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Mesenchymal stem cells immunomodulation: The road to IFN-γ licensing and the path ahead

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ABSTRACT

Mesenchymal Stem Cells (MSCs) have gained prominence as an important tool in cell therapy, especially considering their capacity to control the immune system. Due to this property, the application of MSCs has been investigated for the treatment of several immune disorders, such as diabetes, rheumatoid arthritis, Crohn's disease, systemic lupus erythematosus, and graft-versus-host-disease (GvHD). The application of MSCs to treat inflammatory diseases has led to impressive results. However, individual response to treatment is still heterogeneous, and the number of cells required to treat humans is very high. The possibility of increasing the immunosuppressive potential of MSCs is seen at this point as a promising alternative to overcome such limitations. One of the most exploited strategies for this purpose has been the licensing of MSCs prior to clinical application. In this review, we will discuss the mechanisms by which MSCs modulate the immune response and the main advances in the licensing of these cells, with a special focus on the use of interferon gamma (IFN-γ). Also, we will address the main challenges ahead before licensed MSCs are finally used successfully in clinical practice.

1. Introduction

Mesenchymal Stem Cells (MSCs) have been defined as multipotent progenitors, with fibroblastoid morphology and the capacity to give rise to several mesodermal cell types, such as adipocytes, osteoblasts and chondrocytes [1]. MSCs were first isolated from the bone marrow, but it was later shown that they can be obtained from basically all tissues in humans, as well as mice [2,3].

So far, a specific marker for MSCs is still lacking, therefore the International Society of Cell Therapy (ISCT) proposed that MSCs must be characterized following three basic criteria, namely: plastic adherence when cultured; ability to differentiate *in vitro* into adipocytes, chondrocytes and osteocytes, and; specific immunophenotype, combining the absence of hematopoietic markers (CD11, CD14, CD34 and CD45), as well as costimulatory molecules (CD40, CD80 and CD86), with the presence of CD105, CD73 and CD90 surface markers [4]. Despite the harmonization of MSC identification procedure, an increasing number of reports have demonstrated that MSCs constitute a heterogeneous cell population. In this sense, much effort has been made to isolate and evaluate the biological properties of MSC subpopulations, based on specific membrane markers, including Stro-1, SSEA-4, CD271, CD146 and MSCA-1 [5,6]. Additionally, MSC morphology has also been identified as a relevant parameter to identify MSC subpopulations with different properties, *in vitro* [7]. Still, since most articles published so far predominantly consider MSCs as a single (yet heterogeneous) population, we will follow the same perspective in the present review.

Since their original description [8], MSCs have gained prominence as an important tool in the cell therapy-based regenerative medicine field, due to their innumerable biological characteristics, associated to the ease of their obtention and *in vitro* expansion. As knowledge regarding MSC regenerative property accumulates, increasing awareness of their clinical potential stands out. For instance, MSCs remarkably induce cell proliferation and angiogenesis. Furthermore, MSCs also display antiapoptotic, immunoregulatory and antimicrobial functions. Importantly, the therapeutic effects of MSCs observed *in vivo* indicate that the clinical efficacy depends on the microenvironment of MSCs. According to the stimuli received, MSCs may secrete higher or lower amounts of a broad variety of growth factors and chemokines [9,10]. The best conditions to boost MSCs therapeutic potential may, therefore, vary among different diseases, and are subject of active investigation.

Interestingly, despite the initial focus of applying MSCs for regenerative purposes, one of the most important therapeutic functions

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attributed to MSCs at this point is their immunoregulatory capacity. The first clinical evidence of the high immunomodulatory potential of MSCs, published by Le Blanc and colleagues [11], paved the way for the use of MSCs for the treatment of several immune disorders, such as diabetes [12], rheumatoid arthritis [13], Crohn's disease [13,14], systemic lupus erythematosus [15], and graft-versus-host-disease (GvHD) [16,17] to cite but a few. The application of MSCs to treat inflammatory diseases has led to impressive results, yet individual response to treatment is still heterogeneous, and the number of cells required to treat humans is high.

Considering the several different approaches performed at this point with the aim of maximizing MSCs immunomodulatory function, as well as the mixed results published so far, we will discuss, in the present review: the main mechanisms used by MSCs to control the immune response of T cells; the consequences of licensing MSCs with inflammatory cytokines, especially with interferon gamma (IFN-γ); and the challenges ahead prior to clinical application.

2. MSCs, T-cells and the immune response

2.1. Revisiting MSC immune privileged status

There is little controversy that, upon stimulation, MSCs exert immunomodulatory effects over humoral and cellular components of the immune system. Importantly, *in vitro* expanded MSCs express low levels of MHC class I, and no MHC class II nor co-stimulatory molecules CD40, CD80 and CD86. This particular composition of surface markers added to the demonstration that these cells were capable of inhibiting the proliferation of MHC mismatched lymphocytes *in vitro* [18], and contributed to the view of these cells as immune privileged.

However, the notion that MSCs are immune evasive and not immune privileged has emerged based on other pieces of evidence. For example, after *in vivo* infusion, allo-MSCs present lower survival rate compared to syngeneic MSCs. Also, allo-MSCs–treated mice show increased levels of memory T-cells and more rapidly eliminate immunogenic donor cells, compared to "naive" recipients [19–21]. Furthermore, after *in vivo* infusion, allo-MSCs have been observed in higher amounts at immunosuppressed sites. Such evidence contradicts previous observations acquired *in vitro*, which consistently revealed strong immunosuppressive behavior of MSCs. Since the ratio between MSCs and immune cells *in vitro* is artificial, it has been hypothesized that MSCs are not immune privileged, but may benefit from immunosuppressed sites, and may also overcome immune activation, when in sufficient amounts [22]. In fact, MSCs secrete an arsenal of immunomodulatory molecules able to suppress the function of all immune cells, from B cells and T cells, to neutrophils, dendritic (DCs), and natural killer (NK) cells.

