Chapter

The Dual Nature of Mesenchymal Stem Cells (MSCs): Yin and Yang of the Inflammatory Process

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Abstract

The well-known reparative properties of mesenchymal stem cells (MSCs) make them an attractive source for cell-based therapy. In vitro and in vivo studies support an anti-inflammatory role of MSCs by directly targeting immune cells or via the secretion of immunomodulatory factors. MSCs have been isolated from several human normal tissues, even from pathological biopsies and blood samples; in these cases, MSCs displayed peculiar characteristics, suggesting a phenotype transition into a pathological state. Indeed, MSCs derived from inflamed tissues acquired a pro-inflammatory behaviour. In this view, MSCs may be crucial players of many pathways involved in human diseases, especially during the inflammatory cascade. The present chapter will minutely describe the basic biology of human MSCs derived from normal and pathological arteries, focusing on their dual nature as cellular switchers of the inflammatory setting. We will also discuss the emerging role of miRNAs in regulating MSC functions and their potential use as alternative strategies to manipulate MSC efficacy.

Keywords: mesenchymal stem cells (MSCs), immunomodulation, inflammatory process, phenotype switching, vascular wall

1. Introduction

Mesenchymal stem cells (MSCs) are adult, multipotent stem cells endowed of self-renewal, a process of continuous divisions essential to maintain the stem cell pool. Meanwhile, MSCs can be activated under the action of growth factors, chemokines and cytokines which are normally released during the physiological tissue renewal or in pathological conditions in the presence of tissue damage. Specific signals stimulate the MSC migration at the damaged site and their differentiation into specialized cell types belonging to the mesodermal lineage. The homing and the differentiation potential allow MSCs to be actively involved in the tissue homeostasis as well as in the repair process. MSCs have been firstly identified as a non-hematopoietic, adherent and spindle-shaped cell subset of the human bone marrow stroma [1]. From their first isolation in 1970, MSCs have been extensively characterized, gaining the increasing interest of the scientific community, and lots of studies issued the biology and the inner properties of these promising cells. One of the most intriguing and studied functions of MSCs is the immunomodulation, that is, the ability to repress inflammation; however, little is known about the reversal of

this property that has been observed in some disease models. The present chapter will review the differentiation and immunomodulatory capabilities of MSCs, will discuss the contradictory face of MSCs and will focus on the vascular wall setting.

2. Mesenchymal stem cells (MSCs): source, phenotype and properties

In vivo and in vitro data have demonstrated the unique reparative potential of MSCs, which are now considered as the most attractive and functional source for cell-based therapies in the field of regenerative medicine. First of all, the ease of MSC isolation and in vitro propagation excited researchers, who addressed their efforts to find novel sources for MSCs and to characterize them. MSCs have been efficiently isolated and characterized from different human tissues, other than their native site, i.e. peripheral blood [2], umbilical cord (UC) blood [3], fat [4], Wharton's jelly (WJ) [5], synovial membrane [6] and vascular wall [7, 8]. The lack of standard markers, differences in laboratory procedures, type and age of the source tissue may affect the purity of MSC pool and impair their effectiveness for clinical applications. In order to fill this gap and provide a consensus statement for MSC definition, the International Society for Cellular Therapy (ISCT) postulated the following minimal criteria [9]:

- Adherence to plastic substrate in vitro
- Expression of surface markers CD90, CD105 and CD73 (mesenchymal linage) and lack of CD34, CD19, CD45 and CD11a (hematopoietic lineage), CD31 (endothelial lineage) and HLA-DR (human leukocyte antigen)
- Multilineage differentiation ability into the mesodermal lineage (chondrogenic, adipogenic, osteogenic commitments)

Even if researchers concur with the consideration of CD34 as distinctive marker of endothelial and hematopoietic cells, it has been detected also in MSCs correlating with advanced progenitor properties [10, 11]. Moreover, adipose tissue-derived MSCs positive to CD34 lost its expression after in vitro propagation suggesting that the absence of CD34 may be a result of in vitro culture [12, 13]. Thus, the immunophenotype is not per se sufficient to identify MSCs, but functional assays aimed at testing the stemness properties, like the ability to form colonies, and the differentiation potential are necessary. At this regard, MSCs also manifest the capacity to differentiate into ectoderm- and endoderm-derived cell types, i.e. endothelial cells, neurons and hepatocytes.

