Stem Cells International

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Editorial Board

Contents

Hindawi
Stem Cells International
Volume 2017, Article ID 2489041, 2 pages
https://doi.org/10.1155/2017/2489041

Editorial

Cellular and Molecular Mechanisms of Mesenchymal Stem Cell Actions

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Received 25 September 2017; Accepted 26 September 2017; Published 6 December 2017

The clinical interest in the therapeutic use of mesenchymal stromal/stem cells (MSC) is further increasing as their versatility in animal trial settings becomes more and more obvious. Indeed, screening the official review site for clinical trials (http://www.clinicaltrials.gov) using the search term "Mesenchymal Stem Cells" reveals 611 records (September, 2017). The large diversity of medical indications for treatment with MSC comprises hematological malignancies, diabetes type 1, neurologic diseases, joint and bone diseases, organ transplantation, or liver diseases, just to mention a small selection. This indicates the big potential of MSC to act beneficially both on chronic and acute diseases of either local or systemic origin. However, albeit we are only starting to understand the mode of action of MSC in certain disease conditions, there is still hesitation on the safety, upscale feasibility and, effectiveness of clinical MSC application.

MSC from different tissues and organs feature rather similar phenotypic characteristics when put in culture. These include the capacity of plastic adherence, multiple differentiation potential, and surface marker profiles, which comprise the minimal definition criteria for MSC [1]. But gaining knowledge of molecular signatures by global gene expression analyses currently reveals that heterogeneity exists between different MSC populations depending on their origin, isolation and propagation procedures, and on their status of differentiation [2–5]. The actions mediated by MSC may comprise two principally different mechanisms. The one is based on the functional integration of differentiated MSC into diseased host tissue after transplantation as has been shown for liver regeneration after partial hepatectomy or toxic injury [6, 7]. The second mechanism comprises paracrine or cellular support of self-restoration of the diseased tissue or organ [8, 9]. The impact of MSC on the regulation of both the innate and the acquired immune system was intensively investigated and sparked the application in the setting of organ transplantation, where immunosuppression of alloreactivity is essential to prevent rejection [10, 11]. It is mainly the involvement of key molecules like prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase, cytokines, and other growth factors, which act on cells of the immune system to activate or modulate their activity state and thus to impact on the immune status of the organ or organism as a whole.

Knowledge on the cellular targets of MSC actions is emerging. Yet, gain of knowledge still remains limited. Especially, effects on cell cycle and metabolism remain elusive which however are essential to predict potential adverse effects in the treatment of tumorigenic diseases like hepatitis [12] or diseases associated with the metabolic syndrome like diabetes type 2. Facing the pleiotropic properties of MSC like modulation of immune responses and alleviation of inflammation and tissue damage, as well as

stimulation of tissue regeneration, it will be the goal of future efforts using relevant cell or animal model systems to unequivocally elucidate the molecular and cellular impact of a defined MSC population on a specified disease environment before their clinical application. In this special issue of Stem Cells International on the cellular and molecular mechanisms of MSC actions (CMMM), we provide a collection of work stepping towards this goal in order to complement gaps of knowledge before unequivocal use of MSC in clinical settings

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Hindawi
Stem Cells International
Volume 2017, Article ID 8076529, 12 pages
https://doi.org/10.1155/2017/8076529

Review Article

Mesenchymal Stem Cell Benefits Observed in Bone Marrow Failure and Acquired Aplastic Anemia

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Received 4 May 2017; Revised 15 August 2017; Accepted 10 September 2017; Published 3 December 2017

Academic Editor: Marc Hendrik Dahlke

Acquired aplastic anemia (AA) is a type of bone marrow failure (BMF) syndrome characterized by partial or total bone marrow (BM) destruction resulting in peripheral blood (PB) pancytopenia, which is the reduction in the number of red blood cells (RBC) and white blood cells (WBC), as well as platelets (PLT). The first-line treatment option of AA is given by hematopoietic stem cell (HSCs) transplant and/or immunosuppressive (IS) drug administration. Some patients did not respond to the treatment and remain pancytopenic following IS drugs. The studies are in progress to test the efficacy of adoptive cellular therapies as mesenchymal stem cells (MSCs), which confer low immunogenicity and are reliable allogeneic transplants in refractory severe aplastic anemia (SAA) cases. Moreover, bone marrow stromal cells (BMSC) constitute an essential component of the hematopoietic niche, responsible for stimulating and enhancing the proliferation of HSCs by secreting regulatory molecules and cytokines, providing stimulus to natural BM microenvironment for hematopoiesis. This review summarizes scientific evidences of the hematopoiesis improvements after MSC transplant, observed in acquired AA/BMF animal models as well as in patients with acquired AA. Additionally, we discuss the direct and indirect contribution of MSCs to the pathogenesis of acquired AA.

1. Introduction

Red bone marrow (BM) is a gluey, complex, and heterogeneous tissue found in the medullary cavity of long bone and spongy bone cavities of the body. It is anatomically made up of the stromal cells (fibroblasts, adventitial reticular cells, adipocytes, and others) responsible for the tissue structure [1] and the parenchymal cells (hematopoietic cells—blood-producing cells) [2, 3]. To fabricate these blood-producing cells, BM contains a pool of hematopoietic stem cells (HSCs), which are self-renewing cells, differentiate into red (erythrocytes) and white (leukocytes) blood cells, and generate megakaryocytes and these produce platelets (PLT) [2–4]. Only mature hematopoietic cells enter the bloodstream. With age, red BM tends to be substituted with yellow BM, which is mostly made up of fat cells [5, 6].

BM stroma is a key element of hematopoiesis that provides the structural and physiological support for blood cell production. It also consists of a heterogeneous population of different cell types among which is a rare population of nonhematopoietic skeletal progenitor cells named bone marrow stromal cells (BMSC) [7, 8]. Red BM (hematopoietic marrow) and stroma are crucial components of the hematopoietic microenvironment as they interact and produce

together—or individually—humoral growth and/or inhibitory factors necessary to maintain normal hematopoiesis, which is essential for life and human health.

BM can be susceptible to two types of failure syndromes: inherited or acquired. The inherited bone marrow failure (BMF) syndromes are a group of disorders usually diagnosed in childhood and passed down from parent to child through the association with some genetic abnormality [9], which may cause the aplastic anemia (AA) and cancer predisposition [10]. Young people and adults usually may develop the acquired BMF, which can be caused by different extrinsic and intrinsic factors including chemicals, irradiation, chemotherapy treatments, and immune system harms [11, 12].

Initially, BMF syndromes were denominated as "idiopathic AA" because at first, etiology was unknown. Nowadays, the term "AA" encompasses a heterogeneous BMF disorders which are characterized by BM cellular component ablation [13, 14].

Among BMF diseases, the acquired AA is more common. The treatment of acquired AA depends on the patient's age, health, and the severity of the disease. Treatment of moderate cases of acquired AA is indicated blood transfusions and supportive care with an antibiotic. However, many moderate cases may progress to severe AA (SAA) [10]. Therefore, to treat acquired SAA, HSC transplant from matched sibling donor is a matter of choice, which in some cases is satisfactorily effective [15]. It can be used in combination or not with immunosuppressive (IS) therapies. However, most patients have no access to immediate HSC transplant due to the lack of a matched sibling donor. Frequently, extensive time is needed to find a suitable unrelated donor for HSC transplant in SAA patients [16, 17].

Allogeneic transplant of MSCs can be a potential supplementary alternative to treat refractory SAA, since these cells are hypoimmunogenic, thus displaying low expression levels of human leukocyte antigen (HLA) class I, no expression of HLA class II [18]. Potentially, these cells may also be an addition to IS therapies because they possess broad immunomodulatory properties, secreting several biological molecules that influence both adaptive and innate immune responses [19]. Some studies showed that MSCs can prevent graft-versus-host disease (GVHD) and improve hematopoiesis when coinfused with HSCs [20, 21]. Hence, animal models have been developed to assess the response of MSCs in acquired AA as well as the hematologic cell amelioration [22–24] to find conditions to improve HSC transplant regimens or even to evaluate its own effect to reverse BMF and consequently to enhance survival rates of the patients.

This review aims to critically evaluate the potential of MSCs, focusing mainly of BMSC, on acquired BMF/AA in animal models and in recent AA reported clinical cases.

2. AA: Origin, Causes, Diagnostic, and Treatment

AA was first described in 1888 by Paul Erich merely as an "empty" BM with replacement by fat cells [25] and now is defined by decreased hematopoietic precursors in the BM, resulting in BM hypoplasia, peripheral blood (PB) pancytopenia, and precocious fat replacement [26, 27].

The etiology of BM precursor destruction remained elusive for decades [13]. Currently, heterogeneous origin of this disease is accepted. Some inherited disorders can damage the BM cells and lead to AA, mostly as Fanconi anemia (FA), Shwachman-Diamond syndrome (SDS), and dyskeratosis congenita (DC) [9]. The acquired AA can be induced by many different factors such as antineoplastic drugs, antibiotics, nonsteroidal anti-inflammatory drugs, and pesticides, as well as active viral infections (Epstein Barr, hepatitis virus, human immunodeficiency virus, and parvovirus) and radiation exposure [17, 28].

Most of the acquired AA is the result of an immunomediated process that leads to apoptosis of BM cells triggered by cytotoxic T cells [17, 29]. This process occurs as the result of an imbalance between $CD8^+$ and $CD4^+$ T cells, including T helper (Th) type 1 (Th1), Thtype 2 (Th2), regulatory T cells (Treg) and Th type 17 (Th17) cells, natural killer (NK) cells, and NK T cells. Besides that, there is abnormal production of cytokines including interferon- (IFN-) γ, tumor necrosis factor- (TNF-) α, and transforming growth factor (TGF) [30–34].

For acquired AA diagnostic, the pancytopenia is evaluated using three main criteria: neutrophil count lower than 0.5×10^9 cells/L, platelet count lower than 20×10^9 cells/L, and reticulocyte count lower than 1% [35]. Patients with acquired AA often present symptoms of anemia purpura or hemorrhage, and, frequently, infection that may worsen the symptoms [35].

The treatment of acquired AA depends on the severity of the disease. As already mentioned, treatment for moderate cases is based on red blood cell (RBC) transfusions to treat anemia, on platelet transfusions to prevent bleeding, and on supportive care in association with antibiotics [36] aiming to reestablish blood cell volume and prevent secondary infections.

Many moderate cases may progress to severe pancytopenia [35]. Moreover, for severe cases, the first-line treatment to date is HSC transplants from matched sibling donor, more efficient in young patients [15] and IS therapies, most commonly used due to lack of histocompatible sibling donors (HLA) and indicated for older patients [17].

Nevertheless, the success of HSC transplant is limited due to late complications, such as graft rejection and relapse due to resurgent autoimmune attack, and more often due to development of GVHD [15, 37], whereas lack of response, relapse, and clonal evolution limit the success of IS drugs [38].

3. MSCs and Mechanisms of Action

BMSCs are a natural component of stromal BM cellular environment, which are found at low frequency (0.001–0.01%) [39]. When isolated in vitro culture, they show fibroblast-like cell morphology with capacity to form colonies and are able to differentiate mainly into mesoderm derivatives. Moreover, only BMSCs have been shown to self-renew *in vivo* [40, 41].

More recently, similar mesenchymal stem cells (MSC) to BMSC were found in umbilical cord (UC) blood and Wharton's jelly [42], in adipose tissue (AT) [43] in dental pulp (DP) tissue [44], and in amniotic fluid [45] and other fetal and postnatal tissues [46, 47]. According to the International Society of Cellular Therapy (ISCT), MSCs, firstly, must be plastic-adherent when maintained in standard culture conditions. Second, they are characterized by expression of cell surface antigens (CD105, CD73, and CD90), lack of expression of CD34, CD45, CD14 or CD11b, CD79α, and HLA-DR surface molecules, and third, they showed the capacity to differentiate in vitro into adipocytes, osteoblasts, and chondroblasts [48]. However, MSCs derived from different sources have similar immune profile after in vitro culture expansion. On the other hand, it can possess a distinct differentiation potential and biological function, which depend on their embryonic and adult tissue origin [49, 50]. Moreover, profound differences in development potential between MSC sources were found, which are not dependent on donor age and may implicate with MSC clinical use [49, 50].

Paracrine mechanism of BMSC action was first evidenced by their capacity to support HSC growth and differentiation in vitro [51]. Furthermore, MSCs derived from adipose tissue (AT) have also been demonstrated as being able to support hematopoietic niche *in vitro* and *in vivo* [52]. Many studies focused on BMSC's ability to secrete a series of bioactive molecules, as cytokines and growth factors in response to injury into BM microenvironment [53–56]. BMSCs interact with HSC niche secreting such bioactive molecules to support proliferation and long-term growth of HSCs, thus influencing hematopoiesis [57]. Therefore, C-X-C motif chemokine ligand 12 (CXCL12) is responsible for regulation of adhesion, expansion, migration, and homing of HSCs. The Flt-3 ligand (FLT3LG), interleukin-6 (IL-6), and thrombopoietin (TPO) influence HSC proliferation, differentiation, and self-renewal, while stromal cell-derived factor 1 (SDF-1) reduces the production of inflammatory cytokines and chemokines [58–61].

In addition to paracrine effect, general MSCs demonstrate immunomodulatory activity *in vitro* [62, 63]. MSCs interact with various immune cells and secrete soluble mediators [53]. They express several adhesion molecules, including vascular cell adhesion molecule- (VCAM-) 1, intercellular cell adhesion molecule- (ICAM-) 1, and lymphocyte function-associated antigen- (LFA-) 3 involved in T cell interactions, which provide signaling of immunomodulatory response. MSCs suppress T cell proliferation and activation and regulate the differentiation of Th cells [64]. MSCs are capable to inhibit B cell activation, as well as, dendritic cells (DCs) and their precursor proliferation, differentiation, and maturation [65]. Moreover, MSCs modulate the immune responses by generation of Tregs to prevent immune intolerance. It is an important mechanism which could prevent GVHD [66]. Therefore, MSCs have significant clinical implications in BMF, such as acquired AA and related disorders [63].

4. BMSCs and AA

Although acquired AA is considered to affect mainly blood-producing cells, aplastic BM shows significant reduction in endosteal cells, vascular cells, and perivascular cells—pericytes [67, 68]. There are also growing evidences in the scientific literature that MSCs, which showed pericyte-like properties [69, 70]. When isolated from the SAA, patients are affected by this disease. They may present aberrant morphology, impaired osteogenic potential, changes in gene expression, and reduced ability to support hematopoiesis in vitro [71–73]. The number of $CD146^+$ cells is reduced in aplastic BM [74]. This marker is expressed in bone marrow pericyte cells [70, 75–77] and BMSCs [76, 78–80] which can maintain the long-term repopulation potential of HSCs in vitro [81]. MSCs isolated from the patients with acquired AA patient were prone to differentiate into adipocytes rather than osteoblasts. These cells demonstrate downregulation of transcription factor (TF) GATA-2, which is expressed in hematopoietic progenitors, including early erythroid cells, mast cells, and megakaryocytes and overexpression of TF peroxisome proliferator-activated receptor gamma (PPARγ) [82], which has multiple roles in MSCs obtained from AT of the patients with acquired AA [83, 84]. These alterations contribute to the abnormal AT deposit, thus affecting BM tissue remodeling and repair. A low expression level of basic fibroblastic growth factor 2 (FGF2) gene in BMSCs of AA patients was also reported [85, 86]. It is well known that BMSCs are the genuine source of FGF2, which directly influences the HSCs and their precursors in vitro [87, 88]. Furthermore, BMSCs from AA patients were impaired in maintaining the immune homeostasis associated with $CD4^+$ T cells in vitro, which might cooperate with BMF [89]. In contrast to previous observations, one recent study shows that MSCs from patients diagnosed as moderate–severe AA did not present any alteration in morphology, osteogenic potential, gene expression, and ability to support hematopoiesis in vitro [90]. Figure 1 summarized recent studies that show the key role of MSCs in hematopoiesis and in AA pathogenesis, as well as demonstrate possible benefits from allogeneic MSC transplant in this disease.

5. Animal Models for the Study of Acquired AA

Animal models have greatly contributed to elucidate different aspects of BMF and acquired AA. Initially, the attempt to mimic AA has used exposure to toxic/chemical agents and pharmacological drugs that result in BMF through a direct toxic effect [13, 91–97], which were then replaced by physical and biological agents, as irradiation [22, 23] and lymph node infusion [98–102]. The administration of toxical/chemical and pharmacological agents results in BMF in attempt to mimic AA [13, 103]. However, the use of toxic drugs did not provide the immune-mediated destruction of the animal BM, which is commonly observed in human AA disease [13].

In turn, the model which employs infusion of lymph node cells in preirradiated animal shares many pathophysiological features with human immune-mediated AA. These animals develop BM hypoplasia rapidly, which is followed by severe peripheral pancytopenia, adipose cell invasion in BM, and hematopoietic cell reduction [102]. The changes in T lymphocyte subsets and IFN-γ ratio also occur [99].

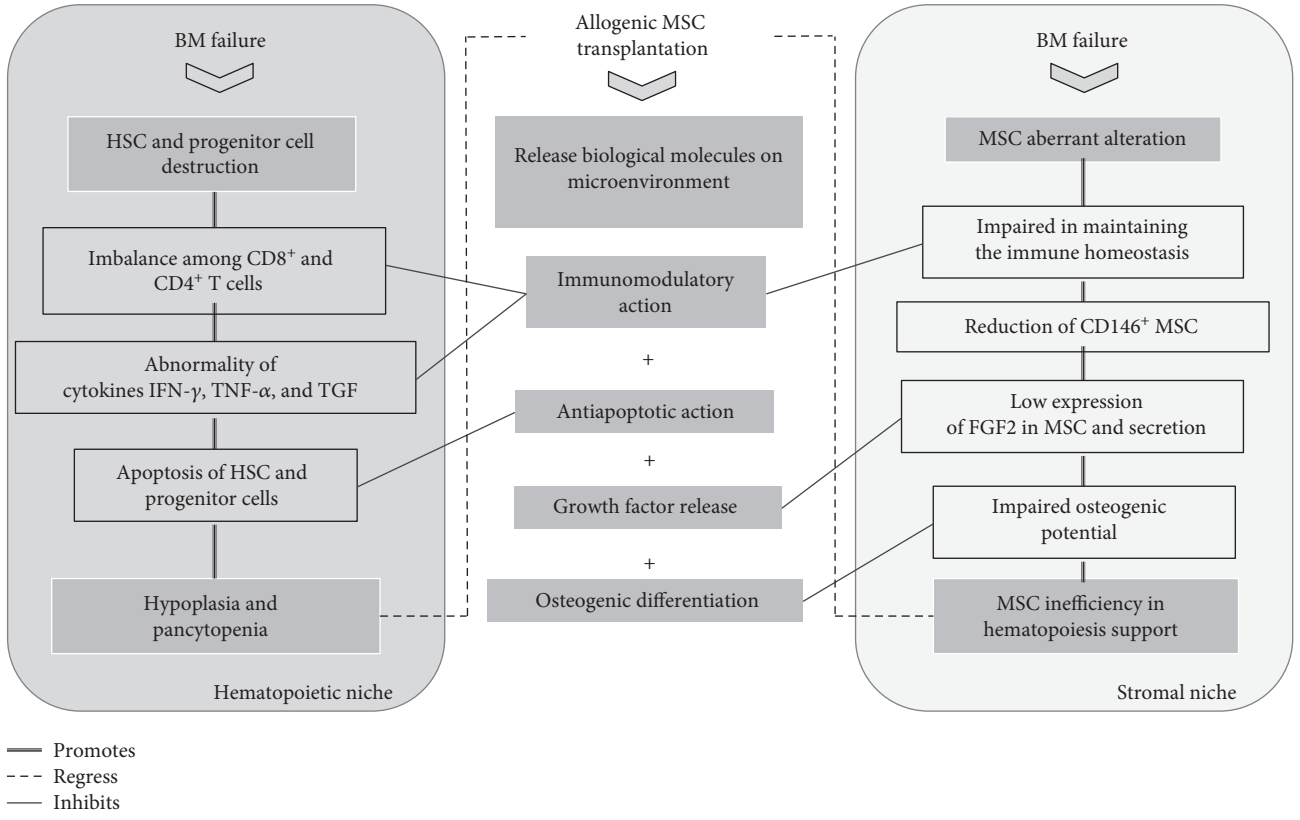

Figure 1: Effect of mesenchymal stem cell (MSC) transplant on bone marrow failure (BMF) etiology and progression. Acquired aplastic anemia (AA) is caused by destruction of hematopoietic stem cell (HSC) and progenitor cells associated with MSC abnormalities, caused by hematotoxic agents (drugs, chemical agents, radiation, and virus). These events lead to imbalance among $CD8^+$ and $CD4^+$ T cells and abnormal cytokine secretion, which correlates apoptosis of HSC and progenitor cells and consequently bone marrow (BM) aplasia and pancytopenia. Additionally, in turn of BM, imbalance aberrant alteration on MSC from BM niches arises. MSC aberrant alteration is observed by, impaired in maintaining the immune homeostasis, reduction of $CD146^+$ MSC and low expression of FGF2 in MSC and its secretion, which lead to MSC inefficiency in hematopoiesis support and collaborate to progress of disease. According to the literature for AA, MSCs improve engraftment of HSC and prevent apoptosis in BM failures. BMF improvements occur as a result of MSC transplant through very similar mechanisms, such as immunomodulation, release growth factors, and osteogenic support. Although in vivo improvement on hematopoiesis was not demonstrated, several properties of MSCs, as well as its association with AA, justify the use of MSC in BM failures.

Irradiation alone also causes BMF in animals. HSCs and committed BM progenitor cells present rapid cell turnover, thus being more sensitive to irradiation, when compared with other cell types [104–106]. The total body irradiation (TBI) using different doses of gamma irradiation ranging from two to eight grays (Gy) induces high expression of the apoptosis regulator gene (BAX gene) causing rapid lymphocyte death [107]. Low doses, around 2 Gy cobalt-60 gamma rays, result in decreased lymphocyte concentration and immune suppression in mouse. Medium and high doses of radiation (5 to 8 Gy) lead to BMF, neutropenia, thrombocytopenia, and anemia, as well as to low count of colony-forming unit granulocyte/macrophage (CFU-GM) and colony-forming unit fibroblast (CFU-f) [22]. Higher (above 8 Gy) doses of radiation may cause lethal hemorrhage or infections and death [108]. Therefore, care should be taken to choose the dose of radiation to induce BMF with minimized side effects and eventually low death incidence.

The murine models mimicking AA have improved over time. The development of an immune-mediated model in the destruction of the BM was important, being more closely modeling human AA [13]. However, human AA is extremely heterogeneous disease. Therefore, different stem cell therapeutic strategies, as source of the stem cell, doses, concentration, and periodicity, as well as the administration route, should be considered to treat different AA pathologies [109, 110]. MSCs, in turn, although showed similar basic characteristics, have different embryonic origin that may reflect on their medicinal paracrine properties [111, 112].

6. Benefits of MSC Transplant in BMF and AA Animal Models

Despite the immune-mediated animal model being considered the closest to mimic the pathophysiology of human AA, most studies choose the BMF animal model, which has failure induction mainly from irradiation strategy, to assess the mechanism of MSCs. The term "bone marrow failure" encompasses any primary failure condition at the HSCs, resulting to the decrease of one or more circulating blood cell

lineage [113]. Table 1 summarizes the current knowledge regarding MSC transplantation into BMF and AA animal models. In the literature, the studies have used mouse irradiation doses ranging from 4 Gy to 8 Gy [22–24, 100, 114–116]. In immune-mediated AA animal model, the preirradiated (4 Gy) mouse received 1×10^6 lymph node cells to induce acquired AA [98, 100]. MSCs from different sources were used in order to assess its possible therapeutic benefits, such as BMSC [22], umbilical cord-derived mesenchymal stem cells (UC-MSCs) [23, 24], adipose-derived mesenchymal stem cell (AD-MSCs) [115, 116], and multiplacenta-pooled cells, which contain MSCs derived from placenta, umbilical cord (UC), and UC blood [100]. Additionally, MSCs were infused in AA animal model in combination with HSCs [115] or extracellular superoxide dismutase (ECSOD), which is an extracellular searcher of superoxide ($O_2^{\cdot -}$) and the main regulator of nitric oxide (NO) in the blood vessel wall and other organs [23]. On the majority of published works, a single MSC transplant was used and doses ranged from 1×10^6 to 2.5×10^7 cells per mouse. The cells were mostly administrated by endovenous (EV) [22, 23, 98, 116] and less by intraperitoneal (IP) route [100]. Only one study analyzed and showed engraftment of MSCs in BM after EV route [23]. Most in vivo studies did not use IS drugs before or during cell transplant [22, 24, 100, 114–116].

The preclinical studies report the increase of the levels of WBC [22, 24, 98], PTL [22, 23], and hemoglobin in PB [23, 100] after MSC transplant in comparison with the BMF control group that did not receive MSCs. Different studies show BM recovery and demonstrate an increased number of BM cells in vivo [22–24, 116] as well as an increased CFU-f [22, 116] and CFU-GM in vitro capacity [22, 98, 114]. Additionally, an increased megakaryocyte concentration was observed in BM after MSC transplantation [23, 116]. Besides, the increase of PB and BM cells, hematopoietic cytokines, as FLT3LG and TGF-beta1, was reported. These cytokines, which are secreted by MSCs, are important to HSC proliferation and differentiation process [24].

Although TBI led to HSCs and progenitor cells in BM apoptosis, few reports showed MSCs' antiapoptotic effect on HSC and hematopoietic progenitor cell preservation [22, 116]. The exact mechanism of MSCs' antiapoptotic effect is still under investigation. However, it has been shown that MSC transplant leads to a reduction of BAX gene expression in BM cells [116].

It is known that the increased levels of IFN-γ and TNF-α in irradiated mouse activate the Th1 and Th2 cells [24]. On the other hand, MSCs present immunoregulatory properties that could be used to attenuate the imbalance of immunologic system after radiation exposure [114, 117]. Hence, one study showed that MSCs reduce irradiation-induced hematopoietic toxicity. MSCs improved lymphocyte-mediated inhibition of CFU-GM and induced additional immunoprotective effects by expanding the Tregs, regulating chemokine receptor expression, and promoting the Th1/Th2 balance toward anti-inflammatory Th2 polarization [114].

Recent publications focused on preclinical studies demonstrated that MSCs could recover BMF by its antiapoptotic and immunoregulatory properties [22, 23, 114, 116]. However, all these studies evaluated MSC benefits after short-term experiments (from 24 hours to 30 days post-MSC injection); therefore, long-term benefits and stability on MSC transplant still need to be assessed.

7. Transplant of MSC in AA Patients

The clinical use of the MSCs in the hematological diseases has received special attention because of their inhibitory effects on the proliferation and cytotoxic activity of immune system cells in patients, which developed GVHD in response to allogeneic HSC transplantation [118, 119]. In AA patient transplant of allogeneic BM—or UC—MSCs were performed alone or in combination with HSCs [120–125] (Table 2). Some studies are registered at the National Institutes of Health (NIH) clinical trial database [126, 127]. The patients enrolled in these clinical trials presented severe stage of AA disease and did not respond to IS therapy (exhibit refractory stages). In addition, many patients prior to MSC transplant had already received treatment with HSC or BM cell transplant without clinical amelioration [121–123, 125]. In these studies, MSC doses ranged from 1×10^6/kg to 1×10^7/kg per transplant and the patients received one to two transplants per month.

Most of the studies did not evaluate whether MSCs engraft into host BM after EV transplant [121, 123–125]. Only one study showed MSC chimerism in a patient's BM microenvironment after MSC transplant. The chimerism study was performed by real-time PCR for the SRY gene for detection of male DNA in whole BM sample from a woman patient. This study showed improvement of BM stromal niche in a patient with SAA refractory to ATG and cyclosporine who was ineligible for allogeneic HSCT. After receiving two allogeneic transplants of MSCs, the biopsy demonstrated reduction of necrotic areas, but the BM improvement was not observed [120].

Cotransplant of MSC and HSC therapy also shows hematopoietic recovery in AA in humans [123, 128]. Six patients were treated, and two of them presented a hematopoietic recovery in both BM and PB three months after transplant [123].

All clinical studies used immunosuppression protocol [120–125]. In spite of this, some patients manifested adverse events such as mild self-limited febrile reactions, headaches, hypoxemia, mild dyspnea, and diarrhea after MSC transplant. All these adverse events were observed during or after MSC infusions and were mild and self-limited [123, 125]. Three studies reported a few deaths of SAA patients after the second or third MSC transplant alone [120, 125] or combined with HSCs [124]. However, these deaths occurred as a result of natural complications of AA disease [120, 124, 125]. Besides, no study reported occurrence of tumor after MSC transplant during the follow-up studies.

There are few clinical cases which use the therapy with MSCs on the AA disease, and then only in the most severe cases which did not respond to conventional treatment. And these studies show that the treatment was safe, but not enough to alone recover the BM. This observation can

Table 1: MSC transplantation in BMF and AA animal models.

Reference	Mice model	Gender	Age (weeks)	BMF induction method	Cell source	MSC profile	Number of transplanted MSC	A.R.	Evidence of MSC efficacy
[22]	Balb/c	F	6-7	Irradiation (5.5 Gy)	BM-MSC	$CD34^-$, $CD45^-$, $CD105^+$, $CD29^+$, $CD44^+$, and $Sca\text{-}1^+$	2.5×10^7	EV	↑ WBC and PLT in PB, CFU-F, and CFU-GM ↑ BMC ↓ apoptotic cells in BM
[23]	Balb/c	M	6	Irradiation (5.8 Gy)	UC-MSC + ECSOD	$CD14^-$, $CD73^+$, $CD90^+$, $CD105^+$, $CD44^+$, $CD29^+$, $CD34^-$, $CD45^-$, $CD19^-$, and $HLA\text{-}DR^-$	1×10^6	EV	↑ WBC, PLT, RBC, HB Attenuate upregulation of apoptotic genes (p16, p21, p53, and NOX4) ↑ BM cells and megakaryocyte ↓ apoptotic cells
[24]	Balb/c	F	6	Irradiation (7 Gy)	hUC-MSC	$CD105^+$ and $CD34^-$	NI	NI	↑ WBC in PB and BMC ↑ levels of hematopoietic cytokines (Flt3L and TGF-β1)
[100]	Balb/c	F	8	Irradiation (4 Gy) + lymph node cell infusion	Multiplacentas pooled cells	NI	1×10^7	IP	Higher survival ↑ HB
[114]	Balb/c	NI	NI	Irradiation (8 Gy)	CB-MSC	$CD45^-$, $CD34^+$, $CD29^+$, $CD44^+$, $CD117^-$, and $Sca\text{-}1^+$	NI	NI	↑ survival and gain body weight ↑CFU-GM ↑ Treg cells ↓ suppressed Th1 immunity and regulation of T cell chemokine receptor expressions (CCR7 upregulation and CXCR3/CCR5 downregulation)
[115]	B6D2F1	NI	10–12	Irradiation (5–7 Gy)	HSC coinfusion of AD-MSC	$CD29^+$, $CD44^+$, $CD73^+$, $CD90.2^+$, $CD105^+$, $CD106^+$, $CD144^+$, $CD166^+$, $CD34^-$, $CD45.1^-$, $CD80^-$, and $Sca\text{-}1^-$	1×10^6	NI	BM reconstitution Facilitating and homing of HSC to recipient BM
[116]	Balb/c	M	6–8	Irradiation (4 Gy)	AD-MSC	$CD29^+$, $CD31^-$, $CD34^-$, $CD45^-$, and $CD90^+$	1×10^6	EV	↑ CFU-F, CFU-MK, and megakaryocytes in BM ($cd41^+$ cells) Recovery of BM cells ↓ apoptotic cells

A.R.: administration route; NI: noninformed; F: female; M: male; BMF: bone marrow failure; MSC: mesenchymal stem cell; BMSC: bone marrow stromal cell; hUC-MSC: human umbilical cord-derived mesenchymal stem cell; UC-MSC: umbilical cord-derived mesenchymal stem cell; CB-MSC: compact bone-derived mesenchymal stem cell; ECSOD: extracellular superoxide dismutase; HSC: hematopoietic stem cell; AD-MSC: adipose-derived mesenchymal stem cell; CD: cluster differentiation; Sca-1: stem cell antigen-1; HLA-DR: human leucocyte antigen-D related; EV: endovenous infusion; IP: intraperitoneal infusion; WBC: white blood cell; PLT: platelet; PB: peripheral blood; CFU-F: colony-forming unit fibroblast; CFU-GM: colony-forming unit granulocyte-macrophage; BMC: bone marrow cell; BM: bone marrow; Flt3L: FMS-like tyrosine kinase 3 ligand; TGF-β: transforming growth factor beta; CCR7: C-C chemokine receptor type 7; CXCR3: C-X-C motif receptor 3; CCR5: C-C chemokine receptor type 5; RBC: red blood cell; HB: hemoglobin; NOX 4: nicotinamide adenine dinucleotide phosphate oxidase 4; CFU-MK: colony-forming unit megakaryocytes.

Table 2: MSC transplantation in reported clinical cases with SAA.

Reference	Cell source	MSC profile	Number of patients	Age of patient (years)	Gender	Disease stage	Previous HSC or BM transplant	Number of transplants	A.R.	Number of MSC/kg	Immunossupressive treatment	Adverse event	Follow-up (months)	Death (patients)	Evidence of MSC efficacy
[120]	BMSC	$SH2^+$, $SH3^+$, $CD34^-$, and $CD45^-$	1	68	F	Refractory SAA	–	2	EV	2×10^6 and 6×10^6	CsA	NI	NI	1	MSC engraftment Partial recovery of BM stromal niche ↑ CFU-F ($n = 1$)
[121]	BMSC	NI	1	26	M	SAA	+	2	EV	1×10^6	CTX, ATG, TBI, FAMP, and ALS	NI	NI	None	Partial recovery of BM niche ($n = 1$)
[122]	UC-MSC coinjection HSC	$CD13^+$, $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD14^-$, and $CD31^-$, $CD34^-$, $CD45^-$, and $HLA\text{-}DR^-$	2	11 and 13	F	SAA	+	1	NI	1×10^6	CsA, ATG, and Methylprednisolone	None	NI	None	Enhance the HSC engraftment ($n = 2$)
[123]	BMSC	$CD29^+$, $CD73^+$, $CD90^+$ $CD105^+$, $CD34^-$, $CD45^-$, and $CD14^-$	14/4	16–56	M/F	Refractory SAA or NSAA	–	4–6	EV	6×10^5	CsA and ATG	Transient fever and headache ($n = 2$)	12	None	Recovery of three hematopoietic cell line ($n = 2$) Recovery RBC and hemoglobin level ($n = 2$) Recovery PLT ($n = 2$)
[124]	UC-MSC coinjection HSC	VEGFR2/$Flk1^+$, $CD166^+$ $CD105^+$, $CD44^+$, $CD29^+$, and HLA class I^+ $CD34^-$, $CD45^-$, $CD14^-$, and HLA class II^-	14/23	15	F/M	SAA	+	1	EV	1×10^6	CTX, FAMP, and ATG	None	60	9	↑Neutrophil ↑PLT Enhance the HSC homing and engrafting
[125]	BMSC	$CD73^+$, $CD90^+$, $CD\ 29^+$, $CD13^+$, $CD44^+$, $CD49e^+$, $STRO1^+$, HLA class I^+;$CD34^-$, $CD14^-$, $CD45^-$, glycophorin A^-, $CD31^-$, $cadherin^-$, KDR^-, and HLA class II^-	9	19–50	F and M	Refractory SAA or NSAA	–	5	EV	2.7×10^6	CsA and ATG	Fever, hypoxemia, mild dyspnea, and diarrhea	20	4	Partial hematologic response ($n = 2$)

MSC: mesenchymal stem cells; BMSC: bone marrow stromal cell; hUC-MSC: human umbilical cord-derived mesenchymal stem cell; HSC: hematopoietic stem cells; CD: cluster differentiation; HLA-DR: human leucocyte antigen-D related; HLA: human leucocyte antigen; VEGFR2: vascular endothelial growth factor receptor 2; SAA: severe aplastic anemia; NSAA: nonsevere aplastic anemia; EV: endovenous infusion; CsA: cyclosporine A; ATG: antithymocyte globulin; ASL: antilymphocyte serum; TBI: total body irradiation; FAMP: fludarabine; BM: bone marrow; CFU-F: colony-forming unit fibroblast; RBC: red blood cell; PLT: platelets; NI: noninformed; CTX: cyclophosphamide; SH2: Src homology 2; SH3: Src homology 3; VEGFR2/Flk1: vascular endothelial growth factor receptor 2.

suggest that therapy with MSCs is promising but still needs to be in combination with HSC transplant [120, 125].

8. Final Considerations

Regulatory agencies require that investigators provide robust data on in vivo efficiency of new biological products. They recommend the use of well-characterized animal models to predict the response in humans. In general, transgenic animals are more indicated for this purpose. However, as we mentioned above, in the case of acquired AA, an immune-mediated animal model is well accepted [102]. MSC therapeutic potential was assessed using two BMF models: immune-mediated and irradiation-induced model [22–24, 100, 114–116]. Although these studies helped to demonstrate the several benefits of MSCs on acquired AA, they present limitation—natural reversibility of AA pathogenesis following long periods of evaluation [129].

On the other hand, clinical studies which use MSCs, demonstrate that patients with a very severe form of AA were enrolled, as well as each study includes very limited number of patients. Another drawback of clinical studies includes the use of IS drugs [120–125] which hinder the interpretation of results, as these drugs may ameliorate and even recover BMF alone [130, 131]. In addition, IS drugs could negatively influence the therapeutic action of the MSCs [132].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Vivian Fonseca Gonzaga, Cristiane ValverdeWenceslau, and Gustavo Sabino Lisboa contributed equally.

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Hindawi
Stem Cells International
Volume 2017, Article ID 6720594, 13 pages
https://doi.org/10.1155/2017/6720594

Review Article

Mesenchymal Stem Cells in Myeloid Malignancies: A Focus on Immune Escaping and Therapeutic Implications

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Received 7 April 2017; Revised 6 June 2017; Accepted 20 July 2017; Published 21 August 2017

Academic Editor: Marcella Franquesa

The importance of the bone marrow microenvironment forming the so-called niche in physiologic hemopoiesis is largely known, and recent evidences support the presence of stromal alterations from the molecular to the cytoarchitectural level in hematologic malignancies. Various alterations in cell adhesion, metabolism, cytokine signaling, autophagy, and methylation patterns of tumor-derived mesenchymal stem cells have been demonstrated, contributing to the genesis of a leukemic permissive niche. This niche allows both the ineffective haematopoiesis typical of myelodysplastic syndromes and the differentiation arrest, proliferation advantage, and clone selection which is the hallmark of acute myeloid leukemia. Furthermore, the immune system, both adaptive and innate, encompassing mesenchymal-derived cells, has been shown to take part to the leukemic niche. Here, we critically review the state of art about mesenchymal stem cell role in myelodysplastic syndromes and acute myeloid leukemia, focusing on immune escaping mechanisms as a target for available and future anticancer therapies.

1. Introduction

Bone marrow stromal cells include mesenchymal stem cells (MSCs), adipocytes, osteoblasts, fibroblasts, endothelial cells, tissue macrophages, and osteoclasts. Recent evidences support the notion that patients with myeloid malignancies may present bone marrow microenvironment alterations in terms of abnormal hematopoietic-to-stromal cell interactions, relative deficiency of hematopoietic growth factors, and aberrant release of inhibitors [1]. Nevertheless, the level of MSC involvement in myeloid malignancies remains controversial. MSC molecular and genetic alterations in this context have been demonstrated, and cytogenetic abnormalities in MSC derived from myeloid malignancy patients have been reported [2–4], while other studies [5] failed to find any significant quantitative or qualitative alterations in myelodysplastic syndrome- (MDS-) derived MSCs.

Leukemogenesis is the result of multistep alterations involving both the genetic and the epigenetic levels; moreover, the immune system, far to be an innocent bystander, plays an active role in leukemic immune escaping mechanisms. In addition, it has not been completely elucidated whether cancer-associated MSCs belong primarily to the abnormal clone or emerge after leukemic stem cell induced environmental damage.

We therefore aimed to synthetically describe the state-of-the-art MSC alterations in myelodysplastic syndromes and acute myeloid leukemia, focusing on biological evidences about MSCs pathophysiologic role in immune escape, that may represent a possible target both for present and future anticancer therapies.

2. Mesenchymal Stem Cell Physiology

MSCs are adult multipotent cells that can be isolated from the bone marrow, umbilical cord blood, placenta, or adipose tissue [6] and represent fundamental actors in the formation, organization, and function of the hematopoietic niche [7–9]. Given their heterogeneity, the International Society for Cellular Therapy (ISCT) position statement suggested to use the term "mesenchymal SCs" only for cells that are plastic adherent in culture and express CD73, CD90, and CD105, but not CD14, CD34, CD45, CD79α, and human leukocyte antigen-D-related (*HLA-DR*). Moreover, they should be able

to differentiate fibroblasts, osteoblasts, adipocytes, and chondroblasts in vitro [10] and to transdifferentiate tissues of neuroectodermal origin such as neurons or glial cells. MSC tissue of origin remains a matter of debate, and both mesodermal and neuroectodermal embryonal sheets are possible candidates [11]. The vascular tissues may be considered as a source of MSCs, as it is well known that marrow endothelial cells protect and maintain the repopulating capacities of hematopoietic precursors *in vivo* [12] and regulate proliferation and differentiation by tight spatial colocalization with perivascular cells [13] and through E-selectin secretion [14]. Cytokine and chemokine release [15] and crosstalk molecules expression, such as Jagged1 and CXCL12 [16–18], play important roles in the regulation of these interactions.

MSCs display systemic immunoregulatory and immunosuppressive properties [19–24] and influence both adaptive and innate immune responses. One of the immunomodulatory mechanisms is the expression of cell surface molecules with immunosuppressive capacity, such as programmed death ligand 1 (PD-L1) and Fas ligand, on MSC surface, so that they are able to directly deliver inhibitory signals to immune cells expressing PD-L1 and/or Fas, via cell-to-cell contact mechanisms [25, 26]. In fact, MSCs can repress Th1 and Th17 polarization [27, 28] via PD-L1 upregulation/constitutive expression [29]. In this context, their impairment has been implicated in tumor immune escaping, as described below.

Moreover, it seems that MSCs may inhibit erythropoiesis in favor of myeloid differentiation, through soluble factor production [30], including interleukin (IL) 6, which was shown to expand myeloid progenitors blocking erythroid development [31]. In this context, elevated IL-6 and TNFα levels have been correlated with adverse survival in patients with acute myeloid leukemia (AML) [32]. Another player engaged in the niche regulation is the autonomic nervous system that accompanies marrow blood vessels through adrenergic fibers. An interaction of adrenergic fibers with the MSC microenvironment has been described, and deregulation of this system has been implicated in impaired hematopoiesis which is a hallmark of several hematologic diseases [32, 33].

3. Mesenchymal Stem Cell Behaviour in Myeloid Malignancies

MSC role in MDS and AML, the two overlapping/evolving models of myeloid malignancies, will be discussed focusing on these two biological and clinical conditions which, although very similar and belonging to a unique disease spectrum, show deep differences in both cellular/molecular and outcome aspects. Recent evidences, reviewed by Pleyer et al. [34], show that MDS cells are heavily dependent on their "dysplastic niche." MDS-derived MSCs display enhanced supportive capacities for clonal hematopoiesis by decreased expression of cell surface molecules [35], including CD44 and CD49e (α5-integrin) [36]. Moreover, in patients with low-risk disease displaying a hypermethylated phenotype, circulating endothelial precursors are increased and exhibit downregulation of members of the wingless-int (Wnt) signaling pathway and failure to adequately sustain normal hematopoiesis [37]. Keeping on with endothelial-associated MSCs, CD271-positive MSCs are increased in MDS/AML marrow, especially in low-risk MDS compared to high-risk MDS or AML, and show higher CXCL12 expression. CXCL12-expressing cell density, in turn, correlated with marrow blast counts and disease progression [38]. Finally, the TGFβ pathway is constitutively activated in marrow blasts from patients with MDS, suggesting TGFβ implication in the pathogenesis of the dysplastic niche [39]. As described for MDS, in AML, MSC-derived endothelial cells are significantly increased, especially in cases with rapidly proliferating disease, further suggesting MSCs derived cell implication in leukemic niche building. Furthermore, AML blasts have been shown to modulate endothelial cell expansion, proliferation, and activation through the upregulation of E-selectin adhesion molecule [40]. AML blasts may then adhere to the stroma and be sequestered in a quiescent status becoming chemo-resistant, constituting a pool of residual disease which will possibly lead relapse [41]. Adhesion and chemotaxis have been also evaluated in a recent study, where AML MSCs from AML patients showed similar β1 integrin, CD44, CD73, CD90, and E-cadherin but decreased monocyte chemoattractant protein-1 levels compared to MSCs from healthy donors. AML MSCs showed chromosomal aberrations, but no significant differences in gene expression were detected [42]. Moreover, AML MSCs display constitutive TGFβ signaling, which may be inhibited by the transcription factor FOS upregulation, and secrete lower levels of IL-6 and granulocyte macrophage colony-stimulating factor levels, resulting in diminished supportive capacity for healthy marrow precursors [40]. Keeping on with MSC interaction with AML blasts, it has been shown that both cells constitutively release several soluble mediators, and when cocultured normal MSCs had an antiapoptotic and growth-enhancing effect on primary human AML cells in 51 unselected AML patients, this was associated with increased phosphorylation of mTOR and its downstream targets. The authors concluded that the cytokine-mediated effect of the MSCs is growth enhancement/apoptosis inhibition [43]. A recent interesting finding is that different cytogenetic/clinical AML subsets may show differences also in MSC niche, as elegantly demonstrated by Rodrigues Lopes et al., who characterized MSC cytokine expression in patients with AML with myelodysplasia-related changes (MRC), a well-recognized clinical subtype of secondary AML, and de novo AML. They found that AML-MRC MSCs presented higher IL-6 expression, whereas de novo AML MSCs presented increased expression of VEGFA, CXCL12, RPGE2, IDO, IL-1β, IL-6, and IL-32, and decreased IL-10 expression. Interestingly, IL-32 was shown to promote stromal proliferation, chemotaxis, and crosstalk AML blasts [44].

Finally, altered adrenergic regulation is observed in leukemic niche and this AML-induced neuropathy (i.e., sympathetic denervation of marrow arterioles and reduced sympathetic tone) reinforces leukemia progression through depletion of arteriole-associated pericytic mesenchymal cells [45].

4. MSC Molecular Pathway Alterations in MDS and AML

As described in murine experimental models, microenvironment molecular alterations may contribute to the induction of hematopoietic disorders. Some examples are the activating mutation in β-catenin and the deletion of miRNA processor gene DICER1 in osteoblasts that resulted in the development of MDS and AML in mice [46, 47]. Interestingly, as reported by Diaz de la Guardia et al., marrow MSCs, although clearly linked to disease activity and treatment outcome, do not carry tumor-specific cytogenetic/molecular alterations [48]. Recently, von der Heide et al. [2] studied genetic, transcriptional, and DNA methylation alterations in human AML MSCs and observed a nonspecific mutation pattern with variable frequency of synonymous and nonsynonymous single-nucleotide variations as well as insertions/deletions of specific genes. Interestingly, the number of variants per sample lowered from diagnosis to relapse. The only mutation with high variant allele frequency involved the PLEC gene (R1801Q), encoding the cytoskeleton linker protein plectin. PLEC mutation analysis confirmed that it is present at an early stage of AML-MSC expansion. Genome-wide DNA methylation analysis also showed globally altered DNA methylation pattern in AML MSCs. Generally, CpG methylation showed skewing toward hypomethylation in AML MSCs. Other authors [49] also reported transcriptional alterations in MDS MSCs using RNAseq analyses, contributing to multiple pathway deregulation, including adhesion molecules and metabolic pathways as well as endocytosis [50–54].

Another process involved in MSC leukemic phenotype acquisition is that of autophagy that is responsible of senescent cell molecule elimination and turn over. Reduced expression of autophagy genes was found in human AML blasts, whereas autophagy pathway is upregulated in blasts from low-risk MDS cases [55–58], suggesting a role in preventing progression to high-risk disease or AML evolution.

The gene TWIST, a transcriptional regulator contributing to MSC self-renewal and differentiation, was shown to be upregulated in MDS human blasts, and its expression was demonstrated to be altered by stroma contact and to correlate with disease stage and p53-mediated apoptosis [59–61]. In this context, TWIST tumor suppressor function, exerted by p21 activation, appears epigenetically silenced by hypermethylation in 31% of adult AML patients, providing leukemic cells with proliferation and survival advantages [62].

Finally, Notch/Jagged1 abnormalities have been described in human MSCs, leading to impaired differentiation and plasticity and contributing to MDS pathogenesis [63], while constitutively active *β-catenin* expression favored AML induction in murine models [46]. Moreover, Geyh et al. [64] showed that MSCs from AML patients exhibit Kit-ligand and Jagged1 pathway alterations, causing growth deficiency and impaired osteogenic differentiation capacity, partially reversible and correlating with a disease status.

5. MSCs and Tumor Immune Escape

5.1. Immune System in Myeloid Malignancies: A Naive Surveillant? Innate and adaptive immune pathways demonstrate aberrant activation in the hematopoietic niche of MDS playing a role in the increased rate of apoptosis of marrow precursors which is the hallmark of marrow failure. Moreover, an increased incidence of autoimmune diseases (e.g., primary immune thrombocytopenia, vasculitis, hypothyroidism, and rheumatoid arthritis) in patients with MDS has been reported in epidemiological studies [65], and recently, [66] the existence of a spectrum of pathophysiologic entities, spanning from chronic autoimmune attack to frank MDS and AML, through idiopathic cytopenia/dysplasia of undeterminate significance (ICUS/IDUS), has been hypothesized. Focusing on cellular immunity, naïve T cells (CD3+) exhibit shorter telomere length and have significantly less proliferative potential in MDS than in normal controls; oligoclonal T cells, often derived from the malignant MDS clone, may be present and seem to inhibit hematopoiesis *in vitro,* possibly through MHC class I molecules targeting on benign and malignant hematopoietic precursors, as a part of tumor surveillance. As the targeting of these mechanisms through immune-suppressive therapies has not been associated to AML progression, it is thought that MDS cells escape immune patrolling through a cytokinic permissive stromal pattern. These aspects are also shared byaplastic anemia, another genotypical (as these patients harbor mutation characteristic of MDS and ICUS/IDUS in almost half of cases) and phenotypical models bridging autoimmunity and malignancy, in which T cell-mediated marrow suppression leads to bone marrow failure with an increased risk of AML evolution [67]. Altogether, these alterations may reduce the "naive" immune surveillance on malignant transformation, modulating those mechanisms that, although various and articulated, may be eluded. In MDS patients, an increased expression of inflammatory T helper 1 cytokines has been reported, contributing to systemic symptoms and increased apoptosis [67]. In particular, tumor necrosis factor alpha (TNF-α) overexpression is inversely correlated to hemoglobin and survival, as it favors Fas-mediated- and TNF-related apoptosis-inducing ligand- (TRAIL-) driven ineffective hemopoiesis. Moreover, IFN regulatory factor 1 (IRF-1) mRNA, a tumor suppressor gene involved in T cell maturation and inflammatory responses, has been found upregulated in MDS patients with autoimmune phenomena compared to those without [68, 69]. Transforming growth factor-β (TGF-β) has been implicated in MDS hemopoietic suppression directly or mediated by the production of other myelosuppressive cytokines (e.g., IL-6, IL-32, IFN-g, and TNF), leading to decreased B cell proliferation, natural killer dysfunction, and propagation of cell autophagy or apoptosis. Inhibition of TGF-β receptor I kinase was shown to decrease apoptosis and improve erythroid and myeloid colony formation in vitro. T-helper 17, involved in the development/prevention of autoimmunity and inflammation, is significantly increased in low-risk MDS [69]. Finally, innate immunity is highly active in MDS cases due to overexpression of TLR activators, such as MYD88, TIRAP, IRAK1/4, and TRAF,

and downregulation of inhibitory factors, such as microRNAs miR145 and miR146a [70].

In AML, the immune system may play a role in enabling blast proliferation originating from the leukemic stem cell which in turn escapes surveillance. Cellular-mediated immune killing is driven by T CD8+ recognition of aberrant/exogenous molecules presented on MHC Class I complex, and the loss/mutation of MHC Class I is a demonstrated escape strategy in solid tumours. In AML, MHC Class I loss is rare, but allows NK cells to escape through killer cell immunoglobulin-like receptors (KIR). Moreover, expression of nonclassical HLA molecules, as reported for soluble HLA-G detected in AML sera, may contribute to immune suppression. CD4+ T cells and antibody-driven immunity rely on MHC Class II recognition of presented extracellular antigens; these molecules present variable expression on AML cells, and their total loss on promyelocytic leukemia cells may represent a possible escaping strategy. Currently, great interest is being given to AML-associated antigens (e.g., antigens aberrantly expressed by AML cells) that may elicit immune responses. For example, Wilms' tumour protein 1 (WT1), a zinc finger transcription factor overexpressed in AML cases, especially in leukemic stem cells, provides a target to eliminate the quiescent neoplastic cells. Moreover, some AML-specific genetic alterations may lead to aberrant antigen recognition through both MHC Classes I and II, as observed for FLT3-ITD, PML-RARalpha, BCR-ABL, DEK-CAN, and NPM1 mutations. As described for MDS, both innate and adaptive immunities are eluded by AML blasts through different mechanisms: (1) suppression of NK cell-mediated cytotoxicity by the inability to lyse AML cells, production of cytotoxic cytokines, aberrant expression of VEGFC, and by immature NK cell inhibition of T cell activation. (2) T cell suppression through aberrant gene expression in T cells, through the inability to form effective immune synapses with AML blasts, and by the coexpression of immunosuppressive proteins (TIM-3 and PD-13). Higher proliferation of Tregs facilitates blast expansion by T effector cell suppression through secretion of immunosuppressive cytokines (e.g., TGF-beta). In addition, Tregs increase the production of adenosine, an immunosuppressant for T and NK cells. (3) Secretion/expression of immunosuppressive factors by AML cells, including indoleamine 2,3-dioxygenase 1 (IDO) expression, arginine metabolism, and secretion of reactive oxygen species [71]. It is noteworthy to mention that immunologic milieu in AML is dynamic, as it is altered and modulated not only by the disease itself, but even by therapeutic interventions. As a matter of fact, AML in remission after chemotherapy shows decreased innate (neutrophils and monocytes) and specific immune activities (B cell activation) and cytokine pattern may also be changed. Even deeper is the change after allogeneic transplant, where immune reconstitution is a long and complicated process implying the adoption/maturation of the donor immunocompetent cells and the persistency of the recipient memory cells. In a recent study, evaluating the functional capacity of the immune system in 10 adult patients with AML using the response to seasonal influenza vaccination as a surrogate, only 2 patients generated protective titers in response to vaccination and a majority of patients had abnormal frequencies of transitional and memory B cells, with B cell repertoire showing little evidence of somatic hypermutation. Conversely, T cell populations were similar to healthy controls, and cytotoxic T cells demonstrated antigen-specific activity after vaccination, with T-effector cells showing increased PD-1 expression with possible therapeutic implications (see specific paragraph) [72].

