, OJL 358, December 1997; National Institutes of Health Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Animals were housed in a constant temperature room with a 12-h dark/12-h light cycle and fed a standard diet.

MSC isolation and expansion

Bone marrow (BM) was obtained from 2-mo-old B6C3 or B6 mice. Briefly, mice were killed and femurs and tibias were aseptically removed. BM was flushed from the shaft of the bone with DMEM (Sigma-Aldrich) containing 5% FCS (Invitrogen) and then filtered through a 100-μm sterile filter to produce a single-cell suspension. MSC were recovered from BM by their tendency to adhere tightly to the plastic culture dish and were isolated as previously described (33). Filtered BM cells were plated in DMEM/10% FCS and allowed to adhere for 6 h. Adherent cells were then cultured for 2–3 wk with medium change every 3 days. Thereafter, primary MSC cultures were collected and immunodepleted of CD45+ and CD11b+ cells. After blocking with PBS/0.5% BSA, cells were incubated for 20 min with rat anti-mouse CD45 and rat anti-mouse CD11b Abs (0.2 μg/106 cells; Caltag Laboratories, San Francisco, CA). Cells were then washed, and antibodies were then isolated by a MACS system (Miltenyi Biotec). CD45+ CD11b- MSC were then isolated by a MACS system (Miltenyi Biotec). FACS analysis (FACSort; BD Biosciences) on purified MSC confirmed the absence of CD45 and CD11b expression (>95% CD45+ CD11b- cells). MSC properties to differentiate toward osteoblasts, adipocytes, and chondroblasts in vitro have been routinely assayed as previously described (33).

Hematopoietic stem cell (HSC) isolation

For HSC isolation, total BM cells were incubated with rat anti-mouse mAbs specific for the following lineage markers: CD4, CD8, CD45R/ B220, CD11b, Gr-1, and Ter-119 (Caltag Laboratories). After washing, labeled cells were incubated with magnetic microbeads and depleted by magnetic cell sorting (Miltenyi Biotec) as previously described (33). The obtained lineage-negative cells (Lin-) were then incubated with rat anti-mouse CD117 (c-KIT) PE conjugate (Caltag Laboratories). Positive cells were then isolated by cell sorting (FACSaria; BD Biosciences) to obtain purified Lin- c-KIT- HSC preparations (97%).

Detection of donor MSC in recipient tissues

For in vivo tracking experiments, male B6C3 or B6 MSC were labeled with PKH26 according to the manufacturer’s protocol (Red Fluorescence Cell Linker Kit; Sigma-Aldrich) and infused into B6 mice. Labeling efficacy was found to be >90% by FACS analysis. At sacrifice, single-cell suspensions were obtained from spleen, lymph nodes (cervical, mesenteric, and iliac), thymus, bone marrow, and blood and analyzed by FACS for the presence of PKH26-labeled MSC. As negative controls, single-cell suspensions from naive mice (n = 3) were run in parallel. From each sample, 500,000 cells were analyzed and the number of events falling in the PKH26 fluorescence window was recorded. No positive events were found in the PKH26 fluorescence window with negative control cells. The total number of PKH26+ cells in each tissue was calculated by relating the number of PKH26+ events in 500,000 cells to the total number of cells in each tissue and the percentage of PKH26+ MSC/total MSC infused was calculated as follows: number of PKH26+ cells in each tissue/total number of infused PKH26-labeled MSC (500, 000) × 100.

To evaluate MSC engraftment in the liver and lungs, the organs from PKH26+ MSC-infused mice were fixed in paraformaldehyde, impregnated with sucrose, and rapidly frozen. Tissues were then sectioned on a cryostat (10 μm), fixed with acetone, stained with 4′,6-diamidino-2-phenylindole (1 μg/ml; Sigma-Aldrich), and analyzed by fluorescence confocal microscopy. For each tissue, three consecutive sections were analyzed and PKH26+ cells in each section were counted. Results are expressed as mean number of PKH26+ cells per section.

Heterotopic heart transplant

B6C3, C3, or BALB/c hearts were transplanted into the abdomen of B6 recipients (34). Mice were anesthetized by inhalation of isoflurane (Abbott Laboratories). Donor hearts were perfused with chilled, heparinized saline via the inferior vena cava and harvested after ligation of the superior vena cava and pulmonary veins. The aorta and pulmonary artery of donor hearts were anastomosed to the abdominal aorta and inferior vena cava of recipient mice, respectively, using a microsurgical technique. Recipients were kept on a warming blanket and under a heating lamp to recover postoperatively. Ischemic time during the surgical procedure was routinely 30 min. Graft survival was followed by palpation at least three times per week. Rejection was defined by complete cessation of palpable contraction confirmed by direct visualization.

Graft histology

Fragments of cardiac allografts were fixed in 10% neutral formalin, embedded in paraffin, sectioned, and stained with H&E. Sections were examined microscopically and graded (from mild to severe) for acute rejection changes: presence of vasculitis, infarction, lymphocytic infiltration, thrombosis, and hemorrhage. The development of chronic rejection was evaluated by the presence of vessels, including coronary arteries and arterioles, affected by obliterator vasculopathy.

Adaptive cell transfer

Spleen cells or electronically sorted splenic CD4+ T cells, CD4+ T cell subsets, and CD8+ T lymphocytes were obtained from mice with long-term graft survival, from infused and naive mice. Cells were then cultured in PBS and injected into the tail vein of naive mice the day before heart transplantation. No immunosuppressive drugs were given to the animals.

MLR and proliferation studies

Spleen cell suspensions were obtained by passing the spleen through a 70-μm stainless steel strainer and the erythrocytes were depleted by hypotonic lysis. CD4+ T cells were isolated using mouse CD4+ T cell isolation kit (Miltenyi Biotec) and used as responders (0.5 × 106 cells) in the MLR. Either 4000-rad irradiated splenocytes or irradiated mesenchymal stem cells (0.5 × 106 cells) were used as stimulators. Cells were resuspended in complete RPMI 1640 supplemented with 10% heat-inactivated FCS and antibiotics (Invitrogen) or in conditioned medium from primary MLR with MSC. MLR were conducted in 96-well plates and cells were cultured for 72 h. Cell proliferation was determined by pulsing the cells with [3H]thymidine during the last 14–16 h of culture and measuring the radioactivity incorporated by liquid scintillation counting. Proliferative response was expressed as Δcpm by subtracting the cpm recorded in the control syngeneic combination from the cpm of allogeneic combinations. T cell proliferation in response to anti-CD3/anti-CD28 Abs was assessed by coating 96-well flat-bottom plates with anti-CD3 mAb (0.5 μg/well, hamster anti-mouse CD3, clone 7D169; Serotec) overnight at 4°C. Wells were then washed and spleen cells (0.5 × 106/well) were added with 0.2 μg/well anti-CD28 mAb (hamster anti-mouse CD28, clone 37.51.1; Caltag Laboratories). For experiments studying the induction of anergy, 100 IU/ml IL-2 (mouse rIL-2; BD Biosciences) was added to the wells. Cell proliferation was determined by pulsing the cells with [3H]thymidine during the last 14–16 h of culture and measuring the radioactivity incorporated by liquid scintillation counting. Proliferative response was expressed as cpm.

