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Molecular Mechanisms Responsible for Anti-inflammatory and Immunosuppressive Effects of Mesenchymal Stem Cell-Derived Factors

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Abstract

Mesenchymal stem cells (MSCs) are self-renewable cells capable for multilineage differentiation and immunomodulation. MSCs are able to differentiate into all cell types of mesodermal origin and, due to their plasticity, may generate cells of neuroectodermal or endodermal origin in vitro. In addition to the enormous differentiation potential, MSCs efficiently modulate innate and adaptive immune response and, accordingly, were used in large number of experimental and clinical trials as new therapeutic agents in regenerative medicine. Although MSC-based therapy was efficient in the treatment of many inflammatory and degenerative diseases, unwanted differentiation of engrafted MSCs represents important safety concern. MSC-based beneficial effects are mostly relied on the effects of MSC-derived immunomodulatory, pro-angiogenic, and trophic factors which attenuate detrimental immune response and inflammation, reduce ischemic injuries, and promote tissue repair and regeneration. Accordingly, MSC-conditioned medium (MSC-CM), which contains MSC-derived factors, has the potential to serve as a cell-free, safe therapeutic agent for the treatment of inflammatory diseases. Herein, we summarized current knowledge regarding identification, isolation, ontogeny, and functional characteristics of MSCs and described molecular mechanisms responsible for MSC-CM-mediated anti-inflammatory and immunosuppressive effects in the therapy of inflammatory lung, liver, and kidney diseases and ischemic brain injury.

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Keywords
Immunosuppression · Inflammatory diseases · Mesenchymal stem cells · Soluble factors · Therapy

Abbreviations

(EMT)	Epithelial-to-mesenchymal transition
AF-MSCs	Amniotic fluid-derived MSCs
AT-MSCs	Adipose tissue-derived MSCs
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BM-MSCs	Bone marrow-derived MSCs
BMP	Bone morphogenetic protein
BPD	Bronchopulmonary dysplasia
C/EBPα	CCAAT/enhancer-binding protein alpha
CCL	CC chemokine ligand
c-MYC	Avian myelocytomatosis virus oncogene cellular homolog
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
ERK	Extracellular signal-regulated kinases
ESCs	Embryonic stem cells
FABP4	Fatty acid-binding protein 4
FAS	Fatty acid synthase
FasL	First apoptosis signal ligand
GLUT4	Glucose transporter type 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hMSCs	Human MSCs
HO-1	Heme oxygenase-1
IDO	Indoleamine 2,3-dioxygenase
IFN-β	Interferon beta
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL-1Ra	Interleukin 1 receptor antagonist
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte growth factor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
M-CSF	Monocyte colony-stimulating factor
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MSC-CM	MSC-conditioned medium
MSCs	Mesenchymal stem cells
mMSCs	Murine MSCs
MZ	Marginal zone
NECs	Neuroepithelial cells
NK	Natural killer
NKT	Natural killer T cells
NKTregs	Regulatory NKT cells
PAX	Paired box
PGE2	Prostaglandin E2
PL-MSCs	Placenta-derived MSCs
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
RUNX2	Runt-related transcription factor 2
SCF	Stem cell factor
Sox9	Sex-determining region Y-box 9
SSEA	Stage-specific embryonic antigen
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-beta
TIMP-1	Tissue inhibitor of metalloproteinase-1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRA-1–60	Tumor resistance antigen 1–60
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	T regulatory cells
TSG-6	TNF-α-stimulated gene/protein 6
UC-MSCs	Umbilical cord-derived MSCs

1 Introduction

Stem cells, as self-renewable cells with capacity for pluri- or multilineage differentiation, have raised enormous expectations among healthcare professionals and patients due to their biological importance and therapeutic potential (Volarevic et al. 2011a, 2018). Therapy of many incurable diseases is in the focus of stem cell-based research, and, currently, stem cell-derived tissues, products, and biomaterials represent new hope in regenerative medicine (Volarevic et al. 2011a).

Because of their regenerative and immunomodulatory characteristics, including self-renewability, rapid proliferation, multilineage differentiation, and production of immunosuppressive and pro-angiogenic factors, mesenchymal stem cells (MSCs) are, among stem cells, most usually used in clinical trials as new therapeutic agents for the treatment of inflammatory, degenerative, and ischemic diseases (Gazdic et al. 2017; Markovic et al. 2018; Arsenijevic et al. 2017; Volarevic et al. 2014). MSCs can be easily derived from almost all adult tissues and, accordingly, represent highly accessible cell source with great potential for autologous transplantation (Volarevic et al. 2011b). Moreover, MSCs do not express major histocompatibility complex (MHC) molecules class II and are considered immune-evasive cells capable to engraft in the tissues of MHC-mismatched recipients. Accordingly, MSCs represent valuable cell source for safe allogeneic transplantation (Gazdic et al. 2015). MSC-dependent regeneration of injured tissues is relied on their unlimited differentiation potential. These stem cells differentiate into the cells of mesodermal origin in vivo, while in vitro (under specific culture conditions) MSCs may generate cells of ectodermal and endodermal origin, as well (Volarevic et al. 2011b; Chamberlain et al. 2007). Immediately after transplantation, MSCs are able to migrate toward the site of injury where, through the production of immunomodulatory, pro-angiogenic, and trophic factors, they regulate immune response, induce generation of new blood vessels, and promote tissue repair and regeneration (Volarevic et al. 2017). Since MSCs represent vehicles for the delivery of immunosuppressive and trophic factors, their engraftment attenuates inflammation, encourages endogenous regeneration, and results in repopulation of injured cells (Gazdic et al. 2017; Markovic et al. 2018; Arsenijevic et al. 2017; Volarevic et al. 2014, 2017).

In this chapter we summarized current knowledge about origin, phenotypic, and functional characteristics of MSCs with particular focus on molecular mechanisms which are responsible for beneficial effects of MSC-derived factors in the therapy of chronic inflammatory diseases.

