

Exploring the Role of Soluble Factors Associated with Immune Regulatory Properties of Mesenchymal Stem Cells

Ênio José Bassi · Danilo Candido de Almeida ·
Pedro Manoel Mendes Moraes-Vieira ·
Niels Olsen Saraiva Câmara

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Abstract Mesenchymal stem cells (MSCs) are characterized as multipotent stromal cells with the capacity for both self-renewal and differentiation into mesodermal cell lineages. MSCs also have a fibroblast-like phenotype and can be isolated from several tissues. In recent years, researchers have found that MSCs secrete several soluble factors that exert immunosuppressive effects by modulating both innate (macrophages, dendritic and NK cells) and adaptive (B cells and CD4⁺ and CD8⁺ T cells) immune responses. This review summarizes the principal trophic factors that are related to immune regulation and secreted by MSCs under both autoimmune and inflammatory conditions. The understanding of mechanisms that regulate immunity in MSCs field is important for their future use as a novel cellular-based immunotherapy with clinical applications in several diseases.

Keywords Mesenchymal stem cells · Immune regulation · Immune system · Soluble factors · Immunotherapy · Inflammation

Ê. J. Bassi · P. M. M. Moraes-Vieira · N. O. S. Câmara
Laboratory of Transplantation Immunobiology,
Department of Immunology, Institute of Biomedical Sciences IV,
Universidade de São Paulo-USP,
05508-000 São Paulo, Brazil

D. C. de Almeida
Nephrology Division, Medicine Department,
Universidade Federal de São Paulo-UNIFESP,
04039-000 São Paulo, SP, Brazil

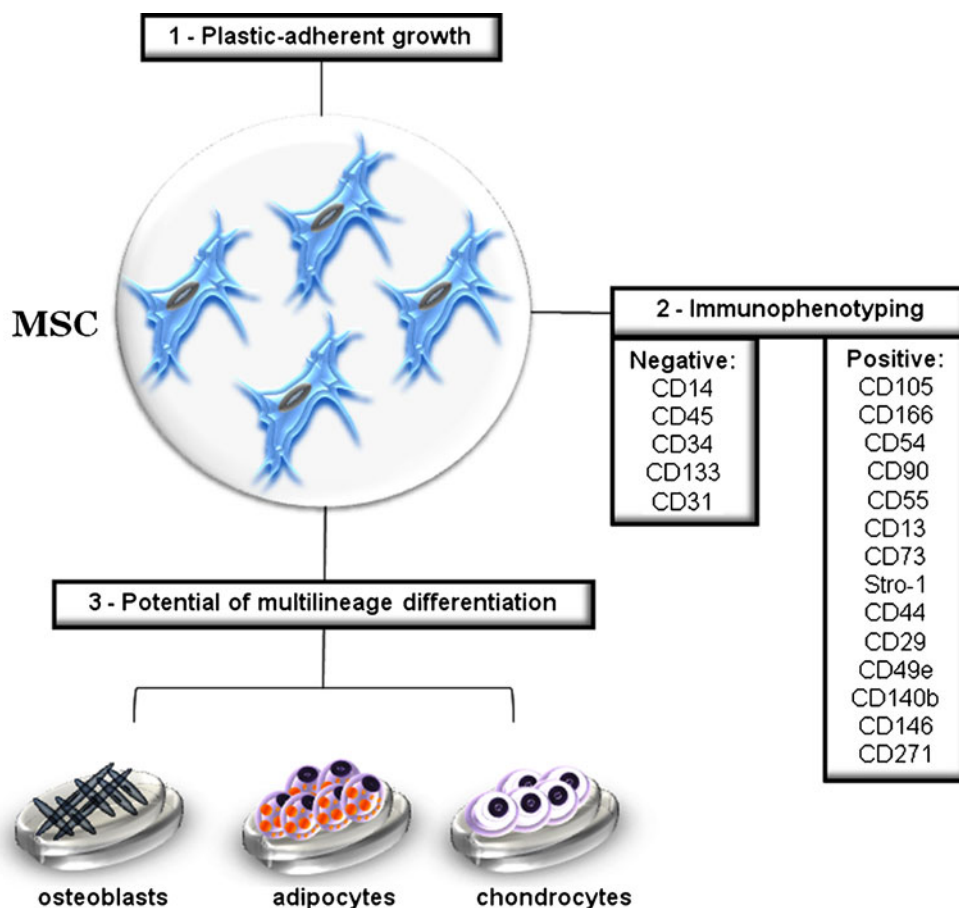
N. O. S. Câmara (✉)
Department of Immunology, Institute of Biomedical Sciences IV,
University of São Paulo,
Av. Prof. Lineu Prestes, 1730,
05508-000 São Paulo, SP, Brazil
e-mail: niels@icb.usp.br

Introduction

Multipotent stromal cells or mesenchymal stem cells (MSC) are adult, adherent non-hematopoietic stem cells that were first isolated by Friedenstein et al. in 1970 from bone marrow stroma characterized by long-term self-renewal [1]. In recent years, these cells have been isolated from various post-natal tissues, including bone marrow, placenta, tooth pulp, skin, adipose tissue, the nervous system and kidney [2]. Morphologically, MSCs are cells with fibroblast-like properties (fusiform) and are characterized by their ability to form fibroblast colony-forming units (CFUs) in their early growth in vitro and the potential to differentiate into osteocytes, adipocytes and chondrocytes. Moreover, these cells do not express the hematopoietic surface markers CD14, CD45, CD34, CD133 and endothelial marker CD31 and are positive for CD105, CD166, CD54, CD90, CD55, CD13, CD73, Stro-1 and CD44 [3]. Although MSCs can be isolated from several sources, according to the International Society for Cellular Therapy [4], every MSC should have a plastic-adherent growth pattern, expression of specific surface antigens and the potential for multilineage differentiation (osteogenic, chondrogenic and adipogenic) (Fig. 1) [5]. Moreover, the term “multipotent mesenchymal stromal cells” has been generally used instead of “mesenchymal stem cells” [5].

