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## Mesenchymal stromal cells for Systemic sclerosis treatment

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## ABSTRACT

Systemic sclerosis (SSc) is a rare chronic autoimmune disease characterized by vasculopathy, dysregulation of innate and adaptive immune responses, and progressive fibrosis. SSc remains an orphan disease, with high morbidity and mortality in SSc patients. The mesenchymal stromal cells (MSC) demonstrate *in vitro and in vivo* pro-angiogenic, immuno-suppressive, and anti-fibrotic properties and appear as a promising stem cell therapy type, that may target the key pathological features of SSc disease.

This review aims to summarize acquired knowledge in the field of :1) MSC definition and *in vitro* and *in vivo* functional properties, which vary according to the donor type (allogeneic or autologous), the tissue sources (bone marrow, adipose tissue or umbilical cord) or inflammatory micro-environment in the recipient; 2) preclinical studies in various SSc animal models , which showed reduction in skin and lung fibrosis after MSC infusion; 3) first clinical trials in human, with safety and early efficacy results reported in SSc patients or currently tested in several ongoing clinical trials.

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### Keywords:

systemic sclerosis, mesenchymal stromal cells, immune modulation, angiogenesis, fibrosis, clinical trials

## INTRODUCTION

Systemic sclerosis (SSc) is a rare chronic autoimmune disease with a prevalence of 38-341 cases per million adults. It is characterized by a pathogenic triad of 1) early endothelial damage and small vessel vasculopathy, 2) dysregulation of innate and adaptive immune responses, and 3) progressive fibrosis primarily affecting the skin, gastrointestinal tract, lungs, heart, and kidneys, resulting in multi-organ dysfunction [1,2]. Health-related quality of life (HRQoL) is considerably impaired for these patients [3,4]. Disease presentation is heterogeneous, with high morbidity and a standardized mortality ratio of 3.5, compared to the general population [5]. The leading causes of death are cardiac [6] and pulmonary [7,8] involvement, and the 8-10-year mortality rate is over 20-30% [5,6,8]. In cases of rapid and diffuse progression, which accounts for 10 to 20% of the SSc population, the 3-5-year survival rate is estimated at 50-70% [9,10], depending on the type and extent of organ involvement.

SSc remains an orphan disease with high unmet therapeutic needs. The recommended treatments are mostly symptomatic for Raynaud's phenomenon and gastro-esophageal reflux [11]. Standard immunosuppressive drugs, including cyclophosphamide and mycophenolate mofetil, for early severe or rapidly progressive forms, have modest effects without improving survival [12]. Several immunotherapies that specifically target cytokine signaling (eg.: Interleukin (IL)-6), B cells (with anti-CD20 therapy), T cells (with inhibition of T-cell co-stimulation), or other specific fibrosis signaling pathways, such as anti-transforming growth factor (TGF)- $\beta$  agents are under investigation, but have yet to be approved as disease-modifying therapies in SSc.

In this context, stem cell therapies are emerging as a new therapeutic option for SSc. Autologous hematopoietic stem cell transplant (AH SCT) was the first intervention to have disease modifying properties [13–15], including regression of skin [16] and lung [14,17] fibrosis, allowing improvement in both overall survival (OS) and event-free survival (EFS) up to 5-7 years after transplant. However, the procedure is contraindicated in cases with advanced visceral involvement [18,19], and its use is restricted to the most severely affected patients, which represent 20 to 30 % of the SSc patient population. Mesenchymal stromal cells (MSC), first identified in the bone marrow (BM-MSC) 25 years ago by Friedenstein [20], have been extensively characterized [21–24]. MSC can be easily harvested from various other sources including adipose tissue (AD-MSC), and umbilical cord or Wharton jelly (UC-MSC). These multipotent progenitor cells modulate both innate [25] and adaptive [26] immune systems, and have pro-angiogenic and anti-fibrotic properties, all providing a strong rationale for their use to target the SSc pathogenic triad [27,28].

## **1. PATHOPHYSIOLOGY OF SYSTEMIC SCLEROSIS**

Although the exact cause of SSc remains undetermined, numerous studies show that a complex interplay between genetic susceptibility and environmental factors (such as exposure to silica dust and organic or occupational solvents) triggers disease onset with consequent endothelial damage and microvascular injury, inflammation, and autoimmune activation [1,2]. These factors contribute to fibroblast activation, enhanced collagen and extracellular matrix protein production, with consequent fibrosis, and disease progression.

### **1.1 Genetic susceptibility**

SSc is not a monogenic disease [29]. Multiple gene polymorphisms have been associated with a predisposition to developing SSc. While the SSc concordance rate between monozygotic twins is low (4.2%) compared to other autoimmune diseases [30], the relative risk of SSc is ~1% among relatives of SSc patients. These individuals also have a higher frequency of other autoimmune disorders [31]. Candidate gene studies and genome-wide associated studies (GWAS) [32], immunochip data, and whole exome sequencing (WES) have identified many SSc-associated polymorphisms (reviewed in [29,33–35]). Genetic susceptibilities are insufficient to explain disease entirely, underlining the importance of environmental and epigenetic dysregulation, as recently reviewed [29,33,35,36]. Evidence supporting the latter include: 1) abnormal DNA methylation of the promoter region of genes encoding for costimulatory molecules, such as CD70 on activated lymphocytes or CD40LG on activated T cells, FOXP3 on peripheral blood CD4+ cells [29,33,35,36], Friend Leukemia-Integration (FLI-1) in keratinocytes, endothelial cells, and lung fibroblasts, and Bone morphogenetic protein receptor type II (BMPR 2) and nitric oxide synthase 3 (NOS3) in endothelial cells; 2) abnormal histone acetylation resulting in Krüppel-Like Factor 5 (KLF5) and Friend leukemia integration 1 transcription factor (FLI1) epigenetic repression and increasing connective tissue growth factor (CTGF) in fibroblasts from SSc patients, and abnormal histone methylation associated with abnormalities in Transforming Growth Factor- $\beta$  (TGF- $\beta$ )-SMAD signaling pathways; 3) changes in non-coding RNA expression in immune cells, fibroblasts, and in serum levels of micro RNAs that correlate with disease phenotypes and activity.