5.2. MSCs Interact with Microenvironment Immunologic Landscape. MSCs have immunoregulatory activities exerted by interaction with a large number of effector cells, including T cell subsets, B cells, NK cells, monocyte-derived dendritic cells, and neutrophils, by direct cell-to-cell adhesion and/or secretion of soluble molecules, the so-called MSC secretome, which includes soluble molecules and extracellular vesicles (EVs) released by the MSCs into the extracellular milieu. Moreover, terminally differentiated mesenchymal cells prevent both proliferation and apoptosis of activated T cells. MSC-mediated immune regulation encompasses several mediators including interferon-γ (IFN-γ), interleukin-1β (IL-1β), transforming growth factor-β1, indoleamine-2,3-dioxygenase (IDO), IL-6, IL-10, prostaglandin-E2, hepatocyte growth factor, tumor necrosis factor (TNF)-α, nitric oxide (NO), heme oxygenase-1, and HLA-G5. These suggest a wide variety of targets for both immune inhibition and escaping mechanisms. Concerning innate immunity, MSCs are able to inhibit neutrophil proinflammatory activities suppressing the respiratory burst and prolonging survival through IL-6 and STAT-3 pathways. Moreover, they inhibit mature DC differentiation while promoting IL-10-producing plasmacytoid DCs. In a recent report, bone marrow MSCs were able to inhibit mouse DC activity by decreasing expressions of TLR3 and TLR9, confirming the immunomodulatory role of MSCs in a cell-based therapy [73]. Finally, MSCs suppress NK proliferation, soluble factor production, and cytotoxic activity. As long as adaptive immunity is concerned, MSCs promote quiescent T cell survival, but induce anergy of activated T cells and inhibit proliferation in favor of Tregs development driven by IL-10 secretion. B cell proliferation/differentiation is also suppressed both directly and through activated CD4+ T cell suppression. These immunoregulatory properties are exerted both directly and through MSC-derived exosomes, which have been used for therapeutic purposes as later discussed [74]. The abilities of MSCs to modulate immune responses may contribute to their therapeutic activity in models of autoimmune/inflammatory disorders. As an example, in rheumatoid arthritis, human adipose tissue-derived MSCs were able to inhibit IL-17 and IL-21 secretions by mononuclear cells and to increase TGF-β expression with consistent immunomodulatory effects [75].

5.3. MSCs Contribute to Leukemia Permissive Niche Also by Immune-Mediated Mechanisms in Lymphoid and Myeloid Models. Tumor microenvironment is thought to be inflammatory and "educated" by the neoplastic cells to be permissive in favor of the neoplastic clone growth. MSCs from the neoplastic niche are then in turn able to switch their phenotypes from MSC Type 1 (proinflammatory) to "tumor-

educated" MSC Type 2 cells (anti-inflammatory) that exhibit stronger immunosuppressive and migratory properties and drug resistance and promote proliferation [76, 77]. Interestingly, transcriptome profiling assays revealed a proinflammatory signature in bone marrow MSCs, with deregulation of immune and inflammatory modulators of the prostaglandin synthesis, largely depending on the genomic instability characteristic of oncogenic transformation, which is more typical of solid tumor than of AML, that is known to show limited number and type of identifiable driver/passenger mutations [78, 79]. The phenomenon of epithelial to mesenchymal transition, typical of solid tumors and linked to metastatization ability, is another evidence of mesenchymal contribution to tumor escape: malignant epithelial cells modify their transcriptional programme losing cell–cell attachments and acquiring mesenchymal-like features and motility, through deregulation of molecular pathways that include TGFβ, Wnt, Notch, hedgehog, and tyrosine kinase receptors. This phenotype has been associated in solid tumors with poor prognosis and resistance to radio- and chemotherapy. A minority of these mesenchymal-switched-circulating tumor cells migrate and survive in the bloodstream, evading immune surveillance, extravasate, and are able to colonize target organs or tissues [79]. Similarly, chronic lymphocytic leukemia-derived exosomes actively promote disease progression by modulating several functions of surrounding stromal cells that acquire features of cancer-associated fibroblasts. More in details, CLL-derived exosomes are actively incorporated by endothelial and mesenchymal stem cells that show enhanced proliferation, migration, and secretion of inflammatory cytokines, contributing to a tumor-supportive microenvironment [80, 81]. Cancer-associated MSCs have greater chemotactic activity on mononuclear cells and Tregs and inhibit T cell cytotoxicity and B cells and NK cells, as observed in solid and hematologic malignancies [82–85]. As mentioned above, MSCs secrete IDO and PGE2. The IDO enzyme activates dendritic cells and macrophages, helping to create an environment that favors suppression and tolerance. This molecule has been shown to play a relevant role in promoting solid/liquid tumor tolerance in vitro and in vivo murine models [86–88]. In addition, many human tumors have been shown to produce IDO [89]. In a recent report, MSCs isolated from 20 bone marrow AML samples showed higher expression of IDO and increased Tregs compared to control subjects, with a positive correlation between IDO expression and Tregs, responsible for immunosuppressive microenvironment [90]. Neoplastic MSCs in MDS and AML decrease tumor immunosurveillance through downregulation of costimulatory molecules (CD40, CD80, and CD86) [91], increase immunosuppressive cytokine production (TGFβ, IL-6, and HGF) with consequent T cell and DC suppression and Tregs and MSC type 2 increases. These alterations are more markedly observed in high-risk than in low-risk MDS [92, 93]. Constitutive overexpression of IDO observed in AML blast cells and sera [94] correlates with decreased relapse-free and overall survival [95]. Moreover, high IDO levels in MDS cases correlate with cytopenias severity [96].

Recent evidences support the role of T helper (Th) cells in the pathogenesis of hematological malignancies, including Th1/Th2 unbalance, increased Treg and Th17 levels, as well as the recently identified Th22 cells, that produce IL-22, which belongs to IL-10 cytokine family involved in the pathogenesis of autoimmune diseases and MDS. Tian et al., studied T helper subsets in AML patients and found that, while Th1, Th2, Th17, and Th22 cells and IL-22 were significantly reduced, Treg cells were increased in newly diagnosed AML patients compared to patients in remission or controls and chemotherapy ameliorated these variations [97]. MSC-mediated alteration of immune environment may be reversible with leukemia treatment, as also shown in patients with juvenile myelomonocytic leukemia, whose MSCs showed differential mRNA expression, including genes involved in immunomodulation and cell–cell interaction, that normalized during remission after successful hematopoietic stem cell transplantation [98].

As far as innate immunity is concerned, coculture of AML cells with MSC significantly protects leukemic blasts from NK cell-mediated lysis, mainly through cell-to-cell contact with supportive MSC, implying a relevant role of MSC in the immune response against AML blasts [99]. Moreover, overexpression of toll-like receptor- (TLR-) regulated genes has been described in MDS, resulting in excessive apoptosis with consequent cytopenias and decreased erythropoiesis in lower risk stages. In fact, TLR1, 2, and 6 expressions are higher in CD34+ blasts from patients with lower risk compared to higher risk MDS, whereas TLR2 and 4 expressions were similar in AML patients and healthy controls [100–102]. Concerning clinical correlations, higher expression of TLR2 is associated with prolonged survival, whereas higher expression of TLR6, TLR7, and MYD88 (a key mediator of TLR signaling) might confer a worse prognosis. On the contrary, in AML, stimulation of TLR2 and 4 resulted in induction of immune escape mechanisms such as upregulation of PD-L1, which protected AML cells from cytotoxic T lymphocyte lysis in vitro [103, 104]. Finally, recent evidences also show antileukemic effects for TLR8 activation, independently from its immunomodulating properties [105].

On the contrary, in lymphoid malignancies, a host beneficial immunomodulatory effect mediated by MSCs has been shown in low/intermediate risk acute lymphoblastic leukemia patients, where MSCs promoted an efficient NK cell response including cytokine production, phenotypic activation, and cytotoxicity [106].

Recently, Giallongo et al. identified a population of myeloid-derived suppressor cells (MDSC) in chronic myeloid leukemia (CML) patients that is part of the tumor clone and provides a leukemic friendly microenvironment mediated by ARG1, NOS2, reactive species of oxygen (ROS), cyclooxygenase 2 (COX2), TGFβ and immunosuppressive cytokine production, inhibition of NK function, and Treg expansion. When MSC-educated MDSC from healthy donors and CML patients were generated by coculturing MSC with peripheral blood-mononucleated cells, only CML-MSC-educated MDSC exhibited a suppressive ability on autologous T lymphocytes and overexpressed TGFβ, IL-6, and IL-10, thus contributing to CML immune escape [107].

6. Mesenchymal Stem Cells: Therapeutic Implications

6.1. Targeting Immune Escape. The immune disregulation of the dysplastic and leukemic niches is an interesting target for biological therapies. Recently, growing evidences support the use of immune checkpoint blockers as well as engineered immunocompetent cells and monoclonal antibody therapies engaging specific T cells in hematologic malignancies. Immune checkpoints are regulatory pathways that are induced in activated T cells and regulate the amplitude as well as the quality of T cell antigen responses. However, cancer can exploit these immune cell-intrinsic checkpoints for escaping immune-mediated destruction, by upregulation and activity of checkpoint molecules on T cells, leading to reduction or elimination of antitumor immune activity. CTLA-4 and PD-1 are two of the most actively studied inhibitory receptors expressed by activated T cells. The PD-1 pathway not only suppresses functions of effector T cells, lytic capacity of NK cells and B cell antibody production, but also promotes Treg stability and functions, thus contributing to the maintenance of immune suppression in the microenvironment. PD1/PDL1 upregulation may be targeted with immune checkpoint blockers. As a matter of fact, immune checkpoint inhibitors may enhance cytotoxicity of cytokine-induced killer cells against human myeloid leukaemic blasts [108], suggesting that also AML may benefit from checkpoint inhibition, as it has been demonstrated in solid cancers.

In a recent study, vaccination with MSCs exposed to microgravity inhibited proliferation and promoted apoptosis of tumor tissue, by inducing Th1-mediated cytokine response and CD8-dependent cytotoxic response and by increasing MHC1 and HSP protein expressions. The enhanced antitumor immune response of MSCs was strongly associated with the higher expression of MHC class I molecule on DCs that made tumor molecules more cross-presentable to the host DCs to generate protective antitumor activity [109].

Another interesting approach consisted in the adoptive transfer of gene-modified MSCs, which produce and secrete tumor-directed monoclonal antibodies continuously in the body of the patient, as bispecific autoantibodies have short half-lives *in vivo* and are rapidly cleared from circulation. As MSCs have limited immunogenicity and tend to accumulate in close proximity of the tumor, they can be used as a platform for the targeted delivery of anticancer agents. Aliperta et al. recently demonstrated that gene-modified MSCs are able to express the CD33-CD3 bispecific antibody at high levels and to mediate an efficient lysis of AML blasts by human T cells of both healthy donors and AML patients [110]. The same mechanism was explored in a murine model of disseminated ALL, to selectively deliver measle virus to leukemic cells [111]. As regards leukemic MSC role in adapted immunity, they seem to favor chemotaxis of mononuclear cells and Tregs and to inhibit T cell cytotoxicity and B cell and NK cell activities, possibly through the secretion of IDO and PGE2 [86]. This observation may provide a further rationale for the use of IDO inhibitors in myeloid malignancies. Recently, Ninomiya et al. showed that tumor microenvironment suppresses chimeric antigen receptor T (CART) cell activity through IDO immune escape in a xenograft lymphoma model. Moreover, they report that fludarabine and cyclophosphamide downregulate IDO expression and improve CART antitumor activity [112].

MSCs of MDS overexpress TLR-regulated genes, resulting in excessive apoptosis with consequent cytopenias and decreased erythropoiesis. Preliminary *in vitro* observations favour the rationale of targeting aberrant TLR signaling in this setting [113]. Furthermore, TLR2 and 4 are upregulated in AML, resulting in induction of immune escape mechanisms such as upregulation of PD-L1, which protects AML cells from cytotoxic T lymphocyte [114], with a negative prognostic impact. Consequently, TLR agonists have been used as immunotherapy with the intent of inducing blast-derived dendritic cell maturation, both in vitro [115–119] and in clinical trials [120].

As regards antibodies and antibody-derived agents such as antibody drug conjugates and bispecific agents, CD33 is a valuable and clinically validated target; a prototypical agent, gemtuzumab ozogamycin, was shown to be effective in AML treatment, but was withdrawn from the clinical use because of side toxicities, mostly due to the chemical linker connecting the toxin component to the antibody carrier, even if in early 2017 a new request for US and EU approval was submitted. Moreover, antibodies specific for CD123 are under evaluation and seem to target the leukemia stem cells. New CD33-directed agents include new toxins coupled with improved linkers to the antibody (e.g., SGN-CD33a), bispecific antibodies recruiting effector cells like NKs or T cells (e.g., AMG-330), and single-chain triplebodies (e.g., 123–16-33 and 33–16-33) with a double receptor for the AML blast and one recruiting NKs. Immune reconstitution has a key role in enabling immune-mediated therapies in AML patients in remission to eliminate leukemic stem cells and MRD. NKs recovered from AML patients at diagnosis are often reduced and show reduced cytolytic activity; a recent study compared blood titers and cytolytic function of NKs from an AML patient with those of a healthy monozygotic twin. The Authors found that NK functions measured in the AML patient after disease remission were comparable to those of his healthy twin brother; moreover, ex vivo cytolytic activities mediated by triplebody SPM-2 were also maintained, making triplebodies promising new agents for the treatment of AML [121].

Graft versus host disease (GVHD) has been one of the autoimmune targets for MSC therapies in mouse models. These models are unfortunately different from the human counterparts as murine MSCs are more prone to spontaneous immortalization and transformation and IDO is not involved in mouse MSC-mediated immunoregulation. In mice, a single infusion of MSCs at the time of transplant does not prevent GVHD, whereas multiple injections may be beneficial, and engraftment of MSCs at GVHD sites is low. MSC cotransplantation does not promote engraftment in T cell-replete transplants, but might be beneficial in T cell-depleted transplants. MSC treatment of corticosteroid-refractory acute GVHD showed controversial results in human, with a European trial providing 30 out of 55 complete responses,

whereas another study failed to demonstrate any durable benefit. Recently, a phase II randomized study showed that repeated infusion of MSCs may inhibit chronic GVHD symptoms and reverse the Th1/Th2 cell ratio imbalance [74].

An interesting therapeutic approach is adoptive immunotherapies in myeloid malignancies that include, among others, bone marrow allogeneic transplant, donor lymphocyte infusion (DLI), and chimeric antigen receptor T (CART) cells [122]. These approaches are currently aimed at leukemic blast targeting, but MSC patterns might be new targets in the next future. In particular, CART cells, engineered with synthetic polypeptides consisting of an extracellular variable fragment directed to a tumor antigen and an intracellular signaling domain, potentiated by the addition of costimulatory molecules in new generation models, have shown great success in relapsed/refractory ALL and may find a role in AML too, possibly targeting AML-associated/specific antigens like CD33, CD123, Lewis Y antigen, and folate receptor beta [123]. DLI is effective in reinducing response in AML residual/relapsing disease after allogeneic stem cell transplant and is currently investigated as a way to eradicate minimal residual disease (MRD), which correlates to higher relapse rate and reduced survival in AML [124].

Considering MDS, the therapeutic efficacy of lenalidomide is of great interest and is being moved from 5q syndrome to all low-risk cases. The drug, while directly inducing proliferation inhibition and apoptosis of MDS cells, plays important immunomodulatory activities by inducing T cell-mediated cytotoxicity, through CD28 costimulation and interferon-γ- and interleukin-2-increased productions, and by NK cell activation. At the same time, lenalidomide decreases inflammation by inhibiting the production of proinflammatory cytokines such as TNF-alfa and interleukin-1,-6,-12 and eliciting anti-inflammatory cytokine release, like interleukin-10, thus contrasting the inflammatory microenvironment that is detrimental to normal hematopoiesis [125].

6.2. Other MSC-Related Targets. The observation that cell-to-cell contact is necessary for "stemness" maintenance has led to the evaluation of adhesion mechanisms as possibly targetable players in malignant bone marrow milieu. In this context, signaling pathway of adhesion protein HSP90α/β has been shown to give proliferative advantage to MDS MSCs in patients with advanced-stage MDS, [126] being a potential therapeutic target in MDS. A recent study demonstrated the inhibitory effects of BIIB021, an orally available Hsp90 inhibitor, on an imatinib-resistant chronic myeloid leukemia cell lines, with significant growth inhibition and apoptosis and autophagic response [127]. HSP90 also safeguards proteins and is deacetylated by histone deacetylases 6; recent data show that histone deacetylase inhibitor-induced acetylation of HSP90 might control oncologically relevant proteins, especially in leukemic cells [128]. Moreover, various experiments indicate that [129, 130] CD44 adhesion pathway is of great interest in human AML and therapeutic blocking of this molecule in AML cells has been evaluated in murine xenografts, with some promising results [131]. For example, Li et al. reported that the anti-CD44 monoclonal antibody A3D8 inhibited proliferation of acute leukemia cell line HL-60. The A3D8 treatment increased the percentage of G0/G1 cells [132]. However, other in vitro experiments showed that MSCs may escape this targeted therapy and that leukemic stem cells become less microenvironment dependent in advanced-stage AML, so that targeting of CD44 may be less successful than expected. Anyway, some phase 1 trials are testing antibody against CD44v6 (bivatuzumab mertansine) in solid tumors, and MDS/AML setting might be a future field of application [133, 134].

As described in a recent study from von der Heide et al. [2], plectin, a cytoskeleton linker protein, is highly overexpressed in AML BM MSCs and its mutations are present at diagnosis, at the time of response and at relapse. This protein is also a biomarker for pancreatic ductal adenocarcinoma cells and a recent study [135] designed a plectin-1 selective drug delivery to pancreatic malignant cells. As immunohistochemical staining of plectin in bone marrow showed high proportion of plectin-positive cells in AML, a rationale may exist for such therapeutic strategies in this disease too. The targeted drug delivery that uses MSC for the spatial control of drug release is a growing field that will possibly have further implication in AML niche-directed therapies.

One of chemotaxis is another potentially targetable pathway: inhibition of CXCL12 stromal cells, and/or the CXCR4/CXCL12 axis, has shown promising results in mice and is currently being explored in clinical trials of MDS and AML [136–139]. Peng et al. report that a humanized anti-CXCR4 monoclonal antibody, LY2624587, blocked SDF-1 binding to CXCR4 in human lymphoma and leukemia cells, inhibiting cell migration, cell signaling including activation of MAPK and AKT, and mediating receptor internalization and CXCR4 downregulation on the cell surface. LY2624587 caused dose-dependent apoptosis in vitro and in mouse xenograft models, providing significant survival benefit [140]. Besides receptor antagonists that directly inhibit leukemic cell proliferation, preclinical and clinical studies demonstrate that CXCR4 inhibition mobilizes leukemic-lymphoma cells from their niches, improving conventional chemotherapy efficacy. An interesting study recently highlighted that runt-related transcription factor 3 (RUNX3) is involved in MSC-mediated protection of leukemia cells from As2O3- (arsenic trioxide-) induced apoptosis. In particular, in the presence of MSCs, As2O3-induced expression of RUNX3, modulated by CXCL12/CXCR4 signaling, was reduced. Furthermore, overexpression of RUNX3 restored the sensitivity of leukemic cells to As2O3. Therefore, RUNX3 is a promising target for therapeutic approaches to overcome MSC-mediated drug resistance [141].

Again, in the field of soluble niche molecules, high levels of TGFβ are secreted by leukemic MSCs, possibly contributing to impaired normal hematopoiesis in MDS. The TGFβ pathway, which is constitutively active in MDS blasts, may be targeted by specific inhibitors. Suppression of TGFβ signaling in murine models and in human bone marrow samples in vitro was shown to be able to restore normal myelopoiesis and erythropoiesis [30, 40]. Moreover, Naka et al. demonstrated the therapeutic efficacy of EW-7197, an orally bioavailable TGFβ signaling inhibitor, combined with

tyrosine kynase inhibitors, in eliminating chronic myeloid leukemia initiating cells in vivo: the combination significantly delayed disease relapse and prolonged survival [142]. In another study, TGF-β-transduced MSCs were able to suppress T cell proliferation and IFN-γ release and to reduce expressions of CD40, CD86, and MHC II as well as TNF-α secretion by dendritic cells, while IL-4 secretion was enhanced. TGF-β-transduced MSCs could provide a promising tool for treatment of clinical conditions such as organ transplantation, GVHD, and autoimmune disorders [143].

As regards DNA methylation in myeloid malignancies, altered epigenetic modifications in myeloid blasts are thought to be highly pathogenetic in these diseases, providing a rationale for hypomethylating drug use and efficacy. These drugs form nowadays the backbone for anticancer therapies in the elderly and in those patients ineligible for intensive chemotherapy. As a matter of fact, recent studies demonstrated that leukemic MSCs harbor highly specific differences in methylation patterns in comparison to healthy volunteers, indicating differential activity and function of MSCs in these diseases. In this context, the epigenetically regulated Wnt/β-catenin pathway was shown to be altered in MSCs from myeloid malignancies and associated with adverse prognosis [144–146]. Hypomethylating agents are able to demethylate Wnt antagonist gene promoters in vitro [147], leading to Wnt reexpression and normal myelopoiesis restoration. Similar results were described for the above mentioned gene TWIST, implicated in the pathophysiology of clonal myeloid diseases [148], whose reexpression in vitro occurred after treatment with hypomethylating agents [149]. Regarding autophagy, which confers a sort of cell protection in lower risk MDS and becomes defective along with disease progression, it could be another possible target for hypomethylating agents. In fact, downregulation of autophagy-associated genes was shown to be due to promoter hypermethylation in higher risk MDS and AML, and reexpression of autophagy-associated genes has been observed in AML cell lines [55, 56].

7. Conclusions

Current knowledge highlights the importance of a leukemic permissive bone marrow niche in supporting abnormal myeloid precursor clonal proliferation. MSC- and MSC-derived cells show morphologic, cytogenetic, and molecular abnormalities in myeloid malignancies encompassing cell adhesion, cytokine signaling, autophagy, and methylation patterns, leading to and favoring differentiation arrest, proliferation, and clonal selection. Moreover, the immune system, both adaptative and innate, participates in the leukemic permissive milieu and immune escape correlates with disease relapse and refractoriness to standard therapies. Finally, these patterns are currently being investigated as suitable targets for already available and future anticancer therapies. The development of immune escape-directed treatments will have to consider the cross-talk between immune cells and MSCs, particularly considering disease characteristics, phase (onset, remission, and MRD status), immune reconstitution, and therapeutic goal.

Disclosure

The authors state that this manuscript contains original unpublished work and is not being submitted for publication elsewhere at the same time.

Conflicts of Interest

The authors have no personal relationships between themselves and others that might bias their work to disclose.

Authors' Contributions

All authors have made substantial contributions to the conception and design of the study, to the acquisition and interpretation of the data, and to the article drafting. Finally, all authors revised the paper for important intellectual content and approved the final version to be submitted.

Acknowledgments

Fondazione Beat Leukemia Dr. Alessandro Cevenini Onlus, via Bellini 27, 20900 Monza (MB), http://www.beat-leukemia.org, mail: info@beat-leukemia.org, gifted a grant to cover publication fees.

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Hindawi
Stem Cells International
Volume 2017, Article ID 9717353, 9 pages
https://doi.org/10.1155/2017/9717353

Research Article

Mesenchymal Stromal Cells Accelerate Epithelial Tight Junction Assembly via the AMP-Activated Protein Kinase Pathway, Independently of Liver Kinase B1

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Received 30 March 2017; Accepted 21 May 2017; Published 11 July 2017

Academic Editor: Bruno Christ

Background. Mesenchymal stromal cells (MSC) are fibroblast-like multipotent cells capable of tissue-repair properties. Given the essentiality of tight junctions (TJ) in epithelial integrity, we hypothesized that MSC modulate TJ formation, via the AMP-activated kinase (AMPK) pathway. Liver kinase-β1 (LKB1) and Ca^{2+}-calmodulin-dependent protein kinase kinase (CaMKK) represent the main kinases that activate AMPK. *Methods*. The in vitro Ca^{2+} switch from 5 μM to 1.8 mM was performed using epithelial Madin-Darby canine kidney (MDCK) cells cultured alone or cocultured with rat bone marrow-derived MSC or preexposed to MSC-conditioned medium. TJ assembly was measured by assessing ZO-1 relocation to cell-cell contacts. Experiments were conducted using MDCK stably expressing short-hairpin-RNA (shRNA) against LKB1 or luciferase (LUC, as controls). Compound STO-609 (50 μM) was used as CaMKK inhibitor. *Results*. Following Ca^{2+} switch, ZO-1 relocation and phosphorylation/activation of AMPK were significantly higher in MDCK/MSC compared to MDCK. No difference in AMPK phosphorylation was observed between LKB1-shRNA and Luc-shRNA MDCK following Ca^{2+} switch. Conversely, incubation with STO-609 prior to Ca^{2+} switch prevented AMPK phosphorylation and ZO-1 relocation. MSC-conditioned medium slightly but significantly increased AMPK activation and accelerated TJ-associated distribution of ZO-1 post Ca^{2+} switch in comparison to regular medium. *Conclusions*. MSC modulate the assembly of epithelial TJ, via the CaMKK/AMPK pathway independently of LKB1.

1. Introduction

Epithelial tight junctions (TJ) form a seal at the superior pole of the lateral plasma membrane when cells differentiate and acquire polarity [1]. TJ regulate the passage of ions and small molecules through the paracellular pathway [2] and also restrict the diffusion of membrane proteins between the apical and basolateral compartments. TJ are made of at least 40 different proteins including transmembrane proteins, such as claudins and occludins, and adaptor proteins, such as members of the MAGUK (membrane-associated guanylate kinase) family, ZO-1, ZO-2, and ZO-3 [3, 4]. At the time of TJ assembly, ZO-1 and ZO-2 have essential roles in both organizing TJ components and targeting them to their proper location [5]. Many factors have been identified as modulators of TJ assembly/disassembly, including extracellular Ca^{2+} [6]. Extracellular Ca^{2+} is essential for both the development of new junctions [7] and the stabilization of mature junctions [8, 9] between epithelial cells [10]. The dependence of TJ assembly on Ca^{2+} is probably attributable to the stabilization of the cell adhesion molecule E-cadherin in its adhesive state. Numerous pathways have been implicated in TJ regulation, including the AMP-activated protein kinase [1, 11–16].

AMPK is an ubiquitous heterotrimeric complex made of 1 catalytic α-subunit and 2 regulatory β- and γ-subunits

[17]. AMPK activity is modulated by the intracellular AMP-to-ATP ratio, as well as by the activity of upstream AMPK kinases, such as liver kinase-β1 (LKB1) and Ca^{2+}-calmodulin-dependent protein kinase kinase (CaMKK) [18–20]. An increased ratio of AMP/ATP induces AMP binding to the γ-subunit, thereby promoting AMPK phosphorylation at a threonine residue (Thr-172) and its activation [21]. Additionally, in case of energy stress, LKB1 phosphorylates and activates AMPK via the formation of a complex with the pseudokinase STRAD and the scaffolding protein MO25 [22]. CaMKK activates AMPK in an AMP-independent manner in response to increased cytosolic calcium concentration [23, 24]. Note that AMPK autophosphorylation at β-subunit Thr-148 has been reported [25]. Activated AMPK promotes ATP production by favoring catabolism and switching off anabolic pathways. Interestingly, the pharmacological activation of AMPK by AICAR induces TJ assembly, independently of extracellular Ca^{2+} or energy deprivation [11, 18]. This effect might be achieved by strengthening the *trans* interactions mediated by cell adhesion molecules involved in the nectin–I-afadin system [12, 26] and/or by modulating cytoskeleton dynamics near the cell membrane [12]. Furthermore, preactivation of AMPK by metformin or AICAR helps preserve the functional integrity of epithelial cells in the face of ischemia and energy depletion, as demonstrated in vitro and in vivo [27–29]. TJ disruption is indeed considered as one of the earliest hallmarks of epithelial injury, leading to the loss of cell polarity and tissue disorganization.

Cumulative evidence in the field of epithelial injury supports that mesenchymal stromal cells (MSC) are capable of tissue-repair properties [30]. MSC represent a heterogeneous population of adult fibroblast-like multipotent cells [31]. In addition to their beneficial immunomodulatory and anti-inflammatory abilities [32], MSC may help epithelial cells survive, proliferate, and differentiate following injury [30, 33–35]. Hence, recent in vitro observations highlighted the role of MSC in wound healing of airway epithelium, via direct cell-cell contacts and paracrine activation of the epidermal growth factor receptor [36–38]. Also, MSC are known to release membrane vesicles (MVs) of various size and composition into the extracellular environment [39]. MSC-derived MVs may help transfer cytosolic components, including proteins, lipids, RNA, and organelles, from MSC to neighboring cells, which accelerate tissue repair [40, 41].

In the present study, we first investigated which AMPK kinases were responsible for AMPK phosphorylation and activation at the time of a Ca^{2+} switch. Next, we questioned the impact of MSC on epithelial TJ regulation in a coculture system of Ca^{2+}-induced TJ assembly in MDCK cells. Finally, we studied the impact of MSC-conditioned medium on epithelial TJ assembly. This paper was presented at MiSOT 2016—The 6th Expert Meeting on Therapeutic MSCs for Immune Modulation.

2. Materials and Methods

2.1. MDCK Culture Conditions. MDCK cells were grown to confluence in α-MEM supplemented with 10% FBS, 1% L-glutamine (Lonza), and 1% penicillin (Lonza), in a humidified atmosphere containing 5% CO_2 at 37°C. MDCK shRNA for *LKB1* and *luciferase* (*Luc,* used as controls) were generated using pSUPER/retro-puro vector, as previously reported [24]. Stable populations were maintained using puromycin (2 μg/mL; Sigma) as selection agent.

2.2. Isolation and Characterization of Bone Marrow-Derived MSC. Bone marrow cells were flushed from both femurs and tibias of male 9-week-old *Lewis* rats using phosphate-buffered saline (PBS, Lonza). After homogenization, cell suspension was filtered and centrifuged at 1200 rpm for 10 min. Cells were resuspended in α-MEM medium (Lonza) and gently sieved through Ficoll (Healthcare Life Sciences). After an additional 1500 rpm centrifugation for 45 min at room temperature (RT), mononuclear cells were removed from the gradient interface and suspended in α-MEM solution before final 1200 rpm centrifugation for 10 min. The cells were then plated in 75 cm^2 culture flask containing α-MEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin. MSC were maintained at 37°C in a humidified 5% CO_2 incubator. Supplemented α-MEM was changed twice a week. Cells were trypsinised at 80% of confluence for maximum 8 passages. At confluence, fresh culture medium was poured, collected at day 3, centrifuged at 1.800 rpm for 5 min, and stored at −80°C for further use. MSC phenotype was tested according to the criteria of the International Society of Cell Therapy: (i) plastic adherence, (ii) (non)expression of conventional surface markers using flow cytometry; and (iii) differentiation into adipogenic, osteogenic, and chondrogenic lineages [42]. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences), using Alexa Fluor-conjugated anti-rat CD29 antibody (BD Pharmingen), APC-conjugated anti-rat CD90 antibody (BD Pharmingen), V450-conjugated anti-rat CD45 antibody (BD Horizon), FITC-conjugated anti-rat CD11b (BD Pharmingen), and PE-conjugated anti-CD79a antibody (Abcam).

2.3. MSC/MDCK Coculture System. Cell populations were mixed and seeded on 6-well plates at density 1.5×10^5 cells/well. The seeding ratio of MSC : MDCK was 1 : 3. All experiments were performed at confluence. Alternatively, MDCK cells were incubated with 2 mL of α-MEM medium preexposed to MSC for 3 days.

2.4. Ca^{2+} Switch Experiments. Following steady state, cells were rinsed and incubated in Ca^{2+}-free S-MEM supplemented with 5% dialyzed FBS ($[Ca^{2+}]$, 5 μM) for 16 h before being switched back to normal medium (α-MEM; $[Ca^{2+}]$, 1.8 mM) for the indicated times. Compounds STO-609 (50 μM; Sigma) and dorsomorphin/compound C (50 μM; Sigma) were used as CaMKK and AMPK inhibitors, respectively. Increasing concentrations of dorsomorphin/compound C were tested in order to fully block AMPK activation (Supplementary Figure, panel A, available online at https://doi.org/10.1155/2017/9717353). Alternatively, MDCK cells were exposed to MSC-conditioned medium for 24 h, before exposure to S-MEM for 16 h. The Ca^{2+} switch

was then realized using MSC-conditioned α-MEM for the indicated times.

2.5. Western Blot Analysis. Cells were lysed on ice in RIPA lysis buffer including protease and phosphatase inhibitors (Roche). Cell lysates were obtained by centrifugation at 13.000 rpm for 30 min at 4°C. Supernatant was collected. Protein concentration was determined using Bradford method. Protein lysates were mixed with Laemmli buffer (1 : 4) and heated for 2 min at 95°C. Equal amounts of protein (30 μg/lane) were loaded onto stain-free SDS electrophoresis gels and separated at 100 V (Bio-Rad). Gels were exposed to UV light for 5 min (ChemiDoc MP system, Bio-Rad). Proteins were transferred to PVDF membranes (previously activated by ethanol) using the Trans-Blot Turbo transfer system for 7 min at RT. Blots were blocked with 5% milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h and incubated overnight at 4°C with primary antibodies: pAMPK (T172) (Cell Signaling), AMPK (Cell Signaling), and pACC (Cell Signaling) antibodies. Blots were rinsing 5 times with TBS-T for 5 min and incubated with HRP-conjugated anti-rabbit secondary antibodies (1/4000) for 90 min at RT. After rinsing, chemiluminescent signals were captured by ChemiDoc MP system after applying chemiluminescent substrate (Femto, Thermoscientific) on blots. Image data were analyzed and quantified ($n = 4$ for each experimental condition) using Image Lab 4.1 software. Representative samples were then run on the same stain-free SDS gels for the sake of publication, in agreement with the ASBMB policy.

2.6. Immunofluorescence and Quantification of ZO-1 Deposits. Cells grown on coverslips were rinsed twice with PBS and fixed in cold methanol for 12 min. After blockade with PBS/BSA 5% dilution for 60 min at RT and incubated for 90 min with anti-ZO-1 (ThermoFisher Scientific) and followed by 60 min of incubation with Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes), cells were visualized on an FSX-100 (Olympus Life Science). Contrast, brightness, and focus settings were chosen so that all pixels were in the linear range. To quantify the average ZO-1 length per cell, 4 fields were randomly selected, and the total length of ZO-1 in each field was outlined manually on Photoshop, followed by measurement using Image J software (NIH) [11–13]. Cell numbers were counted for each field with the DAPI Fluoromount-G (SouthernBiotech) slide mounting.

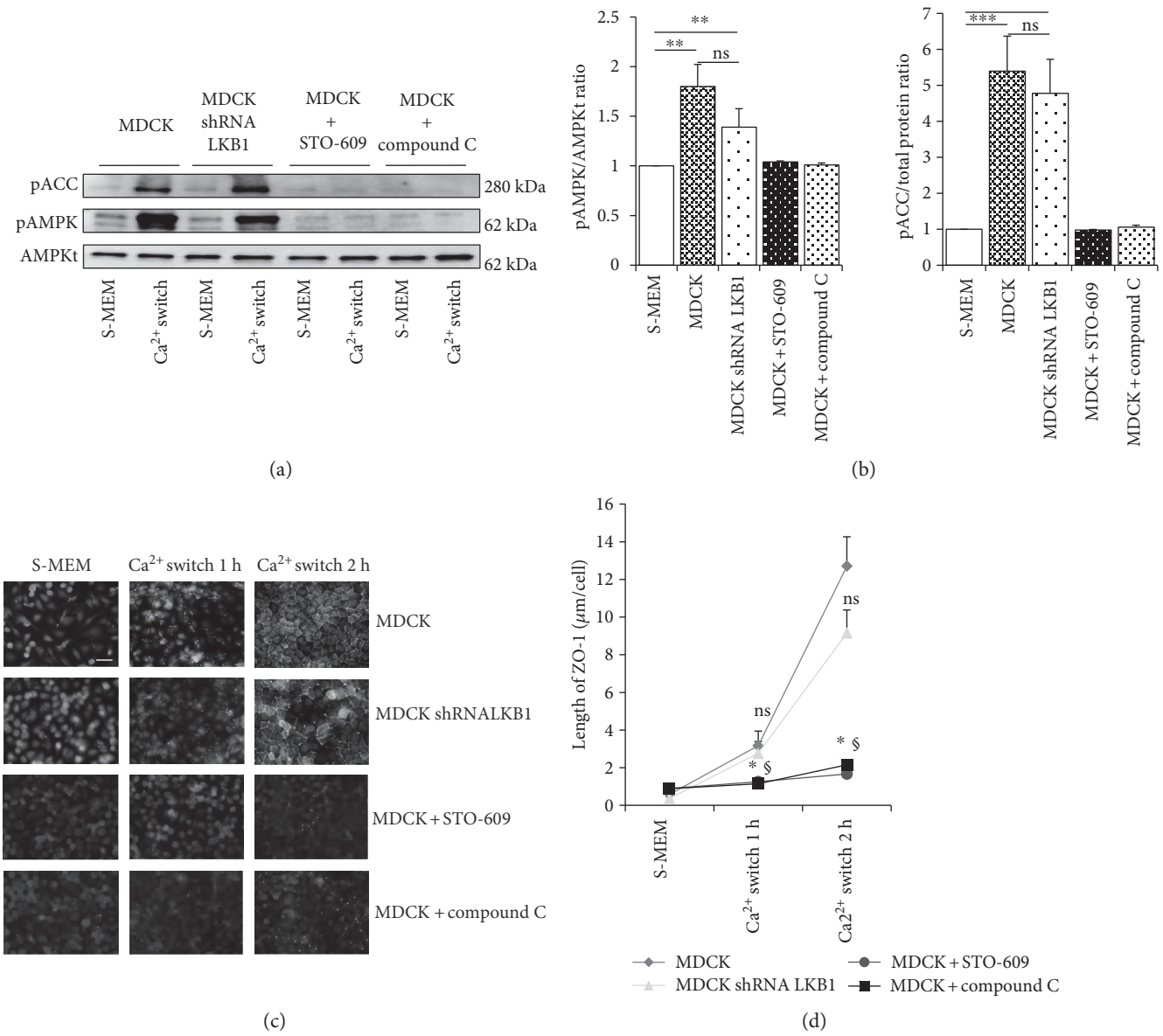
2.7. Statistical Analyses. Data were expressed as mean ± 1 standard deviation (SD). One-way analysis of variance, Mann–Whitney, and Student t-test were appropriately performed, with a significant p value set at 0.05 (MedCalc software).

3. Results

3.1. The Phosphorylation and Activation of AMPK Following a Ca^{2+} Switch Depend on CaMKK in MDCK Cells, Independently of LKB1. Following a Ca^{2+} switch, we observed a mean 1.75-fold increase of pAMPK compared to S-MEM medium ($n = 4$, $p < 0.05$) whereas total AMPK remained unchanged, as previously reported [11] (Figures 1(a) and 1(b)). Mean levels of pACC, a typical substrate of AMPK, followed a similar pattern, with a 5.3-fold increase following Ca^{2+} switch ($n = 4$, $p < 0.05$). LKB1 and CaMKK are considered as the 2 major AMPK kinases [23]. In LKB1-shRNA MDCK cells, mean levels of pAMPK and pACC were 1.4-fold and 4.7-fold increase, respectively, in comparison to S-MEM ($n = 4$, $p < 0.05$), with no significant difference with control Luc-shRNA ($n = 4$, not significant (ns)) (Figures 1(a) and 1(b)). Of important note, Luc-shRNA MDCK cells behave similarly as MDCK cells regarding AMPK phosphorylation/activation and ZO-1 relocation following Ca^{2+} switch ($n = 4$, data not shown). Conversely, pharmacological inhibition of CaMKK using STO-609 prevented AMPK phosphorylation and activation after Ca^{2+} switch, with mean levels of pAMPK and pACC similar to S-MEM conditions ($n = 4$, ns). Incubation of MDCK with AMPK inhibitor, dorsomorphin/compound C (50 μM) prevented AMPK autophosphorylation classically induced by Ca^{2+} switch (Figures 1(a) and 1(b)). These observations suggest that CaMKK plays a role in Ca^{2+}-induced AMPK activation in MDCK cells, independently of LKB1.

3.2. Pharmacological Inhibitions of AMPK or CaMKK Prevent Ca^{2+}-Induced TJ Relocation of ZO-1. During a Ca^{2+} switch, the translocation of TJ-associated protein ZO-1 from cell cytosol to cell-cell junctions represents a key and early step of TJ assembly [5]. Hence, we monitored the length of ZO-1 membrane deposits following a Ca^{2+} switch in MDCK cells exposed to various experimental conditions [11–13]. In normal conditions, readdition of Ca^{2+} causes a 4-fold increase of ZO-1 length compared to S-MEM conditions (Figures 1(c) and 1(d)). After 2 h of Ca^{2+} switch, TJ were largely assembled as a classical chicken-wire network. In LKB1-shRNA MDCK cells, ZO-1 relocation followed a pattern similar to control MDCK ($n = 4$, *not significant*). By contrast, pharmacological inhibition of CaMKK (using STO-609) ($n = 4$, $p < 0.05$) or AMPK (dorsomorphin/compound C) ($n = 4$, $p < 0.05$) prevented ZO-1 relocation induced by the Ca^{2+} switch (Figures 1(c) and 1(d)). These observations suggest that AMPK and CaMKK kinase activity participates in Ca^{2+}-induced ZO-1 deposits in MDCK cells, independently of LKB1.

3.3. The Phosphorylation and Activation of AMPK in MDCK Cells Are Enhanced in the Presence of MSC, Which Is Associated with Faster Relocation of ZO-1 to Cell-Cell Contacts. TJ assembly in epithelial cells may be modulated by nonepithelial cells [43, 44]. As an example, lymphocytes have been shown to boost Ca^{2+}-induced activation of AMPK and accelerate TJ formation [43]. Similarly, we postulated that MSC may participate in TJ formation, and we investigated whether AMPK was implicated in such a process. In comparison to MDCK alone, phosphorylation and activation of AMPK was significantly increased in MDCK/MSC coculture, as demonstrated by mean levels of pAMPK/AMPK ratio ($n = 4$, $p < 0.05$) and pACC ($n = 4$, $p < 0.05$) (Figures 2(a) and 2(b)). Of important note, immunoreactive signals for AMPK activation and pACC were undetectable in MSC alone, which suggest that only MDCK AMPK activation pathway is tested



(a) (b) (c) (d)

Figure 1: Role of the AMPK kinases, LKB1 and CaMKK, in AMPK activation and ZO-1 relocation following a Ca^{2+} switch in MDCK cells. Representative immunoblotting (a) and quantifications (b) of phospho-acetyl-Coa carboxylase (pACC), phospho-AMP-activated protein kinase (pAMPK), and total AMPK (AMPKt) in low Ca^{2+} conditions (S-MEM) and following Ca^{2+} switch using MDCK cells or LKB1-shRNA MDCK cells. Compounds STO-609 and C were used as CaMKK and AMPK inhibitors, respectively. Quantifications of immunoreactive signals were performed by stain-free method after normalization to total protein content of each lane. Quantifications of phospho-ACC, phospho-AMPK, and AMPKt signals following Ca^{2+} switch were calculated and expressed by the ratio to the immunoreactive signal of SMEM condition in each individual experiment (a). For the sake of bar-graph clarity (b), SMEM values of all experiments were normalized to 1 in order to represent mean ratios of phospho-ACC/total protein content and phospho-AMPK/AMPKt in different experimental conditions (b). Data are presented as mean ± SD; ns: not significant, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Representative immunofluorescence (c) and quantifications (d) of ZO-1 deposits at increasing time points following Ca^{2+} switch in similar conditions as in (a) and (b) (scale bar: 16 μm). No statistically significant difference was observed between MDCK and LKB1-shRNA MDCK (ns: not significant). MDCK exposed to compound C ($^{*}p \leq 0.01$) or STO-609 ($^{\S}p \leq 0.01$) showed a significant reduction of ZO-1 relocation in comparison to control MDCK. Data are presented as mean ± SD.

in our model [45] (Supplementary Figure, panel B). In line with these observations, we monitored the time-course of ZO-1 relocation to cell-cell contacts following Ca^{2+} switch in the presence or absence of MSC (Figures 2(c) and 2(d)). After 16-hour deprivation of Ca^{2+}, the length of TJ-associated ZO-1 per cell was 3x higher in MDCK/MSC coculture compared to MDCK cells alone ($n = 4$, $p < 0.05$) (Figures 2(c) and 2(d)). After 1 h of Ca^{2+} switch, ZO-1 relocation to cell-cell contacts was twice higher in MDCK/MSC than in MDCK alone ($n = 4$, $p < 0.05$). Still, at 2 hours post Ca^{2+} switch, the

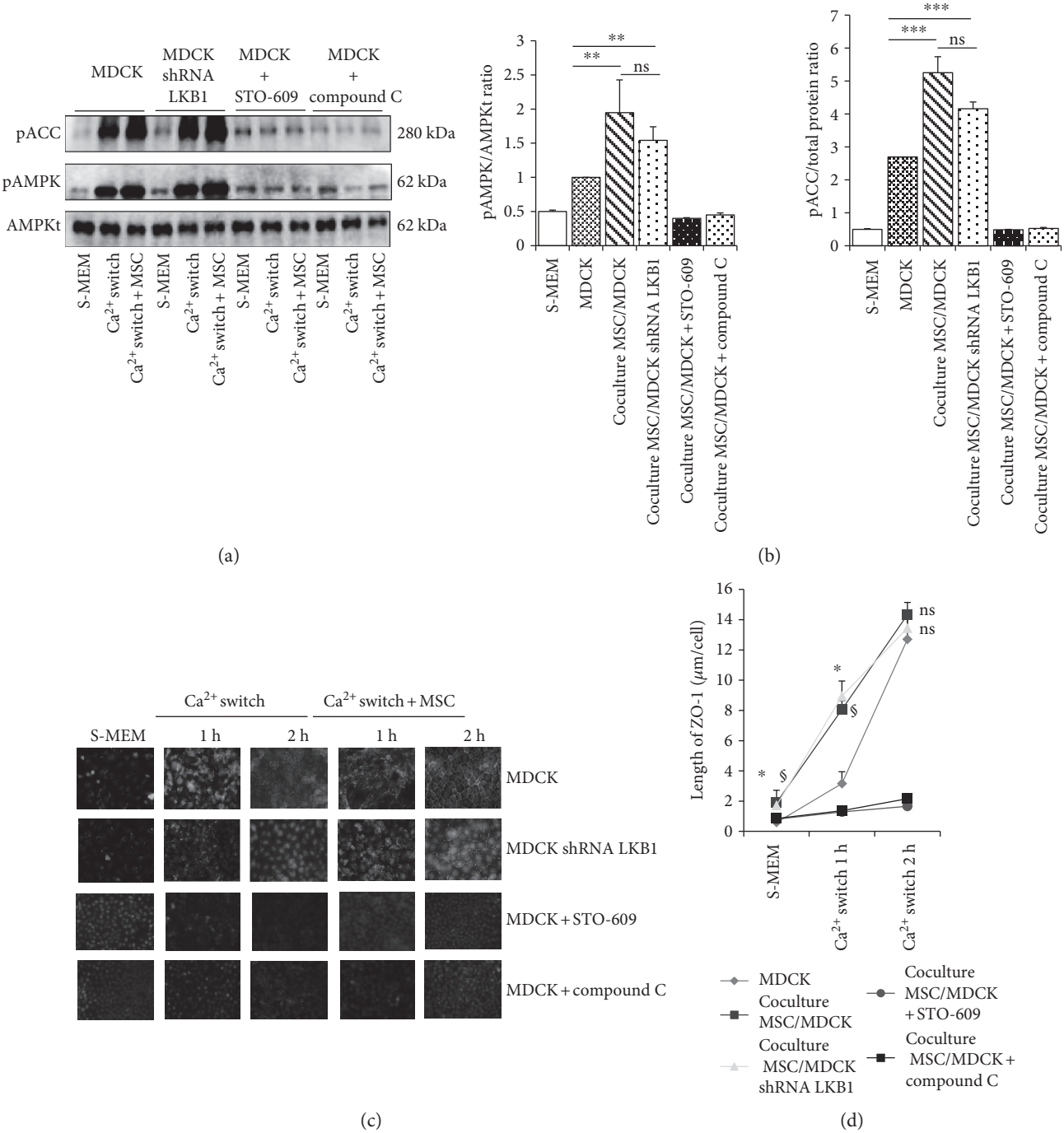


Figure 2: Impact of mesenchymal stromal cells (MSC) on AMPK activation and ZO-1 relocation following a Ca^{2+} switch in MDCK cells. Representative immunoblotting (a) and quantifications (b) of phospho-acetyl-Coa carboxylase (pACC), phospho-AMP-activated protein kinase (pAMPK), and total AMPK (AMPKt) in low Ca^{2+} conditions (S-MEM) and following Ca^{2+} switch using MDCK cells or LKB1-shRNA MDCK cells, with versus without MSC. Compounds STO-609 and C were used as CaMKK and AMPK inhibitors, respectively. Quantifications of immunoreactive signals were performed by stain-free method after normalization to total protein content of each lane. Data are presented as mean ± SD; ns: not significant, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Representative immunofluorescence (c) and quantifications (d) of ZO-1 deposits at increasing time points following Ca^{2+} switch in similar conditions as in (a) and (b) (scale bar: 16 μm). MSC/MDCK (i.e., MDCK ($^{\S}p \leq 0.01$) or MDCK LKB1-shRNA ($^{*}p \leq 0.01$)) cocultures show significantly increased ZO-1 deposits at 1-hour post Ca^{2+} switch in comparison to MDCK alone. At 2 hours post Ca^{2+} switch, no significant (ns) difference in ZO-1 lengths is observed between MDCK and MSC/MDCKs. Data are presented as mean ± SD.

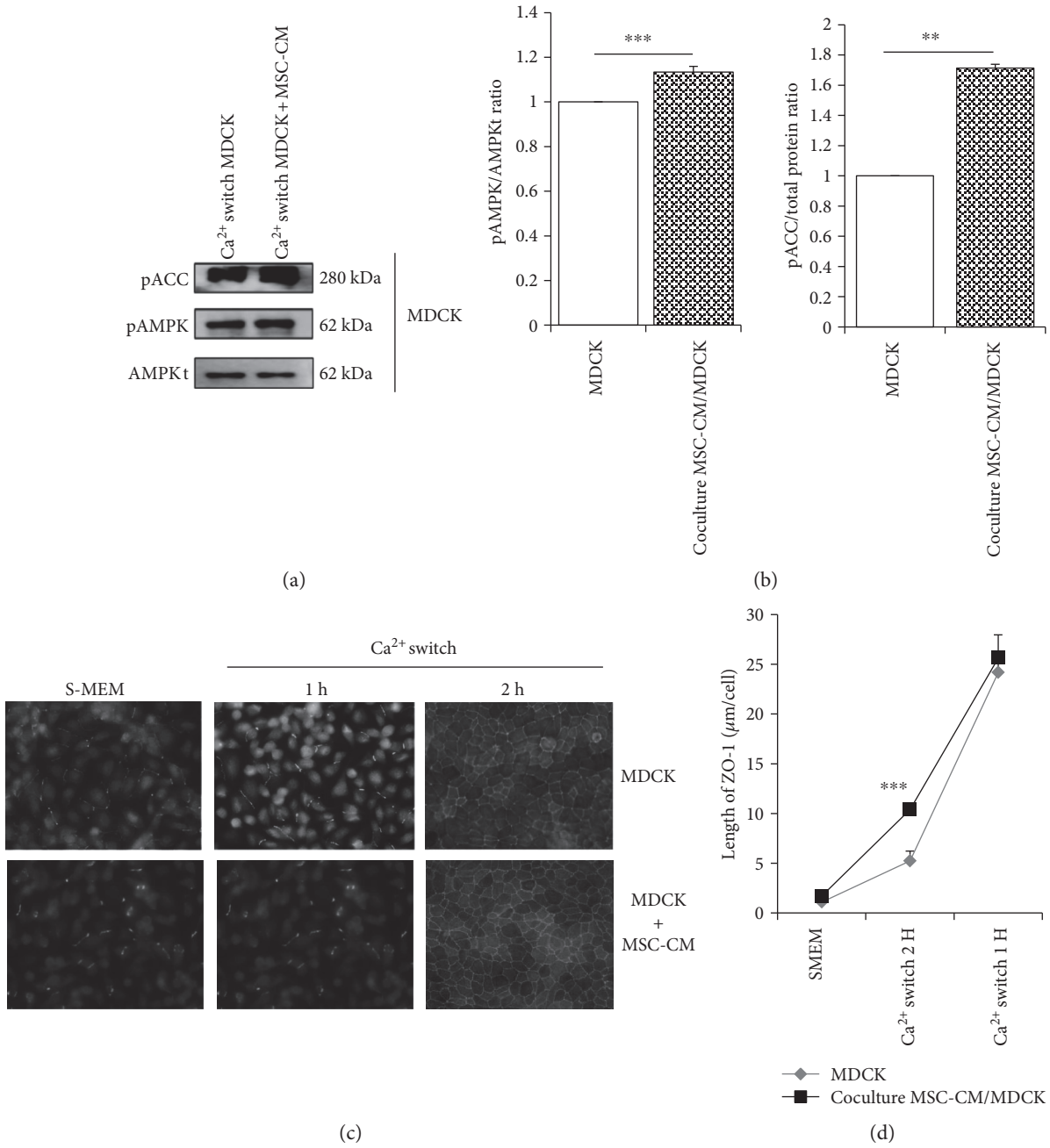


Figure 3: Impact of mesenchymal stromal cell- (MSC-) conditioned medium (CM) on AMPK activation and ZO-1 relocation following a Ca^{2+} switch in MDCK cells. Representative immunoblotting (a) and quantifications (b) of phospho-AMP-activated protein kinase (pAMPK) and total AMPK (AMPKt) in low Ca^{2+} conditions (S-MEM) and following Ca^{2+} switch using MDCK cells exposed to regular versus MSC-preexposed medium. Quantifications of immunoreactive signals were performed by stain-free method after normalization to total protein content of each lane. Representative immunofluorescence (c) and quantifications (d) of ZO-1 deposits at increasing time points following Ca^{2+} switch in similar conditions as in (a) and (b) (scale bar: 16 μm). Data are presented as mean ± SD; $^{***}p \leq 0.001$.

length of membrane-associated ZO-1 per cell was similar in both groups ($n = 4$, ns) (Figures 2(c) and 2(d)). Of note, immunofluorescence signal for ZO-1 was undetectable in MSC cultured alone, which suggests that ZO-1 quantification only reflects ZO-1 deposits in MDCK cells in our model (Supplementary Figure, panel C). As a whole, these results indicate that MSC may accelerate ZO-1 deposition to cell-cell contacts at the time of TJ assembly in MDCK cells.

