REVIEW

Mesenchymal Stromal Cells: A New Tool against Graft-versus-Host Disease?

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Mesenchymal stromal cells (MSCs) represent a heterogeneous subset of multipotent cells that can be isolated from several tissues including bone marrow and fat. MSCs exhibit immunomodulatory and anti-inflammatory properties that prompted their clinical use as prevention and/or treatment for severe graft-versus-host disease (GVHD). Although a number of phase I-II studies have suggested that MSC infusion was safe and might be effective for preventing or treating acute GVHD, definitive proof of their efficacy remains lacking thus far. Multicenter randomized studies are ongoing to more precisely assess the impact of MSC infusion on GVHD prevention/treatment, whereas further research is performed in vitro and in animal models with the aims of determining the best way to expand MSCs ex vivo as well as the most efficient dose and schedule of MSCs administration. After introducing GVHD, MSC biology, and results of MSC infusion in animal models of allogeneic hematopoietic cell transplantation, this article reviews the results of the first clinical trials investigating the use of MSC infusion as prevention or treatment of GVHD.

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GRAFT-VERSUS-TUMOR (GVT) EFFECTS AND GRAFT-VERSUS-HOST DISEASE (GVHD)

Allogeneic hematopoietic cell transplantation (HCT) is the treatment of choice for many patients with life-threatening hematologic diseases such as patients with acquired lack of marrow function, inborn errors, and hematologic malignancies [1]. In the latter case, eradication of malignancies depends not only on the high-dose chemo/radiotherapy given in the conditioning regimen, but also on donor T and natural killer (NK) cells present in the graft (GVT effect) [2-7]. Initial evidence for GVT effects in humans came from studies reporting reduced leukemic relapse rates in allografted patients who developed acute and/or chronic GVHD (aGVHD, cGVHD, vide infra) compared with those who did not [8,9], and higher risk of relapse in patients given T cell–depleted grafts or grafts from syngeneic donors [10-13]. Further, direct evidence for antitumor effects of allogeneic cells came from observations that infusion of donor lymphocytes could induce complete remissions in a number of patients with hematologic malignancies who had relapsed after allogeneic HCT [4,14-16]. These observations were the basis for the development of allogeneic HCT following reduced-intensity (RIC) or truly nonmyeloablative conditioning regimen, in which the burden for tumor eradication relies mainly (RIC) or nearly exclusively (nonmyeloablative conditioning) on GVT effects [17-26].

Unfortunately, donor-versus-host alloreactivity is not always limited to destruction of tumor cells, but can also be the cause of GVHD, a potentially life-threatening complication of allogeneic HCT, in which donor lymphocytes destroy host organs [27]. GVHD has been classically divided into 2 syndromes: aGVHD, occurring within 100 days after transplantation, and cGVHD developing thereafter [27]. However, GVHD with characteristics of the chronic form can occur as early as 50 days after HCT, whereas aGVHD may occur beyond day 100 after HCT in patients given nonmyeloablative or RIC [28], often upon discontinuation of postgrafting immunosuppression or at the time of conversion of mixed donor T cell chimerism to full donor T cell chimerism [29,30]. These observations prompted the development of

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a new GVHD classification proposed by the National Institutes of Health Consensus Conference [31]. This classification recognized 2 categories of GVHD: *aGVHD* defined as GVHD without features consistent with cGVHD comprising *classic aGVHD* occurring before day 100, and *late a GVHD* occurring after day 100; and *cGVHD* comprising *classic cGVHD* defined as cGVHD without signs of aGVHD and *overlap syndrome* in which features of both aGVHD and cGVHD coexist [31]. Interestingly, 3 recent reports have observed that classic cGVHD was significantly associated with GVT effects after allogeneic HCT following RIC or nonmyeloablative conditioning, whereas aGVHD and late aGVHD were not [32-34].

In mice, the pathogenesis of aGVHD includes 3 sequential phases [35,36]. In the first phase, the conditioning regimen (and in particular total body irradiation [TBI]) induces tissue damages that activate host tissues. Activated host cells secrete several inflammatory cytokines and growth factors, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) ("cytokine storm"), leading to increased expression of adhesion and cell surface recognition molecules by host cells, thereby enhancing the recognition of host minor or major histocompatibility (MHC) antigens by mature donor T cells. Antigen presentation (mainly by host dendritic cells that are essential to induce GVHD in mice [37]), as well as activation, proliferation and differentiation of donor T cells occur in the second phase. Finally, in the third phase, activated T cells and TNF-α induce organ damage and the clinical manifestations of aGVHD [35,36].

Although several reports have observed an association between the intensity of the cytokine storm and the probability of GVHD in humans [38-41], the observation that donor lymphocyte infusions given without any preceding conditioning induced GVHD in one-half of the patients demonstrated that aGVHD could occur without any cytokine storm in humans, contrary to what has been observed in many murine models.

The pathophysiology of cGVHD remains not fully understood. It is generally accepted that donor T cells (and particularly $CD4^+$ T cells [42]) are largely involved in the pathophysiology of cGVHD, because profound T cell depletion leads to a very low incidence of cGVHD even in the HLA haploidentical setting [43], and because infusion of peripheral blood stem cells (PBSC, containing 10 times more T cells than marrows) or addition of donor buffy coat cells increased the incidence of cGVHD in comparison to patients receiving allogeneic marrows alone [44,45]. In addition, donor lymphocyte infusion induces cGVHD in approximately 60% of the recipients [4]. HLA disparities between donor and recipient are also a risk factor of cGVHD (although to a lesser extent than for aGVHD [46]), suggesting that cGVHD manifestations are because of donor T cells recognizing allogeneic antigens (such as major or minor histocompatibility antigens). The role of minor histocompatibility antigen disparities is supported by the higher incidence of cGVHD in male patients given grafts from female donors versus other gender combinations [46], because the Y chromosome encodes the HY antigen that can act as a minor histocompatibility antigen [47]. Host thymus integrity could also play a role, as suggested by the lower incidence of cGVHD in younger recipients [27,46]. A possible role of the thymus could be the deletion of "auto" reactive clones during negative selection, and perhaps the generation of regulatory T cells [48,49]. However, a recent study observed a similar incidence of cGVHD in patients with thymic function (assessed by measuring signal joint T cell receptor excision circle levels) below or above the median on day 100 after HCT [50]. It has also been suggested that B cells might play a role in the physiology of cGVHD [51]. Indeed, deposition at the dermoepidermal junction of IgM and complement was frequently observed in patients with chronic cutaneous GVHD [52], whereas antibodies directed against HY proteins have been found in male cGVHD patients given allo-HCT from female donors [53]. More arguments in favor of a role of B cells in the physiology of cGVHD include a lower incidence of cGVHD in patients given rituximab (a monoclonal antibody directed against the CD20) [54], and reports of improvement of chronic skin GVHD after rituximab administration [55].

Depending on the extent of HLA matching between donor and recipient, the intensity of the conditioning regimen, the stem cell source, the graft composition, the patient age, and the gender combination, the incidents of grade II-IV aGVHD and extensive cGVHD have varied from 10% to 70%. The current standard of care for GVHD prevention in patients receiving grafts from HLA-matched donors has remained in most centers the combination of a calcineurin inhibitor (cyclosporin or tacrolimus) with an antimetabolite (short methotrexate in case of myeloablative bone marrow/PBSC transplantation or mycophenolate mofetil in case of nonmyeloablative or cord blood transplantation) [23,56-59]. Further progress in GVHD prevention might involve the use of antithymocyte globulin (ATG) [60-63], or mammalian Target of Rapamycin (mTOR) inhibitors such as sirolimus [64].

The treatment for aGVHD generally involves high-dose steroids [27,65]. Specifically, the most common primary therapy for aGVHD consists of methylprednisolone, 2 mg/kg/day for 7 to 14 days, followed by gradual dose reduction if the GVHD improves, although 1 recent paper has suggested that initial treatment with 1 mg/kg/day methylprednisolone was as efficient as initial treatment with 2 mg/kg/day in patients with grade II aGVHD [66]. Corticosteroids produce durable complete responses in 20% to 75% of

the patients with grade II-IV aGVHD [66-68], and less in patients with lower gut aGVHD [68]. In patients not responding to steroids, outcome is dismal [27]. Specifically, patients with grade II, III, and IV steroid-refractory aGVHD had median survivals of 4.1, 3.6, and 2.7 months, respectively, after salvage therapy with ATG [69], whereas no other second-line treatments have proven to do better than what has been achieved with ATG for gut or liver steroid-refractory aGVHD [65]. First-line treatment for extensive cGVHD is generally based on steroids, often combined with cyclosporine or tacrolimus [70]. Unfortunately, with this regimen only 20% to 50% of the patients achieve complete resolution of GVHD and withdrawal of all systemic treatment within 2 to 3 years [71,72]. Even though a number of immunosuppressive agents have demonstrated therapeutic activity in steroid-refractory cGVHD, the prognosis of patients with steroid-refractory cGVHD remains unsatisfactory with 2-year survival ranging from 41 to 85 with salvage therapy [73-75]. These data stress the need for novel therapies for both aGVHD and cGVHD.

