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# Hitting the right spot with mesenchymal stromal cells (MSCs)

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## Abstract

Mesenchymal stromal cells or mesenchymal stem cells (MSCs) have captured considerable scientific and public interest because of their potential to limit physical and immune injury, to produce bioactive molecules and to regenerate tissues. MSCs are phenotypically heterogeneous, and distinct subpopulations within MSC cultures are presumed to contribute to tissue repair and the modulation of allogeneic immune responses. As the first example of efficacy, clinical trials for prevention and treatment of graft-versus-host disease (GVHD) after hematopoietic cell transplantation show that MSCs can effectively treat human disease. The view of the mechanisms whereby MSCs function as immunomodulatory and reparative cells has evolved simultaneously. Initially, donor MSC were thought to replace damaged cells in injured tissues of the recipient. More recently, however, it has become increasingly clear that even transient MSC engraftment may exert favorable effects through the secretion of cytokines and other paracrine factors, which engage and recruit recipient cells in productive tissue repair. Thus, an important reason to investigate MSCs in mechanistic preclinical models and in clinical trials with well defined end-points and controls is to better understand the therapeutic potential of these multifunctional cells. Here, we review the controversies and recent insights into MSC biology, the regulation of alloresponses by MSCs in preclinical models, as well as clinical experience with MSC infusions and the challenges of manufacturing a ready supply of highly defined transplantable MSCs.

### Keywords

mesenchymal stromal cells; mesenchymal stem cells; mesenchymal stem cell transplantation; hematopoietic stem cell transplantation; graft-versus-host disease; regenerative medicine

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> "… with each problem we solve, we not only discover new and unsolved problems, but we also discover that where we believed that we are standing on firm and safe ground, all things are, in truth, insecure and in a state of flux."
>
> K.R. Popper

## I. Biology

Non-hematopoietic cells in bone marrow have been known since the 1960s to have special properties including a remarkable capacity to expand rapidly *in vitro*[1]. For large periods of the 20th century however, stem cell biology was dominated by discoveries of the molecular circuitry that dictates the identity of bone marrow hematopoietic stem cells and their progressively more differentiated progeny. As a new conception of the functional progenitor cell hierarchy and regulatory network was being assembled from experimentation in animal models[2–3], it became clear that transplantation of relatively few hematopoietic stem cells was able to fully reconstitute the lymphohematopoietic system in conditioned recipients. While a complete understanding of the molecular mechanisms underlying successful hematopoietic cell transplantation (HCT) in animals remained elusive, its clinical application in blood and marrow transplantation for selected malignant and fatal non-malignant diseases over the last four decades has been an impressive success[4]. This was a hard act to follow. Nonetheless, the same goals of detailed understanding and clinical translation, have been extended to non-hematopoietic bone marrow cells.

### Central Dogma

Based on the initial definition these spindle-shaped cells derived from bone marrow attach to tissue culture plastic and form fibroblast colonies which enumerate progenitor cells termed colony-forming unit-fibroblast (CFU-F)[1]. They have been isolated predominantly from hematopoietic tissue, such as bone marrow, peripheral blood and umbilical cord blood but also from parenchymal non-hematopoietic tissues such as muscle, fat or liver[5–9]. They express surface proteins CD29 (integrin beta 1), CD44 (hyaluronate receptor), CD73 (SH-3/SH-4), CD90 (Thy- 1) and CD106 (vascular cell adhesion molecule-1) while they typically do not express hematopoietic cell markers, such as CD14 (monocyte surface protein), CD34 (mucosialin) and CD45 (common leukocyte antigen). As they can differentiate *in vitro* into cells resembling bone, cartilage and fat cells[10], their precursors in a differentiation hierarchy or continuum analogous to the one envisaged for the marrow hematopoietic compartment[11–12], were termed "mesenchymal stem cells" (MSCs).

### Areas of Uncertainty

The precise model illustrated above is complicated by the evidence that the majority of cells fitting the above criteria are not true long-lived self-renewing stem cells but rather a mixture of diverse cell types of uncertain proliferative and differentiation potential. Even though rare cells capable of mesenchymal trilineage differentiation into osteocytes, chondrocytes and adipocytes on a clonal level are present in early cultures, the majority of MSCs are bipotent or unipotent[6, 13–15]. The limitation of the unified MSC model is further evidenced by multiple terms used to describe these cells, such as marrow stromal cells, mesenchymal stem cells, mesenchymal stromal cells or multipotent stromal cells, as well as by efforts of several groups to separate and define MSC subpopulations with superior "stemness", such as unrestricted somatic stem cells, embryonic-like stem cells and very small embryonic–like cells[16–18].