MSCs have the potential to provide effective treatment for a wide range of diseases and can be used in several applications in regenerative medicine such as tissue repair and gene delivery. In addition, MSCs were among the first stem cells to be introduced into clinical practice. Interestingly, these cells can modulate the immune response by both inhibiting the maturation/differentiation of dendritic cells and by suppressing the activation and/or function of T and B lymphocytes and NK cells in several autoimmune

Fig. 1 Characterization of MSCs isolated from several sources. For the characterization of MSCs three criteria should be considered: (1) adherence to plastic surfaces upon in vitro expansion; (2) expression of mesenchymal stem cell surface antigens verified by immunophenotyping; and (3) in vitro potential of differentiation into osteogenic, adipogenic and chondrogenic cell lineages



and inflammatory diseases. Over the past few years, MSC isolated from bone marrow, adipose tissue and umbilical cord tissue have been used as the main sources for tissue regeneration or cellular-based therapy, and their immune regulatory effects have been explored [6, 7]. Interestingly, both umbilical cord and adipose tissue can provide an interesting alternative source of MSCs due their efficient potential for expansion and differentiation. In addition, the process of obtaining these cells is less invasive compared to classical bone marrow aspiration [6, 8, 9]. Moreover, adipose tissue-derived MSCs have generally been associated with immunomodulatory activities and umbilical cord-derived MSC with a greater angiogenic potential [10–13].

In this review, the main soluble factors that are secreted by MSCs and related to the immune regulatory effect will be discussed. Understanding the mechanisms that are involved in regulating immunity by these cells both in vitro and in vivo is important for expanding its use in cell-based therapies of several autoimmune and inflammatory conditions.

MSCs and Immune Regulatory Properties

MSCs are considered to be “immunologically privileged”, as they express a relatively small complement of the

molecules that are required for fully activating T cells. These cells have reduced expression of both class I and II major histocompatibility complex (MHCs) as well as a lack of surface expression of co-stimulatory molecules (CD80, CD86 and CD40). These properties allow the use of mismatched MSC in vivo and do not promote a proliferative T cell response in an allogeneic mixed-lymphocyte reaction in vitro [14]. In addition, MSCs can reduce the expression of certain activation markers (e.g., CD25, CD38 and CD69) in in vitro stimulated T lymphocytes and can suppress the proliferation of both CD4⁺ and CD8⁺ T cells [15]. MSCs can also attenuate the differentiation of naïve CD4⁺ T cells into T helper 1 (Th1) effector cells by decreasing their production of IFN- γ and promoting a shift toward the Th2 immune response. Interestingly, when co-cultured with antigen-specific T cells, MSCs induce the expansion of regulatory T cells (Tregs)—which are a specialized T cell subpopulation that suppress immune system activation—thereby maintaining homeostasis and tolerance to self antigens [16]. Furthermore, it was recently reported that MSCs inhibits the in vitro differentiation of human naïve CD4⁺ T cells into Th17 cells and promotes forkhead box p3 (FOXP3) expression and IL-10 production. The production of IL-17, IL-22, IFN- γ and TNF- α by fully differentiated Th17 cells was decreased when they

were co-cultured with MSCs [17]. Th17 cells are linked to the immune response against extracellular pathogens that are encountered at mucosal surfaces and play an important role in the inflammatory process that is associated with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and psoriasis [18].

MSCs also regulate the immune response by interacting with B lymphocytes. When bone marrow-derived MSCs and B lymphocytes from the peripheral blood of healthy human donors were co-cultured with various B cell stimuli, both the proliferation of B lymphocytes and the production of immunoglobulins were inhibited by the MSCs [19]. Tabera et al. [20] showed that MSCs increased B cell viability and inhibited their proliferation by arresting the cell cycle in the G0/G1 phase. Interestingly, in another study, MSCs that were derived from adipose tissue were more efficient at suppressing immunoglobulin production by mitogen-stimulated B cells than MSCs that were derived from bone marrow [20].

In addition to the immunosuppressive properties that they confer to T and B lymphocytes, MSCs can also inhibit the differentiation and activation of dendritic cells (DCs). Specifically, MSCs alter the cytokine profile of DCs by stimulating the secretion of regulatory cytokines, such as IL-10, and by inhibiting the production of pro-inflammatory cytokines, such as IFN- γ , IL-12 and TNF- α [21]. Furthermore, molecules that are related to antigen presentation, such as CD1a, CD40, CD83, CD80, CD86 and HLA-DR, were all inhibited after the maturation of DCs in the presence of MSCs.

Over the past few years, several studies using conditioned medium or a transwell culture system have identified several soluble factors that underlie the immunosuppressive properties that MSCs confer on various cells in the immune system (Table 1). These studies were primarily *in vitro* experiments that used neutralizing antibodies, silencing RNAs or MSCs that were derived from animals that were genetically deficient in a specific target molecule, with the goal of identifying putative trophic factors implicated in regulating the immune system. This cross-talk between MSCs and several cells of the immune system will be extensively discussed in this review. Figure 2 summarizes the principal immune regulatory molecules that are secreted by MSCs and will be thoroughly reviewed hereafter.

Mechanisms that are Associated with Immune Regulation by MSC-derived Soluble Factors

Transforming Growth Factor β

In mammals, transforming growth factor beta (TGF- β) has three isoforms: TGF- β 1, TGF- β 2 and TGF- β 3 [22]. These

growth factors are immunosuppressive cytokines that are secreted by MSCs and are important in the regulation of the immune system [23]. In this sense, MSCs that were isolated from dental pulp produced TGF- β and suppressed the proliferation of peripheral blood mononuclear cells (PBMCs). Interestingly, this effect could be either reversed by the addition of anti-TGF- β neutralizing antibodies or increased by the addition of a TLR-3 agonist, which potentiated the secretion of TGF- β by the MSCs [24]. Another study reported that allogeneic bone marrow MSCs could induce the expression of the Treg markers Foxp3 and CD25 in PBMCs in a TGF- β —and prostaglandin E2 (PGE2)—dependent manner [25]. Although the MSC-induced increase in Treg frequency can be considered an important protective effect in autoimmune diseases, in other situations, this effect could support the growth and/or development of cancers by protecting cancer cells from immune clearance. In an *in vitro* study, Patel et al. [26] showed that MSCs suppressed the proliferation of PBMCs against breast cancer cells by decreasing the levels of granzyme B in NK and cytotoxic CD8+ T cells. Moreover, MSC-derived TGF- β 1 was largely responsible for the increase in Treg frequency as shown in knockdown studies, thereby contributing to the support of breast cancer development [26].

