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TGF-β and mesenchymal stromal cells in regenerative medicine, autoimmunity and cancer

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ABSTRACT

Multipotent mesenchymal stromal cells (MSCs) represent a promising cell-based therapy in regenerative medicine and for the treatment of inflammatory/autoimmune diseases. Importantly, MSCs have emerged as an important contributor to the tumor stroma with both pro- and anti-tumorigenic effects. However, the successful translation of MSCs to the clinic and the prevention of their tumorigenic and metastatic effect require a greater understanding of factors controlling their proliferation, differentiation, migration and immunomodulation *in vitro* and *in vivo*. The transforming growth factor(TGF)-β1, 2 and 3 are involved in almost every aspect of MSC function. The aim of this review is to highlight the roles that TGF-β play in the biology and therapeutic applications of MSCs. We will discuss the how TGF-β modulate MSC function as well as the paracrine effects of MSC-derived TGF-β on other cell types in the context of tissue regeneration, immune responses and cancer. Finally, taking all these aspects into consideration we discuss how modulation of TGF-β signaling/production in MSCs could be of clinical interest.

1. Multipotent mesenchymal stromal cells (MSCs): discovery and definition(s)

The discovery of MSCs dates back to the 1960 s when Friedenstein, Owen and colleagues showed that the bone marrow contains clonogenic adherent cells that can differentiate into bone and cartilage *in vitro* and give rise to a bone/marrow ossicle (composed of bone and hematopoiesis-supportive stroma of donor origin, and hematopoiesis of recipient origin) when transplanted *in vivo* [1]. These cells were named "bone marrow osteogenic stem cells" and represented, at the time, the second example (after hematopoietic stem cells) of an adult tissue-specific stem cell. These cells were later defined as perivascular $CD34^{-}CD45^{-}CD146^{+}$ cells with a critical role in maintenance of hematopoietic stem cells and bone formation/remodeling [2,3].

Based on the work of Friedenstein, Arnold Caplan proposed the existence of a common progenitor of mesodermal tissue which he named "mesenchymal stem cells". These cells should be able to give rise, not only to skeletal tissue, but also to dermis, muscle, tendon and ligaments, representing a new therapeutic technology of "self-cell repair" [4], a notion that greatly increased the interest in MSCs. The term "mesenchymal stem cells" was applied to unfractionated, plastic adherent fibroblastic cells which could be found in virtually all tissues and organs [5]. However, due to the lack of convincing data to support the stemness of these cells, the International Society for Cellular Therapy (ISCT) suggested that the term "multipotent mesenchymal stromal cells" (MSCs) should be used when referring to these cells. In addition, the ISCT proposed a set of minimal criteria to define human MSCs, consisting of (i) plastic-adherence in standard culture conditions, (ii) expression of CD73, CD90, CD105 while being negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules and (iii) the ability to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [6]. However, it is now becoming clear that, while MSCs isolated from distinct tissues adhere to the ISCT criteria when cultured *in vitro*, their gene expression, DNA methylation profile and *in vivo* differentiation potential depend on their tissue origin, suggesting that MSCs represent tissue-specific progenitor/stem cells rather than a common progenitor of mesodermal tissue [7].

Regardless of their stem cell properties, *in vitro* expanded, unfractionated MSCs have been shown to promote endogenous tissue regeneration and neovascularization [8] and inhibit inflammatory/autoimmune responses *in vitro* and *in vivo* in preclinical models [9–11] and in human disease [12–14]. This has made MSCs an attractive tool for

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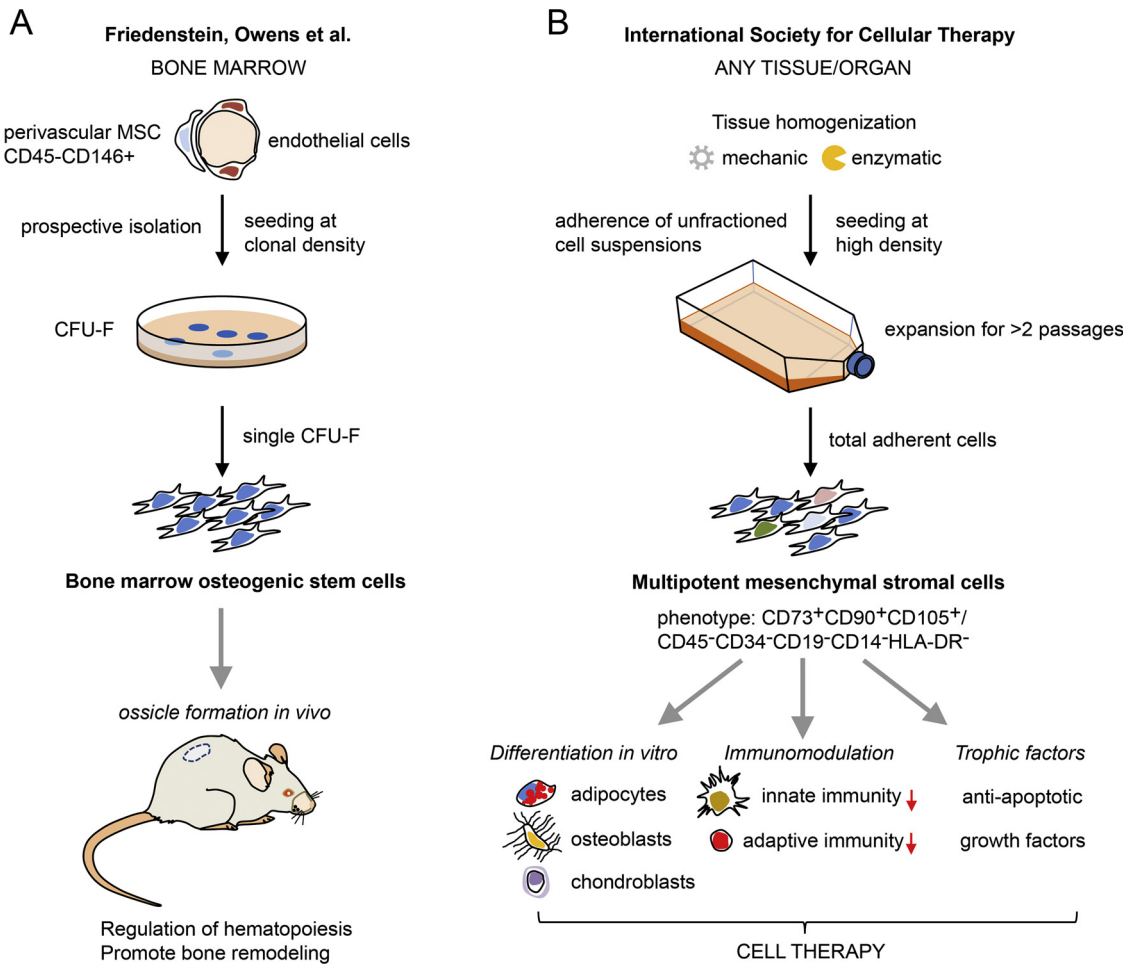