3.4. MSC-Associated AMPK Activation and ZO-1 Relocation in MDCK Cells Following Ca^{2+} Switch Are Prevented by AMPK and CaMKK Inhibitors Independent of LKB1. Using LKB1-shRNA and Luc-shRNA MDCK cells cocultured with MSC, we assessed the role of LKB1 in MSC-enhanced AMPK activation and ZO-1 relocation. In LKB1-shRNA MDCK cells, mean levels of pAMPK/AMPK ratio ($n = 4$, $p < 0.05$) and pACC ($n = 4$, $p < 0.05$) were, respectively, 1.5-fold increased and 2-fold in the presence versus absence of

MSC, to a similar extent of Luc-shRNA MDCK cells (Figures 2(a) and 2(b)). Furthermore, after a 16-hour deprivation of Ca^{2+}, the length of ZO-1 remaining at TJ sites per cell was 4x higher in MSC cocultured with LKB1-shRNA MDCK in comparison to MDCK alone ($n = 4$, $p < 0.05$) (Figures 2(c) and 2(d)). After 1 h of Ca^{2+} switch, the length of ZO-1 membrane deposits per cell was twice longer in the presence versus absence of MSC ($n = 4$, $p < 0.05$). In strong contrast, incubation of MSC/MDCK or MDCK cells alone with CaMKK (STO-609) or AMPK (dorsomorphin/compound C) inhibitors prevented MSC impact on AMPK activation and ZO-1 distribution, both after Ca^{2+} deprivation and Ca^{2+} switch (Figures 2(a) and 2(d)). These data suggest that, in a coculture system, MSC modulate Ca^{2+}-induced CaMKK-mediated AMPK activation at the time of TJ assembly epithelial cells, independently of LKB1.

3.5. MSC-Conditioned Culture Medium Slightly but Significantly Enhances AMPK Activation and ZO-1 Relocation Following Ca^{2+} Switch in MDCK Cells. Mechanisms of MSC properties involve both direct cell-cell contacts and indirect impacts via paracrine factors [46]. To assess whether the impact of MSC on Ca^{2+}-induced TJ assembly in MDCK cells requires direct cell-cell interactions, we performed the Ca^{2+} switch using an α-MEM culture medium preexposed to MSC for 3 days. Hence, we observed that MSC-conditioned medium slightly (1.14-fold) but significantly ($n = 4$, $p < 0.05$) increased AMPK phosphorylation. Thus, pACC was also increased by 1.7-fold in the coculture ($n = 4$, $p < 0.05$). The relocation of ZO-1 was 1.7-fold accelerated at 1-hour post Ca^{2+} switch in comparison to untreated α-MEM ($n = 4$, $p < 0.05$) (Figures 3(a) and 3(d)). There was no difference between MSC-conditioned and untreated α-MEM at 2 hours post Ca^{2+} switch (Figures 3(b) and 3(c)).

4. Discussion

The present in vitro observations suggest that bone marrow-derived MSC modulate epithelial TJ at the time of their Ca^{2+}-induced assembly. The relocation of TJ-associated adaptor protein, ZO-1, to MDCK cell-cell contacts was indeed significantly accelerated in the presence of MSC. Furthermore, AMPK phosphorylation and activation at the time of Ca^{2+}-induced epithelial TJ assembly were significantly enhanced when MDCK cells were cocultured with MSC, which could be prevented by the pharmacological inhibition of CaMKK. Conversely, the depletion of LKB1 did not significantly influence AMPK phosphorylation following Ca^{2+} switch, with or without MSC coculture.

AMPK activity is modulated by 2 major upstream kinases, that is, LKB1 and CaMKK [18–20, 47]. Still, the respective contribution of each of these AMPK kinases in AMPK activation at the time of a Ca^{2+} switch remains unknown [11, 18]. LKB1 provides a high basal level of AMPK phosphorylation, which is modulated by the binding of AMP to the AMPK γ-subunit. AMP binding to the γ-subunit allosterically activates AMPK, making it more susceptible for phosphorylation of the α-subunit activation loop (at residue Thr172) by LKB1 [48]. Note that AMPK activation associated with Ca^{2+}-induced TJ assembly is independent of changes in AMP/ATP ratio or energy privation [11, 18]. Conversely, CaMKK kinase has been shown to trigger AMPK phosphorylation on Thr172 in response to increased intracellular Ca^{2+} concentration with no necessary changes in AMP or ADP levels [21, 49]. Our present in vitro observations further support a role for CaMKK in the activation of AMPK during a Ca^{2+} switch, independently of LKB1 activity. Hence, the pharmacological inhibition of CaMKK hampered AMPK phosphorylation and ZO-1 relocation when culture conditions were shifted from low to high Ca^{2+} concentration, whereas the inactivation of LKB1 did not significantly influence these processes.

Circulating factors and cells have been shown to modulate TJ formation and maintenance in epithelia [50, 51]. Hence, lymphocytes accelerate TJ assembly in a coculture in vitro model compared to epithelial cells alone [43]. This acceleration was found to be mediated by AMPK, independently of changes in cellular ATP levels. Furthermore, it was found to be activated by the proinflammatory cytokine TNF-alpha [43]. In line with these observations, coculturing endometrial epithelial cells with peripheral blood leukocytes improves both the survival of leukocytes and the epithelial barrier function, as reflected by a 4-fold increase in the transepithelial resistance as compared to epithelial cells alone [44]. In this study, direct cell-cell contacts were required for the beneficial impact of immune cells. In our model, we hypothesized that MSC may also influence TJ of epithelial cells given the previous reports about their tissue-repair properties in various organs and tissues [31, 32, 52, 53]. MSC effects are known to be mediated by both direct cell-cell contacts and paracrine secretion of MVs [54, 55]. Using a classical model of Ca^{2+}-induced TJ assembly [8], we found that the presence of MSC was associated with a significantly faster deposition of ZO-1 to cell-cell contacts. Furthermore, MSC influence was abrogated in case of cell incubation with CaMKK or AMPK inhibitors, suggesting a key role of the AMPK pathway in such a process. Of important note, AMPK-dependent and independent roles of compound C have been reported in a context-dependent manner [56].

These observations could be partly reproduced by incubating epithelial cells with MSC-conditioned medium, which supports a fractional role for MSC-derived MVs in epithelial TJ regulation.

5. Conclusion

As a whole, we report on the role of CaMKK as AMPK kinase at the time of Ca^{2+}-induced assembly of epithelial TJ, independently of LKB1. Moreover, we highlight the impact of MSC in the AMPK-mediated regulation of epithelial TJ, via both direct cell-cell contacts and MSC-derivated particles and MSC-derived MVs. These findings open novel research avenues in the deciphering of MSC repair properties.

Conflicts of Interest

All the authors declared that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

P. Erpicum and F. Jouret are fellows of the Fonds National de la Recherche Scientifique (FNRS) and received support from the University of Liège (Fonds Spéciaux à la Recherche and Fonds Léon Fredericq) and the FNRS (research credits 2013 and 2016).

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Hindawi
Stem Cells International
Volume 2017, Article ID 3282656, 13 pages
https://doi.org/10.1155/2017/3282656

Research Article

Galectin-3 Knockdown Impairs Survival, Migration, and Immunomodulatory Actions of Mesenchymal Stromal Cells in a Mouse Model of Chagas Disease Cardiomyopathy

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Received 10 February 2017; Revised 27 May 2017; Accepted 5 June 2017; Published 10 July 2017

Academic Editor: Luc van der Laan

Therapies based on transplantation of mesenchymal stromal cells (MSC) hold promise for the management of inflammatory disorders. In chronic Chagas disease cardiomyopathy (CCC), caused by chronic infection with *Trypanosoma cruzi*, the exacerbated immune response plays a critical pathophysiological role and can be modulated by MSC. Here, we investigated the role of galectin-3 (Gal-3), a beta-galactoside-binding lectin with several actions on immune responses and repair process, on the immunomodulatory potential of MSC. Gal-3 knockdown in MSC did not affect the immunophenotype or differentiation potential. However, Gal-3 knockdown MSC showed decreased proliferation, survival, and migration. Additionally, when injected intraperitoneally into mice with CCC, Gal-3 knockdown MSC showed impaired migration in vivo. Transplantation of control MSC into mice with CCC caused a suppression of cardiac inflammation and fibrosis, reducing expression levels of CD45, TNFα, IL-1β, IL-6, IFNγ, and type I collagen. In contrast, Gal-3 knockdown MSC were unable to suppress the immune response or collagen synthesis in the hearts of mice with CCC. Finally, infection with *T. cruzi* demonstrated parasite survival in wild-type but not in Gal-3 knockdown MSC. These findings demonstrate that Gal-3 plays a critical role in MSC survival, proliferation, migration, and therapeutic potential in CCC.

1. Introduction

Mesenchymal stromal cells (MSC) are multipotent stem cells with the ability to differentiate into mesoderm-derived cell lineages, such as chondrocytes, osteocytes, and adipocytes [1]. Described by Friedenstein and colleagues in 1970 [2], MSC are plastic-adherent cells presenting fibroblast-like morphology and are characterized by the expression of specific surface markers and demonstration of trilineage differentiation potential. MSC can be easily obtained from different organs and tissues of adult individuals, being presently among the most studied cell types in cell therapies [1].

The potential use of MSC to treat inflammatory and autoimmune disorders is based on several described immunomodulatory actions, including inhibition of the activation of T and B lymphocytes, NK cells, and dendritic cells and stimulation of regulatory T cell differentiation [3]. The anti-inflammatory actions of MSC are well studied and found to be mediated by IL-10, TGF-β, PGE2, HGF, and IDO (for human cells) or iNOS (for mouse cells). Galectin-3 (Gal-3) has also been suggested as a critical mediator of immunomodulatory actions of human MSC [4, 5].

Galectins are a group of galactoside-binding lectins that regulate various biological processes. Gal-3 is present in the

extracellular and intracellular compartments, being involved in cell adhesion, migration, apoptosis, inflammation, and tissue repair [6]. Expression of Gal-3 in fibroblasts is associated with proliferation and synthesis of extracellular matrix components, contributing to scar formation [7–9]. In endothelial precursor cells, Gal-3 promotes proliferation and angiogenesis [10]. While the role of Gal-3 in immune cells has been extensively studied, the actions affected by Gal-3 expression in MSC are not well established. Being highly expressed in inflammatory and fibrogenic microenvironments in tissues [11], Gal-3 is likely to affect MSC biology and response, naturally or in a cell therapy scenario.

Cell therapy has been investigated as a potential alternative treatment for Chagas disease cardiomyopathy, a relevant cause of chronic heart failure in Latin America which results from *Trypanosoma cruzi* infection [12, 13]. An exacerbated immune response directed against the parasite and to host antigens plays a central role in the pathogenesis of CCC, leading to progressive cardiomyocyte loss, fibrosis, arrhythmia, and loss of ventricular function [13]. Previously, it was demonstrated that transplantation of MSC into mice chronically infected with *T. cruzi* caused a reduction of myocarditis and modulation of fibrosis [14–16]. Additionally, we have shown that Gal-3 expression is increased in the hearts of chronic chagasic mice and in human samples [17, 18]. *T. cruzi* infection induces increased Gal-3 expression in different cell types, which favors parasite adhesion, migration, invasion, and reduces antiparasitic immune responses [19–23]. Here, we investigated the potential involvement of Gal-3 in the ability of MSC to migrate and exert immunomodulatory actions in a mouse model of CCC, also investigating potential actions in parasite-host cell interactions.

2. Materials and Methods

2.1. Animal Procedures. Six- to eight-week-old female C57BL/6 mice were used in this study. All animals were raised and maintained at the animal facility of the Center for Biotechnology and Cell Therapy, São Rafael Hospital, in rooms with controlled temperature ($22 \pm 2^{\circ}$C) and humidity ($55 \pm 10\%$), continuous air flow, and 12 h light/12 h dark cycles (6 am–6 pm) and provided with rodent diet and water ad libitum. Mice were handled according to the NIH guidelines for animal experimentation, and the study received prior approval by the animal ethics committee at São Rafael Hospital.

2.2. Isolation and Culture of MSC. Bone marrow cells were obtained from the tibiae and femurs by flushing and were cultured in Dulbecco's modified Eagle's medium (DMEM; ThermoFisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (ThermoFisher Scientific), and 1% penicillin/streptomycin (ThermoFisher Scientific) in a humidified incubator at 37°C with 5% atmospheric CO_2. The medium was changed every 2-3 days and, when the culture reached 90% confluency, the cells were passaged with trypsin-EDTA 0.25% solution (ThermoFisher Scientific).

2.3. Galectin-3 Knockdown. Stable Gal-3 knockdown was achieved by MSC or J774 macrophages by transduction with lentiviral vectors carrying a shRNA sequence targeting *Lgals3* gene or scrambled control (Lgals3_shRNA1 5′-GAT TTCAGGAGAGGGAATGAT-3′; one Lgals3_scrbl_shRNA 5′-AGGTATGAGTCGAGATTGAGA-3′), as previously described [18]. Culture medium was replaced and the cells were cultured for an additional 48 h, being assessed for GFP reporter gene expression by using an inverted fluorescence microscope (Eclipse Ti-E; Nikon, Tokyo, Japan). The cells were expanded and knockdown efficiency for each shRNA was evaluated by confocal microscopy and qPCR analyses.

2.4. Flow Cytometry Analysis. For immunophenotyping, MSC lines were passaged and centrifuged and the pellet was resuspended in PBS. A total of 5×10^5 cells was used for labeling with the following antibodies in the concentration 1/50: Sca1PE-Cy7 (BD Biosciences, San Jose, CA, USA), CD45-PerCP (eBioscience, San Diego, CA, USA), CD44-PE (BD Bioscience), CD90-APC (BD Bioscience), CD34-AlexaFluor647 (BD Bioscience), and control isotypes. Cells were incubated in 100 μL of binding buffer (ThermoFisher Scientific) with annexin-V-FITC and 7-AAD (BD Biosciences, San Jose, CA, USA) for 15 minutes in the dark at RT. After the incubation period, cells were washed twice with PBS, and the data acquisition and analysis were performed using a LRSFortessa flow cytometer (BD Biosciences). At least 10,000 events were acquired and analyzed.

2.5. Trilineage Differentiation Assay. Adipogenic, osteogenic, and chondrogenic differentiations were performed using commercially available kits, following the manufacturer's instructions (ThermoFisher Scientific). For adipogenic differentiation, cells were cultured in 24-well plates in an adipogenic induction medium, StemPro Adipogenesis Differentiation Kit. Lipid inclusions were detected on differentiation day 14, by fixation in 4% paraformaldehyde and staining with Oil red solution. For osteogenic differentiation, the cells were cultured in a specific osteogenic differentiation medium, StemPro Osteogenesis Differentiation Kit. Half the differentiation medium was changed every two days. Calcium-rich matrix deposition was observed by staining with Alizarin red 2%. For chondrogenic differentiation, cells were cultured for 21 days in chondrogenic differentiation medium, StemPro Chondrogenesis Differentiation Kit. Proteoglycan synthesis was evaluated after staining with Alcian Blue solution. The images were captured with an inverted microscope (Eclipse Ti, Nikon, Tokyo, Japan).

2.6. Endothelial Cell Differentiation. Differentiation of MSC to endothelial cells was performed by incubating the cells with EGM-2 medium (Lonza, Basel, Switzerland), as previously described [15]. Endothelial tube formation assay was performed to observe capillary-like 3-D structures by plating the differentiated cells on Matrigel (Corning, Corning, NY, USA). The images were captured using an inverted microscope (Eclipse Ti, Nikon).

2.7. Proliferation Assay. For comparative evaluation of the proliferation rate among different MSC lines, the cells were plated in 96-well plates, at a density of 10^4 cells/well, in a final volume of 200 μL, in triplicate, and cultured in DMEM supplemented with 10% FBS. After 24 h, plates were pulsed with 1 μCi of methyl-^{3}H thymidine (PerkinElmer) for 18 h, and proliferation was assessed by measurement of ^{3}H-thymidine uptake by using a Chameleon β-plate counter (Hydex; Turku, Finland).

2.8. Cell Migration Analyses. MSC were plated in wells of a 24-well plate, at a cell density of 5×10^4 cells/cm^2. Live cell imaging was performed using the Operetta High Content Imaging System (Perkin Elmer) under controlled temperature (37°C) and atmospheric CO_2 (5%). Digital phase-contrast images were acquired at 10x magnification (10x high NA objective) using Operetta's automatic digital phase-contrast algorithm. Image acquisition interval was set to 10 min during 16 h. Images were segmented using the Find Cells building block of the Harmony 3.5.2 software (Perkin Elmer), which provides a dedicated algorithm for segmenting digital phase-contrast images. The segmented cells were subjected to cell tracking using the Track Objects building block. Properties that describe cell migration per time point were calculated, such as displacement. Representative graphs of mean square displacement for each well is shown. For in vitro wound healing assay, MSC were cultured in a 6-well plate until a monolayer was formed. A pipette tip was used to make a scratch along the well, and the area was photographed at time point 0 and after 3 days for gap distance measurements.

2.9. T. cruzi Infection and Cell Transplantation. Trypomastigotes of the myotropic Colombian *T. cruzi* strain were obtained from culture supernatants of infected LLC-MK2 cells, as previously described [24]. Then, C57BL/6 mice were infected by intraperitoneal injection with 1000 *T. cruzi* trypomastigotes in PBS. Infection was confirmed by following parasitemia at different time points after infection.

Six months after infection, mice were randomly assigned into three groups: control MSC, Gal-3 knockdown MSC, or saline. The administration regimen consisted of one weekly intraperitoneal injection of a suspension of 10^6 MSC, or equal volume of saline (100 μL). Mice were euthanized by cervical dislocation under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), on the 7th week after the beginning of the treatment, for analysis.

For in vitro infections, MSC or J774 macrophages were incubated with *T. cruzi* trypomastigotes (MOI = 10) for 24 h. Then, the wells were washed and the medium replaced. Cells were fixed, stained with DAPI for parasite quantification in the Operetta system (PerkinElmer), or submitted for transmission electron microscopy processing and analysis. For ultrastructural analysis, cells were fixed at 4°C for 12 h in a solution of 3% glutaraldehyde (Sigma-Aldrich) in PBS, washed with 0.1 M sodium cacodylate buffer, and postfixed in osmium tetroxide 1% for 30 min. Dehydration was performed by using a graded series of acetone solutions, then the samples were embedded in epoxy resin Polybed812 (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections were obtained using EM UC7 ultramicrotome (Leica, Wetzlar, Germany) and contrasted with uranyl acetate and lead citrate. The sections were analyzed using a transmission electron microscope JEM1230 JEOL (Tokyo, Japan) at 80 kV.

2.10. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-qPCR). Dissociated cells, heart, and spleen samples were subjected to total RNA extraction using TRIzol reagent (Thermo Scientific). The RNA concentration was determined by spectrophotometry. Next, cDNA was synthetized, starting with 1 μg RNA using High Capacity cDNA Reverse Transcription Kit (Thermo Scientific), following the manufacturer's instructions. RT-qPCR assays were performed to detect the expression levels of *Tbet* (Mm_00450960_m1), *Tnf* (Mm_00443258_m1), *Ifng* (Mm_00801778_m1), *Col1a1* (Mm_0801666_g1), *Il1b* (Mm_0043228_m1), *Il6* (Mm_00446190_m1), and *Ptprc* (Mm_01293577_m1). The RT-qPCR amplification mixtures contained 20 ηg template cDNA, Taqman Master Mix (10 μL), and probes in a final volume of 20 μL (all from Thermo Scientific). The reactions were run in duplicate on an ABI7500 Sequence Detection System (Thermo Scientific) under standard thermal cycling conditions. The mean Ct (cycle threshold) values from duplicate measurements were used to calculate the expression of the target gene, with normalization to an internal control—*Gapdh* (mm99999915_g1), using the 2−DCt formula. Experiments with coefficients of variation greater than 5% were excluded. A nontemplate control and non-reverse transcription controls were also included.

2.11. Histology and Morphometric Analyses. Hearts were collected and fixed in 10% buffered formalin. Heart sections were analyzed by light microscopy after paraffin embedding, followed by standard hematoxylin and eosin (H&E), or Sirius red staining. Sirius red-stained sections were entirely digitalized using a confocal microscope A1+ (Nikon). The percentage of fibrosis was determined by analysis of whole sections stained with Sirius red-stained heart sections and semiautomatic morphometric quantification using Image Pro Plus v.7.0. Two blinded investigators performed the analyses.

2.12. Immunofluorescence Analysis. Immunostainings for detection of Gal-3 expression were performed in MSC plated on coverslips. The cells were fixed with paraformaldehyde 4% and incubated overnight at 4°C with the primary antibody goat anti-Gal-3, diluted 1 : 400 (Santa Cruz Biotechnology, Dallas, TX, USA). On the following day, sections were incubated for 1 h with phalloidin conjugated with Alexa Fluor 488 (1 : 200; ThermoFisher Scientific) mixed with the secondary antibody anti-goat IgG Alexa Fluor 568 (1 : 1000; ThermoFisher Scientific). Proliferating cells were evaluated by KI67 staining (anti-Ki67 1 : 1000; ThermoFisher Scientific), followed by anti-rabbit IgG Alexa Fluor 568 (1 : 1000 ThermoFisher Scientific). Dead cells were stained with PI (BD Biosciences). Nuclei were stained with 4,6-diamidino-2-phenylindole (VECTASHIELD

mounting medium with DAPI H-1200; Vector Laboratories, Cambridgeshire, UK). The presence of fluorescent cells was determined by observation using an A1+ confocal microscope (Nikon).

2.13. Statistical Analyses. Continuous variables are presented as means ± SEM. Parametric data were analyzed using Student's unpaired *t*-test, for comparisons between two groups, and 1-way ANOVA, followed by Bonferroni post hoc test for multiple-comparison test, using Prism 6.0 (GraphPad Software). Values of $P < 0.05$ were considered statistically significant.

3. Results

Bone marrow-derived MSC lines were generated by transduction with lentiviral vectors containing the shRNA sequence targeting Gal-3 gene or a nontargeting scrambled sequence. The MSC lines were assessed for Gal-3 expression, in order to confirm the knockdown efficiency by confocal microscopy and qPCR analysis (Figures 1(a), 1(b), and 1(c)). Gal-3 was expressed in the cytoplasm and inside the nuclei of wild-type (Figure 1(a)) and control vector-transduced MSC lines. Cells transduced with the vector containing the shRNA sequence for Gal-3 knockdown showed a marked reduction of Gal-3 expression (Figure 1(b)). This finding was confirmed quantitatively at the mRNA level by RT-qPCR analysis (Figure 1(c)).

MSC lines were then characterized in order to ensure the maintenance of the phenotype and biological properties that define MSC. Immunophenotyping by flow cytometry showed a similar pattern of expression of surface markers by the different cell lines, with a positive staining for the MSC markers CD44, CD90, and Sca-1, and low frequency of cells expressing hematopoietic lineage markers CD45 and CD34 (Figure 1(d)). Next, we assessed the multipotential of MSC by a trilineage differentiation assay in vitro. Upon induction by specific culture media, Gal-3 knockdown and control MSC lines were able to efficiently undergo osteogenic, chondrogenic, and adipogenic differentiation (Figure 2(a)). Additionally, knockdown of Gal-3 in MSC did not interfere with their ability to form capillary-like structures when cultured in endothelium-inducer medium (Figure 2(b)).

Next, MSCs were analyzed regarding proliferation rate and survival. We found that Gal-3 knockdown MSC present decreased proliferation rate when compared to controls, as measured by ^{3}H-thymidine incorporation (Figure 3(a)). Moreover, the number of cells undergoing apoptosis was higher in Gal-3 knockdown MSC, when compared to controls, after incubation with 10 μM H_2O_2 (Figure 3(b)).

Galectin 3 is known to affect cell-extracellular matrix protein binding and cell migration processes [25, 26]. To investigate whether Gal-3 knockdown interferes with migration of MSC, we assessed the in vitro migratory ability of Gal-3 knockdown MSC and control cell lines in vitro. Gal-3 knockdown caused a decreased migration in vitro in a wound healing assay when compared to control MSC (Figures 4(a), 4(b), 4(c), 4(d), and 4(e)). Additionally, by using displacement cell tracking by time-lapse image analysis, we found that Gal-3 knockdown caused a reduction in the mobility of MSC when compared to controls (Figures 4(f), 4(g), and 4(h)).

In order to test if Gal-3 knockdown could impair MSC therapeutic actions in an in vivo setting, the cells were administered i.p. into mice chronically infected with *T. cruzi*, a model of chronic Chagas disease cardiomyopathy (Figure 5(a)). First, the ability of MSC to migrate to the spleen and heart was evaluated shortly after transplantation, by qPCR analysis of GFP mRNA expression. GFP expression was detected in the spleens as early as 30 min after cell transplantation and increased at the 3 h time point. However, significantly lower levels of GFP mRNA expression were detected in the spleens of mice transplanted with Gal-3 knockdown MSC when compared to control MSC, both 30 min and 3 h after the cell administration. Negligible levels of GFP were detected in the hearts at the same time points (Figure 5(b)).

Next, we investigated the long-term effects of cell transplantation in *T. cruzi*-infected mice. Groups of mice received weekly i.p. injections of 10^6 MSC—wild-type or Gal-3 knockdown cell line—for five weeks. A vehicle control group was injected with equal volumes of saline solution (Figure 5(a)). Seven weeks after the beginning of the treatment, mice were euthanized for histological and molecular evaluations.

Histological analysis of heart sections revealed the presence of multifocal inflammatory infiltrates predominantly composed by mononuclear cells in *T. cruzi*-infected mice (Figures 5(c), 5(d), 5(e), and 5(f)). The levels of PTPRC—which encodes for CD45, a pan-leukocyte marker—in heart samples were decreased in the hearts of mice treated with wild-type MSC, but not with Gal-3 knockdown MSC (Figure 5(g)). Similarly, treatment with wild-type MSC, but not with Gal-3 knockdown MSC, reduced the expression of genes in the heart which are associated with inflammation, such as IL-1β, IL-6, and TNFα (Figures 5(h), 5(i), and 5(j)). The levels of expression of IFNγ and T-bet, associated with Th1 responses, were significantly reduced by treatment with wild-type MSC. However, treatment with Gal-3 knockdown MSC did not reduce IFNγ or T-bet expression, when compared to infected controls (Figures 5(k) and 5(l)).

The analysis of Sirius red-stained heart sections of *T. cruzi*-infected mice showed extensive areas of fibrosis (Figures 6(a), 6(b), and 6(c)). While the fibrosis content in the heart was not changed between the groups, collagen synthesis, as measured by collagen type I (*Col1a1*) gene expression, was reduced with wild-type MSC, but not with Gal-3 knockdown MSC (Figures 6(d) and 6(e)).

Since Gal-3 has been previously associated with the process of infection by *T. cruzi* [27], we hypothesized that Gal-3 is required for parasite life cycle also in MSC. In order to test that hypothesis, the MSC lines were submitted to in vitro *T. cruzi* infection. We found that Gal-3 expression is increased 48 and 72 h after *T. cruzi* infection in wild-type MSC (Figure 7(a)). Moreover, both wild-type MSC and Gal-3 knockdown MSC were successfully infected by *T. cruzi*, presenting a similar percentage of infection and number of

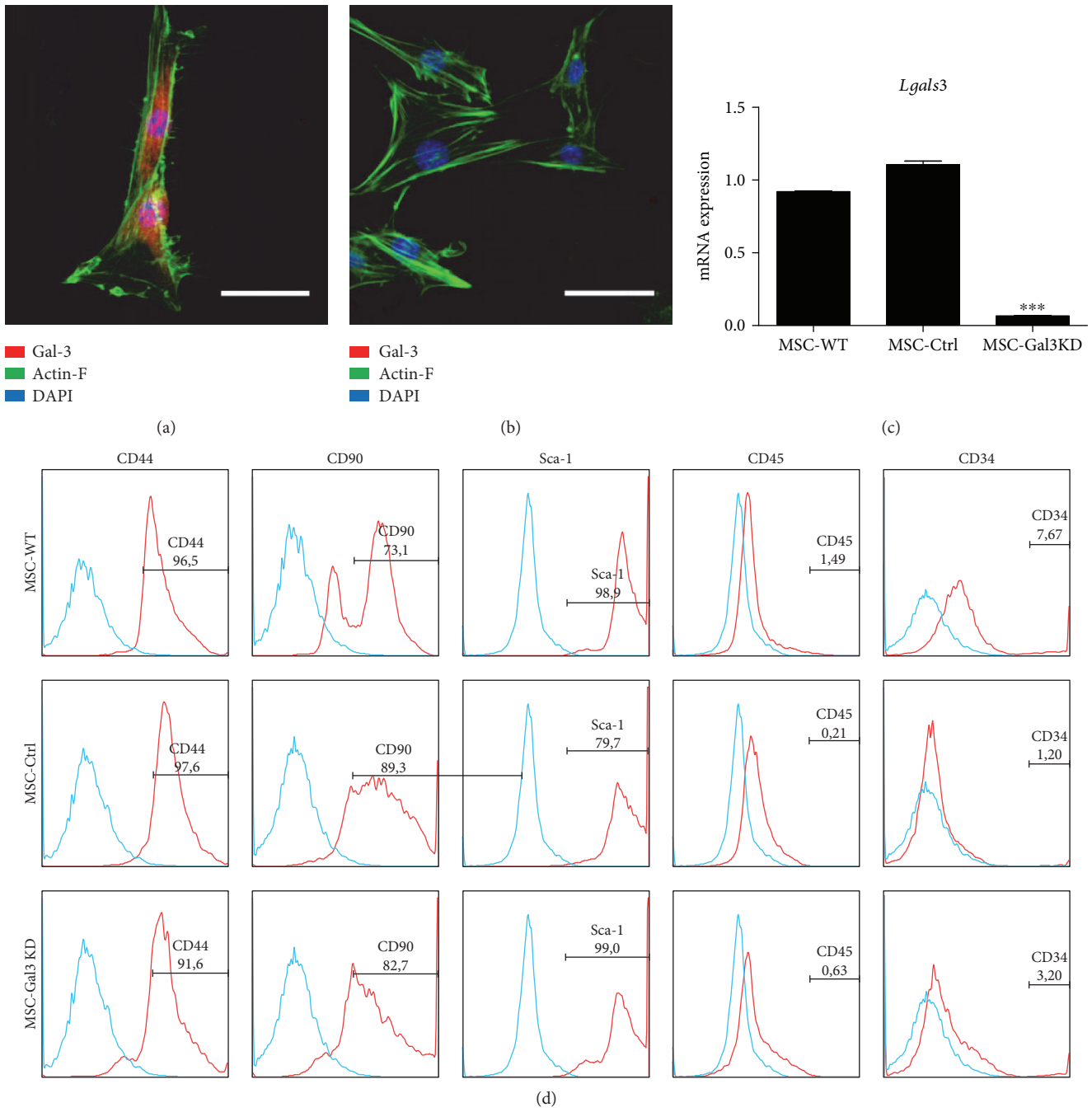

FIGURE 1: Evaluation of Gal-3 knockdown efficiency and characterization of MSC lines. Confocal microscopy images showing Actin-F (green), Gal-3 (red), and nuclei stained with DAPI (blue) in wild-type (a) and Gal-3 knockdown MSC (b). Scale bars = 20 μm. (c) Gene expression analysis of *Lgals3* by qRT-PCR. $^{***}P < 0.001$, compared to the other groups. (d) Histograms demonstrating expression of surface marker characteristics of MSC and low expression of hematopoietic markers, analyzed by flow cytometry.

parasites per cell 24 h after infection. However, at 48 and 72 h after infection, Gal-3 knockdown MSC presented a significantly lower percentage of infection and number of parasites per cell (Figure 7(b)). At the same time points evaluated, no differences were observed regarding the percentage of proliferating ($KI67^+$) and dead cells (PI staining), between the infected MSC lines (data not shown). In order to evaluate if this was a cell-type specific effect, infection was performed also in J774 macrophages. Gal-3 knockdown was also associated with a lower percentage of infection and number of parasites per cell in J774 macrophages (Figures 7(c) and 7(d)).

Ultrastructure analysis by transmission electron microscopy was performed in MSC infected with *T. cruzi*, showing that *T. cruzi* efficiently evade the parasitophorous vacuoles and multiply in the cytosol in wild-type MSC

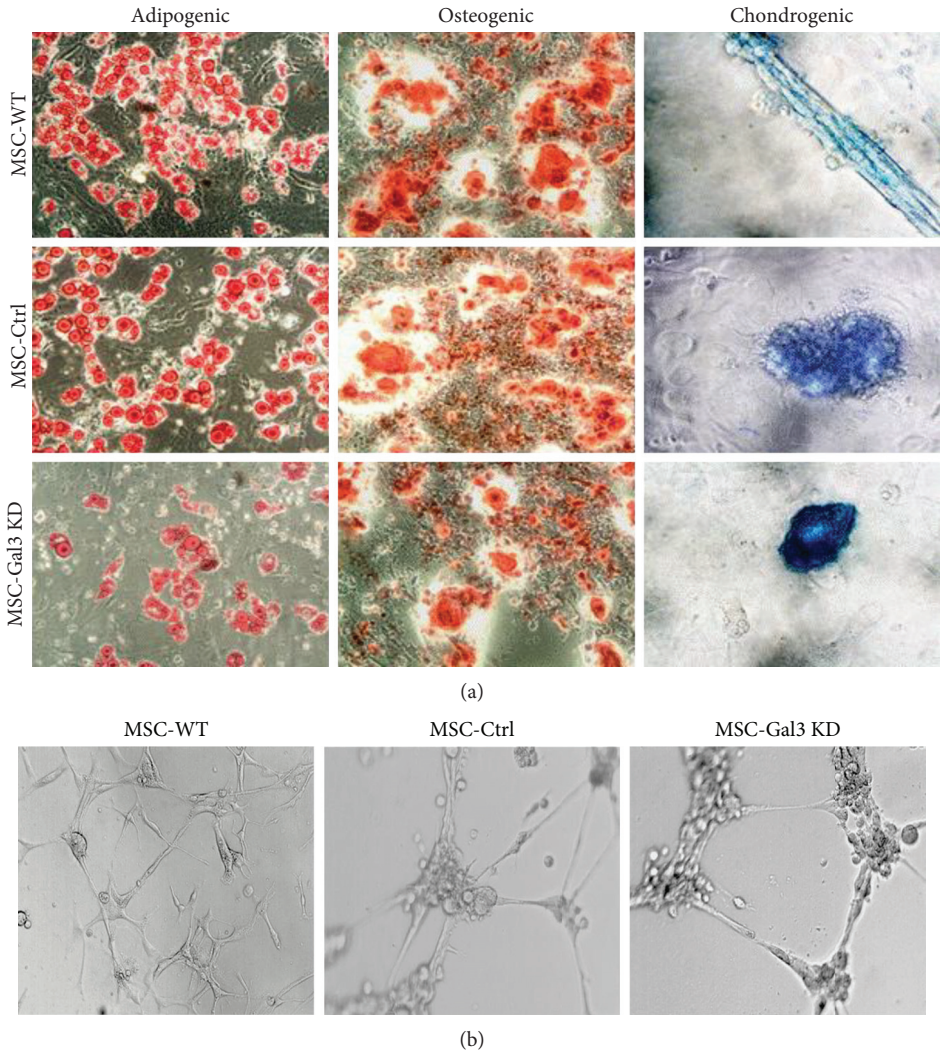

Figure 2: Characterization of MSC lines by differentiation assays. (a) Trilineage differentiation assay performed in MSC lines to generate adipocytes, visualized by Oil red staining, osteocytes, visualized by alizarin red staining, and chondrocytes, visualized by Alcian blue staining, respectively. (b) Angiogenic ability demonstrated by endothelial tube formation assay on Matrigel. MSC-WT = wild-type MSC; MSC-Ctrl = MSC transduced with a nontargeting shRNA vector; MSC-Gal3KD = Gal-3 knockdown MSC. Magnification = 200x.

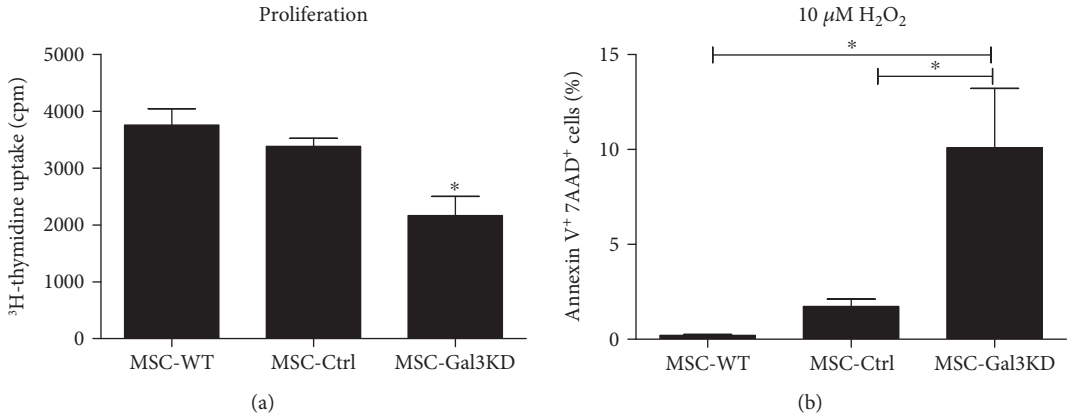

Figure 3: Effects of Gal-3 knockdown on cell proliferation and survival. (a) Proliferation rate in different MSC lines, evaluated by ^{3}H-thymidine incorporation assay. $^*P < 0.05$, compared to the other groups. (b) Apoptosis analysis by Annexin V/7-AAD assay, comparing the rate of cells undergoing apoptosis after incubation with 10 μM H_2O_2. MSC-WT = wild-type MSC; MSC-Ctrl = MSC transduced with a nontargeting shRNA vector; MSC-Gal3KD = Gal-3 knockdown MSC. $^*P < 0.05$.

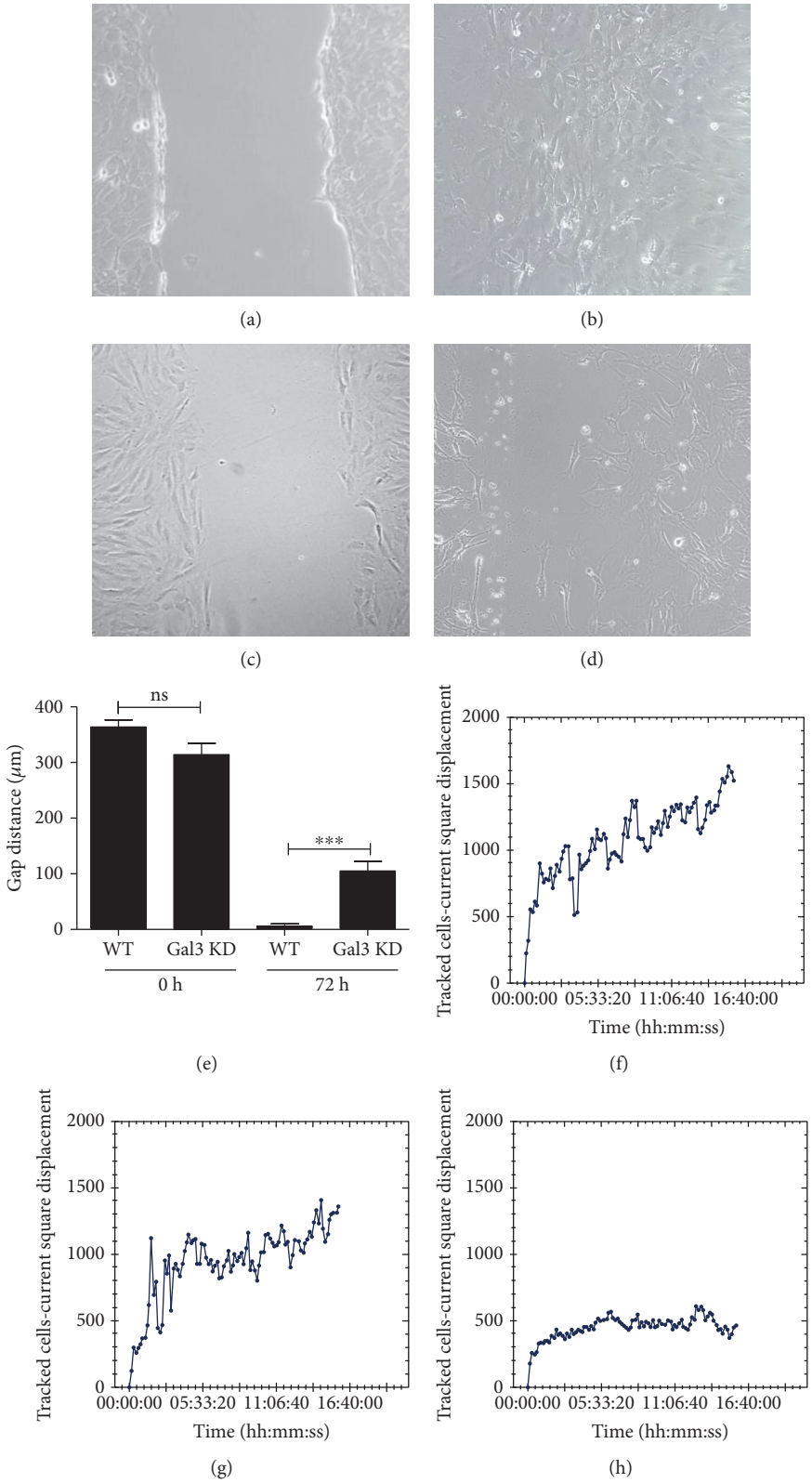

Figure 4: Gal-3 knockdown MSC exhibit defective migration and displacement in vitro. Migration was evaluated by the wound healing assay. Phase contrast representative images showing scratch area at day 0 for wild-type MSC (a) and Gal-3 knockdown MSC (c), and at day 3 for wild-type MSC (b) and Gal-3 knockdown MSC (d). (e) Gap distance was evaluated at 72 h and compared to time 0. $^{***}P < 0.001$. (f–h) Mean square displacements were obtained by individually tracked cells at various time points, from the time of the first position, until the end of the overnight incubation of MSC-WT (d), MSC-Ctrl (e), and MSC-Gal3KD (f). MSC-WT = wild-type MSC; MSC-Ctrl = MSC transduced with a nontargeting shRNA vector; MSC-Gal3KD = Gal-3 knockdown MSC.

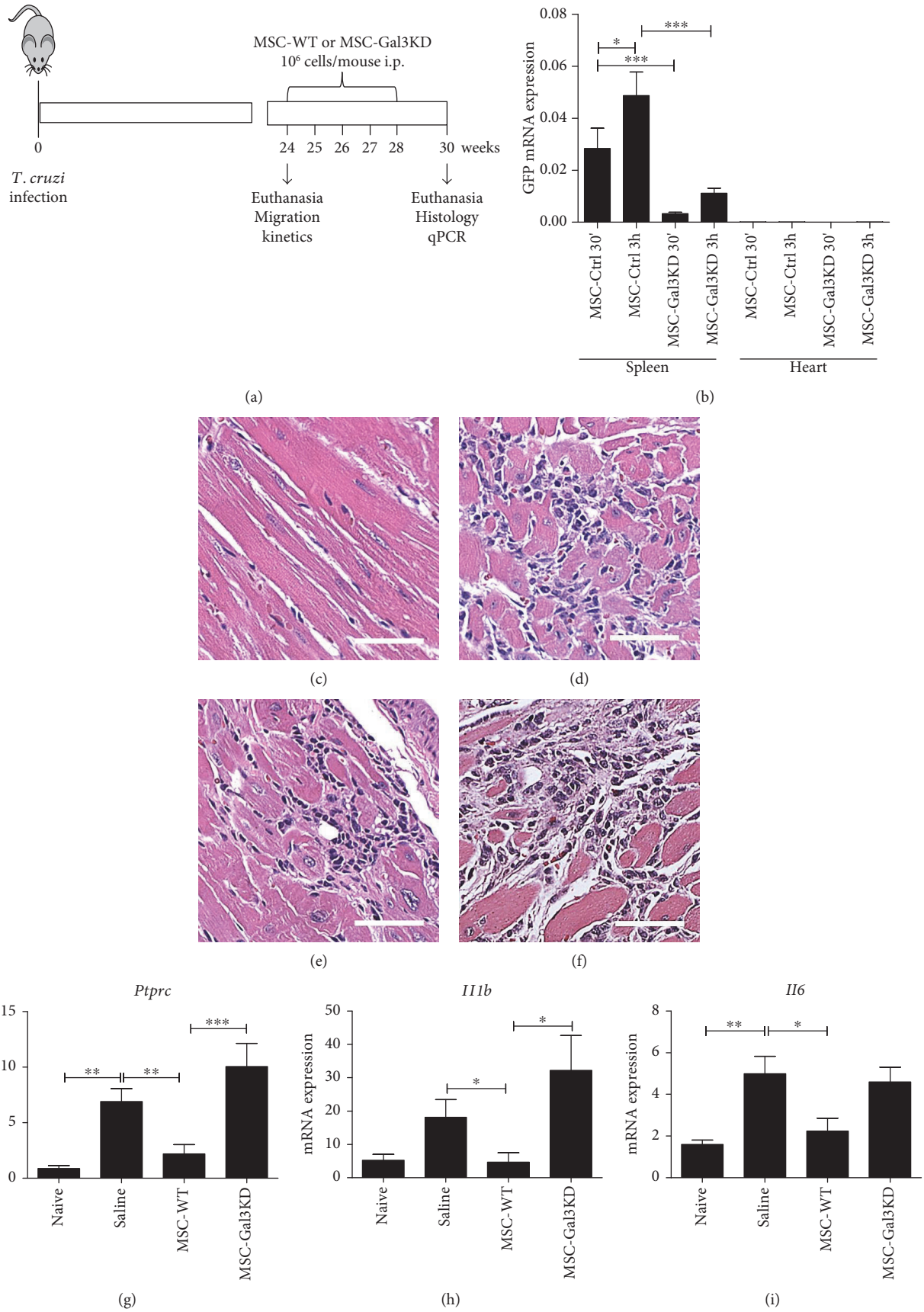

Figure 5: Continued.

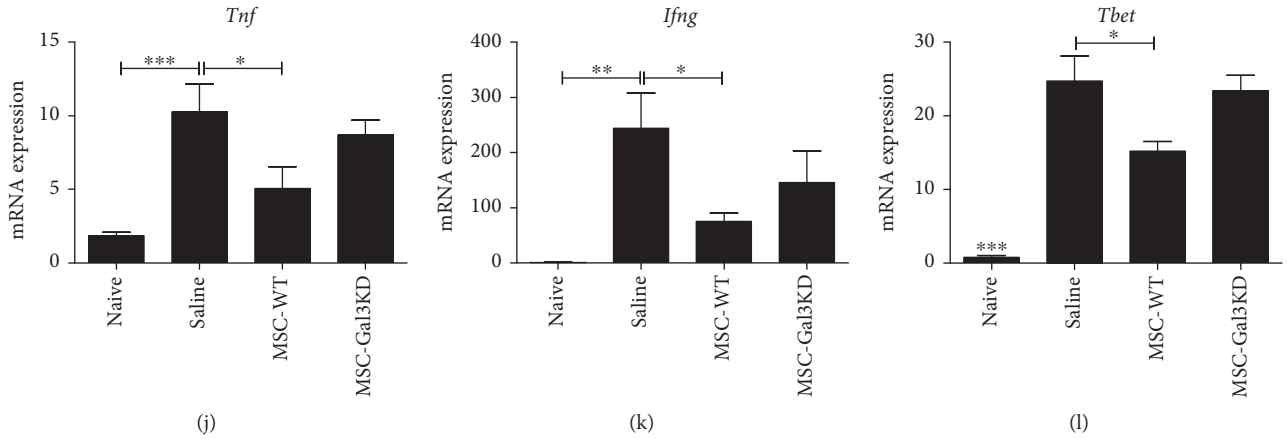

FIGURE 5: Effects of the transplantation of MSC lines in a mouse model of chronic *T. cruzi* infection. (a) Study design. (b) Cell migration and homing to spleens and hearts were evaluated by amplification of GFP mRNA by qRT-PCR. (c–f) Representative images of H&E stained heart sections of naïve mice (c), infected and administered with saline (d), MSC-WT (e), or MSC-Gal3KD (f). Quantification of mRNA expression levels of CD45 coding gene (PTPRC), evaluated qRT-PCR (g). RTqPCR analysis of gene expression in the heart tissue of the cytokines IL1-β (h), IL-6 (i), TNF-α (j), IFN-γ (k), and Th1-associated transcription factor T-bet (l). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. MSC-WT = wild-type MSC; MSC-Ctrl = MSC transduced with a nontargeting shRNA vector; MSC-Gal3KD = Gal-3 knockdown MSC.

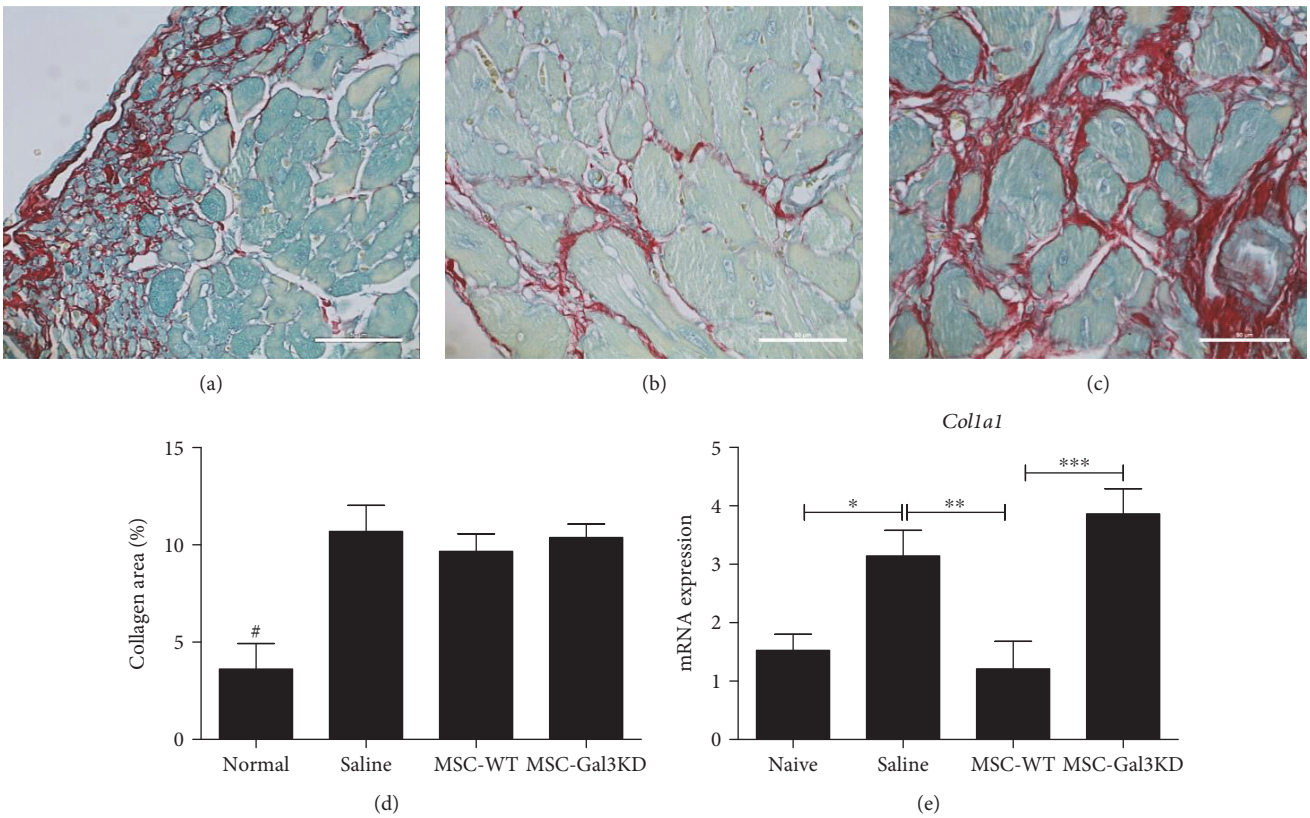

FIGURE 6: Modulation of collagen synthesis in the heart after administration of MSC. Representative images of Sirius red stained heart sections of *T. cruzi* infected mice administered with saline (a), MSC-WT (b), or MSC-Gal3KD (c). (d) Quantification of the collagen-stained area by morphometry. (e) Type I collagen (*Col1a1*) gene expression analysis by qRT-PCR in the heart tissue. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#}P = 0.01$, compared to the other groups.

(Figures 8(a), 8(c), and 8(e)). In contrast, *T. cruzi* remained inside the vacuoles in Gal-3 knockdown MSC and were frequently observed destroyed in the following days (Figures 8(b), 8(d), and 8(f)).

4. Discussion

Gal-3 is a multifunctional lectin with diverse, concordant, and occasionally opposing actions, when expressed by

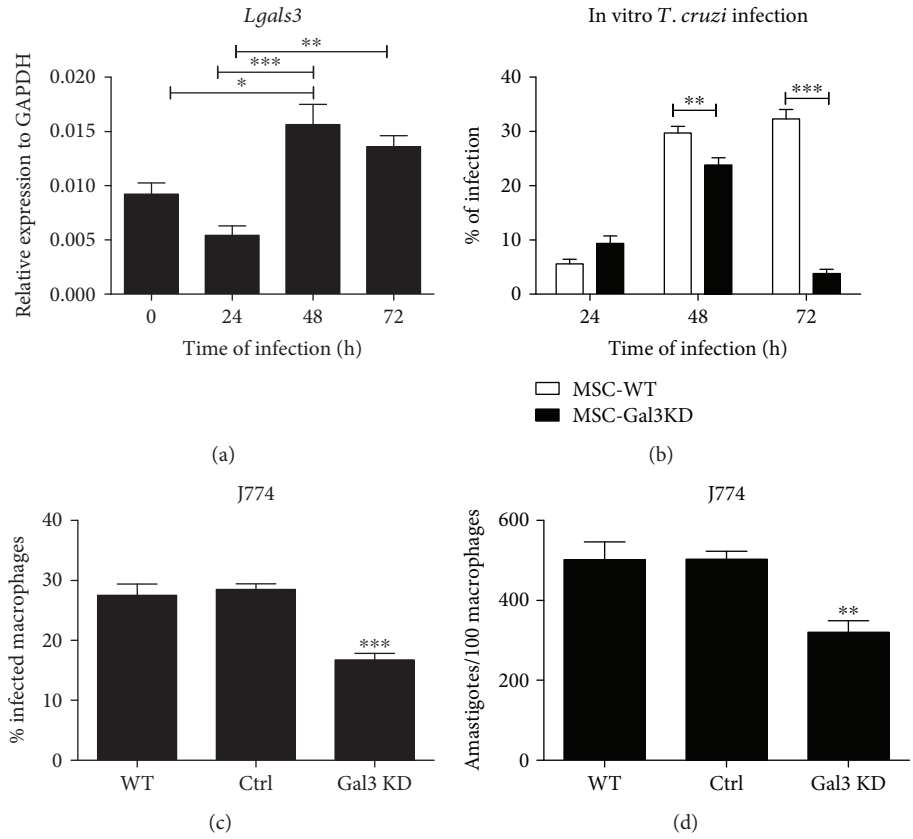

Figure 7: Gal-3 knockdown impairs *T. cruzi* infectivity in vitro. (a) Gene expression of Gal-3 is increased 48 h and 72 h after infection of MSC-WT with *T. cruzi*. (b) Percentage of *T. cruzi* infection in MSC-WT and MSC-Gal3KD lines during the first 72 h. (c) Percentage of infection and (d) number of parasites per cell in J774 macrophages nontransduced (WT) or transduced with control vector (Ctrl) or Gal-3 shRNA (Gal3 KD). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

different cell types and either in extracellular or intracellular compartments [6]. Adhesion, proliferation, and migration are processes that are consistently favored by Gal-3 expression in different cell types, and increased Gal-3 expression play a role in migration and invasion by neoplastic cells [28]. In the present study, we showed that Gal-3 knockdown in MSC was associated with decreased migration and proliferative capacity. These results are in accordance with a recent study using bone marrow-derived MSC obtained from miniature pigs [29]. Here, we showed that Gal-3 plays key roles supporting cell proliferation, migration, and survival, with an impact in therapeutic effects observed after transplantation in a mouse model of chronic Chagas disease cardiomyopathy.