### **1.2 Vasculopathy**

Vascular injury and endothelial cell damage occur early in SSc [37], with infiltration and accumulation of immune cells in affected tissues [1,2,38,39]. The primary trigger is thought to increase reactive oxygen species (ROS) and cause mild oxidative stress [2,37]. Endothelial cells undergo vacuolization and apoptosis, resulting in break-down of the endothelial lining [39]. The interaction between leukocytes

and endothelial cells increases endothelial cell permeability, with upregulation of the expression of cell adhesion molecules, and secretion of chemokines and mediators of trans-endothelial leukocyte migration by the activated endothelial cells [38]. Perivascular infiltration and proliferation of monocytes, macrophages, mast cells, T-lymphocytes [40], and autoantibody-producing cells all contribute to cytokine release [41]. Autoantibodies against the native platelet-derived growth factor (PDGF) receptor in scleroderma patients [42], were shown to trigger tyrosine phosphorylation and ROS accumulation and to stimulate type I collagen expression as well as conversion of normal human primary fibroblast into myofibroblast. Angiogenesis is also altered in SSc, with inadequate proliferation and differentiation of endothelial cells in response to endothelial injury [37,38]. Endothelial damage, dysfunctional angiogenesis, and sustained inflammation lead to progressive structural alterations and loss of small blood vessels, particularly arterioles [37]. Vascular smooth-muscle cells and pericytes proliferate actively in early SSc, with progressive thickening of the perivascular wall. Pericytes infiltrate the perivascular space and can transdifferentiate into vascular smooth-muscle cells, fibroblasts, or myofibroblasts [37].

### **1.3 Activation of the innate and the adaptive immune responses**

Activation of the innate immune response has been shown for long in SSc, with the presence of monocytes, macrophages [43], mast cells, and dendritic cells (DC) in skin [44–47] and lung [48] biopsies, even before the detection of vasculopathy and altered endothelial cells. Activation of the adaptive immune response [49] is present at early stages, with predominant T lymphocyte infiltration [40,50,51] in the skin and lung, and auto-antibody production [52,53], which all may precede disease symptoms. Immune system activation contributes to large amount of cytokine release [41] and further promote inflammation and fibrosis [54]. More recently transcriptome analysis within the peripheral blood [55] and skin [56–58] samples have shown variables degrees of upregulation of the immune response signatures, according to disease and activity stages [59].

In the early inflammatory phase of SSc, Toll-like receptors (TLR) signaling in innate immune cells, which can be triggered by pathogens, cellular damage, or stress, is an important driver of inflammation [60,61]. TLR activation in macrophages and dendritic cells triggers production of interleukins (IL)-1, IL-6 and tumor necrosis factor (TNF)- $\alpha$  [62], and results in the release of inflammatory cytokine release, including type I interferons (IFNs). IFN- $\alpha$  induces inflammation and activation of other innate immune cells, such as classical DC (cDC), which in turn lead to increased production of cytokines (IL-6, TNF- $\alpha$ ), chemokines (CXCL10, CCL4), and cell adhesion molecules (P selectin, glycoprotein ligand (PSGL-1)). DC promote inflammation, myofibroblast transformation, and extracellular matrix (ECM) deposition in

affected tissue from SSc patients [47,63]. Upregulated CXCL4 production by plasmacytoid DC (pDC) can modulate monocyte differentiation into monocyte-derived inflammatory DC with increased TLR-mediated cytokine production, superior T-cell stimulation, and a profibrotic phenotype [47,63]. TLR-4 expression is elevated in SSc skin and lung biopsies, with robust TLR4 staining in fibroblast and vascular cells of affected tissues, and correlates with disease progression [64]. A high level of skin TLR-9 expression and downstream pathway activation contributes to endogenous TGF $\beta$  production and fibrosis. pDC produce high levels of IFN $\alpha$  [65], CXCL4 and TLR-8, and activated cDC are the most efficient inducers of T cell activation.

T cells play a critical role in the pathogenesis of SSc and can also influence autoantibody synthesis [50]. CD4<sup>+</sup> and CD8<sup>+</sup> T-cells have been identified in skin [66–68] and lung [69,70] of SSc patients and the two subpopulations may contribute to different disease stages [68]. CD4<sup>+</sup> T cells could differentiate into various functionally and phenotypically different subtypes, including Th1, Th2, Th17, Treg (T regulatory cells), and Tfh (T-follicular helper), depending on the cytokine context [41,71–73]. Although the exact role of IL-17 in fibrinogenesis remains controversial (ie.: trans-differentiation of human fibroblast into myofibroblast, collagen synthesis), Th17 cells and IL-17 production were found to be elevated in the peripheral blood, skin, and lungs from SSc patients, and to participate in and exacerbate early inflammatory responses [74–76]. Restoration of the Th1/Th2/Th17/Treg cytokine balance is an important goal of therapy.

At early stages of SSc, macrophages and Th1 cells are the predominant inflammatory cell types to induce inflammation, which is associated with IFN-type I upregulation. Tissue resident macrophages are activated by Interferon (M1, classically activated) and IL-4 (M2, alternatively activated), and become fibrotic with exposure to type 2 cytokines, such as IL-6 [62] and IL-13 [77,78]. Reduced numbers [79,80] and altered suppressive functions [81–84] of Treg cells also contribute to immune activation [78,84]. B cell activation is associated with phenotypic and functional changes and contributes to autoantibody production [85]. Cell surface expression of CD19 is upregulated in peripheral blood B cells [86], which regulates B cell receptor-induced signaling and antibody production. CD95, CD80 and CD86 are also upregulated in memory B cells [86–88], as well as B cell activating factor (BAFF) [89], which also contribute to the regulation of B cell-induced immunoglobulin production and secretion [52]. In addition, B cells can also act as antigen-presenting cells to T cells and induce dendritic cell maturation, promoting Th2 profibrotic responses [90].