BIOLOGY OF MSC

The bone marrow microenvironment is composed of different elements that support hematopoiesis and bone homeostasis. Among them, bone marrow stroma contains an adherent fibroblast-like population, representing 0.01% to 0.001% (depending on age) of marrow cells that, under appropriate conditions, retain the ability to differentiate into a number of cell lineages, including bone, cartilage, tendon, muscle, or adipose tissue [76,77]. These cells, first identified by Friedenstein et al. [78,79] in 1968, have been termed bone marrow "stromal cells," "mesenchymal stem cells," or "multipotent mesenchymal stromal cells" (MSCs) [80,81]. After ex vivo expansion, human MSCs have a fibroblastic-like morphology, and are uniformly positive for CD73 (SH3 or SH4), CD90, CD105 (SH2), CD29, CD44, CD71, CD106, CD120a, CD124, and CD166, but are negative for common hematopoietic markers like CD14, CD45, or CD34 [80-83]. In addition, human MSCs express HLA-class I and can be induced to express HLA-class II by interferon gamma (IFN-γ) [80]. However, MSCs are only weakly immunogenic in humans, even when infused after allogeneic HCT [84,85].

In recent years, further interest in MSCs has been raised by the observation that they exhibit profound immunosuppressive abilities in vitro and in vivo [80]. In vitro, MSCs inhibited T cell proliferation in mixed lymphocyte reactions or induced by mitogens [86,87], and inhibited naive and memory T cell responses to their cognate antigens [88]. Interestingly, 1 study showed that MSCs had little impact on T cell responses to cytomegalovirus and Epstein-Barr virus, while exhibiting strong immunosuppressive effects on alloreactive T cells [89]. The degrees of inhibition were dose-dependent and independent of HLA-matching. Further, MSCs could induce regulatory T cells, as evidenced by the increased proportion of $CD4^+CD25^+FoxP3^+$ cells in mixed lymphocyte cultures in the presence of MSCs [82,90]. MSCs also inhibited IL-2 (or IL-15) induced NK cell proliferation, although the inhibition was only partial [91], whereas the impact of MSCs on B cell proliferation has remained debated [92,93]. How MSCs modulate T cell activation and immune response has remained an open question. Several groups have reported that T cell modulation was contact independent and involved soluble factors [92,93]. Because MSC culture medium was only mildly immunosuppressive [94], one could hypothesize that MSCs produce these immune factors in crosstalk with immune cells. Potential soluble factors involved could include transforming growth factor beta (transforming growth factor-β), hepatocyte growth factor, bone morphogenetic protein 2, prostaglandin E2, indoleamine 2,3 dioxygenase, nitric oxide, IL-10, heme oxygenase-1, HLA-G5, and galectin-1 [81,82,95-98]. It has been shown that the inhibition of lymphocyte proliferation in vitro by nonactivated MSCs was neither because of induction of lymphocyte apoptosis nor induction of T cell tolerance, but instead due to induction of anergy of activated T cells that could be reinitiated after MSCs withdrawal [86,88,99]. Finally, MSCs inhibited differentiation of both $CD34^+$ cells and monocytes into mature dendritic cells, decreased their ability to activate T cells, and favored the induction of regulatory antigen-presenting cells [90,100-102].

In mice, MSC infusion prolonged cardiac allograft survival through the generation of regulatory T cells [103]; it also prevented the rejection of allogeneic B16 melanoma cells ($H\text{-}2^{b/b}$) in immunocompetent C3H mice ($H\text{-}2^{k/k}$) [104], improved autoimmune enteropathy and experimental colitis [105,106], and ameliorated experimental autoimmune encephalomyelitis by inducing T cell anergy [107]. In contrast, MSCs failed to inhibit activated T cells in a mouse model of collagen-induced arthritis [108]. Finally, infusion of ex vivo expanded donor or third-party MSCs prolonged survival of histoincompatible skin grafts in baboons [109].

MSCs IN ANIMAL MODELS OF HCT

Inbred Mice Models

Models of engraftment

In bone marrow niches, MSCs act as paracrine mediator, producing chemokines, cytokines, and extracellular matrix proteins that support survival, proliferation, and engraftment of hematopoietic stem cells [110-114]. There is little evidence thus far that MSCs

can migrate to bone marrow niches, although it has been shown that some MSCs express the CXCR4 receptor, which might contribute to MSC homing via stem cell–derived factor-1 gradients [115]. In contrast, MSCs can migrate to inflammatory sites and to tumors [116,117]. Using immunodeficient mice models of engraftment, several groups of investigators have demonstrated that cotransplantations of human MSCs improved engraftment of human hematopoietic stem ($CD34^+$) cells into nonobese severe combined immunodeficiency (NOD/SCID) mice when low numbers of $CD34^+$ cells were injected [118-120].

Because engraftment of allogeneic hematopoietic cells depends on dampening immune-mediated host-versus-graft reactions [121], Nauta et al. examined the in vivo immunomodulatory properties of MSCs in various murine models of transplantation [122]. Using a multiple minor histocompatibility antigen mismatched model (BALB/b [H-$2^{b/b}$] into B6 [H-$2^{b/b}$]) where B6 mice were given T cell–depleted marrows from BALB/b mice after sublethal irradiation, the authors observed that B6-derived MSC infusions on days 4, 7, 10, and 14 resulted in a significant increase in the percentage of engrafted mice (44% versus 82%; $P < .05$) [122]. In contrast, all recipient B6 mice that received transplanted BALB/b bone marrows and BALB/b-derived MSCs failed to engraft and developed memory T cell responses against BALB/b cells. The same was true when only 1 single dose of BALB/b-derived MSCs was infused. Further, the lower engraftment when allogeneic donor MSCs were infused was also observed in a major MHC mismatch transplantation model (B6 bone marrows transplanted into BALB/c [H-$2^{d/d}$] recipients) (13% engraftment versus 50% with syngeneic MSCs). Finally, the authors observed that infusion of third-party MSCs had no significant impact on the engraftment of allogeneic marrow after sublethal irradiation. They concluded that murine MSCs were capable of inducing immune responses in vivo that could result in graft rejection when donor-derived allogeneic MSCs were infused, whereas MSCs promoted engraftment when they were not rejected as the result of an allo-immune response (ie, when syngeneic MSCs were infused). It should be stressed that these observations in murine models differ from what has been observed in human allogeneic HCT recipients, where MSC infusions were only weakly immunogenic, because HCT recipients given MSCs showed no response to infused MSCs before and up to 6 months after MSC infusion [85].

Models of GVHD

A number of studies have analyzed the ability of MSCs to prevent GVHD in mice (Table 1). However, it should be stressed that murine and human MSCs differ in several instances. First, the in vitro immunosuppressive activity of murine MSCs seems to be lower than that of human MSCs [123]. Second, ex vivo production of purified MSCs is much faster in humans than in mice [123]. Third, murine MSCs are more prone to undergo immortalization and transformation in culture than human MSCs [124]. Fourth, in contrast to human MSCs, murine MSCs failed to express functional indoleamine 2,3 dioxygenase enzyme, even with maximal cytokine activation [125].

Using an MHC mismatched model of GVHD (C57BL/6 [H-$2^{b/b}$] into BALB/c [H-$2^{d/d}$]), Sudres et al. [123] observed that MSCs (derived from bone marrows from C57BL/6 mice) added to bone marrow transplants at MSC/T cell ratios of 1 of 1 to 8 of 1 (a ratio that provided strong inhibition of T cell in vitro) did not decrease in vivo T cell activation and failed to decrease GVHD incidence or severity [123]. The lack of effect of MSCs was not because of rejection of MSCs, because MSCs could be detected up to 6 days after transplantation in bone marrows and lungs of grafted animals. However, only traces of MSCs could be detected in GVHD target organs, perhaps explaining their failure to suppress GVHD.

In contrast to what was observed by Sudres et al. [123], Li et al. [126] showed that MSCs added to bone marrow transplants at MSC/splenocyte cell ratios of 1 of 10 or 1 of 20 somewhat delayed aGVHD in a similar GVHD model (C57BL/6 [H-$2^{b/b}$] into BALB/c [H-$2^{d/d}$]), although all transplanted mice eventually died from GVHD within the first 13 days after transplantation.

Using another MHC mismatched model (BALB/c [H-$2^{d/d}$] into C57BL/6 [H-$2^{b/b}$]), Polchert et al. [127] observed that bone marrow BALB/c-derived MSCs infused i.v. on day 0 failed to decrease GVHD incidence or severity, confirming observations by Sudres et al. [123]. However, MSCs given either on day 2 or on day 20 after transplantation significantly improved survival and decreased GVHD symptoms [127]. Although mice given 0.1×10^6 MSCs or 0.5×10^6 MSCs on day 2 had similar survival, animals given MSCs on day 20 had higher survival when given 0.5×10^6 MSCs than when given 0.1×10^6 MSCs. This could be because of a higher number of activated T cells present on day 20 (at the time of GVHD) than on day 2 after HCT. Interestingly, interferon-γ activation of MSCs was required to prevent GVHD in that model, and interferon-γ activated MSCs given on day 0 were able to abrogate GVHD mortality.

