Mesenchymal stromal cells transiently alter the inflammatory milieu post-transplant to delay graft-*versus*-host disease

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ABSTRACT

Background
Multipotent mesenchymal stromal cells suppress T-cell function *in vitro,* a property that has underpinned their use in treating clinical steroid-refractory graft-*versus*-host disease after allogeneic hematopoietic stem cell transplantation. However the potential of mesenchymal stromal cells to resolve graft-*versus*-host disease is confounded by a paucity of pre-clinical data delineating their immunomodulatory effects *in vivo*.

Design and Methods
We examined the influence of timing and dose of donor-derived mesenchymal stromal cells on the kinetics of graft-*versus*-host disease in two murine models of graft-*versus*-host disease (major histocompatibility complex-mismatched: UBI-GFP/BL6 [H-2^b]→BALB/c [H-2^d] and the sibling transplant mimic, UBI-GFP/BL6 [H-2^b]→BALB.B [H-2^b]) using clinically relevant conditioning regimens. We also examined the effect of mesenchymal stromal cell infusion on bone marrow and spleen cellular composition and cytokine secretion in transplant recipients.

Results
Despite T-cell suppression *in vitro*, mesenchymal stromal cells delayed but did not prevent graft-*versus*-host disease in the major histocompatibility complex-mismatched model. In the sibling transplant model, however, 30% of mesenchymal stromal cell-treated mice did not develop graft-*versus*-host disease. The timing of administration and dose of the mesenchymal stromal cells influenced their effectiveness in attenuating graft-*versus*-host disease, such that a low dose of mesenchymal stromal cells administered early was more effective than a high dose of mesenchymal stromal cells given late. Compared to control-treated mice, mesenchymal stromal cell-treated mice had significant reductions in serum and splenic interferon-γ, an important mediator of graft-*versus*-host disease.

Conclusions
Mesenchymal stromal cells appear to delay death from graft-*versus*-host disease by transiently altering the inflammatory milieu and reducing levels of interferon-γ. Our data suggest that both the timing of infusion and the dose of mesenchymal stromal cells likely influence these cells' effectiveness in attenuating graft-*versus*-host disease.

Key words: stem cell transplantation, graft-*versus*-host disease, mesenchymal stromal cells, IFNγ.

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The online version of this article has a Supplementary Appendix.

Introduction

Graft-*versus*-host disease (GVHD) remains an unavoidable complication of allogeneic hematopoietic stem cell transplantation (HSCT). Current treatment options for GVHD focus mainly on donor T cells. These therapies can result in systemic immunosuppression, rendering patients susceptible to infection, graft failure and relapse of the underlying hematologic malignancy. There is, therefore, a need to identify immunosuppressive therapies that will control GVHD and maintain anti-leukemic and anti-infectious immunity.

Multipotent, mesenchymal stromal cells (MSC) prevent T-cell proliferation *in vitro* and secrete a number of soluble factors that modulate the immune response, including transforming growth factor-β,[1] indoleamine, 2,3-dioxygenase[2] and nitric oxide.[3] Clinical exploitation of these cells has yielded mixed results. Several studies have reported striking resolution of steroid-refractory GVHD after MSC infusion,[4-7] particularly in patients with gut and liver involvement, while a recent phase III clinical trial by Osiris on the treatment of steroid-refractory GVHD reported an overall response to MSC (Prochymal™) that was not different from that to placebo overall (35% treated *versus* 30% in controls, n=260).[8] These reports highlight that while much enthusiasm has surrounded the effectiveness of MSC as a therapeutic for GVHD, further research is required to validate and optimize the use of these cells for anti-GVHD therapy.

One significant problem is that although the immunosuppressive nature of MSC has been delineated *in vitro*, little is known about the ability of these cells to modulate the immune response *in vivo*. Such studies are likely to help understand how MSC could be most effectively used to suppress immune responses that underlie GVHD while maintaining the anti-leukemic effect of HSCT. We, therefore, used murine models of GVHD to investigate the *in vivo* effects of MSC.

Design and Methods

Mice

Female BALB/c [H-2^d] and BALB.B [H-2^b] recipient mice; and C57BL/6 [H-2^b] donor mice[9] were used between 6-8 weeks of age and were obtained from the Animal Resources Center (WA, Australia). Breeding pairs of UBI-GFP/BL6 (H-2^b) mice (BL/6 mice transgenic for green fluorescent protein [GFP] under the control of the ubiquitin promoter) were obtained from Dr. David Curtis and Prof. Alex Bobik (Baker Institute, VIC, Australia). Mice were housed under specific pathogen-free conditions and allowed to acclimatize for 1 week prior to commencement of experimental work. All animal work was approved by the University of Queensland Animal Ethics Committee.

Mesenchymal stromal cell isolation, characterization and preparation

Mononuclear cells were obtained from crushed femur, tibia and hip bones of UBI-GFP/BL6 mice by collagenase digestion (3 mg/mL type I collagenase, Worthington Biosciences, NJ, USA) followed by density centrifugation (Lympholyte-M, Cedarlane, Canada). After two passages, contaminating cells were removed by depletion of CD45$^+$ and CD11b$^+$ cells by magnetic activated cell separation (MACS, Miltenyi Biotec). The remaining MSC expressed Sca-1, CD90 and CD44 and lacked expression of CD45, CD11b and CD31. Mesodermal differentiation assays confirmed the adipogenic, osteogenic and chondrogenic potential of the MSC. All MSC used for experiments were between passage 8-13 to provide sufficient numbers of cells for *in vivo* infusion. A typical doubling time of these MSC was 2.5 days. Passage number did not affect the characteristic properties of MSC, including the phenotype, immunosuppressive capacity or mesodermal differentiation of MSC (*data not shown*).

