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Mesenchymal Stromal Cells Fail to Prevent Acute Graft-versus-Host Disease and Graft Rejection after Dog Leukocyte Antigen-Haploidentical Bone Marrow Transplantation

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Mesenchymal stromal cells (MSCs) have been shown to have immunosuppressive effects in vitro. To test the hypothesis that these effects can be harnessed to prevent graft-versus-host disease (GVHD) and graft rejection after hematopoietic cell transplantation (HCT), we administered a combination of 3 different immortalized marrow-derived MSC lines (15-30 $\times$ 10^6 MSCs/kg/day, 2-5 times/week) or third-party primary MSC (1.0 $\times$ 10^6 MSCs/kg/day, 3 times/week) to canine recipients (n = 15) of dog leukocyte antigen–haploidentical marrow grafts prepared with 9.2 Gy of total body irradiation. Additional pharmacological immunosuppression was not given after HCT. Before their in vivo use, the MSC products were shown to suppress alloantigen-induced T cell proliferation in a dose-dependent, major histocompatibility complex–unrestricted, and cell contact–independent fashion in vitro. Among 14 evaluable dogs, 7 (50%) rejected their grafts and 7 engrafted, with ensuing rapidly fatal acute GVHD (50%). These observations were not statistically different from outcomes obtained with historical controls (n = 11) not given MSC infusions (P = .69). Thus, survival curves for MSC-treated dogs and controls were virtually superimposable (median survival, 18 vs 15 days, respectively). Finally, outcomes of dogs given primary MSCs (n = 3) did not appear to be different from those given clonal MSCs (n = 12). In conclusion, our data fail to demonstrate MSC-mediated protection against GVHD and allograft rejection in this model.

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KEY WORDS: Hematopoietic cell transplantation, MSC, GVHD, Rejection, Canine model

INTRODUCTION

Mesenchymal stromal cells (MSCs) suppress proliferation of alloantigen-activated lymphocytes in vitro in a dose-dependent, cell contact–independent, and major histocompatibility complex (MHC)-unrestricted fashion [1-4]. Motivated by the immunosuppressive properties observed in vitro, MSCs derived from bone marrow have been evaluated in animal models and in human patients for treatment and prevention of graft-versus-host disease (GVHD) and allograft rejection [4-12]. Thus far, most of the evidence documenting the in vivo immunosuppressive effects of MSC is based on case reports, small case series, and phase II studies [13]. Although results have not been overwhelmingly positive, no acute or long-term adverse events after MHC infusion, including ectopic tissue formation, have been reported [14-18].

One obstacle that may limit the effectiveness of MSCs in vivo is the relatively low numbers of MSCs that can be generated for clinical applications. In a recent phase II study of MSCs for treatment of steroid-resistant, severe acute GVHD, 55 patients were treated with allogeneic MSCs at a median dose of 1.4 $\times$ 10^6 cells (range, 0.4-9 $\times$ 10^6 cells) per infusion [17]. Some 89% of patients in this study received a total of only 1 or 2 MSC infusions. Even though the survival of patients who responded to MSCs in this study seemed higher (52% at 2 years) than described previously for patients with similarly severe acute GVHD, randomized controlled trials unequivocally proving the usefulness of MSCs for GVHD prevention or treatment have yet to be published. One recently

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completed phase III study of MSCs for treatment of steroid-refractory acute GVHD has thus far been presented only in abstract form and showed no benefit of this intervention with respect to the prospectively defined primary endpoint of a durable complete response for ≥28 days [19]. A subgroup analysis, however, suggested a possible therapeutic benefit for patients with liver and gut involvement.

Reports of the effectiveness of MSC infusion in treating or preventing GVHD have been inconsistent. We hypothesized that these inconsistencies could be attributed to several variables, including (1) suboptimal numbers of MSCs infused, (2) interdonor variations in the quality of the marrow harvest, or (3) varying ratios of MSC subpopulations, leading to functional differences in bulk cultures. To address this concern, we used immortalized clonal populations of canine MSCs to provide a consistent product for infusion. First, we demonstrated that their in vitro immunosuppressive potential was comparable to that of primary MSCs. Next, we tested the canine MSCs' ability to prevent GVHD and allograft rejection in a canine dog leukocyte antigen (DLA)-haploidentical hematopoietic cell transplantation (HCT) model. Our results indicate that even though clonal MSC could be produced with high efficiency and were infused frequently and in high numbers, they failed to either mitigate or prevent GVHD, or to decrease the likelihood of rejection.

MATERIALS AND METHODS

Immortalized Clonal Populations of Bone Marrow–Derived MSCs

Clonal MSC lines were generated from marrow derived from 1 donor dog according to procedures described previously for human MSC lines [20]. In brief, canine marrow-derived mononuclear cells were depleted of hematopoietic lineage cells by immune absorption after incubation with anti-CD45 antibodies. The remaining cells were cultured until confluent and then immortalized with a retrovirus containing the human papilloma virus E6/E7 genes. Infected cells were plated at low concentrations, and growing clones were isolated with cloning rings. Twenty cloned dog MSC lines, designated DS 1-20, were established. Five DS lines were subsequently analyzed in more detail for their ability to suppress the allogeneic mixed leukocyte culture (MLC) in vitro. DS1-3, the 3 lines with the strongest suppressive activity in vitro yet the greatest phenotypic differences between one another, were chosen for further in vivo experiments. The differences in mRNA profile, immunephenotype and cytokine phenotype among DS1-3 are shown in Figure 1, and Tables 1 and 2.