A recent study using a model of ragweed-induced asthma showed that MSCs use TGF- β to suppress the allergic response [27]. The injection of MSCs protected the mice from the majority of asthma-specific pathological effects such as eosinophil infiltration and excess mucus production in the lungs. This beneficial effect was associated with a decrease in the levels of Th2 cytokines (e.g., IL-4, IL-5 and IL-13) in bronchial lavage aspirates, decreased serum Th2 immunoglobulins (IgG1 and IgE) and an increase in the frequency of Treg cells. To explore the underlying mechanism of action, MSCs were isolated from several knockout (KO) mice and neutralizing antibodies were used to block cytokines *in vivo*. Interestingly, IL-4 and/or IL-13 were able to activate the STAT6 pathway in MSCs, which led to increased TGF- β secretion. TGF- β secreted by MSCs could either alone or by recruiting Treg cells to confer the beneficial effects that were observed after treatment, thereby playing a pivotal role in the mechanism of *in vivo* MSC immune regulation. Furthermore, it has been suggested that inflammatory cytokines that are produced during the development of asthma can modulate MSCs, improving their secretion of immune regulatory factors. Thus, based on several studies, TGF- β 1 is now accepted as being responsible for the immunosuppressive effect of MSCs on CD4+ T lymphocytes both *in vitro* and *in vivo*. However, their effect can be also associated with other trophic factors, such as hepatocyte growth factor (HGF) and prostaglandin E2 (PGE2).

Table 1 Soluble factors that are associated with the immune regulatory properties of MSCs. *TGF- β 1* transforming growth factor- β 1; *HGF* hepatocyte growth factor; *PGE2* prostaglandin E2; *IDO*: indoleamine 2,3-dioxygenase; *NO* nitric oxide; *HO-1* heme oxygenase-1; *IL-10* interleukin-10; *IL-6* interleukin-6; *MMP-2*

metalloproteinase-2; *MMP-9* metalloproteinase-9; *HLA-G5* human leukocyte antigen-5; *CCL2* CC-chemokine ligand 2; *CCL5* chemokine (C-C motif) ligand 5 (also known as RANTES); *LIF* leukemia inhibitory factor; *TIMP-2* tissue inhibitor of metalloproteinase-2; *TIMP-3* tissue inhibitor of metalloproteinase-3

SOLUBLE FACTORS	SOURCE OF MSC	REFERENCES
TGF- β 1	Bone marrow, tooth pulp and adipose tissue	[23–27, 75, 83]
HGF	Bone marrow and adipose tissue	[30, 31]
PGE2	Bone marrow, umbilical cord and adipose tissue	[25, 39–46]
IDO	Bone marrow and umbilical cord	[49–51]
NO	Bone marrow	[56–59]
HO-1	Bone marrow	[62, 63]
IL-10	Bone marrow and adipose tissue	[72–75]
IL-6	Bone marrow	[79–83]
MMP-2, MMP-9, TIMP-2 and TIMP-3	Bone marrow	[90–95]
HLA-G5	Bone marrow	[101]
CCL2 and CCL5	Bone marrow	[104, 107–109]
LIF	Bone marrow and adipose tissue	[113, 114]

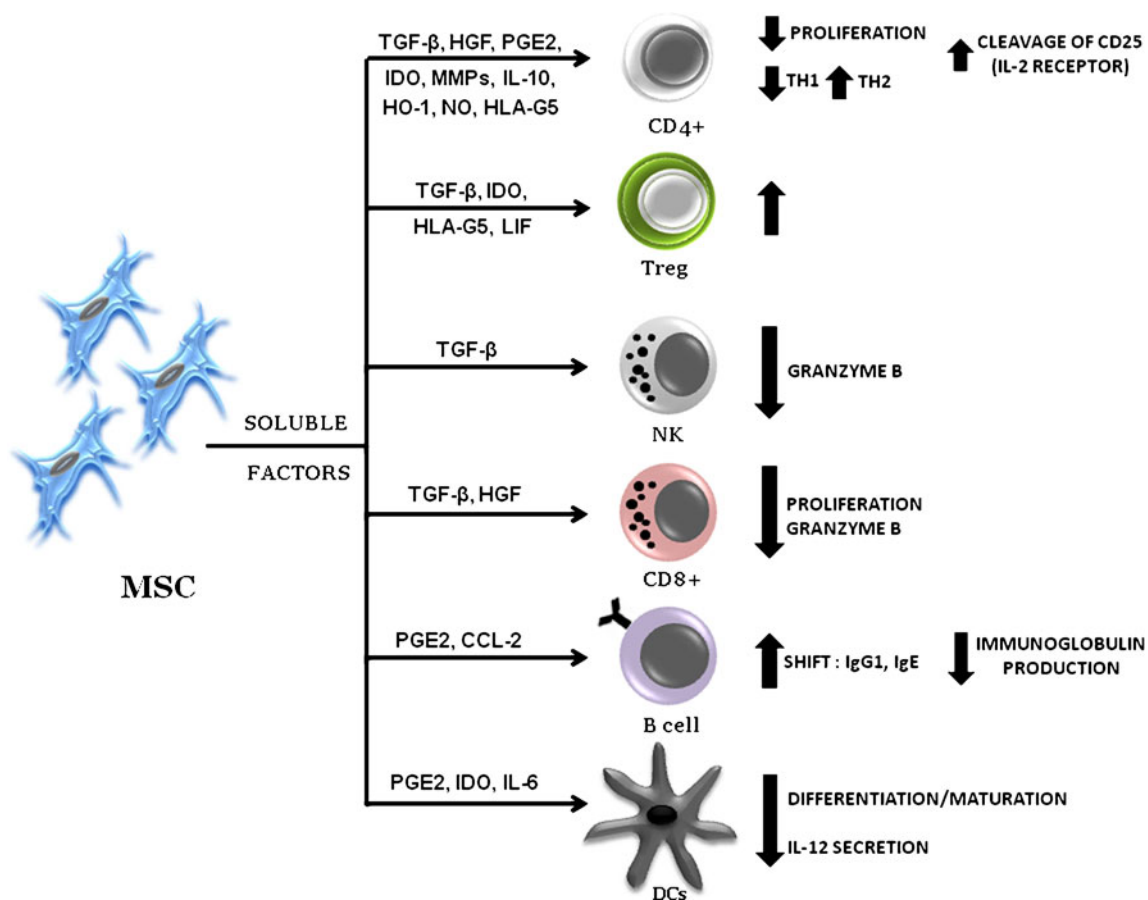


Fig. 2 Immune regulatory effects of MSC-secreted soluble factors in several cells of the immune system. Cytokines (e.g., IFN- γ), chemokines and toll-like receptor (TLR) agonists have been shown to activate and/or modulate the immune suppression properties of MSCs.

