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ABOUT COVER

Editorial Board Member of *World Journal of Stem Cells*, Dr. Mohammed Grawish is a Distinguished Professor at Mansoura University and Vice-Dean for Community Services and Environmental Affairs at Delta University for Science and Technology (Egypt). Dr. Grawish received his Bachelor's degree (1990), Master's degree in Oral Biology (1998), and his PhD (2003) from the Faculty of Dentistry, Mansoura University. After, he worked as Lecturer in the Al-Gabl Al-Garby University (2005-2008; Gehrian, Libya) and as Associate Professor in the King Saud University (2011-2013; Riyadh, Saudi Arabia). His ongoing research interests focus mainly on the appropriate therapeutic use of stem cells in dentistry, the design and characterization of biomaterials as scaffold materials for loading stem cells, and the application of complementary and alternative medicine as an adjunctive treatment to traditional medicine for oral diseases. (L-Editor: Filipodia)

AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

INDEXING/ABSTRACTING

The *WJSC* is now indexed in Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, BIOSIS Previews, Scopus, PubMed, and PubMed Central. The 2020 Edition of Journal Citation Reports® cites the 2019 impact factor (IF) for *WJSC* as 3.231; IF without journal self cites: 3.128; Ranking: 18 among 29 journals in cell and tissue engineering; Quartile category: Q3; Ranking: 113 among 195 journals in cell biology; and Quartile category: Q3. The *WJSC*'s CiteScore for 2019 is 4.9 and Scopus CiteScore rank 2019: Histology is 15/60; Genetics is 124/324; Genetics (clinical) is 35/90; Molecular Biology is 177/381; Cell Biology is 143/274.

RESPONSIBLE EDITORS FOR THIS ISSUE

Novel insights for improving the therapeutic safety and efficiency of mesenchymal stromal cells

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Abstract

Mesenchymal stromal cells (MSCs) have attracted great interest in the field of regenerative medicine. They can home to damaged tissue, where they can exert pro-regenerative and anti-inflammatory properties. These therapeutic effects involve the secretion of growth factors, cytokines, and chemokines. Moreover, the functions of MSCs could be mediated by extracellular vesicles (EVs) that shuttle various signaling messengers. Although preclinical studies and clinical trials have demonstrated promising therapeutic results, the efficiency and the safety of MSCs need to be improved. After transplantation, MSCs face harsh environmental conditions, which likely dampen their therapeutic efficacy. A possible strategy aiming to improve the survival and therapeutic functions of MSCs needs to be developed. The preconditioning of MSCs *ex vivo* would strength their capacities by preparing them to survive and to better function in this hostile environment. In this review, we will discuss several preconditioning approaches that may improve the therapeutic capacity of MSCs. As stated above, EVs can recapitulate the beneficial effects of MSCs and may help avoid many risks associated with cell transplantation. As a result, this novel type of cell-free therapy may be safer and more efficient than the whole cell product. We will, therefore, also discuss current knowledge regarding the therapeutic properties of MSC-derived EVs.

Key Words: Cell-therapy; Mesenchymal stromal cells; Safety; Extracellular vesicles; Efficiency; Preconditioning

Core Tip: Mesenchymal stromal cells (MSCs) have a strong capacity to regenerate

injured tissues by displaying trophic and immunomodulatory functions. The tissue environment plays a significant role during the reparative process of MSCs. Preconditioning of MSCs with a specific signal promotes the adequate response. As the therapeutic functions of MSCs are mainly linked to their secretome, the use of extracellular vesicles as an acellular tool is strongly recommended. Thus, new insights on the properties of MSCs as well as their mechanisms of actions will help to develop a safe and efficient therapeutic strategy.

INTRODUCTION

Due to the high prevalence of tissue injury and damage, new therapeutic strategies should be considered[1]. Tissue regeneration is a complex process encompassing several interplays between tissue progenitor/stem cells, local environment, and immune cells. The healing ability is generally attributable to a pool of undifferentiated cells capable of replacing damaged cells in order to guarantee the integrity of the tissue[2]. These cells can sense several signals of injury and damage, which can in turn modulate their biological features. However, the pool of tissue-specific stem/stromal and progenitor cells may show functional exhaustion. Depending on the injury and damage, these endogenous cells may display low efficiency regarding their healing mechanism. Impairments of cell self-renewal, function, differentiation capacity, and homing may thus be observed[3].

New therapies using *ex vivo*-expanded stem/stromal and progenitor cells have been developed to enhance the healing process. In this context, mesenchymal stromal cells (MSCs) have been reported as an attractive candidate for cell therapy. Originally identified within the bone marrow niche, MSCs were subsequently identified in different tissues. Morphologically similar to fibroblasts, MSCs are characterized *in vitro* by plastic-adherent proliferation, a specific immunophenotype and multipotency as defined by the International Society for Cellular Therapy (ISCT)[4]. They express CD73, CD90 and CD105 and lack the expression of CD14, CD19, CD34, CD45 and human leukocyte antigen (HLA)-DR. To date, their multipotent capabilities have not been clearly demonstrated *in vivo*, particularly because of the lack of methods to identify and define differentiated populations. However, the trophic and immunomodulatory properties of MSCs are likely the key attributes that mediate tissue repair[5,6].