The process of migration and homing of MSC inflammatory sites is still poorly understood and may involve different adhesion molecules, chemokines, and receptors, such as the CXCR4/SDF-1 axis [30]. Gal-3 was recently found to promote migration of MSC through inhibition of RhoA-GTP activity, enhancement of p-AKT (ser473) expression, and regulation of p-Erk1/2 levels [29]. Based on these data and in our findings, it is reasonable to suggest that Gal-3 plays a significant role in MSC migration and homing, which could have several implications in a cell transplantation setting.

In the chronic Chagas disease model, Gal-3 expression by MSC was associated with increased migration from the peritoneal cavity to the spleen. The spleen was also characterized as a reservoir for inflammatory monocytes that emigrate from the subcapsular red pulp and populate inflammatory sites [31]. By reaching the spleen, MSC may be able to exert immunomodulatory actions, with systemic repercussions, as observed previously [32]. Besides regulating lymphocyte populations, MSC also were shown to promote expansion of regulatory populations of monocytes and granulocytes, known as myeloid-derived suppressor cells (MDSC), through HGF secretion [33]. By migrating to heart tissues of *T. cruzi* mice, MDSC were shown to suppress T lymphocytes present in the inflammatory infiltrate [34]. Indeed, i.p.-transplanted MSC showed negligible migration to the heart, but were still able to promote immunomodulation with detectable effects in the heart disease. In mice transplanted with Gal-3 knockdown MSC, however, in which a reduced cell migration to the spleens was observed, inflammation and fibrosis remained at the level of saline controls.

During the chronic phase of Chagas disease cardiomyopathy, different mechanisms are associated with the exacerbated immune response found in the heart, including parasite persistence and autoimmunity [13]. The ability of transplanted MSC to decrease cardiac inflammation in

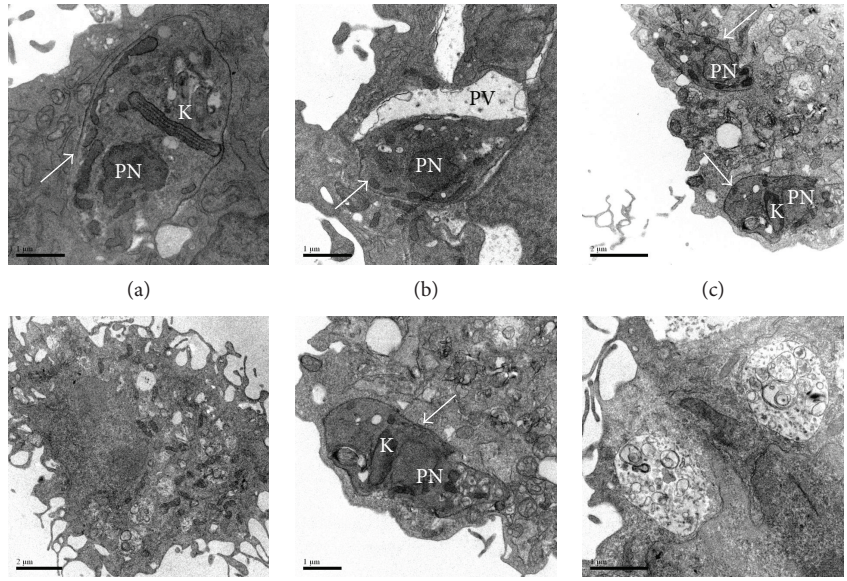

Figure 8: Ultrastructural analysis of *T. cruzi* infected MSC. Viable parasites were found in the cytosol of MSC-WT (a) and inside parasitophorous vacuoles of MSC-Gal3KD (b), 24 h after infection. (c and e) Viable parasites are seen in the cytosol of MSC-WT 72 h after infection. (d and f) Absence of viable parasites and presence of large vacuoles containing degraded material in the MSC-Gal3KD 72 h after infection. White arrows = viable parasites'; K = kynetoplast; PN = parasite nucleus; PV = parasitophorous vacuole.

experimental *T. cruzi* infected mice was shown before in studies that applied systemic and local delivery routes for cell transplantation [14–16, 35]. Here, we demonstrated that transplanted MSC caused downregulation of inflammatory cytokines directly involved in the disease pathogenesis, such as TNF-α and IFN-γ [13]. The immunomodulatory effects observed in the heart tissue were not associated with a high recruitment and homing of MSC to the cardiac tissue, favoring the hypothesis that these cells exert a systemic modulatory action at lymphoid organs such as the spleen, where we did observe migration of MSC. This is corroborated by our finding that Gal-3 knockdown MSC had a significantly lower migration efficiency to the spleen and exerted a lower immunomodulatory action than wild-type MSC.

Regarding parasite persistence, in addition to its presence in the heart, it has been demonstrated that tissues rich in stromal cells, such as the adipose tissue, are reservoirs of *T. cruzi* [13, 36]. A role for MSC as reservoirs for *T. cruzi* in the human disease setting is possible, but has yet to be determined. Here, we show that MSC are efficiently infected by *T. cruzi*, which replicates with time of infection in vitro. Moreover, we found that Gal-3 does not interfere in the invasion process, but it is involved in the further steps of the parasite life cycle. Our data is in accordance with previous work that describes a role for Gal-3 in the step of parasite evasion from the parasitophorous vacuole to the cytosol in macrophages, a critical step for *T. cruzi* life cycle [21].

Gal-3 expression was increased by *T. cruzi* infection in the host cell in the present study, and in previous reports [27]. It has been demonstrated that Gal-3 overexpression induced by infection is important for the parasite cycle, since it can facilitate processes such as adhesion to extracellular matrix, host cell entry, and evasion from parasitophorous vacuole [20, 37]. However, Gal-3 overexpression induced by *T. cruzi* has been also associated with modulation of different aspects of the antiparasitic immune response, by inhibiting plasma cell differentiation and production of immunoglobulins [22] and promoting the release of immature thymocytes [23]. Whether increased Gal-3 expression by MSC contributes or not to the modulation of immune responses in the acute infection by *T. cruzi* is a question that needs further investigation.

5. Conclusion

In conclusion, Gal-3 is involved in the mechanisms of infection by *T. cruzi* and is a mediator of the immunomodulatory actions performed by MSC in a chronic Chagas disease cardiomyopathy model. Gal-3 knockdown decreased MSC survival, migration, and engraftment capabilities, leading to decreased therapeutic effects. Therefore, Gal-3 has the potential to be applied as a predictive biomarker, as part of the quality control on cell preparations to be therapeutically applied, but this merits further investigation.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work was supported by funding from CNPq, FAPESB, and FINEP. The authors would like to thank Dr. Cláudio Pereira Figueira for the technical assistance in the transmission electron microscopy analysis.

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Hindawi
Stem Cells International
Volume 2017, Article ID 8492797, 12 pages
https://doi.org/10.1155/2017/8492797

Research Article

Intrinsic Variability Present in Wharton's Jelly Mesenchymal Stem Cells and T Cell Responses May Impact Cell Therapy

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Received 10 February 2017; Accepted 26 April 2017; Published 5 July 2017

Academic Editor: Marc Hendrik Dahlke

Wharton's jelly mesenchymal stem cells (WJ-MSC) exhibit immunomodulatory effects on T cell response. WJ-MSC are easy to collect, process, and proliferate rapidly in culture, but information on the variability of individual cell samples impacting upon in vitro expansion, immunomodulatory potential, and aging processes is still lacking. We propose to evaluate the immunomodulatory cytokine profile and capacity to inhibit T cell proliferation of WJ-MSC progressing to replicative senescence in order to analyze if expected responses are affected. Our results show that the gene expression of immunomodulatory molecules varied among samples with no specific pattern present. In coculture, all WJ-MSC were capable of inhibiting mitogen-activated $CD3^+$ T cell proliferation, although to different degrees, and each PBMC responded with a different level of inhibition. Thus, we suggest that each WJ-MSC displays unique behavior, differing in patterns of cytokine mRNA expression and immunomodulatory capacity. We believe that variability between samples may play a role in the effectiveness of WJ-MSC employed therapeutically.

1. Introduction

Mesenchymal stem cells (MSC) are multipotent cells with the ability to proliferate, self-renew, and differentiate into different cell types [1, 2]. Minimal criteria from the International Society of Cellular Therapy establish that human MSC must be plastic adherent, exhibit a specific cell-surface expression profile, and differentiate into osteocytes, adipocytes, and chondrocytes in vitro [3, 4]. Bone marrow (BM) is deemed the "gold standard" for MSC derivation and use in clinical trials [5, 6]. However, MSC obtained from the umbilical cord wall, known as Wharton's jelly (WJ-MSC), can easily be isolated and processed. The cells proliferate rapidly in culture with the added value of being of very young age (neonatal), environment protected, and from a source with low ethical concerns.

Along with their capacity of differentiating into mesodermal cells, MSC also display an important feature, namely, their immunomodulatory capacity [7, 8]. Indeed, the current consensus is that the more promising benefits occur in patients presenting acute pathologies with a strong inflammatory component. In these conditions, in response to proinflammatory cytokines, MSC start to produce immunoregulatory factors that downsize the immune response [9]. The effect is most likely due to soluble factors secreted by the MSC [10], such as transforming growth factor β-1 (TGF-β1) [11], interleukin-10 (IL-10) [12], hepatocyte growth factor (HGF) [13], prostaglandin E2 (PGE2) [14], and indoleamine 2,3-dioxygenase- (IDO-) mediated tryptophan depletion [10], which result in crosstalk between MSC and immune cells. The effects of MSC on the cells of the immune system are usually anti-inflammatory and have been observed on many cell types. MSC induce M1 to M2

phenotype macrophage transformation [15], preserve neutrophil viability and function [16], modulate dendritic cell (DC) generation and maturation [17], affect B cell proliferation and maturation [18], block natural killer (NK) cell activation and cytotoxicity [19], inhibit T cell proliferation [20, 21], suppress allogeneic T cell responses [22], and induce proliferation of regulatory T cells (Treg) [23]. Moreover, studies have shown that BM-MSC licensed with IFN-γ (activated to produce anti-inflammatory cytokines) before coculture with T cells have their suppressive capacity increased [24–26].

Due to their immunosuppressive potential and ability to maintain and repair tissues, MSC have recently emerged as a promising tool for cell therapy [27, 28]. However, MSC have a limited lifespan in vitro, exhibiting a progressive reduction in their capacity for self-renewal that usually ends in the irreversible arrest of cell division or replicative senescence [29, 30]. The result of this process is the loss of stem cell functionality, which limits its use for therapeutic purposes. In addition, senescent MSC have been associated with enhanced progression of age-related diseases [31]. Conversely, Helman et al. have shown that p16- (CDKN2A-) expressing beta cells from aged human islets become more efficient when progressing to senescence. The senescent beta cells showed increased insulin secretion in vitro indicating a role in the control of normal cellular function during tissue aging [32].

Intrinsic variability between samples obtained from different WJ-MSC donors has been demonstrated by our group [33]. In order to study the variability of each donor during aging, we evaluated neonatal WJ-MSC, where we show that these cells obtained after cesarean delivery from healthy, end-term pregnant mothers of similar age, isolated, and expanded always with the same techniques exhibit different population doubling rates and reach senescence at different passages. Thus, despite the known immunomodulatory capacity of MSC, it remains to be seen how the different profiles affect the proper function of MSC and how aging impacts upon their immunosuppressive properties and the expected therapeutic efficiency.

Based on these results, the aim of the present study is to investigate if WJ-MSC from different donors, after a varying number of passages in culture, maintain the same immunomodulatory potential and if this differs from donor to donor. We chose to monitor the following molecules known for their immunosuppressive capacity: IL-10, IL-11, IDO, HGF, TGF-β, and LIF, and the following standard cytokines produced by MSC: IL-1α, IL-1β, IL-6, and IL-8 produced by WJ-MSC, the last two considered biomarkers of cell senescence. We also evaluated if WJ-MSC were capable of inhibiting phytohaemagglutinin (PHA) mitogen-stimulated T cells after being licensed by IFN-γ and if these different cytokine profiles impact upon the immunosuppressive potential of WJ-MSC.

2. Materials and Methods

2.1. WJ-MSC Isolation. Umbilical cords (UC) from healthy donors of full-term births were obtained from caesarean section deliveries ($n = 3$). Only healthy WJ-MSC samples seronegative for hepatitis A, B, and C; HIV I and II; HTLV I and II; cytomegalovirus; toxoplasmosis; hemoglobin electrophoresis; Chagas disease; and syphilis were included (ANVISA/RDC No. 153/2004), obeying the evaluation criteria of the Public Umbilical Cord Blood Bank of Hospital Israelita Albert Einstein (HIAE). According to our previously published enzymatic protocol [33], cords were minced to pieces to a maximum size of 5 mm^3 with a scalpel. Enzymatic digestion was achieved by incubating for one hour at 37°C under gentle shaking, in 4% type I collagenase (200 units/mg) dissolved in nonsupplemented DMEM. Next, 50% FBS in DMEM was added and the material was filtered through a 150 μm pore size mesh to remove tissue debris. Cells were centrifuged, the pellet was further washed with supplemented DMEM, and the cells were seeded accordingly.

2.2. Cell Culture. WJ-MSC were cultured at 37°C in DMEM low glucose (Invitrogen, San Diego, CA), supplemented with 10% fetal bovine serum (FBS), L-glutamine 2 mM/mL, and antibiotic-antimycotic solution 100x (100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL de amphotericin B) in a humidified atmosphere with 5% CO_2. WJ-MSC were seeded onto 25 or 75cm^2 tissue flasks (Corning, St. Louis, MO) maintaining a density of 4000 cells/cm^2 in all passages. Standard protocols included flow cytometry for cell-surface markers: CD105, CD73, CD44, CD29, CD166 and CD90, CD14, CD34, CD45, CD117, CD133, CD31, CD106, CD133, HLA-DR and osteogenic and adipogenic differentiation.

2.3. Isolation and Culture of PBMC Obtained from Leukoreduction Chambers. PBMC (peripheral blood mononuclear cells) were isolated from leukoreduction chambers collected, after informed consent, from healthy volunteer platelet donors, regular donors at the Blood Bank of HIAE. Samples were diluted 1 : 5 in PBS, and Ficoll gradient was added. After centrifugation at 970g for 30 minutes, mononuclear cells were collected and washed with 50 mL of PBS. Pellets were dissociated, recombined, and frozen in 1×10^7 cells/mL aliquots. PBMC samples were cultured at 37°C in DMEM low glucose (Invitrogen, San Diego, CA), supplemented with 10% fetal bovine serum (FBS), L-glutamine 2 mM/mL, and antibiotic-antimycotic solution 100x (100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL de amphotericin B) in a humidified atmosphere with 5% CO_2.

2.4. Basal Cytokine Profile Analysis. WJ-MSC were seeded onto 6 well plate (Corning, St. Louis, MO) maintaining a density of 4000 cells/cm^2 in all passages. Samples were collected when cells reach 70% of confluence. Experiments were performed at an early stage (passage 5), at an intermediate stage (passage 15), and in replicative senescence (when cells stop proliferating). mRNA was extracted, for subsequent analysis of immunomodulatory molecules by real-time PCR. The supernatant was collected and frozen immediately in liquid nitrogen for further analysis of secreted cytokines by CBA.

2.5. Coculture Assay. For the coculture assays, 3×10^4 WJ-MSC cells were seeded per well in adherent 48-well plates.

At first, cells were incubated for 24 hours with 50 ng/mL of human recombinant interferon-γ (IFN-γ). Cells were then incubated for 72 h with 3×10^5 PBMC (ratio 1 : 10) in the absence or presence of PHA (Gibco, Carlsbad, CA). WJ-MSC were tested on passages 5 (P5) and 10 (P10). After 3 days, PBMC were collected to evaluate T cell proliferation by flow cytometry and WJ-MSC were collected for RNA extraction, for subsequent analysis of immunoregulatory molecules by real-time PCR. The supernatant was collected and frozen immediately in liquid nitrogen for further analysis of secreted cytokines by CBA.

2.6. Real-Time Quantitative RT-PCR. WJ-MSC total RNA was extracted using an RNeasy Micro Kit (Qiagen, Venlo, NV) according to the manufacturer's instructions, and cDNA was synthesized from 500 ng of total RNA using a SuperScript III Reverse Transcriptase kit (Invitrogen, San Diego, CA) with oligo dT primers. Real-time PCR was performed according to the Taq Man Master Mix assay protocol (Applied Biosystems, Foster City, USA) using the Sequence Detector ABI PRISM 7500 (Applied Biosystems, Foster City, USA). The gene-specific probes were selected using the TaqMan probe database from Life Technologies (Carlsbad, CA). We used a 2-step amplification protocol with a denaturing temperature of 94°C and an annealing-extension temperature of 60°C. HPRT gene expression was used as an internal reference for each individual sample. The relative gene expression was calculated from cycle threshold (Ct) using the $\Delta\Delta$Ct method [34].

2.7. Cell Proliferation Assay. PBMC were labeled with the Click-iT® EdU Pacific Blue™ flow cytometry assay kit (Life Technologies, Carlsbad, CA), a label using a thymidine analogue (EdU) that incorporate in the DNA of proliferating cells. The protocol was performed according to the manufacturer's instructions. In addition, the cells were labeled with anti-CD3, anti-CD4, and anti-CD8 antibodies to evaluate the effect of WJ-MSC on each T cell subpopulation. Data were acquired using BD Fortessa (BD Biosciences, San Jose, CA) flow cytometer and analyzed using the FCS express flow cytometry data analysis (De Novo Software, Glendale, CA), after acquisition of a 10000 events per sample on a LOG fluorescence scale.

2.8. Cytokine Secretion. To evaluate cytokines produced by WJ-MSC, we used the Cytometric Bead Assay (CBA) Flex technique that allows the detection of multiple cytokines simultaneously in the same sample. The measure of IL-1α, IL-1β, IL-6, IL-8, IL-10, and IL-11 was performed according to the manufacturer's instructions.

2.9. Statistical Analysis. All data analyses were performed using SAS (SAS Institute, 2001). Statistically significant differences between the groups were evaluated by linear regression. When the normal distribution criteria were not reached, logarithmic transformations were used. The Tukey-Kramer posttests were used to adjust for multiple comparisons. The model included IFN-γ treatment, PBMC sample, cell passage, and WJ-MSC sample and, when significant, also included their interactions. Values are presented as mean ± SEM of triplicate wells, for each WJ-MSC sample. In all analyses, the level of significance was considered as $p < 0.05$.

3. Results

3.1. WJ-MSC Display Different Basal Patterns of Cytokine mRNA Expression and Protein Secretion. To determine the basal profile of immunomodulatory cytokines produced by WJ-MSC and based on previous literature, we chose the following soluble molecules: IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-11, HGF, LIF, TGF-β, and IDO. mRNA from all genes except IL-10 was detected. IL-1β mRNA was increased at passage 15 but decreased as cells reached senescence. HGF and IL-11 gene expression decreased along passages. Surprisingly, senescence led to diminished production of both IL-6 and IL-8 mRNA. IL-1α, IDO, and LIF gene expression varied among samples with no specific pattern (Figure 1(a)). We observed different profiles in each sample. WJ-MSC1 expressed both proinflammatory (IL-1β, IL-6) and anti-inflammatory molecules (IDO); WJ-MSC2 expressed predominantly IDO while WJ-MSC3 mainly expressed proinflammatory cytokines and did not express IDO. Only IL-6, IL-8, and IL-11 were secreted in detectable amounts. IL-11 secretion decreased after several passages, and IL-6 and IL-8 showed variable results (Figure 1(b)). In some instances, replicate samples were analyzed in parallel yielding similar results (data not shown).

3.2. All WJ-MSC Samples Present the Ability to Inhibit Mitogen-Activated $CD3^+$ T Cell Proliferation but Differed between Samples. To assess the immunomodulatory potential of WJ-MSC, we performed a long established functional assay [35, 36] aiming to measure the suppression of T cell proliferation. Replicate samples from two platelet donor PBMC were stimulated with PHA for 72 hours and tested against 3 different WJ-MSC. This experimental design aimed to, while eliminating responder cell (PBMC) variability, evidence differences between the WJ-MSC donors.

All WJ-MSC samples demonstrated the ability to inhibit mitogen-activated $CD3^+$ T cell proliferation (Figure 2). It is possible to observe that each WJ-MSC sample behaves differently depending on which PBMC was used for the challenge but always showing a higher level of inhibition on PBMC2 ($p < 0.0001$). Compared to WJ-MSC alone or cocultured with PBMC1, IL-10 mRNA was also increased in the presence of PBMC2 (data not shown).

On the other hand, though the suppressive effect occurred on both PBMC, each WJ-MSC exhibited different results. PBMC1 proliferation was less pronounced when cocultured with WJ-MSC1 (39%) while WJ-MSC2 induced greater inhibition on PBMC2 (Figure 2), indicating each WJ-MSC has a unique pattern of response.

3.3. T Cell Responses Also Present Different Profiles according to Each PBMC Sample. We also evaluated the immunomodulatory effect of WJ-MSC on subpopulations of $CD3^+$ $CD4^+$ helper and $CD3^+$ $CD8^+$ cytotoxic T cells. WJ-MSC were able to inhibit proliferation of both $CD3^+$ $CD4^+$ ($p < 0.0001$)

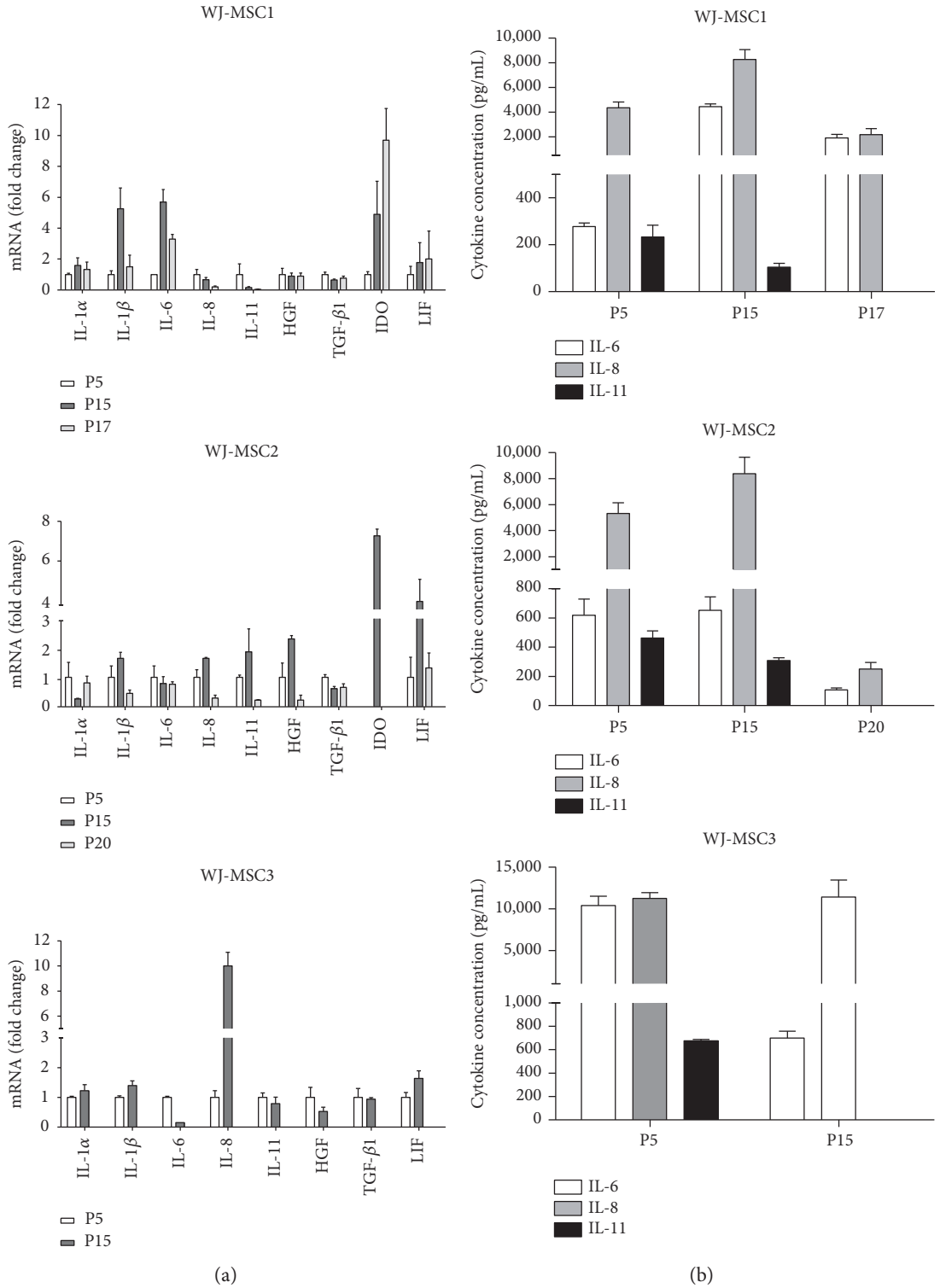

Figure 1: WJ-MSC gene expression of immunomodulatory molecules. Cells were seeded at a density of 4000 cells per cm^2, and samples were collected at 3 different passages. After reaching 80% of confluence, cells were lysed, total RNA extracted, and real-time PCR performed. IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-11, HGF, TGF-β1, IDO, and LIF gene expression and protein secretion from WJ-MSC1, WJ-MSC2, and WJ-MSC3. (a) Gene expression and (b) protein secretion. Gene expression was normalized by housekeeping gene GAPDH and expressed as fold change compared to the control—passage 5. Experiments were performed in triplicate. Results are represented as mean ± SD ($n = 3$).

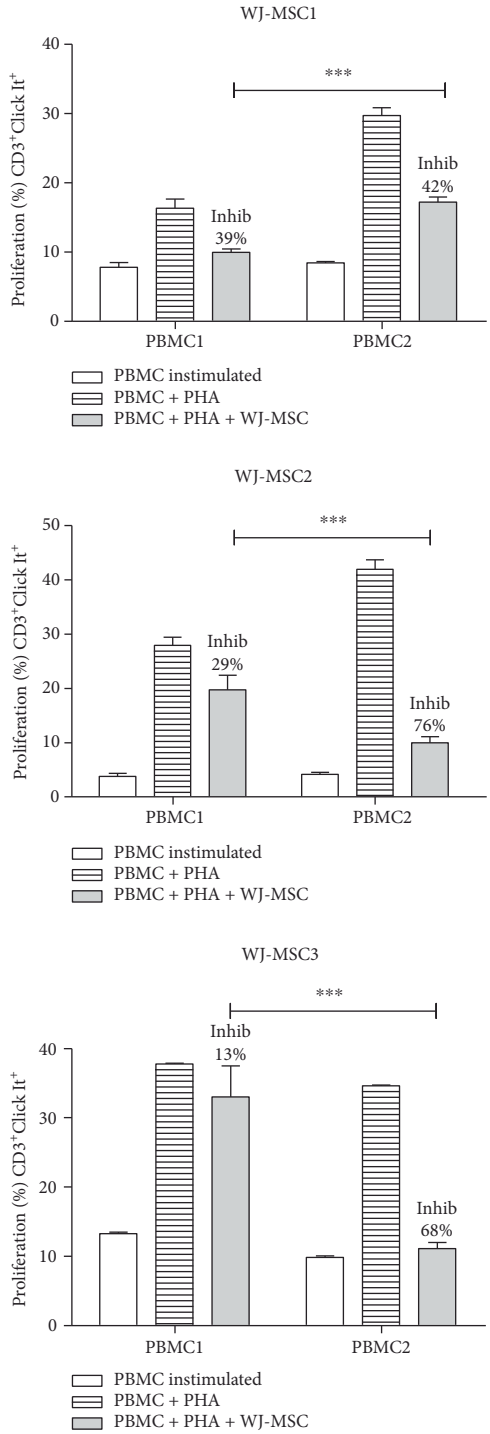

Figure 2: WJ-MSC inhibit $CD3^+$ T cell proliferation. WJ-MSC were seeded, after 24 hours. PBMC were added and stimulated with PHA for 3 days in the presence of MSC; the ratio used was 1 : 10 (WJ-MSC : PBMC). The WJ-MSC passages used in experiments were either P5 or P6. T cells were collected and stained with anti-CD3 antibody and proliferation measured by flow cytometry using the Click It Kit. The percentage of inhibition (Inhib) was calculated using the percentage of proliferation of PBMC + PHA + WJ-MSC compared to the control PBMC + PHA. Experiments were performed in triplicate. Results are represented by mean ± SD. Statistically significant differences are shown as $^{***}p < 0.0001$ $(n = 3)$.

(Figure 3(a)) and $CD3^+$ $CD8^+$ ($p < 0.0001$) (Figure 3(b)) cells when cocultured with PBMC2, regardless of the WJ-MSC sample used. However, in spite of similar CD4 and CD8 T cell counts in both PBMC (data not shown), WJ-MSC did not inhibit $CD4^+$ and $CD8^+$ T cell subpopulations harvested from PBMC1. In other words, this result confirms that the extension of inhibition of the proliferative response differs markedly between PBMC samples.

3.4. Passage Number and Licensing with IFN-γ Does Not Impact upon the Immunomodulatory Capacity, Which Varies according to the WJ-MSC Donor. Studies have shown that IFN-γ is required to activate MSC and to enhance their efficiency when in contact with the immune system [24, 37]. However, it is not clear whether the immunosuppressive capacity is maintained after several passages. These experiments were performed using passages 5 and 10. P10 was chosen because usually, MSC are not completely senescent in P10 and, after 10 passages, it is still possible to obtain sufficient amount of cells to use in cell therapy. In clinical trials, $1\text{-}2 \times 10^6$ cells/kg body weight are injected in patients [38]. Therefore, we investigated whether using different passages and pretreatment with IFN-γ interferes in WJ-MSC function. Three different WJ-MSC at passages 5 and 10 were licensed with IFN-γ for 24 hours. After treatment, WJ-MSC were challenged using further two samples of PBMC, in the presence or absence of PHA for 72 hours. We evaluated the ability of WJ-MSC to suppress $CD3^+$ T cell proliferation (Figure 4), and also of the corresponding $CD4^+$ and $CD8^+$ subpopulations (Supplementary data available online at https://doi.org/10.1155/2017/8492797).

WJ-MSC were able to inhibit PBMC3 and PBMC4 just as observed with the non IFN-γ-treated PMBC2, but at different levels ($p < 0.0001$). WJ-MSC1 showed the best suppressive capacity on both PBMC3 and PBMC4 (90 and 92%), with no significant differences observed between passages or as a result of licensing with IFN-γ. In contrast, the immunosuppressive effect was decreased when WJ-MSC2 and 3 samples were cocultured with PBMC3 and PBMC4 at passage 10. It is possible that in this case, a more advanced senescence stage impacted on their suppressive capacity. As we have shown previously, WJ-MSC do progress to senescence with differing patterns [33].

Finally, inhibition patterns when analyzing the $CD4^+$ T cell subpopulation were similar to the $CD3^+$ data. $CD8^+$ T cells, however, did not show a consistent pattern (Supplementary data).

In summary, our results reinforce our premise that the immunosuppression potential varies according to the WJ-MSC sample, in addition to the passage used or licensing with IFN-γ (Figure 4).

3.5. IL-10 and IDO Expression Are Upregulated in All WJ-MSC Samples Cocultured with PBMC but Vary according to the WJ-MSC Donor. In an attempt to increase our understanding of the mechanisms associated with the immunomodulatory potential, we investigated whether molecules involved in the immunosuppressive effect by WJ-MSC are altered after challenging with allogeneic PBMC. To this

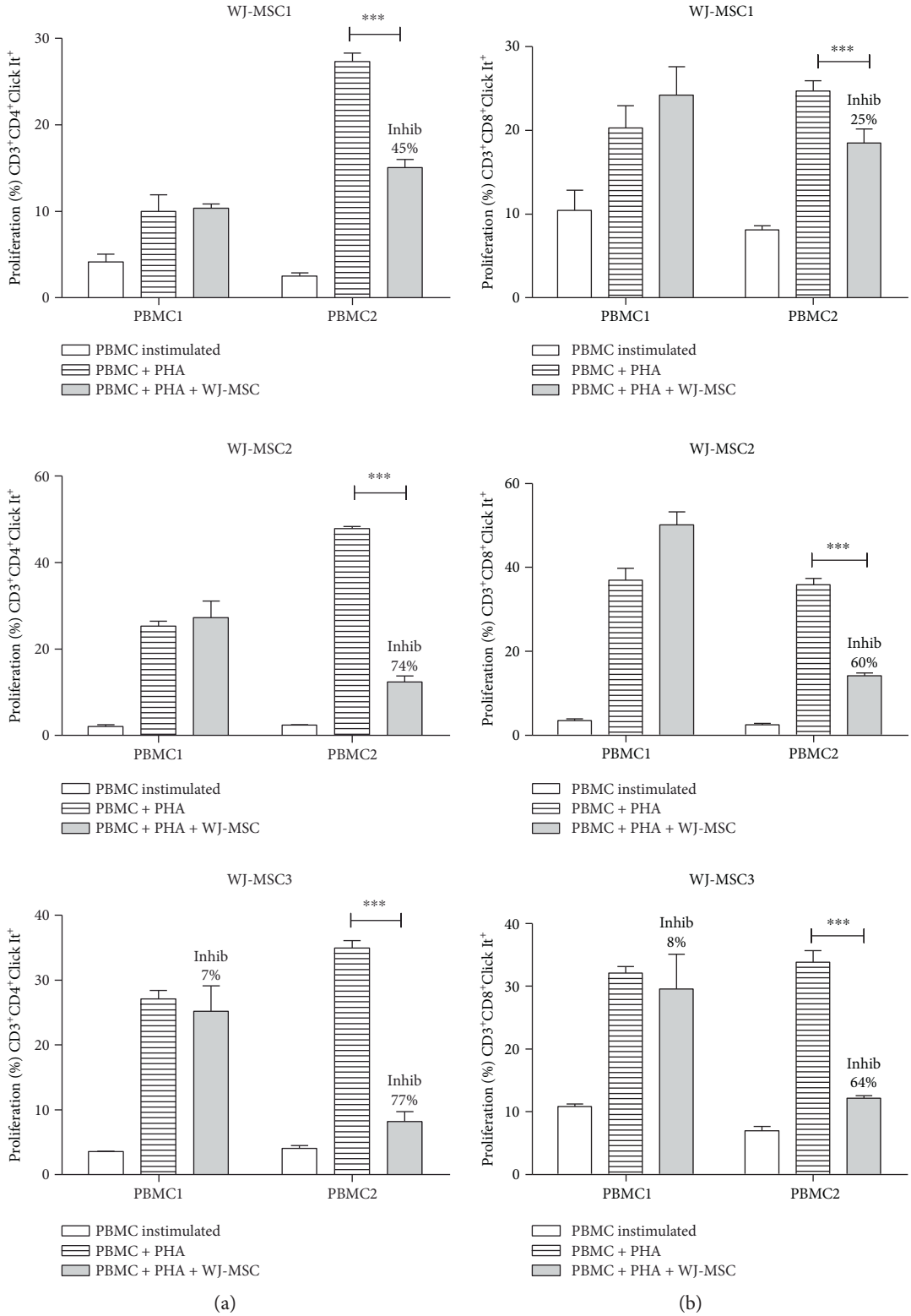

Figure 3: WJ-MSC inhibit $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cell proliferation. WJ-MSC were seeded, after 24 hours. PBMC was added and stimulated with PHA for 3 days in the presence of WJ-MSC; the ratio used was 1 : 10 (WJ-MSC : PBMC). The WJ-MSC passages used in experiments were either P5 or P6. T cells were collected, stained with anti-CD3, anti-CD4, and anti-CD8 antibodies, and proliferation was measured by flow cytometry using the Click It Kit. (a) % proliferation of $CD3^+CD4^+$Click It^+ T cell and (b) % proliferation of $CD3^+CD8^+$Click It^+ T cell. The percentage of inhibition (Inhib) was calculated using the percentage of proliferation of PBMC + PHA + WJ-MSC compared to the control PBMC + PHA. Experiments were performed in triplicate. Results are represented by mean ± SD. Statistically significant differences are shown as $^{***}p < 0.0001$ ($n = 3$).

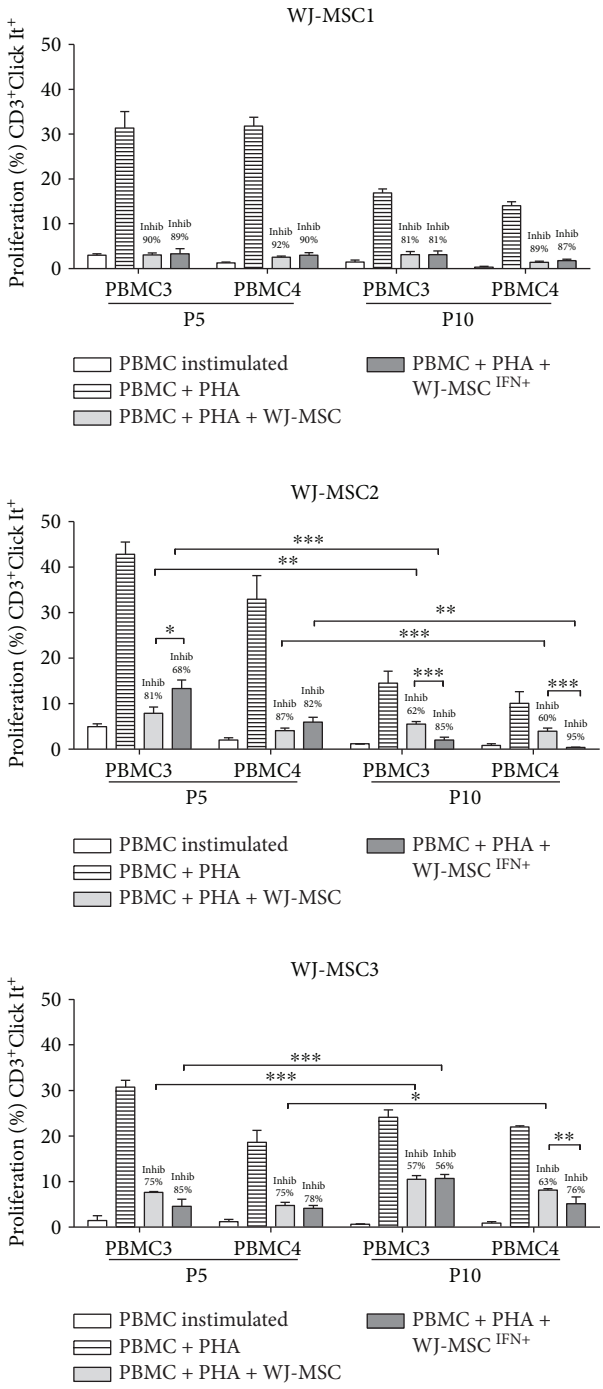

Figure 4: WJ-MSC licensed with IFN-γ inhibit $CD3^+$ T cell proliferation. WJ-MSC were seeded, and IFN-γ was added for 24 hours. PBMC were stimulated with PHA for 3 days in the presence of WJ-MSC; the ratio used was 1 : 10 (WJ-MSC : PBMC). The WJ-MSC passages used in the experiments were P5 and P10. T cells were collected, stained with anti-CD3, anti-CD4, and anti-CD8 antibodies, and proliferation was measured by flow cytometry using the Click It Kit. The percentage of inhibition (Inhib) was calculated using the percentage of proliferation of PBMC + PHA + WJ-MSC compared to the control PBMC + PHA. Experiments were performed in triplicate. Results are represented by mean ± SD. Statistically significant differences are shown as $^*p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0001$ ($n = 3$).

end, we carried out gene expression analysis. IL-1α, IL-1β, IL-6, IL-8, IL-11, TGF-β, LIF, and HGF gene expression did not exhibit any pattern shared between WJ-MSC samples (detailed results are available upon request).

Absent or very low in the o basal profile, IDO and IL-10 were increased when WJ-MSC were cocultured with PBMC compared to WJ-MSC alone. Upon coculture, all WJ-MSC increased IDO gene expression, albeit each cell is with a unique profile. Treatment with IFN-γ increased IDO mRNA expression both in the presence or absence of coculture ($p < 0.0001$), in both passages 5 and 10 (Figure 5(a)).

IL-10 response exhibited a different pattern. There was no IL-10 expression in WJ-MSC alone even after licensing with IFN-γ. However, after coculture, all three WJ-MSC started to express IL-10 at different levels (Figure 5(b)). Cells in contact with PBMC4 consistently expressed more IL-10 than the cells cocultured with PBMC3 ($p < 0.0001$). No significant correlation of IDO and IL-10 mRNA expression with inhibition of T cell proliferation could be found.

4. Discussion

Understanding the changes occurring in the WJ-MSC immunomodulatory properties during the progress to senescence is an important step to achieve improved application of these cells in therapeutic approaches. The present study investigated if intrinsic variability of WJ-MSC impacts upon their immunomodulatory potential. We evaluated the effects of cell aging and IFN-γ licensing on WJ-MSC function, as measured by T cell proliferation and production of molecules involved in immunosuppression.

Several studies have shown that MSC may exhibit immunosuppressive or proinflammatory profiles [39–42]. It has been reported that MSC secrete cytokines either spontaneously or after induction by other cytokines, and it is believed that the effects are determined by the local microenvironment condition. Waterman et al. showed that in an inflammatory environment with high levels of TNF-α and IFN-γ, MSC may exhibit an anti-inflammatory profile secreting PGE2, IDO, TGF-β, and HGF that suppress T cell proliferation and induce activation of Treg cells [39]. When MSC are in a noninflammatory environment with low levels of TNF-α and IFN-γ, they may acquire a proinflammatory phenotype, secreting chemokines that recruit T cells to the site of inflammation and increase the immune responses [40, 41]. The balance between these opposite profiles may be important to promote homeostasis preventing tissue damage and supporting tissue regeneration and repair.

In accordance with our previous study that showed intrinsic variability in expansion capacity and cell longevity [33], our results show that each WJ-MSC sample exhibits a unique basal profile of immunomodulatory molecules. WJ-MSC1 showed increase in both proinflammatory and immunosuppressive molecules, WJ-MSC2 exhibited a greater expression of immunosuppressive molecules, and WJ-MSC3 showed enhanced expression of genes related to a proinflammatory activity. After IFN-γ stimulation, the cytokine pattern was also quite variable in WJ-MSC samples at early and later passages (Supplementary data). Of note, all samples

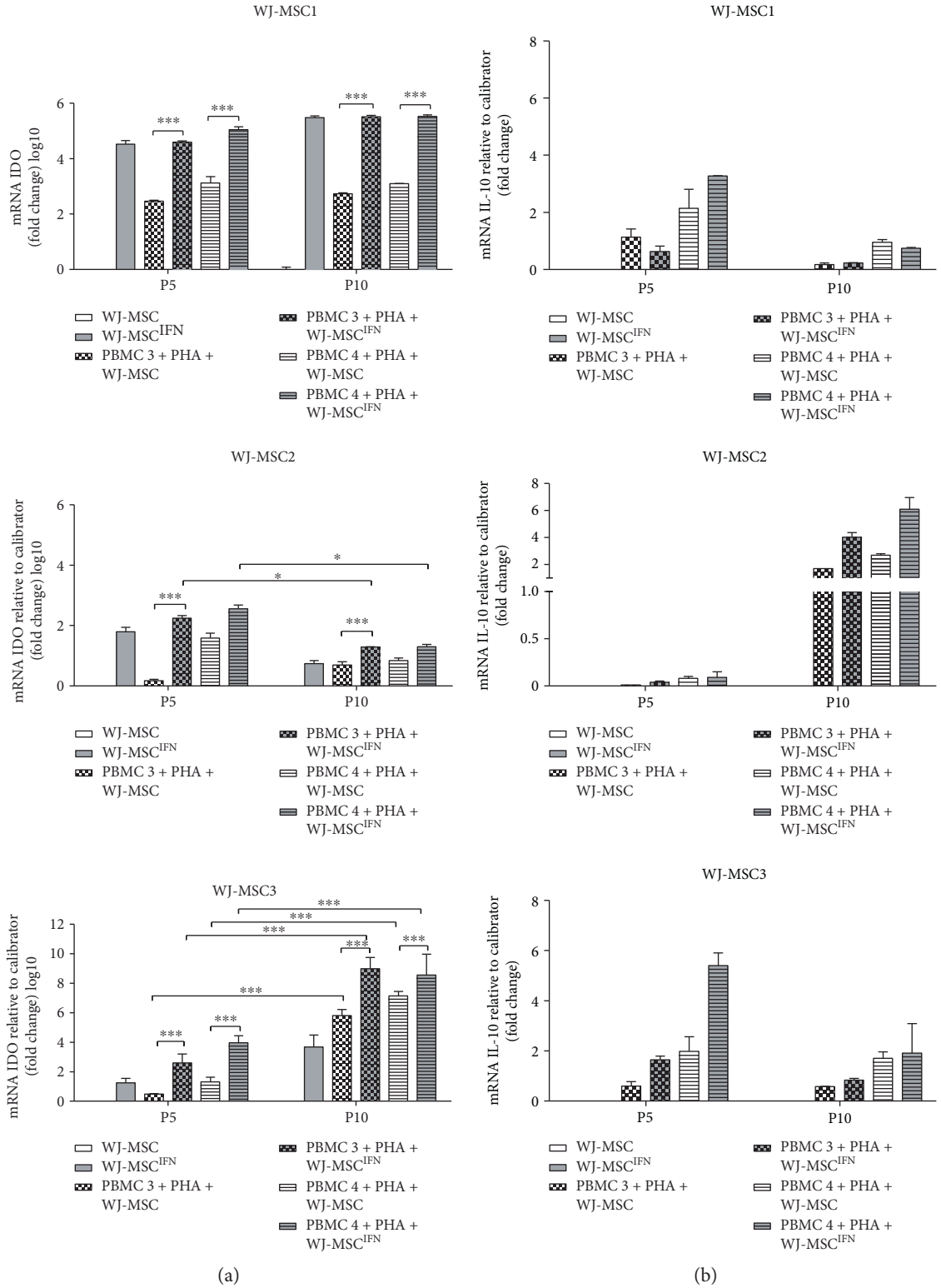

Figure 5: Gene expression of IL-10 and IDO is increased in WJ-MSC after coculture with PBMC. WJ-MSC were seeded, after 24 hours. PBMC was added and stimulated with PHA for 3 days in the presence of MSC; the ratio used was 1 : 10 (MSC : PBMC). The WJ-MSC passages used in the experiments were P5 and P10. After 3 days, WJ-MSC were lysed, total RNA extracted, and real-time PCR performed. (a) IDO and (b) IL-10 gene expression of WJ-MSC1, WJ-MSC2, and WJ-MSC3. Gene expression was normalized by GAPDH and expressed as fold change compared to the control WJ-MSC. Experiments were performed in triplicate. Results are represented as mean ± SD. Statistically significant differences are shown as $^{*}p < 0.05$, $^{***}p < 0.0001$ $(n = 3)$.

were cultivated following the same standard protocols and using uniform reagents (including donor PBMC). In addition, from the same neonatal source, donor age previously identified as a source of variability is eliminated [43, 44].

To confirm if the different profiles impact on the immunomodulatory capacity of WJ-MSC, we performed functional assays, using coculture with PBMC to measure T cell proliferation and gene expression. Our data are in accordance with other authors [21, 22, 45] that have shown that MSC in coculture, in our case obtained from umbilical cord wall, are able to inhibit $CD3^+$ T cell proliferation. Najar et al. compared the immunomodulatory capacity of MSC obtained from adipose tissue, bone marrow, and Wharton's Jelly. The MSC immunosuppressive effect was not restricted to a specific T cell population and the different MSC which equally inhibited $CD4^+$ and $CD8^+$ T cell proliferation [46]. However, our results show there was a different pattern of PBMC responses when $CD3^+$, $CD4^+$, and $CD8^+$ T cell subpopulations were analyzed. Surprisingly, in spite of a clear, although smaller effect on $CD3^+$ T cells by all three WJ-MSC samples, no significant inhibition of $CD4^+$ and $CD8^+$ T proliferation was observed with PBMC1. Our findings suggest that the efficacy of WJ-MSC immunomodulatory capacity is also dependent on the recipient cell profile. Other studies have shown that human BM-MSC not only inhibit T cell proliferation but also impact upon cytokine production by $CD4^+$ and $CD8^+$ T cells [47, 48], but in our study, we measured only basal cytokine production.

Senescence is an important biological process that happens in many cell types, including MSC, and consists of irreversible cell growth arrest [49]. Nevertheless, a clear understanding how this process can affect WJ-MSC immunomodulatory potential and whether IFN-γ can enhance the suppressor effect of aging WJ-MSC is still lacking. In order to study the relationship between aging and the immunoregulatory activity of WJ-MSC, we focused our experiments on IFN-γ licensing to evaluate the effect of aging on WJ-MSC. A previous study has shown that radiation-induced senescent human BM-MSC lose their protective immunoregulatory function in a mouse model of sepsis, though a partial capacity to inhibit T cell proliferation and the ability to regulate the inflammatory response on macrophages in vitro were retained [50]. Our data indicate that the immunomodulatory potential is maintained after 10 passages in vitro. However, the activity by WJ-MSC2 and WJ-MSC3 was reduced at passage 10 when compared with passage 5. Interestingly, in some experiments, IFN-γ licensing enhanced WJ-MSC suppressor activity at the later passage (see WJ-MSC2 in Figure 4).

Recent reports also studied the immunomodulatory potential of MSC after proinflammatory stimuli. Szabo et al. showed that mouse BM-MSC exhibit differences between the clones in their ability to inhibit T cell proliferation, but after MSC pretreatment with proinflammatory cytokines, these differences disappear [51]. Fuenzalida et al. demonstrated that pretreatment with a TLR3 ligand (poly I:C) enhances UC-MSC immunosuppressive capacity [52]. Unlike these studies, we observed that the variability is maintained after pretreatment with IFN-γ and that this proinflammatory stimulus does not seem to potentiate the inhibitory capacity of WJ-MSC upon T cells. However, it should be taken into account that Szabo et al. performed their study on murine BM-MSC and that Fuenzalida et al. showed no differences between UC-MSC cocultured with PBMC in the presence/absence of LPS (TLR4 ligand), also a proinflammatory stimulus. We have not tested TLR ligands on our human samples but chose instead to use the standard activation by IFN-γ. It remains to be seen if results would have been comparable.

Another study published in 2013 [53] comparing samples from different MSC sources showed that in a same MSC sample, there are 2 different MSC subpopulations, which are $CD106^+$ and $CD106^-$. CD106 is an adhesion molecule involved in cell-cell contact and, hence, plays an important role in MSC-mediated immunosuppression [54]. MSC expressing CD106 on their surface have greater expression of IL-1α, IL-1β, COX-2, IL-6, IL-8, IDO, and PGE2 and higher suppressor capacity when compared to $CD106^-$ cells. When we evaluated the expression of IDO in WJ-MSC samples after coculture with PBMC, we observed that WJ-MSC1 and WJ-MSC3 expressed more IDO than WJ-MSC2. Moreover, after treatment with IFN-γ, a significant decrease in immunosuppressive capacity of WJ-MSC2 cocultured with PBMC3 at P5 was observed. We also evaluated CD106 expression; our data demonstrated increased basal expression of CD106 in 2 samples, WJ-MSC1 (13.7% $CD106^+$) and WJ-MSC3 (4.9% $CD106^+$), but lower in WJ-MSC2 (1.6% $CD106^+$) at P5 (data not shown). This could help us understand the heterogeneity in our samples since there was a varied expression of CD106 in our WJ-MSC samples. Thus, our results suggest that indeed the increase in CD106 expression in WJ-MSC seems to be related to the expression of IDO immunomodulatory gene.

Previous studies have found that WJ-MSC in basal conditions do not express IL-10 [42, 55, 56]. In line with those reports, we found that WJ-MSC alone or stimulated with IFN-γ do not produce IL-10. However, WJ-MSC start to express IL-10 after being cocultured with PBMC. Even though MSC are plastic adherent and T cells are suspended in the supernatant, after coculture with contact, all precautions were taken trying to avoid cross-contamination during WJ-MSC total RNA extraction. We believe that even if any T cell had remained, it would be in a very small amount compared to the amount of WJ-MSC in the culture and would not alter results significantly.

Taken together, our findings though consistent with previous studies [57] show that MSC inhibit T cell proliferation but that this capacity may vary from cell to cell and also as cells age.

5. Conclusion

Our results show that neonatal, environment-protected WJ-MSC display different basal patterns of cytokine mRNA expression and protein secretion. WJ-MSC are able to inhibit $CD3^+$ T cell proliferation after INF-γ licensing, but at different levels. In addition, T cell responses also presented different profiles according to the PBMC donor. It remains to be

seen if markers of cell aging may aid in identifying the best donor-recipient pairs, an issue currently still under study. Taken together, our data indicate that the therapeutic use of WJ-MSC may be impacted by the intrinsic variability present in donors (WJ-MSC) and recipients (monocytes and lymphocytes).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Anna Carla Goldberg was responsible for the design and coordinated the present study. Fernanda Vieira Paladino and Luiz Roberto Sardinha performed the experiments. Fernanda Vieira Paladino, Luiz Roberto Sardinha, and Carla Azevedo Piccinato were responsible for analyzing the data. Fernanda Vieira Paladino and Anna Carla Goldberg wrote the manuscript. Fernanda Vieira Paladino, Luiz Roberto Sardinha, Carla de Azevedo Piccinato, and Anna Carla Goldberg were responsible for discussing the results and reviewing the manuscript.

Acknowledgments

The authors would like to acknowledge the contribution of Isis Mozetic and Juliana de Moraes Rodrigues who have helped make this study possible. They thank Dr. Andreia Kondo and the team of nurses at our local Public Umbilical Cord Blood Bank for their help to obtain the samples. They thank Dr. Luciana Marti and Dr. Andrea Sertié for expert technical assistance and insightful suggestions. FAPESP and CAPES have financed graduate fellowships. Anna Carla Goldberg is a recipient of personal fellowship from CNPq. They are indebted to UC donors and acknowledge the generous support by the Ruhman family.