2.2. MSCs x immune cells: identified mechanisms of immunosuppression

MSCs are considered to be an effective tool to control immunity, due to their ability to act over an impressive amount of targets. Considering the battle between MSCs and B-cells, it has been shown that MSCs inhibit B-cell differentiation, proliferation and activation [23,24]. More recently, another important effect of MSCs over B-cells was clarified by the demonstration that MSCs can induce the generation of IL-10 secreting regulatory B-cells [25,26], which possess immunosuppressive capacity and can support immunological tolerance [27]. Mechanisms used by MSCs to modulate B-cell function include paracrine mechanisms, e.g. production of Indoleamine 2,3-dioxygenase (IDO) and tryptophan breakdown [28], and Transforming Growth Factor-beta (TGF-β) production [29]. Nevertheless, it is important to emphasize that, when unstimulated, MSCs may fail to modulate B-cell activation [30].

The role of contact-dependent, or at least the proximity-dependent mechanisms, of MSC influence over B-cell function is still contentious [28]. The influence of MSC on B-cell behavior results from direct mechanisms, but, importantly, may also indirectly reflect the MSC properties of inhibiting the differentiation and function of DC derived from either CD34+ cells or monocytes [31,32]. Indeed, it has been observed that, during MSCs and monocytes co-culture experiments, not only DC failed to differentiate, but also monocytes began to secrete high levels of IL-10, explaining, at least in part, the suppressive effect of MSCs over other cell types [33,34].

MSCs also control the proliferation of freshly isolated NK cells and inhibit the effector functions of these cells, including cytotoxic activity and cytokine production [35]. However, recently, it was shown that this MSC-NK interaction is rather complex and depends upon the cytokines used to activate NK cells. For instance, when stimulated with IL-2, NK cells can generate ROS and promote MSC death [36]. Importantly, MSCs can also engage in a cross-talk with phagocytic granulocytes, enhancing their antibacterial activity, inhibiting their apoptosis and prolonging their survival [36–39].

The first demonstrations that MSCs are able to promote T-cell immunosuppression were published in 2002. In that year, using MSCs isolated from Baboons, Bartholomew and colleagues showed that these cells could inhibit T-cell proliferation *in vitro,* and prolonged skin graft survival *in vivo* [40]. During the same year, the first evidence that human MSCs were also able to modulate T-cell function was published. Di Nicola et al. showed that both autologous and allogeneic bone marrow derived MSCs (BMSCs) could suppress CD4 and CD8 T-cell proliferation, and that this effect was dose dependent. Furthermore, the observed effect occurred even when MSCs and T-cells were physically separated using a transwell system. Finally, TGF-β and hepatocyte growth factor (HGF) were also shown to have important roles in MSC-mediated T-cell immunosuppression [41]. Since the demonstration that MSCs are able to control T-cell immune response, the scientific community has sought to elucidate how the communication between MSCs and T-cells occurs, and also how MSCs become immunoregulatory. In parallel, part of the scientific community continued to struggle to elucidate the molecular mechanisms used by MSCs to suppress the immune response.

The immunomodulatory properties of MSCs are not constitutive and, during the immune response, MSCs communicate with immune cells. The inflammatory microenvironment is thus crucial to direct the anti-inflammatory state of MSCs, untangling the roles played by IFN-γ [42] and interleukin-1 beta (IL-1β) [43] in this context. Although, adding complexity into the scenario, our group has found that the conditioned medium obtained from resting (adipose tissue-derived) MSCs controls T-cell proliferation, even in the absence of any inflammatory stimuli [44]. Others have also demonstrated that inactivated MSCs, initially unable to respond to inflammatory signals or secrete immunomodulatory factors, are able to control the inflammatory response independently of their secretome or active cross-talk with immune cells [43,45]. These results point towards the need for more studies to investigate the intrinsic *versus* induced capacity and/or alternative routes used by MSCs to control the immune response. Glennie et al. reported that activated T-cells co-cultured with MSCs show arrested cell cycle at the G1 phase and inhibition of cyclin D2 expression. Unexpectedly, no changes in the expression of the activation markers CD25 and CD69 accompanied the lack of T-cell proliferation [46]. MSC effects over T-cell activation markers are contradictory in the literature, being that other reports have demonstrated that MSCs significantly reduce the activation state of T-cells, altering CD25 and CD69 expression [43,47]. Similarly, while initial observations indicated that the immunosuppressive effects of MSCs over activated lymphocytes were independent of apoptosis [42], it was later reported that MSCs may actually induce apoptosis when controlling T-cell proliferation [48,49]. More recently, it was proposed that the levels of IL-7 in the coculture system of MSCs and T-cells can be determinant to prevent T-cells from undergoing apoptosis [50]. Despite the conflicting results, it is well known that MSCs control T-cell responses through varied mechanisms, including the generation of soluble

immunosuppressive molecules, cell-cell interaction, and induction of regulatory T-cells (Tregs), as discussed below.