Flow cytometry analysis

Mesenchymal cell surface phenotypic analysis was performed by flow cytometric analysis using FACSort. The following Abs were used: FITC-conjugated mouse anti-mouse H-2Kb, R-PE-conjugated rat anti-mouse I-A/
I-E (BD Biosciences), R-PE-conjugated rat anti-mouse CD44 (clone IM7; Biologend), and R-PE-conjugated rat anti-mouse CD86 (clone GL1; BD Biosciences), FITC-conjugated rat anti-mouse CD4 (clone CT-CD4; Caltag Laboratories), allopurinol-conjugated rat anti-mouse CD25 mAb (Biologend), R-PE- conjugated rat anti-mouse CD8a/Ly-2 mAb (Southern Biotechnology Associates), and R-PE-conjugated anti-mouse/rat forkhead box p3 (Foxp3) staining set (clone FJK-16s; Bioeisence) were used for splenocyte phenotypic analysis and cell sorting (FACS aria; BD Biosciences). To block nonspecific binding, a 30-min preincubation with 5% rat serum was performed. All stainings include negative controls with isotype Abs. Light scattering parameters were set to exclude dead cells and debris.

Real-time quantitative PCR for Foxp3, IFN-γ, and IL-10 in cardiac allograft tissues

Total RNA was obtained from cardiac tissue by homogenization followed by TRIzol extraction (Invitrogen). RNA was treated with DNase and reverse transcribed to cDNA by Superscript II (Invitrogen). Quantitative real-time PCR was performed on a TaqMan Applied Biosystems Prism 7000 Sequence Detection System with Power SYBR Green Master Mix and the following specific primers: mouse Foxp3 (GenBank sequence NM_054039): forward (300 nM) 5′-GGCG AAAT GTGA GAG AGTG TAT T-3′; reverse (300 nM) 5′-TTC CAA GTC TCT GCT GAA GGC-3′; mouse IFN-γ (GenBank sequence NM_008357): forward (300 nM) 5′-TGA ATT CAT GAT TAT CGA AA T-3′; reverse (300 nM) 5′-GCT TCC TGA GGC TGG ATT CC-3′; and mouse IL-10 (GenBank sequence NM_010548): forward (300 nM) 5′-GGG CTG AGG CCG TGT C-3′; reverse (300 nM) 5′-GTC CTT GCT ATT TTC ACA GG-3′. GAPDH served as housekeeping gene. The ΔΔCt equation was used to compare the target gene expression in each sample with the expression in control mouse lymph node tissues taken as calibrator (set to 1 arbitrary unit).

Immunohistochemical analysis

We analyzed in situ intragraft CD4+ T cells by an immunofluorescence technique on frozen tissue sections. Cardiac sections (8 μm) were cut with a cryostat, air-dried, and fixed with acetone. We incubated the sections with the Abs: FITC-conjugated rat anti-mouse CD4 (100 μg/ml, clone RM4-5; BD Pharmingen) and rat anti-mouse Foxp3 mAbs (20 μg/ml, clone MF333F; Alexis) followed by Cy3-conjugated goat anti-rat IgG (6 μg/ml; Jackson ImmunoResearch Laboratories). The double immunofluorescence staining was analyzed by an inverted confocal laser scanning microscope (LS 510; Meta; Zeiss). The numbers of total single- and/or double-positive cells were counted in at least 10 randomly selected high-power fields. For each animal, the total CD4+ cell counts and the percentage of CD4+Foxp3+ on CD4+ were calculated.

ELISPOT assays

ELISPOT assays were performed using BD ELISPOT mouse IFN-γ and IL-10 reagents. Responder splenocytes were placed in 96-well ELISPOT plates (Millipore) precoated with capture anti-IFN-γ or anti-IL-10 Ab at the concentration of 100,000/well for IFN-γ or 250,000/well for IL-10 assays. The same number of stimulator cells (irradiated splenocytes) from B6C3, BALB/c, and B6 (self-competition) mice were added to the wells and the plates were incubated at 37°C in 5% CO2 for 48 h. Aliquots of responder splenocytes were also incubated with medium alone (negative controls). Each combination was run in triplicate wells. The assays were then conducted according to the manufacturer’s instructions. The resulting spots were counted on a computer-assisted Immunospot image analyzer (Aelvis ELISPOT Scanner System). Results are the mean value of spots per 100,000 (IFN-γ) or spots per 250,000 (IL-10) recipient splenocytes stimulated with B6C3 or BALB/c cells after subtracting spots in negative controls (usually 2 or less).

Statistical analysis

Data are reported as mean ± SEM. Survival data were compared using the log-rank test. All of the other data were analyzed by ANOVA. Differences with a p < 0.05 were considered significant.

Results

MSC characterization

MSC, isolated from BM by their adherence to plastic, consisted of a heterogeneous cell population with a spindle-shaped morphology. At this stage, FACS analysis of MSC preparation revealed a 15–35% of contaminating cells that expressed CD45 and CD11b hematopoietic markers. After further purification by MACS, >95% of cells were CD45 CD11b+. Murine CD45 CD11b+ MSC expressed low levels of MHC class I and MHCII and were positive for CD44 (on average 44%) and negative for CD86, as shown in Fig. 1A.

MSC are not immunogenic and suppress T cell proliferation in vitro

As shown in Fig. 1B, murine MSC themselves did not elicit a proliferative response by allogeneic CD4+ T cells. Indeed, B6 CD4+ T cell proliferation against B6C3 MSC was negligible and significantly lower than the proliferation induced by mature splenocytes from B6C3 mice. B6C3 MSC added at a 1:10 ratio (MSC:CD4+ T cells) to naive MLR cultures significantly reduced the proliferative response of allogeneic B6 CD4+ T cells to splenocytes from the same MSC donor strain (Fig. 1B). B6C3 MSC at the same concentration also significantly lowered the proliferation of allogeneic B6 CD4+ T cells elicited by third-party splenocytes from BALB/c mice (Fig. 1B). When naive MLR were repeated in the presence of conditioned medium (diluted 1/10) collected from the above MSC-MLR cocultures, the CD4+ T cell proliferative allosresponse was not affected, whereas 20–30% reduction was observed with conditioned medium diluted 1/5 (Fig. 1B). These results would suggest that in our experimental conditions the MSC immunomodulatory effect was mainly cell-contact dependent.

To test whether MSC were capable of suppressing the proliferation of autologous CD4+ T cells in MLR as well, graded doses of B6 MSC were added to a naive MLR with B6 CD4+ T cell responders and B6C3 splenocytes as stimulators. For comparison, additional MLR with graded doses of B6C3 MSC added to allogeneic B6 CD4+ T cell responders and B6C3 splenocyte stimulators were performed. MSC were able to suppress the proliferation of both allogeneic and autologous CD4+ T cells to alloantigens in a dose-dependent manner and to a comparable extent (Fig. 1C).