The presence of MSCs with self-renewal and multilineage differentiation properties within adult tissues suggests their intrinsic participation to the regular tissue homeostasis and cell turnover. Cytokines and chemokines that are released from the injured tissue act as recruiting factors of MSCs from their niche, allowing their mobilization and trafficking. The ability of differentiate into tissue-specific cell types is the major mechanism through which MSCs replace dead cells; in addition, MSCs secrete soluble factors that include hepatocyte growth factor (HGF), transforming growth factor (TGF) β1 and vascular endothelial growth factor (VEGF) and contribute to cutaneous wound healing [14].

The migration and the differentiation abilities support the clinical use of MSCs for the cure of degenerative diseases but, if uncontrolled or impaired, could become prerequisites to the occurrence of pathological conditions. Further, the tissue source constitutes a discriminating factor among MSCs in terms of differentiation potency, migration and effectiveness in tissue repair. The differentiation potency can also

be regulated on epigenetic basis; at this regard, the methylation status of the main regulators of MSC fate crucially drives the differentiation program. This condition has been demonstrated in a study by Xu et al., where BM-MSCs resulted more effective in osteogenic differentiation than adipose tissue (AT)-MSCs, which displayed the opposite trend and were mainly addressed towards the adipogenic commitment [15]. Similarly, perivascular MSCs isolated from the UC blood exhibited higher angiogenic potency than umbilical artery and WJ- MSCs, with implications for the cure of ischemic injury [16]. Therefore, the clinical use of MSCs is a promising and undeniable chance for regenerative medicine, but it needs to be optimized because of the MSC multifaceted nature

2.1 MSC immunomodulation

A large body of experimental and clinical studies showed that MSCs modulate the immune response, both innate and adaptive with possible implications in the management of transplantation, autoimmune and inflammatory disorders [17]. MSCs have been historically considered as immune-privileged cells, because of their poor immunogenicity. Indeed, the low levels of human leukocyte antigen (HLA) histocompatibility complex-I and the lack the complex HLA-II allow MSCs to elicit the immune recognition. Additionally, MSCs do not express the Fas ligand and the co-stimulatory molecules CD40, CD80 (B7-1) and CD86 (B7-2), which are necessary for effector T-cell activation [18, 19]. On the other hand, it has been widely demonstrated that MSCs affect the immune system, both through cell–cell interactions and by the paracrine secretion of anti-inflammatory factors.

2.2 MSC-immune cell interactions and in vitro immunomodulatory assays

The majority of studies use a mixed lymphocyte reaction (MLR) assay and explore the immunosuppressive effects of MSCs on allogeneic T cells in the MLR reaction. Peripheral blood mononuclear cells (PBMCs) are obtained by density gradient separation and cultured on a feeder layer of irradiated MSCs generally for 3 or 5 days. PBMCs are stimulated by the addition of mitogens to the culture media like phytohaemagglutinin (PHA), which markedly induces CD8 T-cell proliferation [20]. The coculture protocol can be executed in a direct manner for testing effects mediated by physical cell-cell interactions; alternatively, it is possible to evaluate the influence of MSC paracrine secretion in a separate coculture system by the use of a Transwell insert of 0.4 μm that inhibits PBMC migration and maintains the two cell compartments separated. At the end of the experimental coculture, PBMCs are collected and analyzed in terms of proliferation and activation. Cell cycle analysis and specific proliferation test, like the incorporation rate of bromodeoxyuridine (BrdU) into DNA, are executed for T-cell survival together with the analysis of T-cell subpopulation percentage. On the other hand, MSCs are characterized for immunomodulatory markers, such as HLA-G, interleukin (IL)-10 and HGF, through a wide range of techniques including flow cytometry, immunofluorescence and Western blot. A pictorial description of the basic immunomodulatory assay can be observed in **Figure 1a**. MSCs inhibit naïve $CD4^+$ T helper cell proliferation by inducing the cell cycle arrest at G0 and by hindering the T helper cell differentiation into T_H1 and T_H17 subsets. In addition to direct cell-cell interactions, the MSC immunomodulation is exerted through the secretion of soluble anti-inflammatory mediators, such as nitric oxide (NO), TGF-β1, interleukin (IL)-10, indoleamine 2,3-dioxygenase (IDO), HGF and prostaglandin E2 (PGE2). HLA-G is another crucial component involved into the MSC immunosuppressive system, allowing the induction of regulatory T cells (Treg) and inhibiting natural killer (NK) cell cytotoxicity and

