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EDITORIAL

Immune regulatory properties of multipotent mesenchymal stromal cells: Where do we stand?

Ênio José Bassi, Carlos Alberto Mayora Aita, Niels Olsen Saraiva Câmara

Ênio José Bassi, Niels Olsen Saraiva Câmara, Laboratory of Transplantation Immunobiology, Department of Immunology, Universidade of São Paulo, 05508-900 São Paulo, Brazil
Carlos Alberto Mayora Aita, Centro de Ciências Biológicas e da Saúde, Pontifícia Universidade Católica do Paraná, 80215-901 Curitiba, Brazil
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Correspondence to: Niels Olsen Saraiva Câmara, MD, Professor, Laboratory of Transplantation Immunobiology, Department of Immunology, Universidade of São Paulo, Av. Prof. Lineu Prestes, 1730, 05508-900 São Paulo, Brazil. niels@icb.usp.br
Telephone: +55-11-30917388 Fax: +55-11-30917224
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Abstract

Multipotent mesenchymal stromal cells (MSC) can be isolated and efficiently expanded from almost every single body tissue and have the ability of self-renewal and differentiation into various mesodermal cell lineages. Moreover, these cells are considered immunologically privileged, related to a lack of surface expression of costimulatory molecules required for complete T cell activation. Recently, it has been observed that MSC are capable of suppressing the immune response by inhibiting the maturation of dendritic cells and suppressing the function of T lymphocytes, B lymphocytes and natural killer cells in autoimmune and inflammatory diseases as a new strategy for immunosuppression. The understanding of immune regulation mechanisms by MSC is necessary for their use as immunotherapy in clinical applications for several diseases.

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Key words: Immunosuppression; Mesenchymal stem cell; Immune system; Inflammation; Autoimmune disease

Peer reviewer: Guangwen Ren, PhD, Department of Molecular Genetics, Microbiology and Immunology, Robert Wood Johnson Medical School-UMDNJ, 675 Hoes Lane West, Piscataway, NJ 08854, United States

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INTRODUCTION

Multipotent mesenchymal stromal cells (MSC) are adult multipotent non-hematopoietic stem cells capable of self-renewal and generation of different cell lines. Friedenstein and colleagues in 1970 were the first to isolate and report a population of adherent stem cells from the bone-marrow stroma[1]. Although initially encountered in the bone marrow, they are now shown to reside in almost every type of connective tissue and can be isolated from various post-natal tissues such as bone marrow, cornea and retina, placenta, tooth pulp, skin, nervous system and kidney[2]. Due to the facility of isolation and extensive differentiation potential, MSC are among the first stem cells to be introduced in clinical practice with a great potential in cell therapy.

Isolation, differentiation and expansion capacity

Generally, these cells can self-renew and are multipotent and therefore have a potential of differentiation more limited than embryonic stem cells which are pluripotent. MSC have been shown to be able to differentiate *in vitro* and *in vivo* into various mesodermal cell lineages including osteocytes, adipocytes, chondrocytes, muscle and myelo-sup-

portive stroma[3,4]. In addition, some studies have reported the ability of MSC to differentiate *in vitro* into tissues from other germ layers such as ectoderm (neurons) and endodermal (hepatocytes), a phenomenon denominated as plasticity, although these findings are still controversial[5,6]. *In vitro*, MSC can be efficiently expanded as adherent cells, can clonally regenerate and can give rise to differentiated progeny but generally have a limited *in vitro* lifespan due to a lack of activity of immortalizing enzyme telomerase, a phenomenon called "replicative senescence"[7,8]. Moreover, recent studies suggested that MSC could become neoplastic after long-term *in vitro* culture, enhancing tumor growth in some experimental models[9].

MSC are traditionally obtained by gradient centrifugation of bone marrow aspirates to isolate mononuclear cells that are then seeded in tissue culture plates in medium containing fetal bovine serum. Then, MSC adhere to plastic surfaces and can be expanded in culture plates while non-adherent cells are removed in the culture medium. It has been estimated that MSC represent a small fraction of the total nucleated cells isolated from bone marrow (0.001%-0.01%) through a Percoll gradient of a density of 1.073 g/mL[3]. Taking advantage of their plastic adherence characteristic and, in some cases associated with enzymatic tissue digestion and density gradient centrifugation methods, these cells may also be isolated from various tissues such as skeletal muscle, adipose tissue, synovial membranes, placenta, peripheral and cord blood[2,10-13].