Several MSC-secreted soluble factors have been identified as being responsible for their immune regulation properties in dendritic cells (DCs), and T and B cells and include TGF- β , PGE2, HGF, LIF, IDO, IL-10, IL-6, NO, HLA-G5, MMPs, CCL2, CCL5 and HO-1

Hepatocyte Growth Factor

HGF is a potent hepatocytic mitogen and is related to liver regeneration. This multi-function cytokine has important roles in multiple diverse biological pathways, such as morphogenesis, carcinogenesis and anti-apoptotic activity, in several cell types. Importantly, HGF can be secreted by MSCs and is related to tissue repair/regeneration in several cell types following injury by activating a tyrosine kinase signaling cascade [28, 29]. In vitro, autologous or allogeneic human MSCs suppressed CD4⁺ and CD8⁺ T lymphocyte proliferation that was induced by allogeneic or polyclonal stimuli in an HGF- and TGF- β 1—dependent manner, as simultaneously neutralizing both cytokines with monoclonal antibodies restored T cell proliferation [30]. In another study, the levels of HGF and TGF- β 1 were significantly increased in the conditioned medium of canine adipose-derived MSCs that were co-cultured with leukocytes, suggesting a role for HGF in the immune regulatory mechanism [31].

Although preliminary in vitro studies have suggested an immune regulatory effect of HGF (primarily when associated with TGF- β 1) the in vivo role of this molecule as a possible mechanism of MSC immunosuppression has been not yet investigated. Interestingly, aside from its immune regulatory effect, MSC-secreted HGF exhibits regenerative potential (e.g., a pro-angiogenic effect) that could promote tissue regeneration following injury, thus contributing to the therapeutic effect of MSCs in several diseases.

Prostaglandin E2

PGE₂, an eicosanoid that is enzymatically derived from fatty acids, has a short half-life and is the most abundant prostaglandin in the body. The biosynthesis of PGE₂ is regulated by two cyclooxygenase (COX) enzymes. COX-1 is constitutively expressed, whereas COX-2 is induced by cytokines, endotoxins and pro-inflammatory agents such as IL-1 β and TNF- α [32]. PGE₂ has an important role in regulating the inflammatory immune response, as it mediates hyperalgesia and arterial dilation and enhances microvascular permeability. PGE₂ exerts its actions by binding to one (or a combination) of its four G-protein coupled receptor subtypes called EP₁, EP₂, EP₃ and EP₄ [33]. A recent study showed that PGE₂ regulates Th17 cell differentiation through cAMP and EP₂/EP₄ receptor signaling, thereby possessing inflammatory potential [34]. However, it was also shown that PGE₂ can have anti-inflammatory properties both by inhibiting Th1 proliferation and their production of IFN- γ and by enhancing the production of Th2 cytokines (e.g., IL-4, IL-5 and IL-10) [35]. In B cells, PGE₂ stimulates an immunoglobulin isotype class switch to IgG1 and IgE in a cAMP-

dependent mechanism [36]. In addition, PGE₂ inhibits the maturation and antigen-presenting capacity of macrophages and DCs by inducing their expression of IL-10 and inhibiting their expression of IL-12, TNF- α and IL-1 β [37, 38].

Interestingly, MSCs constitutively produce PGE₂, and human MSC proliferation is regulated by this prostaglandin through differential activation of cAMP-dependent protein kinase isoforms [39]. Furthermore, PGE₂ plays a key role in mediating the immunosuppressive effects of MSCs over DCs, T lymphocytes and inflammatory cytokines such as IFN- γ , TNF- α and IL-1 β that up regulate both the expression of COX2 and the subsequent production of PGE₂ by MSCs [40, 41]. In vitro, blocking PGE₂ restores T lymphocyte proliferation and plasmacytoid DC maturation when co-cultured with MSCs [42, 43]. Inhibition of CD4⁺ and CD8⁺ T-cell proliferation and activation by MSCs are mediated by the expression of COX1/COX2 enzymes and the production of PGE₂ [44]. In an in vitro study, monocytes cultured in the presence of MSCs did not acquire the surface phenotype of DCs (CD1a⁺, CD80⁺, CD86⁺) after the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 [45]. Moreover, DCs that were co-cultured with MSCs did not produce IL-12 and failed to induce T cell activation/proliferation in a PGE₂-dependent manner. Inhibiting PGE₂ production with the COX-2 inhibitor NS-398 restored DC differentiation and function [45]. In an in vivo study, Nemeth et al. [46] showed that injection of MSCs in mice attenuated sepsis by reducing mortality and improving organ function via secretion of PGE₂. Interestingly, macrophages that were isolated from septic lungs of treated mice produced higher levels of IL-10 compared with macrophages from untreated mice, and the therapeutic effect of MSCs was abolished by macrophage depletion or by neutralizing antibodies against IL-10. These results suggest that PGE₂ that is secreted by MSCs upon activation by LPS or TNF- α can reprogram macrophages through EP₂ and EP₄ receptor binding, thereby increasing their IL-10 production. In addition, MSCs that lack either toll-like receptor 4 (TLR-4), myeloid differentiation primary response gene-88 (MyD88) or TNF receptors lack their in vitro immunosuppressive effects.

In conclusion, PGE₂ seems to play a key role in the immune regulatory effect of MSCs primarily through DCs and CD4⁺ cells as shown by several in vitro studies using neutralizing antibodies or pharmacological and genetic inhibition of the cyclooxygenase signaling pathway. Moreover, the in vivo role of PGE₂ as a trophic factor that confers MSCs with their immunosuppressive effect in inflammatory conditions is well established. Thus, it has been suggested that PGE₂ secretion by MSCs can be modulated by inflammatory signals (e.g., TLR agonists and inflammatory cytokines) that are present in the in vivo

microenvironment of several immune-mediated disorders, and the capacity for MSCs to regulate the inflammatory process can be improved.

Indoleamine 2,3-Dioxygenase

Several studies have demonstrated a role for indoleamine 2,3-dioxygenase (IDO) in MSC-mediated immune suppression. IDO is the rate-limiting enzyme in the catabolism of the essential amino acid tryptophan into kynurenine and is expressed in several tissues such as lymphoid organs and placenta. The production of IDO is generally enhanced in an inflammatory response and can be induced by LPS and cytokines (e.g., IFN- γ) [47, 48]. In MSC, the IFN- γ /IDO axis is essential in the modulation of lymphocyte proliferation [40, 49]. In MSCs that were derived from umbilical cord, IFN- γ induced IDO and increased the expression of B7-H1, a molecule that negatively regulates T cell activation. Moreover, *in vitro*, these factors that are expressed by MSC inhibited T cell proliferation, inhibited the differentiation/maturation of PBMC-derived DCs and stimulated the generation of Tregs [50]. In a kidney transplant model, IDO that was produced by MSCs was responsible for the induction of kidney allograft tolerance in mice through the generation of Tregs [51]. Tolerant animals exhibited both higher levels of circulating kynurenine (that resulted from tryptophan catabolism by IDO) and a higher frequency of CD4⁺/CD25⁺/Foxp3⁺ T cells in the spleen and in the grafts. Importantly, antibody-induced CD25⁺ T cell depletion confirmed the critical role of Tregs in MSC-induced tolerance. However, this effect was not observed in renal allograft recipients that were treated concomitantly with an IDO inhibitor (1-methyl-tryptophan) or with MSCs that were isolated from IDO KO mice, showing that functional IDO is necessary for the immunosuppressive effect of MSCs. IDO is closely associated with the *in vivo* mechanism of graft tolerance induction by MSCs primarily through the expansion of Tregs. Hence, inducing immunological tolerance by MSCs in kidney, liver or heart allograft transplantation and autoimmune diseases (e.g., type I diabetes, Crohn's disease and multiple sclerosis) could be a promising clinical application for these cells.

