Chapter 7
Immunomodulatory Properties of MSCs

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Abstract Mesenchymal stromal cells (MSCs) are multipotent cells that can be isolated from several human tissues and expanded *ex vivo* for clinical use. They comprise a heterogeneous population of cells, which, through production of growth factors, cell-to-cell interactions and secretion of matrix proteins, play a key role in the regulation of haematopoiesis. In recent years, several experimental studies have shown that MSCs are endowed with potent immunomodulatory properties directed in vitro at all cells involved in immune responses. Due to their immunomodulatory and engraftment-promoting properties, MSCs have been tested in the clinical setting both to facilitate haematopoietic engraftment and to treat steroid-resistant acute graft-versus-host disease (GvHD). More recently, experimental findings and clinical trials have focused on the ability of MSCs to home to injured tissues and to produce paracrine factors with anti-inflammatory properties, resulting in functional recovery of damaged tissues. The mechanisms through which MSCs exert this pleomorphic

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therapeutic potential rely on some key properties of these cells: the capacity to home to sites of injury, the ability to blunt exaggerated immune responses and the ability to secrete soluble factors capable of stimulating both the survival and recovery of injured cells. This chapter focuses on recent advances in MSC biology and summarises the clinical studies on their immunomodulatory and anti-inflammatory properties, particularly in the setting of allo- and autoimmune disorders.

Introduction

In addition to haematopoietic stem cells (HSCs), the bone marrow (BM) also contains mesenchymal stromal cells (MSCs). These cells were first recognised more than 40 years ago by Friedenstein et al. who described a population of adherent cells from the BM which were non-phagocytic, exhibited a fibroblast-like appearance and could differentiate in vitro into bone, cartilage, adipose tissue, tendon and muscle [1]. Moreover, after transplantation under the kidney capsule, these cells gave rise to the different connective tissue lineages [2]. Human MSCs were first identified in postnatal BM and later in a variety of other human tissues, including periosteum, muscle connective tissue, perichondrium, adipose tissue (AT), umbilical cord blood (UCB) and fetal tissues, amniotic fluid and placenta [1, 3–8]. One of the hallmarks of MSCs is their multipotency, defined as the ability to differentiate into several mesenchymal lineages [9]; usually trilineage differentiation into bone, adipose tissue and cartilage is taken as a criterion for multipotentiality. Recently, the existence of pluripotent cells has been reported that have the ability to differentiate into cells of the mesodermal lineage but also into endodermal and neuroectodermal cell types, including neurons, hepatocyte and endothelium [10–12]. MSCs have been also demonstrated to display chemotactic ability, to migrate to sites of inflammation and injury [13], as well as to secrete paracrine mediators able to reverse acute organ failure [14]. MSCs have been successfully used in repairing tissue injury, occurring after allogeneic haematopoietic stem cell transplantation (HSCT) [15]. In view of their immunosuppressive properties, as well as of their role in sustaining tissue repair and trophism, MSCs represent a promising tool in immunoregulatory and regenerative cell therapies [16, 17].

MSC *Ex Vivo* Expansion

Due to the low frequency of mesenchymal progenitors in human tissues, in vivo use of MSCs requires that the cells be extensively *ex vivo* manipulated to achieve the numbers that are necessary for their clinical application [18–20]. Standard conditions for *ex vivo* expansion of MSCs are based on the presence of 10% fetal bovine serum (FBS), and serum batches are routinely prescreened in order to guarantee both the optimal growth of MSCs and the biosafety of the cellular product [18–20].

However, the use of FBS raises concerns when utilised in clinical grade preparations, because it might theoretically be responsible for the transmission of zoonoses as well as cause immune reactions in the host, especially if repeated infusions are needed. This may lead to the risk of rejection of the transplanted cells [21, 22]. In view of these considerations, serum-free media, appropriate for extensive expansion and devoid of the risks connected with the use of animal products, are being developed.

Both autologous and allogeneic human serums have been tested for in vitro expansion of MSCs [23]; several serum-free media, based on the use of cytokines and growth factors, such as basic fibroblast growth factor (b-FGF) and transforming growth factor β (TGF-β), have been proposed in experimental conditions [24, 25]. Platelet lysate (PL) has been demonstrated to be a powerful substitute for FBS in MSC expansion, especially in terms of cell growth due to its high concentration of *natural* growth factors [26–28]. Doucet et al. first demonstrated that the growth factors contained in PL are able to promote MSC expansion in a dose-dependent manner [26]. This was further substantiated by data published by other groups, showing that culture medium with 5% PL added is superior to 10% FBS in terms of clonogenic efficiency and proliferative capacity of MSCs, while preserving MSC immunomodulatory functions [27, 28]. It has, however, to be emphasised that clinical data on the safety and efficacy of MSCs have been obtained, so far, mainly with cells expanded in the presence of FBS, whereas relatively little in vivo experience is available with MSCs cultured in alternative medium supplements. Therefore, cells expanded in the presence of alternative expansion media require extensive experimental and clinical testing before being safely and effectively employed to substitute cells generated in the presence of FBS-based media.

MSC Surface Markers and Prospective Isolation

Little is known about the characteristics of the primary mesenchymal precursors in vivo; this has been mainly due to the inability to prospectively isolate the most primitive mesenchymal cells from bulk cultures because of their low frequency and the lack of specific markers. To date, MSC isolation/identification has relied mainly on morphology and adherence to plastic; immunophenotyping by flow cytometry has been applied to identify *ex vivo*-expanded MSCs and to define purity. No specific marker has been shown to identify true MSCs, and *ex vivo*-expanded cells are characterised by a combination of both positive (CD105, CD73, CD90, HLA class I) and negative (CD34, CD45, CD14, CD31) markers [9, 29], at least in case of BM-derived cells (indeed, a proportion of AT-derived MSCs express CD34) [5, 9, 29].

