Mesenchymal stromal cells, from indifferent spectators to principal actors. Are we going to witness a revolution in the scenario of allograft and immune-mediated disorders?

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Two different articles in this issue of the journal are focussed on mesenchymal stem cells (MSCs), with particular emphasis on their immune-regulatory properties and homing capacity, respectively.[1,2] In the first study, Prevosto *et al*. demonstrate that short-term co-culture of human MSCs with resting lymphocytes induces the differentiation of a pool of different T-cell subsets with a powerful immune suppressive activity.[1] The second manuscript, by Shi *et al*., provides evidence to support the hypothesis that one of the most important mechanisms at the basis of the tissue-specific homing capacity of human MSC, namely MSC recruitment to the bone marrow (BM), is mediated by the interaction between the CXCR4 chemokine receptor, expressed on MSC, and its ligand, the stromal cell-derived factor-1 (SDF-1).[2] Furthermore, this latter study demonstrates that it is feasible to consistently increase CXCR4 expression on *in vitro* propagated MSC, endowed with long-term BM-engraftment capacity, by using a tailored cocktail of cytokines in the expansion procedure.[2]

Both articles further our understanding of MSC biology and reflect the huge interest that the scientific community has in these particular cells. In fact, they are attracting increasing attention from many researchers involved in the optimization of different approaches for reparative/regenerative cell therapy, as well as in the perspective of modulating immune response against alloantigens or even to autoantigens.[3-12]

What we know about MSC: from the historical perspective to the more recent insights into their biological characteristics

Friedenstein *et al*. first drew our attention to BM stromal cells which they also called mechanocytes.[13] However, these cells then remained neglected until Prockop reawakened the interest in them.[14] This is remarkable, since Friedenstein's group had already demonstrated that with MSC the hematopoietic microenvironnement could be transferred. This showed that MSC provide a substantial contribution to the creation of what is now defined as the *niche*, namely the most appropriate habitat for the hematopoietic stem cell (HSC). However, for many years, MSC continued to be considered mainly a component of marrow stroma, without any special function, and endowed only with structural support properties. The crucial role played by MSCs in sustaining hematopoiesis was subsequently described in two seminal papers. One showed that a deficiency in the environment could cause anemia and was the origin of the subsequent identification of the stem cell factor.[15] The other reported that stromal cells are able to sustain hematopoiesis *in vitro* and represented the basis to set up and standardize the system for long-term *in vitro* culture of hematopoietic cells.[16] Our knowledge of the hematopoietic microenvironment or *niche* has substantially improved over time and in particular over the last decade. We now know that it provides a spatial structure where HSC are stored in a quiescent state, allowing their self-renewal, while inhibiting cell-differentiation. The microenvironment of mammalian bone marrow is composed of several different elements, including macrophages, fibroblasts, adipocytes, osteoprogenitors, endothelial cells reticular cells and the multipotent MSC. All these elements contribute to support hematopoiesis and bone homeostasis through a molecular crosstalk between HSC and cells that comprise the *niche*, involving a large number of molecules, including cadherins, integrins, chemokines and cytokines.[17-20] BM-derived MSC play a crucial role in the development and differentiation of the lympho-hematopoietic system by secreting a number of growth factors and regulatory cytokines, and by promoting cell-to-cell interactions. This is because they are present in the hematopoietic *niche* in close contact with HSC, endothelial cells and osteoblasts which in turn differentiate directly from MSCs through a coordinated process.[21] Some osteoblast-derived molecules might also be of paramount importance for maintaining quiescent HSC in an undifferentiated state.[20]

Although BM remains the primary source for obtaining these stem cells, MSC can also be isolated from other tissues, including cord blood, fetal liver, fetal lung, amniotic fluid, teeth and adipose tissue.[22-24] They have the capacity to differentiate *in vitro* into cells of the mesodermal lineage, including osteoblasts, adipocytes, chondrocytes, as well as tendon and muscle cells.[25,26] In some experimental conditions, MSC have also been shown to be able to differentiate into cells of other lineages, including neural cells.[25] The frequency of MSC in human BM can be estimated in the order of approximately 0.001-0.01% of nucleated cells. Adult MSC can be readily isolated, exploiting their adhesion ability, and extensively expanded. In the absence of a particular marker, MSC are typically defined by a combination of phenotypic and functional characteristics. By using

flow cytometry, MSC are negative for hematopoietic markers CD14, CD34 and CD45, while they stain positive for a number of adhesion molecules including CD73, CD90, CD105 and CD166. The hallmark of MSC is their *in vitro* tri-lineage differentiative potential, i.e. the ability to differentiate into bone, cartilage and fat in appropriate *in vitro* conditions.