2 Developmental Origin and Characterization of MSCs

Developmental biology has witnessed controversies concerning origin and characterization of MSCs (Fitzsimmons et al. 2018). Most findings support the hypothesis that there are several subpopulations of MSCs that originated from different precursor cells during embryogenesis (Fitzsimmons et al. 2018). Epithelial-to-mesenchymal transition (EMT)-derived cells have a functional resemblance to bone marrow-derived MSCs (BM-MSCs), in terms of antigenic profile, multipotency, and homing capacity, and, accordingly, were proposed as possible precursor cells of MSCs (Battula et al. 2010). Several other studies indicated neural crest origin of MSCs by providing evidence that Sox1+ neuroepithelial cells (NECs) are precursors of MSCs (Takashima et al. 2007a, b; Quirici et al. 2002; da Silva Meirelles et al. 2008). This hypothesis was supported by the findings that MSCs, similar as Sox1 + NECs, expressed receptor for nerve growth factor and were able to differentiate in neuroectodermal (neurons/glias) cells in vitro (Quirici et al. 2002; da Silva Meirelles et al. 2008), while Sox1+ NECs, similar as MSCs, could generate osteoblasts, chondroblasts, and adipocytes (Takashima et al. 2007b). Accordingly, it was suggested that Sox1 + NECs represent the earliest population of MSCs that reside in prenatal tissues, while later in postnatal development, MSCs could be derived

from the cells of nonneural crest origin (Takashima et al. 2007a). In accordance to their multipotency and capacity for spontaneous differentiation in cells of mesodermal origin, lateral plate mesoderm-derived mesoangioblast cells from the embryonic dorsal aorta were proposed as nonneural crest source of MSCs (da Silva Meirelles et al. 2008; Sheng 2015). Additionally, several studies have shown that the blood vessel walls represent an important reservoir of MSC-like stem/progenitor cells (Crisan et al. 2008; Chen et al. 2013). These blood vessel-derived precursor cells, isolated from multiple organs, give rise to cells with typical MSC markers and exhibit capacity for differentiation into osteoblasts, chondrocytes, and adipocytes (Crisan et al. 2008; Chen et al. 2013). Additionally, similarities between MSCs and pericytes in terms of ontogeny, phenotypic and functional characteristics, suggest that these two cell populations originated from the same precursor cell (Harrell et al. 2018a). In line with these findings, it is not an easy task to precisely identify and characterize pure population of MSCs in perivascular tissues (Harrell et al. 2018a).

Having in mind that many diverse antigens have been found on the surface of MSC, but none of them were unique for MSCs (Volarevic et al. 2011a), researchers from International Society for Cellular Therapy focused their attention on morphological and functional properties that were specific for MSCs and managed to define three minimal criteria for characterization of MSCs. First, MSCs have to adhere to plastic culture dishes under standard in vitro conditions. Second, more than 95% of cell population must express CD105 (endoglin, also identified as SH2, a component of the receptor complex of transforming growth factor-beta (TGF-β) involved in proliferation, differentiation, and migration), CD73 (SH3/4, ectoenzyme that regulates the purinergic signaling through the hydrolysis of adenosine triphosphate (ATP)), and CD90 (Thy-1, regulates differentiation of MSCs). MSCs must lack expression of CD45 (pan-leukocyte marker), CD34 (marker of hematopoietic cells), CD14 or CD11b (markers of monocytes), CD79a or CD19 (marker of B lymphocytes), and MHC class II molecules (marker of professional antigen-presenting cells) (Dominici et al. 2006). Third, cells must be able to spontaneously differentiate into adipocytes, osteoblasts, and chondrocytes under standard in vitro differentiating conditions (Dominici et al. 2006). In addition to these well-defined phenotypic and functional characteristics, MSCs constitutively express several adhesion molecules, CD44 (hyaluronan receptor), CD166 (vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule), and CD49b (integrin alpha-2), that enable their migration toward the site of the injury (Dominici et al. 2006).

3 MSC Subpopulations: Phenotype and Functional Characteristics

MSCs reside in perivascular niches of many diverse adult, fetal, and neonatal tissues (bone marrow, adipose tissue, peripheral blood, dental pulp, amniotic fluid, placenta, umbilical cord, etc.) (Hass et al. 2011). Differences in extracellular milieu (influence of neighboring cells and their products, hypoxia) as well as intracellular conditions (expression of certain microRNAs) significantly affect function and therapeutic potential of MSCs. Accordingly, MSCs are considered as heterogeneous group of stem cells that consist of several subpopulations with variable morphological and functional characteristics (Hass et al. 2011).

Among all subpopulations of MSCs, BM-MSCs are best explored and most usually used in experimental and clinical trials. In the bone marrow, MSCs regulate lifelong turnover and growth of bone (Bianco 2014; Wu et al. 2018) and, as major source of stem cell factor (SCF), represent an important cellular component of the hematopoietic stem cell niche (Savickienė et al. 2017). BM-MSC-derived osteocytes promote hematopoiesis, while BM-MSC-derived adipocytes inhibit expansion of hematopoietic progenitors (Bethel et al. 2013). Main biologic

characteristics of BM-MSCs which favor their therapeutic use are rapid proliferation in vitro, reduced expression of MHC molecules and, accordingly, potential for safe allogeneic transplantation, genomic stability after long-term propagation, capacity for spontaneous trilineage (osteogenic, chondrogenic, and adipogenic) differentiation, and suppression of detrimental immune response (Volarevic et al. 2017). However, the derivation of BM-MSCs involves harvesting of bone marrow that is a highly invasive procedure (Nishida et al. 1999; Mueller and Glowacki 2001; Stenderup et al. 2003). Therefore, several alternative tissue sources for isolation of MSCs have been strongly pursued including the adipose tissue, amniotic fluid, umbilical cord, and placenta (Zhou et al. 2014a).

The adipose tissue contains a significant number of MSCs that are easy to harvest by liposuction (Lee et al. 2016). Compared to BM-MSCs, adipose tissue-derived MSCs (AT-MSCs) have similar phenotype, greater proliferation capacity, higher potential for adipogenic differentiation, and inferior potential for osteogenesis and chondrogenesis (Lee et al. 2016; Zuk et al. 2002).