Pre-transplant conditioning regimen

Cyclophosphamide (Baxter Healthcare, Deefield, IL, USA) was injected into mice intraperitoneally at a dose of 60 mg/kg/day on day –3 and day –2 pre-transplant, followed by 1000 cGy (BALB/c) or 850 cGy (BALB.B) of total body irradiation (^{137}Cs source – Gammacell 40), administered in two doses separated by 3 h to minimize gut toxicity, 1 day prior to the transplant.[10]

Preparation and transplantation of donor cells and mesenchymal stromal cells

Donor bone marrow cells (1×10^7/mouse) were prepared by flushing femora and tibiae with media. Donor splenocytes (1×10^7/mouse) were prepared by passing spleens through a steel mesh. Cell suspensions were combined and injected intravenously into conditioned mice on day 0 (day of the transplant). Two allogeneic HSCT models were used: (i) the major histocompatibility complex (MHC)-mismatched model (UBI-GFP/BL6 [H-2^b] → BALB/c [H-2^d]), and (ii) MHC-matched, minor histocompatibility antigen (miHA) mismatched model (UBI-GFP/BL6 [H-2^b] → BALB.B [H-2^b]), the latter mimicking an HLA-identical sibling HSCT.[10] Prior to infusion, UBI-GFPBL/6 MSC were thawed and re-suspended in sterile medium supplemented with DNase (5 μg/mL) and heparin (50 U/mL). MSC were administered intraperitoneally with a 26 gauge needle. Control mice received medium alone. In some experiments cohorts of mice received conventional immunosuppression with either the murine monoclonal antibody, KT3 (0.1 mg/mouse on days +1, +3 and +5) which targets T cells, or daily injections of cyclosporine (50 mg/kg).[11]

Graft-versus-host disease monitoring

Conditioned and transplanted mice were monitored daily for the onset and severity of GVHD as previously described.[10] Briefly, mice were given a score from 0-2 (or 2.5 in the case of weight loss) for a series of clinical parameters indicative of GVHD including posture (hunching), activity, fur texture, skin and eye integrity and diarrhoea. Any animals that scored 2.0 for either activity or diarrhea, 2.5 for weight loss or achieved a cumulative score of 8.0 were considered to have severe GVHD. These mice were sacrificed and their organs harvested for analysis. Otherwise mice were monitored for up to 3 months after HSCT and scored in a blinded manner to eliminate bias. At the time of sacrifice we evaluated the effects of MSC on onset and severity of GVHD, donor chimerism, pro-inflammatory cytokine production, dendritic cells, macrophages, granulocytes, B cells, T cells including T-regulatory cells in the bone marrow and spleen, using the techniques described below. Hematoxylin- and eosin-stained sections of skin, liver, small and large intestine and lungs were assessed by a pathologist with no knowledge of treatment groups using a previously described scoring system.[12]

Flow cytometry

Cellular phenotype analysis was performed by flow cytometry using fluorescently labeled rat anti-mouse antibodies as follows (except where stated): MHC class II (I-A/I-E) phycoerythrin (PE) (M5/114.15.2, IgG2b), hamster anti-mouse CD11c allophycocyanin (APC) (HL3, IgG1), CD45R (B220) allophycocyanin-cya-

nine 7 dye (APC-Cy7) (RA3-6B2, IgG2a), Ly-6G and Ly-6C (Gr-1) APC-Cy7 (RB6-8C5, IgG2b), CD31 APC (MEC13.3, IgG2a), Sca-1 PE (D7, IgG2a), FoxP3 PE (NRRF030, IgG2a) and F4/80 APC (BM8, IgG2a). All antibodies were purchased from Becton-Dickinson (BD PharMingen, San Jose, CA, USA) or Ebioscience (in the case of FoxP3 and F4/80) as were appropriate isotype controls. Viability was assessed by 7-amino-actinomycin D incorporation (BD Viaprobe cell viability solution). Intracellular staining for the identification of regulatory T cells was performed using the Ebioscience (CA, USA) mouse FoxP3$^+$ regulatory T-cell staining kit according to the manufacturer's instructions. Donor engraftment was determined by positive expression of GFP. Fifty thousand events were collected using a BD LSRII (BD, San Jose, CA, USA) and analyzed using FlowJo (TreeStar, OR, USA). Cells subsets were defined as follows: immature dendritic cells = CD11c$^+$ MHCII$^-$; mature dendritic cells = CD11c$^+$MHCII$^+$; macrophages = CD11b$^+$,F4/80$^+$; granulocytes = CD11b$^+$,Gr-1$^+$; T cells = CD3$^+$, CD4$^+$ or CD8$^+$ and regulatory T cells = CD3$^+$, CD4$^+$, CD25$^+$, Fox P3$^+$; B cells = CD19$^+$.

Cytokine measurement

Extracellular fluids or serum were analyzed for T_H1/T_H2 cytokine release using the Cytokine Bead Array (CBA Th1/Th2 mouse, BD). Extracellular fluids was obtained by collecting 2 mL of supernatant from cell suspensions. Serum was obtained via cardiac puncture.

Mixed lymphocyte reaction

T-cell proliferation was measured by a mixed lymphocyte reaction (MLR) as previously described.[10] Responder T cells derived from C57BL/6 were purified using a pan T-cell isolation kit (Miltenyi Biotec, Gladbach, Germany). Stimulator cells (2×10^5 cells/well) and responder T cells (3×10^5 cells/well) were co-cultured (complete α-MEM-10) in a humidified 37°C, 5% CO_2 incubator. MSC (3000 per well) were plated, cultured overnight, and then irradiated (3000 cGy) prior to co-culture with responders and stimulators. Proliferation was assessed by [^{3}H]-thymidine incorporation (1 μCurie/well) (Amersham Biosciences, UK) after a total of 96 h of culture. Cells were harvested using the TOMTEC 96-well Mach III Harvester (Perkin-Elmer, Vic, Australia) and counts per minute (cpm) were measured on a 1450 MICROBETA TRILUX β-scintillation counter (Perkin-Elmer, Vic, Australia). [^{3}H]-thymidine was not added to some cultures so that the resultant T-cell phenotype could be analyzed by flow cytometry.

Real time polymerase chain reaction

GFP expression in the hind leg bones, large intestine, spleen and inguinal and mesenteric lymph nodes was identified by quantitative real time polymerase chain reaction (qRT-PCR). After harvest, organs were snap-frozen on dry ice, homogenized using a mortar and pestle and the DNA extracted using a QIAamp DNA Mini Kit (Qiagen) according to manufacturer's protocol. Aliquots of DNA (1–1.5 μg) were analyzed using fluorescence FAM/Sybr probes (5' primer; CTGCTGCCCGACAACCA, 3' primer; TGTGATCGCGCTTCTCGTT, probe: FAM-CCCAGTCCGCCCTGAGCAAAGAC-Sybr). To generate a standard curve, genomic DNA was isolated from known numbers of murine UBI-GFP/BL6 MSC. Engrafted cell numbers were calculated from the standard curve with the aid of the Rotorgene-6 software (Rotorgene, Corbett Research). The total number of nucleated cells was calculated using primers based on β2-microglobulin (5' primer; TCATTAGGGAGGAGCCAATG, FRET probe; CCTGCCACCTAGGGAATTGC, 3' primer; ATCCCCTTTCGTTTT-TGCTT).