MSC as a tool to accelerate hematopoietic recovery and to cure some selected inherited disorders

Human MSC have been shown to promote engraftment of cord blood-derived HSC in NOD-SCID mice and in fetal sheep.[23,27,28] The enhancing effect, involving cells of myeloid, lymphoid and megakaryocytic lineages, is particularly prominent when the dose of hematopoietic cells is low.[27] These experimental data, together with the known physiological role played by MSC in sustaining hematopoiesis, have provided the rationale for testing the capacity of these cells to accelerate hematologic recovery in patients receiving myeloablative therapy with HSC support. The first clinical trial on the use of MSC for accelerating hematologic recovery was conducted in women undergoing autologous transplantation for breast cancer. Twenty-eight breast cancer patients were infused with $1–2\times10^6$ MSC/kg, and rapid hematopoietic recovery occurred without any undesired reaction related to the infusion of MSC.[29] Subsequently, a multicenter trial explored the safety of MSC infusion also in recipients of allogeneic HSC. Forty-six patients received HSC and culture-expanded MSC from their HLA-identical siblings. MSC were cultured starting from 30 mL of donor bone marrow aspirate, while HSC were obtained either from donor BM or from peripheral blood after mobilization. The planned MSC dose escalation scheme was 1×10^6, 2.5×10^6 , and 5×10^6/kg in both patients receiving HSC from BM or peripheral blood. MSC were infused 4 hours before HSC transplantation. Hematopoietic recovery was prompt for most patients and moderate to severe acute graft-versus-host disease (GvHD) was observed in 28% of the patients. MSC infusion caused no acute or long-term MSC-associated adverse events. These data further supported the hypothesis that the infusion of culture-expanded MSC, together with HSC transplantation, is a safe procedure and could potentially enhance marrow recovery after myeloablative treatment.

The *in vivo* infusion of MSCs has also found a place in the context of *osteogenesis imperfecta*, a genetic disease characterized by production of defective type I collagen, the principal protein in bone. Patients with this disorder experience painful fractures, retarded bone growth with progressive bone deformation and, in the most severe form of the disease, usually do not survive beyond the second decade of life. Horwitz *et al*. first demonstrated that transplantation of BM cells from an HLA-identical sibling could, at least transiently, ameliorate the clinical conditions of patients with *osteogenesis imperfecta*. This was because donor-derived, mesenchymal progenitors contained in transplanted BM could migrate to bone and give rise to osteoblasts that determined an improvement of bone structure.[30,31] The same group has subsequently proven that the infusion of purified allogeneic MSC may enhance the therapeutic benefits of allogeneic BM transplantation in the treatment of *osteogenesis imperfecta*,[32] confirming the possibility of exploiting the osteogenic potential of MSC in clinical trials.

MSC and immune modulation: it takes more than two to tango

Certainly most of the present popularity of MSC must be attributed to the discovery of their immune modulatory properties. While in the beginning most studies investigated the effects of MSC on T lymphocytes,[9,11,33-35] with accumulating data, it has become evident that MSC may display their effect on all the cells involved in an immune response, including B lymphocytes, dendritic cells and natural killer (NK) cells.[6,10,12] To further complicate the situation, it must be underlined that MSC possess the dual ability to both suppress and/or activate immune responses, depending on the stimulus to which they are exposed.

MSC express low levels of human HLA class I molecules and, normally do not express class II molecules. These can, however, be induced on the cell surface by incubation with interferon-γ (γ-IFN).[7,10,11,36,37] In addition, MSC do not express the CD40, CD80 or CD86 co-stimulatory molecules and are unable to induce proliferation of allogeneic lymphocytes.[7,10,11,36] As a result of the low expression of MHC class I molecules, MSC can escape detection by T cells. For years, they have been considered cells which can be potentially ignored by the immune system.[36] However, recent *in vivo* data in mice challenge the concept of the immunoprivilege of MSC, because allogeneic MSC infused into MHC-mismatched mice were sometimes rejected.[38,39] Furthermore, both autologous and allogeneic interleukin 2-activated NK cells can kill MSC efficiently due to the low levels of human MHC class I antigens on the latter cells and to the surface expression of ligands recognized by activating NK receptors.[40]