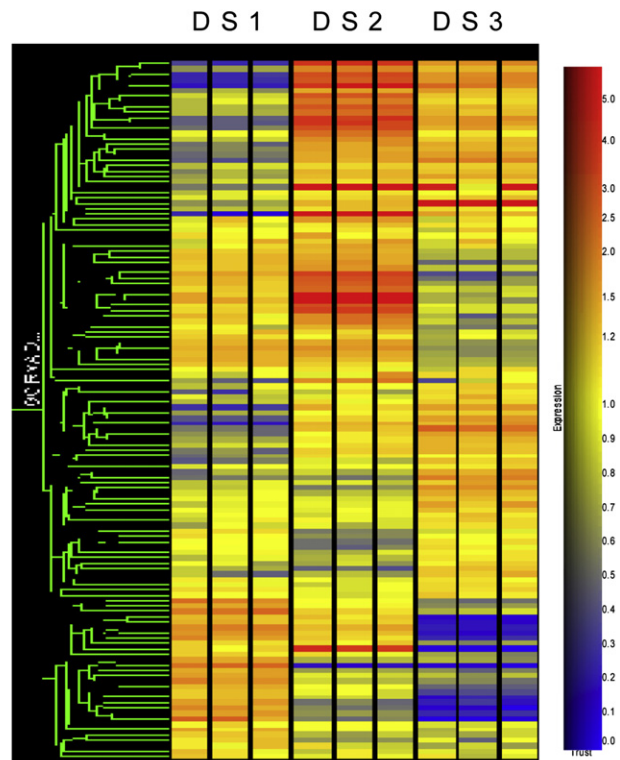

Figure 1. Clustering analysis of genes expressed by DS1-3. Three separate mRNA preparations were analyzed for each DS line. First, genes on the canine mRNA arrays (Affymetrix) with raw expression levels >100 were selected. Second, a group of 130 genes with functional annotations allowing them to be classified as "extracellular" by Gene Ontology were chosen and were found to include secreted factors, cell surface receptors, and extracellular matrix proteins. The clustering analysis by the Agilent GeneSpring GX7.3.1 program was based on average linkage, using the Pearson correlation. DS2 and DS3 differ from DS1 and from each other.

Transcriptome Comparison of DS Lines

MSC lines DS1-3 were grown to semiconfluency, and RNA was extracted [21]. Using canine mRNA arrays (Affymetrix, Santa Clara, CA), 3 separate mRNA preparations were processed for each line. Only those genes with absolute expression values >100 and adequate annotation to be classified as "extracellular," "chemokines/growth factors," or "receptors" by Gene Ontology (GO slims) were considered for further analysis. The clustering analysis by the Agilent GeneSpring GX7.3.1 program was based on average linkage, using the Pearson correlation [21].

Primary MSC Cultures (Long-Term Stromal Cultures)

Ex vivo culture conditions for primary canine MSCs were adapted from methods described by Le Blanc et al. [2] and Gartner and Kaplan [22]. In brief, buffy coat cells from marrow aspirates were plated in T-75 flasks (Costar, Cambridge, MA) at 1-2 × 10^6/mL. Adherent cells were grown in long-term marrow culture (LTC) medium containing Iscove's modified Dulbecco's medium, 12.5% horse serum, 12.5% fetal

Table 1. Phenotypic Characterization of Immortalized Dog Marrow Stromal Cell Lines (DS1-3) and Randomly Selected Primary Marrow Stromal Cultures (LTCs 1-5)

		MSC Type Tested							
Marker	Detection Method	DS1	DS2	DS3	LTC1	LTC2	LTC3	LTC4	LTC5
MHC class II	FC	-	-	-	ND	ND	ND	ND	ND
CD10	RT-PCR	+	+	+	+	+	++	+	+
CD13	RT-PCR	-	-	-	++	++	+++	-	+++
CD14	FC	-	-	-	ND	ND	ND	ND	ND
CD29	RT-PCR	+	+++	+	++	+++	+++	++	++
CD34	FC	-	-	-	ND	ND	ND	ND	ND
CD45	FC/ RT-PCR	-	-	-	+++	++	-	-	+
CD73	RT-PCR	++	+++	+++	++	++	++	+	++
CD90	FC/ RT-PCR	+++	+++	+++	++	++	++	-	-
CD105	RT-PCR	++	+	++	+++	+++	+++	-	-
CD106	RT-PCR	+	-	-	+	-	++	-	-

ND indicates not done.

Expression levels of informative stromal cell- and lineage-specific markers by marrow-derived stromal cell lines (DS1-3) and 5 randomly selected primary marrow stromal cultures (LTC 1-5) were assessed by semiquantitative RT-PCR and flow cytometry (FC) as described in Materials and Methods. Expression levels of markers of interest were estimated in relation to that of the housekeeping gene, *G3PDH*, and isotype-matched control antibodies for RT-PCR and FC, respectively. Expression levels: (+++), strong; (++) intermediate; (+) weak; (-) absent.

calf serum, L-glutamine (0.4 mg/mL), sodium pyruvate (1 mmol/L), penicillin (100 U/mL), streptomycin sulfate (100 pg/mL), hydrocortisone sodium succinate (10^{-6} mol/L), and P-mercaptoethanol (10^{-4} mol/L) and fed weekly by demi-depletion. Stromal layers were maintained at 37°C in an atmosphere of 5% CO_2. After reaching confluency, the adherent layers were trypsinized once and grown to confluency again in a T-225 flask to deplete hematopoietic cells before harvest and infusion. Experiments were performed with 3-week-old LTC after cells were confluent.

Phenotypic Characterization of Stromal Cell Cultures

Based on the availability of canine cell–specific monoclonal antibodies, expression levels of informative stromal and cell lineage–specific markers were assessed by flow cytometry or semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR). For RT-PCR, mRNA was extracted from stromal cell cultures and transcribed into cDNA using the µMACS One-Step cDNA Kit (Miltenyi Biotec, Auburn, CA). PCR reactions were performed using 1.1× PCR Master Mix plates (Thermo Scientific, Hudson, NH) containing Thermoprime Plus DNA Polymerase, 1.5 mM $MgCl_2$, and 75 mM Tris-HCl. Then 100 ng of cDNA template and 2.5 µM of primers (Supplemental Table 1) were added to each reaction. cDNA was amplified over 40 cycles at 94°C for 30 seconds, annealing at 58-62°C for 45-60 seconds, and extension at 72°C for 1 minute. Amplified products were separated on 2% agarose gels and visualized by ethidium bromide fluorescence for gene expression. Canine G3PDH was used as a control for input cDNA and was equivalent in all samples. Expression levels of markers determined by flow cytometry (Supplemental Table 1) were compared with those of isotype-matched control antibodies.