Statistical analysis

Survival of transplant recipients, as a function of the MSC treatment, was analyzed using Kaplan-Meier estimation and stratified Cox regression analysis. Unpaired T tests or one way ANOVA were used to analyze potential differences in various parameters. All experiments comprised three distinct experiments unless otherwise stated. Results are expressed as mean ± SEM and were considered statistically significant if the *P* value was 0.05 or less. Statistical analysis was performed using Graphpad Prism 5.0 analysis software (CA).

Results

Mesenchymal stromal cells potently suppress T-cell proliferation and inflammatory cytokine production in vitro

The suppressive potential of MSC was confirmed *in vitro* prior to the commencement of *in vivo* studies. MSC, at a ratio of 1 MSC:100 T cells significantly suppressed T-cell proliferation ($P<0.001$, Figure 1A). This suppression was further enhanced if the number of MSC was increased such that the ratio of MSC:T cells was 1:10 ($P<0.0001$). MSC-mediated suppression of T-cell proliferation could not be enhanced further by pre-treatment of MSC with pro-inflammatory cytokines (tumor necrosis factor-α [TNFα] or interferon-γ [IFNγ])[13,14] (Figure 1B). Furthermore, these results were replicated in a third party MLR, in which the responders, stimulators and MSC were all mismatched at the MHC (*Online Supplementary Figure S1*). Together, these results demonstrate that MSC strongly suppress T-cell proliferation and mediate their suppressive effects in an MHC-independent manner.

To elucidate how MSC exert their immunosuppressive

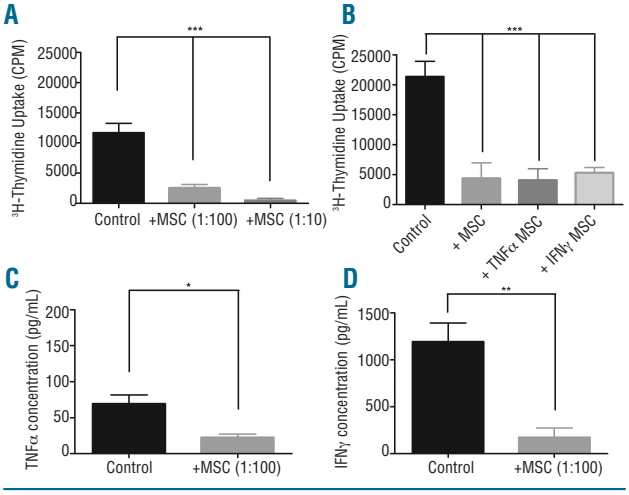

Figure 1. MSC suppress T-cell proliferation and inflammatory cytokines *in vitro*. Irradiated UBI-GFP/BL6 MSC were co-cultured in a mixed lymphocyte reaction with irradiated BALB/c (host) stimulators and UBI-GFP/BL6 purified T cells (donor). T-cell proliferation, measured by ^{3}H-thymidine incorporation, was significantly reduced in the presence of MSC (A, $P<0.001$, n=11), and was not further reduced by cytokine pre-treatment (B). The inflammatory cytokines TNFα (C) and IFNγ (D) were assessed in supernatants by cytokine bead array post MLR (n=3-4 independent experiments, IFNγ: $P<0.01$; TNFα: $P<0.05$). Data presented as mean ± SEM.

effects on T cells, the phenotype of the resultant cells at the end of the MLR (in the absence of ^{3}H-thymidine) was assessed by flow cytometry and supernatants assayed for the presence of T_H1/T_H2 cytokines (TNFα, IFNγ, interleukin [IL]-2, IL-4, and IL-5). Inflammatory cytokines such as TNFα and IFNγ are important mediators of the clinical manifestations of GVHD, due to their toxic effects on the gastrointestinal tract. In the presence of MSC, levels of TNFα in MLR supernatants in both donor-derived and third party MLR (*data not shown*) were significantly reduced. Donor-derived MSC reduced the presence of TNFα 3-fold; from a mean of 69.15±12.54 pg/mL in controls to 22.57±4.56 pg/mL in MSC-treated cultures (P<0.05, Figure 1C). IFNγ levels in the MLR culture were reduced 10-fold from 1193.16 ± 199.5 pg/mL in controls to 173.16±101.2 pg/mL in donor-derived MSC-treated cultures (P<0.01, Figure 1D). Secretion of T_H2 cytokines, IL-2, IL-4 and IL-5, was, however, minimal and not different between cultures with or without MSC (*data not shown*).

Despite a significant reduction in T-cell proliferation (control cpm: 11700±1536 *versus* MSC at a 1:100 ratio: 2547±616), the presence of MSC in the MLR did not alter the proportions of $CD4^+/CD8^+$ T cells or induce $FoxP3^+$ regulatory T cells (T-reg) (*data not shown*), suggesting that the mechanism of suppression by MSC was not due to induction of T-reg.

Donor-derived mesenchymal stromal cells delay but do not prevent graft-versus-host disease in vivo after major histocompatibility complex-mismatched hematopoietic stem cell transplantation

As MSC suppress T-cell proliferation *in vitro* significantly, we assessed their effect on GVHD severity after MHC-mismatched HSCT. Intraperitoneal injection of 4×10^5 donor-derived MSC 24 h post-transplant was sufficient to significantly delay death from GVHD (Figure 2A, P<0.0001). The mean day of death was postponed from 6.57±0.13 days (untreated controls, n=28) to 12±1.73 days (MSC-treated mice, n=18, P<0.001) and one MSC-treated transplant recipient survived for 35 days. Overall GVHD scores (Figure 2B) were not different between the untreated controls and mice treated with MSC. Scores at day +3 (Figure 2D) were not different; however, at day +6 post-transplant we observed a small but significant reduction in GVHD score: the mean clinical score for MSC-treated transplant recipients was 6.8±0.23 compared to 7.46±0.09 in untreated controls (Figure 2D, P<0.01).