In vivo tissue distribution of MSC

We next evaluated in vivo the distribution of either allogeneic B6C3 or syngeneic B6 PKH26-labeled MSC (0.5 × 106) infused either in the portal vein or in the tail vein of B6 mice. After 1, 7, and 21 days, single-cell suspensions were obtained from bone marrow, thymus, spleen, lymph nodes, and blood and analyzed by FACS for the presence of PKH26+ MSC. The tissue distribution and the degree of engraftment of allogeneic MSC was similar to those of syngeneic MSC both after tail vein and after portal vein injection (Fig. 2A). FACS analysis of blood from mice in all experimental group did not reveal any PKH26+ cells (data not shown). In both syngeneic and allogeneic combinations, infusion via tail vein was associated with higher percentages of MSC localizing in secondary lymphoid tissues as compared with intraportal injection. Indeed, 1 day after tail vein injection, ~12–19% of the overall PKH26+ MSC infused localized in the spleen and 4–5% localized in lymph nodes, as compared with 5–7% and 1.4% found in spleen and lymph nodes of mice given the cells via the portal vein. On the other hand, the amount and distribution of MSC in bone marrow and thymus were not influenced by the site of injection (Fig. 2A). Seven and 21 days after infusion, PKH26+ MSC were hardly or not detectable in bone marrow and lymphoid tissues of recipient animals, either in allogeneic or in syngeneic settings (Fig. 2A), respectively from the site of injection.

We next evaluated MSC engraftment in lungs and livers by fluorescence microscopy. After intraportal injection of either allogeneic or syngeneic PKH26+ MSC, approximately six to eight cells per section and four to five cells per section were detected in...
the liver 1 and 7 days after infusion, respectively (Fig. 2, B–D). Lower but detectable numbers of allogeneic and syngeneic MSC were found in the liver 1 day after tail vein injection, whereas at 7 days PHK26-labeled MSC were negligible (p/H11021 0.05 vs portal vein infusion; Fig. 2). No PKH26 MSC were found in lungs from mice receiving either syngeneic or allogeneic MSC via the portal vein (Fig. 2). Few positive cells were found in lungs at 1 day (Fig. 2, B and E) but not at 7 days after tail vein injection. No PKH26 MSC cells were found in liver and lungs taken 21 days after infusion in any experimental group (data not shown).

To evaluate the distribution and compartmentalization of MSC after transplantation, additional mice received double infusion (portal vein, day 7; tail vein, day 1) of either allogeneic B6C3 or syngeneic B6 PKH26-labeled MSC (0.5 × 10^6 each infusion) and were transplanted with a B6C3 heart at day 0 or left untreated. Tissues were collected and analyzed 7 days later (i.e., 15 days after the first MSC infusion). As shown in Fig. 2A, both allogeneic and syngeneic MSC were hardly detectable or even absent in bone marrow and lymphoid tissues, with no apparent difference between transplanted and untransplanted animals studied at the same time point. Few PKH26 cells were found in the livers of either transplanted or untransplanted mice (Fig. 2B), whereas MSC were virtually absent in lung tissues (Fig. 2B).

Donor-derived MSC infusion prolongs the survival of a semiallogeneic heart transplant

To evaluate whether MSC had immunomodulatory effects in vivo, we infused B6C3 (H-2b,k) MSC (0.5 × 10^6) into the portal vein of semiallogeneic B6 (H-2b) mice. Injection into the portal vein was done on the basis of previous studies showing that the liver is a privileged organ for donor allogeneic mature (splenocytes) or stem cell (bone marrow or embryonic stem cells) engraftment and development of tolerance (5, 35–37). Seven days later, mice were sacrificed and splenocytes isolated to perform MLR assays. CD4 T cells from the spleen of MSC-infused mice had a reduced MLR proliferative response toward either donor B6C3 or third-party
BALB/c (H-2d) stimulators as compared with the response of CD4+ T cells from naive mice run in parallel (Fig. 3A). When splenocytes from infused mice were stimulated with anti-CD3/anti-CD28 Abs, they were unable to proliferate (Fig. 3B) even in the presence of a high dose of IL-2 (Fig. 3B), indicating that MSC administration induced profound T cell hyporesponsiveness.

Based on striking inhibition of in vitro and ex vivo T cell activation by MSC, we sought to address whether MSC infusion had tolerogenic properties in a murine model of solid organ transplantation using the semiallogeneic combination of B6C3 donor hearts in B6 recipients. Experimental groups are shown in Table I. Donor-derived B6C3 MSC (0.5 × 10^6) were intraportally injected in mice 7 days before transplantation (group 1). To evaluate the possible contribution of donor BM cells other than MSC in inducing long-term graft acceptance, an additional group of mice receiving intraportal donor total BM cells (0.5 × 10^6, group 2) was studied. No immunosuppression was given to recipient mice.

Untreated B6 recipients (group 4) rejected a B6C3 heart within 13 days (Table I and Fig. 4A). Mice receiving an intraportal infusion of donor MSC showed a significant (p < 0.05 vs untreated mice) prolongation of graft survival. Of note, in 33% of MSC-infused recipient mice, graft survival reached more than 100 days, indicating that graft acceptance was achieved in a subgroup of allograft recipients. At variance, slightly accelerated cardiac allograft rejection was observed in group 2 mice given donor total BM cells (p < 0.05 vs untreated mice; Table I and Fig. 4A).

To evaluate whether a single infusion into a peripheral vein was effective in prolonging heart graft survival like portal vein infusion, naive B6 mice (group 3) received 0.5 × 10^6 B6C3 MSC into the tail vein 7 days before a cardiac B6C3 transplant. All mice rejected their cardiac graft within 15 days (Table I and Fig. 4A). These results suggest that liver is the favored site for tolerance induction by MSC (5, 35–37).

In an attempt to enhance the in vivo tolerogenic properties of MSC, recipient B6 mice were given two doses of 0.5 × 10^6 B6C3 MSC; an intraportal injection 7 days and an i.v. injection (tail vein) 1 day before transplantation of a B6C3 heart (group 5). Mice receiving two injections of donor total BM cells (group 6) were also...
was added at a concentration of 100 U/ml. Results are mean ± SE.

### Table I. Experimental groups and heart graft survival

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart</th>
<th>Recipient</th>
<th>Site and Timing</th>
<th>Injected Cells</th>
<th>Graft Survival (Days)</th>
<th>MST</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>B6C3</td>
<td>B6</td>
<td>Portal vein, day −7</td>
<td>B6C3 MSC</td>
<td>10, 12, 13, 18, 25, 56, 78, &gt;100 × 4</td>
<td>40bd</td>
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<tr>
<td>2</td>
<td>B6C3</td>
<td>B6</td>
<td>Portal vein, day −7</td>
<td>B6C3 BM</td>
<td>7 × 3</td>
<td>7c</td>
</tr>
<tr>
<td>3</td>
<td>B6C3</td>
<td>B6</td>
<td>Tail vein, day −7</td>
<td>B6C3 MSC</td>
<td>8, 10 × 3, 15 × 2</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>B6C3</td>
<td>B6</td>
<td>Untreated</td>
<td>B6C3 MSC</td>
<td>9 × 3, 10, 12 × 2, 13</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>B6C3</td>
<td>B6</td>
<td>Portal vein, day −7; tail vein, day −1</td>
<td>B6C3 MSC</td>
<td>8, 9 × 2, 10, 11 × 2, 20, 32, 50, &gt;100 × 5</td>
<td>26cd</td>
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<tr>
<td>6</td>
<td>B6C3</td>
<td>B6</td>
<td>Portal vein, day −7; tail vein, day −1</td>
<td>B6C3 BM</td>
<td>8, 11, 14</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>B6C3</td>
<td>B6</td>
<td>Portal vein, day −7; tail vein, day −1</td>
<td>B6C3 MSC + HSC</td>
<td>7, 8 × 3, 13</td>
<td>8</td>
</tr>
</tbody>
</table>

