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Mesenchymal stem cells and innate tolerance: biology and clinical applications

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Summary

The properties of mesenchymal stem cells (MSC) have been widely investigated during the last decade, from their differentiation capacity to their immunosuppressive effect on any type of immune cell. These properties have been successfully harnessed for the treatment of inflammatory diseases such as graft versus host disease (GvHD). Different mechanisms have been proposed for their immunosuppressive properties, although it seems likely that they are used in concert. The inflammatory environment to which MSC are exposed plays a pivotal role in activating their functions.

Conversely, the interplay of MSC with the immunoregulatory networks recruited during inflammation is fundamental to the delivery of immunosuppression. Since other types of terminally differentiated stromal cells share these properties, it is plausible that stemness is not a required feature. Therefore these functions may be involved in the physiological control of acute inflammation in various tissues. These notions highlight the importance of investigating the role of stromal cells as modulators of immune responses.

Mesenchymal stem cells (MSC) are multipotent progenitor cells of stromal origin, originally isolated from adult bone marrow and subsequently from other tissues including in both adult and foetal life [1–9]. Even though MSC are defined according to their ability to differentiate into various tissues of mesodermal origin (osteocytes, chondrocytes, adipocytes), there are unconfirmed reports that they can also differentiate into endothelial cells [10], as well as neural cells [11], and cells of endodermal origin [12].

The identification of MSC with the use of specific markers remains elusive. They are commonly described as expressing CD73, CD105, CD90 and negative for the haematopoietic (CD45) and vascular (CD31) markers [13]. In mouse MSC markers of embryonic origin such as SSCA-1 [14] and SSCA-4 [15] have been identified, but more recently the co-expression of PDGFRα and Sca-1 [16] appears to be particularly effective at selectively identifying MSC because the vast majority of cells with progenitor activity resides in this subset. The efforts at detecting markers of human MSC have not delivered consistent results, but have indicated that they may preferentially express markers of neuronal lineage like low-affinity nerve growth factor receptor-1 (LNFGR1) [17] and ganglioside GD2 [18]. Although these markers have not been entirely confirmed, the notion of the neuroepithelial origin of MSC has recently been supported by an elegant study showing that $Sox1^+$ neuroepithelial cells supply the earliest wave of MSC differentiation during embryogenesis [19].

Pre-MSC type cells with characteristics of pluripotency have been isolated in the bone marrow or in foetal/perinatal tissues. Good examples are multipotent adult progenitor cells (MAPC), which differentiate into various lineages *in vitro* using defined cytokine combinations, and when transplanted they directly contribute to haematopoiesis *in vivo* and generate long-term repopulating haematopoietic stem cells and the full repertoire of haematopoietic progenitors [20].

The relative ease with which MSC can be isolated from adult tissues and the lack of ethical concern have probably been the main reason for their popularity. MSC have been successfully tested for their ability to protect from a variety of tissue injuries both in experimental [21–23] and clinical [24] settings.

Key words: *Mesenchymal; stem cells; immune responses; immunoregulation; graft-versus-host disease; autoimmune diseases*

The immunosuppressive activity of MSC

A further aspect that makes MSC of particular interest is the finding that they exert immunoregulatory activities. MSC from various species (humans, rodents and primates) can suppress the response of T cells to mitogenic and poly-

clonal stimuli [25, 26] and to their cognate peptide [27]. Such an effect is not cognate dependent because it can still be observed using MSC from third-party donors fully mismatched for the MHC haplotype of the responder T cells [28] or MSC which are constitutively negative for MHC molecule expression [27]. MSC-induced unresponsiveness lacks any selectivity, as it similarly affects memory and naïve T cells [27] as well as $CD4^+$ and $CD8^+$ subsets [29].

The characterisation of MSC-induced anergic T cells showed that the inhibitory effect of MSC is directed mainly at the level of T cell proliferation. T cells stimulated in the presence of MSC are arrested at the G1 phase as a result of cyclin D2 downregulation. The expression of CD25 and CD69 markers of T cell activation is completely unaffected by MSC co-culture, and inhibition of T cell effector functions can be reversed by MSC removal [29]. Whilst MSC induce an unresponsive T cell profile, they can prevent the apoptosis of activated T cells [30], indicating that MSC-mediated immunosuppression results from an induced division arrest anergy.