Collection of umbilical cord-derived MSCs (UC-MSCs) is a noninvasive, painless, and safe procedure that has not been encumbered with ethical problems (Nagamura-Inoue and He 2014). MSCs have been isolated from several compartments of the umbilical cord including Wharton's jelly, vein, arteries, UC lining, and subamnion and perivascular regions (Nagamura-Inoue and He 2014). There is no significant difference in the proliferation rate among the cells derived from various compartments of UC, and, importantly, all subpopulations of UC-MSCs exhibit a significantly higher frequency of colony-forming unit fibroblasts than BM-MSCs (Majore et al. 2011; Baksh et al. 2007; Lü et al. 2008). Regarding the differentiation ability, UC-MSCs have higher potential for chondrogenic differentiation than BM-MSCs, but show delayed and insufficient differentiation into osteocytes and adipocytes (Hsieh et al. 2010; Mennan et al. 2013). Interestingly, UC-MSCs may be considered as pluripotent cells, since they express several genes associated with pluripotency: Oct-3/4, Nanog, Sox2, and KLF4 (Greco et al. 2007).

Amniotic fluid-derived MSCs (AF-MSCs) are isolated from amniotic fluid samples obtained through amniocentesis under ultrasonographic control (Tsai et al. 2004; Savickienė et al. 2017; Spitzhorn et al. 2017). Accordingly, amniotic fluid can serve as a rich and advantageous source of MSCs in terms of number of potential donors (Tsai et al. 2004; Moraghebi et al. 2017; Bitsika et al. 2012). Phenotype and differentiation potential of AF-MSCs are similar to both BM-MSCs and embryonic stem cells (ESCs) (De Coppi et al. 2007). AF-MSCs express cell surface antigens CD105, CD90, and CD73 that are expressed on BM-MSCs, and at the same time, AF-MSCs display intracellular and extracellular markers of ESCs, such as Oct-3/4, Nanog, SSEA-3, and SSEA-4, and alkaline phosphatase (Tsai et al. 2004; Moschidou et al. 2013; Joerger-Messerli et al. 2016; Kim et al. 2007; Prusa et al. 2003; Klemmt et al. 2011; Perin et al. 2010). Accordingly, AF-MSCs avian myelocytomatosis virus oncogene cellular homolog (c-MYC), tumor resistance antigen 1–60 (TRA-1–60), and stage-specific embryonic antigen (SSEA) may generate cells of all three germ layers and have notably higher capacity for differentiation than BM-MSCs. It should be highlighted that, in contrast to ESCs, AF-MSCs have stable genotype and are non-tumorigenic in vivo suggesting their potential for safe clinical application (De Coppi et al. 2007; Zhou et al. 2014b).

Several lines of evidence suggest that MSCs derived from placental tissues have superior cell biological properties such as improved proliferative capacity, life span, and differentiation potential than MSCs derived from the bone marrow and other adult tissues. Additionally, ethical concerns related to the derivation of placenta-derived MSCs (PL-MSCs) should be disregarded by the fact that placental tissues are normally considered medical waste and can be recovered without harm to the donor or fetus (Moore et al. 2017). Importantly, PL-MSCs have a higher expansion and engraftment capacity than BM-MSC (Hass et al. 2011), and similar as UC-MSCs and AF-MSCs, PL-MSCs express

pluripotent genes and are able to generate cells of all three germ layers (Hass et al. 2011; Kil et al. 2016; Cho et al. 2018; Jiang et al. 2017).

High-density oligonucleotide microarrays and functional network analyses demonstrated that set of core gene expression profiles has been preserved in all subpopulations of MSCs. This core signature transcriptome includes genes involved in the regulation of osteogenic, adipogenic, and chondrogenic differentiation potential and capacity for immunomodulation (Tsai et al. 2007).

4 Role of Signaling Pathways in Differentiation of MSCs

Signaling pathways involved in differentiation of MSCs toward chondrocytes, adipocytes, and osteocytes have been extensively investigated during the last decade.

Fully differentiated MSC-derived chondrocytes express high levels of runt-related transcription factor 2 (RUNX2), collagen type X, alpha I, and low levels of sex-determining region Y-box 9 (Sox9), which is expressed in early phase of chondrogenesis and is proposed as the main transcription factor responsible for successful differentiation of MSCs into chondrocytes (Akiyama et al. 2002). Members of the TGF-β superfamily (TGF-β1 and bone morphogenetic protein (BMP)) attach to their receptor serine/threonine kinases and activate Smad cascade (Danišovič et al. 2012). Activated Smad1, Smad5, and Smad8 associate with Smad4 and translocate to the nucleus to induce expression of Sox9, collagen type II alpha I, and aggrecan, which are crucially important for the functional properties of chondrocytes (Ikeda et al. 2004; Yu et al. 2012). TGF-β can also induce chondrogenic differentiation of MSCs via the activation of mitogen-activated protein kinase (MAPK) proteins (p38, extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinase (JNK)) (Tuli et al. 2003; Zhang 2009; Mu et al. 2012). Unlike TGF-β signaling pathway, the Wnt proteins have been shown to suppress chondrogenic differentiation of MSCs, by reducing expression of SOX9 and collagen type II alpha 1 (Day et al. 2005). In line with these findings, blocking of canonical Wnt signaling in murine as well as human MSCs upregulated expression of collagen type II, alpha 1, and SOX9 and promoted differentiation of MSCs into chondrocytes (Day et al. 2005; Im and Quan 2010).

Key transcriptional factor for adipogenic differentiation of MSCs is peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Rosen et al. 1999). Binding of insulin and insulin-like growth factor 1 (IGF-1) to their receptors play crucial role for the induction of PPAR-γ expression (Muruganandan et al. 2009). Activation of PPAR-γ by insulin/IGF-1 results in increased expression of CCAAT/enhancer-binding protein alpha (C/EBPα), which in turn generates a positive feedback loop and causes further expression of PPAR-γ (Wu et al. 1999). Activated PPAR-γ induces the expression of genes involved in lipid synthesis and storage such as fatty acid synthase (FAS), glucose transporter type 4 (GLUT4), lipoprotein lipase (LPL), and fatty acid-binding protein 4 (FABP4) resulting in the generation of functional adipocytes (Frith and Genever 2008; Oger et al. 2014).