- Recipient B6 mice received 0.5 × 10⁶ B6C3 cell infusion.
- p < 0.05 vs groups 2 and 3.
- p < 0.05 vs group 4.
- p < 0.05 vs groups 6 and 7.
- Lin− c-Kit+ HSC (0.25 × 10⁶) were injected with MSC.

studied as controls. The double dose of donor MSC resulted in significant prolongation of heart allograft survival as compared with that observed after two injections of total BM cells or in untreated recipients (Table I and Fig. 4B). There was, however, no significant difference in graft survival between animals given one or two MSC injections.

Histological analysis of rejected not beating allografts (n = 2) taken 9–10 days after transplant from untreated B6 recipients of a B6C3 heart revealed an intense interstitial mononuclear cell infiltrate, many apoptotic and degenerating myocytes, scattered areas of hemorrhage, and total or subtotal vascular luminal obliteration (Fig. 5A). A similar histological picture was found in nonbeating allografts taken at 10–12 days after transplant from the subgroup of donor MSC-infused mice that rejected their grafts (n = 2 from group 1 in Table I; Fig. 5B). Grafts obtained at >100 days after transplantation from donor-derived MSC-tolerized mice (n = 3) demonstrated mild, diffuse mononuclear cell infiltrate, viable myocytes but also showed moderate signs of chronic allograft vasculopathy (Fig. 5C), indicating that MSC infusion did not prevent chronic rejection.

### FIGURE 3.

Splenocytes from B6 mice receiving B6C3 MSC infusion display hyporesponsiveness ex vivo. A, Isolated CD4⁺ T cells (0.5 × 10⁶) from either naive (n = 5) or B6C3 MSC-infused B6 mice (sacrificed 7 days after infusion, n = 5) were cultured for 3 days with irradiated (4000-rad) B6C3, BALB/c or syngeneic B6 splenocytes. MLR proliferative results (mean ± SE) are expressed as Δcpm by subtracting cpm of syngeneic MLR from allogeneic combinations. #, p < 0.05 vs naive mice. B, Results of anti-CD3/anti-CD28 mAb stimulation of total splenocytes from naive (n = 5) or B6C3 MSC-infused B6 mice (n = 5). IL-2 was added at a concentration of 100 U/ml. Results are mean ± SE. #, p < 0.05 vs naive.

### FIGURE 4.

Donor MSC induce long-term cardiac allograft survival. A, Either B6C3 MSC (portal vein, n = 12; tail vein, n = 6) or total BM cells (portal vein, n = 3) were injected into recipient B6 (H-2b) mice 7 days before cardiac B6C3 (H-2Bb) transplantation. Untreated B6 mice (n = 7, no cell infusion) rejected B6C3 cardiac grafts within 13 days. Only donor MSC infusion into the portal vein induced a significant prolongation of cardiac allograft survival. §, p < 0.05 vs no cell infusion; ○, p < 0.05 vs total BM cell infusion and MSC tail vein. B, B6 recipients of B6C3 cardiac grafts received two pretransplant infusions (portal vein at day −7 and tail vein at day −1 before surgery) of donor MSC (n = 14), total BM cells (n = 3), MSC + Lin− c-Kit+ HSC (n = 5) or no cell infusion (n = 7). Only mice receiving donor MSC showed prolonged survival of cardiac allografts while recipients of total BM cells or MSC plus Lin− c-Kit+ HSC promptly rejected B6C3 cardiac grafts. #, p < 0.05 vs no cell infusion, total BM cells, and MSC + HSC.

Donor-derived HSC antagonize MSC tolerogenic effects

MSC isolated using a plastic adherence method from rodent BM usually contain heterogeneous cell populations including HSC.
To evaluate whether MSC and HSC could exert either synergistic or antagonistic effects on tolerance induction, an additional group of B6 mice received two injections of $0.5 \times 10^6$ B6C3 MSC plus $0.25 \times 10^6$ B6C3 Lin−c-Kit+ HSC (group 7) given 7 days (portal vein) and 1 day (tail vein) before B6C3 heart transplantation. No prolongation of cardiac allograft survival was observed with the combined cell treatment, indicating that HSC do not synergize but instead hamper the in vivo tolerogenic properties of MSC (Table I and Fig. 4B). The presence of residual hematopoietic cells in MSC preparations may at least in part explain failure of some studies in showing an immunomodulatory effect of MSC in organ transplantation in rodents (22).

**Donor-derived MSC induce in vivo formation of Treg**

To clarify whether MSC infusion induced the formation of Treg, adoptive transfer experiments were performed. Forty million splenocytes obtained from B6 mice sacrificed 7 days after intraportal infusion of $0.5 \times 10^6$ B6C3 MSC were given i.v. to B6 naive mice 1 day before donor B6C3 heart transplantation. As shown in Fig. 6, all B6 mice adoptively transferred with splenocytes from B6C3 MSC-treated mice showed prolongation of donor B6C3 cardiac allograft survival ($p < 0.05$ vs naive recipients) that was indefinite in 60% of animals.

We next investigated whether Treg had also a role in the maintenance phase of MSC-induced tolerance. To this purpose, mice with long-term graft survival (>100-day survival of a B6C3 heart) by either intraportal or combined intraportal and tail vein B6C3 MSC infusion were used as splenocyte donors for adoptive transfer experiments. Naive B6 mice receiving splenocytes ($40 \times 10^6$ i.v.) from mice with long-term graft survival the day before transplantation of a B6C3 heart had significant prolongation of graft survival ($p < 0.05$ vs mice receiving naive splenocytes; Table II). Four of five mice had indefinite (>100 days) survival of the allograft (Table II). Notably, spleen cells from these secondary transplanted mice were able to transfer tolerance to other naive B6 mouse recipients of a new B6C3 heart (Table III). Once these latter animals were tolerant for more than 100 days, their splenocytes transferred tolerance to additional naive recipients (Table III), further documenting the infectious properties of MSC-induced Treg (40). To document the donor-specificity of Treg, naive B6 mice receiving splenocytes from animals tolerizing a B6C3 heart were transplanted with fully allogeneic donor-specific C3 hearts. Two of three mice showed a prolongation of heart survival (Table II). In contrast, naive B6 mice receiving splenocytes from animals tolerizing to a B6C3 heart promptly rejected a fully allogeneic third-party BALB/c heart (Table II).

Altogether these data indicate that both the induction and the maintenance phase of pretransplant donor MSC infusion-induced graft acceptance were associated with the formation of Treg. Of note, Treg isolated during the maintenance phase of MSC-induced tolerance were donor specific.