MSC are reportedly beneficial for the resolution of clinical acute GVHD in the gut.[4] In our murine HSCT model, weight loss represents an important objective component of the overall GVHD score. Analysis of the weight loss percentage in the control and MSC-treated transplant recipients showed a similar pattern to that seen with the overall GVHD scores (Figure 2C). However no significant difference in weight loss was observed at day +3 (mean –16.53±0.46% in controls *versus* –16.38±0.60% in MSC-treated mice) or day +6 (mean -30.95±0.51% in controls *versus* –29.73±0.73% in MSC-treated mice, Figure 2E). This suggests that while MSC appear to exert their immunosuppressive effects early post-transplant, they do not have a protective effect on the gut, and consequently do not attenuate weight loss. Furthermore, these observations also suggest that in this transplant setting, MSC attenuate GVHD via a mechanism that does not directly heal damaged gut tissue.

Assessment of the cellular composition of bone marrow (Figure 2F) and spleen (Figure 2G) at the time of sacrifice did not reveal any significant differences in donor engraftment. Despite MSC-mediated *in vitro* suppression of T-cell proliferation, no differences in the percentage of donor or host T cells, or their subsets were observed in the spleen (or the bone marrow, *data not shown*) between MSC-treated and control mice (Figure 2H). There were also no differences in B cells, granulocytes or macrophages (*data not shown*). However MSC-treated mice had less mature host dendritic cells in the spleen (Figure 2I, P<0.05) when compared with controls, suggesting that MSC may exert their immunosuppressive effects via host dendritic cells.

Mesenchymal stromal cells alter the inflammatory milieu early after major histocompatibility complex-mismatched hematopoietic stem cell transplantation

Given the importance of dendritic cells in driving GVHD, we used time course experiments to directly compare differences in engraftment, cellular composition and cytokine secretion in the bone marrow, lymph nodes and spleen between controls or mice treated with MSC 24 h after HSCT. We found that the cellular composition in bone marrow and spleen remained unchanged between control mice and mice that received MSC. There were also no significant differences in engraftment levels in the bone marrow, spleen or lymph nodes, or in percentages of total T cells (Figure 3A and 3B), B cells or NK cells between mice administered MSC and controls at any of the time points assessed (*data not shown*). This was in stark contrast to our *in vitro* results, which showed significant suppression of T-cell proliferation. Despite the observation of decreased mature host dendritic cells at the time of sacrifice (Figure 2H), and studies that suggested that MSC alter dendritic cell maturation and function,[15-17] the percentages of total mature dendritic cells between control mice and mice treated with MSC post-transplant were comparable on both day +3 and day +6 (Figure 3C,D).

Similar to what we observed *in vitro*, there were no significant alterations in IL-2, IL-4, IL-5 (*data not shown*) or TNFα in any of the samples assessed from the different experimental cohorts (Figure 3E,F). When recipient mice were sacrificed on day 0, limited IFNγ secretion was observed in both the serum and spleen extracellular fluid (Figure 3G,H). However, when mice were sacrificed on day +1 (when mice receiving MSC would normally receive their MSC infusion), we observed significantly more IFNγ compared to that detected in mice sacrificed on day 0 (Figure 3E). Less IFNγ was also present in the serum of mice that received MSC post-transplant compared to controls on day +6 (Figure 3G, P<0.05). Furthermore, there was a trend towards a significant reduction in splenic levels of IFNγ on day +6 (Figure 3H, P=0.059). These results suggest that the delay in death from GVHD after MSC infusion is at least in part associated with a reduction in serum levels of IFNγ.

Mesenchymal stromal cells do not contribute to healing of graft-versus-host disease target organs after major histocompatibility-mismatched hematopoietic stem cell transplantation

Previous reports suggest that MSC exert beneficial anti-GVHD effects by homing to sites of inflammation, and either recruiting other reparative cells or undergoing direct

differentiation to repair damaged tissue.[18] To assess the migratory capacity of MSC post-transplant, we transplanted conditioned BALB/c mice with wild-type C57BL/6 cells as the source of bone marrow and spleen cells on day 0 and 1×10^6 GFP$^+$ MSC administered intraperitoneally on day +1. The expression of GFP$^+$ genomic DNA was assessed in the bones, large bowel, spleen and lymph nodes. Despite infusing 1×10^6/mouse GFP$^+$ MSC, very few MSC were detected even within 1 h after infusion (Figure 4A). The largest number of detected MSC were in the spleen. However, even 24 h after the MSC infusion, when the majority of MSC were found (mean 3632±1695 cells), only 0.2% of the MSC infused were accounted for (although only GVHD-target organs were assessed; Figure 4B). This suggests that when MSC are administered via intraperitoneal injection they either die, or are dispersed widely throughout the body, the latter hypothesis being supported by other studies.[19-21] Other data from our laboratory have shown that intravenous infusion of MSC does not increase MSC detection (except in the lungs) (Kollar *et al.* 2010, *unpublished data*), suggesting that our inability to detect MSC following intraperitoneal injection was not specifically due to the route of MSC injection. Our results demonstrate that death from GVHD can be delayed if MSC are infused post-transplant, yet MSC were not readily detected in quantities that would suggest a direct effect of MSC on wound healing, or T-cell suppression. This suggests that the immunosuppressive effect of MSC on GVHD kinetics is via a transient paracrine effect rather than their engraftment into GVHD target organs.