Fig. 1. Bone marrow osteogenic stem cells *versus* multipotent mesenchymal stromal cells. (A) The bone marrow osteogenic stem cells described by Friedenstein, Owen and colleagues are defined as $CD45^{-}CD46^{+}$ perivascular cells that can generate CFU-F *in vitro* (self-renewal) and generate a bone ossicle upon heterotopic transplantation in mice. Such $CD45^{-}CD146^{+}$ cells can be isolated from various organs and tissues but their gene expression and *in vivo* differentiation potential differs, suggesting that they could represent tissue-specific progenitor cells. Bone marrow osteogenic stem cells regulate hematopoiesis, participate in bone remodeling and recent evidence suggest that these cells can home to tumors and incorporate into the tumor stroma. (B) Multipotent mesenchymal stromal cells, defined by the international society for cellular therapy, is the name of unfractionated, plastic adherent, fibroblastic cells that adopt a CD73 + CD90 + CD105 + CD45-CD34-CD19-CD14-HLA-DR- phenotype upon *in vitro* culture which can differentiate into adipocytes, osteoblasts and chondroblasts *in vitro*. These cells are being used as a cell-therapy in regenerative medicine and for the treatment of inflammatory/autoimmune diseases due to their differentiation and immunomodulatory capacities and their secretion of trophic factors.

cell-therapy in regenerative medicine and for the treatment of inflammatory/autoimmune diseases (Fig. 1). Recently, it has also been appreciated that endogenous MSCs can migrate to tumors and participate in cancer progression [15]. This necessitates the evaluation of the protumorigenic effects of *in vitro* expanded MSCs but also presents the opportunity to use MSCs as vectors for anti-cancer drug delivery.

2. Transforming growth factor (TGF)-β

The TGF-β1, 2 and 3 are pleiotropic molecules involved in multiple biological processes, including development, carcinogenesis, wound healing, hematopoiesis and immune responses. The TGF-β isoforms induce similar responses *in vitro* but exhibit distinct functions *in vivo*, which is evident in their respective knockout mouse model which display non-overlapping phenotypes with defects in the immune system (TGF-β1), development of skeleton, heart, eyes, ears, and organs of the urogenital tract (TGF-β2) and palate fusion (TGF-β3) [16]. In humans, mutations in genes involved in TGF-β signaling give rise to several diseases associated with skeletal defects and cardiovascular manifestations, including Camurati-Engelmann disease, Marfan syndrome and Loeys-Dietz syndrome [3].

The TGF-βs are produced as disulfide-linked homodimeric precursor polypeptides, consisting of the latency associated peptide (LAP) domain and the mature TGF-β peptide. The endoprotease furin cleaves off LAP which remains non-covalently bound to TGF-β, forming the small latency complex (SLC). The SLC normally associate with the latent TGF-β binding proteins (LTBP) 1–4, forming the large latency complex (LLC), which targets latent TGF-β to the extracellular matrix (ECM) [17]. However, latent TGF-β can also be secreted by the cell or remain at the cell surface in association with several membrane-bound molecules, including glycoprotein A repetitions predominant (GARP) [18,19] and neuropilin-1 [20].

Activation of TGF-β is a highly regulated process and refers to the dissociation of TGF-β from LAP, which allows TGF-β to bind to its receptors and induce signaling. This is achieved through multiple mechanisms, including proteolytic cleavage of LAP (plasmin, matrix metalloproteinases (MMP)-2 and -9), reactive oxygen species (ROS) and interaction with αVβ6 and αVβ8 integrins and thrombospondin (TSP)-1 [21].

3. MSCs and TGF-β - production and activation

Human MSCs (hMSCs) isolated from bone marrow (BM-MSCs), adipose tissue (ASCs), dental pulp, Whartons´s jelly (WJ-MSCs) and placenta produce TGF-β1 [22–26]. In addition, hMSCs have also been shown to secrete the TGF-β2 and TGF-β3 isoforms [27]. Undifferentiated hBM-MSCs cultured *in vitro* secrete latent TGF-β1 mainly in association with LTBP-1 and LTBP-3 [28]. Interestingly, it has been suggested that MSCs can activate TGF-β *via* LTPB-3 and TSP-1 *in vitro* in a cell intrinsic manner [28,29] and MSCs overexpressing TGF-β1 show a constitutive activation of SMAD2/3 [30]. Recently, MSCs have also been revealed to express membrane-bound TGF-β1 (mbTGF-β1) and its involvement in immunomodulation and cancer will be discussed below.

It is not clear whether hMSCs from different tissue origins produce similar levels of TGF-β1 and contradictory results have been obtained which could be due to donor variation and culture/experimental conditions [26,31,32]. In addition, the production of TGF-β by hMSCs can be increased by proinflammatory cytokines [22], TLR agonists [24], substrate rigidity [33], glucose levels [34] and hypoxia. This could explain why MSCs isolated from patients suffering from inflammatory diseases exhibit altered TGF-β1 production compared to MSCs from healthy individuals [35,36].

4. MSCs respond to TGF-β

MSCs respond to TGF-β which has a great impact on their biology and therapeutic potential. The members of the TGF-β family induce signaling through the formation of heteromeric complexes of transmembrane type I (TβRI/activin-like receptor kinase (ALK)1-7) and type II (TβRII) serine/threonine kinase receptors. The TGF-β homodimer binds to the TβRII which allows the recruitment of the TβRI and the subsequent phosphorylation of the SMAD proteins, which move to the nucleus and initiate transcription [37]. In most cell types, TGF-β signals *via* TβRII and ALK5 which activate SMAD2/3/4 but TGF-β can also signal *via* ALK1 and ALK2 which activate SMAD1, SMAD5 and SMAD8 [38]. In addition to the SMADs, TGF-β can activate several non-canonical pathways, including the extracellular signal-regulated kinase (ERK), JNK/p38, small GTPase and PI3K/Akt pathways and it is the cooperation between these SMAD and non-SMAD pathways that determine the final response to TGF-β [39].

BM-MSCs [40], ASCs [41] and WJ-MSCs [42] express TβRII and ALK5 and respond to TGF-β1-3 *via* SMAD2/3 phosphorylation [41,43,44]. Furthermore, TGF-β has also been shown to signal *via* ALK1/SMAD1/5/8 in hBM-MSCs [40]. Finally, hMSCs express several TGF-β co-receptors, including CD105 (endoglin) [45] which also modulate their responses to TGF-β as will be discussed below.

5. The effect of TGF-β on MSC proliferation

The expansion of MSCs for clinical use is time consuming and decreases their therapeutic efficacy. Several groups have demonstrated that recombinant TGF-β1 promotes the proliferation of hBM-MSCs [46,47] and retroviral overexpression of TGF-β1 strongly enhanced the proliferation of human synovium-derived MSCs [48]. Jian et al. [49] reported that TGF-β1 induced a SMAD3-dependent nuclear accumulation of β-catenin promoting hBM-MSC proliferation. In another study, Kim et al. [50] found that TGF-β1 induced Runx1 expression in human prostate-derived MSCs which was required for their cell cycle progression and proliferation. In contrast, other studies have shown that both TGF-β1 [51] and TGF-β2 [52] inhibit proliferation and promote senescence in hBM-MSCs. Interestingly, Zhou et al. [53], using prostatic stromal cells, and Ng et al. [54], using hBM-MSCs, found that low doses of TGF-β1 (≤ 0.25 ng/ml) increased MSC proliferation, while higher doses of TGF-β1 (≥ 1 ng/ml) inhibited their proliferation. These data suggest that TGF-β could exert a biphasic, dose-dependent effect on MSC proliferation, similar to that seen for hematopoietic stem cells [55] which could, in part, explain the contradictory results obtained for TGF-β and MSC proliferation.