Hence, from the practical standpoint experimental data have to be interpreted cautiously since the same term, MSCs, may denote cells that are very different from each other due to the isolation technique used, variations in the cell expansion protocol and passage number

(e.g., the progeny of 10 cells cultured on a surface of 1 $cm^2$ or in a large bioreactor both represent a single passage), and topographical specifics, i.e., MSCs isolated from different tissues and organs appear distinct[19–20]. Furthermore, extrapolation of the multi-differentiation potential of MSCs *in vitro* to their *in vivo* behavior has been lacking, and, despite similarities with cells located on the abluminal site of blood vessels (pericytes) and the concept of MSCs as parenchymal tissue-resident stem cells, the identity and function of MSCs *in vivo* remains an enigma[21–25]. Just as importantly, despite several intriguing possibilities[26], there are no definitive human markers that have been widely used for prospective isolation of all MSC populations.

## Paradigm Lost

The convenient but unfortunate term "MSCs" has been used to describe virtually any *ex vivo* expanded stromal cell population. Thus, MSC cultures are internally heterogenous, different from each other and potentially biologically distinct from the in vivo populations from which they were obtained. Critically and as discussed below, these committed progenitors with admixture of self-renewing, multipotential stem cells do not have to be pure stem cells to be clinically useful[27]. In fact, this can make them safer to use. It is primarily to avoid over-interpretation of experimental findings, that new descriptors that better characterize cell subtypes within the array of cells termed "MSCs" will be needed to supplant the ones in use. Despite years of effort to illuminate the functional complexity of specific cellular subpopulations concealed in the bulk MSC cultures the term "MSCs" is likely here to stay for now. Thus, we prefer to use the term "mesenchymal stromal cells" and reserve "mesenchymal stem cells" for true self-renewing stem cells (and abbreviate as MSCs for either)[10].

The prospect of MSCs remains exciting and, in another parallel with the hematopoietic cell biology and HCT, the conceptual simplification should not detract from the significant clinical expectations associated with functional aspects of MSCs, namely, immune modulation (discussed in the next sections), their reparative potential and their capacity to be gene-modified for purposes of disease-specific and patient-specific cellular therapy.

## Tissue Repair

It has been known that MSCs home to injured myocardium, lung, pancreas, skin and bone, and aid in tissue regeneration[19, 24]. It was once thought that donor MSCs repair injured organs primarily by robust replacement of the damaged cells of the recipient. We now know that at least in the setting of acute injury this is not the case, at least not entirely. Prockop and others showed that in response to injury, MSCs secrete large quantities of bioactive molecules, such as cytokines, antioxidant and pro-angiogenic substances, trophic factors, and other proteins (e.g., a peptide hormone stanniocalcin) able to mediate productive repair by limiting stress response and apoptosis and by recruiting the immune and reparative cells of the recipient[19, 28–29].

While the two mechanisms of action (paracrine effect of MSCs and cell replacement by MSC-derived cells) are not exclusive of each other, the difference is fundamental, as the former concept clears the way to isolate the MSC-derived "healing" factors, and infuse these factors after injury without the risks of cellular transplantation to induce and reinforce fast and transient tissue repair.

## MSC Transplantation

Reasoning that, for other clinical indications–such as for systemic correction of soluble or structural protein deficiency[30–33]–a more permanent effect is desired, the secretory nature of MSCs can be harnessed to establish a platform for patient-specific transplantation with

gene-corrected MSCs that can be administered in vivo and provide a source of missing or defective protein for patients with congenital or acquired deficiency disorders (e.g., enzymopathies, hemophilia and extracellular matrix disorders). However, there are obstacles to be overcome. For example, the choice of the optimal vector for safe transgenesis by viral or non-viral means remains a matter of debate, and the persistence and efficiency with which donor MSCs and their progeny can integrate into the targeted tissues and ameliorate disease are unknown.

First among these is the capacity of some MSC cultures to form tumors, such as teratomas and sarcomas[34–35]. Importantly, no tumors have been found in the human recipients of MSCs to-date, and remarkably, even aneuploid MSCs may not give rise to tumors[36]. Second, it remains controversial whether MCSs stimulate growth of other tumors[37–38].Lastly, due to their immunomodulatory and anti-inflammatory capacities[39] critical to the favorable effects in GVHD (discussed below), infusion of MSCs may lead to immunosuppression and, perhaps, higher risk of infections.

## II. MSC regulation of alloresponses in preclinical models

The immune regulatory properties of MSCs have been reviewed[40]. We will summarize the properties of MSCs most relevant to regulating alloresponses, especially in models of bone marrow transplantation (BMT) including GVHD and bone marrow graft rejection.