To confirm the hypothesis that MSC do not contribute to healing of GVHD target organs in the allogeneic transplant model, sections of the skin, liver, large intestine, small intestine and lungs were harvested throughout the

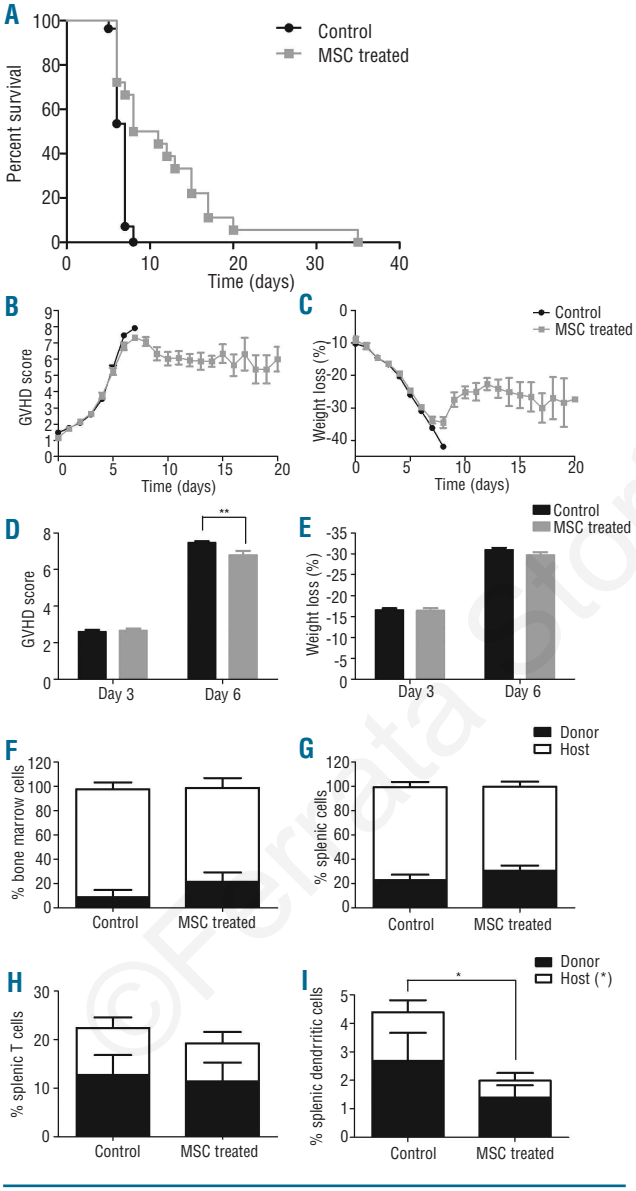

Figure 2. MSC administered 24 h post-transplant delay death from GVHD after MHC-mismatched HSCT. BALB/c mice (n=28 controls, n=18 MSC treated, 4 independent experiments) were transplanted with UBI-GFP/BL6 bone marrow and splenocytes and treated mice administered donor-derived MSC via intraperitoneal injection on day +1. Mice were then monitored daily for GVHD. Overall survival (A, $P<0.0001$), GVHD scores (B) and weight loss (C). GVHD scores (D) and weight loss (E) on day 3 and day 6. Engraftment (total and cellular subtypes) was assessed by FACS analysis of donor (GFP$^+$) *versus* host (GFP$^-$) cells in the bone marrow (F, bone marrow and G, spleen), T cells (H) and dendritic cells (I). Results are expressed as mean ± SEM.

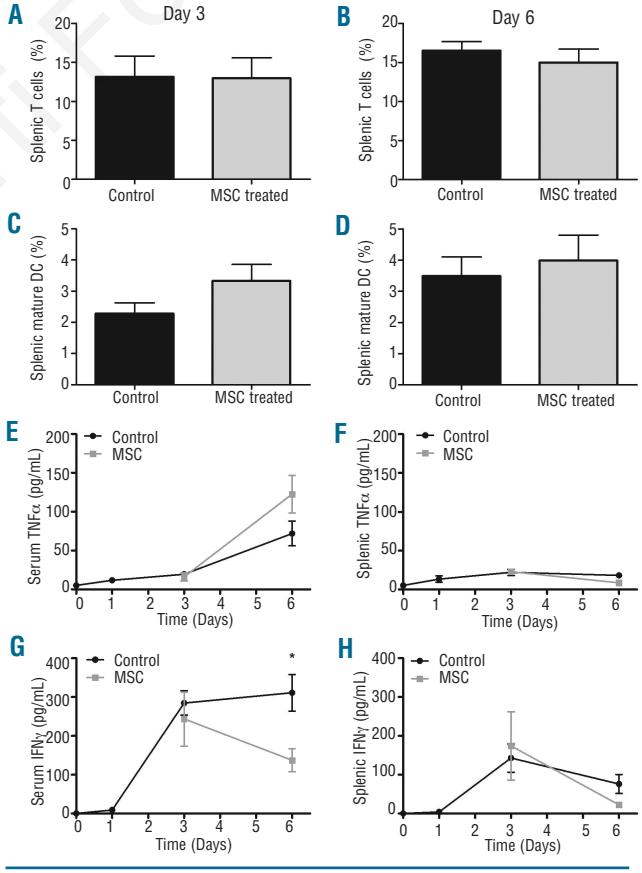

Figure 3. MSC cause changes in IFNγ after MHC-mismatched HSCT. BALB/c mice were transplanted with UBI-GFP/BL6 bone marrow and splenocytes ± MSC on day +1. Splenic T cells (A and B) and splenic mature dendritic cells (DC) (CD11c$^+$MHCII$^+$, C and D) were assessed by FACS on day 3 and day 6 post-transplant. TNFα (E and F) or IFNγ (G and H) were detected using the cytokine bead array in either serum (E and G) or spleen extracellular fluids (F and H). N=5 per time point where results are expressed as mean ± SEM. Day 6 serum IFNγ: controls *versus* MSC, $P<0.05$.

timed sacrifice experiment for histological analysis. Although most organ damage was observed on day +6, there was no difference in histopathology scores between control and MSC-treated allogeneic HSCT recipients (Figure 4E). Minimal damage was seen in the lungs or the skin in the MHC-mismatched transplant recipients regardless of the administration of MSC (*data not shown*).

Mesenchymal stromal cells are less effective than traditional immunosuppressive therapies

In all reported clinical studies, MSC have been administered to patients in conjunction with immunosuppression. Given the success of T-cell targeted therapies in controlling clinical GVHD, the efficacy of MSC alone or in combination with traditional therapeutic immunosuppressive agents (cyclosporine and T-cell depletion [KT3]) were evaluated for their ability to delay death from GVHD after MHC-mismatched HSCT. Transplant recipients who received either cyclosporine alone[11] or KT3 alone survived an average of 11-14 days longer than saline-treated controls and 8-11 days longer than MSC-treated mice (mean survival - with cyclosporine: 18.17±3.28 days; - with KT3: 21.33±3.21 days, controls: 6.67±0.26 days, MSC-treated mice 9.5±1.02 days, Figure 5A). However we did not observe any further survival advantage when these anti-T cell therapies were used in combination with MSC. These results highlight the efficacy of traditional immunosuppressants and although administered at the optimal time and dose, MSC as therapy for GVHD in this model were significantly less effective (MSC *versus* KT3: $P<0.01$; MSC *versus* cyclosporine: $P<0.05$).