6. The effects of TGF-β on MSC differentiation

6.1. Osteogenesis

Since the discovery of the bone forming potential of BM-MSCs by Friedenstein and colleagues, it has been established that endogenous MSCs also play a key role in bone remodeling during normal bone homeostasis and fracture repair *via* intricate instructions from TGF-β. The importance of TGF-β in bone formation is evident in TGF-β2 deficient mice, which exhibits skeletal malformations, and in humans where mutations in genes involved in TGF-β signaling or activation result in skeletal disorders such as Camurati–Engelmann disease (CED), Loeys-Dietz syndrome and Marfan syndrome [3]. Under physiological conditions, a controlled gradient of TGF-β1 recruits endogenous BM-MSCs to sites of bone remodeling where they undergo osteoblast differentiation in response to local osteotropic factors [56]. Dysregulation of TGF-β activation leads to an abnormal migration and differentiation of BM-MSCs which results in cartilage degradation and inflammation [57]. While TGF-β1 promotes the recruitment and proliferation of MSCs and their differentiation into early osteoprogenitors, several studies have shown that TGF-β inhibits their maturation, mineralization and transition into osteocytes *via* canonical (ALK5/SMAD3) [43] and non-canonical (MAPK-ERK) signaling which represses Runx2 [58] (Fig. 2)

Due to the innate osteo/chondrogenic potential of endogenous MSCs, much effort has been made to develop MSC-based cell-therapies for clinical bone and cartilage repair [1,59]. *In vivo* expanded hBM-MSCs have successfully been used in the repair of large bone defects [13] and to stimulate growth in children with osteogenesis imperfecta [60]. However, donor variation and loss multipotency *in vitro* severely reduce the osteogenic capacity of MSCs [61,62] warranting strategies overcoming these obstacles. To this end, it has been shown that overexpression of BAMBI, which is an inhibitor of TGF-β/SMAD signaling, significantly enhanced the osteogenic capability of MSCs *in vitro* [63]. Also, $CD105^-$ MSCs are more prone to osteogenic differentiation *in vitro* [64] and *in vivo* [65], in part due to their lower responsiveness to TGF-β1.

6.2. Chondrogenesis

The TGF-βs are potent regulators of the chondrogenic differentiation of MSCs [66] through the SMAD2/3/4-mediated induction of the master chondrogenic transcription factor SOX9 [67]. TGF-β promotes cartilage matrix synthesis while inhibiting terminal chondrocyte differentiation and hypertrophy [58]. Ahn et al. [42] demonstrated that the levels of ALK5 and TβRII on hWJ-MSCs varied between donors and that ALK5/$T\beta RII^{high}$ MSCs exhibited a greater chondrogenic capacity upon TGF-β1 stimulation *in vitro* and *in vivo*. In the same line, Fan et al. [68] observed a heterogeneous expression of CD105 on human synovial MSCs and a better chondrogenic potential of $CD105^+$MSCs due to an increased SMAD2 signaling. The chondrogenic differentiation potential of hBM-MSC, in response to either TGF-β1 or TGF-β3 is in general greater than hASCs [66]. Higher concentrations of TGF-β were needed to induce chondrogenesis in hASCs compared to hBM-MSCs which correlated with a lower expression of ALK5 on hASCs [69,70]. Although TGF-β1 is widely used to induce chondrogenesis in hMSCs, several studies have demonstrated that TGF-β2 and TGF-β3 are more efficient chondrogenic factors through the induction of higher levels of collagen II and aggrecan production and glycosaminoglycan deposition [71]. In addition to the selection of MSC origin, MSC subpopulations and TGF-β isotype/dose, several groups have improved the chondrogenic potential of MSCs through the overexpression of TGF-β1 [72] or its receptors [73]. Preclinical studies have shown that BM-MSCs overexpressing TGF-β1 are more efficient in repairing articular cartilage defects in rats

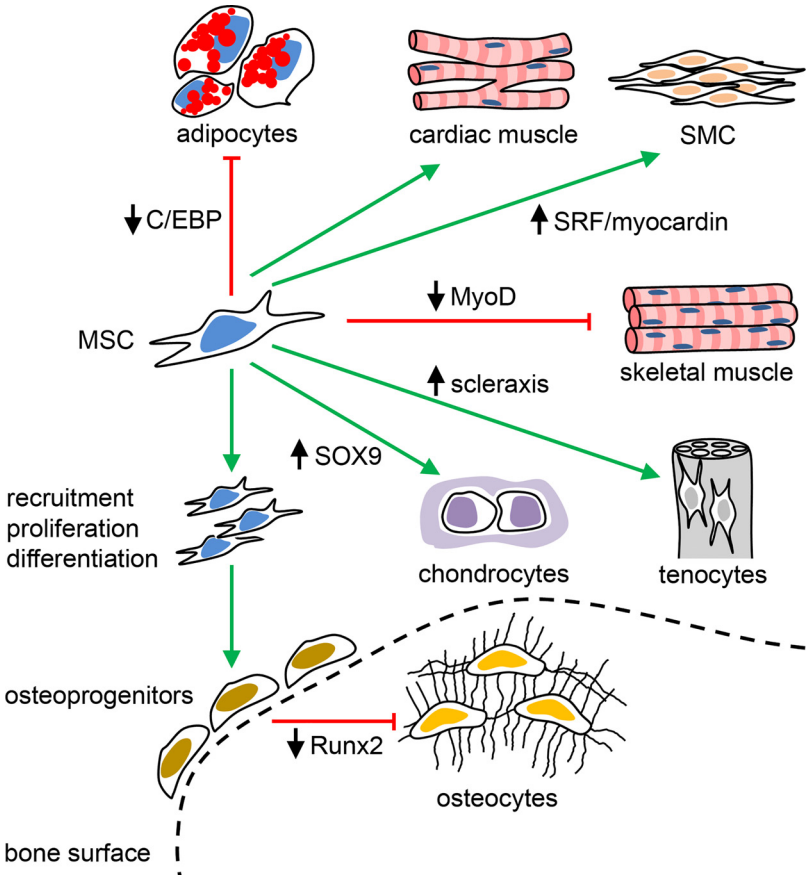

Fig. 2. The effects of TGF-β1 on MSC differentiation. MSCs have the capacity to differentiate into several mesodermal lineages, including osteoblasts and chondroblasts, adipocytes, myocytes and tenocytes. Arrow-headed green lines indicate activation whereas bar-headed red lines indicate inhibition by TGF-β. TGF-β plays an important role in bone resorption/formation, promoting the proliferation and differentiation of MSCs into osteoprogenitors which are recruited to the site of bone remodeling. However, TGF-β inhibits osteocyte differentiation through the suppression of Runx2. TGF-β also inhibits adipogenesis through the inhibition of CBP/E, while promoting the SOX9-mediated differentiation of MSCs into chondrocytes and the scleraxis-dependent differentiation into tenocytes. While TGF-β inhibits the differentiation of MSCs into myoblasts and prevents myofibril generation, *via* the inhibition of MyoD, TGF-β promotes the differentiation of MSCs into smooth muscle cells (SMC) and cardiac muscle cells.

[74] and rabbits [75]. Although, several clinical trials are using hMSCs for repair of knee cartilage, to date no study has evaluated the effects of TGF-β or other growth factors on cartilage regeneration in patients.