RUNX2 is transcription factor crucially important for differentiation of MSCs into osteocytes (Chen et al. 2014). Activated RUNX2 induce transcription of numerous genes that are important for osteocyte function including collagen type I, alkaline phosphatase, osteocalcin, and bone sialoprotein (Roach 1994; Aubin et al. 1995). However, increased expression of RUNX2 in MSCs is not sufficient to induce generation of functional osteocytes. Accordingly, simultaneous activation of RUNX2 and several other transcriptional factors is needed for successful osteogenic differentiation of MSCs. BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 bind to their receptors and activate Smad1/5/8 as well as ERK, JNK, and p38 kinases of MAPK signaling cascade resulting in increased expression of alkaline phosphatase and osteocalcin in differentiated MSCs (Kang et al. 2009; Dorman et al. 2012; Wang et al. 1993; James 2013; Lai and Cheng 2002). Additionally, RUNX2 may be also a target of Wnt/β-catenin pathway which, through the activation of T-cell factor 1 (TCF1), promotes differentiation of MSCs into functional osteocytes (Gaur et al. 2005).

5 Two-Edged Sword of MSC-Based Immunomodulation

In addition to the potential for multilineage differentiation, MSCs have capacity to modulate innate and adaptive immune response. This crosstalk between MSCs and immune cells within the tissue microenvironment in which MSCs were transplanted is important for modulation of immune cell functions, but is also essential for changes in phenotype and function of MSCs (Gazdic et al. 2015). A huge number of studies showed that MSCs were not constitutively immunosuppressive and that immunomodulatory activities of MSCs were regulated by concentration of inflammatory cytokines secreted by neighboring immune cells.

After engraftment in the tissue with low concentration of inflammatory cytokines (particularly tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ)), MSCs develop inflammatory phenotype and promote host defense to infections (Gazdic et al. 2015; Bernardo and Fibbe 2013). During the onset of inflammation, microbes activate toll-like receptor (TLR)-4 on MSCs and induce polarization of MSCs in pro-inflammatory cells which, in interleukin (IL)-6/signal transducer and activator of transcription (STAT)3, interferon beta (IFN-β), or granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent manner, prevent apoptosis of neutrophils (Raffaghello et al. 2008; Cassatella et al. 2011). Additionally, pro-inflammatory MSCs produce IL-8 and macrophage migration inhibitory factor (MIF) which are responsible for enhanced phagocytic ability and increased recruitment of neutrophils from the circulation into the inflamed tissue. During the early phase of inflammatory response, MSCs might also increase proliferation and activation of T and B lymphocytes (Bernardo and Fibbe 2013; Traggiai et al. 2008; Griffin et al. 2013; Rasmusson et al. 2007a). MSCs primed with low concentrations of IFN-γ and TNF-α secrete CCL5, CXCL9, and CXCL10 which recruit activated T cells to the sites of inflammation and promote T-cell-driven immune response (Bernardo and Fibbe 2013). In tissues where lipopolysaccharides (LPS) or viral antigens induced a weak inflammatory response, MSCs induce expansion and differentiation of B lymphocytes in immunoglobulin-secreting plasma cells and stimulate production of immunoglobulin (Ig)G in paracrine, IL-6-dependent manner, resulting in enhanced humoral immune response (Traggiai et al. 2008; Rasmusson et al. 2007a).

On the contrary, MSCs generate anti-inflammatory phenotype after engraftment in the tissue where inflammatory cytokines, particularly TNF-α and IFN-γ, are present in high concentrations (Gazdic et al. 2015). Enhanced production of these cytokines by inflammatory immune cells promotes generation of immunoregulatory phenotype in MSCs, induces enhanced secretion of MSC-derived immunosuppressive soluble factors, and, accordingly, augments MSC-based suppression of immune response and inflammation (Li et al. 2012; Dazzi and Krampera 2011).

6 MSC-Derived Factors as New Agents in Immunosuppression of Inflammatory Diseases

Most usually, MSCs alter phenotype and function of immune cells in paracrine manner. MSCs, through the production of soluble factors, suppress maturation and antigen-presenting function of dendritic cells (DCs) and macrophages; inhibit proliferation and effector functions of Th1, Th2, and Th17 lymphocytes; attenuate antibody production and class switching in B cells; and suppress cytotoxicity of natural killer (NK) and natural killer T (NKT) cells (Fig. 1).

6.1 Modulation of Antigen-Presenting Cells by MSC-Derived Factors

MSCs, in paracrine manner, affect generation, maturation, proliferation, and capacity for antigen presentation of DCs (Nauta et al. 2006). Among MSC-derived factors, prostaglandin E2 (PGE2), IL-6, and monocyte colony-stimulating factor