### T cell responses

MSCs are themselves poor stimulators of an in vitro allogeneic T cell response and when present with naive T cells, will inhibit their proliferation to mitogenic stimuli, including allogeneic antigen-presenting cells (APCs)[41–45] except under in vitro conditions of exposure to low interferon gamma (IFNg) concentrations that results in major histocompatibility complex (MHC) class II upregulation[46–47]. MSC-induced changes in APCs include reduced expression of MHC class I and II antigens and costimulatory molecules[39, 48–51]. Because APC function is a critical element for maximum acute and chronic GVHD initiation[52–55], MSCs should suppress in vivo alloreactive donor anti-host T cell responses with the caveat that acute GVHD typically results in high levels of IFNg release that may increase MHC class II expression on MSCs and hence augment GVHD[56–57]. By preventing the differentiation/maturation of monocytes to immature dendritic cells (DCs) and subsequently to mature myeloid DCs, MSCs may render APCs unable to maximally support a T cell response, pushing APCs away from a pro-inflammatory (e.g., tumor necrosis factor alpha and interleukin-12, IL-12) phenotype and toward an anti-inflammatory (e.g., IL-10 production) phenotype[39, 48–49, 58–59]. As a result, T cell responses skew away from a type I response (i.e., IFNg production) and toward a type 2 response (i.e., IL-4 production)[60]. However, these effects on cytokine response alone are not able to predict whether MSCs will reduce or augment GVHD as type I responses sometimes can inhibit and type 2 responses augment GVHD lethality[61] because the cytokine level, timing of production, duration of exposure, and strain combination (or clinical condition) may all influence GVHD outcome.

### Cytokine production

MSCs also can secrete soluble immune suppressive molecules such as prostaglandin-E2 (PGE2)[39], transforming growth factor beta-1 (TGF-b1)[42], IL-10 and human leukocyte antigen G isoform (HLA-G5)[62], known to be anti-proliferative for naive T cells[40]. Upregulation of intracellular pathways such as the essential amino acid catabolic pathway, indoleamine 2,3 dioxygenase (IDO) by MSCs[63] results in a state of amino acid starvation (tryptophan depletion) and the accumulation of potentially toxic metabolites known as

kyneurinines that suppress T cell immune responses[64]. IDO expression by host APCs and epithelial cells has been shown in preclinical models to diminish GVHD lethality[65–67]. Upregulation of stress response pathways such as inducible nitric-oxide synthetase[68–69] and heme-oxygenase-1[70] contribute to MSC-induced immune suppression. The net effects of MSCs on the innate and adaptive immune response are inhibition of CD4+ and CD8+ T cells[42], inhibition of resting natural killer cell (NK) cytotoxic function[71–72], defective neutrophil respiratory burst and survival[73–74], and the generation of innate and adapative immune regulatory cell populations[39, 62, 75]. Most but not all studies indicate MSCs can suppress B cell proliferative responses, along with antibody production[76–78], that are often ascribed to the etiopathogenesis of chronic GVHD[79–81].

## T regulatory cells

Antigen-reactive T cells exposed to MSCs fail to efficiently progress through cell cycle, similar to the profile of anergic (antigen hyporesponsive) T cells that do not receive the critical costimulatory (accessory) signals for a productive proliferative response[76, 82]. As can be seen in anergic conditions, MSCs can generate immune regulatory cells such as CD4+25+FoxP3+ T regulatory cells (Tregs). Tregs may be produced by the thymus (classical or natural Tregs) or induced from CD4+25-FoxP3- T cells by, for example, exposure to plasmacytoid DCs that produce IDO or IL-10[83]. MSCs induced production of IL-10 by plasmacytoid DCs may favor inducible Treg development in vivo[39, 75]. Because Tregs can suppress T cell proliferation, IFNg secretion in vitro or in vivo, and GVHD lethality[84–85], the immune suppressive properties of MSCs may depend in part upon their effects on Treg generation or function. Additional immune regulatory cells that are induced by MSCs include regulatory DCs, “alternative” anti-inflammatory M2 macrophages, and myeloid derived suppressor cells, each of which can suppress T cell proliferative and proinflammatory cytokine responses and GVHD lethality.