Nitric Oxide

Nitric oxide (NO) is an important cellular signaling molecule that is involved in inflammatory reactions. The production of NO from L-arginine is catalyzed by several types of nitric oxide synthase (NOS), including neuronal, endothelial and inducible NOS [52]. Inducible nitric oxide synthase (iNOS or NOS2) is expressed in cells of the immune system such as macrophages under inflammatory and oxidative conditions. The role of iNOS-generated NO

is complex, and antimicrobial, antiparasitic and immunomodulatory properties have been described [53]. NO that is derived from macrophages inhibits T-cell proliferation by decreasing the phosphorylation of Stat5, an important signaling cascade that is activated via IL-2 receptors [54].

Thus, soluble NO is an attractive candidate for T-cell suppression by MSCs, as these cells can produce this molecule [55]. Sato et al. [56] detected the induction of iNOS in MSCs that were co-cultured with T lymphocytes. Moreover, MSC-secreted NO suppresses Stat5 phosphorylation in CD4⁺ and CD8⁺ T cells, thereby resulting in an inhibition of T cell proliferation. Interestingly, MSCs that were isolated from iNOS KO mice were deficient in suppressing T cell proliferation [56]. In addition, NO also plays an important role in the modification of Th1 differentiation by MSCs. The addition of a NOS inhibitor restored both proliferation and IFN- γ production in Th1 CD4⁺ T cells that were co-cultured with MSCs. Moreover, IFN- γ and NF- κ B activation were critical for NO production by MSCs [57]. *In vivo*, the injection of MSCs from mice—but not from IFN receptor 1 KO or iNOS KO mice—prevented graft-versus-host disease and inhibited delayed-type hypersensitivity in mice, and these beneficial effects were abolished by an anti-IFN- γ neutralizing antibody or iNOS inhibitors [58]. Pro-inflammatory cytokines such as IFN- γ , TNF- α or IL-1, which are generally related to the inflammatory microenvironment, were required to induce immune suppression by MSCs through the action of NO. However, this phenomenon is a key mechanism of the *in vitro* and *in vivo* anti-inflammatory properties of murine MSCs. Ren et al. [59] observed that *in vitro* immune suppression of human- or monkey-derived MSCs was mediated by IDO, whereas murine MSCs used NO under the same culture and stimulus conditions, thus demonstrating species variability in the mechanism of MSC-mediated immune suppression. Therefore, to better understand their therapeutic actions, both *in vitro* and *in vivo* studies using human MSCs are needed to determine whether NO is also related to their immunosuppressive properties similarly to murine MSCs.

Heme Oxygenase-1

The inducible form of heme oxygenase (HO-1) is the rate-limiting enzyme that catabolizes a heme group to biliverdin, CO and free divalent iron [60]. This enzyme has anti-inflammatory, anti-apoptotic, pro-angiogenic and immunosuppressive properties and is generally induced by oxidative stress and pro-inflammatory cytokines [61]. Regarding the role of HO-1 in MSC function, the inhibition of T cell proliferation by human MSCs was reversed after *in vitro* HO-1 inhibition [62]. In addition, injecting MSCs delayed heart allograft rejection, and inhibiting HO-1 or iNOS reversed this beneficial effect [62]. Interestingly, MSCs that

were isolated from HO-1 KO mice had reduced secretion of growth and pro-angiogenic factors, such as stromal cell-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF) and HGF, thereby exhibiting decreased angiogenic potential. Moreover, conditioned medium from cultured HO-1 KO MSCs was ineffective at ameliorating acute kidney injury that was induced by cisplatin, whereas conditioned medium from wild-type MSCs conferred renoprotection [63]. In conclusion, HO-1 has been associated with the immune regulatory and regenerative properties of human MSCs. In addition, pre-clinical studies using several experimental models of tissue injury have suggested a key role for this enzyme in the therapeutic action of these cells. Interestingly, HO-1 expression in MSCs can be readily induced *in vitro* (e.g., using hemin) and could therefore be an interesting approach to improving their immunosuppressive and regenerative potential in several disorders.

Interleukin-10

Interleukin-10 was first described as a cytokine produced by murine Th2 cells that could inhibit the activation of IFN- γ -producing cells [64]. It has been shown that this cytokine inhibits macrophage NO production and the expression of class II MHC, IL-12 and CD80/CD86 [65, 66]. Moreover, IL-10 has an important role in immune tolerance, DC development and B cell differentiation [67–69]. Thus, this cytokine plays a key role in the control of inflammation and has been associated with susceptibility to several infectious and autoimmune diseases [70].

In vitro studies have shown that splenocytes that were stimulated with alloantigen in the presence of MSC-conditioned medium produced significant levels of IL-10. Moreover, blocking IL-10 or its receptor reversed the inhibition of T cell proliferation that was induced by MSC-conditioned medium [71, 72]. Interestingly, in co-cultures of T lymphocytes and MSCs with cell-to-cell contact, the expression levels of IL-10 and TGF- β were higher than in cultures grown in a transwell system, suggesting that in the presence of cell contact, MSC-mediated immune suppression is enhanced [73]. In an *in vivo* study, bone marrow mononuclear cells contributed to cardiac protection after myocardial infarct by decreasing T lymphocyte accumulation, reactive hypertrophy and myocardial collagen deposition via IL-10 secretion [74]. However, it is important to note that in certain situations such as cancer, the expression of IL-10 by MSCs can lead to cancer growth and/or development. In this regard, IL-10 and TGF- β 1 expression levels were higher in adipose-derived MSCs from breast cancer patients compared with healthy individuals [75]. Therefore, MSCs play a crucial role in breast tumor growth and/or progression by inducing regulatory molecules within the tumor's microenvironment [75].