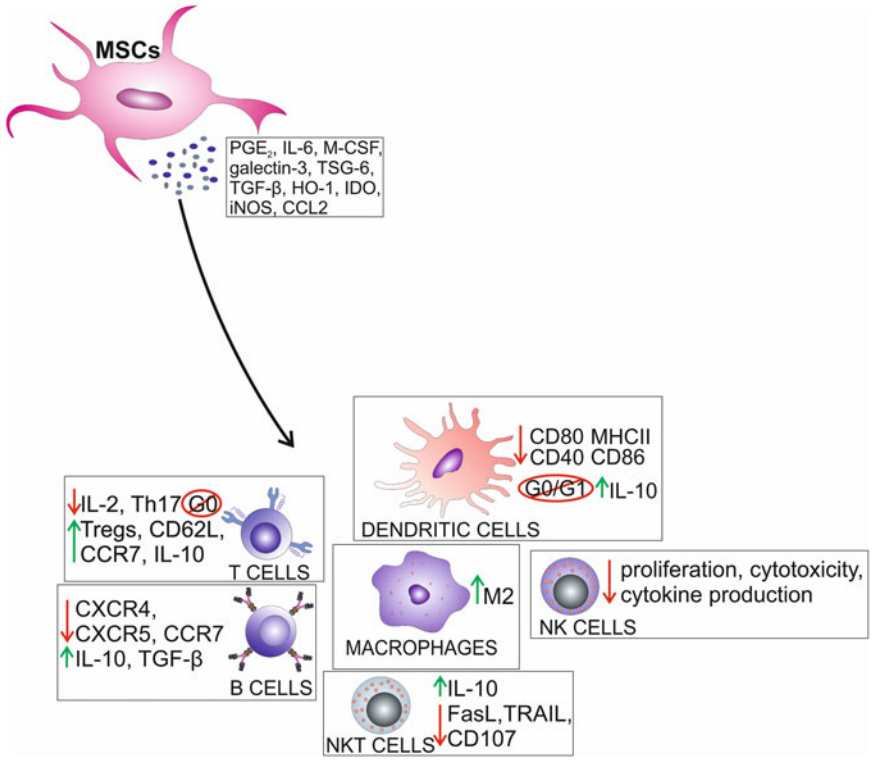

Fig. 1 The effects of MSC-derived factors on phenotype and function of immune cells. Through the production of soluble factors (PGE2, IL-6, galectin 3, TGF-β, PGE2, HO-1, CCL2) and due to the increased iNOS and IDO activity, MSCs induce G0/G1 cell cycle arrest of DCs and T cells, impair antigen presentation ability of DCs and promote their conversion in tolerogenic phenotype, attenuate capacity of plasma cells for antibody production, promote conversion of inflammatory B cells into IL-10+ and TGF-β + regulatory B cells, inhibit proliferation and cytotoxicity of NK and NKT cells, and promote generation of immunosuppressive M2 macrophages, Tregs, and NKTregs

(M-CSF) were considered as the most important molecules for MSC-mediated modulation of DC phenotype and function. MSCs, in IL-6- and M-CSF-dependent manner, inhibit differentiation of DCs from CD34+ progenitor cells and attenuate their proliferation by inducing G0/G1 cell cycle arrest through the decreased expression of cyclin D2 (Nauta et al. 2006; Ramasamy et al. 2007). DCs, cultured with MSCs in transwell systems (which physically separated these two populations), failed to upregulate co-stimulatory molecules CD40, CD80, and CD86 and MHC molecule class II (Zhang et al. 2004; Jiang et al. 2005). These MSC-altered DCs were notably impaired in their ability to present antigen to naïve T cells (Nauta et al. 2006). We recently provided the first evidence that MSCs, through the secretion of immunomodulatory galectin 3, inhibited production of inflammatory cytokines (IL-1β, IL-12, IL-6, and TNF-α) in DCs and attenuated expression of co-stimulatory molecules on their membranes significantly reducing their capacity for activation of naïve T cells (Nikolic et al. 2018). Additionally, MSCs in PGE2-dependent manner suppressed capacity of DCs to induce proliferation and activation of allogeneic T cells (Spaggiari et al. 2009). MSC-derived IL-6 promoted generation of IL-10-producing monocytes (Melief et al. 2013a). Accordingly, MSCs, in IL-6-dependent manner, polarized DCs from inflammatory into tolerogenic, IL-10-producing cells that suppressed cytokine production in effector T cells (Spaggiari et al. 2009; Beyth et al. 2005).

In the inflammatory environment, MSCs were able to actively modulate phenotype of macrophages by suppressing production of inflammatory and by enhancing secretion of immunoregulatory cytokines in these cells (Gazdic et al. 2015). LPS-, IFN-γ-, or TNF-α-primed MSCs, through the production of PGE2, TNF-α-stimulated gene/protein 6 (TSG-6), and IL-6, induced conversion of inflammatory, IL-12- and TNF-α-producing M1 macrophages into anti-inflammatory, alternatively activated M2 cells (Melief et al. 2013a, b; Eggenhofer and Hoogduijn 2012; Németh et al. 2009; Choi et al. 2011; François et al. 2012). M2 macrophages, on turn, through the secretion of IL-10 and CCL18, induced generation of T regulatory cells (Tregs) and enhanced their migration in inflamed tissues, contributing to the creation of immunosuppressive microenvironment (Melief et al. 2013b; Selmani et al. 2008).

6.2 MSC-Derived Factors Suppress Proliferation and Effector Functions of Lymphocytes

Plenty of evidence suggested that MSC-derived soluble factors are crucially important for inhibition of T-cell proliferation. MSCs block interleukin-2-dependent autocrine proliferation in T cells through the secretion of TGF-β, PGE2, and heme oxygenase-1 (HO-1) (Aggarwal and Pittenger 2005; Ghannam et al. 2010a; Bright et al. 1997; Kalinski 2012). These mediators promote G1 cell cycle arrest by suppressing production of IL-2 and by downregulating expression of IL-2 receptor resulting in inhibition of Janus kinase (JAK)-STAT and ERK/MAPK kinase pathways in T cells (Aggarwal and Pittenger 2005; Ghannam et al. 2010a; Bright et al. 1997; Kalinski 2012; Pae et al. 2004). Additionally, in PGE2-dependent manner, MSCs downregulate expression of cyclin D2 and increase expression of the cyclin-dependent kinase inhibitor p27kip1 in T lymphocytes leading to cell cycle arrest (Glennie et al. 2005).