2.3. MSC secretome and T-cell suppression

A recent report investigated the proteomic profile of MSC secretome by mass spectrometry and demonstrated that the proteomic signature of these cells contains 457 proteins, the majority of which are related to angiogenesis, inflammation and immune response. Interestingly, this work also revealed that MSCs submitted to an inflammatory *milieu* present altered composition of their secretome, especially regarding the expression of proteins related to inflammation [51]. The secretion of bioactive molecules by MSCs is essential for these cells to control the immune response and T-cell function. It is well known that protein and non-protein molecules participate in the MSCs elicited T-cell immunomodulation, including HGF, TGF-?? [41], IDO [52], prostaglandin E2 (PGE2) [53], interleukin 6 (IL-6) [54], interleukin 10 (IL-10) [55], semaphorin-3A, galectin (Gal)-1 [56] and Gal-9 [57] to cite but a few. In this point, it is important to mention that there was a consensus that, while murine MSCs produce nitric oxide (NO) as immunosuppressive molecule to control the immune response, human MSCs mostly rely on IDO to do so [58,59]. However, some authors have shown production of NO by human MSCs [60] and that this molecule is also involved in the immunoregulatory potential of these cells [61].

In recent years, the field of cellular communication has witnessed a dramatic increase in interest in the role of extracellular vesicles, such as microvesicles and exosomes in different contexts, including immunoregulation. Extracellular vesicles are generally classified according to their size, exosomes having approximately 50–150 nm in size, while microvesicles generally present 200–1000 nm diameter [62]. Extracellular vesicles are mediators of cellular paracrine signaling and it has been suggested that, after internalization, extracellular vesicles transfer proteins, bioactive lipids, mRNAs and microRNAs to target cells. Recent studies have investigated the content of MSC-derived extracellular vesicles by means of high throughput techniques. For instance, Kim et al. performed a proteomic analysis by LC–MS/MS of MSC-derived microvesicles and found 730 proteins, including surface receptors, signaling molecules, cell adhesion components and MSC-associated antigens [63]. In addition, Haraszti et al. evaluated the proteomic signature of both microvesicles and exosomes derived from MSCs. Importantly, they identified 719 proteins in exosomes and 1357 proteins in microvesicles, concluding that exosomes are enriched for proteins related to immune response regulation, compared to microvesicles [64]. Corroborating such observations, a growing body of evidence has been published reporting functional properties of extracellular vesicles derived from MSCs [62,65,66]. For instance, MSC-derived extracellular vesicles exert immunoregulatory effect over T-cells by inducing Treg generation and promoting apoptosis of T-cells, despite failing to control T-cell proliferation [67–69]. Others have shown that MSC-extracellular vesicles exert their immunoregulatory function by promoting a reduction of proinflammatory factors secreted by peripheral blood mononuclear cells (PBMCs) and controlling both CD4+ and CD8+ T-cell proliferation, in a dose dependent manner [44,70–72]. The different observations are not conflicting, and may arise from different methods of extracellular vesicle isolation and experimental design. Regardless of the discrepancies at this point, there is little controversy that MSC-derived extracellular vesicles, similar to their cellular progenitors, possess the capacity to control T-cell function.

2.4. MSCs and T-cell contact-dependent interaction

In the context of MSC and T-cell interaction, contact-dependent mechanisms are highly relevant. It has been shown that, to exert their immunoregulatory function, MSCs attract T-cells, taking advantage of T-cell chemokine ligand (CXCL) expression. Using those and other receptors, MSCs bind and remain attached to T-cells [73,74]. Currently, the best investigated mechanism of immunoregulation in this context includes the interaction between MSCs and T-cells through intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1) and inhibitory molecule programmed death 1 (PD-1) [75,76]. Nevertheless, it is important to emphasize that the cell surface signature of MSCs is composed of approximately 888 membrane proteins. Cell adhesion like integrins, cadherins, some adhesion molecules of the immunoglobulin superfamily, as well as receptors involved in cell signaling, including platelet-derived growth factor (PDGF)-receptor, epidermal growth factor (EGF)-receptor, TGF-β–receptor, tumor necrosis factor (TNF)-receptor and ephrin-receptors are but a few examples of such 888 proteins [77]. Therefore, existing knowledge may be but a glimpse of a complex scenario.

The importance of adhesion molecules as another immunoregulatory tool in the MSC toolbox was first demonstrated by Di Nicola and colleagues, who after physically separating MSCs and T-cells, noticed a partial inhibition of the immunosuppressive effect of the former over the latter [41]. Interestingly, under basal conditions, MSCs express low levels of ICAM-1, but this expression is strongly enhanced under inflammatory conditions, mainly as a response to IFN-γ and TNF-α [78]. Mice and human MSCs seem to differ in this sense, though. While murine MSCs show compromised immunosuppression upon ICAM-1 blockade [79], their human counterparts are not significantly influenced by such treatment [80]. More recently, Rubtsov et al. demonstrated that ICAM-1 has an important role in the activation of the immunosuppressive function of human MSCs from adipose tissue [81]. Furthermore, the importance of ICAM-1 in the immunosuppressive function of human MSCs was strengthened by the demonstration that it is essential for MSC interaction with M1 macrophages, and that such interaction leads to an increased immunosuppressive potential of MSCs [82].

Similar to ICAM-1, VCAM-1 is also overexpressed on murine MSC surface under inflammatory conditions and its inhibition also compromises MSC-mediated immunoregulation [79]. In humans, this molecule has heterogeneous expression on MSCs, and VCAM-1^+ MSCs display higher immunosuppressive potential compared to VCAM-1^- [83].

Another cell-cell contact mechanism involved in T-cell immunosuppression by MSCs is mediated by PD-1/B7-H1 pathway. When in contact with MSCs, T-cells enhance the expression of PD-1 protein at their surface and may undergo apoptosis [84]. In this context, MSCs also enhance the expression and secretion of PD-L1 to suppress T-cell function and induce its apoptosis [85].