Interferon-γ pre-treatment of mesenchymal stromal cells does not enhance their efficacy

Although MSC were significantly less effective than KT3 and cyclosporine monotherapy and could not prolong survival when used in combination with these therapies, Polchert *et al.*[12] recently showed that administration of IFNγ pre-treated MSC prevented death from GVHD in mice after allogeneic HSCT. We, therefore, sought to determine whether the efficacy of MSC could be enhanced by IFNγ pre-treatment in our model. We showed that transplant recipients that received MSC pre-treated for 48 h with IFNγ survived for 10.67±1.38 days,

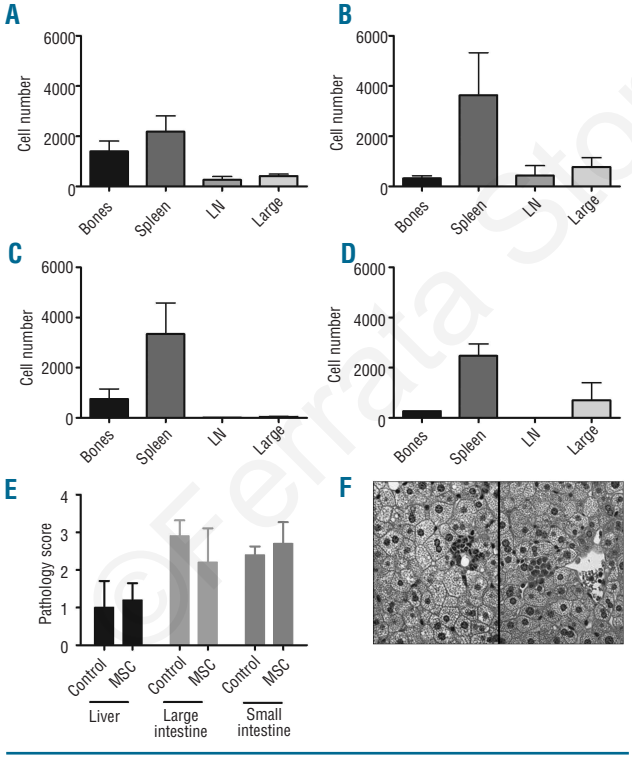

Figure 4. MSC do not contribute to healing of GVHD target organs. BALB/c mice were transplanted with C57BL/6 bone marrow and splenocytes and administered a $1x10^6$ (A-D) or $4x10^5$/mouse (E-F) dose of MSC on day +1 post-transplant by intraperitoneal injection. Three mice per time-point were sacrificed post infusion at 1 h (A), 24 h (day 2 post-HSCT) (B), 48 h (day 3) (C) or 120 h (day 6 post-HSCT) (D) and whole organs (bones, spleen, large intestine and lymph nodes) were removed and analyzed. Quantitative PCR was performed for detection of GFP. Organs were also scored for GVHD in untreated controls and mice treated with MSC on day 6 (E, n=5). Example of GVHD induced liver damage in control (F, left) or MSC treated mice (F, right).

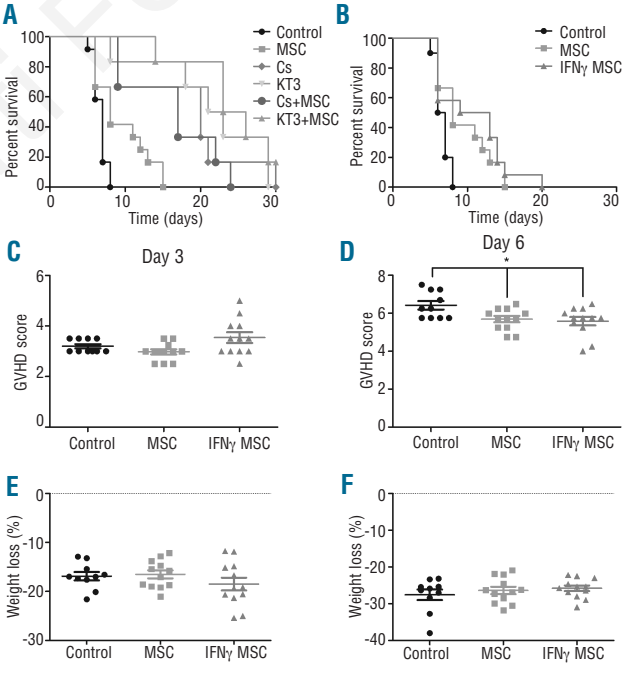

Figure 5. Impact of IFNγ pre-treatment of MSC, T-cell depletion or cyclosporine on survival after MHC mismatched HSCT. (A) BALB/c mice (n=12 controls, n=12 MSC treated, n=6 per remaining treatment groups, 2 independent experiments) were transplanted with UBI-GFP/BL6 bone marrow and splenocytes and MSC treated mice administered donor-derived MSC via intraperitoneal injection 24 h later. Cyclosporine (Cs, 50 mg/kg/day) was administered from day 0 until day of death. The T-cell-depleting antibody KT3 (0.1 mg/day) was administered on days +1, +3 and +5. (B) BALB/c mice were transplanted with UBI-GFP/BL6 bone marrow and splenocytes and administered $4x10^5$/mouse MSC on day 1 post-transplant via intraperitoneal injection. IFNγ MSC were pre-treated for 48 h with 1000 IU/mL of recombinant mouse IFNγ. Mice were monitored daily for GVHD. Survival (B). GVHD scores on day 3 (C) and day 6 (D). Weight loss percentage day 3 (E) and day 6 (F). N=10 controls, n=12 MSC treated (both cohorts), two independent experiments. Controls *versus* MSC, $P<0.05$; control *versus* IFNγ MSC, $P<0.05$.

compared to untreated mice that survived a mean of 6.6±0.31 days. Mice treated with unmanipulated MSC survived a mean of 9.5±1.02 days (Figure 5B). Administration of IFNγ pre-treated MSC to HSCT recipients did not translate into a significant overall increase in survival compared to that seen in mice receiving unmanipulated MSC (*P*=NS). GVHD scores were not different between groups on day +3, but at day +6 were significantly reduced in both MSC-treated groups compared to in controls (Figure 5D, *P*<0.05). No differences in weight loss were observed between groups at either day +3 (Figure 5E) or day +6 (Figure 5F), suggesting that IFNγ pre-treatment did not increase the efficacy of MSC in our model.