#### **1.4 Fibrosis**

Early vascular inflammation is eventually replaced by a fibrotic stage. CD4<sup>+</sup> and CD8<sup>+</sup> T cells undergo expansion in response to cytokine release [51,66,67], which in turn activates fibroblast and stimulates collagen production via direct or indirect mechanisms. Fibroblasts can be activated by the two major pro-fibrotic cytokines, transforming growth factor- $\beta$  (TGF- $\beta$ ) [91] and connective tissue growth factor (CTGF), as well as through interactions with the other immune mediators and growth factors. Fibroblast activation results in excessive deposition of collagen and remodeling of other ECM components associated with progressive diffuse fibrosis, loss of small vessels, hypoxia, and consequent alterations in skin and internal organ architecture and function [92,93]. Plasma levels of Th2/Tc2 cytokines (IL-4 and IL-13) are elevated in SSc patients compared with healthy controls [41]. Fibroblasts can be recruited by chemotaxis to sources of TGF- $\beta$ , IL-4, TNF- $\alpha$ , PDGF and ET-1, and will proliferate in response to IL-1, IL-4, IL-6, IL-17, ET-1, PDGF, or TGF- $\beta$ . These profibrotic cytokines, as well as TGF- $\beta$ , stimulate differentiation and activation of myofibroblasts, with increased ECM production leading to fibrosis. Disruptions in the ECM vary with disease progression; changes in different collagens, proteoglycans, and fibrillins [94] occur typically earlier in the disease, with type I collagen accumulating in later disease stages.

## 2. DEFINITION AND CHARACTERISATION OF MESENCHYMAL STROMAL CELLS (MSC)

*In vitro* expanded MSC are defined a minima since 2006 by the International Society for Cellular Therapy (ISCT) MSc committee, as : 1) a plastic-adherent polyclonal population with fibroblast-like morphology, 2) expressing CD73, CD90 and CD105 (in > 95% MSC), 3) in the absence of hematopoietic and endothelial markers, and 4) able to differentiate *in vitro* into osteoblasts, adipocytes, and chondroblasts [21]. Importantly, MSC are not equivalent to mesenchymal stem cells, referring to cells with validated *in vitro* and *in vivo* self-renewal and multi-lineage differentiation potential at the clonal level [95]. Some MSC effects are mediated by direct cell-cell contact, but most of their action is exerted through secretion of soluble factors, which are not constitutively expressed by MSC, but are induced by several proinflammatory cytokines in the local milieu.

MSC secrete growth factors, cytokines, and hormones [96–99], which are central to MSC paracrine activities, all contributing to tissue regeneration in various diseases, including for SSc patients [100,101] [96–99]. Together with this short-lasting bystander trophic activity, the clinical potential of MSC has been proposed to depend on their wide immunosuppressive and anti-inflammatory potential, suggesting that immunomodulation facilitates host tissue regeneration [102,103]. Numerous parameters have been proposed to modulate MSC functional properties, including the tissue source and the production process, and also the inflammatory environment, since MSC immune properties are



essentially not constitutive, but licensed (also called MSC priming) by inflammatory stimuli (e.g. IFN- $\gamma$   $\pm$  TNF- $\alpha$ , IL-1 $\alpha$  or IL-1 $\beta$ ) [104–106]. Therefore, the expression of surface markers and MSC functional features may change according to culture conditions, cryopreservation, and inflammatory status [104], and these properties are critical for clinical application and the interpretation of MSC studies [107,108]. Importantly, MSC priming also increases the expression of major histocompatibility complex (MHC) class I and induces MHC class II molecules and MSC are recognized and killed by activated immune cells. The early concept of MSC as being immune-privileged is thus more accurately described as immune-evasive [23,109].

## **2.1 Different sources of MSC**

MSC, originally identified in the bone marrow (BM) stem cell niche [20,24], were subsequently isolated from adipose tissue, dental pulp, synovial membranes, placenta, umbilical cord, and Wharton's jelly [110,111]. The most frequent sources of MSC for therapeutic use have been bone marrow (BM-MS), adipose tissue (AD-MS) [112], and fetal tissues [111] [umbilical cord (UC-MS) tissue or blood, Wharton's jelly, and placenta]. While MSC from these sources share many biological features, they differ in terms of their proliferation potential, multilineage capacities, overall transcriptional profile, and functionality [107,113–117].

BM-MS were first considered as the main source of MSC used in clinical trials, although their capacity to proliferate and differentiate changes significantly over time, decreasing with age, and varies extensively according to donor sources [107,118]. It requires a painful invasive procedure to obtain BM sample in the donor and there is a risk of viral exposure. Adipose-derived MSC (AD-MS) isolated from the stromal vascular fraction (SVF) of subcutaneous adipose tissue can yield up to a 500-fold higher number of MSC compared to bone marrow [119]. Recently, the first transcriptomic, phenotypic and functional analyses on paired BM-MS and AD-MS samples from the same healthy donor, showed stronger inhibition of immune responses by AD-MS and lower immunogenicity, providing a new rationale to support the use of adipose tissue as a source of MSC for the treatment of immune-mediated diseases [120]. UC-MS as well as MSC from other perinatal tissues (ie.: amniotic membrane, chorionic membrane, placental decidua and Wharton Jelly) are an interesting alternative cell therapy product, as they can be isolated and cryopreserved in a non-traumatic and ethical way (reviewed in [121]). Of interest, UC-MS may be less immunogenic due to a lack of MHC class II upregulation in response to IFN- $\gamma$  [122–124]. In addition, they display a unique gene expression profile, including genes involved in immunomodulation, angiogenesis, and wound healing, compared to BM-MS as evaluated by single cell RNA seq technology [125].

These three types of MSC all secrete angiogenic, antiapoptotic, and anti-inflammatory cytokines with different cytokine profiles according to tissue source and donor type [126]. Which tissue is best for a particular therapeutic application is a question that is currently under evaluation in SSc patients. Indeed, a comprehensive comparison of UC-MSC versus AD-MSC and BM-MSC is still lacking.

In addition to MSC tissue source, the culture conditions, including culture medium and scale of expansion, and the use of cryopreservation were shown to affect MSC functions [126]. In particular, both the onset of replicative senescence associated with huge *in vitro* expansion and the use of cryopreservation were shown to impact the immunosuppressive functions and the *in vivo* persistence of clinical-grade MSC, which can be partly restored by *in vitro* licensing by inflammatory stimuli [127–131]. Each of these numerous sources of heterogeneity during the MSC production process [107] and their release for therapeutic use has to be considered when designing clinical trials [126].

## **2.2 MSC immunomodulatory and immunosuppressive properties**

MSC exert their immunomodulatory and immunosuppressive effects on both innate and adaptive immune cells through a wide panel of mechanisms [100,101].