Altogether, there is an increasing awareness regarding the importance of MSC surface-proteins for the control of immune response, beyond cell contact-dependent mechanisms. For example, our group has shown that CD39 and CD73 present in the MSC surface are also relevant for T-cell suppression by promoting adenosine production [86–88].

2.5. Induction of Tregs

The immunosuppressive function of MSCs occurs through a myriad of mechanisms, which include the cooperation with classical Tregs ($CD4^+CD25^+FOXP3^+$) [89,90]. Importantly, the production of classical Tregs is induced by MSCs by means of both cellular contact with T-cells, as well as soluble factors, including PGE2, TGF-β, insulin growth factor (IGF). MSCs also promote Treg expansion by secreting HLA-G5 [91–93]. Of note, this already complex scenario gained another layer of complexity when English et al. showed another route by which MSCs would promote Treg generation with the participation of monocytes [91,94]. Interestingly, the dynamic interaction of MSCs and Tregs reveals that, besides promoting Treg generation and expansion, MSCs also recruit these cells, maintain their phenotype and ensure that Tregs stabilize their immunosuppressive potential for long periods [90]. It has been proposed that the enhanced Treg capacity to suppress the immune response when influenced by MSCs is dependent on PD-1 upregulation

[95]. Interestingly, during the generation of Tregs, MSCs from amniotic membrane also modulate the proinflammatory response by down-regulating Th1 and Th17 T-cell subsets [96]. It is important to mention that, in addition to classical Treg generation, MSCs also induce and regulate the function of non-classical Tregs, including $CD8^+CD28^-$ and suppressive $CD69^+$ T-cells [97,98].

As can be seen, MSCs play their immunosuppressive role through a multilevel network of mechanisms that probably occur simultaneously, complementarily, and even synergistically. More studies are needed to elucidate how the different immunosuppressive mechanisms of MSCs connect to each other.

3. MSCs licensing

The immunosuppressive effect of MSCs over T-cells has been thoroughly demonstrated, and important advances have been made regarding the understanding of the associated mechanisms. Nevertheless, several researchers are actively searching an ideal protocol to maximize the immunosuppressive potential of MSCs. It is hypothesized that an optimized procedure would decrease the number of cells required for clinical applications, and, potentially, guarantee more homogenous clinical outcomes. Interestingly, Krampera and colleagues indicated that IFN-γ treatment could have a distinct role in the regulation of MSC-mediated immunosuppression, by demonstrating that IFN-γ secretion by T-cells has a paramount role in activating the MSC immunomodulatory program [42]. In fact, several publications followed Krampera`s seminal work and have confirmed that IFN-γ has a central role in the initiation and intensity of MSC-mediated immunosuppression, as discussed below.

3.1. IFN-γ licensing of MSCs

IFN-γ is a gene product of the type II IFN family, synthesized by T- and NK cells. In the context of MSC and T-cell interaction, the IFN-γ secreted by T-cells binds to its receptor, which is present in MSC membrane. Interestingly, it has been demonstrated that the production of IFN-γ by T-cells is actually needed for MSC immunosuppression [42,99]. Building upon Krampera`s observation, an increasing amount of studies have attempted to increase current knowledge regarding how IFN-γ impacts the immunoregulatory potential of MSCs. For instance, it has been shown that IFN-γ regulates the production of several molecules important for MSC-mediated immunosuppression, such as HGF, TGF-β and IDO [42,99]. At this point, it is important to mention that even though most studies point to the central immunoregulatory role of IDO in the immunomodulation promoted by MSCs, according to Gieseke et al., these cells are able to control T-cell proliferation in the absence of IFN-γ receptor signaling and IDO [100].

Following the recognition of the importance of IFN-γ in promoting the immunosuppressive function of MSCs, came the investigation of IFN-γ treatment as a licensing (or activation) strategy to boost MSC immunosuppressive properties. Polchert and colleagues developed a pioneering work in this field, demonstrating that, in a murine model of GvHD, IFN-γ-treated MSCs became immediately active and suppressed GvHD more efficiently than their non-licensed counterparts [101]. Soon after, Rafei et al. tested the same rationale, but failed to find any beneficial effect of using IFN-γ-licensed MSCs to treat a murine model of experimental autoimmune encephalomyelitis, compared to non-licensed MSCs [102]. Despite the initial mixed results regarding the efficacy of IFN-γ licensing of MSCs, several other publications, based on *in vitro* and *in vivo* models, point to a therapeutic advantage of using this licensing strategy (Table 1). In this line, additional studies which focused on GvHD also revealed that IFN-γ-licensed MSCs inhibit T-cell proliferation, reduce target organ pathology, and significantly prolong mice survival [103,104]. IFN-γ-licensing was also revealed to be effective in an animal model of colitis [105].

These *in vivo* demonstrations of IFN-γ-licensed MSC treatment on different disease models have been accompanied by the search for a better understanding of the mechanisms involved in the immunosuppressive potency of licensed cells. Importantly, IFN-γ promotes increased expression of VCAM-1 and ICAM-1 on the surface of the MSCs, which reinforces their immunosuppressive potential [79]. Furthermore, IFN-γ-licensing increases the capacity of MSCs to generate different subtypes of Tregs [106], and decreases cryopreserved MSC susceptibility to lysis by activated T-cells [107]. Therefore, the importance of MSC licensing seems to exceed initial expectations (Fig. 1).