As adherent cells isolated from these explants are a heterogeneous population, evidenced by the different morphology and functional potentials observed, and also, because sometimes they do not meet the criteria of a stem cell, the International Society for Cellular Therapy (ISCT) recently reclassified these cells as "multipotent mesenchymal stromal cells"[14]. In order to create a consensus and more uniformly characterize these cells, later the ISCT also published a position statement to propose a standard set of criteria to define the identity of a MSC[15].

Characterization and definition

Morphologically, human MSC are cells with fibroblast-like format (fusiform) characterized by the ability to form fibroblastic colony-forming units (CFU) in their early growth *in vitro*. These cells are negative for hematopoietic surface markers CD14, CD45, CD34, CD133 and positive for CD105, CD166, CD54, CD90, CD55, CD13, CD73, Stro-1 and CD44[16]. But, as no single antigen is exclusively expressed by human MSC, three criteria have been proposed by the ISCT for their characterization: (1) Adherence to plastic surfaces; (2) Potential to differentiate into osteocytes, adipocytes and chondrocytes; and (3) Expression of stem cell surface antigens.

Firstly, MSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Secondly, ⩾ 95% of the MSC population must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (⩽ 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class Ⅱ. Thirdly, the cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions[15].

Although many MSC studies have been developed recently, several questions remain unanswered about the origin of these cells and their relationship to other stromal cells such as fibroblasts. Recent studies showed evidence that MSC and fibroblasts share more similarities than previously recognized with respect to cell size, morphology, growth property, cell surface phenotype and immunomodulatory function[17]. For example, fibroblasts are plastic adherent cells expressing CD73, CD105 and negative for hematopoietic markers, a MSC property. Moreover, fibroblasts could be differentiated into osteoblastic, chondrogenic and adipogenic cell lineages[18,19], a MSC potentiality. A global comparative analysis of RNA expression between MSC and fibroblasts showed that the expression profiles of MSC and fibroblasts are highly similar; however, genes encoding transmembrane proteins (EPHA3 and FGFR2, tyrosine kinases receptors; GPR177, a G-protein-coupled receptor) or associated with tumors were differently expressed in MSC, providing a molecular basis for the discovery of novel MSC-specific biomarkers[20]. In addition, recently, a subset of MSC were identified *in vivo* and prospectively isolated from adult mouse bone marrow by phenotypical, morphological and functional criteria as PDGFRα+ Sca-1+ CD45- TER119- cells, providing a useful method to identify MSC[21].

MSC AND THE IMMUNE SYSTEM

MSC appear to have a major advantage over many other cell types used for cellular therapy because they are considered "immunologically privileged". This property is related to a reduced expression on class Ⅰ and Ⅱ MHC antigens in addition to a lack of surface expression of CD40, CD80 and CD86, costimulatory molecules required for activation of T cells. Generally, the use of fully mismatched MSC does not provoke a proliferative T-cell response in an allogeneic mixed lymphocyte reaction *in vitro,* as demonstrated by some studies where MSC was transplanted across MHC barriers due to their immunosuppression property[22]. In addition, some *in vitro* studies suggested a greater immunosuppressive effect of allogeneic MSC compared with autologous MSC[23]. This mechanism of allogeneic escape may be of therapeutic value because transplantation of allogeneic MSC would be readily available, as opposed to a culture of autologous or donor-related cells to each patient.

Over the last few years it has been observed that MSC are capable of suppressing the immune response by inhibiting the maturation of dendritic cells and suppressing the function of T lymphocytes, B lymphocytes and NK cells[24-27] .

Dendritic cells

MSCs can inhibit the differentiation, maturation and activation of dendritic cells (DCs), generating immature DCs.

They can alter the secretory cytokine profile of DCs by stimulating the secretion of regulatory cytokines such as IL-10 and by inhibiting pro-inflammatory cytokines such as interferon (IFN)-γ, IL-12 and tumor necrosis factor (TNF)-α[28]. Molecules related to antigen presentation such as CD1a, CD40, CD83, CD80 (B7-1), CD86 (B7-2) and HLA-DR could be inhibited during the maturation of DCs in the presence of MSC[27]. Moreover, DCs isolated from co-cultures with MSC showed a reduced potential to activate proliferation of CD4+ T cells[29].