*In vitro* data showed that MSC modulate the immunological activity of the different cell populations engaged in the pathogenesis of systemic sclerosis (Figure 1), which led us and others to evaluate MSC potential therapeutic values in SSc patients [28]. First, MSC inhibit DC differentiation, maturation, cytokine expression and capacity to present antigens to T lymphocytes [132,133]. They influence antigen presentation by DC via downregulation of DC cell surface expression of MHC class II, CD11c, and CD83, which modulate their anti-inflammatory action [134]. MSC directly modulate T cell activation, proliferation, differentiation, and effector function. MSC inhibit the proliferation of both naïve and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells through arrest in the G0/G1 phase of the cell cycle [135], and abrogate T cell activation [116,136–138]. Interestingly, MSC also influence the differentiation of naïve CD4<sup>+</sup> T helper cells by promoting anti-inflammatory immune responses and affect the balance of T cell polarization [139]. Thereby, in an inflammatory setting, MSC appear to increase the number and activity of Treg cells and IL-10 expression, while suppressing Th1, Th2, and Th17 cells [140,141]. MSC can reduce the release of pro-inflammatory cytokines from different T cell populations, including interferon IFN- $\gamma$ , TNF, IL-6, and IL-7, and increase anti-inflammatory cytokines, such as IL-4 and IL-10 [139,142]. MSC can also inhibit IL-2-induced proliferation of resting NK cells and partially inhibit the proliferation of activated NK cells and thereby NK mediated cytotoxicity [143]. Besides, MSC also inhibit B cell proliferation, antibody production, and chemotaxis under inflammatory conditions [116,144], and favor

Breg expansion. Inhibition of B cell proliferation appears to be indirect, as it requires the presence of CD4<sup>+</sup> and CD8<sup>+</sup> T cell lymphocytes [145].

*MSC effects are mediated in part by direct cell-cell contact.* In particular CD106/VCAM-1 and CD54/ICAM-1, that are both upregulated on MSC by TNF $\alpha$ , play a critical role in the immunosuppressive capacities of MSC by favoring adhesion to T cells [146]. In addition, CD54 is specifically upregulated and enriched at the contact area between inflammatory macrophages and MSC, thus increasing their immunosuppressive capacities [147]. MSC were also shown to directly promote apoptosis of activated T cells via the Fas/Fas ligand pathway [148] and to suppress T cell proliferation via engagement of the inhibitory molecule programmed death 1 (PD-1) by its ligands PD-L1 and PD-L2 [149]. PD-1/PD-L1 axis was also proposed as involved in the repression of Th17 differentiation program by MSC [150]. Interestingly, human MSC also express several ligands for activating NK cell receptors such as ULBP3, poliovirus receptor (PVR) and nectin 2 (also known as PVRL2). In agreement, they can be lysed by activated NK cells but not by freshly isolated NK cells [151–153]. Interestingly, treatment of MSC with IFN- $\gamma$  leads to the upregulation of MHC class I expression and the downregulation of ULBP3 expression [97,143,154], making them more resistant to NK cell cytotoxicity [153].

*Most MSC effects are paracrine and exerted through secretion of soluble factors.* Typically, the mechanism of action of MSC first involves the release of chemokines allowing attraction of activated T cells [98], that in turn produce proinflammatory cytokines responsible for the priming of MSC towards an immunosuppressive phenotype. In turn, MSC secrete growth factors, cytokines, enzymes and hormones (e.g., VEGF, PDGF, ANG-1, IL-11, PGE2, TSG-6, SDF-1, HGF, IGF-1, IDO) [96–99], which are central to MSC paracrine activities. Immunosuppressive soluble factors secreted by MSC *in vitro* include indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes transformation of tryptophane to kynurenine [143,155], which is involved in inhibition of B cell and T cell proliferation and in the polarization of monocytes into IL-10-secreting M2 macrophages [156]. Non-inflammatory resting MSC, that do not express IDO, induce expansion of IL-10-expressing Breg, instead of inhibiting B cell proliferation and differentiation, through poorly characterized soluble factors [144,157]. Apart from the central role of IDO/iNOS, other soluble factors have been proposed to contribute to the inhibition of T cell proliferation, including galectin-1 and 3 [158,159], or IL-10 [160]. Conversely, the capacity of MSC to induce Treg is mediated by TGF- $\beta$  [161] and soluble HLA-G5 [162], with IL-10, IL1R $\alpha$ , and PGE2 further impacting the Th17/Treg balance [163]. HLA-G5 and PGE2 have both been shown to be involved in the suppression of NK function [143,162]. Moreover, PGE2 abrogates the monocyte differentiation into DC, and contributes to the repolarization of macrophages into anti-inflammatory M2 macrophages

producing IL-10 [164,165]. Other immunosuppressive activities of MSC on myeloid cells are driven by soluble factors, such as CCL2 and CXCL12 that cooperate as a heterodimer to upregulate IL-10 in CCR2pos macrophages [166], tumor necrosis factor-stimulated gene 6 (TSG-6) that promotes the early inhibition of neutrophil and macrophage activity at sites of inflammation and inhibits CXCL8-dependent neutrophil transendothelial migration and chemotaxis [167–169], and IL6 that is responsible for the blockade of monocyte differentiation into DC [170]. Finally, HGF displays both anti-inflammatory and anti-fibrotic effects [171] and has been proposed to favor the development of myeloid-derived suppressor cells (MDSC) together with PGE2 [172,173].

*MSC also produce extracellular vesicles (EVs), including exosomes, microvesicles and apoptotic bodies, which are small (44-100 nm in diameter) membrane vesicles.* These exosomes have immunosuppressive and immunomodulatory activity, and have been found to favor Treg expansion [174,175], potentially mediated by an increased secretion of IL-10 and TGF- $\beta$ 1 by DC [176]. Human MSC-EVs were shown to polarize macrophages to a regulatory phenotype in a PGE2-dependent manner *in vitro* [177]. In addition, MSC-derived exosomes support the differentiation of suppressive M2 macrophages in a mouse model of breast cancer, via carrying high levels of TGF- $\beta$ , semaphorins, and complement factors, [178]. Exosomes from IFN- $\gamma$ -activated MSC were also shown to improve the clinical score in a mouse model of colitis, due to increased amounts of micro ribonucleic acid family miR-125a and miR-125b, which repress Th-17 differentiation by targeting stat3 [179]. MSC-derived EVs in various preclinical models, together with their immunosuppressive and regenerative activity, encourages their use in clinical setting, as cell-free products with highly scalable/quality-controllable production process and interesting safety profiles [180].