The ability of parental or recipient MSCs i.v. infusion to prevent GVHD in a haploidentical model of GVHD (C57BL/6 [H-$2^{b/b}$] → CB6F1 [H-$2^{b/d}$]) was assessed by Prighozina et al. [128]. Repeated i.v. infusions of parental or recipient bone marrow MSCs ($0.5\text{-}5 \times 10^5$ MSC/mice) on days 0, 7, and 14 after transplantation failed to reduce GVHD-related mortality.

Yanez et al. [129] compared the properties of human adipocyte-derived MSCs and human bone

Table 1. Impact of MSC Infusions in Murine Models of GVHD

Authors (References)	Mice Model	MSC Source, Dose, and Schedule of Administration	Main Observations
Sudres et al. [123]	C57BL/6 (B6; H-$2^{b/b}$) → BALB/c (H-$2^{d/d}$)	MSCs derived from BM from C57BL/6 mice and given on day 0 at an MSC/T cell ratio from 1/1 (5×10^5MSC and $CD3^+$ T cells) to 8/1.	- MSCs cotransplantation failed to prevent GVHD. - On day 6 after transplantation, MSCs were mainly present in the bone marrows and lungs (where they were trapped), whereas only traces of MSCs were detected in target organs of GVHD and in secondary lymphoid organs.
Li et al. [126]	C57BL/6 (B6; H-$2^{b/b}$) → BALB/c (H-$2^{d/d}$)	MSCs derived from BM from C57BL/6 mice and given on day 0 at an MSC/splenocyte cell ratio from 1/10 (2×10^6 MSC and 2×10^7 splenocytes) to 1/1000.	- MSCs given at the dose of 2×10^6 or 1×10^6 cells somewhat delayed aGVHD, but all mice died of GVHD within the first 13 days after transplantation. - MSC infusion increased the number of T cells in secondary lymphoid organs and decreased the migration of effector T cells in GVHD target tissues.
Polchert et al. [127]	BALB/c (H-$2^{d/d}$) → C57BL/6 (B6; H-$2^{b/b}$)	BM-derived MSCs from BALB/c mice were infused at the doses of 1×10^5 or 5×10^5 at various timing after transplantation.	- MSCs infused on day 0 prevented GVHD only when previously activated with high-doses of IFN-γ (500 U/mL). - MSCs infused on day 2 or on day 20 at the dose of 1×10^5 or 5×10^5 MSCs/mice each prolonged survival. - MSCs infused on day 2 failed to prolong survival when splenocytes from IFN-γ knockout mice were used to induce GVHD.
Prigozhina et al. [128]	C57BL/6 (B6; H-$2^{b/b}$) →$CB6F_1$ (H-$2^{b/d}$)	BM-derived MSCs from C57BL/6 or $CB6F_1$ infused on days 0, 7, and 14 at the dose of 0.5×10^5 or 5×10^5 MSCs/mice.	- Neither parental nor recipient MSCs decreased GVHD.
Yanez et al. [129]	C57BL/6 (B6; H-$2^{b/b}$) (10^7 BM cells + 2×10^7 splenocytes) → B6D2F1 (H-$2^{b/d}$)	5×10^4 MSCs derived from adipose tissue from B6D2F1 infused on days 0, 7, and 14 or on days 14, 21, and 28.	- Decreased GVHD and prolonged survival in mice given MSCs on days 0, 7, and 14. - No impact of MSCs when administered on days 14, 21, and 28.
Badillo et al. [130]	C57BL/6 (B6; H-$2^{b/b}$) (10^7 BM cells + 3×10^7 splenocytes) → B6 x BALB/c F1 (H-$2^{b/d}$)	1.5×10^5-1×10^6 MSCs/mice derived from bone marrows from C57BL/6 mice infused on single dose either on day 0, 2, 10, or on day 21 after transplantation, or 0.5×10^5 MSCs infused on days 0, 7, and 14 after transplantation (serial infusion).	- MSCs failed to prevent GVHD and to prolong survival in all tested schedules.
Christensen et al. [131]	- UBI-GFP/BL6 (H-$2^{b/b}$) → BALB/c (H-$2^{d/d}$) and - UBI-GFP/BL6 (H-$2^{b/b}$) → BALB.B (H-$2^{b/b}$)	4×10^5 MSCs derived from bone marrows from UBI-GFP/BL6 mice infused i.p. on day 1 after transplantation.	- MHC-mismatched model: MSC infusion postponed death from GVHD from 6.6 ± 0.13 days to 12.0 ± 1.7 days ($P < .001$), but did not prevent death from GVHD. - Minor histocompatibility mismatched model: MSC infusion postponed mortality from GVHD from 31.6 ± 2.6 days to 50.5 ± 8.8 days ($P < .05$) and prevented death from GVHD in 30% of MSCs infused mice.
Tisato et al. [132]	Human PBMC (20×10^6) i.v. → NOD/SCID mice	3×10^6 human cord blood–derived MSCs injected on day 0, or on days 0, 7, 14, and 21, or 4 times every 3 days at the time of GVHD.	- Cotransplantation of MSCs on day 0 only failed to prevent GVHD. - MSC infusions on days 0, 7, 14, and 21 prevented human lymphocyte expansion and GVHD. - MSC infusions at the time of GVHD failed to resolve GVHD.
Bruck et al.* [133]	Human PBMC (30×10^6) i.p. → NOD/SCID/γc^{null} mice	3×10^6 human bone marrow-derived MSCs injected in i.p. on days 0, 7, 14, and 21 after PBMC injection.	- Repeated infusions of MSCs failed to prevent death from GVHD.

BM indicates bone marrow; INF, interferon.
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marrow–derived MSCs, and then assessed the ability of murine adipocyte-derived MSCs to prevent GVHD in an MHC-haploidentical model of GVHD (C57Bl/6 [H-$2^{b/b}$] into B6D2F1 [H-$2^{b/d}$]) [129]. The authors observed that human adipocyte-derived and bone marrow–derived MSCs exhibited comparable phenotype and immunoregulatory properties. Further, murine adipocyte-derived MSCs given i.v. at the dose of 5×10^4 MSCs (MSC/splenocyte ratio of 1:400) on days 0, 7, and 14 after transplantation improved survival and decreased GVHD. In contrast, MSCs given at the dose of 5×10^4 MSCs on days 14, 21, and 28 failed to prevent GVHD mortality. The authors concluded that adipocyte-derived MSCs could effectively control MHC-haploidentical GVHD in mice, but that the timing and the number of MSC infusions played a major role in GVHD prevention.

Badillo et al. [130] assessed the ability of bone marrow–derived MSCs to prevent or treat GVHD in an MHC-haploidentical model of GVHD (C57Bl/6 [B6; H-$2^{b/b}$] into B6x BALB/cF1 [H-$2^{b/d}$]). MSCs infused at various dose and timing after transplantation failed to prevent or treat GVHD, or to prolong survival.

Christensen et al. [131] examined the impact of timing and dose of donor-derived MSCs on kinetics of GVHD in 2 murine models of GVHD (MHC-mismatched model: UBI-GFP/BL6 [H-$2^{b/b}$] into BALB/c [H-$2^{d/d}$], and minor histocompatibility mismatched model: UBI-GFP/BL6 [H-$2^{b/b}$] into BALB.B [H-$2^{b/b}$]) [131]. Bone marrow–derived MSCs from UBI-GFP/BL6 mice were infused i.p. at the doses of 4×10^5 or 1×10^6 MSCs/mice. In the MHC-mismatched model, i.p. infusion of 4×10^5 donor MSCs/mice on day 1 after transplantation delayed GVHD mortality from 6.6 ± 0.13 days to 12.0 ± 1.7 days ($P < .001$). In the minor histocompatibility mismatched model, i.p. infusion of 4×10^5 donor MSCs/mice on day 1 after transplantation postponed mortality from GVHD from 31.6 ± 2.6 days to 50.5 ± 8.8 days ($P < .05$), and even prevented death from GVHD in 30% of MSCs infused mice. Interestingly, mice given 1×10^6 donor MSCs/mice i.p. on day 1 died sooner than controls (21.7 ± 1.2 days versus 31.6 ± 2.6 days, $P < .01$). Finally, MSC infusions were unable to resolve established GVHD.

Tisato et al. [131] assessed the ability of cord blood–derived human MSCs to prevent GVHD in a model of xenogeneic GVHD into NOD/SCID mice. All mice given 20×10^6 human peripheral blood mononuclear cells (PBMCs) i.v. after sublethal irradiation (2.5 Gy TBI), and no MSCs developed signs of xenogeneic GVHD. Similar results were seen in mice receiving a single i.v. injection of human MSCs (3×10^6 MSCs/mice) on day 0. In contrast, mice given 4 doses of MSCs at weekly intervals (total dose of 12×10^6 MSCs) did not develop GVHD and had significantly less activated human T cells than mice not given MSCs or given MSCs only at day 0. However, no therapeutic benefit was observed when MSCs were administered at onset of GVHD.