6.3. Adipogenesis, myogenesis and tenogenesis of MSCs

The TGF-βs have also been found to modulate adipogenic, myogenic and tenogenic differentiation of MSCs. Firstly, TGF-β1 inhibits the adipogenic differentiation through the SMAD3/SMAD4-dependent inhibition of C/EBP [76]. Blocking TGF-β signaling using SB431542 [46] or reducing the expression of TβR2 [41] significantly increased adipogenesis by MSCs. In addition, $CD105^-$ASCs exhibit an increased adipogenic potential compared to $CD105^+$ASCs *in vitro* [64,65]. Secondly, TGF-β/SMAD2 signaling induces the differentiation of hBM-MSCs [77] and hASCs into smooth muscle cells (SMC) *in vitro*, *via* the serum response factor (SRF)/myocardin-mediated induction of α-smooth muscle actin (α-SMA) and calponin [78]. In contrast, TGF-β inhibits myoblast differentiation *via* the SMAD3-dependent inhibition of MyoD and prevents the formation of multinucleated myofibers [58]. Thirdly, both TGF-β1 [79] and TGF-β2 [80] have been shown to promote tenogenic differentiation of MSCs. The transcription factor mohawk [80] was shown to induce TGF-β2 expression in MSCs, resulting in an upregulation of *scleraxis* and tenogenesis.

7. TGF-β and the immunomodulatory properties of MSCs

A great body of work has shown that MSCs can inhibit innate and adaptive immune responses *in vitro* and *in vivo* [10,11,81,82]. Importantly, administrations of MSCs have shown clinical benefits with immunomodulation in patients suffering of multiple sclerosis (MS) [12], graft-*versus* host disease (GvHD) [83] and refractory Crohn's disease [14,84]. It is becoming clear that both the response to, and production of TGF-β by MSCs are involved in their immunomodulatory capacity. Several studies have shown that active TGF-β, released from the ECM due to injury and inflammation, recruits MSCs which participate in tissue repair and immune regulation. Firstly, TGF-β1 is important for the mobilization of MSCs from the BM into peripheral blood and their subsequent recruitment to sites of arterial repair/remodeling [85]. Secondly, aberrant activation of TGF-β1 in inflammatory prostatic hyperplasia was shown to recruit $nestin^+$MSCs to the prostatic stroma. *In vivo* neutralization of TGF-β1 or knockdown of TβRII in the $nestin^+$-lineage reduced both MSC recruitment and the hyperplasia [86]. Thirdly, TGF-β1 acted as a key pro-migratory factor for recruiting MSCs to the airways in a mouse model of asthma [87]. Dubon et al. [88] recently showed that TGF-β1 promotes the migration of BM-MSC through N-cadherin-dependent intercellular interactions and the activation of the non-canonical ERK-1/2, Akt and p38 pathways.

Once the MSCs reach their target tissue they are further activated by inflammatory mediators (especially IFN-γ, TNF-α and IL-1β) which induce immunosuppressive enzymes such as inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO) [89] and increase the expression of immunosuppressive cytokines, including TGF-β1 [22,90]. Importantly, MSC-derived TGF-β1 has been shown to modulate the activity of T cells, NK cells [91], mast cells [92], macrophages/microglia which will be discussed more in detail below (Fig. 3).

7.1. Inhibition of T cell proliferation

TGF-β1 is a fundamental regulator of the adaptive immune system [93] but the specific role of MSCs-derived TGF-β1 in inhibiting T cell activation/proliferation is not clear. Several groups have demonstrated that hBM-MSCs [82,94], dental pulp MSCs [24] and umbilical-cord (UC)-MSC [95] suppress T cell proliferation *in vitro*, partly through the production of TGF-β1. Recently, Gao et al. [96] found that induction of autophagy in BM-MSCs increased their secretion of TGF-β1 which

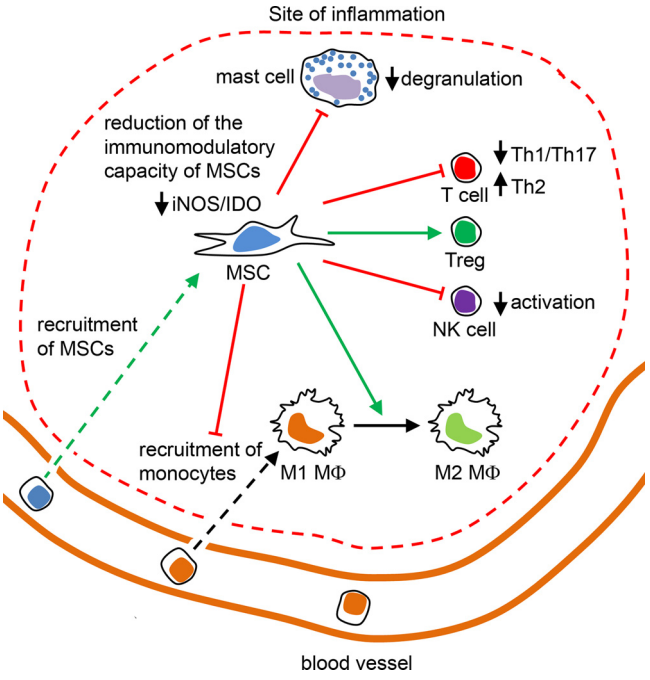

Fig. 3. TGF-β1 promotes and inhibits the immunomodulatory potential of MSCs. Arrow-headed green lines indicate activation whereas bar-headed red lines indicate inhibition by TGF-β. The immunomodulatory properties of MSCs make them a potent tool for cell therapy in inflammatory/autoimmune diseases. Firstly, TGF-β1 can recruit MSCs to the site of inflammation/tissue remodeling where MSC-derived TGF-β1 exerts anti-inflammatory effects through the inhibition of mast cell degranulation, Th1/Th17 responses, and NK cell activation and proliferation while promoting Th2 responses and Treg induction. In addition, it has been demonstrated that MSC-derived TGF-β1, possibly through their above mentioned immunomodulatory effects, can inhibit the recruitment of CD68+ monocytes to the site of inflammation. Importantly, MSC-derived TGF-β1 has also been shown to promote an anti-inflammatory/wound healing-promoting M2 activation state of macrophages. Interestingly, TGF-β1 signaling in murine and human MSCs can reduce their immunomodulatory capacity of through the inhibition of inducible nitric oxide synthase (iNOS) and indolamine 2,3-dioxygenase (IDO), respectively.

improved their inhibition of $CD4^+$ T cell activation. In the same direction, Groh et al. [97] showed that IL-1β-activated monocytes induced TGF-β1 expression in hBM-MSCs which inhibited T cell responses. At the same time, several studies have failed to show a role for TGF-β1 in MSC-mediated suppression of T cells *in vitro* [22,89,90], suggesting a redundant and/or assay-dependent role of TGF-β1. However, the above studies, regardless of outcome, have used similar protocols to induce T cell proliferation (peripheral blood leukocytes or peripheral blood mononuclear cells stimulated with mitogens or mixed leukocyte reactions). Therefore, the discrepancies are not due to the presence/absence of antigen presenting cells but to other factors such as differences in MSC origin and passage number, culture or other experimental conditions.