*Other mechanisms for MSC immunosuppressive actions were recently discovered using in vivo models.* Among these, MSC mitochondrial transfer increases the phagocytic capacity of lung macrophages, and thereby favors bacterial clearance in sepsis [181,182]. In addition, artificial mitochondrial transfer from MSC to T cells was shown to trigger transcriptomic, metabolic, and functional reprogramming and to favor Treg differentiation [183]. Efferocytosis, the process by which apoptotic cells are engulfed, has been associated with the capacity of MSC to upregulate PGE2 and IL-10 [184]. When MSC are infused intraperitoneally in mice with Graft versus Host Disease (GvHD), efferocytosis also accounts for their immunosuppressive activity, leading macrophage toward IDO expression. However, MSC improve GvHD only when infused intraperitoneally [99], and the efficacy of apoptotic MSC in this model was substantially lower than that of live MSC [185].

*MSC immunopotency assays.* In 2016, the MSC committee of the ISCT published guidelines addressing the characterization of MSC immune modulatory properties [23]. Since MSC deploy a panel of immunomodulatory and regenerative properties, largely dependent on their interaction with their microenvironment. Standard functional markers of MSC potency as well as release potency assays have been defined for conducting advanced clinical studies and their potential registration [23,186]. Standardized methods have been proposed to assess MSC functional properties [187]. The preferred analytic methods for matrix assays evaluating the immunosuppressive and immunomodulatory capacities of MSC [188,189] currently include: a) quantitative RNA analysis of selected gene products [190], b) flow cytometry analysis of functionally relevant surface markers, and c) protein-based assay of secretome. Recently, a combination of transcriptome and secretome analyzes has been proposed as predictive of T cell immunosuppressive properties [191] but additional quantitative assays are urgently required to capture the whole MSC immunosuppressive, anti-inflammatory, and regenerative potential that could synergistically improve SSc patient behavior. Similarly, standardized high-throughput multiparametric immunomonitoring tools have to be set up to characterize the patient immunological status and the various immune cell subsets before and after MSC treatment, to identify responders and thereby optimize clinical trials design.

### **2.3 MSC proangiogenic and antifibrotic properties: *in vitro* evidence**

The angiogenic potential of MSC was first demonstrated by their capacity to differentiate towards an endothelial cell lineage [192]. Adult human BM-MSC can be expanded *in vitro* and differentiated into cells with phenotypic and functional features of endothelial cells able to form capillary-like structures [193–195]. A broad complement of angiogenic factors has been identified in the BM-MSC secretome, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, placental growth factor (PLGF), angiopoietin (Ang)-1, monocyte chemoattractant protein-1 (MCP-1), and IL-6 [196–199]. Human AD-MSC can also give rise to CD34<sup>+</sup> and CD13<sup>+</sup> populations, which can spontaneously differentiate into endothelial cells *in vitro* and participate in the formation of vascular structures in a Matrigel model [192]. AD-MSC secrete angiogenic and antiapoptotic factors, such as VEGF, hepatocyte growth factor (HGF) [200], leptin [201], basic fibroblast growth factor (bFGF), Ang-1, Ang-2, platelet derived growth factor (PDGF) [202–204], as well as mesenchymal stem cell-like protein (MSCP1) and stromal cell-derived factor-1 (SDF1), which are essential for vascular remodeling [205]. The combination of VEGF and shear stress can enhance endothelial differentiation of AD-MSC [206]. More recently the angiogenic/angiostatic signaling pathways by which AD-MSC support neo-vessel formation and stabilization were described, elucidating the functional interaction between AD-MSCs and endothelial

cells [207–211], and AD-MSC appear to be more effective in this regard than their counterparts from bone marrow. Placental MSC also secrete angiogenic factors such as IL-6 [212]. The secretion of angiogenic factors by MSCs can be upregulated according by different chemokines and local hypoxia [206,213]. In BM-MSC, TGF $\beta$  induces secretion of several growth factors, including VEGF, HGF, PDGF-BB, Ang-2, IL-6, and IL-8 [213].

The contribution of the MSC secretome to inhibiting or reversing fibrosis in the SSc pathologic microenvironment is not well understood. MSC-conditioned medium can inhibit proliferation of lung fibroblasts *in vitro* and improve regeneration of injured lung epithelium in an alveolar epithelial wound repair assay [214]. There is currently no consensus on which assays should be used to assess anti-fibrotic activity of MSC *in vitro*, which is crucial for the selection of MSC products to be used in the therapeutic setting, including for clinical trials.

### 3. MSC IN EXPERIMENTAL ANIMAL MODELS OF SYSTEMIC SCLEROSIS

MSC have been investigated in several animal models of SSc [92,215], although none of these encompass all the features of the disease [92]. Table 1 summarizes common animal models of SSc.

Subcutaneous injection of bleomycin, an antitumor antibiotic agent that arrests cells in the G2 phase, is used as a model of skin and lung fibrosis, with mononuclear cell infiltration, antinuclear antibody production, and dermis thickening at sites of injection and in alveolar walls. Reactive oxygen species (ROS) produced by bleomycin cleave DNA, and induce oxidative RNA degradation and lipid peroxidation. Cell damage induced by free-radicals, subsequent recruitment of immune cells, and release of pro-fibrotic mediators appear to underlie the fibrotic response. SMA-positive myofibroblasts observed in the skin gradually increase with dermal sclerosis. Using this model, BM-MSC from male bleomycin-resistant Balb/C mice transplanted into female C57BL/6 mice exposed to intratracheal bleomycin [216] were localized by *in situ* hybridization to areas of bleomycin-induced injury, consistent with MSC homing. MSC infused immediately after bleomycin exposure reduced the extent of bleomycin-induced inflammation and collagen deposition in the lung, but not when MSC infused 7 days after exposure to bleomycin. In rats, injection of BM- MSC 4 days after bleomycin exposure reduced neutrophil infiltration and collagen deposition, as well as nitric oxide metabolites and cytokines [217]. In this model, two weeks after rat BM- MSC injection [218], TGF $\beta$ 1, PDGF-A, PDGF-B, and IGF-I mRNA expression in lung tissue was decreased, as measured by real-time PCR,.

The effect of a myelosuppressive regimen with busulfan prior to similar intratracheal bleomycin exposure 6 hours before injection of BM-MSC derived from GFP-positive C57BL/6 mice was examined in

C57BL/6 recipient mice [219]. Myelosuppressive conditioning significantly decreased 14-day survival following bleomycin exposure, but all MSC-treated animals survived, whether they were myelosuppressed or not. Injured lung cells cultured in vitro secreted factors that increased MSC proliferation and homing to the injured lung cells in a migration assay. Similarly, AD-MSC infusion 24 hours after intratracheal bleomycin exposure in C57BL/6 mice reduced lung and dermis fibrosis, and accelerated wound healing [220].