Finally, we evaluated the ability of bone marrow–derived human MSCs to prevent xenogeneic GVHD in NOD/SCID/γ_C^{null} (NSG) mice infused i.p. with 30×10^6 human PBMC after sublethal irradiation (n = 30) [133]. Although MSCs exhibited potent immunosuppressive properties in vitro, i.p. injection of 3×10^6 bone marrow–derived human MSCs at time of PBMC injection and on days 7, 14, and 21 thereafter (for a total of 12×10^6 MSCs) failed to reduce in vivo T cell proliferation and failed to prevent death from GVHD.

Taken together, these studies suggest that a single injection of (nonactivated) MSCs given on day 0 failed to prevent GVHD in most mice models, whereas repeated MSCs injections at the time of and after transplantation showed clinical benefits in some (but not all) GVHD models, depending on the origin of MSCs, the timing of infusion, as well as the dose of MSCs infused. Further, engraftment of MSCs at sites of GVHD was either very low or completely absent, suggesting that beneficial effects of MSCs were not mediated by differentiation of MSCs at the site of GVHD reactions.

Preclinical Dog Model

Although mouse studies have proven unevaluable for studying GVHD biology and physiology [134,135], it has been known since the late 1960s that GVHD reactions in humans and random-bred large animals are more violent than in inbred rodents [136]. The wide genetic diversity, well-mixed gene pool, and the outbred nature of dogs have made them a particularly suitable model for preclinical transplantation studies including understanding of graft-versus-host and host-versus graft reactions, and developing clinically relevant regimens for allogeneic HCT and for GVHD prevention [136]. This prompted Lee et al. [137] and Mielcarek et al. [94] to investigate the impact of MSC infusions on both engraftment and GVHD in dogs.

As observed with human bone marrow–derived MSCs, canine bone marrow–derived MSCs could be obtained after 2 to 3 weeks of culture, expressed CD29, CD44, CD73, CD90, and CD106 but not CD45, CD34, or CD14, and strongly inhibited mixed lymphoid reactions independently of dog leukocyte-antigen (DLA) matching between MSCs and effector cells, and independently of cell-to-cell contact but requiring communication between PBMCs and MSCs [137]. However, in contrast to human MSCs, dog MSCs did not express CD105.

Mielcarek et al. [94] infused ^{111}In-labeled MSCs into a beagle and analyzed their in vivo distribution using a gamma camera. Although ^{111}In-labeled MSCs accumulated in the lung immediately after infusion,

they left the lungs after 24 hours and preferentially redistributed to liver and spleen where residual label could be detected up to 9 days after infusion. Weaker signals were detected in gut and bone marrow.

Model of engraftment in the MHC-identical setting after nonmyeloablative conditioning

Consistent and sustained mixed donor–host chimerism has been achieved in dogs given DLA-identical marrows after a small dose of 2 Gy TBI and postgrafting immunosuppression with cyclosporine for 35 days plus either mycophenolate mofetil (MMF) or rapamycin (sirolimus) for 28 days [138,139]. When the TBI dose was decreased to 1 Gy in this setting, all dogs engrafted initially but eventually experienced graft rejection, demonstrating a delicate balance between host-versus-graft and graft-versus-host reactions [138,139]. The most likely role of TBI in that model was host immunosuppression rather than creation of marrow space (reviewed in [140]). Indeed, successful sustained allografts were accomplished in dogs given 1 Gy TBI conditioning, which had been "sensitized" against donor PBMC in the presence of T cell postimulatory blockade with CTLA4-Ig [141] or with an antibody directed against CD154 [142]. Also, dogs conditioned with 4.5 Gy irradiation to the cervical, thoracic, and upper abdominal lymph node chain instead of TBI [143], and those conditioned with T cell ablation using a bismuth-213-labeled (α emitter) anti-T cell receptor-$\alpha\beta$ monoclonal antibody [144] had sustained marrow engraftment. Further, a single donor lymphocyte infusion given 28 to 36 days either with (n = 5) or without (n = 4) preceding treatment with the immunosuppressive drug pentostatin allowed sustained engraftment in 3 of 9 dogs given DLA-identical marrows after 1 Gy TBI and postgrafting immunosuppression with cyclosporine and MMF [145].

Based on these observations, Lee et al. [137] investigated whether donor bone marrow–derived MSC infusions on the day of marrow grafting (1.2-1.8 $\times$ 10^6 MSCs/kg) and on day 35 (the day of cyclosporine discontinuation, 1.1-1.3 $\times$ 10^6 MSCs/kg) prevented graft rejection in dogs given DLA-identical marrows after 1 Gy TBI and postgrafting immunosuppression with cyclosporin and MMF. Even though the MSCs infused strongly inhibited lymphocyte culture reactivity in vitro (median inhibition index of 71%, range: 32%-92%), they failed to prevent graft rejection in vivo in all 4 studied dogs (Figure 1A).

Model of GVHD in the MHC-haploidentical setting

Mielcarek et al. [94] assessed the ability of canine MSCs to prevent graft rejection and GVHD in dogs given marrows from DLA-haploidentical donors after 9.2 Gy TBI without pharmacologic postgrafting immunosuppression. MSCs consisted of either a combination of 3 different immortalized MSC lines (15-30 $\times$ 10^6 MSCs/kg/day administered i.v. 2-5 times/week, n = 12) or third-party primary MSCs (1 $\times$ 10^6 MSCs/kg/day administered i.v. 3 times/week, n = 3). Graft rejection occurred in 7 of 14 analyzable dogs (including 2 of 3 dogs given third-party primary MSCs), whereas the 7 remaining dogs succumbed to

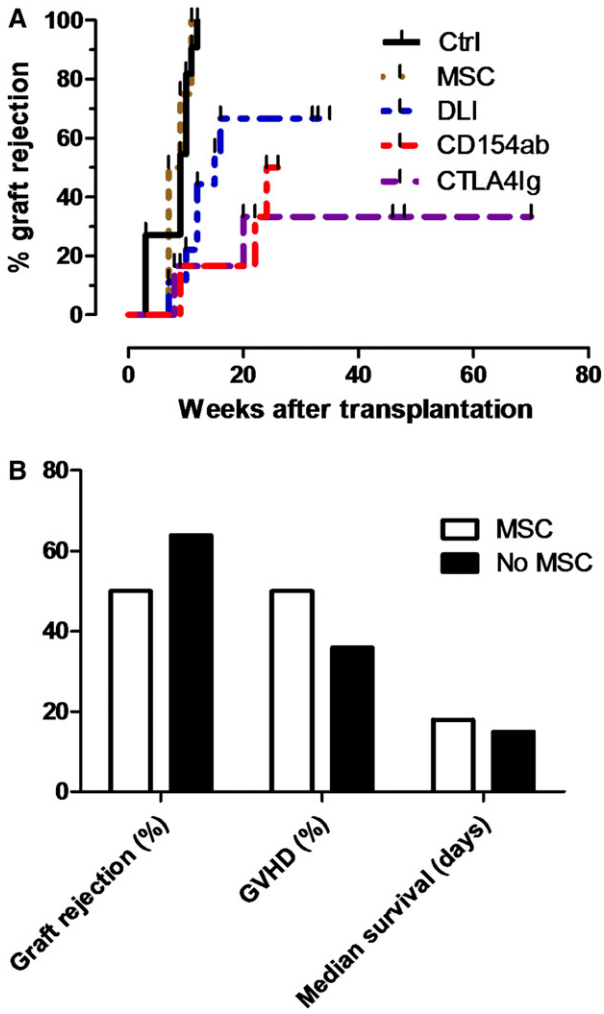

Figure 1. (A) Cumulative incidents of graft rejection in dogs given DLA-identical marrows after 1 Gy TBI and postgrafting immunosuppression with cyclosporin plus mycophenolate mofetil (or rapamycin) according to various additional approaches of cellular therapy [Ctrl, no additional therapy, n = 11 [138,139]; MSC, infusions of mesenchymal stem cells (1.1-1.8 $\times$ 10^6 MSC/kg) on days 0 and 35 after transplantation, n = 4 [137]; DLI, donor lymphocyte infusion (1-2.6 $\times$ 10^8 CD3/kg) around day 35 with or without preceding pentostatin, n = 9 [145]; CTLA4Ig, injection of donor PBMC before transplantation with costimulation blockade with CTLA4Ig, n = 5 [141]; and injection of donor PBMC before transplantation with costimulation blockade with CD154 antibodies, n = 6 [142]. Although CTLA4Ig ($P < 0.001$), CD154ab ($P = 0.001$), and DLI ($P = 0.001$) somewhat increased the rate of stable engraftment, graft rejection occurred at a similar rate in dogs given MSCs and in control dogs ($P = .7$). (B) Incidents of graft rejection and of GVHD as well as median number of days of survival in dogs given DLA-haploidentical marrows after 9.2 Gy TBI with or without added MSC infusions (2-3 infusions/week of 1-30 $\times$ 10^6 MSC/kg) after transplantation [94].