Different subsets of T cells with regulatory properties have been reported in literature, including CD4+CD25+ Treg, CD4+CD25− Treg, and certain CD8+ T cell subsets, which may be induced in vivo by protolerogenic strategies (41).

Additional experiments were done to identify the subset of Treg responsible for MSC-induced infectious tolerance. CD4+ and CD8+ T cells were transferred into naive recipients of a B6C3 heart from animals with long-term graft survival, and spleen cells from these secondary transplanted mice were able to transfer tolerance to additional naive recipients (Table III). Once these latter animals were tolerant for more than 100 days, their splenocytes transferred tolerance to additional naive recipients (Table III), further documenting the infectious properties of MSC-induced Treg (40). To document the donor-specificity of Treg, naive B6 mice receiving splenocytes from animals tolerizing a B6C3 heart were transplanted with fully allogeneic donor-specific C3 hearts. Two of three mice showed a prolongation of heart survival (Table II). In contrast, naive B6 mice receiving splenocytes from animals tolerizing to a B6C3 heart promptly rejected a fully allogeneic third-party BALB/c heart (Table II).

**FIGURE 5.** Histological analysis of cardiac graft tissues. Representative images of grafts harvested from rejecting untreated mice 10 days after transplantation (A), from donor MSC-infused mice acutely rejecting the graft 10 days after transplantation (B), and from donor MSC-tolerized mice >100 days after transplantation (C).

**FIGURE 6.** Splenocytes from donor MSC-infused mice transfer tolerance into naive mice. Survival time of cardiac grafts in naive B6 mice adoptively transferred with splenocytes ($40 \times 10^6$) from B6C3 MSC-infused mice sacrificed 7 days after cell injection are shown. Donor B6C3 hearts had prolonged survival ($n = 5$) as compared with donor B6C3 ($n = 3$) hearts in B6 mice receiving naive splenocytes. §, $p < 0.05$ vs B6 mice given naive splenocytes.

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**Table II. Results of adoptive transfer experiments with spleen cells or splenic T cell subpopulations from mice made tolerant by donor MSC to a B6C3 heart**

<table>
<thead>
<tr>
<th>Cell injection</th>
<th>Heart</th>
<th>Graft Survival (Days)</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolerant splenocytes</td>
<td>B6C3</td>
<td>69, &gt;100 × 4</td>
<td>&gt;100$^b$</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>B6C3</td>
<td>&gt;100 × 3</td>
<td>&gt;100$^b$</td>
</tr>
<tr>
<td>CD4+CD25+ T cells</td>
<td>B6C3</td>
<td>&gt;100 × 3</td>
<td>&gt;100$^b$</td>
</tr>
<tr>
<td>CD4+CD25− T cells</td>
<td>B6C3</td>
<td>14, 27, 38</td>
<td>27</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>B6C3</td>
<td>13, 15, 14</td>
<td>14</td>
</tr>
<tr>
<td>Tolerant splenocytes</td>
<td>C3</td>
<td>11, &gt;40 × 2</td>
<td>&gt;40$^b$</td>
</tr>
<tr>
<td>Tolerant splenocytes</td>
<td>BALB/c</td>
<td>8, 8, 11</td>
<td>8</td>
</tr>
<tr>
<td>Naive splenocytes</td>
<td>B6C3</td>
<td>11, 12, 12</td>
<td>12</td>
</tr>
<tr>
<td>Naive splenocytes</td>
<td>C3</td>
<td>7, 7, 7</td>
<td>7</td>
</tr>
<tr>
<td>Naive splenocytes</td>
<td>BALB/c</td>
<td>8, 9, 10</td>
<td>9</td>
</tr>
</tbody>
</table>

$^a$ B6C3 and C3 donor and BALB/c third-party hearts were transplanted into B6 recipients the day after splenocyte or splenic T cell subpopulation injection.

$^b$ $p < 0.05$ vs naive splenocytes.
CD8+ T cell subpopulations from splenocytes of mice with long-term graft survival were sorted and transferred into naive B6 animals the day before transplantation. Six million total CD4+ T cells, 1.5 × 10^6 CD4+CD25+ T cells, 2.5 × 10^6 CD4+CD25− T cells, and 4 × 10^6 CD8+ T cells, that correspond to the number of cells in each subset isolated by sorting 40% of total CD4+ T cells from mice with long-term graft survival, were infused i.v.

Results are summarized in Table II. Naïve B6 mice receiving either total CD4+ T cells or CD4+CD25+ T cells from mice with long-term graft survival had indefinite survival of the cardiac B6C3 allograft, whereas mice infused with CD8+ T cells acutely rejected the donor heart within 15 days (Table II). Mice given CD4+CD25− T cells showed a slight prolongation of cardiac allograft survival, but eventually rejected the graft within 38 days.

To confirm the phenotype of MSC-induced Treg, we stained splenocytes from mice, made tolerant to B6C3 hearts by B6C3 MSC infusion, with anti-CD4, anti-CD25, and anti-Foxp3 Abs. We found a higher percentage of cells that coexpressed CD4, CD25, and Foxp3 in splenocytes from MSC-tolerized mice than in naïve mice (Fig. 7, A and B, p < 0.05 vs naïve mice).

Real-time PCR of Foxp3 mRNA and immunohistochemical analysis of CD4+Foxp3+ cells were also performed in cardiac allograft tissues to document Treg recruitment at the graft site. Foxp3 mRNA levels were higher in MSC-tolerized heart allografts than in rejected grafts from untreated recipients (Fig. 8A). Approximately 30% of CD4+ cells in beating heart allografts taken at 9–10, 21, and >100 days posttransplantation (Fig. 8, C and D) from mice tolerized by donor MSC infusion expressed Foxp3. By contrast, few CD4+ cells in rejected nonbeating heart allografts from untreated mice showed a positive staining for Foxp3 (Fig. 8, B and D; p < 0.05 vs MSC tolerized).

We next investigated whether expansion of Treg in mice with long-term graft survival was associated with a Th1/Th2 shift. The frequencies of anti-donor IFN-γ-producing splenocytes from mice studied at >100 days after transplant were lower than those from untreated mice rejecting their graft at 7–10 days after transplant (Fig. 7C). The frequency of IFN-γ-producing splenocytes against BALB/c was comparable in both groups (Fig. 7C). In contrast, the frequency of anti-donor IL-10-producing splenocytes was comparable in MSC-tolerized (11.5 ± 4.4 spots/250,000 responders,

### Table III. Survival of B6C3 heart transplants after serial adoptive transfers of splenocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Source of Adoptively Transferred Splenocytes</th>
<th>Heart Survival (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (first adoptive transfer)</td>
<td>B6 recipient taken at &gt;100 days after B6C3 MSC infusion and primary</td>
<td>&gt;100 × 3</td>
</tr>
<tr>
<td>2 (second adoptive transfer)</td>
<td>B6C3 heart transplant</td>
<td>&gt;100 × 3</td>
</tr>
<tr>
<td>3 (third adoptive transfer)</td>
<td>Group 1 taken &gt;100 days after B6C3 secondary heart transplant</td>
<td>&gt;100 × 3</td>
</tr>
<tr>
<td></td>
<td>Group 2 taken &gt;100 days after B6C3 tertiary heart transplant</td>
<td>&gt;100 × 3</td>
</tr>
</tbody>
</table>
Recipient-derived MSC prolong the survival of semiallogeneic cardiac allografts and induce the formation of Treg