## MSCs in experimental transplantation

Although MSCs are poised to blunt alloresponses in vivo, preclinical data do not indicate that MSCs are uniformly efficacious in preventing adverse responses following BMT. For example, whereas host-type MSCs were found to improve the engraftment of allogeneic bone marrow given to sublethally irradiated recipients, surprisingly donor MSCs caused graft rejection[86]. Other studies have shown that allogeneic MSCs can be immunologically rejected which was associated with the generation of memory CD4+ and CD8+ T cells in rechallenge experiments[87]. In several murine BMT studies, allogeneic or syngeneic MSCs have failed to suppress donor anti-host alloresponses in vivo or reduce GVHD lethality. In our own studies, allogeneic MSCs did not home to secondary lymphoid organs and were unable to reduce GVHD lethality. Even upon intrasplenic injection of allogeneic MSCs, GVHD lethality was unimpacted, in contrast to the reduced GVHD lethality conferred by the intrasplenic but not systemic injection of a different but related non-hematopoietic progenitor population (multipotent adult progenitor cells) that has a similar in vitro immune suppressive potency as assessed in an allogeneic mixed lymphocyte culture reaction[51]. Not all rodent GVHD studies with MSCs have been negative[88–92]. For example, whereas MSCs given on day 0 post-BMT were ineffective in GVHD prevention, day 2 administration significantly reduced lethality, possibly due to the cytokine milieu present at that time that favored MSC mediated suppressor function, homing/migration pattern or their persistence[90]. Donor T cell production of IFNg was found to be essential for the MSC protective effect, likely due to the known function of IFNg on augmenting MSC production of inhibitory intracellular pathways (e.g. indoleamine 2,3 dioxygenase), upregulation of cell surface antigens (e.g PD-1 and its ligands)[77] or secreted molecules that downmodulate immune responses (e.g. PD-1 ligand; PGE2) and perhaps alteration in the expression of homing and adhesion molecules as well as chemokines and their receptors[93].

Thus, location of the immune suppressive population and their persistence in the sites of GVHD initiation are likely to be critical determinants of their anti-GVHD potency. Additional factors such as timing of MSC infusion and presence of proinflammatory and anti-inflammatory cytokines, especially within the context of the microenvironment in which MSCs reside in vivo, also likely substantially influence the biological potency of MSCs on inhibiting alloresponses in BMT recipients.

## III. Clinical experience of MSC infusion

In the setting of allogeneic hematopoietic cell transplantation (HCT), MSCs have been brought to the clinic mainly to promote hematopoietic engraftment and for immunosuppression in graft-versus-host disease. In addition, MSCs have been given to promote healing of regimen-related toxicity, and to correct inborn errors of metabolism[32–33, 94]. Intravenous administration of MSC appears safe, and no infusional toxicity or ectopic tissue formation has been reported in any of the studies described below.

### MSC infusion to enhance hematopoietic stem cells engraftment and prevent graft-versus-host disease

The first recipients of culture-expanded MSCs were given autologous cells as a safety trial[95]. Subsequent trials in patients undergoing myeloablative therapy for breast cancer indicated that autologous MSCs were not easily grown from patients treated with chemotherapy[96]. The marrow stroma is damaged by high-dose chemoradiotherapy and reconstitutes poorly[97–98]. A conventional bone marrow graft contains few MSCs, and the stroma remains of recipient origin post transplant[99–100]. Even though ex vivo expanded MSCs also have a limited capacity for reconstituting the marrow microenvironment, infusion of MSC promote hematopoietic cell engraftment in experimental animal models[101–104]. Several mechanisms could explain how MSCs facilitate hematopoietic stem cell (HSC) engraftment. Constitutively, MSCs secrete growth factors important for HSC expansion and differentiation. In addition, the immunosuppressive properties of MSCs may protect HSCs, particularly when transplanted in an allogeneic or xenogeneic environment. Haploidentical MSCs have been infused to promote hematopoietic recovery in a patient with refractory severe aplastic anemia and another patient with primary graft failure after autologous HCT[105–106]. Donor MSC engraftment was detected by polymerase chain reaction (PCR) in the endosteum, but not in marrow aspirates suggesting that MSCs are primarily located in the bone tissue and can persist in HLA disparate individuals. The first patient showed histologic improvement in the marrow microenvironment while the second patient also recovered peripheral neutrophil and platelet counts.

Hypothesizing that co-transplantation of HLA-matched sibling derived HSCs and MSCs could facilitate engraftment and, through their immunosuppressive properties, also prevent severe GVHD, 46 patients undergoing myeloablative HCT were infused with MSCs in escalating doses from 1 to $5\times10^6$/kg[107]. Stromal cell chimerism was demonstrated in two of 19 examined patients at 6 and 18 months post transplant. Moderate to severe acute GVHD was observed in 28% of the patients, and chronic GVHD was seen in 61%. MSC infusion caused no acute or long-term MSC-associated adverse events. A lower incidence of GVHD in the MSC-treated group was observed in a similar set of patients, in a small open-labeled randomized trial[38]. However, in contrast to previous trials, an increased risk of particularly early relapse after MSC infusion was suggested, resulting in discontinuation of the trial.