GVHD 13 to 18 days after transplantation. These results are comparable to what has been achieved in dogs given DLA-haploidentical marrows after 9.2 Gy TBI without MSCs administration (Figure 1B). Interestingly, without previous ex vivo expansion of stromal elements, MSCs could not be detected by polymerase chain reaction in the dog tissues at necropsy. Further, MSCs could be detected in the bone marrow, spleen, and lung of only 2 of 6 dogs after previous ex vivo expansion of stromal elements collected in the dog tissues at the time of necropsy, suggesting that the frequency of tissue-based MSCs was very low.

Taken together, these data show that, although canine MSCs had phenotype and immunosuppressive abilities comparable to human MSCs, they failed to promote engraftment or prevent aGVHD, even when repeated injections of high doses of MSCs were used.

MSCs IN HUMANS

In the HCT setting, MSCs have been infused with the aims of promoting engraftment, preventing/treating GVHD, or promoting healing of regimen-related toxicity (recently reviewed in [146]). As observed in murine models, there has been no evidence in humans that i.v. injection of MSCs was followed by MSCs engraftment and differentiation in organs affected by GVHD. Instead, it has been postulated that MSC infusions could modulate inflammation and immune responses by secreting soluble mediators, and perhaps by inducing immune tolerance because activated MSCs express HLA-class II molecules without expressing costimulatory molecules [97,147].

Importantly, i.v. infusion of MSCs appeared to be safe, with no infusional toxicity (and, in particular, no report of pulmonary embolism), MSC-derived malignancy, or ectopic tissue formation being reported thus far in any of the studies discussed below. However, long-term follow-up of patients given MSCs is important in order to detect possible late effects.

Ex Vivo Expansion of Human MSCs

It should be emphasized that human MSCs are not a homogeneous population and that some properties of human MSCs do change with increasing numbers of cell divisions [120]. In the early 1980s, Mets et al. observed that the cells that initially adhere to tissue culture surfaces in early passage, low-density cultures were spindle shaped and very rapidly proliferating [148]. Those cells were termed type 1 cells or rapidly self-renewing MSCs. The author then observed that rapidly self-renewing MSCs gave rise to larger cells, termed type 2 cells or slowly replicating MSCs. Lee et al. [149] recently identified 2 antibodies, 1 recognizing PODXL and another recognizing CD49f, that allowed identifying the early progenitors in cultures of MSCs. $PODXL^{hi}/CD49f^{hi}$ cells were more efficient than $PODXL^{lo}/CD49f^{lo}$ cells both in generating single cell-derived colonies and in differentiating in culture, but they were less likely to produce lethal pulmonary emboli and survived longer in the lung of SCID mice after intravenous infusion. Unfortunately, the impact of MSC composition (type 1 versus type 2) on their efficacy to prevent or treat GVHD has not been assessed thus far.

Another concern with long-term culture of MSCs is the observation that human MSC senescence could occur as early as after 2.6 cumulative population doubling (4 "passages") with some MSC donors, with senescent cells having lost most of their plasticity and clonogenicity properties [150]. These data should be kept in mind when analyzing preliminary data of MSC infusions in humans because some studies transplanted MSCs after 2 to 3 passages in plates [151], whereas in other studies, MSCs were expanded more extensively in large bioreactors [152].

Because MSCs are believed to act mainly by secreting soluble factors, the impact of MSC preparation (MSC origin, culture media, type of serum supplementation, and extent of ex vivo expansion) on their ability to produce specific soluble factors deserves further investigations. Further, given that activation of MSCs by IL-1 impacts on their cytokine expression and production [153], the inflammatory status of patients at the time of MSC infusion is also likely to influence the biological activities of MSCs.

European centers interested in clinical trials of MSC infusion have created a European Group for Blood and Marrow Transplantation (EBMT) "MSC expansion consortium," to define common protocols for MSCs isolation and expansion procedures, as well as common release criteria, enabling large-scale multicenter trials with comparable MSC products [151]. Briefly, this procedure consists of culture of bone marrow adherent mononuclear cells in 10% fetal bovine serum (FBS) containing medium. Cells are passaged when cultures are near confluence and then are harvested at passage 1 to 4. Release of MSC criteria include absence of visible clumps, spindle-shape morphology, absence of contamination by pathogens, viability >95%, and immune phenotyping showing expression of CD73, CD90, and CD105 surface molecules (>90%) and absence of CD34, CD45, CD14, and CD3 [151]. MSCs could be either infused freshly or after cryopreservation in 10% dimethyl sulfoxide (DMSO).

Several studies discussed below infused MSC products termed Prochymal® produced by Osiris Therapeutics®, Inc. (Columbia, MD). In 1 of these studies, all MSCs infused (124 infusions in 12 children) were derived from bone marrows of 4 healthy volunteer donors [154], suggesting quite extensive expansion of MSCs ex vivo. Adherent cells were grown as

symmetric fibroblastic colonies, leading to a homogeneous cell population positive for surface antigens including CD29, CD73, CD90, and CD105 and negative hematopoietic markers including CD14, CD34, and CD45. The cells were cryopreserved in 5% human serum albumin and 10% DMSO-containing medium. Final lots were tested for potential viral pathogens, mycoplasma, sterility, endotoxin, cell identity, purity, and viability before being released for clinical use.

Although many regulatory agencies have not forbidden the use of FBS in the culture medium of MSCs, several groups of investigators have developed FBS-free MSC expansion protocols based on the use of human plasma, or human platelet lysate [155-157]. For example, Lucchini et al. [156] expanded MSCs from nucleated cells isolated from the washouts of sealed bone marrow collection bags and filters in platelet-lysate-containing medium. Release criteria included: lack of detectable microbial contamination, cell viability ≥90%, endotoxin levels in the final product ≤5 EU/kg, normal karyotype, and inability to grow without anchorage in a semisolid fluid. Phenotyping release criteria were less stringent than those used by other groups of investigators and included ≥70% expression of CD73, CD90, and CD105 and ≤10% expression of CD14, CD34, and CD45.

Given that these differences in MSC isolation and culture expansion conditions are likely to influence MSC behavior after infusion to patients [158,159], one should remain cautious when comparing results of clinical studies performed with MSC products prepared with different protocols.

Engraftment of Donor MSCs after Allogeneic HCT

Although marrow grafts contain approximately 10,000 MSCs/kg of recipient weight [76,77], stromal progenitor cells remain host in origin after allogeneic HCT in adults, even after 27 years of complete donor hematopoietic chimerism [160,161], suggesting that donor MSCs have a limited role in reconstituting the marrow microenvironnement after HCT in adults. In contrast to what has been observed in adult recipients, engraftment of donor MSCs has been demonstrated in approximately 30% of pediatric patients given allogeneic cord blood or marrow grafts (Figure 2) [162]. Why MSCs sometimes engraft in children but not in adults has remained unexplained thus far.

Cotransplantation of MSCs and Autologous HCT

Koc et al. [163] analyzed the safety and feasibility of infusing bone marrow–derived autologous MSCs in combination with autologous PBSC (containing a median of 13.9×10^6 $CD34^+$ cells/kg) in 32 patients

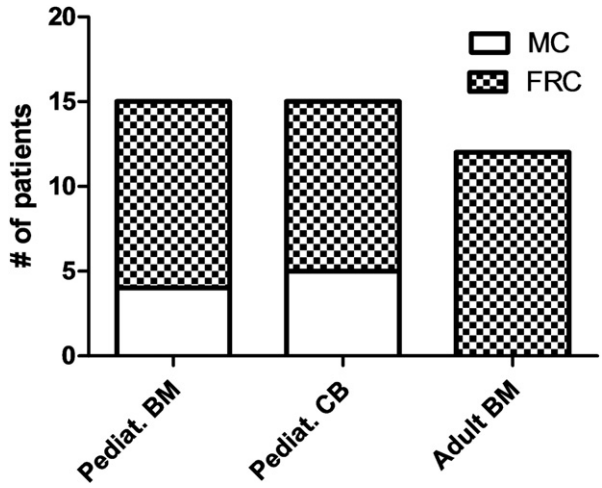

Figure 2. MSCs chimerism 1-192 (median 5) months after allogeneic bone marrow or cord blood transplantation in the study reported by Pozzi et al. [162]. MSCs were analyzed after a median of 4 passages and were positive (≥98% positivity) for CD73, CD105, CD106, CD29, CD13, and CD44 and negative (≤1%) for CD34, CD45, and CD14. MSC chimerism was determined by amelogenin assay (in case of female donor to male recipient) or by STR PCR. Pediat., pediatric recipients; BM, bone marrow; CB, umbilical cord blood; MC, mixed donor/host chimerism; FRC, full recipient chimerism.

given high-dose chemotherapy as treatment for breast cancer. Expansion cultures allowed obtaining $>1 \times 10^6$ MSCs/kg for all patients after 2 to 6 passages. Twenty-eight patients were given 1 to 2.2×10^6 autologous MSCs/kg intravenously. Clonogenic MSCs were detected in venous blood up to 1 hour after infusion in 13 of 21 tested patients (62%). No toxicities related to MSC infusions were observed. Median times to achieve a neutrophil count >500/μL and a platelet count ≥20,000/μL were 8 days (range: 6-11 days) and 8.5 days (range: 4-19 days), respectively. Based on these observations, the authors concluded that MSC infusions might have a positive impact on hematopoiesis after autologous HCT and should be tested in phase III randomized studies. Unfortunately, as of yet, no large randomized studies aimed at testing this hypothesis have materialized.