We and others recently demonstrated importance of indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS) for immunosuppressive activity of MSCs (Ren et al. 2009; Gazdic et al. 2018a, b; Milosavljevic et al. 2017). Final products of IDO activity (kynurenine, quinolinic acid, and 3-hydroxyanthranillic acid) negatively affect proliferation or induce apoptosis of T and NKT cells (Ren et al. 2009; Gazdic et al. 2018a, b; Milosavljevic et al. 2017). iNOS generate highly reactive NO which inhibits phosphorylation of STAT5 in T cells, leading to the cell cycle arrest (Sato et al. 2007). Ren and colleagues suggested that MSC-based modulation of T-cell-dependent immune response varied among species (Ren et al. 2009). They proposed that human MSCs (hMSCs) suppress proliferation and effector functions of T cells in IDO-dependent manner, while murine MSCs (mMSCs) overexpress iNOS and, through the production of NO, inhibit expansion and activation of T lymphocytes (Ren et al. 2009). Nevertheless, NO is highly unstable, it only acts locally (Sato et al. 2007) and, accordingly, could not be responsible for systemic and endocrine effects of mMSC-based therapy. By using animal model of acute liver injury, we recently demonstrated that under Th1 inflammatory conditions (in the presence of elevated levels of IFN-γ), mMSCs initially produce NO which, in autocrine manner, induces increased IDO activity in mMSCs, resulting with enhanced production of kynurenine that suppresses proliferation and effector functions of NKT cells (Gazdic et al. 2018b). Importantly, the same molecular mechanism (interplay between NO and IDO) was crucially important for hMSC-mediated suppression of activated human peripheral blood lymphocytes (Gazdic et al. 2018b).

In line with these findings, we demonstrated that NO- and IDO-dependent attenuation of acute liver injury in MSC-treated mice was accompanied with reduced presence of liver-infiltrated inflammatory (IFN-γ- and IL-17-producing) NKT cells and with an increased influx of immunosuppressive IL-10-producing CD4 + CD25 + FoxP3+ Tregs and FoxP3+ regulatory NKT cells (NKTregs) in the injured livers (Gazdic et al. 2018a; Milosavljevic et al. 2017). MSCs managed to prevent expansion of

inflammatory IL-17-producing Th17 and NKT17 cells by promoting their conversion in immunosuppressive Tregs and NKTregs (Gazdic et al. 2018a; Milosavljevic et al. 2017; Duffy et al. 2011; Ghannam et al. 2010b). Additionally, MSCs, in paracrine, NO- and IDO-dependent manner, significantly increased expression of CD62L and CCR7 and increased production of immunosuppressive IL-10 and TGF-β in Tregs enhancing their migratory and immunosuppressive capacities (Gazdic et al. 2018a). In similar, NO- and IDO-dependent manner, MSCs suppressed expansion of NKT cells, reduced expression of apoptosis-inducing ligands (first apoptosis signal ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL)), downregulated expression of CD107 (marker of degranulation), and polarized NKT cells from inflammatory, TNF-α-, IFN-γ-, and IL-17-producing cells into immunosuppressive, IL-10-producing cells contributing to the attenuation of NKT cell-dependent cytotoxicity and inflammation (Milosavljevic et al. 2017; Gazdic et al. 2018b). 1-Methyl-DL-tryptophan (specific IDO inhibitor) or L-NG-monomethyl arginine citrate (specific iNOS inhibitor) completely abrogated immunoregulatory capacity of MSCs in vitro and in vivo and restored pro-inflammatory cytokine production and cytotoxicity of NKT cells, suggesting that increased activity of iNOS and IDO was crucially important for MSC-mediated suppression of NKT cells (Milosavljevic et al. 2017; Gazdic et al. 2018b).

MSCs efficiently modulated proliferation, activation, and cytotoxicity of NK and cytotoxic T lymphocytes (CTLs) in paracrine manner. MSC-derived IDO, PGE2, and TGF-β1 were able to reduce expression of the activating receptors (NKp30, NKp44, and NKG2D), attenuate cytotoxicity, inhibit production of inflammatory cytokines (IFN-γ, TNF-α), and suppress IL-2-dependent proliferation of CTLs and NK cells (Rasmusson et al. 2007b; Li et al. 2014; Sotiropoulou et al. 2006; Spaggiari et al. 2008).

In line with these findings, MSCs, in paracrine, NO- and IDO-dependent manner, suppressed influx of IL-6- and TNF-α-producing, inflammatory B cells in the liver and increased presence of liver-infiltrated immunosuppressive (IL-10- and TGF-β-producing) marginal zone (MZ)-like regulatory B cells (CD23-CD21 + IgM+) resulting in the significant attenuation of acute liver inflammation (Gazdic et al. 2018a). Additionally, through the activation of IDO/kynurenine pathway, MSCs induce apoptosis of B cells (Corcione et al. 2006) and block ERK1/2 phosphorylation resulting in division arrest and anergy of B cells (Tabera et al. 2008). MSCs downregulate expression of chemokine receptors (CXCR4, CXCR5, and CCR7) on naïve and activated B cells, affecting their homing and migratory capacities (Corcione et al. 2006), but did not alter expression of co-stimulatory molecules (CD80 and CD86) on B cells and, accordingly, did not reduce their capacity for antigen presentation (Corcione et al. 2006). MSC-derived CC chemokine ligand (CCL)2 suppresses immunoglobulin production in plasma cells by modulating expression of paired box (PAX)5 and STAT3 (Rafei et al. 2008).

7 MSC-CM as New, Cell-Free Therapeutic Agent in Regenerative Medicine

Despite the fact that MSCs have proved their therapeutic potential in a large number of studies, unwanted differentiation of engrafted MSCs in vivo is still the most important safety concern related to MSC-based immunomodulation and regeneration (Volarevic et al. 2018). Various growth factors, produced in the tissue microenvironment where MSCs are engrafted, may induce spontaneous and unwanted differentiation of MSCs toward the cells of mesodermal origin, most usually the cartilage and bone (Volarevic et al. 2018). Accordingly, a large number of studies investigated immunomodulatory and regenerative potential of MSC-CM, as MSC-derived cell-free therapeutic agent which can bypass many of the limitations of MSC-based therapy, including safety concern related to unwanted differentiation of engrafted MSCs (Table 1).