Recently, the identification and prospective isolation of mesenchymal progenitors, both in murine and human adult BM, have been reported, based on the expression of specific markers [30–40]. Anjos-zfonso et al. have reported the identification, isolation and characterisation of a population of multipotent mesenchymal cells in

murine BM, based on the expression of the stage-specific embryonic antigen-1 (SSEA-1) [30]. In human cells, with the aim to prospectively isolate MSCs, surface markers such as SSEA-4, STRO-1, the low affinity nerve growth factor receptor (CD271) and MCAM/CD146 (melanoma cell adhesion molecule) [31–38] have been employed. Battula et al. have recently isolated by flow cytometry MSCs from human BM, using antibodies directed against the surface antigens CD271, mesenchymal stem cell antigen-1 (MSCA-1), CD56 and SSEA-3, and identified novel MSC subsets with distinct phenotypic and functional properties [38, 39]. In particular, CD271, which has been employed for prospective isolation of MSCs from BM, has been reported to define a subset of MSCs with immunosuppressive and lympho-haematopoietic engraftment-promoting properties in vivo [35]. Moreover, it has been shown that only $CD271^{bright}$, but not $CD271^{dim}$, cells give rise to clonogenic MSCs and these populations differ considerably in their morphological appearance [34, 35, 39]. Similarly, a 100-fold enrichment in fibroblast colony-forming cells (CFU-F) was found in the STRO-1^{+} population in the bone marrow [33]. MCAM/CD146 molecule, which has been shown to allow for CFU-F enrichment, was expressed on both MSCs and pericytes [37]. A STRO-4 monoclonal antibody has been demonstrated to be specific for mesenchymal precursors cells from human and ovine tissues, being capable of providing enrichment in CFU-F when employed for MSC isolation from BM [40].

Despite the identification of these new MSC markers [30–40], none presently available has demonstrated to be, by itself, capable of identifying the true mesenchymal stem cell. Whether culture-expanded MSCs differ from their progenitors in vivo is uncertain, as proliferation on plastic surfaces and culture conditions may induce both phenotypic and functional changes. Future research should focus on the identification of MSC-specific markers which will hopefully allow to dissect the developmental hierarchy of MSCs and facilitate the generation of homogenous cellular products.

MSC Tissue Sources for Clinical Use

As previously mentioned, MSCs, after their first identification in BM [1], have been isolated from a variety of other human tissues. Although similar MSCs can be cultured from different fetal and adult tissues [3–8], clinical experience has been mainly gained with *ex vivo*-expanded BM-derived cells; only few studies have employed different sources, such as AT [41]. The frequency of mesenchymal progenitors, their proliferative capacities and differentiation potential, as well as their immunophenotype and immunomodulatory properties have been shown to vary in different sources [42, 43]. Intrinsic diversities of MSCs residing in a tissue, as well as their physiological role in that tissue, might influence the properties of a specific source, as compared to other MSC sources. The frequency of cells with lineage-specific differentiation capacity may differ between tissue sources, and therefore, MSCs with the ability to differentiate into bone-forming cells might be present with higher

frequency in BM rather than in fetal lung or placenta. Also the culture conditions employed for MSC *ex vivo* expansion might influence their biological properties, leading to the commitment of MSCs towards a specific function or cell lineage.

These differences should be taken into account when considering the clinical application of MSCs in the various clinical settings, together with the method of collection from a specific tissue (invasive procedure for BM and AT vs. noninvasive collection of UCB) and their isolation efficiency (100% success rate when isolating MSCs from BM and AT vs. 20–63% success rate when culturing MSCs from UCB) [44–47]. Whether one specific source might be more useful in a defined clinical setting depending on its biological and functional properties needs to be further investigated.

Safety Data on Malignant Transformation

It has been suggested that *ex vivo* manipulation of both human and murine MSCs may alter the functional and biological properties of the cells, leading to the accumulation of genetic alterations [48–52]. A high susceptibility to malignant transformation was also reported in murine BM-derived MSCs by different groups [50, 52]. Other researchers, using human MSC, did not confirm a propensity to develop morphological and genetic changes [27, 53–55]. In particular, both BM- and UCB-derived human MSCs, expanded in the presence of FBS or PL, could be safely cultured for long term without losing their phenotypical and functional characteristics and without showing the presence of chromosomal abnormalities [27, 53, 54]. French researchers have reported the presence of aneuploidy in a number of MSC preparations for clinical use; by further characterising these genetic abnormalities, they found that these alterations were not related to cell transformation, but rather to senescence of the cells [55]. While earlier reports indicated that human AT- and BM-derived MSCs are prone to undergo malignant transformation after long-term *ex vivo* expansion [48, 51], recently, it was shown that the tumour cells that they had described were unrelated to the original MSCs and were derived from contaminating tumour cell lines in these laboratories [56, 57]. Human bone marrow-derived MSCs have been long term expanded until senescence or until independent clones emerged. These cultures represented 8–15 passages and 33–55 population doublings, and no independent clones emerged. The likelihood of malignant transformations was estimated to be $<10^{-9}$. Altogether these data indicate that under the commonly used culture conditions, tumorigenesis is likely to be an extremely uncommon event [58]. This incident highlights the risk of cross-contamination and emphasises on the importance of cell line verification with DNA fingerprinting.

In light of these observations, phenotypic, functional and genetic assays, although known to have limited sensitivity, should be routinely performed on MSCs before in vivo use to verify whether the clinical application of *ex vivo*-expanded MSCs is safe.