In parallel, harsh environments within damaged and diseased tissues such as ischemia, inflammatory/immunological reactions, oxidative stress, and infectious episodes may influence the fate and functions of cells[7]. By sensing such signals, MSCs respond by migrating, proliferating, and initiating regenerative mechanisms. MSCs will try to reconstitute the immune microenvironment and empower tissue stem/progenitor cells and other resident cells, thereby facilitating tissue repair[8]. A rapid detection of the primary insult allows to display the adequate response attempting repair[9]. In general, a tight regulation of the balance between the native and activated states of MSCs appears essential for their functional properties during tissue repair. It has been shown that MSCs require specific signals to display adequate therapeutic potential[10]. Such behavior indicates that MSCs possess functional plasticity, and that, under specific conditions, it is possible to polarize their biological response toward an adequate response. Depending on tissue injury or disease conditions, the secretome may change allowing MSCs to exert different effects. These changes generally modulate the type and level of cytokines and growth factors important for the tissue repair process[11]. The results obtained from clinical applications of MSCs indicate that therapeutic outcomes could be improved. The secretome of MSCs is mainly responsible for the modulation of immune responses and the function empowerment of local progenitors. It consists of a diverse range of growth factors, cytokines, chemokines, and extracellular vesicles (EVs) that govern different paracrine signaling. MSCs convey regulatory messages to recipient cells to

initiate tissue repair[6]. The release of EVs has emerged as an important mediator of cell-to-cell communication. They have been shown to effectively mimic the effects of MSCs, thus participating in their therapeutic process. In fact, EVs may transfer proteins, lipids, and genetic material, which also contribute to the modulation of the biology and fate of recipient cells, without requiring the presence of MSCs. The use of this cell-free tool would certainly enhance the safety of MSC-based therapy (Figure 1).

The safety and efficiency of MSCs are the two major aspects that need to be managed to improve the therapeutic effects of MSCs[4]. These two goals are closely linked to their acellular component and preconditioning strategy, respectively. A broader understanding of the tissue environment characteristics and components that allow to modulate the plasticity of MSCs and therefore set up more specific MSC responses by using preconditioning strategies. Here, we focus on the influences of inflammatory and infectious signals, mostly found within the injured tissue, on several features of MSCs. The secretome of MSCs, in particular EVs, make them an attractive tool as cell-free based therapy. More insight into the biology, production, composition and role of EVs would increase the quality and safety of the therapy. Of note, preconditioning should be applied to enhance the production of EVs as well as empower their effects by maximizing their contents of regulatory and trophic factors. This deeper understanding would improve the capacity to manipulate MSCs and ultimately lead to safer and more efficient therapeutic effects (Figure 2).

PRECONDITIONING STRATEGIES OF MSCS

The cellular and molecular tissue microenvironment is a dynamic compartment where several signals can modulate MSC features[12]. The balance between the physiological and pathological states of the tissue critically influences the reparative process. After injection, MSCs can migrate into the injured tissues where they encounter an unfavorable environment coupled with death signals, infections, oxidative stress, and immune/inflammatory responses[13]. Moreover, clinical data suggest that the outcomes of MSC-based therapy are affected by medication or the presence of infections[14]. Hurdles including poor homing, survival, engraftment, and functions may hamper the therapeutic effect of transplanted MSCs[15]. In the quest to circumvent these challenges, several strategies can be utilized including preincubation with growth factors, cytokines, receptor agonists, optimization of culture conditions (*e.g.*, hypoxia, glucose), and the use of bioactive or biomimetic scaffolds[16,17]. Also called licensing or priming, these strategies may be considered as cell preconditioning (Figure 3).

It consists of boosting cells for a specific function in a specific challenging environment[18]. The goal is to not only maintain, but also enhance the inherent biological activities and therapeutics effects of MSCs with respect to migration, homing to target site, tissue engraftment, survival, and improved reparative/trophic functions[19-24]. Thus, these features should be carefully evaluated *in vitro* and *in vivo*[24]. Overall, MSCs should withstand the inhospitable environment in the recipient tissue (transplanted cell-preconditioning). At the same time, the damaged tissue becomes more receptive to the transplanted cells favoring their engraftment and interplay (tissue-preconditioning)[21]. Such development may accelerate the process to translate experimental evidence from preclinical studies to daily clinical practice[22].

Optimized culture conditions

After isolation, MSC are typically cultured under normoxic conditions (21% oxygen). However, the physiological niches for MSC in the bone marrow and other sites have much lower oxygen tension. When used as a therapeutic tool to repair tissue injuries, MSCs cultured in standard conditions must adapt from 21% oxygen in culture to less than 1% oxygen in the ischemic tissue[23]. Several data suggest that preculturing MSCs under hypoxic conditions prior to transplantation improves their tissue regenerative potential. Interestingly, hypoxia pretreatment significantly increased the proliferation and chondrogenic differentiation of adipose tissue-derived stem cells but decreased their osteogenic differentiation in a vascular endothelial growth factor (VEGF)-dependent manner[25]. In parallel, hypoxia-preconditioned olfactory mucosa mesenchymal stem cells inhibits pyroptotic and apoptotic death of microglial cell in response to cerebral ischemia/reperfusion insult by activating hypoxia-inducible factor-1 (HIF-1) *in vitro*[26]. Hypoxia preconditioning of human dental MSCs empowers their immunoregulatory functions. Overexpression of HIF-1α leads to the modulation of the adaptive and innate immune responses. Following an increase of C-C motif chemokine ligand 2 (CCL2, also referred to as monocyte chemoattractant protein-1)