Canine Mixed Lymphocyte Cultures and Mitogen Stimulation

For this, 10^5 responder peripheral blood mononuclear cells (PBMCs) were cultured with 10^5 irradiated (24 Gy), allogeneic, DLA-mismatched stimulator PBMCs at a concentration of 10^6/mL for 6 days according to established methods [23]. In some experiments, 10^5 PBMCs were stimulated with concanavalin A (conA) at a concentration of 20 µg/mL for 72 hours. Defined numbers (3-50 × 10^3) of irradiated (24 Gy)

Table 2. Constitutive Production of Selected Cytokines by Immortalized Dog Marrow Stromal Cell Lines (DS1-3) and a Primary Marrow Stromal Culture (LTCs)

	GM-CSF	IL-4	IL-6	IL-8	IL-10	MCP-1
DS-1	ND	47.6	95.7	7857	11.8	787.7
DS-2	ND	55.6	72.2	6002	10.4	2168
DS-3	ND	47.6	116.3	ND	11.5	27.4
LTC	ND	11.0	87.5	ND	10.3	311.6
Culture medium	16.8	17.1	ND	166.1	5.0	20.2

ND indicates not detectable.

Concentrations of cytokines in 5-day CM from immortalized canine marrow stromal cell lines DS1-3 and primary marrow stromal cultures (LTCs) were determined using the Lincoplex system (Millipore, Billerica, MA). Concentrations are given in pg/mL. Culture medium incubated without stromal cells (bottom row) served as a control.

clonal MSCs or primary marrow stromal cells were added to MLC or conA stimulation cultures at the beginning of culture. Cells were labeled with 1 μCi/well of ^{3}H-thymidine for the final 16 hours of culture, harvested onto glass fiber filters, and counted for isotope incorporation.

To determine whether MSC-conditioned medium (CM) was suppressive in MLC, CM was produced from MSC lines (5-day culture), concentrated 10-fold, and then added to allogeneic MLC at a 1:9 ratio with fresh medium. To further assess whether cell contact was required for MSC-mediated suppression of T cell proliferation, irradiated MSCs were cocultured with responder and irradiated stimulator PBMCs in allogeneic MLC established on 24-well plates. In these experiments, MSCs were either cultured in direct contact with responders/stimulators or separated by a 0.3-μm porous membrane on Transwell inserts (Corning, Lowell, MA).

Hematopoietic Cell Transplantation

Dogs

Litters of random-bred dogs were either raised at the Fred Hutchinson Cancer Research Center (FHCRC) or purchased from commercial Class A vendors licensed by the US Department of Agriculture. The dogs weighed 7.4-14.4 kg (median, 11.4 kg) and were age 7-56 months (median, 9 months). All dogs were enrolled in a veterinary preventive medicine program that included routine antihelminthic agents and a standard immunization series [24]. The study was approved by the Institutional Animal Care and Use Committee of FHCRC, which has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Littermate donor–recipient pairs were selected on the basis of complete family studies showing haploidentity for highly polymorphic MHC-associated class I and II microsatellite markers [25] and for DLA-DRB1 alleles determined by direct sequencing [26].

Transplantation regimen

On day 0, donor marrow was harvested and infused intravenously into allogeneic recipients after a single 9.2-Gy dose of total body irradiation (TBI) delivered at 7 cGy/min (Varian Clinac 4; Varian Medical Systems, Palo Alto, CA). The median number of nucleated marrow cells infused was 3.8×10^8 cells/kg (range, $2.1\text{-}9.7 \times 10^8$ cells/kg) (Table 2). Recipients were not given pharmacologic immunosuppression for prevention of GVHD after transplantation.

MSC preparation and infusion

MSC lines DS1-3 and primary LTC-derived MSCs were expanded to at least semiconfluency ex vivo using T250 culture flasks (Corning, Kennebunk, MN). In an adaptation of previously described methods for culture of human MSCs [20,27], 10% heat-inactivated fetal calf serum was substituted for pooled dog serum. MSCs were then trypsinized, harvested, washed twice, and suspended in 0.9% NaCl before infusion. The first MSC infusion was given within 30 minutes after marrow graft infusion. Three different MSC regimens were used (Table 2):

(1) Seven recipients; DS1/DS2/DS3 in a 1:1:1 ratio; total dose per infusion, 30×10^6/kg recipient weight; given 3 days/week during the first week post-HCT and on 2 days/week starting the second week post-HCT.
(2) Five recipients; DS1/DS2/DS3 in a 1:1:1 ratio; total dose per infusion, 15×10^6/kg recipient weight; given 5 days/week.
(3) Three recipients; primary marrow stromal cells pooled from 7 donors; total dose per infusion, 1×10^6/kg recipient weight; given 3 days/week.

Supportive care and study termination

Prophylaxis with oral antibiotics was given from the day of TBI up to the end of the study. Broader antibiotic coverage was administered when neutrophil counts declined to $<0.5 \times 10^3$/μL or fever developed. Intravenous fluids were administered when dehydration occurred. Irradiated blood transfusions were given either when platelet count declined to $<5 \times 10^3$/μL or when petechiae and ecchymoses of the skin and mucous membranes were observed. Clinical signs of acute GVHD included diarrhea, skin erythema, and elevated liver enzymes [28]. Dogs were euthanized if they were in poor clinical condition, and complete necropsies were performed, which allowed histopathological distinction between GVHD and regimen-related toxicities.

Statistical considerations

This study was designed to use the fewest dogs necessary to have adequate power to detect differences deemed clinically meaningful in comparison with our historical results [29]. Based on 11 dogs given haploidentical grafts and no pharmacologic immunosuppression after transplantation, the median survival was 15 days, and long-term survival (>100 days) did not occur. It was predetermined that a group size of 7 dogs treated with MSCs provided 80% power to detect an increase in median survival to 37 days, at a one-sided .05 level of significance. Seven dogs also provided 80% power to detect an increase in the proportion of long-term survivors from 5% to 38%, based on an exact binomial test at a 1-sided .05 level of significance.

Engraftment and Chimerism

Hematopoietic engraftment was assessed by increases in granulocyte and platelet counts following postirradiation nadirs, marrow histology from autopsy specimens, documentation of donor-type hematopoiesis in peripheral blood and marrow by variable number tandem repeat (VNTR) polymorphisms [30,31], and clinical and histological evidence of GVHD.

In Vivo Distribution of ^{111}In-Labeled DS1 Cells

DS1 cells were labeled in vitro with ^{111}In (30 Bq/cell) [32] before injection. Labeled cells were infused into one recipient (G-943) immediately after administration of 8 Gy of TBI (delivered at 7 cGy/min as a single fraction). Single-photon emission computed tomography images were obtained immediately after injection and at 1, 2, 4, and 9 days after injection.