UC-MSC isolated from Wharton's jelly were infused 24 hours after intranasal bleomycin exposure in severe combined immunodeficiency (SCID) mice to minimize potential rejection of human cells [221]. UC-MSC were localized at sites of injury and fibrosis in lung two weeks after infusion and were no longer found in lung 28 days after infusion. MSC infusion reduced bleomycin-induced pneumonitis and lung fibrosis. Quantitative PCR identified a decreased expression of IL-10, TNF- $\alpha$ , TGF- $\beta$ , and IFN- $\gamma$  in lung with MSC treatment. In mice simultaneously receiving infusions of AD-MSCs isolated from C57/BL6 GFP-positive mice ( $2 \times 10^6$  in 100  $\mu$ l), dermal thickness and hydroxyproline content were significantly reduced 4 weeks after transplant [222].

In the SSc hypochlorite (HOCl)-injected mouse model, daily subcutaneous injections of HOCl in BALB/c mice produce ROS, anti-DNA topoisomerase 1 antibodies, progressive collagen deposition, and early and continued skin and lung fibrosis that best reproduce human pathological findings [223]. In this model, a single infusion of allogeneic BM- MSC immediately after HOCl exposure in BALB/c mice ( $2.5 \times 10^5$ ,  $5 \times 10^5$ ,  $5 \times 10^6$  cells) significantly reduced skin and lung fibrosis [224]. The lowest dose ( $2.5 \times 10^5$ ) examined was the most effective, with sustained reduction of fibrosis, advanced oxidative protein products and total collagen content until 42 days. Markers of fibrosis and cytokine expression were lower in lung tissue after MSC infusion. Only fibrosis marker expression was significantly reduced in skin and only with the lowest dose. Serum levels of anti-scl-70 auto-antibodies were also lower in all mice that received MSC infusions. MSC treatment transiently improved skin thickness in HOCl-exposed mice, with a rate of disease progression similar to untreated mice after day 21 and no additional treatment benefit after this point. In mice that received a second MSC infusion on day 21, a significantly slower progression of skin thickening between day 21 and 42 was observed. In mice with established SSc, the effects of a late single MSC infusion were observed from 1 week after treatment onward, with significant reductions in skin and lung fibrosis. Both allogeneic and xenogeneic MSC were examined in the SSc (HOCl)-induced mouse model [225]. BALB/c mice exposed to HOCl were infused with syngeneic BALB/c MSCs, allogeneic C57BL/6 MSC, or xenogeneic human MSC isolated from either bone marrow or adipose tissue. In each of these condition MSC were collected from 3 donors. All the sources of MSC appeared to have similar



therapeutic effects, including a decrease in skin thickness, collagen levels in skin and lung, and expression of collagen 1, collagen 3, and  $\alpha$ Sma [225,226].

Various labeling methods have been used to analyze MSC biodistribution and homing, but these techniques cannot confirm sustained cell viability, in either animals or humans [227–229]. While studies have identified mechanism by which MSCs can migrate to sites of injury and participate in tissue repair [230], studies in mice have reported that MSC are rapidly removed from circulation (~24 hours) after iv injection; the cells are first trapped in lung, then transmigrate beyond vascular spaces, where most MSC are rapidly phagocytosed by lung-resident tissue macrophages [164]. The adhesion molecules VLA-4/VCAM-1 influence interactions between MSCs and endothelial cells during transmigration in the lung. The fucosylation of CD44 to HCELL, a highly active E-selectin ligand on MSC, may also modulate MSC homing to bone marrow endothelium [231]. The term “medicinal signaling cells” (MSC) proposed by Caplan [24] reflect that *in vivo* MSC “release therapeutic agents *in situ* at site of injury, disease, or inflammation” due to their secretory properties.

#### **4. USE OF MSC IN SSC PATIENTS: CLINICAL STUDIES**

Although we and others initially reported that MSC obtained from SSc and healthy controls inhibit proliferation of mixed peripheral blood mononuclear cells and T cell proliferation at similar rates [232,233], when specifically co-cultured with PHA-conditioned T lymphocytes [232], disease-specific abnormalities in MSC from SSc patients later emerged [234,235].

##### **4.1 Abnormal MSC in SSc patients**

BM-MSc from SSc patients have increased TGF $\beta$ -R2 on their cell surface, and a higher sensitivity to TGF $\beta$ , resulting in excessive production of collagen 1, contributing to tissue fibrosis [236]. BM-MSc samples from SSc patients also have reduced clonogenicity (assessed in colony-forming unit fibroblast (CFU-F) assay) [234], and undergo early senescence (increased  $\beta$ -Gal activity), with a significantly decreased proliferation rate (lower ki67 gene expression, higher p21 transcript level) [237]. SSc BM-MSc also demonstrate a reduced capacity to differentiate into endothelial progenitor cells, osteoblasts, and adipocytes, as well as angiogenic dysfunction [235]. When co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> lymphocytes, TGF- $\beta$  expression is significantly higher in MSC derived from SSc patients, compared to healthy controls. Cell surface expression of TGF $\beta$ -RII is also increased SSc MSC, resulting in a higher sensitivity to TGF- $\beta$  [237]. In the presence of TGF- $\beta$ , SSc MSC significantly increase collagen 1 $\alpha$  synthesis and Smad-3 phosphorylation, which contribute to tissue fibrosis [236]. A recent study confirmed that the proliferation rate, metabolic activity, and migration and invasion potential of AD-MSc was decreased in



SSc patients compared to normal controls [238]. These data support the use of MSC from umbilical cord or other allogeneic (instead of autologous) MSC sources for SSc treatment.