B cells

MSC may also regulate the immune response through interaction with B lymphocytes. When bone marrow MSCs and B lymphocytes from peripheral blood of healthy donors were co-cultured with stimuli to B cells activation, the proliferation of B lymphocytes and immunoglobulin production (IgM, IgG and IgA) were inhibited by the secretion of soluble factors by MSC. Moreover, the chemotactic property of B cells was affected since CXCR4, CXCR5, CXCL12 and CXCR4 ligand were significantly down-regulated by MSC[24]. However, depending on the level of stimulation (e.g. by lipopolysaccharide or viral antigens), the IgG secretion by activated B cells could be stimulated or inhibited after the addition of MSC[30] and these variable results might reflect the different experimental conditions.

The secretome of MSC suppressed plasma cell immunoglobulin production as a result of MSC-derived CC chemokine ligands CCL2 and CCL7 processed by the activity of matrix metalloproteinases (MMPs). The neutralization of CCL2 or inhibition of MMP enzymatic activity abolished their suppressive effect and the MMP-processed CCL2 suppressed the STAT3 activation in plasma cells. Furthermore, MSC could decrease antihuman factor Ⅷ (hFⅧ)-IgG levels in hemophilic B6 mice[31].

NK cells

NK cells are cytotoxic cells that mainly target cells that lack or down-regulate the expression of class Ⅰ HLA. It was also reported that MSC inhibited the IFN-γ production by IL-2 stimulated NK cells. At low NK-MSC ratios, MSC could modify the phenotype of NK cells and suppress proliferation, cytokine secretion and cytotoxicity against class Ⅰ -expressing HLA targets. However, MSC were susceptible to lysis by activated NK cells[32]. In another study, it was shown that non-classic human leukocyte antigen class Ⅰ molecule (HLA-G) secreted by human MSC inhibited NK cell-mediated cytolysis and IFN-γ secretion[33].

T cells

The immunomodulatory effect of MSC on T cells has only been described recently and is based on the observation that bone marrow MSC suppressed T-cell proliferation *in vitro*[25]. Moreover, on *in vivo* infusion, MSC prolonged skin engraftment in baboons[34] and the suppression of T cell proliferation did not require MHC restriction since it was mediated by allogeneic MSC, although little is known about the molecular mechanisms involved. MSC were capable of reducing the expression of some activation markers such as CD25, CD38 and CD69 on *in vitro* stimulated lymphocytes and suppressing the proliferation of CD4+ and CD8+ cells[35]. In addition, it has been reported that MSC induced T cell anergy that is only partly reversed by exogenous IL-2[36] and both naïve and memory T cell could be inhibited[37].

MSC can interfere with naïve CD4+ T cell differentiation into T helper 1 (Th1) effector cells by decreasing the production of IFN-γ and inducing a Th-2 shift toward an increased IL-4 production to induce a more anti-inflammatory phenotype. In addition, after co-culture with antigen-specific T cells, MSC can induce the expansion of regulatory T cells, a specialized sub population of cells that suppress activation of the immune system maintaining homeostasis and tolerance to self antigens[26]. Recently, it was observed that MSC prevented the *in vitro* differentiation of naïve CD4+ T cells into Th17 cells and inhibited the production of IL-17, IL-22, IFN-γ and TNF-α by fully differentiated Th17 cells, inducing the expression of fork head box p3 (Foxp3) and IL-10 production[38]. Moreover, MSC can suppress the lysis mediated by CD8+ and prevent the development of cytotoxic T cells[39], although this effect could not be observed after activation of cytotoxic T cells.

Many factors produced by MSC which promote lymphocyte suppression such as transforming growth factor (TGF)-β, hepatocyte growth factor (HGF), iNOS, indoleamine 2,3-dioxygenase (IDO), PGE_2, HLA-G5 and IL-10 were characterized as possible molecules responsible by this immunomodulation and will be discussed below.

MECHANISMS OF ACTION: WHERE DO WE STAND?

Over the last few years, several studies have shown possible soluble factors related to the immunosuppressive effect of MSC and the mechanisms have only started to be elucidated. Many soluble factors have been identified as responsible for inhibition of proliferation/differentiation of immune cells and are shown in Table 1.

It is important to note that the immune suppression by MSC may be caused by different mechanisms and contradictory studies are found since different T cells stimulus (e.g. mitogens or allogeneic cells) were used. Moreover, the suppressive factor(s) is (are) not constitutively secreted by MSC because generally cell culture supernatants do not suppress T-cell proliferation.