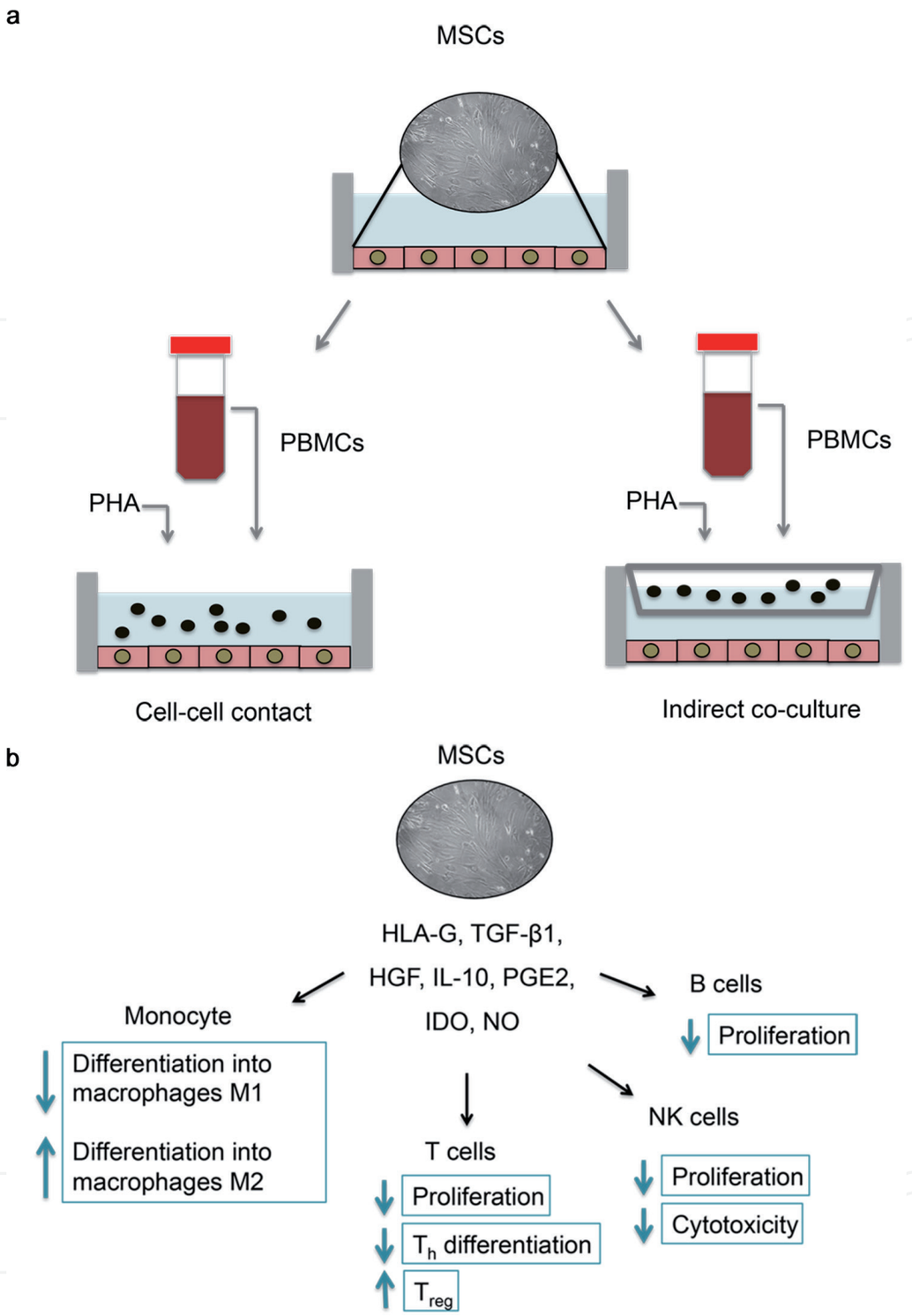

Figure 1.
MSC immunomodulation: (a) schematic description of the immunomodulatory procedure for testing the MSC effects on PBMCs; and (b) overview of the main mechanisms MSC-dependent on immune cells. Abbreviations: PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; HLA-G, human leukocyte antigen; HGF, hepatocyte growth factor; TGF-β1, transforming growth factor; IL-10, interleukin; NO, nitric oxide; IDO, indoleamine 2,3-dioxygenase; PGE2, prostaglandin E2.

dendritic cell (DC) maturation [21, 22]. MSCs further exert their immunosuppressive effects on B cells, blocking their proliferation. The scheme in **Figure 1b** summarizes the main mechanisms relative to the MSC-driven immunomodulation.