Interestingly, there are also data indicating that TGF-β signaling in MSCs can lower their immunomodulatory potential. Xu et al. [98] showed that TGF-β1 blocked the IFN-γ/TNF-α-mediated induction of iNOS/nitric oxide (NO) in MSCs *via* a SMAD3-dependent mechanism, thereby reducing their capacity to inhibit T cell proliferation. In the same line, we reported that $CD105^-$mASCs were less responsive to TGF-β1, produced more NO and were better at inhibiting T cell proliferation *in vitro* [64]. The suppressive capacity of hMSCs depends on IDO, rather than iNOS [89]. However, TGF-β1 inhibits the IFN-γ-induced IDO activity in human fibroblasts [99] and IDO mRNA in hUC-MSCs, suggesting that TGF-β can also reduce the immunomodulatory capacity of hMSCs. Finally, Lerrer et al. [100] recently showed that TGF-β1 and TNF-α cooperatively promoted a pro-inflammatory MSC phenotype, characterized by CCL2, IL-8 and COX-2 expression. These data show that the effect of TGF-β on the immunomodulatory capacity of MSCs is highly context dependent.

7.2. Induction of Tregs and modulation of Th1, Th2 and Th17 subset composition

The establishment of immunological tolerance is the key to obtain a long-term resolution of inflammatory/autoimmune diseases and TGF-β1 is an important inducer of regulatory T cells (Tregs) both in the thymus and in the periphery [101]. Human BM-MSCs [102] and ASCs [103] induce $Foxp3^+$Tregs *in vitro via* TGF-β1 and Wang et al. [104] demonstrated that TGF-β1-deficient hUC-MSCs could not generate Tregs *in vitro*. Recently, Tang et al. [105], using a liver transplantation model in rats, showed that TGF-β1-overexpressing BM-MSCs increased the frequency of $Foxp3^+$Tregs in the graft and reduced its rejection compared to control MSCs. In the same line, Park et al. [106], using a model of collagen-induced arthritis in mice, found that TGF-β1-overexpressing mBM-MSCs induced higher levels of splenic type II collagen-specific $Foxp3^+$Tregs and were more effective in ameliorating disease compared to control MSCs.

MSC-derived TGF-β1 also modulates the differentiation of T helper (Th) subsets. Both murine and rat BM-MSCs promoted Th2 responses while inhibiting Th17 responses *in vitro via* TGF-β1 [107,108]. Overexpression of TGF-β1 in mBM-MSCs increased their therapeutic potential in a model of type 1 diabetes which correlated with an increased Th2 response [109]. In contrast, Nemeth et al. [110] showed that mBM-MSC-derived TGF-β1 was crucial in suppressing allergic responses, decreasing lung histology score and reducing the Th2 response in a mouse model of ragweed-induced asthma. *In vivo* neutralization of TGF-β abolished the beneficial effects of mBM-MSCs, while injection of $TGF\text{-}\beta1^{KO}$ mBM-MSCs did not have any effect on disease. Similarly, Xu et al. [111] showed that MSCs-derived TGF-β1 attenuated cockroach allergen-induced lung inflammation, reducing the levels of IL-4/IL-13 and IL-17.

7.3. Modulation of macrophage/microglia activation

TGF-β can induce anti-inflammatory M2 macrophages which participate in wound healing and inhibition of immune responses [112]. Noh et al. [113] reported that MSC-derived TGF-β1 could inhibit the LPS-induced production of proinflammatory cytokines in microglia. Similarly, Yoo et al. [114] demonstrated that transplantation of MSCs into the ischemic brain reduced immune reactions *via* TGF-β1 secretion. Transplantation of TGF-β1-silenced MSCs failed to attenuate the infiltration of $CD68^+$ monocytes into the brain and was associated with only minor improvements in motor function. Using an ovalbumin-induced allergic asthma model, Song et al. [115] showed that the injection of hBM-MSCs ameliorated disease which correlated with an increase in M2 macrophages in the lungs. *In vivo* inhibition of TGF-β signaling abrogated the induction of M2 macrophages and significantly abolished the therapeutic effect of the MSC transplantation.

8. Cancer and MSCs

Tumor progression is no longer viewed as a cancer cell autonomous event but involves multiple cells types present in the tumor microenvironment (TME), including endothelial, immune and stromal cells, such as cancer associated fibroblasts (CAFs). A high stroma:tumor cell ratio correlates with poor clinical outcomes in cancer patients suffering from solid tumours [116]. Importantly, the TGF-β signaling pathway in the tumor stroma carry prognostic value and can represent a therapeutic target in cancer [117,118].

MSCs from both the BM and local adipose tissue migrate towards the tumor [15,119], incorporate into the TME and promote tumor

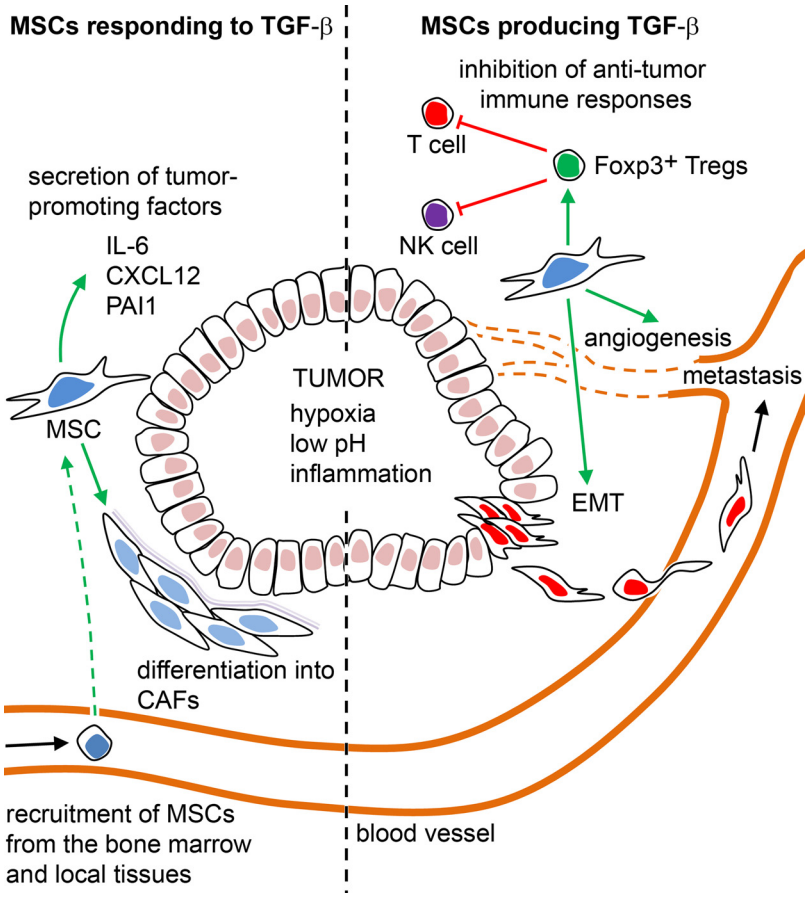

Fig. 4. MSCs regulate cancer progression and metastasis through their production and responses to TGF-β. Tumor-derived TGF-β1 can recruit MSCs from the bone marrow and adjacent local tissues and differentiate them into tumor-promoting cancer associated fibroblasts (CAFs) or inducing their production of pro-tumorigenic factors, including IL-6, CX and PAI. In parallel, MSC-derived TGF-β1 promotes (1) the induction of Tregs can inhibit anti-tumor immune responses, (2) angiogenesis and (3) induction of the epithelial-to-mesenchymal transition (EMT), all of which increases tumor growth and spread.

growth and metastasis through differentiation into CAFs and the expression of growth factors/immunomodulatory cytokines that can (i) promote angiogenesis and tumor growth, (ii) suppress anti-cancer immune responses and (iii) induce epithelial to mesenchymal transition (EMT) in cancer cells [120]. However, some studies have reported anti-tumor properties of MSCs *in vitro* and *in vivo* [121]. In order to establish MSCs as a safe cell-therapy for inflammatory autoimmune diseases and a vector for anti-cancer drug delivery, it is important to understand the mechanisms behind their tumor promoting/inhibiting effects. While some studies have suggested a tumor inhibitory role of MSC-derived TGF-β [122,123], a growing body of evidence show that MSCs promote tumor growth and metastasis through their production and responses to TGF-β1. Below, we will summarize the latest data on the role of MSCs and TGF-β in cancer (Fig. 4).