The effects of MSC on immune responses are not confined to T cells. Although they are susceptible to recognition and lysis by IL-2 activated cells and natural killer (NK) cells *in vitro*, due to their low expression of HLA class I [31, 32], MSC have been demonstrated to be capable of inhibiting the proliferation of interleukin-2 (IL-2) or IL-15 stimulated NK cells [31–33]. Whilst there is agreement on the immunosuppressive ability of MSC on NK cells, their influence on NK cell-mediated cytotoxicity remains controversial. Initial data suggested that MSC could inhibit the cytolytic activity of IL-2 activated NK cells [33], but more recent studies have shown that lysis of HLA I positive allogeneic targets by freshly isolated NK cells is not inhibited by MSC [31]. NK cells' cytokine production is also influenced by MSC, which are able to induce the release of IFN-γ [32, 34] and TNF-α [34].

The effect of MSC on B cell proliferation remains controversial. Studies in the mouse [29] and humans [35] showed that MSC inhibit B cell proliferation, inducing a block in G0/G1 phase of the cell cycle. MSC have also been shown to inhibit the differentiation of B cells to antibody secreting cells [35, 36] as well as downregulating CXCR4, CXCR5 and CCR7 chemokine receptors [35]. In contrast, other studies have suggested that human MSC promote the proliferation and differentiation of B cells from healthy donors and patients with systemic lupus erythematosus [37]. Although apparently in contradiction, the opposing results of these studies can be reconciled by the different conditions in which B cells have been stimulated. As a result of different B cell stimulation, the secreted cytokines could in fact polarise MSC towards a proinflammatory phenotype. This concept is well established for other cell types with regulatory functions, such as monocytes/macrophages [38].

The immunosuppressive properties of MSC can also target antigen-presenting cells (APC). The same effects exerted on cell cycle progression in T cells have been documented to affect monocytes [39]. MSC inhibit the differentiation of monocytes or $CD34^+$ haematopoietic progenitors into mature dendritic cells (DC) [40]. DC precursors exposed to MSC lose their ability to stimulate alloresponses and acquire regulatory features producing large amounts of interleukin-10 [41].

Besides their effect on immune cells, MSC exhibit an antiproliferative activity *in vitro* on different tumour cell lines, whereas *in vivo* they facilitate tumour engraftment and growth [42]. Since MSC contribute to the stem cell niche, these findings indicate that MSC can also provide the niche for cancer stem cells [43] and may influence the course of malignant diseases not only by creating an immunosuppressive environment within the tumour but also protecting tumour cells from apoptosis and facilitating its spread [44].

Mechanisms involved in the immunosuppressive effect

The mechanisms by which MSC exert their antiproliferative effect have still to be fully elucidated, although several candidate molecules have been proposed that are likely to act in concert and/or in alternate fashion depending on the environmental conditions to which MSC are exposed. Studies in both animal and human systems have shown that, although the effect requires an initial cell contact phase, the ultimate signal is mediated by several factors, which include transforming growth factor β-1 (TGF-β1) [26], indoleamine 2,3-dioxygenase (IDO) [45], prostaglandin E_2 (PGE_2) [41], nitric oxide (NO) [46], heme oxygenase-1 (HO-1) [47], and insulin-like growth factor-binding proteins [48].

The role of these molecules is different in the mouse and in humans, as in human MSC the effect of IDO is prominent, whereas in murine MSC NO seems to play a major role.

Transforming growth factor β-1 (TGF-β1) and hepatocyte growth factor (HGF) were the first molecules to be described as mediators of the immunosuppressive properties of MSC [26]. Recently it has been proposed that TGF-β gene expression is modulated in a contact-dependent mechanism by MSC [49].

Indoleamine 2,3-dioxygenase (IDO) is one of the immunosuppressive mechanisms believed to control T cell responses to autoantigens and alloantigens [50, 51], because its activity causes tryptophan depletion and kynurenine synthesis, capable of inhibiting the growth and function of immune cells by depleting nutrients and/or direct toxic activity of their catabolites [52]. IDO has been observed to be produced by MSC under inflammatory conditions such as exposure to IFNγ, and has been implicated in the inhibition of T-cell [45], NK-cell [32] and activated B-cell [33] proliferation.

Another mediator with immunosuppressive potential secreted by MSC is prostaglandin E_2 (PGE_2). Inhibitors of PGE_2 synthesis mitigate the overall human MSC suppressive effects [32, 41], with IDO as a synergistic partner [32]. It has been shown that MSC-derived PGE_2 is involved in skewing an inflammatory environment into an antiinflammatory environment, altering the cytokine secretion profile of dendritic cell subsets (DC1 and DC2) and T-cell subsets (Th1, Th2, or T_{regs}) [41].