The successful application of MSCs has been reported in several clinical studies; among these, MSCs resulted effective in graft-versus-host disease (GvHD) patients even in those resistant to steroid treatment. Moreover, beneficial effects were observed in patients affected by systemic lupus erythematous (SLE) and Crohn's disease [23].

However, the effectiveness and safety of MSC application are still under debate, and some data suggest that the immunomodulatory function is tightly regulated. Moreover, many studies have elucidated differences in terms of immunomodulatory potency among MSCs isolated from distinct sources. At this regard, a study by Mattar et al. compared the immunosuppressive properties of BM-MSCs, AT-MSCs, CB-MSCs and WJ-MSCs on PHA-activated T cells, showing that MSCs from tissues alternative to bone marrow were effective in inhibiting T-cell proliferation [24]. Similarly, Li et al. demonstrated that WJ-MSCs exhibited the highest immunosuppressive force and lowest levels of immunogenic factors than AT-, BM- and PL-MSCs, thus resulting as the most promising for potential therapeutic application [25, 26]. Further comparative analysis aimed at deepen functional characteristics of MSCs from multiple sources is necessary to improve their translation into the clinic.

2.3 MSCs like macrophages: switch from pro-inflammatory to anti-inflammatory phenotype

Several studies have addressed the intrinsic mechanisms associated with MSC immunomodulation, and increasingly evidences have demonstrated the plastic nature of this intriguing property. The effectiveness of MSC immunomodulation can depend on the external microenvironment; different studies support the hypothesis that MSCs can both reduce and strengthen the inflammatory process but is the inflammatory context itself at determining the immunosuppressive function of MSCs. Thus, MSCs need to be 'licensed' for their activation and regulate the immune response [23]. At this regard, MSCs can switch from a pro-inflammatory to an anti-inflammatory phenotype, characterized by a different soluble factor and cytokine panel. This paradigm mimics the macrophage polarization model. Macrophages are immune cells that derive from the differentiation of their precursor cells, monocytes, and represent key players of the immune response. Monocytes, once arrived at the damaged site, can differentiate into M1- or M2-type macrophage, depending on the microenvironment stimuli. The M1 macrophage releases a series of inflammatory cytokines, contributing to the local inflammation; conversely, the M2 macrophage mainly secretes IL-10 and TGF-β1 that are anti-inflammatory. The local microenvironment critically triggers MSC polarization, and the Toll-like receptors (TLRs) are of crucial importance in this regulatory mechanism. TLRs represent a conserved family of pattern recognition receptors (PRRs) able to detect a wide spectrum of pathogen-associated patterns (PAMPs) and activate the immune cells. The expression of TLRs in MSCs changes according to the tissue source but also depends on the microenvironment stimuli. According to Bunnel et al., the activation of TLR4 triggers MSCs skew into a pro-inflammatory phenotype, releasing factors like IL-6 and IL-8 that contribute to tissue injury [27]. Conversely, the stimulation of TLR3 shifts MSCs to the anti-inflammatory phenotype accompanied by the secretion of IL-4, IDO and PGE-2 [27, 28]. Other studies support the contribution of nitric oxide (NO) to the anti-inflammatory activity of MSCs; indeed, the inhibition or the genetic ablation of NO synthase (iNOS) stimulates T-cell proliferation after being cultured with MSCs [29]. Thus, the poor activation of MSC anti-inflammatory profile leads to the worsening of the inflammatory process and of the tissue damage. Defects of the immunomodulatory functions have been observed in some diseases, like a study on a mouse model of collagen-induced arthritis (CIA) demonstrated. MSCs exposed to an inflammatory environment, like in the CIA model, displayed impaired immunomodulation and, after TNF-α addition, lost the ability to regulate T-cell proliferation [30]. Also, MSCs isolated from the bone marrow of multiple myeloma patients were ineffective in vitro, reflecting an aberrant T-cell function in vivo [31]. The therapeutic application of MSCs for treating inflammatory conditions is

really promising; nevertheless, the effectiveness and safety of their administration in vivo are still controversial, representing a challenging issue. The diversity of MSC biology and mechanism of action implicate knowledge gaps that need to be filled for a clinical application of MSCs on large scale [32, 33]. For this reason, novel studies should address all the technical concerns related to the use of MSCs and extend their investigation field to MSCs from human disease models.