The effects of IFN-γ over the morphology and proteome of MSCs have been investigated. Using high-content imaging, Klinker et al. noticed that IFN-γ induces morphological modifications on MSCs, and that these alterations significantly correlate with increased immunosuppressive capacity of these cells. The authors showed that, after IFN-γ stimulation, the MSCs with high immunosuppressive capacity present relatively low cell perimeter, low cell maximum feret diameter, and high nucleus/cytoplasm ratio [108]. In contrast, Dae Seong Kim and colleagues did not report significant changes in the morphology of MSCs using conventional microscopy [104]. Unexpectedly, the significant functional and morphological alterations induced by IFN-γ licensing are related to modified expression of only a small subset of 210 proteins. Among them, proteins associated to antigen processing and presentation, cell adhesion and immunoregulation are more abundant in IFN-γ-licensed MSCs than in untreated cells. In this publication, not only the classical immunoregulatory proteins, such as IDO, ICAM-1 and VCAM-1 were differentially expressed, but less discussed proteins, such as PTGS2, PTGIS, FAM20A, FAM 20C and LRRC32/ GARP [109].

To summarize, there is a growing body of evidence regarding the increased potency of IFN-γ-licensed MSCs to control the immune response. However, other studies are necessary to better clarify the mechanisms involved in the immunosuppression promoted by IFN-γ licensed MSCs, as well as the clinical relevance of this strategy.

3.2. Do other inflammatory factors contribute to the licensing effect promoted by IFN-γ?

Considering the complexity of the immune response, which involves a wide variety of growth factors and cytokines, a series of studies were performed, seeking to associate other factors with IFN-γ, in the attempt to enhance the potentialities of MSCs. The main cytokines investigated at this point are TNF-α, IL-1α, IL-1β and IL-17. Considering TNF-α, IL-1α and IL-1β, even though they failed to enhance MSC immunomodulatory properties when used solely, after combination with IFN-γ, a synergistic effect was detected [110]. The combination of IL-1α and IL-1β also led to higher G-CSF release, a decrease of proinflammatory markers and increased production of anti-inflammatory factors by MSCs [111].

Currently, TNF-α is the main inflammatory factor investigated as adjuvant of IFN-γ in the context of MSC licensing. Treating MSCs with a combination of IFN-γ and TNF-α renders MSCs more suppressive [112], increases the expression of programed death ligand 2 (PDL2) and stimulates the generation of Treg subsets by MSCs [113]. Interestingly, using microarray and single cell qRT-PCR, Ping Jin and colleagues showed that the combination of IFN-γ and TNF-α polarized MSCs to a uniform Th1 phenotype and induced the expression of the immunosuppressive molecules IL-4, IL10, IDO and CD274/PD-L1 [114]. Recently, an additional immunoregulatory mechanism has been revealed in this scenario, which is the production of soluble PDL1 and PDL2 induced by IFN-γ and TNF-α-licensing of MSCs. Such soluble compounds suppress the activation status and effector function of T-cells [85,114].

A possible role of IL-17 as another MSC-licensing promoter is currently under investigation. Since IL-17 is one of the most potent proinflammatory cytokines, it was hypothesized that it might lead to superior results compared to IFN-γ when used to license MSCs. Unexpectedly, IL-17 failed to enhance the immunosuppressive potential

Table 1
Effect of INF-γ licensing over Mesenchymal Stem Cells properties.

Cell type/ Source	Assay model	Dosage	Licensing duration	Observed Effects	Mechanism	Reference
eBM-MSCs	In vitro	100 ng/mL	24h	↓T cell proliferation	↑ MHC I and II expression, NOS2, IDO, PTGS2, IL-6, CCL2, CXCL10 and ↓ IL-8	[135]
hAD-MSCs	In vitro	50 ng/mL	48 h	↓T cell proliferation, Maintain MSCs Phenotype	↑ICAM-1, IDO, IL-1β, TNF-α, ↓GAL-1	[44]
hBM-MSCs hUC-MSCs	In vitro	10 ng /mL	120 h	↓T cell proliferation	↑ IDO, HLA-ABC, HLA-DR, Treg cells	[136]
hBM-MSCs	In vitro	10-50 ng/mL	24 h	Induce MSCs Morphological changes and ↑immunossupressive capacity	ND	[108]
hBM-MSCs	In vitro	20 ng/mL	48 h	MSCs Protection from NK cytotoxicity, ↓ NK cytolytic and degranulation ability	↑ IDO, HLA-ABC and HLA-E expression, ↑ PGE-2 secretion	[137]
hBM-MSCs	In vitro	20 ng/mL	48 h	↓T cell proliferation and degranulation, ↑ thawed MSC's survival and immunosuppressive properties	Preserves IDO protein expression and activity	[107]
hBM-MSCs	In vitro	30 ng/mL	20 h	↓T cell proliferation, ↑ Tregs generation	↑ VCAM-1, ICAM-1 PD-L1, IDO, BST-2	[138]
hBM-MSCs	In vitro	30 ng/mL	20h	↑ Antigen processing and presentation, Cell adhesion molecules and Arachidonic acid metabolism proteins expression	↑ VCAM-1, ICAM-1 PD-L1, IDO, BST-2	[109]
hBM-MSCs	In vitro	40 ng/mL	24 h	↑ glycolytic activity, ↓ mitochondrial electron transport activity, positively regulates ROS level, ↓T cell proliferation	↑ IDO, COX-2 expression, ↑ PGE-2 secretion	[139]
hBM-MSCs	In vitro	50 ng/mL	48 h	↓ IFN-g production and T cells degranulation	↑ MHC I and II, B7H1 and B7DC expression	[76]
hBM-MSCs	In vitro	200 ng/mL	24 h	↓T cell proliferation	↑ IDO, HGF, TGF-B1	[99]
hBM-MSCs	In vivo	500 IU/mL	48 h	↓ mice aGVHD-related weight loss, gut and liver cell infiltration, ↑ mice time survival, ↓ CD4 + T cells expansion	↓ TNF-α in serum	[103]
hBM-MSCs	In vitro	500 IU/mL	120 h	Induce MSCs Morphological changes, ↓T cell proliferation and activation	↑ IDO, MHC I and II, PD-L1, COX-1, ↓ PGE-2, INF-Ɣ, TNF-α, IL-2, IL-6 and IL-10 secretion	[117]
hPD-MSCs	In vitro	10-30 ng/mL	24 h	↓ MSCs proliferation and migration, ↑ Tregs generation	↑ IL-10, TGF-b and ↓ INF-Ɣ secretion	[106]
hBM-MSCs	In vitro	50 ng/mL	48 h	↓ NK activation and protection from NK cytotoxicity	↑ IDO and PGE-2	[140]
hWJ-MSCs hBM-MSCs hAD-MSCs hUC-MSCs	In vivo	200 IU/mL	24 h	↑ GvHD mice survival, ↓ clinical symptoms and immune cell infiltration into the skin and small intestine	ND	[104]
hWJ-MSCs hBM-MSCs hAD-MSCs hUC-MSCs	In vitro	200 IU/mL	24 h	↓ PBMC proliferation	↑ IDO, CXCL9, CXCL10, CCl8	[104]
hWJ-MSCs	In vitro	1000 IU/mL	24 h	↓T cell proliferation	ND	[141]
mBM-MSCs	In vivo	2 ng/mL	24 h	Loss of suppressive activity	↑ MHC I and II, CCl2, INF-Ɣ and IL-17	[102]
mBM-MSCs	In vivo	500 IU/mL	ND	↑ GvHD mice survival	ND	[101]