Detection of DS Cells In Vivo

MSC lines DS 1-3 were generated from one donor dog (DLA-DRB1 3/6), which allowed their detection in blood and tissue samples by VNTR-based polymorphism analysis [30,31]. For this purpose, DNA was extracted from blood and tissue preparations. To detect viable MSCs, blood or tissue obtained from recipients at different time points after MSC infusion were cultured in RPMI medium supplemented with 10% heat-inactivated dog serum, which allowed for outgrowth of the immortalized cells. After 72 hours of initial culture, nonadherent cells and debris were rinsed off, and adherent cells were expanded for 7-14 days. Adherent layers were then trypsinized before DNA extraction and chimerism analysis.

RESULTS

Transcriptome Comparison of DS Lines

In DS lines 1-3, 130 genes with absolute expression values >100 and adequate annotation to be classified as "extracellular," "chemokines/growth factors," or "receptors" by Gene Ontology (GO slims) were considered for analysis. The clustering analysis of the relative expression of these 130 selected genes shows that the 3 DS lines differ significantly from one another in terms of their mRNA expression profiles (Figure 1). The complete dataset showing transcriptomes for these lines using the Affymetrix microarray platform can be accessed at http://webapps.fhcrc.org/labs/graf/grantdata.html.

Phenotypic Characterization of DS Lines and Primary Marrow Stromal Cultures

Although primary marrow stromal cultures (LTCs) showed variable expression of hematopoietic lineage markers CD13, CD14, and CD45, these markers were undetectable in DS lines 1-3 (Table 1). In contrast, markers widely associated with cultured human MSCs [33,34], including CD29 (integrin β1-chain), CD73 (ecto-5′-nucleotidase), and CD90 (Thy-1), were consistently expressed by DS 1-3 and by most LTCs. Moreover, CD105 (endoglin) was variably expressed by the different LTCs tested and consistently expressed by all 3 DS lines. The 3 DS lines did not express DLA class II or CD34.

Constitutive Production of Cytokines by DS Cells and Primary Marrow Stromal Cells

The 3 DS lines were assayed for cytokine production using the Lincoplex system (Millipore, Billerica, MA). All 3 lines expressed low but consistent levels of interleukin (IL)-4, IL-6, and IL-10 and did not secrete detectable levels of granulocyte macrophage colony-stimulating factor, interferon γ, CXCL1, IL-2, IL-15, IL-18, or tumor necrosis factor α (Table 2). DS1 and DS2 expressed high levels of IL-8, whereas DS3 produced undetectable levels of IL-8. DS1 and DS2 showed a 3-fold difference in monocyte chemotactic protein 1 (MCP-1) production. Thus, in terms of production of this limited panel of cytokines, DS1 and DS2 were more similar, differing considerably from DS3. The 12 cytokines assayed were also represented on the Affymetrix microarrays; the constitutive expression levels of two of these, IL-8 and MCP-1, differed in DS3 compared with the other 2 lines. Thus, the transcriptome analysis lends weight to the cytokine results and supports the concept of functional heterogeneity among the generated clonal MSC lines. Except for nondetectable levels of IL-8, the cytokine profile of primary marrow stromal cells (LTCs) was similar to that of DS1-3.

Suppression of Alloantigen-Driven T Cell Proliferation by DS Cells

DS1, DS2, or DS3 cells were added at the initiation of allogeneic MLCs at a 1:2 DS cell/responder cell ratio (Figure 2A). The results indicate that the 3 DS lines had variable inhibitory effects on T cell proliferation, with DS3 resulting in >80% suppression of MLCs and DS1 or DS2 resulting in 50%-70% suppression of MSCs.

MSC-CM was then tested for its ability to suppress T cell proliferation in allogeneic MLCs. For this purpose, 5-day CM was produced and concentrated 10-fold before being added to MLCs at the initiation of culture (final MSC-CM concentration, 1×). As shown in Figure 2B, MSC-CM was only minimally effective in suppressing the allogeneic reaction, with DS3-CM having the strongest impact (~25% suppression). CM produced by coculture of the 3 DS lines was equally ineffective in suppressing the proliferative response as CM derived from culture of individual DS lines (Figure 2B).