The SDF-1/CXCR4 axis is involved in MSC recruitment at sites of injury and in angiogenesis. Compared to healthy controls, SDF-1 is upregulated in MSC derived from SSc patients, although CXCR4 expression is comparable [239]. TGF $\beta$ 1 expression is similar between MSC isolated from SSc patients and healthy controls, but VEGF or SDF-1 stimulation increases TGF $\beta$ 1 secretion by SSc MSC only, not healthy controls. In addition, TGF $\beta$ RI expression is significantly lower in SSc MSC compared to healthy controls [239]. Stimulation with VEGF, TGF $\beta$ , and SDF-1 only increase TGF $\beta$ RII expression in MSC from SSc patients, not healthy controls. Using *in vitro* assays of capillary morphogenesis on dermal microvascular endothelial cells (MVECs) [239], MSC-conditioned medium from SSc patients was shown to significantly increase capillary morphogenesis compared to MSC-conditioned medium from healthy controls, with pro-angiogenic effects mediated by VEGF and SDF-1.

#### **4.2 Autologous MSC in SSc patients**

In a 24 year-old SSc patient refractory to all conventional first-line treatments, with extensive critical limb ischemia and gangrene, autologous MSC ( $1 \times 10^6$ /Kg) was infused at Day 0, 30, and 60. Post-infusion angiography showed new vessel formation [240]. In another SSc patient, intramuscular injection of autologous bone marrow-derived mononuclear cells into ischemic limbs reduced the size and number of digital ulcers [241]. Some autologous BM–MSC transplants have had positive outcomes, but results are conflicting.

#### **4.3 Allogeneic MSC in SSc patients**

The feasibility of allogeneic BM-MSC is supported by several small studies in severe SSc patients, with follow-up varying between 6 and 44 months. Improvement in skin ulcers and reduction in skin fibrosis has been reported in 6 patients to date [242,243]. One clinical case reported an allogeneic BM-MSC transplant from a father to his 41-year old daughter with diffuse cutaneous SSc [242]. Vascular ultrasound 6 months after transplantation revealed a marked improvement in perfusion of hands and fingers, and revascularization of the patient's extremities was confirmed on angiography. A follow-up study by the same group investigated MSC in 5 patients with severe SSc, with follow-ups ranging from 6 to 44 months, without any major adverse events [243]. Two of the patients received fresh MSC, and 3 were injected with MSC isolated from cryopreserved tissue. One patient died of cardiac arrest related to disease progression 18 months after treatment.

One Chinese study investigated the combination of plasmapheresis (PE) and allogeneic MSC in systemic sclerosis [244]. Fourteen patients received three repeated PE treatments with subsequent pulse

cyclophosphamide on days 1, 2, and 5. UC-MSC infusion on day 8. At 12-month follow-up, patients exhibited improvements in the modified Rodnan skin score ( $20.1 \pm 3.1$  versus  $13.8 \pm 10.2$ ;  $P < 0.001$ ). A subset of patients with interstitial lung disease experienced an improvement in lung function and on computed tomography (CT) scans. Scl70 antibodies, serum TGF- $\beta$ , and EGF levels were also significantly decreased at 12-month follow-up.

A phase I/II dose escalation trial of a single IV infusion of allogenic BM-MSC in diffuse SSc refractory to standard therapy is ongoing in France (ClinicalTrials.gov Identifier: NCT02213705), with 20/20 patients included as of September, 2020. There are 4 additional trials of MSC for SSc patients in November 2020 registered at clinicaltrials.gov, of which 2 involve BM-MSC (in Netherlands, NCT03211793, using intramuscular injections for severe ischemia and the other in China through IV infusion, NCT00962923, started in 2009 with unknown status), 1 involves autologous AD-MSC (in France, NCT04356755) through digital infusion, and 1 involves UC-MSC (in Canada, (NCT04356287)) through IV infusion.

## CONCLUSION

*In vitro* studies have demonstrated that MSC have the potential to target the three pathological disease components in SSc progression. Pre-clinical studies in animal models of SSc confirmed that MSC transplant can reduce inflammation, as well as skin and lung fibrosis. The characterization of the MSC secretome and standardization of functional markers of potency, release potency assays, and tests for assessing MSC functional properties have paved the way for conducting clinical trials with reproducible results. Early human trials in SSc patients are showing that MSC transplant can result in regression of skin fibrosis, reductions in skin ulcers and recovery of circulation in the extremities, as well as improvements in lung function. To increase the success of MSC based clinical trials in SSc, the design and validation of relevant preclinical models and potency assays with good predictive value are required, with adequate clinical study design, based on immune profiling strategies allowing patient screening and follow-up. Ongoing studies will help establish the appropriate choice of MSC source in different clinical settings, optimal MSC characteristics and priming, as well as the method of delivery.

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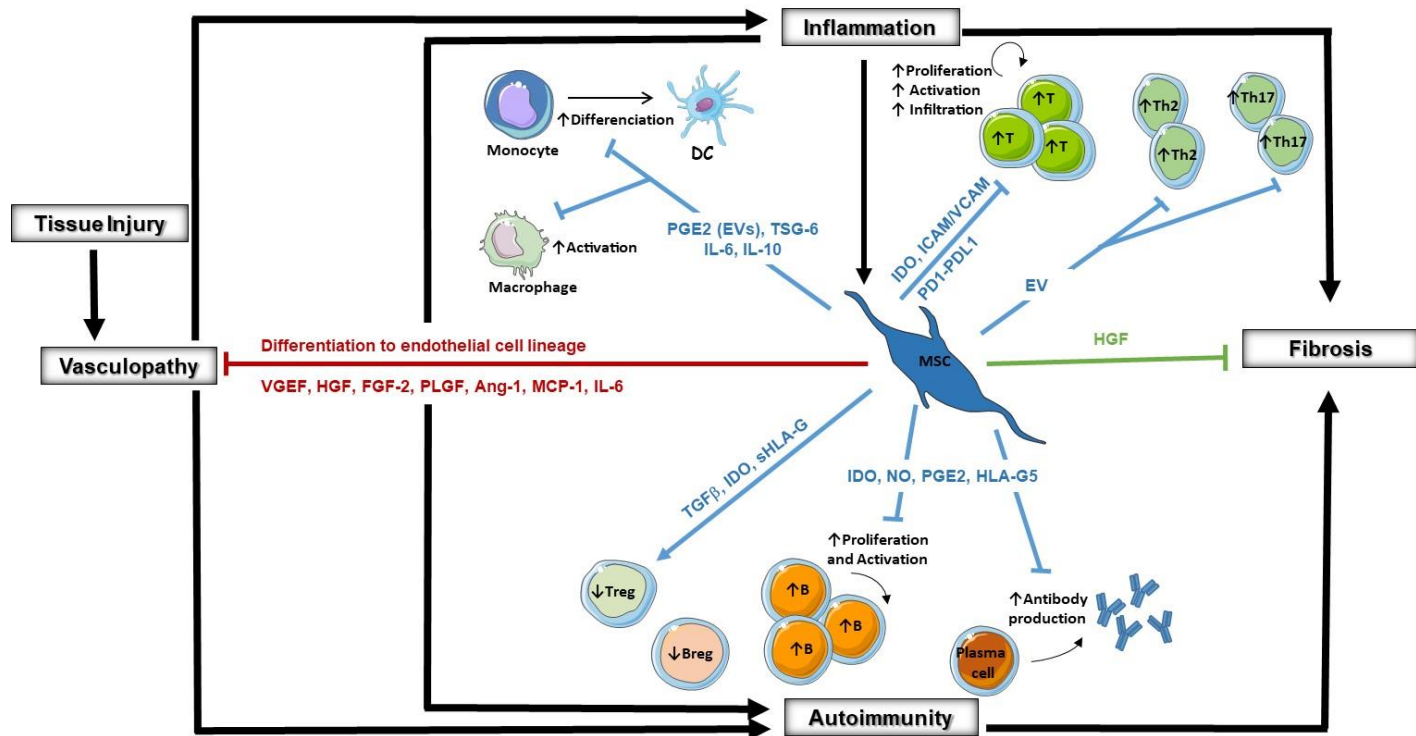
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Table 1 Animal models of Systemic Sclerosis