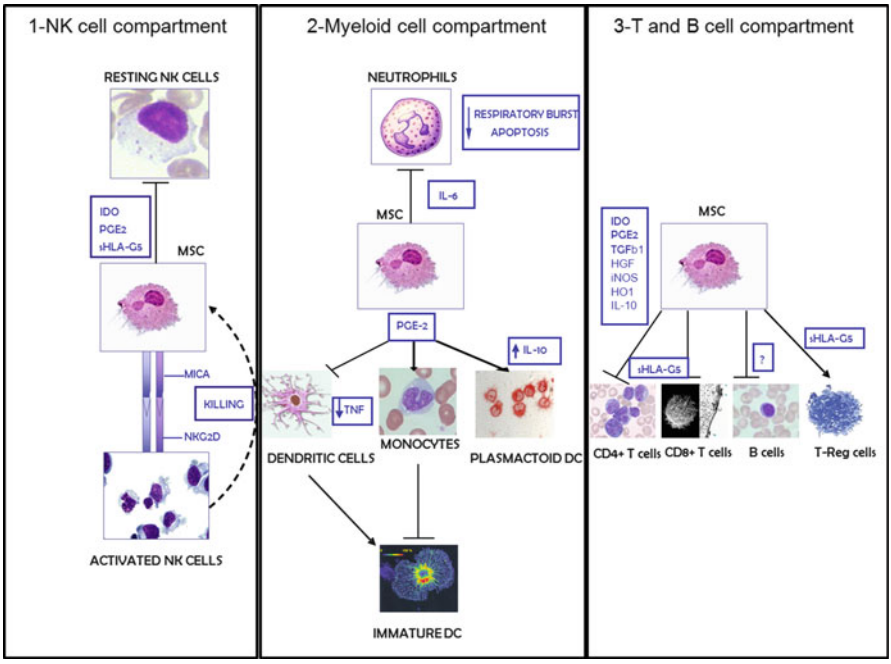

Fig. 7.1 Possible mechanisms of interaction between MSCs and cells of the immune system. (1) Mesenchymal stromal cells (*MSCs*) can inhibit the proliferation and cytotoxicity of resting natural killer (*NK*) cells; these effects are mediated by indolamine 2,3-dioxygenase (*IDO*), prostaglandin E2 (*PGE2*) and soluble HLA-G5 (*sHLA-G5*) released by *MSCs*. Killing of MSCs by cytokine-activated NK cells involves the engagement of activating receptors expressed by NK cells and of their ligands expressed by MSCs. (2) MSCs inhibit the differentiation of monocytes into immature myeloid dendritic cells (*DCs*), skew mature DCs to an immature DC state, inhibit tumour necrosis factor (*TNF*) production by DCs and increase *IL-10* production by plasmacytoid DCs. MSC-derived PGE2 is involved in all these effects. MSCs dampen the respiratory burst and delay the spontaneous apoptosis of neutrophils by constitutively releasing *IL-6*. (3) Direct inhibition of $CD4^+$ T-cell function depends on the release by MSCs of soluble molecules (IDO, PGE2, transforming growth factor-b1 (*TGFb1*), hepatocyte growth factor (*HGF*), inducible nitric oxide synthase (*iNOS*), heme oxygenase-1 (*HO-1*) and IL-10). MSCs inhibit $CD8^+$ T-cell cytotoxicity and stimulate the differentiation of regulatory T cells a.o. through the production of sHLA-G5. MSC-mediated inhibition of B-cell function involves both cell-to-cell contact and soluble mediators. → indicates a stimulatory effect; ⊥ indicates an inhibitory effect (Adapted from Uccelli et al. [62])

Immunomodulatory Properties of MSCs In Vitro

MSCs display broad and potent immunomodulatory properties that have been first demonstrated in vitro and, subsequently, in vivo both in animal models and in humans. Initially, most studies focused on the effects of MSCs on T lymphocytes; however, it is now evident that these cells display their effects on other cells involved in immune response, including B lymphocytes, dendritic cells (DCs) and natural killer (NK) cells [59–61]. See Fig. 7.1.

MSCs and T Cells

MSCs were first demonstrated to suppress in vitro T lymphocyte proliferation induced by alloantigens [63], mitogens [64], CD3 and CD28 agonist antibodies [65, 66]. MSCs have been reported to inhibit the effects of cytotoxic T cells (CTLs), probably due to suppression of CTL proliferation [67]. The inhibition of T-cell proliferation and cytotoxicity mediated by MSCs is not HLA restricted; in fact, MSCs are able to induce a similar degree of inhibition in the presence of both autologous and allogeneic responder cells [63, 66]. This observation supports the concept that MSCs can be considered *universal suppressors*. Since the separation of MSCs and PBMCs by transwell experiments does not completely abrogate the suppressive effect, most human MSC-mediated immunosuppression on activated T lymphocyte has been attributed to the secretion of antiproliferative soluble factors, such as TGF-β, hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO, an enzyme causing depletion of tryptophan, an essential factor for lymphocyte proliferation), nitric oxide (NO), heme oxygenase-1 (HO-1) and interleukin (IL)-10 [59–61, 68]. In particular, more recently, the stress-inducible enzyme HO-1 which is able to exert potent anti-inflammatory, anti-oxidative and anti-apoptotic activities has been found to be expressed in rat and human MSCs and to be involved in MSC-mediated immunosuppression [69]. However, published data do not exclude that a part of the immunosuppressive effect exerted by human MSCs on alloantigen-induced T-cell activation be dependent on cell-to-cell contact mechanisms. Of interest, the calcineurin inhibitors, cyclosporine-A and tacrolimus, employed to both prevent and treat graft-versus-host disease (GVHD), enhance the immunosuppressive effect of human MSCs, in particular the in vitro activation of alloantigen-specific and the T-cell-mediated cytotoxicity [70]. Some authors have shown that the unresponsiveness of T cells in the presence of MSCs is transient and that T-cell proliferation can be reinitiated after MSC removal [59, 64, 66]. Inhibition of lymphocyte proliferation by MSCs has not been associated with the induction of apoptosis, but is rather interpreted as due to inhibition of cell division, thus preventing T lymphocyte capacity to respond to antigenic triggers while maintaining these cells in a quiescent state [64, 66, 71]. Indeed, T cells in the presence of MSCs remain in the G0/G1 phase of the cell cycle, and this, at a molecular level, translates into the inhibition of cyclin D2 expression [71].

MSCs are also capable of inducing in vitro regulatory T cells (Treg), as demonstrated by the increase in the population of $CD4^+CD25^+FoxP3^+$ cells in mixed lymphocyte reactions (MLRs) in the presence of MSCs [72, 73]. Very recently, the cytoprotective enzyme HO-1, produced by MSCs, has been shown to promote differentiation of IL-10^+ Tr1 and TGF-β^+ Th3 Treg subsets in a MLR system, as well as to produce IL-10, a suppressive cytokine produced by regulatory cells [74]. It is conceivable that suppression of T-cell proliferation and induction of Tregs are related events.

MSCs and DCs

MSCs have been reported to interfere in vitro with DC differentiation, maturation and function. Differentiation of both monocytes and CD34+ progenitors into $CD1a^+$-DCs is inhibited in the presence of MSCs, and DCs generated in this latter condition are impaired in their function, in particular in their ability to induce activation of T cells [75, 76]. Transwell experiments have demonstrated that the suppressive effect of MSCs on DC differentiation is at least partly mediated by soluble factors, namely, IL-6, macrophage-colony stimulating factor (M-CSF), PGE2 and IL-10 [76].

Incubation of MSCs with mature DCs reduces the latter's expression of HLA class II and co-stimulatory molecules, inhibits TNF production and impairs antigen presentation, therefore favouring the induction of regulatory APCs through which they could indirectly suppress T-cell proliferation [75, 77]. Moreover, MSCs could act as non-professional antigen-presenting cells early in immune responses, in the presence of low levels of interferon γ (IFN-γ). However, the increase in the levels of INF- γ leads MSCs to later switch to their immune suppressive function [77, 78].