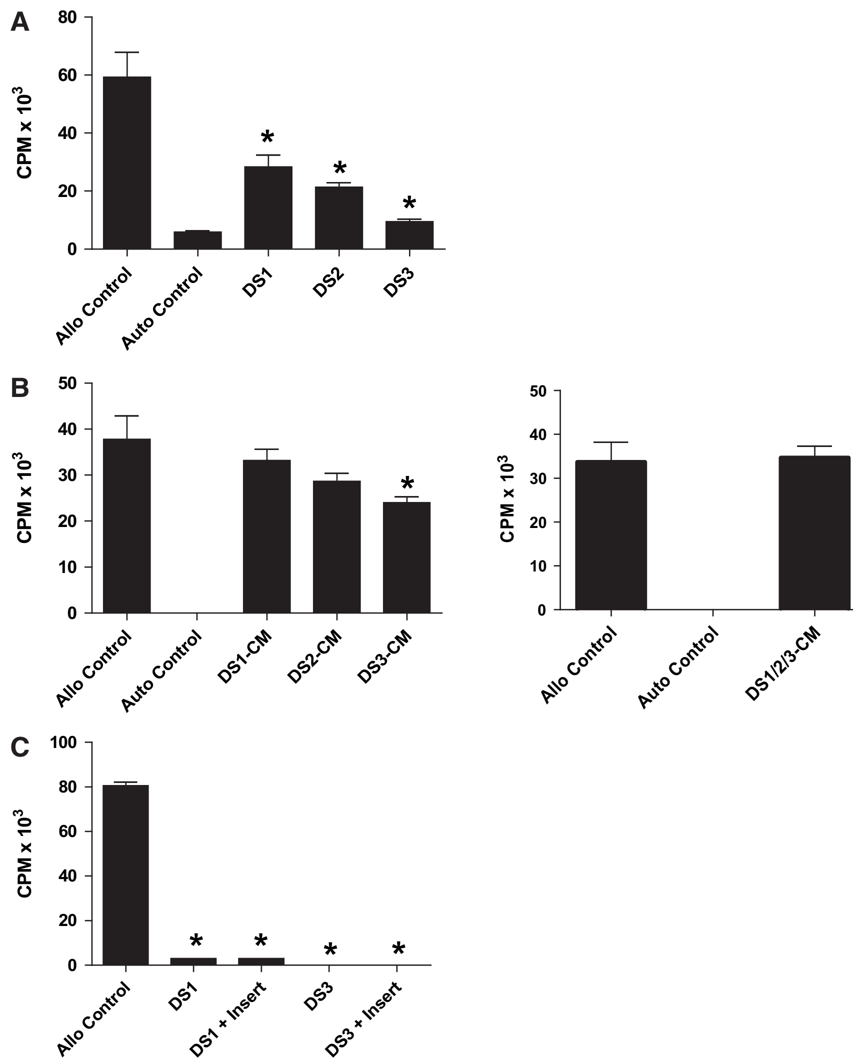

Figure 2. Inhibition of T cell proliferation in canine allogeneic MLC by different marrow stromal cell lines (DS1-3) under contact and noncontact conditions, and by stromal cell–conditioned medium. (A) Standard MLC was set up using PBMCs from unrelated DLA-mismatched dog pairs. Different MSC lines, designated DS1-DS3, were irradiated and added at the initiation of MLC at a 2:1 responder/MSC ratio. All 3 DS lines tested had suppressive effects on T cell proliferation. (B) Five-day DS cell CM was produced, concentrated 10-fold, and then added to allogeneic MLC in a 1:9-ratio with fresh medium. The left panel shows results for CM from individual DS lines. The right panel shows results for CM produced by coculture of the 3 DS lines (DS1/2/3 CM). (C) DS1 or DS3 cells were irradiated and cocultured with responder and irradiated stimulator PBMCs in allogeneic MLC established on 24-well plates. DS cells were either cultured in direct contact with responders/stimulators or separated by a 0.3-µm porous membrane on Transwell inserts (+ insert). "Allo Control" refers to allogeneic MLC without addition of DS cells; "Auto Control" refers to MLC without addition of DS cells using autologous responder and stimulator cells. One of 3 representative experiments is shown. Results for each experimental condition represent the mean ± standard error of the mean of 6 replicate wells. An asterisk indicates a statistically significant decrease in proliferative activity compared with Allo Controls ($P < .05$, 2-tailed, Mann-Whitney U test).

Suppression of T Cell Proliferation by DS Cells Is Contact-Independent

To determine whether the relative lack of suppression of T cell proliferation with MSC-CM was due to the lack of cell contact, Transwell experiments were conducted in which DS1 or DS3 cells were set up in the upper chamber and separated by a porous 0.3-µm membrane from the MLCs in the lower chamber (Figure 2C). DS cells separated from responder and stimulator cells were as effective in suppressing the allogeneic MLCs as were DS cells added directly to responder and stimulator cells.

DS Cells Suppress Alloantigen- and Mitogen-Stimulated T Cell Proliferation in a Dose-Dependent Fashion

DS1, DS2, and DS3 cells, used singly or in combination (DS1/DS2/DS3 in a 1:1:1 ratio; DS-Mix), were added to allogeneic MLCs (Figure 3A) or mitogen-stimulated PBMCs (Figure 3B) in graded numbers. The inhibition of T cell proliferation by DS cells was cell dose–dependent, with DS3 cells and the DS-Mix having the strongest suppressive effects. Allogeneic MLC performed with titrated numbers of DS1/DS2/DS3 (DS-Mix) in the presence of the prostaglandin

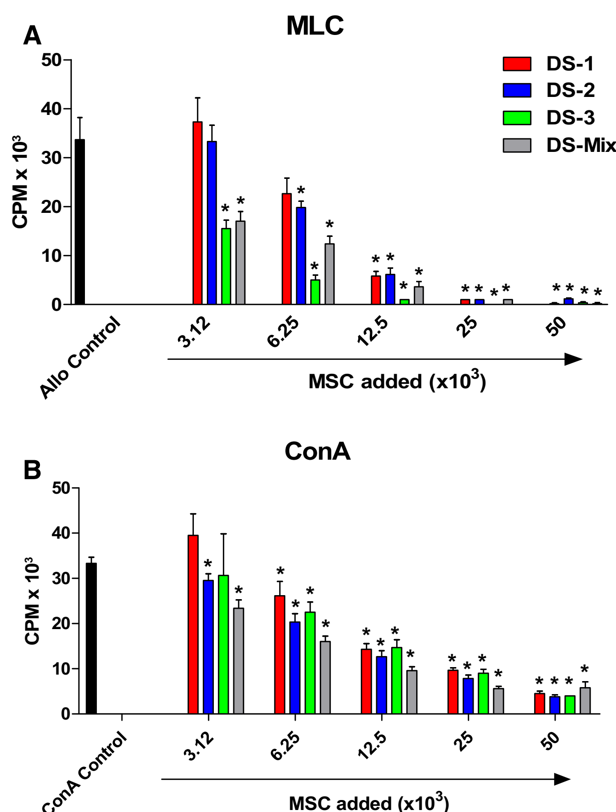

Figure 3. Canine MSC lines DS1, DS2, and DS3 inhibit alloantigen- and mitogen-induced T cell proliferation in a dose-dependent fashion. Different numbers (3.12-50.0 $\times$ 10^3) of DS1, DS2, and DS3 cells, used singly or in combination (DS-Mix), were irradiated and added to 10^5 canine PBMCs that were stimulated with 10^5 irradiated allogeneic stimulator PBMCs in allogeneic MLC or Con A. Shown are 1 of 5 representative experiments using individual DS lines, and 1 of 2 representative experiments using the DS-Mix. Results for each experimental condition represent the mean $\pm$ standard error of the mean of 6 replicate wells. An asterisk indicates a statistically significant decrease in proliferative activity compared with controls cultured without DS cells (P <.05, 2-tailed, Mann-Whitney U test).

synthesis inhibitor indomethacin (20 μM) reversed DS cell–mediated suppression of T cell proliferation by 32%-56%, suggesting a partial role of prostaglandin E in DS cell–mediated suppression of T cell proliferation.