9. Effect of TGF-β1 on MSCs in cancer

9.1. Recruiting MSCs to the tumor microenvironment

Cancer has been likened to a chronic wound and similar to inflammation and ECM perturbations/wound healing, TGF-β1 has emerged as an important cytokine recruiting MSCs to the TME. Human glioma [124] and prostate carcinoma [125] cell lines attract hBM-MSCs *in vitro via* TGF-β1. Shinojima et al. [124] demonstrated in an elegant study that hBM-MSCs could home to human glioma xenografts *in vivo* but not to TGF-β1^{KO} xenografts. Silencing of either TβRII or CD105 in the hBM-MSCs decreased their migration towards glioma xenografts *in vivo*. TGF-β1 also mediated the homing of hBM-MSCs to xenografts of patient-derived glioma cancer stem cells (GSCs) and the rate of MSC migration correlated with the TGF-β expression levels by the GSCs.

9.2. Differentiation of MSCs into cancer-associated fibroblasts (CAFs)

CAFs, which include α-SMA$^+$ myofibroblasts and FSP-1$^+$ fibroblasts are key players in tumorigenesis [126]. Several tissues contribute to the CAF pool and Quante et al. [15] showed that at least 20% of the CAFs originated from BM-MSCs whereas Kidd et al. [119] found that a large part of α-SMA$^+$ myofibroblasts originated from neighboring adipose tissue. BM-MSCs and hASCs can differentiate into pro-tumorigenic CAFs in response to TGF-β1 secreted by the tumor/tumor stroma [125] or TGF-β1-expressing cancer cell-derived exosomes [127]. Pre-treatment of MSCs with TGF-β1 increased their expression of CAF-associated genes (α-SMA and tenascin C) and increased their growth-promoting effect on pancreatic tumor cells *in vivo* compared to control MSCs [128]. Overexpressing an inhibitor of TGF-β/SMAD signaling, BAMBI, in MSCs blocked their differentiation into CAFs and abolished their pro-tumorigenic effects *in vivo* [63]. In the same direction, Quante et al. demonstrated that the inhibition of TGF-β *in vivo* reduced the differentiation of MSCs into α-SMA$^+$CAFs and reduced tumor growth compared to control mice [15]. Finally, Calon et al. [117] presented evidence that TGF-β signaling in CAFs could promote the initiation of metastases in colorectal cancer.

9.3. Production of tumor promoting/inhibiting factors by MSCs

TGF-β signaling is also important for the pro-tumorigenic effects of BM-MSCs as it increases the expression of multiple tumor-promoting factors such as plasminogen activator inhibitor type 1 (PAI1/SERPINE1), and IL-6 [15,129]. In a recent publication, Yu and colleagues demonstrated that control MSCs, but not TβRII-silenced MSCs, could promote the formation of lung metastases in a model of murine breast cancer. The authors showed that TGF-β1 blocked the expression of CXCL12 by MSCs, which resulted in an upregulation of CXCR7 in the

breast cancer cell lines, promoting their metastatic capacity [130]. These data show that TGF-β can modulate the production of both pro- and anti-tumorigenic factors by MSCs.

10. Effect of MSC-derived TGF-β1 in cancer

10.1. Induction EMT and chemoresistance in tumor cells

TGF-β inhibits cell proliferation and induces apoptosis in early stage tumors, acting as a potent tumor suppressor. However, deletions/mutations in the components of the TGF-β signaling pathways, changes in matrix rigidity and hypoxia converts TGF-β into a promoter of tumor progression and metastasis [131,132]. MSC-derived TGF-β1 has been shown to induce EMT in tumor cells, promoting their invasive/metastatic capacity [133]. Inflammation, characterized by high levels of IL-1, TNF-α and IL-6, hypoxia and acidic extracellular pH are major features of the TME. IL-1α-stimulated MSCs promoted prostate cancer cell growth *in vivo* and shRNA-mediated silencing of TGF-β1 in these cells abrogated their tumor-promoting capacity [134]. Similarly, stimulation of hBM-MSCs with IFN-γ and TNF-α increased their production of TGF-β1 which induced EMT in the human hepatocellular carcinoma cell line SMMC-7721 [135] and pancreatic adenocarcinoma cell lines [136] *in vitro* and increased their metastatic capacity *in vivo*. Both studies demonstrated that silencing of TGF-β1 in MSCs using shRNA-LVs, blocked their tumor promoting effect *in vitro* and *in vivo* [135,136]. Hypoxia (1% O_2) also increased TGF-β1 expression by BM-MSCs and promoted the growth, motility, and invasive ability of the breast cancer cell lines MCF-7 and MDA-MB-231 *in vitro* [137]. Similarly, extracellular acidity increased the TGFβ-1 expression by MSCs and augmented their ability to induce EMT in melanoma cells [138]. TME-associated MSCs are believed to promote the development of chemoresistance of tumor cells through various mechanisms, including the induction of EMT described above [139]. In addition, MSC-derived TGF-β1 can promote chemoresistance in tumors by increasing their resistance to apoptosis [140] and promoting angiogenesis [141].

10.2. Inhibition of anti-tumor immune responses

A strong infiltration of $CD8^+$ memory T cells and Th1 cells into the tumor has been correlated with a good clinical outcome in numerous studies whereas the presence of Tregs correlates with poor survival [142]. As previously discussed, MSCs can inhibit both innate and adaptive immunity and their presence in the TME could thus be detrimental to an efficient anti-tumor response. Patel and colleagues showed that hBM-MSC, *via* TGF-β1 secretion, induced Tregs which could protect breast cancer cells against CTL- and NK cell-mediated lysis *in vitro* [143]. In the same direction, Musso et al. [144] demonstrated that MSCs isolated from lymph nodes (LN-MSCs) of non-Hodgkin's lymphomas inhibited the anti-tumor activity of Vδ2T lymphocytes *in vitro*. Pretreatment of the LN-MSCs with the aminobisphosphonates pamidronate or zoledronate inhibited their production of TGF-β1 and abrogated their suppressive function.