MSC-CM contains broad range of MSC-derived immunomodulatory factors, and, accordingly, injection of MSC-CM showed

Table 1 Therapeutic potential of MSC-CM

MSC-CM as a cell-free therapeutic agent		
Disease	Outcomes	Mechanism of action
Acute lung injury (Ionescu et al. 2012a; Lee et al. 2009)	Improvement of lung endothelial barrier, reduction of pulmonary edema, suppression of inflammatory response	Insulin-like growth factor, keratinocyte growth factor
Bronchopulmonary dysplasia (Monsel et al. 2016)	Prevention of blood vessel remodeling, reduction of neutrophil and macrophages influx in the lungs, decreased expression of pro-inflammatory cytokines (IL-6 and IL-1β)	Macrophage-stimulating factor 1, osteopontin, stanniocalcin-1
Chronic inflammatory lung diseases (Ionescu et al. 2012b; Abreu et al. 2017; Harrell et al. 2018b)	Prevention of airway hyperresponsiveness, reduction of peribronchial inflammation and airway remodeling, attenuated production of Th2 cytokines (IL-4 and IL-13), expansion of Tregs and M2 macrophages	Adiponectin exosomes containing IL-1Ra, IL-27, CXCL14, CXCL16
Acute liver injury (Gazdic et al. 2018a, b; Huang et al. 2016; Milosavljevic et al. 2017; Parekkadan et al. 2007; van Poll et al. 2008; Xagorari et al. 2013)	Suppression of hepatocyte apoptosis, attenuation of liver inflammation, reduced hepatotoxicity and total number of IFN-γ+ and IL-17+ NKT cells, increased presence of IL10+ Tregs and NKTregs	IL-6, fibrinogen-like protein 1, IDO
Liver fibrosis (Milosavljevic et al. 2018)	Expansion of protective FoxP3 + IL-10+ Tregs, suppressed activation of pro-fibrogenic Th17 cells and stellate cells	IDO
Acute kidney injury (Bi et al. 2007; Liu et al. 2018; Simovic Markovic et al. 2017; Overath et al. 2016)	Decreased serum levels of creatinine, IL-1, and IL-6; decreased influx of activated neutrophils, TNF-α + DCs, and IL-17+ CTLs in the kidneys; increased presence of tolerogenic DCs and Tregs	iNOS
Ischemic brain injury (Jiang et al. 2018; Egashira et al. 2012; Faezi et al. 2018)	Recovery of motor functions, reduction of infarct volume and brain edema	Tissue inhibitor of metalloproteinase-1 (TIMP-1) progranulin insulin-like growth factor, brain-derived neurotrophic factor

beneficial effects in the treatment of inflammatory lung, liver, and kidney diseases (Pierro et al. 2013; Ionescu et al. 2012a, b; Lee et al. 2009; Monsel et al. 2016; Aslam et al. 2009; Sutsko et al. 2013; Abreu et al. 2017; Cruz et al. 2015; Du et al. 2018; Harrell et al. 2018b; Ortiz et al. 2007; Tan et al. 2018; van Poll et al. 2008; Xagorari et al. 2013; Parekkadan et al. 2007; Huang et al. 2016; Milosavljevic et al. 2018; Bi et al. 2007; Liu et al. 2018; Simovic Markovic et al. 2017; Overath et al. 2016). Additionally, due to the presence of trophic and pro-angiogenic factors, MSC-CM efficiently protected brain tissue from ischemic injury (Jiang et al. 2018; Egashira et al. 2012; Faezi et al. 2018).

MSC-CM administration exerts short- as well as long-term therapeutic effects in immune-mediated lung injury (Pierro et al. 2013). Intratracheal application of MSC-CM (concentrated 25x) significantly reduced pulmonary edema and inflammation in animal model of LPS-induced acute lung injury (Ionescu et al. 2012a). MSC-derived IGF-1, contained in MSC-CM, induced conversion of macrophages

toward immunosuppressive M2 phenotype resulting in notable attenuation of inflammation (Ionescu et al. 2012a), while MSC-derived keratinocyte growth factor (KGF) was crucially important for the regenerative effects of MSC-CM (Lee et al. 2009). In similar manner, allogeneic human MSC-CM improved lung endothelial barrier and restored alveolar fluid clearance in an ex vivo perfused human lungs injured by LPS (Lee et al. 2009).

Several studies confirmed beneficial effects of MSC-CM in a murine model of bronchopulmonary dysplasia (BPD) (Monsel et al. 2016). MSC-CM prevented blood vessel remodeling and alveolar injury and significantly reduced influx of neutrophils and macrophages in the lungs of hyperoxia-exposed mice (Aslam et al. 2009). MSC-CM-based improvement of lung structure in hyperoxic pups was associated with decreased expression of pro-inflammatory cytokines (IL-6 and IL-1β), followed by attenuation of ongoing inflammation (Sutsko et al. 2013). Macrophage-stimulating factor 1, osteopontin, and antioxidant stanniocalcin-1, all present at high levels in MSC-CM, were responsible for beneficial immunomodulatory and therapeutic effects of MSC-CM in the therapy of experimental BPD (Aslam et al. 2009).

MSC-CM efficiently attenuated acute and chronic asthma in several experimental studies (Ionescu et al. 2012b; Abreu et al. 2017). MSC-CM attenuated production of Th2 cytokines (IL-4 and IL-13) and promoted expansion of IL-10-producing Tregs and M2 macrophages contributing to the creation of anti-inflammatory microenvironment within the asthmatic lungs (Ionescu et al. 2012b). Adiponectin, an anti-inflammatory adipokine found in MSC-CM, was suggested as important MSC-derived factor for the prevention of airway hyperresponsiveness, peribronchial inflammation, and airway remodeling (Ionescu et al. 2012b).