Nitric oxide (NO) is synthesised by the inducible isoform of the NO synthase (iNOS), which is induced in MSC

by contact with activated $CD4^+$ or $CD8^+$ lymphocytes [46]. The proliferation of T cells is inhibited by NO-mediated suppression of phosphorylation of Stat5, a transcription factor crucial for T cell activation and proliferation [53]. Sato *et al.* have shown that $iNOS^{-/-}$ MSC are less effective in suppressing T cell proliferation than wild type MSC, and this is demonstrated also by the blocking effect of *N*-nitro-L-arginine methyl ester, a specific inhibitor of the iNOS, on MSC immunomodulatory potential [46].

It has also been shown that HLA-G, a nonclassical MHC class I, is involved in immunomodulation by MSC [54]. Dendritic cells, NK, and T cells present inhibitory receptors that interact with both the membrane-bound and the soluble isoform of HLA-G, which are expressed by human MSC. The soluble isoform HLA-G5, secreted by MSC after IL-10 stimulation, can inhibit the cytolitic activity of NK and $CD8^+$ T cells, shift the T cell response to a Th2 cytokine profile and induce the expansion of regulatory T cells [55]. Taken together these data suggest that all these factors may be involved, depending on the environmental conditions to which MSC are exposed, and are crucial for their antiproliferative activity.

Stem/progenitor cells exhibit a particularly active transcriptional activity that might account for the several properties described in undifferentiated cells, including immunosuppression [56]. However, it has been shown that the immunosuppressive effect of MSC is not a property confined to mesenchymal progenitor cells, but is rather a function exerted by most mesenchymal cells including those terminally differentiated. This has been demonstrated on primary articular human chondrocytes [57] and fibroblasts from synovial joints, lung and skin [30, 58].

All the immunosuppressive activities described so far are not a constitutive property of MSC. For the MSC to inhibit immune responses, they need to be "licensed". In fact, Jones and colleagues showed that only the supernatants obtained from co-cultures of stromal cells and activated T cells displayed an immunosuppressive effect when added to secondary cultures of proliferating T cells [30]. The immunosuppressive function of MSC is elicited by IFN-γ [33] and other proinflammatory cytokines such as TNF-α, IL-1α, or IL-1β [59].

MSC-mediated immunosuppression is not exclusively the result of a direct inhibitory effect but involves the recruitment of other regulatory networks. MSC act in concert with monocytes because the magnitude of the effect seems to be proportional to the number of monocytes in culture [60, 61]. Furthermore, MSC can also activate and expand regulatory T cells (T_{regs}) [62], although T_{regs} themselves are not required as a unique component to effect MSC immunosuppressive activity [27].

These findings suggest a crosstalk between MSC and the environment whereby first inflammatory monocytes 'license' MSC to acquire their immunosuppressive properties, and in turn MSC skew the inflammatory environment into an antiinflammatory environment both directly and through the effect on immunoregulatory circuits involving monocytes and T_{regs}.

Clinical applications

The tissue repair function and the inhibitory effect on the cell cycle on immune and nonimmune cells are properties that lend themselves to therapeutic exploitation, and there are a variety of disorders for which the use of MSC has been proposed. One of the first preclinical studies in baboons transplanted with allogeneic skin grafts showed that the *in vivo* administration of donor MSC to MHC-mismatched recipient prolonged the survival of third-party skin grafts [25]. On the same lines are the findings in islet transplantation. The use of MSC has been tested in a rat model of streptozotocin-induced diabetes with a view to producing haematopoietic chimerism in concomitance with allogeneic islet transplantation [63].

Further preclinical studies have shown that MSC could be successfully exploited in autoimmune diseases. MSC can ameliorate experimental autoimmune encephalomyelitis (EAE), reducing central nervous system inflammation and demyelination through the induction of peripheral T cell tolerance [64], and reduce the relapse rate of EAE impairing pathogenic T and B cell responses directed against the immunising antigen [65]. Whilst a first report on cell therapy for collagen induced arthritis (CIA) using MSC showed that their use was not beneficial in curing arthritis, suggesting that activation of the TNFα inflammatory pathway in the injured tissues might reverse their immunomodulatory effect [66], it has recently been demonstrated that a single injection of allogeneic MSC in a CIA model can prevent the occurrence of irreversible damage to bone and cartilage [67]. On the basis of their ability to skew the inflammatory environment to an antiinflammatory environment, MSC have also been investigated in sepsis [68] and colitis [69]. Activated MSC can induce *in vivo* the production of higher amounts of IL-10 from macrophages [68, 69] by releasing PGE_2, thus preventing neutrophils from migrating into tissues and causing oxidative damage [68].