Interleukin-6

IL-6 is associated with both antigen-specific immune responses and the inflammatory reaction by either inducing T cell differentiation or increasing the production of IL-2 [76]. Importantly, IL-6 is produced during the acute phase of the inflammatory reaction and plays a key role in the induction of C-reactive protein, fibrinogen and serum amyloid A protein. In the chronic inflammatory process, this cytokine also stimulates T and B lymphocytes [77].

MSCs secrete high levels of IL-6 and can inhibit the differentiation of DCs through an IL-6-dependent mechanism [78]. However, at low concentrations, IL-6 that is secreted from MSCs can promote T cell proliferation and inhibit lymphocyte apoptosis [79, 80]. Guo et al. [81] co-cultured MSCs that were derived from fetal bone marrow with human CD4+ T cells and observed higher levels of IL-6 and IL-1 that were associated with an increase in CD4+/IL17+ cells in the presence of MSCs. Consistent with this study, administering Flk-1+ MSCs aggravated the clinical symptom score and histological evaluation of collagen-induced arthritis by up-regulating IL-6 secretion, thereby promoting Th17 differentiation [82]. In this context, using a model of experimental autoimmune encephalomyelitis (EAE), researchers found that MSCs modulate antigen-stimulated T cells to differentiate into Th17 by a mechanism involving TGF- β and IL-6, thereby ameliorating the disease's progression [83]. Bai et al. [84] showed that bone marrow-derived MSCs home to the central nervous system and reduced the extent of the damage in lesioned areas. In addition, the expression of inflammatory IFN- γ and IL-17 was reduced in CD4+ cells, whereas the expression of anti-inflammatory cytokines (e.g., IL-4) was increased.

Because MSCs can secrete TGF- β and IL-6, and because these cytokines can act in the Treg/Th17 axis, the role of MSCs in promoting the differentiation of Tregs/Th17 cells in several autoimmune conditions should now be carefully evaluated. As discussed previously, in an inflammatory microenvironment, MSCs can be stimulated by TLR agonists or other inflammatory cytokines (e.g., IFN- γ), which would increase TGF- β secretion and thereby favor Treg expansion. However, additional studies are needed to better comprehend the combination of these two cytokines (IL-6 and TGF- β) that are secreted by MSCs and the course of the immune response in various autoimmune and proinflammatory conditions.

Matrix Metalloproteinases and Their Tissue Inhibitors

Matrix metalloproteinases (MMPs) are enzymes that require a zinc ion in their active site for catalytic activity. These enzymes can degrade the components of the

extracellular matrix, such as fibronectin, laminin and proteoglycans, and play a crucial role in embryogenesis, wound healing and tissue remodeling [85]. In addition, MMPs regulate both the immune response and cell proliferation, due to their broad range of substrates that include cytokines and inflammatory mediators [86]. Recent studies showed that MMPs suppress inflammation by degrading biologically active molecules such as chemokines, cytokines and growth factor receptors [87–89]. A suppressive role for MMP-2 and a pivotal role for MMP-9 in the development of inflammatory joint disease were demonstrated in rheumatoid arthritis [89].

MSCs secrete soluble forms of MMP-2 and MMP-9. Moreover, these enzymes play a role in MSC-dependent inhibition of T cell responses by cleaving the CD25 receptor at the T cell's surface. In an *in vitro* study, blocking MMP-2 and MMP-9 completely restored T-cell proliferation and the expression of CD25 [90]. In addition, in a model of islet transplantation in diabetic mice, administering MSCs significantly reduced the delayed-type hypersensitivity response to allogeneic antigens by increasing the survival of the islet grafts. Similarly, this effect was completely reversed by inhibiting MMP-2 and MMP-9 *in vivo* [90].

Interestingly, MSCs can also express tissue inhibitors of metalloproteinases (TIMPs), a family of protease inhibitors that comprises 4 members, TIMP-1 to TIMP-4. Shen et al. [91, 92] showed that the tissue inhibitor of metalloproteinase-3 (TIMP-3) expressed by MSCs in mouse bone marrow can regulate hematopoietic stem cell proliferation, differentiation and *in vivo* trafficking. In addition, *in vitro* studies suggested that inflammatory chemokines and cytokines involved in the regulation of the inflammatory process can modulate MMPs/TIMPs and promote the migration of MSCs through the extracellular matrix into damaged tissues [93, 94]. MSCs can also inhibit both endogenous and exogenous MMPs via secreted TIMP-1 and TIMP-2 and thus protect the perivascular niche from high levels of MMPs [92]. Moreover, the transplantation of MSCs overexpressing heme oxygenase (HO-1; discussed previously) in a rat myocardium infarct model could normalize the ratio of MMPs/TIMPs (TIMP-2 to MMP-2 and TIMP-3 to MMP-9), thus contributing to the reversion of myocardial extracellular remodeling [95].

Although MMPs and TIMPs have emerged as promising new molecules that are secreted by MSCs and associated with immune regulatory properties, their *in vivo* role has just begun to be elucidated, primarily with regard to MMP-2, MMP-9, TIMP-2 and TIMP-3. Because these enzymes can degrade chemokines, cytokines and growth factor receptors, thereby suppressing the inflammatory reaction, the use of MSCs in exploring this striking effect could provide an interesting alternative in the treatment of inflammatory diseases such as rheumatoid arthritis.

Human Leukocyte Antigen-G5

Human leukocyte antigen-G (HLA-G) is a non-classic major histocompatibility complex class I-b molecule that is randomly spliced to yield seven proteins isoforms, four of which (HLA-G1, HLA-G2, HLA-G3, HLA-G4) are membrane-bound and three of which (HLA-G5, HLA-G6 and HLA-G7) are soluble [96]. HLA-G is primarily expressed in trophoblast cells, thymus, cornea, nail matrix, pancreatic islets and erythroid and endothelial precursor cells. However, in several pathological conditions (e.g., transplantation, tumors, viral infection and autoimmune disease), as well as after whole-organ transplantation, the ectopic expression of HLA-G may be up regulated on the cell surface of monocytes and other inflammatory cells [97].

The first evidence that HLA-G can exhibit immunomodulatory properties was observed on the surface of cytotrophoblast cells that displayed an immunological tolerance of the fetus by the mother via an NK cell-dependent mechanism [98]. Subsequently, another study reported that HLA-G favors *in vivo* graft tolerance and decreases the incidence of rejection in patients who underwent heart transplantation [99].