Non-myeloablative HCT depends on a graft-versus-tumor effect for eradication of the leukemia and should, at least theoretically, be the setting where an MSC-mediated increase in relapse would be most prominent. Twenty patients undergoing reduced-intensity treatment and transplanted with HLA-mismatched hematopoietic stem and progenitor cells

were co-infused with third party, HLA-disparate MSCs and compared to 16 historic controls[108]. Hematopoietic cell engraftment was prompt in both groups but the overall survival at one year was significantly higher in the MSC-treated group. The incidence of relapse was similar to that of controls and the difference in survival attributed to a lower risk of death from either GVHD or GVHD-related infection in MSC-treated patients.

Larger studies are required to assess whether MSCs affect the risk of graft failure. In theory, an immunosuppressive effect of MSCs in vivo could interfere with the GVHD reactions required to establish donor hematopoiesis and thereby increase the risk of graft failure. On the other hand, MSCs could also mitigate the host-versus-graft effect and possibly the production of HLA-antibodies associated with rejection and thus facilitate engraftment. Several pilot trials suggest a possible beneficial effect of MSC treatment when the risk of poor engraftment is increased. HLA–identical or haploidentical MSCs have been successfully regrafted after primary or secondary graft failure[109–110]. In all patients, co-transplantation resulted in stable hematopoietic engraftment and 100% donor chimerism within 3 months. One of the patients diagnosed with aplastic anemia had graft-failure after her first transplantation and severe Henoch-Schonlein purpura[109]. After retransplantation, she recovered from both the Henoch-Schonlein purpura and aplasia.

Haploidentical HCT is associated with an increased risk of graft failure. Donor-derived MSCs were co-transplanted with HLA-disparate CD34+ cells from a relative in 14 children. While graft failure in 47 historic controls was 15%, all patients given MSCs showed sustained hematopoietic engraftment without any adverse reactions or increased number of infections[111]. Meuleman et al. treated 6 patients with graft failure post transplant with MSC infusions[112]. All patients were donor chimeras with a marrow cellularity of less than 10%. Two of the patients, both transplanted in first complete remission, showed prompt hematopoietic recovery within several weeks of the MSC infusion, whereas patients transplanted at later stages of their disease were unresponsive. Similarly, following blood group incompatible transplantation, pure red cell aplasia caused by anti-ABO antibodies produced by persistent B cells of donor origin was corrected in two patients following infusion of adipose-derived MSCs[113].

MSC infusion may also be beneficial in cord blood transplantation but the studies published so far include too few patients to draw conclusions. The outcome of 21 pediatric and 9 adult patients has been reported and compared to historic controls[114–116]. None of the studies indicate that co-infusion of MSCs reduces the time to platelet or neutrophil recovery. The study by Bernardo et al reported the incidence of severe GVHD to be significantly lower in the MSC group. No patients in the study by Gonzalo-Daganzo et al. developed grade III–IV GVHD compared to 6 of 46 patients in the control group. However, larger studies are required to determine a preventive effect of MSC treatment. This would be particularly true for adult patients where the risk of poor engraftment, due to a low HSC dose, and the risk of GVHD is higher.

## MSC infusion to treat GVHD

Steroids represent the first-line treatment for established acute GVHD, but when the GVHD is unresponsive to steroids, survival is poor. As MSCs promote tissue repair in animal models and have immunomodulatory effects on human lymphocytes *in vitro*, it was hypothesized that they could have beneficial effects on already established GVHD. Haploidentical MSCs were first infused into a 9 year old boy with treatment-resistant severe GVHD of the gut and liver[117]. Response in terms of improved liver values and intestinal function was prompt. Upon discontinuation of immune suppressive medication, the patient's acute GVHD recurred but remained responsive to a second MSC infusion.

A subsequent report included eight patients with similar steroid-refractory acute GVHD[118]. A complete response to MSC-treatment was seen in six patients. Their survival rate was better than that of 16 controls. In one patient, DNA from both MSC donors (one haplo-identical and one mismatched unrelated) could be detected at low levels in the colon and lymph nodes of the gastrointestinal tract on month after infusion.

A beneficial effect was corroborated in a multicenter non-randomized trial of the European Blood and Marrow Transplant MSC consortium, using a shared expansion protocol for the cells and common reagents[119]. Twenty-five pediatric and 30 adult patients were treated with HLA-identical, haplo-identical or mismatched MSCs for GVHD. The patients included in the study were severely ill, mainly with GVHD of the gastrointestinal tract and liver. A single MSC infusion was given to 27 patients and the remaining patients were treated with 2 or more infusions. Thirty patients showed a complete response to MSC infusion; of these, 27 were complete responders already after a single MSC infusion. Only 5 patients were treated with HLA-matched MSCs and, due to the low number of infusions with completely matched or haploidentical MSCs, an efficacy analysis regarding the importance of HLA-matching between MSC donor and recipient was not possible. There was a trend for a better response in the pediatric patients, with a statistically better survival.