MSCs and B Cells

The ability of MSCs to inhibit B cell proliferation was first reported in murine studies [71]. Thereafter, human MSCs have been demonstrated to suppress in vitro the proliferation of B cells activated with anti-Ig antibodies, soluble CD40 ligand and cytokines, as well as to interfere with differentiation, antibody production and chemotactic behaviour of B lymphocytes [79]. Corcione et al. also demonstrated that MSCs do not induce apoptosis, but determine a block of B cells in the G0/G1 phases of the cell cycle, as already shown for T cells [79]. Krampera et al. have reported that MSCs are able to reduce the proliferation of B cells in vitro in the presence of IFN-γ, thanks to its ability to induce IDO activity by MSCs [80]. In contrast with these observations, Traggiai et al. have reported that BM-derived MSCs are able to promote proliferation and differentiation into immunoglobulin-secreting cells of transitional and *naive* B cells isolated from both healthy donors and paediatric patients with systemic lupus erythematosus (SLE) [81].

These conflicting in vitro results on MSC effect on B lymphocyte function/proliferation may partly reflect the differences in experimental conditions employed by the different authors, although, overall, the majority of reports suggest that in vivo B cell proliferation, as well as differentiation and expression of cytokines are inhibited by MSCs [79, 80]. Moreover, as T cells orchestrate B cell function, whatever be the ultimate effects of MSCs on B cell functions are, B cells are likely to be significantly influenced by the MSCs-mediated T-cell inhibition.

MSCs and NK Cells

It has been reported that MSCs are able to suppress NK-cell proliferation after stimulation with IL-2 or IL-15 [67, 82]. Indeed, while MSCs do not inhibit the lysis of freshly isolated NK cells [67], these latter cells when cultured for 4–5 days with IL-2 in the presence of MSCs display a reduced cytotoxic potential against K562 target cells [80]. Transwell experiments have suggested that the suppression of IL-15-driven NK-cell proliferation as well as of their cytokine production by MSCs is mediated by soluble factors [80, 82]. On the contrary, the inhibitory effect displayed by MSCs on NK-cell cytotoxicity required cell-cell contact [82].

Although MSCs were initially considered immunoprivileged and therefore capable of escaping lysis by freshly isolated NK cells [67], recent experiments have demonstrated that IL-2 activated both autologous and allogeneic NK cells are capable of effectively lysing MSCs [83]. Although MSCs express normal levels of MHC class I that should protect against NK-mediated killing, they display ligands that are recognised by activating NK receptors that, in turn, trigger NK alloreactivity [83]. Moreover, it has been recently demonstrated that MSCs can be lysed also by cytotoxic T lymphocytes or antibodies, when infused into MHC-mismatched mice, resulting in their rejection [84].

MSC Mechanisms of Action In Vitro

Several studies have demonstrated that MSCs, in vitro, are capable of modulating the function of different cells active in the immune response, although a clear view of MSC mechanisms of action has yet to be obtained. Cell-cell contact and soluble factors are thought to be required for the induction of MSC-mediated immunosuppression [62]. Primary contact between MSCs and the target cell is initiated by adhesion molecules [85]. Most studies demonstrate that soluble factors are involved, as the separation of MSCs and peripheral blood mononuclear cells (PBMCs) by a transwell permeable membrane does not prevent the inhibition of proliferation [64]. It has been demonstrated that MSCs release several soluble molecules either constitutively or following crosstalk with other cells [62]; these include TGF-β, PGE2, IDO, IL-10, NO and HO-1 [59–61, 68, 69]. Release of IFN-γ by target cells induces the release of IDO by MSCs, which, in turn, depletes tryptophan, an essential amino acid for lymphocyte proliferation [80, 86]. IDO is necessary to inhibit proliferation of Th1 cells and, together with PGE2, inhibits NK-cell function [68, 80]. IFN-γ can, in a murine model where pro-inflammatory cytokines are added, stimulate chemokine production by MSCs, resulting in T-cell attraction and increased inducible NO synthase (iNOS) [87]. T cells are inhibited by the subsequent production of NO [88]. Moreover, cytokines produced by target cells can increase the release of some of these MSC-derived soluble factors [62]. Soluble HLA-G5 (sHLA-G5) released by MSCs suppresses T-cell proliferation, as well as $CD8^+$ T cell and NK-cell cytotoxicity [88]. Conversely, MSCs through the release of sHLA-G5 initiate the up-regulation

of $CD4^+CD25^+FoxP3^+$ cells [88, 89], although their depletion has no effect on the inhibition of T-cell proliferation by MSCs [90].

The complexity and mechanisms whereby MSCs interact with cells of both the adaptive and innate immune system are schematically represented in Fig. 7.1. Whether these effects are displayed through real suppression of immune responses or through a nonspecific antiproliferative effect is still unclear. The mechanisms by which MSCs display their immunosuppressive effect are largely restricted to in vitro studies. The in vivo biological relevance of the in vitro observations needs to be addressed in appropriate in vivo models.

The Importance of Host Factors: Pro-inflammatory Environment

The clinical potential of MSCs might be also influenced by host factors; it has been suggested that MSCs need to be activated in the host environment in order to mediate their immunomodulatory effect [91]. In this sense, MSCs are not constitutively inhibitory, but they acquire their immunosuppressive functions after being exposed to an inflammatory environment [91]. This became clear after the observation that anti-IFN-γ receptor antibodies can block the suppressive effect of MSCs. The various techniques employed to activate an immune response in vitro may involve the release of IFN-γ which, in turn, activates the immunosuppressive activity of MSCs [80, 92]. Moreover, the level of IFN-γ and the contemporary presence of other inflammatory cytokines, such as TNF-α and IL-1ß, can influence the immunosuppressive effect of MSCs, as well as induce changes in their immunophenotype [93]. Indeed, IFN-γ, TNF-α or IL-1ß are able to induce the up-regulation of HLA class I. How about HLA-II? ICAM-1 and VCAM-1 on MSC surface, while IFN-γ alone can induce the activity of IDO [93]. Different cytokine combinations, and consequently the heterogeneity in the host environment, can produce different effects on MSC function; this may explain the variability of response that is observed in patients enrolled in clinical trials and treated with MSCs.