In Vivo Distribution of ^{111}In-Labeled MSCs

To better understand the in vivo distribution of immortalized marrow stromal cells, DS1 cells were labeled with ^{111}In and injected intravenously into a beagle dog. The distribution of the label was then monitored over time using a gamma camera. Based on the assumption that the detected radioactivity was associated with DS1 cells, we found that DS1 cells accumulated in the lung immediately after infusion (day 0), but then left the lung within 24 hours and preferentially redistributed to liver and spleen; weaker signals were detectable in bone marrow and gut (Figure 4). Residual label in liver and spleen could be detected as long as 9 days after infusion.

Infusions of DS Cells or Primary Marrow Stromal Cells after DLA-Haploidentical Marrow Transplantation Do Not Prevent GVHD or Graft Rejection

The dogs given DLA-haploidentical marrow grafts without pharmacologic postgrafting immunosuppression (n = 11) had a median survival of 15 days (range, 13-25 days) (Table 3) [29]. Seven recipients (64%) rejected their grafts, and 4 (36%) engrafted with ensuing lethal GVHD. Based on this historical experience, we examined whether postgrafting MSC infusions, by virtue of their putative immunosuppressive effects, might increase rates of engraftment and mitigate GVHD after engraftment. Thus, MSCs were infused after transplant according to the 3 regimens detailed in Materials and Methods (Table 3). Three dogs (dogs 13-15) were given primary LTC-derived MSCs that had been pooled from 7 different marrow donors. Even though the doses of primary MSCs were substantially lower than those of DS1-3 cells administered (3 $\times$ 10^6/kg/week vs 60-90 $\times$ 10^6/kg/week), they were consistent with MSC doses given in previous clinical GVHD treatment studies [17,18].

Compared with historical controls that underwent transplantation in accordance with similar supportive care standards, postgrafting MSC infusions did not

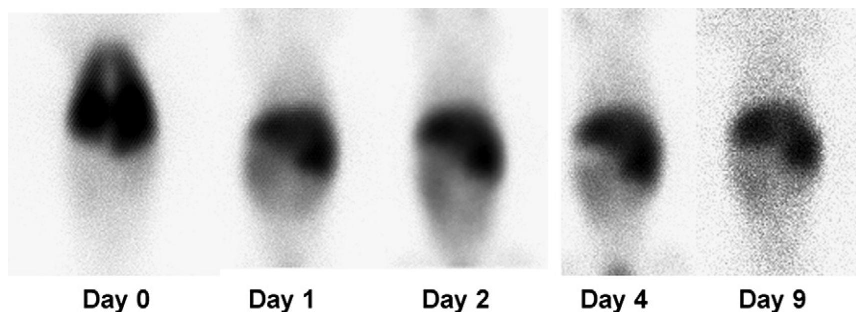

Figure 4. In vivo distribution of ^{111}In-labeled DS1 cells in a beagle dog (G-943). DS1 cells were labeled with ^{111}In in vitro and then injected into the dog immediately after 8 Gy TBI (delivered at 7 cGy/min as a single fraction). The dose of labeled and subsequently injected DS1 cells was 28 $\times$ 10^6/kg recipient weight. Single-photon emission computed tomography images were obtained immediately after injection and at 1, 2, 4, and 9 days after injection. Shown are anterior views in craniocaudal (top to bottom) orientation.

Table 3. Graft Composition and Outcome of DLA-Haploidentical Marrow HCT With and Without MSC Infusion Following 9.2 Gy TBI

				DLA-DRB1			Bone Marrow Cell Dose			MSC Treatment				Clinical and Histological GVHD				
Dog	Donor ID	Recipient ID	Recipient Weight, kg	Donor	Recipient	MSC	TNC ($\times 10^8$/kg)	CD34 ($\times 10^6$/kg)	CD3 ($\times 10^7$/kg)	MSC Type	Doses/ Week	Single Dose ($\times 10^6$ cells/kg)	Rejection	Skin	Liver	Gut	Survival, Days	Hematopoietic Chimerism at Necropsy
1	G-897	H-003	13.2	3/6	3/9	3/6	3	0.9	1.02	DS1/2/3‡	2-3	30	No	Yes	Yes		17	Donor
2	G-993	G-988	12.6	6/9	9/17	3/6	2.1	0.42	1.28	DS1/2/3	2-3	30	No	Yes	Yes	Yes	18	Donor
3	G-896	H-006	14.3	9/20	6/20	3/6	3.8	2.28	1.44	DS1/2/3	2-3	30	Yes				18	Host
4	H-030	H-029	13.4	3/22	20/22	3/6	5.5	1.1	2.53	DS1/2/3	2-3	30	No	Yes	Yes		18	Donor
5	H-098	H-097	9.1	2/15	2/17	3/6	3.6	0.36	1.22	DS1/2/3	2-3	30	Yes				18	Host
6	H-095	H-096	10.6	2/3	2/15	3/6	3.8	1.52	2.55	DS1/2/3	2-3	30	Yes				16	Host
7	H-092	H-093	7.4	3/9	9/15	3/6	7.1	4.26	4.47	DS1/2/3	2-3	30	NE				3	NE
8	H-101	H-100	11.5	9/17	9/15	3/6	6.8	5.44	3.67	DS1/2/3	5	15	Yes				18	Host
9	H-139	H-130	7.8	15/22	19/22	3/6	5.0	6.5	2.8	DS1/2/3	5	15	Yes				>280†	Host
10	H-053	H-048	9.2	20/22	3/22	3/6	9.7	5	0.97	DS1/2/3	5	15	No	Yes			14	Donor
11	G-550	H-098	10.4	2/9	2/15	3/6	8.6	19.8	2.84	DS1/2/3	5	15	No	Yes			13	Donor
12	G-550	H-099	14.5	2/9	2/15	3/6	2.3	1.16	2.07	DS1/2/3	5	15	No	Yes	Yes	Yes	18	Donor
13	H-178	H-176	11.4	14/15	6/14	*	3.7	2.22	2.52	Primary§	3	1	No	Yes	Yes		14	Donor
14	H-180	H-175	9.5	6/9	6/14	*	3.5	2.8	3.74	Primary	3	1	Yes				25	Host
15	H-180	H-179	11.5	6/9	6/14	*	5	5	4.9	Primary	3	1	Yes				24	Host
	Median		11.4				3.8	2.3	2.5								18	

Without MSC infusions: historical data [29]

Dogs Studied	Recipient Weight, kg	Bone Marrow Cell Dose, Median (Range) TNC ($\times 10^8$/kg)	CD34 ($\times 10^6$/kg)	CD3 ($\times 10^7$/kg)	Rejection, n (%)	GVHD, n (%)	Median Survival, Days (Range)
11	10.1 (6.5-15)	3.1 (1.0-4.4)	2.8 (2.0-4.9)	1.3 (1.2-2.9)	7 (64)	4 (36)	15 (13-25)

TNC indicates total nucleated cells; NE, not evaluable.