3. Tissue specificity of MSCs: normal versus diseased arteries

As described in the second paragraph, MSCs are markedly represented in several adult human tissues other than bone marrow and fat; the rationale to this wide distribution comes from the existence of the vasculogenic zone, which is a stem cell niche within the vascular wall.

In this field, our research team has reached a 10-year experience in isolating MSCs from a broad range of vascular segments of small and large size, including healthy and diseased arteries. As regards the technical concerns, the isolation protocol consists of the enzymatic digestion of both fresh and cryopreserved arteries collected at the Cardiovascular Tissue and Cord Blood Bank (S. Orsola-Malpighi Hospital, Bologna, Emilia Romagna-Italy) and belonged to different vascular districts (carotid bifurcation, aortic arch, abdominal/thoracic aorta, femoral artery). In spite of the extreme temperature conservation in liquid nitrogen for about 10 years, these vascular tissues were vital and resulted as an unusual source of clonogenic and highly proliferative MSCs endowed of chondrogenic, adipogenic, osteogenic and smooth muscle differentiation capability [34]. Interestingly, these human vascular wall-MSCs (hVW-MSCs) possess angiogenic potential, as demonstrated by the capacity of forming a capillary-like network when seeded onto a semi-solid matrix (Matrigel) and by the expression of endothelial markers after VEGF stimulation. The capacity of VW-MSCs to differentiate into endothelial-like cells befits with their location within the arterial wall, suggesting their function as cell reservoir during the normal tissue renewal. The angiogenic potential and the migratory capacity of VW-MSCs were explored as crucial aspects for the healing of vascular ulcers and resulted boosted under the effects of recombinant HGF [35]. Another intriguing finding supporting the therapeutic force of VW-MSCs regarded the immunomodulatory capability. The vessel wall is prone to inflammatory infiltration following the endothelial dysfunction; thus, it is reasonable to hypothesize that vascular resident progenitors are able to contain this phenomenon. hVW-MSCs exerted a significant suppressive effect on PHA-PBMCs proliferation, partially mediated by the expression of HLA-G. These data support the existence of MSC cells within the vascular wall and their participation in the normal tissue homeostasis as well as in the arterial repair. During the early phase of the atherogenic process, monocytes cross the endothelial barrier and differentiate into macrophages within the media where the release of matrix metalloproteinases (MMPs), cytokines and chemokines recruits other monocytes and lymphocytes. This vascular inflammation is the licencing factor that activates vascular MSCs into the immunosuppressive phenotype; any intimate disturbance to this mechanism may skew MSCs into the opposite trend, failing in vascular healing.

3.1 Functional deregulation of MSCs within the injured artery

As reported in some disease models, like osteo-chondrogenic disorders or hematological affections, MSCs can undergo a deregulation of their reparative properties that can be enhanced or defected with undeniable pathological implications. Our team investigated the contradictory face of MSCs, extending the analysis to the field

of cardiovascular diseases like abdominal aortic aneurysm (AAA), atherosclerotic carotid plaque and arteriovenous fistula. AAA is a chronic dilatation of the aortic wall, due to the exaggerated degradation of extracellular matrix proteins by MMPs resulting in the loss of elastin and collagen; in addition, the inflammatory infiltrate occurs and contributes to the degeneration of the media tunica, together with further release of MMPs and the destabilization of the wall. Published data have shown for the first time that, in spite of the complete disorganization of the tissue, MSCs isolated from the AAA wall (named AAA-MSCs) were comparable to the healthy counterpart in morphology, growth rate and immunophenotype [36]. Even more, these pathological MSCs displayed altered functions in vitro; consistent with the chronic inflammatory setting of the AAA disease, AAA-MSCs displayed low HLA-G expression and resulted ineffective in modulating the PBMC proliferation. This low efficacy may reflect the switch of vascular MSCs into the pro-inflammatory phenotype and the worsening of the aortic wall conditions. Angiogenesis represents another distinctive hallmark of AAA pathology, significantly affecting the wall stability. AAA-MSCs were able to differentiate into endothelial-like cells, as demonstrated by the formation of a vascular network onto Matrigel and by the positivity to CD31 marker. Nevertheless, we observed a reduced expression of CD146, a pericyte marker, suggesting the instability and immaturity of the AAA-MSC-derived neo-vessels [37]. A representative image of AAA-MSC functional characteristics is reported in **Figure 2**.