Effect of mesenchymal stromal cells after major histocompatibility complex-matched, minor histocompatibility antigen-mismatched hematopoietic stem cell transplantation

Published murine transplant models assessing the effectiveness of MSC as a therapeutic agent for GVHD have most commonly used either the complete MHC mismatched model or the F1 (MHC-haploidentical) model of GVHD.[12,22-28] To assess the effectiveness of MSC in a more clinically relevant model we used an MHC matched, minor histocompatibility antigen (miHA)-mismatched transplant model mimicking an HLA-identical sibling transplant.

Conditioned BALB.B [H-2^b] mice that received UBI-GFP/BL6 BM [H-2^b] and splenocytes on day 0 and 4×10^5 MSC 24 h post-HSCT had a significant increase in survival compared to controls (mean day of death: controls=31.55±2.55, MSC=50.5±8.76; *P*<0.05). At the end of the experiment, 90 days post-HSCT, 30% of MSC recipients had not succumbed to GVHD (Figure 6A). In contrast, conditioned BALB.B mice that received UBI-GFP/BL6 BM and splenocytes on day 0 and 1×10^6 MSC 24 h post-HSCT survived a significantly shorter time (21.67±1.2 days) compared to controls (Figure 6A, *P*<0.01). Conditioned BALB.B mice that received UBI-GFP/BL6 BM and splenocytes on day 0 and either 4×10^5 or 1×10^6 MSC on day 7 showed no improvement in survival compared to controls at either dose (Figure 6B, *P*=NS). Similar observations were made in cohorts of mice that received MSC on day 14 (Figure 6C, *P*=NS), suggesting that low dose MSC must be infused early (24 h) post-transplant to effectively influence the course of GVHD. When comparing GVHD scores and weight loss post-transplant in the miHA-mismatch model, we observed that early post-transplant (up to day 7), scores and weight loss were similar among all groups, suggesting that administration of MSC did not attenuate conditioning-related toxicity. Divergence between controls and MSC-treated mice started from day 18 as reflected in the survival curves; however, over time, no obvious differences were observed in either weight loss (Figure 6D-F) or GVHD scores of either cohort (Figure 6G-I).

Given the reduction in serum levels of TNFα and IFNγ

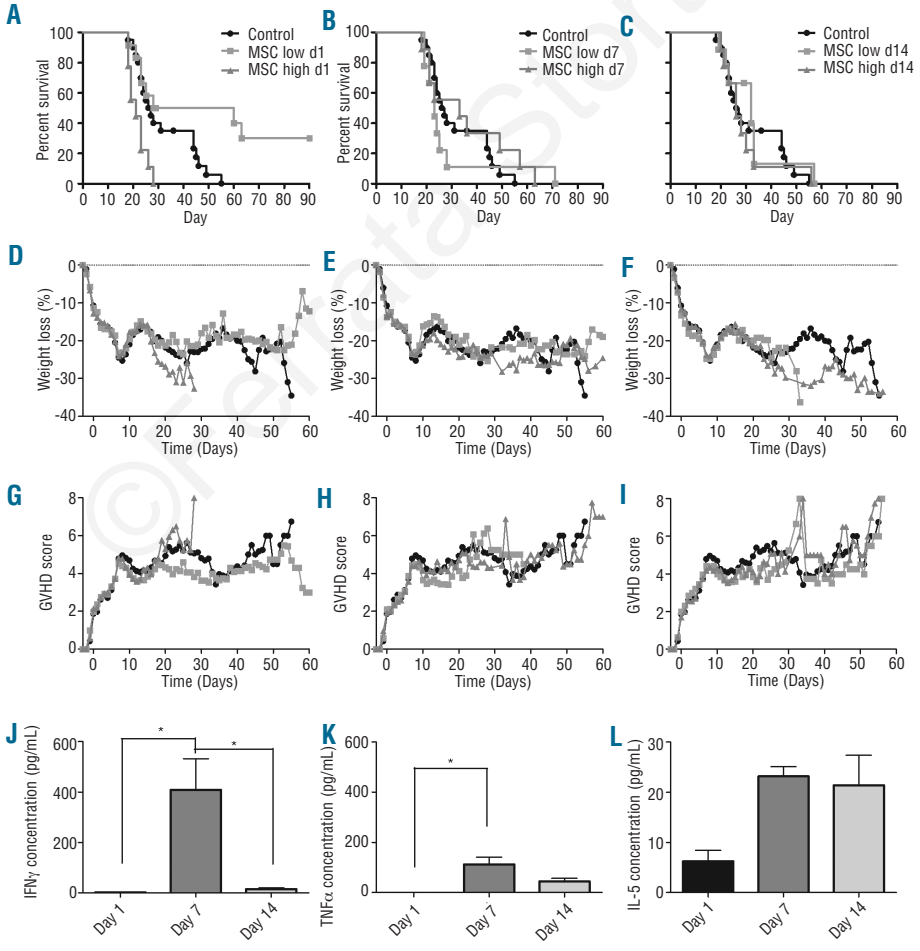

Figure 6. Survival is increased after low dose MSC are infused 24 h after MHC-matched, miHA mismatched HSCT. BALB.B mice were transplanted with UBI-GFP/BL6 bone marrow and splenocytes and administered at a low (4x10^5/mouse) or high (1x10^6/mouse) dose of MSC on day +1 (A), +7 (B) or +14 (C) post-transplant via intraperitoneal injection. Mice were then monitored daily for GVHD. (D) Weight loss and (G) GVHD scores of mice administered MSC on day +1; (E) weight loss and (H) GVHD scores from day +7 MSC injection and (F) weight loss and (I) GVHD scores of mice given MSC on day +14. N=19 controls, n=12 low dose day +1, n=9 for all remaining cohorts, four independent experiments. NB: controls in each graph were pooled from the four experiments, although treatment groups are separated into the three different days for visual clarity. Control *versus* MSC low dose day +1 *P*<0.05. Levels of serum IFNγ (J), TNFα (K) and IL-5 (L) were assessed via cytokine bead array in control mice on days +1, +7 and +14 (n=3 per time-point).