How HLA-matching and the expansion procedure influence the beneficial effect of MSCs on GVHD is unclear. In the study by Le Blanc et al., MSCs were expanded in fetal bovine serum (FBS) and cultured for an average of 2–3 passages in the presence of FBS. Intravenous infusion of MSCs generated in the presence of FBS has so far been safe, but unfavourable immune responses toward FBS may occur[120–121]. Other possible risks with the use of FBS include bacterial infections and prions[122–124]. For these reasons, MSCs expanded with protocols where FBS is replaced by frozen human platelets have been attempted in HCT patients[125–126]. MSCs generated in platelet lysate were given to 13 adult patients to treat GVHD[125]. Two patients responded to MSCs, and an additional 5 patients improved after MSC infusion followed by additional salvage immunosuppressive therapy. Overall response after 28 days was 54%, with the best responses seen in patients with gut and liver GVHD. Comparative studies will be required in the future to evaluate whether the apparent lower response rate seen results from differences in the MSC expansion protocols, the fact that only adult patients were included, or other factors. Early MSC therapy, at the time of GVHD diagnosis and initiation of steroid therapy, has also been attempted[127]. Thirty-two adult patients were randomized to receive 2 or 8 million third party MSC/kg in combination with corticosteroids for de novo GVHD. The MSC used in this study were derived from 6 donors and extensively expanded in FSC to generate the final cell product. Seventy-seven percent of patients responded to therapy, including 89% of patients with gut GVHD. There was no difference in response to intervention between the high and low MSC dose groups.

Preliminary data of a phase III trial using a similar approach of generating a large number of cells from a limited number of unrelated donors to treat steroid-refractory GVHD, have recently been presented[128]. The results suggest that MSC are safe. In a phase III trial for treating steroid resistant acute GVHD, subsets of patients with steroid-resistant liver or gastrointestinal GVHD had an improved response to MSCs.

## IV. Clinical grade cell manufacture

Given that more than 100 MSC-related clinical research protocols are listed in www.clinicaltrials.gov and, in all probability, more than 2,000 patients have been treated with MSCs worldwide, it is not surprising that considerable variation exists in the mode and stringency of the manufacture of these cells. Many involve protocols for single arm studies

with small numbers of subjects for whom the MSCs were generated in HCT processing laboratories or similar facilities. Other protocols employ current Good Manufacturing Practice (cGMP) standards in which the cells are manufactured under the highest standards of sterility, quality control and documentation. The impact of these differences is unknown, but clinical prudence and regulatory requirements in many countries mandate at the least, that the manufacturing is conducted under a good laboratory practice (GLP) standard. The cGMP standard is at a higher level and although it involves a similar stringency in the implementation of standard operating procedures (SOPs), additional requirements include a formal and independent quality assurance program.

A number of factors in the cell manufacturing process influence the nature and, probably, the function of MSCs. For example, under identical culture conditions, the prevention of cell adhesion alters the immunophenotype of the cells considerably, and possibly their biodistribution[129]. Other factors include the oxygen tension, temperature, and composition of the culture medium. As discussed above, more recent protocols eschew FBS in favor of human plasma or human platelet lysate. While many regulatory agencies tolerate the presence of FBS in the culture medium of MSCs for phase I trials, later phase studies tend to require serum-free medium. It will be important to determine immunophenotypic, genotypic, and functional changes in MSCs when culture media are modified.

Although the International Society for Cellular Therapy (ISCT) has established the definition of MSCs[10, 130], release criteria were not dictated, tend to be protocol-dependent, and are determined in conjunction with regulatory agencies. It is especially important, for example, when employing allogeneic MSCs (in contrast to autologous MSCs) to ensure that B or T cell contamination is low or absent in order to eliminate the possibility of GVHD[131]. A major challenge in establishing release criteria is the lack of an accepted functional assay. However, given the wide range of potential clinical effects of MSCs—from the treatment of specific tissue injury to immunosuppression for GVHD—any such assay(s) will need to be specific to the particular indication or clinical trial. Another confounding issue is that some MSC products may be distinguished from other MSC products by differences in immunophenotype or function in vitro, in part to employ unique MSC cell types for purposes of intellectual property protection. Because very few comparative studies have been done, it is difficult to assess the importance of these differences on clinical outcomes. There may also be significant differences among MSC products from different tissue sources. The most extensive comparisons have involved adipose-derived MSCs versus MSCs from bone marrow[132]. The source of MSCs may influence the ability of the cells to differentiate along, for example, osteogenic, chondrogenic or myogenic lineages. Functional differences appear to exist between MSCs derived from human umbilical cord perivascular cells and those from bone marrow (personal observation of one of us, AK). Cell manufacturing protocols must therefore take into account the variability in the characteristics of the MSCs, including their proliferative and differentiative capacities.