Cotransplantation of MSCs and Allogeneic HCT

A number of studies have assessed the impact of MSC infusion at the time of HCT on engraftment and GVHD (Table 2). Lazarus et al. [164] conducted a study of cotransplantation of MSCs and HLA-identical sibling marrows or PBSC after myeloablative conditioning in 46 patients. Postgrafting immunosuppression consisted of cyclosporine and methotrexate (the latest given at days 1, 3, and 6). MSCs (cultured from bone marrow aspirates from the hematopoietic stem cell donor) were administered 4 hours before HCT in a dose escalation scheme at 1.0 (n = 20), 2.5 (n = 21), or 5.0 (n = 5) $\times 10^6$ MSCs/kg. There was no impact of the dose of MSCs infused on the speed

Table 2. Studies of Cotransplantation of Allogeneic Hematopoietic Stem Cells and MSCs in Humans

Authors [References]	No. of Patients/Study Phase	Stem Cell Source	MSC Dose $\times 10^6$/kg	Main Observations
Lazarus et al. [164]	46/I-II, single arm	BM or PBSC from HLA-identical siblings	1-5	- MSCs cotransplantation feasible and safe. - MSCs donor chimerism (>1%) was evinced in 2 of 18 patients in whom BM MSCs chimerism was assessed after HCT.
MacMillan et al. [165]	15/I-II, single arm	Unrelated cord bloods	0.9-5.0*	- MSC cotransplantation feasible and safe.
Ball et al. [166]	14/I-II, single arm	HLA-disparate $CD34^+$ cells	1-3.3	In comparison to a group of 47 matched historical controls: - faster leukocyte (and NK cell) recovery - a trend for lower risk of graft rejection (0% versus 15%)
Bernardo et al. [167]	13/I-II, single arm	Unrelated cord bloods	1-3.9	In comparison to a group of 39 matched historical controls: - comparable risk of graft rejection - less grade III-IV aGVHD ($P = .05$) - similar survival and relapse incidence
Ning et al. [168]	30 (10 given MSC); II randomized	PBSC and/or BM from HLA-identical siblings	0.03-1.5	- Lower incidence of grade II-IV aGVHD but higher risk of relapse ($P = .02$) in MSC patients (n = 10) than in controls (n = 15).
Baron et al. [169]	20/I-II, single arm	PBSC from HLA-mismatched donors	1-2	- Cotransplantation of HLA-mismatched PBSC and third-party MSC is feasible with an acceptable nonrelapse mortality (10% at 1 year).

BM indicates bone marrow.
*Three patients received a second MSC infusion on day 21.

of hematopoietic engraftment. The incidents of grade II-IV and grade IV aGVHD were 28% and 4%, respectively, whereas 22% of patients who survived at least 90 days after HCT experienced extensive cGVHD. Two-year overall survivals (OS) and progression-free survivals were 78% and 53%, respectively. Interestingly, the authors were able to demonstrate MSCs donor chimerism in only 2 of 18 patients in whom MSC chimerism was assessed from posttransplantation bone marrow aspirates.

In an attempt to speed hematopoietic recovery after umbilical cord blood transplantation, 15 pediatric patients with high-risk acute leukemia were enrolled by MacMillan et al. [165] in a phase I-II clinical trial in which MSCs from HLA-haploidentical parental donors were infused at the time of transplantation. Seven patients did not receive MSC infusions because transplantation did not occur (n = 3) or occurred at a different center (n = 1) or because of insufficient MSC availability at the time the patient was ready for transplantation (n = 3). The median number of MSCs infused on day 0 was 2.1×10^6 (range: 0.9-5.0×10^6) MSCs/kg, whereas 3 patients received a second dose ranging from 0.06 to 5×10^6 MSCs/kg on day 21 after HCT. The 8 MSC patients achieved neutrophil engraftment at a median of 19 days (range: 9-28 days) after HCT. Probability of platelet engraftment was 75%, at a median of 53 days (range: 36-98 days) after HCT. Three of 8 patients experienced grade II aGVHD, whereas no patient developed cGVHD. Interestingly, no evidence of MSC donor chimerism was detectable in any patient at any time point after HCT.

Ball et al. [166] cotransplanted donor MSCs in 14 children undergoing transplantation of $CD34^+$ selected cells from HLA-disparate related donors. MSCs (isolated from HSC donor bone marrows) were collected at passage 3 or less and given at a dose of 1.0-3.3 $\times 10^6$ cells/kg. No MSC infusion-related toxicity was observed. All 14 patients achieved sustained engraftment, whereas graft failure occurred in 7 of 47 comparable historic patients not given MSCs ($P = .14$). Further, counts of NK cells were higher in MSC-treated than in historical patients 1 month after HCT (497 versus 252/μL, $P = .02$). The authors concluded that MSC cotransplantation might reduce the risk of graft failure after allogeneic HCT with HLA-disparate donors, possibly because of their immunosuppressive effects on recipient immune cells having survived the conditioning regimen.

The same group of investigators cotransplanted parental HLA-disparate MSCs in 13 children with hematologic disorders undergoing unrelated cord blood transplantation after myeloablative conditioning [167]. HCT outcomes were compared with those observed in a cohort of 39 matched historic controls. The cumulative incidence of graft rejection was 15% in the MSC group versus 3% in the historic group (NS), whereas the tempo of neutrophil and platelet engraftments was comparable between the 2 groups. The incidence of grade III-IV aGVHD was 0% in the MSC group versus 26% in the historic group ($P = .05$), whereas the cumulative incidence of cGVHD was 0% in the MSC group versus 11% in the historic group (NS). Finally, 3-year OSs were 63% and 64% in the MSC and control groups,

respectively, whereas the 3-year cumulative incidents of relapse were 25% and 23%, respectively.

Ning et al. [168] performed a randomized clinical trial including 30 patients given marrows and/or PBSC from HLA-identical siblings with (n = 15) or without (n = 15) added MSCs after myeloablative conditioning. The median dose of MSCs given was relatively low (3.4 [range: 0.3-15.3] $\times$ 10^5/kg), and the authors failed to grow sufficient numbers of MSCs in 5 of the 15 patients randomized to the MSC arm. The authors compared the outcomes of the 10 patients given MSCs with those observed in the 15 patients randomized in the control arm. The only patient with acute myelogenous leukemia in relapse at transplantation was in the MSC arm. One of 10 patients in the MSC arm versus 8 of 15 in the control arm experienced grade II-IV aGVHD. However, patients given MSCs had higher risk of relapse (60% versus 20% at 3 years; $P = .02$), and lower disease-free survival (30% versus 67% at 3 years; $P = .035$) than those in the control arm.

Baron et al. [169] reported the results of a pilot study analyzing the safety of MSC cotransplantation in 20 patients given HLA-mismatched PBSC after fludarabine and 2 Gy TBI [169]. MSCs were collected after 3 or less passages and given at doses of 1-2 $\times$ 10^6 MSCs/kg. The primary endpoint was safety, defined as a 100-day incidence of nonrelapse mortality <35%. The 100-day cumulative incidence of grade II-IV aGVHD was 35%, whereas 65% of the patients experienced moderate/severe cGVHD. One-year nonrelapse mortality (10%), relapse (30%), OS (80%), progression-free survival (60%), and 1-year incidence of death from GVHD or infection with GVHD (10%) were encouraging. Transplantation outcomes in these 20 patients were compared with those observed in a historic group of 16 patients given HLA-mismatched PBSC (but no MSCs) after the same nonmyeloablative conditioning. One of 20 patients in the MSC group versus 0 of 16 patients in the historic group experienced graft rejection. The 1- and 2-year probabilities of dying from GVHD or infection while on treatment for GVHD were 10% and 10%, respectively, in the MSC group, versus 31% and 38%, respectively, in the historic group ($P = .04$).