e- equine; h-Human; m-murine; AD-MSC – Adipose Derived Mesenchymal Stem cells; BM-MSCs – Bone Marrow Mesenchymal Stem Cells; PD-MSCs – Placenta Derived Mesenchymal Stem Cells; UC-MSCs – Umbilical Cord Mesenchymal Stem Cells; WJ-MSCs – Wharton's Jelly Mesenchymal Stem Cells; PBMC – Peripheral Blood Mononuclear Cells; GvHD – Graft-versus-host disease; ND - not described in the manuscript.

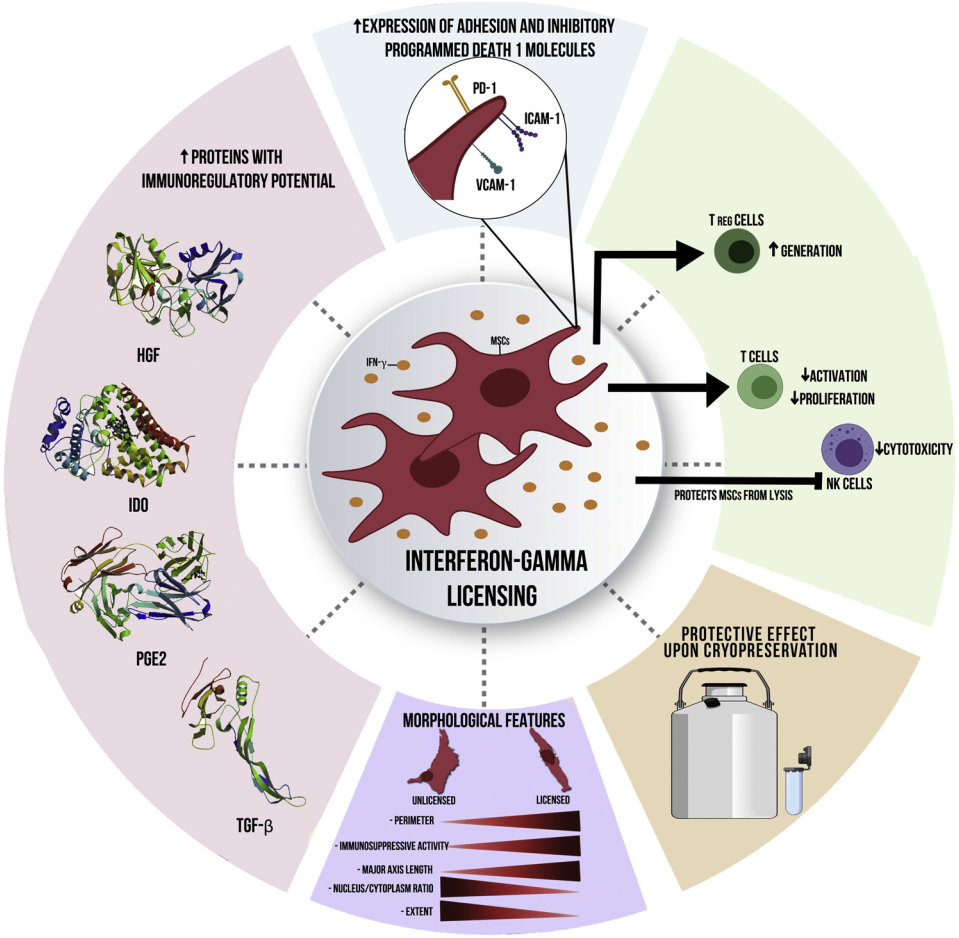

Fig. 1. Effects of IFN-γ licensing over MSCs. IFN-γ licensing induces increased expression of adhesion molecules on MSC surface, stimulate the secretion of immunoregulatory molecules, favor immunomodulatory phenotype identified by morphological aspects of these cells, promotes a protective effect upon cryopreservation and boosts the immunomodulatory effects of these cells over immune cells.

of murine MSCs when used alone. Nevertheless, when used in combination with IFN-γ and TNF-α, it stimulated the expression of inducible nitric oxide synthase (iNOS) and superior immunosuppression by MSCs [115]. Interestingly, in human cells, licensing with IL-17 enhanced the immunosuppressive potential and induced the generation of Tregs. In this context, it is important to mention that an advantage has been attributed to IL-17 as a strategy for the licensing of MSCs, because, unlike IFN-γ, this cytokine does not induce alteration in the expression of HLA and costimulatory molecules [116,117].