Several independent groups have shown that cultured MSC can suppress T-cell proliferation induced by allogeneic peripheral blood mononuclear cells and by mitogens, such as phytohemagglutinin, concanavalin A and anti-CD3/anti-CD28 antibodies, in a dose-dependent manner.[6,7,9-11,33-37] Inhibition of lymphocyte proliferation by MSC has not been associated with the induction of apoptosis but is thought to be due to inhibition of cell division. This prevents T-lymphocyte capacity to respond to antigenic triggers while maintaining these cells in a quiescent state.[6] MSC also strongly inhibit *in*

vitro activation of alloantigen-specific cytotoxic lymphocytes at a higher ratio than the effector cells. At a low ratio they may either decrease or increase alloantigen-specific cell-mediated cytotoxic activity.[11,41] Inhibition of T-cell proliferation and cytotoxicity did not require MHC compatibility between MSC and responder lymphocytes. This supports the hypothesis that MSC can be considered *universal suppressors*. Most human MSC-mediated immune suppression on activated T-lymphocytes has been attributed to the secretion of anti-proliferative soluble factors, such as hepatocyte growth factor, prostaglandin E2, transforming growth factor-β1 (TGF-β1), indoleamine 2,3-dioxygenase (IDO), which causes depletion of tryptophan, an essential factor for lymphocyte proliferation), nitric oxide and interleukin (IL)-10.[6,7,9-11,33-37] However, published data do not exclude that a part of the immunosuppressive effect exerted by human MSC on alloantigen-induced T-cell activation could be dependent on cell-to-cell contact mechanisms. Interestingly, the calcineurin inhibitors, cyclosporine-A and tacrolimus, currently employed to prevent or treat GvHD, enhance the immune suppressive effect of human MSC.[35]

As already mentioned, the effect played by MSC on the immune system is pleiotropic. In fact, it has been recently reported that MSC co-cultured with purified $CD19^+$ B cells in the presence of a cocktail of stimuli significantly inhibited B-cell proliferation and that this effect was mainly due to soluble factors.[12] Human MSC display an inhibitory effect on alloantigen-induced dendritic cell differentiation and on antigen presenting cell maturation.[42] This could be related to their capacity to produce anti-inflammatory cytokines, such as TGF-β,[33] known to inhibit *in vitro* activation and maturation of dendritic cells.[43]

Some studies have reported that the interaction between MSC and human lymphocytes favors the differentiation of $CD4^+CD25^+$ T lymphocyte subsets displaying a regulatory phenotype (Treg), in particular Foxp3 and CTLA4 expression, together with a suppressive function on other T-lymphocyte subpopulations.[10,11,44] As previously mentioned, data presented by Prevosto *et al*. in this issue of the Journal[1] offer further insight into Treg cell generation under the effect of MSCs, demonstrating that short-term co-culture of human MSCs with resting lymphocytes favors the emergence of this suppressive lymphocyte subpopulation. The paper also raises two other interesting points, namely (a) that a high level of Foxp3 expression is not the only characteristic hallmark of a powerful suppressor T-lymphocyte subset; and (b) that secretion of anti-proliferative cytokines or metabolites (prostaglandin E2), enzyme activation (such as IDO), or differentiation of $Foxp3^+$/$CTLA4^+$ Treg are not the only mechanisms responsible for immune suppressive activity of human MSCs. The hypotheses raised by the study by Prevosto *et al*.[1] will be useful to stimulate further investigations aimed at better defining the role played by human MSC in the development of peripheral immune tolerance.

MSC and GVHD: a revolutionary treatment

The multiple immune suppressive properties of MSC provide the biological explanation of the efficacy of MSC in the treatment of patients with acute GvHD, and even those refractory to conventional treatment. After the seminal report by Le Blanc *et al*. on a patient rescued from acute liver and gut GvHD resistant to multiple lines of immune suppressive therapy,[8] other reports involving a substantial number of patients have supported the view that MSC may represent a valuable option for allogeneic HSC transplantation recipients suffering from acute GvHD.[45,46] The real efficacy of MSC infusion in the management of patients with GvHD remains to be proven in a randomized trial comparing this innovative treatment with more conventional approaches. Other still unsolved questions include the number of infusions to be performed, the optimal dose of MSC to be administered for each infusion, and the possible synergisms of MSCs with other therapies demonstrated to be active in patients with acute GvHD. Likewise, it remains to be determined whether MSC may also have a role in the prevention of GvHD, given that MSC failed to prevent GvHD in an animal model.[47] In addition, the negative aspect of the immune suppressive effects of MSC could be that they facilitate either leukemia recurrence or occurrence of lethal infections. However, there are still no data to show this.