Taken together, these data suggest that MSC cotransplantation does not seem to promote engraftment or prevent graft rejection in the setting of T cell–replete transplantation, whereas it might be beneficial in the setting of HLA-disparate T cell–depleted HCT, perhaps because of their immunosuppressive effects on recipient immune cells that survived the conditioning regimen. Further, 3 studies [167-169] observed lower risks of aGVHD in patients cotransplanted with MSCs than in historic (n = 2) or concurrent (n = 1) controls, suggesting that MSCs might help reduce aGVHD. However, these observations should be taken with some caution given the small number of patients included in these pilot studies. Prospective double-blind randomized studies are ongoing in order to more definitely assess the impact of MSC cotransplantation on GVHD incidence and severity.

MSCs as Front-Line Treatment for Grade II-IV aGVHD

As mentioned above, corticosteroids produce durable complete responses in 20% to 75% of the patients with grade II-IV aGVHD [66-68] and less in patients with lower gut aGVHD [68]. Many attempts at intensifying the front-line treatment for grade II-IV aGVHD failed. Specifically, the addition of ATG, daclizumab, etanercept, denileukin diftitox (denileukin), or pentostatin to methylprednisolone failed to significantly improve response rates, and were often associated with higher transplant-related mortality [170-172]. A phase II 4-arm randomized study was recently performed with the aim of identifying the most promising agent (in addition to standard corticosteroids) for initial therapy for grade II-IV GVHD [172]. Patients were randomized to receive methylprednisolone 2 mg/kg per day plus etanercept, MMF, denileukin, or pentostatin. Day 28 complete response rates were etanercept 26%, MMF 60%, denileukin 53%, and pentostatin 38%, suggesting that MMF plus corticosteroids was the most promising regimen to compare against corticosteroids alone in a definitive phase III trial.

A recent randomized multicenter phase II study has evaluated 2 different doses of MSCs (Prochymal®) given in combination with standard corticosteroid therapy for the initial treatment of aGVHD [152]. MSCs derived from 6 different donors were expanded for a total of 5 cell passages. Thirty-two adult patients with grade II (n = 21), grade III (n = 8), or grade IV (n = 3) aGVHD were randomized to receive 2 doses of either 2 or 8 million MSCs/kg each in combination with corticosteroids. One patient withdrew consent on study day 10 and was not included in the analysis. The first MSC infusion was given within the 48 hours following diagnosis of grade II-IV aGVHD, whereas the second MSC infusion was given 3 days after the first. A total of 62 MSC products were infused. Ninety-four percent of patients achieved complete (77%) or partial (16%) responses to MSCs and corticosteroids. Interestingly, the response rates were similar in patients given 2 or 8 million MSCs/kg (Figure 3A), although the trial was not designed to detect a potential difference between the 2 MSC doses. The authors concluded that the addition of MSCs to corticosteroids might induce a high response rate in patients with grade II-IV aGVHD, and that a dose of 2 $\times$ 10^6 MSC/kg should be tested in a phase III placebo-controlled trial. Preliminary results of such a randomized multicenter phase III trial with injection

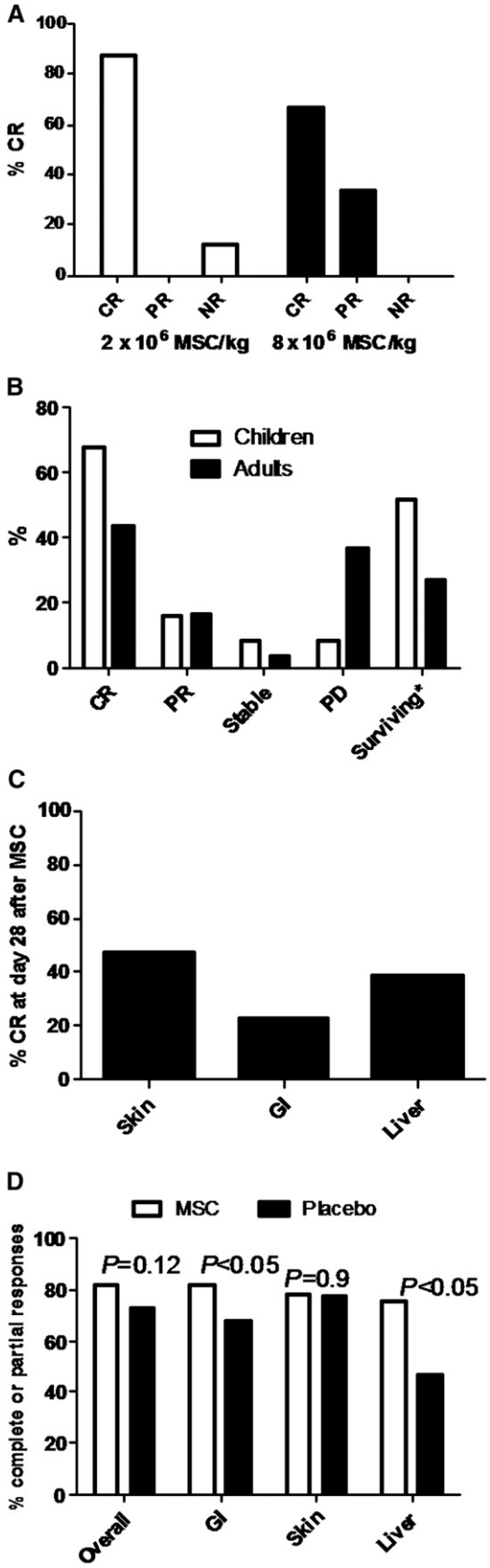

Figure 3. (A) Complete response (CR), partial response (PR), and no response (NR) rates in patients given methylprednisolone (2 mg/kg) plus MSCs (prochymal^{R}) at the dose of 2 $\times$ 10^6/kg (n = 16) or of 8 $\times$ 10^6/kg (n = 15) as first-line treatment for grade II-IV aGVHD [152]. A second infusion of MSCs at the same dose was done 3 days following the first MSC infusion. (B) Percentage of responses to MSC infusion (27 patients received 1 infusion, 22 received 2 infusions, and 6 received ≥3 infusions of a median dose of 1.4 [range: 0.4-9] $\times$ 10^6 MSCs/kg) in adults or children with steroid refractory aGVHD reported by Le Blanc et al. (EBMT survey) [151]. (C) Complete response rate according to the organ affected in 59 pediatric patients with steroid-refractory aGVHD treated with MSCs (produced by Prochymal®; 8 infusions of 2 $\times$ 10^6 MSC/kg over 4 weeks followed by an additional 4 infusions administered weekly after day 28 in patients who had a partial response) (this study has been reported only in an abstract form [176] thus far). (D) Overall complete or partial response rates in patients with steroid-refractory grade II-IV aGVHD treated by second-line therapy with added MSCs (produced by Prochymal®; 8 infusions of 2 $\times$ 10^6 MSC/kg over 4 weeks followed by an additional 4 infusions administered weekly after day 28 in patients who had a partial response) or placebo (this study has been reported only in an abstract form [178] thus far).

of MSCs (Prochymal; at the dose of 2 $\times$ 10^6 MSC/kg) versus placebo in addition to standard corticosteroid therapy as primary treatment for patients with grade II-IV aGVHD have been released (although the results of final analysis are pending); the trial failed to reach the primary endpoint of durable complete response ≥28 days [173].

MSCs as Treatment for Steroid-Refractory aGVHD

Although dynamics of treatment responses differ between target organs (faster improvement in skin than in liver or gut aGVHD), criteria for steroid refractory GVHD have included: progression of GVHD on day 3 after initiation of steroids, no improvement of GVHD on day 7 after initiation of steroids, absence of complete resolution of aGVHD on day 14 after initiation of steroids, and/or relapse of aGVHD during or after steroid taper.

Based on the immunosuppressive abilities of MSCs, and on the apparent safety of infusion of ex vivo expanded MSCs, Le Blanc et al. [174] infused such cells in a 9-year-old boy who had refractory grade IV gut and liver aGVHD after HLA-matched unrelated HCT. Although his GVHD was refractory to (methyl)prednisolone, cyclosporine, infliximab, and daclizumab, infusion of 2 $\times$ 10^6 MSCs/kg from his HLA-haploidentical mother resulted in remarkable improvement, with normalization of stools and decline in bilirubin levels. However, long-term tolerance was not induced by MSC infusion because GVHD recurred after discontinuation of postgrafting immunosuppression, although, remarkably, GVHD symptoms resolved again after a second MSC infusion. This case report suggested that MSC infusion could be helpful in selected patients with steroid-refractory aGVHD.