Other few works have issued the pathological role of MSCs residing within the vasculogenic zone. At this regard, the first hypothesis on MSC contribution to vascular diseases was proposed in 2012 by Tang et al., who showed that rodent MSCs undergo increased proliferation and migration upon vascular injury [38]. In 2016, a study of Kramann et al. performed on ApoE−/− mice with chronic kidney disease demonstrated the involvement of a population of MSC-like cells to the onset of athero- and arteriosclerosis as well as to the differentiation into osteoblast-like cells [39]. An aberrant differentiation program of MSCs can be crucial in triggering the complications of the atherosclerotic plaque, like ectopic bone formation,

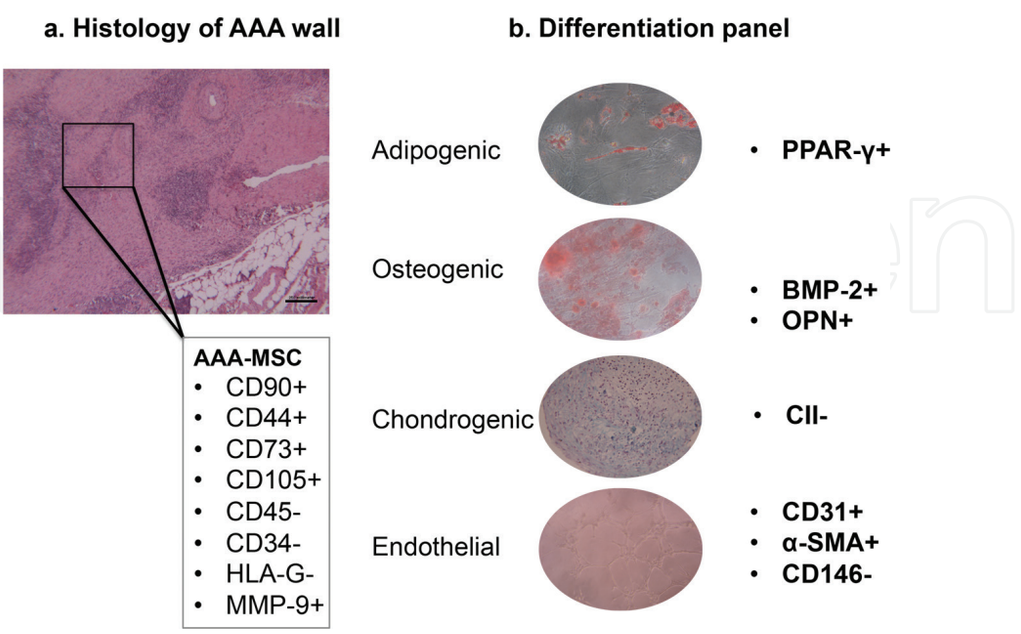

Figure 2.
Immunophenotype and differentiation assays of AAA-MSCs: (a) AAA-MSCs positive to CD90, CD44, CD73, CD105 and MMP-9 and negative to CD45 and CD34 were isolated from the aorta affected by aneurysm, inflammation and atherosclerotic plaque. (b) AAA-MSCs were characterized for adipogenic, osteogenic, chondrogenic and endothelial differentiation abilities. Abbreviations: HLA, human leukocyte antigen; MMP, matrix metalloproteinase; PPAR, peroxisome proliferator-activated receptor; BMP, bone morphogenetic protein; OPN, osteopontin; CII, collagen type 2; SMA, smooth muscle actin.