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Hindawi
Stem Cells International
Volume 2017, Article ID 1653254, 13 pages
https://doi.org/10.1155/2017/1653254

Review Article

Trophic Activity and Phenotype of Adipose Tissue-Derived Mesenchymal Stem Cells as a Background of Their Regenerative Potential

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Received 10 February 2017; Revised 28 April 2017; Accepted 14 May 2017; Published 5 July 2017

Academic Editor: Mustapha Najimi

There has been an increased interest in mesenchymal stem cells from adipose tissue, due to their abundance and accessibility with no ethical concerns. Their multipotent properties make them appropriate for regenerative clinical applications. It has been shown that adipose-derived stem cells (ASCs) may differ between the origin sites. Moreover, a variety of internal and external factors may affect their biological characteristics, as what we aimed to highlight in this review. It has been demonstrated that ASCs secrete multiple trophic factors that are capable of stimulating cell proliferation and differentiation and migration of various cell types. Particular attention should be given to exosomes, since it is known that they contribute to the paracrine effects of MSCs. Secretion of trophic agents by ASCs is thought to be in a greater importance for regenerative medicine applications, rather than cells engraftment to the site of injury and their differentiation ability. The surface marker profile of ASCs seems to be similar to that of the mesenchymal stem cells from bone marrow, although some molecular differences are observed. Thus, in this review, we have attempted to define trophic activity, as well as phenotypic characterization of ASCs, as crucial factors for therapeutic usage.

1. Introduction

Stem cells reside in almost all tissues within the human body where they exhibit various potential. These cells reveal self-renewal capacity, long-term viability, and ability to undergo multiple lineage differentiation in an appropriate microenvironment. They are of great importance for application in regenerative medicine because they control homeostasis, regeneration, and healing [1–3]. The stem cells should be accessible in large quantities, and the procedure of collection and harvesting of them should be non or minimally invasive, so then they can be used in regenerative medicine approaches. In addition, the differentiation of stem cells along multilineage pathways can be carried out in a reproducible manner. Then, the transplantation of them to autologous or allogeneic host is safe and effective, and their manufacturing is performed in accordance with current Good Manufacturing Practice guidelines [1, 3]. According to the origin, classification of stem cells is the following: embryonic stem cells (ES cells) [4], fetal stem cells [5], and adult (postnatal) stem cells [2, 6]. Although embryonic stem cells display enormous potential related to their pluripotency, many restrictions as well as ethical concerns are hindering their clinical applications. Facing such limitations, the need to generate an alternative source of pluripotent stem cells has emerged. The efforts succeeded in 2006, when Takahashi

and Yamanaka announced the derivation of induced pluripotent stem (iPS) cells from mouse somatic cells by transduction of four defined transcription factors [7]. These adult cells reprogrammed to embryonic-like states offer a great perspective for regenerative medicine, enabling the development of patient-specific therapies [8]. There has been also an increased interest in adult stem cells as a promising tool for tissues repairing. Numerous studies have focused on bone marrow as a primary source of human adult stem cells [1, 3]. The bone marrow is considered to contain two major stem cell populations: hematopoietic (HSCs) and mesenchymal stem cells (MSCs). The latter exhibit the plastic adherent growth and extensive expansion under specific culture conditions [6, 9, 10]. However, the presence of MSCs has been also identified in other tissues and organs, such as umbilical cord blood, peripheral blood, skin, adipose tissue, skeletal muscle, gut, liver, lung, and brain [1]. In response to appropriate culture conditions, the MSCs have the ability to differentiate into mesodermal cells—osteocytes, chondrocytes, and adipocytes [6]. The capacity of mesenchymal stem cells to differentiate into other cell types of mesodermal and nonmesodermal origin remains a matter of debate [11], although differentiation into endothelial cells [12], cardiomyocytes [13], hepatocytes [14], and neural cells [15] has been reported. Such multipotential properties are not universally accepted because of the lack of globally standardized methods for their isolation, expansion, and identification, as well as the range in assays used to define terminally differentiated, functionally mature populations. Additionally, it has been described that bone marrow-derived mesenchymal stem cell cultures contribute to many tissues upon transplantation not through differentiation into mature cell types but through fusion with endogenous cells [16], making the claims for in vivo differentiation potential into other cell types controversial. Thus, it remains elusive which multipotential properties the mesenchymal stem cells really possess [11]. The endogenous roles of MSCs are the maintenance of the hematopoietic stem cell niche, organ homeostasis, wound healing, and aging. For all these reasons, MSCs are an attractive source of stem cells for therapeutic usage, and their transplantation may have a promising potential in organ repair.

In the context of clinical applications, mesenchymal stem cells originated from human adult fat depots, known as adipose-derived stem cells (ASCs), are of great importance, because of their high accessibility with minimal invasiveness and no ethical limitations. Besides the fact that mesenchymal stem cells from adipose tissue are more heterogeneous [17], they exhibit immunomodulatory properties [9, 18, 19] and differentiation ability similar to bone marrow-derived MSCs [20]. Importantly, the adipose tissue contains higher densities of adult mesenchymal stem cells, comparing to bone marrow [6]. Therefore, in this article, we have reviewed the medical literature describing the adipose tissue-derived mesenchymal stem cells as they seem to have a promising potential in regenerative medicine. Our goal was to highlight the variety of factors which may affect ASC behavior. We also noted that ASCs from distinct locations within human body may differ in their functions and characteristics. Due to the fact that the usage of ASCs in clinical applications requires the well-defined and homogenous cell population, we have tried to summarize, based on current knowledge, the molecular characterization of ASCs. We have particularly focused on trophic activity, since this feature is believed to be in a greater importance for regenerative medicine applications, rather than cell engraftment to the site of injury and their differentiation ability.

2. Adipose Tissue-Derived Mesenchymal Stem Cell Characteristics

2.1. Nomenclature. Mesenchymal stem cells isolated from adipose tissue are described by various terms which include adipose-derived stem/stromal cells (ASCs), adipose-derived adult stem (ADAS) cells, adipose-derived adult stromal cells, adipose-derived stromal cells (ADSCs), adipose stromal cells (ASC), adipose mesenchymal stem cells (AdMSCs), lipoblasts, pericytes, preadipocytes, and processed lipoaspirate (PLA) cells. Therefore, to prevent a confusion in the literature related to the use of different nomenclature, the International Fat Applied Technology Society reached a consensus to adopt the term "adipose-derived stem cells" (ASCs) to identify the isolated, plastic-adherent, multipotent cell population [1, 3]. According to these recommendations, we use the name "ASCs" in this review.

2.2. The Variety of Fat Depots as the Sources of Mesenchymal Stem Cells. Adipose tissue is a highly complex tissue of mesodermal origin. It comprises multiple cell types, including mature adipocytes, preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages, and lymphocytes [21, 22]. At present, it is known that apart from energy storage function the adipose tissue is an important endocrine tissue and a source of multipotent mesenchymal stem cells.

According to the developmental origin, adipose tissue can be classified into two main categories: brown and white adipose tissue, BAT and WAT, respectively [23]. Brown adipose depots are responsible for energy expenditure, whereas white adipose tissue stores energy and provides insulation. The main localizations of these two types of adipose tissue within the human body are presented in Figure 1. BAT occurs in axillary, cervical, perirenal, and periadrenal regions in fetus and newborn and is transformed to WAT during aging. Although BAT has been believed to have an insignificant function in adults, some data indicate that metabolically active brown fat can be found in the cervical, supraclavicular, axillary, paravertebral, and suprarenal regions of adult individuals [24, 25]. In turn, depots of white adipose tissue are dispersed in diverse locations such as intraabdominal and subcutaneous sites and may exhibit differentiation potential differences. For instance, Kim et al. [26] have observed the higher proliferation and adipogenic differentiation capacity in subcutaneous ASCs, compared to ASCs from intraabdominal region. The main intraabdominal WAT depots are located around the omentum, intestines, and perirenal areas, and subcutaneous depots are present in the abdomen,

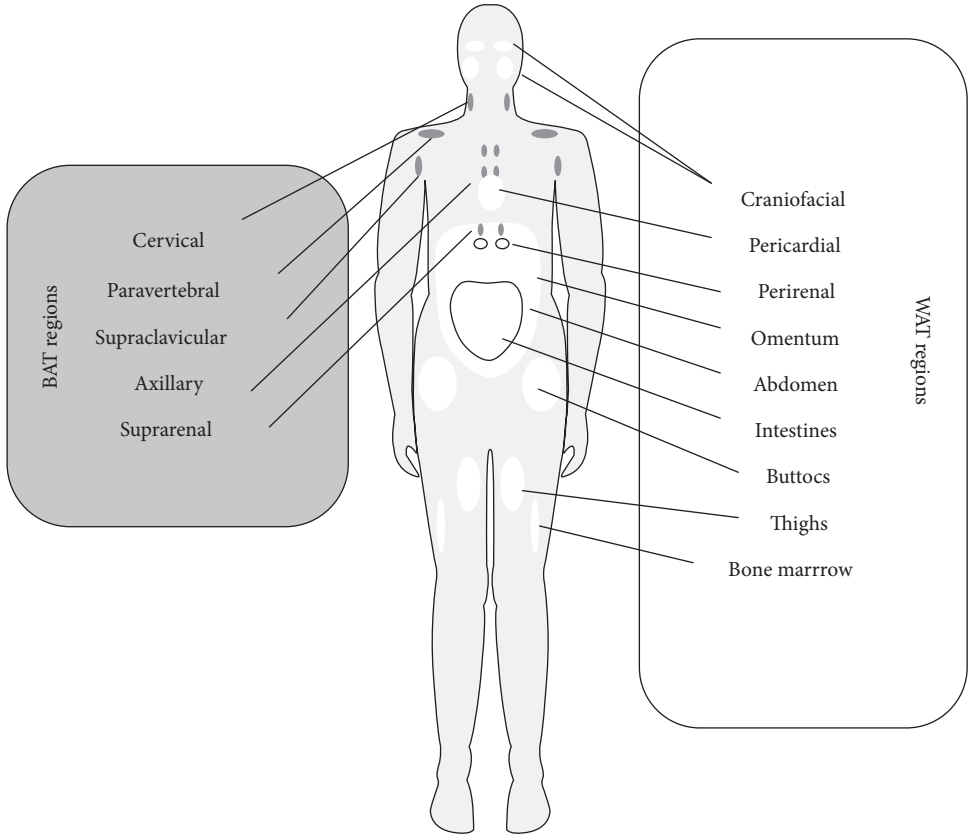

Figure 1: Distribution of brown (BAT) and white (WAT) adipose tissues within the human adult body [3, 23–25].

buttocks, and thighs. However, WAT is also located in other regions, such as retroorbital space, on the face, and extremities, supporting the eye, hand, and other critical structures. Additionally, WAT depots are found within the bone marrow, where they both occupy space no longer required for hematopoiesis and represent an energy reservoir and cytokine source for osteogenesis and hematopoiesis [3, 24]. Among various fat areas, the subcutaneous depots have been in a particular interest due to their availability, abundance, and renewability. There are two different localizations of subcutaneous fat in the abdominal region, namely superficial adipose tissue (SAT) and deep adipose tissue (DAT), and mesenchymal stem cells from these sources may be different. Di Taranto et al. [27] have noted that ASCs from subcutaneous regions displayed a slightly higher osteogenic potential, indicated by higher expression level of osteogenic marker genes, in comparison to ASCs derived from deep adipose tissue. However, adherent cells isolated from SAT and DAT showed comparable proliferation capacity and adipogenic potential. They have also found that SAT contained a higher stromal tissue compound, along with a higher proportion of CD105-positive cells, than DAT from the same harvesting site. What is more, based on the significantly higher expression levels of stemness-related transcription factors, the cells isolated from SAT were thought to exhibit increased multipotency and stemness properties [27].

Indeed, adipose tissue is promoted as an attractive stem cell source for clinical applications, due to the possibility of ASCs obtaining in significant quantities under local anesthesia using a minimally invasive procedure with no ethical concerns. As we previously mentioned, adult mesenchymal stem cells occur in higher densities in adipose tissues, rather than in the bone marrow [6]. The yield of these cells is from ~40- [18] even up to 500-fold greater in fat depots, compared to bone marrow [28, 29].

2.3. Factors Affecting Adipose Tissue-Derived Mesenchymal Stem Cells Characteristics. As a reservoir of adult mesenchymal stem cells, the adipose tissue is usually harvested via two different procedures: standard en bloc resection or lipoaspiration. What is important, the viability, yield, and growth characteristics of ASCs are affected by the type of harvesting procedure. Vermette et al. [30] demonstrated that cell yield obtained at the time of extraction was 1.8 times greater for lipoaspiration-derived cells, and they proliferated similarly or slightly better in culture than cells derived from resection. However, it has been shown that ultrasound-assisted liposuction resulted in a lower frequency of proliferating ASCs, as well as a longer population doubling time, compared with resection [31]. Alharbi et al. [32] compared conventional Coleman [33] versus microfat-harvesting procedure, in the context of the influence of these two fat harvesting techniques on such biological properties of ASCs as cell yield, viability, secreted growth factors concentrations or migration, and adhesion rate. The study has shown only slight differences in the yields of ASCs from abdominal subcutaneous

fat tissue, obtained by these two methods. Likewise, no significant effects on the in vitro viability of lipoaspirates were found. However, the viability and migration of isolated ASCs gained from microharvested lipoaspirates were significantly higher. Furthermore, the study has revealed a significant high adherence rate of isolated ASCs from the microfat-harvesting technique onto collagen matrices. In addition, significantly higher contents of growth factors such as insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), but not platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF), were observed in conventionally obtained lipoaspirates. The authors indicated the different sizes and surface/volume ratios of pieces of fatty tissue obtained using different cannula sizes as the factors responsible for the observed effects. Similarly, the study carried out by Trivisonno et al. [34] has determined the significantly higher ASCs yield in samples collected with the microcannula, compared to a standard cannula.

Summarizing, simple surgical procedure as well as easy to perform isolation and culturing protocols promotes adipose tissue to be used in clinical applications as MSC source. However, the isolation methods differ between laboratories. Mostly, the cell preparation is based on the procedure described by Rodbell [35–37]. The first step of isolation is tissue mincing, followed by enzymatic digestion with collagenase type I. The heterogeneous fraction of cells obtained immediately after collagenase digestion, which constitutes a population of adult mesenchymal stem cells and endothelial progenitor cells, is named the stromal-vascular cell fraction (SVF). After differential centrifugation, the pelleted SVF cells are placed in culture and the adherent cell population is then expanded.

Adult stem cells are influenced by many biochemical and biophysical stimuli in their in vivo microenvironment, including fluid shear stress, hydrostatic pressure, and trophic factors. It is worth to note that multiple external factors may also affect the ASC biology. Thus, it is very important to apply proper culturing conditions, such as plating density, media composition, and time of contact to plastic surface, which may influence both proliferation rate and differentiation potential of ASCs [38]. Due to the fact that ASCs exhibit surface adherence, the seeding density seems to be one of the main critical conditions affecting their biological functions. The lower seeding densities are associated with maintenance of high proliferation rate and multipotentiality. Sequential passages may influence on the quality of cultured MSCs, with progressive senescence, slowed proliferation rate, and cells progressively experiencing loss of multipotentiality [38]. Therefore, finding the correlation between seeding density and optimal cell proliferation as well as appropriate limiting of the population doublings number is useful in both laboratory studies and clinical applications because it allows the cell culturing procedure to be less time consuming, with a lower risk of cell culture contamination, and more cost effective [38, 39]. It is worth to emphasize that in terms of time of culture and functions the culture media are not equivalent and may have an impact on the behavior of the final product. Thus, the safety and efficacy of MSCs produced by distinct culture media should be carefully tested and documented [38]. It has been shown that different serum-free media based on cocktails of growth factors can maintain the main phenotypic and functional properties of cultured mesenchymal stem cells, but they require upgrading for clinical usage [40]. For example, the growth properties as well as neurotrophic and angiogenic effects of ASCs cultured in a defined xeno-free, serum-free medium were investigated by Brohlin et al. [41]. At early passage, ASCs performed better proliferation in serum-free medium, compared with standard α-MEM-containing fetal calf serum. However, CFUs were significantly lower in serum-free medium. ASCs cultured in serum-free medium continued to expand faster than cells grown in serum, contrary to BM-MSCs, which exhibited senescence features. In addition, stimulated ASCs and BM-MSCs expanded in serum-free medium displayed high levels of neurotrophic and angiogenic activity [41].

It has been suggested that a long-term culture with a high number of population doublings may result in undergoing of expanded MSCs to senescence and genetic instability, contributing to an increased risk of transformation or chromosomal aberrations. Indeed, there were two reports presenting spontaneous transformation and/or aneuploidy of ASCs, following long-term in vitro culture [42, 43]. However, they were retracted based on data indicating tumor cell cross-contamination artifacts. Nevertheless, testing the genetic stability of expanded mesenchymal stem cells is very relevant, both for the correct interpretation of biological outcomes and for ensuring the safety of potential stem cell therapy. It has been also hypothesized that physiological stress or in vitro culture conditions may significantly lead to the occurrence of cell or chromosomal abnormalities [44]. For instance, the enzymatic cell dissociation such as trypsinization raises more concerns in relation to abnormalities than mechanical dissociation. Thus, the culture conditions should be well defined and regularly controlled, to avoid the occurrence of karyotypic alterations [45].

It seems to be obvious that the attachment and proliferation rate are more prominent in ASCs from younger patients compared to older donors. To confirm, in the study performed by Choudhery et al. [46], aged ASCs exhibited reduced viability and proliferation when compared to cells obtained from young donors. Aged ASCs displayed increased senescent features, indicated by higher expression of senescence markers—p16 and p21 genes. These features were also associated with significantly reduced osteogenic and chondrogenic differentiation potentials in aged ASCs compared to young ASCs. It has been considered that stem cell aging is largely affected by the epigenetic modification of the genome such as DNA methylation and/or chromatin remodeling [47]. It has been suggested that DNA methylation plays a crucial role in adult stem cell aging, as DNA methylation increases with donor age in ASCs. Yan et al. [48] have found a decrease in proliferation rate as well as an impaired osteogenic differentiation potential of ASCs from aged donors, which was accompanied by a strong DNA methylation. Jurgens et al. [49] have found that adipose tissue-harvesting site may be responsible for the differences in the yield of ASCs, but no impact on total number of nucleated cells in the SVF or the ASC proliferation and differentiation

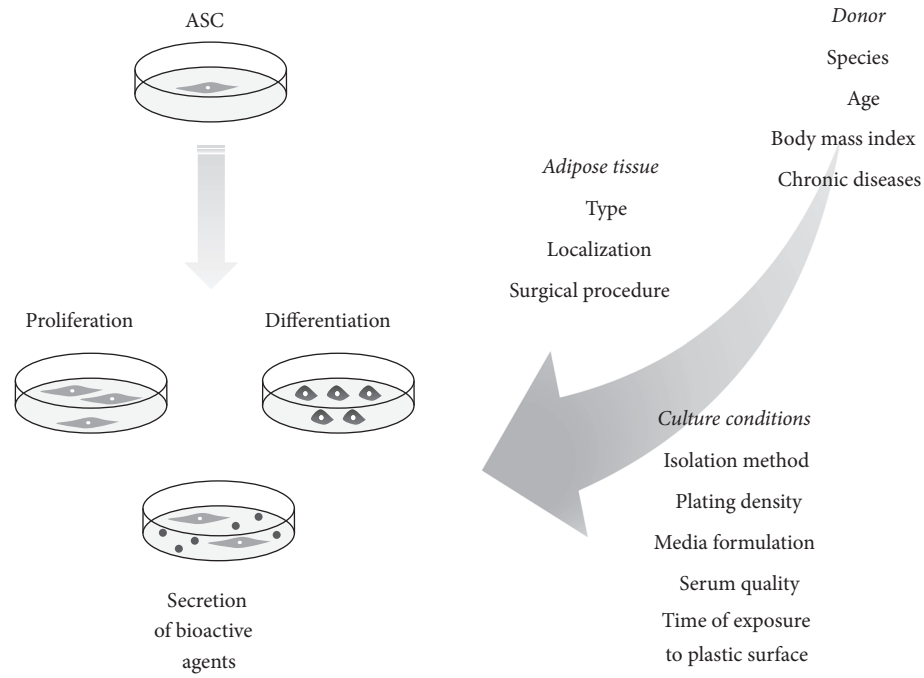

Figure 2: A variety of factors influencing adipose tissue-derived mesenchymal stem cell properties (proliferation capacity, differentiation potential, and trophic effects) [19, 32, 34, 38, 41–46, 48–55].

capacities was observed. They indicated abdomen as a more preferable site for harvesting ASCs than the hip/thigh region. Padoin et al. [50] have compared the cell concentration of processed lipoaspirate cells in 6 commonly used donor sites for fat grafting such as the upper abdomen, lower abdomen, trochanteric region, inner thigh, knee, and flank. They have evidenced that both lower abdomen and inner thigh have higher concentrations of processed lipoaspirate cells, what points to these regions as the better donor sites of ASCs. Another factor, which may affect ASC yield, is BMI. Aust et al. [51] have shown a significant negative correlation between the number of cells obtained per milliliter of lipoaspirate with the BMI. The research group of van Harmelen et al. [52] has found that although there was an increase in the total number of fat cells and stromal cells with BMI, there was a negative correlation between BMI and number of fat cells and stromal cells per gram of adipose tissue. This decrease of the number of cells per gram of adipose tissue might be explained by the enlargement of the fat cells with increasing BMI. In addition, the chronic disease may also have an impact on ASC characteristics. For example, stem cell phenotypes may be negatively impacted by diabetes [53], and diabetic ASCs display an impaired neovascular potential in vitro [54, 55].

Thus, as we mentioned above, the type and localization of adipose tissue as well as surgical procedure may also have an impact on ASCs biology. The factors influencing ASC biological processes are presented in Figure 2.

2.4. Trophic Activity of Adipose Tissue-Derived Mesenchymal Stem Cells. The MSCs promote damaged tissue recovery by proliferating and differentiating cells, which are progeny of the engrafted cells. This feature was initially thought to be the most crucial for stem cell-based therapies' success. However, it has been reported that the implanted cells may stimulate the endogenous healing potential by their trophic activity, which attracts host progenitor cells and leads to tissue regeneration by local and invading cells [56]. Therefore, the MSC capacity to supply reparative molecules such as growth factors and cytokines to repairing tissue is believed to be in a greater importance for regenerative medicine applications, rather than MSC engraftment to the site of the lesion and their differentiation ability. It has been confirmed that ASCs are superior in secretion of bioactive factors that may stimulate cell proliferation, differentiation, and migration of various cell types such as fibroblasts, endothelial, and epithelial cells [57]. In addition, ASCs have ability to deliver protective and/or supportive factors, which may reduce apoptosis, fibrosis, and inflammation [58], in significantly higher quantities and numbers than MSCs from bone marrow [59]. For instance, Hsiao et al. [60] have found that of all examined MSC populations, ASCs represented the most attractive cell type for promoting angiogenesis in tissue engineering applications, expressing at higher levels some paracrine factors such as insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor-D (VEGF-D), and interleukin-8 (IL-8). ASCs showed a significantly greater angiogenic potential compared with BM-MSCs in a study performed by Kim et al. [61]. Moreover, the factors released by MSCs may suppress the local immune system, by modulating T and B cells and inducing the expression of anti-inflammatory factors, such as interleukin-10 (IL-10), IL-1 receptor antagonist (IL-1Ra), or prostaglandin E2 (PGE2) [62–64].

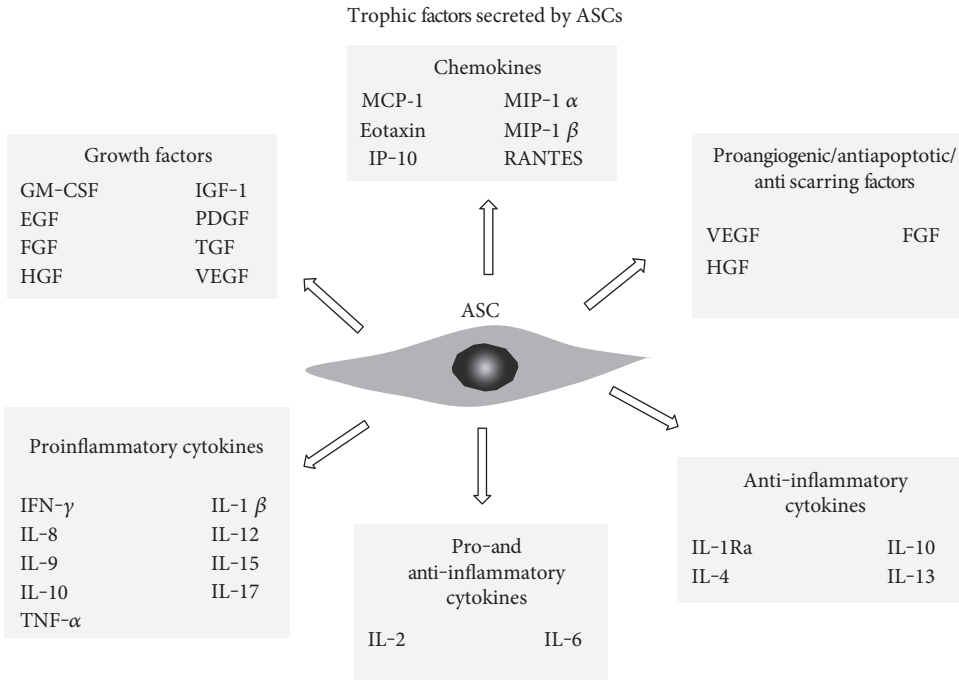

Figure 3: Different types of trophic factors released by adipose tissue-derived mesenchymal stem cells [27, 65–69, 76, 99].

The ASCs produce a larger number of growth factors than bone marrow MSCs, which include granulocyte-macrophage colony stimulating factor (GM-CSF) [58, 59], granulocyte-colony stimulating factor (G-CSF), IL-1Ra, IL-8, and HGF [59]. Additionally, they produce a large number of other factors, such as transforming growth factor (TGF) [58], vascular endothelial growth factor (VEGF) [59, 65], platelet-derived growth factor (PDGF) [66], fibroblast growth factor (FGF) [67], hepatocyte growth factor (HGF) [68], and members of epidermal growth factor family (EGF) [69] (Figure 3). Secretion of angiogenic and antiapoptotic growth factors at bioactive levels makes the subcutaneous ASCs a novel source for cardiovascular therapies. In fact, some clinical trials using ASCs in cardiac therapy have been succeeded [70]. The first study, A Randomized Clinical Trial of AdiPOse-derived Stem ceLLs in the Treatment of Patients With ST-elevation myOcardial Infarction—the APOLLO Trial (NCT00442806), investigated the safety and feasibility of intracoronary infusion of autologous adipose-derived stem and regenerative cells in acute myocardial infarction patients after successful revascularization. The data reported at the 7th International Symposium on Stem Cell Therapy and Cardiovascular Innovation showed the improvement in the left ventricular ejection fraction (LVEF), reduction in infarct size, and improvement in myocardial perfusion [71]. The ADVANCE Study (NCT01216995) further evaluated the efficacy of this approach defined as reduction in infarct size at 6 months. Another clinical trial targeted to patients with chronic ischemic heart disease, MyStromalCell Trial (NCT01449032), completed in 2014, was evaluating the efficacy and safety of intramyocardial delivery of VEGF-A_{165}-stimulated autologous adipose tissue-derived MSCs to improve myocardial perfusion and exercise capacity and reduce symptoms. The outcomes of another trial, the PRECISE trial (https://www.clinicaltrials.gov/NCT00426868), suggested that adipose-derived regenerative cells may preserve ventricular function, myocardial perfusion, and exercise capacity in patients suffering from chronic ischemic cardiomyopathy [72].

In turn, Sawada et al. [73] have reported the importance of the trophic effects of adipose tissue-derived multilineage progenitor cells (ADMPCs) in the periodontal tissue regeneration. The growth factors released by ADMPCs included HGF, VEGF, and insulin-like growth factor binding protein 6 (IGFBP6). Among those, HGF participates in the proliferation and migration of vascular endothelial cells. The second factor, VEGF, plays critical roles in blood vessel formation through endothelial cell proliferation and migration. Furthermore, the expression of VEGF and its receptors has been confirmed in periodontal ligament cells (PDLs) and gingival fibroblasts in periodontal tissues. It is also known that VEGF promotes proliferation and migration of human periodontal ligament cells (HPDLs) and their differentiation into osteoblasts [74, 75]. For these reasons, HGF and VEGF secreted by ADMPCs are suggested to contribute to periodontal tissue regeneration in a non-cell-autonomous manner. IGFBP6, the most highly presented in ADMPC-conditioned medium, was shown to stimulate the differentiation of HPDLs to mineralized tissue-forming cells [73].

In addition to growth factors, the ASCs produce a variety of other molecules, such as cytokines and chemokines. For example, proinflammatory cytokines secreted by ASCs include IFN-γ, IL-1β, IL-7, IL-8, IL-9, IL-12, IL-15, IL-17, and TNF-α, anti-inflammatory cytokines—IL-1Ra, IL-4, IL-10, and IL-13 [59]. Moreover, the ASCs produce IL-2 and IL-6 that are considered to display both pro- and

anti-inflammatory effects under different conditions. In turn, the ASCs secrete some chemokines such as MCP-1, eotaxin, IP-10, MIP-1α, MIP-1β, and RANTES [59, 76] (Figure 3). However, it has been found that the cytokine secretion profiles vary between ASCs from diverse locations. For instance, Naftali-Shani et al. [77] have demonstrated that human mesenchymal stromal cells isolated from patients with ischemic heart disease from the right atrium and epicardial fat secreted the highest amounts of trophic and proangiogenic factors, namely HGF, basic FGF, and PDGF, in comparison to pericardial and subcutaneous fat depots. Moreover, the immunomodulatory cytokines, such as TNF-α, tissue inhibitors of metalloproteinase 2, and IL-13, were produced in the highest amounts also by MSCs from epicardial fat and the right atrium of those patients. However, despite the higher levels of trophic and angiogenic factor secretion, MSCs from epicardial fat and the right atrium exerted the inferior effect on cardiac remodeling and function, as evidenced by the highest inflammation score in the infarcted heart, compared to subcutaneous fat MSCs [77]. Furthermore, whereas the accumulation of visceral fat is associated with increased prevalence of insulin resistance, metabolic syndrome, and related cardiovascular complications [78], factors secreted by subcutaneous fat MSCs may mediate the antiatherogenic effects of subcutaneous fat [79]. In turn, the experiments performed by Mazurek et al. [80] have shown that epicardial adipose tissue is a source of several inflammatory mediators in high-risk cardiac patients. In case of suffering from coronary artery disease (CAD), the epicardial adipose tissue displayed significantly higher levels of chemokine (MCP-1) and several inflammatory cytokines (IL-1β, IL-6, IL-6sR, and TNF-α), comparing to subcutaneous adipose tissue. The inflammatory mediators present in epicardial adipose tissue could lead to amplification of vascular inflammation, plaque instability via apoptosis (TNF-α), and neovascularization (MCP-1) [80].

Particular attention should be given to exosomes, since it is known that they contribute to the paracrine effects of MSCs [81]. Exosomes are small, intraluminal vesicles of multivesicular bodies released when they fuse with the plasma membrane [82]. It has been suggested that these vesicles are secreted by a variety of cell types and can function as intercellular transmitters of mRNA, microRNA, and proteins [83]. They are thought to mimic the roles played by mesenchymal stem cells from which they originate [84, 85]. The importance of ASC-secreted exosomes in promoting tissue repair has been reported. For instance, Hu et al. [86] have indicated that exosomes of adipose tissue may promote beneficial effect for soft tissue wound healing. They have found that exosomes released by ASCs could be internalized by fibroblasts to stimulate cell migration, proliferation capacity, and collagen synthesis in a dose-dependent manner. In turn, the exosomes from ASCs have been considered as a therapeutic agent for the treatment of inflammation-related diseases by Blazquez et al. [87], due to their inhibitory effect in the differentiation and activation of T cells as well as a reduced T cell proliferation and IFN-γ release on in vitro stimulated cells. Furthermore, Katsuda et al. [88] have demonstrated that exosomes secreted by ASCs contained enzymatically active neprilysin (neutral endopeptidase: NEP or CD10), involved in the degradation of β-amyloid peptide (Aβ) whose accumulation in the brain plays a critical role in Alzheimer's disease pathogenesis. Ascending evidence have suggested that exosomes might be the main components of paracrine factors, thus, they may represent a novel therapeutic tool in regenerative approaches. However, prior to clinical applications, the oncogenic risks that may be associated with MSC-derived exosomes should be overcome [84].

2.5. Surface Markers of Adipose Tissue-Derived Mesenchymal Stem Cells. Depending on the source of origin, the MSCs show differences in gene expression, surface epitopes, clonogenicity, ability to differentiate, and therapeutic potential. Surface marker profile of MSCs varies between species. Furthermore, these cells express different molecules according to the isolation and culture procedure [89, 90]. Expression of surface markers can be also conditioned by factors produced by the accessory cells in the initial culture period. In addition, the expression of some molecules may vary in vitro and in vivo [91]. In tissue, MSCs reside at various stages of differentiation. Therefore, in culture conditions, these cells exhibit heterogeneity in marker expression, renewal capacity, and differentiation potential [92]. The MSCs have been identified by a set of nonspecific surface antigens, but there are no definitive surface markers for the exclusive isolation of these cells. Therefore, prior to clinical applications, the challenges concerning the isolation, identification, and purification of stem cells must be overcome. The defining of MSC specific marker proteins, to use the well-identified and homogeneous cell population, is pivotal.

Phenotypically, MSCs have been described as CD29, CD44, CD90, and CD105 positive and negative for hematopoietic lineage markers and HLA-DR [18]. There are some attempts to well define the surface markers specific to ASCs, distinguishing them from BM-MSCs (Figure 4). It has been demonstrated that ASCs expressed classical MSC markers such as CD29-beta-1 integrin, involved in therapeutic angiogenesis, CD44-hyaluronate receptor, which is crucial in the development of neoextracellular matrix and is involved in many pathologic and physiologic phenomena, and were absent for HLA-DR and c-kit expression. Other surface molecules expressed by both ASCs and BM-MSCs include CD13, CD49e, CD54, CD63, and CD146 [17, 19] (Figure 4). Furthermore, cultured adipose tissue-derived stem cells display the expression of classical bone marrow MSC surface markers, such as CD73, CD90, CD105, and the lack of expression of haematopoietic (CD14, CD45) and endothelial markers (CD31) [9]. However, it has been revealed by Mitchell et al. [93] that the latter was expressed on SVF cells and did not change significantly with serial passage. What is more, they observed that other endothelial cell-associated markers, such as CD144 (VE-cadherin), VEGFR-2, and von Willebrand factor, were expressed on crude SVF cells. The levels of these molecules did not change much through the culture period [93]. Importantly, no expression of hematopoietic markers such as CD3, CD11b, CD14, CD15, CD16,

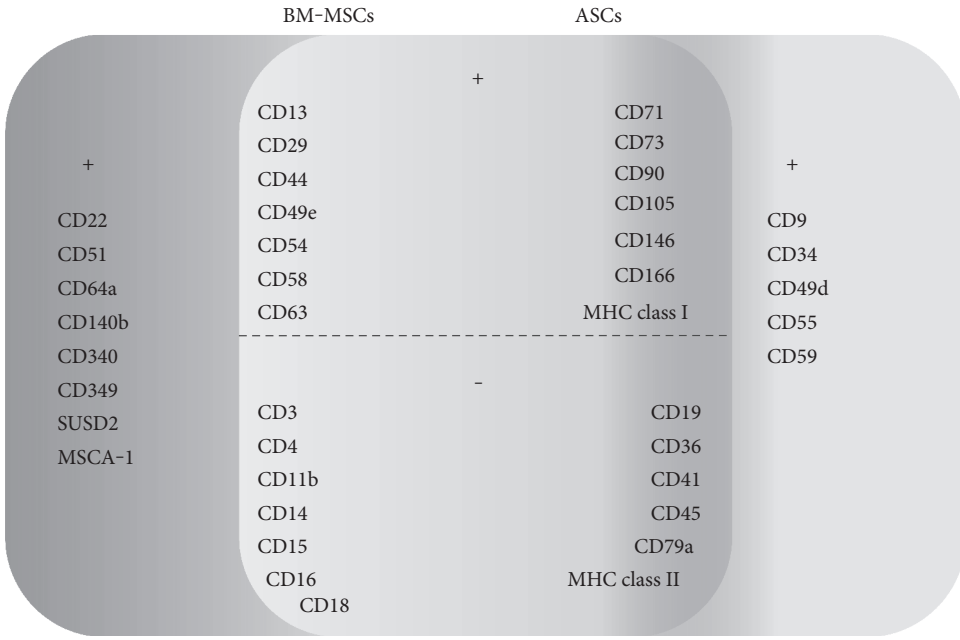

Figure 4: Comparison of surface marker profile of mesenchymal stem cells from two distinct origins, bone marrow (BM-MSCs) and adipose tissue (ASCs) [1–3, 36, 93–98].

CD18, and CD41 was observed [21]. Other molecules not expressed by both ASCs and BM-MSCs include CD4, CD19, CD36, and CD79a [9, 21]. The studies performed by Mitchell et al. [93] have shown that the immunophenotype of ASCs has progressively changed with adherence and passage. The levels of stromal cell-associated markers, including CD13, CD29, CD44, CD63, CD73, CD90, and CD166, were initially low on SVF cells and enhanced significantly with serial passage. The ALDH, used to identify the hematopoietic stem cells, was present at a high level (more than 70%) to passage 4 [93]. In addition, there is a report indicating the expression of CD71 and MHC class I by both ASCs and BM-MSCs [94]. It is worth to note that the data referring to the surface molecule characteristic for ASCs are inconsistent. For instance, CD106 has been described in the literature both as expressed [21] and not expressed [31] by adipose-derived stem cells. It has been demonstrated that native ASCs belong to the CD34+ cell fraction of the adipose tissue SVF [95]. However, other groups also suggest the existence of mesenchymal stem cell population derived from CD34 cells within adipose tissue [96]. Mitchell et al. [93] have observed that about 60% of the initial SVF cells showed expression of CD34, but the percentage of positive cells reduced in successive passage. Likewise, studies performed by Maumus et al. [95] have shown that CD34 expression decreased during the culture period and was negatively correlated with cell proliferation rate. Moreover, some data have revealed that ASCs are CD166 (activated lymphocyte common adhesion molecule) and STRO-1 positive [20]. However, it has been found that the latter, which is the best known MSC marker, is expressed at higher levels by ASCs cultured beyond the first passage or in case of inducing them to differentiate into endothelial cells. This indicates that STRO-1 is intrinsically an endothelial antigen, and its expression in MSC might be inducible [97]. Interestingly, ASCs in their native microenvironment were negative for CD140b and NG2 pericyte markers but the expression of them was induced by culture process [95].

There are some attempts to distinguish the population of bone marrow mesenchymal stem cells, from those of adipose tissue origin. Busser et al. [98] have demonstrated the lack of expression of SUSD2 and MSCA-1 by mesenchymal stem cells from adipose tissue in situ, in contrast to MSCs from bone marrow. What is more, BM-MSCs have been shown to be positive for CD22, CD51, CD64a, CD340, and CD349, comparing to ASCs [94]. In turn, the researchers have observed that other marker, CD271, allows to define adipose tissue cell subsets with particular abilities, but only in lipoaspiration samples and not in abdominoplasty samples [98]. It has been also reported that CD58 and CD117 were expressed by both bone marrow and adipose tissue mesenchymal stem cell populations [94]. On the other hand, some reports exert that all types of MSCs are characterized by lack of CD117 expression [9]. In contrast to cells derived from bone marrow, the ASCs exhibited the expression of CD49d. This receptor forms a heterodimer with CD29 to create VLA-4 (very late activation antigen-4), the main cognate ligand for vascular cell adhesion molecule-1 [VCAM-1]–CD106 ligand [94]. Moreover, it might be suggested that CD9, CD49d, CD55, and CD59 are molecules distinguishing mesenchymal stem cells derived from adipose tissue and bone marrow [20, 94]. In comparison to MSCs from bone marrow, the ASCs display high levels of CD54 (intercellular adhesion molecule-1 [ICAM-1]) [20]. This protein, a member of the immunoglobulin superfamily, can be upregulated in response to some inflammatory mediators and cytokines.

3. Conclusion

Over the last few years, there has been an increasing demand on therapeutic usage of stem cells in regenerative medicine. Mesenchymal stem cells can be easily obtained from patient's own tissues, isolated ex vivo, expanded, and transplanted back into the patient as an autologous transplant. Additionally, MSCs are real candidates for cellular therapy in allogeneic approaches, because of their immunosuppressive properties. Adipose tissue may serve as an abundant and accessible source of adult stem cells that can be used as an alternative to bone marrow mesenchymal stem cells in regenerative medical therapies. The ASCs are one of the cell populations found in stromal vascular fraction of adipose tissue. The adipose tissue-derived mesenchymal stem cells are capable of differentiating along multiple pathways. In addition, isolation and culture procedures are easy to perform.

Noteworthy, ASC property of secreting or making other cells in the vicinity to secrete functionally active agents is particular. It is believed that this feature is especially important for the success of tissue regeneration and repair after injury. ASCs are known to provide trophic immunosuppressive and anti-inflammatory effects through production of a variety of growth factors, cytokines, and chemokines (Figure 3). However, there are some reports indicating that secretion of these bioactive factors may vary between different fat regions [77, 80]. Moreover, the differences in trophic activity of ASCs may even occur within the same adipose tissue region, such as in the superficial and deep adipose tissues which both belong to abdominal region of subcutaneous fat [27]. It has been also observed that the production of trophic agents, namely G-CSF, GM-CSF, IL-6, IL-7, IL-8, IL-15, HGF, NGF, VEGF, IP-10, eotaxin, and IL-1Ra, reveals donor to donor variations [59]. This means that microenvironmental cues, like stimuli from growth factors, have a significant impact on stem cell "behavior". Thus, the cells forming the niche are no less important than stem cells that occupy and respond to this microenvironment.

Mesenchymal stem cells have been determined by a set of nonspecific surface proteins (Figure 4). However, for clinical applications, the identification of surface markers for the exclusive isolation of these cells is crucial. Importantly, to precisely define the MSC population, a combination of surface antigens and gene expression parameters is required; therefore, a single marker might not be sufficient. It has been indicated that some conditions may cause differences in the surface molecules expression. For instance, diverse collection and processing methods, quality of serum, or donor specificity may lead to differences in the composition and characteristics of MSC populations. Furthermore, surface marker expression profile is also known to be dependent on species. Although ASCs express surface markers similar to BM-MSCs, some variations occur. Such differences may also be related to the age of donors.

The literature reports concerning the immunophenotype of ASCs remain inconsistent. Primarily, it is worth noting that the expression pattern of markers analyzed in vitro does not always reflect the characteristics in vivo. For instance, while during culture period ASCs expressed CD140b, a cell surface tyrosine kinase receptor for members of the PDGF family, in vivo the lack of expression was observed [95]. Therefore, the expression of some molecules might be inducible. As it was mentioned above, during the culture period, the alterations of surface molecule expression level may occur. Some stromal cell-associated markers are initially expressed on a low level by SVF cells, whereas with serial passage, their increase is reported. On the other hand, it has been shown that a CD34, predominantly regarded as a marker of hematopoietic stem and progenitor cells, is expressed by most of the SVF cells but its level decreases during culture [93, 95]. Some endothelial markers, such as CD31, are expressed by crude SVF cells but not by cultured ASCs. It might be due to heterogeneity of SVF population, which besides the mesenchymal stem cells consists of endothelial progenitor cells. Taken together, a comprehensive and comparative analysis with other types of stem cell preparations, as well as a variety of terminally differentiated cell types, is necessary to specify a subset of reliable molecular markers.

To conclude, the unique properties of ASCs, such as self-renewal, differentiation into specialized tissues and organs, and secretion of trophic factors, in combination with supplying them to repairing tissue, make this type of mesenchymal stem cells a great tool for tissue engineering applications. Nevertheless, multiple aspects can influence adipose-derived stem cell properties and, thereby, affect the success of healing. The developmental fate of a stem cell depends on a general predetermined potential as well as on microenvironmental signals. This interaction of stem cells with their physiological environment deserves a particular attention, in the context of stem cell-based therapy development.

Abbreviations

ALDH: Aldehyde dehydrogenase
AT: Adipose tissue
CD: Cluster of differentiation
CFU: Colony-forming unit
HLA: Human leukocyte antigens
IFN-γ: Interferon gamma
IGF-1: Insulin-like growth factor 1
IL: Interleukin
IP-10: IFN-gamma-inducible protein 10
MCP-1: Monocyte chemoattractant protein-1
MIP: Macrophage inflammatory proteins
MSCA-1: Mesenchymal stromal cell antigen-1
Oct-4: Octamer-binding transcription factor 4
RANTES: Regulated on activation, normal T cell expressed and secreted
SOX2: (Sex determining region *Y*)-box 2
SUSD2: Sushi domain containing 2
TNF-α: Tumor necrosis factor alpha.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Beata Kocan designed and drafted the manuscript, prepared Figures 1–4, coordinated and revised the manuscript to the final version to be published. Aleksandra Maziarz participated in the manuscript draft and sequence alignment and was involved in the preparation of Figures 1–4. Jacek Tabarkiewicz revised the manuscript critically for important intellectual content. Takahiro Ochiya participated in the manuscript design and draft. Agnieszka Banaś-Ząbczyk provided the main idea, participated in its design and draft, coordinated and revised the manuscript critically for important intellectual content, and gave the final approval of the version to be published. All authors read and approved the final manuscript.

Acknowledgments

The study was performed within the project "Centre for Innovative Research in Medical and Natural Sciences" realized by the University of Rzeszow, cofinanced within the Regional Operational Programme for the Podkarpackie Province for the years 2007–2013, contract number UDA-RPPK.01.03.00-18-004/12-00.

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Hindawi
Stem Cells International
Volume 2017, Article ID 2352954, 11 pages
https://doi.org/10.1155/2017/2352954

Research Article

Safety and Tolerance of Donor-Derived Mesenchymal Stem Cells in Pediatric Living-Donor Liver Transplantation: The MYSTEP1 Study

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Received 8 February 2017; Accepted 8 May 2017; Published 27 June 2017

Academic Editor: Jay L. Vivian

Background. Calcineurin inhibitors (CNI) have significantly improved patient and graft survival in pediatric liver transplantation (pLT). However, CNI toxicity leads to significant morbidity. Moreover, CNIs cannot prevent long-term allograft injury. Mesenchymal stem (stromal) cells (MSC) have potent immunomodulatory properties, which may promote allograft tolerance and ameliorate toxicity of high-dose CNI. The MYSTEP1 trial aims to investigate safety and feasibility of donor-derived MSCs in pLT. *Methods/Design*. 7 to 10 children undergoing living-donor pLT will be included in this open-label, prospective pilot trial. A dose of 1×10^6 MSCs/kg body weight will be given at two time points: first by intraportal infusion intraoperatively and second by intravenous infusion on postoperative day 2. In addition, participants will receive standard immunosuppressive treatment. Our primary objective is to assess the safety of intraportal and intravenous MSC infusion in pLT recipients. Our secondary objective is to evaluate efficacy of MSC treatment as measured by the individual need for immunosuppression and the incidence of biopsy-proven acute rejection. We will perform detailed immune monitoring to investigate immunomodulatory effects. *Discussion*. Our study will provide information on the safety of donor-derived MSCs in pediatric living-donor liver transplantation and their effect on immunomodulation and graft survival.

1. Background

In recent decades, pediatric liver transplantation has evolved into a state-of-the-art procedure improving prognosis and quality of life for children and adolescents with terminal liver disease. Immunosuppressive pharmacotherapy including calcineurin inhibitors (CNIs) allows the transplantation of solid organ grafts with reasonable patient and graft survival rates [1–3]. However, long-term continuous exposure to immunosuppressive drugs, such as CNIs, mTOR inhibitors, and steroids, carries with it significant clinical side effects. These include renal dysfunction, arterial hypertension, glucose intolerance, posttransplant lymphoproliferative disorder, and opportunistic infections [4, 5]. These side effects

account for significant morbidity after liver transplantation [1, 6, 7]. Children are more affected than adults by the chronic toxicity of immunosuppressive medications, in particular by the toxicity of CNIs [8]. In addition, CNIs are not effective in preventing chronic de novo hepatitis in transplanted allografts. This long-term graft injury is associated with a high risk of developing progressive graft fibrosis after 10 years, and up to 25% of patients need retransplantation [9]. Recently, studies have focused on immunological complications after pediatric liver transplantation, such as humoral graft rejection [10] leading to late allograft failure. Therefore, we need novel immunomodulating approaches to limit the risk of immunosuppressive therapy while continuously improving outcome, achieving optimal cognitive and physical development, and maintaining a high quality of life for liver-transplanted children.

1.1. Mesenchymal Stem Cells. Mesenchymal stem cells or, according to the terminology of the International Society of Cellular Therapy (ISCT), multipotent mesenchymal stromal cells [11], are adult progenitor cells originating in the neural crest and mesoderm. MSCs can be isolated from the bone marrow and many other sources, including adipose tissue and Wharton's jelly. MSCs can differentiate into mesenchymal cells like osteoblasts, chondrocytes, and adipocytes; in addition, MSCs may potentially differentiate into cell types normally derived from the ectoderm or endoderm, such as hepatocytes [12]. According to ISCT criteria, human MSCs are characterized by their ability to adhere to plastic, their differentiation potential, the presence of stromal cell markers, and the absence of hematopoietic cell markers [11]. MSCs can be used either fresh after culture or after cryopreservation without loss of phenotype or differentiation potential [13], supporting easy clinical application.

Numerous experimental studies imply that MSCs play a role in modulation of immune responses: in mixed lymphocyte cultures, human MSCs have a significant suppressive effect on T-cell proliferation by means of cell-cell interaction and secretion of soluble factors [14]. Mediators secreted by MSCs include, for example, galectin-1 [15]; TGF-β1 (transforming growth factor); hepatocyte growth factor [14]; soluble human leucocyte antigen-G5; TNF-stimulated gene 6 protein [16]; and prostaglandin E2. The enzyme indoleamine-2,3-dioxygenase (IDO) is another important factor of immune regulation by MSCs [17]. Together, these factors are able to modulate immune activation of T-cells [18–20], B-cells [21], and macrophages [22], which, in turn, adopt a regulatory phenotype. Furthermore, MSCs affect the differentiation, maturation, and function of dendritic cells [23].

Preclinical models have confirmed these beneficial immunomodulatory effects of MSCs [18, 24, 25]. The first clinical applications have been to treat allo- and autoimmune disorders such as steroid-resistant graft-versus-host disease [26] and Crohn's disease [27]. These applications have been shown to be safe and very promising in terms of clinical efficacy.

1.2. MSCs and Solid Organ Transplantation. Mesenchymal stem (stromal) cells (MSCs) may represent an attractive therapeutic option in solid organ transplantation because they modulate immune response and promote regeneration [28]. Systemic application to patients has shown them to be very well tolerated. Clinical studies focusing on the use of autologous [29–31] and allogeneic MSCs [32, 33] in kidney transplantation have been completed, demonstrating safety and feasibility. These studies have shown lower incidence of acute rejection and improving renal function one year after transplantation, illustrating the immunosuppressive properties of MSCs. In adult liver transplantation, the first clinical phase I/II studies of the use of MSCs are ongoing (NCT01841632, NCT01429038, and NCT01844063). Preliminary reports showed that systemic and intraportal infusions of allogeneic MSCs after deceased and living-donor liver transplantation were safe and well tolerated by transplant recipients [34, 35]. None of the previous studies using comparable cell dosage demonstrated any significant side effects of MSC infusion.

Due to the aforementioned toxicity of CNI and insufficient long-term outcome results after liver transplantation, children may benefit from alternative approaches to immunomodulation that will prolong graft survival while reducing CNI toxicity, improving quality of life and promoting long-term allograft tolerance. However, in the context of pediatric living-donor liver transplantation, the safety and feasibility of intraportal and intravenous application of MSCs remain to be proven.

2. Methods and Design

2.1. Objectives and Endpoints. The primary objective of this pilot trial is to assess the safety of donor-derived MSC infusions in children undergoing LDLT. Safety will be determined by the following:

(1) Incidence, timing, and severity of acute complications related to MSC infusion, using a specific toxicity scoring system (MYSTEP score, Figure 1)

(2) Incidence of severe adverse events (SAEs) and their relation to investigational treatment

(3) Graft integrity and function after liver transplantation, as measured by aminotransferase and gamma glutamyl transferase activity, bilirubin, albumin, and INR.

Further, this study aims to evaluate the following:

(1) Efficacy

  (a) Feasibility and safety of tapering immunosuppressive medication according to standard guidelines [36] and according to the step-wise tapering protocol, beginning 6 months after pLT (Figure 2)

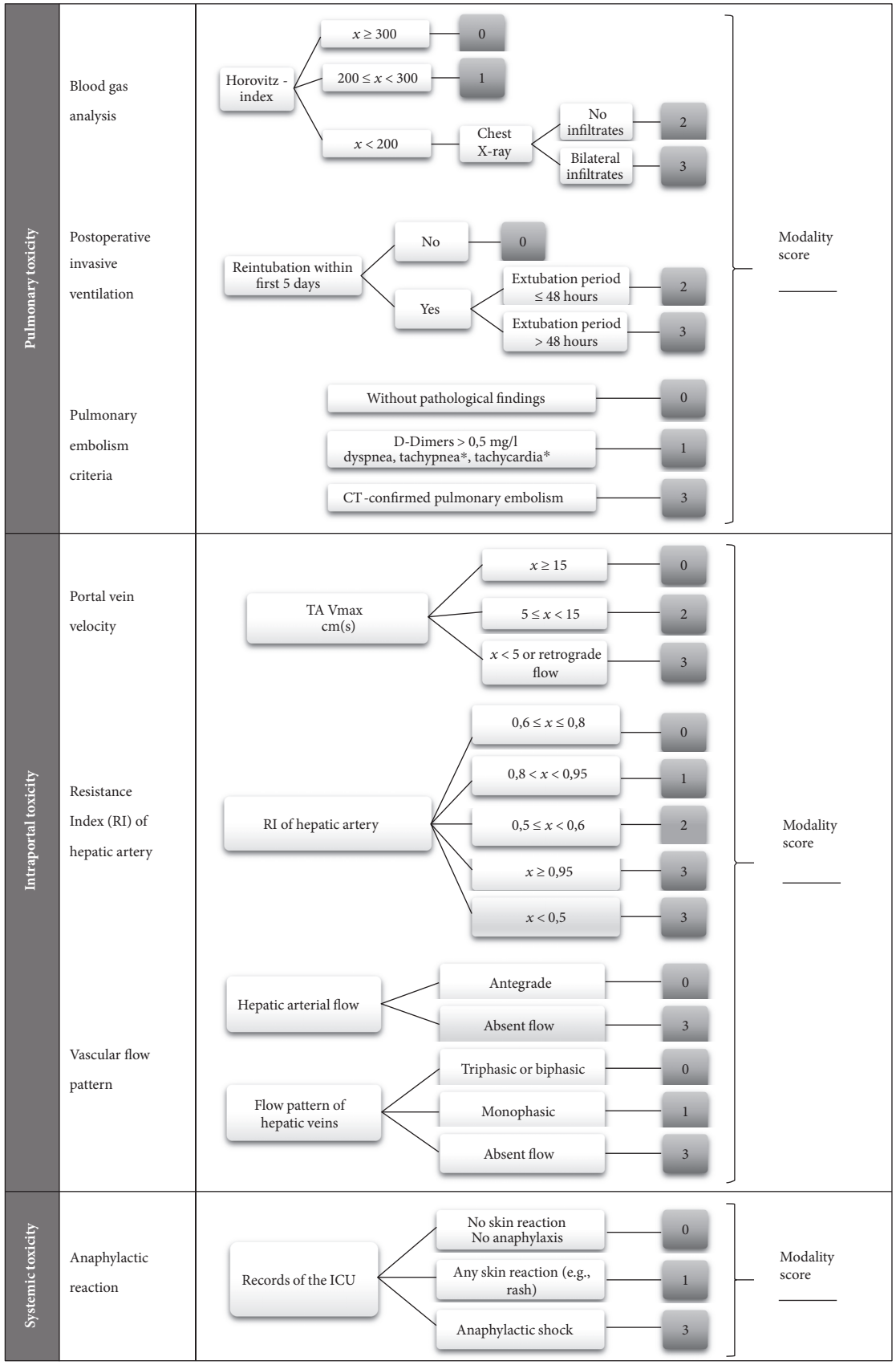

Figure 1: MYSTEP score monitoring infusional toxicity. Cut-off levels are defined upon current publications in adult [61, 62] and pediatric [63, 64] liver transplantation. *Tachycardia or tachypnea are defined as elevation of age-related range.