The most studied therapeutic application for MSC is graft-versus-host disease (GvHD), a severe condition that develops after allogeneic haematopoietic stem cell transplantation (HSCT). The clinical efficacy of MSC in GvHD was initially observed in a 9-year-old boy suffering from steroid-resistant grade IV acute GvHD who received haploidentical third-party MSC [70]. Subsequently a phase II trial involving 55 patients with the same condition demonstrated that the infusion of MSC could significantly improve overall survival [71]. A previous multicentre phase I/II clinical trial in which MSC were given at the time of HSCT before any sign of GvHD produced a different outcome, no difference being observed in the incidence of GvHD between the group receiving MSC and the controls [72]. The discrepancies might be explained by the findings of the preclinical studies. Initial studies reported that a single infusion of MSC at the time of the transplant did not prevent the development of GvHD in MHC-mismatched donor-recipient pairs [73]. The work of Tisato and colleagues, although confirming the ineffectual activity of MSC injected in a single dose at the beginning of HSCT, showed that GvHD could be totally prevented by multiple doses [74]. Polchert *et al.* observed that MSC could significantly increase the survival rate of recipient mice only

when given at day +2 or +20 when IFN-γ levels are at their peak, MSC efficacy being dependent on the presence of IFN-γ in the environment [75]. Timing is therefore essential for MSC to exert their inhibitory effect, due to the need for the appropriate inflammatory environment to 'licence' the MSC. The role of IFN-γ [33, 59] and of the inflammatory environment [30] in activating MSC had also been previously described *in vitro*, as already discussed. Another suggested role for the inflammation derived from *in vivo* studies [23, 74, 76] is the recruitment of MSC. IFN-γ might be able to cause the accumulation of antigen-specific T cells at the site of inflammation by inducing MHC molecule expression on the endothelium [77]. IFN-γ could therefore promote accumulation of MSC and recruitment of antigen-specific T cells at the same site, retaining suppressive and effector cells in the same anatomical compartment [78].

Besides their use to modulate immune responses, MSC have been employed to promote tissue repair. It is likely that the antiproliferative and antiapoptotic activity of MSC on parenchymal cells [42] effect a cytoprotective action that preserves residual stem cells from further destruction thus favouring their recovery and spontaneous tissue repair. The anti-inflammatory activity also favours the generation of antiinflammatory macrophages, which are crucial for promotion of tissue repair [79]. MSC has therefore been used to promote the expansion and development of islet β-cells in diabetes therapy. Repeated transplantation of human MSC-induced repair of pancreatic islets and renal glomeruli in NOD/*scid* mice suffering from STZ-induced diabetes [80]. By coinjecting sex mismatched bone marrow cells (BMC) and syngeneic or allogeneic MSC it was possible to demonstrate that tissue repair was not the result of trans-differentiation but rather the consequence of an endogenous repair process initiated by the graft and the suppression of T cell-mediated immune response against newly formed β-cells by donor MSC [81]. A recent field of investigation is represented by the therapeutic potential of MSC in acute renal failure [82] in which a paracrine activity mediated by MSC appears to play the main role in tissue repair [23]. Similarly, in a model of bleomycin-induced pulmonary fibrosis, inflammation and collagen deposition were significantly reduced after MSC administration via a mechanism involving IL-1 receptor antagonist [22].

Conclusions

The immunosuppressive properties of MSC have aroused keen interest in the last few years. Current data indicate that MSC utilise a number of synergistic mechanisms to non-specifically control immune responses and activate further immunosuppressive circuits to boost MSC action. *In vitro* and *in vivo* studies have suggested that the inflammatory environment is crucial to enable MSC to exert their antiproliferative and antiapoptotic effects, thus highlighting the importance of dissecting the molecular features of the microenvironment to maximise the therapeutic impact. This notion is also important in improving our understanding of the function of tissue stroma as effector of innate tolerance to rapidly modulate immune response and, with overlapping mechanisms, to protect tissue progenitors from dying, activate their self-renewal programme and contribute to tissue repair.

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