Properties	Source	
	Healthy arteries	Pathological aorta
Morphology	Fibroblast-like	Fibroblast-like
Immunophenotype	CD90+, CD105+, CD73+, CD44+, CD34-	CD90+, CD105+, CD73+, CD44+, CD34-
Multilineage differentiation		
Adipogenic	++	++
Osteogenic	+	+++
Chondrogenic	++	-
Angiogenic potency	+	++
	CD31+, α-SMA+, CD146+	CD31+, α-SMA+, CD146-
Immunomodulation		
T cell proliferation	++	-
HLA-G expression	+	-

Table 1.
Phenotypic and functional characteristics of healthy versus pathological vascular MSCs.

and represents the early stage during calcification process [40]. AAA-MSCs also exhibited a marked osteogenic ability, correlating with the vascular calcium levels as measured by angio-CT in the enrolled patients [37]. Thus, it can be postulated that MSCs are key players during the renewal as well as the pathological conditions affecting the vascular wall. The MSC behaviour can be seen as fine balance between two opposite forces, which is strongly influenced by the external microenvironment and the interaction with the neighboring cells. At this regard, the immune cells and the cytokines released during inflammation are key factors in exacerbating the osteogenic differentiation of healthy VW-MSCs [37].

The most remarkable characteristics of AAA-MSCs and their comparison to the hVW-MSCs are summarized in **Table 1**.

4. miRNA regulation of MSC immunomodulatory capacity

Micro-RNAs (miRNAs) constitute a class of single-stranded non-coding RNAs of approximately 18–22 nucleotides that function as endogenous regulators of gene expression through the degradation of the target mRNA or the inhibition of the transcription process. Over the last decade, the growing interest for miRNA applications elucidated their involvement into many biological mechanisms, like cell growth and proliferation. Based on the observation that miRNAs are differentially regulated in the presence of pathological conditions like cancer or immune diseases, many researchers have proposed their use as diagnostic markers or therapeutic targets. Several studies revealed the miRNA involvement into the hematopoietic stem cell (HSC) system, driving aspects like cell survival, self-renewal and differentiation. Moreover, these 'immuno-miRs' orchestrate crucial steps of both innate and adaptive immune cell development and function [41]. miR-21, miR-146a and miR-155 are included in this category and are induced upon T-cell receptor (TCR)

activation through the NF-kB cascade. miRNAs have also been shown to regulate the stem cell behaviour, self-renewal and differentiation; therefore, investigating immuno-miRs in human MSCs could be suggestive of their reparative properties like differentiation and immunomodulatory potency. Some of the described immuno-miRs target the TLR pathway in MSCs or immune cells suppressing or enhancing TLR activation by targeting adapter molecules, cytokines and transcription factors. At this regard, a work by Matysiak et al. demonstrated the upregulation of several miRNAs in differentiated BMSCs that had lost immunomodulation, including miR-146a, together with a low expression of PGE2 [42]. The role of miR-155 in interfering with MSC immunomodulation has been also reported; indeed, miR-155 decreases the iNOS production in cytokine-activated MSCs, partially targeting TGF-beta-activated kinase 2 (TAB2), an adapter protein involved in TLR pathways [43]. As recently demonstrated, many of the therapeutic effects of MSCs are mediated by the extracellular vesicles (EVs), which are membrane-bound vesicles that serve as vehicle of mRNAs and proteins. Moreover, EVs are enriched of miRNAs that are released in the circulatory system. The EV-released miRNAs, such as Let-7b, miR-1180 and miR-183, induce macrophage polarization into M2 phenotype and mitigate inflammation by reducing TLR4 [44]. The mechanisms regulating the miRNA-MSCs interplay are complex and require further investigations; the use of miRNA-enriched EVs derived from human MSCs could be the promising therapeutic cell-free alternative for the cure of GvHD and inflammatory diseases.

5. Conclusions

The regenerative and reparative properties of MSCs are certainly undisguised even though many efforts are necessary to ensure their use for clinical therapy. The contradictory inflammatory activity of MSCs is a result of their plastic nature and represents a critical issue that needs to be addressed. Tissue-resident MSCs can represent the optimal target of stem cell-reprogramming therapies aimed at restoring their native reparative properties. The emerging role of miRNAs in regulating the MSC functions is promising and requires further investigations for miRNA manipulation in order to address MSC towards a more efficient and safe reparative activity.

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