*The DLA-DRB1 types of the different primary MSC donors were as follows: 1/6, 3/9, 3/15, 6/9, 9/17, 9/27, and 15/22.

†Dog rejected and, after a long duration of profound pancytopenia, survived with autologous hematopoietic recovery.

‡DS cell lines 1-3 were always infused at a 1:1:1 ratio.

§Primary MSC products in all 3 treated dogs were generated from 7 different donors as described in Materials and Methods, pooled, and infused at approximately equal ratios.

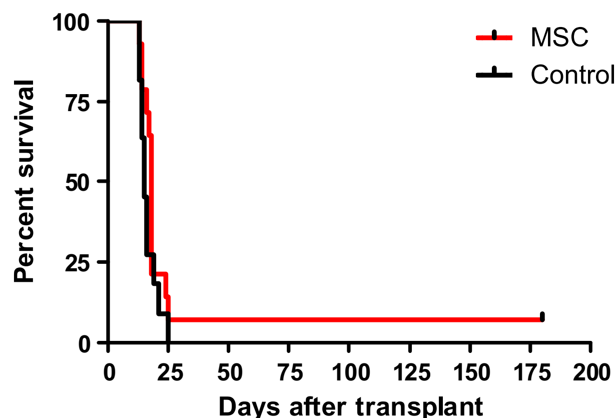

Figure 5. Survival of dogs prepared with 9.2 Gy TBI followed by bone marrow transplantation from a DLA-haploidentical donor. No pharmacologic immunosuppression was administered after transplantation. MSC: MSC infusions were given after transplantation (n = 15). Control: No MSC infusions were given after transplantation (n = 11) [29]. One dog given MSCs (H-130; see Table 2) rejected the graft and, after prolonged myelosuppression, survived with autologous hematopoiesis (absolute neutrophil and platelet counts exceeding $0.5 \times 10^3/\mu L$ and $20 \times 10^3/\mu L$ on days 40 and 56 after transplantation, respectively). Survival did not differ significantly between the 2 groups ($P = .18$, log-rank test).

significantly improve survival (15 days [range, 13-25 days] vs 18 days [range, 3≥280 days]), rates of rejection (64% vs 50%) and rates of GVHD (36% vs 50%) ($P = .69$, Fisher's exact test) (Table 3). One dog (H-093) died on day 3 posttransplantation from acute gastrointestinal toxicity related to the conditioning regimen and thus was not evaluable for rejection or GVHD. One dog treated with DS cell infusions (H-130) rejected the marrow graft and survived with autologous hematopoietic reconstitution. Even though the numbers were too small for meaningful intergroup comparisons, no MSC regimen appeared to be effective in preventing the two major endpoints of this clinical study, rejection and GVHD. Whereas 3 of 12 recipients of DS cell infusions received DLA-haploidentical MSCs (Table 3), 9 recipients received fully mismatched MSCs. There was no indication that DLA compatibility between the MSC product and the recipient had an effect on rates of rejection and GVHD. The Kaplan-Meier survival curves for MSC-treated recipients and historical controls are shown in Figure 5.

Detection of MSCs in Blood and Tissues

Of the 12 recipients given combined infusions of DS1-3 cells, 7 recipients were analyzed for in vivo detection of MSCs after infusion (Table 4). Immediately after intravenous infusion, DS cells were detected in the blood by PCR in only 1 of 7 recipients evaluated. At 24, 48, and 72 hours after infusion, PCR products specific for DS cells could be detected in 3 of 6, 1 of 4, and 1 of 4 recipients evaluated, respectively. However, after 7-14 days, DS signals could be detected in the blood of all 7 recipients. Tissue samples obtained from 6 recipients at necropsy were also subjected to PCR analysis (Table 4). Without previous ex vivo expansion of stromal elements, DS cell–specific signals were not detected in any of the samples tested; however, after ex vivo expansion, DS cell–specific signals were found in the bone marrow, lung, and spleen in 2 of 6 dogs evaluated. None of the dogs infused with DS cells showed clinical or histopathological evidence of tumor formation even though cells were immortalized and clonal in nature.

DISCUSSION

By administering third-party bone marrow–derived MSCs in large numbers and at high frequency after DLA-haploidentical bone marrow transplantation, we hoped to prevent or at least mitigate acute GVHD. In addition, by giving bone marrow that was not supplemented with a T cell–rich buffy coat and by not using pharmacologic immunosuppression after transplantation, this well-established preclinical model allowed us to use graft rejection as an additional readout for MSC-mediated immunosuppression in vivo.

The majority of recipients were given a combination of 3 different marrow stromal cell lines (derived from one donor dog) that shared phenotypical features associated with human MSCs [33,34] and had the ability to suppress allogeneic T cell proliferation in vitro. In fact, their T cell suppressive potential in vitro was similar to or greater than that observed with primary LTC-derived MSCs [12]. Analogous to what has been reported previously with primary MSCs in vitro, T cell suppression by DS cells was unrestricted by antigens encoded by the MHC and was contact-independent. Even though DS cells suppressed the T cell proliferative response to alloantigen in a contact-independent manner, DS cell–conditioned medium was only minimally suppressive. This finding suggests that soluble factors released during the allogeneic immune response might induce MSCs to release a soluble mediator that interferes with T cell function. Several soluble factors (including transforming growth factor β, hepatocyte growth factor, and prostaglandin E2, among others) have been proposed to mediate the suppressive effects of human MSCs [35-37] and canine MSCs [12] in vitro. Our findings also suggest that prostaglandins are involved in DS-mediated immunosuppression in vitro.