	Inflammation	Vascular	Autoimmunity	Fibrosis
<b>Spontaneous mutations</b>				
Type 1 tight skin mouse (Tsk1) <i>fibrillin 1 gene</i>	--	<ul style="list-style-type: none"> <li>No vasculopathy</li> <li>Endothelial dysfunction</li> </ul>	✓	Skin
UCD-200/206 chickens <i>Homozygous at the major histocompatibility complex [B-locus]</i>	<ul style="list-style-type: none"> <li>Inflammatory cell infiltration</li> </ul>	<ul style="list-style-type: none"> <li>Microvascular endothelial cell apoptosis</li> </ul>	✓	Esophagus Lung Kidney
<b>Transgenic models</b>				
Type 2 tight skin mouse (Tsk2) <i>(Type III collagen)</i>	<ul style="list-style-type: none"> <li>Inflammatory cell infiltration</li> </ul>	<ul style="list-style-type: none"> <li>No vasculopathy</li> </ul>	✓	Skin
Conditional TGFβRI <sup>ca</sup> Col1a2 <sup>cre-ER</sup> <i>Conditional overexpression of Type I TGF-β receptor (TGFβRI) by fibroblasts at postnatal day 14</i>	<ul style="list-style-type: none"> <li>Systemic inflammation</li> </ul>	<ul style="list-style-type: none"> <li>Thickening of smaller arteries in lung, kidney,</li> </ul>	--	Skin Lung Kidney
Dominant negative TGFβRIIΔk <i>Overexpression of a dominant-negative kinase-deficient type II TGF-β receptor in fibroblasts</i>	<ul style="list-style-type: none"> <li>Minimal</li> </ul>	<ul style="list-style-type: none"> <li>Microvascular alterations in lung</li> </ul>	--	Skin Lung gut
KLF5 <sup>-/+</sup> /Fli1 <sup>-/+</sup> <i>Kruppel-like factor 5 / Friend leukemia integration 1</i>	<ul style="list-style-type: none"> <li>B cell activation</li> </ul>	<ul style="list-style-type: none"> <li>Vasculopathy</li> </ul>	✓	Kidney Heart
uPAR/CD87 <sup>-/+</sup> <i>Urokinase-type plasminogen activator receptor</i>	--	<ul style="list-style-type: none"> <li>Microvascular alterations</li> <li>Impaired angiogenesis</li> </ul>	--	Skin Lung
Fra-2 <i>Fos-related antigen 2</i>	<ul style="list-style-type: none"> <li>Systemic inflammation</li> </ul>	<ul style="list-style-type: none"> <li>Small blood vessel loss in skin and lung</li> <li>Endothelial cell apoptosis</li> </ul>	--	Skin Lung
<b>Chemically-induced models</b>				
Bleomycin	<ul style="list-style-type: none"> <li>Inflammatory cell infiltration</li> </ul>	<ul style="list-style-type: none"> <li>Endothelial cell apoptosis</li> </ul>	✓	Skin Lung
Vinyl chloride	<ul style="list-style-type: none"> <li>Inflammatory cell infiltration</li> </ul>	--	✓	Skin
Hypochlorite (HOCl)	<ul style="list-style-type: none"> <li>Inflammatory cell infiltration in lung</li> </ul>	<ul style="list-style-type: none"> <li>Endothelial cell damage</li> </ul>	✓	Skin Lung
<b>Mismatched immune cell transplant</b>				
Scl-GVHDv1	<ul style="list-style-type: none"> <li>T cells, monocyte, and mast cell infiltration</li> </ul>	--	✓	Skin Lung Kidney
Scl-GVDH 2	<ul style="list-style-type: none"> <li>Inflammatory cell infiltration</li> </ul>	--	✓	Skin Lung Kidney

Figure 1. Mesenchymal Stromal Cells (MSC) for the treatment of Systemic Sclerosis



Due to a dysregulation of adaptive and innate immune responses, SSc leads to a pro-inflammatory microenvironment with high systemic levels of inflammatory cytokines. These pro-inflammatory stimuli shift MSC toward an immunomodulatory state, activating immunomodulatory pathways, such as the production of metabolic enzymes (e.g.: IDO), cytokines (e.g.: IL-6, IL-10, PGE-2), and upregulation of specific membrane proteins (e.g.: ICAM, PDL-1). Activation of these pathways results in the modulation of immune responses through reduction of inflammation and promotion of tissue repair. MSC Immunosuppressive (blue), pro-angiogenic (red) and antifibrotic (green) properties can target the 3 pathogenic pathways of Systemic Sclerosis

## Mesenchymal stromal cells for Systemic sclerosis treatment

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Systemic Sclerosis (SSc) is a rare chronic autoimmune orphan disease.

SSc pathogenesis associates vasculopathy, immune dysregulation and fibrosis.

Mesenchymal Stromal Cells (MSC) properties can target the SSc pathogenic triad.

*In vitro and in vivo* MSC properties vary with donor sources and local inflammation.

MSC appear as a new promising stem cell therapy for SSc patients.