seen in the MHC-mismatched HSCT recipients, we monitored the cytokine milieu that MSC were entering at the time of infusion in miHA HSCT recipients. Blood samples were taken from untreated controls on day +1, day +7 and day +14 to measure T_H1 and T_H2 serum cytokine levels. IFNγ showed the most significant fluctuation, rising from virtually undetectable levels on day 1 (mean 1.6±1.34 pg/mL) to a sharp peak on day +7 (409±123.3 pg/mL), and returning to low levels by day +14 post-transplant (mean 14.47±4.87 pg/mL, $P<0.05$, Figure 6J). TNFα also increased significantly from day +1 (none detected) to day +7 (mean 112.4±28.6 pg/mL, $P<0.05$, Figure 6K) and then decreased again by day +14. Levels of IL-5, a potent stimulator of B-cell proliferation and differentiation, were increased on day +7 (mean 23.19±1.96 pg/mL, $P<0.01$) and day +14 (21.38±5.99 pg/mL; $P<0.01$) compared to day +1 (6.25l±2.19 pg/mL, Figure 6L).

Discussion

MSC show significant immunosuppressive capacity *in vitro* and infusion of these cells is, therefore, being tested in trials as a novel therapy to ameliorate clinical GVHD. Although this treatment has been reported to be effective in several studies,[5,7,29,30] a recent trial has questioned their efficacy.[8] Understanding how MSC exert their immunosuppressive effects in clinically relevant *in vivo* models is essential to evaluate the therapeutic potential of MSC against GVHD. However MSC-mediated control of GVHD is not reliably reproduced in animal models. This may be because MSC are administered as monotherapy in murine models of GVHD using radiation alone as opposed to clinically relevant conditioning regimens, while in the clinic MSC are invariably administered in combination with conventional immunosuppressive drugs such as cyclosporine.

To address this, we used two murine models of GVHD in combination with clinically relevant conditioning (cyclophosphamide + total body irradiation) as the preparative regimen. We found that a single infusion of 4×10^5 MSC (equivalent to 2×10^7/kg) on day +1 after MHC-mismatched HSCT significantly delayed death from GVHD. When we used cyclosporine or T-cell depletion (KT3) alongside MSC, which more accurately replicates a potential clinical scenario, we found that cyclosporine and KT3 were more effective than MSC alone in delaying death from GVHD. In this model, MSC offered no additional survival benefit when they were combined with these traditional therapies. The lack of improved efficacy of these combination therapies may be a consequence of decreased IFNγ production due to the reduction in donor T-cell numbers post-transplant. If there are fewer activated T cells, less IFNγ is produced and the observed effects of MSC are diminished. Using MSC in combination with other classes of pharmacological immunosuppressants that have less effect on IFNγ may provide increased synergy.

When we used the MHC-matched, miHA-mismatched HSCT model, which replicates HLA-identical allogeneic sibling transplantation, similar to our MHC mismatched model, we found that MSC were most effective at delaying the onset of GVHD when 4×10^5 MSC/mouse were administered on day +1.

Despite the postponement of death from GVHD in our HSCT models, we only observed minimal differences in the cellular composition of the bone marrow and spleen between MSC-treated and control mice. We observed a reduced percentage of mature host dendritic cells at the time of death, although this may simply have reflected a temporal difference because these dendritic cell subsets were not altered between control and MSC-treated mice in timed sacrifice experiments. Interestingly, the percentage of T cells remained unchanged, which highlights differences between the re-created *in vitro* environment in which MSC block T-cell proliferation and the *in vivo* milieu. Clinical studies found that MSC-treated patients experience a higher incidence of relapse[30] and incidence of infection;[5] our observations suggest that the efficacy of MSC is not T-cell-dependent, allowing us to speculate that MSC will not compromise the beneficial graft-*versus*-leukemia effect of the transplant.

The notable observation that may explain why MSC delay GVHD in our murine transplant recipients was that IFNγ levels were reduced in the presence of MSC. Because MSC do not alter the composition of the T-cell populations in these HSCT models, these data do not support the hypothesis that MSC are licensed by IFNγ to suppress T-cell proliferation but instead suggest that MSC may delay GVHD progression by altering the inflammatory milieu. In the MHC-matched, miHA-mismatched transplant experiments, we observed that MSC were only effective if they were added on day +1, but were not effective if they were added on day +7 or +14. This may be because the levels of IFNγ at these later time points were too high to be abrogated by MSC, or that the effects of increased IFNγ may have already caused the GVHD to progress beyond a stage that could be controlled by infusion of MSC. This hypothesis was supported by the inability of MSC to resolve established GVHD (*Online Supplementary Figure S2*) and the lack of efficacy of multiple infusions of MSC (on days 1, 7 and 14; *data not shown*) on the onset of GVHD.

Our observations concord with those of other studies showing beneficial effects of MSC on GVHD;[12,22,26,28] however, survival in the MHC-mismatched HSCT model was only extended in 50% of mice and all mice eventually succumbed to GVHD. This suggests that the immunosuppressive effects of MSC are only transient. This may explain why MSC did not show clinical resolution of overall GVHD in the recent Osiris trial despite a sub-analysis which showed that MSC were beneficial to patients with steroid-refractory GVHD involving the gut or liver.[31] These data are in contrast with those of several other pre-clinical and clinical studies. The significant differences between our study and those which showed a prolonged survival benefit from MSC in pre-clinical models[12,22,23,26,28] may result from variations in MSC isolation and culture methods, the degree of H-2 disparity between the donor and host and our use of a clinically relevant conditioning regimen. The lack of congruity between our results and the majority of clinical studies[5,7,29,30] may be due to the fact that MSC are administered to highly immunosuppressed patients and the use of combination therapy may mask the effect of MSC on GVHD suppression and resolution, a variable not taken into consideration in pre-clinical transplant models.

In conclusion, while MSC may have a clinically relevant role in the attenuation of GVHD, the dose and timing of their administration will directly affect their efficacy. Given the lack of consensus from pre-clinical models and

the recent Osiris trial results, further research into the mechanism of action is required before MSC become conventional therapy in the setting of allogeneic HSCT. Importantly, our observation of an association between increased systemic IFNγ and increased efficacy of MSC-mediated immunosuppression could provide a key to define the time patients at risk of GVHD are most likely to benefit from MSC administration.

Authorship and Disclosures

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