Although all 3 DS lines used in our study suppressed alloantigen- and mitogen-induced T cell proliferation in vitro, they differed from one another with respect to their immune phenotype, mRNA expression profile, and repertoire of secreted cytokines. The use of MSC products composed of 3 phenotypically different MSC lines isolated from the same LTC was done

Table 4. Detection of Third-Party DS Cells in Blood and Tissues

Dog	Recipient ID	Blood or Tissue Enriched for MSC before Detection by VNTR	MSC Detection									Necropsy		
			In Blood, Hours after Infusion				In Tissue at Necropsy							
			0	24	48	72	BM	Lung	Liver	Spleen	LN	Day after HCT	BM Cellularity, %	Chimerism
1	H-003	No	Yes	No			No	No	No	No	No	17	50	Donor
		Yes	Yes	Yes			Yes	Yes	ng	No	No			
2	G-988	No	No				No	No	No	No	No	18	50	Donor
		Yes	Yes				No	ng	ng	ng	No			
3	H-006	No	No	No	No		No	No	No	No	No	18	5	Host
		Yes	Yes	Yes	Yes		Yes	Yes	ng	Y es	ng			
4	H-029	No	No	No		No	No	No	No	No	No	18	80	Donor
		Yes	Yes	Yes		Yes	No	No	No	No	No			
5	H-097	No	No	No	No	No	No	No	No	No	No	18	0	Host
		Yes	Yes	No	No	No	ng	ng	ng	ng	ng			
6	H-096	No	No	No	No	No	No	No	No	No	No	16	0	Host
		Yes	Yes	No	No	No	No	No	No	No	No			
7	H-130	No	No	No	No	No	NA	NA	NA	NA	NA	Alive	NA	Host
		Yes	Yes	No	No	No								

BM indicates bone marrow; LN, lymph node; ng, no growth; NA, not applicable.
MSC lines DS1-3 were generated from one donor dog, which allowed their detection in blood and tissue samples by VNTR-based polymorphism analysis. For this purpose, DNA was extracted from unmodified or MSC-enriched white blood cell (WBC) and tissue preparations. To enrich for viable MSCs, WBCs obtained from recipients at different time points after MSC infusion, or minced tissue samples obtained at necropsy were cultured in RPMI medium supplemented with 10% heat-inactivated dog serum on 12-well plates. After 72 hours of initial culture, nonadherent cells and debris were rinsed off, and adherent cells were expanded for 7-14 days. Adherent layers were then trypsinized before DNA extraction and subjected to chimerism analysis as described in Materials and Methods. In cases where DS cells were detectable by chimerism analysis after ex vivo expansion of adherent stromal cells ("Yes"), DS cell–specific signals consistently exceeded 80%.

to provide an unlimited amount of a consistent cell product for experimental use that would mimic the heterogeneous composition of primary MSCs.

Our results in this established canine model show that compared with historical controls (n = 11) given 9.2-Gy TBI and DLA-haploidentical marrow grafts without MSC infusions after transplantation, recipients of MSCs (n = 15) experienced similar rates of marrow graft rejection. Moreover, all 7 MSC recipients that had donor marrow engraftment (50%) developed vicious and rapidly fatal acute GVHD, identical to the experience with historical controls. As a result, survival curves for MSC-treated dogs and controls were virtually identical. Even though a meaningful subgroup analysis was limited by small numbers, no MSC regimen appeared to be effective in preventing the two major endpoints of this preclinical study, rejection and GVHD. Primary MSCs also were ineffective in preventing or treating GVHD and rejection. This finding is consistent with a recent study by Lee et al. [12] showing that donor-derived primary MSCs did not prevent marrow graft rejection after DLA-identical HCT. There was also no indication in our study that the degree of DLA compatibility between MSC products and recipients affected the rates of rejection and GVHD. Finally, it is important to emphasize that because no therapeutic benefits associated with MSC infusions were found, a significant bias related to the use of historical controls appears to be unlikely.

Additional analysis showed that ^{111}In-labeled DS1 cells accumulated in the lungs immediately after intravenous infusion and within 24 hours redistributed to the liver, spleen, and bone marrow. Thus, the in vivo distribution of DS1 cells was similar to that described for primary marrow–derived MSCs studied in other animal models [38,39]. In addition, the in vivo distribution of DS1 cells in this irradiated dog was not different from that in 2 nonirradiated dogs studied previously (data not shown). A limitation of this analysis, however, is the fact that the distribution of the ^{111}In label might not have been indicative of viable cells because detection of ^{111}In signals in parenchymal organs could be due to retention of free ^{111}In label by macrophages. Thus, we sought to detect viable DS cells in various organs at the time of necropsy. For this purpose, stromal cells were expanded from organ samples before subjecting them to DS cell–specific PCR analyses. Even though the expansion of stromal cells from organ samples was not uniformly successful, DS cells could be expanded from the lungs, spleen, and marrow of 2 dogs. These limited data suggest that after high-dose irradiation of recipients, allogeneic DLA-mismatched DS cells could migrate to and survive in parenchymal organs for several days after infusion. Given that detection of DS cells was virtually impossible in organ samples and PBMCs without ex vivo expansion of stromal cells, the relative frequency of circulating and tissue-based DS cells after infusion appeared to be low.

In conclusion, our results obtained in the clinically relevant canine model of allogeneic HCT indicate that marrow-derived clonal and primary MSCs, although

strongly immunosuppressive in vitro, have no identifiable immunosuppressive activity in vivo. MSCs were administered frequently and in doses typically undeliverable in clinical transplantation; however, the MSC regimens used in this study failed to prevent graft rejection and GVHD, the two major immunologic barriers of allogeneic transplantation. It is possible that the DS lines used in our study were not representative of primary MSCs used in and associated with successful treatment of GVHD in clinical studies [17]. However, outcomes with primary MSCs were not different from those with DS cells. Due to the rigorous nature of our model, it is also possible that the addition of MSCs to a regimen of pharmacologic immunosuppression might have been more successful. At the very least, these data emphasize a disconnect between in vitro and in vivo data. Given that the medical literature tends to favor positive study results, our study should serve as a counterpoint emphasizing that the in vivo immunosuppressive effects of MSC have not yet been defined unequivocally.

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AUTHOR CONTRIBUTIONS

Marco Mielcarek designed the study, analyzed and interpreted the data, and wrote and finally approved the manuscript. Ludmila Golubev and Alla Nikitine expanded MSCs ex vivo and performed all in vitro assays. Billanna Hwang performed phenotypical MSC analysis. Beverly Torok-Storb and Rainer Storb contributed to study design and data interpretation and edited the manuscript. George E. Georges and Richard A. Nash assisted with data interpretation and edited the manuscript.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbmt.2010.08.015.

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