MSCs also express a large number of toll-like receptors (TLRs), and their stimulation has been shown to affect MSC immunomodulatory properties [94]. TLRs are non-catalytic receptors that recognise molecules derived from microbes and mediate the activation of immune responses of both innate and adaptive immunity [95]. In analogy with the functional status of monocytes/macrophages, Waterman et al., by using a short-term TLR-priming protocol, identified two functionally different MSC populations: the TLR4-primed MSC population which exhibits a pro-inflammatory profile (MSC1) and the TLR3-primed MSC population which delivers immunosuppressive signals (MSC2). In accordance with this theory, T-cell inhibition is achieved only in case of co-culture with MSC2, whereas T-lymphocyte activation takes place following MSC1 co-culture [96].

MSCs may exert direct antiproliferative effects on T cells, NK cells and B cells and in this way direct suppress effector immune mechanisms. At the same time, they may exert indirect modulatory activities by inducing tolerogenic immune

responses though the induction of regulatory T cell and tolerogenic dendritic cells. A pro-inflammatory environment may lead to the activation of MSC and may be critical for the induction of suppressive mediators.

Based on these findings which underline the importance of host factors, it has been proposed to mimic the in vivo pro-inflammatory environment by activating MSCs in vitro with the addition of cytokines and to use these activated cells for the treatment of allo- and autoimmune disorders, as well as in the repair of tissue damage.

Immunomodulatory Properties of MSCs In Vivo in Animal Models

The immunomodulatory and reparative/anti-inflammatory properties of MSCs have been tested in a variety of animal models (see Table 7.1).

Animal Models of HSC Engraftment

MSCs have been reported to secrete cytokines important for haematopoiesis and to promote engraftment of haematopoietic stem cells (HSCs) in experimental animal models, especially when the dose of transplanted HSCs was low [97, 98]. Systemic infusion of allogeneic BM-derived MSCs from baboons has been demonstrated to suppress lymphocyte proliferation and prolong the survival of allogeneic skin grafts, as compared to animals not receiving MSCs [122]. Almeida-Porada et al. observed that co-transplantation of human MSCs into pre-immune fetal sheep resulted in enhanced long-term engraftment of human cells in the BM and in higher levels of donor cells in the circulation [97]. Another study performed in NOD/SCID mice demonstrated that co-infusion of fetal lung-derived MSCs and cord blood-derived $CD34^+$ cells is associated with enhancement of engraftment of human HSCs in the BM of the animals, the effect being particularly evident when relatively low doses of HSCs were transplanted [98]. In NOD/SCID mice, co-transplantation of placenta-derived MSCs resulted in both enhanced engraftment of double umbilical cord blood transplantation (UCBT) and reduced single cord predominance [99]. In non-human primates, co-transplantation of MSCs improved HSC engraftment after autologous intra-BM transplantation, and this was associated with increased chimerism in the peripheral blood [100]. Kuci et al. demonstrated that CD271-positive MSCs were capable of promoting significantly greater lymphoid engraftment, as compared to an unselected population of plastic-adherent MSCs, when co-transplanted with CD133+ HSCs in NOD/SCID mice [35].

It has been demonstrated that allogeneic MSCs are not intrinsically immunoprivileged, since, under appropriate conditions, they can induce an immune response, resulting in their rejection when infused into MHC-mismatched mice [84]. In contrast, infusion of syngeneic host-derived MSCs resulted, in the same model, in enhanced engraftment of allogeneic haematopoietic cells [84]. These observations

Table 7.1 Immunomodulatory properties of MSCs in vivo in animal models

Animal model	Outcome	Ref. no.
Part A: HSC engraftment		
Pre-immune fetal sheep	Enhancement of human HSC engraftment	[97]
NOD-SCID mouse	Enhancement of human HSC engraftment	[98]
NOD-SCID mouse	Enhanced engraftment of double UCBT, reduced single-donor predominance	[99]
Non-human primate	Enhancement of autologous HSC engraftment	[100]
NOD-SCID mouse	Promoted lymphoid engraftment	[35]
MHC mismatched mouse	Promotion of graft rejection	[84]
MHC mismatched mouse	Promotion of engraftment	[84]
Part B: GvHD		
NOD-SCID mouse (MSC at weekly intervals)	Prevention of GvHD; mice increased survival	[101]
NOD-SCID mouse (1 MSC dose, d + 0 after Tx)	No effect on GvHD prevention	[102]
NOD-SCID mouse (MSC at weekly intervals)	Prevention of GvHD	[103]
NOD-SCID mouse (1 MSC dose, d + 2 or d + 20)	Prevention of GvHD; mice increased survival	[104]
Part C: AID and regenerative medicine		
Mouse, EAE	Prevention of EAE development	[105, 106]
Mouse, SLE	Ameliorated signs and symptoms of SLE	[107]
Mouse, STZ diabetes	Ameliorated diabetes and kidney disease	[108–110]
Rat, glomerulonephritis	Stimulated glomerular healing	[111]
Mouse, AKI	Ameliorated renal function and tubular cell injury	[112, 113]
Rat, experimental colitis	Stimulated intestinal mucosa healing	[114–116]
Rat, acute hepatic failure	Protected against hepatic injury	[14, 117]
Mouse, rat, pig, myocardial infarction	Improved cardiac function	[118–120]
Mouse, CIA	No beneficial effect; accentuation of Th1 response	[121]

HSC haematopoietic stem cells, *MSC* mesenchymal stromal cells, *NOD-SCID* nonobese diabetic severe combined immunodeficient mice, *UCBT* umbilical cord blood transplantation, *GvHD* graft-versus-host disease, *Tx* transplantation, *AID* autoimmune diseases, *EAE* experimental autoimmune encephalomyelitis, *SLE* systemic lupus erythematosus, *STZ* streptozotocin, *AKI* acute kidney injury, *CIA* collagen-induced arthritis, *Ref. n.* reference number

suggest that MSCs may promote engraftment, provided that they survive in vivo and are not rejected as the result of an alloimmune response. See also Table 7.1, part A.

Animal Models of Graft-Versus-Host Disease (GVHD)

Several animal studies have addressed the issue of the suppressive effect of MSCs in the context of GVHD prevention/treatment; however, conflicting results have been published, in particular on the role of MSCs in GVHD prevention.