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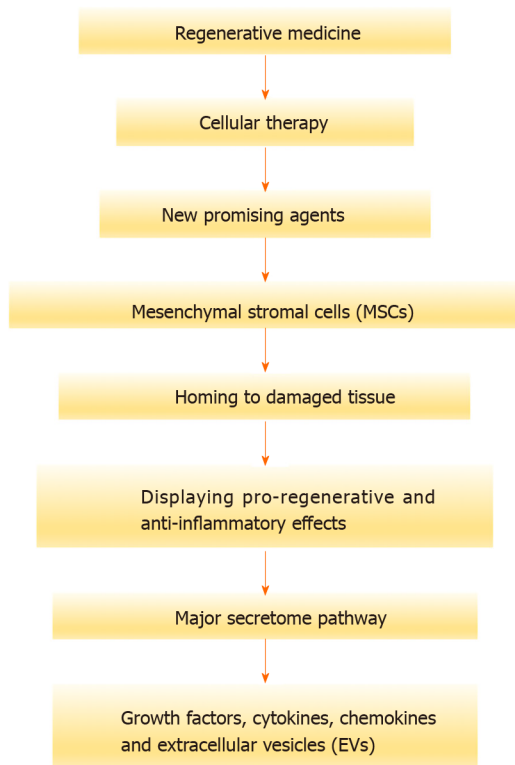
Figure 1 A flow chart showing the use of the cell-free approach to enhance the safety of mesenchymal stromal cell-based therapy. MSC: Mesenchymal stromal cell; EVs: Extracellular vesicles.
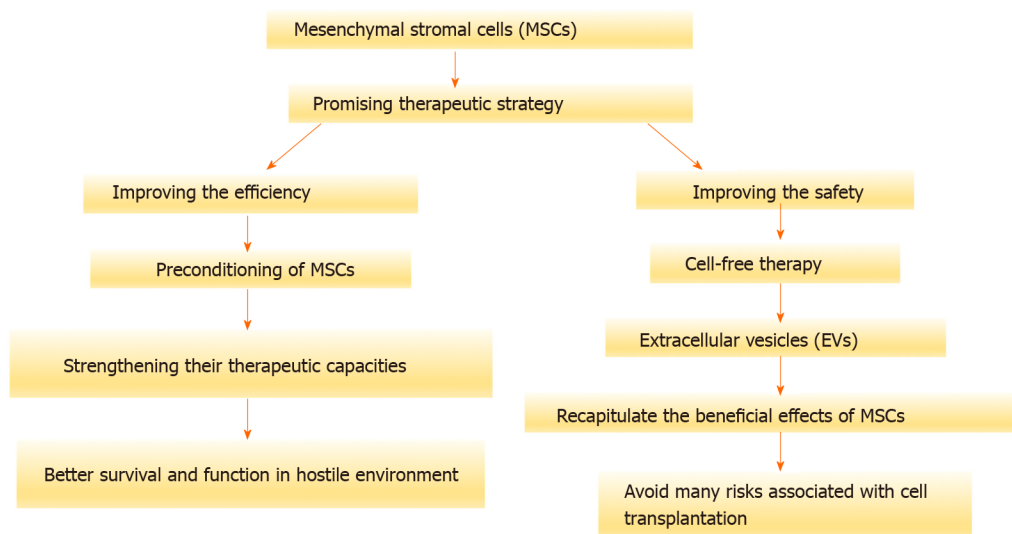
Figure 2 Mesenchymal stromal cells as a promising therapeutic strategy with improved efficiency and safety. MSC: Mesenchymal stromal cell; EVs: Extracellular vesicles.

production, monocytes are significantly recruited and induced to acquire M2 macrophage phenotypes known for their immunosuppressive properties[27]. These effects could be linked to the expression of interleukin (IL)-10 and Fas-ligand. Indeed, the IL-10/CD210 axis is functionally relevant during immunomodulation by regulating several features of T cell response[28].

Because melatonin exhibits antioxidant and anti-inflammatory properties, it has been used as a preconditioning tool to optimize the response of MSCs within a unreceptive microenvironment[29]. Preconditioning of rat bone marrow (BM)-MSCs by
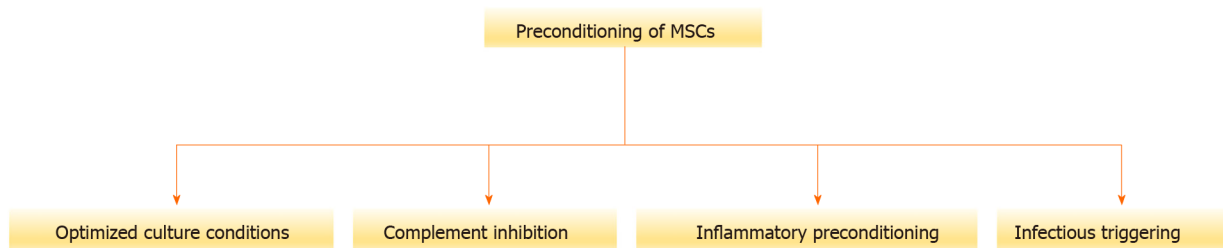
Figure 3 Strategies applied in the preconditioning of mesenchymal stromal cells. MSCs: Mesenchymal stromal cells.

omentin-1, a fat depot-specific secretory adipokine, allowed to display improved therapeutic functions. Such a condition promotes proliferation, inhibits apoptosis, increases secretion of angiogenic factors, and enhances the ability to stimulate tube formation by human umbilical vein endothelial cells *via* the PI3K/AKT signaling pathway[30]. In line with this, *ex vivo* antioxidant preconditioning by using n-acetylcysteine and ascorbic acid 2-phosphate improved the viability of MSCs and protected them in the presence of diabetic wound fluid[31]. The preconditioning of MSCs by glutathione-allylsulfur conjugates can enhance their survival after post-ischaemic myocardial implantation. In a concentration dependent manner, such treatment increased the proliferation, migration, and differentiation of cardiac lineage marker-negative/stem cell antigen-1-positive human mesenchymal stem cells. These beneficial effects are consecutive to the upregulation of proteins involved in oxidative stress protection, cell-cell adhesion, and commitment to differentiation[32].

Recently, treatment of human decidua basalis MSCs with a high level of glucose was shown to enhance the engraftment and therapeutic potential of MSCs. Preconditioning with glucose increased gene expression related to survival, proliferation, migration, invasion, and immunomodulatory properties[33]. Glutamine (GLUT) is a nonessential amino acid that can become conditionally essential under stress conditions, being able to participate in the modulation of the immune responses in several ways. GLUT has been reported to enhance the immunosuppressive properties of MSCs. In a dose dependent manner, the addition of GLUT augmented the proliferation of MSCs, reduced lymphocyte and macrophage proliferation. This effect was probably reached by decreasing levels of pro-inflammatory cytokines and by increasing levels of anti-inflammatory cytokines[34].

The use of biomaterial scaffolds may lead to higher clinical benefits in patients treated by MSCs. Triggering the expression of cytoprotective genes that aim at enhancing the longevity of MSCs and the duration of their regulatory effects is a very promising strategy[10]. MSC-biomaterial constructs retain MSCs *in situ* and prolong their survival. Moreover, biomaterial scaffolds can both preserve the tissue architecture and provide a three-dimensional biomimetic milieu for embedded MSCs, which enhance their paracrine functions[35]. In accordance with these achievements, our group accomplished a cutting-edge feat by developing a feasible, safe and physiological process that increases the level of MSCs in the BM niche with a specific commitment[36]. Injection of autologous platelet-rich plasma activated by the recombinant human soluble tissue factor in the iliac crest, enhanced the production of autologous MSCs. This strategy avoids the use of allogeneic MSCs and the need of their expansion through *ex vivo* culture with the accompanying risks of contamination and cell differentiation.