Furthermore, HLA-G isoforms exhibit immunosuppressive activity by down-regulating several immune cells, including NK cells and T and B cells. In addition, HLA-G promotes Treg cell expansion and induces a Th2 cytokine profile [97, 98]. These immunomodulatory effects are strictly dependent upon HLA-G proteins interacting with their specific inhibitory receptors on the surfaces of NK (e.g., ILT2, KIR2DL4 and CD94/NKG2A receptors), DC (ILT4 receptors) and endothelial (CD160 receptors) cells, as well as CD4⁺ (ILT2 and ILT4 receptors) and CD8⁺ (ILT2 and CD8 receptors) T cells. This association between HLA-G proteins and their specific receptors activates inhibitory intracellular pathways in the target cells, thereby restricting immunosuppression [98, 100].

HLA-G5 is the soluble counterpart of membrane-bound HLA-G1 and can be secreted by MSCs [101]. In addition, through a recently characterized mechanism called trogocytosis, the HLA-G-bound membrane can be transferred from MSCs to other cells (e.g., lymphocytes) and thus can expose a cell to cell-specific immunosuppression [102]. Hence, some immunoregulatory properties of MSCs are closely related to HLA-G functions [96]. In this context, MSC-secreted HLA-Gs can protect themselves from attack by NK cells and inhibit IFN- γ release. Additionally, the expression of HLA-G is also strongly correlated with IL-10 synthesis. MSCs that were pre-treated with IL-10 secreted higher levels of HLA-G5 *in vitro*, and experiments showed that blocking HLA-G5 dramatically decreased IL-10 production in the supernatants of MSC/T

cell co-cultures in a dose-dependent manner [101]. Similarly, T cells that were co-cultured with MSCs exhibited higher levels of IL-10 and TGF- β transcripts and high expression levels of HLA-Gs. These findings might reflect an important positive feedback loop between HLA-G5 and IL-10.

By influencing HLA-G, MSCs also inhibit the proliferation of alloactivated T cells and suppress NK cytotoxicity, and this effect is dependent on and up-regulated by cell-cell contacts. Additionally, MSC-secreted HLA-G5 has a strong influence on the generation and expansion of CD4⁺/CD25^{high}/Foxp3⁺ Treg cells [101]. MSC-generated Tregs were found to be hyporesponsive to allogeneic stimuli and inhibited T-cell proliferation in an in vitro suppression assay. Interestingly, treating with anti-HLA-G antibodies abrogated this effect, demonstrating that the HLA-G molecule contributes actively to MSC immunomodulation [101].

Based on these studies, soluble HLA-G5 has emerged as an important factor secreted by human MSCs that interacts with IL-10 cytokine as a possible mechanism to protect these cells from the immune response. Moreover, HLA-G5 acts primarily by suppressing the activity of CD4⁺ T cells and NK cells and also promotes the expansion of Tregs. However, all of the studies on the immune suppressive effect of MSC-secreted HLA-G5 were performed under in vitro conditions. Therefore, in vivo studies using experimental models of autoimmune diseases are still needed to elucidate the immune regulatory effect of this MSC-secreted trophic factor secreted under inflammatory conditions.

CCL2, CCL5 and Other Chemokines

Soluble chemokines constitute a large family of peptides that are structurally similar to cytokines. Most chemokines are secreted in response to an inflammatory signal and have important roles in recruiting lymphocytes, monocytes and neutrophils. For example, CC-chemokine ligand 2 (CCL2), a member of the CC subclass of chemokines, and its receptor CCR2 have a key role in the early inflammatory process. Interestingly, CCL2 has been shown to be induced in several diseases such as multiple sclerosis, rheumatoid arthritis, atherosclerosis, and insulin-resistant diabetes [103]. This chemokine is produced by a variety of cell types (endothelial, epithelial, smooth muscle, mesangial, astrocytic, monocytic, and microglial cells as well as fibroblasts), including MSCs [104]. It is important to note that the ligand-receptor interaction between CCL2 and CCR2 has a pleiotropic action in both pro-inflammatory and anti-inflammatory profiles. The inflammatory property is predominantly dependent on antigen-presenting cells (APCs) and T cells, whereas the anti-inflammatory function

is controlled by regulatory T cells [103]. Moreover, there is evidence suggesting an association between CCL2 and Th2 cell development by activation of the IL-4 promoter via T cell receptor (TCR) signaling, thereby reducing IL-12 production by APCs and leading to an anti-inflammatory phenotype [103, 105].

MSCs secrete high levels of CCL2 and also a variety of chemoattractants, such as CCL3 (MIP-1a), CCL4 (MIP-1b), CCL5 (RANTES), CCL7 (MCP-3), CCL20 (MIP-3a), CCL26 (eotaxin-3), CX3CL1 (fractalkine), CXCL5 (ENA-78), CXCL11 (i-TAC), CXCL1 (GRO α), CXCL12 (SDF-1), CXCL8 (IL-8), CXCL2 (GRO β) and CXCL10 (IP-10) [106]. These chemokines target monocytes, eosinophils, neutrophils, basophils, memory and naïve T cells, B cells, NK cells, DCs and hematopoietic and endothelial progenitors. A recent study showed that MSCs modulate immunoglobulin production in plasma cells by suppressing STAT3 in a CCL2-dependent manner. This signaling invariably induces the transcription factor PAX5, resulting in the inhibition of immunoglobulin synthesis [104]. In another study, the administration of MSCs was shown to ameliorate experimental autoimmune encephalomyelitis (EAE) by inhibiting CD4⁺ Th17 cells [107]. Interestingly, MSC-conditioned medium inhibited EAE-derived CD4⁺ T cell activation by suppressing STAT3 phosphorylation in a CCL2-dependent manner. Moreover, the key role of MSC-derived CCL2 was confirmed when the therapeutic effect of MSCs was abolished by administering MSCs that were isolated from CCL2 KO mice to EAE mice [107].

The chemokine (C-C motif) ligand 5 (CCL5), also known as RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), is a member of the CC chemokine subfamily that is also associated with inflammatory conditions and plays an important role in immune regulatory mechanisms. The CCL5 cytokine can also be secreted in high levels by MSCs, and their expression level can increase following co-culture with breast cancer cells. Karnoub et al. [108] showed that breast cancer cells stimulated the secretion of CCL5 by MSCs, which in turn acted on the cancer cells to enhance their motility, invasion and metastasis. This effect was also conferred by MSCs that were derived from adipose tissue in that these cells produce CCL5, which then promotes in vitro cancer cell invasion. Moreover, this effect was diminished by the addition of anti-CCL5 antibodies [109]. At the same time, CCL5 can inhibit the anti-tumor activity of activated T cells and can contribute to the immunosuppressive phenotype that is characteristic of inflammation-associated malignant diseases [110].