In one study, AT-derived MSCs have been infused systemically in mice early after transplantation of haploidentical HSCs and were able to rescue the animals from lethal GvHD [101]. Sudres et al. have reported that a single dose of BM-derived MSCs at time of allogeneic BM transplantation did not affect the incidence and severity of GVHD in mice [102], whereas UCB-derived MSCs administered at weekly intervals were able to prevent GVHD development after allogeneic transplantation of human PBMCs in NOD/SCID mice [103]. The same cells were not effective when administered prophylactically right after PBMC infusion, as well as when infused late in the course of GVHD development [103]. Polchert et al. tested the ability of MSCs to prevent GVHD by administering a single dose of the cells at different time points: only when MSCs were infused at day +2 or +20 after the allograft they were able to significantly increase the survival of the recipient mice. At these time points, the levels of IFN-γ were found to be particularly high in the animals, this corroborating the observation that MSCs need to be activated by inflammatory cytokines present in the host microenvironment to deliver their immunosuppressive effect [104]. See also Table 7.2, part B. The studies suggest that MSCs may prevent GVHD following allogeneic stem cell transplantation, but are not effective in the treatment of acute GVHD. These results are in contrast with preliminary results of clinical studies, where MSCs have been used to treat acute GVHD (see section "Clinical Trials of MSC Infusion to Treat GvHD").

Animal Models of Autoimmune Diseases and Regenerative Medicine

Due to their ability to home to inflamed sites and to repair injured tissues, together with their immunomodulatory and anti-inflammatory properties, MSCs have been also tested in animal models of tissue injury and autoimmune disorders (see Table 7.1, part C) [14, 105–121].

Murine MSCs have been demonstrated to ameliorate experimental autoimmune encephalomyelitis (EAE), a model of post-vaccinal encephalitis with many aspects resembling those of human multiple sclerosis (MS), through the induction of peripheral T-cell tolerance against the central nervous system (CNS)-restricted antigens [105, 106]. In a murine model of SLE, MSCs were able to inhibit autoreactive T and B cells, thus ameliorating the signs and symptoms of the disease [107]. MSCs have been also employed for the experimental treatment of diabetes in a mouse model, and their infusion was associated with an increase in the number of pancreatic islets and insulin-producing β cells, as well as with the repair of renal glomeruli [108]. Moreover, the administration of congeneic MSCs in a murine model of type 1 diabetes was shown to suppress both diabetogenic T-cell proliferation and generation of myeloid/inflammatory DCs, resulting in long-term reversal of hyperglycemia [109, 110]. The infusion of rat MSCs in an experimental model of glomerulonephritis was able to stimulate glomerular healing, resulting in the repair of the damaged renal tissue [111]. Intravenous infusion of MSCs in immunodeficient mice with

Table 7.2 Clinical applications of MSCs in phase I–II studies

Clinical context	Outcome	Ref. no.
Part A: HSC engraftment		
Breast cancer; autologous HSCT	No tox. Rapid haematopoietic recovery	[123]
Haematological malignancy; allogeneic HSCT	No tox. Prompt haematopoietic recovery	[124]
Haematological disorders; haploidentical T-cell-depleted HSCT	No tox. Graft rejection prevention. Accelerated leukocyte recovery	[18]
Haematological disorder (1 pt); double UCBT	Alleviated single-donor predominance	[125]
Haematological disorders; UCBT	No tox. Prompt haematopoietic recovery	[126]
Haematological disorders; UCBT	No tox. No effect on engraftment and haematopoietic recovery. GvHD prevention	[19]
Haematological disorders; UCBT + 3rd PD HSC	No tox. No effect on kinetics of engraftment and GvHD	[127]
Part B: aGvHD		
Grade IV aGvHD after allogeneic HSCT (1 pt)	Complete resolution of grade IV acute GVHD	[128]
Grade II–IV aGvHD after allogeneic HSCT/DLI (55 pt)	Overall response rate: 69%; improved OS in responders	[20]
Grade III–IV aGvHD after allogeneic HSCT/DLI (37 peds)	CR 59%; improved OS if early treatment	a
Part C: AID and regenerative medicine		
MS and ALS	Demonstration of safety + increase in Treg	[129, 130]
Fistulizing refractory CD (intrafistular injection)	Demonstration of safety + fistula healing + increase in Treg	[41, 131]
Refractory CD (intravenous injection)	Safety + some clinical response + increase in Treg	[132]
Cirrhosis	Safety + improved clinical conditions	[133]
Myocardial infarction (intravenous infusion)	Improvement in overall health + increase of LVEF	[134]
Myocardial infarction (intracoronary infusion)	Improvement of LVEF not maintained over time	[135]

HSC haematopoietic stem cells, *HSCT* haematopoietic cell transplantation, *tox*. toxicity, *pt* patient, *UCBT* umbilical cord blood transplantation, *PD* party donor, *aGvHD* acute graft-versus-host disease, *DLI* donor lymphocyte infusion, *peds* paediatric patients, *CR* complete response, *OS* overall survival, *MS* multiple sclerosis, *ALS* amyotrophic lateral sclerosis, *Treg*, regulatory T cells, *CD* Crohn's disease, *LVEF* increase of the left ventricular ejection fraction

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cisplatin-induced acute kidney injury ameliorated both renal function and tubular cell injury and prolonged survival due to the inhibition of oxidative damage [112, 113]. Topical implantation of BM-derived MSCs has been shown to be beneficial in promoting the healing process of experimental colitis in rats, confirming the ability of these cells to induce tissue repair [114]. In similar models of experimental colitis,

MSCs of different tissue origin alleviated the signs and symptoms of the disease by displaying immunomodulatory functions and ameliorating inflammation-related tissue destruction [115, 116]. In a rat model, MSC-derived conditioned medium proved to be effective in reversing fulminant hepatic failure, this suggesting that MSC-derived molecules are able to promote regeneration of hepatocytes [14, 117]. Recent preclinical studies have suggested that MSCs could be employed to mediate cardiac repair after myocardial infarction, as well as after chronic progressive cardiac failure. In particular, human MSCs have been shown to differentiate into cells with a cardiomyocyte phenotype in the adult murine heart [118] and to improve cardiac function after transplantation in porcine and rat models [119, 120].

While the vast majority of reports indicate a favourable role of MSCs in the promotion of tissue repair, infusion of MSCs had no beneficial effects on collagen-induced arthritis (CIA), a murine model of rheumatoid arthritis. In particular, in this context, MSC treatment was associated with an enhanced Th1 response, although MSCs could not be detected in the articular environment [121].