Cruz and co-workers suggested that MSC-derived exosomes (nano-sized extracellular vesicles that deliver proteins, lipids, DNA fragments, and microRNA to the tissue-resident and immune cells) were responsible for attenuation of airway allergic inflammation (Cruz et al. 2015). Similar conclusions were drawn by Du and colleagues who confirmed that MSC-derived exosomes alleviated airway inflammation, enhanced proliferation and immunosuppressive properties of Tregs, and enhanced production of anti-inflammatory cytokines (IL-10 and TGF-β) in peripheral blood mononuclear cells obtained from asthmatic patients (Du et al. 2018). In line with these results are our findings related to the therapeutic potential of "Exosomes d-MAPPS," which activity was based on PL-MSC-derived exosomes containing interleukin 1 receptor antagonist (IL-1Ra) and several other immunomodulatory cytokines and chemokines (IL-27, CXCL14, CXCL16) (Harrell et al. 2018b). When MSC-derived IL-1Ra binds to the IL-1 receptor (IL-1R) on lung epithelial cells, various pro-inflammatory events initiated by IL-1:IL-1R binding become inhibited (including the synthesis and releases of inflammatory cytokines that attract neutrophils, macrophages, and lymphocytes in injured lungs), resulting in the attenuation of lung inflammation (Ortiz et al. 2007). Accordingly, results, obtained in a pilot trial with small number of patients, revealed notably attenuated lung inflammation and significantly improved pulmonary function parameters in Exosomes d-MAPPS-treated patients with chronic lung inflammation (Harrell et al. 2018b). Similar results, related to the efficacy of MSC-derived exosomes in the therapy of lung injury and fibrosis, were obtained by Tan and co-workers who found that MSC-derived exosomes attenuated fibrosis, recovered pulmonary function, and enhanced endogenous lung repair (Tan et al. 2018).

Similar as it was observed in MSC-based therapy of lung inflammation, MSC-CM efficiently attenuated inflammation and fibrosis in the liver (Gazdic et al. 2017). MSC-CM dramatically reduce total number and cytotoxicity of liver-infiltrated immune cells, attenuated apoptosis of hepatocytes, and increased their proliferation resulting in significantly improved survival of MSC-CM-treated animals (van Poll et al. 2008; Xagorari et al. 2013; Parekkadan et al. 2007;

Huang et al. 2016). Several lines of evidence demonstrated that IL-6 and fibrinogen-like protein 1 were responsible for MSC-CM-based suppression of apoptosis and enhanced regeneration of hepatocytes, while enhanced activity of IDO/kynurenine pathway was mainly responsible for MSC-CM-mediated attenuation of detrimental immune response in the liver (Gazdic et al. 2017, 2018a, b; Milosavljevic et al. 2017; Xagorari et al. 2013; Parekkadan et al. 2007). MSC-CM significantly reduced hepatotoxicity and total number of inflammatory IFN-γ- and IL-17-producing NKT cells and notably increased presence of FoxP3 + IL-10+ Tregs and NKTregs cells in the livers of mice with acute hepatitis (Milosavljevic et al. 2017). This phenomenon was completely abrogated in the presence of IDO inhibitor, confirming that IDO/kynurenine pathway was responsible for MSC-CM-based suppression of acute liver inflammation (Milosavljevic et al. 2017). Similar cellular and molecular mechanisms were involved in MSC-CM-mediated attenuation of liver fibrosis. MSC-CM promoted expansion of protective FoxP3 + IL-10+ Tregs and suppressed activation of pro-fibrogenic Th17 cells and stellate cells in IDO-/kynurenine-dependent manner (Milosavljevic et al. 2018).

MSC-CM significantly reduced apoptosis of tubular cells, improved renal function, and increased survival of mice suffering from acute kidney injury and renal fibrosis (Bi et al. 2007; Liu et al. 2018). MSC-CM efficiently attenuated cisplatin-induced nephrotoxicity by reducing the influx and capacity of DCs and T lymphocytes to produce inflammatory cytokines (Simovic Markovic et al. 2017). NO was mainly responsible for MSC-CM-mediated renoprotective effects since inhibition of iNOS activity in MSCs and, accordingly, lack of NO in MSC-CM resulted in increased influx of inflammatory, TNF-α-producing DCs and IL-17-producing CTLs and decreased presence of IL-10-producing tolerogenic DCs and Tregs in cisplatin-treated mice (Simovic Markovic et al. 2017). In line with these findings, Overath and co-workers showed that injection of MSC-CM significantly attenuated cisplatin-induced acute kidney injury and inflammation as demonstrated by downregulated serum levels of creatinine, IL-1, and IL-6 and reduced presence of activated neutrophils in injured kidneys (Overath et al. 2016).

MSC-CM-based therapy protected brain tissue from ischemic injury and promoted functional recovery after stroke in experimental mice and rats (Jiang et al. 2018; Egashira et al. 2012). Intracerebroventricular administration of MSC-CM markedly reduced infarct volume and brain edema in tissue inhibitor of metalloproteinase-1 (TIMP-1) and progranulin-dependent manner and in IGF-1 and brain-derived neurotrophic factor (BDNF)-dependent manner significantly improved cognitive and motor skills (Egashira et al. 2012). MSC-CM notably reduced neuronal loss by affecting expression of caspase-3, Bax, and Bcl-2 in motor cortex and accordingly resulted in recovery of motor functions in experimental animals (Faezi et al. 2018).

8 Conclusions

Due to their immunomodulatory and regenerative abilities, MSCs and their secretomes represent potentially new therapeutic agents in regenerative medicine. Although MSC-based therapy was efficient in the treatment of many inflammatory and degenerative diseases, unwanted differentiation of engrafted MSCs represents important safety concern. Beneficial immunoregulatory effects of MSCs are mainly relied on the effects of their soluble factors which act through multiple mechanisms affecting maturation, phenotype, and function of naïve and effector immune cells of innate and acquired immunity. Accordingly, MSC-CM has the potential to serve as a cell-free therapeutic agent for the treatment of immune cell-mediated diseases. However, it should be noted that MSC-CM contains broad number of immunosuppressive and pro-angiogenic factors, and its therapeutic use could inhibit immune surveillance of tumor cells and may promote uncontrolled growth and expansion of tumor cells. Accordingly, previous history of malignant

diseases has to be considered as an important exclusion criteria for the use of MSC-CM. In line with these observations, new experimental and clinical studies have to determine the exact protocols for therapeutic application of MSC-CM and should focus their attention on long-term safety issues related to MSC-CM-based therapy before this MSC-derived product could be broadly used in regenerative medicine.

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