FIGURE 8. Treg accumulation and reduced IFN-γ mRNA expression in the graft of MSC-tolerized mice. A, mRNA was extracted from cardiac allograft tissues from untreated B6 recipient mice (n = 3, □) or from B6 mice tolerized (>100 days) by donor-derived B6C3 MSC (n = 3, □) or by recipient-derived B6 MSC (n = 3, □). mRNA was also extracted from native cardiac tissue of B6C3 mice (negative control). Foxp3 expression was evaluated by real-time PCR and expressed as AU. ○, p < 0.05 vs rejecting. B and C, Representative images of intragraft immunostaining of CD4+ cells (green) and CD4+ Foxp3+ cells (yellow/red) in cardiac grafts from untreated rejecting mice (B) or from donor MSC-tolerized mice (>100 days posttransplant (C). Original magnification, ×400. D, Total CD4+ graft-infiltrating lymphocyte counts (cells per high-power field) and percentages of CD4+ Foxp3+ on total infiltrating CD4+ cells from naive (n = 3), rejecting (n = 3) or donor MSC-tolerized mice (double portal/tail vein infusion) with a beating heart allograft taken at 7 (n = 3), 21 (n = 3) or >100 days (n = 4) post transplant. *p < 0.05 vs naive and rejecting. E, mRNA was extracted from cardiac allograft tissues of recipient B6 mice tolerized by donor MSC (>100 days posttransplant, n = 3; □) or from untreated rejecting mice (n = 4; □). mRNA was also extracted from native cardiac tissue of B6C3 mice (negative control). IFN-γ expression was evaluated by real-time PCR and expressed as AU. "p < 0.05 vs rejecting.

n = 7) and in rejecting untreated mice (11.9 ± 2.9 spots/250,000 responders, n = 5).

These data indicate that tolerance induced by MSC infusion is characterized by reduced Th1 effector cells without Th2 shift.

Consistently, we found less IFN-γ mRNA expression in cardiac grafts from mice with long-term graft survival as compared with rejecting mice (Fig. 8E). The evaluation of graft IL-10 mRNA expression did not reveal any difference between long-term graft survival and rejecting mice (mice with long-term graft survival, 0.74 ± 0.31 arbitrary units (AU), n = 4; rejecting, 1.10 ± 0.23 AU, n = 4, p = NS).

No IFN-γ, IL-10, and Foxp3 mRNA expression was found in cardiac tissues from naive B6C3 mice.

Altogether these results indicate that tolerance induced by pretransplant donor MSC infusion is associated with the accumulation of CD4+CD25+Foxp3+ Treg in lymphoid organs and in the graft and a reduction of anti-donor Th1 effector cells.

Recipient-derived MSC prolong the survival of semiallogeneic cardiac allografts and induce the formation of Treg

On the basis of in vitro data that MSC were capable of suppressing the proliferation of autologous T cells against alloantigens (Fig. 1C), we next evaluated whether infusion of recipient-derived MSC prolonged the survival of a semiallogeneic heart transplant. B6 mice were given 0.5 × 106 B6 MSC into the portal vein 7 days and a second dose into the tail vein 1 day before heart transplant from B6C3 mice (Table IV). The infusion of recipient-derived MSC induced tolerance to B6C3 cardiac allografts in 50% of mice (Table IV). These results are very comparable to those obtained with donor-derived MSC infusion (Table I).
To investigate whether HSC of recipient origin hamper the tolerogenic potential of MSC, an additional group of B6 mice received 0.5 × 10^6 B6 MSC along with 0.25 × 10^6 B6 Lin−c-Kit+ HSC (Table IV) 7 days (portal vein) and 1 day (tail vein) before a B6C3 heart transplantation. Two of four mice showed prolonged cardiac graft survival (>40 days; Table IV), a percentage that was the same obtained with the sole injection of recipient-derived MSC, indicating that recipient-derived HSC did not antagonize the tolerogenic potential of MSC infusion, at variance with donor-derived HSC.

To verify whether in this setting tolerance was associated with the emergence of Treg, adoptive transfer experiments with splenocytes from B6 mice made tolerant to B6C3 heart by B6 MSC infusion were performed. Forty million splenocytes from tolerized animals had no effect on heart allograft survival (survival: 15 and 18 days; median survival time (MST), 16.5 days, n = 2). However, infectious tolerance was obtained by increasing the amount of infused splenocytes to 70 × 10^6 (survival: 46, 91, and >115 days; MST, 91 days, n = 3). In contrast, the same amount of splenocytes did not prolong the survival of BALB/c hearts to any extent (survival: 8, 9, and 9 days; MST, 9 days, n = 3). FACs analysis of spleen cells from mice with long-term graft survival revealed a higher percentage of CD4+CD25+Foxp3+ Treg than splenocytes from naive animals (Fig. 7A; p < 0.05 vs naive). Moreover, RT-PCR analysis of heart allografts tolerized by recipient-derived MSC showed a well-detectable Foxp3 expression (Fig. 8A). However, both the percentage of Treg in the spleen and the level of Foxp3 expression in heart grafts from these animals were lower than those observed in B6 mice made tolerant to B6C3 cardiac allografts by donor-derived MSC infusion (Figs. 7A and 8A). These results indicate that recipient-derived MSC infusion induced the formation of Treg, although to a lesser extent than donor-derived MSC.

Pretransplant infusion of recipient MSC is more effective than peritransplant infusion to prolong allograft survival

With the perspective of clinical application of the tolerogenic procedure, experiments were also performed changing the MSC infusion protocol. We first tested the efficacy of a double tail vein dose of 0.5 × 10^6 recipient-derived MSC given to B6 mice 7 and 1 days before a B6C3 heart transplant. The latter protocol had similar tolerogenic potential as the double intraportal/tail vein infusion (Table IV). To shorten the timing between MSC infusion and transplantation, 0.5 × 10^6 B6 MSC were infused in B6 mice, either into the portal vein during surgery (peritransplant) or into the tail vein 1 day after transplantation of B6C3 heart grafts. As shown in Table IV, peritransplant infusion of recipient-derived MSC via the portal vein induced indefinite allograft survival in two of six mice, whereas tail vein infusion of B6 MSC 1 day after transplantation failed to prolong heart allograft survival to any extent (Table IV). Thus, peritransplant infusion of recipient-derived MSC via the portal vein had a lower tolerogenic effect than pre-transplant infusion, while a single tail vein MSC infusion 1 day after transplantation was ineffective.

Recipient-derived MSC do not prolong fully allogeneic cardiac allograft survival

We then investigated whether infusion of recipient-derived MSC was able to tolerate a fully MHC-mismatched cardiac allograft. To this purpose, a group of B6 mice (n = 4) was given a double infusion of 0.5 × 10^6 recipient-derived B6 MSC (day –7 in the portal vein and –1 in the tail vein) before receiving a BALB/c allograft. MSC failed to prolong the survival of fully MHC-mismatched cardiac allografts (survival times: MSC-treated mice: 7, 7, 9, and 12 days; MST, 8 days; naive B6 mice: 8, 9, and 10 days; MST, 9 days).