Other factors that may influence the function and safety of MSC preparations include age and sex of the donor and the number of cell doublings necessary to arrive at the final product. To mitigate malignant transformation of human MSCs, meticulous attention must be taken to prevent cell senescence and ensure that preferably fewer than 25–30 cell doublings occur (Darwin J. Prockop, Malcolm Brenner, Willem E. Fibbe, Edwin Horwitz, Katarina LeBlanc, Donald G. Phinney, Paul J. Simmons, Luc Sensebe, and Armand Keating, submitted). Despite these measures, potential genetic instability remains a concern, hence many centers advocate the demonstration of a normal karyotype as part of the release criteria for MSCs.

Two approaches can be taken to make the MSC product available quickly: the use of allogeneic cells that have been cultured, tested, cryopreserved and ready for release and administration after thawing; or the rapid culturing of autologous MSCs by aspirating, under local anesthesia, a large volume of bone marrow (100–150mL), that will require fewer passages to achieve the desired number of cells but in medium supplemented with cytokines, such as fibroblast growth factor alpha. The latter approach could provide autologous MSCs within two weeks. Another strategy is to grow the MSCs more rapidly in bioreactors. The final approach will be dictated by the research protocol and the clinical importance of an autologous versus allogeneic source. There is a perception that trials with autologous cells may receive more rapid approval by regulatory agencies, although this is not certain and the decision might be more appropriately reached by considerations of feasibility and the underlying pathophysiology of the disease targeted.

## Future Clinical Trials

Although perhaps as many as several thousand patients have been treated with MSC to date, no infusional toxicity or immediate adverse out comes have been reported, suggesting MSC infusion to be safe. However, rare adverse event and late complications of the treatment can only be detected in large cohorts of patients with long follow up. The long experience of cooperative groups such as the CIBMTR and the EBMT to collect data on patient treated with HSCT and evaluate long term patient outcome provide an excellent infrastructure that can be employed to patients treated with novel cellular therapies, such as MSC, also to avoid publication bias. In fact, a registry specific for novel cellular therapies has already been established in the EBMT and efforts to establish a similar registry are ongoing in the CIBMTR[133].

Efficacy of MSC treatment, however, remains to be established for most indications. Pilot trials aim at establishing safety, but comparative studies are needed to show a beneficial effect of MSC. Reproducibility of patient responses in several centers and by MSC produced in different labs is best shown in collaborative multicenter studies adhering to similar protocols for generation of MSC. Unbiased comparisons of the clinical effect of MSC derived from donors of various degrees of HLA-matching, generated in different growth media and after various periods of in vitro culturing will further be essential to optimize MSC treatment.

Protocol design for tissue regeneration with MSCs was based on the assumption that the cells differentiated into the cells of the injured organ (e.g., MSCs introduced after acute myocardial infarction differentiated into cardiomyocytes). This notion is now considered unlikely[134] and has been replaced by myriad mechanisms to explain the objective improvements that have been documented in some cases[135]. The presence of the MSCs at sites of injury in pre-clinical animal models generally has been transient (days rather than weeks) and paracrine mechanisms have been invoked[29]. Such findings raise a number of challenging issues in the design of MSC trials in the future. First, it will be important to determine whether threshold effects occur and if an MSC dose response and/or infusion duration actually exists for a particular indication or end-point. Secondly, it will be useful to correlate biodistribution of MSCs with therapeutic response. Finally, real time imaging and tracking studies of MSCs in patients will provide an enormous impetus in moving the field forward. The most feasible imaging agent to enter human clinical trials is likely to be a form of superparamagnetic iron. It is hoped that a suitable iron formulation will be available in the near future for tracking the marked cells by magnetic resonance imaging (MRI).

Given the very high costs of conducting early phase cell therapy protocols, including those with MSCs, it is important to optimize the information obtained even from phase I clinical trials to gain a better understanding of the mechanisms by which MSCs mediate immune

suppression or tissue regeneration. There is the added issue that it has frequently not been possible to garner relevant data on human MSC-immune interactions from xenogeneic models to inform the design of subsequent trials in patients.