Important candidates which have been extensively studied are TGF-β and HGF. The immunosuppressive effect induced by human MSC on effector T cells against peripheral blood mononuclear cells (PBMCs) could be abrogated in the presence of high concentrations of neutralizing antibodies to TGF-β1 and hepatocyte growth factor (HGF)[25]. However, the neutralization of each factor separately resulted in a partial restoration of T cell proliferation, excluding a single role for TGF-β in MSC-induced suppression.

Another mechanism that has been investigated is the

Table 1 Potential candidates responsible for immunoregulation by mesenchymal stromal cells

Soluble factors	Measured response	Ref.
TGF-β and/or HGF	Proliferation, IFN-γ production	[25]
IDO	Proliferation	[40]
PGE2	Proliferation, IFN-γ and TNF-α production	[26,42]
HLA-G5	Proliferation and expansion of CD4+CD25+Foxp3+ regulatory T cells	[33]

TGF: Transforming growth factor; HGF: Hepatocyte growth factor; IDO: Indoleamine 2,3-dioxygenase; IFN: Interferon; TNF: Tumor necrosis factor.

expression of IDO by MSC stimulated with IFN-γ. IDO promotes the depletion of tryptophan in the medium since it catalyzes the conversion of tryptophan to kynurenine which reduced lymphocyte proliferation. It was observed that the addition of tryptophan could restore proliferation in T cells stimulated with PBMC co-cultured with MSC[40]. However, another study excluded a role for IDO since the addition of tryptophan or an IDO inhibitor showed no effect on MSC suppression[41].

MSC constitutively express both cyclooxygenases (COX-1 and COX-2) and PGE2 production increased when MSC were co-cultured with T cells. Moreover, inhibitors of PGE2 synthesis (e.g. indomethacin or NS-398) could restore the proliferation of stimulated T cells[26,42,43]. The addition of a PGE2 inhibitor restored DC differentiation and function that was inhibited by MSC. Moreover, PGE2 added directly to cultures of monocytes blocked their differentiation toward DCs in a manner similar to MSC, suggesting a major role for this prostaglandin in the MSC inhibitory effect[44].

Another important soluble molecule involved in MSC immune regulation is the non-classic class I human leukocyte antigen (HLA) molecule 5 (HLA-G5). The soluble isoform of HLA-G5 secreted by MSC after cell-to-cell contact with allo-stimulated T cells is responsible for their immunomodulatory properties of suppression of T-cell proliferation and expansion of CD4+CD25+Foxp3+ regulatory T cells as well as NK-cell mediated cytolysis and IFN-γ secretion[33].

Recently, it was shown that the inhibition of inducible nitric-oxide synthase (iNOS) was sufficient to restore T-cell proliferation in mixed co-cultures of MSC and activated anti-CD3 splenocytes, showing that nitric oxide (NO) has an important role in immune suppression by MSC. It was found that the immunosuppressive function of MSC is elicited by IFN-γ and the concomitant presence of any of three other pro-inflammatory cytokines (TNF-α, IL-1a or IL-1b) which promotes the high expression of several chemokines and iNOS. These chemokines drive T cell migration into proximity with MSC where T cell responsiveness is suppressed by NO[45].

Although many studies have identified several molecules to explain the possible mechanisms of immune regulation by MSC, the inhibition of any of these molecules generally does not result in a complete loss of MSC suppressor activity. Furthermore, the roles of these identified molecules are variable and sometimes contradictory in different studies, suggesting that MSC immune regulation is a complex phenomenon and may include different inhibitory and stimulatory mechanisms mediated by several molecules. Importantly, the mechanism of MSC-mediated immunosuppression may be different among different species. For example, under the same culture conditions, immunosuppression by human- or monkey-derived MSC was mediated by IDO whereas mouse MSC used NO[46].

A summary of the main mechanisms of immunosuppression by MSC is shown in Figure 1.

MSC AND AUTOIMMUNE AND INFLAMMATORY DISEASES: A NEW STRATEGY FOR IMMUNOSUPPRESSION?

The *in vivo* immune suppression property of MSC was first observed in a study where allogeneic MSC prolonged skin-graft survival in baboons[34]. Moreover, it was demonstrated that MSC could be used for treatment of severe graft-versus-host disease (GvHD) as a novel strategy of immunosuppressive therapy[47,48] and their infusion in mice transplanted with haploidentical hematopoietic grafts controlled the lethal GvHD[49]. However, the mechanism responsible for the clinical improvement remains speculative and has been studied in animal models. Interestingly, it was showed IFN-$\gamma^{-/-}$ T cells did not respond to MSC treatment in GvHD. Moreover, MSC pre-treated with IFN-γ are activated and can suppress GvHD more efficiently (fivefold more) than non- IFN-γ-activated MSC[50].