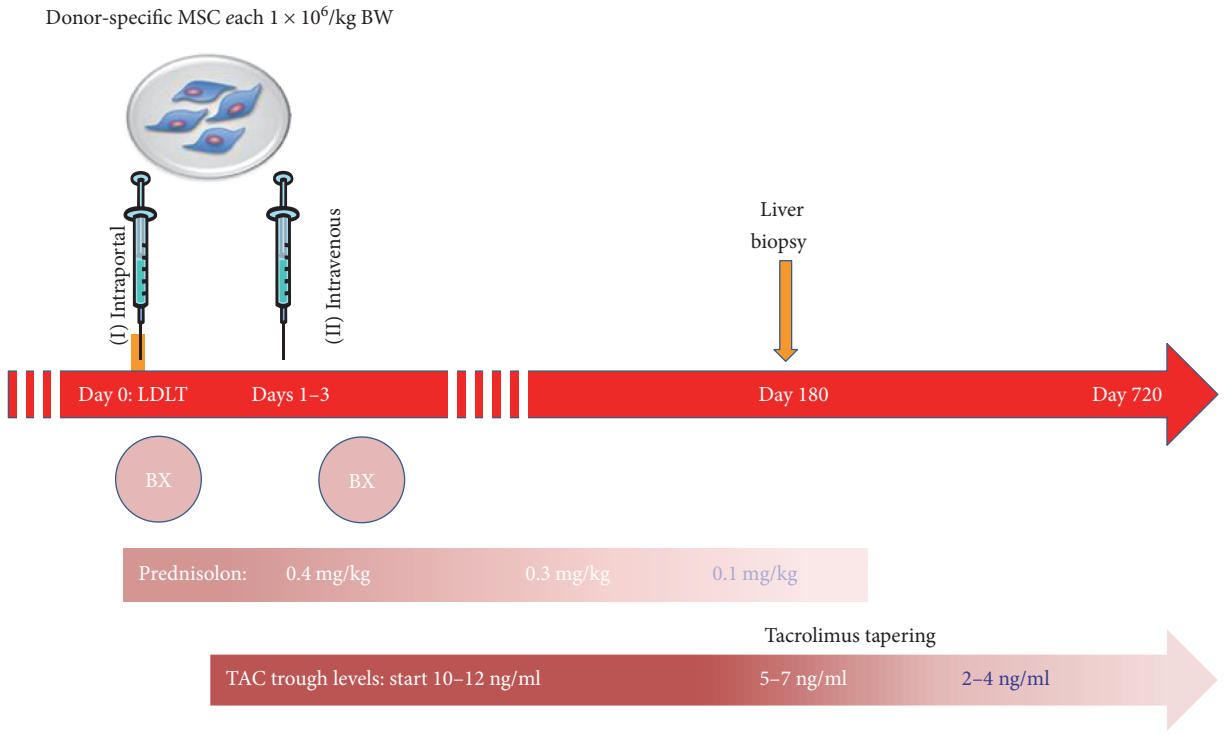

Figure 2: Scheme of investigational treatment and IS tapering strategy. BX = basiliximab; TAC = tacrolimus.

(b) Time to first biopsy-proven acute rejection (BPAR).

(2) Hematologic and immunologic function, as measured by characterization and quantification of mononuclear cell populations, detection of donor-specific antibodies (DSA) and liver autoantibodies, and analysis of a protocol transplant biopsy.

(3) Patient and graft survival at 1 and 2 years posttransplantation.

2.2. Study Design. The MYSTEP1 (Mesenchymal Stem Cells in Pediatric Liver Transplantation) trial is a 24-month, nonrandomized, open-label, prospective, single-center pilot trial. In total, a minimum of 7 de novo liver recipients, 0–17 years of age, will be recruited at the University Hospital of Tübingen. We will enroll them in the study upon the consent of their legal representatives and upon meeting the eligibility criteria. The study group may be expanded to as many as 10 patients after consulting the data safety monitoring board (DSMB), which will monitor study progress. The board comprises a pediatrician, a transplant surgeon, and a biometrician not otherwise involved in the trial. We have obtained regulatory approval from the Ethical Committee of the University of Tübingen and from the German Federal Institute for Vaccines and Biomedicines (Paul-Ehrlich-Institut; EudraCT number 2014-003561-15). This trial is registered with Clinicaltrials.gov NCT02957552.

2.3. Inclusion Criteria. Patients eligible for inclusion in this study must fulfill all of the following criteria:

(1) Patient and both parents and/or legal guardian must have given written informed consent.

(2) Patients will undergo living-donor liver transplantation for chronic terminal liver failure.

(3) Age ≥ 8 weeks and ≤18 years.

(4) Body weight > 5 kg.

2.4. Exclusion Criteria. Patients fulfilling any of the following criteria are not eligible in this study:

(1) Living donor not suitable according to donor and recipient criteria

(2) Pregnant or breastfeeding

(3) Refusal of adequate contraception (if appropriate)

(4) Acute liver failure or highly urgent transplantation

(5) Receiving any form of solid organ retransplantation

(6) Multi-organ-transplantation

(7) Active autoimmune disease

(8) Preexisting renal failure with eGFR < 50 ml/min/1.73 m^2 or require hemodialysis

(9) Reduced pulmonary function (lung function test in children older than 6 years: FEV1 and FVC < 70% of age-appropriate norm) or clinical suspicion of pulmonary disease affecting the patient's physical

performance, requiring invasive or noninvasive mechanical ventilation

(10) History of pulmonary embolism

(11) Pulmonary hypertension and/or right ventricular load in echocardiography

(12) Reduced cardiac function: left ventricular shortening fraction < 25%

(13) Clinically significant systemic infections

(14) Undergoing critical care treatment like mechanical ventilation, dialysis, or vasopressor agents

(15) Seropositivity for HIV, HTLV, or hepatitis B/C

(16) Hepatobiliary malignancies or history of any extrahepatic malignancy

(17) Thrombophilia

(18) Budd-Chiari syndrome

(19) Preexisting thrombosis of portal vein

(20) Doppler-sonographic evidence for relevant portosystemic shunts, for example, persistent ductus venosus

(21) Cold ischemia time > 90 min

(22) Known abuse of drugs or alcohol

(23) Known allergy to DMSO.

2.5. MSC Culture. Donor-derived mesenchymal stem cells will be obtained 4 weeks before the planned LDLT via bone marrow puncture of the living donor. All donors will undergo routine examination and screening tests including an extensive infectious disease work-up, according to our transplant center's donor screening protocol [37, 38]. Donors must also gain approval from the living donation committee. For MSC preparation, about 20 ml of bone marrow will be taken from the iliac crest under local anesthesia. The processing and expansion of the cells will take place at the Good Manufacturing Practice (GMP) facility of University Children's Hospital, Tübingen. MSCs are cultivated in cell culture flasks using animal-free cell media. For MSC culture, we use human albumin, plasma, and platelets that are obtained from healthy blood donors according to German Blood Transfusion Law. Normally, cultivation takes about 20 days and 2-3 passages are needed. With our culture method, we obtain about 50×10^6 cells with 20 ml bone marrow. Only in rare occasions, more bone marrow is needed for MSC therapy in pediatric recipients.

The cell product can only be released if the following criteria have been fulfilled: regular surface marker expression (CD105+CD73+CD90+CD45-HLA-DR-cells > 90%; CD3+, CD19+, and CD14+ cells < 0.5%); spindle-shaped morphology; a colorless cell suspension; viability of cells of >80%; absence of microbial contamination using culture, mycoplasma PCR, and endotoxin testing; and absence of cell aggregates. The MSC product is cryopreserved with 10% DMSO until designated application (storage at <−150°C in gas phase of liquid nitrogen). On the day of cell transfusion, the assigned dose of mesenchymal stem cells will be thawed at the GMP stem cell laboratory, washed, and suspended in an appropriate volume of isotonic saline with 0.5% albumin at a cell concentration ranging between about 1 and 1.5×10^6 cells/ml.

2.6. Study Treatment. The investigational treatment will consist of two transfusions of donor-derived mesenchymal stem cells, each dose $\sim 1 \times 10^6$ MSC/kg body weight, with the first infusion intraoperatively (day 0) and the second infusion postoperatively, on days 1–3 after living-donor liver transplantation (Figure 2). Further, all study participants will initially continue on the standard immunosuppressive regimen consisting of basiliximab, corticosteroids, and tacrolimus (tacrolimus trough level 5–10 ng/ml after 6 months), in accordance with our center's pediatric LT protocol. If the protocol liver biopsy 6 months post LT is unremarkable and there is no history of rejection, immunosuppressive medication can be reduced according to the stepwise tapering protocol aiming for a tacrolimus trough level of 2–4 ng/ml (Figure 2). In close collaboration with the responsible regulatory authorities, we have planned a staggered approach: the first three patients will receive study treatments one at a time, with a safety interval of 30 days. Consultation with the DSMB will be required before we resume patient recruitment.

Postoperative venous thrombosis prophylaxis is mandatory using low-dose heparin. After about 10 days, prophylaxis should be switched to aspirin and maintained for 3 months after LDLT. Prophylaxis against bacterial, fungal, and viral infections will adhere to our center's pLT protocol.

2.7. Data Collection. Children enrolled in this study will undergo a standard pretransplant work-up, which consists of baseline clinical data (demographics, medical history, current medication, physical examination, laboratory examinations, thrombophilia screening test, urinalysis, electrocardiogram, abdominal ultrasound, and chest X-ray). Pregnancy tests will be performed using a test for β-hCG in serum on all female participants with childbearing potential (those age 9 years or older) during the screening visit.

2.8. MSC Infusion. The first administration of 1×10^6 cells/kg body weight will be performed intraoperatively via portal infusion after complete liver allograft reperfusion (day 0). MSC suspension will be administered via a small venous catheter into the portal vein for 20 minutes while gently waving the syringe to keep cells in suspension. In addition to using Doppler ultrasonography, we will measure portal flow by transit time flow measurement (Medistim®) during cell infusion [39, 40]. Cell infusion should be discontinued if the portal flow decreases significantly (below 20 ml/min/100 g liver weight). The second MSC administration, dosing 1×10^6 cells/kg body weight, will be performed via a systemic intravenous route on postoperative day 2 (time window ± 1 day). MSC suspension will be transfused via a central venous catheter or peripheral venous catheter for 20 minutes under

Table 1: Assessment schedule MYSTEP1 study.

Visit	BL	MSC1	MSC2	Follow-up visits									
Days after LT	−28	0	2 ± 1	4	7	10	28	90	180	270	360	540	720
Informed consent Inclusion and exclusion criteria	x												
Concomitant medication			x	x	x	x	x	x	x	x	x	x	x
TAC dosage			x	x	x	x	x	x	x	x	x	x	x
Anthropometric parameters	x	x					x	x	x	x	x	x	x
Vital signs	x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination	x	x	x	x	x	x	x	x	x	x	x	x	x
Tacrolimus blood trough level			x	x	x	x	x	x	x	x	x	x	x
Routine laboratory including liver parameters	x	x	x	x	x	x	x	x	x	x	x	x	x
eGFR	x				x		x	x	x	x	x	x	x
Virus PCR: EBV, HCMV, and ADV	x				x		x	x	x	x	x	x	x
HHV-6	x				x		x						
Doppler ultrasonography	x	x	x	x	x	x	x	x	x	x	x	x	x
Infusional toxicity score		x	x	x	x	x	x						
TNF-α and IL-6 serum levels	x		x	x	x								
Immune monitoring	x				x		x		x		x		x
Antibodies: DSA; ANA, SMA, and LKMA	x								x		x		x
Percutaneous liver biopsy									x				
MSC administration		x	x										

BL: baseline; MSC1: first intraoperative MSC infusion; MSC2: second MSC infusion on postoperative day 2.

sterile conditions. During cell infusions, we will continuously monitor the patient's pulse, blood pressure, oxygen saturation, respiratory rate, and body temperature.

2.9. Follow-Up Visits. We will see patients frequently for follow-up visits during the first 28 days after transplantation. Study visits will consist of regular clinical examinations, Doppler sonography, and blood tests aimed at early detection of treatment-emergent events. We will assess the patient's MYSTEP toxicity score on days 0, 2, 4, 7, 10, and 28 after LDLT. Additional study visits will be performed up to 720 days after LDLT to assess allograft survival, incidence of rejection, incidence of (opportunistic) infections, kidney function, and individual need for immunosuppressive medication (Table 1). If the patient follows an unremarkable clinical course and a normal protocol liver biopsy, immunosuppressive drugs will be gradually reduced after six months (Figure 2), aiming at tacrolimus trough levels of 4 ng/ml or below after 12 months. After termination of the study, participants will be followed in our outpatient clinic for another five years. We will monitor the patients for long-term allograft function, extrahepatic organ function, and long-term complications, particularly the occurrence of malignancy.

2.9.1. MYSTEP Score. In order to evaluate and quantifiy treatment-emergent adverse events of MSC infusion, we defined a pediatric infusional toxicity score that adopts the MiSOT-I score for adults [41]. The score focuses on three independent modalities reflecting injury to the lungs and to the liver allograft, for example, by thrombembolism, and systemic reactions, such as anaphylaxis (Figure 1). For each of these three modalities, degrees of severity between 0 (no treatment-emergent adverse event) and 3 (severe treatment-emergent adverse event) were defined. Clinical data, blood gas analysis, chest X-ray, and doppler-ultrasound will be obtained on designated study visits (Table 1). The occurrence of two consecutive grade 3 events will be reported as severe adverse event. The MYSTEP score was validated retrospectively by analysing our cohort of pediatric LT recipients without investigational treatment [42].

2.9.2. Protocol Liver Biopsy. In this study, we will perform a protocol liver biopsy 6 months after LDLT and MSC infusion. A percutaneous liver biopsy will be performed under sonographic control and in analgosedation, according to recommendations of the ESPGHAN Hepatology Committee [43] and local standards. Additionally, we will perform protocol liver biopsies routinely every 5 years after LT. Liver tissue will be processed for immunohistochemistry (hematoxylin and eosin staining; staining for CD3, CD4, and CD20). Biopsies will be scored according to the Banff criteria and the liver allograft fibrosis scoring system [44]. Further, expression of anti- and proinflammatory cytokines will be measured by real-time RT-PCR in liver tissue.

2.9.3. Immune Monitoring. Additional blood samples will be collected to investigate surrogate markers of the participant's immune response status (Table 1). This immunological monitoring will include lymphocyte proliferation assay to evaluate antidonor reactivity, flow cytometry to describe the recipients' leucocyte phenotypes and presence of donor leucocytes (HLA chimerism), and serum analysis to screen

for DSA, liver-directed autoantibodies, and inflammatory cytokines. Further, we will screen for serological markers of iron hemostasis, that is, ferritin and hepcidin, which presumably play a role in development of operational tolerance after liver transplantation [45].

2.10. Risk-Benefit Assessment. MSCs may support induction of allograft tolerance and help to achieve long-term tolerance [25]. Patients may need smaller amounts of immunosuppressive drugs, which are associated with a risk of toxicity and in many cases prove fail to prevent long-term damage of the allograft. In addition, MSCs have the potential to foster regeneration of transplanted organs, for example, following ischemia-reperfusion injury [46, 47]. The associated risks of MSC therapy in pediatric LT recipients are unknown. For indications other than solid organ transplantation, the systemic application of MSCs in children has been shown to be safe, and no treatment-emergent adverse events have been reported [26, 48]. However, potential risks include transmission of infectious disease, thromboembolism, portal vein thrombosis, anaphylaxis, and carcinogenic effects [49]. These risks require preventive measures and continuous monitoring. In our own experience, more than 100 applications of MSCs in children in the setting of treatment for GvHD and Crohn's disease have shown MSCs to be well tolerated without occurrence of severe adverse events. Further, infusion of donor-derived MSCs bear a theoretical risk of sensitization by donor antigens, which could lead to formation of de novo DSAs and rejection. However, clinical studies using donor-derived MSCs after kidney transplantation [32, 33] demonstrated fewer rejection episodes and better allograft function one year after transplantation. On the basis of current experience, we believe that the potential for beneficial effects of MSC administration after liver transplantation and the limited potential risks of adverse side effects justify participation in this study.

3. Discussion

Introduction of current standard immunosuppressive therapies including CNIs has had a major impact on reduction of acute mortality after pediatric liver transplantation [2, 50]. However, life-long exposure to chronic pharmacological immunosuppressants impairs quality of life and reduces long-term survival. Children are particularly affected by chronic drug toxicity and long-term allograft failure [5]. These limitations motivate our search for alternative cellular treatment strategies for achieving allograft tolerance after pediatric liver transplantation. The immunomodulatory properties of mesenchymal stem cells, tested in both in vitro and in vivo models and in early clinical trials, may make them suitable for improved immunomodulation in pLT. The addition of MSCs to current immunosuppressive strategies can help to reduce the level of toxic CNIs and limit ischemia/reperfusion injury [47] while improving graft survival. Preclinical data suggest that MSCs may contribute to long-term allograft tolerance by induction of tolerogenic regulatory T-cells [18–20] and macrophages [22]. The first clinical studies of kidney transplantation have demonstrated the safety of MSC infusion and indicated the efficacy of MSCs in reducing allograft rejection and interstitial fibrosis [30, 31]. Further, clinical studies of the efficacy of allogeneic MSCs in liver transplantation are pending. Preliminary results underline a beneficial safety profile upon clinical application of this cell type [34, 35].

To our knowledge, this is the first clinical trial of immunomodulating therapy with mesenchymal stem cells in pediatric solid organ transplantation. In this pilot study, we aim primarily to determine safety and feasibility of intraportal and intravenous infusion of donor-derived MSC in children undergoing LDLT. We will assess safety based on incidence of acute infusion-related complications measured by the MYSTEP score, on occurrence of severe adverse events and on allograft function after pLT. We have designed our study protocol to optimize prevention of adverse events and to identify any that arise early. For example, using a "staggered approach" to patient recruitment and ensuring intraoperative quantitative monitoring of graft perfusion by transit time flow measurement will help ensure a very high level of safety for the children who participate. In adults, no toxicity has been observed during intravenous and intraportal infusion to date [51].

Previous studies of MSCs in kidney transplantation have shown contradictory data on the risk of overimmunosuppression, which may lead to opportunistic infections [30, 31]. This safety issue might play an important role in pediatric liver recipients, since they are more frequently naïve for EBV or HCMV infections compared to adult LT recipients. Therefore, as part of follow-up after pLT and MSC infusion, it is essential to frequently and accurately monitor infectious complications and TAC trough levels.

Many details of the mechanisms of immunoregulation by MSCs in transplant recipients remain unknown. It is evident, however, that an increase in the percentage of Foxp3 positive regulatory T-cells is one important mode of MSC action in transplant patients [28, 29, 35, 52]. Other cell types which are supposed to mediate the immunomodulatory and regenerative effects are dendritic cells, monocytes, macrophages [22], and MDSCs [20]. In the MYSTEP1 study, immune monitoring will therefore focus on quantitative analysis of leukocyte subpopulations using validated protocols [35, 53]. We will also analyze, in liver tissue, RNA expression of pro- and anti-inflammatory cytokines mediating immunomodulatory effects. Further, eventual humoral alloreactive responses will be monitored by detecting HLA and liver-specific antibodies.

The optimal donor source of MSCs is still unclear. In this trial, we will use bone marrow-derived MSCs obtained from the solid-organ donor. Donor-derived MSCs may contribute to donor-derived allograft tolerance [54]. Living-donor liver transplantation is a standard procedure in children and offers the opportunity to obtain MSCs and graft tissue from an identical living donor, frequently a parent. However, sensitization of the recipient and formation of de novo DSAs may constitute an adverse effect of allogeneic MSCs. A study of renal transplantation in an animal model reported increased allograft rejection and an increase of DSAs when donor-derived MSCs were administered 4 days before

transplantation [55]. This supports the hypothesis that timing of infusions and initial concomitant immunosuppressive treatment are crucial [56]. In this regard, previous clinical studies administering allogeneic, donor-derived MSCs at the time of kidney transplantation [32, 33] showed fewer rejection episodes and better allograft function one year after transplantation. In the MYSTEP1 trial, we will regularly screen for the occurrence of de novo DSA.

In addition to the source of MSCs, the time and route of cell application may influence the effectiveness of MSCs. Direct infusion of the cells into the graft can make use of the tissue-repair capacity of MSCs to treat ischemia reperfusion injury. Furthermore, preclinical models showed that in addition to systemic effects, local mechanisms were responsible for transplant tolerance by MSCs [19]. One important mechanism was attenuation of allostimulatory dendritic cells [23]. After intravenous application, the largest fraction of MSCs were pooled in the lungs [57] and lymph nodes and did not reach the liver. Still, most studies in solid organ transplantation apply intravenous MSC infusion. The observed immunomodulatory effects may be mediated longer term by other host cells [58]. Based on these considerations, in the MYSTEP1 trial, we will administer the first dose of MSCs intraportally at the end of the LDLT procedure and administer the second dose intravenously on postoperative day two [49].

In keeping with our focus on safety and feasibility, all participants will be treated in combination with the center's standard immunosuppressive regime, which consists of basiliximab, tacrolimus, and steroids. Preclinical studies suggested that CNIs and glucocorticoids may affect MSC morphology, migration, and immunomodulatory behavior, possibly affecting the success of the cell therapy [59, 60]. However, current studies of adult kidney transplant recipients that have applied MSCs in combination with steroids and CNIs [29–33] have demonstrated potential effectiveness in spite of the concomitant use of CNIs and steroids. To address questions about the synergistic or counteractive effects of immunosuppressive medication on MSC function, a planned phase 2 clinical study will focus on the optimization of an immunosuppressive treatment regime in combination with MSC infusions.

In conclusion, MSCs have the potential to become part of an array of novel treatment options for pediatric LDLT recipients aimed at promoting allograft tolerance and improving long-term allograft survival while reducing toxicity of chronic IS treatment. A positive outcome of the MYSTEP1 trial in terms of safety and allograft survival would constitute a major advancement in pediatric solid organ transplantation. Subsequently, we intend to conduct a second, larger multicenter trial to study the immunomodulatory efficacy of MSC treatment protocols for improving long-term allograft tolerance in pediatric liver transplant recipients.

Abbreviations

ADV: Adenovirus
ANA: Antinuclear antibody
BPAR: Biopsy-proven acute rejection
DSA: Donor-specific HLA-antibody
HCMV: Human cytomegalovirus
DMSO: Dimethylsulfoxide
DSMB: Data safety monitoring board
EBV: Epstein-Barr virus
INR: International normalized ratio
IS: Immunosuppression
ICU: Intensive care unit
ISCT: International Society of Cellular Therapy
LDLT: Living-donor liver transplantation
LKMA: Anti-liver-kidney microsomal antibody
LT: Liver transplantation
MDSC: Myeloid-derived immunosuppressive cell
MSC: Mesenchymal stem (stromal) cell
pLT: Pediatric liver transplantation
RI: Resistance index
RT-PCR: Reverse transcription polymerase chain reaction
TA Vmax: Time-averaged maximum velocity
SAE: Severe adverse event
SMA: Anti-smooth muscle antibody.

Ethical Approval

The MYSTEP1 study was approved by the Medical Ethics Committee of the University of Tübingen (Ref. 003/2016AMG1).

Disclosure

The funding bodies have no influence on the study design and collection, analysis, and interpretation of data.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Steffen Hartleif and Ekkehard Sturm designed the study and will conduct the study as principal investigators. Michaela Döring, Alfred Königsrainer, Silvio Nadalin, Marc H. Dahlke, and Rupert Handgretinger contributed to the idea and design of the study. Michael Schumm, Peter Lang, and Rupert Handgretinger set up and coordinate the MSC culture and quality controls in the GMP facility. Michaela Döring, Markus Mezger, and Steffen Hartleif contributed to the design and implementation of immune monitoring. All authors have read and approved the final manuscript.

Acknowledgments

The authors thank Cathy Shufro for the text editing. This study is supported by the Center for Rare Diseases, Tübingen; the University of Tübingen; and the Astellas European Foundation.

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Hindawi
Stem Cells International
Volume 2017, Article ID 8178643, 11 pages
https://doi.org/10.1155/2017/8178643

Research Article

PGE2 Promotes the Migration of Mesenchymal Stem Cells through the Activation of FAK and ERK1/2 Pathway

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Received 28 November 2016; Revised 3 February 2017; Accepted 29 March 2017; Published 28 May 2017

Academic Editor: Luc van der Laan

A critical step of MSCs therapy is dependent on its ability to migrate into the sites of injury, so various approaches have been introduced to boost the migratory ability of MSCs. PGE2 is the major prostaglandin generated by COX enzymes and has been implicated in inflammatory response. Evidence indicates that PGE2 can facilitate MSCs migration. Further exploration of the underlying molecular mechanism participating in the promigratory ability of PGE2 may provide a novel strategy to improve MSC transplantation efficacy. In this study, our findings suggested that EP2 prostanoid receptor promotes MSCs migration through activation of FAK and ERK1/2 pathways. Furthermore, MSCs migration induced by PGE2 was blunted by FAK or ERK1/2 inhibitors. EP2-mediated MSCs migration depends on the activation of FAK and ERK1/2. However, the current study did not investigate the migration of MSCs over a blood vessel endothelial barrier. In conclusion, our findings reveal EP2-mediated FAK and ERK1/2 activation was essential for MSCs migration induced by PGE2, indicating that activation of EP2 receptor and FAK/ERK pathways may be a promising strategy to accelerate homing efficiency of MSCs, which in turn enhances therapeutic potential of MSCs transplantation.

1. Introduction

Mesenchymal stem cells (MSCs) are pluripotent nonhematopoietic cells derived from multiple sources, including bone marrow, adipose tissue, and umbilical cord blood [1–3]. Owing to their immunoregulatory, anti-inflammatory and proregenerative properties, MSCs are becoming an appealing candidate in treating various diseases [4, 5]. However, the therapeutic efficacy of MSCs is contingent upon their migratory capacity to sites of injury [6, 7]. Given only a small fraction of transplanted cells engrafted into injured tissue after MSC injection, various approaches have been introduced to facilitate engrafting efficiency, such as overexpression of chemokine receptor CXCR4 [8], or MSC cultured under hypoxic condition [9], both resulting in a higher retention of transplanted MSCs compared with control, offering incremental benefits.

PGE2 is the major prostaglandin generated by COX-1and COX-2 enzymes and exerts distinct actions in a broad array of physiologic and pathologic settings [10, 11]. PGE2 synthesis is markedly increased in the inflammatory setting [12]. It is well documented that PGE2 mediates both pro- and anti-inflammatory responses via binding to its four receptors, namely EP1-EP4 [13]. Aside from its role in inflammatory response, PGE2 also participates in proliferation and migration in several cell types [14–16]. In addition, Yun and his colleagues suggested that MSC migration can be boosted by PGE2 stimulation [17]. Among the four receptor subtypes, EP2 receptor activation was identified to link PGE2-induced MSC migration.

Focal adhesion kinase (FAK), also known as cell adhesion kinase, was initially identified in 1992 as a member of nonreceptor cytoplasmic tyrosine kinase [18]. The structure of FAK comprises an N-terminal region, a C-terminal region, and a central kinase region [19]. The N-terminal region of FAK serves as a linker between membrane integrin receptors and various growth factor receptors, transducing extracellular signaling input to cellular cytoskeleton. The C-terminal domain of FAK containing a ~100 sequence so called FAT and two proline-rich regions. FAT mediates binding with integrin-binding protein paxillin and talin, facilitating the adhesion of FAK to focal contact [20].

Over the last decades, accumulating evidence indicates that FAK functions in promoting cell migration in diverse array of normal and tumor cells [21, 22]. In addition, it has been proposed that the underlying mechanism FAK facilitated cell migration was through its promotion of cytoskeletal rearrangements and focal contact formation. Using fibroblast cell line, researchers demonstrated that FAK-deficient cells migrate poorly in response to growth factor stimulation [23]. Conversely, re-expression of FAK rescues the motility defect of fibroblast cell. Additionally, overexpression of FAK in ovary cells exhibited accelerated cell motility. Taken together, considering FAK signaling implicated in cell motility in various different cell lines, its function in the migration of MSCs is of potential importance.

2. Methods

2.1. Cell Line. Bone marrow-derived MSCs were purchased from Cyagen Biosciences (Guangzhou, China). CD45−, CD73+, CD90+, and CD105+ MSCs were confirmed by flow cytometry as we previously described [24]. We also evaluated the osteogenic differentiation and adipogenic differentiation capacity of MSCs using specific differentiation media. In our study, MSCs were obtained from one donor and undergone culture expansion in vitro to purify and generate sufficient numbers for transduction and experiment.

2.2. MSCs Culture. MSCs were seeded at 1×10^5 cells/cm^2 in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. MSCs were cultured at 37°C in a humidified atmosphere in an incubator with 5% CO_2. Media were changed every 3 days. Adherent MSCs were washed with phosphate-buffered saline (PBS) and harvested by trypsinization with 0.25% trypsin. MSCs on the fourth to sixth passages were used for experiments.

2.3. Construction of MSCs Overexpressing EP2 Receptor. The EP2 plasmid was constructed as described previously [24]. Then, human embryonic kidney 293T cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were transfected recombinant lentiviral vectors with EP2 or GFP. MSCs from passages 4 to 6 were transduced with high-titer recombinant lentiviral vectors with EP2 or GFP. Because MSCs expressed both the EP2 and GFP genes (EP2-MSCs), the transduction efficiency was assessed with a fluorescence microscope. EP2 expression was assessed using Western blotting and PCR, as we described previously [24]. After MSCs were successfully transduced with recombinant EP2 plasmids, we evaluated cell surface markers (CD73, CD90, CD105, and CD45) and the osteogenic differentiation ability of MSC-EP2. In addition, we evaluated the migratory ability of MSCs via transwell assay following the completion of EP2 overexpression.

2.4. Migration Assay. MSCs migratory ability was assessed by a 24-well transwell system inserting a 8 μm pore size filter membrane. 2×10^4 cells in a serum-free medium of 200 μL volume were added to the upper chamber, and 600 μL serum-free medium or PGE2 (1 μmol/L) was added to the bottom chamber. Subsequently, MSCs were incubated at 37°C for 6 h in serum free medium with PGE2 in the presence or absence of FAK inhibitor PF573228 (10 μmol/L) or ERK1/2 inhibitor PD98059 (10 μmol/L). 600 μL serum-free medium in the presence or absence of PGE2 was added to the bottom chamber. After 6 h incubation at 37°C, the cells on the upper surface of the filter membrane were removed, whereas the migrated cells on the undersurface of the filter membrane were fixed with 4% paraformaldehyde, and cells on the undersurface of the filter were subsequently stained in crystal violet. Cells migrated to the lower surface of the filter were imaged using a microscope. Five fields from each filter were randomly chosen to determine the number of migrated cells.

2.5. Wound Healing Migration Assay. Migration was also evaluated by a wound healing migration assay. MSCs were cultured in 6-well plates until reached a confluent monolayer. Monolayers of MSCs were scratched with a disposable pipette tip and then wells were washed with phosphate buffered saline to remove the cell debris. Subsequently, MSCs were incubated at 37°C for 24 h in serum-free medium with PGE2 in the presence or absence of FAK inhibitor PF573228 (10 μmol/L) or ERK1/2 inhibitor PD98059 (10 μmol/L). The scratched area was photographed with a digital camera attached to a light microscope. Means were taken from five fields in the scratched area of each group. Migration assay was performed 3 separate times.

2.6. Western Blot Analysis. For Western blot, total protein from the cultured cells were extracted with RIPA reagents and stored at −80°C. Equal amounts of protein were electrophoresed on a 8% or 5% SDS-PAGE, then transferred to polyvinylidene fluoride membrane. Subsequently, membranes were blocked with TTBS (Tris-buffered saline with 0.1% Tween 20) containing 5% dry milk for 1 h at room temperature. After washing, PVDF membranes were incubated with indicated primary antibody for overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated goat antirabbit IgG for 1 h at room temperature. Immunoreactive proteins were detected using a chemiluminescence kit. Band intensities were quantified using ImageJ software. Each experiment was performed 3 separate times.

2.7. Statistical Analysis. Data were presented as mean ± standard deviation (SD). Comparisons between two groups

were made using an unpaired t-test. Differences among groups were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's correction. The SPSS software 17.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Characterization of MSCs. The cultured MSCs presented a uniformly spindle shape. MSCs were demonstrated to differentiate into adipocytes and osteocytes. MSCs exhibited very high expression of CD105, CD73, and CD90 but negligible expression of CD45 as previously reported [24].

3.2. MSCs Were Successfully Transduced with Recombinant EP2 Plasmids. Seven days after transduction, 97% of MSCs-EP2 were GFP positive, indicating the MSCs were stably transduced with lentivirus expressing either GFP or both GFP and EP2. The expression of GFP in the MSCs was detected under fluorescence also confirmed that MSCs were successfully transduced with recombinant EP2 plasmids. The Western blots showed that EP2 protein and mRNA expression in the EP2-MSCs was higher than that in the MSCs that had only received vector (MSC-vec) and the control MSCs, the detailed results reported in our published paper [24].

3.3. EP2 Overexpression Does Not Affect MSCs Surface Marker and Differentiation Ability. To explore the impact that over-expression of EP2 has on the functional characteristics of MSCs, we evaluated cell surface markers (CD73, CD 90, CD105, and CD45) of MSC-EP2 via flow cytometry. Furthermore, we evaluated the osteogenic and adipogenic differentiation ability of MSCs following the completion of EP2 overexpression. The MSCs-EP2 were positive for CD73, CD90, and CD105 and negative for CD45 (Figure 1(a)), suggesting that EP2 overexpression does not change cell surface receptor phenotype. In addition, overexpression of EP2 does not impact MSCs osteogenic differentiation capability (Figure 1(b)).

3.4. EP2 Overexpression Does Not Affect MSC Migration. To investigate whether EP2 overexpression affect MSC migration, we evaluated the migratory ability of MSCs via transwell assay following the completion of EP2 overexpression and found that EP2 overexpression alone did not affect MSCs migration (Figure 1(c)).

3.5. PGE2 Promotes Migration of MSCs. To examine whether PGE2 plays a role in the migration of MSCs, confluent MSCs were wounded and incubated for additional 24 h in serum-free medium with or without PGE2 (1 μmol/L). In contrast to control, PGE2 evoked a substantial migration of the cells into the scratched area. Transwell filters were also used to further evaluate the migratory ability of MSCs stimulated with or without PGE2 (1 μmol/L); PGE2-treated MSCs almost filled the scratched area and the width distance was significantly narrower than that of MSCs without PGE2 stimulation (Figure 2(a)). To clarify the activation of EP2 receptor which may underlie PGE2-induced MSCs migration. Transwell assay revealed that the number of MSCs-EP2 traversing the membrane was significantly more than the control when stimulated with PGE2 (Figure 2(b)).

3.6. FAK Signaling Contributes to PGE2-Mediated Migration of MSCs. To investigate whether FAK signaling participated in PGE2-mediated migration of MSCs, the activation status of FAK was assessed by Western blot. Immunoblotting revealed that the phosphorylated expression level of FAK is much higher in PGE2-treated MSCs compared to the counterparts without PGE2 stimulation. We also examine whether EP2 receptor participated in PGE2-induced activation of FAK. The expression of FAK in MSCs-EP2 was also tested. Immunoblotting revealed that the phosphorylated expression level of FAK is much higher in MSCs-EP2 treated with PGE2 compared to the MSCs stimulated with PGE2 (Figures 3(a) and 3(b)).

3.7. Involvement of ERK1/2 Pathways in PGE2-Induced MSCs Migration. ERK1/2 plays a crucial role in regulating cell migration via relaying signal from FAK in several cell types. To investigate the involvement of ERK1/2 in PGE2-induced MSCs migration, we detected the effect of PGE2 on ERK1/2 expression. MSCs were stimulated with 1 μmol/L PGE2. MSCs treated with PGE2 showed an increased expression of ERK1/2. We also examine whether EP2 receptor participated in PGE2-induced activation of ERK1/2. The expression of ERK1/2 in MSCs-EP2 was also tested. Immunoblotting revealed that the phosphorylated expression level of ERK1/2 is much higher in MSCs-EP2 treated with PGE2 compared to the MSCs stimulated with PGE2 (Figures 3(c) and 3(d)).

3.8. FAK Inhibitor Suppressed PGE2-Induced MSCs Migration. To further elucidate the role of FAK in MSCs migration, FAK inhibitor was added at the indicated time. Wound-healing assay demonstrated that MSCs and MSCs-EP2 migration was markedly reduced in the presence of FAK inhibitor PF573228 (Figure 4(a)). Transwell assay revealed that the number of treated MSCs traversing the membrane was significantly less than the untreated control when incubated with PGE2 (Figure 4(b)). Taken together, FAK signaling is a required component for MSCs migration.

3.9. ERK1/2 Inhibitor Suppressed PGE2-Induced MSCs Migration. Furthermore, we confirmed that PGE2-induced ERK1/2 activation was essential for promoting migration and wound healing of MSCs by performing transwell migration assay and wound healing experiment in the presence of PD98059, a specific inhibitor of the ERK1/2 pathway. Inhibition of ERK1/2 by PD98059 also blocked the ability of PGE2 to promote migration of MSCs and fill a wound in in vitro wound-healing assays (Figure 5(a)). In agreement with these findings, Transwell migration assay demonstrated that MSCs and MSCs-EP2 migration was markedly reduced in the presence of PD98059 (Figure 5(b)).

3.10. FAK and ERK1/2 Inhibitor Successfully Blocked FAK and ERK1/2, Respectively. To demonstrate that the inhibitors

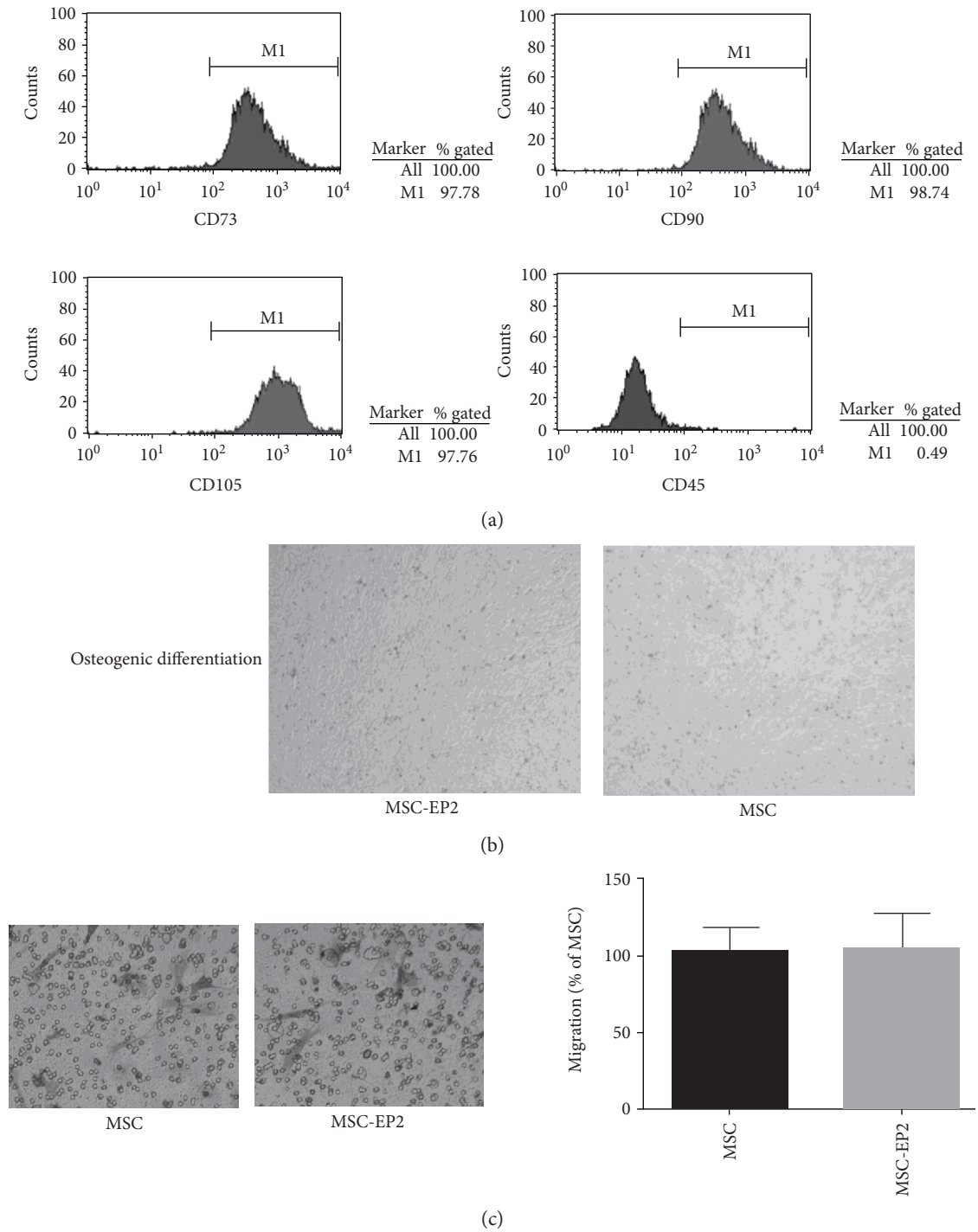

Figure 1: (a) The MSCs-EP2 were positive for CD73, CD90, and CD105 and negative for CD45. (b) Overexpression of EP2 does not impact MSCs osteogenic differentiation capability. (c) Transwell assay demonstrated that EP2 overexpression does not affect MSC migration, $P > 0.05$ (MSC versus MSC-EP2).

successfully blocked FAK and ERK1/2, respectively, we evaluated the activation state of FAK and ERK1/2 following the supplementation of FAK and ERK1/2 inhibitors via Western blot. Our results demonstrated that FAK and ERK1/2 inhibitors successfully blocked FAK and ERK1/2, respectively (Figures 6(a) and 6(b)).

4. Discussion

Mesenchymal stem cells have emerged as a leading candidate for treating numerous diseases due to their multipotent capacity, immune privilege properties, and their ability to secrete trophic factors. Beneficial effects induced by MSCs

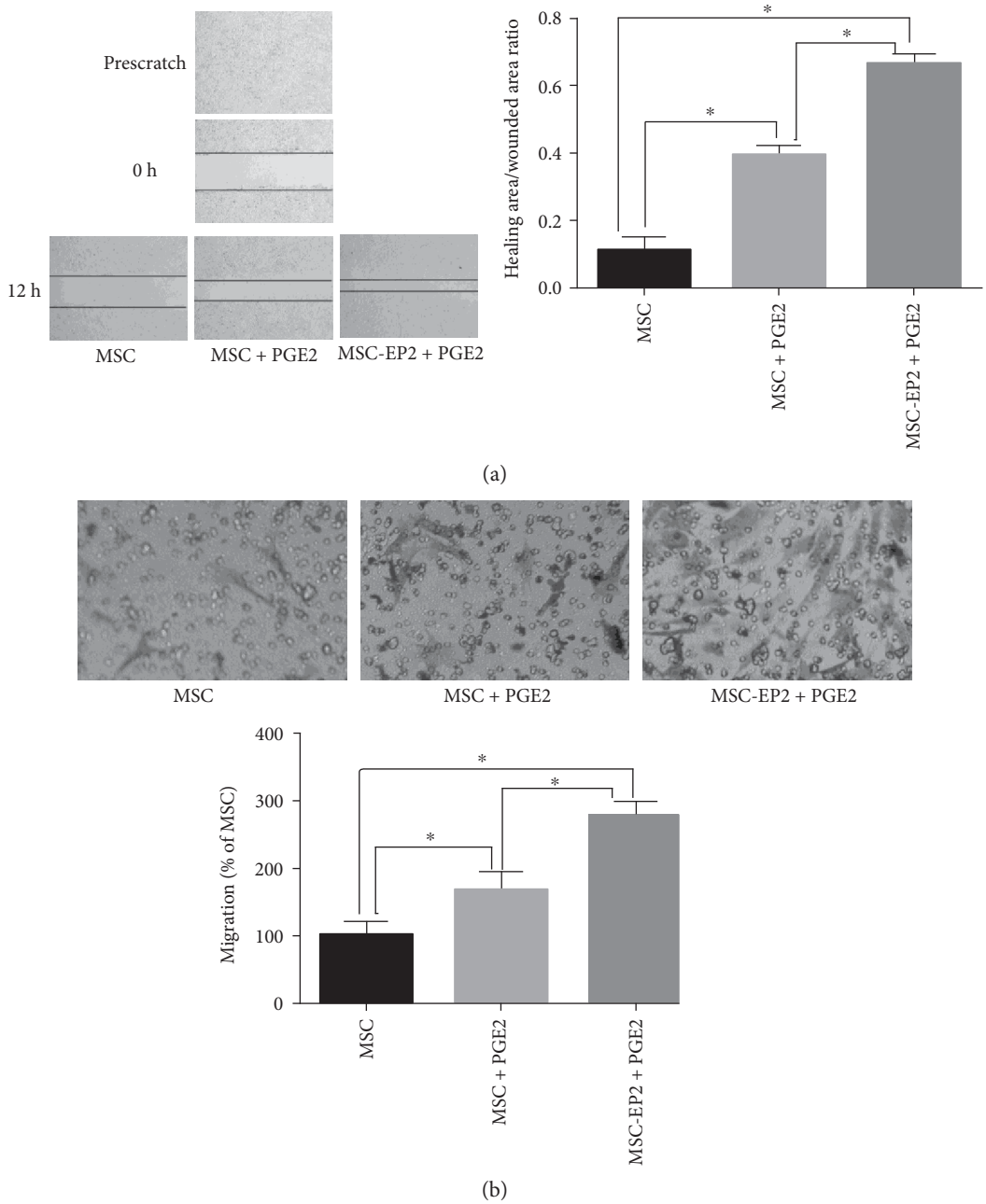

Figure 2: (a) Wound-healing assay demonstrated PGE2 facilitated MSCs and MSCs-EP2 migration. (b) Transwell assay demonstrated that PGE2 facilitated MSCs and MSCs-EP2 migration, $^{*}P < 0.05$.

are mainly resulted from their ability to secrete a great variety of paracrine factors rather than the degree of MSC differentiation. Low engraftment of transplanted MSCs in the site of injury is one of the obstacles MSCs face for clinical application [25]. Methods to ensure the sustained expression of cytoprotective cytokines in the injured tissues confer incremental efficacy. A growing body of evidence demonstrated that further increase in the migration ability after MSC delivery produces a pool of trophic factors within the damaged tissue, consequently leading to the enhanced therapeutic outcome [26].

PGE2 plays a critical role in the regulation of inflammation, the modulation of immune process, and the maintenance of intestinal mucosa. COX-derived PGE2 can be produced by many cell types, including macrophage and dendritic cell. The complex roles of PGE2 are mediated by four G-protein coupled receptors (EP1–EP4), delivering different physiologic and pathophysiologic effects. Over the last decade, increasing attention has been paid to the role of PGE2 in the regulation of cellular migration. PGE2 facilitating cellular migration has been well established using several normal and tumor cells [27]. Short-term ex vivo exposure of

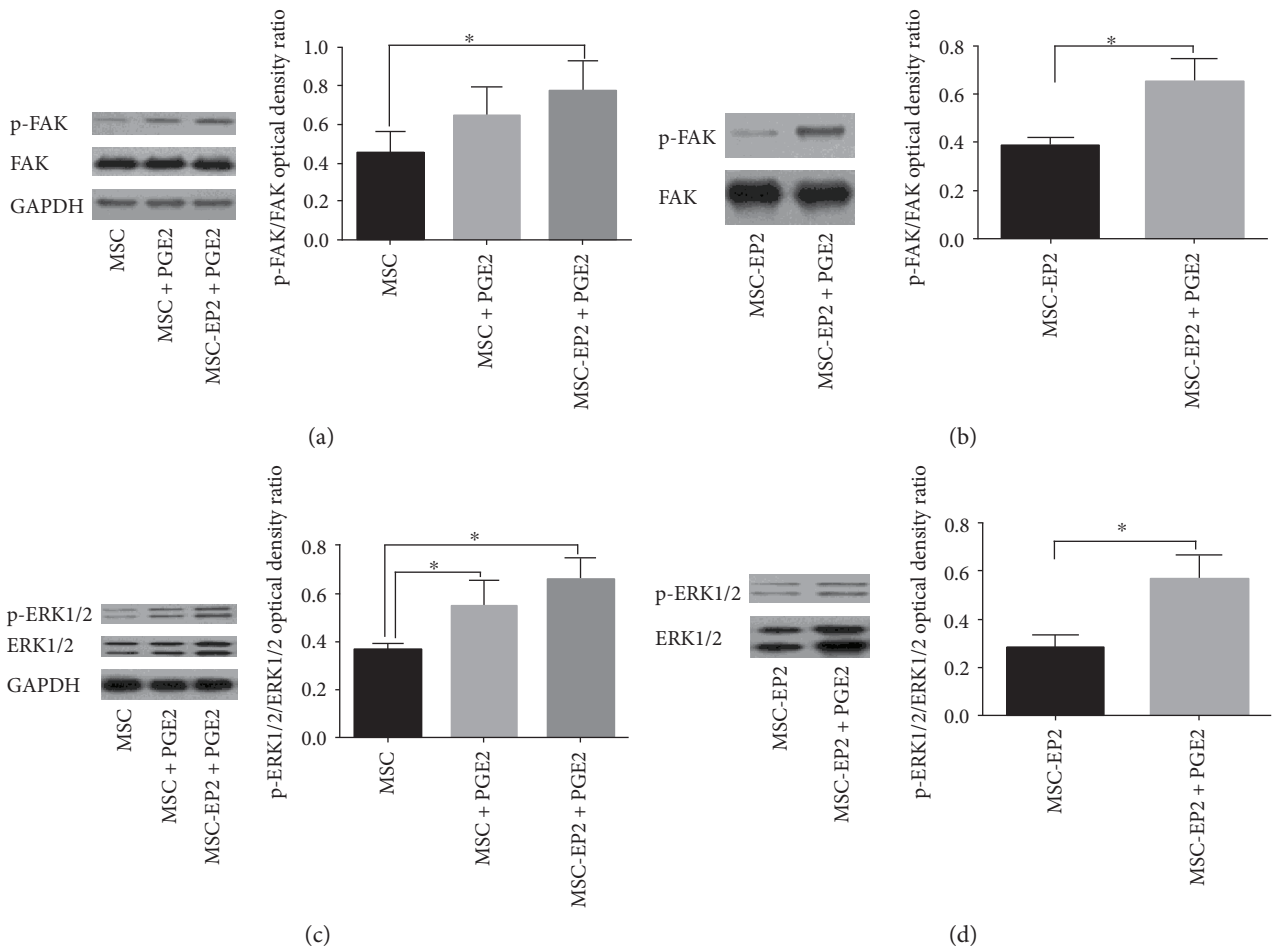

Figure 3: (a) Immunoblotting revealed that the phosphorylated expression level of FAK is much higher in MSCs-EP2 treated with PGE2 compared to the MSCs stimulated with PGE2. (b) Immunoblotting revealed that the phosphorylated expression level of FAK is much higher in PGE2 treated MSCs-EP2 compared to the counterparts without PGE2 stimulation. (c) Immunoblotting revealed that the phosphorylated expression level of ERK1/2 is much higher in MSCs-EP2 treated with PGE2 compared to the MSCs stimulated with PGE2. (d) Immunoblotting revealed that the phosphorylated expression level of ERK1/2 is much higher in PGE2-treated MSCs-EP2 compared to the counterparts without PGE2 stimulation, $^{*}P < 0.05$.

HSCs to PGE2 was shown to be correlated with increased homing of HSCs after transplantation. Moreover, Donnini et al. have demonstrated that treatment with PGE2 markedly increased the proliferation and migration of squamous carcinoma cells [28].

Prostaglandin E2 (PGE2) is the prominent prostaglandins generated from arachidonic acid by cyclooxygenases and PGE2 sysnthases. PGE2 is a critical mediator that modulates diverse physiologic and pathophysiologic processes via an autocrine or paracrine fashion. PGE2 can potentiate the migratory capacity of MSC to target tissue. A critical step of MSCs therapy is dependent on its ability to migrate into the sites of injury. Further exploration of the underlying molecular mechanism participating in the promigratory ability of PGE2 may provide a novel strategy to improve MSC transplantation efficacy. This may represent a novel strategy to accelerate MSC transplantation efficiency. PGE2/EP2 plays a crucial role in MSC homing to sites of injury. PGE2 serves as a homing signal for MSCs expressing EP2 to navigate MSC to reach the sites of tissue damage. We utilized virus-mediated EP2 transduction to upregulate the expression of EP2 on the surface of MSCs, which resulted in increased migration of MSCs to PGE2 when compared to control MSCs.

Elevated FAK expression was observed after MSCs treated with PGE2. Our results also revealed that FAK inhibitor-treated MSCs exhibited decreased cell motility and a slower wound closure, suggesting that FAK signaling is a critical component in the regulation of MSCs migration. FAK was thought to play a prominent role in the regulation of cell migration, as evidenced in several different cell settings [29]. FAK-null fibroblast exhibit a slower motility as compared with that observed in normal fibroblast. Additionally, carcinoma cells using antisense treatment to reduce FAK expression indicate that these cells exhibit cell motility defect. Conversely, increased cell motility was observed after FAK overexpression in squamous cell. Taken together, FAK may be a required component for MSCs migration similar to that observed in other cell lines.