Complement inhibition

The viability and/or function of MSCs seems to be altered as they may undergo a complement-dependent lysis. Results indicate that the complement system is integrally involved in recognizing and injuring MSCs after their infusion[37]. MSCs activate the complement system, which triggers complement-mediated lymphoid and myeloid effector cell activation in blood. MSCs were found to present complement component (C3)-derived fragments inactivated C3b (iC3b) and C3dg and to generate complement-derived anaphylatoxins (C3a and C5a) with chemotactic activity[38]. It has been suggested that complement anaphylatoxins C3a and C5a participate in activation and recruitment of MSCs to sites of tissue damage and repair[39]. Of importance, complement bound to MSC enhanced their phagocytosis by classical and intermediate

monocytes which may explain, at least in part, why MSCs are not found in the blood circulation after infusion[40]. The inhibition of complement activation has been proposed for improving the outcome of MSC-based therapy. Thus, cell-surface engineering of MSCs with heparin has been shown to improve their viability and enhance their function after infusion. Heparin by directly inhibiting the complement protein and by recruiting factor H inhibited complement activation on MSCs[41]. Of note, treatment of MSCs with all-trans retinoic acid protected them from immune thrombocytopenia by regulating the complement-IL-1β loop. All-trans retinoic acid increased the number and improved the function of the complement-positive MSCs by upregulating DNA hypermethylation of the IL-1β promoter[42].

Inflammatory preconditioning

The role of the cytokine environment: Tissue injury is usually associated with inflammation, cell-damage products release and subsequent infiltration of neutrophils and macrophages. The inflammatory response is thought to act as a regulator of tissue stemness either by directly affecting tissue stem cells or by shifting differentiated cells toward a stem-like cell character. During the phagocytosis of damaged cells, pro-inflammatory cytokines mainly interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-1 are secreted[43]. Other pro-inflammatory mediators (*e.g.*, various chemokines, leukotrienes, and free radicals) are released by immune cells and may contribute to inflammation. In this context, MSCs are known to preferentially migrate to the sites of inflammation and tissue damage which are typically characterized by cytokine outburst[44]. The specific cytokine milieu is pivotal in determining the fate and behaviors of MSCs (Figure 4).

Accordingly, we reported that inflammation may induce critical changes in the phenotype, multilineage potential, hematopoietic support, and immunomodulatory capacity of MSCs[45].

IFN-γ as a master regulator: One of the most used strategy for this purpose has been the IFN-γ preconditioning of MSCs prior to clinical application. This approach enhances both the immunomodulatory properties and viability of MSCs[46]. In depth proteomic analysis of IFN-γ treated BM-MSCs indicated that approximately 30% of the altered proteins were linked to immunoregulation[47]. The expression levels of selected candidates including indoleamine 2,3-dioxygenase 1 (IDO-1), programmed death ligand 1 (PD-L1), vascular cell adhesion molecule 1, intercellular adhesion molecule 1 (ICAM-1) and bone marrow stromal antigen 2 (BST-2) were markedly increased following IFN-γ treatment. These factors may contribute to the immunoregulatory activities of MSCs. In addition, there are significant immunological and immunoregulatory mediators that are not currently described as IFN-γ responsive [*e.g.*, prostaglandin-endoperoxide synthase 2, prostaglandin I2 synthase, FAM20A (FAM20A golgi-associated secretory pathway pseudokinase), FAM20C (FAM20C golgi-associated secretory pathway kinase) and glycoprotein A repetitions predominant (GARP)/leucine rich repeat containing 32] that were upregulated. Moreover, MSCs isolated from the dental pulp of human exfoliated deciduous teeth and fragments of the orbicularis oris muscle showed enhanced immunomodulatory properties probably linked to increased HLA-G expression[48] in the presence of IFN-γ. Indeed, a recent study highlighted the key role of the *HLA-G* gene in modulating the immune response, and the importance of its polymorphisms and alleles associated with the outcome of the transplants[49].

A pro-inflammatory cytokine cocktail: INF-γ has also been used in combination with other potent inflammatory cytokines namely TNF-α and IL-1-β. We have shown that preconditioning BM-MSCs with a pro-inflammatory cytokine cocktail increased the expression of cyclooxygenase (COX)-2, leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), IL-11, IL-8, and IL-6, while it reduced that of COX-1, galectin-1, and transforming growth factor-beta (TGF-β)[50]. BM-MSCs may inhibit complement activation by producing factor H, which could be another mechanism underlying the broad immunosuppressive capabilities of MSCs. Of note, the production of factor H by MSCs is upregulated by inflammatory cytokines TNF-α and IFN-γ in both a dose- and a time-dependent manner[51]. Using such a preconditioning strategy may empower the immune fate of MSCs and, as a result, enhance their value for cell-based immunotherapy. Under IFN-γ and TNF-α preconditioning, BM-MSCs have been shown to synergistically express PD-L1 and PD-L2, which suppress T-cell activation and functions[52]. Interaction of PD-L1 with its known ligands, PD-L1/B7-H1 and PD-L2/B7-DC, provides an inhibitory signal in regulating cellular activation and proliferation. MSCs also secrete significantly higher levels of sPD-L2 compared to sPD-
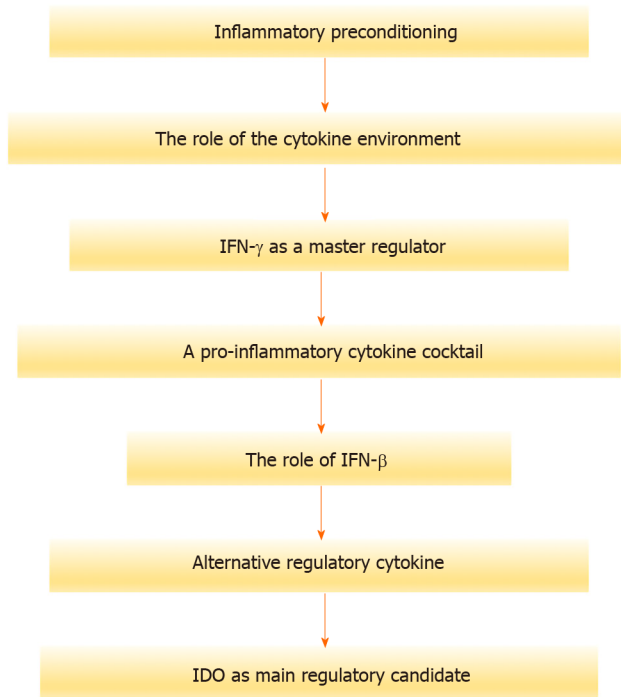
Figure 4 The inflammatory specific cytokine environment is pivotal in determining the fate and behaviors of mesenchymal stromal cells.
IFN-γ: Interferon-γ; IFN-β: Interferon-β; IDO: Indoleamine 2,3-dioxygenase.