The Developmental Committee of the European Group for Blood and Marrow Transplantation [151] reviewed data from 55 patients given MSCs (median dose 1.4 [range: 0.4-9] $\times$ 10^6 MSCs/kg) as treatment for steroid-refractory grades II (n = 5), III (n = 25), or IV (n = 25) aGVHD in a phase II study. No side

effects were seen after MSC infusions. Twenty-seven patients received 1 infusion, 22 received 2 infusions, 4 received 3 infusions, 1 received 4 infusions, and 1 received 5 infusions (total of 92 infusions). MSC donors were either HLA-identical siblings (n = 5), HLA-haploidentical relatives (n = 18), or third-party HLA-mismatched individuals (n = 69). Among the 55 patients, 30 had complete responses and 9 showed improvement. Responses were somewhat more frequent in children than in adults (Figure 3B; $P = .07$). Median time from MSC infusion to complete response was 18 (range: 3-63) days. Three patients had recurrent malignant disease and 1 developed de novo acute myelogenous leukemia of recipient origin. Complete responders to MSCs were more likely to be alive 2 years after HCT than patients with partial or no responses (52% versus 16%; $P = .018$). Interestingly, in the subset of patients given MSCs at the Karolinska Institutet, achievement of responses correlated to the number of MSC expansion passages. Specifically, patients given first- (n = 1) or second- (n = 7) passage MSCs had a higher response rate and a higher OS (50% versus 8% at 1 year, $P = .02$) than those given MSCs from passage 3 to 4 (n = 14) [175].

Prasad et al. [154] reported the results of a multicenter compassionate protocol evaluating the safety and efficacy of MSC (Prochymal®) infusions in 12 children with severe steroid-refractory aGVHD. MSCs (8×10^6 cells/kg/dose in 2 patients and 2×10^6 cells/kg/dose in the 10 remaining patients) were scheduled to be infused twice a week for 4 weeks, whereas partial and mixed responders received subsequent weekly therapy for 4 weeks. At the time of first MSC infusion, 5 patients had grade III and 7 patients grade IV gastrointestinal aGVHD. All 12 patients were steroid refractory and had failed a median of 3 (range: 2-5) other immunosuppressive therapies. A total of 124 MSC doses were administered with a median of 8 (range: 2-21) doses per patient. At day 32 after first MSC infusion, 9 patients had complete (n = 1) or partial (n = 8) responses, whereas overall, 9 patients achieved complete (n = 7) or partial (n = 2) responses. Two-year survival from first MSC infusion was 40% in the whole group (68% for the 7 patients who achieved complete responses, whereas the 5 patients who failed to achieve complete responses died within 100 days after first MSC infusion). The same group of investigators recently reported, in abstract form, data from 59 children with grade II (n = 6), grade III (n = 20), or grade IV (n = 33) steroid-refractory aGVHD given MSCs (Prochymal®) under a Food and Drug Administration expanded access program [176]. Patients received 8 infusions of 2×10^6 MSC/kg over 4 weeks, with an additional 4 infusions administered weekly after day 28 in patients who had partial responses. At day 28, overall response rate (defined as organ improvement of ≥1 stage without worsening in any other) was 64%, whereas 17% of patients had stable disease or mixed responses, and 19% had GVHD progression. The complete response rate per organ is shown in Figure 3C.

Von Bonin et al. [177] gave MSCs expanded in platelet lysate-containing medium in 13 patients with grade III (n = 2) or grade IV (n = 11) steroid refractory aGVHD. Median dose of MSCs was 0.9 (range: 0.6-1.1) $\times 10^6$/kg, and median number of MSC infusions was 2 (range: 1-5). MSC products had a median purity >97% in FACS analyses. Responses to first MSC infusion were observed in only 2 of 13 patients (1 complete response and 1 partial response on day 28 after first MSC infusion), whereas the remaining 11 patients required additional immunosuppressive therapy.

Lucchini et al. [156] treated 11 pediatric patients with steroid-refractory aGVHD or cGVHD by a total of 21 MSC infusions. MSCs were isolated from the washouts of the bone marrow collection bags and filters of a single donor and expanded in platelet lysate-containing medium. The median MSC dose infused was 1.2 (range: 0.7-3.7) $\times 10^6$/kg. Four of 11 patients achieved complete responses, but 2 of the 4 complete responders experienced GVHD recurrence 46 days and 95 days after MSC infusion.

The potential role of MSCs (Prochymal®) was also evaluated in addition to standard of care, including institutionally selected second-line treatment, in a randomized (2:1) trial in patients with steroid-refractory grade II-IV aGVHD. Patients received 8 infusions of 2×10^6 MSC/kg each over 4 weeks (or volume equivalent for placebo), with an additional 4 infusions administered weekly after day 28 in patients who had partial responses. Two-hundred forty-four patients with gastrointestinal (n = 179), skin (n = 144), and/or liver (n = 61) refractory grade II-IV aGVHD were included. There were more patients with grade IV aGVHD in the MSC arm (27 versus 16%), and therefore less patients with grade II (22 versus 26%) or III (51 versus 58%) aGVHD. Preliminary results have been reported in abstract form [178]. The study failed to demonstrate a significant difference in the rate of durable (for ≥28 days) complete responses between the 2 groups (primary endpoint; 35% in the MSCs versus 30% in the placebo group ($P = .3$) in intent to treat analysis, and 40% versus 28% ($P = .08$), respectively, in per protocol analysis). Looking at secondary endpoints, the overall complete or partial response rates at day 28 were higher in the MSCs than in the placebo group for patients with gastrointestinal and for those with liver aGVHD, but not for those with skin aGVHD (Figure 3D). The incidents of infection, relapse, and toxicities were similar in the MSCs and the placebo arms. These data suggest that the addition of MSCs might help improve patients with visceral steroid-refractory GVHD without inducing specific toxicities, but raise the question of the durability of GVHD responses achieved with MSCs.

Although most MSC studies in humans have investigated marrow-derived MSCs, Fang et al. [179] investigated the feasibility of infusing i.v. adipose tissue-derived MSCs in patients with severe aGVHD. Six patients with steroid-refractory grades III (n = 2) or IV (n = 4) aGVHD received 1 (n = 5) or 2 (n = 1) i.v. infusions of 1.0×10^6 MSCs/kg. No side effects were noted after the infusions. Five of 6 patients achieved complete responses, 4 of whom were still alive after a median follow-up of 40 months after MSC infusion.

Taken together, these studies suggest that MSC infusion has some beneficial activities in patients with steroid-refractory grade II-IV aGVHD, although one should be cautious about a possible bias for publishing preferentially positive studies. Further large randomized studies are ongoing in order to better define the impact of MSC infusion in patients with steroid-refractory aGVHD.

MSCs as Treatment for cGVHD

There are few data published thus far on the impact of MSC infusion in patients with cGVHD. Zhou et al. [180] reported data from 4 patients with severe sclerodermic cGVHD who received MSCs from unrelated donors expanded ex vivo. MSCs were injected into the bone marrow of the anterosuperior iliac spine at total doses of $1\text{-}2 \times 10^7$ cells for 4 to 8 infusions within a 22- to 52-day period. Concomitant medications for cGVHD were individualized for each patient and included tacrolimus, prednisolone, thalidomide, or MMF. The doses of these medications were tapered significantly after MSC infusion, whereas GVHD symptoms gradually improved in all 4 patients. More recently, Weng et al. [181] analyzed the impact of MSC infusion in 19 patients with refractory cGVHD. Bone marrow–derived MSCs from HLA-matched donors or HLA-disparate third-party adult donors and expanded in FBS-containing media were infused i.v. at a median dose of 0.6 (range: 0.2-1.4) $\times 10^6$ MSCs/kg. Patients received a median of 2 (range: 1-5) MSC infusions with a median duration between the first and second MSC infusion of 6 months. Fourteen of the 19 patients achieved partial (n = 10) or complete (n = 4) responses after MSC infusions, whereas the 2-year survival rate from the first MSC infusion was 78%. Taken together, these 2 reports might serve as the basis of prospective randomized studies assessing the impact of MSC infusions versus placebo in patients with steroid-refractory cGVHD.

CONCLUSIONS AND PERSPECTIVES

During the last decade, there has been a growing excitement by immunologists and clinicians for the use of MSCs in the prevention or treatment of GVHD. Although MSCs were originally thought to improve GVHD by promoting tissue healing through engraftment, differentiation, and long-term survival in injured tissues, it is now accepted that MSCs do not engraft in affected tissues but might instead decrease inflammation and perhaps induce immunomodulation by secreting soluble factors. Preclinical animal models of GVHD prevention or treatment by MSCs have yielded conflicting results. Specifically, repeated MSC infusions prevented death from GVHD or delayed GVHD mortality in some but not all murine models, whereas repeated MSC infusion failed to prevent GVHD in a preclinical canine model of transplantation. Similarly, it remains difficult to draw definitive conclusions about the efficacy of MSCs in the prevention or treatment of severe aGVHD in humans given that most published positive results are from phase I-II or phase II studies, whereas a large randomized study of MSC versus placebo infusions in patients with steroid-refractory aGVHD failed to demonstrate a higher durable GVHD complete response rate in the MSC arm. Further double-blinded randomized studies are ongoing to assess the impact of MSC infusion on GVHD prevention/treatment. Other studies are focused on determining the most efficient culture condition, dose, and schedule of MSC administration [182], and on analyzing the impact on MSC infusion on immune recovery after HCT. Because there is at yet no proof for higher efficacy of MSC over conventional approaches for preventing or treating GVHD, the use of MSCs should remain restricted to patients who are included in clinical trials.

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