The downstream signal by which FAK contributes to PGE2-induced MCSs migration was unclear. Extracellular

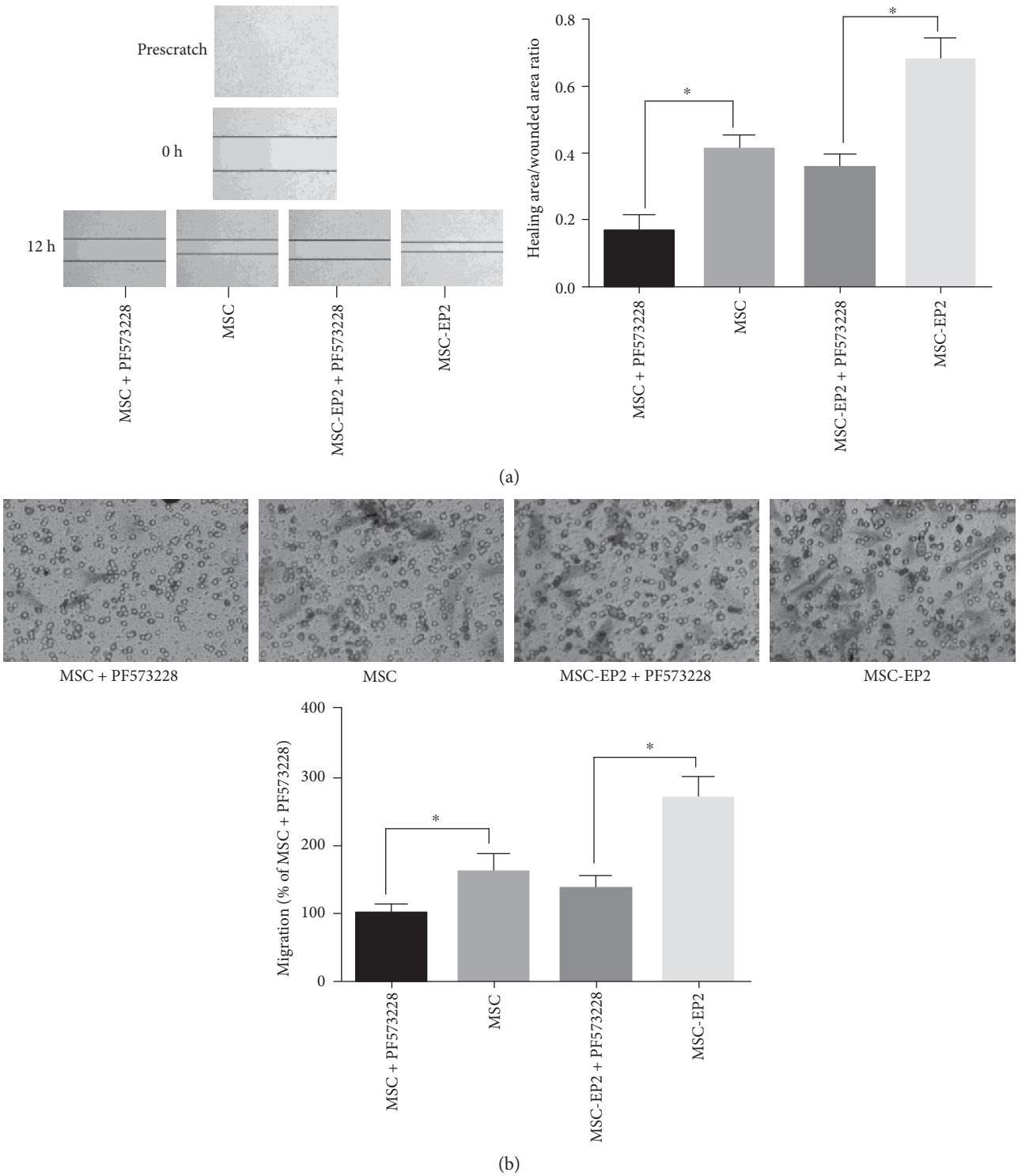

Figure 4: (a) Wound-healing assay demonstrated that MSCs and MSCs-EP2 migration was markedly reduced in the presence of FAK inhibitor PF573228. (b) Transwell assay revealed that the number of FAK inhibitor PF573228 treated MSCs and MSCs-EP2 traversing the membrane was significantly less than the untreated control when incubated with PGE2, $^{*}P < 0.05$.

signal-regulated kinase (ERKs) is the subfamily of serine/threonine protein kinases, namely mitogen-activated protein kinases (MAPKs). Two isoforms ERK-1 and Erk-2 have been identified [30]. Erk1/2 has been participating in numerous cell functions, such as cellular proliferation and apoptosis. In particular, evidences have shown that ERK1/2 plays a crucial role in regulating cell migration via relaying signal from FAK in several cell types [31].

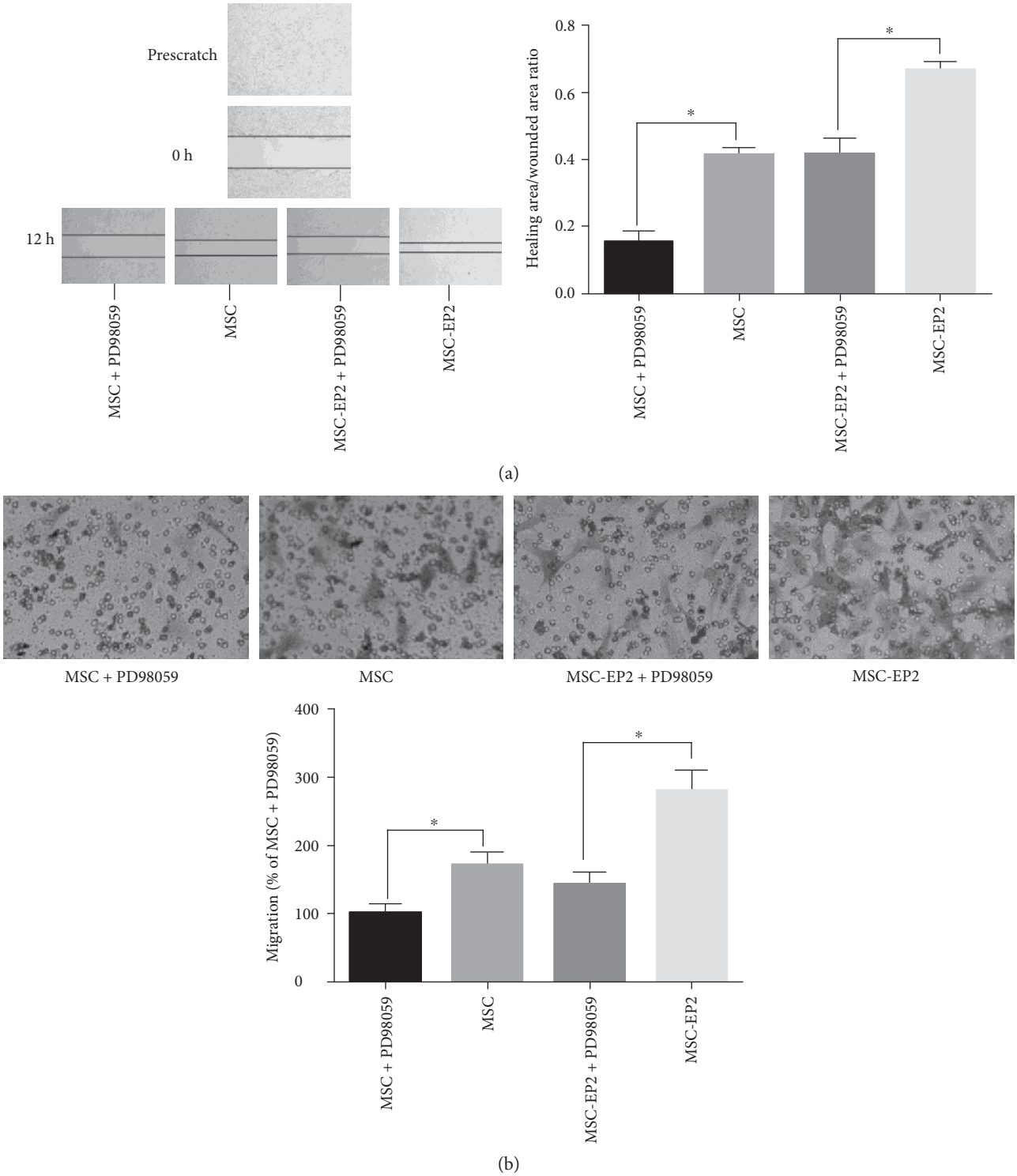

Figure 5: (a) Wound-healing assay demonstrated that MSCs and MSCs-EP2 migration was markedly reduced in the presence of ERK1/2 inhibitor PD98059. (b) Transwell assay revealed that the number of ERK1/2 inhibitor PD98059 treated MSCs and MSCs-EP2 traversing the membrane was significantly less than the untreated control when incubated with PGE2, $^{*}P < 0.05$.

The study by Cuevas et al. demonstrated that ERK1/2 was an essential component for fibroblasts migration. Additionally, human osteoblastic cells exhibit reduced cell motility after ERK antisense treatment of these cells [32]. In our study, the expression of ERK1/2 was upregulated in MSCs in response to PGE2 stimulation. Furthermore, exposure of MSCs to specific ERK1/2 inhibitor effectively blocks PGE2-induced migration of MSCs. Taken together, our findings suggested that ERK1/2 signaling plays a key role in regulating of MSC migration.

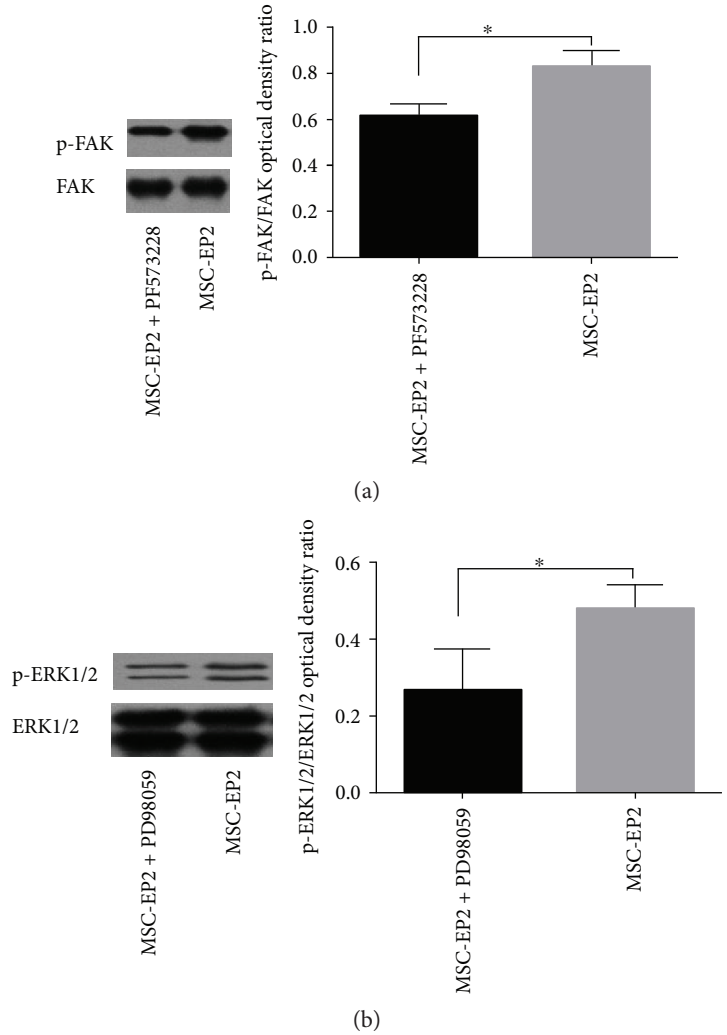

Figure 6: (a) Western blot demonstrated that FAK inhibitor PF573228 successfully blocked FAK. (b) Western blot demonstrated that ERK1/2 inhibitor PD98059 successfully blocked ERK1/2, $^*P < 0.05$.

Several limitations in our study deserve consideration. First, one clear limitation of the current study is that it does not investigate the migration of MSCs over a blood vessel endothelial barrier. Second, pharmacological inhibitors were used to reduce FAK and ERK1/2 expression, deletion of FAK and ERK1/2 by genetic knockdown may make the results more robust. Third, endogenous PGE2 produced from MSCs plays a vital role in inflammatory setting and whether endogenous PGE2 contributes to MSCs migration is still unclear. In addition, evidences indicate that cytoskeletal dynamic was involved in the cellular migration process, and to investigate the change of cellular shape and the formation of stress-fiber may further understand the role of PGE2 in regulating MSCs migration. Thus, future studies are needed to address the above questions.

In conclusion, our findings reveal PGE2 binding to its subtype receptor, EP2 in MSCs, and subsequent activation of FAK and ERK1/2 signaling, thereby facilitating MSCs migration, indicating that activation of EP2 receptor and FAK/ERK pathways may be a promising strategy to accelerate homing efficiency of MSCs, which in turn enhances therapeutic potential of MSCs transplantation.

Disclosure

Xiaomin Lu and Jibin Han are co-first authors.

Conflicts of Interest

All authors declare no conflict of interest.

Acknowledgments

This work is financed by the National Natural Science Foundations of China (81201489, 81372093, 81571874, 81501705, 81300060, and 81471843), Clinical Medicine Science and Technology Projects in Jiangsu province (BL2013030).

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Hindawi
Stem Cells International
Volume 2017, Article ID 5251313, 12 pages
https://doi.org/10.1155/2017/5251313

Review Article

Roles of Mesenchymal Stem Cells in Spinal Cord Injury

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Received 20 January 2017; Accepted 24 April 2017; Published 28 May 2017

Academic Editor: Luc van der Laan

Spinal cord injury (SCI) represents one of the most complicated and heterogeneous pathological processes of central nervous system (CNS) impairments, which is still beyond functional regeneration. Transplantation of mesenchymal stem cells (MSCs) has been shown to promote the repair of the injured spinal cord tissues in animal models, and therefore, there is much interest in the clinical use of these cells. However, many questions which are essential to improve the therapy effects remain unanswered. For instance, the functional roles and related molecular regulatory mechanisms of MSCs in vivo are not yet completely determined. It is important for transplanted cells to migrate into the injured tissue, to survive and undergo neural differentiation, or to play neural protection roles by various mechanisms after SCI. In this review, we will focus on some of the recent knowledge about the biological behavior and function of MSCs in SCI. Meanwhile, we highlight the function of biomaterials to direct the behavior of MSCs based on our series of work on silk fibroin biomaterials and attempt to emphasize combinational strategies such as tissue engineering for functional improvement of SCI.

1. Introduction

Spinal cord injury (SCI) usually results in severe neural dysfunction below the injury site. Moreover, mammals are unable to regenerate their spinal cords after injury which can lead to lifelong disability and loss of independence. After a primary damage of spinal cord tissue by a direct mechanical force, a series of secondary events involving various pathological responses accelerate the tremendous cell loss, release of cytotoxic factors, and cystic cavitation [1, 2]. Furthermore, excessive extracellular matrices produced by activated astrocytes, called glial scarring, together with the hostile microenvironment, severely inhibit cell migration and axonal regrowth [3]. Although many experimental and clinical studies have been tested, it still lacks effective treatment until now [4–6]. The neuropathological outcome of SCI is complicated, and therefore, several challenging objectives, such as decreasing neural cell death, reducing scarring and cavitation, regaining healthy neural cells, and stimulating functional axonal regeneration, remolding the injury niche should be taken into consideration [7–11].

Numerous studies have demonstrated that stem cells might provide a source of neural cells as well as exerting neuroprotective effects after SCI. Among them, mesenchymal stem cells (MSCs) emerged as one of the most promising types of stem cells due to a favorable ethical profile and better safety [12]. The present data revealed that recovery after MSC implantation therapy is comparatively low possibly because of uncertain neural plasticity and limited capacity for the axonal regeneration of MSCs in the spinal cord [13, 14]. The therapeutic application of MSCs in SCI is still in its infancy. It is of considerable interest as to how stem cells respond to the local environment and play functional roles in vivo, which will provide important information for improving the therapy effects and designing better therapeutic strategies.

2. The Biological Behavior of MSCs In Vivo

2.1. Migration of MSCs. A few points need to be taken into account to obtain more effective stem cell therapy outcomes. For instance, it is important for transplanted cells to arrive

and migrate into the injured spinal cord tissue after intravenous infusion. It has been demonstrated that MSC homing toward injured tissue is not an efficient process; very few cells reach the injury site [15]. Some of the transplanted cells were trapped into the lung and other organs while many cells were sacrificed during the journey [16]. And only a small percentage of cells were verified to have high homing ability since the transplanted MSCs are always mixed cell populations. There are experimental data that support that MSCs possess high migratory potential and higher ability to help neural regeneration. In this case, it is believed that the insufficient number of migratory cells will partly account for the decreased number of transplanted MSCs and further decreased the cell therapy effects.

On the other hand, it is also crucial for MSCs to migrate and integrate into the host spinal cord tissue after cells are injected into a lesion, or close to a lesion area. It is not surprising that people may feel confused: Why do cells need to migrate if they are already in the lesion area? We noticed that cells would die quickly if they stayed in the injection site by in situ MSC transplantation after SCI. Actually, MSCs were observed to be migrating away from the injection site in the first 1 hour after cell transplantation. By 7 days, the cells had migrated across the injury site to form a cellular scaffold, suggesting migration toward the injury sites [17]. Also, some cells with neuronal marker expression were observed in the injured and surrounding tissues after MSC transplantation [18]. However, the engraftment potential of MSCs was low which was verified by many experiments. Indeed, MSCs delivered via injection largely remained restricted to the lesion site and were not seen to contact significant amounts of the host spinal cord tissue. The numbers of the engrafted cells are dramatically decreased after transplantation by either in situ injection or intravenous infusion [19]. It was reported that there were small numbers, even less than 0.001% to 0.002%, of the transplanted MSCs left, and few functional neurons were detected after cell transplantation [20–23].

There are studies showing that the migratory and homing capacities of MSCs are closely related to their engraftment and regeneration ability. After transplantation, grafted MSCs, which possess higher migratory ability, exhibited greater survival at the periphery of the lesion. Consistently, the motor functions of the rats that had received these grafts improved significantly [21]. These data establish the fact that better recovery of damaged tissues via stem cell therapy demands sufficient recruitment of transplanted cells to the target tissue. Interestingly, it was shown that the migratory behaviors of Drosophila stem cells are closely related to their regeneration ability too. For example, hindgut stem cells of Drosophila would begin to differentiate and replace the damaged cells and tissues as long as they migrate to arrive at the right place, which is controlled by the Wnt and Hh signaling pathways [24, 25]. The mechanisms of MSC migration and homing were extensively investigated too. Studies have demonstrated that MSCs strongly respond to inflammatory or chemotactic stimuli released from injured tissues including chemokines and various growth factors like vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and SDF-1α/CXCR4 axis [26, 27]. Studies indicate that MSCs with enhanced migratory ability to the lesion site following SCI enhance the antiapoptotic effects by upregulating the expression of stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) axis. In one investigation, impaired expression of CXCR4 and cell engraftment was observed in populations of bone marrow MSCs [28]. Consistently, the SDF-1α/CXCR4 axis enhances cell migration toward injured tissues and promotes recovery after SCI by mediating bone marrow MSCs [29, 30]. Besides, substance P that acts as a neurotransmitter was able to mobilize MSCs from the bone marrow and subsequently enter into the impaired tissues [31]. Granulocyte-colony-stimulating factor (G-CSF) was also known to promote mobilization of MSCs to the injured tissue [32]. Our recent study demonstrates that calcitonin gene-related peptide (CGRP) is one of the key factors that regulate the homing of transplanted MSCs to sites of SCI [21]. It looks like there are many factors that regulate the migratory behavior of MSCs. Usually, MSC cultures are initiated with a heterogeneous, poorly defined cell population. It is unknown which MSC populations are expanded and how this process affects homing capacity. There is evidence that only a small percentage of MSCs are able to migrate toward different chemotactic stimuli. We found that MSCs in varying neural differentiation states display different chemotactic responses to HGF. In addition, the phosphorylation levels of PI3K/AKT or MAPK signaling were closely related to the migration efficiency of MSCs [33]. Other authors reported that a population of $CD34^-$ adult bone marrow-derived stem cells do not express functional CXCR4, or only a small proportion of MSCs express functionally active CXCR4 [34]. Probably, different mechanisms are involved to induce cell migration for different subpopulations of mixed MSC cultures. Precise "homing" mechanism of the transplanted cells to the lesion site is still largely unknown, which is of great interest for future study.

2.2. Differentiation of MSCs. Morphological studies showed that neuronal and oligodendroglia cell protein markers are expressed in transplanted MSCs after SCI [35]. For example, small amounts of fluorescent-tagged MSCs can be found in the blood vessels in the area of SCI where they can differentiate into NSE-positive neurons, indicating that MSCs can migrate into the injured area and differentiate into neuron-like cells. In another study, expression of βIII-tubulin at the injury site was verified indicating the potential for functional regeneration. Moreover, grafted MSC can differentiate into myelin-forming cells in the completely transected rat spinal cord [36]. However, it was reported that transplanted cells were identified adjacent to neurons and astrocytes after SCI, but no cells were seen to be labeled with any neural markers at any time [37]. Although some groups have found neuronal differentiation of MSCs in vivo, the survival number of grafted and differentiated neurons were too small to be considered to contribute to functional recovery after SCI [38, 39]. Moreover, these cells, sometimes, do not show specific neuronal electrophysiological properties [40]. Indeed, controversial opinions are coexisted regarding the neural

differentiation capacity of MSCs in vivo. Many experimental data support the opinion that the ability of MSCs to secrete soluble factors or vesicles rather than engrafting and transdifferentiating plays an important role in SCI repair [41–43].

2.3. Gene Therapy to Increase Nerve Regeneration of Transplanted MSCs after SCI. Efforts were made to increase the regeneration efficacy of MSC therapy for SCI. A previous study has shown that MSCs expressing the Shh transgene could increase cell survival after transplantation [44]. At day 28 after treatment, more MSCs were present in the injured tissue in the Shh-MSC group than in the MSC group. Furthermore, the transplanted cells expressing Shh exhibit enhanced functional recovery of neurological function after SCI in rats. Kumagai et al. verified that transplantation of MSCs expressing MNTS1, a multineurotrophin that binds TrkA, TrkB, and TrkC and p75NTR receptors, led to recovery of sensory function, promoting axonal growth after SCI [45]. Similarly, a series of studies indicated that NT3 or other neurotrophin gene-transfected MSCs are an effective approach to improve nerve regeneration and functional recovery after SCI [46–52].

Recently, central roles for microRNAs (miRNAs) as core regulators of gene expression during central nervous system (CNS) pathologies were revealed by many studies [53, 54]. It has been shown that overexpression of miRNA-21 dramatically downregulates expressions of caspase-3, Fas ligand, and programmed cell death (PDCD4), improves the survival of intact motor neurons, and exerts neuroprotective effects on spinal cords against ischemia-reperfusion injury [55]. More recently, both in vitro and in vivo studies found that miR-133b promotes neurite outgrowth and improve functional recovery after SCI while the detailed mechanisms need to be evaluated further [56]. The polypyrimidine tract-binding proteins (PTBPs) are one of the important RNA-binding protein family members, which are thought to be involved in cell-specific alternative splicing. PTBP1 and its brain-specific homologue polypyrimidine tract-binding protein 2 (PTBP2) regulate neural precursor cell differentiation [57]. Experimental data demonstrated that specific miRNA, like miR-124, could promote the productivity of neurogenic cells (NSE-positive cells) by increasing PTBP2 expression of stem cells. Moreover, neurogenic cells derived from miR-12-overexpressed stem cells successfully participate in neural restoration after SCI [58, 59]. These findings provide important regulatory roles of miRNAs in response to CNS damage and encourage novel therapy targeting miRNAs and their target genes for SCI in the future.

3. Function of MSC Transplantation after SCI

3.1. Animal Model. MSC implantation exerts a therapeutic effect on experimental SCI animal models, which is supported by evidence of functional recovery [12]. However, the precise function of MSC transplantation has not been clarified until now. It is expected that after cell transplantation, MSCs would be able to differentiate into specialized neuronal and glial cell lineages. The neural differentiation ratio is low and these kinds of neurons did not show specific neuronal electrophysiological properties sometimes. Although there are controversies, the present data support that the efficacy of MSCs is mainly based on paracrine and neuroprotection functions like secreting numerous growth factors and trophic factors rather than differentiation [42, 60–62].

In general, the function of MSC transplantation includes both structural and functional benefits. Recent data show that MSC transplantation prevented cavity formation due to SCI and resulted in subsequent motor recovery after SCI [63–65]. At the same time, MSC admission promotes recovery of bladder and hindlimb function after SCI in rats [66]. Matsushita et al. suggest that intravenously delivered MSCs have important effects on reducing blood spinal cord barrier leakage, which could contribute to their therapeutic efficacy too [67]. MSCs are immune-privileged cells that may cross human leukocyte antigen barriers to facilitate transplantation [41, 64, 68, 69]. In other studies, reduction of inflammatory infiltrates and decrease of cell apoptosis at the lesion epicenter of the spinal cord are observed after MSC transplantation [61, 70–73]. MSCs are able to reprogram macrophages from a proinflammatory M1 phenotype toward an anti-inflammatory M2 phenotype and also able to regulate immune response in the injured spinal cord to provide a permissive environment for axonal extension and functional recovery [74]. Proteomic analysis of the conditioned medium of MSCs reveals a novel set of inducers for anti-inflammatory M2-like macrophages, such as monocyte chemoattractant protein-1 (MCP-1) [75]. Depletion of MCP-1 from conditional medium decreases MSCs' abilities to induce M2 macrophages and recovery from SCI. Hence, the therapeutic effect of MSC transplantation is partly based on MSCs' paracrine function, such as their ability to secrete trophic factors. Besides MCP-1, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and many other growth factors are also increased after MSC transplantation for SCI [42, 76].

Generally, most stem cell therapy studies have focused on the acute or subacute phase, while there are a limited number of studies evaluating treatment efficacy during the chronic phase of SCI. There are data indicating enhanced therapeutic effects of MSC transplantation at 9 days postinjury period rather than the transplantation immediately after injury. Indeed, subacute intraparenchymal grafting of syngeneic MSCs has only a minor effect on functional recovery [77]. The function of stem cell transplantation approach for SCI might be different depending on the different time phases [16]. For chronic SCI, MSCs were transplanted 8–10 weeks after the induction of SCI and an improved functional recovery and neural regeneration was verified [78, 79]. The systemic infusion of MSCs resulted in functional improvement, which is associated with structural changes, including stabilization of the blood-spinal cord barrier (BSCB), axonal sprouting/regeneration, and remyelination. However, anti-inflammation strategies would be needed to further improve the chronically injured spinal cord, which could be a challengeable mission of MSC transplantation for chronic SCI treatment.

3.2. Clinical Trials. Based on preclinical experiments in SCI animal model showing MSC transplantation in the improvement of functional recovery after SCI, a series of clinical trials were performed. These experiments showed that the grafting of such cells is safe and brings benefits for some patients by using different cell application methods and transplantation procedures [80]. Collectively, autologous MSC transplantation has been shown to be an overall safe and well-tolerated procedure. Intralesional transplantation of autologous MSCs in subjects with complete SCI is safe, is feasible, and may play some roles to promote neurological improvements [81, 82]. Consistently, an approach to personalized cell therapy in chronic SCI indicated that all patients experienced improvement, primarily in sensitivity and sphincter control, while intralesional motor activity, according to clinical and neurophysiological studies, obtained an improvement by more than 50% of the total 12 patients [83]. A case report indicated that MSC transplantation can partially promote recovery of deep sensory pathways as demonstrated by somatosensory evoked potential (SSEP) recording and alleviate neuropathic pain of a patient with traumatic complete cervical SCI [84].

However, this is not always the case. Recently, a study reported a clinical trial which made an attempt to track bone marrow-derived MSCs in a patient with a chronic cervical SCI. The results suggested that tagged bone marrow-derived stem cells were detected at the patient's cervical spinal cord with magnetic resonance imaging at 48 hours, which faded after two weeks, and then disappeared after one month. Unfortunately, no clinical improvement of the neurological function had occurred at the end of this study [85]. Similarly, there are also data indicating that there is no significant improvement in Basso, Beattie, and Bresnahan (BBB) score after MSC transplantation for SCI [86, 87]. Pal et al. reported that there is no effectiveness of the treatment involved after MSC injection for a of total 20 SCI patients during 1–3 years follow-up [88]. So, there is a common concern regarding the efficiency and reproducibility of the therapeutic use of MSCs for SCI patients. It is necessary to ensure the efficacy of MSCs as therapeutic agents for SCI before recommending clinical application of this treatment at this time.

Among various strategies for SCI treatment, it is generally accepted that stem cell transplantation is a good candidate approach leading to recovery of neural function [89, 90]. MSC transplantation shows some improvements in a varying degree of functional recovery after SCI. However, there are many concerns before MSC application into SCI patients extensively [1]. Until now, many neuroprotection roles of MSC transplantation were reported for SCI treatment. Here comes the question: which one or none of them plays a central role? The answer to this question is important for us to understand the repair mechanisms of stem cells, consequently ensuring the effectiveness of cell therapy and developing new strategies for SCI treatment. In general, MSCs from the bone marrow or other sources are mixed with different cell populations, which display complex antigen expression profiles. Thus, we have no idea about which specific cell population produces the best therapy effects. Moreover, MSC therapy only partly improves neurological function, which is not good enough when being applied to treat chronic SCI. Hence, MSC transplantation is not an effective and reliable therapy for SCI so far. More studies need to be done before massive clinical therapy is applied.

4. MSC and Tissue Engineering

Considering the complex environment and cell interactions within the spinal cord, a combination of stem cells with other treatment strategies, like application of biomaterials, might bring up better results [16, 91, 92]. Generally speaking, suitable biomaterials should have some special characteristics, such as biocompatibility, porosity and permeability for the diffusion of ions, nutrients, and waste products, and biodegradability. More importantly, biomaterials should have the capacity of mimicking the extracellular matrix (ECM) of CNS tissues, which provides a more permissive environment for cell survival, growth, migration, and differentiation [93]. Therefore, they are expected to provide an adequate environment for the regeneration of the injured tissues. Taking into account the well-known capacity of MSCs to secrete paracrine factors and the neural protection function when transplanted into spinal cord lesion models, their combination with a 3D matrix holds great promise to SCI repair [94–97].

Silks are naturally occurring polymers that have been used clinically as sutures for centuries. Silk fibroin in various formats has been shown to support cell adhesion, proliferation, and differentiation in vitro with a variety of cells and promote tissue repair in vivo. The work from our groups found that electrospun silk fibroin (SF) nanofibers support the adhesion and growth of neural cells. Interestingly, our data indicated that nanofibers could help neurons form the three-dimensional network by providing the supported substrate. At the same time, SF nanofibers promote neurite outgrowth and astrocyte migration [98]. Furthermore, we proposed that the diameter of biodegradable SF polymer could influence the growth behavior of cells in vitro. In conclusion, our in vitro data demonstrate that smaller diameter and aligned electrospun tussah silk fibroin represent valuable scaffolds for supporting and promoting growth and migration of stem cells, thus raising the possibility of manipulating SF scaffolds to enhance growth, homing, and therapeutic potential of stem cells in cellular therapy [99].

Besides natural biomaterials, biodegradable synthetic scaffolds have been used to support and improve the stem cell regenerative performance too. Hydrogels are particularly appealing for neural tissue repair because of their special physical properties such as being injected into the body in a localized and noninvasive manner [100, 101]. In a more recent study, a new agarose/carbomer-based hydrogel which combines different strategies to optimize MSC viability was evaluated. The study demonstrates that a combination of MSCs and biomimetic hydrogel is able to immunomodulate the proinflammatory environment in a SCI mouse model and promote a favorable regeneration environment in situ significantly [96]. This study presents the ability of a 3D ECM deposition to increase adherence and viability of loaded human MSCs.

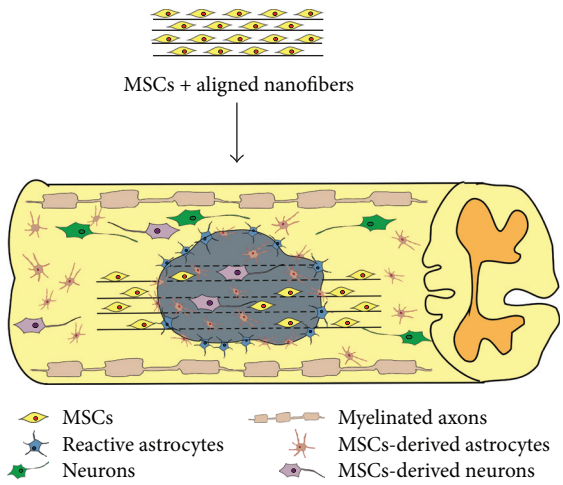

Figure 1: Biomaterials with different topographies have the capacity of mimicking the ECM of the CNS tissue and further influencing the growth behavior of transplanted stem cells. The aligned nanofibers were supposed to improve the migration and differentiation of cells after SCI.

As mentioned previously, the biological behavior of MSCs in vivo is closely related to their regeneration ability. For instance, the migratory and differentiation capacities of MSCs are closely related to their engraftment and regeneration ability. Therefore, one of the principal functions of nerve tissue-engineered scaffolds is to direct neural cell behavior such as growth, cell spreading, migration, and differentiation and to respond to the environment in a targeted implantable tissue. Hyatt et al. showed that MSCs delivered via scaffold formed longitudinally aligned layers growing over the spinal cord lesion site [102]. Host neurites within the spinal cord tissue were found to migrate into the graft. In addition, the layered architecture of the scaffold appeared to induce cell/tissue polarity and promote longitudinal growth of neurites within the graft [103]. Also, multichannel/laminin (LN) silk scaffolds could mediate cell migration, stimulate blood capillary formation, and promote axonal extension, suggesting a strong correlation between scaffold topography and growth behavior of stem cells [104]. More intensive studies are required for the investigation of the activities of stem cells after being combined with biomaterials to offer insights into the design and development of nerve tissue engineering scaffold especially for SCI (Figure 1) [105].

5. MSC-Derived Exosomes as a Promising Therapeutic Vesicle for SCI

Secretomes, also called extracellular vesicles, are several groups of secreted vesicles, which could be classified as exosomes, microvesicles (MVs), and apoptotic bodies. Exosomes (30–100 nm) can be distinguished from MVs (100–1000 nm) and apoptotic bodies (1000–5000 nm) according to their size, morphology, origin, composition, and density [106]. They are membrane-bound vesicles which are secreted naturally by many types of cells. Exosomes contain proteins, lipids, and various nucleic acids, including mRNAs, miRNAs, and long noncoding RNAs (lncRNAs) [107]. These exosomal RNAs can be taken up by distant cells and lead to the protein translation in the target cells. Thus, exosomes function as natural carriers of signal molecules and further act as physiological regulators of cell-to-cell communication. Recently, several studies indicate that lncRNAs in cancer exosomes can act as diagnostic and prognostic biomarkers [108, 109]. The discovery of their regulatory roles on distinct physiological or pathological conditions has brought increasing attention to exosomes.

MSC exosomes, like exosomes in general, carry exosome-associated markers such as Alix, tetraspanins (CD9, CD63, and CD81), and heat-shock proteins including Hsp60, Hsp70, and Hsp90. Besides, the other distinct composition of MSC exosomes depends on cell sources (which tissue MSCs were isolated from) and their physiological states [93]. There are around 857 unique gene products and more than 150 kinds of miRNAs expressed in MSC exosomes, suggesting that exosomal proteins and RNAs could form different functional RNA-protein complexes to perform diverse cellular responses [110].

Exosomes derived from MSCs may have a comparable therapeutic potential as cells themselves. Studies showed that exosomes derived from MSCs have therapeutic potential for many kinds of diseases [111]. For example, exosomes derived from MSCs exert protective effects on myocardial ischemia/reperfusion injury. MSC-derived exosomes can reverse the degeneration of neurons and astrocytes, as well as synaptic loss in hippocampus of diabetic mice [112]. Zhang et al. demonstrated that exosomes derived from MSCs can promote axonal growth of cortical neurons, indicating a potential therapeutic strategy to enhance axonal growth after CNS injury [113]. Moreover, MSC exosomes contribute to the improvement of impaired neurological functions, implying their potential clinical applications [114]. These results raise a possibility that exosomes derived from MSCs might be a promising therapeutic tool for SCI. However, there still lack direct experimental evidence that administration of cell-free exosomes generated from MSCs promotes axonal growth and improves neurological functions after SCI.

In general, MSCs represent the most promising source of exosomes for the neurotherapeutic applications. MSCs were found to produce large amounts of exosomes and could be used as the source to produce commercially sustainable production of exosomes. Exosomes are less immunogenic, more biocompatible and stable, compared to other existing viral or liposome-based gene delivery. It has been proposed that exosomes may cross the blood-brain barrier and enter into the CNS via intercellular junctions of endothelial cells. In addition, exosomes can be modified with genetic engineering, which will improve their therapeutic efficiency. These characteristics suggest that exosomes can be developed as an ideal vehicle for therapeutic delivery. However, exosomes contain a diverse array of signaling molecules with complicated functions, which could raise multiple safety issues. Therefore, it is critical for future studies to engineer exosome delivery systems containing high density of the defined therapeutic molecules, which target specific cells on the given situations.

6. Molecular Mechanisms after MSC Transplantation for SCI

It is well known that MSCs can produce various growth factors, neuroprotective cytokines and chemokines, including HGF, VEGF, fibroblast growth factor (FGF), BDNF, and NGF, which could indeed underlie functional benefits associated with MSC transplantation [115, 116]. Recent studies demonstrated that MSCs are an efficient source of HGF and suggest that the therapeutic effects of MSC transplantation are partly mediated by HGF secreted by these cells [117]. HGF blocked secretion of transforming growth factor-β (TGF-β) from activated astrocytes and prevented expression of specific chondroitin sulfate proteoglycan (CSPG) species. Transplantation of HGF-overexpressing MSCs markedly decreased Neurocan expression and glycosaminoglycan chain deposition around hemisection lesions in the spinal cord. Animals treated with HGF-MSCs showed increased axonal growth and improvement in functional recovery [118], which is consistent with the view that HGF have been identified as attractive signals for guidance of motor axons to the target tissue [119]. In addition, HGF has been reported to provide therapeutic effects in central nerve injury, such as the suppression of demyelination, apoptosis, and blood-brain barrier disruption, through the c-Met receptors that are upregulated after injury in rat neurons, oligodendrocytes, and astrocytes [120].

Besides growth factors which act as paracrine signaling, immunological cytokines are also involved in the process of stem cell therapy after SCI. For instance, transplantation of MSCs into a lesion spinal cord reduced the secretion of TNFα, IL-4, IL-1β, IL-2, IL-6, and IL-12 when compared to that of the saline-treated controls [121–123]. Particularly, implantation of MSCs prevents second-phase neuronal injury by suppressing lymphocyte and microglia effects and reduces the inflammatory reaction in the local environment after SCI [124]. These results indicate that neuronal survival after lesion might occur through cytokine release and immunomodulation followed by MSC administration.

Previous studies reveled that MSC implantation modulates glial scar formation after SCI. One of these reports concludes that MSC treatment after SCI upregulates matrix metalloproteinase- (MMP-) 2 levels and reduces the formation of the glial scar thereby creating an environment suitable for endogenous regeneration mechanisms [125]. In addition, it was shown that human MSCs deposit fibronectin (FN) following SCI, which is a well-known inducer of axonal growth, as well as a component of the extracellular matrix (ECM) [126]. Importantly, it has been shown that FN secreted by MSCs are essential for neurite elongation of neuronal differentiating MSCs as well as nerve fiber regeneration after SCI. Laminin is a well-known inducer of axonal growth too, as well a component of the ECM associated to neural progenitors. Laminin and TGF-β expression have also been increased in the injured spinal cord after MSC admission for SCI. The in vivo data suggest that laminin can be the paracrine factor mediating the proregenerative effects of MSCs in spinal cord injury [127].

Apoptosis-related pathways have been found involved in SCI after MSC transplantation. Recent findings suggest that caspase-3-mediated apoptosis on both neurons and oligodendrocytes following SCI was significantly downregulated by MSCs, which was regulated through stimulation of endogenous survival signaling pathways, PI3K/Akt, and the MAPK/ERK1/2-cascade [128]. Extracellular-adjusting protein kinases 1 and 2 (ERK1/2) are important intracellular signaling molecules that are members of the MAPK family. Consistently, Wang et al. showed that transplanting MSCs activates ERK1/2 in spinal cords of ischemia-reperfusion injury rats and improves nerve function [129]. At the same time, Bcl2 expression increased, whereas Bax expression decreased following stem cell transplantation. There are also data indicating that transplantation of MSCs for neurological disorders inhibited apoptosis and the protein expression of c-Jun N-terminal kinase and p38 as well triggered the phosphorylation of P-42/44 ERK1/2 [130]. However, it remains undetermined whether MAPK/ERK1/2-cascade participates in other mechanisms beyond inhibition of apoptosis, such as secretion of various neurotrophic factors that promote the regeneration or improving the axon regeneration microenvironment.

It has been demonstrated that Wnt/β-catenin signaling plays a key role in promoting the differentiation of MSCs toward a neuronal fate. Wnt-7a enhanced neuronal differentiation in MSCs via both canonical and noncanonical signaling pathways [131]. Contusion spinal cord injury induced a time-dependent increase in Wnt expression from 6 hours until 28 days postinjury. Specially, after an initial decrease by 1 day, an increase in phosphorylation of the Wnt coreceptor, low-density lipoprotein receptor-related protein 6 (LRP6), and an increase in active β-catenin protein were shown, indicating that canonical Wnt signaling is active in the adult spinal cord and in cells around the wound epicenter after SCI [132]. There is some evidence that spinal radial glia, neural progenitors in zebrafish, exhibit canonical Wnt/β-catenin activity as they undergo neurogenesis following spinal cord transection [133]. Wnt/β-catenin signaling may promote axon regrowth either directly or through induction of secondary pathways in radial glia, suggesting important regulating roles in neural regeneration. In addition, overexpression of Dkk1b, an inhibitor of Wnt/β-catenin signaling, hampers locomotor recovery, axon regeneration, and glial bridge formation in the regenerating spinal cord of adult zebrafish. However, it is still undetermined in mammals that whether Wnt/β-catenin signaling is the activated response to SCI after MSC implantation, which might be explored in the near future (Figure 2).

7. Conclusions

MSCs are considered as the most promising sources for cellular therapies following SCI. The mechanisms underlying the biological behavior of MSCs and their complicated function in vivo are not fully understood, which is very important for improving the therapeutic effects and for designing better therapeutic strategies. A combination of MSCs with nerve tissue-engineered scaffolds can direct cell behavior such as

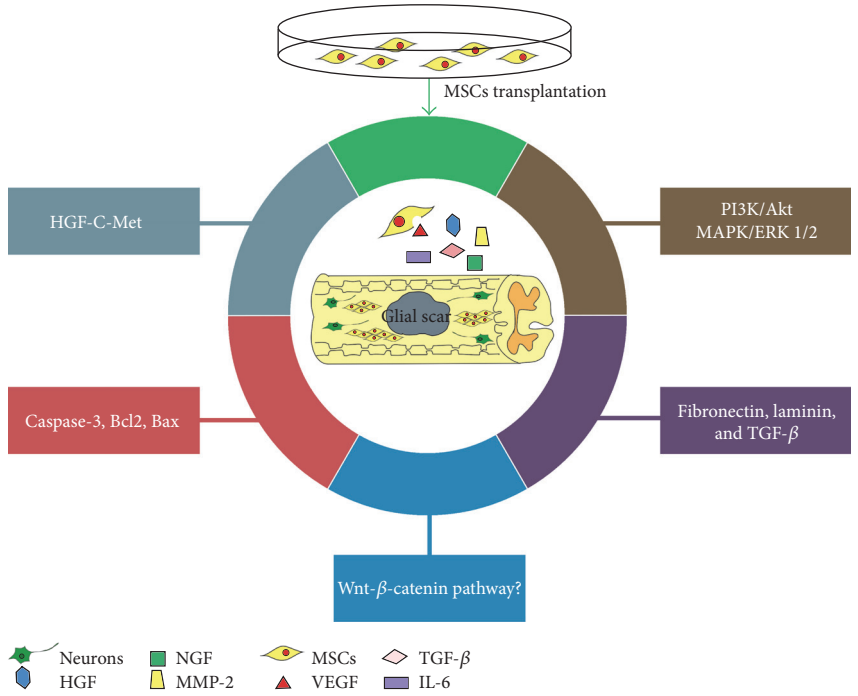

Figure 2: Regulatory molecular mechanisms involved in SCI after MSC transplantation.

growth, cell spreading, migration, and differentiation and respond to the local environment after SCI. More intensive studies are required for the investigation of the activities of cells after combined with biomaterials to offer insights into the design and development of nerve tissue-engineering scaffold for SCI. MSCs represent the most promising source of exosomes for the neurotherapeutic applications, and exosomes derived from MSCs may have a comparable therapeutic potential as cells themselves. Notably, MSCs respond to the local environment in multiple ways. MSCs produce various growth factors, neuroprotective cytokines and chemokines, reduce the inflammatory reaction by suppressing lymphocyte effects, modulate glial scar formation, downregulate Caspase-3 mediated apoptosis by activating ERK1/2-cascade, and so forth. In addition, Wnt/β-catenin signaling pathway might also play important regulatory roles for MSC behavior after SCI. In conclusion, it is of considerable interest to investigate the biological behavior and function of MSCs, especially after SCI treatment. The regulatory mechanisms directing MSC behavior in molecular details will undoubtedly provide valuable insights in improving the MSC-mediated therapy effects and designing better therapeutic strategies.

Conflicts of Interest

All authors have none to declare.

Authors' Contributions

Huanxiang Zhang designed study and made the decision to submit the paper for publication, and Jing Qu wrote the paper.

Acknowledgments

This work was supported by the Natural Science Foundation of Jiangsu Province (Grant no. BK20141198).

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Hindawi
Stem Cells International
Volume 2017, Article ID 2389753, 15 pages
https://doi.org/10.1155/2017/2389753

Research Article

Allogeneic Adipose-Derived Mesenchymal Stromal Cells Ameliorate Experimental Autoimmune Encephalomyelitis by Regulating Self-Reactive T Cell Responses and Dendritic Cell Function

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Received 17 October 2016; Accepted 18 December 2016; Published 30 January 2017

Academic Editor: Marcella Franquesa

Multipotent mesenchymal stromal cells (MSCs) have emerged as a promising therapy for autoimmune diseases, including multiple sclerosis (MS). Administration of MSCs to MS patients has proven safe with signs of immunomodulation but their therapeutic efficacy remains low. The aim of the current study has been to further characterize the immunomodulatory mechanisms of adipose tissue-derived MSCs (ASCs) in vitro and in vivo using the EAE model of chronic brain inflammation in mice. We found that murine ASCs (mASCs) suppress T cell proliferation in vitro via inducible nitric oxide synthase (iNOS) and cyclooxygenase- (COX-) 1/2 activities. mASCs also prevented the lipopolysaccharide- (LPS-) induced maturation of dendritic cells (DCs) in vitro. The addition of the COX-1/2 inhibitor indomethacin, but not the iNOS inhibitor L-NAME, reversed the block in DC maturation implicating prostaglandin (PG) E_2 in this process. In vivo, early administration of murine and human ASCs (hASCs) ameliorated myelin oligodendrocyte protein- (MOG_{35-55}-) induced EAE in C57Bl/6 mice. Mechanistic studies showed that mASCs suppressed the function of autoantigen-specific T cells and also decreased the frequency of activated ($CD11c^+CD40^{high}$ and $CD11c^+TNF\text{-}\alpha^+$) DCs in draining lymph nodes (DLNs). In summary, these data suggest that mASCs reduce EAE severity, in part, through the impairment of DC and T cell function.

1. Introduction

Multipotent mesenchymal stromal cells (MSCs) are non-hematopoietic, perivascular cells which support hematopoiesis and are thought to participate in tissue repair in vivo [1–3]. MSCs can be obtained from virtually all tissues and organs of the body and have been defined in vitro as plastic adherent cells that express CD73, CD90, and CD105 while lacking expression of CD45, CD34, and CD14, which can differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [4]. Although the proportion of *bona fide* stem cells within MSC preparations is likely to be low [5], it has been clearly shown that in vitro-expanded MSCs possess potent immunomodulatory properties. Firstly, MSCs can modulate the activation of cells of both the innate and adaptive immune system in vitro. Depending on species and immune cell studied, this immunomodulatory effect is achieved through a combination of mechanisms including cytokines (IL-10, TGF-β1, and IL-6), intracellular enzymes (inducible nitric oxide synthase (iNOS) in murine MSCs and indoleamine 2,3-dioxygenase (IDO) in human MSCs), growth factors (hepatocyte growth factor, vascular endothelial growth factor), membrane bound molecules (programmed death ligand-1, FASL), and prostaglandin E2 (PGE_2) [6–8]. Secondly, injection of

MSC preparations has shown delay allograft rejection [9, 10] and ameliorating disease in several animal models of inflammation/autoimmunity by inhibiting the deleterious immune responses against self-antigens [11–14]. Based on their success in experimental models, the clinical potential of human autologous and allogeneic MSCs for the treatment of autoimmune/inflammatory diseases has been assessed in various phase I/II and III clinical trials [15–19].

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) where autoreactive T cells and macrophages attack and disrupt the communication between neurons which result in a multitude of neurological symptoms [20]. Several clinical trials have utilized MSCs for the treatment of MS and a few of these studies have analyzed the effects of injected MSCs on the immune status of patients with MS. In a phase 1/2 open-safety clinical trial for MS, injections of autologous MSCs resulted in a rapid inhibition of the immune system [17] while another study observed increased foxp3 transcript levels in peripheral blood mononuclear cells in MSC-treated patients [21]. These data suggest that the therapeutic effect of MSCs in MS depends, at least partially, upon their immunomodulatory capacity. However, while the administration of MSCs has been proven safe, their capacity to ameliorate MS remains poor [16, 17, 19, 22]. Therefore, it is important to further study how MSCs can modulate the immune system both in vitro and in vivo in order to improve MSC-based therapies for MS [23].

Thus, the objective of the current study has been to characterize the immunomodulatory properties of murine adipose tissue-derived MSCs (mASC) in vitro and in vivo using the experimental autoimmune encephalomyelitis (EAE) model of CNS autoimmunity. EAE is a well-accepted animal model of MS sharing both its immunological and pathological features [24]. Injection of MSCs at distinct stages of disease has been shown to inhibit disease severity in various models of EAE, including relapsing/remitting EAE, chronic EAE, and disease induced by adoptive transfer of encephalitogenic T cells [11, 25–27]. We show that mASC inhibited T cell proliferation in vitro via iNOS and COX-1/2 activities. Both allogeneic and xenogeneic (human) ASCs ameliorated MOG_{35-55}-induced chronic EAE in C57Bl/6 mice. Murine ASCs reduced EAE severity through the inhibition of the autoimmune T cell response with no increase in foxp3 Tregs. Importantly, mASC inhibited the maturation of dendritic cells (DCs) in vitro via COX-1/2 activity and reduced the percentage of activated DCs in the draining LNs of EAE mice. Our data suggests that MSC, through their modulation of T cell and DC function, can ameliorate inflammatory/autoimmune CNS disease.

2. Materials and Methods

2.1. Animals. Male Balb/c (6–10 weeks) and female C57Bl/6 (6–8 weeks) (Charles River, Barcelona, Spain) were used to initiate cultures of mASCs and for the induction of EAE, respectively. Experimental protocols were performed according to the National/EU Guidelines for the Care and Use of Laboratory Animals in Research with the approval of the local ethical committee at the Institute for Parasitology and Biomedicine "López-Neyra" and the central ethical committee at the Consejo Superior de Investigaciones Cientificas (CSIC). Mice were anesthetized before immunization using intraperitoneal injections of ketamine-HCl (dose at 100 mg/kg) mixed with xylazine-HCl (10 mg/kg). Mice were sacrificed using CO_2 when extracting the spinal cord for histological analyses. In all other cases, mice were sacrificed using cervical dislocation.

2.2. Isolation and Expansion of mASCs. Mesenchymal stromal cells were isolated from adipose tissue as previously described [28]. Cells were resuspended in MesenCult (Stem Cell, Grenoble, France) containing 20% mouse mesenchymal supplements (Stem Cell) and penicillin/streptomycin (Invitrogen, Carlsbad, CA), plated at a density of $2\text{-}3 \times 10^4$ cells/cm^2 and cultured at 37°C in hypoxia at 5% O_2, 5% CO_2. Nonadherent cells were removed after 24 hours in culture. Subsequent passages were plated at 10^4 cells/cm^2 and maintained at 5% O_2, 5% CO_2. mASCs were used at passages 3–9. The phenotypic characterization and differentiation capacity into adipocytes, osteocytes, and chondrocytes were performed as previously described ([28]; data not shown). Human ASCs were obtained from Cellerix SA, Tres Cantos (Madrid), Spain, and cultured in advanced DMEM supplemented with 10% FCS (Invitrogen), Glutamax (GIBCO, Life Technologies, CA), and 100 U/mL penicillin/streptomycin (GIBCO, Life Technologies) as previously described [14].

2.3. Assessment of mASC-Mediated Inhibition of T Cell Proliferation In Vitro. mASCs were treated with mitomycin C (50 μg/mL, Sigma Aldrich, St. Louis, MO) for 20 minutes at 37°C and washed 3 times with complete RPMI1640 (2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 50 μM 2-mercaptoethanol, and 10% heat-inactivated FCS, all from Invitrogen). Mitomycin C-treated mASCs were plated in flat bottomed 96-well plates and allowed to adhere for 3-4 hours. Mitomycin C treatment of mASCs did not impact on their ability to inhibit T cell proliferation in vitro (see Figure S1 in Supplementary Material available online at https://doi.org/10.1155/2017/2389753). Spleens from female Balb/c mice were homogenized and erythrocytes lysed using ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA at pH 7.4) and 2×10^5 cells were added to wells with or without mASCs. Concanavalin A (ConA, 2.5 μg/mL, Sigma Aldrich) was added to cultures as a mitogenic stimulus of T cells. L-NAME (1 mM), indomethacin (20 μM), and L-norvaline (10 mM) (all from Sigma Aldrich) were added at the initiation of the cocultures. After 3 days, cells were pulsed with 0.5 μCi/well [^{3}H]-thymidine (Perkin Elmer, Waltham, MA) for 6 hours and harvested onto glass fiber filters using a FilterMate 96 well-harvester (Perkin Elmer). Uptake of [^{3}H]-thymidine was measured on a 1450 Microbeta Trilux scintillation counter (Wallac Oy, Turku, Finland).

2.4. Measurement of Nitrite Production. To assess iNOS activity in mASCs, supernatants from control/stimulated mASCs and mASC: DC cocultures were assayed for nitrite

contents using the Griess assay. In brief, 100 μL of Griess reagent (a 1/1 mixture of 1% p-aminobenzene-sulfonamide in 5% H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride in distilled H_2O; Sigma Aldrich) was added to 100 μL culture supernatants and standard ($NaNO_2$) in 96-well plates. Plates were incubated at room temperature for 10 min, and absorbance was measured at 550 nm.

2.5. Measurement of Arginase Activity. Arginase activity was measured as previously described [29]. Urea was used for the standard curve. The protein concentrations of cell lysates were measured using the bicinchoninic acid (BCA) assay [30]. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 mmol of urea per min.