11. The expression of membrane-bound TGF-β on MSCs and its possible involvement in immune modulation and cancer

Several cytokines and growth factors, including IL-1, IL-15, TNF-α, M-CSF and TGF-β exist in both secreted and membrane-bound (mb) forms [145]. In contrast to their secreted form, the response to membrane-bound cytokines/growth factors requires cell-cell contact which can result in a different response due to a higher local concentration of the ligand in the synapse [146] and to the presence of additional cell-cell interactions that modulate their responses in a manner which the secreted cytokine/growth factor cannot mimic. Ostroukhova et al. [147] reported that mbTGF-β1 expression on Tregs mediated their immunosuppressive function *in vitro* and *in vivo* through the activation of the NOTCH1-HES1 axis in responder T cells. $CD4^+$T cells lacking mbTGF-β1, but secreting similar levels of soluble TGF-β1, were unable to trigger NOTCH1 activation and were not immunosuppressive.

Expression of mbTGF-β1 has been found on hUC-MSCs [148], hBM-MSCs [149], hASCs [23] and also on microvesicles derived from mBM-MSCs [150] and hBM-MSCs [151]. We recently reported that GARP, also named leucine rich repeat containing 32 (LRRC32), binds latent TGF-β1 to the surface of mASCs and hASCs [23]. GARP was initially found to bind latent TGF-β1 to the surface of activated Tregs [18,19] and has been shown to be crucial for their immunomodulatory function *in vivo* [152]. In addition to GARP, several other molecules have also been implicated in the attachment of TGF-β1 to the plasma membrane, including heparan sulfate [153], neuropilin-1 (Nrp-1/CD304) [20] and GRP78 [154]. However, if these molecules also bind TGF-β1 to the surface of MSCs is not known.

Whether the mode of activation of mbTGF-β differs from that of ECM-bound and soluble TGF-β is not clear. While GARP is important for the cell intrinsic activation of TGF-β by Tregs *via* integrin αvβ8 [155,156], we found that silencing of GARP in mASCs increased their secretion and activation of TGF-β1 [23]. One explanation of these results could be that GARP, by retaining latent TGF-β1 on the cell surface, prevents the LTBP-3-mediated targeting of latent TGF-β1 to the ECM and its subsequent activation. As a continuation, we will discuss the evidences that suggest that mbTGF-β1 on MSCs could play a role in immunomodulation and cancer (Fig. 5).

11.1. mbTGF-β1 in immune modulation

MSCs inhibit T cells through soluble mediators and cell-cell contact. However, whether mbTGF-β1 on MSCs plays a role in the MSC-mediated modulation of T cell responses has hardly been addressed. Interestingly, MSCs upregulated TGF-β mRNA only upon cell-cell contact with T cells, suggesting a specific role for MSC-derived TGF-β1 in the cell contact-dependent inhibition of T cells [94]. In accordance, hBM-MSCs, *via* TGF-β1, were shown to induce $Foxp3^+CD25^+$ expression in purified $CD4^+$ T cells upon cell-cell contact, but not when separated using transwells [102]. We found that the silencing of GARP in MSCs reduced their ability to inhibit T cell proliferation *in vitro*, in spite of an increased secretion of TGF-β1 [23]. As mentioned, Ostroukhova et al. [147] reported that Tregs suppressed responder T cells through a mbTGF-β1-dependent mechanism that resulted in NOTCH1/HES1 signaling in responder T cells. However, we could not detect increased HES1 expression in T cells in our MSC:T-cell suppression assays and the addition of an inhibitor of the HES1 signaling pathway did not affect the MSC-mediated suppression of T cell proliferation. Instead, we observed that GARP-silenced ASCs produced less NO, possibly due to an increased autocrine TGF-β signaling, which correlated with their decreased suppressive capacity [23,98]. Further studies are required to understand if and how MSCs employ mbTGF-β1 when modulating the activity of immune cells.

Microvesicles (MVs) secreted by MSCs have also been found to express mbTGF-β and modulate T cell activation. Mokarizadeh et al. [150] demonstrated that mBM-MSC-derived MVs expressed mbTGF-β which increased upon IL-1β stimulation. Interestingly, MVs from activated MSCs had a greater capacity of inducing $Foxp3^+$ T cells *in vitro* compared to MVs derived from non-stimulated MSCs. Similarly, Favaro et al. [151] found mbTGF-β1 on MVs from hBM-MSCs which could inhibit T cell responses and induce Tregs through mechanisms involving TGF-β.

11.2. mbTGF-β and MSCs in cancer

As mentioned above, mbTGF-β on cancer exosomes can promote the differentiation of MSCs into tumor promoting CAFs and mbTGF-β on MSCs can inhibit T cell responses and induce Tregs. However, role of MSC-derived mbTGF-β in cancer is not well known and warrants

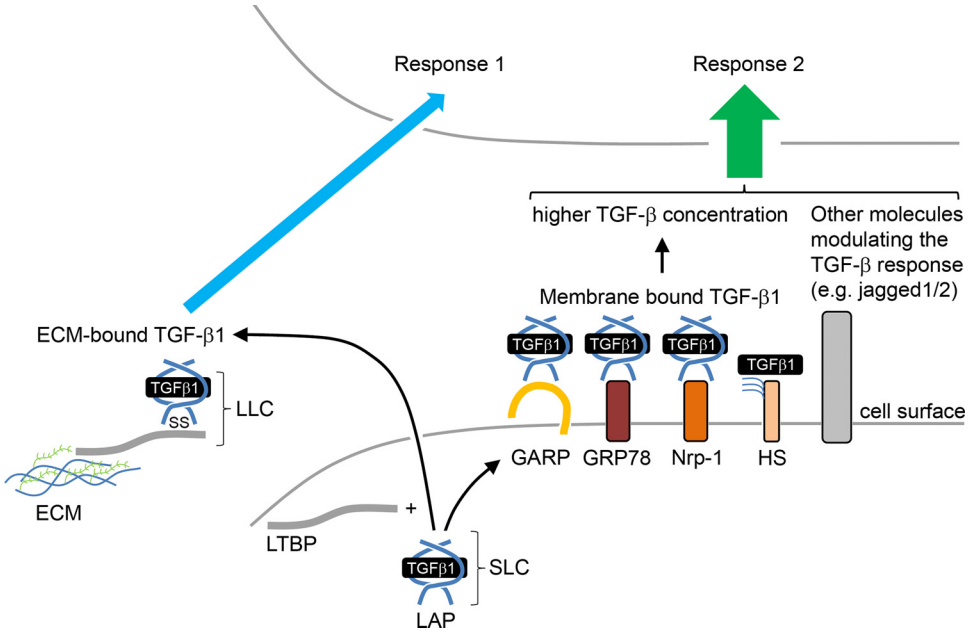

Fig. 5. Membrane-bound(mb) TGF-β1 has the potential to induce a qualitatively distinct response compared to ECM-bound TGF-β1. The small latency complex (SLC) comprise of the latency associated peptide (LAP) which is non-covalently bound to the mature TGF-β homodimer. The SLC binds to latent TGF-β binding protein (LTBP), forming the large latency complex (LLC) which subsequently is secreted from the MSCs and binds to the extracellular matrix (ECM). Activation of TGF-β, *i.e.* the dissociation of TGF-beta from LAP, allows it to bind to its receptors and induce signaling (Response 1). However, GARP can compete with LTBP for the binding of SLC and present it on the cell membrane. Other membrane proteins, including GRP78 and NRP-1 can also bind SLC to the cell membrane whereas heparan sulphate (HS) binds active TGF-β1 to the cell surface. It has been demonstrated that mbTGF-β can induce a response that is qualitatively distinct from that of soluble SLC or ECM-bound TGF-β1. This is believed to be due to the required cell-cell contact, which can protect active TGF-β1 from degradation, increasing its concentration in the synapse. Furthermore, signaling through additional cell surface receptors can modulate the final outcome of the TGF-β1-induced signaling in the target cell (Response 2).

further study. Fonseka et al. [148] reported that mbTGF-β1 expression was induced on hUC-MSCs upon coculture with the human erythromyeloblastoid leukemic cell line K562. Interestingly, the hUC-MSCs inhibited the proliferation of the K562 cells through a cell-cell contact dependent mechanism but whether mbTGF-β was important for the inhibitory effects observed was not addressed. In another study, Mele et al. [149] found that mbTGF-β was induced on hBM-MSCs upon coculture with the colon cancer cell lines LS180, HTC116 and HT-29. Importantly, the hBM-MSCs induced EMT in the tumor cells *in vitro via* a mbTGF-β1-dependent mechanism.