Discussion

In the present study, we confirmed that MSC have immunosuppressive properties in vitro and in vivo and found that infusion of donor MSC is effective in prolonging the survival of semiallogeneic heart transplants in unconditioned recipient mice. This tolerogenic effect was not shared by total BM cells. Confusion of HSC abrogated the tolerogenic effect of MSC. More importantly, we found that MSC from the recipient strain are capable of prolonging a donor cardiac allograft survival as well. The tolerogenic potential of both donor-derived and recipient-derived MSC was associated with the expansion of CD4+CD25+Foxp3+ Treg.

The in vitro immunosuppressive effect of murine MSC was dose dependent and not donor specific. Indeed, MSC inhibited the proliferative response of both autologous and allogeneic CD4+ T cells to either donor or third-party alloantigens in MLR, which is consistent with most published studies in human, baboon, and rodent MSC (13, 14, 18, 21–26).

The role of either soluble factors or cell contact-dependent mechanisms in suppression of T cell response by MSC is still an unsolved issue. Immunosuppression by human MSC has been reported to be mediated by soluble factors such as hepatocyte growth factor (13) TGF-β1 (13), PGE2 (42), IDO-mediated tryptophan deletion (43), and NO (44). In contrast, T cell inhibition by murine MSC was either dependent on soluble factors (23, 24, 43) or required cell contact (14, 23–25, 43). In this study, we found that, in vitro, the conditioned medium of murine MSC-T cell cultures only partially affected T cell alloreactivity, which would suggest a role of cell contact in MSC-mediated T cell suppression. Discrepancies in different studies may be explained by the different experimental conditions used, such as the ratio between MSC and responder cells. Indeed, evidence is available that MSC-derived soluble factors played a role at high MSC:responder ratio (1:1) (23), whereas at lower ratios, as the one used in the present study (1:10), cell contact was required to inhibit immune cell response (25). In addition, most studies used total splenocytes as responders, which

Table IV. Effect of recipient-derived B6 MSC infusion on B6C3 cardiac allograft survival in B6 mice

<table>
<thead>
<tr>
<th>Site and Timing</th>
<th>Injected Cells</th>
<th>Graft Survival (Days)</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portal vein, day –7; tail vein, day –1</td>
<td>MSC</td>
<td>10, 10, 12, 25, &gt;100 × 4</td>
<td>&gt;62.5*</td>
</tr>
<tr>
<td>Portal vein, day –7; tail vein, day –1</td>
<td>MSC + HSC</td>
<td>7, 15, &gt;40 × 2</td>
<td>&gt;27.5*</td>
</tr>
<tr>
<td>Tail vein, day –7; tail vein, day –1</td>
<td>MSC</td>
<td>10, 19, &gt;100 × 2</td>
<td>&gt;59.5*</td>
</tr>
<tr>
<td>Tail vein, day –1</td>
<td>MSC</td>
<td>9, 9, 12</td>
<td>9</td>
</tr>
<tr>
<td>Portal vein, day 0</td>
<td>MSC</td>
<td>8, 14, 17, 28, &gt;100 × 2</td>
<td>22.5*</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td>9, 9, 10, 12, 12, 13</td>
<td>10</td>
</tr>
</tbody>
</table>

*Recipient B6 mice received 0.5 × 10^6 B6 MSC and were transplanted with a B6C3 heart.

p < 0.05 vs untreated.

B6 Lin−c-Kit+ HSC (0.25 × 10^6) were injected with MSC.
include disparate subsets of cells that may be potential MSC targets. In this regard, B lymphocytes were sensitive to the inhibitory action of MSC-released soluble factors, whereas the inhibition of T cell proliferation by MSC was cell contact dependent (24). The latter finding is consistent with a cell contact-dependent mechanism as suggested by our coculture experiments in which purified CD4+ T cells were used as targets.

Several studies in animal models and in humans have demonstrated that MSC are capable of long-term engraftment after i.v. injection, although only a small fraction of the infused cells underwent vascular emigration and tissue distribution in bone marrow, lungs, heart, kidneys, and lymphoid organs (29, 30, 45–48). By in vivo infusion experiments, we found that MSC tissue distribution was influenced by the site of injection but not by the donor-recipient match. After infusion, both allogeneic and syngeneic MSC rapidly but transiently localized in secondary lymphoid organs with a higher number of cells observed after tail vein than after portal vein injection. MSC were well detected in the recipient liver after portal vein injection, whereas after tail vein infusion MSC could be detected transiently in the liver and in lower numbers than when given via portal vein.

The different in vivo distribution of MSC following portal vein vs tail vein infusion influenced the MSC tolerogenic properties. Indeed, a single intraportal infusion of MSC caused reduction of T cell alloreactivity and prolonged the survival of semiallogeneic cardiac transplants, indicating that MSC when given via portal vein are capable to modulate immune cell response in vivo. By contrast, a single donor-derived MSC infusion into the tail vein was not enough to prolong allograft survival.

Altogether, these results are in line with previous studies indicating that the liver is an immune privileged organ for tolerance induction by donor cell infusion. Intraportal but not systemic i.v. infusions of donor splenocytes or bone marrow cells could extend the survival time of skin, islet, cardiac, and kidney transplants (37, 49–51). All of these models are consistent with the idea that the contact between donor-derived cells and the host immune system in the liver results in immune inactivation. The nature of this interaction is not clear, although it was hypothesized that either sinusoidal endothelial cells (52) or the liver’s large population of sinusoidal macrophages, the Kupffer cells, may have a role (53).

Several studies in rodents (3, 6, 7) have documented the possibility to achieve allogeneic chimerism and tolerance to solid organ allotransplantation by infusion of either BM cells or HSC, although different manipulations of the host immune system were required to achieve BM engraftment and tolerance (6, 7). In this study, we found that injection of total donor BM cells was not associated with any prolongation of allograft survival in an unconditioned host, whereas MSC were effective in this setting, which would support a more potent protolerogenic potential of MSC than total BM cells. More importantly, the protolerogenic effect of MSC was impaired when MSC were injected along with donor-derived but not recipient-derived HSC, suggesting that allogeneic HSC may have a negative impact on MSC immunomodulatory properties. The presence of allogeneic HSC in donor-derived MSC preparation could alter the delicate equilibrium of MSC and recipient T cell interaction by generating an allogeneic immune reaction.

Different mechanisms have been proposed to mediate the immunosuppressive properties of MSC, which include veto functions on effector T cells (14) and blunting effects on the maturation of professional APC (42, 54, 55). MSC can also elicit T cell anergy, reversible by adding exogenous IL-2 (30). In an experimental model of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in mice, MSC infusion caused T cell hyporesponsiveness to MOG-peptide, which was restored by IL-2 (30). In contrast, MSC inhibition of mouse T cell response against the male HY minor histocompatibility Ag was not overcome by IL-2 added to the cell culture (26). In line with the latter study, we found that the hyporesponsiveness of T cells from MSC-infused animals was not reverted by high-dose IL-2, excluding that recipient T cells were anergized by MSC infusion. Consistently, administration of IL-2 in donor-derived MSC-infused animals made tolerant to a semiallogeneic heart graft did not revert tolerance (data not shown).