## V. Summary

Taken altogether, we know with considerable certainty that the diverse non-hematopoietic cell types present in bone marrow, collectively termed MSCs, hold the promise of fulfilling major unmet needs in tissue repair, cell therapy and tissue engineering. There is tremendous enthusiasm for the development of patient-specific or off-the-shelf prototypic cellular therapy tailored to variety of clinical scenarios. A cycle of bench to bedside and back to the bench is particularly pertinent for MSC trials, whatever the therapeutic goal.

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## Table 1

Clinical experience of mesenchymal stem cells in hematopoietic stem cell transplantation

| Disease | No. patients | Source of MSCs/HCT | Outcome | Study |
|---|---|---|---|---|
| Hematologic malignancies | 15 | Autologous | No adverse events | Lazarus et al.[95] |
| Breast cancer | 28 | Autologous in autologous HCT | IV infusion safe; rapid autologous hematopoietic recovery | Koç et al.[96] |
| Inborn errors of metabolism | 11 | HLA-identical from HCT donor | No immune response; improved nerve- conduction velocity | Koç et al. [33] |
| Osteogenesis imperfecta | 5 | HLA-identical from HCT donor | Gene-marked MSCs engrafted; new dense bone formation; few fractures | Horwitz et al.[30] |
| Severe plastic anemia | 1 | Haploidentical MSCs | Engraftment; improved stroma | Fouillard et al.[105] |
| Severe acute GVHD | 1 | Haploidentical MSCs; matched unrelated SCT | Resolution of grade IV acute GVHD | Le Blanc et al.[117] |
| Leukemia | 46 | HLA-identical sibling | Safe, stable HSC engraftment | Lazarus et al.[107] |
| Severe acute GVHD | 8 | Matched or mismatched allogeneic | Complete response in 6 of 8 patients | Ringdén et al.[118] |
| Graft failure | 1 | Haploidentical MSCs, autologous HCT | Stable hematopoietic reconstitution | Fouillard et al.[106] |
| Leukemia | 7 | Matched allogeneic HCT, HLA-identical or haploidentical MSCs | Safe, stable hematopoietic engraftment | Le Blanc et al.[109] |
| Malignant and non-malignant disorders | 14 | Haploidentical HCT and MSCs | Stable hematopoietic engraftment, reduced engraft failure | Ball et al. 2007[111] |
| Tissue toxicity, hemorrhagic cystitis | 10 | Matched or mismatched MSCs; allogeneic HCT | Safe | Ringdén et al.[94] |
| Leukemia | 25 | HLA-identical from HCT donor | Increased relapse in MSCs recipients | Ning et al. [38] |
| Severe acute GVHD | 55 | Matched or mismatched MSCs, allogeneic HCT | Improved survival in responder patients | Le Blanc et al.[119] |
| Leukemia | 7 | HLA-identical or haplo MSCs, allogeneic HCT | Safe | Müller et al.[126] |
| Aplastic anaemia, graft failure | 2 | Haploidentical MSC, HLA identical sibling HCT | Stable engraftment | Fang et al.[110] |
| Leukemia, graft failure | 6 | HLA identical or haploidentical from HCT donor | Hematopoietic recovery in 2 of 6 patients | Meuleman et al.[112] |
| Pure Red Cell Aplasia after allogeneic HCT | 2 | Haplo or matched sibling MSCs, matched allogeneic HCT | Recovery from aplasia | Fang et al.[113] |
| Leukemia | 8 | Haploidentical MSCs, umbilical cord HCT | Safe, stable engraftment | Macmillan et al.[114] |
| Leukemia, acute GVHD | 9 | Haploidentical MSCs, cord blood + haplo CD34+ HCT | Safe, stable engraftment | Gonzalo-Daganzo et al.[115] |
| Severe acute GVHD | 13 | Allogeneic mismatched MSCs, allogeneic matched HCT | Overall response day 28, 54% | Von Bonin et al.[125] |
| De novo acute GVHD | 31 | Allogeneic mismatched MSCs, matched allogeneic HCT | 94% response to MSCs and steroids | Kebriaei et al.[127] |
| Leukemia | 20 | Related or unrelated MSCs; matched reduced intensity HCT | Safe, stable hematopoietic engraftment | Baron F et al.[108] |

| Disease | No. patients | Source of MSCs/HCT | Outcome | Study |
|---|---|---|---|---|
| Hematologic disease | 13 | Haplo MSCs, umbilical cord blood HCT | Safe, low incidence of severe acute GVHD | Bernardo et al.[116] |

HCT, hematopoietic cell transplantation; MSCs, mesenchymal stem cells; HLA, human leukocyte antigen; IV, intravenous; GVHD, graft-versus-host disease; haplo, HLA haploidentical.