4. Licensed MSCs and challenges for clinical application

Considering the knowledge acquired during the last two decades or so, it is rather surprising to acknowledge that even though more than 600 trials have been performed using MSCs, less than 20 of them were industry sponsored phase III trials (clinicaltrials.gov). Therefore, this field is actually quite young and inexperienced. Even though the field of MSC therapy has faced many challenges, it has also progressively evolved, speaking to the important lessons which have been learned. Nevertheless, the challenge of fulfilling the clinical potential of MSCs, which was anticipated by preclinical research, is still to be overcome.

As in many different areas, knowledge regarding MSC biology and clinical potential derive from animal and *in vitro* experiments. Translation to the bedside, though, has been more challenging than expected. Several factors differ between the highly controlled and syngeneic scenario of preclinical animal research, compared to the almost chaotic clinical settings, as discussed before [118]. Along the way towards the clinical application of MSCs, the importance of several other factors has also arisen.

4.1. Cell dose and delivery optimization

The amount of stem cells used for therapy is still considered a challenge to be overcome, considering that, while an average of 50 million MSCs/kg are used in rodents, usually 1–2 million MSCs/kg are used in human trials [118]. In this scenario, increasing MSC dose in humans is simply proposed but hardly performed. MSC production costs, as well as scale up complexity, are impeditive for the commercial viability of significantly increasing the amount of cells infused into the patient [119,120]. Combined with the dosing challenge, comes another issue: how to infuse the MSCs into the patient. The choice of the route of stem cell delivery adds complexity into the treatment. Usually, two immediate alternatives present themselves in this sense: to deliver cells in the site of lesion, or to inject cells systemically. Even though delivering cells in the lesion site seems the most obvious option in order to potentialize MSCs therapeutic effects, the results from NCT01768702 clinical trial, which aimed to use MSCs to treat congestive heart fail, point to the fact that MSC injection *per se* may cause significant tissue damage [121]. Therefore, the option of delivering MSCs systemically may be advantageous in some scenarios, at least. On the other hand, after being delivered systemically, MSCs are rapidly trapped into the lungs, spleen, and liver [122], explaining why local application is sometimes preferred. There is no common sense at this point regarding how important it actually is for MSCs to be present at the injury site in

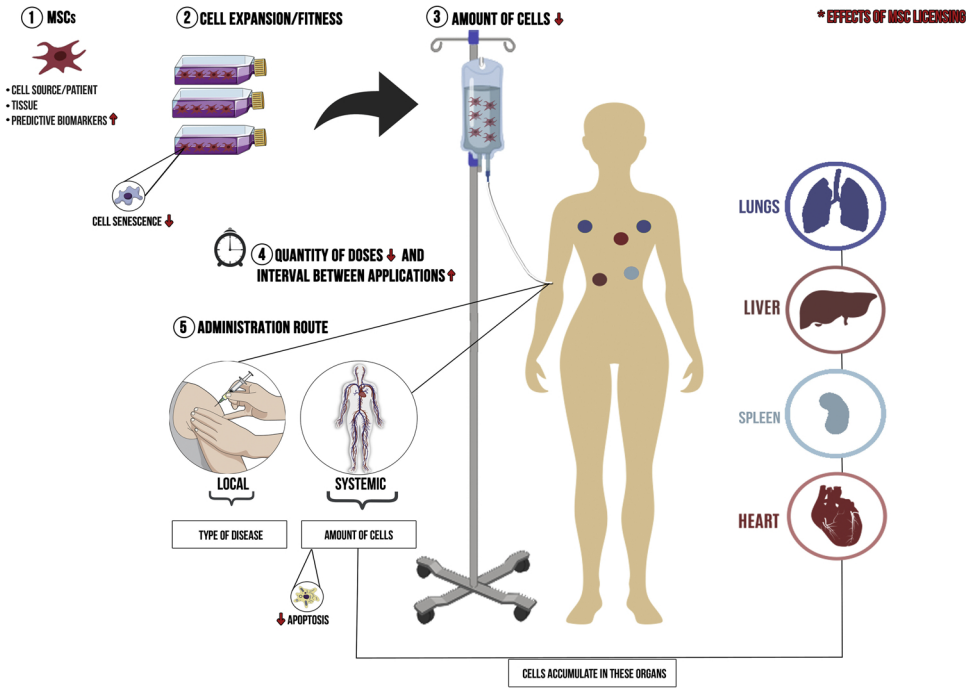

Fig. 2. Potential benefits of IFN-γ licensed MSCs in the clinical scenario. By optimizing MSC immunomodulatory properties, IFN-γ licensing may potentially increase predictive biomarkers of MSCs used for therapy, increase MSC fitness, promote cell survival following infusion, decreasing the number of cells required for therapy and even altering dose regimen.

order to promote therapeutic benefit. Conflicting data poses doubt regarding the real importance of the site of MSC injection, akin to the increasing recognition of paracrine signaling as a paramount mechanism of MSCs therapeutic effect. As shown by Braid et al. [123], intramuscular injected MSCs are still metabolically active after 100 days of injection and may be beneficial to treat remote lesions. More strikingly, Preda et al. [124] showed that subcutaneously injected MSCs are still protective to the injured myocardium. A safe partial conclusion at this point is that MSC dosing and delivery will have to be differently optimized for each clinical application. In this scenario, MSC licensing would be extremely beneficial, since it may decrease the amount of MSCs required for successful clinical application, facilitating commercial viability and delivery (Fig. 2).