L1 in preconditioning conditions, indicating the potential importance of sPD-L2 in MSC immunomodulation. In line with this, we have shown that the percentage of adipose tissue-derived MSCs expressing PD-L1 (*i.e.*, CD274) significantly increased upon pro-inflammatory stimulation reaching around 90% of the whole cell population[53]. CD274 is known to downregulate T-lymphocyte proliferation and cytokine secretion by binding to the PD-L1 receptor. Interestingly, IFN-γ priming of human amnion-derived MSCs boosted their immunomodulatory potential through different mechanisms. They induced a remodeling of the immune response toward an anti-inflammatory phenotype. In this context, a significant production of chemokines such as C-X-C motif ligand 10 [CXCL10, also known as IFN-γ-induced protein 10 (IP-10) or small-inducible cytokine B10], CXCL9 [also known as monokine induced by gamma interferon (MIG)], C-C motif ligand 3 [CCL3, also known as macrophage inflammatory protein 1-alpha (MIP-1-α)] and CCL4 (*i.e.*, MIP-1-β) was involved in the chemotaxis of innate immune cells. The suppression of the immune response was linked to the expression of PD-L1, IL-10, IDO, and prostaglandin E2 (PGE2), as well as release of different immunomodulatory exosome-derived miRNAs[54]. Within these exosomes, 46 miRNAs were upregulated and 114 were downregulated. Moreover, a miRNA-target network analysis revealed that these miRNAs were associated with immune and inflammatory responses/regulation pathways. Indeed, they were involved in the regulation of both T cell activation/anergy and monocyte differentiation pathways.

In order to increase the efficiency of the preconditioning, encapsulated MSCs with IFN-γ was thus developed. Tethering IFN-γ to the hydrogel increased the retention of IFN-γ within the biomaterial while increasing their immunomodulatory functions[55]. However, such strategy does not work universally as some progenitor cells showing immune-modulatory activities are less responsive to TNF-α and IFN-γ[56]. In comparison to BM-MSCs, human fetal cartilage progenitor cells preconditioned with either TNF-α or IFN-γ did not affect the expression LIF, TNF-stimulated gene-6 (TSG-6), IDO, and HGF known for their immunoregulatory roles. In contrast, adult-derived human liver stem/progenitor cells significantly induced the expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα, CCL5, IL-12a, IL-12b, IL-23p19, IL-27p28, and EBI-3) as well as immunoregulatory cytokines (IL1-RA, IDO-1, and TDO-1) under an inflammatory setting[57]. Within this context, liver derived stem/progenitor cells demonstrate marked immunological plasticity that has main importance for future cell therapy application.

The role of IFN-β: On the other hand, IFN-β was reported to enhance the immunomodulatory functions of MSCs isolated from mouse BM. This increase was linked to the induction of secretory leukocyte protease inhibitor and HGF following a rapid and transient phosphorylation of the transcription factors of signal transducer and activator of transcription (STAT) 1 and 3[58]. In a recent study, a long-lasting induction of mammalian target of rapamycin signaling supported by IFN-β was associated with an increased glycolytic capacity in umbilical cord blood-derived MSCs (UCB-MSCs). These UCB-MSCs also showed an improved ability to control T-cell proliferation[59].

Alternative regulatory cytokine: The preconditioning of BM-MSCs with macrophage-derived soluble factors, either TNF-α or IL-10, enhanced their immunomodulatory potential through increased PGE2 secretion[60]. Cell-to-cell contact with pro-inflammatory macrophages increased TSG-6 production by MSCs, thereby enhancing the suppressive regulation of T cells and switching macrophages to M2. Both cells are associated with wound healing and tissue repair by reducing immune inflammation and infiltration.

Moreover, the expression of CD200 was increased on MSCs thus facilitating the reprogramming of macrophages towards an anti-inflammatory skew through the interaction of CD200 with CD200 receptor on pro-inflammatory macrophages[61]. CD200 is differentially expressed and modulated on MSCs depending on their tissue of origin and culture conditions. A high constitutive expression of CD200 by Wharton's jelly (WJ)-MSCs may represent a distinctive marker of MSCs[62]. In parallel, GARP is proposed as a novel biomarker and new target to improve the therapeutic efficiency of MSCs. Results showed that GARP is constitutively expressed on mouse BM-MSCs and is required to activate locally produced latent TGF-β1, thus inhibiting the proliferation of T-cells and inflammatory cytokine production[63]. However, the presence or absence of GARP on adipose-derived MSCs (ASCs) was noted to display distinct TGF-β responses with diametrically opposing effects on ASC proliferation and survival[64]. Inflammation induces the balance towards a more pronounced tissue regenerative and anti-inflammatory phenotype. Indeed, IFN-γ preconditioning of ASCs enhanced their anti-inflammatory potency. Within the secretome, sixty miRNAs accounted for > 95% of the genetic message that resulted to be chondroprotective and M2 macrophage polarizing[65].