Potential MSC Mechanisms of Action In Vivo

The mechanisms through which MSCs exert their therapeutic potential, although not fully established, might rely on some key properties of the cells: (i) the ability to secrete soluble factors capable of stimulating both survival and functional recovery of injured cells; (ii) the ability to home to sites of damage; and (iii) the ability to modulate immune responses. In most of the reported studies, the therapeutic effect of MSCs was not associated with their differentiation into the resident cell types, but, rather appeared to be mostly related to antiproliferative and anti-inflammatory effects, as well as to the capacity to stimulate survival and functional recovery in injured organs, likely through paracrine mechanisms [14, 111–113, 117]. It is conceivable that the therapeutic benefit is due to the release of soluble factors (such as HGF, insulin-like growth factor, PGE2, NO, IDO) produced by the cells and/or by the local microenvironment and that MSC survival is not strictly necessary for the clinical effect [14, 117]. Also the engraftment-promoting effect might be obtained through the secretion of paracrine factors produced by MSCs, which might promote the creation of a favourable microenvironment for the survival, proliferation and engraftment of HSCs.

Experimental and clinical data obtained so far indicate that sustained engraftment of MSCs does not occur or it is limited to a small number of cells. In this regard, studies in baboons using a green fluorescent retroviral construct suggest engraftment of MSCs in the gastrointestinal tract and in various tissues in the range of 0.1–2.7%, with comparable results for both autologous and allogeneic cells [136]. Although little is known about MSC homing to target tissues after infusion, it might be largely regulated by chemokines and growth factors released during systemic and/or local inflammatory conditions and be mediated by the interaction with integrins and selectins expressed on the surface of MSCs. In this respect, Wynn et al.

showed that homing of MSCs to BM depends on stromal-derived factor-1 (SDF-1) which interacts with CXCR4 on the MSC surface, thus promoting their migration [137]. Similar mechanisms have been shown to regulate migration of MSCs to pancreatic islets [138] and ischemic tissues [139]. In view of these experimental data, a possible strategy to facilitate homing of MSCs involves the modification of surface structures that play a role in migration to specific tissues, as suggested by Sackstein et al. [140]. These authors converted the native CD44 glycoform expressed on MSCs into E-selectin/L-selectin ligand (HCELL) (expressed on HSCs) using fucosyltransferase. Intravital microscopy in NOD/SCID mice showed BM infiltration by HCELL(+) MSCs within several hours after intravenous infusion [140].

The inhibition of inflammatory and immune responses by MSCs might also be due to the generation of regulatory T cells, as shown in an experimental murine model of Crohn's disease, in which MSC infusion was efficacious in both preventing and curing colitis, probably through the induction of FoxP3^{+} regulatory T cells [116].

Clinical Applications of MSCs

Clinical Trials of MSC Infusion to Promote Engraftment

The first clinical trial on the use of MSCs for accelerating haematological recovery was performed in 28 patients with breast cancer given autologous transplantation of peripheral blood HSCs and co-infused with $1–2 \times 10^{6}$ MSCs/kg body weight. No MSC-related toxicity was recorded, and rapid haematopoietic recovery was noted [123]. After this study, a multicenter phase I/II trial aimed at evaluating the safety of MSC infusion was conducted in 46 patients affected by haematological malignancies and receiving allogeneic HSCT from an HLA-identical sibling [124]. MSC co-infusion was not associated with adverse events, and haematopoietic recovery was prompt for most patients; moderate to severe acute GVHD was observed in 28% of the patients. In a phase I/II, multicenter clinical trial, infusion of MSCs proved to be safe in children given a T-cell-depleted allograft from an HLA-disparate relative [18]. All patients given MSCs showed sustained haematopoietic engraftment without any adverse reaction, this finding comparing favourably with 20% graft failure rate observed in the historical controls. Leukocyte recovery was faster in children given MSCs, as compared to the historical controls. In the setting of UCBT, MSCs were first employed in a single patient transplanted with UCB-derived cells with the aim of improving the outcome of double-unit UCBT [125]. In this patient, MSCs were administered without clinical adverse effects, and the single unit predominance described after multiple UCBT was not observed. In a paediatric, phase I–II clinical trial, including eight children given co-transplantation of unrelated donor UCB cells and *ex vivo*-expanded third-party MSCs, infusion of MSCs proved to be safe and patients had neutrophil recovery a median time of 19 days after the allograft [126]. In another paediatric, phase I/II clinical study,

the safety of co-transplantation of parental MSCs was confirmed in 13 paediatric patients given UCB-derived HSCs [19]. In contrast with preclinical results [98] and the experience reported in the haploidentical transplants [18], no difference was found in engraftment rate and speed of haematological recovery between study patients and controls receiving UCBT alone, although much less study patients were given granulocyte-colony stimulating factor (G-CSF) as compared to controls. Interestingly, MSC co-infusion significantly prevented the incidence of life-threatening acute GVHD and GVHD-associated transplantation-related mortality (TRM), as compared to controls [19]. In adult patients receiving UCBT with co-infusion of third-party donor mobilised HSCs, MSC administration at time of transplantation had no effect on the kinetics of UCB cell engraftment, as well as on GVHD prevention [127]. See Table 7.2, part A.

Altogether these data indicate that co-transplantation of HSCs and MSCs is safe, whereas the efficacy of MSCs on promoting engraftment of donor cells and accelerating the speed of haematological recovery remains to be demonstrated. In some contexts (such as T-cell-depleted allograft from an HLA-disparate relative), MSCs may modulate host alloreactivity and/or promote a better engraftment of donor haematopoiesis, reducing the risk of graft failure. The difference between the haploidentical and UCBT settings may be related to the mechanisms underlying graft failure in UCBT, which might be inherent to the low numbers of HSCs infused in UCBT and/or to altered homing mechanisms.

Despite reports indicating engraftment of MSCs after systemic infusion in animal models [97, 141], the transplantability and sustained engraftment of MSCs in humans has not been demonstrated. A number of studies have documented that marrow stroma remains of host origin after allogeneic HSCT in the majority of patients [142–144], whereas others have shown limited engraftment capacity of MSCs following HSCT in both adult and paediatric patients [18, 19, 22, 145, 146].