MSC and autoimmune disease: a new hope

Given their capacity to modulate immune responses and promote tissue repair, MSC have also been suggested as a potentially useful treatment for many autoimmune diseases.[6,48] Indeed, MSC have been used for the treatment of experimental animal models of multiple sclerosis, diabetes, systemic lupus erythematosus, and, with less success, of rheumatoid arthritis.[6] Of particular relevance are the studies on the effect of MSC on experimental autoimmune encephalomyelitis (EAE), a murine model of human multiple sclerosis. In this animal model, it has been demonstrated that MSC, infused intravenously following the onset of disease, can ameliorate EAE through the induction of T-cell tolerance, occurring at the level of the peripheral lymphoid organs, and the suppression of pathogenic B-cell responses *in vivo*.[6,49] Besides EAE, infusion of MSC has been thought to play a role in the protection of neurons from damage occurring in other conditions, such as spinal cord injury, stroke, and amyotrophic lateral sclerosis.[50-52] A common feature of all these studies is that the therapeutic effect of MSC does not seem to be associated with differentiation into neural cells. In fact, despite a large body of evidence suggesting that

MSC can differentiate into virtually any cell type *in vitro*, the therapeutic effect observed following *in vivo* administration of these stem cells seems to be mostly related to their immune modulatory capacity and stimulation of survival and functional recovery of resident cells in injured organs. This latter goal can be obtained mainly through paracrine mechanisms, supporting the recruitment of local precursor cells and production of trophic factors promoting the survival and repair of injured cells.

In fact, despite promising experimental results, the use of MSCs in the clinical treatment of autoimmune diseases is far from being a standardized and accepted therapy. Well conducted clinical trials are urgently warranted to investigate what looks useful in animal models. Defining the role and function of resident MSCs, as well as the tissue-specific homing capacity of *in vitro* expanded, adoptively transferred MSC, will be of great importance in our understanding of the therapeutic role of MSCs in autoimmune diseases. The results provided by the study of Shi *et al*., published in this issue of the Journal,[2] could help in the design of approaches to cell therapy. However, as Shin *et al*. used a rare and particular subset of human fetal BM-derived MSC, characterized by the expression of adherent fetal liver kinase-1 ($Flk1^+$) molecule,[2] further investigation is warranted to confirm these data, using more easily obtainable human MSC, such as cord blood-derived or BM-derived adult MSC.

MSC and safety

Concerns that adult human MSCs are prone to malignant transformation have been raised recently. In fact, human adipose tissue-derived MSCs have been shown to undergo spontaneous transformation after long-term *in vitro* culture.[53] The same phenomenon was also noted in murine BM-derived MSCs.[54] After numerous passages in culture, these increased telomerase activity and proceeded to malignant transformation. Both these findings emphasize the need for accurate studies aimed at investigating the bio-safety of these cells using appropriate and sensitive tests. Indeed, the absence of transformation potential in cultured MSC must be documented before considering infusion of these cells into patients. This is particularly important in the case of immune-compromised subjects, in whom failure of immune surveillance mechanisms might further favor the development of tumors *in vivo*. We have recently investigated the susceptibility to transformation of human BM-derived MSC at different *in vitro* culture time points.[55] The genetic characterization of MSCs was investigated through array-comparative genomic hybridization (array-CGH), conventional karyotyping, and subtelomeric fluorescent *in situ* hybridization (FISH) analysis both before and after prolonged culture. MSC were tested for the expression of telomerase activity, and hTERT transcripts. BM-derived MSC expanded *in vitro* usually displayed a progressive decrease in proliferative capacity until reaching senescence and did not show chromosomal abnormalities. Telomerase activity and hTERT transcripts were not expressed in any of the examined cultures and telomeres shortened during the culture period. These results suggest that BM-derived MSC can be safely expanded *in vitro* and are not susceptible to malignant transformation.

Finally, it must be mentioned that MSC are currently expanded *in vitro*, either under experimental or clinical grade conditions, in the presence of 10-20% fetal calf serum (FCS), which is considered crucial for the *ex vivo* expansion of MSC.[56,57] FCS batches are routinely pre-screened to guarantee both the optimal growth of MSC and the bio-safety of the cellular product. Despite this, the use of FCS raises some concerns when used in clinical grade cellular preparations, since the administration of animal products to humans might theoretically cause the transmission of prions and still unidentified zoonoses. Furthermore, bovine proteins or peptides might be incorporated by MSC during culture procedures[58,59] and cause immune reactions in the host, especially if repeated infusions are needed, with consequent rejection of the transplanted cells.[32] We and others have recently demonstrated that platelet lysate may represent a useful alternative to replace FCS in the generation and expansion of MSC in cell-therapy protocols.[44,60] Indeed, MSCs prepared in the presence of platelet lysate are comparable to MSC generated using FCS in their capacity to: i) decrease alloantigen-induced cytotoxic activity; ii) favor differentiation of $CD4^+$ T-cell subsets expressing a Treg phenotype; and iii) increase early secretion of IL-10 in the supernatant of a mixed lymphocyte reaction. Furthermore, in the clinical setting, the use of platelet lysate appears to provide very efficient expansion in a time-frame of 2-3 weeks, instead of the 4-5 weeks necessary with current protocols.

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