IDO as the main regulatory candidate: IDO is seemingly the most convincing candidate for a crucial role in T-cell inhibition by MSCs. The induction of IDO, which converts tryptophan to kynurenine, is substantially dependent on the preconditioning of MSCs with IFN-γ. Decreased concentration of tryptophan and increased kynurenine, both interfere with effective immune response[66]. It was demonstrated that IDO-mediated tryptophan starvation triggered by MSCs inhibited T-cell activation and proliferation through induction of cellular stress[67]. Both BM-MSCs and dental pulp MSCs were shown to inhibit T-cell activation and proliferation through IDO-mediated tryptophan starvation[67]. Also, preconditioned-adipose tissue-derived MSCs by INF-γ presented a higher immunosuppressive potential probably due to an elevated expression of IDO and ICAM-1[68]. An important immunoregulatory link between IDO and TSG-6 expression was observed in human umbilical cord-derived MSCs. Kynurenic, a metabolite of IDO, controls the TSG-6-mediated anti-inflammatory therapeutic effects of MSCs[69]. In agreement with these observations, we recently demonstrated that pro-inflammatory cytokines-stimulated skin-derived precursors (SKP) were able to suppress the graft-*vs*-host response when co-transplanted with human peripheral blood mononuclear cells in severe-combined immune deficient mice[70]. Significant changes in the expression and secretion profile of chemo/cytokines and growth factors involved in the immunomodulatory effects of SKP were also reported. Of importance, IFN-γ-preconditioned MSCs were shown to take up and process antigens as well as upregulate HLA class II expression, thus granting the conditions necessary to transform BM-MSCs into unconventional antigen-presenting cells. These MSCs maintained their ability to inhibit lymphocyte proliferation in an antigen-specific manner, without overtly increasing the immunogenicity of allogeneic MSCs[71]. These encouraging findings pave the way to use activated HLA-haplotype matched allogeneic MSCs as a cellular immune intervention therapy by combining their immunomodulatory properties with their antigen-presenting features.

Infectious triggering

Toll-like receptors as active sentinels: Infection of the wound triggers the body's immune response, causing inflammation and tissue damage, as well as slowing the healing process. In parallel to innate cells, there are other cells spread within the tissue that may be involved in pathogen detection. These may express sensors and transducers that can actively monitor invasion by viruses and bacteria[72]. The presence of microorganisms could be detected by germline-encoded pattern recognition receptors (PRRs) such as toll-like receptors (TLRs). These receptors are responsible for the sensing of structures conserved among microbial species called pathogen-associated molecular patterns. After this recognition, these PRRs may activate different signaling pathways resulting in the secretion of different cytokines and chemokines involved in the immune response against the present infection[73]. PRR ligation may also trigger the activation of phagocytic cells and the phagocytosis of damaged cells. TLRs represent one of the bridges that regulate the crosstalk between the innate and adaptive immune systems. TLRs interact with molecules shared and preserved by the pathogens of origin but also with endogenous molecules (damage/danger-associated molecular patterns) that derive from injured tissues[74]. By being in the surrounding area, MSCs will act as sensors of various signals and accordingly regulate the tissue repair process[75]. We have extensively described how infections such as obtained following TLR-agonist engagement may modulate MSCs at different levels. The phenotype, multilineage potential, hematopoietic support and immunomodulatory capacity of MSCs may thus present relevant changes, which could further affect their therapeutic potential[76]. Moreover, several environmental conditions modulate the pattern and function of TLRs expressed by MSCs (Figure 5).

Specific effects of TLRs: TLR activation may affect MSCs immunomodulatory functions by modulating their cytokine profile. Indeed, a decrease in the immunosuppressive capabilities of BM-MSCs is observed following TLR3 and TLR4 activation by poly(I:C) and lipopolysaccharides (LPS), respectively. Moreover, TLR3 activation augmented IL-6, IL-12p35, IL-23p19, and IL-27p28 transcription, whereas TLR4 activation increased IL-23p19 and IL-27p28 transcription[77]. In a comparative study, TLR3 [poly(I:C)] and TLR4 (LPS) ligation have differentially affected the suppressive functions of BM-, WJ- and adipose tissue (AT)-MSCs[54]. Remarkably, the immunosuppressive potential of WJ- and AT-MSC was not affected while BM-MSC showed reduced ability to inhibit lymphocyte activation. Differences in the levels of HGF and PGE2 secreted by MSCs following TLR activation have been hypothesized to underline these changes[78]. Similarly, the expression profile and function of TLR3 and TLR4 in human olfactory ecto (OE)- and AT-MSCs were reported to be different[79]. Both cell types differentially responded to their agonist engagement by presenting distinct cytokine and chemokine profiles. OE-MSCs exhibited significantly higher levels of chemokine CCL5, IL-8, and TGF-β production, in comparison with AT-MSCs. IL-6 secretion by AT-MSCs was considerably more elevated than that by OE-MSCs. These changes may explain why OE-MSCs exhibited a high proliferative potential and AT-MSCs a more immunomodulatory function. In parallel, injection of LPS-preconditioned MSCs into wounds resulted in a significant acceleration of wound closure. Sensing an infected microenvironment and subsequently activating several innate immune cells by adipose MSCs is linked to TLR4 expression which are triggered by LPS[80]. Preconditioned MSCs with LPS not only trigger innate immune cells by recruiting and activating neutrophils and macrophages, but also initiate a cascade of cellular events which contribute to accelerated repair of skin injury. A network of genes mediating the adaptive immune response including chemokine CXCL6 [also known as granulocyte chemotactic protein 2 (GCP-2)], IL-8, and IL-1β was upregulated through the TLR4 pathway and may be responsible for the acceleration of wound healing. Of note, MSCs isolated from the mouse's abdominal adipose tissues presented a secretome with a dipeptidyl peptidase-IV (DPP-IV)/CD26 and aminopeptidase N/CD13 activity that was decreased following stimulation of MSCs by TLR4 agonist. The DPP-IV/CD26 are ubiquitous ectoenzymes, which can digest various substrates including some chemokines and neuropeptides that are involved in inflammatory conditions[81].