4.2. MSC fitness

When therapeutic cells are injected in a diseased tissue, they frequently encounter a refractory *milieu*, and may face immunological response, lack of nutrition, apoptosis signaling, etc. Indeed, it has been observed that an important fraction of infused cells die soon after injection. Therefore, the use of healthy, and probably more resistant/resilient cells is of great interest in order to promote the highest therapeutic potential as possible. Even though dead/apoptotic MSCs exert immunomodulatory and regenerative effects, thawed cells are less potent than fresh cells, which are considered the fittest for *in vivo* application. Yet, the use of fresh cells significantly increases the complexity of the logistics involved in large scale trials and commercialization. Once again, MSC licensing with IFN-γ may contribute for MSC fitness, as revealed by Chinnadurai et al., who described that IFN-γ licensing decreases cryopreserved MSC susceptibility to lysis by activated T-cells [107].

4.3. MSCs predictive biomarkers

MSCs are currently recognized as a heterogeneous cell population, in which some pose better beneficial effects than others [125]. Accordingly, if only the highest fitness and most potent cells could be selected for patient treatment, it is anticipated that higher therapeutic effects would be obtained. Such a hypothesis has actually been tested using animal preclinical experiments, and proved to be largely correct [126,127].

In line with this conception, several aspects of MSCs are being considered in order to anticipate therapy success. Senescence status, CXCL5, IL-8, and VEGF secretion [127], TSG6 expression [128], TWIST1 expression [129]; hTERT mRNA (in hTERT-transfected cells) [130]; secretome content [44] are but a few parameters already correlated to superior MSC immunomodulation and/or therapeutic efficacy. Interestingly, the validation of such biomarkers enables the optimization of MSC treatment, in which only the cell batches with the best characteristics are infused to the patient. According to Boregowda and Phinney [131], different biomarkers may predict therapeutic outcome for different MSCs applications.

5. MSC licensing - the path ahead for clinical application

The search for the fittest and most efficient MSCs has promoted the development of *in vitro* treatments designed to boost MSC *in vivo* therapeutic potential. Priming, activation or licensing strategies are currently under development and go hand-in-hand with the concept of MSCs fitness, in the sense that treating MSCs *in vitro* prior to injection may increase the expression of fitness markers and, ultimately, may engender superior therapeutic outcome.

As aforementioned, the concept of MSC licensing emerged in 2006, when Krampera et al. revealed the importance of IFN-γ stimulation for MSC immunoregulatory function. Since the concept of licensing is very appealing, it is quite surprising that the clinical experience with licensed MSCs is still extremely limited. To the best of our knowledge, only one clinical experience has been published at this point. The clinical study was performed by Taddio and collaborators [132], and, surprisingly, was considered a failure. The patient which received the two infusions of 2.10^6 million cells/kg was suffering from a "case of refractory, drug-resistant, steroid-dependent childhood-onset" Crohn`s Disease. Initially, after licensed MSC treatment, the patient presented clinical evidence for amelioration, with an improvement in the Crohn's Disease Activity Index (CDAI) and indirect evidence obtained from patient`s blood samples. Despite these observations, though, the patient required rescue therapy with infliximab soon after cell therapy.

The lack of other clinical reports involving licensed MSCs may

involve different aspects. First, literature inconsistency may still provoke a resistance to perform clinical trials; second, it is important to bear in mind that MSC licensing imposes a significant increase in the manipulation process of the cells. Therefore, requiring additional layers of control prior to clinical experience.

Importantly, before letting the frustrated experience of Taddio and collaborators discourage further research, it is important to recognize that the choice of the patient may have significantly impacted outcome, as discussed before [118]. Previous experience has shown that the field must evolve in this sense. For instance, among GvHD patients, *in vitro* behavior of patient T- and NK cells was partially correlated with therapeutic outcome of MSC therapy [133]. Also for GvHD treatment, children respond better than adults for Prochymal treatment [134], and also specific GvHD types are more amenable to treatment than others. Therefore, the report by Taddio and coauthors [132] may be but a first lesson in the pathway towards the successful clinical application of licensed MSCs for immune diseases.

6. Conclusions and perspectives

Important progress has been made to optimize MSC treatment for immune diseases. In this context, the prominence of IFN-γ as an effective strategy to boost MSC immunomodulatory properties is beyond dispute. Nevertheless, there is still a long path ahead and numerous aspects to be improved considering several aspects of clinical application of MSCs, including MSC-, patient- and clinical study design-related aspects. Considering the rapid development of MSC therapy over the last 20 years, clinical experience with licensed MSCs is set to expand. The results of such efforts are eagerly anticipated, as they will likely build ground for an exciting generation of licensed MSCs, endorsing licensing as an effective strategy to pave the way for growth and innovation in the cell therapy industry.

Declarations of interest

None.

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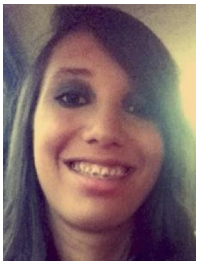

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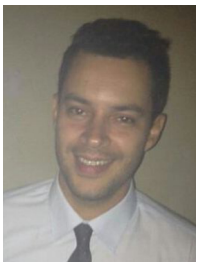

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