2.6. Induction of EAE. Female C57Bl/6 mice (6–8 weeks-old) were immunized s.c. in the flanks with 150 μL of an emulsion (75 μL/flank) containing 150 μg MOG_{35-55} (Genscript, Hong Kong, China) in PBS mixed with an equal volume of complete Freund's adjuvant (CFA) supplemented with 4 mg/mL *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Mice were injected i.p. with 200 ng pertussis toxin (Sigma Aldrich) in PBS on the day of immunization and 2 days later. Immunized mice were randomly distributed in different groups. Group 1: control mice ($n = 8$) were injected i.p. with PBS at the onset of disease (clinical scores: 0-1). Group 2: control mice ($n = 13$) were injected i.p. with PBS at the acute phase of disease (clinical scores: 1–3). Group 3: Mice ($n = 9$) were treated i.p. with allogeneic mASCs (10^6 cells obtained from Balb/c mice and expanded in hypoxia) at the onset (clinical scores: 0-1). Group 4: mice ($n = 7$) were treated i.p. with allogeneic mASCs (10^6 cells obtained from Balb/c mice and expanded in hypoxia) at the acute phase of the disease (clinical scores: 2-3). Group 5: mice ($n = 7$) were treated i.p. with hASCs (10^6 cells) at the acute phase of disease (clinical scores: 1-2). Clinical symptoms of EAE were scored daily using a 0–8 scale as follows: 0, no detectable signs of EAE; 1, affected tail tonus; 2, tail paralysis; 3, mild hind leg paresis; 4, severe hind leg paresis; 5, one hind leg paralysis; 6, complete hind leg paralysis; 7, complete hind leg paralysis and foreleg paresis; and 8, death. For the acquisition of cells and tissues, another set of mice were used and sacrificed 7 days after treatment with PBS or mASCs as described below. Mice were scored daily for disease symptoms. Water gel products providing water and moistened food pellets were placed on the cage floor in Petri dishes which were changed daily to prevent dehydration. Mice were euthanized if exhibiting severe hind leg paralysis and foreleg paresis (a clinical score of 7).

2.7. Histological Analysis of Cell Infiltration and Demyelinization. Spinal cords from EAE mice treated i.p. with PBS ($n = 4$) or allogeneic mASC ($n = 4$) at the onset of disease (clinical scores: 0-1) were removed 7 days after treatment and processed for immunohistochemistry and Klüver-Barrera staining. For light microscopy, cervical and lumbar spinal cord segments were fixed with buffered 10% formalin for 48 h and processed for paraffin inclusion and sectioning. Transversal sections (4 μm thickness) were stained with Luxol fast blue, cresyl violet, and hematoxylin following the Klüver-Barrera technique [31] and were analyzed for the presence of areas of demyelination and cell infiltration using a light microscope (Olympus, Tokyo, Japan). For immunohistochemistry, spinal cord sections were obtained as described for paraffin processing followed by blocking steps with peroxidase blocking reagents, heat-treated in 1 mM EDTA buffer pH 8 at 95°C during 20 min for antigenic unmasking, and incubated for 30 min at room temperature with polyclonal anti-myelin basic protein Ab (Master Diagnostica, Granada, Spain). The immunohistochemical study was done on an Autostainer 480 (Thermo Fisher Scientific Inc., Waltham, MA) using the polymer-peroxidase-based method and developed with diaminobenzidine.

2.8. Assessment of Autoreactive T Cell Responses in EAE Mice. Spleen and draining lymph node (DLN) cells from EAE mice treated i.p. with PBS (control, $n = 4$) or allogeneic mASC ($n = 4$) at the onset of disease (clinical scores: 0-1) were isolated 7 days after mASC injection and stimulated with MOG_{35-55} (50 μg/mL) or anti-CD3 (1 μg/mL; 145-2C11; BD Pharmingen, San Diego, CA) at 1.5×10^6 cells/mL in flat bottomed 96-well plates for proliferation and at 10^6 cells/mL in 24-well for cytokine determination. Supernatants were collected after 48 hours and assayed for cytokine levels by ELISA. After 3 days, cell proliferation was determined by [^{3}H]-thymidine uptake as described above. For intracellular cytokine staining, spleen and DLN cell suspensions (1×10^6 cells/mL) were stimulated with PMA (50 ng/mL) and ionomycin (0.5 μg/mL; both from Sigma Aldrich) for 6 hours in the presence of 3 μM monensin (eBioscience, San Diego, CA) during the last three hours. Cells were processed for FACS analysis as described below.

2.9. Isolation and Characterization of DCs from EAE Mice. To determine the effect of mASCs on DC phenotype and function in vivo, EAE mice were treated i.p. with PBS (control, $n = 4$) or with allogeneic mASC ($n = 4$) after the onset of disease (clinical scores: 1-2), and 7 days later, DLNs were isolated and digested with 1.6 mg/mL collagenase type IV and 0.1% DNAse I (Sigma Aldrich) in RPMI1640 medium without supplements at 37°C for 30 minutes. For intracellular TNF-α staining, DLN cells were washed twice with complete RPMI1640 and 2×10^6 cells/mouse were plated in 12-well plates in the presence of 3 μM monensin for 4 h. Cells were gently harvested using cell scrapers, washed using FACS buffer, and processed for intracellular staining as described below. For TNF-α ELISA, $CD11c^+$ DCs were immunomagnetically purified using CD11c-microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) from collagenase type IV-digested DLNs and plated at 2.5×10^5 cells/mL in the presence of LPS (1 μg/mL). Supernatants were collected after 48 hours for cytokine determinations.

2.10. Generation of Bone Marrow-Derived DCs (BM-DCs) and mASC Cocultures. BM-DCs were generated as previously described [32]. Briefly, 0.4×10^6 BM cells/mL from C57Bl/6

mice were cultured in complete RPMI1640 containing 20 ng/mL GM-CSF (Peprotech, London, UK). After 6–8 days, nonadherent cells were harvested and $CD11c^+$ immature DCs were immunomagnetically purified using CD11c-microbeads (Miltenyi Biotech) according to the manufacturer's instructions. Cell preparations consisted of >95% $CD11c^+$ DCs. To assess the effect of mASCs on the maturation of DCs, different numbers of mASCs were plated in 12-well plates and allowed to adhere for at least 6 hours. $CD11c^+$ DCs (0.4×10^6 cells/well) were seeded into wells with or without mASCs and LPS (1 μg/mL) was added for 48 hours to induce DC maturation/activation. In some experiments L-NAME (1 mM) or indomethacin (20 μM) was added to the DC: mASC cocultures during maturation. DCs were harvested from the cocultures by gently collecting the nonadherent DCs from the adherent mASC monolayer. The acquired cells consisted of >95% $CD11c^+$ DC. Supernatants were collected and assayed for cytokine levels by ELISA or NO_2 levels by Griess assay. Cell suspensions were processed for flow cytometry analysis of cell surface antigens or used for MLR as described below.

2.11. Mixed Leukocyte Reaction. Spleens from BALB/c (H-2^d) were homogenized and erythrocytes lysed using the Ammonium-Chloride-Potassium (ACK) lysis buffer. Cells (4×10^6 cells/mL) were plated in Petri dishes and allowed to adhere at 37°C for 90 minutes. Nonadherent cells were collected and used as responders. C57Bl/6 (H-2^b) DCs from the DC: mASC cocultures or purified $CD11c^+$ DCs from the DLNs of untreated or mASC-treated EAE mice were added in flat bottomed 96-well plates (15000 DCs/well) together with 3×10^5 responder cells. After 4 days, cell proliferation was determined by ^{3}H-thymidine uptake as described above.

2.12. Flow Cytometry. Collagenase/DNAse-treated DLN cell suspensions or mASC-cultured DCs were preincubated with anti-FcγRII/FcγRIII mAb at 2.5 μg/mL (2.4G2; BD Pharmingen) and 7-aminoactinomycin D (2 μg/mL; Sigma Aldrich) and then stained with the following antibodies: CD11c-APC (N418; eBioscience), CD40-PE (2/23), CD80-PE (16-10A1), and/or CD86-PE (GL-1) (BD Pharmingen). For intracellular cytokine staining cells were fixed, permeabilized, and stained for IFN-γ-FITC (XMG1.2), IL-17-PE (TC11-18H10), TNF-α-PE (MP6-XT22), IL-4-PE (BVD4-1D11), and IL-10-PE (JES5-16E3) (all from BD Biosciences, San Diego, CA) using the BD cytofix/cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Each sample was stained with appropriate isotype controls. For the analysis of foxp3 expression cells were stained with CD4-FITC (GK1.5; BD Biosciences) and subsequently processed for foxp3 staining using the foxp3 staining kit (eBioscience) according to the manufacturer's instructions. Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

2.13. Cytokine and PGE_2 ELISAs. The cytokine content in supernatants from the mASC: splenocyte and DC cultures were determined by sandwich ELISAs using capture/biotinylated detection antibody pairs for IFN-γ, TNF-α, IL-12p40 (BD Pharmingen), IL-10, IL-17, and CXCL10 (eBioscience). Plates were developed using peroxidase-labelled streptavidin (Sigma) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and read at 405 nm. PGE_2 levels in supernatants were measured by ELISA (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instruction.

2.14. Gene Expression Analysis. Total RNA was purified using Ultraspec (Biotecx, Huston, TX) from spinal cords isolated from PBS-treated (control, $n = 4$) or allogeneic mASC-treated ($n = 4$) EAE mice (7 days after treatment) or from mASCs stimulated with LPS (1 μg/mL, Sigma) or TNF-α (10 ng/mL, Peprotech) and IFN-γ (10 ng/mL, BD Biosciences) for 6, 12, and 24 hours. Total RNA (1 μg/sample) was reverse transcribed using M-MuLV RT (Roche Diagnostic, Basel, Switzerland) and random hexamer primers. Semiquantitative PCR and qPCR were performed using Taq polymerase (Biotools, Madrid, Spain) or Supermix (Bio-Rad, Hercules, CA), respectively. Primer pairs include the following: iNOS FW: 5′-GTTCTCAGCCCAACAATACAAGA-3′; iNOS RV: 5′-GTGGACGGGTCGATGTCAC-3′; MCP-1 FW: 5′-TTAAAAACCTGGATCGGAACCAA-3′; MCP-1 RV: 5′-GCATTAGCTTCAGATTTACGGGT-3′; TGF-β1 FW: 5′-TGCGCTTGCAGAGATTAAAA-3′; TGF-β1 RV: 5′-AGCCCTGTATTCCGTCTCCT-3′; IL-10 FW: 5′-GGTTGCCAAGCCTTATCGGA-3′; IL-10 RV: ACCTGCTCCACTGCCTTGCT; IDO FW: 5′-GGCTAGAAATCTGCCTGTGC-3′; IDO RV: 5′-AGAGCTCGCAGTAGGGAACA-3′; COX-2 FW: 5′-GGGTTGCTGGGGGAAGAAATGTG-3′, COX-2 RV: 5′-GGTGGCTGTTTTGGTAGGCTGTG-3′; Arginase I FW: 5′-CAGAAGAATGGAAGAGTCAG-3′; Arginase I RV: 5′-CAGATATGCAGGGAGTCACC-3′; Foxp3 FW: 5′-TTCATGCATCAGCTCTCCAC-3′; Foxp3 RV: 5′-CTGGACACCCATTCCAGACT-3′; IFN-γ FW: 5′-ACACTGCATCTTGGTTTGC-3′; IFN-γ RV: 5′-TTGCTGATGGCCTGATTGTC-3′; β-actin FW: 5′-AATCGTGCGTGACATCAAAG-3′; β-actin RV: 5′-ATGCCACAGGATTCCATACC-3′.

2.15. Statistical Analysis. All results are expressed as mean (SEM) of at least 3 independent experiments unless otherwise stated in the figure legends. The Mann–Whitney U-test was applied on all in vivo results and cell-culture experiments to compare nonparametric data for statistical significance. A p value < 0.05 was considered significant.

3. Results

3.1. Immunomodulatory Mechanisms of mASCs In Vitro. Acquiring high numbers of low passage MSCs with potent immunosuppressive capacity is crucial for their successful use as a therapy for inflammatory/autoimmune diseases [33]. In agreement with previous studies [34, 35], we found that mASC expanded at low oxygen tension (5% O_2) proliferated at a higher rate compared to mASCs expanded at normoxia (Figure 1(a)). Thus, we decided to culture the ASCs at 5% O_2 and use these cells for the subsequent characterization of their immunomodulatory functions in vitro and in vivo. Whereas mASCs constitutively expressed TGF-β1, COX-2,

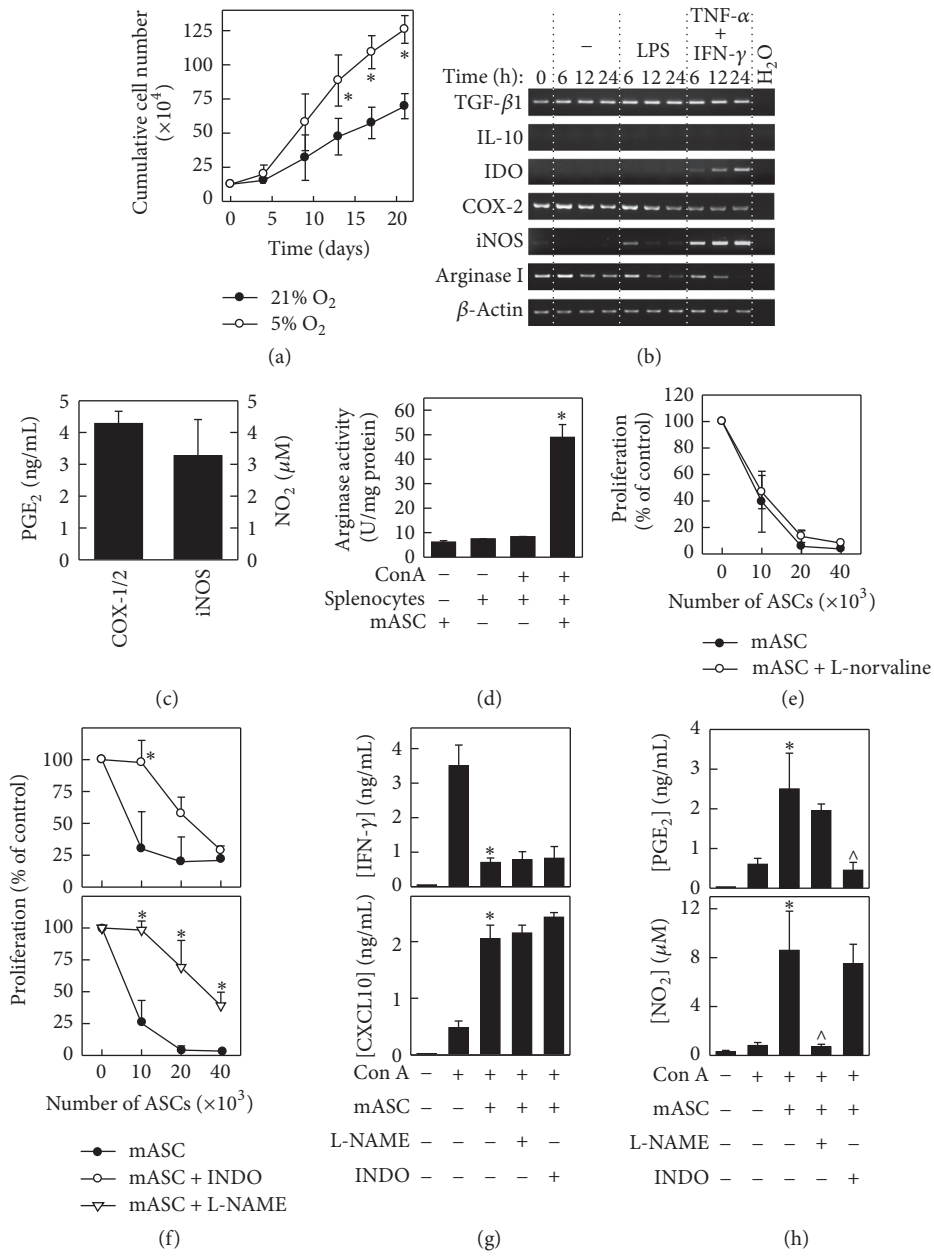

Figure 1: mASCs expanded in hypoxia maintain their immunosuppressive activities. (a) Proliferative response of mASCs expanded in normoxia (21% O_2) or in hypoxia (5% O_2). mASCs were seeded at 5000 cells/cm^2 in T25 flasks and counted at different time points when reaching 70–80% of confluence. Data are shown as mean (SD) of one representative experiment out of three. $^*p < 0.05$ versus 21% O_2. ((b) and (c)) Immune phenotypic characterization of mASCs. mASCs were expanded under hypoxic conditions and then cultured with medium (unstimulated) or stimulated with LPS (1 μg/mL) or TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) for different time points (24 hours for NO_2 determination). Gene expression of different immune markers was analyzed using semiquantitative PCR. iNOS activity was measured in TNF-α/IFN-γ-stimulated mASCs by the detection of NO_2 in culture supernatants using Griess assay. COX-1/2 activity was determined by measuring the PGE_2 levels in culture supernatants from unstimulated mASCs. (d) Arginase activity is induced in mASC: splenocyte cocultures. Splenocytes were cultured with or without mASCs and stimulated with ConA for 3 days. Defined quantities of cell lysates were then subjected to an in vitro arginase activity assay. Data are shown as mean (SEM) of 3 independent experiments. $^*p < 0.05$ versus splenocytes + ConA. ((e), (f), and (g)) mASCs suppress T cell proliferation in vitro via iNOS and COX-1/2 but not arginase activity. Splenocytes (10^6 cells/mL) were stimulated with ConA (2.5 μg/mL) in the presence of different numbers of mitomycin C-treated mASCs (ratio 20 : 1 for IFN-γ and CXCL10 determination). ((e) and (f)) The iNOS inhibitor L-NAME (1 mM), the COX-1/2 inhibitor indomethacin (20 μM), and the arginase inhibitor L-norvaline (10 mM) were added to cultures when indicated. After 3 days, cell proliferation was measured by [^{3}H]-thymidine incorporation and (g) IFN-γ and CXCL10 contents in the supernatants were determined by ELISA. Data are shown as mean (SEM) of 3 independent experiments. $^*p < 0.05$ versus mASC in (f) and $^*p < 0.05$ versus ConA in (g). (h) iNOS and COX-2 activities are increased in mASC: splenocyte cocultures. ConA-stimulated splenocytes (10^6 cells/mL) were cultured with mitomycin C-treated mASCs (ratio 20 : 1) in the presence of L-NAME or indomethacin for 3 days and the PGE_2 and NO_2 contents measured in the culture supernatants. Results are shown as mean (SEM) of 3 separate experiments. $^*p < 0.05$ versus ConA; $^\wedge p < 0.05$ versus mASC + ConA.

and arginase I mRNAs and PGE_2, the expression of both IDO and iNOS mRNA and NO_2 production was induced upon stimulation with TNF-α and IFN-γ, but not LPS (Figures 1(b) and 1(c), Figure S2). Interestingly, we found a significant induction of arginase activity in the splenocyte: mASCs cocultures in comparison to mASCs or splenocytes cultured alone (Figure 1(d)). Although arginase activity has been shown to inhibit the proliferation of T cells [36], we found that the addition of the arginase inhibitor L-norvaline did not significantly revert the suppressive activity of mASCs in concanavalin A- (ConA-) induced splenocyte proliferation (Figure 1(e)). In contrast, inhibition of iNOS activity with L-NAME or of COX-1/2 activities with indomethacin significantly alleviated the mASC-mediated suppression of T cell proliferation (Figure 1(f)). However, none of these inhibitors affected the mASC-mediated suppression of IFN-γ or the induction of CXCL10 in these cocultures (Figure 1(g)). Since inhibition of iNOS may decrease COX-2 expression and PGE_2 secretion [37, 38], we measured the PGE_2 levels in mASC: splenocyte cocultures treated with L-NAME. We found that L-NAME did not affect the production of PGE2 by mASCs and indomethacin did not modulate their iNOS activity. In summary, these data show that low oxygen tension increases the expansion of mASCs in vitro and that these cells are effective in suppressing immune responses in vitro, using both constitutive (COX-1/2) and inducible (iNOS) mechanisms.

3.2. Therapeutic Effect of Allogeneic and Xenogeneic ASC on Chronic EAE. We next wanted to assess the capacity of allogeneic and xenogeneic ASCs to inhibit immune responses in vivo. To this end, we induced chronic EAE in C57Bl/6 mice and administered mASCs to the animals at different time points after immunization. A single injection of allogeneic mASC at the onset or at the acute phase of disease significantly reduced the disease severity compared to control animals receiving PBS (Figure 2(a)). Both early and late injections of mASCs significantly reduced the cumulative disease index (Figure 2(b)). Xenogeneic hASCs injected into EAE mice during the acute phase of the disease (animals with a mean score of 1.5) also reduced the mean clinical score and significantly decreased the cumulative disease index compared to control mice (Figures 2(c) and 2(d)).

3.3. Allogeneic mASCs Reduce Demyelinization and Inflammatory Infiltration in the Central Nervous System during EAE Progression. We then wanted to analyze CNS tissue sections from control and mASC-treated EAE mice for the presence of inflammatory infiltrates and demyelinating plaques. To this end, mASCs were injected intraperitoneally into EAE mice after the onset of the clinical symptoms (average score 1.9 (0.4)) and spinal cords were obtained 7 days later from mASC-treated mice (average score 2.2 (1.9)) and control mice (average score 3.5 (1.1)) and processed for staining. We found that mASC-treated mice exhibited little cell infiltration into the CNS parenchyma and subsequently few demyelinating plaques compared to control EAE mice (Figures 3(a) and 3(b)). Consistent with the reduced leukocyte infiltration, we detected lower levels of IFN-γ, but also TGF-β1, IL-10, and foxp3 mRNAs in mASC-treated mice compared to control EAE mice (Figure 3(c)).

3.4. Allogeneic mASCs Inhibit MOG-Specific Immune Responses but Do Not Induce foxp3+ Tregs. In both EAE and MS, autoreactive Th1 and Th17 cells producing IFN-γ and IL-17, respectively, infiltrate the CNS and promote disease, whereas treatments that induce a skewing towards an IL-4 dominated Th2 response generally suppress EAE [39]. mASC treatment significantly reduced the number of IFN-γ- and IL-17-secreting cells in both DLNs and spleen in EAE mice (Figure 4(a) and representative gating strategy in Figure S3). Moreover, there was a significant reduction in the MOG_{35-55}-specific recall response, with respect to both proliferation and production of IFN-γ and IL-17 compared to control EAE mice. The effect was antigen specific since activation of total T cells with anti-CD3 Abs which resulted in similar proliferation and cytokine production by both groups (Figures 4(b) and 4(c)). However, we could not detect an increase in the anti-inflammatory cytokines IL-10 and IL-4 in mASC-treated mice compared to control mice (Figure 4(a)), suggesting that the mASCs did not induce a skewing towards a Th2 response or IL-10 producing Tregs. In addition, we could not detect any change in the size of the $CD4^+foxp3^+$ T cell population in DLNs or spleens of mASC-treated EAE mice compared to control mice (Figure 4(d)).

3.5. mASCs Inhibit the Maturation/Activation of Dendritic Cells In Vitro and In Vivo. Considering the importance of DCs in the initiation of the autoimmune response seen in EAE and MS [40], we decided to evaluate the effect of mASC on the DC activation in vitro and in vivo. To this end, we cocultured BM-derived immature DCs with different numbers of mASCs in the presence of LPS. We found that mASC significantly inhibited the LPS-induced expression of CD40 and TNF-α while not affecting the induction of CD80 and CD86 or the production of IL-10 or IL-12 by BM-DCs (Figure 5(a) and representative gating strategy in Figure S4). This partial block of DC maturation resulted in a significant decrease in their T cell activating capacity (Figure 5(b)). Since both iNOS and PGE_2 inhibited the proliferation of splenocytes in vitro and can modulate DC activation [41–44], we set out to investigate their involvement in the mASC-mediated inhibition of DC maturation. We found that addition of indomethacin but not L-NAME significantly reversed the mASC-mediated suppression of TNF-α production and CD40 expression resulting in a significant increase in their immunostimulatory capacity (Figure 5(c)). These effects were observed despite the presence of iNOS activity in the mASC: DC cocultures (Figure S2).

To study DC activation in vivo, we purified DCs from DLNs of mASC-treated and control EAE mice 7 days after mASC injection and analyzed their expression of costimulatory markers and TNF-α production. Consistent with the in vitro data, DCs from mASC-treated EAE mice expressed significantly lower levels of CD40, but not CD86, compared to DCs from control EAE mice (Figure 5(d)). In addition, intracellular staining of TNF-α on whole LN suspensions revealed that mASC-treated EAE mice had fewer $CD11c^+TNF\text{-}\alpha^+$ DCs

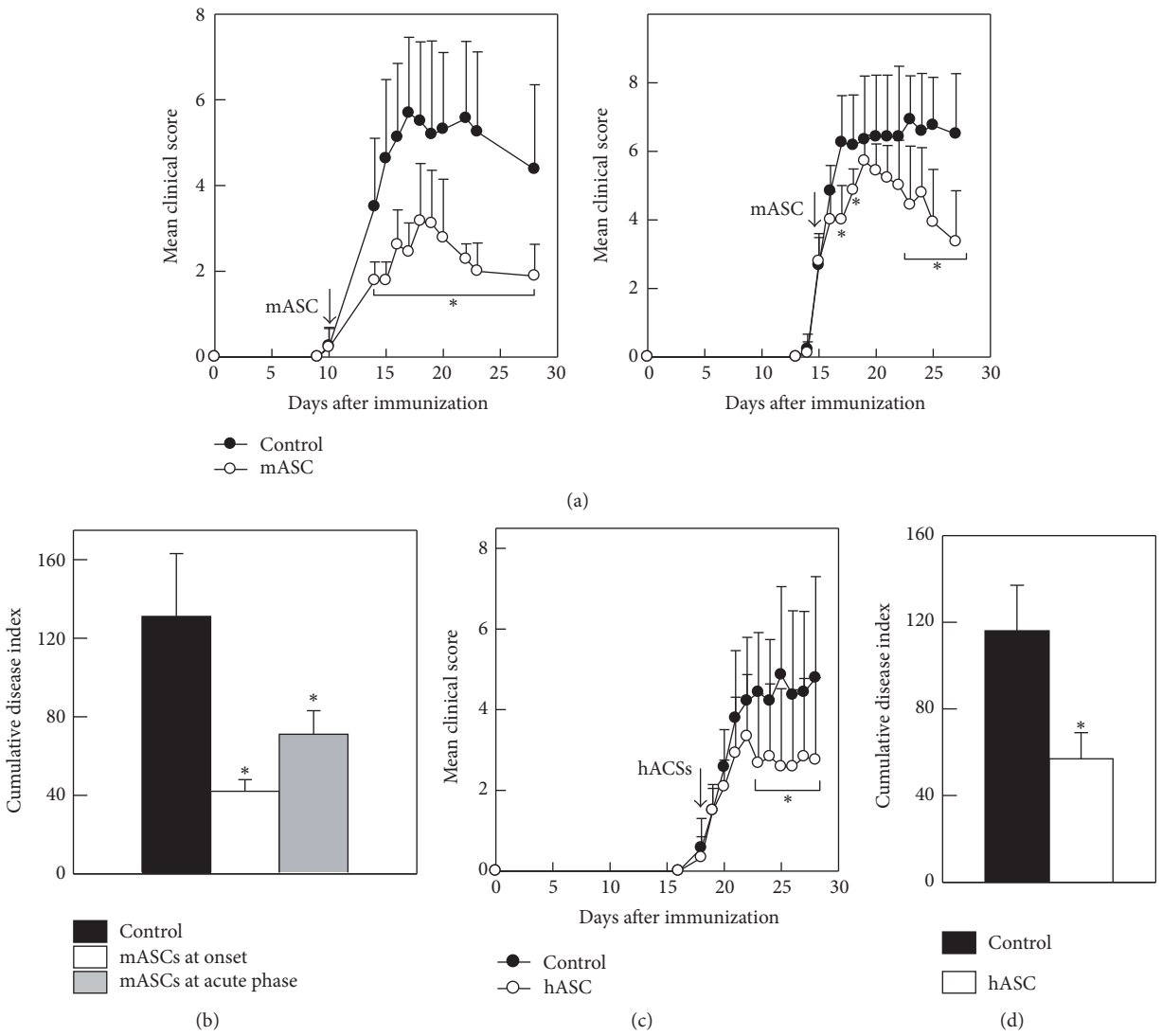

Figure 2: Allogeneic and xenogeneic ASC reduce EAE severity. Chronic progressive EAE was induced in C57Bl/6 mice by immunization with $MOG_{35–55}$. Animals were injected intraperitoneally with PBS (control) or with hypoxia-expanded allogeneic mASCs (10^6 cells/mouse) either at the onset of EAE (day 11 after immunization) when the mean clinical score was 0.25 in the control group ($n = 8$ mice) and 0.22 in the mASC-treated group ($n = 9$ mice) or at the acute phase of EAE (day 15 after immunization) when the mean clinical score was 2.7 in the control group ($n = 6$ mice) and 2.8 in the mASC-treated group ($n = 7$ mice). (a) Clinical symptoms were scored daily in the group treated at onset (left panel) and at the acute phase of EAE (right panel). (b) Cumulative disease index is the sum of daily clinical scores observed between days 20 and 40. Results are shown as mean (SD). $^*p < 0.05$ versus control. (c) Human ASCs ameliorate EAE. Mice with MOG-induced EAE were intraperitoneally injected with PBS (control, $n = 7$) or with hASCs expanded in hypoxia (10^6 cells/mouse, $n = 6$ mice) during the acute phase of the disease (arrow) when the mean clinical score was 1.5 in both groups. Clinical symptoms were scored daily. (d) Cumulative disease index is the sum of daily clinical scores observed between days 20 and 40. Results are shown as mean (SD). $^*p < 0.05$ versus control.

compared to control EAE mice (Figure 5(d) and representative dot plots in Figure S5). In order to further analyze DC function we purified $CD11c^+$ cells from the DLNs of control and mASC-treated EAE mice and assessed their TNF-α producing capacity upon LPS restimulation in vitro. We found that DCs from mASC-treated animals produced less TNF-α upon LPS stimulation in vitro compared to control DCs (Figure 5(d)). Taken together, these data suggest that mASC could suppress EAE partly by preventing DC function in vivo.

4. Discussion

MSCs have emerged as a promising therapy for MS with some studies suggesting an MSC-mediated modulation of the aberrant immune response [17, 21]. However, the efficacy has so far been modest warranting the search for improved MSC culture protocols and a greater understanding of the mechanisms behind the immunomodulatory and trophic effects of MSCs in vitro and in vivo [16, 17, 19]. Our analysis of the immunomodulatory mechanisms employed by mASCs

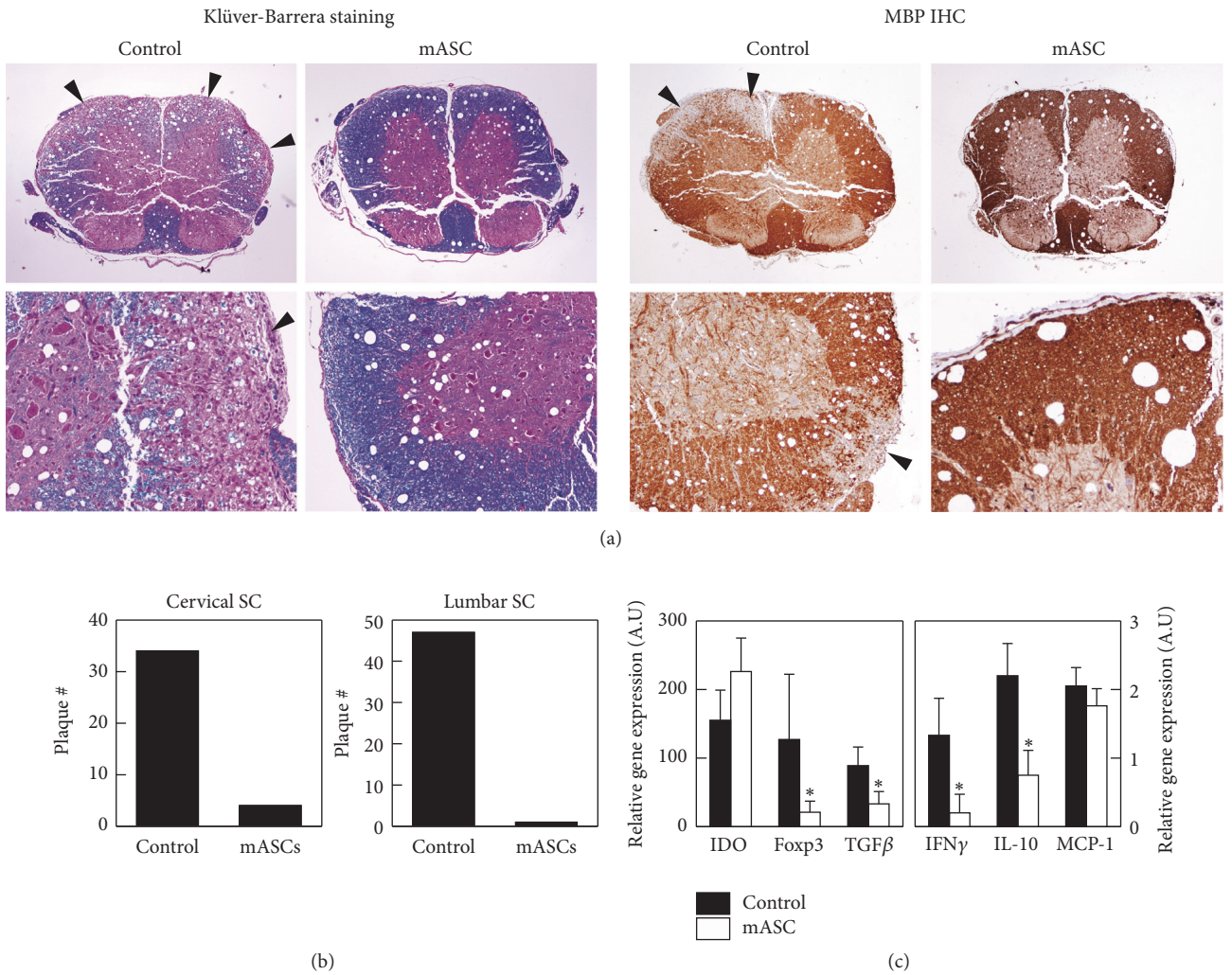

Figure 3: Allogeneic mASCs reduce demyelinization and inflammatory infiltration in the central nervous system during EAE progression. Mice with MOG-induced EAE were intraperitoneally injected with PBS (control, $n = 4$) or with mASCs expanded in hypoxia (10^6 cells/mouse, $n = 4$) after the onset of the clinical symptoms, and spinal cords were obtained at the peak of the disease 7 days later. ((a) and (b)) Transverse sections of cervical and lumbar regions of spinal cord were randomly analyzed for the presence of inflammatory infiltrates and plaques of demyelinization using the Klüver-Barrera staining (left panels) and anti-myelin basic protein immunohistochemistry (right panels). Arrows point to areas of demyelinization. (c) Gene expression of different inflammatory markers was determined by PCR in mRNAs isolated from the spinal cords. Results are shown as mean (SD) of 4 mice per group. $^*p < 0.05$ versus control.

cultures at 5% O_2 showed that both their iNOS and COX-1/2 activities are involved in the inhibition of T cell proliferation in vitro. Although iNOS is used only by murine MSCs, COX-1/2 activity has been shown to be important for the immunomodulatory capacity of both murine and human MSCs [29, 45, 46]. In addition to iNOS and COX-1/2, we also detected a significant induction of arginase activity in the mASC: splenocyte cultures, which was found predominantly in the adherent splenocyte fraction and not in the mASC population (Figure S6). This is in agreement with our recent finding that MSCs can induce arginase I^+ regulatory macrophages in vitro [29]. Although arginase activity does not appear to be important for the MSC-mediated inhibition of T cell proliferation in vitro, the role of MSC-educated macrophages and/or microglia in controlling autoimmune-mediated inflammation in the CNS remains to be elucidated [47, 48].

In agreement with previous studies [11, 27], we found that early injection of mASCs reduced the activation of MOG_{35-55}-specific T cells and significantly inhibited EAE progression and severity.

Regulatory T cells (Tregs) control the severity of EAE through the prevention of T cell expansion in the DLNs [49] and by inhibiting activated T cells in the CNS [50]. Several studies have reported an increased frequency of Tregs in the LNs of EAE animals treated with MSCs [26, 51]. Interestingly, two studies observed an increase in Treg number [17] or foxp3 mRNA [21] in PBMCs from MS patients after MSC administration. In contrast, we did not observe an increased

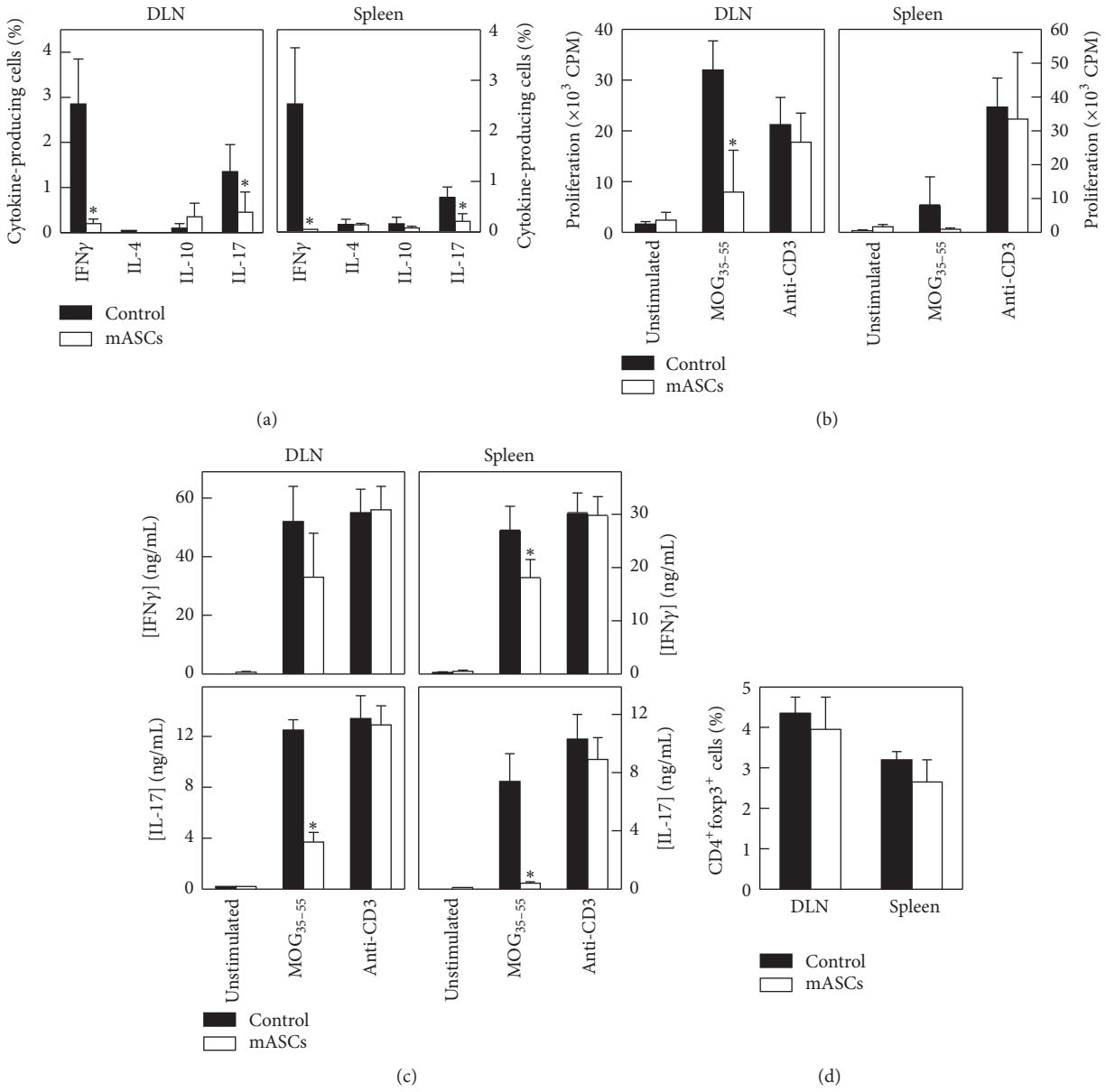

Figure 4: Allogeneic mASCs inhibit MOG-specific immune responses but do not induce foxp3$^+$ Tregs. mASCs were injected intraperitoneally in MOG-induced EAE mice after the onset of the clinical symptoms and spleens and DLNs were isolated at the peak of the disease. Untreated EAE mice were used as controls. (a) The percentages of cytokine-producing cells in spleen and DLNs were determined by intracellular flow cytometry as described in Materials and Methods. ((b) and (c)) DLN and spleen cells were cultured with medium (unstimulated) or stimulated with MOG_{35-55}(50 μg/mL) or anti-CD3 (1 μg/mL, used as unspecific stimulation). Proliferation was measured after 3 days by [^{3}H]-thymidine incorporation (b). The content of cytokines was determined in culture supernatants after 48 hours (c). (d) The percentages of CD4$^+$Foxp3$^+$ T cells in spleen and DLNs were determined by flow cytometry. Results are shown as mean (SD) from 4 mice per group. $^*p < 0.05$ versus control.

frequency of CD4$^+$foxp3$^+$ T cells or IL-10$^+$ cells in spleen and lymph nodes nor increased foxp3 mRNA levels in the CNS after mASC injection into EAE mice which is in accordance with other studies on MSCs and EAE [27, 52, 53]. These discrepancies could be due to differences in the immunomodulatory mechanisms employed by murine and human MSCs, underlying differences in the pathophysiological mechanisms between EAE and MS, and the timing of MSC injection relative to disease progression and to the numbers of MSCs reaching the DLNs [26, 51]. These differences could also partially explain why the clinical efficacy of MSCs in MS is not quite established. Importantly, in another MS-trial, Connick et al. [54] observed a cessation of the protective effects six months after intravenous infusion of MSC, suggesting that a possible induction of Tregs by MSCs was not enough to restore immune homeostasis in MS patients. Future studies

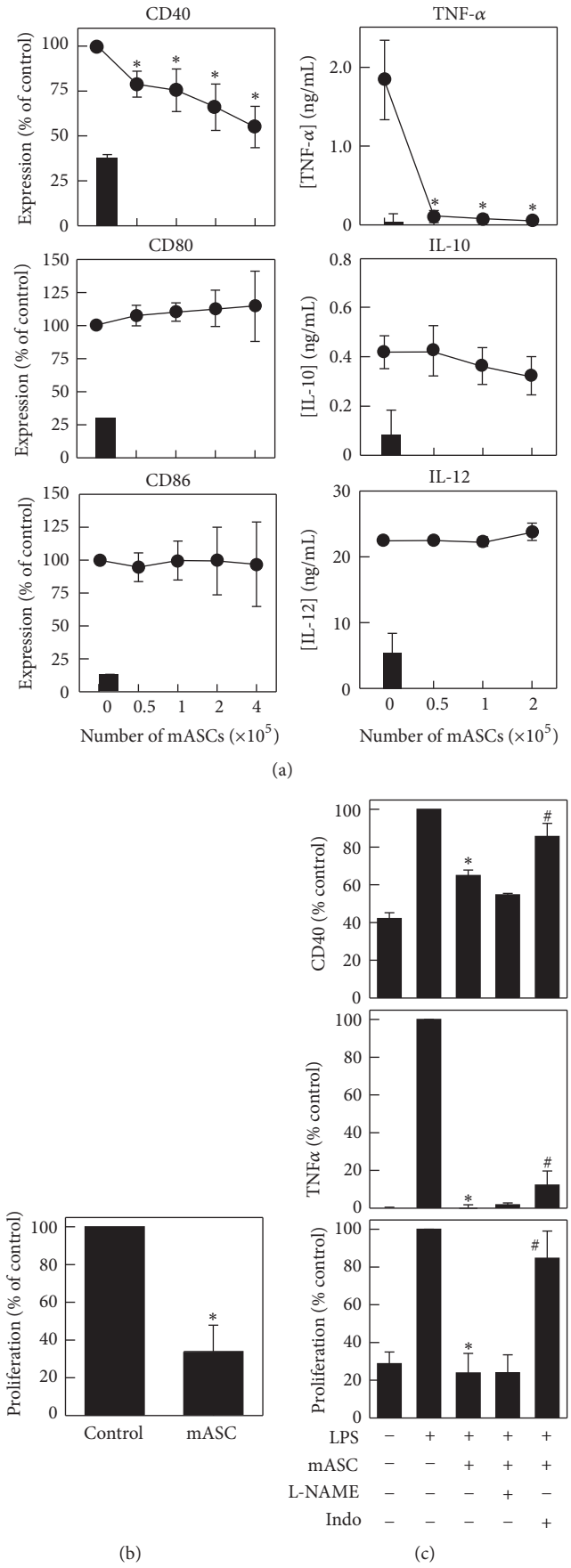

Figure 5: Continued.

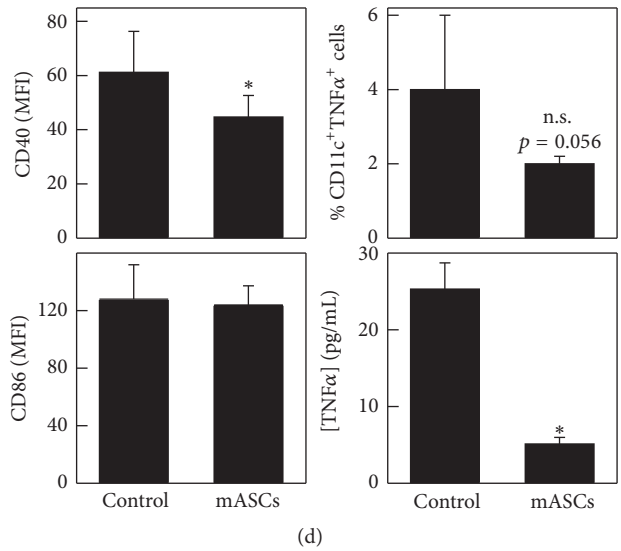

Figure 5: mASCs inhibit the maturation/activation of dendritic cells in vitro and in vivo. (a) mASCs inhibit the maturation of DCs in vitro. Bone marrow-derived DCs from C57BL/6 mice (4×10^5 cells/well) were matured/activated with LPS (1 μg/mL) in the absence or presence of different numbers of mASCs. After 48 hours, nonadherent or loosely adherent cells were harvested (>95% $CD11c^+$ DCs) and analyzed for surface expression of CD40, CD80, and CD86, and the culture supernatants were analyzed for TNF-α, IL-10, and IL-12 content by ELISA. Data for nonstimulated immature DCs are shown as black bars. Results are shown as mean (SEM) of 3 (CD40, CD80, and CD86 FACS and IL-12 ELISA) or 4 (IL-10 and TNF-α ELISA) independent experiments. $^*p < 0.05$ versus control (no mASCs). (b) mASC-treated DCs show impaired costimulatory activity on T cells. C57Bl/6 DCs (4×10^5 cells) were LPS-matured in the absence (control) or presence of mASCs (2×10^5 cells) for 48 hours and then added to allogeneic BALB/c splenocytes (used as responders). The proliferation in the MLR was measured by [^{3}H]-thymidine incorporation. Results are shown as mean (SEM) of 3 independent experiments. $^*p < 0.05$ versus control. (c) COX-1/2 but not iNOS activity is partly responsible for the effect of mASCs on DC maturation. C57Bl/6 DCs (4×10^5 cells) were LPS-matured in the absence or presence of mASCs (2×10^5 cells) and L-NAME (1 mM) or indomethacin (20 μM) for 48 hours. Expression of CD40 was determined in $CD11c^+$ cells by flow cytometry (5 independent experiments) and the levels of TNF-α in culture supernatants were measured by ELISA (3 independent experiments). The costimulatory activity of the mASC-treated DCs was determined in a MLR using BALB/c splenocytes as responders (4 independent experiments). Results are expressed as percentage of values found with control samples treated with LPS alone in the absence of mASCs and shown as mean (SEM) of the independent experiments. $^*p < 0.05$ versus control; $^\#p < 0.05$ versus mASCs. (d) mASC treatment impairs DC function in DLNs of EAE mice. C57BL/6 mice with initial EAE symptoms (scores 1-2) were injected intraperitoneally with allogeneic mASCs (10^6 cells/mouse) isolated from BALB/c mice. After 7 days, DLN cells from untreated (mean score 4.6 (1.0)) and mASC-treated mice (mean score 2.1 (1.0)) were analyzed for the expression of surface CD11c, CD40 ($n = 8$ mice/group), and CD86 ($n = 5$ mice/group) and intracellular TNF-α ($n = 4$ mice/group) by flow cytometry as described in Materials and Methods. $CD11c^+$ DCs were purified by magnetic separation from DLNs from untreated (control) or mASC-treated mice ($n = 4$ mice/group), restimulated (2.5×10^5 cells/mL) with LPS (1 μg/mL) for 24 hours, and the TNF-α levels in supernatants are determined by ELISA (lower right panel). Results are shown as mean (SD). $^*p < 0.05$.

are needed to elucidate if/how MSCs can induce functional Tregs in human MS patients and to design strategies to increase their induction.

DCs are specialized antigen presenting cells that, upon maturation by toll like receptor ligands and cytokines, upregulate costimulatory molecules (CD40, CD80, and CD86) and produce cytokines (TNF-α, IL-12), in order to induce appropriate T cell responses [55]. However, DCs also play fundamental roles in the induction of EAE and the severity of CNS damage correlates with the activation status of DCs [56–58]. In MS patients, DCs can be found in CNS lesions [59] and circulating DCs exhibit an activated $CD40^{high}$TNF-α^+ phenotype [60]. Importantly, several first-line drugs for MS, such as IFN-β [61] and glatiramer acetate [62], inhibit DC function, suggesting that DCs could represent a target for therapeutic modulation. It is thus important to understand if and how MSCs can affect DC function both in vitro and in vivo. PGE_2 has been shown to inhibit T cell proliferation directly or indirectly through its effect on DCs [63]. Spaggiari et al. [43] showed that BM-MSC-derived PGE_2 could inhibit early DC differentiation but not LPS-induced DC maturation. In contrast, Yañez et al. [64] described that hASC inhibited DC maturation in a PGE_2-independent manner. We found that the mASC-derived COX-1/2, but not iNOS activity, could inhibit the LPS-induced CD40 and TNF-α expression by DCs. These contradictory results could be due to species differences, source of DCs, and methodological setup as well as differences in the readout for the DC maturation state. We found that although the expression of CD80, CD86, IL-12, and IL-10 was unaffected, mASC-educated DCs were significantly less effective in inducing T cell proliferation. This is in accordance with the study reported by Sá-Nunes et al. [65] showing that exogenous PGE_2 could inhibit the LPS-induced TNF-α secretion and CD40, but not CD80 and CD86, expression on

DCs which resulted in a decreased T cell-stimulatory ability. In addition to PGE_2, other COX1/2-dependent prostanoids, including PGD_2 and its metabolite 15-deoxy-delta12,14-PGJ_2 have been shown to inhibit LPS-induced production of proinflammatory cytokines by DCs [66, 67]. Although it is conceivable that PGE_2 is the main suppressive prostanoid produced by MSCs, further studies should assess the presence of other prostanoids and PGE_2-derived metabolites [68] and include specific inhibitors for the PGE_2 receptors, EP1-4. Interestingly, a recent study showed that BM-MSCs can produce PGE_2 and prostacyclin (PGI2) [69]. The expression of both anti-inflammatory prostanoids was lost upon oncogenic transformation of the BM-MSCs and this was paralleled with their inability to inhibit immune responses in vitro and in vivo. However, the role of BM-MSC-derived PGI2 in the modulation of DC activation has not been investigated.

As discussed above, MSCs can inhibit DC function in vitro but whether this is also true in vivo is less known. To date only one clinical trial has assessed the effects of MSCs on DCs in vivo and found a reduction in circulating CD40+ myeloid DCs shortly after MSC injection [17]. Using a mouse model based on the adoptive transfer of T cells and LPS-activated DCs, Chiesa et al. showed that BM-MSCs could inhibit the migration and T cell priming capacity by the injected DCs in vivo [70]. Another study using a model of bacteria-induced hepatitis showed that MSC administration induced a population of $CD80^{low}CD86^{low}$ tolerogenic DCs in vivo [71]. However, only two studies have analyzed the activation state of endogenous DCs in CFA/MOG-induced EAE after MSC infusion with contradictory results [27, 53]. In our study, we found that $CD11c^+$ DCs from DLNs of mASC-treated mice expressed lower levels of CD40, but not CD86, and intracellular TNF-α compared to EAE control mice corroborating our in vitro data. In addition, purified LN-DCs from mASC-treated mice produced less TNF-α upon LPS restimulation in vitro. These data suggest that MSCs can inhibit the activation of DCs in vivo. Although not addressed in the current study, we and others have found that injected MSCs reach DLNs in CFA/MOG-induced EAE [27, 52]. Thus, we believe that the reduction of activated DCs in the DLNs of MSC-treated mice is most likely a combination of (i) reduced DC migration from the immunization site and (ii) a direct MSC-mediated inhibition of DC activation in the DLNs, although the relative contribution of both mechanisms requires further studies.

5. Conclusions

Our data suggest that allogeneic ASCs expanded in hypoxia could represent a promising treatment of autoimmune diseases, including MS, through their effects on DC activation and autoimmune responses. It is becoming clear that DCs represent important effector cells, mediating the beneficial effects of MSCs in vivo. Further studies should address in more detail the effects of MSCs on DC function in MS patients and how to increase the MSC-mediated inhibition of DCs. Moreover, the elucidation of the molecular mechanisms involved in the immunomodulatory activity of ASCs, especially the activation of COX-2, suggests that the administration of COX-2 inhibitors (i.e., indomethacin or ibuprofen) during the treatment with ASCs of patients with MS or other autoimmune disorders could impair their therapeutic efficacy.

Competing Interests

No competing financial interests exist

Acknowledgments

This work has been financed by the Instituto de Salud Carlos III, Spain (http://www.isciii.es) and Fondo Europeo de Desarrollo Regional (FEDER, http://ec.europa.eu/regional_policy/es/funding/erdf/) from the European Union, through the Research Grants PI15/00794, CP09/00228, and CPII15/00032 (Per Anderson); PI12/01097, PI15/02015, and ISCIII Red de Terapia Celular (RD12/0019/0006, http://www.redtercel.com/) (Francisco Martin); and PS09-00928 (Mario Delgado). Mario Delgado was supported by a grant (PSE-010000-2009-3) from the Ministerio de Ciencia e Innovación, Spain (http://www.idi.mineco.gob.es/), and P09-CTS-4723 from the Junta de Andalucia (Proyecto de Excelencia). Francisco Martin is funded by the Fundación Progreso y Salud (Consejería de Salud, Junta de Andalucía, http://www.juntadeandalucia.es/fundacionprogresoysalud/).

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