Paradoxically, another study showed that TLR3 is predominantly upregulated in UCB-MSC and its activation leads to their senescence. Additionally, Janus kinase 1 (JAK1) was identified as a key regulatory factor linked to the cellular senescence triggered by TLR3 activation in MSCs. JAK1 activation mediates the IFN-β increased-TLR3 expression, thereby reinforcing TLR3-mediated MSC senescence[59]. In a separate study, TLR ligation was reported to differentially affect the *in vitro* osteogenic potential of MSCs depending on their tissue origin. Indeed, TLR3 [Poly(I:C)] or TLR4 (LPS)
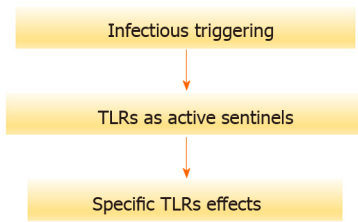
Figure 5 The infectious environment may modulate mesenchymal stromal cells through different pattern and function of toll-like receptors. TLRs: Toll-like receptors.

triggering increased the osteogenesis in AT-MSCs and, to a lesser extent, in BM-MSCs. However, WJ-MSCs constitutively disclosed a lower osteogenic potential as compared with other MSCs, which is not affected by TLR[82]. Further findings support that triggering TLR2 may enhance the proliferation and immunomodulatory functions of BM-MSCs. More specifically, the increased immunomodulatory capacity of MSCs was associated with an increased expression of anti-inflammatory cytokine expression and diminution of pro-inflammatory cytokine expression[83]. MSCs can potently regulate the functions of immune cells by attracting them or by migration to inflammatory sites. TLR3-stimulated MSCs exhibit increased migration and chemotaxis within and toward damaged tissues through the expression of forkhead box protein O1-activated genes[84]. The understanding of TLR involvement in the response of stem/progenitor cell to specific tissue damage and in the reparative processes is decisive for the development of new therapeutic strategies[75].

In line with this, we reported approaches using empowered MSCs for the treatment or prevention of different immunological diseases. Preconditioning by new licensing stimuli will empower the immune fate of MSCs and, therefore, promote a better and more efficient biological response[50]. We reported several new pathways induced by inflammatory signals such as fibrinogen-like 2, GAL, semaphorin (SEMA) 4D, SEMA7A, and IDO-1 and can contribute to immunomodulation. These pathways allow distinct MSC populations to display enhanced immunomodulatory effects, particularly during inflammation[85]. Our data also demonstrated that MSCs depending on their tissue-source, may present several relevant receptors potentially involved in the regulation of inflammatory and immunological responses. The expression of advanced glycation end-product receptors, C-type lectin receptors (including DECTIN-1, DECTIN-2 and MINCLE), leukotriene B4 receptors (BLT1 and BLT2) and cysteinyl leukotrienes receptors (CYSLTR1 and CYSLTR2) was substantially influenced by inflammatory conditions[86]. These data strongly support that inflammatory signals are important for the immunological plasticity of MSCs allowing them to display specific responses.

It is important to increase our knowledge about these preconditioning methods and better understand the molecular and cellular mechanisms underlying their effects. It is noteworthy that inadequate manipulation of MSC plasticity may lead to aberrant immune hemostasis and tissue regeneration[87]. The development of the most suitable strategy should not only aim at enhancing the therapeutic value of MSCs but also confirm the absence of any potential adverse effect.

Cell-free based therapeutics
The therapeutic potential of MSCs for tissue repair and wound healing is essentially based on their paracrine effects[88]. The secretome is defined as series of factors including chemokines, cytokines, extracellular matrix molecules and growth factors that are secreted into extracellular space. Once collected, the secretome is considered as "cell-free therapeutics" because it is derived from MSCs and devoid of cells[89]. Moreover, some advantages such as the easy storage/thaw process with a preserved quality and function of the factors are associated with the use of the secretome[90]. The deep analysis of the secretome composition will enable us to evaluate its utility as a new therapeutic option[91]. Thus, advances in the characterization of the secretome composition pointed out the presence of EVs with functional activities.

EVs are small lipid membrane-enclosed particles released by cells into the extracellular space. EVs are very heterogeneous in size and content and can be released naturally or after specific induction. They contain a variety of bioactive components which promote cell-to-cell communication both in a healthy and diseased

state[92]. EVs facilitate intracellular communication by carrying and delivering biological materials, such as proteins, lipids, and nucleic acids[93]. Depending on their cellular origin, EVs cover different structures such as exosomes, microvesicles (MVs), and apoptotic bodies[94]. Exosomes are the smallest secreted vesicles (40-140 nm) which are formed by the invagination of the membrane of the multivesicular bodies and fusion with the plasma membrane, to be finally released into the extracellular environment. MVs (50-1000 nm) are budded directly off the plasma membrane and apoptotic bodies (1-5 μm) released from cells undergoing apoptosis[95]. More specifically, MSCs may secrete at least three types of EVs based on their affinities for membrane lipid-binding ligands. As their cargo load are different for each EV type, they are likely to have a different biogenesis pathway and possibly different functions[96]. Several scientific societies (such as SOCRATES, International Society For Extracellular Vesicles, ISCT and International Society of Blood Transfusion) propose to optimize the methods and harmonize the criteria that identify and characterize MSC-derived EVs[97]. These preparations should be defined physically, biochemically, and functionally by quantifiable features and using reproducible and standardized assays[97].