In the context of MSCs, chemokines have recently been included in their mechanisms of immune regulation. Therefore, it has been suggested that chemokines (e.g., CCL2 and CCL5) can drive T cell migration into the

proximity of MSCs, where T cell responsiveness can be suppressed by soluble factors (e.g., TGF- β , PGE2 and NO) that are expressed by the MSCs. However, it is important to note that this hypothetical mechanism of MSC chemokines is based solely on *in vitro* studies. Because MSCs secrete a wide variety of chemokines, future studies that test the role of these molecules in particular will be important to elucidate their immune regulatory functions in an inflammatory microenvironment.

Leukemia Inhibitor Factor

Leukemia inhibitory factor (LIF) is classified as a type VI interleukin. The glycoprotein cytokine LIF is so named because of its ability to induce terminal differentiation or inhibit the proliferation of a myeloid leukemia cell line. In addition, LIF regulates the growth and differentiation of distinct target cells and modulates the humoral and cellular immune responses [111]. Importantly, LIF plays an essential role in establishing pregnancy by facilitating fetal implantation and thereby avoiding fetal rejection by the mother. In addition, LIF has also been associated with regulatory transplantation tolerance, as reviewed by Metcalf et al. [112].

Interestingly, both LIF mRNA and protein levels were increased in MSC by 40% after co-culture with bone marrow derived mononuclear cells [113]. *In vitro*, the addition of LIF-neutralizing antibodies restored lymphocyte proliferation. Moreover, LIF has also been associated with the generation of Foxp3⁺ Treg cells after co-culturing MSCs with lymphocytes. Importantly, the same effect was reported for MSCs that were derived from both adipose tissue-derived and Wharton's jelly [114].

Thus, these preliminary data suggest that LIF is an important factor secreted by human MSCs that may act primarily on T lymphocytes. However, it is important to note that *in vivo* experiments evaluating LIF's association with other soluble factors and its action on other cells of the immune system (including DCs, NK cells, macrophages and B cells) should be performed.

Questions, Future Challenges and New Insights

Over the past few years, MSCs have been extensively studied for their potential to provide effective treatment for several inflammatory processes, autoimmune diseases and to promote long-term survival in organ transplantation. However, our understanding of the immune regulatory effect on immune cells by the secretion of trophic factors such as growth factors, chemokines and cytokines has just begun to be elucidated and may play a decisive role in realizing its full therapeutic potential.

As previously discussed in this review, several soluble factors that are secreted by MSCs can be responsible for immune regulatory effect of these cells. However, due to the complexity of the immune system, it has been alleged that the combination of synergism/antagonism among these soluble factors should be considered if we are to understand the MSC immunosuppressive process as a whole. In addition, in some cases, prior cell-cell contacts or signaling through cytokines or Toll-like receptors appear to be required to initiate the immunomodulatory process. Likewise, most of the studies of the immune properties of MSC-derived soluble factors were performed solely under *in vitro* conditions. In some situations, this approach may not reflect the *in vivo* "real milieu" of cytokines, cells and other factors that are present in the inflammatory process. Furthermore, MSCs can have features that are specific to the species (e.g., rat, mouse, monkey or human), the tissue origin (e.g., fat, liver, lung or bone marrow) and the isolation method used (e.g., enzymatic, mechanic or sorting). In this regard, the immune regulation mechanism that one observes with murine bone marrow-derived MSCs (for example) cannot be considered equivalent to human adipose-derived MSC mechanisms.

Another key question is which type of cell source therapy (i.e., allogeneic vs. autologous MSC transplantation) is more efficient in promoting the immune regulatory effect. MSCs are considered "immunologically privileged" because they express a limited complement of the molecules that are required for full activation of T cells, thus allowing their use *in vivo* in class I and/or class II MHC mismatched MSC engraftment. Nevertheless, future studies are needed to evaluate whether the immune suppressive properties of MSCs are sufficient to control or block completely the humoral and/or cellular immune rejection that is elicited by allogeneic cells.

In recent years, several preclinical and clinical MSC studies have been performed to analyze their safety in a short period following administration. However, long-term evaluation should also be considered, primarily due to the potential risks that are associated with MSC systemic transplantation. It is tempting to speculate that soluble factors secreted by MSCs can suppress the immune system by impairing the "immune surveillance state", thus leading to the development of opportunistic infections (either viral or bacterial). Importantly, this phenomenon must be carefully considered in special situations, for example in immunosuppressed individuals. Additionally, the secretion of immune suppressive cytokines (e.g., IL-10 and TGF- β), growth and pro-angiogenic factors (e.g., VEGF and HGF) and MMPs by MSCs could stimulate and support tumor growth, thereby leading to tumorigenesis or cancer development. Thus, the use of MSCs in certain situations should be carefully evaluated to determine their *in vivo* safety.

Another interesting property of MSCs is their ability to home to injured tissues, followed by their secretion of soluble factors, thereby allowing them to regulate the immune response at the injury site. In this regard, tissue-specific homing has been demonstrated in injured kidney and ischemic myocardium. On the other hand, it has been suggested that long-term MSC engraftment upon systemic administration rarely occurs. Thus, comprehending the mechanisms that underlie tissue-specific homing and the secretion of anti-inflammatory molecules are important to understand how MSCs act in a dynamic in vivo system. In an attempt to enhance this property, the overexpression of key molecules that are related to the migratory property of MSCs, for example chemokine receptors (e.g., CXCR1, -2, -3, -4, -5 and -7) could improve the therapeutic effect of MSCs by increasing their ability to home to injured tissues, followed by their secretion of trophic factors.

In addition, several interesting studies have suggested that MSCs can be modulated by inflammatory cytokines (e.g., IFN- γ and IL-1 β) activated by TLRs binding (TLR -2, -3, -4, -7 and -9), thereby leading to an increase in the secretion of immune regulatory trophic factors. Regardless of the stimulus, the in vitro pre-activation of MSCs holds promise as an interesting tool to improve their immunomodulatory effects, thus conferring a therapeutic advantage in several diseases.

Despite the challenges that must be overcome, pre-clinical and basic studies have undoubtedly shown encouraging results in virtually every type of MSC-based therapy that involves damaged or injured tissues in several diseases. In summary, additional studies are needed to provide a more comprehensive understanding of MSC immune suppression pathways in order to ensure their safe use in future clinical applications for several autoimmune and inflammatory diseases.

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