There is increasing evidence that T cells with regulatory function play a central role in the control of both reactivity to self-Ags and alloimmune response (56). Namely, CD4+CD25+Foxp3+ Treg contribute to prevent allograft rejection in many animal models of tolerance induced by either donor cell infusion or costimulatory blockade (57–59).

The role of Treg in MSC-induced immunomodulation is controversial. Data exist that human MSC-mediated immune inhibition is not reversed by removing Treg from cocultured MLR (25). Others have reported that the CD4+CD25+ Treg population increased significantly in MLR when MSC were present (20, 42, 60, 61). In this study, we demonstrated that the induction and maintenance phases of tolerance after donor-derived MSC infusion in mice are associated with the emergence of functional Treg (Fig. 9), since splenocytes harvested either 7 days after MSC injection or at >100 days after transplantation from mice with long-term graft survival were capable of transferring tolerance to secondary naive recipients. Increased numbers of CD4+CD25+Foxp3+ cells in lymphoid organs and in the graft of animals with long-term graft survival along with the ability of CD4+CD25+ cells to transfer tolerance to naive secondary hosts indicate that expansion of this specific cell subset played a role in MSC-induced tolerance. We hypothesize that, despite the rather low degree of MSC engraftment into lymphoid organs and in the liver, these cells are capable of initiating an active tolerogenic process that self-sustains by interacting with the resident T cell population and favoring the generation of CD4+CD25+ Treg. Regulation, once established, becomes the dominant mechanism that limits Th1 cell priming and prevents the rejection of subsequent semiallogeneic and fully allogeneic donor-specific allografts. MSC-induced tolerogenic mechanisms in vivo were donor specific, since mice adoptively transferred with splenocytes taken from donor-derived MSC-tolerized animals acutely rejected a third-party heart. In line with this interpretation are published data showing that Treg, isolated from ABM mice that spontaneously accepted bm12 heart allografts, suppressed rejection of alloantigen-specific bm12 but not of third-party skin allografts when coinfused with effector T cells into nude mice (62).

Of note, heart allografts taken at >100 days from MSC-infused mice showed signs of chronic allograft vasculopathy. Why MSC-treated mice were not protected from chronic allograft rejection despite an enhanced number of Treg in lymphoid organs and in the graft remains matter of speculation. One possibility is that the number of Treg did not reach the threshold level to properly suppress the complex pathways of effector T cells. Another possibility could be that MSC-induced Treg were specific for directly presented donor Ags but did not suppress the indirect pathway, which appears to play a main role in chronic rejection. In this regard, in a recent report in irradiated mice receiving donor bone marrow, infusion of Treg specific for directly presented donor Ags prevented acute but not chronic rejection of skin and heart allografts, whereas regulatory T cells specific for both directly and indirectly presented alloantigens prevented both acute and chronic rejection (63).
Another major finding of this study is that pretransplant infusion of B6 MSC of recipient origin was as effective in inducing long-term acceptance of semiallogeneic B6C3 cardiac allografts as donor-derived MSC (Fig. 9). Of note, recipient-derived MSC did not prolong the survival of a fully MHC-mismatched BALB/c heart (Fig. 9). We hypothesize that in the semiallogeneic setting, B6 MHC molecules on MSC interacted with B6-restricted TCR of recipient T cells and caused expansion of naturally occurring Treg (Fig. 9). These cells recognized B6 Ags in the graft and prevented a host T cell response against C3 Ags as well, through linked suppression, but did not recognize fully mismatched H2d Ags on BALB/c transplant and graft rejection occurs.

In contrast, a single recipient-derived MSC infusion given peritransplant was marginally effective, and a single MSC dose given 1 day after transplantation was not effective at all. These results indicate that MSC should have already activated protolerogenic mechanisms at the time the recipient immune system comes in contact with graft alloantigens to induce graft acceptance. Relevant to this interpretation are data that MHCI expression on MSC requires stimulation by low IFN-γ levels, whereas in the presence of high IFN-γ levels MSC lose their expression of MHCI (64). This may suggest that during an ongoing immune response, in the presence of high levels of IFN-γ released by effector T cells, MSC lose their ability to interact with CD4+ T cells and to induce Treg differentiation. This possibility is supported by published studies (20, 65) showing that administration of anti-MHCII-blocking Abs prevented MSC-mediated T cell inhibition in vitro (20) and tolerance induction in vivo (65). However, the possibility that treating the recipient with multiple MSC infusion postransplant could have tolerogenic potential cannot be excluded on the basis of the present data.

In summary, we documented that MSC may act as a biological immunosuppressive agent in vivo through the induction of donor-specific CD4+CD25+Foxp3+ Treg. Recipient-derived MSC are equally effective as donor-derived MSC in prolonging allograft survival, which makes this strategy clinically applicable to cadaveric organ transplantation. However, the requirement of a partial MHC match between the donor and the recipient and of pretransplant infusion for MSC tolerogenic effects should be taken into account in designing clinical studies in the setting of solid organ allotransplantation.

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Disclosures
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References
2006. Mesenchymal stem cells inhibit generation and function of both CD34+-
57. Cavinato, R. A., F. Casiraghi, N. Azzollini, P. Cassis, D. Cugini, M. Mister,
peripheral blood mononuclear cells infusion induces transplantation tolerance by
generating regulatory T cells. Transplantation 79: 1034–1039.
58. Lechler, R. I., O. A. Garden, and L. A. Turka. 2003. The complementary roles of
deletion and regulation in transplantation tolerance. Nat. Rev. Immunol. 3:
147–158.
regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immu-
60. Maccario, R., M. Podesta, A. Moretta, A. Cometa, P. Cornoli, D. Montagna,
mesenchymal stem cells with cells involved in alloantigen-specific immune re-
sponse favors the differentiation of CD4+ T-cell subsets expressing a regulatory/
61. Di Ianni, M., B. Del Papa, M. De Ianni, L. Moretti, E. Bonifacio, D. Cecchini,
P. Sportoletti, F. Falzetti, and A. Tabilio. 2008. Mesenchymal cells recruit and
62. Sanchez-Fueyo, A., S. Sandner, A. Habicht, C. Mariat, J. Kenny, N. Degaude,
X. X. Zheng, T. B. Strom, L. A. Turka, and M. H. Sayegh. 2006. Specificity of
CD4+CD25+ regulatory T cell function in alloimmunity. J. Immunol. 176:
329–334.
63. Joffre, O., T. Santolari, D. Calise, T. Al Saati, D. Hudrisier, P. Romagnoli, and
J. van Meerwijk. 2008. Prevention of acute and chronic allograft rejection with
64. Chan, J. L., K. C. Tang, A. P. Patel, L. M. Bonilla, N. Pierobon, N. M. Poncio,
and P. Rameshwar. 2006. Antigen-presenting property of mesenchymal stem
cells occurs during a narrow window at low levels of interferon-γ. Blood 107:
4817–4824.
essential for induction of transplantation tolerance by bone marrow cells. J. Im-
munol. 164: 4452–4457.