Donor-to-donor variability and heterogeneity of MSC therapeutics are significant factors that interfere with their sustainable translation to the clinic. The use of EVs has multiple advantages in overcoming the limitations and adverse events observed with cell-based therapy[98]. Moreover, the use of MSCs may raise some concerns regarding the purity, viability, identity, recovery after cryopreservation, functional and genomic stability of the cellular product. The choice of the cell source, phenotype, infusion-related toxicities and immunological complication (donor graft-*vs*-host disease and recipient graft rejection), ectopic tissue formation represent risks associated with the use of MSCs[99]. Thus, MSC-derived EVs can be evaluated as cell-free based therapeutics. EVs have been shown to effectively mimic the therapeutic effects of MSCs, in several preclinical models[100]. Indeed, MSC-derived EVs exert both immunomodulatory as well as pro-regenerative effects[101]. Several observations indicate that EVs may suppress pro-inflammatory processes and reduce oxidative stress. By switching the pro-inflammatory response into a tolerogenic one, MSC-derived EVs are likely to promote tissue regeneration by creating a pro-regenerative environment allowing endogenous stem and progenitor cells to successfully repair affected tissues[102]. Accumulating evidence indicates that MSC-derived EVs exhibit immunomodulatory effects by delivering biologically active nucleic acid and proteins. There is a strong correlation between the expression levels of TGF-β1, pentraxin-related protein-3 (also known as TNF-inducible gene 14 protein), let-7b-5p, or miR-21-5p in MSC-derived EVs and their suppressive function[103]. Moreover, a recent study demonstrated that BM-MSC-derived EVs were incorporated by lymphocytes and decreased their proliferation and differentiation. Interestingly, MSC-derived EVs, by increasing forkhead box P3 expression and reducing the production of IFN-γ, generated a subpopulation of regulatory T cells with suppressive properties. Mechanistically, MSC-derived EVs decreased miR-23a-3p expression and increased expression of its target gene, transforming growth factor beta receptor 2, suggesting active participation of TGF-β pathway in this regulation[104]. Furthermore, early indications suggest that the favorable effects of MSC-derived EVs could be further enhanced by modifying the way in which the donor MSCs are cultured[105]. In general, the preconditioning of MSCs by an inflammatory milieu is the most used method to increase the immunotherapeutic function of MSC-derived EVs[106]. Exosomes derived from BM-MSCs preconditioned with a low dose of dimethyloxaloylglycine (DMOG) were reported to exert enhanced proangiogenic activity during bone regeneration[107]. Several classical pathways related to proliferation and osteogenic and angiogenic differentiations, including yes-associated protein of the Hippo pathway, JAK of the JAK/STAT pathway, p65 of the NF-κB pathway, and β-catenin of the wnt pathway were significantly elevated. In contrast, PTEN and key factors that regulate apoptosis such as p53 and p21 were significantly downregulated[107]. To mimic hypoxic conditions, DMOG degrades peroxisomes by selectively activating pexophagy in a HIF-2α dependent manner involving autophagy receptor p62[108]. In addition, thrombin preconditioning of human UCB-MSCs significantly accelerated EV biogenesis and enriched their cargo contents largely *via* protease-activated receptor-1-mediated pathways[109]. Recently, MSCs from rat BM were preconditioned with melatonin resulting in an improved therapeutic potential for acute liver ischemic injury[110].

Depending on both MSC origin and environment, EVs may display stimulatory or suppressive immune functions. It is likely that the composition of EVs is a critical determinant of their functions. As previously reported, both the immunomodulatory capabilities of distinct vesicle preparations and the responsiveness of individual

patients towards MSC-derived EVs are critical factors for a successful therapeutic application of MSC-derived EVs preparations[111]. Previous studies reported that EVs derived from hypoxic-primed MSCs are packaged with numerous metabolites that are directly associated with immunomodulation, including M2 macrophage polarization and regulatory T lymphocyte induction[112]. The expression of some cytokines, chemokines, and chemokine receptors such as IL-10, HGF, LIF, CCL2, VEGF-C, CCL20, chemokine CXCL2, CXCL8, CXCL16, defensin alpha 1, human hect domain and RLD 5, and interferon induced transmembrane protein 2 were reported to underline the interplay between EVs and their cellular targets[112]. These mediators may participate in migration to the site of injury, regulation of inflammation/immune response and modulation of the repair and regeneration process[113,114]. As we discussed, both pro- and anti-inflammatory signals are present during tissue healing. A critical requisite of MSC-derived EVs is their capacity to support the diametrically opposite pro- and anti-inflammatory functions at the appropriate time. Recently, preconditioned MSC-derived EVs were described as a novel cell-free strategy for expanding hematopoietic stem cells (HSCs) *in vitro*[115]. Therefore, MSC-derived EVs contribute to the BM homeostasis but may also display multiple roles in the induction and maintenance of abnormal hematopoiesis[116].

Currently, the number of clinical trials involving MSC-derived EVs as listed in www.clinicaltrials.gov is substantially limited. Future experimental designs are still required to deeply understand the therapeutic effects of EVs and their mechanisms of action[100]. Of importance, the current coronavirus disease 2019 (COVID-19) pandemic, which leads to severe and increased worldwide morbidity and mortality, has rapidly promoted MSCs as potential therapeutic agents to suppress COVID-19. In this context, some registered clinical trials using EVs from MSCs are now under investigations. The rationale for these approaches is supported by a growing body of evidence showing that MSC-derived EV preparations are therapeutically safe and efficient in a wide range of diseases[117].

More highlights on optimal production, isolation, storage, therapeutic dose, and schedule of EV administration, will increase the safety and efficiency of the therapy[118]. We also need to consider the state of health of the patient before injection, the immune-biology of MSCs and adequate pre-clinical models[99]. The use of a cell-free preparation based on EVs makes MSCs an important material for the development of new approaches for non-cell-based therapies. Moreover, modulating or preconditioning MSCs to achieve a specific composition of the EV cargo for the *ex vivo* expansion of HSCs is, therefore, a promising strategy that can overcome several challenges associated with the use of naïve/unprimed MSCs.

CONCLUSION

The research field of MSCs is innovating but needs more studies to address optimal therapeutic efficiency and safety. MSCs hold great promise in the field of immunotherapy and regenerative medicine. Accordingly, a better understanding of MSC immune biology will improve their therapeutic value and use[4]. The development of a cell-free product derived from MSCs such as EVs is the most promising tool to increase the safety of such therapy. EVs should be well-identified in terms of phenotype, functions, and effects. In parallel, enhancing the survival and functions by evaluating the optimal preconditioning strategy, will improve the therapeutic beneficial actions of MSCs. It is of the utmost importance that this feature, which is closely dependent on disease environment and patient health status, be identified and understood. The development of both features should enhance the value of MSCs by providing a therapeutic product with high quality, safety, and efficiency.

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