The immunosuppressive effect of MSC has also been used in autoimmune diseases such as diabetes, arthritis, multiple sclerosis and systemic lupus erythematosus. In experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis in mice, systemic injection of MSC at disease onset ameliorated the inflammatory infiltrates (T cells, B cells and macrophages) and demyelination and inhibited T-cell response to myelin oligodendrocyte glycoprotein (MOG)[51]. The conditioned medium of MSC inhibited EAE-derived CD4+ T cell activation by suppressing STAT3 phosphorylation. In addition, CD4 T cell infiltration of the spinal cord of MSC-treated mice was decreased along with reduced plasma levels of IL-17 and TNF-α[52]. In another study, administration of allogeneic Balb/c-derived MSC to C57BL/6 mice with pre-established EAE led to a significant disease score decrease that was correlated with a significant blunting of immune

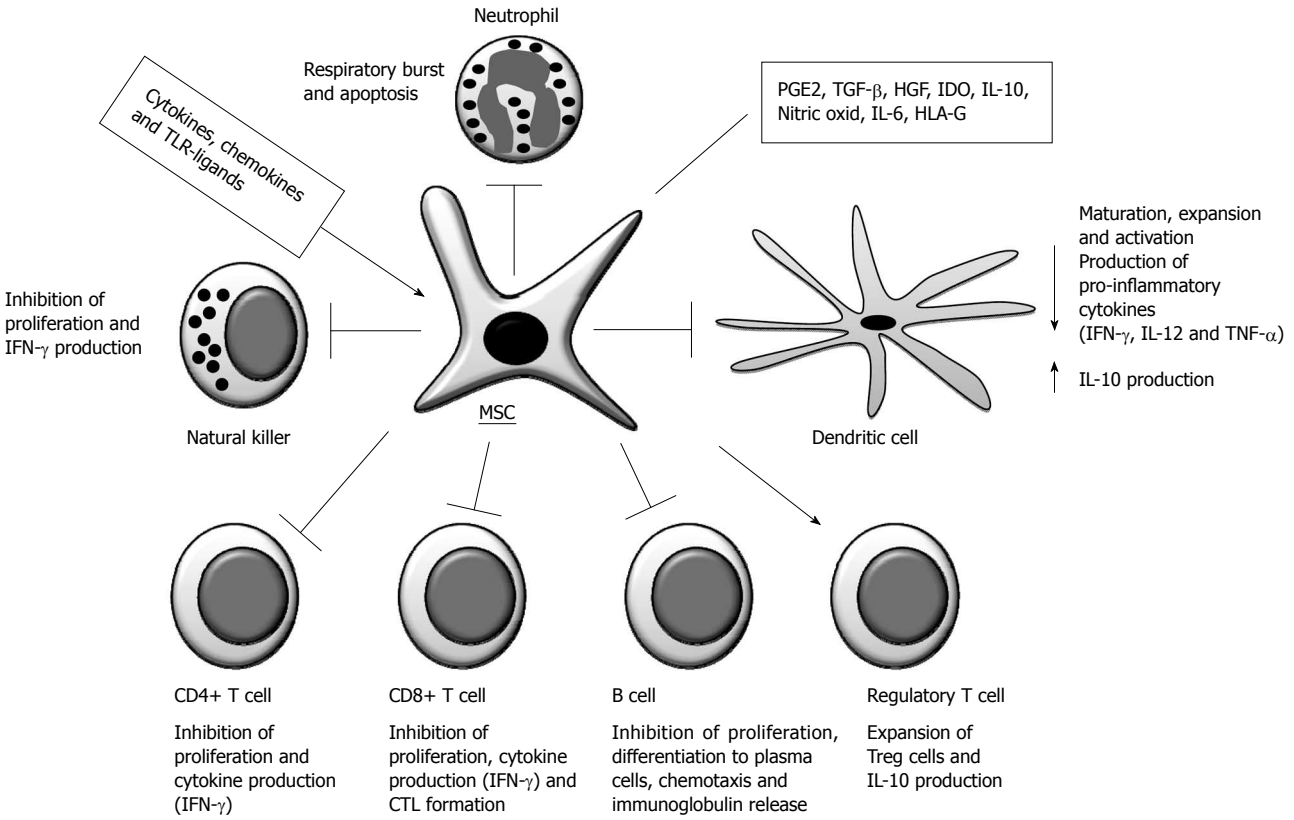

Figure 1 Immune modulation effects of mesenchymal stromal cells on various cells of the immune system. Several soluble factors have been identified to play a major role in immunosuppressive effects of mesenchymal stromal cells (MSC) including prostaglandin E2 (PGE2), transforming growth factor (TGF-β), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), interleukin-10 (IL-10), nitric oxide, IL-6 and HLA-G. Moreover, cytokines (e.g. IFN-γ), chemokines and Toll-like receptors (TLR) ligands have been shown to modulate/activate immune suppression by MSC.