12. Concluding remarks

MSCs represent a promising cell-based therapy in regenerative medicine and for the treatment of inflammatory/autoimmune diseases. MSCs have been approved in Canada, New Zeeland (Prochymal) and Japan (TEMCELL®) for the treatment of steroid-resistant GvHD and several clinical trials are currently evaluating their efficacy in various human pathologies (www.clinicaltrials.gov). However, many questions remain to be answered, such as how to expand MSCs *in vitro* without losing their therapeutic activity and safety, their mechanism of action (homing and engraftment of MSCs *in vivo),* how to avoid unwanted differentiation of MSCs *in vitro/in vivo* and the role of MSCs (endogenously and administered) in cancer. Understanding the role of the TGF-βs in the biology and therapeutic efficacy of MSCs could be of advantage when designing MSC-based therapies for regenerative medicine, inflammatory /autoimmune diseases and cancer.

Although TGF-β1 appears to have a redundant role in MSC-mediated immunomodulation *in vitro*, several studies have shown a therapeutically non-redundant role of MSC-derived TGF-β1 *in vivo*. Genetically engineered MSCs, overexpressing TGF-β1, could represent one strategy to increase their therapeutic potential in autoimmune and inflammatory diseases, including rheumatoid arthritis and asthma, by promoting the induction of immunological tolerance. Such cells could also be useful for neurodegenerative diseases such as MS and ALS where TGF-β1 has been found to protect neurons from cell death and for the repair/generation of cartilage. However, uncontrolled TGF-β signaling in MSCs can result in aberrant differentiation, induction of stress fibers [30] and an inhibition of proliferation and a possible induction of senescence [23,157]. In addition, autocrine TGF-β1 signaling in MSCs, in combination with proinflammatory stimuli has been shown to reduce their immunomodulatory capacity. These data suggest that, while MSC-derived TGF-β could be beneficial, actions to reduce their responses to TGF-β, either by selecting for $CD105^-$cells or genetically disrupting/silencing TβRI/II, would improve the efficacy of MSCs as a cell-therapy for inflammatory/autoimmune diseases.

In humans, systemic injections of MSCs have been shown to increase the frequency of circulating $CD4^+CD25^+$ Tregs in MS [12] and SLE patients [104]. However, any connection between MSC-derived TGF-β1 and the effects observed in clinical studies can only be drawn from preclinical studies or *in vitro* experiments using patient PBMCs. Perhaps clearer evidence has been obtained by Kim et al. [158], who treated ALS patients with autologous BM-MSCs and showed that the beneficial response correlated with the secretion levels of TGF-β by the MSCs *in vitro*. More clinical trials are needed to understand the role of MSC-derived TGF-β1 in ameliorating human autoimmune/inflammatory diseases.

We must also consider that MSCs isolated from different sources and individuals can have different properties. In particular, it has been shown that MSCs from MS and myelodysplastic syndromes (MDS) patients expressed lower levels of TGF-β1 and exhibited decreased suppressive function [35]. In addition, Serena et al. recently showed that the ASCs isolated from Crohn´s disease patients secrete significantly lower levels of TGF-β1 and are less effective in inducing a M2 macrophage phenotype [159]. Since the majority of MSC-based clinical studies have used autologous MSCs (clinicaltrials.gov), it is important to clarify to which extent the therapeutic efficacy of MSCs can be influenced by the disease state. The alternative would be to use fully characterized allogeneic MSCs from healthy donors.

It is becoming clear that the responses and production of TGF-β by MSC that incorporate into the TME are coupled to a poor prognosis in cancer. Importantly, many inflammatory/autoimmune conditions are associated with an increased risk of cancer in the organs/tissues targeted by the aberrant immune response such as inflammatory bowel disease and rheumatoid arthritis [160]. This aspect is of importance for the use of MSCs as cell therapy for inflammatory/autoimmune diseases and as a delivery vector of anti-cancer drugs. Such approached could potentially be dangerous if one ignores the tumor-promoting properties

of MSCs. Interestingly, MSCs derived from induced pluripotent stem cells (iPSCs) secrete lower levels of TGF-β and other related pro-tumorigenic factors compared to BM-MSCs and would therefore have less potential to promote tumors [161]. iPSCs-derived MSCs also maintain tumor tropism and could represent an interesting tool as a Trojan horse for anti-cancer therapies. Finally, a recent study showed that hUC-MSC-derived exosomes promoted cancer cell EMT and migration while, at the same time, inhibiting the proliferation and enhancing apoptosis of the tumor cells. Silencing TGF-β1 expression in hUC-MSCs abrogated the EMT promoting effects of the while enhancing their anti-tumor effects [162]. This again shows the benefits of inhibiting the production of TGF-β in MSCs before using them in anti-cancer therapies. However, due to the context-dependent role of TGF-β1 in cancer and the fact that TGF-β signaling is involved in many normal physiological functions these therapies are not easy to design. Indeed, in some cases, inhibition of TGF-β has been shown to facilitate tumor progression and metastases in mouse tumor models [163,164], whereas other studies have demonstrated that TGF-β blockade decreased tumor growth and metastasis while improving cancer drug penetration in carcinoma mouse models [165]. As mbTGF-β1 on MSCs appears to play a role in tumor development and metastasis, it is important to further characterize the different TGF-β-anchoring molecules on MSCs, such as GARP and nrp-1 and analyze their effect on tumor progression. These TGF-β1-anchoring molecules most likely display a more restricted temporal and spatial expression pattern compared to TGF-β1 and could serve as more specific targets for cancer treatments. In fact, the expression of GARP is more restricted [156] and we envision GARP as a possible molecular target for therapeutic intervention in cancer. In addition, such a therapy would also target tumor-promoting Tregs present in the TME [152] and GARP-expressing tumor cells [166].

In conclusion, we have tried to demonstrate the importance of secreted TGF-β and mbTGF-β for the different roles that MSCs play in health and disease. MSC-derived TGF-βs can have beneficial or harmful effects when administered into a patient and the final outcome will depend on the condition being treated and the levels of the different TGF-βs and their receptors on the MSCs administered. In general, TGF-β is important for the beneficial effects of MSCs in autoimmune/inflammatory disorders and in regenerative medicine. However, the effect of MSC-derived TGF-β can be deleterious favoring tumor progression. The continued research on the responses of MSCs to TGF-βs and the paracrine effects of MSC-derived TGF-β will be important for their successful application or targeting in human disease. The funders had no role in the preparation and the decision to publish the manuscript.

Conflicts of interest

None.

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