Clinical Trials of MSC Infusion to Treat GVHD

The most impressive clinical effect of MSCs in vivo has been observed in the treatment of acute GvHD (aGvHD) developing after allogeneic HSCT or donor lymphocyte infusion (DLI). The first striking report of this effect was reported by Le Blanc et al. who described a paediatric patient experiencing grade IV aGvHD of the liver and gut after allogeneic HSCT from an unrelated volunteer, resistant to multiple lines of immunosuppressive therapy. The child was rescued by the infusion of BM-derived MSCs isolated from the mother [128]. More recently, the benefit deriving from the infusion of MSCs in patients with steroid-resistant aGvHD has been confirmed in a study reporting 55 adult and paediatric patients, treated in six different institutions. Infusion of MSCs appeared to be safe, and no major toxicities were observed. Treatment with MSCs resulted in a response in the majority of patients, this resulting into a significant difference in survival between complete responders and partially responding/nonresponding patients [20]. When compared to adults,

children seemed to have a better response rate and a greater probability of overall survival [20].

The outcome of 37 children receiving MSCs for grade III–IV acute GVHD refractory to steroids have been recently reported [147]. A median of two infusions were administered, with a median cell dose of 2×10^6/kg; MSCs were from third party HLA-mismatched donors in the majority of the patients. Complete response (CR) was observed in 22 children (59% of the overall population), transplantation-related mortality (TRM) being 14%. Fifteen children had either no ($n=6$) or partial ($n=9$) response to MSCs, TRM in this group being 60% ($p=0.005$). With a median follow-up of 2.3 years, overall survival (OS) was 62%, the values for patients who did or did not achieve CR after MSCs being 87 and 27%, respectively ($p<0.001$). Children treated after 2009 had received less second-line treatment and had received MSCs earlier after onset of steroid treatment (mean day 8 vs. day 24 for children treated before 2009). This translated into a significantly better OS for children enrolled in the study after 2009 (93% vs. 65% for those treated before 2009; $p<0.05$). These data indicate that MSCs are a safe and valuable therapy for children with severe, refractory aGVHD, better results being obtained when treatment is employed early in the disease course. See Table 7.2, part B.

The real efficacy of MSC infusion in the management of patients with GVHD will be tested in a randomised controlled trial being conducted in Europe.

Clinical Trials of MSC Infusion in Autoimmune Disorders and Tissue Repair

Following the numerous reports showing a beneficial effect of MSC treatment in experimental models of autoimmunity and acute tissue injury [14, 105–120], clinical data on the use of MSCs in regenerative medicine have become available (see Table 7.2, part C) [129–135, 148–150].

A phase I clinical study including ten patients with multiple sclerosis and treated with autologous MSC infusion has shown the feasibility and the safety of the approach [130]. In 15 patients with MS and 19 patients with amyotrophic lateral sclerosis treated with intrathecal and/or intravenous MSC infusion, the procedure was found to be safe and was associated with an increased proportion of $CD4^+CD25^+$ regulatory T cells in the peripheral blood of the patients [131], suggesting that the possible effects of MSCs involve the induction of regulatory T cells.

In a phase I clinical trial, autologous, AT-derived MSCs have been successfully employed to treat complex perianal fistulas of cryptoglandular origin or associated with Crohn's disease with promising results [41]. Sustained closure of fistula tracks, together with a parallel reduction of Crohn's disease and perianal disease activity indexes, has been obtained in patients with refractory fistulizing Crohn's disease through local injections of autologous BM-derived MSCs [132]. In another phase I/II study, intravenous infusion of autologous MSCs proved to be feasible and safe in nine patients with Crohn's disease refractory to conventional treatments, three

of them showing clinical response [133]. In these latter two studies, an increase in regulatory $CD4^+CD25^+FoxP3^+$ T cells in mucosal biopsies was found after MSC treatment, as compared with what observed before treatment initiation [132, 133].

MSCs have been also employed to treat liver cirrhosis in a limited number of patients. Kharaziha et al. reported a phase I–II clinical trial in which eight patients with end-stage liver cirrhosis were treated with autologous injection of MSCs via either a peripheral vein or the portal vein; preliminary results confirm the safety of the approach and suggest some improvement in the clinical conditions of the patients [148]. Similar findings were obtained by Mohamadnejad et al. who also showed that MSCs are superior to HSCs in treating liver cirrhosis [134, 149].

A randomised placebo-controlled clinical trial has been conducted in patients within 10 days following acute myocardial infarction [135]. As compared to patients treated with placebo, patients receiving MSC intravenous infusion of MSCs experienced an improvement in overall health, coupled with an increase of left ventricular ejection fraction (LVEF) 1 year after treatment. However, patients treated with intracoronary administration of MSCs after MI did not maintain a significant improvement in LVEF over controls at the 18 months follow-up evaluation [150]. The heterogeneity in the route of administration, timing of MSC infusion after myocardial infarction and number of cells administered render definitive conclusions difficult to draw on the efficacy of this approach.

Additional studies have been initiated in other autoimmune and inflammatory disorders, such as type 1 diabetes mellitus, systemic sclerosis and SLE, acute kidney injury, gastrointestinal (autoimmune enteropathy) and pulmonary (chronic obstructive pulmonary disease) disorders [151–155]. The results of these studies will become available in the near future.

Conclusions

In addition to their regenerative properties, MSCs have been shown to exert immunomodulatory effects. Extensive *ex vivo* studies have indicated that they affect a broad range of immune functions, including those of T cells, B cells, DCs and NK cells, mainly through the secretion of soluble mediators. These mediators may act directly on immune cells, to inhibit their proliferation or to inhibit apoptosis. They may also act indirectly through intermediate cells, including monocytes, to induce regulatory responses that result in the induction of regulatory T cells or tolerogenic DCs.

Further investigations aimed at better defining the role played in vivo by human MSCs in developing peripheral immune tolerance are desirable. Relatively little is known about the functional differences between MSCs derived from different tissue sources, i.e. BM-derived versus AT-derived MSCs. It is reasonable to hypothesise that different MSC subsets may be responsible for specific functional activities in vivo. Few surface markers are nowadays available for the prospective identification of MSC subsets, and it is still uncertain to what extent functional

properties are preserved/modified following *ex vivo* expansion. The importance of the host tissue microenvironment has recently become apparent as being a possible determinant of in vivo function of MSCs. It is conceivable that host factors play a crucial role in activating or priming MSCs to exert their immunomodulatory properties. Identification of such factors may lead to novel strategies to functionally activate MSCs prior to infusion in order to enhance/optimise their therapeutic effects.

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