cell infiltration to the central nervous system and low levels of IFN-γ and IL-17 in the blood[53].

In a model of rheumatoid arthritis (RA) collagen-induced in mice, the systemic infusion of human adipose-derived MSC (hADMSC) significantly reduced the incidence and severity of disease by down-regulating the Th1-driven autoimmune and inflammatory response. In addition, hADMSC decreased the antigen-specific Th1/Th17 cell expansion and induced the production of anti-inflammatory IL-10 cytokine in lymph nodes and joints generating antigen-specific CD4+CD25+Foxp3+ cells[54]. *In vitro*, hADMSC suppressed the collagen antigen-specific response of T cells from patients with RA by inhibiting the proliferative response and the production of inflammatory cytokines and by increasing the levels of IL-10 producing T cells. Moreover, hADMSC also stimulated the generation of regulatory T cells with capacity to suppress collagen-specific T cell response and down-regulated matrix-degrading enzymes by synovial cells isolated from patients with RA[55]. However, in another study, Flk-1+ MSC (a population of MSC with a defined phenotype) aggravated arthritis in mice by up-regulating the secretion of IL-6 which promotes Th17 differentiation[56].

In a model of acute renal failure, administration of MSC ameliorates the renal function through the inhibition of pro-inflammatory cytokines (IL-1β, TNF and IFN-γ)[57]. The role of MSC in fibrogenesis in chronic kidney disease was investigated in a remnant model in rats. An amelioration of functional parameters and reduced levels of fibrosis were observed in MSC-treated animals whereas renal IL-6 and TNF-α were significantly decreased. Moreover, anti-inflammatory cytokines such as IL-4 and IL-10 expression levels were increased[58]. In an experimental model of lung fibrosis, MSC inhibited the inflammation within the lungs. Interestingly, it was shown that MSC secrete interleukin-1 receptor antagonist as a potential mediator of TNF-α and IL-1 neutralization[59].

In autoimmune type I diabetes, allogeneic murine MSC delayed the disease onset when administered to pre-diabetic (non-obese diabetic) NOD mice by promoting a shift towards Th2-immune response[60]. Prevention of auto-immune β-cell destruction and subsequent diabetes was observed after a single intravenous injection of MSC and this effect was related to the induction of regulatory T cells[61]. *In vitro*-expanded syngeneic bone marrow-derived MSC homed to the pancreas and enhanced insulin secretion that sustained normoglycemia into a rat model of streptozotocin-induced β-cell injury. In addition, islets expressed high levels of both PDX-1 (pancreatic and duodenal homeobox-1) and insulin which confirmed β-cell activation in MSC-treated animals. Interestingly, peripheral T cells exhibited a shift toward IL-10/IL-13 production (Th2-immune response) and higher frequencies of CD4+/CD8+ Foxp3+ cells[62].

A major bottleneck of current MSC application is their low engraftment *in vivo* since in most studies the cells are infused intravenously (i.v.) into mice or rats and are rapidly trapped in lung as microemboli and in the liver[63]. In a model of myocardial infarction in mice, i.v.-infused human MSC produced a functional improvement by decreasing inflammatory responses and then reducing the infarct size. Interestingly, the cells were trapped in lungs and were activated to produce the anti-inflammatory factor TNF-α-induced protein 6 (TSG-6). Moreover, the beneficial effect was not observed when MSC transduced with TSG-6 silencing RNA (siRNA) were used[64].

CONCLUSION

MSC can provide effective treatments for a wide range of diseases and in several applications in regenerative medicine such as tissue repair and gene delivery. In addition, recently, the immunomodulatory potential of MSC has been extensively studied for application in various inflammatory responses, auto-immune disorders and organ transplantation. However, the pathways involved in their property of immune regulation on various immune cells are not fully elucidated. Thus, additional pre-clinical studies still need to be performed for the safe use of MSC in future clinical applications.

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